

Review

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Post-Translational Modifications of Cysteine Residues in Plant Proteins and Their Impact on the Regulation of Metabolism and Signal Transduction

[Charlie Boutin](#) , [Camille Clément](#) , [Jean Rivoal](#) *

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Review

Post-Translational Modifications of Cysteine Residues in Plant Proteins and Their Impact on the Regulation of Metabolism and Signal Transduction

Charlie Boutin, Camille Clément and Jean Rivoal *

Institut de Recherche en Biologie Végétale, Université de Montréal, 4101 Rue Sherbrooke est, Montréal, QC, Canada H1X 2B2; charlie.boutin@umontreal.ca (CB); camille.clement@umontreal.ca (CC)

* Correspondence: jean.rivoal@umontreal.ca; Tel.: (+1 514 343 2150)

Abstract: Cys is one of the least abundant amino acids in proteins. However, it is often highly conserved and usually found in important structural and functional regions of proteins. Its unique chemical properties allow it to undergo several post-translational modifications, many of which mediated by reactive oxygen, nitrogen, sulfur or carbonyl species. Thus, in addition to their role in catalysis, protein stability and metal binding, Cys residues are crucial for redox regulation of metabolism and signal transduction. In this review, we discuss Cys post-translational modifications (PTMs) and their role in plant metabolism and signal transduction. These modifications include oxidation of the thiol group (*S*-sulfenylation, *S*-sulfinylation and *S*-sulfonylation), the formation of disulfide bridges, *S*-glutathionylation, persulfidation, *S*-cyanylation, *S*-nitrosation, *S*-carbonylation, *S*-acylation, prenylation, CoAlation as well as the formation of thiohemiacetal. For each of these PTMs, we discuss the origin of the modifier, the mechanisms involved in PTM, as well as their reversibility. Examples of the involvement of Cys PTMs in the modulation of protein structure, function, stability, and localization are presented to highlight their importance in the regulation of plant metabolic and signaling pathways.

Keywords: post-translational modification; cysteine; regulation; signal transduction; metabolism; thiol; redox modification

1. Introduction

Protein post-translational modifications (PTMs) are chemical or enzymatic modifications of proteins that can affect various aspects of protein activity through changes in structure, function, regulation, localization, interactions, or stability to name a few examples [1]. A large number of PTMs have been described in plants, similarly to other living organisms [1–5]. Due to their sessile lifestyle, PTMs are especially important for plants in which they serve as efficient regulatory mechanisms, allowing rapid and often reversible cellular responses to changes in homeostasis, as well as adjustments to alterations in metabolism, physiology or external stimuli [2,5,6]. In plants, one of the most widespread consequence resulting from modifications in their biotic or abiotic environment is an alteration in the cell redox status [7–10]. Such changes govern a variety of redox signaling events that are involved in adjusting plant metabolism or signaling pathways [7,9,11,12]. Redox signaling occurs notably via the oxidative modification of Cys residues in proteins [13].

Cys is thought to be a comparatively recent addition to the genetic code [14] and is involved in important structural and functional regions of proteins [15,16]. Since two codons translate to Cys, the latter should theoretically represent 3.3% of the amino acids in proteins [17]. However, it is underrepresented in organisms from all kingdoms of life [17]. Interestingly, Cys content in proteins increases with the complexity of the organisms, ranging from 0.4–0.5% in Archaea to around 2.3% in mammals [17,18]. When it comes to plants, analyses show a similar trend, with a Cys content ranging from ~1.5% in green algae to ~1.9% in land plants [18,19]. The vast majority of proteins contain Cys. For instance, more than 92% of total plant protein sequences contain at least one Cys, with a median of six residues/protein [18]. In most organisms, Cys distribution in protein sequences is also peculiar,



with a preference for the CXXC sequence pattern [17]. The CXXC motif is usually found in oxidoreductase and metal-binding domains [17]. However, in contrast to other organisms, plant proteins do not exhibit a high representation of this specific motif [17]. Cys also displays a distinctive conservation pattern. In most proteins, Cys is either more than 90% conserved, or less than 10% conserved, indicating a strong selective pressure to both maintain important functional Cys and remove other ones [20]. The negative selection of Cys residues appears to be stronger for isolated Cys on protein surfaces [20].

Cys has unique physico-chemical properties among all protein amino acids. Its side chain carries a thiol group (-SH) which can deprotonate as a consequence of various interactions with its environment. This loss of a proton generates a thiolate (-S⁻), increasing the nucleophilicity of the side chain [16]. This is a key determinant in Cys reactivity. The average pKa of protein Cys residues exposed to the solvent is around 7.5, a much lower value than that of buried Cys (around 9.5). This low pKa contributes to the fact that surface Cys are much more reactive [20]. Indeed, all protein Cys residues are not equally reactive. With a pKa value of 8.45 found for free Cys thiols, most Cys would be expected to exist in their protonated form [21]. However, the protein microenvironment can significantly affect Cys thiols pKa. For instance, basic amino acids, such as His, Arg or Lys, as well as metal ions adjacent to a Cys residue tend to lower the pKa of Cys thiol thereby stabilizing its thiolate form and promoting its reactivity [22,23]. The end-positioning of Cys residues on α -helices is another factor that affects their pKa. It has long been known that α -helices behave as dipoles [24]. Studies of the thioredoxin superfamily have shown that the dipole properties of an α -helix, together with the localization of a Cys at the N-terminus of the helix contributes to lowering the thiol pKa, thereby enhancing its reactivity [25,26]. Another way by which the protein microenvironment might increase the reactivity of a thiol group is by decreasing the activation energy during the transition state of the reaction involving the thiol. This has been demonstrated in the case of mechanistic studies of thiol/disulfide exchange [27,28]. In enzyme catalyzed reactions, contrary to a polar solvent, the hydrophobic environment provided by the protein does not stabilize the reactants in relation to the reaction transition state. This process reduces the activation energy required for the reaction to proceed allowing a faster reaction rate [28].

The reactivity of thiols is a crucial factor responsible for the involvement of Cys residues in multiple facets of protein function, including folding, catalysis, proteostasis and signal transduction [29]. The Cys sulfur atom possess oxidation states ranging from -2 to +4 [30,31], that allow a variety of redox post-translational modifications (PTMs). Disulfide-base reversible modifications of Cys residues, such as disulfide bridge or mixed disulfide with low-molecular weight thiols is often referred to as redox or thiol switches [29,32]. These play an important role in the modulation of protein activity, function, and localization in response to external stimuli. Redox signaling can involve reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive carbonyl species (RCS) and reactive sulfur species (RSS) [33]. PTMs on Cys residues include S-sulfenylation, S-sulfinylation and S-sulfonylation, disulfide bridge (S-S) formation, S-glutathionylation, persulfidation, S-cyanylation, S-nitrosation, S-carbonylation, S-acylation, prenylation, CoAlation and thiohemiacetal formation. An analysis of the literature shows the increasing complexity of Cys modifications in plants and reveals evidence of a growing recognition of the importance of these PTMs in the regulation of metabolism and signal transduction. For reference, a searchable database catalogs modified proteins and PTM sites in several model plants (Plant PTM Viewer 2.0, <https://www.psb.ugent.be/webtools/ptm-viewer/>) [4]. Another database, based on deep learning framework was recently established to help facilitate protein Cys modifications in eukaryotes (pCysMod, <http://pcysmod.omicsbio.info/>) [34]. Unsurprisingly, many plant proteins are identified as targets for multiple PTMs. Indeed, since multiple molecules present in the same cell compartment at the same time are able to interact with reactive thiols, multiple Cys modifications can compete with each other *in vivo*, adding a layer of complexity in the understanding of Cys-mediated signaling in plant cells [33,35–37].

The aim of this review is to provide a comprehensive survey of the various PTMs of Cys residues identified in plants. For each modification, we evaluate the current state of knowledge on

mechanisms facilitating PTM and its reversion, whether spontaneous or enzymatic. For each PTM, examples of targets involved in metabolism and/or signal transduction are identified and the implication of the modification in the regulation of these targets are critically discussed in relation to their function. In some cases, gaps in knowledge and possible future avenues of research on Cys PTMs in plants are also identified.

2. S-sulfenylation

Under normal conditions, aerobic metabolic processes, such as photosynthesis and respiration, constantly produce low levels of ROS as by-products. ROS are present in the cell in different forms, such as $O_2^{\bullet-}$ (superoxide radical), H_2O_2 (hydrogen peroxide) and $\bullet OH$ (hydroxyl radical) [38]. Various biotic and abiotic stresses can disrupt redox homeostasis by promoting significant increases in ROS, leading to oxidative stress. Basal and stress-induced ROS production and detoxification in plants has been extensively reviewed and the reader is therefore invited to consult relevant publications and references within [39–48]. ROS greatly differ in their reactivity, diffusion rate and concentration in cells [38]. They can also cause a range of reversible and irreversible damage to lipids, DNA and proteins, altering their function in the cells [41,42]. Although they were initially thought to be exclusively toxic molecules leading to oxidative distress, it is now widely recognized that ROS can also generate oxidative eustress, or ‘good stress’ [49] by having essential signaling functions [33,37,38,50].

2.1. S-Sulfenylation is Promoted by Oxidative Conditions and is a Stepping-Stone Towards Other Cys Redox PTMs

Among ROS, H_2O_2 has the longest half-life and the highest capacity for diffusion [41], which makes it highly suitable for redox signaling. Indeed, H_2O_2 acts as a second messenger in plants by diffusing in cells and across membranes via aquaporins, thereby allowing both autocrine and paracrine signaling [45]. H_2O_2 is relatively stable and its reaction with reduced free Cys or glutathione (GSH) is slow compared to some other ROS and RCS [49]. H_2O_2 generally reacts more easily with protein Cys but its reactivity for thiol oxidation is highly dependent on a favorable protein microenvironment that reduces activation energy [49]. This dependency on protein structure for reactivity allows specificity of H_2O_2 mediated redox signal [49]. Thiolates, which are more reactive than thiols towards H_2O_2 , can perform a nucleophilic attack on H_2O_2 , causing a reversible two electrons oxidation to a sulfenic acid (-SOH) [51], potentially altering enzymes function and activity (Figure 1) [42]. It is noteworthy that in addition to H_2O_2 , natural or artificial hydroperoxides and peroxy nitrite can also cause thiol oxidation to sulfenic acid [31,52]. The latter is usually considered highly unstable and acts as an intermediate towards several Cys redox PTMs (Figure 1A), including, as discussed below, S-sulfinylation, S-sulfonylation, S-glutathionylation, S-S formation, or persulfidation [22]. As discussed in the section below, there is also an enzymatic pathway responsible for the generation of S-sulfenylated Cys. This occurs in instances where a S-sulfenylated Cys can be reduced by a sulfiredoxin (SRX) [53]. The stability of the S-sulfenylated Cys is mainly determined by its molecular environment. Improvement of sulfenic acid stability is determined by decreased solvent accessibility, absence of a proximal Cys that could induce the formation of a S-S and stabilization of the sulfenate by an H-bond network with adjacent amino acids [22]. In addition, the sulfenic acid has a unique reactivity since it can act both as a nucleophile and an electrophile [31]. For instance, the nucleophilic reactions of sulfenic acid include its overoxidation to sulfinic acid [31]. For this, the sulfenic acid performs a nucleophilic attack on H_2O_2 , leading to irreversible sulfinic and sulfonic forms of oxidation discussed below. Electrophilic sulfenic acid reactions lead for example to Cys persulfidation which involves a reaction with H_2S and cannot occur with a non-oxidized thiolate (see section below, [54]). As well, sulfenic acid can react with a thiol to create an intramolecular or intermolecular S-S or a mixed disulfide [31] (see S-S and S-glutathionylation sections below). The ability of sulfenic acid to act as an electrophile has also been exploited by using its reactivity towards 5,5-dimethyl-1,3-cyclohexanedione (dimedone). This highly selective reaction has been used to develop chemoselective dimedone-based probes enabling the detection of sulfenylated proteins in

cells [55]. More recently, a more reactive benzo[c][1,2]thiazine-based (BTD) probe [56] was used to identify the *Arabidopsis thaliana* (Arabidopsis) sulfenome [57]. Chemosselective methods for surveying and identifying the different levels of Cys thiol oxidation have been recently reviewed [58].

2.2. S-Sulfenylation is a Reversible Primary Cys Oxidation

The reversion of Cys thiol oxidation from its sulfenic acid back to the thiol form is possible (Figure 1). *In vitro*, it can be achieved using a variety of reducing agents such as dithiothreitol (DTT), arsenite [31] or ascorbate in the case of 1-Cys peroxiredoxins (PRXs) [59,60]. The pool of ascorbate in normally highly reduced in plants [61]. Thus, the *in vitro* activity of ascorbate for the reduction of Cys sulfenic acid may have some relevance *in vivo*, although this remains to be seen. Nevertheless, the role of ascorbate in this process has been known for some time in animals [62]. The reduction of sulfenic acid can also occur using other mechanisms [63]. Methionine sulfoxide reductase B1 (MSRB1) is a 1-Cys reductase involved in the reduction of Met sulfoxide back to Met, allowing proteins that carry oxidized Met to return to their basic state. In the process, the catalytic Cys of MSRB1 becomes oxidized to a sulfenic acid. The reaction of the S-sulfenylated catalytic Cys with GSH leads to its S-glutathionylation (see section below for mechanistic details). The S-glutathionylated Cys can then be reduced back to its thiol form by a glutaredoxin (GRX) [63]. The study demonstrated that GRXC4 and GRXS12 could carry out the reaction *in vitro*. In addition to GSH, some TRX can act as an electron donor for the reduction of S-sulfenylated Cys. While the latter work showed a lack of capacity for TRXh1 to reduce S-sulfenylated MSRB1 [63], MSRB2 was a substrate. A subsequent study provided strong evidence that S-sulfenylated MSRB1 could be reduced by TRX CDSP32 under physiological conditions in a process that involved the formation of a S-S [64]. More recently, the enzymatic reduction of protein sulfenic acid has also been demonstrated *in vitro* for EF-Tu, a redox sensitive chloroplastic translation elongation factor of *Arabidopsis* [65]. The study identified that TRX f1 mediated the reduction of S-sulfenylated Cys¹⁴⁹. Thus, various pathways may contribute to the conversion of Cys sulfenic acid to its thiol form, and much remains to be investigated concerning the possible differences in efficiency of the various actors in the process.

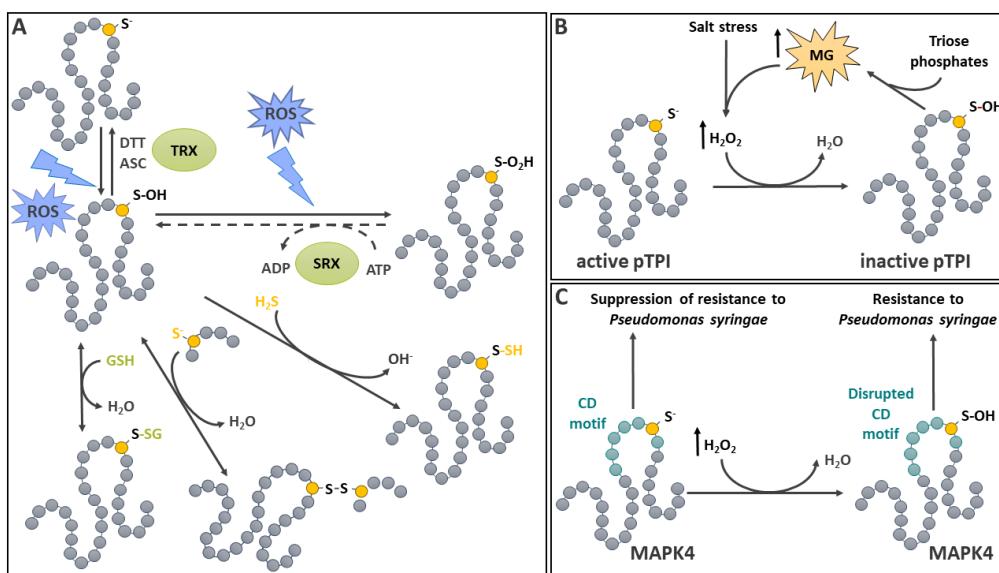


Figure 1. Protein S-sulfenylation in plants. A: The transition of Cys residues from thiolate to S-sulfenylation state. A Cys residue (yellow dot) in deprotonated state (thiolate, S⁻) (top left corner) undergoes oxidation to its sulfenic acid form (S-OH) upon exposure to oxidative conditions caused by reactive oxygen species (ROS). Reductants such as dithiothreitol (DTT), ascorbate (ASC) or thioredoxin (TRX) have been shown to reverse this reaction. The S-sulfenylated form allows further Cys modifications, such as generation of a reversible mixed disulfide with glutathione (GSH) to yield an S-glutathionylation (S-SG); a reaction with a protein Cys in thiolate form, to generate a reversible disulfide bridge (S-S); and a higher degree of

oxidation towards the sulfinic acid form (SO_2H). The latter reaction can be reversed with an ATP-dependent sulfiredoxin (SRX). B: Inhibition of plastidial triose phosphate isomerase (pTPI) by *S*-sulfenylation. Under high H_2O_2 conditions created by salt stress, pTPI becomes inactive due to *S*-sulfenylation. The inability of pTPI to process triose phosphates leads to the formation of methylglyoxal, which acts as a promoter of H_2O_2 formation. C: Regulation of MAPK4 by *S*-sulfenylation. MAPK4 is a negative regulator of resistance to *Pseudomonas syringae*. Under normal conditions, the protein is active, and suppresses resistance to the pathogen. Under oxidative stress conditions, a Cys residue of the protein's Common Docking (CD) motif becomes *S*-sulfenylated, thereby disrupting MAPK4 function and enabling resistance to the pathogen. See text for additional details.

2.3. *S*-sulfenylation as a Redox-Control Mechanism in Plant Primary Metabolism

ROS-mediated oxidation modulates the activity of many enzymes involved in metabolism and stress response. Cellular oxidative conditions, as discussed above, are conducive to protein *S*-sulfenylation. A survey of *Arabidopsis* cell culture sulfenylome revealed that more than 1,000 proteins were *S*-sulfenylated in response to treatments with H_2O_2 (up to 400 μM), with an average of 1.5 modification/protein [57]. The study noted a particular enrichment in protein classes belonging to important metabolic pathways, while several *S*-sulfenylated sites were predicted or proved to have a functional importance. Among these *in vivo* targets, cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) had been previously shown to be strongly inhibited by H_2O_2 [66]. In the case of GAPDH, *S*-sulfenylation can also lead to inhibitory *S*-glutathionylation (see below). A decrease in cytosolic GAPDH activity, such as documented in an *Arabidopsis* knockout line leads to decreased ATP and tricarboxylic cycle intermediates pools [67]. This is consistent with the fact that oxidative stress generally inhibits several aspects of respiratory metabolism [6]. Moreover, in plants and other systems, oxidative inhibition of triose phosphate metabolism enzymes in glycolysis has been linked to a redirection of C flux to the oxidative pentose phosphate pathway (OPPP) to serve for NADPH generation. [6,68]. This can be used by enzymes in the redoxin family in order to restore redox homeostasis. The evolutionary conservation of this redox-controlled metabolic switch between glycolysis and the OPPP may indicate an ancient origin for the strategy.

Lately, there has been other examples illustrating the potential for Cys *S*-sulfenylation as a regulatory mechanism for metabolic enzymes in the chloroplast. A first investigation has shown that the plastidial triosephosphate isomerase in *Arabidopsis* is inhibited by Cys⁷⁴ *S*-sulfenylation resulting from H_2O_2 accumulation induced by salt stress (Figure 1B) [69]. A consequence of this inhibition was an accumulation of methylglyoxal [69], which is known to promote H_2O_2 formation [70], thus creating a feedback loop. More recently, examination of the redox properties of plastidial NAD-dependent malate dehydrogenase revealed four redox-active Cys [71]. It was determined that, following *in vitro* oxidative treatment, a great proportion of Cys¹²⁹ was *S*-sulfenylated. This residue was further found responsible for the reversible oxidative inhibition of the enzyme using a directed mutagenesis study. Interestingly, reduced or oxidized nicotinamide adenine dinucleotide cofactors offered a relatively high level of protection against plastidial NAD-dependent malate dehydrogenase inhibition [71]. This proposed *S*-sulfenylation regulatory mechanism could impact stromal compartment dicarboxylate metabolism in a redox dependent manner. However, this remains to be further established using *in vivo* approaches.

2.4. Involvement of Protein *S*-Sulfenylation in Stress Signal Transduction

In the past years, several proteomic surveys have illustrated the importance of *S*-sulfenylation-dependent mechanisms in plant stress signaling [57,72–74]. Current approaches to study this topic take advantage of above-mentioned dimedone-based sulfenate probes *in vivo*. A recent publication illustrates the role played by Respiratory Burst Oxidase Homolog (RBOH) in protein *S*-sulfenylation during the response to pathogen [74]. RBOH is a plasma membrane localized NADPH oxidase that generates $\text{O}_2^{\bullet-}$ in the apoplast [75]. H_2O_2 is then formed upon detoxification of $\text{O}_2^{\bullet-}$ by a superoxide dismutase present in the extracellular space [76]. RBOH is an important player involved in the

recognition of Pathogen-Associated Molecular Patterns (PAMPs) [77]. Upon recognition by pathogens-derived signals, plasma membrane receptors trigger a phospho-relay signaling cascade that leads to the activating phosphorylation of RBOH by Ca^{2+} -dependent protein kinases and Botrytis-Induced Kinase 1 (BIK1) [77,78]. Mitogen Activated Protein kinases (MAPKs) cascades are also activated in this process, resulting in the transcriptional activation of PAMP-related genes [79]. ROS production occurring during pathogen-triggered RBOH activation plays a role in setting off cellular oxidative conditions that govern plant pathogen responses. This can lead to hormonal signaling, metabolic reprogramming, protein redox-PTMs or cell death related to hypersensitive response (HR) [74,80]. In *Nicotiana benthamiana*, pathogen derived signals induced a dramatic increase in protein S-sulfenylation, which was attenuated by silencing of *RBOHB* [74]. This strongly supports an important role for RBOH in the control over S-sulfenylation in response to pathogen stress. The study further demonstrated that pathogen signals induced HR in leaves, which was sensitive to dimedone. Since, as stated earlier, dimedone reacts with S-sulfenylated residues, it was concluded that signaling via S-sulfenylation is key for HR in response to pathogens.

MAPK cascades mediate signal transduction in response to pathogens and a variety of other stresses in plants. Several MAPKs are activated as a result of an increase in cellular or extracellular H_2O_2 or other oxidative conditions [75]. In most cases however, the underlying mechanistic details remain to be established. Arabidopsis proteomic surveys aiming at identifying *in vivo* targets of S-sulfenylation revealed that several MAPKs, and in particular MAPK4, are modified in response to H_2O_2 [57,72]. Following Arabidopsis cell culture exposure to H_2O_2 , MAPK4 was shown to be modified on Cys¹⁸¹ which resides in the Common Docking (CD) motif, a signature MAPK protein interaction domain (Figure 1C) [57]. Using a site directed mutagenesis approach, Cys¹⁸¹ was demonstrated to be critical for *in vitro* maximal kinase activity [57]. Further analyses were conducted using transgenic plants transformed with MAPK4 variants where Cys¹⁸¹ was replaced by Ser (oxidation insensitive) or Asp (putative S-sulfenylation-mimic) [81]. This study demonstrated that the Cys¹⁸¹ to Asp mutation disrupted the proper function of the CD domain and that Cys¹⁸¹ is essential for adequate signal transduction *in vivo*. MAPK4 is involved in mediating plant response to cold, salt and pathogens as well as cell division [82–84]. In particular, MAPK4 is a known suppressor of resistance to *Pseudomonas syringae* [82]. A transgenic plant carrying the Cys¹⁸¹ to Asp variant phenocopied an *mapk4* insertion line, while the one carrying Cys¹⁸¹ to Ser variant behaved as the WT [81]. The study also further documented the importance of MAPK4 Cys¹⁸¹ in plant growth and development. The studies discussed above offer evidence that S-sulfenylation is an important mode of signal transduction in plants and the approaches used in these works, including the use of S-sulfenylation-mimic variants offer a potent tool to study the *in vivo* relevance of this redox PTM. Nevertheless, while significant progress has been made, component(s) involved in the reduction of Cys¹⁸¹ remain to be identified.

3. S-Sulfinylation and S-Sulfonylation

Sustained oxidative conditions can lead to the spontaneous overoxidation of the sulfenic acid to sulfinic and sulfonic acid forms, which are typically associated to oxidative distress (Figure 2A). Sulfonic acid, which is the most highly oxidized species of thiol, is completely irreversible. The sulfinate level of oxidation is generally irreversible, except in the specific case of PRXs. The latter are thiol-dependent enzymes that decompose peroxides using a peroxidatic (catalytic) Cys (Cys_P) (Figure 2B) [53]. During the catalytic cycle of a PRX, its Cys_P becomes oxidized to the sulfenic acid form, which is normally reduced by a resolving Cys (Cys_R) [85]. This leads to the formation of a S-S between Cys_P and Cys_R, which is further reduced by a thioredoxin (TRX) to complete the catalytic cycle [85,86]. Interestingly, under oxidative conditions, the sulfenic Cys_P can become further oxidized to a sulfinic acid [53,85]. Such overoxidized Cys_P is catalytically inactive, but can be reduced by an ATP-dependent SRX [87,88] which returns the Cys_P to its sulfenic form [53,85]. However upon overoxidation, plant PRX oligomerizes and acquires a novel function as a chaperone (Figure 2B) [86,89], as documented in other systems [90]. This chaperone activity in overoxidized, high molecular weight PRX has been documented *in vitro* using insulin and citrate synthase as substrates [91,92]. The physiological relevance of PRX overoxidation and oligomerization has been studied. In most

physiological stress condition, there is little change in PRX status, whereas treatment with the herbicide methyl viologen, an efficient ROS inducer [93], can effectively increase PRX molecular weight [94]. In addition, PRX can be modified by other PTMs besides overoxidation [53,95]. Thus, further research will be needed to understand the interplay between PRX PTMs, stress conditions and PRX functions.

In addition to the above-described non-enzymatic oxidation of sulfenic acid, plant cysteine oxidases (PCOs), can catalyze the addition of two oxygen atoms on a thiol group to form a sulfinic acid [96]. PCOs can be classified into two groups [97]. Group A PCOs are ubiquitous in plants and are not regulated by O₂ tension, whereas PCOs of Group B are specific to spermatophytes and induced by O₂ deficiency [97]. In *Arabidopsis*, a family of five PCOs (AtPCO1 to AtPCO5) have been described, with a higher affinity for Cys residues localized at the N-termini of proteins [96]. Recent progress has been made in the resolution of the structure of PCOs and the elucidation of their catalytic mechanism [98,99]. So far, evidence suggests that PCOs from Group A (e.g. AtPCO4 and AtPCO5) and Group B (e.g. AtPCO2) have significant similarities in the structure of their catalytic site with a cupin-like double-stranded β -helix containing a triad of His residues coordinating a metal cofactor [98,99]. PCOs are involved in the oxidation of N-terminal Cys residues of specific proteins leading to N-degron pathway dependent proteolysis. Oxidation of N-terminal Cys to the sulfinic acid form can lead to protein destabilization and degradation through the N-end rule pathway, which is conserved in mammals, bacteria and plants [100]. Indeed, overoxidation of N-terminal cysteine is required for its arginylation which then induces proteosomal degradation [100,101]. The role of Cys oxidation in the N-end rule pathway in plants was recently reviewed [102].

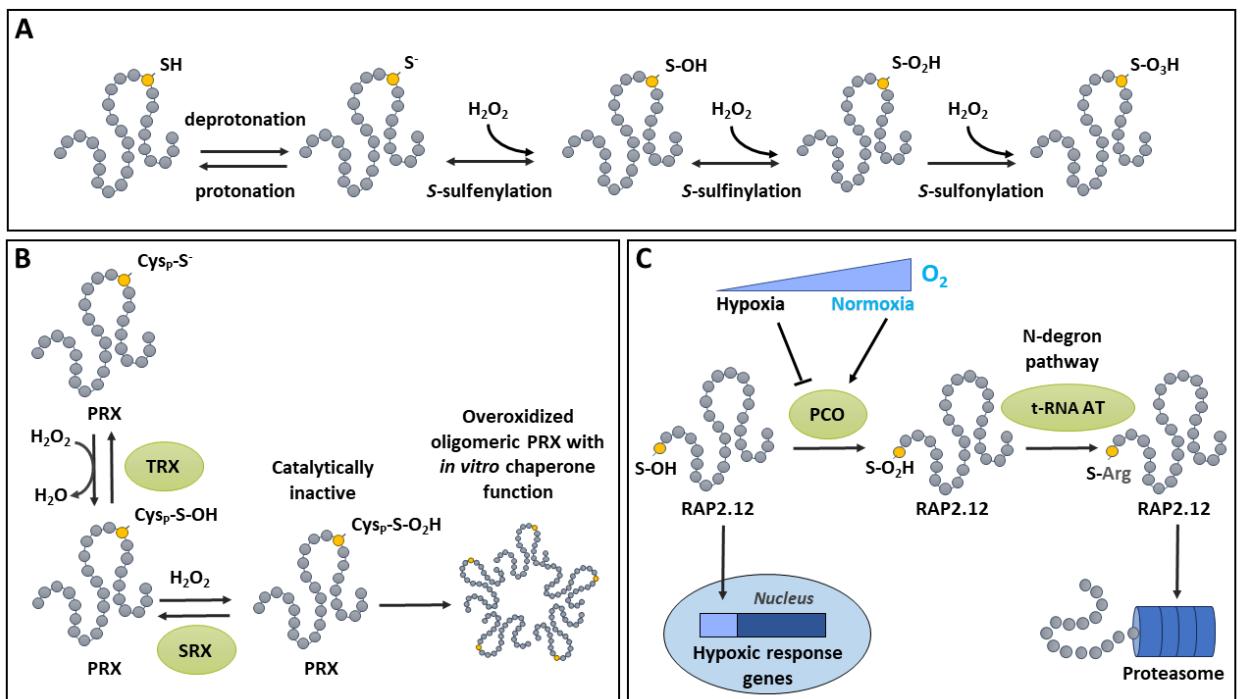


Figure 2. Protein S-sulfinylation and S-sulfonylation in plants. A: The transition of Cys residues from thiol to S-sulfonylated state. When fully protonated (left), Cys residues (yellow dots) are in the thiol state (SH). Depending on conditions prevailing in the environment, the thiol can deprotonate to form a nucleophilic thiolate (S⁻) sensitive to oxidation by H₂O₂. Sequential oxidations of the thiolate by H₂O₂ (left to right) lead to its S-sulfenylation (SOH), S-sulfinylation (SO₂H) and S-sulfonylation (SO₃H). The last step is considered irreversible. B: Modulation of peroxiredoxin functions by oxidation status. Peroxiredoxin (PRX) detoxifies H₂O₂ to H₂O using a peroxidatic Cys (Cys_p). The reaction oxidizes Cys_p to its sulfenic acid form, which can be reduced back with the help of a thioredoxin (TRX). In the presence of H₂O₂, the sulfenylated Cys_p can be further oxidized to a sulfinic acid. This step is reversible in a reaction catalyzed by sulfiredoxin (SRX). Sulfinylated PRX can lead to the formation of an oligomeric, catalytically inactive PRX displaying in vitro chaperone function. C: The N-degron pathway and hypoxic response genes. Hypoxia (low O₂) leads to the activation of PCO (Protein Cysteine Oxidase) which converts Cys_p-S to Cys_p-SO₂H. This intermediate is then converted to Cys_p-SO₃H by RAP2.12. The Cys_p-SO₃H form is recognized by t-RNA AT, which facilitates arginylation (S-Arg). The resulting N-degron is targeted to the Proteasome for degradation. The N-degron pathway is also involved in the regulation of Hypoxic response genes in the Nucleus.

C: Involvement of plant cysteine oxidases in O₂ status signal transduction. The transcription factor RAP2.12 positively regulates plant gene expression in response to hypoxia. Under normoxic conditions, RAP2.12 has low stability because it is oxidized by a plant cysteine oxidase (PCO), leading to sulfinylation of an N-terminal Cys, which is then arginylylated by a tRNA-ARGINYL-TRANSFERASE (t-RNA AT). This process leads RAP2.12 to its degradation by the proteasome. Under hypoxia, PCO is inactivated, leading to the stabilization of RAP2.12, allowing the promotion of hypoxic-responsive genes expression. See text for additional details.

The implication of Cys oxidation by PCOs in the signal transduction of oxygen deficiency is supported by studies on RAP2.12, a member of group VII ETHYLENE RESPONSE FACTORs (ERF-VIIs) (Figure 2C). ERF-VIIs are important transcription factors that promote the response to low O₂ stress (hypoxia) [103]. They contain a highly conserved Cys residue at the N-terminal [104]. In *Arabidopsis*, when O₂ becomes limiting for the maintenance of aerobic metabolism, AtPCO1 and AtPCO2 become less efficient for the oxidation of RAP2.12. The kinetic properties of AtPCOs make them less active under the physiological conditions prevailing in the hypoxic cell (pH, O₂ concentrations), making them excellent candidates to act as plant O₂ sensors [105]. Thus, under normoxia, PCOs are active and oxidize the N-terminal Cys residue of ERF-VIIs, thereby promoting their arginylylation by tRNA-ARGINYL-TRANSFERASE and their degradation by the proteasome [98,106,107]. Low O₂ availability lowers PCO activity, resulting in an increased stability of ERF-VIIs, which can then fulfill their function as transcriptional activators of the hypoxic response [107]. Furthermore, it was recently demonstrated that this PCO/ERF-VII pathway is under the control of the metabolic energy sensor Target Of Rapamycin through a mechanism that allows the coordination of ERF-VII-mediated response to hypoxia with the energy status of the cell [108].

Oxygen sensing and signaling via PCO and the Cys/Arg branch of the N-degron pathway also play important roles in plant development [109]. Indeed, due to lack of internal O₂ transport, plant tissues display diffusion-dependent O₂ gradients and some tissues, such as the shoot apical meristem (SAM), are normally in an hypoxic state [109]. This situation may allow O₂ concentration-dependent stabilization of transcription factors in different cell types and thus, regulation of plant development [110], as documented in the case of LITTLE ZIPPER 2 (ZPR2) [109]. ZPR2 has a conserved Cys at position 2 and is a substrate of PCO after removal of the N-terminal Met by a Met-aminopeptidase [109]. ZPR2 functions as an activator of leaf initiation that acts by regulating class-III homeodomain-leucine zippers (HD-ZIP III), which are necessary to initiate new primordia from SAM. Thus, hypoxic conditions prevailing in the SAM stabilize ZPR2 and regulate SAM activity [109].

Another important plant process regulated by overoxidation of Cys and the N-end rule pathway is vernalization. Recombinant AtPCO1-5 can catalyze VERNALIZATION 2 (VRN2) oxidation to sulfenic acid on its N-terminal Cys² residue in vitro [111], leading to its destabilization and degradation. VRN2 is a major regulator of vernalization in *Arabidopsis* [112]. It is constitutively expressed and stays enclosed in the meristems in aerobic conditions and warm temperature [111]. Low temperatures reduce O₂ diffusion and therefore its availability for both respiration and the enzymatic activity of PCOs. Cold also inhibits PCO activity and VRN2 oxidation [111]. These conditions lead to VRN2 stabilization and its subsequent accumulation in plant organs in response to hypoxia and long-term cold exposure [111]. PCOs therefore contribute to stress response signaling during hypoxia and cold exposure. Hence, the regulation of transcription factor stability by catalyzed overoxidation provides an example of how, despite being usually associated to oxidative distress, S-sulfinylation can play essential roles in oxidative eustress.

4. Disulfide Bridge Formation

Two protein Cys residues can form a covalent bond called S-S (Figure 3). The importance of S-S as a key element in protein structure and function has long been recognized. A number of kinetically distinct pathways involving inter- or intramolecular reactions can lead to their generation [113]. These include (i) a two-electron Cys oxidation; (ii) a one-electron Cys oxidation involving the formation of a thiy radical and (iii) a thiol disulfide exchange with a preformed S-S. In order for such

covalent bonding to occur, the two intervening Cys residues must come in close proximity (within a few nm) [114]. Once formed, the S-S is a stable covalent bond between two sulfur atoms. Interestingly, the distance between the sulfur atoms is significantly lower for stable structural disulfides compared to reversible, usually regulatory ones (respectively 2.05 Å *vs* 2.18 Å) [115]. The generation of the S-S can be facilitated by thiolation of one of the Cys residues [28,113]. It can also be promoted by S-glutathionylation of one Cys, which can promote S-S formation with an adjacent Cys [116] or by S-nitrosation of an adjacent Cys (see S-glutathionylation and S-nitrosation sections below). As well, an oxidative environment, such as the conditions prevailing in the lumen of the eukaryotic endoplasmic reticulum (ER), favors S-S formation and proper protein folding [113,117]. Pathways and mechanisms of oxidative protein folding have recently been thoroughly reviewed for plants [118]. In the ER lumen, oxidative protein folding of nascent proteins is catalyzed by protein disulfide isomerase (PDI). This process consists in a disulfide relay system in which PDI is first oxidized by an oxidized ER oxidoreductin (ERO) [119]. ERO uses O₂ as an electron acceptor, producing H₂O₂ in the process. Reduced PDI can also catalyze the reduction of S-S on misfolded proteins (Figure 3A) [120].

S-S notably contribute to maintain protein structure and stability [15], but also serve vital regulatory purposes [121]. An important aspect of the latter function is the dynamic and reversible nature of S-S formation. Disulfide stability in proteins varies depending on a few parameters, one of which being the dihedral angle of the bond [28]. This feature is influenced by the tertiary structure of the protein. The most stable angle is found at 90°, and there is increasing strain on bonding as the angle diverges from this value [28]. However, the most important impact on disulfides in proteins is due to redox mechanisms responsible for S-S formation by oxidation and their elimination by reduction (Figure 3A). Since the early works on plant enzyme redox modifications, it has been recognized that S-S reduction is mainly catalyzed by thioredoxins (TRXs) [122]. The *Arabidopsis* genome contains 41 TRX genes [123]. The plant TRX system and its role in metabolism and signaling has been a major area of research and the subject of several extensive reviews in recent years, therefore the reader is directed to these resources for a more complete overview of the topic [124–127]. In addition to TRXs, there is now evidence that GRXs can reduce protein S-S [128]. Several reaction mechanisms, all using GSH as reductant, have been proposed [128].

4.1. Disulfide Bridge Reduction is an Important Regulatory Mechanism that Links Light Harvesting and CO₂ Fixation in the Chloroplast

Redox regulation of metabolic enzymes by reversible S-S generation provides sensing of environmental conditions and is especially important in the chloroplast stroma (Figure 3B). This compartment contains the assimilatory enzymes of the Calvin-Benson-Bassham (CBB) cycle, as well as enzymes of the glycolysis and the pentose phosphate pathway (PPP), which use the products of photosynthesis. Plants thus need a strict control over these enzymes in order to quickly tune CO₂ fixation to changes in excitation pressure and to the light/dark cycle [129,130]. Four CBB cycle enzymes are redox-regulated through reversible S-S formation: phosphoribulokinase (PRK) [131,132], heterotetrameric GAPDH [133], fructose-1,6-bisphosphatase (FBPase) [134], and sedoheptulose-1,7-bisphosphatase [135]. In all cases, enzyme activity is inhibited by S-S formation in dark conditions. In addition, homotetrameric GAPDH and PRK are also regulated by interaction with the redox-sensitive CP12 scaffold protein [136]. In this instance, S-S formation on CP12 serves to initiate an interaction between GAPDH bound to NAD⁺ and CP12, and results in a small decrease in GAPDH activity [137]. The formation of the a ternary PRK/GAPDH/CP12 complex is then possible with oxidized PRK, resulting in a much larger decrease in activity for both enzymes [137]. Upon illumination, the chloroplast electron transport chain provides electrons for the subsequent reduction of ferredoxin (Fd) used by Fd-dependent TRX reductase (FTR) to reduce TRX [126,138]. Through a disulfide exchange mechanism, the TRX system mediates the reduction of S-S on target enzymes [126]. A comparative study of the reduction of different targets by the FTR/TRX system suggests that the final electron transfer from TRX to the target enzyme is a rate limiting step in this redox regulatory process [139]. As noted above, CBB- cycle enzymes are activated by S-S reduction. In contrast, the first enzyme in the PPP, plastidic glucose-6-phosphate dehydrogenase is active upon formation of a

S-S between Cys¹⁴⁹ and Cys¹⁵⁷ [140]. This oxidation promotes a change in conformation that improves enzyme efficiency. Conversely, the reduction of S-S mediated by reduced TRX *f1* deactivates the enzyme.

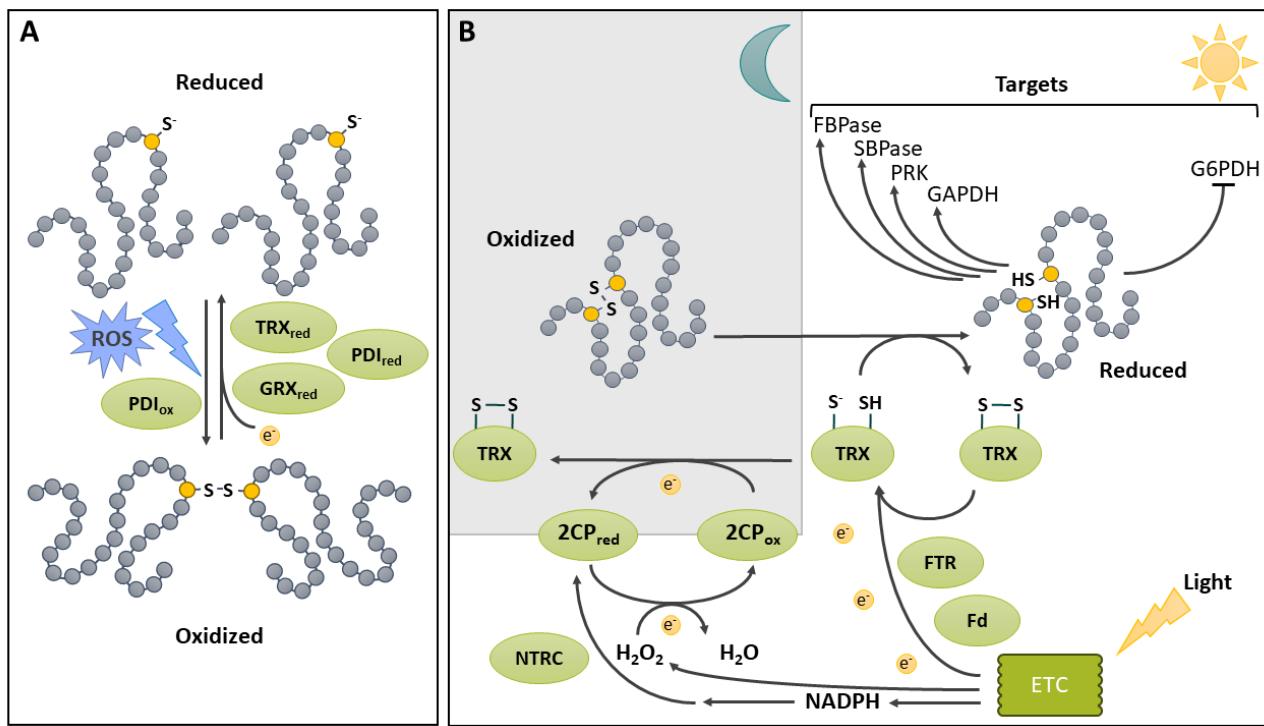


Figure 3. Reversible disulfide bridge formation in plants. A: Reversible formation of disulfide bridge (S-S). Oxidative conditions promoted by ROS and oxidized protein disulfide isomerase (PDI_{ox}) in the endoplasmic reticulum favour S-S formation between two adjacent Cys (yellow dots). Thioredoxin (TRX) and glutaredoxins (GRX) reduce S-S, concomitantly with consumption of reducing power (e⁻). In the ER, reduced PDI (PDI_{red}) can reduce S-S on misfolded proteins. B: Importance of disulfide bridge formation for metabolic regulation in the light and the dark. In the light (white background), the photosynthetic electron transport chain (ETC) generates reducing power for the reduction of thioredoxins (TRX) via ferredoxin (Fd) and ferredoxin thioredoxin reductase (FTR). Reduced TRX reduces disulfide bridges on targets in the chloroplast stroma. Activated targets (pointed arrow) include enzymes of the Calvin-Benson-Bassham (CBB) cycle: fructose-1,6-bisphosphatase (FBPase); sedoheptulose-1,7-bisphosphatase (SBPase), phosphoribulo kinase (PRK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The oxidative pentose phosphate pathway enzyme glucose-6-phosphate dehydrogenase (G6PDH) is inactivated (blunted arrow). At the onset of dark conditions (grey background), targets become oxidized (S-S). In these conditions, reduced TRX becomes inactive by transferring electrons to 2-Cys peroxiredoxin (2CP), thereby terminating activation of CBB cycle enzymes. Note that, in the light, 2CP detoxifies H₂O₂ generated at the level of ETC and is maintained reduced by NADP-thioredoxin reductase C (NTRC) using photosynthetic NADPH. Subscripts red and ox respectively symbolize reduced and oxidized state. See text for additional details.

4.2. Disulfide Bridge Formation in the Chloroplast under Dark Conditions

As illustrated above, the reduction of chloroplast enzymes upon illumination is relatively well characterized. However, oxidation mechanisms upon a switch to dark conditions remain poorly understood. Nonetheless, recent studies are providing information that help identification of players responsible for oxidation of reduced targets in the dark (Figure 3B). As seen above, chloroplast redox regulation is highly dependent on TRXs, which must be reduced to catalyze target disulfide reduction in the light. In chloroplasts, there is a second redox pathway, which uses NADPH-dependent TRX reductase C (NTRC). NTRC is implicated in the antioxidant capacity of the chloroplast by reducing 2-Cys peroxiredoxins (2CPs), which gets oxidized while scavenging H₂O₂ [141]. 2CPs can also be

reduced, although less efficiently, by other plastidic TRX [142]. In the *Arabidopsis ntrc* mutant, reduction of 2CPs is therefore mediated by the Fd/FTR/TRX system, which causes a depletion in reduced TRX, indirectly affecting the regulation of TRX targets [142]. Decreasing the level of 2CPs in the *ntrc* mutant background enabled recovery of the WT phenotype, indicating the important role of NTRC in chloroplast redox homeostasis by regulating 2CPs [142]. Furthermore, a study using genetically encoded redox probes provided further support for a key role of 2CPs in the oxidative inhibition of CBB cycle function [143]. Thus, the Fd/FTR/TRX system for regulation of CBB cycle enzymes and the NTRC/2CPs for H₂O₂ detoxification are linked by the redox status of 2CPs [142]. By draining electrons from TRXs, 2CPs allow fast oxidation of TRXs in the dark enabling inactivation of CBC enzymes within 15 min of darkness [144].

5. S-Glutathionylation

Glutathione synthesis and degradation in plants was recently extensively reviewed [47]. Briefly, the Glu-Cys ligase (GSH1) conjugates the γ -carboxyl group of Glu and the amino group of Cys. Glutathione synthase (GSH2) then uses the resulting γ -glutamylcysteine and Gly to produce glutathione. GSH2 activity is present in the cytosol and the plastid whereas the step catalyzed by GSH1 is solely localized in the chloroplast and redox regulated [47]. Thus, glutathione synthesis is linked to the plastid redox state. This sensitivity to redox is mediated by the formation of intramolecular S-S between Cys¹⁷⁸ and Cys³⁹⁸, which activates *Arabidopsis* GSH1 and has been proposed to act as a redox switch for glutathione synthesis [145].

Glutathione is usually present in mM concentrations in plants, mostly in its monomeric reduced form (GSH) [47]. Oxidative stress promotes the accumulation of its dimeric oxidized form (GSSG). GSSG can be recycled to its reduced form by glutathione reductase (GR), using the reducing power of NADPH [47]. GSH is involved in the cellular redox buffer and the provision of electrons to the Foyer-Halliwell-Asada cycle during H₂O₂ detoxification [48]. The value of the GSH/GSSG ratio is therefore linked to the removal of H₂O₂. In absence of stress, this ratio is normally very high [48]. The maintenance of an appropriate GSH/GSSG ratio is dependent on GRs. Indeed, lack of this activity in the cytosol or organellar compartments leads to accumulation of GSSG that can be documented using genetically encoded redox sensors [146,147].

A low cellular GSH/GSSG ratio promotes Cys S-glutathionylation [48,51]. This formation of a mixed S-S between glutathione and an accessible protein Cys residue can occur spontaneously [6]. However, with a pK_a of 8.8, GSH is highly protonated and thus weakly reactive in the physiological pH range, specially towards thiols [148]. Thus, the S-glutathionylation reaction (Figure 4A) can involve GSH and a sulfenic acid, or result from a disulfide exchange between GSSG and a thiolate residue [6,148,149]. Nitrosoglutathione (GSNO) has also been shown to act as a mediator of protein S-glutathionylation [150]. When tested as a S-glutathionylation agent, GSNO was differently effective on various targets [150]. In animals, S-glutathionylation appears to be at least partially catalyzed. A study on human glyoxalase II revealed that this enzyme could mediate in vitro S-glutathionylation of specific targets [151]. The involvement of glyoxalase II in S-glutathionylation has not been explored in plants so far. In addition, animal glutathione S-transferase Pi (GST Pi) also promotes protein S-glutathionylation in vivo and in vitro [152]. In contrast to animals, plants lack GST Pi [153]. There is nevertheless a study that has documented the catalysis of S-glutathionylation in plants. In this research, plant GRXC2 stimulated S-glutathionylation of the Leu-rich receptor Ser/Thr protein kinase BAK1 using GSSG as substrate [154]. By this means, GRXC2 inhibited BAK1 kinase activity [154]. This mechanism could potentially allow a redox regulation of the brassinosteroid signaling pathway, in which BAK1 is active [154,155]. However, the in vivo significance of BAK1 S-glutathionylation remains to be established. There are several examples of a regulatory role of protein S-glutathionylation in glycolytic and respiratory metabolism, as reviewed lately [6].

S-glutathionylation is fully reversible (Figure 4A). *In vitro*, strong reductants such as DTT are commonly used to induce non-enzymatic protein deglutathionylation [66,156]. *In vivo*, reducing conditions such as high GSH/GSSG ratio promote removal of glutathione (deglutathionylation) [157]. A study on human PDIs showed a limited capacity for deglutathionylation in vitro [158], however,

this has not been explored in plants. Some evidence for protein deglutathionylation by cyanide has also been provided in mammalian cells [159] but, so far, this possibility does not appear to have been reported in plants. Deglutathionylation is most likely catalyzed by GRXs *in vivo* [48,160,161]. Plant genomes encode a large GRX gene family, from approximatively 30 genes in *Arabidopsis*, rice and poplar to 85 in wheat [162,163]. Two catalytic mechanisms have been described for the removal of the glutathione moiety on proteins by GRXs [160]. The reduction of the mixed S-S between glutathione and a protein first involves a nucleophilic attack of the modified Cys by a thiolated GRX Cys active site. In the monothiol mechanism, the resulting *S*-glutathionylated GRX is subsequently reduced by GSH, generating GSSG in the process. In the dithiol mechanism, a second Cys attacks the mixed S-S between the GRX and the glutathione, resulting in the formation of a S-S between the two Cys of the GRX and the liberation of GSH. A later reduction of the S-S on the GRX allows it to become active for a new catalytic cycle. In addition to GRXs, TRXs have also been implicated in the deglutathionylation of plant proteins *in vitro* [66,161]. TRXs and GRXs are related proteins involved in thiol-disulfide exchange [164]. Their substrate specificity is considered to be broad and they may exhibit some limited overlap. The precise determinants of GRX and TRX substrate specificity remain poorly understood. Nevertheless, a recent modeling study has shown that electrostatic complementarity could play an important role in determining interactions between the different redoxins isoforms and their interaction partners [165]. Studies conducted *in vitro* on two *Arabidopsis* cytosolic GAPDH isoforms show that TRX can catalyze deglutathionylation of GAPDH in a GSH independent manner, although less efficiently than GRX [66].

5.1. *S*-Glutathionylation as a Means of Protecting Metabolic Enzymes against Irreversible Oxidation

Because of its reversibility, and the fact that deglutathionylation restores an intact thiol, *S*-glutathionylation has long been recognized as a means to protect protein Cys against irreversible oxidation of thiols due to *S*-sulfonylation (Figure 4A) [149,166]. This protective function has been documented for plant metabolic enzymes. Between a few tens and a few hundreds of proteins have been identified as *S*-glutathionylation targets in various plant proteomic surveys [167,168] [169–171]. Among these, metabolic enzymes are usually abundantly represented. An example of *S*-glutathionylation serving as a protective mechanism against irreversible oxidation comes from a study of ascorbate peroxidases (APXs) in the red alga *Galdieria partita* and in tobacco [172]. APX is responsible for H₂O₂ detoxification in the Foyer-Halliwell-Asada cycle, but may become inactive in absence of ascorbate due to irreversibly oxidized Cys residues [172]. *S*-glutathionylation of several APX Cys residues was demonstrated *in vitro* in the presence of H₂O₂ and GSH, and a protective role of *S*-glutathionylation under oxidative stress was suggested to occur *in vivo* [172]. *S*-glutathionylation was also shown to protect *Arabidopsis* chloroplastic α -amylase 3 (AMY3) activity from overoxidation *in vitro* [161]. It is thought that this mechanism could allow the recovery of AMY3 function (stress-induced starch degradation) after exposure to oxidative conditions generated under stress [161]. Excess H₂O₂ can also cause the irreversible inactivation of GAPDH, a key glycolytic enzyme which is also involved in signaling. This enzyme possesses a catalytic Cys that is highly sensitive to inactivation by H₂O₂. In this case also, *S*-glutathionylation was shown to mitigate the effects of oxidative distress *in vitro* [66].

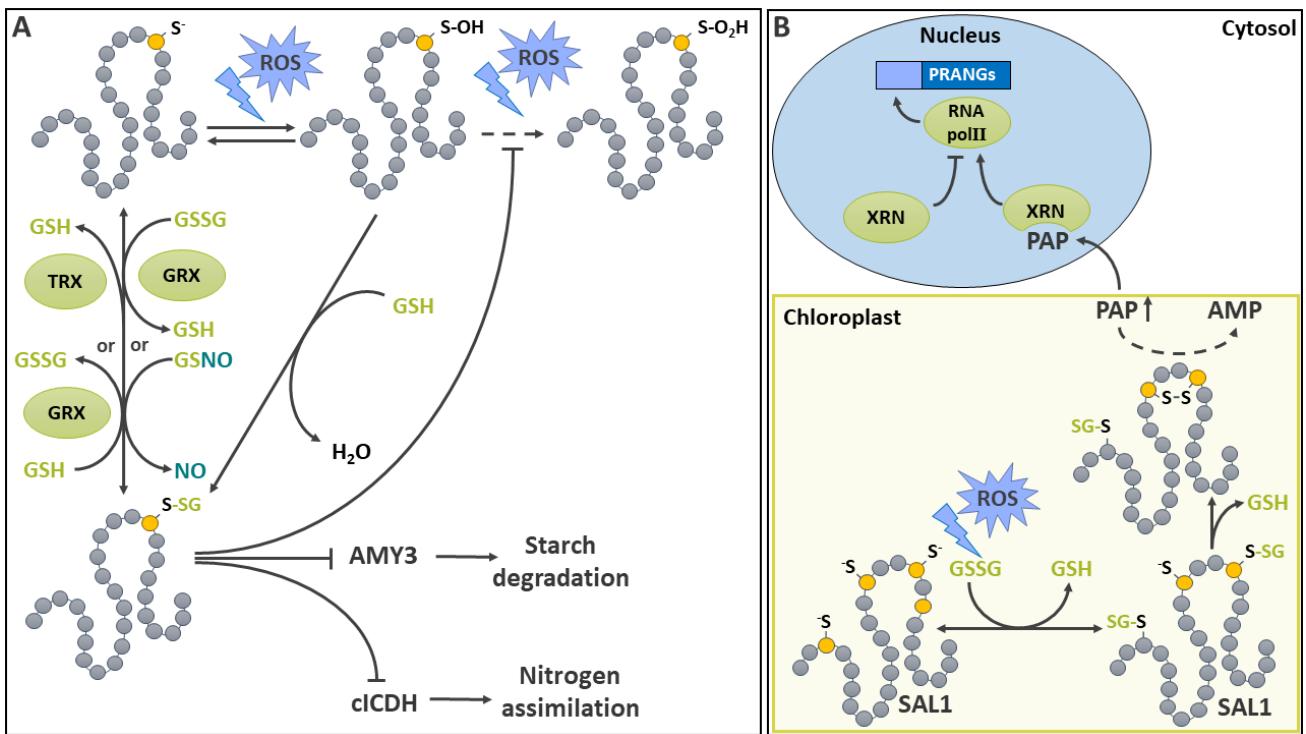


Figure 4. Protein S-glutathionylation in plants. A: Mechanisms of S-glutathionylation/deglutathionylation and regulation by S-glutathionylation. S-glutathionylation (S-SG) occurs on deprotonated (S) Cys (yellow dot) in a glutaredoxin (GRX) catalyzed reaction with oxidized glutathione (GSSG), or spontaneously in a reaction involving nitrosoglutathione (GSNO). It can also occur spontaneously between a sulfenylated Cys (S-OH) and reduced glutathione (GSH). Deglutathionylation is catalyzed by GSH-dependent GRX or by thioredoxin (TRX). S-glutathionylation of a Cys residue protects the protein from ROS-dependent oxidation towards the S-sulfinylated form (-SO₂H, top of the panel). S-glutathionylation protects amylase 3 (AMY3) from overoxidation and inhibits its activity towards starch degradation. AMY3 activity is recovered after stress. It also has an inhibitory effect on cytosolic NADP-dependent isocitrate dehydrogenase (cICDH), which is active in nitrogen assimilation. B: Involvement of S-glutathionylation of SAL1 in expression of plastid redox-associated nuclear genes. SAL1 is a 3'-phosphoadenosine 5'-phosphate (PAP) phosphatase that controls the degradation of PAP to AMP in the chloroplast. In oxidative stress conditions, S-glutathionylation of SAL1 induces the formation of an intramolecular disulfide bridge (S-S) which participates in increasing PAP levels. According to a model, PAP is exported to the nucleus, where it binds 5'-3' exoribonucleases (XRN) involved in inhibition of RNA polymerase II (RNA polII) transcription of plastid redox-associated nuclear genes (PRANGs). PAP binding to XRN relieves inhibition of RNA polII, allowing PRANGs transcription. See text for additional details.

5.2. Metabolic Enzymes Targeted by Regulatory S-Glutathionylation under Oxidative Conditions

There are numerous examples of S-glutathionylated enzymes in plant primary metabolism. As seen above, there are multiple cases of redox regulation in chloroplast metabolism. Enzyme sensitivity to reducing power in the organelle is also mediated by S-glutathionylation [51]. An analysis of the conservation of S-glutathionylated sites in chloroplast proteins has provided evidence for evolutionary conservation for some target proteins [171]. This indicates an ancient origin for the implication of S-glutathionylation in chloroplastic stress response. As an example, S-glutathionylation of at least three chloroplastic AMY3 Cys residues has been described in *Arabidopsis* (Figure 4A) [161]. Among these, Cys⁴⁹⁹ and Cys⁵⁸⁷ were previously shown to be involved in a regulatory S-S reversible by TRX, in a process similar to redox-modified CBB cycle enzymes [173]. In a proposed model, S-glutathionylation of one Cys in the pair led to the formation of the S-S, resulting in spontaneous deglutathionylation of the other [161]. AMY3 deglutathionylation and S-S reduction was respectively promoted by GRX and TRX [161].

Implications of reversible *S*-glutathionylation in the regulation of glycolytic and respiratory metabolism was reviewed a short while ago [6]. More recently, the cytosolic NADP-dependent isocitrate dehydrogenase (cICDH) was shown to be subject to regulatory *S*-glutathionylation (Figure 4A) in a study that provides an example of GSNO as *S*-glutathionylation agent [174]. By generating 2-oxoglutarate used as a carbon skeleton in N assimilation, cICDH plays a key function at the interface between C and N metabolisms [175]. The sensitivity of cICDH was demonstrated by decreases in extractable cICDH activity from leaves of the *Arabidopsis* mutants impacted in H₂O₂ detoxification or GSSG reduction [174]. Furthermore, *in vitro* cICDH activity was inhibited in the presence of GSSG, GSNO, or treatment with H₂O₂ plus GSH [174]. In these assays, GSNO appeared to be particularly effective. Detailed analyses revealed that GSNO induced *S*-glutathionylation of cICDH on Cys³⁶³. Following treatments with GSNO, there was also evidence for *S*-nitrosation of the protein, although the targeted Cys residue(s) could not be identified. ICDH activity could be restored by GRXC1 and GRXC2 and, less efficiently, by TRXs [174].

5.3. Involvement of *S*-Glutathionylation in Signaling

3'-phosphoadenosine 5'-phosphate (PAP) is a product of sulfotransferases [176,177]. In some instances, PAP has been described as a potent retro-inhibitor for these enzymes [178]. It is also a product and an inhibitor of the stromal acyl carrier protein synthase [179]. PAP also partakes in retrograde signaling between the chloroplast and the nucleus, as it regulates Plastid Redox Associated Nuclear Genes (PRANGs) [180]. PAP is degraded to AMP by the chloroplastic PAP phosphatase SAL1 [181,182]. Under normal conditions, low levels of PAP are therefore controlled by SAL1 activity in a process that involves *S*-glutathionylation (Figure 4B). Under stress-induced oxidative conditions, *Arabidopsis* SAL1 activity decreases in conjunction with its dimerization, *S*-glutathionylation and the formation of an intramolecular S-S between evolutionary conserved Cys¹⁶⁷ and Cys¹⁹⁰ [183]. Treatment of SAL1 with GSSG *in vitro* promoted *S*-glutathionylation of Cys¹¹⁹ and Cys¹⁹⁰ and down-regulated monomeric and dimeric SAL1 activity. These experiments also revealed the existence of a mechanism in which a prior Cys *S*-glutathionylation induced the formation of a S-S between Cys¹⁶⁷ and Cys¹⁹⁰ by means of a thiol disulfide exchange, leading to the down regulation of SAL1 [183]. This inhibition of SAL1 leads in turn to an increased steady state level of PAP which acts as a chloroplast-to-nucleus retrograde signal [181]. Later studies have led to the development of a model where PAP accumulation allows it to bind and inhibit 5'-3' exoribonucleases involved in PRANGs expression by degradation of uncapped RNAs, interference with RNA polymerase II function and/or silencing [182].

6. Protein Persulfidation

Hydrogen sulfide (H₂S) is an important intermediate in the plant sulfate assimilatory pathway [184] as well as a gaseous pollutant that can be absorbed by plants [184–186]. It has recently emerged as a signaling molecule in plants [35,187,188]. The modification of a protein thiol by H₂S is called persulfidation. In the literature, it is also sometimes referred to as protein persulfuration or protein sulfhydration. However, this latter terminology is considered incorrect since no hydration reaction is involved [54]. This PTM allows H₂S-based signal transduction. The production of H₂S occurs in several subcellular compartments. During sulfate assimilation, it is generated in plastids by sulfite reductase [184] and transferred on O-acetylserine (OAS) by OAS(thiol)lyases (OSTLs) to generate Cys (Figure 5A) [184,189,190]. Apart from the sulfur assimilation pathway, the production of intracellular H₂S by several other plant pathways has been recently reviewed [189,190]. These mainly involve the catabolism of Cys by L-Cys desulfhydrases (LCD), D-Cys desulfhydrases (DCD) and the cytosolic OSTL homolog DES1 (Figure 5A). LCDs and DCDs produce H₂S, ammonia and pyruvate in the cytosol while DES1 breaks down Cys into H₂S and OAS. Other enzymes are involved, such as the mitochondrial β -cyanoalanine synthase (β CS), which catalyzes the conversion of Cys and hydrogen cyanide (HCN) to H₂S and β -cyanoalanine. In addition, a small family of 3-mercaptopyruvate sulfurtransferases (MSTs) characterized in *Arabidopsis* [191] also generate H₂S using 3-mercaptopyruvate as sulfur donor and reduced TRX or GRX as electron donors (Figure 5A).

Furthermore, upon reaction with reduced Cys or GSH, they respectively generate Cys persulfide (Cys-SSH) and glutathione persulfide (GSSH) [191].

6.1. Addition of Sulfide on Cys Results from Direct Persulfidation or Transpersulfidation

Several mechanisms can lead to the spontaneous persulfidation of Cys (Figure 5A). In aqueous solution, H₂S dissociates in hydrosulfide (HS⁻, pK_a = 7.0 at 25°) and sulfide anions (S²⁻, pK_a = 17-19 at 25°C) [54]. Thus, even if we use the term H₂S in this review, the more nucleophilic HS⁻ is probably the most abundant form at physiological pH [54]. HS⁻ can perform a nucleophilic attack on an oxidized thiol, such as a sulfenic acid or a disulfide, but cannot react with a reduced thiol [192]. Other means of protein persulfidation have been discussed [54], such as those involving radical sulfur species (RSSH^{•-}) or inorganic polysulfide (-S-S_n-S-) however, the relevance of these reactions in plants still remains to be clearly established. Experimentally, p-methoxyphenyl(morpholino)phosphinodithioic acid (GYY4137) and NaHS are used as sulfide donors for protein modification and physiological studies [185,193-195].

In *Arabidopsis*, the ability of MSTs to catalyze a transpersulfidation reaction (transfer of a sulfide from one protein to another, Figure 5A) has been demonstrated in vitro using roGFP2 as a model protein substrate [191]. In this reaction, MST becomes persulfidated on its catalytic Cys following interaction with its substrate 3-mercaptopyruvate and in absence of TRX or GRX. The transfer of sulfide occurs from MST to a thiolate residue on roGFP2. Following a rearrangement, the persulfidated Cys forms a disulfide bridge with a nearby thiol, resulting in the generation of H₂S [191]. Interestingly, the catalytic Cys of MST is also subject to inhibitory oxidation by H₂O₂. The persulfidation of this residue also has a protective role against irreversible oxidation of the MST [191].

Cys modification by persulfidation is reversible in vitro with artificial reducing agents such as DTT and tris(2-carboxyethyl)phosphine (TCEP) [194,196]. In animals, redoxins can reduce protein persulfides and their levels are controlled by the thioredoxin system [54,197]. It is quite possible that this process also takes place in plants, however, it remains to be formally demonstrated.

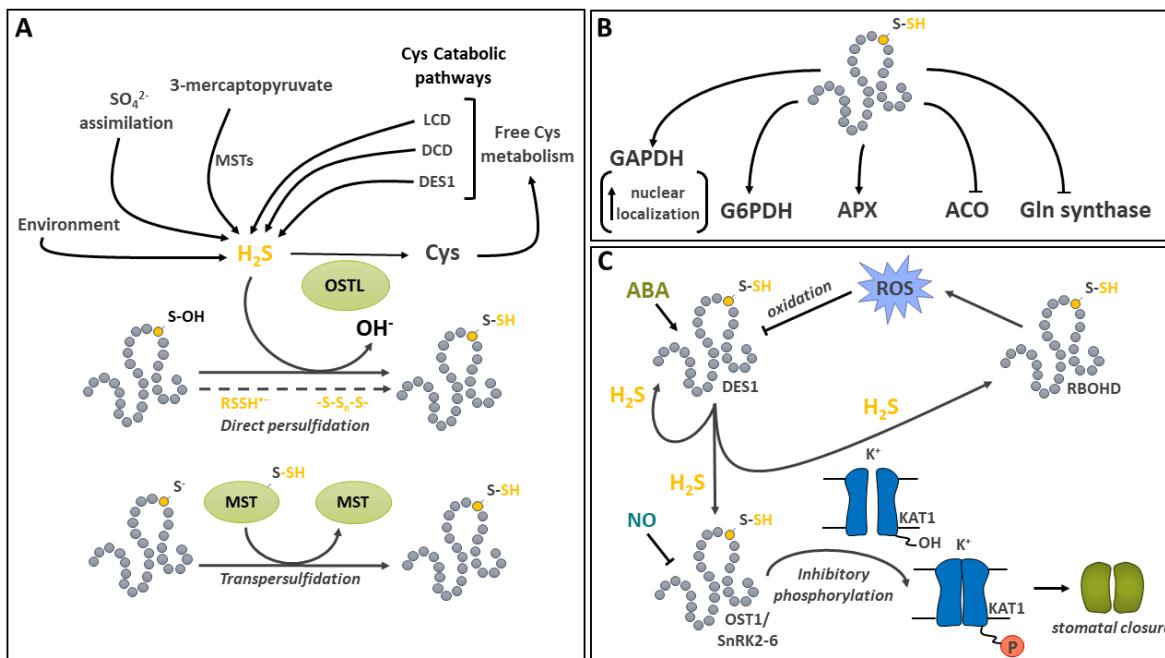


Figure 5. Protein persulfidation in plants. A: Pathways controlling cellular H₂S together with mechanisms of persulfidation. In the top of the panel, pathways that promote cellular H₂S levels are represented. These include uptake from the environment, SO₄²⁻ assimilation, 3-mercaptopyruvate sulfurtransferases (MSTs), and the catabolism of free Cys by L-Cys desulphydrases (LCD), D-Cys desulphydrases (DCD) and the cytosolic O-acetylserine (thiol)lyase (OSTL) homolog DES1, while OSTL consumes H₂S for Cys synthesis. Note that H₂S is used, although HS⁻ is probably present in physiological conditions. The bottom of the panel represents the two pathways implicated in protein

persulfidation. The direct persulfidation pathway modifies Cys residues (yellow dots) in the S-sulfenylated form (S-SH) using free H₂S. Other mechanisms of direct persulfidation (dotted arrow) do not have strong experimental support. The transpersulfidation pathway allows the transfer of a persulfide group from MST to a thiolated Cys (S). B: Metabolic targets of persulfidation in plants. The effect of persulfidation is represented (pointed arrow: increased activity, blunt arrow: inhibition). Some target names are abbreviated: ACO, aminocyclopropane-carboxylic acid oxidase; APX: cytosolic ascorbate peroxidase; G6PDH, glucose-6-phosphate dehydrogenase; GAPDH: cytosolic glyceraldehyde 3P dehydrogenase. Increased nuclear localization has also been reported as a result of GAPDH persulfidation. C: Involvement of persulfidation in signal transduction. H₂S serves to activate DES1 by persulfidation. Upon abscisic acid (ABA) stimulation, DES1-derived H₂S modifies Open Stomata 1/Sucrose nonfermenting 1-RELATED PROTEIN KINASE2.6 (OST1/SnRK2-6), which is inhibited by NO signaling and responsible for the inhibitory phosphorylation of the KAT1 potassium channel in the guard cell membrane. Phosphorylation of KAT1 inhibits potassium transport, leading to stomatal closure. In a regulatory negative feedback loop, modification of Respiratory Burst Oxidase Homolog D (RBOHD) by H₂S activates it, leading to ROS formation and inhibition of DES1. See text for additional details.

6.2. Metabolic Targets of Cys Persulfidation

Over the past decade, high-throughput proteomic methods have been developed and used to survey the extent of protein persulfidation in plants [198–201]. Based on a biotin tag switch method, a large number of persulfidated proteins have been identified in *Arabidopsis* showing widespread occurrence of this PTM in leaves and roots as well as its regulation by environmental conditions [199,200]. Comparison of various proteomic studies revealed that persulfidation seems far more abundant in *Arabidopsis* than S-nitrosylation or S-glutathionylation [202].

Persulfidation has been implicated in the regulation of key enzymes in the metabolic pathways such as Gln synthetase in N assimilation, G6PDH in the OPPP, GAPDH in glycolysis, cytosolic ascorbate peroxidase (APX) in the ascorbate-glutathione cycle and aminocyclopropane-carboxylic acid oxidase (ACO) in ethylene synthesis (Figure 5B) [194,203]. In a study of APX, in vitro activity was shown to be modestly stimulated by persulfidation, whereas Gln synthetase was inhibited by a treatment with NaHS at nM concentration [194]. For ACO, incubation of recombinant protein with NaHS led to the reversible inhibition by persulfidation of Cys⁶⁰, and treatments of *Solanum lycopersicum* (tomato) plants with the H₂S donor further supported in vivo enzyme activity inhibition by persulfidation [203]. In a recent study on *Arabidopsis* and tomato G6PDHs, persulfidation was shown to play an important role in the regulation of the activity of this enzyme, which catalyzes C entry in the OPPP [204]. G6PDH modification was detected on Cys¹⁵⁵ in *Arabidopsis* G6PDH6 and Cys¹⁵⁹ in tomato G6PDHC. The same residues were also shown to be sensitive to oxidation by H₂O₂. G6PDHs are structurally relatively well conserved between plants and animals. The fact that the persulfide-modified residues are only found in cytosolic isoforms suggests a plant- and isoform-specific modification [204]. In vitro and in vivo treatments with NaHS inducing persulfidation of G6PDH6 and G6PDHC enhanced enzyme activity. This effect was reversed in the presence of DTT. Further analyses indicated that G6PDH persulfidation increased affinity of NADP used as substrate and promoted enzyme oligomerization towards the formation of tetramers. In addition, exposure of *Arabidopsis* seedlings to salt stress caused an oxidation of Cys¹⁵⁵ and resulted in decreased enzyme activity. However, in the presence of NaSH, a competition between oxidation and persulfidation occurred, highlighting the potential of Cys¹⁵⁵ persulfidation in the protection of G6PDH activity under oxidative conditions.

Persulfidation of *Arabidopsis* cytosolic GAPDH C1 was shown to reversibly increase enzyme activity in vitro [194]. Proteomic surveys have shown that cytosolic and chloroplastic GAPDH isoforms are modified in vivo by persulfidation [199]. The relative localization to the nucleus and the cytoplasm for GAPDH isoforms C1 and C2 was compared in WT and *des1* mutant of *Arabidopsis* [205]. Decreased localization to the nucleus was reported in the *des1* background whereas treatment of the mutant with NaHS increased nuclear localization. These results support the hypothesis of a preferential nuclear localization upon GAPDH persulfidation [205]. Moonlighting functions and

nuclear localization have been reported before for animal and plant GAPDHs under various stress conditions [206]. In *Arabidopsis*, persulfidation of GAPDH appears likely to promote its migration to the nucleus. However, its nuclear function still remains to be deciphered [205,206].

6.3. Cys Persulfidation Involvement in ABA-Mediated Stomatal Movement

A wide variety of signaling functions have been shown to be impacted by persulfidation in plants, including in abiotic stress tolerance [35,188,207]. In particular, it has been implicated in drought stress signaling by regulating ABA-mediated stomatal movement (Figure 5C) [208–210]. ABA regulation of guard cell function implicates a complex network of signals comprising protein kinases, H₂O₂, NO and H₂S-regulated steps [208,211]. Initial investigations showed that in the guard cell, ABA induces a production of ROS which was linked to the activity of NADPH oxidase RBOHD and F [212]. The synthetic H₂S donor GYY4137 was then shown to inhibit the activity of *Nicotiana tabacum* inward-rectifying K⁺ channel [193]. Among the protein kinases involved in ABA signal transduction, Open Stomata 1 /Sucrose nonfermenting 1-RELATED PROTEIN KINASE2.6 (OST1/SnRK2.6) is responsible for mediating the phosphorylation of Thr³⁰⁶ on the inward K⁺ channel KAT1, thereby reducing K⁺ uptake by the guard cell and promoting stomatal closure in *Arabidopsis* [213]. Recently, it has been demonstrated that ABA signaling induces the persulfidation of Cys⁴⁴ and Cys²⁰⁵ on DES1, which is recognized as a major source of H₂S in the cytosol [209]. This PTM leads to the enhancement of DES1 activity in an autoactivating mechanism. The production of H₂S by DES1 leads to the persulfidation of RBOHD on Cys⁸²⁵ and Cys⁸⁹⁰, thereby stimulating its activity and promoting the production of ROS [209]. In turn, oxidation of the persulfidated Cys residues on DES1 due to the rise of ROS provides a negative feedback mechanism leading to a decrease in DES1 activity [209]. DES1 mediated H₂S production in guard cells also contributes to the mediation of ABA signaling by promoting the persulfidation of OST1/SnRK2.6 on Cys¹³¹ and Cys¹³⁷ [208]. Interestingly, persulfidation of this key protein kinase increases its activity [208] while S-nitrosylation on Cys¹³⁷, which is close to the catalytic site, is inhibitory [214]. This complex cross-talk between H₂S and NO signals acting as second messengers in various aspects of plant physiology has been recently reviewed [215]. So far, there is strong evidence that, in guard cells, the two molecules collaborate in the fine regulation of components of ABA signaling [187].

7. S-Cyanylation

S-cyanylation is a PTM resulting from the reaction of HCN on a protein Cys residue (Figure 6). HCN is a pollutant naturally present at low levels in the environment where it often is the result of human activity [216]. It is volatile and can dissociate to H⁺ and CN⁻ when dissolved in aqueous solutions ($pK_a = 9.2$). Therefore, in the physiological pH range, HCN mainly occurs in its undissociated form. HCN can be formed enzymatically or non-enzymatically in a variety of living systems, from bacteria to mammals [217–219]. It is widely recognized as a poisonous compound due to its enzyme inhibitory effects, most importantly on cytochrome c oxidase [220], although a physiological function as a gasotransmitter is also currently debated [219].

In plants, a number of pathways, many of which related to stress defense, are implicated in the generation of HCN (Figure 6). The latter is produced during the synthesis of ethylene by 1-aminocyclopropane-1-carboxylate (ACC) oxidase [221]. Therefore, biotic and abiotic stresses, as well as developmental situations that promote ethylene synthesis [222] result in HCN formation. The hydrolysis of cyanogenic glycosides by β -glycosidase is another source of HCN in a relatively large number of plant families [217,220]. This so-called cyanogenesis mechanism is thought to be an effective deterrence strategy upon wounding or attack by herbivores [220]. HCN is also formed during the synthesis of the phytoalexin camalexin [223] and in a reaction that uses glyoxylate and hydroxylamine [224]. However, the enzymatic mechanism responsible for this reaction remains elusive. Plant cellular HCN can be detoxified by β -cyanoalanine synthase (CAS) [225,226]. However, this reaction also produces HS⁻, which, at high concentration can inhibit cytochrome c oxidase [227]. In addition, as seen above, HS⁻ is also a protein Cys modifier.

In animal and plant systems, HCN has been reported to form protein adducts *in vivo* by *S*-cyanylation [228,229]. HCN nucleophilic properties allow a non-enzymatic attack of S-S, including those present in proteins [219,230,231]. As a result, the bond is broken and *S*-cyanylation most likely occurs on the Cys residue which is the farthest from an electrophilic group [230,232]. It is thought that HCN can also attack the mixed disulfide bridge between GSH and Cys, resulting in the deglutathionylation of the Cys. *In vitro*, protein *S*-cyanylation is promoted by the use of an oxidative treatment (e.g. H₂O₂), presumed to induce S-S formation [229]. Although the CN adduct can be eliminated *in vitro* [233], *S*-cyanylation is regarded as an irreversible PTM in living systems [229].

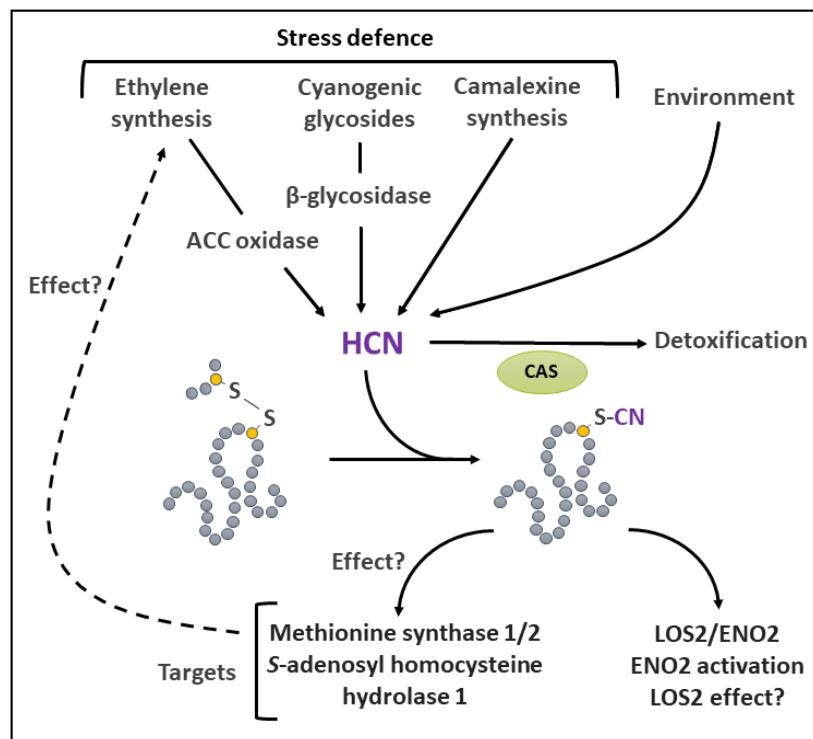


Figure 6. Protein *S*-cyanylation in plants. The main pathways involved in the generation of HCN are represented. HCN can also be taken from the environment. The reaction of β -cyanoalanine synthase (CAS) consumes HCN in plants. *S*-cyanylation (purple symbols) occurs mainly on Cys residues (yellow dots) engaged in disulfide bonds. Among the few targets of *S*-cyanylation identified in plants, the glycolytic enzyme Enolase 2 (ENO2) was shown to be activated by the modification. Other targets were identified, and some are involved in pathways leading to ethylene synthesis. As indicated by question marks, many effects of *S*-cyanylation are still largely unknown. See text for additional details.

There is still limited information available on the occurrence and the physiological relevance of protein *S*-cyanylation in plants. Nevertheless, in a ground-breaking study, feeding of an *Arabidopsis* CAS null mutant with ACC was used to increase endogenous HCN levels in order to detect *S*-cyanylated proteins [229]. This strategy allowed the identification of 163 targets. Among the modified proteins, there was an enrichment in metabolic enzymes involved in non-photosynthetic and photosynthetic carbon metabolism. One of the identified targets was Enolase 2 (ENO2), which catalyzes the penultimate step of the cytosolic glycolytic pathway. ENO2 was shown to be activated by *S*-cyanylation on Cys³⁴⁶, hinting to the possible involvement of HCN in the regulation of glycolytic flux (Figure 6). Interestingly, the locus *LOS2/ENO2*, which encodes for ENO2 also produces a truncated form of the protein, C-MYC BINDING PROTEIN1 (LOS2) which serves as a transcriptional regulator. The effect of HCN on this protein is however unknown. Among the other *S*-cyanylation targets identified in this study were Met synthase 1 and 2, as well as *S*-adenosyl-homocysteine

hydrolase 1 (Figure 6). These enzymes are involved in Met and S-adenosyl Met metabolism. Their modification by S-cyanylation could therefore impact methylation reactions, gene silencing or ethylene synthesis [229]. However, this still remains to be established. More recently, another proteomic study using the CAS null mutant confirmed that enzymes involved in Met and S-adenosyl Met metabolism are preferred targets of S-cyanylation in plant roots [234].

Obviously, much remains to be done to further understand the physiological relevance of these findings. This will require a careful examination of the function of S-cyanylation targets. In addition, future research efforts will need to consider the fact that S-cyanylation only affects oxidized Cys residues (involved in S-S or mixed disulfide bonds).

8. S-Nitrosation

Nitric oxide (NO) is a gaseous free radical that can easily diffuse across membranes. In plants, NO synthesis can be achieved through several pathways, most of which involve the reduction of nitrite [235]. Several details in plant NO production are not yet fully understood. Although sequences with homology to animal nitric oxide synthase (NOS) have been found in plants, the role of NOS in plants NO synthesis remains elusive [235]. It appears that metabolic routes involving the reduction of NO_2^- contribute more likely to the production of plant NO (Figure 7A). A reaction involving nitrate reductase (NR), which catalyzes the reduction of NO_3^- to NO_2^- , has been implicated in the process [235]. To produce NO, electrons from NR are supplied to NO-forming nitrite reductase (NOFNiR) leading to the reduction of NO_2^- to NO (Astier et al., 2018). Hence, NR mutants (e.g. *nia1* and *nia2* in *Arabidopsis*) are often used to decrease NO production *in planta* [236]. Another pathway, in which the reduction of NO_2^- to NO by the mitochondrial electron transport chain under anaerobic conditions has been documented [237]. Following its formation, NO can be scavenged by plant hemoglobins (Phytoglobins, Pgb), which are induced by O_2 deficiency. Evidence for this was initially provided by studies in transgenic *Medicago sativa* (alfalfa) roots [238] and *Zea mays* (maize) cell cultures [239] with different *Pgb* expression levels. Under O_2 deficiency conditions, NO levels were inversely correlated to Pgb expression level in both cases.

8.1. Mechanisms Involved in Protein S-Nitrosation

NO can react directly with protein Cys to cause a PTM called S-nitrosation. This denomination is used here to describe the modification of a Cys residue by NO instead of the commonly used S-nitrosylation, as the latter refers to the modification of a metal by NO [240]. NO plays an important role in plants, animals and bacteria as a second messenger and controls a wide variety of biological functions [241]. Direct protein S-nitrosation (Figure 7A) occurs via different reaction mechanisms of NO with protein Cys and has been reviewed in some detail [240,242]. These reactions are complex and probably facilitated by additional redox reactions under physiological conditions [242]. Briefly, direct S-nitrosation can be the result of (i) a thiolate reaction with the nitrosonium cation (NO^+) formed by NO oxidation by transition metals, (ii) the reaction of NO with a protein thyl radical or (iii) a Cys thiol reaction with NO, in which the participation of a second NO molecule leads to the production of a nitrosated Cys and HNO [243]. A transnitrosation reaction can also lead to S-nitrosation [241]. The latter can be associated to the denitrosation of the NO donor [244]. Although all these mechanisms promote protein S-nitrosation, it is important to note that S-nitrosation induced by different reactions (e.g. radical reaction and transnitrosation) do not necessarily have the same protein target specificity [241,245]. However, this issue is understudied in plants.

In plant tissues GSNO, acts as a stable NO reservoir for spontaneous protein S-nitrosation [246]. GSNO is recognized as the main NO donor for transnitrosation reactions (Figure 7B), although some enzymes, such as human GAPDH, may also possess a trans-nitrosase activity [247]. Mechanisms affecting cellular GSNO will therefore indirectly impact S-nitrosation. The spontaneous reaction of the glutathione thyl radical with NO has been described as a pathway leading to the formation of GSNO [247]. In plants, it is assumed that the main enzyme regulating levels of GSNO is S-nitrosoglutathione reductase (GSNOR), a cytosolic class III alcohol dehydrogenase implicated in GSNO catabolism [246,248,249]. GSNOR catalyzes the NADH-dependent reduction of GSNO to

GSSG and NH₃ [248]. This enzyme has three conserved solvent-accessible Cys that are sensitive to redox modifications. A study on *Lotus japonicus* revealed that LjGSNOR1 and LjGSNOR2 can be modestly activated by persulfidation and inhibited by S-glutathionylation [250]. However, the latter reaction was slow (several h) and required mM concentrations of GSSG. It is thus unclear if S-glutathionylation of GSNOR is relevant *in vivo*. *Arabidopsis* GSNOR is also inhibited by H₂O₂ *in vitro* and by conditions promoting oxidative stress *in vivo* [251]. Interestingly, tomato GSNOR is reversibly inhibited by S-nitrosation of solvent-accessible Cys²⁷¹ [252,253]. This inhibition has been proposed to be involved in a mechanism that would allow proper NO signaling during nitrosative burst by allowing initial GSNO accumulation [252]. More recently, enzymes belonging to the aldo-keto reductase (AKR) family were identified as NADPH dependent GSNO reductase in mammals [254] and *Arabidopsis* [255]. Two *Arabidopsis* AKR4C are significantly upregulated in a GSNOR null mutant, which displays a higher NADPH-dependent GSNO reduction than the WT, suggesting that AKR4Cs are involved in GSNO homeostasis and compensate for the loss of GSNOR [255].

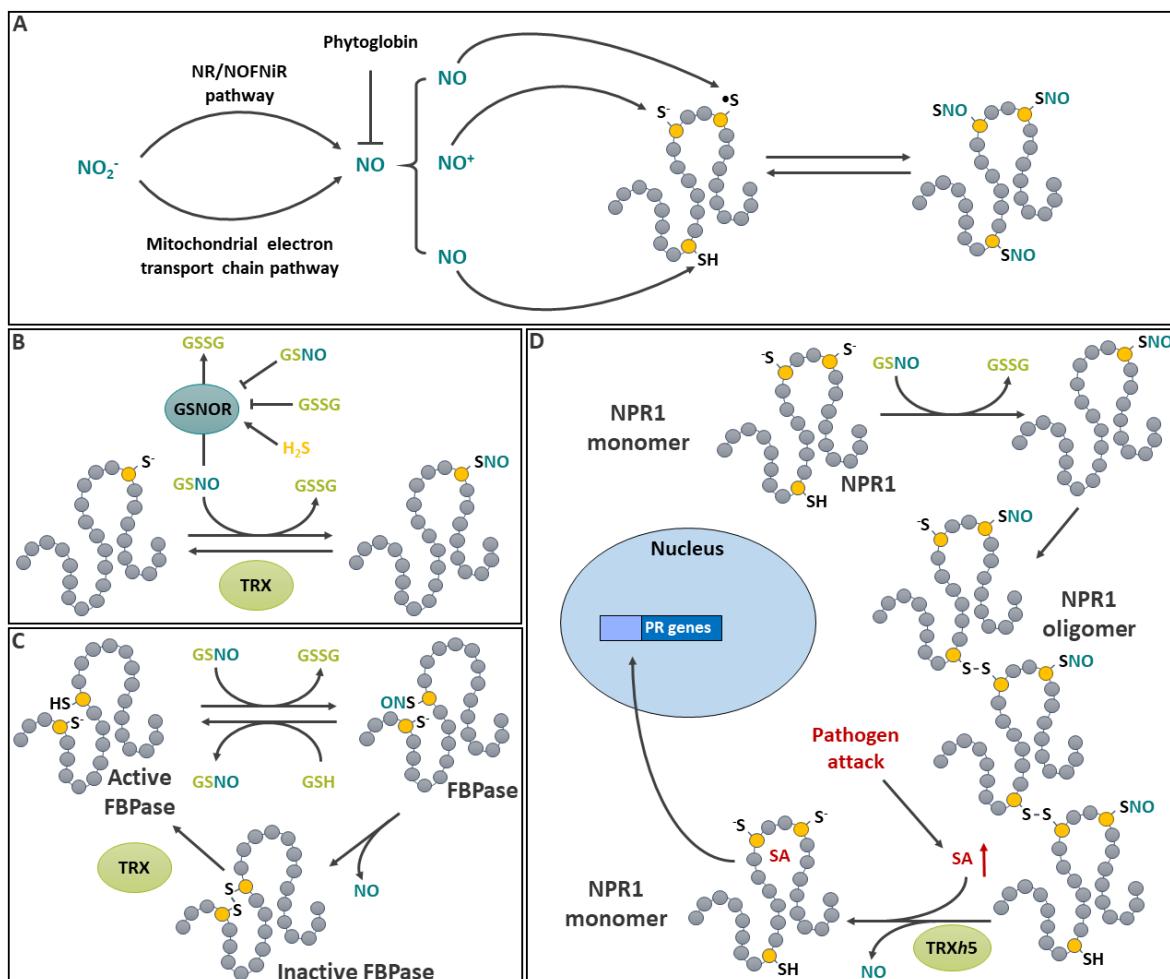


Figure 7. Protein S-nitrosation in plants. A: The generation of NO in plants and the modification of Cys by direct S-nitrosation. NO can be formed from NO_2^- by a pathway that involves nitrate reductase (NR) and NO forming nitrite reductase (NOFNiR). It can also be formed by reduction of NO_2^- by the mitochondrial electron transport chain. NO can be scavenged by phytochrome. Direct reactions of NO with Cys (yellow dots) thiolate (S⁻), thylid (•S) or thiol (SH) can lead to S-nitrosation (SNO). B: Protein transnitrosation by S-nitrosoglutathione. S-nitrosoglutathione (GSNO) is a stable form of NO which can be used in transnitrosation of proteins. GSNO is catabolized by GSNO reductase (GSNOR), which is susceptible to inhibition by GSNO and oxidized glutathione (GSSG) and stimulated by H₂S. C: Regulation of fructose-1,6-bisphosphatase (FBPase) by reversible S-nitrosation. In its reduced form (SH, top left), FBPase is active. High GSNO promotes the S-nitrosation of FBPase (top right), whereas high glutathione (GSH) reverts it. The S-nitrosated FBPase can undergo the formation of a disulfide bond (S-S). D: Pathogen attack triggers the formation of S-nitrosated (SNO) NPR1 monomers, which then oligomerize. TRXh5, a thiol reducing agent, maintains the reduced state of the NPR1 monomers.

bridge (S-S, bottom) that inhibits FBPase activity. S-S reduction by thioredoxin (TRX) occurs in the light and restores FBPase activity. D: Regulation of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) by S-nitrosation. NPR1 is a receptor for the phytohormone salicylic acid (SA), which transcriptionally regulates systemic acquire resistance through the induction of pathogenesis-related (PR) genes. In absence of pathogen stress, NPR1 is S-nitrosated by GSNO and sequestered in the cytosol in the form of large oligomers with intersubunit S-S. Upon pathogen attack, TRXh5 promotes monomerization of NPR1 by reducing inter-subunit S-S and catalyzing its denitrosation. In turn, reduced monomeric NPR1 can enter the nucleus where it promotes PR genes transcription. See text for additional details.

8.2. Protein Denitrosation

Current knowledge on plant denitrosation was recently extensively reviewed [256]. Briefly, protein denitrosation can involve enzymatic or non-enzymatic reactions (Figures 7B, 7C, 7D). In *Arabidopsis*, cytosolic TRXh5 can catalyze denitrosation of specific proteins [257]. Implication of other TRXs in plant protein denitrosation remains to be tested, however the high amino acid sequence similarity between TRX isoforms suggest that it is likely [256,257]. Despite their sequence similarities, TRX isoform differ in their subcellular localization and specific interactions, which could influence their substrate specificity [256]. PRX IIE is involved in a trans-denitrosation mechanism for the bZIP67 transcription factor [244]. Protein denitrosation activity was documented for animal SRX [258] and GRX [259] but the relevance of these enzymes in plant protein denitrosation remains to be investigated. Some enzymes, such as GAPDH [260] and GSNOR [261] can be denitrosated in an enzyme-independent way by direct reaction between GSH and the S-nitrosated thiol, resulting in GSNO production (see example below).

8.3. Targets of Protein S-Nitrosation in Plant Metabolism

Plant protein S-nitrosation has now been studied for over 20 years with proteomic surveys employing the biotin switch technique [262–264]. Several hundreds of S-nitrosated proteins, together with S-nitrosation sites have been identified. Among these, the *Arabidopsis* GAPC1 isoform of cytosolic GAPDH is known to be strongly inhibited by GSNO mediated S-nitrosation on its catalytic Cys¹⁴⁹ [260]. GAPC1 denitrosation is promoted *in vitro* by GSH, but not by the TRX system [260]. Furthermore, the GSH-dependent denitrosation is influenced by the GSH/GSNO ratio, but unaffected by the GSH/GSSG ratio [260]. These results contrast with the mechanism taking place with animal GAPDH, which is effectively denitrosated by TRX and resistant to GSH-mediated denitrosation [265,266]. Although S-nitrosation is currently more studied than denitrosation, the latter certainly deserves to be more thoroughly investigated, as there appears to be some protein and/or organism specific mechanisms at work.

S-nitrosation can also be involved in cooperative Cys modification for metabolic regulation. This remarkable mechanism has been documented with pea FBPase. This CBB cycle enzyme can be S-nitrosated by GSNO in light conditions (Figure 7C), when the enzyme is normally present in its reduced form (see Figure 3B) [267]. When fully reduced, FBPase is active. A high GSNO/GSH ratio promotes S-nitrosation of FBPase Cys¹⁵³. This S-nitrosation can be reversed if GSH concentration increases (*i.e.* under low GSNO/GSH), leading to the formation of GSNO. If conditions do not favor GSH-dependent denitrosation, the presence S-nitrosated Cys¹⁵³ induces a rapid S-S between Cys¹⁵³ and adjacent Cys¹⁷³ with the concomitant loss of NO. The newly formed S-S effectively inactivates the enzyme. In absence of TRX activity, such as in dark conditions, the enzyme is kept in this oxidized and inactive form. Under conditions where light favor TRX reduction, S-S can then be reduced and the enzyme becomes active [267]. FBPase, which has long been known to be regulated by reversible S-S formation. The effects of its S-nitrosation provide additional sensitivity to environmental factors that may affect NO status and the chloroplastic GSNO/GSH ratio to fine-tune the function of the CBB cycle.

8.4. Involvement of S-Nitrosation in Signal Transduction Pathways

S-nitrosation also plays an important role in signal transduction by affecting other major PTMs (e.g. sumoylation, phosphorylation or acetylation) that are involved in biochemical regulation. This topic was recently reviewed [268]. At the physiological level, it is now recognized that S-nitrosation plays a role in important aspects of plant hormone signaling [269]. It is for example the case for the ABA signaling during stomatal closure. As discussed above, the activity of protein kinase OST1/SnRK2.6 which is involved in the regulation of the KAT1 inward K⁺ channel is stimulated by persulfidation (Figure xC). Investigations into the regulation of this protein kinase demonstrated a negative regulation of ABA signaling by NO [270]. In particular, *gsnor1-3*, an *Arabidopsis* mutant with increased NO accumulation, was insensitive to the closure of stomata induced by ABA. Further investigations demonstrated that OST1/SnRK2.6 activity was inhibited in vitro in the presence of the NO donor GSNO. This inhibition was caused by S-nitrosation of conserved Cys¹³⁷, which was stimulated in vivo as a result of ABA treatment. It is possible that this work has even deeper significance. Indeed, S-nitrosation of Cys¹³⁷ could be relevant to the regulations of other evolutionary-related protein kinases [270].

A second example of the importance of S-nitrosation in signaling is found in SA signal transduction. In this process, NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) is regulated by reversible S-nitrosation (Figure 7D). *NPR1* is a key regulator of SA signaling and Systemic Acquired Resistance (SAR), was first identified using a genetic screen that aimed to identify genetic lesions causing a lack of systemic response normally induced by *Pseudomonas syringae* [271]. SA, which is involved in the positive regulation of SAR [272], binds to NPR1 [273]. Under normal conditions, NPR1 can be found as a high molecular weight oligomer with inter-subunit S-S (Figure 7D) [274]. NPR1 is S-nitrosated by GSNO on Cys¹⁵⁶, which promotes its oligomerization and sequestration in the cytoplasm [275]. Upon pathogen attack, there is an increase in endogenous SA and SA binding to NPR1. Upon SA accumulation, TRXh5 catalyzes the conversion of NPR1 oligomers to monomers, which are translocated to the nucleus to activate the expression of pathogenesis-related genes [274]. The monomerisation of NPR1 involves TRXh5 in two ways: it reduces the inter-subunit S-S [275] and catalyzes its denitrosation [257].

9. S-Carbonylation by Reactive Carbonyl Species

Membrane lipids are a major target of oxidation, especially under stress, due to their abundance and proximity with ROS and free radical production sites, such as RBOH, mitochondrial and chloroplastic electron transport chains [41,276–278]. This leads to an oxidative degradation of polyunsaturated fatty acids (PUFA) and subsequent oxylipin production (Figure 8A). Oxylipins are also produced enzymatically, mainly by lipoxygenases (LOX) that act on linoleic acid and α-linolenic acid (Figure 8A) [276,279]. This enzymatic pathway can lead to the production of important stress signaling molecules such as cis-(+)-12-oxophytodienoic acid (OPDA) and jasmonic acid (JA) [276,279]. Oxylipins also comprise more than a dozen species which contain a reactive electrophilic α,β-unsaturated carbonyl moiety that are collectively termed reactive carbonyl species (RCSs) [280]. RCSs include, but not exclusively, compounds such as acrolein, OPDA, 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) [280]. In this review, we follow a restrictive definition of RCSs given in the review by Mano [280]. Nevertheless, it is important to note that RCS definition appears to vary between authors and that species such as methylglyoxal or some aldehydes are regularly referred to as RCSs, although they are not included in the previous definition [280]. The latter publication also specifies the terms reactive electrophile species and oxylipins, which are sometimes interchanged with RCSs in the literature [280].

Since lipid composition and abundance of PUFAs varies between subcellular membranes, some, such as the chloroplast appear to be a major RCS production site [281,282]. Furthermore, the hypothesis of stress- or compartment-specific RCS signatures (usually referred to as oxylipin signature) is increasingly supported by the literature [283,284] [279,285]. RCS production, effect, target specificity and detoxification vary between members of the RCS group and were recently reviewed [281,286].

In plants, RCS can be detoxified enzymatically. Various enzymes are involved and they display some specificity towards the structure of their RCS substrate(s). Enzyme activities such as aldehyde dehydrogenase, aldehyde reductase, aldo-keto reductase, 2-alkenal reductase, alkenal/one oxidoreductase and glutathione transferase Tau (GST τ) have been implicated in RCS detoxification [287]. In a survey of the in vitro activity of 23 GST τ isoforms with different RCSs, acrolein and HNE were the preferred substrate of 11 isoforms [287]. Non-enzymatic RCS scavenging also occurs and involves the formation of a conjugate between RCS and polyphenols [288] or GSH. RCS detoxification by the GSH pool (Figure 8A) under conditions of high GSH/GSSG ratio is illustrated in a study of *Arabidopsis* overexpressing GR. Overexpression of GR led to higher levels of GSH and GSH/GSSG ratio, which was associated with enhanced RCS detoxification capacity compared to the WT [289].

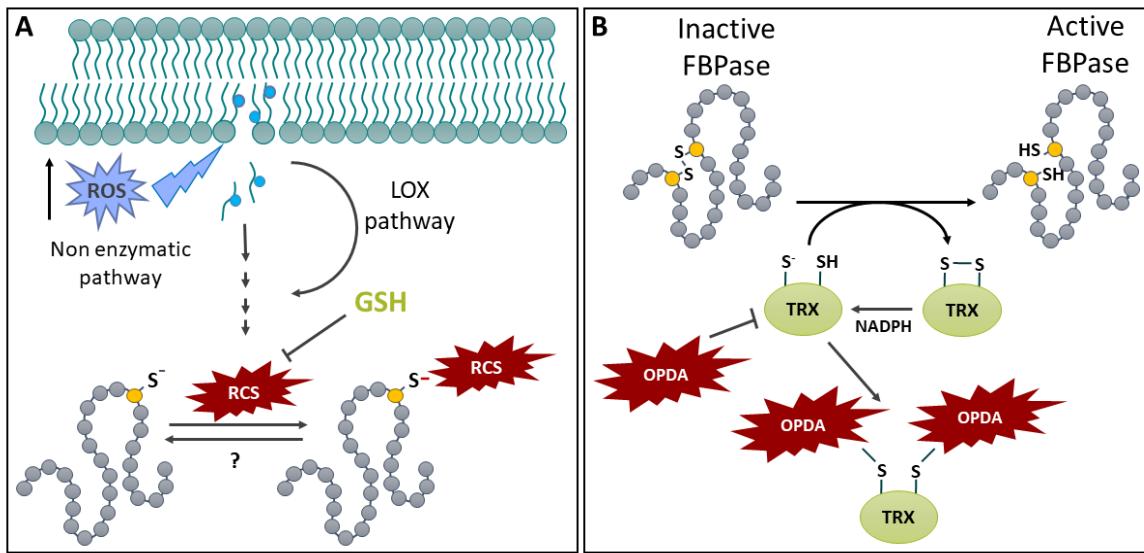


Figure 8. Protein S-carbonylation in plants. A: generation of reactive carbonyl species (RCS) and their reaction with protein Cys residues. Two main pathways are involved in the generation of RCS. The non-enzymatic pathway results from the attack of membrane-derived unsaturated fatty acids (marked with blue dots) by reactive oxygen species. The enzymatic pathway proceeds via lipoxygenase (LOX). Both pathways can produce a variety of reactive electrophilic α,β -unsaturated carbonyl compounds collectively termed RCSs. Detoxification of RCS can occur and often associated with reduced glutathione (GSH consumption). RCS can modify Cys residues (yellow dots) in thiolate form ($-S^-$), leading to protein S-carbonylation. The modification is widely considered irreversible as the decarbonylation reaction, indicated with a question mark, is still hypothetical in plants. B: Regulation of thioredoxin by S-carbonylation. The RCS cis-(+)-12-oxophytodienoic acid (OPDA) is formed enzymatically in the LOX pathway. Thioredoxin f1 (TRX) is responsible for the reduction of a disulfide bridge on fructose-1,6-bisphosphatase (FBPase) thereby activating the enzyme. In vitro modification of TRX with OPDA (S-OPDAylation) renders TRX unable to activate FBPase. See text for additional details.

9.1. Interrelations between RCS and ROS Signaling

RCS are produced downstream of ROS and increase during oxidative stress [281]. Like ROS, RCS can cause both eustress (signaling) and distress (damage) [286,290]. In a study on *Chlamydomonas reinhardtii*, acrolein treatment at low dose significantly upregulated genes involved in GSH, S and ascorbate metabolism in addition to redox homeostasis enzymes leading to acclimation to ROS [290]. However, RCS distress, caused by higher doses of acrolein, led to loss of cell viability [290]. RCSs could thus act downstream of ROS as signal in plant stress response [282,286,291–294]. However, it remains to be seen whether any of these effects involve Cys carbonylation.

Nevertheless, there is evidence that RCSs impact the redox network. RCSs may affect cellular glutathione contents and redox state, as illustrated with high acrolein treatments [290,294,295]. As

discussed above, RCS enzymatic or non-enzymatic detoxification that consumes GSH could deplete the GSH pool and increase the GSSG/GSH ratio. This could indirectly impact redox-sensitive Cys PTMs, for instance, by altering protein *S*-glutathionylation [295]. However, a formal demonstration of this effect has yet to be provided.

RCSs have a longer half-life than ROS and their shared characteristics with lipid allows them to diffuse through membranes [296]. The greater diversity of RCS compared to ROS could contribute to allow for a greater specificity of downstream ROS signaling [291]. Thus, RCS should be considered in the pursuit of understanding how stress-specific and compartment specific signaling pathways are generated [282,297].

9.2. Protein Thiols Modification by RCSs and *S*-OPDAylation

RCS electrophilic β carbon allows covalent modification of targets such as nucleic acids and proteins [282]. Because of its reactivity, Cys is a prominent RCS target, although other protein amino acids (e.g. His, Lys) can also be modified [298,299]. RCS can modify Cys residues via a Michael reaction mechanism, resulting in a primary protein carbonylation [279]. The reactivity of the RCS modifier, as well as its availability which is controlled by its formation/scavenging rates thought to control the nature of protein carbonyls [281,282]. Furthermore, protein carbonyls can undergo secondary reactions leading to irreversible and complex protein modifications [282]. Thus, protein carbonylation is traditionally considered as being irreversible. However, some reports in animals show some support for thiol-dependent decarbonylation [300] [301]. In plants, protein decarbonylation still appears poorly characterized. In a report, MDA-induced protein carbonylation was partially reversed, but the mechanism remains elusive [302]. It is possible that the reversibility of Cys carbonylation varies in function of the target protein and/or the primary and secondary modifications induced by RCSs.

The effect of RCS modifications on specific targets of the redox network in plants was recently reviewed [279] and Cys modifications of specific targets are beginning to be identified [303,304]. Nevertheless, progress in this area is still limited, perhaps due to the complexity of the detection of primary and secondary modifications. The cytotoxic and reactive species acrolein and HNE are among the most studied RCSs [278,295]. There are reports that these compounds may cause specific enzyme inhibition in plants [295,305]. So far, no direct evidence links these inhibitions to Cys modification. However, this hypothesis certainly deserves consideration since the enzyme activity of several reported RCS targets [295] is known to be sensitive to Cys oxidation.

OPDA is structurally related to cyclopentenone prostaglandin hormones found in animals [304]. Prostaglandins are known to exert some of their physiological functions through oxidation of Cys thiols [306]. In plants, recent studies demonstrated that OPDA can react with protein thiols, resulting in *S*-OPDAylation [304]. In vitro TRXf1 modification by OPDA was detected and Cys modification was documented by mass spectrometry. This *S*-OPDAylation led to an inhibition of TRXf1 to reductively activate fructose-1,6-bisphosphatase (Figure 8B) [304]. Subsequent inhibitory *S*-OPDAylation of chloroplast TRXm4 and cytosolic TRXh3 and h5 was also found [307]. The data suggest a unified mode of action for OPDA on TRXs. However, individual *S*-OPDAylated residues were not confirmed by mass spectrometry in the latter study.

ODPA is a precursor of jasmonic acid, a phytohormone implicated in response to biotic and abiotic stress responses [308]. Similarly to OPDA, which is reported to modulate key elements in plants redox regulation [307], cyclopentenone prostaglandins target essential components of the redox regulation network in animal cells under stress [306,309]. The fact that prostaglandin regulatory mechanisms by protein redox PTM provides a perspective and a possible working model worth exploring for the action mode of OPDA in plants.

10. *S*-Acylation

In plants, the chloroplast is the main site for de novo fatty acids production [310]. *S*-acylation (also known as *S*-palmitoylation) consists of the reversible modification of a Cys residue by a fatty acid, usually palmitic acid (C16) or stearic acid (C18), through a thioester bond [311]. Among the

known lipid modifications of proteins, S-acylation is unique because of its reversibility, thus making it a dynamic means of regulation [311,312]. S-acylation is not a trivial PTM in plants. A recent study in different *Arabidopsis* tissues identified 1849 Cys residues modified by S-acylation on 1094 different proteins using an acyl-resin-assisted capture (acyl-RAC) strategy [313]. The meristem and the siliques contained the most acylated proteins. While about half of protein acylation were contributed by a single tissue, only 6.5% were found in all tissues studied, indicative of a certain tissue specificity. S-acylation is catalyzed by a family of enzymes called Protein S-acyl transferases (PATs, also called DHHCs), that contain a Asp-His-His-Cys (DHHC) motif shown to be necessary for activity [314]. This motif is conserved in PATs found in microorganisms, animals and plants [315–317]. PATs are membrane proteins that are distributed in several different cellular membrane compartment (Figure 9A) [318]. The *Arabidopsis* genome contains 24 PATs, with half of them being targeted to the plasma membrane [318] while the rest localize to different endomembrane systems. This situation contrasts with yeast and mammals PATs, which are predominantly localized to the ER and Golgi [318]. Another unique feature of S-acylation is that it does not occur on conserved sites or consensus sequence motifs [319]. It is rather thought that it is influenced by the ability of the catalytic site of PATs to access their target Cys [320]. It is currently unknown if PATs are redox regulated, but possible modification of the conserved Cys in the DHHC motif could have an impact on S-acylation activity. Recently, a proteomic survey identified PAT24 as a persulfidation target in *Arabidopsis* roots [200]. PAT24 mutants display an altered root hair phenotype, indicating that S-acylation, catalyzed by PAT24, plays an important role in root hair growth [321].

10.1. S-Acylation Appears to be Critical for Target Proteins Membrane Localization

The effect of S-acylation on proteins that lack transmembrane helices (TMHs) is an increase in their hydrophobicity, thereby promoting their association with membranes (Figure 9A). For example, among all S-acylation target proteins identified in *Arabidopsis*, the vast majority (73%) lacked TMH [313]. Accordingly, this suggests that S-acylation may significantly influence cellular membrane distribution for target proteins. However, many proteins already associated with membranes can also be S-acylated [322]. It is thus unlikely that the function of protein S-acylation would be limited to membrane anchoring. Early studies suggested that S-acylation of a Cys residue in close proximity to a transmembrane domain (TMD) may serve to influence the conformation of the TMD within the membrane [323]. However, recent investigations of this effect in plants suggest that a careful validation of the epitope-tag methods used to study these phenomenon is warranted before drawing strong conclusions [311]. Nevertheless, several studies document the impacts of S-acylation lie in the regulation of signal transduction and cell wall metabolism.

The cellulose synthase complex (CSC) is responsible for the synthesis of the most abundant plant polymer. The CSC is a large transmembrane protein complex which consists of at least 18 cellulose synthase (CESA) subunits [324]. It is located at the plasma membrane where it consumes UDP-Glucose on the cytoplasmic side and generates nascent cellulose microfibrils on the apoplastic side. Multiple CESA subunits were found to be S-acylated [325], with an estimated 100 S-acylated Cys per CSC. Out of the twenty-six Cys in *Arabidopsis* CESA7, four Cys of the Variable Region 2 (VR2) and two others, found at the C-terminal end (CT) are S-acylated and were studied using a site directed mutagenesis approach in transgenic lines. This indicated that VR2 region Cys residues were important for plant cellulose contents [325]. And that, while VR2 and CT Cys are not essential for CSC assembly at the endomembrane level up to the Golgi vesicles, they are required for the correct localization to the plasma membrane [325]. Thus, S-acylation of the CSC subunits may control cellulose production by allowing the correct intracellular trafficking of the complex to the proper site for cellulose production after its assembly on endomembranes.

The importance of protein S-acylation can further be illustrated by its impact on several signaling pathways in plants, as previously reviewed [319]. In recent years, there has been mounting evidence that Ca^{2+} sensing and signaling proteins are S-acylated. It was shown that S-acylation of several Ca^{2+} sensors Calcineurin B-Like proteins (CBLs) is required for their membrane positioning and their signaling function in ion homeostasis. For example, the proper localization of *Arabidopsis* CBL6 was

shown to depend on S-acylation of several Cys residues at the N-terminus of the protein [326]. This also impacted the correct targeting of CBL6-interacting protein kinase in planta, implying that modification of CBL6 is responsible for the targeting of the protein complex [326].

In another study, CBL5 and the Ca^{2+} -regulated protein kinase CPK6 were shown to be subject to a double lipid modification (N-myristoylation and S-acylation), which were required for proper localization of the two proteins to the plasma membrane (Figure 9B) [327]. The dual modification allowed the recruitment of Calcineurin B-like protein-interacting protein kinase 11 (CIPK11) by CBL5 in a process that is involved in the regulation of guard cell Slow anion channel 1 (SLAC1) (Figure 9B). SLAC1 is a key element in abscisic acid-dependent stomatal closure. Further studies using heterologous expression in *Xenopus* oocytes indicate that the dual lipid modification of CBL5 is critical for the activation of SLAC1 by phosphorylation by the CBL5/CIPK11 complex [327]. Thus, this example illustrates the impact of N-myristoylation and S-acylation in the regulation of stomatal function.

S-acylation is also involved in plant response to phosphate (Pi) deficiency (Figure 9C). *Arabidopsis* non-specific phospholipase C4 (NPC4) is a promiscuous enzymes that hydrolyzes membrane phospholipids, generating a free head group and diacylglycerol which is involved in signaling [328]. The importance of NPCs in plant signaling for growth development and stress has recently been reviewed [329]. In NPC4, S-acylation of Cys⁵³³, located at the C-terminal extremity the protein was shown to be necessary for its plasma membrane association [330]. Palmitate was identified as the main modifier. Using a mutation analysis strategy, S-acylation of Cys⁵³³ was shown to be responsible for successfully restoring NPC4 function in a *npc4* mutant [330]. These data strongly support the involvement of a single S-acylation site in the proper localization of NPC4, thereby allowing it to access its substrate(s) and fulfill its signaling functions.

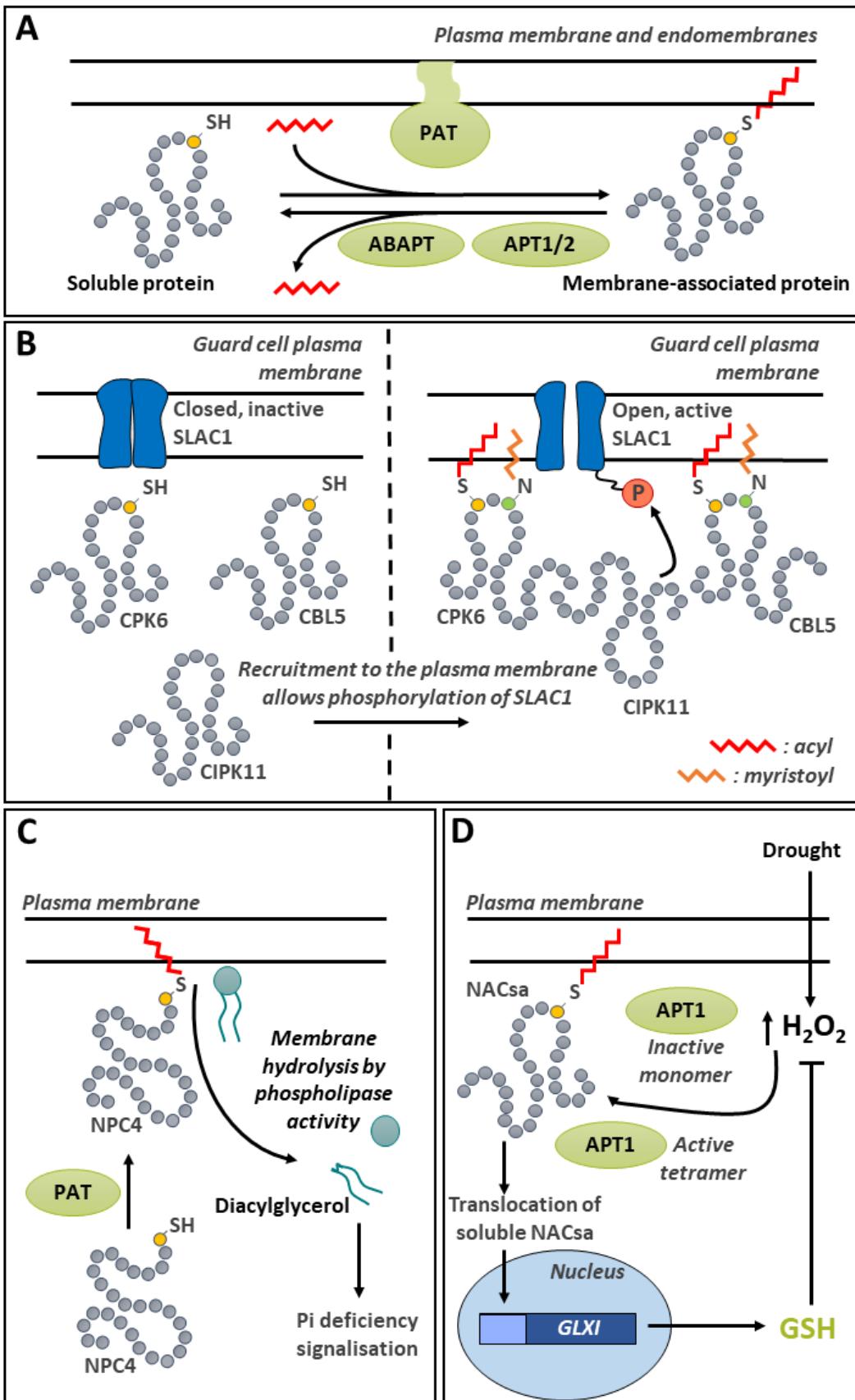


Figure 9. Protein S-acylation in plants. A: S-acylation and deacylation. Membrane-localized protein S-acyl transferase (PAT) catalyzes the addition of an acyl group (red jagged line) on a Cys (yellow dot) thiol present on a soluble protein. The addition allows the S-acylated protein to become membrane-associated. The reverse reaction (deacylation) is catalyzed by ABHD17-like acyl protein

thioesterases (ABAPT) or acyl protein thioesterases (APT), which release the acyl group and solubilize the protein. B: Involvement of S-acylation in the regulation of SLAC1. In the guard cell plasma membrane, the Slow anion channel 1 (SLAC1) is a key element in abscisic acid-dependent stomatal closure. The channel is activated by phosphorylation. A double lipid modification (N-myristoylation on a Gly (green dot) and S-acylation) allow the membrane localization of otherwise soluble Calcineurin B-Like protein 5 (CBL5) and Ca^{2+} -regulated protein kinase CPK6. This allows the recruitment of Calcineurin B-like protein-interacting protein kinase 11 (CIPK11) by CBL5 and the phosphorylation (activation) of SLAC1. C: Involvement of S-acylation in Pi deficiency signalization. The non-specific phospholipase C4 (NPC4) is S-acylated on a C-terminal Cys. Its phospholipase activity allows it to generate diacylglycerol that acts in Pi deficiency signalization. D: Involvement of de-S-acylation of transcription factor NACsa in signal transduction. Under normal conditions, the transcription factor NACsa is sequestered to the plasma membrane by S-acylation. Upon drought, an increase in H_2O_2 activates monomeric acyl protein thioesterase 1 (APT1) to its tetrameric form. Tetrameric APT1 deacylates NACsa and releases it from the membrane, allowing its translocation to the nucleus where it activates the transcription of *Glyoxalase 1* (GLX1), which promotes glutathione build-up acting to inhibit H_2O_2 accumulation. See text for additional details.

10.2. De-S-Acylation Players and Evidence of Their Involvement in Plant Signal Transduction

In animals, two distinct types of proteins, acyl protein thioesterases (APT) [331] and ABHD17-family depalmitoylases [332], have been implicated in the de-S-acylation of proteins by cleaving the Cys-fatty acid thioester bond. A group of 11 *Arabidopsis* hydrolases, named ABHD17-like acyl protein thioesterases, (ABAPTs) was recently identified. They share a conserved ABHD region with mammalian ABHD17 proteins and were shown to catalyse de-S-acylation in plants and act using a similar catalytic mechanism (Figure 9A) [333].

Plants seemingly share poor sequence homology with mammalian APT1 and APT2 [311]. Nonetheless, a few studies support a role for APT1 in plant de-S-acylation (Figure 9A, 9D) [334,335]. In *Medicago* species, NACsa is a transcription factor localized at the plasma membrane through Cys²⁶ S-acylation (Figure 9D) [334]. Under drought stress condition, APT1 catalyzes NACsa de-S-acylation, thereby promoting its translocation to the nucleus and the transcription of glyoxalase I (GLXI). GLXI activation increases the GSH/GSSG ratio [334]. These data indicate that APT1 is a mediator of cellular redox potential under drought. Interestingly, APT1 is itself redox-regulated. Its sequence contains three redox sensitive Cys; Cys²⁰, Cys²² and Cys²³, that can be S-glutathionylated in control conditions [335]. S-glutathionylation of APT1 promotes its inactive, monomeric form. Drought increases intracellular H_2O_2 , leading to oxidative stress and promoting the formation of a tetrameric APT1 complex with de-S-acylase activity. The de-S-acylation of NACsa leads increased GLXI and a higher GSH/GSSG ratio, contributing to the restoration of redox homeostasis and promoting S-glutathionylation and inactivation of APT1 [335]. As a result, APT1 behaves as an effective redox sensor during drought.

Recently, de-S-acylation was identified as a key mechanism in hormone cross talk. Brassinosteroids (BRs) are important plant hormones mainly involved in plant growth and development [336], while salicylic acid (SA) is associated with plant defense and stress response [337]. BRs signaling involves the interaction of brassinosteroid insensitive 1 (BRI1) receptor and brassinosteroids signaling kinase (BSKs) at the plasma membrane [336]. BRs binding to BRI1 leads to its activation and phosphorylation of BSKs, initiating the BRs signaling cascade [336]. In normal conditions, BSKs are maintained at the plasma membrane by S-acylation, allowing their interaction with BRI1 [338]. Stresses, such as pathogen infection, induce an important increase in SA biosynthesis and signaling [337]. SA increases the expression of ABAPT11 [338]. This promotes the de-S-acylation of BSKs, reducing their membrane localisation, thus impairing their function BRs signalisation [338].

11. Prenylation

Protein prenylation has been known to take place in plants for quite some time (see the review by [339]). Protein prenylation is the irreversible covalent modification of a Cys residue by

isoprenoids. The known modifiers are 15- (farnesyl moiety) or 20- (geranylgeranyl moiety) carbon chains. These isoprenoids are formed by condensation of 5-carbon units (isopentenyl pyrophosphate and its isomer dimethylallyl pyrophosphate) that are produced by two plant isoprenoid biosynthetic routes, the mevalonate pathway in the cytoplasm and the methylerythritol 4P pathway in the chloroplast [340]. Protein modification by isoprenoids is catalyzed by protein prenyltransferases. These enzymes are classified as farnesyl transferases (FT), geranylgeranyl transferases (GGT-1) and Rab-geranylgeranyl transferases (RGGT, also called GGT-2), depending on their specificity for various isoprenoids [341]. Protein prenylation is widely considered as a means of anchoring proteins in membranes, in particular in the secretory pathway [342,343]. Prenylation occurs at the C-terminal end of proteins on a consensus site. In the case of FT and CCT-1, they modify the consensus Cys-a-a-X motif, where a represents an aliphatic residue and X any amino acid [344]. This prenylation step directs the modified protein to the endoplasmic reticulum (ER) where it is followed by the proteolytic removal of the aaX sequence is catalyzed by STE24 or RCE1 endoproteases [343]. The resulting free carboxyl residue of the prenylated Cys then becomes available for methylation by a protein-S-isoprenylcysteine O-methyltransferase (ICMT) [343]. The subcellular localization of the post-prenylation processing machinery supports an ER membrane localization for proteins with the Cys-a-a-X motif, however, they can also follow the secretory pathway [343].

The RGGT/CCT2 class recognizes targets that are bound to the Rab Escort Protein and carry consensus sequences such as Cys-Cys, Cys-X-Cys, Cys-Cys-X, Cys-Cys-X-X and Cys-Cys-X-X-X, in which both Cys present in the motif can be modified [345]. Despite the above described classification of protein prenyltransferases in two categories distinguished by consensus sequences, protein substrate recognition may not be strictly exclusive [346]. An extensive searchable protein prenylation database that includes plants is available to facilitate research on this topic (<https://mendel.imp.ac.at/PrePS/index.html>) [347].

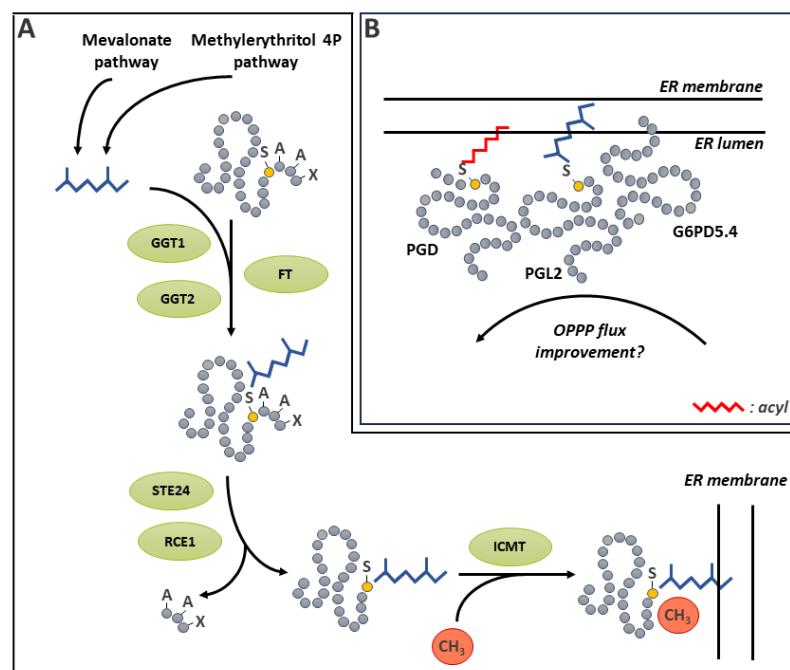


Figure 10. Protein prenylation in plants. A: Pathways responsible for prenyl group synthesis and mechanism of protein prenylation. Prenyl groups (blue colored structure representing isoprenoid C15 or C20 chains) are synthesized by the mevalonate or the methylerythritol 4P pathways. Farnesyl transferases (FT), geranylgeranyl transferases (GGT-1) and Rab-geranylgeranyl transferases (RGGT/GGT-2) modify a Cys (yellow dot) present in a Cys-A-A-X (A = aliphatic and X = any amino acid) motif located at the C terminus, directing the protein to the ER membrane. A proteolytic removal of C-terminal the AAX sequence is catalyzed by STE24 or RCE1 endo-proteases, and a methylation of the prenylated Cys is done by a protein-S-isoprenylcysteine O-methyltransferase (ICMT). B: Involvement of prenylation in the formation of an oxidative pentose phosphate pathway (OPPP) in

the ER. Prenylated 6-phosphogluconolactonase (PGL2) is localized to the ER membrane and interacts with ER S-acylated (red jagged line) phosphogluconate dehydrogenases (PGD). PGL2 recruits glucose-6P dehydrogenase 5.4 (G6PD5.4) to the membrane. The result constitutes a metabolon containing the first three steps of the OPPP in ER the lumen. Such organization could improve C flux in the OPPP. See text for additional details.

Plant protein prenylation has been the subject of numerous reviews, including a recent one on its involvement in plant stress responses [344] and another on signaling aspects [348]. We chose to only focus on a single recent study exemplifying the impact of prenylation in metabolism. This concerns the organization of the oxidative pentose phosphate pathway (OPPP) enzymes. OPPP commits carbon the for the generation of NADPH and carbon skeletons used in redox homeostasis and anabolic pathways [349]. Traditionally, this pathway has been thought to operate in the cytosol and the plastid stroma [349], while some steps have also been found in the peroxisome [350]. In *Arabidopsis*, one of the isoforms of the second OPPP enzyme (6-phosphogluconolactonase, PGL2) contains a C-terminal Cys-a-a-X motif [351] indicating its possible prenylation. Additionally, all three phosphogluconate dehydrogenases (PGD, the third step of the OPPP) can be S-acylated[313,351]. In an intriguing study, the presence of PGL2 at the ER was documented. Moreover, this subcellular localization was shown to be dependent on the Cys residue of the Cys-a-a-X motif. Furthermore, there is evidence that PGL2 recruits G6PD5.4 in specific ER subdomains. G6PD5.4 is a functional isoform of glucose-6P dehydrogenase arising by alternative splicing of *G6PD5* and carrying an N-terminal extension serving as ER membrane anchor. Additional evidence suggests that S-acylated PGD2 may interact with the first two OPPP enzymes [351]. Thus, a combination of G6PD5.4N-terminal ER membrane targeting, together with PGL2 prenylation and PGD2 S-acylation allows the constitution of a metabolon regrouping the first three steps of the OPPP on the cytosolic side of the ER membrane. It is hypothesized that such organization should improve metabolic flux in the OPPP. Since the significance of this organization also depends on the proportion of OPPP enzymes involved in the ER metabolon relative to other subcellular localizations, the study by Linnenbrügger [351] opens up exciting future research opportunities.

12. CoAlation

Coenzyme A (CoA) is an ubiquitous cofactor in prokaryotes and eukaryotes [352]. Its five steps biosynthetic pathway from panthenate has been elucidated for *Arabidopsis* [353,354]. Protein modification by CoA (CoAlation) was first described in animal [355] cells and shortly after in bacteria and Amoebozoa [356,357]. These studies were greatly facilitated with the use of an anti-CoA antibody able to detect protein CoAlation. This PTM can occur spontaneously. It was promoted by oxidative stress conditions *in vivo* and fully reversible *in vitro* using DTT [355–357]. A large number of CoAlation targets were identified, many of which being involved in metabolic processes [355,356]. Such widespread, and evolutionary ancient occurrence of protein CoAlation suggests it could also occur in plants. However, evidence for this is still extremely limited. A study on soybean protein Tyr phosphatase by mass spectrometry suggested the possible CoAlation of the recombinant enzyme produced in *E. coli*, in addition to *S*-glutathionylation [167]. The characterization of this PTM does not appear to have been pursued. It is unknown so far if this finding are only a reflection of the experimental conditions [167].

13. Thiohemiacetal Formation

Thiohemiacetal formation is the result of a Cys nucleophilic attack on an acyl group. It is a reversible reaction for example involved in the catalytic mechanism of a number of dehydrogenases [358] [359]. In this case, the thiohemiacetal is a reaction intermediate involving a covalent bond between an enzyme's catalytic Cys and its substrate [359]. In less common instances, however, thiohemiacetal formation may have regulatory functions. For instance, in plants, thiohemiacetal formation has been involved in the regulation of betaine aldehyde dehydrogenase (BADH). BADH catalyzes the oxidation of betaine aldehyde (BAL), which constitutes the last step in the biosynthetic

pathway of the major plant osmoprotectant glycine betaine (GB) [360]. BADH catalytic Cys²⁹¹ forms a thiohemiacetal as part of its catalytic mechanism. Interestingly, in a structural study of *Spinacia oleracea* BADH, it was shown that Cys⁴⁵⁰, a non-catalytic residue also undergoes thiohemiacetal formation with BAL [361]. This reaction hinders substrate binding, effectively inhibiting BADH activity. This mechanism was discussed as a possible regulatory mechanism that would prevent excessive NAD⁺ consumption during GB synthesis [361].

Studies on plant mitochondrial alternative oxidase (AOX) provide other evidence for the possible involvement of thiohemiacetal formation in regulatory mechanisms. AOX is an inner mitochondrial membrane dimeric terminal oxidase. It diverts electrons from the cytochrome pathway, bypassing complexes III and cytochrome c oxidase, thereby reducing respiratory ATP yield [362]. The reversible formation of an inter-subunit S-S is involved in AOX regulation. AOX carrying the S-S is inactive. When NAD(P)H levels are high in the mitochondrial matrix, there is reduction of the disulfide bridge, leading to an AOX with basal activity. Pyruvate, as well as various matrix α -ketoacids such as glyoxylate, oxaloacetate or α -ketoglutarate then interact with the reduced thiols and fully activate AOX. The formation of a thiohemiacetal between the reduced Cys residues and the α -keto acid has long been suspected to be involved in this regulation [363,364]. A site directed mutagenesis study has led to the proposition of a model for the activation of *Arabidopsis* AOX by pyruvate [364]. In this model, the S-S involving Cys⁷⁸ on both subunits would block access of pyruvate to a reaction with this residue, whereas reduction of the bond would allow the Cys⁷⁸ thiol to interact with pyruvate. This is consistent with the increased AOX activity obtained after substitution of Cys⁷⁸ by Glu, which possess a side chain able to mimic a thiohemiacetal [364,365]. This mutant is also insensitive to pyruvate [364]. However, the detection of a thiohemiacetal by mass spectrometry remains elusive in AOX and its crystal structure is not available [365]. Thus, although converging results suggest that AOX regulation by pyruvate occurs via the formation of a thiohemiacetal, the exact mechanism still remains to be elucidated.

14. Conclusion

The chemical properties of Cys residues make them reactive to a wide variety of PTMs. With this review, we wanted to cover the range of Cys PTMs present in plants. In some cases, for example with the reversible formation of S-S, substantial research advances have been made over the years and now provide an already broad overview of the involvement this process in plant biochemistry. For several other PTMs, such as, persulfidation, S-cyanilation, S-glutathionylation, S-nitrosation, S-carbonylation or CoAlation, we are still very much in the early steps in our exploration of their implication in the molecular physiology of plants. Future progress on these issues is undoubtedly going to benefit from current advances in specialized proteomic and chemistry techniques, the setting up of resources like specialized PTM databases and the development of analytical and predictive bioinformatics tools. A point of particular interest is the fact that certain Cys residues can be modified by multiple PTMs under a variety of conditions (see, for example the GAPDH catalytic Cys mentioned in this review). This exemplifies the sometimes-complex interactions that can take place between different PTMs for a given Cys residue. We believe that we are currently only scratching the surface of this complexity.

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