Review

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Protein persulfidation in plants: mechanisms and functions beyond a simple stress response

https://doi.org/10.1515/hsz-2024-0038
Received March 1, 2024; accepted August 28, 2024;
published online September 23, 2024

Abstract: Posttranslational modifications (PTMs) can modulate the activity, localization and interactions of proteins and (re)define their biological function. Understanding how changing environments can alter cellular processes thus requires detailed knowledge about the dynamics of PTMs in time and space. A PTM that gained increasing attention in the last decades is protein persulfidation, where a cysteine thiol (-SH) is covalently bound to sulfane sulfur to form a persulfide (-SSH). The precise cellular mechanisms underlying the presumed persulfide signaling in plants are, however, only beginning to emerge. In the mitochondrial matrix, strict regulation of persulfidation and H_2S homeostasis is of prime importance for maintaining mitochondrial bioenergetic processes because H_2S is a highly potent poison for cytochrome c oxidase. This review summarizes the current knowledge about protein persulfidation and corresponding processes in mitochondria of the model plant Arabidopsis. These processes will be compared to the respective processes in non-plant models to underpin similarities or highlight apparent differences. We provide an overview of mitochondrial pathways that contribute to H_2S and protein persulfide generation and mechanisms for H_2S fixation and de-persulfidation. Based on current proteomic data, we compile a plant mitochondrial persulfidome and discuss how persulfidation may regulate protein function.

Keywords: posttranslational modification; cysteine; protein persulfidation; hydrogen sulfide; mitochondria

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1 Introduction

Posttranslational modifications (PTMs) are key mechanisms to increase proteomic diversity as they can change the behavior of proteins in multiple form, such as activity, localization and interactions, and can thus (re)define a protein's biological function (Conibear 2020). Elucidation of how cellular processes adapt to changing environments to maintain homeostatic condition within the cell thus requires knowledge about which proteins are posttranslationally modified, as well as how and when. Thiols are one of the most versatile functional groups that can be modified in multiple forms including so-called persulfidation (Corpas et al. 2022; Zhou et al. 2023). This PTM in which a sulfur atom is covalently bound to a cysteine thiol (-SH) to form a persulfide (-SSH), gained increasing attention over the last two decades.

Protein persulfidation occurs in Bacteria, Archaea, and Eukarya and proteomic analyses have shown that up to 5,000 functionally diverse proteins can get persulfidated in the model plant *Arabidopsis thaliana*, suggesting a widespread role of protein regulation through persulfidation in plants (Aroca et al. 2017a; García-Calderón et al. 2023; Jurado-Flores et al. 2023; Ogata et al. 2023; Zivanovic et al. 2019). Similar to other PTMs, persulfidation can activate or inactivate proteins like alcohol dehydrogenase 5 (ADH5) or 1-Cys peroxiredoxin alkyl hydroperoxide reductase E (*Mt*AhpE), respectively, and help to adapt the protein function to changing conditions (Aroca et al. 2021; Cuevasanta et al. 2019; Kasamatsu et al. 2023). Indeed, protein persulfidation increases under various stress conditions like H_2O_2-induced oxidative stress in mammalian cells or drought stress in plant cells (Gao et al. 2015; García-Calderón et al. 2023) and has been proposed to protect thiols against irreversible oxidation caused by elevated intracellular H_2O_2 levels (Dóka et al. 2020; Wang et al. 2023; Zivanovic et al. 2019). Despite the increasingly recognized role of persulfidation in plants and other species, the cellular mechanisms behind persulfide formation remain largely unknown. Several non-enzymatic reactions have been discussed that all include the formation of hydrogen sulfide (H_2S). H_2S, which under most physiological pH conditions will be present predominantly as

hydrosulfide anion (HS^-), can then, for instance, further react with sulfenylated cysteine residues (-SOH) to form persulfides (Cuevasanta et al. 2015; Filipovic et al. 2018). In plants, exogenous application of sulfide has been shown to promote flowering and to have positive effects on plant growth and fruit ripening, resulting in increased fresh weight and yield as well as increased resilience to drought, salinity, and heavy metal (e.g. cadmium) toxicity. Based on these observations, persulfides are considered candidate species for the transduction of signals triggered by the application of H_2S (Dooley et al. 2013; Jurado-Flores et al. 2023; Ma et al. 2023, 2024; Mostofa et al. 2015).

To generate sulfide endogenously, plants employ several reactions with cysteine as sulfur source (García et al. 2010; Koprivova and Kopriva 2014). It remains unclear, however, whether non-enzymatic persulfidation through H_2S alone is sufficiently specific and fast to be physiologically relevant *in planta*. When, for example, the two-step reaction of protein thiols with H_2O_2 and H_2S is considered, it has to be noted that the respective reaction rates are quite slow. While the reaction rate of a thiol group with H_2O_2 to generate a sulfenic acid is $8.2 \times 10^4\,M^{-1}\,s^{-1}$ for the *Mycobacterium tuberculosis* 1-Cys peroxiredoxin (1-Cys Prx) alkyl hydroperoxide reductase E (*Mt*AhpE) or $13\,M^{-1}\,s^{-1}$ for human serum albumin (HSA) (Bonanata et al. 2017; Hugo et al. 2009), the reaction of H_2S with sulfenylated *Mt*AhpE or HSA is around $1.4 \times 10^3\,M^{-1}\,s^{-1}$ or $4.7 \times 10^2\,M^{-1}\,s^{-1}$, respectively (Cuevasanta et al. 2015, 2019). Although this two-step reaction might be feasible under oxidative stress with strongly elevated H_2O_2 levels, endogenous amounts of H_2S and H_2O_2 under non-stress condition are in the nanomolar to low micromolar range and therefore unlikely to be important for basal persulfidation (Huang et al. 2016; Foyer and Noctor 2016; Furne et al. 2008; Seaver Lauren and Imlay James 2001). It has thus been hypothesized that persulfidation could be enzymatically facilitated by protein persulfidases like 3-mercaptopyruvate sulfurtransferases or cysteinyl-tRNA synthetases (Akaike et al. 2017; Moseler et al. 2021; Pedre et al. 2023). Persulfidation can further occur on low molecular weight molecules like cysteine or glutathione that form cysteine persulfide (Cys-SSH) and glutathione persulfide (GSSH), respectively. To account for the variety of molecules with catenated sulfur-sulfur bonds, Akaike and colleagues have recently introduced the term 'supersulfides' that include persulfides (RSSHs) and polysulfide species (Barayeu et al. 2023a; Kasamatsu et al. 2023; Matsunaga et al. 2023). The diverse biological functions of such supersulfides are only beginning to emerge and we here review our current understanding of protein persulfidation, the impact of this PTM and sulfide homeostasis in plant mitochondria. A specific focus will be on Arabidopsis protein persulfidases and plant proteins that are candidates for being regulated by persulfidation.

2 Proteins undergo persulfidation: exploring biological significance

Cysteine is a relatively rare amino acid that in yeast, maize, tomatoes or humans constitutes just 1.62–2.26 % of the amino acids in proteins. Yet, 92 % of human or *Saccharomyces cerevisiae* proteins contain at least one cysteine (Miseta and Csutora 2000). These cysteine residues frequently link vital redox reactions to biological structures and functions and are therefore more highly conserved compared to other amino acids (Go et al. 2015; Huang et al. 2023; Nietzel et al. 2020; Sies et al. 2024). Protein persulfidation as a prevalent and reversible oxidative cysteine modification gained increasing attention as it was shown to be involved in many biological processes, some of which are detailed below. The respective mechanisms for regulation of different processes include protection against cysteine overoxidation and influencing the protein activity.

2.1 Protective effect of persulfides by enhanced oxidant scavenging

A crucial role of persulfides based on their chemical properties is to protect proteins from excessive and concomitant irreversible oxidation. The chemical properties of persulfides have been summarized in many excellent reviews (see *e.g.* Benchoam et al. 2019; Filipovic et al. 2018; Ono et al. 2014; Vignane and Filipovic 2023). Briefly, low molecular weight persulfides have a lower pK_a than the respective thiol resulting in predominant presence of the RSS^- anionic species at physiological pH (Benchoam et al. 2020; Cuevasanta et al. 2015). For example, a pK_a of 8.94 was determined for glutathione (GSH) whereas the pK_a of glutathione persulfide (GSSH) is 5.45 (Benchoam et al. 2020). In proteins, where the molecular microenvironment impacts on the reactivity of redox-sensitive cysteine residues, this correlation may not always hold true (Trost et al. 2017). The persulfidated form of human thiosulfate sulfurtransferase, for instance, has a pK_a of 9.38, whereas that of the thiolated form is 6.5 (Benchoam et al. 2024). Furthermore, persulfides are more nucleophilic than the respective thiols. This persulfide nucleophilicity is based on the presence of a sulfur atom with free electron pairs that increases the nucleophilicity of the outer or terminal sulfur atom via the so-called α-effect (Benchoam et al. 2020; Ida et al. 2014). These two characteristics, lower pK_a

and higher nucleophilicity, result in a faster reactivity with two-electron oxidizing electrophiles such as H_2O_2 and peroxynitrite or one-electron oxidants like peroxyl radicals compared to the respective thiol (Chauvin et al. 2016; Cuevasanta et al. 2015; Ida et al. 2014). Hence, due to the enhanced oxidant scavenging of persulfides, especially low molecular weight molecules like GSSH, they can be considered to have a protective effect. GSSH for example, reacts 22 times faster *in vitro* with oxidizing H_2O_2 and even 97 times faster with peroxynitrite compared to GSH (Benchoam et al. 2020). In addition, it was shown *in vitro* that GSSH reduces radicals. Forming stable perthiyl radicals (GSS•) and recombining into tetrasulfides (GSSSSG), GSSH prevents the propagation of radical chain reactions, thereby eliminating radicals from the system. This was further confirmed *in vivo* where increased amounts of persulfides result in decreased radical amounts and radical-driven lipid peroxidation (Barayeu et al. 2023b).

It should be noted though that the described faster reactivity of persulfides with oxidants compared to the respective free thiol cannot be generalized. Exceptions of antioxidant enzymes exist where persulfidation of the active site cysteine results in a decreased reduction of specific endogenous substrates. This was shown for *Mt*AhpE, which in its persulfidated form was found to be less reactive towards H_2O_2 and peroxynitrite (Cuevasanta et al. 2019; Reyes et al. 2018). Peroxidatic cysteines of peroxidases are characterized by a low pKa, which is achieved through stabilization of the thiolate form by surrounding amino acids that depends on the correct topology of the active site. Based on molecular dynamic simulations, it was suggested that persulfidation in this case results in significant disruption in the topology of the active site altering key interactions involved in catalysis of the Prx substrates (Cuevasanta et al. 2019).

2.2 Protective effect of persulfides for protein thiols

In vivo, several studies show a direct connection between elevated H_2O_2 and protein persulfidation levels. In Arabidopsis, it was shown that under non-photorespiratory conditions both, H_2O_2 levels as well as protein persulfidation levels, increase (García-Calderón et al. 2023). Also, in mouse embryonic fibroblasts and HeLa cells an increased amount of persulfides was detected after H_2O_2 treatment (Zivanovic et al. 2019). Notably, H_2S cannot directly react with a cysteine thiol but needs a preceding oxidation step such as sulfenylation. The reaction of sulfide with sulfenic acids is considered to render H_2S as a cellular defense against cysteine overoxidation (Figure 1A). While the formation of sulfenic acid is a reversible key reaction in cellular redox signaling, the subsequent oxidation to sulfinic (Cys-SO_2H) or sulfonic (Cys-SO_3H) acid would render the oxidation irreversible and thus result in permanent inhibition of the respective protein, although some exceptions exist for reduction of sulfinylated proteins, particularly through ATP-dependent reduction by sulfiredoxins (Biteau et al. 2003; Iglesias-Baena et al. 2011; Woo et al. 2003). In contrast, enzymatic activity of persulfidated proteins can be recovered after oxidation. The respective persulfide derivatives perthiosulfenic acid (Cys-SSOH), perthiosulfinic acid (Cys-SSO_2H), and perthiosulfonic acid (Cys-SSO_3H) can be reduced back by the thioredoxin (TRX) system or other reductants that attack the disulfide bridge as it was shown *in vitro* for HSA and peroxiredoxin Prx2 as well as for the 3-mercaptopyruvate sulfurtransferases (MSTs) from rat and Arabidopsis after H_2O_2 treatment (Dóka et al. 2020; Moseler et al. 2021; Nagahara et al. 2012). Also, in bovine and human cells, in which the TRX reductase was downregulated or inhibited chemically by auranofin, an increased amount of persulfidated proteins is

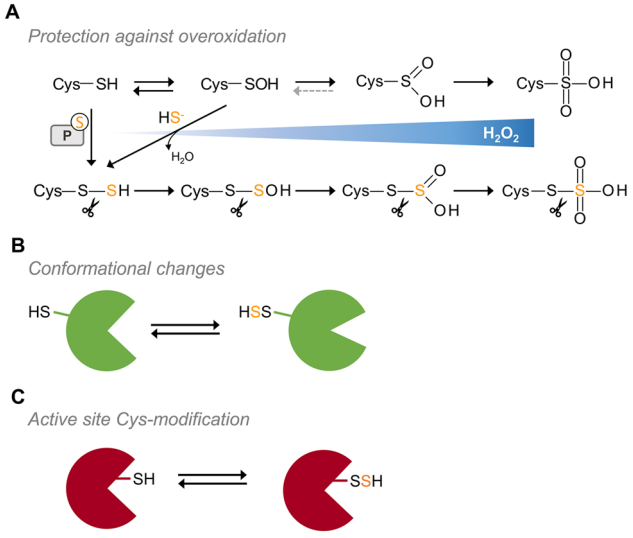

Figure 1: Impact of persulfides on proteins. (A) Protection against overoxidation. Under oxidative stress, cysteine thiols can be oxidized to sulfenic acid. This reaction is reversible, while the further oxidation to sulfonic acid is irreversible. Thiols can get persulfidated either enzymatically (indicated with P) or sulfide (HS^-) reacts with sulfenic acid. Because of the intrinsic disulfide bridge the oxidized persulfides can get reduced back by enzymes such as thioredoxins (indicated by scissors) resulting in recovery of the native thiol. (B + C) Regulation of enzymatic activity. The persulfidation can occur outside of the active site causing conformational changes of the protein (B). These changes can result in different outcomes like enhancing protein-protein interaction, substrate affinity, subcellular localization or oligomerization. On the other hand, persulfidation can occur directly at the active site cysteine (C), where it can have inhibitory or enhancing effects.

detected (Dóka et al. 2016; Wedmann et al. 2016). In human cells, a large number of proteins are perthiosulfenylated even though they are not facing elevated H_2O_2 levels resulting from activation of NADPH oxidases DUOX1 and NOX2. This suggests possible functions of persulfide oxidation beyond a protective role of persulfidation (Heppner et al. 2018). The role of this persulfide derivative in redox signaling, however, awaits further characterization.

Besides the reduction of persulfide derivatives and subsequent recovery of the free thiol moieties, several examples for proteins exist, whose persulfidated form provides protection against oxidation. For the cytosolic pentose phosphate pathway enzyme glucose-6-phosphate dehydrogenase 6 (G6PD6) it was shown that persulfidation of a conserved cysteine (C159 in Arabidopsis and C155 in tomato) alleviates oxidative protein inactivation caused by elevated levels of H_2O_2 under salt stress. On the one hand, the persulfidation induces structural changes in the G6PD6 homotetramer that support stabilization of enzyme activity and promote the affinity to $NADP^+$. On the other hand, the persulfidation mitigates oxidative modifications of G6PD6 at C159/C155 that would result in enzyme inhibition (Wang et al. 2023).

In addition, persulfide levels are linked to protective physiological effects. For the antioxidant N-acetylcysteine, it was shown that its cytoprotective effect is based on the generation of supersulfides predominantly in mitochondria (Ezeriņa et al. 2018; Pedre et al. 2021). Furthermore, it was shown in human fibroblasts, *Caenorhabditis elegans* and mice that organisms with elevated persulfide levels had a longer lifespan compared to the respective control (Zivanovic et al. 2019). Additionally, in older individuals decreased amounts of persulfides but increased sulfinylation levels were detected supporting the idea that increased persulfidation improves the capacity to cope with stress stimuli and reactive oxygen species-related aging (Redman et al. 2018; Zivanovic et al. 2019). Arabidopsis null mutants lacking the L-cysteine desulfhydrase DES1 are characterized by a decreased sulfide content and a decreased level of protein persulfidation (Álvarez et al. 2010, 2012a; Aroca et al. 2017a). Phenotypically, these biochemical parameters correlate with premature leaf senescence. While so far only correlative, these observations may pave the way towards molecular analysis of the underlying mechanistic processes.

2.3 Identification of persulfidated cysteines and impact of persulfidation on protein activity

Basal protein persulfidation comprises nearly 5 % of the Arabidopsis proteome under non-stress conditions indicating other roles of persulfidation beyond mere protection of cysteines against oxidation (Aroca et al. 2017a; García-Calderón et al. 2023; Jurado-Flores et al. 2023). Several methods are available to detect persulfides *in vivo* based on their nucleophilic nature; each of them facing limitations like specificity or sensitivity (reviewed in Filipovic et al. 2018; Huang and Xie 2023; Pantaleno and Scuffi 2024). To detect protein persulfides, thiols and persulfides are frequently first alkylated by biotin-labeled alkylating agents like EZ-Link Iodoacetyl-PEG2-Biotin and pulled down from the protein mixture. Subsequently persulfides are separated in a reduction step leaving the non-reducible thiol-tagged biotin conjugates bound to the streptavidin beads (Dóka et al. 2016, 2021; Longen et al. 2016). Some alkylating agents like N-ethylmaleimide and iodoacetamide are, however, unsuitable as they convert the persulfides into thioethers (Schilling et al. 2022, 2023). The currently preferred method to detect protein persulfidation is the dimedone-switch method (for a detailed protocol see Aroca et al. 2022 and Zivanovic et al. 2019). The disadvantage of both methods is that they do not identify the persulfidated cysteine itself, but only the protein, which leaves ambiguities regarding the persulfidated thiols if more than one cysteine is present.

To identify specific persulfidated cysteine residues and their effect on enzyme activity, often recombinant proteins or cell lysates are treated with sulfide salts like NaHS and Na_2S or other sulfide donors like morpholino–phosphino–dithioic acid (GYY4137). These sulfide donors initially form polysulfides in solution, which subsequently are most likely the source for persulfidation of the respective cysteines rather than the sulfide donor itself (Greiner et al. 2013). Whether the thiol modification is specific for distinct cysteine thiols or also occurs non-specifically on other thiols needs to be demonstrated *in vivo*. A prominent example for a protein that is prone to artificial persulfidation is the cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For GAPDH from mouse liver lysates or recombinant human GAPDH treated with NaHS, it was shown that persulfidation of the catalytic cysteine C150 enhances the GAPDH activity (Mustafa et al. 2009). Similar treatment of recombinant human GAPDH with NaHS resulted in persulfidation of C247, whereas treatment with potassium polysulfide resulted in persulfidation of C247 and C156. The cysteine residue C152, which corresponds to C150 in mouse GAPDH, was not persulfidated in this case. After treatment with polysulfide, human GAPDH did not show an increased but rather a decreased activity (Jarosz et al. 2015). Hence, apart from improving specificity and sensitivity, another prerequisite for the detection of biologically meaningful protein persulfidation is the application of methods that directly identify persulfidated cysteine residues. This prerequisite is fulfilled

by the quantitative thiol reactivity profiling (low pH-QTRP) method in which the reduction step after alkylation is omitted to retain the labeled probes during digestion into tryptic peptides. Probe-labelled peptides are subsequently conjugated with a biotin-tag, captured by streptavidin beads and analyzed by mass spectrometry (Fu et al. 2020).

The effect of persulfidation on enzyme activity depends on the protein and the complexity of persulfide-induced changes bars making general statements about the respective consequences. The persulfidation can occur outside the active site and induce conformational changes (Figure 1B). These changes can attenuate the binding of the respective proteins to interaction partners, regulate oligomerization, affect substrate affinity or enhance certain subcellular sequestration (Aroca et al. 2017b; Chen et al. 2020; Ma et al. 2023; Wang et al. 2023). Similar to C156 in the human GAPDH, also the homologous residue C160 in the Arabidopsis orthologue GAPC1 can get persulfidated. NaHS treatment of Arabidopsis plants resulted in the persulfidation of the protein and translocation to the nucleus (Aroca et al. 2017b). A similar nuclear sequestration was also observed in cadmium-stressed plants suggesting a role of nuclear GAPC1 in oxidative stress signaling or protection (Vescovi et al. 2013). OPEN STOMATA 1 (OST1)/SNF1-RELATED PROTEIN KINASE2.6 (SnRK2.6) is expressed in guard cells and involved signaling pathways mediating stomatal closure. Two cysteines (C131 and C137) adjacent to the catalytic loop of the kinase were identified to be persulfidated *in vitro* after incubation of recombinant SnRK2.6 with either NaHS or the alternative sulfide donor GYY4137 (Chen et al. 2020). Furthermore, the persulfidation of SnRK2.6 was confirmed in Arabidopsis *ost1* null mutants by analyzing the differential SnRK2.6 persulfidation levels after complementation of *ost1* with wild-type SnRK2.6 or a mutated SnRK2.6$^{C131S\ C137S}$ variant. Persulfidation alters the SnRK2.6 structure bringing the pivotal phosphorylation site S175 closer to the phosphate-acceptor D140, which increases the kinase activity (Chen et al. 2020, 2021). Cys/Ser substitution variants expressed in the mutant background were not able to complement the drought-sensitive phenotype of *ost1* indicating an essential role of both Cys residues in regulating the protein activity (Chen et al. 2020, 2021). An example for persulfidation affecting the binding to interaction partners was shown recently for the transcription factor WRKY6 in tomato (Zhang et al. 2024). Persulfidation of WRKY6 at C396 attenuates binding to the W-box in the promoter of the chlorophyll degradation-related gene *SGR1* and the senescence-related gene *SAG12* in unripe tomato. During fruit ripening H_2S levels decrease and concomitantly the degree of WRKY6 persulfidation also decreases. Subsequently, the enhanced binding to the promotor of the respective WRKY6 target genes results in increased *SGR1* and *SAG12* expression, which leads to fruit ripening and plant senescence. Expression of *WRKY6 C396A* in the *wrky6* background resulted in enhanced expression of *SGR1* and *SAG12* accompanied with early fruit ripening. On the other hand, increasing H_2S levels either exogenously by fumigation or endogenously by overexpression of the cytosolic H_2S-generating enzyme L-cysteine desulfhydrase (LCD1) significantly delayed tomato fruit ripening indicating that persulfidation is important for the regulation of fruit ripening (Zhang et al. 2024).

Beside persulfidation of proteins outside their catalytic center, active site cysteines can get persulfidated resulting in increased or decreased activity (Figure 1C). The autophagy-related (ATG) Cys protease AtATG4a is persulfidated at C170 that is part of the catalytic Cys-His-Asp triad. This persulfidation inhibits AtATG4 activity based on conformational changes and intramolecular rearrangements of the catalytic site (Laureano-Marín et al. 2020). Also, for *S. cerevisiae* and *Chlamydomonas reinhardtii* ATG4 orthologues a redox regulation was shown, but so far no connection to persulfidation was made (Pérez-Pérez et al. 2014, 2016). For the cytosolic ascorbate peroxidase 1 (APX1) persulfidation at C32 was detected, which results in an increased activity (Aroca et al. 2015). Treatment of tomato plants with NaHS also increased APX activity in the respective crude extracts (Li et al. 2020).

2.4 Persulfidated proteins in Arabidopsis mitochondria

Although little is known about the effects of persulfidation on mitochondrial proteins, more than 80 mitochondrial proteins were identified in the reported Arabidopsis persulfidome (García-Calderón et al. 2023; Jurado-Flores et al. 2023) (Table 1). Mitochondria are the hub for central metabolic processes including the tricarboxylic acid cycle (TCA cycle), amino acids biosynthesis, as well as the production of several cofactors like iron sulfur cluster, lipoic acid or the precursor of molybdenum cofactor. Hence, a careful and precise regulation of metabolic dynamics in order to adapt metabolism and signal transduction has to be considered. The current proteomic data for the persulfidome in plants represents a valuable starting point for deciphering the actual targets of persulfidation and to further elucidate the molecular function of this cysteine modification.

Based on mitochondrial functions, it is not surprising that a global KEGG analysis (at least 7 proteins have to cluster) of the respective proteins associate them with glyoxylate metabolism, pyruvate metabolism, the TCA cycle, oxidative phosphorylation or the propanoate pathway

Table 1: Persulfidated mitochondrial proteins in Arabidopsis.

Persulfidated mitochondrial proteins found in García-Calderón et al. (2023) and Jurado-Flores et al. (2023)	Protein description	Protein ID
At1g11860	Aminomethyltransferase (glycine cleavage system T protein, GLDT)	O65396
At1g48520	Glutamyl-tRNA(Gln) amidotransferase subunit B (GATB)	Q9FV81
At5g14780	Formate dehydrogenase (FDH1)	Q9S7E4
At2g20420	Succinate-CoA ligase subunit β	O82662
At5g08300	Succinate-CoA ligase subunit *α*-1	P68209
At5g23250	Succinate-CoA ligase subunit *α*-2	Q8LAD2
At3g07770	Heat shock protein 90-6 (HSP90.6)	F4JFN3
At5g26830	Threonine-tRNA ligase 1 (SYT1)	O04630
At1g79230	3-Mercaptopyruvate sulfurtransferase 1 (MST1)	O64530
At3g61440	L-3-cyanoalanine synthase/cysteine synthase C1 (CYSC1)	Q9S757
At1g29880	Glycine-tRNA ligase 1 (GlyRS-1)	O23627
At4g13850	Glycine-rich RNA-binding protein 2 (GRP2)	Q9SVM8
At5g20080	NADH-cytochrome b5 reductase-like protein (B5R)	P83291
At4g02930	Elongation factor Tu	Q9ZT91
At5g10860	CBS domain-containing protein (CBSX3)	Q9LEV3
At5g07440	Glutamate dehydrogenase 2 (GDH2)	Q38946
AtMg01190	ATP synthase subunit *α* (ATP1)	P92549
At3g22200	Gamma-aminobutyrate transaminase POP2 (GABA-T)	Q94CE5
At4g08390	Stromal L-ascorbate peroxidase (sAPX)	Q42592
At4g00570	NAD-dependent malic enzyme 2 (NAD-ME2)	Q8L7K9
At2g13560	NAD-dependent malic enzyme 1 (NAD-ME1)	Q9SIU0
At2g47510	Fumarate hydratase 1 (fumarase 1, FUM1)	P93033
At2g05710	Aconitate hydratase 3 (aconitase 3, ACO3)	Q9SIB9
At3g59760	Cysteine synthase (O-acetylserine (thiol)-lyase, OAS-TL C)	Q43725
At3g13860	Chaperonin CPN60-like 2 (HSP60-like 2)	Q93ZM7
At3g02090	Mitochondrial-processing peptidase subunit *β* (MPPBETA)	Q42290
At1g32470	Glycine cleavage system H protein (GDC-H1)	Q9LQL0
At4g39660	Alanine-glyoxylate aminotransferase 2 (AGT2)	Q940M2
At2g17130	Isocitrate dehydrogenase II (IDH-II)	P93032
At2g26080	Glycine dehydrogenase 2 (Glycine cleavage system P protein 2, GLDP2)	O80988
At4g33010	Glycine dehydrogenase 1 (glycine cleavage system P protein 1, GLDP1)	Q94B78
At5g03290	Isocitrate dehydrogenase V (IDH-V)	Q945K7
At3g09810	Isocitrate dehydrogenase VI (IDH-VI)	Q8LG77
At1g17290	Alanine aminotransferase 1 (ALAAT1)	F4I7I0
At4g26970	Aconitate hydratase 2 (aconitase 2, ACO2)	Q94A28
At5g26780	Serine hydroxymethyltransferase 2 (SHMT2)	Q94C74
At4g35260	Isocitrate dehydrogenase I (IDH-I)	Q8LFC0
At1g26460	Pentatricopeptide repeat-containing protein	Q9FZD1
At2g33210	Chaperonin CPN60-like 1 (HSP60-like 1)	Q8L7B5
At2g33040	ATP synthase subunit gamma (ATP3)	Q96250
At4g33650	Dynamin-related protein 3A (dynamin-like protein 2, ADL2)	Q8S944
At4g08900	Arginine amidohydrolase 1 (ARGAH1)	P46637
At4g08870	Arginine amidohydrolase 2 (ARGAH2)	Q9ZPF5
At4g37910	Heat shock 70 kDa protein 9 (HSP70-9)	Q8GUM2
At4g15940	Fumarylacetoacetate hydrolase domain-containing protein 1 (FAHD1A)	Q93ZE5
At1g53580	Persulfide dioxygenase (sulfur dioxygenase, ETHE1)	Q9C8L4
At5g09590	Heat shock 70 kDa protein 10 (HSP70-10)	Q9LDZ0
At2g14170	Methylmalonate-semialdehyde dehydrogenase (MM-ALDH)	Q0WM29
At1g72330	Alanine aminotransferase 2 (ALAAT2)	Q9LDV4
At5g08680	ATP synthase subunit *β*-3	Q9C5A9
At2g35370	Glycine cleavage system H protein (GDCH)	P25855
At4g37930	Serine hydroxymethyltransferase 1 (SHMT1)	Q9SZJ5
At4g11600	Glutathione peroxidase-like 6 (GPX6)	O48646
At4g13360	3-Hydroxyisobutyryl-CoA hydrolase-like protein 3	Q9T0K7

Table 1: (continued)

Persulfidated mitochondrial proteins found in García-Calderón et al. (2023) and Jurado-Flores et al. (2023)	Protein description	Protein ID
At4g02580	NADH dehydrogenase flavoprotein 2	O22769
At3g15640	Cytochrome c oxidase subunit 5b-1 (COX5b-1)	Q9LW15
At1g47420	Succinate dehydrogenase subunit 5 (SDH5)	Q9SX77
At5g66760	Succinate dehydrogenase subunit 1 (SDH1-1)	O82663
At4g34700	NADH dehydrogenase 1 β subcomplex subunit 9 (CIB22)	Q945M1
At1g22840	Cytochrome c-1 (CYTC-1)	O23138
At1g51980	Insulinase (peptidase family M16) protein	Q9ZU25
At1g79440	Succinate-semialdehyde dehydrogenase (SSADH1)	Q9SAK4
At2g35010	Thioredoxin O1, (TRXo1)	O64764
At3g06050	Peroxiredoxin IIF (PRXIIF)	Q9M7T0
At1g53240	Malate dehydrogenase 1 (MDH1)	Q9ZP06
At3g17240	Dihydrolipoyl dehydrogenase 2 (mLPD2)	Q9M5K2
At1g48030	Dihydrolipoyl dehydrogenase 1 (mLPD1)	Q9M5K3
At3g15020	Malate dehydrogenase 2 (MDH2)	Q9LKA3
At5g50850	Pyruvate dehydrogenase E1 component subunit β-1 (PDHE1-B)	Q38799
At3g13930	Pyruvate dehydrogenase subunit 2-2 (mtE2-2)	Q8RWN9
At1g54220	Pyruvate dehydrogenase subunit 2–3 (mtE2-3)	Q5M729
At2g44350	Citrate synthase 4 (ATCS)	P20115
At1g59900	Pyruvate dehydrogenase E1 component subunit α-1 (PDHE1-A)	P52901
At3g10920	Manganese superoxide dismutase (MSD1)	O81235
At5g65620	Organellar oligopeptidase A (OOP)	Q94AM1
At3g48000	Aldehyde dehydrogenase family 2 member B4 (ALDH2B4)	Q9SU63
At2g20360	NADH dehydrogenase 1 α subcomplex subunit 9	Q9SK66
At5g51970	Sorbitol dehydrogenase (SDH)	Q9FJ95
At4g29130	Hexokinase-1 (HXK1)	Q42525
At5g47770	Farnesyl pyrophosphate synthase 1 (FPS1)	Q09152
At1g50200	Alanine-tRNA ligase (Alanyl-tRNA synthetase, ALATS)	P36428
At3g25660	Glutamyl-tRNA amidotransferase subunit A (Glu-AdT subunit A)	Q9LI77

Persulfidated proteins have been independently detected in two indicated publications with the dimedone switch method and subsequently identified by LC–MS/MS analysis (Aroca et al. 2022). The respective protein IDs in both publications were screened for mitochondrial localization (Consortium et al. 2023) and only included here if they were consistently detected in both data sets. Additional proteins that appeared in only one data set (38 in García-Calderón et al. (2023) and 76 in Jurado-Flores et al. (2023) were excluded from the Table.

(Figure 2A). A more detailed gene ontology (GO) analysis of the biological processes (at least 3 proteins have to cluster) indicate that nearly 50 % of the proteins are involved in amino acid catabolism, pyruvate metabolism or cellular respiration (Figure 2B). The other GO categorizations revealed that the persulfidated proteins play a role in several important biological processes like photorespiration. Also, the GO term 'response to cadmium (Cd) ion' is present.

This can potentially be linked to previous reports in which Arabidopsis mutants lacking mitochondrial D-cysteine desulfhydrase (DCDES1, see 3.4 D-cysteine desulfhydrase), which is involved in H_2S production, where shown to be more sensitive to Cd, while transgenic lines overexpressing *DCDES1* were more tolerant to Cd stress when compared with wild-type plants (Zhang et al. 2020). Under normal conditions, the content of H_2S was similar in wild-type plants and *dcdes1* null mutants, but H_2S production was impaired in *dcdes1* plants under cadmium stress. Cd stress stimulated the expression of DCDES1 in a process mediated by the Cd-induced transcription factor WRKY13, which binds to the *DCDES1* promoter (Zhang et al. 2020). Also in alfalfa (*Medicago sativa*) increased DCDES activity was observed in less than 1 h after exposure to Cd-stress (Cui et al. 2014).

3 H_2S and protein persulfide-generating enzymes in mitochondria

Mitochondria are key hubs of plant metabolism and continuously need to modulate their function to cope with changing environmental conditions. Changes frequently occur on short time scales, i.e. within seconds, and

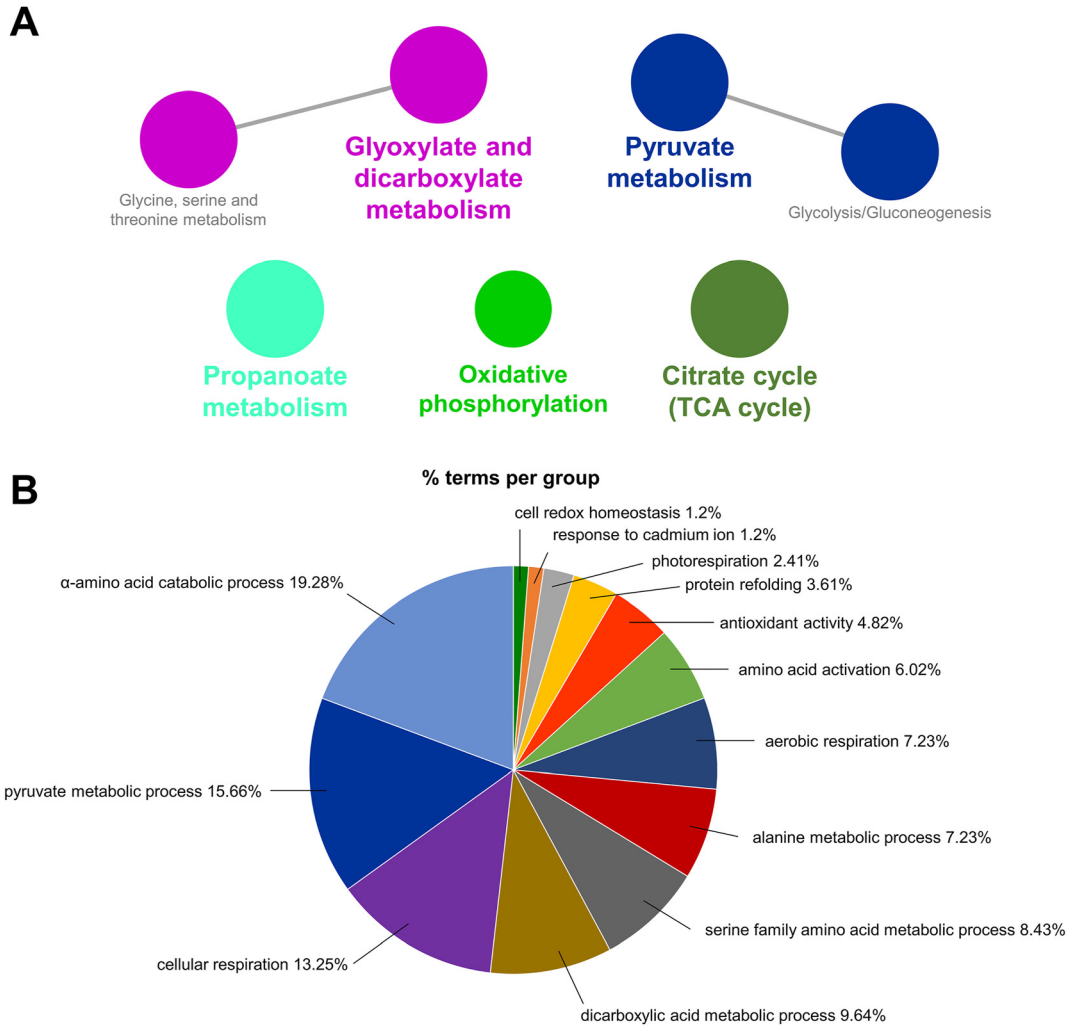

Figure 2: Functional classification of persulfidated proteins localized to mitochondria. (A) KEGG pathways associated to the mitochondrial proteins listed in Table 1. At least seven proteins had to cluster to represent a pathway. (B) Functional classification of gene ontology terms categorized by biological processes. At least three proteins had to cluster to define a group. All results were created with ClueGO v2.5.10 using Cytoscape (https://cytoscape.org/).

redox-associated PTMs are one attractive way to provide the respective adaptability (Møller et al. 2020) In eukaryotic organisms, enzymes that are involved in the generation of supersulfides are located in the cytosol and mitochondria (Figure 3). In most photosynthetic organisms, the sulfide-generating enzyme sulfite reductase (SIR) is exclusively localized in plastids where it reduces sulfite to sulfide predominantly with electrons provided by the photosynthetic machinery (Khan et al. 2010; Patron et al. 2008). In Arabidopsis mitochondria, putative candidates for supersulfide production are the β-cyanoalanine synthase (CAS-C1), MST, cysteinyl-tRNA synthetase (CARS), and D-cysteine desulfhydrase (DCDES). The abundances of these proteins in mitochondria isolated from 7-day old Arabidopsis suspension culture cells differ substantially: CAS-C1 is one of the most abundant mitochondrial proteins with 12,218 copies per organelle, followed by MST (2,776 copies), CARS (119 copies) and lastly DCDES with only 95 copies (Fuchs et al. 2020).

3.1 β-Cyanoalanine synthase

A pathway for H_2S production present in plants and bacteria is the conversion of cyanide and cysteine into β-cyanoalanine and H_2S catalyzed by the pyridoxal 5′-phosphate (PLP)-dependent β-cyanoalanine synthase CAS-C1 (syn. CYSC1; At3g61440; EC 4.4.1.9) (Arenas-Alfonseca et al. 2018; Machingura et al. 2016; Yamaguchi et al. 2000). Also, in some arthropod lineages, like *Lepidoptera* and some mites, a gene with putative CAS function was identified (Wybouw et al. 2014). Closely related to O-acetylserine (thiol) lyases, that synthesize cysteine from O-acetylserine and sulfide, CAS

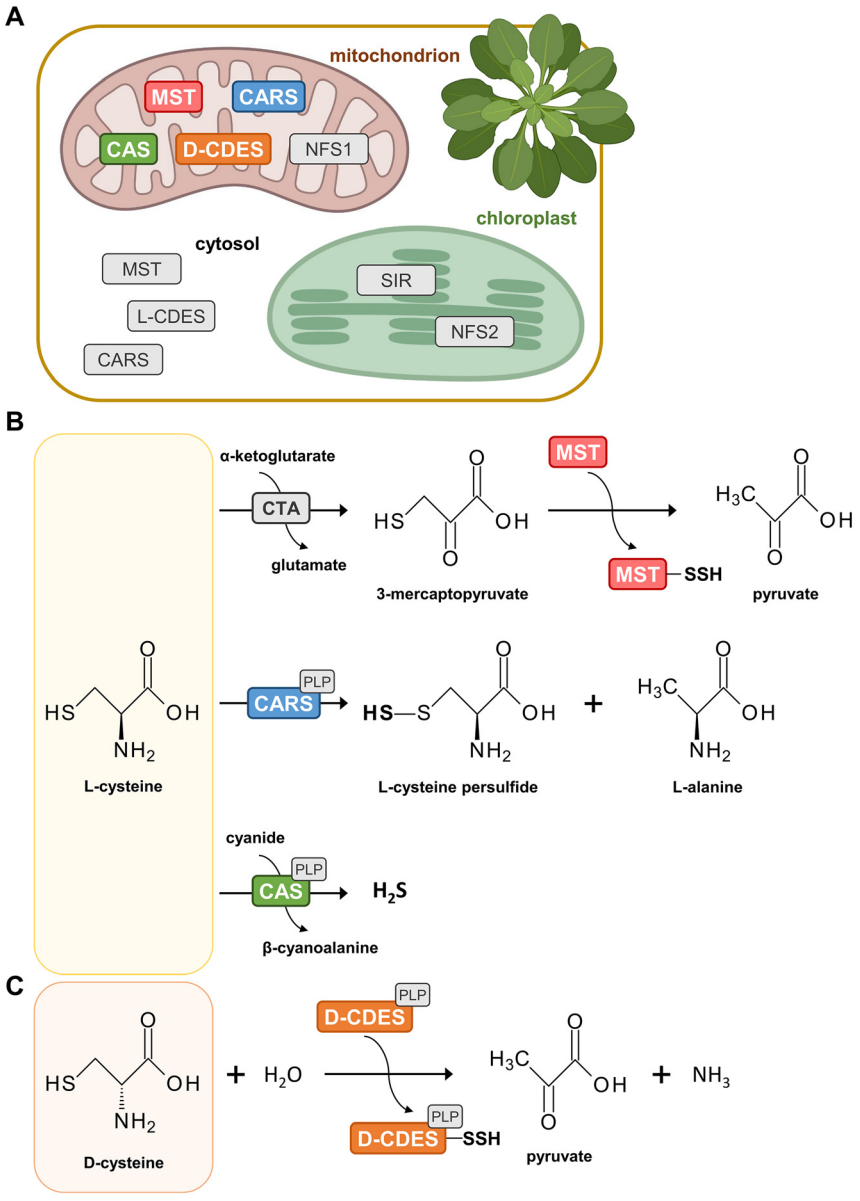

Figure 3: Plant proteins that contribute to sulfide and persulfide generation. (A) Subcellular localization of proteins that are implicated to play a role in sulfide generation or direct persulfidation of target thiols in plant cells. NFS1 and NFS2 are cysteine desulfurases delivering sulfide in form of a persulfide for the iron-sulfur cluster assembly. As this pathway is not contributing to protein persulfidation, it was excluded from this review although another role of these proteins cannot be excluded. The figure was partially generated with Biorender.com. (B) Reaction mechanism of MST, CARS and CAS-C1 using L-cysteine as substrate. The cysteine transaminase in plants is not identified yet. (C) Reaction mechanism of D-CDES using D-cysteine as substrate. Generation of the persulfidated D-CDES is based on findings on L-CDES (Shen et al. 2020). MST = 3-mercaptopyruvate sulfurtransferase, CAS = β-cyanoalanine synthase (CAS-C1), CARS = cysteinyl-tRNA synthetase, NFS1 = cysteine desulfurase 1 (Syn. Nitrogen Fixation S (NIFS)-like 1), D-CDES = D-cysteine desulfhydrase, L-CDES = L-cysteine desulfhydrase (DES1), SIR = sulfite reductase, NFS2 = cysteine desulfurase 2, CTA = cysteine transaminase.

enzymes from various plant species (Arabidopsis, spinach, lupins, potato, rice) unanimously favor CAS activity over cysteine synthase activity (Hatzfeld et al. 2000; Hendrickson and Conn 1969; Lai et al. 2009). Based on a crystal structure of soybean CAS, this functional specialization was linked to structural differences in the PLP binding sites of these enzyme classes (Yi et al. 2012). Mitochondria are the major target of cyanide which inhibits cytochrome c oxidase (COX) by irreversibly binding to the heme iron (Antonini et al. 1971). In plants, cyanide derives from either the catabolism of cyanogenic glycosides or from ethylene biosynthesis, where cyanide is a degradation product of 1-aminocyclopropane-1-carboxylate (ACC) (Siegień and Bogatek 2006). Interestingly, it was shown in tomato that persulfidation of ACC oxidases 1 and 2 negatively regulates ethylene biosynthesis (Jia et al. 2018). Plants harness the toxicity of cyanide for protection against herbivores by hydrolyzing cyanogenic glycosides upon herbivory damage (Machingura et al. 2016). In *Brassicaceae*, the degradation of glucosinolates can also contribute to cyanide generation (Machingura et al. 2016). The main pathway for cyanide detoxification occurs via the mitochondrial CAS-C1 (Machingura et al. 2016). The *cas-c1* null mutant has no obvious shoot phenotype but is defective in root hair formation and accumulates cyanide in root tissues. The observed root hair phenotype is likely due to the accumulation of cyanide since the root hair defect is phenocopied in WT plants treated with cyanide. Furthermore, an increase of the alternative oxidase pathway in the *cys-c1* mutant was

observed, indicating an inhibition of COX by cyanide (García et al. 2010). Considering the high abundance of the protein in mitochondria, but low cyanide concentrations, since the IC_{50} for COX is 7 nM cyanide, CAS-C1 is probably a key enzyme for cyanide detoxification rather than supersulfide generation (Birke et al. 2012; Fuchs et al. 2020). No information, however, is available to which extent this pathway contributes to supersulfide generation leaving this question open for further investigations.

3.2 3-Mercaptopyruvate sulfurtransferases

3-Mercaptopyruvate sulfurtransferases (EC 2.8.1.2, MSTs) belong to the sulfurtransferase (STR) family and contain two characteristic rhodanese domains connected by a short linker peptide (Spallarossa et al. 2004). The active site locates at the interface between these domains and the catalytic cysteine residue is part of a canonical CG[S/T]GVT motif within the C-terminal domain (Yadav et al. 2013). Across kingdoms, MSTs are highly conserved and in Arabidopsis represented by two sulfurtransferases MST1 (syn. STR1; At1g79230) in mitochondria and MST2 (syn. STR2; At1g16460) in the cytosol (Nakamura et al. 2000; Moseler et al. 2020). All photosynthetic organisms (including cyanobacteria) possess at least one MST. In all cases where only one gene coding for MST is present, the MST contains an N-terminal extension that is predicted to function as a target peptide for mitochondrial localization (Moseler et al. 2020). The cytosol and the mitochondria of human cells both contain an MST (syn. tRNA thiouridin modification protein 1, TUM1) which are derived from two splice variants of the same gene (Fräsdorf et al. 2014). While good evidence exists for the function of MSTs in supersulfide generation in bacteria and vertebrates (Lec et al. 2018; Pedre and Dick 2021; Pedre et al. 2023; Yadav et al. 2020), little is known about their counterparts in plants. Arabidopsis *mst1* null mutants show an ambiguous shrunken seed phenotype with ~87 % of embryos arrested at heart stage (Mao et al. 2011). The remaining 13 % mature without any phenotypic aberrations compared to wild-type controls. In contrast, *mst2* null mutants generally appear like the wild type (Mao et al. 2011; Nakamura et al. 2000). The *mst1 mst2* double mutant, however, is embryonically lethal at heart stage indicating functional redundancy of both MSTs in an essential metabolic process (Mao et al. 2011).

In vitro, Arabidopsis MSTs catalyze the desulfuration of 3-mercaptopyruvate (3-MP) to pyruvate while generating a persulfide on their catalytic cysteine (Moseler et al. 2021). Subsequently, the outer sulfur atom of the persulfide is transferred to an acceptor molecule, either a low-molecular weight thiol or a thiol-containing protein. So far, protein-protein interactions of Arabidopsis MSTs were observed only with TRXs through bimolecular fluorescence complementation (BiFC) (Henne et al. 2015). The presumed transsulfuration reaction from the MST persulfide to the dithiol TRX and the subsequent substitution of sulfide by the second TRX cysteine should lead to the formation of H_2S. This principal possibility has been demonstrated with the release of H_2S from 3-MP in the presence of the TRX system comprising the TRX, NADPH-dependent thioredoxin reductase (NTR) and NADPH or other reductants *in vitro* (Figure 4) (Moseler et al. 2021). Besides TRXs, low molecular weight thiols like glutathione or cysteine can also act as sulfur acceptors of MSTs (Höfler et al. 2016; Kimura et al. 2017; Moseler et al. 2021). For Arabidopsis MST1, the catalytic efficiency with mitochondrial TRXo1 as acceptor was, however, 100-fold higher when compared to GSH ($1.3 \times 10^6\,M^{-1}\,s^{-1}$ and $2.7 \times 10^4\,M^{-1}\,s^{-1}$, respectively) (Moseler et al. 2021). The binding affinity between MST1 and TRXo1 (K_m = 5.3 µM) falls into a physiological meaningful range and compares well with that of human MSTs (MPST1 and MPST2) with thioredoxin TXN (Yadav et al. 2020). GSH concentrations in mitochondria of plant cells are in the low millimolar range (Fricker et al. 2000) and exceed the binding affinity between MST1 and GSH (K_m = 200 µM), suggesting that MST1 is continuously saturated with GSH to generate GSSH. However, the total pool of accessible protein thiols in mitochondria may reach concentrations comparable to that of GSH (Requejo et al. 2010). Considering the higher binding affinities of MSTs to protein clients (Moseler et al. 2021; Yadav et al. 2020), an MST-mediated protein persulfidation is thus possible despite comparatively high GSH concentrations. Indeed, for MST from *S. cerevisiae*, it was shown that protein clients are the most efficient sulfur acceptors *in vitro*, with the exception of sulfite (Pedre et al. 2023). In the absence of other putative interaction partners, both Arabidopsis MSTs can transfer the persulfide to redox-sensitive green fluorescent protein 2 (roGFP2) revealing them as putative protein persulfidases (Moseler et al. 2021). Recently, it was shown that partial depletion of MST in human cells significantly lowers cellular protein persulfidation levels (Pedre et al. 2023). Seven of the 64 identified persulfidation targets of human MST are predicted to be localized in mitochondria (Consortium et al. 2023; Pedre et al. 2023). All seven human proteins have homologs in Arabidopsis with five of them sharing conserved cysteines. One of these, isocitrate dehydrogenase (IDH3A) was also found to be persulfidated in Arabidopsis, highlighting potential similarities in persulfidation pattern (García-Calderón et al. 2023; Jurado-Flores et al. 2023). Also in the heart tissue of MST knock-out mice, a reduced persulfidation of 24 proteins was found

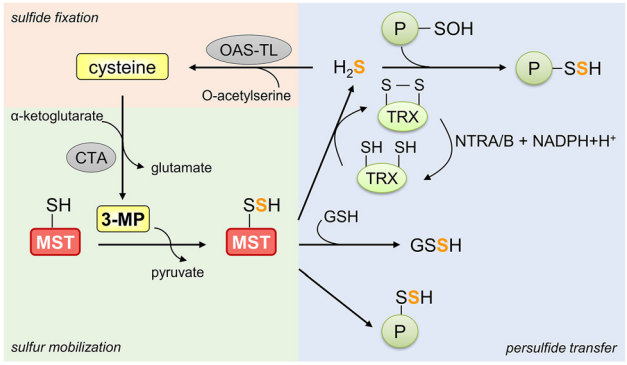

Figure 4: The putative role of Arabidopsis MST1 in persulfide formation. Cysteine is deaminated to 3-mercaptopyruvate (3-MP) by a yet unknown cysteine transaminase (CTA). Subsequently, MST1 subtracts the sulfur from 3-MP by forming a persulfide on its active site cysteine along with the release of pyruvate. The persulfide can then be transferred to several acceptors. One possibility are low molecular weight molecules like GSH to form glutathione persulfide (GSSH). Other presumed persulfide acceptors are proteins (P), but so far, no evidence for direct persulfidation of distinct proteins exist except for thioredoxins (TRX). In the latter case, the persulfide is transferred from MST1-SSH to the N-terminal cysteine in the CxxC active site cysteine of the TRX. Subsequently, the C-terminal resolving cysteine of the active site motif attacks the persulfide to release H_2S and oxidized TRX. The TRX disulfide is then reduced by thioredoxin reductases (NTRA/B) with NADPH + H^+ as their electron source. H_2S can react with sulfenylated (-SOH) proteins resulting in the protein persulfidation. Excess H_2S can be detoxified via OAS-TL c, which converts sulfide and O-acetylserine to cysteine. Green indicates the sulfur mobilization, blue the persulfide transfer and red the sulfide fixation.

(Drekolia et al. 2024). Comparison with the mitochondrial Arabidopsis persulfidome (Table 1) revealed several homologs to be persulfidated also in Arabidopsis like the malate dehydrogenases (MDH) or succinate dehydrogenase (SDH1-1).

Plant MSTs thus are candidates to produce H_2S and also candidates to act as persulfidases that persulfidate proteins or LMW molecules without releasing H_2S. The presence of two isoforms, MST1 and MST2, in Arabidopsis in two compartments and yet partially redundant function calls for further genetic and biochemical characterization of these enzymes to clarify their function *in vivo*.

3.3 Cysteinyl-tRNA synthetases

Other highly conserved enzymes related to supersulfide formation in both cytosol and mitochondria are the cysteinyl-tRNA synthetases (CARSs, Cysteine-tRNA ligase; EC 6.1.1.16) (Ogata et al. 2023). Generally, CARS plays a role in protein synthesis by producing cysteinyl-tRNA using cysteine and aminoacyl-tRNA as substrate. CARS can, however, also convert cysteine to Cys-SSH and Cys-S(S)$_n$H using cysteine as sulfur donor, which is described as cysteine persulfide synthase (CPERS) activity of CARS (Akaike et al. 2017). In Arabidopsis, three CARSs are present. CARS1 (At3g56300) and CARS2 (At5g38830) are cytosolic, CARS3 (syn. SYCO; At2g31170) is dual-localized in plastids and mitochondria, and, in addition, a splice variant is predicted to code for a cytosolic isoform (Peeters et al. 2000). While there is no information on the role of CARS1 and CARS2, an essential function of CARS3 in plant development is evident from embryo lethality of homozygous *cars*3 null mutants. This appears to be consistent with the situation in mice where deletion of mitochondrial CARS2 is lethal (Akaike et al. 2017). In Arabidopsis, the lethality is based on the inability of the two haploid polar nuclei to fuse in the central cell of the female gametophyte (Kägi et al. 2010). Furthermore, *cars*3 mitochondria of the central cell lacked regular cristae underpinning the role of mitochondria during nuclear fusion (Kägi et al. 2010; Portereiko et al. 2006).

Bacterial CARS as well as mitochondrial CARSs from human and mice are able to generate cysteine persulfides using a PLP-cofactor (Akaike et al. 2017). In this reaction, the PLP-cofactor is coordinated by lysine residues including a $_{124}$KIIK$_{127}$ and a $_{317}$KMSK$_{320}$ motif (numbering is based on human CARS2). In Arabidopsis CARS3, the KMSK motif is conserved while the KIIK motif is converted to KIIA. Although only the residues K124 and K317 are involved in PLP-binding, it was shown for *Escherichia coli* CARS that individual K-to-A substitutions of all four lysine residues have no effect on protein synthesis but result in decreased CPERS activity (Akaike et al. 2017). This observation leads to the question whether the corresponding Arabidopsis protein does have CPERS activity at all? Nevertheless, in yeast or zebrafish CARS2, the PLP-binding site reads KIIL or KIIQ rather than the KIIK (Ogata et al. 2023). Yeast CARS2 was shown to exhibit a CPERS activity indicating some possible variations in the motif (Nishimura et al. 2024).

In *S. cerevisiae*, mutation of the first lysine in the KIIK motif to alanine causes the same enzymatic changes observed in *E. coli*. Notably, this yeast mutant showed an impaired mitochondrial energy metabolism with decreased ATP levels (Nishimura et al. 2024). Also, for human cells, the knockdown of mitochondrial CARS2, which resulted in decreased Cys-SSH content, alters mitochondrial morphogenesis and bioenergetics. Knockdown of cytosolic CARS1, however, showed no significant decrease of Cys-SSH. The observations together demonstrate that the formation of Cys-SSH occurs more frequently in mitochondria (Akaike et al. 2017). An interesting aspect is that Cys-SSH generated by CARS2 in human cells can serve as direct acceptor for electrons from the mitochondrial electron transport chain (ETC) resulting in HS^- production. Induction of

mitochondrial DNA deprivation as well as specific inhibition of complex III by antimycin A decreased HS^- levels, indicating that Cys-SSH is directly reduced by electrons from the ETC. HS^- is subsequently used by sulfide quinone oxidoreductase (SQR) generating GSSH, which can be further oxidized to sulfite by the persulfide dioxygenase ETHE1 (ETHYLMALONIC ENCEPHALOPATHY 1). On the one hand, this mechanism prevents excessive generation of ROS by leaked electrons from the ETC and on the other hand it prevents sulfide toxicity (Akaike et al. 2017; Barayeu et al. 2023a). In plants, however, SQR is not present excluding thereby this supersulfide-mediated electron flow (see Section 4. Protein depersulfidation and refixation of sulfide).

A new aspect that was introduced by Akaike and colleagues is that CARS are not posttranslational modifiers of cysteine residues, but can co-translationally affect protein synthesis through the generation of persulfidated cysteinyl-tRNA and subsequent incorporation of cysteine persulfide and polysulfides into the nascent polypeptide chain during translation (Akaike et al. 2017). The function and stability of persulfidated cysteine residues in the native polypeptide chain, however, remains elusive.

3.4 D-Cysteine desulfhydrase

Cysteine desulfhydrases (CDES) are PLP-dependent enzymes that decompose cysteine to produce pyruvate, ammonium, and H_2S. Cysteine desulfhydrases are widely conserved in bacteria and plants and were shown to use L-cysteine (L-cysteine desulfhydrases; LCDES; EC 4.4.1.28) and/or D-cysteine (D-cysteine desulfhydrases; DCDES; EC 4.4.1.15) as substrates (Nagasawa et al. 1985; Riemenschneider et al. 2005b; Schmidt 1982; Todorovic and Glick 2008). The Arabidopsis genome encodes at least four proteins with reported CDES activity: LCD (At3g62130) and DES1 (At5g28030) that are thought to reside in the cytosol and to exclusively accept L-cysteine as substrate (Álvarez et al. 2010; Shen et al. 2012) and DCDES1 (At1g48420) and DCDES2 (At3g26115) that accept D-cysteine as substrate (Riemenschneider et al. 2005a). While the subcellular localization of DCDES2 has not been experimentally determined, several lines of evidence support DCDES1 mitochondrial localization (Riemenschneider et al. 2005b; Senkler et al. 2017; Fuchs et al. 2020). DCDES1 thus likely contributes to mitochondrial H_2S production. Indeed, Arabidopsis *dcdes1* null mutants display a significantly decreased H_2S level (Li et al. 2018). Furthermore, alterations of the *DCDES1* transcript level result in phenotypes that were also linked with exogenous H_2S application on plants, including cadmium tolerance and fruit ripening (Jia et al. 2016; Mostofa et al. 2015; Yao et al. 2018; Zhang et al. 2020; Zhao et al. 2023). Growth of Arabidopsis seedlings on cadmium can induce DCDES1 expression and elevate the total H_2S production rate (Zhang et al. 2020), effects that were previously also shown for the cytosolic DES1 (Jia et al. 2016). Similarly, constitutive heterologous expression of Arabidopsis *DCDES1* in *E. coli* lead to increased H_2S levels and enhanced cadmium tolerance (Shen et al. 2012). Notably, Arabidopsis DCDES1 can apparently be persulfidated (Jurado-Flores et al. 2023), allowing to speculate about the integration of DCDES1 in regulatory loops as it was shown for DES1 (Shen et al. 2020). Worth mentioning is that it was shown that elevated H_2S levels improved plant growth under Cd-stress by reduction of cadmium-induced oxidative stress in rice and alfalfa (Cui et al. 2014; Mostofa et al. 2015). In rice plants suffering Cd-stress, $O_2^{\cdot-}$ and H_2O_2 levels were reduced when treated with NaHS (Mostofa et al. 2015). Considering the protective effects of persulfides as mentioned above (2.1. Protective effect of persulfides by enhanced oxidant scavenging) this could explain these results although they have to be confirmed for the mitochondrial compartment.

Despite these correlative observations, our understanding of D-cysteine's physiological importance and the mechanistic links between increased H_2S levels and the observed phenotypes remains largely limited. While conservation of *DCDES* genes across the green linage argues for a functional significance, the source of D-cysteine, as substrate to produce H_2S, is unclear. In mammals, D-cysteine was suggested to be endogenously produced by serine racemase (SR), although recombinantly produced mammalian SR showed no measurable L- to D-cysteine conversion activity *in vitro* (Semenza et al. 2021; Shibuya et al. 2013). Arabidopsis possesses an SR homolog (At4g11640), but no L- to D-cysteine racemase activity has been demonstrated yet. While other D-amino acids, such as D-alanine, were shown to be present in soil and to be taken up directly by Arabidopsis, no such uptake data has been described for D-cysteine (Brodowski et al. 2005; Gördes et al. 2011).

4 Protein depersulfidation and refixation of sulfide

4.1 Depersulfidation of proteins via cellular reducing systems

To harness persulfidation for the dynamic switching of protein behavior and characteristics, cells must also be able to selectively depersulfidate proteins. In mammals, the thioredoxin (Trx)/thioredoxin reductase (TrxR) system was

shown to play a major role in protein depersulfidation (Dóka et al. 2016; Wedmann et al. 2016) as mentioned above (Section 2.2. Protective effect of persulfidation). TrxR, in concert with human Trx1 and the Thioredoxin-related Protein of 14 kDa (TRP14), was shown to reduce bovine serum albumin persulfide (BSA-SSH) *in vitro* and chemical/genetic interference with the Trx/TrxR system led to global increases of persulfidation levels in human cells (Dóka et al. 2016; Wedmann et al. 2016). Mammalian selenocysteine (Sec)-TrxR itself exhibits polysulfide-reducing activity *in vitro* that is improved by the presence of Trxs (Dóka et al. 2016). In contrast, Arabidopsis NADPH-dependent thioredoxin reductase NTRB, which does not possess a selenocysteine residue, has no depersulfidation activity (Moseler et al. 2021). Whether plant TRXs also regulate cellular persulfidation by restoring reduced thiols has not yet been tested. At least *in vitro*, cytosolic TRXh1 and mitochondrial TRXo1 and TRXo2 were able to interact with MSTs to release H_2S and restore the activity of persulfidated MSTs after oxidative stress by H_2O_2 treatment (Moseler et al. 2021). Canonical Trxs are characterized by the presence of two cysteine residues in a CxxC motif in their active site, both of them being indispensable for their disulfide and also persulfide reducing activity. Accordingly, a decreased H_2S release was observed in the presence of Trx variants mutated for either cysteines (Moseler et al. 2021). Trx exerts its depersulfidation activity through two possible mechanisms (Libiad et al. 2018; Vignane and Filipovic 2023). The first possibility is that the outer sulfur of the persulfide is transferred in a transpersulfidation reaction to the catalytic cysteine of the Trx to transiently form Trx-SSH. Trx-SSH is then attacked by the resolving cysteine of Trx to displace HS^- and form an intramolecular disulfide bond within Trx. Alternatively, one of the two Trx cysteines could directly attack the inner persulfide sulfur to eliminate H_2S and form a mixed Trx-target disulfide. The Trx-target disulfide would then further react with the resolving cysteine of Trx to substitute the target and form an intramolecular disulfide bond within Trx. Because thiol-disulfide exchange reactions occur through a 180° attack of the free thiol on a disulfide, it can be assumed the mechanism through which the reaction proceeds is determined by steric hindrance (Lillig and Berndt 2013). In human cells, it was shown that Trx1 forms mixed disulfides with pyruvate carboxylase and GAPDH indicating a preference for the second mechanism with an attack on the inner sulfur atom (Ju et al. 2016). Also, the glutaredoxin (GRX) system including a class I GRX, GSH, glutathione reductase and NADPH is able to reduce protein persulfides (Dóka et al. 2016; Moseler et al. 2021). Similar to TRXs, class I GRXs contain two cysteines in their active site. In the reaction mechanism, however, only the catalytic cysteine is required (Moseler et al. 2021). In plant mitochondria, no class I GRX is present excluding therefore this mechanism of protein depersulfidation (Schlößer et al. 2024). Importantly, independent of the reducing system involved, protein depersulfidation can generate free sulfide.

4.2 Detoxification of sulfide

Sulfide is a potent, non-competitive, reversible inhibitor of cytochrome *c* oxidase (COX; syn. complex IV) in the mitochondrial ETC. It binds to the oxidized states of the binuclear center of COX and the inhibited state is assumed to have one sulfide bound to the reduced copper ion Cu_B and the other bound to oxidized heme a_3 (Hill et al. 1984; Nicholls et al. 2013). Sulfide inhibits Arabidopsis COX efficiently at low nanomolar concentrations (IC_{50} = 6.9 nM; (Birke et al. 2012)) and animal COX at higher nanomolar concentrations (e.g. IC_{50} = 130 nM; K_i = 200 nM (Petersen 1977; Roth et al. 1998)). Animal and plant mitochondria thus require sulfide detoxification systems to maintain ATP production by the ETC. These systems help to keep sulfide levels in animal tissues in the low nanomolar range (Furne et al. 2008).

In fission yeast and animals, sulfide is consumed by the inner mitochondrial membrane-bound sulfide:quinone oxidoreductase (SQR) (Hildebrandt and Grieshaber 2008; Shahak and Hauska 2008). SQRs are additionally found in many bacteria and were, from an evolutionary perspective, essential for early life forms in the pre-oxygenic world where sulfide served as an energy source (Olson and Straub 2015). SQRs catalyze two half-reactions: transfer of electrons to the quinone pool of the ETC to ultimately reduce oxygen and sulfur transfer to an thiophilic acceptor, primarily GSH with GSSH as product (Figure 5A) (Landry et al. 2017, 2019, Libiad et al. 2014; Jackson et al. 2019). GSSH has been reported to be exported from the mitochondrial matrix via the ATP-binding cassette transporter ATM3 in the inner mitochondrial membrane (Schaedler et al. 2014). Alternatively, GSSH is consumed by persulfide dioxygenase ETHE1 in the mitochondrial matrix which produces sulfite. The resulting sulfite gets either oxidized to sulfate by sulfite oxidase (SO) or metabolized to thiosulfate by the thiosulfate sulfurtransferase (TST; syn. rhodanese) (Filipovic et al. 2018). Notably, in mice and human cells up to 1 % of the GSH pool is persulfidated indicating that GSSH is not just a side product (Barayeu et al. 2023b; Ida et al. 2014). It has been suggested that GSSH has cytoprotective effects and can scavenge endogenously generated free radicals and, thereby, suppress lipid peroxidation and ferroptosis (Barayeu et al. 2023b).

Plants lack SQR and rhodanese homologs (Table 2) and thus require alternative routes for sulfide detoxification

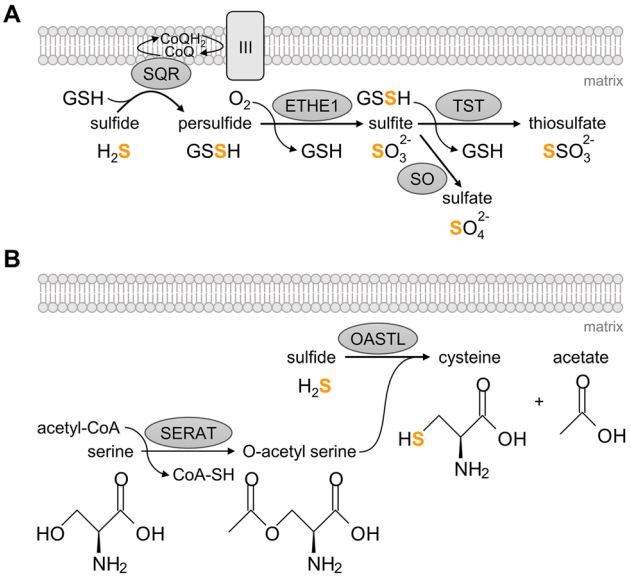

Figure 5: Key sulfide detoxification mechanisms in animals and plants. (A) In animals, the sulfide:quinone reductase (SQR) at the inner mitochondrial membrane transfers the electrons from sulfides to the quinone (coenzyme Q; CoQ) pool and the sulfur to reduced glutathione (GSH) to produce glutathione persulfide (GSSH). GSSH is consumed by oxygen-dependent ETHE1 with sulfite and glutathione as product. Sulfite is either oxidized to sulfate by sulfite oxidase (SO) or metabolized to thiosulfate by the thiosulfate sulfurtransferase (TST; syn. rhodanese). III: complex III of the mitochondrial electron transport chain. (B) In plants, sulfide is used to synthesize cysteine through the cysteine synthase complex (CSC). Serine acetyltransferase (SERAT) produces the cysteine precursor O-acetylserine (OAS) from serine and acetyl coenzyme A and O-acetylserine (thiol) lyase (OAS-TL) incorporates sulfide into OAS to form cysteine.

(Moseler et al. 2020; Theissen et al. 2003). Although plants contain homologs of ETHE1, it is unclear whether plant ETHE1, in the absence of SQR, substantially contributes to consumption of sulfide. Efficient removal of sulfide through plant ETHE1 would require that sulfide efficiently forms a persulfide with oxidized glutathione (GSSG) in a first step. The embryo-lethality of an *ethe1* knock-out, and the severe embryo-developmental effects of an *ethe1-1* knock-down, are accordingly thought to be associated with the role of ETHE1 in cysteine catabolism (Holdorf et al. 2012; Höfler et al. 2016; Krüßel et al. 2014). The key mechanism that plants use to detoxify mitochondrial sulfide is thought to be the mitochondrial matrix-localized cysteine synthase complex (CSC). The CSC consists of serine acetyltransferase2;2 (SERAT2;2) and O-acetylserine (thiol) lyase C (OAS-TL C) (Álvarez et al. 2012b; Birke et al. 2015; Wirtz et al. 2010). SERAT produces the cysteine precursor O-acetylserine (OAS) from serine and acetyl coenzyme A and OAS-TL incorporates sulfide into OAS to form cysteine (Figure 5B). Arabidopsis possesses two further OAS-TL isoforms, A and B, that were shown to reside in the cytosol and plastids, respectively (reviewed in Hell et al. (2002)). It is, however, unlikely that they contribute a major detoxification capacity for mitochondrial sulfide since sulfide predominantly exists as charged hydrosulfide anion (HS^-) in the alkaline mitochondrial matrix and can as such not freely permeate across the mitochondrial membrane. Although mitochondrial OAS-TL C contributes only little to net cysteine synthesis of the plant cell, *oas-tl c* null mutants suffer from inhibition of COX activity (Birke et al. 2012), demonstrating the role of OAS-TL C in clearance of toxic sulfide. Notably, the affinity of Arabidopsis OAS-TL C for sulfide is in the low micromolar range (Wirtz et al. 2004) and thus much higher than the IC_{50} of COX for sulfide. Efficient removal of sulfide from plant mitochondria may thus require additional detoxification systems. Recently, the cytosolic superoxide dismutase SOD1 from yeast was shown to contribute to sulfide detoxification (Switzer et al. 2023). Plants contain mitochondrial homologs of yeast SOD1, but their role in sulfide clearance is currently unclear.

Table 2: Presence of rhodanese (TST), sulfide quinone reductase (SQR) and O-acetylserine (thiol) lyase (OAS-TL) in selected organisms.

Organism	TST	SQR	OAS-TL
Mus musculus	P52196	Q9R112	–
Homo sapiens	Q16762	Q9Y6N5	–
Danio rerio	–	F1QYT2	–
Drosophila melanogaster	P22978	Q9VZF6	–
Caenorhabditis elegans	–	J7SF87	CYSK1 (Q93244), CYSK2 (O45679), CYSK3 (O01592)
Escherichia coli	–	–	CYSK (P0ABK5), CYSM (P16703)
Bacillus subtilis	–	–	CYSK (P37887), CYSM (O34476)
Saccharomyces cerevisiae	–	–	CYS4 (P32582), MCY1 (P53206)
Schizosaccharomyces pombe	Q10215	O94284	–
Chlamydomonas reinhardtii	–	–	A8ISA9, A0A2K3DH20, A0A2K3DX63
Physcomitrium patens	–	–	A9SEU2, A0A2K1J3F3, A0A2K1IZX4
Arabidopsis thaliana	–	–	OASTL-C (syn. CYSKM; Q43725), OASTL-A (syn. CYSKP; P47998), OASTL-B (syn. CYSK1; P47999)
Zea mays	-	–	B4FR08, B8A367, CYSK (P80608)

Human TST and SQR as well as Arabidopsis OAS-TL C sequences were used to identify protein orthologues in indicated organisms. Sequences were retrieved from the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) using BLAST®.

5 Future perspectives

The beneficial effects of exogenous application of H_2S in various aspects of plant physiology sparked the interest of its endogenous function. Persulfidation of critical thiols has been recognized as the main mechanism with protein persulfidation as a reversible modification in order to adapt metabolism and signaling transduction. Our mechanistic understanding of how H_2S synthesis and protein persulfidation is regulated is still rather limited in plants and many open questions remain to be answered. How specificity is maintained for the persulfidation of proteins and which cysteine residues exactly are persulfidated are one of the major questions. With the identification of the persulfidated cysteine the next questions arise including the spatio-temporal distribution of the persulfidation and the consequences for the protein. The persulfidation as reversible modification implies also a depersulfidation, but how this is regulated and if other proteins than TRXs can act as depersulfidase remain unanswered so far. Hence, there is no doubt that the current knowledge of persulfidation is a stepping stone to more exciting research to decipher the role of persulfides.

Acknowledgments: A.M. and S.W. are grateful to the University of Bonn for funding.
Research ethics: Not applicable.
Author contributions: A.M. conceptualized the review and wrote the original draft together with S.W. A.J.M. reviewed and edited the draft. All authors have accepted the entire content of the submitted manuscript and approved submission.
Competing interests: The authors declare no conflicts of interest regarding this article.
Research funding: This work was supported by the Open Access Publication Fund of the University of Bonn and the Deutsche Forschungsgemeinschaft (DFG) (grant MO 4527/3-1 to A.M.).
Data availability: Not applicable.

References

Akaike, T., Ida, T., Wei, F.-Y., Nishida, M., Kumagai, Y., Alam, M.M., Ihara, H., Sawa, T., Matsunaga, T., Kasamatsu, S., et al. (2017). Cysteinyl-tRNA synthetase governs cysteine polysulfidation and mitochondrial bioenergetics. *Nat. Commun.* 8: 1177.

Álvarez, C., Calo, L., Romero, L.C., García, I., and Gotor, C. (2010). An O-acetylserine(thiol)lyase homolog with L-cysteine desulfhydrase activity regulates cysteine homeostasis in Arabidopsis. *Plant Physiol.* 152: 656–669.

Álvarez, C., García, I., Moreno, I., Pérez-Pérez, M.E., Crespo, J.L., Romero, L.C., and Gotor, C. (2012a). Cysteine-generated sulfide in the cytosol negatively regulates autophagy and modulates the transcriptional profile in Arabidopsis. *The Plant Cell* 24: 4621–4634.

Álvarez, C., García, I., Romero, L.C., and Gotor, C. (2012b). Mitochondrial sulfide detoxification requires a functional isoform O-acetylserine(thiol)lyase C in *Arabidopsis thaliana*. *Mol. Plant* 5: 1217–1226.

Antonini, E., Brunori, M., Rotilio, G.C., Greenwood, C., Malmström, B.G., and Rotilto, G.C. (1971). The interaction of cyanide with cytochrome oxidase. *Eur. J. Biochem.* 23: 396–400.

Arenas-Alfonseca, L., Gotor, C., Romero, L.C., and García, I. (2018). ß-cyanoalanine synthase action in root hair elongation is exerted at early steps of the root hair elongation pathway and is independent of direct cyanide inactivation of NADPH oxidase. *Plant Cell Physiol.* 59: 1072–1083.

Aroca, A., Benito, J.M., Gotor, C., and Romero, L.C. (2017a). Persulfidation proteome reveals the regulation of protein function by hydrogen sulfide in diverse biological processes in Arabidopsis. *J. Exp. Bot.* 68: 4915–4927.

Aroca, A., Jurado-Flores, A., Filipovic, M.R., Gotor, C., and Romero, L.C. (2022). Detection of protein persulfidation in plants by the dimedone switch method. *Methods Enzymol.* 676: 385–402.

Aroca, A., Schneider, M., Scheibe, R., Gotor, C., and Romero, L.C. (2017b). Hydrogen sulfide regulates the cytosolic/nuclear partitioning of glyceraldehyde-3-phosphate dehydrogenase by enhancing its nuclear localization. *Plant Cell Physiol.* 58: 983–992.

Aroca, A., Serna, A., Gotor, C., and Romero, L.C. (2015). S-sulfhydration: a cysteine posttranslational modification in plant systems. *Plant Physiol.* 168: 334–342.

Aroca, A., Yruela, I., Gotor, C., and Bassham, D.C. (2021). Persulfidation of ATG18a regulates autophagy under ER stress in Arabidopsis. *Proc. Natl Acad. Sci. USA* 118, https://doi.org/10.1073/pnas.2023604118.

Barayeu, U., Sawa, T., Nishida, M., Wei, F.-Y., Motohashi, H., and Akaike, T. (2023a). Supersulfide biology and translational medicine for disease control. *Br. J. Pharmacol.*: 1–16, https://doi.org/10.1111/bph.16271.

Barayeu, U., Schilling, D., Eid, M., Xavier da Silva, T.N., Schlicker, L., Mitreska, N., Zapp, C., Gräter, F., Miller, A.K., Kappl, R., et al. (2023b). Hydropersulfides inhibit lipid peroxidation and ferroptosis by scavenging radicals. *Nat. Chem. Biol.* 19: 28–37.

Benchoam, D., Cuevasanta, E., Möller, M.N., and Alvarez, B. (2019). Hydrogen sulfide and persulfides oxidation by biologically relevant oxidizing species. *Antioxidants* 8: 48.

Benchoam, D., Cuevasanta, E., Roman, J.V., Banerjee, R., and Alvarez, B. (2024). Acidity of persulfides and its modulation by the protein environments in sulfide quinone oxidoreductase and thiosulfate sulfurtransferase. *J. Biol. Chem.* 300: 107149.

Benchoam, D., Semelak, J.A., Cuevasanta, E., Mastrogiovanni, M., Grassano, J.S., Ferrer-Sueta, G., Zeida, A., Trujillo, M., Möller, M.N., Estrin, D.A., et al. (2020). Acidity and nucleophilic reactivity of glutathione persulfide. *J. Biol. Chem.* 295: 15466–15481.

Birke, H., De Kok, L.J., Wirtz, M., and Hell, R. (2015). The role of compartment-specific cysteine synthesis for sulfur homeostasis during H_2S exposure in Arabidopsis. *Plant Cell Physiol.* 56: 358–367.

Birke, H., Haas, F., De Kok, L., Balk, J., Wirtz, M., and Hell, R. (2012). Cysteine biosynthesis, in concert with a novel mechanism, contributes to sulfide detoxification in mitochondria of *Arabidopsis thaliana*. *Biochem. J.* 445: 275–283.

Biteau, B., Labarre, J., and Toledano, M.B. (2003). ATP-dependent reduction of cysteine–sulphinic acid by *S. cerevisiae* sulphiredoxin. *Nature* 425: 980–984.

Bonanata, J., Turell, L., Antmann, L., Ferrer-Sueta, G., Botasini, S., Méndez, E., Alvarez, B., and Coitiño, E.L. (2017). The thiol of human serum albumin: acidity, microenvironment and mechanistic insights on its oxidation to sulfenic acid. *Free Radical Biol. Med.* 108: 952–962.

Brodowski, S., Amelung, W., Lobe, I., and Du Preez, C.C. (2005). Losses and biogeochemical cycling of soil organic nitrogen with prolonged arable cropping in the South African Highveld – evidence from D- and L-amino acids. *Biogeochemistry* 71: 17–42.

Chauvin, J.-P.R., Haidasz, E.A., Griesser, M., and Pratt, D.A. (2016). Polysulfide-1-oxides react with peroxyl radicals as quickly as hindered phenolic antioxidants and do so by a surprising concerted homolytic substitution. *Chem. Sci.* 7: 6347–6356.

Chen, S., Jia, H., Wang, X., Shi, C., Wang, X., Ma, P., Wang, J., Ren, M., and Li, J. (2020). Hydrogen sulfide positively regulates abscisic acid signaling through persulfidation of SnRK2.6 in guard cells. *Mol. Plant* 13: 732–744.

Chen, S., Wang, X., Jia, H., Li, F., Ma, Y., Liesche, J., Liao, M., Ding, X., Liu, C., Chen, Y., et al. (2021). Persulfidation-induced structural change in SnRK2.6 establishes intramolecular interaction between phosphorylation and persulfidation. *Mol. Plant* 14: 1814–1830.

Conibear, A.C. (2020). Deciphering protein post-translational modifications using chemical biology tools. *Nat. Rev. Chem* 4: 674–695.

Consortium, T.U., Martin, M.J., Orchard, S., Magrane, M., Ahmad, S., Alpi, E., Bowler-Barnett, E.H., Britto, R., Bye-A-Jee, H., Cukura, A., et al. (2023). UniProt: the universal protein knowledgebase in 2023. *Nucleic Acids Res.* 51: D523–D531.

Corpas, F.J., González-Gordo, S., Rodríguez-Ruiz, M., Muñoz-Vargas, M.A., and Palma, J.M. (2022). Thiol-based oxidative posttranslational modifications (OxiPTMs) of plant proteins. *Plant Cell Physiol.* 63: 889–900.

Cuevasanta, E., Lange, M., Bonanata, J., Coitiño, E.L., Ferrer-Sueta, G., Filipovic, M.R., and Alvarez, B. (2015). Reaction of hydrogen sulfide with disulfide and sulfenic acid to form the strongly nucleophilic persulfide. *J. Biol. Chem.* 290: 26866–26880.

Cuevasanta, E., Reyes, A.M., Zeida, A., Mastrogiovanni, M., De Armas, M.I., Radi, R., Alvarez, B., and Trujillo, M. (2019). Kinetics of formation and reactivity of the persulfide in the one-cysteine peroxiredoxin from *Mycobacterium tuberculosis*. *J. Biol. Chem.* 294: 13593–13605.

Cui, W., Chen, H., Zhu, K., Jin, Q., Xie, Y., Cui, J., Xia, Y., Zhang, J., and Shen, W. (2014). Cadmium-induced hydrogen sulfide synthesis is involved in cadmium tolerance in *Medicago sativa* by reestablishment of reduced (homo)glutathione and reactive oxygen species homeostases. *PLOS ONE* 9: e109669.

Dóka, É., Arnér, E.S.J., Schmidt, E.E., Dick, T.P., van der Vliet, A., Yang, J., Szatmári, R., Ditrói, T., Wallace, J.L., Cirino, G., et al. (2021). Comment on "Evidence that the ProPerDP method is inadequate for protein persulfidation detection due to lack of specificity". *Sci. Adv.* 7, https://doi.org/10.1126/sciadv.abe7006.

Dóka, É., Ida, T., Dagnell, M., Abiko, Y., Luong, N.C., Balog, N., Takata, T., Espinosa, B., Nishimura, A., Cheng, Q., et al. (2020). Control of protein function through oxidation and reduction of persulfidated states. *Sci. Adv.* 6: eaax8358.

Dóka, É., Pader, I., Bíró, A., Johansson, K., Cheng, Q., Ballagó, K., Prigge, J.R., Pastor-Flores, D., Dick, T.P., Schmidt, E.E., et al. (2016). A novel persulfide detection method reveals protein persulfide- and polysulfide-reducing functions of thioredoxin and glutathione systems. *Sci. Adv.* 2: e1500968.

Dooley, F.D., Nair, S.P., and Ward, P.D. (2013). Increased growth and germination success in plants following hydrogen sulfide administration. *PLOS ONE* 8: e62048.

Drekolia, M.-K., Karantanou, C., Wittig, I., Li, Y., Fuhrmann, D.C., Brüne, B., Katsouda, A., Hu, J., Papapetropoulos, A., and Bibli, S.-I. (2024). Loss of cardiac mitochondrial complex I persulfidation impairs NAD^+ homeostasis in aging. *Redox Biol.* 69, https://doi.org/10.1016/j.redox.2023.103014.

Ezeriņa, D., Takano, Y., Hanaoka, K., Urano, Y., and Dick, T.P. (2018). N-Acetyl cysteine functions as a fast-acting antioxidant by triggering intracellular H_2S and sulfane sulfur production. *Cell Chem. Biol.* 25: 447–459.

Filipovic, M.R., Zivanovic, J., Alvarez, B., and Banerjee, R. (2018). Chemical biology of H_2S signaling through persulfidation. *Chem. Rev.* 118: 1253–1337.

Foyer, C.H. and Noctor, G. (2016). Stress-triggered redox signalling: what's in pROSpect? *Plant Cell and Environ.* 39: 951–964.

Fräsdorf, B., Radon, C., and Leimkühler, S. (2014). Characterization and interaction studies of two isoforms of the dual localized 3-mercaptopyruvate sulfurtransferase TUM1 from humans. *J. Biol. Chem.* 289: 34543–34556.

Fricker, M.D., May, M., Meyer, A.J., Sheard, N., and White, N.S. (2000). Measurement of glutathione levels in intact roots of Arabidopsis. *J. Microsc.* 198: 162–173.

Fu, L., Liu, K., He, J., Tian, C., Yu, X., and Yang, J. (2020). Direct proteomic mapping of cysteine persulfidation. *Antioxid Redox Signal* 33: 1061–1076.

Fuchs, P., Rugen, N., Carrie, C., Elsässer, M., Finkemeier, I., Giese, J., Hildebrandt, T.M., Kühn, K., Maurino, V.G., Ruberti, C., et al. (2020). Single organelle function and organization as estimated from Arabidopsis mitochondrial proteomics. *The Plant J.* 101: 420–441.

Furne, J., Saeed, A., and Levitt, M.D. (2008). Whole tissue hydrogen sulfide concentrations are orders of magnitude lower than presently accepted values. *Am. J. Physiol.-Regulatory, Integrative and Comp. Physiol.* 295: R1479–R1485.

Gao, X.-H., Krokowski, D., Guan, B.-J., Bederman, I., Majumder, M., Parisien, M., Diatchenko, L., Kabil, O., Willard, B., Banerjee, R., et al. (2015). Quantitative H_2S-mediated protein sulfhydration reveals metabolic reprogramming during the integrated stress response. *eLife* 4: e10067.

García, I., Castellano, J.M., Vioque, B., Solano, R., Gotor, C., and Romero, L.C. (2010). Mitochondrial β-cyanoalanine synthase is essential for root hair formation in *Arabidopsis thaliana*. *The Plant Cell* 22: 3268–3279.

García-Calderón, M., Vignane, T., Filipovic, M.R., Ruiz, M.T., Romero, L.C., Márquez, A.J., Gotor, C., and Aroca, A. (2023). Persulfidation protects from oxidative stress under nonphotorespiratory conditions in Arabidopsis. *New Phytol.* 238: 1431–1445.

Go, Y.-M., Chandler, J.D., and Jones, D.P. (2015). The cysteine proteome. *Free Radical Biol. Med.* 84: 227–245.

Gördes, D., Kolukisaoglu, Ü., and Thurow, K. (2011). Uptake and conversion of D-amino acids in *Arabidopsis thaliana*. *Amino Acids* 40: 553–563.

Greiner, R., Pálinkás, Z., Bäsell, K., Becher, D., Antelmann, H., Nagy, P., and Dick, T.P. (2013). Polysulfides link H_2S to protein thiol oxidation. *Antioxid Redox Signal* 19: 1749–1765.

Hatzfeld, Y., Maruyama, A., Schmidt, A., Noji, M., Ishizawa, K., and Saito, K. (2000). β-Cyanoalanine synthase is a mitochondrial cysteine cynthase-like protein in spinach and Arabidopsis. *Plant Physiol.* 123: 1163–1172.

Hell, R., Jost, R., Berkowitz, O., and Wirtz, M. (2002). Molecular and biochemical analysis of the enzymes of cysteine biosynthesis in the plant *Arabidopsis thaliana*. *Amino Acids* 22: 245–257.

Hendrickson, H.R. and Conn, E.E. (1969). Cyanide metabolism in higher plants. IV. Purification and properties of the β-cyanolanine synthase of blue lupine. *J. Biol. Chem.* 244: 2632–2640.

Henne, M., König, N., Triulzi, T., Baroni, S., Forlani, F., Scheibe, R., and Papenbrock, J. (2015). Sulfurtransferase and thioredoxin specifically interact as demonstrated by bimolecular fluorescence complementation analysis and biochemical tests. *FEBS Open Bio* 5: 832–843.

Heppner, D.E., Hristova, M., Ida, T., Mijuskovic, A., Dustin, C.M., Bogdándi, V., Fukuto, J.M., Dick, T.P., Nagy, P., Li, J., et al. (2018). Cysteine perthiosulfenic acid (Cys-SSOH): a novel intermediate in thiol-based redox signaling? *Redox Biol.* 14: 379–385.

Hildebrandt, T.M. and Grieshaber, M.K. (2008). Three enzymatic activities catalyze the oxidation of sulfide to thiosulfate in mammalian and invertebrate mitochondria. *The FEBS J.* 275: 3352–3361.

Hill, B.C., Woon, T.C., Nicholls, P., Peterson, J., Greenwood, C., and Thomson, A.J. (1984). Interactions of sulphide and other ligands with cytochrome c oxidase. An electron-paramagnetic-resonance study. *Biochem. J.* 224: 591–600.

Höfler, S., Lorenz, C., Busch, T., Brinkkötter, M., Tohge, T., Fernie, A.R., Braun, H.-P., and Hildebrandt, T.M. (2016). Dealing with the sulfur part of cysteine: four enzymatic steps degrade L-cysteine to pyruvate and thiosulfate in Arabidopsis mitochondria. *Physiol. Plant.* 157: 352–366.

Holdorf, M.M., Owen, H.A., Lieber, S.R., Yuan, L., Adams, N., Dabney-Smith, C., and Makaroff, C.A. (2012). Arabidopsis ETHE1 encodes a sulfur dioxygenase that is essential for embryo and endosperm development. *Plant Physiol.* 160: 226–236.

Huang, B.K., Stein, K.T., and Sikes, H.D. (2016). Modulating and measuring intracellular H_2O_2 using genetically encoded tools to study its toxicity to human cells. *ACS Synth. Biol.* 5: 1389–1395.

Huang, J., Staes, A., Impens, F., Demichev, V., Van Breusegem, F., Gevaert, K., and Willems, P. (2023). CysQuant: simultaneous quantification of cysteine oxidation and protein abundance using data dependent or independent acquisition mass spectrometry. *Redox Biol.* 67, https://doi.org/10.1016/j.redox.2023.102908.

Huang, J. and Xie, Y. (2023). Hydrogen sulfide signaling in plants. *Antioxid Redox Signal* 39: 40–58.

Hugo, M., Turell, L., Manta, B., Botti, H., Monteiro, G., Netto, L.E.S., Alvarez, B., Radi, R., and Trujillo, M. (2009). Thiol and sulfenic acid oxidation of AhpE, the one-cysteine peroxiredoxin from *Mycobacterium tuberculosis*: kinetics, acidity constants, and conformational dynamics. *Biochemistry* 48: 9416–9426.

Ida, T., Sawa, T., Ihara, H., Tsuchiya, Y., Watanabe, Y., Kumagai, Y., Suematsu, M., Motohashi, H., Fujii, S., Matsunaga, T., et al. (2014). Reactive cysteine persulfides and S-polythiolation regulate oxidative stress and redox signaling. *Proc. Natl Acad. Sci. USA* 111: 7606–7611.

Iglesias-Baena, I., Barranco-Medina, S., Sevilla, F., and Lázaro, J.-J. (2011). The dual-targeted plant sulfiredoxin retroreduces the sulfinic form of atypical mitochondrial peroxiredoxin. *Plant Physiol.* 155: 944–955.

Jackson, M.R., Loll, P.J., and Jorns, M.S. (2019). X-ray structure of human sulfide:quinone oxidoreductase: insights into the mechanism of mitochondrial hydrogen sulfide oxidation. *Structure* 27: 794–805.e794.

Jarosz, A.P., Wei, W., Gauld, J.W., Auld, J., Özcan, F., Aslan, M., and Mutus, B. (2015). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is inactivated by S-sulfuration *in vitro*. *Free Radical Biol. Med.* 89: 512–521.

Jia, H., Chen, S., Liu, D., Liesche, J., Shi, C., Wang, J., Ren, M., Wang, X., Yang, J., Shi, W., et al. (2018). Ethylene-induced hydrogen sulfide negatively regulates ethylene biosynthesis by persulfidation of ACO in tomato under osmotic stress. *Front. Plant Sci.* 9, https://doi.org/10.3389/fpls.2018.01517.

Jia, H., Wang, X., Dou, Y., Liu, D., Si, W., Fang, H., Zhao, C., Chen, S., Xi, J., and Li, J. (2016). Hydrogen sulfide-cysteine cycle system enhances cadmium tolerance through alleviating cadmium-induced oxidative stress and ion toxicity in Arabidopsis roots. *Sci. Rep.* 6: 39702.

Ju, Y., Wu, L., and Yang, G. (2016). Thioredoxin 1 regulation of protein S-desulfhydration. *Biochem. Biophys. Rep.* 5: 27–34.

Jurado-Flores, A., Aroca, A., Romero, L.C., and Gotor, C. (2023). Sulfide promotes tolerance to drought through protein persulfidation in Arabidopsis. *J. Exp. Bot.* 74: 4654–4669.

Kägi, C., Baumann, N., Nielsen, N., Stierhof, Y.-D., and Groß-Hardt, R. (2010). The gametic central cell of Arabidopsis determines the lifespan of adjacent accessory cells. *Proc. Natl Acad. Sci. USA* 107: 22350–22355.

Kasamatsu, S., Nishimura, A., Alam, M.M., Morita, M., Shimoda, K., Matsunaga, T., Jung, M., Ogata, S., Barayeu, U., Ida, T., et al. (2023). Supersulfide catalysis for nitric oxide and aldehyde metabolism. *Sci. Adv.* 9: eadg8631.

Khan, M.S., Haas, F.H., Allboje Samami, A., Moghaddas Gholami, A., Bauer, A., Fellenberg, K., Reichelt, M., Hansch, R., Mendel, R.R., Meyer, A.J., et al. (2010). Sulfite reductase defines a newly discovered bottleneck for assimilatory sulfate reduction and is essential for growth and development in *Arabidopsis thaliana*. *The Plant Cell* 22: 1216–1231.

Kimura, Y., Koike, S., Shibuya, N., Lefer, D., Ogasawara, Y., and Kimura, H. (2017). 3-Mercaptopyruvate sulfurtransferase produces potential redox regulators cysteine- and glutathione-persulfide (Cys-SSH and GSSH) together with signaling molecules H_2S_2, H_2S_3 and H_2S. *Sci. Rep.* 7: 10459.

Koprivova, A. and Kopriva, S. (2014). Molecular mechanisms of regulation of sulfate assimilation: first steps on a long road. *Front. Plant Sci.* 5: 589.

Krüßel, L., Junemann, J., Wirtz, M., Birke, H., Thornton, J.D., Browning, L.W., Poschet, G., Hell, R., Balk, J., Braun, H.-P., et al. (2014). The mitochondrial sulfur dioxygenase ethylmalonic encephalopathy protein1 is required for amino acid catabolism during carbohydrate starvation and embryo development in Arabidopsis. *Plant Physiol.* 165: 92–104.

Lai, K.W., Yau, C.P., Tse, Y.C., Jiang, L.W., and Yip, W.K. (2009). Heterologous expression analyses of rice *Os*CAS in Arabidopsis and in yeast provide evidence for its roles in cyanide detoxification rather than in cysteine synthesis *in vivo*. *J. Exp. Bot.* 60: 993–1008.

Landry, A.P., Ballou, D.P., and Banerjee, R. (2017). H_2S oxidation by nanodisc-embedded human sulfide quinone oxidoreductase. *J. Biol. Chem.* 292: 11641–11649.

Landry, A.P., Moon, S., Kim, H., Yadav, P.K., Guha, A., Cho, U.-S., and Banerjee, R. (2019). A catalytic trisulfide in human sulfide quinone oxidoreductase catalyzes coenzyme A persulfide synthesis and inhibits butyrate oxidation. *Cell Chem. Biol.* 26: 1515–1525.e1514.

Laureano-Marín, A.M., Aroca, A., Pérez-Pérez, M.E., Yruela, I., Jurado-Flores, A., Moreno, I., Crespo, J.L., Romero, L.C., and Gotor, C. (2020). Abscisic acid-triggered persulfidation of the Cys protease ATG4 mediates regulation of autophagy by sulfide. *The Plant Cell* 32: 3902–3920.

Lec, J.-C., Boutserin, S., Mazon, H., Mulliert, G., Boschi-Muller, S., and Talfournier, F. (2018). Unraveling the mechanism of cysteine persulfide formation catalyzed by 3-mercaptopyruvate sulfurtransferases. *ACS Catal.* 8: 2049–2059.

Li, J., Chen, S., Wang, X., Shi, C., Liu, H., Yang, J., Shi, W., Guo, J., and Jia, H. (2018). Hydrogen sulfide disturbs actin polymerization via S-sulfhydration resulting in stunted root hair growth. *Plant Physiol.* 178: 936–949.

Li, J., Shi, C., Wang, X., Liu, C., Ding, X., Ma, P., Wang, X., and Jia, H. (2020). Hydrogen sulfide regulates the activity of antioxidant enzymes through persulfidation and improves the resistance of tomato seedling to copper oxide nanoparticles (CuO NPs)-induced oxidative stress. *Plant Physiol. Biochem.* 156: 257–266.

Libiad, M., Motl, N., Akey, D.L., Sakamoto, N., Fearon, E.R., Smith, J.L., and Banerjee, R. (2018). Thiosulfate sulfurtransferase-like domain-containing 1 protein interacts with thioredoxin. *J. Biol. Chem.* 293: 2675–2686.

Libiad, M., Yadav, P.K., Vitvitsky, V., Martinov, M., and Banerjee, R. (2014). Organization of the human mitochondrial hydrogen sulfide oxidation pathway. *J. Biol. Chem.* 289: 30901–30910.

Lillig, C.H. and Berndt, C. (2013). Glutaredoxins in thiol/disulfide exchange. *Antioxid Redox Signal* 18: 1654–1665.

Longen, S., Richter, F., Köhler, Y., Wittig, I., Beck, K.-F., and Pfeilschifter, J. (2016). Quantitative persulfide site identification (qPerS-SID) reveals protein targets of H_2S releasing donors in mammalian cells. *Sci. Rep.* 6, https://doi.org/10.1038/srep29808.

Ma, T., Xu, S., Wang, Y., Zhang, L., Liu, Z., Liu, D., Jin, Z., and Pei, Y. (2024). Exogenous hydrogen sulphide promotes plant flowering through the Arabidopsis splicing factor AtU2AF65a. *Plant, Cell & Environ.*: 1–15, https://doi.org/10.1111/pce.14849.

Ma, Y., Li, F., Yi, Y., Wang, X., Li, T., Wang, X., Sun, H., Li, L., Ren, M., Han, S., et al. (2023). Hydrogen sulfide improves salt tolerance through persulfidation of PMA1 in Arabidopsis. *Plant Cell Rep.* 42: 1265–1277.

Machingura, M., Salomon, E., Jez, J.M., and Ebbs, S.D. (2016). The β-cyanoalanine synthase pathway: beyond cyanide detoxification. *Plant, Cell & Environ.* 39: 2329–2341.

Mao, G.H., Wang, R.G., Guan, Y.F., Liu, Y.D., and Zhang, S.Q. (2011). Sulfurtransferases 1 and 2 play essential roles in embryo and seed development in *Arabidopsis thaliana*. *J. Biol. Chem.* 286: 7548–7557.

Matsunaga, T., Sano, H., Takita, K., Morita, M., Yamanaka, S., Ichikawa, T., Numakura, T., Ida, T., Jung, M., Ogata, S., et al. (2023). Supersulphides provide airway protection in viral and chronic lung diseases. *Nat. Commun.* 14: 4476.

Miseta, A. and Csutora, P. (2000). Relationship between the occurrence of cysteine in proteins and the complexity of organisms. *Mol. Biol. Evol.* 17: 1232–1239.

Møller, I.M., Igamberdiev, A.U., Bykova, N.V., Finkemeier, I., Rasmusson, A.G., and Schwarzländer, M. (2020). Matrix redox physiology governs the regulation of plant mitochondrial metabolism through posttranslational protein modifications. *The Plant Cell* 32: 573–594.

Moseler, A., Dhalleine, T., Rouhier, N., and Couturier, J. (2021). *Arabidopsis thaliana* 3-mercaptopyruvate sulfurtransferases interact with and are protected by reducing systems. *J. Biol. Chem.* 296, https://doi.org/10.1016/j.jbc.2021.100429.

Moseler, A., Selles, B., Rouhier, N., and Couturier, J. (2020). Novel insights into the diversity of the sulfurtransferase family in photosynthetic organisms with emphasis on oak. *New Phytol.* 226: 967–977.

Mostofa, M.G., Rahman, A., Ansary, M.M.U., Watanabe, A., Fujita, M., and Tran, L.-S.P. (2015). Hydrogen sulfide modulates cadmium-induced physiological and biochemical responses to alleviate cadmium toxicity in rice. *Sci. Rep.* 5, https://doi.org/10.1038/srep14078.

Mustafa, A.K., Gadalla, M.M., Sen, N., Kim, S., Mu, W., Gazi, S.K., Barrow, R.K., Yang, G., Wang, R., and Snyder, S.H. (2009). H_2S signals through protein S-sulfhydration. *Sci. Signaling* 2: ra72.

Nagahara, N., Nirasawa, T., Yoshii, T., and Niimura, Y. (2012). Is novel signal transducer sulfur oxide involved in the redox cycle of persulfide at the catalytic site cysteine in a stable reaction intermediate of mercaptopyruvate sulfurtransferase? *Antioxid. Redox Signal* 16: 747–753.

Nagasawa, T., Ishii, T., Kumagai, H., and Yamada, H. (1985). D-Cysteine desulfhydrase of *Escherichia coli*. *Eur. J. Biochem.* 153: 541–551.

Nakamura, T., Yamaguchi, Y., and Sano, H. (2000). Plant mercaptopyruvate sulfurtransferases. *Eur. J. Biochem.* 267: 5621–5630.

Nicholls, P., Marshall, D.C., Cooper, C.E., and Wilson, M.T. (2013). Sulfide inhibition of and metabolism by cytochrome *c* oxidase. *Biochem. Soc. Trans.* 41: 1312–1316.

Nietzel, T., Mostertz, J., Ruberti, C., Née, G., Fuchs, P., Wagner, S., Moseler, A., Müller-Schüssele, S.J., Benamar, A., Poschet, G., et al. (2020). Redox-mediated kick-start of mitochondrial energy metabolism drives resource-efficient seed germination. *Proc. Natl Acad. Sci. USA* 117: 741–751.

Nishimura, A., Yoon, S., Matsunaga, T., Ida, T., Jung, M., Ogata, S., Morita, M., Yoshitake, J., Unno, Y., Barayeu, U., et al. (2024). Longevity control by supersulfide-mediated mitochondrial respiration and regulation of protein quality. *Redox Biol.* 69, https://doi.org/10.1016/j.redox.2023.103018.

Ogata, S., Matsunaga, T., Jung, M., Barayeu, U., Morita, M., and Akaike, T. (2023). Persulfide biosynthesis conserved evolutionarily in all organisms. *Antioxid Redox Signal* 39: 983–999.

Olson, K.R. and Straub, K.D. (2015). The role of hydrogen sulfide in evolution and the evolution of hydrogen sulfide in metabolism and signaling. *Physiology* 31: 60–72.

Ono, K., Akaike, T., Sawa, T., Kumagai, Y., Wink, D.A., Tantillo, D.J., Hobbs, A.J., Nagy, P., Xian, M., Lin, J., et al. (2014). Redox chemistry and chemical biology of H_2S, hydropersulfides, and derived species: implications of their possible biological activity and utility. *Free Radical Biol. Med.* 77: 82–94.

Pantaleno, R. and Scuffi, D. (2024). Posttranslational modifications triggered by H_2S in plant cells. In: *H_2S in plants*. Academic Press, Cambridge, Massachusetts, US, pp. 169–191.

Patron, N.J., Durnford, D.G., and Kopriva, S. (2008). Sulfate assimilation in eukaryotes: fusions, relocations and lateral transfers. *BMC Evol. Biol.* 8: 39.

Pedre, B., Barayeu, U., Ezeriņa, D., and Dick, T.P. (2021). The mechanism of action of N-acetylcysteine (NAC): the emerging role of H_2S and sulfane sulfur species. *Pharmacol. Ther.* 228, https://doi.org/10.1016/j.pharmthera.2021.107916.

Pedre, B. and Dick, T.P. (2021). 3-Mercaptopyruvate sulfurtransferase: an enzyme at the crossroads of sulfane sulfur trafficking. *Biol. Chem.* 402: 223–237.

Pedre, B., Talwar, D., Barayeu, U., Schilling, D., Luzarowski, M., Sokolowski, M., Glatt, S., and Dick, T.P. (2023). 3-Mercaptopyruvate sulfur transferase is a protein persulfidase. *Nat. Chem. Biol.* 19: 507–517.

Peeters, N.M., Chapron, A., Giritch, A., Grandjean, O., Lancelin, D., Lhomme, T., Vivrel, A., and Small, I. (2000). Duplication and quadruplication of *Arabidopsis thaliana* cysteinyl- and asparaginyl-tRNA synthetase genes of organellar origin. *J. Mol. Evol.* 50: 413–423.

Pérez-Pérez, M.E., Lemaire, S.D., and Crespo, J.L. (2016). Control of autophagy in *Chlamydomonas* is mediated through redox-dependent inactivation of the ATG4 protease. *Plant Physiol.* 172: 2219–2234.

Pérez-Pérez, M.E., Zaffagnini, M., Marchand, C.H., Crespo, J.L., and Lemaire, S.D. (2014). The yeast autophagy protease Atg4 is regulated by thioredoxin. *Autophagy* 10: 1953–1964.

Petersen, L.C. (1977). The effect of inhibitors on the oxygen kinetics of cytochrome c oxidase. *Biochim. Biophys. Acta Bioenerg.* 460: 299–307.

Portereiko, M.F., Sandaklie-Nikolova, L., Lloyd, A., Dever, C.A., Otsuga, D., and Drews, G.N. (2006). NUCLEAR FUSION DEFECTIVE1 encodes the Arabidopsis RPL21M protein and is required for karyogamy during female gametophyte development and fertilization. *Plant Physiol.* 141: 957–965.

Redman, L.M., Smith, S.R., Burton, J.H., Martin, C.K., Il'yasova, D., and Ravussin, E. (2018). Metabolic slowing and reduced oxidative damage with sustained caloric restriction support the rate of living and oxidative damage theories of aging. *Cell Metab.* 27: 805–815.e804.

Requejo, R., Hurd, T.R., Costa, N.J., and Murphy, M.P. (2010). Cysteine residues exposed on protein surfaces are the dominant

intramitochondrial thiol and may protect against oxidative damage. *FEBS J.* 277: 1465–1480.

Reyes, A., Cuevasanta, E., De Armas, M.I., Mastrogiovanni, M., Zeida, A., Radi, R., Alvarez, B., and Trujillo, M. (2018). Interactions of hydrogen sulfide with AhpE from *Mycobacterium tuberculosis*: formation and reactions of a model peroxiredoxin persulfide. *Free Radical Biol. Med.* 128: S57.

Riemenschneider, A., Bonacina, E., Schmidt, A., and Papenbrock, J. (2005a). Isolation and characterization of a second D-cysteine desulfhydrase-like protein from Arabidopsis. In: *Sulfur transport and assimilation in plants in the post genomic era*. Backhuys Publishers, Kerkwerve, Netherlands, pp. 103–106.

Riemenschneider, A., Wegele, R., Schmidt, A., and Papenbrock, J. (2005b). Isolation and characterization of a D-cysteine desulfhydrase protein from *Arabidopsis thaliana*. *The FEBS J.* 272: 1291–1304.

Roth, R.A.N.S.H., Brookes, A.Z.J.Z.J., and Bennington, B.S.R. (1998). Inhibition of respiratory and bioenergetic mechanisms by hydrogen sulfide in mammalian brain. *J. Toxicol. Environ. Health, Part A* 54: 491–507.

Schaedler, T.A., Thornton, J.D., Kruse, I., Schwarzländer, M., Meyer, A.J., van Veen, H.W., and Balk, J. (2014). A conserved mitochondrial ATP-binding cassette transporter exports glutathione polysulfide for cytosolic metal cofactor assembly. *J. Biol. Chem.* 289: 23264–23274.

Schilling, D., Barayeu, U., Steimbach, R.R., Talwar, D., Miller, A.K., and Dick, T.P. (2022). Commonly used alkylating agents limit persulfide detection by converting protein persulfides into thioethers. *Angew. Chem., Int. Ed.* 61: e202203684.

Schilling, D., Ditrói, T., Barayeu, U., Jurányi, E.P., Nagy, P., and Dick, T.P. (2023). The influence of alkylating agents on sulfur–sulfur bonds in per- and polysulfides. *Curr. Opin. Chem. Biol.* 76, https://doi.org/10.1016/j.cbpa.2023.102368.

Schlößer, M., Moseler, A., Bodnar, Y., Homagk, M., Wagner, S., Pedroletti, L., Gellert, M., Ugalde, J.M., Lillig, C.H., and Meyer, A.J. (2024). Localization of four class I glutaredoxins in the cytosol and the secretory pathway and characterization of their biochemical diversification. *The Plant J.* 118: 1455–1474.

Schmidt, A. (1982). A cysteine desulfhydrase from spinach leaves specific for D-cysteine. *Z. Pflanzenphysiol.* 107: 301–312.

Seaver Lauren, C. and Imlay James, A. (2001). Hydrogen peroxide fluxes and compartmentalization inside growing *Escherichia coli*. *J. Bacteriol.* 183: 7182–7189.

Semenza, E.R., Harraz, M.M., Abramson, E., Malla, A.P., Vasavda, C., Gadalla, M.M., Kornberg, M.D., Snyder, S.H., and Roychaudhuri, R. (2021). D-cysteine is an endogenous regulator of neural progenitor cell dynamics in the mammalian brain. *Proc. Natl Acad. Sci. USA* 118, https://doi.org/10.1073/pnas.2110610118.

Senkler, J., Senkler, M., Eubel, H., Hildebrandt, T., Lengwenus, C., Schertl, P., Schwarzländer, M., Wagner, S., Wittig, I., and Braun, H.P. (2017). The mitochondrial complexome of *Arabidopsis thaliana*. *The Plant J.* 89: 1079–1092.

Shahak, Y. and Hauska, G. (2008). Sulfide oxidation from cyanobacteria to humans: sulfide-quinone oxidoreductase (SQR). In: *Sulfur metabolism in phototrophic organisms*. Springer, Berlin, pp. 325–342.

Shen, J., Zhang, J., Zhou, M., Zhou, H., Cui, B., Gotor, C., Romero, L.C., Fu, L., Yang, J., Foyer, C.H., et al. (2020). Persulfidation-based modification of cysteine desulfhydrase and the NADPH oxidase RBOHD controls guard cell abscisic acid signaling. *The Plant Cell* 32: 1000–1017.

Shen, J.J., Qiao, Z.J., Xing, T.J., Zhang, L.P., Liang, Y.L., Jin, Z.P., Yang, G.D., Wang, R., and Pei, Y.X. (2012). Cadmium toxicity is alleviated by *At*LCD and *At*DCD in *Escherichia coli*. *J. Appl. Microbiolo.* 113: 1130–1138.

Shibuya, N., Koike, S., Tanaka, M., Ishigami-Yuasa, M., Kimura, Y., Ogasawara, Y., Fukui, K., Nagahara, N., and Kimura, H. (2013). A novel pathway for the production of hydrogen sulfide from D-cysteine in mammalian cells. *Nat. Commun.* 4: 1366.

Siegień, I. and Bogatek, R. (2006). Cyanide action in plants — from toxic to regulatory. *Acta Physiol. Plant.* 28: 483–497.

Sies, H., Mailloux, R.J., and Jakob, U. (2024). Fundamentals of redox regulation in biology. *Nat. Rev. Mol. Cell Biol.*, https://doi.org/10.1038/s41580-024-00730-2.

Spallarossa, A., Forlani, F., Carpen, A., Armirotti, A., Pagani, S., Bolognesi, M., and Bordo, D. (2004). The "rhodanese" fold and catalytic mechanism of 3-mercaptopyruvate sulfurtransferases: crystal structure of SseA from *Escherichia coli*. *J. Mol. Biol.* 335: 583–593.

Switzer, C.H., Kasamatsu, S., Ihara, H., and Eaton, P. (2023). SOD1 is an essential H_2S detoxifying enzyme. *Proc. Natl Acad. Sci. USA* 120, https://doi.org/10.1073/pnas.2205044120.

Theissen, U., Hoffmeister, M., Grieshaber, M., and Martin, W. (2003). Single eubacterial origin of eukaryotic sulfide:quinone oxidoreductase, a mitochondrial enzyme conserved from the early evolution of eukaryotes during anoxic and sulfidic times. *Mol. Biol. Evol.* 20: 1564–1574.

Todorovic, B. and Glick, B.R. (2008). The interconversion of ACC deaminase and D-cysteine desulfhydrase by directed mutagenesis. *Planta* 229: 193–205.

Trost, P., Fermani, S., Calvaresi, M., and Zaffagnini, M. (2017). Biochemical basis of sulphenomics: how protein sulphenic acids may be stabilized by the protein microenvironment. *Plant, Cell & Environ.* 40: 483–490.

Vescovi, M., Zaffagnini, M., Festa, M., Trost, P., Lo Schiavo, F., and Costa, A. (2013). Nuclear accumulation of cytosolic glyceraldehyde-3-phosphate dehydrogenase in cadmium-stressed Arabidopsis roots. *Plant Physiol.* 162: 333–346.

Vignane, T. and Filipovic, M.R. (2023). Emerging chemical biology of protein persulfidation. *Antioxid Redox Signal* 39: 19–39.

Wang, X., Shi, C., Hu, Y., Ma, Y., Yi, Y., Jia, H., Li, F., Sun, H., Li, T., Wang, X., et al. (2023). Persulfidation maintains cytosolic G6PDs activity through changing tetrameric structure and competing cysteine sulfur oxidation under salt stress in Arabidopsis and tomato. *New Phytol.* 240: 626–643.

Wedmann, R., Onderka, C., Wei, S., Szijártó, I.A., Miljkovic, J.L., Mitrovic, A., Lange, M., Savitsky, S., Yadav, P.K., Torregrossa, R., et al. (2016). Improved tag-switch method reveals that thioredoxin acts as depersulfidase and controls the intracellular levels of protein persulfidation. *Chem. Sci.* 7: 3414–3426.

Wirtz, M., Birke, H., Heeg, C., Mueller, C., Hosp, F., Throm, C., König, S., Feldman-Salit, A., Rippe, K., Petersen, G., et al. (2010). Structure and function of the hetero-oligomeric cysteine synthase complex in plants. *J. Biol. Chem.* 285: 32810–32817.

Wirtz, M., Droux, M., and Hell, R. (2004). O-acetylserine (thiol) lyase: an enigmatic enzyme of plant cysteine biosynthesis revisited in *Arabidopsis thaliana*. *J. Exp. Bot.* 55: 1785–1798.

Woo, H.A., Chae, H.Z., Hwang, S.C., Yang, K.-S., Kang, S.W., Kim, K., and Rhee, S.G. (2003). Reversing the inactivation of peroxiredoxins caused by cysteine sulfinic acid formation. *Science* 300: 653–656.

Wybouw, N., Dermauw, W., Tirry, L., Stevens, C., Grbić, M., Feyereisen, R., and Van Leeuwen, T. (2014). A gene horizontally transferred from bacteria protects arthropods from host plant cyanide poisoning. *ELife* 3: e02365.

Yadav, P.K., Vitvitsky, V., Carballal, S., Seravalli, J., and Banerjee, R. (2020). Thioredoxin regulates human mercaptopyruvate sulfurtransferase

at physiologically-relevant concentrations. *J. Biol. Chem.* 295: 6299–6311.

Yadav, P.K., Yamada, K., Chiku, T., Koutmos, M., and Banerjee, R. (2013). Structure and kinetic analysis of H_2S production by human mercaptopyruvate sulfurtransferase. *J. Biol. Chem.* 288: 20002–20013.

Yamaguchi, Y., Nakamura, T., Kusano, T., and Sano, H. (2000). Three Arabidopsis genes encoding proteins with differential activities for cysteine synthase and β-cyanoalanine synthase. *Plant Cell Physiol.* 41: 465–476.

Yao, G.-F., Wei, Z.-Z., Li, T.-T., Tang, J., Huang, Z.-Q., Yang, F., Li, Y.-H., Han, Z., Hu, F., Hu, L.-Y., et al. (2018). Modulation of enhanced antioxidant activity by hydrogen sulfide antagonization of ethylene in tomato fruit ripening. *J. Agric. Food Chem.* 66: 10380–10387.

Yi, H., Juergens, M., and Jez, J.M. (2012). Structure of soybean β-cyanoalanine synthase and the molecular basis for cyanide detoxification in plants. *The Plant Cell* 24: 2696–2706.

Zhang, M., Hu, K., Ma, L., Geng, M., Zhang, C., Yao, G., and Zhang, H. (2024). Persulfidation and phosphorylation of transcription factor *Sl*WRKY6 differentially regulate tomato fruit ripening. *Plant Physiol.* 196: 210–227.

Zhang, Q., Cai, W., Ji, T.-T., Ye, L., Lu, Y.-T., and Yuan, T.-T. (2020). WRKY13 enhances cadmium tolerance by promoting D-cysteine desulfhydrase and hydrogen sulfide production. *Plant Physiol.* 183: 345–357.

Zhao, Y.-Q., Hu, K.-D., Yao, G.-F., Wang, S.-Y., Peng, X.-J., and Zhang, H. (2023). A D-cysteine desulfhydrase, *Sl*DCD2, participates in tomato fruit ripening by modulating ROS homoeostasis and ethylene biosynthesis. *Hortic. Res.* 10, https://doi.org/10.1093/hr/uhad014.

Zhou, H., Huang, J., Willems, P., Van Breusegem, F., and Xie, Y. (2023). Cysteine thiol-based post-translational modification: what do we know about transcription factors? *Trends Plant Sci.* 28: 415–428.

Zivanovic, J., Kouroussis, E., Kohl, J.B., Adhikari, B., Bursac, B., Schott-Roux, S., Petrovic, D., Miljkovic, J.L., Thomas-Lopez, D., Jung, Y., et al. (2019). Selective persulfide detection reveals evolutionarily conserved antiaging effects of S-sulfhydration. *Cell Metab.* 30: 1152–1170.