Hydrogen sulfide signaling in plant adaptations to adverse conditions: molecular mechanisms

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Highlights: This review focuses on recent advances relating to H$_2$S signaling mechanisms. Interconnections between H$_2$S and H$_2$O$_2$ at the posttranslational modification level and with ABA in stomatal movement are highlighted.
Abstract

Hydrogen sulfide (H$_2$S) is a signaling molecule that regulates critical processes and allows plants to adapt to adverse conditions. The molecular mechanism underlying the H$_2$S action relies on its chemical reactivity, and the mainly characterized is the persulfidation, which involves the modification of protein thiol groups, resulting in the formation of persulfide groups. This modification derives a change of protein function, altering catalytic activity or intracellular location and inducing important physiological effects. H$_2$S cannot react directly with thiols but instead can react with oxidized cysteine residues; therefore, H$_2$O$_2$ signaling through sulfenylation is required for persulfidation. A comparative study performed in this review reveals 82% of identity between the sulfenylome and persulfidome. With regard to abscisic acid (ABA) signaling, widespread evidence shows an interconnection between H$_2$S and ABA in the plant response to environmental stress. Proteomic analyses have revealed persulfidation of several proteins involved in the ABA signaling network and have shown that persulfidation is triggered in response to ABA. In guard cells, a complex interaction of H$_2$S and ABA signaling has also been described, and the persulfidation of specific signaling components seems to be the underlying mechanism.

Keywords: Abscisic acid, persulfidation, proteomics, redox modifications, stomatal movement, sulfenylation
**Abbreviations:** ABA, abscisic acid; CO, carbon monoxide; H₂O₂, hydrogen sulfide; NO, nitric oxide; PEG, polyethylene glycol; ROS, reactive oxygen species; -SOH, sulfenic; -SO₂H, sulfinic; -SO₃H, sulfonic
Introduction

Hydrogen sulfide (H$_2$S) is a colorless gas with a characteristic unpleasant odor. In nature, H$_2$S is present in volcanic gas, hot springs, rock salts, and natural gas, as well as in emissions produced as a result of industrial activity. In biological systems, H$_2$S can be considered an ancient molecule since it originates from bacterial anaerobic metabolism. In the absence of oxygen, sulfur-reducing microorganisms use different forms of oxidized sulfur as electron acceptors during the degradation of simple organic matter, producing H$_2$S and CO$_2$ (Offre et al., 2013). H$_2$S is additionally used by sulfur-oxidizing bacteria as an electron donor in anoxicogenic photosynthesis to produce oxidized sulfur compounds (Johnston et al., 2009).

Hydrogen sulfide has long been considered a toxic molecule dangerous to the environment and complex biological organisms. In mammals, the presence of sulfide in mitochondria causes the inhibition of cytochrome c oxidase of the respiratory chain, as does the presence of carbon monoxide (CO) and nitric oxide (NO) (Cooper and Brown, 2008). However, below a specific concentration threshold, CO, NO and H$_2$S affect various cellular events and are currently considered to be signaling molecules that function as physiological gasotransmitters (Wang, 2014). In plants, H$_2$S is also recognized to have the same relevance as other signaling molecules, such as NO and hydrogen peroxide (H$_2$O$_2$) (Aroca et al., 2020; Aroca et al., 2018; Calderwood and Kopriva, 2014). All these molecules, including H$_2$S, show toxicity/signaling duality, depending on the concentration threshold.

Although H$_2$S is known to be present in mammalian tissues, it was first established in the late 20th century its intracellular production and signaling function as neuromodulator (Abe and Kimura, 1996). H$_2$S is produced endogenously by cells through different enzymes involved in cysteine metabolism, both in mammals and in plants. In plants, H$_2$S is also produced in the photosynthetic sulfate assimilation pathway in the chloroplast (Gotor et al., 2019). Intensive research on H$_2$S has been carried out in recent years both in animals and in plants, and an impressive exponential increase in the number of original publications and reviews has been occurred. Consequently, the number of biological functions in which sulfide is known to be involved has rapidly increased. In plants, H$_2$S has been shown to be essential in regulating a wide range of vital processes. H$_2$S improves the tolerance and protection of plants to numerous adverse environmental conditions, and in this way, it allows plant adaptability and viability, and its beneficial effects play a role in important aspects of development (Zhang et al., 2021b; Zhou et al., 2021b). Sulfide also regulates critical processes, including autophagy and abscisic acid (ABA)-dependent stomatal movement (Gotor et al., 2019; Laureano-Marín et al., 2020; Zhang et al., 2020). In addition, the interplay of H$_2$S with other signaling molecules and phytohormones in multiple physiological processes has been extensively described (Aroca et al., 2020).
Despite the very large number of plant studies that are continuously being conducted, studies on the molecular mechanisms through which sulfide exerts its regulatory effects are still scarce. We believe that this aspect deserves specific attention and this review highlights the progress obtained in understanding the mechanism of action of sulfide in plant systems. The most recent outcomes on the mechanism of the sulfide control of guard cell ABA signaling are also highlighted.

**Hydrogen sulfide action**

The reaction mechanism in which H$_2$S participates and exerts regulatory and signaling function is complex, and it is necessary to take into account the complex reactivity of this molecule. Hydrogen sulfide encompasses neutral H$_2$S and anionic forms (hydrosulfide, HS$^-$, and sulfide, S$^{2-}$) with pK$_{a1}$ and pK$_{a2}$ values of 6.9 and >12, respectively (Kabil and Banerjee, 2010). Therefore, in aqueous solution, H$_2$S exists in equilibrium with its H$^+$ and HS$^-$ anionic forms, these latter are unable to cross organelle membranes. Under physiological pH conditions, two-thirds of hydrogen sulfide exists in the form of HS$^-$. However, the lipid solubility of H$_2$S and its membrane permeability promote the biological distribution of sulfide species within cells (Cuevasanta et al., 2012).

The mechanism of action of H$_2$S is related to the characteristics of acid-base behavior and chemical reactivity with other biochemical molecules, such as low-molecular weight thiols, protein thiols, protein metal centers and biological oxidants. Among these oxidants, hydroxyl radical (HO$^\cdot$), nitrogen dioxide (NO$_2$)$^\cdot$, superoxide radical (O$_2^-$)$^\cdot$, hydrogen peroxide (H$_2$O$_2$), peroxynitrite (ONOOH) and hypochlorite (HOCl) can support H$_2$S oxidation (Li and Lancaster, 2013; Zaffagnini et al., 2019).

Metalloproteins are well-established biochemical targets of H$_2$S that covalently attach to heme porphyrins. Thus, H$_2$S acts as a potent inhibitor of mitochondrial cytochrome c oxidase, inhibiting mitochondrial respiration, releasing cytochrome c during apoptosis and stimulating procaspase 9 persulfidation (Vitvitsky et al., 2018). H$_2$S can also react quickly and reversibly with other ferric heme proteins such as methemoglobin and leghemoglobin to reduce their iron center and form a complex (Boubeta et al., 2020; Jensen and Fago, 2018). In addition to modifying heme proteins, H$_2$S can also modify Zn-finger proteins but leads to persulfidation and rapid thiol oxidation (Lange et al., 2019).

A second mechanism of action of H$_2$S that is well established in mammalian and plant systems is the modification of proteins by the oxidation of cysteine residues to form corresponding persulfides.
Protein thiol persulfidation has been widely described for numerous proteins, and it was initially described as S-sulfhydration in mouse liver. Susceptibility of several proteins to modification by sulfide has been determined (Filipovic et al., 2018; Mustafa et al., 2009). In plants, three high-throughput proteomic analyses also revealed the presence of persulfidation in the Arabidopsis proteome, showing more than 3400 and 5214 proteins susceptible to persulfidation in leaf and root tissue, respectively (Aroca et al., 2017a; Aroca et al., 2015; Jurado-Flores et al., 2021). Different studies on this posttranslational modification of specific proteins have shown that this modification results in changes to the function of the proteins, altering their catalytic activity or intracellular location and inducing important physiological effects ranging from regulation of autophagy, ABA-dependent stomatal closure, ethylene biosynthesis and root hair growth to resistance to oxidative stress (Table 1). Persulfides on specific Cys residues have been described in different Arabidopsis proteins, including abscisic acid insensitive 4 (ABI4) (Zhou et al., 2021a), cytosolic ascorbate peroxidase 1 (APX1) (Aroca et al., 2015), cytosolic glyceraldehyde-3-phosphate dehydrogenase (GapC1) (Aroca et al., 2017b), actin 2 (ACT2) (Li et al., 2018), L-cysteine desulphydrase (DES1), respiratory burst oxidase homolog protein D (RBOHD) (Shen et al., 2020), SNF1-related protein kinase SnRK2.6 (Chen et al., 2020) and the autophagic proteins ATG4a and ATG18a (Aroca et al., 2021a; Laureano-Marin et al., 2020). In addition, tomato 1-aminocyclopropane-1-carboxylic acid oxidases 1 and 2 (LeACO1 and LeACO2, respectively) (Jia et al., 2018) and tomato antioxidant enzymes (Li et al., 2020) have also been demonstrated to be persulfidated on specific Cys residues (Table 1).

Numerous biochemical and genetic data have undoubtedly established the signaling effect of H$_2$S in cells through persulfidation, with important consequences for numerous physiological and pathological processes in mammals and plants (Aroca et al., 2020; Paul and Snyder, 2018; Yuan et al., 2017). However, the precise mechanism that leads to the modification and the sulfur species that produces the protein persulfide formation is the subject of extensive debate and study. H$_2$S, or its ionic forms, HS$^-$ and S$_2^-$, cannot react directly with protein thiols and requires the presence of an oxidant. Thus, H$_2$S can react with oxidized cysteine residues as sulfenic acid (R-SOH), but also with protein nitrosothiols (R-SNO) to give protein persulfides, but this latter process is thermodynamically unfavorable (Filipovic et al., 2012). H$_2$S can also chemically react with disulfides (R-S-S-R) but seems unlikely due to the low level of intracellular H$_2$S and the slow reaction rate (Filipovic, 2015). Therefore, the reaction of H$_2$S with protein sulfenic acid to form protein persulfide seems the most plausible explanation for H$_2$S action. Cysteine residue oxidation represents a way for redox control of protein function; and therefore, H$_2$O$_2$ signaling takes place via the oxidation of cysteine to sulfenic acid, and the direct outcome of proteins is protein sulfonylation (Cuevasanta et al., 2015; Willems et al., 2020; Zivanovic et al., 2019). Although sulfenylated residues (R-SOH) can be reversed to reduced thiol by the action of a diverse set of reducing enzymes, stress conditions can lead to the...
overoxidation of cysteine residues originating the sulfinic (-SO$_2$H) or sulfonic (-SO$_3$H) motifs that are irreversible (Zaffagnini et al., 2019). However, in the catalytic cycle of peroxiredoxins it was shown that the sulfinic Cys can be reduced by sulfiredoxin in the presence of ATP via the formation of a phosphoryl intermediate (Sevilla et al., 2015). Protein sulfenic acid residues can react either with low molecular weight (LMW) thiols or with H$_2$S (HS$^-$ ionic form) but the later shows significant higher rate constant (Fig. 1). In fact, protein sulfenic residues react two orders of magnitude faster with H$_2$S than with glutathione (Cuevasanta et al., 2015). Since H$_2$S reacts with selenylated residues to form persulfide, protein persulfidation may play a role in H$_2$O$_2$-based signal transduction by preventing the overoxidation of cysteine residues, resulting in the loss of protein function. Under persistent oxidation stress, persulfidated proteins can react with ROS to form perthiosulfenic acids (-SSOH), and in the presence of excess oxidant, perthiosulfenic acid can be oxidized to perthiosulfinic (-SSO$_2$H) and perthiosulfonic acid (-SSO$_3$H) (Aroca et al., 2018; Filipovic et al., 2018). These oxidized perthiol residues can be reduced back to thiol by the action of glutathione and thioredoxin systems, as has been demonstrated in mouse liver (Doka et al., 2020; Zivanovic et al., 2019). The protective effect of persulfidation against overoxidation has also been shown in different cell types, where protein persulfidation increases following a phase-shifted curve after an increase in protein sulfenylation (Zivanovic et al., 2019) (Fig. 1).

Redox regulation has been shown to be involved in many signaling processes that regulate environmental (biotic and abiotic) stress responses (Alvarez et al., 2012; Hancock, 2019), development (Deng et al., 2020; Jia et al., 2015) or autophagy and cell death (Gotor et al., 2013; Perez-Perez et al., 2014; Xie et al., 2014) processes where H$_2$S is also involved. There is much evidence that there is an overlap between ROS and H$_2$S; therefore, together, protein cysteine oxidation and persulfidation may represent a mechanism for the modulation of signaling processes induced by developmental or environmental stress events. In recent years, many proteins have been described as sulfenylation targets in Arabidopsis in several works, revealing more than 2000 targets for this modification (De Smet et al., 2019; Huang et al., 2019; Wei et al., 2020). A comparison performed between the sulfenylated and the previously identified persulfidated proteins, more than 6000 targets (Aroca et al., 2017a; Aroca et al., 2015; Jurado-Flores et al., 2021; Laureano-Marin et al., 2020) revealed that 82% of the sulfenylated proteome described in Arabidopsis also undergoes persulfidation (Fig. 2A). A total of 1701 proteins are targets for either sulfenylation or persulfidation, a number that must be underestimated taking into account that the Arabidopsis samples were very different in all these proteomic analyses. Despite the probable differences in their metabolism, the number of common proteins in both proteomes is considerably high, revealing the role of these modifications in the finely tuned balance between H$_2$O$_2$-based signal transduction and protection against overoxidation. Gene ontology (GO) enrichment analysis of these proteins showed that several of these targets are associated with abiotic stress response-related GO terms, such as response to
cadmium (170), metal ion (180) and zinc (12), response to oxidative stress (61) and cellular response to oxidative stress (17), response to cold (57), response to heat (51), response to reactive oxygen species (24) and hydrogen peroxide (11), among others (Fig. 2B). Included in these targets, three L-ascorbate peroxidases, four dehydroascorbate reductases, three glutaredoxins, ten thioredoxins, two nitrate reductases, and numerous FAD/NAD(P)-oxidoreductases were found to be regulated by persulfidation and sulfenylation (Table S1 at Zenodo; 10.5281/zenodo.4727057; Aroca et al., 2021b), underlying the signaling role of these modifications in the activation of the antioxidant system against oxidative stress. Besides, GO enrichment showed that among those proteins regulated by persulfidation and sulfenylation they were found targets involved in response to biotic stress, hormones, signaling, other posttranslational modifications and transport (Fig. 2B).

Role of hydrogen sulfide in ABA signaling

In plants, precise mechanisms have been developed to perceive environmental stress. ABA, an important plant hormone, is involved in the regulation of growth and developmental processes and defense against various environmental stresses. ABA is a central regulator that triggers complex signaling networks and is also involved in stomatal movement. Under certain conditions, ABA concentrations increase to activate these signaling pathways, and consequently, ABA binds to the ABA receptor protein family members Pyrabactin Resistance 1 (PYR1)/ PYR1-Like (PYL)/ Regulatory Component of ABA receptor (RCAR), and inhibits the activity of clade A protein phosphatases (PP2Cs) (Fujii et al., 2009; Ma et al., 2009; Park et al., 2009). This process then results in the release of sucrose nonfermenting 1 (SNF1)-related protein kinase 2s (SnRK2s) from suppression by the PP2Cs, enabling the activation of SnRK2 protein kinases. These kinases subsequently phosphorylate and activate dozens of downstream targets (Hauser et al., 2017).

Extensive and convincing evidence published in the last decade has shown a close interrelation between H₂S and physiological processes regulated by the hormone ABA, suggesting crosstalk occurs between both molecules in regulation and signaling in plants (Aroca et al., 2020; Gotor et al., 2019). It has been widely reported that H₂S plays a role in stomatal closure, and the latest data are discussed in detail below. In addition to its role in plant growth and development, ABA plays a crucial role in plant responses to environmental stresses such as drought, salinity, osmotic stress and heat stress, processes in which H₂S has shown a protective effect alleviating the oxidative stress associated with these adverse conditions (Gotor et al., 2019). There is a large amount of additional evidence that
interconnects H$_2$S signaling with other plant processes regulated by ABA beyond mere antioxidant defenses. For example, it has been observed that the response to drought or heat mediated by ABA induces the accumulation of intracellular H$_2$S, and exogenous H$_2$S addition increases plant tolerance to these stresses (Jin et al., 2011; Li and Jin, 2016). It has also been observed that ABA shows an opposite effect on the transcriptional regulation of the cytosolic L-cysteine desulphydrase (DES1) that catalyzes the desulfuration of cysteine to generate H$_2$S, depending on the tissue, inhibiting its transcription in mesophyll cells and increasing its transcription in guard cell-enriched tissues (Scuffi et al., 2014). In general, sulfate availability affects the ABA content and germination response to ABA and salt stress, highlighting the importance of sulfur for stress tolerance (Cao et al., 2014). From a molecular point of view, the most extensive proteomic analysis published to date on protein persulfidation has shown that several proteins involved in ABA signaling, such as the pyrabactin resistance receptor 1 (PYR1) and pyrabactin resistance-like receptor (PYL), SnRK2.2 protein kinase, and protein phosphatase HAB2, are capable of being persulfidated (Aroca et al., 2017a).

Recently, a publication about ABA-triggered persulfidation of proteins (Laureano-Marín et al, 2020) revealed nearly 800 proteins that undergo persulfidation in response to ABA treatment in comparison with an untreated control (Table S2 at Zenodo). Data can be obtained from ProteomeXchange Consortium via PRIDE (Vizcaíno et al., 2016) partner repository with the identifier PXD019802. The GO enrichment data of the persulfidated proteins induced by ABA treatment were processed using AgriGO, (Table S3 at Zenodo). The GO term associated with response to stimulus, which contained 778 proteins, was analyzed to identify the most enriched GO terms (Fig. 3A), and it included 23 proteins in response to osmotic stress, 32 in response to temperature stimulus, 19 in response to oxidative stress, 24 in response to cold, 22 in response to salt stress and 13 in response to water deprivation. Besides, other 52, 19 and 36 proteins were involved in defense response, wounding and biotic stimulus, respectively. All the ABA-induced persulfidated proteins involved in abiotic stress are listed in Table S4 at Zenodo.

Overall, these results show that ABA treatment triggers persulfidation of a high number of proteins, and some of them aim to activate a cellular response to combat abiotic and biotic stresses. In addition, a total of 276 of these abscisic acid-induced persulfidated proteins have been described as being sulfenylated. Further analysis of these targets shows that there are proteins involved in the response to abiotic stresses that are also susceptible to persulfidation and sulfenylation. The GO enrichment data of the persulfidated proteins induced by ABA treatment, which can be also targets for sulfenylation were processed using AgriGo tool (Table S5 at Zenodo), and a selection of the GO enriched terms associated with the stress response was constructed to identify the most enriched GO terms (Fig. 3B). Those GO terms more represented were response to endoplasmic reticulum stress (GO:0034976) with a p-value of 0.000042 and a FDR of 0.00076, including 6 proteins in this GO term; response to cadmium (GO:0046686) with scores of $4.2 \times 10^{-16}$ and $8.8 \times 10^{-14}$ of p-value and FDR,
respectively; and response to heat (GO:0009408) and cold (GO:0009409) with p-values of 0.00094 and 0.0017, and FDR of 0.012 and 0.02, respectively. Nevertheless, as shown in Figure 3B, other important GO terms, such as response to osmotic stress, signal transduction and response to hormone are overrepresented. These results highlight the existence of crosstalk between sulfenylation and persulfidation in response to certain abiotic stresses and that protein posttranslational modifications play an important role in regulating these responses.

Role of hydrogen sulfide in guard cell ABA signaling

As pointed out previously, the activation of ABA signaling pathways induces downstream targets that, in conjunction with ROS, Ca\(^{2+}\) and Ca\(^{2+}\)-dependent protein kinases (CDPKs), activate ion channels to mediate stomatal closure and reduce water loss from transpiration (Mustilli et al., 2002; Papanatsiou et al., 2015). The participation of H\(_2\)S in stomatal closure has also been described previously (Garcia-Mata and Lamattina, 2010; Jin et al., 2013). An initial study showed that ABA cannot induce the stomatal closure of des knockout mutants deficient in cytosolic DES1, which produces H\(_2\)S in the cytosol (Alvarez et al., 2010), while the addition of an exogenous H\(_2\)S donor restored the closure. Moreover, ABA-dependent stomatal closure was partially blocked by an inhibitor of L-cysteine desulphhydrase and a scavenger of H\(_2\)S, DL-propargylglycine (PAG) and hypotaurine (HT), respectively, suggesting that H\(_2\)S participates in ABA-triggered stomatal movement (Scuffi et al., 2014). Although DES1 is expressed at all growth stages, at the tissue level, GFP expression driven by the DES1 promoter is very high in guard cells (Laureano-Marín et al., 2014). It is also noteworthy that DES1 gene expression level in the RNA extracts of epidermal cells was several-fold higher than that in the mesophyll cell-enriched samples upon ABA treatment (Scuffi et al., 2014), which provides a clue that the high expression level of DES1 in epidermal cells may largely be due to the proportion of guard cells. Recently, genetic evidence also indicated that guard cell-specific expressed DES1 is required for in situ H\(_2\)S production and is sufficient in regulating ABA-induced stomatal closure (Zhang et al., 2020).

The synthesis of ABA is a central response to stress. Interestingly, guard cells contain the complete suite of ABA biosynthesis pathway components. The molybdenum cofactor sulfurase ABA3 that mediated ABA synthesis in guard cells is sufficient to induce stomatal closure and relieve leaf wilting (Bauer et al., 2013). Several studies have revealed that H\(_2\)S is involved in ABA synthesis. Exogenous application of NaHS was shown to increase the transcript levels of ABA biosynthesis-
related genes during PEG treatment in both wheat leaves and wheat roots (Ma et al., 2016). Consistently, it was found that the transcription of genes related to ABA biosynthesis, such as the nine-cis-epoxycarotenoid dioxygenases NCED2, NCED3, and NCED5, sharply increased in rice seedlings under drought stress conditions, and pretreatment with NaHS further strengthened this inductive effect (Zhou et al., 2020). Recently, Zhang et al. (2021a) demonstrated that the accumulation of all of the transcripts involved in ABA synthesis in leaves increased in wild type but not in des1 mutants, indicating that DES1 is essential for dehydration-induced ABA synthesis. Moreover, the H₂S content was lower in aba3 mutant than in wild type, suggesting that DES1-produced H₂S is regulated by ABA synthesis. In addition, H₂S participates in NO- and ethylene-induced stomatal closure (Hou et al., 2013; Scuffi et al., 2014).

Proteomic analyses have revealed that various proteins involved in ABA signaling are susceptible to persulfidation and that ABA treatments trigger the persulfidation of a considerable number of protein targets (Aroca et al., 2017a; Laureano-Marin et al., 2020), thus suggesting that H₂S regulates ABA signaling pathways through persulfidation of specific targets, including those within guard cells. In this way, the DES1-mediated guard cell ABA cascade is attributable to H₂S signaling through persulfidation of open stomata 1 (OST1)/SNF1-RELATED PROTEIN KINASE 2.6 (SnRK2.6) at Cys131 and Cys137, which enhances ABA signaling (Chen et al., 2020).Remarkably, SnRK2.6/OST1 is also nitrosylated by NO at Cys137, leading to the inhibition of its activity and further negatively regulating guard cell ABA signaling (Wang et al., 2015). Crosstalk between H₂S and NO has been previously described in the ABA signaling network in guard cells (Lisjak et al., 2010; Scuffi et al., 2014), and SnRK2.6/OST1 could be one of the targets driving this interplay.

DES1 itself is also activated by H₂S through autopersulfidation at Cys44 and Cys205, which leads to transient H₂S overproduction and the amplification of H₂S signals in guard cells (Shen et al., 2020). Activated DES1 persulfidates NADPH oxidase RBOHD at Cys825 and Cys890 to rapidly induce a ROS burst that results in stomatal closure. Interestingly, persulfidation of both DES1 and RBOHD is redox dependent, and ROS accumulation at high levels induces persulfide-oxidation, which inhibits the activity of these proteins, leading to ABA desensitization. The oxidized persulfides can be reduced back to thiol groups by thioredoxin and prevent the continuous activation of the ABA signaling. Thus, these processes form a negative feedback loop through H₂S- and ROS-mediated modification that finely tunes guard cell redox homeostasis and ABA signaling. In addition, the accumulation of ROS induced by H₂S was also found to stimulate Ca²⁺ influx in guard cells (Wang et al., 2016). Other element involved in guard cell DES-mediated ABA signaling has been recently defined, the transcription factor ABA insensitive 4 (ABI4). The DES1-dependent H₂S accumulation induced by ABA generates the persulfidation of ABI4 at Cys250, promoting the MAPKKK18 transactivation and thus, propagating the MAPK signaling cascade in response to ABA (Zhou et al., 2021a). Together, these findings hint at the complexity of the H₂S signaling in stomatal movement.
The control of the stomatal closure or opening relies on the activity of ion channels and ion transport proteins in the plasma and vacuolar membranes. The regulation of inward-rectifying $K^+$ channels by $H_2S$ was shown in the sense that inactivation of the current associated with these channels induces stomatal closure by submicromolar concentrations of $H_2S$ (Papanatsiou et al., 2015). Proof was also provided by low-concentration-$H_2S$ activation of $S$-type anion currents in guard cells, the process of which requires elevated free cytosolic $Ca^{2+}$ levels and OST1 function (Wang et al., 2016). All these data highlight the complexity of the relationship between $H_2S$ and ion channels in the regulation of guard cell movement. Other secondary messengers that interact with $H_2S$ in the guard cell signaling network have been elucidated. In addition to the above-described ROS burst produced by NADPH oxidases, phospholipase D-derived phosphatidic acid is needed (Liu et al., 2021; Scuffi et al., 2018).

In summary, the $H_2S$ signaling network of stomatal movement is highly complex, and interactions among many different components of ABA-dependent signaling have been demonstrated (Pantaleno et al., 2021). Moreover, accumulative evidence indicates that the $H_2S$ molecular mechanism involves persulfidation (Fig. 4).

Conclusions and future perspectives

During past years, immense number of plant studies describing the role of $H_2S$ in the regulation of essential processes has enabled $H_2S$ to be consider a signaling molecule of the same significance as NO and $H_2O_2$. Moreover, recent reports have permitted to have a considerable insight of the molecular mechanism involved in the $H_2S$ action in some specific processes such as the ABA-dependent stomatal closure, and importantly, to know the specific protein targets of persulfidation. Nevertheless, it is no doubt that the current challenges on the $H_2S$ field are, in one regard, to deepen on the knowledge of the molecular mechanism involved in the $H_2S$ action, and in the other, to ascertain what is the bona-fide sulfurating molecule that modifies the thiol group on proteins. Regarding the $H_2S$ action, while an important effort has been made to establish chemical methods to label and enrich persulfidated proteins and technical improvement of mass spectrometers have allowed to identify an increasing number of plant proteins, mainly in Arabidopsis, the knowledge of the $H_2S$ mechanism of action in a particular process is still scarce. As described above, insight on this mechanism has only been revealed more or less profoundly in the ABA-dependent stomatal closure.
process, and in the regulation of autophagy by $\text{H}_2\text{S}$, although in the case of autophagy, the information is still limited.

With respect to the nature of the sulfurating species, this aspect is subjected to a great debate, even in animal systems. Due to the chemical nature of $\text{H}_2\text{S}$, it cannot react directly with the thiols in proteins, and several scenarios to lead to the formation of persulfidated proteins have been proposed. Thus, $\text{H}_2\text{S}$ can react with oxidized cysteine residues such as sulfenylated or nitrosylated cysteines or disulfides, and therefore, under specific oxidative conditions the sulfurating species can be $\text{H}_2\text{S}$, or its ionic forms, $\text{HS}^-$ and $\text{S}^{2-}$. Other sulfurating molecules proposed have been polysulfides, which contain the form of sulfur named sulfane with the oxidation state of 0 and they have the ability to attach reversibly to other sulfur atoms (Ida et al., 2014). We can hypothesize that depending on the specific condition/microenvironment of the target protein or the biological process in which the protein is involved, a particular sulfurating species or a mixture of them can be the responsible of the protein persulfidation and it would be very difficult to differentiate between them. In addition, it has been also described a prokaryotic and mammalian cysteinyl-tRNA synthetase that synthesizes persulfidated Cys for direct incorporation to proteins (Akaike et al., 2017). Another interesting point is to correlate the protein persulfidation pattern/level in one specific tissue/condition with the level of the sulfurating molecules. Perhaps in the context of high level of persulfidation, it would be possible discriminate which sulfurating species is the responsible of performing persulfidation.
Data availability

https://zenodo.org/record/4727058

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Conflicts of interest

The authors declare that they have no conflicts of interest.
Author contributions

A.A., J.Z. and L.C.R. wrote the first draft; Y.X. reviewed the manuscript; and C.G. conceived the study and reviewed and finalized the manuscript.
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Table 1. Plant proteins persulfidated at specific cysteine residues

| Protein                                                                 | Accession Number | No. Cys residues | Persulfidated Cys | Effect of the modification                                                                 | Reference                      |
|-------------------------------------------------------------------------|------------------|------------------|------------------|--------------------------------------------------------------------------------------------|--------------------------------|
| Arabidopsis Abscisic Acid Insensitive 4 (ABI4)                          | At2g40220        | 3                | Cys250           | MAPKKK18 transactivation/ increase the MAPK cascade signal in response to ABA               | (Zhou et al., 2021a)           |
| Arabidopsis Actin2 (ACT2)                                               | At3g18780        | 4                | Cys287           | Inhibition of actin polymerization/ depolymerization of F-actin bundles/ inhibition of root hair growth | (Li et al., 2018)              |
| Arabidopsis Cytosolic Ascorbate Peroxidase1 (APX1)                      | At1g07890        | 5                | Cys32            | Increase of enzyme activity                                                                | (Aroca et al.)                 |
| Arabidopsis Autophagy-related protein Cysteine Protease 4a (ATG4a)      | At2g44140        | 12               | Cys170           | Inhibition of proteolytic activity/ repression of autophagy                                | (Laureano-Marin et al., 2020)  |
| Arabidopsis Autophagy-related protein ATG18a                            | At3g62770        | 8                | Cys103           | Activation of ATG18a binding capacity to specific phospholipids/repression of autophagy     | (Aroca et al., 2021a)          |
| Arabidopsis L-Cysteine Desulhydrase1 (DES1)                             | At5g28030        | 3                | Cys44 and Cys205 | Increase of enzyme activity/induction of H$_2$S production/ABA-dependent stomatal closure  | (Shen et al., 2020)            |
| Arabidopsis Cytosolic Glyceraldehyde 3-Phosphate Dehydrogenase C1 (GapC1) | At3g04120        | 2                | Cys160           | Enhanced nuclear localization                                                              | (Aroca et al., 2017b)          |
| Arabidopsis Open Stomata1/SNFI-Related Protein Kinase2.6 (OST1/SnRK2.6) | At4g33950        | 6                | Cys131 and Cys137| Increase of enzyme activity/enhanced interaction with ABA response factor ABF2/ ABA-dependent stomatal closure | (Chen et al., 2020)            |
| Arabidopsis NADPH Oxidase Respiratory Burst Oxidase Homolog Protein D (RBOHD) | At5g7910         | 10               | Cys825 and Cys890| Increase of enzyme activity/induction of H$_2$O$_2$ production/ABA-dependent stomatal closure | (Shen et al., 2020)            |
| Tomato 1-Aminocyclopropane-1-Carboxylic Acid Oxidases 1 and 2 (ACO1 / 2) | NP_001234024/NP_001316842 | 4               | Cys60            | Inhibition of enzyme activity/repression of ethylene biosynthesis                          | (Jia et al., 2018)             |
| Tomato Cytosolic Ascorbate Peroxidase1 (cAPX1)                          | NP_001234782.1   | 6                | Cys168           | Increase of enzyme activity/enhanced resistance to oxidative stress                        | (Li et al., 2020)              |
| Tomato Catalase1 (CAT1)                                                 | NP_001234827.1   | 10               | Cys234           | Inhibition of enzyme activity/enhanced resistance to oxidative stress                      | (Li et al., 2020)              |
| Tomato Peroxidase 5 (POD5)                                              | XP_004235031.1   | 10               | Cys46 and Cys61  | Increase of enzyme activity/enhanced resistance to oxidative stress                        | (Li et al., 2020)              |

Shown as the accession number, the number of cysteine residues in the amino acid sequence, the specific persulfidated cysteines and the effects and biological consequences of the posttranslational modification of the proteins. The references of the studies are below.
Figure legends

**Fig. 1.** Schematic representation of the temporal dynamic of protein S-sulfenylation (P-SOH) and persulfidation (P-SSH) in different cell types (adapted from Zivanovic et al., 2019). After a transient ROS production induced by developmental or stress signal, the levels of S-sulfenylation in proteins are increased, accompanied by a raise in the activity of sulfide-generating enzymes and/or induction of low molecular weight (LMW) thiols, followed by a transient increase in protein persulfidation reversed by the action of reducing enzymes such as thioredoxin system (Trx/TrxR). The rate constants for the reaction of R-SOH with LMW thiols and H₂S at physiological pH 7.4 are shown (Cuevasanta et al., 2015).

**Fig. 2.** Comparison of persulfidated and sulfenylated proteins. A) Venn diagram showing the number of proteins. B) Fold-change enrichment of GO terms of common proteins modified by sulfenylation and persulfidation. Analysis was performed with PANTHER software. The numbers beside the bars indicate the number of proteins associated with each GO term for the input set.

**Fig. 3.** (A) Gene Ontology enrichment of ABA-induced persulfidated proteins involved in response to stimulus. (B) GO enrichment of persulfidated targets in response to ABA susceptible to be sulfenylated. P-value for each GO term is annotated in red numbers.

**Fig. 4.** Graphical model of interconnections between H₂S- and ABA-signaling networks in guard cells through the persulfidation of specific proteins. Under various environmental stress conditions, in guard cells, ABA concentrations increase and trigger the DES1 activity to induce the production of H₂S to persulfidate specific protein targets. DES1 itself is persulfidated at Cys44 and Cys205, and causes the persulfidation of open stomata 1 (OST1) at Cys131 and Cys137, the NADPH oxidase RBOHD at Cys825 and Cys890, and ABI4 at Cys250. Persulfidated RBOHD produces a ROS burst that results in stomatal closure. Overaccumulation of ROS induces persulfide oxidation leading to ABA desensitivility. Persulfidated ABI4 promotes the MAPKKK18 transactivation and MAPK signaling.
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