REVIEW ARTICLE

Hydrogen sulfide signaling in plants

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Abstract

Significance: Hydrogen sulfide (H_2S) is a multitasking potent regulator that facilitates plant growth, development, and responses to environmental stimuli.

Recent Advances: The important beneficial effects of H_2S in various aspects of plant physiology aroused the interest of this chemical for agriculture. Protein cysteine persulfidation has been recognized as the main redox regulatory mechanism of H_2S signaling. An increasing number of studies, including large-scale proteomic analyses and function characterizations, have revealed that H_2S-mediated persulfidations directly regulate protein functions, altering downstream signaling in plants. To date, the importance of H_2S-mediated persufidation in several abscisic acid signaling-controlling key proteins has been assessed as well as their role in stomatal movements, largely contributing to the understanding of the plant H_2S-regulatory mechanism.

Critical Issues*:* The molecular mechanisms of the H_2S sensing and transduction in plants remain elusive. The correlation between H_2S-mediated persulfidation with other oxidative posttranslational modifications of cysteines are still to be explored.

Future Directions: Implementation of advanced detection approaches for the spatiotemporal monitoring of H_2S levels in cells and the current proteomic profiling strategies for the identification and quantification of the cysteine site-specific persulfidation will provide insight into the H_2S signaling in plants.

Keywords: hydrogen sulfide, persulfidation, abscisic acid, stomatal movement

Introduction

Sulfur is the 10th most abundant chemical element in the universe and is essential for all living organisms (Räisänen, 2005). It occupies a unique position in the reduction–oxidation (redox) biology due to its availability to reach many distinct oxidation states, ranging from –2 to + 6 (Fig. 1). As the planet got oxidized, sulfate (+6) became the most abundant inorganic form of sulfur on earth. Hydrogen sulfide (H_2S), the most reduced inorganic form of sulfur (-2) (Fig. 1), is a colorless, but flammable, gas, smelling of rotten eggs that is naturally released from volcanic emissions or other geothermal activities and from decaying plant and animal proteins.

As H_2S is a weak acid with the dissociation constants pK_{a1} of 6.9 (Pomeroy, 1941) and pK_{a2} of between 12 and 17 (Ellis and Golding, 1959, Meyer et al., 1983), it can be dissociated into hydrosulfide (HS^-) and sulfide (S^{2-}) anions in aqueous solutions. H_2S usually stands for all species, including H_2S, HS^-, and S^{2-} (Paulsen and Carroll, 2013). Nevertheless, in solutions at an approximately physiological pH of 7.4, H_2S releases negligible amount of S^{2-} and exists primarily as HS^-(Hughes et al., 2009). Given the basic chemical properties of H_2S and HS^- with the lowest oxidation state of −2, they both can only be oxidized. Whereas H_2S is a gasotransmitter that can diffuse freely across cellular membranes, HS^- needs specific ion channels to move between different subcellular organelles or cells (Kabil and Banerjee, 2010). Accordingly, the H_2S and HS^- might regulate cellular functions differently. H_2S, HS^-, and S^{2-}, together with various chemically reactive forms of cysteine thiols (see below) and other sulfur-containing compounds that either reduce or oxidize biomolecules, can be classified as reactive sulfur species (RSS).

H_2S has been implicated in the origin of life (Filipovic et al., 2018; Olson and Straub, 2016). Life began 3.8 billion years ago (bya) in a anoxic and ferrous ion (Fe^{2+})-rich ocean. Cyanobacteria, the first photosynthetic oxygen-generating organisms, are believed to have then evolved and contributed to the Great Oxygenation Event around 2.5 bya (Demoulin et al., 2019; Fournier et al., 2021; Planavsky et al., 2014). Along with the slightly increased oxygen level, sulfur oxidized to sulfate, which was further reduced to sulfide by ubiquitous Fe^{2+} present in the ocean, greatly increasing the H_2S level and, consequently, leading to a anoxic and sulfidic ocean (Cortese-Krott et al., 2017). The first eukaryotes appeared and adapted under this condition using H_2S as their major energy source (Olson and Straub, 2016). Green algae, one of the earliest photosynthetic eukaryotes of the Plantae kingdom that contained primary chloroplasts, derived from endosymbiosis with cyanobacteria, are assumed to have evolved as

early as 1.0 bya (Tang et al., 2020). The combined activity of cyanobacteria and algae tremendously increased the level of ambient oxygen approximately 0.6 bya, sequentially, land plants have evolved. The antioxidant enzymes, such as superoxide dismutase (Cannio et al., 2000; Miller, 2012), catalase (Zamocky et al., 2008), glutathione peroxidase (Margis et al., 2008), peroxiredoxins (Dietz, 2011; Knoops et al., 2007), thioredoxins (TRXs) (Balsera and Buchanan, 2019), and glutaredoxins (GRXs) (Alves et al., 2009), were already present in an anoxic and high H_2S environment as early as 2.0 bya (Cortese-Krott et al., 2017). Therefore, these redox regulation systems most probably evolved primarily to use H_2S as energy source or to deal with RSS, which was later amended to regulate reactive oxygen species (ROS) (Olson and Straub, 2016; Cortese-Krott et al., 2017). Along with the evolution and increasing complexity of organisms, the ancient mechanisms of H_2S metabolism and regulation had to be adapted. For example, the sulfate transport system in chloroplasts has been suggested to undergo several adaptions, spanning the evolution of green algae, liverworts, and flowering plants (Kopriva et al., 2015; Mendoza-Cózatl et al., 2005; Takahashi et al., 2012). In brief, H_2S metabolism as well as regulatory mechanisms in living organisms have evolved as the result of environmental adaptation, while the fundamental principles might remain conserved (Yamasaki and Cohen, 2016).

Besides its implied vital role in evolution, H_2S has been recognized as a potent signaling molecule in the regulation of critical cellular processes (Wang, 2002, 2014). Increasing evidence has shown that H_2S is not only involved in plant growth, development, reproduction processes (Baudouin et al., 2016; Chen et al., 2011; Ma et al., 2021), and promotion of nodulation in the rhizobium-legume symbiosis (Zou et al., 2019), but also facilitates tolerance to various environmental stresses, such as drought (Jin et al., 2011; Shen et al., 2013), salinity (Christou et al., 2013; Li et al., 2014a), heavy metals (Fu et al., 2019, Zhang et al., 2020b), and extreme temperatures (Du et al., 2021; Li et al., 2012). Furthermore, H_2S has been reported to improve the quality maintenance during postharvest storage of fruit (Ge et al., 2017; Hu et al., 2012), vegetables (Li et al., 2014b), and flowers (Zhang et al., 2011). Given the important beneficial effects of H_2S in multiple aspects of plant physiology, exogenous H_2S seems to be a promising biotechnological strategy with a great agronomic interest.

Numerous studies have uncovered the positive physiological impact of H_2S on plants (Corpas, 2019; Corpas and Palma, 2020; Zhang et al., 2021), but how plants sense and transduce H_2S signals remains elusive. H_2S can modify proteins through posttranslational modification (PTM), a process named persulfidation. Persulfidation on critical proteins plays an important role in activating downstream signaling as demonstrated by both proteomic analyses and

functional characterizations (Aroca et al., 2015, 2017b, 2021a, 2021b; Fu et al., 2020a; Laureano-Marín et al., 2020; Zivanovic et al., 2019). Recently, H_2S-induced protein persulfidation has been found to regulate stomatal movements in the abscisic acid (ABA) signaling pathway (Chen et al., 2020; Shen et al., 2020; Zhou et al., 2021), contributing to the understanding of the molecular mechanism of H_2S signaling in plants.

In this review, we provide a wide perspective of H_2S in plants, including H_2S biosynthesis, exogenous application, endogenous detection methods, and the mechanisms and identification strategies for protein persulfidation. We further give insights into the molecular mechanisms of persulfidation in the regulation of ABA-mediated stomatal closure by highlighting several recent functional studies.

H_2S biosynthesis in plants

Plants generate H_2S endogenously through several biosynthesis pathways in different subcellular organelles (Fig. 2). The major H_2S source is associated with the photosynthetic sulfate assimilation pathway in chloroplasts. Plants take up sulfate from the environment through sulfate transporters (Takahashi et al., 2011), a protein class with high sulfate affinity that facilitates sulfate trafficking across membranes, into the chloroplasts, where H_2S is mainly generated through sulfur metabolism. Sulfate is reduced by ATP sulfurylase to form the adenosine 5'-phosphosulfate (APS) intermediate that is further reduced to sulfite by the APS reductase. H_2S is then produced from sulfite in the reaction catalyzed by sulfite reductase (Takahashi et al., 2011) (Fig. 2). H_2S reacts with *O*-acetylserine (OAS), generating cysteine via catalyzation by OAS (thiol)lyase (OAS-TL) (Fig. 2). Based on an *in vitro* activity assay, OAS-TL has been suggested to catalyze the reverse reaction to break down cysteine into H_2S and OAS (Burandt et al., 2001). However, negligible amounts of H_2S were formed when compared to the cysteine production, indicating that the OAS-TL reaction is a net H_2S-consuming reaction (Bloem et al., 2004). Moreover, the production of endogenous H_2S *in planta* by OAS-TL remains unclear. In plants, the main OAS-TLs responsible for cysteine synthesis are the cytosolic OAS-TL A1, the chloroplastic OAS-TL B, and the mitochondrial OAS-TL C (Fig. 2). Recently, the H_2S level was found to be higher in an *oas-tl a1* mutant than that of wild-type *Arabidopsis thaliana* plants, confirming the major biological function of OAS-TL in cysteine biosynthesis rather than in H_2S generation (Li et al., 2018).

Cysteine desulfhydrase (CDes) was the first studied H_2S-producing enzyme (Harrington and Smith, 1980; Tishel and Mazelis, 1966). Because of its ubiquitous activity in various

physiological processes of different plant species (Zhao et al., 2020), CDes is considered to be the most critical enzymatic source of H_2S in plants. There are two type of CDes: L-CDes that degrades L-cysteine and D-CDes that uses D-cysteine as substrate. In the cytosol, L/D-cysteine is catalyzed by L/D-CDes to produce H_2S, NH_3, and pyruvate (Fig. 2). L-CDes 1 (DES1), an OAS-TL homolog located in the cytosol, had originally been thought to have a function similar to that of OAS-TL A1, until its CDes activity had been proven (Álvarez et al., 2010). The *Arabidopsis DES1* gene is ubiquitously expressed at all developmental stages in plants (Laureano-Marín et al., 2014) and its function has been extensively investigated in the context of H_2S signaling in ABA-mediated stomatal movement (see below). Moreover, the nitrogenase Fe–S cluster (NifS), localized both in chloroplasts and mitochondria, is also a putative H_2S-producing enzyme due to its L-CDes-like activity (Pilon-Smits et al., 2002; Van Hoewyk et al., 2008). In addition, the mitochondrial β-cyanoalanine synthase (CAS) detoxifies cyanide that appeared in the cells to β-cyanoalanine in the presence of cysteine, along with the production of H_2S (Hatzfeld et al., 2000) (Fig. 2).

In mammals, 3-mercaptopyruvate sulfurtransferase (MST) that belongs to the sulfurtransferase (STR) family, is one of the most important H_2S-producing enzymes, but information about MST in plants is scarce. Recently, two *Arabidopsis* MSTs, the mitochondrial STR1 and the cytosolic STR2, were characterized by means of an *in vitro* activity assay, revealing their H_2S-producing capability in the presence of reducing systems, such as TRXs and GRXs (Moseler et al., 2021). Nevertheless, the *in planta* contribution to H_2S biosynthesis from STR1 and STR2 awaits to be further investigated.

Exogenous H_2S application in plants

Currently, investigation of the physiological roles of H_2S is mainly based on studies applying exogenous H_2S donors. To date, various H_2S donors have been developed (Powell et al., 2018; Yang et al., 2022) and have been widely used in plant studies (Corpas, 2019; Corpas and Palma, 2020; Liu et al., 2021). Here we provide a short discussion and an update on the these donors.

Sulfide salts, such as sodium hydrosulfide (NaHS) and sodium sulfide (Na_2S), are inorganic compounds that release H_2S by hydrolysis. To date, NaHS is the most popular and widely applied H_2S donor in various plant species (Corpas, 2019; Corpas and Palma, 2020; Liu et al., 2021). The use of NaHS as H_2S donor has greatly improved our understanding of the biological function of H_2S, but the NaHS shortcoming is that it does not mimic the biological effects of the physiological H_2S generation. Indeed, NaHS hydrolyzes immediately in aqueous solutions

and instantaneously releases large amount of H_2S, HS^-, and S^{2-} species, a process very different from the endogenously slow and continuous H_2S enzymatic production. Therefore, chemicals with a slow H_2S-releasing rate are required.

The morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate (GYY4137) is such a slow releasing H_2S compound that had initially been synthesized and evaluated with a vasodilatorory and antihypertensive activity (Li et al., 2008). Due to the commercial availability and application feasibility, GYY4137 is the most widely used H_2S donor, besides from sulfide salts in the mammalian field (Powell et al., 2018). In plants, GYY4137 often applied in parallel with NaHS that has similar effects. For example, similarly to NaHS, GYY4137 application could induce stomata closure in *Arabidopsis* (García-Mata and Lamattina, 2010; Honda et al., 2015) and *Nicotiana tabacum* (tobacco) (Papanatsiou et al., 2015) and could improve growth of *Pisum sativum* (pea), *Lactuca sativa* (lettuce), and *Raphanus sativa* (radish) (Carter et al., 2018).

Recent environmentally friendly slow H_2S-releasing chemicals, dialkyldithiophosphates and disulfidedithiophosphates, have been shown to improve the growth of *Zea mays* (maize) plants, hinting at potential applicability in agriculture (Brown et al., 2021; Carter et al., 2019). Another novel H_2S donor is a class of nitric oxide (NO)–hydrogen sulfide-releasing hybrid (NOSH) compounds that release NO and H_2S simultaneously, which was designed for its extreme effectiveness in growth inhibition of human cancer cell lines (Kodela et al., 2012). NOSH and its aspirin hybrid (NOSH-aspirin) that additionally releases acetylsalicylic acid have been shown to improve drought tolerance of *Medicago sativa* (alfalfa) (Antoniou et al., 2020), suggesting NOSH might be a promising plant priming agent against environmental stresses.

H_2S has been reported to act as electron donor for respiration and to contribute to ATP production in mitochondria of prokaryotes (Sakurai et al., 2010) and in a variety of species, such as California killifish (*Fundulus parvipinnis*) (Bagarinao and Vetter, 1990), marine mussel (*Geukensia demissa)* (Doeller et al., 1999, 2001; Parrino et al., 2000), sandworm *(Arenicola marina*) (Vökel and Grieshaber, 1997), chicken (*Gallus gallus)* (Yong and Searcy, 2001), and human (*Homo sapiens*) (Goubern et al., 2007). A mitochondria-specific H_2S donor, (10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yl)phenoxy)decyl) triphenylphosphonium bromide (AP39) (Le Trionnaire et al., 2014) can be used as a useful tool to specifically investigate the biological function of H_2S in mitochondria. AP39 was initially used in murine microvascular endothelial cells, had an antioxidant and cytoprotective impact under oxidative stress conditions (Szczesny et al., 2014), and later improved the mitochondrial function in the nematode *Caenorhabditis elegans* (Fox et al., 2021). H_2S is certainly tightly linked to the mitochondrial electron transport

chain (ETC) activity in the above mentioned species. However, the knowledge of this aspect in plants remains modest. Recently, AP39 was applied in *Arabidopsis* to investigate stomata movement (Pantaleno et al., 2023). In addition to the induction of stomatal closure, AP39 could modulate mitochondrial ETC activity and redox homeostasis of guard cells, providing the first piece of evidence that H_2S modulates mitochondrial energetics in plants.

In vivo **detection of H_2S in plants**

The important physiological functions of H_2S have attracted attention in plant research, compelling the development of detection techniques in living cells, tissues, and different organisms, but the direct detection of endogenous H_2S in plants remains a challenge. Traditional detection methods for H_2S, such as colorimetric assays (Siegel, 1965), gas chromatography (Hannestad et al., 1989), high-performance liquid chromatography (Shen et al., 2011), polarographic H_2S sensor (Doeller et al., 2005), and ion-selective electrode (ISE) (Li et al., 2000), typically require sample destruction and are limited to *in vitro* detection. Fluorescent probes are emerging as tools for the noninvasive study of reactive species *in situ* in different biological systems, because of their high cell permeability and specificity. Different fluorescent approaches, including chemical and genetically encoded probes, have been used for H_2S detection (Chen et al., 2012; Chen et al., 2013b; Lin et al., 2015; Liu et al., 2011a; Youssef et al., 2019). Despite the considerably fewer reports in plants than in the mammalian field, fluorescent probes are considered effective and noninvasive tools for the real-time detection and imaging of H_2S in plants.

As the methods for H_2S detection have been extensively reviewed (Filipovic et al., 2018; Kong et al., 2022; Lin et al., 2015; Luo et al., 2022; Zeng et al., 2021; Zhao et al., 2020), we will discuss the fluorescent probes recently used to discover endogenous H_2S in different plant species and organisms. In general, the various strategies can be categorized in three groups based on the reaction types, namely azide/nitro/nitroso reduction, copper sulfide precipitation, and nucleophilic reaction-based methods (Luo et al., 2022). The fluorescent probes used in plant studies are mainly based on azide reduction and nucleophilic reaction (Fig. 3).

AzMC

The azide reduction-based chemical probe, 7-azido-4-methylcoumarin (AzMC), is commercially available. The strong electron-withdrawing ability of the azide group of AzMC

quenches its fluorescence, whereas H_2S reduces the azide to amine, thus turning on the fluorescence (Kong et al., 2022). Initially, AzMC has been used in photoaffinity labeling of the substrate-binding site of the human phenol sulfotransferase (Chen et al., 1999) and later for H_2S detection in living cells and cardiac tissues (Chen et al., 2013a). In plants, AzMC was first applied to measure the H_2S level in tomato (*Solanum lycopersicum*) guard cells in response to ethylene signal transduction upon osmotic stress (Jia et al., 2018), Recently, this probe has been utilized to determine H_2S levels in *Arabidopsis* guard cells from wild-type and *des1* mutant plants, which are deficient in cytosolic H_2S generation due to the lack of DES1, in response of ABA-induced--stomatal closure (Shen et al., 2020; Zhang et al., 2020a). The ABA-induced H_2S accumulation in guard cells of wild-type plants was abolished in the *des1* mutant plants, whereas the H_2S donor NaHS could clearly induce H_2S in plants of both genotypes (Fig. 3), indicating that DES1 is responsible for the sensitivity of ABA-induced stomatal closure (Zhang et al., 2020a).

SiND-ANPA-N_3

Another azide reduction-based chemical probe is the silicon nanodots-4-azido-N-alanine-1,8-naphthalimide (SiND-ANPA-N_3**)**. This probe contains three moieties, the two-photon fluorophore dye *N*-alanine-1,8-naphthalimide, the azido adduct responsible for reduction-activated fluorescence, and the attached silicon nanodot (SiND) that increases water solubility and cell permeability. This probe has been tested in the inner-layer epidermal tissues of onion (*Allium cepa*) and evaluated as a potential probe for H_2S detection in aqueous media and living cells (Fu et al., 2020b) (Fig. 3).

WSP-1

The Washington state probe-1 (WSP-1) is a nucleophilic reaction-based chemical probe (Liu et al., 2011a) that contains a pyridyl disulfide moiety and a fluorophore group. H_2S reacts with the pyridyl disulfide and generates a persulfide intermediate that undergoes a spontaneous intramolecular cyclization to release the fluorophore. By using WSP-1 in tomato roots, an increased level of H_2S was detected upon an exogenous NO donor treatment, which was inhibited by applying the NO scavenger cPTIO (Li et al., 2014c) (Fig. 3). Until now, WSP-1 was utilized for endogenous H_2S detection in the roots of turnip (*Brassica rapa*) and the H_2S level decreased upon selenium treatment (Chen et al., 2014) (Fig. 3).

SSP4

The nucleophilic reaction-based sulfane sulfur probe, sulfane sulfur probe 4 (SSP4), has been developed based on the original design of the SSP1 and SSP2 probes (Chen et al., 2013b). Sulfane sulfur reacts with the nucleophilic thiols in the nonfluorescent SSP4 to form a persulfide intermediate that reacts with electrophilic ester groups, leading to spontaneous intramolecular cyclization and release of the fluorophore. Recently, SSP4 has been used for endogenous H_2S detection during the root nodule symbiosis in the legume *Lotus japonicus* and an increased level of H_2S has been observed during nodulation (Fukudome et al., 2020) (Fig. 3). SSP4 is commercially available and has a relatively high selectivity and sensitivity to sulfane sulfurs. However, sulfane sulfur probes are not specific for H_2S, because they also react with persulfides and polysulfides.

SSNIP

Another sulfane sulfur probe that shares a thiophenol moiety and an ester linker with the SSP4 probe is attached to an near-infrared (NIR) fluorophore, designated sulfane sulfur NIR probe (SSNIP). The SSNIP probe has been tested in *Arabidopsis* roots (Fig. 3) during different growth stages (Jiang et al., 2019).

HBTP-H_2S

The 2-(2-hydroxyphenyl) benzothiazole-based H_2S probe, HBTP-H_2S, is a recent NIR fluorescent probe and contains a dinitrophenyl (DNP) ether that undergoes thiolysis under nucleophilic attacks by H_2S, releasing the fluorophore. This NIR fluorescent probe has been applied for *in situ* bioimaging of endogenous H_2S in rice (*Oryza sativa*) roots and revealed an increased level of H_2S under Al^{3+} and flooding stresses (Wang et al., 2021) (Fig. 3).

Genetically encoded H_2S sensors

Since 2012, reaction-based genetically encoded fluorescent H_2S sensors have been studied and a probe based on *p*-azidophenylalanine (pAzF) was originally developed and tested by expressing the cpGFP-Tyr66pAzF in Hela cells (Chen et al., 2012). This pAzF-based genetic

probe has been optimized by modification with pAzF of the chromophore of a circularly permutated, superfolder green fluorescent protein (cpsGFP) to derive the cpsGFP-pAzF, which subsequently served as a Förster resonance energy transfer acceptor to an enhanced blue fluorescent protein EBFP2 (Youssef et al., 2019). Thus far, the H_2S genetic probe has not been utilized in plant research, whereas other genetic sensors, such as HyPer or the roGFP series for hydrogen peroxide (H_2O_2) sensing, and SoNar or Peredox for NADH/NAD^+ sensing (Müller-Schüssele et al., 2021), have been extensively used for monitoring the redox states in living plant cells. The development or application of genetic probes for real-time H_2S monitoring in plant research would be greatly beneficial for understanding the H_2S-regulatory mechanisms in plants.

H_2S signaling via protein persulfidation

Protein persulfidation, also called S-sulfhydration, as a type of oxidative PTM of cysteines, has been increasingly recognized as the main redox mechanism directly regulating diverse biological processes in H_2S signaling, such as protein function, structure, and subcellular localization. Protein cysteine thiols (–SH) are very susceptible to H_2O_2 and can undergo different oxidative PTMs. Initially, thiol reacts with H_2O_2 to form sulfenic acid (–SOH) (Allison, 1976) that is intrinsically unstable and an intermediary *en route* to other PTMs. In the presence of H_2O_2 excess, –SOH forms the relatively more stable sulfinic acid (–SO_2H) and sulfonic acid (–SO_3H) (Cremlyn, 1996), which are generally considered to cause protein overoxidation (Fig. 4). The –SO_3H formation is irreversible, whereas –SO_2H can be reduced via an ATP-dependent reaction by sulfiredoxin of certain proteins (Akter et al., 2018; Biteau et al., 2003). Alternatively, –SOH can react with proximal –SHs from proteins and with glutathione (GSH), forming intra/intermolecular disulfide (–SS–) (Nagy and Winterbourn, 2010, Turell et al., 2021) and *S*-glutathione adduct (–SSG) (Turell et al., 2008), respectively (Fig. 4). Disulfides and glutathione adducts can be enzymatically reduced to –SH by thiol reductases, so-called redoxins, such as TRXs and GRXs (Huang et al., 2018; Willems et al., 2021). Besides H_2O_2, reactive nitrogen species, which mainly refer to NO, trigger the formation of S-nitrosothiols (–SNO) (Hess et al., 2005) that can also be reduced by redoxins.

H_2S reacts with oxidized, not reduced, thiols, –SOH, and disulfides specifically, to form persulfides (–SSH) (Cuevasanta et al., 2015). The kinetics of the H_2S reactions with low-molecular weight albumin disulfides and –SOH have been determined. The rate constant of H_2S with –SOH for the formation of persulfides is 270 $M^{-1}s^{-1}$, significantly higher than that of

disuldfides (0.6 $M^{-1}s^{-1}$), implying that the formation of protein persulfides might mainly occur through reaction of H_2S with –SOH. However, extremely high reaction rates of protein disulfides with H_2S have been detected in some special cases, such as human sulfide quinone oxidoreductase (Cuevasanta et al., 2017). Furthermore, protein disulfides are relatively more stable than the labile –SOH in the environment; hence, the generation of protein persulfides via the H_2S reaction with disulfides cannot be excluded. Persulfides might be formed by reaction of H_2S with –SNO or –SSG (Francoleon et al., 2011; Iciek et al., 2015; Mishanina et al., 2015), but this reaction mainly remains hypothetical and needs further investigation.

Protein persulfides can be oxidized by H_2O_2 to form perthiosulfenic acid (–SSOH), perthiosulfinic acid (–SSO_2H), and perthiosulfonic acid (–SSO_3H). In contrast to the hardly reversible –SO_2H and irreversible –SO_3H formed upon H_2O_2 overoxidation, the corresponding –SSH and its oxidative derivatives can be reduced by redoxins (Dóka et al., 2020; Filipovic, 2015; Ju et al., 2016; Wedmann et al., 2016) (Fig. 4).

Protein persulfidation detection in plants

In the past decade, a variety of detection methods for efficient persulfidation labeling and identification have been developed to investigate the H_2S signaling executed via persulfidation. Protein persulfidation can be directly detected by means of mass spectrometry (MS), because of the mass increase of 31.972 Da by the addition of one sulfur atom, but is difficult to distinguish from other modification, such as –SO_2H due to the addition of two oxygen atoms (mass increase of 31.99 Da). Thus, specific labeling with chemical probes is required to persulfidation detection. Initially, a modified biotin switch method had been applied in human cells, in which methyl methanethiosulfonate (MMTS) was believed to specifically block –SH, whereafter –SSH was targeted and enriched with *N*-[6-(biotinamido) hexyl]-3'-(2'-pyridyldithio)propionamide (biotin-HPDP) (Mustafa et al., 2009). In plants, this method was first utilized in *Arabidopsis* leaf extracts and 106 persulfided proteins were identified (Aroca et al., 2015). However, because the reactivity toward MMTS of SSH was higher than that of thiols (Pan and Carroll, 2013), this method was questioned and should be used with caution.

The most challenging aspect of persulfidation detection is the discriminative labeling from thiols, because of their similar reactivity to commonly used reagents through alkylation, such as maleimide, *N*-ethylmaleimide (NEM), and iodoacetamide (IAM) (Pan and Carroll, 2013). Due to their greater nucleophilicity, persulfides react even much faster than thiols to thiol labeling reagents (Cuevasanta et al., 2015). Nevertheless, because alkylation of thiols yield

thioethers, whereas persulfides generate disulfides, many thiol label-based detection approaches have been exploited based on this characteristic.

At first, a fluorescent probe, designated red maleimide, had been used to study the persulfidation of the p65 subunit of mice NF-κB (Sen et al., 2012). Both −SH and −SSH are labeled with red maleimide and followed with dithiothreitol (DTT) reduction, so that only the labeled −SSH, namely the R−S−S−maleimide-red adducts, are reduced (Fig. 5A). The protein samples are subsequently separated by gel electrophoresis and detected by in-gel fluorescence. The loss of fluorescence can be calculated for evaluation of the persulfidation level. This method revealed the persulfidation of recombinant human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein at Cys150, which enhances its catalytic activity (Gao et al., 2015).

Later, another thiol label-based probe, a maleimide compound containing a peptide arm, designated maleimide peptide (MalP, 1.95 kDa), was used to study the persulfidation of the iron-sulfur cluster machinery in mammalian proteins (Parent et al., 2015). Similarly as with the red maleimide fluorescent probe, both −SH and −SSH are labeled with MalP and only the labeled −SSHs are further reduced by the subsequent DTT incubation and release the succinimide-peptide moiety (the product of the maleimide reaction with a sulfhydryl). As a result, the mobility shift in the protein migration can be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 5A).

The two aforementioned approaches offered many advantages, such as relative application simplicity and radiometric read outs (−SSH versus −SH) giving extra quantification information. Nevertheless, these method are restricted to biochemical characterizations and are rather not applicable to large-scale MS-based proteomic analyses.

A MS-coupled thiol labeling-based proteomic approach, termed biotin thiol assay (BTA), had initially been utilized for mapping protein persulfidation in mammalian cells by means of maleimide-biotin (Cuevasanta et al., 2015), maleimide-PEG2-biotin (Gao et al., 2015), or iodoacetyl-PEG2-biotin (Dóka et al., 2016, 2019). In this method, −SH and −SSH are first labeled with biotin-tagged alkylating reagents that are sequentially enriched on an avidin column, whereafter the labeled −SSHs are selectively eluted by reduction via DTT or tris(2-carboxyethyl)phosphine (Fig. 5B). By labeling the eluted persulfide-derived thiols by isotope-labeled (D5, heavy) or normal (H5, light) maleimide, a quantitative analysis under different conditions could be achieved (Gao et al., 2015). Recently, a BTA assay combined with the iodoacetyl isobaric tandem mass tag system allowed the quantitative cysteine site-specific identification of persulfidation (Gao et al., 2020).

Additionally, a 'tag-switch' method was developed for persulfidation detection (Zhang et al., 2014). Here, methylsulfonyl benzothiazole (MSBT) was used to block both −SH and −SSH, whereafter solely MSBT disulfide adducts (–SS–MSBT) could react with cyanoacetate biotin (CN-biotin), hence designated 'tag switch' (Fig. 5C). This method was further improved by attaching cyanoacetate with the fluorescent BODIPY moiety (CN-BOT) or the Cy3 dye (CN-Cy3), so that persulfidation could be visualized *in situ* by fluorescence confocal microscopy or in-gel fluorescent detection from the cell lysates (Kouroussis et al., 2019; Wedmann et al., 2016). In plants, the 'tag switch' method was applied in wild-type and *des1* mutant *Arabidopsis* plants (Aroca et al., 2017a). In total, 2,015 and 2,130 persulfidated proteins were identified in the wild-type and *des1* plants, respectively, suggesting that a large fraction of the *Arabidopsis* proteome undergoes persulfidation even under nonstressed conditions (Aroca et al., 2017a). Recently, the application of the 'tag switch' method on *Arabidopsis* root tissue identified 5,214 –SSH proteins (Jurado-Flores et al., 2021).

A variant of this tag-switch method for the –SSH identification is called the 'dimedone switch' assay (Zivanovic et al., 2019) (Fig. 5C). In this assay, –SSH, –SH, and –SOH first react with 4-chloro-7-nitrobenzofurazan (NBF-Cl), whereafter a dimedone-based probe, such as a biotin-conjugated analog DCP-Bio1 (Poole et al., 2007) or azide-conjugated analog DCP-N_3, selectively switches with –SS–NBF disulfides. The biotin-tagged persulfides are subsequently enriched and identified by MS and by in-gel fluorescence or confocal microscopy when DCP-Bio1 and DCP-N_3 and Cy5-alkyne click mix are used, respectively (Zivanovic et al., 2019). This dimedone switch method recently applied in *Arabidopsis* revealed persulfidaton of Cys103 of the autophagy-related protein 18a, thereby activating its phospholipid-binding activity in a reversible manner and, hence, regulating autophagy under endoplasmic reticulum stress (Aroca et al., 2021a).

Currently, a direct persulfidation detection method was developed for proteomic analysis by labeling –SSH at pH 5.0 by means of an alkyne-linked IAM, *N*-propynyliodoacetamide (IPM) (Fu et al., 2020a) (Fig. 5D). Given the lower pK_a of persulfide (4.3) than that of thiol (8.29), persulfides maintain a relatively high reactivity at pH 5.0 when compared to protonated thiols. Hence, efficient labeling of –SSH can be achieved by labeling proteome extracts with IPM at a low pH, resulting in disulfide adducts (SS-IPM), in addition to thioether adducts (S-IPM) due to the unavoidable –SH labeling. IPM-labeled peptides are further biotinylated by reaction with Az-UV-biotin reagents through a click reaction, the addition of biotin facilitating peptide enrichment. Two types of probe-modified peptides, including the disulfide forms derived from –SSH and the thioether forms derived from –SH, can be analyzed by MS (Fu et al., 2020a).

H_2S-mediated persulfidation in ABA-regulated stomatal movement

Stomata are pores on the epidermis of plant leaves surrounded by a pair of guard cells. Stomatal movement regulates gas and water exchange between the plants and the environment and is important for plant growth, development, and response to environmental stimuli (Kim et al., 2010). That H_2S induced stomatal closure and participated in ABA signaling was first evidenced by application of NaHS in epidermal strips of *Vicia faba* (broad bean), *Arabidopsis thaliana* and *Impatiens walleriana* (impatiens) (García-Mata and Lamattina, 2010). In contrast, H_2S was reported to cause stomatal opening in *Arabidopsis* (Lisjak et al., 2010) and pepper (*Capsicum annuum*) (Lisjak et al., 2011). The reason of the opposite effects of H_2S on stomata movement remains inconclusive. However, an increasing number of studies subsequently revealed that H_2S is a key regulator of stomatal closure triggered by different environmental stresses, such as drought (Jin et al., 2017), cold (Du et al., 2019), and mediated by phytohormones, such as ABA that accumulates under drought stress (Jin et al., 2013), ethylene (ET) (Hou et al., 2016; Liu et al., 2011b), salicylic acid (SA) (He et al., 2020), and jasmonic acid (JA) (Deng et al., 2020) (Fig. 6). To date, the H_2S signaling function has been best characterized in the ABA-regulated stomatal movement. ABA has been generally recognized as eliciting the *DES1* expression in guard cells that increases the endogenous level of H_2S, because DES1-mediated H_2S production is required for downstream NO signaling (Scuffi et al., 2014) and respiratory burst oxidase homolog (RBOH)-dependent H_2O_2 signaling (Scuffi et al., 2018) to activate stomatal closure (Fig. 6). Besides DES1 and RBOH (Shen et al., 2020), ABSCISIC ACID INSENSITIVE 4 (ABI4) (Zhou et al., 2020), and SNF1-RELATED PROTEIN KINASE2.6 (SnRK2.6), also known as Open stomata 1 (OST1) (Chen et al., 2020), have also been found as key proteins involved in H_2S signaling in ABA-regulated stomatal movement (see below). In addition, by means of pharmacological and genetic approaches, phospholipase D and mitogen-activated protein kinase 4 were shown to participate in H_2S-mediated guard cell signaling (Scuffi et al., 2018) and to be an important downstream signal of H_2S in stomatal movement in response to drought stress, respectively (Du et al., 2019).

Persulfidation on several key proteins in plants have been characterized, such as the critical antioxidant enzyme ascorbate peroxidase 1 (Aroca et al., 2015), the moonlighting cytosolic GAPDH protein (Aroca et al., 2017b), the autophagy-related protein 4 (Laureano-Marín et al., 2020) and 18 (Aroca et al., 2021a). For a recent review, see Aroca et al. (2021b). Here, we focus

on the most recent proteins, *i.e.*, DES1, RBOHD, OST1, and ABI4, that are involved in H_2S signaling in ABA-regulated stomatal closure (Fig. 7).

The ABA level in guard cells under normal conditions remains low, but increases upon stress stimuli. When ABA is absent, the protein phosphatase type 2C (PP2C) binds to the SnRK2.6 kinase domain and inhibits the kinase activity by dephosphorylation. In the presence of ABA, it binds to the ABA receptor PYRABACTIN RESISTANCE/PYR-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR (PYR/PYL/RCAR) to form a complex that binds to PP2C and inhibits the catalytic activity of PP2C (Fig. 7), thereby activating SnRK2.6 because of its dissociation from PP2C and autophosphorylation (Soon et al., 2012). The activated SnRK2.6 then transmits the ABA signal by phosphorylating downstream factors, such as RBOH, ultimately inducing rapid ROS production and cellular Ca^{2+} influx and activating specific ion channels that trigger stomatal closure (Fig. 7).

ABA induces the *DES1* expression transcriptionally through an unclear mechanism, thereby inducing the H_2S production in guard cells (Scuffi et al., 2014). The ABA-induced H_2S production results in the persulfidation of SnRK2.6 at Cys131 and Cys137, which are adjacent to the catalytic loop of the kinase and the pivotal phosphorylation site Ser175 (Chen et al., 2020) (Fig. 7). Persulfidation of SnRK2.6 promotes its activity and interacts with the transcription factor ABA response element-binding factor 2 (ABF2) that enhances the cytosolic Ca^{2+} signaling. Persulfidation of SnRK2.6 at Cys131 and Cys137 alters its protein structure, hence bringing the Ser175 residue closer to the phosphate-acceptor Asp140 and improving its activity (Chen et al., 2021). In contrast, NO negatively regulates ABA signaling in guard cells by inhibiting SnRK2.6 through formation of -SNO at Cys137 (Wang et al., 2015).

SnRK2 phosphphorylates the downstream transcription factor Related to ABI3/VP1-Like 1 (RAV1) to inhibit the expression of ABI4 (Feng et al., 2014). ABI4 has been considered as a versatile activator or repressor of its downstream target genes (Wind et al., 2013). The DES1-mediated H_2S production has been reported to induce the persulfidation of ABI4 at Cys250, which is essential for the ABI4 function in the regulation of plant responses to ABA (Zhou et al., 2021). The persulfidation of ABI4 at Cys250 enhances its transactivation activity on *Mitogen-Activated Protein Kinase Kinase Kinase 18* (*MAPKKK18*), thereby activating a MAPK cascade (Fig. 7). Furthermore, transactivation of *DES1* could be mediated by ABI4 persulfidation, hinting at the existence of a regulatory loop.

In addition, we recently demonstrated that H_2S-mediated persulfidation is involved in ABA signaling in guard cells by directly regulating the activity of both ROS and H_2S-producing enzymes (Shen et al., 2020). Upon ABA treatment, DES1-mediated H_2S triggers its

autopersulfidation at Cys44 and Cys205, leading to a burst of H_2S in guard cells. The accumulation of H_2S further induces the persulfidation of RBOHD Cys825 and Cys890, enhancing the RBOHD activity and leading to ROS overproduction (Fig. 7). NO was demonstrated to negatively regulate *Arabidopsis* RBOHD by forming -SNO at Cys 890 during the hypersensitive response in plant immunity (Yun et al., 2011). Nevertheless, NO-mediated modifications on RBOHD still need to be investigated regarding the impact on the ABA signaling in guard cells. Here, the interplays between H_2S, ROS, and NO involved in the regulatory ABA signaling mechanism in guard cells occur most probably through redox PTMs of key proteins.

Summary and Perspectives

In the past decades, numerous studies revealed the multitasking capacity of H_2S that is involved in many physiological and pathological processes in mammals (Dilek et al., 2020; Kimura et al., 2012; Murphy et al., 2019; Shatalin et al., 2011) and growth, development, and response to environmental stimuli in plants (Baudouin et al., 2016; Chen et al., 2011; Jin et al., 2013, 2017; Li et al., 2014a; Zou et al., 2019). Nevertheless, our current knowledge on the molecular mechanisms by which H_2S executes its signaling function remains limited. The emerging studies focusing on H_2S-mediated persulfidations in plants, especially the recently reported cases showing the key function of persufidation in ABA-regulated stomatal movements, have greatly contributed to the understanding of the H_2S regulatory mechanism in plants. Although several aspects regarding H_2S signaling in plants still await to be assessed, we believe that they will be solved in the near future by the application of advanced techniques, such as quantitative proteomics, real-time imagining, and structural biology.

Intra/inter disulfide formation and S-glutathionylation are known to be the major redox mechanisms that protect –SH from overoxidation, with GSH being the most important low molecular weight antioxidant in the cells. Recently, the reaction rate of SOH with H_2S has been reported to be 2700 $M^{-1}s^{-1}$ (Cuevasanta et al., 2015), which is much faster than that of –SH (21.6 $M^{-1}s^{-1}$) or that of GSH (2.9 $M^{-1}s^{-1}$) for the formation of disulfides and -SSG, respectively (Turell et al., 2008). In addition, increased persulfidation has been observed as a response to H_2O_2 stress in mammalian cells (Wedmann et al., 2016), indicating that the persulfidation chemistry involves the preferential addition of H_2S to a –SOH protein. Furthermore, H_2S might play an important role as an antioxidant through persulfidation in protecting thiols from irreversible overoxidations, such as $–SO_3H$. To compare the antioxidant role of H_2S-mediated

persulfidation with that of others PTMs, such as GSH-mediated S-glutathionlyation, several features need to be taken into account, including the cellular concentration of H_2S in the microenvironment. The concentrations of H_2O_2 and GSH in plant cells have been well documented (Cheeseman, 2006; Hasanuzzaman et al., 2017; Smirnoff and Arnaud, 2019) and measurements at a spatiotemporal resolution of H_2O_2 and GSH have been improved by means of genetically encoded sensors (Niemeyer et al., 2021; Nietzel et al., 2019; Ugalde et al., 2021a, 2021b, 2022). Unfortunately, the information on the cellular H_2S content is scarce. Although the available H_2S fluorescent probes provide useful tools to detect H_2S production *in situ*, the noninvasive application of chemical fluorescent probes *in planta* for real-time H_2S measurements remains a challenge because plant tissues are rather rigid. In our opinion, the implementation of genetic sensors for spatiotemporal monitoring of H_2S levels in cells would greatly advance our understanding of H_2S signaling in plants.

Besides GSH, ascorbate, another abundant antioxidant in plant cells, occurs in all subcellular compartments with particularly high levels in the chloroplasts (Smirnoff and Wheeler, 2000). Ascorbate-GSH cycle has been recognized as one of the major redox regulation pathways to detoxify H_2O_2 in plant cell (Foyer and Noctor, 2011). Although ascorbate was thought to selectively reduce –SNO formation and had been used extensively for the –SNO protein identification in proteomic studies (Willems et al., 2021), it has been found to also reduce –SOH of several proteins, including 1-Cys Prx (Monteiro et al., 2007) and the thiol-specific antioxidant enzyme 2 (Anschau et al., 2020) from *Saccharomyces cerevisiae* and papain from *Mus musculus* (Zito et al., 2012). Therefore, concern about the selectivity of ascorbate toward –SNO in terms of proteomic analysis is increasing. More importantly, these findings hint at an alternative pathway of thiol redox regulation via ascorbate. As H_2S is known to react with –SOH to form –SSH, ascorbate might presumably affect persulfidation of certain proteins indirectly via reduction of –SOH. Nevertheless, the direct correlation between ascorbate and H_2S-mediated persulfidation remains elusive. In addition to ascorbate itself, ascorbate peroxidase (APX), the enzyme catalyzing the conversion of H_2O_2 into H_2O in the presence of ascorbate, undergoes several types of thiol-based PTMs. The enzymatic activity of cytosolic APX1 in *Arabidopsis* is enhanced by NO-triggered –SNO formation (Begara-Morales et al., 2014; Yang et al., 2015) or H_2S-induced –SSH formation (Aroca et al., 2015) at Cys32. Large-scale proteomic studies revealed that tAPX and sAPX from chloroplast were also sensitive to thiol modifications (Huang et al., 2019, Wei et al., 2020). However, which are the exact types of thiol-based PTMs and how they regulate the biological function of chloroplastic APXs still await to be explored by functional analysis.

To better understand the function of H_2S-mediated persulfidation, it is crucial to find out the occupancy of –SSH and its correlation with other thiol-based PTMs. Such a correlation was evidenced by a comparative analysis based on proteomics data between –SSH and –SOH or –SH events in mammalian studies (Fu et al., 2020a; Zivanovic et al., 2019) and between –SSH, –SOH, and –SNO in *Arabidopsis* (Aroca et al., 2021b; Zhang et al., 2021; Zhou et al., 2020). Likewise many thiol-based PTMs, –SSH sites have been mapped at the whole-proteome level in mammals (Fu et al., 2020a), but remain uncharted territory in plants. Adoption of advanced proteomic profiling strategies for identification and quantification of the complete repertoire of persulfidation-undergoing Cys sites in plants will prove a promising future endeavor.

Authors' Contributions

J.H. conceived and wrote the article, Y.X. revised and submitted the manuscript.

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Figure legends

FIG. 1. Sulfur oxidation states in some biologically relevant compounds. The oxidation states of sulfur in different organic and inorganic compounds range from –2 to +6. R refers to the remainder of the molecule and symbolizes the variable side chain in protein structures.

FIG. 2. H_2S biosynthesis in plant cells. H_2S is produced in different subcellular organelles in the plant cells via various enzymes. The assimilated sulfate in plant cells is transported to the chloroplasts, where the sulfate is reduced first to APS, then to sulfite that is subsequently reduced to sulfide (H_2S) via SiR. Sulfide can be further catalyzed by OAS-TL in the presence of OAS to generate cysteine. In chloroplasts, this reaction is catalyzed by OASB, while the same reaction is catalyzed by OASA1 in the cytosol and by OASC in mitochondria. In the cytosol, L-cysteine and D-cysteine are degraded by L-CDes and D-CDes, respectively, to produce H_2S, pyruvate, and NH_3. Thus far, DES1 is the best H_2S-producing enzyme. NifS has been suggested to catalyze cysteine to H_2S in chloroplasts and mitochondria. In mitochondria, CAS catalyzes the reaction of cyanide and L-cysteine to produce H_2S and β-cyanoalanine. Abbreviations: APS, adenosine 5'-phosphosulfate; CAS, β-cyanoalanine synthase; DES1, L-cysteine desulfhydrase 1; D-CDes, D-cysteine desulfhydrase; H_2S, hydrogen sulfide; L-CDe, L-cysteine desulfhydrase; NH_3, ammonia; NifS, nitrogenase Fe–S cluster; OAS, O-acetylserine; OAS-TL, O-acetylserine (thiol)lyase; SiR, sulfite reductase.

FIG. 3. H_2S detection with fluorescent probes in plants. Various fluorescent probes have been used to visualize and determine the H_2S level in different plant species. In the chemical structures, the red star highlights the reaction moiety for H_2S and the yellow color marks the fluorophore group. AcMZ is a azide (N_3)-based chemical probe, used to determine the H_2S level in guard cells induced by ABA in wild-type *Arabidopsis* (accession Columbia-0 [Col-0]), but not in *des1* mutant plants, whereas the H_2S donor NaHS induced H_2S production in the guard cells of both genotypes (Zhang et al., 2020a). Another azide-based probe, SiND-ANPA-N_3, had been tested in inner-layer epidermal tissues of onion (Fu et al., 2020b). A nucleophilic reaction-based chemical probe, WSP-1, containing a pyridyl disulfide moiety as H_2S reaction sites, has been used in tomato roots to determine H_2S induction upon nitric oxide donor and scavenger treatments, and in the root of turnip for the observation of H_2S production repression upon selenium treatment (Chen et al., 2014). Two sulfane sulfur probes, SSP4 and SSNIP, both contain thiophenol moiety. The SSP4 probe has been used to reveal the H_2S production during

symbiosis in the nodules of *Lotus japonicus* (Fukudome et al., 2020). SSNIP is a recent NIR probe used in *Arabidopsis* roots (Jiang et al., 2019). The most recent fluorescent probe applied in plants is the NIR-based probe, HBTP-H_2S, showing an increased H_2S level upon Al^{3+} and flooding stresses (Wang et al., 2021). Abbreviations: ABA, abscisic acid; AcMZ, 7-azido-4-methylcoumarin; Al^{3+}, aluminum ion; BF, bright field; HBTP-H_2S, 2-(2-hydroxyphenyl) benzothiazole--based H_2S probe; H_2S, hydrogen sulfide; NIR, near-infrared; SiND-ANPA-N_3, silicon nanodots-4-azido-N-alanine-1,8-naphthalimide; SSP4, sulfane sulfur probe 4; SSNIP, sulfane sulfur near-infrared probe; WSP-1, Washington State probe-1.

FIG. 4. Overview of cysteine thiols undergoing different oxidative posttranslational modifications. Protein cysteine –SH reacts with H_2O_2 to form –SOH that can be further oxidized by H_2O_2 to –SO_2H and –SO_3H, both considered protein overoxidations. Via an ATP-dependent reaction –SO_2H can be reduced by SRX, whereas –SO_3H is an irreversible modification. The –SOH protein can react with –SH or with GSH to form –SS– or –SSG, respectively. The proteins –SS– and –SSG can be reduced by redoxins, including TRXs and GRXs. The protein cysteine –SH reacts with NO to form –SNO that can also be reduced by redoxins. H_2S reacts with –SOH and –SS– to form –SSH, which has been suggested to be formed also by the reaction of H_2S with –SNO or –SSG. The protein –SSH can be oxidized by H_2O_2 to form –SSOH, –SSO_2H, and–SSO_3H and –SSH and its oxidative derivatives can be reduced by redoxins. Abbreviation: GSH, glutathione; H_2O_2, hydrogen peroxide; H_2S, hydrogen peroxide; NO, nitric oxide; –SH, thiol; –SOH; sulfenic acid; –SO_2H, sufinic acid; –SO_3H, sulfonic acid; SRX, sulfiredoxin; –SS–, intra/intermolecular disulfides; –SSG, glutathione adduct; –SSOH, perthiosulfenic acid; –SSO_2H, perthiosulfinic acid; –SSO_3H, perthiosulfonic acid; –SNO, S-nitrosothiol.

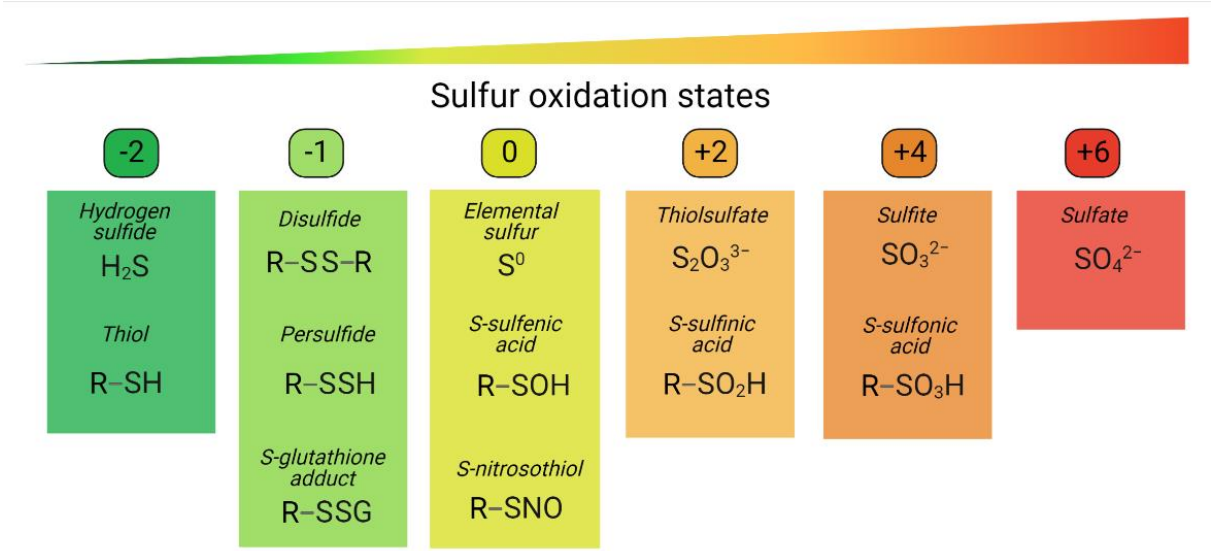
FIG. 5. Persulfidation detection approaches. (**A**) Red maleimide and MalP methods. Both –SH and –SSH are initially labeled, but only the labeled –SSH can be reduced by DTT. In the red maleimide method, –SSH is detected by SDS-PAGE and displays fluorescence loss. As the Mal-P labeling results in an molecular mass increase of 1.95 kDa, –SSH is discovered as a decreased mobility shift in SDS-PAGE. (**B**) BTA method for proteomic analysis. Both –SH and –SSH are labeled with maleimide-biotin and enriched by streptavidin magnetic beads. The enriched –SSH proteins are eluted by DTT reduction, digested with trypsin, and subjected to MS analysis. (**C**) Tag switch and dimedone switch methods. In the tag switch method, MSBT is used to block both –SH and –SSH, the latter forming –SS–MSBT that further reacts with CN-

biotin to be biotinylated, which is further enriched by streptavidin AP, digested by trypsin, and subjected to MS analysis. Alternatively, CN-BOT and CN-Cy3 are used instead of CN-biotin to visualize –SSH *in situ* by confocal microscopy and in-gel fluorescent detection, respectively. In the dimedone switch assay, NBF-Cl reacts with –SSH, –SH, and –SOH, whereafter DCP-Bio1 or DCP-N3 is added to selectively switch with the –SS–NBF adducts. When DCP-Bio1 is utilized, the –SSH proteins are subsequently enriched with streptavidin AP and identified by MS. When DCP-N_3 and the Cy5-alkyne click mix are applied, the –SSH is detected by in-gel fluorescent assay or with confocal microscopy. (**D**). Direct labeling method at low pH for proteomic analysis. Both –SH and –SSH are labeled by IPM at pH 5.0, resulting in proteins with S-IPM and SS-IPM adducts that are further digested by trypsin. The digested peptides are biotinylated by a click reaction with Az-UV-biotin reagents, enriched by streptavidin AP, cleaved by UV light to remove the biotin moiety, and subjected to MS analysis. Abbreviations: AP, affinity purification; Az-UV-biotin, UV cleavable biotin-azide; BTA, biotin thiol assay; CN-biotin, cyanoacetate biotin; CN-BOT, cyanoacetate with fluorescent BODIPY moiety; CN-Cy3, cyanoacetate with Cy3-dye; DCP-Bio1, dimedone-based biotin-conjugated analog; DCP-N_3, dimedone-based azide-conjugated analog; DTT, dithiothreitol; IPM, *N*-propynyliodoacetamide; MalP, maleimide-peptide; MSBT, methylsulfonyl benzothiazole; NBF-Cl, 4-chloro-7-nitrobenzofurazan; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; –SH, thiol; –SSH, persulfide; –SOH, sulfenic acid.

FIG. 6. Simplified scheme of H_2S-triggered stomatal closure in ABA-regulated stomatal closure under drought stress. Under drought or cold stress, ABA or other phytohormones, such as ET, SA, and JA, accumulate in the cells and regulate stomatal movement via H_2S signaling. ABA induces the expression of *DES1*, increasing the H_2S production. DES1-catalyzed H_2S signals activate downstream H_2O_2 and NO signaling to trigger stomatal closure. Abbreviations: ABA, abscisic acid; DES1, L-cysteine desulfhydrase 1; ET, ethylene; H_2S, hydrogen sulfide; H_2O_2, hydrogen peroxide; JA, jasmonic acid; NO, nitric oxide; SA, salicylic acid.

FIG. 7. H_2S-triggered persulfidation in ABA-regulated guard cell movement. When ABA is present, it binds PYR/PYL/RCAR to form a receptor complex that interacts with and inhibits the catalytic activity of PP2C, activating SnRK2.6. SnRK2.6 undergoes H_2S-triggered persulfidation, thereby promoting its activity and interaction with ABF2. SnRK2 phosphphorylates downstream the transcription factor RAV1, hampering the expression of

ABI4, whereas DES1-mediated H_2S production induces ABI4 persulfidation that enhances its transactivation activity on *MAPKKK18*, thereby initiating a MAPK cascade. DES1-mediated H_2S triggers its autopersulfidation, leading to a H_2S burst. The H_2S accumulation induces RBOHD persulfidation, thereby increasing its activity and leading to ROS overproduction. The ROS accumulation increases the Ca^{2+} influx, contributing to stomata closure in response to ABA. Abbreviations: ABA, abscisic acid; ABF2, ABA response element-binding factor 2; ABI4, ABA insensitive 4; Ca^{2+}, calcium cation; DES1, L-cysteine desulfhydrase 1; H_2S, hydrogen sulfide; MAPK, mitogen-activated protein kinase; *MAPKKK18, MAPK kinase kinase 18;* PP2C, protein phosphatase type 2C; PYR/PYL/RCAR, PYRABACTIN RESISTANCE/PYR-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR; RAV1, ABI3/VP1-like 1; RBOHD, respiratory burst oxidase homolog D; SnRK2.6, SNF1-RELATED PROTEIN KINASE2.6; ROS, reactive oxygen species.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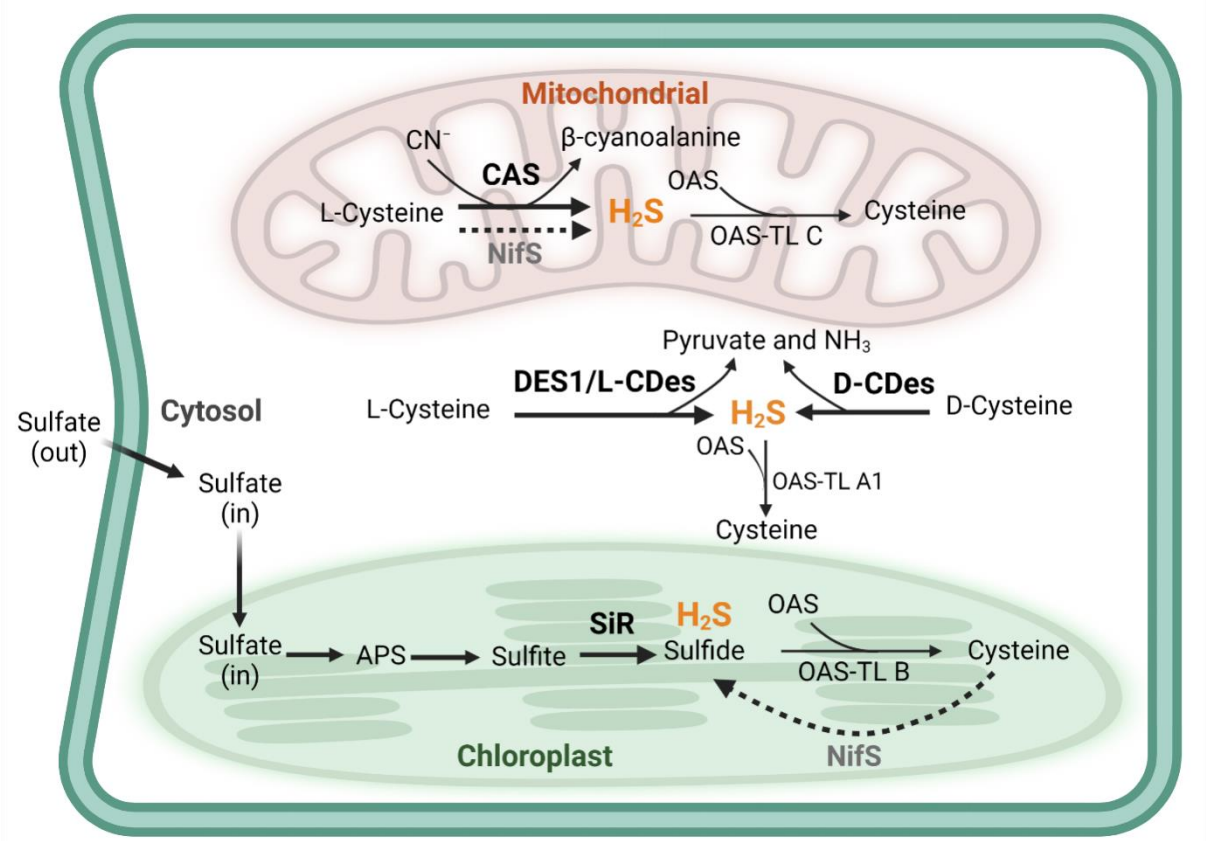
H_2S
Thiol
R–SH
-1
Disulfide
R–SS–R
Persulfide
R–SSH
S-glutathione adduct
R–SSG
0
Elemental sulfur
S^0
S-sulfenic acid
R–SOH
S-nitrosothiol
R–SNO
+2
Thiolsulfate
$S_2O_3^{3-}$
S-sulfinic acid
$R–SO_2H$
+4
Sulfite
SO_3^{2-}
S-sulfonic acid
$R–SO_3H$
+6
Sulfate
SO_4^{2-}

Fig. 1.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OAS
L-Cysteine
H2S
Cysteine
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NifS
Pyruvate and NH3
DES1/L-CDes
D-CDes
Sulfate
(out)
Cytosol
L-Cysteine
H2S
D-Cysteine
OAS
OAS-TL A1
Sulfate
(in)
Cysteine
SiR
H2S
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(in)
APS
Sulfite
Sulfide
Cysteine
OAS-TL B
Chloroplast
NifS

Fig. 2.

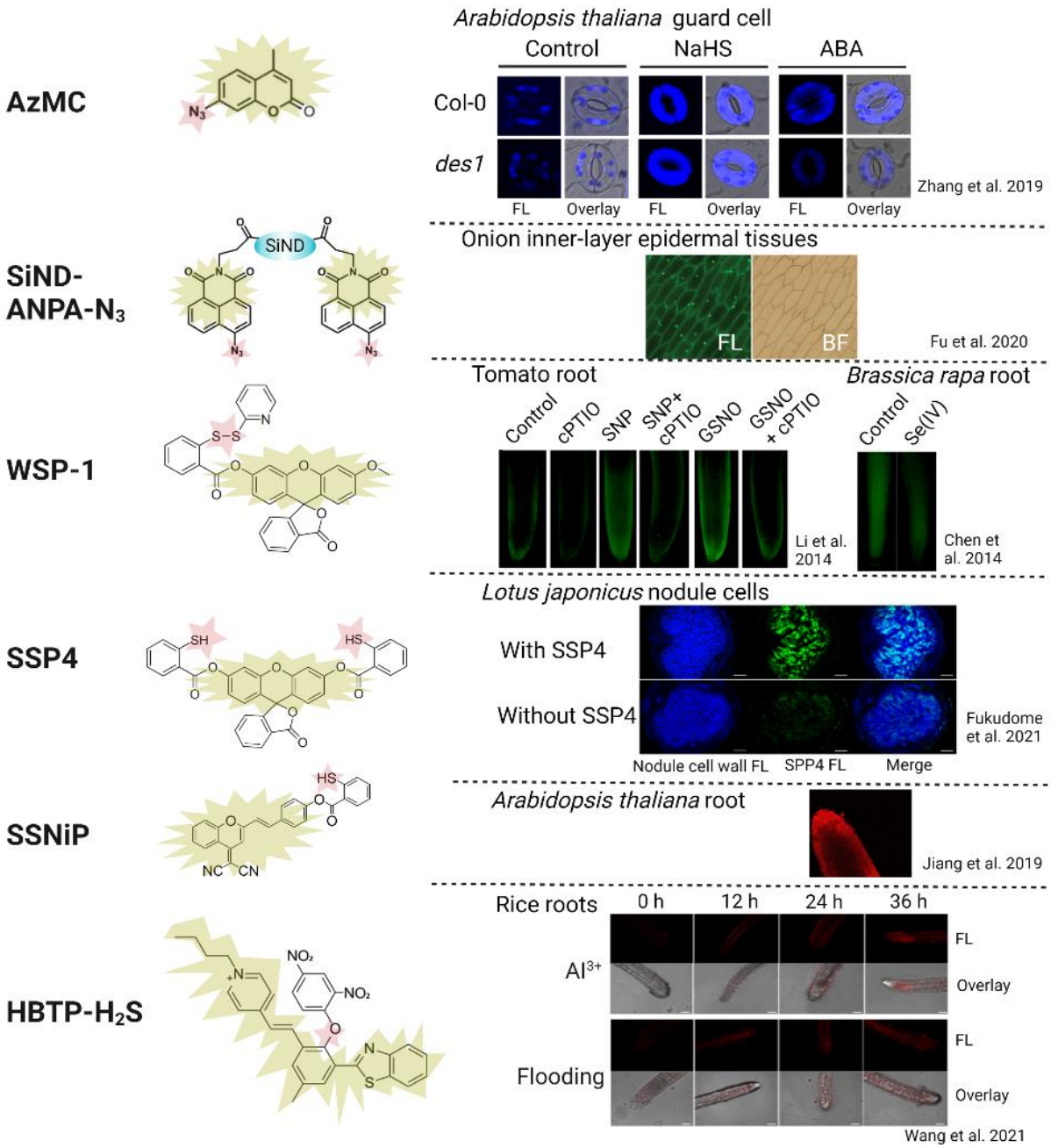
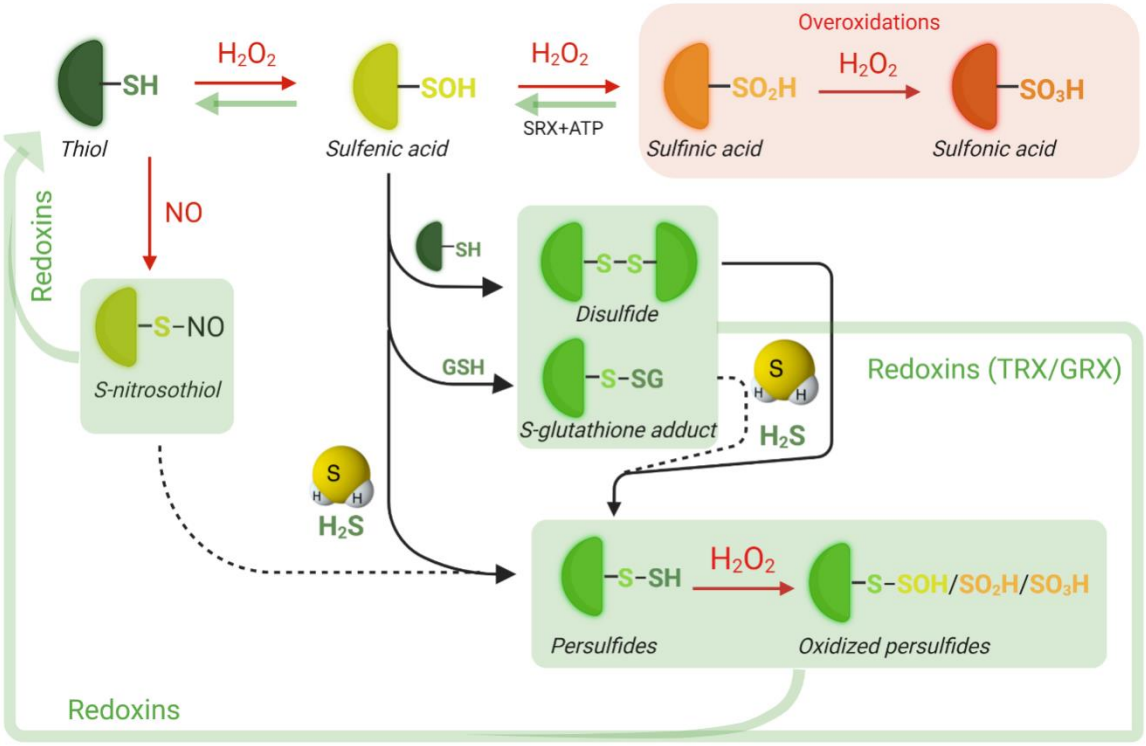

AzMC
Arabidopsis thaliana guard cell
Control
NaHS
ABA
Col-0
des1
FL
Overlay
Zhang et al. 2019
SiND-ANPA-N3
SiND
Onion inner-layer epidermal tissues
FL
BF
Fu et al. 2020
WSP-1
Tomato root
Brassica rapa root
Control
cPTIO
SNP
SNP+ cPTIO
GSNO
GSNO + cPTIO
Li et al. 2014
Control
Se(IV)
Chen et al. 2014
SSP4
Lotus japonicus nodule cells
With SSP4
Without SSP4
Nodule cell wall FL
SPP4 FL
Merge
Fukudome et al. 2021
SSNiP
Arabidopsis thaliana root
Jiang et al. 2019
HBTP-H2S
Rice roots
0 h
12 h
24 h
36 h
Al3+
Flooding
FL
Overlay
Wang et al. 2021

Fig. 3.


Overoxidations
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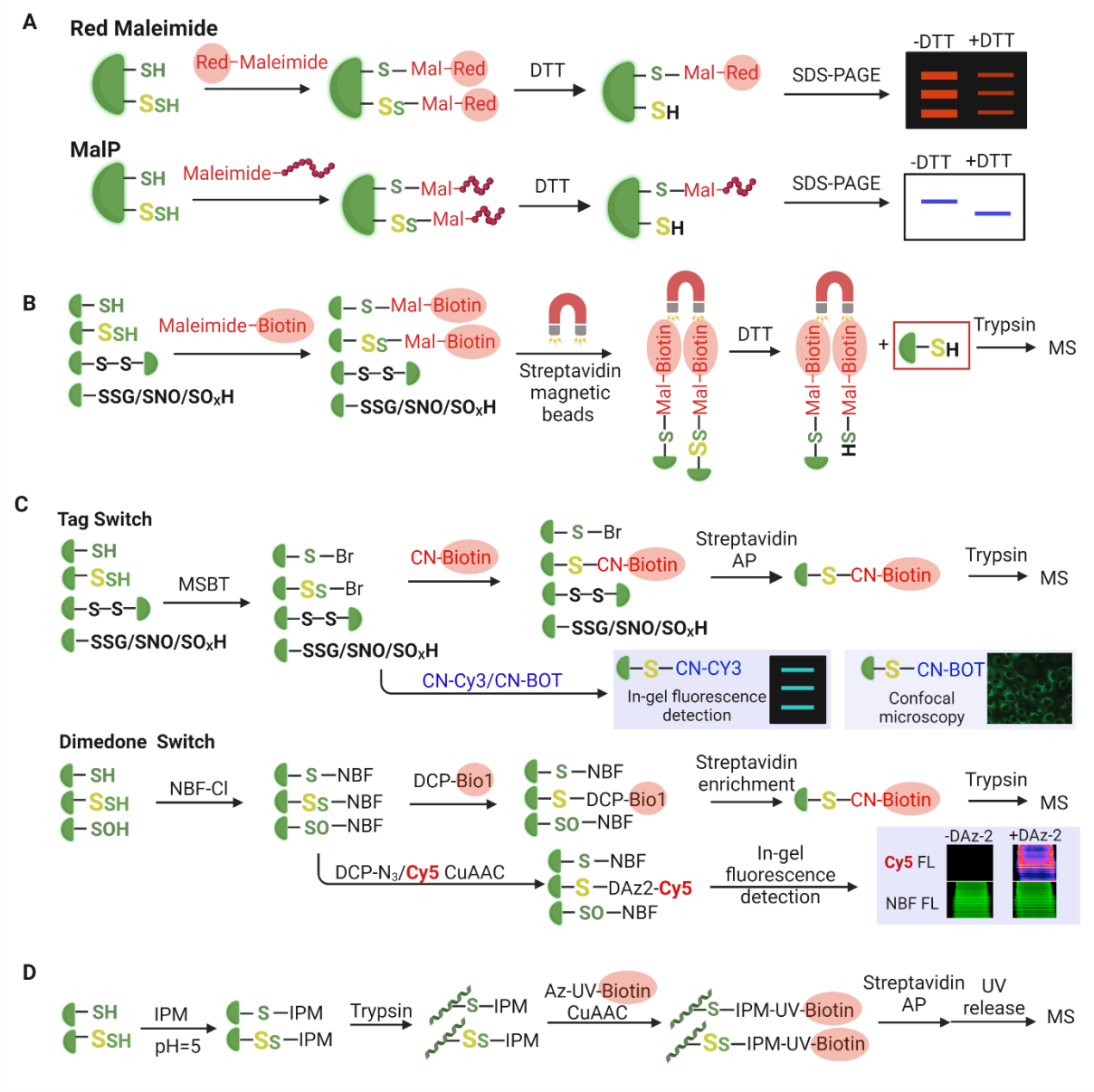
Thiol
H_2O_2
SOH
Sulfenic acid
H_2O_2
SRX+ATP
SO_2H
Sulfinic acid
H_2O_2
SO_3H
Sulfonic acid
Redoxins
NO
S-NO
S-nitrosothiol
SH
S-S
Disulfide
GSH
S-SG
S-glutathione adduct
S
H
H
H_2S
Redoxins (TRX/GRX)
S
H
H
H_2S
S-SH
Persulfides
H_2O_2
S-SOH/SO_2H/SO_3H
Oxidized persulfides
Redoxins

Fig. 4.


A
Red Maleimide
SH
SSH
Red-Maleimide
S—Mal-Red
SS—Mal-Red
DTT
S—Mal-Red
SH
SDS-PAGE
-DTT +DTT
MalP
SH
SSH
Maleimide-
S—Mal-
SS—Mal-
DTT
S—Mal-
SH
SDS-PAGE
-DTT +DTT
B
SH
SSH
S–S–
SSG/SNO/SOxH
Maleimide-Biotin
S—Mal-Biotin
SS—Mal-Biotin
S–S–
SSG/SNO/SOxH
Streptavidin
magnetic
beads
Mal-Biotin
Mal-Biotin
S—
SS—
DTT
Mal-Biotin
Mal-Biotin
S—
HS—
+
SH
Trypsin
MS
C
Tag Switch
SH
SSH
S–S–
SSG/SNO/SOxH
MSBT
S—Br
SS—Br
S–S–
SSG/SNO/SOxH
CN-Biotin
S—Br
S—CN-Biotin
S–S–
SSG/SNO/SOxH
Streptavidin
AP
S—CN-Biotin
Trypsin
MS
CN-Cy3/CN-BOT
S—CN-CY3
In-gel fluorescence
detection
S—CN-BOT
Confocal
microscopy
Dimedone Switch
SH
SSH
SOH
NBF-Cl
S—NBF
SS—NBF
SO—NBF
DCP-Bio1
S—NBF
S—DCP-Bio1
SO—NBF
Streptavidin
enrichment
S—CN-Biotin
Trypsin
MS
DCP-N3/Cy5 CuAAC
S—NBF
S—DAz2-Cy5
SO—NBF
In-gel
fluorescence
detection
-DAz-2
+DAz-2
Cy5 FL
NBF FL
D
SH
SSH
IPM
pH=5
S—IPM
SS—IPM
Trypsin
S—IPM
SS—IPM
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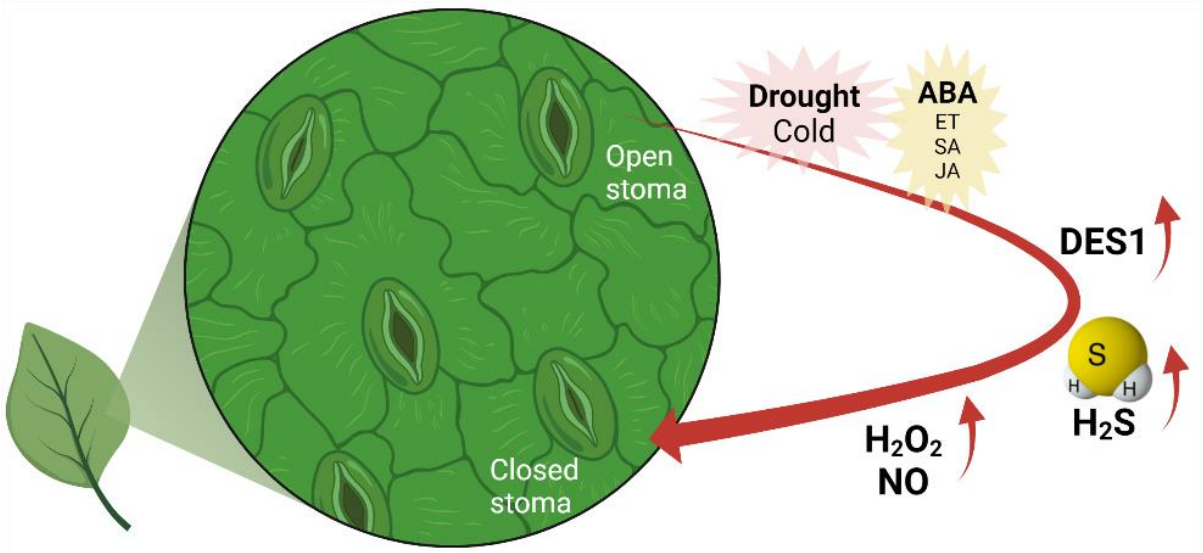
Streptavidin
AP
UV
release
MS

Fig. 5.


Drought
Cold
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ET
SA
JA
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stoma
DES1
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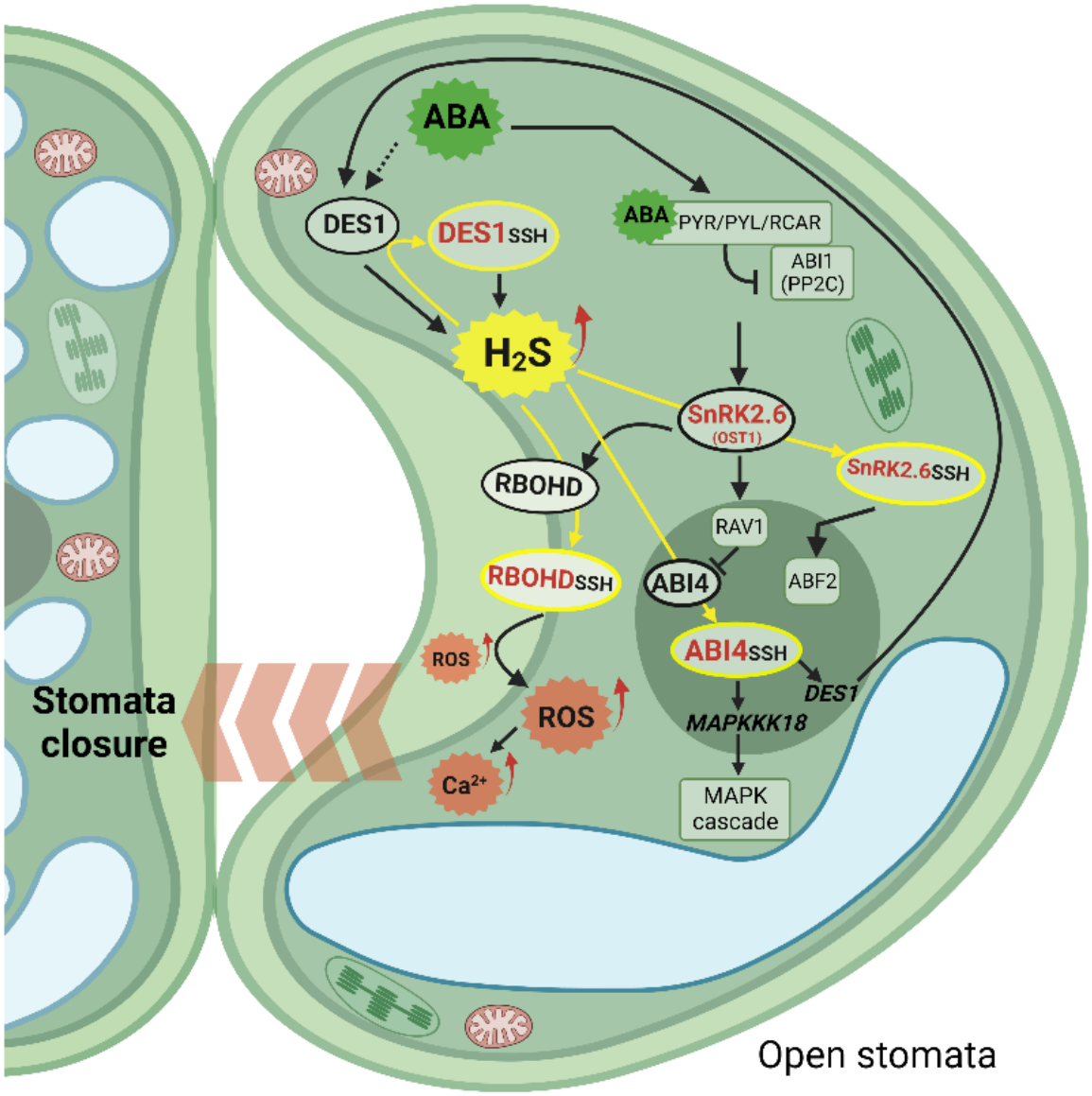
H
H
H_2S
H_2O_2
NO
Closed
stoma

Fig. 6.



1275

1276

1277 **Fig. 7.**