



Review

# Biological Functions of Hydrogen Sulfide in Plants

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**Abstract:** Hydrogen sulfide (H<sub>2</sub>S), which is a gasotransmitter, can be biosynthesized and participates in various physiological and biochemical processes in plants. H<sub>2</sub>S also positively affects plants' adaptation to abiotic stresses. Here, we summarize the specific ways in which H<sub>2</sub>S is endogenously synthesized and metabolized in plants, along with the agents and methods used for H<sub>2</sub>S research, and outline the progress of research on the regulation of H<sub>2</sub>S on plant metabolism and morphogenesis, abiotic stress tolerance, and the series of different post-translational modifications (PTMs) in which H<sub>2</sub>S is involved, to provide a reference for future research on the mechanism of H<sub>2</sub>S action.

**Keywords:** hydrogen sulfide; resistance; cysteine residues; plant growth regulator; crosstalk; post-translational modification

## 1. Introduction

Hydrogen sulfide (H<sub>2</sub>S) is a colorless, flammable, toxic, and corrosive gas that gives rotten eggs their distinctive odor; it is involved in numerous physiological processes in the plant, such as seed germination [1], stomatal movement [2], root morphogenesis [3], photosynthesis [4], senescence and yield [5], and fruit ripening and quality [6]. One of the major targets of H<sub>2</sub>S signaling in eukaryotic cells is the mitochondria. In nature, sulfate (SO<sub>4</sub><sup>2-</sup>)-reducing bacteria (SRB) use SO<sub>4</sub><sup>2-</sup> as a terminal electron acceptor, rather than oxygen (O<sub>2</sub>), to perform anaerobic respiration, resulting in the production of H<sub>2</sub>S as a major respiratory byproduct. They have a symbiotic relationship with aerobic sulfide-oxidizing bacteria, which can oxidize H<sub>2</sub>S back to SO<sub>4</sub><sup>2-</sup> and may, thus, be used continuously by SRB [7,8]. Initially, H<sub>2</sub>S was found to inhibit cytochrome c oxidase (COX) in mitochondria, and the research into H<sub>2</sub>S focused on its toxic effects [9]. Research into plant mitochondria has made some progress with the advent of deeper studies. Mitochondria are one of the compartments wherein H<sub>2</sub>S is produced [10]. At the subcellular level, H<sub>2</sub>S determines the ability of mitochondria to regulate energy production and prevent cellular senescence, thereby preventing leaf senescence in plants under drought-stress conditions [11]. H<sub>2</sub>S protects plants from salt stress by reducing hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) accumulation, regulating membrane stability and antioxidant systems in mitochondria [12]. These reports show that H<sub>2</sub>S has a positive effect on plant physiology.

On the other hand, H<sub>2</sub>S is involved in the defense mechanisms of plants against various abiotic stresses, including osmotic stress [13], drought stress [14], salt stress [15], extremely high- or low-temperature stress [16], and metalloids stress [17], by reducing reactive oxygen species (ROS) accumulation and actively mobilizing bioactive proteins, such as post-translational modification (PTM), as represented by S-sulphydration [18,19]. H<sub>2</sub>S is capable of interacting with many plant hormones, such as abscisic acid (ABA) [20], auxin (AUX) [21], gibberellins (GAs) [22], ethylene (ETH) [6], salicylic acid (SA) [23], jasmonic acid (JA) [24], and brassinosteroids [25]. The H<sub>2</sub>S-mediated PTM targets are protein residues, particularly cysteine (Cys) residues. Structural changes in proteins can cause changes in protein activity. Subsequent changes in hormone signaling in which the protein is involved will then follow, for example, changes in respiratory burst oxidase homolog protein D



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(RBOHD) [26], Open Stomata 1 (OST1)/Sucrose nonferme-1(SNF1)-RELATED PROTEIN KINASE2.6 (SnRK2.6) [27], ABSCISIC ACID INSENSITIVE 4 (ABI4) [28], etc.

In this review, we describe H<sub>2</sub>S synthesis and the metabolic pathways, donors, and detection methods for H<sub>2</sub>S research, the specific functions of H<sub>2</sub>S in the plant life cycle, and regulation by H<sub>2</sub>S on tolerance against abiotic stresses. In addition, the progress of research on a range of different post-translational modifications/signaling systems regulated by H<sub>2</sub>S in plants is also summarized.

## 2. Biosynthesis of H<sub>2</sub>S in Organisms

### 2.1. Biosynthesis Pathway of H<sub>2</sub>S

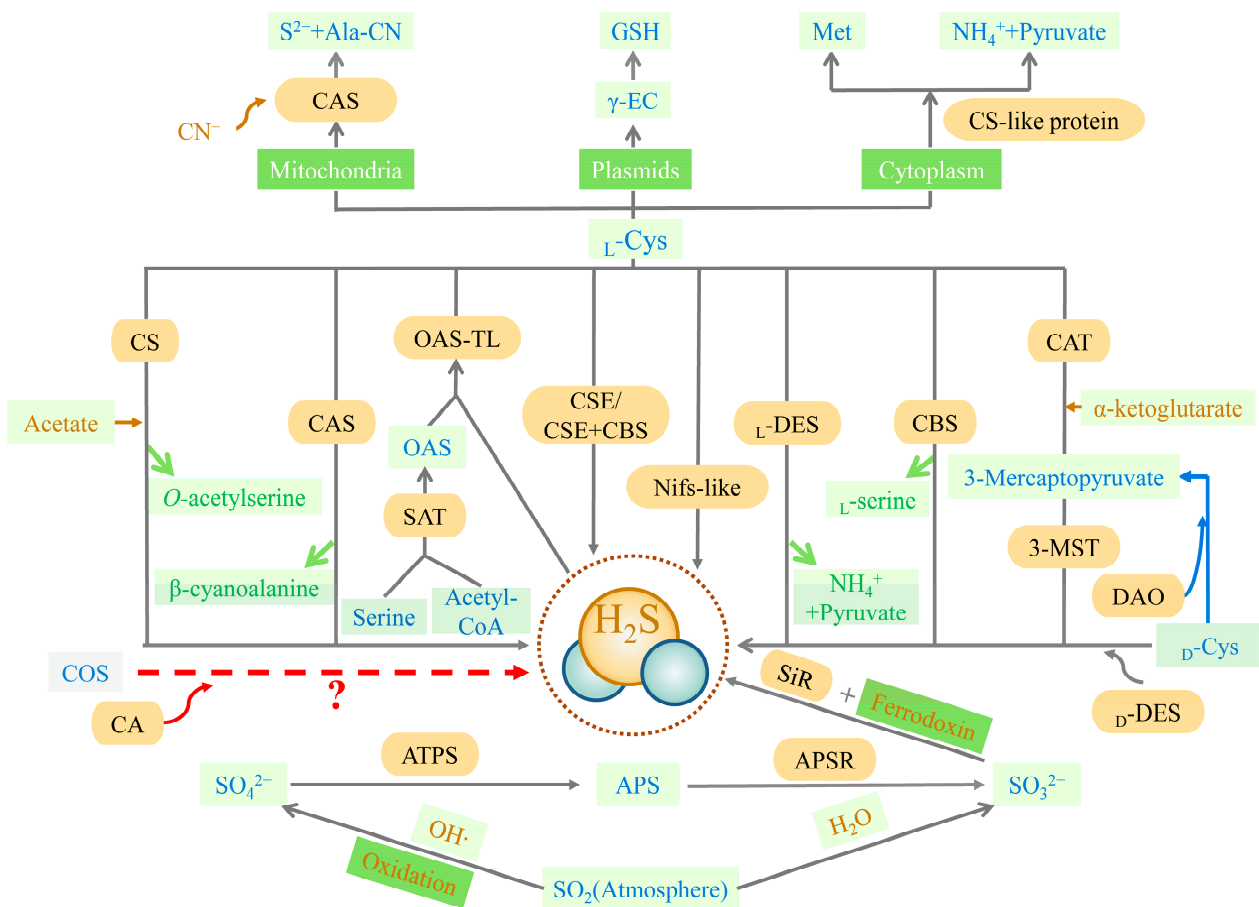
The biosynthesis of H<sub>2</sub>S includes both non-enzymatic and enzymatic pathways.

#### 2.1.1. Non-Enzymatic Pathway

The non-enzymatic pathway of H<sub>2</sub>S biosynthesis occurs due to the reaction of thiols or thiol-containing compounds with other molecules [18,29–31]. Glutathione (GSH) reduces inorganic polysulfides or hydrolyzed inorganic sulfide salts, i.e., sodium sulfide (Na<sub>2</sub>S) or sodium hydrosulfide (NaHS) with water to produce H<sub>2</sub>S [29–31]. Cysteine is the preferred substrate for the non-enzymatic production of H<sub>2</sub>S, and the process is catalyzed by iron and vitamin B<sub>6</sub> [32].

#### 2.1.2. Enzymatic Pathway

The trans-sulfuration pathway is the primary source of endogenous H<sub>2</sub>S and is the only way to produce endogenous cysteine, involving cellular sulfur metabolism and redox regulation [33] (Figure 1). Methionine, acting as a substrate, is catalyzed to produce cysteine eventually, with H<sub>2</sub>S as the byproduct. Cystathionine-β-synthase (CBS, EC 4.2.1.22), cystathionine-γ-lyase (CSE, EC 4.4.1.1), cysteine aminotransferase (CAT, EC 2.6.1.3), and 3-mercaptopyruvate sulfurtransferase (3-MST, EC 2.8.1.2) are all involved in this pathway. CBS and CSE are only located in the cytoplasm, while CAT and 3-MST can be present in the cytoplasm and mitochondria [34]. In the first irreversible step of converting methionine to cysteine, CBS catalyzes the condensation of homocysteine with serine (Ser) or cysteine to form cystathionine. The main role of CSE in the trans-sulfuration pathway is in the conversion of cystathionine to cysteine and α-ketobutyrate. CBS can produce H<sub>2</sub>S through β-substitution reactions [34–36]. Similarly, CSE can produce H<sub>2</sub>S via the β-elimination reaction with cysteine or the γ-replacement reaction between two homocysteine molecules. There is also a mechanism for H<sub>2</sub>S production, mediated by CAT and 3-MST in the transsulfuration pathway. CAT catalyzes L-cysteine and α-ketoglutarate to form 3-mercaptopyruvate (3-MP) and glutamate. The sulfur group of 3-MP is then transferred to 3-MST-accepting nucleophilic Cys247 in the presence of 3-MST, to produce 3-MST-bound persulfide and pyruvate. After this stage, the MST-persulfide reacts with thiols or is reduced by thioredoxin (Trx) to form H<sub>2</sub>S [37,38]. In addition, 3-MST transfers the sulfur group from 3-MP to cyanide, to form thiocyanate [39]. Similar to the CAT/3-MST pathway, there is also a D-amino acid oxidase (DAO, EC 1.4.3.3)/3-MST pathway to generate H<sub>2</sub>S (Figure 1). DAO metabolizes D-cysteine into 3-MP, which is metabolized into H<sub>2</sub>S by 3-MST [40].



**Figure 1.** Synthesis of H<sub>2</sub>S. APS (adenosine 5'-phosphosulfate), APSR (APS reductase, EC 1.8.99.2), ATPS (ATP sulfurylase, EC 2.7.7.4), CA (carbonic anhydrase, EC 4.2.1.1), CAS (L-3-cyanoalanine synthase, EC 4.4.1.9), CAT (cysteine aminotransferase, EC 2.6.1.3), CBS (cystathionine-β-synthase, EC 4.2.1.22), COS (carbonyl sulfide), CN<sup>-</sup> (cyanide), CS (O-acetyl-L-serine via cysteine synthase, EC 4.2.99.8), CSC (hetero-oligomeric cysteine synthase complex), CSE (cystathionine-γ-lyase, EC 4.4.1.1), DAO (D-amino acid oxidase, EC 1.4.3.3), D-Cys (D-cysteine), D-DES (D-cysteine desulfhydrase, EC 4.4.1.15), γ-EC (γ-glutamylcysteine), GSH (glutathione), HO· (hydroxyl radicals), H<sub>2</sub>O (water), H<sub>2</sub>S (hydrogen sulfide), L-Cys (L-cysteine), L-DES (L-cysteine desulfhydrase, EC 4.4.1.1), Met (methionine), 3-MST (3-mercaptopyruvate sulfurtransferase, EC 2.8.1.2), NH<sub>4</sub><sup>+</sup> (ammonium), Nifs-like (nitrogenase Fe-S cluster-like), OAS (O-acetyl-L-serine), OSATL (O-acetylserine(thiol)lyase, EC 2.5.1.47), S<sup>2-</sup> (Sulfide), SAT (serine acetyltransferase, EC 2.2.1.30), SiR (sulfite reductase, EC 1.8.7.1), SO<sub>2</sub> (sulfur dioxide), SO<sub>3</sub><sup>2-</sup> (sulfite), SO<sub>4</sub><sup>2-</sup> (sulfate). The closed lines filled with yellow represent enzymes. Rectangles represent substance/organelle reaction conditions. Rectangles filled with light green represent substances (wherein the blue-lettered cells are pivotal substances, yellow-lettered cells are involved in the synthesis of substrates, and green-lettered cells are reaction synthesis byproducts). Rectangles filled with dark green represent substance organelles/reaction conditions (wherein the white-lettered cells are organelles and the yellow-lettered cells are reaction conditions). Yellow arrows represent the substrates involved in the reaction, green arrows represent byproducts of the reaction, and red arrows represent the need for verification by further research. The reactions involved in the blue arrows are not currently found in plants.

In plants, the synthesis pathway of H<sub>2</sub>S can be divided into five types, according to the different substrates. These are cysteine degradation and sulfite reduction, cyanide detoxification, iron-sulfur cluster turnover, and carbonyl sulfide (COS) conversion [41] (Figure 1). Similar to animals, the metabolism of cysteine is the main source of endogenous H<sub>2</sub>S production in plants. The plants also have a characteristic sulfate reduction

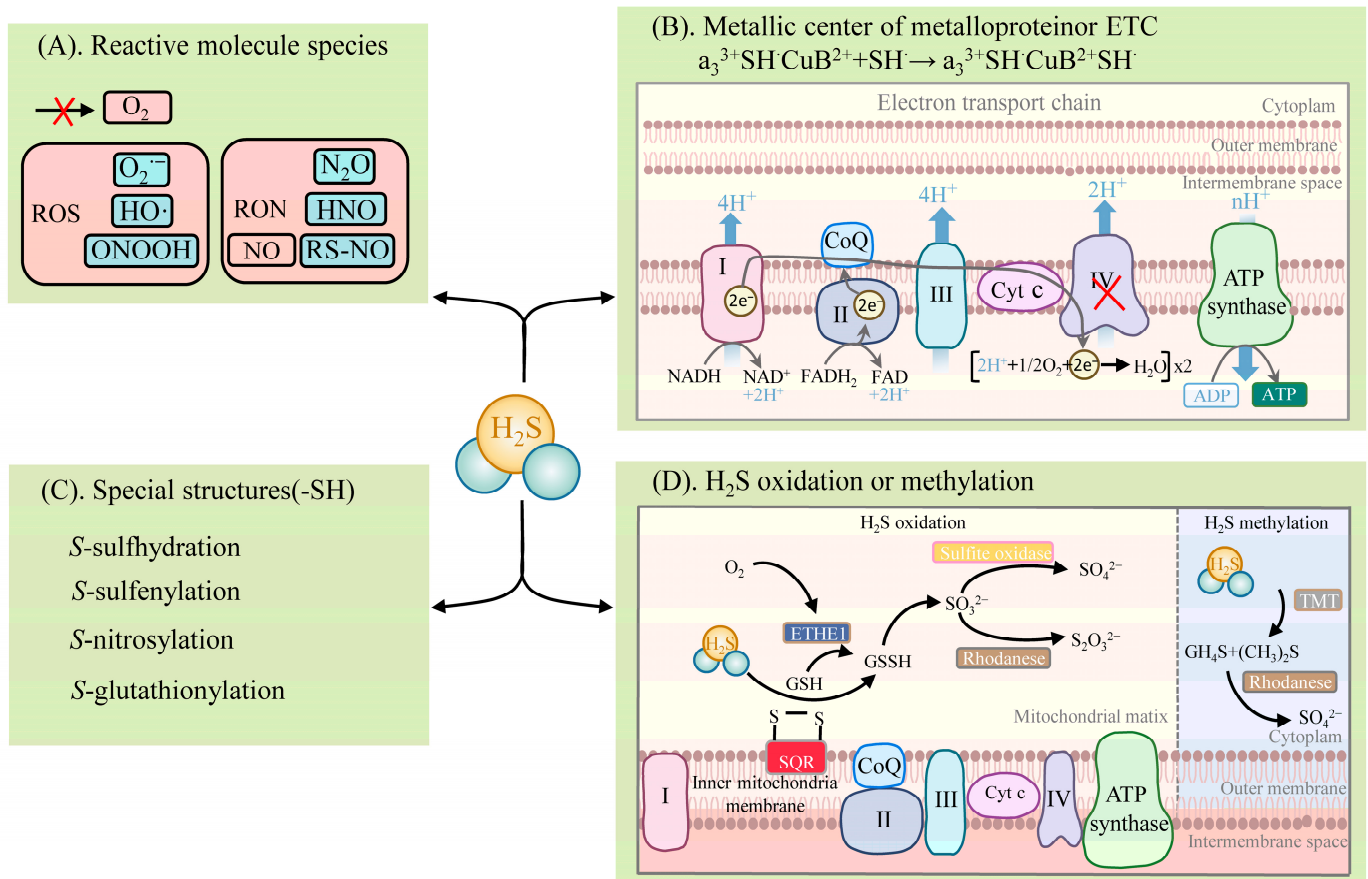
assimilation of H<sub>2</sub>S production. H<sub>2</sub>S synthesis occurs in chloroplasts, cytoplasm, and mitochondria [42]. First, H<sub>2</sub>S is mainly derived from cysteine degradation in the plant, catalyzed by different cysteine-degrading enzymes, including L-cysteine desulfhydrase (L-DES, EC 4.4.1.1), D-cysteine desulfhydrase (D-DES, EC 4.4.1.15), and L-3-cyanoalanine synthase (CAS, EC 4.4.1.9) [43]. Second, H<sub>2</sub>S is derived from the reductive assimilation of sulfite (SO<sub>3</sub><sup>2-</sup>) in the plants (Figure 1). These two pathways of H<sub>2</sub>S synthesis are closely linked. Sulfate or atmospheric sulfur dioxide (SO<sub>2</sub>) is the source of SO<sub>3</sub><sup>2-</sup> production in the plant in the presence of adenosine 5'-phosphosulfate (APS) reductase (APSR, EC 1.8.99.2). Atmospheric SO<sub>2</sub> can also produce SO<sub>3</sub><sup>2-</sup> spontaneously via non-enzymatic interaction with water. Sulfite reductase (SiR, EC 1.8.7.1) reduces SO<sub>3</sub><sup>2-</sup> to H<sub>2</sub>S in the presence of chloroplast enzymes and ferredoxin [44,45]. Under alkaline conditions in the chloroplast stroma, plants spontaneously transport HS<sup>-</sup> (a dissociated form of H<sub>2</sub>S) into the cytoplasm (cytosol). With pyridoxal phosphate as a cofactor, L/D-cysteine is catalyzed by L/D-DES to produce pyruvate, NH<sub>4</sub><sup>+</sup>, and H<sub>2</sub>S in the cytoplasm, chloroplasts, and mitochondria. Third, the nitrogenase Fe-S cluster-like (NFS/NifS-like) protein, which has similar activity to L-cysteine desulfurases, also catalyzes the conversion of cysteine to alanine and sulfur or sulfide using L-Cys as a substrate. It is also a possible source of H<sub>2</sub>S in plants [46,47]. The cyanide detoxification mechanism is also an important source of H<sub>2</sub>S in the plant (Figure 1). Ser and Acetyl-CoA are used to synthesize the intermediate reaction of O-acetyl-L-serine (OAS), catalyzed by Ser acetyltransferase (SAT, EC 2.2.1.30). O-acetyl-serine(thiol)lyase (OASTL, EC 2.5.1.47), also known as cysteine synthase, catalyzes the insertion of a particular sulfide (in this case, H<sub>2</sub>S) into the carbon skeleton via an elimination reaction, and produces cysteine [48]. CAS involves cyanide detoxification and regulates the production of H<sub>2</sub>S in mitochondria. H<sub>2</sub>S is both an intermediate reduction product of sulfate assimilation and a substrate for the synthesis of cysteine. The biosynthesis of cysteine in plastids implies a transition between a reduction in the assimilated sulfate-reducing pathway and actual metabolism [48]. The iron-sulfur cluster located in *Arabidopsis* mitochondria is capable of assembling the NIF system and presents cysteine desulfurase activity, which may also offer a potential source of H<sub>2</sub>S [49]. Besides the four H<sub>2</sub>S synthesis pathways described above, carbonic anhydrase (CA, EC 4.2.1.1) catalyzes the hydrolysis of COS to produce carbon dioxide (CO<sub>2</sub>) and H<sub>2</sub>S. The plants absorb COS from the air and achieve the efficient use of sulfur assimilation via CA [50,51], which may also be a source of endogenous H<sub>2</sub>S in the plant.

## 2.2. Metabolic Pathways of H<sub>2</sub>S

H<sub>2</sub>S exhibits diverse physiological and signaling roles, mainly in four distinct biochemical ways (Figure 2): (1) reacting with reactive molecule species, such as ROS, reactive nitrogen species (RNS), hypochlorite (HOCl), and reactive carbonyl species (RCS) [52–54]; (2) binding to the metal center of metalloproteins or the reduction of the hemoglobin center [55–57]; (3) post-translationally modifying proteins with specific structures (e.g., proteins containing cysteine residues (-SH)), which are mainly via S-sulfhydration [18,26,28,58]. The other PTMs are described in detail below [18]; (4) activities involving the oxidative and methylation pathways [33,59].

H<sub>2</sub>S reacts with the reactive molecule species, including ROS and RNS [18] (Figure 2). H<sub>2</sub>S can react with several biological oxidants, including superoxide radicals (O<sub>2</sub><sup>•-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radicals (HO·), nitrogen dioxide (NO<sub>2</sub>), peroxyxynitrite (ONOOH), and many others. H<sub>2</sub>S reacts readily with HOCl to form polysulfides (-S-S<sub>n</sub>-S-) [52]. Excessive ROS and RNS levels lead to oxidative stress when plants are exposed to adversity. In turn, H<sub>2</sub>S significantly impacts the products of the plant's defensive system. Nitric oxide (NO) is an important signaling molecule. H<sub>2</sub>S can react with NO, leading to the formation of various nitrogen (nitrous oxide (N<sub>2</sub>O), nitroxyl (HNO), S-nitrosothiols (RS-NO/SNO), and sulfur derivatives (e.g., S<sup>0</sup>, S<sup>-</sup>), which are thus involved in physiological signaling. NO converts the adversity-induced O<sub>2</sub><sup>•-</sup> to the less toxic ONOO<sup>-</sup>. H<sub>2</sub>S further reacts with ONOO<sup>-</sup> to form thionitrate (HSNO<sub>2</sub>) [54]. This property of H<sub>2</sub>S to

actively scavenge  $\text{ONOO}^-$  provides strong support for the inference that it synergizes with NO to reduce ROS oxidative stress. In addition,  $\text{H}_2\text{S}$  can react directly with NO to produce HNO and also react with RS-NO to form thionitrous acid (HSNO) [54]. HSNO can be metabolized to provide  $\text{NO}^+$ , NO, and NO species, thus acting as a transportable NO reservoir in the organism that is involved in NO signaling.



**Figure 2.** Metabolism of  $\text{H}_2\text{S}$ : (A) reaction of  $\text{H}_2\text{S}$  with reactive molecule species; (B) binding or electron transfer of  $\text{H}_2\text{S}$  to the metal center of a metalloprotein; (C) reaction of  $\text{H}_2\text{S}$  with proteins with specific structures (involved in post-translational modifications); (D) metabolic pathways of  $\text{H}_2\text{S}$  oxidation and methylation. Note: I (mitochondrial complex I), II (mitochondrial complex II), III (mitochondrial complex III), IV (mitochondrial complex IV), Cyt c (Cytochrome c), CoQ (coenzyme Q/ubiquinone),  $\text{CH}_4\text{S}$  (methanethiol),  $(\text{CH}_3)_2\text{S}$  (dimethyl sulfide), ETHE1 (ethylmalonic encephalopathy 1 protein), GSH (glutathione), GSSH (glutathiol), HNO (nitroxyl),  $\text{H}_2\text{S}$  (hydrogen sulfide),  $\text{HO}\cdot$  (hydroxyl radicals),  $\text{N}_2\text{O}$  (nitrous oxide), NO (nitric oxide),  $\text{O}_2$  (oxygen),  $\text{O}_2^{\bullet-}$  (superoxide radical), ONOOH (peroxynitrite), RON (reactive nitrogen species), ROS (reactive oxygen species), RS-NO (*S*-nitrosothiols),  $\text{SO}_4^{2-}$  (sulfate),  $\text{S}_2\text{O}_3^{2-}$  (thiosulfuric acid),  $\text{SO}_3^{2-}$  (sulfite), SQR (sulfide quinone reductase), TMT (thiol *S*-methyl-transferase), TST (thiosulfate sulfurtransferase). A red cross indicates an inhibitory effect on enzyme activity or the production of a substance.

$\text{H}_2\text{S}$  binds to the metal centers of metalloproteins or participates in electron transfer [18] (Figure 2).  $\text{H}_2\text{S}$  involves the physiological regulation of the oxidative phosphorylation of the electron transfer chain (ETC) by means of binding to components of the ETC. It is mainly the direct binding of  $\text{H}_2\text{S}$  to COX that affects ETC function, and the reduction of COX by  $\text{H}_2\text{S}$  leads to the formation of  $\text{HS}\cdot$  or  $\text{S}^{\bullet-}$  (which can interact with protein sulfhydryl groups (thiol)), affecting other components of the ETC. ETC complex IV, also known as COX, consists of two redox centers, Cyt a,  $\text{Cu}_A$ , and Cyt  $\text{a}_3$ ,  $\text{Cu}_B$ .  $\text{H}_2\text{S}$  associates with the COX component, hemoaxylin  $\text{a}_3$  (heme  $\text{a}_3$ ), and the  $\text{Cu}_B$  center, thus

participating in the electron transfer of the ETC [55,56]. H<sub>2</sub>S favors the formation of a polar environment (tyrosine (Tyr) residues and Cu<sub>B</sub> centers) around the heme a<sub>3</sub> subunit, while H<sub>2</sub>S promotes heme a<sub>3</sub> reduction to achieve an increase in COX enzyme activity at low concentrations. At high concentrations, H<sub>2</sub>S can bind directly to the component a<sub>3</sub> and Cu<sub>B</sub> centers of COX, resulting in the formation of the stable H<sub>2</sub>S-Cu<sub>B</sub> and unstable hemoglobin H<sub>2</sub>S-Fe<sup>2+</sup> inhibitory groups. In this case, the stability of the H<sub>2</sub>S-Fe<sup>2+</sup> group is dependent on H<sub>2</sub>S concentration. However, the inhibition of COX by H<sub>2</sub>S can behave differently, depending on the concentration. Unlike medium concentrations, a high concentration of H<sub>2</sub>S is accompanied by the formation of stable hemoglobin a<sub>3</sub> H<sub>2</sub>S-Fe<sup>3+</sup> inhibitory groups, the inhibitory effect of which is irreversible [55–57]. In addition, the reduction of COX by H<sub>2</sub>S promotes increased ATP synthesis (which can bypass complex III to promote ETC activity) and the accumulation of reactive sulfur [33].

H<sub>2</sub>S can post-translationally modify proteins by converting the thiol group of cysteine residues to the persulfide group (-SSH) [18,26,28,58] (Figure 2). This modification is named S-sulfhydration [18]. The increased nucleophilicity of the converted persulfides, compared to the thiol group, highlights the highly reactive nature of S-sulfhydration. This also explains the potential for persulfides to act as mediators of sulfide signaling.

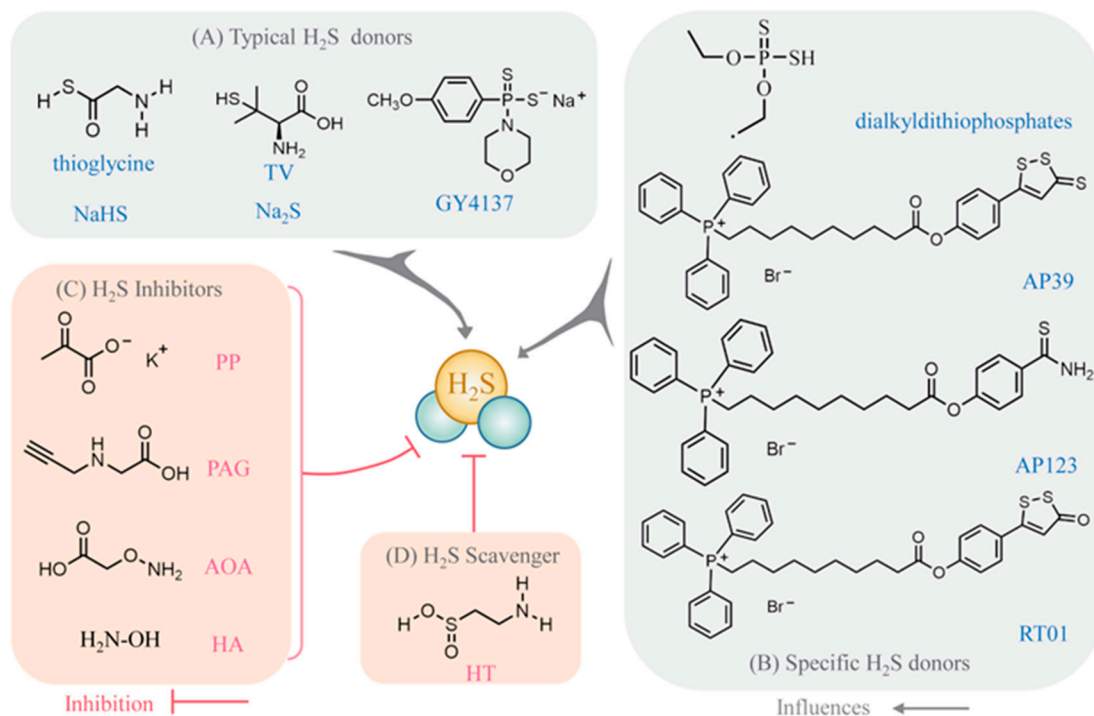
The metabolic pathways of H<sub>2</sub>S include oxidation and methylation [59] (Figure 2). The oxidation of H<sub>2</sub>S occurs in the mitochondria and involves several enzymes, such as sulfide quinone reductase (SQR) and the ethylmalonic encephalopathy 1 protein (ETHE1, also known as persulfide dioxygenase), thiosulfate sulfurtransferase (TST, also known as rhodanese), and mitochondrial sulfite oxidase. SQR oxidizes H<sub>2</sub>S in the inner mitochondrial membrane to produce persulfide species (e.g., glutathiol (GSSG)). At the same time, electrons released by SQR are captured by ubiquinone and transferred from H<sub>2</sub>S to coenzyme Q and to ETC at complex III. The persulfide is further oxidized by ETHE1 to produce SO<sub>3</sub><sup>2-</sup>, which is further oxidized by sulfite oxidase to SO<sub>4</sub><sup>2-</sup>, or by TST to S<sub>2</sub>O<sub>3</sub><sup>2-</sup> [59]. The metabolism of H<sub>2</sub>S by methylation occurs more as a complementary mechanism to oxidation and takes place in the cytoplasm. Then, thiol S-methyltransferase converts H<sub>2</sub>S to methanethiol (CH<sub>4</sub>S) and dimethyl sulfide (CH<sub>3</sub>)<sub>2</sub>S, which is further oxidized by rhodanese to produce thiocyanate and SO<sub>4</sub><sup>2-</sup> [33].

### 3. H<sub>2</sub>S Donors, Inhibitors (Including Scavengers), and Detection Methods

#### 3.1. Selection of Pharmacological Agents for H<sub>2</sub>S Pharmacology Experiments

At present, studies on H<sub>2</sub>S are based on the use of the donors and/or inhibitors (scavengers) of H<sub>2</sub>S. Typical H<sub>2</sub>S donors include Na<sub>2</sub>S, NaHS, morpholin-4-ium 4-methoxyphenyl (morpholino) phosphinodithioate (GYY4137), L-thiovaline (TV), and thioglycine (Figure 3) [14,60,61]. Notably, the specific targeting of mitochondrial H<sub>2</sub>S donors, including 10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yl) phenoxy) decyl triphenylphosphonium bromide (AP39), 10-(4-carbamthioylphenoxy)-10-oxodecyl triphenylphosphonium bromide (AP123), and RT01, have received much attention recently [62]. Unlike the acting mechanism of the traditional H<sub>2</sub>S donors, AP39 (a mitochondria-specific H<sub>2</sub>S donor) plays a direct role. The mitochondrial effects of Na<sub>2</sub>S are dependent on the NO/cyclic guanosine monophosphate (cGMP)/cGMP-dependent protein kinase (PKG) pathway. The inhibition of NO synthesis prevents the mitigating effects of Na<sub>2</sub>S and TV regarding cellular oxidative damage. This means that AP39 can be used for research even if upstream intracellular signaling (e.g., the NO/cGMP/PKG signaling pathway) is blocked due to environmental changes [60]. In terms of the mitigation of oxidative damage by H<sub>2</sub>S donors, their mitigation capacity can be listed in descending order of strength: AP39, TV, Na<sub>2</sub>S, and GYY4137 [60]. In addition, other characteristic H<sub>2</sub>S donors have been identified (Figure 3). Dialkyldithiophosphate is a new environmentally friendly H<sub>2</sub>S-releasing agent with 3 advantages. Firstly, it slowly degrades to release H<sub>2</sub>S. Secondly, the degradation products are natural, non-toxic, and can be used as fertilizer. Thirdly, only 0.28 mg of H<sub>2</sub>S is released when 1 mg of this substance is added to the soil near the seeds, avoiding growth inhibition at high concentrations [63]. Exogenous H<sub>2</sub>S inhibitors that act in the opposite direction

include potassium pyruvate (PP), DL-propargylglycine (PAG), AOA, and hydroxylamine (HA) (Figure 3). Similar effects are reported with the use of exogenous H<sub>2</sub>S scavengers, such as hypotaurine (HT) (Figure 3) [64].



**Figure 3.** The donors of H<sub>2</sub>S (inhibitor (scavenger)): (A) Traditional H<sub>2</sub>S donor; (B) special H<sub>2</sub>S donor; (C) inhibitor of H<sub>2</sub>S; (D) scavenging of H<sub>2</sub>S. Note: AP39 (anethole dithiolethione/(10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yl)phenoxy)decyl) triphenylphosphonium bromide), AP123 (hydroxythiobenzamide), AOA (aminoxyacetic acid), GY4137 (morpholin-4-ium 4-methoxyphenyl (morpholino) phosphinodithioate), HA (hydroxylamine), HT (hypotaurine), NaHS (sodium hydrosulfide), Na<sub>2</sub>S (sodium sulfide), PAG (DL-propargylglycine), PP (potassium pyruvate), RT01 (dialkyldithiophosphates), TV (L-thiovaline).

### 3.2. H<sub>2</sub>S Detection Methods

Depending on the purpose of the experiment, many methods measuring H<sub>2</sub>S have been developed successively, including photometric methods, gas chromatography, electrochemical methods, and biotransformation analysis [65,66]. The difference between these approaches is that proteomics studies need to consider the need to maintain protein function, while organelle studies focus more on in vivo-specific localization.

Fluorescent probes are also important in detecting H<sub>2</sub>S, including intracellular and in vitro detection. The former is mainly used for the in vivo imaging of H<sub>2</sub>S, while the latter is mostly used for biological sample detection. The selection of fluorescent probes should take into account their response rate and selection specificity. The ratiometric fluorescent probe eliminates most of the environmental factors' interference by the ratio of fluorescence intensity at two wavelengths (i.e., self-correction), thus enabling the quantitative detection of the species under test when the probe concentration is unknown. Based on these advantages, the technique is considered to be an accurate method of measuring substances [67]. A large number of ratiometric fluorescent probes have been reported for the determination of H<sub>2</sub>S, e.g., the Cy-N<sub>3</sub> H<sub>2</sub>S probe [68], the CouMC H<sub>2</sub>S probe [67], the coumarin-merocyanine dyad (CPC) H<sub>2</sub>S probe [69], the ratio-H<sub>2</sub>S 1/2 probe [70], and AcHS-2 ratiometric two-photon fluorescent probes [71]. In addition, the use of targeted mitochondrial H<sub>2</sub>S donors greatly simplifies studies at the subcellular level, such as those into AP39 [72], AP123 [73], and RT01 [62].

### 3.3. Detection of Protein S-Sulfhydration Modifications by H<sub>2</sub>S

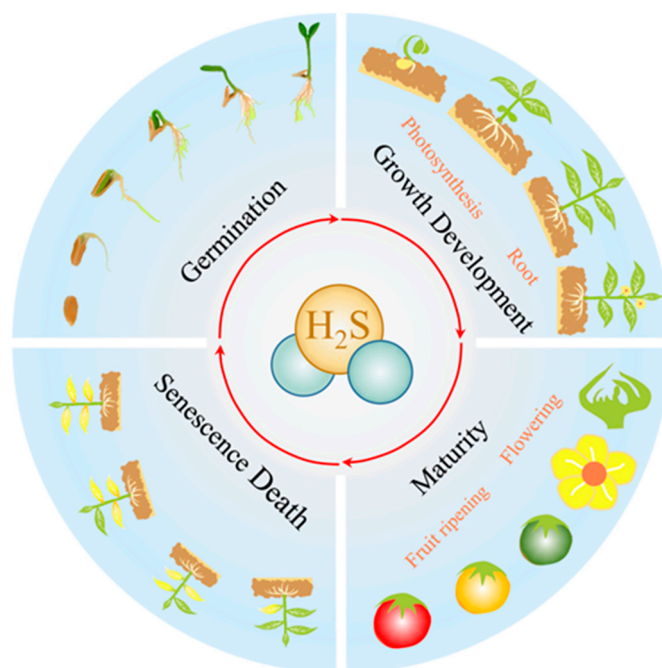
Studies of H<sub>2</sub>S-mediated S-sulfhydration proteins are currently being carried out, following the biotin conversion approach as the basic process. The primary process has two steps involving the closure of the thiol-blocking reagent and the persulfide by an electrophile reagent and the release of the persulfide by a reducing agent. Specifically, there are four methods commonly used for S-sulfhydration testing. The first method is the classic electrophile S-methyl methanethiosulfonate (MMTS) method. MMTS is first used as a sulfhydryl blocker, followed by the labeling of persulfides with N-[6-(biotinamido) hexyl]-3'-(2'-pyridyldithio) propionamide (biotin-HPDP). This method includes S-nitrosation, in addition to S-sulfhydration labeling [74]. In the second method, iodoacetic acid (IAA) accurately detects the presence of sulfhydryl groups in the peroxidized proteins. The target groups (-SH and -SSH) in the protein are alkylated by IAA, thus achieving the blockage of the free thiol with protein persulfides. DTT specifically cleaves the -SSH group in the protein that is alkylated. Iodoacetamide-linked biotin (IAP) is labeled against the cleaved moiety by dithiothreitol (DTT). This method effectively eliminates the effect of intramolecular disulfides and intermolecular disulfides [75]. The third method, using N-ethyl maleimide, is the one that is most widely and commercially used. N-ethyl maleimide, which is linked to both the target groups (-SH and -SSH) in the protein, is used as a sulfhydryl group sealer. DTT specifically cleaves the target moiety (-SSH), which has been released by linking Cy5-conjugated maleimide to the site. The in-gel fluorescence signal is reduced in the samples containing persulfide [76]. The fourth method is the tag-switch test (tag-switch): a two-step reaction involving sulfhydryl blocking (SH-blocking (BR)) and tag-switching reagent substitution. The tag-switching reagent contains a reporting molecule (R) and a nucleophile (Nu). Nucleophiles differ in their reactivity toward persulfide adducts, due to their reactive properties. The selection of suitable nucleophilic reagents for this property allows for the specific detection of disulfide bonds in persulfides. For example, in the first step, methylsulfonyl benzothiazole (MSBT) is used as a sulfhydryl-blocking substance for SH-blocking (BR). Next, labeling is completed by binding to labeled tagged cyanoacetate derivatives, which effectively label the persulfides [77]. Recently, a new method for selective persulfide detection, named the dimedone-switch method, has been developed, which has the advantages of being specific, rapid, and stable [78]. The method is based on using dimedone probes for chemoselective persulfuration labeling. Since protein persulfides (PSSH) are very reactive, their reactivity is similar to that of cysteine residues. Therefore, it becomes difficult and important to design tools for selective labeling. The dimedone-switch method is roughly divided into two steps. In the first step, 4-chloro-7-nitrobenzofurazan (NBF-Cl)-treated material was used to block and detect thiols, amines, and sulfenic acids. Then, -S-S-signal detection is conducted, based on the dimedone/dimethyl ketone probe. NBF-Cl not only reacts with PSSH and thiols but also blocks sulfenic acids. NBF-Cl is key to the specificity of this method for the detection of persulfides. Conventional methods rely on the closure of sulfhydryl groups and persulfides with electrophilic reagents, followed by the release of the latter by means of reducing agents. These methods require a great deal of attention and are tedious and complex [79].

## 4. H<sub>2</sub>S Is Involved in Plant Growth and Development

### 4.1. H<sub>2</sub>S and Germination

Seed germination is crucial for the initiation of the plant's life cycle (Figure 4). H<sub>2</sub>S delays seed germination in a dose-dependent manner [80]. This regulation does not act directly but probably occurs via a cascade involving other substances. Exogenous H<sub>2</sub>O<sub>2</sub> can enhance the promotion of seed germination [81], and H<sub>2</sub>S acts upstream of H<sub>2</sub>O<sub>2</sub> in seed germination [82]. The regulation of seed germination by H<sub>2</sub>S may be achieved by the persulfide modification of key proteins (ABI4) [1]. H<sub>2</sub>S is also able to indirectly regulate the transcription of AOX genes, thereby regulating seed germination [83].





**Figure 4.** H<sub>2</sub>S is involved in the growth and development of plants.

H<sub>2</sub>S may promote germination by mitigating the adverse effects of multiple stresses on seeds. Exogenous H<sub>2</sub>S enhances endosperm amylase activity in germinating seeds, effectively reducing the accumulation of malondialdehyde (MDA) and H<sub>2</sub>O<sub>2</sub> and promoting seed germination [84]. The application of H<sub>2</sub>S donors alleviates high-temperature-induced tissue viability, reduces MDA accumulation, and improves germination rates [85]. Exogenous H<sub>2</sub>S can promote the NO-induced salt resistance pathway and enhance salt tolerance in alfalfa seeds under salt stress [86]. In summary, H<sub>2</sub>S has a potential regulatory role in relation to seed germination. Low concentrations of H<sub>2</sub>S promote seed germination, while high concentrations of H<sub>2</sub>S inhibit seed germination [1,81,82]. This may be related to the properties of H<sub>2</sub>S itself. H<sub>2</sub>S inhibits COX in mitochondria [9]. From the perspective of energy metabolism, H<sub>2</sub>S is positive in terms of reducing energy consumption. H<sub>2</sub>S-mediated S-sulfhydration is one of the most important safeguarding mechanisms to protect proteins from peroxidation. From this point of view, it may be beneficial for seed germination. Toxicity studies have been a prominent feature of H<sub>2</sub>S since its discovery. This may be the reason for showing the inhibition of seed germination in some of the studies.

#### 4.2. H<sub>2</sub>S and Growth Development

##### 4.2.1. H<sub>2</sub>S and Root Development

The root system is an essential source of water and minerals for plants (Figure 4) [87]. The factors affecting root development can be divided into intrinsic regulation and extrinsic inter-rooting influences (Table 1).

H<sub>2</sub>S involves the intrinsic regulation of root development through H<sub>2</sub>S-NO-carbon monoxide (CO) crosstalk [88]. Changes in root morphology and the regulation of root development are accomplished through various signaling networks. AUX is a vital growth hormone that regulates root cell replication, root elongation, and root morphological changes. PIN3- and PIN7-mediated changes in auxin allocation are responsible for the bending of roots toward the horizontal side of high AUX. AUX induces cell expansion through the synergistic activity of small auxin-up RNAs (SAUR), *Arabidopsis* H<sup>+</sup>-ATPase (AHA), and cell-wall-modifying proteins [89]. NO is actively involved in the regulation of the root system. Exogenous NO leads to adventitious root (AR) formation by mediating the growth hormone response in cucumber [90], is involved in the induction of root tip elongation in *Lupinus luteus* [91], regulates the formation of ARs [92] and lateral roots

(LRs) [93], may also influence root lignification and growth hormone-dependent cell cycle gene regulation [94,95]. NO may be indirectly involved in root regulation as a component of the relevant signaling pathway. For example, NO is a downstream signaling molecule for the induction of LR by CH<sub>4</sub> [96,97]. Similar to NO, exogenous CO treatment can also promote LR and AR formation. Exogenous CO induces LR and AR formation and the use of ZnPPIX (a CO scavenger) can reverse this effect. NO-CO crosstalk regulates root development. CO induces the formation of LRs that may be mediated by the NO/NOS pathway, and NO may act downstream of CO signaling [98]. The heme oxygenase isoenzyme-1 (HO-1) pathway is the primary source of endogenous CO in plants. This means that the oxidation of heme as a substrate to CO, free iron (Fe<sup>2+</sup>), and biliverdin (BV) is catalyzed by HOs [88]. NO modulates the activity of HOs and the transcriptional level of *HO-1* in soybean [99]. Under both normal and stress conditions, H<sub>2</sub>S has a precise regulatory effect on the primary, lateral, and adventitious roots of plants [88]. H<sub>2</sub>S causes the inhibition of filamentous actin (F-actin) bundles through the modification of F-actin by S-sulfhydration, which leads to the inhibition of root hair growth [88]. There are other mechanisms by which H<sub>2</sub>S regulates root polarity transport in plants through its effect on F-actin. Actin is essential for the transport and distribution of PIN proteins. Actin-binding proteins (ABPs) act as the downstream effectors of H<sub>2</sub>S signaling, thereby regulating the assembly and depolymerization of F-actin in root cells. H<sub>2</sub>S inhibits growth hormone transport by altering the polar subcellular distribution of PIN proteins, thereby regulating root development [100]. H<sub>2</sub>S-NO crosstalk regulates root growth and development. Numerous biological compounds are produced by H<sub>2</sub>S (nucleophilic) and NO (electrophilic), via oxidation or nitrosation reactions, and these substances are also actively involved in physiological regulation. H<sub>2</sub>S can be involved in physiological regulation through a cascade of signals, in common with NO [101]. Exogenous NaHS alleviates the adverse effects of salt-stress-induced oxidative damage in *Medicago sativa* roots, while the application of c-PTIO (a NO scavenger) reverses these effects [86].

H<sub>2</sub>S regulates root development by actively participating in signaling events to plant-bacteria symbiosis [102–105]. Aqueous solutions of H<sub>2</sub>S with a weakly acidic pH may affect the activity of inter-rooted microorganisms. Recent studies have also found that exogenous H<sub>2</sub>S can modify the expression of target genes and achieve an improved root structure by mediating the microRNA (miRNA) [15].

In addition, root growth is promoted by H<sub>2</sub>S at low concentrations and inhibited by H<sub>2</sub>S at high concentrations, which is typical of the effect of H<sub>2</sub>S on root development [106,107]. High concentrations of H<sub>2</sub>S regulate the root system architecture (RSA) by affecting the transport of growth hormones [106]. High concentrations of H<sub>2</sub>S activated the ROS-(MITOGEN-ACTIVATED PROTEIN KINASE 6) MPK6-NO signaling pathway and inhibited primary root (PR) growth. During this process, ROS production, activated by exogenous H<sub>2</sub>S, is required for NO generation, and MPK6 mediates H<sub>2</sub>S-induced NO production [107].

#### 4.2.2. H<sub>2</sub>S and Photosynthesis Photomorphogenesis

Photosynthesis is the process by which plants convert light energy into chemical energy. H<sub>2</sub>S involves plant photosynthesis by regulating the redox state, the non-stomatal factors, and stomatal movement [27,108–110].

Both the photoreaction centers and photosystem II (PSII) reaction centers are largely affected by redox states [111]. Exogenous H<sub>2</sub>S enhances the scavenging of ROS, regulating the activities of antioxidant enzymes (superoxide dismutase (SOD), ascorbate peroxidase (APX), and peroxidase (POD), catalase, inhibiting the accumulation of H<sub>2</sub>O<sub>2</sub> and MDA, and improving the stress tolerance of the plant [108,109,112]. H<sub>2</sub>S maintains high guaiacol peroxidase (GPX), POD, APX, catalase, and GSH reductase (GR) activities and down-regulates the chlorophyll degradation-related genes, *BoSGR*, *BoCLH2*, *BoPaO*, and *BoRCCR* in broccoli [113].

The amount and state of the relevant components of photosynthesis (non-stomatal factors) are also limiting factors for photosynthesis. Exogenous H<sub>2</sub>S alleviates the net photosynthetic rate ( $P_n$ ), stomatal conductance ( $G_s$ ), intercellular CO<sub>2</sub> concentration ( $C_i$ ), transpiration rate ( $E$ ), the maximal quantum yield of PSII photochemistry ( $F_v/F_m$ ), the effective quantum yield of PSII photochemistry ( $\Phi_{PSII}$ ), the photochemical burst of ribulose-1,5-bisphosphate ribulose carboxylase, and the decrease in electron transport rate ( $ETR$ ) in low light-stress (LL)-induced tall fescue seedlings [110]. Further analysis reveals that  $P_n$  follows an opposite trend to  $C_i$ , implying that the high level of photochemical efficiency maintained by H<sub>2</sub>S may be dominated by non-stomatal factors [110]. NaHS effectively increases chlorophyll content and ribulose1,5-bisphosphate carboxylase (RuBISCO) activity [114].

H<sub>2</sub>S regulates photosynthesis by participating in stomatal movement. In *Arabidopsis*, H<sub>2</sub>S is involved in stomatal closure through induction of the NO-mediated 8-nitro-cGMP/8-mercapto-cGMP synthesis pathway (one of the classical mechanisms of NO-induced stomatal closure). Conversely, the application of NO scavengers and NO-producing enzyme inhibitors can inhibit H<sub>2</sub>S-induced stomatal closure. H<sub>2</sub>S may be upstream of the NO-induced stomatal closure pathway [115]. ROS changes are early marker events for the stomatal closure response. ROS are important signaling molecules involved in stomatal movement [116]. H<sub>2</sub>S triggers the production of ROS via NADPH oxidase (NADPHox) and phospholipase D (PLD) [117]. Crosstalk between H<sub>2</sub>S and other signaling molecules (ABA, NO, ROS) is the leading method of stomatal movement. H<sub>2</sub>S-mediated post-translational modification, SnRK2.6, by S-sulfhydration is a novel regulatory mechanism for ABA signaling and ABA-induced stomatal closure [27]. In addition, the H<sub>2</sub>S-mediated modification of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO) by S-sulfhydration becomes a way of regulating ABA stomatal regulation [118]. Modifying target proteins by H<sub>2</sub>S S-sulfhydration regulates stomatal movement and is described in Section 5.3.

Stomatal movement is also closely related to the redox state of photosynthetic components. The redox state of the PSII receptor, lateral plastid quinone (PQ), may be transmitted to the stomatal guard cells in leaves through signal transduction, thereby regulating the stomatal opening and closing and affecting the stomatal conductance [119]. The above findings suggest that H<sub>2</sub>S can promote the onset of photosynthesis and the expression of photosynthetic enzymes in plants. The reason for this is presumed to be that H<sub>2</sub>S induces the expression of genes involved in photosynthetic electron transfer via thiol redox modification [114].

#### 4.3. H<sub>2</sub>S and Maturity

##### 4.3.1. H<sub>2</sub>S and Flowering

Flowering is a sign that the plant has changed from nutritional to reproductive growth (Figure 4). Suppressor of Overexpression of Constant 1 (*SOC 1*), Flowering Locus T (*FT*), and Flowering Locus C (*FLC*) are essential genes for the regulation of flowering. *FLC* is a MADS-box transcription factor. *FLC* achieves the regulation of the vernalization pathway by controlling *SOC 1* and strongly represses the expression of genes that promote plant flowering. Exogenous 100  $\mu\text{mol L}^{-1}$  H<sub>2</sub>S promotes flowering in heading Chinese cabbage via S-sulfhydration, by weakening or eliminating the binding capacity of BraFLCs to the downstream promoters [120]. It suggests that H<sub>2</sub>S is actively involved in the flowering process of plants.

##### 4.3.2. H<sub>2</sub>S and Fruit Ripening

Ripening and completion are two critical stages in the fruit life cycle (Figure 4). From ripening to completion, a series of changes occur within the fruit: starch is converted into sugar, organic acids are broken down, the acidity decreases, the pectinase activity increases, causing pectin differentiation, and the pulp becomes soft. *ETH* is an essential factor in the regulation of fruit-ripening initiation [121,122].

H<sub>2</sub>S maintains fruit quality and delays fruit senescence and decay by antagonizing the effects of ETH [6]. Exogenous H<sub>2</sub>S maintains high levels of nutrient-related metabolites (e.g., anthocyanins, starch, soluble proteins, ascorbic acid, flavonoids, total phenols, etc.) in fruit, enabling the maintenance of fruit quality during storage [6,123,124]. Inhibiting ETH synthesis and signal transduction are crucial in how H<sub>2</sub>S is involved in the fruit-ripening process. H<sub>2</sub>S inhibits the expression of ETH biosynthesis (*MdACS1*, *MdACS3*, *MdACO1*, and *MdACO2*) and signal transduction (*MdETR1*, *MdERS1*, *MdERS2*, *MdERF3*, *MdERF4*, and *MdERF5*)-related genes in apples [124]. In addition, ETH induces the production of H<sub>2</sub>S in guard cells during osmotic stress; ETH-induced H<sub>2</sub>S negatively regulates ETH biosynthesis through the S-sulphydration of LeACO [118]. These findings suggest that there is a crosstalk between H<sub>2</sub>S and ETH.

The H<sub>2</sub>S-induced attenuation of respiration is a critical way of delaying fruit senescence. Mitochondria are the place where respiration occurs and the leading site of ROS production [125]. The lipoxygenase (LOX, EC 1.13.11.12) catalyzes and mediates the oxylipin synthesis pathway, also known as the fatty acid pathway, the primary method by which fruit aroma substances are formed. The enzymes downstream of LOX, hydroperoxide lyase (HPL), allene oxide synthase, alcohol dehydrogenase (ADH), and alcohol acyltransferases (AAT), are critical in the synthesis of alcohols, aldehydes, esters, and other derivatives in plants [126]. The excessive accumulation of ROS induces autophagy and programmed cell death (PCD) in plant cells (this is not described in detail here, but as described in Section 5.3). Decreased LOX activity and reduced ROS production are specific manifestations of reduced respiration. Improving the ability to scavenge ROS is essential for plants, offering a way to slow down senescence [127]. It is also a strategy for H<sub>2</sub>S to slow down ripening by maintaining high levels of antioxidant enzymes, such as APX, catalase, GPX, POD, and GR, and by reducing the activities of LOX, polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), and protease in a dose-dependent manner, inhibiting the post-harvest senescence and decay of the fruit [113,124,128].

H<sub>2</sub>S can also regulate post-harvest ripening and decay by directly affecting the degradation of the cell wall [129,130]. The cell wall is an essential structure for maintaining plant morphology and defense against fungi. Pectin methylesterase (PME, EC 3.1.1.11), polygalacturonase (PG, EC 3.2.1.15), and endo- $\beta$ -1,4-glucanase (EGase, EC 3.2.1.4) are crucial factors influencing the process of post-harvest cell wall softening [121]. Chitinase (CHI, EC 3.2.1.14) and beta-1,3-glucanase (GNS, EC 3.2.1.6) are critical factors in fruit decay [129]. In strawberry plants, exogenous H<sub>2</sub>S significantly inhibited fruit softening by decreasing the activities of PME, PG, and EGase [129]. Similarly, in the Chilean strawberry, 0.2 mmol L<sup>-1</sup> NaHS could reduce the expression of pectin degradation-related genes (*FcPG1*, *FcPPL1*, *FcEXP2*, *FcXTH1*, *FcEG1*) and alleviate pectin degradation, thereby prolonging fruit shelf-life [130].

#### 4.4. H<sub>2</sub>S and Senescence

Plant senescence is the process of the decline in life activity that leads to natural plant death. In this process, the plant transports stored material or even disintegrated protoplasm to new organs or other organs, facilitating the survival of the individual plant or the continuation of the species [131]. From this point of view, senescence is not a purely passive and reactive process; instead, there is precise regulation via antagonistic regulatory substances in the plant [132]. The following section on plant senescence refers specifically to leaf senescence.

The antagonistic regulation of PCD by H<sub>2</sub>S and GAs provides a precise regulatory mechanism for seed germination. The PCD of the pasteurized layer cells contributes to the maintenance of the stability of the internal environment [133]. During mid-seed development, the embryo-surrounding region (ESR) is degraded to provide space for the growing embryo [134]. GAs positively regulate PCD in the cells of the pasteurized layer. It has been reported that GAs induce ROS production via NADPHox in barley and that ROS promote the expression of *GAMYB* in dextrin cells, which, in turn, induces the expression

of amylase [135,136]. Nucleases and proteases that are found downstream of ROS are associated with dextrin vesiculation and cell death [137]. ROS may not directly control the PCD of the paste powder layer, but instead, feed back the GAs signal by disrupting the SLN1. GAs affects the breakdown of starchy endosperm (SE) by targeting the DELLA protein, SLENDER1 (SLN1), for degradation and by inducing the expression of secreted amylase [136]. Unlike the action of GAs, exogenous H<sub>2</sub>S delays PCD and enhances the secretion of alpha-amylase [138]. In addition, GAs inhibits L-DES activity in dextrin cells, thereby reducing the synthesis of endogenous H<sub>2</sub>S [139].

H<sub>2</sub>S regulates plant senescence through cascade crosstalk with other plant hormones and signaling molecules [5,140]. Exogenous H<sub>2</sub>S inhibits the yellowing process by down-regulating the transcription of chlorophyll-degrading genes (*BoSGR*, *BoCLH2*, *BoPaO*, and *BoRCCR*) and maintaining high levels of chlorophyll, carotenoids, anthocyanins, and ascorbic acid in leaves [113]. It is well known that ETH is involved in the regulation of the senescence process in plants and that it mainly plays a role in accelerating maturation and senescence [141]. Leaf abscission is an important physiological phenomenon of senescence. H<sub>2</sub>S plays an antagonistic role in ETH-promoted petiole abscission [142]. NO also inhibits ETH synthesis in a dose-dependent manner [143]. The ETH-NO-H<sub>2</sub>S signaling pathway becomes an important pathway for regulating plant maturation.

Furthermore, the nuclear factor-E2-related factor (Nrf2) is the primary regulator of the antioxidant response in cells, whereas the Kelch-like ECH-associated protein 1 (Keap1) is a negative regulator of Nrf2. H<sub>2</sub>S is post-translationally modification of (Keap1), induces Keap1 to segregate Nrf2, enhances Nrf2 nuclear translocation, and stimulates Nrf2-targeted downstream genes to prevent senescence [144]. H<sub>2</sub>S also prevents senescence by regulating the signaling of the oxidative stress pathways.

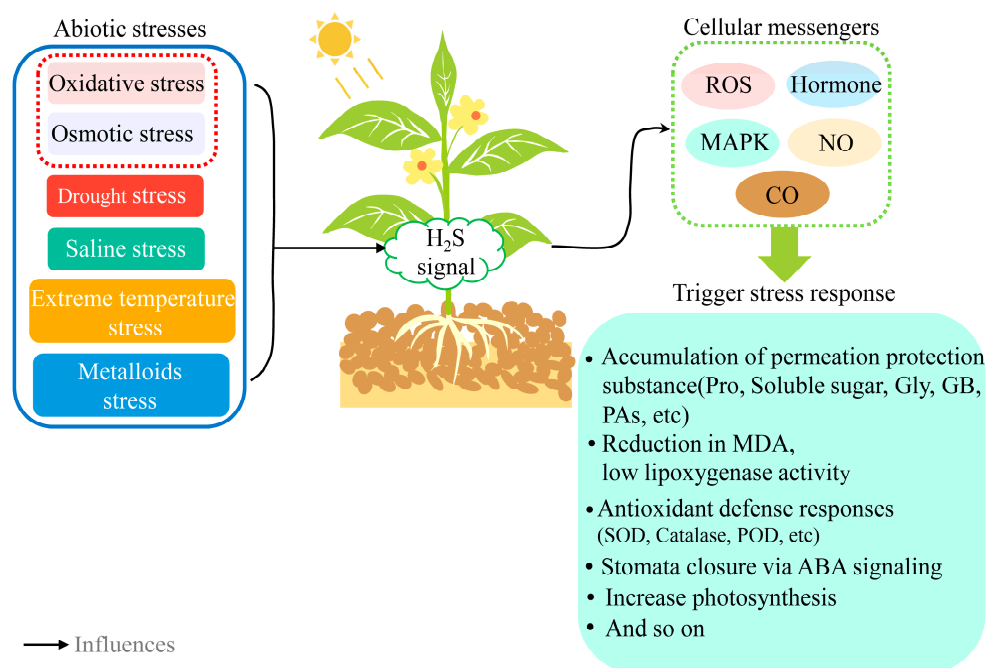
**Table 1.** H<sub>2</sub>S is involved in plant growth and development.

Development	Plant Species	H <sub>2</sub> S Doses	Effects	References
Germination	<i>Arabidopsis thaliana</i>	12 µmol L <sup>-1</sup> NaHS	Activated AOX Mediated cyanide-resistant respiration pathway	[83]
	<i>Arabidopsis thaliana</i>	0.1 mmol L <sup>-1</sup> NaHS	Maintained the protein stability of ABSCISIC ACID-INSENSITIVE 4	[1]
Root development	Peach ( <i>Prunus persica</i> (L.) Batsch)	0.2 mmol L <sup>-1</sup> NaHS	Increased the concentration of endogenous auxin Upregulated the expression of <i>LATERAL ORGAN BOUNDARIES DOMAIN 16</i>	[145]
	Cucumber ( <i>Cucumis sativus</i> L.)	100 µmol L <sup>-1</sup> NaHS	Promoted the occurrence of adventitious roots Regulated osmotic substance content (proline) and enhanced antioxidant ability	[106]
Photosynthesis	Wheat ( <i>Triticum aestivum</i> L.)	200 µmol L <sup>-1</sup> NaHS	Increased photosynthesis and carbohydrate metabolism	[109]
	Garlic ( <i>Allium sativum</i> )	200 µmol L <sup>-1</sup> NaHS	Increased the values of net photosynthetic rate, transpiration rate and stomatal conductance	[108]
Flowering	Heading Chinese cabbage ( <i>B. rapa</i> L. syn. <i>B. campestris</i> L. ssp. <i>chinensis</i> Makino var. <i>pekinensis</i> (Rupr.) J. Cao et Sh. Cao)	100 µmol L <sup>-1</sup> NaHS	Promoted plant flowering by weakening or eliminating the binding abilities of BraFLCs to downstream promoters via S-sulphydration	[120]
Maturity and senescence	Strawberry ( <i>Fragaria chiloensis</i> (L.) Mill.)	0.2 mmol L <sup>-1</sup> NaHS	Maintained fruit firmness Delayed pectin degradation Downregulated the expression of polygalacturonase, pectate lyase, and expansin	[130]
	Goji berry ( <i>Lycium barbarum</i> L.)	1.4 mmol L <sup>-1</sup> NaHS	Improved postharvest quality Increased bioactive compounds accumulation Boosted antioxidant capacity	[128]

NaHS: sodium hydrosulfide; AOX: alternative oxidase.

## 5. H<sub>2</sub>S Improves Plant Stress Tolerance

Unlike animals, the ability to tolerate the environment is crucial for plants. Even if the factors of the environment are different, the same adverse effects can be induced. Abiotic stresses, such as salinity, low temperature, and metalloids induce the onset of oxidative and osmotic stress, causing the accumulation of ROS in plants, the onset of membrane lipid peroxidation, impaired cell membrane stability, and impaired root and leaf function, leading to the onset of cellular water loss and photo-inhibition in plants. H<sub>2</sub>S can counteract the adverse effects of abiotic stress by regulating several critical physiological and biochemical processes (Figure 5). Among these, the main functions induced by H<sub>2</sub>S are typically to up-regulate antioxidant metabolic activity, accumulate osmotic regulation protective substances, and enhance stomatal movement/non-stomatal photosynthesis [14,146] (Table 2).



**Figure 5.** The effect of H<sub>2</sub>S on plant stress tolerance. ABA (abscisic acid), CO (carbon monoxide), GB (glycine betaine), Gly (glycine), H<sub>2</sub>S (hydrogen sulfide), MAPK (mitogen-activated protein kinase), MDA (malondialdehyde), PAs (polyamines), POD (peroxidase), Pro (proline), ROS (reactive oxygen species), SOD (superoxide dismutase).

### 5.1. H<sub>2</sub>S and Oxidative Stress

Exposure of plants to various abiotic stresses, such as drought, salinity, temperature extremes, toxic metals, and hypoxia, causes an imbalance in endogenous redox homeostasis or oxidative stress. The excessive accumulation of ROS, such as O<sub>2</sub><sup>•−</sup> and H<sub>2</sub>O<sub>2</sub>, is the first step in the onset of oxidative stress, triggering autophagy and PCD [134]; it also causes protein oxidation, membrane lipid peroxidation, and the subsequent blockage of membrane-related physiological processes [147].

Changes in ROS levels in cells can alter the structure and function of a variety of proteins, thereby affecting many different signal transduction pathways. Under stress, interactions between ROS production and scavenging in the different compartments of plants can generate specificity [148]. Among them, peroxisomes are subcellular compartments that are important for ROS metabolism in plants. In plant peroxisomes, xanthine oxidoreductase (XOR) activity produces uric acid, which is accompanied by the production of O<sub>2</sub><sup>•−</sup>. Nitric oxide synthase (NOS)-like enzyme generates NO by using L-arginine and Ca<sup>2+</sup> in substrate activity. NO reacts with O<sub>2</sub><sup>•−</sup>, thereby converting it to the less toxic ONOO<sup>−</sup> [149]. H<sub>2</sub>O<sub>2</sub>-producing enzymes (Catalase, GOX) are regulated by NO-mediated S-nitrosation

and tyrosine nitration [150].  $O_2^{\bullet-}$  can also be converted to  $H_2O_2$  by CuZnSOD. In addition, glycolate oxidase (GOX), acyl-CoA oxidase (AOX), urate oxidase (UO), polyamine oxidase, copperamine oxidase (CuAO), sulfite oxidase (SO), and sarcosine oxidase (SOX) are important sources of the peroxisomal  $H_2O_2$  pool. In the peroxisome, catalase (located in the substrate) works with APX (located in the membrane) to break down  $H_2O_2$  [149]. In plants, both catalase [151] and GOX [152] activities can be inhibited by  $H_2S$ . Studies have found that  $H_2S$  is present in plant peroxisomes [151]. Evidently,  $H_2S$  and NO play an important role in the metabolism of ROS in the peroxisome. Antioxidant enzymes also play an important role in ROS scavenging. Proteomics studies have shown that antioxidant enzymes are important targets of  $H_2S$ -mediated S-sulfhydration, including matrix sAPX and cystoid thylakoid tAPX, catalase 1/2/3, DHAR1/2/3, GR1/2, etc. [153]. Chen et al. speculated that  $H_2S$  may interact with ROS homeostasis by acting against oxidases and/or other targets in the signaling pathway [154]. From the above examples, it is clear that  $H_2S$  is involved in all ROS metabolism processes.

The interaction between  $H_2S$  and other signaling molecules jointly regulates ROS. Both NO and  $H_2S$  enhance plant antioxidant capacity by reducing the excess production of ROS, decreasing lipid peroxidation, and increasing the activity of major antioxidant enzymes [155]. The pharmacological experiments showed that  $H_2S$  donors increased NO content, and vice versa, accompanied by the upregulation of several antioxidant enzyme activities (e.g., SOD, catalase, and APX) [156]. In maize, 0.2 mmol  $L^{-1}$  SNP (a NO donor) promotes the activity of endogenous  $H_2S$  synthases (L-DES, CAS, and CS) and  $H_2S$  accumulation [157]. Similarly, in the tomato, NO was observed to stimulate  $H_2S$  accumulation by upregulating the transcript levels of  $H_2S$  biosynthetic enzymes and acting downstream of NO to reduce oxidative stress [158]. Exogenous  $H_2S$  reduced NO levels and controlled salinity-induced oxidative and nitrosative cell damage [159]. Carbon monoxide (CO) has been shown to protect plants from the harmful effects of excess ROS [160]. Heme oxygenase-1 (HO-1) is an important source of endogenous CO in plants [161]. In pepper, NaHS induces the *CsHO-1* gene and *CsHO-1* protein expression in a time-dependent manner [162]. Hemoglobin (CO scavenger) treatment enhanced  $H_2S$  biosynthesis (L-cysteine desulfhydrase, L-DES), which induced the accumulation of  $H_2S$  in tobacco cells [163].

## 5.2. $H_2S$ and Osmotic Stress

Plants grow in a sedentary manner and therefore need to respond and adapt to harsh environmental conditions to survive. Similar to oxidative stress, osmotic stress can be induced by numerous abiotic stresses. The immediate consequence of osmotic stress in plants is water deficit. Osmoregulation is an essential response made by plants undergoing osmotic stress, which is manifested by the massive accumulation of osmoprotective substances, such as proline (Pro), soluble sugars (glucose, sucrose, and alginate), glycine betaine (GB), glycine (Gly), polyamines (PAs), and sugar alcohols. The accumulation of these osmoprotective substances contributes to the plant's ability to resist stress. Among them, Pro is the typical osmoprotective substance, the accumulation of which mitigates the adverse effects of abiotic stresses. The physiological functions of this substance are shown in four aspects: relieving physiological dehydration and maintaining plasma membrane stability; forming hydrocolloids to protect protein molecules; effectively removing ROS; offering an effective means of energy storage under abiotic stress [164,165].

$H_2S$  acts as a downstream signaling molecule that regulates stomatal closure, actively participating in stomatal closure and reducing water dissipation, thereby improving tolerance to osmotic stress. PLD is a significant lipid hydrolase that hydrolyses the membrane phospholipids to produce phosphatidic acid (PA) [166]. PLD- $\delta$  is required for ABA-mediated stomatal closure and is essential for stabilizing the membrane system and balancing osmotic pressure when plants experience hyperosmotic stress [167].  $H_2S$  is located downstream of PLD- $\delta$  and promotes stomatal closure in response to osmotic stress [168], alleviating osmotic stress by increasing PLD and inhibiting the ROS [169].

In addition, the H<sub>2</sub>-H<sub>2</sub>S pathway has been found to regulate the stomata. In *Arabidopsis*, H<sub>2</sub>S is involved in osmotic stress tolerance as a downstream molecule of H<sub>2</sub>-regulated stomatal closure [170]. In cereals, osmotic stress induces the production of endogenous H<sub>2</sub>S, the accumulation of which enhances osmotic stress tolerance by mediating DNA methylation [13]. ACO is a key enzyme in ETH biosynthesis in plants. When tomato plants suffer osmotic stress, ACO is activated, and ETH is produced in large quantities. In one study, the application of 200 μmol L<sup>-1</sup> of NaHS and ETH both induced stomatal closure and water retention. An HT supply counteracted the effects of ETH or osmotic stress on stomatal closure. On the other hand, ETH-induced H<sub>2</sub>S negatively regulates ETH biosynthesis via the S-sulfhydration of LeACO1/2. H<sub>2</sub>S also indirectly inhibited the transcription of *LeACO1* and *LeACO2*. This showed that endogenous H<sub>2</sub>S was downstream of osmotic stress signaling. H<sub>2</sub>S can be involved in osmotic stress signaling through either direct or indirect means [118].

### 5.3. H<sub>2</sub>S and Drought Stress

Drought stress is followed by a range of adverse plant effects, including water deficit, osmotic stress, oxidative stress, impaired cell integrity, and damage to PS II [14].

H<sub>2</sub>S promotes the accumulation of osmoprotective substances and alleviates the adverse effects of osmotic stress (induced by drought stress) [171,172] (Figure 5). Water deficit is the result of a combination of root water uptake rate and leaf transpiration rate, which is essentially caused by osmotic stress. The typical response to water deficit in plants is osmoregulation, mainly in the form of osmoprotective substances such as Pro, soluble sugars (glucose, sucrose, and alginate), GB, Gly, PAs, sugar alcohols, etc. The accumulation of these osmoprotective substances contributes to improving plant stress tolerance. During drought stress, exogenous H<sub>2</sub>S promotes the accumulation of Pro, GB, and trehalose in wheat [173], increases the levels of soluble sugars, PAs, Pro, and betaine in spinach seedlings, and up-regulates several genes related to PAs and soluble sugar biosynthesis, enhancing drought tolerance [174].

H<sub>2</sub>S mitigates damage from drought stress by regulating stomatal movement [175] (Figure 5). The ABA response is the classic response mechanism for stomatal movement [176,177]. Under drought stress, L-DES catalyzes the degradation of cysteine as an essential pathway for H<sub>2</sub>S production. After NaHS treatment, ABA-induced stomatal closure is attenuated in *lcd* mutants, and the transcript expression of ABA receptor candidates is up-regulated; in *aba3* and *abi1* mutants, stomatal density is reduced, and L-DES expression and H<sub>2</sub>S synthesis are decreased [178]. Drought stress-induced *DES1* expression is abolished in the *aba3* mutant. The addition of ABA or the expression of *ABA3* or *DES1* to the guard cells of *aba3/des1* double-mutants cannot alter the wilting phenotype of these mutants. However, with the application of the NaHS treatment, the wild-type phenotype is completely restored [179]. These findings suggest that H<sub>2</sub>S is involved in ABA-mediated stomatal movement via crosstalk with ABA. A growing number of reports have revealed its mechanisms. In guard cells, ABA regulates the activities of several ion channels in promoting stomatal closure and inhibiting stomatal opening, leading to changes in guard cell expansion pressure and stomatal closure, which is the central modulation of stomatal movement [180]. H<sub>2</sub>S may be an essential link in the regulation of stomata by ABA through ion channels, while influencing the expression of ABA receptor candidates and thereby regulating stomatal closure in guard cells [178]. H<sub>2</sub>S can also be involved in ABA-mediated stomatal movement, via the regulation of MPK4, to alleviate the water deficit caused by drought stress. In the guard cells, ROS acts as a second messenger for ABA-regulated stomatal closure signals [181]. In *Arabidopsis thaliana*, ROS production by the NADPHox pathway (e.g., *Arabidopsis thaliana* respiratory burst oxidase homolog protein D and F (*AtROHD* and *AtROHF*)) is the rate-limiting link in ABA signal transduction [182]. H<sub>2</sub>S increases the endogenous H<sub>2</sub>O<sub>2</sub> in guard cells by affecting NADPHox activity and enhancing its ability to produce ROS [117]. This effect of H<sub>2</sub>S may be related to the transcriptional activation of NADPHox respiratory burst oxidase homolog (*RBOH*) and



the modification of RBOH by S-sulphydration [26]. Sucrose nonferme-1(SNF1)-RELATED PROTEIN KINASE2 (*SnRK2*), a significant switch for downstream ABA signaling in guard cells, can be activated by ABA [183]. ABA-induced endogenous H<sub>2</sub>S is involved in stomatal closure via peroxide-modified SnRK2 activity and improves drought tolerance [27]. In *Arabidopsis thaliana*, endogenous H<sub>2</sub>S induces the opening of the K<sup>+</sup> channel (the outward K<sup>+</sup>-channel), which acts as the main osmoregulatory channel in response to drought stress, causing K<sup>+</sup> efflux and Ca<sup>2+</sup> and Cl<sup>-</sup> efflux, leading to stomatal closure [180]. With the activation of the S-type anion channels (SLAC1), anions efflux out, playing a vital role in stomatal closure [184]. Exogenous H<sub>2</sub>S activates SLAC1, which occurs due to dependence on SnRK2.6, and elevates the intracellular free Ca<sup>2+</sup> levels in *Arabidopsis* [185]. In addition, H<sub>2</sub>S enhances drought resistance by means of ABA non-dependent stomatal closure. In tobacco plants, H<sub>2</sub>S-induced stomatal closure in the guard cells is caused by the specific inactivation of inward-rectifying K<sup>+</sup> channels (I<sub>KIN</sub>) [186]. The above finding suggests that a complex signaling network of ABA, H<sub>2</sub>O<sub>2</sub>, and H<sub>2</sub>S cascades provides the regulatory mechanism for stomatal closure under drought stress, and that stomatal movement is accompanied by changes in the flux of several ions across the plasma membrane, carried by ion channels. In addition, in *Arabidopsis*, ABI4 acts downstream of L-cysteine desulfurase1 (DES1) to control the ABA response. The S-sulphydration of ABI4 occurs at Cys250, triggered by ABA in a time-dependent manner, while the loss of function of DES1 weakens this process [28].

H<sub>2</sub>S alleviates drought stress-induced photosynthetic decline. Drought stress directly inhibits chlorophyll synthesis, reduces PSII-related light-trapping pigments, and decreases ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) activity [187]. Exogenous H<sub>2</sub>S promotes the rapid conversion of the D1 protein and thereby restores PSII activity [188]. Aquaporins (AQP) are channels in the biological membranes that facilitate the movement of water across membranes, and their status and activities are key factors affecting the ability of plants to cope with drought stress. In addition, stomatal movement under drought stress provides a guarantee for the smooth progress of photosynthesis. Drought stress closes stomata and reduces C<sub>i</sub>, leading to an inhibition of the photosynthetic carbon reduction cycle, presenting a hindrance to photosynthesis [189]. This is potentially also the reason for the high photosynthetic capacity maintained by exogenous H<sub>2</sub>S under drought stress.

Exogenous H<sub>2</sub>S increases water channel protein expression (*SoPIP1;2*) in spinach seedlings [174], thereby improving drought resistance. The mechanism of the AQP channel opening and closing is regulated by PTMs, such as phosphorylation, methylation, acetylation, deamidation, and glycosylation. H<sub>2</sub>S is a potential source of these PTMs, but the exact mechanism of the regulation of AQP remains to be further elucidated [190].

#### 5.4. H<sub>2</sub>S and Saline Stress

H<sub>2</sub>S maintains intracellular homeostasis when plants suffer salt stress (Figure 5). Salt stress mainly disrupts the thermodynamic balance of water and ions in the plant cells, leading to osmotic stress, ionic imbalance, and ionic toxicity [191,192]. The maintenance of intracellular homeostasis (ionic and osmotic balance) in plants is one of the most important strategies to counteract the adverse effects of salt stress. In barley, H<sub>2</sub>S is able to maintain low Na<sup>+</sup> levels in the cytoplasm by enhancing the transcript levels of PM H<sup>+</sup>-ATPase (*HvHA1*) and the Na<sup>+</sup>/H<sup>+</sup> reverse transporter protein (*HvSOS1*) to achieve Na<sup>+</sup>/H<sup>+</sup> homeostasis, which is mediated by NO signaling [193]. Studies in *Cyclocarya paliurus* also found that H<sub>2</sub>S alleviated the adverse effects induced by salt stress in close correlation with NO levels [194]. The stomatal limitation is caused by salt stress reducing the water content in the leaves, triggering the closure of stomata, which leads to a decrease in C<sub>i</sub> and a reduction in the plant's photosynthetic rate. Exogenous H<sub>2</sub>S upregulates the expression of PM H<sup>+</sup>-ATPase, *SOS1*, and *SKOR* so as to maintain Na<sup>+</sup> and K<sup>+</sup> homeostasis and alleviate salt-stress-induced stomatal limitation-induced reductions in the photosynthetic properties, chlorophyll fluorescence, and stomatal parameters in cucumber plants [195]. In mutants lacking H<sub>2</sub>S and JA, stomatal density and stomatal index values are increased,

while exogenous NaHS reverses this phenomenon [196]. H<sub>2</sub>S inhibits ETH synthesis by inhibiting the activity of ACO via S-sulphydration [118]. It has also been found that the MT tolerance of iron deficiency (ID) and salt stress may be related to downstream signaling crosstalk between NO and H<sub>2</sub>S [197].

### 5.5. H<sub>2</sub>S and Extreme Temperature Stress

Extreme temperature is an important factor limiting plant growth and productivity. Unlike animals, plants are more likely to only tolerate temperature extremes passively. Improving their tolerance to temperature extremes is, therefore, even more critical. Depending on the temperature range in which the stress occurs, extreme temperature stress can be classified into freezing stress, chilling stress, and heat stress [198,199]. The effects of extreme temperatures on plant physiology have commonalities, including altered membrane fluidity, cellular water loss, protein denaturation, disruption of the RNA secondary structure, and, in particular, photo-inhibition and impaired metabolic homeostasis [200]. It is important to note that the protein denaturation caused by high temperatures is often irreversible. Freezing stress is accompanied by mechanical damage from ice crystals [198,201].

H<sub>2</sub>S improves plant resistance to low-temperature stress (Figure 5). Chilling stress promotes the activity of L-DES and D-DES in grapes, resulting in a significant increase in H<sub>2</sub>S content, suggesting that this may be a protective response by the plant to chilling stress [202]. The transcriptional response to cold stress can be divided into two phases: early and late. The early genes that respond mainly encode the transcription factors, while the late genes that respond belong to a core group of cold-inducible genes. The early encoded transcription factors, such as the C-repeat binding factor (CBF) or dehydration response element-binding factor 1 (DREB1s), are essential. Late cold-induced gene products that are directly involved in stress protection and the maintenance of cellular homeostasis include cold-regulated (COR), cold-induced (KIN), low-temperature-induced (LTI), responsive to dehydration (RD), and late embryo genesis-abundant (LEA) products [203]. These molecules and proteins, including ions, lipids, protein kinases, and transcription factors, are interconnected and form the basis of the plant's response to cold stress. H<sub>2</sub>S upregulates cold stress-related mitogen-activated protein kinase (MAPK), especially MPK4, in *Arabidopsis* in response to cold stress by mediating the regulation of *ICE1*, *CBF3*, *COR15A*, and *COR15B* by MPK4, while inhibiting stomatal opening [204]. H<sub>2</sub>S can also directly influence S-sulphydration MPK4 and increase MPK4 kinase activity in response to cold stress [205]. The effect of H<sub>2</sub>S on low-temperature stress is multifaceted. The exposure of cucumber root systems to chilling stress results in H<sub>2</sub>S promoting the expression of plasma membrane proton pump isoforms (*CsHA2*, *CsH4*, *CsH8*, *CsH9*, and *CsHA10*). The modulation of ATPase activity by H<sub>2</sub>S is more pronounced than that by NO and H<sub>2</sub>O<sub>2</sub> [206]. The effects of exogenous NO, NO scavengers, and NO synthesis inhibitors on H<sub>2</sub>S metabolism differ during the low-temperature storage of peaches, implying that the regulation of endogenous H<sub>2</sub>S metabolism by exogenous NO is not a simple linear regulation [207]. NaHS alleviates plant photo-inhibition and membrane damage induced by exposure to chilling stress [208]. H<sub>2</sub>S may cause this effect by triggering downstream signals (e.g., GSH) regarding its chilling tolerance [209]. H<sub>2</sub>S also upregulated the expression of antioxidant enzyme genes (*CaSOD*, *CaPOD*, *CaCAT*, *CaAPX*, *CaGR*, *CaDHAR*, and *CaMDHAR*) to achieve improved cold tolerance [210].

In terms of heat stress, H<sub>2</sub>S crosstalks with signaling molecules (GTs) [211] and plant hormones [212,213]. In tobacco plants, CO improves cellular heat resistance, while NaHS enhances CO-induced heat resistance but can be attenuated by PAG, a specific inhibitor of H<sub>2</sub>S biosynthesis, or its scavenger, HT [163]. The application of NaHS and GYY4137 enhances SNP-induced heat tolerance; H<sub>2</sub>S may be a downstream signaling molecule for NO-induced heat tolerance in maize seedlings [214]. Heat stress activates H<sub>2</sub>S biosynthesis in plants and induces H<sub>2</sub>S accumulation. S-nitrosoglutathione reductase (GSNOR) is a key enzyme associated with the NO cycle in plants. H<sub>2</sub>S locates upstream of GSNOR and effectively eliminates RNS and ROS by upregulating GSNOR activity [215]. GSNOR

may be a vital component linking NO to H<sub>2</sub>S. High temperature-induced photo-inhibition can be mitigated well by NO with H<sub>2</sub>S [211]. SA is a hormone that is involved in plant growth and development and that actively participates in plant heat tolerance. In maize (*Zea mays* L.), PAG and HT attenuate SA-induced heat tolerance. SA increases L-DES activity, which induces the accumulation of endogenous H<sub>2</sub>S, implying a positive role for SA and H<sub>2</sub>S crosstalk in plant heat tolerance [212]. Cellular water deficit, caused by heat stress, is a significant cause of injury; ABA-regulated stomatal closure is vital for managing water loss in leaves. In tobacco plants, ABA upregulates L-DES activity and induces the accumulation of endogenous H<sub>2</sub>S. ABA-induced heat resistance is enhanced by NaHS and is conversely weakened by PAG and HT [213]. In addition, exogenous H<sub>2</sub>S induces heat-shock proteins (HSP70, HSP80, and HSP90) and water channel proteins (PIP), while activating a coordinated network, associated with heat shock defense at the transcriptional level, that favor the formation of protective molecules [216].

### 5.6. H<sub>2</sub>S and Metalloids Stress

Metalloids, e.g., nickel (Ni), molybdenum (Mo), zinc (Zn), copper (Cu), chromium (Cr), lead (Pb), and cadmium (Cd) in soil are not entirely harmful; plant micronutrients (e.g., Ni, Zn, Mo, Cu, and Fe) are not toxic at low concentrations [217]. At specific concentrations, metalloids, represented by Ni, can significantly prevent the plants' uptake and use of crucial mineral elements [218]. Conversely, some of the metallic elements, such as Cr, lead (Pb), and Cd, and mercury (Hg), are toxic whenever they are present [217]. The exposure of plants to metalloids stress induces the endogenous production of H<sub>2</sub>S [219]. Once generated, H<sub>2</sub>S can move freely across the plant membrane as a form of resistance to metalloids stress [220].

The mechanism by which H<sub>2</sub>S affects plant tolerance to metalloids is similar to oxidative stress and osmotic stress. H<sub>2</sub>S enhances the antioxidant system (via the upregulation of POD, SOD, catalase, and APX) to counteract oxidative stress (via the downregulation of MDA, H<sub>2</sub>O<sub>2</sub>, and EL) [17,221] and improves physiological processes, such as photosynthesis (Figure 5) [222]. The difference is that cell-wall metabolism is an integral part of H<sub>2</sub>S resistance to metalloids stress. The plant cell wall is an important barrier to the movement of metalloids across the membrane [223]; metal-tolerant proteins, represented by metallothionein and phytochelatin, are vital in maintaining cation homeostasis in the plant cell wall [224,225]. Recently, it has been found that exogenous H<sub>2</sub>S stimulates endogenous metal-binding protein activity, which, in turn, alters the accumulation of Cd in the cell wall, thereby reducing the mobility of intracellular metal ions [225].

In addition, the crosstalks between H<sub>2</sub>S and other signaling molecules (e.g., GTs), plant hormones, and plant growth regulators (PGRs) are involved in the related stress responses. H<sub>2</sub>S crosstalks with other GTs (NO, CO, CH<sub>4</sub>) to mitigate the damage to HMs [222]. In particular, H<sub>2</sub>S and NO both have similar defense responses: increased antioxidant metabolism, inhibition of the accumulation of HMs, and the triggering of Ca<sup>2+</sup> [222]. Exogenous NO induces endogenous H<sub>2</sub>S, enhances reactive oxygen metabolism and photosynthesis, and eliminates the adverse effects of Cr (VI) on tomato seedlings [226]. Ca<sup>2+</sup> is the second messenger of cellular resistance signaling. The calcium-dependent protein kinases (CDPKs) are important components of Ca<sup>2+</sup> signaling, and these CDPKs are Ca<sup>2+</sup>-dependent. CDPKs are upstream of H<sub>2</sub>S and can be modulated to improve Cd stress tolerance in plants [227]. SA is a phenolic compound that has a positive effect on plant stress tolerance. The exogenous administration of SA with H<sub>2</sub>S triggers NO signaling in vivo while increasing these osmoregulatory substances alleviates the toxic effects of lead stress. H<sub>2</sub>S-SA crosstalk favors the expression of ASA-GSH metabolic activity [228]. Methyl jasmonate (MeJA) alleviates Cd stress-induced damage and enhances the expression of homeostasis-related genes (*MTP1*, *MTP12*, *CAX2*, and *ZIP4*), which process is mediated by H<sub>2</sub>S [24]. Thiamine (vitamin B1) is an important coenzyme that is involved in many metabolic pathways, especially in those processes related to energy metabolism (carbon assimilation and respiration) and is considered a plant growth regulator that is involved in

plant responses to adversity [229]. H<sub>2</sub>S and NO are involved in THI-induced tolerance to Cd toxicity [230].

**Table 2.** H<sub>2</sub>S improves plant stress tolerance.

Plant Species	Stressors	H <sub>2</sub> S Doses	Protective Effects	References
<i>Arabidopsis thaliana</i>	Oxidative	0.5 mmol L <sup>-1</sup> NaHS	Repressed glycolate oxidase activities	[152]
<i>Arabidopsis thaliana</i>	Osmotic	150 mmol L <sup>-1</sup> NaHS	Involved in osmotic stress-triggered stomatal closure	[168]
Safflower	Drought: 70 and 50% field capacity	0.5 and 1.0 mmol L <sup>-1</sup> NaHS	Increased the accumulation of secondary metabolites Strengthened the antioxidant capacity Regulated elemental uptake	[171]
Wheat ( <i>Triticum aestivum</i> L.)	Drought: 30% field capacity	10 mg m <sup>-3</sup> SO <sub>2</sub>	Triggered proline accumulation Activated antioxidant enzymes Changed expression level of transcription factors Increased H <sub>2</sub> S content	[172]
<i>Cyclocarya paliurus</i>	Salinity: 100 mmol L <sup>-1</sup> NaCl	0.5 mmol L <sup>-1</sup> NaHS	Maintained chlorophyll fluorescence Regulating nitric oxide level Improved antioxidant capacity	[194]
Wheat ( <i>Triticum aestivum</i> L.)	Heat: 40 °C	200 µmol L <sup>-1</sup> NaHS	Reduced glucose sensitivity Increased the activities of SOD, catalase, and the AsA-GSH cycle	[211]
Pepper ( <i>Capsicum annuum</i> L.)	Chilling: 10 °C/5 °C day/night	1 mmol L <sup>-1</sup> NaHS	Enhanced the antioxidant capacity Increased the enzyme transcription levels Reduced the contents of O <sub>2</sub> <sup>•-</sup> , H <sub>2</sub> O <sub>2</sub> , and MDA	[210]
Wheat ( <i>Triticum aestivum</i> L.) Rice ( <i>Oryza sativa</i> L. var.)	Metalloids: 20 µmol L <sup>-1</sup> Cr(VI)	15 µmol L <sup>-1</sup> NaHS	Maintained fruit firmness Delayed pectin degradation Downregulated the expression of polygalacturonase, pectate lyase, and expansin	[221]

SOD: Superoxide dismutase; AsA-GSH: ascorbate-gluthathione cycle; MDA: malondialdehyde; O<sub>2</sub><sup>•-</sup>: superoxide radical; H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide.

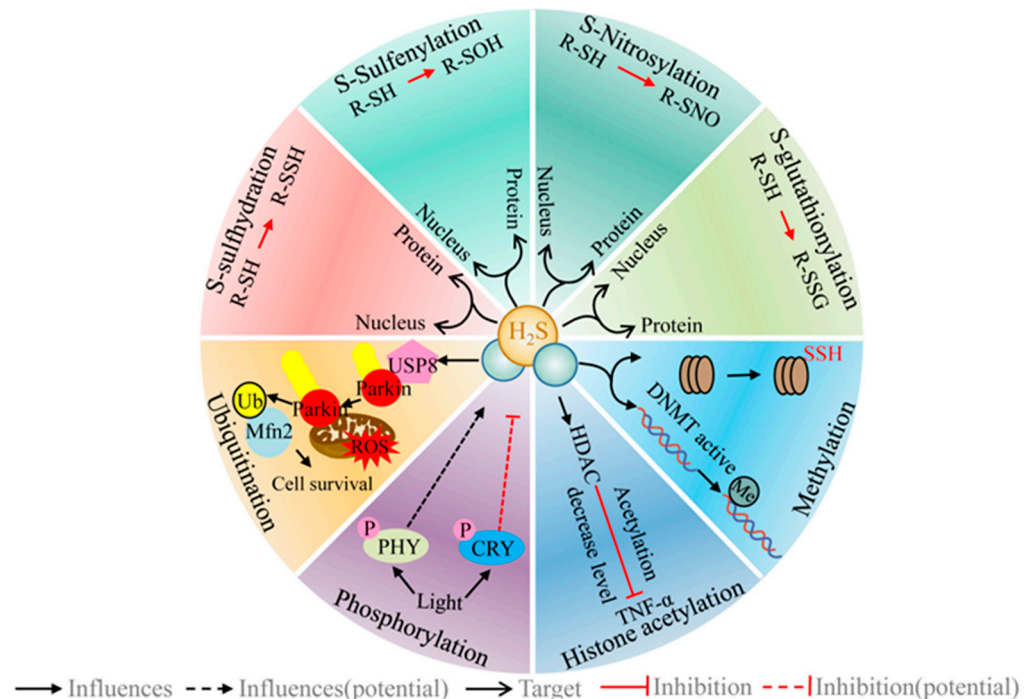
## 6. H<sub>2</sub>S Regulates a Range of Different PTMs/Signaling Systems

The PTM of proteins offers an important pathway by which to exert biological effects related to life activities, environmental responses, and epigenetics. Emerging evidence suggests that H<sub>2</sub>S exerts its function by regulating various PTMs. Here, some of these recent developments are highlighted.

### 6.1. H<sub>2</sub>S Regulates the Post-Translational Modification of Protein Cysteine Residues (R-SH)

The common oxidative post-translation modifications (oxPTMs) of protein cysteine sulfhydryl groups include S-sulfhydration, S-sulfenylation, and S-nitrosylation. H<sub>2</sub>S is directly or indirectly involved in these oxPTMs [231,232] (Figure 6). The exposure of plants to stress causes the accumulation of ROS and RNS, which ultimately leads to oxidative stress. Originally, cysteine thiols may undergo different oxPTMs, for example, S-nitrosylation and S-sulfenylation. The products of the above two modifications are mostly oxidized compounds that can be reduced in cells via reducing agents (GSH, thioredoxin, and glutaredoxin) [233]. S-sulfhydration has a protective effect on peroxidation. Upon a further increase in stress, the protein will react with ROS/RNS and form an adduct (RSSO<sub>3</sub>H) that can be restored to free thiols by thioredoxin [231]. In *Arabidopsis* and pea, the Cys32 residue, as exemplified by peroxidase, can be targeted by two PTMs (S-nitrosylation and

S-sulfhydration) [234,235]. Therefore, the regulatory effect of H<sub>2</sub>S on oxPTMs is complex and nonlinear. Chemically speaking, S-sulfhydration usually increases the reactivity of the target protein, while S-nitrosylation usually decreases the protein activity [236].



**Figure 6.** H<sub>2</sub>S regulates a range of different PTMs/signaling systems. CRY (cryptochromes), DNMTs (DNA methylation transferases), HDACs (histone deacetylases), Mfn2 (mitofusin-2), PHY (phytochromes), TNF- $\alpha$  (tumor necrosis factor- $\alpha$ ), Ub (ubiquitin), USP8 (ubiquitin-specific peptidase 8).

### 6.1.1. H<sub>2</sub>S and S-Sulfhydration

S-sulfhydration (persulfidation) involves the modification of protein cysteine residues to form persulfides (cysteine thiols (RSH) are mercapturised to mercaptothiols (RSSH)), which results in the production of persulfide groups (-SSH) [237]. The synthesis of persulfides results from both the enzymatic and direct non-enzymatic production of persulfides from H<sub>2</sub>S. The primary enzymatic sources of persulfides are: (1) formations catalyzed by mercaptopyruvate sulfurtransferase (MST); (2) the sulfur oxidation pathway, in which the persulfide product is produced in a two-step reaction. Sulfide-quinone oxidoreductases (SQRs) catalyze the oxidation of H<sub>2</sub>S to sulfane sulfur, which remains covalently attached to the enzyme. This sulfane sulfur can then be transferred to the sulfite to form thiosulfate [238]. (3) Formed in the enzymatic cleavage of cystine by CBS or CSE, H<sub>2</sub>S is directly and non-enzymatically generated as a peroxisulfide, a precursor to highly reactive sulfur-containing compounds, including sulfenic acids, S-nitrosated cysteines, pre-existing inter- or other inter- or intramolecular disulfides, or via the facilitation of the formation of persulfides from H<sub>2</sub>S and protein sulfhydryl groups in the presence of metal ions. In addition, persulfides can be used as carriers of sulfane sulfur and participate in the “trans-S-sulfhydration” reaction.

S-sulfhydration is one crucial way in which H<sub>2</sub>S is involved in life activities [139] (Figure 6). In eukaryotes, cellular autophagy is a highly conserved mechanism of material degradation (the ability to spontaneously eliminate cellular components) [239]. This mechanism is of great significance for differentiation, development, and cell survival. Autophagy-related proteins (ATG) are a group of critical proteins that are involved in the autophagic process. The H<sub>2</sub>S target, ATG, offers the most robust evidence for its direct involvement in cellular autophagy. In animals, H<sub>2</sub>S modifies the Cys150 residue of GAPDH by S-sulfhydration, to achieve the regulation of cellular autophagy. Similarly, in plants, H<sub>2</sub>S can modify the

residues of Cys170 of ATG4 and Cys103 of ATG18 via *S*-sulfhydration to achieve the inhibition of cellular autophagy [239]. This mechanism of inhibition of autophagy is independent of redox conditions [240]. The energy sensor, Snf1-related protein kinase 1 (SnRK1), the kinase target of rapamycin (TOR), ATG1 kinase complex, and the endoplasmic reticulum stress sensor inositol-requiring enzyme-1 (IRE1) are autophagy regulators that have been identified in plants [241]. The *S*-sulfhydration-based modification of these target proteins by H<sub>2</sub>S is another important way in which they are involved in cellular autophagy [239]. ABA signaling is the classical mechanism that regulates stomatal movement [242]. Similarly, H<sub>2</sub>S modifies specific cysteine residues through *S*-sulfhydration, thereby affecting stomatal closure [26,28,175], for example, ABI4 [28] and RBOHD [26]. Notably, the complex signaling network of the H<sub>2</sub>S, ABA, and H<sub>2</sub>O<sub>2</sub> cascade crosstalk is an important way to activate the stomatal closure regulatory mechanisms [27,117,181]. In addition, potential target proteins for H<sub>2</sub>S occur in redox homeostasis, energy status, and cytokinesis-related enzymes, including APX [235], catalase [151], RBOHD [26], glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [235], NADP-isocitrate dehydrogenase (NADP-ICDH) [243], NADP-malic enzyme (NADP-ME) [244], actin [245], etc. This implies that H<sub>2</sub>S is involved in the above metabolic activities via *S*-sulfhydration-based modifications.

H<sub>2</sub>S cannot react directly with thiols but can react with oxidized cysteine residues [246]. *S*-sulfhydration requires H<sub>2</sub>O<sub>2</sub> signaling via sulfenylation. H<sub>2</sub>S can react with oxidized cysteine residues to obtain sulfenic acid (R-SOH) or with protein nitrosothiols (R-SNO) to obtain protein persulfides, but the latter process is thermodynamically unfavorable [54]. It is evident that H<sub>2</sub>S-mediated *S*-sulfhydration does not exist completely independently. The study of specific protein targets for *S*-sulfhydration remains the subject of intense debate.

#### 6.1.2. H<sub>2</sub>S and *S*-Sulfenylation

The occurrence of H<sub>2</sub>S-mediated *S*-sulfhydration may be predicated on *S*-sulfenylation [19,236]. H<sub>2</sub>S finds it difficult to directly reduce the disulfide bonds inside the protein-forming *S*-sulfide, from the thermodynamic and kinetic points of view [247]. Thiols are easily oxidized, and the presence of intracellular ROS can react with free thiols to undergo *S*-sulfenylation. *S*-sulfenylation will then produce oxidized cysteine. The presence of this class of residues is a direct target to enable the action of H<sub>2</sub>S to occur [236]. H<sub>2</sub>S participates in the endogenous cycle of mercaptan (RSH), *S*-sulfenylation, and *S*-sulfhydration (Figure 6). When a protein containing a thiol group [242] is exposed to ROS, the thiol group is reversibly converted to sulfenic acid (RSOH), at which point the protein is considered to undergo *S*-sulfenylation. RSOH is highly reactive, scavenging ROS and converting them to disulfides. Firstly, the ROS are scavenged, and these ROS can further irreversibly oxidize RSOH to form sulfinic acid (RSO<sub>2</sub>H) or sulfonic acids (RSO<sub>3</sub>H). Secondly, RSOH can react with H<sub>2</sub>S to produce water and RSSH. Similarly, RSSH can scavenge ROS in two steps to form RSH (the initial product is an adduct (RSSO<sub>3</sub>H) and RSSO<sub>3</sub>H is dependent on Trx activity for the further reaction to form RSH), or it is dependent on Trx activity to achieve direct conversion back to RSH [231]. The propensity of cysteine residues to undergo oxidation is mainly influenced by three aspects: (1) thiol nucleophilicity; (2) the surrounding protein micro-environment; (3) the proximity of the target thiol to the ROS source [248]. Cysteine residues with low pK<sub>a</sub> content exist as thioanions under normal conditions; they are more susceptible to attack by various oxidants and are also susceptible to *S*-sulfhydrate [18].

#### 6.1.3. H<sub>2</sub>S and *S*-Nitrosylation

H<sub>2</sub>S is actively involved in the modulation of *S*-nitrosylation products. NO-mediated PTMs represent the primary mode of NO bioactivity, including tyrosine nitration [249], *S*-nitrosylation, and metal-nitrosylation [250], of which *S*-nitrosylation is the central part. *S*-nitrosylation is the specific linkage of NO to the protein thiol group [242] of a cysteine residue, to form SNO [251]. *S*-nitrosylation is not only closely related to the NO signaling molecules but is also actively involved in gene expression. Both NO and H<sub>2</sub>S lipophilic

molecules have some important properties in common: they can diffuse to the cell membrane, react with thiol groups, and mediate two important PTMs, namely, *S*-nitrosylation and *S*-sulfhydration. The thiol group of the cysteine residue associates H<sub>2</sub>S with NO. H<sub>2</sub>S influences NO production and its metabolites by affecting NO synthase, and NO alters the bioavailability of H<sub>2</sub>S by acting on H<sub>2</sub>S-producing enzymes [252–254]. *S*-nitrosylation is naturally affected as NO mediates the typical PTMs. H<sub>2</sub>S can react with the *S*-nitrosylated product SNO to form nitrosopersulfide (SSNO<sup>−</sup>), which is regarded as an intermediate in the crosstalk between NO and sulfide [255]. That is because SSNO<sup>−</sup>, spontaneously and multi-step reversibly, forms a variety of inorganic polysulphides, including HSNO (HNO and HSSNO, etc., are involved in the modification of cysteine residues). H<sub>2</sub>S reacts with SNO to form HSNO (the smallest of the RSNO), which acts as a NO carrier to diffuse freely between cells, entering the cell and promoting the nitrosylation of proteins [54]. H<sub>2</sub>S is reversible with HSNO to form HNO, and HNO mediates the synergistic effect of sulfide and NO on TRPA1 channel activation [256]. The pKa of HSSNO is approximately 5, implying the insensitivity of the molecule to a sulfhydryl-mediated reduction in cells (at a physiological pH) [257], which avoids reduction by effective enzyme systems in the organism (the NADPH-driven bio-reduction mechanisms of Trx and GR systems). SSNO-derived products can be decomposed to sulfane sulfur, so that SSNO<sup>−</sup> becomes a stable carrier for sulfane sulfur delivery [255].

#### 6.1.4. H<sub>2</sub>S and *S*-Glutathionylation

Similar to the above modifications (*S*-sulfhydration, *S*-sulfenylation, and *S*-nitrosylation), *S*-glutathionylation is also a PTMs for the reaction of a cysteine residue (R-SH) with a specific substrate; the difference is that the substrate is GSH [232].

The deglutathionylation of proteins can be catalyzed by glutaredoxin (Grx) and Trx. *S*-glutathionylation is a defense mechanism to avoid the excessive oxidation of cysteine residues and is mainly dependent on the status of GSH/GSSG. *S*-glutathionylation is, thus, a regulator of the redox state of cells, particularly in mitochondria [232]. Under oxidative stress, glutathionylation protects Trx from ROS-induced irreversible oxidation [258]. H<sub>2</sub>S can also convert R-SSG to R-SSH [232]. When CBS (the key enzyme for endogenous H<sub>2</sub>S synthesis) is modified by *S*-glutathionylation, it enhances the synthesis of H<sub>2</sub>S [259]. H<sub>2</sub>S is important for maintaining cellular GSH and glucose homeostasis in C<sub>2</sub>C<sub>12</sub> myotubes and enhances total protein *S*-glutathionylation [260]. The above study demonstrated that H<sub>2</sub>S interacts with *S*-glutathionylation. In addition, *S*-glutathionylation appears to mediate the inhibition induced by *S*-nitrosylation in the presence of GSNO in vitro, suggesting that *S*-sulfenylation is most likely part of the signaling involved in *S*-nitrosylation, but the exact manner of involvement is yet to be investigated at this time [261].

#### 6.2. H<sub>2</sub>S and Phosphorylation

H<sub>2</sub>S-mediated protein phosphorylation regulates plant physiological activity. Protein phosphorylation is the most widespread PTM in living organisms, mainly through the action of protein kinases and phosphatases on specific amino acid residues (typically, the hydroxyl groups of Ser, Thr, and Tyr), adding or removing one or more phosphate groups, thereby effectively altering the structure and activity of the substrate protein [262]. The phosphorylation modifications of proteins are closely associated with a variety of biological processes. In *Arabidopsis thaliana*, PSM and hinge multisite auto-phosphorylation are key to regulating protein degradation and Pr-to-Pfr transformation [263]. Phytochromes (PHY) and cryptochromes (CRY) are important photoreceptors for plant photomorphogenesis. PHY regulatory effects are strongly influenced by R/FR light-induced phosphorylation. The CRY regulatory effect is strongly influenced by blue light-induced phosphorylation. Red light (RL) exposure showed the transient activation of cysteine desulfurase activity, the key enzyme for endogenous H<sub>2</sub>S, whereas white light (WL) or blue light (BL) exposure inhibited the activity of this enzyme. L-DES is phosphorylated after exposure to RL or BL. Therefore, light can modulate the production of H<sub>2</sub>S. This process relies on the

phosphorylation modification of L-DES [264]. In the root system, the  $H^+$  gradient generated by PM  $H^+$ -ATPase is an important driver for maintaining ion homeostasis by the plasma membrane  $Na^+/H^+$  anti-porter (SOS1). Under salt stress,  $H_2S$  regulates PM  $H^+$ -ATPase gene expression and phosphorylation status [265]. CDPKs regulate cellular recognition and signal transduction through reversible protein phosphorylation and increase Cd tolerance in *Arabidopsis thaliana* by enhancing  $H_2S$  signaling [227]. The rapid recovery of the D1 protein facilitates the alleviation of photo-inhibition. Under drought stress,  $H_2S$  alleviates photo-inhibition by mediating the phosphorylation of the D1 protein and accelerating the D1 protein turnover [188]. *SnRK2.6* is predominantly expressed in the guard cells. SnRK2.6-mediated ABA-induced stomatal closure is an important stress response of plants in response to drought. Crosstalk exists between phosphorylation and S-sulfhydration. The S-sulfhydration of SnRK2.6 increased the phosphorylation level of S175 residues. The phosphorylation status of S267 affects the peroxisulfation of SnRK2.6 in vivo and in vitro. Phosphorylation promotes S-sulfhydration, which alters the structure and increases the activity of SnRK2.6, ultimately protecting the guard cells [266].

### 6.3. $H_2S$ and Ubiquitination (Ub)

Ub is one of the mechanisms of PTMs that is prevalent in living organisms; it adds ubiquitin to the substrate protein and thereby labels the target protein. Its primary biochemical function is to provide a marker for the subsequent selective degradation of the protein. The ubiquitin-proteasome system (UPS), of which Ub is the core component, is the main pathway for intracellular protein degradation and consists of Ub, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin-protein ligase (E3), the proteasome and its substrate (protein) [267].

$H_2S$  can be involved in regulating protein degradation in cells by modifying ubiquitinated and deubiquitinated components [40,268] (Figure 6). E1 uses the energy generated by ATP hydrolysis to create a thioester bond between the sulfhydryl group of its own catalytic site, cysteine, and the carboxyl group of ubiquitin. The activated ubiquitin is then transferred to the sulfhydryl group of E2. Finally, the transfer of ubiquitin to a specific substrate is carried out by E3. In the UPS, E3 confers specificity to protein degradation reactions. E3 ubiquitin ligases are divided into the homologous type and the E6-AP carboxyl terminus (HECT)-type, really interesting new gene (RING)-type, and RING-between-RING (RBR)-type [269]. In humans, exogenous  $H_2S$  modifies ubiquitin-specific peptidase 8 (USP8) via S-sulfhydration to promote the USP8-mediated deubiquitination of parkin (an RBR-type E3 ubiquitin ligase) for the effective elimination of dysfunctional mitochondria [268]. Similarly,  $H_2S$  regulates parkin activity and enhances its neuroprotective activity by incorporating bound sulfane sulfur into the cysteine residues [40].

### 6.4. $H_2S$ and Histone Acetylation

The covalent modification of acetyl groups (acetyl groups) from acetyl CoA to the  $\epsilon$ -amino group of the N-terminal lysine residue of the histone molecule is called histone acetylation [270].

The mutually antagonistic activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs) is an important way of regulating histone acetylation in plants. HATs catalyze the acetyl transfer of acetyl coenzyme A on histone lysine residues, to achieve acetylation [271]. Plant HATs are classified into four categories, including p300/CREB, TATA-binding protein-associated factors, the MYST family of proteins (MOZ, Ybf2/Sas3, Sas2, and Tip60), and the general control non-repressible 5 (Gcn5)-related N-acetyltransferases (GNATs) [272]. In contrast, HDACs remove the acetyl group from the histone tails and achieve deacetylation, leading to chromatin condensation and reduced gene expression activity [273]. Plant HDACs are divided into three categories, including reduced potassium dependency 3/histone deacetylase 1 (RDP3/HDA1), the silent information regulator 2 (SIR2), and histone deacetylase 2 (HD2) [270].



H<sub>2</sub>S can regulate cellular function by affecting the level of histone acetylation [274–276] (Figure 6). In animals, H<sub>2</sub>S can regulate cellular function by affecting the acetylation and deacetylation of histones. For example, H<sub>2</sub>S upregulates *HDAC3* expression, inhibits histone acetylation levels, and thereby reduces transcription of pro-inflammatory factors (IL6 and TNF- $\alpha$ ) [274]. H<sub>2</sub>S inhibits *HDAC6* expression, suppresses endothelial dysfunction, and prevents the development of hypertension [275,276]. Sirtuin-1 (SIRT1) is a histone deacetylase. Endogenous H<sub>2</sub>S directly sulfates SIRT1, enhancing the binding of SIRT1 to zinc ions, which then promotes its deacetylation activity and enhances the stability of SIRT1 [277]. Acetyl coenzyme a is important in the performance of the physiological role of acetylases [278,279]. Exogenous H<sub>2</sub>S inhibits the accumulation of acetyl coenzyme a, implying that H<sub>2</sub>S may also be involved in protein acetylation via transcription [280].

### 6.5. H<sub>2</sub>S and Methylation

Methylation is an important form of chromatin remodeling, including DNA and histone methylation. DNA methylation is one of the most important pathways of epigenetic modification. The DNA methylation transferases (DNMTs)-dependent methyl group transfer is the main way in which methylation occurs. Based on the characteristics of their catalytic structural domains, DNMTs in plants are divided into three families, including methyltransferase (MET), chromomethylase (CMT), and domain-rearranged methyltransferases (DRM) [13]. Methylation generally occurs when some cytosine bases are methylated at the 5' position to become 5-methyl-cytosine (5mC) [270]. There are three types of methylation sites: "CG", "CHG", and "CHH" C-base (H for A, C, or T), depending on the sequence of the methylation site. CG methylation relies on DNA methyltransferase 1 (MET1); CHG methylation relies on chromomethylase 3 (CMT3) and CMT2; CHH methylation relies on structural domain rearranged methyltransferase 2 (DR methyltransferase 2 (DRM2), CMT2 and CMT3 [270]. There are two regulatory pathways for DNA methylation levels: the family of DNMTs favors methylation levels. DNMT-3a and DNMT-3b are used for ab initio methylation, while DNMT-1 is used to maintain the methylation that is present [281]. Conversely, ten–eleven translocation (Tet) enzymes facilitate the removal of 5-methylcytosine [281]. Similarly, demethylases play similar roles, including DEMETER (DME) and the Repressor of Silencing 1 (ROS1) [13]. DNA, which is methylation-dependent on the activity of DNMTs, was based on S-adenosyl-L-methionine (SAM) as the methyl donor. Similarly, histone methylation is also dependent on the activities of the histone methyltransferase (HMT) family, mainly including histone lysine methyltransferases (HKMTs) and protein arginine methyltransferases (PRMTs), with SAM as the methyl donor [270].

H<sub>2</sub>S is closely associated with methylation phenomena [13,282–284] (Figure 6). Osmotic stress induces the production of endogenous H<sub>2</sub>S in *Setaria italica* L., accompanied by the upregulation of DNMTs activity, causing the expression of drought-resistant TFs (*AREB1*, *DREB2A*, *ZIP44*, *NAC5* expression) and ultimately increasing the resistance to stress [13]. Pretreatment with 2  $\mu\text{mol L}^{-1}$  NaHS significantly inhibited ETH release and pectin synthesis, increased pectin methylation, and reduced the Al accumulation in the cell wall in rice [282]. Methylation at protein arginine methyltransferase 5 (PRMT5) in *Arabidopsis* increased the enzymatic activity of AtLCD, thereby enhancing the endogenous H<sub>2</sub>S signaling and improving Cd<sup>2+</sup> tolerance [283]. These findings suggest a potential link between H<sub>2</sub>S and DNA methylation.

## 7. Conclusions and Prospects

The pathways for the synthesis and metabolism of biologically endogenous H<sub>2</sub>S are summarized in Section 2. At present, H<sub>2</sub>S generation by the DAO/3-MST pathway has only been found in animals, and it is worthy of further study to establish whether a similar pathway exists in plants.

With the function of H<sub>2</sub>S in plants gradually being revealed, its donor has been used extensively to reveal a variety of roles in the plant. H<sub>2</sub>S studies are all based on pharmacological experiments that are carried out through the use of combinations of H<sub>2</sub>S donors,

inhibitors, and scavengers. It is important to select the right H<sub>2</sub>S donor, depending on the purpose of the study. In this paper, the selection of H<sub>2</sub>S donors and inhibitors (scavengers) was summarized. The classical donors of Na<sub>2</sub>S, NaHS, GYY4137, thioglycine, and TV are more widely used in plant studies. Research at the subcellular level is inevitable in the future, but it is clear that the substances mentioned above are not appropriate. AP39, AP123, and RT01 represent H<sub>2</sub>S donors that specifically target mitochondria and are more suited to the relevant cellular-level studies. If H<sub>2</sub>S is to be used in practical production, the donor needs to be water-soluble, stable in action, highly controllable, and non-toxic. Conventional H<sub>2</sub>S donors are equally unsuitable. The new environmentally friendly hydrogen sulfide-releasing agents, represented by dialkyldithiophosphates, can be a good solution. However, these substances are not very suitable for facility cultivation (hydroponics). It is evident that the research and development of new H<sub>2</sub>S donors still need to be enhanced. The concentration of endogenous H<sub>2</sub>S is very important for the physiological regulation of plants; finding the means to accurately determine the amount of H<sub>2</sub>S in plants is a problem that needs to be overcome in the relevant studies. In terms of the functional impact of H<sub>2</sub>S on proteins, the basic process generally follows the biotin conversion method. The N-ethyl maleimide method is the most commercially successful method. Unlike proteomic studies, organelle studies focus more on in vivo-specific localization. The study of fluorescent probes for H<sub>2</sub>S provides technical support for research in this direction. The optimization of measurement methods and techniques has a positive effect on the research.

H<sub>2</sub>S plays an important role as a gaseous transmitter throughout the life cycle of plants. The persistence of plants (sessile organisms) in situ leads to stress tolerance becoming necessary for plants to face adversity. Numerous reports have shown that H<sub>2</sub>S has a positive impact on improving plant stress tolerance. However, the topic deserves further study. For example, H<sub>2</sub>S crosstalk exists with other signaling molecules (e.g., GTs), plant hormones, and PGRs. Exactly how they regulate one another and also their functions remain to be determined. In particular, the precise determination of the location of H<sub>2</sub>S in the signaling pathway is a crying need, and the direction needs to be verified in a large number of future experiments. Numerous experiments have now confirmed that H<sub>2</sub>S has a positive effect on the improvement of plant stress resistance, and the main mode of its regulatory action is PTMs, but most of the studies on this modifying mode in plants are related to phenotypic traits that are visible to the naked eye (e.g., wilting or senescence). The mechanism of the cascade crosstalk relationship between this modification mode and other signaling molecules (e.g., NO and CO) at the cellular and subcellular levels needs to be further clarified to provide a reference for elucidating the mechanism of action studies in the future.

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