Interplay between hydrogen sulfide and other signaling molecules in the regulation of guard cell signaling and abiotic/biotic stress response

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ABSTRACT

Stomatal aperture controls the balance between transpirational water loss and photosynthetic carbon dioxide (CO₂) uptake. Stomata are surrounded by pairs of guard cells that sense and transduce environmental or stress signals to induce diverse endogenous responses for adaptation to environmental changes. In a recent decade, hydrogen sulfide (H₂S) has been recognized as a signaling molecule that regulates stomatal movement. In this review, we summarize recent progress in research on the regulatory role of H₂S in stomatal movement, including the dynamic regulation of phytohormones, ion homeostasis, and cell structural components. We focus especially on the cross talk among H₂S, nitric oxide (NO), and hydrogen peroxide (H₂O₂) in guard cells, as well as on H₂S-mediated post-translational protein modification (cysteine thiol persulfidation). Finally, we summarize the mechanisms by which H₂S interacts with other signaling molecules in plants under abiotic or biotic stress. Based on evidence and clues from existing research, we propose some issues that need to be addressed in the future.

Keywords: hydrogen sulfide, stomatal guard cell, phytohormone, persulfidation, abiotic/biotic stress, signaling molecule

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INTRODUCTION

The stoma, a unique motile apparatus in higher terrestrial plants, is situated in the epidermis and surrounded by a pair of guard cells. The opening and closing of stomata regulate gas and water exchange between plants and the environment, thereby affecting plant growth and development (Schroeder et al., 2001). At the same time, stomata also play important roles in sensing biotic and abiotic environmental stresses and transmitting signals to the plant (Daszkowska-Golec and Szarejko, 2013; Ou et al., 2014; Zheng et al., 2018; Kollist et al., 2019). In this process, stomatal movement is closely associated with a series of complex signal perception, transduction, and regulation processes in guard cells. Many previous studies have shown that a variety of plant hormones, osmolytes, signal receptors, protein kinases, reactive oxygen species (ROS), Ca²⁺ signals, and ion channels are involved in the precise regulation of stomatal movement (Blatt and Grabov, 1997; MacRorie, 1997; Pandey et al., 2007; Kim et al., 2010; Hua et al., 2012; Roelfsema et al., 2012; Lee et al., 2016; Singh et al., 2017; Sussmich et al., 2019; Li et al., 2020). In addition, several gaseous signaling molecules also participate in the regulation of stomatal movement (García-Mata and Lamattina, 2001; She and Song, 2008; Song et al., 2011).

Hydrogen sulfide (H₂S) is a noxious gas and has been considered toxic for over 300 years since its discovery (Lefer, 2019). Excessive inhalation of H₂S not only causes nerve palsy and health damage in humans and mammals, but also disrupts plant cell structure and inhibits plant growth and development (De Kok et al., 1989). In recent decades, H₂S has been recognized as a new endogenous gaseous signaling molecule, like nitric oxide (NO) and carbon monoxide (CO), that participates in signal regulation and transduction in organisms (Lisjak et al., 2013; Wang, 2012). H₂S-producing enzymes have been identified in both mammals and plants, and several reviews have provided a comprehensive introduction to these enzymes ( Cuevasanta et al., 2017; Filipovic et al., 2018; Pandey and Gautam, 2020; Papenbrock et al., 2007; Singh et al., 2020; Wang, 2012). The enzymes that produce H₂S in plants include L-cysteine desulphhydrase 1 (DES1, EC 4.4.1.1), L-cysteine desulphhydrolase (LCD, EC 4.4.1.28), D-cysteine desulphhydrase (DCD, EC 4.4.1.15), and cyanoalanine synthase (CAS, EC
of cysteine, sulfide is also oxidized to slightly toxic sulfite. Similarly, cyanoalanine can produce H$_2$S in a reaction catalyzed by CAS. In some special cases, plants can assimilate sulfur-containing gas molecules, such as H$_2$S, SO$_2$, and COS, into the sulfur metabolic pathway through the stomata. Solid arrow, existing pathway; dotted arrow, predicted pathway; APR, APS reductase; APS, adenosine 5'-phosphosulfate; ATPS, ATP sulfurylase; CAS, cyanoalanine synthase; CS26, S-sulfocysteine synthase; Cys, cysteine; DES1, L-cysteine desulhydrase 1; OAS, O-acetylserine; OAS-A1/B/C, O-acetylserine (thiol) lyase A1/B/C; PLP, pyridoxal 5'-phosphate; SAT, serine acetyltransferase; Ser, serine; SiR, sulfite reductase; STR, sulfur transferase; SDO, sulfur dioxygenase; Sulr, sulfite transporter.

4.4.1.9) (Gotor et al., 2019; Liu et al., 2021). Apart from these H$_2$S-producing enzymes, the main source of H$_2$S in plants is plastid sulfur metabolism, which involves the three-step enzymatic reduction of sulfate. ATP sulfurylase (ATPS, EC 2.7.7.4) catalyzes the adenylation of sulfate to form adenosine 5’-phosphosulfate (APS), which is then reduced to sulfite by APS reductase (APR, EC 1.8.99.2) and further reduced to sulfide by sulfite reductase (SiR, EC 1.8.7.1) (Kopriva, 2006; Takahashi et al., 2011) (Figure 1). Sulfide acts as a signaling molecule in cells at low levels but is toxic at high levels. Another enzyme, O-acetylserine (thiol)lyase (OAS-TL) (OAS-A1/B/C), with different SATs to provide OAS is produced by serine acetyltransferase (SAT, EC 2.3.1.30). SAT and OAS-TL can interact and form a heterooligomeric cysteine synthase complex that regulates cysteine synthesis and probably acts as a sulfide sensor (Wirtz and Hell, 2006; Wirtz et al., 2010, 2012; Hell and Wirtz, 2011). In mitochondria, sulfide inhibits the activity of cytochrome c oxidase, which is vital for the cellular respiratory electron transport chain. Two enzymes, OAS-TL C and the sulfur dioxygenase ethylmalonic encephalopathy protein 1, contribute to sulfide detoxification in the mitochondria of Arabidopsis (Birke et al., 2012, 2015b; Krübel et al., 2014) (Figure 1).

In mammal research, physiological concentrations of H$_2$S can not only improve cardiovascular capacity, liver function, and nerve cell activity, but also regulate the activities of various proteases and ion channels through direct protein post-translational modification (PTM), which will be introduced in a later section (Wang, 2012; Filipovic et al., 2018; Kimura, 2019). In plants, correlation studies have revealed that H$_2$S is involved in seed germination (Zhang et al., 2008; Baudouin et al., 2016; Chen et al., 2019a), root hair growth (Li et al., 2018), flowering (Zhang et al., 2011; Ma et al., 2021), senescence (Li et al., 2014b; Liu et al., 2017; Jin et al., 2018), fruit ripening (Ge et al., 2017; Mukherjee, 2019; Hu et al., 2020), and other growth and developmental processes (Zhang et al., 2020b). It also participates in responses to abiotic and biotic stresses, including salt (Li et al., 2014a), drought (Jin et al., 2011, 2018; Shen et al., 2013), hypoxia (Cheng et al., 2013; Peng et al., 2016), toxic metals (Fang et al., 2017; He et al., 2018), and pathogen infection (Bloom et al., 2012a; Tian et al., 2019).

In the past decade, the physiological and molecular mechanisms by which H$_2$S regulates the stomatal movement of plants have gradually been revealed (Pantaleno et al., 2021). This review summarizes recent progress in research on the interplay between H$_2$S and other signaling molecules in the regulation of stomatal guard cell signaling and plant abiotic and biotic stress responses.

**EXOGENOUS H$_2$S INDUCES STOMATAL MOVEMENT**

Garcia-Mata and Lamattina (2010) reported that exogenous application of the H$_2$S donors NaHS and GYY4137 (morpholin-4-ium-4-methoxyphenyl (morpholin) phosphinodithioate) in aqueous solutions promoted stomatal closure, whereas application of the H$_2$S scavenger hypotaurine inhibited it, suggesting a positive role for exogenous H$_2$S in the regulation of stomatal
Regulation of guard cell signaling and stress response

closure. By contrast, Li et al. (2010) found that both NaHS and GYY4137 caused stomatal opening in the light and inhibited stomatal closure in the dark. Another interesting study revealed that GYY4137 could induce stomatal closure, which reached a peak after 150 min at 1 μM GYY4137 or after 90 min at 10 and 100 μM GYY4137. Stomata then gradually opened with further GYY4137 treatment (Honda et al., 2015). These inconsistent findings may be ascribed to different plant growth conditions, experimental methods, concentrations of exogenous H2S donors, and treatment durations. It should be noted that more recent studies have reported the induction of stomatal closure by H2S in Arabidopsis (Jin et al., 2013; Shen et al., 2020a; Chen et al., 2020b). These studies also revealed that other hormones and signaling molecules, including abscisic acid (ABA), NO, hydrogen peroxide (H2O2), and 8-mercapto-cGMP, participate in H2S-induced stomatal movement. However, H2S exposure does not affect stomatal movement in the monocot plant maize (Auszma et al., 2020). We will discuss the cross talk between H2S and other signaling molecules in the following sections.

H2S INTERACTS WITH PHYTOHORMONES TO REGULATE STOMATAL MOVEMENT

H2S plays an important and positive physiological role in the regulation of plant growth and adaptation to environmental stress. The induction of stomatal closure by H2S is associated with various phytohormones, including ABA (Garcia-Mata and Lamattina, 2010; Jin et al., 2013; Scuffi et al., 2014; Hsu et al., 2021), ethylene (ET) (Hou et al., 2013), salicylic acid (SA), and jasmonic acid (JA) (Hou et al., 2011; Li et al., 2015; Deng et al., 2020).

H2S interacts with ABA signaling to regulate stomatal closure

ABA plays pivotal roles in seed maturity and dormancy, stomatal closure, and plant growth (Chen et al., 2020a). It promotes stomatal closure and inhibits stomatal opening, thereby enabling plants to retain water under drought stress (Hsu et al., 2021; Kim et al., 2010). Garcia-Mata and Lamattina’s prior work has revealed the participation of H2S in ABA-dependent induction of stomatal closure: scavenging of H2S or inhibition of the enzyme responsible for endogenous H2S synthesis partially blocked ABA-dependent stomatal closure (Garcia-Mata and Lamattina, 2010). In Arabidopsis, H2S is produced enzymatically through the activity of cytosolic DES1, which has been identified as being mainly responsible for the degradation of cysteine to generate H2S in the cytosol (Alvarez et al., 2010, 2012). Application of exogenous ABA can enhance the activity and expression of the DES1 enzyme, thereby increasing the content of endogenous H2S (Chen et al., 2020b; Sun et al., 2016; Zhang et al., 2020a). The transfer DNA insertion mutants lcd and des1 showed greater stomatal aperture, insensitivity to ABA-induced stomatal closure, and lower tolerance to drought stress relative to wild-type plants (Jin et al., 2013; Scuffi et al., 2014). In addition, a recent study revealed that LONG HYPOCOTYL 1 (HY1), a member of the heme oxygenase family, is involved in ABA- and DES1/H2S-induced stomatal movement. Its mutant hy1 is hypersensitive to ABA stimulation and displays intensified DES1 expression and H2S production (Zhang et al., 2020a). To study ABA signaling in Arabidopsis, the ABA biosynthesis enzyme mutant aba3, the clade-A protein phosphatase 2C mutant abi1, and the ABA receptor mutants pyrabactin-resistant 1 (pyr1)/pyrabactin-like 1 (pyl1)/pyl2/pyl4 were used for physiological experiments to measure stomatal conductance under exogenous H2S treatment. Stomatal closure was responsive to exogenous H2S in pyl1/pyl2/pyl4 but not in abi1-1, suggesting an interaction of H2S with ABA signaling (Jin et al., 2013; Scuffi et al., 2014). A recent study has demonstrated that in situ synthesis of ABA and DES1 expression in guard cells synergistically controls drought-induced stomatal closure (Zhang et al., 2021a). Another study showed that H2S positively regulates ABA signaling through the persulfidation of OPEN STOMATA 1 (OST1)/SNF1-RELATED PROTEIN KINASE 2.6 (SnRK2.6) in guard cells. Persulfidation, a type of PTM, and its related mechanism of action will be introduced in a later section. Almost simultaneously, Shen et al. (2020a) reported that H2S promotes ABA signaling by the PTM of persulfidation. The in vivo synthesis of ABA is induced upon the perception of drought stress, promoting the self-activation of DES1 via persulfidation and the amplification of guard cell H2S signals. Subsequently, the induced DES1/H2S further activates RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD) through an analogous modification, which in turn leads to stomatal closure (Shen et al., 2020a). A recent study showed that ABSICIC ACID INSENSITIVE 4 (ABI4) acts downstream of DES1 and H2S in guard cells; furthermore, MITOGEN-ACTIVATED PROTEIN KINASE KINASE 18 (MAPKKK18) acts downstream of the DES1–ABI4 module in ABA-triggered stomatal closure (Zhou et al., 2021). All the above results indicate that H2S acts downstream of ABA signaling to regulate stomatal closure. In addition, H2S also has the potential to promote ABA biosynthesis (Batool et al., 2018). As a type of sulfide, H2S has been shown to promote the synthesis of cysteine, which serves as a substrate for molybdenum cofactor sulfurase ABA3 (EC 2.8.1.9) in the activation of abscisic aldehyde oxidase 3 (AAO3, EC 1.2.3.14) (Bittner et al., 2001). Cysteine levels are directly related to ABA synthesis and affect AAO activity in vivo (Cao et al., 2014). Elevated levels of cysteine could also increase the transcript level of 9-cis-epoxy-carotenoid dioxygenase 3 (NCED3; EC 1.13.11.51), which promotes the synthesis of ABA (Batool et al., 2018). These findings suggest that H2S probably has the ability to boost ABA synthesis in stomatal closure (Figure 2). In a recent study, H2S failed to induce stomatal closure in a cysteine-biosynthesis-depleted mutant, which disrupted ABA biosynthesis (Rajab et al., 2019). The study also revealed that sulfate and sulfide are incorporated into cysteine to induce stomatal closure, and OST1, ABI1, and ROS signals all participate in sulfate-induced stomatal closure (Rajab et al., 2019).

Under drought stress, sulfur metabolism tends to differ between roots and leaves (Ahmad et al., 2016). Sulfate transported from roots to leaves in the xylem interacts with ABA to regulate transpiration rate and stomatal movement at an early stage of drought stress (Ernst et al., 2010). A further study showed that sulfate in the xylem sap promotes ABA synthesis in the guard cells and activates QUAC1/ALMT12 to trigger stomatal closure (Malcheska et al., 2017). Another recent study showed that most sulfate transporter subfamily 3 members, which are responsible for the uptake of sulfate in chloroplasts, have positive effects on ABA and cysteine synthesis and on...
sulfate-induced stomatal closure (Chen et al., 2019b). Sulfate can be reduced to sulfide in the plastids, and sulfide (H₂S) has a clearer relationship with ABA in triggering stomatal closure.

**H₂S interacts with ET-, JA-, and SA-induced stomatal closure**

In addition to ABA, ET has also been reported to have physiological functions in stomatal movement (Acharya and Assmann, 2009; Wilkinson and Davies, 2010) and was found to have an interesting “frenemy” relationship with H₂S. H₂S plays an antagonistic role with ET during fruit ripening, preventing ET synthesis as well as ET-induced fruit ripening and senescence processes (Hu et al., 2014; Jia et al., 2018). However, there seems to be a more harmonious relationship between H₂S and ET in the regulation of stomatal movement and seed germination. According to Liu et al. (2011) and Hou et al. (2013), treatment with 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ET, led to increases in H₂S production and D/L-cysteine desulphydrase (D/L-CDes) activity in Arabidopsis leaves. Promoter analysis of AtDCD1 showed that it contains the ET-responsive cis element ERE and the abiotic stress responsive cis elements MBS, LTR, and ABRE (Hou et al., 2016a). Analysis of GUS activity in pAtDCD1::GUS lines revealed that the section of the AtDCD1 promoter from –697 to –408 bp was responsive to ET (Hou et al., 2016a). Several D/L-CDes inhibitors, such as aminooxy acetic acid, NH₂OH, and C₅H₅NO₃ + NH₃, significantly inhibit ET-induced stomatal closure in Arabidopsis. These results suggest that D/L-CDes-generated H₂S is involved in the regulation of ET-induced stomatal closure in Arabidopsis (Figure 3). The same physiological mechanism also appears to operate in ET-induced stomatal closure of Vicia faba L. (Liu et al., 2012).

SA is involved in a variety of physiological functions, especially the induction of stomatal closure to improve plant disease resistance. The exogenous application of SA could significantly increase NO and H₂O₂ levels in the cytoplasm of V. faba and Commelina communis guard cells (Liu et al., 2003; He et al., 2007). Recent research has revealed that SA induces the expression of the ACC synthases ACS6 and ASC11 and thus enhances ET synthesis in Arabidopsis guard cells (Sun and He, 2017). Another study revealed a role for H₂S in the regulation of abiotic stress response downstream of SA (Li et al., 2015). All these results suggest that H₂S is probably involved in SA-induced stomatal closure; however, there is still a lack of solid evidence to support this hypothesis.

There are also some reports of the involvement of H₂S in the regulation of stomatal movement by JA. One report suggested that H₂S may function downstream of H₂O₂ in JA-induced stomatal closure in V. faba (Hou et al., 2011) (Figure 3). In addition, recent research has revealed that JA negatively regulates stomatal development by promoting LCD expression and H₂S biosynthesis (Deng et al., 2020). H₂S accumulation inhibits the initiation of stomatal formation, suggesting that H₂S has dual functions in the control of stomatal movement and development.

**H₂S, NO, AND H₂O₂ CROSS TALK IN THE REGULATION OF STOMATAL MOVEMENT**

Micromolar levels of H₂S can play a signaling role in abiotic stress acclimation, growth and development, and specific plant physiological processes through interaction with ROS and other signaling compounds such as NO. Abundant evidence suggests that NO is a key messenger in plants and exhibits cross talk with H₂O₂ in guard cell signaling networks (Jannat et al., 2020; Lv et al., 2018; Shi et al., 2015b; Wang et al., 2020). Recent studies have shed light on the close link between H₂S and NO signaling. García-Mata and Lamattina (2001) used a
pharmacological approach to demonstrate that NO donors induce stomatal closure in both monocots and dicots. Nine years later, the same lab reported that H2S donors induce stomatal closure in the separated epidermis of different plant species such as Arabidopsis, V. faba, and Impatiens walleriana, indicating that H2S has effects similar to those of NO and H2O2 in the stimulation of stomatal closure (García-Mata and Lamattina, 2010). Scuffi et al. (2014) demonstrated that both H2S and NO are involved in ABA-induced stomatal closure. H2S promotes NO generation, and NO acts downstream of H2S. The increase in NO (and total S-nitrosothiols) may be due to the enhancement of nitrate reductase and glyoxalase I and II activities and the inhibition of S-nitrosogluthathione reductase activity by H2S (Che et al., 2015; Cheng et al., 2018; Janicka et al., 2018; Ziegas et al., 2015) (Figure 4). Another study showed that the H2S donor GYY4137 promotes NO production in guard cells and also induces NO-mediated 8-nitro-cGMP/8-mercaptopo-cGMP synthesis, which triggers stomatal closure (Honda et al., 2015). However, Ljšjak et al. (2010) proposed that H2S donors decrease the NO level, enhance stomatal opening under light, and inhibit stomatal closure in the dark. In addition, a number of reports have also demonstrated that NO can increase the H2S level by enhancing the activity of H2S-producing enzymes in the process of promoting plant resistance to abiotic stresses (da-Silva et al., 2018; Ye et al., 2020). Therefore, H2S and NO signaling pathways in guard cells may be more complex than expected (Corpas et al., 2019). Moreover, the potential direct chemical reactions between H2S and NO are also a source of valuable research ideas. HSNO, which is produced by oxidized NO and H2S accumulated on the cell membrane, can enhance the plasma membrane permeability of NO and H2S (Cuevasanta et al., 2012; Lancaster, 2017). Recent research revealed that pre-treatment with HSNO donors (NOSH and NOSH-aspirin) could effectively alleviate water deficit in Medicago sativa L. under drought stress (Antoniou et al., 2020).

In addition to exhibiting cross talk with NO, H2S affects stomatal movement by regulating the dynamic balance of ROS, especially H2O2 and the superoxide anion, in guard cells. The production of ROS is a common response to most stimuli, and ROS serve as signaling molecules that modify target proteins. Numerous studies have shown that the application of exogenous H2S at submicromolar concentrations can reduce ROS by direct redox reaction (Koppennol and Bounds, 2017; Filipovic et al., 2018) and by enhancing the activity of ROS scavenging enzymes and the transcript levels of their corresponding genes (Yao et al., 2018; Ye et al., 2020). These enzymes include superoxide dismutase, catalase, ascorbate peroxidase (APX), peroxidase (POD), glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase, and guaiacol peroxidase. Their activities are triggered by H2S in different tissues and organs of various plants (Shen et al., 2013; Wei et al., 2017; Shan et al., 2018; Li et al., 2019b; Qi et al., 2019), thereby reducing the overaccumulation of ROS during lateral root formation, leaf senescence, fruit ripening, and response to abiotic stresses such as toxic metals, salinity, drought, and hypoxia (Li et al., 2014b; Hu et al., 2014; Ge et al., 2017; Kharbech et al., 2017; Liu et al., 2017; Yao et al., 2018). However, the same mechanisms may not necessarily allow H2S to regulate ROS in guard cells and further mediate stomatal movement. Scuffi et al. (2018) combined pharmacological and genetic approaches to reveal the involvement of H2S in stomatal closure and document the interplay between H2S and the NADPH oxidase isoforms RBOHD and RBOHF. Guard-cell-specific microarray analysis showed that only 2 (RBOHD and RBOHF) of 10 isoforms were specifically expressed in Arabidopsis guard cells (Yang et al., 2008). H2S stimulates RBOHD- and RBOHF-dependent H2O2 production in the guard cells (Scuffi et al., 2018). H2O2 scavengers and synthesis inhibitors suppressed the induced H2S level, D/L-CDes activity, and stomatal closure, implying that there are dynamic upstream or downstream relationships between H2S and ROS (Hou et al., 2016b). Recent research further demonstrated that H2S directly activates RBOH by PTM to produce H2O2; however, with increasing levels of H2O2, the activity of DES1 was inhibited, and the H2S level in guard cells was reduced (Shen et al., 2020a). These findings suggested that H2S acts as an upstream regulator to mediate the H2O2 level and that an increase in H2O2 level reduces the H2S level in a feedback loop (Shen et al., 2020a). These results indicate that H2S and H2O2 can regulate each other.

Figure 3. H2S is involved in ET-, JA-, and SA-induced stomatal closure.

ET induces H2S production and stomatal closure by increasing D/L-CDes transcript level and activity. SA can promote the production of ET by enhancing the activity of ACS and enhancing the signal of H2O2 by promoting the production of phosphatidic acid (PA), both of which indirectly cause an increase in H2S. Similarly, JA affects the level of H2S by regulating H2O2 content. By contrast, H2S increases the production of ET by enhancing the activity of ACS and ACO via PTM.
to maintain a suitable balance in guard cells (Figure 4), and the concentration ratio of the two signaling molecules largely determines the mode of stomatal movement.

PERSULFIDATION IS A UNIQUE POST-TRANSLATIONAL MODIFICATION BY WHICH H₂S INFLUENCES STOMATAL MOVEMENT

Based on its chemical properties, H₂S is a nucleophilic molecule and can react with oxygen, H₂O₂, or peroxynitrite, suggesting that it exerts its functions by reducing cellular oxidative stress (Fukuto et al., 2012; Kabil and Banerjee, 2010). In addition, H₂S is known to participate in the PTM of protein cysteine residues to form persulfide (R-SSH) groups (Mustafa et al., 2009; Paul and Snyder, 2012) in a process called persulfidation (previously known as S-sulfhydration). Persulfidation is a distinctive protein modification initiated by H₂S; it is similar to S-nitrosylation, in which cysteine residues are modified by NO to form S-nitroso-Cys residues (Aroca et al., 2015; Filipovic et al., 2018). In Arabidopsis, Aroca et al. (2015) employed an artificial biotin switch method, first described for the protein analysis of mouse liver lysates (Mustafa et al., 2009), to screen H₂S-triggered proteins that could be modified by S-nitrosylation. Subsequently, the same team updated a comparative and quantitative proteomic analysis approach for the detection of endogenous persulfidated proteins in wild-type Arabidopsis and des1 mutant leaves using the tag-switch method (Aroca et al., 2017, 2018). More than 5% of the entire Arabidopsis proteome, including approximately 2330 potential target proteins, may undergo persulfidation under baseline conditions (Aroca et al., 2017). Bioinformatic analysis revealed that persulfidated thiols participate in a wide range of biological functions, regulating critical physiological processes such as energy metabolism, abiotic or biotic stress response, plant growth and development, and RNA translation (Aroca et al., 2017). Among the screened kinase targets were SnRK2.2 and SnRK2.6/OST1, which play essential roles in the ABA-dependent regulation of stomatal movement (Aroca et al., 2017). A recent study showed that H₂S persulfidates SnRK2.6/OST1 to respond to ABA signaling and promote ABA-induced stomatal closure (Chen et al., 2020b). ABA induces the production of H₂O₂ indirectly by increasing ATP production and secretion, PLD synthesis, phospholipid acid signaling, Ca²⁺ signaling, and NO synthesis. Notably, H₂S, NO, and H₂O₂ can promote one another and function together in the regulation of stomatal closure. Arrow, induction; flat arrow, inhibition; solid arrow, existing pathway; dotted arrow, predicted pathway; orange arrow, persulfidation; red arrow and red ball with “P”, phosphorylation; “-SH” in dotted line, predicted persulfidation; CysXXX, existing persulfidation/S-nitrosylation (orange/blue). ABF, ABRE binding factor; ACA, autoinhibited Ca²⁺-ATPase; CAX, cation calcium exchanger; CBL, calcineurin B-like protein; CDPK, Ca²⁺-dependent protein kinase; CIPK, CBL-interacting protein kinase; CPP, cell wall protein precursor; eATP, extracellular ATP; EGase, endo-β-1,4-glucanase; GLOI/II, glyoxalase I/II; GORK, gated outwardly rectifying K⁺ channel; GSNOR, S-nitroso glutathione reductase; HSNO, nitro-sosulfane or (hydridosulfanido)oxidonitrogen; KAT1, potassium channel in Arabidopsis thaliana 1; MRP4/5, multidrug-resistance-associated proteins 4/5; NR, nitrate reductase; PC, phosphatidylcholine; PG, polygalacturonase; PIP2:1, plasma membrane intrinsic protein 2:1; PLD, phospholipase D; RBOHD, respiratory burst oxidase homolog D; SLAC1, slow-type anion channel-associated 1; OST1, open stomata 1; TCH4, xyloglucan endotransglycosylase; TPC1, two-pore-segment channel 1.

Figure 4. H₂S interacts with other signaling molecules in the guard cell signaling network.

Exogenous H₂S can pass through the phospholipid membrane, and the synthesis of HSNO with NO may enhance membrane permeability. In the cytoplasm, DES1 uses cysteine as a substrate to produce H₂S, which amplifies the signal by inducing DES1 persulfidation and initiates downstream signaling through persulfidation of SnRK2.6, RBOHD, and ABI4. H₂S directly or indirectly enhances K⁺ out and Cl⁻ out transport and inhibits K⁺ in transport on the plasma membrane, and the opposite regulation of ion currents may exist on the vacuolar membrane. In addition, H₂S promotes the production of H₂O₂ indirectly by increasing ATP production and secretion, PLD synthesis, phospholipid acid signaling, Ca²⁺ signaling, and NO synthesis. Notably, H₂S, NO, and H₂O₂ can promote one another and function together in the regulation of stomatal closure. Arrow, induction; flat arrow, inhibition; solid arrow, existing pathway; dotted arrow, predicted pathway; orange arrow, persulfidation; red arrow and red ball with “P”, phosphorylation; “-SH” in dotted line, predicted persulfidation; CysXXX, existing persulfidation/S-nitrosylation (orange/blue). ABF, ABRE binding factor; ACA, autoinhibited Ca²⁺-ATPase; CAX, cation calcium exchanger; CBL, calcineurin B-like protein; CDPK, Ca²⁺-dependent protein kinase; CIPK, CBL-interacting protein kinase; CPP, cell wall protein precursor; eATP, extracellular ATP; EGase, endo-β-1,4-glucanase; GLOI/II, glyoxalase I/II; GORK, gated outwardly rectifying K⁺ channel; GSNOR, S-nitroso glutathione reductase; HSNO, nitro-sosulfane or (hydridosulfanido)oxidonitrogen; KAT1, potassium channel in Arabidopsis thaliana 1; MRP4/5, multidrug-resistance-associated proteins 4/5; NR, nitrate reductase; PC, phosphatidylcholine; PG, polygalacturonase; PIP2:1, plasma membrane intrinsic protein 2:1; PLD, phospholipase D; RBOHD, respiratory burst oxidase homolog D; SLAC1, slow-type anion channel-associated 1; OST1, open stomata 1; TCH4, xyloglucan endotransglycosylase; TPC1, two-pore-segment channel 1.
Regulation of guard cell signaling and stress response

can attack during S-nitrosylation, which inhibits SnRK2.6/OST1 and negatively regulates ABA signaling in guard cells (Wang et al., 2015b) (Figure 4). Zhou et al. (2020) reviewed the specific regulatory mechanism of persulfidation in guard cells, analyzed the existing plant persulfidation-modified proteomic data (Aroca et al., 2015, 2017, 2018), and predicted the potential persulfidated target proteins in the guard cell ABA signaling pathway. Many important ABA signaling components, such as the ABA receptor pyrabactin resistance 1-like, calcium-dependent protein kinases, SnRK2s, protein phosphatase 2A subunits, and probable protein phosphatase 2Cs (Zhou et al., 2020), are potential targets of PTM and deserve further investigation.

Here, it is important to mention the intricate relationships between S-nitrosylation, persulfidation, and even oxidation such as S-sulfenylation (thiol group modification by ROS). For proteins, activable cysteine residues are hotspots for the aforementioned multiple redox modifications. Once cysteine residues are exposed to large amounts of ROS, cysteine thiols can be modified to sulfenic acid (RSHO) via oxidation. RSHO seems to be an active state of cysteine residues; it can be further oxidized to form irreversible sulfenic (RSO₂H) and sulfonic acids (RSO₃H) (Filipovic and Jovanovic, 2017) and can also react with NO and H₂S to generate RSN and RSSH (Koppenol and Bounds, 2017). NO can react directly with thiols to form RSSN (Wong et al., 1998), but it remains unclear whether a similar reaction occurs between H₂S and thiols. Moreover, high concentrations of H₂S can promote the conversion of nitrosylated RSNO to RSSH (Hancock and Whiteman, 2016), which initiates the function of SnRK2.6/OST1 and terminates that of APX, although the reverse reaction is unclear. When persulfidated thiol (RSSH) encounters excess ROS, it will react with the ROS to form an adduct (RSSO₂H) (Wedmann et al., 2016), which will block the regulation of persulfidation-modified proteins, with the side effect of reducing the damage caused by excessive ROS. In addition, thioredoxin can reduce oxidized RSO₂H and RSSO₂H to RSSH and RSSSH (Wedmann et al., 2016; Filipovic and Jovanovic, 2017), which jointly construct the endogenous H₂S cycle.

A large number of plant proteins can be both persulfidated and nitrosylated (Aroca et al., 2017). Recently, Shen et al. (2020a) revealed that persulfidation acts as a specific and reversible signaling mechanism in the ABA response of Arabidopsis guard cells. ABA stimulates the persulfidation of DES1 at Cys44 and Cys205 in a redox-dependent manner, which amplifies the cells. ABA stimulates the persulfidation of DES1 at Cys44 and signaling mechanism in the ABA response of revealed that persulfidation acts as a specific and reversible proteins can be modified by persulfidation, such as POD, actin, Gin synthetase, ACC oxidases, and MAPKs (Aroca et al., 2015, 2018; Jia et al., 2018; Huang et al., 2019). Research on the potential mechanism of persulfidation in H₂S-dependent stomatal movement is still in an early stage, especially with regard to its relationship with NO-induced S-nitrosylation and ROS-induced S-sulfenylation. Protein kinases, phosphatases, defense-response molecules, and proteasome complex components are most likely to be the potential targets of H₂S-mediated persulfidation and to participate in H₂S-regulated stomatal closure.

H₂S REGULATES ION CHANNELS IN STOMATAL GUARD CELLS

Activities of ion channels in guard cells and their influence on the turgor of guard cells that surround the stoma are essential to the control of stomatal movement. When signals stimulate stomatal closure, large amounts of ions and osmotically active solutes are redistributed on both sides of the guard cell membrane, depolarizing its membrane potential to reduce the electrochemical gradient. Intracellular potassium ions (K⁺), anions, and solutes flow out through outward potassium (K⁺out) channels and anion channels in the guard cells, resulting in the discharge of water from inside the cells, decreasing turgor pressure, and finally causing stomatal closure (Kim et al., 2010; García-Mata and Lamattina, 2013). In an opposite pattern, stomatal opening stimuli activate plasma membrane H⁺-ATPases, leading to plasma membrane hyperpolarization and the activation of inward-rectifying K⁺ (IKIN) channels in guard cells (Kim et al., 2010). Recent studies have revealed that H₂S regulates these ion channels and ion equilibria in guard cells. In the RT-PCR experiments of Jin et al. (2013), the expression of genes encoding Ca²⁺ channels (ACA9, ACA11, and CAX1) and outward-rectifying K⁺ channels (GORK, SKOR, and KCO1) was reduced in an LCD-deletion mutant whose endogenous H₂S concentration was lower than that of the wild type. By contrast, the expression of genes encoding inositol channels (AKT1, AKT2, KC1, and KAT1) and two pore segment channel 1 (TPC1) increased. The activation of the vacuolar Ca²⁺ channel CAX3, one of the candidate targets of persulfidation (Aroca et al., 2015), was suppressed in the des1 mutant (Álvarez et al., 2012).

To further explore the mechanism by which H₂S affects stomatal movement, the two-electrode voltage clamp and patch-clamp techniques have been employed to test the ion channels in the plasma membranes of guard cells. In parallel with its stimulation of stomatal closure, H₂S selectively inhibits the IKIN channels of tobacco (Nicotiana tabacum L.) guard cells but has no significant effect on outward-rectifying K⁺ channels (Papanatsiou et al., 2015). Increases in cytosolic Ca²⁺ ([Ca²⁺]cyt) are involved in the ABA inhibition of IKIN (Siegel et al., 2009). By contrast, H₂S inhibits IKIN in a [Ca²⁺]cyt-independent manner (Papanatsiou et al., 2015). Slow-type anion channels (SLAC) have a central function in stomatal closure (Negi et al., 2008; Vahisalu et al., 2008). Wang et al. (2016) provided direct evidence that H₂S activates the slow-type anion current in Arabidopsis guard cells, which is mediated by SLAC1 channels (Figure 4). Moreover, the key molecules in ABA signaling, protein kinase SnRK2.6/OST1 and [Ca²⁺]cyt, both participate in H₂S-induced activation of the SLAC1 current and stomatal closure (Wang et al., 2016).
Plant Communications

At present, there is a lack of experimental evidence for the specific mechanisms by which ion channels are involved in H2S-regulated stomatal movement. Many reports in mammals may provide important clues; for example, H2S and polysulfide can directly regulate the transport activity of ion channels by promoting S-persulfidation (Lefer, 2019; Yang et al., 2020). Also, there may be an additional pathway by which H2S can alter the activities of K+ channels by increasing the ROS content of guard cells (Shen et al., 2020a).

**POTENTIAL COMPONENTS THAT MAY FUNCTION IN GUARD CELL H2S SIGNALING**

Until now, we have discussed some signaling molecules and protein modulations involved in the regulation of stomatal movement by H2S. However, there are still many possible candidates that may be modulators closely associated with H2S signals. Here, we propose some hypotheses. In Arabidopsis, extracellular ATP (eATP) has been reported to participate in the regulation of stomatal movement with a biphasic dose effect: it induces stomatal opening at lower levels but leads to stomatal closure at higher levels (Clark et al., 2011, 2013). Che et al. (2015) found that H2S induces an increase in eATP by regulating the activity of ATP-binding cassette (ABC) transporters. Cytosolic ATP is transported out of the protoplast membrane by ABC transporters (AtMRP4 and AtMRP5, from the MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN family of ABC transporters) that are located in guard cells and function in stomatal movement (Gaedeke et al., 2001; Klein et al., 2003). Thus, eATP promotes outward K+ currents by stimulating NADPH oxidases to produce H2O2 (Wang et al., 2015a). Similarly, G proteins are also involved in the H2S regulation of stomatal movement, as they act upstream of H2O2 (Zhang et al., 2013). H2S can induce stomatal closure as well as the expression of G-PROTEIN α SUBUNIT (GPA) and β SUBUNIT (AGB) genes in the leaves of wild-type Arabidopsis, but it has no significant effect on stomatal movement in mutants deficient in G-protein subunits (gpa1-3, gpa1-4, agb1-1, and agb1-2) (Zhang et al., 2013). This is consistent with the lack of stomatal response to ABA signaling in gpa1 and gpb1 mutants (Fan et al., 2008) and also indirectly indicates that the involvement of H2S in ABA-induced stomatal closure is associated with G proteins. Also, a low concentration of eATP promotes stomatal opening in Arabidopsis through the heterotrimeric G protein α subunit (Hao et al., 2012), further indicating that H2S plays a central role in this process.

The stability of both the cell membrane and the cell wall is critical to stomatal movement. As a lipophilic molecule, H2S, like NO and CO, can permeate the cell membrane freely without a special requirement for channel mediation. Zhou et al. (2019) have summarized recent advances in research on the essential roles of the guard cell wall in stomatal functions, including the roles of cellulose, xylan, pectin, lignin, and cell wall relaxation factors in maintaining stomatal guard cell function. H2S can inhibit the activities of polygalacturonase and endo-β-1,4-glucanase, thereby maintaining the integrity of the cell wall in Fragaria ×ananassa and Actinidia deliciosa (Gao et al., 2013; Zhang et al., 2014). Microarray hybridization analysis was used to compare the transcriptional differences between wild-type and des1 seedlings. The results showed that the expression levels of cell wall protein precursor and xyloglucan endotransglycosylase, which are related to cell-wall formation, were suppressed in des1 mutants. In addition, previous research on the role of phosphatidic acid (PA) in stomatal movement revealed that the PA produced by phospholipase D (PLD) could induce the production of H2O2 through interaction with RBOHD/F, and ROS would trigger the subsequent cascade reaction (Zhang et al., 2009), which occurs in the process of stomatal closure induced by SA and ABA (Zhang et al., 2009; Kalachova et al., 2013; Wang et al., 2019). Also, genetic evidence has shown that phospholipase C is involved in stomatal movement and ABA signaling (Zhang et al., 2018). The effect of H2S donors on stomata was partially eliminated by the phospholipase C inhibitor neomycin and PLD-dependent formation of the PA inhibitor butanol-1 (Yastreb et al., 2019). Combined with a recent study that indicates an interplay between H2S and PLD activity in the regulation of ROS production and stomatal movement, it is reasonable to speculate that PA is involved in H2S-induced stomatal movement (Scuffi et al., 2018). It is noteworthy that PA can regulate the capacity for K+ ion transport through direct interaction with OsAKT2 in rice (Shen et al., 2020b), similar to the regulation of channel activity by PA in animals (Poveda et al., 2019). There may be an unknown pathway by which H2S regulates ion channels in guard cells through PA, and this possibility is worthy of further exploration (Shen et al., 2020b; Maron, 2020).

The microfilaments and microtubules that compose the cytoskeleton may also be involved in H2S-induced stomatal movement. Depolymerization of microfilaments and microtubules in guard cells is essential during the process of stomatal closure induced by ABA, NO, and H2O2 (Li et al., 2006; Eisinger et al., 2012). It has been reported that H2S regulates the stability of microtubules by per sulfidation of actins and tubulins in mammals (Mustafa et al., 2009). Recently, Li et al. (2018) reported that H2S induces S-persulfidation to affect actin dynamics and root hair growth in Arabidopsis. It remains to be determined whether H2S-induced stomatal movement involves dynamic changes in microfilaments and microtubules.

**H2S INTERACTS WITH OTHER MOLECULES IN RESPONSE TO ABIOTIC/BIOTIC STRESSES**

A number of studies have shown that H2S plays a positive role in assisting plants in response to drought (Jin et al., 2011; Ziogas et al., 2015), salinity (Christou et al., 2013; Mostofa et al., 2015), heat (Li et al., 2012; Chen et al., 2019a), chilling (Du et al., 2017; Liu et al., 2019), heavy metals (Zhang et al., 2008, 2020c; Fang et al., 2017), and other stresses (Cheng et al., 2013; Hao et al., 2020). Recently, several reviews have summarized related research progress (Caldewood and Kopriva, 2014; Li et al., 2016; Corpas, 2019a; Chen et al., 2020c; Ahmed et al., 2021; Zhang et al., 2021b; Huang et al., 2021; Liu et al., 2021). Here, we will briefly summarize the role of H2S in the response to abiotic or biotic stresses in plants.

The valence of sulfur varies from −2 to +6. The sulfur in H2S has the lowest valence of −2, which makes it a good electron donor. Thus,
Regulation of guard cell signaling and stress response

H2S can act as a strong reductant to alleviate oxidative stress, which may be one of the most effective ways to enhance the abiotic stress tolerance of plants. A common feature of plants under either abiotic or biotic stress is the high accumulation of ROS, although the mechanisms of ROS production may differ in response to different adverse environmental factors (Apel and Hirt, 2004). H2S primarily reduces ROS levels via enzymatic systems such as superoxide dismutase, catalase, POD, APX, and glutathione reductase, or non-enzymatic systems such as glutathione (GSH) (Chen et al., 2020c), although H2S acts synergistically with H2O2 in guard cell signaling (Scuffi et al., 2018; Shen et al., 2020a). In addition to its close relationship with ROS signals, H2S also coordinates with NO in response to abiotic stress (Corpas, 2019b; Corpas et al., 2019). Increasing evidence has shown that H2S interacts with plant hormones such as ABA, ET, and SA in response to abiotic stress. For example, H2S interacts with ABA signaling to promote the drought tolerance of wheat (Ma et al., 2016). H2S plays a positive role in SA-induced heat tolerance in maize (Li et al., 2015), as well as SA-regulated Cd tolerance in Arabidopsis (Qiao et al., 2015). More recent data show that H2S-mediated persulfidation is an important mechanism in its interplay with other signaling molecules when plants experience abiost stress (Filipovic and Jovanovic, 2017; Arroca et al., 2018; Jia et al., 2018; Hancock, 2019; Palma et al., 2020), including the aforementioned interaction with other molecules in guard cell signaling (Zhou et al., 2020).

H2S participates in plant sulfur metabolism together with GSH and cysteine, both of which confer pathogen resistance on plants (Bloem et al., 2007; Kruse et al., 2007). In the cytosol, the balance of H2S and cysteine levels is regulated by DES1 and OAS-TL. The des1 knockout mutant, which contains high levels of SA and shows induction of WRKY54 and pathogenesis-related protein 1 (PR-1), showed high resistance to biotrophic and necrotrophic pathogens. By contrast, the OAS-A1 knockout mutant oas-a1 was sensitive to these pathogens (Álvarez et al., 2012). Another study also showed that OAS-A1 plays an indispensable role in the biotic stress response of plants (Tahir et al., 2013). These results suggest that the homeostasis of cysteine and H2S plays an important role in the response of plants to biotic stress.

In response to infection with Pyrenopeziza brassicae, Brassica napus showed increased levels of GSH, cysteine, and DES1 activity, which promoted the release of H2S (Bloem et al., 2004, 2012b). H2S has an antifungal effect on Aspergillus niger and Penicillium italicum that grow on fruits (Fu et al., 2014; Hu et al., 2014). Shi et al. (2015a) dissected the function of H2S in the regulation of abiotic and biotic stress resistance in Arabidopsis. H2S was found to upregulate the transcript abundance of abiotic and biotic stress-related genes and inhibit ROS levels. Moreover, H2S upregulates SA-related genes and modulates MIR393-mediated auxin signaling (Shi et al., 2015a). At present, there is limited information about the interaction of H2S with other signaling molecules in response to biotic stress.

CONCLUDING REMARKS AND PERSPECTIVES

Appropriate stomatal movement can help plants better adapt to environmental changes, more efficiently obtain carbon sources, maintain water balance, and resist the challenges of a harsh environment. As a recently recognized gaseous signaling molecule, H2S has been shown to play a key role in the regulation of stomatal movement. During this process, the H2S-related enzyme DES1 is activated by both upregulation at the transcriptional level and modification at the protein level, promoting the production and accumulation of cytosolic H2S in guard cells. Biotic or abiotic stress promotes the D/L-CDes/H2S process through the synthesis of stress-related hormones (ABA, SA, JA, and ET) and ROS accumulation, resulting in the generation and accumulation of H2S in guard cells. Elevated H2S levels then directly or indirectly affect secondary signals and components such as NO, H2O2, Ca2+, and eATP, thereby promoting stomatal closure (Figure 4). This process is related to the dynamic redox balance between H2S, NO, and ROS, and to the regulation of a variety of enzymes of secondary signaling by S-persulfidation. H2S directly or indirectly regulates transmembrane transporters and ion channels, changing the osmotic potential and turgor pressure of guard cells and resulting in stomatal closure. In addition, it can alter guard cell morphology by affecting the stability of the cell membrane, cytoplasm, and cell wall.

Based on our current knowledge, our understanding of the regulatory role played by H2S in stomatal movement is still relatively poor, and some more sophisticated mechanisms remain to be dissected by further exploration. Some of the points presented below may be important directions for future research.

(i) Engineered fluorescent proteins can detect changes in cellular signals in vivo. These proteins include Yellow Cameleon fluorescent protein for Ca2+ (Allen et al., 1999; Loro et al., 2016), roGFP2-Orp1 for H2O2 (Gutscher et al., 2009; Scuffi et al., 2018), ABAleon for ABA (Waadt et al., 2014), and PAleon for PA (Li et al., 2019a). However, to the best of our knowledge, there is not yet a suitable specific fluorescent protein for monitoring variations in H2S in vivo. Engineering such fluorescent proteins for dynamic monitoring of H2S levels may greatly promote our understanding of H2S signaling in plant cells.

(ii) The most commonly studied H2S-producing enzyme is DES1, but several other enzymes are involved in H2S metabolism, including SiR, OAS-TL, CAS, and DCD (Filipovic and Jovanovic, 2017; Corpas, 2019a). Arroca et al. (2017) compared the proteomes of wild-type Arabidopsis and the des1 mutant. Their results showed that over 2000 proteins were persulfidated, suggesting that other enzymes in addition to DES1 may be involved in persulfidation or that other regulatory elements participate in the protein modulation. To date, among the H2S metabolism-related enzymes, only DES1 has been extensively reported to be involved in stomatal regulation, and its activity and self-persulfidation have been found to play roles in ABA-induced stomatal closure (Jin et al., 2013; Scuffi et al., 2014; Shen et al., 2020a; Zhang et al., 2020a). However, the factors that control DES1 expression and the other elements that regulate DES1 activity remain largely unknown, and the possibility that other enzymes function in the control of stomatal movement requires further validation.
Plant Communications

(iii) H\textsubscript{2}S initiates mercapto (-SH) modification. Protein persulfidation seems unlikely to occur through a direct reaction between thiol groups and H\textsubscript{2}S, as this reaction is thermodynamically unfavorable (Filipovic and Jovanovic, 2017; Aroca et al., 2018; Filipovic et al., 2018). Whether some persulfidases may trigger the persulfidation of thiol groups by H\textsubscript{2}S remains to be determined. Moreover, how H\textsubscript{2}S selectively induces the persulfidation of specific thiol groups remains largely unclear.

(iv) The activities of ion channels, including I\textsubscript{KCa} and SLAC1, in guard cells can be regulated during H\textsubscript{2}S-induced stomatal closure. It was reported that the persulfidation of the Kir6.1 subunit of the K\textsubscript{ATP} Channel by H\textsubscript{2}S in smooth muscle cells induces channel opening with K\textsuperscript{+} influx, resulting in smooth muscle relaxation (Mustafa et al., 2011; Wang, 2012). A similar phenomenon has been reported for K\textsubscript{Ca} channels, Ca\textsuperscript{2+} channels, Cl\textsuperscript{-} channels, and TPR channels (Lefler, 2019; Yang et al., 2019). It will be interesting to determine whether ion channels in guard cells are persulfidated during H\textsubscript{2}S-induced stomatal movement. Furthermore, the functions of other ion channels and transporters in the H\textsubscript{2}S signaling pathway in guard cells remain largely unknown.

As the redox pioneer professor Hideo Kimura once commented on the study of H\textsubscript{2}S, “It is fortunate to come across a secret of nature and pick it up” (Lefler, 2019). We acknowledge his view and want to admire the fact that “H\textsubscript{2}S, like the aroma of roses, has far-reaching influence on stomatal movement, and has a long meaning on plant resistance.”

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Regulation of guard cell signaling and stress response

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