Hydrogen sulfide induced by hydrogen peroxide mediates darkness-induced stomatal closure in *Arabidopsis thaliana*

Yinli Ma (✉ mayinli1978@163.com)

Shanxi Normal University <https://orcid.org/0000-0001-7040-3209>

Luhan Shao

Shanxi Normal University

Jiao Niu

Shanxi Normal University

Research article

Keywords: hydrogen sulfide, hydrogen peroxide, stomatal closure, darkness, *Arabidopsis thaliana*

Posted Date: September 23rd, 2019

DOI: <https://doi.org/10.21203/rs.2.14712/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Pakistan Journal of Botany on June 1st, 2021. See the published version at [https://doi.org/10.30848/PJB2022-1\(31\)](https://doi.org/10.30848/PJB2022-1(31)).

Abstract

Background Whether hydrogen sulfide (H₂S) mediates darkness-induced stomatal closure in *A. thaliana* is unknown, and the interaction between H₂S and hydrogen peroxide (H₂O₂) in the process needs to be elucidated.

Results Our results indicated that H₂S modulators hypotaurine (HT), aminoxy acetic acid (AOA), hydroxylamine (NH₂OH) and potassium pyruvate (N₃H₃KO₃)+ammonia (NH₃) all inhibited darkness-induced stomatal closure, H₂S generation and L-/D-cysteine desulfhydras (L-/D-CDes) activity increase in wild-type *A. thaliana* leaves. Darkness induced stomatal closure in wild-type plants, but failed in *Atl-cdes* and *Atd-cdes* mutants. Additionally, H₂S content and L-/D-CDes activity were significantly decreased after application with H₂O₂ modulators ascorbic acid (ASA), catalase (CAT), diphenylene iodonium (DPI), and salicylhydroxamic acid (SHAM) in darkness, but there is almost no effects on H₂O₂ levels when in presence of HT, AOA, NH₂OH, and C₃H₃KO₃+NH₃ in darkness in wild-type plants. Moreover, darkness couldn't increase H₂S content and L-/D-CDes activity of *AtrbohF* and *AtrbohD/F* mutants leaves, but the levels of H₂O₂ increased in guard cells of *Atl-cdes* and *Atd-cdes* mutants.

Conclusions The results suggest that L-/D-CDes-generated H₂S mediates darkness-induced stomatal closure, and functions downstream of H₂O₂ in *A. thaliana*.

Background

Hydrogen sulfide, a novel gasotransmitter, has a similar function with nitric oxide (NO) and carbon monoxide (CO). Early research is devoted to its toxicity studies, while neglecting its physiological functions in plants and animals. Nowadays, it has been reported that H₂S is a new gas signal molecule and plays an important role in signal transduction in animals and plants. For example, in animals, H₂S is involved in brain development, inflammation, neuronal antioxidant protection, the regulation of the heart and nervous system, diastolic cardiovascular and digestive tract smooth muscle [1–3]. Compared with the degree of research in animals, the understanding of the function of H₂S in plant growth and development is far from enough. However, with the increasing attention of researchers, the physiological role of H₂S in plants also has many new discoveries. H₂S not only promotes the growth and development of plants, such as increasing seed germination rate, promoting root development, enhancing photosynthesis, prolonging florescence and delaying senescence, but also enhances plant resistance to abiotic stresses [4–9]. In addition, it is found that H₂S is an important signaling molecule regulating stomatal movement [10]. H₂S interact with abscisic acid (ABA) and participate in the regulation of stomatal movement in *A. thaliana* and it is located upstream of ATP-binding cassette (ABC) transporter to regulate ABA-induced stomatal closure [6]. H₂S is involved in ethylene-induced stomatal closure and act downstream of NO in the process [11].

Hydrogen peroxide (H₂O₂), another signal molecule, is one of the main reactive oxygen species (ROS) produced in the process of plant cell metabolism. Its molecular properties are relatively stable [12]. H₂O₂

is generated via enzymatic sources such as NADPH oxidases (NOX), polyamine oxidases or cell wall peroxidases in plant cells [13–15]. A large number of studies have shown that plant tissue can resist all kinds of abiotic or biological stresses by producing H_2O_2 , including extreme temperature, ABA, ultraviolet-b radiation (UV-B), darkness, ethylene and bacterial invasion etc [16–21]. Moreover, H_2O_2 mediates various physiological processes in plants, including plant growth and development, resistance to adversity, defense response to pathogenic bacteria, gene expression, stomatal movement, and programmed cell death, all of which have important regulatory roles [16, 22–25].

It has been reported that both H_2O_2 and H_2S mediate darkness-induced stomatal closure, and H_2S acts downstream of H_2O_2 in the process in *Vicia faba* (*V. faba*) [14, 26]. However, whether H_2S is involved in darkness-induced stomatal closure in *A. thaliana* is unknown, the interaction between H_2S and H_2O_2 and the enzymatic pathways of them in the process need to be elucidated. To address these questions, *A. thaliana* genotypes (wild-type, *Atl-cdes*, *Atd-cdes*, *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants) were used to investigate the importance and interactions of H_2S and H_2O_2 in darkness-induced stomatal closure.

Results

Involvement of H_2S in darkness-induced stomatal closure

Effects of darkness on stomatal movement of wild-type *A. thaliana*, *Atl-cdes* and *Atd-cdes* mutants

In order to determine whether H_2S mediates darkness-induced stomatal closure, the effects of H_2S scavenger HT, H_2S synthesis inhibitors AOA and NH_2OH , $C_3H_3KO_3+NH_3$ and the producer of L-/D-cysteine desulfhydrase (L-/D-CDes) on the stomatal aperture of the wild-type *A. thaliana* (Col-0), and the effect of darkness on stomatal aperture of *Atl-cdes* and *Atd-cdes* T-DNA insertion lines were detected. The presence of HT, AOA, NH_2OH , and $C_3H_3KO_3+NH_3$ was found to inhibit darkness-induced stomatal closure. When HT, AOA, NH_2OH , and $C_3H_3KO_3+NH_3$ were applied in light, stomatal closure was not induced (Fig. 1a). In addition, darkness could not induce stomatal closure of *Atl-cdes* and *Atd-cdes* mutants (Fig. 1b). These results indicated that L-/D-CDes-generated H_2S might be involved in darkness-induced stomatal closure in *A. thaliana*.

Effects of HT, AOA, NH_2OH , and $C_3H_3KO_3+NH_3$ on darkness-induced H_2S content and L-/D-CDes activity in wild-type plants leaves

To identify further the metabolic pathways involved in H_2S synthesis, H_2S content and the activity of L- and D-CDes in wild-type plants leaves were determined. The content of H_2S in darkness was significantly higher than that in light, while HT, AOA, NH_2OH , and $C_3H_3KO_3+NH_3$ could reduce darkness-induced H_2S production (Fig. 2a). Similarly, darkness caused a marked increase in L- and D-CDes activity, and the activity of L- and D-CDes were reduced in the presence of HT, AOA, NH_2OH , and $C_3H_3KO_3+NH_3$ in darkness

(Fig. 2b and 2c). However, when HT, AOA, NH_2OH , and $\text{C}_3\text{H}_3\text{KO}_3+\text{NH}_3$ were applied in light, no effect was observed (Fig. 2a-c). These results suggested that darkness-induced H_2S biosynthesis might function via L- and/or D-CDes.

The relationship between H_2S and H_2O_2 in darkness-induced stomatal closure in *A. thaliana*

Effects of H_2O_2 scavenger and synthesis inhibitors on darkness-induced H_2S content and L-/D-CDes activity

To analyze the relationship between H_2S and H_2O_2 during darkness-induced stomatal closure in *A. thaliana*, H_2S content and activity of L- and D-CDes in leaves of wild-type plants were examined with application of H_2O_2 modulators ASA [27], CAT, DPI (an inhibitor of NADPH oxidase)[28, 29], and SHAM (an inhibitor of inhibitor of cell wall peroxidase) in darkness. Treatment with ASA, CAT, DPI, and SHAM in darkness decreased H_2S content and reduced the activity of L- and D-CDes in wild-type plants leaves (Fig. 3a-c). We proposed that both NADPH oxidase-derived and peroxidase-derived H_2O_2 might represent a novel upstream component of H_2S signaling cascade during darkness-induced stomatal closure in *A. thaliana*.

Effects of darkness on H_2S content and L-/D-CDes activity in the leaves of *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants

To further investigate the relationship between H_2S and H_2O_2 in *A. thaliana*, H_2S content and L- and D-CDes activity were detected in the leaves of NADPH oxidase mutants *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants. H_2S content in leaves of wild-type plants and *AtrbohD* in darkness was significantly higher than that in the light, and L- and D-CDes activity of leaves was also significantly enhanced (Fig. 4 a-c). However, darkness couldn't induced increase in H_2S content and L- and D-CDes activity in *AtrbohF* and *AtrbohD/F* mutants (Fig. 4 a-c). The results further suggested that H_2S acted downstream of H_2O_2 in darkness-regulated stomatal closure in *A. thaliana*.

Effects of H_2S scavenger and synthesis inhibitors on darkness-induced H_2O_2 levels in guard cells of the wild-type *A. thaliana*

To further confirm the relationship between H_2S and H_2O_2 , H_2O_2 fluorescence in guard cells after application of HT, NH_2OH , AOA, and $\text{N}_3\text{H}_3\text{KO}_3+\text{NH}_3$ in darkness were examined by $\text{H}_2\text{DCF-DA}$, which previously used to measure H_2O_2 production in darkness-induced stomatal closure by Allan and Fluhr (1997) [30]. Darkness caused a marked increase an intense in the H_2O_2 levels of guard cells (Fig. 5b). However, treatment with HT, NH_2OH , AOA, and $\text{N}_3\text{H}_3\text{KO}_3+\text{NH}_3$ in darkness had no clear effects on H_2O_2 levels (Fig. 5c-f). Therefore, these results consolidated the fact that H_2S functioned downstream of H_2O_2 in darkness-induced stomatal closure in *A. thaliana*.

Effects of darkness on the levels of H₂O₂ in guard cells of *Atl-cdes* and *Atd-cdes* mutants

To confirm that H₂S as a downstream factor of H₂O₂ mediated stomatal closure induced by darkness, we detected the levels of H₂O₂ in guard cells of *Atl-cdes* and *Atd-cdes* mutants. In darkness the levels of H₂O₂ in guard cells of *Atl-cdes* and *Atd-cdes* mutants were significantly stronger than that in light (Fig. 6c-f), and there was no significant difference from the wild-type plants (Fig. 6a, b). The above data further demonstrated that the H₂S, as a downstream factor of H₂O₂, mediated darkness-induced stomatal closure in *A. thaliana*.

Discussion

Stomata are important structures for the exchange of gases and water in higher plants and they respond to different environmental factors by controlling their aperture. The mechanism of regulating stomatal movement is extremely complex. In addition to the classical theory of cell turgor pressure, the stomatal closure is also affected by cytoplasmic calcium concentration changes, pH, protein phosphorylation and K⁺ and anion channel regulation [31]. During the continuous exploration of the mechanism of stomatal movement, some essential regulators of signal transduction are gradually emerging, such as NO, H₂O₂, H₂S and so on.

As the third gasotransmitter, H₂S has become a new star in plant signal transduction process, and plays a vital role in plant stomatal movement. Lisjak et al. (2010, 2011) reported that exogenous H₂S released by NaHS induces stomatal opening [32, 33]. However, García-Mata and Lamattina (2010) also reported that H₂S induces stomatal closure in different plants [10]. It has been shown that H₂S mediates ABA, ethylene and darkness-regulated stomatal movement [6, 11, 26, 34]. It is well known that darkness is one of the important external factors regulating stomatal closure. It has been proved that sphingosine-1-phosphate (S1P), phytosphingosine-1-phosphate (Phyto-S1P), cytosolic alkalization [35–37], NO, CO, H₂O₂ and intracellular calcium [17, 18, 38–40] mediate darkness-induced stomatal closure. However, the mechanism of H₂S in darkness-regulated stomatal closure is still unclear. The present results showed that darkness-induced stomatal closure is significantly blocked by H₂S scavenger HT, the inhibitors of H₂S biosynthesis NH₂OH and AOA, and the products of L- and/or D-cysteine desulfhydrase C₃H₃KO₃+NH₃ in wild-type *A. thaliana* (Fig. 1a). Darkness could induce stomatal closure in wild-type *A. thaliana*, but had no effect on stomatal aperture of *Atl-cdes* and *Atd-cdes* mutants (Fig. 1b). Additionally, HT, AOA, NH₂OH, and C₃H₃KO₃+NH₃ reduced darkness-induced H₂S production (Fig. 2a), and diminished the activity of L-CDes (Fig. 2b) and D-CDes in darkness (Fig. 2c). These results indicated that H₂S is required for darkness-regulated stomatal closure in *A. thaliana*, and L-/D-CDes is one of the key enzymes for its synthesis, which is in agreement with the results of Ma in *V. faba* [26].

Hydrogen peroxide (H₂O₂) is another important signal molecule involved in the signal transduction of plant cells. As a second messenger in plants, H₂O₂ participates in many physiological processes

including stomatal movement. McAinsh et al. (1996) proved for the first time that H_2O_2 had an effect on stomatal movement in plants, exogenous H_2O_2 could promote the increase of calcium level in guard cells of *V. faba*, leading to stomatal closure [41]. In recent years, the role of H_2O_2 in stomatal movement has been studied more deeply. It has been proved that guard cells accumulate H_2O_2 to promote stomatal closure under the stimulation of drought, salt, high concentration of CO_2 , ethylene, ABA, UV-B, brassinosteroid, as well as darkness [16, 18, 19, 30, 42–47]. Data from Desikan et al. (2004) showed that stomatal closure is related to H_2O_2 accumulation in the guard cells of pea, and the large gp91phox subunit (rboh genes) which encoding NOX were present in pea guard cells, likely to mediate the darkness and ABA responses [17]. It has been reported that soaking with H_2O_2 could increase L-CDes activity, promote H_2S production of, and improve germination rate of *Jatropha curcas* seeds [48]. Additionally, Li and He (2015) showed that H_2O_2 acts downstream of H_2S in seed germination of *Vigna radiata* [49]. Wang et al. (2015) showed that H_2O_2 is involved in exogenous H_2S -induced stomatal closure [50]. H_2O_2 has been reported to function upstream of H_2S in darkness and salt stress-induced stomatal closure in *V. faba* [26, 51]. It has been proved that H_2O_2 mediates darkness-induced stomatal closure [17, 18, 26]. However, the interaction between H_2O_2 and H_2S is still unclear in darkness-induced stomatal closure in *A. thaliana*. The data presented here showed that H_2O_2 modulators ASA, CAT, DPI and SHAM could significantly inhibit darkness-induced H_2S production and L-/D-CDes activity enhancement in *A. thaliana* wild-type leaves (Fig. 3), H_2S scavenger HT and its synthesis inhibitors AOA, NH_2OH and $C_3H_3KO_3+NH_3$ have no significant effects on H_2O_2 levels of wild-type guard cells in darkness (Fig. 5). Compared with light treatment, darkness had no significant effects on H_2S content and L-/D-CDes activity in leaves of *AtrbohF* and *AtrbohD/F* mutants (Fig. 4), while darkness could increase H_2O_2 levels in guard cells of *Atl-cdes* and *Atd-cdes* mutants (Fig. 6). These data indicated that the action of NADPH oxidase-derived and peroxidase-derived H_2O_2 could be upstream of L-/D-CDes-generated H_2S in stomatal closure induced by darkness in *A. thaliana*, which is the same as the results of Ma et al. (2018) in *V. faba* [26].

Conclusions

In this study, we explored the interaction between H_2S and H_2O_2 during darkness-induced stomatal closure. Our results suggested that darkness induces H_2O_2 synthesis via promotion of NADPH oxidase and peroxidase activity, which leads to L-/D-CDes-derived H_2S production and stomatal closure in *A. thaliana*. Additionally, studies have shown that L-/D-CDes-generated H_2S functions downstream of H_2O_2 in *A. thaliana*. Our work enriches the signal transduction network for the regulation of stomatal movement induced by darkness, and it provides experimental evidences in plant physiology, cell biology and genetics for the relationship between H_2S and H_2O_2 in stomatal movement of plants.

Methods

Chemicals

The molecular probe H₂DCF-DA was obtained from Biotium (Hayward, CA, USA). 2-(*N*-morpholino) ethanesulfonic acid (MES), hypotaurine (HT), aminooxy acetic acid (AOA), hydroxylamine (NH₂OH), potassium pyruvate (C₃H₃KO₃), ammonia (NH₃), ascorbic acid (ASA), catalase (CAT), diphenylene iodonium (DPI), salicylhydroxamic acid (SHAM), L-cysteine, D-cysteine, dimethyl sulfoxide (DMSO), *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride and dithiothreitol (DTT) were obtained from Sigma-Aldrich (St Louis, MO, USA). Unless stated otherwise, the remaining chemicals were of the highest analytical grade available from various Chinese suppliers.

Plant materials

A. thaliana ecotype Columbia (Col-0) was used throughout this study. Seeds of L-/D-cysteine desulfhydrase deletion mutants of *AtL-CDes* T-DNA insertion line (N541918, designated *Atl-cdes*), *AtD-CDes* T-DNA insertion line (CS853264, designated *Atd-cdes*), and NADPH oxidase gene single mutant lines (N9555, designated *AtrbohD*) and (N9557, designated *AtrbohF*) and of the homozygous transposon insertion double mutant line (N9558, designated *AtrbohD/F*) were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC, Nottingham, UK). The mutant *Atl-cdes*, *Atd-cdes* and *AtrbohD*, *AtrbohF*, *AtrbohD/F* has been identified by PCR and RT-PCR [52–54]. Wild-type and mutants seeds of *A. thaliana* were surface-sterilized and sown on sterilized vermiculite. Seedlings were stratified in darkness for 2–4 d at 4 °C. After growing 4 euphylla, seedlings were grown in controlled-environment chamber with a humidity of 80%, a 16-h light/8-h dark cycle, and a day/night temperature cycle of 22 °C/ 18 °C with a photon flux density of 100 μmol·m⁻²·s⁻¹ PAR generated by cool white fluorescent tubes (Philips, New York, NY, USA). Fully expanded leaves were harvested for immediate use at 4–6 weeks.

Stomatal bioassays

Stomatal bioassay was performed as described by McAinsh et al. (1996) with minor modifications [41]. The freshly prepared epidermal strips were treated with MES-KCl buffer (10 mM MES, 50 mM KCl, 100 μM CaCl₂, pH 6.15) alone or containing various compounds or inhibitors in light (100 μmol·m⁻²·s⁻¹) or darkness. And then the stomatal apertures were recorded with a light microscope and an eyepiece graticule previously calibrated with a stage micrometer. In each treatment, we scored 30 randomly-selected apertures per replicating and treatments were repeated at least three times. The data presented are the mean ± s.e. of 90 measurements.

Measurement of H₂S emission

Measurement of H₂S emission was determined by formation of methylene blue, which was performed as described by Sekiya et al. (1982) and Hou et al. (2013) with slight modifications [34, 55]. Fully expanded leaves were used to measure H₂S emission. Firstly, the leaves were treated with MES-KCl buffer alone or containing various scavengers or synthesis inhibitors in light (100 μmol·m⁻²·s⁻¹) or darkness for 3h, and

then took 0.1 g of them for grinding by addition 0.9 mL 20 mM Tris-HCl (pH 8.0) buffer. After centrifugation, the supernatant and a trap with 1% of zinc acetate was put in a test tube, meanwhile the test tube was sealed quickly with a Parafilm. After H₂S was absorbed for 30 min at 37 °C, 100 µL 20 mM *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 M HCl and 100 µL 30 mM FeCl₃ dissolved in 1.2 M HCl was added into the trap. Finally, the absorbance was measured at 670 nm. A calibration curve was made with known concentrations of Na₂S solution. Each treatment was repeated 3 times, and the data presented are the mean ± s.e.

L-/D-cysteine desulfhydrase activity measurements

To further investigate the L-/D-cysteine desulfhydrase (L-/D-CDes) activity, we determined the H₂S which was released from L-/D-cysteine within a certain period of time [34, 56]. The assay contained in a total volume of 1mL: 100 µL 0.8 mM L-/D-cysteine, 400 µL 2.5 mM DTT, 400 µL 100 mM Tris-HCl, and 100 µL supernatant. Then added 100 µL 20 mM *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 M HCl and 100 µL 30 mM FeCl₃ dissolved in 1.2 M HCl into the trap after reaction for 30 min at 37 °C. And the rate of H₂S released was showed by the determined of absorbance at 670 nm. The activity of L-CDes and D-CDes was determined by the same method, but the pH of Tris-HCl buffer that the former used was 9, and the latter was 8. Each treatment was repeated 3 times, and the data presented are the mean ± s.e.

Measurement of endogenous H₂O₂

H₂O₂ levels were measured with 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) by the method of Allan and Fluhr (1997) with minor modifications [30]. To study the effects of H₂S scavenger and synthesis inhibitors on darkness-induced H₂O₂ production in guard cells, the epidermal strips were incubated in MES-KCl buffer alone in light or in MES-KCl buffer alone or containing ASA, CAT, DPI, and SHAM in darkness for 3 h, and then were immediately loaded with 50 µM H₂DCF-DA in Tris-KCl buffer (10 mM Tris, 50 mM KCl, pH 7.2) for 10 min in darkness. To study the effects of darkness on H₂O₂ levels in guard cells of *Atl-cdes* and *Atd-cdes* mutants, the epidermal strips were incubated in MES-KCl buffer alone in light or in MES-KCl buffer alone in darkness for 3 h, and then were immediately loaded with 50 µM H₂DCF-DA in Tris-KCl buffer for 10 min in darkness. Then excess dye was washed off with fresh Tris-KCl loading buffer in darkness, and the epidermal strips were immediately examined by TCS SP5 laser-scanning confocal microscopy (Leica Lasertechnik GmbH, Heidelberg, Germany) with the following settings: excitation 488 nm, emission 530 nm, power 10%, zoom about 4, normal scanning speed, and frame 512×512 pixels. Images thus acquired were analyzed with Leica image software and processed with Photoshop 7.0 (Adobe, San Jose, CA, USA). Each treatment was repeated at least 3 times. The confocal images depicted here represent similar results from all three replications.

Statistical analyses

The statistical significance of treatments was checked using one-way ANOVA followed by Duncan's multiple range test. The data were considered statistically significant when *P*-values were below 0.05. The figures were plotted by Origin6.1 (Microcal Software, Nothampton, MA, USA) and processed with Photoshop 7.0 (Adobe, San Jose, CA, USA).

Abbreviations

ABA abscisic acid

ABC ATP-binding cassette

AOA aminooxy acetic acid

ASA ascorbic acid

A. thaliana *Arabidopsis thaliana*

CAT catalase

CO carbon monoxide

DPI diphenylene iodonium

DTT N,N-dimethyl-*p*-phenylenediamine dihydrochloride and dithiothreitol

H₂DCF-DA 2',7'-dichlorodihydrofluorescein diacetate

H₂O₂ hydrogen peroxide

H₂S hydrogen sulfide

HT hypotaurine

L-/D-CDes L-/D-cysteine desulfhydrase

NaHS sodium hydrosulfide

N₃H₃KO₃ potassium pyruvate

NH₃ ammonia

NH₂OH hydroxylamine

NO nitric oxide

NOX NADPH oxidase

ROS reactive oxygen species

SHAM salicylhydroxamic acid

UV-B ultraviolet-b radiation

V. faba Vicia faba

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by grants from the Innovative Training Program for College Students in Shanxi, China (No.2019229). The funding bodies had no role in the experimental design, data analysis, decision to publish, or preparation of the manuscript.

Authors' contributions

YM designed the experiments and wrote the manuscript. LS and JN performed the experiments. YM, LS and JN analyzed data. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

References

1. Wang R. Two's company, three's a crowd: can H₂S be the third endogenous gaseous transmitter. *FASEB*. 2002;16:1792–98.
2. Linden DR, Levitt MD, Farrugia G, Szurszewski JH. Endogenous production of H₂S in the gastrointestinal tract: still in search of a physiologic function. *Antioxid Redox Sign*. 2009;12:1135–46.
3. Hosoki R, Matsuki N, Kimura H. The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem Biophys Res Commun*. 1997;237:527–31.
4. Zhang H, Tang J, Liu XP, Wang Y, Yu W, Peng WY, et al. Hydrogen sulfide promotes root organogenesis in *Ipomoea batatas*, *Salix matsudana* and *Glycine max*. *J Integr Plant Biol*. 2009;51:1086–94.
5. Chen J, Wu FH, Wang WH, Zheng CJ, Lin GH, Dong XJ, et al. Hydrogen sulphide enhances photosynthesis through promoting chloroplast biogenesis, photosynthetic enzyme expression, and thiol redox modification in *Spinacia oleracea* seedlings. *J Exp Bot*. 2011;62:4481–93.
6. Jin ZP, Xue SW, Luo YN, Tian BH, Fang HH, Li H, et al. Hydrogen sulfide interacting with abscisic acid in stomatal regulation responses to drought stress in *Arabidopsis*. *Plant Physiol Biochem*. 2013;62:41–46.
7. Li ZG, Gong M, Xie H, Yang L, Li JB. Hydrogen sulfide donor sodium hydrosulfide-induced heat tolerance in tobacco (*Nicotiana tabacum* L) suspension cultured cells and involvement of Ca²⁺ and calmodulin. *Plant Sci*. 2012a;185–186:185–89.
8. Wang YQ, Li L, Cui WT, Xu S, Shen WB, Wang R. Hydrogen sulfide enhances alfalfa (*Medicago sativa*) tolerance against salinity during seed germination by nitric oxide pathway. *Plant Soil*. 2012;351:107–19.
9. Mostofa MG, Saegusa D, Fujita M, Tran LSP. Hydrogen sulfide regulates salt tolerance in rice by maintaining Na⁺/K⁺ balance, mineral homeostasis and oxidative metabolism under excessive salt stress. *Front Plant Sci*. 2015;6:1055.
10. García-Mata C, Lamattina L. Hydrogen sulphide, a novel gasotransmitter involved in guard cell signalling. *New Phytol*. 2010;188:977–84.
11. Liu J, Hou ZH, Liu GH, Liu LX. Hydrogen sulfide may function downstream of nitric oxide in ethylene-induced stomatal closure in *Vicia faba* L. *J Integr Agric*. 2012;11:1644–53.
12. Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol*. 2004;55:373–99.
13. Barr R, Sandelius AS, Crane FL, Morré DJ. Redox reactions of tonoplast and plasma membranes isolated from soybean hypocotyls by free-flow electrophoresis. *Biochim Biophys Acta*. 1986;852:254–61.

14. Rubinstein B, Luster DG. Plasma membrane redox activities: components and role in Plant processes. *Ann Rev Plant Physiol Plant Mol Biol.*1993;44:131–55.
15. Crane FL, Morré DJ, Löw HE. Oxidoreduction at the plasma membranerelation to growth and transport: volume 2: relation to growth and transport. Bosa Roca: Crc Press;1990.p.267–70.
16. Neill S, Desikan RJ, Hancock J. Hydrogen peroxide signalling. *Curr Opin Plant Biol.* 2002;5:388–95.
17. Desikan R, Cheung MK, Clarke A, Golding S, Sagi M, Fluhr R, et al. Hydrogen peroxide is a common signal for darkness- and ABA-induced stomatal closure in *Pisum sativum*. *Funct Plant Biol.* 2004;31:913–20.
18. She XP, Song XG, He JM. Role and relationship of nitric oxide and hydrogen peroxide in light/dark-regulated stomatal movement in *Vicia faba*. *Acta Bot Sin.* 2004;46:1292–300.
19. He JM, Xu H, She XP, Song XG, Zhao WM. The role and the interrelationship of hydrogen peroxide and nitric oxide in the UV-B-induced stomatal closure in broad bean. *Funct Plant Biol.* 2005;32:237–47.
20. Desikan R, Last K, Harrettwilliams R, Tagliavia C, Harter K, Hooley R, et al. Ethylene-induced stomatal closure in *Arabidopsis* occurs via AtrbohF-mediated hydrogen peroxide synthesis. *Plant J.* 2006;47:907–16.
21. Larkindale J, Huang B. Thermotolerance and antioxidant systems in *Agrostis stolonifera*: Involvement of salicylic acid, abscisic acid, calcium, hydrogen peroxide, and ethylene. *J Plant Physiol.* 2004;161:405–13.
22. Laloi C, Apel K, Danon A. Reactive oxygen signalling: the latest news. *Curr Opin Plant Biol.* 2004;7:323–28.
23. Ren D, Yang H, Zhang S. Cell death mediated by MAPK is associated with hydrogen peroxide production in *Arabidopsis*. *J Biol Chem.* 2002;277:559–65.
24. Li S, Xue L, Xu S, Feng H, An L. Hydrogen peroxide involvement in generation and development of adventitious roots in cucumber. *Plant Growth Regul.* 2007;52:173–80.
25. Potikha TS, Collins CC, Johnson DI, Delmer DP, Levine A. The involvement of hydrogen peroxide in the differentiation of secondary walls in cotton fibers. *Plant Physiol.* 1999;119:849–58.
26. Ma YL, Niu J, Wu X. Hydrogen sulfide may function downstream of hydrogen peroxide in mediating darkness-induced stomatal closure in *Vicia faba*. *Funct Plant Biol.* 2018;45:553–60.
27. Noctor G and Foyer CH. Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Mol Biol.* 1998;49:249–79.
28. Cross AR, Jones OTG. The effect of the inhibitor diphenylene iodonium on the superoxide-generating system of neutrophils. Specific labelling of a component polypeptide of the oxidase. *Biochem J.* 1986;237:111–16.
29. Lee S, Choi H, Suh S, Doo IS, Oh KY, Choi EJ, et al. Oligogalacturonic acid and chitosan reduce stomatal aperture by inducing the evolution of reactive oxygen species from guard cells of tomato and *Commelina communis*. *Plant Physiol.* 1999;121:147–52.

30. Allan AC, Fluhr R. Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. *Plant Cell*. 1997;9:1559–72.
31. Blatt MR, Grabov A. Signal redundancy, gates and the integration in the control of ion channels for stomatal movement. *J Exp Bot*. 1997;48:529–37.
32. Lisjak M, Srivastava N, Teklic T, Civale L, Lewandowski K, Wilson I, et al. A novel hydrogen sulfide donor causes stomatal opening and reduces nitric oxide accumulation. *Plant Physiol Biochem*. 2010;48:931–35.
33. Lisjak M, Teklić T, Wilson ID, Wood M, Whiteman M, Hancock JT. Hydrogen sulfide effects on stomatal apertures. *Plant Signaling Behav*. 2011;6:1444–46.
34. Hou ZH, Wang LX, Liu J, Hou LX, Liu X. Hydrogen sulfide regulates ethylene-induced stomatal closure in *Arabidopsis thaliana*. *J Integr Plant Biol*. 2013;55:277–89.
35. Ma YL, She XP, Yang SS. Sphingosine–1-phosphate (S1P) mediates darkness induced stomatal closure through raising cytosol pH and hydrogen peroxide (H₂O₂) levels in guard cells in *Vicia faba*. *Sci China Life Sci*. 2012;55:974–83.
36. Ma YL, She XP, Yang SS. Cytosolic alkalization-mediated H₂O₂ and NO production are involved in darkness-induced stomatal closure in *Vicia faba*. *Can J Plant Sci*. 2013;93:119–30.
37. Ma YL, Niu J. The role of phytosphingosine–1-phosphate (Phyto-S1P) and its relationships with cytosolic pH and hydrogen peroxide (H₂O₂) during stomatal closure by darkness in broad bean. *S Afr J Bot*. 2017;108:237–42.
38. Schwartz A. Role of Ca²⁺ and EGTA on stomatal movements in *Commelina communis* L. *Plant Physiol*. 1985;79:1003–05.
39. She XP, Song XG. Carbon monoxide-induced stomatal closure involves generation of hydrogen peroxide in *Vicia faba* guard cells. *J Integr Plant Biol*. 2008;50:1539–48.
40. Song XG, She XP, Zhang B. Carbon monoxide-induced stomatal closure in *Vicia faba* is dependent on nitric oxide synthesis. *Physiol Plant*. 2008;132:514–25.
41. McAinsh MR, Clayton H, Mansfield TA, Hetherington AM. Changes in stomatal behavior and guard cell cytosolic free calcium in response to oxidative stress. *Plant Physiol*. 1996;111:1031–42.
42. An GY, Ding XY, Wu GL, Li HW, Song CP. ECS1 mediates CO₂-induced stomatal closure and the production of H₂O₂ in *Arabidopsis thaliana*. *Chinese Bull Bot*. 2012a;47:209–16.
43. An GY, Li BZ, Wu GL. H₂O₂ could act as root source signal to mediate stomatal closure induced by salt stress of *Vicia faba* L. *Plant Physiology Journal*. 2012b;48:265–71.
44. Yao Y, Liu X, Li Z, Ma X, Rennenberg H, Wang X. Drought-induced H₂O₂ accumulation in subsidiary cells is involved in regulatory signaling of stomatal closure in maize leaves. *Planta*. 2013;238:217–27.
45. Lü D, Zhang X, Jiang J, An GY, Zhang LR, Song CP. NO may function in the downstream of H₂O₂ in ABA-induced stomatal closure in *Vicia faba* L. *Plant Physiology Journal*. 2005;31:62–70.

46. Bright J, Desikan R, Hancock JT, Weir IS, Neill SJ. ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H₂O₂ synthesis. *Plant J.* 2006;45:113–22.
47. Shi CY, Qi C, Ren HY, Huang AX, Hei SM, She XP. Ethylene mediates brassinosteroid-induced stomatal closure via Gα protein-activated hydrogen peroxide and nitric oxide production in Arabidopsis. *Plant J.* 2015;82:280–301.
48. Li ZG, Gong M, Liu P. Hydrogen sulfide is a mediator in H₂O₂-induced seed germination in *Jatropha Curcas*. *Acta Physiol Plant.* 2012b;34:2207–13.
49. Li ZG, He QQ. Hydrogen peroxide might be a downstream signal molecule of hydrogen sulfide in seed germination of mung bean (*Vigna radiata*). *Biologia.* 2015;70:753–59.
50. Wang LX, Ma XY, Che YM., Hou LX, Liu X, Zhang W. Extracellular ATP mediates H₂S-regulated stomatal movements and guard cell K⁺ current in a H₂O₂-dependent manner in Arabidopsis. *Chinese Sci Bull.* 2015;60:419–27.
51. Ma YL, Zhang W, Niu, J, Ren Y, Zhang F. Hydrogen sulfide may function downstream of hydrogen peroxide in mediating salt stress-induced stomatal closure in *Vicia faba*. *Funct Plant Biol.* 2019;46:136–45.
52. Hou LX, Zhu D, Ma Q, Zhang DD, Liu X. H₂S synthetase AtD-CDes involves in ethylene and drought regulated stomatal movement. *Sci Bull.* 2016;61:1171–75.
53. Hou ZH, Wang LX, Liu J, Hou LX, Liu X. Hydrogen Sulfide Regulates Ethylene-induced Stomatal Closure in Arabidopsis thaliana. *J Integr Plant Biol.* 2013;55:277–89.
54. Torres MA, Dangl JL, and Jones JDG. Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. *PNAS.* 2002;99:517–22.
55. Sekiya J, Schmidt A, Wilson LG, Filner P. Emission of hydrogen sulfide by leaf tissue in response to L-cysteine. *Plant Physiol.* 1982;70:430–36.
56. Riemenschneider A, Nikiforova V, Hoefgen R, De Kok LJ, Papenbrock J. Impact of elevated H₂S on metabolite levels, activity of enzymes and expression of genes involved in cysteine metabolism. *Plant Physiol Biochem.* 2005;43:473–83.

Figures

Figure 1

Effects of HT, AOA, NH₂OH and C₃H₃KO₃+NH₃ on darkness-induced stomatal closure in wild-type leaves (a), and effects of darkness on stomatal movement in *Atl-cdes* and *Atd-cdes* mutants (b). Isolated epidermal strips were incubated in MES-KCl buffer alone or containing 15 μM HT, 0.4 mM AOA, 0.4 mM NH₂OH, and 0.4 mM C₃H₃KO₃+0.4 mM NH₃ in light(white columns) or in darkness (black columns) for 3 h, respectively. The data are presented as means±s.e.(n=90) of three independent experiments. Means denoted by different letters in (a), and (b) differ significantly at P<0.05 according to Duncan's multiple range test.

Figure 2

Effects of HT, NH₂OH, AOA, C₃H₃KO₃+NH₃ on darkness-induced H₂S content (a) and L-CDes activity (b) and D-CDes activity (c) in wild-type plants leaves. Fully expanded leaves of 4-6 weeks old seedlings were treated with MES-KCl buffer alone or containing 15 μ M HT, 0.4 mM NH₂OH, 0.4 mM AOA, and 0.4 mM C₃H₃KO₃+0.4 mM NH₃ in light (white columns) or in darkness (black columns) for 3 h, and then were used to measure H₂S content (a), L-CDes activity (b) and D-CDes activity (c). The data are presented as

the means±s.e. of three independent experiments (n=9). Means denoted by different letters in (a), (b), and (c) differ significantly at P<0.05 according to Duncan's multiple range test.

Figure 3

Effects of ASA, CAT, DPI, and SHAM on darkness-induced H₂S content (a), L-CDs activity (b), and D-CDs activity (c) in wild-type. Fully expanded leaves of 4-6 weeks olds seedlings were treated with MES-KCl buffer alone or containing 100 μM ASA, 100 units·mL⁻¹ CAT, 10 μM DPI, and 10 μM SHAM in light

(white columns) or in darkness (black columns) for 3 h, and then were used to measure H₂S content (a), L-CDes activity (b) and D-CDes activity (c). The data are presented as the means±s.e. of three independent experiments (n=9). Means denoted by different letters in (a), (b) and(c) differ significantly at P<0.05 according to Duncan's multiple range test.

Figure 4

Effects of darkness on H₂S content (a), L-CDes activity (b), and D-CDes activity (c) in the leaves of NADPH oxidase mutants *AtrbohD*, *AtrbohF* and *AtrbohD/F*. Fully expanded leaves of 4-6 weeks olds seedlings were treated with MES-KCl buffer alone in light (white columns) or in darkness (black columns) for 3 h, and then were used to measure H₂S content (a), L-CDes activity (b) and D-CDes activity (c). The data are presented as the means±s.e. of three independent experiments (n=9). Means denoted by different letters in (a), (b) and (c) differ significantly at P<0.05 according to Duncan’s multiple range test.

Figure 5

Effects of HT, NH_2OH , AOA, and $\text{C}_3\text{H}_3\text{K}_3\text{O}_3+\text{NH}_3$ on darkness-induced H_2O_2 production in guard cells of wild-type Guard cells were treated for 3h as follows: (a) with MES-KCl buffer alone in light; (b) MES-KCl buffer alone, (c) containing $15\text{ }\mu\text{M}$ HT, (d) 0.4 mM NH_2OH , (e) 0.4 mM AOA, and (f) 0.4 mM $\text{C}_3\text{H}_3\text{K}_3\text{O}_3+0.4\text{ mM}$ NH_3 in darkness. After treatments, epidermal strips were immediately loaded with $50\text{ }\mu\text{M}$ $\text{H}_2\text{DCF-DA}$ in Tris-KCl buffer in darkness for 10 min, then excess dye was removed and the strips were examined by laser-scanning confocal microscopy. (g) Average fluorescent intensity of guard cells in images (a–f), and (a')–(f') are enlarged graphs of (a)–(f). data of fluorescence pixel intensities are displayed as means $\pm$ s.e. of three independent experiments (n=9). Means in (g) denoted by different letters differed significantly at $P<0.05$ according to Duncan's multiple range test. Bars in (f) = $40\text{ }\mu\text{m}$ and in (f') = $25\text{ }\mu\text{m}$ for all images.

Figure 6

Effects of darkness on the level of H₂O₂ in guard cells of *Atl-cdes* and *Atd-cdes* mutants. Isolated epidermal strips of wild-type were incubated in MES-KCl buffer alone in light (a), or in darkness (b), *Atl-cdes* in light (c), or in darkness (d) and *Atd-cdes* in light (e), or in darkness (f). Three hours later, epidermal strips were immediately loaded with 50 μM H₂DCF-DA in Tris-KCl buffer in darkness for 10 min, then excess dye was removed and the strips were examined by laser-scanning confocal microscopy. (g)

Average fluorescent intensity of guard cells in images (a–f), and (a')–(f') are enlarged graphs of (a)–(f). data of fluorescence pixel intensities are displayed as means $\pm$ s.e. of three independent experiments (n=9). Means in (g) denoted by different letters differed significantly at $P<0.05$ according to Duncan's multiple range test. Bars in (f) = 40 μ m and in (f') = 25 μ m for all images.