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HYDROGEN SULFIDE GENERATED BY HYDROGEN PEROXIDE MEDIATES DARKNESS-INDUCED STOMATAL CLOSURE IN *ARABIDOPSIS THALIANA*

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Abstract

Hydrogen sulfide (H_2S) plays an important role in the regulation of stomatal movement in plants. Here we present the relationships and functions of H_2S and hydrogen peroxide (H_2O_2) in stomatal movement by darkness in *Arabidopsis thaliana*. H_2S synthesis inhibitors and scavengers inhibited darkness-induced stomatal closure, H_2S generation and L-/D-cysteine desulfhydrase (L-/D-CDes) activity increase in wild-type leaves. Darkness induced stomatal closure in wild-type plants, but failed in L-/D-CDes deletion mutants *Atl-cdes* and *Atd-cdes.* Additionally, both L-/D-CDes activity and H_2S content were significantly decreased after applying H_2O_2 synthesis inhibitors and scavengers, but there was almost no effects on H_2O_2 levels in the presence of H_2S synthesis inhibitors and scavengers in wild-type leaves in darkness. Moreover, darkness couldn't increase H_2S content and L-/D-CDes activity of mutant lines of NADPH oxidase gene *AtrbohF* and *AtrbohD/F* mutants leaves, but increased H_2O_2 levels in *Atl-cdes* and *Atd-cdes* guard cells. Taken together, we conclude that both H_2S and H_2O_2 are involved in darkness-induced stomatal closure, and H_2S functioned downstream of H_2O_2 in *A. thaliana*. Darkness induced H_2O_2 synthesis via promoting the activity of NADPH oxidase, and further led to the production of L-/D-CDes-derived H_2S and stomatal closure in *A. thaliana*. NADPH oxidase gene *AtrbohF* participated in the process.

Key words: Gasotransmitter, Stomatal movement, Darkness, *Arabidopsis*.

Abbreviations:

ABA	Abscisic acid;
ABC	ATP-binding cassette
AOA	Aminooxy acetic acid
ASA	Ascorbic acid
CAT	Catalase
CO	Carbon monoxide
DPI	Diphenylene iodonium
DTT	N, N-dimethyl-*p*-phenylenediamine dihydrochloride and dithiothreitol
H_2DCF-DA	2′, 7′-dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
HT	Hypotaurine
L-/D-CDes	L-/D-cysteine desulfhydrase
NaHS	Sodium hydrosulfide
$N_3H_3KO_3$	Potassium pyruvate
NH_3	Ammonia
NH_2OH	Hydroxylamine
NO	Nitric oxide
NOX	NADPH oxidase
ROS	Reactive oxygen species
SHAM	Salicylhydroxamic acid
UV-B	Ultraviolet-bradiation

Introduction

Hydrogen sulfide (H_2S), a novel gasotransmitter, has a similar function with carbon monoxide (CO) and nitric oxide (NO). Early research is devoted to its toxicity studies, while neglecting its physiological functions in plants and animals. Nowadays, H_2S is reported as a new type of gas signal molecule, which regulates many physiological processes of animals and plants. For example, in animals, H_2S is involved in brain development, regulation of heart and nervous system, diastolic cardiovascular and digestive tract smooth muscle (Wang, 2002; Kimura, 2002). Compared with the research degree in animals, the understanding of the function of H_2S in plant growth and development is far from enough. However, with the increasing attention of researchers, there are also many new discoveries about the physiological role of H_2S in plants. H_2S not only promotes the growth and development of plants, for example, increasing the seed germination rate, promoting the root development, enhancing the photosynthesis, prolonging the florescence and delaying senescence, but also enhances the resistance of plant to abiotic stresses (Zhang *et al*., 2009; Li *et al*., 2012a; Wang *et al*., 2012; Jin *et al*., 2013; Duan *et al*., 2015; Mostofa *et al*., 2015; Ding *et al*., 2019). In addition, it is also found that H_2S is an important signaling molecule regulating stomatal movement (García-Mata & Lamattina, 2010). H_2S interacts with abscisic acid (ABA) and participates in regulating stomatal movement in *Arabidopsis thaliana*, located upstream of ATP-binding cassette (ABC) transporter regulating stomatal closure by ABA (Jin *et al*., 2013). H_2S also participates in stomatal closure by ethylene, and NO acts upstream of it (Liu *et al*., 2012).

Hydrogen peroxide (H_2O_2), another kind of signal molecule in plant cells, is one of the main reactive oxygen species (ROS) produced in many metabolism processes with relatively stable molecular properties (Apel & Hirt, 2004). H_2O_2 is generated by enzymatic sources including cell wall peroxidases in plant cells, NADPH oxidases (NOX), or polyamine oxidases (Veal *et al*., 2007). A large number of studies have shown that plant tissue can resist all kinds of abiotic or biological stresses via the production of H_2O_2, including extreme temperature, ABA, ultraviolet-b radiation (UV-B), darkness, ethylene and bacterial invasion etc (Neill *et al*., 2002; Desikan *et al*., 2004; Larkindale & Huang, 2004; She *et al*., 2004; He *et al*., 2005; Desikan *et al*., 2006; He *et al*., 2017; Zhang *et al*., 2017). Moreover, H_2O_2 also mediates different physiological processes in plants, and resistance to adversity, defense response to pathogenic bacteria, gene expression, stomatal movement, and programmed cell death, all of which have important regulatory roles

(Potikha *et al*., 1999; Neill *et al*., 2002; Ren *et al*., 2002; Laloi *et al*., 2004; Li *et al*., 2007).

Both H_2O_2 and H_2S have been reported to mediate stomatal closure by darkness, and H_2S functions downstream of H_2O_2 during the process in *Vicia faba* (She *et al*., 2004; Ma *et al*., 2018). However, it is unclear that whether H_2S participates in stomatal closure by darkness in *A. thaliana*. The interaction between H_2S and H_2O_2 and their enzymatic pathways in the process needs to be elucidated. To address these questions, *A. thaliana* genotypes (*Atl-cdes*，*Atd-cdes*, *AtrbohD*, *AtrbohF*, *AtrbohD/F* mutants and wild-type) were adopted to investigate the significance and interactions between H_2S and H_2O_2 in stomatal closure by darkness.

Methods

Chemicals: The molecular probe H_2DCF-DA was bought from Biotium (Hayward, CA, USA), while 2-(*N*-morpholino) ethanesulfonic acid (MES), salicylhydroxamic acid (SHAM), potassium pyruvate ($C_3H_3KO_3$), aminooxy acetic acid (AOA), hypotaurine (HT), hydroxylamine (NH_2OH), catalase (CAT), ammonia (NH_3), diphenylene iodonium (DPI), D-cysteine, dimethyl sulfoxide (DMSO), ascorbic acid (ASA), L-cysteine, dithiothreitol (DTT) and *N*, *N*-dimethyl-*p*-phenylenediamine dihydrochloride were acquired from Sigma-Aldrich (Located in St Louis, MO, USA). Unless stated otherwise, the other chemicals were purchased from various Chinese suppliers with highest analytical grade.

Plant materials: *A. thaliana* ecotype Columbia (Col-0) was applied throughout this study. Seeds of L-/D-cysteine desulfhydras deletion mutants of *AtL-CDes* T-DNA insertion line (N541918, designated *Atl-cdes*), *AtD-CDes* T-DNA insertion line (CS853264, designated *Atd-cdes*), NADPH oxidase gene single mutant line (N9555, designated *AtrbohD* and N9557, designated *AtrbohF*), and homozygous transposon insertion double mutant line (N9558, designated *AtrbohD/F*) were provided by Nottingham *Arabidopsis* Stock Centre (NASC, Nottingham, UK). The mutants *Atd-cdes, Atl-cdes* and *AtrbohF, AtrbohD, AtrbohD/F* have been respectively identified by PCR and RT-PCR. 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, they were transferred in a controlled-environment chamber with a humidity of 80%, 16-h light/8-h dark cycle, and day/night temperature cycle of 22°C/18°C with a photon flux density of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR generated by cool white fluorescent tubes (Philips, New York, NY, USA). Fully expanded leaves were harvested at 4-6 weeks for immediate use.

Stomatal bioassays: Stomatal bioassay was performed as described by McAinsh *et al*., (1996) with minor modifications. The epidermal strips newly prepared were treated with MES-KCl buffer (10 mM MES, 50 mM KCl, 100 μM $CaCl_2$, pH 6.15) alone or containing various compounds or inhibitors in light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or darkness. And then the stomatal apertures were recorded by an optical microscope and eyepiece graticule previously calibrated with a stage micrometer. In each treatment, 30 randomly-selected apertures were scored per replicating and the treatment was repeated three times at least. The data provided are the mean ± s.e. of 90 measurements.

Measurement of H_2S emission: Measurement of H_2S emission was determined by the formation of methylene blue, which was performed as described by Hou *et al*., (2013) with slight modifications. Fully expanded leaves were utilized to measure H_2S emission. Firstly, the leaves were treated with MES-KCl buffer alone or containing various scavengers or synthesis inhibitors in light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or darkness for 3h, and then 0.1 g of them was taken for grinding by adding 0.9 mL 20 mM Tris-HCl (pH 8.0) buffer. After the centrifugation, the supernatant and a trap with 1% of zinc acetate were put into a test tube, and then the tube was quickly sealed with a Parafilm at the same time. Then 100 μL 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 M HCl and 100 μL 30 mM $FeCl_3$ dissolved in 1.2 M HCl were added into the trap after the absorption of H_2S for 30 min at 37°C. Finally, the absorbance was measured at 670 nm. In addition, a calibration curve was also drawn with known concentrations of Na_2S solution. Each treatment was repeated three times, and all the data presented are the mean ± s.e.

L-/D-cysteine desulfhydrase activity measurements: H_2S was determined to further study the activity of L-/D-cysteine desulfhydrase (L-/D-CDes), which was released from L-/D-cysteine within a certain period of time (Riemenschneider *et al*., 2005; Hou *et al*., 2013). The assay contained in the total volume of 1mL includes 100 μL 0.8 mM L-/D-cysteine, 400 μL 100 mM Tris-HCl, 400 μL 2.5 mM DTT, and 100 μL supernatant. Then 100 μL 20 mM *N*, *N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 M HCl and 100 μL 30 mM $FeCl_3$ dissolved in 1.2 M HCl were added into the trap after reaction for 30 min at 37°C. And the rate of H_2S released was presented by the determination of absorbance at 670 nm. Besides, the activity of L-CDes and D-CDes was also confirmed by the same method, but the pH of Tris-HCl buffer used previously was 8, and the latter was 9. Each treatment was repeated three times, and the data presented were the mean ± s.e.

Measurement of endogenous H_2O_2: H_2O_2 levels were measured with 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) by the method of Allan & Fluhr (1997) with minor modifications. In order to find the influence of H_2S scavenger and synthesis inhibitors on darkness-induced H_2O_2 production in guard cells, the epidermal strips were incubated in MES-KCl buffer alone in light or MES-KCl buffer alone or containing HT, AOA, NH_2OH and $C_3H_3KO_3$ + NH_3 in darkness for 3 h, and then immediately loaded with 50 μM H_2DCF-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 H_2O_2 levels in guard cells of *Atl-cdes* and *Atd-cdes* mutants, the epidermal strips were incubated in MES-KCl buffer alone in light or MES-KCl buffer alone in darkness for 3h, and then immediately loaded with 50 μM H_2DCF-DA in Tris-KCl buffer for 10 min in darkness. After that, 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 following settings: excitation 488 nm, emission 530 nm, power 10%, zoom about 4, normal scanning speed, and frame 512×512 pixels. Leica image software and Photoshop 7.0 (Adobe, San Jose, CA, USA) were used to analyze and process the images acquired. Each treatment was repeated at least three times. The depicted confocal images represent similar results from three replications.

Statistical analyses

The statistical importance of treatments was checked by one-way ANOVA as well as Duncan's multiple range test. The data was considered to be statistically important when *P*-values were below 0.05. All the figures were plotted by Origin 6.1 (Microcal Software, Nothampton, MA, USA) and processed with Photoshop 7.0 (Adobe, San Jose, CA, USA).

Results

Involvement of H_2S in stomatal closure by darkness

Influences of darkness on stomatal aperture in wild-type, *Atl-cdes* and *Atd-cdes*: To analyze whether H_2S mediates stomatal closure by darkness, the influences of H_2S synthesis inhibitors AOA, NH_2OH, $C_3H_3KO_3$+NH_3, H_2S scavenger HT and the producer of L-/D-cysteine desulfhydrase (L-/D-CDes) on stomatal aperture of wild-type (Col-0), as well as the influence of darkness on stomatal aperture of *Atl-cdes* and *Atd-cdes* which are T-DNA insertion lines were detected. Moreover, it is found that the presence of HT, AOA, NH_2OH, and $C_3H_3KO_3$+NH_3 inhibited darkness-induced stomatal closure. The stomatal closure was not induced when NH_2OH, AOA, HT, and $C_3H_3KO_3$+NH_3 were applied in light (As shown in Fig. 1a). Actually, *Atl-cdes* and *Atd-cdes* stomatal aperture could not be changed by darkness (Fig. 1b). From the results, we found that L-/D-CDes generated H_2S might participate into stomatal closure by darkness in *A. thaliana*.

Effects of H_2S modulators on darkness-induced L-/D-CDes activity and H_2S content in wild-type: To confirm the metabolic pathways participated in H_2S systhesis, L- and D-CDes activities and H_2S content in wild-type were determined. In fact, the content of H_2S in darkness was greatly 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, there was a remarkable increase in activity by darkness, and L- and D-CDes activities were reduced in the presence of HT, AOA, NH_2OH, and $C_3H_3KO_3$+NH_3 in darkness (Fig. 2b and 2c). However, no effect was observed when HT, AOA, NH_2OH, and $C_3H_3KO_3$+NH_3 were applied in light (Fig. 2a-c). These results suggested that darkness-induced H_2S biosynthesis might function via L- and/or D-CDes in *A. thaliana.*

Relationship between H_2O_2 and H_2S in stomatal closure by darkness

Effects of H_2O_2 modulators on L-/D-CDes activity, and H_2S content in darkness: For the sake of analyzing the relationship between H_2O_2 and H_2S during stomatal closure by darkness, H_2S content and L-and D-CDes activities in leaves of wild-type plants were examined with application of H_2O_2 synthesis inhibitors SHAM, DPI, and H_2O_2 scavenger ASA, CAT in darkness. Moreover, the treatment with SHAM, ASA, DPI, and CAT in darkness not only decreased H_2S content, but also reduced L- and D-CDes activities in wild-type (Fig. 3a-c). We proposed that both NADPH oxidase-derived and peroxidase-derived H_2O_2 might be a novel upstream component of H_2S signaling cascade during stomatal closure by darkness in *A. thaliana*.

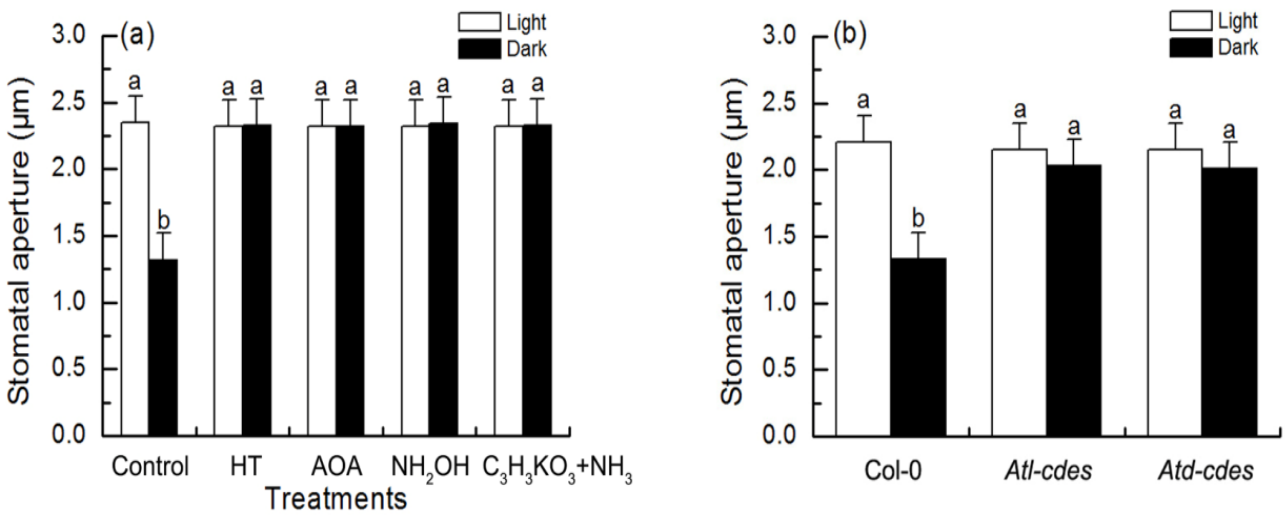

Fig. 1. Effects of HT, AOA, NH_2OH and $C_3H_3KO_3$+NH_3 on stomatal closure by darkness in wild-type (a), and effects of darkness on stomatal aperture in *Atl-cdes* and *Atd-cdes* (b). (a) Control: with MES-KCl buffer, 15 μM HT, 0.4 mM AOA, 0.4 mM NH_2OH, and 0.4 mM $C_3H_3KO_3$+0.4 mM NH_3 in light (white columns) or in darkness (black columns) for 3 h. (b) Col-0, *Atl-cdes* and *Atd-cdes* in light (white columns) or in darkness (black columns) for 3 h. Means in Fig.1a and 1b is from three in dependent determinations; different letters indicate significant differences (Duncan's multiple range test, $p<0.05$).

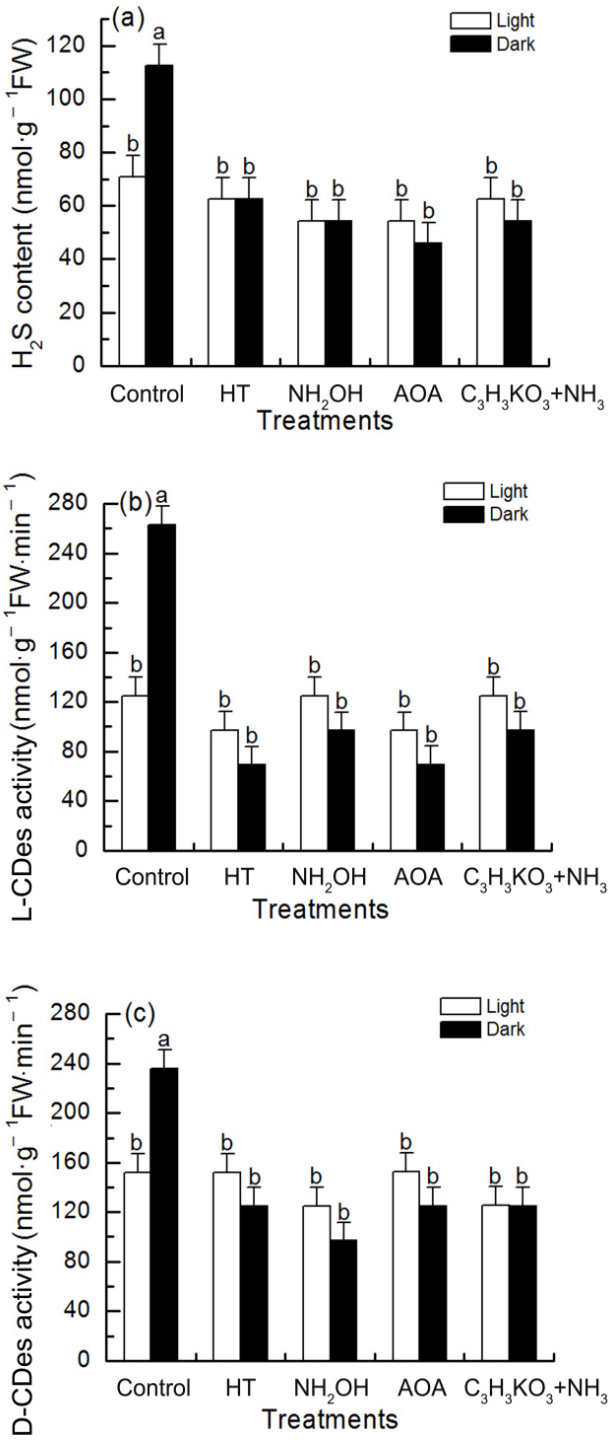

Fig. 2. Effects of HT, NH_2OH, AOA, $C_3H_3KO_3$+NH_3 on darkness-induced H_2S content (a), L-CDes activity (b) and D-CDes activity (c) in wild-type. (a-c) Control: with MES-KCl buffer, 15 μM HT, 0.4 mM NH_2OH, 0.4 mM AOA, and 0.4 mM $C_3H_3KO_3$+0.4 mM NH_3 in light (white columns) or in darkness (black columns) for 3 h. Further illustration in Fig. 2a-c is the semblable as in Fig. 1a.

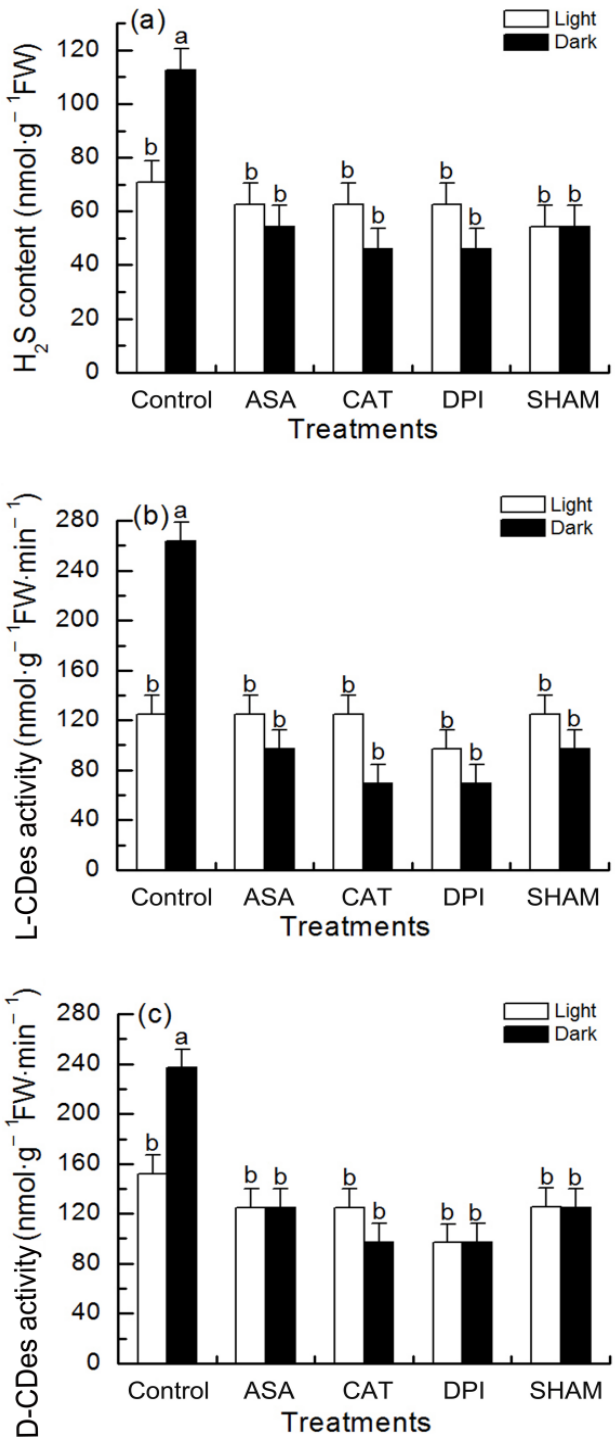

Fig. 3. Effects of ASA, CAT, DPI, and SHAM on darkness-induced H_2S content (a), L-CDes activity (b), and D-CDes activity (c) in wild-type. (a-c) Control: with MES-KCl buffer, 100 μM ASA, 100 units·mL^{-1} CAT, 10 μM DPI, and 10 μM SHAM in light (white columns) or in darkness (black columns) for 3h. Further illustration in Fig. 3a-c is the semblable as in Fig. 1a.

Influences of darkness on L-/D-CDes activity and H_2S content in *AtrbohD*, *AtrbohF*, and *AtrbohD/F*: To further investigate the interaction between H_2O_2 and H_2S, L- and D-CDes activities, as well as H_2S content, were detected in the leaves of *AtrbohD*, *AtrbohF*, and *AtrbohD/F*. H_2S content in leaves of wild-type and *AtrbohD* in darkness was apparently higher than that in the light, and L-and D-CDes activities of leaves was significantly enhanced as well (Fig. 4a-c). However, darkness couldn't induce any increase in H_2S content and L-and D-CDes activities in *AtrbohF* and *AtrbohD/F* (Fig. 4a-c). The results further suggested that H_2S acted downstream of H_2O_2 in darkness-regulated stomatal closure in *A. thaliana*.

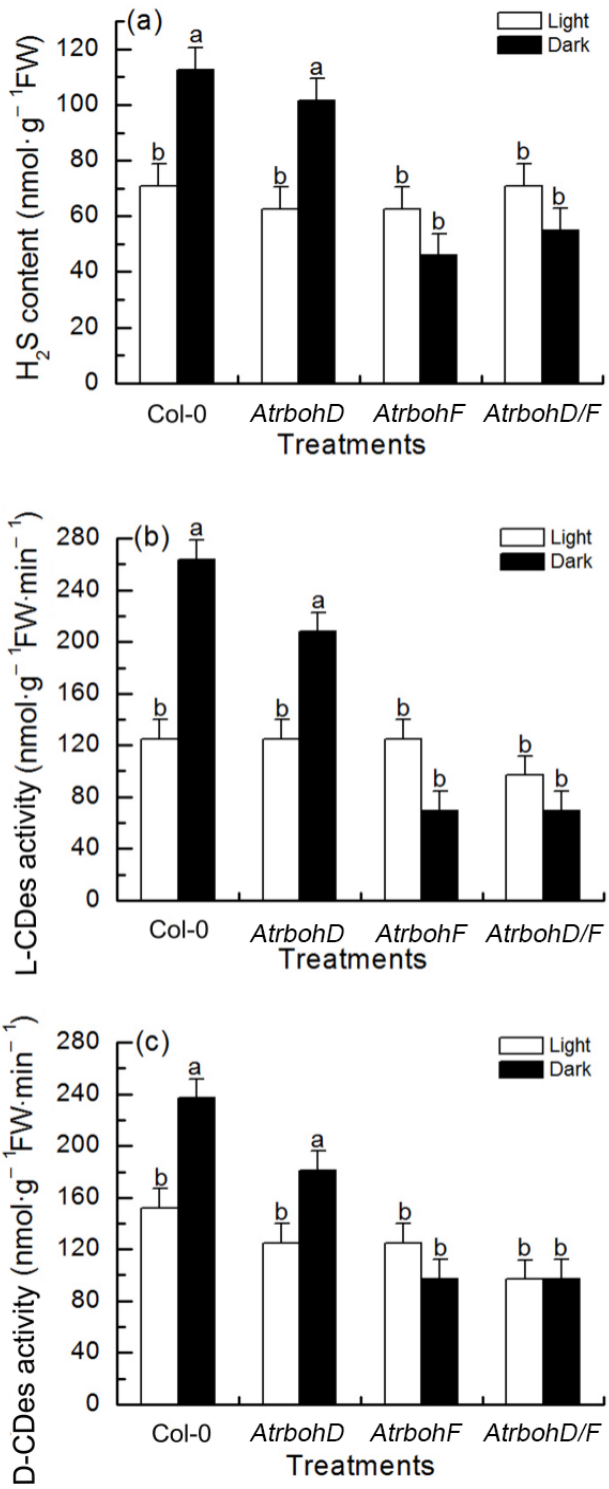

Fig. 4. Effects of darkness on H_2S content (a), L-CDes activity (b) and D-CDes activity (c) in the leaves of NADPH oxidase mutants *AtrbohD, AtrbohF* and *AtrbohD/F.* MES-KCl buffer alone in light (white columns) or in darkness (black columns) for 3 h. Further illustration in Fig. 4a-c is the semblable as in Fig. 1a.

Influences of H_2S modulators on the guard cells H_2O_2 levels by darkness in wild-type: For the sake of further validating the relationship between H_2O_2 and H_2S, the fluorescence of H_2O_2 in guard cells after using HT, NH_2OH, AOA, and $N_3H_3KO_3$+NH_3 in darkness was examined by H_2DCF-DA, which was previously used to measure the production of H_2O_2 in stomatal closure by darkness by Allan & Fluhr (1997). A great increase was caused by darkness in H_2O_2 levels of guard cells (Fig. 5b). However, treatment with HT, NH_2OH, AOA, and $N_3H_3KO_3$+NH_3 in darkness had no clear effects on H_2O_2 levels (Fig. 5c-f). Therefore, a fact was consolidated by these results, H_2S functioned downstream of H_2O_2 in stomatal closure by darkness in *A. thaliana.*

Influences of darkness on H_2O_2 production in *Atl-cdes* and *Atd-cdes*: To confirm that H_2S mediated darkness-induced stomatal closure as a downstream factor of H_2O_2, H_2O_2 production of *Atl-cdes* and *Atd-cdes* was detected. In darkness, H_2O_2 fluorescence in *Atl-cdes* and *Atd-cdes* guard cells were significantly stronger than that in light (Fig. 6c-f) without significant difference from the wild-type (Fig. 6a, b). Additionally, the above data further demonstrated that H_2S, as a downstream factor of H_2O_2 mediated darkness-induced stomatal closure in *A. thaliana.*

Discussion

Stomata are important structures to exchange gases and water in higher plants, which respond to different environmental factors by controlling their aperture. The mechanism of regulating stomatal movement is extremely complex. Other than the classical theory of cell turgor pressure, the stomatal closure is also affected by cytoplasmic calcium concentration changes, pH, protein phosphorylation, K^+ and anion channel regulation (Blatt & Grabov, 1997). During the continuous exploration of stomatal movement mechanism, some essential regulators of signal transduction emerged gradually, such as NO, H_2O_2, H_2S and so on.

As the third gasotransmitter, H_2S has become a new star in the transduction process of plant signal. Lisjak *et al*., (2011) reported that exogenous H_2S released by NaHS caused stomatal opening. However, García-Mata & Lamattina (2010) also reported that H_2S induced the stomatal closure in diferent plants. It has been shown that H_2S mediates ABA, ethylene and darkness-regulated stomatal movement (Liu *et al*., 2012; Jin *et al*., 2013; Ma *et al*., 2018), and that NO, CO, and intracellular calcium mediate stomatal movement by darkness (Schwartz, 1985; She *et al*., 2004; She & Song, 2008). However, the mechanism of H_2S in stomatal movement by darkness is unclear. Our results suggested that stomatal closure by darkness was significantly inhibited by H_2S scavenger HT, the inhibitors of H_2S biosynthesis NH_2OH and AOA, and the products of L- and/or D-cysteine desulfhydrase $C_3H_3KO_3$+NH_3 in wild-type *A. thaliana* (Fig. 1a). Darkness could induce stomatal closure in wild-type without influence on stomatal aperture of *Atl-cdes* and *Atd-cdes* (Fig. 1b). Additionally, HT, AOA, NH_2OH, and $C_3H_3KO_3$+NH_3 not only reduced the production of darkness-induced H_2S (Fig. 2a), but also diminished L-CDes activity (Fig. 2b) as well as D-CDes activity in darkness (Fig. 2c). From these results, we could find that H_2S was needed for darkness-regulated stomatal closure in *A. thaliana*, and L-/D-CDes was one of the key enzymes for its synthesis, which was consistent with the results of Ma *et al*. (2018) in *V. faba*.

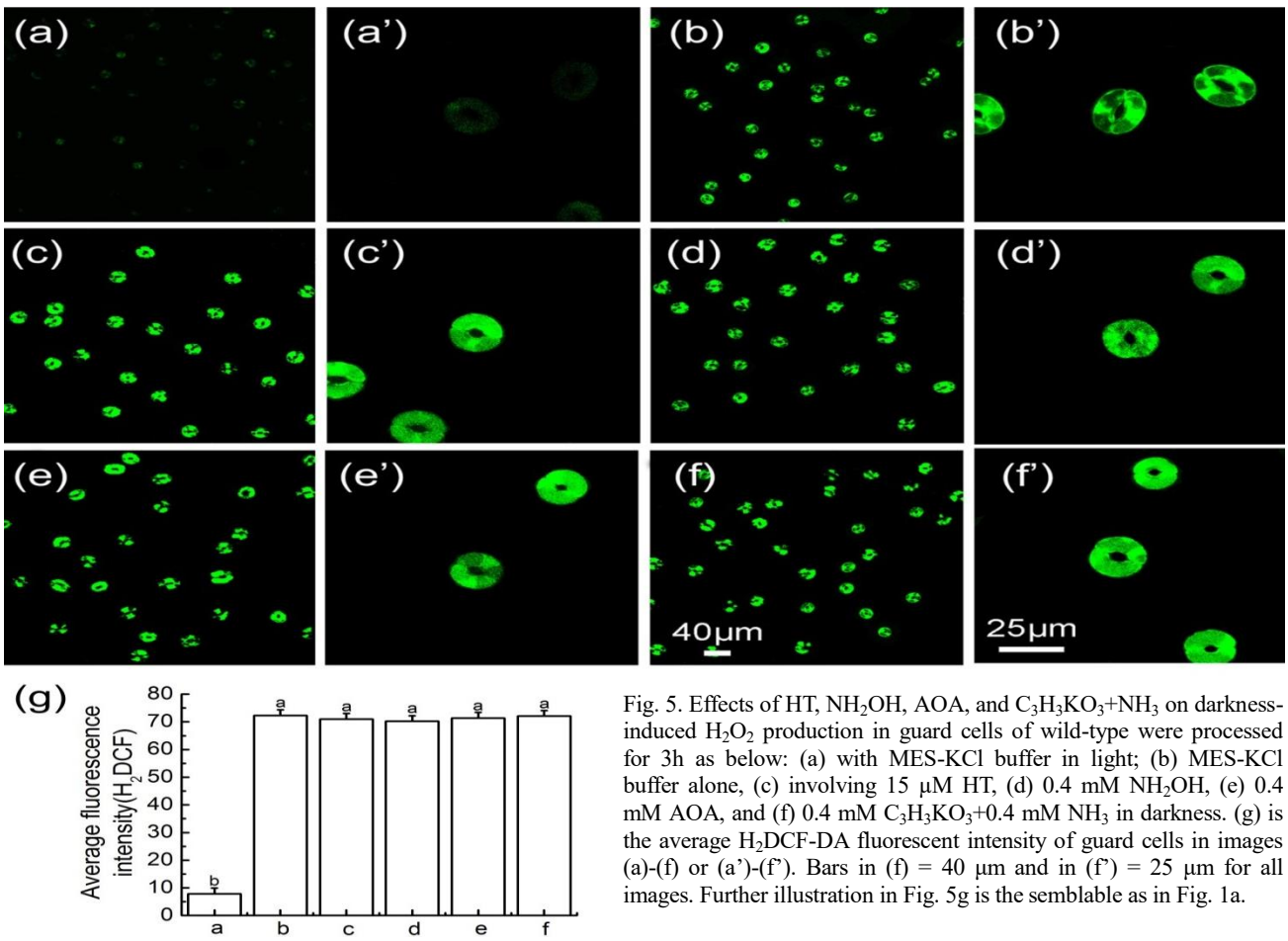

Fig. 5. Effects of HT, NH_2OH, AOA, and $C_3H_3KO_3$+NH_3 on darkness-induced H_2O_2 production in guard cells of wild-type were processed for 3h as below: (a) with MES-KCl buffer in light; (b) MES-KCl buffer alone, (c) involving 15 μM HT, (d) 0.4 mM NH_2OH, (e) 0.4 mM AOA, and (f) 0.4 mM $C_3H_3KO_3$+0.4 mM NH_3 in darkness. (g) is the average H_2DCF-DA fluorescent intensity of guard cells in images (a)-(f) or (a')-(f'). Bars in (f) = 40 μm and in (f') = 25 μm for all images. Further illustration in Fig. 5g is the semblable as in Fig. 1a.

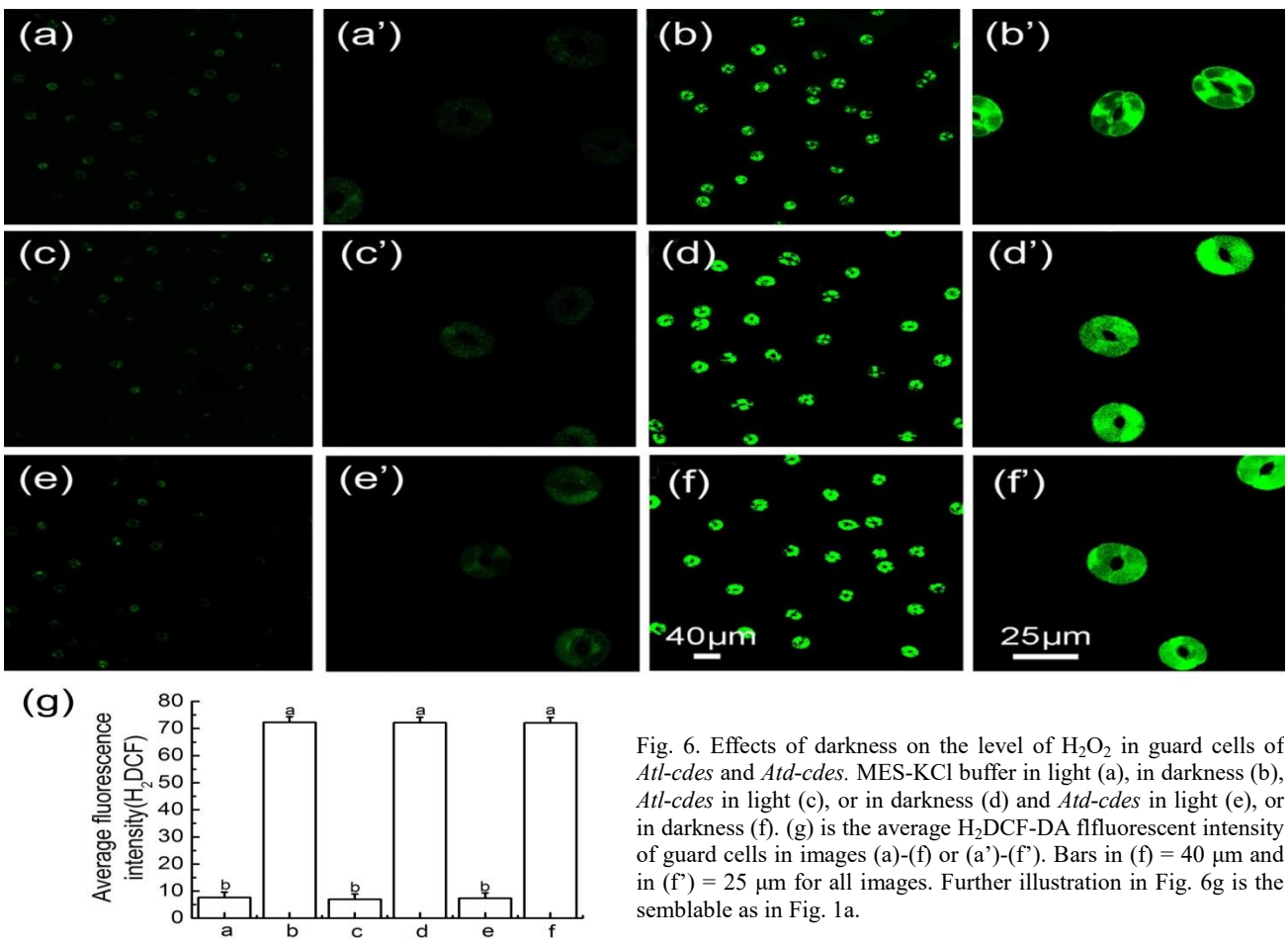

Fig. 6. Effects of darkness on the level of H_2O_2 in guard cells of *Atl-cdes* and *Atd-cdes.* MES-KCl buffer in light (a), in darkness (b), *Atl-cdes* in light (c), or in darkness (d) and *Atd-cdes* in light (e), or in darkness (f). (g) is the average H_2DCF-DA flfluorescent intensity of guard cells in images (a)-(f) or (a')-(f'). Bars in (f) = 40 μm and in (f') = 25 μm for all images. Further illustration in Fig. 6g is the semblable as in Fig. 1a.

Hydrogen peroxide (H_2O_2) is another important signal molecule involved in the signal transduction of plant cells. As the second messenger in plants, H_2O_2 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, and exogenous H_2O_2 could promote calcium level increase, leading to stomatal closure. For the past few years, deep research on the function of H_2O_2 has been carried out in stomatal movement. 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 (Allan & Fluhr, 1997; Pei *et al*., 2000; Neill *et al*., 2002; She *et al*., 2004; He *et al*., 2005; An *et al*., 2012, Yao *et al*., 2013; Shi *et al*., 2015; Ma *et al*., 2019a). In addition, H_2O_2 also mediates darkness-induced stomatal closure (Desikan *et al*., 2004; She *et al*., 2004; Ma *et al*., 2018). Data from Desikan *et al*., (2004) indicated that stomatal closure was related to H_2O_2 accumulation in guard cells of *pea*, and the large $gp91^{phox}$ subunit (rboh genes) encoding NOX was appeared in guard cells of *pea*, which probably participated in ABA responses and darknes. It has been reported that soaking with H_2O_2 could not only increase L-CDes activity, promote H_2S production, but also improve the germination rate of *Jatropha curcas* seeds (Li *et al*., 2012b). Li & He (2015) showed that H_2O_2 acted downstream of H_2S in seed germination of *Vigna radiata*. Wang *et al*., (2015) indicated that H_2O_2 was involved in exogenous H_2S-induced stomatal closure. Ma *et al*., (2019a) showed that H_2O_2 acted upstream of H_2S in NaCl-induced stomatal closure in *V. faba*. Additionally, H_2O_2 has been reported to function upstream of H_2S in $CdCl_2$-induced stomatal closure in *Vigna radiata* (Ma *et al*., 2019b). The latest research showed that H_2S induced by H_2O_2 mediates EBR-induced stomatal closure of *A. thaliana*, and H_2S production was catalyzed by AtL-CDes/AtD-Cdes and H_2O_2 production depended on AtrbohF (Ma *et al*., 2021). Our previous research H_2O_2 has been reported to function upstream of H_2S in darkness-induced stomatal closure in *V. faba*, and H_2S production is catalyzed by AtL-CDes/AtD-CDes, H_2O_2 production depends on NADPH oxidase and cell wall peroxidase (Ma *et al*., 2018). However, the interaction between H_2O_2 and H_2S is still unclear in darkness-induced stomatal closure in *A. thaliana*. The data displayed here suggested that H_2O_2 modulators ASA, CAT, DPI and SHAM could significantly inhibit the production of darkness-induced H_2S 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 had no significant effects on H_2O_2 levels of wild-type guard cells in darkness (Fig. 5). Compared with light treatment, there was no significant effect of darkness on H_2S content and L-/D-CDes activity in *AtrbohF* and *AtrbohD/F* leaves (Fig. 4), but could increase H_2O_2 levels in *Atl-cdes* and *Atd-cdes* (Fig. 6). These data indicated that H_2O_2 acts upstream of H_2S in stomatal closure induced by darkness in *A. thaliana*, and H_2S production was catalyzed by AtL-CDes/AtD-CDes, H_2O_2 production depends on cell wall peroxidase and NADPH oxidase gene AtrbohF. Our results are consistent with the previous reports (Ma *et al*., 2018).

Conclusions

In this study, we explored the interaction between H_2O_2 and H_2S in stomatal closure by darkness. Our results indicated that darkness induced H_2O_2 synthesis via promoting the activity of NADPH oxidase and peroxidase, and further led to the production of L-/D-CDes-derived H_2S and stomatal closure in *A. thaliana*. In fact, our work not only enriches the signal transduction network to regulate the stomatal movement induced by darkness, but also provides experimental evidences in plant physiology, cell biology and genetics for the interaction between H_2O_2 and H_2S in stomatal movement.

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