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HYDROGEN SULFIDE INDUCED BY HYDROGEN PEROXIDE MEDIATES STRIGOLACTONES-TRIGGERED STOMATAL CLOSURE IN *ARABIDOPSIS THALIANA*

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Abstract

The function of hydrogen sulfide (H_2S) and interaction with hydrogen peroxide (H_2O_2) during stomatal movement caused by strigolactones (SLs) in *Arabidopsis thaliana* were examined. SL triggered stomatal closure and induced rise in H_2S content, L-cysteine desulfhydrase (L-/D-CDes) and D-cysteine desulfhydrase (D-CDes) activity increase in leaves of the wild type, but these effects were prohibited by H_2S scavenger or synthesis inhibitors. However, SL couldn't significantly change stomatal aperture in *Atl-cdes* and *Atd-cdes* mutants. Moreover, H_2O_2 scavengers and synthesis inhibitor obviously prohibited SL-caused H_2O_2 production and stomatal closure of the wild type, but SL couldn't close stomata and couldn't cause H_2O_2 production of guard cells in *AtrbohD*, *AtrbohF* and *AtrbohD*/*F* mutants. Furthermore, H_2O_2 scavengers and synthesis inhibitor prevented SL-caused H_2S synthesis, L-CDes and D-CDes activity increase in leaves of the wild type, but H_2S scavenger or synthesis inhibitors couldn't inhibit SL-caused H_2O_2 synthesis. SL increased H_2O_2 levels in *Atl-cdes* and *Atd-cdes* mutants' guard cells. However, SL couldn't cause H_2S synthesis, L-CDes and D-CDes activity increase in leaves of *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants. All the data suggested that SLs-caused stomatal closure required H_2O_2 synthesis derived from NADPH oxidase and subsequent L-/D-CDes-sourced H_2S production.

Key words: Strigolactones, Stomatal closure, Hydrogen sulfide, Hydrogen peroxide.

Abbreviations: HT = hypotaurine; AOA = aminooxy acetic acid; NH_2OH = hydroxylamine; $C_3H_3KO_3$ = potassium pyruvate; NH_3 = ammonia; ASA = ascorbic acid; DPI = diphenylene iodonium; CAT = catalase; DTT = dithiothreitol; L-/D-Cdes = L-/D-cysteine desulfhydrase; H_2S = hydrogen sulfide; H_2O_2 = hydrogen peroxide

Introduction

Strigolactones (SLs) have been firstly proved to be able to stimulate seed germination (Shaw *et al*., 1962; Cook *et al*., 1966). Subsequently, it has been shown that SLs play a key role in hyphal branching and root colonization (Akiyama *et al*., 2005; Besserer *et al*., 2006). SLs, as a kind of signal component, are indicated to be one of hormones produced by root and can move to shoot and inhibit branch of root (Gomez-Roldan *et al*., 2008). Moreover, SLs have been indicated to mediate various processes including photomorphogenesis, stomatal movement, etc. (Shen *et al*., 2007; Lv *et al*., 2017). SLs also mediate responses to many stimuli (Ha *et al*., 2014; Liu *et al*., 2015). Some evidence showed that SLs and abscisic acid (ABA) interact in adapting abiotic stresses (Ha *et al*., 2014; Cardinale *et al*., 2018). Furthermore, SLs-caused stomatal closure required hydrogen peroxide (H_2O_2), nitric oxide (NO), and SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) anion channel activation in an abscisic acid (ABA)-independent way (Lv *et al*., 2017).

More evidence indicates that hydrogen sulfide (H_2S) is a gas acting as a signaling molecule, which is the same as carbon monoxide (CO) and NO (Wang, 2002). In animals, H_2S participates in regulating many physiological processes, such as regulating nervous system and cardiac function, relaxing blood vessels and smooth muscle of digestive tract, etc (Linden *et al*., 2010; Kimura *et al*., 2011). In plants, H_2S synthesis mainly depends on the catalyzed reaction of L-/D-cysteine desulfhydrase (L-/D-CDes) (Papenbrock *et al*., 2007). H_2S mediates the responses to environmental stresses (Cui *et al*., 2014; Zhou *et al*., 2020). It has been shown that H_2S can regulate many biological processes including photosynthesis, stomatal movement, etc. (Chen *et al*., 2011; Ma *et al*., 2018a, 2018b). H_2S has been proved to participate in stomatal movement induced by ABA, salt stress and BR, etc. (Wang *et al*., 2012a; Hou *et al*., 2013a; Ma *et al*., 2018a, 2018b, 2021, 2022). H_2O_2-mediated H_2S synthesis participates in stomatal closure caused by ethylene, ABA and salt stress, etc. (Hou *et al*., 2013b; Ma *et al*., 2018a, 2018b, 2021, 2022).

It has been indicated that high concentration of H_2O_2 causes damage to plants, and low concentration of H_2O_2 acts as a signaling molecule, which functions in responses to various stresses and regulates many physiological processes in plants (Neill *et al*., 2002; Li *et al*., 2007; Jin *et al*., 2013; Li & He, 2015). Moreover, H_2O_2, as an important signal component, mediates darkness-, ABA-, SLs-, salt stress-, and 2, 4-epibrassinolide (EBR, a bioactive BR)-causes stomatal closure (Desikan *et al*., 2004; Lv *et al*., 2017; Ma *et al*., 2018a, 2021). BR can close stomata by causing AtrbohF-dependent H_2O_2 synthesis and Nia1-derived NO production (Shi *et al*., 2015). Darkness-caused stomatal closure requires AtRBOHD/F-derived H_2O_2 synthesis and NO production (Zhang *et al*., 2017). SLs-triggered stomatal closure is proved to be dependent on H_2O_2 production and NO synthesis (Lv *et al*., 2017). Until now, it is unknown whether H_2S functions in SLs-triggered stomatal movement, and it is still unclear whether H_2S interacts with H_2O_2 in the process. This study provided strong evidence that H_2S participates in SLs-triggered stomatal closure in *Arabidopsis thaliana*, H_2O_2 synthesis mediates H_2S production in the physiological process.

Material and Methods

Chemicals: The molecular probe H_2DCF-DA was purchased from Biotium (Hayward, CA, USA), and 2-(N-morpholino) ethanesulfonic acid (MES), (3aR*,8bS*,E)-3-(((R*)-4-methyl-5-oxo-2, 5-dihydrofuran-2-yloxy) methylene)-3, 3a, 4, 8b-tetrahydro-2H-indeno [1, 2-b]furan-2-one (GR24), L-cysteine, D-cysteine, hypotaurine (HT), aminooxy acetic acid (AOA), hydroxylamine (NH_2OH), potassium pyruvate ($C_3H_3KO_3$), ammonia (NH_3), ascorbic acid (ASA), diphenylene iodonium (DPI), catalase (CAT), dithiothreitol (DTT) and *N*, *N*-dimethyl-*p*-phenylenediamine dihydrochloride were obtained from Sigma-Aldrich. Unless stated otherwise, all other chemicals were of the highest analytical grade purchased from various Chinese suppliers.

Plant materials: Seeds of *A. thaliana* ecotype Columbia (Col-0) were used in 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*), 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). Seeds were surface-sterilized and sown on sterilized vermiculite. Seeds were stratified in darkness for 2-4 d at 4°C, placed in a growth chamber at a day/night temperature cycle of 22°C/18°C, 80% RH, and 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ under a 16 h /8 h day/night photoperiod, after growing four euphylla, seedlings were potted in soil and continued to grow. The epidermis was carefully peeled from the abaxial surfaces of the youngest, fully expanded leaves of 4-6-week-old seedlings.

Stomatal bioassays: Stomatal bioassays were performed as described by McAinsh *et al*., (1996) with minor modifications. Briefly, the freshly prepared epidermal strips were incubated in MES-KCl buffer (10 $mmol \cdot L^{-1}$ MES, 50 $mmol \cdot L^{-1}$ KCl, 0.1 $mmol \cdot L^{-1}$ $CaCl_2$, pH 6.15) for 3 h at 22 ± 2°C in light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in order to promote stomatal opening. After incubation the epidermal strips were transferred to MES-KCl buffer or GR24 alone or MES-KCl containing various compounds or inhibitors in light for 3 h at 22 ± 2°C and then the stomatal apertures were recorded with light microscope and eyepiece graticule previously calibrated with a stage micrometre. In each treatment, we scored 30 randomly selected apertures per replicating and treatments were repeated at least three times. The data presented are the means ± s.e. of 90 measurements.

Measurement of H_2S emissions: Measurement of H_2S emission was determined by the formation of methylene blue, which was performed as described by Sekiya *et al*., (1982) and Hou *et al*., (2013a) with slight modifications. Fully expanded leaves of 4-5-week-old seedlings in the wild type, *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants were used to measure the emission of H_2S. Firstly, 0.1 g leaves were cut and treated with MES-KCl buffer or containing GR24 alone or various scavengers or synthesis inhibitors in light (100 $\mu mol\ m^{-2}\ s^{-1}$) at 22 ± 2°C for 3 h, and then the leaves were taken out and ground by addition of 0.9 mL 20 $mmol \cdot L^{-1}$ Tris-HCl (pH 8.0) buffer. After centrifugation, the supernatant and a trap with 1% of zinc acetate were put into a test tube and sealed quickly with a Parafilm. After H_2S was absorbed for 30 min at 37°C, 100 µL 20 $mmol \cdot L^{-1}$ N, N-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 $mol \cdot L^{-1}$ HCl and 100 µL 30 $mmol \cdot L^{-1}$ $FeCl_3$ dissolved in 1.2 $mol \cdot L^{-1}$ HCl were added into the trap. Finally, the absorbance was measured at 670 nm, and a calibration curve was made with known concentrations of Na_2S solution. Each treatment was repeated three times, and the data presented are the means ± s.e.

L-/D-cysteine desulfhydrase activity measurements: To further investigate the activity of L-/D-CDes, we determined H_2S released from L-/D-cysteine within a certain period of time (Riemenschneider *et al*., 2005; Hou *et al*., 2013a). 0.1 g leaves were cut and ground using a mortar and pestle. The soluble proteins were extracted over a period of 4 h by addition of 0.9 mL of 20 mmol/L Tris-HCl (pH 8.0) to 0.1 g plant material. After centrifugation, the protein content of the supernatant was adjusted to 100 µg/mL to provide an equal amount of protein in each assay sample. L-cysteine desulfhydrase activity was measured by the release of H_2S from L-cysteine in the presence of DTT. The 1 mL assay included: 100 µL 0.8 $mmol \cdot L^{-1}$ L-/D-cysteine, 400 µL 2.5 $mmol \cdot L^{-1}$ DTT, 400 µL 100 $mmol \cdot L^{-1}$ Tris-HCl, and 100 µL supernatant. Then 100 µL 20 $mmol \cdot L^{-1}$ N, N-dimethyl- *p*-phenylenediamine dihydrochloride dissolved in 7.2 $mol \cdot L^{-1}$ HCl and 100 µL 30 $mmol \cdot L^{-1}$ $FeCl_3$ dissolved in 1.2 $mol \cdot L^{-1}$ HCl were added into the trap after reacting for 30 min at 37°C. The rate of released H_2S was indicated by the determination of absorbance at 670 nm. Moreover, the activities of L-CDes and D-CDes were determined by the same method, but the pH of Tris-HCl buffer used by the former and latter was 9 and 8, respectively. Each treatment was repeated for three times.

Measurement of endogenous H_2O_2: H_2O_2 levels were measured with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) using the method by Allan & Fluhr (1997) with minor modifications. To study the effects of H_2O_2 and H_2S scavengers and synthesis inhibitors on GR24-induced H_2O_2 production in guard cells of the wild type, the epidermal strips were incubated in MES-KCl buffer or GR24 alone, or containing ASA, CAT, DPI, HT, AOA, NH_2OH, and $C_3H_3KO_3$ + NH_3 at 22 ± 2°C in light for 3 h, and then immediately loaded with 50 $\mu mol \cdot L^{-1}$ H_2DCF-DA in Tris-KCl buffer at 25 ± 2°C in darkness for 10 min. To study the effects of GR24 on H_2O_2 levels of guard cells in *AtrbohD*, *AtrbohF* and *AtrbohD/F*, *Atl-cdes* and *Atd-cdes* mutants, the epidermal strips were incubated in MES-KCl buffer or GR24 alone at 22 ± 2°C in light for 3 h, and then immediately loaded with 50 $\mu mol \cdot L^{-1}$ H_2DCF-DA in Tris-KCl buffer (10 $mmol \cdot L^{-1}$ Tris, 50 $mmol \cdot L^{-1}$ KCl, pH 7.2)

at 25 ± 2°C in darkness for 10 min. The excessive dye was washed off with fresh Tris-KCl loading buffer, and the epidermal strips were immediately examined under luminescence microscope (OLYMPUS BX53, U-RFLT50, JAPAN) with following settings: 450 nm of excitation, 490 nm of emission. 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 three times. The images depicted here represent similar results from all three replications.

Statistical analyses

All data were taken from at least three independent experiments. 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 <0.05. 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

SLs close stomata in *A. thaliana*: To explore if SLs can close stomata of *A. thaliana*, we observed the effects of GR24, a synthetic analogue of SLs, on stomatal aperture of the wild type. GR24 could close stomata, the best effects on stomata under GR24 treatment were 1 μmol·L^{-1} and 3 h, respectively (Fig. 1a). Fig. 1b showed that the influence of GR24 to stomata was not irreversible effects from an elution treatment. The data suggested that SLs could close stomata of *A. thaliana*.

H_2S mediates SL-triggered stomatal closure: To declare if H_2S mediates SL-triggered stomatal closure, the effects of H_2S modulators on GR24-caused stomatal closure, H_2S synthesis and L-/D-CDes activity change of leaves in the wild type were measured. HT (a H_2S scavenger), AOA and NH_2OH (H_2S synthesis inhibitors), and $C_3H_3KO_3$ and NH_3 (the producer of L-/D-CDes) significantly prevented GR24-triggered stomatal closure, and AOA, NH_2OH, and $C_3H_3KO_3$ and NH_3 prohibited rise in H_2S content of leaves (Fig. 2a-d, Fig. 3a). Moreover, GR24 significantly induced L-CDes and D-CDes activity increase, the effects were significantly prohibited by AOA, NH_2OH, and $C_3H_3KO_3$ and NH_3 (Fig. 3b, c). However, treatment with AOA, NH_2OH, and $C_3H_3KO_3$ and NH_3 alone couldn't increase H_2S content, L-CDes and D-CDes activity of leaves (Fig. 3a-c). The data suggested that H_2S synthesis might mediate SL-triggered stomatal closure, and H_2S synthesis depended on L-CDes and D-CDes.

To further examine whether H_2S synthesis mediated SL-triggered stomatal closure, we determined the effects of GR24 on *Atl-cdes* and *Atd-cdes* mutants' stomata. Fig. 4 showed that GR24 triggered stomatal closure in the wild type, while it couldn't significantly change stomatal aperture of *Atl-cdes* and *Atd-cdes* mutants, suggesting that H_2S synthesis really mediated SL-triggered stomatal closure, and H_2S synthesis was dependent on both AtL-CDes and AtD-CDes in the process.

H_2O_2 mediates SL-triggered stomatal closure: To study whether H_2O_2 regulates SL-triggered stomatal closure, the effects of H_2O_2 modulators including ASA (a substrate for H_2O_2 removal), CAT (a H_2O_2 scavenger enzyme) and DPI (an inhibitor of NADPH oxidase) on GR24-iuduced stomatal closure in the wild type were analyzed. H_2O_2 modulators all significantly prohibited GR24-triggered stomatal closure (Fig. 5a). Subsequently, we detected levels of H_2O_2 in guard cells of the wild type by using special H_2O_2 detection fluorescence dye H_2DCF-DA. In agreement with the epidermal bioassay, H_2O_2 production of guard cells induced by GR24 was significantly abolished by H_2O_2 scavengers and synthesis inhibitor (Fig. 6a-e). From the results, we inferred that H_2O_2 might participate in SL-triggered stomatal closure.

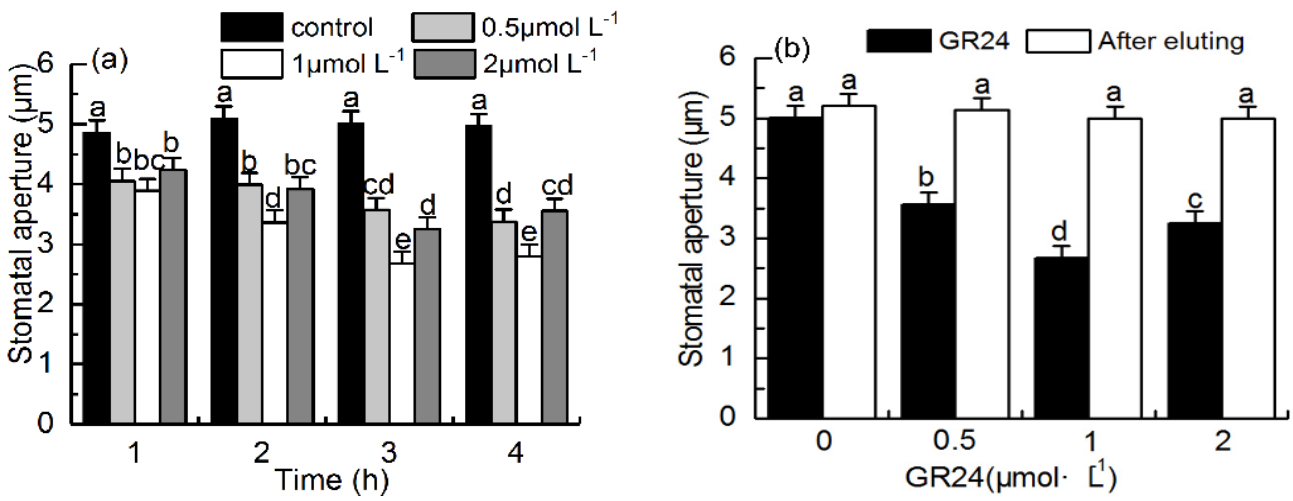

Fig. 1. Effects of GR24 on stomatal aperture in the wild type. (a) Isolated epidermal strips were incubated in MES-KCl buffer alone or containing different concentrations of GR24 in light at 22 ± 2°C for 1 h, 2 h, 3 h and 4 h, then stomatal apertures were recorded. (b) After the incubation in MES-KCl buffer containing different concentrations of GR24 in light for 3 h (black columns), the strips were treated with MES-KCl buffer in light at 22 ± 2°C for another 3 h (white columns), then apertures were measured. The data are presented as means ± s.e. of three independent experiments (n = 90), and means denoted by different letters in (a) and (b) differ significantly at p<0.05 according to Duncan's multiple range test.

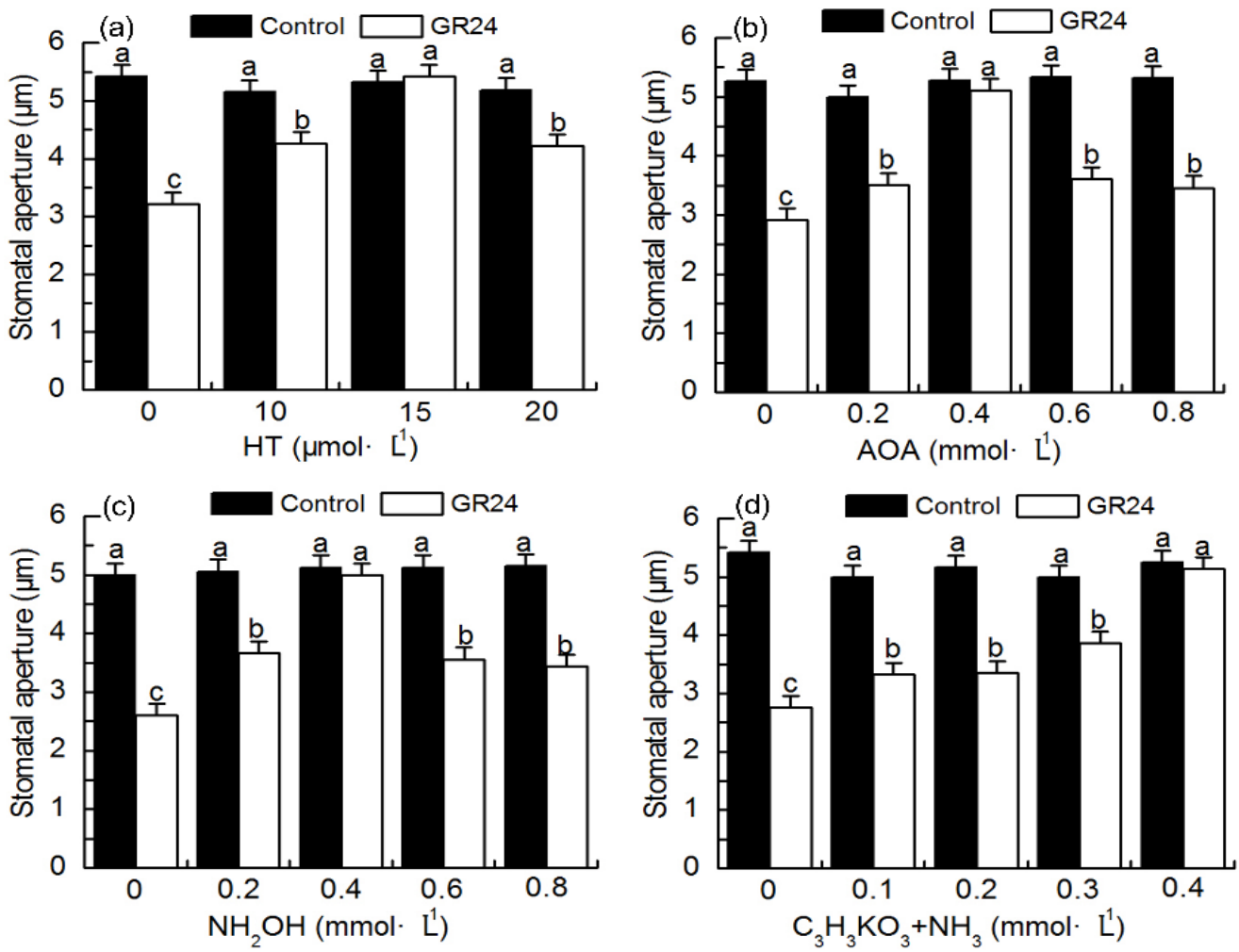

Fig. 2. Effects of HT, AOA, NH_2OH, and $C_3H_3KO_3$ + NH_3 on GR24-induced stomatal closure in the wild type. Isolated epidermal strips were incubated in MES-KCl buffer alone, or containing (a) HT (0, 10, 15, 20 μmol·L^{-1}), (b) AOA (0, 0.2, 0.4, 0.6, 0.8 mmol·L^{-1}), (c) NH_2OH (0, 0.2, 0.4, 0.6, 0.8 mmol· L^{-1}, and (d) $C_3H_3KO_3$ (0, 0.1, 0.2, 0.3, 0.4 mmol·L^{-1}) + NH_3 (0, 0.1, 0.2, 0.3, 0.4 mmol·L^{-1}) in the absence (black columns) or presence of 1 μmol·L^{-1} GR24 (white columns) in light at 22 ± 2°C for 3 h, respectively, then apertures were measured. Data presented are means ± s.e. of three independent experiments (n=90). See Fig. 1a for further experimental details.

To further confirm that H_2O_2 functions in SL-triggered stomatal movement signal network, we measured stomatal aperture and H_2O_2 levels in guard cells of the wild type, *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants after GR24 treatment. GR24 closed stomata of the wild type, but GR24 couldn't close stomata of *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants (Fig. 5b). Moreover, GR24 obviously increased H_2DCF fluorescence in guard cells of the wild type, but failed to stimulate H_2O_2 production in *AtrbohD*, *AtrbohF* and *AtrbohD/*F mutants (Fig. 6b, f-h). From the data, it was concluded that AtrbohD- and AtrbohF-catalyzed H_2O_2 synthesis participated in SL-triggered stomatal closure.

H_2S interacted with H_2O_2 during SL-triggered stomatal closure

H_2O_2 modulators prohibited SL-caused rise in H_2S content and L-/D-CDes activity increase: To declare the interrelationship between H_2S and H_2O_2 during SLs-triggered stomatal closure, we firstly examined the effects of H_2O_2 modulators ASA, CAT, and DPI on GR24-caused rise of H_2S synthesis, L-CDes and D-CDes activity change in leaves of the wild type. As shown in Fig. 7, GR24 could cause H_2S synthesis and increased L-CDes and D-CDes activity, these effects were significantly prohibited by ASA, CAT, and DPI (Fig. 7a-c). The data showed that H_2O_2 synthesis might mediate SL-triggered stomatal closure by inducing H_2S production.

SL couldn't increase H_2S level, L-CDes and D-CDes activity of leaves in *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants: To further confirm H_2O_2 synthesis mediated SL-triggered stomatal closure by inducing H_2S production, the effects of GR24 on H_2S level, L-CDes and D-CDes activity in *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants were detected. GR24 could increase H_2S level, L-CDes and D-CDes activity, but the effects observed in the wild type were blocked in *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants (Fig. 8a-c). Our data provided evidence that H_2O_2 synthesis mediated SL-triggered stomatal closure by inducing H_2S production.

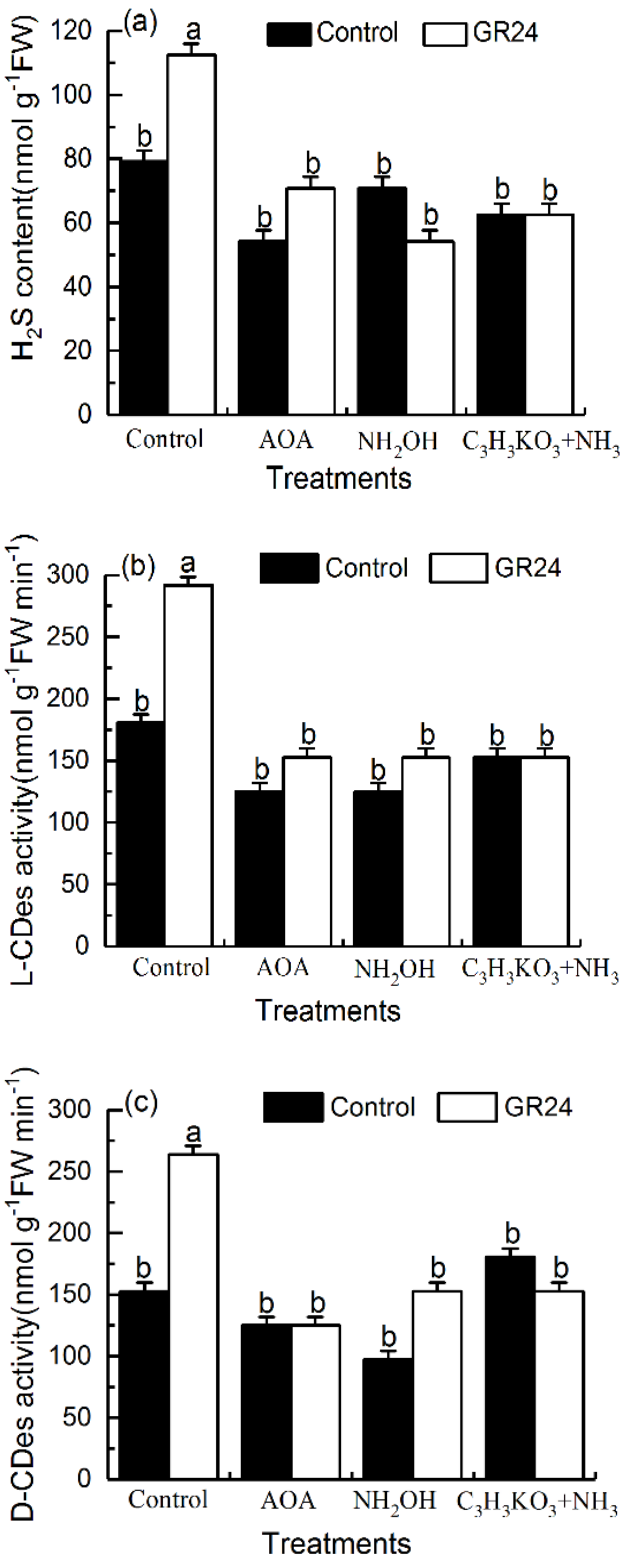

Fig. 3. Effects of AOA, NH_2OH, and $C_3H_3KO_3$ + NH_3 on GR24-induced H_2S synthesis, L-CDes activity and D-CDes activity change of leaves in the wild type *A. thaliana*. Isolated epidermal strips were incubated in MES-KCl buffer alone, or containing 0.4 mmol·L^{-1} AOA, 0.4 mmol·L^{-1} NH_2OH, and 0.4 mmol·L^{-1} $C_3H_3KO_3$ + 0.4 mmol·L^{-1} NH_3 in the absence (black columns) or presence of 1 μmol·L^{-1} GR24 (white columns) in light at 22 ± 2°C for 3 h, respectively, then H_2S content (a), L-CDes activity (b), and D-CDes activity (c) were measured. Data presented means ± s.e. of three independent experiments (n=9). See Fig. 1a for further experimental details.

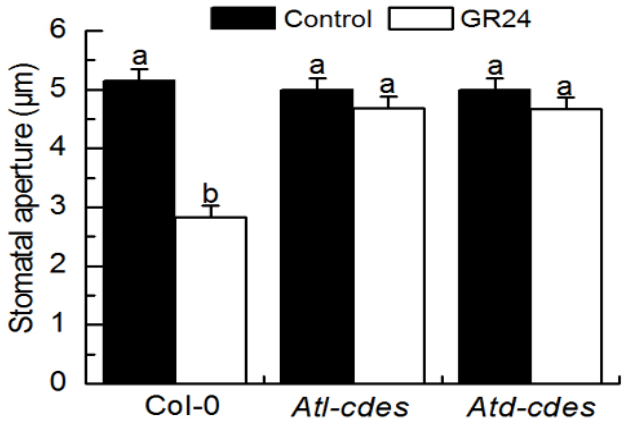

Fig. 4. Effects of GR24 on stomatal movement in the wild type, *Atl-cdes* and *Atd-cdes* mutants. Isolated epidermal strips of the wild type, *Atl-cdes* and *Atd-cdes* mutants were incubated in MES-KCl buffer alone (black columns) or containing 1 μmol·L^{-1} GR24 in light at 22 ± 2°C for 3 h (white columns), respectively, then apertures were measured. Data presented means ± s.e. of three independent experiments (n=90). See Fig. 1a for further experimental details.

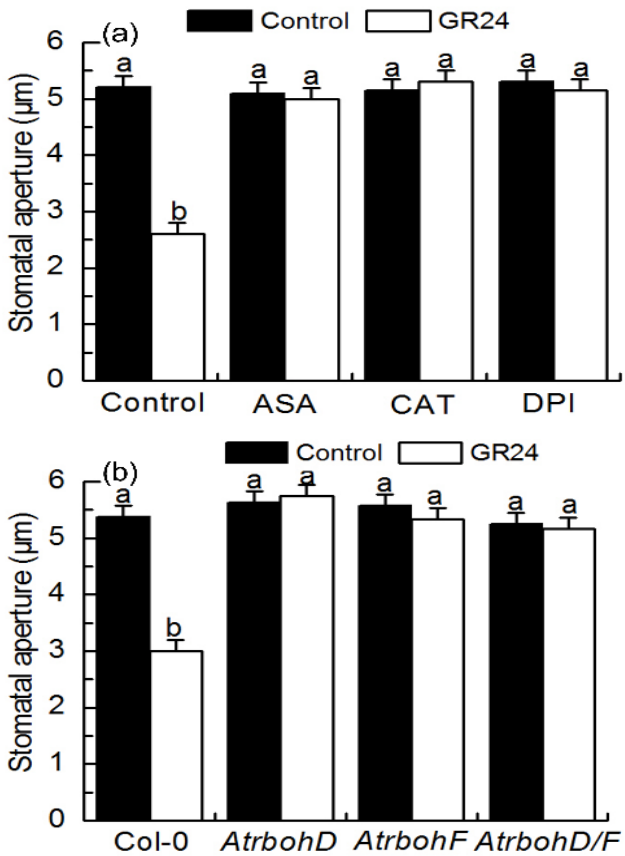

Fig. 5. Effects of ASA, CAT, and DPI on GR24-induced stomatal closure in the wild type (a), and effects of GR24 on stomatal movement in the wild type, *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants (b). (a) Isolated epidermal strips were incubated in MES-KCl buffer alone, or containing 100 μmol·L^{-1} ASA, 100 units·mL^{-1} CAT and 10 μmol·L^{-1} DPI in the absence (black columns) or presence of 1 μmol·L^{-1} GR24 (white columns) in light at 22 ± 2°C for 3 h, respectively, then apertures were measured. (b) Isolated epidermal strips of the wild type, *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants were incubated in MES-KCl buffer alone (black columns) or containing 1 μmol·L^{-1} GR24 in light at 22 ± 2°C for 3 h (white columns), respectively, then apertures were measured. Data presented means ± s.e. of three independent experiments (n=90). See Fig. 1a for further experimental details.

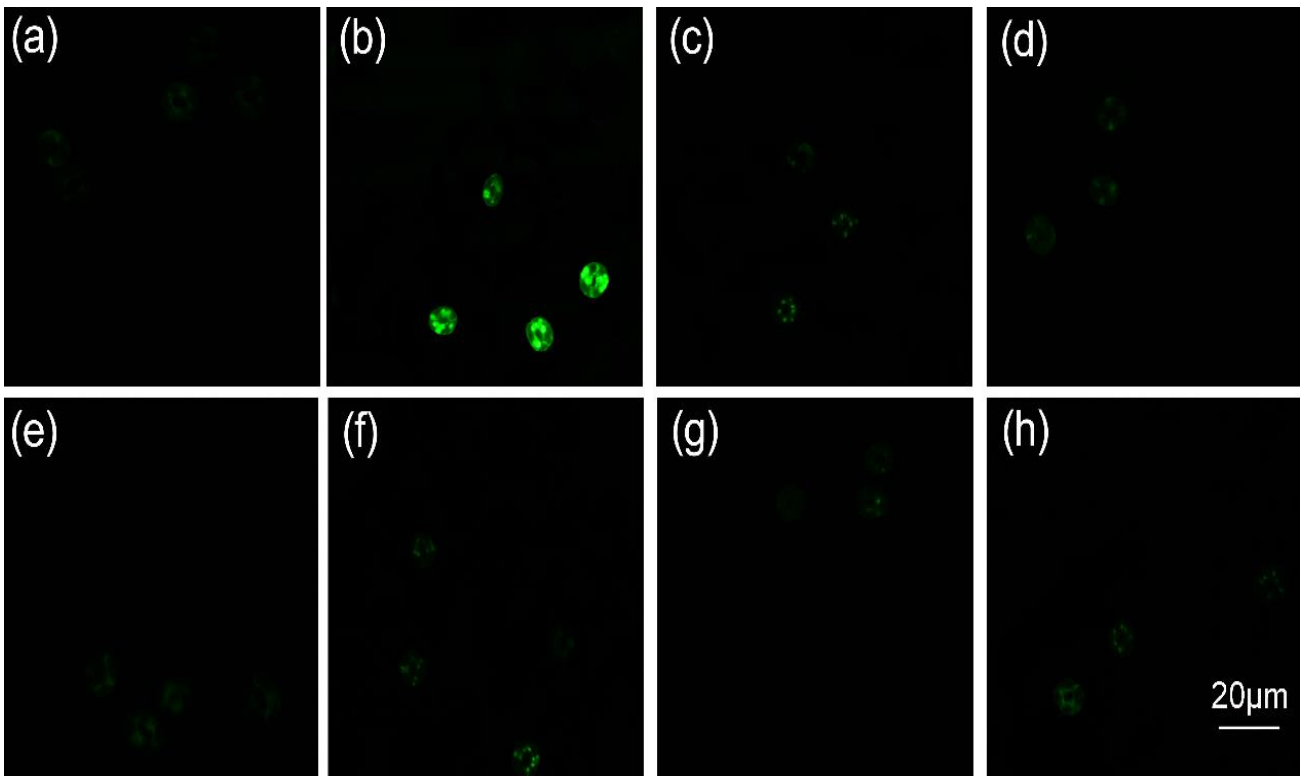

Fig. 6. Effects of ASA, CAT, and DPI on GR24-induced H_2O_2 production in guard cells of the wild type, and effects of GR24 on H_2O_2 levels of guard cells in the wild type, *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants. Guard cells were treated in light at 22 ± 2 °C for 3 h as follows: (a) with MES-KCl buffer alone in light; (b) containing 1 μmol·L^{-1} GR24, (c) 1 μmol·L^{-1} GR24 +100 μmol·L^{-1} ASA, (d) 1 μmol·L^{-1} GR24 +100 units·mL^{-1} CAT, (e) 1 μmol·L^{-1} GR24 +10 μmol·L^{-1} DPI, respectively. (f-h), Guard cells of *AtrbohD*, *AtrbohF* and *AtrbohD*/*F* mutants were treated with 1 μmol·L^{-1} GR24, respectively. After treatments, epidermal strips were immediately loaded with 50 μmol·L^{-1} H_2DCF-DA in Tris-KCl buffer in darkness for 10 min, then excess dye was removed and the strips were examined by luminescence microscope. Scale bar = 20 μm for all images.

H_2S modulators couldn't prohibit SL-caused H_2O_2 synthesis in the wild type: To further establish that H_2S synthesis mediated SL-triggered stomatal closure by inducing H_2O_2 production, H_2DCF fluorescence after cotreatment with H_2S modulators and GR24 were measured. H_2DCF fluorescence of guard cells after treatment with GR24 was more intense than that of the control (Fig. 9a, b), and HT, AOA, NH_2OH, and $C_3H_3KO_3$ and NH_3 couldn't inhibit GR24-induced rise in H_2DCF fluorescence intensity (Fig. 9c-f). The data indicated that H_2S synthesis really mediated SL-triggered stomatal closure by inducing H_2O_2 production.

SL induced H_2O_2 production in *Atl-cdes* and *Atd-cdes* mutants: To consolidate the conclusion that H_2S synthesis really mediated SL-triggered stomatal closure by inducing H_2O_2 production, we further measured GR24-triggered H_2DCF fluorescence change of guard cells in *Atl-cdes* and *Atd-cdes* mutants. Treatment with GR24 induced intense H_2DCF fluorescence of guard cells in the wild type, *Atl-cdes* and *Atd-cdes* mutants (Fig. 9g, h), which reinforced the conclusion that H_2O_2 represented a novel component upstream of H_2S in SL-triggered stomatal closure.

Discussion

Stomata regulates water and carbon cycles in plants (Heherington & Woodward, 2003). Light, drought, salt stress, and various phytohormones (such as JA, ABA, BR, etc) can affect stomatal aperture (Desikan *et al*., 2004; Acharya & Assmann, 2009; Jin *et al*., 2013; Ma *et al*., 2018a, 2021). Stomata responds to environmental stresses by inducing endogenous signals' production and defending cell's perception of environment (Acharya & Assmann, 2009). Therefore, stomatal movement is essential for the survival of plants. In recent years, many signal molecules such as H_2S, H_2O_2, NO and ATP have been indicated to be involved in stomatal movement of plants (Wang *et al*., 2015; Ma *et al*., 2022).

SLs are plant hormones that can regulate various physiological processes in plants including seed germination, secondary growth, development, etc. (Shaw *et al*., 1962; Agusti *et al*., 2011; Brewer *et al*., 2013). SLs have been reported to act as a co-regulator in regulating stomatal aperture. SLs is integrated with ABA in responding to stress adaptation (Visentin *et al*., 2016). Lv *et al*., (2017) reported that SLs can close stomata via H_2O_2 production and NO synthesis, and SLAC1 anion channel activation in *A. thaliana* in ABA-independent manner. GR24 caused stomatal closure of *Vicia faba* (Zhang *et al*., 2018). However, the signal network in SLs-triggered stomatal movement is still unclear. In our study, GR24 could close stomata, and 1 μmol·L^{-1} GR24 treatment for 3 h showed the optimum effects (Fig. 1a). The results are consistent with the previous results (Lv *et al*., 2017).

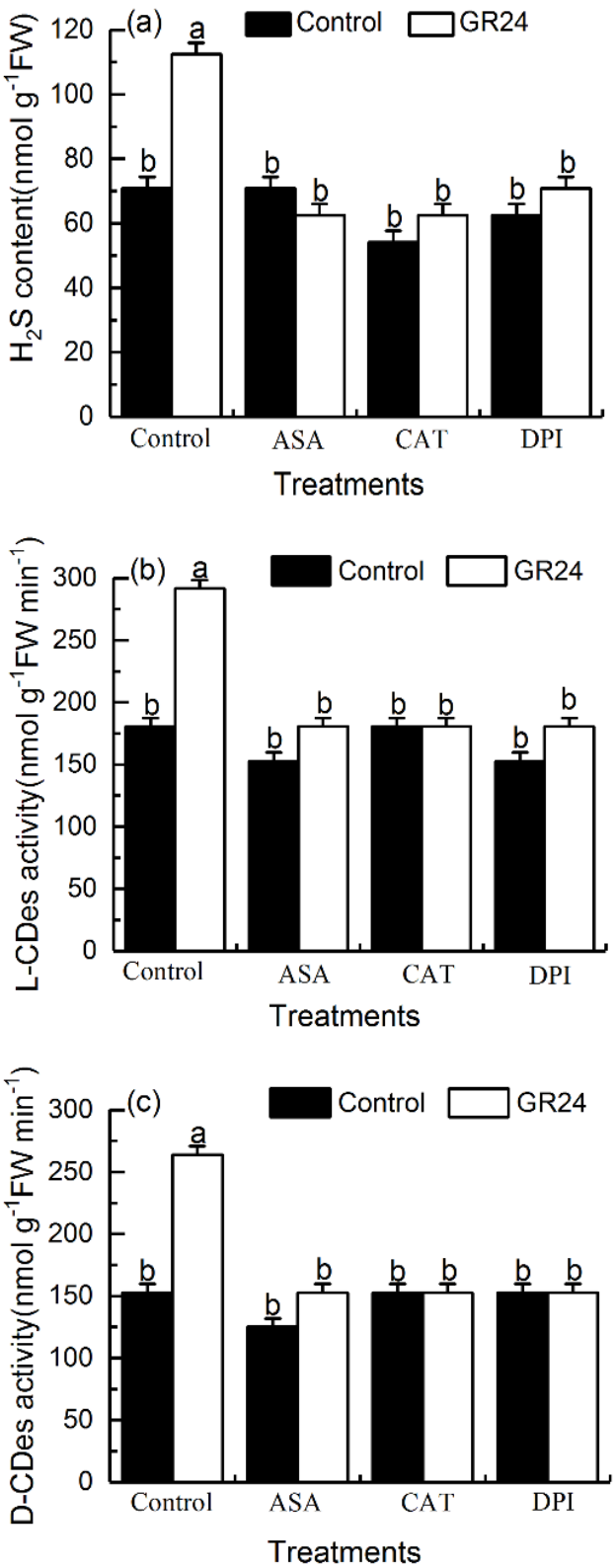

Fig. 7. Effects of ASA, CAT, and DPI on GR24-induced H_2S synthesis, L-CDes and D-CDes activity increase in leaves of the wild type. Isolated epidermal strips were incubated in MES-KCl buffer alone, or containing 100 µmol·L^{-1} ASA, 100 units·mL^{-1} CAT and 10 µmol·L^{-1} DPI in the absence (black columns) or presence of 1 µmol·L^{-1} GR24 (white columns) in light at 22 ± 2°C for 3 h, respectively, then H_2S content (a), L-CDes activity (b), and D-CDes activity (c) were measured. Data presented are means ± s.e. of three independent experiments (n=9). See Fig. 1*a* for further experimental details.

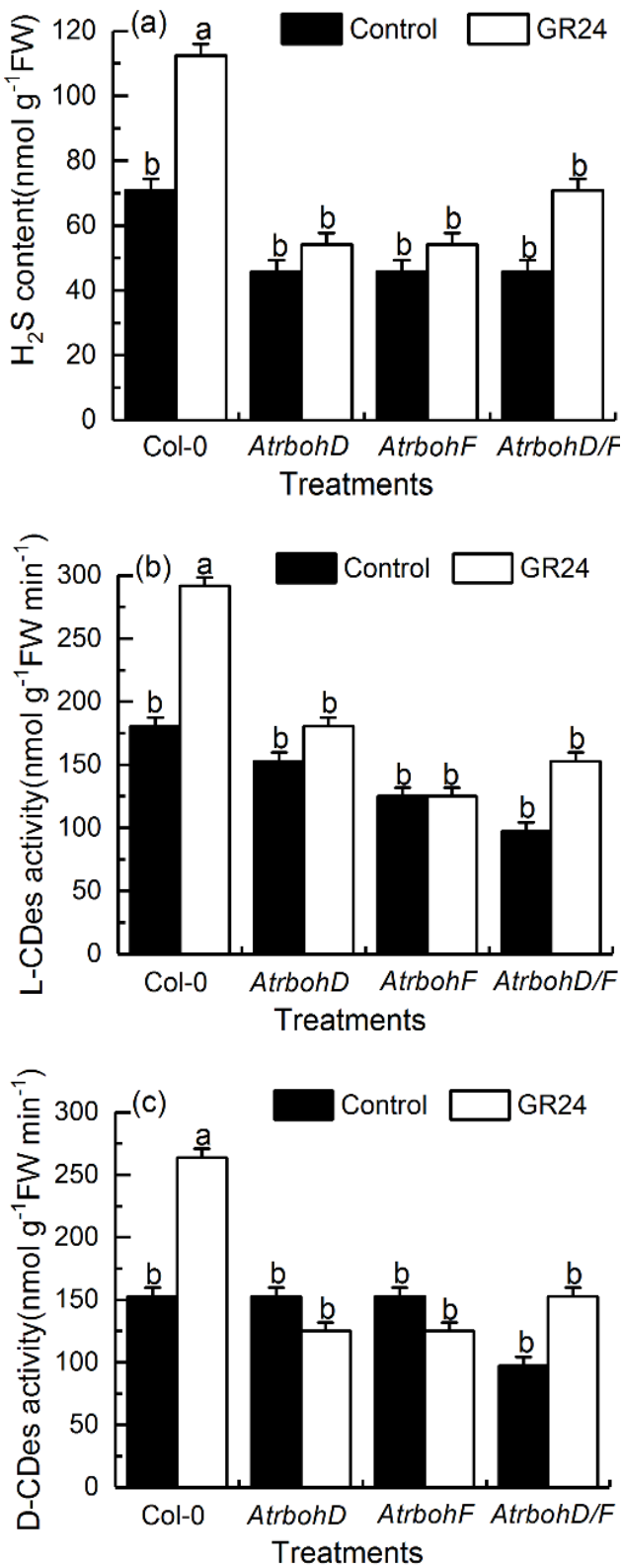

Fig. 8. Effects of GR24 on H_2S content, L-CDes activity and D-CDes activity in leaves of the wild type, *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants. Isolated epidermal strips of the wild type, *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants were incubated in MES-KCl buffer alone (black columns), or containing 1 µmol·L^{-1} GR24 in light at 22 ± 2°C for 3 h (white columns), respectively, then H_2S content (a), L-CDes activity (b), and D-CDes activity (c) were measured. Data presented are means ± s.e. of three independent experiments (n=9). See Fig.1a for further experimental details.

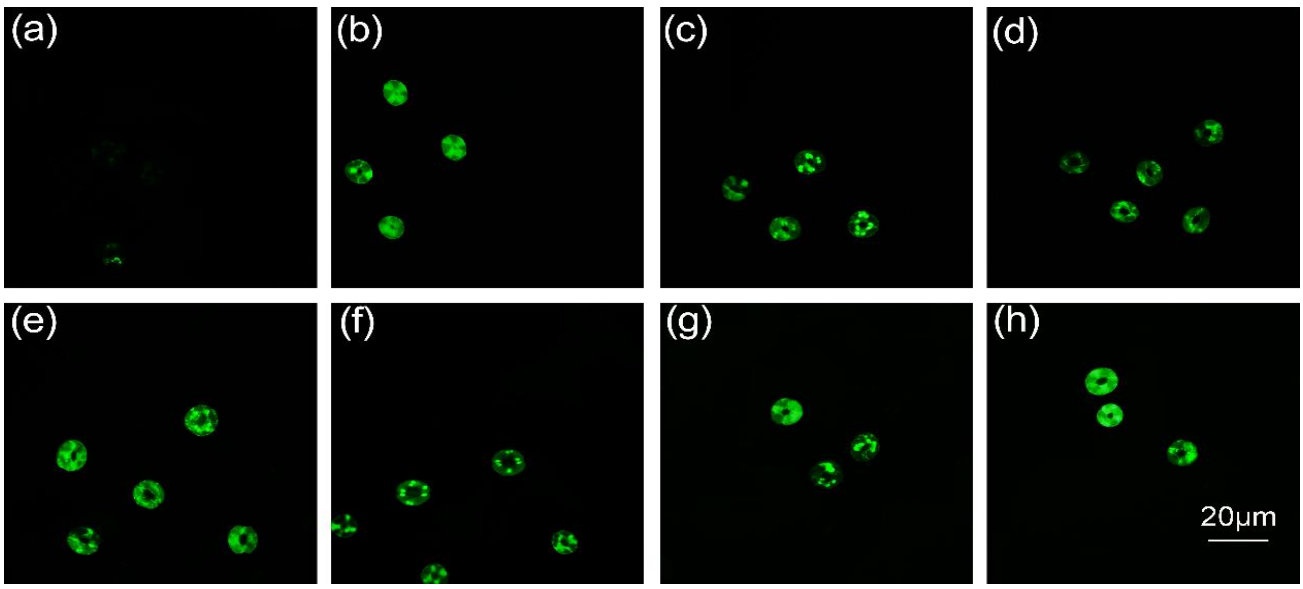

Fig. 9. Effects of HT, AOA, NH_2OH, and $C_3H_3KO_3$ + NH_3 on GR24-induced H_2O_2 production in guard cells of the wild type, and effects of GR24 on H_2O_2 levels of guard cells in the wild type, *Atl-cdes* and *Atd-cdes* mutants. Guard cells were treated in light at 22 ± 2°C for 3 h as follows: (a) with MES-KCl buffer alone; (b) containing 1 μmol·L^{-1} GR24, (c) 1 μmol·L^{-1} GR24 +15 μmol· L^{-1} HT, (d) 1 μmol·L^{-1} GR24 +0.4 mmol·L^{-1}AOA, (e) 1 μmol·L^{-1} GR24 +0.4 mmol·L^{-1} NH_2OH, (f) 1 μmol·L^{-1} GR24 +0.4 mmol·L^{-1} $C_3H_3KO_3$+0.4 mmol·L^{-1} NH_3, respectively. (g-h), Guard cells of the *Atl-cdes* and *Atd-cdes* mutants were treated with 1 μmol·L^{-1} GR24, respectively. After treatments, epidermal strips were immediately loaded with 50 μmol·L^{-1} H_2DCF-DA in Tris-KCl buffer in darkness for 10 min, then excess dye was removed and the strips were examined by luminescence microscope. Scale bar = 20 μm for all images.

H_2S, as an important signaling molecule, has been shown to participate in various physiological processes in plants (Zhang *et al*., 2008; Ma *et al*., 2022). H_2S functions in alleviating environmental stresses (Wang *et al*., 2012b; Zhou *et al*., 2020). It has been shown that exogenous H_2S can close stomata (García-Mata *et al*., 2010). More evidence has demonstrated that H_2S participates in darkness-, salt stress-, BR- and $CdCl_2$-triggered stomatal movement (Ma *et al*., 2018a, 2018b, 2019, 2021, 2022). Until now, whether H_2S, as a signaling molecular, functions in mediating SLs-regulated stomatal movement is unknown. Here, we showed that H_2S participated in GR24-triggered stomatal closure. HT, AOA, NH_2OH, and $C_3H_3KO_3$ and NH_3 could prevent GR24-triggered stomatal closure (Fig. 2a-d). Additionally, we investigated the source of H_2S in SLs-triggered stomatal movement. Treatment with GR24 significantly increased H_2S level, L-CDes and D-CDes activity of leaves in the wild type, cotreatment with AOA, NH_2OH, and $C_3H_3KO_3$ and NH_3 and GR24 prevented the effects (Fig. 3a-c). Furthermore, GR24 closed stomata of the wild type, while it couldn't change *Atl-cdes* and *Atd-cdes* mutants' stomatal aperture (Fig. 4). The results proved that H_2S soured from L-CDes- and D-CDes mediated SL-triggered stomatal closure in *A. thaliana*, both AtL-CDes and AtD-CDes mediated H_2S synthesis in the process.

H_2O_2, as another signaling molecule in plants, has been implicated in stomatal movement triggered by SLs, ABA, and BR, etc (Desikan *et al*., 2004; Shi *et al*., 2015; Lv *et al*., 2017). McAinsh *et al*., (1996) first proved that the rise of calcium level is necessary for exogenous H_2O_2-induced stomatal closure. In *A. thaliana*, salt stress-triggered H_2O_2 production depends on AtrbohD and AtrbohF **(**Ma *et al*., 2012**)**. H_2O_2 functions in SL-triggered stomatal closure (Lv *et al*., 2017). Our results showed that H_2O_2 modulators (ASA, CAT, and DPI) inhibited GR24-triggered stomatal closure (Fig. 5a) and enhancement of H_2DCF fluorescence of guard cells in the wild type (Fig. 6c-e). But GR24 couldn't close the stomata of *AtrbohD*, *AtrbohF* and *AtrbohD*/*F* mutants (Fig. 5b), and it failed to stimulate H_2O_2 production of guard cells in *AtrbohD*, *AtrbohF* and *AtrbohD*/*F* mutants (Fig. 6f-h). These results proved that H_2O_2 was derived from NADPH oxidase pathway and participated in SL-triggered stomatal closure, H_2O_2 is sourced from AtrbohD and AtrbohF, the results are the same as the previous reports (Lv *et al*., 2017).

H_2S and H_2O_2 interact in many physiological processes in plants, and exogenous H_2S can reduce H_2O_2 level to resist oxidative damage (Zhang *et al*., 2008). H_2S participates in ethylene-caused stomatal closure depending on H_2O_2 synthesis in *A. thaliana* (Hou *et al*., 2016). Moreover, H_2S is a component downstream of H_2O_2 in stomatal movement triggered by darkness, NaCl stress, and BR, etc (Ma *et al*., 2018a, 2018b, 2021, 2022). Until now, it is unknown whether H_2S is related to H_2O_2 in SL-triggered stomatal closure. In the present work, we showed that ASA, CAT and DPI prohibited GR24-caused H_2S production, L-CDes and D-CDes activity increase in the wild type (Fig. 7a-c), but GR24 couldn't change H_2S content, L-CDes and D-CDes activity of leaves in *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants (Fig. 8a-c). Furthermore, HT, AOA, NH_2OH, and $C_3H_3KO_3$ and NH_3 couldn't inhibit GR24-induced rise in H_2DCF fluorescence intensity (Fig. 9c-f), and GR24 induced intense H_2DCF fluorescence in *Atl-cdes* and *Atd-cdes* mutants (Fig. 9g, h). From these data, we concluded that H_2S derived from L-CDes and D-CDes represented a novel component downstream of H_2O_2 production from NADPH oxidase pathway in SL-triggered stomatal closure in *A. thaliana.*

Conclusions

In the present study, it was concluded that SLs induced NADPH oxidase-derived H_2O_2 production, then caused L-CDes and D-CDes-sourced H_2S synthesis, and finally led to stomatal closure in *A. thaliana* (Fig. 10). During SLs-triggered stomatal closure, both AtrbohD and AtrbohF were responsible for H_2O_2 production, H_2S synthesis depended on both AtL-CDes and AtD-CDes. However, the interaction between H_2S and other signaling molecule, such as ethylene, G protein, CO or Ca^{2+} remains to be further investigated.

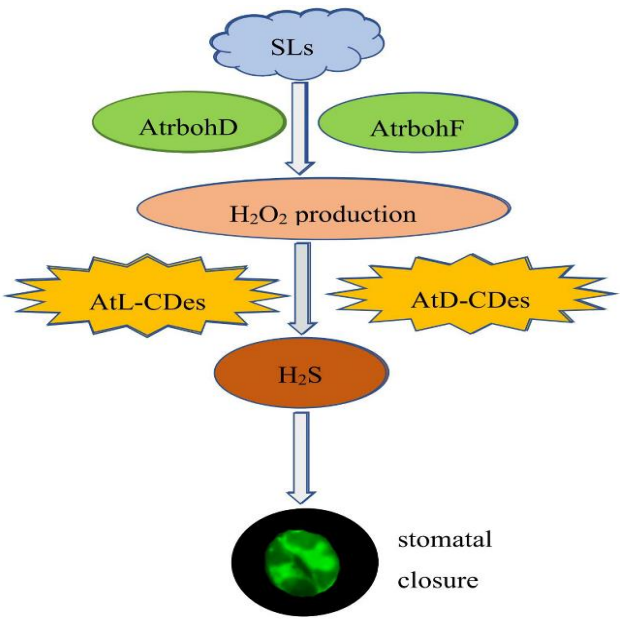

Fig. 10. Model of possible signaling pathway for SLs-triggered stomatal closure in *A. thaliana*. SLs promotes H_2O_2 production by AtrbohD and AtrbohF, subsequently increases AtL-CDes-/AtD-CDes-catalysed H_2S synthesis, and finally closes the stomata.

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