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Hydrogen sulfide induces hydrogen peroxide and nitric oxide mediation of salt stress-caused stomatal closure in *Arabidopsis thaliana* (L.) Heynh.

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The roles of H_2S, hydrogen peroxide (H_2O_2) and nitric oxide (NO) in signaling transduction of stomatal movement response to salt stress in *Arabidopsis thaliana* (L.) Heynh. are still unknown. The role of H_2S and its relationship with H_2O_2 and NO in salt stress-caused stomatal closure by using pharmacological, spectrophotographic and fluorescence microscopic approaches in *A. thaliana* were investigated. Our results will provide evidence for further elucidating the signal transduction mechanism of stomatal movement caused salt stress in plants. Salt stress caused stomatal closure in wild-type and *AtrbohD* mutant, these effects were prohibited by H_2S modulators, H_2O_2 modulators, NO modulators in wild-type, respectively. However, salt stress couldn't significantly change the stomatal aperture of *Atl-cdes*, *Atd-cdes*, *Atnoa1*, *nia1-2*, *nia2-1*, *nia1-2/nia2-5*, *AtrbohF* and *AtrbohD/F* mutants. Salt stress caused rise of H_2S content and *L-/D*-cysteine desulfhydrase (*L-/D*-CDes) activity of leaves in wild-type and *AtrbohD* mutant, but these effects were prevented by H_2S modulators in wild-type. H_2O_2 modulators and NO modulators significantly inhibited salt stress-caused H_2O_2 production and NO synthesis in wild-type, respectively. These results suggested that H_2S, H_2O_2 and NO participate in salt stress-caused stomatal closure. H_2O_2 modulators and NO modulators prevented salt stress-caused H_2S synthesis and *L-/D*-CDes activity increase in leaves of wild-type, but H_2S modulators couldn't inhibit salt stress-caused H_2O_2 production and NO synthesis in wild-type. Salt stress increased H_2O_2 and NO levels in *Atl-cdes* and *Atd-cdes* mutants, but failed to cause H_2S synthesis and L-/D-CDes activity increase in *AtrbohF*, *AtrbohD/F*, *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants. All the data suggested that salt stress induced H_2O_2 and NO production, subsequent caused H_2S synthesis, and finally closed stomata in *A. thaliana*.

Keywords: Abiotic stress, H_2O_2 synthesis, H_2S synthesis, L-/D-CDes activity, Mouseear cress, NO production, Rockcress, Salt stress, Thale cress

Abiotic stresses are known to affect plant growth[1]. Soil salinization can affect global ecological environment, crop growth and reduce agricultural production[2]. Salt stress can influence many physiological processes and cause serious damage to plants[3-5]. Stomata play an important role in controlling photosynthesis and transpiration in plants[6]. It can induce stomatal movement and influence transpiration[7], and also cause stomatal closure as reported in *Vicia faba*[8] and *Arabidopsis thaliana*[9].

Hydrogen sulfide (H_2S) has been indicated to be a gaseous signaling molecule after carbon monoxide (CO) and nitric oxide (NO)[10], and higher plants are known to emit H_2S[11]. H_2S can mediate many physiological processes in animals[12] and in plants[13,14], and improve resistance to environmental stresses[15,16]. Yang *et al*.[17] showed that H_2S can alleviate salt stress through auxin signaling in Arabidopsis. It has been demonstrated that H_2S regulates stomatal closure induced by many factors[16,17-22]. Additionally, H_2S interacts with NO and hydrogen peroxide (H_2O_2), one of the reactive oxygen species (ROS) and an endogenous signal molecule, in adapting to environmental stresses and regulating many physiological processes including stomatal movement in plants[20,23]. H_2O_2 is known to play a role in adaptation to various stresses and regulation of many physiological processes including stomatal closure in plants[24-26]. It has been documented that *AtrbohD/F*-sourced H_2O_2 mediates plant defense response and stomatal closure[27-30]. Likewise, NO, as a fat-soluble small signaling molecule, also play a role in regulation in many physiological processes including stomatal closure in plants[26,31-33] and responses to various stresses[34]. Exogenous NO can close stomata[35]. NO interacts with H_2O_2 and Ca^{2+}. Cytosolic alkalization-mediated H_2O_2 and NO synthesis mediates darkness-induced stomatal closure[36]. However, it is unknown whether H_2S, H_2O_2 and NO function in salt stress-caused stomatal closure in *A. thaliana*, and whether

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H_2S directly interact with H_2O_2 and NO is unclear. Here, we investigated participation of H_2S in salt stress-caused stomatal movement in the Rock cress or Thale cress, *Arabidopsis thaliana* (L.) Heynh., and also the interrelationship of H_2S and H_2O_2, NO in the process.

Materials and Methods

Chemicals

The molecular probe H_2DCF-DA was purchased from Biotium (Hayward, CA, USA). The following chemicals were procured from Sigma-Aldrich (Located in St Louis, MO, USA): 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), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), sodium tungstate dihydrate (Na_2WO_4), N^G-Nitro-*L*-arginine methyl ester, hydrochloride (*L*-NAME), NO specific fluorescent probe 4, 5-diaminofluorescein diacetate (DAF-2DA), L-cysteine (L-Cys), D-cysteine (D-Cys), *N*, *N*-dimethyl-*p*-phenylenediamine dihydrochloride, and 2-(N-morpholine) ethane sulfonic acid (MES). Unless stated otherwise, all other chemicals were of the highest analytical grade purchased from various local suppliers.

Plant materials

Seeds of *A. thaliana* ecotype Columbia (Col-0), *L-/D*-cysteine desulfhydrase deletion mutants of *Atl-cdes* T-DNA insertion line (N541918, designated *Atl-cdes*), *Atd-cdes* T-DNA insertion line (N853264, 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). *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants were donated by Prof. Junmin He (Shanxi Normal University). Seeds were surface-sterilized and sown on sterilized vermiculite. Plates were stratified in darkness for 2-4 days at 4°C, and then transferred to a growth chamber at a day/night temp. cycle of 22°C/18°C, 80% RH, and 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ under a 16/8 h day/night photoperiod. After growing four euphylla, seedlings were potted in a mixture of 1:1 vegetative soil and vermiculite and continued to grow in the same condition. Mutants and WT plants of 4-6 week old at equivalent development stages were used for the following experiments. The epidermis was carefully peeled from the abaxial surfaces of the youngest, fully expanded leaves of seedlings and cut into pieces of 5×5 mm.

Stomatal bioassays

Stomatal bioassays were performed as described by McAinsh *et al*.[37] with minor modifications. Briefly, the freshly prepared epidermal strips from the wild type and various mutants 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 induce stomatal opening. After incubation the epidermal strips were transferred to MES-KCl buffer or NaCl alone or MES-KCl containing various scavengers or inhibitors in light for 1 h at 22 ± 2°C, and then stomatal aperture were recorded with a light microscope and an 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 ± SE. of 90 measurements.

Measurement of H_2S emission

Measurement of H_2S emission was performed as described by Sekiya *et al*.[38] and Hou *et al*.[18] with minor modifications. The emission of H_2S was measured by fully expanded leaves of the wild-type, *Atnoa1*, *nia1-2*, *nia2-1*, *nia1-2/nia2-5*, *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants. Firstly, 0.1 g leaves were cut and treated with MES-KCl buffer for 3 h, then transferred to MES-KCl buffer or NaCl alone or contained various scavengers or synthetic inhibitors in light (100 $\mu mol\ m^{-2}\ s^{-1}$) for 1 h at 22±2°C and take out them and ground the presence of 0.9 mL 20 $mmol \cdot L^{-1}$ Tris-HCl (pH 8.0) buffer. After grinding and centrifuging for 15 min, the supernatant and a trap with 3 mL 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 ± SE.

L-/D-cysteine desulfhydrase activity measurements

L-/D-cysteine desulfhydrase activity measurements were performed as described by Riemenschneider *et al*.[39].

The content of each component in the total volume of 1 mL includes: 100 µL 0.8 mmol·L^{-1} *L-/D*-cysteine, 400 µL 2.5 mmol·L^{-1} DTT, 400 µL 100 mmol·L^{-1} Tris-HCl, and 100 µL supernatant. Then 100 µL 20 mmol·L^{-1} *N*, *N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 mol·L^{-1} HCl and 100 µL 30 mmol·L^{-1} $FeCl_3$ dissolved in 1.2 mol·L^{-1} HCl were added into the trap after reacting for 30 min at 37°C. The rate of H_2S released was indicated by the determination of absorbance at 670 nm. Moreover, the activities of *L*-CDes and *D*-CDes was 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 thrice.

Measurement of endogenous H_2O_2 or NO levels

To study the effects of *Atl-cdes* and *Atd-cdes* mutnts on salt stress-induced H_2O_2 and NO production in guard cells, H_2O_2 levels were measured with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) and NO levels were measured with 4,5-di-aminofluorescein diacetate (DAF-2DA) using the method by Allan & Fluhr[40], and Gonugunta *et al*.[41] with minor modifications. The freshly prepared epidermal strips from the wild type and *Atl-cdes*, *Atd-cdes*, *AtrbohD*, *AtrbohF*, *AtrbohD/F*, *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants were incubated in MES-KCl buffer for 3 h, in order to stimulate stomatal opening, then the epidermal strips were incubated in MES-KCl buffer, NaCl alone or containing various scavengers or inhibitors for 1 h, and then immediately loaded with 50 µmol·L^{-1} H_2DCF-DA in Tris-KCl buffer (10 mmol·L^{-1} Tris, 50 mmol·L^{-1} KCl, pH 7.2) for 10 min or 10 µmol·L^{-1} DAF-2DA in Tris-KCl buffer for 30 min in darkness at 25°C. The excessive dye was washed off with fresh Tris-KCl loading buffer, and the epidermal strips were immediately examined by luminescence microscope (Olympus BX53, U-RFLT50, JAPAN) with following settings: 450 nm of excitation, 490 nm of emission. Images thus acquired were analysed with Leica image software and processed with Photoshop 7.0 (Adobe, San Jose, CA, USA). Each treatment was repeated thrice. The images depicted here represent similar results from all three replications.

Statistical analysis

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 image was drawn by Origin 6.1 (Microcal Software, Nothampton, MA, USA) and processed with Photoshop 7.0.

Results

Salt stress caused stomatal closure in *A. thaliana*

To study whether salt stress affects stomatal movement of *A. thaliana*, the effects of NaCl on stomatal aperture of wild-type were observed. NaCl could close stomata, the best effects on stomata under NaCl treatment were 100 mmol·L^{-1} and 1 h (Fig. 1A). The results of an elution treatment showed that stomatal aperture could be restored, indicating that the influence of NaCl to stomata was not irreversible effects (Fig. 1B). These results demonstrated that NaCl could close stomata of *A. thaliana*.

H_2S mediates salt stress-caused stomatal closure

To investigate whether H_2S mediates salt stress-caused stomatal closure of *A. thaliana*, the effects of H_2S modulators including HT (a H_2S scavenger), AOA and NH_2OH (H_2S synthesis inhibitors), and $C_3H_3KO_3$ and NH_3 (the products of L-/D-CDes) on salt stress-caused stomatal closure in wild-type were measured. Treatment with 100 mmol·L^{-1} NaCl significantly induced stomatal closure (Fig. 2A). Additionally, NaCl treatment could induce rise of H_2S content of leaves (Fig. 2B). H_2S modulators significantly prohibited NaCl stress-caused stomatal closure (Fig. 2A) and H_2S production (Fig. 2B) in *A. thaliana* leaves. The data showed that H_2S synthesis mediated salt stress-caused stomatal closure, and H_2S was sourced from *L*-CDes and *D*-CDes.

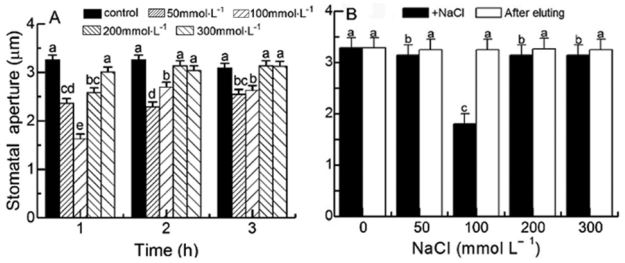

Fig. 1 — Effects of salt stress on stomatal aperture in wild-type. (A) Isolated epidermal strips were incubated in MES-KCl buffer alone, or containing different concentrations of NaCl in light at 22 ± 2°C for 1, 2 and 3 h, and stomatal apertures were measured; (B) After the incubation in MES-KCl buffer containing different concentrations of NaCl in light at 22 ± 2°C for 1 h (black columns), the strips were treated with MES-KCl buffer in light at 22 ± 2°C for another 1 h (white columns), and stomatal apertures were measured. [Data are mean ± SE of 30 stomata in each of three independent experiments (n = 90), means denoted by different letters in A and B differed significantly at *P* <0.05 according to Duncan's multiple range test]

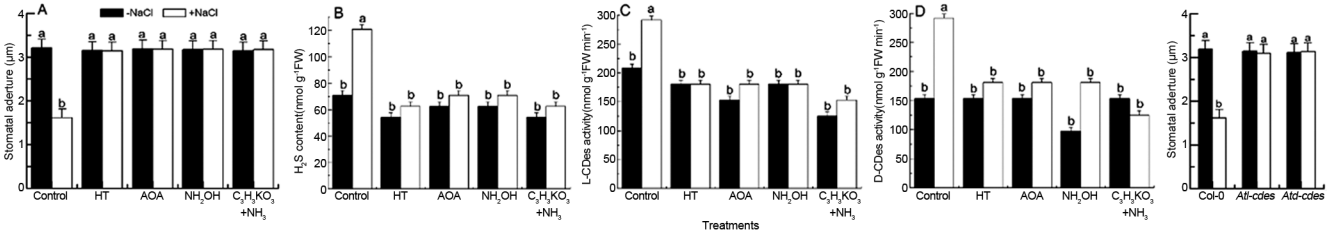

Fig. 2 — Effects of H_2S modulators in (A) salt stress-induced stomatal closure; (B) H_2S content; (C) *L*-CDes; and (D) *D*-CDes activity in *A. thaliana* leaves; and (E) effects of salt stress on stomatal aperture in wild-type, *Atl-cdes* and *Atd-cdes* mutants. [(A) Isolated epidermal strips were incubated in MES-KCl buffer alone, or containing 15 μmol·L^{-1} HT, 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 100 mmol·L^{-1} NaCl (white columns) in light at 22 ± 2°C for 1 h, and stomatal apertures were measured. (B-D) Treatments as Fig. 2A, and then the leaves were used to measure (B) H_2S content; (C) L-CDes activity; and (D) D-CDes activity. Data are means ± SE. of three independent experiments (n = 9). (E) Isolated epidermal strips of wild-type, *Atl-cdes* and *Atd-cdes* mutants were incubated in MES-KCl buffer alone (black columns), or containing 100 mmol·L^{-1} NaCl in light at 22 ± 2°C for 1 h (white columns), and stomatal apertures were measured. Data in A and E are mean ± SE. of 30 stomata in each of three independent experiments (n = 90), means denoted by different letters in A-E differed significantly at P <0.05 according to Duncan's multiple range test]

In order to explore the metabolic pathways of H_2S, *L*-CDes and *D*-CDes activity of leaves in wild-type were tested. Salt stress induced increase of *L*-CDes and *D*-CDes activity, but H_2S modulators decreased *L*- and *D*-CDes activity under salt stress (Fig. 2 C and D). However, when H_2S modulators were applied without NaCl, there was no obviously effect on *L*-CDes and *D*-CDes activity (Fig. 2 C and D). The data implied that both *L*-CDes and *D*-CDes were responsible for salt stress-induced H_2S synthesis in *A. thaliana*.

To further prove whether H_2S synthesis mediated salt stress-caused stomatal closure, we determined the effects of NaCl on stomata of *Atl-cdes* and *Atd-cdes* mutants (*AtL-CDes* and *AtD-CDes* T-DNA insertion line). Fig. 2E showed that salt stress caused stomatal closure of wild-type, but salt stress couldn't significantly change stomatal aperture of *Atl-cdes* and *Atd-cdes* mutants, indicating that H_2S was involved in salt stress-caused stomatal closure of *A. thaliana*, both *AtL-CDes* and *AtD-CDes* were responsible for H_2S synthesis in the process.

H_2O_2 mediates salt stress-caused stomatal closure

To examine whether H_2O_2 mediates salt stress-caused stomatal closure of *A. thaliana*, 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 salt stress-induced stomatal closure, and the effects of NaCl treatment on stomatal movement in NADPH oxidase mutants *AtrbohD*, *AtrbohF* and *AtrbohD/F* were detected. NaCl stress-induced stomatal closure was significantly inhibited by H_2O_2 modulators in wild-type (Fig. 3A). NaCl stress significantly induced stomatal closure of wild-type and *AtrbohD* mutant, but couldn't close the stomata of *AtrbohF* and *AtrbohD/F* mutants (Fig. 3B). From the data, we inferred that H_2O_2 sourced from NADPH oxidase was involved in salt stress-caused stomatal closure, and *AtrbohF* might be responsible for H_2O_2 synthesis in the process.

To further confirm H_2O_2 functions in salt stress-caused stomatal movement signal network, we examined the effects of H_2O_2 modulators on H_2O_2 levels of guard cells in wild-type, and the effects of NaCl treatment on H_2O_2 levels of *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants. Compared with light treatment (Fig. 3C), NaCl treatment increased H_2DCF fluorescence intensity of wild-type (Fig. 3D). However, NaCl-induced H_2O_2 production were abolished by H_2O_2 modulators (Fig. 3 E-G). NaCl treatment could increase H_2O_2 levels in *AtrbohD* mutant (Fig. 3H), but failed to stimulate H_2O_2 production in *AtrbohF* and *AtrbohD/F* mutants (Fig. 3 I and J). The data further confirmed that AtrbohF-derived H_2O_2 participated in salt stress-caused stomatal closure.

NO mediates salt stress-induced stomatal closure

To explore whether NO mediates salt stress-induced stomatal closure, the effects of NO modulators (NO scavenger c-PTIO, NR inhibitor Na_2WO_4, and NOS inhibitor *L*-NAME) on salt stress-caused stomatal closure were examined in wild-type. NO modulators significantly prohibited stomatal closure caused by salt stress in wild-type (Fig. 4A). When c-PTIO, Na_2WO_4, and *L*-NAME treated wild-type epidermal peels alone, stomatal closure could not be induced (Fig. 4A). NaCl treatment failed to close stomata of *Atnoa1*, *nia1-2*, *nia2-1*, *nia1-2/nia2-5* mutants (Fig. 4B). These results suggested that NO sourced from NOS and NR probably

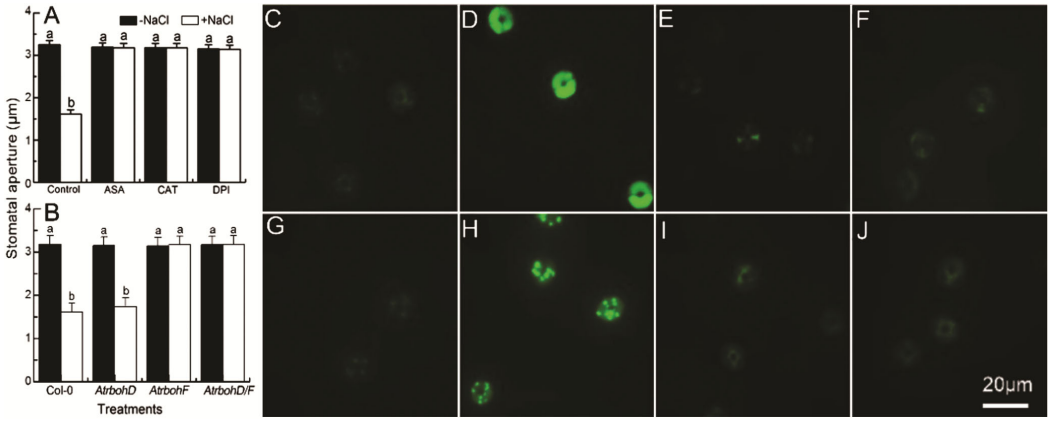

Fig. 3 — Effects of H_2O_2 modulators on (A) salt stress-induced stomatal closure in wild-type; (B) effects of salt stress on stomatal aperture in wild-type, *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants; (C-G) effects of H_2O_2 modulators on salt stress-induced H_2O_2 synthesis of guard cells in wild-type; and (H-J) effects of salt stress on H_2O_2 synthesis of guard cells in *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants. [(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 100 mmol·L^{-1} NaCl (white columns) in light at 22 ± 2°C for 1 h, then apertures were measured. (B) Isolated epidermal strips of wild-type, *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants were incubated in MES-KCl buffer alone (black columns), or containing 100 mmol·L^{-1} NaCl in light at 22 ± 2°C for 1 h (white columns), then apertures were measured. Data are mean ± SE. of 30 stomata in each of three independent experiments (n = 90), means denoted by different letters in A and B differed significantly at P <0.05 according to Duncan's multiple range test. (C-G) Guard cells were treated in light at 22 ± 2°C for 1 h as follows: (C) with MES-KCl buffer alone in light; (D) containing 100 mmol·L^{-1} NaCl; (E) 100 mmol·L^{-1} NaCl + 100 μmol·L^{-1} ASA; (F) 100 mmol·L^{-1} NaCl + 100 units·mL^{-1} CAT; and (G) 100 mmol·L^{-1} NaCl + 10 μmol·L^{-1} DPI. (H-J) Guard cells of *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants were treated with 100 mmol·L^{-1} NaCl in light at 22 ± 2°C for 1 h. After treatments, epidermal strips were immediately loaded with 50 μmol·L^{-1} H_2DCF-DA in Tris-KCl buffer in darkness at 25 ± 2°C for 10 min, then excess dye was removed and the strips were examined by laser-scanning confocal microscopy. Scale bar in (J) represents 20 μm for images from (C) to (J)]

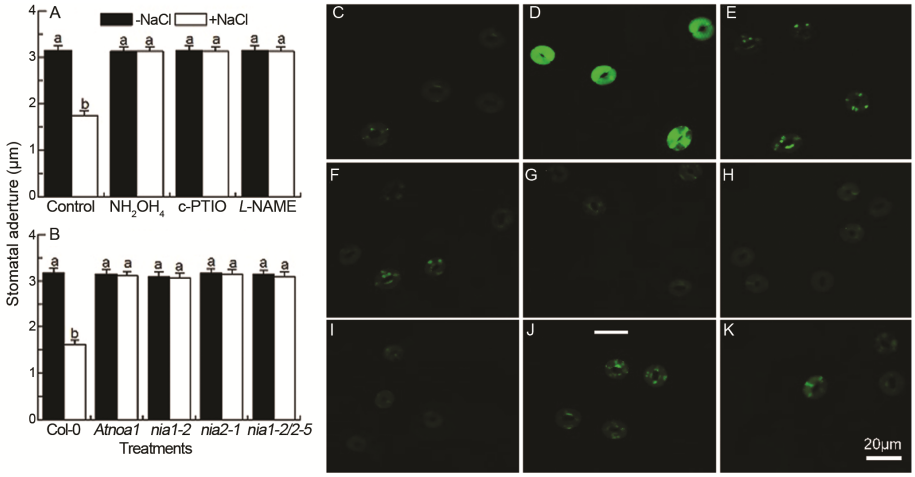

Fig. 4 — Effects of (A) NO modulators on salt stress-induced stomatal closure in wild-type; (B) effects of salt stress on stomatal aperture in *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants; (C-G) effects of NO modulators on salt stress-induced NO synthesis of guard cells in wild-type; and (H-K) effects of salt stress on NO synthesis of guard cells in *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants. [(A) Isolated epidermal strips were incubated in MES-KCl buffer alone, or containing 200 μmol·L^{-1} c-PTIO, 100 μmol·L^{-1} Na_2WO_4, and 25 μmol·L^{-1} *L*-NAME in the absence (black columns) or presence of 100 mmol·L^{-1} NaCl (white columns) in light at 22 ± 2°C for 1 h, then apertures were measured. (B) Isolated epidermal strips of wild-type, *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants were incubated in MES-KCl buffer alone (black columns), or containing 100 mmol·L^{-1} NaCl in light at 22 ± 2°C for 1 h (white columns), then apertures were measured. Data are mean ± SE. of 30 stomata in each of three independent experiments (n = 90), means denoted by different letters in A and B differed significantly at P <0.05 according to Duncan's multiple range test. (C-K) Guard cells were treated in light at 22 ± 2°C for 1 h as follows: (C) with MES-KCl buffer alone in light; (D) containing 100 mmol·L^{-1} NaCl; (E) 100 mmol·L^{-1} NaCl + 200 μmol·L^{-1} c-PTIO; (F) 100 mmol·L^{-1} NaCl + 100 μmol·L^{-1} Na_2WO_4; (G), 100 mmol·L^{-1} NaCl + 25 μmol·L^{-1} *L*-NAME; and (H-K) Guard cells of *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants were treated with 100 mmol·L^{-1} NaCl in light at 22 ± 2°C for 1 h. After treatments, epidermal strips were immediately loaded with 10 μmol·L^{-1} DAF-2DA in Tris-KCl buffer at 25 ± 2°C for 30 min, then excess dye was removed and the strips were examined by luminescence microscope. Scale bar in (K) represents 20 μm for images from (C) to (K)]

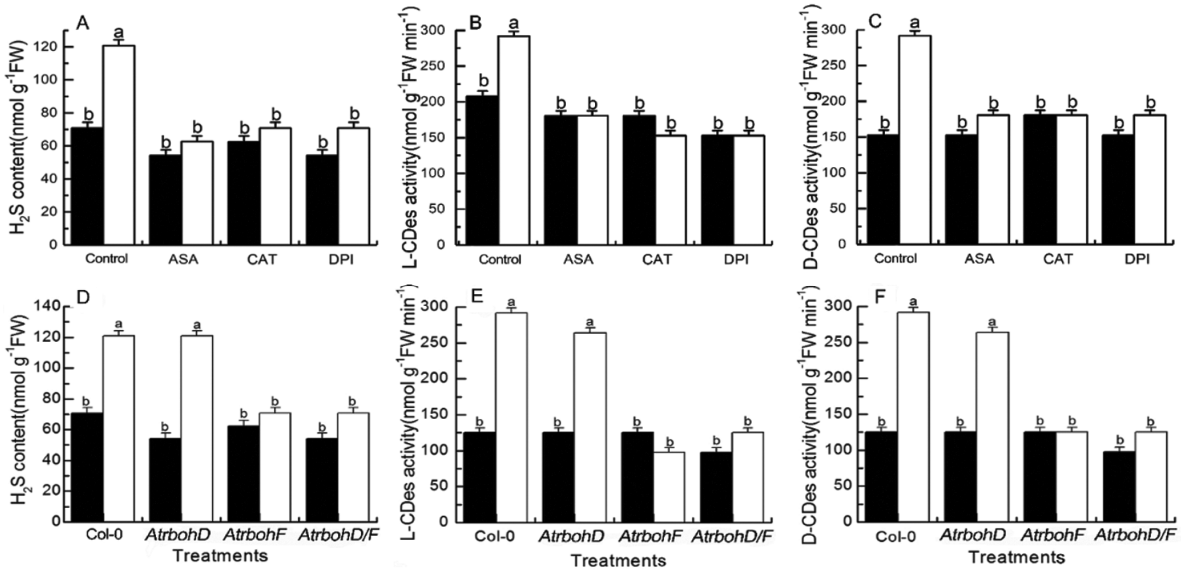

Fig. 5 — (A-C) Effects of H_2O_2 modulators on salt stress-induced H_2S content, *L*-CDes and *D*-CDes activity in wild-type leaves; and (D-F) effects of NaCl on H_2S content, *L*-CDes activity and *D*-CDes activity in leaves of wild-type, *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants. [(A-C) Isolated 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 100 mmol· L^{-1} NaCl (white columns) in light at 22 ± 2°C for 1 h, then the leaves were used to measure (A) H_2S content; (B) *L*-CDes activity; and (C) *D*-CDes activity. Data are means ± SE. of three independent experiments (n = 9). (D-F) Isolated epidermal strips of wild-type, *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants were incubated in MES-KCl buffer alone (black columns), or containing 100 mmol· L^{-1} NaCl in light for 1 h (white columns), and then the leaves were used to measure (D) H_2S content; (E) *L*-CDes activity; and (F) *D*-CDes activity. Data are means ± SE. of three independent experiments (*n*=9), means denoted by different letters in A-F differed significantly at $P < 0.05$ according to Duncan's multiple range test]

mediated salt stress-caused stomatal closure, *Atnoa1*, *nia1* and *nia2* participated in NO production in the process in *A. thaliana*.

In addition, to further confirm NO mediated stomatal closure caused by the salt stress, NO levels were monitored using the specific NO fluorescent probe diaminofluorescein diacetate (DAF-2DA) and luminescence microscopy[42]. Compared with light treatment (Fig. 4C), NaCl treatment induced intense DAF-2 fluorescence of guard cells in wild-type (Fig. 4D). After treatments with c-PTIO, Na_2WO_4 and *L*-NAME in the presence of NaCl, NO levels obviously decreased in guard cells (Fig. 4 E-G). Furthermore, NaCl treatment could not increase NO levels in *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants compared with wild-type (Fig. 4 D and H-J). The data further confirmed the conclusion that NO generated from NOS and NR pathways participated in salt stress-caused stomatal closure, *Atnoa1*, *nia1* and *nia2* were responsible for NO production in the process in *A. thaliana*.

Interaction between H_2S and H_2O_2 during salt stress-induced stomatal closure

H_2O_2 modulators can inhibit salt stress-induced H_2S synthesis and L-/D-CDes activity increase

To confirm the interrelationship between H_2S and H_2O_2, effects of H_2O_2 modulators on the change of H_2S level and activity of *L*-CDes and *D*-CDes in wild-type leaves under salt stress were examined. Salt stress caused H_2S synthesis and increased the activity of *L*-CDes and *D*-CDes in wild-type leaves, H_2O_2 modulators prohibited the effects caused by salt stress (Fig. 5 A-C). It suggested that H_2O_2 synthesis probably mediated salt stress-caused stomatal closure through inducing H_2S production.

Effects of salt stress on H_2S content, L-CDes and D-CDes activity of leaves in wild-type, AtrbohD, AtrbohF and AtrbohD/F mutants

To further prove H_2O_2 mediated salt stress-caused stomatal closure through inducing H_2S synthesis, we detected H_2S content, *L*-CDes and *D*-CDes activity in wild-type, *AtrbohD*, *AtrbohF* and *AtrbohD/F* mutants under salt stress. H_2S content, *L*-CDes and *D*-CDes activity of leaves in wild-type and *AtrbohD* mutant under salt stress were significantly increased (Fig. 5 D-F). However, the effects observed in wild-type were blocked in *AtrbohF* and *AtrbohD/F* mutants (Fig. 5 D-F). The data provided evidence that H_2O_2 synthesis mediated salt stress-caused stomatal closure through inducing H_2S synthesis, and AtrbohF participated in H_2O_2 production in the process.

H_2S modulators can't prevent salt stress-caused H_2O_2 production in wild-type

To further establish that H_2S synthesis mediated salt stress-caused stomatal closure through inducing H_2O_2 production, H_2DCF fluorescence under salt stress in presence of H_2S modulators were measured. The H_2DCF fluorescence intensity of guard cells under salt stress was significantly intense than that in light treatment (Fig. 6 A and B). Moreover, H_2S modulators couldn't prohibit salt stress-caused rise in H_2DCF fluorescence intensity (Fig. 6 C-F). These data provided evidence that H_2O_2 really acted upstream of H_2S mediating salt stress-caused stomatal closure.

Salt stress can cause H_2O_2 synthesis in Atl-cdes and Atd-cdes mutants

To further consolidate the conclusion that H_2O_2 acted upstream of H_2S in salt stress-caused stomatal closure, we measured H_2DCF fluorescence in *Atl-cdes* and *Atd-cdes* mutants' guard cells under salt stress. Salt stress induced intense H_2DCF fluorescence in wild-type, *Atl-cdes* and *Atd-cdes* mutants (Fig. 6 B and G-H), which reinforced the conclusion that H_2O_2 represented a novel component upstream of H_2S in salt stress-caused stomatal closure.

Interaction between H_2S and NO during salt stress-induced stomatal closure

NO modulators prevent salt stress-caused H_2S synthesis and L-/D-CDes activity increase

To explore the interrelationship between H_2S and NO in salt stress-caused stomatal closure, the effects of NO modulators on change of H_2S synthesis, *L*-CDes and *D*-CDes activity induced by salt stress in wild-type were examined. The results showed that salt stress caused a significant increase in H_2S content, and *L*-CDes and *D*-CDes activity of leaves, c-PTIO, Na_2WO_4, and *L*-NAME significantly reduced H_2S content, *L*-CDes and *D*-CDes activity under NaCl stress (Fig. 7 A-C). The results indicated that NO might function upstream of H_2S in salt stress-caused stomatal closure *in A. thaliana*.

Effects of salt stress on H_2S content, and L-CDes and D-CDes activity of leaves in wild-type, Atnoa1, nia1-2, nia2-1 and nia1-2/nia2-5 mutants

To further prove NO functioned upstream of H_2S in salt stress-caused stomatal closure, we detected H_2S content, *L*-CDes and *D*-CDes activity in *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants' leaves under salt stress. NaCl treatment increased H_2S content, *L*-CDes and *D*-CDes activity of leaves in wild-type, but the effects were blocked in *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants (Fig. 7 D-F). The results further indicated that H_2S might function downstream of NO during stomatal closure caused by salt stress in *A. thaliana*.

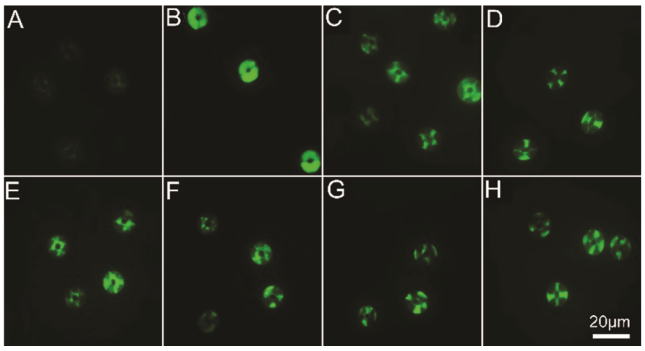

Fig. 6 — (A-F) Effects of H_2S modulators on salt stress-induced H_2O_2 synthesis of guard cells in wild-type; and (G-H) effects of salt stress on H_2O_2 synthesis of guard cells in *Atl-cdes* and *Atd-cdes* mutants. [Guard cells were treated in light at 22 ± 2°C for 1 h as follows: (A) with MES-KCl buffer alone in light; (B) containing 100 mmol·L^{-1} NaCl; (C) 100 mmol· L^{-1} NaCl + 15 μmol·L^{-1} HT; (D) 100 mmol·L^{-1} NaCl + 0.4 mmol·L^{-1} AOA; (E) 100 mmol·L^{-1} NaCl + 0.4 mmol·L^{-1} NH_2OH; and (F) 100 mmol·L^{-1} NaCl + 0.4 mmol·L^{-1} $C_3H_3KO_3$ + 0.4 mmol·L^{-1} NH_3. (G-H) Guard cells of *Atl-cdes* and *Atd-cdes* mutants were treated with 100 mmol·L^{-1} NaCl in light at 22 ± 2°C for 1 h. After treatments, epidermal strips were immediately loaded with 50 μmol·L^{-1} H_2DCF-DA in Tris-KCl buffer at 25 ± 2°C for 10 min, and then excess dye was removed and the strips were examined by luminescence microscope. Scale bar in (H) represents 20 μm for images from (A) to (H)]

H_2S modulators can't prevent salt stress-caused NO production of guard cells in wild-type

To further establish that H_2S functioned downstream of NO during stomatal closure caused by salt stress, DAF-2 fluorescence in presence of H_2S modulators under salt stress were measured. The DAF-2 fluorescence intensity of guard cells under salt stress was significantly intense than that in light treatment (Fig. 8 A and B), but H_2S modulators couldn't inhibit salt stress-caused rise in DAF-2 fluorescence intensity (Fig. 8 C-F). The results provided evidence that NO really functioned upstream of H_2S during salt stress-caused stomatal closure.

Salt stress can increase NO levels of guard cells in Atl-cdes and Atd-cdes mutants

To further establish the conclusion that NO acted upstream of H_2S in salt stress-caused stomatal closure, we measured DAF-2 fluorescence in *Atl-cdes* and *Atd-cdes* mutants' guard cells under salt stress. Salt

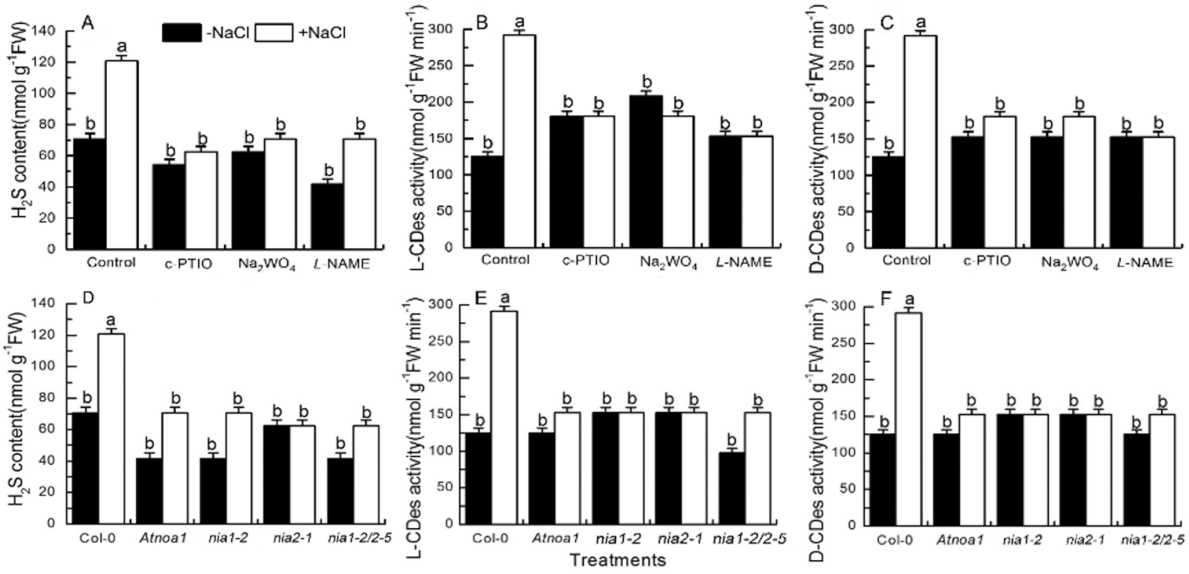

Fig. 7 — (A-C) Effects of NO modulators on salt stress-induced H_2S synthesis and *L*-CDes and *D*-CDes activity increase in wild-type leaves; and (D-F) effects of NaCl on H_2S content and *L*-CDes and *D*-CDes activity in the leaves of wild-type, *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants. [Isolated epidermal strips were incubated in MES-KCl buffer alone, or containing 200 μmol·L^{-1} c-PTIO, 100 μmol·L^{-1} Na_2WO_4, 25 μmol·L^{-1} *L*-NAME in the absence (black columns) or presence of 100 mmol ·L^{-1} NaCl (white columns) in light at 22 ± 2°C for 1 h, and then the leaves were used to measure (A) H_2S content; (B) *L*-CDes activity; and (C) and *D*-CDes activity. (D-F) Isolated epidermal strips of wild-type, *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants were incubated in MES-KCl buffer alone (black columns), or containing 100 mmol·L^{-1} NaCl in light at 22 ± 2°C for 1 h (white columns), then the leaves were used to measure (D) H_2S content, (E) *L*-CDes activity, and (F) *D*-CDes activity. Data are means ± SE. of three independent experiments (n = 9), means denoted by different letters in A-F differed significantly at P <0.05 according to Duncan's multiple range test]

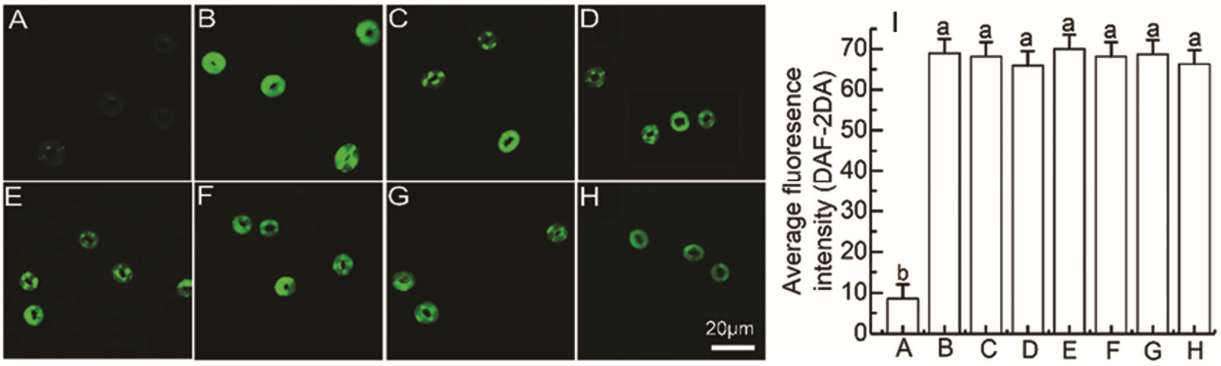

Fig. 8 — (A-F) Effects of H_2S modulators on salt stress-induced NO synthesis of guard cells in wild-type; and (G-H) effects of salt stress on NO synthesis of guard cells in *Atl-cdes* and *Atd-cdes* mutants; and (I) Average fluorescent intensity of guard cells in images. [(A-G)Guard cells were treated in light at 22 ± 2°C for 1 h as follows: (A) with MES-KCl buffer alone in light; or (B) containing 100 mmol·L^{-1} NaCl; (C) 100 mmol·L^{-1} NaCl +15 μmol·L^{-1} HT; (D) 100 mmol·L^{-1} NaCl + 0.4 mmol·L^{-1} AOA; (E) 100 mmol·L^{-1} NaCl + 0.4 mmol·L^{-1} NH_2OH; and (F) 100 mmol·L^{-1} NaCl + 0.4 mmol·L^{-1} $C_3H_3KO_3$ + 0.4 mmol·L^{-1} NH_3. (G-H) Guard cells of *Atl-cdes* and *Atd-cdes* mutants were treated with 100 mmol·L^{-1} NaCl in light at 22 ± 2°C for 1 h. After treatments, epidermal strips were immediately loaded with 50 μmol·L^{-1} H_2DCF-DA in Tris-KCl buffer at 25 ± 2°C for 10 min, and then excess dye was removed and the strips were examined by luminescence microscope. Scale bar in (H) represents 20 μm for images from (A) to (H). For (I), data of fluorescence pixel intensities are displayed as means ± SE of three independent experiments (n = 9). Means followed by different letters in (I) are significantly different at P <0.05 according to Duncan's multiple range test]

stress induced intense DAF-2 fluorescence in wild-type, *Atl-cdes* and *Atd-cdes* mutants (Fig. 8 B and G-H), which consolidated the conclusion that NO represented a novel component upstream of H_2S in salt stress-caused stomatal closure.

Discussion

Salt stress can cause damage to plants through osmotic stress, ion poisoning and oxidative stress[1]. Salt stress can affect diverse processes in plants[3,4]. Plants can reduce the damage caused by salt stress

and drought stress through adjusting stomatal aperture, synthesizing osmotic substances, separating excessive ions and scavenging reactive oxygen species, and can maintain ion and osmotic balance and activity of antioxidant enzymes, and finally adapt environmental stresses[43]. Stomata are very sensitive to environmental change, responding quickly to various stimuli. The change of stomatal aperture is critical for plants to adapt to environmental change. It has been shown that salt stress can induce stomatal closure[8]. It has been shown that NaCl treatment can induce stomatal closure in *V. faba*[8]. However, the mechanism during salt stress-caused stomatal movement in *A. thaliana* is still unclear. Our results showed that NaCl treatment induced stomatal closure in *A. thaliana*, the best effects of NaCl on stomatal closure were 100 $mmol \cdot L^{-1}$ and 1 h, respectively (Fig. 1A), and the results from the elution experiment showed that NaCl treatment had reversible effects on stomatal aperture (Fig. 1B), which is consistent with the previous reports[8].

H_2S is the third signal molecule, which has positive physiological effects and plays a key role in organisms. More evidence has shown that H_2S, as an important gaseous mediator, plays a vital role in many physiological processes in plants[12,15,16]. Previous studies have shown that H_2S can alleviate the damage caused by abiotic stresses[16,44]. Moreover, H_2S has been proved to mediate stomatal closure caused by drought, ethylene, darkness, salt stress, $CdCl_2$, brassinosteroid and abscisic acid (ABA)[8,16-21,25,45], etc. Whether H_2S functions as a signaling molecule participating in salt stress-caused stomatal closure in *A. thaliana* remains unknown. Our data showed that salt stress induced stomatal closure and increased H_2S content of leaves, these effects were significantly prohibited by H_2S modulators HT, AOA, NH_2OH, and $C_3H_3KO_3$ and NH_3 (Fig. 2 A and B), implying that H_2S is involved in salt stress-caused stomatal closure in *A. thaliana*, which is similar to the reported results[8]. Moreover, NaCl treatment significantly increased *L*-CDes and *D*-CDes activity in leaves of wild-type, cotreatment with H_2S modulators and NaCl prohibited the effects (Fig. 2 C and D). Furthermore, NaCl treatment could close stomata of wild-type, but failed to close stomata of *Atl-cdes* and *Atd-cdes* mutants (Fig. 2E). The results suggested that both *L*-CDes and *D*-CDes were responsible for H_2S production in salt stress-caused stomatal closure in *A. thaliana*.

H_2O_2 plays an important role in regulating stress response, stomatal motility and plant development[24,25,27]. H_2O_2 is proved to mediate stomatal movement induced by NaCl[8], darkness[19] and UV-B[46], etc. H_2O_2 sourced from *AtrbohD* mediates ABA-induced stomatal closure in *A. thaliana*[21]. Our results showed that H_2O_2 modulators ASA, CAT and DPI significantly inhibited salt stress-induced stomatal closure in wild-type (Fig. 3A). NaCl treatment significantly induced stomatal closure of wild-type and *AtrbohD* mutant, but failed to close stomata of *AtrbohF* and *AtrbohD*/*F* mutants (Fig. 3B). NaCl treatment could induce intense H_2DCF fluorescence of guard cells in wild-type (Fig. 3 C and D), H_2O_2 modulators prohibited the effects under NaCl treatment (Fig. 3 E-G). Furthermore, NaCl treatment intensified H_2DCF fluorescence in *AtrbohD* mutant, but couldn't induced intense H_2DCF fluorescence of *AtrbohF* and *AtrbohD/F* mutants (Fig. 3 H-J). These results proved that NADPH oxidase pathway-derived H_2O_2 was involved in salt stress-caused stomatal closure in *A. thaliana*, which is the same as the reports[8], and H_2O_2 was sourced from *AtrbohF* in the process.

More evidence has shown that NO, as an endogenous signaling molecule in plants, regulates various physiological processes[16,31,32]. NO has been shown to mediate stomatal closure induced by ethylene, darkness, EBR and UV-B[23,26,29,46], etc. Until now, whether NO mediates salt stress-caused stomatal closure in *A. thaliana* remains unclear. Our results showed that NO modulators c-PTIO, Na_2WO_4, and *L*-NAME could significantly inhibit stomatal closure caused by salt stress in wild-type (Fig. 4A). Compared with the wild-type, salt stress had no significant influence on stomatal aperture of *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants (Fig. 4B). Additionally, salt stress could induce an intense DAF-2 fluorescence of guard cells compared with light treatment in wild-type (Fig. 4 C-D), but the effects were significantly prohibited by c-PTIO, Na_2WO_4, and *L*-NAME (Fig. 4 E-G). Moreover, there was no significant difference in DAF-2 fluorescence in *Atnoa1*, *nia1-2*, *nia2-1*, and *nia1-2/nia2-5* mutants from that in wild-type (Fig. 4 C and H-J). The data indicate that NO derived from NOS and NR participated in salt stress-caused stomatal closure, *Atnoa1*, *nia1* and *nia2* were responsible for NO production in the process in *A. thaliana*.

It has been proved that H_2S and H_2O_2 interact in various physiological processes in plants. H_2O_2 is proved to mediate H_2S-induced carbon assimilation and photoprotection in cucumber seedlings[47]. H_2O_2-mediated H_2S synthesis participates in darkness-, $CdCl_2$- and NaCl-induced stomatal movement[8,18,45]. Recent research showed that EBR induces stomatal closure through inducing H_2O_2 synthesis and subsequent H_2S production in *A. thaliana*, H_2O_2 and H_2S are sourced from *AtrbohF* and *AtL-CDes*-/*AtD-CDes*, respectively[19]. Whether H_2S and H_2O_2 interact in salt stress-caused stomatal closure in *A. thaliana* is still unclear. The data presented here showed that H_2O_2 modulators inhibited salt stress-caused H_2S production and increase in *L*-CDes and *D*-CDes activity in wild-type (Fig. 5 A-C), and salt stress couldn't change H_2S content, *L*-CDes and *D*-CDes activity in *AtrbohF* and *AtrbohD/F* mutants (Fig. 5 D-F). In addition, H_2S modulators couldn't change H_2DCF fluorescence under salt stress in wild-type (Fig. 6 C-F), while salt stress could induce rise in H_2DCF fluorescence intensity in *Atl-cdes* and *Atd-cdes* mutants (Fig. 6 G-H). These results suggested that H_2S sourced from *L*-CDes and *D*-CDes functions downstream of H_2O_2 derived from *AtrbohF* in salt stress-caused stomatal closure in *A. thaliana.* However, ABA induced H_2S synthesis catalysed by *L*-CDes, subsequent caused *AtrbohD*-derived H_2O_2 production, and finally closed stomata in *A. thaliana*[21], which is different from our results. The divergence of the results probably is related to differences in stress condition.

It has been shown that H_2S interacts with NO in multiple physiological processes[48]. But H_2S has been shown to act downstream of NO derived from NR and mediates stomatal closure[23,33,34]. However, whether H_2S interacts NO in salt stress-caused stomatal closure in *A. thaliana* is still unknown. Our results showed that salt stress significantly increased H_2S levels, *L*-CDes and *D*-CDes activity of leaves in wild-type, but the effects were prohibited by NO modulators (Fig. 7 A-C). Salt stress significantly increased H_2S levels, *L*-CDes and *D*-CDes activity in wild-type leaves, but there were no obvious changes in H_2S levels, *L*-CDes and *D*-CDes activity in *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants compared with that in wild-type (Fig. 7 D-F). In addition, salt stress induced an intense DAF-2 fluorescence in guard cells compared with light treatment in wild-type (Fig. 8 A and B), but salt stress-induced increase in NO levels of guard cells didn't change when epidermal strips were treated by H_2S modulators under NaCl stress (Fig. 8 C-F). Salt stress increased NO levels of guard cells in *Atl-cdes* and *Atd-cdes* mutants (Fig. 8 G and H), and there was no significant difference from NO levels in wild-type (Fig. 8B). From the data, we suggest that H_2S functions downstream of NO in salt stress-induced stomatal closure in *A. thaliana*, both NOS and NR are necessary for NO production, and *L*-CDes and *D*-CDes are responsible for H_2S synthesis in the process. In addition, NO sourced from *Atnoa1*, *nia1* and *nia2* mediates salt stress-induced stomatal closure, which is similar to the results from Hao *et al*.[49]. However, H_2S promotes NO generation in ABA-induced stomatal closure, and NO acts downstream of H_2S in the process, in which the divergence with our results probably is related to differences in stress conditions.

Conclusion

The above results suggest that salt stress promotes NADPH oxidase-derived H_2O_2 production and NOS- and NR-sourced NO synthesis, and further causes *L*-CDes- and *D*-CDes-catalysed H_2S synthesis, and finally leads to stomatal closure in the rockcress or thale cress, *Arabidopsis thaliana*. During salt stress-induced stomatal closure, *AtrbohF* was responsible for H_2O_2 production, NO synthesis depends on *Atnoa1*, *nia1* and *nia2*, both *AtL-CDes* and *AtD-CDes* are responsible for H_2S synthesis. Our results provide evidence that H_2S, H_2O_2 and NO mediate salt stress-caused stomatal closure, and H_2S acts downstream of H_2O_2 and NO in the process in *A. thaliana*. Whether or not other signals such as CO, cytosolic pH, or protein kinases mediate salt stress-induced stomatal closure, and the interrelationship among them need to be further investigated.

Conflict of interest

Authors declare no competing interests.

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