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NITRIC OXIDE FUNCTIONS UPSTREAM OF HYDROGEN SULFIDE IN STRIGOLACTONES-TRIGGERED STOMATAL CLOSURE IN *ARABIDOPSIS THALIANA* (L.) HEYNH

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Abstract

Strigolactones (SLs)-induced stomatal closure involves nitric oxide (NO) and hydrogen sulfide (H_2S) in *Arabidopsis thaliana* (L.) heynh. H_2S synthesis inhibitors and NO modulators prohibited SL-triggered stomatal closure. SL caused stomatal closure of *nia2-1* mutant, but failed to close stomata of *Atl-cdes*, *Atd-cdes*, *nia1-2*, *nia1-2/nia2-5* and *Atnoa1* mutants. SL induced NO production in wild-type and *nia2-1* mutant, but not in *Atnoa1*, *nia1-2* and *nia1-2/nia2-5* mutants, NO modulators inhibited the effects induced by SL in wild-type. Furthermore, SL promoted H_2S synthesis and L-/D-CDes activity in wild-type and *nia2-1* mutant, NO modulators prevented the effects induced by SL in wild-type. The induction of H_2S synthesis and L-/D-CDes activity by SL was abolished in *Atnoa1*, *nia1-2* and *nia1-2/nia2-5* mutants. However, H_2S synthesis inhibitors could not inhibit SL-induced NO production in wild-type, *Atl-cdes* and *Atd-cdes* mutants exhibited normal NO levels in guard cells. The results suggested that NO functioned upstream of H_2S synthesis in SLs-triggerred stomatal closure in *A. thaliana*.

Introduction

Stomata play an important role in the process of gas and water exchange between plants and the external environment. Many factors can regulate stomatal movement, such as darkness (Ma *et al.* 2018), plant hormones (Shi *et al.* 2015) and $CdCl_2$ stress (Ma *et al.* 2019). It has been proved that strigolactones (SLs), as plant hormones, can regulate plant development and stomatal movement (Al-Babili and Bouwmeester 2015, Lv *et al.* 2018, Ma *et al.* 2024). SLs play a positive role in stress acclimatization including drought and salt stress (Ha *et al.* 2014). Lv *et al.* (2018) reported that hydrogen peroxide (H_2O_2) and nitric oxide (NO) are involved in SLs-induced stomatal closure in an ABA-independent manner. However, the mechanism of signaling transduction in SLs-induced stomatal closure is still unclear.

Hydrogen sulfide (H_2S) and NO, as endogenous signaling molcules, mediate many physiological processes in plants, such as photosynthesis (Chen *et al.* 2011) and stomatal movement (Scuffi *et al.* 2014, Zhang *et al.* 2020, Ma *et al.* 2022), etc. Both H_2S and NO are involved in responses to abiotic stresses in plants (Liu *et al.* 2008, Jin *et al.* 2013). Lv *et al.* (2018) reported that NO is involved in SLs-induced stomatal closure. NO has been proved to regulate 2, 4-epibrassinolide (EBR, a bioactive BR)-caused stomatal closure through inducing H_2S synthesis (Ma *et al.* 2022). Ma *et al.* (2024) showed that H_2S functions downstream of H_2O_2 in SLs-induced stomatal closure. However, it is unclear whether H_2S relates to NO in SLs-induced stomatal closure. In this study, we provided evidence that NO functions upstream of H_2S synthesis in SLs-induced stomatal closure in *Arabidopsis thaliana* (L.) heynh by using pharmacological, spectrophotographic and fluorescence microscope approaches. The findings provide important insights into the signaling mechanism of SLs-regulated stomatal movements in plants.

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Materials and Methods

Seeds of *A. thaliana* wild-type (Col-0) and L-/D-cysteine desulfhydrase (L-/D-CDes) deletion mutants (*Atl-cdes*, *Atd-cdes*) were purchased from Nottingham Arabidopsis Stock Center (NASC, Nottingham, UK). NOS-like gene mutant *Atnoa1* and NR gene mutants *nia1-2*, *nia2-1* and *nia1-2/nia2-5* were gifted by Professor He Junmin from Shaanxi Nornal University. *A. thaliana* wild-type and mutants were grown in the conditions as described by Ma *et al.* (2024), and the epidermis strips were prepared as described by Ma *et al.* (2024).

Stomatal bioassay was performed as described by McAinsh *et al.* (1996) and Ma *et al.* (2024). In brief, freshly prepared epidermal strips were treated with MES-KCl buffer (10 mmol/L MES/KOH, 50 mmol/L KCl, 100 μmol/L $CaCl_2$, pH 6.15) alone or containing various compounds or inhibitors in light (300 μmol/m^2·sec) at 25 ± 2°C for 3 hrs. and then stomatal apertures were recorded with a light microscope and an eyepiece graticule previously calibrated with a stage micrometer. Each treatments were repeated at least three times, and the data presented are the means ± standard errors (SEs) (n = 90).

Measurement of H_2S emission was determined by formation of methylene blue, which was performed as described by Ma *et al.* (2024). Firstly, 0.1 g treated leaves were taken out and ground in the presence of 0.9 mL 20 mmol/l 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/l N, N-dimethyl-phenylene diamine dihydrochloride dissolved in 7.2 mol/l HCl and 100 μL 30 mmol/L $FeCl_3$ dissolved in 1.2 mol/L 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. To investigate L-/D-CDes activity, we determined H_2S which was released from L-/D-cysteine within a certain period of time (Riemenschneider *et al.* 2005, Ma *et al.* 2024). Fully expanded leaves of 4-week-old seedlings were treated and used to measure H_2S emission and L-/D-CDes activity. The data presented are means ± SEs of three independent experiments (n = 9).

NO in guard cells was monitored by using fluorescent indicator dye DAF-2 DA, as previously described (Ma *et al.* 2022). After treatments, the epidermal strips were incubated in Tris-KCl buffer (Tris 10 mmol/l and KCl 50 mmol/l, pH 7.2) containing 10 μmol/l DAF-2 DA for 30 min in darkness at 25 ± 2°C. Then excess dye was washed off with Tris-KCl buffer in darkness, the epidermal strips were immediately examined by fluorescence microscope (OLYMPUS BX53, U-RFLT50, JAPAN) with the following settings: 450 nm of excitation, 490 nm of emission. Each experiment was repeated at least three times. The selected confocal images represented the same results from three replications.

The statistical significance of treatments was checked using one-way ANOVA followed by Duncan's multiple range test. The data were considered statistically significant when *P*-values were below 0.05. The means denoted by different letters in figures differ significantly at $P < 0.05$ according to Duncan's multiple range test.

Results and Discussion

Our previous data indicated that SLs can close stomata of *A. thaliana* (Ma *et al.* 2024). The results in the study showed that H_2S synthesis inhibitors AOA and NH_2OH, $C_3H_3KO_3$+NH_3 (the product of L-/D-CDes), and NO specific scavenger c-PTIO (García-Mata and Lamattina 2001), mammalian nitric oxide synthase (NOS) inhibitor *L*-NAME (Neill *et al.* 2003) and nitrate reductase (NR) inhibitor Na_2WO_4 all significantly prohibited stomatal closure triggered by GR24 (a synthetic analogue of SLs) (Fig. 1A). Additionally, GR24 could cause stomatal closure of wild-type and *nia2-1* mutant, but couldn't change the stomata of *Atl-cdes*, *Atd-cdes*, *nia1-2*, *nia1-*

2/nia2-5 and *Atnoa1* mutants (Fig. 1B). The results suggested that both H_2S and NO might mediate SLs-triggered stomatal closure, H_2S might be produced by L-/D-CDes pathway (AtL-CDes, AtD-CDes), and NO synthesis might be catalyzed by NOS and NR (Nia1).

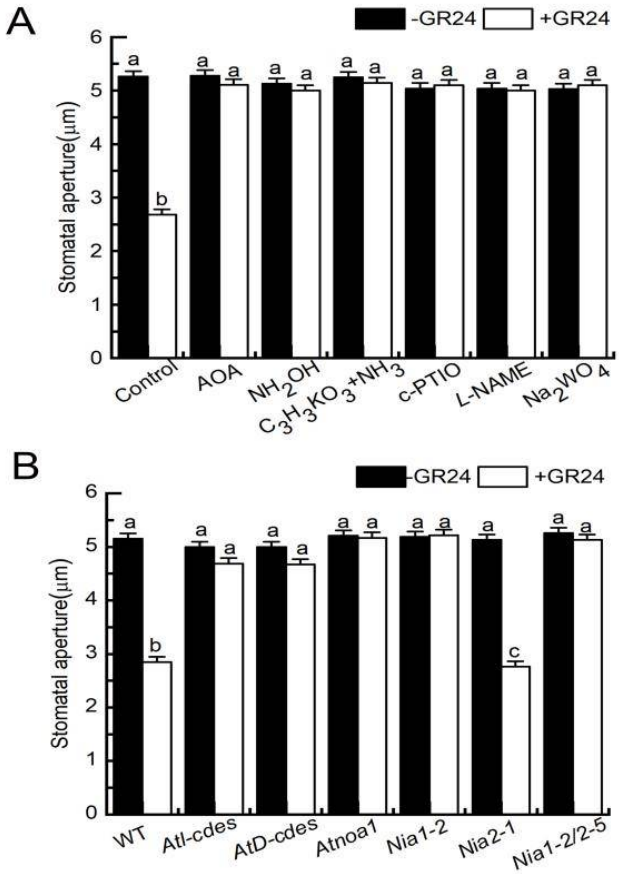

Fig. 1. H_2S synthesis inhibitors and NO modulators inhibit GR24-induced stomatal closure in wild-type (A), and effects of GR24 on stomatal aperture in *Atl-cdes*, *Atd-cdes*, *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants (B). A. Isolated epidermal strips of wild-type were incubated in MES/KCl buffer alone or containing 0.4 mmol/L AOA, 0.4 mmol/l NH_2OH, 0.4 mmol/l $C_3H_3KO_3$ + 0.4 mmol/L NH_3, 200 μmol/l c-PTIO, 25 μmol/l *L*-NAME and 100 μmol/l Na_2WO_4 in the absence (black columns) or presence of 1 μmol/l GR24 (white columns B. isolated epidermal strips of wild-type, *Atl-cdes*, *Atd-cdes*, *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants were incubated in MES/KCl buffer alone (black columns), or containing 1 μmol/l GR24 (white columns) in light for 3 hrs., respectively, then apertures were measured.

Next, NO-specific fluorescent dye DAF-2DA was used to detect NO levels in guard cells of wild-type, *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants. The results of Fig. 2 showed that GR24 significantly induced NO production in guard cells compared with the control (Fig. 2A, B and J) in wild-type, while c-PTIO, *L*-NAME and Na_2WO_4 obviously prohibited the effects (Fig. 2C-E and J). GR24 increased NO levels in guard cells of *nia2-1* mutant (Fig. 2H and J), but had no significant effect on NO levels in *Atnoa1*, *nia1-2* and *nia1-2/nia2-5* mutants (Fig. 2F, G, I and J). Combined with the results in Fig. 1, the data further indicated that NO mediated SLs-triggered stomatal closure, NO synthesis depended on AtNOA1 and NR (Nia1) in the process in *A. thaliana.*

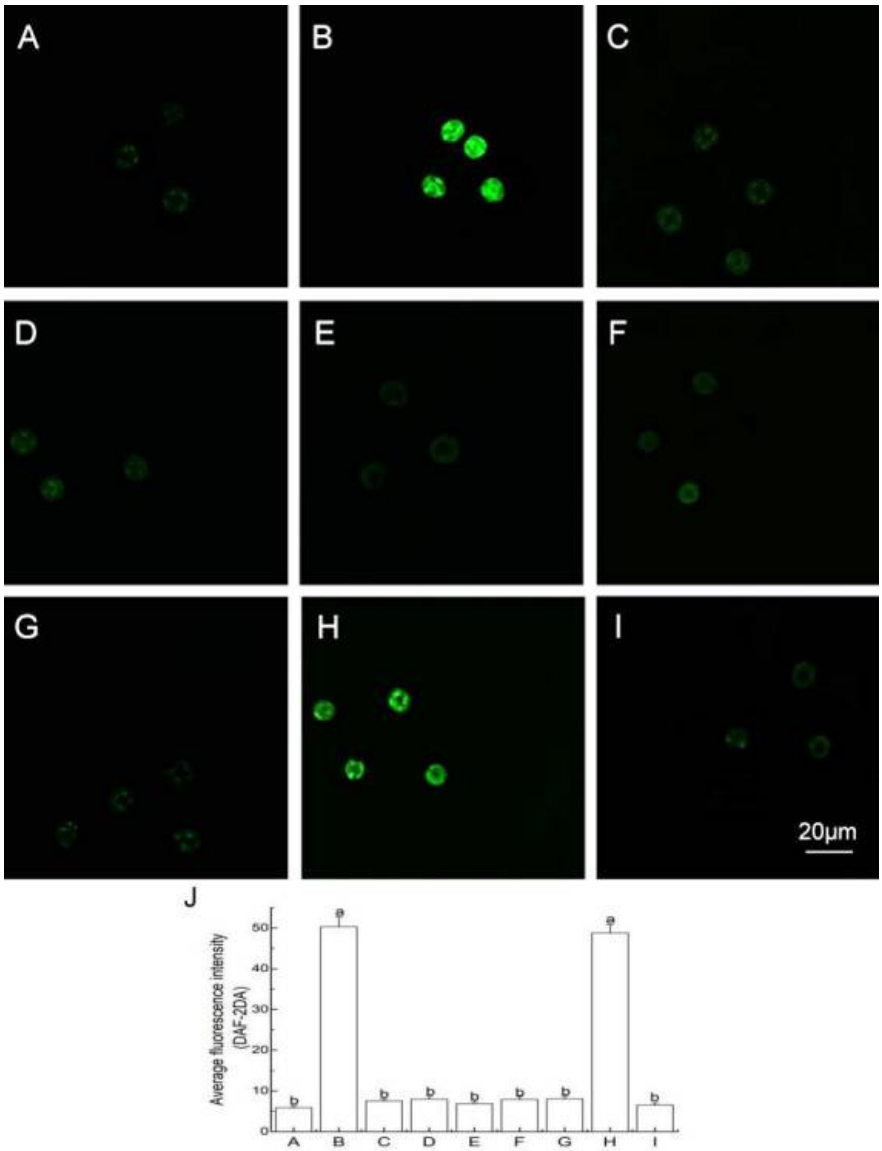

Fig. 2. Effects of NO modulators on GR24-induced NO production in wild-type, and effects of GR24 on NO levels in *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants. (A) Guard cells were treated with MES/KCl buffer alone, or containing (B) 1 μmol/L GR24, (C) 200 μmol/L c-PTIO + 1 μmol/L GR24, (D) 25 μmol/L *L*-NAME + 1 μmol/L GR24, (E) Na_2WO_4 + 1 μmol/L GR24; and (F-I), guard cells of *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants were incubated in 1 μmol/L GR24 in light for 3 hrs., respectively. (J) Average fluorescent intensity of guard cells in images (A) to - (I); data are means ± SEs of three independent experiments (n = 3). Scale bar in (I) represents 40 μm for all images.

We further explored the relationship between H_2S and NO in SLs-triggered stomatal closure. The results showed that c-PTIO, *L*-NAME and Na_2WO_4 significantly prevented GR24-induced H_2S synthesis and L-/D-CDes activity increase of leaves in wild-type plants (Fig. 3A-C). GR24 could obviously increase H_2S content and L-/D-CDes activity of leaves in *nia2-1* mutant, but had

no significant effects on H_2S content and L-/D-CDes activity in *Atnoa1*, *nia1-2* and *nia1-2/nia2-5* mutants (Fig. 3D-F). These results suggested that NO might act as an upstream signal component of H_2S in SLs-triggered stomatal closure in *A. thaliana*

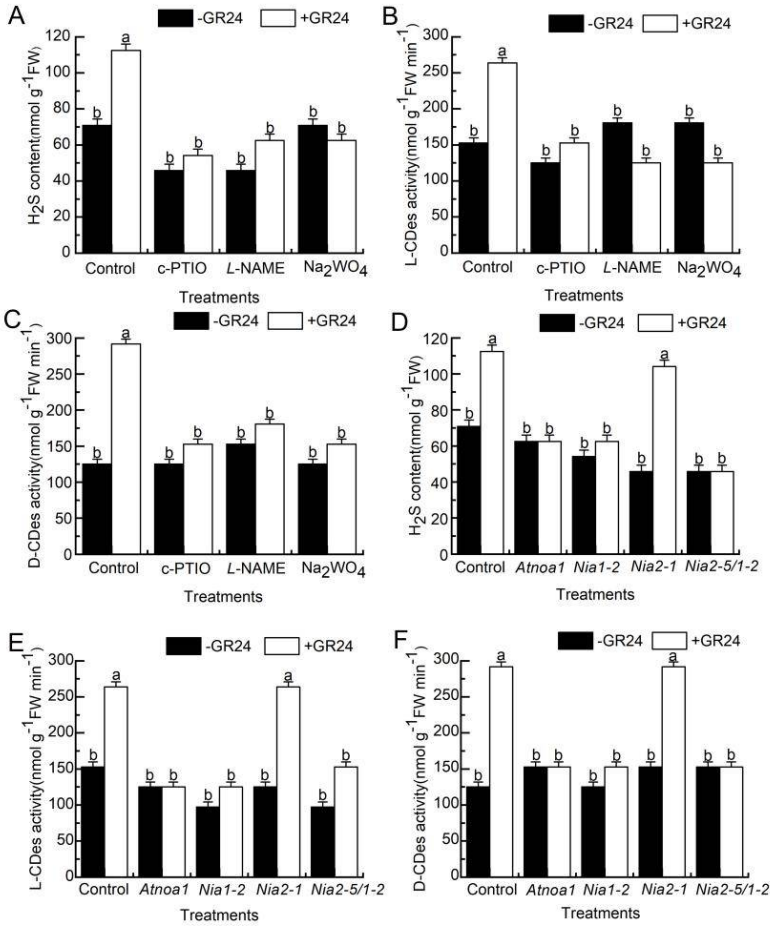

Fig. 3. NO modulators prevent GR24-induced H_2S synthesis and L-/D-CDes activity increase in wild-type (A-C), and effects of GR24 on H_2S content and L-/D-CDes activity in *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants (D-F). A-C, The leaves of wild-type were incubated in MES/KCl buffer alone, or containing 200 μmol/L c-PTIO, 25 μmol/L *L*-NAME, and 100 μmol/L Na_2WO_4 in the absence (black columns) or presence of 1 μmol/L GR24 (white columns) in light for 3 hrs., respectively; D-F, the leaves 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 1 μmol/L GR24 in light for 3 hrs. (white columns), respectively, then H_2S content (A and D) and L-/D-CDes activity (B, C, E, and F) were measured.

Finally, we detected NO levels of guard cells in wild-type, *Atl-cdes* and *Atd-cdes* mutants. Compared with the control, GR24 significantly caused NO production in guard cells of wild-type (Fig. 4A, B and H). AOA, NH_2OH and $C_3H_3KO_3$+NH_3 couldn't prevent these effects induced by GR24 (Fig. 4C-E and H). In addition, NO levels in *Atl-cdes* and *Atd-cdes* mutants under GR24 treatment showed no difference from that in wild-type (Fig. 4B, F-G and H). These results further proved that NO functioned upstream of H_2S in SLs-triggered stomatal closure in *A. thaliana*.

SLs, as plant hormones, are synthesized from carotenoids (Waldie *et al.* 2014, Al-Babili and Bouwmeester 2015). SLs can regulate plant development (Al-Babili and Bouwmeester 2015), stomatal movement (Zhang *et al.* 2018, Lv *et al.* 2018, Ma *et al.* 2024). SLs can respond to various stresses (Liu *et al.* 2015, Visentin *et al.* 2016). However, the mechanism of SLs-regulated stomatal movement is still unknown. H_2S and NO, as two important signal molecules, mediate diverse aspects of physiological processes in plants (Liu *et al.* 2012, Wang *et al.* 2012, Jin *et al.* 2013, Ma *et al.* 2022). Our results showed that H_2S sourced from L-/D-CDes pathways mediated SLs-triggered stomatal closure in *A. thaliana*, which is consistent with the previous results (Ma *et al.* 2024). Additionally, NO synthesis catalyzed by NOS and NR (Nia1) was involved in SLs-induced stomatal closure in *A. thaliana*, which is the same as the previous results of Lv *et al.* (2018).

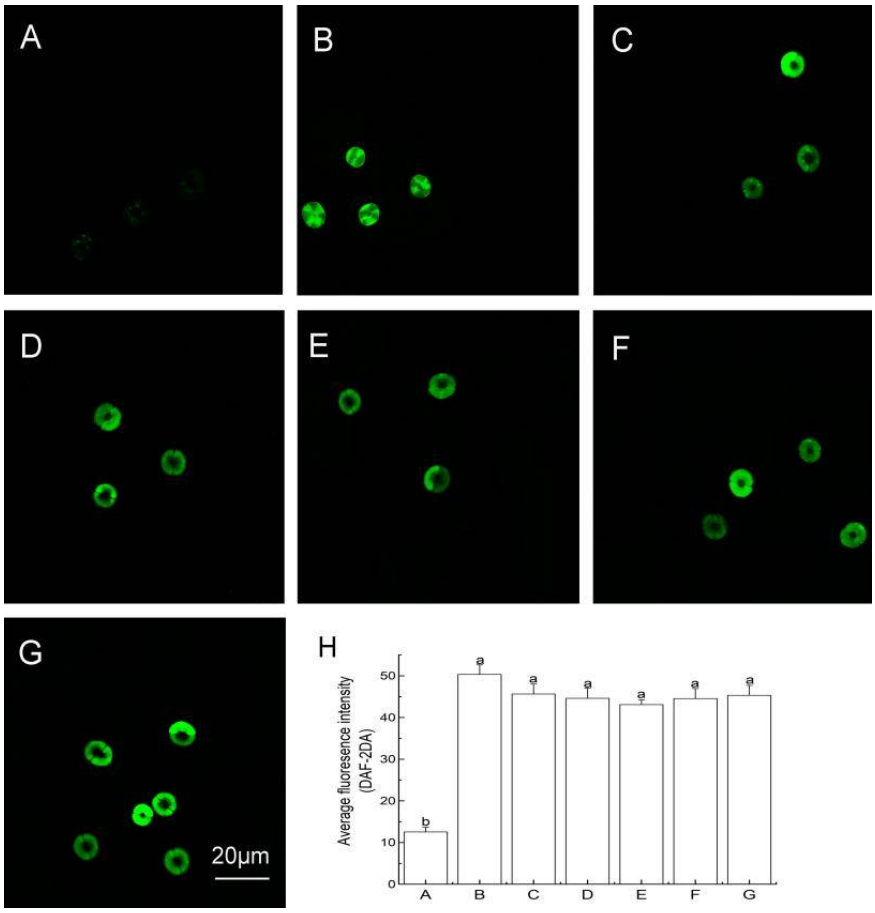

Fig. 4. Effects of H_2S synthesis inhibitors on GR24-induced NO production in wild-type, and effects of GR24 on NO levels in *Atl-cdes* and *Atd-cdes* mutants. (A) Guard cells were treated with MES/KCl buffer alone, or containing (B) 1 μmol/l GR24, (C) 0.4 mmol/l AOA + 1 μmol/l GR24, (D) 0.4 mmol/L NH_2OH + 1 μmol/l GR24, (E) 0.4 mmol/l $C_3H_3KO_3$ + 0.4 mmol/L NH_3 + 1 μmol/l GR24; and (F-G), guard cells of *Atnoa1*, *nia1-2*, *nia2-1* and *nia1-2/nia2-5* mutants were incubated in 1 μmol/l GR24 in light for 3 hrs., respectively. (H) Average fluorescent intensity of guard cells in images (A)-(G); data are means ± SEs of three independent experiments (n=3). Scale bar in (G) represents 40 μm for all images.

H_2S and NO have been proved to mediate ethylene-, EBR-, and ABA-induced stomatal closure (Liu *et al*. 2012, Scuffi *et al*. 2014, Ma *et al.* 2022). However, whether H_2S interacts to NO during SLs-induced stomatal movement is still unclear. Our results showed that NO functioned upstream of H_2S in the signal transduction pathway of SLs-induced stomatal closure in *A. thaliana.*

Altogether, the results provided evidence that H_2S and NO participated in SLs-induced stomatal closure in *A. thaliana*, and H_2S functioned downstream of NO in the physiological process. H_2S production was catalyzed by L-/D-CDes, and NO was derived from NOS and NR (Nia1) pathways in SLs-induced stomatal closure. In the present study, our results confirmed the effects of SLs on stomatal movement and clarified the interaction of H_2S and NO in the process. However, whether H_2S interacts with other signal molecules such as G protein, CO or Ca^{2+} remains to be further investigated.

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