Article

Hydrogen sulfide induced by nitric oxide mediates ethylene-induced stomatal closure of *Arabidopsis thaliana*

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Pharmacological, laser scanning confocal microscopic (LSCM), real-time PCR and spectrophotographic approaches are used to study the roles of hydrogen sulfide (H_2S) and nitric oxide (NO) in signaling transduction of stomatal movement response to ethylene in *Arabidopsis thaliana*. In the present study, inhibitors of H_2S synthesis were found to block ethylene-induced stomatal closure of *Arabidopsis*. Treatment with ethylene induced H_2S generation and increased *L-/D*-cysteine desulfhydrase (pyridoxal-phosphate-dependent enzyme) activity in leaves. Quantitative PCR analysis showed *AtL-CDes* and *AtD-CDes* transcripts were induced by ethylene. It is suggested that ethylene-induced H_2S levels and *L-/D*-cysteine desulfhydrase activity decreased when NO was compromised. The data clearly show that ethylene was able to induce H_2S generation and stomatal closure in *Atnoa1* plants, but failed in the *Atnia1,nia2* mutant. Inhibitors of H_2S synthesis had no effect on ethylene-induced NO accumulation and nitrate reductase (NR) activity in guard cells or leaves of *Arabidopsis*, whereas ethylene was able to induce NO synthesis. Therefore, we conclude that H_2S and NO are involved in the signal transduction pathway of ethylene-induced stomatal closure. In *Arabidopsis,* H_2S may represent a novel downstream indicator of NO during ethylene-induced stomatal movement.

hydrogen sulfide, nitric oxide, *L-/D*-cysteine desulfhydrase, *Arabidopsis thaliana*, ethylene, stomatal closure

Stomata are by far the most influential components in gas exchange and their movements control transpiration [1]. Consequently, stomata are important regulators of plant growth and development. Although previous studies have shown stomata respond to a variety of environmental stresses, such as drought, cold, high CO_2 concentration and phytohormone [2], little is known about the signal transduction mechanisms that function in guard cells. The effects of some newly identified signal molecules on regulation of stomatal aperture have not been studied yet.

Ethylene is best known for its roles in the regulation of many developmental processes throughout the plant's life cycle, from germination to senescence, and is also involved in responses to environmental stimuli such as stress and pathogen attack. It was reported ethylene induces stomatal closure in detached epidermal strips from leaves of *Arabidopsis thaliana*, and hydrogen peroxide (H_2O_2) production that originated from NAD(P)H oxidase pathway plays an important role in the ethylene signal transduction leading to stomatal closure [3]. Recently, Liu et al. [3] demonstrated that H_2O_2-regulated nitric oxide (NO) production involves ethylene-induced stomatal closure, NO production was associated with nitrate reductase (NR) activity, and the nitric oxide synthase (NOS) inhibitor *L*-NAME had little effect on ethylene-induced NO synthesis. It is indicated that transient changes of calcium [4] and alkalization of the cytoplasm [5] enhanced NO generating in ethylene-induced stomatal closure. The signal transport pathway for regulation of

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stomatal movement by ethylene is a complex signaling network, which includes calcium, protons, NO, H_2O_2, and several other components. Cross reactions are known to exist between these signaling molecules. Further studies of signal transduction mechanisms of ethylene-induced stomatal closure are needed to understand the mechanism of stomatal closure.

Hydrogen sulfide (H_2S) and its counterparts NO and carbon monoxide (CO) are increasingly recognized as "gasotransmitters". Accumulating evidence shows that H_2S participates in a variety of biological processes in plants [6–8]. In higher plants, cysteine desulfhydrase catalyzes the formation of *L*-alanine and elemental sulfur or H_2S from *L*-cysteine. For this reaction, two mechanisms are possible: elemental sulfur is released enzymatically from *L*-cysteine and reduced to sulfide non-enzymatically with *L*-cysteine or dithiothreitol contained in the reaction mixture. Recently, a review of H_2S in plants was published, and H_2S emission by higher plants has been observed to inhibit the damage of pathogen attack [9]. However, it remains unclear whether H_2S serves as a signal molecule during regulated stomatal movement. It was reported that NO donors induce H_2S synthesis in rat aortic tissue and enhance the expression of cystathionine-γ-lyase (CSE) [10]. Zhang et al. [11] reported the process of H_2S-induced adventitious root formation was probably mediated by NO signals and H_2S might act upstream of NO signal pathways. Thus the regulation of stomatal conductance is extremely complex. Is there an interaction between H_2S, NO and other signaling molecules? To address this question, *Arabidopsis* genotypes (wild type, *Atl-cdes*, *Atd-cdes*, *Atnoa1* and *Atnia1,nia2* mutants) were used to assess the importance and interactions of H_2S and NO during stomatal responses to ethylene.

1   Materials and methods

1.1   Chemicals

The following buffers and solutions were used: MES-KOH buffer (10 mmol/L MES, 50 mmol/L KCl, 0.1 μmol/L $CaCl_2$, pH 6.1); 20 mmol/L Tris-HCl; 2.5 mmol/L dithiothreitol (DTT); 0.8 mmol/L *L*-cysteine (Sigma, USA); 0.8 mmol/L *D*-cysteine (Sigma); 30 mmol/L $FeCl_3$; 20 mmol/L *N*,*N*-dimethyl-*p*-phenylenediamine   dihydrochloride;   0.2 mmol/L 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl imidazoline-1-oxyl-3-oxide potassium salt (cPTIO; Sigma); 0.025 mmol/L *Nω*-nitro-*L*-arginine methyl ester (*L*-NAME; Sigma); 10 μmol/L 4,5-diaminofluorescein diacetate (DAF-2DA; Sigma); 0.004% ethephon (Sigma); 0.1 mmol/L Na_2WO_4; 0.4 mmol/L aminooxy acetic acid (AOA; Sigma); 0.2 mmol/L potassium pyruvate ($C_3H_3KO_3$); 0.2 mmol/L NH_3; and 0.4 mmol/L NH_2OH.

1.2   Plant materials

Arabidopsis thaliana ecotype Columbia (Col-0) was used

throughout this study. Seeds of the *AtL-CDes* T-DNA insertion line (SALK_041918, designated *Atl-cdes*) and *AtD-CDes* T-DNA insertion line (CS853264, designated *Atd-cdes*) were obtained from The *Arabidopsis* Information Resource (TAIR, USA). *Atnoa1* was obtained from Prof. Yingzhang Li (China Agricultural University), and the *Atnia1,nia2* mutant was obtained from Prof. Yuling Chen (Hebei Normal University). Seeds were surface-sterilized with 10% bleach and sown on Murashige and Skoog (MS) medium. Plates were stratified in darkness for 2–4 d at 4°C and then transferred to a tissue culture room at 22–25°C under a 16 h/8 h day/night photoperiod. After 1 week, seedlings were potted in soil and placed in a growth chamber at 22–25°C, 70% RH, and 120 μmol $m^{-2}s^{-1}$ under a 16 h/8 h day/night photoperiod.

1.3   Stomatal bioassay

Stomatal bioassays were performed as described by Liu et al. [3] with minor modifications. The abaxial epidermis was peeled from the leaves of 3- to 4-week-old plants. Epidermal peels were floated, peeled-side down, in MES-KOH buffer and incubated under light (200 μmol m^{-2} s^{-1}) to stimulate stomatal opening. Epidermal peels with preopened stomata were transferred to the same buffer supplemented with different treatments (ethephon, ethephon with $C_3H_3KO_3$+NH_3, NH_2OH, AOA) for 30 min. The width of the stomatal aperture was measured using a light microscope with the help of a precalibrated ocular micrometer. Ten apertures were monitored at random in three different epidermal strips from each treatment. The data presented are the means ± SE of 90 measurements.

1.4   Quantitative RT-PCR analysis

Total RNA were extracted with the TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. For real-time PCR analysis, first-strand cDNA was synthesized from 3 μg total RNA using M-MLV reverse transcriptase (TaKaRa, Japan). After synthesis of the first-strand cDNA using oligo d(T)18 primer (TaKaRa), real-time PCR was performed using the MyiQ Real-Time PCR Detection System (Bio-Rad, USA) in the presence of SYBR Green I (BioWhittaker Molecular Applications) in the amplification mixture according to the manufacturer's protocols. Specific primer sets were designed for *AtL-CDes* (forward primer 5′-TGTATGTGAGGAGGAGGC-3′, reverse primer 5′-GTTCATACTGATGCTGCTC-3′) and *AtD-CDes* (forward primer 5′-ATAGAAGCAGCAAGGGAA-3′, reverse primer 5′-TGAGGCTCTTACTAATGCT-3′). Amplification of actin transcripts served as the internal standard. The data were analyzed using MyiQ software (Bio-Rad).

1.5   Measurement of H_2S emission

Measurement of H_2S emission was performed as described by Sekiya et al. [12] with minor modifications. Plant mate-

rials were frozen in liquid nitrogen and crushed with a mortar and pestle. The soluble proteins were extracted by addition of 0.9 mL of 20 mmol/L Tris-HCl (pH 8.0) to 0.1 g plant material, which was treated by 0.004% ethephon for periods of 0, 1, 2, 4, 6, or 8 h. After centrifugation, the protein content of the supernatant was adjusted to a concentration of 100 μg/mL to obtain equal amounts of protein in each assay sample. Then H_2S was absorbed into a zinc acetate trap located at the bottom of the test tube. After reaction for 30 min at 37°C, 100 μL of 30 mmol/L $FeCl_3$ dissolved in 1.2 mol/L HCl was added to the trap. A volume of 100 μL of 20 mmol/L *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 mol/L HCl was injected into the trap. The amount of H_2S in the zinc acetate trap was determined colorimetrically at 670 nm.

1.6   *L-/D*-cysteine desulfhydrase activity measurements

L-/D-cysteine desulfhydrase activity measurements were performed as described by Riemenschneider et al. [13]. Plant material was frozen in liquid nitrogen and crushed 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: 0.8 mmol/L *L*-cysteine, 2.5 mmol/L DTT, 100 mmol/L Tris-HCl (pH 9.0), and 10 μg protein. The reaction was initiated by the addition of *L*-cysteine. After incubation for 15 min at 37°C, the reaction was terminated by addition of 100 μL of 30 mmol/L $FeCl_3$ dissolved in 1.2 mol/L HCl and 100 μL of 20 mmol/L *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 mol/L HCl. The formation of methylene blue was determined at 670 nm.

    D-cysteine desulfhydrase activity was determined using the same method, except that *D*-cysteine was used instead of *L*-cysteine and the pH of the Tris-HCl buffer was 8.0.

    All results are expressed as means ± SE of three duplications.

1.7   Detection of NO in guard cells

Measurement of NO in guard cells was performed as dtscribed by Gonugunta et al. [14] with minor modifications. The abaxial epidermis was peeled from rosette leaves of 3- to 4-week-old plants and treated with MES-KOH buffer. The epidermal strips were incubated in the dark for 20 min with 10 μmol/L of the fluorescent probe DAF-2DA (Sigma) to detect NO. Excess dye was removed by three washes with incubation buffer. Cells were observed using a confocal laser scanning microscope equipped with a camera (excitation at 488 nm, emission at 505–530 nm; Zeiss LSM 510 META). Images were analyzed using LSM 5 Image

Browser software. We observed 15–30 guard cells per experiment in each treatment. Each experiment consisted of at least three independent replicates.

1.8   NO level and NOS activity in leaves

We determined NO level and NOS activity in the leaves using a kit provided by the Nanjing Jiancheng Bioengineering Institute of China.

1.9   NR activity measurement in leaves

The activity of NR in the leaves was determined using the method described in Ref. [15] with minor modifications. All results are expressed as the mean ± SE of three duplicates.

2   Results

2.1   Involvement of H_2S in ethylene-induced stomatal closure

(i) Effects of H_2S synthesis inhibitors on ethylene-induced stomatal closure, H_2S concentrations and *L-/D*-cysteine desulfhydrase activity.   Treatment with 0.004% ethephon caused stomatal closure (Figure 1(a)). In addition, ethephon caused a marked increase in the H_2S level of *A. thaliana* leaves (Figure 1(b)). Inhibitors of H_2S synthesis and the production of *L-/D*-cysteine desulfhydrase reduced ethylene-induced stomatal closure (Figure 1(a)) and H_2S production (Figure 1(b)) in *A. thaliana*.

    To identify further the metabolic pathways involved in H_2S synthesis, *L*- and *D*-cysteine desulfhydrase activity was determined. The presence of AOA, NH_2OH, or $C_3H_3KO_3 +$ NH_3 blocked the ethylene-induced synthesis of H_2S and caused a marked increase in *L*- and/or *D*-cysteine desulfhydrase activity. However, when inhibitors of H_2S synthesis were applied without ethylene, no effect was observed (Figure 1(c),(d)). These data imply that both *L*- and *D*-cysteine desulfhydrase activities are implicated in ethylene-induced H_2S production in *A. thaliana*.

    (ii) Effects of ethylene on stomatal aperture size in wild-type *Arabidopsis thaliana*, *Atl-cdes* and *Atd-cdes* mutants. To evaluate further the effects of H_2S on ethylene-induced stomatal closure in *A. thaliana*, epidermal peels of *Atl-cdes* and *Atd-cdes* mutants were treated with 0.004% ethephon. The *Atl-cdes* and *Atd-cdes* T-DNA insertion lines showed no difference in stomatal aperture size from wild-type plants (Figure 2).

2.2   NO requirement for ethylene-induced H_2S emission

(i) Effects of NO scavenger and NO synthesis inhibitors on ethylene-induced H_2S concentration and *L-/D*-cysteine desulfhydrase activity. H_2S and NO are involved in the signal transduction pathway of ethylene-induced stomatal closure. To analyze the interactions between H_2S and NO, H_2S content and activities of *L*- and *D*-cysteine desulfhydrase were determined after application of cPTIO, Na_2WO_4,
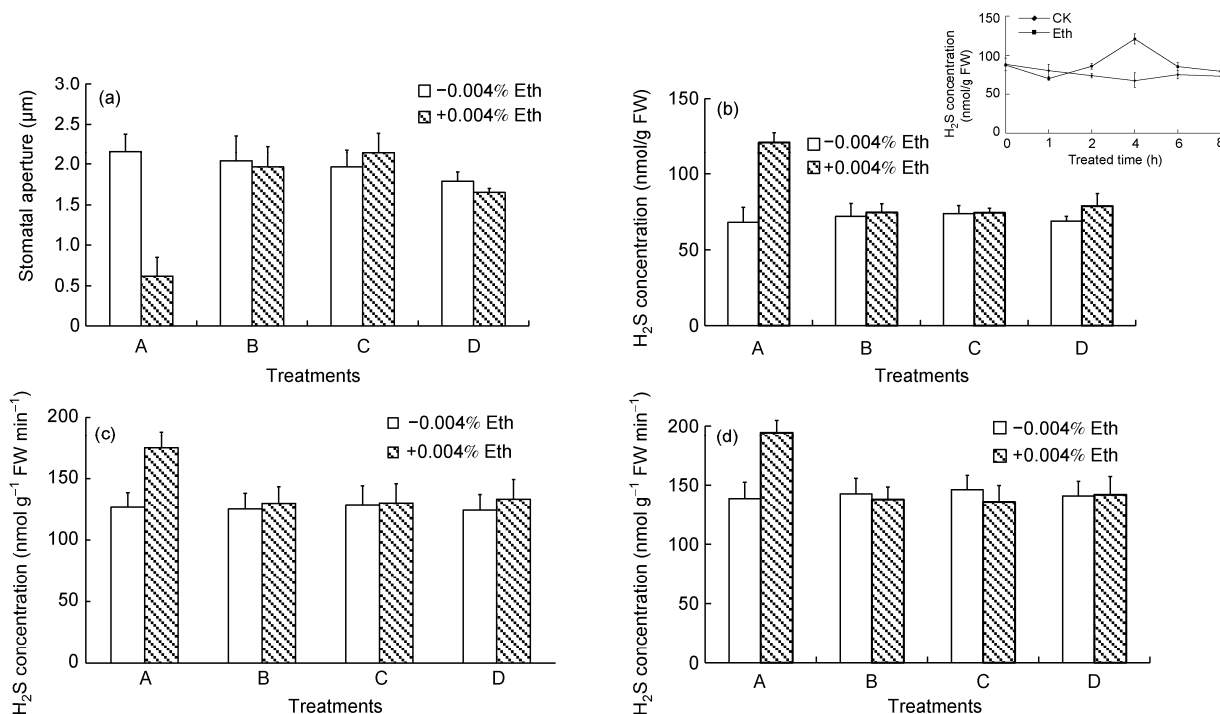
Figure 1 Effects of H$_2$S synthesis inhibitors on ethylene-induced stomatal closure (a), H$_2$S concentration (b) and *L-/D*-cysteine desulfhydrase activity (c and d) in *Arabidopsis thaliana*. Treatments: A, CK; B, 0.4 mmol/L AOA; C, 0.4 mmol/L NH$_2$OH; D, 0.2 mmol/L C$_3$H$_3$KO$_3$ + 0.2 mmol/L NH$_3$. Iconograph: Time course of the effect of 0.004% ethyleneon on H$_2$S content in *Arabidopsis thaliana* leaves.
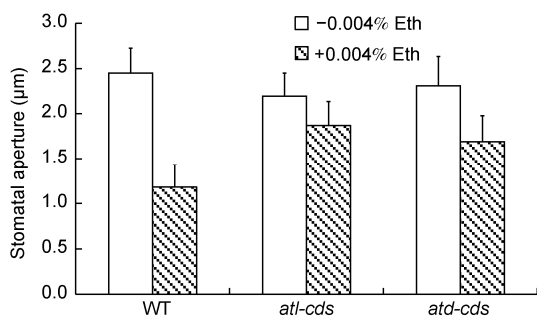

Figure 2 Effects of 0.004% ethphon on stomatal aperture size in wild-type *Arabidopsis thaliana, Atl-cdes* and *Atd-cdes* mutants.
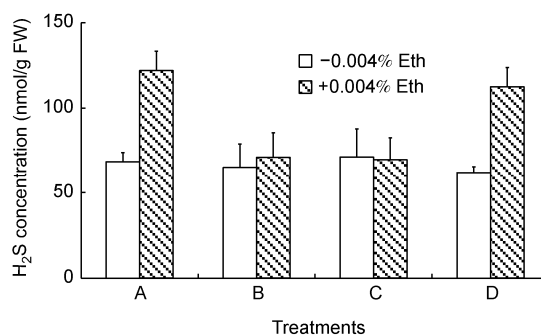

Figure 3 Effects of NO scavenger and NO synthesis inhibitors on ethylene-induced H$_2$S concentration. Treatments: A, CK; B, 0.2 mmol/L cPTIO; C, 0.1 mmol/L Na$_2$WO$_4$; D, 0.025 mmol/L *L*-NAME.

and *L*-NAME. Both cPTIO and Na$_2$WO$_4$ decreased H$_2$S production (Figure 3) and reduced the *L-/D*-cysteine desulfhydrase activity (Figure 4) in the leaves. In contrast, *L*-NAME had no significant effect on ethylene-induced H$_2$S synthesis.

(ii) Effects of NO scavenger and NO synthesis inhibitors on expression of *AtL-CDes* and *AtD-CDes* in leaves exposed to ethephon. Expression of *AtL-CDes* and *AtD-CDes* was determined after application of a NO donator, SNP, a NO scavenger, cPTIO, a NO inhibitor, Na$_2$WO$_4$, and a NOS inhibitor, *L*-NAME. The data showed that *AtL-CDes* and *AtD-CDes* expression were increased after treatment with 0.004% ethylene, and maximum express level of *AtL-CDes* and *AtD-CDes* were reached at 120 and 30 min respectively (data not shown). SNP enhanced this effect, cPTIO and Na$_2$WO$_4$ inhibited *AtL-CDes* and *AtD-CDes* expression by

ethylene, while *L*-NAME had little effect (Figure 5).

(iii) Effects of ethylene on H$_2$S concentrations in the wild-type and *Atnoa1* and *Atnia1,nia2* mutants. In response to ethephon treatment, the H$_2$S level in leaves of the *Atnoa1* mutant was comparable to that of wild-type plants. However, ethephon had no clear effects in the *Atnia1,nia2* mutant (Figure 6).

2.3 NO function downstream of H$_2$S in ethylene-induced stomatal closure

(i) Effects of H$_2$S synthesis inhibitors on ethephon-induced NO production, NR activity, and NOS activity in *Arabidopsis thaliana* leaves. To investigate further the interaction between NO and H$_2$S in ethylene-induced stomatal closure,
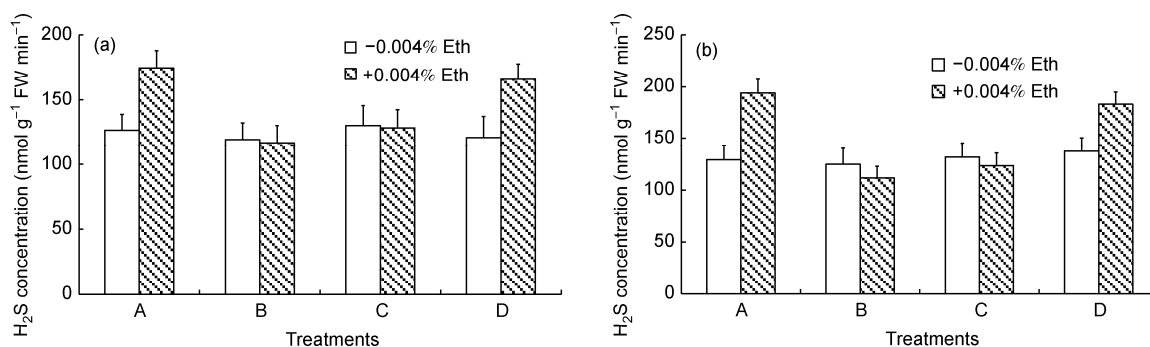
Figure 4 Effects of NO scavenger and NO synthesis inhibitors on ethylene-induced *L*-(a)/*D*-(b)cysteine desulfhydrase activity. Treatments: A, CK; B, 0.2 mmol/L cPTIO; C, 0.1 mmol/L Na$_2$WO$_4$; D, 0.025 mmol/L *L*-NAME.
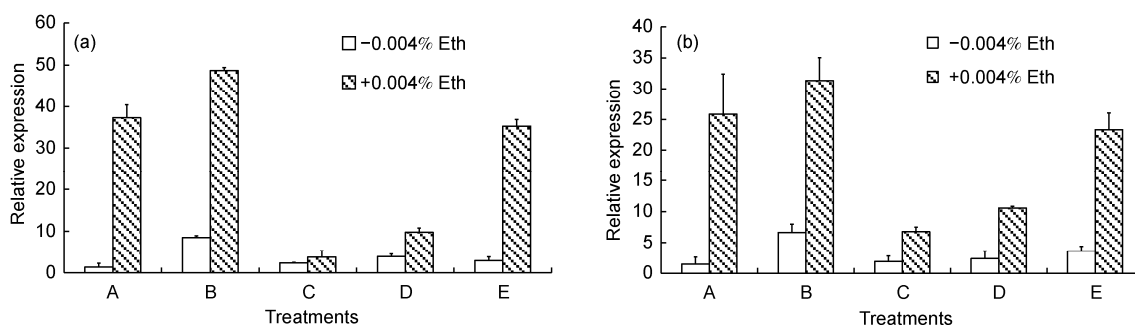

Figure 5 Effects of NO scavenger and NO synthesis inhibitors on expression of *AtL-CDes* (a) and *AtD-CDes* (b) in leaves of *Arabidopsis thaliana* exposed to 0.004% ethephon. Treatments: A, CK; B, 0.1 mmol/L SNP; C, 0.2 mmol/L cPTIO; D, 0.1 mmol/L Na$_2$WO$_4$; E, 0.025 mmol/L *L*-NAME.
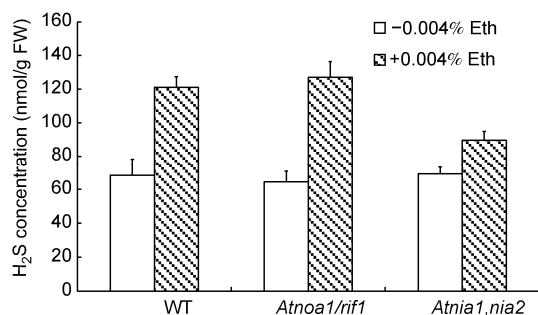

Figure 6 Effects of ethylene on H$_2$S concentration in leaves of wild-type *Arabidopsis thaliana*, and *Atnoa1* and *Atnia1,nia2* mutants.

NO content and activities of NR and NOS were determined after application of AOA, NH$_2$OH and C$_3$H$_3$KO$_3$+NH$_3$. None of these compounds affected ethylene-induced NO level in guard cell (Figure 7(a)) and in leavers (Figure 7(b)), or enhanced NR activity (Figure 8(a)). The data indicated that the action of NO could be upstream of H$_2$S. However, ethylene cannot enhance NOS activity (Figure 8(b)), supporting that ethylene induces NO synthesis via promotion of NR activity.

(ii) Effects of ethylene on NO levels in guard cells of the wild-type, and *Atl-cdes* and *Atd-cdes* mutants. The fluorescence probe DAF-2DA was used to determine the changes in NO levels in guard cells of the wild type, and *Atl-cdes* and *Atd-cdes* mutants, exposed to ethephon.

Treatment with ethephon caused a marked increase in the NO levels of guard cells (Figure 9). Ethephon caused stomatal closure in the wild-type. In contrast, ethephon had no effect on stomatal closure in the *Atl-cdes* and *Atd-cdes* mutants (Figure 9).

3   Discussion

That H$_2$S might operate as an endogenous neurotransmitter was first suggested in 1996 by Abe and Kimura [16] who described the enzymatic mechanism of H$_2$S production in human brain. Similar to NO and CO, H$_2$S is lipophilic and freely permeates plasma membranes, although because of partial dissociation membranes are relatively less permeable to H$_2$S than to both other gases [16]. H$_2$S is increasingly recognized as a member of a growing group of "gasotransmitters", together with its two counterparts, NO and CO. However, little is known about H$_2$S as a signaling molecule in plants. Recently, it was shown a novel H$_2$S donor caused stomatal opening [17]. It is well known that ethylene causes stomatal closure in *A. thaliana* [2]. However, it is not known whether H$_2$S is involved in the process of ethylene-induced stomatal closure. In the present work, it is shown that the levels of *AtL-CDes* and *AtD-CDes* transcripts reached maximum values after treatment with ethylene (Figure 5). The presence of inhibitors of H$_2$S synthesis
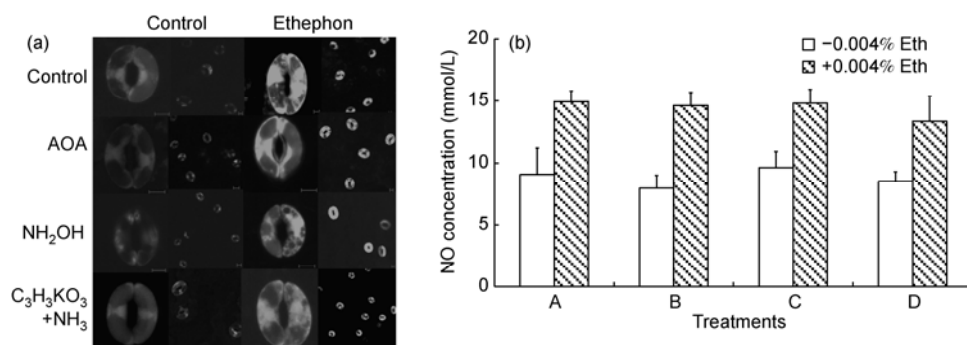
Figure 7 Effects of H$_2$S synthesis inhibitors on ethephon-induced NO production in guard cells (a) or in leaves of *Arabidopsis thaliana* (b). Treatments: A, CK; B, 0.4 mmol/L AOA; C, 0.4 mmol/L NH$_2$OH; D, 0.2 mmol/L C$_3$H$_3$KO$_3$ + 0.2 mmol/L NH$_3$.
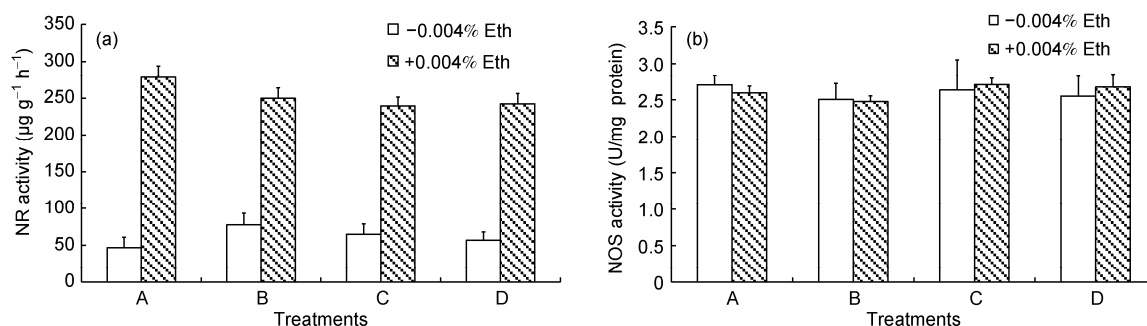

Figure 8 Effects of H$_2$S synthesis inhibitors on ethephon-induced NR activity (a) and NOS activity (b) in *Arabidopsis thaliana* leaves. Treatments: A, CK; B, 0.4 mmol/L AOA; C, 0.4 mmol/L NH$_2$OH; D, 0.2 mmol/L C$_3$H$_3$KO$_3$ + 0.2 mmol/L NH$_3$.
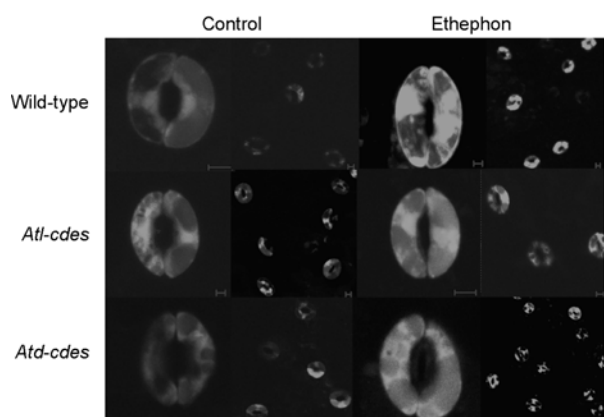

Figure 9 Effects of 0.004% ethephon on NO levels in guard cells of wild-type *Arabidopsis thaliana*, and *Atl-cdes* and *Atd-cdes* mutants.

(AOA and NH$_2$OH) and of production of *L*- and *D*-cysteine desulfhydrase (C$_3$H$_3$KO$_3$ + NH$_3$) decreased ethylene-induced stomatal closure (Figure 1(a)) and diminished H$_2$S production (Figure 1(b)). Ethephon had no effect on stomatal aperture size in the *Atl-cdes* and *Atd-cdes* mutants (Figure 2). These results imply that H$_2$S is required for ethylene-induced stomatal closure.

As a signaling molecule, NO has been demonstrated to be involved in many developmental, physiological and metabolic processes in plants [18]. Using *Arabidopsis* [3] and *Vicia faba* [4], we investigated whether NO that acted

via a NR-independent route is involved in ethylene-induced stomatal closure. NO and H$_2$S have wide-ranging effects in diverse aspects of physiological processes, which is indicative of a close relationship between these two signaling molecules in animals. Although exogenously applied H$_2$S alone relaxed smooth muscles, much lower concentrations of H$_2$S greatly enhanced smooth muscle relaxation induced by NO in the thoracic aorta [19]. However, it remains unclear whether there is an interaction between H$_2$S and NO in ethylene-induced stomatal closure in *A. thaliana*. The data presented herein clearly show that the NO scavenger cPTIO and the NR inhibitor Na$_2$WO$_4$ prevented H$_2$S production (Figure 3) and *L*-/*D*-cysteine desulfhydrase activity (Figure 4), as well as the transcript levels of *AtL-CDes* and *AtD-CDes* (Figure 5), induced by ethylene. After treatment with ethylene the H$_2$S level in leaves of the *Atnoa1* mutant was comparable to that of wild-type plants, whereas no significant effect was observed in the *Atnia1,nia2* mutant (Figure 6). In addition, AOA, NH$_2$OH, and C$_3$H$_3$KO$_3$ + NH$_3$ had no significant effect on ethylene-induced NO synthesis (Figure 7) or NR activity (Figure 8(a)), and no effect in the *Atl-cdes* and *Atd-cdes* mutants (Figure 9). These results indicate that the action of NO could be upstream of H$_2$S during stomatal closure induced by ethylene. However, Zhang et al. [11] reported that the process of H$_2$S-induced adventitious root formation was probably mediated by NO signals and H$_2$S might act upstream of NO signaling pathways [11]. In con-

clusion, our results indicate that ethylene induces NO synthesis via promotion of NR activity, which leads to L-/D-cysteine desulfhydrase-derived H_2S production and stomatal closure.

The signal transport pathway for regulation of stomatal movement by ethylene is reported to be a complex signaling network, which includes calcium, protons, NO, H_2O_2, and several other components. For instance, the marked prevention of ABA-induced stomatal closure by EGTA (a calcium chelator) suggested that cytosolic Ca^{2+} is necessary during stomatal closure by ethylene [4]. Ethylene-induced H_2O_2 production that originated from NAD(P)H oxidase and cell wall peroxidase activity plays an important role in the ethylene-induced NO synthesis that leads to stomatal movement [3]. NO generated upstream during alkalization of the cytoplasm also regulates ethylene-induced stomatal closure [5]. Cross-reactions are known to exist between these signaling molecules. It is not known whether additional signaling molecules are involved in the process of stomatal closure induced by ethylene. It is reported that H_2S counteracts the oxidative burst generated by H_2O_2 production in response to different stresses by reducing H_2O_2 concentration and increasing the activity of antioxidant enzymes [11,20,21]. This result indicates that an interaction occurs between H_2S and H_2O_2 in signal pathways. Ethylene-induced NO synthesis appeared to occur later than that of Ca^{2+} increase and alkalization of the cytoplasm, which indicates H_2S production is not an early ethylene-signaling event such as alkalization of the cytoplasm and Ca^{2+} increase; as stated earlier, H_2S is probably acting on different targets in guard cells.

Further studies are needed to explore further the mechanism of H_2S synthesis. The other challenges for future research will be investigation of other components involved in this process or other sources of H_2S in ethylene-induced stomatal closure.

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