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Research article

Hydrogen sulfide interacting with abscisic acid in stomatal regulation responses to drought stress in *Arabidopsis*

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ABSTRACT

Hydrogen sulfide (H_2S) plays a crucial role in the regulation of stomatal closure in plant response to drought stress, and L-cysteine desulfhydrase (LCD) has been identified as being mainly responsible for the degradation of cysteine to generate H_2S. In view of the similar roles to abscisic acid (ABA), in this study, the *lcd*, *aba3* and *abi1* mutants were studied to investigate the close inter-relationship between H_2S and ABA. The *lcd* mutant showed enlarged stomatal aperture and more sensitivity to drought stress than wild-type plants. Expression of Ca^{2+} channel and outward-rectifying K^+ channel coding genes decreased in the *lcd* mutant, and conversely, expression of inward-rectifying K^+ increased. The stomatal aperture of *aba3* and *abi1* mutants decreased after treatment with NaHS (a H_2S donor), but stomatal closure in responses to ABA was impaired in the *lcd* mutant. The expression of *LCD* and H_2S production rate decreased in both the *aba3* and *abi1* mutants. Transcriptional expression of ABA receptor candidates was upregulated in the *lcd* mutant and decreased with NaHS treatment. The above results suggested that H_2S may be an important link in stomatal regulation by ABA via ion channels; H_2S affected the expression of ABA receptor candidates; and ABA also influenced H_2S production. Thus, H_2S interacted with ABA in the stomatal regulation responsible for drought stress in *Arabidopsis*.

1. Introduction

Hydrogen sulfide (H_2S), as the third gas transmitter in mammalian cells, is involved in many areas including vasorelaxation, neurotransmission, smooth-muscle relaxation and innate immune response. Cystathionine β-synthase (CBS, EC4.2.1.22) and cystathionine γ-lyase (CSE, EC4.4.1.1) have been consistently demonstrated to produce H_2S in mammalian tissues with L-cysteine as the main substrate [1–3]. In plants, specific desulfhydrases have also been identified and functionally characterized – respectively localized in the cytosol, plastids and mitochondria. L-Cysteine desulfhydrase (LCD, EC4.4.1.1), a homolog of CSE in mammals, is mainly responsible for generating H_2S in plants [4]. Riemenschneider et al. identified a gene encoding a putative D-cysteine desulfhydrase (DCD) in *Arabidopsis thaliana*, based on high homology to an *Escherichia coli* protein called YedO that has DCD activity [4]. NFS/Nifs is also potentially involved in H_2S production in *Arabidopsis* due to its LCD-like activity [5]. In addition, the exploration of the physiological importance of H_2S in plants over the last 5 years or so has led to several conclusions: promotion of seed germination and root formation [6,7]; regulation of plant maturation and flower senescence [8]; and protection of multiple plants against abiotic stresses, such as drought [9], heat [10] and heavy metals [11–13].

Abscisic acid (ABA) plays important regulatory roles in various aspects of plant growth and development throughout the plant life

Abbreviations: AAO3, ABA-aldehyde oxidase 3; ABA, abscisic acid; ABAR, abscisic acid receptor; ACA, adenylyl cyclase-associated protein; AKT, *Arabidopsis* potassium transporter; CAX, calcium exchanger; CBS, cystathionine β-synthase; CHLH, H subunit of the Mg-chelatase; CSE, cystathionine γ-lyase; GCR2, G-protein coupled receptor 2; GORK, guard cell outward-rectifying K^+ channel; GTGs, GPCR-type G proteins; GUN5, genome uncoupled 5; H_2S, hydrogen sulfide; KAT1, potassium channel in *Arabidopsis thaliana* 1; KC1, potassium channel 1; KCO, outward-rectifying K^+ channel; LCD, L-cysteine desulfhydrase; NCED3, 9-*cis*-epoxycarotenoiddioxygenase 3; NFS, nitrogenase Fe–S cluster; PYR, pyrabactin resistant protein; PYLs, PYR-like proteins; RCARs, regulatory components of ABA receptor; ROS, reactive oxygen species; RT-PCR, reverse transcriptase polymerase chain reaction; SKOR, SKI family transcriptional corepressor; SLAC1, slow anion channel associated 1; TPC1, two pore segment channel 1; WT, wild type.

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cycle, particularly in the ability to sense and respond to various abiotic stresses, including drought, salt and cold stresses during vegetative growth [14]. The enzymatic biosynthesis pathway of the sesquiterpenoid, ABA, from C_{40} carotenoids has been well characterized biochemically and genetically [15]. A rapid increase in ABA concentration in response to abiotic stresses can be partly explained by transcriptional induction of ABA biosynthesis genes such as the rate-limiting step enzyme NCED3 [16], AAO3 and AAO2, which is a substitute for AAO3 [17]. Two types of ABA mutants have been studied: insensitivity mutants *abi1–abi5* and synthesis defective mutants *aba1–aba3*, most of which exhibit stomatal opening [14].

ABA regulates stomatal movement in response to drought stress in plants [18]. When guard cells perceive increased ABA levels, their turgor and volume are reduced by efflux of anions and potassium ions and by gluconeogenic conversion of malate into starch, causing membrane depolarization and stomatal closure. Stomatal opening, in contrast, is mediated by the increasing K^+, anion and malate concentrations in the guard cells [19]. It is thus clear that ion channels are critical mechanisms for controlling stomatal movement, which facilitate the identification of genes encoding specific ion channel and transporter activities. *Arabidopsis* guard cells express several of the inward-rectifying channels: AKT1, AKT2, KAT1, KAT2 and the regulatory subunit KC1, which mediate hyperpolarization-induced K^+ influx. In particular, KAT1 contains 75% of the activity of K^+ channel currents [20]. The outward-rectifying channel SKOR was first described, when it was found to be expressed in the root vascular cylinder, where K^+ was released into the xylem. GORK is the major outward-rectifying K^+ channel in guard cells and KCO1 was the first K^+ channel to be localized to the plant tonoplast [21].

Vacuoles serve as a major Ca^{2+} store, which can accumulate to millimolar levels, and this accumulation is promoted by the high affinity P-type Ca^{2+}–ATPase and the moderate affinity Ca^{2+}–H^+ cation antiporter CAX, each of which are coded by multigene families in *Arabidopsis*. The voltage-dependent Ca^{2+} channel homolog TPC1 has been well characterized and is expressed in the vacuolar membrane to release Ca^{2+} to cytoplasm. Recent genetic screens showed that SLAC1 was a member of a novel plant anion/Cl^- channel family in the plasma membrane. SLAC1 has a central role in regulating stomatal aperture, and MRP5 is a plasma membrane-localized negative regulator of anion channels.

Research on ABA in recent years has focused on identification of ABA receptors and action mechanisms. The discovery of a protein family with 14 members in *Arabidopsis*, PYR1/PYLs/RCARs, was a major advance in understanding ABA signaling and has led to identification of the core ABA signaling module [22,23]. Binding of ABA to the ABA receptors PYR1/PYLs/RCARs leads to inactivation of type 2C protein phosphatases. The protein phosphatases seem to function as coreceptors and their inactivation launches SNF1-type kinase action which targets ABA-dependent gene expression and ion channels [22,23]. In addition, the other classes were found to specifically bind ABA with high affinity and mediate all major aspects of ABA responses: *Arabidopsis* CHLH/ABAR/GUN5, GCR2 and GTGs. However, their exact role in ABA signaling requires further investigation due to some conflicting experimental evidence [24].

It is well known that ABA induces stomatal closure through ion channels. The role of H_2S in the regulation of stomatal closure in drought stress was shown in our previous report [9]. Involvement of K_{ATP} channel activities in H_2S-induced vasorelaxation was determined in mammals [3]. Thus, we proposed that H_2S-induced stomatal closure also through ion channels in plants. In the present study, the function of H_2S in stomatal movement and the relationship between H_2S and ABA in signaling transduction is discussed.

2. Results

2.1. Identification and characterization of the Arabidopsis lcd mutant

The SALK_082099 mutant designated *lcd* was obtained from ABRC for further analysis. The T-DNA was inserted in 3′-UTR of the *LCD* genomic DNA (Fig. 1A). At first, heterozygous seeds of the *lcd* mutant were planted and homozygosis lines identified by genotyping in the offspring (Fig. 1B). Then, RT-PCR analysis was conducted on the homozygous mutant plants using specific primers P1 and P4, and weak *LCD* expression was detected in the *lcd* mutant (Fig. 1C). Meanwhile, the H_2S production rate of the *lcd* mutant was determined to be about 50% of the WT ($P < 0.01$) (Fig. 1D). These results suggest that *LCD* had been knocked-down in the *lcd* mutant, in accordance with significant decreases in H_2S production rate.

2.2. The lcd mutation increased Arabidopsis sensitivity to drought stress

The *lcd* mutant had no significant phenotypic differences to WT under normal growth conditions, either in soil or on solid MS medium in Petri dishes. When 3-week-old plants were subjected to drought stress for 10 days by completely restricting water, the difference between WT and the *lcd* mutant was remarkable (Fig. 1E). WT plants were greener and showed more turgidity than *lcd* mutants, which were seriously wilted after 10 days. After

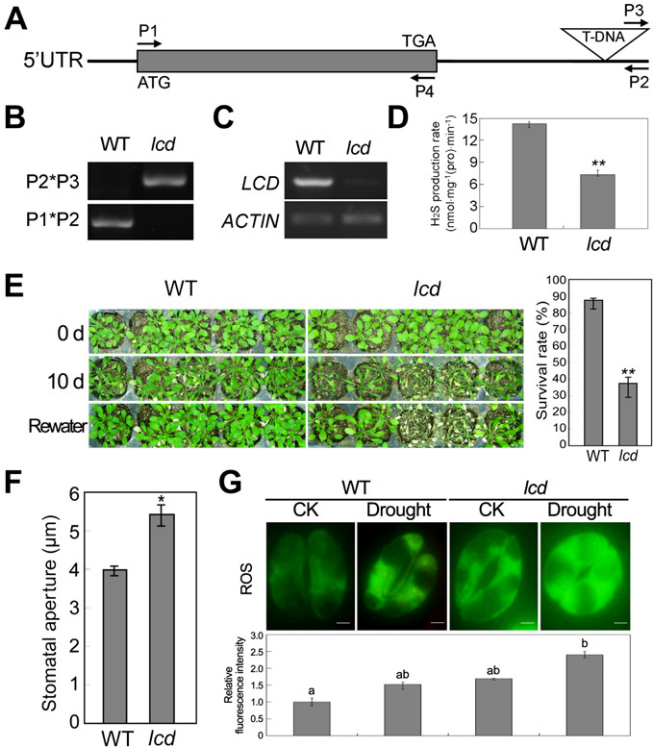

Fig. 1. Experimental analysis of the *lcd* mutant. (A) T-DNA insertion site in the *lcd* mutant (SALK_082099). Gray box denotes coding region, and solid lines indicate the 5′- and 3′-UTR, respectively. Arrows P1–P3 represent the primer locations for genotyping. (B) Genotyping analysis of the *lcd* mutant. (C) The expression of *LCD* in the WT and the *lcd* mutant. (D) H_2S production rate in the WT and the *lcd* mutant. (E) Phenotype analysis of the *lcd* mutant compared with the WT under normal and drought stress conditions (left), and the survival rate of the WT and the *lcd* mutant under drought stress (right). (F) Stomatal aperture of WT and the *lcd* mutant. (G) ROS in leaves of WT and the *lcd* mutant, determined *in vivo* by fluorescence microscopy with DCFH-DA under normal and drought stress conditions (400×). Bars = 10 μm.

rewatering the soil to saturation, we quantified the survival rate between the total number of living seedlings to the total number of sown seedlings. Using this metric, seedlings of the *lcd* mutant had a lower survival rate of 37.51% compared to 87.52% for WT ($P < 0.01$). This result suggested that the *lcd* mutant was more sensitive to drought stress than WT.

To investigate the reason for the weakened drought resistance in the *lcd* mutant, the stomatal aperture sizes were measured. Leaves of the *lcd* mutant showed a significant opening of the stomatal aperture compared to control (Fig. 1F); with mean stomatal aperture size of 5.42 μm, which was about 20% larger than that of the WT. ROS imaging (specifically stained for H_2O_2) *in vivo* using fluorescence microscopy was performed on the leaves of the *lcd* mutant and the WT; this revealed higher ROS content in the *lcd* mutant than in the WT, both before and after drought stress (Fig. 1G). These results suggest that H_2S deficiency resulted in larger stomatal aperture and more serious injury in the *lcd* mutant.

2.3. H_2S affected the ion channels related to stomatal closure

It is well known that stomatal movement is controlled by turgor pressure, which is determined by concentrations of K^+ and anions in guard cells. To investigate the effects of H_2S in ion channels, expression levels of channel-protein coding genes were compared between the *lcd* mutant and the WT. As expected, expression of genes of the Ca^{2+} channel TPC1 decreased, whereas Ca^{2+}–ATPase ACA9, 11 and Ca^{2+}–H^+ cation antiporter CAX1 were elevated in the *lcd* mutant (Fig. 2A); this was in accordance with stronger Ca^{2+} fluorescence in the WT than in the *lcd* mutant (Fig. 2C). The expression levels of inward-rectifying K^+_{in} channels (AKT1, AKT2, KC1 and KAT1) (Fig. 2B) were clearly upregulated. By contrast, the outward-rectifying K^+_{out} channels (GORK, SKOR and KCO1) (Fig. 2B) and the anion channel regulator MYP5 were downregulated (Fig. 2A). The anion channel SLAC1 (Fig. 2A) showed no significant change between WT and *lcd*. These results show that H_2S was involved in the expression regulation of ion-channel genes, and may be a signaling component of stomatal movement regulated by ion channels.

2.4. Deficiency of H_2S in the lcd mutant weakened the ABA induction of stomatal closure

Considering that the regulation of stomatal behavior by H_2S was very similar to that of ABA, it was necessary to determine the relationship between H_2S and ABA. The stomatal aperture size in *aba3* and *abi1* mutants and WT treated with NaHS decreased by 40.54, 50.06 and 51.78%, respectively (Fig. 3A). Addition of 10 and 50 μM ABA in the incubation solution led to stomatal aperture of the *lcd* mutant decreasing by 21.89 and 34.39%, respectively, compared to corresponding values of 26.02 and 54.72% in the WT. Thus the aperture size of the *lcd* mutant was significantly larger than for the WT (Fig. 3B). These results indicate that deficiency of ABA did not weaken the induction of H_2S on stomatal closure, while inhibition by ABA of stomatal opening was impaired in the *lcd* mutant. Thus, the induction by ABA of stomatal closure was partially dependent on H_2S.

2.5. H_2S might be involved in the ABA signaling through an ABA receptor

To further investigate how H_2S participated in ABA signal transduction, study of the expression of ABA receptors or candidates was conducted. It is noteworthy that the expression level of ABA receptor candidates was enhanced in the *lcd* mutant (Fig. 3C),

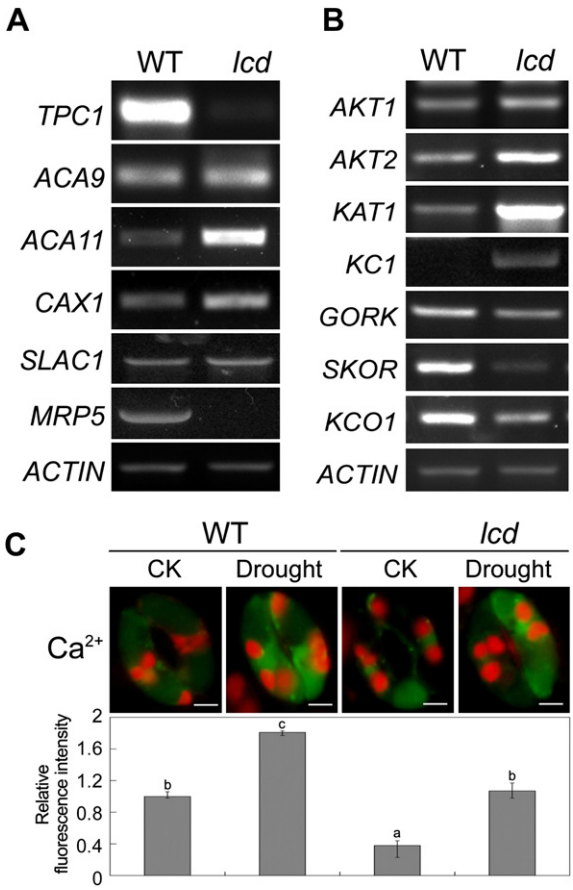

Fig. 2. Effect of hydrogen sulfide on ion channels in leaves. (A) Expression of Ca^{2+}-related genes *TPC1*, *ACA9*, *ACA11* and *CAX1*, and anion channel *SLAC1* and anion channel regulator *MYP5* in the WT and the *lcd* mutant. (B) Expression of K^+-related genes *AKT1*, *AKT2*, *KC1*, *KAT1*, *GORK*, *SKOR* and *KCO1* in the WT and the *lcd* mutant, with *ACTIN* as an internal control. (C) Content of Ca^{2+} in leaves of the *lcd* mutant and WT, determined *in vivo* by fluorescence microscopy with Fluo-3AM under normal and drought stress conditions (400×). Bars = 10 μm.

and conversely the expression was reduced with NaHS treatment (Fig. 3D). Thus, H_2S was involved in expression of coding genes of ABA receptors. The influence of H_2S may begin upstream of the ABA signaling pathway, and not limited in the linear relationship.

Study of the biosynthetic pathway was also conducted using mutants. Both *AAO3* and *NCED3*, the key enzymes of ABA synthesis, showed no difference between the *lcd* mutant and WT (Fig. 3E), and *AAO2* expression was not detected in either. However, expression of *LCD* decreased in the *aba3* and *abi1* mutants (Fig. 3F). Subsequently the production rate of H_2S was determined, and was significantly different in WT – about double, with 14.20 nmol mg^{-1} (pro)·min^{-1}– compared to *aba3* and *abi1* mutants (Fig. 3G). The expression in transcription level seemed consistent with the H_2S production rate. The results suggest that ABA had an impact on transcriptional expression of *LCD*.

3. Discussion

In recent years, study of the roles of H_2S in the plant kingdom has greatly advanced. However, knowledge of the mechanisms of actions of H_2S in plant systems is still limited. Following the pioneering studies of the regulation of ATP-sensitive K_{ATP} channels by H_2S in mammals [3,25], a similar physiological mechanism is gradually emerging in the study of plant response to abiotic stress,

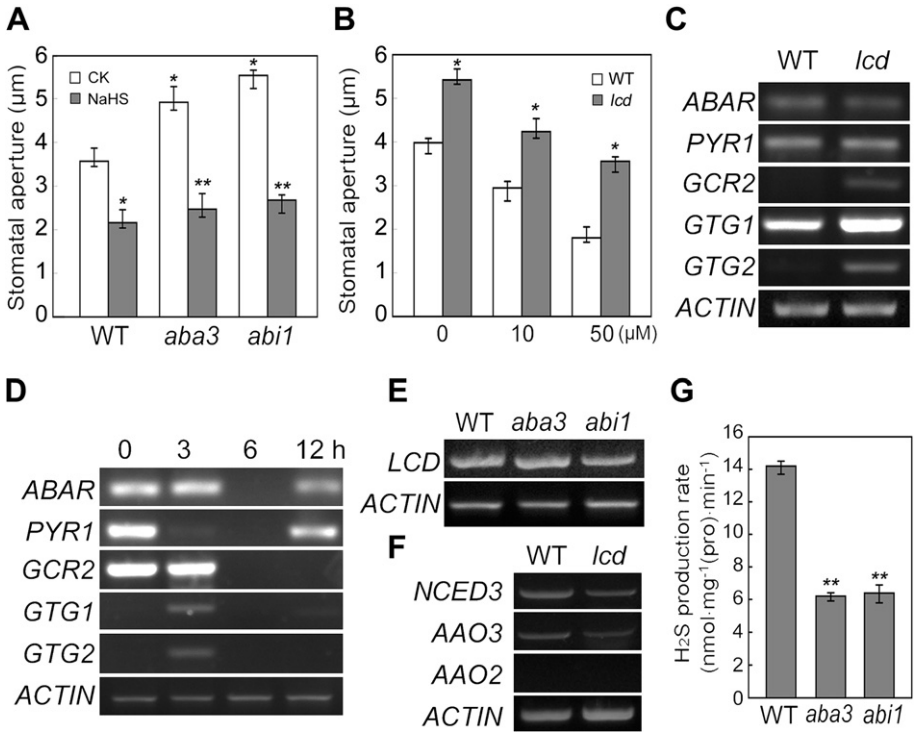

Fig. 3. Interaction of hydrogen sulfide and abscisic acid. (A) H_2S-induced stomatal closure in *aba3* and *abi1* mutants. (B) ABA-induced stomatal closure in the *lcd* mutant. (C) Transcriptional expression of ABA receptors in WT and the *lcd* mutant. (D) Expression patterns of ABA receptors during NaHS fumigation. (E) Expression of *LCD* in *aba3* and *abi1* mutants compared with WT. (F) Expression of ABA synthesis genes in the *lcd* mutant compared with WT. (G) H_2S production rate in WT and *aba3* and *abi1* mutants.

especially drought. Stomatal movement is the best characterized physiological function of H_2S, and so in this study we primarily focused on K^+, anion and Ca^{2+} channels in guard cells. The results show that expression of ion-channel coding genes for Ca^{2+} and outward-rectifying K^+_{out} channels decreased in the *lcd* mutant, while inward-rectifying K^+_{in} and anion channels increased. Thus, deficiency of H_2S in the *lcd* mutant resulted in changed expression of ion-channel protein coding genes, which might vary the ion concentration in guard cells, and so induce stomatal movement. In fact, the phenotype of the *lcd* mutant with increased stomatal opening and sensitivity to drought stress confirmed our initial proposal. Also, it confirms that the regulation of H_2S on ion channels in guard cells is a signaling mechanism in plants.

Despite the constant advances made in understanding ABA signaling mechanisms, new aspects are still emerging. In this work we present evidence of H_2S participation in ABA signaling from a new perspective: a deficiency of H_2S weakened the ability of ABA-induced stomatal closure, and affected the expression of ion channels and the ABA receptor. Meanwhile, in two mutants related ABA signaling and synthesis, both the expression of *LCD* and the H_2S production rate decreased. It seems that there was no simple upstream or downstream linear relationship between these two signal molecules, although there may be complex crosstalk. In plants, however, relationships between H_2S and the messengers of ABA signal transduction need to be further investigated.

In our previous study, exogenous H_2S released by its donors induced stomatal closure in *Arabidopsis* [9]. This observation is consistent with a previous report that exogenous H_2S donors (NaHS and GYY4137) induced stomatal closure in different plant species [26]. They also showed the H_2S donor induced stomatal closure in a dose-dependent manner, reaching the maximum effect at 100 μM NaHS [26]. Liu et al. recently showed that H_2S induced by NO mediates ethylene-induced stomatal closure [27]. In contrast, M. Lisjak et al. obtained a different result that NaHS-treated plants displayed stomatal opening in the light and the dark [28,29]. In fact, in this report, stomatal apertures increased firstly and then decreased as the H_2S treatment time length and concentration increased [28]. The seemingly opposite effect might be due to the different processing mode.

The expression level of ABA receptor candidates was enhanced in the *lcd* mutant (Fig. 3C), but was reduced with the H_2S donor fumigation (Fig. 3D). However, there were different expression patterns for ABA receptor candidates with H_2S treatment. Following the major suppression observed after 6 h, there was up-regulation again after 12 h for *ABAR*, *PYR1* and *GTG1* (Fig. 3D). One possibility is the different regulation mechanisms on different receptors. Furthermore, ABA regulates many physiological processes, and the functions of H_2S are similar to ABA in certain respects. For example, H_2S as signal of drought stress may affect the expression levels of ABA receptors in a certain period of time, but it can also be related to other physiological processes of ABA related regulation, in which may exist in the feedback regulation mechanisms to upregulate the expression of the receptors. Further investigation should be conducted to explore the underlying mechanism.

Research on H_2S is entering a phase of exponential increase [2]. However, in many cases H_2S acts as a double-edged sword, and so it is necessary to determine the physiological concentration of H_2S to examine its physiological roles. Some detection methods have been reported to measure endogenous H_2S content indirectly, such as by spectrophotometry, nanotube-based sensors, sulfur ion-specific electrodes, polarographic H_2S sensors and chromatography [2]. However, these methods all have different shortcomings. Fluorescent staining is an important means of identifying the other two gas transmitters, and can directly reflect the endogenous concentration and distribution, but H_2S has lacked a specific fluorescent dye. However, recently, an appropriate dye was reported [30], and so we look forward to the emergence of methods to increase our knowledge of H_2S.

4. Materials and methods

4.1. Plant materials and growth conditions

A. thaliana ecotype Columbia (Col-0) was used in this study. Seeds of a T-DNA insertion mutant of *LCD* (*lcd*; SALK_082099) were obtained from the *Arabidopsis* Biological Resource Center (ABRC, http://www.arabidopsis.org/abrc/); and *abi1* and *aba3* mutants were kindly provided by Xiaofeng Cao (Institute of Genetics and Developmental Biology, CAS, China). For each experiment, seeds were incubated for 2–4 days at 4 °C and then grown in pots containing a soil/perlite/vermiculite (1/1/1, v/v/v) mixture that was maintained at 23 °C and 60% relative humidity. They were subjected to a 16/8 h (light/dark) photoperiod with light illumination at 160 μE m^{-2} s^{-1}, and watered with a Murashige and Skoog (MS) nutritive medium. For investigation of the expression level of each associated gene in the mutants, leaves of 4-week-old plants were collected, immediately frozen in liquid nitrogen and stored at −80 °C.

4.2. Stress and chemical treatments

The dehydration treatment and NaHS fumigation were described previously [9]. The 3-week-old *lcd* and wild type (WT) were subjected to drought stress when grown in pots by withholding water for 10 d, and then re-watered until the substrate was saturated.

4.3. Genotyping and RT-PCR analysis

To obtain a homozygosis *lcd* strain, heterozygous seeds of *lcd* were sown, and after 2 weeks DNA was extracted for genotyping. The specific primers were defined as P1, P2 and P3. Total RNA samples were isolated from leaves of 4-week-old plants, and RT-PCR was analyzed as described previously [9]. The appropriate primer pairs were presented in Supplemental Table S1.

4.4. Determination of H_2S production rate

LCD activity was ascertained by measuring the production rate of H_2S from L-cysteine. The total protein of 4-week-old plants was extracted following the protocol described previously [4].

4.5. Assay of stomatal aperture

Stomatal apertures were measured as described previously [9]. For stomatal closing assays, rosette leaves from 4-week-old plants were harvested and then floated in solutions containing 50 mM KCl, 10 mM MES–KOH (pH 6.15), and kept at 23°C under light (160 μE m^{-2} s^{-1}) for 2.5 h, and then incubated for 2 h with or without the addition of 80 μM NaHS for *aba3* and *abi1*, 10 and 50 μM ABA for *lcd* in the incubation medium. The abaxial epidermis was peeled from the treated leaves, and stomata on the epidermal strips were photographed using an EclipseTi (Nikon, Tokyo, Japan). The stomatal aperture was measured using IMAGEJ analysis software (NIH, Bethesda, MD).

4.6. Detection of reactive oxygen species (ROS) and Ca in guard cells

Measurement of ROS and Ca in guard cells was performed as follows: the abaxial epidermis was peeled from rosette leaves of 4-week-old plants and treated with MES–KOH buffer for 2 h with or without the addition 30%PEG in the light. They were then incubated in darkness for 20 min, with 10 μM of the fluorescent probe DCFH-DA (Sigma) used to detect ROS and 5 mM of the fluorescent probe Fluo-3AM (Beyotime) to detect Ca^{2+}. Excess dye was removed by three washes with incubation buffer. Cells were observed using a fluorescent microscope at 488 nm. Images were analyzed using LSM 5 Image Browser software. Image-Pro Plus (IPP) analysis software was used to measure fluorescence signal values of guard cells. Thirty guard cells per experiment were observed in each treatment. The relative fluorescence of each treatment group = fluorescence signal value/value of control group.

4.7. Statistical analysis

Each experiment was independently replicated at least three times. Values are expressed as the mean ± SE. Data were analyzed using Statistica (Stat Soft Inc, Tulsa, OK) software. All mean comparisons were carried out using *t*-tests for independent samples. For stomatal opening assays, measurements were subjected to a one-way analysis of variance (ANOVA) to evaluate significant treatment effects at $P < 0.05$. Different letters within columns indicate significant differences ($p < 0.05$), according to Tukey's multiple range test.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plaphy.2012.10.017.

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