ABA-triggered guard cell L-cysteine desulfhydrase function and in situ H₂S production contributes to heme oxygenase-modulated stomatal closure

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Abstract:	Recent studies have demonstrated that hydrogen sulfide (H ₂ S) produced through the activity of L-cysteine desulfhydrase (DES1) is an important gaseous signaling molecule in plants that could participate in abscisic acid (ABA)-induced stomatal closure. However, the coupling of the DES1/H ₂ S signaling pathways to guard cell movement has not been thoroughly elucidated. The results presented here provide genetic evidence for a physiologically relevant signaling pathway that governs guard cell-in situ DES1/H ₂ S function in stomatal closure. We discovered that ABA-activated DES1 produces H ₂ S in guard cells. The impaired guard cell ABA phenotype of <i>des1</i> mutant can be fully complemented when DES1/H ₂ S function has been specifically rescued in guard cells and epidermal cells, but not mesophyll cells. This research further characterized DES1/H ₂ S function in the regulation of LONG HYPOCOTYL1 (HY1, a member of the heme oxygenase family) signaling. ABA-induced DES1 expression and H ₂ S production are hyper-activated in the <i>hy1</i> mutant, both of which can be fully abolished by the addition of H ₂ S scavenger. Impaired guard cell ABA phenotype of <i>des1/hy1</i> can be

	restored by H2S donors. Taken together, this research indicated that guard cell-in situ DES1 function is involved in ABA-induced stomatal closure, which also acts as a pivotal hub in regulating HY1 signaling.

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ABA-triggered guard cell γ -cysteine desulphydrase function and *in situ* H_2S production contributes to heme oxygenase-modulated stomatal closure

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34

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36

37

38 ABSTRACT

39 Recent studies have demonstrated that hydrogen sulfide (H₂S) produced
40 through the activity of L-cysteine desulfhydrase (DES1) is an important gaseous
41 signaling molecule in plants that could participate in abscisic acid (ABA)-
42 induced stomatal closure. However, the coupling of the DES1/H₂S signaling
43 pathways to guard cell movement has not been thoroughly elucidated. The
44 results presented here provide genetic evidence for a physiologically relevant
45 signaling pathway that governs guard cell-*in situ* DES1/H₂S function in stomatal
46 closure. We discovered that ABA-activated DES1 produces H₂S in guard cells.
47 The impaired guard cell ABA phenotype of the *des1* mutant can be fully
48 complemented when DES1/H₂S function has been specifically rescued in guard
49 cells and epidermal cells, but not mesophyll cells. This research further
50 characterized DES1/H₂S function in the regulation of LONG HYPOCOTYL1
51 (HY1, a member of the heme oxygenase family) signaling. ABA-induced *DES1*
52 expression and H₂S production are hyper-activated in the *hy1* mutant, both of
53 which can be fully abolished by the addition of H₂S scavenger. Impaired guard
54 cell ABA phenotype of *des1/hy1* can be restored by H₂S donors. Taken together,
55 this research indicated that guard cell-*in situ* DES1 function is involved in ABA-
56 induced stomatal closure, which also acts as a pivotal hub in regulating HY1
57 signaling.

58

59 **Keywords:** hydrogen sulfide, guard cell, HY1, DES1, ABA

61 1 INTRODUCTION

62 Drought stress has been considered to be one of the most severe
63 environmental factors; this stress dramatically restricts plant growth and
64 development, resulting in a reduction in grain yield (Geiger et al., 2009; Fedoroff
65 et al., 2010; Zhu, 2002; 2016). To adapt to this hostile environment, plants have
66 developed a series of mechanisms for activating adaptive responses, such as
67 the signaling pathway of the phytohormone abscisic acid (ABA; Fujita et al.,
68 2005; Park et al., 2009; He et al., 2012; Saruhashi et al., 2015) and the
69 regulation of stomatal movement to limit water loss (Geiger et al., 2010; Brandt
70 et al., 2015; Grondin et al., 2015; Hauser et al., 2017) are the most fully
71 understood. Stomata are located in the plant epidermis and consist of two guard
72 cells. Stomatal pores transfer water vapor out of the plant and absorb carbon
73 dioxide for plant photosynthesis (Schroeder et al., 2001). The phytohormone
74 ABA can be biosynthesized immediately after water shortage occurs and
75 regulates stomatal movement through intricate signaling networks (Brandt et al.,
76 2015; Grondin et al., 2015). In guard cells, several second messengers involved
77 in ABA-dependent signaling cascades have been identified, such as calcium
78 (Ca^{2+} ; Yuan et al., 2014; Song et al., 2018; Voss et al., 2018), nitric oxide (NO;
79 Scuffi et al., 2014), and reactive oxygen species (ROS; He et al., 2012), and
80 new members are still emerging.

81 Recent evidence has demonstrated that hydrogen sulfide (H_2S) acts as an
82 important messenger that regulates plant growth development and stress
83 responses (Ju et al. 2013; Olas, 2015; Gotor et al., 2015; Guo et al., 2016;
84 Gotor et al., 2019; Shen et al., 2019). The active form of H_2S in biological
85 systems has not been specified to date; therefore, H_2S usually stands for both
86 H_2S and HS^- . By exogenously using its donor, H_2S was reported to confer
87 positive effects in multiple physiological processes, such as root organogenesis
88 (Zhang et al., 2009), plant responses to phytohormones (Hou et al., 2013; Scuffi
89 et al., 2014), and tolerance towards drought, salt, oxidative and heavy-metal

stresses (Zhang et al., 2009; Jin et al., 2013; 2017). Several independent groups have reported the participation of H₂S in ABA- and ethylene-dependent stomatal closure induction (García-Mata and Lamattina, 2010; Hou et al., 2013; Jin et al., 2013; Du et al., 2019). It has long been noted that higher plants have L-Cys desulhydrase (DES) activity with the release of H₂S, pyruvate, and ammonia during its catalyzed L-Cys degradation. (Harrington and Smith, 1980; Papenbrock et al., 2007). Until 2010, a Cys synthase-like gene (At5G28030), a member of the O-acetyl-Ser(thiol)lyase family was characterized as a true DES and named DES1 (Alvarez et al., 2010). Although the null mutants of DES1 showed an approximately 30% decrease in total DES activity, it was clearly demonstrated that DES1 expression was hormonally regulated. Importantly, DES1-produced H₂S acts early in ABA-dependent stomatal closure and interacts with other second messengers or protein kinases to relay the guard cell signaling network, such as NO, ROS, and MAP kinase as well (Scuffi et al., 2014; 2018; Corpas et al., 2019; Du et al., 2019).

At the cellular level, the precise expression profiles and the accumulation level of DES1 in various tissues have been investigated. DES1 is expressed ubiquitously in different organs of mature Arabidopsis and was detected in the cytoplasm of both epidermal and mesophyll cells, including guard cells (Laureano-Marin et al., 2014). Interestingly, the transcript abundance of *DES1* was remarkably activated in ABA-treated guard cell-enriched epidermal strips but not in mesophyll cell-enriched samples (Scuffi et al., 2014), suggesting that guard cell-autonomous H₂S production may be involved in the modulation of ABA-dependent stomatal closure. H₂S possesses a high level of permeability through the lipid membrane, and its solubility is five times greater in lipophilic solvents than in water (Wang et al., 2003). However, it is not understood how the H₂S produced by DES1 in different tissues/organs is integrated to regulate complex physiological responses, such as stomatal closure, and how the strongly varying H₂S levels in different tissues modulate ABA-dependent signaling.

Retrograde signaling was found to be associated with environmental stress, such as drought and excess sunlight (Li et al., 2009; Estavillo et al., 2011; Xiao et al., 2012). Early detection of chloroplast-to-nucleus retrograde signaling was found in etiolated barely seedlings (Rocca et al., 2001). Subsequently, in Arabidopsis, five genomes uncoupled (*gun*) genes involved in plastid-to-nucleus signaling have been first identified by applying norflurazon (NF), which is an inhibitor of carotenoid biosynthesis (Chan et al., 2016). For example, *GUN2*, which participates in the tetrapyrrole pathway and can catabolize heme to CO, free iron, and biliverdin IXa (BV), functions as heme oxygenase (HO; EC 1.14.99.3; Chi et al., 2013). Interestingly, HOs have been found to play a protective role against various abiotic stresses (Mahawar and Shekhawat 2018). Our previous result showed that loss of HY1 (LONG HYPOCOTYL1; a member of the HO family) function enhanced, but gain of HY1 function reduced the plant resistance towards drought stress (Xie et al., 2016). In addition, we showed that under drought stress, a plastid-to-nucleus retrograde HY1-ABI4 signaling occurs in the guard cells, and HY1 can negatively regulate ABA-induced stomatal closure through the activation of transcription of ABI4. However, the specific signaling mechanism of this process has not been determined. Whether DES1 participates in the HY1-ABI4 signaling pathway and what kind of role H₂S plays under drought stress are not well understood.

In this work, we investigated the contribution of ABA-activated DES1 expression in guard cell H₂S production, as well as the signaling steps in ABA-triggered stomatal closure in which DES1/H₂S is involved. By creating transgenic lines that expressed DES1 in a tissue-specific fashion, we observed that guard cell-*in situ* DES1 signaling is involved in the ABA-induced physiological molecular responses in guard cells. Using mutants of the DES1 and HY1 genes and their double mutants, we conclude that guard cell DES1 function and *in situ* H₂S production modulate ABA-dependent stomatal closure through the HY1 signaling pathway.

2 MATERIALS AND METHODS

2.1 Plant materials and growth conditions

The *Arabidopsis* (*Arabidopsis thaliana*) *des1* (SALK_103855; Col-0) *hy1-100* (CS236, Col-0) and *hy1-1* (CS67, Ler) mutants were obtained from the *Arabidopsis* Biological Resource Center (<http://www.arabidopsis.org/abrc>). The *Des* (SALK_205358C) mutant was obtained from Yanxi Pei, Shanxi University. *pDES1:GFP* lines were from Cecilia Gotor, Spanish National Research Council. The 35S:HY1 lines were constructed in our lab previously (Xie et al., 2011). The *hy1-100/des1* double mutant lines were constructed by crossing single mutant *hy1-100* and *des1*. Homozygous mutants were identified by sequencing combined with PCR-based genotyping and corresponding phenotypes (including elongated hypocotyl, yellow and etiolated in appearance; increased apical dominance and reduced leaf size; exhibits extremely spindly and weak seedling).

Seeds were surface-sterilized, washed three times with sterile water for 20 min and then cultured in Petri dishes on solid MS medium (pH 5.8). Plants were grown in a growth chamber with a 16/8-h (23°C/18°C) day/night regime at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiation.

2.2 For expression in planta

The promoter regions of *CAB3* (2000 bp), *ML1* (1500 bp) and *MYB60* (1500 bp) were amplified by PCR and cloned into the pCAMBIA 1305 vector between *SacI* and *SpeI* sites, and the PCR-amplified fragments of *AtDES1* full-length were introduced into the *pCAB3/pML1/pMYB60-pCAMBIA 1305* vectors, using the ClonExpress®II One Step Cloning Kit (Vazyme), with the enzyme digestion sites of *NdeI* and *XhoI*. All procedures followed the manufacturer's manual. Transgenic plants were selected on 1/2 MS medium containing 50 $\mu\text{g/ml}$ hygromycin B. Identification of transgenic plants was conducted by PCR and fluorescence detection. Primers are presented in Table S1.

178

179 2.3 Quantitative RT-PCR (qRT-PCR)

180 Total RNA from the leaves of 1-month-old plants and synthesized cDNA was
181 isolated using the One-Step gDNA Removal and cDNA Synthesis SuperMix Kit
182 (TransGen Biotech). Quantitative reverse transcription-PCR (qRT-PCR) was
183 performed using TransStart Top Green qPCR SuperMix (TransGen Biotech)
184 according to the manufacturer's instructions. The reactions were run in Applied
185 Biosystems™ 7500 real-time PCR systems. The relative expression level of the
186 target genes was analyzed with the delta-delta Ct method and normalized to
187 the *ACTIN7* (At5g09810) and *UBQ10* (AT4G05320) expression levels. All
188 experiments were repeated two times (biological repeats with 3 technical
189 repeats for each experiment), and similar results were obtained. Primers are
190 presented in Table S1.

191

192 2.4 Measurement of guard cell H₂S production and GFP fluorescence

193 For H₂S production in guard cells, epidermal strips of each ecotype were
194 preincubated for 3 h in opening buffer (10 mM MES, pH 6.15, and 50 mM KCl)
195 under light (120 $\mu\text{E m}^{-2}\text{s}^{-1}$) and loaded with 7-Azido-4-methylcoumarin (10 μM ;
196 a fluorogenic probe useful for the detection of H₂S; Sigma-Aldrich; Jia et al.,
197 2018) for 40 min before washing in MES buffer three times for 15 min each.
198 Subsequently, samples were treated for 1 hr with different chemicals indicated
199 in the figure legends. For GFP fluorescence in guard cells, epidermal strips
200 peeled from 4-week-old transgenic Arabidopsis *pDES1:GFP* were treated with
201 10 μM ABA in 10 mM MES buffer, pH 6.15, and the GFP fluorescence
202 examined every 10 min using a Zeiss LSM 880 laser scanning confocal
203 microscope (LSCM; Zeiss, excitation at 405 nm, emission at 454-500 nm for
204 the detection of 7-Azido-4-methylcoumarin fluorescence; excitation at 488 nm,
205 emission at 500–530 nm for detection of GFP fluorescence; 25°C $\pm$ 1°C). The
206 fluorescence of the integrated guard cells was calculated using ImageJ
207 software. Corresponding confocal data are provided, taking the fluorescence

level for control samples as 100%. Values are expressed as relative units $\pm$ SE of total fluorescence/area of the stomata of three independent experiments. Each value represents the mean of at least 90 stomata taken from different leaves. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple comparison. Additionally, the age-oxidized NaHS treatment was used as the negative control by maintaining the separated solution of NaHS (100 μ M) for at least 10 d in the light in a specific open tube to eliminate the entire H_2S .

2.5 Stomatal aperture assays

Stomatal aperture measurements were carried out as described previously (Xie et al. 2016). Epidermal strips were soaked in opening buffer containing 10 μ M ABA, 100 μ M HT (H_2S scavenger), 100 μ M NaHS (H_2S donor) or 10 μ M ABA+100 μ M HT for 1 h. All manipulations were performed at 25 ± 1 °C. Data were analyzed by using ImageJ software. Each value represents the mean of at least 90 stomata taken from three independent experiments, and bars represent the SE of each treatment.

2.6 Statistical analysis

Data are means $\pm$ SE from three independent experiments with replicated measurements as described. For statistical analysis, Duncan's multiple comparison ($P < 0.05$) or the t-test ($P < 0.05$, 0.01 or 0.001; *, ** or ***) was chosen.

3 RESULTS

3.1 ABA induces guard cell *in situ* DES1 expression and H₂S production

Previous genetic and pharmacological studies have shown the involvement of H₂S in stomatal closure in Arabidopsis and that DES1 acts early in ABA-mediated guard cell signal transduction (Scuffi et al., 2014). Although DES1 is ubiquitously expressed at all developmental stages (Laureano-Marín et al., 2014), the fact that DES1 expression is selectively activated in guard cells by ABA suggested that guard cell-autonomous H₂S production is involved in the regulation of stomatal movement. To examine the spatial and temporal regulation of DES1 gene expression by ABA, an Arabidopsis transgenic line expressing the GFP protein under the control of the *DES1* promoter was adopted (*PromDES1-GFP*; Laureano-Marín et al., 2014). In this study, the stomata were viable as we could not find any typical autofluorescence in the walls surrounding the pore (Figure. S1). Subsequently, a significant GFP fluorescence signal was detected in Arabidopsis guard cells, and the level of this fluorescence intensity was markedly induced as early as 10 minutes after ABA treatment (Figure. 1). It was further observed that the GFP signal intensity of ABA-treated guard cells continued to increase and remained significantly elevated throughout the series of measurements, further indicating that ABA-elevated *DES1* transcript abundance and its function are predominantly located in guard cells.

To verify the possibility that DES1 was physiologically associated with H₂S production, the production of endogenous H₂S levels in guard cells was examined using a H₂S fluorescent probe (7-Azido-4-methylcoumarin). As expected, compared with the wild-type, the basal or ABA-induced 7-Azido-4-methylcoumarin-associated fluorescence levels were significantly lower in guard cells of two *des1* mutant alleles, respectively (Figure 2a, b). It was further demonstrated that the detected fluorescence was specifically associated with H₂S production because (1) the induced fluorescence detected in ABA-treated

wild-type or *des1* mutant guard cells was significantly reduced in the presence of HT, a H₂S scavenger. (2) H₂S donor NaHS could clearly induce 7-Azido-4-methylcoumarin-associated fluorescence in both the wild-type and *des1* mutant lines; (3) By contrast, age-oxidized NaHS, the degradation product of NaHS, had no such inducible effect on the fluorescence intensity.

In agreement with previous results (García-Mata and Lamattina, 2010), it was observed that the ABA-induced stomatal closure of wildtype was impaired by the addition of HT and largely reduced by the mutation of DES1 in two *des1* mutant alleles (Figure 2c). Further experiments revealed that NaHS markedly triggered stomatal closure in all Arabidopsis lines tested, whereas no significant response was observed when age-oxidized NaHS was applied that was unable to behave as a sulfide donor. Together, these findings suggest that DES1-dependent H₂S production in guard cells is involved in ABA-induced stomatal closure.

3.2 Complementation of the impaired ABA-dependent stomatal closure and wilted phenotypes of the *des1* mutant by guard cell *in situ* DES1 expression

It was reported that *DES1* is expressed in the cytosol of both epidermal and mesophyll cells, including the guard cells (Laureano-Marín et al., 2014). To elucidate the tissue-related DES1 function, we expressed DES1-GFP protein in the *des1* mutant background under the control of *pML1*, *pCAB3* and *pMYB60* promoters that have already been used in multiple studies to express proteins of interest in epidermal cells (including guard cells), mesophyll cells and guard cells, respectively (Mitra et al., 1989; Cominelli et al., 2011; Bauer et al., 2013; Bernula et al., 2017). At least three independent transgenic lines were created in this study, and the existence of GFP fusion protein was validated by confocal laser scanning microscopy (CLSM). As expected, GFP fluorescence was successfully detected in the epidermal cells of *pML1:DES1-GFP/des1*, mesophyll cells of *pCAB3:DES1-GFP/des1* and guard cells of *pMYB60:DES1-*

291 *GFP/des1*, suggesting that the DES1-GFP fusion proteins were successfully
292 expressed in these tissue-specific transgenic lines (Figure 3a).

293 Next, to investigate the function of the DES1 protein located in different
294 tissues, we performed stomatal bioassay analysis using these tissue-specific
295 complementation lines together with the corresponding wild-type and *des1*
296 mutant in the condition of either ABA or NaHS treatment. Subsequent results
297 revealed that supplemental ABA induced stomatal closure in wild-type plants,
298 whereas the *des1* mutant was considerably less responsive, in agreement with
299 previous results (Scuffi et al., 2014). Importantly, transgenic plants
300 *pMYB60:DES1-GFP/des1* and *pML1:DES1-GFP/des1* showed pronounced
301 ABA-induced stomatal closure similar to that of wild-type plants, suggesting the
302 guard cell *in situ* DES1 function in the ABA-regulated stomatal movement
303 (Figure 3b). However, ABA treatment failed to induce stomatal closure in the
304 *pCAB3:DES1-GFP/des1* line, indicating that the H₂S produced by DES1 in
305 mesophyll cells was not involved in ABA-induced stomatal closure. In
306 agreement with this observation, compared with the wild type guard cells, the
307 guard cell responses to NaHS, a well-known H₂S donor, were similar in all
308 transgenic lines, illustrating that the H₂S in the treatment solution rather than in
309 mesophyll cells could permeate into the guard cells (Figures 2 and 3).

310 To obtain more insight into the guard cell *in situ* function of DES1 expression
311 and H₂S production, DES1-GFP protein was also expressed in the wild-type
312 background under the control of the *proMYB60* promoter (*pMYB60:DES1-GFP*).
313 Time course stomatal bioassay analysis revealed that guard cell-specific
314 expression of DES1 could fully complement the phenotype of the *des1* mutant,
315 suggesting that guard cell *in situ* DES1 function is required for ABA-triggered
316 stomatal closure (Figure 4a; S2). Moreover, compared with that of wild-type,
317 ABA-induced stomatal closure or reduced water loss after detachment was
318 considerably accelerated in either young (Figure 4a) and old (Figure S3) rosette
319 leaves in *pMYB60:DES1-GFP* individual transgenic lines. Leaves from all
320 transgenic lines were further excised to investigate whether guard cell DES1

function and H₂S production is required for stomatal closure and water loss (Figure 4b; S4). NaHS treatment resulted in the significant recovery of the wilted phenotypes in all tested genotypes, whereas *des1* leaves still wilted after ABA treatment, which was fully recovered in both *pMYB60:DES1-GFP/des1* and *pMYB60:DES1-GFP* transgenic lines.

Compared with the *des1* mutant, the guard cell 7-Azido-4-methylcoumarin-associated fluorescence was induced by ABA in both the *pMYB60:DES1-GFP/des1* and *pMYB60:DES1-GFP* transgenic lines (Figure. 4c and S5). However, these fluorescence levels were largely reduced by the addition of HT. Accordingly, ABA-dependent guard cell responses in the *des1* mutant were restored by guard cell complementation of DES1 expression, which was fully abolished by HT, further indicating the guard cell-specific function of H₂S production by DES1 in ABA-triggered stomatal closure (Figure 4d).

3.3 ABA-induced DES1 expression is amplified by the mutation of *HY1*

As we reported previously, disruption of *HY1* enhances, but overexpression of this gene reduces ABA-induced stomatal closure and Arabidopsis drought tolerance (Xie et al., 2016). However, whether DES1 participates in HY1-related guard cell ABA signaling is unknown. With this aim, we used two null mutants deficient in HY1 protein (*hy1-100* and *hy1-1*) together with three independent HY1-overexpression lines (*35S:HY1-3/4/5*) to investigate the participation of DES1 in HY1-related ABA signaling.

First, the changes in the *DES1* transcript abundance in response to ABA treatment were compared between the *HY1* mutant alleles and the corresponding wild-type background (Figure 5a). As expected, the transcriptional abundances of *DES1* were both moderately induced within a short period after ABA treatment, followed by a stronger decrease in both Col-0 and Ler wild-type, which was in agreement with the observation that ABA induced DES1 promoter-driven GFP fluorescence predominantly in guard cells (Figure 1). In contrast, these induced tendencies were greatly strengthened in

both the *hy1-100* and *hy1-1* mutant alleles. *DES1* induction peaked at 20 min after ABA treatment, followed by a gradual decrease within the rest of the treatment period, suggesting that HY1 function negatively regulates ABA-induced *DES1* expression in Arabidopsis leaves. This deduction was further validated by functional analysis of *DES1* expression in three *HY1* transgenic overexpressing lines, namely, *35S:HY1-3*, *35S:HY1-4*, and *35S:HY1-5* (Figure 5b). Similarly, slight induction of *DES1* transcripts was observed in all *HY1*-overexpressing lines after 20 min of ABA treatment. However, we did not find any significant changes compared with that of wild-type. Taken together, these genetic results suggested that the induction of *DES1* at the transcriptional level in ABA signaling was negatively regulated by the loss of HY1.

3.4 HY1 negative regulation of the ABA stomatal response requires DES1-generated H₂S production in guard cells

In a previous work, we presented evidence showing that HY1 negatively regulates ABA responses in stomatal movement, regarding ABA-promoted stomatal closure and ABA-inhibited stomatal opening (Xie et al., 2016). Subsequently, the differences in sulfide levels in guard cells among HY1-related mutants with or without ABA treatment were further compared. It was observed that the ABA-induced 7-Azido-4-methylcoumarin-associated fluorescence levels in guard cells of two *hy1* mutant alleles were much higher with respect to those of the ABA-treated wild-type cells, whereas these levels were largely quenched in three independent *HY1*-overexpressing lines (Figure 6a-c). These results revealed that HY1 negatively regulated ABA-induced *DES1* expression and H₂S production in guard cells. Subsequent pharmacological results revealed that the strong induction of ABA-triggered 7-Azido-4-methylcoumarin-associated fluorescence levels in *hy1* mutant alleles was fully blocked by the addition of HT, a H₂S scavenger (Figure 6b). Accordingly, while ABA treatment failed to increase the fluorescence levels in *HY1*-overexpressing lines, these levels were increased after treatment with

NaHS. We also noticed that 7-Azido-4-methylcoumarin-associated fluorescence levels in *hy1* mutant lines were slightly higher than in the corresponding wild-type lines under basal conditions. Combined with the data of the amplified or diminished *DES1* expression induced by ABA in *HY1*-related lines (Figure. 5), our results clearly showed that HY1 acts upstream of DES1/H₂S in ABA-induced stomatal closure and negatively regulates ABA activated DES1 expression and H₂S production in guard cells.

Consistent with a previous report (Scuffi et al., 2014), we observed that ABA failed to close the stomata in *des1* mutants, but this effect was restored by the application of exogenous H₂S (Figure. 2). The differences in stomatal behaviors among *HY1*-related mutants were further investigated. In parallel with a previous report, loss of function in *HY1* resulted in ABA-hypersensitive phenotypes regarding stomatal closure, while gain of function in *HY1* resulted in hyposensitive phenotypes (Xie et al., 2016), both of which could be reversed by the application of HT or NaHS, respectively (Figure 6d and 6e).

To further confirm these results, we crossed *des1* with *hy1-100* and obtained a *des1/hy1-100* homozygous line. Subsequently, we examined the responses of double mutant *des1/hy1-100* to ABA and NaHS. Interestingly, the ABA phenotypes in the *des1/hy1-100* mutant were more comparable to its parent line *des1* than to *hy1-100* (Figure 7). The hypersensitivity of single mutant *hy1-100* to ABA-induced H₂S production and stomatal closure were largely abolished in this double mutant. Meanwhile, the double mutant *des1/hy1-100* behaved similarly to the single mutants for *DES1* in terms of either ABA-induced stomatal closure or H₂S production in guard cells. In contrast, the stomatal response of *des1/hy1-100* to NaHS was not altered compared with that of wild-type guard cells. These genetic findings clearly placed DES1 downstream of HY1 expression in ABA signaling and revealed that HY1 could negatively regulate ABA-induced H₂S production in guard cells and subsequent stomatal closure by controlling DES1 activation.

410 4 Discussion

411 A previous report characterized DES1 as an O-acetylserine(thiol)lyase
412 (OASTL) homolog with an L-cysteine desulfhydrase activity in Arabidopsis. It
413 has been reported that DES1 catalyzes the desulfuration of L-cysteine to H₂S,
414 ammonia and pyruvate in the cytosol (Álvarez et al., 2010), and the former is a
415 highly reactive molecule that has been proposed as gasotransmitter in
416 mammals (Mazza et al., 2013). In higher plants, the increasing evidence has
417 shown that DES1/H₂S is involved in adaptations and phytohormone responses
418 (García-Mata and Lamattina, 2010; Lisjak et al., 2010; Munaron et al., 2013;
419 Guo et al., 2017; Scuffi et al., 2018). DES1/H₂S has been identified as an
420 essential component of the complex signaling network in multiple ABA
421 responses (Jin et al., 2013; Scuffi et al., 2014). For example, stomatal aperture
422 assays showed that H₂S could function in ABA-induced stomatal closure, which
423 could be partially impaired by both propargylglycine (PAG) and hypotaurine
424 (HT), the inhibitor of L-cysteine desulfhydrase and the scavenger of H₂S,
425 respectively (García-Mata et al., 2010). Genetic evidence also revealed that the
426 response to ABA was impaired in the *DES1* knockout mutant lines, and ABA
427 could not close the stomata in isolated epidermal strips of *des1* mutants (Scuffi
428 et al., 2014). However, whether guard cell *in situ* H₂S production is involved in
429 ABA-induced stomatal closure has not been determined.

430 It is noteworthy that ABA-induced *DES1* expression levels in the epidermal
431 cell RNA extracts were approximately 7-fold higher than those in the mesophyll
432 cell-enriched samples upon ABA treatment (Scuffi et al., 2014). One could
433 assume that the high expression level of *DES1* in epidermal cells was due to
434 the large proportion of guard cells. However, genetic evidence remains lacking.
435 To address the scientific question of whether ABA could induce *DES1*
436 expression tissue-specifically, the Arabidopsis transgenic line containing a
437 *DES1* promoter drive the expression of the GFP gene was applied in this study
438 (Laureano-Marín et al., 2014). Upon ABA treatment, it was clearly observed

that the guard cell GFP fluorescence intensity was swiftly activated at the early time stage (Figure 1), which clearly establishes the localization of *DES1* transcription and helps to characterize its physiological relevance in stomatal ABA responses in guard cells. Interestingly, it was further found that in contrast to the continuous activation of the GFP fluorescence signal in ABA treated *pDES1:GFP* guard cells, the induction of the *DES1* transcript upon ABA treatment was relatively weak in leaves (Figures 1 and 5). These results strongly indicated that other unknown components may be involved in the ABA-activated *DES1* transcription.

Subsequently, a H₂S-specific fluorescent probe (7-Azido-4-methylcoumarin) was adopted to monitor the changes in H₂S production in the guard cells (Jia et al., 2018), which displayed high selectivity for H₂S in animal cells. Clearly, the fluorescence intensity was reduced in two independent *des1* mutants but enhanced in the *pMYB60:DES1* transgenic line, which could be increased by the application of H₂S donor in both Col-0 and *des1* mutant lines (Figure 2a; 4c). Moreover, the promoted fluorescence intensity observed in ABA-treated guard cells was sharply reduced in the presence of H₂S scavenger (Figure 2a, b). In contrast, treatment with age-oxidized NaHS (unable to behave as a sulfide donor) had no effect on fluorescence intensity changes (Figure 2). Taken together, the observed induction of either GFP or H₂S probe fluorescence intensity triggered by ABA was associated with guard cell *DES1* expression and *in situ* H₂S production.

In agreement with database searching results that showed that *DES1* is ubiquitously expressed throughout all growth and developmental stages, analysis of transgenic *pDES1-GFP* plants revealed that GFP fluorescent signals can be detected in the cytosol of both epidermal and mesophyll cells, including guard cells (Laureano-Marín et al., 2014). In this study, the GFP fluorescent signal was predominantly intensified by ABA treatment in guard cells (Figure 1). Consistently, the guard cell *DES1*-rescued line *pMYB60:DES1-GFP/des1* displayed fully complemented ABA-induced stomatal closure and

469 water loss resistance after detachment, suggesting that guard cell DES1 is
470 required for the regulation of stomatal ABA responses (Figure 4). However, no
471 significant differences in ABA-induced stomatal behaviors have been found
472 between *pML1:DES1-GFP/des1* and *pMYB60:DES1-GFP/des1*, in which
473 DES1 protein had been specifically expressed in epidermal and guard cells,
474 separately. Meanwhile, results from *pCAB3:DES1-GFP/des1* showed that
475 mesophyll-specific expression of DES1 fails to complement the impaired guard
476 cell ABA phenotype of the *des1* mutant. In contrast, the guard cell responses
477 to NaHS of all these tissue-specific DES1-transgenic lines were not altered
478 compared with those of wild-type and *des1* mutant lines. Based on these data,
479 we can exclude the possibility that DES1 in mesophyll cells has an impact on
480 ABA-triggered guard cell movement, and we conclude that guard cell *in situ*
481 H₂S production is required for triggering downstream events in ABA signaling.
482 Our results show that stomata are capable of responding to ABA in a cell-
483 autonomous way, and this is mediated through the highly specific localized H₂S
484 produced by DES1 activity. The guard cell *in situ* biological function of
485 DES1/H₂S is involved in the ABA-induced stomatal closure.

486 HY1 is the most highly expressed HO isoform in Arabidopsis and is
487 involved in the modulation of light signaling, salt acclimation, and drought
488 tolerance (Shekhawat and Verma 2010; 2018). The physiological significance
489 of Arabidopsis heme oxygenase HY1 involved in Arabidopsis stomatal
490 movement has been investigated. Mutation of *HY1* promoted, but
491 overexpression of this gene impaired ABA-induced stomatal closure in
492 Arabidopsis. In this work, it was further observed that ABA-induced H₂S
493 fluorescence intensity in guard cells of two independent HY1-knockout mutant
494 lines (*hy1-100* and *hy1*) was approximately twice as high as that of wild-type,
495 but was severely impaired in that of HY1 over-expressing lines (*35S:HY1-3/4/5*),
496 with an 80% reduction compared with the level in wild-type (Figure 5).
497 Meanwhile, the promoted level of ABA-induced H₂S fluorescence in *hy1*
498 mutants was largely impaired by H₂S scavenger, whereas NaHS treatment

could increase guard cell H₂S fluorescence levels in *HY1* overexpressing lines (Figure 6). Simultaneously, ABA-induced *DES1* expression was further strengthened in two independent *HY1*-knockout mutant lines, whereas no significant changes were found in *HY1* overexpressing lines (Figure 5). These results are consistent with a previous study showing that HY1 negatively regulates ABA signaling in guard cells and indicates that cell DES1 expression and *in situ* H₂S production is involved in *hy1*-promoted stomatal closure triggered by ABA. The contributions and interrelationships of HY1 and DES/H₂S within ABA-induced stomatal movement in Arabidopsis were further examined by creating a *des1/hy1-100* double mutant line. Interestingly, *des1/hy1-100* behaved similarly to the single mutants for *DES1* in terms of either ABA-/NaHS-induced H₂S production in guard cells and stomatal closure as well (Figure 7). These results extend our observation that DES1-produced H₂S is negatively regulated by HY1 expression in guard cells and is required for ABA-induced stomatal closure.

Taken together, our results provide genetic evidence showing that rather than in mesophyll cells, H₂S production in guard cells is required for ABA-induced stomatal closure and its controlled water loss, as well. Thus, guard cell *in situ* DES1/H₂S function not only allows an individual stoma to respond to change upon stresses that use ABA as a signal but also permits its self-regulation to be exquisitely controlled at a highly local level. Our data also demonstrate DES1/H₂S function in the regulation of LONG HYPOCOTYL1 (HY1) retrograde signaling to control ABA-induced stomatal closure, helping to elucidate how plants couple photosynthesis and water loss to changes in their environment.

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530

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- 697

FIGURE LEGENDS

Figure 1. ABA-dependent accumulation of GFP fluorescence in stomata from *pDES1:GFP* plants.

(a) Representative transmitted light and GFP images from one experiment where the stomata are marked with asterisks. Bar=50 μ m. (b) The integrated fluorescence of the stomata responding to ABA calculated using ImageJ software. Epidermal strips peeled from 4-week-old transgenic Arabidopsis *pDES1:GFP* were treated with 10 μ M ABA in 10 mM MES buffer, pH 6.15, and the GFP fluorescence observed every 10 min. Values are expressed as relative units $\pm$ SE of total fluorescence/area of the stomata of three independent experiments.

Figure 2. Effect of ABA and NaHS on the guard cell H₂S production and stomatal closure of wild-type and *des1* mutants.

(a) Representative images of the guard cell H₂S fluorescence intensity and stomatal apertures of wild-type, *des1* mutant and *des* mutant plants. Bar=10 μ m. (b-c) Epidermal strips from 4-week-old wild-type, *des1* mutant and *des* mutant were soaked in to the opening buffer, 100 μ M NaHS (the donor of H₂S), 100 μ M age-oxidized NaHS (without H₂S), 10 μ M ABA, 100 μ M HT (the scavenger of H₂S), or 10 μ M ABA+100 μ M HT (the scavenger of H₂S) for 1 h. The integrated fluorescence of the stomata was determined using ZEISS software, and the stomatal apertures were measured using ImageJ software. Each value represents the means of at least 90 stomata taken from different leaves. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple comparison.

Figure 3. Complementation of the *des1* mutant lines by tissue-specific expression of DES1.

(a) Representative GFP images of lines of *des1* mutant rescued by guard cells/pavement cells/mesophyll-specific expression of DES1 lines. (b) Stomatal apertures of the *des1* mutant lines rescued by tissue-specific expression of DES1. Epidermal strips from *des1* mutant and lines of *des1* mutant rescued by tissue-specific expression of DES1 lines were soaked in to the opening buffer, 10 μ M ABA or 100 μ M NaHS. The stomatal apertures were measured using ImageJ software. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple comparison. Data are presented as the means $\pm$ SE of 90 guard cells.

736

Figure 4. Guard cell *in situ* H₂S production is involved in ABA-dependent stomatal movement.

(a) Stomatal closure rate under ABA treatment of wild-type, *des1* mutant, transgenic lines with guard cell-specific expression of DES1. (b) The wilted phenotype of excised young rosette leaves from 1-month-old wild-type, *des1* mutant, and transgenic lines with guard cell-specific expression of DES1 before (upper line of detached leaves) and after spraying distilled water, 10 μ M ABA or 100 μ M NaHS for 1 hr (lower line of detached leaves). Bar=1 cm. (c-d) The guard cell H₂S fluorescence intensity and stomatal apertures of wild-type, *des1* mutant, and transgenic lines with guard cell-specific expression of DES1. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple comparison. Data are presented as the means $\pm$ SE of 90 guard cells.

750

Figure 5. ABA-induced DES1 expression in wild-type and HY1-loss- and gain-of-function mutant leaves.

(a) Expression of the *DES1* gene in 1-month-old wild-type (Col-0 and Ler) and *hy1* mutant (*hy1-100* and *hy1-1*) leaves under ABA treatment within 1 hr. (b) Expression of the *DES1* gene in 1-month-old wild-type, *hy1* mutant and 35S:*HY1* (-3, -4 and -5) transgenic lines leaves under ABA treatment for 20

min. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple comparison.

Figure 6. HY1 negatively regulates stomatal response to ABA

(a) Representative images of the guard cell H_2S fluorescence intensity and stomatal apertures of wild-type (Col-0 and Ler), *hy1* mutant (*hy1-100* and *hy1-1*) and *35S:HY1* (-3, -4 and -5) transgenic lines. Bar=10 μm . The fluorescence intensities or stomatal apertures of each ecotype was shown in b-c or d-e, respectively. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple comparison. Data are presented as the means $\pm$ SE of 90 guard cells.

Figure 7. Guard cell phenotype of *des1/hy1-100* mutant

(a) Representative images of the guard cell H_2S fluorescence intensity and stomatal apertures of wild-type and *des1/hy1-100* double mutant lines. Bar=10 μm . (b-c) The fluorescence intensities and stomatal apertures of each line. Within all the treatments of each line, bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple comparison.

SUPPORTING INFORMATION

Supplemental Information includes 1 table and 5 figures

Table S1 Primers used in this study.

Figure S1 Fluorescence without 7-Azido-4-methylcoumarin probe in wild-type guard cells.

Figure S2 Stomatal closure rate within 10 mins under ABA treatment of wild-type, *des1* mutant, *transgenic* lines with guard cell specific expression of DES1 shown in Figure 4a.

Figure S3 The wilted phenotype of excised old rosette leaves from 1-month-old transgenic lines of *pMYB60:DES1-GFP*.

786 **Figure S4** Real water content assay for excised leaves shown in Figure 4 (b).
787 Statistical comparisons were performed by independent samples t test.
788 Asterisks represented significant differences between the *des1* or
789 *pMYB60:DES1/des1-1* mutants and wild type values (*P < 0.05, **P < 0.01,
790 ***P < 0.001).

791 **Figure S5.** Representative guard cell 7-Azido-4-methylcoumarin fluorescence
792 pictures for *des1* and *pMYB60:DES1/des1* with or without ABA treatment. The
793 integrated fluorescence of the stomata was determined using ZEISS software,
794 measured by Image J, and presented in Figure 4c.

795

Figure 1

(a)

(b)

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Figure 4

Figure 5

Figure 6

Figure 6

Figure 7

revised figure 7

SUPPORTING INFORMATION**Table S1 Primers used in this study.**

pML1- <i>Sac</i> I -F	catgattacgaattcgagctcATAACATTA AAACAACCTTTTAATACTATTTTC	for promter ML1-pCAMBIA1305-GFP
pML1- <i>Spe</i> I -R	agctccggacttaagactagtCTTACAAAA TTTGACTAATAAAACCAGAAT	
PCAB3- <i>Sac</i> I -F	catgattacgaattcgagctcTCAAGAACG GAAGATTGGCTATG	for promter CAB3-pCAMBIA1305-GFP
pCAB3- <i>Spe</i> I -R	agctccggacttaagactagtTGAAACTTT TTGTGTTTTTTTTTTTTT	
pMYB60- <i>Sac</i> I -F	catgattacgaattcgagctcTGGTTGCAC TAAGTTCGGTTTTAC	for promter MYB60-pCAMBIA1305-GFP
pMYB60- <i>Spe</i> I -R	agctccggacttaagactagtATGTAAACA AATAAGCCAGTTTAGGG	
DES1- <i>Xba</i> I -F	aagtcggagctagctctagaATGGAAGA CCGCGTCTTGATC	for DES1-pCAMBIA1305-GFP
DES1- <i>Bam</i> H I -R	gcccttgctcaccatggatccTTCAACTGG CAAATTCTCAGCTT	
qDES1-F	TCGAGTCAGTCAGATATGAAGCT	
qDES1-R	TGTAACCTTGGTACCAACATCTC T	
qUBQ10-F	GGCCTTGTATAATCCCTGATGAA TAAG	for qRT-PCR
qUBQ10-R	AAAGAGATAACAGGAACGGAAAC ATAGT	
qACTIN-F	GGACCTGACTCATCGTACTC	
qACTIN-R	TACAGTGTCTGG ATCGGAGG	

Figure S1

Figure S1. Fluorescence without 7-Azido-4-methylcoumarin probe in wild-type guard cells.

Do not distribute

Figure S2

Figure S2. Stomatal closure rate within 10 mins under ABA treatment of wild-type, *des1* mutant, *transgenic* lines with guard cell specific expression of DES1 shown in Figure 4a.

Figure S3

Figure S3. The wilted phenotype of excised old rosette leaves from 1-month-old transgenic lines of *pMYB60:DES1-GFP*.

Figure S4

Figure S4. Real water content assay for excised leaves shown in Figure 4 (b). Statistical comparisons were performed by independent samples t test. Asterisks represented significant differences between the *des1* or *pMYB60:DES1/des1-1* mutants and wild type values (*P < 0.05, **P < 0.01, ***P < 0.001).

Figure S5

Figure S5. Representative guard cell 7-Azido-4-methylcoumarin fluorescence pictures for *des1* and *pMYB60:DES1/des1* with or without ABA treatment. The integrated fluorescence of the stomata was determined using ZEISS software, measured by Image J, and presented in Figure 4c.