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The coordination of guard-cell autonomous ABA synthesis and DES1 function *in situ* regulates plant water deficit responses

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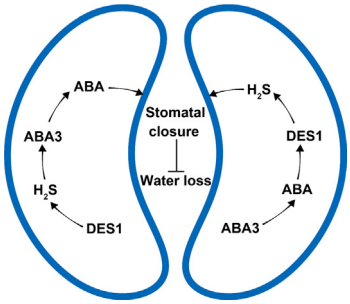
GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Drought stress triggers the synthesis and accumulation of the phytohormone abscisic acid (ABA), which regulates stomatal aperture and hence reducing plant water loss. Hydrogen sulfide (H_2S), which is produced by the enzyme L-cysteine desulfhydrase 1 (DES1) that catalyzes the desulfuration of L-cysteine in Arabidopsis, also plays a critical role in the regulation of drought-induced stomatal closure. However, little is known about the regulation of DES1 or the crosstalk between H_2S and ABA signaling in response to dehydration.
Objectives: To demonstrate the potential crosstalk between DES1-dependent H_2S and ABA signaling in response to dehydration and its regulation mechanism.
Methods: Firstly, by introducing guard cell-specific *MYB60* promoter, to produce complementary lines of *DES1* or *ABA3* into guard cell of *des1* or *aba3* mutant. And the related genes expression and water loss under ABA, NaHS, or dehydration treatment in these mutant or transgenics lines were determinate.
Results: We found that dehydration-induced expression of *DES1* is abolished in the abscisic acid deficient 3 (*aba3*) mutants that are deficient in ABA synthesis. Both the complementation of *ABA3* expression in guard cells of the *aba3* mutants and ABA treatment rescue the dehydration-induced expression of *DES1*, as well as the wilting phenotype observed in these mutants. Moreover, the drought-induced expression of ABA synthesis genes was suppressed in *des1* mutants. While the addition of ABA or the

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expression of either *ABA3* or *DES1* in the guard cells of the *aba3/des1* double mutant did not alter the wilting phenotype of these mutants, the wild type phenotype was fully restored by the expression of both *ABA3* and *DES1*, or by the application of NaHS.

Conclusion: These results demonstrate that the coordinated synthesis of ABA and *DES1* expression is required for drought-induced stomatal closure in Arabidopsis.

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Introduction

Plants have developed various mechanisms to perceive water deficits and relay stress signals locally and systemically. Stomatal movements, which are controlled by the guard cells, regulate the gas exchange with the environment and water loss through transpiration. These responses are particularly important to sustain plant growth under stressful or changing environmental conditions [1,2]. The synthesis and accumulation of the phytohormone abscisic acid (ABA) is a central response to drought stress [3]. ABA participate in a complex signaling network that regulates stomatal movement and thus controls water loss in response to drought.

In Arabidopsis, ABA is synthesized primarily in vascular tissues and also in seeds [4,5]. The pathway of ABA synthesis involves multiple steps, starting with the metabolism of carotenoid precursors in the plastids. This pathway involves the epoxidation, isomerization and cleavage of zeaxanthin to produce xanthoxin, which is subsequently exported from the plastids to the cytosol, where it is converted into abscisic aldehyde. The molybdenum cofactor containing sulfurase ABA3 (AT1G16540) converts abscisic aldehyde to ABA [4,6]. ABA3 is the rate-limiting step of ABA biosynthesis, and it plays an important role in drought stress responses. The expression of *ABA3* is induced by drought. In addition, knock-out mutants in this gene exhibit decreased ABA accumulation and are more sensitive to drought than the wild-type [7,8,9]. Interestingly, guard cells contain the complete ABA biosynthesis pathway, facilitating "guard cell-autonomous", ABA synthesis, a process that controls stomatal closure in response to changing environmental conditions, particularly water and CO_2 availability [10,11,12]. ABA3 mediated-guard cell ABA synthesis alone is sufficient to stomatal closure and regulate wilting, as demonstrated by complementation of ABA3 in guard cell of *aba3* mutants [11].

Hydrogen sulfide (H_2S), which has long been considered only as a toxic gas, has been known in recent years to be an important gaseous signaling molecule that regulates plant growth, development and stress responses [13,14,15,16]. Pretreatment with exogenous NaHS (a H_2S donor) alleviates drought stress responses by increasing ABA synthesis through the expression of ABA synthesis genes in wheat [17]. The involvement of H_2S in control of stomatal movement has been widely reported [18,19]. The application of NaHS alters the size of the stomatal aperture compared to untreated controls. Moreover, H_2S-induced stomatal closure involves the control of K^+ efflux, and Ca^{2+} and Cl^- influxes in response to drought in Arabidopsis [20]. L-cysteine desulfhydrase (DES1; At5G28030) catalyzes the desulfuration of L-cysteine to H_2S, pyruvate, and ammonia is the major source of H_2S in plants [16,21]. A recent study has shown that while stomatal closure in the *des1* mutants was not influenced by ABA treatment, it was fully rescued by the administration of NaHS, a finding that suggests a positive role of H_2S in ABA signaling [22]. Moreover, the specific expression of DES1 in guard cells and the *in situ* H_2S production leads to the persulfidation of NADPH oxidase RBOHD [23,24]. Taken together, these finding suggest that stomatal movements are finely controlled by an *in situ* guard cell signal network that involves both ABA and H_2S.

Materials and methods

Plant materials and growth conditions

Seeds of the Arabidopsis (*Arabidopsis thaliana*) *des1* (SALK_103855) mutants (in the Col-0 backgroud) was purchased from the Arabidopsis Biological Resource Center (http: www.arabidopsis.org abrc). Seeds of the *aba3* and *pMYB60:ABA3 aba3* mutant lines were obtained from Julie Gray at the University of Sheffield. The double mutant lines *aba3 des1* and *pMYB60:ABA3 aba3 des1* were obtained by crossing the *aba3 or pMYB60:ABA3 aba3* with *des1,* respectively. Homozygous mutants were identified by sequencing combined with PCR-based genotyping and typical phenotypes of *ABA3*-deficient mutant (slightly darker green plants, reduced growth, reduced plant size and vigor etc). The *pMYB60: DES1 aba3 des1* and *pMYB60:ABA3 pMYB60:DES1 aba3 des1* lines were produced by expression of the *DES1* gene under the control of the guard cell-specific *MYB60* transcription factor promoter [11,23]. Independent transgenic T3 transgenic lines that had been selected using the antibiotic hygromycin B used for the following analyses.

Seeds were surface sterilized and placed in petri dishes containing 1/2 Murashige and Skoog agar medium. They were incubated at 4 °C for 2–4 d and then transferred to soil and grown in pots in a growth chamber with a 16/8 photoperiod at an irradiance of 120 μmol m^{-2} s^{-1} and a 23 °C/18 °C day night regime.

Dehydration stress treatment and water loss assay

Wild-type, mutant, and transgenic plants were grown in soil at 22 °C for 1 month at an irradiance of 120 μmol m^{-2} s^{-1} and a 23 °C/18 °C day night regime. The leaves of 1-month-old plants were excised and exposed to dehydration stress as follows. Excised leaves were placed in eppendorf tubes. Photographs of the detached leaves were taken before and after exposure to dehydration stress. The fresh weight of each leaf was measured immediately upon harvest and these values were set as 100% water content. Water loss was calculated thereafter from the decreases in the fresh weight of the tissues measured every 5 min for 50 min and expressed as a percentage.

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from the leaves of 1-month-old wild-type and mutant plants using the One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech). The quantitative reverse transcription-PCR (qRT-PCR) reactions were performed using Applied BiosystemsTM 7500 real-time PCR systems using TransStart Top Green qPCR SuperMix (TransGen Biotech) according to the manufacturer's instructions. The relative expression levels were analyzed using the delta-delta Ct method, after normalization to the *ACTIN7* (At5g09810) and *UBQ10* (AT4G05320) internal standards. The primers used in this study are presented in Table 1.

Table 1
Primers used in this study.

Primer	Sequence (5′-3′)	Description
des1 -LP	GCGGTCTTTTGTCTCTTCTTC	Identification for *des1* mutant
des1 -RP	AGTAACCGTTCCACCAGTTCC	
LBb1.3	ATTTTGCCGATTTCGGAAC	
pMYB60-*Sac*I-F	catgattacgaattcgagctcTGGTTGCACTAAGTTCGGTTTTAC	for promoter pMYB60-pCAMBIA1305-GFP
pMYB60-*Spe*I-R	agctccggacttaagactagtATGTAAACAAATAAGCCAGTTTAGGG	
DES1-*Xba*I-F	aagtccggagctagctctagaATGGAAGACCGCGTCTTGATC	for DES1-pCAMBIA1305-GFP
DES1-*Bam*HI-R	gcccttgctcaccatggatccTTCAACTGGCAAATTCTCAGCTT	
pMYB-DES -F	ACTATTGGGTTATTTGCC	Identification for *pMYB60:DES1-pCAMBIA1305-GFP* lines
pMYB-DES -R1	CCAGAGCCTATTCCTTG	
pMYB-ABA3 -F	TAAACAGAGGCAACGAGA	Identification for *pMYB60:ABA3-pCAMBIA1305-GFP* lines
pMYB-ABA3 -R	CCAAGCCCAGTAGGATAA	
qDES1-F	TCGAGTCAGTCAGATATGAAGCT	for RT-PCR
qDES1-R	TGTAACCTTGGTACCAACATCTCT	
qABA3-F	TTTCCGTGGACCCAAGACAG	
qABA3-R	ATTGCACTCCGAGGGGAAAG	
qNCED3-F	CTCAGCCGCCATTATCGTCT	
qNCED-R	GTGTGACACGACTGGCCATA	
qZEP-F	TCTTCGGATGTTGGTGGTGG	
qZEP-R	GGATAGAATCCCCGAGCAGC	
qAAO3-F	TACGAAAGCCTCCGCAAGTT	
qAAO3-R	CCTTGTCCCACCTCAATCCC	
qACTIN7-F	GGACCTGACTCATCGTACTC	
qACTIN7-R	TACAGTGTCTGG ATCGGAGG	
qUBQ10-F	GGCCTTGTATAATCCCTGATGAATAAG	
qUBQ10-R	AAAGAGATAACAGGAACGGAAACATAGT	

Determination of endogenous H_2S content

Endogenous H_2S content was measured by the formation of methylene blue, according to the method previously published [25]. Briefly, Arabidopsis leaf samples (0.2 g) were extracted in 1 ml of phosphate buffer solution (pH 6.8, 50 mM) containing 0.1 M EDTA and 0.2 M AsA. The supernatant was mixed with 0.5 ml of 1 M HCl in a test tube to release H_2S, following by absorbing in 5 ml 1% (w/v) zinc acetate trap. After 30 min reaction, 0.3 ml 5 mM N,N-dimethyl-p-phenylenediamine dihydrochloride (dissolved in 3.5 mM H_2SO_4), and 0.3 ml of 50 mM ferric ammonium sulphate (dissolved in 100 mM H_2SO_4) was added. After leaving the mixture for 15 min at room temperature, the amount of H_2S in zinc acetate traps was measured at 670 nm by ultraviolet spectrophotometry. Solutions with different concentrations of NaHS were treated in the same way as the assay samples, and used for the quantification of H_2S.

Statistical analysis

Error bars represent ± SD three biological replicates, analyzed using SPSS version 23.0. The comparison of two groups was performed by using Student's *t*-test. * and ** represent $P < 0.05$ and $P < 0.01$, respectively. Different letters indicate significantly different at $P < 0.05$ according to Duncan's multiple range test ($P < 0.05$).

Results and discussion

De-novo ABA synthesis is critical for dehydration-induced DES1 expression and responses to dehydration stress

We have recently demonstrated that the abundances of *DES1* transcript is induced by ABA treatment of wild-type Arabidopsis thaliana leaves [23]. To investigate the potential role of ABA in dehydration-induced *DES1* expression, we first analyzed the expression of *DES1* gene in response to dehydration stress in the wild-type and *aba3* mutants, which are defective in the last step of ABA synthesis in plants [7,8]. As predicted, *DES1* was induced in wild-type rosette leaves within 6 h after detachment. However, the induction of *DES1* expression was not observed in the detached *aba3* mutant (Fig. 1A). Treatment with ABA, but not NaHS, stimulate *DES1* expression in both wild-type and *aba3* mutant leaves (Fig. 1B and C). This observation suggests ABA3-meidated ABA synthesis is required for the induction of *DES1* expression in response to dehydration stress.

Although ABA can be transferred from the vascular tissues to the guard cells [4,26], guard cells house the complete ABA synthesis pathway [11]. Moreover, ABA3 mediated-guard cell ABA synthesis is required for stomatal closure. To test whether *de novo* ABA synthesis in the guard cells is required for *DES1* expression and the related dehydration responses, we further use *pMYB60:ABA3 aba3* plants in which ABA synthesis was rescued in the mature guard cells of the *aba3* mutants [12]. *DES1* expression was significantly increased in *pMYB60:ABA3 aba3* lines after 6 h dehydration, similar to wild-type (Fig. 1D). This result suggests that ABA3-mediated-guard cell ABA synthesis is sufficient to trigger dehydration induced-*DES1* expression. DES1 is involved in stomatal closure and related drought tolerance responses [15,18,20]. To further evaluate the possible link between the functions of ABA3 and DES1 in drought stress responses, *DES1* was specifically expressed in the guard cell of *aba3* mutant under control of guard cell-specific MYB60 promoter, to produce *pMYB60:DES1 aba3* lines. In agreement with previous results [11], the complementation of ABA3 in guard cells rescued the wilting phenotype of the *aba3* mutants. Moreover, the *pMYB60:ABA3 aba3* plants showed a similar response to dehydration stress as the wild-type (Fig. 1E) However, there were no significant differences between the *pMYB60:DES1 aba3* plants and the *aba3* mutants, indicating that ABA3-mediated guard-cell ABA synthesis is sufficient for dehydration-induced DES1 expression and related dehydration tolerance.

DES1 is involved in dehydration-induced ABA synthesis and responses to dehydration

Cysteine and/or the products of cysteine metabolism might trigger ABA synthesis and contribute to the regulation of stomatal closure in response to drought [27]. Cysteine is also the substrate of

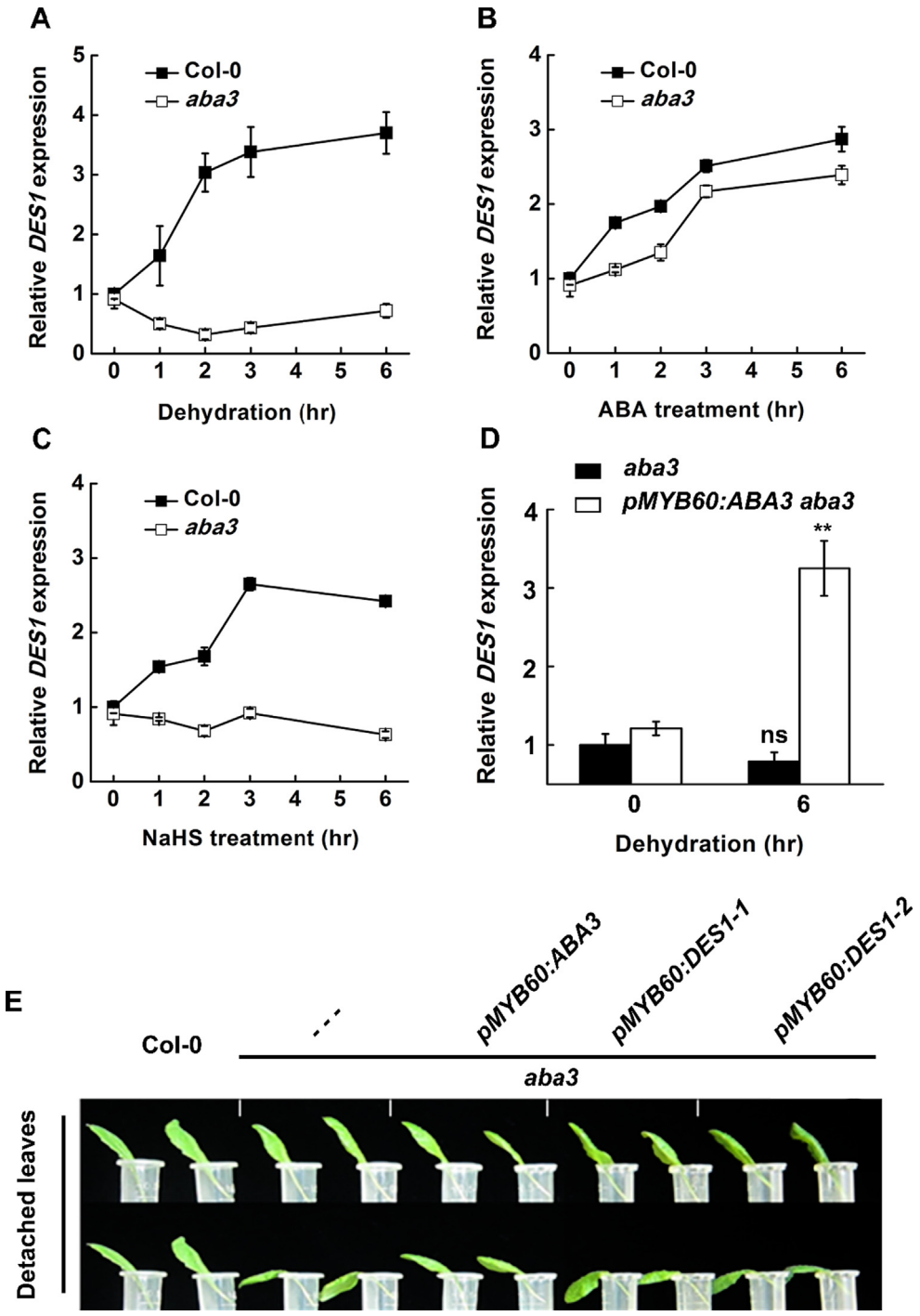

Fig. 1. ABA *denovo* synthesis is critical for drought induced DES1 expression and plant drought tolerance**.** The induction of *DES1* expression in *aba3* mutant. *DES1* expression in 1-month-old wild type and *aba3* mutant leaves after detachment (A), detachment with ABA (10 μM) (B) or NaHS (100 μM) (C) pretreatment in 6 hr were detected. (D) The induction of *DES1* in *aba3* mutant and *aba3* mutant carrying *ABA3* in the guard cells. Relative transcript levels are shown using *actin7* and *UBQ10* as internal control. (E) Relative dehydration stress phenotype of wild type, *aba3*, and *aba3* mutant seedlings carrying *ABA3* or *DES1* in the guard cells. The excised leaves of 1-month-old Col-0, *aba3*, and *aba3* mutant seedlings carrying *ABA3* (*pMYB60:ABA3*) or *DES1* (*pMYB60:DES1*) in the guard cells were put into the eppendorf tubes immediately, with photo (the upper panel) taken as 0 hr. The lower panel photo was taken 1 hr later. Bar = 0.5 cm. Error bars represent ± SD three biological replicates. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, Student's *t* test.

the Moco-sulfurylase ABA DEFICIENT3 required for activation of ALDEHYDE OXIDASE 3 (AAO3). To investigate the possible role of DES1, which degrades cysteine to produce H_2S, we first checked the expression profiles of genes encoding proteins responsible for ABA synthesis in the leaves wild-type plants and *des1* mutants, including *ZEAXANTHIN EPOXIDASE* (*ZEP*), *9-cis-EPOXYCAROTENOID DIOXYGENASE* (*NCED3*), *ALDEHYDE OXIDASE 3* (*AAO3*), and *ABA DEFICIENT3* (*ABA3*). Exposure to dehydration increased the accumulation of all of these transcripts in the wild-type but not in the *des1* mutants (Fig. 2), These results indicate that *DES1* is required for dehydration-induced ABA synthesis. Combined with the results shown in Fig. 2, these data suggest that the downstream products of DES1-mediated cysteine metabolism, trigger drought-induced ABA synthesis rather than cysteine per se. In support of

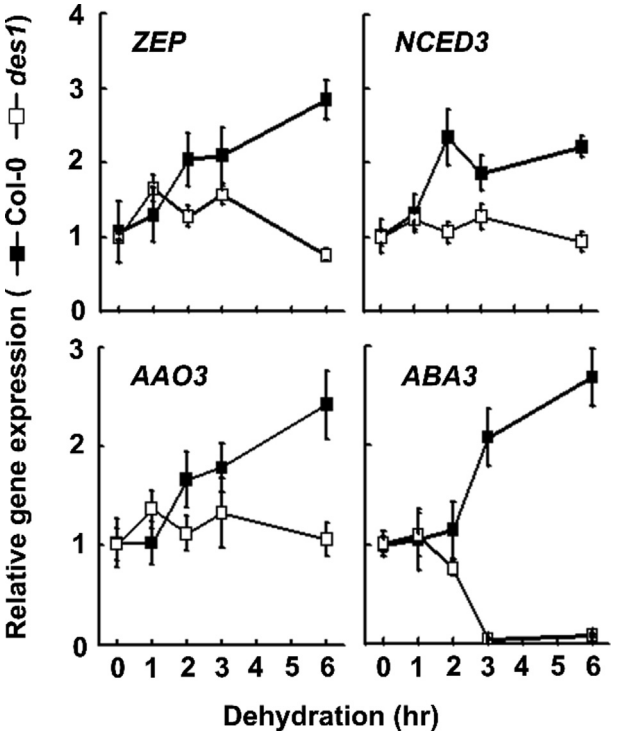

Fig. 2. DES1 is involved in drought-induced ABA synthesis**.** The induction of genes involved in ABA synthesis were abolished in *des1* mutant. Expression profile of ABA-synthesis genes in 1-month-old wild type and *des1* mutant leaves after detachment were detected within 6 hr. Relative transcript levels are shown using *actin7* and *UBQ10* as internal control.

this conclusion, it has been reported that ABA synthesis contributes to H2S-induced drought tolerance in wheat [17].

To confirm the function of DES1-produced H_2S in ABA-mediated dehydration responses, we crossed the *aba3* and *des1* mutants to obtain *aba3 des1* double mutants. Functional characterization revealed that *aba3 des1* plants possessed much lower endogenous H_2S levels than either parental lines or wild-type (Fig. 3A; [25]). We also noticed that the H_2S level was relatively lower in *aba3* mutant than wild-type, suggesting that DES1-produced H_2S is regulated by ABA synthesis. In this study, drought-induced ABA biosynthesis was impaired in *des1* mutant, and *vice versa* (Figs. 1, 2). Importantly, the levels of cysteine accumulation were about 20–25% higher in the des1 mutant leaves than the wild-type regardless of the growth stage of the plant [21]. These results implied DES1-H_2S signals might be divergent from cysteine assimilation [27], both of which may fine-tune ABA synthesis and signal in plants.

Subsequently, we observed that the rate of water loss was greater in the *aba3 des1* double mutant than those of either the *aba3* or *des1* single mutants, or the wild-type leaves following detachment (Fig. 3B). This result suggests that DES1 is involved in ABA3-meidated ABA synthesis during plant responses to dehydration. Taken together, these results increase our understanding of the intrinsic crosstalk between the DES1/H2S and ABA signaling pathways and shed more light onto the regulatory role of H_2S in plant cells.

The coordination of guard-cell autonomous ABA synthesis and *in situ* DES1 function in controlling plant dehydration responses.

DES1 is expressed in the cytosol of mesophyll and epidermal cells, including the guard cells at all stages of development [23].

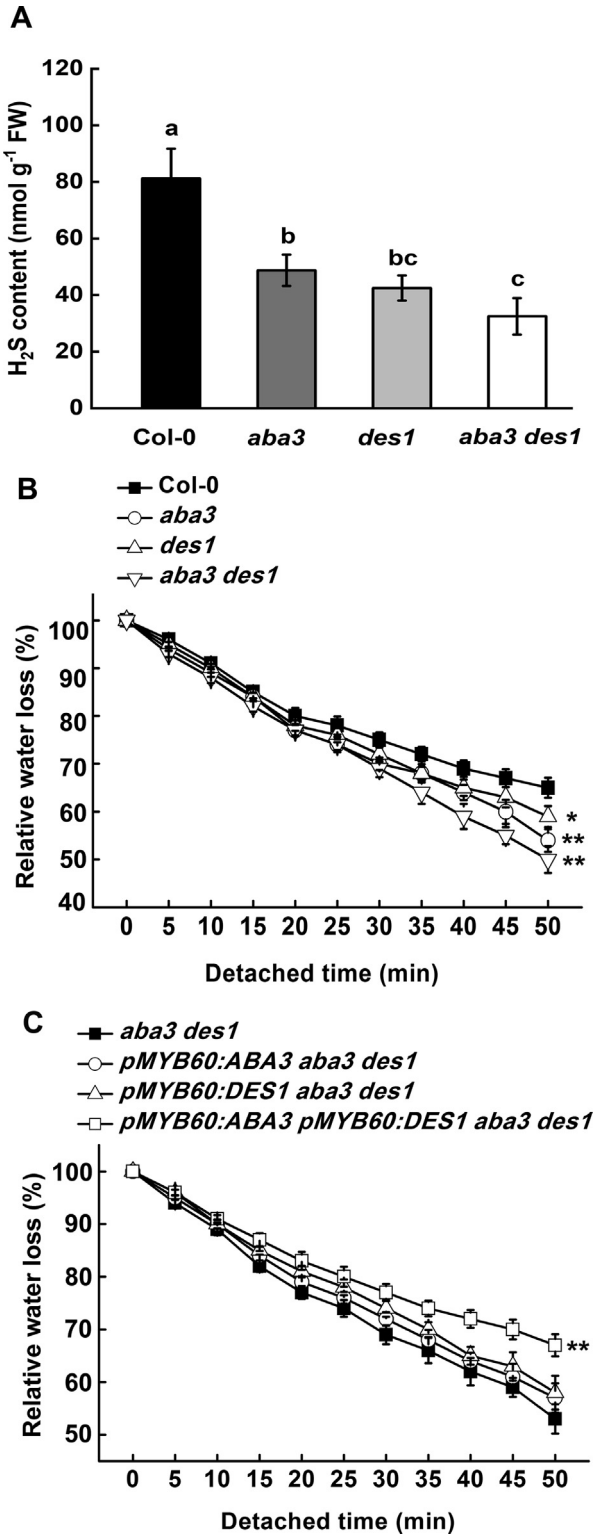

Fig. 3. Endogenous H_2S production and relative water content in ABA3 and DES1 related mutant and complementation lines. (A) Endogenous H_2S production in 1-month-old leaves of wild type, *aba3*, *des1* mutant and *aba3 des1* double mutants. (B) Relative water loss of wild type, *aba3*, *des1* mutant and *aba3 des1* double mutant leaves within 50 min. (C) Relative water loss of *aba3 des1*, *pMYB60:DES1 aba3 des1*, *pMYB60:ABA3 aba3 des1*, and *pMYB60:ABA3 pMYB60:DES1 aba3 des1* leaves within 50 min. The 1-month-old leaves were excised, and fresh weight was measured every 5 min within 50 min. The fresh weight was measured immediately, setting as 0% water loss. Error bars represent ± SD three biological replicates. Different letters indicate significantly different at $P < 0.05$ according to Duncan's multiple range test ($P < 0.05$). * $p < 0.05$; ** $p < 0.01$; Student's *t* test.

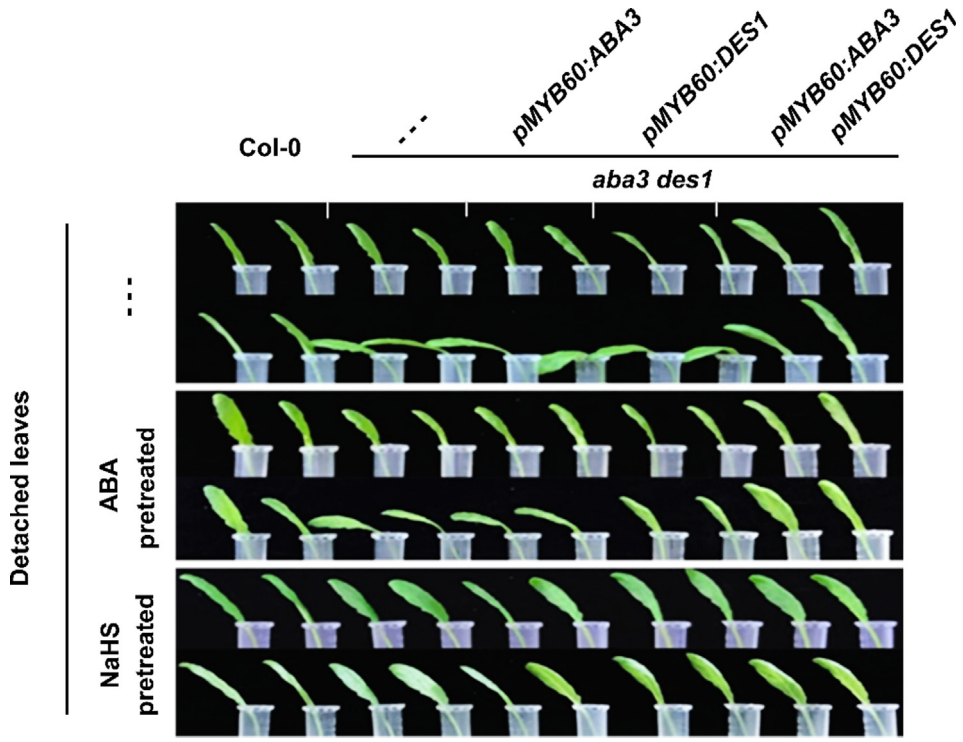

Fig. 4. Relative dehydration stress phenotype of 1-month-old *aba3 des1*, and *aba3 des1* mutant carrying *ABA3*, *DES1* or both *ABA3* and *DES1* in the guard cells. The excised leaves of 1-month-old Col-0, *aba3 des1*, and *aba3 des1* mutant seedlings carrying *ABA3* (*pMYB60:ABA3*), *DES1* (*pMYB60:DES1*) or both (*pMYB60:ABA3 pMYB60:DES1*) in the guard cells were put into the eppendorf tubes sprayed with distilled water, NaHS (100 μM) or ABA (10 μM) individually, and the photo (the upper panel) was taken as 0 hr. The lower panel photo was taken 1 hr later. Bar = 1 cm.

We have recently reported that DES1 is required for *in situ* H_2S production in guard cells and the related control of stomatal closure [22]. To evaluate the contribution of guard cell specific-DES1 expression in the ABA3-mediated dehydration response, *DES1 or ABA3* were introduced into the *aba3 des1* double mutants under the control of the *pMYB60* promoter, to produce *pMYB60:ABA3 aba3 des1* and *pMYB60:DES1 aba3 des1* lines respectively, as well as *pMYB60:ABA3 pMYB60:DES1 aba3 des1* plants. The *pMYB60: ABA3 aba3 des1* and *pMYB60:DES1 aba3 des1* plants exhibited similar rates of dehydration-induced water loss to the *aba3 des1* double mutants. Hence, complementation with either DES1 or ABA3 in guard cell could not prevent the dehydration-induced water loss in the *aba3 des1* double mutants. The *pMYB60:ABA3 pMYB60:DES1 aba3 des1* leaves displayed a significant decrease in dehydration-induced water loss compared to the *aba3 des1* mutants (Fig. 3C). These results suggest that DES1 and ABA3 in the guard cells interact synergistically to control water loss in response to dehydration. The leaves of the *aba3 des1* double mutants wilt after detachment. The expression of either *ABA3* or *DES1* in the guard cells of the *aba3 des1* mutants did not prevent wilting. However, the leaves of the *pMYB60:ABA3 pMYB60:DES1 aba3 des1* plants showed a similar wilting behavior to the wild-type following detachment (Fig. 4). It is worthy to note that the guard cell specific overexpression of *DES1* delayed the wilting phenotype and reduced water loss following exposure to dehydration stress [23]. Both the *pMYB60: DES1 aba3 des1* and *pMYB60:DES1 aba3* plants exhibit greater wilting in response to dehydration than the wild-type (Figs. 1 and 4). These finding suggest that ABA synthesis in guard cells is sufficient to support DES1 functions in the dehydration stress response. Most importantly, the wilting phenotype of *pMYB60:DES1 aba3 des1* plants can be fully restored by ABA pretreatment. This was not observed in the *aba3 des1* and *pMYB60:ABA3 aba3 des1* mutant line. In contrast, NaHS pretreatment rescued the wilting phenotype in all of the mutant lines, a finding that indicates that DES1 is required in guard cells for ABA-triggered dehydration responses. Taken together, these results suggest that the coordination of guard-cell autonomous ABA synthesis and DES1 functions control the dehydration response in *Arabidopsis*.

Conclusions

In summary, the findings presented here demonstrate that ABA3-mediated guard-cell autonomous ABA synthesis is required (and is sufficient) for *DES1* expression and related dehydration responses. DES1 is involved in dehydration-induced ABA synthesis. Moreover, the *in situ* DES1 function of guard cell are required for ABA-triggered dehydration responses. Using transgenic lines that specifically express ABA3 and DES1 in the guard cells, we found that the expression of either *ABA3* or *DES1* in the guard cells of the *aba3*/*des1* mutants failed to alter wilting, the wild-type phenotype was fully restored by simultaneous expression of *ABA3* and *DES1,* or following treatment with NaHS but not ABA. Taken together, these results suggest that the coordination of guard-cell autonomous ABA synthesis and DES1 functions control the dehydration stress response in *Arabidopsis*. These findings not only extend our current knowledge of H_2S function in guard cells and ABA signaling, but they also provide new targets for plant improvement strategies, seeking to produce crops that are better able to withstand changing environmental conditions.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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