

# Hydrogen Sulfide Generated by L-Cysteine Desulfhydrase Acts Upstream of Nitric Oxide to Modulate Abscisic Acid-Dependent Stomatal Closure<sup>1[C][W]</sup>

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Abscisic acid (ABA) is a well-studied regulator of stomatal movement. Hydrogen sulfide (H<sub>2</sub>S), a small signaling gas molecule involved in key physiological processes in mammals, has been recently reported as a new component of the ABA signaling network in stomatal guard cells. In *Arabidopsis* (*Arabidopsis thaliana*), H<sub>2</sub>S is enzymatically produced in the cytosol through the activity of L-cysteine desulfhydrase (DES1). In this work, we used *DES1* knockout *Arabidopsis* mutant plants (*des1*) to study the participation of *DES1* in the cross talk between H<sub>2</sub>S and nitric oxide (NO) in the ABA-dependent signaling network in guard cells. The results show that ABA did not close the stomata in isolated epidermal strips of *des1* mutants, an effect that was restored by the application of exogenous H<sub>2</sub>S. Quantitative reverse transcription polymerase chain reaction analysis demonstrated that ABA induces *DES1* expression in guard cell-enriched RNA extracts from wild-type *Arabidopsis* plants. Furthermore, stomata from isolated epidermal strips of *Arabidopsis* ABA receptor mutant *pyrabactin-resistant1* (*pyr1*)/*pyrabactin-like1* (*pyl1*)/*pyl2*/*pyl4* close in response to exogenous H<sub>2</sub>S, suggesting that this gasotransmitter is acting downstream, although acting independently of the ABA receptor cannot be ruled out with this data. However, the *Arabidopsis* clade-A PROTEIN PHOSPHATASE2C mutant *abscisic acid-insensitive1* (*abi1-1*) does not close the stomata when epidermal strips were treated with H<sub>2</sub>S, suggesting that H<sub>2</sub>S required a functional ABI1. Further studies to unravel the cross talk between H<sub>2</sub>S and NO indicate that (1) H<sub>2</sub>S promotes NO production, (2) *DES1* is required for ABA-dependent NO production, and (3) NO is downstream of H<sub>2</sub>S in ABA-induced stomatal closure. Altogether, data indicate that *DES1* is a unique component of ABA signaling in guard cells.

Abscisic acid (ABA) regulates diverse physiological and developmental processes in plants, among which seed dormancy and stomatal movement are the most studied. Stomata are pores bordered by pairs of specialized cells

named guard cells located in the epidermis of the aerial part of most land plants. Due to the waxy cuticle of plants, stomatal pores regulate approximately 90% of all the gas exchange (i.e. the uptake of CO<sub>2</sub> required for photosynthesis and the loss of water vapor during transpiration) between the plant and the environment (Hetherington and Woodward, 2003). Thus, stomatal movement is a key process for the regulation of plant water status and biomass production. In guard cells, ABA induces an increase of intracellular calcium concentrations, which, in turn, induces an efflux of anions that causes membrane depolarization. In this voltage milieu, ion uptake is blocked through the inactivation of inward-rectifying potassium channels, and ion efflux is induced through activation of outward-rectifying potassium channels. This solute relocation drives water out of the guard cells and closes the stomatal pore as a result of a reduction in guard cell turgor (Blatt, 2000; Kim et al., 2010). The ABA-dependent signaling network in guard cells involves numerous second messengers, including calcium, K<sup>+</sup>, protein phosphatases (mainly PP2C), guanylate cyclase/cyclic ADP ribose, hydrogen peroxide, and nitric oxide (NO) among others. The complexity of the interactions resembles that of a scale-free network (Hetherington and Woodward, 2003). Numerous loci have been identified to be regulated by ABA in guard cells either upstream or downstream of the

<sup>1</sup> This work was supported by grants from the Universidad Nacional de Mar del Plata, Consejo Nacional de Investigaciones Científicas y Técnicas, and Agencia Nacional de Promoción Científica y Tecnológica and by the European Regional Development Fund through the Ministerio de Economía y Competitividad (grant no. BIO2013-44648), the Junta de Andalucía (grant no. CVI-7190), and fellowship support through the program Junta para la Ampliación de Estudios (to C.Á.).

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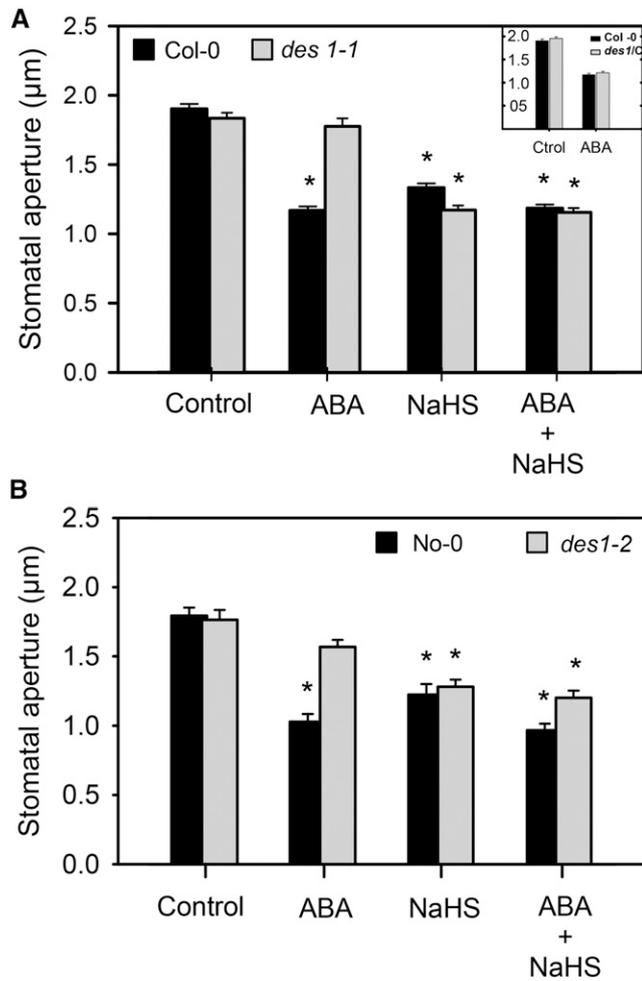
D.S., C.G., and C.G.-M. designed the experiments and analyzed the data; D.S. performed most of the experiments; C.Á. performed some of the qPCR experiments and analyzed the data; N.L. provided technical assistance; L.L. and C.G.-M. conceived the project; L.L. supervised and complemented the writing; C.G.-M. wrote the article.

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<sup>[W]</sup> The online version of this article contains Web-only data.

[www.plantphysiol.org/cgi/doi/10.1104/pp.114.245373](http://www.plantphysiol.org/cgi/doi/10.1104/pp.114.245373)



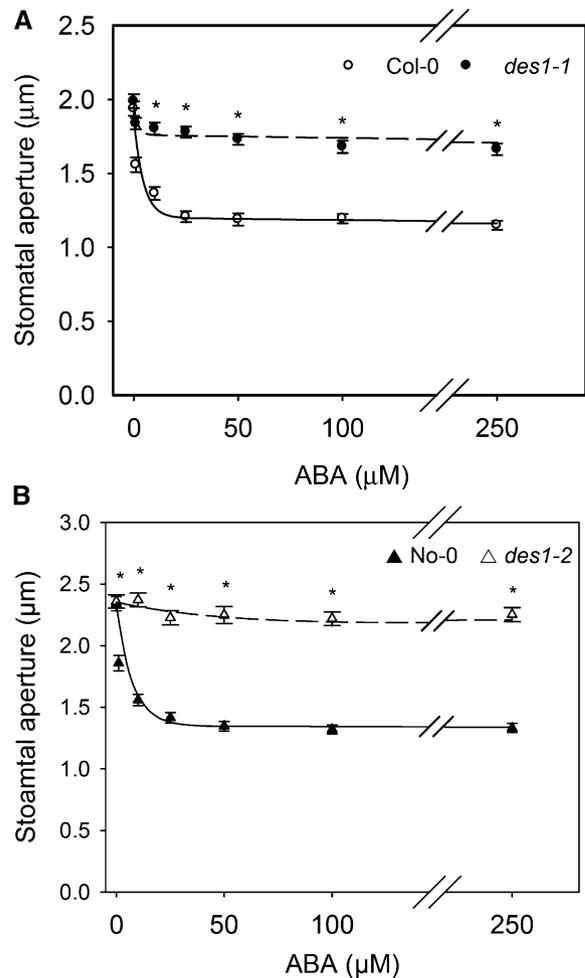
**Figure 1.** DES1 is involved in ABA-dependent stomatal closure. Epidermal strips, peeled from *Arabidopsis* mutants *des1-1* (A), *des1-2* (B), *des1-1* complemented with *DES1* full cDNA (*des1/C*, inset), and their genetic backgrounds, were preincubated for 3 h in opening buffer (10 mM K-MES, pH 6.1, and 10 mM KCl) under light. Strips were subsequently treated for 90 min under light with opening buffer (Control), 100  $\mu$ M of the H<sub>2</sub>S donor NaHS, 50  $\mu$ M ABA, or 100  $\mu$ M NaHS plus 50  $\mu$ M ABA. The values are expressed as the means  $\pm$  SE and represent the mean of 20 to 40 stomata per experiment from at least three independent experiments ( $n = 60$ –160). The asterisks denote significant differences with respect to the control treatments (Student's *t* test,  $P < 0.05$ ). Ctrol, Control (inset).

receptor complex (Leonhardt et al., 2004; Cutler et al., 2010); however, unique components are still emerging.

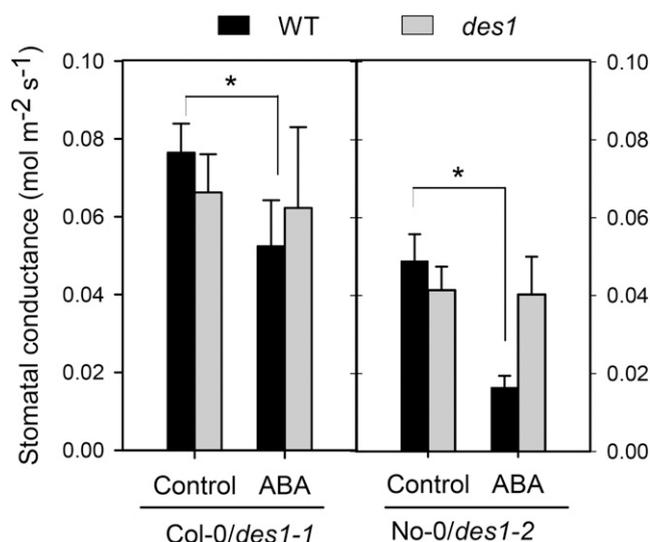
Hydrogen sulfide (H<sub>2</sub>S) is a small and reactive water-soluble gas. In aqueous solutions (pH 7.4), the ratio between the hydrosulfide anion (HS<sup>-</sup>) and H<sub>2</sub>S is 3 to 1 (Kabil and Banerjee, 2010). The active form of H<sub>2</sub>S in biological systems has not been specified yet; therefore, H<sub>2</sub>S usually stands for H<sub>2</sub>S/HS<sup>-</sup>. H<sub>2</sub>S can freely permeate lipid membranes, as its solubility is 5 times greater in lipophilic solvents than in water (Wang, 2002).

H<sub>2</sub>S has been proposed as the third gasotransmitter in animal cells, after NO and carbon monoxide, due to its high level of permeability through biological membranes,

its effect at low concentrations, and toxicity at high doses (Mancardi et al., 2009). H<sub>2</sub>S has been implicated in different physiological processes such as blood vessel relaxation, neurotransmission (Li et al., 2006), insulin signaling (Yang et al., 2005), angiogenesis (Coletta et al., 2012), and inflammation (Szabó, 2007), among others. In mammals, most of the endogenous H<sub>2</sub>S is produced through the activity of two pyridoxal-5'-P-dependent enzymes cystathionine  $\beta$ -synthase (EC 4.2.1.22) and cystathionine  $\gamma$ -lyase (EC 4.4.1.1). It has long been known that plants have L-Cys desulfhydrase (DES) activity (Harrington and Smith, 1980; Papenbrock et al., 2007). However, no bona fide *DES* gene was reported until 2010, when the *Cys synthase-like* gene (*At5G28030*), a member of the *O*-acetyl-Ser(thiol)lyase



**Figure 2.** Stomata from *Arabidopsis des1* mutants show reduced sensitivity to ABA. Epidermal strips peeled from *des1-1* (A) or *des1-2* (B) plants and their genetic backgrounds were preincubated for 3 h in opening buffer (10 mM K-MES, pH 6.1, and 10 mM KCl) under light and subsequently treated with increasing concentrations of ABA (0, 1, 10, 25, 50, 100, and 250  $\mu$ M) for 90 min under light. The curves were fitted to an exponential linear combination function. The values are expressed as the means  $\pm$  SE and represent the mean of 20 to 40 stomata per experiment from at least three independent experiments ( $n = 60$ –160). Asterisks denote significant differences with respect to the wild-type plants (Dunn's post hoc test,  $P < 0.05$ ).



**Figure 3.** Stomatal conductance is less responsive to ABA in Arabidopsis *des1* mutants. The effect of ABA on the stomatal conductance was measured in planta in leaves of both *des1* mutant plants and in their respective genetic backgrounds. The plants were sprayed with water (Control) or 50  $\mu$ M ABA, and the stomatal conductance was measured 3 h after treatment from a fully expanded leaf in a closed chamber under a constant CO<sub>2</sub> concentration for 5 min using an IRGA. The values are expressed as the means  $\pm$  SD of at least three independent experiments. The asterisks indicate significant differences with respect to each control (Student's *t* test,  $P < 0.05$ ). WT, Wild type.

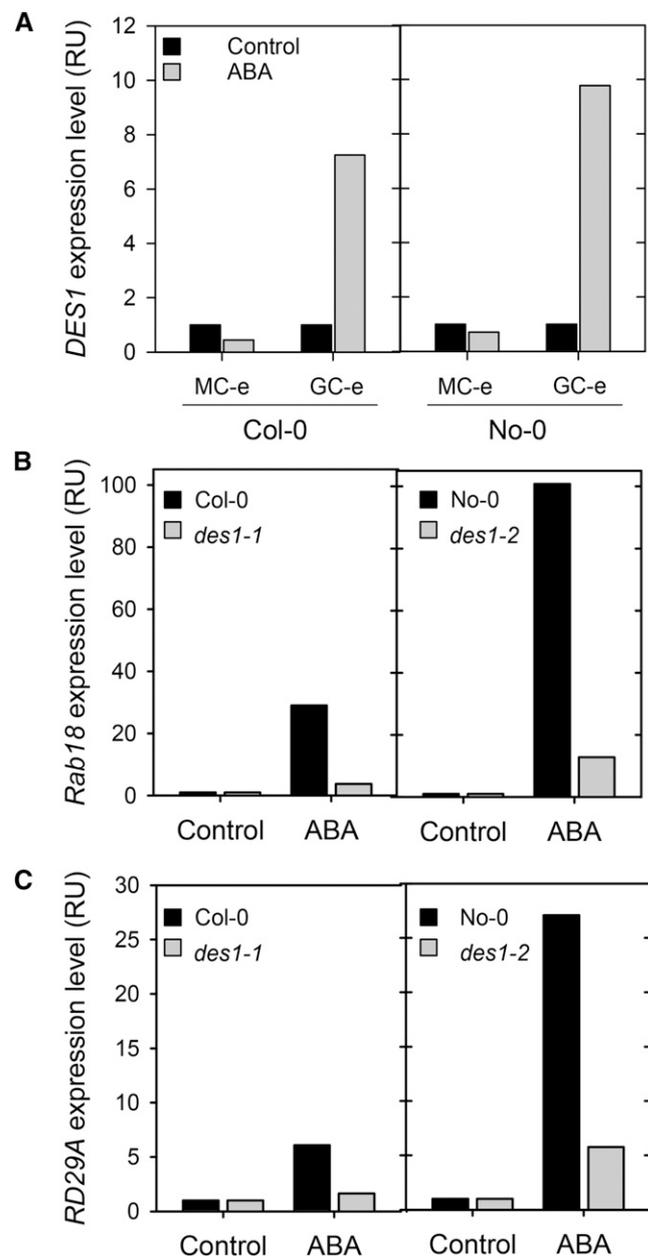
family was identified as a true *DES* and named *DES1* (Alvarez et al., 2010). *DES1* releases H<sub>2</sub>S, pyruvate, and ammonia during L-Cys degradation (Alvarez et al., 2010). Early studies concerning H<sub>2</sub>S emission in plants were associated with the plant response to pathogens as part of a so-called Sulfur Induced Resistance (Bloem et al., 2004). Using a H<sub>2</sub>S-releasing compound, H<sub>2</sub>S was later reported to confer a protective effect against oxidative (Zhang et al., 2009b, 2010b) and cadmium (Sun et al., 2013) stresses, alleviate aluminum toxicity (Zhang et al., 2010c), increase antioxidant activity, and participate in root organogenesis (Zhang et al., 2009a). Several independent groups have recently reported the participation of H<sub>2</sub>S in ABA- and ethylene-dependent stomatal closure induction (García-Mata and Lamattina, 2010; Liu et al., 2011, 2012; Jin et al., 2013). In this study, we used the characterized *DES1* knockout Arabidopsis (*Arabidopsis thaliana*) mutant plants *des1-1* and *des1-2* to obtain new insights on the cross talk between H<sub>2</sub>S and NO and further evidence supporting the involvement and requirements of H<sub>2</sub>S in ABA-induced signaling cascade leading to stomatal closure.

## RESULTS

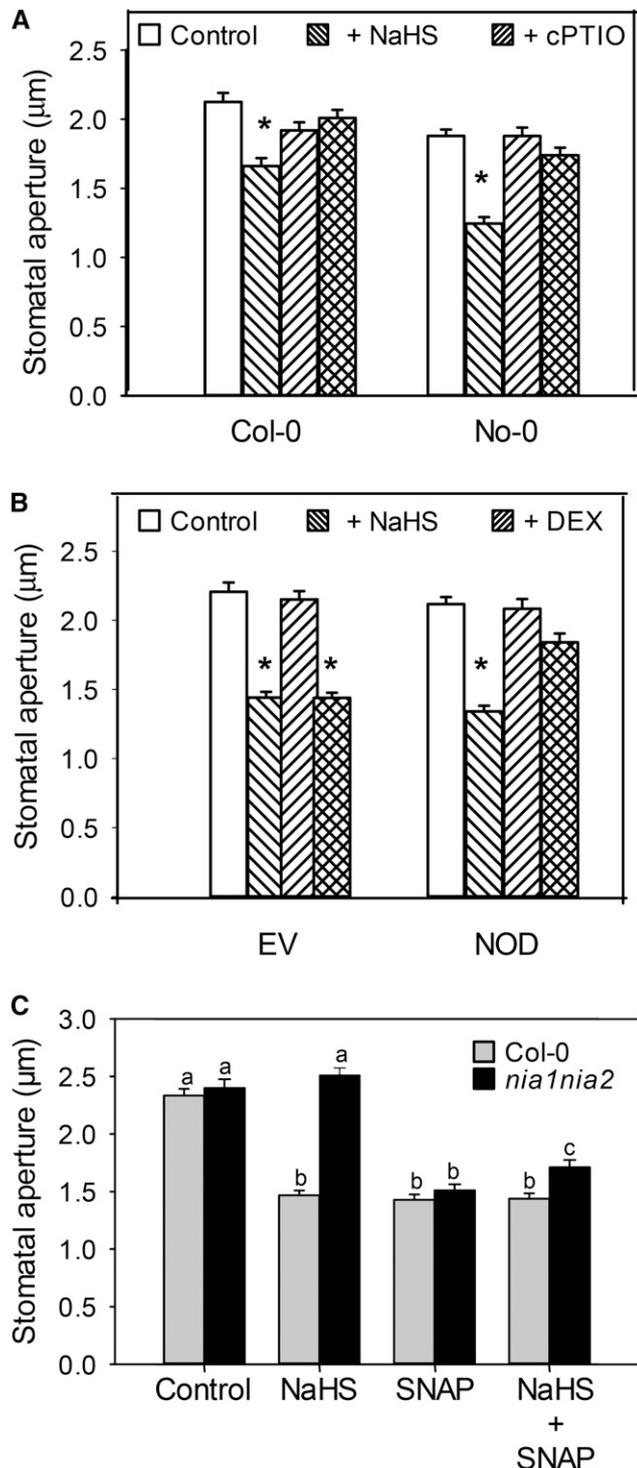
### DES1 Is Required for ABA-Dependent Stomatal Closure

In a previous work, we presented pharmacological evidence showing that H<sub>2</sub>S might be part of the signaling network leading to ABA-dependent stomatal

closure in different plant species (García-Mata and Lamattina, 2010). This was recently confirmed for Arabidopsis by Jin et al. (2013) using a transfer DNA insertion mutant of the gene AT3G62130 that codes for an L-Cys desulfhydrase. In this work, by contrast,



**Figure 4.** Analysis of the expression levels of *DES1* and ABA-responsive genes in GC-e and MC-e RNA extracts of wild-type and *des1* mutant plants. Epidermal strips and mesophyll tissue prepared from *des1* plants and their respective genetic background were preincubated for 3 h in opening buffer (10 mM K-MES, pH 6.1, and 10 mM KCl) and then treated with or without 50  $\mu$ M ABA under light. After 90 min of treatment, qRT-PCR analysis of *DES1* gene expression was performed in GC-e RNA extracted from Col-0 and No-0 (A). qRT-PCR analysis of ABA-responsive genes *RD29A* (B) and *RAB18* (C) was performed for GC-e RNA extracted from the wild type and *des1* mutants. The values are expressed as percentages with respect to the control treatments. RU, Relative unit.

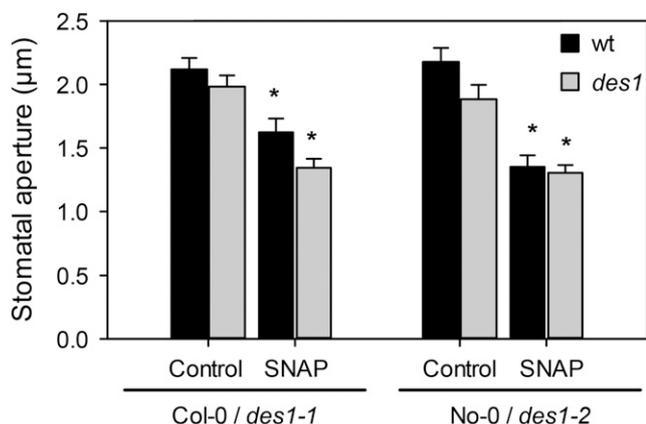


**Figure 5.** NO is required for the H<sub>2</sub>S-induced stomatal closure. A, Epidermal strips peeled from Arabidopsis leaves were preincubated for 3 h in opening buffer (10 mM K-MES, pH 6.1, and 10 mM KCl) under light. Subsequently, strips were treated for 90 min under light with 100 μM NaHS in the absence or presence (+cPTIO) of 200 μM of the NO scavenger cPTIO. B, Arabidopsis Col-0 plants transformed with empty vector (EV) or NOD were sprayed with water (mock) or 0.01% (v/v) DEX. After 48 h of the DEX induction, epidermal strips were peeled, preincubated for 3 h in opening buffer under light, and then treated for

we used two null mutants deficient in the DES1 protein to demonstrate the participation of DES1 in ABA signaling in stomata. Previously, the recombinant DES1 protein was expressed in bacteria and was enzymatically characterized as L-Cys desulfhydrase (Alvarez et al., 2010). With that aim, epidermal strips from Arabidopsis *DES1* null mutant plants (*des1-1* and *des1-2*) and their genetic backgrounds (ecotypes Columbia [Col-0] and Nössen [No-0], respectively) were prepared and treated with or without 50 μM ABA for 90 min. As expected, epidermal strips from the wild-type plants closed the stomata in response to exogenous ABA application (Fig. 1); however, ABA-dependent stomatal closure was strongly inhibited in both *des1-1* (Fig. 1A) and *des1-2* (Fig. 1B) epidermal strips, indicating that *DES1* is required for ABA-dependent stomatal closure. The lack of response of *des1-1* to ABA was restored in epidermal strips of *des1-1* knockout mutant complemented with the full-length *DES1* complementary DNA (cDNA; Fig. 1, inset). Moreover, the addition of exogenous H<sub>2</sub>S as 100 μM of the H<sub>2</sub>S donor sodium hydrosulfide (NaHS) together with ABA treatment also restored the stomatal response to ABA in both *des1-1* and *des1-2* mutants, suggesting that the lack of response was due to reduced levels of endogenous H<sub>2</sub>S (Fig. 1, A and B). Interestingly, an ABA dose-response experiment showed that *des1* mutant plants remain insensitive, even when the epidermal strips were treated with 250 μM ABA, suggesting that this effect was not dependent on ABA concentration (Fig. 2). Consistent with our previous report (García-Mata and Lamattina, 2010), epidermal strips from both genetic backgrounds No-0 and Col-0 responded to the H<sub>2</sub>S donor in a dose-dependent manner, showing maximal stomatal closure induction at 100 μM of the donor NaHS (Supplemental Fig. S1A). Interestingly, high doses of the H<sub>2</sub>S donor (500 μM) did not induce stomatal closure, probably due to rather toxic effects (Supplemental Fig. S1A). The fact that morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate (GYY 4137, another H<sub>2</sub>S donor) induced stomatal closure in both wild-type plants and *des1* mutants (Supplemental Fig. S1B) and that the H<sub>2</sub>S scavenger hypotaurine (HT) blocked the effect of the donor (Supplemental Fig. S1C) confirms that the response was due to the released H<sub>2</sub>S and not by any by-product of the donor molecule.

To determine if the *des1* mutants also show reduced sensitivity to ABA at the whole-plant level, Arabidopsis *des1* mutant plants and their respective genetic background

90 min with opening buffer (Control) or 100 μM NaHS. C, Epidermal strips peeled from Arabidopsis double mutant *nia1nia2* leaves were preincubated for 3 h in opening buffer under light and then treated for 90 min with 100 μM NaHS, 100 μM of NO donor SNAP, or 100 μM NaHS plus 100 μM SNAP. The values are expressed as the means ± SE and represent the mean of 20 to 40 stomata per experiment from at least three independent experiments ( $n = 60-160$ ). The asterisks indicate significant differences with respect to each control, and different letters indicate significant differences between treatments (Dunn's post hoc test,  $P < 0.05$ ).



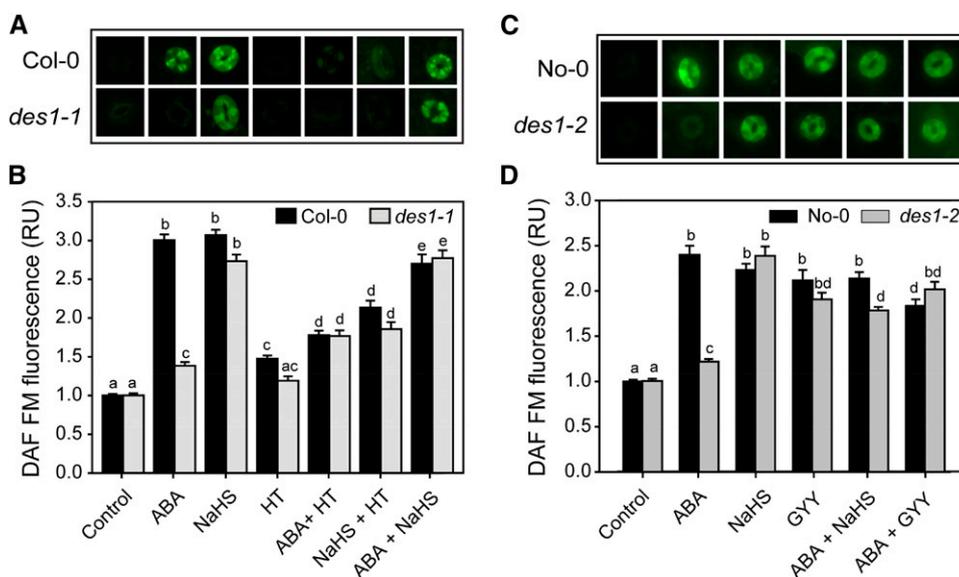
**Figure 6.** NO induces stomatal closure in Arabidopsis *des1* mutants. Epidermal strips peeled from Arabidopsis mutants *des1* and their genetic backgrounds were preincubated for 3 h in opening buffer (10 mM K-MES, pH 6.1, and 10 mM KCl) under light and subsequently treated for 90 min under light with 100  $\mu$ M SNAP. The values of stomatal aperture are expressed as the means  $\pm$  SE and represent the mean of 20 to 40 stomata per experiment from at least three independent experiments ( $n = 60$ –160). Asterisks denote statistical differences with respect to the control treatment of each genetic background (Dunn's post hoc test,  $P < 0.05$ ). wt, Wild type.

were sprayed with water or 50  $\mu$ M ABA for 3 h, and then stomatal conductance measurements were performed using an infrared gas analyzer (IRGA). Figure 3

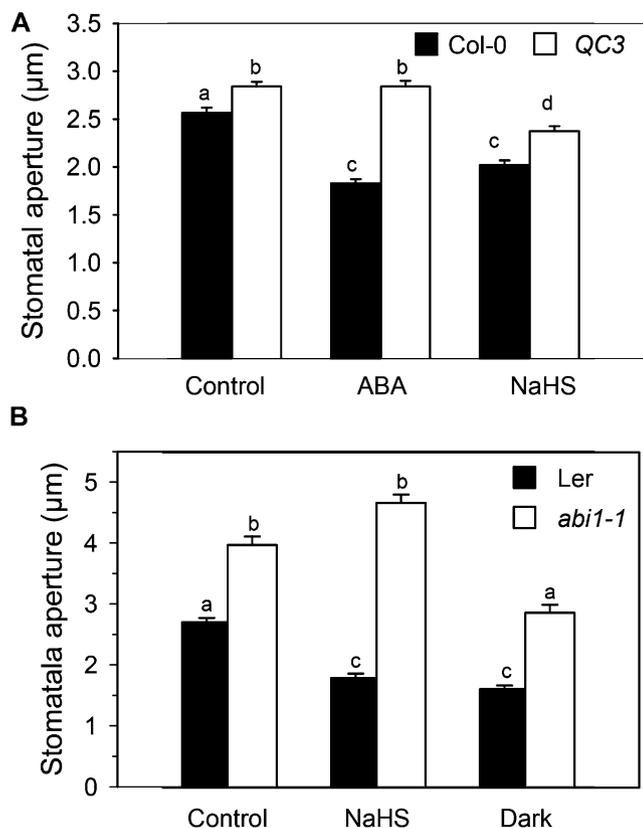
shows that ABA treatment induced a significant reduction of stomatal conductance in wild-type plants, while *des1* mutants were less sensitive to ABA treatment. This result indicates that the reduced response to ABA observed in stomata from *des1* mutants correlates with the response at the whole-plant level.

#### ABA Increases *DES1* Expression in Arabidopsis

Both pharmacological and genetic data indicate that *DES1* is involved in ABA-induced stomatal closure. To obtain further evidence for ABA-*DES1* cross talk, we analyzed the expression levels of the *DES1* gene in epidermal strips from Arabidopsis wild-type plants. The RNA extracted from the epidermal strips was considered as guard cell-enriched (GC-e) RNA because 90% to 95% of the living cells in epidermal strips are guard cells. We also extracted RNA from the rest of the leaf (without the abaxial epidermis layer), and we considered this extract as mesophyll cell-enriched (MC-e) RNA. Marker genes were used in reverse transcription PCR (RT-PCR) assays to confirm that we have both GC-e and MC-e extracts. *ECERIFERUM2* (*CER2*; Leonhardt et al., 1999; Wang et al., 2011) was used as a marker gene for guard cells, and  $\beta$ -Carbonic anhydrase1 (*Canh1*; Pandey et al., 2002) as a marker gene for the mesophyll cells (Supplemental Fig. S2). To assess if *DES1* is regulated by



**Figure 7.** H<sub>2</sub>S is required for the ABA-induced NO production in Arabidopsis guard cells. Epidermal peels from Arabidopsis *des1* mutant and their genetic backgrounds were preincubated for 3 h in opening buffer (10 mM K-MES, pH 6.1, and 10 mM KCl) under light and subsequently loaded with 10  $\mu$ M of the NO-specific fluorescent dye DAF-FM-DA. After washing, the strips were treated for 15 min under light with opening buffer (Control) or with the following treatments: 50  $\mu$ M ABA, 100  $\mu$ M NaHS, 200  $\mu$ M of the H<sub>2</sub>S scavenger HT, ABA plus HT, NaHS plus HT, or ABA plus NaHS for Col-0 and *des1-1* plants (A and B) and 50  $\mu$ M ABA, 100  $\mu$ M NaHS, 100  $\mu$ M of the H<sub>2</sub>S donor GYY 4137, ABA plus NaHS, or ABA plus GYY for No-0 and *des1-2* plants (C and D). Images depict one representative picture of stomata from epidermal peels corresponding to three independent experiments. The green fluorescence pixel intensity is expressed as relative unit (RU) with respect to the control treatment. The values are expressed as means  $\pm$  SE and represent the mean of 40 to 50 stomata per experiment from three independent experiments ( $n = 120$ –150). Different letters denote significant differences (Dunn's post hoc test,  $P < 0.05$ ). [See online article for color version of this figure.]



**Figure 8.** Position of H<sub>2</sub>S in a PYR/PYR/RCAR cascade. Epidermal strips, peeled from the Arabidopsis mutants in ABA receptor *pyr1/pyr11/pyl2/pyl4* (QC3; A), the PP2C mutant *abi1-1* (B), and their genetic backgrounds, were preincubated for 3 h in opening buffer (10 mM K-MES, pH 6.1, and 10 mM KCl) under light. Strips were subsequently treated for 90 min under light with opening buffer (Control), 100 µM of the H<sub>2</sub>S donor NaHS (A and B), or 50 µM ABA (A) or shifted to the dark for 90 min (B). The values are expressed as the means ± SE and represent the mean of 20 to 40 stomata per experiment from at least three independent experiments ( $n = 60\text{--}160$ ). Different letters indicate significant differences among treatments (Dunn's post hoc test,  $P < 0.05$ ). Ler, Landsberg erecta.

ABA at the transcriptional level, we treated the epidermal strips and mesophyll tissue from wild-type plants with stomatal opening buffer or 50 µM ABA for 90 min. Subsequently, GC-e and MC-e RNA were extracted from the samples, and *DES1* gene expression was assessed using quantitative reverse transcription (qRT)-PCR analysis. The results shown in Figure 4A reveal only marginal variations in *DES1* expression levels in MC-e; however, GC-e showed a dramatic increase of *DES1* transcript levels upon ABA treatment in both ecotypes (7- and 9-fold as compared with the control treatment for Col-0 and No-0, respectively), indicating that *DES1* was significantly regulated by ABA in the guard cells. Furthermore, we performed a qRT-PCR analysis of the expression levels of the ABA-responsive genes *RESPONSIVE TO DEHYDRATION A* (*RD29A*) and *RESPONSIVE TO ABA18* (*RAB18*) in GC-e RNA extracts from wild-type and *des1* plants. The results shown in Figure 4, B and C,

reveal that both *des1* mutant lines have a significant reduction of the expression of both *RD29A* and *RAB18* genes in response to ABA treatments with respect to the wild types. Taken together, these results suggest that *DES1* is regulated by ABA at the transcriptional level in Arabidopsis guard cells and that an active *DES1* is required to attain a full expression of ABA-responsive genes.

### H<sub>2</sub>S Participates in ABA-Dependent NO Production during Stomatal Closure Induction

It has been demonstrated that, in animal systems, H<sub>2</sub>S and NO might interact either in an agonistic or antagonistic way, depending on the biological system and physiological process (Li et al., 2009; Kajimura et al., 2010; Yong et al., 2010). In plants, it has been recently reported that H<sub>2</sub>S acts downstream of NO in ethylene-induced stomatal closure (Liu et al., 2011, 2012). It was also reported that H<sub>2</sub>S reduces ABA-dependent NO production in Arabidopsis and *Capsicum annuum* guard cells (Lisjak et al., 2010, 2011). To add some knowledge to the NO-H<sub>2</sub>S cross talk in Arabidopsis guard cells, we first performed a pharmacological assay to assess whether NO is involved in H<sub>2</sub>S-dependent stomatal closure. Epidermal strips from Arabidopsis wild-type leaves were treated with or without the NO-specific scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). In Figure 5A, it is shown that cPTIO impaired H<sub>2</sub>S-dependent induction of stomatal closure in both wild-type ecotypes. This result was supported by genetic assays using epidermal strips from Col-0 plants expressing the NO dioxygenase (NOD; Zeier et al., 2004), which were proven to have reduced levels of NO after inducing NOD expression with dexamethasone (DEX; Zeier et al., 2004; Tossi et al., 2011). Exogenous application of H<sub>2</sub>S induced stomatal closure in water-sprayed NOD plants, but no stomatal closure was observed in DEX-sprayed NOD plants, confirming the NO requirement for H<sub>2</sub>S-dependent stomatal closure (Fig. 5B). To further confirm the interaction of H<sub>2</sub>S and NO, we used *NITRATE REDUCTASE1 / NITRATE REDUCTASE2* double mutant (*nia1/nia2*) plants, which produced very low levels of NO (Desikan et al., 2002; Lozano-Juste and León, 2010). As shown in Figure 5C, NaHS was not able to induce stomatal closure in the *nia1/nia2* mutant, an effect that was restored by exogenous addition of the NO-specific donor *S*-nitroso-*N*-acetylpenicillamine (SNAP). All together, the pharmacological and genetic evidence indicate that the depletion of endogenous NO blocks H<sub>2</sub>S-mediated induction of stomatal closure, demonstrating the interaction of these two gasotransmitters in stomatal closure processes.

The lack of response to H<sub>2</sub>S in NO-depleted epidermal strips prompted us to investigate whether NO was able to induce stomatal closure in the *des1* mutants. With that aim, stomatal aperture experiments were performed in epidermal strips from the wild type and both *des1* mutants in presence or absence of 100 µM of the

NO donor SNAP. Exogenous addition of NO induced stomatal closure to the same extent in epidermal strips from both wild-type and *des1* mutant plants (Fig. 6), confirming again that NO is downstream of DES1 in the ABA signaling pathway, leading to stomatal closure.

Several reports have shown that ABA increases endogenous NO production in guard cells (García-Mata and Lamattina, 2002; Neill et al., 2002). The results presented in this work indicate that H<sub>2</sub>S acts upstream of NO in ABA-dependent stomatal closure; therefore, we used the fluorescent dye 4,5-diaminofluorescein diacetate (DAF-FM-DA) to test whether H<sub>2</sub>S has any effect on endogenous NO production. Figure 7 shows that guard cells from wild-type epidermal strips loaded with DAF-FM-DA display significant increases in endogenous NO levels when treated with H<sub>2</sub>S donors NaHS or GYY (Fig. 7, A and C). However, this increase was not evident either in *des1* mutants or in Col-0 plants treated with ABA together with the H<sub>2</sub>S scavenger HT (Fig. 7, A and B), indicating that DES1 is required for ABA-induced NO formation. Moreover, the impairment of *des1-1* and *des1-2* mutants to produce NO in response to ABA was rescued by the addition of exogenous H<sub>2</sub>S using either NaHS or GYY, supporting that DES1 acts upstream of NO in ABA-dependent stomatal closure (Fig. 7).

### H<sub>2</sub>S Acts Upstream of ABI1 in ABA-Dependent Stomatal Closure

The mutants of the recently identified ABA receptor *PYRABACTIN RESISTANT/PYRABACTIN-LIKE/REGULATORY COMPONENTS OF ABA RECEPTOR (PYR/PYL/RCAR)* show a strong insensitivity to ABA and failure to close stomata (Nishimura et al., 2010) but do not show insensitivity to downstream signaling components such as extracellular Ca<sup>2+</sup> (Nishimura et al., 2010; Wang et al., 2013) or hydrogen peroxide (Wang et al., 2013). Therefore, we assayed H<sub>2</sub>S-dependent stomatal closure in epidermal strips of the quadruple mutant of the ABA receptor *pyr1/pyl1/pyl2/pyl4* (hereafter, called QC3). Figure 8A shows that H<sub>2</sub>S was able to close the stomata both in the wild type and, to a lesser extent, in the QC3 quadruple mutant epidermal strips. These data suggest that H<sub>2</sub>S is acting downstream or independently of the ABA receptor. It is noteworthy that exogenous application of the H<sub>2</sub>S donor NaHS did not induce stomatal closure in epidermal strips of the ABA-insensitive *abi1-1* mutant (Fig. 8B), but it does induce stomatal closure in its genetic background Landsberg *erecta*, indicating that a functional ABI1 is required for DES1/H<sub>2</sub>S action in ABA-dependent guard cell signaling.

## DISCUSSION

The gasotransmitter H<sub>2</sub>S has rapidly emerged as a hot topic in animal physiology, where it is known to have an active role in cardioprotection, neurotransmission, and O<sub>2</sub> sensing, among others (Kabil and Banerjee, 2010;

Peng et al., 2010). In plants, although originally associated with plant-pathogen interaction, it is now known to have an active role in diverse physiological processes such as oxidative stress, germination, and heat tolerance (Zhang et al., 2009a, 2009b, 2010a, 2010b, 2010d; Li et al., 2013a, 2013b). Recently, H<sub>2</sub>S has been proven to participate in ABA- or ethylene-induced stomatal closure in different plant species (García-Mata and Lamattina, 2010; Liu et al., 2011, 2012; Jin et al., 2013). Moreover, it has been reported also that H<sub>2</sub>S induces stomatal opening (Lisjak et al., 2010, 2011).

In this work, we present new insights into the regulation of the H<sub>2</sub>S-generating enzyme DES1 and *DES1* gene expression by ABA, providing evidence supporting the participation of DES1 in ABA-induced stomatal closure. It is known that DES1 plays a role in leaf senescence and modulates the progression of autophagy (Alvarez et al., 2010; Álvarez et al., 2012); however, no data are available concerning the role of hormones in the regulation of *DES1*. In a previous report, it was shown that the pharmacological inhibition of DES results in a partial blockage of ABA-dependent stomatal closure (García-Mata and Lamattina, 2010). In this work, using a genetic approach, we show that two independent knockout mutants of the Arabidopsis *DES1*, *des1-1* and *des1-2*, failed to close the stomata in response to increasing concentrations of ABA. The lack in the sensitivity to ABA of *des1* mutants was restored through either the exogenous addition of H<sub>2</sub>S donors or by the complementation of the *des1-1* mutant with the full-length *DES1* cDNA, indicating that DES1 participates in ABA-dependent stomatal closure.

We demonstrate that, at the time scale used for stomatal aperture experiments (90 min of treatment), *DES1* is up-regulated by ABA in guard cells but not in mesophyll cells, supporting the participation of DES1 in the signaling events leading to stomatal closure. Guard cell-specific gene expression has been already reported by microarray analysis using guard cell protoplasts (GCPs) versus mesophyll cell protoplasts (MCPs; Leonhardt et al., 2004) and epidermal strips versus leaf tissue (Wang et al., 2011). In both works, the authors have identified three groups of genes: (1) those equally regulated in GCP and MCP, (2) those with altered expression in MCPs and not in GCPs, and (3) those with modified expression in GCPs and not in MCPs. We have found that *DES1* expression is selectively activated in guard cells by ABA. Transcriptional cell specificity regulation in response to ABA may reflect a differential distribution of ABA in the different cell types or to a differential utilization of ABA-specific promoters or transcription factors in each cell type (Leonhardt et al., 2004). Further measurements of enzyme activity in guard cells will be needed to unequivocally correlate the up-regulation of *DES1* in GC-e extracts with an increase in DES-dependent H<sub>2</sub>S production in guard cells; however, we could not obtain enough mass of protein from the isolated epidermal strips to assay the DES1 activity. Nevertheless, we did analyze the effect of ABA treatments on DES activity

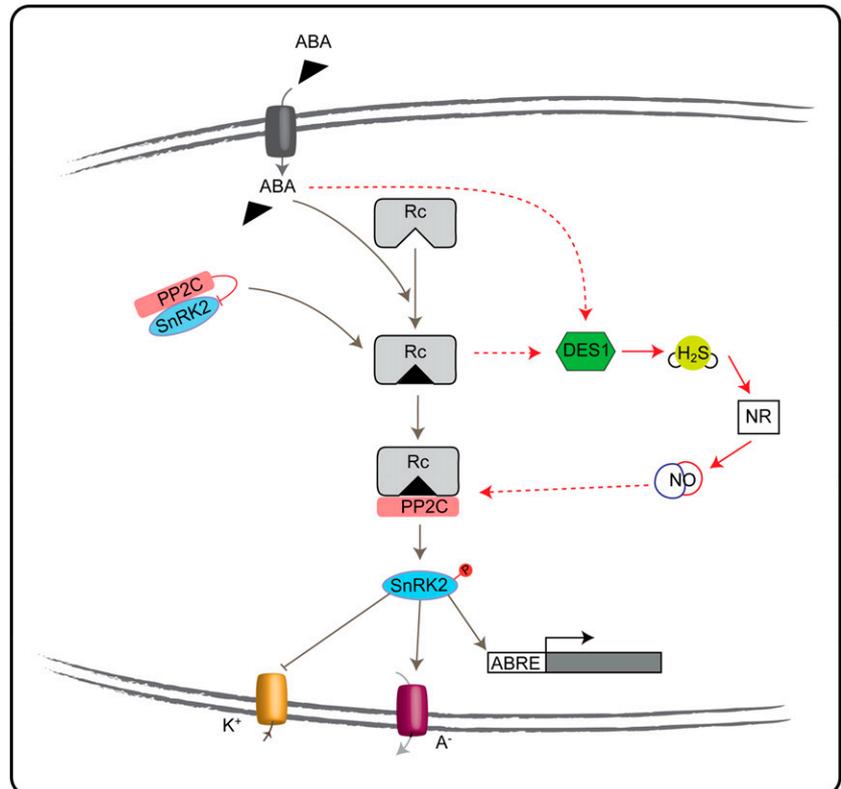
levels in whole-leaf extracts from wild-type *Arabidopsis* plants, and we observed a positive correlation between *DES1* expression and DES activity but at over longer periods of treatments (Supplemental Fig. S3, A and B). Although further work is needed to unveil the mechanism by which ABA activates *DES1* transcription, the presence of a Dc3 Promoter-Binding Factor3 (DPBF3) and DPBF2 and a Dehydration-Responsive Element-Like in the promoter region of *DES1* let us speculate on the possibility that ABA is directly regulating the expression of the gene (Supplemental Fig. S4).

Lately, the interest on the biology of gasotransmitters has been oriented toward the interactions between these three gases on the different biological systems (García-Mata and Lamattina, 2013; Kolluru et al., 2013). In animal systems, there are many reports on the interaction of  $H_2S$  and NO, although the nature of this interaction is still poorly understood. Some reports have shown a positive interaction between  $H_2S$  and NO, where  $H_2S$  increases the rates of NO release from small nitrosothiols such as S-nitrosoglutathione (Ondrias et al., 2008) or even forms new nitrosothiol species with NO that might act as biological regulators of the availability of both gasotransmitters (Whiteman et al., 2006; Filipovic et al., 2013). Contrasting evidence, however, has come from observations that: (1) *in vitro*,  $H_2S$  inhibits all the three isoforms of mammalian NO synthases (Kubo et al., 2007) and (2)  $H_2S$  reacts with NO to form a new compound that exhibits contrasting effects in the heart (Yong et al., 2010). In addition, it was recently

reported that both gasotransmitters are mutually required for regulation of vascular function (Coletta et al., 2012).

The data presented here indicate that there is a cross talk between  $H_2S$  and NO during ABA-dependent stomatal closure. The depletion of NO, by chemical or genetic means, blocked the  $H_2S$ -dependent stomatal closure, suggesting that NO is acting downstream of  $H_2S$  in this particular physiological response. Accordingly, none of the *des1* mutants produce NO in response to ABA treatment, although they do close the stomata in response to exogenous application of NO donors. This result is in agreement with a recently annotated microarray data where both *NIA1* and *NIA2* isoforms of the nitrate reductase are up-regulated upon  $H_2S$  treatment (data accessible at National Center for Biotechnology Information Gene Expression Omnibus database, accession no. GSE32566), which supports the lack of response of the *nia1/nia2* double mutant to the exogenous addition of  $H_2S$  observed in this study. As for animal systems, in plants, there is controversial evidence about  $H_2S$ -NO interaction. On one hand, it was shown that  $H_2S$  acts upstream of NO in root organogenesis and heavy metal toxicity (Zhang et al., 2009a; Li et al., 2012). On the other hand,  $H_2S$  was reported to act downstream of NO in the ethylene-induced stomatal closure and in the resistance to heat stress (Liu et al., 2012; Li et al., 2013b). Interestingly, Jin et al. (2011) have recently reported that, in *Arabidopsis* whole-plant extracts, the production rate of  $H_2S$  increased upon drought stress treatment.

**Figure 9.** Simplified model showing the interaction of *DES1*/ $H_2S$  with the core ABA signaling pathway in guard cells. ABA enters the guard cells through ATP-binding cassette transporters. Once in the guard cells, it binds to the PYR/PYL/RCAR receptor (Rc). The ABA PYL/PYL/RCAR receptor binds the clade A PP2C inhibiting its phosphatase activity and, through it, releases the SnRKs protein kinases and the signaling elements downward. *DES1* is up-regulated by ABA, releasing  $H_2S$ , which increases endogenous NO via nitrate reductase (NR) activity. NO might regulate the binding of ABI1 to the PYR/PYL/RCAR receptor and through the downstream response. Arrow lines indicate activation, blunt lines indicate inhibition, dashed red lines indicate cross talk proposed in this work (hypothetical), gray lines indicate already published.  $K^+$ , Potassium uptake;  $A^-$ , anion efflux; ABRE, ABA-responsive element transcription factors.



Lisjak et al. (2010, 2011) have proposed a negative interaction between H<sub>2</sub>S and NO in ABA-induced stomatal closure in *Arabidopsis* and *C. annuum*. Based in pharmacological evidence, the authors speculate that the NO produced by ABA is removed by H<sub>2</sub>S, and thus, the depletion of endogenous NO increases stomatal aperture in the dark (Lisjak et al., 2010). Differences in methodological procedures concerning the isolation of epidermal strips and the timing of the treatments may result in the differential response of the guard cells to the H<sub>2</sub>S donors between Lisjak et al. (2010) and this study.

Liu et al. (2011) showed that stomatal closure but not NO synthesis is impaired in an *Arabidopsis* mutant of the L-Cys desulfhydrase *AtL-CDES* gene. Therefore, they suggest that NO is acting upstream of H<sub>2</sub>S in ethylene-induced stomatal closure. However, some considerations must be taken into account. First, the locus mutated in *Atl-cdes* codes for a mitochondrial/chloroplastic Cys desulfurase (At5g65720), which is a NITROGEN FIXATION S (NIFS)-LIKE1 protein involved in the biosynthesis of different cofactors such as the iron-sulfur clusters (Van Hoewyk et al., 2008). This NifS-like protein catalyzes the conversion of Cys to Ala and elemental sulfur instead of the conversion to pyruvate, ammonium, and sulfide, as has been unequivocally established for *DES1* (At5g28030; Alvarez et al., 2010). Second, despite it having been reported that a convergence exists between ABA-dependent and ethylene-dependent signaling pathways (Ribeiro et al., 2009), the interaction of these two players is still poorly understood. Therefore, the data presented by Liu et al (2011) is not enough to rule out an ABA-induced, H<sub>2</sub>S-independent NO production.

The characterization of the central ABA signaling pathway in stomata, which involves the participation of the PYR/PYL/RCAR receptors, Clade I PP2C (including ABI1, ABI2, and HOMOLOGUETOABI1), and ABA-activated SNF1-RELATED KINASE2 (SnRK2) (Fujii et al., 2009; Ma et al., 2009; Park et al., 2009; Santiago et al., 2009; Umezawa et al., 2009), has become a milestone in the study of ABA signaling. A challenge for identifying the new components that are emerging in guard cell ABA signaling, such as *DES1*, is to understand how these new pathways are associated with the central ABA signaling pathway. The quadruple mutant of the ABA receptor QC3 generated by Nishimura et al. (2010) was showed to be highly insensitive to ABA; however, it still will close the stomata in response to downstream elements (Nishimura et al., 2010; Wang et al., 2013). Accordingly, guard cells from QC3 plants do not produce endogenous reactive oxygen species or NO in response to ABA (Yin et al., 2013). Likewise, exogenous H<sub>2</sub>S is able to induce stomatal closure in the QC3 mutant, suggesting that it acts downstream of the ABA receptor, although a PYL/PYR/RCAR-independent pathway cannot be ruled out with this data. Our data show that exogenous H<sub>2</sub>S does not induce stomatal closure in the gain-of-function mutants of ABI1 that is a negative regulator of ABA-induced stomatal closure (Merlot et al., 2001; Saez et al., 2004; Kuhn et al., 2006; Rubio et al., 2009), suggesting that H<sub>2</sub>S is acting

upstream of this ABA signaling element. Interestingly, previous reports show that (1) NO is produced in guard cells from *Arabidopsis* wild-type plants in response to ABA (García-Mata and Lamattina, 2002; Lozano-Juste and León, 2010) but not in the QC3 quadruple mutant (Yin et al., 2013), and (2) exogenous addition of NO induces stomatal closure in wild-type *Arabidopsis* plants but not in *abi1-1* and *abi1-2* (Desikan et al., 2002; Dubovskaya et al., 2011), suggesting that NO is acting downstream of the PYR/PYL/RCAR receptor and upstream of ABI1. Considering the evidence presented here indicating that *DES1*/H<sub>2</sub>S is acting upstream of NO, a simplified model is shown in Figure 9 to propose the position of *DES1*/H<sub>2</sub>S in the ABA signaling network. The nature of the interaction between NO and ABI1 is yet to be clarified. It can be speculated that NO might be regulating the binding of ABI1 to PYR/PYL/RCAR through the modification of either ABI1 protein or the ABI1 binding domain at the receptor. Another possibility is the existence of a signaling element positioned between NO and ABI1. A candidate for this signaling component might be the phospholipid signal phosphatidic acid, whose production is increased by NO in guard cells (Distéfano et al., 2008) and who was reported to regulate ABI1/PP2C activity preventing ABI1 translocation from the cytosol to the nucleus (Zhang et al., 2004).

Recent evidence demonstrates the H<sub>2</sub>S can regulate protein activity through the direct interaction of H<sub>2</sub>S with the thiol group of the target protein yielding a hydroper-sulfide moiety (Mustafa et al., 2009). Further studies will be needed to see if this is the mechanism by which H<sub>2</sub>S regulates ABA-signaling components. Overall, this study presents compelling evidence supporting *DES1* as a unique component of ABA signaling in guard cells, mediating H<sub>2</sub>S production and acting upstream of NO to induce stomatal closure.

## MATERIALS AND METHODS

### Plant Material

*Arabidopsis* (*Arabidopsis thaliana*) wild-type Col-0 and No-0, *des1-1* (SALK\_103855) and *des1-2* (RIKEN RATM13-27151\_G; Alvarez et al., 2010; Álvarez et al., 2012; Supplemental Fig. S5), *abi1-1* (Arabidopsis Biological Resource Center stock CS22), *nia1nia2* (Arabidopsis Biological Resource Center stock CS6512), and *pyr1/pyl1/pyl2/pyl4* (QC3, kindly provided by Sean Cutler, University of California, Riverside) mutants were used in this work. NOD and EV *Arabidopsis* seeds were kindly provided by Jurgen Zeier (University of Fribourg). NOD *Arabidopsis* plants expressing the bacterial flavohemoglobin (Col-0 background) were generated by *Agrobacterium tumefaciens*-mediated plant transformation using the DEX-inducible expression vector pTA7001 as described in Zeier et al. (2004). Plants transformed with empty expression vector pTA7001 EV were used as controls (EV). Plants were grown in soil:perlite:vermiculite: (1:1:1, v/v/v) at 25°C under a 16-h-light/8-h-dark photoperiod and watered with *Arabidopsis* salt (Wilson et al., 1990) nutritive medium.

To generate the *des1-1* complementation line, a 972-bp cDNA fragment containing the full-length coding sequence of *DES1* was obtained by RT-PCR amplification using the proofreading Platinum Pfx DNA polymerase (Invitrogen) and the primers *DES1F* and *DES1R* (Supplemental Table S1). The fragment was cloned into the pENTR/D-TOPO vector (Invitrogen) and transferred into the pMDC32 vector (Curtis and Grossniklaus, 2003) using the Gateway system (Invitrogen) according to the manufacturer's instructions. The final construct was generated by transformation into *A. tumefaciens* and then introduced into *des1-1* null plants using the floral dip method (Clough

and Bent, 1998). Four transgenic lines were generated, showing complementation, and the line used in this work is a homozygous T4.

## Chemicals and Treatments

NaHS, HT, and ABA were purchased from Sigma, (*p*-methoxyphenyl) morpholino-phosphinodithioic acid (GYY 4137) was purchased from Cayman Chemicals, cPTIO and SNAP were purchased from Molecular Probes, and 3-amino-4-(*N*-methylamino)-2',7'-difluorofluorescein diacetate (DAF-FM-DA) was purchased from Calbiochem. The stomatal aperture treatments were performed on epidermal strips excised from the abaxial side of fully expanded Arabidopsis leaves. Immediately after stripping, the epidermal peels were floated in opening buffer (10 mM K-MES, pH 6.1, and 10 mM KCl) for 3 h. The strips were subsequently maintained in the same opening buffer and exposed to different treatments. After 90 min, stomata were digitized using a Nikon DS-Fi 1 camera coupled to a Nikon Eclipse Ti microscope. The stomatal aperture width was measured using ImageJ analysis software (National Institutes of Health).

## Stomatal Conductance Measurements

Arabidopsis wild-type Col-0 and No-0 and *des1* mutant plants were sprayed with water (control) or 50  $\mu$ M ABA. After 4 h of treatment, the leaf gas exchange was measured in planta using a S151 IRGA (Qubit Systems), and the leaf temperature was measured using a S171 Leaf Chamber Thermistor (Qubit Systems) according to the manufacturer's instructions.

## RNA Isolation, RT Reaction, and PCR and Real-Time PCR Analysis

### Whole Leaf

Three-week-old wild-type Col-0 and No-0 plants were floated for 1, 2, 5, 7, 9, and 24 h in water as controls or treated with different ABA concentrations. Total RNA was extracted from the leaves using an RNeasy Plant Mini Kit (Qiagen). The RNA was reverse transcribed using an oligo(dT) primer and the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the recommended protocols.

### GC-e

Arabidopsis abaxial epidermal peels (GC-e) were floated in opening buffer (10 mM K-MES, pH 6.1, and 10 mM KCl) for 3 h and then treated in the same buffer with or without 50  $\mu$ M ABA for 90 min. Samples of the epidermal peels were loaded with the viability fluorescent probe fluorescein diacetate. None of the analyzed samples showed stained mesophyll cells; however, epidermis showed roughly 10% of pavement cells that survived. Therefore, we state that we have a GC-e extraction. To assess if the epidermal cell contamination or any unnoticed mesophyll cell contamination has any effect on the expression levels of the selected transcripts, we collected leaf cuts after the extraction of the abaxial epidermal layer (MC-e), we floated them in opening buffer (10 mM K-MES, pH 6.1, and 10 mM KCl) for 3 h, and then we subjected them to the same treatments assayed for GC-e. Total RNA was extracted using Trizol reagent (Invitrogen). Subsequently, 2  $\mu$ g of total RNA was used for the RT reaction and 1  $\mu$ g of total RNA for qRT-PCR using an oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Promega).

### RT-PCR Reactions

An aliquot of the cDNA was amplified in subsequent PCR reactions using the following primers: ActinF/ActinR for the constitutive *Actin* (At4g05320) gene, CER2F/CER2R for the guard cell marker *CER2* (At4g24510) gene, and Canh1F/Canh1R for the mesophyll cell marker *Canh1* (At3g01500) gene (Supplemental Table S1). For the amplification of *CER2* and *Canh1*, the following amplification conditions were used: a denaturation cycle of 5 min at 94°C; 32 amplification cycles of 30 s at 94°C, 50 s at 60°C, and 1 min 30 s at 72°C; and an extension cycle of 5 min at 72°C. For the *Actin* gene, 24 amplification cycles were performed.

## Quantitative Real-Time RT-PCR to Analyze *DES1* Gene Expression

First-strand cDNA was synthesized as described above. The specific primers for this gene and the constitutive POLYUBIQUITIN10 (*UBQ10*) used

as a control were designed using the Vector NTI Advance 10 software (Invitrogen; Supplemental Table S1). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad), and the signals were detected on an iCycler (Bio-Rad) according to the manufacturer's instructions. The cycling profile consisted of 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Subsequent to the PCR cycling, a melt curve from 60°C to 90°C was performed. The expression levels of *DES1* were normalized to that of the constitutive *UBQ10* gene by subtracting the cycle threshold value of *UBQ10* from the cycle threshold value of the gene ( $\Delta$ CT). The gene expression level was calculated as  $2^{-\Delta\text{CT}}$  (López-Martín et al., 2008). The results shown are given as means  $\pm$  SD of at least three independent RNA samples. For *RD29A* and *Rab18* gene analysis, the specific primers for this gene and the constitutive *ACTIN2* (*ACT2*) used as a control were designed using the Vector NTI Advance 10 software (Invitrogen; Supplemental Table S1). The Fast Universal SYBR Green Master mix from Roche was employed, using a Step-One Real-Time PCR machine from Applied Biosystems. The standard amplification program was used. The expression levels of the gene of interest were normalized to that of the constitutive *ACT2* gene as described above.

## DES Activity

Plant leaf material was ground in 20 mM Tris-HCl (pH 8) using a mortar and pestle with liquid nitrogen. After centrifugation at 15,000g for 15 min at 4°C, the resulting supernatant was used as plant-soluble extract for measuring DES activity. The total amount of protein in the extracts was determined by using the Bradford method. The DES activity was measured by the release of sulfide from L-Cys as described previously (Alvarez et al., 2010). The assay contained 1 mM dithiothreitol, 1 mM L-Cys, 100 mM Tris-HCl, pH 8.0, and enzyme extract in a total volume of 1 mL. The reaction was initiated upon the addition of L-Cys. After incubation for 15 min at 37°C, the reaction was terminated upon the addition of 100  $\mu$ L of 30 mM FeCl<sub>3</sub> dissolved in 1.2 N HCl and 100  $\mu$ L of 20 mM *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 N HCl. The formation of methylene blue was determined at 670 nm, and the enzyme activity was calculated using the extinction coefficient of  $15 \times 10^6$  cm<sup>2</sup> mol<sup>-1</sup>.

## Fluorescence Microscopy

NO was visualized using the specific NO dye DAF-FM-DA. Arabidopsis epidermal strips preincubated in opening buffer for 3 h in the dark were loaded with 10  $\mu$ M DAF-FM-DA for 30 min. The strips were washed three times with fresh opening buffer and exposed to different treatments for 15 min. Fluorescent images were obtained using a Nikon DS-Fi 1 digital camera coupled to a Nikon Eclipse Ti epifluorescence microscope (excitation, 495 nm; emission, 515–555 nm). The green fluorescence was quantified as the pixel intensity of a fixed area for all guard cells using ImageJ analysis software (National Institutes of Health). The fluorescence values are presented as relative units with respect to the control treatments and are expressed as the means  $\pm$  SE. Fifteen to 30 guard cells were observed per experiment in each treatment for at least four independent replicates.

## Statistical Analysis

Data analyses were performed using Sigmaplot for Windows (Systat Software). The statistically significant differences were analyzed using one-way ANOVA, or the Student's *t* test, as indicated in the figure legends.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number At5G28030.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** H<sub>2</sub>S induces stomatal closure in a dose dependent manner in Arabidopsis.

**Supplemental Figure S2.** Gene expression analysis of Arabidopsis guard cell- vs. mesophyll cell-enriched extracts.

**Supplemental Figure S3.** ABA regulates *DES1* transcript levels and DES enzyme activity in whole leaf.

**Supplemental Figure S4.** Promoter region of *DES1* contains ABA-related motifs.

**Supplemental Figure S5.** Structure of the *DES1* gene in the insertion mutants.

**Supplemental Table S1.** Sequences of oligonucleotides used in this work.

Received June 17, 2014; accepted September 23, 2014; published September 29, 2014.

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