

Running head: *H₂S requires NO to modulate ABA-induced stomatal closure*

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Hydrogen sulfide generated by L-cysteine desulphydrase acts upstream of nitric oxide to modulate ABA-dependent stomatal closure

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ABSTRACT

Abscisic acid (ABA) is a well-studied regulator of stomatal movement. Hydrogen sulfide (H₂S), a small signaling gas molecule involved in key physiological processes in mammals, has been recently reported as a new component of ABA signaling network in stomatal guard cells. In Arabidopsis, H₂S is enzymatically produced in the cytosol through the activity of L-cysteine desulphydrase (DES1). In the present work, we used *DES1* knock-out Arabidopsis mutant plants (*des1*) to study the participation of DES1 in the crosstalk between H₂S 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 H₂S. qRT-PCR 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 *pyr1/pyl1/pyl2/pyl4* close in response to exogenous H₂S, suggesting that this gasotransmitter is acting downstream, although acting independently of ABA receptor cannot be rule out with the present data. However Arabidopsis clade-A PP2C mutant *abi1-1* do not close the stomata when epidermal strips were treated with H₂S suggesting that H₂S required a functional ABI1. Further studies to unravel the cross-talk between H₂S and NO indicate that: (i) H₂S promotes NO production, (ii) DES1 is required for ABA-dependent NO production, and (iii) NO is downstream of H₂S in ABA-induced stomatal closure. Altogether, data indicate that DES1 is a novel component of ABA signaling in guard cells.

INTRODUCTION

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 CO₂ 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 ($[Ca^{2+}]_{cyt}$), 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 (K^+_{in}) channels, and ion efflux is induced through activation of outward rectifying potassium (K^+_{out}) 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^+ , protein phosphatases (mainly PP2C), guanylate cyclase/cyclicADPR (GC/cADPR), hydrogen peroxide (H₂O₂), 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 up or downstream of the receptor complex (Leonhardt et al., 2004; Cutler et al., 2010); however, novel components are still emerging.

Hydrogen sulfide (H₂S) is a small and reactive water soluble gas. In aqueous solutions (pH 7.4) the ratio between the hydrosulfide anion (HS⁻) and H₂S is 3:1 (Kabil and Banerjee, 2010). The active form of H₂S in biological systems has not been specified yet; therefore H₂S usually stands for H₂S/HS⁻. H₂S can freely permeate lipid membranes as its solubility is 5 times greater in lipophilic solvents than in water (Wang, 2002).

H₂S has been proposed as the third gasotransmitter in animals cells, after NO and carbon monoxide (CO), due to its high level of permeability through biological membranes, its effect at low concentrations and toxicity at high doses (Mancardi et al., 2009). H₂S 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 H₂S is produced through the activity of two pyridoxal-5'-phosphate-dependent enzymes cystathionine β-synthase (CBS; EC 4.2.1.22) and cystathionine γ-lyase (CSE; EC 4.4.1.1). It has long been known that plants have L-cysteine desulfhydrase (DES) activity (Harrington and Smith, 1980; Papenbrock et al., 2007). However, no *bona fide* DES gene was reported until 2010 when the *CS-LIKE* gene (At5G28030), a member of the O-acetylserine(thiol)lyase (OAS-TL) family was identified as a true DES and named *DES1* (Alvarez et al., 2010). DES1 releases H₂S, pyruvate and ammonia during L-cysteine degradation (Alvarez et al., 2010). Early studies concerning H₂S emission in plants were associated with the plant response to pathogens as part of a so called Sulfur Induced Resistance (SIR) (Bloem et al., 2004). Using a H₂S releasing compound, H₂S was later reported to confer a protective effect against oxidative (Zhang et al., 2009b; Zhang et al., 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 H₂S in ABA- and ethylene-dependent stomatal closure induction (García-Mata and Lamattina, 2010; Liu et al., 2011; Liu et al., 2012; Jin et al., 2013). In the present study, we used the characterized *DES1* knock out Arabidopsis mutants plants *des1-1* and *des1-2* to obtain new insights on the cross-talk between H₂S and NO and further evidences supporting the involvement and requirements of H₂S in ABA-induced signaling cascade leading to stomatal closure.

RESULTS

The L-Cysteine Desulfhydrase 1 (DES1) Is Required for ABA-Dependent Stomatal Closure

In previous work, we presented pharmacological evidence showing that H₂S 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 and colleagues (2013) using a T-DNA insertion mutant of the gene AT3G62130 that codes for a L-Cys desulfhydrase (lcd). In the present work, by contrast, 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 (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* knock out mutant complemented with the full-length *DES1* cDNA (Fig. 1 inset). Moreover, the addition of exogenous H_2S , as 100 μM of the H_2S donor 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_2S (Fig. 1A, 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 (Garcia-Mata and Lamattina, 2010), epidermal strips from both genetic backgrounds No-0 and Col-0 responded to the H_2S 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_2S donor (500 μM) did not induce stomatal closure, probably due to rather toxic effects (Supplemental Fig. S1A). The fact that GYY 4137 (another H_2S donor) induced stomatal closure in both wild type plants and *des1* mutants (Supplemental Fig. S1B), and that the H_2S scavenger hypotaurine (HT) blocked the effect of the donor (Supplemental Fig. S1C) confirms that the response was due to the released H_2S and not by any by-product of the donor molecule.

To determine if the *des-1* mutants also show reduced sensitivity to ABA at the whole plant level, Arabidopsis *des1* mutant plants and their respective genetic background were sprayed with water or 50 μM ABA for 3h 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 since 90-95% 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 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 *β Carbonic anhydrase 1* (*Canh1*) (Pandey et al., 2002) as a marker gene for the mesophyll cells (Supplemental Fig. S2). In order to assess if *DES1* is regulated by 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 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-fold 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 *RD29A* and *RAB18* in GC-e RNA extracts from wild type and *des1* plants. The results shown in Figure 4B 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.

H₂S Participates in ABA-Dependent NO Production During Stomatal Closure Induction

It has been demonstrated that in animal systems H₂S 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 H₂S acts downstream of NO in ethylene induced stomatal closure (Liu et al., 2011; Liu et al., 2012). It was also reported that H₂S reduces ABA-dependent NO production in Arabidopsis and *Capsicum annuum* guard cells (Lisjak et al., 2010; Lisjak et al., 2011). To add some knowledge to the NO-H₂S crosstalk in Arabidopsis guard cells, we first performed

a pharmacological assay to assess whether NO is involved in H₂S-dependent stomatal closure. Epidermal strips from Arabidopsis wild type leaves were treated with or without the NO-specific scavenger cPTIO (2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide). In Figure 5A it is shown that cPTIO impaired H₂S-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 nitric oxide 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 H₂S induced stomatal closure in water sprayed NOD plants, but no stomatal closure was observed in DEX-sprayed NOD plants, confirming the NO requirement for H₂S-dependent stomatal closure (Fig. 5B). To further confirm the interaction of H₂S and NO we used nitrate reductase (NR) 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, effect that was restored by exogenous addition of the NO-specific donor SNAP (*S*-nitroso-*N*-acetylpenicillamine). All together, the pharmacological and genetic evidence indicate that the depletion of endogenous NO blocks H₂S-mediated induction of stomatal closure demonstrating the interaction of these two gasotransmitters in stomatal closure processes.

The lack of response to H₂S 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 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 H₂S acts upstream of NO in ABA-dependent stomatal closure; therefore, we used the fluorescent dye DAF-FM-DA (4,5-diaminoflorescein diacetate) to test whether H₂S 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 H₂S donors NaHS or GYY (Fig 7A,C). However this increase was not evident either in *des1* mutants or in Col-0 plants treated with ABA together with the H₂S

scavenger hypotaurine (Fig. 7A, 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 H₂S using either NaHS or GYY, supporting that DES1 acts upstream of NO in ABA-dependent stomatal closure (Fig. 7).

H₂S acts upstream of ABI1 in ABA-dependent stomatal closure

The mutants of the recently identified ABA receptor PYL/PYR/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 Ca²⁺ (Nishimura et al., 2010; Wang et al., 2013) or H₂O₂ (Wang et al., 2013). Therefore we assayed H₂S-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 H₂S was able to close the stomata of both wild type and, to a lesser extent, in the QC3 quadruple mutant epidermal strips. These data suggest that H₂S is acting downstream or independently of the ABA receptor. It is noteworthy that exogenous application of the H₂S donor NaHS did not induce stomatal closure in epidermal strips of the ABA insensitive (ABI) *abi1-1* mutant (Fig. 8B), but it does induce stomatal closure in its genetic background Landsberg erecta (Ler), indicating that a functional ABI1 is required for DES1/H₂S action in ABA-dependent guard cell signaling.

Discussion

The gasotransmitter H₂S has rapidly emerged as a hot topic in animal physiology where it is known to have an active role in cardioprotection, neurotransmission and O₂ 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; Zhang et al., 2009b; Zhang et al., 2010a; Zhang et al., 2010b; Zhang et al., 2010d; Li et al., 2013a; Li et al., 2013b). Recently, H₂S 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; Liu et al., 2012; Jin et al., 2013). Moreover, it has been reported also that H₂S induces stomatal opening (Lisjak et al., 2010; Lisjak et al., 2011).

In this work we present new insights into the regulation of the H₂S-generating enzyme L-cysteine desulphydrase1 (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 (Garcia-Mata and Lamattina, 2010). In this work, using a genetic approach we show that two independent knock-out 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 H₂S 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 upregulated 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) vs. mesophyll cell protoplasts (MCPs) (Leonhardt et al., 2004) and epidermal strips vs. leaf tissue (Wang et al., 2011). In both works the authors have identified three groups of genes: i) those equally regulated in GCP and MCP; ii) those with altered expression in MCPs and not in GCPs and iii) 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). Enzyme activity measurements will be needed to unequivocally correlate the upregulation of *DES1* in GC-e extracts with an increase DES-dependent H₂S production in guard cells; however, we could not obtain enough mass of protein from the isolated epidermal strips in order 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 figure S3A and B). Although further work is needed to unveil the mechanism by which ABA activate DES1 transcription, the presence of a Dc3 Promoter-

Binding Factor-1 and 2 (DPBF1&2) and a Dehydration-Responsive Element-like (DRE-like) in the promoter region of *DES1*, let us speculate on the possibility that ABA is directly regulating the expression of the gene (Supplemental S4).

Lately, the interest on the biology of gasotransmitters has been oriented towards the interactions between these three gases on the different biological systems (García-Mata and Lamattina, 2013; Kolluru et al., 2013). In animal system there are many reports on the interaction of H₂S and NO, although the nature of this interaction is still poorly understood. Some reports have shown a positive interaction between H₂S and NO where H₂S increases the rates of NO release from small nitrosothiols such as GSNO (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: i) *in vitro*, H₂S inhibits all the three isoforms of mammalian nitric oxide synthases (NOS) (Kubo et al., 2007), and ii) that H₂S 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 H₂S and NO during ABA-dependent stomatal closure. The depletion of NO, by chemical or genetic means, blocked the H₂S-dependent stomatal closure suggesting that NO is acting downstream of H₂S 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 H₂S treatment (data accessible at NCBI GEO database, accession GSE32566), which supports the lack of response of the *nia1/nia2* double mutant to the exogenous addition of H₂S observed in this study. As for animal systems, in plants there is controversial evidence about H₂S-NO interaction. On one hand it was shown that H₂S acts upstream of NO in root organogenesis and heavy metal toxicity (Zhang et al., 2009a; Li et al., 2012). On the other hand, H₂S 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 and colleagues have recently reported that in *Arabidopsis* whole plant extracts the production rate of H₂S increased upon drought stress treatment (Jin et al., 2011).

Lisjak and colleagues have proposed a negative interaction between H₂S and NO in ABA-induced stomatal closure in *Arabidopsis* and *Capsicum annuum* (Lisjak et al., 2010; Lisjak et al., 2011). Based in pharmacological evidence, the authors speculate that the NO produced by ABA is removed by H₂S, 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 H₂S donors between Lisjak et al (2010) and this study.

Liu and co-workers (2011) showed that stomatal closure but not NO synthesis is impaired in an *Arabidopsis* mutant of the L-cysteine desulfhydrase *AtL-CDES* gene. Therefore, they suggest that NO is acting upstream of H₂S 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 cysteine desulfurase (At5g65720) which is a NifS-like protein involved in the biosynthesis of different cofactors like the iron-sulfur clusters (Van Hoewyk et al., 2008). This NifS-like protein catalyzes the conversion of cysteine to alanine 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, from the data presented by Liu et al (2011) it cannot be ruled out an ABA-induced H₂S-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 HAB1) and ABA-activated 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 and colleagues (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 ROS or NO in response to ABA (Yin et al., 2013). Likewise, exogenous H₂S is able to induce stomatal closure in the QC3 mutant, suggesting

that it acts downstream of the ABA receptor, although a PYL/PYL/RCAR –independent pathway cannot be ruled out with the current data. Our data show that exogenous H₂S do not induce stomatal closure in the gain of function mutants of ABI1 that is a negative regulator of ABA-induced stomatal closures (Merlot et al., 2001; Saez et al., 2004; Kuhn et al., 2006; Rubio et al., 2009), suggesting that H₂S is acting upstream of this ABA signaling element. Interestingly, previous reports show that i) 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 ii) exogenous addition of NO induce 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/H₂S is acting upstream of NO, a simplified model is shown in Fig. 9 to propose the position of DES1/H₂S in 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 (PA) which production is increased by NO in guard cells (Distéfano et al., 2008) and was reported to regulate ABI1/PP2C activity preventing ABI1 translocation from the cytosol to the nucleus (Zhang et al., 2004).

Recent evidence demonstrate the H₂S can regulate protein activity through the direct interaction of H₂S with the thiol group of the target protein yielding a hydropersulfide (-SSH) moiety (Mustafa et al., 2009). Further studies will be needed to see if this is the mechanism by which H₂S regulates ABA-signaling components. Overall, the current study presents compelling evidence supporting DES1 as a novel component of ABA signaling in guard cells, mediating H₂S production and acting upstream of NO to induce stomatal closure.

MATERIALS AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*) wild type ecotypes Columbia (Col-0) and Nossen (No-0), *des1-1* (SALK_103855) and *des1-2* (RIKEN RATM13-27151_G) (extensively described in Alvarez et al., 2010; 2012), *abi1-1* (ABRC stock CS22), *nialnia2* (ABRC stock CS6512) and *pyr1/pyl1/pyl2/pyl4* (QC3, kindly provided by Sean Cutler, University of California, Riverside) mutants were used in this work. Nitric oxide dioxygenase (NOD) and empty vector (EV) *Arabidopsis* seeds were kindly provided by Jurgen Zeier (University of Fribourg, Fribourg, Switzerland). NOD *Arabidopsis* plants expressing the bacterial flavohemoglobin *Hmp* (Col-0 background) were generated by *Agrobacterium*-mediated plant transformation using the Dexamethasone (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 16:8 h light:dark photoperiod and watered with *Arabidopsis thaliana* salt (ATS) (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 *Agrobacterium 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

Sodium hydrosulfide (NaHS), hypotaurine (HT) and abscisic acid (ABA) were purchased from Sigma (St Louis, MO, USA), (p-methoxyphenyl)morpholino-phosphinodithioic acid (GYY 4137) was purchased from Cayman Chemicals (Ann Arbor, MI, USA), 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) were purchased from Molecular Probes (OR, USA) and 3-Amino-4-(*N*-methylamino)-2', 7'-difluorofluorescein diacetate (DAF-FM-DA) was purchased from Calbiochem (San Diego, CA, USA). 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, 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 (Nikon, Tokyo, Japan). The stomatal aperture width was measured using ImageJ analysis software (NIH, Bethesda, MD, USA).

Stomatal Conductance Measurements

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

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

Whole leaf: Three-week-old wild-type ecotypes Col-0 and No-0 plants were floated for 1, 2, 5, 7, 9 and 24 h in water (H₂O) 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 (RT) using an oligo(-dT) primer and the Superscript™ First-Strand, Synthesis System for RT-PCR (Invitrogen) according to the recommended protocols.

Guard cell-enriched (GC-e): Arabidopsis abaxial epidermal peels (GC-e) were floated in opening buffer (10 mM K-MES, pH 6.1, 10 mM KCl) for 3 h and then treated in the same buffer with or without 50 μ M ABA for 90 min. Samples of the epidermal peels were loaded with the viability fluorescent probe fluorescein diacetate (FDA). 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 guard cell enriched extraction. In order 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 (mesophyll cell-

enriched, MC-e), we floated them in opening buffer (10 mM K-MES, pH 6.1, 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, Gaithersburg, MD). Subsequently, 2 µg of total RNA was used for the RT reaction and 1 µg of total RNA for qRT-PCR using an oligo(-dT) primer and M-MLV 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 *ECERIFERUM2* (CER2; At4G24510) gene, Canh1F/Canh1R for the mesophyll cell marker β -*Carbonic Anhydrase 1* (Canh1; At3g01500) gene (Supplemental Table S1). For the amplification of *ECERIFEMUM2* and *Carbonic anhydrase b* 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 *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 CT value of the gene (Δ CT). The gene expression level was calculated as $2^{-(\Delta$ CT)} (Lopez-Martin et al., 2008). The results shown are given as means \pm standard deviation (SD) of at least three independent RNA samples. For *RD29A* and *Rab18* genes analysis: The-specific primers for this gene and the constitutive *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 (Mannheim, Germany) was employed, using a Step-one Real-time PCR machine from Applied Biosystems (California, USA). 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 15,000 $\times g$ 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 μ l of 30 mM FeCl₃ dissolved in 1.2 N HCl and 100 μ 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 $15 \cdot 10^6 \text{ cm}^2 \text{ mol}^{-1}$.

Fluorescence Microscopy

Nitric oxide (NO) was visualized using the specific NO dye DAF-FM-DA. *A. thaliana* epidermal strips pre-incubated in opening buffer for 3 h in the dark were loaded with 10 μ 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 (Nikon, Japan) 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 (NIH, Bethesda, MD, USA). The fluorescence values are presented as relative units (RU) with respect to the control treatments and are expressed as the means \pm standard error (SE). Fifteen to thirty 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, Inc., Chicago, IL, USA). The statistical significant differences were analyzed using one-way ANOVA, or the Student's *t*-test, as indicated in the figure legends.

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FIGURE LEGENDS>

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 pre-incubated for 3 h in opening buffer (10 mM K-MES, pH 6.1, 10 mM KCl) under light. Strips were subsequently treated for 90 min under light with: Opening buffer (Control), 100 μ M of the H₂S donor NaHS, 50 μ M ABA or 100 μ M NaHS + 50 μ M ABA. The values are expressed as the means \pm SE and represent the mean of 20–40 stomata per experiments from at least three independent experiments (n = 60–160). The asterisks denote significant differences with respect to the control treatments (*t-test* p<0.05).

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 pre incubated for 3 h in opening buffer (10 mM K-MES, pH 6.1, 10 mM KCl) under light and subsequently treated with increasing concentrations of ABA (0, 1, 10, 25, 50, 100 and 250 μ 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 20–40 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 μM ABA, and the stomatal conductance was measured 3h after treatment from a fully expanded leaf in a closed chamber under a constant CO_2 concentration for 5 min using an infrared gas analyzer (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 (*t-test* $p < 0.05$).

Figure 4: Analysis of the expression levels of *DES1* and ABA-responsive genes in guard cell-enriched (GC-e) and mesophyll cell-enriched (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 3h in opening buffer (10 mM K-MES, pH 6.1, 10 mM KCl), and then treated with or without 50 μM 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 and MC-e RNA extracted from wild type and *des1* mutants. The values are expressed as percentages with respect to the control treatments.

Figure 5: Nitric oxide is required for the H₂S-induced stomatal closure. (A) Epidermal strips peeled from Arabidopsis leaves were pre-incubated for 3 h in opening buffer (10 mM K-MES, pH 6.1, 10 mM KCl) under light. Subsequently, strips were treated for 90 min under light with 100 μM NaHS in the presence (+ cPTIO) or absence (- cPTIO) of 200 μM of the NO scavenger cPTIO. (B) Arabidopsis Col-0 plants transformed with an empty vector (EV) or nitric oxide dioxygenase (NOD) were sprayed with water (mock) or 0.01% (v/v) Dexametasone (DEX). After 48 of the DEX induction, epidermal strips were peeled, pre-incubated for 3 h in opening buffer under light and then treated for 90 min with opening buffer (Control) or 100 μM NaHS. (C) Epidermal strips peeled from Arabidopsis double mutant *nial/nia2* leaves were preincubated for 3 h in opening buffer under light and then treated for 90 min with 100 μM NaHS or 100 μM of NO donor SNAP or 100 μM NaHS + 100 μM SNAP. The values are expressed as the means ± SE and represent the mean of 20–40 stomata per experiments from at least three independent experiment (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 pre-incubated for 3 h in opening buffer (10 mM K-MES, pH 6.1, 10 mM KCl) under light and subsequently treated for 90 min under light with 100 μM SNAP. The values of stomatal aperture are expressed as the means ± SE and represent the mean of 20–40 stomata per experiments 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).

Figure 7: H₂S is required for the ABA-induced NO production in Arabidopsis guard cells. Epidermal peels from Arabidopsis *des1* mutant and their genetic backgrounds were pre-incubated for 3 h in opening buffer (10 mM K-MES, pH 6.1, 10 mM KCl)

under light and subsequently loaded with 10 μM of the NO-specific fluorescent dye DAF-FM DA. After washing, the strips were treated for 30 min under light with opening buffer (Control) or with the following treatments: 50 μM ABA, 100 μM NaHS, 200 μM of the H_2S scavenger Hypotaurine (HT), ABA + HT, NaHS + HT or ABA + NaHS for Col-0 and *des1-1* plants (A and B) and 50 μM ABA, 100 μM NaHS, 100 μM of de H_2S donor GYY 4137, ABA + NaHS or ABA + 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 units (RU) with respect to the control treatment. The values are expressed as means \pm SE and represent the mean of 40–50 stomata per experiment from three independent experiments ($n = 120\text{--}150$). Different letters denote significant differences (Dunn's post hoc test $p < 0.05$).

Figure 8: Position of H_2S in a PYR/PYR/RCAR cascade. Epidermal strips, peeled from the Arabidopsis mutants in ABA receptor *pyr1/pyl1/pyl2/pyl4* (QC3) (A), the PP2C mutant *abi-1* (B) and their genetic backgrounds, were pre-incubated for 3 h in opening buffer (10 mM K-MES, pH 6.1, 10 mM KCl) under light. Strips were subsequently treated for 90 min under light with: Opening buffer (Control), 100 μM of the H_2S 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 \pm SE and represent the mean of 20–40 stomata per experiments from at least three independent experiments ($n = 60\text{--}160$). Different letters indicate significant differences among treatments (Dunn's post hoc test $p < 0.05$).

Figure 9: Simplified model showing the interaction of DES1/H₂S with the core ABA signaling pathway in guard cells. ABA enters in the guard cells through ABC transporter, once in the guard cells it binds to the PYR/PYL/RCAR receptor (Rc). The ABA PYL/PYR/RCAR binds the clade A protein phosphatases 2C (PP2C) inhibiting its phosphatase activity, and though it releases the SnRKs protein kinases and the signaling elements downward. L-cysteine desulphydrase (DES1) is upregulated by ABA releasing H₂S, which increases endogenous NO via nitrate reductase (NR) activity. NO might regulate the binding of ABI1 to Rc, and though the downstream response. Arrow lines: activation; blunt lines: inhibition; color lines: crosstalk proposed in this work; grey lines: already published and dashed lines hypothetical. K⁺, Potassium uptake, A⁻, Anion efflux, ABRE, ABA responsive element transcription factors.

Figure 1

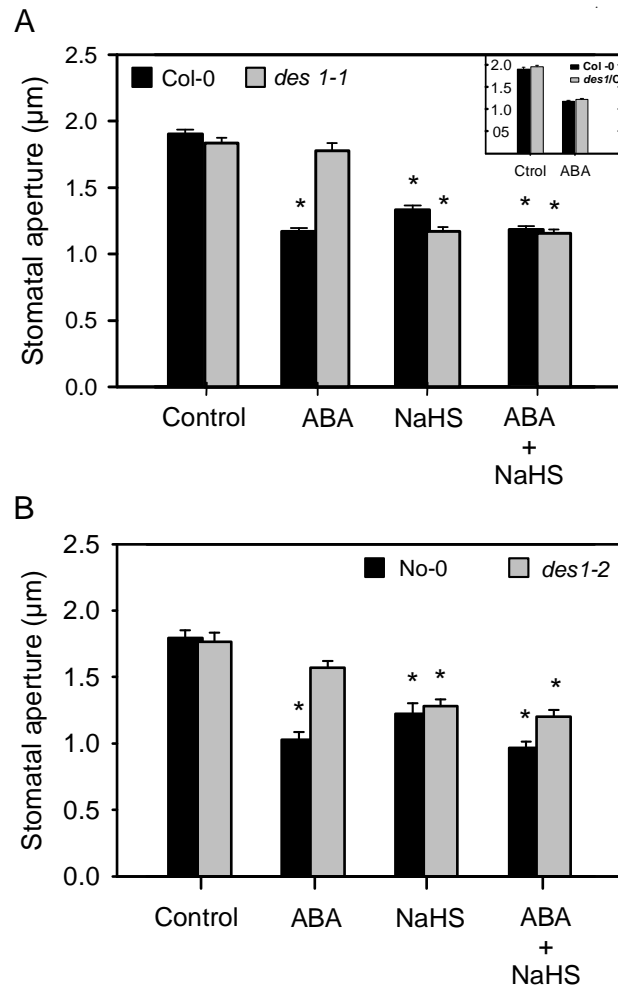


Figure 1: DES1 is involved in ABA-dependent stomatal closure. Epidermal strips, peeled from Arabidopsis mutants *des1-1* (A), *des1-2* (B), *des1-1* complemented with *DES1* full cDNA (*des1/C*; inset) and their genetic backgrounds, were pre-incubated for 3 h in opening buffer (10 mM K-MES, pH 6.1, 10 mM KCl) under light. Strips were subsequently treated for 90 min under light with: Opening buffer (Control), 100 μM of the H₂S donor NaHS, 50 μM ABA or 100 μM NaHS + 50 μM ABA. The values are expressed as the means ± SE and represent the mean of 20–40 stomata per experiments from at least three independent experiments (n = 60–160). The asterisks denote significant differences with respect to the control treatments (*t-test* p<0.05).

Figure 2:

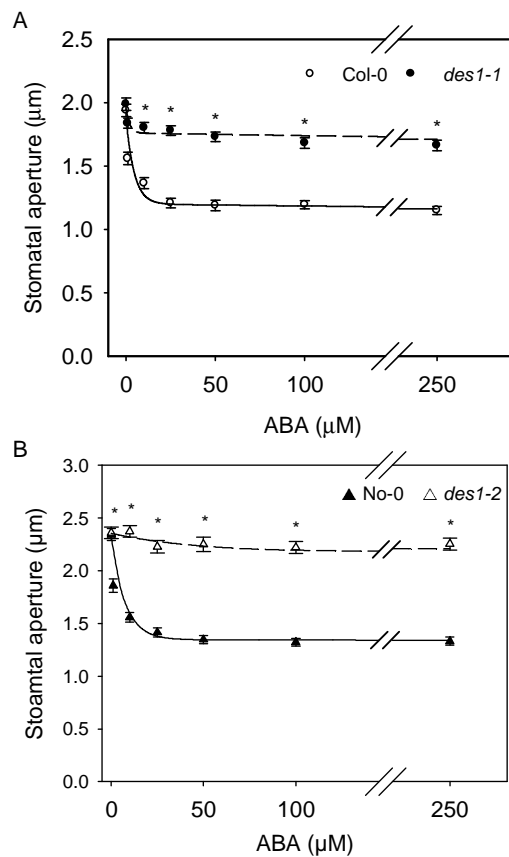


Figure 2: Stomata from *Arabidopsis des1* mutants show reduced sensitivity to ABA. Epidermal strips peeled from *des1-1* (A) or *des1-2* (B) plants, and their genetic backgrounds were pre incubated for 3 h in opening buffer (10 mM K-MES, pH 6.1, 10 mM KCl) under light and subsequently treated with increasing concentrations of ABA (0, 1, 10, 25, 50, 100 and 250 μM) 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 20–40 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:

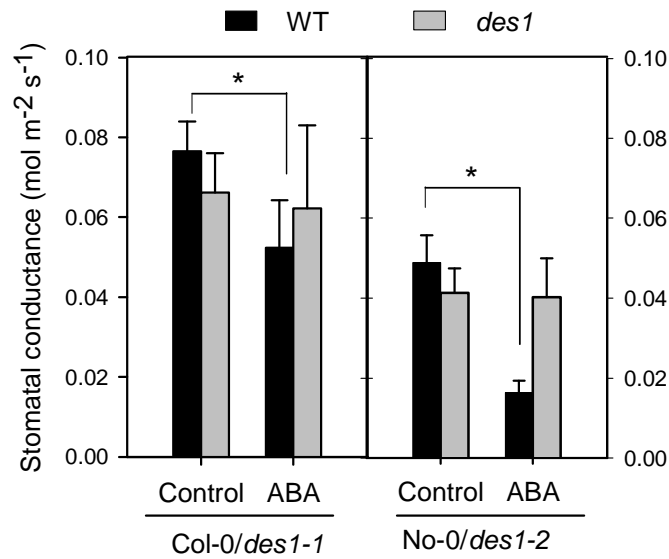


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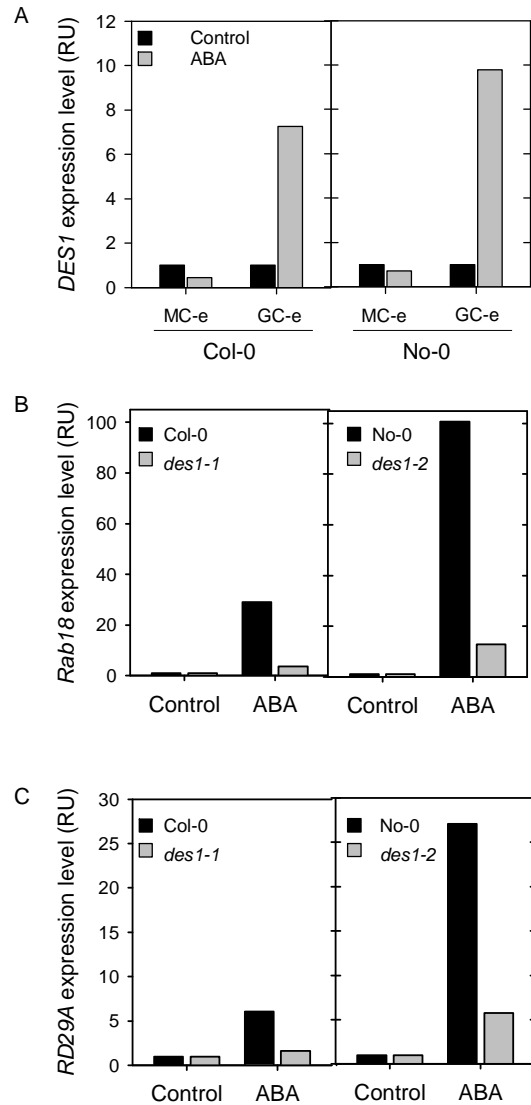


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Figure 5

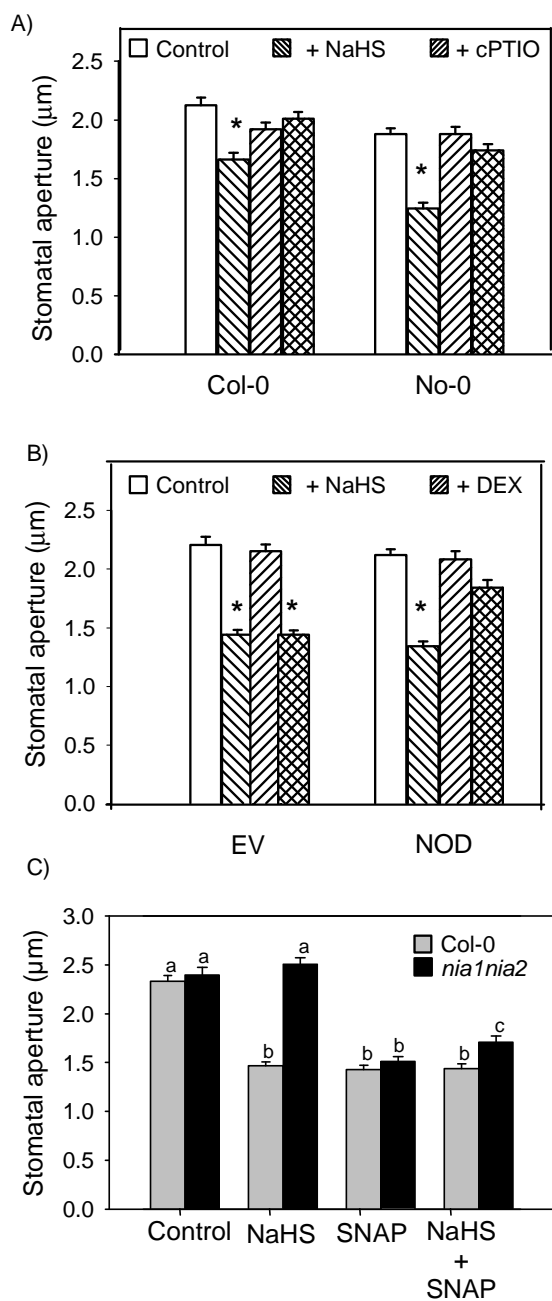


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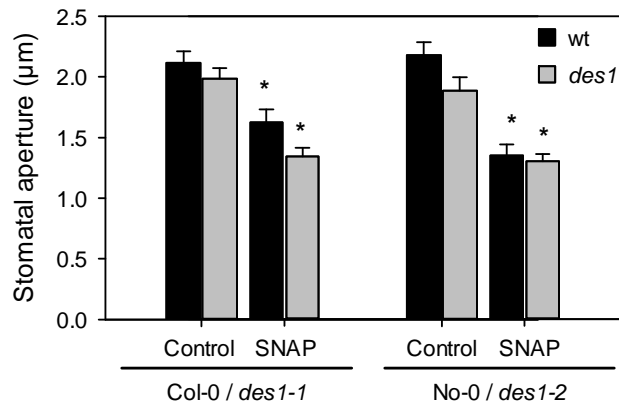


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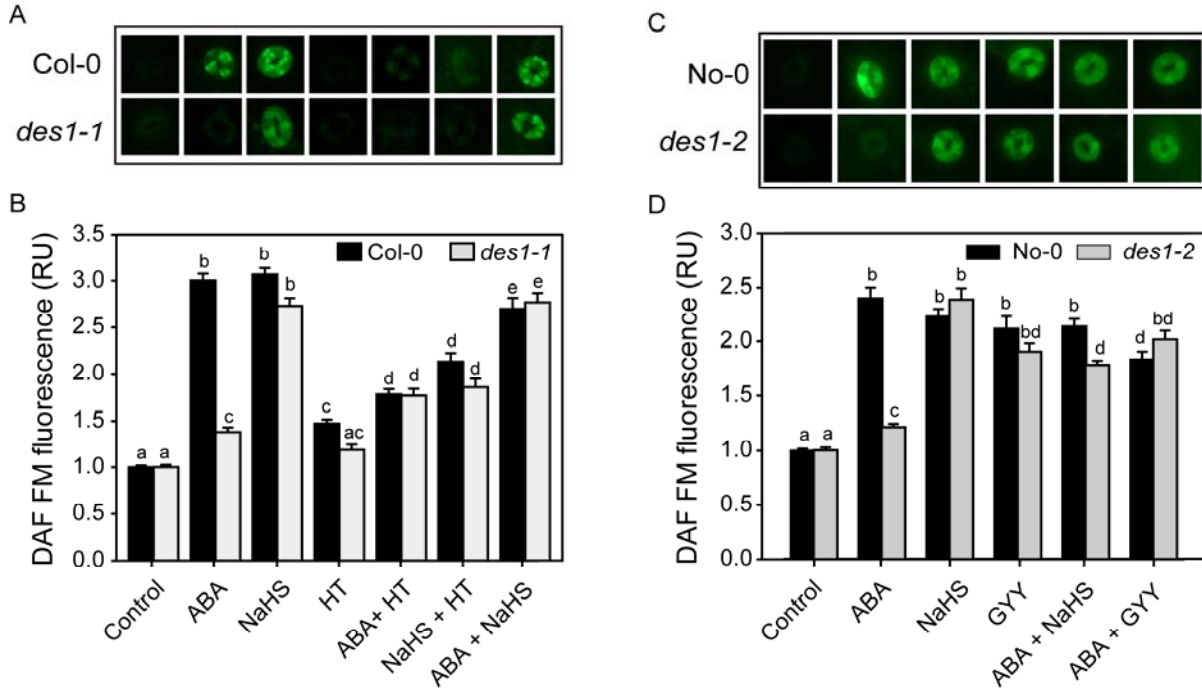


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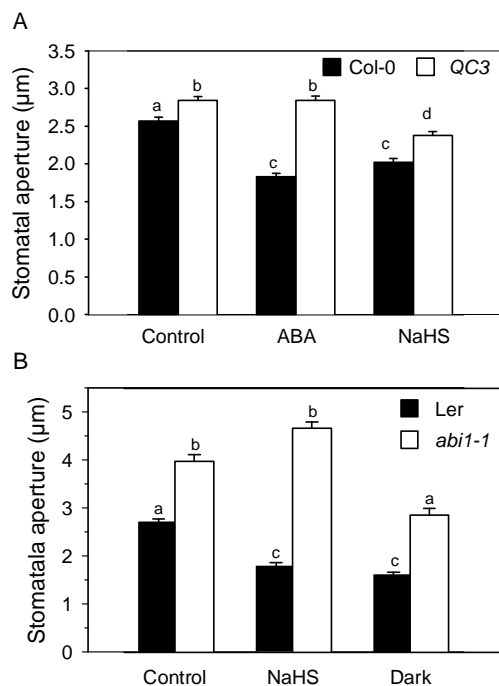


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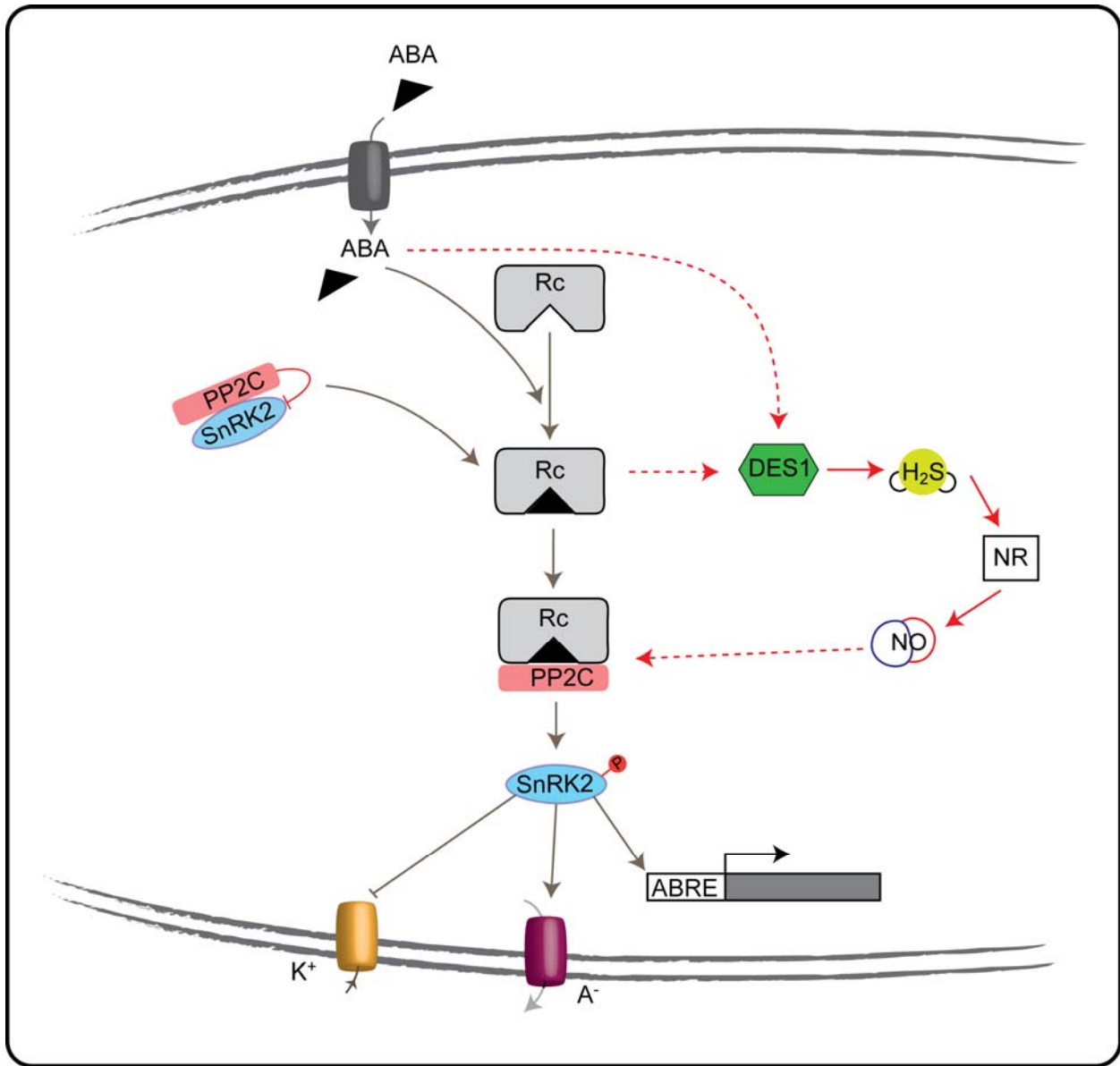


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