

Hydrogen Sulfide Increases Production of NADPH Oxidase-Dependent Hydrogen Peroxide and Phospholipase D-Derived Phosphatidic Acid in Guard Cell Signaling¹

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Hydrogen sulfide (H₂S) is an important gaseous signaling molecule in plants that participates in stress responses and development. L-Cys desulfhydrase 1, one of the enzymatic sources of H₂S in plants, participates in abscisic acid-induced stomatal closure. We combined pharmacological and genetic approaches to elucidate the involvement of H₂S in stomatal closure and the interplay between H₂S and other second messengers of the guard cell signaling network, such as hydrogen peroxide (H₂O₂) and phospholipase D (PLD)-derived phosphatidic acid in *Arabidopsis thaliana*. Both NADPH oxidase isoforms, respiratory burst oxidase homolog (RBOH)D and RBOHF, were required for H₂S-induced stomatal closure. In vivo imaging using the cytosolic ratiometric fluorescent biosensor roGFP2-Orp1 revealed that H₂S stimulates H₂O₂ production in *Arabidopsis* guard cells. Additionally, we observed an interplay between H₂S and PLD activity in the regulation of reactive oxygen species production and stomatal movement. The PLD α 1 and PLD δ isoforms were required for H₂S-induced stomatal closure, and most of the H₂S-dependent H₂O₂ production required the activity of PLD α 1. Finally, we showed that H₂S induced increases in the PLD δ -derived phosphatidic acid levels in guard cells. Our results revealed the involvement of H₂S in the signaling network that controls stomatal closure, and suggest that H₂S regulates NADPH oxidase and PLD activity in guard cells.

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Stomata are formed by pairs of specialized guard cells that sense and integrate multiple stimuli (such as light, CO₂, humidity, and hormones) to modulate stomatal pore size. Guard cells have become a model for studying signal transduction in plants. During stomatal closure, guard cells undergo depolarization of the plasma membrane through the inhibition of H⁺-ATPases, and an increase of cytosolic Ca²⁺ concentration. This response generates a net loss of osmotically active solutes (mainly K⁺) through regulation of inward and outward K⁺ rectifier channels, providing an intracellular environment that drives water out of the cells to reduce guard cell volume (Blatt, 2000; Schroeder et al., 2001). These processes are controlled by a complex signaling network, the architecture of which resembles that of a scale-free network (Hetherington and Woodward, 2003). Some of the pathways that constitute this network have been well studied, such as those triggered by abscisic acid (ABA) or CO₂, while for others, our understanding is still lacking.

The production of reactive oxygen species (ROS) by the guard cells is a response common to most of the

stimuli that induce stomatal closure (Song et al., 2014). Cytosolic ROS are generated primarily in the apoplast by NADPH oxidase (NADPHox) respiratory burst oxidase homolog (RBOH), which generates superoxide that dismutates to H₂O₂ (Kwak et al., 2003). In *Arabidopsis thaliana*, the RBOH family contains 10 members. Isoforms D (RBOHD) and F (RBOHF) are involved in the regulation of stomatal movement (Sierla et al., 2016). RBOH is composed of 6 transmembrane domains, a C-terminal tail containing the NADPH and FADH binding sites, and an N-terminal region, which contains two EF-hands and several phosphorylation sites, both of which are involved in activation (Suzuki et al., 2011; Sierla et al., 2016). Apart from the canonical regulation, other messengers can regulate the activity of RBOH. The Cys-890 amino acid residue of RBOHD is susceptible to modification by nitric oxide (NO; S-nitrosylation), which reduces the activity of NADPHox during the defense response to *Pseudomonas syringae* (Yun et al., 2011).

Another messenger that regulates NADPHox activity in guard cells is the signaling phospholipid, phosphatidic acid (PA; Zhang et al., 2009). PA, produced by phospholipase D (PLD), binds to RBOHD and RBOHF to induce ABA-dependent ROS production (Zhang et al., 2009). In plants, PA is generated via phospholipase C (PLC) activity in concert with diacylglycerol kinase, or directly through hydrolysis of structural lipids by PLD. PA has emerged as a key player in plant signaling (Testerink and Munnik, 2005; Wang et al., 2006). The *Arabidopsis* genome encodes 12 PLDs, which are divided into 6 subgroups [$\alpha(3)$, $\beta(2)$, $\gamma(3)$, δ , ϵ , and $\zeta(2)$]. Only PLD α 1 and PLD δ are associated with regulation of stomatal closure, and, in the ABA-dependent response, PLD α 1 is required for ROS production (Zhang et al., 2009), while PLD δ is involved in NO-dependent regulation of stomatal closure (Distéfano et al., 2012).

Studies of signal transduction in plants and animals have revealed gasotransmitters as central messengers (Mustafa et al., 2009; Zhang, 2016), which are also evident in the guard cell signaling network (Scuffi et al., 2016). The first gasotransmitter described to participate in the modulation of stomatal movement was NO, which modulates the induction of stomatal closure and the inhibition of stomatal opening (García-Mata and Lamattina, 2001; Garcia-Mata and Lamattina, 2007). Recently, H₂S has been recognized as a stomatal closure inductor and ascribed an active role in the signaling response to different stimuli (Scuffi et al., 2016). In plants, H₂S is enzymatically produced by a Cys desulfhydrase in the catalyzed conversion of Cys to pyruvate, H₂S, and NH₃⁺. In *Arabidopsis*, the cytosolic protein DES1 was recently characterized as a novel γ -Cys desulfhydrase (Alvarez et al., 2010). DES1 is involved in the response to heavy metal stress, plant immunity, autophagy, and stomatal closure (Alvarez et al., 2010, 2012a, 2012b; Scuffi et al., 2014). Null mutants of *DES1* show an approximately 30% decrease in γ -Cys desulfhydrase activity (Alvarez et al., 2010),

suggesting that other sources also contribute to H₂S production. Although other enzymes may produce endogenous H₂S, we have shown that DES1 activity is required for ABA-dependent stomatal closure, and DES1 acts early in ABA-mediated signal transduction (Scuffi et al., 2014).

This raises the question of whether there is an interplay between H₂S and other components of the guard cell signaling network, such as H₂O₂ production and PLD activity.

RESULTS

H₂S Requires RBOHF and RBOHD for the Induction of Stomatal Closure

To analyze the putative interaction between H₂S and H₂O₂, we assessed the ability of H₂O₂ to induce stomatal closure in H₂S synthesis mutants, and the ability of H₂S to induce stomatal closure in RBOHF and RBOHD mutants. First, we prepared isolated epidermal peels (epidermal strips) from wild-type *Arabidopsis* plants and from DES1 knockout mutants (*des1-1* and *des1-2*). To induce stomatal opening, the epidermal strips were preincubated in opening buffer for 3 h in white light. Then, the strips were treated with 100 μ M H₂O₂ for 90 min. H₂O₂ induced a 25% reduction of the stomatal pore width in both the wild type and *des1* mutants, indicating that H₂O₂ acts either downstream or independently of H₂S (Fig. 1, A and B). The NADPHox isoforms RBOHD and RBOHF are responsible for the majority of guard cell H₂O₂ production (Kwak et al., 2003; Nühse et al., 2007; Zhang et al., 2007). Accordingly, we analyzed H₂S-induced stomatal closure in the *rboh*d and *rboh*f mutants. Epidermal strips from the wild type and *rboh* mutants were preincubated in opening buffer, and then treated with 100 μ M of sodium hydrosulfide (NaHS) and morpholin-4-ium4 methoxyphenyl(morpholino) phosphinodithioate (GYY4137), which are H₂S donors. Both H₂S donors induced stomatal closure by about 20% in the wild type, confirming previous observations (Scuffi et al., 2014); however, no closure was evidenced in the *rboh*-mutant lines (Fig. 2, A–D). Moreover, stomatal closure was impaired in wild-type epidermal strips treated with 100 μ M NaHS in the presence of the flavoenzyme inhibitor diphenyleneiodonium (DPI), used to inhibit NADPHox (Fig. 2E), validating our observations in the NADPHox mutants. These results indicated that there is interplay between H₂S and H₂O₂ and suggested that NADPHox acts downstream of H₂S.

H₂S Increases H₂O₂ Production in Guard Cells

To analyze H₂O₂ production in response to H₂S inside the guard cells, we used the fluorescent protein biosensor roGFP2-Orp1, which consists of the yeast peroxidase Orp1 fused to redox-sensitive roGFP2 to generate a proximity-based thiol relay that passes on

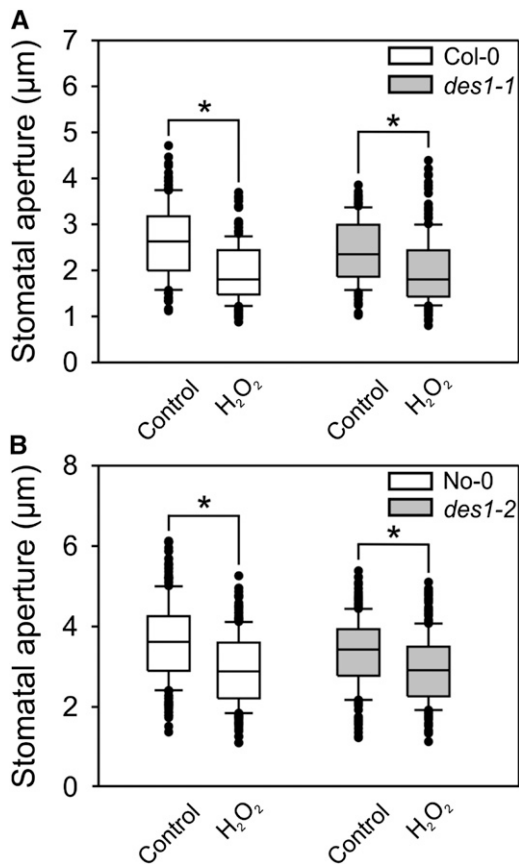


Figure 1. H₂O₂ induces stomatal closure in *des1* mutants. Epidermal strips from two independent Arabidopsis mutants, *des1-1* (A) and *des1-2* (B), and their genetic backgrounds, Col-0 and No-0, respectively, were pre-incubated for 3 h in opening buffer (5 mM K-MES, pH 6.1, and 50 mM KCl) under light and subsequently treated for 90 min with 100 μ M H₂O₂. The values of stomatal aperture are expressed in microns and represented in the box plot where the box is bounded by the 25th to 75th percentile, whiskers span 10th to 90th percentile, and the line in the middle is the median. The individual points represent outliers. Data are from at least three independent experiments. Asterisks denote statistical differences with respect to the control treatment (Mann-Whitney rank sum test, $P < 0.001$).

the H₂O₂-induced oxidation of Orp1 to roGFP2 by thiol-disulfide exchange (Schwarzländer et al., 2016). This biosensor can dynamically monitor intracellular H₂O₂ fluctuations in vivo (Gutscher et al., 2009). We generated stable Arabidopsis lines expressing roGFP2-Orp1 in the cytosol. Since H₂S is a reductant and H₂O₂ is an oxidant, and both are likely to affect the cellular thiol systems, we also used plants expressing the biosensor Grx1-roGFP2 to monitor the cytosolic glutathione redox potential (E_{GSH}). This construct is a fusion of roGFP2 and human glutaredoxin 1 (Grx1) for rapid equilibration with the local glutathione pool to provide a readout for E_{GSH} in real time (Gutscher et al., 2008). Epidermal strips from plants expressing the H₂O₂ biosensor roGFP2-Orp1 and from plants expressing the E_{GSH} biosensor Grx1-roGFP2 were used to assess the effect of the H₂S donors on H₂O₂ production and E_{GSH} . We observed that immediately after peeling, both roGFP2-Orp1 and Grx1-roGFP2 biosensors

showed pronounced oxidation, probably as a response to the mechanical stress associated with the procedure (Supplemental Fig. S1). Therefore, we extended the pre-incubation time in opening buffer to allow for the recovery of guard cell redox physiology (Supplemental Fig. S1). Oxidation started to decrease only after 4 h, reaching steady-state levels comparable to the intact leaf after 7 h of preincubation (Supplemental Fig. S1). Accordingly, a 7-h preincubation was used for the assays. We then treated epidermal strips with 100 μ M NaHS. This H₂S donor led to a rapid oxidation of roGFP2-Orp1 in guard cells (Fig. 3, A and B); however, E_{GSH} also responded as indicated by an oxidative shift of the Grx1-roGFP2 sensor (Fig. 3, D and E). We then assayed GYY4137 as an alternative, tissue-permeating, slow release H₂S donor (Li et al., 2008). GYY4137 also induced oxidation of roGFP2-Orp1 and Grx1-roGFP2 (Fig. 3, A, C, D, and F).

Epidermal strips have been a valuable and widely used model to study guard cell dynamics, but our observation of transient oxidation suggests that epidermal strips have a weakened redox buffering capacity, even after the extended recovery period. To test whether the observed oxidative shifts in guard cells of epidermal strips also occur in intact leaves, we assessed H₂S treatments in intact leaves. Leaves from plants expressing roGFP2-Orp1 or Grx1-roGFP2 were incubated in opening buffer for 30 min and then treated with NaHS and GYY4137. Both sensors were reduced under the control conditions and NaHS showed no oxidative effect in the guard cells (Fig. 4, A, B, D, and E). By contrast, GYY4137 induced a rapid increase in roGFP2-Orp1 oxidation (Fig. 4, A and C), whereas the redox status of Grx1-roGFP2 was unchanged compared to the control (Fig. 4, D and F). Interestingly, when the roGFP2-Orp1 fluorescence ratio was analyzed in the pavement cells, there was no response to the H₂S treatment (Supplemental Fig. S2), indicating that oxidation occurred with some specificity in guard cells. These observations indicated that oxidation of roGFP2-Orp1 in the guard cells is due to the production of H₂O₂ in H₂S-treated leaves.

In an independent set of experiments, we used the ROS-sensitive probe H₂DCF-DA to examine ROS levels in H₂S-treated plants. First, epidermal strips from wild-type plants were loaded with H₂DCF-DA and then treated with 100 μ M of NaHS. H₂S induced a 2-fold increase in DCF fluorescence compared to the control (Supplemental Fig. S3, A and B). To determine if NADPHox activity is required for the increase of ROS production in guard cells after H₂S treatment, the epidermal strips were treated with NaHS in the presence of DPI. DPI inhibited the H₂S-induced ROS production, indicating that H₂S induces ROS production through the activity of NADPHox (Supplemental Fig. S3, A and B). Taken together, our results suggest that H₂S induces the production of H₂O₂ in guard cells.

H₂S Requires PLD α 1 and PLD δ for the Induction of Stomatal Closure

PLD α 1 and PLD δ are the two members of the PLD multigene family expressed in guard cells.

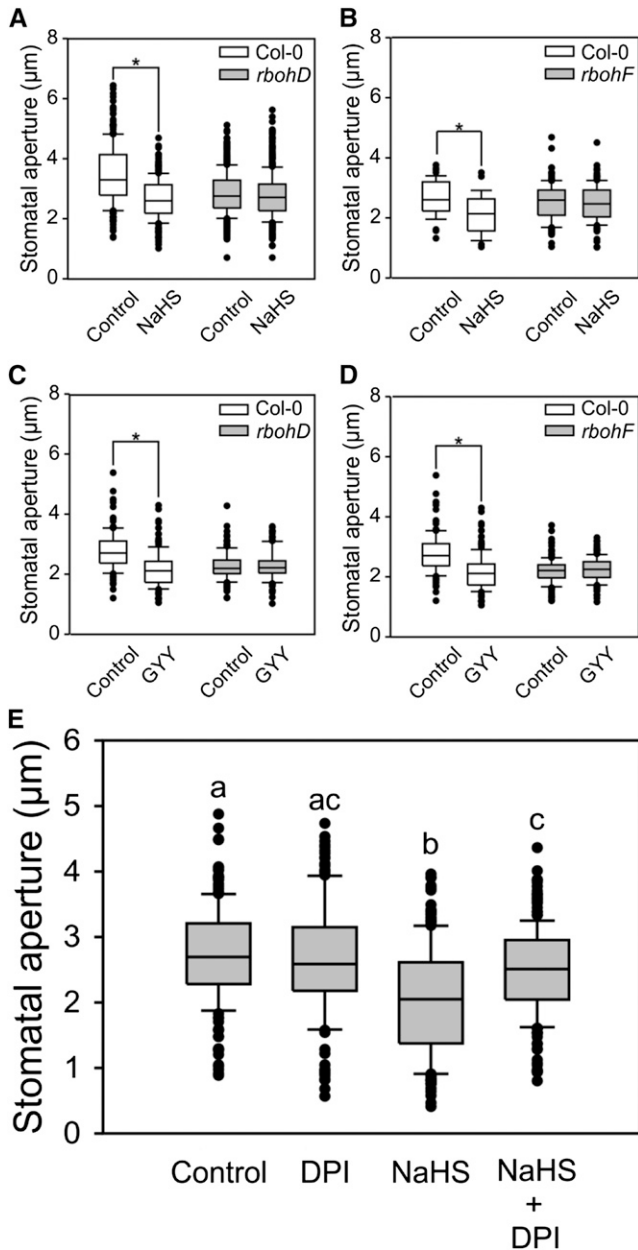


Figure 2. NADPH oxidase is involved in H₂S-induced stomatal closure. Epidermal strips from *Arabidopsis* plants were incubated in opening buffer (5 mM MES, pH 6.1, and 50 mM KCl) under light for 3 h and subsequently subjected to different treatments for 90 min under light. Epidermal strips from the *rbohD* (A and C) and *rbohF* (B and D) mutants were treated for 90 min under light with 100 µM of H₂S donors (NaHS [A and B] and GYY4137 [C and D]). Epidermal strips from the wild type (Col-0) were treated with 10 µM of the flavoenzyme inhibitor DPI, 100 µM of the H₂S donor NaHS, or both (E). The values of stomatal aperture are expressed in microns and represented in the box plot where the box is bound by the 25th to 75th percentile, whiskers span 10th to 90th percentile, and the line in the middle is the median. The individual points represent outliers. Data are from at least three independent experiments. Asterisks denote statistical differences with respect to the control treatment (Mann-Whitney rank sum test, $P < 0.001$). Different letters indicate statistical differences among treatments (Dunn's post hoc test, $P < 0.05$).

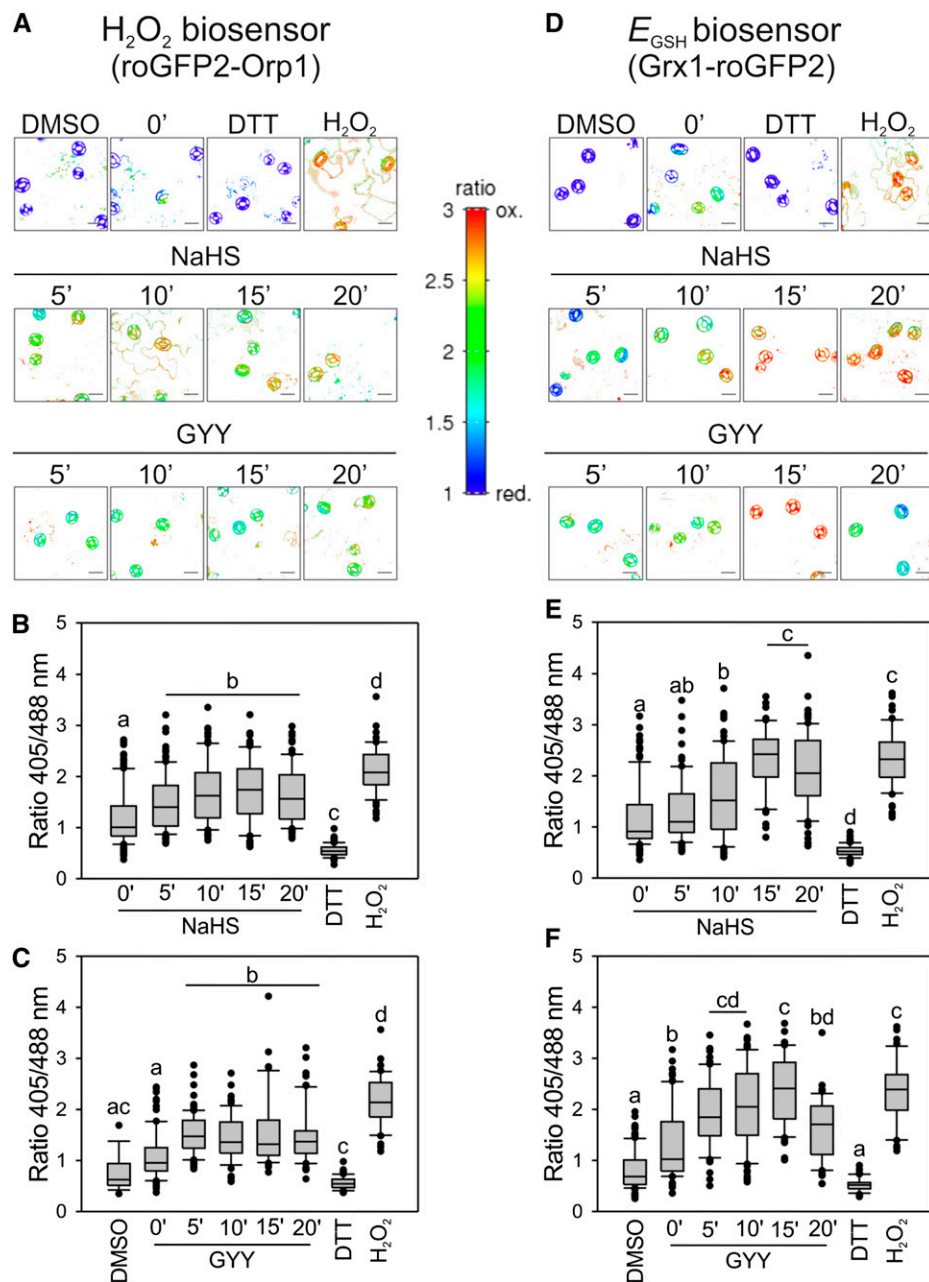
Both isoforms participate in ABA-dependent stomatal closure, although only one of them, PLD α 1, is involved in the regulation of ROS production (Zhang et al., 2009; Distéfano et al., 2012). Therefore, we assessed the participation of the two PLDs in the regulation of H₂O₂ production in response to H₂S. Epidermal peels from the *pld* mutant plants expressing roGFP2-Orp1 (*pld α 1 x roGFP2-Orp1 and *pld δ X roGFP2-Orp1) were incubated in opening buffer for 7 h and subsequently treated with NaHS and GYY4137 for 15 min. The H₂S donor GYY4137 shifted the redox state of roGFP2-Orp1 to around 50% sensor oxidation in the guard cells of wild-type plants and to 90% in the *pld δ mutants. In addition, the NaHS treatment caused an increase in sensor oxidation in the guard cells of the wild type and the *pld δ mutants, but not in the *pld α 1 mutants (Fig. 5). In contrast, the *pld α 1 background, despite showing some biological variability, displayed a 20% shift in the redox state of roGFP2-Orp1 in response to H₂S treatment (Fig. 5). In addition, when epidermal strips were loaded with H₂DCF-DA and then treated with 100 µM NaHS, the wild type and the *pld δ mutants, but not the *pld α 1 mutants, showed increased levels of DCF fluorescence (Supplemental Fig. S3, A and C). These results suggest that PLD α 1, but not PLD δ , is required for H₂S-induced H₂O₂ production.********

To further investigate the participation of PLDs in H₂S-dependent signaling, we assessed the stomatal closure response to H₂S in epidermal strips from the *pld α 1 and *pld δ mutants. Despite our finding that only PLD α 1 is involved in the regulation of ROS production, H₂S-dependent stomatal closure was blocked in both *pld α 1 and *pld δ lines (Fig. 6, A and B), suggesting that both PLD isoforms are required for H₂S-mediated stomatal closure.****

We also assessed the effect of H₂S on the production of PA as the active messenger generated by the PLDs. Radioactive labeling and quantification of guard cell phospholipids in epidermal strips (where guard cells represent around 90% of the living cells) was previously performed in broad bean (*Vicia faba*), since the leaf size and straightforward epidermal extraction procedures facilitate obtaining the required epidermal tissue mass for the analysis (Distéfano et al., 2008). Yet, the repertoire of genetic tools for *V. faba* is limited and suitable PLD mutants have not been reported. Hence, we used *Arabidopsis* epidermal strips from wild-type and PLD mutant plants to quantify PA production. Epidermal strips were labeled with ³²P_i for 3 h in opening buffer under white light and then treated for 1 h with 100 µM NaHS under the same conditions. Lipids were extracted, separated, and quantified using a phosphor imaging. H₂S induced a 40% increase in PA levels in epidermal strips of wild-type and *pld α 1 plants (Fig. 7). However, NaHS treatment did not increase PA levels in epidermal strips of the *pld δ mutants. These results indicated that H₂S requires the activity of PLD δ for the induction of PA.**

We next analyzed the production of PA in response to H₂S at the whole-leaf level. *Arabidopsis* leaf discs were labeled overnight with ³²P_i in opening buffer, and then treated with 100 µM NaHS for 1 h. After 1 h of H₂S

Figure 3. H₂S donors lead to oxidation of roGFP2-Orp1 and Grx1-roGFP2 in guard cells. Epidermal peels from *Arabidopsis* leaves expressing the H₂O₂ biosensor, roGFP2-Orp1 (A–C), and the E_{GSH} biosensor, Grx1-roGFP2 (D–F), in the cytosol were incubated in opening buffer (5 mM MES, pH 6.1, and 50 mM KCl) for 7 h under light and then treated with: 0.1% (v/v) DMSO for 15 min, 100 μM of NaHS or GYY4137 for 0, 5, 10, 15, or 20 min, 20 mM DTT for 10 min, and 10 mM H₂O₂ for 10 min under the same conditions. Pseudocolor ratio images of representative peels (A and D). Scale bar = 10 μm. The values are expressed as the ratio of 405/488 nm and are represented in the box plots where the box is bound by the 25th to 75th percentile, whiskers span 10th to 90th percentile, and the line in the middle is the median. The individual points represent outliers (B, C, E, and F). Data are from at least three independent experiments. Different letters denote statistical differences between treatments (Dunn's post hoc test, *P* < 0.05).



treatment, PA levels increased by about 40% in the wild type (Supplemental Fig. S4). To identify the source of the PA increase, we performed similar experiments using leaf discs from both *pld* mutants. Both the wild type and the *plda1* mutant showed a comparable increase of PA, while PA levels in the *pldd* mutant remained unchanged (Supplemental Fig. S4). These results indicated that PLD δ is also required for the production of PA at the whole-leaf level, similar to our observations in the epidermal strips.

Previous reports postulated that PLD δ acts downstream of ROS production in guard cells (Distéfano et al., 2012; Guo et al., 2012). To investigate if H₂S-induced

PLD δ activity is also RBOH dependent, we analyzed PLD δ activity in *rboh* mutant leaf discs. Increased PA levels were found in leaf discs of the wild type and, to a lesser extent, in the *rbohF* mutants (about 30%; Fig. 8). This increase was not observed in the *rbohD* or *rbohD/F* double mutant plants, suggesting that H₂S requires RBOHD to induce PLD δ -derived PA.

DISCUSSION

The activities of the different isoforms of NADPHox, which participate in the process of stomatal closure, can vary according to the stimulus that triggers

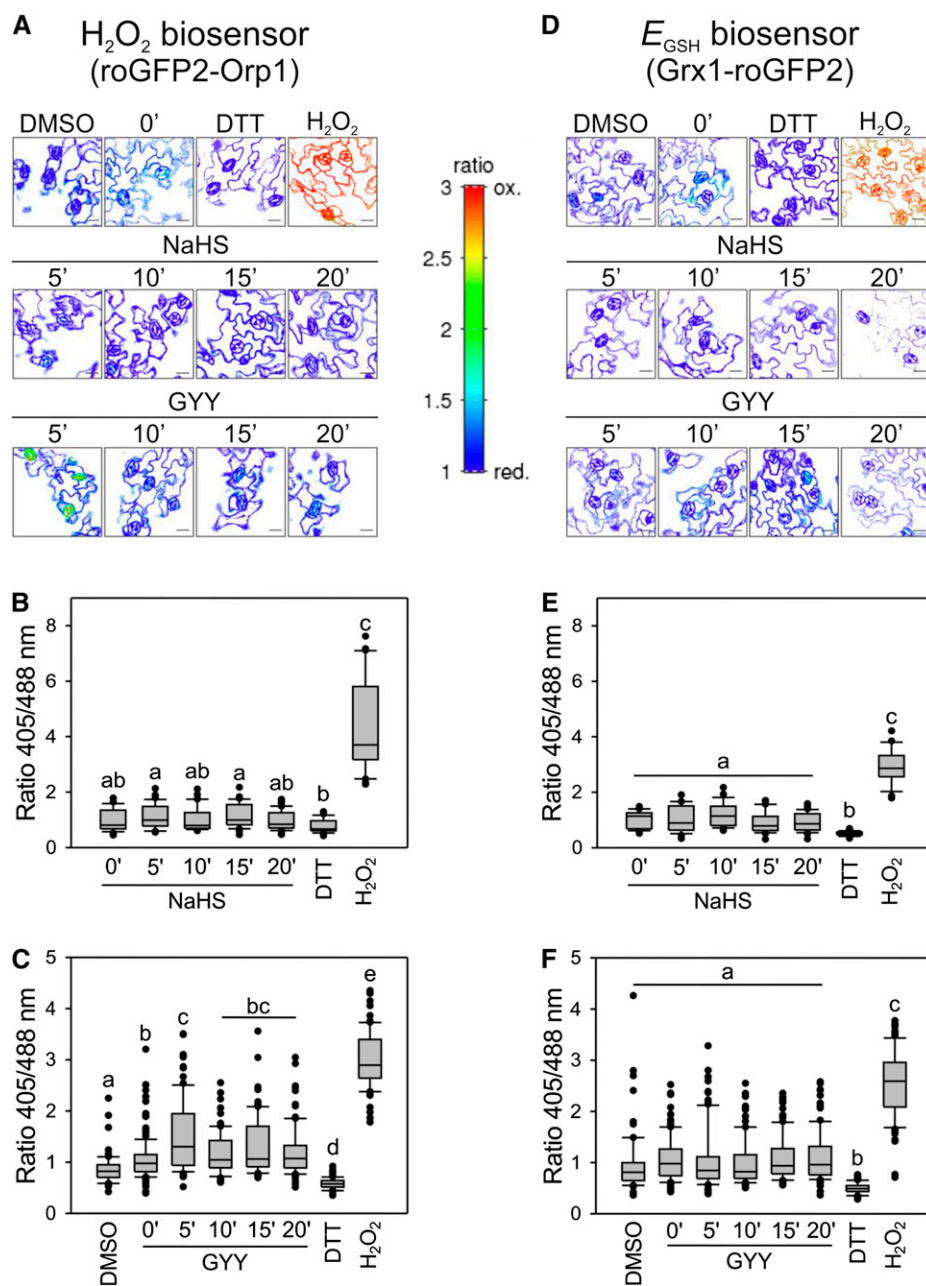


Figure 4. The GYY4137 treatment leads to oxidation of the roGFP2-Orp1 sensor specifically in guard cells. Whole Arabidopsis leaves expressing the H₂O₂ biosensor, roGFP2-Orp1 (A–C), and the E_{GSH} biosensor, Grx1-roGFP2 (D–F), in the cytosol were incubated in opening buffer (5 mM MES, pH 6.1, and 50 mM KCl) for 30 min under light and then treated with: 0.1% (v/v) DMSO for 15 min, 100 μM of NaHS or GYY4137 for 0, 5, 10, 15, or 20 min, 20 mM DTT for 10 min, and 10 mM H₂O₂ for 20 min under the same conditions. Pseudo-color ratio images of representative leaves (A and D). Scale bar = 10 μm. The values are expressed as the ratio of 405/488 nm and are represented in the box plots where the box is bound by the 25th to 75th percentile, whiskers span 10th to 90th percentile, and the line in the middle is the median. The individual points represent outliers (B, C, E, and F). Data are from at least three independent experiments. Different letters denote statistical differences between treatments (Dunn's post hoc test, $P < 0.05$).

the response. In pathogen-associated molecular pattern-induced stomatal closure, ROS production is dependent on RBOHD activity (Nühse et al., 2007; Zhang et al., 2007), whereas ROS production in the ABA-induced response depends mostly on RBOHF activity (Kwak et al., 2003). The stomatal responses to H₂S treatments showed additional differences. Our data indicate that H₂S requires the RBOHD and RBOHF isoforms to induce stomatal closure. The role of RBOHF acting downstream of H₂S matches our previous finding that H₂S participates in ABA-dependent stomatal closure (Scuffi et al., 2014). In addition, the fact that RBOHD is required for the induction of stomatal

closure suggests that H₂S could be a key component in those responses for which ROS production is largely dependent on RBOHD activity, such as plant-pathogen interactions. Preliminary data obtained in our laboratory indicate that the H₂S/DES1 pathway participates in the response to the bacterial elicitor flagellin. The H₂S scavenger hypotaurine partially blocked stomatal closure in response to the 22-amino acid flagellin fragment (flg22). In addition, *des1* mutant plants do not close their stomata in response to treatment with flg22 (Supplemental Fig. S5), supporting our hypothesis that H₂S can act in different signaling pathways that lead to stomatal closure.

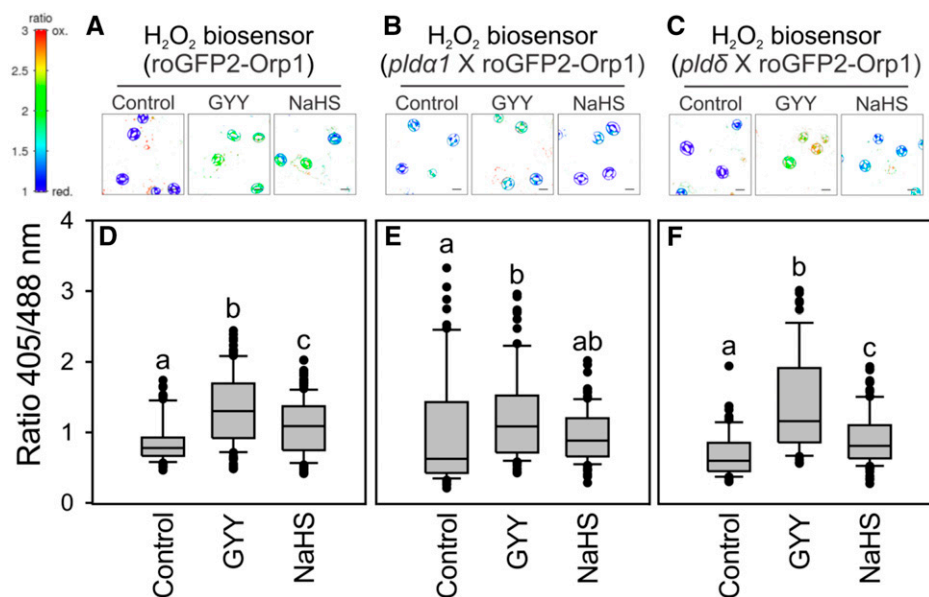


Figure 5. PLD α 1 is required for H₂S-dependent induction of H₂O₂ levels in guard cells. Epidermal peels from *Arabidopsis* Col-0 (roGFP2-Orp1) (D), *pld α 1* (*pld α 1* × roGFP2-Orp1) (E), and *pld δ* (*pld δ* × roGFP2-Orp1) (F) leaves expressing the H₂O₂ biosensor, roGFP2-Orp1, in the cytosol were incubated in opening buffer (5 mM MES, pH 6.1, and 50 mM KCl) for 7 h under light and then treated with opening buffer (control), or 100 μ M of the H₂S donors, GYY4137 (GYY) or NaHS, for 15 min. Pseudo-color ratio images of representative peels (A–C). Scale bar = 10 μ m. The values are expressed as the ratio of 405/488 nm and are represented in the box plots where the box is bound by the 25th to 75th percentile, whiskers span 10th to 90th percentile, and the line in the middle is the median. The individual points represent outliers (D–F). Data are from at least three independent experiments. Different letters denote statistical differences between treatments (Dunn's post hoc test, $P < 0.05$).

H₂DCF-DA is the most widely used probe for intracellular detection of ROS in plants. However, the probe has numerous limitations and is prone to measurement artifacts and inappropriate interpretation of the results (Bonini et al., 2006). The lack of alternative techniques to monitor intracellular ROS has encouraged the development of new tools for *in vivo* H₂O₂ monitoring. A particularly elegant concept has been the utilization of H₂O₂-sensitive proteins, such as peroxidases, to generate genetically encoded sensors (Winterbourn, 2014; Buettner, 2015). In this study, we used the fluorescent biosensor roGFP2-Orp1 to monitor H₂O₂ production in guard cells. roGFP2-Orp1 is a ratiometric (i.e. self-normalizing) biosensor, and its reactivity and selectivity for H₂O₂ has been demonstrated *in vitro* as well as *in vivo* in yeast and animal cells (Gutscher et al., 2009; Albrecht et al., 2011). The pH stability of the sensor is an important advantage over H₂O₂ sensors of the HyPer family, which can introduce pH artifacts to *in vivo* measurements even through minor intracellular pH changes. Yet, similar to the HyPer sensors, the dynamic response of roGFP2-Orp1 also depends on the endogenous thiol redox systems (discussed in detail by Schwarzlander et al., 2016). We observed that the preparation of the epidermal strips gives rise to sensor oxidation, similar to the response to tissue wounding (Meyer et al., 2007). The occurrence of the oxidation event revealed by our study should be considered when guard cell physiology, and the involvement of

redox-related events in particular, are investigated using epidermal peels. However, the oxidation event observed in our study was transient, allowing measurements to be performed after recovery. Although NADPHox-dependent ROS are produced in the apoplast, we monitored intracellular ROS and redox dynamics, since we expressed roGFP2-based biosensors in the cytosol. The influence of apoplastic ROS production on rapid guard cell cytosolic signaling was supported by different reports showing that ROS can rapidly permeate into the cytosol through aquaporins. Two members of the plasma membrane intrinsic proteins (AtPIP2;1 and AtPIP1;4) were shown to permeate H₂O₂ in response to ABA and pathogen-associated molecular patterns (Grondin et al., 2015; Tian et al., 2016). Moreover, the permeation of H₂O₂ into guard cells through PIP2;1 in response to ABA and *flg22* was recently confirmed using the HyPer biosensor (Rodrigues et al., 2017). To validate the results obtained using roGFP-Orp1, we measured the production of ROS using H₂DCF-DA. Although we only measured fluorescence in the cytosol, we observed a high signal from guard cell chloroplasts under H₂S treatment. This phenomenon has been reported in ozone-treated guard cells (Vahisalu et al., 2010). Targeting of roGFP-Orp1 to different organelles in future studies may help to better understand H₂O₂ dynamics in different subcellular compartments.

Since roGFP2-based biosensors contain redox-sensitive Cys residues, this biosensor qualifies as a

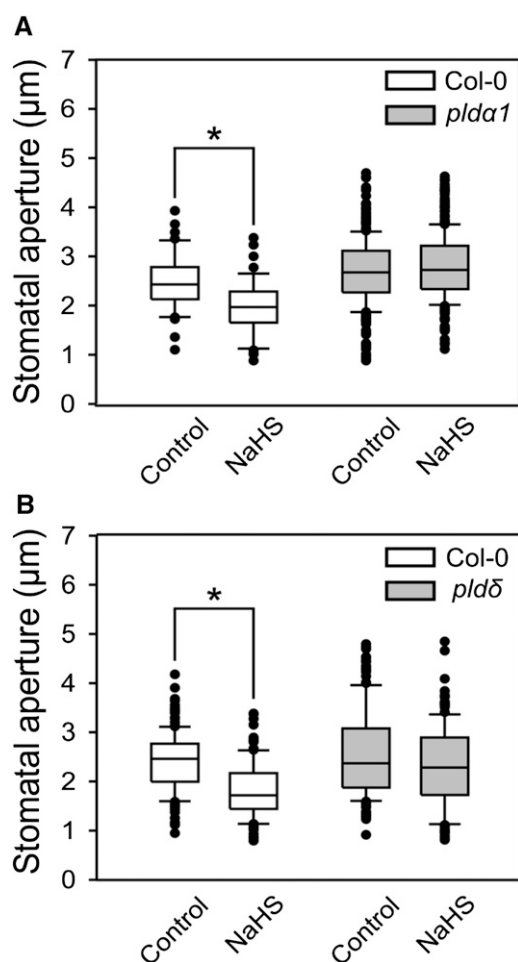


Figure 6. PLD α 1 and PLD δ are required for H₂S-induced stomatal closure. Epidermal strips from *Arabidopsis pld α 1* (A) and *pld δ* (B) mutants and wild-type (Col-0) plants were preincubated in opening buffer (5 mM MES, pH 6.1, and 50 mM KCl) under light for 3 h and then treated for 90 min under light with 100 μ M of the H₂S donor, NaHS. The values of stomatal closure are expressed in microns and are represented in the box plots where the box is bound by the 25th to 75th percentile, whiskers span 10th to 90th percentile, and the line in the middle is the median. The individual points represent outliers. Data are from at least three independent experiments. Asterisks denote statistical differences with respect to the control treatment (Mann-Whitney rank sum test, $P < 0.001$).

potential target for H₂S, and we cannot rule out the possibility of direct sensor modification by H₂S. H₂S acts as a reductant inducing protein S-sulphydration, while oxidation (i.e. formation of an intramolecular Cys disulfide) of the biosensor was observed. This makes it unlikely that the observed response was the result of direct sensor modification and supports H₂S-triggered H₂O₂ production as a logical and sufficient interpretation. The responses of the biosensors in the epidermal strips may be explained by H₂O₂ oxidizing the glutathione pool, which serves as a source of reductant for several peroxidase systems. The absence of glutathione oxidation, as observed in whole-leaf guard cells, may be

due to a higher redox buffering capacity. Differences in the maximal in vivo response of a sensor to saturating conditions have been consistently observed and can vary between tissues and compartments (Schwarzländer et al., 2008, 2016).

The existing models of guard cell signaling suggest that in addition to phosphorylation and to calcium binding, other mechanisms are involved in the regulation of NADPHox in guard cells (for a recent review, see Sierla et al., 2016). For example, the activation of PLDs and their product, PA, were suggested as regulators of ROS production in guard cells (Zhang et al., 2009; Distéfano et al., 2012; Uraji et al., 2012; Kalachova et al., 2013). PA binds to the stomatal closure-related proteins ABI1 and NADPHox (Zhang et al., 2004, 2009), to glyceraldehyde-3-phosphate dehydrogenase (Guo et al., 2012), MAPK6 (Yu et al., 2010), and microtubules (Zhang et al., 2012). In response to ABA, PA produced by PLD α 1 generates ROS via NADPHox, and downstream ROS production acts as a signal to produce PA via PLD δ activity in an NO-dependent manner (Distéfano et al., 2012; Uraji et al., 2012). In this study, we showed that: (1) H₂S induced the production of PA via the activity of PLD δ in a RBOHD-dependent pathway, (2) under our experimental conditions, PLD α 1-derived PA in guard cells did not contribute to the PA pool measured after H₂S treatments, and (3) the responses generated by H₂S in the guard cells were not always identical to those generated by ABA, as was previously reported (Papanatsiou et al., 2015).

Several plausible scenarios may explain the differences with the model proposed for ABA-dependent

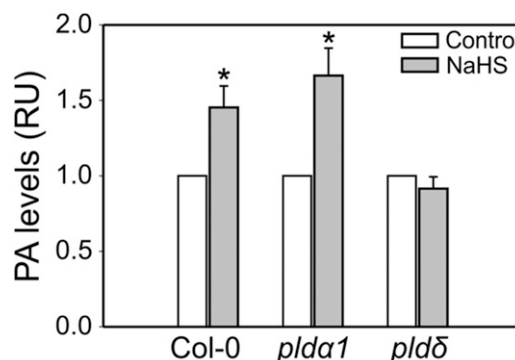


Figure 7. H₂S induces PA levels through PLD δ activity in *Arabidopsis* guard cells. Epidermal peels from the wild type (Col-0), and *pld α 1* and *pld δ* mutant *Arabidopsis* plants were incubated in opening buffer (5 mM MES, pH 6.1, and 50 mM KCl) in the presence of ³²P_i for 3 h under light in a wet chamber and then treated with 100 μ M of the H₂S donor, NaHS, for 1 h. Lipids were extracted, separated by EtAc TLC, and PA levels were quantified and expressed as a fold increase with respect to the controls. The values are expressed as means \pm SE as relative units (RU) from at least three independent experiments. Asterisks denote statistical difference with respect to the control treatment (Mann-Whitney rank sum test; $P = 0.003$ for Col-0, $P = 0.305$ for *pld δ* and t test; $P = 0.006$ for *pld α 1*). The percentage of PA in control treatments were: Col-0 = 6.6340 ± 0.8 , *pld α 1* = 7.5380 ± 0.7570 , and *pld δ* = 8.5170 ± 0.6320 .

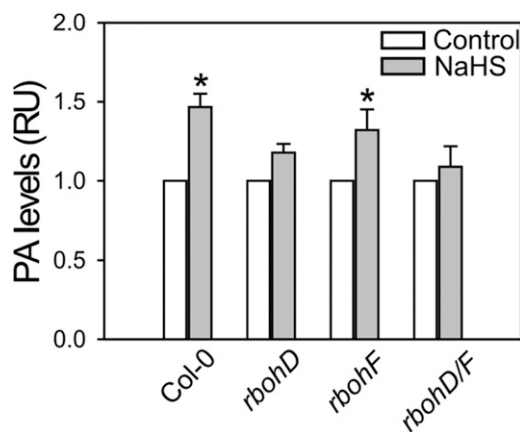


Figure 8. H₂S requires RBOHD to induce PA levels in Arabidopsis leaf discs. Leaf discs from the wild type (Col-0), and *rbohD*, *rbohF*, and *rbohD/F* double mutant Arabidopsis plants were labeled with ³²P_i overnight in opening buffer (5 mM MES, pH 6.1, and 50 mM KCl) under light in a wet chamber and then treated with 100 μM of the H₂S donor, NaHS, for 1 h. Lipids were extracted and separated by EtAc TLC. PA levels were quantified and expressed as a fold increase in relation to the controls. Error bars represent SE of means ($n \geq 3$). Asterisks denote statistical differences with respect to each control treatment (Student's *t* test, $P < 0.001$ for Col-0, $P = 0.111$ for *rbohD*, $P = 0.013$ for *rbohF*, and $P = 0.717$ for *rbohD/F*). The percentage of PA in control treatments were: Col-0 = 0.4690 ± 0.0358 , *rbohD* = 0.5779 ± 0.0403 , *rbohF* = 0.5602 ± 0.0402 , and *rbohD/F* = 0.4827 ± 0.0553 .

signaling. One may be due to the differences in the subcellular localization of PLD proteins and NADPHox in response to H₂S. This “spatial mismatch” has been observed before. The Arabidopsis PLD δ isoform is found in the subcellular fraction corresponding to the plasma membrane, and PLD α 1 was also detected in the plasma membrane, but predominantly in the soluble fraction (Wang and Wang, 2001). Another study reported that tomato PLD β 1 relocalized from the cytosol to punctuate structures close to the plasma membrane, while PLD α 1 remained in the cytosol in suspension-cultured tomato cells treated with xylanase (Bargmann et al., 2006). Thus, PA derived from different PLDs will be differentially localized, giving specificity to the signal that triggers the response. The fact that we could not detect PLD α 1-derived PA may be due to the techniques and experimental conditions used to measure PA, related with localization, quantity, and/or timing.

Another explanation is related to the organization of the guard cell cytoskeleton, a key structure for the modulation of stomatal movement (Higaki et al., 2010). Given that PLD δ modulates the cytoskeleton by forming physical bridges between the microtubules and the plasma membrane (Andreeva et al., 2009; Ho et al., 2009), and that H₂S regulates microtubule organization in root hairs (Jia et al., 2015), it can be postulated that PLD δ -derived PA might affect stomatal closure in response to H₂S via modulation of microtubule organization.

We previously found that PLD and PLC activity are required for NO to induce stomatal closure (Distéfano

et al., 2008, 2012) and that H₂S induces NO production in ABA-mediated responses (Scuffi et al., 2014). These data are not enough to establish if the PLC/diacylglycerol kinase pathway is linked to H₂S-induced stomatal closure, but NO appears as a probable link between both pathways.

In summary, H₂S appears to induce stomatal closure via the participation of RBOHD, RBOHF, PLD α 1, and PLD δ through a bifurcated pathway. It can be concluded that H₂S induces ROS production via the activation of PLD α 1 and the production of PA occurs via deactivation of PLD δ and that the absence of either of these two pathways prevents H₂S from inducing stomatal closure.

MATERIALS AND METHODS

Plant Materials, Chemicals, and Stomatal Assays

Arabidopsis (*Arabidopsis thaliana*) Columbia-0 (Col-0) and Nössen (No-0) wild types, and the *des1-1* (SALK_103855) and *des1-2* (RIKEN RATM13-27151_G) mutants were kindly provided by Dr. Cecilia Gotor; The *pld δ* (SALK_023247) and *pld α 1* (SALK_067533) mutants were kindly provided by Dr. Munnik, and the *rbohD* and *rbohF* mutants were kindly provided by Prof. Jonathan Jones at The Sainsbury Laboratory, Norwich. Arabidopsis sensor lines with stable, cyto-nuclear expression of the Grx1-roGFP2 biosensor were used as described by (Marty et al., 2009). To generate the roGFP2-Orp1 Arabidopsis lines, the roGFP2-Orp1 sequence (Cys-36 and Cys-82; Gutscher et al., 2009) was amplified, and Gateway cloning sites were attached by PCR with the primers 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGTGAGCAA-GGCGGAGGAG-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTCTATCCACCTCTTTCAAAGTTC-3'. The PCR product was cloned into the pDONR207 vector (Thermo Fisher Scientific). After sequencing, the roGFP2-Orp1 insert of the Gateway entry clone was transferred to the pH2GW7 vector (Karimi et al., 2002) under the control of a CaMV35S promoter. The destination vector carrying the roGFP2-Orp1 insert was electroporated into *Agrobacterium tumefaciens* strain C58C1 (Deblaere et al., 1985). Arabidopsis plants were transformed by the floral dip method (Clough and Bent, 1998). Seeds were screened for fluorescence and propagated to the T3 generation to select homozygous plants. 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. Mutant *pld* plants (*pld α 1* and *pld δ -1*) were crossed with the roGFP2-Orp1 biosensor lines. The F4 generation (*pld α 1* X roGFP2-Orp1 and *pld δ* X roGFP2-Orp1) plants used were previously characterized by fluorescence for roGFP2-Orp1 and by PCR for T-DNA insertions according to Distéfano et al. (2012).

NaHS, DPI, and hypotaurine were purchased from Sigma. Chemicals for lipid extraction and silica-60 thin-layer chromatography (TLC) plates were purchased from Merck. 2',7'-dichlorodihydro-fluorescein diacetate (H₂DCF-DA) was purchased from Thermo Fisher. flg22 (QRLSTGSRINSKDDAAGLQIA) was synthesized by Genbiotech.

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 (5 mM K-MES, pH 6.1, and 50 mM KCl) for 3 h in the light. 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).

Epifluorescence Microscopy

H₂O₂ was visualized using the ROS-sensitive probe H₂DCF-DA. Arabidopsis epidermal strips were preincubated in opening buffer for 3 h under light and then loaded in the dark with the dye in 10 mM Tris buffer, pH 7.2, for 20 min. The strips were washed three times with fresh 10 mM Tris buffer, pH 7.2, and exposed to different treatments for 10 min in opening buffer. Fluorescence images were obtained using a Nikon DS-Fi 1 digital camera coupled to a Nikon Eclipse E200 epifluorescence microscope with excitation at 488 nm and emission at 505 to 530 nm.

The green fluorescence was quantified using ImageJ analysis software (National Institutes of Health) as the pixel intensity of guard cells, except for the chloroplasts, and subtracting the pixel intensity of the background. The fluorescence values are presented as relative units with respect to the control treatments and are expressed as the means + SE.

Confocal Laser Scanning Microscopy

Epidermal peels from Arabidopsis leaves expressing the H₂O₂ biosensor, roGFP2-Orp1, or the E_{GSH} biosensor, Grx1-roGFP2, in the cytosol were incubated in opening buffer for 30 min, 2 h, 4 h, or 7 h for recovery, or for 7 h and subsequently treated with 100 μM of the H₂S-donors under the same conditions. Whole Arabidopsis leaves were incubated in opening buffer for 30 min under light before treatment with 100 μM of the H₂S-donors. To determine the dynamic range of the spectral response of the biosensors in situ, we used treatments with 10 mM H₂O₂ and 20 mM DTT for full oxidation and reduction, respectively, of the sensors. The epidermal peels and whole leaves were mounted under a Zeiss confocal microscope LSM 780 (Carl Zeiss MicroImaging) as described previously (Wagner et al., 2015). Images were collected with a 63× lens (Plan-Apochromat, 1.40 numerical aperture, oil immersion) and the biosensors were excited sequentially at 405 and 488 nm (line-switching mode) and emission was detected at 508 to 535 nm.

Analysis of Ratiometric Images

Ratiometric images were analyzed using a custom MatLab program package (Fricker, 2016). A region of interest in the cytosol was defined for each guard or pavement cell.

Lipid Labeling, Extraction, and Quantification

Guard Cell Phospholipids Labeling

Guard cell phospholipids were labeled by floating epidermal peels in 600 μL of opening buffer containing 0.06 μCi ³²P_i μL⁻¹ (carrier free) in a 12-well plate in a wet chamber under light for 3 h. Subsequently, epidermal peels were subjected to different treatments for 1 h under light, as indicated. The treatments were stopped by transferring the epidermal peels to a 2-mL Eppendorf tube containing a mixture of 170 μL of opening buffer plus 20 μL of 50% perchloric acid (v/v).

Whole-Leaf Phospholipids Labeling

Whole-leaf phospholipids were labeled by floating leaf discs in opening buffer containing 0.06 μCi ³²P_i μL⁻¹ (carrier free) in a 2-mL Eppendorf tube under light in a wet chamber overnight. Treatments were performed for 1 h in the same tube under light, as indicated.

Lipid Extraction and Quantification

Lipids were extracted by adding 750 μL of CHCl₃/MeOH/HCl (50:100:1, v/v/v) and vortexing for 5 min. The samples were processed as described previously (Munnik and Laxalt, 2013). Lipids were chromatographed using an ethyl acetate solvent system as the mobile phase [the organic upper phase consisted of ethyl acetate/isooctane/formic acid/water (13:2:3:10, v/v/v/v)]. Radiolabeled lipids were visualized by autoradiography (BioMax XAR; Kodak) and quantified by phosphor imaging (Storm; Molecular Dynamics). For each treatment, radioactivity levels of ³²PA were normalized to the amount of radioactivity in the phosphatidylethanolamine and phosphatidylglycerol spots. Finally, ³²PA levels were expressed as a fold increase relative to the control treatment for at least three independent experiments.

Statistical Analyses

Data analyses were performed using SigmaPlot11 for Windows (Systat Software). The statistically significant differences were analyzed using one-way ANOVA, or Student's *t* test, as indicated in the figure legends.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *DESI* (At5g28030), *RBOHD* (At5g47910), *RBOHF* (At1g64060), *PLDα1* (At3g15730), and *PLDδ* (At4g35790).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Redox recovery of epidermal peels in opening buffer.

Supplemental Figure S2. H₂S does not affect cytosolic redox balance in pavement cells.

Supplemental Figure S3. NADPH oxidase activity and PLDα1 are both required for H₂S-dependent induction of ROS production in guard cells.

Supplemental Figure S4. H₂S induces PA production through PLDδ activity in Arabidopsis leaf discs.

Supplemental Figure S5. H₂S is involved in flagellin-induced stomatal closure.

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