Aquaporins facilitate hydrogen peroxide entry into guard cells to mediate ABA- and pathogen-triggered stomatal closure

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Significance

Guard cells play a crucial role in controlling transpiration and the plant water status. Here, we show that the Arabidopsis thaliana plasma membrane aquaporin PIP2;1 is involved in stomatal closure triggered by abscisic acid (ABA) or the pathogen-associated molecular pattern flg22. The use of a genetic probe for hydrogen peroxide (H2O2) revealed that PIP2;1 is also required for intracellular accumulation of H2O2 after flg22 or ABA treatment. Our data lead to a model whereby flg22 and ABA activate PIP2;1 through phosphorylation at a conserved site to facilitate transport of both water and H2O2 and promote stomatal closure. This study fills a gap in our understanding of stomatal regulation and suggests a general signaling role of aquaporins in contexts involving H2O2.


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Stomata are specialized pores formed by two guard cells at the surface of plant aerial parts. Stomata mediate gas exchange between the plant and atmosphere, thereby acting on both the rate of photosynthesis and plant water status (1). Their opening and closing, as triggered by numerous endogenous and environmental stimuli, involve combined movements of ions and water across the guard cell plasma membrane, which, in turn, alter guard cell turgor and volume (2). Abscisic acid (ABA), a key hormone in plant response to water deficit, is a potent inducer of stomatal closure (3). ABA binds to PYR/PYL/RCA receptors, which capture protein phosphatases 2C (4), leading to activation of Snf1-related protein kinases 2 such as SnRK2.6 (OST1) (5). This protein kinase, in turn, activates several types of membrane proteins involved in stomatal closure such as NADPH oxidases (6, 7); the anion channels SLAC1, SLAHL1, and SLAHL3 (8–10); and the plasma membrane aquaporin (AQP) AtPIP2;1 (11).

Stomata are also a potential entry gate for pathogens. While plants have the capacity to close their stomata after perception of pathogen-associated molecular patterns (PAMPs) or damaged associated molecular patterns (DAMPs) (12), some pathogens can, in turn, thwart the stomatal closure by means of effectors such as coronatine (12) or HoPM1 (13). Signaling pathways involved in guard cell response to pathogens have been the focus of recent studies (14). Notably, flg22 (a PAMP from the bacterium Pseudomonas syringae pv. tomato) is perceived by the receptor kinase FLS2 which, in interaction with BAK1 and BIK1 protein kinases (15), activates NADPH oxidases (16). In conjunction with superoxide dismutases (SOD) and cell wall peroxidases (17), the latter triggers apoplastic production of reactive oxygen species (ROS) (18) and, as a consequence, marked accumulation of hydrogen peroxide (H2O2) in the guard cell cytoplasm. Alternative signaling mechanisms acting downstream of flg22 perception have been proposed. Flg22 would target the same SLAC1 anion channel as ABA does, but through an oxylinin-dependent ABA-independent pathway (19) that merges at OST1 (20).

A role for AQPs was recently established in Arabidopsis thaliana guard cells (11). Plants lacking AtPIP2;1 showed defects in ABA-triggered stomatal closure in epidermal peel assays. This phenotype was associated to cellular defects in both plasma membrane water transport and hormone signaling (ROS accumulation). Furthermore, ABA was found to activate AtPIP2;1 through OST1-mediated phosphorylation of a key cytoplasmic residue (Ser121), this modification being mandatory for ABA-induced stomatal closure (11).

Recent studies have revealed that the function of plant AQPs extends beyond water transport (21). For instance, members of the plasma membrane intrinsic protein (PIP) subfamily facilitate carbon dioxide (CO2) (22) or H2O2 (23, 24) transport in...
heterologous systems. A contribution of AtZIP2:1 to guard cell CO₂ transport was recently proposed, based on functional reconstitution of CO₂ signaling in Xenopus oocytes (25). The significance of H₂O₂ transport by plant AOs with respect to ROS metabolism and detoxification or ROS-dependent signaling in guard cells has not yet been elucidated. By contrast, a role for AtZIP1:4-mediated H₂O₂ transport in plant immunity against the bacterial pathogen Pseudomonas syringae was recently uncovered (26).

In the present work, we used the context of stimulus-induced guard cell movements to explore a putative role of AOs in plant cell signaling. A key point was to express the genetically encoded fluorescent H₂O₂ sensor, HyPer (27), in plant lines altered in AtZIP2:1 function and regulation. Our data establish the significance of H₂O₂ transport by plant AOs during both ABA- and flg22-induced stomatal closure and uncover common signaling components acting on AQP activity.

**Results**

**HyPer Allows Monitoring of H₂O₂ Abundance in Guard Cells.** The expression and subcellular localization of HyPer in guard cells was followed by fluorescence microscopy on isolated leaf epidermis. HyPer fluorescence was essentially observed (Fig. S1A) in the nucleus, perinuclear areas, and close to the plasma membrane, in regions where the cytoplasm is reduced to a thin layer due to large vacuoles (28). HyPer oxidation at a maximal rate of cytoplasmic H₂O₂ accumulation can be monitored by the ratio of fluorescence emission at 530 nm (R) after excitation at 475 nm and 438 nm. In control conditions, R was 0.25 ± 0.1, indicating that HyPer was strongly reduced. Addition of exogenous H₂O₂ (50 μM) on Col-0 epidermal peels induced an increase in R relative to its initial value (R₀), with similar amplitude and kinetics between the three different areas of preferential HyPer expression, with a peak R/R₀ value from 1.12 ± 0.02 to 1.15 ± 0.04 (Fig. S1C).

Thus, the subcellular heterogeneity of HyPer localization in guard cells does not interfere with intracellular H₂O₂ monitoring. Exposure of guard cells to various external H₂O₂ concentrations also showed that HyPer can detect time- and dose-dependent changes in H₂O₂ concentration with a maximal R/R₀ (2.5 ± 0.1) at 2 s after addition of 200 μM H₂O₂, where most of HyPer is oxidized, and a subsequent decrease in signal in the following minute, likely due to cytoplasmic HyPer reduction (Fig. S2). A much fainter and slower transient signal was observed in response to 50 μM H₂O₂.

**ABA- and flg22-Induced Guard Cell Accumulation of H₂O₂ Depends on AtZIP2:1.** We exposed the leaf epidermal peels of Col-0 and two allelic pip2;1 mutants (pip2;1-1, pip2;1-2) to 50 μM ABA by using 0.1% ethanol as a mock (control) treatment. The changes in R/R₀ seen under the latter conditions were subtracted to the R/R₀ changes induced by ABA (Fig. S3), yielding a stimulus-specific HyPer fluorescence signal [Δ(R/R₀)]. In Col-0 plants, ABA induced a transient decrease in signal, by 7 ± 0.3% after 5 min, followed by a steady increase up to 10% after 25–30 min (Fig. L4, Fig. S4 A–D, and Movie S1). No significant difference in Δ(R/R₀) was determined between wild-type and the two pip2;1 genotypes at 5 min after the ABA treatment [pip2;1-1: Δ(R/R₀) = −10.7 ± 0.3%; pip2;1-2: Δ(R/R₀) = −4.7 ± 0.2%]. However, pip2;1 stomata did not show any subsequent increase in Δ(R/R₀) but rather a steady decrease, down to −16% and −12% for pip2;1-1 and pip2;1-2, respectively (Fig. L4, Fig. S4 A and E–G and Movie S2).

To determine the contribution of Δ(R/R₀) of apoplastic H₂O₂, we pretreated Col-0 epidermal peels by catalase (200 U) (Fig. S5). This treatment abolished the intracellular H₂O₂ accumulation observed when ABA was applied, with Δ(R/R₀) decreasing by 17 ± 3% after 30 min while it increased by 10 ± 1% when ABA was applied in the absence of catalase (Fig. S5A). The overall data indicate that AtZIP2:1 is necessary for ABA-dependent accumulation of H₂O₂ in guard cells, this accumulation being contributed by apoplastic H₂O₂.

**Role of AtZIP2:1 in flg22-Induced Stomatal Closure.** We next investigated whether the defect in flg22-induced H₂O₂ accumulation seen in pip2;1 plants could be associated with a defect in stomatal closure in response to flg22, as observed for ABA (11). Stomata of Col-0 and pip2;1 plants and of a pip2;1 complemented mutant line (pip2;1-1 PIP2;1) showed a similar opening response to a light pretreatment (Fig. 2A). However, stomata of pip2;1-1 and pip2;1-2 plants did not close in response to 1 μM flg22, whereas stomata from Col-0 and pip2;1-1 pip2;1-2 reduced their aperture by almost 40% after 2 h. Thus, AtZIP2:1 is required for flg22-induced stomatal closure.

We previously showed that ABA activates AtZIP2:1-mediated guard cell water transport (11). To determine if a similar mechanism
operates in response to flg22, we investigated the effect of flg22 on the $P_t$ of guard cell protoplasts isolated from Col-0, pip2;1-1, pip2;1-2, and pip2;1-1 PIP2;1 plants. In the absence of flg22, all protoplast types had similar $P_t$ in the range of 50–60 μm·s⁻¹ (Col-0: $P_t = 55 ± 10$ μm·s⁻¹). Treatment with 1 μM flg22 increased twofold the $P_t$ of Col-0 (103 ± 10 μm·s⁻¹) and pip2;1-1 PIP2;1 guard cell protoplasts (Fig. 2B). In contrast, the $P_t$ of pip2;1-1 and pip2;1-2 guard cell protoplasts was totally unresponsive to flg22. Thus, flg22, similar to ABA, increases the water transport activity of AtPIP2;1 in guard cells.

**Contribution of AtPIP2;1 To Guard Cell Transport of H₂O₂ in Response to flg22 and ABA.** In view of the activation by ABA and flg22 of AtPIP2;1-dependent $P_t$, we investigated whether AtPIP2;1-mediated H₂O₂ transport is also ABA- and flg22-dependent. Epidermal peels were first pretreated by flg22 (1 μM), ABA (10 μM), or their respective control solution (water or 0.02% ethanol, respectively). Kinetic variations of guard cell $R/R_o$ were then monitored, following sudden exposure to exogenous H₂O₂ (100 μM) (Fig. 3). When epidermal peels of Col-0, pip2;1-1, or pip2;1-2 plants were submitted to control pretreatments, exogenous H₂O₂ induced a similar slow and progressive increase in $R/R_o$ up to a maximum of 1.4, with a slight decay after 30–40 s (Fig. 3 A–F). Col-0 epidermal peels pretreated by flg22 (Fig. 3A) showed a faster HyPer oxidation response to H₂O₂, with a peak $R/R_o$ value of 1.69 ± 0.05 reached at 24 s after H₂O₂ addition. In contrast, pip2;1-1 and pip2;1-2 guard cells pretreated with flg22 (Fig. 3 B and C) showed an HyPer oxidation response similar to that after a control pretreatment, with a maximum $R/R_o$ value reached for both genotypes after 42 s of exposure to exogenous H₂O₂.

ABA also enhanced the HyPer oxidation response of Col-0 guard cells to exogenous H₂O₂, with $R/R_o$ reaching a maximum of 1.67 ± 0.02 after 37 s (Fig. 3D). By comparison, $R/R_o$ in ethanol-pretreated peels showed a maximum of 1.16 ± 0.03 at 45 s following addition of H₂O₂. At variance with Col-0, pip2;1-1 and pip2;1-2 guard cells (Fig. 3 E and F) showed similar and low-amplitude HyPer oxidation response to exogenous H₂O₂ whether pretreated or not with ABA. The data show that pretreatments with flg22 or ABA promote the accumulation of exogenously supplied H₂O₂ in Col-0 guard cells. The lack of such effects in pip2;1 plants suggests that ABA and flg22 activate AtPIP2;1 to increase the guard cell membrane permeability to H₂O₂.

**Protein Kinases Involved in PAMP and ABA Signaling Are Crucial for AtPIP2;1 Function During flg22-Induced Stomatal Closure.** To determine the PAMP signaling components involved in activation of AtPIP2;1 by flg22, we investigated the effect of the peptide on the $P_t$ of guard cell protoplasts isolated from Col-0, fbs2 efr, snrk2.6, and bak1-5 plants (Fig. 4), considering that bak1-5 is a semidominant allele of BAK1 with a specific phenotype related to PAMP responsiveness (29). In the absence of flg22, all protoplast types had similar $P_t$ in the range of 53–65 μm·s⁻¹ (Col-0: $P_t = 60 ± 10$ μm·s⁻¹). While treatment with 1 μM flg22 increased twofold the $P_t$ of Col-0 (113 ± 13 μm·s⁻¹), the $P_t$ of fbs2 efr, bak1-5, and snrk2.6 guard cell protoplasts was totally unresponsive to flg22. The $P_t$ of guard cell protoplasts was also insensitive to 10 μM ABA in snrk2.6, whereas it was enhanced by twofold in Col-0 (132 ± 8 μm·s⁻¹; ref. 11). The overall data indicate that, in guard cells, flg22 increases AtPIP2;1 water transport activity by acting through its receptor (FLS2) and interacting coreceptor (BAK1). Interestingly, OST1 is involved in activation of AtPIP2;1-mediated water transport by both ABA and flg22.

**Role of AtPIP2;1 Ser121 in flg22-Induced Guard Cell Functions.** Phosphorylation of AtPIP2;1 at Ser121 is mandatory for stimulation of both guard cell protoplast $P_t$ and stomatal closure by ABA (11). In vitro phosphorylation (11) and genetic analyses (Fig. 4) suggest that this effect is mediated by OST1. Because the effects of flg22 on guard cell water transport also depend on OST1, we investigated the possible role of Ser121 phosphorylation in this mechanism. We used a pip2;1-2 line expressing phosphorylation-deficient (S121A) or phosphomimetic (S121D) forms of PIP2;1 (11). S121A protoplasts displayed moderate $P_t$ values that were insensitive to a flg22 treatment (Control, $P_t = 57 ± 2$ μm·s⁻¹; flg22, $P_t = 57 ± 3$ μm·s⁻¹) and similar to those in pip2;1-2 plants or Col-0 plants in control conditions (Fig. S8). S121D plants displayed significantly higher $P_t$ values which, however, were also insensitive to flg22 (Control, $P_t = 82 ± 3$ μm·s⁻¹; flg22, $P_t = 86 ± 2$ μm·s⁻¹).

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Fig. 2. Stomatal movement and water transport responses of Col-0, pip2;1-1, pip2;1-2, and pip2;1-1 PIP2;1 to flg22. (A) Epidermal peels from the indicated genotypes were placed in a bathing solution for 3 h under light and further incubated in the absence (white bars) or in the presence of 1 μM flg22 (green bars). Stomatal aperture was measured after 2 h. Data from three independent plant cultures, each with 60 stomata per genotype. Error bars represent SEs. The letters indicate statistically different values (ANOVA, Newman-Keuls: $p < 0.05$). (B) Guard cell protoplasts were isolated from the indicated genotypes and incubated under light in the absence (white bars) or presence (green bars) of 1 μM flg22. Their $P_t$ was measured as described in Materials and Methods. Data from three independent experiments, with a total of n = 12–17 protoplasts per condition. Same conventions as in A.

Fig. 3. Influx of exogenously supplied H₂O₂ in guard cells of Col-0, pip2;1-1, and pip2;1-2 plants. Epidermal peels from Col-0 (A and D), pip2;1-1 (B and E) or pip2;1-2 (C and F) plants were placed under light for 3 h and subsequently treated for 6 min by flg22 (1 μM) (green) or water (yellow) (A–C) or by ABA (10 μM) (black) or ethanol (0.02%) (yellow) (D–F) before application of exogenous H₂O₂. Kinetic changes in HyPer fluorescence ($R/R_o$) were recorded before and after the application of 100 μM H₂O₂ (red arrow at t = 5 s). Error bars represent the SEs from measurements cumulating three independent plant cultures, with a total between 30 and 40 guard cells per genotype.
These data indicate that phosphorylation of AtPIP2;1 on Ser121 is necessary for stimulation of guard cell P$_i$ by flg22. Because of the crucial role of BAK1 in flg22-dependent activation of AtPIP2;1, we investigated the ability of recombinant BAK1 to modify AtPIP2;1 peptides in an in vitro phosphorylation assay with $^{32}$P-labeled ATP (Fig. S9A). In this assay, BAK1 efficiently labeled the generic protein kinase substrate MBP. A C-terminal AtPIP2;1 peptide containing two well-described phosphorylation sites at Ser280 and Ser283 was poorly phosphorylated by BAK1 (Fig. S9A), whereas a 29-residue peptide covering the entire AtPIP2;1 loop B was markedly labeled. While this peptide includes Ser121 and two other Ser/Thr residues, no radiolabeling was observed when Ser121 was substituted by an Ala residue (S121A). The dose dependency of peptide labeling by BAK1 indicated an apparent $K_a$ of the protein kinase for the loop B peptide of 18.2 ± 5 μM (Fig. S9B). These data indicate that, albeit with a lower affinity than OST1, BAK1 can phosphorylate AtPIP2;1, preferentially at Ser121. We next wondered if the AtPIP2;1-dependent H$_2$O$_2$ transport activity observed in response to flg22 (Fig. 3A and C) also depends on Ser121 phosphorylation. We expressed HyPer in the S121A and S121D lines and monitored guard cell HyPer oxidation kinetics. S121A guard cells showed variations of R/R$_0$ in response to exogenous H$_2$O$_2$ that were similar and of low amplitude, whether the epidermis was pretreated or not with flg22 (Fig. S10B). This profile is reminiscent of that seen in pip2;1-2 plants (Fig. 3B and C). Flg22 pretreatment did not alter the HyPer oxidation signal to exogenous H$_2$O$_2$ in S121D guard cells either (Fig. S10C). However, these plants showed, both in the absence or presence of a flg22 pretreatment, high R/R$_0$ peak values of 1.82 ± 0.01 and 1.68 ± 0.02, respectively, at 26 s after exposure to exogenous H$_2$O$_2$ (Fig. S10B and C). The data strongly suggest that Ser121 phosphorylation mediates the stimulating effects of flg22 on the guard cell permeability to H$_2$O$_2$.

We next investigated the significance of this AtPIP2;1 regulation mechanism in integrated responses of stomata to flg22. The peptide induced a marked H$_2$O$_2$ accumulation in both Col-0 and S121D stomata (Fig. 5) with, after 30 min, a maximal increase in Δ(R/R$_0$) of 37% and 46%, respectively. In contrast, S121A guard cells, similar to pip2;1-2, lacked this response and showed a Δ(R/R$_0$) decreasing by 6% after 30 min. With regard to flg22-induced stomatal closure, expression of the Ser121A form of AtPIP2;1 was not able to complement the defect of pip2;1-2 plants whereas expression of the S121D form restored a stomatal closure response similar to Col-0 plants (Fig. S11). In addition, application of catalase on Col-0 or S121D epidermal peels fully abolished the stomatal closure observed in the presence of flg22, thereby mimicking the lack of stomatal response of pip2;1 plants to flg22 (Fig. S12). Altogether, these data pinpoint the requirement of AtPIP2;1 Ser121 phosphorylation for flg22-induced accumulation of H$_2$O$_2$ in guard cells and subsequent stomatal closure.

**Discussion**

**Signaling Function of AtPIP2;1 in Guard Cells.** We previously established an essential role of AtPIP2;1 in ABA-induced stomatal closure (11). In this initial study, we screened abiotic stimuli acting on stomatal movements and found no obvious involvement of AtPIP2;1 in guard cell response to CO$_2$, light, or darkness. In line with AtPIP2;1 contribution to ABA-induced stomatal closure, the P$_i$ of guard cell protoplasts was enhanced by ABA through activation of AtPIP2;1. Assays using H$_2$DCFDA, a generic ROS probe, also revealed a defect of pip2;1 plants in ABA-dependent ROS signaling, indicating that the role of AtPIP2;1 in guard cells may go beyond its canonical water channel function. Independent growth tests and transport assays using H$_2$DCFDA have established, indeed, that AtPIP2;1 can facilitate ROS diffusion in yeast (24, 30). In addition, a role in plant defense was recently attributed to the AtPIP2;1 homolog, based on its ability to transport H$_2$O$_2$ in the mesophyll (26). Thus, we assumed that AQPs and AtPIP2;1 in particular may play a general role in H$_2$O$_2$-dependent signaling. Here, we used the guard cell system and investigated stimuli which, besides ABA, involve H$_2$O$_2$ signaling. The role of AtPIP2;1 in flg22-induced stomatal closure was therefore uncovered.

Another key point was to use the genetically encoded H$_2$O$_2$ sensor HyPer for kinetic monitoring of intracellular H$_2$O$_2$ in various genetic backgrounds. This approach was instrumental to show that both ABA and flg22 trigger within a few minutes an accumulation of H$_2$O$_2$ in the guard cell cytoplasm. We also showed that this accumulation was not due to possible confounding effects of the AQP on cytosolic pH but originates from H$_2$O$_2$ produced in the apoplast and requires AtPIP2;1.

Another important analogy between ABA and flg22 is that they both enhance within minutes the water permeability P$_i$ of the guard cell plasma membrane. We therefore assumed that the associated activation of AtPIP2;1 may also play a role in H$_2$O$_2$ transport. Although our assay cannot be considered as a genuine measurement of H$_2$O$_2$ membrane permeability, the finding that flg22 and ABA pretreatments favor the influx of exogenous H$_2$O$_2$ in an AtPIP2;1-dependent manner provides strong evidence that AtPIP2;1 transports H$_2$O$_2$ through the guard cell plasma membrane.
thereby contributing to ABA and flg22 signaling during stomatal closure. AtPIP2;1 also plays a signaling role during PAMP-triggered immunity (26), but whether this aquaporin is also activated during this process remains unknown. As AtPIP2;1 is the most abundant PIP2 in guard cells, we speculate that transport by PIP1s of water and/or H\(_2\)O\(_2\) at the plasma membrane may require heteromerization with PIP2s, and preferentially AtPIP2;1, thereby explaining the strong stomatal phenotype of the single pip2;1 mutants. Altogether, these findings are reminiscent of results obtained in animal cells. A pioneering work using HyPer unraveled the role of AQP3 in H\(_2\)O\(_2\) transport and epidermal growth factor (EGF) signaling (31). This function was recently extended to NF-κB signaling in keratinocytes (32) or in response to environmental stresses in colon epithelia (33). Similarly, AQP8 facilitates cellular accumulation of H\(_2\)O\(_2\) after VEGF stimulation, thereby enhancing PI3K activity and phosphorylation of MAPKs, two essential processes for plant cell proliferation (34).

Combined with our previous work (11), the present study indicates that the contribution of AtPIP2;1 to guard cell responses to ABA and flg22 involves both a signaling and a hydraulic function. Interestingly, pip2;1 plants showed impaired stomatal movements in response to ABA (11) and flg22 (this study) instead of a reduced rate of closure, as could be expected from a simple decrease in cell water permeability. This suggests that the signaling function of AtPIP2;1 may somewhat dominate in these contexts. However, a hydraulic and a signaling role are not exclusive. As H\(_2\)O\(_2\) and water share the same permeation path within single AQP monomers (35), mechanisms acting on AQP function, such as phosphorylation, similarly enhance water and H\(_2\)O\(_2\) transport. Thus, AtPIP2;1 may facilitate H\(_2\)O\(_2\) influx into the guard cell during the early phase of ABA or flg22 perception and, subsequently, facilitate water efflux from the guard cell, thereby contributing to stomatal closure. The ROS signaling function of AtPIP2;1 may also be relevant in other tissues or organs where AtPIP2;1 operates such as bundle sheaths (36) or roots (37). In the latter case, AtPIP2;1 was shown to facilitate the emergence of lateral roots, a process known to involve ROS (38). These ideas are not exclusive of other cell signaling functions of AtPIP2;1, such as extracellular CO\(_2\) transport and signaling in guard cells (25). In this case, however, parallel transport of CO\(_2\) through the lipid membrane or another AtPIP isoform may have prevented the detection of a defective stomatal response to CO\(_2\) in pip2;1 plants (25).

**Signaling Specificity and Cross-Talks in Guard Cells.** Signaling pathways inducing stomatal closure in response to ABA and pathogens are increasingly well documented (3, 14). We recently proposed that phosphorylation of AtPIP2;1 at Ser121, by OST1 and possibly other protein kinases, is critical for increasing guard cell water transport in response to ABA (11). The present study extends these observations showing the essential role of AtPIP2;1 at Ser121 phosphorylation in flg22-induced guard cell transport of water and H\(_2\)O\(_2\). Accordingly, AtPIP2;1 Ser121 phosphorylation was required for stomatal closure in response to both ABA (11) and flg22 (Fig. S11). Interestingly, the corresponding residue (Ser126) of a barley PIP homolog seems to be crucial for H\(_2\)O\(_2\) transport in yeast (39, 40).

In the case of pathogen infection, PAMPs and DAMPs are perceived as general signals for stomatal closure, thereby limiting plant infection. Early signaling elements, which include H\(_2\)O\(_2\), nitric oxide, or calcium lead to activation of RbohD NADPH oxidase and SLAC1 anion channel, are shared among the PAMP, DAMP, and ABA response pathways. In agreement with earlier studies proposing a role for OST1 in guard cell responses to flg22, including activation of SLAC1 (12, 20), the protein kinase was also required for flg22-dependent activation of AtPIP2;1. Knowing that OST1 is activated by BAK1 during guard cell response to ABA (41), it may be regulated in a similar way in response to flg22. This model fits with the idea that BAK1 acts as a relay between the flg22 receptor FLS2 and downstream components. In these respects, it was somewhat surprising that recombiant BAK1 can also phosphorylate AtPIP2;1 on Ser121. Because BAK1 showed a fivefold higher \(K_m\) than OST1 in this assay and flg22-dependent activation of \(P_I\) was cancelled in both bak1-5 and snrk2.6, we rather favor the idea that AtPIP2;1 is activated through a nonredundant pathway whereby BAK1 activates OST1 which, in turn, phosphorylates AtPIP2;1 at Ser121.

Although our study points to commonalities between ABA and flg22 signaling, with H\(_2\)O\(_2\) acting as a central hub, distinct patterns of ROS can be observed in response to specific stimuli (42). In molecular terms, flg22 activates ABA-independent signaling components, such as oxylipins and salicylic acid, together with specific protein kinases (19). These include BIK1 and CPK5, which were recently shown to phosphorylate RbohD (18, 43), or CPK4, CPK6, and CPK11, which function as positive regulators of the PAMP-induced ROS burst (44). Along with these lines, our study suggests that ABA and flg22 induced distinct kinetics and intensities of H\(_2\)O\(_2\) accumulation in the guard cell cytoplasm. In particular, ABA induces an AtPIP2;1-independent decrease in HyPer signal after 5 min, which was not observed upon flg22 treatment. This ABA-specific response, whether of extracellular or intracellular origin, may reflect distinct modes of RbohD activation by ABA and flg22, or alternatively, distinct effects of the two stimuli on cytosolic pH. Finally, our work highlights the importance of intracellular H\(_2\)O\(_2\) signaling in guard cells. While key proteins such as glutathione peroxidase 3 (AtGPX3) (45) or ABI2 protein phosphatase (46) are known to be regulated through ROS-dependent oxidation, other cellular targets of H\(_2\)O\(_2\) may play an important role during stomatal closure and not restricted to guard cell responses to flg22 and ABA. Ethylene and methyl jasmonate (MeJA) also induce H\(_2\)O\(_2\) production (14, 47) to promote stomatal closure, thereby protecting the plant from dehydration and/or pathogen attacks. While AtPIP2;1 is the only detected PIP2 expressed in guard cells (48), several PIP1s are also expressed, which may transport H\(_2\)O\(_2\) (24, 30). Thus, a potential role of other AQP isoforms in ethylene and MeJA-induced stomatal closure remains to be investigated.

In conclusion, this work has improved our general knowledge of plant cell signaling, by showing that an AQP can have a signaling function, here in the context of ABA- and flg22-induced stomatal closure. In addition, the activating role of specific protein kinases was uncovered. The use of HyPer, a specific H\(_2\)O\(_2\) probe, opens perspectives to address more generally the role of other AQP isoforms in H\(_2\)O\(_2\) transport, a process that is attracting a growing interest in physiology. For instance, H\(_2\)O\(_2\) was proposed to mediate long-distance signaling in plant tissues (49). Together with NADPH oxidases, AQP isoforms may be crucial for signal propagation, in analogy with the role of ion channels in electrical signaling.

**Materials and Methods**

**Plant Materials.** All experiments were performed in A. thaliana Col-0 or its derivatives. The aquaporin genotypes (pip2;1-1, pip2;1-2, pip2;1-PP2;1, S121A, S121D) and signaling mutants (fls2c efr-1, bak1-5, snrk2.6) were as described in ref. 11 and refs. 29, 50, and S1, respectively. Aquaporin lines expressing a cytoplasmic form of HyPer under the control of a double enhanced cauliflower mosaic virus 35S promoter (28) were obtained by crossing as described in SI Materials and Methods.

**Physiological Responses.** Stomatal aperture was measured on epidermal peels excised from the abaxial side of leaves of 3- to 4-wk-old plants as described (11). Guard cell protoplasts were prepared from approximately 50 leaves (11), and their osmotic \(P_f\) was measured by using an osmotic swelling assay according to a described procedure (52). Additional information on plant growth conditions or measurements of stomatal aperture or \(P_f\) can be found in SI Materials and Methods.

**Guard Cell Fluorescence Imaging.** Epidermal fragments isolated from leaves of 3-wk-old Arabidopsis plants were attached to microscope coverslips by using a silicone adhesive (Telesis S; Paris Berlin) and incubated in a bathing...
solution (30 mKCl, 10 mM Mes/Tris, pH 6.0) for 3 h under constant light (−300 μm−2 s−1). Guard cells expressing MyPer were analyzed by using an inverted fluorescence microscope (Zeiss Axioplan) with a 40x immersion oil objective. Excitation light was produced by a monochromator (Lumenor) at 475/428 nm and 438/424 nm. The two excitation wavelengths were delivered as alternating pulses (100 ms), and the emitted light deflected by dichroic mirrors (HC BS 506) was collected through emission filters (BP 536/540). Images were acquired using a CCD camera (Cooled SNAP HQ, Photometrics). Synchronization of the monochromator and CCD camera was performed through a control unit run by a Fluorescence Ratio Imaging Software (MetaFluor). Image analysis was performed with an ImageJ software. For time course experiments, fluorescence intensity in guard cells was determined overall regions of interest, at 530 nm after excitation at 438 nm or 475 nm (E438 and E475). Background fluorescence signals were measured in regions outside the cell, using similar excitation and emission wavelengths (E438 and E475), and subtracted from corresponding fluorescence values measured in guard cells. A fluorescence ratio R was calculated as R = (E475−E438)/(E438+E478). Changes in fluorescence over time were expressed with respect to the initial ratio R0 as R/R0. Imaging of the ratiometric pH sensitive probe BCECF was performed by a similar approach as described in SI Materials and Methods.

**In Vitro Phosphorylation.** Phosphorylation assays using recombinant BAK1 and AtPKP1.2 peptides were as described in SI Materials and Methods.

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**References.**


