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Rapid Structural Changes and Acidification of Guard Cell Vacuoles during Stomatal Closure Require Phosphatidylinositol 3,5-Bisphosphate[C][W]

Gwangbae Bak,[a] Eun-Jung Lee,[b] Yuree Lee,[a,2] Mariko Kato,[c] Shoji Segami,[c] Heven Sze,[d] Masayoshi Maeshima,[c] Jae-Ung Hwang,[a,1] and Youngsook Lee[b,1,3]

[a] POSTECH-UZH Cooperative Laboratory, Department of Life Sciences, Pohang University of Science and Technology, Pohang 790-784, Korea

[b] POSTECH-UZH Global Research Laboratory, Department of Integrative Bioscience and Biotechnology, World Class University Program, Pohang University of Science and Technology, Pohang 790-784, Korea

[c] Laboratory of Cell Dynamics, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan

[d] Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, Maryland 20742-5815

Rapid stomatal closure is essential for water conservation in plants and is thus critical for survival under water deficiency. To close stomata rapidly, guard cells reduce their volume by converting a large central vacuole into a highly convoluted structure. However, the molecular mechanisms underlying this change are poorly understood. In this study, we used pH-indicator dyes to demonstrate that vacuolar convolution is accompanied by acidification of the vacuole in fava bean (*Vicia faba*) guard cells during abscisic acid (ABA)–induced stomatal closure. Vacuolar acidification is necessary for the rapid stomatal closure induced by ABA, since a double mutant of the vacuolar H^+-ATPase *vha-a2 vha-a3* and vacuolar H^+-PPase mutant *vhp1* showed delayed stomatal closure. Furthermore, we provide evidence for the critical role of phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2] in changes in pH and morphology of the vacuole. Single and double *Arabidopsis thaliana* null mutants of phosphatidylinositol 3-phosphate 5-kinases (PI3P5Ks) exhibited slow stomatal closure upon ABA treatment compared with the wild type. Moreover, an inhibitor of PI3P5K reduced vacuolar acidification and convolution and delayed stomatal closure in response to ABA. Taken together, these results suggest that rapid ABA-induced stomatal closure requires PtdIns(3,5)P_2, which is essential for vacuolar acidification and convolution.

INTRODUCTION

Water conservation is critical for a plant's survival in fluctuating environments. When water supply becomes limited, guard cells rapidly close stomata to reduce transpiration. To bring about stomatal closure, the stress hormone abscisic acid (ABA) triggers the release of anions and K^+ from guard cells (Keller et al., 1989; MacRobbie, 1998; Schroeder et al., 2001; Lebaudy et al., 2007). The decrease of guard cell osmotic pressure results in water release, collapse of the guard cell vacuole, and stomatal closure.

The perception and transduction of signals underlying ABA-induced stomatal closure have been extensively studied, and a number of molecular components involved in this process have been identified. Stomatal closure is characterized by changes in protein phosphorylation status, an increase in cytosolic pH and [Ca^{2+}], activation of anion channels and outward K^+ channels, activation of phospholipases, reorganization of the cytoskeleton, and changes in membrane trafficking (Kim et al., 1995; Hwang et al., 1997; Hetherington, 2001; Pandey et al., 2007; Roelfsema et al., 2012). This complex signaling pathway at the molecular level eventually leads to structural changes in guard cells; guard cells lose as much as 20% of their volume and surface area of their plasma membrane within an hour of stomatal closure (Tanaka et al., 2007). The volume decrease in guard cells is mainly due to the reduction in vacuolar volume, which precedes the total cell volume change and is achieved by convolution of the central vacuole to vesicle-like bodies and tubular structures (Gao et al., 2005; Tanaka et al., 2007). Surprisingly, despite the volume decrease, the vacuolar surface area increases by 20% during stomatal closure (Tanaka et al., 2007), indicating the occurrence of active membrane flow and dynamic reorganization of the vacuolar membrane during the morphological changes that take place during stomatal closure. Despite a detailed cytological description of the changes in vacuolar morphology that occur during stomatal closure, the identity of the molecules involved in these changes and their mechanisms of action are poorly understood.

Clues to the mechanisms underlying vacuolar dynamics can be obtained from studies of yeast vacuoles and mammalian lysosomes, organelles with some similarity to plant vacuoles.

[1] These authors contributed equally to this work.

[2] Current address: Department of Plant Molecular Biology, University of Lausanne, Quartier Sorge, 1015 Lausanne, Switzerland.

[3] Address correspondence to ylee@postech.ac.kr.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Youngsook Lee (ylee@postech.ac.kr).

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Rab-GTPase Ypt7p, the homotypic fusion and vacuole protein sorting complex, the Ccz1p-Mon1p complex, and the vacuolar proton ATPase (V-ATPase) complex are known to be important components of the vacuolar fusion machinery (Wang et al., 2001; Baars et al., 2007). By contrast, V-ATPase proton pump activity and vacuolar acidification are important for vacuolar fission (Yamamoto et al., 1995; Gary et al., 1998; Augsten et al., 2002; Baars et al., 2007). Phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2] is also reported to be a critical factor in the structural changes of yeast vacuoles. Components that contribute to both membrane fusion and vacuolar fission may function in the guard cells of closing stomata because guard cell vacuoles lose volume but increase surface area by convolution, a process that is unique to plant guard cells.

PtdIns(3,5)P_2 is generated from phosphatidylinositol 3-phosphate (PtdIns3P) by a PtdIns3P 5-kinase (PI3P5K) known as Fab1p (formation of aploid and binucleate cells) in yeast and PIKfyve in mammals (Cooke et al., 1998; Gary et al., 1998; Odorizzi et al., 2000; Morishita et al., 2002). The yeast *fab1* mutant, which exhibits compromised Fab1p activity, has abnormally enlarged vacuoles (Gary et al., 1998). A similar phenomenon was reported in mammals. Overexpression of the kinase-deficient PIKfyve mutant in mammalian cells results in abnormally enlarged late endosomes/lysosomes (Ikonomov et al., 2001). In plants, too, there is emerging evidence that PtdIns(3,5)P_2 plays a conserved and critical role in regulating vacuolar dynamics. Knockout of *Arabidopsis thaliana FAB1* genes (*FAB1A* and *FAB1B*), which encode PI3P5Ks, causes severe defects in vacuolar biogenesis during pollen development, which results in a failure to form fertile mature pollen (Whitley et al., 2009).

In this study, we show that ABA-induced stomatal closure is accompanied by acidification of the guard cell vacuole and that the dynamic structural changes of the vacuole require normal levels of PtdIns(3,5)P_2. Pharmacological inhibition of PtdIns(3,5)P_2 synthesis delayed the ABA-induced changes in vacuolar pH, vacuolar dynamics, and stomatal closure. *Arabidopsis* mutants that do not express the putative PI3P5Ks were unable to close stomata as fast as the wild type. These results suggest a molecular mechanism by which vacuolar dynamics are regulated in guard cells and reveal the importance of PtdIns(3,5)P_2 and lipid kinases in this process. We thereby establish a novel aspect of the regulation of stomatal movement.

RESULTS

The Guard Cell Vacuole Becomes Highly Convoluted during Stomatal Closure

To understand the mechanisms underlying changes in vacuolar structure, we first visualized the vacuolar membrane using a green fluorescent protein (GFP)–tagged vacuolar proton pyrophosphatase (V-PPase), which localizes exclusively to the vacuolar membrane (Robinson et al., 1996). We transiently expressed GFP-fused *Arabidopsis* V-PPase (VHP1-sGFP) in fava bean (*Vicia faba*) guard cells by particle bombardment. Bombarded fava bean leaves were incubated on 30 mM KCl/10 mM MES buffer for 3 h under white light to induce full stomatal opening. In most guard cells of fully opened stomata, a large central vacuole was observed, occupying the majority of the intracellular space (Figure 1A). Upon 10 μM ABA treatment for 1 h, this smooth inflated vacuolar membrane became convoluted, forming a folded structure, which resultantly looked as if there were many small vacuoles at the median focal plane, when observed by confocal microscopy (Figures 1B and 1C). The extent of vacuolar membrane convolution was quantified by counting the number of apparent small vacuoles (named vacuolar compartments) in images of guard cells captured at the median focal plane. Guard cells of fully opened stomata typically had fewer than four vacuolar compartments (Figure 1D). However, the mean number of vacuolar compartments increased to 21 within 30 min of ABA treatment and to 37 within 60 min, as stomatal closure progressed in response to the treatment (Figure 1D). This result confirms that reported by Tanaka et al. (2007).

The Lumenal pH of Guard Cell Vacuoles Decreases during Stomatal Closure

To assess whether the vacuolar lumen of guard cells is acidified during stomatal closure, we used two different pH-sensitive fluorescent dyes, Acridine orange (AO) and LysoSensor Green DND-189 (lysosensor) (Figure 2). AO is cell permeable and has an emission maximum of 530 nm (green light) in its neutral form. However, when AO enters acidic compartments, such as lysosomes and vacuoles, it becomes protonated, remains sequestered in the compartment, and emits orange light (emission maximum at 655 nm) when excited by blue light (Millot et al., 1997; Han and

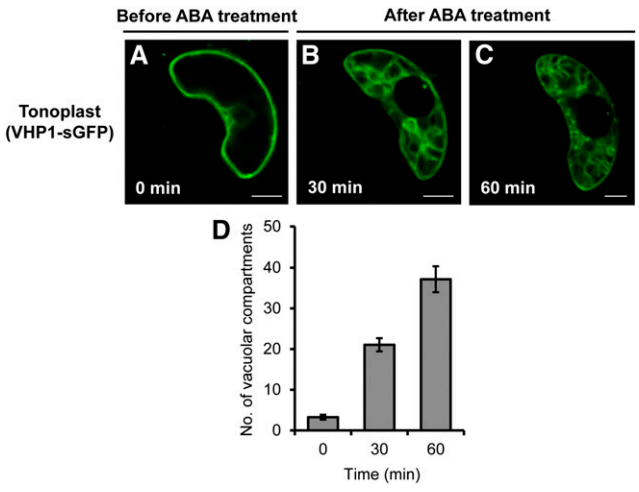

Figure 1. The Vacuoles of Stomatal Guard Cells Become Highly Convoluted during ABA-Induced Stomatal Closure.

ABA-induced vacuolar convolution was observed in fava bean guard cells expressing VHP1-sGFP, a vacuolar membrane marker.

(A) to **(C)** Representative mid-plane confocal images of guard cells expressing VHP1-sGFP before **(A)** and after (**[B]** and **[C]**) treatment with 10 μM ABA. Bars = 10 μm.

(D) The extent of vacuolar convolution was quantified as the number of vacuolar compartments observed in mid-plane confocal images. The results are from four independent experiments (mean ± SE, $22 \leq n \leq 35$). [See online article for color version of this figure.]

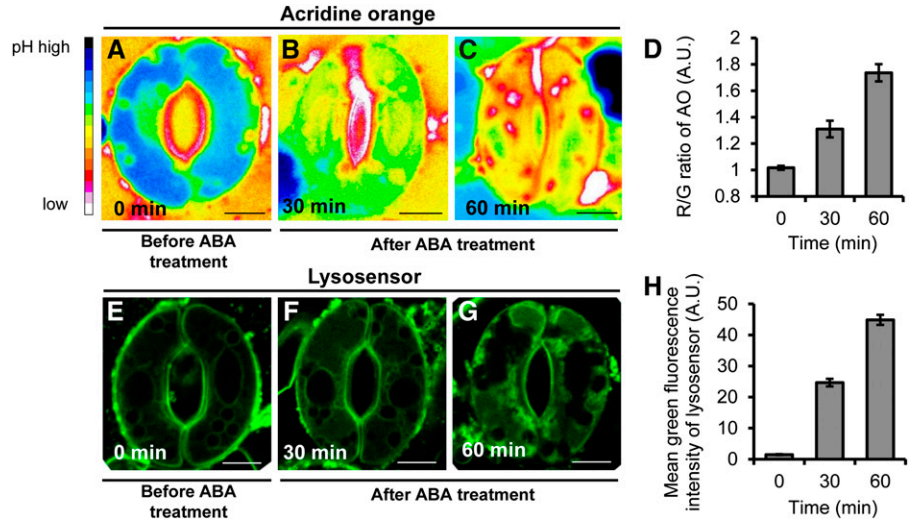

Figure 2. ABA Induces Vacuolar Acidification in Fava Bean Stomatal Guard Cells.

(A) to **(D)** Vacuolar acidification as observed using acridine orange (AO). Guard cells were stained with 50 μM AO for 100 min before ABA treatment and the fluorescence ratio of red (R) to green (G) emissions was obtained using a Zeiss LSM510 Meta microscope. Representative R/G ratio images obtained at the indicated time points are shown (**[A]** to **[C]**). The R/G ratio is displayed in pseudocolor. Regions with the highest R/G ratio (more acidic) are in white, while those with the lowest R/G ratio are in dark blue. Bars = 10 μm.

(D) Vacuolar acidification was quantified as the mean values of R/G ratios in vacuolar lumens. The results are from three independent experiments (mean ± SE, $30 \leq n \leq 44$). A.U., arbitrary unit.

(E) to **(G)** Vacuolar acidification observed with LysoSensor Green DND-189 (lysosensor). Guard cells were stained with lysosensor (4 μM) for 20 min before analysis. Representative confocal images of guard cells stained with lysosensor without (0 min) or with (30 and 60 min) 10 μM ABA treatment are presented. Bars = 10 μm.

(H) The extent of vacuolar acidification was quantified as the mean values of green fluorescence intensity of lysosensor in vacuolar lumens. Higher values indicate more acidic conditions. The results are from six independent experiments (mean ± SE, $71 \leq n \leq 84$).

Burgess, 2010). Therefore, we assessed the vacuolar acidification of guard cells by measuring the ratio of the red-to-green emissions (R/G) of AO (Figures 2A to 2C). Fava bean epidermal layers with fully opened stomata were stained with 50 μM AO for 100 min and then treated with 10 μM ABA. The fluorescence emissions of AO in the red and green channels (615 to 660 nm and 530 to 540 nm, respectively) were monitored by confocal microscopy. As stomatal closure progressed, the R/G ratio in the vacuoles of guard cells increased significantly, exhibiting a 1.3- and 1.7-fold increase within 30 and 60 min of ABA treatment, respectively (Figure 2D).

Lysosensor is an acidotropic probe that accumulates in acidic compartments, where it is protonated (Han and Burgess, 2010). The protonation relieves the fluorescence quenching of the dye by its weak base side chain, resulting in an increase in fluorescence intensity. Therefore, lysosensor exhibits a gradual increase in fluorescence intensity upon acidification over the pH range of 6.5 to 4.5 (Han and Burgess, 2010; Zhang et al., 2010). Guard cells were treated with lysosensor (4 μM) for 20 min, and fluorescence was observed by confocal microscopy. In guard cells of fully opened stomata, the lumen of the large central vacuole exhibited only a low level of lysosensor fluorescence (Figure 2E). Upon exposure to 10 μM ABA, the level of green fluorescence inside the vacuolar lumen gradually increased and stomata closed (Figures 2F and 2G). We quantified the extent of vacuolar acidification by measuring the level of green fluorescence intensity in the vacuolar lumen (see Methods). The mean green fluorescence intensity of the vacuolar lumen increased to ~16 times the initial value within 30 min of treatment with ABA and to 30 times within 1 h ($n > 70$ guard cells; Figure 2H). Thus, our experimental results using two different pH dyes suggest that the vacuolar lumen is acidified during stomatal closure in response to ABA.

Loss of Vacuolar H^+-Pump Activities Delays Stomatal Closure in Response to ABA

To test whether vacuolar acidification is a necessary step for stomatal closure in response to ABA, we examined the effect of ABA on mutants defective in either V-ATPase or the V-PPase. *vha-a2 vha-a3* double knockout (KO) mutants lack vacuolar H^+-pumping V-ATPase activity and thus exhibit a vacuolar pH of 6.4 instead of the normal 5.9 in the wild type (Krebs et al., 2010). The vacuolar pH of the V-PPase mutant, *vhp1*, is increased by 0.25 units relative to the wild type (Ferjani et al., 2011). The rosette leaves of wild-type and mutant *Arabidopsis* plants were first illuminated with white light for 3 h to open the stomata fully and then treated with 4 μM ABA. At the indicated time points, the abaxial epidermis was peeled off and the stomatal apertures were measured. The stomata of both vacuolar proton pump mutants closed more slowly than those of the wild type in response to ABA (Figure 3A), supporting the idea that vacuolar acidification is necessary for ABA-induced stomatal closure.

V-ATPases are critical for the maintenance of vacuolar acidity in mature tissues. However, it is unclear whether V-PPases play a substantial role in modulating vacuolar acidity in mature tissues. Thus, we tested whether ABA-induced vacuolar acidification is indeed compromised in *vhp1*. Although the green fluorescence of lysosensor increased upon treatment with ABA in the *vhp1* guard cells, the increase was less than that in the wild type (Figure 3B), indicating that vacuoles of the *vhp1* mutant are acidified to a lesser extent during stomatal closure than are wild-type vacuoles under our experimental conditions. This result supports a role for V-PPase in vacuolar lumenal acidification of guard cells during stomatal closure.

An Inhibitor of PtdIns(3,5)P_2 Biosynthesis Suppresses Vacuolar Lumenal Acidification during ABA-Induced Stomatal Closure

We hypothesized that PtdIns(3,5)P_2 plays an important role in the regulation of vacuolar acidification in guard cells, since this lipid has been reported to be essential for the acidification of yeast vacuoles (Gary et al., 1998). To test this idea, we examined the effect of a PI3P5K inhibitor (PIKfyve inhibitor) on vacuolar acidification in guard cells during stomatal closure.

PIKfyve inhibitor is a cell-permeable PI3P5K inhibitor widely used in animal and yeast studies; it selectively inhibits murine PIKfyve at IC_{50} = 33 nM and yeast Fab1 at IC_{50} > 5 μM (Jefferies et al., 2008; Ikonomov et al., 2009). Plants contain numerous members of the Fab1/PIKfyve family (Whitley et al., 2009), and *Arabidopsis* has four Fab1/PIKfyve homologs with evolutionarily conserved lipid kinase domains (Mueller-Roeber and Pical, 2002). PIKfyve has only been tested on animal and yeast PI3P5Ks; we therefore tested whether PIKfyve inhibitor inhibits plant PI3P5K activity (Figure 4A). *Arabidopsis* FAB1C and its conserved lipid kinase domain were expressed in bacteria, and PtdIns(3,5)P_2 synthesis was examined in vitro. Both the full-length enzyme and lipid kinase domain successfully produced PtdIns(3,5)P_2 from the substrate PtdIns3P under our experimental conditions. Treatment with PIKfyve inhibitor (1 to 10 μM) significantly inhibited the production of PtdIns(3,5)P_2 by both the full-length enzyme and the lipid kinase domain (Figure 4A), indicating that PIKfyve inhibitor is an effective blocker of plant PI3P5K.

We then tested whether PIKfyve inhibitor suppressed vacuolar acidification (Figures 4B to 4K). Fava bean epidermal fragments stained with 50 μM AO for 100 min were treated with 10 μM ABA in the absence or presence of 1 μM PIKfyve inhibitor. The vacuoles of control fava bean guard cells not subjected to PIKfyve inhibitor treatment exhibited a 1.3- and 1.7-fold increase in R/G ratio 30 and 60 min after ABA treatment, respectively (Figures 4B and 4C). In the presence of 1 μM PIKfyve inhibitor, however, the increase was significantly suppressed (Figures 4D and 4E). Guard cells treated with PIKfyve inhibitor exhibited only a 1.1- and 1.2-fold increase relative to the initial level at the same time points (Figure 4F), indicating that the PIKfyve inhibitor suppressed the vacuolar acidification induced by ABA.

This result was verified using lysosensor (Figures 4G to 4K). Fava bean guard cells were treated with 10 μM ABA in the presence of 1 μM PIKfyve inhibitor. Then, the cells were treated with 4 μM of lysosensor for 20 min before observation. Compared with control cells treated with ABA alone, the increase in mean green fluorescence intensity was greatly reduced in the vacuoles of inhibitor-treated guard cells (Figures 4G to 4J). The untreated control guard cells exhibited a 16- and 30-fold increase in green fluorescence 30 and 60 min after ABA treatment, respectively, whereas guard cells treated with PIKfyve inhibitor exhibited only a 7- and 17.5-fold increase relative to the initial level at the same time points (Figure 4K). These results indicate that PtdIns(3,5)P_2 is required for ABA-induced vacuolar acidification in stomatal guard cells.

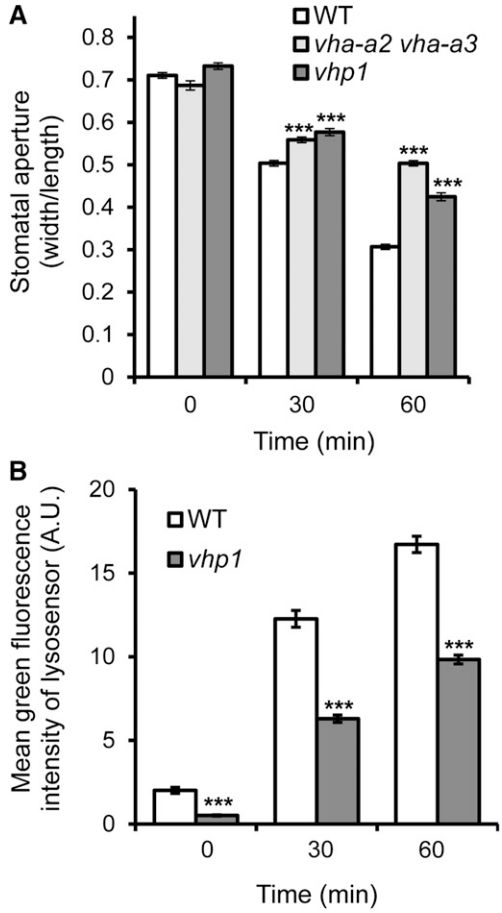

Figure 3. Loss-of-Function Mutants of Vacuolar Proton Pumps Are Delayed in ABA-Induced Stomatal Closure and Vacuolar Acidification.

(A) ABA-induced stomatal closure in *Arabidopsis* mutant plants defective in vacuolar proton pump activities. Leaves from the wild type (WT) and vacuolar proton pump mutants (*vha-a2 vha-a3* and *vhp1*) were incubated on buffer under white light for 3 h and then transferred to fresh buffer containing 4 μM ABA. Stomatal apertures were measured 30 and 60 min after the transfer. The results are from three independent experiments (mean ± SE, 200 ≤ *n* ≤ 260). Asterisks indicate significant differences at P < 0.0001 compared with the wild type.

(B) Impaired vacuolar acidification in *vhp1* guard cells. Stomata of 21-d-old wild-type and *vhp1 Arabidopsis* plants were stained with lysosensor (4 μM) 20 min before observation. The extent of vacuolar acidification was quantified as the mean values of green fluorescence intensity of lysosensor in vacuolar lumens. The results are from four independent experiments (mean ± SE, 55 ≤ *n* ≤ 75). Asterisks indicate significant differences at P < 0.0001 compared with the wild type. A.U., arbitrary unit.

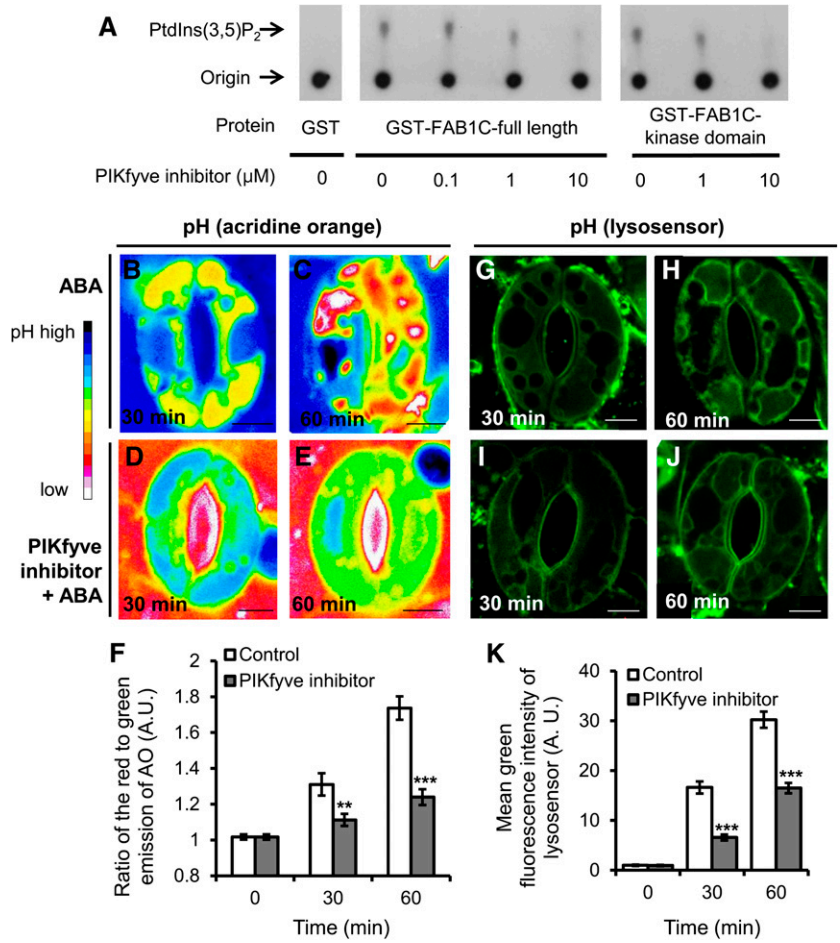

Figure 4. Treatment with a PI3P5K Inhibitor (PIKfyve Inhibitor) Suppresses Vacuolar Acidification during ABA-Induced Stomatal Closure.

(A) PIKfyve inhibitor reduces the PI3P5K activity of *Arabidopsis* FAB1C in a concentration-dependent manner. Bacterially expressed GST-FAB1C (full length, 1 to 1648 amino acids) and GST-FAB1C kinase domain (856 to 1645 amino acids) were tested for PI3P5K activity in vitro. The production of radiolabeled PtdIns(3,5)P_2 from PtdIns3P and [γ-^{32}P]ATP was detected using thin layer chromatography and autoradiography. The results shown are representative of three replicate experiments. GST, glutathione *S*-transferase.

(B) to **(E)** PIKfyve inhibitor suppresses ABA-induced vacuolar acidification, as determined by AO. Representative R/G ratio images of fava bean guard cells after treatment with 10 μM ABA in the absence (**[B]** and **[C]**) or presence (**[D]** and **[E]**) of PIKfyve inhibitor. Bars = 10 μm.

(F) The extent of vacuolar acidification was quantified as the means of the R/G ratios. The results are from three independent experiments (mean ± SE, $30 \leq n \leq 44$). Asterisks indicate significant differences at $P < 0.001$ (**) and $P < 0.0001$ (***) between samples treated or not with PIKfyve inhibitor. A.U., arbitrary unit.

(G) to **(J)** Suppression of ABA-induced vacuolar acidification by PIKfyve inhibitor, as observed with lysosensor. Representative confocal images of fava bean guard cells stained with lysosensor after treatment with 10 μM ABA in the absence (**[G]** and **[H]**) or presence (**[I]** and **[J]**) of PIKfyve inhibitor. Bars = 10 μm.

(K) The extent of vacuolar acidification was quantified as the mean values of green fluorescence intensity of lysosensor measured in vacuolar lumens. The results are from six independent experiments (mean ± SE, $71 \leq n \leq 84$). Asterisks indicate significant differences at $P < 0.0001$ between samples treated or not with PIKfyve inhibitor.

PIKfyve Inhibitor Suppresses ABA-Induced Stomatal Closure and Vacuolar Convolution

If vacuolar acidification is essential for the stomatal closure induced by ABA and if PtdIns(3,5)P_2 is important for vacuolar acidification, it follows that inhibition of PtdIns(3,5)P_2 synthesis would also inhibit ABA-induced stomatal closure. To test this idea, stomata were induced to open fully by irradiating fava bean leaves with white light for 3 h. Leaves were then treated with 10 μM ABA in the presence or absence of PIKfyve inhibitor. The inhibitor suppressed ABA-induced stomatal closure in a dose-dependent manner (Figure 5A), suggesting that a normal level of PtdIns(3,5)P_2 is required for rapid ABA-induced stomatal closure.

We then tested whether the PIKfyve inhibitor also inhibited vacuolar convolution under the same conditions. In the presence of 1 μM PIKfyve inhibitor, ABA-induced vacuolar convolution, visualized using VHP1-sGFP, was significantly suppressed and delayed

(Figures 5B to 5E). The untreated guard cells exhibited 21 and 37 vacuolar compartments per cell when observed at 30 and 60 min after ABA treatment, respectively. By contrast, the inhibitor-treated guard cells exhibited only 13 and 23 vacuolar compartments at the same time points (Figure 5F). These results suggest a connection between the acidification and convolution of vacuoles and the importance of PtdIns(3,5)P_2 in these processes.

Knockout of *PI3P5Ks* (*FAB1s*) Inhibits ABA-Induced Stomatal Closure in *Arabidopsis*

To obtain genetic evidence for the role of PtdIns(3,5)P_2 in stomatal closure, we examined plants with mutations in PI3P5Ks, the enzymes that catalyze PtdIns(3,5)P_2 synthesis. *Arabidopsis* has four genes encoding PI3P5Ks (Figure 6A; *FAB1A*, *FAB1B*, *FAB1C*, and *FAB1D*). FAB1A and FAB1B are conventional PI3P5Ks that contain all of the evolutionarily conserved functional domains of PI3P5Ks: FYVE, the Cpn60_TCP1 (HSP chaperonin_T-complex protein 1) homology domain, and the lipid kinase domain. However, FAB1C and FAB1D lack the FYVE domain and are thus called unconventional PI3P5Ks, which are unique to plants (Whitley et al., 2009).

We first determined which *FAB1* genes are expressed in mature stomatal guard cells (Figure 6B). RNA was isolated from the guard cell–enriched *Arabidopsis* epidermis, and the transcript levels of *FAB1* genes were assessed using quantitative RT-PCR with gene-specific primers. *FAB1B* was expressed at the highest level. *FAB1A* and *FAB1C* were also expressed at high levels, whereas *FAB1D* transcript was not detected (Figure 6B). This result is supported by three independent microarray studies that show expression of A, B, and C types of *FAB1* genes in guard cells (Yang et al., 2008; Bauer et al., 2013), though their expression is not induced by ABA (Wang et al., 2011). The expression of phosphoenolpyruvate carboxylase, which is a marker of mesophyll cells (Ueno, 2001; Gousset-Dupont et al., 2005), was not detected in our guard cell preparation (Figure 6B), indicating that the preparation was sufficiently pure. Since the *fab1a fab1b* double mutation is lethal (Whitley et al., 2009), we analyzed the effects of reduced PI3P5K activity on ABA-induced stomatal closure using loss-of-function mutants of FAB1B and FAB1C.

We isolated the T-DNA insertion KO mutants of *FAB1B* (*fab1b-1*, SALK_048293; *fab1b-2*, SALK_066673) and *FAB1C* (*fab1c-1*, SAIL_254_G09; *fab1c-2*, SK_13557) (see Supplemental Figure 1 online) and generated the *fab1b-1 fab1c-1* double mutant. We then investigated ABA-induced stomatal closure in the mutants. Compared with the wild type, *fab1b-1* and *fab1c-1* stomata closed significantly more slowly upon treatment with 4 μM ABA (Figure 6C). *fab1b-1 fab1c-1* stomata closed the slowest, indicating that

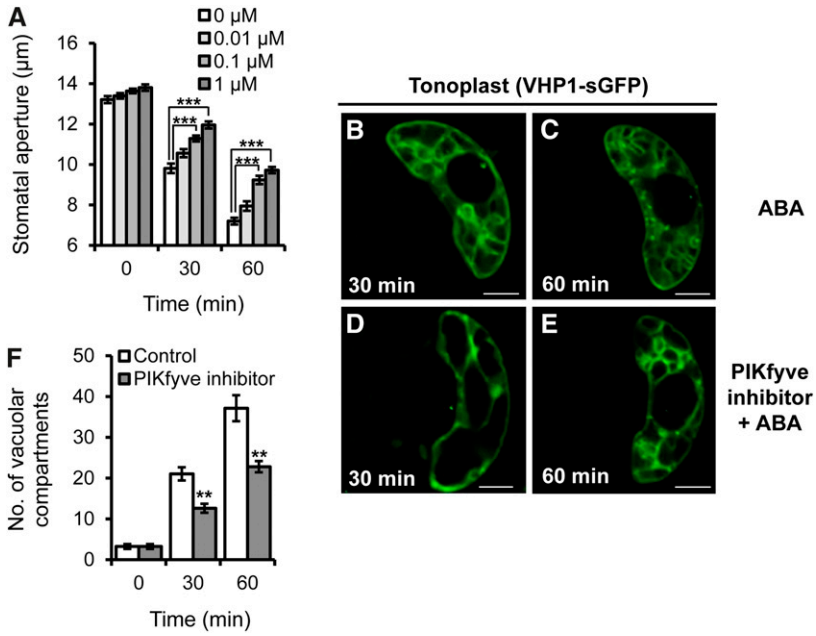

Figure 5. Treatment with a PIKfyve Inhibitor Suppresses ABA-Induced Stomatal Closure and Vacuolar Convolution.

(A) PIKfyve inhibitor suppresses ABA-induced stomatal closure in fava bean. Leaves were incubated on 10 mM MES/10 mM KCl buffer containing 10 μM ABA with various concentrations of PIKfyve inhibitor (0 to 1 μM). The results are from four independent experiments (mean ± SE, $130 \leq n \leq 298$). Asterisks indicate significant differences at $P < 0.0001$ between samples treated or not with PIKfyve inhibitor.

(B) to **(E)** PIKfyve inhibitor suppresses ABA-induced vacuolar convolution. Representative mid-plane confocal images of fava bean guard cells expressing VHP1-sGFP after treatment with ABA (10 μM) in the absence (**[B]** and **[C]**) or presence (**[D]** and **[E]**) of PIKfyve inhibitor. Bars = 10 μm.

(F) The extent of vacuolar convolution was quantified as the number of vacuolar compartments observed in mid-plane confocal images. The results are from four independent experiments (mean ± SE, $22 \leq n \leq 35$). Asterisks indicate significant differences at $P < 0.001$ between samples treated or not with PIKfyve inhibitor.

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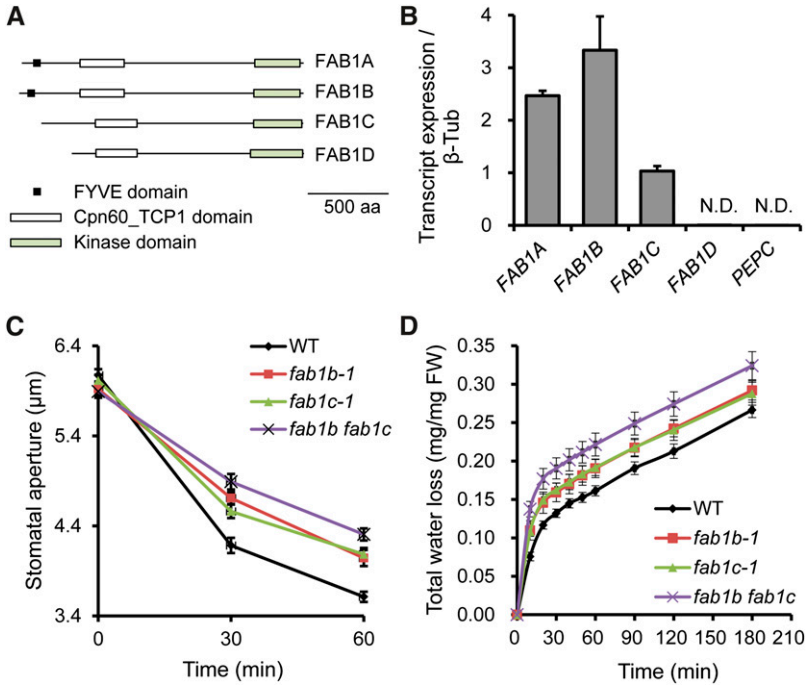

Figure 6. *Arabidopsis* Null Mutants of FAB1s Exhibit Delayed ABA-Induced Stomatal Closure and Accelerated Water Loss.

(A) Structure of *Arabidopsis* FAB1 PI3P5Ks. aa, amino acids.

(B) Transcript levels of FAB1 PI3P5Ks in mature *Arabidopsis* guard cells. Expression was analyzed using quantitative RT-PCR. β-*Tubulin* was used as an internal control. Phosphoenolpyruvate carboxylase is a marker gene of mesophyll cells and its expression was not detected (N.D.) in our guard cell–enriched epidermal sample. These results are from three independent experiments (mean ± SE).

(C) Stomatal closure of *fab1* mutants is delayed in response to ABA. Wild-type (WT) and *fab1b*, *fab1c*, and *fab1b fab1c* mutant *Arabidopsis* leaves were incubated under white light for 3 h and then transferred to fresh buffer containing 4 μM ABA. The results are from three independent experiments (mean ± SE, $150 \leq n \leq 255$).

(D) *fab1* mutant leaves exhibit increased water loss. Water loss is presented as the decrease in weight relative to the initial value after exposure to dry air. The results are from seven independent experiments (mean ± SE). FW, fresh weight.

the inhibitory effects of *fab1b-1* and *fab1c-1* on stomatal closure are additive (Figure 6C). The independent alleles of *fab1b* (*fab1b-2*) and *fab1c* (*fab1c-2*) also exhibited delayed stomatal closure upon treatment with 4 μM ABA (see Supplemental Figures 2A and 2B online). We then tested whether *Arabidopsis* guard cells exhibited the same response to the PIKfyve inhibitor as did those of fava bean (Figure 5A). Indeed, *Arabidopsis* stomata were delayed in ABA-induced stomatal closure in the presence of physiological concentrations of PIKfyve inhibitor (see Supplemental Figure 3 online). Taken together, these results support the hypothesis that PtdIns(3,5)P_2 is important for rapid stomatal closure in response to ABA.

Arabidopsis FAB1 PI3P5K Activity Is Required for Water Use Efficiency

If the impaired stomatal response to ABA caused by *fab1* mutations is physiologically meaningful (Figure 6C), then *fab1* mutant plants would lose water faster than wild-type plants. Indeed, water loss (decrease in fresh weight relative to the initial value) was more rapid in *fab1b-1* and *fab1c-1* than in the wild type and fastest in *fab1b-1 fab1c-1*, when leaves were exposed to dry air (Figure 6D). The independent KO alleles *fab1b-2* and *fab1c-2* also exhibited accelerated water loss relative to the wild type (see Supplemental Figures 2C and 2D online). These results indicate that normal FAB1 activity is necessary to minimize water loss from leaves.

PtdIns(3,5)P_2 Binds to V-PPase

Our results suggest that PtdIns(3,5)P_2 is required for rapid vacuolar acidification in response to ABA treatment, which in turn leads to rapid vacuolar reorganization and stomatal closure (Figures 1 to 5). An important question remaining is how PtdIns(3,5)P_2 regulates vacuolar acidification. One possibility is that PtdIns(3,5)P_2 physically interacts with and regulates important proton transporters at the tonoplast, including V-ATPases and V-PPase. To test this possibility, we investigated whether PtdIns(3,5)P_2 interacts with V-PPase (Figure 7). Using membranous strips spotted with various types of phospholipids (PIP Strips), we tested whether PtdIns(3,5)P_2 interacts with V-PPase more strongly than with other membrane lipids. As shown in Figure 7, V-PPase bound to PtdIns(3,5)P_2, phosphatidylinositol 4,5-bisphosphate (PtdIns[4,5]P_2), and phosphatidylinositol 3,4,5-triphosphate (PtdIns[3,4,5]P_3) but not to other phospholipids. PtdIns(4,5)P_2 is mainly localized to the plasma membrane (Mueller-Roeber and Pical, 2002; Lee et al.,

2007), and PtdIns(3,4,5)P_3 has not been found in plants (Mueller-Roeber and Pical, 2002), excluding the possibility that either of these lipids interact with V-PPase at the vacuolar membrane. The enzyme that produces PtdIns(3,5)P_2 was shown to localize to the vacuolar membrane, prevacuolar compartment, and endosomes (Cabezas et al., 2006; Michell et al., 2006; Hirano et al., 2011). Therefore, it is highly likely that V-PPase interacts specifically with PtdIns(3,5)P_2 in vivo.

Vacuolar Acidification Is Accompanied by Cytosolic Alkalization of Guard Cells during ABA-Induced Stomatal Closure

We wondered if vacuolar acidification would affect cytosolic pH. We tested whether ABA-induced cytosolic alkalization in guard cells observed before (Irving et al., 1992; Suhita et al., 2004; Gonugunta et al., 2008; Islam et al., 2010) depends on a normal change in vacuolar pH using a PIKfyve inhibitor to suppress vacuolar pH change. Cytosolic pH was monitored by a fluorescent dye BCECF (2′,7′-bis-[2-carboxyethyl]-5-[and-6]-carboxyfluorescein), which has pH-independent excitation maximum at 458 nm (blue light) and pH-dependent excitation maximum at 488 nm (cyan light). Fava bean leaf fragment with fully opened stomata were treated with 10 μM ABA in the presence or absence of 1 μM PIKfyve inhibitor. Then, the cells were incubated with 20 μM acetoxymethyl ester derivative of BCECF (BCECF-AM) for 30 min, and the fluorescence emissions of BCECF produced by cyan and blue excitation were monitored by confocal microscopy. As stomatal closure progressed, the ratio of the fluorescence emitted by excitations at cyan to blue in the cytosol of guard cells increased significantly, exhibiting a 1.4- and 2.6-fold increase at 30 and 60 min of ABA treatment, respectively; whereas inhibitor-treated cells exhibited 1- and 1.3-fold increase at the same time points (Figure 8). This result is consistent with a previous report of cytosolic alkalization of closing stomatal guard cells (Islam et al., 2010) and indicates that normal acidification of vacuolar pH is connected with alkalization of cytosolic pH.

We then tested whether blocking the cytosolic alkalization affects vacuolar acidification during stomatal closure. For this experiment, we used a weak acid, butyrate, to inhibit cytosolic alkalization, as described previously (Blatt and Thiel, 1994; Suhita et al., 2004; Gonugunta et al., 2008; Islam et al., 2010). Fava bean guard cells were pretreated with 0.5 mM butyrate and ABA-induced vacuolar acidification in these cells was followed using lysosensor. As shown in Supplemental Figure 4 online, the vacuolar acidification was significantly suppressed in the presence of butyrate. Together, these results suggest that cytosolic alkalization and vacuolar acidification are interdependent during ABA-induced stomatal closure.

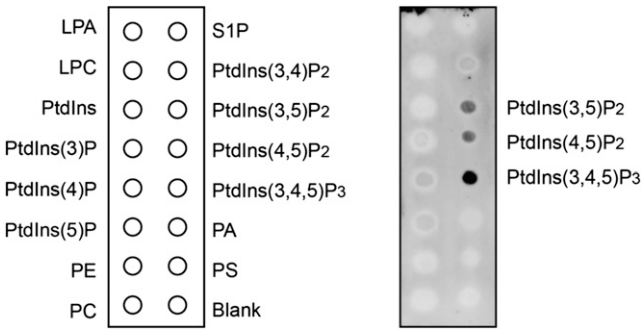

Figure 7. PtdIns(3,5)P_2 Binds to V-PPase.

A PIP strip, a hydrophobic membrane spotted with various phospholipids, was overlaid with purified V-PPase and immunoblotted with anti-V-PPase antibody. V-PPase interacted with PtdIns(3,5)P_2, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3. LPA, lysophosphatidic acid; LPC, lysophosphocholine; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns5P, phosphatidylinositol 5-phosphate; PE, phosphatidylethanolamine; PC, phosphatidylcholine; S1P, sphingosine 1-phosphate; PtdIns (3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PA, phosphatidic acid; PS, phosphatidylserine.

DISCUSSION

Vacuolar Acidification Is Necessary for the Rapid Stomatal Response to ABA

Changes in the guard cell plasma membrane as the stomatal pore closes in response to water stress or ABA treatment have been well characterized; however, the responses of intracellular membranes during stomatal closure are largely unknown. A few studies have highlighted the dynamic nature of the vacuolar membranes (Gao et al., 2005; Tanaka et al., 2007), though the molecular bases underlying these changes have not been explored. Here, we used a combination of cell biology, biochemical, and molecular genetic tools to unravel this question. This study provides two lines of evidence that vacuolar acidification is important for stomatal closure. First, we showed that the vacuolar lumen is acidified during ABA-induced stomatal closure, using the fluorescent pH sensors AO and lysosensor (Figure 2). Second, when we inhibited such acidification, either by loss-of-function mutations of vacuolar H^+-pumps or pharmacologically with the PIKfyve inhibitor, stomatal closure was delayed (Figures 3A and 5A).

The change in vacuolar pH progressed in pace with the reduction of stomatal aperture (Figure 2), though the time course of vacuolar pH change in our experiments is slow relative to changes in stomatal conductance of intact leaves. We used leaf fragments floating on buffer solution and found that stomata closed halfway at 60 min after ABA application. In gas exchange experiments using intact leaves, stomatal conductance reached halfway 10 to 15 min after ABA application (Hedrich et al., 2001), and guard cells of intact leaf and epidermal peels were found to differ in calcium dynamics (Levchenko et al., 2008). In addition, many other factors, including pH of the extracellular space, oxygen availability, and hydrodynamics, may vary, causing the difference in the speed of the change.

The pH homeostasis of the vacuole is regulated by the proton transport activities of tonoplast-localized transporters, including two proton pumps (V-ATPase and V-PPase) and Na^+/H^+ and Ca^{2+}/H^+ antiporters (Yamaguchi et al., 2001; Cheng et al., 2005). Our results indicate that V-ATPase and V-PPase are critical for rapid stomatal closure in response to ABA (Figure 3). V-ATPase is a highly conserved, multisubunit proton pump that translocates protons from the cytosol into endomembrane compartments by

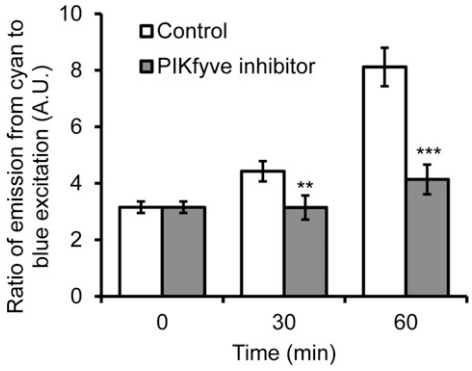

Figure 8. Treatment with a PIKfyve Inhibitor Suppresses Cytosolic Alkalization during ABA-Induced Stomatal Closure.

Cytosolic alkalization was observed using BCECF-AM. Fava bean guard cells were incubated with 20 μM BCECF-AM for 30 min before observation and the emission ratio with cyan (488 nm) to blue (458 nm) excitation was obtained using a Zeiss LSM510 Meta microscope. Cyan-to-blue ratios of fava bean guard cells after treatment with 10 μM ABA in the absence or presence of PIKfyve inhibitor were compared. The results are from five independent experiments (mean ± SE, $59 \leq n \leq 87$). Asterisks indicate significant differences at $P < 0.05$ (**) and $P < 0.001$ (***) between samples with or without PIKfyve inhibitor. A.U., arbitrary unit.

hydrolyzing ATP (Sze, 1985; Hedrich et al., 1989; Sze et al., 1992). V-PPase also pumps protons from the cytosol into the vacuole but uses energy from the hydrolysis of PPi and is thus less efficient than V-ATPase in terms of transport rate (Rienmüller et al., 2012). Recent studies suggest that V-ATPase plays a more prominent role in acidifying the vacuole than does V-PPase (Ferjani et al., 2011). Our observation that stomatal closure was delayed to a greater extent in the V-ATPase mutant *vha-a2 vha-a3* than in the V-PPase mutant *vhp1* (Figure 3A) supports this previous report. However, *vhp1* also exhibited substantial delays in ABA-induced vacuolar acidification and stomatal closure (Figure 3), which indicates that V-PPase contributes to vacuolar acidification in mature stomatal guard cells. Thus, V-PPase seems to play a role in the ABA-induced acidification of guard cell vacuoles and in stomatal closure, but a smaller role than does V-ATPase. This function of V-PPase in mature plant cells is surprising, since V-PPases were reported to be important in young, growing cells, and less significant in mature cells (Martinoia et al., 2007; Ferjani et al., 2011). However, guard cells are not the only mature plant cells in which V-PPase functions as a significant vacuolar proton pump; V-PPase contributes to the acidification of pear (*Pyrus communis*) (Shiratake et al., 1997) and grape (*Vitis vinifera*; Terrier et al., 1998) vacuoles.

How Does Vacuolar Acidification Contribute to Stomatal Closure?

Our data revealed a strong correlation between vacuolar acidification, vacuolar convolution, and stomatal closure (Figures 2 to 5). Based on analogies from studies on yeast cells that showed that acidification of the vacuole is a requirement for proper membrane dynamics of this organelle (Yamamoto et al., 1995; Gary et al., 1998; Augsten et al., 2002; Baars et al., 2007), we speculate that acidification of the vacuole facilitates its convolution and thereby leads to rapid loss of its volume in guard cells.

We envisage two possible mechanisms by which vacuolar acidification contributes to structural changes of the organelle. First, the proton gradient across the vacuolar membrane may directly induce physical changes in membrane structure. Membrane curvature can be affected by changes in the charge and conformation of the membrane lipids and proteins, which depend on the proton gradient across the membrane (Hope et al., 1989; Farge and Devaux, 1992; Matsuo et al., 2004). Second, the regulation of endomembrane acidification is tightly linked to membrane trafficking and protein sorting, as shown in yeast (Hurtado-Lorenzo et al., 2006) and in mammalian cells (Casey et al., 2010). Membrane dynamics depend on diverse protein–protein associations as well as dissociations that could be sensitive to pH homeostasis.

Acidic vacuolar pH might also regulate ion fluxes (Figure 9) by providing a driving force for K^+ flux into the cytosol through the vacuolar membrane–located KT/HAK/KUP K^+/H^+ symporter (Rodríguez-Navarro, 2000; Bañuelos et al., 2002; Rodríguez-Navarro and Rubio, 2006). Anions can also be easily released from the vacuole to the cytosol when vacuolar pH is low. The release of ions from the vacuole into the cytosol is a prerequisite for water release from the vacuole during stomatal closure (Y. Wang et al., 2012; Laanemets et al., 2013).

Finally, vacuolar acidification contributes to alkalization of the cytosol, which is essential for ABA-induced stomatal closure (Irving et al., 1992; Suhita et al., 2004; Gonugunta et al., 2008). The cytosolic pH starts to rise in guard cells as early as 2 min after an application of ABA and remains alkaline during the ABA-induced stomatal closure (Islam et al., 2010). It remains unclear how the cytosolic pH is maintained alkaline throughout the stomatal closing process. Our result (Figure 8) indicates that the vacuolar acidification is necessary to maintain cytosolic pH alkaline during stomatal closing. Interestingly, the converse was also true: Inhibition of cytosolic alkalization inhibited vacuolar acidification (see Supplemental Figure 4 online). Thus, there is a close interdependence between cytosolic pH and vacuolar pH in guard cells, which is not surprising, considering the large volume occupied by vacuoles, which are separated from the cytosol by only a single membrane. The mode of the coupling between the pH of the two compartments may be direct and/or indirect and requires further in-depth investigation.

PtdIns(3,5)P_2 Is Necessary for Vacuolar Acidification and Convolution during Stomatal Closure Induced by ABA

Our study indicates that PtdIns(3,5)P_2 is important for vacuolar dynamics and stomatal closure (Figures 4 to 6). Inhibition of PtdIns(3,5)P_2 synthesis by PIKfyve inhibitor and *fab1* mutations induced a substantial delay in the decrease in vacuolar lumenal pH and vacuolar convolution in stomatal guard cells (Figures 4 to 6; see Supplemental Figure 3 online). Moreover, leaves of *fab1* mutants lost water more rapidly under dry air conditions than did those of the wild type (Figures 6D; see Supplemental Figure 2 online). The function of PtdIns(3,5)P_2 in guard cells is reminiscent of its functions in animal and yeast cells, where it regulates the size, shape, and acidity of endosomal and

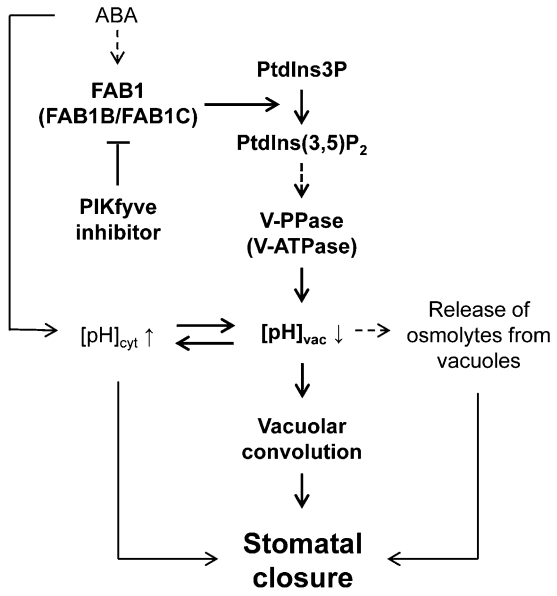

Figure 9. Model Showing the Mechanism by Which PtdIns(3,5)P_2 May Regulate Stomatal Closure.

A stomatal closure signal (e.g., ABA) may induce the activation of the PI3P5Ks (e.g., FAB1B and FAB1C), causing an increase in (PtdIns[3,5]P_2). PtdIns(3,5)P_2 may activate vacuole membrane-localized proton pumps and thereby acidify the vacuolar lumen. The acidified vacuole undergoes structural changes and releases osmolytes, allowing rapid stomatal closure. The vacuolar acidification likely contributes to the maintenance of alkalized cytosolic pH and vice versa. Solid lines, relationships based on experimental data; dashed lines, hypothesized relationships. [pH]vac, vacuolar pH; $[pH]_{cyt}$, cytosolic pH.

lysosomal/vacuolar compartments and membrane and protein trafficking to and from these compartments (Gary et al., 1998; Odorizzi et al., 1998; Efe et al., 2005; Michell et al., 2006; Dove et al., 2009). In fact, many phosphoinositides play major roles in membrane trafficking by acting as membrane identifiers and serving as sites for protein binding (Cremona and De Camilli, 2001; Krauss and Haucke, 2007; Clague et al., 2009). It is tempting to speculate that PtdIns(3,5)P_2 provides a docking point for trafficking of small compartments that fuse to or bud from the vacuole when membrane flows from the shrinking plasma membrane to expanding vacuolar membrane during stomatal closure.

PtdIns(4,5)P_2 has been reported to have multiple roles in ABA-induced stomatal closing (Lee et al., 1996; Jacob et al., 1999; Ma et al., 2009). PtdIns(4,5)P_2 is hydrolyzed by phospholipase C and activates phospholipase D, producing second messengers inositol 1,4,5-trisphosphate and phosphatidic acid, respectively. A protein that binds PtdIns(4,5)P_2, which presumably interferes with the interaction of the lipid with its partners, inhibits ABA-induced stomatal closing (Jung et al., 2002). However, the role of PtdIns(3,5)P_2 in vacuolar acidification does not seem to be related to that of PtdIns(4,5)P_2 because PtdIns(4,5)P_2 is exclusively localized at the plasma membrane in guard cells (Lee et al., 2007). Moreover, PI3P5K is unlikely to produce PtdIns(4,5)P_2, which is synthesized by PI4P5K activity in vivo, as PI3P5K cannot complement the phenotype of cells that lack PI4P5K (McEwen et al., 1999). Thus, the vacuolar acidification mediated by PI3P5K is not directly related to PtdIns(4,5)P_2 and/or the second messengers produced by its degradation.

The level of PtdIns(3,5)P_2 was reported to increase rapidly and transiently in plant cell systems exposed to hyperosmotic stress (Meijer et al., 1999). A similar phenomenon was observed in budding yeast exposed to salt stress (Dove et al., 1997; Cooke et al., 1998; Gary et al., 1998; Bonangelino et al., 2002). Thus, it is likely that the level of PtdIns(3,5)P_2 increases in stomatal guard cells experiencing drought stress. However, this question may not be resolved soon, as it is technically challenging to accurately quantify the endogenous level of PtdIns(3,5)P_2, especially in guard cells alone; the lipid is of low abundance in both yeast and animal cells, accounting for <0.1% of total inositol phospholipids in unstressed cells (Dove et al., 1997; Michell et al., 2006; Ho et al., 2012). Although the absolute amount of PtdIns(3,5)P_2 has not been quantified in plants, it is presumed to be very low, even when elevated in response to stimuli (Meijer et al., 1999; Meijer and Munnik, 2003).

V-PPase Is a Putative Effector of PtdIns(3,5)P_2 in the Control of Vacuolar Acidification

PtdIns(3,5)P_2 appears to have multiple effector proteins (Michell et al., 2006; Dong et al., 2010). Our study suggests that V-PPase is an effector protein of PtdIns(3,5)P_2 in stomatal guard cells. First, our binding assay revealed that PtdIns(3,5)P_2 directly binds (Figure 7); second, we found that normal V-PPase function is necessary for vacuolar acidification and stomatal closure induced by ABA (Figure 3). A previous report revealed that phospholipids are necessary for mung bean (*Vigna radiata*) V-PPase (Maeshima and Yoshida, 1989) to be maximally active. In particular, PtdIns(3,5)P_2 regulates the ion transport activity of the endolysosome-localized mucolipin transient receptor potential channel (Dong et al., 2010) and endosome- and lysosome-localized mammalian two-pore channel proteins (X.Wang et al., 2012). Moreover, plant TPC1 and nonselective slow vacuolar channel contributed to the K^+ efflux from vacuole to cytosol, which is requisite for stomatal closure (Ivashikina and Hedrich, 2005). These facts suggest the possibility that PtdIns(3,5)P_2 might directly activate channel activity for stomatal closure. Future research is necessary to determine how PtdIns(3,5)P_2 affects the activity of V-PPase.

Another potential effector of PtdIns(3,5)P_2 is V-ATPase, which has been shown to be necessary for both fusion and fission of yeast vacuoles (Baars et al., 2007). In support of this possibility, V-ATPase is the most important factor in the acidification of vacuoles, and acidification is tightly coupled with the convolution of vacuoles in guard cells (Figures 3 to and 5). In addition, a previous report revealed that phospholipids are necessary for maximal activity of purified yeast V-type H^+-pumping ATPase (Uchida et al., 1988). However, we could not find any clue as to which subunit of this large transporter with at least 12 subunits might interact with PtdIns(3,5)P_2 and thus could not test whether it directly binds to the lipid.

Homologs of other putative PtdIns(3,5)P_2 effectors found in yeast and animal systems are found in plants, too. They include the seven-bladed β-propeller PROPPIN proteins and the epsin-like proteins (Michell et al., 2006); however, these homologs have not yet been studied. Future investigation is necessary to

identify the effectors of PtdIns(3,5)P_2 and to understand how the lipid regulates them. Taken together, our results support the possibility that PtdIns(3,5)P_2 interacts with proton transporters such as V-PPase and V-ATPase and that such an interaction may activate rapid proton-pumping into the vacuolar lumen of guard cells, which in turn facilitates stomatal closure.

In summary, we found that ABA-induced vacuolar convolution is accompanied by the acidification of guard cell vacuolar lumen, which is necessary for rapid stomatal closure. Furthermore, we demonstrated that vacuolar acidification, stomatal closure, and consequent water conservation require PtdIns(3,5)P_2, the product of PI3P5Ks. Finally, we identified V-PPase as a putative effector protein of PtdIns(3,5)P_2. This study thus contributes to our understanding of the biochemical basis of vacuolar dynamics in guard cells during stomatal closure, which is critical for water conservation in plants. It will be an interesting future study to examine how vacuolar acidification is related with other events in the signaling network in guard cells.

METHODS

Plant Growth Conditions

Fava bean (*Vicia faba*) was grown for 3 to 4 weeks in a greenhouse with light/dark cycles of 16/8 h at 22°C. *Arabidopsis thaliana* plants were grown on half-strength Murashige and Skoog agar plates supplemented with 1.5% Suc in a controlled growth room with a 16/8-h light/dark cycle at 22°C for 2 weeks. The plants were transferred to soil and grown for an additional week before their leaves were used in experiments.

Biolistic Gene Bombardment into Fava Bean Guard Cells

Vectors expressing sGFP-fused VHP1 were introduced into fava bean guard cells using biolistic bombardment (Biolistic PDC-1000/He particle delivery system; Bio-Rad) as described by Park et al. (2003) with minor modifications. Briefly, 1.25 mg of gold particles (1 μm diameter; Bio-Rad) were coated with 10 μg of plasmid DNA and bombarded into healthy fava bean leaves at a He pressure of 1350 p.s.i. and under a 28-inch Hg vacuum. Transformed leaves were kept in darkness for 15 to 20 h at 22°C. Green fluorescence was observed using a laser scanning confocal microscope (Fluoview/FV 1000; Olympus).

Vacuolar Lumenal Acidification Measurement

The acidification of guard cell vacuoles was observed using the pH-dependent fluorescent dyes AO (Invitrogen) and LysoSensor Green DND-189 (referred to here as lysosensor; Invitrogen). For vacuolar acidification analysis using AO, fava bean epidermis with fully opened stomata was stained with 50 μM AO for 100 min and then transferred to fresh buffer (10 mM KCl/10 mM MES, pH 6.05) containing 10 μM ABA at 23°C. Fluorescence emissions of AO in the red and green channels (615 to 660 nm and 530 to 540 nm, respectively) after excitation with a 488-nm laser were obtained using a Zeiss LSM 510 META system and processed and analyzed with an AxioVision 4.8.2 analysis program (Zeiss) and ImageJ software (NIH Image). For analysis using lysosensor, guard cells were treated with lysosensor (4 μM) for 20 min at room temperature before observation. The green fluorescence of the dye was observed using an Olympus Fluoview FV 1000 laser scanning confocal microscope at excitation/emission wavelengths of 458 nm/505 to 530 nm. The extent of vacuolar acidification was quantified by measuring the intensity of dye fluorescence in vacuolar lumens using the AxioVision 4.8.2 analysis program. Fluorescence intensities of the whole cell area except the nucleus and chloroplast were measured from mid-plane confocal images of a pair of guard cells, and their mean values were calculated.

Cytosolic Alkalization Measurement

Cytosolic pH was assessed using BCECF-AM (Invitrogen). Fava bean leaf fragment was treated with 20 μM BCECF-AM for 30 min before observation. Ratio of fluorescence emissions of BCECF at 505 to 550 nm with dual excitation (488- and 458-nm lasers) was obtained using a Zeiss LSM 510 META system and processed and analyzed with an AxioVision 4.8.2 analysis program.

Stomatal Aperture Measurement

Abaxial epidermal fragments of fava bean or intact *Arabidopsis* leaves were incubated on 30 mM KCl/10 mM MES-KOH, pH 6.05, buffer for 3 h under white light to open stomata fully. Fava bean leaf epidermal fragments or *Arabidopsis* leaves were then transferred to fresh 10 mM KCl/10 mM MES, pH 6.05, buffer containing 10 or 4 μM ABA. The optimum concentrations of ABA for the assay of stomatal closing movement were experimentally determined for *Arabidopsis* and for fava bean guard cells to be 4 and 10 μM, respectively. To test the effect of PIKfyve inhibitor (YM201636; Calbiochem) on stomatal closure, PIKfyve inhibitor was added to the 10 mM KCl/10 mM MES buffer containing 10 or 4 μM ABA. Stomata were observed and photographed using a Zeiss Axioskop 2 microscope equipped with a charge-coupled device camera. Aperture size was analyzed using the AxioVision 4.8.2 analysis program.

Quantitative RT-PCR Analysis of FAB1 Expression in Guard Cells

The abaxial epidermal layers were peeled off *Arabidopsis* leaves. Epidermal strips were soaked in 10 mM KCl/10 mM MES-KOH, pH 6.05, for 30 min to stabilize the cells and then sonicated for 30 to 40 s to remove mesophyll cells. Epidermal cells other than the guard cells were removed by treating the epidermal layers with phosphate/citrate buffer, pH 4.5, for 30 min with gentle shaking.

Total RNA was isolated from the guard cell–enriched epidermal layers using an RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. The cDNA was synthesized using GoScript reverse transcriptase (Promega), and quantitative PCR was performed using a Takara quantitative PCR kit (SYBR premix EX Taq) and a Takara thermal cycler (TP800) programmed to the following three-step cycling protocol: 30 s at 95°C for one cycle and then 5 s of denaturation at 95°C, 10 s of annealing at 58°C, and 20 s of extension at 72°C for 45 cycles. The primer sets used in this study are listed in Supplemental Table 1 online. Transcript expression levels were normalized to those of β-*Tubulin* in the same experimental setup, and data were calculated according to the $\Delta\Delta C_T$ (cycle threshold) value.

Isolation of FAB1 KO *Arabidopsis* Plants

T-DNA insertion lines of *FAB1B* (*fab1b-1*, SALK_048293; *fab1b-2*, SALK_066673) and *FAB1C* (*fab1c-1*, SAIL_254_G09; *fab1c-2*, SK_13557) were obtained from the ABRC. Homozygote mutant plants were identified using PCR analysis of genomic DNA. The primer sequences used are listed in Supplemental Table 2 online. The absence of FAB1B and FAB1C expression in these KO mutants was confirmed using RT-PCR analysis of transcripts with the gene-specific primer sets listed in Supplemental Table 3. The *fab1b fab1c* double KO mutant was generated by crossing isolated *fab1b-1* with *fab1c-1*.

Water Loss Test

The fully expanded young rosette leaves of wild-type and *fab1* KO mutant (*fab1b*, *fab1c*, and *fab1b fab1c*) plants were excised and their initial fresh

weights were measured. The leaves were exposed to dry air in a controlled growth room (22°C ± 2°C; relative humidity, 40%), and water loss was measured every 10 min for the first 1 h and then every 30 min and calculated as the decrease in weight relative to the initial value.

Bacterial Expression of GST-FAB1C

The full-length FAB1C coding sequence and the coding sequence of the kinase domain alone (856 to 1648 amino acids) were cloned into a pGEX-5X-1 vector to yield GST-FAB1C and GST-FAB1C kinase domain fusions. GST-FAB1C and GST-FAB1C kinase domain fusion proteins were expressed in *Escherichia coli* strain Rosetta (DE3) and purified using GSH-sepharose 4B beads (GE Healthcare) according to the manufacturer's instructions.

Lipid Kinase Activity Assay

GST-FAB1C or GST-FAB1C kinase domain bound to GSH-sepharose beads (0.5 μg) was mixed with the substrate PtdIns3P (10 μg; Echelon) in lipid kinase assay buffer (final volume, 50 μL) containing 25 mM HEPES-KOH, pH 7.4, 1.5 mM $MgCl_2$, 120 mM NaCl, 5 mM 2-glycerophosphate, 1 mM DTT, 10% (w/v) sodium cholate, and 0 to ~10 μM PIKfyve inhibitor (Calbiochem). The reaction was initiated by adding 50 μM cold ATP with 10 μCi of [γ-^{32}P]ATP (Perkin-Elmer) and continued at 25°C for 15 min. For a lipid kinase-negative control, GST proteins bound to GSH-sepharose beads were used instead.

The reaction was terminated by adding 190 μL of chloroform:methanol: concentrated HCl (50:100:1, v/v), and 189 μL of chloroform and 50 μL of 1 N HCl were then sequentially added for phase separation. After the mixture was centrifuged for 1 min in a tabletop centrifuge, the upper phase was removed and the lower chloroform phase was transferred to a new 1.5-mL tube. The separated lower phase was washed three times with an equal volume of chloroform:methanol:1 N HCl (3:48:47, v/v/v). In every washing step, the lower phase was transferred to a new 1.5-mL tube. The final lower phase was concentrated and dried under a stream of N_2 gas, and the dried lipids were dissolved in 30 μL of chloroform:methanol (1:1, v/v).

The dissolved lipids were spotted onto potassium oxalate (1%) pre-coated silica gel H thin layer chromatography plates (Analtech) and separated using developing solution consisting of chloroform:methanol:25% ammonia:water (45:35:2:8, v/v/v/v). Thin layer chromatography plates were then autoradiographed using an x-ray film (Ortho CP-G plus; Agfa). The bands of PtdIns(3,5)P_2 product were identified by comigration with cold PtdIns(3,5)P_2, which was run in a parallel lane on the same thin layer chromatography plate.

Lipid-Protein Binding Assay

Phospholipid-coated strip (PIP strips; Echelon) was incubated with TBST buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, and 0.1% [w/v] Tween 20) supplemented with 3% fatty acid–free BSA for 1 h at 25°C to block the strip. The strip was then treated with 3 μg/mL purified mung bean (*Vigna radiata*) V-PPase (Maeshima and Yoshida, 1989) in TBST at 4°C for 16 h and washed with TBST. V-PPase bound to phospholipids was detected by immunoblot using anti-V-PPase antibody.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: *FAB1A* (At4g33240), *FAB1B* (At3g14270), *FAB1C* (At1g71010), and *FAB1D* (At1g34260).

Supplemental Data

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

Supplemental Figure 1. Characterization of *fab1b* and *fab1c Arabidopsis* Mutants.

Supplemental Figure 2. ABA-Induced Stomatal Closure and Water Loss in *fab1b-2* and *fab1c-2 Arabidopsis*.

Supplemental Figure 3. PIKfyve Inhibitor Suppresses ABA-Induced Stomatal Closure in *Arabidopsis*.

Supplemental Figure 4. Pretreatment with Butyrate Suppresses Vacuolar Acidification during ABA-Induced Stomatal Closure.

Supplemental Table 1. Primers Used for Quantitative RT-PCR to Check *FAB1* Expression Level in Guard Cells.

Supplemental Table 2. Primers Used in Genomic DNA PCR to Isolate *FAB1* Knockout Mutants.

Supplemental Table 3. Primers Used in RT-PCR Analysis of Transcripts to Isolate *FAB1* Knockout Mutants.

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AUTHOR CONTRIBUTIONS

G.B., Y.L., J.-U.H., and Y.L. designed the research. G.B., E.-J.L., M.K., and S.S. performed the experiments. All authors analyzed the data. G.B., J.-U.H., M.M., H.S., and Y. L. wrote the article.

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