Phosphatidylinositol 3- and 4-phosphate modulate actin filament reorganization in guard cells of day flower

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ABSTRACT
Phosphatidylinositol 3-kinases (PtdIns 3-kinases) that produce phosphatidylinositol (3,4,5) triphosphate (PtdIns(3,4,5)P3) are considered to be important regulators of actin dynamics in animal cells. In plants, neither PtdIns(3,4,5)P3 nor the enzyme that produces this lipid has been identified. However, a PtdIns 3-kinase that produces phosphatidylinositol 3-phosphate (PtdIns3P) has been identified, suggesting that PtdIns3P, instead of PtdIns(3,4,5)P3, regulates actin dynamics in plant cells. Phosphatidylinositol 4-kinase (PtdIns 4-kinase) is closely associated with the actin cytoskeleton in plant cells, suggesting a role for this lipid kinase and its product phosphatidylinositol 4-phosphate (PtdIns4P) in actin-related processes. Here, we investigated whether or not PtdIns3P or PtdIns4P plays a role in actin reorganization induced by a plant hormone abscisic acid (ABA) in guard cells of day flower (Commelina communis). ABA-induced changes in actin filaments were inhibited by LY294002 (LY) and wortmannin (WM), inhibitors of PtdIns3P and PtdIns4P synthesis. Expression of PtdIns3P- and PtdIns4P-binding domains also inhibited ABA-induced actin reorganization in a manner similar to LY and WM. These results suggest that PtdIns3P and PtdIns4P regulate actin dynamics in guard cells. Furthermore, we demonstrate that PtdIns3P exerts its effect on actin dynamics, at least in part, via generation of reactive oxygen species (ROS) in response to ABA.

Key-words: cytoskeleton; phosphoinositides; ROS; stomata.

INTRODUCTION
The actin cytoskeleton plays an important role in stomatal movements (Kim et al. 1995; Eun & Lee 1997). In illuminated open stomata, actin filaments are arranged transversely at the guard cell cortex. When these cells are treated with abscisic acid (ABA), a signal for stomatal closure, the actin cytoskeleton rapidly fragments and reorganizes into a random orientation (Eun & Lee 1997). This change in actin organization appears to modulate stomatal movements. When guard cells are treated with phalloidin, a chemical that stabilizes actin filaments, both light-induced stomatal opening and ABA-induced stomatal closing are reduced (Kim et al. 1995). Treatment of guard cells with cytochalasin D (CD), which depolymerizes actin filaments, causes partial opening of dark-closed stomata and enhances stomatal opening under white light irradiation (Kim et al. 1995; Eun & Lee 1997). These actin antagonists also alter inward K+ channel activities (i.e. K+ influx is inhibited and facilitated by phalloidin and CD, respectively) and stretch-activated calcium channels, suggesting that actin filaments regulate stomatal movements by modulating ion channel activity (Hwang et al. 1997; Zhang, Fan & Wu 2007). Potential upstream regulators of actin dynamics include protein phosphatase activity and monomeric G proteins. In Arabidopsis ab1 mutant plants, in which a member of the protein phosphatase 2C subfamily is disrupted, actin dynamics are abnormal and stomata do not close in response to ABA (Eun, Bae & Lee 2001; Lemichez et al. 2001). Expression of a dominant-negative form of the small G protein AtRAC1 causes depolymerization of actin filaments in guard cells and stomatal closure, whereas a constitutively active form of AtRAC1 blocks ABA-mediated effects on actin and stomatal closure (Lemichez et al. 2001). However, the identities of direct regulators of actin dynamics, as well as the precise mechanism(s) underlying their regulation, remain unclear.

In eukaryotic cells, phosphoinositides constitute a minor fraction of total lipid but play many important roles (Drøbak, Dewey & Boss 1999; Stevenson et al. 2000). Phosphoinositides participate in signal transduction processes in guard cells of plants; consistent with this activity, phosphatidylinositol (4,5) bisphosphate (PtdIns(4,5)P2) and PtdIns4P levels fluctuate rapidly in response to ABA (Lee et al. 1996). PtdIns3P and PtdIns4P have been detected in guard cells (Parmar & Brearley 1993, 1995) and are important for normal stomatal movements (Jung et al. 2002). Overexpression of PtdIns3P-(EBD, endosome-binding domain of human EEA1) or PtdIns4P-(FAPP1PH)-binding proteins enhanced light-induced stomatal opening and decreased ABA-induced stomatal closing. It has been suggested that PtdIns3P mediates oscillations in the levels of Ca2+ (Jung et al. 2002), and generation of reactive oxygen species (ROS) in response to ABA (Park et al. 2003). It remains to be determined how PtdIns4P modulates guard cell movements, and whether there are additional mechanisms of action for PtdIns3P in guard cells.
The enzyme PtdIns 3-kinase phosphorylates the D-3 position of the inositol head group on phosphatidylinositol (PtdIns), PtdIns4P and PtdIns(4,5)P_2, and produces PtdIns3P, PtdIns(3,4) bisphosphate and PtdIns(3,4,5)P_3, respectively. In animal cells, three different types of PtdIns 3-kinases exist and they are involved in diverse processes including cell proliferation (Katso et al. 2001), polarization, assembly of actin filaments (Niggli & Keller 1997) and chemotaxis (Higaki et al. 1996; Katso et al. 2001). PtdIns 3-kinase in animal cells regulates actin dynamics predominantly through the production of PtdIns(3,4,5)P_3 (Chung, Potikyan & Firtel 2001; Funamoto et al. 2001; Iijima & Devreotes 2002). However, in Arabidopsis, only one PtdIns 3-kinase (AtVPS34), which exclusively generates PtdIns3P has been identified, and PtdIns(3,4,5)P_3 has not been found. Therefore, an unanswered question is whether the plant PtdIns 3-kinase and its product PtdIns3P regulate actin dynamics, similarly to their counterparts in animal cells. In plants, PtdIns 3-kinase has been reported to be involved in vesicle trafficking (Kim et al. 2001), membrane proliferation (Hong & Verma 1994), vacuolar sorting (Matsuoka et al. 1995), nod factor signalling (Kelly-Skupek & Irving 2006) and endocytosis (Leschem, Seri & Levine 2007). AtVPS34 seems to be necessary for essential functions in normal growth, as plants transformed with an anti-sense construct show severe defects in growth and development (Welters et al. 1994). PtdIns 3-kinase activity has been demonstrated in guard cells and the PtdIns 3-kinase inhibitors LY294002 (LY) and wortmannin (WM) enhance light-induced stomatal opening and decrease ABA-induced stomatal closing, in a manner similar to that of the PtdIns3P-binding protein EBD (Jung et al. 2002).

PtdIns4P is synthesized by PtdIns 4-kinase, which catalyzes phosphorylation of the D-4 position of the inositol ring of PtdIns to form PtdIns4P. In Arabidopsis, 12 putative PtdIns 4-kinases have been identified (Mueller-Roeber & Pical 2002). These kinases are important for membrane biogenesis and vesicle trafficking from the endoplasmic reticulum to the Golgi, and from the Golgi to the plasma membrane (Roth 1999). In a number of mouse cell lines, PtdIns 4-kinase activity has been linked to the actin cytoskeleton and its activity increases upon treatment with epidermal growth factor (Payrastre et al. 1991). In plant cells, PtdIns 4-kinase activity is present in many different locations including the plasma membrane, cytosol and cytoskeleton (Xu et al. 1992; Drøbak et al. 1999; Davis et al. 2007). The close association between PtdIns 4-kinase and the actin cytoskeleton in many types of cell suggests conservation of the role played by this lipid kinase and its product (PtdIns4P) in actin-related processes.

A growing body of evidence implicates phosphoinositides in the modulation of actin dynamics via regulation of actin-binding proteins such as profilin, ADF/cofilin and villin/gelsolin, as well as capping protein (Drøbak, Franklin-Tong & Staiger 2004). Guard cells represent an excellent system for elucidating the role of phosphoinositides in the regulation of actin organization, but the mode of action for these signalling phospholipids, if any, has not yet been studied. Here, we present evidence that PtdIns3P and PtdIns4P modulate actin reorganization in guard cells. We also present data indicating that ROS, which are generated by a pathway involving PtdIns3P, are secondary messengers required for actin fragmentation or reorientation.

**MATERIALS AND METHODS**

**Plant materials and chemicals**

Day flower (Commelina communis L.) plants were grown in a greenhouse under cycles of 16 h light and 8 h dark at 22 ± 2 °C. Plants were watered with Hyponex solution (1 g L⁻¹) (Hyponex Corp., Marysville, OH, USA). In all experiments, tissue samples were taken from fully expanded second leaves of 3- to 4-week-old plants. LY, WM, dimethyl sulfoxide (DMSO), ABA, p-phenylendiamined (PPD), ascorbic acid and catalase were purchased from Sigma (St. Louis, MO, USA). Rhodamine-phalloidin and rose bengal diacetate were purchased from Molecular Probes (Eugene, OR, USA).

**Biologic gene transfer**

We constructed a fusion protein using sequences encoding two FYVE (Fab1, YOTB, Vca1 and EEA1) domains from the mouse Hrs protein linked by the sequence QGQGS and attached to the N-terminus of green and red fluorescent proteins (plasmid mGFP and monomeric RFP, respectively) as described previously (Gillooly et al. 2000). GFP : 2xFYVE, mRFP : 2xFYVE, GFP : FAPP1PH, GFP : PLCδ1PH, GFP alone and mRFP alone were introduced into C. communis guard cells with the bombardment technique (Particle Delivery System-1000/He; Bio-Rad, Hercules, CA, USA). In brief, 10 μg of plasmid DNA was mixed with 1.25 mg of 1.0-μm-diameter gold particles (Bio-Rad) in a 25 μL aqueous solution. We then added 1.25 mM CaCl₂ and 20 mM spermidine to the DNA-gold particle mixture, vortexing vigorously. This mixture was resuspended in ethanol and applied to a plastic macrorcarier. Healthy young leaves from 3- to 4-week-old day flower plants were placed on wet filter papers in petri dishes. A vacuum was pumped to 28.5 inch-Hg and DNA-coated gold particles were shot into the leaves at 1350 psi He pressure. Bombardment-treated leaves were maintained in darkness for 16–24 h.

**Stomatal aperture measurements**

The epidermal fragments of day flower leaves were floated on 30 mm KCl and 10 mM MES-KOH (pH 6.1) bathing medium under 0.15 to 0.16 mmol m⁻²s⁻¹ white light at 29 °C for 2.5 h. The epidermal tissues were then treated for 1 h with 10 μM ABA or DMSO added to the bathing medium. Stomatal apertures were measured using an Axio vision 3.0 (Zeiss, Jena, Germany).

**Visualization of actin filaments**

After bombardments or pretreatments, epidermal fragments were fixed for 4 h at room temperature in PMSE
buffer [50 mM PIPES (1,4-piperazinediethanesulfonic acid), 2 mM MgSO4, 5 mM EGTA, 0.25% (v/v) DMSO and 0.0025% (v/v) Triton X-100] containing 0.2 mM m-maleimidobenzoyl N-hydroxysuccinimide ester (MBS). Actin filaments in guard cells were then stained by incubation overnight at room temperature in phosphate-buffered saline containing rhodamine-phalloidin (1 U/100 μL), 0.05% (v/v) Triton X-100 and 0.1% (v/v) PPD. Actin filaments were observed and photographed using either a fluorescence microscope (Axioskop 2; Zeiss) or a confocal microscope (FLUOVIEW FV1000; Olympus, Tokyo, Japan). All confocal images were obtained using a 60x oil immersion objective. The excitation wavelength was 488 nm for GFP and 543 nm for rhodamine-phalloidin. Emission was detected for GFP between 500 and 530 nm, and for rhodamine-phalloidin between 555 and 655 nm. In each experiment, more than 10 guard cell pairs (transformed and its non-transformed sister) from at least two epidermal fragments were observed per sample, and results from three to five independent experiments were used for statistical analyses (Student’s t-test).

**RESULTS**

**LY and WM inhibit ABA-induced actin reorganization**

To elucidate the roles of phosphoinositide lipids in dynamic changes in actin reorganization, we pretreated *C. communis* guard cells with phosphatidylinositol kinases inhibitors (LY or WM) and observed ABA-induced changes in actin filaments. For quantitative analyses of guard cell actin filament arrays, we identified three patterns (types 1, 2 and 3). Type 1 arrays comprise long filaments at the cortical area of the cell, radiating out from the stomatal pore side in transverse directions and surrounding the cytoplasm in a hoop-like pattern; they are present in open guard cells under light irradiation (Fig. 1a). Type 2 arrays are short and fragmented; they are found in stomata in transition from an open to closed state (Fig. 1b). Type 3 arrays are randomly distributed in the cortical, subcortical and central areas of the cell; they are typically found in closed stomata following

![Figure 1](image-url)
treatment with ABA for about 1 h (Fig. 1c). This classification represents a simplification of that used by Hwang & Lee (2001), in that it amalgamates the transitional patterns of the original types 2 and 3 into type 2.

Firstly, we confirmed that treatment with 10 µM ABA for 1 h altered actin patterns in guard cells from a predominantly transverse to a random orientation (Fig. 1d); the proportion of cells with type 1 actin decreased from 75 ± 3% (average ± SD) (untreated control) to 10 ± 2% (ABA), whereas cells with a type 3 arrays increased from 5 ± 2% to 70 ± 5%, respectively. Then we pretreated the samples with the inhibitors 100 µM LY or 10 µM WM, and observed that the proportion of cells maintaining type 1 arrays increased from 10 ± 2% (ABA) to 52 ± 6% (ABA + LY) and 47 ± 4% (ABA + WM), respectively. Conversely, the same pre-treatment decreased the proportion of cells that formed type 3 actin from 70 ± 5% (ABA) to 26 ± 8% (ABA + LY) and 33 ± 5% (ABA + WM). Treatment with LY or WM alone for 30 min did not induce a significant change in the proportion of actin patterns observed (Fig. 1d; P > 0.05).

Thus, these results suggest that LY or WM pretreatment inhibits ABA-induced reorganization of actin filaments.

**Biolistically introduced phosphatidylinositol-binding proteins have effects similar to LY and WM on ABA-induced stomatal closing and actin reorganization**

For blocking signal transduction downstream of PtdIns3P, we employed a biolistic gene delivery system and transiently expressed the PtdIns3P-binding protein GFP : 2xFYVE in *C. communis* guard cells. The FYVE domain has been reported to bind specifically and with high affinity to PtdIns3P in yeast, mammals and plants (Gillooly et al. 2000; Voigt et al. 2005; Vermeer et al. 2006). Although *C. communis* is one of the best systems for guard cell research, particularly for actin dynamics, it is a difficult plant for genetic transformation, and thus there has been no report of successful transformation of its guard cells.

Firstly, we examined the distribution of GFP : 2xFYVE in guard cells and observed that it co-localized with (or close to) large and small vesicles, as well as tonoplast membranes (Fig. 2a), similar to patterns reported for guard cells of *Arabidopsis* (Vermeer et al. 2006). We then compared the half-stomatal apertures of transformed guard cells expressing GFP : 2xFYVE (At; Fig. 2b) with those of non-transformed guard cells (An; Fig. 2b). The non-transformed sister guard cells served as a good control because a pair of guard cells is produced from division of a single guard mother cell. Although overexpression of GFP : 2xFYVE accelerated light-induced stomatal opening under our normal conditions (10 mM KCl and 23 °C), transformed and non-transformed stomata were induced to open to the same extent by using 30 mM KCl and increasing the temperature to 29 °C (as described by Jung et al. 2002). Subsequent treatment with 10 µM ABA for 1 h reduced the half stomatal aperture of both transformed and non-transformed guard cells. However, the aperture of transformed cells was

**Figure 2. Abscisic acid (ABA)-induced stomatal movements and actin reorganization in guard cells expressing GFP : 2xFYVE are inhibited.** Half-stomatal apertures of stomata bordered by GFP : 2xFYVE-transformed (At) guard cells are larger than those of non-transformed (An) guard cells at 1 h after treatment with 10 µM ABA (b,c). Images show GFP : 2xFYVE fluorescence (a) and bright-field (b) photograph of a live guard cell transiently transformed with GFP : 2xFYVE and its neighbour non-transformed guard cell. Results shown are averages ± SD from three independent experiments. Asterisk indicates significant difference from non-transformed sister guard cells (P < 0.01, n = 51). Actin filaments display a random pattern in the majority of the non-transformed guard cells, but the ABA-induced change in actin pattern is suppressed in guard cells transformed with GFP : 2xFYVE (d–g). Images shown are GFP : 2xFYVE fluorescence (d), actin filaments visualized by rhodamine-phalloidin staining (e), and bright field image of the same cells (f). Guard cells biolistically transformed with GFP : 2xFYVE and their sister guard cells were irradiated with white light for 3 h, treated with 10 µM ABA for 1 h, then fixed for observation of actin filaments. Only the guard cell that shows green fluorescence is transformed with GFP : 2xFYVE (d). The cells shown in (a) and (b) are live whereas those in (d), (e) and (f) are fixed for visualization of actin filaments. Results shown are averages ± SD from five independent experiments (n = 82). Bar = 10 µm.
larger than that of non-transformed cells (1.2 ± 0.1 μm and 2.1 ± 0.2 μm for non-transformed and transformed guard cells, respectively, \( P < 0.01, n = 51, \text{Fig. 2c} \)). This result shows that the FYVE domain inhibited ABA-induced stomatal closing, and confirms the previous report of the same effect of another PtdIns3P-binding domain EBD in *Vicia faba* guard cells (Jung *et al.* 2002).

To observe the effects of the PtdIns3P-binding domain on ABA-induced actin patterns, we bombarded *C. communis* guard cells with the GFP : 2xFYVE construct, and after 16 h, irradiated with white light for 3 h, treated with 10 μM ABA for 1 h, then labelled with rhodamine-phalloidin for visualization of actin filaments. Many non-transformed guard cells (83 ± 5% of all labelled cells) had type 3 actin filament arrays typical of ABA treatment (guard cell on the left, Fig. 2e, right, \( n = 82 \)), whereas only 30 ± 8% of guard cells expressing 2xFYVE were able to form type 3 arrays. Conversely, type 1 actin arrays were found in only 6 ± 2% of non-transformed sister guard cells, whereas they were present in 37 ± 8% of transformed guard cells (guard cell on the right, Fig. 2e, left). These results demonstrate that binding of PtdIns3P (with consequent reduction of free PtdIns3P) inhibits ABA-induced actin reorganization in guard cells.

Transformation of guard cells with GFP alone did not alter the ABA-induced actin reorganization (\( n = 63, \text{Fig. 3} \)). At 1 h after treatment with 10 μM ABA, the majority of GFP-transformed guard cells showed type 3 actin arrays, and less than 10% of the cells showed type 1 arrays (Fig. 3d). This actin pattern closely matched that found in the non-transformed sister guard cells (Fig. 3d). These results indicate that GFP does not interfere with actin organization in guard cells, and thus is not responsible for the effect of GFP : 2xFYVE on ABA-induced actin dynamics (Fig. 2g).

To block signal transduction downstream of PtdIns4P, we bombarded guard cells with a vector for transient expression of the PtdIns4P-binding protein GFP : FAPP1PH (PI-four-phosphate adaptor protein-1 pleckstrin homology domain). FAPP1PH has been reported to bind specifically to PtdIns4P in vitro (Dowler *et al.* 2000; Jung *et al.* 2002), and we observed previously that FAPP1PH localizes at or near the plasma membrane and nucleus in guard cells, and inhibits ABA-induced stomatal closing (Jung *et al.* 2002). To observe the effects of GFP : FAPP1PH on actin reorganization during the ABA response, guard cells bombarded with the GFP : FAPP1PH construct were irradiated with white light for 3 h, treated with 10 μM ABA for 1 h, then stained with rhodamine-phalloidin. During the ABA response, GFP : FAPP1PH exhibited a similar (but slightly less pronounced) effect on actin reorganization when compared with that of GFP : 2xFYVE (Fig. 4). Type 3 random filament arrays were found in the majority (86 ± 5% of all labelled cells, \( n = 89 \); left panel, Fig. 4d) of non-transformed guard cells (guard cell on the left, Fig. 4b). In guard cells which were transformed with FAPP1PH (guard cell on the right, Fig. 4b), the population with type 3 actin decreased to 47 ± 8% (right panel, Fig. 4d). In contrast, the population with type 1 actin increased significantly (\( P < 0.05 \)) from 4 ± 2% in non-transformed cells to 23 ± 8% in cells expressing GFP : FAPP1PH. These results indicate that binding of PtdIns4P (and the consequent reduction in free PtdIns4P) inhibits ABA-induced actin reorganization in guard cells.

PtdIns(4,5)P2 has been implicated in actin dynamics in many plant cells (Kost *et al.* 1999; Stevenson *et al.* 2000; Allwood *et al.* 2002), although its effect on guard cell actin organization has never been tested. To test whether PtdIns(4,5)P2 has effects on actin organization in guard cells, we transformed the cells with GFP : PLCδ1PH (phospholipase Cδ1 pleckstrin homology domain), which binds specifically to PtdIns(4,5)P2, and has been used to visualize PtdIns(4,5)P2 at the plasma membrane (Stauffer, Ahn & Meyer 1998; Lee *et al.* 2007). At 1 h after ABA treatment, GFP : PLCδ1PH did not exhibit a similar effect on actin reorganization (Fig. 5) when compared with GFP : 2xFYVE (Fig. 2) or GFP : FAPP1PH (Fig. 4). Patterns of actin filaments were not different in guard cells transformed with GFP : PLCδ1PH and their non-transformed sister cells (\( P > 0.05 \) by Student’s paired
After 1 d, were treated with ABA for 1 h, followed by an ROS staining dye H₂-dichlorofluorescein (DCF)-DA. The cells expressing mRFP:2xFYVE were identified by their red fluorescence (Fig. 6a), and their DCF intensity (Fig. 6b) was compared to that of neighbouring non-transformed guard cells according to the method described previously (Zhang et al. 2001; Park et al. 2003). After 1 h of ABA treatment, the DCF intensity of transformed guard cells was lower (69 ± 3%, P < 0.01, n = 42–69; Fig. 6g) than that of their non-transformed neighbours. As a control, mRFP alone was expressed in guard cells using the same method (Fig. 6d,e). Guard cells overexpressing the mRFP construct alone did not exhibit significantly different ROS generation from their non-transformed neighbours (P > 0.05; Fig. 6g). These results confirmed that an additional PtdIns3P-binding domain inhibits ABA-induced ROS generation in guard cells. WM and LY also have been shown to inhibit ABA-induced ROS generation (Park et al. 2003). Because the three treatments that reduce the level of free PtdIns3P (LY, WM and 2xFYVE) inhibited both ABA-induced ROS generation and actin dynamics, we hypothesized that ROS is a mediator of PtdIns3P effect on actin dynamics in guard cells.

**Effects of ROS on actin filaments organization and stomatal movements**

A possible mechanism for PtdIns3P action in ABA-induced actin reorganization is via regulation of ROS, a previously reported downstream target of PtdIns3P in both animal and plant cells (Ellson et al. 2001; Park et al. 2003). In contrast, PtdIns4P was shown not to be involved in ABA-induced ROS generation in guard cells (Park et al. 2003). Firstly, we examined whether or not expression of the 2xFYVE domain in guard cells inhibited ROS generation following ABA treatment, as had been observed previously with the PtdIns3P-binding domain EBD (Park et al. 2003). A gene construct expressing the 2xFYVE domain fused to mRFP (mRFP:2xFYVE) was bombarded into guard cells, which, after 1 d, were treated with ABA for 1 h, followed by an ROS staining dye H₂-dichlorofluorescein (DCF)-DA. The cells expressing mRFP:2xFYVE were identified by their red fluorescence (Fig. 6a), and their DCF intensity (Fig. 6b) was compared to that of neighbouring non-transformed guard cells according to the method described previously (Zhang et al. 2001; Park et al. 2003). After 1 h of ABA treatment, the DCF intensity of transformed guard cells was lower (69 ± 3%, P < 0.01, n = 42–69; Fig. 6g) than that of their non-transformed neighbours. As a control, mRFP alone was expressed in guard cells using the same method (Fig. 6d,e). Guard cells overexpressing the mRFP construct alone did not exhibit significantly different ROS generation from their non-transformed neighbours (P > 0.05; Fig. 6g). These results confirmed that an additional PtdIns3P-binding domain inhibits ABA-induced ROS generation in guard cells. WM and LY also have been shown to inhibit ABA-induced ROS generation (Park et al. 2003). Because the three treatments that reduce the level of free PtdIns3P (LY, WM and 2xFYVE) inhibited both ABA-induced ROS generation and actin dynamics, we hypothesized that ROS is a mediator of PtdIns3P effect on actin dynamics in guard cells.

**Figure 4.** Abscisic acid (ABA)-induced actin filament reorganization in guard cells expressing GFP:FAPP1PH is inhibited. Images shown are fluorescence of GFP:FAPP1PH (a), actin filaments visualized by rhodamine-phalloidin staining (b) and bright field image of the same cells (c). Biolistically transformed guard cells and their sister cells were irradiated with white light for 3 h, and then treated with 10 μM ABA for 1 h. Actin filaments in non-transformed sister cells are in random pattern, whereas those in transformed guard cells remain transverse (b). Actin patterns in populations of non-transformed and transformed guard cells treated with 10 μM ABA for 1 h indicate that expression of GFP:FAPP1PH inhibited ABA-induced reorganization of actin filaments (d). Results shown are averages ± SD from five independent experiments (n = 89). Bar in (a) = 10 μm.

**Figure 5.** Abscisic acid (ABA)-induced actin filament reorganization in guard cells expressing GFP:PLCδ1PH is not significantly inhibited. GFP:PLCδ1PH fluorescence in a biolistically transformed guard cell, irradiated with white light for 3 h, and then treated with 10 μM ABA for 1 h (a). Actin filaments of the same guard cell shown in (a) and its non-transformed sister guard cell (b). Bright field image of the same cells shown in panels a and b (c). Actin patterns in non-transformed and transformed guard cells treated with 10 μM ABA for 1 h are not significantly different (b,d). Results shown are averages ± SD from five independent experiments (n = 63). Bar in (a) = 10 μm.
To test this hypothesis, we investigated whether or not ROS alone can induce actin reorganization in the absence of ABA treatment. Epidermal strips of *C. communis* leaves were floated for 1 h on medium containing rose bengal diacetate, which permeates through the plasma membrane and is cleaved by endogenous esterases to produce singlet oxygen. Singlet oxygen is very unstable and rapidly converted to other ROS species in plant (Foyer, Lelandais & Kunert 1994) and animal cells (Ishibashi, Lee & Okabe 1996). Rose bengal diacetate-treated guard cells showed elevated levels of DCF fluorescence (Fig. 7a,b), indicating that the treatment indeed increased the level of H$_2$O$_2$ in the cells. The epidermal strips were then stained with rhodamine-phalloidin. In epidermal tissues treated with 200 µm rose bengal diacetate, we observed significantly higher proportions of guard cells containing type 2 (46 ± 7% versus 30 ± 4%, $P < 0.05$) and type 3 (36 ± 8% versus 13 ± 5%, $P < 0.05$; Fig. 7c) actin filaments than in non-treated tissue.

If ROS are necessary for ABA-induced actin reorganization, reductions in ROS levels may inhibit actin reorganization, even in the presence of ABA. To test this possibility we used ascorbic acid (a reducing agent) and catalase (an enzyme that degrades H$_2$O$_2$), that have been shown to reduce stomatal closing induced by ABA (Zhang et al. 2001) and elicitors (Lee et al. 1999), via reducing ROS in guard cells (Zhang et al. 2001). Epidermal fragments of *C. communis* were floated on buffer solution and irradiated with white light for 2.5 h until their stomata open wide, then they were pretreated with ascorbic acid or catalase for 30 min. Subsequently, they were treated with 10 µm ABA for 1 h, then fixed and stained for visualization of actin filaments. In ABA-treated guard cells, treatment with either ascorbic acid or catalase increased the proportion of cells with type 1 actin filament arrays from 13 ± 5% (ABA

![Figure 6](image_url). Expression of mRFP: 2xFYVE in guard cells inhibits abscisic acid (ABA)-induced reactive oxygen species generation. Epidermal tissue showing a guard cell transiently transformed with mRFP: 2xFYVE (a,b,c). Note that the three other guard cells in this field of view are not transformed (a,c). Epidermal tissue showing a guard cell transiently transformed with free mRFP (d,e,f). The three other guard cells in this field of view are not transformed (d,f). (a,d) Red fluorescence of RFP from guard cells expressing mRFP: 2xFYVE or free mRFP (b,e). Green fluorescence images of guard cells shown in (a) and (d), respectively, loaded with H$_2$-dichlorofluorescein (DCF) solution. (c,f) Bright field images corresponding to fluorescence images (a,b) and (d,e), respectively, Following ABA treatment, relative DCF intensity level of guard cells expressing mRFP: 2xFYVE to their neighbouring guard cells is lower than that of guard cells expressing free mRFP (g) (*$P < 0.01$, $n = 42–69$ from three independent experiments). Bar in (a) = 10 µm.

![Figure 7](image_url). Reactive oxygen species induce depolymerization of actin filaments in guard cells. The guard cells were treated without (a) or with (b) 200 µm rose bengal, and then loaded with H$_2$-dichlorofluorescein (DCF). The bright DCF fluorescence in (b) indicates H$_2$O$_2$ level elevated by the treatment. Compared to controls, the rose bengal-treated guard cells show increased proportions of types 2 and 3, and decreased proportion of type 1 actin arrays (c). Results shown are averages ± SD from five independent experiments ($P < 0.05$, $n = 103–132$). Bar in (b) = 20 µm.
decreased the medium pH to 5.2. Using HCl to provide proper control for ascorbic acid which inhibited ABA-induced actin filament reorganization in guard cells. The results and our discussions are summarized in Fig. 7, and together, they support the idea that ROS play a role in reorganization of actin filaments in guard cells. Upon exposure to ABA, guard cells initiate ABA signalling pathway, which activates PI3K and PI4K. The kinases produce PtdIns3P and PtdIns4P, respectively, which bind to actin-binding proteins (ABPs) and typically inhibit their activity. PtdIns3P is involved in generation of reactive oxygen species (ROS), which in turn activates Ca$^{2+}$ channels. Elevated Ca$^{2+}$ level also alters ABP activities directly (e.g. villin) or indirectly (e.g. profilin). Some ABPs bind to actin filaments and stabilize them against depolymerization (e.g. villin1 and fimbrin). Thus, elevation of PtdInsPs will inactivate these ABPs and facilitate depolymerization of actin filaments in the cortical area, and perhaps subsequently permit repolymerization in subcortical areas of the cell. Wortmannin (WM) and LY294002 (LY) inhibit lipid kinases as indicated.

**DISCUSSION**

In this study, we used lipid kinase inhibitors and biolistic gene transfer to investigate the roles of PtdIns3P and PtdIns4P in dynamic changes of actin organization in plant guard cells. The results and our discussions are summarized in Fig. 9. The PtdIns 3- and PtdIns 4-kinase inhibitors (LY and WM) inhibited ABA-induced reorganization of actin filaments in guard cells (Fig. 1). Biolistically transformed cells expressing the PtdIns3P-binding domain (2xFYVE) showed inhibition of ABA-induced stomatal closing (Fig. 2), and the actin organization showed similar effects to treatment with lipid kinase inhibitors (Fig. 2). The same PtdIns3P-binding domain inhibited ABA-induced ROS generation (Fig. 6), leading to the hypothesis that ROS are necessary intermediates for PtdIns3P-mediated actin reorganization. Indeed, ROS reduced the proportion of cells that maintained transverse actin filaments (Fig. 7). In contrast, agents that reduce ROS levels increased the proportion of cells with transverse actin after ABA treatment (Fig. 8). They also inhibited the formation of random actin filaments following ABA treatment (Fig. 8). Thus, we propose that ROS are the downstream effector molecules of PtdIns3P in ABA-induced actin reorganization. Biolistically transformed cells expressing the PtdIns4P-binding domain also exhibited similar, but less pronounced effects on actin dynamics when compared with the lipid kinase inhibitors and the PtdIns3P-binding domain (Fig. 4). Collectively, these results suggest that PtdIns3P and PtdIns4P are important factors in ABA-induced actin reorganization and stomatal closing movements in *C. communis* guard cells. To the best of our knowledge, this is the first direct experimental evidence for roles of PtdIns3P and PtdIns4P as regulators of actin dynamics in plant cells. The use of these monophosphorylated inositides to modulate actin dynamics is not common in animal cells which more often

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**Figure 8.** Ascorbic acid and catalase inhibit abscisic acid (ABA)-induced actin filament reorganization in guard cells. The cells were pretreated with 5 mM ascorbic acid (a) or 3 mg mL$^{-1}$ catalase (b) for 30 min prior to 1 h ABA treatment, then fixed and processed for visualization of actin filaments. Results shown are averages ± SD from five independent experiments ($P < 0.01$, $n = 120–185$). In (a), the pH of all bath solutions was adjusted to 5.2 using HCl to provide proper control for ascorbic acid which decreased the medium pH to 5.2.

**Figure 9.** A model for the roles of PtdIns3P and PtdIns4P in abscisic acid (ABA)-induced actin filament reorganization in guard cells. Upon exposure to ABA, guard cells initiate ABA signalling pathway, which activates PI3K and PI4K. The kinases produce PtdIns3P and PtdIns4P, respectively, which bind to actin-binding proteins (ABPs) and typically inhibit their activity. PtdIns3P is involved in generation of reactive oxygen species (ROS), which in turn activates Ca$^{2+}$ channels. Elevated Ca$^{2+}$ level also alters ABP activities directly (e.g. villin) or indirectly (e.g. profilin). Some ABPs bind to actin filaments and stabilize them against depolymerization (e.g. villin1 and fimbrin). Thus, elevation of PtdInsPs will inactivate these ABPs and facilitate depolymerization of actin filaments in the cortical area, and perhaps subsequently permit repolymerization in subcortical areas of the cell. Wortmannin (WM) and LY294002 (LY) inhibit lipid kinases as indicated.
use PtdIns(3,4,5)P$_3$ for this function. It is tempting to speculate that the diverse functions of PtdIns(3,4,5)P$_3$ in animal cells is carried out by PtdIns3P in plant cells, where the type I PtdIns 3-kinase and its product PtdIns(3,4,5)P$_3$ have not been found. Consistent with this hypothesis, AtPDK1, a counterpart to human PDK1 which binds PtdIns(3,4,5)P$_3$, binds PtdIns3P (Deak et al. 1999).

PtdIns3P is reported to modulate a variety of downstream factors in a number of signalling pathways. In order to determine whether or not it plays a role in actin reorganization of guard cells, we attempted to isolate a PI3K knockout mutant in Arabidopsis. However, the self-fertilized heterozygous T-DNA insertion plants (Salk_007281, which has T-DNA inserted at positions +2388 relative to the ATG of At(VPS34) produced progeny that segregated 1:1 for wild type and heterozygous plants, and no viable homozygous knockout plants were obtained (Y. Lee, unpublished results). As an alternative approach, we used PtdIns 3-kinase inhibitors and transient expression of a PtdIns3P-binding domain (2xFYVE), to inhibit PtdIns3P synthesis and interaction with downstream effector molecules. In most guard cells of C. communis, both treatments inhibited ABA-induced stomatal closing and actin reorganization (Figs 1 & 2). Thus, PtdIns3P is an important factor in ABA-induced actin reorganization in guard cells. PtdIns 3-kinase inhibitors and 2xFYVE may have inhibited stomatal closing movements (Fig. 2c) in a manner similar to phalloidin, which is an actin-stabilizing agent that reduces stomatal closing (Kim et al. 1995).

PtdIns4P also appears to be involved in regulation of actin dynamics in guard cells during the ABA response, because expression of the PtdIns4P-binding protein FAPP1PH inhibits actin reorganization following ABA treatment (Fig. 4). Although PtdIns 4-kinase activity is associated with the cytoskeleton in plant cells (Xu et al. 1992; Davis et al. 2007), and therefore it was postulated to be involved in regulating actin dynamics, no direct evidence for such a role for the enzyme was available before. It has been suggested that in animal cells, PtdIns4P and PtdIns(4,5)P$_2$ play an important role in actin polymerization via binding of actin-severing proteins (Janmey et al. 1987; Janmey & Stossel 1989). In plants, actin regulatory proteins are often functionally conserved (Drøbak et al. 2004). Plant capping protein, profilin and actin depolymerizing factor (ADF)/cofilin bind to and are regulated by PtdIns(4,5)P$_2$ in vitro (Staiger et al. 1997; Kovar et al. 2001; Allwood et al. 2002; Huang et al. 2003, 2006; Drøbak et al. 2004). ADF/cofilin is the only plant actin-binding protein published to interact with PtdIns4P (Gungabissoon et al. 1998; Allwood et al. 2002), but most others have not yet been tested. Generally, phosphoinositide binding inhibits the ability of actin-binding proteins to interact with actin. Inhibition of ADF/cofilin activity by phosphoinositides would likely result in more stable actin filaments and lower rates of actin filament turnover; thus, this seems an unlikely mechanism for ABA-induced filament elongation and reorientation in guard cells. On the other hand, plant fimbrin or villin1 which binds to and stabilizes actin filaments (Kovar et al. 2000; Huang et al. 2005), might be more likely targets for lipid regulation in guard cells. Both AtFIM1 and AtFIM2 are regulated by binding to PtdIns3P and PtdIns4P, among other phosphoinositides; specifically, their F-actin binding and bundling activity is inhibited by a broad range of lipid species (L. Y. Gao et al., unpublished results). And, AtVLN1 binds to several phosphoinositides on protein-lipid overlays, with a marked preference for those phosphorylated at the 3-OH position of the inositol headgroup (S. Huang & C. J. Staiger, unpublished results). Elevation of cellular PtdIns3P and/or PtdIns4P could result in fimbrin (or villin1) release from actin filaments, thereby allowing ADF/cofilin access to filaments and stimulation of depolymerization (Huang et al. 2005).

PtdIns(4,5)P$_2$ has been thought to play a role in actin dynamics through interactions with many actin-binding proteins (Staiger et al. 1997; Stevenson et al. 2000; Allwood et al. 2002; Drøbak et al. 2004). In Arabidopsis, AtP1PK1, a phosphatidylinositol phosphate kinase that produces PtdIns(4,5)P$_2$, directly binds actin filaments and recruits AtP14K1 to the actin cytoskeleton (Davis et al. 2007). Therefore, it is surprising that in guard cells PtdIns(4,5)P$_2$ did not show any dramatic effect on ABA-induced actin reorganization (Fig. 5). The question as to why PtdIns4P does, and PtdIns(4,5)P$_2$ does not regulate actin pattern in this cell type is an intriguing question that remains to be determined.

Experiments with a PtdIns3P-binding domain (EBD) indicate that ROS are downstream targets of PtdIns3P in ABA-induced stomatal closing movements (Park et al. 2003). Using an alternative PtdIns3P-binding domain (2xFYVE), we confirmed that PtdIns3P is involved in ABA-induced ROS generation (Fig. 6). To test whether or not ROS are involved in actin reorganization, we used both a ROS generation system and agents that reduce cellular ROS levels. Actin filaments in guard cells treated with rose bengal diacetate were fragmented and randomly arranged (Fig. 7). Although prolonged treatments with a highly concentrated ROS may have harmful effects on cells, the concentration range and treatment time of rose bengal diacetate employed in this experiment have been used previously to demonstrate the role of ROS in signalling without deleterious effects on cells (Green & Fluhr 1995; Allan & Fluhr 1997; Narusaka et al. 2003), and we did not observe any toxic effect of the drug on guard cells, which exclude the possibility that the observed effect is due to damage to the cells. ABA-induced depolymerization and subsequent formation of random actin filaments was inhibited by ascorbic acid (an ROS scavenging anti-oxidant) and catalase (a catalytic anti-oxidant; Fig. 8). These agents have been shown to reduce stomatal closing induced by ABA (Zhang et al. 2001) and elicitors (Lee et al. 1999). The effect of catalase was shown to be due to reduction in ROS level in and around the guard cells (Zhang et al. 2001). Although catalase was not expected to cross the plasma membrane, H$_2$O$_2$ has been shown to permeate through the membrane efficiently (Yamasaki, Sakihama & Ikehara 1997). Thus, our results showed that ROS generation and reduction effected
opposing responses, which strongly support the suggestion that ROS are regulators of actin organization.

Interestingly, comparison of ABA and ROS effects on actin reorganization (Figs 2g & 4d, left panels; Fig. 7c, right panel) revealed that ROS alone could not completely substitute for ABA in inducing actin reorganization. In epidermal tissues treated with ROS, a larger proportion of guard cells maintained transverse (type 1) actin arrays than in ABA-treated epidermis (18 ± 5% in Fig. 7c, right panel and 6 ± 2% in Fig. 2g, left panel, respectively). A great difference in the proportion of cells that formed type 3 random actin filament arrays was observed between ROS and ABA treatments (36 ± 8% in Fig. 7c, right panel and 83 ± 5% in Fig. 2g, left panel, respectively), too. Therefore we suggest that, in addition to ROS, ABA-induced fragmentation of transverse actin filaments and formation of random actin filaments requires another as yet unidentified mediator.

How can ROS regulate actin organization? In animal cells, ROS depolymerize actin filaments via weakening of inter-monomer bonds (Milzani, DalleDonne & Colombo 1997). In plants, ROS are known to be involved in various signalling pathways via Ca\(^{2+}\) channel activation (Mori & Schroeder 2004) and ROS generation in response to ABA opens Ca\(^{2+}\) channels in guard cells (Pei et al. 2000). It has been suggested that Ca\(^{2+}\) is important for regulation of actin organization: in pollen tubes, low and high concentrations of Ca\(^{2+}\) cause actin depolymerization and polymerization, respectively (Wang et al. 2004). Some actin-binding proteins bind actin in a Ca\(^{2+}\)-dependent manner. For example, villin arranges actin filaments into bundles at low [Ca\(^{2+}\)] but regulates actin dynamics through capping and depolymerization at high [Ca\(^{2+}\)] (Yokota et al. 2005). Moreover, profilin activity is indirectly regulated by [Ca\(^{2+}\)]; the profilin-Ca\(^{2+}\)-G-actin complex adds poorly to filament barbed ends, thereby enhancing the apparent sequestering effect of profilin in the presence of physiological [Ca\(^{2+}\)] (Kovar et al. 2001). Therefore, it is possible that the elevated levels of ROS generated by ABA treatment induce actin depolymerization by directly weakening inter-monomer bonds or indirectly through regulating Ca\(^{2+}\) channel activity.

Another factor that may regulate actin dynamics in guard cells is a small G protein, the Rho-related GT-pase Rop, which regulates ROS generation in guard cells (Park et al. 2003). It also regulates ROS and the Ca\(^{2+}\) gradient in pollen tube cells (Li et al. 1999). In fact, this family of monomeric GTPases is a key regulator of the actin cytoskeleton in yeast and animal cells (Hall 1998). In many animal cells, Rho-related small G proteins are downstream targets of PtdIns(3,4,5)\(_3\) in processes that require dynamic changes in actin filament organization (Katso et al. 2001). Rop activity regulates the actin cytoskeleton in plant cells (Kost et al. 1999). In guard cells, ABA-induced inactivation of AtRac1 is essential for stomatal closure and actin depolymerization (Lemichez et al. 2001). A constitutively active form of AtRac1 blocks ABA-mediated effects on the actin cytoskeleton and stomatal closure, and a dominant-negative AtRac1 mutant exhibits ABA-like effects in the absence of the hormone. Rop2 is also known to regulate actin organization during root hair initiation (Jones et al. 2002), in the formation of cortical fine F-actin arrays during organogenesis (Fu, Li & Yang 2002) and in tip-localized F-actin in pollen tubes (Fu, Wu & Yang 2001). Further investigations will be required to determine whether or not Rop activity is affected by PtdIns3P in plant cells, similarly as Rho small G proteins are regulated by PtdIns(3,4,5)\(_3\) in animal cells (Katso et al. 2001), and if this lipid interacts with any other members of its family.

In this paper, we have demonstrated that PtdIns3P and PtdIns4P are required for actin reorganization in guard cells and the stomatal closing movement induced by ABA. Our results establish roles for these mono-phosphorylated inositol lipids in actin dynamics of guard cells, one of the most frequently used plant cell-signalling systems. PtdIns3P and PtdIns4P may modulate actin dynamics by interacting with other factors such as actin-binding proteins, and further investigation will be necessary to identify these interaction partners, as well as to understand how these lipids regulate actin dynamics in plant cells.

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