A Role for Phosphatidylinositol 3-Phosphate in Abscisic Acid-Induced Reactive Oxygen Species Generation in Guard Cells

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Guard cells generate reactive oxygen species (ROS) in response to abscisic acid (ABA), which leads to stomatal closing. The upstream steps of the ABA-induced ROS generation pathway remain largely unknown. In animal cells, ROS generation in neutrophils is activated by phosphatidylinositol 3-phosphate (PI3P). Stomatal guard cells contain PI3P and PI 3-kinase activity. In this study, we tested whether PI3P has a role in ROS generation in guard cells exposed to ABA. We found that PI 3-kinase inhibitors wortmannin or LY294002 inhibited ABA-induced ROS generation and stomatal closing. Endosome-binding domain (of human EEA1), which specifically binds to PI3P, also inhibited ABA-induced ROS generation and stomatal closing when overexpressed in guard cells. Hydrogen peroxide partially reversed the effects of wortmannin or LY294002 on ABA-induced stomatal closing. These results support a role for PI3P in ABA-induced ROS generation and stomatal closing movement.

Many plant cells generate reactive oxygen species (ROS) during various physiological and pathological processes. Maize (Zea mays) root cells generate ROS during gravitropic response (Joo et al., 2001), whereas suspension-cultured tomato (Lycopersicon esculentum) cells generate ROS in response to elicitors of the mold pathogen Cladosporium fulvum (Vera-Estrella et al., 1992). Guard cells also generate ROS in response to elicitors (Lee et al., 1999) and phytohormone abscisic acid (ABA; Pei et al., 2000; Zhang et al., 2001c). In guard cells, ROS generated by ABA play an important role as signal mediators for the activation of multiple downstream events that are important for signal-induced stomatal movements, including the opening of Ca^{2+} channels (Pei et al., 2000), intracellular alkalization (Zhang et al., 2001b), and closure of inward potassium channels (Zhang et al., 2001a).

The mechanism of ROS generation and the molecules involved have been well studied in animal cells, particularly in neutrophils. The NADPH oxidase complex, which consists of many components, is responsible for ROS generation in neutrophil cells, and is activated by the binding of phosphatidylinositol 3-phosphate (PI3P) to one of the components (Ellson et al., 2001). Similar ROS-generating mechanisms have been suggested to exist in plants based on the biochemical characteristics of ROS generation (Levine et al., 1994; Xing et al., 1997). However, it has not been determined whether PI3P regulates ROS generation in plant systems.

PI3P is a product of phosphatidylinositol 3-kinase (PI3K), which phosphorylates the D-3 position of phosphoinositides. Three types of PI3K with different substrate specificities have been reported in animal cells (Toker and Cantley, 1997). In plants, only type III PI3-kinase, which makes PI3P from phosphoinositide, has been reported (Bunney et al., 2000), and it has been shown to be involved in vesicle trafficking (Kim et al., 2001). Broad bean (Vicia faba) guard cells have type III PI3-kinase activity, and PI3P is necessary in ABA-induced stomatal closing (Jung et al., 2002). Guard cells overexpressing PI3P-binding protein showed decreased stomatal closing in response to ABA, and the same effects were observed in guard cells treated with the PI3K inhibitors wortmannin (WM) and LY294002 (LY; Jung et al., 2002). These inhibitors suppressed Ca^{2+} oscillation, which indicates that PI3K may act upstream of Ca^{2+} signaling. Hydrogen peroxide (H_{2}O_{2}) is also involved upstream of Ca^{2+} signaling (Pei et al., 2000). Therefore, we hypothesized that PI3P, as found in animal cells, activates H_{2}O_{2} generation during ABA-induced stomatal closing. In this paper, we investigated this...
possibility using WM, LY, and overexpression of a PI3P-binding protein.

RESULTS

WM and LY Inhibit ABA-Induced ROS Generation in Broad Bean Guard Cells

We examined the effect of two PI3-kinase inhibitors of different action mechanisms, WM and LY, on ABA-induced ROS production in guard cells, using the ROS indicator dihydrorhodamine-123 (Joo et al., 2001) and H$_2$-dichlorofluorescin (DCF; Zhang et al., 2001c), which produce fluorescent rhodamine-123 and DCF, respectively, upon oxidation. WM and LY have been shown to inhibit PI3K in guard cell-enriched preparations, and LY was found to have a high specificity to PI3K, whereas WM has a broad specificity for several lipid kinases in this system (Fig. 3 in Jung et al., 2002). The level of ROS was quantified by measuring the green fluorescence intensity of rhodamine-123 or DCF from microscopic images using Photoshop software (Adobe Systems, Mountain View, CA). The fluorescence of ABA-treated guard cells was observed in chloroplasts and the cytosol (inset of Fig. 1A, data not shown for DCF) as reported previously (Zhang et al., 2001c). Guard cells were treated with 10 $\mu$M ABA or 0.2% (w/v) dimethyl sulfoxide (DMSO; solvent control), and their fluorescence levels were compared 10 min later. The fluorescence emission was 21% and 36% higher in ABA-treated samples compared with the DMSO-treated control as assessed using rhodamine-123 (Fig. 1B) and DCF (Fig. 1C), respectively. Preincubation with 10 $\mu$M WM abolished this ABA-induced ROS level increase (Fig. 1, B and C). WM alone did not change the fluorescence level of control guard cells (Fig. 1, B and C). In the LY experiments, the fluorescence emission was 12% and 21% higher in ABA-treated samples compared with the DMSO-treated control in assays using rhodamine-123 (Fig. 1D) and DCF (Fig. 1E), respectively. Preincubation with 100 $\mu$M LY abolished this ABA-induced ROS level increase (Fig. 1, D and E), whereas LY alone did not change the fluorescence level of control guard cells (Fig. 1, D and E). These results suggest that PI3-kinase activity is necessary for ABA-induced ROS generation in guard cells.

Overexpression of PI3P-Binding Domain Suppresses ABA-Induced ROS Generation

We further tested the importance of PI3K in ABA-induced ROS generation by overexpressing endosome-binding domain (EBD), which binds specifically to PI3P (Kim et al., 2001). Overexpression of PI3P-binding proteins in guard cells has been suggested to block PI3P function by competing with endogenous PI3P-binding proteins (Jung et al., 2002). Guard cells expressing red fluorescent protein (RFP):EBD were identified under a fluorescence microscope (Fig. 2, A and B), and their ROS levels were quantified from the fluorescence intensity of DCF as described above for Figure 1. The DCF fluorescence from the neighbor nontransformed guard cell served as a good control because a pair of guard cells is produced from division of a single guard mother cell. Among the guard cell pairs that had only one member transformed, we selected those in which the nontransformed control cell showed an increase of ROS after ABA treatment, which was 69% of the total guard cell pairs observed (data not shown). The extent of fluorescent change in the guard cells transformed with RFP:EBD was 56% ± 12% (average ± st; $P < 0.01$; Fig. 2M) of that of the nontransformed guard cells (Fig. 2, C and D). As a control for RFP:EBD expression, we expressed a mutated form of RFP:EBD, RFP:EBDC1358S (Fig. 2, E and F), which does not bind to PI3P (Kim et al., 2001). Guard cells that overexpress this construct did not differ in ROS response compared with their nontransformed neighbor guard cells (Fig. 2, G, H, and N; $P > 0.2$).

In Jung et al. (2002), phosphatidylinositol 4-phosphate (PI4P), as well as PI3P, was found to be necessary in ABA-induced stomatal closing. Therefore, we investigated whether PI4P is also involved in ABA-induced ROS generation by overexpressing RFP:PI-four-P adaptor protein-1 pleckstrin homology domain (FAPP1PH), which binds specifically to PI4P (Jung et al., 2002; Fig. 2, I and J). Guard cells that overexpress this construct did not differ in ABA-induced ROS generation compared with their nontransformed neighbor guard cells (Fig. 2, K, L, and O; $P > 0.2$). These results suggest that PI3P and not PI4P is involved in ABA-induced ROS generation in guard cells.

$H_2O_2$ Rescues the Inhibitory Effect of WM or LY on ABA-Induced Stomatal Closing

If ROS generation is the primary function of PI3K in the ABA-induced stomatal closing process, then ROS should be able to bypass a deficiency of PI3-kinase activity and induce stomatal closing. Indeed, $H_2O_2$, a form of ROS, partially restored ABA-induced stomatal closure even in the presence of WM or LY (Fig. 3). WM pretreatment increased the control stomata aperture and inhibited ABA-induced stomatal closure (Fig. 3A), and this effect was partially reversed by 1 mM $H_2O_2$ treatment. The stomatal aperture of guard cells treated with ABA, WM, and $H_2O_2$ was significantly reduced compared with that of guard cells treated with only ABA and WM ($P < 0.05$). LY pretreatment did not change the control stomatal aperture, but it did inhibit ABA-induced stomatal closure (Fig. 3B). This effect was almost completely reversed by 10 $\mu$M $H_2O_2$ treatment. The stomatal aperture of guard cells treated with ABA, LY, and $H_2O_2$ was significantly reduced compared
with that of guard cells treated with only ABA and LY (P < 0.05). The viability of guard cells treated with 1 mM H₂O₂ was verified by reopening the stomata with 1 μM fusicoccin. Fluorescein diacetate staining also supported the viability of guard cells incubated in 1 mM H₂O₂-containing solution (data not shown).

**DISCUSSION**

In this study, we investigated the role of PI3P in ABA-induced ROS generation using inhibitors and a biolistic gene transfer technique. We propose that PI3P is important in ABA-induced ROS generation based on three lines of evidence. First, two PI3-kinase inhibitors (PI3K) impaired the generation of ROS in guard cells treated with ABA. Second, the ROS levels were significantly reduced when guard cells were treated with PI3-kinase inhibitors before ABA treatment. Third, the expression of PI3P was increased in guard cells treated with ABA, while PI3-kinase inhibitors reduced this expression. These findings suggest that PI3P is a key player in ABA-induced ROS generation in guard cells.
inhibitors of different action mechanisms, WM or LY, commonly inhibited ABA-induced ROS generation (Fig. 1). Second, the PI3P-binding protein EBD also inhibited ABA-induced ROS generation when over-expressed in guard cells, whereas the overexpression of a mutated form of EBD that does not bind to PI3P and the overexpression of RFP:FAPP1PH, which binds to PI4P, did not show any inhibition (Fig. 2).

Figure 2. Overexpression of RFP:EBD inhibits ABA-induced ROS generation in broad bean guard cells. A through D, A guard cell transiently transformed with RFP:EBD. Bar = 10 μm. E through H, A guard cell transiently transformed with RFP:EBDC1358S. I through L, A guard cell transiently transformed with RFP:FAPP1PH. B, F, and J, Bright-field images corresponding to fluorescence images A, E, and I, respectively. C, G, and K, Fluorescence images of guard cells loaded with H$_2$DCF solution before ABA treatment. D, H, and L, Fluorescence images of the cells shown in C, G, and K after ABA treatment. M, The green fluorescence of guard cells expressing RFP:EBD does not change as much as that of their neighboring guard cells after ABA treatment (**$P<0.01$, $n=39$). N, The green fluorescence of guard cells expressing RFP:EBDC1358S changes as much as that of their neighboring guard cells after ABA treatment ($P>0.2$, $n=22$). O, The green fluorescence of guard cells expressing RFP:FAPP1PH changes as much as that of their neighboring guard cells after ABA treatment ($P>0.2$, $n=41$). Only the guard cells on the right side were transiently transformed in these photographs (A, E, and I).
Third, H$_2$O$_2$, a common form of ROS in plant and animal cells, reversed the inhibitory effect of the PI3K inhibitors on ABA-induced stomatal closing (Fig. 3).

In guard cells, NADPH oxidase has been suggested to be a ROS-generating enzyme during ABA signaling (Murata et al., 2001). If ROS in guard cells are generated at the plasma membrane by NADPH oxidase, the inhibitory effect of RFP:EBD on ROS generation indicates that it is localized at the plasma membrane, where NADPH oxidase is located, or that it inhibits PI3P targeting to the plasma membrane.

We did not observe RFP:EBD at the plasma membrane, but rather found it at the tonoplast and on small vesicular structures in the cytosol (Fig. 2A), confirming previous reports (Kim et al., 2001; Jung et al., 2002). This distribution of RFP:EBD did not change in response to ABA (data not shown). RFP:EBD may exist in guard cells at the plasma membrane area at very low levels that cannot be observed microscopically, and this small amount may be sufficient to block PI3P from activating NADPH oxidase. Alternatively, RFP:EBD located on small vesicles in the cytosol may disturb the delivery of PI3P to the plasma membrane, thereby inhibiting the interaction of PI3P with NADPH oxidase. ROS was detected mainly in chloroplasts and the cytosol, as indicated by the sites of rhodamine-123 and DCF green fluorescence in Figures 1 and 2, respectively, which was consistent with a previous report (Zhang et al., 2001c). ROS in the cytosol may have diffused from the plasma membrane where it is generated by NADPH oxidase, and from the chloroplasts, as suggested by Zhang et al. (2001c). This diffused ROS may have oxidized cytosolic dihydrorhodamine-123 and H$_2$DCF into fluorescent rhodamine-123 and DCF, respectively.

WM and LY had slightly different effects on stomatal aperture (Fig. 3). LY did not change the control stomatal aperture, whereas WM increased it. The concentrations of H$_2$O$_2$ required to reverse the inhibitory effects of WM and LY on ABA-induced stomatal closing were also different. WM-treated guard cells required at least 1 mM H$_2$O$_2$, whereas LY-treated guard cells required only 10 μM H$_2$O$_2$. This difference may be due to the much more effective inhibition of PI3K by WM than by LY (Fig. 2 in Jung et al., 2002).

It has been well established that intracellular calcium level oscillation is important in ABA signaling in guard cells. We previously showed that WM and LY inhibited Ca$^{2+}$ oscillation induced by ABA (Jung et al., 2002), which supports the role of PI3P in ABA-induced Ca$^{2+}$ oscillation. Ca$^{2+}$ oscillation is controlled by Ca$^{2+}$ channels that have been reported to open in response to ROS (Pei et al., 2000). Our data, taken together with these previous reports, suggest that PI3P activates ROS generation, thereby opening Ca$^{2+}$ channels, which in turn contributes to Ca$^{2+}$ oscillation during ABA response in guard cells.

In neutrophils, PI3P regulate H$_2$O$_2$ production by binding to the noncatalytic component p40phox of the NADPH oxidase (Ellson et al., 2001). However, a plant homolog of p40phox has not been reported. Therefore, the detailed mechanism of action of PI3P during ROS generation awaits further investigation.

In summary, we demonstrate a role for PI3P in ABA-induced ROS generation in broad bean guard cells using PI3K inhibitors and by expressing a PI3P-
binding protein. To the best of our knowledge, PI3P is the first lipid component to be identified that activates ROS-generating machinery in guard cells. Therefore, our findings reveal a mechanism of regulating synthesis of ROS, an important signal mediator in guard cells. It may also have more general importance because ROS is important in many aspects of plant physiology and pathology.

MATERIALS AND METHODS

Plant Materials and Chemicals

Broad bean (Vicia faba) plants were grown in a greenhouse with 16-h light and 8-h dark cycles at 22°C ± 2°C. Plants were watered with hypoxen solution (1 g L⁻¹). In all experiments, the youngest fully expanded leaves from 3- to 4-week-old plants were used. WM, LY, DMSO, ABA, and p-phenylenediamine (PPD) were purchased from Sigma (St. Louis). H₂DCF-diacetate (H₂DCF-DA) and dihydrorhodamine-123 were purchased from Molecular Probes (Eugene, OR).

ROS Bioassay

Broad bean epidermal strips or intact leaves were illuminated for 3 h in a bathing medium containing 10 mM KCl and 10 mM MES-KOH (pH 6.15). They were illuminated with 0.15 approximately 0.16 mmol m⁻² s⁻¹ white light. Ten micromoles WM or 100 μM LY was added to the bathing medium on which epidermal strips floated during the last 1 h of the 3-h illumination. In experiments where intact leaves were used, the abaxial epidermis was peeled after 2.5 h of illumination and was incubated for 30 min to remove any ROS that stripping might have caused. The samples were then treated with 10 μM ABA in 1 mM KCl and 10 mM MES-KOH for 10 min. The epidermal strips were floated on a 50-μm H₂DCF-DA solution including 1:100 diluted 10% (w/w) PPD or, on a 0.01% (w/v) dihydrorhodamine-123 solution in 10 mM Tris-KCl (pH 7.2) for 10 min. Finally, guard cells were observed under a fluorescence microscope (Axioskop 2; Zeiss, Jena, Germany), and pictures of fluorescent and bright-field images of epidermal strips were taken with a CCD camera (Axio Cam; Zeiss). To quantify the fluorescence level in guard cells, we used Adobe Photoshop 5.5 software (Adobe Systems). We first delineated regions of individual pairs of guard cells from the pictures of epidermal strips, and we then chose the image → histogram menu, which graphs the number of pixels at each color intensity level, and obtained the mean of green fluorescence intensity in these regions.

Fluorescent Gene Constructs

The construction of RFP:EBD has been previously reported (Kim et al., 2001). To generate the RFP:EBD:EBD13588 construct, the EBD13588 fragment was cut using EcoRI from the corresponding pBluescript SK⁺ construct (Kim et al., 2001) and was cloned as a translational fusion to the C terminus of RFP in p33:RFP. The construction of RFP:FPAP1PH followed the method described previously for the construction of GFP:FPAP1PH (Jung et al., 2002), except that this construct had the RFP coding region instead of a GFP coding one.

A Role for Phosphatidylinositol 3-Phosphate in ROS Generation

Biologic Gene Transfer

RFP:EBD, RFP:EBD13588, and RFP:FPAP1PH were introduced into broad bean guard cells using a bombardment technique (Particle Delivery System-1000/He; Bio-Rad, Hercules, CA). In brief, 20 to 30 μg of plasmid DNA were mixed with 2.5 mg of 1.0-μm gold particles (Bio-Rad) in a 50-μl aqueous solution. Then, 1.25 mM CaCl₂ and 20 mM spermidine were added to the DNA-gold particle mixture while vortexing vigorously. Subsequently, this mixture was resuspended in ethanol and applied onto a plastic macrocarrier. Young and healthy leaves from 3- to 4-week-old broad bean plants were placed on wet filter papers in petri dishes. Vacuum was pumped to 28 inch-Hg and DNA-coated gold particles were shot into the leaves at 1,350 psi He pressure. The bombarded leaves were kept under darkness for 2 to 3 d before microscopic observations.

Stomatal Aperture Measurement

Young leaves of 3- to 4-week-old broad bean plants were blended in distilled water for 5 s and epidermal fragments were collected on 220-μm nylon mesh. The epidermal fragments were floated on 10 mM KCl and 10 mM MES-KOH (pH 6.15) bathing medium. To test the stomatal closing response to ABA, stomata were at first induced to open by irradiation with 0.15 approximately 0.16 mmol m⁻² s⁻¹ white light for 3 h. Ten micromoles WM or 100 μM LY was added to the bathing medium for the final 30 or 60 min, respectively, of the 3-h opening period. Then, ABA or DMSO (solvent control) with or without H₂O₂ was added to the bathing medium, and the samples were incubated for an additional 1 h. To accelerate closing, 10 μM CaCl₂ was added to the bathing medium at the beginning of the 1-h closing period. Stomatal apertures were measured with an eyepiece micrometer.

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LITERATURE CITED


