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Arabidopsis PLDζ2 Regulates Vesicle Trafficking and Is Required for Auxin Response [W]

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Phospholipase D (PLD) and its product, phosphatidic acid (PA), play key roles in cellular processes, including stress and hormonal responses, vesicle trafficking, and cytoskeletal rearrangements. We isolated and functionally characterized *Arabidopsis thaliana PLDζ2*, which is expressed in various tissues and enhanced by auxin. A *PLDζ2*-defective mutant, *pldζ2*, and transgenic plants deficient in *PLDζ2* were less sensitive to auxin, had reduced root gravitropism, and suppressed auxin-dependent hypocotyl elongation at 29°C, whereas transgenic seedlings overexpressing *PLDζ2* showed opposite phenotypes, suggesting that PLDζ2 positively mediates auxin responses. Studies on the expression of auxin-responsive genes and observation of the β-glucuronidase (GUS) expression in crosses between *pldζ2* and lines containing DR5-GUS indicated that PLDζ2, or PA, stimulated auxin responses. Observations of the membrane-selective dye FM4-64 showed suppressed vesicle trafficking under *PLDζ2* deficiency or by treatment with 1-butanol, a PLD-specific inhibitor. By contrast, vesicle trafficking was enhanced by PA or *PLDζ2* overexpression. Analyses of crosses between *pldζ2* and lines containing PIN-FORMED2 (PIN2)–enhanced green fluorescent protein showed that *PLDζ2* deficiency had no effect on the localization of PIN2 but blocked the inhibition of brefeldin A on PIN2 cycling. These results suggest that PLDζ2 and PA are required for the normal cycling of PIN2-containing vesicles as well as for function in auxin transport and distribution, and hence auxin responses.

INTRODUCTION

Phospholipase, the key protein of the phosphatidylinositol signaling pathway, is classified into phospholipase D (PLD), PLC, PLA_2, and other types based on the different hydrolysis sites of phospholipids. PLD hydrolyzing phosphatidylcholine to phosphatidic acid (PA) and choline has been detected in bacteria, fungi, plants, and animals (Morris et al., 1996). Both PLD and PA play key roles in plant growth and development, stress responses, and hormone effects (Wang, 2005). PLD-deficient plants or plants treated with the PLD-specific inhibitor 1-butanol showed arrested growth and development, inhibited seed germination and emergence of radicle and cotyledons, and disorganized networks of microtubules and actin (Dhonukshe et al., 2003; Gardiner et al., 2003; Motes et al., 2005; Hirase et al., 2006). *PLDζ1*-deficient plants specifically showed altered patterns of root hair formation, whereas *PLDζ1* overexpression resulted in branched and swollen root hairs (Ohashi et al., 2003). PA serves as a second messenger and regulates multiple developmental processes (seedling development, root hair growth and pattern formation, pollen tube growth, leaf senescence, and fruit ripening) and various stress responses (high salinity and dehydration, pathogen wounding, cold, and freezing) (Munnik, 2001; Wang, 2005; Testerink and Munnik, 2005).

In *Arabidopsis thaliana*, 12 PLD isoforms have been identified that fall into two subfamilies, C2-PLDs and PXPH-PLDs, based on biochemical characteristics and domain structures (Qin and Wang, 2002). Ten members (α, β, γ, δ, and ε types) belong to the C2-PLD subfamily and contain a conserved Ca^{2+}-dependent phospholipid binding C2 (for protein kinase C–conserved 2) domain at the N terminus and exhibit Ca^{2+}-dependent enzymatic activity (Qin and Wang, 2002; Wang, 2005). The PXPH-PLD members, PLDζ1 and PLDζ2, contain PX (for Phox homology) and PH (for Pleckstrin homology) domains at the N terminus and have Ca^{2+}-independent enzymatic activity similar to that of PLDs of yeast and mammalian cells (Qin and Wang, 2002).

Mammalian PLDs and PA are critical in vesicle trafficking, such as vesicle budding from the Golgi apparatus, vesicle transport, exocytosis, endocytosis, and vesicle fusion (Liscovitch et al., 2000; Freyberg et al., 2003; Jenkins and Frohman, 2005). Inhibition of PA biosynthesis by treatment with 1-butanol resulted in inhibited vesicle secretion and complete fragmentation of Golgi membranes in vitro (Siddhanta et al., 2000; Sweeney et al., 2002). PA also regulates vesicle secretion in plants (Monteiro et al., 2005). During pollen tube tip growth, 1-butanol treatment seriously decreased PA amount and the accumulation of PA-mediated secretory vesicles in the apex, resulting in a loss of apical polarity in the pollen tube and inhibited tip growth (Monteiro et al., 2005).

PLD and PA are involved in signaling pathways of plant hormones such as abscisic acid, gibberellic acid, ethylene, and cytokinin. PLDα1 and PA participate in abscisic acid–regulated processes through tethering of ABI1 (for ABA-INSENSITIVE1, a

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negative regulator in the abscisic acid signaling pathway) to the plasma membrane, resulting in reduced ABI1 translocation to the nucleus and inhibited ABI1 function (Zhang et al., 2004). In barley (*Hordeum vulgare*) aleuronic cells, PLD and PA mediate abscisic acid inhibition of gibberellic acid–promoted α-amylase production (Ritchie and Gilroy, 1998). Ethylene stimulates *PLDα1* expression (Fan et al., 1997), and CTR1, a negative regulator in the ethylene response, is a potential target of PA (Munnik, 2001). 1-Butanol partially blocks cytokinin-induced ARR5–β-glucuronidase (GUS) expression, indicating PLD involvement in the cytokinin signaling pathway (Romanov et al., 2002). PLD may also play a role in wound-induced jasmonic acid accumulation and jasmonic acid–regulated gene expression (Wang et al., 2000). However, there are still no reports of the participation of PLD in the responses of auxin and brassinosteroids.

Auxin is involved in multiple plant developmental processes, such as the establishment of root and shoot architecture, organ pattern formation, vascular development, gravitropic responses, and cell division/elongation (Cleland, 2004; Woodward and Bartel, 2005). Recent studies showed that auxin regulates the transcription of target genes such as ARF by promoting the degradation of AUXIN/INDOLE ACETIC ACID (Aux/IAA) transcriptional repressors through the auxin receptor TIR1 (Dharmasiri et al., 2005; Kepinski and Leyser, 2005) and ubiquitin-mediated proteolysis (Gray et al., 2001; Tiwari et al., 2001). Mutation or deficiency of auxin signaling proteins such as TIR1, AXR1, and AXR2/IAA7 results in the alteration of auxin-regulated growth processes, including hypocotyl elongation and lateral root formation (Lincoln et al., 1990; Ruegger et al., 1998; Nagpal et al., 2000).

Auxin can be polarly transported from synthesis sites to action sites in a strict directionality through influx and efflux carrier proteins. Influx of auxin is mediated by the influx carrier AUX1, a presumptive auxin permease exhibiting a basal plasma membrane localization (Bennett et al., 1996; Swarup et al., 2001). Efflux of auxin is mediated by several efflux carriers, of which PIN1 is essential for IAA acropetal transport in root tissues (Galweiler et al., 1998) and PIN-FORMED2 (PIN2) is involved in auxin redistribution in root gravitropism (Muller et al., 1998; Shin et al., 2005; Abas et al., 2006). Polar auxin transport is regulated by many factors, including phytotropins (1-*N*-naphthylphthalamic acid [Katekar and Geissler, 1980]), phosphorylation and dephosphorylation (PID1 [Benjamins et al., 2001] and RCN1 [Rashotte et al., 2001]), and plant hormones (auxin [Paciorek et al., 2005] and brassinosteroids [Li et al., 2005]). Additionally, polar auxin transport depends on actin and vesicle trafficking (Geldner et al., 2001) and is reduced by cytochalasin and the vesicle trafficking inhibitor brefeldin A (BFA) (Cande et al., 1973; Butler et al., 1998; Petrasek et al., 2003). BFA treatment blocks the cycling of PIN1 and PIN2 (Geldner et al., 2001, 2003; Grebe et al., 2003; Shin et al., 2005) between the plasma membrane and the proposed internal pools as well as their accumulation within BFA-induced compartments. Treatment with cytochalasin D, a drug that depolymerizes actin filaments, results in blocked PIN1 accumulation in response to BFA treatment and restoration of PIN1 localization after BFA washout (Geldner et al., 2001).

The mechanism of PIN1 cycling is very similar to the cycling of Glut4 (for glucose transporter 4) in mammalian cells, and both depend on vesicle trafficking and actin (Lachaal et al., 1994; Geldner et al., 2001; Muday and Murphy, 2002). Human PLD1 partially colocalizes with Glut4 and is activated by insulin signaling and trafficking from intracellular membrane storage sites to exocytic sites in Glut4-containing vesicles. Additionally, PA is critical for the process of vesicle fusion to the plasma membrane (Huang et al., 2005). In addition, the inhibition of PA production resulted in severely disorganized actin in *Arabidopsis* seedlings (Motes et al., 2005), probably because PA binds to *ARABIDOPSIS THALIANA* CAPPING PROTEIN (ATCP) and inhibits its activity, resulting in enhanced actin polymerization (Huang et al., 2006). Both vesicle trafficking and actin dynamics are important for PIN cycling (Geldner et al., 2001, 2003; Grebe et al., 2003; Shin et al., 2005; Jaillais et al., 2006); however, no plant PLD has been implicated in this process to date.

We show here that PLDζ2 and PA stimulate auxin responses, root gravitropism, and the expression of early auxin-responsive genes, whereas *PLDζ2* dysfunction results in the opposite responses. Deficiency of *PLDζ2* and 1-butanol treatment reduce auxin accumulation and vesicle trafficking, which could be rescued by exogenously supplied PA. In addition, enhanced *PLDζ2* and PA increase vesicle trafficking and repress BFA compartment formation, stimulating PIN protein cycling and auxin transport. Our data suggest that PLDζ2 and PA modulate auxin responses through the regulation of vesicle trafficking and auxin transport.

RESULTS

Isolation and Structural Analysis of PLDζ2, a Putative PXPH-PLD

A putative *Arabidopsis* PLD-encoding gene (*At3g05630*) was identified by analyzing the *Arabidopsis* genomic sequence. Specific primers were designed, and a full-length cDNA, designated *PLDζ2* (accession number AM182458) and encoding a 1046–amino acid peptide (~118.8 kD), was isolated by PCR-based screening of a cDNA library constructed from *Arabidopsis* hypocotyl tissues. *PLDζ2* is a single-copy gene, and its deduced protein product PLDζ2 is 74.4% identical to PLDζ1, but with little similarity to other *Arabidopsis* PLD members, including PLDα1 (42.4%), PLDβ1 (39%), PLDγ1 (37.5%), and PLDδ (41.05%).

Structural organization analysis identified several highly conserved domains in PLDζ2, including two conserved HKD domains (positions 472 to 499 and 847 to 874, with conserved sequences [HxKxxxxD] present in and necessary for all known eukaryotic PLDs) and PX and PH domains (positions 45 to 209 and 216 to 343, respectively) (Figure 1A), suggesting that PLDζ2 is a PXPH-PLD. In addition, a PIP2 binding domain, a Gα-interacting DRY motif, and a conserved IYIENQFF motif were also detected (Figure 1A).

PLDζ2 Is Expressed in Various Tissues and Enhanced by IAA

RT-PCR and promoter-reporter gene fusion studies were performed to study the expression pattern of *PLDζ2*. RT-PCR analysis revealed the expression of *PLDζ2* in seedlings, roots, leaves, stems, and flowers (Figure 1B, top panel). Analysis of GUS activities in several transgenic lines harboring the *PLDζ2*

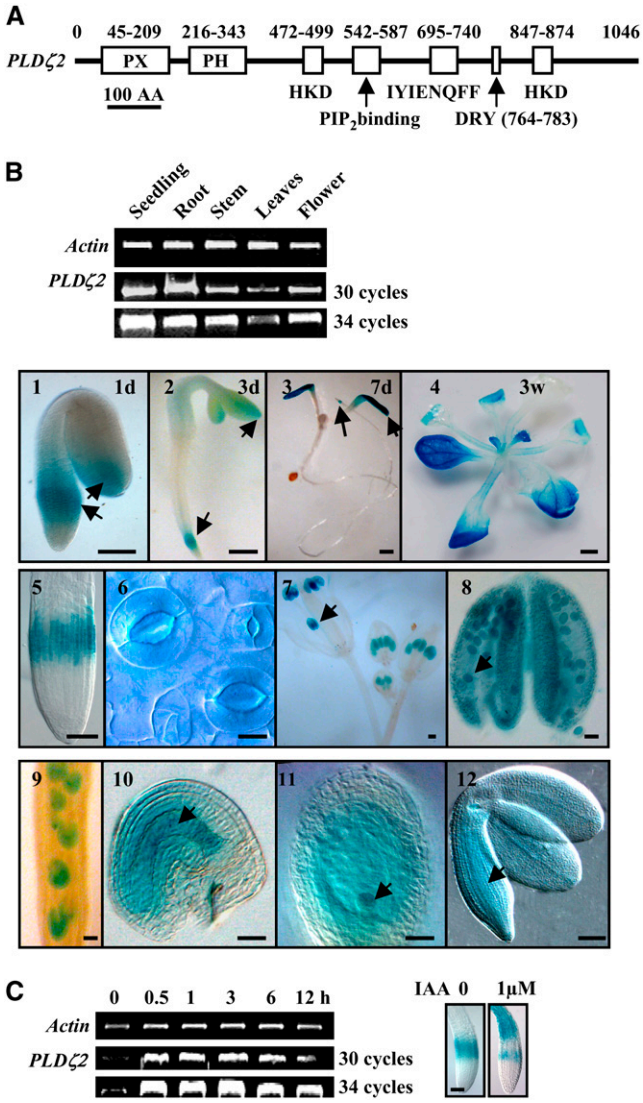

Figure 1. Structural Organization and Expression Pattern Analysis of PLDζ2.

(A) Diagram illustrating conserved domains and motifs present in the deduced protein of PLDζ2. AA, amino acid; PX, Phox-homology domain; PH, Pleckstrin-homology domain; HKD, H(X)K(X)(4)D conserved hydrolysis domain; DRY, Gα-interacting motif.

(B) Expression pattern analysis of *PLDζ2*. RT-PCR analysis revealed the expression of *PLDζ2* in multiple tissues (top). *Arabidopsis Actin2* was used as an internal positive control. Promoter-GUS fusion experiments (bottom) indicated *PLDζ2* expression in seedlings, especially roots and cotyledons (panels 1 to 4, arrows), the elongation zone of primary roots (panels 1 to 3 and 5), guard cells of cotyledons (panel 6), anthers (panel 7), pollen grains (panel 8), and immature seeds (panel 9). *PLDζ2* was also expressed at different stages of embryo development, including the early stage after fertilization (panel 10), the globular stage (panel 11), and the mature cotyledon stage (panel 12). Arrows highlight GUS signals. Bars = 200 μm (panels 1 and 5), 20 μm (panels 6 and 8), 500 μm (panels 2 to 4, 7, and 9), and 100 μm (panels 10 to 12).

(C) *PLDζ2* expression was enhanced by IAA. RT-PCR analysis showed that *PLDζ2* expression was stimulated by 100 μM IAA treatment for various times (left panel; 7-d-old seedlings were treated). *Actin2* was used as an internal positive control. Analysis of transgenic *Arabidopsis* plants carrying the *PLDζ2* promoter-GUS fusion construct revealed that *PLDζ2* expression in roots was enhanced by external IAA application (right panel; 7-d-old seedlings were treated with 1 μM IAA for 48 h). Bar = 200 μm.

promoter–GUS cassette (pBI101-P) revealed that *PLDζ2* was expressed mainly in seedling cotyledons and rosette leaves (Figure 1B, panels 1 to 4), distal elongation zones of primary and lateral roots (Figure 1B, panels 1 to 3 and 5), vascular and guard cells (Figure 1B, panels 4 and 6), and seeds (Figure 1B, panel 9). *PLDζ2* was highly expressed in anthers, especially pollen grains at late developmental stages (Figure 1B, panels 7 and 8). *PLDζ2* was expressed in embryos (Figure 1B, panels 10 to 12), especially at early developmental stages. These expression profiles are consistent with the results of digital RNA gel blot analysis (https://www.genevestigator.ethz.ch/; Zimmermann, 2004).

RT-PCR analysis further revealed that *PLDζ2* expression was enhanced by auxin. Treatment with 100 μM IAA for 30 min clearly increased the transcript levels of *PLDζ2*, with the peak increase after 3 h of IAA treatment (Figure 1C, left panel). Further investigations using transgenic lines expressing the *PLDζ2* promoter–GUS cassette confirmed that auxin treatment enhanced *PLDζ2* expression, which can be detected, for example, at elongation regions of primary and lateral roots (Figure 1C, right panel), suggesting that *PLDζ2* may be involved in auxin signaling and/or action.

The PLDζ2 Knockout Mutant, *pldζ2*, Is Less Sensitive to Exogenous Auxin

A putative *PLDζ2* mutant (SALK_119084) was identified (from the T-DNA insertion library database of the Salk Institute: http://signal.salk.edu/cgi-bin/tdnaexpress) (Alonso et al., 2003), and T-DNA insertion was confirmed at the third intron (14 bp to the left of the fourth exon) (Figure 2A, top panel) by PCR analysis using primers located in the T-DNA and flanking the genomic DNA. The homozygous mutant line was further identified and confirmed as a knockout mutant by RT-PCR analysis of the defective *PLDζ2* expression (Figure 2A, bottom panel).

As *PLDζ2* was primarily and highly expressed in roots, we focused our analysis on the potential effects of *PLDζ2* on primary root growth. Phenotypic analysis showed that *pldζ2* roots were shorter than those of wild-type seedlings (see Supplemental Figure 1A online) and, importantly, were less sensitive to exogenous auxin. Compared with wild-type seedlings, whose roots were evidently inhibited by exogenous auxin, *pldζ2* roots exhibited much less inhibition (Figure 2B; see Supplemental Figure 1B online). At an IAA concentration of 10 nM, the root length of wild-type seedlings was ~79.3% of that of untreated seedlings, whereas *pldζ2* roots showed almost no inhibition. When auxin was supplemented at a concentration of 100 nM, wild-type roots were severely inhibited to ~61.3% of the length of untreated seedlings. By contrast, *pldζ2* roots displayed much less inhibition, with root length being ~84.8% of that of untreated controls. However, the resistance of *pldζ2* roots to external auxin was not as strong as that of the auxin-insensitive mutant *tir1-1* (Ruegger et al., 1998) (Figure 2B), indicating that *pldζ2* has reduced sensitivity, rather than being insensitive, to external auxin.

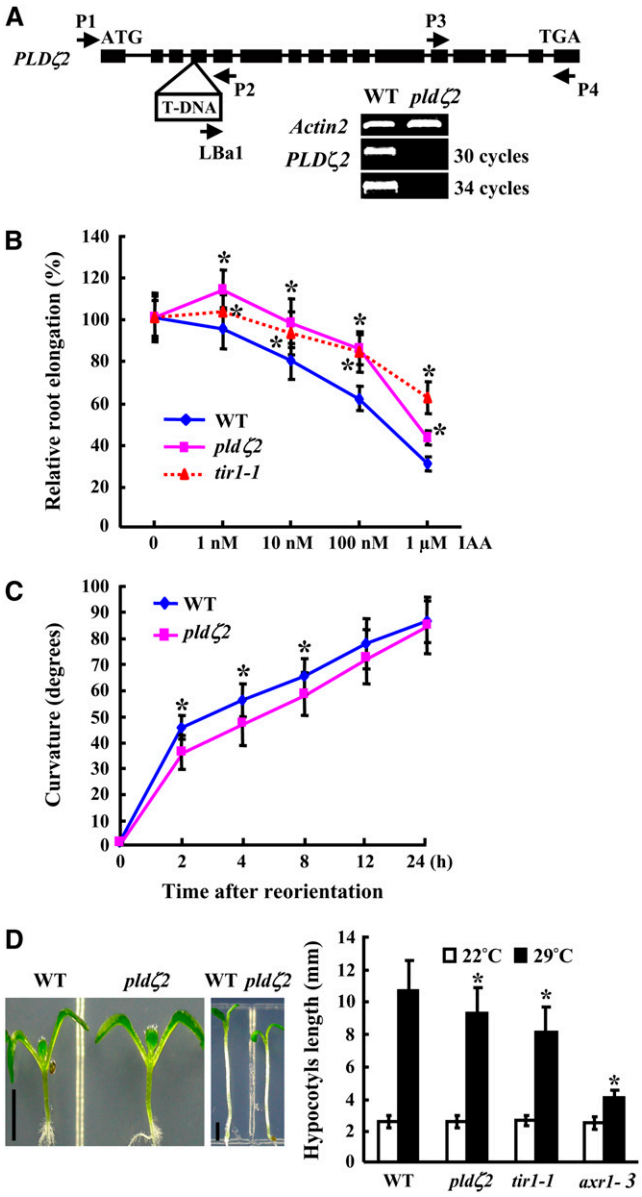

Figure 2. The Knockout Mutant *pldζ2* Showed Reduced Responses to Auxin.

(A) Diagram showing the positions of the T-DNA insertion in the *PLDζ2* genomic sequence and primers used to identify the knockout mutant *pldζ2*. Primers P2 and LBa1, P1 and P2, and P3 and P4 were used to confirm the presence of T-DNA, to screen the homozygous line, and to detect the transcripts of *PLDζ2*, respectively (top). RT-PCR analysis revealed the deficiency of *PLDζ2* transcripts in homozygous mutant plants. *Actin2* was used as an internal positive control (bottom).

(B) Measurement and statistical analysis of relative root growth indicated the reduced auxin responses of *pldζ2* seedlings. The auxin-insensitive mutant *tir1-1* was used as a positive control. Nine-day-old seedlings were used for observation and measurement. Error bars represent SE ($n > 30$), and statistical analysis was performed using a one-tailed Student's *t* test (* significant difference at $P < 0.01$).

(C) *pldζ2* seedlings showed repressed gravity responses compared with wild-type controls. Five-day-old vertically grown seedlings were reoriented by 90°, and curvatures were measured at different times after reorientation. Error bars represent SE ($n > 60$), and a one-tailed Student's *t* test indicated significant differences (*$P < 0.01$).

(D) *pldζ2* seedlings were less sensitive to auxin-dependent hypocotyl elongation under high temperature (left). Hypocotyl lengths of 7-d-old seedlings grown at 22 or 29°C were measured and statistically analyzed (right). The auxin-insensitive mutants *tir1-1* and *axr1-3* were used as positive controls. Bar = 2 mm. Error bars represent SE ($n > 30$), and a one-tailed Student's *t* test indicated significant differences (*$P < 0.01$).

Previous studies have indicated that mutants with altered auxin signaling (*axr2/iaa7* [Nagpal et al., 2000] and *axr5-1/iaa1* [Yang et al., 2004]) or transport (*aux1* [Bennett et al., 1996], *pin2* [Muller et al., 1998], and *rcn1* [Rashotte et al., 2001]) often showed altered gravity responses. Measurement of root curvature in gravity responses showed that *pldζ2* was less sensitive to gravity (*pldζ2* bent more slowly than the wild type; Figure 2C). In addition, stimulation of hypocotyl elongation under high temperature (29°C), an important auxin-dependent response (Gray et al., 1998), was suppressed in *pldζ2*, although not as strongly as in *axr1-3* (Lincoln et al., 1999) (Figure 2D). These observations add evidence for repressed auxin responses of *pldζ2*.

Altered Expression of *PLDζ2* Results in Modified Sensitivities to Auxin

Transgenic approaches were further used to study the physiological functions of *PLDζ2*. The coding region of *PLDζ2* was subcloned into the modified pCAMBIA1301 binary vector (Liu et al., 2003) under the control of the cauliflower mosaic virus 35S promoter in the sense and antisense orientations, and the resulting cassettes were individually transferred into wild-type *Arabidopsis* through genetic transformation. In total, 19 sense and 13 antisense independent T1 transgenic lines were obtained, and transcriptional analysis revealed the enhanced (Figure 3A, left panel) or suppressed (Figure 3A, right panel) expression of *PLDζ2*. Homozygous lines containing single-copy T-DNA insertions were obtained and used for subsequent analysis. As *PLDζ1* shares high similarity with *PLDζ2* in nucleotide sequence (64.67%), *PLDζ1* transcript levels were evaluated, and the results showed that transcript levels of *PLDζ1* in antisense plants did not differ substantially from those of controls (Figure 3A, right panel), indicating the specific antisense suppression of *PLDζ2*.

Similar to *pldζ2*, the primary roots of antisense seedlings were shorter than those of controls, whereas those of sense plants were significantly longer (see Supplemental Figure 2A online). Statistical analysis showed that the inhibitory effects of auxin on root growth were enhanced in *PLDζ2*-overexpressing plants but suppressed in antisense plants (Figure 3B; see Supplemental Figure 2 online). At 10 nM IAA, the primary roots of *PLDζ2*-deficient plants were similar to those of untreated controls, whereas those of control and *PLDζ2*-overexpressing plants were 82.1%, 68.9% (pO-PLDζ2-LO11), and 55.6% (pO-PLDζ2-LO19) as long as those of their respective untreated controls. At 100 nM IAA, primary roots of *PLDζ2*-deficient, control, and *PLDζ2*-overexpressing plants were 80.9% (pA-PLDζ2-LA3), 78.1% (pA-PLDζ2-LA11), 60.6%, 48.7% (pO-PLDζ2-LO11), and 33.8% (pO-PLDζ2-LO19) as long as those of their respective

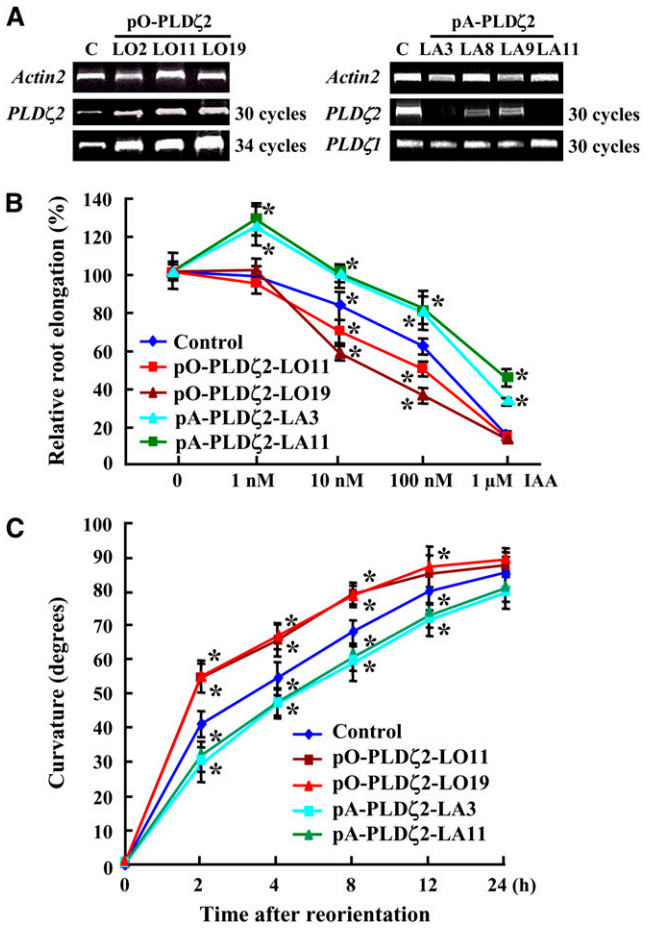

Figure 3. Modified Expression of *PLDζ2* Resulted in Altered Sensitivity to Auxin.

(A) Enhanced expression of *PLDζ2* in transgenic lines (LO2, LO11, and LO19; left) prepared by genetic transformation using construct pO-PLDζ2. Suppressed expression of PLDζ2 in transgenic lines (LA3, LA8, LA9, and LA11; right) developed by genetic transformation using construct pA-PLDζ2. Total RNA samples were extracted from 7-d-old seedlings. *PLDζ2* transcript levels were analyzed using RT-PCR, and *Actin2* was used as an internal control. C, control plants.

(B) Measurement and statistical analysis of relative root elongation of seedlings with altered *PLDζ2* expression grown on medium supplemented with various concentrations of IAA for 9 d confirmed the hypersensitivity to auxin of *PLDζ2*-overexpressing plants and the relative insensitivity of *PLDζ2*-deficient plants. Primary root lengths of untreated plants were set as 100%. Error bars indicate SE ($n > 40$), and statistical analysis was performed using a one-tailed Student's *t* test (*$P < 0.01$).

(C) Time course of curvature in seedling gravity response tests. Curvatures of 5-d-old control and transgenic plants with enhanced or decreased *PLDζ2* expression levels were measured after 90° reorientation and analyzed statistically ($n > 40$). Error bars represent SE, and statistical analysis was performed using a one-tailed Student's *t* test (*$P < 0.01$).

untreated controls. At an even higher auxin concentration (1 μM IAA), primary roots of control and *PLDζ2*-overexpressing plants were ~15% as long as those of their respective untreated controls, whereas those of *PLDζ2*-deficient plants were 31.7% (pA-PLDζ2-LA3) and 44.3% (pA-PLDζ2-LA11) as long as those of the untreated controls (Figure 3B). These results are consistent with the results for *pldζ2* and indicate that deficiency or overexpression of *PLDζ2* results in less sensitive or hypersensitive responses, respectively, to auxin. In addition, compared with controls, *PLDζ2*-overexpressing and -deficient transgenic plants exhibited enhanced and reduced root gravitropism, respectively (Figure 3C).

PA Enhances the Root Gravity Response

Previous studies have indicated reduced PA levels in *pldζ2* (Cruz-Ramirez et al., 2006; Li et al., 2006); thus, we examined the effects of PA on gravitropism. As shown in Figure 4A, PA (10 μM) stimulated gravitropic responses, whereas inhibiting PA production by 1-butanol (0.2 or 0.4%, v/v) resulted in severely decreased root gravitropism. Two analogs of 1-butanol, 2-butanol (0.4%, v/v) and 3-butanol (0.4%, v/v), displayed no effects on root gravitropism (Figure 4B), indicating that PA acts positively on root gravitropism, in agreement with the increased gravity response shown by *PLDζ2*-overexpressing plants.

PLDζ2 Enhances Auxin Distribution and Responses

To gain further insight into physiological function, we investigated auxin distribution and responses under altered *PLDζ2* expression or supplemented PA. *Arabidopsis* seedlings containing the DR5-GUS cassette (the GUS-coding sequence under transcriptional control of a synthetic auxin-inducible promoter), which has been used widely to detect auxin accumulation and distribution (Ulmasov et al., 1997), was used for this series of experiments. Through genetic crossing, the DR5-GUS cassette was transferred into *pldζ2* and *PLDζ2*-overexpressing lines. In control seedlings, GUS signals were detected mainly in root and cotyledon tips, but they were suppressed in the *pldζ2* background, especially in cotyledons. On the other hand, GUS signals were enhanced in the *PLDζ2* overexpression background, with the staining occurring at cotyledon edges and in the root elongation region (Figure 5A).

Compared with controls, PA treatment (10, 20, or 50 μM) enhanced GUS expression in the wild-type background, with the signals detected not only in root columella cells but also in vascular tissues (Figure 5B). Consistent with the observation described above, 1-butanol treatment (0.4 or 0.8%, v/v) of DR5-GUS seedlings for 24 or 48 h severely suppressed GUS expression (Figure 5C), and long-term 1-butanol treatment (48 to 72 h) not only suppressed GUS signals in columella cells but also modified their distribution, with staining appearing in the elongation zone (see Supplemental Figure 3A online). In addition, epidermal cells in the elongation zone were swollen and exhibited intense GUS signals (see Supplemental Figure 3A online). 1-Butanol also suppressed auxin-induced GUS accumulation (10 μM IAA for 3 h; Figure 5D) and inhibited auxin-induced lateral root initiation and growth (1 μM IAA for 3 d; see Supplemental Figure 3B online). As expected, 2-butanol and 3-butanol showed none of the effects listed above, suggesting that PA modifies auxin accumulation and, hence, the effects of PLD on auxin distribution and responses.

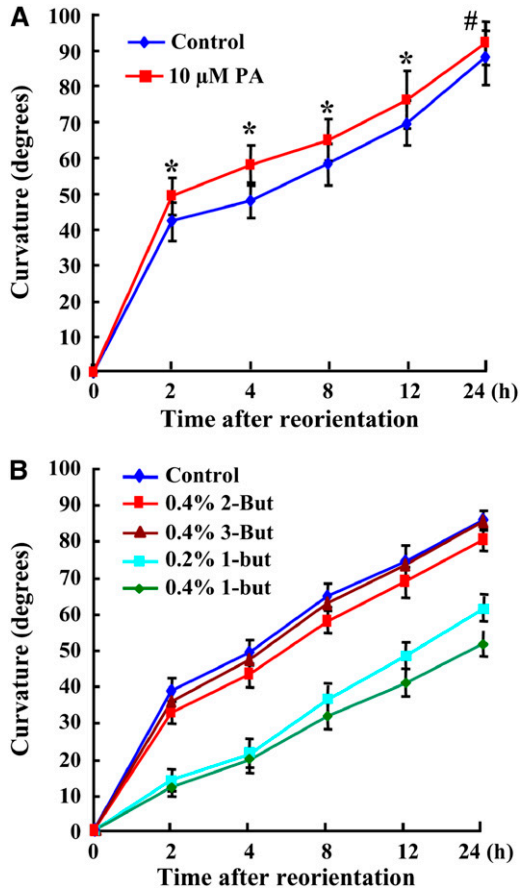

Figure 4. PA Stimulated Root Gravity Responses.

(A) Effects of PA on root gravity response. Five-day-old *Arabidopsis* seedlings were transferred onto medium supplemented with or without 10 μM PA, and seedling curvatures were measured and analyzed statistically ($n > 40$) as described for Figure 3C. Error bars represent SE. * significant difference at $P < 0.01$; # significant difference at $P < 0.05$.
(B) Effects of the PLD-specific inhibitor 1-butanol on root gravity response. Five-day-old seedlings were transferred onto medium supplemented with or without 1-butanol (0.2 or 0.4%, v/v), 2-butanol (0.4%, v/v), or 3-butanol (0.4%, v/v), and seedling curvatures were measured and analyzed statistically ($n > 60$). Results showed that 1-butanol treatment severely suppressed seedling gravity responses, whereas no obvious changes were found with 2-butanol or 3-butanol treatment. Error bars represent SE.

PLDζ2 Activates the Expression of Auxin-Responsive Genes

IAA5, *IAA19*, and *GH3-3*, which belong to the *Aux/IAA* and *GH3* families, were selected to examine whether altered *PLDζ2* expression would affect the transcription of early auxin-responsive genes. Quantitative real-time RT-PCR analysis showed that transcript levels of *IAA5*, *IAA19*, and *GH3-3* were rapidly upregulated after auxin treatment in wild-type plants, consistent with previous reports (Tian et al., 2002; Nakamura et al., 2003). By contrast, in *pldζ2* or *PLDζ2*-deficient antisense plants, the upregulation of these three genes in response to external IAA was suppressed. In *PLDζ2*-overexpressing plants, the auxin-induced transcription increases of *IAA5*, *IAA19*, and *GH3-3* were generally much greater compared with those in wild-type plants (Figure 6), providing further support for the involvement of PLDζ2 in controlling auxin responses.

PLDζ2 and PA Positively Regulate Vesicle Trafficking

The potential involvement of PLDζ2 in vesicle trafficking was investigated using FM4-64, a water-soluble and membrane-selective fluorescent dye that has frequently been used as a membrane marker to monitor endocytosis in mammalian, fungal, and plant cells (Bolte et al., 2004). As depicted in Figure 7, when wild-type seedling roots were submerged in FM4-64 solution, the dye was soon internalized (Figure 7A, panels 1 and 6). But in *pldζ2* and *PLDζ2*-deficient plants, the internalization of FM4-64 into root cells was severely reduced (Figure 7A, panels 2, 5 and 7, 10, respectively). On the contrary, FM4-64 internalization into root cells of *PLDζ2*-overexpressing plants was enhanced, with many more fluorescently labeled vesicles (Figure 7A, panels 3, 4, 8, and 9).

In addition, it was found that PA (50 μM, 3 h of treatment) clearly enhanced FM4-64 internalization in wild-type seedling roots (Figure 7B, panel 4). By contrast, FM4-64 internalization in wild-type seedling roots was substantially inhibited in the presence of 1-butanol (0.4 or 0.8%, treated for 3 or 12 h) (Figure 7B, panels 2, 3, 5, and 6) but not 2-butanol (see Supplemental Figure 4 online, panels 4 to 6). As anticipated, inhibited FM4-64 internalization by 1-butanol (0.4%, 12 h; see Supplemental Figure 4 online, panel 1) could be rescued with enhanced expression of *PLDζ2* (see Supplemental Figure 4 online, panels 2 and 3). These findings suggest that PLDζ2 and PA positively regulate vesicle trafficking in *Arabidopsis* root cells.

PLDζ2 Suppresses BFA Compartment Formation and BFA Inhibition on PIN1 and PIN2 Cycling

BFA is a vesicle transport inhibitor that specifically blocks cell exocytosis but allows endocytosis, resulting in the internalization and accumulation of recycling plasma membrane proteins in BFA compartments (Nebenfuhr et al., 2002). Many plasma membrane proteins, including PIN1, plasma membrane H^+-ATPase (Geldner et al., 2001, 2003), and PIN2 (Geldner et al., 2003; Grebe et al., 2003) have been found in BFA compartments. Based on results showing that altered *PLDζ2* expression or PA levels modified vesicle trafficking, we examined BFA compartment formation in wild-type plants and plants with altered *PLDζ2* levels. After BFA treatment (50 μM) for 2 h, BFA compartments were observed in almost every cell of wild-type seedling roots (Figure 8A, panel 1) and in nearly all root cells of *pldζ2* (Figure 8A, panel 4) and *PLDζ2*-deficient (Figure 8A, panels 5 and 6) seedlings, although the size was much smaller. On the other hand, BFA compartments were much fewer in root cells (~30 to 40%) of *PLDζ2*-overexpressing seedlings (Figure 8A, panels 2 and 3).

BFA inhibits not only vesicle trafficking but also root and hypocotyl elongation, lateral root formation, and gravitropism of roots and hypocotyls (Geldner et al., 2001, 2003). Indeed, compared with wild-type seedlings, statistical analysis showed that *PLDζ2*-overexpressing seedlings were less sensitive to BFA inhibition of elongation of hypocotyls (see Supplemental Figure 5 online) and primary roots, lateral root formation, and root gravity

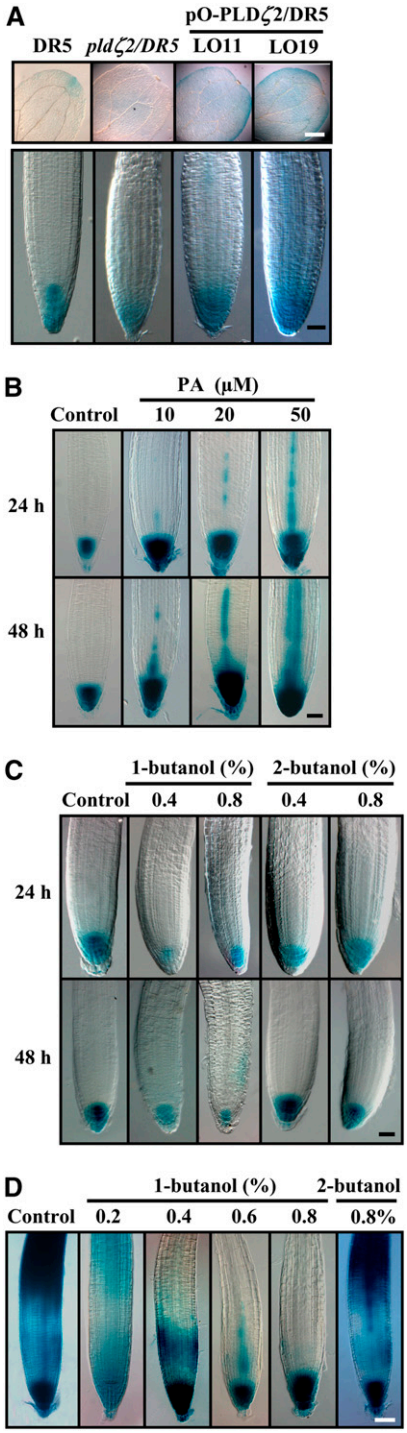

Figure 5. Effects of PLDζ2, PA, and the PLD-Specific Inhibitor 1-Butanol on Auxin Distribution and Response, Evaluated by Detecting DR5-GUS Expression.

For all analyses, the GUS signals shown are representative of >20 independent samples.

(A) *PLDζ2* positively regulates DR5-GUS expression. Five-day-old seedlings were used, and GUS signals were examined in cotyledons (top) and roots (bottom). Left panel, wild-type control; left center panel, *pldζ2*; right center and right panels, *PLDζ2*-overexpressing lines. Bar = 500 μm (top) and 50 μm (bottom).

(B) PA stimulates DR5-GUS expression. Dose concentrations of PA (10, 20, or 50 μM) were supplemented and applied for 24 or 48 h. Roots of 5-d-old seedlings were observed. Bar = 100 μm.

(C) 1-Butanol treatment suppresses DR5-GUS expression. GUS activities were detected under treatment with 1-butanol or 2-butanol (0.4 or 0.8%, v/v) for 24 h (top) or 48 h (bottom). Bar = 100 μm.

(D) 1-Butanol suppresses auxin-induced DR5-GUS expression in the root elongation zone. Seedlings harboring the DR5-GUS construct were treated with 10 μM IAA and different concentrations of 1-butanol (0.2, 0.4, 0.6, or 0.8%, v/v) or 2-butanol (0.8%, v/v) for 3 h. Bar = 50 μm.

response (Figures 8B and 8C). However, *pldζ2* and wild-type seedlings did not differ significantly in their sensitivity to BFA inhibition.

Altered auxin sensitivity and auxin-regulated hypocotyl elongation, root gravity, and DR5-GUS expression in *PLDζ2* mutant or overexpression lines suggested altered auxin signaling or auxin transport. It was shown that cycling of the auxin efflux carriers PIN1 and PIN2 is vesicle-dependent (Geldner et al., 2001, 2003; Grebe et al., 2003; Jaillais et al., 2006) and that BFA treatment reduced auxin transport (Petrasek et al., 2003). As PLDζ2 and PA positively regulate vesicle trafficking and suppress BFA inhibition, the effects of *PLDζ2* deficiency or overexpression on the cycling of PIN2 between plasma membrane and endosomal compartments were then examined. To achieve this, PIN2–enhanced green fluorescent protein (EGFP) (Xu and Scheres, 2005) expression cassettes were individually transferred into a *pldζ2* background and into *PLDζ2*-overexpressing lines through genetic crossing. Figure 9A shows that *PLDζ2* deficiency or overexpression had no effect on the polar localization of PIN2 (Figure 9A, panels 2 to 4). When treated with BFA for 2 h, the cycling of PIN2-EGFP was blocked, with the appearance of two or three large BFA compartments (Figure 9A, panel 5). In *pldζ2* or *PLDζ2*-overexpressing lines, the BFA-induced cytoplasmic aggregation of PIN2-EGFP was generally suppressed (Figure 9A, panels 6 to 8). After washout for 2 h, BFA compartments disappeared and normal polar localization of PIN2-EGFP was detected in the wild-type background (Figure 9A, panel 9) and in *PLDζ2*-overexpressing seedlings (Figure 9A, panels 11 and 12). On the other hand, accumulated PIN2-EGFP in *pldζ2* could not be resumed effectively (Figure 9A, panel 10), suggesting a positive effect of PLDζ2 on PIN2 cycling.

Although BFA effects on PIN2 cycling were alleviated under overexpressed *PLDζ2*, accumulation of PIN2-EGFP (albeit smaller and fewer in number) could still be observed in *PLDζ2*-overexpressing plants under BFA treatment, suggesting partial repression of the BFA inhibitory effect. Indeed, PIN2 cycling was stimulated in *PLDζ2*-overexpressing plants (Figure 9A, panel 12), consistent with previous results indicating that overexpression of *PLDζ2* partially decreased BFA effects on seedling growth (Figure 8B; see Supplemental Figure 5 online).

These observations were further confirmed by investigating PIN2-EGFP localization with PA or 1-butanol treatment. As Figure 9B shows, 1-butanol and PA had no effect on the polar localization of PIN2-EGFP (Figure 9B, panels 1 to 3); however, after pretreatment with 1-butanol or PA (30 min) and application of BFA and 1-butanol or PA (2 h), the BFA inhibition of PIN2-EGFP cycling was completely or largely blocked (Figure 9B, panels 4

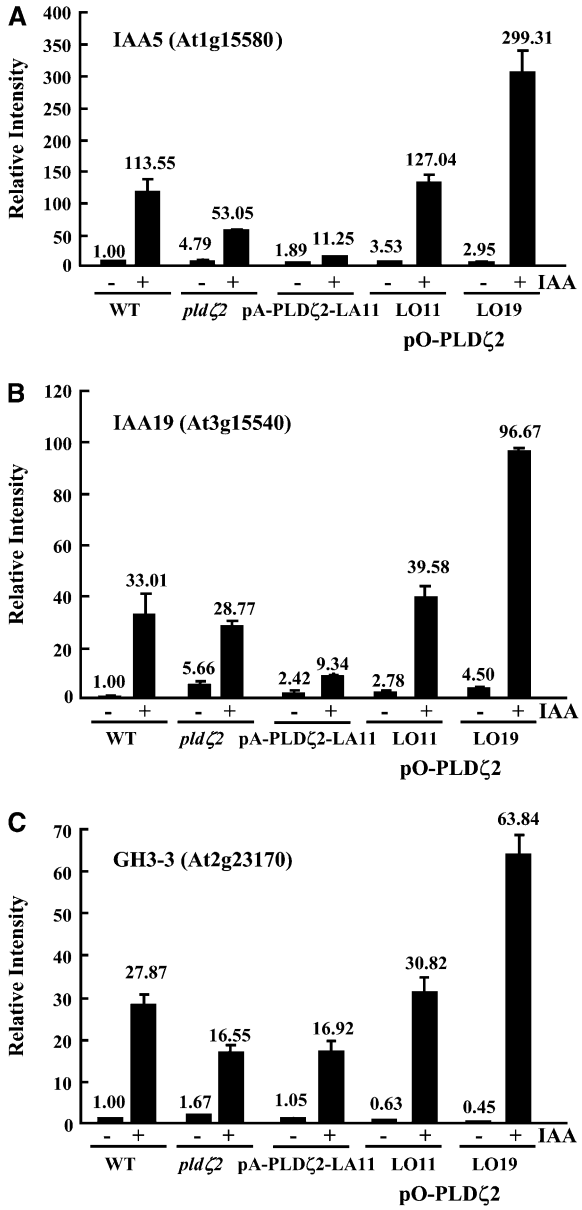

Figure 6. Expression of Auxin-Responsive Genes Was Modulated under Altered Expression of *PLDζ2*.

Relative expression levels of three early auxin-responsive genes, *IAA5* **(A)**, *IAA19* **(B)**, and *GH3-3* **(C)**, were detected in wild-type control, *pldζ2*, and transgenic plants deficient in or overexpressing *PLDζ2* (pA-PLDζ2-LA11, pO-PLDζ2-LO11, and LO19, respectively) in the absence or presence of IAA by quantitative real-time RT-PCR analysis. Induction of three genes by IAA was suppressed in *pldζ2* and transgenic plants deficient in *PLDζ2*, whereas it was enhanced in *PLDζ2*-overexpressing plants. Seven-day-old seedlings were treated with 10 μM IAA for 90 min. Gene transcript abundance was analyzed, and relative intensity was calculated. The experiments were repeated three times, and the data shown are means plus SE. The amplification of *Actin7* was used as a control.

and 6). By contrast, pretreatment with 2-butanol had no obvious influence (Figure 9B, panel 5). In addition, after cells were treated with BFA for 2 h, BFA washout with 1-butanol (2 h) resulted in differential accumulation of PIN2-EGFP compared with that with 2-butanol or PA. The intracellular compartments disappeared, and polar localization of PIN2-EGFP was effectively restored in cells with 2-butanol (Figure 9B, panel 8) or PA (Figure 9B, panel 9), whereas with 1-butanol, intracellular compartments did not disappear completely and the polar localization of PIN2-EGFP was not effectively recovered (Figure 9B, panel 7).

DISCUSSION

Among 12 PLD members present in *Arabidopsis*, PLDζ1 and PLDζ2 are unique in possessing the PX and PH domains. Although considerable progress has been made in understanding

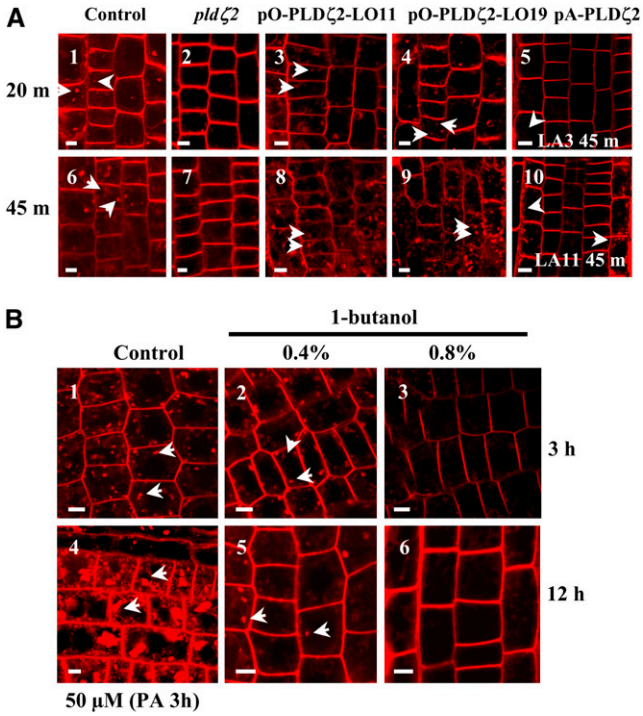

Figure 7. PLDζ2 Stimulated Vesicle Trafficking.

(A) Compared with the wild-type control (panels 1 and 6), deficiency of *PLDζ2* suppressed the internalization of FM4-64 (panels 2, 5, 7, and 10), indicating decreased vesicle trafficking. Alternatively, internalization of FM4-64 was clearly stimulated in *PLDζ2*-overexpressing plants (panels 3, 4, 8, and 9). Roots of 7-d-old seedlings ($n > 15$) were stained with the membrane-selective dye FM4-64 (5 μM, 10 min) and then observed after incubation for 20 or 45 min. Arrowheads highlight the vesicles labeled with FM4-64. Bars = 5 μm.

(B) Compared with the untreated control (panel 1), 1-butanol decreased FM4-64 internalization (panels 2, 3, 5, and 6), whereas PA enhanced it (panel 4). Roots of 7-d-old seedling ($n > 15$) were treated with 1-butanol (0.4 or 0.8%, v/v) for 3 h (panels 2 and 3) or 12 h (panels 5 and 6) or with PA (50 μM, 3 h; panel 4), followed by staining with FM4-64 (5 μM, 10 min), and observed after incubation for 45 min. Arrowheads highlight the vesicles labeled with FM4-64. Bars = 5 μm.

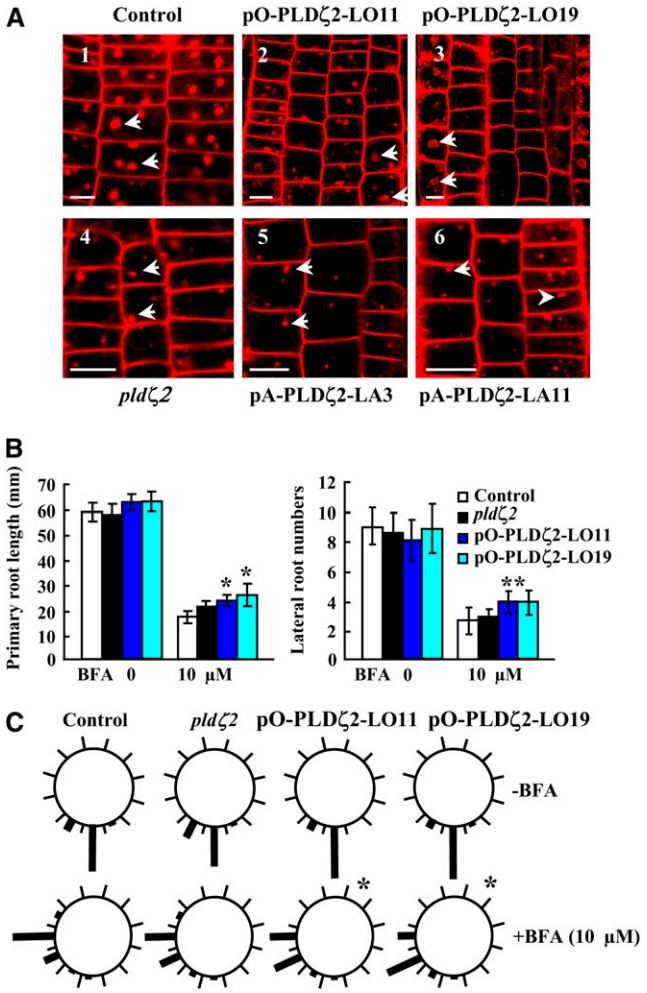

Figure 8. PLDζ2 Suppressed the Inhibitory Effects of BFA on Vesicle Trafficking.

(A) Compared with the wild-type control (panel 1), BFA compartments were detected in only a few cells of *PLDζ2*-overexpressing roots (panels 2 and 3). However, the number of root cells containing BFA compartments was largely indistinguishable between *PLDζ2*-deficient plants (panels 4 to 6) and the wild-type control (panel 1). Roots of 7-d-old seedlings were first treated with BFA (50 μM) for 2 h, then incubated with 50 μM BFA and 5 μM FM4-64 for 30 min, followed by examination by confocal microscopy. Arrowheads highlight BFA compartments labeled with FM4-64. Bars = 10 μm. For all analyses, the results shown are representative of >15 independent samples.

(B) Compared with the wild-type control and *pldζ2*, *PLDζ2*-overexpressing plants (LO11 and LO19) were insensitive to BFA inhibition of primary root elongation (left) and lateral root formation (right). Five-day-old seedlings were transferred onto medium supplemented with (5 μM) or without BFA for another 7 d, and primary root lengths and lateral root numbers were determined and analyzed statistically. Error bars represent SE ($n > 40$). * significant difference ($P < 0.01$).

(C) Compared with control and *pldζ2* seedlings, *PLDζ2*-overexpressing plants (LO11 and LO19) had reduced sensitivity to BFA inhibition of the root gravity response. Five-day-old seedlings were transferred onto medium supplemented with (bottom row; 10 μM) or without (top row) BFA, and root curvatures were measured after 135° reorientation and growth for 36 h. The curvature of each root was assigned to one of 12 30° sectors ($n > 40$).

the genetic function and biochemical properties of PLDζ1, only limited knowledge has been obtained on PLDζ2. Previous investigations revealed the important role of PLDζ2 in phosphate starvation responses of *Arabidopsis* (Cruz-Ramirez et al., 2006; Li et al., 2006). In this work, we show that PLDζ2 is involved in vesicle trafficking in higher plants, and our studies reveal the role and mechanisms of PLDζ2 in regulating auxin responses, providing hints of crosstalk between the phosphatidylinositol signaling and auxin signaling pathways.

PLDζ2 and PA Positively Regulate Auxin Responses

In agreement with previous investigations, we found that *PLDζ2* is expressed in multiple tissues. We also found that *PLDζ2* expression is clearly and significantly increased in response to auxin treatment. Two categories of evidence support the finding that PLDζ2 positively regulates auxin responses in *Arabidopsis*, and this regulation may be achieved through PA, the product of PLDζ2. Genetic analysis revealed that *PLDζ2*-deficient mutants (knockout and antisense lines) exhibited suppressed auxin responses, whereas *PLDζ2*-overexpressing plants showed the opposite phenotype. These results were confirmed by a variety of approaches (i.e., sensitivity to inhibition of root growth by external auxin, alteration in gravity responses, stimulation of hypocotyl elongation at 29°C, size and distribution of the DR5-GUS expression region, and transcriptional pattern of early auxin-responsive genes). In addition, consistent with the positive effects of enhanced *PLDζ2* on auxin responses, treatment of wild-type seedlings with PA stimulated gravitropism and expanded the expression regions of DR5-GUS. The data obtained with PA are relevant to the function of *PLDζ2*, because the stimulating effects on auxin responsiveness observed in PA application were reversed in seedlings treated with 1-butanol and in *PLDζ2*-defective mutants (knockout and antisense plants). This finding provides strong evidence that PLDζ2 regulates auxin responses through its product PA. However, there is still no evidence that PLDζ2 and PA are involved directly in auxin signaling.

At present, we cannot exclude the possibility that, in vivo, PA produced by other PLD or PLC members does not participate in auxin responses. Inhibiting PA by 1-butanol treatment repressed auxin-induced lateral root formation (see Supplemental Figure 3B online) and root gravitropic responses (Figure 4B) more strongly than those of *pldζ2*, indicating that other PLD members or PLC may be involved in auxin responses. Further studies to examine whether other PLD or PLC members contribute to auxin responses and whether there are functional interactions between PLDζ2 and other PLD or PLC members in regulating auxin responses would provide much information.

PLDζ2 and PA Enhance Vesicle Trafficking

Many previous studies have documented that PLDs and PA are involved in vesicle trafficking in both mammalian and plant cells (Liscovitch et al., 2000; Freyberg et al., 2003; Jenkins and Frohman, 2005; Monteiro et al., 2005). In addition, PLD and PA are important for vesicle fusion and aggregation (Jones and Clague, 1997; Ichikawa and Walde, 2004; Huang et al., 2005; Jenkins and Frohman, 2005). In the process of sporulation in

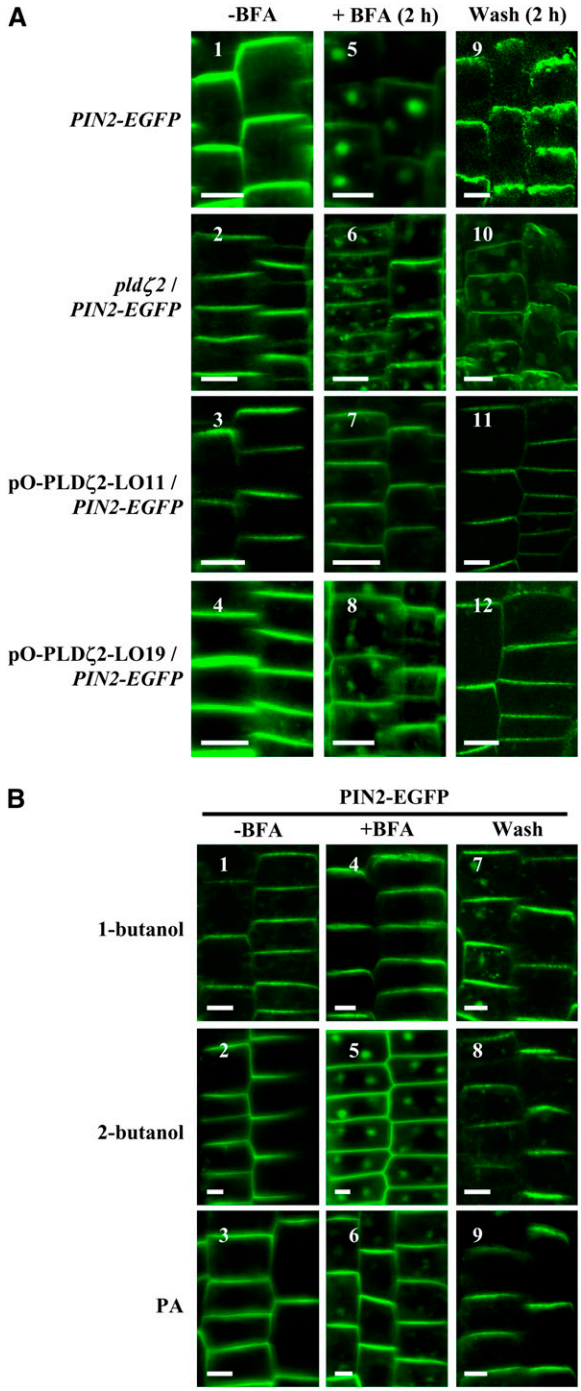

Figure 9. PLDζ2, 1-Butanol, and PA Suppressed BFA Inhibition on PIN2 Cycling.

The PIN2-EGFP cassette was individually transferred into *pldζ2* or *PLDζ2*-overexpression (LO11 and LO19) backgrounds through genetic crosses. The resultant progeny were used in this series of experiments. **(A)** In the absence of BFA, polar localization patterns of PIN2 (panels 1 to 4) were indistinguishable in wild-type, *pldζ2*, or *PLDζ2*-overexpression backgrounds, indicating that manipulation of *PLDζ2* expression levels does not affect the polar localization of PIN2. After BFA treatment (50 μM, 2 h), accumulation of PIN2 in the *PLDζ2*-overexpression background (panels 7 and 8) was much less than that in the wild type (panel 5) and *pldζ2* (panel 6). After BFA washout for 2 h, resumption of PIN2 polar localization was more complete in the *PLDζ2*-overexpression background (panels 11 and 12) than in the wild-type (panel 9) and *pldζ2* (panel 10). For all analyses, roots of 5-d-old progeny were used ($n > 15$). Bars = 10 μm. **(B)** Treatment with 1-butanol, 2-butanol, or PA did not affect the polar localization of PIN2 (panels 1 to 3) in the absence of BFA. In the presence of BFA, pretreatment with 1-butanol or PA (30 min, before the application of BFA and 1-butanol or PA) completely or largely blocked PIN2 accumulation in BFA compartments (panels 4 and 6). By contrast, in cells pretreated with 2-butanol, extensive PIN2 accumulation in BFA compartments was observed (panel 5). In addition, after pretreatment with BFA for 2 h and BFA washout with PA (panel 9) or 2-butanol (panel 8) for 2 h, the polar localization of PIN2 was almost completely restored, whereas there was still accumulated PIN2-EGFP in cells after washout of BFA with 1-butanol for 2 h (panel 7). Roots of 7-d-old *Arabidopsis* seedlings were used for all treatments ($n > 15$). Bars = 5 μm.

yeast, loss of Spo14P, the single phosphatidylcholine-specific PLD, blocks vesicle fusion, resulting in the accumulation of prospore membrane precursor vesicles (Nakanishi et al., 2006). Treatment with 1-butanol or decreased PLD1 activity inhibited early endosome fusion or vesicle fusion to the plasma membrane, which was stimulated by exogenous PLD or lysophosphatidylcholine (Jones and Clague, 1997; Huang et al., 2005). Suppressed vesicle trafficking under *PLDζ2* deficiency might reduce vesicle fusion and aggregation, leading to the formation of small BFA compartments.

We show that PLDζ2, or PA generated by it, stimulates vesicle trafficking in *Arabidopsis* cells. Genetic investigations using *PLDζ2*-deficient or -overexpressing plants demonstrate unequivocally that PLDζ2 is beneficial for internalization of the membrane marker FM4-64 through vesicle trafficking. Significantly, exogenously applied PA mimics the effect of *PLDζ2* overexpression on FM4-64 internalization, whereas 1-butanol treatment diminishes FM4-64 internalization in a way similar to PLDζ2 deficiency. These findings are consistent with previous results showing that decreased PA in the *pldζ2* mutant (Cruz-Ramirez et al., 2006) reduced the accumulation of secretory vesicles in the pollen tube apex. As a widely accepted tracer for endocytic trafficking (Bolte et al., 2004), the reduced internalization of FM4-64 in *pldζ2* and wild-type seedlings treated with 1-butanol suggests that PLDζ2 and PA at least affected endocytosis (Figure 7).

PLDζ2 overexpression significantly counteracts the inhibitory effect of BFA on vesicle trafficking, further confirming PLDζ2 function in stimulating vesicle trafficking. The alleviation of BFA inhibition on vesicle trafficking by *PLDζ2* overexpression correlates well with the significantly improved performance of *PLDζ2*-overexpressing plants in terms of hypocotyl elongation, primary root length, lateral root development, and gravity response on medium containing BFA.

Stimulation of PIN2 Cycling Underlies the Positive Roles of PLDζ2 and PA in Auxin Transport and Enhanced Auxin Responses

Polar auxin transport, which is essential for the execution of auxin responses, is mediated by plasma membrane–localized auxin

influx and efflux carrier proteins, and PINs were the best characterized proteins controlling auxin efflux (Muday and Delong, 2001; Muday and Murphy, 2002). The cycling of PIN proteins (PIN1 and PIN2) between membrane and endosomal compartments is vital for their transport function and is dependent on vesicle trafficking (Geldner et al., 2001, 2003; Grebe et al., 2003; Jaillais et al., 2006). PIN1 cycling and auxin transport require endosomal GNOM activity (Geldner et al., 2003), and recent studies have shown that *Arabidopsis* SORTING NEXIN1, which localizes to an endosomal compartment distinct from GNOM-containing endosomes, was involved in PIN2 endocytic sorting (Jaillais et al., 2006).

Through complementary genetic and cell biological analyses, we deduced that a positive role of PLDζ2 (and PA) in auxin responses is at least partly linked with its beneficial effect on the rapid cycling of PIN2 in cells. Compared with wild-type and *PLDζ2*-deficient cells, the formation of BFA compartments containing PIN2 was substantially suppressed in *PLDζ2*-overexpressing cells. More importantly, after BFA removal, the resumption of PIN2 polar localization was more rapid and complete under enhanced *PLDζ2* expression. The effect of exogenous PA was similar to that of *PLDζ2* overexpression in facilitating PIN2 cycling. Together, these results indicate that PLDζ2 (through its product PA) is required for the normal cycling of PIN2-containing vesicles and regulates auxin transport, which may account for the role of PLDζ2 in positively regulating auxin responses.

It is noteworthy that, although both PA and 1-butanol seem to alleviate the effects of BFA on PIN protein cycling, the relevant mechanisms are different. PA represses BFA inhibition of exocytosis and restores BFA compartments (accumulated PIN proteins) through accelerated vesicle trafficking, whereas the reduced BFA compartments under 1-butanol pretreatment are attributable to inhibited endocytosis. The restoration of BFA compartments after BFA washout further confirms the different mechanisms.

Our analyses showed that PLDζ2 is involved in auxin responses and the regulation of auxin levels and distribution, suggesting that altered responses may be attributable to altered auxin transport and thus changed auxin distribution and levels. This is confirmed by the observation that PIN protein cycling was altered under modulated expression of *PLDζ2*. Altered PIN protein cycling and auxin transport result in altered auxin distribution and levels, consequent to modulated auxin responses, as well as the expression of early auxin-responsive genes. However, there is no evidence that PLDζ2 and PA are involved directly in auxin signaling. These results suggest that PLDζ2 may indirectly control auxin signaling by altering auxin transport and are consistent with the fact that *pldζ2* showed decreased sensitivity to auxin but not the striking phenotype of the auxin signaling–defective mutants *tir1-1* and *axr1-3*.

Mechanistically, PLD and PA may stimulate PIN protein cycling through actin regulation. Inhibited PLD activity resulted in severely disorganized actin structures in *Arabidopsis* seedlings (Motes et al., 2005), implying an essential role of PLD and PA for actin. In addition, PLD can bind to actin (Kusner et al., 2002), and PA can stimulate actin polymerization by inhibiting ATCP activity (Huang et al., 2006). PIN1 cycling is actin-dependent, and treatment with cytochalasin, a drug that depolymerizes actin filaments, reduced auxin transport (Cande et al., 1973; Butler et al., 1998), blocked PIN1 accumulation in response to BFA treatment, and restored PIN1 localization after BFA was washed out (Geldner et al., 2001). Cytochalasin D and 1-butanol have similar inhibitory effects on PIN cycling. Each of them has only a slight effect on the polar localization of PIN, but both inhibit BFA-induced PIN accumulation and PIN trafficking from BFA compartments to the plasma membrane.

PLD and PA may be involved in auxin transport through some other mechanism, such as regulating the activity of RCN1 or PID1. RCN1, a protein phosphatase 2A regulatory subunit involved in auxin transport (Rashotte et al., 2001), is a potential target protein of PA (Testerink et al., 2004). PID1, a Ser/Thr protein kinase, acts as a regulator of polar auxin transport (Bennett et al., 1995), is activated by *Arabidopsis* PDK1 (3-phosphoinositide–dependent protein kinase 1 [Zegzouti et al., 2006]), and its activity is stimulated directly by PA (Anthony et al., 2004). *rcn1* showed increased auxin transport and decreased root gravitropism (Rashotte et al., 2001), and *pid1* has similar phenotypes to those of *pin1* (Bennett et al., 1995). Overexpression of *PID1* results in phenotypes similar to those of auxin-insensitive mutants (Christensen et al., 2000) and induces a basal-to-apical shift of PIN1 localization (Friml et al., 2004). These results suggest that PLD and PA may be involved in auxin transport regulation through modulation of the activity of RCN1 or PID1.

METHODS

Enzymes and Chemical Reagents

All enzymes used for DNA manipulation were purchased from Boehringer. DNA primers were synthesized commercially (Sangon). PA (P9511), IAA (1-2886), and BFA (B-7651) were purchased from Sigma-Aldrich. FM4-64 was purchased from Molecular Probes. 1-Butanol, 2-butanol, and 3-butanol (HPLC-grade) were purchased from Sinopharm Chemical Reagent.

Bacterial and Plant Materials and Growth Conditions

Escherichia coli strain XL1-Blue MRF was used for DNA amplification and subcloning. *Agrobacterium tumefaciens* strain GV3101 was used for plant transformation. *Arabidopsis thaliana* ecotype Columbia was used for transformation by the floral dip method (Clough and Bent, 1998). Seeds were germinated and seedlings were grown in a phytotron set at 22°C with a 16-h light/8-h dark cycle. Ten to 14 d after germination, seedlings were hand-transferred to soil and grown in a phytotron under the same conditions. For hormone treatments, 7-d-old seedlings were treated with 100 μM IAA for various times (0, 0.5, 1, 3, 6, and 12 h).

Isolation of PLDζ2 cDNA

An *Arabidopsis* cDNA clone encoding a putative PLDζ2 was identified through analysis of the *Arabidopsis* genomic sequence. Specific primers PLDζ2-1 (5′-GATCGTTATTCCGCTAC-3′) and PLDζ2-2 (5′-AAGGTTC-CCTCTTGTCTC-3′) were designed and used to screen a cDNA library constructed from *Arabidopsis* hypocotyl tissues through PCR-based screening (Alfandari and Darribère, 1994). Positive clones were converted into pBluescript SK− derivatives using ExAssist helper phage according to the supplier's instructions (Stratagene). The clone with the longest insert was used for further analysis. DNA sequencing was performed by

Genecore, and computational analysis was performed with the programs of the Wisconsin Genetics Computer Group (GCG Package, version 10.1). Domain structure was predicted using Conserved Domain Database searching and gap comparison, and sequence similarity analysis was performed with the GAP program of the GCG Package.

RT-PCR Analysis

RT-PCR analyses were used to study the transcript levels of *PLDζ2* in various tissues. Total RNA samples were isolated with the Trizol reagent (Invitrogen), and 2 μg of RNA was reverse-transcribed with oligo(dT) primers according to the manufacturer's instructions (RNA PCR kit; TaKaRa). Equal amounts of first-strand cDNAs were used as templates for PCR amplification using primers PLDζ2-1 and PLDζ2-2 with different amplification cycles. The *Arabidopsis Actin2* gene (*At3g18780*) was amplified using primers Actin-1 (5′-TCTTCTTCCGCTCTTTCTTTCC-3′) and Actin-2 (5′-TCTTACAATTTCCCGCTCTGC-3′) as internal positive controls for quantifying relative amounts of cDNA. Amplified DNA products were separated using an agarose gel followed by image analysis with GelDoc 2000 (Tanon).

Promoter-GUS Fusion Studies and Histochemical Analysis of GUS Activities

The PLDζ2 promoter region was amplified by PCR using primers PLDζ2-p-1 (5′-CCCAAGCTTTGTACGCAGGGCTCTACT-3′; *Hin*dIII site underlined) and PLDζ2-p-2 (5′-CGGGATCCAAACTCGCCGTTCACGAT-3′; *Bam*HI site underlined) with *Arabidopsis* genomic DNA as template. The resulting 1760-bp fragment was subcloned into vector pBI101.1, and the obtained construct pBI101-P was introduced into *A. tumefaciens* and then used for *Arabidopsis* transformation. Positive transgenic plants were obtained through selection by kanamycin resistance and PCR amplification. T2 and T3 homozygous generations were used for GUS activity detection according to Jefferson et al. (1987) and visualized using Nomarski optics on a Leica DMR microscope with a Leica DC300F digital camera. To observe *PLDζ2* expression under auxin treatment, 7-d-old transgenic seedlings were treated with 1 μM IAA for 48 h.

DR5-GUS seedlings were treated with PA (10, 20, or 50 μM), 1-butanol (0.4 or 0.8%, v/v), 2-butanol (0.4 or 0.8%, v/v), or 3-butanol (0.4%, v/v) for different times, then incubated with GUS solution for 3 h and visualized after removal of chlorophyll.

Identification of a PLDζ2 Knockout Mutant

A putative knockout mutant of PLDζ2, SALK_119084, was identified in the Salk Institute T-DNA insertion library database (http://signal.salk.edu/cgi-bin/tdnaexpress; Alonso et al., 2003) by searching with locus name *At3g05630*. Seeds were obtained from the ABRC (Ohio State University), and seedlings were analyzed individually by PCR amplification to confirm the presence of T-DNA using primer LBa1 (located in the T-DNA) and gene-specific primer P2 (5′-CTCTAGCAAATGAGAGTCTAG-3′). The plants homozygous for T-DNA insertion were checked for lack of *PLDζ2* transcripts using primers P3 (5′-TGATCGTTATTCCGCTAC-3′) and P4 (5′-AAGGTTCCCTCTTGTCTC-3′) by RT-PCR as described above.

Transgenic Modification of PLDζ2 Expression

The *PLDζ2* cDNA coding sequence was subcloned into pCAMBIA1301 (Liu et al., 2003) in the sense orientation, resulting in construct pO-PLDζ2 for overexpressing PLDζ2 in wild-type *Arabidopsis*. A 1-kb region near the 5′ end was subcloned into pCAMBIA1301 in the antisense orientation, producing pA-PLDζ2, which was used to suppress PLDζ2 expression in wild-type *Arabidopsis*. Genetic transformation was conducted as described above, and transgenic plants were confirmed using the following two strategies. First, genomic DNA was extracted with the cetyl-trimethylammonium bromide method (Sambrook et al., 1989), and PCR amplification was performed to confirm the integration of T-DNA into the plant genome. Second, total RNAs were isolated from leaves of putative transformants and RT-PCR was used to examine whether *PLDζ2* expression levels were altered using primers PLDζ2-1 and PLDζ2-2. Primers PLDζ1-1 (5′-GTACAAGAGGATTTTGCGTGCCC-5′) and PLDζ1-2 (5′-TCAATGGAAGACTTGAGGGGAGG-3′) were used to detect *PLDζ1* expression levels in plants transformed with pA-PLDζ2 to ensure that antisense suppression of PLDζ2 was specific. Confirmed transformants were transplanted to soil for self-crossing, and T2 seeds from individual T1 plants were germinated to identify homozygous T2 lines, which were used for further analysis.

Phenotypic Analysis of *pldζ2* and Transgenic Plants, and Gravity Response Measurements

Seeds of wild-type and transgenic plants were germinated on Murashige and Skoog medium supplemented with different concentrations of IAA (0, 1, 10, and 100 nM and 1 μM) for 9 d, and primary root length was measured and calculated. All measurements were performed using triplicate samples, and results presented are averages of at least 30 seedlings. For hypocotyl elongation under high temperature, >30 wild-type and *pldζ2* seedlings were germinated and grown on vertical agar plates at 22 or 29°C for 7 d, followed by measurement of hypocotyl length and statistical analysis.

The root gravity response was determined using 4-d-old light-grown seedlings in the presence or absence of PA, 1-butanol, 2-butanol, or 3-butanol on the medium. The angle of root curvature was measured at 2, 4, 8, 12, and 24 h after reorientation, with the aid of the image-analysis program ImageJ (version 1.34 for Windows; http://rsb.info.nih.gov/ij/).

Expression Pattern Analysis of Auxin-Responsive Genes

Seven-day-old seedlings were treated with IAA (10 μM) for 1.5 h, and leaves were harvested for RNA extraction. PCR reaction was executed using Rotor-Gene real-time thermocycler R3000 (Corbett Research) with a SYBR green probe (SYBR Premix Ex Taq system; TaKaRa). The amount of product was determined at the end of each cycle using Rotor-Gene software (version 6.0.16; Corbett Research). Differences in product levels among the tested samples during the linear amplification phase were used to reveal differential gene expression, which was presented as relative expression levels compared with the amount of *Actin7* transcript. Tested genes were as follows: *IAA5* (5′-ACAGTCTCGAACGGACCAAA-3′ and 5′-ACATCTCCAGCAAGCATCCA-3′), *IAA19* (5′-CTTGATAAGCTCTTCGGTTTC-3′ and 5′-CAGCTCCTTGCTTCTTGTTC-3′), *GH3.3-1* (5′-CTCGGTGCTGCTTGGAAATG-3′ and 5′-TGGGCTGAAGTGTGTAGATA-3′), and *Actin7* (5′-TTCCCGTTCTGCGGTAGTGG-3′ and 5′-CCGGTATTGTGCTCGATTCTG-3′).

FM4-64 Staining and Confocal Microscopy

Roots of wild-type and *PLDζ2*-deficient (knockout and antisense) or overexpression plants were first treated with PA (10, 20, or 50 μM), 1-butanol (0.4 or 0.8%, v/v), or 2-butanol (0.4%, v/v), then stained with 5 μM FM4-64 for 10 min, followed by washing with water three times. After incubation for 20 or 45 min at room temperature, the fluorescence was observed with a confocal laser scanning microscope (FITC488, Zeiss LSM500). FM4-64 fluorescence was excited with a 543-nm argon ion laser and a 600-nm long-pass emission filter. All images within a single experiment were captured with the same gain and exposure settings.

Visualization of BFA Compartments and PIN2 Cycling

Wild-type seedlings and those with altered PLDζ2 expression (knockout, antisense, and overexpression) were treated with 50 μM BFA for 2 h, then

incubated with solution containing BFA (50 μM) and FM4-64 (5 μM) for 30 min, followed by examination of BFA compartments by confocal laser scanning microscopy. Furthermore, 4-d-old wild-type seedlings were pretreated with PA (50 μM), 1-butanol (0.8%, v/v), or 2-butanol (0.8%, v/v) for 30 min, then treated with 0.8% 1-butanol or 2-butanol or 50 μM PA and 50 μM BFA for 2 h and observed after incubation with BFA (50 μM) and FM4-64 (5 μM) for 30 min.

PIN2-EGFP cassettes were individually transferred into *pldζ2* or PLDζ2 overexpression (LO11 and LO19) backgrounds by genetic cross, and homozygous offspring were observed as described above. In addition, seedlings harboring PIN2-EGFP constructs were pretreated with PA (50 μM), 1-butanol (0.8%, v/v), or 2-butanol (0.8%, v/v) for 30 min, treated with 50 μM BFA for 2 h, and observed immediately or after washout with PA (50 μM), 1-butanol (0.8%, v/v), or 2-butanol (0.8%, v/v) for 2 h. Visualization was performed with a confocal laser scanning microscope at a wavelength of 488 nm (EGFP).

Accession Numbers

Sequence data generated or used in this work can be found in the GenBank/EMBL database under the following accession numbers: *PLDζ1* (*At3g16785*, NM_112553), *PLDζ2* (*At3g05630*, AM182458), *IAA19* (*At3g15540*, AY088753), *IAA5* (*At1g15580*, U18407), *GH3-3* (*At2g23170*, AY090371), *Actin7* (*At5g09810*, NM_121018), and *Actin2* (*At3g18780*, NM_112764).

Supplemental Data

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

Supplemental Figure 1. *pldζ2* Is Less Sensitive to Exogenous Auxin.

Supplemental Figure 2. Altered Expression of *PLDζ2* Results in Altered Auxin Effects on Primary Root Growth.

Supplemental Figure 3. 1-Butanol Suppresses DR5-GUS Expression and Auxin-Induced Lateral Root Formation.

Supplemental Figure 4. *PLDζ2* Overexpression Represses 1-Butanol–Suppressed FM4-64 Internalization.

Supplemental Figure 5. PLDζ2 Suppresses the Inhibitory Effects of BFA on Seedling Growth.

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