Phospholipase D (PLD; EC 3.1.4.4) has been proposed to be involved in a number of cellular processes including transmembrane signaling and membrane deterioration. PLD previously described from various plant sources generally requires millimolar ranges of calcium for optimal activity. In this study, we genetically suppressed the expression of this conventional PLD in Arabidopsis by introducing an antisense PLD cDNA. However, both the antisense transgenic and wild-type plants showed comparable PLD activity in the presence of submicromolar concentrations of calcium and phosphatidylinositol 4,5-bisphosphate using phosphatidylcholine as a substrate. This new PLD activity was partially stimulated by phosphatidylinositol 4-phosphate, but not by other phospholipids, including phosphatidylinositol, phosphatidylserine, phosphatidylglycerol, phosphatidic acid, or phosphatidylcholine. Its requirement for polyphosphoinositides was further supported by its ability to be inhibited by neomycin. The polyphosphoinositol-dependent PLD requires calcium for activity, but no magnesium. The calcium stimulation was observed in the nanomolar range and reached a plateau at 5 μM calcium. The findings of this study demonstrate the presence of different PLDs that are regulated in a distinct manner in plants. The potential significance of a PLD that is regulated by polyphosphoinositides and physiological concentrations of Ca²⁺ is discussed.

Phospholipase D (PLD; EC 3.1.4.4) hydrolyzes the terminal phosphodiester bond of phospholipids, generating phosphatidic acid (PA) and a water-soluble head group. PLD-mediated hydrolysis plays an important role in cell signaling and regulation. In mammalian systems, PLD has been proposed to be involved in a broad range of cellular processes including membrane trafficking, cell proliferation, protein secretion, metabolic regulation, and immune and inflammatory responses (reviewed in Ref. 1). In yeast, PLD activity has been shown to be required for sporulation and may also be involved in the adaptation of nutrient utilization (2, 3). In plants, most reports on PLD functional studies have dealt with its possible effects on membrane deterioration in relation to stress injuries, senescence, aging, and pathogenesis (4–6). Recent studies have, however, indicated a role of PLD in signal transduction processes in response to environmental cues (7–9).

In order to understand how PLD is involved in diverse cellular processes, it is important to delineate the mechanisms by which it is regulated. PLD activity was first described in plant extracts about five decades ago (10), and has since been characterized from a number of plant sources (6, 11, 12). The cloning and functional expression of PLD cDNAs have been recently reported (13–15). The distinct feature of those described plant PLDs is that their maximal activity requires non-physiological, millimolar levels of Ca²⁺. This is in stark contrast to the activity of the recently cloned and expressed human and yeast PLDs (2, 3, 16, 17). The human PLD is fully active at micromolar Ca²⁺ levels, and the yeast PLD does not require Ca²⁺ for activity. Furthermore, the PLDs from both human and yeast are activated by PIP₂.

The regulation of PLD activity by PIP₂ is of physiological significance since PIP₂ is an established signaling molecule (18, 19). The hydrolysis of PIP₂ by phospholipase C produces the second messengers diacylglycerol and inositol trisphosphate. In addition, phosphorylation of PIP₂ by phosphoinositide 3-kinase leads to the synthesis of phosphatidylinositol-3,4,5-trisphosphate, which activates Ca²⁺-independent protein kinase C. Furthermore, PIP₂ itself has been shown to regulate the activity of many proteins, including Ras GTPases, DNA polymerase α, gelsolin, and the vandate-sensitive plasma membrane ATPase (19, 20). However, studies using PLD from commercial sources suggested that plant PLD is not regulated by PIP₂ (21).

In this study, we set out to determine the regulation and function of PLD by introducing a PLD antisense gene to suppress PLD expression in Arabidopsis thaliana. During the course of characterizing plants in which the conventional, millimolar Ca²⁺-requiring PLD had been suppressed, we found a novel plant PLD that is characterized by its requirement for PIP₂ or PIP₃ and its ability to function at Ca²⁺ concentrations in the nanomolar to low micromolar range. Results from this study indicate the presence of PLD isoforms that are distinct gene products and regulated differently.

**EXPERIMENTAL PROCEDURES**

**Materials**—PIP₂ and PIP₃ were obtained from Boehringer Mannheim. PEOH, PI, and PE were purchased from Avanti Polar Lipids. All other phospholipids were obtained from Sigma. 1-Palmitoyl-2-oleoyl-[oleoyl-1-³H]-glycerol-3-P-choline, and dipalmitoylglycerol-3-P-[methyl-³H]choline were from DuPont NEN. Silica Gel 60 TLC plates were obtained from Merck (Darmstadt, Germany).

Generation of PLD Antisense Transgenic Arabidopsis—The sequence of a full-length PLD cDNA has been reported previously (14). A 780-base pair Xba/HindIII fragment of the Arabidopsis PLD cDNA was inserted into the Agrobacterium tumefaciens transfer vector, pKYLX7.
(22), in the antisense orientation under the control of the cauliflower mosaic virus 35S promoter. The T-DNA region of pXYL7 was transferred into Arabidopsis plants through Agrobacterium-mediated gene transfer via a vacuum infiltration method (23). Antisense transgenic plants were selected by their resistance to kanamycin, and the presence of transgene-predicted antisense DNA in their genome was verified by Southern blot analysis. Transgenic plants with suppressed expression of the PLD gene were identified by assaying PLD activity in the presence of 25 mM Ca2+ and by measuring PLD protein levels by immunoblotting.

Extraction of PLD Activity—PLD activity was extracted following an adaptation of a previously described procedure (21). Briefly, one part of leaf tissue was ground with a chilled mortar and pestle in three parts of a homogenization buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM KCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol at 4°C. The homogenate was centrifuged at 100,000 × g for 45 min. The supernatant was referred to as the soluble fraction. The pellet was resuspended in a salt-extraction buffer consisting of 0.4 M KCl in the homogenization buffer. After incubation for 1 h at 4°C, the salt-insoluble materials were collected by centrifugation at 100,000 × g for 45 min. This supernatant was designated the membrane-associated fraction and contained the most PIP2-dependent PLD activity. In the subcellular fractionation experiments, the pellet was salt extracted again, and the resulting pellet was resuspended in the salt extraction buffer. The protein content was determined by the Bradford method according to the manufacturer’s instructions (Bio-Rad). Fractions were used directly or stored in aliquots at –80°C.

High Ca2+-dependent PLD Activity Assay—The assay reaction mixture contained 100 mM MES (pH 6.5), 25 mM CaCl2, 0.5 mM SDS, 1% (v/v) ethanol, 5–15 μM of protein, and 2 mM PC (egg yolk) containing dipalmitoylglycerol-3-P-[methyl-3H]phosphate. The substrate preparation, reaction conditions, and product separation were based on previously described procedures (12) with the following changes: the assay volume was reduced to 100 μl and 100 μl of 2 μM KCl was added to the 2:1 (v/v) chloroform:methanol extraction to enhance the phase separation. The release of [3H]choline into the aqueous phase was quantitated by scintillation counting.

PIP2-stimulated PLD Activity Assay—PLD activity was assayed by using either 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphate (1,2-dipalmitoyl-sn-glycerol-3-phosphate or cholesterol) or 1,2-dipalmitoyl-sn-glycerol-3-phosphate with 0.5% (w/v) soybean phosphatidylcholine as substrates. The acylated phospholipid substrate was used for assaying transphosphatidylation activity whereas the choline-labeled PC was used in all other studies. In both cases, 2.5 μCi of [1-14C]phosphatidylcholine was mixed with 3.6 μmol of PC, 0.34 μmol of PIP2, and 0.2 μmol of cold PC in chloroform, and the solvent was evaporated under a stream of N2. In the phospholipid-specificity experiments, PIP2 was replaced with 0.34 μmol of PE, PA, PC, PS, PI, or PIP. The phospholipid substrate was dispersed in 1 ml of H2O by sonication at room temperature. Previously reported conditions were adapted to yield an enzyme assay mixture that contained 100 mM MES (pH 7.0), 5 μM CaCl2, 2 mM MgCl2, 80 mM KCl, 0.4 mM lipase, and 5–15 μM of protein in a total volume of 100 μl (21). The free Ca2+ concentration in the reaction mixture was approximately 100 μM, which was estimated with a free Ca2+-sensitive electrode. The reaction was initiated by addition of substrate and incubated at 30°C for 30 min in a shaking water bath. When choline-labeled PC was used, the reaction was stopped by addition of 1 ml of 2:1 (v/v) chloroform:methanol and 100 μl of 2 μM KCl. After vortexing and centrifugation at 12,000 × g for 5 min, a 200-μl aliquot of the aqueous phase was mixed with 3 ml of scintillation fluid, and the release of [3H]choline was measured by scintillation counting.

When acylated PC was used as the reaction mixture included ethanol to a final concentration of 0.5% (v/v) for assaying the transphospholipid activity of PLD. The reaction was stopped by adding 375 μl of 1:2 (v/v) chloroform:methanol. Additionally, 100 μl of chloroform and 100 μl of 2 μM KCl were added and the sample was vortexed. The chloroform and aqueous phases were separated by centrifugation at 12,000 × g for 5 min. The aqueous phase was removed and the chloroform phase was dried. Thin layer chromatography was conducted as described previously using 65:35:5 chloroform:methanol:NH4OH as the developing solvent (12). Lipids separated on plates were visualized by exposure to iodine vapor. Spots corresponding to the lipid standards, PA, PC, PE, and PEOH, were scraped and radioactivity was measured by scintillation counting. Autoradiography was performed by exposing the dried TLC plate to BIOMAX film (Kodak) for 1.5 days.

Preparation of Ca2+ /Mg2+-EGTA Buffers—In the cation dependence experiments, the concentrations of free Ca2+ and Mg2+ in the reaction mixture were determined using Ca2+ /Mg2+-EGTA buffers at pH 7.5 as described (23). When different Ca2+ concentrations were tested, Mg2+ was maintained at a constant concentration of 2 mM. Likewise, various Mg2+ concentrations were tested, Ca2+ was maintained at a concentration of either 200 nM or 50 μM.

Expression of a Castor Bean PLD cDNA in Escherichia coli—The cloning and expression of a catalytically active PLD from a castor bean cDNA were previously described (13). Briefly, the PLD cDNA was inserted into the pBluescript SK(−) and transformed into E. coli JM109. The production of PLD was induced by addition of isopropyl-1-thio-β-d-galactopyranoside. Cells were pelleted and lysed by sonication in the same homogenization buffer described above for the plant PLD extraction. Cell debris was removed by centrifugation at 10,000 × g for 10 min, and proteins in the supernatant were used for assaying PLD activity.

PAGE Analyses and Immunoblotting—Cytosolic and solubilized-membrane proteins were separated by 8% SDS-PAGE gels following a described procedure (12). After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes and blotted with antibodies that were raised in rabbit against a peptide corresponding to the 12-amino acids of the conventional PLD C terminus. The amino acid sequence was deduced from the cloned Arabidopsis PLD (14). The immunoblot analysis using anti-PLD antibodies was performed as described (12).

The preparation of non-denaturing PAGE was the same as the SDS-PAGE except that 0.05% SDS was omitted (25). Protein samples were adjusted to 5 mM dithiothreitol and 5% (v/v) glycerol prior to loading. After electrophoresis two adjacent lanes on a gel were sliced in parallel. Gel slices from one lane were placed in reaction mixtures for assaying PIP2-stimulated PLD as described above, and those from the other lane were measured for the conventional, PIP2-independent PLD activity in the presence of 25 mM Ca2+ (25). Proteins of another native gel were transferred onto a polyvinylidene difluoride membrane and blotted with specific PLD antibodies. RESULTS

Antisense Suppression of the Conventional Plant PLD in Arabidopsis—A full-length PLD cDNA has been cloned from Arabidopsis (GenBank accession number D45204) based on its high degree of sequence identity (77%) with that of cloned castor bean PLD (14). A 780-base pair fragment near the 3′ end of this Arabidopsis PLD cDNA was used to construct an antisense vector and produce transgenic plants. The leaves of the plant antisense plants exhibited less than 3% of the wild-type PLD activity using previously described assay conditions that included 25 mM Ca2+, 0.5 mM SDS, and PC as substrate (Ref. 12; Fig. 1A). The lack of activity was due to the loss of PLD protein, which was indicated by the absence of the immunoreactive PLD band in the antisense extracts (Fig. 1B). The absence of PLD protein was correlated with a decrease in PLD mRNA (data not shown), indicating the expression of the PLD gene was impaired in the antisense plants. These results have ...

![FIG. 1.  Antisense suppression of conventional PLD in transgenic Arabidopsis. A, PLD activity in wild-type and antisense transgenic plants as assayed in the presence of 25 mM Ca2+, 0.5 mM SDS, 100 mM MES/NaOH (pH 6.5), and 2 mM egg yolk PC with 0.02 μCi of dipalmitoylglycerol-3-P-[methyl-3H]choline. B, immunoblot of PLD in wild-type and transgenic plants using the polyclonal antibody raised against the synthetic 12-amino acid peptide of the C-terminal sequence. Each lane was loaded with 20 μg of protein. S, soluble protein of 100,000 × g supernatant; M, membrane-associated protein solubilized with 0.4 M KCl of the 100,000 × g pellet. Values are representative of three experiments.](image-url)
Supernatant; transphosphatidylation to hydrolysis. PIP2 and 100% PIP2, and 5.1 mol % PC. Reaction products, PA and PEOH, were separated by TLC and monitored by autoradiography (right panels) of the separated PA and PEOH. Right panels: upper, formation of transphosphatidylation product, PEOH; middle, formation of hydrolysis product, PA; bottom, ratio of transphosphatidylation to hydrolysis. S, soluble protein of 100,000 x g supernatant; M, membrane-associated protein solubilized with 0.44 M KCl of the 100,000 x g pellet. Values are means ± S.E. of three experiments.

clearly demonstrated that the expression of the conventional, millimolar Ca2+-requiring PLD was suppressed in the antisense plants. Identification of PIP2-dependent PLD Activity—Unlike yeast, in which the deficiency of PLD interrupted reproductive processes (2, 3), the PLD antisense-suppressed plants grew and developed to maturity. One possible explanation is that other types of PLD exist that compensate for the loss of this PLD developed to maturity. One possible explanation is that other types of PLD exist that compensate for the loss of this PLD.

To confirm that the PA produced resulted from PLD activity, transphosphatidylation activity was measured in the presence of 0.4 mM and PC was kept at a constant 5.1 mol %. The PLD activity was confined to the membrane and pellet fractions, and 70% of that activity could be solubilized by washing the 100,000 x g pellet fractions with 0.44 M KCl (Table I; Fig. 2). These results suggest that the PIP2-dependent PLD activity is peripherally associated with membranes. A majority of PIP2-dependent PLD activity also was found in the particulate fractions in wild-type plants, however, the soluble activity in wild-type was substantially higher than that of the antisense plants.

Effects of Other Phospholipids on PIP2-stimulated Activity—Several common membrane phospholipids were examined to determine their ability to stimulate the new PLD activity (Fig. 3). PIP significantly stimulated the PLD activity in both the antisense and wild-type plants to a level which was about 40–60% of the PIP2 stimulated activity. However, no PLD activity was observed when the PIP2 was replaced with PI. This PLD activity was not appreciably supported by other phospholipids; PS, PG, PA, or PC.

Effect of PIP2 Concentrations on PIP2-dependent PLD—To examine the PIP2 concentration effect on this PLD, the mole % of PIP2 was varied while the total lipid content was maintained at 0.4 mol and PC was kept at a constant 5.1 mol %. The PLD of the antisense extracts displayed virtually no activity until the amount of PIP2 present in the substrate vesicles was greater than 0.1 mol % (Fig. 4). Beyond that point PLD was stimulated by PIP2 in a dose-dependent manner; the maximal stimulation occurred at 7.9 mol %. The activity of extracts from antisense suppressed plants was increased more than 60-fold when the PIP2 content was shifted from 0.1 to 7.9 mol %. On the other hand, higher concentrations of mole % PIP2 were inhibitory to this enzyme; only 30% of the maximal PIP2-dependent PLD activity was observed at 15.8 mol % PIP2. A similar pattern of PIP2 stimulation was observed in the wild-type extracts.

The PIP2 dependence of PLD activity was further demonstrated by its ability to be inhibited by neomycin, a high affinity cationic ligand that is selective for polyphosphoinositides (26, 27). At concentrations below 100 μM the effect of neomycin was not pronounced, suggesting the presence of excess of PIP2 in the assay system (Fig. 4, inset). At concentrations above 100 μM, more severe inhibition was observed, and 2 mM neomycin inhibited the PIP2-dependent PLD by 90% in the antisense extracts. The conventional PLD activity was tested for its sensitivity to neomycin at the assay condition of 25 mM Ca2+, 0.5 mM SDS, and 2 mM PC in the absence of PIP2, and no inhibitory activity was present primarily in the solubilized membrane fraction of the antisense extracts, whereas it was distributed both in the soluble and membranous fractions in the wild-type extracts. To assess the subcellular distribution in more detail,

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from its hydrolytic activity, PLD has the unique ability to utilize primary alcohols instead of water as the acceptor of the cationic ligand that is selective for polyphosphoinositides (26, 27). At concentrations below 100 μM the effect of neomycin was not pronounced, suggesting the presence of excess of PIP2 in the assay system (Fig. 4, inset). At concentrations above 100 μM, more severe inhibition was observed, and 2 mM neomycin inhibited the PIP2-dependent PLD by 90% in the antisense extracts. The conventional PLD activity was tested for its sensitivity to neomycin at the assay condition of 25 mM Ca2+, 0.5 mM SDS, and 2 mM PC in the absence of PIP2, and no inhibitory activity was present primarily in the solubilized membrane fraction of the antisense extracts, whereas it was distributed both in the soluble and membranous fractions in the wild-type extracts. To assess the subcellular distribution in more detail,
PIP2, and 5.1 mol% PC. In this experiment, PIP2 was replaced with 7.9 mol% PE, 0–15.8 mol% PIP2, and 5.1 mol% PC. The relative PLD activity in the wild-type extracts was substantially higher (about 80% of optimal activity) at this pH. The activity in both the antisense and wild-type extracts exhibited an acidic pH optimum (11, 12). The effect of pH on PIP2-dependent PLD activity was investigated over a range of 5 to 9 (Fig. 5). PLD activity was maximal between 5.0 and 7.5, where the Ca2+ concentration approached 100 μM (Fig. 6). This Ca2+ activation profile for the expressor castor bean PLD corresponds to the second phase of Ca2+ dependence in the wild-type extracts and is distinct from that found in the antisense plants, this second peak of PLD activity is likely represent the conventional, millimolar Ca2+-requiring PLD.

The effect of Ca2+ on the conventional PLD was examined using a bacterially expressed PLD from a castor bean cDNA in this Ca2+-dependence study. The castor bean cDNA encodes a conventional PLD that has been shown to possess PIP2-independent activity in millimolar Ca2+ (12, 13). In the present assays containing PIP2, this PLD did not exhibit significant activity in the absence of Ca2+ and Ca2+ stimulation was observed at nanomolar ranges and reached a plateau at 5 μM in the antisense extracts (Fig. 6). The second phase was a sharp increase in PLD activity at millimolar concentrations of Ca2+ (Fig. 6). Because the previously characterized conventional PLD is known to require millimolar levels of Ca2+ for optimal activity (12) and because it is absent in the antisense plants, this second peak of PLD activity is likely represent the conventional, millimolar Ca2+-requiring PLD.

**Fig. 3.** Effects of phosphoinositides and other phospholipids on PIP2-requiring PLD activity. Lipid vesicles (0.4 mM) in the PIP2 assays were composed of 87 mol% PE, 7.9 mol% PIP2, and 5.1 mol% PC. In this experiment, PIP2 was replaced with 7.9 mol% PE, PA, PG, PS, PI, or PIP. Cross-hatched bars represent antisense plants; bars with vertical lines represent wild-type plants. These assays used membrane-associated proteins solubilized with 0.44 M KCl. Values are means ± S.E. of three experiments.

**Fig. 4.** PIP2-stimulated PLD activity of wild-type and antisense plants as a function of mole% PIP2 in lipid vesicles. Lipid vesicles (0.4 mM) in the reaction mixture consisted of 79.1–94.9 mol% PE, 0–15.8 mol% PIP2, and 5.1 mol% PC. Lines with open and filled circles represent antisense and wild-type plants, respectively. Inset, neomycin inhibition of PLD activity. PLD was assayed in the presence of 7.9 mol% PIP2 and 0–2 mM neomycin using protein extracted from antisense plants, and the neomycin inhibition is expressed as percentage of PLD activity in the absence of neomycin. The activity at 100% was 1.81 nmol/min/mg. These assays used membrane-associated proteins solubilized with 0.44 M KCl. Values are means ± S.E. of three experiments.

**Fig. 5.** pH optima of PIP2-stimulated PLD activity in wild-type and antisense plants. MES/NaOH was used for the pH range from 5.0 to 7.5, and Tris/HCl for pH 6.5 to 9.0. Lines with open and filled circles represent antisense and wild-type plants, respectively. These assays used membrane-associated proteins solubilized with 0.44 M KCl. Values are means ± S.E. of two experiments.

The effect of Ca2+ on the conventional PLD was examined using a bacterially expressed PLD from a castor bean cDNA in this Ca2+-dependence study. The castor bean cDNA encodes a conventional PLD that has been shown to possess PIP2-independent activity in millimolar Ca2+ (12, 13). In the present assays containing PIP2, this PLD did not exhibit significant activity until the Ca2+ concentration approached 100 μM (Fig. 6). This Ca2+ activation profile for the expressed castor bean PLD corresponds to the second phase of Ca2+-dependence in the wild-type extracts and is distinct from that found in the antisense plants. The lack of this second phase of Ca2+-dependent activity in the antisense extracts is another indication that the expression of the conventional, PIP2-independent PLD has been suppressed.

Mg2+ caused no significant stimulation of PIP2-dependent PLD activity at any concentration tested (Fig. 6B). When tested at 200 mM Ca2+, at which there was little PLD activity, various Mg2+ levels were unable to stimulate PLD activity in either wild-type or antisense extracts. At 50 μM Ca2+, where the PIP2-dependent PLD activity was maximal, various levels of Mg2+ showed no significant stimulation of the PLD activity. On the other hand, Mg2+ above 2 mM caused a decrease in the PLD activity.

Separation of the PIP2-dependent PLD from the Conventional PLD—To establish whether or not there is a PIP2-dependent PLD isoenzyme separate from the conventional PLD, proteins from Arabidopsis were separated by nondenaturing PAGE and fractionated. PLD activities in gel fractions were assessed for the presence of the PIP2-stimulated and conventional PLDs. When assayed in the presence of PIP2, two peaks of PLD
activity were detected in the gel slices whereas only one peak of PIP2-independent, conventional activity was observed (Fig. 7A). The fast-migrating peak detected in the presence of PIP2 coincided with the conventional PLD peak and was greatly reduced in the antisense plants (data not shown). The conventional PLD has been shown to display some ability to hydrolyze PC in the presence of PIP2 and Ca2+ around the hundred micromolar concentration range (Fig. 6A), and the free Ca2+ level in the assay mixtures was estimated to be approximately 100 μM by use of a Ca2+-sensitive electrode. Thus, this fast-migrating peak is most likely caused by the conventional PLD. On the other hand, the slow-migrating PLD peak represented the PIP2-dependent PLD that was separated from the conventional PLD. In the assays containing PIP2 and 100 μM Ca2+, the PIP2-dependent PLD gave a level of activity (the slow peak) lower than that of the conventional PLD (the fast peak) because the PIP2-dependent PLD was much less stable than the conventional PLD. It was estimated that the recovery of the PIP2-dependent PLD activity after separation was about 5-fold less than that of the conventional activity. Other protein separation methods such as isoelectric focusing and size exclusion chromatography were also used to purify the PIP2-dependent PLD. However, the recovery of this activity was rather low, which hindered further purification of this enzyme. In another study (28), it has been found that the PIP2-dependent PLD requires cytosolic factors, which may explain the instability of this enzyme during purification.

The separation of the conventional and PIP2-dependent PLDs was further confirmed by immunoblot analysis of the proteins resolved by nondenaturing PAGE (Fig. 7B). A dominant band recognized by the antibodies generated against the C terminus of the conventional PLD was observed in the wild-type, but not in the PLD antisense plants (Fig. 7B, lanes 1 and 2). Furthermore, the protein recognized by the conventional PLD antiserum was cross-reacted with the authentic castor bean conventional PLD expressed from its cDNA in E. coli (Fig. 7B, lanes 2 and 3). The position of the immunoreactive band corresponded to that of the conventional PLD activity assayed in the gel slices. When a parallel blot was incubated with the antibodies directed against a newly cloned PIP2-dependent PLD (28), there were two positive bands near the top of the resolving gel, and those bands were observed in both the wild-type and PLD antisense plants (Fig. 7B, lanes 4 and 5). This location of PIP2-dependent PLD protein was in agreement with that of its activity (Fig. 7A). The observation that the PIP2-dependent antibodies and the conventional PLD antibodies recognized different protein bands demonstrates that the two PLDs are not cross-reacted by these antibodies. The finding of more than one band detected by the PIP2-dependent PLD
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antibodies suggests the presence of another unidentified PLD isoform that is more closely related to the PIP$_2$-dependent PLD than to the conventional PLD in *Arabidopsis*.

Unequivocal evidence for the existence of a distinct PLD isoenzyme that possesses PIP$_2$ dependent activity has been obtained recently. Two PLD cDNAs in *Arabidopsis* have been cloned based on their sequence similarity to the castor bean PLD cDNA (13, 14, 28). The two cDNAs gave deduced amino acid sequences with about 80 and 40% sequence identity, respectively, to the castor bean PLD. The cloned castor bean PLD is known to be the conventional plant PLD (12, 13). The *Arabidopsis* cDNA with the higher sequence identity to that of castor bean was used to produce the antisense transgene. The successful suppression of the PLD expression, as documented by the lack of millimolar Ca$^{2+}$-requiring PLD activity and proteins (Fig. 1), demonstrates that this cDNA encodes the conventional PLD. A catalytically active PLD has been expressed from the other cDNA, and this expressed protein has PIP$_2$-dependent, but not conventional, PLD activity. The biochemical properties of the expressed PLD are very similar to those of the PLD characterized from the antisense extracts (28).

**DISCUSSION**

In this study we first generated antisense transgenic *Arabidopsis* plants deficient in the conventional PLD. Given the different cellular roles that have been proposed for this enzyme, it was surprising that the PLD-deficient plants grew and developed to maturity without apparent morphological alterations. Further characterization of the PLD-deficient plants led to the identification of a novel PIP$_2$-dependent PLD activity which was distinct from the previously characterized plant PLD. Unlike the conventional PLD, whose maximal activity requires millimolar amounts of Ca$^{2+}$ (11, 12), the new PLD described here is fully active at micromolar concentrations of Ca$^{2+}$ (Fig. 6) and, moreover, requires PIP$_2$ for activity (Fig. 3).

Several lines of evidence support that the PIP$_2$-dependent PLD activity observed in the antisense plants results from an enzyme separate from the conventional PLD. When assayed for the conventional PLD activity, the antisense plants showed less than 3% of the activity found in the wild-type (Fig. 1). This indicates that the expression of the conventional PLD has been suppressed by introducing an antisense PLD gene. On the other hand, when assayed in the presence of PIP$_2$ and at low micromolar Ca$^{2+}$ concentrations (<50 $\mu$M), the level of PLD activity was virtually indistinguishable between the antisense and wild-type plants (Fig. 6A). These results show that a distinct PLD remains even after the removal of the conventional PLD. Moreover, the PIP$_2$-dependent PLD activity and protein can be physically separated from the conventional PLD (Fig. 7).

Finally, a new PLD cDNA has been cloned from *Arabidopsis*, which encodes a protein possessing PIP$_2$-dependent PLD activity (28). This provides molecular evidence for the presence of this enzyme in plants.

Because the conventional PLD has been suppressed in the antisense plants, the level and properties of PLD activity characterized in the antisense plants represent predominantly PIP$_2$-dependent PLD activity whereas those in the wild-type appear to be a mixture of the conventional and PIP$_2$-dependent activities. When assayed under the respective optimal conditions, the specific activity of the conventional PLD was about 100-fold higher than that of the PIP$_2$-dependent PLD (wild-type activity in Fig. 1 compared with the PLD activity of antisense plants in other figures). However, differences between the two assay conditions made it difficult to compare the two activities directly. A better comparison of the relative levels of the PIP$_2$-dependent and conventional PLDs in plants would come from examining these enzymes under identical conditions. When assayed in the presence of PIP$_2$ and 5 mM Ca$^{2+}$, the wild-type extracts showed a 3-fold higher level of activity than the antisense extracts (Fig. 6). These results indicate that the cellular level of the conventional PLD activity is at least severalfold higher than the PIP$_2$-dependent PLD activity. However, the two PLDs become active at very different Ca$^{2+}$ concentrations (Fig. 6A). The PIP$_2$ dependent activity is sensitive to the changes of Ca$^{2+}$ at nanomolar to low micromolar ranges, whereas the conventional PLD is responsive only to high micromolar to millimolar ranges of Ca$^{2+}$ under the same assay conditions. The distinct Ca$^{2+}$ dependence profiles of the PIP$_2$-dependent PLD and conventional PLD suggest that changing intracellular Ca$^{2+}$ could be an important regulator that differentially activates these PLDs.

The presence of the conventional PLD in wild-type extracts also gave rise to the property differences observed between the antisense and wild-type PLD when assayed in the presence of PIP$_2$. For example, the level of this PIP$_2$-regulated PLD activity in antisense plants was approximately 60% of the wild-type. The conventional PLD is the source of the increased activity observed in the wild-type extracts because it has been demonstrated that the conventional PLD displays the ability, albeit to a much lesser extent, to hydrolyze PC in the presence of PIP$_2$ and Ca$^{2+}$ concentrations around 100 $\mu$M. In addition, PLD in antisense plants was primarily associated with membrane whereas PLD in wild-type plants was present in both soluble and membrane fractions (Fig. 2; Table 1). The conventional PLD has been shown to be localized in both the soluble and membrane fractions (Fig. 1; Ref. 12). Finally, PLD from the antisense extracts had only one pH optimum whereas that in the wild-type extracts exhibited two pH optima (Fig. 5). The acidic peak, around pH 5.5 in wild-type extracts, corresponds to the pH optimum observed for the conventional PLD (11, 12).

The requirement for PIP$_2$ indicates a similarity of some plant PLDs to the cloned yeast and human PLDs, but some properties differ among the three sources. Both the plant and human PIP$_2$-dependent PLDs require physiological concentrations of Ca$^{2+}$ for activity (16), whereas no Ca$^{2+}$ requirement has been reported for the yeast PLD (2, 3, 17). On the other hand, the human PLD has been shown to be specific for PIP$_2$ (21), whereas this plant PLD activity was activated by PIP$_2$ and, to a lesser extent, by PIP. Activation by PIP$_2$ and PIP has also been shown with other plant enzymes such as the vanadate-sensitive ATPase in plasma membranes (20). It has been suggested that this PIP stimulation represents an evolutionary divergence of plant and animal inositol phospholipid regulation (29).

The need of greater than 0.1 mol % PIP$_2$ for the activation of this PLD raises the issue of whether or not such concentrations can be found in vivo. PIP$_2$ has been estimated to constitute not more than 0.05% of the total cellular phospholipids in plants, although there has not been a direct chemical determination of PIP$_2$ content in plant membranes (18). However, PIP$_2$ is likely to be more concentrated in the inner leaflet of plasma membranes. In addition, there is a possibility for the occurrence of PIP$_2$-enriched membrane microdomains, as the domains have been reported in mammalian cells (30). Furthermore, this new PLD is also stimulated by PIP. Therefore, it is possible that some membrane regions may have levels of polyphosphoinositides high enough to activate the PIP$_2$-dependent PLD.

The existence of the PIP$_2$-activated PLD activity strongly suggests that the production of PA may be a key event in the intracellular transduction of signals in plants. In addition to being an important precursor for second messengers, PIP$_2$ itself has been shown to modulate the function of various pro-
teins, which has been suggested to coordinate and control different cellular processes (19). In animal tissues the activation of PIP$_2$-regulated PLD requires active PIP$_2$ synthesis (31). PA, the phospholipid product of PLD hydrolysis, is able to promote PIP$_2$ synthesis via its ability to stimulate PIP 5-kinase. The membrane fusing property of PA and its ability to stimulate PIP$_2$ synthesis have led to proposed roles for PLD and PIP kinase in membrane trafficking (32). In this model, a positive feedback loop between PLD and PIP kinase would lead to a rapid synthesis of PA and PIP$_2$, which would contribute to the turnover of various stimuli (18). Moreover, some studies have found that increased PA production is associated with the turnover of polyphosphoinositides (8), indicating a networking of PLD activation with other signaling cascades.

In summary, the present study has identified a new type of plant PLD whose activity is PIP$_2$-dependent. The Ca$^{2+}$ dependence, pH optimum, and subcellular distribution indicate that the PIP$_2$-dependent PLD is regulated in a different manner from the previously characterized plant PLD. The distinct regulatory mechanisms suggest that these PLDs have different cellular functions. Studies involving the molecular characterization and genetic manipulation of the PIP$_2$-regulated PLD are underway. The transgenic antisense plants should serve as a useful system for sorting out the roles of the different PLDs in cellular signaling and metabolism.

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