A novel plant phospholipase D (PLD; EC 3.1.4.4) activity, which is dependent on phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and nanomolar concentrations of calcium, has been identified in Arabidopsis. This report describes the cloning, expression, and characterization of an Arabidopsis cDNA that encodes this PLD. We have designated names of PLD$\beta$ for this PIP$_2$-dependent PLD and PLD$\alpha$ for the previously characterized PIP$_2$-independent PLD that requires millimolar Ca$^{2+}$ for optimal activity. The PLD$\beta$ cDNA contains an open reading frame of 2904 nucleotides coding for a 968-amino acid protein of 108,575 daltons. Expression of this PLD$\beta$ cDNA clone in Escherichia coli results in the accumulation of a functional PLD having PLD$\beta$, but not PLD$\alpha$, activity. The activity of the expressed PLD$\beta$ is dependent on PIP$_2$ and submicromolar amounts of Ca$^{2+}$, inhibited by neomycin, and stimulated by a soluble factor from plant extracts. Sequence analysis reveals that PLD$\beta$ is evolutionarily divergent from PLD$\alpha$ and that its N terminus contains a regulatory Ca$^{2+}$-dependent phospholipid-binding (C2) domain that is found in a number of signal transducing and membrane trafficking proteins.

Phospholipase D (PLD; EC 3.1.4.4)$^1$-catalyzed hydrolysis of glycerophospholipids produces phosphatidic acid (PA) and a hydrophilic constituent. This activity was first identified in plants and since has been found in animals and microorganisms. PLD in plants was originally proposed to be important in phospholipid catabolism, initiating a lipolytic cascade in membrane deterioration during senescence and stress injuries (1, 2). Recent studies in plants, animals, and yeast indicate that PLD hydrolysis plays a pivotal role in transmembrane signaling and cellular regulation (3–9). Activation of PLD has been proposed to mediate many cellular processes including cell proliferation, membrane trafficking, meiosis, and responses to external and internal stimuli. It has been suggested that multiple forms of PLD are involved in these diverse cellular processes since several studies have shown the presence of PLD variants that are expressed differently (9–12). In castor bean (9) and rice (12), one PLD variant is constitutive whereas the appearance of other variants is associated with specific conditions such as rapid growth, wounding, and senescence. A distinct property shared by these variants is their in vitro requirement of millimolar Ca$^{2+}$ concentrations for optimal activity. Further analyses of the castor bean PLD variants have led to the suggestion that the catalytic activity of these variants results from the same gene product (9–11).

A recent study has provided important evidence for the presence of two plant PLDs that are derived from different gene products and regulated distinctly (13). One PLD requires polyphosphoinositides and submicromolar concentrations of Ca$^{2+}$ for activity and the other is PIP$_2$-independent and is most active in the presence of millimolar amounts of Ca$^{2+}$. The latter is the prevalent form of PLD that has been purified and characterized from a number of plant species (14). Its cDNA has been recently cloned from castor bean (15), Arabidopsis (16), rice and maize (17). We have genetically suppressed the expression of this prevalent plant PLD by introducing a PLD antisense gene into Arabidopsis (13). While they showed less than 3% of the PIP$_2$-independent, millimolar Ca$^{2+}$-requiring PLD activity of wild-type Arabidopsis, the transgenic plants had PIP$_2$-dependent PLD activity comparable to that of wild type at submicromolar calcium. In the present study, we provide molecular evidence for the presence of two distinct PLDs by isolating a new PLD cDNA encoding the PIP$_2$-dependent PLD. Furthermore, analysis of the sequence and expressed protein from the PLD cDNA gives further insights to the activation and function of PLD$\beta$ in plants. Because the regulatory and structural features of the newly identified PLD$\beta$ are distinct from those of the conventional PLD, we have given names of PLD$\beta$ for this PIP$_2$-dependent PLD and PLD$\alpha$ for the previously characterized PIP$_2$-independent PLD that requires millimolar Ca$^{2+}$ for optimal activity.

**EXPERIMENTAL PROCEDURES**

Materials—PIP and PIP$_2$ were obtained from Boehringer Mannheim. Phosphatidylethanol, PI, and PE were purchased from Avanti Polar Lipids. All other phospholipids were obtained from Sigma. 1-Palmitoyl-2-oleoyl-1'-acyl-[g]lycerol-(3) and dipalmitoylglycerol-3-phosphate-[methyl-$^3$H]cholesterol were from DuPont NEN. Silica Gel 60 TLC plates were obtained from Merck (Darmstadt, Germany). PLD cDNA Cloning and Sequencing—Putative expression sequence-tagged Arabidopsis PLD cDNAs were identified by searching the BLAST data base against the castor bean PLD cDNA sequence. These clones were kindly provided by the Ohio State University Arabidopsis Information Center. Strategies for isolating full-length PLD cDNAs are described in the text. The reactions for PCR amplification used DNA purified from an Arabidopsis PRL2 cDNA library (18) as DNA template.
T7 sequence primer as the 5′ primer, and 28 nucleotide bases corresponding to the near 5′ end of the EST cDNA sequence as the 3′ primer. The reaction mixture consisted of 50 pmol of each primer, 0.5 μg of template DNA, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton, 2.5 mM MgCl₂, 0.2 μl of each dNTP, 1 unit of Taq DNA polymerase in a 100 μl volume. The thermal cycling program was an initial denaturing cycle of 5 min at 95 °C. Then 25 to 30 cycles were completed using the following temperature profiles: denaturation at 95 °C for 1 min, annealing for 30 s at 2–5 °C lower than the calculated primer Tₘ, and extension for 1 min at 72 °C. PCR products were cloned into the pGEM-T vector (Promega) according to the manufacturer’s instructions (15).

RACE for 5′-cDNA ends was performed according to the manufacturer’s instructions (Life Technologies, Inc.). The first strand cDNA was synthesized from total RNA isolated from Arabidopsis flowers. After PCR amplification using nested gene-specific primers at the 3′ end and a 5′-RACE anchor primer, the DNA products were cut with KpnI and PsrI and were ligated into pBluescript (SK). The KpnI site was engineered into the 5′ primer, and the PsrI was an internal site of the PLD cDNA near the 3′ end of the RACE product. To isolate full-length PLD cDNAs a ZapII cDNA library, constructed from 3 to 6 kb mRNA isolated from hypocotyls of 3-day-old seedlings, was screened using the 5′ cDNA fragment generated by the 5′-RACE procedure. The hybridization was conducted at 65 °C, and the subsequent DNA manipulation of the positive clones was based on the previously described procedures (15).

To sequence PLD clones, cDNA inserts from positive clones were digested with various restriction enzymes and the fragments were subcloned into the pBluescript plasmids, SK and/or KS. The complete DNA sequence was determined by using the Sequenase 2 kit according to the manufacturer’s instructions (U. S. Biochemical Corp.). Vector pBluescript-based primers, universal forward and reverse, T3, T7, SK, and KS primers were used in most sequencing reactions. PLD cDNA-based primers were also synthesized for clarifying ambiguities. The final sequence was determined from both strands. Phylogenetic analyses, pI calculations, and comparison of PLD nucleic acid and amino acid sequences were done with the Genetics Computer Group software (University of Wisconsin).

**Expression of PLD cDNA in Escherichia coli**—Expression of the PLD gene was performed using pBluescript SK(−) containing the cDNA insert in E. coli JM109. Fifty microliters of an overnight culture of the transconjugant strain were added to 25 ml of LB medium with 50 μg/ml ampicillin. The cells were incubated at 37 °C with shaking for 3 h, and then 25°C and 2 ml was added to a final concentration of 2 μg/ml of chloramphenicol. The cultures were grown overnight at 30 °C, the induced cells were pelleted by centrifugation and then resuspended in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.25 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, and 2 mM dithiothreitol. The cytosolic protein fraction was the supernatant obtained after centrifugation at 10,000 × g for 5 min. Proteins in the supernatant were assayed for PLD activity, and then the soluble fraction was boiled for 5 min followed by centrifugation at 10,000 × g for 5 min. The pellet was extracted with 0.4 M KCl in the homogenization buffer to obtain the salt-solubilized membrane proteins (13). In typical PLD assays, the cytosol containing 3 μg of protein was added to 10 μg of the bacterially expressed PLDβ. In the heat denaturation treatment, the soluble fraction was boiled for 5 min followed by centrifugation for 5 min at 12,000 × g to remove precipitates. The clarified fraction was used directly or treated with different proteases, thermolysin, trypsin, or proteinase K, to digest proteins. After incubating at 37 °C for 30 min, trypsin and proteinase K were inactivated by adding phenylmethylsulfonyl fluoride, and thermolysin was inactivated by adding 2 mM EDTA.

**Southern and Northern Blotting**—Total RNA and genomic DNA were isolated from Arabidopsis tissues (15). Full-length cDNAs of PLDα and PLDβ were used as probes to hybridize the genomic DNA digested with various restriction enzymes at 65 °C under the previously described conditions (15). Total RNA was separated by denaturing formaldehyde-agarose gel electrophoresis, transferred onto a nylon membrane, and hybridized with a full-length PLDβ cDNA at 65 °C (15).

**RESULTS**

**Cloning and Sequences of PLD cDNAs**—Arabidopsis expression sequence-tagged (EST) cDNA clones were identified as putative PLD cDNAs by searching the BLAST data base using the castor bean PLD cDNA sequence. These clones were about 1 kb in length and incomplete PLD cDNAs. The first complete Arabidopsis PLD cDNA was cloned by PCR using nested primers and encodes a protein of 809 amino acids (16). This cDNA has been shown to be PLDα, because introduction of this cDNA as an antisense gene completely abolishes PLD activity in transgenic plants. The antisense plants lost the millimolar Ca²⁺-dependent PLD activity, and exhibited reduced PIP₁-dependent PLD activity comparable to that of wild-type plants at nanomolar Ca²⁺ concentrations (13). In addition, the amino acid sequence of the Arabidopsis PLD cDNA shares a high level of identity (about 80%) with the previously cloned castor bean PLD cDNA whose product displays PIP₁-dependent activity. The cloning of PLDβ cDNA proved to be much more difficult.

1. **Cloning Phosphoinositide-regulated Phospholipase D in Plants**
2. **Generation of PLD Antibodies and Immunoblotting**
3. **Expression of PLD cDNA**
4. **Results**
5. **Discussion**

**Materials and Methods**

**Expression of PLD cDNA**

**Results**

**Discussion**
than that of PLDo. The cloning strategies involved nested PCR, 5'-RACE, and screening of cDNA libraries. A 1.5-kb 5'-fragment of the cDNA was first cloned by PCR amplification using an Arabidopsis PRL2 cDNA library (18) as a DNA template, and screening of cDNA libraries. A 1.5-kb 5'-end fragment of the EST cDNA as the 3'-fragments were not fruitful, so 5'-end sequences have GenBank accession number U36381 and U84568, respectively.

**FIG. 1. Amino acid sequence comparison between PLDj and PLDo from Arabidopsis.**—a) and b) indicate identical and similar amino acids, respectively. Duplicated catalytic HKD motifs are shown by a double asterisk (*). The newly cloned PLD cDNA consists of 3309 nucleotides in size and pI. PLDj from different plant species are very similar in size, ranging from 92 to 96 kDa, whereas the predicted polypeptide from this cDNA has a calculated molecular mass of 109 kDa. This PLD protein consists of 98 amino acids, which is 148 amino acids longer at the N terminus than that of Arabidopsis PLDo (Fig. 1). The newly cloned PLD has a calculated pI of 7.9 whereas all previously cloned plant PLDs display acidic pI values (15–17). Prior to this report, only PLDs from non-plant sources had been reported to have basic pI values (4, 24). This newly cloned cDNA contains a duplicated HXXXXXD motif (amino acids 481–488), which has been conserved in all PLDs and is proposed to be involved in catalysis (24, 25). Additionally, the newly cloned PLD differs from PLDo in size and pI. PLDj from different plant species are very similar in size, ranging from 92 to 96 kDa, whereas the predicted polypeptide from this cDNA has a calculated molecular mass of 109 kDa. This PLD protein consists of 98 amino acids, which is 148 amino acids longer at the N terminus than that of Arabidopsis PLDo (Fig. 1). The newly cloned PLD has a calculated pI of 7.9 whereas all previously cloned plant PLDs display acidic pI values (15–17). Prior to this report, only PLDs from non-plant sources had been reported to have basic pI values (4, 24). This newly cloned cDNA contains a duplicated HXXXXXD motif (amino acids 481–488), which has been conserved in all PLDs and is proposed to be involved in catalysis (24, 25). These results indicate that the newly cloned PLD is a distinct isof orm.

Establishing That the cDNA Encodes a New PIP2-dependent PLDj—To establish unequivocally that the newly cloned cDNA encodes a PLD, protein from this cDNA was expressed in E. coli using pBluescript SK(-) as an expression vector. After IPTG induction, the production of a protein encoded by the cDNA was detected by immunoblotting using antibodies raised against a synthetic peptide corresponding to the C-terminal amino acids of this protein (Fig. 2). No immunoreactive proteins were detected in the protein extracts from E. coli containing vector alone, and a trace amount of PLD was expressed without IPTG induction in the SK construct.

The expressed protein was assayed for both PIP2-dependent and PIP2-independent PLD activity. There were only trace levels of PLD activities in protein extracts from E. coli harboring the SK alone or the vector containing the cDNA insert without IPTG induction. A significant increase in PIP2-dependent PLD activity was observed after IPTG induction (Fig. 2). The levels of PLD were in agreement with the presence or absence of PLD protein detected by immunoblotting using antibodies raised against a synthetic peptide corresponding to the C-terminal amino acids of this protein (Fig. 2). No immunoreactive proteins were detected in the protein extracts from E. coli containing vector alone, and a trace amount of PLD was expressed without IPTG induction in the SK construct.

The expressed protein was assayed for both PIP2-dependent and PIP2-independent PLD activity. There were only trace levels of PLD activities in protein extracts from E. coli harboring the SK alone or the vector containing the cDNA insert without IPTG induction. A significant increase in PIP2-dependent PLD activity was observed after IPTG induction (Fig. 2). The levels of PLD were in agreement with the presence or absence of PLD protein detected by immunoblotting using antibodies raised against a synthetic peptide corresponding to the C-terminal amino acids of this protein (Fig. 2). No immunoreactive proteins were detected in the protein extracts from E. coli containing vector alone, and a trace amount of PLD was expressed without IPTG induction in the SK construct.
to the uninduced samples, showing that the expressed protein is indeed a member of the PLD family.

Stimulation of PLD by a Cytosolic Factor—Recent studies have shown that some mammalian PLDs are stimulated by cytosolic factors (22, 24). To examine whether PLDB could be activated by plant soluble factors, the expressed PLDB was assayed in the presence or absence of a soluble fraction from Arabidopsis. The soluble extract was obtained from transgenic Arabidopsis plants in which the expression of PLDA was antisense suppressed. These plants were used because the 100,000 g supernatant contained virtually no detectable PIP2-dependent nor PIP2-independent PLD activity (13). The soluble extract alone showed little PLD activity (Fig. 3A), but its inclusion increased the PIP2-dependent activity of the expressed protein and this enhancement was dependent upon the concentration of cytosol added (Fig. 3B). These results suggest that the PIP2-dependent PLD expressed in E. coli is stimulated by a soluble factor. In comparison, the cytosolic fraction had no stimulatory effect on PLDA expressed from its cDNA in E. coli (data not shown). Similarly, the PIP2-dependent PLD extracted from the PLDA antisense membranes was insensitive to the addition of cytosol (Fig. 3B). It is possible that the cytosolic stimulator remains bound with the membrane-associated PLD from the antisense plants.

The cytosolic factor was examined by various means to determine its nature. The cloned human PLD is stimulated by small GTP-binding proteins of the ADP-ribosylation factor (ARF) family (24). We tested the activity dependence of various enzymes by it ability to sequester PIP2 (27, 28). PLD was inhibited by greater than 50% at 1 mM neomycin and was nearly abolished at 2 mM neomycin. To determine the influence of Ca2+ and Mg2+ in the reaction mixture were controlled using Ca2+/Mg2+-EGTA buffers at pH 7.5 (23). PIP2-dependent activity was undetectable in the absence of Ca2+, with little activity observed at or below a concentration of 50 nM (Fig. 4D). At 500 nM calcium, PLD activity increased to a maximum and gradually tapered off as millimolar levels of calcium were approached. Under the optimal PIP2 and Ca2+ conditions, the expressed PIP2-dependent PLD showed the highest activity between pH 7.0 and 7.5.

Presence of a Ca2+/Phospholipid-binding (C2) Domain in PLD—PLDA from castor bean was reported to contain a C2 domain at its N terminus (29). The C2 domain is a Ca2+/phospholipid-binding domain present in a number of different proteins involved in signal transduction and membrane trafficking (29, 30). The three-dimensional structure of a C2 domain from the neuronal protein synaptotagmin has been resolved recently by x-ray crystallography and NMR (30, 31). The
FIG. 4. Catalytic properties of PLDβ expressed in E. coli. A, effects of phosphoinositides and other phospholipids on PIP2-stimulated PLD activity. Lipid vesicles (0.4 mM) in the reaction mixture were composed of 87 mol% PE, 7.6 mol% PIP2, and 5.4 mol% PC. PIP2 was replaced with 7.6 mol% PE, PA, PG, PS, or PIP. B, PIP2-stimulated PLD activity as a function of mole% PIP2 in lipid vesicle. Lipid vesicles (0.4 mM) in the reaction mixture consisted of 79.4–94.6 mol% PE, 0–15.2 mol% PIP2, and 5.4 mol% PC. C, neomycin inhibition of PLD activity. PLD was assayed in the presence of 7.6 mol% PIP2 and 0–2 mM neomycin. D, dependence of PLDβ activity on Ca2+. Free Ca2+ and Mg2+ in reaction mixtures were controlled using Ca2+/Mg2+-EGTA buffers at pH 7.5. PLDβ was expressed from its cDNA in E. coli, and each assay used 10 μg of soluble protein containing PLDβ and 3 μg of cytosol proteins from PLDa antisense Arabidopsis. Values are means ± S.E. of three experiments.

FIG. 5. Multiple sequence alignment of the C2 domain. PLDβ and PLDa C2 domain sequences were aligned to the experimentally determined structure of rat synaptotagmin (30). The dashed arrow lines indicate β-sheet structure. Sequences intervening between secondary structural elements are represented by numbers in parentheses. The underlined residues show the positions corresponding to the acidic residues involved in Ca2+ binding in the C2 of synaptotagmin. Syn1, rat synaptotagmin; or, arabidopsis; cb, castor bean.

Crystal structure of a phosphoinositide-specific phospholipase C, which also contains a C2 domain, has been reported recently (32). The C2 domains of synaptotagmin and PLC are comprised of eight-strand sandwich containing 4–5 acidic residues involved in Ca2+ binding. While the eight strands are conserved, the PLDa from castor bean and Arabidopsis possess substitutions within the C2 Ca2+-binding site, indicating a potential loss of Ca2+ affinity (29) (Fig. 5).

An approach similar to that described previously (29) was used to align the sequence of PLDβ with those of synaptotagmin and PLDa. The PIP2-dependent PLDβ contains a C2 domain near its N terminus stretching from amino acid 158 to 279 (Fig. 5). The two most highly conserved segments in different C2-containing proteins are PYV and NPVFNE (30). These two regions have been proposed to maintain the structural integrity of the C2 fold because their residues are largely hydrophobic. In PLDβ the first segment is completely conserved and the second segment, NPVWMQHP, is largely the same with several conservative amino acid substitutions. Furthermore, PLDβ contains the conserved acidic residues (underlined in Fig. 5) that serve to coordinate Ca2+-binding in the C2 domain. This is in contrast to PLDa in which two of the acidic residues are substituted with positively charged or neutral amino acids.

Genomic Organization of PLDβ and PLDa—The molecular organization of PLDβ and PLDa in the Arabidopsis genome was examined by Southern blotting analysis (Fig. 6, A and B). Total genomic DNA was digested with restriction enzymes and hybridized with probes made from full-length PLDβ and PLDa cDNAs. Hybridization of the same DNA with PLDa or PLDβ probes gave unique banding patterns, indicating that the PLDβ and PLDa sequences do not cross-hybridize with each other at high stringency conditions. The PLDβ cDNA has one XhoI site, but no BamHI, KpnI, and XhoI restriction sites, and the digested genomic DNA gave one strong hybridization band (Fig. 6A). The simple banding pattern of hybridization by the PLDβ cDNA indicates that the Arabidopsis genome may contain one gene copy of PLDa. When the same DNA was probed with PLDβ, which contains one KpnI and two BamHI recognition sites, but no XhoI nor XhoI site, the number of hybridization bands was more than that predicted from the BamHI, KpnI, and XhoI digestion of PLDβ cDNA (Fig. 6B). These results could be caused by the presence of these restriction sites in intron sequences of the PLDβ gene and/or by the presence of intron sequences of the PLDα gene and/or by the presence of intron sequences of the PLDβ gene and/or by the presence of intron sequences of the PLDα gene.
another or closely related PLDβ gene. Northern blot analysis using the full-length PLDβ cDNA as a probe detected one RNA band of approximately 3.5 kb (Fig. 6C). The estimated size was in agreement with that of the cloned PLDβ cDNA. However, it was unclear if this band was composed of one PLDβ mRNA species or different PLD transcripts with similar sizes. It is worth noting that another study has shown that two protein bands from Arabidopsis extracts were recognized by the antibodies raised against a PLDβ peptide, suggesting the presence of other PLD(s) closely related to the cloned PLDβ in Arabidopsis (13).

**DISCUSSION**

The present results provide molecular evidence for the presence of two plant PLD isoforms that are distinctly regulated and expressed. PLDα, which was previously cloned and characterized from several plant species, requires millimolar Ca²⁺, but no PIP₂ for activity. Our study involving antisense suppression of the PLDα gene in Arabidopsis has unmasked the presence of a PIP₂-regulated PLD in plants. In this study, we have cloned and functionally expressed the PIP₂-dependent PLD which is designated PLDβ. The biochemical properties of the PIP₂-dependent PLD expressed from the PLDβ cDNA are almost the same as those identified in Arabidopsis protein extracts (13). Specifically, the PIP₂ requirement by the PLD from the plant extracts and the cDNA expressed in E. coli can be partially substituted by PIP, but not by PI, PS, PG, PE, or PA. The optimal pH for the PIP₂-dependent PLD from both sources is around 7 to 7.5. The PLD obtained from both sources requires Ca²⁺ and is fully active at submicromolar ranges of Ca²⁺. These similarities suggest that the cloned PLD is the isoform responsible for the PIP₂-dependent activity measured in the extracts of Arabidopsis.

The only difference between the two enzymes appears in the Ca²⁺-effect at higher concentrations; the activity from PLD expressed in E. coli decreased at millimolar Ca²⁺ whereas the PLD examined in the PLDα-antisense plants showed a sigmoidal response to the increase of Ca²⁺ concentration. However, the plant extract contains more than one type of PLD, and PLDα is known to be most active at millimolar Ca²⁺. Therefore, the stable PLD activity observed in millimolar Ca²⁺ could result from residual PLDα or from any other PLD that is stimulated by millimolar Ca²⁺ and not suppressed by PLDα antisense.

The ability of PLDβ to shift from an inactive state to a highly active state over a narrow range of calcium concentration strongly suggests that changing intracellular concentrations could be a major form of regulation for the enzyme in vivo. This observation is particularly relevant considering that 100 nM and 1 μM are the respective resting and stimulated intracellular calcium concentrations of plants and animals (33, 34). It remains to be elucidated how Ca²⁺ is involved in the PLD-mediated hydrolysis of phospholipids. The finding of a C2 domain near the N-terminal region of several PLDs suggests that one of the roles of Ca²⁺ may be to regulate the enzymes’ binding to phospholipids. The predominant feature of the C2 domain is its ability to mediate Ca²⁺-dependent phospholipid binding. The Ca²⁺/phospholipid-binding domain was first identified in Ca²⁺-dependent protein kinase C isoforms and has since been found in a number of different proteins including intracellular PLA₂ and PIP₂-PLC isoforms. It is believed that the binding of membrane phospholipids by a C2 domain represents a Ca²⁺-dependent translocation mechanism whereby cytosolic proteins become associated with the membrane in a highly regulated manner. Thus for some C2-containing enzymes, phospholipid binding could represent one mechanism of cellular activation. The presence of a C2 domain in PLDs raises the question of whether Ca²⁺-dependent phospholipid binding is involved in the enzyme’s activation, catalysis, or both. Comparison of the C2 domains of PLDs and PLDβ reveals an important difference. The PLDβ C2 domain conserves the acidic residues needed to coordinate Ca²⁺ binding whereas the PLDα C2 domain possesses substitutions, potentially indicating a loss of Ca²⁺ affinity. The difference in the amount of Ca²⁺ needed for activity is one of the most distinct in vitro properties that distinguishes between PLDα and PLDβ. PLDα requires millimolar amounts of Ca²⁺ whereas PLDβ is fully active at low micromolar levels of Ca²⁺ (13). An ongoing study in this laboratory is to determine whether or not the differences in the C2 domain underlie the different Ca²⁺ requirements observed for PLDα and PLDβ. Such studies should help understand the regulatory and catalytic mechanisms for these PLD isoforms.

Sequence analysis indicates that PLDβ and PLDα are evolutionarily divergent and that PLDβ is more closely related to the PLDs cloned from yeast (4) and human (24) than is PLDα. Alignments of these PLD sequences reveals two distinct groups: PLDs from plants and those from human and yeast. Within the plant group PLDβ forms a subgroup distinct from that of PLDα from Arabidopsis, castor bean, maize, and rice. The grouping of PLDs from Arabidopsis, castor bean, maize, and rice suggests that these are more closely related evolutionarily. Phylogram analysis in the unrooted phylogeny places the Arabidopsis PLDβ with the yeast and human PLDs. Furthermore, the phylogenic groupings are supported by comparing the calculated pI values and catalytic properties of these PLDs. PLDα of different plant species all have acidic pI values around 5–6 whereas PLDβ and the yeast and human PLDs have basic pI values of 7.9, 7.6, and 9.3, respectively. PLDα activity is PIP₂-independent and requires millimolar concentrations of Ca²⁺ whereas PLDβ and the cloned PLDs from human and yeast are activated by PIP₂. Both PLDβ and the human PIP₂-dependent PLD are regulated by physiological concentrations of Ca²⁺ and cytosolic factors.

These analyses have clearly shown that there are PLD isoforms in plants that are encoded by different genes and are regulated in a distinct manner. The distinct regulatory mechanisms suggest that these PLDs have different cellular functions. PLDα is the most prevalent plant PLD (13) and it seems

**Fig. 6. Southern blot of PLDβ and PLDα in the Arabidopsis genome and Northern blot analysis of PLDβ.** A and B, autoradiogram of Southern analysis of genomic DNA isolated from Arabidopsis leaves, digested with BamHI (Ba), KpnI (Kp), XhoI (Xh), and HindIII (Xh), and probed with PLDα (A) and PLDβ (B) cDNAs at 65°C. C, autoradiogram of Northern blot analysis of PLDβ mRNA. Total RNA from Arabidopsis leaves (lane 1) and roots (lane 2) was probed with the full-length PLDβ cDNA at 65°C.
to be unique to plants based on the comparison of its catalytic properties with those of mammalian and yeast PLDs. PLDβ, on the other hand, shares some properties with the recently cloned human and yeast PLDs. PLDβ, on the other hand, shares some properties with the recently cloned human and yeast PLDs. It has been shown that the yeast PLD is required for meiosis (4–6). The cloned human PLD is thought to be involved in membrane trafficking and secretion but its role in these processes is unclear (3, 24). We have produced PLDα-suppressed transgenic plants that should be instrumental in defining the function of PLDα. Efforts are underway to produce plants deficient in PLDβ activity and to use these systems to sort out the roles of the different PLDs in growth and development.

Acknowledgment—We thank Dr. A. J. Morris for providing us the recombinant human PLD and ADP-ribosylation factor.

REFERENCES

1. Kates, M. (1954) Can. J. Biochem. Physiol. 32, 571–583
2. Paliyath, G., Lynch, D. V., and Thompson, J. E. (1987) Physiol. Plant. (Sofia) 71, 503–511
3. Exton, J. H. (1994) Biochim. Biophys. Acta 1212, 26–42
4. Rose, K., Rudge, S., Frohman, M. A., Morris, A. J., and Engebrecht, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12151–12155
5. Ella, K. M., Dolan, J. W., Chen, Q., and Meier, K. E. (1996) Biochem. J. 314, 15–19
6. Wang, X. (1993) in Lipid Metabolism in Plants (Moore, T. S., ed) pp. 499–520 CRC Press, Boca Raton, FL
7. Munnik, T., Arisz, S. A., de Vrije, T., and Musgrave, A. (1995) Plant Cell 7, 2197–2210
8. Dyer, J. H. Ryu, S. B., and Wang X. (1994) Plant Physiol. 105, 715–724
9. Dyer, J. H., Zheng, S., and Wang, X. (1996) Biophys. Biochem. Res. Commun. 221, 31–36
10. Ryu, S. B., and Wang, X. (1995) Plant Physiol. 108, 713–719
11. Young, S. A., Wang, X., and Leach, J. E. (1996) Plant Cell 8, 1079–1090
12. Pappan, K., Zheng, S., and Wang, X. (1997) J. Biol. Chem. 272, 7048-7054
13. Heller, M. (1978) Adv. Lipid Res. 16, 267–276
14. Wang, X., Xu, L., and Zheng, L. (1994) J. Biol. Chem. 269, 20312–20317
15. Dyer, J. H., Zheng, L., and Wang, X. (1995) Plant Physiol. 109, 1497
16. Ueki, J., Moriya, K., Komari, T., and Kumashiro, T. (1995) Plant Cell Physiol. 36, 903–914
17. Newman, T., de Bruijn, F. J., Green, P., Keegstra, K., Kende, Hans, McIntosh, L., Ohlrogge, J., Raikhel, N., Somerville, S., Thomashow, M., Retzel, E., and Somerville, C. (1994) Plant Physiol. 106, 1241–1255
18. Kieber, J. J., Rothenberg, M., Roman, G., Feldmann, K. A., and Ecker, J. R. (1993) Cell 72, 427–441
19. Nivison, H. T., and Hanson, M. R. (1987) Plant Mol. Biol. Report 5, 295–309
20. Wang, X., Dyer, J. H., and Zheng, L. (1993) Arch. Biochem. Biophys. 306, 496–494
21. Brown, H. A., Gutowski, S., Mosnow, C. R., Slaughter, C., and Sternweiss, P. C. (1993) Cell 75, 1137–1144
22. Tsien, R., and Puszpan, T. (1989) Methods Enzymol. 172, 230–282
23. Hammond, S. M., Altshuller, Y. M., Sung, T-C., Rudge, S. A., Rose, K., Engebrecht, J., Morris, A. J., and Frohman, M. A. (1995) J. Biol. Chem. 270, 29640–29643
24. Ponting, C. P., and Kerr, I. D. (1996) Protein Sci. 5, 914–922
25. Regad, F., Burtol, C., Tremousayguy, D., Maisan, A., Lescluze, B., and Axell, M. (1993) FEBS Lett. 316, 133–136
26. Schacht, J. (1978) J. Lipid Res. 19, 1063–1067
27. Gabey, E., Kasianowicz, J., Abbott, T., and McLaughlin, S. (1989) Biochim. Biophys. Acta 970, 105–112
28. Ponting, C. P., and Parker, P. J. (1996) Protein Sci. 5, 162–166
29. Sutton, R. B., Davletov, B. A., Berghuis, A. M., Sudhof, T. C., and Sprang, S. R. (1996) Cell 80, 929–938
30. Shao, X., Davletov, B. A., Sutton, R. B., Sudhof, T. C., and Rizo, J. (1996) Science 273, 248–251
31. Essen, L., Perisic, O., Cheung, R., Kata, M., and Williams, R. L. (1996) Nature 380, 595–602
32. Clapham, D. E. (1996) Cell 86, 259–268