The Arabidopsis Phospholipase D Family. Characterization of a Calcium-Independent and Phosphatidylcholine-Selective PLDζ1 with Distinct Regulatory Domains

Chunbo Qin and Xuemin Wang*
Department of Biochemistry, Kansas State University, Manhattan, Kansas 66506

Four types of phospholipase D (PLD), PLDα, β, γ, and δ, have been characterized in Arabidopsis, and they display different requirements for Ca^{2+}, phosphatidylinositol 4,5-bisphosphate (PIP_2), substrate vesicle composition, and/or free fatty acids. However, all previously cloned plant PLDs contain a Ca^{2+}-dependent phospholipid-binding C2 domain and require Ca^{2+} for activity. This study documents a new type of PLD, PLDζ1, which is distinctively different from previously characterized PLDs. It contains at the N terminus a Phox homology domain and a pleckstrin homology domain, but not the C2 domain. A full-length cDNA for Arabidopsis PLDζ1 has been identified and used to express catalytically active PLD in Escherichia coli. PLDζ1 does not require Ca^{2+} or any other divalent cation for activity. In addition, it selectively hydrolyzes phosphatidylcholine, whereas the other Arabidopsis PLDs use several phospholipids as substrates. PLDζ1 requires PIP_2 for activity, but unlike the PIP_2-requiring PLDδ or γ, phosphatidylethanolamine is not needed in substrate vesicles. These differences are described, together with a genomic analysis of 12 putative Arabidopsis PLD genes that are grouped into α, β, δ, γ, and ζ based on their gene architectures, sequence similarities, domain structures, and biochemical properties.

Phospholipase D (PLD) is a prevalent family of phospholipases in plant tissues, and it cleaves phospholipids, producing phosphatidic acid and a free head group such as choline (Wang, 2000). PLD was first identified in plants more than 50 years ago (Hanahan and Chaikoff, 1947). It did not receive widespread attention in other organisms until the 1980s when PLD was revealed to be activated by external stimuli and was later recognized as a lipid-signaling enzyme together with phospholipase A2, phospholipase C, and sphingomyelinase (Cockcroft, 1984; Bocckino et al., 1987). Since the first cloning of a PLD cDNA from castor bean (Ricinus communis; Wang et al., 1994), understanding of PLDs at the molecular, biochemical, and cellular levels has since advanced greatly in plants, animals, and fungi (Frohman et al., 1999; Liscovitch et al., 2000; Wang, 2000; Munnik, 2001). PLD has been proposed to play a pivotal role in many cellular processes such as signal transduction, membrane trafficking, cytoskeletal re-arrangements, and membrane degradation.

Multiple PLDs have been identified in plants. Four types of Arabidopsis PLDs, PLDα, β, γ, and δ, have been characterized at the molecular biological and biochemical levels (Pappan et al., 1997a, 1997b, 1998; Qin et al., 1997; Wang and Wang, 2001). PLDα represents the conventional plant PLD, which does not require phosphoinositides for activity when assayed at millimolar levels of Ca^{2+} (Pappan and Wang, 1999). In contrast, PLD β and γ are phosphatidylinositol 4,5-bisphosphate (PIP_2) dependent and have maximum activity at micromolar levels of Ca^{2+} (Pappan et al., 1997a; Qin et al., 1997). Recently identified PLDδ displays unique biochemical properties; it is activated by free oleic acid and is tightly associated with the plasma membrane (Wang and Wang, 2001).

Despite many differences in the biochemical properties, all of the previously cloned plant PLDs contain a Ca^{2+}-dependent phospholipid-binding C2 domain (protein kinase C-conserved 2 domain) and require Ca^{2+} for activity (Wang, 2000). In addition, they have broad substrate specificity, hydrolyzing several common membrane phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidyl-Ser (PS; Pappan et al., 1998). This study has identified a new type of PLD, PLDζ1, which is independent of Ca^{2+} and selectively hydrolyzes PC. PLDζ1 contains a Phox homology (PX) domain and a pleckstrin homology (PH) domain, but not the C2 domain. The different biochemical properties and domain structures are described, together with a genomic analysis of the evolutionary relationships, sequence similarities, and gene architectures of 12 PLD genes in Arabidopsis.
RESULTS AND DISCUSSION

Genomic Organization and Grouping of the Arabidopsis PLD Gene Family

Twelve PLD genes were identified in the Arabidopsis genome by BLAST searching against the cloned cDNAs. Two of these genes are located on chromosome I, one on chromosome II, three on chromosome III, five on chromosome IV, and one on chromosome V (Fig. 1; Table I). They are grouped into five classes, $\text{PLD}_1$ and $\alpha_2$ are very similar in terms of gene structure and sequence similarity. Their deduced amino acid sequences share about 92% similarity (Table II). Comparison of the cloned PLD cDNAs of castor bean and Arabidopsis PLDa1 with their genomic sequences has shown the presence of an intron in the 5′-untranslated region of PLDa1 (Xu et al., 1997). Arabidopsis EST database searches against these two genes revealed a number of EST clones corresponding to $\alpha_1$, but none for $\alpha_2$ (Table I), indicating that they might differ greatly in abundance despite their high degree of sequence similarity. The complete cDNA for PLDa1 has been isolated (Dyer et al., 1995), and its nucleotide and deduced amino acid sequences show some discrepancies from those based on the genomic exons. Verification of this and other PLD cDNA sequences is under way to determine which differences result from polymorphism or sequencing inaccuracies.

The PLDa3 and $\alpha_4$ genes contain four exons in their coding region (Fig. 1). PLDa3 shares about 70% protein sequence similarity to $\alpha_1$ and $\alpha_2$. However, to date, no EST clone is yet identified for this gene.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Arabidopsis PLD gene structures and genomic organization. Boxes with numbers mark exons, and bars between exons represent introns. The exon and intron junctions are defined based on the comparison between PLD cDNAs and gene sequences and/or predicted intron-exon splicing sites annotated in the GenBank database. The PLD gene sequences were obtained by BLAST searching in GenBank using PLD cDNAs.
The deduced amino acid sequence of PLDa4 is shorter than PLDa1, 2, and 3 (762 versus 810 or 820 amino acids). It shows no more than 55% similarity to the other three αs, or to any other member of Arabidopsis PLDs. EST clones for PLDa4 are present in the database, so it is expressed. PLDa3 and α4 are grouped into the α class because they have four exons, whereas other PLDs have 10 or more exons (Fig. 1). In addition, their overall deduced amino acid sequences are evolutionarily closer to those of PLDa1 and α2 than to other PLDs (Fig. 2). Furthermore, their domain structures involved in catalysis (Fig. 4), Ca\(^{2+}\) binding (Fig. 5), and PIP\(_2\) interaction (Fig. 6) are more closely related to those of PLDa1 and α2 than to the other PLDs. Thus, they may have similar biochemical properties to PLDa. However, the amino acid sequence of PLDa4 is quite distant from all PLDs (Table II), and it might possess unique regulatory and catalytic properties. Therefore, its designation to the PLDa group is tentative, and its appropriate position in the Arabidopsis PLD gene family awaits further experimental characterization of its domain structures and biochemical properties. PLDβ, γ, and δ genes all consist of 10 exons in the coding region. The previously cloned and characterized PLDβ is now designated PLDβ1, and the amino acid sequences of PLDβ1 and β2 are about 89% similar (Table II). The annotated gene structure of β2 in the GenBank contains 11 exons, which is why it was previously designated as a new class (Wang, 2000). However, a close examination of the GenBank annotation indicates that the original prediction for the presence of the first intron is somewhat ambiguous. Inclusion of this intron removes part of an open reading frame that encodes the beginning part of the PLD C2 domain. Changing this region to be part of the first exon would increase the overall similarity between β2 and β1 from 85% to 89%. Thus, the first intron is removed in Figure 1, although ultimate determination of the exon-intron structures of PLDβ2 awaits the cloning of its cDNA. EST database searching against the β2 gene has not identified any expressed sequence. However, northern blotting probed with a β2-specific fragment showed a weak but positive result (C. Qin and X. Wang, unpublished data), suggesting at least that the β2 gene is expressed in low abundance under certain circumstances.

The three PLDγ genes cluster in tandem on chromosome IV with a similar pattern of exon-intron spacing. The three PLDγ genes are very closely related, sharing almost 95% sequence similarities in the deduced amino acid level (Table II). The PLDγ gene whose cDNA was first cloned and characterized was designated γ1. A complete cDNA for the second PLDγ gene, designated γ2, was cloned recently, and some of its biochemical properties are the same as those of γ1 (C. Qin and X. Wang, unpublished data). The third gene in the PLDγ gene cluster is designated as PLDγ3. EST clones corresponding to the PLDγ sequences have been identified, indicating that they all are expressed.

PLDδ is a single gene class (Fig. 1). The gene annotated in the GenBank is actually much longer, with six more exons on the 3′ end that encodes a nuclear localization signal and an RNA-binding region. However, experimental evidence from cDNA cloning and transcript size has shown that the authentic PLDδ gene is shorter than what was annotated and that does not contain the six extra exons at the 3′ end (Wang and Wang, 2001). Two full-length cDNAs of PLDδ have been cloned recently; they differ by 33 nucleotides near the end of the second exon (Katagiri et al., 2001; Wang and Wang, 2001). The cDNA that is
33 nucleotides shorter encodes a catalytically active PLD (Wang and Wang, 2001). It is possible that the two cDNAs come from two transcripts resulting from alternative splicing of the same PLD/H9254 gene. The names PLD/H9254a and PLD/H9254b are proposed to distinguish the two PLD/H9254 transcripts and proteins (Wang and Wang, 2001). The alphabetic affixes distinguish different products of the same gene, whereas the Arabic numerals represent separate PLD genes. A 90-kD Arabidopsis PLD (accession no. AF306345) has recently been shown to be associated with microtubules and has been implicated in regulating cell division (Gardiner et al., 2001). Its cDNA and deduced amino acid sequences exactly match those of PLD/H9254b (accession no. AF322228).

The last two members of putative PLD genes in the Arabidopsis genome are grouped together as PLD/H92561 and PLD/H92562 (Fig. 1) because of their similar protein domain structures (Fig. 3B). Both genes are located on chromosome III. The two PLDs share about 74% sequence similarity in deduced amino acids, and both are quite distant to all the other Arabidopsis PLDs, with no more than 46% sequence similarity (Table II). In addition, their deduced amino acid sequences are 1,096 and 1,039 amino acids in length, respectively, which are longer than the other plant PLD proteins, but in the same range as the mammalian PLD1 (human PLD1 is 1,074 amino acids). Sequence comparison also suggests that PLD/H9256s are closer to the mammalian PLDs than to the other plant ones in terms of amino acid sequence similarity (Table II) and the protein domain structures (Figs. 3–5). EST clones have been identified for both PLD/H9256 genes.

## Domain Structures of the Arabidopsis PLD Proteins

### HKD Motif

The highly conserved domain in the PLD family is the HKD motif, which is used to define the PLD superfamily. It was termed “HKD” because the domain contains the motif HxKxxxxD/E, which is found twice without exception in all cloned PLDs (Hammond et al., 1995; Koonin, 1996; Ponting and Kerr, 1996). The two HKD motifs are far from each other in the primary structure (Fig. 3), but they in-

### Table II. Amino acid sequence similarities among Arabidopsis PLDs

|      | α1   | α2   | α3   | α4   | b1   | b2   | y1   | y2   | y3   | δ    | α1   | α2   | α3   | α4   | b1   | b2   | y1   | y2   | y3   | δ    | hPLD1 | hPLD2 |
|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| α1   | 92.8 | 70.5 | 53.4 | 57.9 | 57.1 | 58.9 | 58.5 | 58.5 | 58.6 | 44.5 | 41.4 | 37.4 | 39.2 |
| α2   | 70.9 | 54.6 | 57.4 | 56.7 | 58.2 | 58.3 | 57.9 | 57.4 | 45.9 | 43.6 | 36.5 | 39.7 |
| α3   | 53.3 | 57.7 | 55.1 | 58.1 | 57.3 | 56.8 | 57.0 | 44.4 | 43.0 | 35.0 | 42.6 |
| α4   | 50.2 | 48.9 | 51.1 | 51.9 | 51.0 | 52.6 | 40.6 | 40.0 | 36.4 | 40.7 |
| β1   |      | 89.1 | 79.7 | 77.2 | 79.4 | 66.5 | 39.3 | 39.3 | 36.8 | 41.4 |
| β2   |      |      | 71.8 | 70.4 | 71.7 | 61.3 | 39.1 | 36.6 | 35.3 | 39.0 |
| γ1   |      |      |      | 93.0 | 95.1 | 64.8 | 42.4 | 42.7 | 36.5 | 38.6 |
| γ2   |      |      |      |      | 93.7 | 64.1 | 40.7 | 38.3 | 36.2 | 39.1 |
| γ3   |      |      |      |      |      | 64.2 | 41.3 | 37.7 | 36.3 | 38.0 |
| δ    |      |      |      |      |      |      | 38.6 | 40.0 | 37.5 | 38.5 |
| ξ1   |      |      |      |      |      |      |      | 74.4 | 47.7 | 51.6 |
| ξ2   |      |      |      |      |      |      |      |      | 46.8 | 50.8 |
| hPLD1|      |      |      |      |      |      |      |      |      | 66.8 |
| hPLD2|      |      |      |      |      |      |      |      |      |      |
teract with each other to form the active site. Point mutagenesis of PLD from several species has revealed that these amino acids are critical for catalysis in vitro and for PLD function in vivo (Sung et al., 1997; Gottlin et al., 1998). This has led to a model for the catalytic cycle using the conserved His for nucleophilic attack on the substrate phosphorous and involving a covalent phosphatidyl enzyme intermediate. The crystal structure of the bacterial endonuclease member Nuc of the PLD superfamily, which encodes a single and very divergent HKD motif, has been determined (Stuckey and Dixon, 1999). Recent structural studies have also led to expansion of the second HKD motif of the PLD family to HxKxxxx-DxxxxxxGSxN. However, definition of the precise role that each amino acid plays will await determination from structural analyses of an eukaryotic PLD crystallized with its normal substrate.

Figure 3. Schematic depiction of Arabidopsis PLD domain structures. A, The structure of previously cloned Arabidopsis PLDα, β, γ, and δ proteins. B, The structure predicted for two Arabidopsis PLDβs and mammalian PLDs. C, Summary of the presence or absence of certain domains in each deduced PLD amino acid sequence. +/xxxx/+/+ marks the number of amino acid residues spacing between the two HKD motifs. Domain structures were determined using Conserved Domain Database (CDD) searching, ProfileScan in PROSITE, and Gap comparison. C2, Protein kinase C-conserved 2 domain.

Figure 4. Sequence comparison of the first HKD domains in Arabidopsis PLDs as well as in human (h) and mouse (m) PLD1s. A, Multiple sequence alignment. The residues HKD are boldfaced. B, Grouping of PLD using a dendrogram generated by the PileUp program in GCG package.

<table>
<thead>
<tr>
<th>Name</th>
<th>C2</th>
<th>PX</th>
<th>PH</th>
<th>HKD1/HKD2</th>
<th>IYIENQFF</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+/322aa/+</td>
<td>+</td>
</tr>
<tr>
<td>α2</td>
<td>+</td>
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<td>-</td>
<td>+/322aa/+</td>
<td>+</td>
</tr>
<tr>
<td>α3</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+/321aa/+</td>
<td>+</td>
</tr>
<tr>
<td>α4</td>
<td>?</td>
<td>-</td>
<td>-</td>
<td>+/302aa/+</td>
<td>+</td>
</tr>
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<td>β1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+/331aa/+</td>
<td>+</td>
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<td>-</td>
<td>-</td>
<td>+/315aa/+</td>
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<td>+</td>
<td>+/368aa/+</td>
<td>+</td>
</tr>
</tbody>
</table>
These duplicated HKD motifs are conserved in deduced amino acid sequences of all the 12 Arabidopsis PLD genes. They are separated by approximately 320 amino acids, except for \( /H9256_1 \) and \( /H9256_2 \), which have longer spacing sequences (Fig. 3C). Sequence alignment reveals that although all the second HKD motif structures look highly identical, the first HKDs and flanking regions display more diversity (Fig. 4A). The 10 exon-containing PLDs, four exon-containing PLD\(_1\)s, and PLD\(_1\)s form three distinct groups (Fig. 4B). The first HKD domains in the \( /H9256 \) group are more similar to that of hPLD1, again indicating a closer relationship between PLD\(_1\) genes and the mammalian PLDs. The dendrogram of the clustering relationship of the HKD1 domains (Fig. 4B) is consistent with the phylogenetic tree of the whole proteins (Fig. 2).

**“IYIENQFF” Motif**

The “IYIENQFF” region between the two HKD motifs is also called conserved region III in mammalian PLD structures and is found only in family members that exhibit bona fide PLD activity (Frohman et al., 1999). Mutagenesis studies have demonstrated that it is almost as critical as the HKD domains (Sung et al., 1997), but the precise role has yet to be determined. This region may be responsible for hydrophobic interactions with the methyl groups of the choline head group, due to the enrichment of aromatic amino acids. An alternative hypothesis is that it might encode the targeting signal for caveolae (Frohman et al., 1999). This “IYIENQFF” motif is the most conserved domain with the least diversity among the 12 Arabidopsis PLDs. For the other PLDs, only the seventh residue, Phe(F), is substituted by Tyr(Y), which should not alter the property of this region.

**Ca\(^{2+}\)/Phospholipid-Binding C2 Domain**

All previously cloned plant PLDs have a C2 domain near the N terminus, which is unique to plant PLDs and is not present in animal or fungal PLDs. The C2 domains are approximately 130 residues in length and can bind Ca\(^{2+}\) and other effectors, including phospholipids, inositol phosphates, and proteins (Nalefski and Falke, 1996; Rizo and Sudhof, 1998; Zheng et al., 2000). The three-dimensional structures and the Ca\(^{2+}\)-binding properties of C2 domains in synatagmin (Shao et al., 1996), cPLA2 (Perisic et al., 1998; Xu et al., 1998), PLC\(_1\) (Essen et al., 1997), and PKC\(_\beta\) (Sutton and Sprang, 1998) have been well studied.

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### Table III. Comparison of biochemical properties of PLD\(_{1}\), \( /H9251 \), \( /H9252 \), and \( /H9253 \)

Data for PLD\(_{1}\), \( /H9251 \), and \( /H9252 \) are based on Pappan et al. (1998) and Pappan and Wang (1999), and those for PLD\(_{2}\) and PLD\(_1\) are from Wang and Wang (2001) and this study, respectively.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ca(^{2+}) Requirement</th>
<th>PIP(_2)</th>
<th>PE</th>
<th>Oleate Stimulation</th>
<th>pH Optimum</th>
<th>Substrate Preference</th>
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<tr>
<td>PLD(_{1})</td>
<td>Micromolar to millimolar range(^a)</td>
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<td>No</td>
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<td>5.5–6.5</td>
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<td>No</td>
<td>Yes</td>
<td>7.0</td>
<td>PE &gt; PC</td>
</tr>
<tr>
<td>PLD(_{1})</td>
<td>None</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>7.0</td>
<td>PC</td>
</tr>
</tbody>
</table>

\(^a\) Depending on pH (Pappan and Wang, 1999).

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Figure 5. Sequence alignment of the Arabidopsis PLD C2 domains with PLC\(_1\) and cPLA2. The conserved amino acid residues involved in Ca\(^{2+}\) binding are boldfaced. The non-Ca\(^{2+}\) ligand residues that occur in positions corresponding to the Ca\(^{2+}\) ligands are underlined. Only the portion involved in Ca\(^{2+}\) binding is shown, and the \( \beta \)-sheet strands and Ca\(^{2+}\)-binding loops are defined according to a previous study (Perisic et al., 1998). CBL, Ca\(^{2+}\)-binding loops.
DRETTPRMPGWRDVGVWVHGAARDLRHTIQWNPWNTKTTAKK-------------------RYKT mPLD2  
DRYSTRPRMPWHDAHSAVHGAARDRVPHIQWNPWNTKIMKS-------------------KYRS hPLD1  
DRKVRPRMPWHQYMTLGEPARLRHFVRQWNYLLRAK-------------------PSLRL Spo14P  
BRKHPRMPWHVHCAWLGPFCRDARVHPWQNYAKKNA-------------------PYED PLD1  
DRKVRPRMPWHDACWLGPFCRDARVHPWQNYAKKNA-------------------PNEQ PLD2  
NLSCGRPFEPWHDHSHKDGPAAYDVLTNFEERW--LKAAPFGSIXYXFL RLDP1  
NVSNCGRPFEPWHDHSHKDGPAAYDVLTNFEERW--LKAAPFGSIXYXFL RLDP2  
TADDGPRLPHSMHIDKDGPAAYDVLANFEERW--MKASPFGIGKSKS LLDy1  
TADDGPRLPHSMHIDKDGPAAYDVLANFEERW--MKASPFGIGKSKS LLDy3  
TEDVGPRPLPHSMHIDKDGPAAYDVLANFEERW--MAKSPFGIGKSKS LLDy2  
GTKA-PRQWPDHLCRDGPAAYDVLIFQERW--RKATRW--KEFLS PLD6  
ITKGGPRPHISRLAPFIAWDVMNYFQERW--SKQGSS--KIDIL VL DL Dix1  
ITKGGGPRPHISRLAPFIAWDVMNYFQERW--SKQGSS--KIDIL VL DL Dix2  
IKKGGGPRPHISRLAPFIAWDVMNYFQERW--MKQGSS--QRFLYL VL DL Dix3  
LSRGGGPRPHISVLAVGAAWDVLKNFQERW--TKQGSS--NNFLVL VL DL Dix4

Figure 6. Alignment of a putative PIP2-binding motif in Arabidopsis PLDs, as well as in mouse (m) PLD2, human (h) PLD1, and yeast PLD (Spo14). The conserved basic amino acids originally identified in mouse PLD2 are in boldface, and some additional basic residues are underlined.

Arabidopsis PLDβs, γs, and δ all have the Ca2+-coordinating acidic residues, whereas the PLDα C2 domains lack one or more of these potential Ca2+-ligands (Fig. 5). Compared with cPLA2, PLDα1 has one, and α2 and α3 have two of them substituted, whereas α4 contains none of the Ca2+-binding residues. The presence of C2 domain in PLDα is not clear, but several reserved hydrophobic amino acids, which have been proposed to maintain the structural integrity of the C2 fold, are present in its corresponding region. On the other hand, Conserved Domain Database (CDD) searching and Gap comparison did not reveal the existence of the C2 domain in the two PLDβs.

The differences in the Ca2+-binding residues suggest that Ca2+-affinity of PLDαs could be lower than that of β, γ, and δ. This lower Ca2+-affinity of PLDα-C2 than PLDβ-C2 domain has been demonstrated experimentally (Zheng et al., 2000). Ca2+- and PC-binding properties of PLDα C2 and PLDβ C2 follow a trend similar to the Ca2+-requirements of the whole enzymes, PLDα1 and PLDβ1, for PC hydrolysis (Table III). Thus, it has been suggested that the C2 domains of PLDα and PLDβ serve as handles by which Ca2+ differentially regulates these PLD activities (Zheng et al., 2000).

**PIP2-Binding Motifs**

PIP2 plays a critical role in PLD activation in mammals and plants. PIP2 is required for activities of Arabidopsis PLDβ and γ replacement of PIP2 by other phospholipids such as PC, PS, PG, PE, and phosphatidylinositol (PI) resulted in loss of PLD activity (Qin et al., 1997). Two putative PIP2-binding motifs were also predicted, flanking the second HKD domain of PLDβ (Pappan et al., 1997b), but the PIP2-binding ability of these motifs in PLD has not been verified experimentally. Recent studies on mouse PLD2 have suggested that a small region containing many conserved basic and hydrophobic residues located between the two HKD motifs is responsible for the PIP2 requirement (Fig. 6; Sciorra et al., 1999). Mutagenesis experiments revealed that this region is required for activation of mammalian and yeast PLDs by PIP2.

Sequence alignments of PLDs from plants, animals, and yeast reveals conservation and variation of the basic residues of this PIP2-binding motif in plant PLDs (Fig. 6). The clustering relationship is quite similar to that revealed by comparison of HKD1 domains. For the five conserved basic amino acids originally identified in mouse PLD2, human PLD1, and yeast PLD (Sciorra et al., 1999), only the last one is substituted by an acidic residue in the two PLDβs (Fig. 6). PLDβs and γs all conserved the last three residues, but the first two are replaced by negatively charged residues or by neutral ones. PLDδ retains two of the conserved residues, whereas PLDαs have only one of them (Fig. 6). A recent study using PLDβ1 has provided experimental evidence for this motif as a PIP2-binding region, and this binding is stimulated by Ca2+ (L. Zheng, R. Krishnamoorthi, and X. Wang, unpublished data). It should be noted that the N-terminal C2 domain of PLDs also binds PIP2, but unlike the PIP2-binding region, the C2-PIP2 interaction is inhibited by Ca2+ (Zheng et al., 2000).

**PX Domain**

A conserved PX domain is present in Arabidopsis PLD ζ1 and ζ2, as well as in mammalian PLDs, but is not found in the other Arabidopsis PLDs (Fig. 3B). The PX domain was originally identified in the protein p47phox, a component of phagocyte NADPH oxidase (Ponting, 1996). It is a novel protein module containing a conserved Pro-rich motif. Recent structural and cell biological studies show that the PX domain can bind phosphoinositides and SH3 domain (Cheever et al., 2001; Hiroaki et al., 2001; Kanai et al.,
Therefore, it may play a critical role in coordinating membrane localization and protein complex assembly during cell signaling. The PX domain is critical for activities of mammalian PLDs (Sung et al., 1999a, 1999b). How it functions is unknown, although potential roles include regulating interactions with factors that promote translocation or activation.

**PH Domain**

PH domain is another potential regulatory module present in Arabidopsis PLD1 and 2, as well as in mammalian PLDs, but not in any other Arabidopsis PLDs (Fig. 3B). PH domains are composed of approximately 120 amino acids found in more than 100 proteins involved in cell signaling, cytoskeletal rearrangement, and other processes (Lemmon and Ferguson, 2000). All PH domains studied to date appear to bind to phosphoinositides, but most bind weakly and nonspecifically. Only a small subclass of PH domains shows strong and specific binding to membrane phosphoinositides (Lemmon and Ferguson, 2000). Deletion analysis of mammalian PLD1 and PLD2 revealed that the PH domain is not required for enzymatic activity nor is the dependence of the enzymatic activity on PIP_{2} affected by deletion of the PH domain (Sung et al., 1999a, 1999b). PH domain could be important in membrane targeting and association with other cellular components. However, its function in PLDs awaits further investigation.

**PLD1 Is a Calcium-Independent, PC-Selective PLD**

To characterize the PX/PH-containing, putative PLD\(_{\alpha}\), the Arabidopsis EST database was searched for putative full-length cDNA clones of PLD\(_{\alpha}\)s using the PLD\(_{\alpha}\) coding sequences annotated in the GenBank database. Several putative PLD1 EST clones were identified, and one (EST no. AV529766) was sequenced completely and found to be a full-length PLD1 cDNA. The cDNA is composed of 3,785 nucleotides, and the nucleotide sequence of the cDNA matches that annotated from the genomic sequencing. The coding region of the PLD1 cDNA starts at nucleotide 248 and ends at 3,538 (GenBank accession no. AF411833). It encodes a protein of 1,096 amino acids with the domain structures depicted in Figure 3B. The calculated molecular mass and pI are 124 kD and 6.27, respectively.

To validate that this cDNA encodes a PLD protein from the cDNA was expressed in *Escherichia coli* using pBluescript SK(-) as expression vector, which has been used successfully to express catalytically active PLD\(_{\alpha}\), \(\beta\), and \(\gamma\) (Wang et al., 1994; Pappan et al., 1997b; Qin et al., 1997). After isopropyl-1-thio-\(\beta\)-galactopyranoside induction, protein extracts from *E. coli* JM109 harboring the SK alone exhibited negligible PLD activity (Fig. 7A). Proteins from *E. coli* harboring the vector with the PLD1 cDNA insert had significant PLD activity. PLD1 required PIP\(_{2}\) for activity; it displayed no activity when PC-only vesicles and PC/Triton X-100 vesicles were used as substrates (Fig. 7A). The PIP\(_{2}\) requirement is a property shared by PLD\(_{\beta}\) and \(\gamma\), as well as mammalian and yeast PLDs, but the activity of PLD\(_{\beta}\) and \(\gamma\) also needs PE (Table III; Pappan et al., 1998). In contrast, PLD\(_{\alpha}\) requires only PIP\(_{2}\) but not PE (Fig. 7A).

Previous analysis of PLD\(_{\beta}\) and PLD\(_{\alpha}\) indicates that the C2 domain is a major determinant for the requirement of different levels of Ca\(^{2+}\) for their activities (Zheng et al., 2000). Accordingly, the absence of the C2 domain in PLD1 (Fig. 3B) could mean that this PLD might not require Ca\(^{2+}\) for activity. To test this hypothesis, 2 mM EGTA and 2 mM EDTA were added to the reaction to chelate Ca\(^{2+}\) and any other divalent cation. In addition, varied concentrations of CaCl\(_{2}\) or MgCl\(_{2}\) were used to examine the cation effect on PLD1. The highest PLD1 activity occurred in the zero to nanomolar concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) (Fig. 7B). The activity decreased gradually at the micromolar range and dropped rapidly when cation concentrations approached millimolar levels (Fig. 7B). These results show that PLD1 is independent of Ca\(^{2+}\) or any other cation for activity. This property is in contrast to all the other cloned plant PLDs that require micromolar or millimolar ranges of Ca\(^{2+}\) for activity (Wang, 2000; Table III).

The substrate specificity was examined next. PLD1 hydrolyzed PC well, but had negligible activity toward PE or PS (Fig. 8A). No PG-hydrolyzing activity was observed (data not shown). This PC-selective activity is distinctively different from other characterized Arabidopsis PLDs, but similar to the cloned mammalian ones. PLD\(_{\alpha}\) hydrolyzes PC, PE, and PG equally well, and PLD\(_{\beta}\) and \(\gamma\) hydrolyze PC, PE, and PS (Pappan et al., 1998; Table III), whereas PLD\(_{\delta}\) uses PE better than PC (C. Qin, C. Wang, and X. Wang, unpublished data). The mammalian PLD1 is PC specific and has no activity toward PE or PI (Hammond et al., 1995), and activity of the cloned mammalian PLD2 toward lipids other than PC has not been reported.

PLD1 functioned at a rather narrow range of pH with a pH optimum at 7. Its activity decreased considerably at pH 6.5 and 7.5, and was virtually abolished at pH 6 and 8 (Fig. 8B). PLD\(_{\beta}\) and \(\gamma\) are also most active at pH 7, but their functional pH is broader than that of PLD1 (Pappan and Wang, 1999; Table III). PLD\(_{\gamma}\) has a comparable activity from pH 5.5 to 8.5. PLD\(_{\beta}\) has a similar activity at pH 6.5 to 7.5, and has still approximately 35% activity at pH 5. PLD\(_{\alpha}\) has acidic pH optima that are influenced by the Ca\(^{2+}\) concentrations. The pH optimum is 4.5 to 5 when assayed in the presence of micromolar levels of Ca\(^{2+}\) and PIP\(_{2}\), but it increases to 5.5 to 6.5 at millimolar levels of Ca\(^{2+}\) (Pappan and Wang, 1999). However, the presence or absence of Ca\(^{2+}\) did not alter the pH optimum of PLD1 (Fig. 8B).
In summary, this study has provided molecular and biochemical evidence for the occurrence of a new class of plant PLD, PLDζ, that is Ca\(^{2+}\) independent and PC selective. These results, together with the genomic analysis of the Arabidopsis PLD family, indicate that different PLDs are subjected to unique controls and that their activation may hydrolyze different membrane lipids in the cell. Also noteworthy is that the biochemical properties, domain structures, and genomic organization of plant PLDs are much more diverse than for other organisms. Thus far, only two PLD genes, PLD1 and PLD2, are identified in mammals, and one PLD gene, SPO14, has been identified in yeast. This is in contrast to the other phospholipase families; more diversity in structures and regulatory properties has been observed for PI-PLC and PLA2 in animals than in plants (Wang, 2001). Could this mean that plants use PLD more than other organisms as part of the regulatory machinery in cellular functions? The occurrence of the C2, PH, and PX domains, which have been demonstrated in many proteins involved in signal transduction, vesicular trafficking, and cytoskeletal rearrangements, hints at important and diverse roles of PLDs in cellular regulation. Studies involving genetic manipulation of specific PLDs have revealed some unique metabolic and physiological function for some PLDs (Wang et al., 2000; Wang, 2001). Genetic redundancy may also occur with some of the PLD genes (Katagiri et al., 2001). Delineation of the unique functions and potential redundancy of the multiple PLDs will be important to a comprehensive understanding of the PLD family and their roles in plant processes.

**MATERIALS AND METHODS**

**Identification of PLDζ and Sequence Analysis**

A putative Arabidopsis EST PLDζ1 cDNA was identified by searching the BLAST database against the PLDζ1 cDNA annotated from its genomic DNA sequence. This EST clone was kindly provided by the Kazusa DNA Research Insti-
Gene-specific primers and the T3 primer from the pBluescript SK(-) vector were used for the sequencing of the insert, which was proved to be a full-length PLD/H9256 cDNA (accession no. AF411833).

The PLD gene sequences were obtained by BLAST searching in GenBank and The Institute for Genomic Research databases using identified PLD cDNAs. This phylogenetic tree was generated using the GrowTree program from the University of Wisconsin Genetics Computer Group. The dendrogram was generated by the PileUp program in GCG. Domain structures were determined using CDD searching and Gap comparison.

Expression of PLD cDNA in *Escherichia coli*

Expression of the PLD/H9256 cDNA was performed using pBluescript SK(-) containing the cDNA insert in *E. coli*. The recombinant plasmid was transformed into *E. coli* JM109. Fifty microliters of an overnight culture of the transformed *E. coli* was added to 25 mL of Luria Bertani medium with 30 μg mL⁻¹ ampicillin. Cells were incubated at 37°C for 3 h with shaking, and then isopropylthio-β-galactoside was added to a final concentration of 0.5 mM. After growing overnight at room temperature, the induced cells were pelleted by centrifugation and were then resuspended in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.25 mM phenylmethylsulfonyl fluoride and were then pelleted by centrifugation. The cells were lysed by sonication in the resuspension buffer, and cell debris was removed by centrifugation at 12,000 g for 5 min. Proteins in the supernatant were assayed for PLD activity.

### PLD Activity Assays

The basic PLD assay mixture contained 100 mM Tris-HCl (pH 7.0), 80 mM KCl, 100 mM Ca²⁺, 0.4 mM lipid vesicles, and 30 μg of pBluescript SK-expressed protein in a total volume of 100 μL. Lipid vesicles were composed of 35 nm of PE, 3 nm PIP₂, and 2 nm PC. PLD-mediated hydrolysis of PC was measured using dipalmitoylglycerol-3-phospho[methyl-3H]choline as substrate (Pappan et al., 1997a). The reaction was initiated by adding enzyme proteins and was incubated at 30°C for 30 min in a shaking water bath. The reactions were stopped by adding 1 mL of chloroform:methanol (2:1) and then 100 μL of 2 mM KCl, and the release of the [³H]choline into the aqueous phase was quantitated by scintillation counting. Control assays were performed using 30 μg of protein from lysed bacteria harboring the pBluescript SK(-) plasmid without a PLD cDNA insert. The background activity from bacteria was negligible and was subtracted from the activity of the samples containing the recombinant PLD. To test the dependence of PLD activity on divalent cations, 2 mM EGTA and 2 mM EDTA were added to the reactions with no CaCl₂ or MgCl₂. In addition, CaCl₂ or MgCl₂ was added to the reaction at indicated concentrations (Fig. 7).

To test the substrate selectivity of PLD/H9256, mixed vesicles composed of PIP₂ (6.5 mol %) and PC, PE, or PS (93.5 mol %) were used as substrates. Hydrolysis of PC, PE, and PS was measured by the release of [³H]choline, [¹⁴C]ethanolamine, or [¹⁴C]Ser. [³H]PC was included at 18 nCi/reaction, and [¹⁴C]PE or [¹⁴C]PS was included at 9 nCi/assay. Divalent cations and chelators were not added to the reactions. Dipalmitoylglycerol-3-P-[methyl-³H]choline, dioleoyl-glycerol-3-P-[ethanolamine-2-¹⁴C]ethanolamine, and dioleoyl-glycerol-3-P-[ser-3-¹⁴C]Ser were obtained from Amersham Biosciences (Piscataway, NJ). PC, PE, and PS were purchased from Avanti Polar Lipids (Birmingham, AL).

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Figure 8. Substrate specificity and pH dependence of PLD/H9256. A, Hydrolysis of PC, PE, and PS in vesicles composed of 6.5 mol % of PIP₂ and 93.5 mol % of PC, PE, or PS. No Ca²⁺ or Mg²⁺ was added to the reactions. B, pH optimum of PLD/H9256 in the presence and absence of 100 μM Ca²⁺. Hydrolysis of PC was monitored using PC/PE/PIP₂ vesicles.


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