N-terminal EF-hand-like domain is required for phosphoinositide-specific phospholipase C activity in *Arabidopsis thaliana*

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Abstract Phosphoinositide-specific phospholipase C's (PI-PLCs) are ubiquitous in eukaryotes, from plants to animals, and catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate into the two second messengers inositol 1,4,5-trisphosphate and diacylglycerol. In animals, four distinct subfamilies of PI-PLCs have been identified, and the three-dimensional structure of one rat isozyme, PLC-δ1, determined. Plants appear to contain only one gene family encoding PI-PLCs. The catalytic properties of plant PI-PLCs are very similar to those of animal enzymes. However, very little is known about the regulation of plant PI-PLCs. All plant PI-PLCs comprise three domains, X, Y and C2, which are also conserved in isoforms from animals and yeast. We here show that one PI-PLC isozyme from *Arabidopsis thaliana*, AtPLC2, is predominantly localized in the plasma membrane, and that the conserved N-terminal domain may represent an EF-hand domain that is required for catalytic activity but not for lipid binding. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Phosphoinositide-specific phospholipase C; Phosphoinositide; EF-hand; Plasma membrane; *Arabidopsis thaliana*

1. Introduction

The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) by phosphoinositide-specific phospholipase C (PI-PLC)[1] results in the production of the two crucial second messengers inositol 1,4,5-trisphosphate and diacylglycerol [1]. In animal cells, PI-PLC isoforms are activated by a wide range of extracellular signals. Four different types of PI-PLCs, β, γ, δ, and ε, have been identified in animal cells [2–4], and their regulation involves heterotrimeric GTP-binding proteins and receptor tyrosine kinases [2]. Five domains are conserved in animal PI-PLC isozymes. From the N-terminus the conserved domains are: a pleckstrin homology (PH) domain, involved in substrate binding, an EF-hand domain, an X and a Y domain that together constitute the catalytic domain of the enzyme, and a C-terminal lipid-binding C2 domain [5,6]. This basic domain arrangement is found in all isozymes, with additional insertions and/or domains being present in β, γ, and ε isozymes [6,3,4].

Plant cells have long been known to contain PI-PLC activity. A classification into two types based on differences in substrate specificity, Ca^{2+} requirement, and subcellular localization has been proposed [7]. Type I would be represented by activities favoring PtdIns as a substrate, requiring millimolar Ca^{2+} concentrations, and localized in the soluble fraction of plant cells, while type II would consist of activities predominantly associated with the plasma membrane, preferring PtdIns(4,5)P_2 as a substrate, and requiring low micromolar levels of Ca^{2+}. PI-PLC activities have been partially purified from several plant species [8–11]. However, no amino acid sequence has been reported for any of the partially purified plant enzymes. Genes encoding biochemically active PI-PLCs with substrate specificities and Ca^{2+} requirement comparable to those of animal PI-PLCs have been identified in *Arabidopsis thaliana* [12], soybean [13], *Nicotiana rustica* [14], and potato [15]. Additional genes encoding PI-PLC-like proteins are found in *Nicotiana tabacum*, pea, *Digitaria sanguinalis*, soybean, and *A. thaliana*. All of the functionally active and putative PI-PLCs from plants are very similar in size, have the same structural organization, and contain the X, Y, and C2 domains conserved in all other PI-PLCs but lack a PH domain. Except for their absolute Ca^{2+} requirement, the regulatory mechanisms of plant PI-PLCs are unknown. Notably, in plant PI-PLCs the X domain is preceded by an N-terminal region of unknown function. It is also still unclear what function the C2 domain serves in these proteins.

We here present results on the characterization of one PI-PLC isoform from *A. thaliana*, AtPLC2. The gene encoding this protein has been described previously [16], but the corresponding protein was not characterized. We show that recombinant AtPLC2 prefers PtdIns(4,5)P_2 as substrate, and requires low micromolar levels of Ca^{2+} for activity. The enzyme is enriched in plasma membranes. Structure prediction and comparison, and analysis of N-terminal deletion mutants of AtPLC2 suggest that plant PI-PLCs possess a N-terminal EF-hand domain necessary for activity.

2. Materials and methods

2.1. Materials

[^{3}H]PtdIns(4,5)P_2 and [^{3}H]PtdIns were purchased from Amersham Pharmacia Biotech. Lipids and thrombin were from Sigma.

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Abbreviations: PI-PLC, phosphoinositide-specific phospholipase C; PtdIns, phosphatidylinositol; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PH, pleckstrin homology

[1] The abbreviation PI-PLC does not include the PtdIns-specific phospholipase C present in prokaryotes such as *Bacillus cereus*.

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2.2. Expression and purification of recombinant proteins

Full-length and deletion variants of *AtPLC2* were amplified by PCR using *Pfu* DNA polymerase, and cloned in the expression vector pGEX-4T-2 (Amersham Pharmacia Biotech). All constructs were checked by DNA automated sequencing. Recombinant GST fusion proteins were expressed in the *Escherichia coli* strain BL21(DE3)pLys, and affinity-purified using glutathione–Sepharose 4B beads according to the manufacturer's instructions. Recombinant proteins were released from their GST tag by incubation of the beads with thrombin. Alternatively, the fusion protein GST–AtPLC2 bound to the Sepharose resin was eluted with 10 mM glutathione, 50 mM Tris–HCl, pH 8.0.

2.3. PI-PLC assay

The PI-PLC assay was performed according to [17] with 0.5 to 3 μg of recombinant protein. Free Ca^{2+} concentrations were maintained using EGTA/$CaCl_2$ buffers according to [18]. All assays were carried out in duplicates.

2.4. Protein binding to lipid vesicles

Protein binding to lipid vesicles was performed as described by [19]. Briefly, a sample of 1.1 mg of a phosphatidylethanolamine/phosphatidylserine/PtdIns(4,5)P_2 mixture (molar ratio 7:3:1) in chloroform was dried under a stream of nitrogen and resuspended in 1.1 ml of LMV buffer (150 mM NaCl, 2 mM EDTA, 20 mM Tris–HCl, pH 7.5) by vortexing. The lipid vesicles formed can subsequently be sedimented by centrifugation. An aliquot of recombinant protein in 300 μl of LMV buffer was mixed with 100 μl of lipid vesicles in the presence of 0.2 mM $CaCl_2$ (final concentration). After 5 min of incubation at room temperature the mixture was centrifuged at 11 600 × *g* for 15 min at 4°C. The pellet was washed once in 300 μl of LMV buffer, dissolved in 35 μl of Laemmli sample buffer and analyzed by SDS–PAGE.

2.5. Antibody production

A peptide KDLGDEEVWGREVPSFIQR (PLC2) corresponding to residues 266–284 of AtPLC2 was synthesized. A Cys residue was added at the C-terminal in order to permit conjugation to KLH. One rabbit was immunized at a 2-weeks interval and serum collected after the second boost. Antibodies directed against the synthetic peptide were affinity-purified using the SulfoLink kit from Pierce (USA) following the manufacturer's instructions.

2.6. Plasma membrane purification

A. thaliana plants were grown in soil in a greenhouse with supplementary light (23 W m^{-2}, 350–800 nm; Philips G/86/2 HPLR 400 W) and with full nutrient supply.

4-week-old plants (everything above soil) were harvested and homogenized in 50 mM MOPS–KOH, pH 7.8, 330 mM sucrose, 0.2% (w/v) casein (boiled hydrolysate), 5 mM EDTA, 5 mM ascorbic acid, 5 mM DTT, and 0.6% (w/v) polyvinylpolypyrrolidone and 1 mM phenylmethylsulfonyl fluoride. The homogenate was filtered and centrifuged at 10 000 × *g* for 15 min. The resulting supernatant was centrifuged at 30 000 × *g* for 1 h giving a supernatant, termed cytosol, and a pellet, termed microsomal fraction. Plasma membranes were purified from the microsomal fraction by aqueous polymer two-phase partitioning in a system composed of (final concentrations): 6.1% (w/w) Dextran T500 (Amersham Pharmacia Biotech, Uppsala, Sweden), 6.1% (w/w) polyethylene glycol (Sigma), 5 mM potassium phosphate, pH 7.8, 3 mM KCl, 1 mM DTT, and 0.1 mM EDTA. Phase partitioning was as described in [20] giving a plasma membrane-enriched upper phase and an intracellular membrane-enriched lower phase. All fractions were frozen and stored in aliquots in liquid nitrogen until use.

2.7. SDS–PAGE and Western blotting

Proteins were separated by PAGE as previously described [21]. They were then transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% (w/v) fat-free milk in TBST (10 mM Tris–HCl, pH 8.0, 160 mM NaCl, 0.05% (w/v) Tween 20) for 1 h at room temperature under gentle shaking, and then incubated with affinity-purified anti-PLC2 antibody diluted 1/500 in TBST overnight at 4°C with shaking. The membrane was then washed with TBST and incubated with a monoclonal anti-rabbit antibody coupled to horseradish peroxidase for 1 h at room temperature with shaking. After three successive washing steps, the protein complexes were revealed using the ECL method from Pierce.

2.8. Protein determination

Protein was determined essentially according to [22] with bovine serum albumin as standard protein.

2.9. Protein sequence analysis

Alignment of amino acid sequences was performed using the ClustalW program available from the ExPASy Molecular Biology Server (www.expasy.ch). Secondary structures were predicted with the PredictProtein server from the Columbia University Bioinformatics Center (http://cubic.bioc.columbia.edu/predictprotein/)

3. Results

3.1. AtPLC2 is a catalytically active PI-PLC enriched in the plasma membrane

Recombinant AtPLC2 was expressed and purified from *E. coli*. The activity of the recombinant protein against PtdIns and PtdIns(4,5)P_2 was measured at different Ca^{2+} concentrations (Table 1). With PtdIns(4,5)P_2 as substrate, maximal hydrolytic activity was reached at 1 μM free Ca^{2+}, while a low PtdIns hydrolysis was observed only at higher Ca^{2+} concentrations (= 100 μM). At physiological cytosolic Ca^{2+} concentrations, PtdIns(4,5)P_2 was the preferred substrate with a 60–350 times higher specific activity than with PtdIns. Comparable results were obtained with other recombinant plant PI-PLCs [12,13,15]. This demonstrates that the *AtPLC2* gene encodes a functional PI-PLC with substrate preference and Ca^{2+} dependence similar to other PI-PLCs.

As mentioned earlier, plant plasma membranes contain PI-PLC activity. The *AtPLC2* gene is expressed constitutively in *A. thaliana* plants, including shoots, leaves and flowers [16]. The cellular distribution of AtPLC2 was investigated using a polyclonal antibody raised against a synthetic peptide specific for this isoform, and various subcellular fractions obtained from *A. thaliana* plants excluding the roots (Fig. 1). The antibody reacted strongly against recombinant GST–AtPLC2 (95 kDa in size) expressed in *E. coli* (Fig. 1, lane 5). The same antibody recognized a polypeptide of approximately 66 kDa in a microsomal fraction and the upper and lower phases obtained after phase partitioning of the microsomal fraction (Fig. 1, lanes 1–3). This corresponds very well with the calculated molecular mass of AtPLC2 (66 142 Da). Pre-immune serum did not show any reaction against any of the fractions analyzed (not shown). The 66-kDa polypeptide was clearly enriched in the plasma membrane fraction (upper phase) as

Table 1
Substrate specificity and Ca^{2+} dependence of AtPLC2

Free Ca^{2+} concentration	PtdIns(4,5)P_2 hydrolysis[a]	PtdIns hydrolysis[a]
0 (2 mM EGTA)	4	0
0.1 μM	19	0.3
1 μM	105	0.3
10 μM	100	0.7
100 μM	95	1
1 mM	65	2.5

[a]PI-PLC activity was assayed as described in Section 2. Activity is expressed as percent of the activity obtained with PtdIns(4,5)P_2 as substrate and 10 μM Ca^{2+} (100% = 66 nmol min^{-1} mg^{-1}). The data presented are representative of one of three independent experiments.

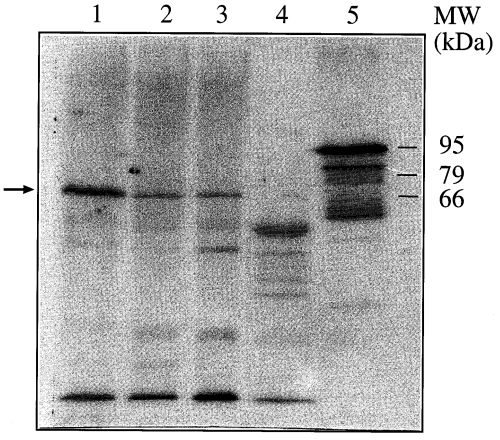

Fig. 1. AtPLC2 is predominantly present in the plasma membrane of *A. thaliana* cells. Subcellular fractions were prepared from *A. thaliana* plants. Plasma membranes were purified by aqueous two-phase partitioning. The fractions were separated by SDS–PAGE and blotted on a polyvinylidene fluoride membrane. The membrane was probed with a polyclonal antibody raised against a synthetic peptide specific to AtPLC2. Antibodies bound to the membrane were detected with a secondary monoclonal antibody coupled to horseradish peroxidase. Lane 1: plasma membrane; lane 2: lower phase; lane 3: microsomal fraction; lane 4: cytosolic fraction; lane 5: GST–AtPLC2 recombinant protein (The GST–AtPIP5K1 protein has a molecular mass of 95 kDa, the additional bands detected by the antibody probably represent proteolytic fragments of the 95-kDa protein). Lanes 1–5 contained 10 μg protein. The position of size markers is indicated on the right. The arrow indicates the position of recombinant AtPLC2 cleaved from the GST tag.

compared to the intracellular membranes (lower phase) and microsomal fraction. Plasma membranes purified from plant tissues by aqueous polymer two-phase partitioning are usually in the right side-out orientation and have a strong affinity for the upper phase. However, inside-out vesicles can represent a small proportion of the total plasma membrane vesicles, and these are recovered in the lower phase upon phase partitioning [23]. This could explain the presence of the 66-kDa polypeptide in the lower phase, although it is also possible that AtPLC2 is present in another compartment than the plasma membrane. Importantly, the cytosolic fraction did not contain any significant amount of this polypeptide (Fig. 1, lane 4). The blurred band of approximately 55 kDa detected in the cytosolic fraction probably represents a non-specific interaction. These results indicate that AtPLC2 is predominantly localized in the plasma membrane.

3.2. PI-PLCs from A. thaliana contain a conserved domain at their N-terminus

Seven genes encoding PI-PLC-like isoforms are present in *A. thaliana* (unpublished observation). Only two of these genes, *AtPLC2* (this work) and *AtPLC1* [12], have to date been demonstrated to encode catalytically active PI-PLCs. The X, Y, and C2 domains conserved in previously characterized PI-PLCs from *A. thaliana*, soybean and potato [12,13,15,16] are also present in the predicted amino acid sequences of the other five PI-PLC genes from *A. thaliana* (Fig. 2). In addition, another domain, named N in Fig. 2, is conserved, although to a lesser degree than the other three domains (Fig. 2A,B). In total, seven residues of the N domain are identical in the seven *A. thaliana* sequences, and an additional 41 residues are conserved (Fig. 2B). Some of these residues are also conserved in rat PLC-δ1. No obvious function can be assigned to the N domain based on amino acid sequence comparison to protein modules with known functions. To examine the role of this domain in AtPLC2, comprising residues 23–96 of this protein, we produced several truncated versions of AtPLC2 that lacked different portions of the N-terminal region: Δ1–22, Δ1–36, and Δ1–100, respectively, the latter lacking the entire N-terminal region preceding the X domain (Fig. 3A). These constructs were expressed individually in *E. coli* (Fig. 3B) and their PI-PLC activity was determined.

3.3. The N-terminal segment of AtPLC2 contains a region necessary for PI-PLC activity

Removal of the complete N-terminal region (i.e. residues 1–100) of AtPLC2 resulted in complete loss of PtdIns(4,5)P_2-hydrolyzing activity (Table 2). When residues 1–22, which are less conserved, were missing, the PI-PLC activity was not affected when compared to that of the full-length protein. When a further 14 residues were removed (Δ1–36) the PtdIns(4,5)P_2-hydrolyzing activity was completely lost (Table 2). This demonstrates that the 22 first residues of AtPLC2 are not required for PI-PLC activity, but that the 14 first residues of the conserved N domain (residues 23–36 of AtPLC2) are needed for activity. Despite these effects on PI-PLC activity, no dramatic alterations in the ability of Δ1–36 and Δ1–100 to bind to PtdIns(4,5)P_2-containing lipid vesicles were observed (Fig. 3C). A recombinant protein comprising residues 1–100 of AtPLC2 (designated N-term) did not bind to lipid vesicles (Fig. 3C). It therefore appears that in the first 14 residues of the conserved N domain, i.e. residues 23–36 of intact AtPLC2, amino acids necessary for catalysis but not for binding to lipid vesicles are present.

3.4. The conserved N-terminal domain of A. thaliana PI-PLCs may represent an EF-hand domain

As mentioned above some of the residues conserved in the N-terminal domain of *A. thaliana* PI-PLCs are also conserved in rat PLC-δ1 (Fig. 2B). In PLC-δ1, these residues belong to the second loop of the EF-hand domain, a domain present in all animal PI-PLCs. The EF-hand domain of PLC-δ1 was not identified by sequence analysis but from the three-dimensional structure of a catalytically active deletion variant of the protein. It consists of two loops, each containing two EF-hand motifs [5]. However, no canonical EF-hand sequences are present in the two EF-hand motifs that form the second loop of the EF-hand domain of PLC-δ1 [5]. The functional role of this domain is obscure as it does not seem to bind Ca^{2+} [5]. The second loop consists of four α-helices forming two consecutive helix–loop–helix units ([5]; Fig. 2B). As shown in Fig. 2B, the N domain of plant PI-PLCs is predicted to contain four α-helices. The arrangements of the four α-helices in the N domain and the second loop of the EF-hand domain of PLC-δ1 are very similar. In addition, the conserved N domain of plant PI-PLCs is placed immediately before the X domain (Fig. 2A), a position similar to that of the second loop of the EF-hand domain in PLC-δ1. We therefore propose that the conserved N domain of plant PI-PLCs represents an EF-hand domain that corresponds to the second loop of the EF-hand of PLC-δ1 (Fig. 2A).

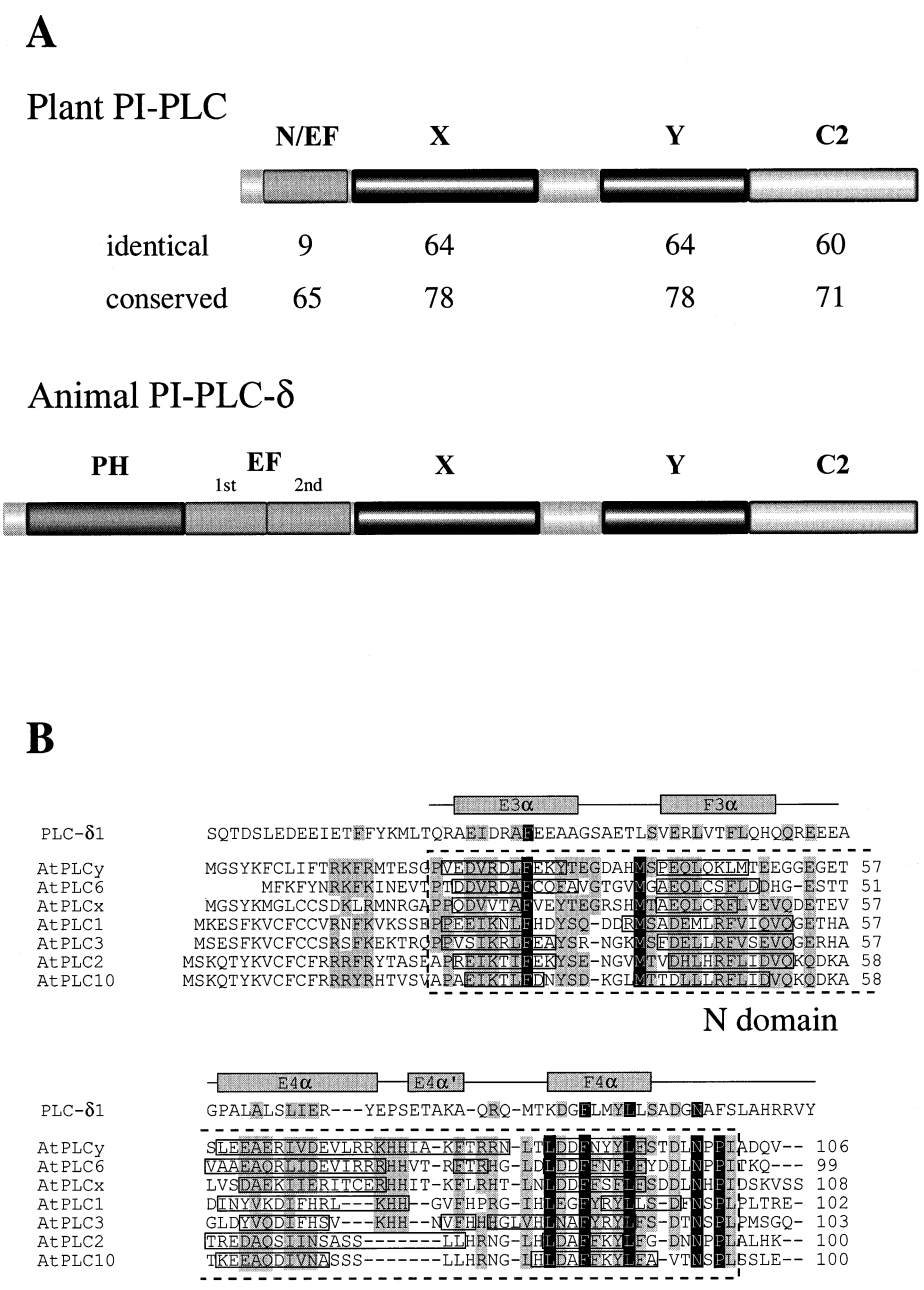

Fig. 2. Schematic representation of plant PI-PLCs and comparison of the N-terminal regions of PI-PLC sequences from *A. thaliana*. A: Structure of plant PI-PLCs and animal PI-PLC-δ. The conserved domains are indicated. The percentages of identical and conserved residues for each domain, and for PI-PLC sequences from *A. thaliana* only, are given under the respective domains. B: The predicted amino acid sequences of the seven *PI-PLC* genes from *A. thaliana* and that of PLC-δ1 from rat were aligned using the ClustalW sequence alignment program and adjusted by hand. The regions preceding the X domains only are shown. Residues identical in all *A. thaliana* sequences appear in white on black background. Conserved residues are on gray background. Numbers on the right side indicate the position of the last amino acid in each sequence. The N domain is marked by a dotted box. The secondary structures of plant sequences were predicted with the PHD set of programs of the PredictProtein server at Columbia University (Bioinformatics Center). The predicted α-helices are indicated by boxes in each *A. thaliana* sequence. The four α-helices of the EF-hand domain of PLC-δ1 are represented by gray boxes over its amino acid sequence. Accession numbers are: D38544 (AtPLC1), D50804 (AtPLC2), AL161593 (AtPLC3), AC007658 (AtPLC6), AB020755 (AtPLCx and AtPLCy), AL163832 (AtPLC10).

4. Discussion

Apart from their similar substrate specificity and Ca^{2+} requirement, the biochemical and molecular characteristics of plant PI-PLCs are much more poorly understood than those of the animal isoforms. We demonstrate here that the *A. thaliana* PI-PLC isoform AtPLC2 is predominantly localized in the plasma membrane. This represents the first subcellular localization of a plant PI-PLC isoform in a non-transgenic plant. These results agree with those reported by [13] for a soybean PI-PLC expressed as a tagged fusion protein in transgenic tobacco plants. This localization is in marked contrast to that of PLCδ, the closest relative of plant PI-PLCs from animal cells. PLCδ has been shown to be present in both the cytosolic and particulate fractions of several different animal cells (e.g. [24]). The PI-PLC activity present in plasma mem-

branes from *A. thaliana* and other plant species shows catalytic properties very similar to those of animal PI-PLCs, i.e. it preferentially hydrolyzes PtdIns(4,5)P_2 and PtdIns4P, and is Ca^{2+}-dependent (present study, [11,17]). Although no sequence for any of the PI-PLCs purified from plant tissues has been reported, it is most likely that they are encoded by a member (or several members) of the only family of *PI-PLCs* identified in plants. Some plant soluble fractions have been shown to contain a phospholipase C activity that preferentially hydrolyzes PtdIns and requires Ca^{2+} in the millimolar range [25,26]. It is unlikely that one of the *PI-PLC* genes identified so far in plants is responsible for such an activity, since they all encode proteins with a domain arrangement identical to that of AtPLC2, and recombinant PI-PLCs with high PtdIns(4,5)P_2- and low PtdIns-hydrolytic activity only have been obtained from these genes [12,13,15]. The *A. thaliana* genome contains two genes whose predicted encoded proteins show significant similarity with the PtdIns-specific phospholipase C from *B. cereus* and *Listeria monocytogenes*. We expressed one of these genes in *E. coli*, but no PtdIns-hydrolyzing activity could be detected with the purified recombinant protein under the different assay conditions tested (unpublished observation).

A distinguishing feature of the plant PI-PLC proteins is that they do not contain a PH domain. In animal PI-PLC-δ, this domain is required for interaction with the plasma membrane [24], and is involved in the binding of the lipid substrate and in processive catalysis [5,27]. However, the C2 domain is also involved in membrane interaction although the present view is that it is not sufficient to position the enzyme in a catalytically active orientation [27]. As plant PI-PLCs lack a PH domain, their interaction with the plasma membrane and PtdIns(4,5)P_2 must clearly differ from PI-PLC-δ. The C2 domain of plant PI-PLCs may be sufficient for membrane and/or lipid binding since, as evidenced in Fig. 3C, only the deletion variant lacking the C2 domain (N-term) was unable to bind lipid vesicles. Apart from their C2 domain, other regions in plant PI-PLCs may be required for plasma mem-

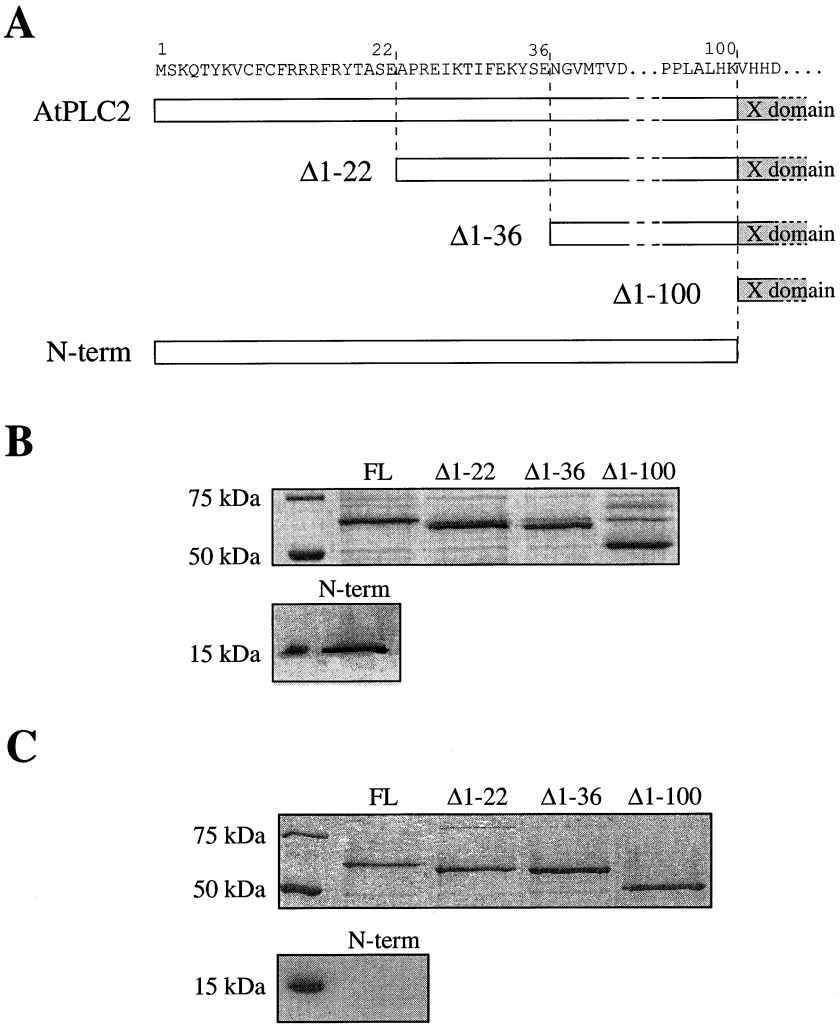

Fig. 3. Description, expression, and lipid-binding properties of AtPLC2, the three deletion mutants, and the N-terminal domain (N-term). A: Representation of AtPLC2 and the three deletion mutants Δ1–22, Δ1–36, and Δ1–100. The amino acid sequence is indicated at the top. B: SDS–PAGE analysis of the different deletion mutant proteins. 10 μg of each recombinant protein were applied. C: Lipid-binding properties of AtPLC2, the three deletion mutants, and N-term. Recombinant proteins, lacking the GST tag, were incubated with lipid vesicles consisting of a mixture of PtdSer, PtdEtn and PtdIns(4,5)P_2 in the presence of 0.2 mM $CaCl_2$ as described in Section 2. Proteins recovered in the pellet after centrifugation were resolved by SDS–PAGE on a 12% polyacrylamide gel. When lipids were absent during the binding assay no proteins were pelleted.

Table 2
Enzyme activity of full-length AtPLC2 and N-terminal deletion mutants

AtPLC2 constructs	PI-PLC activity[a] (nmol PtdIns(4,5)P_2 hydrolyzed min^{-1} mg^{-1})	
	EGTA	10 μM Ca^{2+}
Full-length	0.2 ± 0.2 (10)	209 ± 20 (10)
Δ1–22	0.5 ± 0.3 (5)	216 ± 25 (5)
Δ1–36	0 ± 0 (5)	0 ± 0 (5)
Δ1–100	0 ± 0 (3)	0 ± 0 (3)

[a]Average values ± standard deviation from 3–10 independent experiments (number in parentheses).

brane association, such as hydrophobic moieties, with or without post-translational modification. The absence of a PH domain also suggests that plant PI-PLCs do not exhibit processive catalysis, i.e. the ability to catalyze several cycles of PtdIns(4,5)P_2 hydrolysis.

The determination of the crystal structure of rat PLC-δ1 demonstrated that the X and Y domains form the catalytic domain of the protein, and that the domain present between the PH and X domains forms an EF-hand-like structure. This structure was therefore termed EF-hand domain [5]. PLC-β1 and PLC-γl from rat were predicted to also contain an EF-hand domain [5]. Deletion studies demonstrated that the second loop of this EF-hand is required for rat PLC-δ1 to be active [28]. We here show that AtPLC2, and many other plant PI-PLCs, possess at their N-terminal a domain predicted to be structurally very similar to the second loop of the EF-hand domain of PLC-δ1. The first predicted helix, corresponding to residues 25–33, of this putative EF-hand domain is essential to preserve the PI-PLC activity in AtPLC2 (Table 2). This suggests that the EF-hand of plant PI-PLCs may play a role, by analogy to the suggested function of the second loop of the EF-hand of PLC-δ1, in the formation of a functional catalytic domain. Obtaining a three-dimensional structure of a plant PI-PLC will help in confirming that the conserved N-terminal domain of plant PI-PLCs indeed is an EF-hand domain. Plant PI-PLCs are the smallest of all known functional PI-PLC enzymes, and may represent the ancestral forms of these enzymes. It is however intriguing that animal PI-PLCs may have arisen from an isoform by addition/duplication of two EF-hand motifs to a pre-existing form already containing two EF-hand motifs.

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