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Preparation of polyclonal antibody specific for AtPLC4, an *Arabidopsis* phosphatidylinositol-specific phospholipase C in rabbits

Zhixiang Cao [a,1], Jiewei Zhang [a,1], Yuan Li [a,1], Xiaojing Xu [a], Guoqin Liu [a], Madan K. Bhattacharrya [b], Hailian Yang [a,*], Dongtao Ren [a,*]

[a] *State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing 100094, China*
[b] *Agronomy Department, Iowa State University, Ames, IA 50011-1010, USA*

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Abstract

Phosphoinositide-specific phospholipase Cs (PI-PLCs) are important enzymes in eukaryotes, which catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate into the two second messengers inositol 1,4,5-trisphosphate and diacylglycerol. The *Arabidopsis* genome contains nine putative PI-PLC genes. AtPLC4, an abiotic stress induced gene, has been reported to encode an active PI-PLC isoform. However, the exact roles of putative AtPLC4 in plant remain to be elicited. The first 108 amino acid residues of the N-terminal of AtPLC4, referred to as AtPLC4 N, was expressed as a recombinant protein in *Escherichia coli* and used as antigen in generating antibody. Purified recombinant proteins including AtPLC1 to AtPLC5, AtPLC8, AtPLC9 and AtPLC4 N were transferred onto the same blot to test specificity of the prepared antibody. Western blot result shows that only AtPLC4 and AtPLC4 N can be recognized by the antibody. The antibody recognized a protein of approximately 68 kDa in the plasma membrane fraction and cytosolic fractions prepared from *Arabidopsis thaliana* plants. This corresponds very well with the calculated molecular weight of AtPLC4. The results suggest that AtPLC4 may encode a plasma membrane-associated protein.
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Keywords: *Arabidopsis thaliana*; Phosphatidylinositol-specific phospholipase C; AtPLC4; Polyclonal antibody

Phosphatidylinositol-specific phospholipase C (PI-PLC)[2] is a critical enzyme in various signal- transduction pathways in eukaryotic cells. It hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into two second messengers, inositol 1,4,5-trisphosphate (IP3) and 1,2-diacyl-glycerol (DAG). IP3 induces the release of Ca^{2+} from internal stores, thereby regulating Ca^{2+} and Ca^{2+}/calmodulin-dependent enzymes and channels. In mammals, DAG activates protein kinase C (PKC), which in turn regulates enzymes, receptors, transport and contractile proteins, and cytoskeletal components via phosphorylation [1].

The PI-PLCs have been classified into five subfamilies: β, γ, δ [2], ε [3,4] and ζ (zeta) [5]. Animal PLC β, γ, δ, ε and yeast PLCs are comprised of core sequence including a pleckstrin homology (PH) domain, EF-hand motifs, an X and a Y domain, and a C2 domain [6–8]. This core sequence constitutes the overall structure of δ-isoenzymes, while the β-, γ-, ε-isoenzymes contain specific insertions in the core sequence (see Fig. 1). X and Y domains constitute the catalytic domain of enzyme [6,9]; the PH domain is required for interaction with the plasma membrane, involved in the binding of lipid substrates and in possessive catalysis [7]; the C2 domain is involved in calcium-triggered phospholipid binding; and the EF-hand domain is required for activation of the enzyme [8]. Animal PLC ζ isoforms [5] and all plant PI-PLCs sequences are structurally identical and

* Corresponding authors. Fax: +86 10 62731332.
E-mail addresses: swxyky@cau.edu.cn (H. Yang), ren@cau.edu.cn (D. Ren).
[1] These authors contributed equally to this work.
[2] *Abbreviations used:* PI-PLC, phosphoinositide-specific phospholipase C; DAG, 1,2-diacyl-glycerol; PKC, activates protein kinase C; PH, pleckstrin homology; IPTG, isopropyl-β-D-thiogalactopyranoside.

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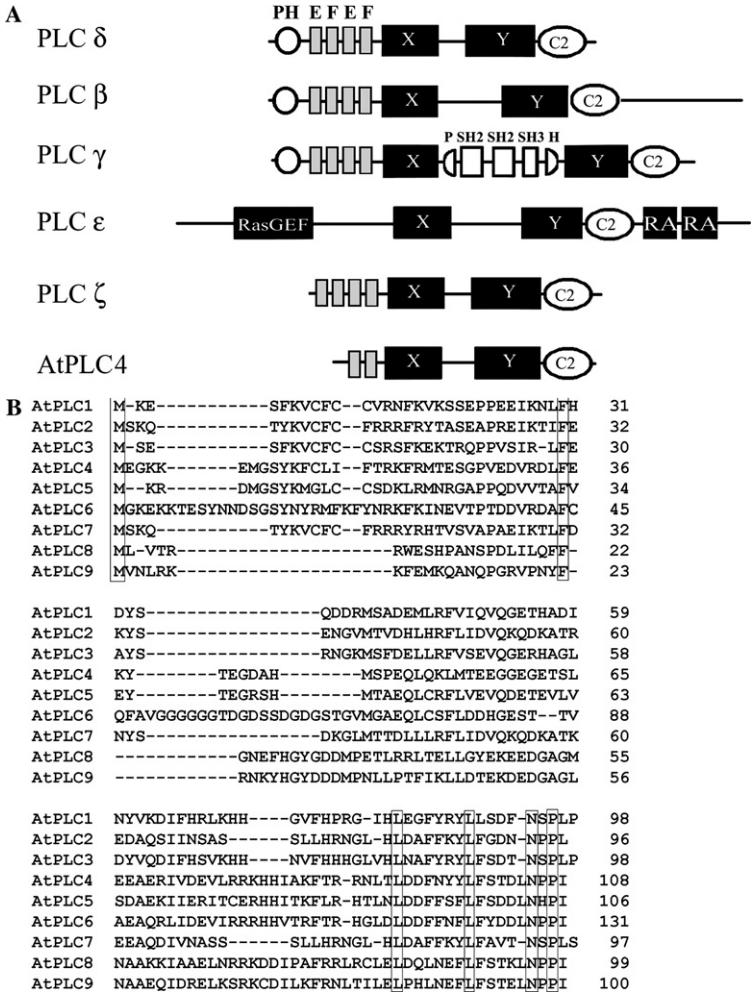

Fig. 1. Schematic representation of the structure of AtPLC4 and comparison of the N-terminal regions using for antibody rising to AtPLC4 with those of other PI-PLCs from *Arabidopsis*. (A) The overall structure of AtPLC4 and PI-PLCs from animals. (B) The predicted N-terminal amino acid sequences of nine PI-PLCs from *Arabidopsis* were aligned using the Cluster W sequence alignment program. Residues identical in all *Arabidopsis* PI-PLC sequences were indicated by boxes. Numbers on the right side indicate the position of the last amino acid in each sequence. Gene loci are: At5g58670 (*AtPLC1*), At3g08510 (*AtPLC2*), At4g38530 (*AtPLC3*), At5g58700 (*AtPLC4*), At5g58690 (*AtPLC5*), At2g40116 (*AtPLC6*), At3g55940 (*AtPLC7*), At3g47290 (*AtPLC8*) and At3g47220 (*AtPLC9*).

composed of an EF-hand motif, an X and a Y domain, and a C2 domain [9].

In plants, the PI-PLC-mediated phosphoinositide signaling pathway has been shown to perform important roles in the cellular responses to extracellular stimuli, such as osmotic stress [10], auxin [11], ABA [12–14], light and gravitational force changes [15,16], pathogen attack [17] and pollination [18]. The first cDNA encoding functional PI-PLC was cloned from animal cells in 1988 [19–22]. Seven years later, the first plant PI-PLC clones were reported from *Arabidopsis* [23] and soybean [24]. To date, a large numbers of plant cDNA clones encoding active PI-PLCs and putative PI-PLCs have since been reported from several plant species [9,25–30].

Nine predicted PI-PLC genes were found in the *Arabidopsis* genome [9]. Except AtPLC1 is known to be required for secondary responses to ABA signals [14], the exact roles of other PI-PLC isoforms in *Arabidopsis* are still obscure. Since the specific antibodies are useful for immuno-detection (such as Western blot analysis, immuno-precipitation and immuno-localization) to distinguish isoform (or isoforms), antibodies that presumably recognize each isoform are valuable. However, right now only reported specific antibody is for AtPLC2 [31]. Here, we describe the induction of polyclonal antibody specific for AtPLC4. Using this antibody, we also show that AtPLC4 may encode a plasma membrane-associated PI-PLC.

Materials and methods

Plant material

Arabidopsis thaliana (Columbia ecotype) plants were grown at 22 °C in a growth room with a 12 h light cycle at a photon flux density of 100 microeinsteins/$m^{-2}\,s^{-1}$. The fully expended leaves from 4-week-old plants were used for experiments. Samples were taken and quickly frozen in liquid nitrogen and stored at −80 °C until use.

Construction of fusion genes

Total RNA was isolated from *Arabidopsis* plants using Trizol reagent (Invitrogen), following the manufacturer's instructions. The reverse transcription was performed using oligo(dT) 16 as the primer and total RNA as template. M-MLV reverse transcriptase (Promega) was used to reverse transcribe the poly(A^+) RNAs. The coding region of AtPLC4 (GenBank Accession No. AF434167) was amplified by reverse transcription PCR using the forward primer AtPLC4-C_3: 5′-GGATCCATGGAAGGAAAAAAAGAG-3′ and the reverse primer AtPLC4-A_2: 5′-ATCTAAATGGATACACGCACCAA-3′. The PCR fragment was purified and ligated into pGEM-T Easy vector (Promega), yielding the intermediate construct pGEM-T-AtPLC4. The *Bam*HI/*Eco*RI fragment from pGEM-T-AtPLC4 was ligated into the *Bam*HI/*Eco*RI sites of pET30a (Novagen) in frame to yield the final construct pET-AtPLC4. A 6× His tag was fused to AtPLC4 at N-terminus. The coding region of AtPLC1, AtPLC2, AtPLC3, AtPLC5, AtPLC8 and AtPLC9 (we could not isolate the full length cDNAs for AtPLC6 and AtPLC7 by RT-PCR from all tissues) were amplified by reverse transcription PCR and cloned into pET30a using the same strategies as the AtPLC4 construct.

Sequence, encoding the N-terminal 108 amino acids of AtPLC4 protein (referred to as AtPLC4 N) to be used for antiserum generating, was PCR amplified by using the primers AtPLC4 C_4: 5′-CATATGGAAGGAAAAAAAGAG-3′ and AtPLC4 A_5: 5′-TTAGATAGGAGGATTAAGAT-3′. The PCR fragment was ligated into pGEM-T easy vector to yield pT-AtPLC4 N. The sequence (5′-CCATGGACTCAAAGGACGACGATGACAAGCATATG-3′) encoding the Flag tag was ligated into *Nco*I/*Nde*I digested pET28a to yield pET28a-F plasmid. The pT-AtPLC4-N was digested with *Nde*I/*Eco*RI, and the *Nde*I/*Eco*RI fragment containing N-terminal region of AtPLC4 was ligated into the *Nde*I/*Eco*RI digested pET28a-F to yield the final construct pET-AtPLC4 N.

All the resultant constructs were confirmed by sequencing, and transformed into *Escherichia coli* strain BL21 by applying heat shock.

Expression and purification of seven Arabidopsis recombinant PI-PLCs protein

A single transformed BL21 colony harboring a recombinant PI-PLC plasmid was grown in 5 ml LB Broth containing 50 mg/L kanamycin overnight at 37 °C. Two milliliters of this culture was transferred into one liter of fresh LB medium containing kanamycin and grown at 37 °C to an OD at 600 nm of 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.1 mM to induce recombinant protein expression. The cultures at 2 h post-induction were centrifuged at 5000*g* for 15 min at 4 °C and 2.88 g for AtPLC1, 2.76 g for AtPLC2, 3.16 g for AtPLC3, 2.08 g for AtPLC4, 2.64 g for AtPLC5, 3.3 g for AtPLC8 and 3.08 g for AtPLC9 of wet weight cells in pellet was harvested. The pellets were washed and stored at −80 °C.

The recombinant cell pellet was resuspended in 20 ml of 20 mM Tris–HCl buffer, pH 8.0, containing 150 mM NaCl and 1 mM PMSF. The tube was placed in an ice bath and sonicated. The sample was centrifuged at 12,000*g* at 4 °C for 30 min. After centrifugation, the recombinant AtPLC1, AtPLC2, AtPLC3, AtPLC8 and AtPLC9 proteins were purified from the resultant pellets by using preparative SDS–polyacrylamide gel electrophoresis and the recombinant AtPLC4 and AtPLC5 proteins were purified from the supernatants by passing the supernatant through columns of Ni^{2+} Chelating Sepharose Fast Flow (Amersham-Pharmacia Biotech) following the manufacturer's instructions.

Antiserum preparation using purified AtPLC4 N

A single transformed BL21 colony harboring the pET-AtPLC4-N plasmid was cultured and induced as described above. Cell pellet (5.28 g) was resuspended in 20 ml of 20 mM Tris–HCl buffer, pH 8.0, containing 150 mM NaCl and 1 mM PMSF. The tube was placed in an ice bath and sonicated. The sample was centrifuged at 12,000*g* at 4 °C for 30 min to collect the pellet. The pellet was resuspended in Tris–HCl buffer and the process of sonication and centrifugation was repeated twice to release trapped proteins. The resultant pellet containing the Flag-AtPLC4 N polypeptide was finally dissolved in 1× SDS loading buffer (62.5 mM Tris–HCl, pH 6.8, containing 1.6% SDS, 10% glycerol, 0.7 M β-mercaptoethanol and 0.002% bromophenol blue). The sample was boiled for 5 min and then centrifuged at 10,000*g* for 15 min. The protein in supernatant was separated by preparative SDS–polyacrylamide gel electrophoresis (SDS–PAGE), using 4.5% stacking gel and 10% separating gel. After electrophoresis, proteins were visualized and the band corresponding to Flag-AtPLC4 N polypeptide was excised, and the polypeptide was eluted electrophoretically from the gel as described by Dager [32]. After dialysis against PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4, 140 mM NaCl and 2.7 mM KCl) at 4 °C for 24 h, the solution containing the purified Flag-AtPLC4 N polypeptide was centrifuged at 12,000*g* at 4 °C for 15 min. The purity of Flag-AtPLC4 N polypeptide in the supernatant was determined on a Coomassie-stained 10% SDS polyacrylamide gel and verified by Western blot using anti-Flag monoclonal antibody. The concentration of the protein was quantified using Bradford reagent (Bio-Rad) using BSA as the protein standard.

The purified Flag-AtPLC4 N polypeptide was injected into rabbits for raising antibody. Two New Zealand White Rabbits were immunized intravenously with 100 μg of the Flag-AtPLC4 N polypeptides per rabbit and followed by a second injection two weeks later. After the second injection, four additional injections (100 μg protein per injection) were done in 15 days. A week after the last injection, sera were collected and used to test the anti-AtPLC4 N antibodies.

The rabbit with the best reactivity toward Flag-AtPLC4 N was sacrificed and the serum was collected. Specific antibodies to AtPLC4 N were purified using Flag-AtPLC4 N polypeptide in a blot purification method [33], and stored as a solution (1 mg/ml IgG in 50 mM PBS containing 30% glycerol) at 4 °C.

Plasma membrane purification

Arabidopsis ecotype Columbia leaves were homogenized in an ice-cold buffer containing 25 mM Tris–Mes, pH 7.5, 0.5 M sucrose, 3 mM DTT, 3 mM EGTA and 0.6% (w/v) polyvinylpolypyrrolidone and filtered through four layers of cheesecloth. The filtrate was centrifuged at 10,000*g* for 10 min to remove debris and the supernatant was re-centrifuged at 50,000*g* for 30 min. The resulting supernatant was used as cytosolic fraction. Plasma membranes were prepared from the pellet by aqueous two-phase partitioning as described by Larsson et al. [34].

SDS–PAGE and Western blot

Proteins were separated by SDS–PAGE according to Laemmli [35], using a 4.5% stacking gel and 10% separating gel. After electrophoresis, the proteins were electronically transferred onto nitrocellulose membranes. Western blotting was performed as described by Towbin et al. [36]. The membranes were incubated with monoclonal anti-Flag antibody, or monoclonal anti-His or the anti-AtPLC4 N primary antibodies, respectively. Following three washings, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse or horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies, respectively. The membranes were visualized by using the Lumi-light Western blotting substrate kit (Roche, Inc.), following the manufacturer's instructions.

Results

N-Terminal amino acid sequences of Arabidopsis PI-PLC isoforms are less conserved

The overall structure of AtPLC4, predicted by the deduced amino acid sequence contains an EF hand, an X and a Y domain and a C2 domain, and is most similar to the animal PI-PLC-ζ isoform (Fig. 1A). Alignment of the N-termini deduced amino acid sequences of nine PI-PLCs of *Arabidopsis* reveals that the N-termini sequences (including the EF hand) are conserved to a lesser degree than the other three domains. Only six residues of the N-terminal sequence are identical (Fig. 1B). Therefore, this region of AtPLC4 was selected as an immunogen for generating polyclonal antibody.

Expression and purification of the recombinant proteins

The *Arabidopsis* PI-PLCs were expressed as 6× His-tag fused recombinant protein in *E. coli* cells and all recombinant proteins were purified. The amounts, purities and yields of the PI-PLC proteins were 1.54 mg, 96.9% and 5.6% for AtPLC1, 1.69 mg, 95.3% and 37.2% for AtPLC2, 0.74 mg, 91.2% and 7.8% for AtPLC3, 5 mg, 94.6% and 41.6% for AtPLC4, 4 mg, 97% and 47.1% for AtPLC5, 0.46 mg, 88.9% and 1.3% for AtPLC8, 0.41 mg, 90% and 3.4% for AtPLC9.

To facilitate the detection of the AtPLC4 protein, antiserum against the expressed Flag-AtPLC4 N polypeptide was raised. For production of this antibody, a Flag-epitope tag instead of a 6× His-tag was fused to *E. coli* expressed AtPLC4 N. This change either facilitated the detection of expressed AtPLC4 N or avoided the possibility that the antibody recognized the recombinant AtPLC4 due to tag peptides. As shown in Fig. 2, the Flag-AtPLC4 N was expressed efficiently in bacteria both in insoluble and soluble forms (Fig. 2, lanes 2, 3 and 4). The Flag-AtPLC4 N in the insoluble fraction of the bacterial lysate was further purified and recovered as a soluble form following the gel elution (Fig. 2, lane 4). The insoluble fraction contained 46.4 mg of crude proteins. It was estimated that the AtPLC4 N protein (52.8% of the insoluble protein as calculated by scanning densitometry) was 24.5 mg. After electro-elution, 5.3 mg of approximately 95.1% pure Flag-AtPLC4 N was obtained. The approximate yield of AtPLC4 N was 21.6%.

The anti-AtPLC4 N antibody is specific for AtPLC4

Five injections (100 μg Flag-AtPLC4 N per injection) were given to two rabbits and the serum containing anti-AtPLC4 N antibody with the stronger specificity was collected. The Western blotting of purified recombinant His-AtPLC4, Flag-AtPLC4 N and the six other His-tagged *Arabidopsis* PI-PLCs (as shown in Fig. 3A, lanes

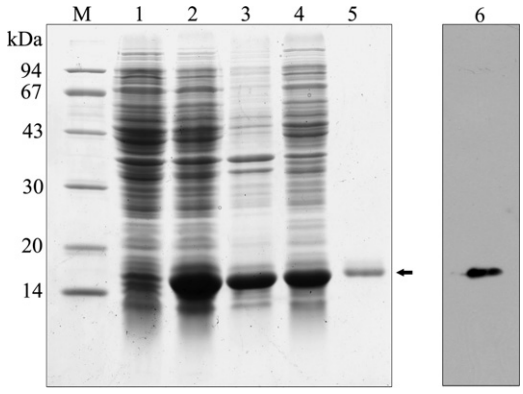

Fig. 2. Analyses of expression and purification of the recombinant AtPLC4 N. Proteins were separated on a 10% SDS–PAGE gel and then stained with Coomassie blue R250 (lanes 1–5), and Western blot using anti-Flag M2 monoclonal antibody (lane 6). Lane 1, total proteins in non-induced bacteria lysate; lane 2, total proteins in induced *E. coli*; lane 3, the insoluble protein in induced *E. coli*; lane 4, the soluble protein in induced *E. coli*; lane 5, the purified recombinant AtPLC4 N protein by preparative SDS–PAGE ; lane M, marker proteins with molecular masses in kilodaltons.

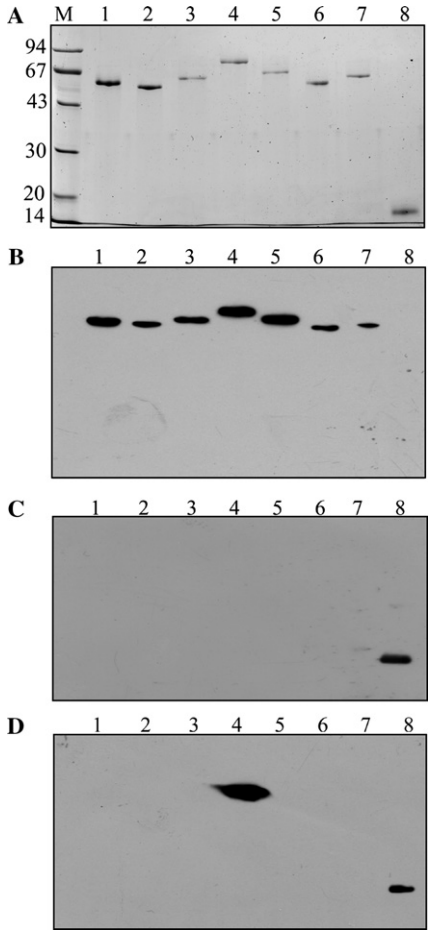

Fig. 3. The obtained anti-AtPLC4 N antibody specifically recognizes expressed Flag-AtPLC4 N polypeptides and His-AtPLC4 recombinant protein. (A) SDS–PAGE of the seven purified AtPLC proteins. (B) Western blot probed with anti-His monoclonal antibody at 1:10,000 dilution. (C) Western blot probed with anti-Flag monoclonal antibody at 1:10,000 dilution. (D) Western blot probed with affinity-purified anti-AtPLC4 N polyclonal antibody. The antibody was used as 0.1 μg/ml in reaction. (A–D) lane 1, purified His-tag fused recombinant AtPLC1 protein; lane 2, purified His-tag fused recombinant AtPLC3 protein; lane 3, purified His-tag fused recombinant AtPLC2 protein; lane 4, purified His-tag fused recombinant AtPLC4 protein; lane 5, purified His-tag fused recombinant AtPLC5 protein; lane 6, purified His-tag fused recombinant AtPLC8 protein; lane 7, purified His-tag fused recombinant AtPLC9; lane 8, purified Flag-tag fused recombinant AtPLC4 N; lane M, marker proteins with molecular masses in kilodaltons.

1–7, we expressed all *Arabidopsis* PI-PLCs as recombinant proteins) has shown that the anti-His monoclonal antibody recognized His-AtPLC4 (Fig. 3B, lane 4) and all other His-tagged AtPLCs (Fig. 3B, lanes 1–3 and lanes 5–7), the anti-Flag M2 monoclonal antibody only recognized Flag-AtPLC4 N (Fig. 3C, lane 8). Furthermore, the affinity-purified AtPLC4 polyclonal antibody specifically recognized both of the AtPLC4 N and AtPLC4 recombinant proteins, but not the other *Arabidopsis* PI-PLC isoforms recombinant proteins (Fig. 3D, lanes 4 and 8). The result suggests that, the generated antibody can be used for further immuno-detection of AtPLC4.

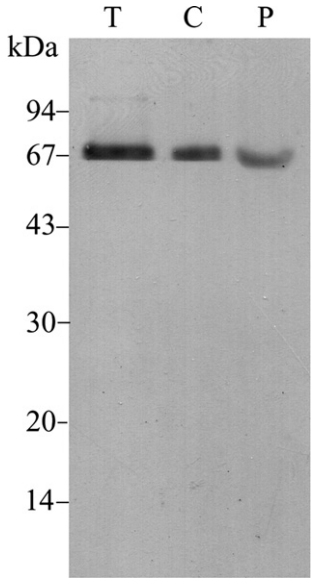

Fig. 4. *Arabidopsis* AtPLC4 is located in both the cytosol and plasma membrane. Cell fractions were prepared from wild type *Arabidopsis* plants. Plasma membranes were purified by partitioning in an aqueous dextran-polyethylene glycerol two-phase system as described in Materials and methods. The proteins were separated by SDS–PAGE and then AtPLC4 protein was detected by Western blot using anti-AtPLC4 antibody. Each lane was loaded with 10 μg protein. Lane T, total protein extracts with 1× SDS sample buffer; lane C, cytosolic fraction; lane P, purified plasma membranes fraction. Molecular mass standard are shown at left.

AtPLC4 is localized in both of the cytosolic and plasma membrane fraction

Both soluble and particulate fractions have been shown to contain PI-PLCs activities in plants [24,31]. In order to determine the subcellular localization of AtPLC4, plasma membrane was prepared and further purified by using the two-phase partitioning method. The cytosolic fractions and purified plasma membranes were analyzed by Western blots using the anti-AtPLC4 N antibody. A single band was revealed in both cytosolic and plasma membrane fractions (Fig. 4). The molecular weight of the band was approximately 68 kDa, which was consistent with the calculated molecular weight based on deduced amino acid sequence of the AtPLC4.

Discussion

Ca^{2+} ion-mediated signal transduction pathways are involved in the regulation of a variety of cellular processes [1] and cell responses to extracellular stimuli [1,10–18]. In animal cells, Ca^{2+} is mainly controlled by a phosphoinositides (referred as PIs) turnover system, in which PI-PLC plays a key role.

All known plant PI-PLCs do not contain PH domain, that is required for the PI-PLCδ interaction with the plasma membrane [7], and involved in the binding of PIP2 and processive catalysis [6]. The mechanism of interaction

between the plant PI-PLC and PIP2 in membranes is thought to differ from that of animal PI-PLCδ. Despite the results that the C2 domain and possibly other regions in plant PI-PLCs are required for lipid, membrane and Ca^{2+} binding [31,37], the regulation of plant PI-PLCs is still obscure. Recently, a novel sperm-specific PI-PLC, PLC ζ has been identified [5,38]. It can trigger Ca^{2+} oscillations in mouse eggs. PLC ζ contains an EF-hand, an X and a Y domain, a C2 domain and lacks a PH domain. By comparing the overall structure, plant PI-PLCs are most similar to animal PLC ζ.

Among the nine *Arabidopsis* PI-PLC, AtPLC1 to AtPLC5 were expressed as recombinant proteins, and all of them shown to have Ca^{2+}-dependent PIP2 hydrolyzing activity [23,29–31]. AtPLC1 is reported to be expressed at a low level in vegetative organs under normal conditions, and induced when plants are exposed to ABA, NaCl and cold [23,30]. Further analysis on the responses of antisense and sense transgenic plants to ABA revealed that, AtPLC1 is required for secondary response to ABA signals [14]. Expression of the AtPLC4 and AtPLC5 are induced by cold, dehydration and NaCl [27,30]. Expression of AtPLC3 was flowering induced and unaffected by stresses [28,30]. Hirayama's results show that AtPLC2 is constitutively expressed in almost all organs except siliques [29], while Hunt et al reported that AtPLC2 is undetectable in flowers [30].

To distinguish the AtPLC4 protein from other isoforms and analyze its subcellular localization, we generated an AtPLC4 antibody using the N-terminal peptides, AtPLC4 N. The AtPLC4 antibody only can recognize AtPLC4 N and AtPLC4 recombinant proteins, but not other PI-PLCs isoforms (at least the six other PI-PLCs recombinant proteins tested). This suggested that the antibody can be used as specific antibody to detect AtPLC4 protein. Western blots on cellular fractions revealed that the antibody recognizes a single band with the predicted size of AtPLC4 in both cytosolic and membrane fractions of *Arabidopsis* leaf extract. The result agrees with many reports that the PI-PLC activity can be detected in both the soluble and the membrane fractions of *Arabidopsis* and other plant cells [24,25,30]. The membranes association can be explained as AtPLC4 binds to PIP2 which is localized in the plasma membrane or other internal membranes as a membrane-associated protein and after PIP2 was hydrolyzed, AtPLC4 can be released into cytosol as a soluble form. We propose here that AtPLC4 is a membrane-associated protein.

Acknowledgments

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