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Subcellular Distribution and Tissue Expression of Phospholipase Dα, Dβ, and Dγ in Arabidopsis[1]

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Three phospholipase Ds (PLDs; EC 3.1.4.4) have been cloned from Arabidopsis, and they exhibit two distinct types of activities: polyphosphoinositide-requiring PLDβ and PLDγ, and polyphosphoinositide-independent PLDα. In subcellular fractions of Arabidopsis leaves, PLDα and PLDγ were both present in the plasma membrane, intracellular membranes, mitochondria, and clathrin-coated vesicles, but their relative levels differed in these fractions. In addition, PLDγ was detected in the nuclear fraction. In contrast, PLDβ was not detectable in any of the subcellular fractions. PLDα activity was higher in the metabolically more active organs such as flowers, siliques, and roots than in dry seeds and mature leaves, whereas the polyphosphoinositide-dependent PLD activity was greater in older, senescing leaves than in other organs. PLDβ mRNA accumulated at a lower level than the PLDα and PLDγ transcripts in most organs, and the expression pattern of the PLDβ mRNA also differed from that of PLDα and PLDγ in different organs. Collectively, these data demonstrated that PLDα, PLDβ, and PLDγ have different patterns of subcellular distribution and tissue expression in Arabidopsis. The present study also provides evidence for the presence of an additional PLD that is structurally more closely related to PLDγ than to the other two PLDs.

Activation of PLD (EC 3.1.4.4) has been proposed as an important step in the signaling of plant responses to ABA, ethylene, and wounding (Ryu and Wang, 1996; Fan et al., 1997; Ritchie and Gilroy, 1998). PLD also has been suggested to play a role in senescence, nutrient starvation, and plant-pathogen interactions (Young et al., 1996; Lee et al., 1998). Recently, it was discovered that there are multiple forms of PLD with distinct regulatory and catalytic properties (Pappan et al., 1997a, 1997b; Qin et al., 1997). Three PLDs, designated PLDα, PLDβ, and PLDγ, have been cloned from Arabidopsis and are encoded by distinct PLD genes. PLDα is the conventional, prevalent form that is polyphosphoinositide independent when assayed at millimolar concentrations of Ca^{2+}. In contrast, PLDβ and PLDγ require a polyphosphoinositide cofactor and are most active at micromolar concentrations of Ca^{2+}. PLDβ and PLDγ hydrolyze phosphatidylserine and *N*-acylphosphatidylethanolamine, but PLDα does not (Pappan et al., 1998). The three PLDs all hydrolyze PC, PE, and phosphatidylglycerol, but the conditions for hydrolysis by PLDα are drastically different from those for hydrolysis by PLDβ and PLDγ. These distinct biochemical properties have led to the hypothesis that these PLDs in plants are regulated differently and may have unique cellular functions (Wang, 1997).

Understanding whether these PLDs are expressed differently in various organs and/or have different subcellular locations may provide further insights into the role and regulatory mechanisms of individual PLDs. The conventional plant PLD, now known as PLDα, is present in soluble and membrane-associated fractions, and its relative distribution between the two fractions varies, depending on the tissues and developmental stages (Dyer et al., 1994). The conventional PLD has been found in plasma membrane, microsomal membranes, mitochondrial membranes, and vacuoles but not in chloroplasts (Brauer et al., 1990; Xu et al., 1996). However, nothing is known about the intracellular distribution and expression of the newly identified PLDβ and PLDγ. Therefore, this study was undertaken to compare how these PLDs are expressed and distributed in different organelles and tissues of Arabidopsis.

MATERIALS AND METHODS

Plant Material

Arabidopsis ecotype Columbia and PLDα-suppressed, antisense Arabidopsis plants were used in this study. The production of the antisense plants has been described (Fan et al., 1997). Seeds were sown in soil and cold treated at 4°C overnight. Plants were grown under 14-h/10-h light/dark cycles with cool-white fluorescent light of 100 μmol m^{-2} s^{-1} at 23°C ± 3°C. Leaves from 6- to 8-week-old plants were used for all subcellular fractionations. All isolation procedures were performed at 4°C unless indicated otherwise.

Protein Extraction and Assay of PLD Activities

Total protein from Arabidopsis tissues was extracted by grinding with an ice-chilled mortar and pestle with buffer A containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, 0.5 mM PMSF, and 2 mM DTT. The homogenate was centrifuged at 10,000*g* for 10 min at 4°C to remove tissue debris, and the supernatant was centrifuged at 100,000*g* for

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Abbreviations: GST, glutathione *S*-transferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLD, phospholipase D.

60 min at 4°C. The resulting supernatant was referred to as the soluble fraction, and the pellet, which was referred to as the membrane fraction, was suspended in buffer A and centrifuged again at 100,000*g* to reduce cytosolic contamination. Protein concentration was determined by the method of Bradford (1976) using a kit (Bio-Rad).

The PIP_2-independent and -dependent PLD activities were determined based on procedures described previously (Pappan et al., 1997a). Briefly, the PIP_2-independent PLD activity was assayed in the presence of 100 mM Mes, pH 6.5, 0.5 mM SDS, 1% (v/v) ethanol, 25 mM $CaCl_2$, 1 mM egg-yolk PC mixed with dipalmtoylglycerol-3-phospho [methyl-^{3}H]choline, and 2 to 10 μg of protein in a total volume of 200 μL. The reaction mixture of the PIP_2-dependent assay included 100 mM Mes, pH 7.0, 100 μM $CaCl_2$, 2 mM $MgCl_2$, 80 mM KCl, 0.4 mM lipid vesicles, and 2 to 10 μg of protein at a total volume of 100 μL. The lipid vesicles were made of PE:PIP_2:PC at the ratio of 85:6.5:8.5 mol %. The PLD-mediated hydrolysis of PC was measured using dipalmtoylglycerol-3-phospho[methyl-^{3}H]choline. Release of [^{3}H]choline into the aqueous phase was quantitated by scintillation counting in both assays.

Antibody Purification and Immunoblotting

Antibodies to PLDα and PLDβ were raised against two 12-amino acid peptides at their respective C termini, and the antibody to PLDγ was raised against a 12-amino acid peptide near its C terminus (Pappan et al., 1997a, 1997b). The expression of GST-fused PLDα was described previously (Wang et al., 1994), and the PLDβ and PLDγ were expressed in the same way. The GST-fusion proteins of PLDα, PLDβ, and PLDγ were extracted and affinity purified using a glutathione-agarose column according to the manufacturer's instructions (Pharmacia LKB Biotech). Each PLD protein was separated by 8% SDS-PAGE and transferred onto a PVDF membrane, which was incubated overnight with the respective antiserum at a dilution of 1:250. After the membrane was washed with 1× PBS buffer, the appropriate membrane strips corresponding to the respective GST-PLD proteins were cut, and bound antibodies were eluted for 3 min with 4 mL of a low-pH (2.69) buffer containing 100 mM Gly, 100 mM NaCl, 0.1% Tween 20, and 0.02% sodium azide. The eluent was neutralized rapidly to pH 7.5 with Tris-HCl buffer, pH 9.0, and the purified antibodies were used immediately for immunoblotting or stored at −80°C until use. For immunoblotting, protein fractions were separated in 8% SDS-PAGE gels and transferred onto PVDF membranes. The membranes were incubated with crude serum or affinity-purified antibodies against PLDα, PLDβ, or PLDγ; this was followed by incubation with a second antibody conjugated with alkaline phosphatase. The antibody-antigen complex was visualized by the alkaline phosphatase reaction (Dyer et al., 1994).

Total RNA and mRNA Isolation and RNA Blotting

Total RNA was isolated from different organs of Arabidopsis with a cetyltrimethylammonium bromide extraction method (Fan et al., 1997). Poly(A^+) RNA was isolated from total RNA using an mRNA purification kit according to the manufacturer's instructions (Pharmacia LKB Biotech). For RNA blotting, 20 μg of total RNA or 1.5 μg of mRNA was separated by denaturing 1% formaldehyde-agarose gel electrophoresis and transferred to nylon membranes. PLDα-, PLDβ-, and PLDγ-specific probes were labeled with [α-^{32}P]dATP by random priming. The hybridization, washing, and visualization were performed as described previously (Fan et al., 1997).

Subcellular Fractionation

Plasma and intracellular membrane were prepared by using an aqueous polymer two-phase system (Larsson et al., 1987; Xu et al., 1996). Total membranes were prepared from fully expanded leaves (20 g) and loaded onto a solution to give a 60-g phase system with a final composition of 6.4% (w/w) dextran T500, 6.4% PEG 3350, 0.25 M Suc, and 5 mM potassium phosphate. After mixing, the two phases were separated by centrifugation. The upper phase, containing the plasma membrane, was washed twice with lower-phase polymer, and the lower phase, containing intracellular membranes, was washed twice with upper-phase polymer to reduce contamination. The washed upper and lower phases were diluted 5-fold with a buffer containing 0.25 M Suc and 5 mM potassium phosphate, and then they were centrifuged at 100,000*g* for 60 min. The pellets were resuspended in buffer A.

Chloroplasts were isolated according to the method of Cline et al. (1985). Briefly, leaves (5 g) were ground with an ice-chilled mortar and pestle with a 20-mL grinding buffer, pH 7.5, containing 50 mM Hepes, 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 5 mM ascorbic acid, and 1% BSA. The homogenate was filtered and then centrifuged in a swinging-bucket rotor at 2000*g* for 3 min. The resulting pellet was suspended in 5 mL of grinding buffer and overlaid on a 20-mL Percoll gradient, which contained 7.5 mL of Percoll, 7.5 mL of 2× grinding buffer, and 1 mg of glutathione, and was prepared by centrifugation at 5000*g* for 40 min. The gradient was centrifuged at 2000*g* for 15 min. The lower green band was collected and diluted 3-fold with a buffer containing 50 mM Hepes-KOH, pH 8.0, and 0.33 M sorbitol. Chloroplasts were then pelleted and washed twice with 25 mL of buffer, and the final pellet was suspended in the same buffer.

Mitochondria were isolated according to an established procedure (Edwards and Gradestrom, 1987). Leaves (20 g) were homogenized with a mortar and pestle in 50 mL of prechilled grinding medium containing 0.3 M mannitol, 50 mM Mes, pH 7.2, 1 mM EDTA, 1 mM $MgCl_2$, 0.2% defatted BSA, 0.5% (w/v) PVP-400, 4 mM Cys, and 10 mM β-mercaptoethanol. The homogenates were filtered through four layers of cheesecloth and centrifuged at 3,300*g* for 20 min. The pellet was suspended in 5 mL of resuspension medium I containing 0.3 M mannitol, 20 mM Mes, pH 7.2, 2 mM potassium phosphate, 1 mM EDTA, 0.1% (w/v) defatted BSA, 2 mM $MgCl_2$, and 14 mM β-mercaptoethanol. The suspension was loaded onto a discontinuous gradient composed of 5 mL of 47%, 6 mL of 26%, and 3 mL of 21% (v/v)

Percoll. The gradients were centrifuged at 58,500*g* for 45 min in a swinging-bucket rotor. The mitochondrial band, located at the interface between the 26% and 47% Percoll layers, was removed and diluted with an equal volume of resuspension medium II containing 0.3 M mannitol, 20 mM Mes, pH 7.2, 1 mM EDTA, 0.1% defatted BSA, 2 mM $MgCl_2$, and 2 mM DTT. The diluted mitochondria (5 mL) were loaded onto a second Percoll gradient composed of 7.5 mL of 47% and 7.5 mL of 26% (v/v) Percoll prepared as in the first gradient. The gradient was centrifuged at 58,500*g* for 30 min. The mitochondrial band, located at the interface between the 26% and 47% Percoll layers, was collected and diluted with 10 volumes of medium II. The mitochondria were pelleted by centrifugation at 18,800*g* for 5 min and resuspended in 1 mL of medium II.

To isolate clathrin-coated vesicles, the total membrane pellet was prepared as described for the plasma membrane isolation with a homogenizing medium containing 0.1 M Mes, pH 6.4, 1 mM EGTA, 3 mM EDTA, 1 mM *o*-phenanthroline, 0.5 mM $MgCl_2$, 2 μM leupeptin, 0.7 μM pepstatin, 1 mM PMSF, and 0.2% (w/v) fatty acid-free BSA (Demmer et al., 1993). The resuspended pellet was loaded onto a Suc step gradient (6 mL of 5% and 4 mL of 30% Suc) and centrifuged in a swinging-bucket rotor at 67,000*g* for 40 min. The 5% layer was then removed, diluted with homogenizing medium, and centrifuged in a fixed-angle rotor at 150,000*g* for 90 min. The pellet was resuspended in the homogenizing medium.

Nuclei were isolated according to methods described previously (Luthe and Quatrano, 1980; Liu and Whittier, 1994), with some modifications. Briefly, about 20 g of leaves was washed, cut into pieces with scissors, and ground in liquid nitrogen. The powder was suspended in 50 mL of a buffer containing 0.5 M Suc, 1 mM spermidine, 4 mM spermine, 10 mM EDTA, 10 mM Tris, pH 7.6, and 80 mM KCl. The homogenate was filtered and then centrifuged at 3000*g* for 5 min in a swinging-bucket rotor. The nuclear pellet was dispersed gently in a suspension buffer containing 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 20% glycerol. The nuclear suspension was loaded onto a discontinuous Percoll gradient composed of 5 mL of 40%, 60%, and 80% (v/v) Percoll on 5 mL of 2 M Suc cushion. All of the Percoll solutions contained 0.44 M Suc, 25 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$. The gradients were centrifuged at 4000*g* in a swinging-bucket rotor for 30 min. The white nuclear band appeared in the 80% Percoll layer above the 2 M Suc and was removed with a Pasteur pipette. After dilution, nuclei were pelleted by centrifugation, washed twice with the grinding buffer, and resuspended in the nuclear resuspension buffer.

Assays of Marker Enzymes for Subcellular Fractions

The NADH-Cyt *c* reductase assay was performed at 25°C in a 3-mL reaction volume containing 100 μL of 50 mM NaCN, 200 μL of 0.45 mM Cyt *c*, 2.5 mL of 50 mM sodium phosphate buffer, pH 7.5, and 10 μg of protein extract (Briskin et al., 1987). Cyt *c* oxidase activities were assayed by monitoring the decrease at A_{550} using dithionite-reduced horse heart Cyt *c* as a substrate at 25°C in 50 mM potassium phosphate buffer, pH 7.2. Fumarase activities were determined spectrophotometrically by monitoring the increase at A_{240}, as described by Cooper and Beevers (1969). Vanadate-sensitive ATPase was assayed in a 1-mL reaction volume containing 3 mM ATP, 3 mM $MgSO_4$, 30 mM Tris-Mes buffer, 50 mM KCl, and 10 μg of protein in the presence or absence of 50 μM Na_3VO_4. The ATP substrate was present as Tris salt after treatment with Dowex 50-W exchange resin (H^+ form). The assay was performed at 38°C for 30 min, and the released Pi was determined by using ammonium molybdate.

RESULTS

Subcellular Localization of PIP_2-Dependent and PIP_2-Independent PLD Activities in Leaves

Fractions enriched in the plasma membrane, intracellular membranes, chloroplasts, mitochondria, nuclei, and clathrin-coated vesicles were prepared from fully expanded Arabidopsis leaves. The identity and purity of each fraction were determined by assaying activities of appropriate marker enzymes (Table I). The plasma-membrane fraction showed the highest activity of its marker enzyme vanadate-sensitive ATPase but little activity for the other enzymes tested. The intracellular membrane fraction had the highest activity of Cyt *c* reductase, a marker enzyme of

Table I. *Distribution of marker enzyme activities in subcellular fractions of Arabidopsis*

Intracellualar fractions were isolated from fully expanded leaves of wild-type and PLDα-suppressed antisense Arabidopsis (anti-PLDα) plants. Data are averages of at least four measurements.

Enzyme	Plant	Chl[a]	Mito[a]	Nu[a]	PM[a]	IM[a]	CCV[a]
ATPase[b]	Wild type	0	0	7.2	45.5	9.1	24.9
	Anti-PLDα	1.7	0	1.2	49.3	8.3	18.2
Cyt *c* reductase[c]	Wild type	0	52.3	0	63.6	439.4	287.7
	Anti-PLDα	0	40.1	0	79.7	398.8	210.4
Cyt *c* oxidase[c]	Wild type	0	540.4	89.9	0	96.8	75.7
	Anti-PLDα	32.8	582.3	92.8	0	78.7	69.1
Fumarase[d]	Wild type	4.6	25.1	0	3.9	0	4.0
	Anti-PLDα	3.3	31.7	0	4.5	0	7.3

[a] Chl, chloroplast; Mito, mitochondria; Nu, nucleus; PM, plasma membrane; IM, intracellular membrane; CCV, clathrin-coated vesicle. [b] nmol phosphate min^{-1} mg^{-1} protein. [c] μmol Cyt *c* min^{-1} mg^{-1} protein. [d] nmol malate min^{-1} mg^{-1} protein.

ER, indicating enrichment of ER in this fraction. The mitochondrial fraction displayed high activities of the mitochondrial marker enzymes Cyt *c* oxidase and fumarase, but it had little ATPase and Cyt *c* reductase activities. Chloroplasts and nuclei showed very little enzymatic activities that are characteristic of other organelles. The identities of the chloroplast and nuclear fractions were confirmed further by microscopic observation and DNA agarose-gel electrophoresis (data not shown). The purity of the clathrin-coated vesicle fraction was less defined than that of the other fractions because of the lack of appropriate marker enzymes. The presence of ATPase and Cyt *c* reductase activities indicated that this fraction contained some plasma membrane and ER membranes.

These subcellular fractions were assayed for PIP_2-independent and -dependent PLD activities. The PIP_2-independent assay used 25 mM Ca^{2+}, SDS, and PC as substrate, and previous studies have established that this assay measures the activity of PLDα but not PLDβ or PLDγ (Qin et al., 1997). PLDα activity was detected in the plasma membrane, intracellular membranes, clathrin-coated vesicles, and mitochondrial fractions (Fig. 1A). The highest specific activity was obtained from the plasma membrane, whereas no PLDα activity was found in chloroplasts and nuclei (Fig. 1A). The relative distribution of the PLD activity corroborated well the level of PLDα in various fractions (see Fig. 3A), suggesting that the different methods of fraction isolation did not interfere significantly with the PLDα assay. To confirm that the PIP_2-independent, PC-hydrolyzing activity came from PLDα and not from PLDβ or PLDγ, the same subcellular fractions were prepared from PLDα antisense, transgenic Arabidopsis in which the expression of the PLDα gene was suppressed genetically (Pappan et al., 1997a). Almost no PLDα activity was detected in the subcellular fractions prepared from the PLDα-suppressed leaves.

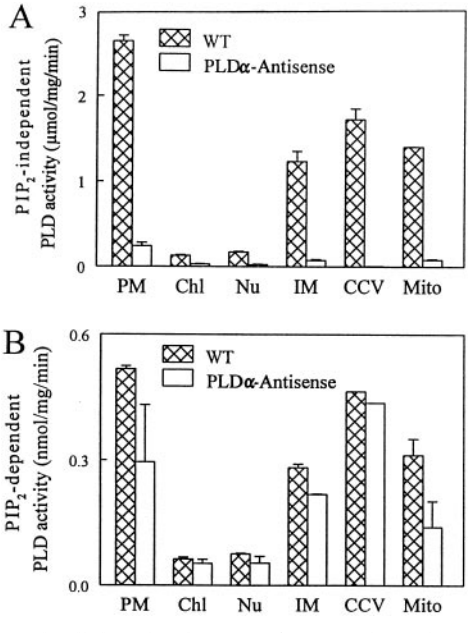

Figure 1. Subcellular distribution of PIP_2-independent (A) and -dependent (B) PLD activities in Arabidopsis leaves. The assay conditions for the two types of PLD activities were as described in "Materials and Methods." Identical methods were used to isolate the intracellular fractions from fully expanded leaves of wild-type (WT) and PLDα antisense Arabidopsis. PM, Plasma membrane; Chl, chloroplast; Nu, nucleus; IM, intracellular membrane; CCV, clathrin-coated vesicle; Mito, mitochondria of wild-type and PLDα antisense plants.

The PIP_2-dependent PLD activity was much higher in the plasma membrane, clathrin-coated vesicles, intracellular membranes, and mitochondria than in chloroplast and nuclear fractions (Fig. 1B). This activity was assayed using 100 μM Ca^{2+} and PIP_2 plus PE and PC vesicles in the absence of SDS. Under these conditions, the activities of PLDβ and PLDγ cloned from Arabidopsis are optimal, whereas PLDα is virtually inactive (Qin et al., 1997; Pappan et al., 1998). To confirm that PLDα did not contribute substantially to this PLD activity, the PIP_2-dependent PLD activity was assayed in the fractions of the PLDα-suppressed transgenic plants. The PIP_2-dependent PLD activity had the same distribution pattern in the PLDα-suppressed plants as in wild-type plants. This result also indicates that the suppression of PLDα in the transgenic plant does not alter the subcellular distribution of the PIP_2-dependent PLDs.

Intracellular Association of PLDα, PLDβ, and PLDγ in Leaves

The PIP_2-dependent PLD activity assays cannot distinguish PLDβ and PLDγ because of their overlapping requirements for PIP_2 and Ca^{2+} (Qin et al., 1997). Thus, the subcellular association of different PLDs was analyzed further by immunoblotting with PLD antibodies raised against 12-amino acid peptides of PLDα, PLDβ, or PLDγ (Pappan et al., 1997b). The specificity of these antibodies to their respective antigens was examined by immunoblotting the purified GST-PLDα, GST-PLDβ, and GST-PLDγ with antibodies to PLDα, PLDβ, and PLDγ. PLDα and PLDβ antibodies reacted only with their respective GST-fusion proteins, whereas the PLDγ antibody cross-reacted with PLDβ but not with PLDα (Fig. 2). Therefore, PLDα and PLDβ antibodies are specific to their respective target proteins, whereas the PLDγ antibody can recognize both PLDβ and PLDγ proteins. These antibodies were affinity purified against the purified, GST-fused PLDα, PLDβ, and PLDγ.

Immunoblotting with PLDα antibodies detected an abundance of PLDα in the plasma membrane, intracellular membranes, clathrin-coated vesicles, and mitochondria, a minute amount in nuclei, and none in chloroplasts (Fig. 3A). As expected, no PLDα protein was detected in the subcellular fractions isolated from the PLDα-suppressed transgenic plant (data not shown). This distribution of PLDα protein was consistent with that of the PIP_2-independent activity (Fig. 1A). PLDγ antibody detected one band in the plasma membrane, intracellular membranes, nuclei, mitochondria, and clathrin-coated vesicles but not in chloroplasts (Fig. 3B). On the other hand, no PLDβ protein was detected in any of the subcellular fractions (data not shown). The titers of the PLDγ and PLDβ antibodies were similar, as estimated by ELISA against the respective synthetic peptides, and the PLDβ antibody was

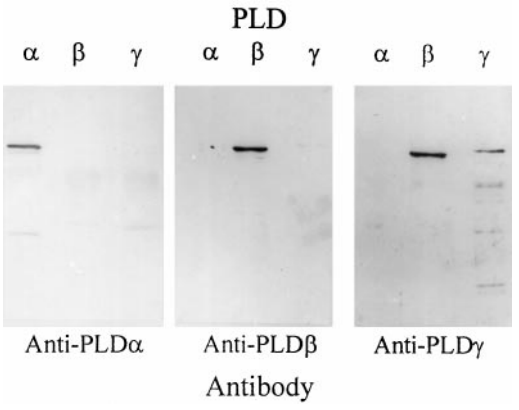

Figure 2. Antibody specificity against PLDα, PLDβ, and PLDγ. Purified GST-PLDα, GST-PLDβ, and GST-PLDγ (0.1 μg per lane) were separated by 8% SDS-PAGE. The blots were incubated with anti-PLDα, anti-PLDβ, or anti-PLDγ polyclonal antibodies (1:1000 dilution) and then the second antibody. PLD-antibody complexes were made visible by alkaline phosphatase.

specific and reacted well to bacterially expressed PLDβ (Fig. 2). Therefore, the inability to detect PLDβ could be attributable to a low level of PLDβ protein in leaves. This result also means that the band detected by the PLDγ antibody can be considered to be PLDγ rather than from PLDβ protein. The relative levels of PLDα and PLDγ proteins in the fractions differed; the banding intensity of PLDα protein was greater in the plasma membrane and clathrin-coated vesicle-enriched fractions, whereas the greatest association of PLDγ protein was with the intracellular membrane fraction. The PLDα and PLDγ bands in the clathrin-coated vesicle fraction migrated more slowly on both blots, and this was found to be caused by a difference in sample-buffer composition.

Organ Distribution of PLDα, PLDβ, and PLDγ

To determine the organ distribution of different PLDs in Arabidopsis, protein extracts from roots, stems, leaves, flowers, siliques, dry seeds, and seedlings were fractionated into soluble and membrane-associated fractions and assayed for PIP_2-independent and -dependent PLD activities. The PIP_2-independent PLDα activity in soluble fractions was high in roots, flowers, and siliques, moderate in stems, and low in seeds, leaves, and seedlings (Fig. 4A). The membrane-associated PLDα activity was highest in siliques, lowest in dry seeds, and intermediate and similar in the other organs (Fig. 4B). In contrast, the specific PIP_2-dependent PLD activity was highest in old, senescing leaves, lowest in dry seeds, and intermediate and similar in the other organs (Fig. 5). The PIP_2-dependent PLD activities were about 2- to 5-fold higher in membrane-associated fractions than in soluble fractions.

The distribution of PLDα protein, as assessed by immunoblotting, was essentially the same as that of PIP_2-independent PLD activity in different organs (Fig. 6). More PLDα protein was present in flowers, stems, roots, and siliques than in other organs. As in subcellular fractions, no PLDβ band was detected using purified PLDβ antibody (data not shown). PLDγ was detected in flowers, stems, roots, and old leaves in soluble fractions (Fig. 7A), whereas weaker signals were found in membrane fractions of flowers, stems, and siliques (Fig. 7B). In addition, two protein bands with estimated molecular masses of 85 and 99 kD were detected by the affinity-purified PLDγ antibody in the soluble fractions of flowers, stems, and old leaves (Fig. 7A), whereas only the lower band was detectable in the membrane fraction. This may suggest the presence of another PLD isoform that is closely related to PLDγ.

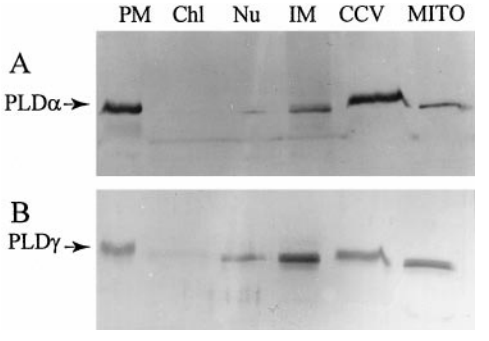

Figure 3. Immunoblot of PLDα (A) and PLDγ (B) in various subcellular fractions. Intracellular fractions were isolated from wild-type Arabidopsis leaves, and equal amounts of proteins (10 μg per lane) were loaded and separated by 8% SDS-PAGE. PLDα and PLDγ were made visible by alkaline phosphatase after blotting with affinity-purified PLDα and PLDγ antibodies, respectively. See Figure 1 for abbreviations.

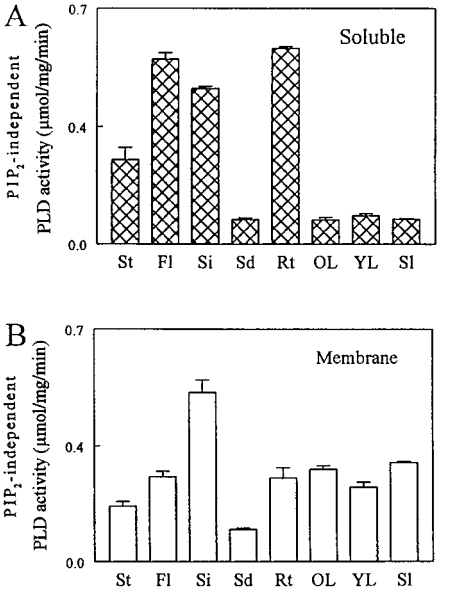

Figure 4. Tissue distribution of PIP_2-independent PLDα activity in Arabidopsis. A, Soluble PLDα activity from 100,000*g* supernatant. B, Membrane-associated PLDα activity from the pellet after centrifugation at 100,000*g* of the 10,000*g* supernatant. St, Stem; Fl, flower; Si, silique; Sd, dry seed; Rt, root; OL, old leaf (bottom leaves of flowering plants with yellowing at the tip); YL, young leaf (not fully expanded, top leaves of 2-month-old plants); Sl, seedling (10 d old).

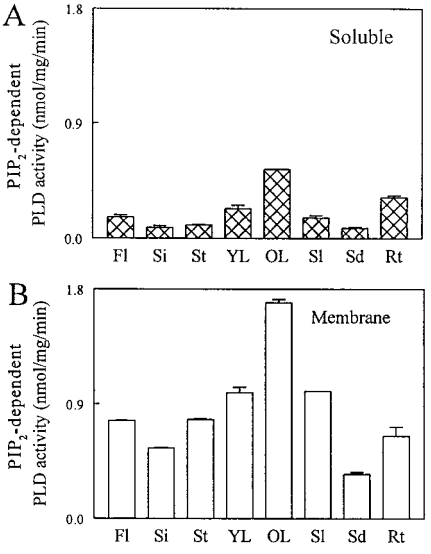

Figure 5. Tissue distribution of PIP_2-dependent PLD activity in Arabidopsis. A and B are soluble and membrane-associated PIP_2-dependent activity, respectively, assayed from various tissues of 2-month-old plants. The protein samples and abbreviations were the same as those used in Figure 4 for assays of PLDα activity.

Tissue Expression of PLDα, PLDβ, and PLDγ Genes

To examine the expression of PLDα, PLDβ, and PLDγ genes in different tissues, RNA-blot analysis was performed using PLDα, PLDβ, and PLDγ cDNAs as probes (Fig. 8). Previous Southern-blot analysis had established that these cDNA probes do not cross-hybridize with one another under highly stringent hybridization conditions (Qin et al., 1997). The level of PLDα transcript was high in roots, stems, and flowers, moderate in leaves, seedlings, and siliques, and undetectable in dry seeds (Fig. 8A). In contrast, the level of PLDγ mRNA was high in roots and flowers, moderate in stems, leaves, and seedlings, low in siliques, and undetectable in seeds (Fig. 8B). Additionally, more than one band was detected on the RNA blot when PLDγ was used as a probe, and the lower band corre-

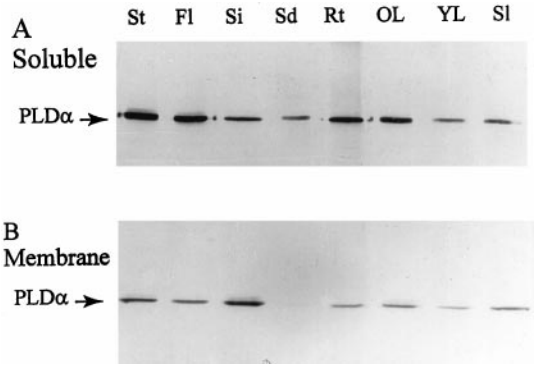

Figure 6. Immunoblot of PLDα in soluble (A) and membrane (B) fractions of different tissues. Equal amounts (10 μg per lane) of soluble and membrane-associated protein were separated by 8% SDS-PAGE. The blots were incubated with the PLDα antibody. The protein samples and abbreviations are the same as those in Figure 4.

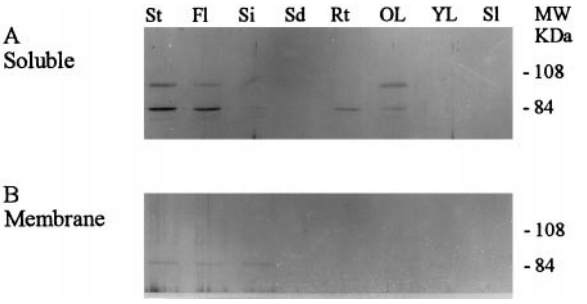

Figure 7. Immunoblot of PLDγ in soluble (A) and membrane (B) fractions of different tissues. Equal amounts (10 μg per lane) of soluble and membrane-associated protein were separated by 8% SDS-PAGE. PLD bands were immunodetected with incubation of the filters with affinity-purified PLDγ antibody. The protein samples and abbreviations are the same as those in Figure 4.

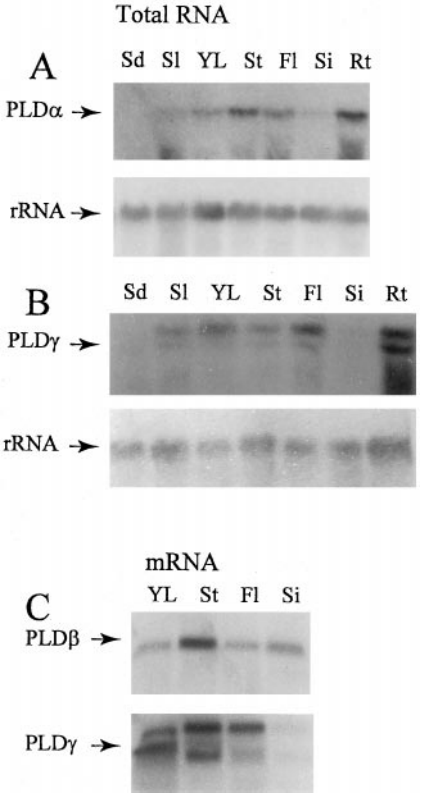

Figure 8. Expression of PLDα, PLDβ, and PLDγ RNA levels in different organs. A, Total RNA (20 μg per lane) isolated from various organs of 2-month-old Arabidopsis was probed with PLDα cDNA. B, Total RNA hybridized with PLDγ cDNA. Blots in both A and B were stripped and hybridized with an rRNA probe to indicate the equal loading of total RNA. C, mRNA (1.5 μg per lane) probed with PLDβ (upper), and the blot stripped and probed with a PLDγ cDNA probe (lower). St, Stem; Fl, flower; Si, silique; Sd, dry seed; Rt, root; YL, young leaf; Sl, seedling (10 d old).

sponded to the size of the cloned PLDγ cDNA. No extra bands were detected when PLDα and PLDβ probes were used, which suggests the presence of another PLD mRNA that is closely related to PLDγ.

The transcripts of PLDα and PLDγ were detected using total RNA. Under the same conditions and using a PLDβ cDNA probe with the same specific radioactivity as the PLDγ probe, however, PLDβ mRNA was not detectable (data not shown). This indicated that the level of the PLDβ mRNA was lower than the levels of PLDα or PLDγ mRNA. When the mRNA from total RNA of leaf, stem, flower, and silique was isolated for blotting, the PLDβ transcript became detectable (Fig. 8C). As a direct comparison, the same mRNA blot was hybridized with PLDγ (Fig. 8C), and the relative distribution of PLDγ in different organs was similar to that on the total RNA blot. The pattern of PLDβ expression was different from that of PLDα and PLDγ. Most noticeably, the mRNA level in siliques relative to the levels in leaves and flowers was much higher for PLDβ than for PLDα and PLDγ.

DISCUSSION

Activation of PLD has been linked to various cellular processes, such as hormonal and developmental signaling, membrane synthesis, remodeling, and lipid degradation (Wang, 1997). The discovery of different forms of PLD (Qin et al., 1997) provides some molecular and biochemical bases for such functional diversity. The present study has shown that PLDα, PLDβ, and PLDγ of Arabidopsis also have different intracellular distribution and expression patterns. At the PLD activity level, one major difference is that the specific PIP_2-dependent activity was higher in membrane-associated fractions than in soluble fractions in all organs, but the distribution of PIP_2-independent activity in the two fractions varied from organ to organ. This result is consistent with a recent report that demonstrated a predominant localization of PIP_2-dependent activity in the membrane-associated fraction of leaves (Pappan et al., 1997a), and it also extends the same distribution pattern of PIP_2-dependent activity to the other organs in Arabidopsis. Another major difference is that the PIP_2-dependent activity showed the highest activity in older leaves, whereas the PIP_2-independent activity was more active in metabolically active tissues such as flowers, siliques, and roots. It is interesting that the levels of both polyphosphoinositides and phosphatidic acid were also found to increase with senescence (Borochov et al., 1994). Regulation of the levels of polyphosphoinositides and the polyphosphoinositide-requiring PLDs may be coordinated.

One of the physiological implications of such a pattern is that the polyphosphoinositide-requiring PLDs may play a more important role than the polyphosphoinositide-independent PLD in senescence. A previous study using PIP_2-independent PLDα-deficient plants also suggested that the PIP_2-independent PLD is not a direct promoter of senescence because antisense suppression of PLDα did not alter natural plant senescence (Fan et al., 1997). However, suppression of PLDα retarded ABA- and ethylene-promoted senescence in detached leaves. PLDα is believed to play a role in phytohormone signaling, and thus its deficiency renders tissues less sensitive to ABA and ethylene treatments (Fan et al., 1997). Such a signaling role of the conventional plant PLD has also been suggested in carrot cells (Lee et al., 1998) and barley aleurone (Richie and Gilroy, 1998).

At the protein level, PLDα was found in all organs, PLDγ was detectable in some organs, but PLDβ was undetectable in any subcellular or tissue fractions. The inability to monitor the PLDβ protein indicates that the amount of this protein is much lower than that of PLDα and PLDγ because the PLDβ antibody has a titer similar to that of the PLDγ antibody and reacted well with bacterially expressed PLDβ (Fig. 2). Consistent with the immunoblot results, RNA blotting showed that the level of PLDβ mRNA was much lower than that of PLDα and PLDγ mRNAs, and PLDβ mRNA could be detected only in isolated mRNA. This suggests that a low level of PLDβ gene expression may be responsible for the small amount of PLDβ. In addition, the pattern of PLDβ mRNA accumulation in different organs was different from that of PLDα and PLDγ mRNAs. Another possible reason for the lack of immunodetection of PLDβ could be proteolytic removal in the cell and/or during isolation of the PLDβ C-terminal peptide to which the antibody was raised. However, nothing is yet known about the posttranslational processing of these PLDs.

Another major difference at the PLD protein level was that a substantial amount of PLDγ, but not PLDα or PLDβ, was found in the nuclear fraction. This nuclear location is particularly interesting because in yeast PLD1 is present in nuclei and its association with nuclear membranes is required for completion of meiosis and subsequent sporulation (Sung et al., 1997). This could mean that PLDγ may play a role in cell division and reproduction. The trace amount of PLDα detected in the nuclei was likely the result of contamination from other fractions (Table I), because a recent immunocytochemical study of castor bean did not find PLDα in the nuclei (Xu et al., 1996).

Recent expression and characterization of cloned PLDs have demonstrated that PLDα is responsible for the PIP_2-independent PLD activity, and PLDβ and PLDγ both possess PIP_2-dependent activity (Qin et al., 1997; Pappan et al., 1998). In this study, the levels of PLDα protein and the PIP_2-independent PLD activity correlated well, but the levels of PLDβ and PLDγ proteins and the PIP_2-dependent PLD activity did not. Specifically, high levels of specific PIP_2-dependent activity are associated with membrane fractions, whereas most PLDγ protein was detected in the soluble fractions and PLDβ was undetectable in any fraction. This discrepancy could result if the membrane-associated PLDγ were more active than the soluble form. The low level of soluble activity might be caused by the presence of PLDγ inhibitors and/or the absence of PLDγ activators in the cytosol. Thus, the membrane-associated PLD is the activated form. Another possibility is that other PLD isoforms contributed significantly to the PIP_2-dependent activities detected in membranes. In fact, two protein bands were detected by the purified PLDγ antibody, and two species of mRNA hybridized specifically to PLDγ cDNA. These results indicate the presence of at least

one additional PLD, whose sequence is more closely related to that of PLDγ than to that of PLDα or PLDβ. Studies are under way to clone and characterize other PLDs from Arabidopsis.

In addition, PLDβ could be another isoform that contributed to the high level of membrane-associated, PIP_2-dependent activity. Although the immunoblot and RNA-blot results indicate a much lower level of expression for PLDβ than for PLDγ, a recent study using PLDs expressed in *Escherichia coli* has shown that PLDβ is much more active toward PC than toward PLDγ (Pappan et al., 1998), and the present study used PC as the substrate for measuring PLD activities. Thus, it is probable that, although the low level of PLDβ eluded immunodetection, it still gave a portion of the membrane-associated PIP_2-dependent activity. Furthermore, the absence of detection of PLDβ could result from a proteolytic removal of its C-terminal peptide to which the PLDβ antibody was raised. Recent analysis in this laboratory has demonstrated that proteolytic deletion of the C-terminal portion does not affect PLDβ activity (K. Pappan and X. Wang, unpublished data). Further studies are warranted to clarify these possibilities

None of the three PLDs was present in chloroplasts, and the absence of PLDα in this organelle is consistent with its localization in other plant species (Xu et al., 1996). On the other hand, this study has shown that substantial amounts of both PIP_2-dependent and -independent PLD activities and PLDα and PLDγ proteins are associated with the mitochondrial fractions of Arabidopsis leaves. Immunocytochemical localization did not find PLDα inside the mitochondria of castor bean leaves (Xu et al., 1996). However, PLD of corn roots was suggested to be associated with mitochondrial membranes (Brauer et al., 1990). The PLDα observed in the mitochondrial fraction likely is associated with mitochondrial membranes, but the exact location of these PLDs in this organelle requires further investigation.

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