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Intracellular Localization of Phospholipase D in Leaves and Seedling Tissues of Castor Bean[1]

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The intracellular distribution of phospholipase D (PLD; EC 3.1.4.4) in castor bean (*Ricinus communis* L.) tissues was investigated by subcellular fractionation and by immuno-electron microscopy. Centrifugal fractionation revealed that most PLD in young leaves was soluble, whereas in mature leaves a majority of PLD was associated with microsomal membranes. Further separation of microsomal membranes by a two-phase partitioning system indicated that PLD was associated with both plasma and intracellular membranes. Sucrose gradient separation of intracellular membranes showed PLD present in the endoplasmic reticulum, a submicrosomal band, and in soluble fractions but not in mitochondria and glyoxysomes of postgermination endosperm. Immunocytochemical studies found high gold labeling in vacuoles in young leaves, suggesting that the high level of soluble PLD in young leaves is due to release of PLD from vacuoles during tissue disruption. In addition to the labeling in vacuoles, gold particles were also found in the cytoplasmic matrices and plasma membrane in leaves and in 2-d postgermination seedlings. Collectively, these results show that PLD in castor bean leaf and seedling tissues is localized in the vacuole and is associated with the endoplasmic reticulum and plasma membrane and that the relative distribution between the soluble and membrane compartments changes during castor bean leaf development.

Hydrolysis of membrane phospholipids by PLD (EC 3.1.4.4) has been thought to play a pivotal role in a number of cellular processes in animal systems (Billah, 1992; Exton, 1994). Many receptor agonists activate PLD, which generates messengers mediating receptor-linked exocytosis, cell proliferation, respiratory burst, actin polymerization, membrane trafficking, and secretion. Several mechanisms, including protein kinase C, G proteins, Ca^{2+} flux, and receptor-linked Tyr kinase, have been suggested for activation of PLD in response to different agonist treatments. A role of PLD has also been implicated in mediating cellular functions in higher plants (Di Nola and Mayer, 1986; Acharya et al., 1991; Munnik et al., 1995).

Most literature on functional studies of plant PLD deals with the role of PLD hydrolysis in membrane deterioration in connection with senescence, aging, and stress injuries (Thompson, 1988; Paliyath and Droillard, 1992; Samama and Pearce, 1993; Voisine et al., 1993). Many plant tissues have active PLD, which can be highly destructive to membrane lipids when assayed in vitro (Quarles and Dawson, 1969; Heller, 1978). Therefore, there must be mechanisms to control or sequester it from its substrates in vivo. Increased association of PLD with microsomal membranes has been proposed to promote PLD-mediated degradation of membrane lipids under some conditions, such as γ-irradiation and senescence (Voisine et al., 1993; Ryu and Wang, 1995). In fruit ripening, decreased fluidity of plasma and microsomal membranes has been reported to activate PLD and increase membrane catabolism (MacCormac et al., 1993). In injuries, such as frost and wounding, massive lipid degradation and membrane deterioration have been suggested to result from the release of PLD from its original stores (Yoshida, 1975; Willemot, 1983), which were often thought to be vacuoles.

The intracellular localization of PLD, however, has not been well defined. PLD has been found in soluble and membrane-associated fractions in various plants, and its relative distribution between these two fractions varies, depending on the species, tissues, and developmental stages of the tissues (Heller, 1978; Wang et al., 1993; Dyer et al., 1994). An early experiment using differential centrifugation suggested that PLD was associated with plastids in several plant species (Kates, 1955). A later study suggested that PLD activity was not associated with chloroplasts and mitochondria (Clermont and Douce, 1970). On the other hand, 80% of PLD in corn roots was proposed to be associated with mitochondrial membranes (Brauer et al., 1990). PLD in mung bean cotyledons was reported to occur in protein bodies (Herman and Chrispeels, 1980), which are thought of as specialized vacuoles. Although the mixed results may reflect tissue- and species-specific distributions of PLD, all of the early studies used subcellular fractionation followed by assaying PLD activity, which might have difficulties in identifying particular fractions and in verifying their purity. In addition, the presence of PLD stimulators and inhibitors might influence the previous results of PLD localization based on its activity distribution. The recent generation of anti-PLD antibodies against purified PLD (Wang et al., 1993) enabled us to detect PLD protein by immunoblotting and immunocytochemistry, which permit the direct measurement of PLD protein and visualiza-

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Abbreviations: g_{av}, average gravity; PC, phosphatidylcholine; PLD, phospholipase D.

tion of PLD-labeled particles in the cell. The present study provides evidence that PLD protein is not only localized in vacuoles but is also associated with the ER and plasma membrane in castor bean.

MATERIALS AND METHODS

Materials

Coatless seeds of castor bean (*Ricinus communis* L. var Hale) were germinated in the dark at 30°C for 4 d. Seedlings were individually transplanted into plastic pots containing a mixture of vermiculite and perlite (1:1, v/v) subirrigated with Hoagland nutrient solution (Hoagland and Arnon, 1950). Castor bean plants were grown under cool fluorescent lights at 23 ± 3°C with a 14-h photoperiod. Polyclonal PLD antibodies were raised in rabbits against purified PLD from 2-d postgermination endosperm of castor bean (Wang et al., 1993). Gold particles (10 nm) conjugated to goat anti-rabbit IgG were purchased from Sigma. Radioisotopes, chemicals, and other reagents were obtained from the sources previously reported (Wang et al., 1993).

Subcellular Fractionation

Young and mature leaves (approximately 1 g each) were ground with a mortar and pestle in an extraction buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM KCl, 1 mM EDTA, 0.5 mM PMSF, and 2 mM DTT on ice (Dyer et al., 1994). The homogenate was centrifuged at 6,000*g* for 10 min at 4°C, and the supernatant was centrifuged at 110,000g_{av} for 60 min. The resulting supernatant and pellet were referred to as the soluble and microsomal fractions. The pellet was suspended in the extraction buffer by grinding with a glass homogenizer. Protein content was determined with a dye-binding assay according to the manufacturer's instructions (Bio-Rad).

For Suc gradient centrifugation of PLD, 15 endosperm halves, after 2 d of imbibition at 30°C, were homogenized by chopping for 15 min with a razor blade in 10 mL of a homogenization medium containing 0.5 M Suc, 0.15 M Tricine-HCl (pH 7.5), 10 mM KCl, 1 mM EDTA, and 10 mM DTT. Procedures for the Suc gradient preparation and organelle separation and identification have been previously reported (Wang and Moore, 1991).

Two-Phase Partitioning of Membranes

Plasma membrane was purified from castor bean leaves and hypocotyls using an aqueous polymer two-phase system (Larsson et al., 1987). Leaves (5 g) were vacuum infiltrated in 20 mL of a cold extraction buffer containing 0.5 M Suc, 50 mM Hepes-KOH (pH 7.5), 5 mM ascorbic acid, 1 mM DTT, 0.6% (w/v) PVP, and 1 mM PMSF for 5 min at 4°C and were then homogenized with a chilled mortar and pestle. Hypocotyls (10 g) (6 d of postgermination) were infiltrated and then homogenized in the same way as above except the buffer (25 mL) contained 0.3 M Suc, 25 mM Tris-Mes (pH 7.5), 1 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, and 1 mM PMSF. The homogenate was centrifuged at 10,000*g* for 10 min at 4°C. A microsomal pellet was obtained from the supernatant by centrifugation at 50,000g_{av} for 30 min at 4°C. The pellet was suspended in a buffer containing 0.33 M Suc, 3 mM KCl, and 5 mM potassium phosphate (pH 6.8). This suspension was loaded to a solution to give a 60-g phase system with a final composition of 6.4% (w/w) Dextran T_{500}, 6.4% (w/w) PEG 3350, 0.25 M Suc, and 5 mM potassium phosphate (pH 6.8). After mixing, the two phases were separated by centrifugation in a swinging bucket rotor at 1,500*g* for 5 min. The upper phase, containing the plasma membrane, was rewashed twice with lower-phase polymer to reduce endomembrane contamination. The lower phase, containing endomembranes, was rewashed twice with upper phase polymer. The washed upper and lower phases were diluted with a solution containing 0.25 M Suc and 5 mM potassium phosphate (pH 6.8) and centrifuged at 100,000g_{av} for 45 min. The membrane pellet was suspended in a buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM KCl, 1 mM EDTA, 0.5 mM PMSF, and 2 mM DTT frozen at −75°C until use. The assays for marker enzymes, Cyt *c* oxidase, NADH-dependent Cyt *c* reductase, and vanadate-sensitive ATPase were performed according to reported procedures (Briskin et al., 1987).

SDS-PAGE and Immunoblotting

SDS-PAGE gels consisted of 8% (w/v) acrylamide (pH 8.8) in the resolving phase and 3.5% (pH 6.8) in the stacking phase (Wang et al., 1993). Protein extracts were mixed with SDS-PAGE loading buffer and heated at 95°C for 3 min before loading. Gels were run at constant voltage of 80 V for 20 min, and the voltage was then increased to 150 V. After electrophoresis, proteins were transferred onto nitrocellulose membranes. The blot was then incubated with PLD antiserum (1:1000 dilution) in PBS containing 5% (w/v) nonfat dry milk. The antigen-antibody complexes were made visible with alkaline phosphatase conjugated with goat antibody against rabbit IgG.

PLD Activity Assay

PLD activity was measured based on the release of radioactive-free choline from the substrate dipalmitoyl-glycero-3-P-[*methyl*-^{3}H]-choline (Wang et al., 1993). Radioactive phosphatidylcholine (PC) (2.5 μCi; 500 μCi/mmol) was mixed with 20 μmol of cold PC (egg yolk) in chloroform, and the mixture was dried under a stream of N_2. The lipid was emulsified in 1 mL of H_2O by sonication at room temperature. A typical enzyme assay mixture contained 100 mM Mes/NaOH (pH 6.5), 25 mM $CaCl_2$, 0.5 mM SDS, 20 μL of substrate (0.4 μmol), and 50 μL of PLD from a gradient fraction in a total volume of 200 μL in a 1.5-mL microcentrifuge tube. The reactions were initiated by adding radioactive PC substrate, incubated in an H_2O bath shaker (200 rpm) at 30°C for 30 min, and terminated by adding 1 mL of chloroform:methanol (2:1, v/v). The mixtures were vortexed vigorously and centrifuged at 12,000*g* for 2 min to separate aqueous and lipid phases. An aliquot (100 μL) of the aqueous phase was mixed with 3 mL of

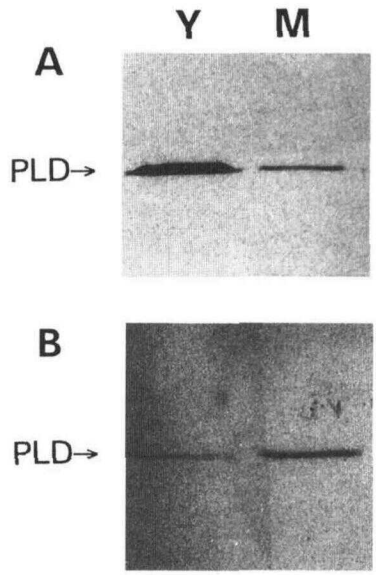

Figure 1. Immunoblot of soluble and microsome-associated PLD in young (lane Y) and mature (lane M) castor bean leaves. Soluble (A) and membrane-associated (B) proteins were 50 μg/lane and 5 μg/lane, respectively. PLD on the blot was made visible using alkaline phosphatase conjugated to goat antibodies against rabbit IgG.

scintillation counting cocktail, and the radioactive choline released from PC was determined by standard scintillation counting.

Immunocytochemistry

The tissue fixation and sectioning were based on a previously described procedure (Stafstrom and Stachelin, 1988) with some modifications. Young and mature (fully expanded) leaf samples were degassed for 30 min in a fixative containing 0.5% glutaraldehyde and 4% paraformaldehyde in a 100-mM sodium phosphate buffer (pH 7.6) and then fixed in the same fixative for 3 h at 4°C with one change of fixative. Endosperm, cotyledon, and hypocotyl of 2 d of postgermination were immersed and rotated in a fixative containing 2% glutaraldehyde and 4% paraformaldehyde in a sodium phosphate buffer (100 mM, pH 7.6) for 5 h at 4°C with one change of fixative. After fixation, the samples were rinsed three times with an ice-cold phosphate buffer (100 mM, pH 7.6) at 4°C, dehydrated in an ascending series of ethanol at 4°C, and then embedded in LR white resin (London Resin Company, London, UK) for sectioning.

Ultrathin sections were cut with diamond knives and mounted on nickel grids. Immunolabeling was carried out by floating grids, section side down, on droplets of blotting solutions on Parafilm (American National Can, Neenah, WI). Preblotting was done in PBS containing 2% BSA and 0.05% Tween 20 for 15 min. The preblotted sections were incubated with PLD antibodies (25 μg/ml) in the preblotting solution overnight at 4°C. The PLD antibodies were purified from antisera with a protein A affinity column (Wang et al., 1993). Unbound PLD antibodies were removed by washing with PBS containing 0.05% Tween 20. The sections on grids were incubated with 10 nm of gold conjugated with anti-rabbit IgG in the blotting solution for 1.5 h followed by two rinses (5 min each) in PBS containing 0.05% Tween 20, two rinses with PBS, and two rinses in distilled H_2O. Controls used preimmune serum with the same dilution as the antiserum. Labeled grids were dried and stained with aqueous uranyl acetate followed by lead citrate.

The sections were examined with a Philips EM201 electron microscope. To quantify the density of label in each cell compartment, gold particles were counted from five grids (30 μm^2) at 15,000× magnification for vacuole and the cytoplasmic matrices. Total counts were taken for individual membrane-bound organelles (plastids, mitochondria, nuclei, and peroxisomes) chosen at random from five grids. The average number per cell compartment was calculated by subtracting the number of particles in control samples from the total in anti-PLD antibody treatments.

RESULTS

PLD Localization by Subcellular Fractionation

Centrifugal fractionation of PLD in castor bean tissues showed that PLD was present in soluble and microsomal fractions, and its distribution in the two fractions differed between young and mature leaves. The relative proportion of PLD in soluble and microsomal fractions as shown in the immunoblot using anti-PLD-specific antibodies (Fig. 1) was consistent with direct PLD activity measurements in the two fractions (Dyer et al., 1994). In the young leaves, most PLD was recovered in 110,000g_{av} supernatant, whereas in the fully expanded, mature leaves PLD was largely present in the pellet between 6,000g and 110,000g_{av} centrifugation.

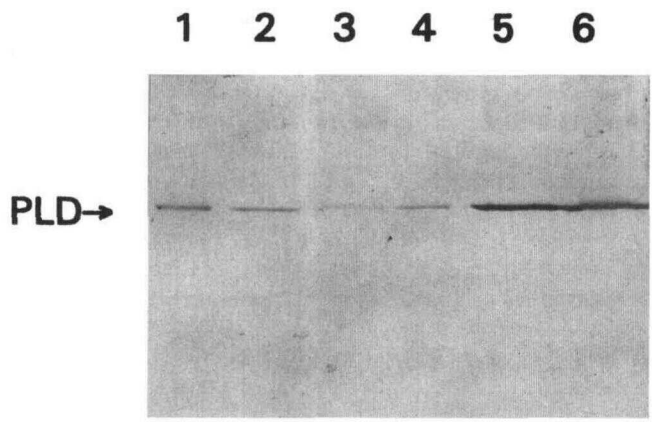

Figure 2. Immunoblot of PLD in the plasma and intracellular membranes obtained from a two-phase partitioning separation of microsomal membranes. Lanes 1 through 3 are plasma, microsomal, and intracellular membranes, respectively, from mature leaves. Lanes 4 through 6 are plasma, microsomal, and intracellular membranes, respectively, from hypocotyls of 6-d postgermination seedlings. Equal amounts of protein from plasma and intracellular membranes (10 μg/lane) were loaded, whereas 20 μg of microsomal protein were loaded onto each lane.

Table I. *Distribution of PLD and marker enzyme activities in plasma and intracellular membranes*

PM and INTRA denote plasma and intracellular membranes, respectively. PLD activity is expressed as 1 nmole product generated min^{-1} mg^{-1} protein, whereas that of Cyt *c* oxidase and NADH-dependent Cyt *c* reductase is expressed as 1 μmole product min^{-1} mg^{-1} protein.

Enzyme	Leaf		Hypocotyl	
	PM	INTRA	PM	INTRA
PLD	19.8	35.5	28.7	68.3
Cyt *c* oxidase	33.3	606.8	0	633.3
NADH-Cyt *c* reductase	26.4	361.9	113.1	2231.8

The microsomal membranes were further separated by an aqueous two-phase system into plasma and intracellular membranes to determine if PLD was associated with plasma membrane. The identity of plasma membrane was verified by the presence of vanadate-sensitive ATPase activity in the upper phase membrane preparation; about 90% of ATPase in the plasma membrane preparations was inhibited by 50 μM Na_3VO_4. Measurements of PLD by immunoblotting and activity assay showed the presence of PLD in both plasma and intracellular membranes (Fig. 2; Table I). PLD-specific activity in the plasma membrane prepared from leaves and hypocotyls was approximately 56 and 42% of that in the intracellular membranes, respectively. The relative amounts of PLD protein in the plasma and intracellular membranes were estimated by immunoblotting analysis using anti-PLD antibodies. Consistent with the result of PLD activity assay, the PLD protein concentration in hypocotyl plasma membrane was lower than that in its intracellular membranes (Fig. 2, lanes 4 and 6). On the other hand, the relative amount of PLD protein in the leaf plasma membrane appeared to be higher than that in its intracellular membranes (Fig. 2, lanes 1 and 3). The higher amount of PLD protein, but lower PLD activity in the leaf plasma membrane than in its intracellular membranes, might result from a loss of PLD activity in the plasma membrane. Most of the PLD found in the plasma membrane was not due to contamination of intracellular membranes because the plasma membrane preparations contained little activities of Cyt *c* oxidase and NADH-dependent Cyt *c* reductase, the marker enzymes for mitochondrial inner membranes and ER, respectively (Table I).

The distribution of PLD in intracellular membranes was investigated by gradient centrifugation of endosperm homogenate followed by measurement of PLD in various fractions. Postgermination castor bean endosperm was used because it is an established model system for subcellular localization of biochemical reactions (Lord et al., 1972). Organelles such as mitochondria, ER, and glyoxysomes formed discrete bands in the gradient and were identified by measurement of various organelle marker enzymes. Markers used for the ER, mitochondria, and glyoxysomes were ethanolamine-phosphate transferase, fumarase, and catalase, respectively (Wang and Moore, 1991). PLD activity assay showed PLD present in the ER, an unidentified membrane band A, and soluble fractions (Fig. 3). Band A was thought to be derived from microsomes, but its identity has not been well defined (Lord et al., 1972). No activity was detected in the fractions of glyoxysomes and mitochondria (Fig. 3). This result was confirmed by immunoblotting analysis, which showed PLD protein in the ER and soluble fractions but not in mitochondria and glyoxysomes (Fig. 3).

Immunocytochemical Localization of PLD

To identify intracellular location of PLD, the IgG-gold labeling technique was used on ultrathin sections of leaves and 2-d postgermination seedlings. In young leaves, the gold particles were concentrated in vacuoles, with some present in the cytoplasmic matrices exclusive of apparent organelle structures (Fig. 4A). In hypocotyls of postgermination bean seedlings, gold particles were detected in the cytoplasmic matrices, vacuoles, and plasma membrane (Fig. 4B). The localization of PLD in the cytoplasmic matrices, vacuoles, and plasma membrane was also observed in mature leaves and postgerminative cotyledons (data not shown). In control experiments using preimmune IgG, very few gold particles were associated with vacuoles, the cytoplasmic matrices, and plasma membrane (Fig. 4C), ruling out a false-positive labeling by PLD antibodies. The number of gold particles found in chloroplasts, mitochondria, glyoxysomes, and nuclei were negligible and resembled the overall nonspecific background labeling. The ab-

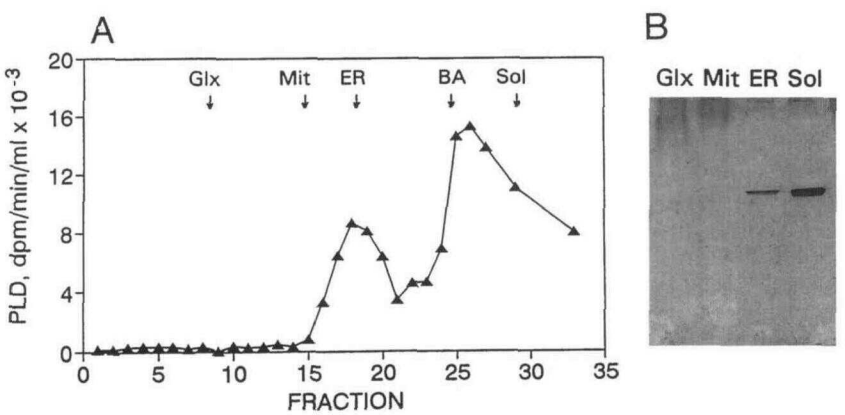

Figure 3. Intracellular location of PLD after linear Suc gradient centrifugation. A, Profile of PLD activity measured in gradient fractions. The fraction numbers are from bottom to top of the gradient. The arrows indicate the positions of glyoxysome (Glx), mitochondria (Mit), ER, membrane band A (BA), and soluble fraction (Sol) in the gradient. B, Immunoblot of PLD in glyoxysome, mitochondria, ER, and soluble fraction. The same amount of protein from each fraction (20 μg) was loaded onto each lane.

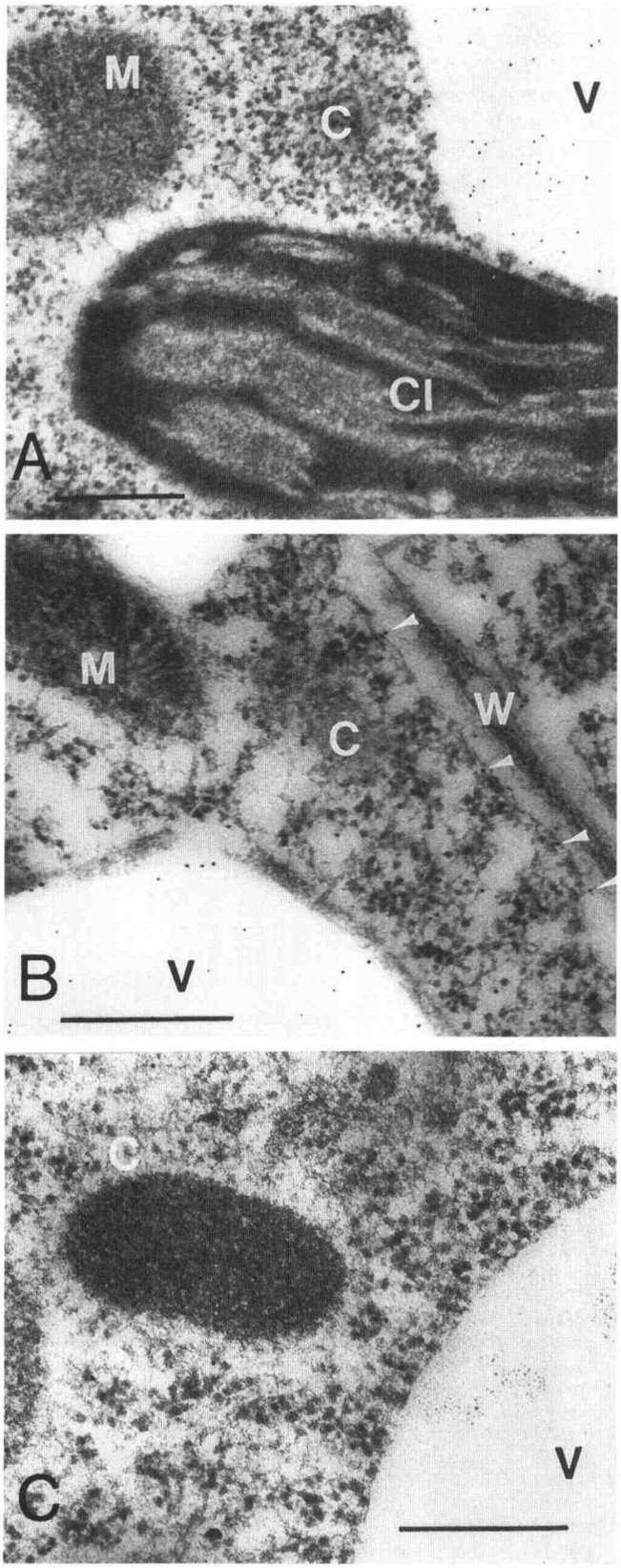

Figure 4. Electron micrographs of immunogold labeling of PLD in castor bean tissues. A, Young leaf cross-section probed with PLD antibodies. B, Cross-section from 2-d postgermination hypocotyl probed with PLD antibodies. C, Cross-section from 2-d postgermination hypocotyl probed with preimmune serum. C, Cytoplasmic matrices; Cl, chloroplast; M, mitochondria; V, vacuole; W, cell wall. The white arrowheads indicate gold labels along the plasma membrane. Bars = 0.5 μm.

sence of PLD labeling in mitochondria (Fig. 4) and glyoxysomes was in agreement with the result of Suc gradient fractionation (Fig. 3). The absence of PLD in chloroplasts (Fig. 4) was also confirmed by immunoblotting and PLD activity analyses of chloroplasts purified by Percoll gradient centrifugation (data not shown).

DISCUSSION

The present study used subcellular fractionation and electron microscopic techniques to localize PLD in castor bean tissues, and the results from the two methods supplement and support one another. Specifically, subcellular fractionation found that PLD was mostly soluble in young leaves, and the immunocytochemical results showed that the most PLD in young castor bean leaves was compartmentalized in vacuoles. These results suggest that soluble PLD in young leaves is due to its release from vacuoles during homogenization. Fractionation results showed that some PLD was microsome associated, and this association was further supported by the gradient separation experiments that showed PLD present in the ER and in a submicrosomal fraction. In the immunocytochemical study, PLD was also found in the cytoplasmic matrices. It is likely that a substantial portion of PLD in the cytoplasmic matrices is ER-associated, based on the results of subcellular fractionation of leaves and endosperm. However, clear visualization of ER structures was difficult in this study because the use of aldehydes as the sole fixative does not impart good contrast to membranes.

One important finding from this study is that the data show, for the first time, to our knowledge, the association of PLD with plasma membrane. The PLD detected in plasma membrane cannot be explained by the contamination of intracellular membranes. This is because the activities of ER and mitochondrial marker enzymes in the plasma membrane preparations are less than 10% of that in intracellular membranes, whereas the amount of PLD protein in the leaf plasma membranes is even higher than that in its intracellular membranes. Furthermore, gold-labeled particles along the plasma membrane are visible in the leaf and hypocotyl tissues.

The localization of PLD in plasma membrane, ER, and vacuoles provides insightful clues as to the roles and regulation of PLD in the cell. For example, because of its destructive effect on membrane lipids, PLD has been thought to be sequestered in vacuoles and to be released upon rupture of cellular structures caused by stresses such as wounding and frost (Yoshida, 1975; Galliard, 1978; Willemot, 1983). A previous report indicated that PLD was associated with protein bodies, which are thought of as specialized vacuoles (Herman and Chrispeels, 1980), but there had not been direct documentation for the vacuolar

localization of PLD in vegetative tissues. The present results provide the evidence for the presence of a substantial amount of PLD in vacuoles. On the other hand, the present data also show clearly that, in addition to the vacuolar PLD, a considerable amount of it is present in plasma membrane and other cytoplasmic regions such as ER. The association of PLD in plasma membrane is consistent with the proposed role of PLD in transmembrane signaling (Exton, 1994; Munnik, et al., 1995). The presence of PLD outside of vacuoles also suggests that not all PLD is sequestered in vacuoles and that there must be mechanisms to regulate the cytoplasmic PLD activity.

A potential regulator of PLD is the change in cytoplasmic Ca^{2+} concentration. Activation of PLD-mediated hydrolysis has been reported in plant response to various stimuli (Di Nola and Mayer, 1986; Thompson, 1988; Acharya et al., 1991; Paliyath and Droillard, 1992; Voisine et al., 1993). It has been known that many perturbations of plants increase cytoplasmic Ca^{2+} concentration. Ca^{2+} at physiological concentrations has been shown to stimulate PLD activity and to promote PLD association with membranes (Paliyath and Thompson, 1987; Yamamoto et al., 1995). Recent cloning of plant PLD has revealed a consensus sequence for a calcium-binding domain present in the enzyme (Ueki et al., 1995; Wang et al., 1994). A study using PLD from *Streptomyces* showed that PLD binds to membrane vesicles in a Ca^{2+}-dependent manner (Yamamoto et al., 1995). It was suggested that the binding of Ca^{2+} was followed by a conformational change of PLD so that the changed PLD has a higher affinity to membranes. Our recent studies have also shown that the amount of PLD associated with castor bean microsomal membranes can be increased with elevated physiological concentrations of free Ca^{2+} in homogenization buffer (S.B. Ryu and X. Wang, unpublished data). The PLD association with membranes appear to be peripheral because a major portion of the membrane-associated PLD can be dissociated from membranes after addition of 0.2 M KCl or 5 mM EGTA and recentrifugation. An increase in the association of PLD with microsomal membranes is thought to activate PLD-mediated hydrolysis under stresses such as γ-ray irradiation and senescence (Thompson, 1988; Paliyath and Droillard, 1992; Voisine et al., 1993; Ryu and Wang, 1995). The presence of PLD in the cytoplasmic matrices as shown in this study would allow its easy access to bind to microsomal membranes such as ER and plasma membranes upon stimulation.

The association of PLD with the different subcellular compartments also raises the question of whether the PLD found in plasma membrane, ER, and vacuoles exists as different molecular forms. Recent studies have shown the presence of three structural variants of PLD in castor bean, and the appearance of specific PLD variants is associated with different stages of plant growth and development (Dyer et al., 1994; Ryu and Wang, 1995). Two forms of PLD were detected in young leaves: PLD 1 is soluble, whereas PLD 2 is found in both soluble and microsomal fractions (Dyer et al., 1994). There is only one form of PLD, PLD 2, detected in mature leaves (Dyer et al., 1994), in which PLD is shown to occur in plasma membrane, intracellular membranes, and soluble fractions based on the present membrane fractionation and immunological localization. These data suggest that, although PLD 1 may be a vacuolar-specific form, the same PLD 2 is distributed among the vacuole, ER, and plasma membrane. The multiple intracellular localizations of one PLD protein may occur through membrane trafficking and/or as a result of cellular regulatory mechanisms. Such mechanisms may include Ca^{2+}- and lipid-mediated intracellular translocation, which has been shown to regulate the activities of several phospholipid-utilizing enzymes, such as phospholipase A_2, phosphatidic acid phosphohydrolase, and protein kinase C (Exton, 1994). However, a defined study on intracellular location of specific PLD isoforms awaits the availability of PLD variant-specific antisera and a better understanding of their molecular origin, both of which are under investigation.

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