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RESEARCH PAPER

In vitro distribution and characterization of membrane-associated PLD and PI-PLC in *Brassica napus*

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Abstract

Two types of phospholipid degrading enzyme, phospholipase D (PLD; EC 3.1.4.4) and phosphatidylinositol-specific phospholipase C (PIP_2-PLC; PI-PLC 3.1.4.11) were studied during the development of seeds and plants of *Brassica napus*. PLD exhibits two types of activity; polyphosphoinositide-requiring (PIP_2-dependent PLD) and polyphosphoinositide-independent requiring millimolar concentrations of calcium (PLDα). Significantly different patterns of activity profiles were found for soluble and membrane-associated forms of all three enzymes within both processes. Membrane-associated PIP_2-dependent PLD activity shows the opposite trend when compared to PLDα, while the highest PI-PLC activity appears in the same stages of development of seeds and plants as for PLDα. In subcellular fractions of hypocotyls of young plants, phospholipases were localized predominantly on plasma membranes. The biochemical characteristics (Ca^{2+}, pH) of all three enzymes associated with plasma membrane vesicles, isolated by partitioning in an aqueous dextran-polyethylene glycol two-phase system, are also described. Direct interaction of PLDα with G-proteins under *in vitro* conditions was not confirmed.

Key words: *Brassica napus*, phospholipases, plasma membrane.

Introduction

Phospholipids play an important role in many signalling pathways in animal and plant cells. Signalling cascades are triggered by the activation of phospholipid cleaving enzymes such as phospholipases C, D and A_2. Their activities result in the formation of second messengers. It is increasingly clear that multiple lipid signalling enzymes often form complex networks that mediate a specific cellular response. PI-PLC releases $InsP_3$ that promotes oscillations and increases in cytoplasmic Ca^{2+} (Staxen *et al*., 1999). The increase of Ca^{2+} may enhance PLD association with membranes, resulting in PLD activation (Wang, 2000; Zheng *et al*., 2000). PI-PLC also produces a diacylglycerol, a well-known effector molecule in animal cells. However, the role of DAG in plants remains unclear. Since phosphatidic acid, a product of PLD action can be dephosphorylated by phosphatidate phosphatase to DAG, both enzymes could be involved in the same signalling cascade. PLD-derived PA may also be a potent stimulator of the phosphatidylinositol 4-phosphate 5-kinase needed for the production of phosphatidylinositol 4,5-*bis*phosphate (PIP_2). In addition to being an activator of one form of PLD and the substrate of PI-PLC, PIP_2 also serves as a membrane attachment site for various proteins involved in membrane trafficking (Blatt, 2000).

Although plant PLDs represent a multiple gene family, only two biochemically distinct forms have been described in plant material and characterized by their requirements for PIP_2 and Ca^{2+} (Wang, 2001). The physiological role of these two types still remains unclear. PIP_2-independent PLD seems to be activated in the early stages of seed and plant development (Ryu *et al*., 1996). To date, the only study distinguishing between the two forms of PLD in the course of tissue development has been done on *Arabidopsis thaliana* (Fan *et al*., 1999) on different parts of 2-month-old plants.

Furthermore, the simultaneous investigation of both types of phospholipases, PI-PLC and PLDs, which are now

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believed to interplay in signal transduction, becomes very important. Activation of PLC by G-protein has been described (Arz and Grambow, 1994) while the evidence for the same mechanism for PLD is much less pronounced (Lein and Saalbach, 2001; Munnik *et al*., 1998)

The purpose of this study was to use previous results obtained in the laboratory on PLD (Novotná *et al*., 1999) and PI-PLC (Crespi *et al*., 1993) and to direct the new project to the common physiological role of these two enzymes in an important crop plant.

Materials and methods

Plant material

Maturing rape seeds (*Brassica napus* L. cv. Lirajet) were collected at weekly intervals starting 34 d after flowering in three consecutive years.

Seeds were germinated at 30 °C for 12 h in the light, then 12 h at 10 °C in the dark. Subsequently, seedlings were grown in continuous light at 25 °C. For the experiments, hypocotyls and cotyledons were separated from other parts of the plants.

Subcellular fractionation

Plant material was homogenized at 4 °C with a mortar and pestle in the buffer (1:4, w/v) containing 70 mM Tris-MES (pH 8.0), 0.25 M sucrose, 3 mM EDTA, 0.2% BSA, 5 mM DTT, and protease inhibitors (0.23 mM PMSF, 0.83 mM benzamidine, 0.7 μM pepstatin, 1.1 μM leupeptin, and 77 nM aprotinin). The homogenate was filtered through the nylon cloth and centrifuged at 6000 *g* for 15 min at 4 °C. The supernatant was filtered through Miracloth (Calbiochem, Switzerland) and diluted (in the ratio 1:3) with suspension buffer containing 1.1 M glycerol, 10 mM Tris-Mes (pH 8.0) and protease inhibitors. After centrifugation at 100 000 *g* for 60 min at 4 °C, the soluble fraction was obtained and pelleted microsomal fraction was resuspended in suspension buffer.

Membrane fractions were further separated by sucrose gradient centrifugation (18–38%, w/w). Sucrose solutions were prepared in suspension buffer (10 ml of each). A preformed gradient was covered with 3 ml of the microsomal fraction (15–20 mg of protein) and centrifuged at 30 000 *g* overnight at 4 °C. Fractions of 2 ml were collected, diluted with suspension buffer (1:4, v/v) and centrifuged at 100 000 *g* for 60 min at 4 °C. Pellets were resuspended in minimal volume of suspension buffer and used immediately for the assays or stored at –70 °C.

Plasma membrane

Plasma membrane was purified from hypocotyls of seedlings by partitioning microsomes in an aqueous dextran-polyethylene glycol two-phase system (Larsson *et al*., 1994). Plant material was homogenized with a mortar and pestle at 4 °C in the homogenization buffer in the ratio 1:3 (w/v). Buffer contained 50 mM HEPES-NaOH, pH 7.5, 0.4 M sucrose, 0.1 M KCl., 0.1 M $MgCl_2$, and protease inhibitors (see above). The homogenate was filtered through nylon net and centrifuged at 10 000 *g* for 10 min. The supernatant was filtered through Miracloth and the microsomal membranes were pelleted by centrifugation at 100 000 *g* for 35 min. Microsomes were resuspended in 5 mM phosphate buffer pH 7.8. From 100 g of fresh plant material about 20 ml of microsomal fraction was obtained for further plasma membrane purification.

The aqueous two-phase system was formed from Dextran T-500 (AP Biotech) and polyethylene glycol 3350, both at a final concentration of 6.1% (w/w), 0.43 mM phosphate buffer pH 7.8, 3 mM KCl, and 0.22 M sucrose. 2 g of the microsomal fraction were applied to the system (14 g), the cuvette content was mixed slowly 40 times and, after the stabilization of the system within 1–2 h at 4 °C, centrifuged at 1500 *g* for 5 min. The plasma membrane fraction (upper phase) was further purified by sequential partitioning against fresh lower phase prepared in another set of cuvettes. Pooled upper phases were diluted five times with 5 mM HEPES-Tris buffer pH 7.8 containing protease inhibitors (see above) and centrifuged at 100 000 *g* for 35 min. The purified plasma membrane pellet was resuspended in the dilution buffer and used immediately or stored at –70 °C.

Membrane marker assays

Microsomal fractions were obtained by sucrose density gradient centrifugation and enriched plasma membrane fractions were characterized by measuring the activity of marker enzymes according to procedures described earlier (Martinec *et al*., 2000).

Endoplasmic reticulum (ER) was detected by antimycin A insensitive NAD(P)H-dependent cytochrome *c* reductase, mitochondria by cytochrome *c* oxidase, for plasma membrane 1,3-β-D-glucan synthase II activity was measured, and, finally, pyrophosphatase was used as a tonoplast marker.

Chlorophyll content

Acetone (80%, v/v) extracts of membrane fractions were measured at 720 nm and 652 nm.

Protein determination

Protein concentration in the samples was determined according to Bradford (1976), using bovine serum albumin as a standard.

PLD assay

PIP_2-independent PLD (PLDα) activity was measured spectrophotometrically using the choline oxidase/peroxidase system for determination of released choline by the procedure described earlier (Sajdok *et al*., 1995). PLD activity was determined for phosphatidylcholine (PC) as a substrate in the presence of 120 mM $CaCl_2$ and 10 mM sodium dodecyl sulphate.

PIP_2-dependent PLD activity was measured radiometrically with [methyl-^{3}H]PC or [methyl-^{14}C]PC (Qin *et al*., 1997). Lipid vesicles were prepared from 3.6 μmol of phosphatidylethanolamine (PE), 0.32 μmol of phosphatidyl inositol 4,5-*bis*phosphate (PIP_2), 0.22 μmol of PC, and 2.5 μCi of labelled PC. Phospholipids were dissolved in chloroform/methanol (2:1, v/v), evaporated under nitrogen and emulsified in 1 ml of water by 30 min sonication in a water bath. The incubation mixture contained 0.1 M MES buffer pH 6.8, 100 μM $CaCl_2$, 80 mM KCl, 2 mM $MgCl_2$, 0.1% Triton, 4–12 μg of proteins, and 0.4 mM lipid vesicles in a total volume 100 μl. After 30 min incubation at 30 °C the reaction was stopped by the addition of 1 ml of the chloroform/methanol mixture (2:1, v/v) and 100 μl of 1 M KCl. Radioactivity was measured in the water phase (200 μl aliquots were mixed with 3 ml of scintillation solution).

PI-PLC (PIP_2-PLC) assay

Activity was estimated using radiolabelled substrate [^{3}H-inositol] PIP_2 followed by biphasic extraction of the reaction product inositol 1,4,5-trisphosphate as described earlier (Crespi *et al*., 1993).

Results

Changes of PLDs and PI-PLC in vitro *activities in the course of seed and plant development*

To determine the distribution of different PLDs and PI-PLC in rape seed, protein extracts from seeds at different

stages of development, dry seeds and the early stages of seedling growth were fractionated into soluble and membrane fractions and assayed for phospholipase activities.

The PIP_2-independent PLD assay was based on the presence of 120 mM Ca^{2+}, SDS, and egg PC as a substrate. PIP_2-independent PLD was found both in soluble and membrane fractions of developing seeds, cotyledons and hypocotyls of seedlings and the mode of distribution differed in developing seeds and plants (Fig. 1A). The specific activities in soluble fractions

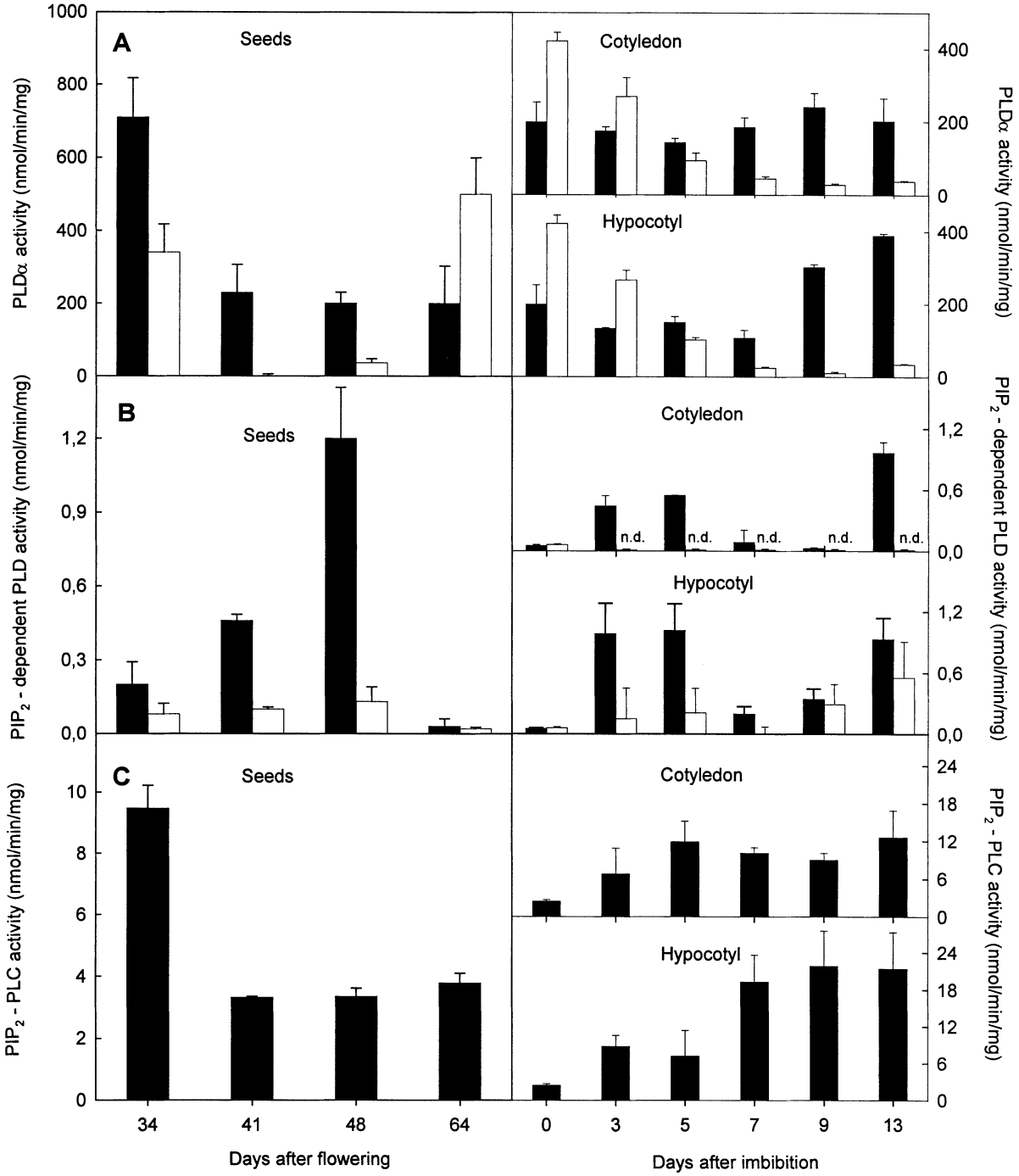

Fig. 1. Changes of phospholipase activities in soluble (white bars) and membrane fraction (black bars) of maturing seeds, cotyledons and hypocotyls of growing seedlings. Membrane fraction was prepared by centrifugation at 100 000 *g* of the 6000 *g* supernatant. (A) Activity of PIP_2-independent phospholipase D referred as PLDα. (B) Activity of PIP_2-dependent PLD. (C) Activity of PIP_2-specific phospholipase C. Values are means ±SE of at least three experiments, activity was measured in duplicate for each experiment.

were significantly higher in the early stages of both processes and dropped markedly in the later stages of development of seeds as well as in plants. Specific activities of membrane-bound forms decreased during seed maturation and increased in the hypocotyls only during the later stages of development.

The PIP_2-dependent form of PLD was assayed in the presence of 100 μM Ca^{2+} and the lipid vesicles were composed of PIP_2, PE and PC in the absence of SDS. Under these conditions, the *in vitro* specific activities of the PIP_2-dependent PLD were lower (200-fold) than those of the PIP_2-independent PLD. PLD specific activities of membrane-associated fractions increased during seed development until day 64. There was very low activity in the mature (64 d) seeds. The specific activities of PLD in hypocotyls and cotyledons were increasing with a significant drop between 5 d and 7 d of seedling growth. The activity in the hypocotyls of young seedlings was 2-fold higher than in the cotyledons (Fig. 1B).

The PI-PLC assay used 0.05 mM Ca^{2+}, sodium deoxycholate and [^{3}H] PIP_2 as a substrate. No soluble activity of PI-PLC was detected. The specific activity in the membrane fraction decreased with the degree of seed maturation and the course was similar to the course of PIP_2-independent PLD activity. The activities in the seedlings were one order of magnitude higher than in developing seeds, increasing in hypocotyls, and decreasing in cotyledon parts (Fig. 1C).

Subcellular localization of phospholipases in seeds

To identify the membrane structures with which phospholipases are associated, the subfractionation of microsomal fraction prepared from developing seeds (48 d after flowering) was performed. The identity and purity of each membrane fraction were determined by assaying activities of appropriate marker enzymes as reported previously (Martinec *et al.*, 2000). 1,3-β-D-glucan synthase II and cytochrome *c* oxidase, the markers for plasma membrane and mitochondria, respectively were concentrated in the higher density fractions, numbers 7–10 (Fig. 2A, B). Pyrophosphatase, the tonoplast marker, was found at the lower density fractions, numbers 2–5 (Fig. 2B). Antimycin A-insensitive cytochrome *c* reductase, the ER marker, was found in the lower density fractions, numbers 1–3, and was not well separated from the tonoplast marker (results not shown). These subcellular fractions were assayed for PIP_2-independent PLD and PIP_2-dependent PLD and PI-PLC activities (Fig. 2C, D). The subcellular localization of PI-PLC and PLDs in developing seeds showed that the highest activities of both enzymes correlated well with the activity of 1,3-β-D-glucan synthase II and cytochrome *c* oxidase.

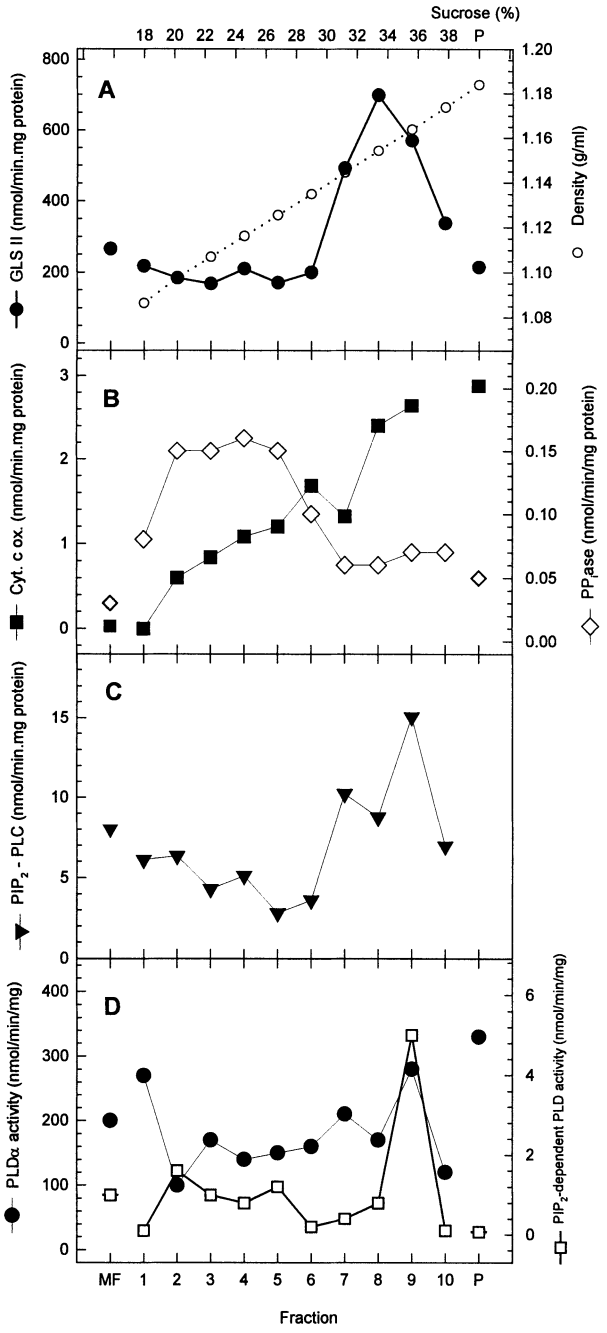

Fig. 2. Microsomal fractions from developing seeds of *B. napus* were separated on an 18% to 38% (w/w) linear sucrose density gradient. Marker enzymes and phospholipase activities were estimated in each fraction as described in the Materials and methods. Fraction 1 represents the top of the gradient, MF is a microsomal fraction, P is a pellet. Data shown are from a typical experiment, similar results were obtained from three other independent experiments. (A) (filled circles) 1,3-β-D-glucan synthase II, plasma membrane marker (open circles), density gradient. (B) (filled squares) cytochrome *c* oxidase, mitochondria marker and (open diamonds) pyrophosphatase, tonoplast marker. (C) (filled inverted triangles) PIP_2-specific phospholipase C. (D) (filled circles) PIP_2-independent phospholipase D, (open squares) PIP_2-dependent phospholipase D.

Table 1. *Characteristics of microsomal fraction (MF), phase-purified plasma membrane vesicles (PM) and residual intracellular membrane fraction (IM) from hypocotyls of rape seedlings*

Data are averages of at least three independent experiments.

Marker	MF	PM	IM
Glucan synthase II[a]	2405.0	5053.0	1295.0
Cytochrome *c* reductase[a]	54.4	17.4	60.9
Cytochrome *c* oxidase[a]	96.0	8.1	135.3
Pyrophosphatase[a]	33.0	15.0	47.0
Chlorophyll[b]	2.6	0.2	3.2

[a] Enzyme activities are given in nmol min^{-1} mg^{-1} protein.
[b] Chlorophyll content is given in mg l^{-1}.

Isolation and characterization of plasma membrane vesicles

The plasma membrane vesicles used in the experiments were highly purified from 5 d hypocotyls for PLD and 9 d hypocotyls for PI-PLC. The enrichment of plasma membrane was achieved by partitioning microsomes in an aqueous dextran-polyethylene glycol two-phase system. The upper phase contained the plasma membranes with only minor contamination by intracellular membranes (Table 1). Typically, the plasma membrane fraction obtained was characterized by at least a 2.5-fold increase of 1,3-β-D-glucan synthase II activity and a decrease of other marker enzyme activities.

Biochemical characteristics of plasma membrane associated forms of PLDs and PI-PLC

The effect of pH on both forms of PLD and PI-PLC was investigated over a range of pH 5.0–8.0 (Fig. 3A, B, C). The PIP_2-independent form exhibited an acidic pH optimum between 5.5 and 6.0. The PI-PLC and the PIP_2-dependent PLD were most active at pH between 6.5 and 7.0.

To determine the influence of divalent cations on phospholipases activities, free Ca^{2+} and Mg^{2+} in the reaction mixture were controlled using Ca^{2+}/Mg^{2+}-EGTA buffers. The activities of both enzymes were undetectable in the absence of Ca^{2+}. The stimulation was observed at a micromolar level for the PI-PLC and the PIP_2-dependent PLD activity (Fig. 4A, B). The sharp increase of PIP_2-independent activity was at millimolar concentrations of Ca^{2+} (Fig. 4C). Mg^{2+}caused no stimulation of PI-PLC at any concentration tested, a slight decrease was observed for the concentrations of Mg^{2+}above 0.2 mM (data not shown).

G-protein activation of phospholipases

To investigate whether GTPase is directly involved in the regulation of phospholipase activities, the effects of GTP-γ-S, a non-hydrolysable analogue of GTP was used. For that purpose the microsomal fraction vesicles and inside-out plasma membrane vesicles were prepared by four times repeated freezing and thawing (DeHahn *et al*., 1997). PLD activities were, in this case, measured using the PC substrate in the form of liposomes prepared by sonication without adding the detergents (Novotná *et al*., 1999). Under these conditions very low PLD activity was detected

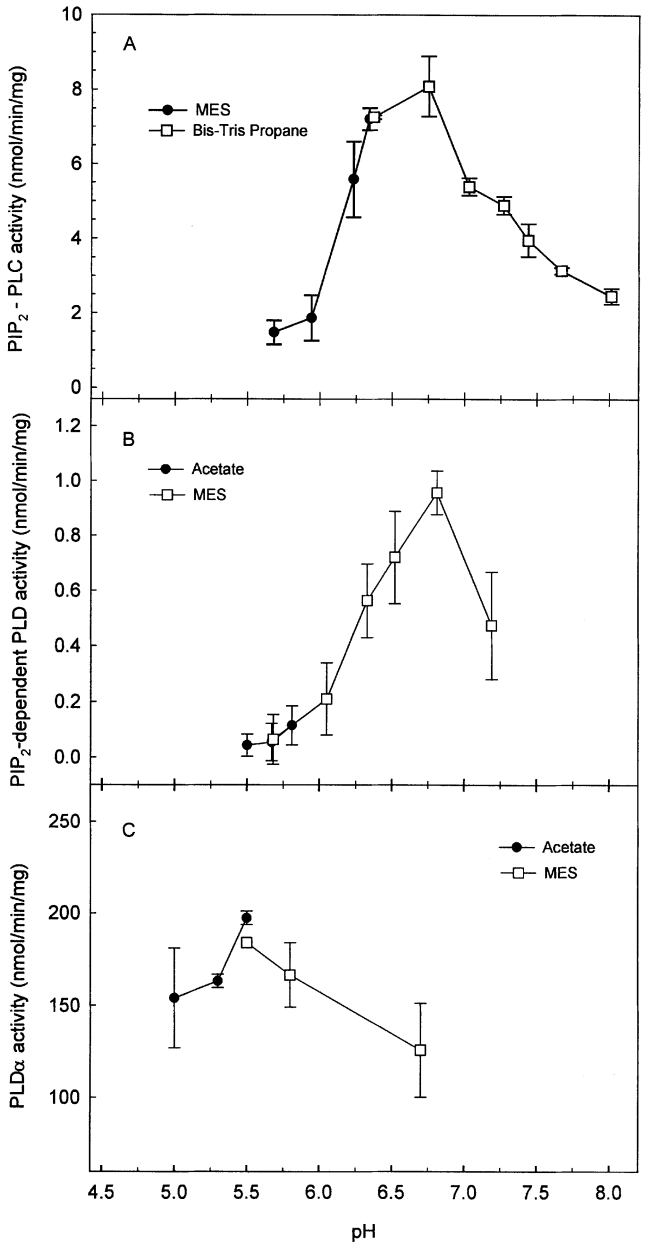

Fig. 3. Effect of pH on activity of plasma membrane associated phospholipases. (A) PI-specific phospholipase C. (B) PIP_2-dependent phospholipase D. (C) PIP_2-independent phospholipase D. Values are means ±SE of two experiments, activity was measured in duplicate for each experiment.

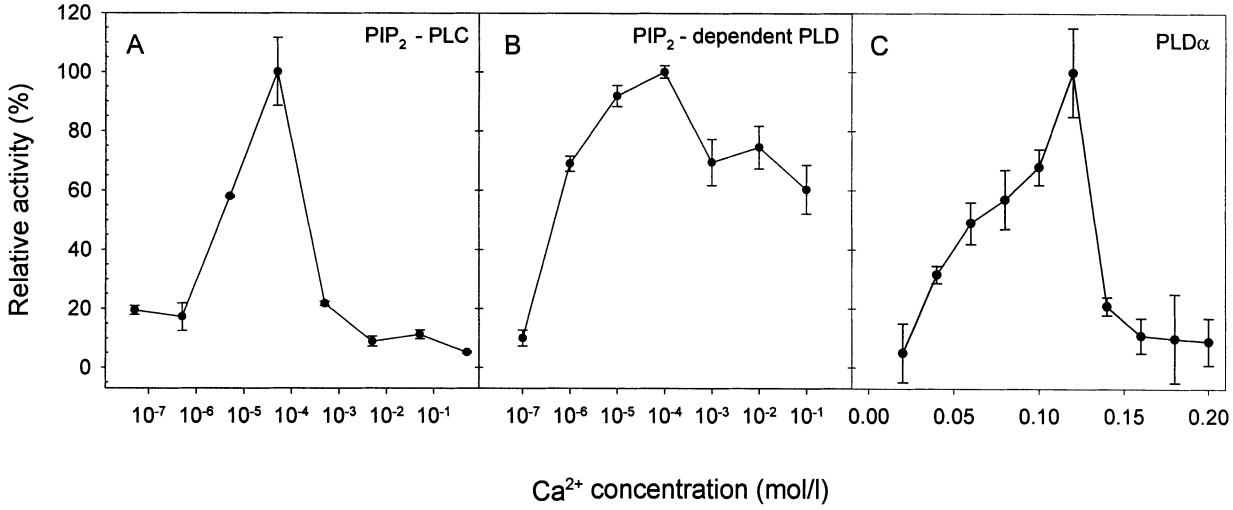

Fig. 4. Effect of Ca^{2+} on the activity of plasma membrane-associated phospholipases. (A) PI-specific phospholipase C. (B) PIP_2-dependent phospholipase D. Free Ca^{2+} in the reaction mixtures was controlled using Ca^{2+}-EGTA buffers. (C) PIP_2-independent phospholipase D. Values are means ±SE of two experiments, activity was measured in duplicate for each experiment.

on the plasma membrane and a very slight increase of about 10% was observed (Fig. 5). The effect was eliminated by the addition of a small amount of SDS (0.5 mM). No effect GTP-γ-S was observed for PIP_2-dependent PLD and PI-PLC (data not shown).

Discussion

In the present study, two types of phospholipid-degrading enzyme activity, PLD and PI-PLC were followed during the development of different tissues, i.e. seeds and plants. Two types of PLD activity, differing in the demands for calcium concentration and effector molecule PIP_2, were screened during these processes. These results indicate that both PLD forms exist in the soluble form and are linked to the membranes, whereas PI-PLC is exclusively associated with a membrane fraction. At the PLD activity level, one major difference is that the specific PIP_2- independent *in vitro* activity was much higher than specific PIP_2-dependent activity. This result is consistent with a recent report that demonstrated the distribution of PLDs in *Arabidopsis* (Fan *et al*., 1999). Another major difference is that the only specific PIP_2-dependent PLD activity of membrane associated enzyme increased 5-fold during seed development while the PIP_2- independent PLD activity decreased in both soluble and membrane fractions. Very low PIP_2-dependent PLD activity was detected in dry seeds. The appearance of PIP_2-independent PLD activity in the cytosol of mature dry seeds could be due to better disruption of the tissue when homogenizing dry seeds. Ryu *et al*. (1996) obtained similar results for extracts from soybean seeds. These authors concluded that PIP_2- independent PLD activity was highest during the early and middle stages of seed development and then declined

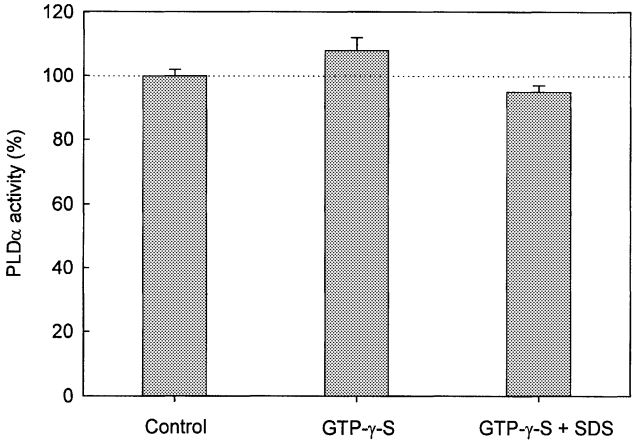

Fig. 5. Effect of GTP-γ-S on the activity of plasma membrane-associated PIP_2-independent phospholipase D. Activity was measured in this case with a different form of substrate (liposomes) prepared by sonication of L-α-phosphatidylcholine in 0.25 M sucrose solution containing 1 mM EDTA. The reaction mixture contained 0.2 M MES pH 6.0, 0.1 M $CaCl_2$, 0.33 mM liposome substrate, 100 μM GTP-γ-S in the absence or presence of 500 μM SDS, and 15 μg of proteins at a total volume of 50 μl.

about 4-fold. In mature dry seeds, specific activity was also increasing, but no PLD mRNA was detected, suggesting that PLD activity comes primarily from the pre-existing PLD rather than *de novo* synthesis.

Compared to seed development, the specific activity of soluble PIP_2-independent enzyme in hypocotyl and cotyledon parts of developing plants, decreased rapidly within 7 d, while specific activity of the membrane-bound form began to increase at this point. One of the possible explanations is that an increase in specific activity of the membrane-bound PIP_2-independent enzyme was due to

the migration of PLD from the cytosol towards the membrane. This tendency was more pronounced in hypocotyls and thus this form of PLD can play a role during rapid tissue development. This sort of redistribution of the soluble (vacuolar) form to the plasma membrane was described for castor bean fully matured leaves compared to 6 d hypocotyls (Xu *et al*., 1996), but not within one tissue.

The presence of PLD in the cytosol suggests that there must be mechanisms to regulate the cytoplasmic PLD activity and an important regulator of PLD is the change in cytoplasmic Ca^{2+}concentration. The calcium-binding domain of PLD was demonstrated to be responsible for the association of the enzyme to the membrane, via an increase in free cellular Ca^{2+} levels (Nalefski *et al*., 1994).

To determine the subcellular origin of the membrane vesicles that possessed phospholipase activity, the crude microsomal membrane preparation was separated using sucrose density gradient centrifugation. Fractionation results showed that PI-PLC and PIP_2-dependent PLD are associated predominantly with the plasma membrane fraction, which contained a small portion of mitochondrial membranes. PIP_2-independent PLD activity was found also in the low-density fractions. These results are in a good agreement with those obtained by Xu *et al*. (1996), demonstrating the intracellular localization of PIP_2-independent PLD in castor bean. Partitioning of microsomal membranes in an aqueous dextran–polyethylene glycol two-phase system is a standard technique for purifying plasma membrane fractions from plant tissues (Larsson *et al*., 1994). During isolation, the plasma membrane vesicles preferentially partition into the polyethylene glycol-rich upper phase, while all contaminating intracellular membranes partition preferentially at the interface or into the lower phase. In the present study, the relative abundance of plasma membrane vesicles in the phase-purified membrane fraction was determined by measuring the activity of 1,3-β-D-glucan synthase II. The presence of contaminating inner membranes in the plasma membrane preparation was identified using marker enzymes and chlorophyll content. The specific activities of markers as well as chlorophyll content are significantly lower than in the corresponding residual inner membrane fraction.

The plasma membrane-associated phospholipases from rape seedlings exhibit distinctive features. Ca^{2+} ions are required for the activities of both enzymes. Unlike the PIP_2-independent PLD, whose maximal activity *in vitro* requires millimolar concentrations of Ca^{2+}, the PIP_2-dependent PLD and PI-PLC are fully active at micromolar concentrations of Ca^{2+} and, moreover, require PIP_2 for activity. The PIP_2-independent PLD was most active at a pH between 5.5 and 6, whereas PIP_2-dependent PLD and PI-PLC were both most active in the pH range between 6.5 and 7.0. These results are consistent with the identification and characterization of PIP_2-dependent PLD activity in *Arabidopsis* (Pappan *et al*., 1997) and PI-PLC activity in *Triticum aestivum* (Arz and Grambow, 1994). These differences suggest that changes in the cellular levels of Ca^{2+}, PIP_2, and pH and in membrane composition, could be an important regulator that differentially activates both enzymes.

The association of phospholipases with the plasma membrane is consistent with the proposed role of phospholipases in transmembrane signalling and appears to be a potential target for the regulation by heterotrimeric G-proteins. Experiments with GTP-γ-S have suggested that plant PLD can be G-protein-regulated (Munnik *et al*., 1998,1995) and Lein and Saalbach (2001) have reported the first indication for a direct interaction between PIP_2-independent PLD and the G-protein α-subunit in plants. Recent studies have demonstrated that GTP-γ-S increases PIP_2-independent PLD and PI-PLC activity in plasma membrane about 3–4-fold in oat cells (Park *et al*., 1996) and 3-fold in light-grown wheat, respectively (Arz and Grambow, 1994). In our experiments such a high increase of the PIP_2-independent PLD was not achieved, probably due to the experimental conditions.

In summary, the basic knowledge of phospholipases concerning changes of their activities during the developmental processes of plant tissues, their localization and characteristics will serve as a starting point for further investigation of their common physiological role.

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