Changes in Phospholipase D Expression in Soybeans During Seed Development and Germination

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ABSTRACT: Activation of phospholipase D (PLD) has been linked to accumulation of nonhydratable phosphatides and lipid degradation leading to soybean seed deterioration during preharvest and postharvest events. This study examined the changes in PLD activity, protein, and mRNA in soybeans during seed development and germination. RNA blotting analysis indicated that expression of the gene that encodes PLD was highest during the early and middle stages of seed development. However, the amount of PLD activity accumulated per cotyledon reached the highest level in mature seeds. During germination and early seedling growth, PLD mRNA was not detected one day after imbibition, while a significant increase in PLD expression occurred in the cotyledons of three- and seven-day seedlings. Similarly, PLD activity and protein concentration showed little change during the first day of imbibition and increased afterward in three- and seven-day seedlings. These results suggested that expression of PLD is developmentally regulated and that the changes in its amount of activity and protein are controlled primarily at the mRNA level. Immunoblotting analysis further revealed the presence of PLD variants that were associated with specific stages of seed development and seedling growth. The PLD variants present in the cotyledons of mature seeds appeared to be distinct from those observed in the early stage of seed development and in young seedlings.

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The phospholipase D (PLD; EC 3.1.4.4) reaction product, phosphatidic acid, is the main component of nonhydratable phosphatides in crude soybean oil (1–3). PLD-catalyzed formation of phosphatidic acid is considered to be the first stage in the rapid deterioration of phospholipids in field- and storage-damaged beans (3–6). PLD activity is thought to initiate a chain of lipolytic activities that release free fatty acids, increase oxidative damage of cell membranes and proteins, and accelerate seed aging and deterioration (7,8). The increased lipid degradation and oxidation may contribute to undesirable taste, color, odor, and instability of soybean oil and products (1,6,9).

The involvement of PLD in membrane deterioration also has been proposed in other plant systems, particularly in connection with senescence, aging, and stress injuries (7,8). However, PLD appears to have multiple functions in cellular metabolism (10). Some studies suggest a role of PLD in lipid mobilization and generation of messengers in signal transduction during seed germination and plant growth (10–12). Multiple forms of PLD have been identified in castor bean seeds, and the appearance of specific PLD variants is associated with developmental and growth conditions, suggesting that different forms of PLD may be involved in different cellular functions (13,14). However, despite the importance of PLD activity in the control of seed oil quality and in plant growth and development, almost nothing is known about the expression and regulation of PLD during seed development and germination. This study was, therefore, undertaken to determine the changes in the activity, protein amount, structural variants, and gene expression of PLD in the soybean cotyledons during seed development, germination, and early seedling growth.

EXPERIMENTAL PROCEDURES

Plant materials. Soybean (*Glycine max* L. Merr cv. KS4390) seeds were imbibed in water for 3 h at 23°C before transferring to plastic pots containing a mixture of moist vermiculite and perlite (1:1, vol/vol). Seedlings were subirrigated with Hoagland nutrient solution and were grown under cool white fluorescent lights at 23 ± 2°C with a 14-h photoperiod. Cotyledons were harvested at the times of 0, 0.125, 1, 3, 7, and 14 d after imbibition. Developing seeds were harvested from soybean plants grown in a greenhouse. Pods at different stages of development, based on cotyledon length, were excised. Seeds were removed from pods and frozen in liquid nitrogen until use.

Protein extraction. The frozen cotyledons were ground in liquid nitrogen with a mortar and pestle. Proteins were extracted with a buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM KCl, 1 mM EDTA, 0.5 M sucrose, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol. All the following steps were carried out at 4°C unless stated otherwise. The homogenate was centrifuged at 6,000 × *g* for 30 min to remove debris and fats, and the resultant supernatant was used either directly or further centrifuged at 20,000 × *g* for 30 min,

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depending upon the experiments. The 6,000 × *g* pellet was resuspended in the homogenization buffer and assayed for PLD activity. Protein content was determined by a dye-binding method (Bio-Rad, Richmond, CA). The protein extracts were used immediately or stored in liquid nitrogen until use.

PLD activity assay. PLD was assayed for its hydrolysis and transphosphatidylation activities using 1-palmitoyl-2-[9,10-^{3}H]palmitoyl-glycero-3-P-choline as substrate. The conditions for substrate preparation, activity assay, and the reaction-product determination were detailed elsewhere (13). Briefly, radioactive phosphatidylcholine (2.5 μCi) and 20 μmol of egg yolk PC were emulsified in 1 mL H_2O by sonication. A standard enzyme assay mixture in a total volume of 0.2 ml contained 100 mM Mes/NaOH (pH 6.5), 25 mM $CaCl_2$, 0.3 mM sodium dodecylsulfate (SDS), 2 mM PC, 1% ethanol, and enzyme preparations in solution or in gel slices. PC, phosphatidic acid, and phosphatidylethanol were separated by thin-layer chromatography and detected by I_2 vapor. Radioactivity in each phospholipids was quantitated by scintillation spectroscopy. The amount of protein used in each assay was typically 10 μg, which was within a linear range for PLD activity under the assay condition.

Polyacrylamide gel electrophoresis (PAGE) analysis. SDS–PAGE analysis used gels containing 8% (wt/vol) acrylamide (pH 8.8) in the resolving phase and 3.5% (pH 6.8) in the stacking phase (15). An equal amount of protein (40 μg) was loaded to each lane. Prestained SDS–PAGE standards (Bio-Rad) were used as molecular weight markers, which consisted of phosphorylase B (106 kD), bovine serum albumin (80 kD), ovalbumin (50 kD), and carbonic anhydrase (33 kD). After electrophoresis, protein on the gels was either transferred on to PVDF membranes for immunoblotting or stained with Coomassie blue.

The preparation of nondenaturing PAGE was the same as the SDS–PAGE except that 0.05% SDS was omitted (13). Protein samples were adjusted to 5 mM dithiothreitol and 5% (vol/vol) glycerol prior to loading, and 50 μg/lane proteins were loaded. Two identical gels were run in parallel at constant voltage of 80 V for 30 min and then increased to 120 V at 4°C for 60 min. After electrophoresis, one gel was immunoblotted using PLD antibodies, and the other was sliced and placed in reaction mixtures for assaying PLD activity as described above.

Purification of PLD antibodies and immunoblotting. Polyclonal antibodies of PLD were raised in rabbits against a 92 kDa protein purified from two-day germinated castor bean endosperm (16). To minimize nonspecific interaction of the antibodies with soybean extracts, the PLD-specific antibodies were isolated using a PLD affinity column in which partially purified PLD from young castor bean leaves was crosslinked to cyanogen bromide-activated Sepharose 4B according to the manufacturer's instruction (Sigma Chemical Co., St. Louis, MO). Briefly, about 7 mg of castor bean, PLD was coupled to 1 mL of the activated gel at room temperature. After uncoupled sites were blocked with 0.2 M glycine, 1 mL of anti-PLD serum was mixed with 10 mL of PLD-coupled gel at 4°C for 3 h, and unbound proteins were washed away with 20 gel bed volumes of the binding buffer [1× phosphate buffered saline (PBS)]. The bound antibodies were eluted with 100 mM glycine (pH 2.5) into tubes containing 1:20 volumes of a neutralizing buffer (1 M potassium phosphate, pH 8.0).

For immunoblotting analysis of SDS–PAGE, proteins were transferred onto polyvinylidine difluoride (PVDF) membranes after electrophoresis. The membranes were incubated with PLD antibodies in PBS (5 μg affinity purified antibodies per mL) containing 5% (wt/vol) nonfat dry milk (15). PLD was made visible with alkaline phosphatase conjugated with goat antibodies against rabbit immunoglobulin (16). PLD protein amounts on immunoblots were estimated by densitometric scanning of the band intensity with a video densitometer (Bio-Rad).

For immunoblot analysis of PLD resolved on nondenaturing PAGE, the gel was immersed in 0.05% SDS in a protein transfer buffer (12.5 mM Tris and 100 mM glycine) for 10 min after electrophoresis (13). The SDS-soaked gel was briefly rinsed with the transfer buffer. Subsequent procedures for transferring the proteins onto PVDF membranes were the same as for the immunoblotting of SDS–PAGE gels (15).

Northern blot analysis. Total ribonucleic acid (RNA) was isolated from cotyledons previously stored in liquid nitrogen using a cetyltrimethylammonium bromide extraction method described previously (16). RNA concentration was determined spectrophotometrically, and the same amount of RNA (20 μg/lane) was subjected to denaturing 1% formaldehyde/agarose gel electrophoresis, and transferred onto a nylon membrane. The equivalency of RNA loading was also verified by ethidium bromide staining of the gel. After transfer, the RNA was fixed on the filter by crosslinking with ultraviolet illumination. The filters were prehybridized in a solution of 0.9 M NaCl, 0.09 M sodium citrate, 0.5% SDS, 5× Denhardt's reagent, and 100 μg/mL salmon sperm DNA at 65°C. The probe was the 2840-bp EcoRI and Kpn I fragment of castor bean PLD cDNA (17). The probes were labelled with [α-^{32}P]deoxyadenosine triphosphate by random priming. Hybridization was performed in the same solution at 65°C overnight. The blots were washed with 150 mM NaCl, 15 mM and 0.1% SDS at 65°C and exposed to x-ray film.

RESULTS AND DISCUSSION

PLD activity, protein, and mRNA changes during seed development. Soybean seeds with different stages of development were grouped based on their cotyledon length. The cotyledon length of 3, 6, 9, 12 mm corresponded approximately to 8–14, 16–22, 24–33, and >35 d after flowering, respectively (18). Accumulation of α and α′, but not β subunits of the storage protein conglycinin, started with 6-mm cotyledons (Fig. 1). The protein composition of 12-mm seeds is similar to dry mature seeds. Thus, the seeds with the cotyledon length at 3, 6, and 9 mm represented the early to middle stages of development, whereas those at 12 mm were at the late stage of development or near maturation.

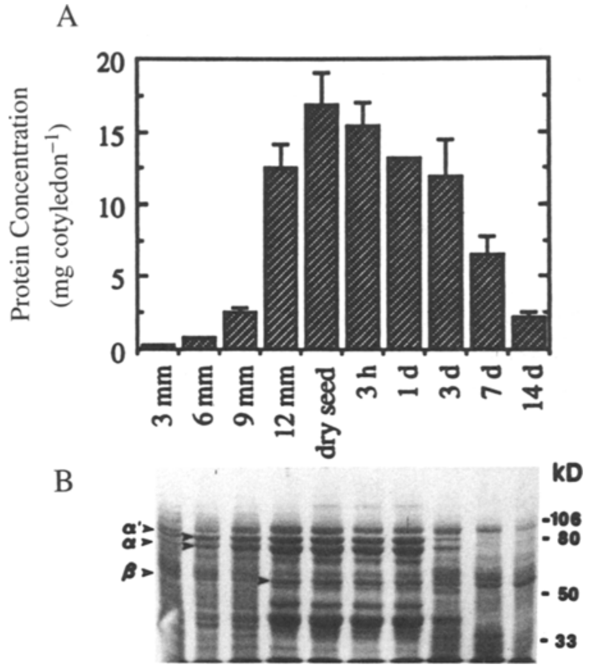

FIG. 1. Change in protein content and composition in the cotyledons during seed development, germination, and seedling growth. A. Protein content per cotyledon as measured in 6,000 × *g* supernatant. B. Protein profile as separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis–PAGE and stained with Coomassie blue.

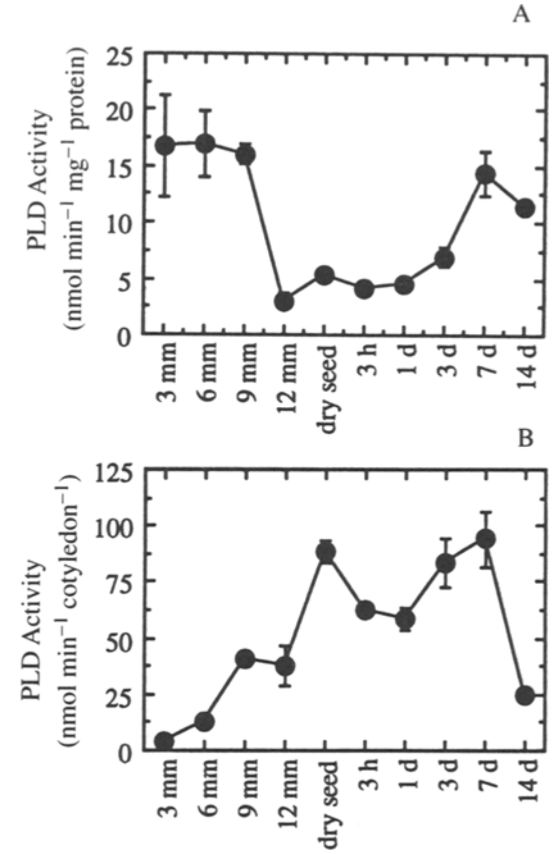

FIG. 2. Change in phospholipase D (PLD) activity in the cotyledons during seed development, germination, and seedling growth. A. Specific PLD activity. B. Total PLD activity per cotyledon. The activity was assayed using 6,000 × *g* supernatant and expressed as nmoles of phosphatidylethanol/min/mg protein or cotyledon. Values are means ± SE of two experiments, and activity was measured in duplicates in each experiment.

Changes in PLD activity among the cotyledons at different stages were determined from 6,000 × *g* supernatant, which contained more than 95% of total PLD activity. The specific activity of PLD was similar in the early and middle stages of development (3, 6, 9 mm), and declined about fourfold in the nearly mature (12 mm) and dry seeds (Fig. 2A). The total PLD activity in the cotyledons of 3, 6, and 9 mm immature seeds increased proportionally to the increase in total protein (Fig. 2B), suggesting that the increase in PLD activity was strongly correlated with seed protein accumulation.

The difference in the level of PLD protein at different developmental stages was monitored by immunoblotting analysis of PLD using the same amount of supernatant proteins (Fig. 3). The PLD antibodies, raised against purified castor bean PLD, crossreacted with soybean PLD, which was estimated about 90 kD and had a similar migration rate to castor bean PLD (data not shown). The PLD antibodies recognized only a 92 kD PLD band in crude castor bean extracts (13,15). In addition to the PLD band, several smaller, weaker protein bands from soybean extracts, however, were immunoreactive with the affinity purified PLD antibodies (Fig. 3). It was unclear whether they were PLD degradation products or other proteins crossreacting to the antibodies.

The level of PLD in the 6,000 × *g* supernatant was similar at 3, 6, 9 mm and declined in near mature and dry seeds (Fig. 3B). This result was in agreement with that of PLD specific activity, suggesting that the change in PLD activity during seed development resulted from change of PLD protein con-

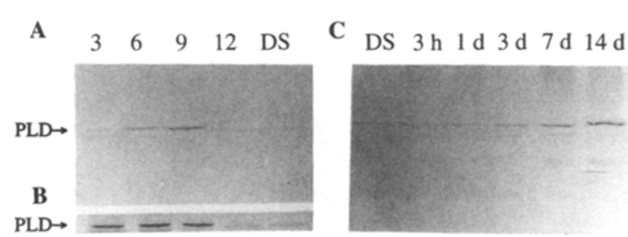

FIG. 3. Immunoblot of soybean PLD separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were immunoblotted with PLD antibodies, and PLD was made visible with alkaline phosphatase. A and B. PLD from 20,000 × *g* and 6,000 × *g* supernatants, respectively, of the cotyledons of developing and dry seeds (40 μg/lane). C. PLD from 20,000 × *g* supernatant of the cotyledons of germinating seeds and young seedlings (40 μg/lane). DS, dry seed. See Figure 2 for abbreviation.

tent rather than the presence of other modulators of PLD, e.g., inhibitors or stimulators. When the 6,000 × g supernatant was centrifuged at 20,000 × g for 30 min, this purification step showed that the level of PLD increased from the 3 to 6 and 9 mm seeds (Fig. 3A). These results indicated that more PLD in the early stages than in the middle stages was removed by the centrifugation.

The accumulation of PLD mRNA, as monitored by RNA blotting using PLD cDNA, appeared to be similar in 3, 6, and 9 mm seeds (Fig. 4). A substantial decline in PLD mRNA level occurred in 12-mm seeds, and no PLD mRNA was detected in dry seeds. This result indicated that expression of the PLD gene is developmentally regulated. Thus, the pattern of PLD mRNA accumulation was similar to that of PLD specific activity, suggesting that the change in the amount of PLD protein is controlled at the mRNA level. The increase in PLD activity in mature seeds could be due to greater disruption of membranes when homogenizing dry seeds.

PLD activity, protein, and mRNA changes during germination and early seedling growth. There was little change in the specific activity of PLD up to the first day after seed imbibition (Fig. 2A). Compared to dry seed, total cellular proteins and PLD activity per cotyledon decreased by about 20% during this period, indicating similar rates of PLD and storage protein degradation during early stages of germination (Figs. 1 and 2). However, little change was found in storage protein composition up to one day after germination (Fig. 1).

Compared to the first day germination treatment, both the specific and total activity of PLD increased by about two-fold by seven days after germination (Fig. 2A). These results indicated that the increase in PLD activity results from an increase in the synthesis and accumulation of PLD protein during germination. However, the specific PLD activity in 14- d seedlings declined about 10% whereas total PLD activity per cotyledon was almost threefold lower than that of the seven days after germination treatment. This result indicated that the relatively high specific activity was largely due to the selective hydrolysis of storage proteins.

The pattern of PLD protein accumulation was similar to that of PLD activity changes in the cotyledons during germination and early seedling growth. The amount of PLD protein remained unchanged up to one day after germination and then increased to the highest level in the 14 d after germination treatment (Fig. 3C). The trend of change in the amounts of PLD protein was the same in the 20,000 × g and 6,000 × g supernatants from the different stages of postgermination (data not shown).

PLD mRNA accumulation also suggested that the increase in PLD protein and activity in three- and seven-day seedlings resulted from increased expression of the PLD gene. As in dry seeds, PLD mRNA was undetected in the cotyledons of three hour and one day of imbibition (Fig. 4), suggesting that PLD activity in the early phase of seed germination comes primarily from the preexisting PLD rather than *de novo* synthesis. A substantial increase in the amount of PLD mRNA was observed in the three- and seven-day seedlings.

PLD variant changes during seed development, germination, and early seedling growth. The occurrence of PLD variants in soybean was tested by nondenaturing PAGE, followed by immunoblotting with anti-PLD antibodies (2). There were two immunoreactive bands in the cotyledons of 9- and 12 mm-length developing seeds, dry seeds, and three-hour, one-day, and three day postgermination cotyledons (Fig. 5A). Under these conditions, there was weak immunoreactivity of PLD from the cotyledons of 3- and 6-mm seeds, and seven- and fourteen-day seedlings. Thus the two bands were not detected in those treatment.

This apparent discrepancy of PLD banding intensity between the native and SDS–PAGE might result from a conformational change in PLD that precluded effective recognition by the PLD antibodies and/or inefficient transfer to blotting membranes under the nondenaturing electrophoresis. To test this possibility and also to verify the immuno-positive bands as PLD proteins, a gel parallel to that used for immunoblotting was run and sliced (2 mm/slice) for measurement of PLD activity in the various gel regions. Three major peaks of PLD activity were detected in the gel at the migration of 8–10, 18–20, and 20–22 mm (Fig. 6). The PLD peak at the 8–10

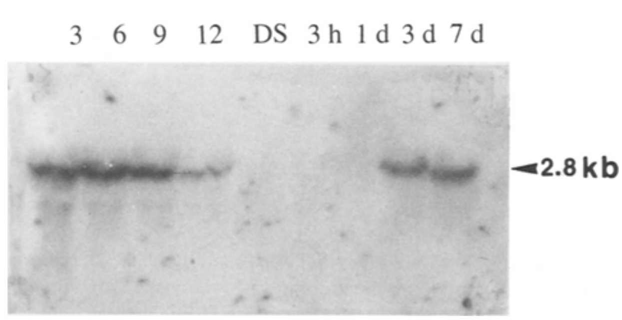

FIG. 4. Northern blot of PLD mRNA isolated from the cotyledons during seed development, germination, and seedling growth. Total RNA (20 μg/lane) was separated by denaturing formaldehyde gel electrophoresis and probed with castor bean PLD cDNA. See Figures 2 and 3 for abbreviations.

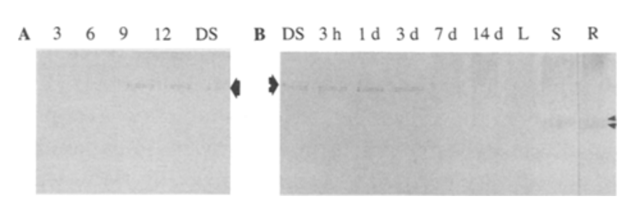

FIG. 5. PLD variants resolved by nondenaturing polyacrylamide gel electrophoresis (8%). A. Immunoblot of PLD from the cotyledons of developing and dry seeds using PLD antibodies. B. Immunoblot of PLD from the cotyledons of germinating seeds and seedlings, and the leaves (L), stems (S), and roots (R) of fourteen-day seedlings. Protein extracts loaded to these gels were 20,000 × g supernatant. An arrow marks the position of the PLD bands at 8 mm and 9.5 mm, and ¤ indicates the PLD bands of 18 mm and 20 mm. See Figures 2 and 3 for abbreviations.

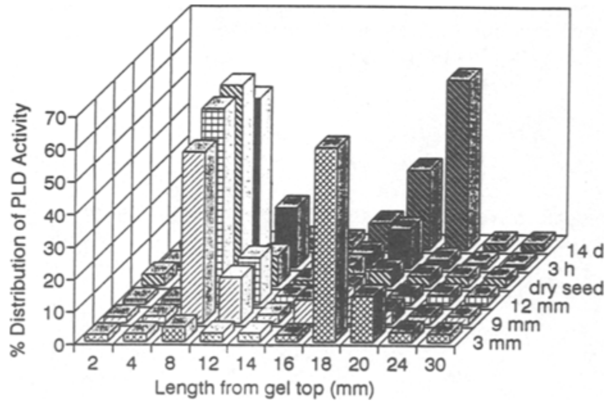

FIG. 6. PLD activity profile assayed in gel slices of nondenaturing polyacrylamide gel electrophoresis gels which were run in parallel to those in Figure 5. See Figure 2 for abbreviation.

mm slice was found only in 9-mm and 12-mm developing seeds, dry seeds, and three-hour imbibition seeds, and the position of the PLD peak coincided with the double immunoreactive bands. This PLD peak was absent in the 8–10 mm slice of the cotyledons from 3-mm developing seeds and fourteen-day seedlings, which also lacked the double immunoreactive bands (Fig. 5). These results indicated that the immuno-positive bands migrating at the 8–10 mm represented the presence of PLD proteins.

The PLD peaks in the 18–20- and 20–22-mm slices were detected in the cotyledons of 3-mm seeds and fourteen-day seedlings, indicating that PLD from the 3-mm and fourteen-day cotyledons migrated faster than that from the other treatments (Fig. 6). While discrete bands immunoreactive to PLD at the 18–22-mm region were not visible from the 3-mm and fourteen day cotyledons, two bands migrating at 18- and 20-mm were detected in the stems and roots of fourteen-day seedlings (Fig. 5B). The amount of PLD protein and specific activity in the stems and roots of fourteen-day seedlings were about three- to four-fold higher than those in the cotyledons and leaves (data not shown). These observations suggested that the two fast-moving PLD variants had a different conformation that was not effectively recognized by the PLD antibodies.

These results indicate the occurrence of possibly four PLD variants in soybean cotyledons, which are associated with specific stages of seed development. The two fast-moving PLD variants were found in the early stages of seed development (3 mm) and late stages of postgermination (fourteen-day seedlings) as well as in vegetative tissues such as stems and roots. The two slow-moving PLD variants were found in the cotyledons of 9-mm developing seeds through three-day postgermination. Therefore, the expression of PLD is developmentally regulated in soybean seeds and likely is controlled at the level of transcription. Furthermore, these findings suggest that the PLD variants present in mature seeds are different from those in embryonic cotyledons and well developed seedlings. Since PLD activity in seeds has deleterious effects on soybean seeds during storage, processing, and handling (1–9), it might be possible to decrease the activity of the storage-specific PLD variants by biotechnological approaches without affecting the PLD function during normal growth and development. However, it should be pointed out that the molecular basis for the PLD variants remains to be elucidated. Experiments are underway to determine whether the PLD variants result from different gene products or from post-translational modifications of one gene product.

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