Promoter Analysis and Expression of a Phospholipase D Gene from Castor Bean

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The expression of a castor bean (Ricinus communis L.) phospholipase D (PLD; EC 3.1.4.4) gene has been studied by examining its promoter activity in transgenic tobacco (Nicotiana tabacum) carrying a PLD promoter-glucuronidase transgene and by monitoring the levels of PLD mRNA in castor bean. Sequence and the 5' truncation analyses revealed that the 5' flanking region from nucleotide -1200 to -730 is required for the regulation and basal function of the PLD promoter. The PLD promoter in vegetative tissues is highly active in the rapidly growing regions such as the shoot apex and the secondary meristem producing axillary buds and vascular tissues of young leaves and stems. The PLD promoter activity in floral tissues was high in stigma, ovary, and pollen grains, but low in petals, sepals, the epidermis of anthers, styles, and filaments. The PLD promoter activity was enhanced by abscisic acid. Northern-blot analysis of PLD in castor bean showed that the PLD mRNA levels were high in young and metabolically more active tissues such as expanding leaves, hypocotyl hooks, developing seeds, and young seedlings, and they decreased in mature tissues such as fully expanded leaves and developed seeds. These patterns of expression suggest a role of PLD in rapid cell growth, proliferation, and reproduction.

PLD (EC 3.1.4.4) is emerging as an important enzyme in regulating a broad range of cellular functions. PLD cleaves glycerophospholipids to generate PA, which along with its derivatives such as diacylglycerol and lysoPA may serve as intracellular and extracellular signaling messengers (English, 1996). Recent work has shown that PLD activity in plants is highly regulated and activation of PLD has been suggested to be part of the signal transduction cascades in wounding (Ryu and Wang, 1996) and plant-pathogen interactions (Young et al., 1996). PLD-catalyzed hydrolysis has been proposed to mediate defflagellation and mating-structure activation in the green alga Chlamydomonas eugametos (Munnik et al., 1995). PLD in animals is believed to be involved in various cellular processes including cell proliferation, vesicular trafficking, secretion, and defense responses (Exton, 1997). In yeast PLD is required for meiosis and sporulation (Rose et al., 1995; Ella et al., 1996; Waksman et al., 1996). In addition, PLD has been implicated in the mobilization of head groups and PA for membrane synthesis during seed germination and seedling growth (Lee, 1989). On the other hand, early studies suggested that PLD is a key lipolytic enzyme contributing to the membrane deterioration of plant tissues during senescence and stress injuries (Paliyath and Droillard, 1992; Voisine et al., 1993).

Some questions important to the understanding of the cellular functions of PLD are, where is PLD expressed and how is the expression of PLD regulated in an organism? The levels of PLD activity have been shown to change in different tissues and developmental stages (Wang et al., 1993; Dyer et al., 1994; Ryu et al., 1996). PLD activity in plants can be affected by different conditions such as senescence, wounding, pathogen infection, and treatments with hormones or mastoparan (Munnik et al., 1995; Ryu and Wang, 1995, 1996; Young et al., 1996). The control of cellular PLD activity is complex, and several mechanisms have been proposed, including Ca2+-mediated intracellular translocation (Ryu and Wang, 1996), increased membrane association (Voisine et al., 1993; Ryu and Wang, 1996), changes in plasma membrane distribution (Young et al., 1996), alteration of PLD isoforms (Dyer et al., 1994; Ryu and Wang, 1995, 1996), and G-protein activation (Munnik et al., 1995). Studies have also indicated that gene expression may be involved in the regulation of PLD. The levels of PLD mRNA changed in soybean seeds during seed development and germination (Ryu et al., 1996), in castor bean (Ricinus communis L.) leaves after treatments with ABA and cytokinin (Ryu and Wang, 1995), and in rice leaves challenged with Xanthomonas oryzae (Young et al., 1996).

The cloning of a castor bean PLD gene has made it possible to analyze the gene promoter that controls its transcription (Xu et al., 1996). In this study, the 5' flanking regions of the castor bean PLD gene were fused with the reporter gene GUS and transformed into tobacco (Nicotiana tabacum), and the transgenic plants were analyzed for the level and tissue distribution of the PLD promoter activity. In addition, the relative accumulation of PLD mRNA was determined in different tissues and growth stages of castor bean. These results reveal the structural and functional...
characteristics of the PLD promoter and the tissue and developmental patterns of the PLD gene expression.

**MATERIALS AND METHODS**

**PLD Promoter-GUS Gene Constructs and Transformation**

The sequence of the 1.2-kb 5′ untranslated region was obtained from an 11-kb genomic fragment containing the castor bean (*Ricinus communis* L.) PLD gene (Fig. 1A) (Xu et al., 1996). PCR was used to generate two 5′ flanking fragments, 1199 and 730 bp in length, and a *HindIII* site was added to the primers for cloning. The 5′ end primers for the two fragments corresponded to nucleotides −1199 to −1185 and −730 to −716, respectively, of the PLD gene (the translation initiation site is designated “+1”). The 3′ end PCR primer was the reverse complement to nucleotides −15 to +1 with an added NcoI site. The DNA template for PCR was a 3-kb genomic subclone containing 2.4 kb of the 5′ flanking region and 0.6 kb of the PLD coding sequences. The thermocycle profiles for PCR were: two cycles of 5 min at 92°C, 2 min at 42°C, and 2 min at 72°C; 40 cycles of 1 min at 92°C, 30 s at 42°C, and 2 min at 72°C; and two cycles of 1 min at 92°C, 30 s at 42°C, and 6 min at 72°C.

The two 5′ flanking fragments were digested with *NcoI* and *HindIII* and inserted in front of the GUS DNA fragment (Fig. 1B). The promoter-GUS cassettes were excised by *EcoRI* and *HindIII* digestion and then cloned into a binary agrobacterial gene transfer vector. The recombinant plasmids were introduced into *Agrobacterium tumefaciens* and transformed into tobacco (*Nicotiana tabacum*) using a leaf disc inoculation method (Horsch et al., 1988). Transgenic plants were selected by resistance to kanamycin.

**ABA, GA, and Cytokinin Treatments**

Seeds of T₁ plants were sterilized with a solution containing 2.63% sodium hypochlorite and 0.1% Tween 20 for 20 min, followed by three washes with sterile water. Seeds were germinated on Murashige-Skoog plates with 70 µg/mL kanamycin under cool-white fluorescent lights at 23 ± 2°C with a 16-h photoperiod. Nine-day-old seedlings were washed briefly in sterile water and then immersed in 50 µM kinetin, ABA, or GA for 24 h at room temperature. After incubation seedlings were briefly rinsed with sterile water twice, and stained with a GUS histochemical solution as described below. Twenty seedlings were examined for each treatment and this experiment was repeated three times.

To grow mature plants, 2- to 3-week-old seedlings were individually transplanted into potting soil and substrigated with Hoagland nutrient solution. Leaf discs were excised with a cork borer (13 mm in diameter) from fully expanded leaves of approximately 8-week-old plants. Leaf discs were placed adaxial side up in Petri dishes (85 mm) on two layers of 3MM filter paper wetted with 3 mL of water and 50 µM ABA, kinetin, or GA. The leaf discs were incubated for 24 h under the same conditions as for growing plants. Four discs, collected from four different plants, were used in each treatment and discs from the same leaf sources were used in all of the treatments.

**Figure 1.** PLD promoter sequence and chimeric gene constructs. A, Nucleotide sequence of the 1.2-kb 5′ flanking region of a castor bean PLD gene. The exon sequence is in uppercase letters and the intron sequence in the 5′ noncoding region is in lowercase, italic letters. The putative CAAT and TATA boxes are in boldface. Two primer sequences for generating the 1.2- and 0.7-kb fragments are underlined. B, Schematic representation of PLD promoter-GUS fusions. Lines represent the 5′ upstream region and introns of the PLD gene and solid boxes are exons of the PLD gene. Open boxes indicate the GUS gene in the 1.2-kb GUS and 0.7-kb GUS chimeric transgenes. The nucleotide at the translational initiation site is designated “+1”.

**Histochemical GUS Staining**

GUS activity localization was performed according to a described procedure (Jefferson et al., 1987). Coatless seeds, seedlings, or tissues at different growth stages or after different treatments were infiltrated in a GUS staining solution containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (cylohexylamine salt), 100 mM phosphate buffer, pH 7.0, 10 mM EDTA, and 0.1% Triton X-100 for 15 min at room temperature. The materials were then incubated at 37°C overnight in the same solution. The samples were dehydrated with an ascending series of ethanol (25–100%) to remove chlorophyll, which often interferes with the detection of blue-colored GUS staining. After decoloration, the samples were rehydrated with a declining series of ethanol and stored in 0.5% NaN₃ solution at 4°C.
Fluorometric GUS Assay

Tobacco tissues were ground with a chilled mortar and pestle in an ice-cold protein extraction buffer containing 50 mM sodium phosphate, pH 7.2, 1 mM EDTA, 0.1% Triton X-100, and 10 mM 2-mercaptoethanol. After ABA, kinetin, or GA treatments leaf discs were briefly rinsed with water and ground with the same ice-cold protein extraction buffer. The homogenates were centrifuged at 6000 g for 15 min at 4°C. Protein concentration in the supernatant was measured with the Bradford method according to the manufacturer’s instructions (Bio-Rad). The fluorometric GUS reaction was performed according to the method of Herbers et al. (1994). The GUS reaction was started by adding supernatant protein (2.5 μg) into 200 μL of an extraction buffer containing 2 mM 4-methylumbelliferyl-β-D-glucuronide. The reaction was incubated at 37°C for 15 min and terminated by adding 0.8 mL of 0.2 M Na₂CO₃. The reaction products were measured fluorometrically (excitation 365 nm, emission 455 nm) with a fluorescence spectrophotometer. The rate of formation was monitored from the initial velocity under the reaction conditions. GUS activity was calculated in nanomoles of product per minute per milligram of protein, and the data were analyzed statistically.

Growth and Sampling of Castor Bean

Coatless seeds of castor bean (cv var Hale) were germinated in moist vermiculite at 30°C in the dark for up to 6 d (Wang et al., 1993). Endosperm was collected 0, 1, 2, 3, 4, 5, and 6 d after beginning imbibition. Cotyledons, roots, and hypocotyls were sampled from seedlings 5 and 6 d after germination. To grow mature plants, 4-d-postgermination seedlings were individually transplanted into plastic pots containing a mixture of vermiculite and perlite (1:1, v/v) subirrigated with Hoagland nutrient solution. Castor bean plants were grown under cool fluorescent lights at 23 ± 3°C with a 14-h photoperiod. Developing seeds were removed from fruits on different days after flowering. All of the above samples were frozen immediately in liquid N₂ until use.

Nucleic Acid Extraction, Southern-Blot Analysis, and Northern-Blot Analysis

Genomic DNA was extracted from young leaves of the wild type and the T₂ generation of transgenic tobacco plants using a modified cetyltrimethylammonium bromide method (Xu et al., 1996). DNA was digested with EcoRI and HindIII, electrophoresed on a 0.7% agarose gel, and transferred to a nylon membrane. The DNA on blots was hybridized with the 2.8-kb, full-length PLD cDNA of castor bean at 65°C (Wang et al., 1994). The relative amount of PLD mRNA on the blots was estimated by densitometric scanning of the band intensity on autoradiograms with a video densitometer.

PLD-Promoter Activity Located in the 1.2-kb 5’ Flanking Region

The presence of several putative transcription factor and cis transcriptional regulatory elements around nucleotide −1175 suggests that this region is important in the regulation of PLD gene transcription. To examine the functional unit of the PLD promoter, two 5’ flanking region fragments of the PLD gene were fused to the reporter gene GUS: one was from nucleotides −1199 to −1, and the other was from nucleotides −730 to −1 (Fig. 1). The former is referred to as the 1.2-kb GUS and included all of the binding sites described in Table I. The latter construct is called the 0.7-kb GUS and contained the first set of TATA box-like and CAAT sequences and excluded most transcription factors and cis regulatory elements. The two constructs were

RESULTS

Sequence Analysis of Transcription Regulatory Elements in the 5’ Flanking Region of the PLD Gene

The 5’ flanking sequence of the PLD gene was searched for the binding sites of various transcription factors and transcriptional regulatory elements. Two TATA box-like sequences are present at nucleotides −629 and −799. In addition, sequences have been found in the 5’ flanking region that are highly similar to those for binding the transcription factor ATF (Lin and Green, 1988) and two subfamily members of the histone promoter-binding proteins, HBP-1b and HBP-1a (Mikami et al., 1994, 1995). In particular, the sequence starting at nucleotide −1174 was perfectly matched with the binding site for HBP-1b (Table I).

There were also sequences sharing a high degree of similarity with several cis transcriptional regulatory elements in the 5’ flanking region (Table I). These include an ACGT box, a JM box (Kim et al., 1992), a GA box (Huttly and Phillips, 1995), a cold-responsive element (Baker et al., 1994), a CATATG box (Kawagoe et al., 1994), CARE, and AP2 (Table I). CARE and AP2 are the cis elements that respond quickly and slowly, respectively, to cAMP in some mammalian cAMP-inducible genes (Roesler et al., 1999). Most of the regulatory sequences overlapped one another in the region around nucleotide −1175, with the ACGT being the core sequence.
plants used in this study were estimated to have two copies of the chimeric PLD promoter-GUS gene in the genome. The presence of the chimeric transgenes in kanamycin-resistant plants was confirmed by Southern-blot analysis of genomic DNA digested with EcoRI/HindIII. This double digestion released two fragments, the 1.2- or 0.7-kb PLD-promoter region and the 2.1-kb GUS region. The 1.2-kb and GUS gene fragments were found in the 1.2-kb GUS transgenic plants, and the 0.7-kb fragment and the GUS gene were present in the 0.7-kb GUS transgenic plants. Neither the 1.2-kb nor the GUS gene fragments were present in untransformed tobacco (data not shown). Transgenic plants used in this study were estimated to have two copies of the chimeric PLD promoter-GUS gene in the genome.

Histochemical GUS staining was used to screen for the PLD promoter activity in T₀ and T₁ transgenic plants carrying the 1.2- and 0.7-kb GUS constructs. Twenty-seven independent transgenic lines of the 0.7-kb GUS construct were generated, and more than 20 seedlings were examined for each line. None of the plants transformed with the 0.7-kb GUS construct displayed detectable GUS activity in different tissues, growth stages, or treatments, whereas plants carrying the 1.2-kb GUS transgene showed blue GUS staining (Fig. 2). These results demonstrate that the first 730 nucleotides in the 5’ flanking region are not sufficient for the PLD promoter activity, and that the region beyond nucleotide −730 is required for the function of the PLD promoter.

**PLD Promoter Activity in Different Vegetative Tissues and Developmental Stages**

The tissue- and developmental stage-expression of the PLD promoter was investigated in the 1.2-kb GUS transgenic tobacco using histochemical and fluorometric GUS assays. GUS activity was low in dry seeds and increased after 1 d of imbibition. At the early stages of seedling growth, GUS activity was present throughout seedling tissues (Fig. 2A). At the later stages high GUS activity was concentrated at the shoot apex and the apical and elongating regions of roots. In more than 100 seedlings examined, tobacco carrying the 0.7-kb GUS transgene exhibited no GUS activity (Fig. 2B).

PLD promoter activity was present in leaves, roots, and stems with defined spatial patterns (Fig. 2, C-G). In young, expanding leaves GUS activity was localized predominately in vascular bundles and, to a smaller extent, in mesophyll cells (Fig. 2C). In fully open, mature leaves GUS activity decreased and was detected only in the vascular bundles (data not shown). Leaf discs from mature leaves did not show GUS staining in the cell layers of the mesophyll immediately along the cut surface (data not shown), suggesting that the high expression in vascular bundles was not caused by preferential uptake of the GUS substrate to this tissue.

In roots GUS activity was mostly localized at the junction between elongation and maturation, where the vascular cylinder was not fully developed (Fig. 2D). Another root region showing GUS activity were the branching points between the primary and lateral roots. Histochemical staining of the transverse sections of stems from flowering plants revealed two zones of high PLD-promoter activity; one corresponding to the region where axillary buds were generated (Fig. 2E) and the other at the internal phloem (Fig. 2F). The PLD promoter was more active in the upper region of stem (Fig. 2F) than in the lower region (Fig. 2G).

A fluorometric GUS assay was used to quantitate PLD promoter activity in different stages of stems, roots, and leaves from flowering plants (Fig. 3). The stem corresponded to the region between the first flower and the top leaf. GUS activity in the stem region was about 2- and 3-fold higher than that of roots and the top leaf (leaf no. 10), respectively. The middle leaves displayed higher GUS activity than the top and bottom leaves. The leaves on the top and the bottom senesce earlier than those at the middle position as tobacco matures. Therefore, our results could mean that the PLD promoter activity declines as the leaves become older or senesce.

**Expression of the PLD Promoter in Reproductive Tissues**

PLD promoter activity was highly active in both developing and mature flowers (Fig. 4A). GUS activity was high in the stigma, including the papillar cells and the subepidermal secretory cells, and throughout the ovary, including the ovules. GUS activity was low or undetectable in petals, sepals, the epidermis of anthers, the style, and filaments (Fig. 4, A–C). Staining of the longitudinal sections of anthers showed that GUS activity was much higher in the pollen sacs than in the epidermis. High GUS staining in the pollen sacs resulted from dense GUS staining of pollen grains (Fig. 4, C and D). In contrast, no positive staining

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**Table 1. Potential binding sites for transcription factors and cis-acting elements in the castor bean PLD gene promoter**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Function</th>
<th>Sequence</th>
<th>Position</th>
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<tbody>
<tr>
<td>ATF</td>
<td>Transcription factor binding site</td>
<td>CCGACGTCG</td>
<td>-1177</td>
</tr>
<tr>
<td>HBP-1b</td>
<td>Histone-promoter binding proteins</td>
<td>ACOTCA</td>
<td>-1174</td>
</tr>
<tr>
<td>HBP-1a</td>
<td>Histone-promoter binding proteins</td>
<td>CGACGT</td>
<td>-1176</td>
</tr>
<tr>
<td>JM</td>
<td>Jasmonate response</td>
<td>CGACGT</td>
<td>-1176</td>
</tr>
<tr>
<td>GA</td>
<td>Gibberelic acid response</td>
<td>GGAGGTGT</td>
<td>-239</td>
</tr>
<tr>
<td>CCGAC</td>
<td>Cold response</td>
<td>CCGAC</td>
<td>-1177</td>
</tr>
<tr>
<td>CATATG</td>
<td>Negative transcription regulator of bean storage proteins</td>
<td>CATATT</td>
<td>-780</td>
</tr>
<tr>
<td>CARE</td>
<td>Quick cAMP response</td>
<td>CACGCTCAC</td>
<td>-1176</td>
</tr>
<tr>
<td>AP2</td>
<td>Slow cAMP response</td>
<td>AACACGAA</td>
<td>-165</td>
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</tbody>
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The nucleotides identical to those reported sequences are shown in boldface.
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Figure 2. Histochemical localization of GUS activity in seedlings and vegetative organs. A and B, GUS staining of seedlings transformed with the 1.2-kb GUS (A) and 0.7-kb GUS (B) constructs. 1, 2, 3, 4, 5, and 6, Number of days after imbibition. C, The top first leaf from a 30-d-old plant. M, Mesophyll; V, veins. D, Roots from a 30-d-old plant. PR, Primary roots; LR, lateral roots. C to G, Tissues from plants transformed with the 1.2-kb GUS transgene. E to G, Transverse sections of upper (E and F) to lower (G) parts of the stem in a flowering plant.

was detected in floral tissues of the 0.7-kb GUS transgenic plants (Fig. 4, E and F).

Effects of ABA, GA, and Cytokinin on PLD-Promoter Activity

The presence of sequences similar to several reported cis transcriptional regulatory elements pointed to the possibility of controlled PLD gene expression. Therefore, the seedlings of the 1.2-kb GUS transgenic plants were treated with ABA, kinetin, and GA, and analyzed for GUS activity (Fig. 5A). In water-treated control seedlings, GUS activity staining was found primarily in the shoot meristem and veins of the 1.2-kb GUS transgenic plants (Fig. 5A). Treatment of the seedlings with growth regulators changed the distribution of the PLD-promoter activity in the leaf. ABA enhanced PLD-promoter activity in mesophyll cells and the shoot and root junction. Kinetin and GA suppressed the PLD-promoter activity in leaves, restricting the promoter activity to the shoot meristem.

ABA, kinetin, and GA treatments were also applied to leaf discs excised from fully expanded leaves, and the effects on PLD-promoter activity were quantitated by a fluorometric GUS assay (Fig. 5B). After the discs were treated with 50 μM concentrations of phytohormones for 24 h, ABA caused about a 2-fold increase in GUS activity, whereas kinetin and GA did not significantly alter GUS activity (Fig. 5B). At the time of sampling, no apparent morphological changes, such as leaf yellowing, were observed among the water- and phytohormone-treated discs. After a longer period of incubation, discs treated with ABA and kinetin displayed different rates of senescence, as indicated by leaf yellowing and retention of chlorophyll. Compared with water-treated control discs, ABA promoted senescence, whereas kinetin attenuated it (data not shown). This result was consistent with previous observations that ABA and kinetin have opposite effects on the senescence of detached leaves (Ryu and Wang, 1995).

Changes in PLD mRNA Levels in Castor Bean Tissues

The above measurements of GUS activity in transgenic tobacco reflected the promoter and, therefore, the transcriptional activity of the castor bean PLD gene. To examine and confirm the pattern of the PLD gene expression,
Figure 4. Histochemical localization of GUS activity in floral tissues. A to D, Floral tissues from plants transformed with the 1.2-kb GUS construct. A, Longitudinal section of a flower. P, Petal; SA, stigma; SY, style; A, anther; AF, anther filament; O, ovary. B, Ovules (OL), style, and stigma. C, Vertical section of anthers. D, Pollen grains. E and F, Pollen grains and longitudinal section of a flower, respectively, from the 0.7-kb GUS transgenic plants.

changes in PLD mRNA levels were monitored in different tissues and developmental stages of castor bean (Fig. 6). In 5- and 6-d-postgermination seedlings, RNA was isolated from roots, endosperm, cotyledons, and hypocotyls. The hypocotyls were divided into hook and elongated regions; the former represents rapidly growing tissue and the latter is a differentiated, relatively slow-growing region.

The size of the PLD mRNA was approximately 2.9 kb, which is in agreement with that derived from the cDNA sequence (Wang et al., 1994) (Fig. 6A). In hypocotyls the amount of PLD mRNA was greater in the hook (Fig. 6A, lane H) than in the elongated regions (Fig. 6A, lane S) in 5-d-postgermination seedlings, and the difference between the two regions became larger in 6-d-postgermination seedlings. The largest amount of PLD mRNA was detected in the hook region and was more than 10-fold greater than that in roots and 18-fold greater than that in endosperm and cotyledons (Fig. 6A). PLD mRNA was undetectable in the endosperm of mature, dry seeds. The level of PLD mRNA increased after 1 d of imbibition, plateaued at 2 d, and decreased by 3 d postgermination (Fig. 6B).

The expression of the PLD gene is developmentally regulated in seeds and leaves; more PLD mRNA accumulated in early than in later developmental stages. The amount of PLD mRNA was much higher in seeds 10 and 15 d after flowering than 30 d after flowering (Fig. 6C). PLD mRNA in the endosperm of seeds 30 d after flowering was detectable only after prolonged exposure of radiographic film, but no PLD mRNA was observed in the endosperm of near-mature seeds (45 d after flowering).

of PLD mRNA was tissue-specific; although the PLD mRNA was readily detectable in the endosperm 15 d after flowering, hybridization of the same amount of total RNA from the seed coats (Fig. 6C, lane SC) and fruit coats (data not shown) at this stage failed to detect PLD mRNA. When the PLD mRNA was analyzed from different leaves of preflowering castor bean plants, the PLD mRNA levels decreased as leaf age increased; the older, bottom leaves had less PLD mRNA than the younger, top leaves (Fig. 6D).

Figure 5. GUS activity in transgenic tobacco treated with water, ABA, kinetin, or GA. A, GUS activity distribution in 9-d-old, 1.2-kb GUS transgenic seedlings after 24-h treatment with water, 50 μM ABA, kinetin, or GA. The seedling shown for each treatment is a representative of 60 seedlings. B, Fluorometric assay of GUS activity in water-, ABA-, kinetin-, or GA-treated leaf discs. Leaf discs were excised from fully expanded leaves from the 1.2-kb GUS transgenic plants and incubated in water, 50 μM ABA, kinetin, or GA for 24 h. GUS activity in each treatment is expressed as a percentage of the GUS activity in discs incubated in water. Values are means ± SE of five experiments. Different letters on the bars indicate significant difference as evaluated by Student's t test.
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Figure 6. Northern-blot analysis of PLD gene expression in castor bean. A, Accumulation of PLD mRNA in hypocotyl hooks (H), cotyledons (C), roots (R), hypocotyl elongated regions (S), and endosperm (E) of 5- and 6-d-postgermination etiolated seedlings. B, Changes in PLD mRNA in endosperm during postgermination: 0, RNA from dry seeds before imbibition; 1, 2, 3, 4, 5, and 6, RNA 1, 2, 3, 4, 5, and 6 d after imbibition. C, Decrease in PLD mRNA during seed development: 10, 15, 30, and 45, endosperm from seeds 10, 15, 30, and 45 d after flowering; SC, RNA from the seed coats 15 d after flowering; SC, RNA from the seed coats 15 d after flowering. D, Accumulation of PLD mRNA in leaves of different ages: 1, 2, 3, and 4, RNA from leaves at positions indicated in the drawing to the right. Total RNA (20 μg/lane) was electrophoresed on a denaturing 1% formaldehyde/agarose gel, transferred to a nylon membrane, and probed with the 2.8-kb PLD cDNA.

DISCUSSION

Increasing evidence has pointed to the involvement of PLD in a variety of physiological processes, ranging from the generation of second messengers to membrane degradation. A molecular understanding of this enzyme is necessary for the delineation of its cellular function. We previously reported the cloning of the first eukaryotic PLD from castor bean (Wang et al., 1994). This paper describes the promoter analysis and the spatial and temporal expression of the castor bean PLD gene. The 1.2-kb 5' flanking region contains the functional and regulatory elements of the PLD promoter, whereas the first 0.7-kb 5' flanking fragment is not sufficient for the promoter activity.

Sequence analysis has also suggested that most of the potential sites for transcriptional regulation reside in the region from nucleotides −1199 to −730. The hexamer ACAGTCA at nucleotide 1175 appears to be the core element, and a number of DNA-binding proteins, such as ATF, HBP-1a, and HBP-1b, interact with this sequence (Lamb and McKnight, 1991; Mikami et al., 1994). ATF is a well-known transcription factor in mammalian cells, and HBP-1a and HBP-1b proteins are likely to be plant transcription factors. The ATF, HBP-1a, and HBP-1b proteins share the same hexamer DNA-binding site. The hexamer or hexamer-like boxes exist in the cis control region of numerous plant genes known to be regulated by environmental and physiological signals (Lamb and McKnight, 1991; Mikami et al., 1994; Yang et al., 1995).

In the present study, deletion of the region from nucleotides −1199 to −731, which includes this hexamer, led to a complete loss of function of the PLD promoter. These findings suggest that the 5' flanking sequences within this region are crucial for the basal and regulated expression of the PLD gene. The hexamer may function as an important binding site for various transcription factors and cis regulatory proteins. The binding of these factors and their interactions after binding may regulate PLD gene transcription.

Using the castor bean PLD promoter-GUS gene fusion system and transgenic tobacco plants, we have shown that the PLD promoter is more active in young, rapidly growing tissues than in mature tissues. High activity of the PLD promoter is found in the shoot apex and in important floral organs such as ovules, pollen grains, and stigmas. The measurements of PLD mRNA accumulation in different tissues and growth stages of castor bean have also shown profiles consistent with those of the promoter activity. The PLD mRNA levels were high in metabolically more active tissues such as expanding leaves, hypocotyl hooks, and young, developing seeds and seedlings, and decreased in mature tissues such as fully expanded leaves and developed seeds.

The levels of PLD protein and activity varied in the same pattern during leaf and endosperm development (Wang et al., 1993; Dyer et al., 1994). For example, the total PLD activity increased 2.5-fold in the endosperm 1 d after imbibition (Wang et al., 1993). Higher amounts of PLD protein were present in young, expanding leaves than in mature leaves (Dyer et al., 1994). The correlated changes in the levels of PLD-promoter activity, mRNA, protein, and enzymatic activity indicate that PLD gene transcription plays an important role in controlling PLD protein levels, and that PLD transcription is developmentally regulated.

The localization of high levels of PLD gene expression in dividing and growing cells suggests that PLD may play an important role in cell growth and proliferation. Recent studies have suggested that activation of PLD is involved in a number of signaling cascades, including cell proliferation, defense response, membrane trafficking, and reproduction (Munnik et al., 1995; Rose et al., 1995; Ryu and Wang, 1996; Young et al., 1996; Exton, 1997). PLD activity is stimulated by a number of key components in signal transduction pathways, including Ca^{2+}, protein kinase C, and G proteins.

The PLD reaction product PA has been proposed to be a mitogen signal that stimulates DNA synthesis and growth-related enzymes (English, 1996). PA is also believed to provide a prolonged supply of diacylglycerol, which is required for sustained signaling in cell proliferation (Exton, 1997). In addition, PA is a central intermediate for the synthesis of glycerolipids. Therefore, PLD may contribute to rapid cell growth and proliferation by providing the signaling messengers and the intermediates for membrane lipid synthesis and remodeling. A role of PLD in membrane remodeling is suggested by the present results showing that the junctions between
primary and lateral roots, at the elongation and maturation regions of shoots and roots, and at axillary buds and main stems (Fig. 2). Cells at these joints must consistently cope with changes and/or injuries resulting from tissue expansion and cell differentiation. High PLD activity in these regions may be involved in rapid lipid turnover and in supplying PA and head groups for membrane repair and remodeling.

The above-suggested roles of PLD seem at variance with a role in membrane lipid degradation, which has been proposed previously (Paliyath and Droillard, 1992). A number of studies have suggested that PLD-mediated hydrolysis of phospholipids is the first and critical step in membrane deterioration during senescence and stress injuries (Willemot, 1983; McCormac et al., 1993; Voisine et al., 1993). PA favors the nonlamellar phase, which may destabilize membranes. It has also been proposed that PA is further catabolized to form lipid peroxides and free fatty acids in deteriorating tissues (Paliyath and Droillard, 1992). However, the roles of PLD in growth and senescence need not be mutually exclusive and may reflect the multiple cellular functions of this enzyme.

In addition, the present study indicates that gene expression plays an important role in the regulation of PLD levels, but the control of PLD activity in the cell may also involve other mechanisms. PLD is present in various organs, including young and senescent tissues. It has been shown that PLD activity increases in response to freezing injuries (Willemot, 1983), mechanical wounding (Ryu and Wang, 1996), pathogen infection (Young et al., 1996), and senescence (McCormac et al., 1993; Voisine et al., 1993). The increase has been proposed to occur through mechanisms such as decompartmentalization, membrane rigidification, enhanced PLD association with membranes, changes in isoform patterns, and modulation by stimulators and inhibitors (McCormac et al., 1993; Voisine et al., 1993; Ryu and Wang, 1995; Young et al., 1996; Exton, 1997; Pappan et al., 1997b). Such activation of PLD-mediated hydrolysis may be important in lipid turnover, membrane repair, and remodeling during adaptation to changing environmental conditions. It may also contribute to lipid destruction during senescence and cell death. Whether PLD acts in stress adaptation or deterioration is likely to be influenced by the nature and severity of the plant stresses and physiological conditions.

As one way to study the responsiveness of PLD to growth conditions, this study examined the effects of phytohormones on the PLD-promoter activity. Previous data have suggested that the gene expression of PLD in detached leaves may be stimulated by ABA, but not by kinetin (Ryu and Wang, 1995). The present data using GUS assays have shown that the PLD-promoter activity in leaf discs is stimulated by ABA, but not by kinetin or GA. These effects are consistent with those observed by direct measurements of PLD mRNA levels in castor bean leaf discs treated with ABA and kinetin (Ryu and Wang, 1995), which suggested that ABA affects PLD expression at the transcriptional level.

Is the ABA effect on PLD gene expression direct or is it a consequence of the physiological changes brought about by the ABA treatment? A well-documented effect of ABA is its promotion of senescence in detached leaves (Ryu and Wang, 1995). The increase in PLD-promoter activity and mRNA was observed 1 d after ABA treatment, at which time no apparent acceleration in senescence was observed. When senescence became apparent after 3 to 5 d of incubation with ABA, PLD gene expression decreased (Ryu and Wang, 1995). The PLD-promoter activity and mRNA level were also reduced during natural senescence (Figs. 3 and 6). This decrease in PLD gene expression in senescing tissues suggests that its increased expression in ABA-treated leaves is not a result of ABA-promoted senescence. It is likely that the ABA-enhanced PLD gene expression results from the action of factors involved in ABA response pathways.

The analysis of the PLD-promoter sequence has identified several regions similar to reported cis transcriptional regulatory elements. In particular, the ACGT box at nucleotide −1177 is a cis control motif found in many plant genes that are regulated by ABA and various stress signals (Baker et al., 1994). This element may represent a target site for ABA control of PLD gene expression. The regulation of PLD expression by ABA implies that PLD is involved in mediating cellular response to ABA.

Another potential regulator of plant PLD gene expression is cAMP, an important regulator in animal cells. Two cis-cAMP-responsive elements, CARE and AP2, are conserved in a number of mammalian cAMP-inducible genes (Roesler et al., 1995). CARE functions as both a basal and an inducible transcriptional enhancer for cAMP-regulated genes (Montminy et al., 1986; Lin and Green, 1988; Roesler et al., 1995). CARE- and AP2-like sequences are present in the promoter region of the castor bean PLD gene. Although cAMP function in plant cells is not well established, there is evidence for the presence of cAMP and enzymes that interact with cAMP in higher plants (Carricarte et al., 1988). Further characterization of the cAMP regulation of PLD gene expression may provide a useful system for investigating the role of cAMP in plant metabolism.

It is worth noting that recent studies have demonstrated the presence of multiple PLD genes in plants, and the cloning of two PLD isoforms, PLDa and PLDb, from Arabidopsis has been reported (Dyer et al., 1995; Pappan et al., 1997a, 1997b). PLDa represents the well-characterized, conventional plant PLD that requires millimolar levels of Ca2+ for optimal activity, whereas PLDb is a novel PLD with the optimal activity of which requires micromolar levels of Ca2+ and phosphatidylinositol 4,5-bisphosphate. PLDa is more prevalent than PLDb in plant tissues, based on the estimates of their activity and protein distributions (Pappan et al., 1997a, 1997b).

The castor bean PLD gene used in this study corresponds to the gene of PLDα (Pappan et al., 1997a, 1997b). The amino acid sequences of PLDα and PLDb are about 50% identical. Studies using Arabidopsis PLDα and PLDb cDNAs have documented that PLDα does not cross-hybridize with PLDb under the high-stringency hybridization conditions used in this study (Pappan et al., 1997b). Therefore, the change in the PLD mRNA from the northern-blot analysis specifically represents the levels of PLDα. An ongoing study in this laboratory is comparing the spatial and temporal expression of different PLD genes.
in plants. Such information will provide insightful clues into the functions of the different PLDs.

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