

Identification and Characterization of Phytochrome-Regulated Phospholipase D in Oat Cells (*Avena sativa* L.)

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(Received August 8, 1996)

Abstract: The activation of phospholipase D (PLD) catalyzes hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) and choline in plants as well as animals. To determine the presence of PLD in oat cells, we prepared inside-out plasma membrane and cytosolic fractions from oat tissues. PLD activities in both cytosol and plasma membrane were detected by ion chromatography method. The activity of PLD in plasma membrane was dependent upon Ca^{2+} concentration and was heat stable. To investigate whether G-protein couples to PLD, the effects of GTP γ S and GDP β S on the PLD activity were measured. PLD activity was dramatically increased 300~400% in the presence of 50 μ M GTP γ S but not in the presence of 50 μ M GDP β S. These results indicate that G-protein may be involved in regulation of PLD activity. To identify whether PLD is regulated by red light receptor, phytochrome, we irradiated red, far-red, or red/far-red/red light on oat protoplasts. PLD activity has increased 5-fold and 3-fold by treatment with red light and red/far-red/red light, respectively. In contrast, irradiation with far-red light had little or no effect on PLD activity. These results suggest that phytochrome regulates PLD activity through activation of G-protein in oat cells.

Key words: G-protein, phospholipase D, phytochrome, signal transduction.

In many cells, phospholipase D (PLD) has been known to play an important role in the regulation of cell responses. PLD hydrolyzes phosphatidylcholine (PC) to choline and phosphatidic acid (PA), then PA is subsequently metabolized by PA phosphohydrolase to form diacylglycerol (DAG), an activator of protein kinase C (Nishizuka, 1992). The metabolites produced by PLD also have crucial roles in the signal transduction pathways of a variety of cells.

Since PLD was first identified in plants (Heller, 1978), PLD has been characterized and purified from plants and animal cells (Lee *et al.*, 1989). It was recently reported that PLD was regulated by ARF (ADP-ribosylation factor), one of the low molecular weight G-proteins, indicating G-protein might regulate PLD activity in HL 60 cells and granulocytes (Brown *et al.*, 1993; Cockcraft *et al.*, 1994; Zhang *et al.*, 1995). Additionally, protein kinase C (PKC) induced maximal activity of PLD with G-protein, suggesting that PLD is under PKC- and G-protein-mediated alternative regulation in HL 60 cells (Geny and Cockcraft, 1992). PLD activity is regulated by cytosolic calcium concentration in citrus callus

tissues (Witt *et al.*, 1987). Interestingly, PLD is activated at micromolar Ca^{2+} concentration in animals but millimolar Ca^{2+} concentration in plants (Kates *et al.*, 1969).

Romero *et al.* (1991) suggested the possibility of a relationship between phytochrome and heterotrimeric G-protein in red light-induced signal transduction pathway. However the regulatory mechanism of G-proteins in phytochrome-mediated signal transduction remains unclear (Romero and Lam, 1993). We recently reported that increased cytosolic Ca^{2+} , phytochrome, and G-protein regulate phospholipase A_2 (Min *et al.*, 1995), phospholipase C (Kim *et al.*, 1995), and protein kinases (Kim *et al.*, 1994), which are key enzymes in the regulation of cell responses in oat cells. These results provide us a possibility that activation of a G-protein(s) by red light irradiation may regulate activity of PLD in oat cells.

In this study, we show that phytochrome regulates PLD through activation of G-protein in oat cells. We also introduce a new PLD assay system, a non-isotopic ion chromatography method.

Materials and Methods

Chemicals

Dextran T-500 was obtained from Pharmacia Fine

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Chemicals (Uppsala, Sweden). PEG 3350, phosphatidylcholine, sorbitol, and Mes were from Sigma Chemical Co. (St. Louis, USA). Cellulase for the preparation of oat protoplasts was from Yakult Honsa (Japan). All used chemicals were standardized in enzyme grade.

Plant materials

Fifty grams of oat (*Avena sativa* L. cv Gary) seeds were soaked at 4°C for 24 h in constant darkness. The seeds were spread on wet vermiculite (100 g/tray) in aluminum trays (35×45 cm) and were grown at 25°C for 7 days in a growth chamber in darkness.

Isolation of plasma membrane

Shoots of 7 days old oats were harvested for the isolation of plasma membrane. All procedures were performed at 4°C. Oat shoots were homogenized with a Blender (Goldstar, Korea) in homogenization buffer (10 mM Tris, pH 7.5, 330 mM sucrose, 1 mM EDTA, 1 mM PMSF, and 70 mM β -mercaptoethanol). The homogenate was filtered through nylon cloth (100 μ m) and centrifuged at 10,000×g for 15 min. The resulting supernatant was centrifuged at 33,500×g for 90 min. The pellet (crude microsomal fraction) was resuspended in two phase buffers (5 mM K⁺-phosphate buffer system containing 6.5% (w/w) Dextran T-500 phase and 6.5% (w/w) PEG 3350 phase). Purification of plasma membrane from the crude microsomal fraction was performed by the method of Widell *et al.* (1982). The purified plasma membrane was resuspended in inside-out buffer (5 mM K⁺-phosphate buffer, pH 7.8, 5 mM KCl, 0.1 mM EDTA, and 1 mM DTT) for preparing inside-out plasma membrane.

Preparation of inside-out plasma membrane

As described by Palmgren *et al.* (1990), the inside-out plasma membrane was prepared by four cycles of freezing (−195°C)/thawing (25°C). The upper phase was diluted with sample buffer and centrifuged at 100,000×g for 60 min. The pellet was resuspended in buffer (100 mM Mes/NaOH, 0.5 mM SDS, pH 6.5) for assaying PLD.

Measurement of phospholipase D activity using ion chromatography

PC (0.2 g) was added to chloroform (1 mL) and then evaporated under N₂. Dried PC was dissolved in 1 mL of 100 mM Mes/NaOH buffer (pH 6.5) supplemented with 0.5 mM SDS by sonication at room temperature. After mixing plasma membrane fraction or cytosolic fraction (each 10 μ g protein/ μ L) with PC (10 μ g/ μ L), the mixture was incubated at 30°C for 30 min. The reaction mixture (200 μ L) was injected into ion

chromatography (CS II column, Dionex Co., USA) using microsyringe and eluted with 5 mM HCl and 5% methanol. Choline was detected by measuring conductivity of sample using conductivity detector.

Preparation of protoplasts

Protoplasts were prepared as previously described (Chae and Han, 1993). Briefly, oat tissues were soaked in 70% ethanol for 5 s and washed with distilled water. Washed tissues were then sliced into 1 mm pieces with a razor blade. Sliced tissues were soaked and incubated in enzyme solution (0.6 M sorbitol, 1 mM CaCl₂, 0.05% BSA, 5% cellulase, pH 6.5) at 26°C for 7.5 h with mild shaking (40 rpm) in the darkness. After enzyme reaction, the reaction mixture was filtered through nylon mesh (100 μ m) and was kept in a cold chamber for 10 min. The filtrate was centrifuged at 1600 rpm for 8 min. Pellet was suspended in the washing buffer (0.6 M sorbitol, 1 mM CaCl₂, and 0.05% BSA, pH 6.5). This washing step was repeated two more times.

Isolation of choline

After protoplasts were treated with appropriate lights, choline extract was obtained and separated by chloroform/methanol (2:1, v/v) (Wang *et al.*, 1993). Water soluble materials including choline were in methanol layer. After evaporating methanol under N₂, choline was dissolved in distilled water and quantified by ion chromatography.

Determination of membrane protein concentration

Protein concentration was determined by modified Maxwell method (Maxwell *et al.*, 1978). Solution A (10 mL of 2% Na₂CO₃ in 0.1 N NaOH, 100 μ L of 2% sodium potassium tartarate, and 100 μ L of 1% CuSO₄) and SDS (final concentration, 1%) were sequentially added to membrane sample. After mixing, 100 μ L of 1:2 diluted phenol reagent was added and incubated at room temperature for 30 min. Protein concentration was determined by measuring absorbance at 660 nm of the reaction mixture.

Results and Discussion

PLD activity has been measured using a radioisotope labeled substrate, the most common and sensitive method for assaying PLD (Wang *et al.*, 1993). In this study, we introduce a new and simple assay system, a non-isotopic ion chromatography method, for measuring PLD activities in oat cells. Merits of the method are as follows; the first, we can analyze choline within 20 min by high performance separator of ion chromatography; the second, we can detect ions less than ppb

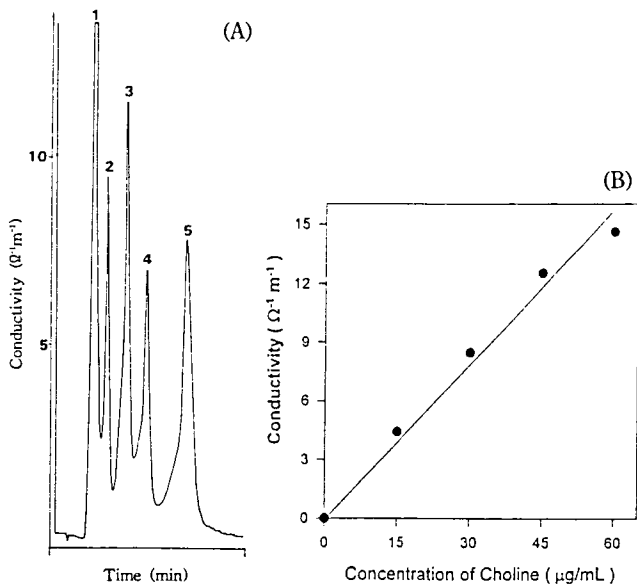


Fig. 1. (A) Ionic chromatogram of monovalent standard cation. Eluent solution was 5 mM HCl and 5% methanol (flow rate; 1 mL/min). The numbers indicate monovalent cations; 1) Li⁺, 2) Na⁺, 3) NH₄⁺, and 5) choline. (B) Changes in conductivity (Ω⁻¹m⁻¹) as a function of choline concentration. Conductivity was detected using conductivity detector. The assay was performed in a reaction mixture (100 mM Mes/NaOH, pH 6.5, and 0.5 mM SDS).

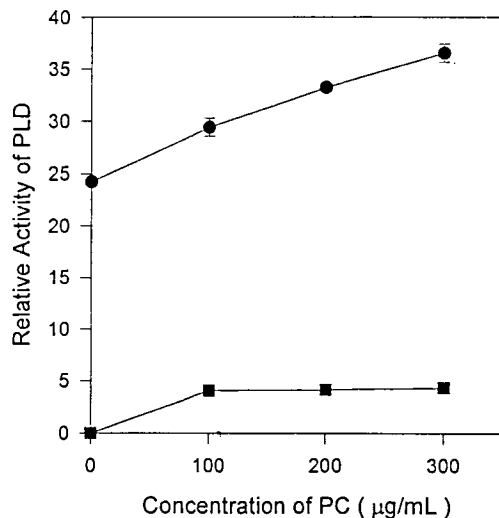


Fig. 2. PLD activity from oat tissues in response to phosphatidylcholine concentration. PC (from 0 to 300 μg) was incubated in 100 mM Mes/NaOH, pH 6.5, 0.5 mM SDS, and either plasma membrane fraction (0.1 mg/mL, closed squares) or cytosolic fraction (0.1 mg/mL, closed circles). See legend of Fig. 1 for more detail assay method.

without stocking sample to install microprocessor in conductivity detector, and the third, we can increase ion selectivity by chemical suppressor. In Fig. 1A, we show an assay of a mixture by ion chromatography (retention time: 17.5 min). Ionic conductivity was linearly dependent upon choline concentration up to 60 μg/mL (Fig.

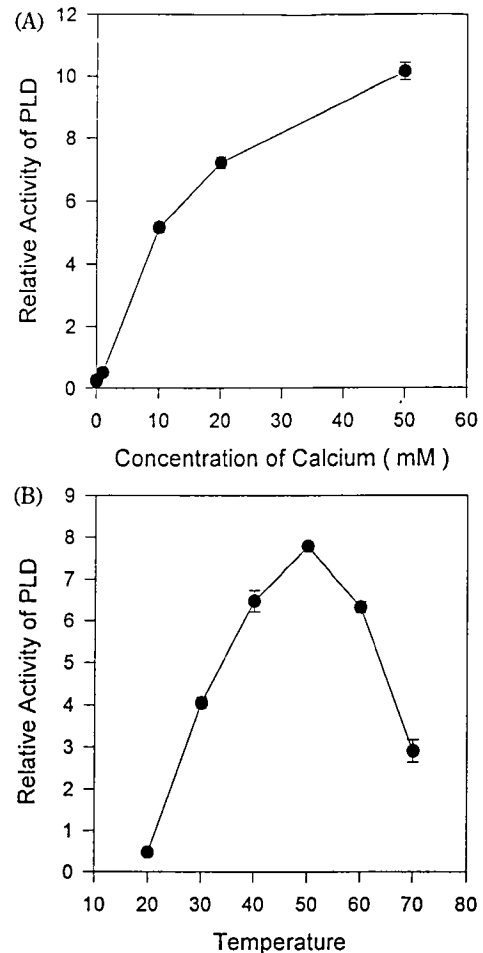


Fig. 3. (A) PLD activity in plasma membrane (0.1 mg protein/mL) in response to external calcium concentrations (from 0.1 μM to 50 mM). (B) The effect of temperature on PLD activity in plasma membrane (0.1 mg protein/mL). See legend of Fig. 1 for more detail experimental procedure.

1B). We have measured PLD activity by this new method in oat cells.

To identify whether oat cells have PLD activity, we prepared cytosolic- and plasma membrane-fractions from oat tissues. PLD activity from the inside-out plasma membrane fraction was dependent on PC concentration and had maximal activity at 100 μg PC/mL (Fig. 2 (closed squares)). Cytosolic fraction also had PLD activity which was linearly dependent on PC concentration (Fig. 2 (closed circles)). PLD from cytosolic fraction showed maximal activity at the highest concentration (300 μg PC/mL). These results indicate that PLD exists in both plasma membrane and cytoplasm in oat cells.

Cytosolic free calcium plays an important role in the regulation of cell responses in a wide variety of cells. Most effector molecules involved in signal transduction are regulated by varied cytosolic calcium concentration. For example, PLC and PLA₂ have maximal activities

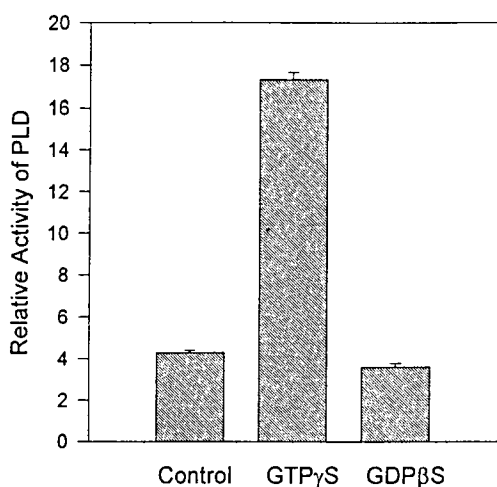


Fig. 4. Effects of GTP γ S and GDP β S on the PLD activity in plasma membrane (0.1 mg/mL). Concentration of GTP γ S or GDP β S was 50 μ M. See legend of Fig. 1 for more detail assay method.

at 100 μ M Ca²⁺ in oat cells (Kim *et al.*, 1995; Min *et al.*, 1995). It was reported that optimal calcium concentration for PLD activity was about 40 mM in soyab cabbage (Wang *et al.*, 1993). PLD is activated by Ca²⁺ and has a maximal activity at Ca²⁺ concentration of 50 mM in citrus callus tissues (Witt *et al.*, 1987). *In vitro*, PLD in plant cells showed a maximal activity at Ca²⁺ concentration from 20 to 100 mM. Heller *et al.* (1975) reported that PLD in peanut seeds did not lose any activity for at least 10 min at 45°C, but lost 50% and 100% of activity at 55°C and 65°C, respectively. PLD activity in response to calcium concentrations (from 0.1 μ M to 50 mM) in plasma membrane fraction from oat tissues are shown in Fig. 3A. PLD activity was increased about 10-fold by 50 mM free calcium. We also measured the effect of temperature on PLD activity from the plasma membrane (Fig. 3B). The optimal activity of PLD in plasma membrane was 50°C. These results indicate that PLD from oat cells is similar to those of other plant cells in Ca²⁺- and temperature-related properties.

G-protein activated by interaction between external stimuli and receptors is an early step in signal transduction pathways. The activated G-protein stimulates effector molecule to generate the second messenger which regulates cell responses. To investigate whether G-protein is involved in the regulation of PLD activity, the effects of GTP γ S, a nonhydrolyzable analogue of GTP, and GDP β S, an inhibitor of the binding of GTP to G-protein, on the PLD activity were tested. GTP γ S increased PLD activity about 3~4 fold but GDP β S had no effect, indicating that G-protein is an early step in regulation of PLD activity in oat cells. These results

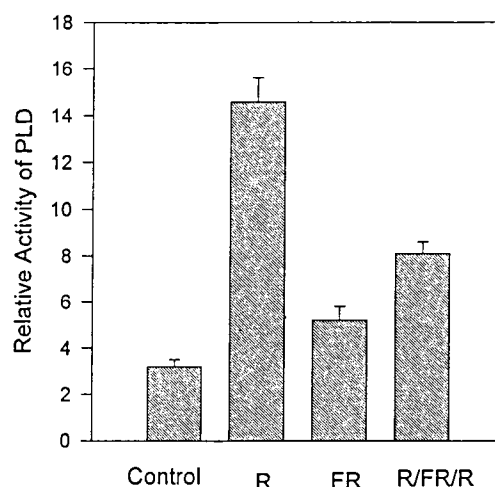


Fig. 5. Phytochrome elicits PLD activity in oat protoplasts. Oat protoplasts (1×10^5 cells/mL) were in suspension medium (1 mM CaCl₂, 0.6 M sorbitol, and 0.5% BSA, pH 6.5). Lights or dark treatments were as follows; Control, constant darkness; R, red light irradiation for 30 s ($280 \text{ mol m}^{-2} \text{ s}^{-1}$); FR, far-red light irradiation for 60 s, and red light irradiation for 60 s in a sequential order. Reaction was terminated by adding organic solvent (chloroform/methanol, 2:1, v/v) to the appropriate light treated protoplasts. After protoplast lysis, PLD activity was measured.

are consistent with our previous studies that G-proteins are involved in regulation of protein kinase-, PLC-, PLA₂-activities in oat cells (Kim *et al.*, 1994; Kim *et al.*, 1995; Min *et al.*, 1995).

To determine whether phytochrome, an important red light receptor for regulating photomorphogenic cell responses in plants, was coupled to regulation of PLD activity, we measured effects of light on the PLD activity in oat protoplasts. We found that irradiation with red light induced an increase of PLD activity about 5-fold but far-red light had little or no effect (Fig. 5). For investigating photoreversibility, one of the special characteristics of phytochrome, we treated red/far-red/red light in a sequential order on oat protoplasts. This treatment increased PLD activity about 3-fold (Fig. 5). These results suggest that phytochrome is involved in regulation of PLD activity in oat cells.

In conclusion, we measured PLD activity in both cytoplasm and plasma membrane from oat cells using a new assay system, a non-isotopic ion chromatography method. Phytochrome, G-protein, and cytosolic calcium are involved in regulation of PLD activity in plasma membrane in oat cells, indicating that PLD is an important enzyme in signal transduction pathways. Further studies are required to show the relationship between cytosolic PLD and plasma membrane PLD, the regulatory mechanism of cytosolic PLD, and physiological roles of PLD in oat cells.

Acknowledgement

This work was supported by research grants from Korean Council for University Education (1995) and Korean Ministry of Education through Research Fund (BSRI-95-416).

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