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Phosphatidylinositol Specific Phospholipase C of Plant Stems[1]

MEMBRANE ASSOCIATED ACTIVITY CONCENTRATED IN PLASMA MEMBRANES

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ABSTRACT

A phosphatidylinositol-specific phospholipase C of plant stems (EC 3.1.4.10) assayed at pH 6.6 and at 30°C cleaved phosphatidylinositol such that more than 85% of the product was inositol-1-phosphate. Other phospholipids were cleaved 5 to 10% or less under these conditions. The phospholipase had both a soluble and a membrane-associated form. The soluble activity accounted for approximately 85 to 90% of the activity and 15% was associated with membranes. The membrane-associated activity was most concentrated in the plasma membranes of hypocotyl segments of both soybean (*Glycine max*) and bushbean (*Phaseolus vulgaris*). The plasma membrane location was verified by analysis of highly purified plasma membranes prepared both by aqueous two-phase partitioning and by preparative free-flow electrophoresis and from the quantitation of the activity in all major cell fractions. Internal membranes also contained phospholipase C activity but at specific activity levels of about 0.1 those present in plasma membranes. Golgi apparatus-enriched fractions from which plasma membrane contaminants were removed by two-phase partition contained the activity at specific activity levels 0.2 those of plasma membrane. Both the soluble and the membrane-associated activity was stimulated by calcium but not by calmodulin, either alone or in the presence of calcium.

There have been periodic reports of soluble phospholipase C activities in plants (8, 9, 11). Additionally, breakdown of membrane-associated phosphatidylinositol has been demonstrated based on analyses using radiolabeled phosphatidylinositol (5, 8, 9, 20). The recent interest in phospholipases has been stimulated by their importance in signal-response transduction mechanisms (3, 18, 22) and the occurrence of phosphoinositol phosphates in plants (4, 7, 21, 25). The latter in carrot cells appear concentrated in plasma membranes (JJ Wheeler, WF Boss, personal communication). In this paper we have examined the subcellular distribution of a phosphatidylinositol-specific phospholipase C of etiolated hypocotyls of soybean and bushbean and the response of the membrane-associated and soluble forms of the activity to calcium and calmodulin. The findings show a clear plasma membrane location of the membrane-associated form of the enzyme and a stimulation of both the soluble and the membrane-associated forms of the enzyme with calcium but not with calmodulin. The calcium-stimulated activity appears to be present in very low levels throughout the system of internal membranes as well.

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MATERIALS AND METHODS

Plant Material. Seeds of soybean (*Glycine max* [L.] Herr., var Williams) or of bushbean (*Phaseolus vulgaris* [L] var Greenpod) were soaked in water 2 to 4 h, planted in moist vermiculite, and grown 4 to 5 d in darkness. One cm long segments, cut 5 mm below the cotyledons, were harvested under dim light and used for isolation of membranes.

Assay of Phospholipase C. The assay contained 200 μM phosphatidylinositol (Sigma) to which were added 25 nCi phosphatidyl-[2-^{3}H]inositol (specific activity 16.3 Ci/mmol, Amersham) in 50 mM potassium maleate buffer (pH 6.6) with 1.2 mM deoxycholate and 0.5 mM $CaCl_2$. The amount of protein was 50 μg for soluble enzyme and 10 to 20 μg for membrane enzyme in a final volume of 500 μl. Reactions were for 30 min at 30°C and stopped by addition of 2 ml chloroform-methanol 2:1 and 50 μl of 4 N HCl, mixed and centrifuged for 10 min at 1000*g* to separate the phases. Portions of the upper phase (200 μl) were added to 5 ml ACS (Amersham) and the radioactivity determined with a Packard model 3255 liquid scintillation spectrometer.

Identification of Products. Products of phosphatidylinositol hydrolysis were separated on Dowex AG 1-X8 formate form according to Berridge *et al.* (2).

Isolation of Plasma Membranes. Plasma membranes were obtained from homogenates prepared by razor blade chopping in 50 mM HEPES (pH 7.5), containing 300 mM sucrose, 100 mM KCl, and 1 mM $MgCl_2$. The homogenates were centrifuged at about 6,000*g* and the pellet discarded. The supernatant was then centrifuged at 50,000*g* for 30 min and this pellet was used for isolation of plasma membrane either by free-flow electrophoresis as described (26) using a VAP-5 free-flow electrophoresis unit (Bender and Hobein, Munich, FRG) or by two phase separation based on Kjellbom and Larsson's (12) adaptation of the general method (1). Isolated crude membrane pellets were mixed with 6.4% (w/w) Dextran T 500 (Pharmacia), 6.4% (w/w) Polyethylene Glycol 3350 (Fisher), 0.25 M sucrose and 5 mM potassium phosphate (pH 6.8). After mixing the contents of the two phase system by 40 inversions of the tubes, the two phases were separated by centrifugation in a swinging bucket rotor at 500*g* for 5 min. The upper phase, enriched in plasma membrane, was rewashed against a fresh lower phase twice, with centrifugation to separate the phases after each set of 40 inversions. Also, the first lower phase was repartitioned against fresh or rewashed upper phases twice. Finally, the combined upper phases with pure plasma membranes were diluted with 5 mM potassium phosphate buffer (pH 6.8), and 0.25 M sucrose and centrifuged at 90,000*g* for 20 min.

Isolation of Other Membranes. Golgi apparatus were prepared by a discontinuous gradient procedure patterned after that of scheme 1 of Morré and Buckhout (19). The homogenization

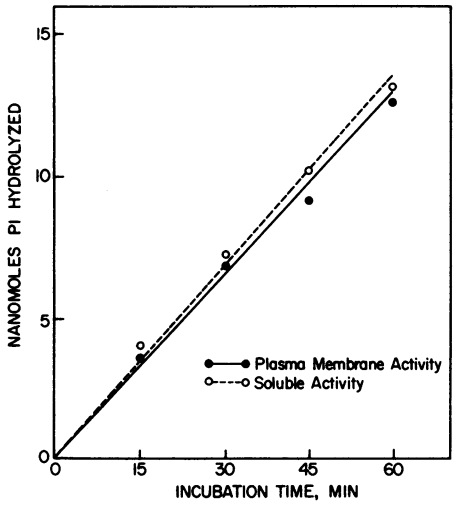

FIG. 1. Hydrolysis of phosphatidylinositol is proportional to time of incubation for both soluble and membrane-associated forms of the activity from soybean when assayed at 50 μg protein for the soluble activity and 20 μg protein for the membrane associated activity. The assays were at pH 6.6 and 30°C in the presence of 0.5 mM calcium. Results with bushbean were similar.

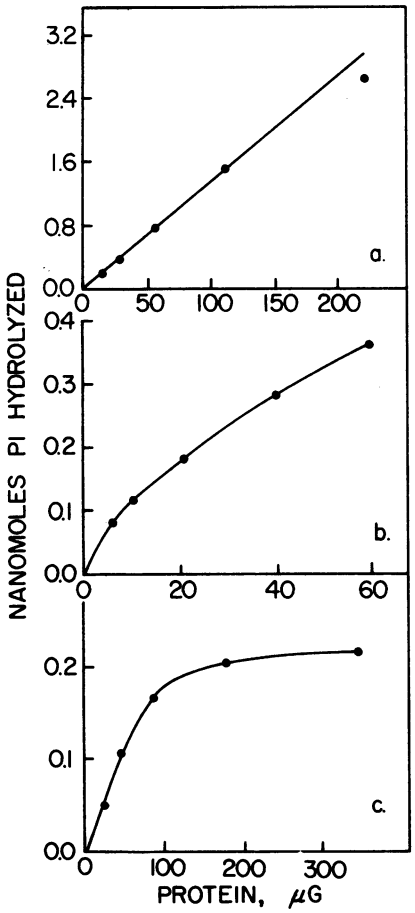

FIG. 2. Hydrolysis of phosphatidylinositol is proportional to protein amount up to 200 μg of protein for the soluble activity (a) and up to about 100 μg protein for the membrane associated form both with plasma membranes (b) and total microsomes (c). The assays were at pH 6.6 and 30°C in the presence of 0.5 mM calcium. Results with bushbean were similar.

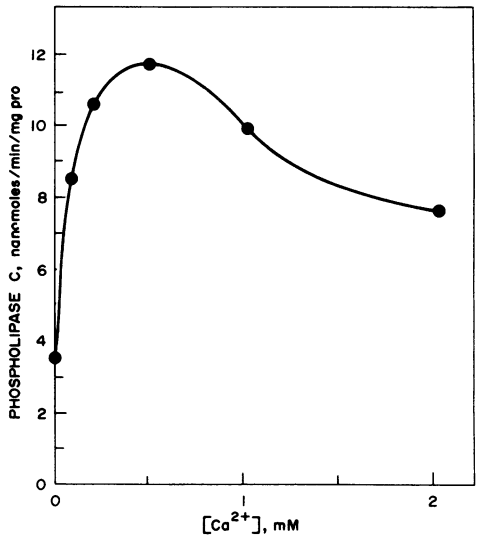

FIG. 3. Hydrolysis of phosphatidylinositol was stimulated by calcium with a broad optimum at 0.5 mM. Values are for the soluble activity of soybean. Results with the membrane-associated form and for bushbean were similar.

Table I. *Distribution of Phosphatidylinositol-Specific Phospholipase C among Highly Purified Various Fractions of Soybean Hypocotyl*

	Specific Activity	Per 10 g Fresh Wt of Tissue	
		Protein[a]	Total activity[b]
	nmol/min·mg protein	*mg*	*nmol/min × 10^{-3}*
Total homogenate	11.1 ± 0.6	52.0	57.7
Plasma membrane	20.5 ± 4.5	1.9	4.0
Golgi apparatus	4.6 ± 0.4	1.8	0.8
Endoplasmic reticulum	2.5 ± 0.2	4.5	1.1
Tonoplast	2.0 ± 0.5	1.5	0.3
Mitochondria	2.7 ± 0.3	2.5	0.7
Nuclear envelope	2.4	0.6	0.2
Etioplasts	1.7 ± 0.4	1.8	0.3
Total microsomes	6.2 ± 0.3	14.6	9.1
Supernatant	12.5 ± 3.2	37.4	46.8

[a] Determined from quantitative electron microscope morphometry coupled with protein analyses as described by Morré and Buckhout (19). [b] Product of the specific activity determined from the analyses of the purified cell fractions (column 1) and the total protein ascribed to the particular fraction (column 2).

medium contained 50 mM HEPES/KOH, 0.4 M sucrose, 10 mM KCl, 3 mM EGTA, 3 mM EDTA, 0.1% BSA and 14 mM mercaptoethanol. Hypocotyl sections were homogenized by smashing in a mortar (1 g fresh weight/ml of medium). Filtered homogenates were freed of unbroken cells and debris by a 10 min centrifugation at 800*g*. The supernatants were layered over a gradient consisting of 20% (w/v) and 35% (w/v) sucrose and centrifuged 30 min at 80,000*g*. The material collecting at the 20%/35% sucrose interface was collected by centrifugation at 60,000*g* for 30 min and resuspended in 5 mM potassium phosphate (pH 6.8) containing 0.25 M sucrose. The Golgi apparatus-enriched pellets were next freed of plasma membrane vesicles by aqueous two phase partition as described for plasma membrane isolation above. The lower phase, was checked for plasma membrane contamination (<0.5%) by staining with phosphotungstic acid at low pH and was used for the enzyme assays.

Rough endoplasmic reticulum and mitochondria were ob-

Table II. *Phosphatidylinositol-Specific Phospholipase C Activity of Soybean and Bushbean Hypocotyl Membranes Purified by Two Phase Aqueous Partitioning*

Fraction	Soybean		Bushbean	
	Specific activity	Plasma membrane	Specific activity	Plasma membrane
	nmol/ min·mg protein	%	*nmol/ min·mg protein*	%
Upper phase				
Plasma membrane rich	20.1 ± 4.3	95 ± 2	22.1	88
Lower phase				
Plasma membrane poor	2.9 ± 0.4	3.8 ± 0.3	5.4	3

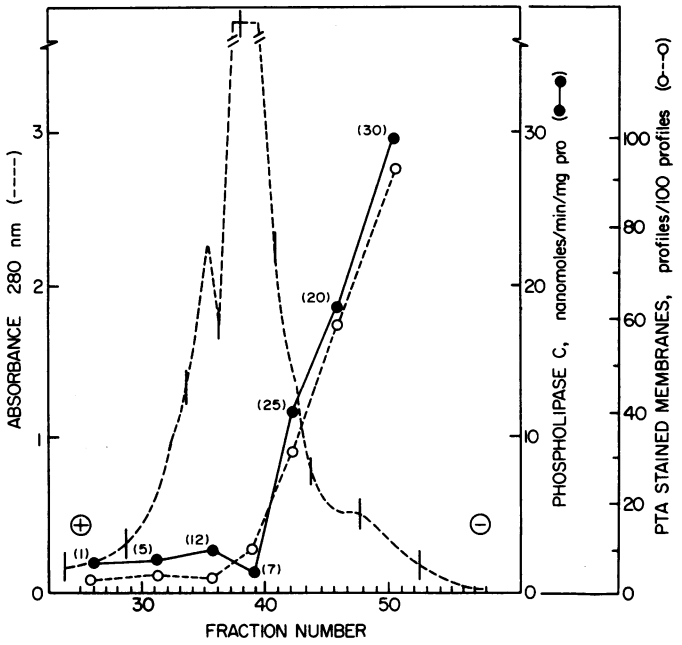

FIG. 4. Free flow electrophoresis separation showing the location of membranes reactive with phosphotungstic acid (PTA) at low pH (○) and the specific activity of the phosphatidylinositol-specific phospholipase C (●) for soybean fractions. Values are for pooled fractions indicated. The numbers in parentheses are the percentages of total phospholipase C activity recovered in each fraction. The total distribution of material along the separation is given by the dotted line. Actual protein values were determined by the Lowry *et al.* (14) procedure for determination of enzyme specific activities. A location of Golgi apparatus markers is centered around fraction 35. Fractions 24 to 33 contain the purified tonoplast membranes. The characteristics of the various free-flow fractions including a detailed evaluation of marker enzymes and identification of tonoplast are given by Sandelius *et al.* (26).

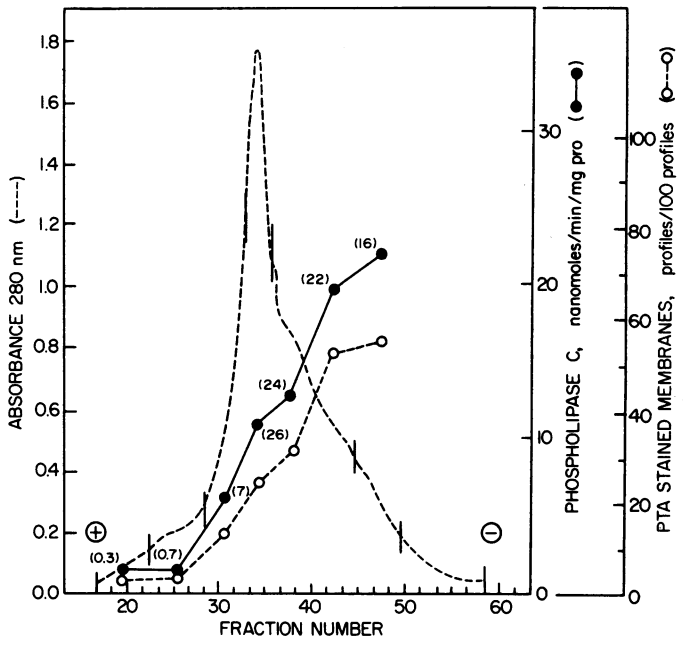

FIG. 5. Free-flow electrophoresis separation showing the location of membranes reactive with phosphotungstic acid (PTA) at low pH (○) (23) and the specific activity of the phosphatidylinositol-specific phospholipase C (●) for bushbean fractions. Values are for the pooled fractions indicated. The numbers in parentheses are the percentages of total phospholipase C activity recovered in each fraction. The total distribution of material along the separation is given by the dotted line. Actual protein values were determined by the Lowry *et al.* (14) procedure for determination of enzyme specific activities.

tained from the lower phase of the aqueous two phase partitioning system used for purification of plasma membrane. The lower phase was washed two times in water to remove the dextran and the resuspended membranes were centrifuged for 10 min at 10,000*g* to yield a pellet highly enriched (more than 85%) in intact mitochondria. The supernatant was centrifuged 30 min at 50,000*g* and the pellet fractionated by free-flow electrophoresis according to Sandelius *et al.* (26) to remove tonoplast. Six electrophoresis fractions (fractions 43–48 = pooled fraction C) were collected and analyzed. The fractions contained about 70% endoplasmic reticulum-derived membranes as evidenced by the presence of membrane vesicles with attached ribosomes and, less than 1% plasma membrane from staining with phosphotungstic acid at low pH. Etioplast membranes were a major contaminant.

Nuclei were isolated by centrifugation of a low speed pellet (500*g*, 900 s) through 2.3 M sucrose (60 min 60,000*g*). Etioplasts were collected by sedimentation at unit gravity (16).

Protein was estimated by the Lowry *et al.* (14) method. BSA was used as the standard.

Electron Microscopy. A small piece of each pellet was fixed in glutaraldehyde (2.5% in 0.01 M sodium phosphate [pH 7.2]) for 15 min at room temperature and placed on ice or refrigerated for at least 1 h. The pellets were rinsed and postfixed in 1% osmium tetroxide in the same buffer. Dehydration was in acetone series with embedment in Epon (15). Thin sections were cut and stained with lead citrate or phosphotungstic acid at low pH (24) for identification of plasma membranes. Morphometric analyses of the electron micrographs was according to the method of Loud (13) using a minimum of three electron micrographs from at least two different preparations.

RESULTS

The phospholipase C assay was proportional to time and protein concentration for both the soluble and membrane-asso-

ciated forms of the activity (Figs. 1 and 2). The pH optimum was 6.6 and the optimum calcium concentration was 0.5 mM (Fig. 3). The assay was proportional to protein concentration over a wide range for the soluble activity (Fig. 2a) but only up to about 60 μg per assay either for the plasma membrane (Fig. 2b) or total microsomes (Fig. 2c). Therefore, for all isolated fractions, proportionality to protein concentration was verified and protein concentrations below 20 μg/assay were used.

Of the total activity of the homogenates approximately 85% was soluble (Table I) and 15% was membrane associated. Both the soluble and the membrane-associated activities were stimulated by calcium in the same manner as total activity with an optimum of 0.5 mM. The products of the reaction for both the soluble and the membrane-associated form of the activity were 85 to 90% inositol-1-phosphate, 5 to 10% free inositol, and 5% glycerophosphorylinositol.

Specificity of the lipase was verified using radiolabeled phosphatidylcholine and nonradioactive phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylserine. Under the same assay conditions as for nearly complete breakdown of phosphatidylinositol, phosphatidylcholine was only 5 to 10% hydrolyzed. No diglyceride was detected from any of the other phospholipids. Sometimes a small amount (<2% of the total) of phosphatidic acid was formed.

When separated by two phase partitioning, the greatest specific activity of the membrane-associated phospholipase C activity specific for phosphatidylinositol was found with the plasma membrane enriched upper phase (Table II). Approximately 50% of the activity remained in the lower phase of low specific activity about 0.15 that of the plasma membrane (Table II). The activities associated both with the plasma membranes of the upper phase and with the internal membranes of the lower phase were intrinsic to the membrane rather than loosely bound or adsorbed from the cytosol since both activities resisted removal with 100 mM Na_2CO_3 according to the criteria of Howell and Palade (10). Under these conditions approximately 25% of the protein was extracted with only a 5% loss in total phospholipase C activity.

To verify a plasma membrane location of the membrane-associated form of the activity, the membranes were resolved by preparative free-flow electrophoresis. Pooled fractions were assayed both for phospholipase C and content of plasma membrane by staining with phosphotungstic acid at low pH (Figs. 4 and 5). The specific activity of the phosphatidylinositol-specific phospholipase C was in proportion to the content of plasma membrane determined from staining with phosphotungstic acid at low pH for both soybean (Fig. 4) and bushbean (Fig. 5). When expressed on the basis of total activity, 75% of the activity for soybean and 66% of the activity for bushbean were recovered from the plasma membrane-enriched fractions in the left hand portion of the separation. These estimates agree with those provided by aqueous two-phase partitioning (Table II) and the balance sheet experiments (Table I) that at least 50% of the membrane-associated phospholipase C activity was plasma membrane associated. The tonoplast enriched peak at the extreme left of the separation showed little or no phospholipase C activity. A small peak in specific activity occurred in the electrophoretic separations in the region of the separation around fraction 35 containing Golgi apparatus.

The distribution of activities among different membrane fractions summarized in Table I was based on analyses of the purified and largely plasma membrane free fractions of Golgi apparatus, endoplasmic reticulum, mitochondria, nuclei, and etioplasts. Removal of plasma membrane from Golgi apparatus, endoplasmic reticulum and mitochondria was by aqueous two phase partitioning and was confirmed by staining of electron microscope sections with phosphotungstic acid at low pH. Less than 0.1% of the membranes of these fractions were reactive. Thus, for the membrane-associated activity about 50% was found with plasma membrane. The remainder was distributed among all internal membranes nearly evenly on a specific activity basis (0.1 that of plasma membrane) except for Golgi apparatus (0.2 that of plasma membrane).

DISCUSSION

In animal tissues, the hormone responsive hydrolysis of phosphatidylinositol is the result of a phospholipase C specific for phosphoinositides (2, 3, 18). In this report we examined the subcellular distribution of a phosphatidylinositol specific phospholipase C from soybean and bushbean hypocotyls. As in mammalian cells, both soluble and membrane-associated forms are found, the majority of the activity being soluble, *i.e.* cytosolic, even though its substrates are membrane bound (17). However, of that fraction which is membrane associated, the greatest activity is recovered with the plasma membrane fraction. The activity is stimulated by calcium but not by calmodulin.

A phospholipase C specific for phosphatidylinositol and associated with the plasma membrane would be of interest in terms of a potential regulatory enzyme for some plasma membrane associated processes of plants such as growth and transport. Phosphatidylinositol breakdown in soybean callus was found to be stimulated by the detergent deoxycholate and by millimolar concentrations of Ca^{2+} (5). A phosphatidylinositol phospholipase C found both in the cytosol and particulate fractions of pollen that increased during germination also has been reported (8, 9). The various C-type phospholipases have been characterized as to their cation and detergent requirements and pH optima and are similar to those reported here for soybean hypocotyls. However, except for the observation of both particulate and soluble enzyme forms, there have been no previous efforts to determine the subcellular distribution of the activities from plants. Even for the mammalian enzyme, we are aware of no detailed balance-sheet analyses for the subcellular distribution of phospholipase C forms nor is it known if the membrane associated forms are distinct from those present in the cytosolic fraction (17).

The present findings show that for plants the phospholipase C specific for phosphatidylinositol is located primarily in the plasma membrane. Plasma membrane fractions contain the activity in 10-fold higher specific activity than most other fractions. Yet, the activity is not entirely absent from internal membranes. It is of low specific activity in tonoplast, mitochondria, etioplasts, endoplasmic reticulum, and Golgi apparatus. In these fractions any contaminating plasma membranes were removed by aqueous two-phase polymer partitioning as verified by staining with phosphotungstic acid at low pH. That approximately 50% of the total membrane-associated activity is distributed among internal membrane is verified by balance-sheet experiments where the amounts of different membrane constituents was determined independently from electron microscope morphometry (19). The latter allowed calculation of total activity distribution based on specific activities of the highly purified cell fractions. Thus, while the phosphatidylinositol-specific phospholipase C is sufficiently concentrated in the plasma membrane to serve as a marker, it appears not to be localized exclusively in the plasma membrane and exhibits a much broader distribution. Among the alternative explanations for the activities present in endoplasmic reticulum and Golgi apparatus for example, would be that they might represent biosynthetic forms of the enzyme en route to the plasma membrane or functional forms of the activity involved in some aspect of cell regulation or both.

At present, while it appears that both polyphosphoinositides and phosphotidylinositol-specific phospholipases are associated with plant membranes, there is little evidence for a role of these activities in the regulation of development or growth or in cell activation in plants. IP_3 (inositol 1,4,5-triphosphate) has been

reported to stimulate calcium efflux *in vitro* (6) and *in vivo* (23). The occurrence of phospholipase C specific for phosphatidylinositol associated with the plasma membrane would be a necessary precondition if the animal paradigm is to extend to plants.

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LITERATURE CITED

1. ALBERTSSON PA, B ANDERSSON, C LARSSON, HE AKERLUND 1982 Phase partition—a method for purification and analysis of cell organelles and membrane vesicles. Methods Biochem Anal 28: 115–150
2. BERRIDGE MJ, RMC DAWSON, CP DOWNES, JP HESLOP, RF IRVINE 1983 Changes in the levels of inositol phosphates after agonist dependent hydrolysis of membrane phosphoinositides. Biochem J 212: 473–482
3. BERRIDGE KJ, RF IRVINE 1984 Inositol triphosphate, a novel second messenger in cellular signal transduction. Nature 312: 315–321
4. BOSS WF, MO MASSEL 1985 Polyphosphoinositides are present in plant tissue culture cells. Biochem Biophys Res Commun 132: 1018–1023
5. CONNETT RJA, DE HANKE 1986 Breakdown of phosphatidylinositol in soybean callus. Planta 169: 216–221
6. DROBAK BK, IB FERGUSON 1985 Release of Ca^{2+} from plant hypocotyl microsomes by inositol-1,4,5-triphosphate. Biochem Biophys Res Commun 130: 1241–1246
7. HEIM S, KG WAGNER 1986 Evidence of phosphorylated phosphatidylinositols in the growth cycle of suspension cultured plant cells. Biochem Biophys Res Commun 134: 1175–1181
8. HELSPER JPFG, PFM DE GROOT, HF LINSKENS, JF JACKSON 1986 Phosphatidylinositol phospholipase C activity in pollen of *Lilium longiflorum*. Phytochemistry 25: 2053–2055
9. HELSPER JPFG, PFM DE GROOT, HF LINSKENS, JF JACKSON 1986 Phosphatidyl monophosphate in *Lilium* pollen and turnover of phospholipid during pollen tube extension. Phytochemistry 25: 2193–2199
10. HOWELL KE, G PALADE 1982 Hepatic Golgi fractions resolved into membrane and content subfractions. J Cell Biol 92: 822–832
11. IRVINE RF 1982 The enzymology of stimulated inositol lipid turnover. Cell Calcium 3: 295–309
12. KJELLBOM P, C LARSSON 1984 Preparation and polypeptide composition of chlorophyll-free plasma membranes from leaves of light-grown spinach and barley. Physiol Plant 62: 501–509
13. LOUD AV 1962 A method for the quantitative estimation of cytoplasmic structures. J Cell Biol 15: 481–487
14. LOWRY OH, N ROSEBROUGH, A FARR, R RANDALL 1951 Protein measurement with the folin phenol reagent. J Biol Chem 193: 265–275
15. LUFT JH 1961 Improvement in epoxy resin embedding method. J Biophys Biochem Cytol 9: 409–414
16. MACDONALD FD, T AP REES 1983 Enzymic properties of amyloplasts form suspension cultures of soybean. Biochim Biophys Acta 755: 81–89
17. MAJERUS PW, TM CONNOLLY, H DECKMYN, TS ROSS, TE BROSS, H ISHII, VS BANSAL, DB WILSON 1986 The metabolism of phosphoinositide-derived messenger molecules. Science 234: 1519–1526
18. MICHELL RH 1982 Stimulated inositol lipid metabolism: an introduction. Cell Calcium 3: 285–294
19. MORRÉ DJ, TJ BUCKHOUT 1979 Isolation of Golgi apparatus. *In* E Reid, ed, Plant Organelles. Ellis Horwood, Chichester, pp 117–134
20. MORRÉ DJ, B GRIPSHOVER, A MONROE, JT MORRÉ 1984 Phosphatidylinositol turnover in isolated soybean membranes stimulated by the synthetic growth hormone, 2,4-dichlorophenoxyacetic acid. J Biol Chem 259: 15364–15366
21. MORSE MJ, RC CRAIN, RL SATTER 1986 Phosphatidylinositol turnover in *Samanea* pulvini: A mechanism of phototransduction. Plant Physiol 80: S-92
22. NISHIZUKA Y 1985 Studies and perspectives of protein kinase C. Science 233: 305–312
23. RINCON M, WF BOSS 1987 Myo-inositol triphosphate mobilizes calcium from fusogenic carrot (*Daucus carota* L.) protoplasts. Plant Physiol 83: 395–398
24. ROLAND JC, CA LEMBI, DJ MORRÉ 1972 Phosphotungstic acid-chromic acid as a selective electron-dense stain for plasma membrane of plant cells. Stain Technol 47: 195–200
25. SANDELIUS AS, M SOMMARIN 1986 Phosphorylation of phosphatidylinositols in isolated plant membranes. FEBS Lett 201: 282–286
26. SANDELIUS AS, C PENEL, G AUDERSET, A BRIGHTMAN, M MILLARD, DJ MORRÉ 1986 Isolation of highly purified fractions of plasma membrane and tonoplast from the same homogenate of soybean hypocotyls by free-flow electrophoresis. Plant Physiol 81: 177–185