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CHARACTERIZATION OF A Ca^{2+}-STIMULATED POLYPHOSPHOINOSITIDE-PHOSPHOLIPASE C IN ISOLATED PLASMA MEMBRANES FROM *SPINACIA OLERACEA* AND *CHENOPODIUM RUBRUM* LEAVES

BY

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ABSTRACT

A polyphosphoinositide-phospholipase C has been identified in highly purified plasma membranes from leaves of *Spinacia oleracea* and *Chenopodium rubrum*. The enzyme hydrolysed phosphatidylinositol, 4-5 biphosphate into a mixture of inositol biphosphate and inositol triphosphate. It was activated by micromolar Ca^{2+} concentration and its optimum pH was between 7 to 7.5. The enzyme activity was also activated by sodium deoxycholate. Preliminary experiments have shown that the enzyme activity was different in plasma membrane isolated from dark-grown or light grown spinach plants. This enzyme may participate in signal transduction of light over plant plasma membrane. Possible implication in flowering process of the light regulation of this enzyme activity will be discussed.

Key-words: *Chenopodium rubrum*, Flowering Induction, Phospholipase C, Plasma membrane, *Spinacia oleracea*.

Abbreviations: $InsP_2$, Inositol 1-4 bisphosphate. $InsP_3$, Inositol 1-4-5 trisphosphate. PIP_2, Phosphatidyl inositol, 4-5 bisphosphate. PLC, Phospholipase C.

RÉSUMÉ

Une polyphospho-inositide-phospholipase C a été identifiée dans des préparations de plasmalemme purifiées à partir de feuilles de *Spinacia oleracea* et de *Chenopodium rubrum*. Cette enzyme hydrolyse le phosphatidyl-inositol, 4-5 biphosphate pour donner un mélange d'inositol-biphosphate et d'inositol triphosphate. Elle est activée par des concentrations micromolaires de Ca^{2+} et possède un pH optimum compris entre 7 et 7,5. Son activité est aussi stimulée par le déoxycholate de sodium. Des expériences préliminaires ont montré chez l'épinard que son activité était différente dans le plasmalemme de plantes de jour court comparée à celle de plantes de jour long. Cette enzyme pourrait participer à la transduction du signal de la lumière via le plasmalemme. Les implications possibles de la régulation, par la lumière, de cette activité enzymatique seront discutées.

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INTRODUCTION

Recently, phospholipase C activity has been reported in isolated plasma membrane of a variety of plant tissues (for review see: LEHLE L, 1990). This enzyme is implicated early in the various steps of the signal transduction mechanism at membrane level (for review see: EINSPAHR K.J. *et al.*, 1990). These mechanisms have been well characterized in animal cells and lead to an increase of cytosolic Ca^{2+} concentration. Unfortunately, little is known about the function of inositol phosphate in plant cells. Only few studies points to a physiologically significant degradation of polyphosphoinositols in plant cells triggered by environmental signals such as osmotic shock (EINSPAHR K. *et al.*, 1989), light (MORSE M.J. *et al.*, 1987) or growth regulators (ETTLINGER C. *et al.*, 1988). There are also in plants some evidences for a link between triphosphate inositol and a release of Ca^{2+} from microsomal (REDDY A.S.N. *et al.*, 1987) or vacuolar (SCHUMAKER K.S. *et al.*, 1987) pools. At least, proteins kinase C have been reported in numerous plant tissues (SCHÄFER A. *et al.*, 1985).

One hypothesis to explain photoinduction of flowering that resulted from investigations with the long day plant *Spinacia oleracea* is that of structural, biochemical and functional modifications in plasma membranes (GREPPIN H. *et al.*, 1990). Early changes of the plasmalemma structure, thickness (AUDERSET G. *et al.*, 1986) and composition, sterols (CRESPI P. *et al.*, 1993), have been demonstrated at the time of floral induction by light or by gibberellic acid. A light dependency of phospholipase C activity has been shown in some experimental system such as the control of leaf movement in *Samanea saman* (MORSE M.J., 1989).

All these recent advances prompt us to undertake investigations on light control of plasmalemma phospholipase C activity. These experiments have been runned on two plant systems with an opposite light requirement for their flowering induction (BERNIER G., 1988; CUMMING B.G. *et al.*, 1965). We here report the presence of Phospholipase C activity in leaves plasmalemma of spinach, a long day plant, and of chenopodium, a short day plant. In both cases, Phospholipase C activity is calcium activated. Phospholipase C activity was also found to be different, compared to control plants, in plasmalemma of spinach leaves after 24 hrs continuous light, a treatment known to provoke floral induction.

MATERIAL AND METHODS

Plant materials

Spinach plants (*Spinacia oleracea* L. cv. Nobel) were grown for 4 weeks in a growth camber with short day illumination (fluorescent tubes, Sylvania: 400 µmol. $m^{-2}.s^{-1}$) of 8 hs daily. The temperature was set at 23°C. The relative humidity was maintained at ca. 8%. Plants were photoinduced by a continuous light period of 24 hrs. Control plants remained under short day condition (8 hours light; 12 hours dark).

Chenopodium plants (*Chenopodium rubrum*), were grown under long day condition for 2 weeks with the same illumination as spinach.

Plasma membrane isolation

Isolation of pure plasmalemma was run by phase partitionning in an aqueous polymer two phase system essentially as described by KJELL BOM P. & LARSSON C. (1984). Briefly, leaves were harvested and homogenised at 4°C in a medium containing 50 mm Hepes-NaOH pH=7.5,500mM sucrose, 10 mM KCl, 1 mM $MgCl_2$ and 10 mM ascorbic acid. After centrifugation at 6000g, the resulting supernatant was centrifuged at 30000g. The crude membrane preparation was then submitted to phase partitionning. Pure plasmalemma was immediatly frozen and stored at minus 80°C until used. For enzyme localization free-flow electrophoresis have been runned on crude microsomal preparation as described by AUDERSET *et al.* (1986). Several enzyme markers and also PLC activity have been tested on pooled fractions of free-flow electrophoresis. Activities of cytochrome C reductase was used as endoplasmic reticulum marker (HODGES T.K. *et al.*, 1974), latent inosine diphosphatase as golgi apparatus marker (QUAIL P.H., 1980), cytochrome C oxydase as mitochondrial marker (WHARTON D.C. *et al.*, 1967) and pyrophosphatase H^+ dependant as tonoplast marker (WANG Y. *et al.*, 1986). The activity of 1,3 β glucan synthase II was used as plasma membrane marker and measured as described by KAUSS H. *et al.* (1985).

Phospholipase C assay

Phospholipase C assay was run essentially as described by MELIN P.M. *et al.* (1987). The standard incubation mixture contained 0.25 mM Bis-Tris Propane (pH=7.5), 50 µM free Ca^{2+}(Ca^{2+}/EGTA mixture), 0.2 mM [^{3}H] labelled PIP_2 (New England Nuclear) in micellar solution and 2 µg of membrane protein in a final volume of 50 µl. The reaction was started by addition of polyphosphoinositides, and performed at 25°C for 4 minutes. Inositol polyphosphates were extracted by phase separation in a chloroform/ methanol/ water system and the upper phase (400 µl) was immediately transferred to a scintillation vial containing 4 ml of Beckman Ready Safe. The radioactivity was measured in a liquid scintillation counter. Values presented have been recalculated to correspond to the total upper phase. Enzyme activities of plasma membrane isolated from dark grown or light grown spinach plants were determined several times on different share of plants. Reaction products have been analysed by ion exchange chromatography on a Whatman partisphere Sax column. An aliquot of the upper phase obtained by phase separation was injected on a Varian high pressure liquid chromatograph. Products of the reaction were then eluted by an NaH_2PO_4 discontinuous gradient at a flow of 1.5 ml. Retention times have been compared with standards of $InsP_2$ and $InsP_3$ from New England Nuclear.

Replication of results

Each phospholipase C activity was determined in duplicate or triplicate. All experiments have been reproduced at least three times.

RESULTS

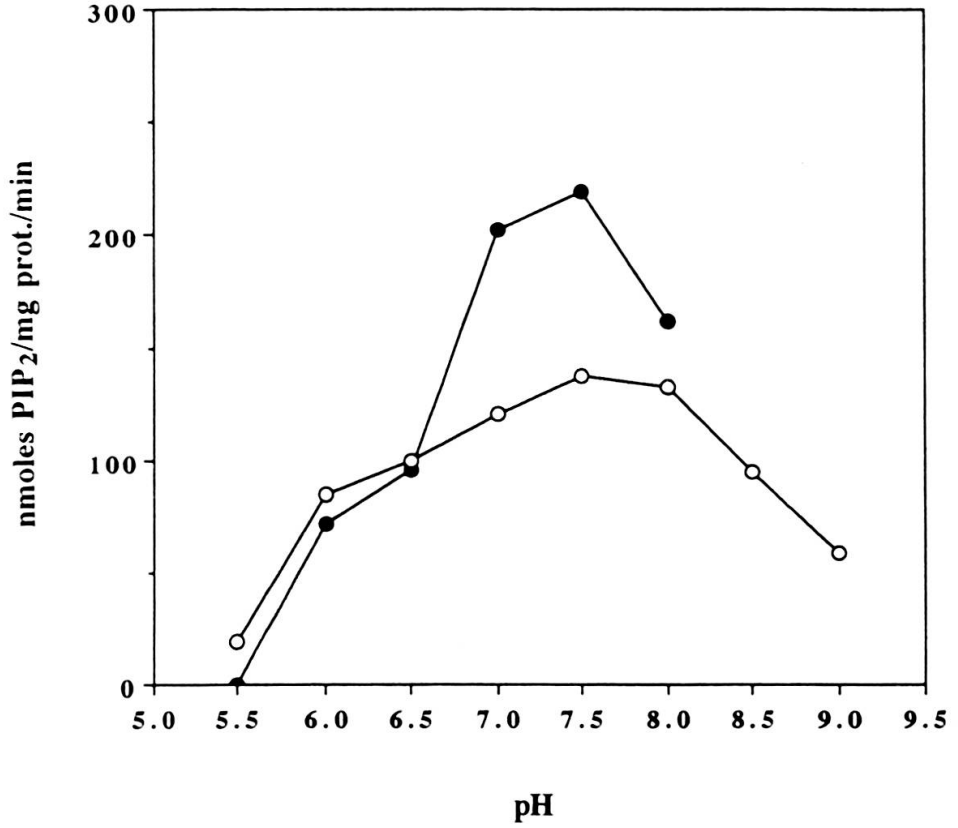
Phospholipase C activities obtained from highly purified plasma membrane of spinach and chenopodium were first tested for their pH dependency. In the two cases, the optimum pH was 7 to 7.5 (Fig. 1). Chenopodium phospholipase C activity was apparently less sensible to acid pH. Effect of sodium deoxycholate was also tested (Fig. 2). The maximum activity was obtained by addition of 0.05% to 0.1% of this detergent. Highest concentrations of sodium deoxycholate were highly inhibitory of plasmalemma phospholipase C activity. This apparent stimulation of PLC activity by deoxycholate was slightly different in spinach than in chenopodium, 210% and 125% respectively and chenopodium seems to be more sensible to high concentration of this detergent. High pressure liquid chromatography analyses of reaction products were showed in Fig. 3. In each cases, a mixture of tri and bis-phosphate inositol was recovered after reaction with phosphatidylinositol bisphosphate as substrate. No inositol phosphate has been detected by this method. Intra-cellular membrane localization of phospholipase C activity have been examined on membrane isolated by free-flow electrophoresis (Fig. 4). Different enzyme markers and phospholipase C activity were tested on pooled fractions (Fig. 4A). Phospholipase C activity showed a good correlation with 1,3 β glucan syntase II activity, an enzyme marker of plasmalemma (Fig. 4B). Enzymatic markers of others endomembranes have a classical distribution as previously described (AUDERSET G. *et al.*, 1986) and have a low activity in fractions were phospholipase C and 1,3 β glucan synthase showed their maximum activities. Ca^{2+} dependency has also been tested (Fig. 5). No phospholipase C activity has been detected in absence of free Ca^{2+} (1mM EGTA) and the activity was found to be maximum for free Ca^{2+} concentration upper to 1 µM. Phospholipase C activity was examined in plant plasma membrane submitted to different light conditions (Table 1). Plasma membrane extracted from spinach plants submited to a continuous light period of 24 hours have a 15% to 30% highest phospholipase C activity than plants maintained under short day conditions.

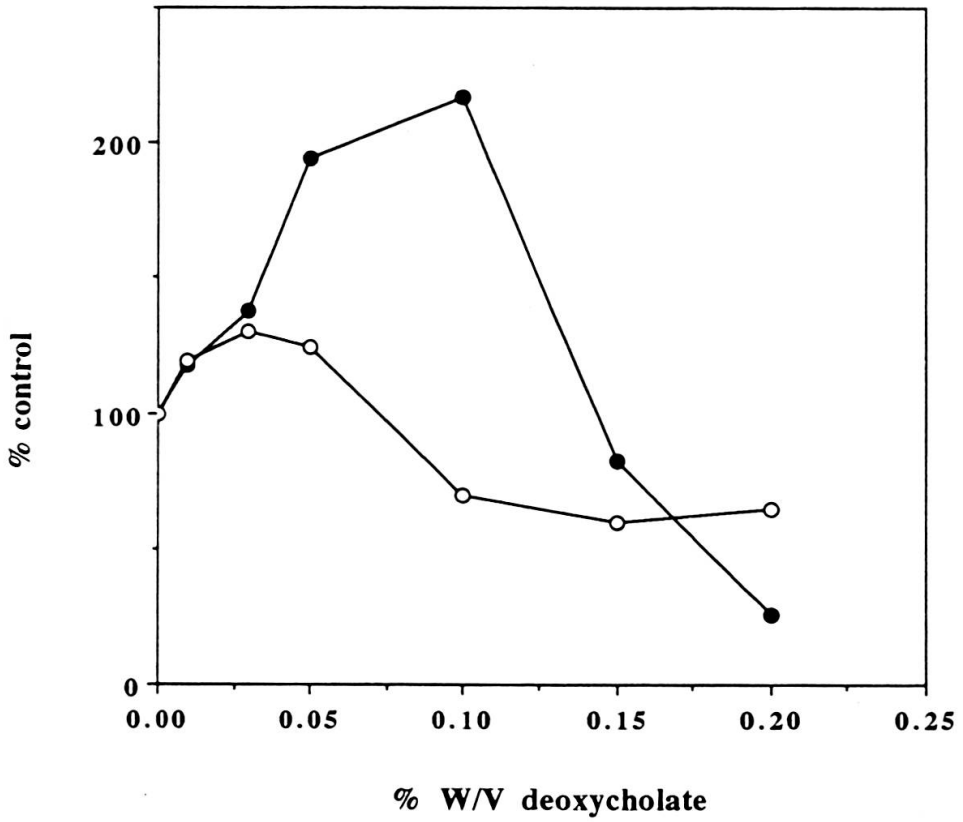
FIG. 1.

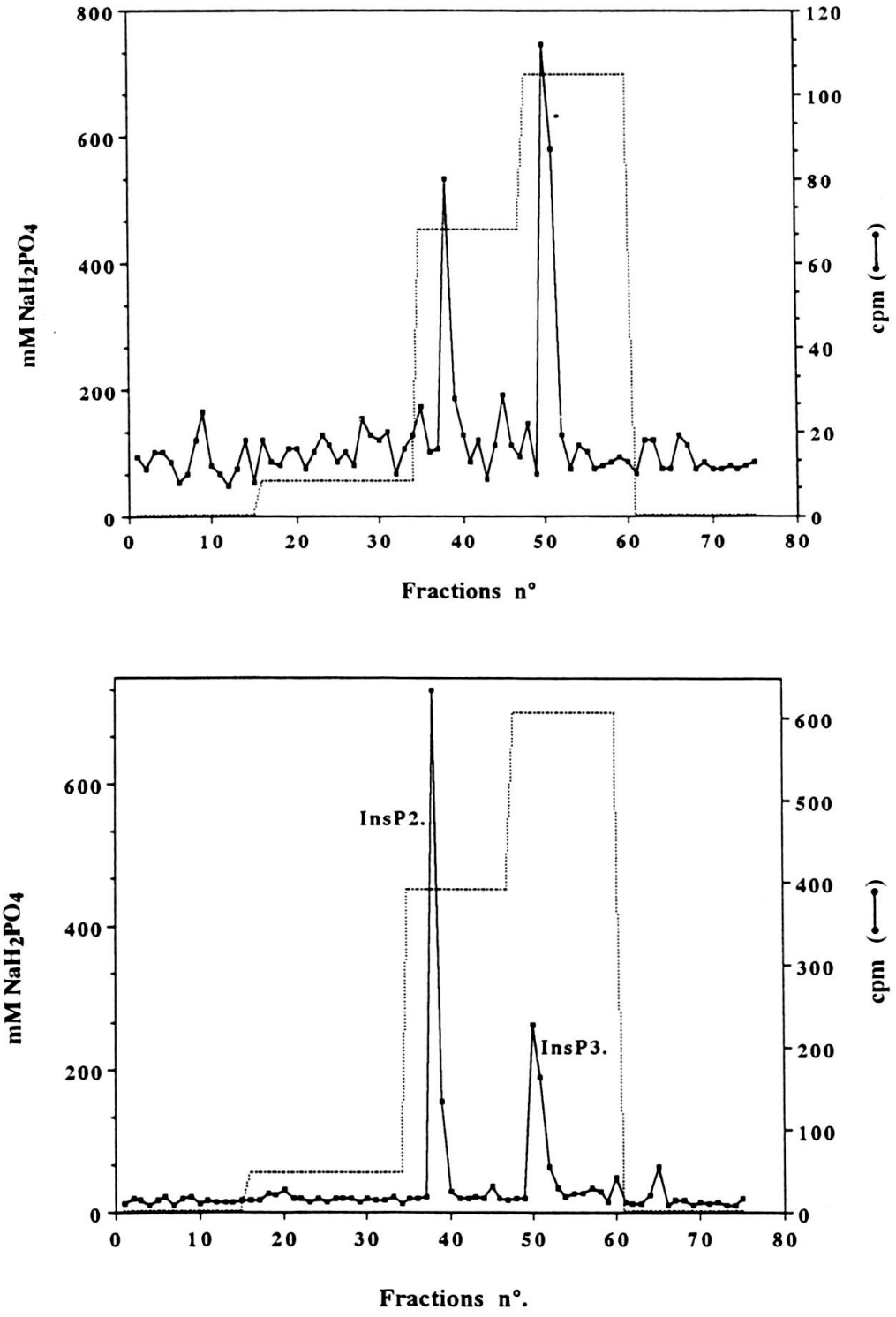
pH dependency of plasmalemma phospholipase C activity. PLC was tested in a Bis-Tris-Propane buffer at a final concentration of 0.25 mM as described in material and methods. Incubation in duplicate has been performed on chenopodium (open circle) and on spinach (close circle) plasma membrane preparations.

FIG. 2.

Sodium deoxycholate effect on phospholipase C activity measured on chenopodium (open circle) and on spinach (closed circle) plasmalemma preparations. Incubation in duplicate were runned at pH=7.5 for 4 minutes at 25°C.


300
200
100
0
nmoles PIP2/mg prot./min
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
pH


200
100
0
% control
0.00
0.05
0.10
0.15
0.20
0.25
% W/V deoxycholate



FIGS 3A & B.

High Pressure Liquid Chromatography profiles of reaction products separation (Fig. 3A) and of $InsP_2$ and $InsP_3$ standards (Fig. 3B). One minute fractions were collected after elution and directly counted in a scintillation counter.

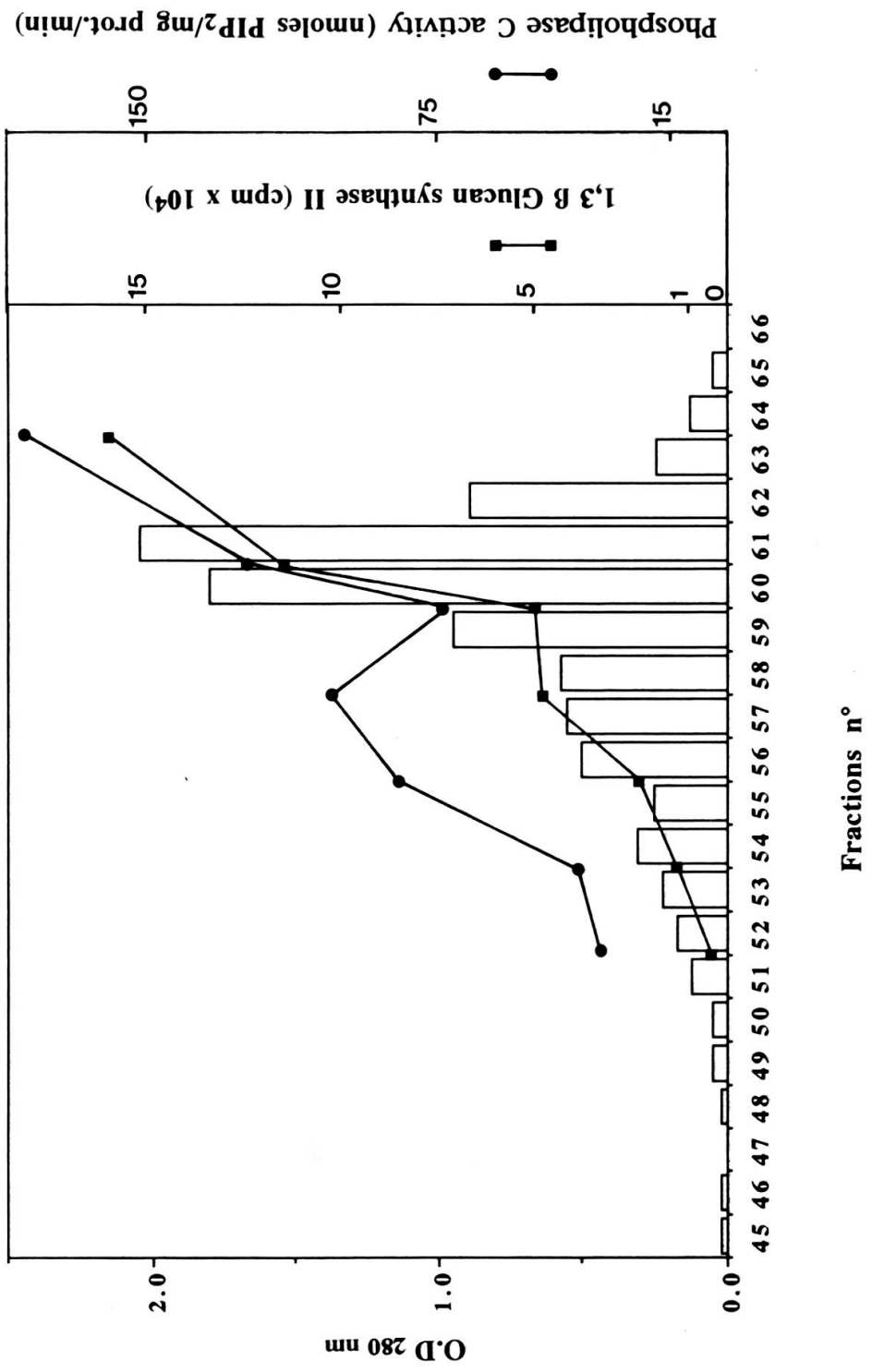


FIG. 4A.

Phospholipase C and 1,3 β Glucan synthase II activities on differents pooled fractions obtained by free-flow electrophoresis spinach membranes separation (bars: Optical Density at 280 nm).

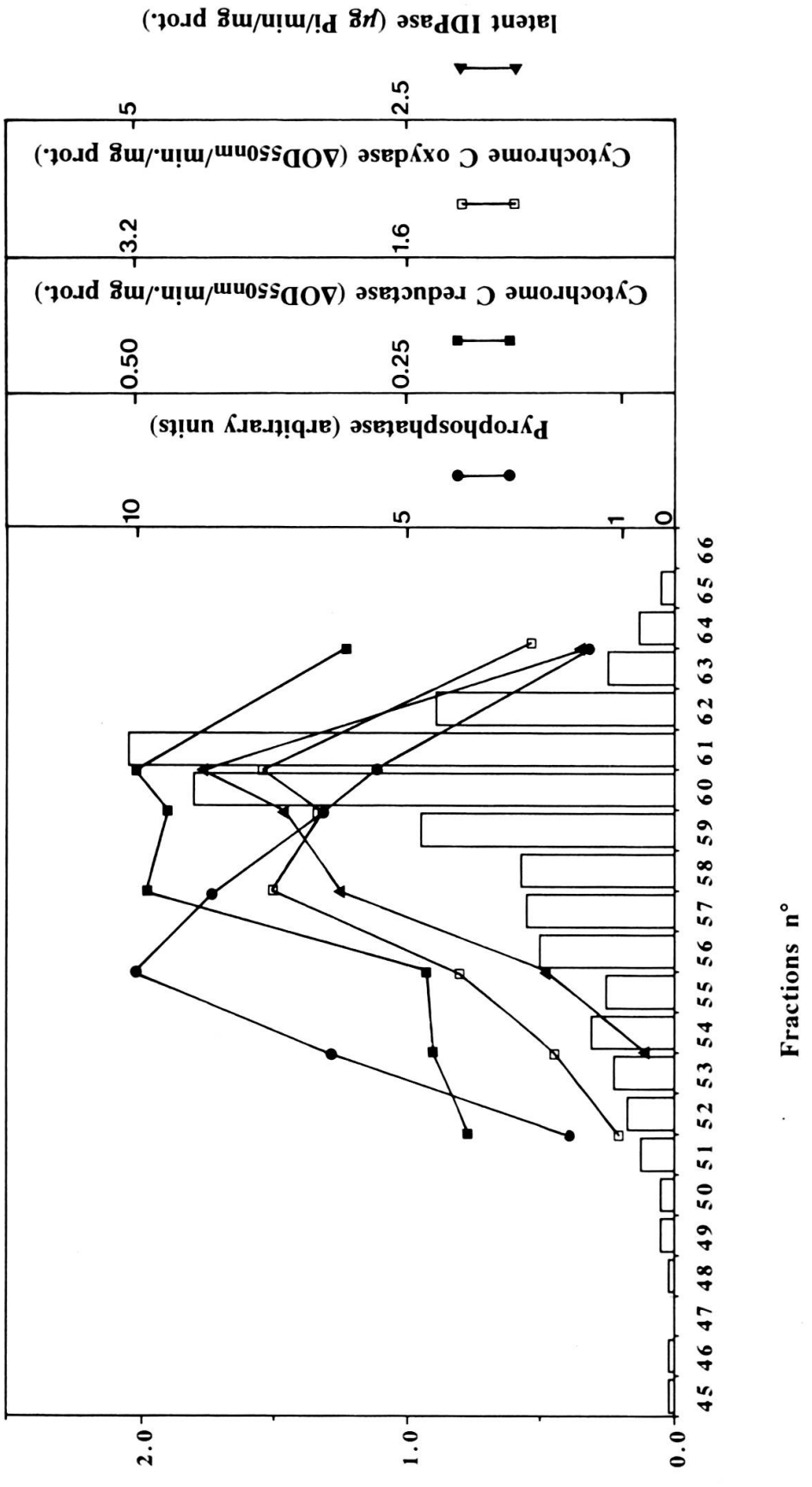


FIG. 4B.

Endomembranes enzymes markers activities found in pooled fractions.

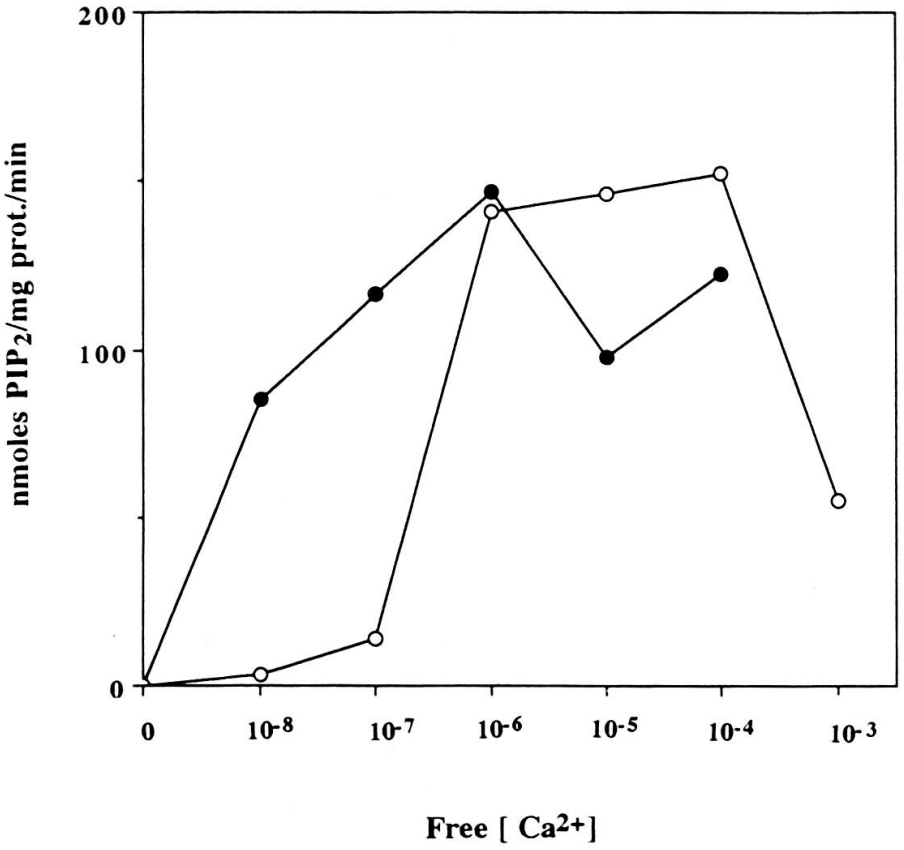


FIG. 5.

Ca^{2+} dependancy of phospholipase C activity. In each cases, *Chenopodium* (open circle) and *Spinacia* (close circle), the maximum activity were found for μM free Ca^{2+} respectively.

TABLE 1.

	experiment n° 1	experiment n° 2	experiment n° 3
control plants	49.8 ± 16	61 ± 4.2	61.9 ± 2.4
induced plants	78.6 ± 6.2	79 ± 3.7	84.2 ± 5.1

Phospholipase C activities (nmoles PIP_2/min./mg prot. ± standard deviation) of spinach plasma membrane isolated from short day and long day plants. Plasmalemma isolation was runned at the end of dark period for the control plants and after 24 hrs. continuous light for long day plants. This table shows three representatives experiments on different shares of plants. Each determination were run in triplicate.

DISCUSSION

The first step of this study was to analyse some caracteristics of phospholipase C activity linked to spinach and chenopodium leaves plasmalemma. The plant enzyme catalysed efficiently the hydrolysis of PIP_2. High pressure liquid chromatography analyses have shown that reaction products correspond to a mix of $InsP_3$ and $InsP_2$. The presence of $InsP_2$ after reaction could be linked to a phosphomonoesterase. Phospholipase C activity presents a Ca^{2+} and a deoxycholate dependency in accordance with the litterature (MELIN P.M. *et al.*, 1992). pH dependancy presents in our plant system a small discrepancy with data of Melin *et al.* for wheat roots and shoots. pH optima were found to be more basic for spinach and for chenopodium too. We have also examined if the phospholipase C activity was only linked to plasma membrane. For this we have realized a free-flow electrophoresis separation of a crude microsomal preparation from spinach leaves. Classically, fractions near the cathode represent a very pure suspension of plasma membrane microsomes (AUDERSET G. *et al.*, 1986). The maximum of phospholipase C activity was found in these fractions. The shoulder present in fractions 54 to 59 could be linked also to plasmalemma microsomes with another orientation. However, phospholipase C and 1,3 β glucan synthase II activities present a good correlation. It means that phospholipase C activity is principally linked to plasma membrane. This result was in accordance with PICAL C. *et al.* (1992) which localized phospholipase C activity on the cytoplasmic surface of the plasma membrane of wheat. This observation could be related to the small discrepancy observed for spinach and chenopodium for sodium deoxycholate stimulation. This could be due to different proportion of inside-in vesicles present in the different preparations.

Investigations on spinach plasmalemma have shown a clear light dependency of phospholipase C activity. This has been also shown in some experimental systems (MORSE M.J. *et al.*, 1987). Unfortunately, no data of light quality effect are available until now, but a direct control of phospholipase C activity by phytochrome or by another light sensitive pigment should not be neglected.

Previous experiments have shown some modifications of spinach and chenopodium plasmalemma in response to different light conditions. These structural, compositionnal and functionnal changes have been demonstrated during the first hours of flowering induction. Calcium has also been implicated in the photoperiodic induction of *Pharbitis nil* (KIYOTOSHI TAKENO, 1993). PLC controled its mobilization at plasma membrane level via $InsP_3$ and this mobilization is the result of stimulation of the cell by growth regulators, mitogenesis agents (WHITMAN M. *et al.*, 1988) and also environmental factors. In photoperiodic control of flowering, it is clearly proved that a message is propagated from the leave to the apical meristem. The nature of this message is still unknown. Several experiments have shown that the plasmalemma could be implicated in the reception and the propagation of this message. Our experiments have shown that PLC, a key enzyme for signal transduction, is modulated by light. Futher experiments should be run to precise this regulation. If PLC could be implicated in the reception of

photoperiodic stimulus, another point should be discussed. Recent experiments have shown that a membrane continuum, via plasmodesmata and endoplasmic reticulum, exist in plant tissues. GRABSKI S. *et al.* (1993) have also demonstrated that the other product of PLC activity, diacylglycerol, can diffuse within the cell using the endoplasmic reticulum continuum. By this way, individual cell could initiate signals for systemic transmission or couple their signaling pathway. Another factor which can diffuse within the cell is Ca^{2+} but it can not promote flowering by itself. It is important to notice that in animal, PLC is not under the regulation of this ion. Ca^{2+} could be more important as a modulator of PLC activity than as a chemical signal through plant tissues. New insight in plasmodesmata research have also implicated Ca^{2+} and $InsP_3$ in its regulation (TUCKER E.B., 1988). All these data suggest that light could produce, at leave cell level, a stimulus for floral induction and/or could control its propagation, through the membrane network, within the plant. These also suggest that the ubiquitous signal transduction pathway, via phospholipase C, is implicated in floral induction process.

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