エリシターによるタバコ培養細胞イノシトールリン脂質代謝の活性化

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Stimulation of Inositol Phospholipid Turnover in Tobacco Suspension Culture Cells by Fungal Elicitor

A dissertation submitted to the Graduate School of Science, University of Tokyo, in fulfillment of the requirement for a Ph. D. degree.

by

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List of Abbreviations

Bistris	bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane
BSA	bovine serum albumine
BTP	1,3-bis(tris(hydroxymethyl)methylamino)propane
$C_{12}E_8$	octaethylene glycol dodecyl ether
cAMP	cyclic AMP
CDPK	Ca^{2+} dependent protein kinase
DG	diacylglycerol
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	glycoletherdiaminetetraacetic acid
ER	endoplasmic reticulum
GUS	β-glucuronidase
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
Ins(1)P	inositol 1-monophosphate
Ins(1,4)P_2	inositol 1,4-bisphosphate
Ins(1,4,5)P_3	inositol 1,4,5-trisphosphate
Mes	2-(N-morpholino)ethane-sulfonic acid
NP-40	nonaethylene glycol octylphenyl ether
PA	phosphatidic acid
PAL	phenylalanine ammonia-lyase
PC	phosphatidylcholine
PI	phosphatidylinositol
PIP	phosphatidylinositol 4-monophosphate

PIP_2	phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
TCA	trichloroacetic acid
TLC	thin layer chromatography
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
Triton X-100	polyoxyethylene octylphenyl ether

General Introduction

"Adapt or perish!" All of living organisms live always adapting themselves to their surroundings. It is essential for their survival to recognize the changes of the environments as signals, transfer the signals into the cells and respond effectively to the environments. Actually they have various means for adaptation. Cellular level study of adaptation mechanism for the environments has been one of exciting areas in biology, and today investigators agree with that the adaptation proceeds as follows; 1) the external (first) signal is perceived by its receptor on the cell surface, 2) then, the receptor activates the effector which convert the signal into "second messenger(s)" which transmit the information in the cell. This process is mediated by the transducer, such as G-protein. 3) The second messengers induce various cellular responses including regulation of enzyme activity and gene expression (Fig. 1). This scheme of signal transduction has been established based on a large accumulation of researches mainly in mammalian or yeast cells. Several signal transduction pathways were found as the results, for example, cAMP cascade, arachidonate cascade, calcium signaling, PI turnover and so on.

PI turnover was firstly reported by Hokin and Hokin (1953), but their original finding had been left forgotten until the middle of 1970s when several investigators proposed and proved the Ca^{2+} mobilization by $Ins(1,4,5)P_3$ (*e.g.* Michell, 1975, Streb et al., 1983). Since the involvement of protein kinase C in PI turnover was found by Nishizuka and his colleagues (1984), the PI turnover has been investigated so actively, that tremendous

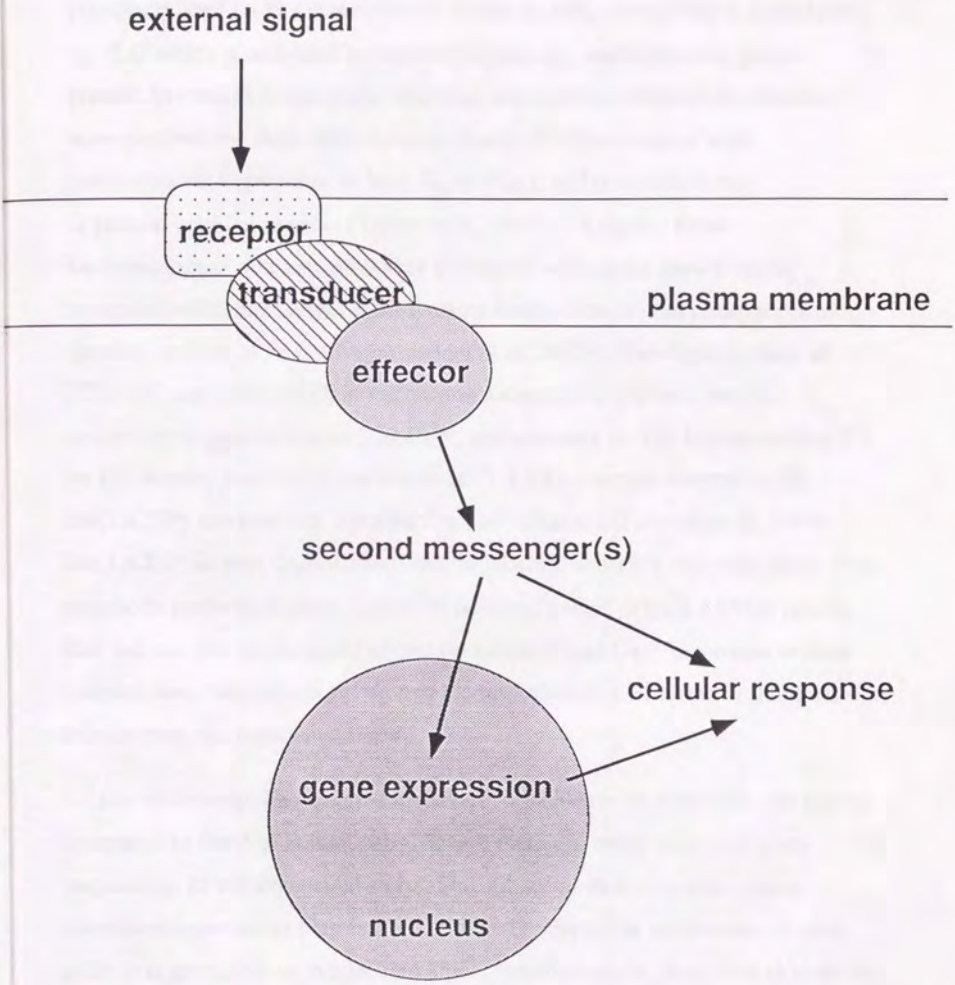


Fig. 1. The model of signal transduction. For details, see the text.

progress in this field has been now achieved. PI in the plasma membrane is phosphorylated by PI kinase and PIP kinase to PIP_2. Then PIP_2 is hydrolyzed by PLC which is activated by external signals *e.g.* hormones and growth factors. Several PLC isozymes which are activated by different mechanisms were purified and their cDNAs were cloned. PLCβ is coupled with heterotrimeric G proteins, at least G_q and G_{11}, and is activated in a G-protein-coupled manner (Taylor et al., 1991). PLCγ has three src-homologous domain and makes a complex with some growth factor receptors which themselves have tyrosine kinase domain and phosphorylate tyrosine residue of PLCγ (Meisenhelder et al., 1989). Two hydrolysates of PIP_2, DG and Ins(1,4,5)P_3 act as second messengers. DG increases the sensitivity of protein kinase C to Ca^{2+}, and activates it. DG is converted to PA by DG kinase. Ins(1,4,5)P_3 activates Ins(1,4,5)P_3 receptor located in ER. Ins(1,4,5)P_3 receptor was identified as Ca^{2+} channel (Furuichi et al.,1989). Ins(1,4,5)P_3 is then dephosphorylated to inositol, and PI is re-synthesized. This metabolic pathway is often called "PI turnover cycle" (Fig. 2.). Observations that indicate the involvement of protein kinase C and Ca^{2+} in various cellular reaction have been accumulated and the importance of PI turnover in signal transduction has been established.

Our knowledge about signal transduction pathways in plant cells are limited compared to those in animal cells, though there are many studies of plant response to its environmental stress. Since it seems sure that some signal transduction pathways play important roles in adaptation mechanism of plant cells, it is inevitable to inquire into signal transduction in plant cells in order to elucidate the adaptation mechanisms of plants. Several investigators

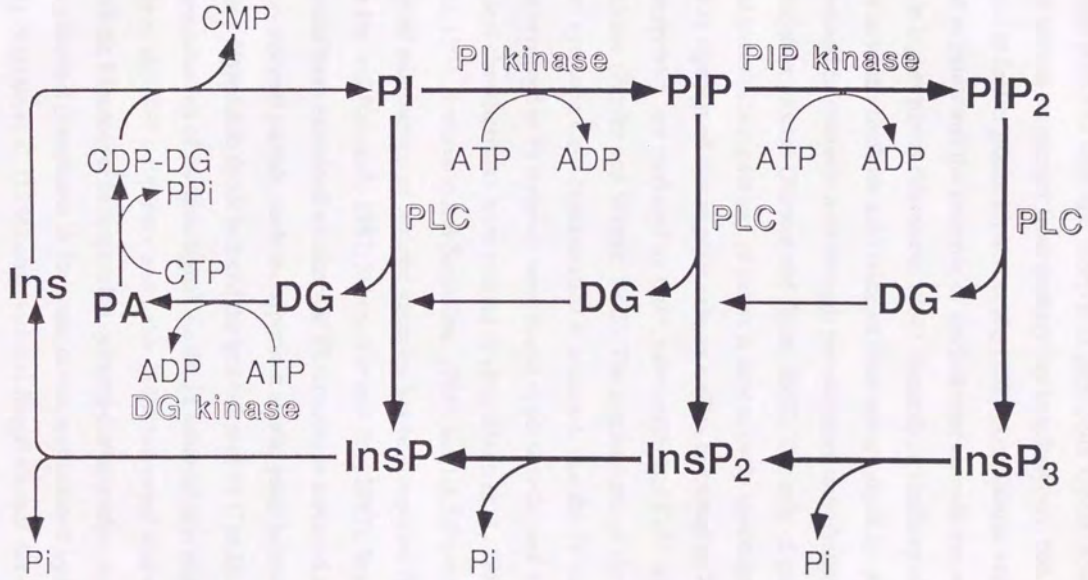


Fig. 2. Pathway of PI turnover cycle.

examined whether some signal transduction pathways functioning in mammalian cells also exist and function in plant cells. Cyclic AMP is the most prevailed second messenger from prokaryotes to eukaryotes, but, its function is questioned in higher plants (Spiteri et al., 1989). Arachidonic acid is very rare fatty acid in plants and the presence of arachidonate cascade has not been focused in higher plants (Harwood, 1980). Recently, a similar pathway was proposed in which linolenic acid released from phospholipid by phospholipase is metabolized to jasmonic acid through peroxidation, dehydration, reduction, and β-oxidation process (Farmer and Ryan, 1992). The role of jasmonic acid in the signal transduction pathway of plants is now actively investigated. Ca^{2+} is regarded as signal mediator in plant cells as well as in animal cells; certain cellular responses are mediated by Ca^{2+}, calmodulin and Ca^{2+} dependent protein kinase (Hepler and Wayne, 1985). The mechanisms of signal-coupled control of cytosolic Ca^{2+} concentration is assumed, like the PI turnover. Enzymes involved in PI turnover were found in plant cells, and their biochemical characteristics were studied *in vitro* (Heim et al., 1987, Kamada and Muto, 1991, Sommarin and Sandelius, 1988). Ins(1,4,5)P_3-induced Ca^{2+} release from microsomes or isolated vacuoles has been reported (Ranjeva et al., 1988, Reddy and Poovaiah, 1987, Schumaker and Sze, 1987). Several investigators have examined whether the PI turnover is involved in responses of plant to external stimuli, such as, hypotonic shock, plant hormones, light, and elicitor. Hypotonic shock induced the activation of PLC in *Dunaliella* cells and the transduction of osmotic signal via the PI turnover was postulated (Einspahr et al., 1988). Ettlinger and Lehle (1988) reported that auxin stimulated the PI turnover in suspension cultured *Catharanthus roseus* cells. Change of inositol phosphates in *Samanea saman* was induced by light (Morse et al., 1987). Strasser et al. (1986) observed that fungal elicitor did not influence

on the PI turnover in cultured parsley or soybean cells. On the other hand, Kurosaki et al. (1987) reported that inositol triphosphate level and phospholipase activity were increased when cultured carrot cells were treated by the elicitor prepared from carrot pectin. However their analytic method of inositol phosphates using Dowex anion exchange column which has been well prevailed in animal materials could not be applied in plant materials, *i.e.* PI turnover products, especially $Ins(1,4,5)P_3$, were co-eluted from Dowex column together with [^{3}H]inositol metabolites other than products of PI turnover (Rincón et al. 1989). Thus the analytical method of PI turnover products must be reconsidered.

I studied the PI turnover of tobacco cells *in vitro* in the studies during my master course I demonstrated that PI kinase, PIP kinase, PLC, and DG kinase exist in the plasma membrane of tobacco suspension culture cells (line BY-2), and investigated their biochemical characteristics. PI kinase and PIP kinase were inhibited and PLC was activated by physiological level of Ca^{2+}, suggesting that increased Ca^{2+} in the cytosol affects these enzymes.

In the present study, I examined whether PI turnover in tobacco cells is involved in the response to external signal. At the beginning there were two problems that I should have overcome; the establishment of analytical method of PI turnover products, and the search for external signals which activate the PI turnover in BY-2 cells. The former would be resolved by improvements of labeling procedure of cells and by HPLC analysis of inositol phosphates. As for the latter, I chose a fungal elicitor, firstly because, the defense system against fungal infection is the best characterized among many signal-response relationships in plants and the defense reaction is easy to determine. Secondly, the relationship between the PI turnover and the defense system still remains to

be elucidated as described above. Before investigating the effect of elicitor on the PI turnover, that the fungal elicitor triggered the defense response in tobacco suspension culture cells must have been demonstrated. Then, if stimulation of PI turnover were induced by the fungal elicitor and defensive response occurred, relationship between the induction and the response, and the existence of another factor which mediates them would be examined.

I. Induction of Phenylalanine Ammonia-lyase by Elicitor

Abstract

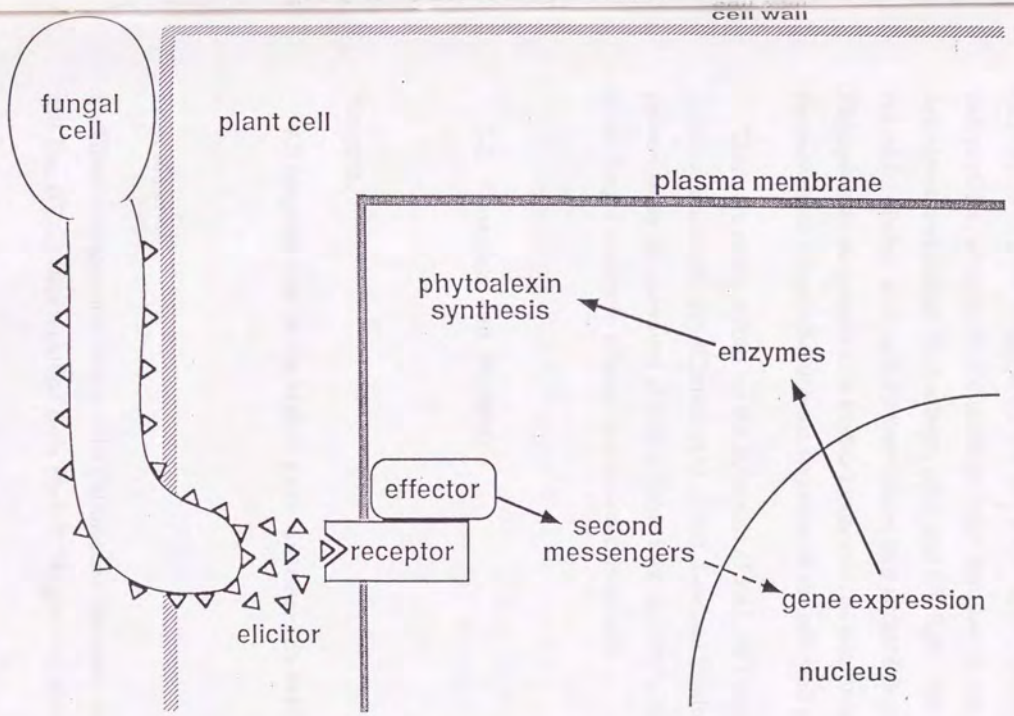
It was investigated whether the elicitor prepared from the cell wall fraction of *Phytophthora nicotianae* var. *nicotianae* induces phenylalanine ammonia-lyase (PAL) activity in tobacco suspension culture cells (cell line BY-2). The induction of PAL activity by the elicitor was observed in tobacco BY-2 cells, and the induction began 2 hr after the addition of the elicitor. Actinomycin D completely inhibited PAL induction suggesting that this induction is regulated at transcriptional level. When the elicitor was removed from the elicited culture by washing treatment 30 min after the addition of the elicitor, PAL activity was still induced. A protein kinase inhibitor K252a strongly inhibited the induction of PAL by the elicitor when added together with the elicitor, however, its effect was reduced by one half when added 1 hr after the elicitation. These results suggest that the elicitor signal transduction system is stimulated within 1 hr after the elicitation.

I-1. Introduction

Higher plants are always subjected to pathogenic microbes and they have various resistance mechanism to prevent themselves from infection. Their resistance mechanisms are effective at different levels in host-parasite interactions and include preformed physical and chemical defense barriers as well as defenses triggered by the invader. One of inducible defense response is the synthesis of phytoalexins. Fig. 3. shows the scheme of phytoalexin induction. The infection of fungal cell is perceived by the host plant as elicitor-binding to its receptor. Then signal transduction pathway is activated

and the enzymes which function defense mechanism *e.g.* phytoalexin synthesis are induced.

Phytoalexins have been defined as antimicrobial compounds with low molecular weight which are synthesized by and accumulated in plants after the exposure of the plant to pathogens. They show the structural complexity typical of higher-plant natural products; predominantly phenylpropanoids, isoprenoids, and acetylenes (Ebel, 1986). Phenylalanine ammonia-lyase (PAL) catalyzes the first step in the sequence of reactions of "general phenylpropanoid metabolism", that is, PAL catalyzes the deamination of L-phenylalanine to yield *trans*-cinnamic acid and ammonia. PAL is one of the key enzymes involved in phenylpropanoid metabolism, and the best characterized among them. PAL protein was purified, and its cDNA and genomic DNA were isolated from various plants. PAL is encoded by gene family, and each gene was induced during development and responding to UV light, wounding, and elicitors.



Fig, 3. The scheme of the induction of plant defense mechanism by fungal infection. For details, see the text.

The term "elicitor" has been commonly used to refer compounds that induce phytoalexin synthesis in plants. Many different types of elicitors for phytoalexin production have been described. Biotic elicitors include complex carbohydrates from fungal and plant cell walls, lipids, microbial enzymes, and polypeptides, whereas abiotic elicitors range from heavy metal salts to detergents, autoclaved ribonuclease, cold, and UV light. The best-characterized microbial elicitor with carbohydrate nature was isolated from the cell wall of *Phytophthora megasperma*, a soybean pathogen. Its minimum structure was determined as a hepta-β-glucoside fragment of the cell-wall polysaccharide.

There are many reports on the induction of PAL by fungal elicitors in suspension culture cells (Cramer et al., 1989, Joos and Hahlbrock, 1992). In the present study we examined at first whether PAL activity is induced responding to the fungal elicitor in tobacco suspension culture cells.

I-2. Materials and Methods

Reagents.

All reagents were of the highest grade commercially available.

Plant Material.

Tobacco suspension culture cells (*Nicotiana tabacum* L. cv. Bright Yellow-2 cell line BY-2) were supplied from Prof. T. Nagata and Prof. T. Kuroiwa,

University of Tokyo, usually propagated according to Nemoto et al. (1988). Briefly, 2.5 ml of 7-day stationary culture was suspended in 97.5 ml of fresh medium, then the obtained culture was shaken for 7 days at 88 rpm by reciprocal shaker at 26 °C.

Fungal Material and Preparation of Elicitor.

Phytophthora nicotianae var. *nicotianae* was donated from Institute for Fermentation, Osaka. Mycelial cells were cultured as described (Keen, 1975), and fungal elicitor was prepared from their mycelial wall fragments as described (Ayers et al., 1976). Briefly, 150 g fresh weight of the fungal mycelial cells were washed and suspended in 0.5 M K-phosphate (pH 7.0), and homogenized by a Polytron homogenizer (Kinematica Co.). The homogenate was passed through a nylon mesh with 40-μm pores, then water-insoluble cell debris was washed by 0.5 M K-phosphate (pH 7.0), chloroform:methanol (1:1 v/v), and acetone to obtain cell wall fraction. After drying the cell wall fraction was added to 300 ml of H_2O and the mixture was autoclaved for 3 hr at 120 °C. The resultant water-soluble cell wall hydrolysate was dried under vacuum and used as the fungal elicitor.

Induction of PAL and Assay of PAL activity.

Fresh tobacco cells (5 ml) in 50 ml culture tubes were shaken at 88 rpm by rotary shaker, at 26 °C for 2 days. They were stimulated by 0.5 ml of 10 mg/ml elicitor dissolved in a medium which had been obtained by filtering the similar culture (2-day medium). To control cell culture 2-day medium was

added. These cultures were incubated for 6 hr, and 1.5 ml of the culture was harvested in an Eppendorf tube, centrifuged at 10,000 rpm for 1 min. The resultant cells were washed once and suspended in 1 ml of 50 mM Tris-HCl (pH 8.8) containing 1 mM DTT and 0.1 mM PMSF, and homogenized by a sonicator (2N-100, Toyo Rikoh, Tokyo). The homogenate was centrifuged at 16,000 x*g* for 10 min and the resultant supernatant solution was passed through a Sephadex G-25 column (12.7 x 1 cm) which had been equilibrated with 50 mM Tris-HCl (pH 8.8) containing 1 mM DTT. Protein fraction was collected and used as enzyme solution.

Enzyme activity of PAL was assayed spectrophotometrically determining the amount of *t*-cinnamic acid formed as described (Minamikawa and Uritani, 1965) with some modifications as follows; The reaction mixture contained 50 mM Tris-HCl (pH 8.8), 1 mM DTT, 0.1 mM PMSF, 10 mM phenylalanine, and 0.2 ml of enzyme solution, in 1 ml. The reaction was carried out at 40 °C for 1 hr, and terminated by the addition of 0.5 ml of ice-chilled 2 N perchloric acid. After mixing well, the reaction mixture was centrifuged at 16,000 x*g* for 10 min at 0 °C. Cinnamic acid in the supernatant solution was determined at A_{280}.

Protein was determined by the method of Bradford (1976) with BSA as standard.

I-3. Results

When added to suspension culture, 0.05 mg/ml elicitor could induce an increase in PAL activity, and the eliciting effect was saturated at 0.5 mg/ml

(Fig. 4a.). The time course of PAL induction was examined with 1 mg/ml elicitor. The PAL induction began 2 hr after the addition (Fig. 4b.). These results indicate that tobacco suspension culture cells recognize the fungal elicitor and induce PAL activity as their defense response.

When tobacco culture was pre-treated with 10 mg/ml actinomycin D or 10 mg/ml cycloheximide, the effect of the elicitor on the PAL induction was reduced (Fig. 5a.), suggesting that the regulation of PAL induction occurred at the transcriptional step.

When the elicited culture was washed with the 2-day medium 30 min after the addition of the elicitor, the PAL activity was induced, while the control cells showed a little induction (Fig. 5a.). A protein kinase inhibitor K252a strongly inhibited the elicitor induction of PAL when added together with the elicitor, however its effect was reduced by one half when added 1 hr after the elicitation and completely when added 2 hr after (Fig. 5b.).

I-4. Discussion

The elicitor treatment induced the activity of PAL of tobacco suspension culture cells suggesting that they respond well to the elicitor prepared from the cell wall fragments of *Phytophthora nicotianae* mycelial cells. This indicates that tobacco BY-2 cells is available to study the signal transduction pathway involved in plant defense reaction. The results from elicitor treatment followed by washing and the inhibitory effect of K252a on the PAL induction suggest that the signal transduction system is stimulated within 1 hr after the elicitor treatment.

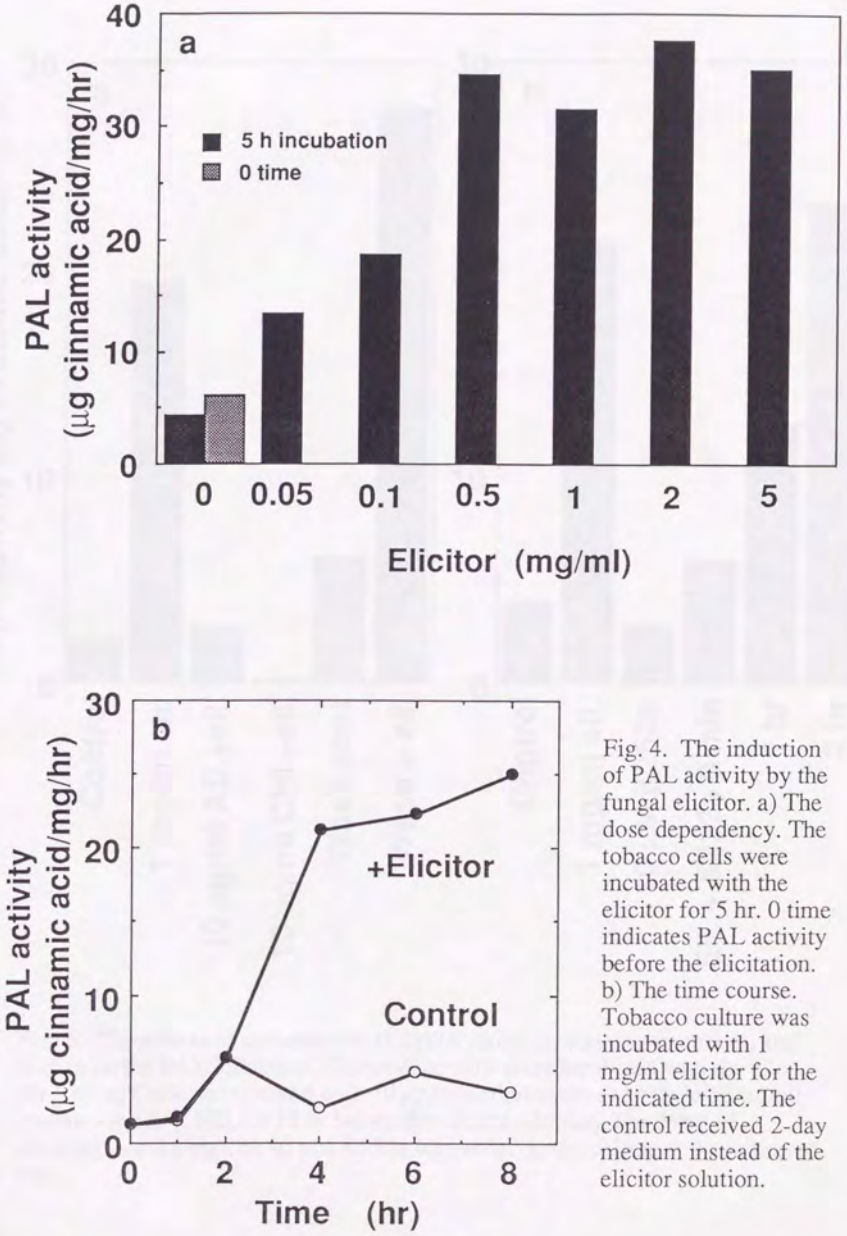


Fig. 4. The induction of PAL activity by the fungal elicitor. a) The dose dependency. The tobacco cells were incubated with the elicitor for 5 hr. 0 time indicates PAL activity before the elicitation. b) The time course. Tobacco culture was incubated with 1 mg/ml elicitor for the indicated time. The control received 2-day medium instead of the elicitor solution.

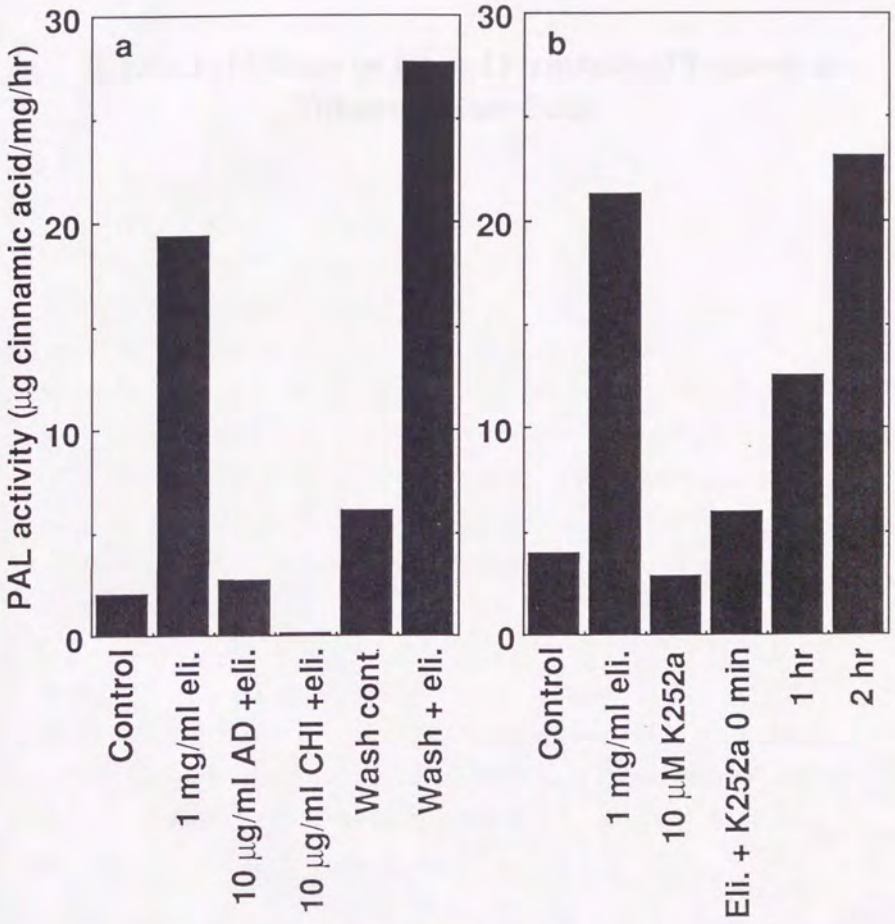


Fig. 5. The effects of actinomycin D, cycloheximide, washing treatment, and K252a on the PAL induction. The tobacco cells were incubated with the elicitor for 6 hr. a) Cells were treated with 10 μg/ml actinomycin D (AD) or 10 μg/ml cycloheximide (CHI) for 12 hr before the elicitor addition. For detail of washing, see the text. b) 10 μM K252a was added to the culture at the indicated time.

II. Effect of Elicitor on Inositol Phospholipid Turnover in Tobacco Culture Cells

Abstract

The effect of the fungal elicitor on phosphatidylinositol (PI) turnover in tobacco cells was examined. Incorporation of [^{3}H]inositol into inositol phosphates in *in vivo* labeling was improved by preculturing tobacco cells with glucuronic acid. Levels of inositol phosphates started to increase 2 min after the elicitor treatment. Inositol 1,4-bisphosphate increased by 15 times as the control, while inositol 1,4,5-trisphosphate by 37.5 %. Incorporation of ^{32}P into phospholipids was also changed by the elicitor. Phosphatidylinositol 4-monophosphate (PIP) began to decrease 10 min after the addition of the elicitor and reached 40 % of the control after 45 min. ^{32}P incorporation into phosphatidylinositol and phosphatidic acid was stimulated by the elicitor. These results suggest that PI turnover cycle was triggered by the elicitor. The activation of phospholipase C (PLC) by the elicitor was assumed, however, PLC was not activated by the elicitor in the isolated plasma membrane. PI kinase in the isolated plasma membrane was activated by the elicitor. Then whether cytosolic proteins were involved in the activation of PLC was tested. The cytosolic proteins did not activate but inhibited PLC activity. This suggests that the activation of PLC by the elicitor may be induced by the release of cytosolic protein factor from PLC.

II-1. Introduction

In Chapter I it was shown that BY-2 cells respond to the elicitor. Now I can examine the involvement of PI turnover in the transduction of elicitor signal.

As mentioned in General Introduction the effect of elicitor on the PI turnover is controversial. Kurosaki et al. (1987) demonstrated that [^{3}H-inositol]PI hydrolysis by the supernatant solution obtained from the cell homogenate by centrifugation at 1,200 x*g* was 30 % stimulated by the elicitor prepared from carrot pectin by partial hydrolysis. When the carrot cells labeled with [^{3}H]inositol for 18 hr were stimulated by the elicitor, then soluble fraction of cell homogenate was analyzed by a Dowex anion exchange column, inositol trisphosphate increased twice within 3 min. However, their analytical method of inositol phosphates was dissatisfied. Rincón et al. (1989) precisely examined the presence of inositol trisphosphate and other inositol phosphates in the carrot cells; the cells were labeled with [^{3}H]inositol for 18 hr and extracted with ice-cold 10 % TCA. The inositol metabolites were separated by a Dowex column and by paper electrophoresis. They found that [^{3}H]inositol metabolites other than inositol triphosphate coeluted with inositol bisphosphate and inositol trisphosphate from the column. They also observed that the [^{3}H]inositol metabolites were abundant in the cell wall fraction, and disturbed the exact analyses of inositol phosphates. On the other hand, Strasser et al. (1986) labeled cultured parsley and soybean cells with [^{3}H]inositol, [^{3}H]glycerol or ^{32}Pi and stimulated with the elicitor prepared from *Alternaria carthami* and *Phytophthora megasperma*, respectively. Inositol phospholipids extracted from them were analyzed with two-dimensional TLC. However, no significant differences of inositol phospholipids were observed in elicitor-treated versus control cells. Toyoda et al. (1992) reported that *in vitro* activities of PI and PIP kinases in the plasma membrane of pea were stimulated by the elicitor prepared from a pea pathogen *Mycosphaerella pinodes*. It should be noted that their experiments were done at 0 °C to prevent breakdown of inositol phospholipids; this is far from the physiological condition.

The purpose of study in this chapter is whether the PI turnover is stimulated by the elicitor in tobacco culture cells. The serious problems in studying the PI turnover in plants are that [^{3}H]inositol is not efficiently incorporated into inositol phosphates and that [^{3}H]inositol was metabolized to precursors of cell wall which disturbed inositol trisphosphate analysis. Since inositol is one of the main precursors of pectin and converted to glucuronate at the first step of metabolism (Fig. 6.) (reviewed by Loewus and Loewus, 1980), I expected that the efficiency of ^{3}H incorporation into inositol phosphates would be increased when cells were precultured with glucuronic acid.

II-2. Materials and Methods

Reagents.

myo-[^{3}H]inositol (2.96 TBq/mmol) was purchased from Amersham, ^{32}Pi (315 TBq/mmol), [inositol-2- ^{3}H]Ins(1,4)P_2, and [inositol-1-^{3}H]Ins(1,4,5)P_3 for HPLC standards were from New England Nuclear. All other reagents used were of the highest grade commercially available.

Plant Material.

Tobacco suspension culture cells were propagated as described in Chapter I, with the medium depleted inositol and precultured for 1 to 2 month(s) in the medium containing 100 mg/l filter-steriled glucuronic acid instead of inositol. This modification of the medium had no obvious effect on either cell growth or lipid analysis (data not shown).

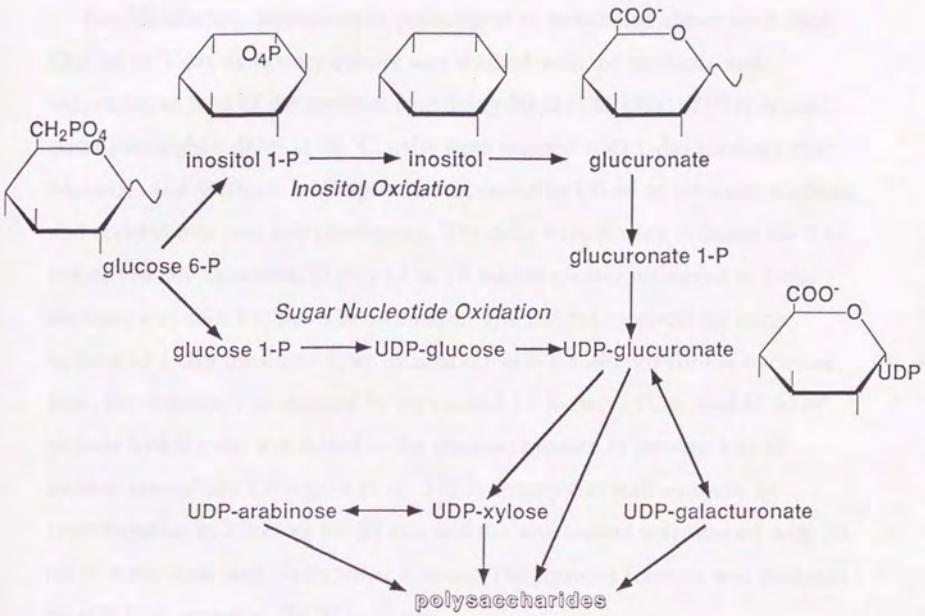


Fig. 6. Inositol metabolism in plant cells.

In Vivo Labeling with [^{3}H]Inositol and Elicitor Stimulation.

For ^{3}H labeling, tobacco cells precultured as mentioned above were used. One ml of 7-day stationary culture was washed with the medium, and suspended in 5 ml of the medium containing 50 µl (185 kBq) of [^{3}H]inositol. After overnight culture at 26 °C, cells were washed with 1-day medium (see Materials and Methods in Chapter I), suspended in 1.6 ml of the same medium, and divided into two 800 µl-aliquots. The cells were further cultured for 2 hr before elicitor treatment. Eighty µl of 10 mg/ml elicitor dissolved in 1-day medium was added to the labeled culture. The control received the same volume of 1-day medium. After incubation with the elicitor for the indicated time, the reaction was stopped by ice-chilled 10 % (w/v) TCA, and 50 µl of phytate hydrolysate was added to the reaction mixture to prevent loss of inositol phosphates (Wreggett et al., 1987). Precipitate was removed by centrifugation at 2,000 ×*g* for 20 min and the supernatant was washed with 10 ml of water-saturated diethylether 4 times. The aqueous fraction was analyzed by HPLC or stored at -20 °C until use.

Analysis of Inositol Phosphates.

Inositol phosphates were analyzed by ion exchange HPLC using a Partisil 10 SAX column (0.46 × 25 cm, Whatman) as described (Dean and Moyer, 1987), with some modifications. Ammonium phosphate buffer (pH 3.8) containing 1 mM EDTA was used, and the gradient used was 10 to 90 mM over 30 min for inositol monophosphates, 209 to 289 mM over 30 min for inositol bisphosphates, 527 to 587 mM over 45 min for inositol trisphosphates and 2 M over 20 min for column wash. The flow rate was 0.7 ml/min. Sample volume

applied was 500 µl. Fraction was collected for each 0.5 min. Radioactivity was measured by liquid scintillation counting. Retention times of inositol phosphates were determined with ^{3}H-labeled authentic compounds.

In Vivo Labeling by ^{32}Pi.

One ml of 7-day culture was washed by phosphate-free medium, suspended in 5 ml of the same medium, and cultured at 26 °C overnight. Five hundred µl of ^{32}Pi diluted to 54 µM by KH_2PO_4 was added to the culture. ^{32}Pi incorporation into the culture was saturated 5 to 10 min after the addition (data not shown). Then elicitor stimulation was carried out as described above. At the indicated time 500 µl of the labeled culture was sampled and lipids were extracted from the cells with 2 ml of cold chloroform:methanol (1:2, v/v) as described (Schacht, 1978). After standing for 20 min at 0 °C, 1.25 ml of chloroform and 1.25 ml of 1.2 N HCl were added to the mixture. After mixing and phase separation, the lower phase was collected and washed with 2 ml of methanol:1.2 N HCl (10:9, v/v). The resulting lipid extracts were dried under N_2 gas stream. The lipid fraction was analyzed by one-dimensional TLC or stored at -20 °C until use.

Analysis of Phospholipids.

Silica gel 60 TLC plate (20 × 20 cm, Merck) was impregnated with 1 % K-oxalate and dried at 110 °C for 45 min prior to use. The solvent system was chloroform:acetone:methanol:acetic acid:H_2O (40:15:13:12:8, v/v/v/v/v). The plate was developed to a height of 15 cm. This development was satisfactorily

used. Each radioactive spot obtained in one-dimensional TLC was confirmed as a single spot in a two-dimensional TLC as follows. In the x-direction with acetone:benzene:methanol:H_2O (8:3:2:1, v/v/v/v) (Sato and Furuya, 1984) to a height of 18 cm, then in the same direction with chloroform:methanol:4 N NH_4OH (9:7:2, v/v/v) to a height of 13 cm, and in y-direction with chloroform:acetone:methanol:acetic acid:H_2O (40:15:13:12:8, v/v/v/v/v) to a height of 15 cm (data not shown). Radioactive phospholipids were visualized by autoradiography. Radioactive spots were scratched off and their radioactivities were determined by liquid scintillation counting.

Isolation of Plasma Membrane.

The plasma membrane was isolated and purified as described (Kamada and Muto, 1991). All steps for membrane preparation were carried out at 0–4 °C. Culture cells were harvested by filtration under suction, and suspended in an isolation medium containing 0.3 M sucrose, 50 mM Mes-Tris (pH 7.6), 5 mM EGTA, 5 mM EDTA, 20 mM NaF, 2.5 mM $Na_2S_2O_5$, 1 mM DTT, 2 mM PMSF, 4 mM salycylhydroxamic acid, and 0.5 % (w/v) BSA at a medium-to-tissue ratio of 2 (v/w). This suspension was homogenized with a French pressure cell at 140 kg/cm^2 and the homogenate was centrifuged for 10 min at 10,000 $\times g$. The resulting pellet was discarded and the supernatant was further centrifuged for 30 min at 100,000 $\times g$ to obtain microsomal fraction, which was suspended in a suspension medium containing 0.25 M sucrose and 10 mM K-phosphate (pH 7.8). The plasma membrane was then purified from the microsomal fraction by the aqueous two-phase partitioning with 5.4 % Dextran T-500 (Pharmacia), 5.4 % polyethylene glycol 3,350 (Sigma) and 0.35 % NaCl. After the partitioning the upper phase a was diluted with 5 mM

Hepes-BTP (pH 7.0), containing 0.25 M sucrose, and 0.1 mM DTT, then centrifuged for 30 min at 160,000 ×*g*. The resulting pellet was suspended in the same medium and used as the plasma membrane. The purity of the plasma membrane has already been estimated showing that contaminations of tonoplasts and mitochondria were very low (Kamada and Muto, 1991).

Preparation of Soluble Protein.

Soluble protein fraction was prepared from the supernatant of the first 100,000 ×*g* centrifugation in the preparation procedure of plasma membrane, with the isolation medium without BSA. The supernatant was centrifuged again for 20 min at 500,000 ×*g* to obtain the soluble fraction. The resultant fraction was passed through a Sephadex G-25 column (1 × 12.7 cm) which had been equilibrated with 50 mM Bistris-HCl (pH 7.0) containing 1 mM DTT, 50 mg/ml leupeptin and 0.5 mM PMSF.

In Vitro Stimulation of PI Kinase Activity by the Elicitor.

PI kinase activity was assayed as described (Kamada and Muto, 1991, Toyoda et al., 1992) with some modifications. Plasma membrane (25 μg protein) was incubated for the indicated time at 30 °C in 200 μl of 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$, 5 mM EGTA, 0.05 % (w/v) Triton X-100, 0.5 mM DTT, 0.1 mM GTP and 0.5 mM [γ-^{32}P]ATP (2,220 MBq/mmol). The reaction was started by the addition of plasma membrane, and terminated by the addition of 1 ml of cold chloroform:methanol (1:2, v/v). After standing for 20 min at 0 °C, 0.75 ml of chloroform and 0.75 ml of 1.2 N

HCl were added to the reaction mixture. After mixing and phase separation, the lower phase was collected and washed with 1 ml of methanol:1.2 N HCl (10:9, v/v). The resulting lipid extracts were dried under N_2 gas stream, then samples were applied to TLC as described above. Radioactive PIP was visualized and determined with a Bio-Imaging scanner system (Bas 2000 system, Fujix).

Assay of Phospholipase C Activity.

PLC activity was assayed as described (Kamada and Muto, 1991) with some modifications. The plasma membrane (10 μg protein) was incubated for 3 min at 30 °C in 80 μl of 50 mM Bistris-HCl (pH 7.0) containing 1 mM $MgCl_2$, 0.5 mM DTT, 0.05 % (w/v) $C_{12}E_8$ (Wako), 50 μg/ml leupeptin (Wako), 0.1 mM ATP, 0.1 mM GTP, and 150 μM [^{3}H]PIP (4,440 dpm/nmol). Elicitor stimulation was done by the addition of 1 mg/ml elicitor dissolved in H_2O to the reaction mixture. The reaction was started by the addition of PIP, and terminated by the addition of 0.5 ml of chloroform:methanol (1:2, v/v). After standing for 20 min at 0 °C, 0.3 ml of chloroform and 0.3 ml of 1.2 N HCl were added to the reaction mixture. After mixing and phase separation, the water-soluble upper phase was collected and its radioactivity was measured by scintillation counting.

II-3. Results

At first, the condition of preculture with glucuronic acid and labeling was examined. Tobacco BY-2 cells were precultured being depleted inositol and

with or without 100 mg/l glucuronic acid. Then the two series of culture were labeled with [^{3}H]inositol in the presence or absence of glucuronic acid. The result was shown in Table 1. The condition that tobacco suspension culture cells were precultured with glucuronic acid and labeled without it was the best among all conditions examined. Thus this method was used to analyze inositol phosphates.

Fig. 7. shows HPLC profiles of ^{3}H-labeled water soluble compounds of both control and elicitor treated cells. Ins(1,4)P_2 greatly and Ins(1,4,5)P_3 slightly increased in the elicited cells, suggesting that the PI turnover was stimulated by the fungal elicitor. No significant change was observed in other compounds. At the same time, the eluates were monitored by A_{254}, but no significant change was detected after elicitation (data not shown). The fact that only inositol phosphates but not other [^{3}H]inositol metabolites or UV-detectable compounds were increased by the elicitor indicates that there is a certain specific pathway, probably activation of PLC, to increase inositol phosphates by the elicitor. Fig. 8. shows time course of changes of Ins(1,4)P_2 and Ins(1,4,5)P_3. In the elicited cells Ins(1,4)P_2 began to increase 2 min after the elicitation, and it achieved 15 times higher than the control cells within 10 min (Fig. 8a.). On the other hand, Ins(1,4,5)P_3 increased only 37.5 % higher than the control cells 10 min after elicitor treatment (Fig. 8b.). The amount of Ins(1,4)P_2 was kept at a high level 1 hr after the elicitor addition, while that of Ins(1,4,5)P_3 returned to the starting level (Fig. 9.). These results suggest that the elicitor stimulated hydrolysis of inositol phospholipids by PLC precedes the PAL induction which occurs after 2 to 4 hr.

In ^{32}Pi labeling experiments, the control cells rapidly incorporated ^{32}P into PIP and PA within a few minutes, and then into PI and PC (Fig. 10.). However, [^{32}P]PIP_2 was hardly detected under this condition (Fig. 11.). Characteristics of

Table 1. The effect of glucuronate on incorporation of ^{3}H into $Ins(1,4,5)P_3$

Preculture conditions	Labeling conditions	Radioactivity of [^{3}H]$InsP_3$ (cpm)
-Inositol	None	25,164 (100%)
-Inositol	+Glucuronate	42,929 (171)
-Inositol, +Glucuronate (2 weeks)	+Glucuronate	20,999 (83)
-Inositol, +Glucuronate (2 weeks)	None	70,164 (279)

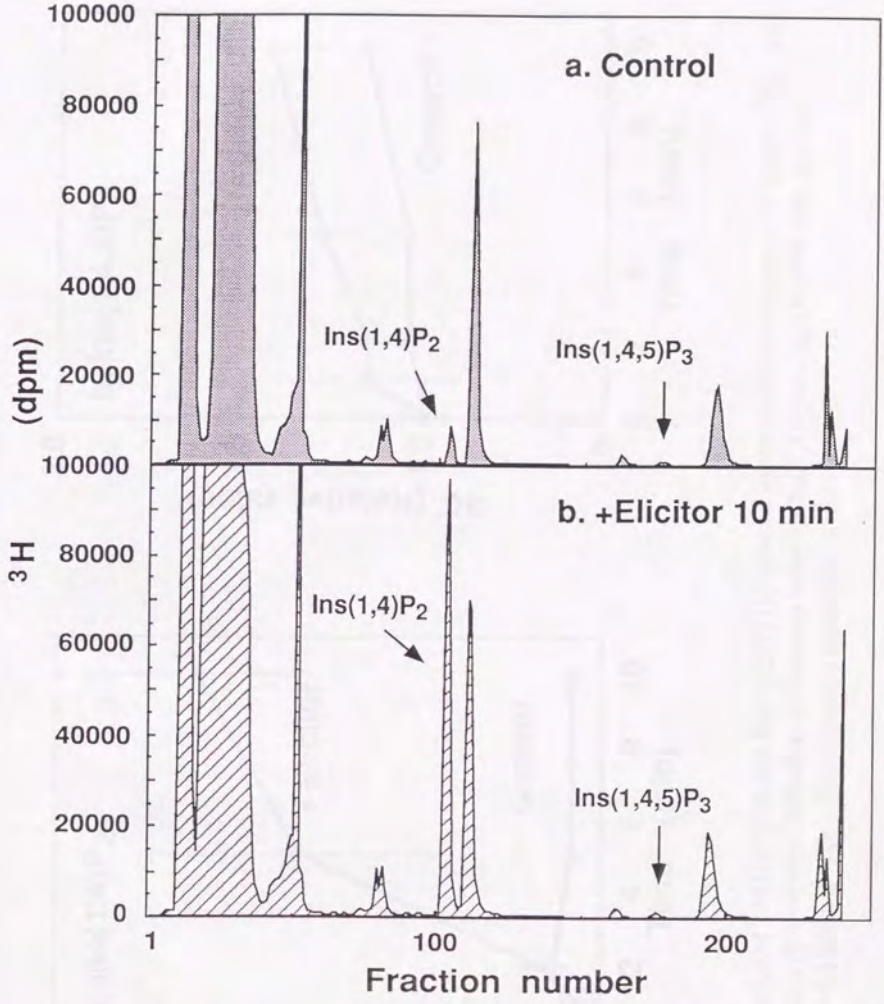


Fig. 7. Change of HPLC profiles of [^{3}H]inositol phosphates by the elicitor. a) control, and b) 10 min after addition of the elicitor.

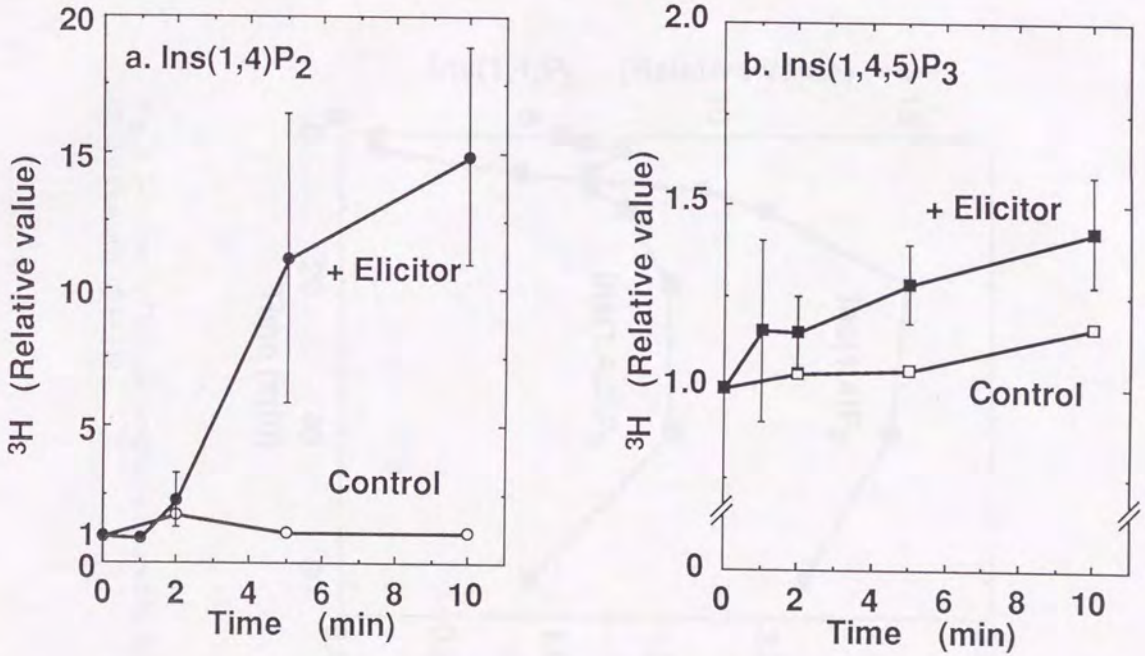


Fig. 8. Formation of $Ins(1,4)P_2$ (a) and $Ins(1,4,5)P_3$ (b) induced by the elicitor treatment up to 10 min. The values before elicitation (0 min) were defined as 1. Standard value for $Ins(1,4)P_2$ are 11,700±900 dpm, and for $Ins(1,4,5)P_3$ are 1,850±250 dpm. Bars represent mean±SE., n=3 to 4.

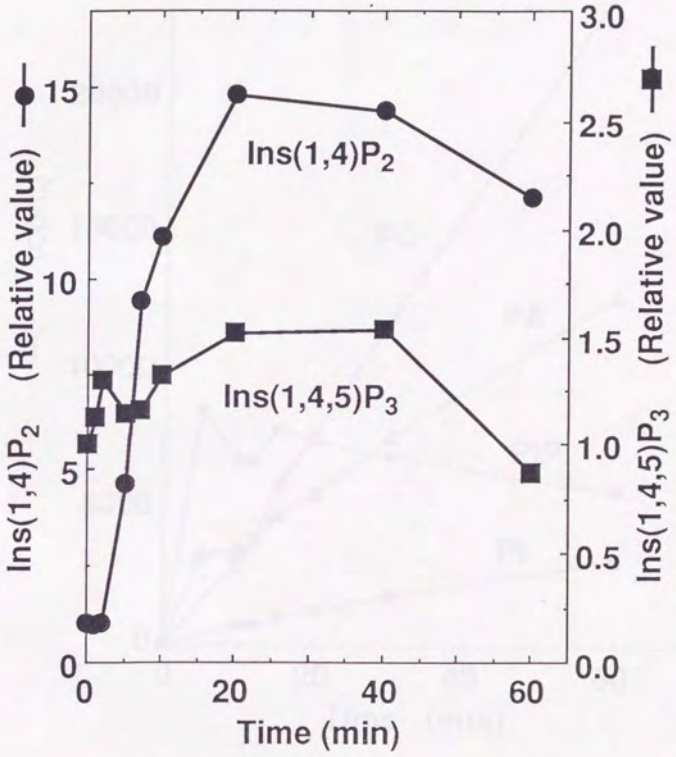


Fig. 9. Formation of inositol phosphates induced by the elicitor treatment up to 1 hr.

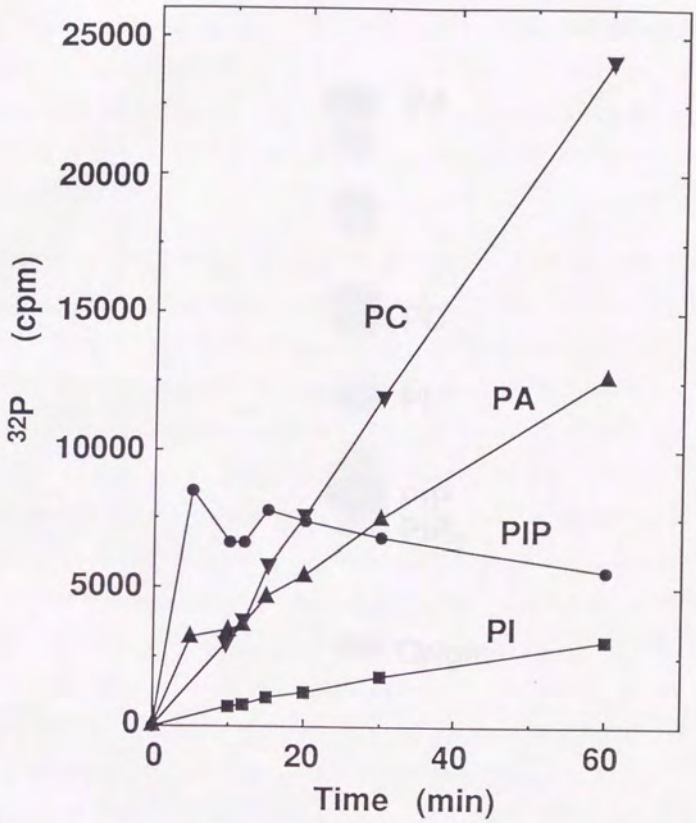


Fig. 10. Incorporation of ^{32}P into phospholipids. This figure shows the typical one of three individual experiments.

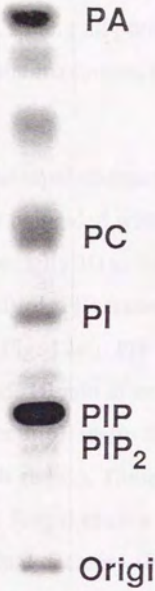


Fig. 11. Autoradiogram of TLC separating [^{32}P]phospholipids.

their labeling patterns may reflect their biosynthetic pathways. ^{32}P labels in PIP and PA were transferred from endogenous [^{32}P]ATP by PI and DG kinases, respectively (see Fig. 2.), while those in PI and PC were incorporated during their *de novo* synthesis. It is of interest that up to 10 min ^{32}P incorporation into PIP was the highest among all phospholipids and then decreased gradually to about 70 % of the maximum, though it is not so abundant in plant cells.

Elicitor-stimulated cells also showed rapid changes in [^{32}P]phospholipids, and the results obtained approximately coincided with those expected (Fig. 12.). Significant changes were observed especially 10 to 50 min after the addition of the elicitor. When compared to the control, PIP started to decrease most markedly 10 min after the elicitation (Fig. 12a.). PIP reached 40 % of the control after 45 min. On the other hand, 10 min after elicitation PA and PI increased to 125 % and 145 % of the control, respectively, then decreased slightly, and stayed in plateau (Fig. 12b and c.). These results suggest that the PI turnover cycle was triggered by the fungal elicitor. PC which is not directly involved in PI turnover showed a gradual decrease (Fig. 12d.). As the PI turnover cycle includes DG and PA which were also precursors of PC, PC synthesis might be affected by the stimulation of PI turnover. Another possibility is that the elicitor directly affects *de novo* synthesis of each phospholipid or also stimulates PC hydrolysis. If the increase of Ins(1,4)P_2 was result from hydrolysis of PIP, the decrease of PIP should begin earlier than the observed decrease, however, such correlation was not observed (see Fig. 8a and 12a.). To investigate the reason for this time gap, *in vitro* study using the isolated plasma membrane was performed. The assay mixture of PI kinase contained EGTA, because PI kinase is inhibited by Ca^{2+}, and *vice versa* Ca^{2+}-dependent PLC was inhibited by EGTA (Kamada and Muto, 1992). In

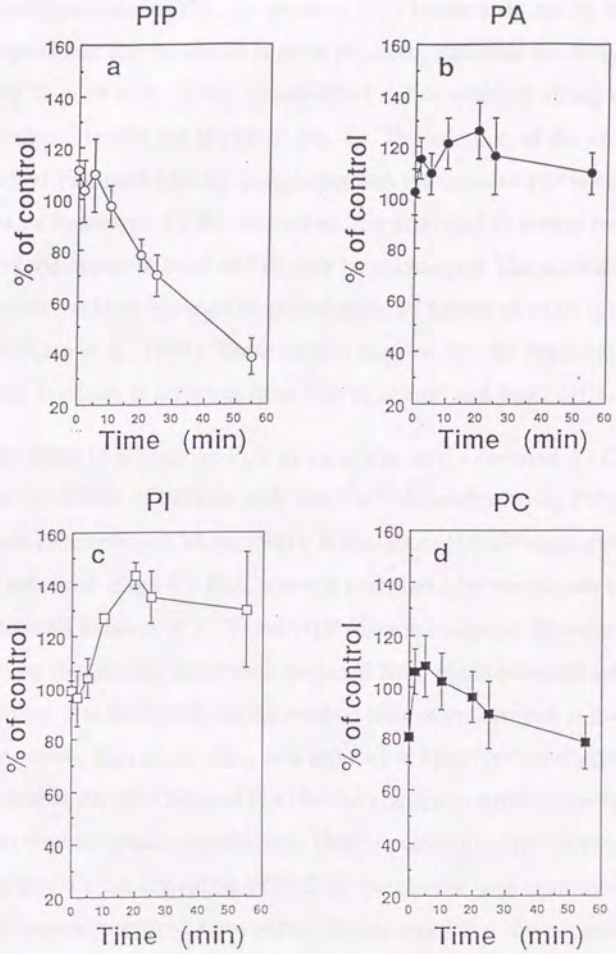


Fig. 12. The effect of the elicitor on individual phospholipids. The changes of ^{32}P incorporation into phospholipids induced by the elicitor are shown as % of the control cells. The ^{32}P incorporation into the control is shown in Fig. 10. a), PIP; b), PA; c), PI; and d), PC.

this condition, labeled PIP, the product of PI kinase may not be further hydrolyzed and this condition is more physiological than the assay condition used by Toyoda et al. (1992) as described in Introduction of this chapter. The experimental results are shown in Fig. 13. The addition of the elicitor stimulated PI kinase activity, suggesting that synthesis of PIP is stimulated as well as its hydrolysis by the elicitation. The activated PI kinase may supplement PIP and the apparent level of PIP may be unchanged. The activation of PI kinase has not been reported in animal cells. PI kinase of yeast is activated by cAMP (Kato et al., 1989). These studies suggest that the regulation of PI turnover in plants is different from that in animal and yeast cells.

The effect of elicitor on PLC *in vitro* was next examined. PLC in the plasma membrane of tobacco cells was Ca^{2+}-dependent using PIP_2 as the substrate (Kamada and Muto, 1991), it also showed Ca^{2+}-dependent using PIP as the substrate (Fig.14.). PLC was not stimulated by the elicitor both in the presence and absence of ATP and GTP (data not shown). However, PLC activity in the plasma membrane prepared from elicitor-treated cells was 30 % higher than that isolated from the control cells when assayed at low Ca^{2+} concentration. This stimulation was reduced at high Ca^{2+} concentration. This suggests that the activation of PLC by the elicitation needs some factors present in other than the plasma membrane. Then a possibility that some cytosolic factors mediate the activation of PLC by the elicitor was examined. Ten μg soluble protein prepared from either elicitor-treated or the control cells was added to the PLC reaction mixture, and PLC activity was assayed. Slight activity of PLC was observed in the cytosolic proteins from both cells. The addition of cytosolic proteins into the reaction mixture markedly inhibited PLC activity in the plasma membranes irrespective of Ca^{2+} concentration. Further

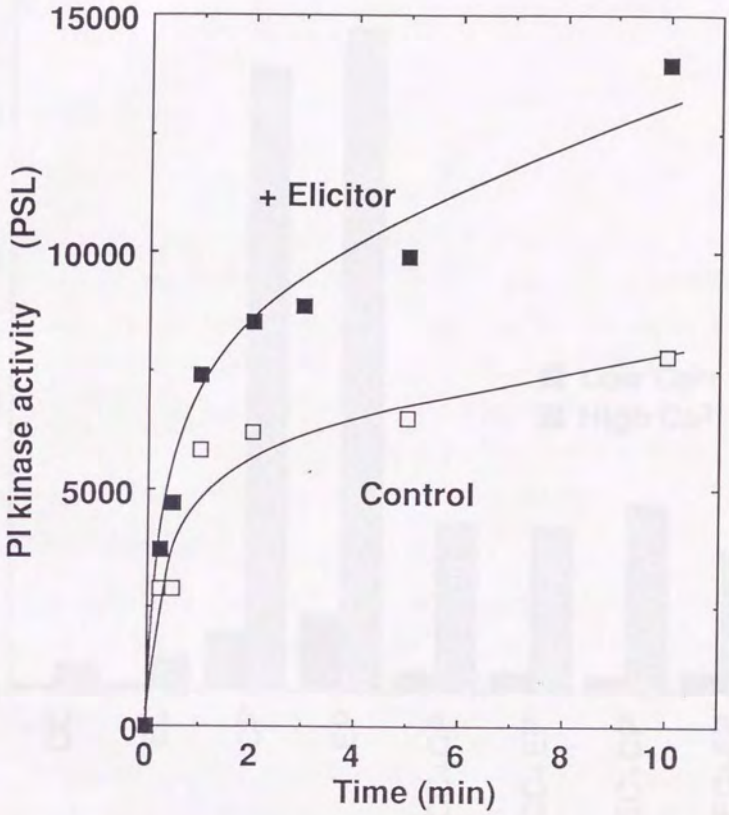


Fig. 13. The activation of PI kinase in vitro by the elicitor. At 0 time the isolated plasma membrane was added to reaction mixture with [γ-^{32}P]ATP in the presence or absence of the elicitor. PSL means dimension of radioactivity used by Bio-Imaging scanner, which is proportional to cpm.

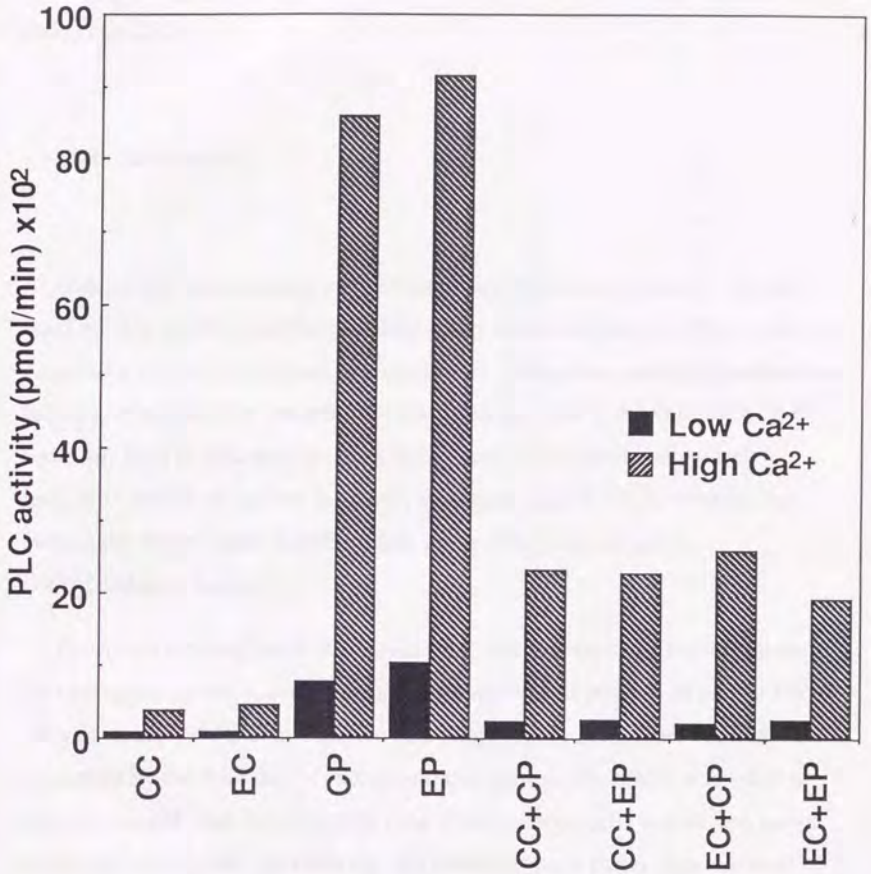


Fig. 14. *In vitro* PLC activity with [^{3}H]PIP as the substrate. Free Ca^{2+} concentrations were adjusted using EGTA-Ca^{2+} buffer, and pCa of low Ca^{2+} and high Ca^{2+} condition were calculated to be 7.3 and 4.4, respectively. CC, 10 μg of cytosolic protein of the control cells; EC, 10 μg of cytosolic protein of the elicited cells; CP, 10 μg of plasma membrane of the control cells; and EP, 10 μg of plasma membrane of the elicited cells. The elicited cells were incubated with the elicitor for 20 min.

addition of the elicitor to the reaction mixture did not activate PLC in both cells (Fig. 15.).

II-4. Discussion

Glucuronate-preculturing method improved the incorporation of ^{3}H into Ins(1,4,5)P_3. HPLC separation established by Dean and Moyer (1987) resolved isomers of inositol phosphates in a single run. After some modification this was satisfactory method for analysis of ^{3}H-labeled Ins(1,4)P_2 and Ins(1,4,5)P_3, however, Ins(1)P was not separated from other [^{3}H]inositol metabolites. Ins(1)P is one of precursors for pectin synthesis. Thus it might be abundant even in the control cells and this might make difficult to detect its elicitor-induced increase.

Phosphate metabolism in BY-2 cells was well characterized by Nagata and his colleagues mainly focusing on phosphate pool and synthesis of plastid DNA (Nagata et al., 1992). They reported that phosphate in the culture medium was consumed by the third day of culture and cytoplasmic phosphate was taken up into the vacuole, that the phosphate pool of the tobacco cells was almost vacant at the stationary phase, and that the cell proliferation is partly dependent on phosphate content of the medium. Therefore, it is assumed that the stationary phase tobacco cells washed with the fresh medium without phosphate continued the stationary phase. However, it is not clear whether the addition of ^{32}Pi would trigger the cell proliferation, because the supply of Pi by the labeling was only micromolar concentration which is much lower than the concentration of phosphate in the standard medium (2.7 mM). Since the addition of ^{32}Pi

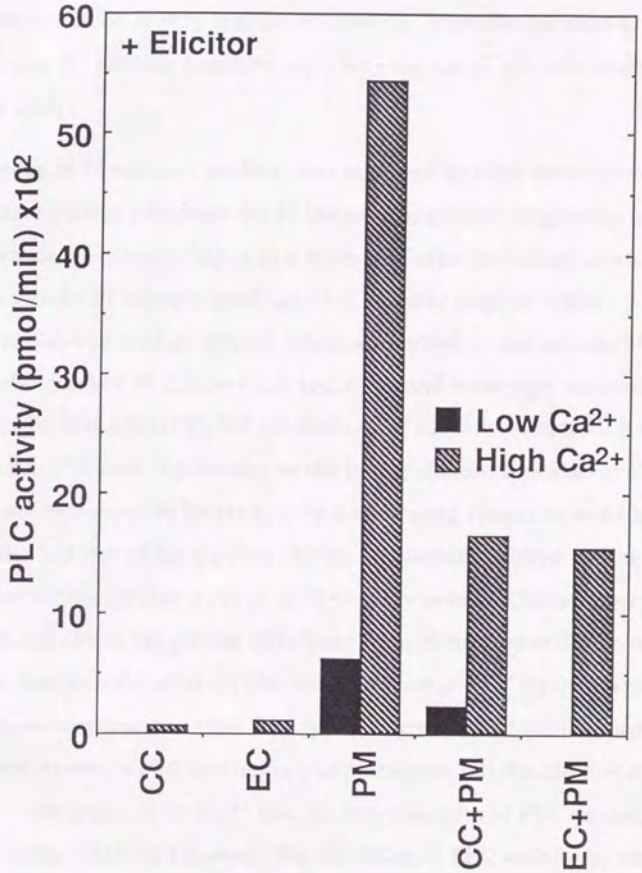


Fig. 15. The effect of the elicitor on PLC activity *in vitro*. PM, isolated plasma membrane. Other abbreviations are as in Fig. 14.

might trigger the cell proliferation and influence on the elicitor signaling, the ^{32}P-labeling condition is very critical. Fortunately, reproducible results were obtained fixing the labeling condition especially the age of the cells throughout the present study.

The change in PI turnover products was observed by elicitation indicating that the fungal elicitor stimulates the PI turnover in tobacco suspension culture cells. Moreover, this change began in a short time after the elicitor treatment suggesting that the PI turnover participates in an early stage of signal transduction pathway in plant defense response. Though it was not identified which metabolite(s) of PI turnover was (were) second messenger molecule(s), it is noteworthy that Ins(1,4)P_2 but not Ins(1,4,5)P_3 greatly increased. It is also interesting that PI kinase is activated by the fungal elicitor. Elucidation of the regulatory mechanism of PI kinase may be a interesting subject to investigate in further study. The use of the purified elicitor or synthetic elicitor will be preferred for further precise study in *in vitro* assay system. On the other hand, though PLC activity in the plasma membrane from elicitor-treated cells was higher than that from the control cells, the activation of PLC by the elicitor could not be demonstrated *in vitro*. The involvement of GTP binding protein and/or protein kinase in PLC activation was postulated, but the addition of ATP, GTP in the presence of Mg^{2+} into the assay mixture of PLC caused no significant change (Data not shown). The inhibition of PLC activity by the soluble protein is very interesting. Activation of PLC by the elicitor may be induced by the release of cytosolic protein factor from PLC. A possibility that it was an artificial effect is not still eliminated. That PLC is Ca^{2+}-dependent should be also considered. If the fungal elicitor induced the increase of cytosolic Ca^{2+} concentration via the activation of Ca^{2+} channel in the plasma

membrane, PLC would be activated. Currently, several investigators are trying to measure cytosolic Ca^{2+} concentration in intact plant cells using fluorescent Ca^{2+} indicators such as Fura-2 and Fluo-3 (Bush and Jones, 1990). The establishment of this technique will be a great help for studying the early stage of PI turnover stimulation in plant cells.

III. Relationship between PAL Induction and PI turnover

Abstract

Relationship between the induction of PAL and the stimulation of the PI turnover by the elicitor was investigated examining the effects of several reagents on these responses. The PAL induction and the stimulation of PI turnover by the elicitor was not affected by pretreatment of the culture with EGTA (10 mM). A23187 (25 μM) did not elicit the induction of PAL and the stimulation of PI turnover. Protein kinase inhibitor K252a and staurosporine (10 μM each) inhibited the induction of PAL and the stimulation of PI turnover by the elicitor when they were added together with the elicitor. Another protein kinase inhibitor H-9 (10 μM) did not inhibit both responses. The facts that several reagents showed the similar effects on the PI turnover and the PAL induction, suggesting that these responses are correlated each other. Furthermore, K252a inhibited PI kinase, and Ca^{2+}-dependent protein kinase (CDPK) in the plasma membrane but did not affect PLC. The effect of K252a on the PAL induction was reduced when it was added 1 hr after the elicitation, suggesting that K252a inhibits the early stage of the elicitor signal transduction. Staurosporine inhibited CDPK in the plasma membrane but did not affect PI kinase and PLC. Inhibitory effect of staurosporine on the PAL induction was maintained for 4 hr after the elicitation, suggesting that it inhibits the late step of the signal transduction. Staurosporine also inhibited the stimulation of PI turnover, but its inhibitory effect was largely reduced when it was added 10 min after the elicitation. This suggests that staurosporine also inhibits the upstream of PI turnover stimulation.

III-1. Introduction

The results described in Chapter I and II propose that PI turnover may be involved in the elicitor signal transduction pathway. To examine this, the relationship between the PI turnover and the PAL induction in tobacco cells was investigated. As Ins(1,4,5)P_3 cannot penetrate the plasma membrane, it was given to the permeabilized cells and induced Ca^{2+} mobilization was proved in animal cells (Streb et al. 1983). However, preparation of permeabilized cells of higher plant has not been established. Then, the effects of several reagents on both the PAL induction and the PI turnover were investigated. The purpose of the experiments in this chapter is to find some association between the PAL induction and the PI turnover and to reveal that the elicitor signal is transmitted by the PI turnover. The interaction of the reagents on them would be investigate in the subsequent experiments. In these following study I chose the increase of Ins(1,4)P_2 as a marker of the stimulation of PI turnover, because it was most out-standing as described in Chapter II.

III-2. Materials and Methods

Materials.

[γ-^{32}P]ATP (111 TBq/mmol) was purchased from New England Nuclear. K252a was purchased from Kyowa Hakko. H-9 was from Seikagaku, and staurosporine and A23187 were from Boehringer Mannheim. Spermidine and PMA were from Sigma. Calyculin A was purchased from Wako. Tobacco cells

were cultured and the fungal elicitor was prepared as described in the previous chapters.

Assays.

The activities of PAL and enzymes of PI turnover, and the level of $Ins(1,4)P_2$ were assayed as described in the previous chapters. All inhibitors were added together with the elicitor to the culture unless otherwise indicated.

Preparation of Crude Nuclear Fraction.

Protoplasts were prepared from 2-day old tobacco suspension culture cells according to Nagata et al. (1981) except that 0.5 M mannitol and 10 mM Tris-Mes (pH 5.8) were used. The protoplasts were washed with and suspended in TAN buffer (Nemoto et al., 1988) with some modifications; 17 % sucrose, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM spermidine, 2 mM DTT, and 0.5 mM PMSF. The protoplasts were then broken by forcing them through nylon mesh with 20-μm pores. NP-40 (final concentration, 0.2 %) was added to the broken protoplasts, and they were centrifuged at 1,000 $\times g$ for 5 min at 0 °C. The resultant pellet which was rich in the nucleus was washed three times by TAN buffer containing 0.2 % NP-40, and the resultant pellet was used as the crude nucleus.

In vitro Phosphorylation of Proteins.

The plasma membrane or the crude nucleus were phosphorylated in the buffer system containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$. Phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP and carried out for 3 min at 30 °C. The reaction was terminated by the addition of

gel electrophoresis sample buffer followed by boiling for 1.5 min. Phosphoproteins were analyzed by electrophoresis in 12.5 % polyacrylamide-SDS gels, according to Laemmli (Laemmli, 1970).

III-3. Results

At first, the effect of dose of the elicitor was examined. PAL induction showed dose dependency as described in Chapter I. Ins(1,4)P_2 also increased depending upon the dose of the elicitor, and its dose dependency looked similar with that of PAL induction (Fig. 16.). This suggests that there are some relationship between the PAL induction and the increase of Ins(1,4)P_2.

To examine the involvement of Ca^{2+} with the PAL induction and the PI turnover, the effects of EGTA and Ca^{2+} ionophore A23187 were given to cells. EGTA (10 mM) was added to tobacco cells 30 min before the elicitor treatment. The fresh culture medium contained 3 mM $CaCl_2$, and free Ca^{2+} concentration after the addition of 10 mM EGTA was calculated to be 2 μM. The induction of PAL and the increase of Ins(1,4)P_2 by the elicitor were not inhibited by EGTA (Fig. 17.). The addition of A23187 alone which causes the artificial increase of cellular Ca^{2+} concentration did not exert any significant influence on the PAL induction or the increase of Ins(1,4)P_2. Stab and Ebel (1987) reported that phytoalexin induction in soybean culture by the fungal elicitor was not inhibited by EGTA and the phytoalexin was induced by A23187 alone, suggesting that Ca^{2+} flux is important for the stimulation of plant defense mechanism. According to them, it seems that the internal Ca^{2+}

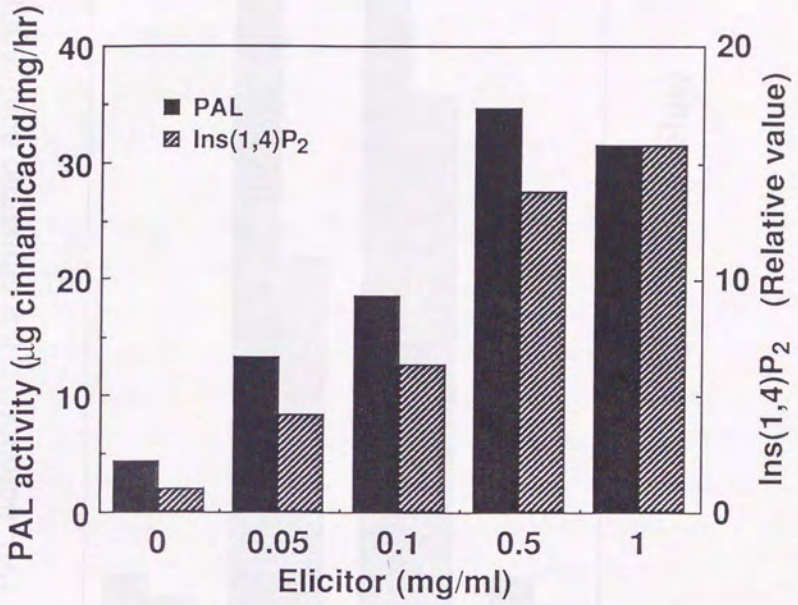


Fig. 16. The effect of elicitor concentration on the induction of PAL activity and the increase of Ins(1,4)P_2. For the assay of PAL activity, the cells were incubated with 1 mg/ml elicitor for 6 hr, and for the analysis of Ins(1,4)P_2, they were incubated for 20 min.

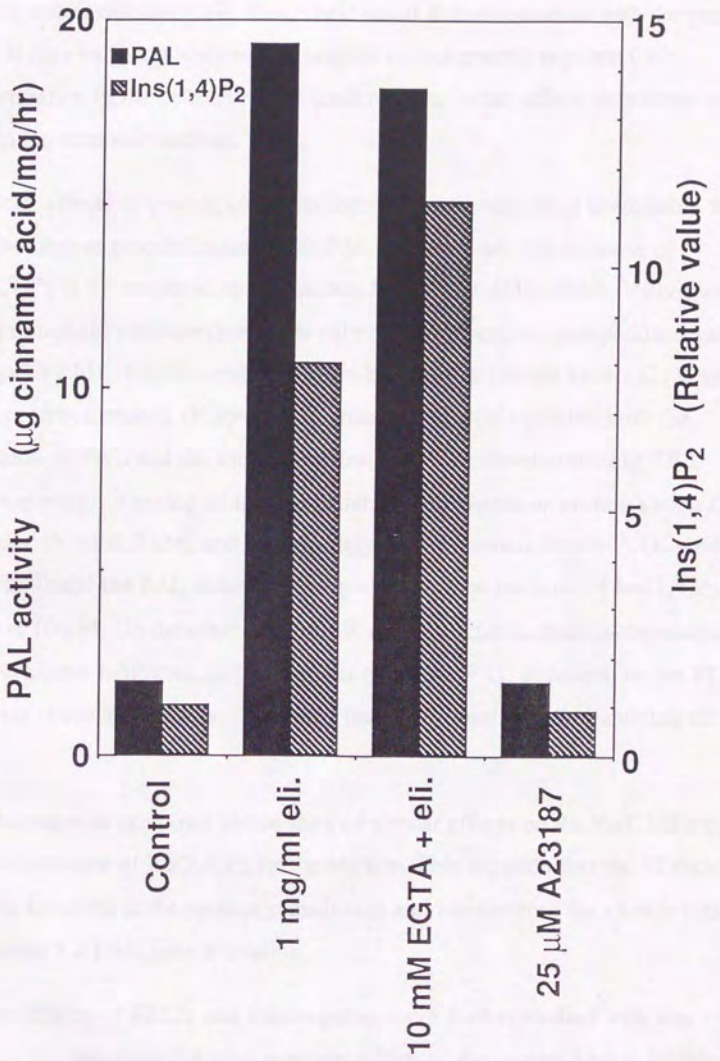


Fig. 17. The effects of EGTA and A23187 on the induction of PAL activity and the increase of Ins(1,4)P_2. 10 mM EGTA was added 30 min before the elicitation. 25 μM A23187 was added at 0 time.

pool is responsible for Ca^{2+} flux. Their result did not coincide with the present data. It may be partly because this reagent cannot exactly regulate Ca^{2+} concentration in the cells and Ca^{2+} concentration in the cells is dependent on that in the external medium.

Next, effects of protein kinase inhibitors were investigated to examine the involvement of protein kinase in the PAL induction and the increase of $Ins(1,4)P_2$ in the response to the elicitor. K252a (10 μM), which firstly found as a calmodulin inhibitor (it inhibits calmodulin-dependent phosphodiesterase (IC_{50}=2.9 μM)), inhibits several protein kinases *e.g.* protein kinase C (IC_{50}=20 nM), protein kinase A (IC_{50}=20 nM), nearly perfectly inhibited both the induction of PAL and the increase of $Ins(1,4)P_2$ by the elicitor (Fig. 18.). Staurosporine, an analog of K252a, which is well known as protein kinase C inhibitor (IC_{50}=2.7 nM) and also strongly inhibits protein kinase A (IC_{50}=8.2 nM), inhibited the PAL induction completely and the increase of $Ins(1,4)P_2$ by 75 % at 10 μM. On the other hand, H- 9, one of cyclic nucleotide-dependent protein kinase inhibitors, did not inhibit either the PAL induction or the PI turnover stimulation. Rather it seemed that H-9 stimulated the induction of PAL.

The reagents examined above showed similar effects on the PAL induction and the increase of $Ins(1,4)P_2$ by the elicitor. This suggests that the PI turnover may be involved in the process transducing and transmitting the elicitor signal to nucleus for PAL gene activation.

The effects of K252a and staurosporine were further studied with this respect. To determine the effective time points of the protein kinase inhibitors on the PAL induction, the inhibitors were added to the cultures at various times after the elicitation. As shown in Table 2, K252a was effective only when it was

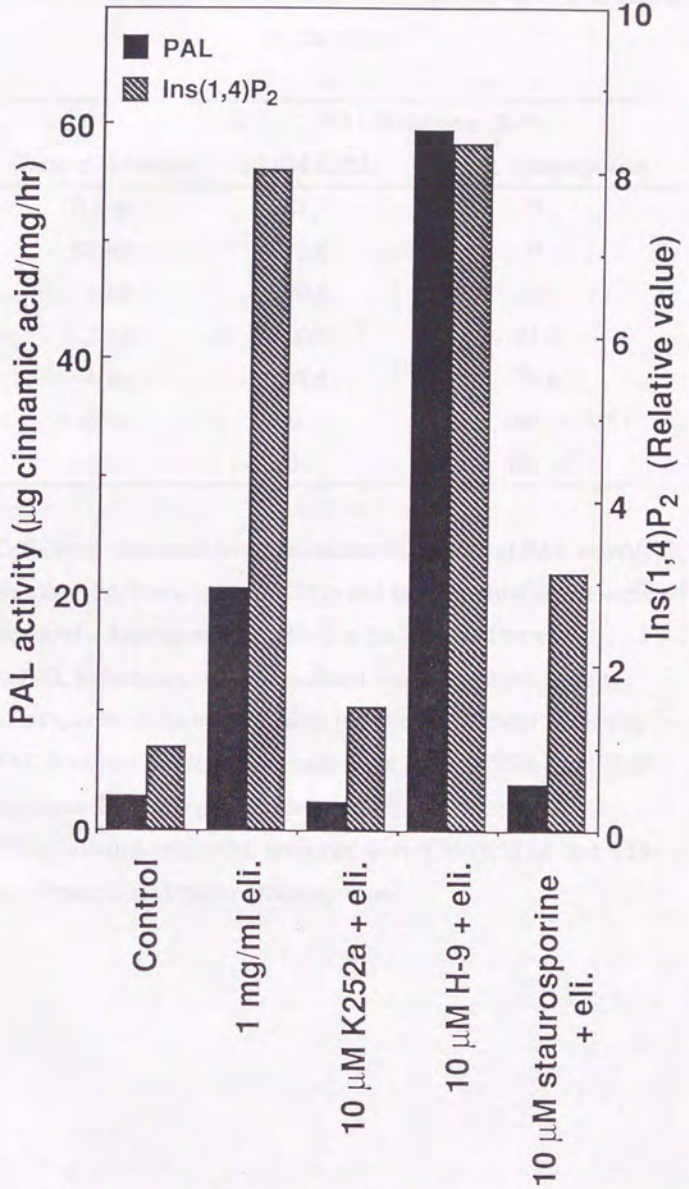


Fig. 18. The effects of protein kinase inhibitors on the induction of PAL activity and the increase of Ins(1,4)P_2. K252a, H-9, and staurosporine were all added at 10 μM together with the elicitor.

Table 2. The Effects of K252a and staurosporine on PAL induction by the elicitor

	PAL Induction (%)*	
Time of Addition	10 μM K252a	10 μM Staurosporine
0 min	11.4	0
30 min	12.6	0
1 hr	49.6	0
2 hr	110.2	21.1
4 hr	101.6	78.6
6 hr	100	100
- eli.**	(0)	(0)

Cells were incubated with the elicitor for 6 hr, and PAL activity was assayed. The effects of K252a and staurosporine were examined separetely. Inhibitors were added at the indicated time.

* PAL induction means the induced increase of PAL activity. Shown as % of the control (with the elicitor, without inhibitor). PAL activities of the control cells were 21.25 (K252a) and 10.83 μg cinnamic acid/mg/hr (staurosporine).

**Non-elicited cells. PAL activities were 3.96 (K252a) and 4.16 μg cinnamic acid/mg/hr (staurosporine).

added within 1 hr after the elicitation. The result indicates that K252a inhibits the early step of the signal transduction. One of candidates for the target of K252a is the PI turnover, because the PI turnover is stimulated by the elicitor in early times. On the other hand, staurosporine inhibited the PAL induction in a different way from K252a. Inhibitory effect of staurosporine was maintained for 4 hr after the elicitation, while in the control cells the PAL induction has already occurred at this time. This suggests that staurosporine may inhibit the later step of the signal transduction, perhaps around PAL gene expression. It is very interesting that the effective time points of K252a and staurosporine were markedly different though they have similar structures. When K252a together with the elicitor was added to the [^{3}H]inositol-labeled cells, the stimulation of PI turnover was inhibited as mentioned above. K252a added to the already-elicited culture also inhibited further increase of Ins(1,4)P_2 (Fig. 19.). If the product(s) of PI turnover mediate the elicitor signal in the cell, and inositol phosphates produced before the addition of K252a are gradually hydrolyzed, it is very reasonable that K252a was effective only at the early stage. Staurosporine also inhibited PI turnover, but its inhibitory effect was largely reduced when it was added 10 min after the elicitation (Fig. 20.). This suggests that staurosporine inhibits also the upstream of PI turnover stimulation.

To identify the enzyme(s) of PI turnover which is sensitive to K252a and staurosporine, a series of *in vitro* studies were done. K252a (10 μM) drastically inhibited PI kinase of tobacco plasma membrane (Fig. 21.), but it did not affect *in vitro* PLC activity, suggesting that at least PI kinase is one candidate of the target of K252a. Staurosporine (10 μM) did not inhibit both enzymes (Fig. 22.). The effects of K252a and staurosporine on protein phosphorylation were next tested. *In situ* protein phosphorylation of the plasma membrane was

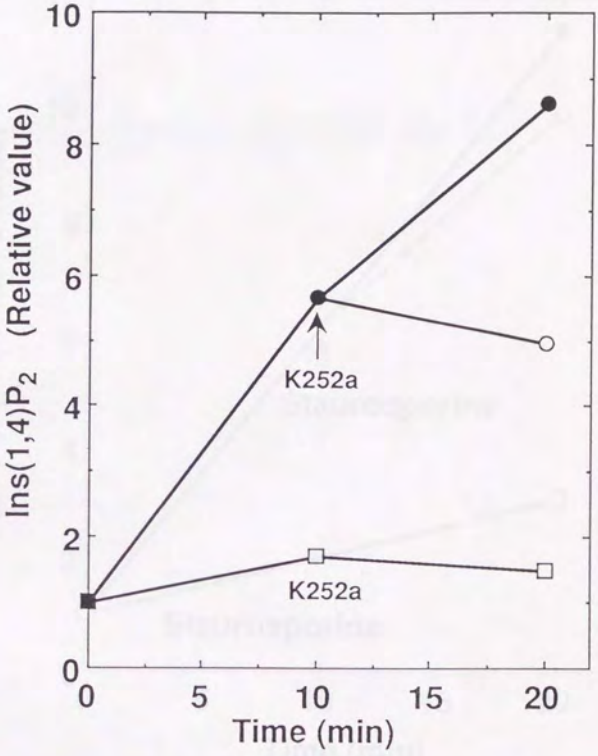


Fig. 19. The inhibition of the increase of $Ins(1,4)P_2$ by 10 μM K252a. The elicitor was added at 0 time. ●, control (elicitor only); □, K252a was added together with the elicitor; ○, K252a was added 10 min after the elicitation (indicated by an arrow).

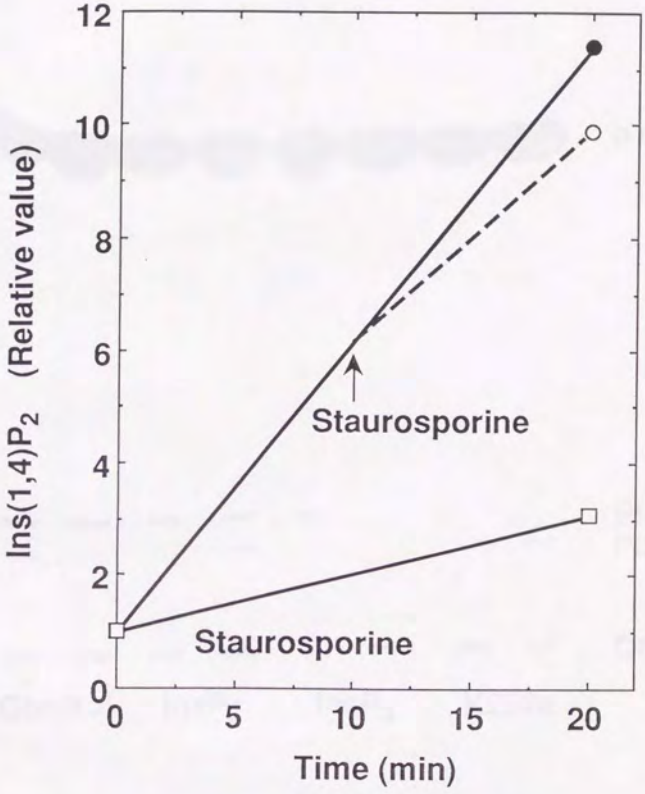


Fig. 20. The inhibition of the increase of $Ins(1,4)P_2$ by 10 μM staurosporine. The elicitor was added at 0 time. ●, the control (the elicitor only); □, staurosporine was added together with the elicitor; ○, staurosporine was added 10 min after the elicitation (indicated by an arrow).

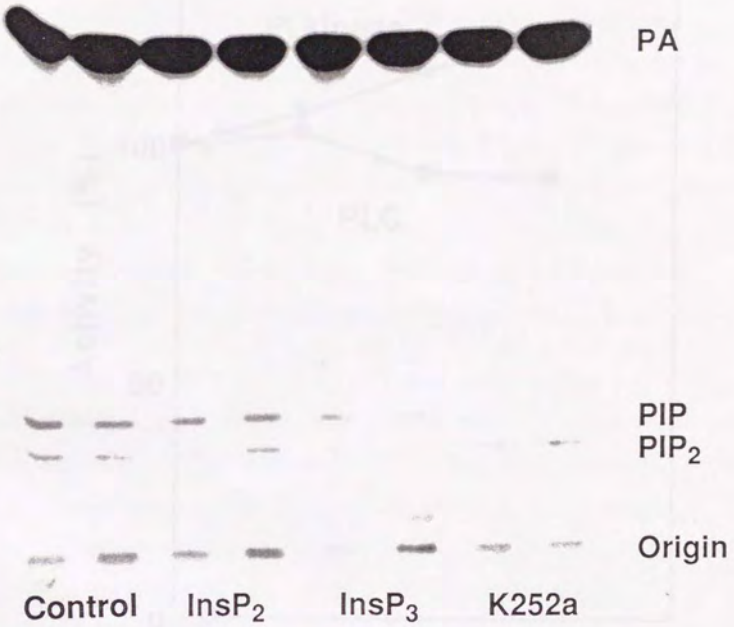


Fig. 21. The effects of K252a and inositol phosphates on PI kinase. 10 μM K252a or 10 μM each inositol phosphates was added. Radioactive lipids were analyzed by TLC.

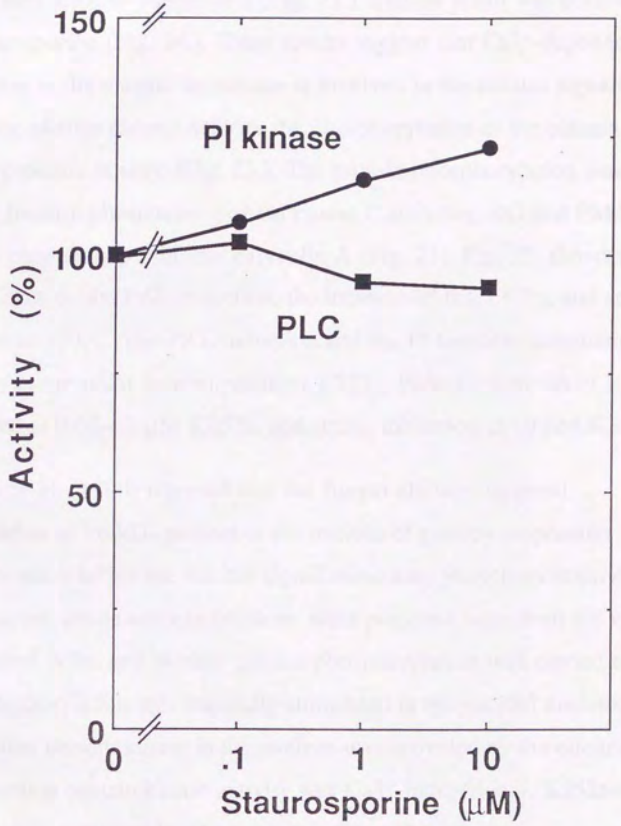


Fig. 22. The effect of staurosporine on PI kinase and PLC activities in the isolated plasma membrane. The activities are shown as % of the control.

inhibited by 10 μM K252a. Most of phosphorylation was inhibited by EGTA suggesting they are Ca^{2+}-dependent (Fig. 23.). Similar result was obtained with 10 μM staurosporine (Fig. 24.). These results suggest that Ca^{2+}-dependent protein kinase in the plasma membrane is involved in the elicitor signaling. However, the elicitor did not activate the phosphorylation of the plasma membrane proteins *in vitro* (Fig. 23.). The protein phosphorylation was not affected by inositol phosphates, protein kinase C activator, DG and PMA, H-9, and protein phosphatase inhibitor calyculin A (Fig. 23). Fig. 25. shows the effect of K252a on the PAL induction, the increase of $Ins(1,4)P_2$, and activities of PI kinase and PLC. The PAL induction and the PI turnover stimulation were inhibited by micromolar concentration of K252a. Partial inhibition of PI kinase was observed at 0.01—1 μM K252a, and strong inhibition at 10 μM K252a.

Dietrich et al. (1990) reported that the fungal elicitor triggered phosphorylation of 26 kDa protein in the nucleus of parsley suspension cultures. To test whether the elicitor signal stimulates phosphorylation of nuclear proteins, crude nuclear fractions were prepared from both the elicited and the control cells, and *in vitro* protein phosphorylation was carried out. Protein phosphorylation was markedly stimulated in the elicited nucleus, suggesting that protein kinase in the nucleus was activated by the elicitor (Fig. 26.). The nuclear protein kinase activity was Ca^{2+}-independent, K252a-resistant and was slightly inhibited by staurosporine (Fig. 27.).

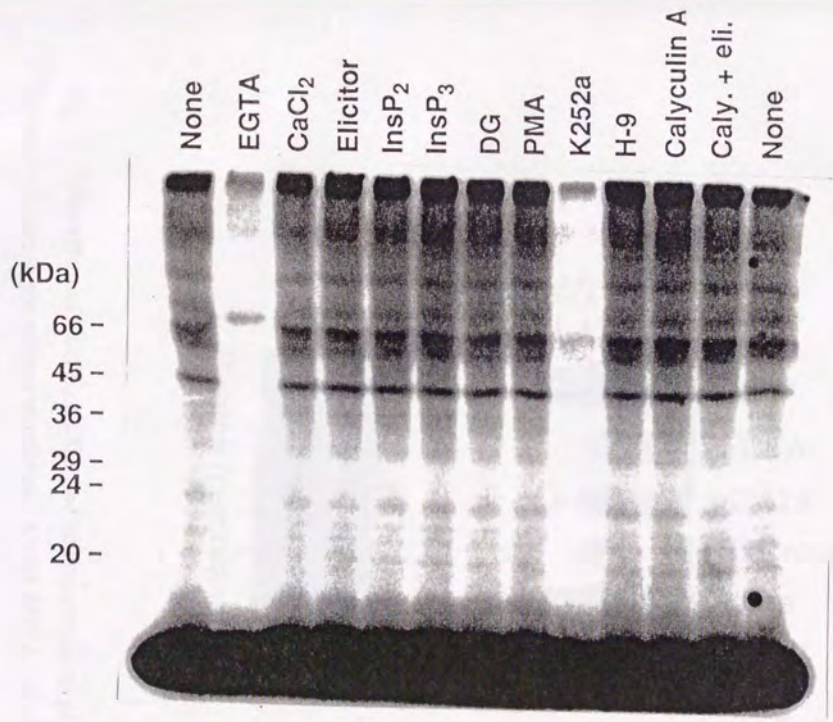


Fig. 23. The effects of various regents on protein phosphorylation of the isolated plasma membrane. The reagents used were; 10 mM EGTA, 0.1 mM $CaCl_2$, 1 mg/ml elicitor, 10 μM $Ins(1,4)P_2$ and $Ins(1,4,5)P_3$, 10 μg/ml DG, 10 μM TPA, K252a, and H-9, 100 nM calyculin A.

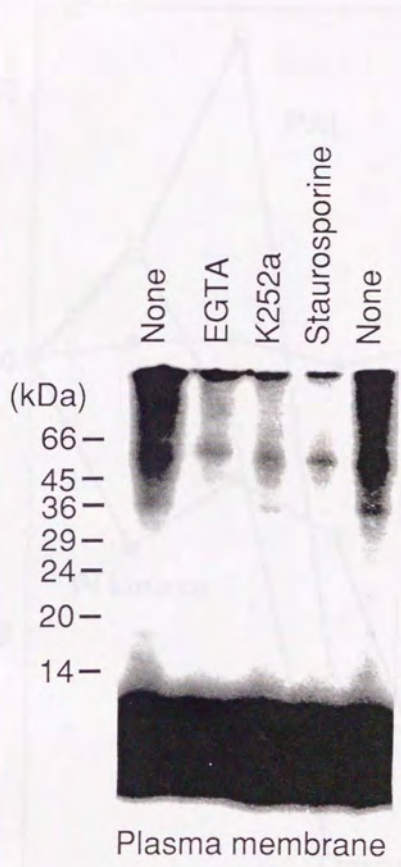


Fig. 24. The effect of EGTA, K252a and staurosporine on protein phosphorylation in the plasma membrane. 5 mM EGTA, 10 μM K252a or 10 μM staurosporine was added to the reaction mixture.

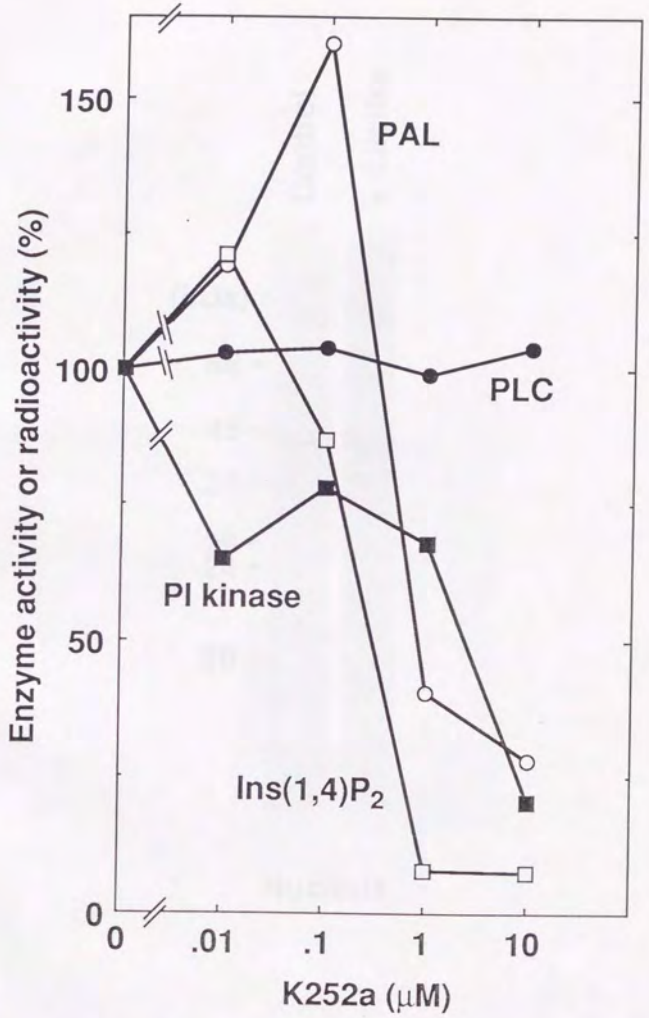


Fig. 25. The effect of K252a on the induction of PAL activity, the formation of Ins(1,4)P_2 in the cell, and PI kinase, PLC activities in the isolated plasma membrane. The activities are shown as % of the control.

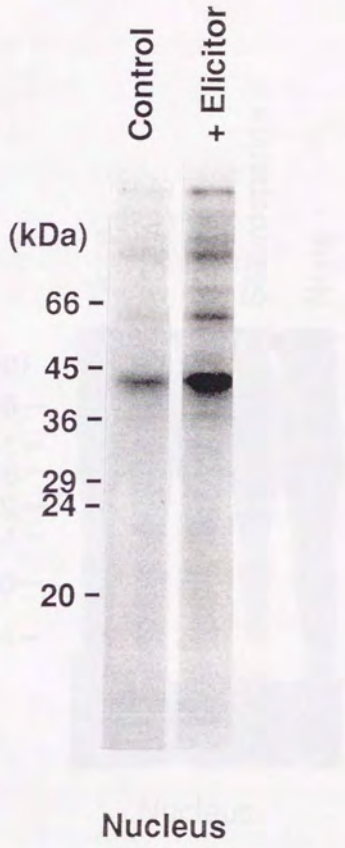


Fig. 26. Protein phosphorylation in the crude nuclear fraction. The nuclear fraction was prepared from the elicited cells (30 min-incubation with the elicitor) or the control cells.

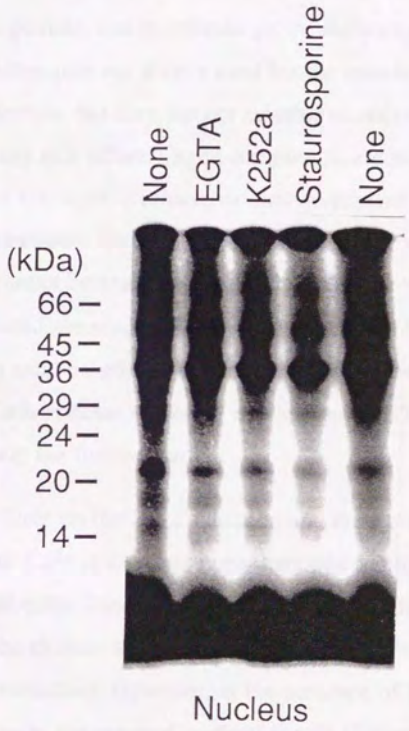


Fig. 27. The effects of EGTA and protein kinase inhibitors on protein phosphorylation in the isolated nuclei. 5 mM EGTA, 10 μM K252a or 10 μM staurosporine was added to the reaction mixture.

III-4. Discussion

Deliveries of inositol phosphates into the cell is very difficult to establish. Several methods are available, for example, electroporation, microinjection, use of caged compounds, and membrane permeabilization. Electroporation and microinjection techniques are widely used for the transfer of large molecules like plasmid or protein, but they are not suitable to observe quick responses of the cell without any side effect. Caged compounds are powerful tool, however, they need flash of UV light to release the active reagents from the "cage". Only limited caged compounds ($Ins(1,4,5)P_3$, Ca^{2+}, and ATP *etc.*) are commercially available. So, it cannot be examined with this technique whether $Ins(1,4)P_2$ functions as a second messenger. Recently, Mimura and Shimmen (1992) reported that they established membrane permeabilization using suspension culture cells of *Catharanthus roseus*. If it is applied to BY-2 cells, this method will be a great help for further study.

The effect of Ca^{2+} on the PAL induction and the PI turnover is very important, because Ca^{2+} is a second messenger and tightly associated with PI turnover in animal cells. Treatment of EGTA did not affect the response of tobacco cells to the elicitor, suggesting that external Ca^{2+} is not needed for the elicitor signal transduction. However, in the presence of 10 mM EGTA free Ca^{2+} concentration in the external medium is still 10 times higher than in the control. In addition, there are abundant Ca^{2+} bound to cell wall. These facts indicate that examination of the effect of EGTA alone is not sufficient. Similar consideration should be given to the effect of A23187, too. Recently, the presence of voltage-dependent Ca^{2+} channels were proven in the plasma membranes and the vacuolar membranes of plants by patch clump technique

(Schroeder and Thuleau, 1991, Johannes et al.,1992, Pantoja et al., 1992, Ping et al., 1992). It is very exciting to examine the effect of the elicitor or inositol phosphates on these channels.

The induction of PAL and the stimulation of PI turnover by the elicitor were inhibited by protein kinase inhibitors strongly suggesting that some protein kinase(s) are involved in the elicitor signaling. Study of plant protein kinases has currently progressed. It is very interesting that neither protein kinase A nor C has been reported so far. Ca^{2+}-dependent protein kinase (CDPK) which is unique in plant cells, is assumed to regulate many cellular responses of plants in a Ca^{2+}-dependent manner. CDPK in the plasma membrane of oat has been reported (Schaller et al., 1992). Protein kinase in tobacco plasma membrane was also Ca^{2+}-dependent. K252a inhibited the early stage of signal transduction of the PAL induction, and also PI kinase and CDPK in the plasma membrane. These facts suggest that PI turnover and CDPK in the plasma membrane are involved in the signal transduction. It is possible that the activated CDPK by the elicitor signal subsequently activates PI kinase. But it is unlikely that the CDPK regulates PI kinase, because staurosporine inhibited CDPK but not PI kinase. Staurosporine inhibited the initiation of the elicitor-induced increase of $Ins(1,4)P_2$ but not the further increase of $Ins(1,4)P_2$. On the other hand, the inhibitory effect of staurosporine on the PAL induction continued during the PAL induction experiment. This suggests that staurosporine effects on two steps of the elicitor signal transduction. One target of staurosporine may lie in the early step of the stimulation process of PI turnover, and the other may position in or near the activation process of PAL gene in the nucleus. Recently it was revealed that some gene expression is regulated by the phosphorylation of transcriptional elements (Hunter and Karin, 1992). Roux and his colleagues indicated that there exist several protein

kinases in the nucleus of plants (Li et al., 1991, Li and Roux, 1992). It is possible that the expression of PAL gene is regulated by staurosporine-sensitive protein kinase, though the inhibition of nuclear protein phosphorylation by staurosporine was not observed in the present study. Phosphoproteins related to the regulation of gene expression may not be abundant proteins in the nucleus and thus may not be easily detected by one-dimensional electrophoresis. Boller and his colleagues observed that 500 nM K252a inhibited the response of tomato cells to fungal elicitors *in vivo* and their microsomal protein kinase *in vitro* (Felix et al., 1991, Grosskopf et al., 1990). Katsuta and Shibaoka (1992) reported that staurosporine and K252a inhibit differently the change of the preprophase band in tobacco BY-2 cells. They concluded that the effects of these reagents were targeted on protein kinase(s), based on only that K252a and staurosporine are well known as protein kinase inhibitors. It should be noted that the effective concentrations of the protein kinase inhibitors in these studies are much higher than concentrations required for the inhibition of protein kinases of animal cells. The concentrations of protein kinase inhibitor(s) required for the inhibition of plant protein kinases are variable; IC_{50} (K252a) and IC_{50} (staurosporine) for CDPK of pea nuclei were 50 nM and 8 nM, respectively (Li et al., 1991), while those for *Dunaliella* were 5 μM (Yuasa and Muto, 1992) and 1 μM (Yuasa and Muto, personl communication), respectively. Results obtained using protein kinase inhibitors thus should be carefully evaluated.

General Discussion

The present study provided evidences that the fungal elicitor stimulates the immediate PI turnover before the induction of PAL activity. This suggests that the PI turnover is involved in the transduction of the elicitor signal. However, the stimulated PI turnover in tobacco cells seems different from that in animal cells. Especially, Ins(1,4)P_2 greatly increased upon the elicitor stimulation, whereas Ins(1,4,5)P_3 increased slightly. It is likely that Ins(1,4)P_2 acts as a second messenger, but no evidence supporting this has been obtained. It is still worth to examine whether the small increase of Ins(1,4,5)P_3 is sufficient to release Ca^{2+} from the internal Ca^{2+} pool as micromolar concentrations of Ins(1,4,5)P_3 were required for *in vitro* Ca^{2+} release. To evaluate the importance of the small increase of Ins(1,4,5)P_3, the volume and shape of cytosolic space and the gradient of Ins(1,4,5)P_3 concentration in cytosol should be determined. DG, the activator of protein kinase C in animal cells, is unlikely to function as a protein kinase activator in plant cells, because protein kinase C has not been found in plant cells and DG is rapidly phosphorylated into PA by DG kinase of which activity is very high in the plant plasma membrane. Not only inositol phosphates, but inositol phospholipids are also candidates for second messengers. Boss and his colleagues (Memon et al., 1989) observed that the addition of inositol phospholipids stimulated ATPase activity in the plasma membrane of carrot cells. They proposed that the decrease of inositol phospholipids causes ionic imbalance and consequently induces cellular

response. However, this could not be demonstrated with the plasma membrane of tobacco cells (data not shown).

The elicitor activated PI kinase but not PLC in the isolated plasma membrane. The examples of *in vitro* activation of PI kinase have been already reported: PI kinase is activated by the elicitor (Toyoda et al., 1992) or auxin (Grabowski et al., 1991). These data suggest that PI kinase is a regulation point in the PI turnover. If this is true, then is PLC activity not really regulated by the elicitor? In the present study, at least three possibilities of the activation mechanism of PLC were proposed. First, since PLC is a Ca^{2+}-dependent enzyme, it will be activated when the cytosolic Ca^{2+} concentration (or at least Ca^{2+} concentration around the plasma membrane) is increased by the elicitation. Second, cytosolic proteins inhibit PLC activity. Then the elicitor may cause the release of inhibitors from PLC, and PLC will be activated. Third, PLC activity was also detected in the cytosolic fraction. Though its specific activity was much lower than that of the plasma membrane, its total activity would be considerably high. Most PLCs purified from mammalian cells were cytosolic, and it is assumed that they translocate to membrane when activated. It is possible that the cytosolic PLC translocate to the plasma membrane when cells perceives the elicitor signal and the hydrolyses of inositol phospholipids are increased. Thus it is necessary to purify plant PLC to demonstrate its translocation.

On the bases of the present study and the study in my master course, a scheme for the elicitor signal transduction is proposed (Fig. 28.). The elicitor bound to its receptor activates the PI turnover. PI kinase is at least one of the enzymes that are activated by the elicitor. I think that the activation of PI

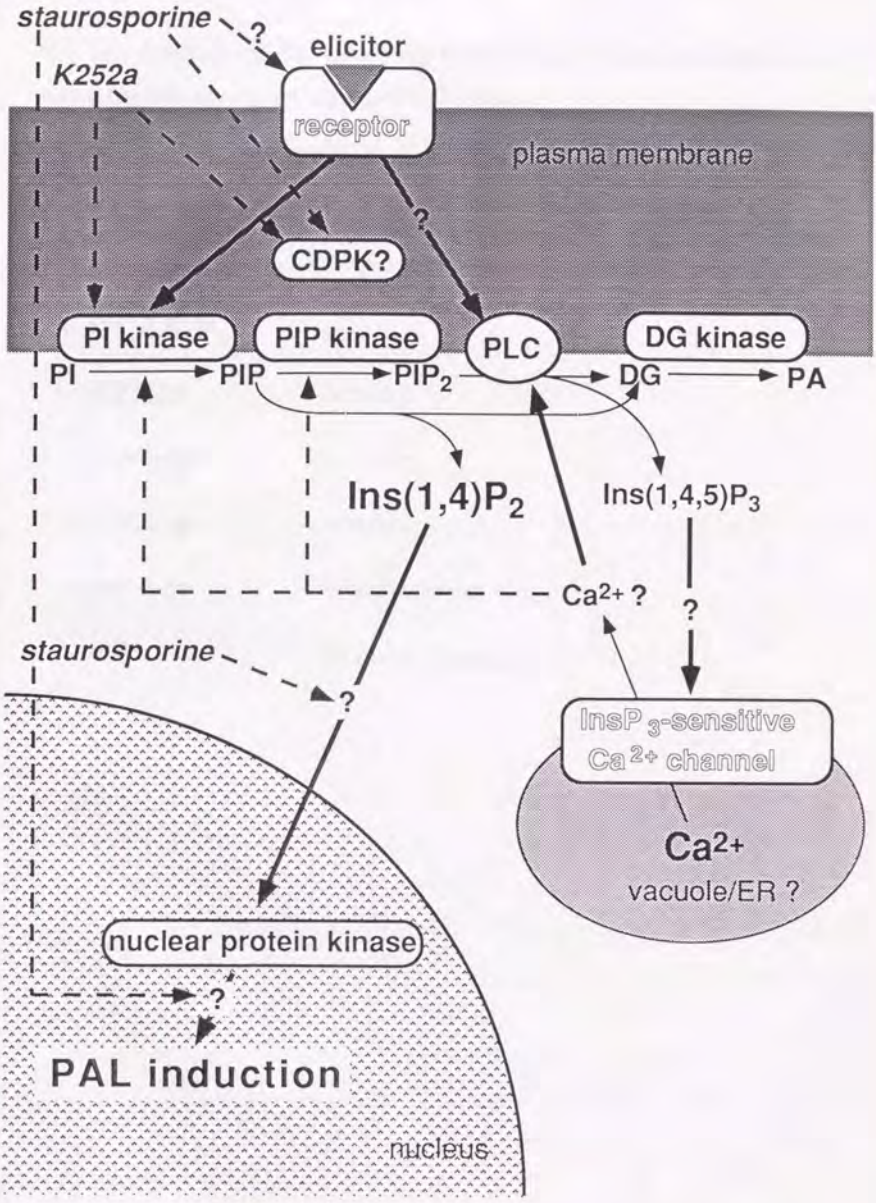
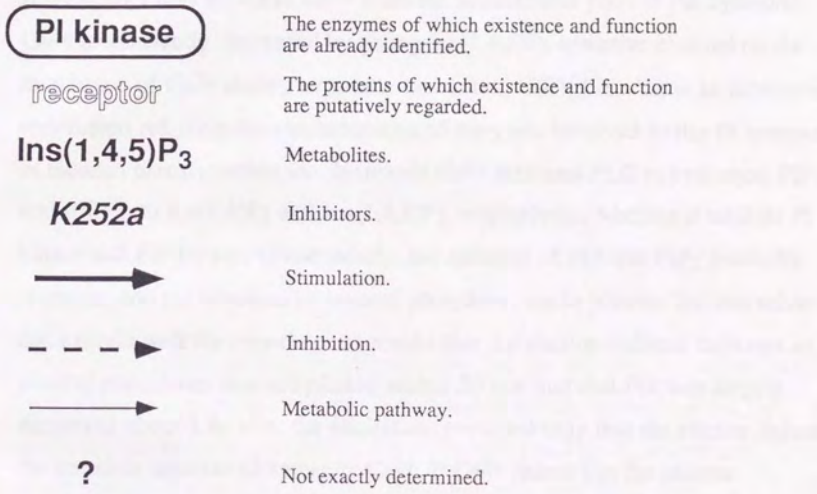

staurosporine
?
elicitor
K252a
receptor
plasma membrane
?
CDPK?
PI kinase
PIP kinase
PLC
DG kinase
PI
PIP
PIP2
DG
PA
Ins(1,4)P2
Ins(1,4,5)P3
Ca2+ ?
?
staurosporine
?
InsP 3-sensitive
Ca 2+ channel
Ca2+
vacuole/ER ?
nuclear protein kinase
?
PAL induction
nucleus

Fig. 28. A scheme of the relationship between the PI turnover and the elicitor signal transduction in the tobacco BY-2 cells.



kinase alone cannot totally explain the great increase of $Ins(1,4)P_2$, though I could not detect *in vitro* activation of PLC by the elicitor ('?' was placed between the receptor and PLC.). Among increased inositol phosphates, $Ins(1,4,5)P_3$ may mobilize Ca^{2+} from the intracellular pool. If the cytosolic Ca^{2+} is transiently increased by gating $Ins(1,4,5)P_3$-sensitive channel on the membrane of Ca^{2+} storing organalle (vacuole or ER), I can draw an interesting conclusion referring the characteristics of enzymes involved in the PI turnover in tobacco plasma membrane. Increased Ca^{2+} activates PLC to hydrolyze PIP and PIP_2 into $Ins(1,4)P_2$ and $Ins(1,4,5)P_3$, respectively, whereas it inhibits PI kinase and PIP kinase. Consequently, the amounts of PIP and PIP_2 gradually decrease, and the increases of inositol phosphates attain plateau. So, this scheme can explain well the experimental results that the elicitor-induced increases of inositol phosphates reached plateau within 20 min and that PIP was largely decreased about 1 hr after the elicitation, provided only that the elicitor induces the transient increase of cytosolic Ca^{2+}. If Ca^{2+} channel in the plasma membrane is activated by the elicitor, it could replace $Ins(1,4,5)P_3$-sensitive Ca^{2+} channel.

$Ins(1,4)P_2$ is a putative second messenger. Its function for example the effects on activities of protein kinase or PI kinase was examined. However, I could not obtain any decisive results. Protein phosphorylation in isolated nucleus was stimulated by the elicitation. Nuclear protein phosphorylation is also a putative step of the elicitor signaling pathway but there is no evidence for that nuclear protein kinase triggers PAL expression. The involvement of Ca^{2+}-dependent protein kinase (CDPK) in the plasma membrane to the activation of PI kinase was denied, because staurosporine inhibited CDPK but not PI kinase. There still remains a possibility that CDPK involves in the elicitor signal transduction.

Two protein kinase inhibitors, K252a and staurosporine inhibit the elicitor signal transduction at the different steps. K252a inhibits the early step, probably events occurring around the plasma membrane, whereas staurosporine inhibits different two steps; an early step probably between the elicitor receptor and the effector (PI kinase and/or PLC) and the later step. As for early step, it is possible that staurosporine-sensitive CDPK mediates the signal transduction between the elicitor receptor and the effector as an activator of the effector. The effective step of staurosporine in the later step is not known. It could be the step before or after the signal reaches the nucleus. It is also necessary to identify whether targets of K252a and staurosporine are really protein kinase.

Development of several experimental method are needed for further study to establish the mechanism of the elicitor signal transduction. They are the analytical method of cytosolic Ca^{2+} concentration, and construction and transformation of PAL promoter-GUS fusion gene into BY-2 cells.

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