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Phosphorylation of Phosphatidylinositols and Production of Lysophospholipid in Pea Plasma Membrane Are Coordinately Regulated by Elicitor and Suppressor from *Mycosphaerella pinodes*

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Effects of elicitor and suppressor from a pea pathogen, *Mycosphaerella pinodes*, on PI metabolism in pea plasma membrane were examined *in vitro*. The elicitor induced rapid phosphorylation of phosphatidylinositols as well as production of lysophospholipid in plasma membranes, but these responses were severely inhibited by the suppressor. These results indicate that a membrane-associated phospholipase A is regulated coordinately by fungal signals, together with PI metabolism, and that it may participate in signal transduction pathways leading to defense responses. To evaluate a possible role of phospholipase A activation in induction of a pea defense response, the effect of free fatty acid on induction of a phytoalexin accumulation was also examined. When pea leaves were treated with linoleic- or linolenic acid, most commonly released in plant cells by phospholipase A, the accumulation of pisatin was induced even in the absence of the elicitor. It is, therefore, conceivable that free fatty acid(s) released from plasma membrane is also implicated in the early stage of elicitor-signal transduction in pea.

Key words : elicitor, pea (*Pisum sativum* L.), phospholipase A, polyphosphoinositide metabolism (PI metabolism), suppressor

Introduction

A pea pathogen, *Mycosphaerella pinodes*, secretes both a glycopeptide elicitor (Mol. wt. > 70 kDa)[15] and glycopeptide suppressors (Mol. wt. < 5 kDa)[23] for defense responses in its pycnospore germination fluid. The elicitor induces diverse host defense responses, such as production of active oxygen species[10] and of infection inhibitor(s)[28], including accumulation of a major phytoalexin of pea, pisatin[22], and some pathogenesis-related (PR) proteins[29]. However, presence of the suppressor from the same fungus inhibits or delays these defenses and induces local susceptibility of pea tissues[18,24]. Recently, it has become clear that the elicitor increases the levels of transcripts for PAL and CHS, key enzymes in the biosynthetic pathway to pisatin, within 1 h, but the concomitant presence of sup-

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Abbreviations
BSA, bovine serum albumin; CHS, chalcone synthase; DAG, diacylglycerol; DAG kinase, diacylglycerol kinase; IP_3, inositol 1,4,5-trisphosphate; PA, phosphatidic acid; LysoPA, lysophosphatidic acid; LysoPIP, lysophosphatidylinositol 4-monophosphate; PAL, phenylalanine ammonia-lyase; PI, phosphatidylinositol; PI kinase, phosphatidylinositol kinase; PI metabolism, polyphosphoinositide metabolism; PIP, phosphatidylinositol 4-monophosphate; PIP kinase, phosphatidylinositol 4-monophosphate kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate.

pressor delays these responses[27]. These findings suggest that the initial action sites of fungal signals may reside upstream of transcriptional activation for defense genes in the nucleus.

Our previous study[25] has shown that PI metabolism in pea epicotyls very rapidly responds to fungal signals. Significant increases in levels of PIP_2 and IP_3 were observed within a few minutes after the start of elicitor-treatment of pea epicotyls, but were effectively negated by the simultaneous addition of suppressor. Moreover, neomycin, an inhibitor of phospholipase C, blocks elicitor-induced increase of IP_3 *in vivo*, as well as accumulation of pisatin in pea epicotyls[25,26]. These results suggest that phospholipid metabolism, especially PI metabolism, may be involved in signal transduction leading to pea defense responses.

In this study, to further confirm the biochemical basis of elicitor-signal transduction, we examine the effects of fungal elicitor and/or suppressor from *M. pinodes* on PI metabolism, using an isolated plasma membrane fraction from pea epicotyls. The physiological relevance of phospholipase A during the early stage of elicitor-signal transduction is also discussed.

Materials and Methods

Chemicals — PI, PIP, PIP_2, PA, LysoPA and fatty acids (linoleic acid and linolenic acid) were purchased from Sigma Chemical Co. [γ-^{32}P] ATP (6000 Ci/mmol) was obtained from Amersham. Primuline was obtained from Tokyo Kasei Co. Other chemicals were from Wako Pure Chemical Inc.

Preparation of elicitor and suppressor from *Mycosphaerella pinodes* — Elicitor and suppressor were prepared from germination fluid of pycnospores of *Mycosphaerella pinodes* (Berk. et Blox.) Verstergren strain OMP-1 (IFO-30342, ATCC-42741) as described previously[22].

Preparation of plasma membrane fraction — Seeds of pea (*Pisum sativum* L., cv. Midoriusui) were sown on moistened vermiculite in a plastic container and grown in darkness at 22 ± 2℃. The plasma membrane fraction was prepared from 9-day-old etiolated epicotyls with an aqueous two-phase partitioning as described previously[30]. The protein content of the fraction was determined, with BSA as the standard, by the dye-binding method with a kit from Bio-Rad.

Phosphorylation of lipids *in vitro* — To confirm lipid phosphorylation *in vitro*, the total volume of reaction mixture was adjusted to 50 μl containing 20 mM Tris/MES (pH 6.5), 15 mM $MgSO_4$, 100 μM GTP, 20 nM [γ-^{32}P] ATP (specific activity, 222 MBq/μmol), 50 μg of plasma membrane fraction and the respective solution of elicitor (100 μg/ml, glucose equiv.) and/or suppressor (100 μg/ml, BSA equiv.). The reaction was initiated by the addition of the plasma membrane fraction, incubated on ice for given times (as described in the legends to Figures), and stopped with 0.2 ml of chilled chloroform/methanol (v/v=1/2).

Extraction and analysis of phosphorylated lipids — Phosphorylated lipids were extracted from plasma membranes that had been treated with elicitor and/or suppressor, according to the method described previously[26]. The lipid fraction was spotted onto a silica gel thin-layer plate (LK5D; Whatman) and the plate was developed with a mixture of chloroform, methanol, 32% ammonia and water (86/76/6/18, by vol.). Phospholipids were visualized by spraying of a 0.02 % solution of primuline in 80 % acetone. Authentic standards were spotted on the same plate and the incorporation of radioactivity from [γ-^{32}P] ATP into PIP, PIP_2 and LysoPA was determined with a Bio-imaging scanner system (Bas 2000 system; Fujix).

Determination of pisatin accumulated in pea leaves — An assay for phytoalexin accumulation was carried out with pea leaves, according to the method by Masuda *et al.*[14]. The detached leaves (6 × 6 mm) were placed on 30 μl of test solution, with the cut surface in contact with the liquid in

each case, and incubated for 18 h. The amount of pisatin accumulated in the tissues was determined by HPLC as described previously[14].

Results and Discussion

In plant cells as well as mammal cells, PI metabolism has been implicated in transduction of diverse extracellular signals such as plant hormones[6,31], light[16,17], hypoosmotic shock[5] and cell-wall degrading enzymes[3], and the components of the pathway are ubiquitously present. This pathway involves sequential phosphorylation of phosphatidylinositols by lipid kinases and subsequent hydrolysis of PIP_2 by inositol-specific phospholipase C, resulting in production of two functional second messengers such as IP_3 and DAG. Although involvement of PI metabolism during induction of a defense response has been described in several plant species[9,11,13,19,25,26], there are few reports that the early signaling event has been directly measured. In this study, we used an *in vitro* system with isolated plasma membranes, to investigate the early stage of signal transduction involved in pea defense responses.

Time course study for phosphorylation of endogenous lipids in isolated plasma membrane fraction showed that the incorporation of radioactivity from [γ-^{32}P] ATP into PIP and PIP_2 was simultaneously detectable, within 5 s, and increased during incubation (Fig. 1). Moreover, formation of ^{32}P-labeled LysoPA, probably generated from [^{32}P] PA, showed the presence of phospholipase A activity in pea plasma membranes (Fig. 2). The results indicate that, in isolated plasma membrane fraction, there exists endogenous substrates for PI-, PIP kinase and DAG kinase. When the phosphorylation was carried out in the presence of the elicitor, the incorporation of radioactivity into PIP and PIP_2 was enhanced as compared to the control (addition of distilled water). Even at 5 s after elicitor-treatment, the rate of incorporation was about 2

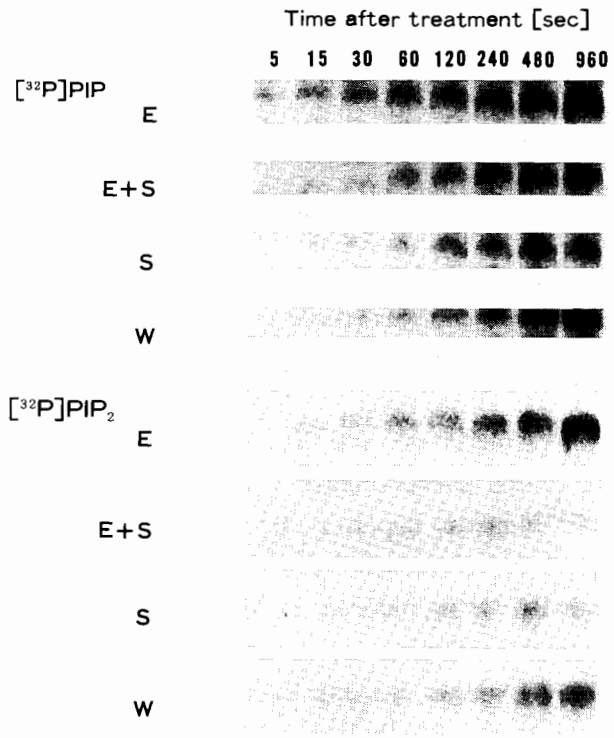

Fig. 1 Phosphorylation *in vitro* of phosphatidylinositols in isolated pea plasma membranes in response to fungal signals. Plasma membrane fractions were treated with the elicitor alone (E), elicitor plus suppressor (E+S), suppressor alone (S) or distilled water (as control; W) in the presence of [γ-^{32}P] ATP. Phosphorylated lipids (PIP and PIP_2) were extracted at 5, 15, 30, 60, 120, 240, 480 and 960 s after the start of incubation, and separated on a silica-gel plate. Radiolabeled lipids were visualized by a Bio-imaging scanner system (Bas 2000 system; Fujix).

-fold higher than those of the control (Fig. 1). However, the concomitant presence of suppressor with the elicitor inhibited such phosphorylation in plasma membranes (Fig. 1). In particular, phosphorylation of PIP to PIP_2 was severely reduced in the presence of suppressor. In a previous study[25], we showed that a rapid and transient increase in level of PIP_2 was induced in elicited pea tissues, prior to accumulation of IP_3. Together with this finding, our present data conclude that phosphorylation of phosphatidylinositols by lipid kinases precedes an activation of phospholipase C.

In the present study, it was also found that a

membrane-associated phospholipase A activity responded to fungal signals from *M. pinodes*. The presence of elicitor induced production of ^{32}P-labeled LysoPA in pea plasma membranes (Fig. 2). In addition, production of ^{32}P-labeled LysoPIP, probably generated from [^{32}P] PIP, was also increased by the elicitor-treatment (not shown). In contrast, these responses were effectively inhibited by the addition of suppressor (Fig. 2). These results indicate that the elicitor also stimulates activity of a membrane-associated phospholipase A as well as those of lipid kinases. Lapetina *et al.*[12] reported that activation of phospholipase A_2, that prefers PIP and PA, was induced in thrombin-stimulated platelets following activation of PI metabolism. Taken together, it seems likely that a membrane-associated phospholipase A is coordinately regulated by fungal signals, together with lipid kinase(s).

Phospholipase A is a lipolytic enzyme that catalyzes hydrolysis of the ester bond at *sn*-2 position of phospholipids, to generate a free fatty acid and the corresponding lysophospholipid. Our present data, that production of LysoPA in plasma membranes increased in response to elicitor, suggest the liberation of free fatty acid(s) during activation of PI metabolism (Fig. 2). To further evaluate a possible role of phospholipase A activation in induction of a pea defense response, the effect of fatty acid on induction of phytoalexin accumulation was also examined. When pea leaves were treated with a solution of linoleic- (18 : 2) or linolenic acid (18 : 3), commonly released in plant cells by phospholipase A, the accumulation of pisatin was induced in a dose-dependent manner, even in the absence of the elicitor (Fig. 3). In plant cells, fatty acids generated from plasma membrane have been shown to be peroxidized by lipooxygenase to generate precursors of jasmonic acid that regulates induc-

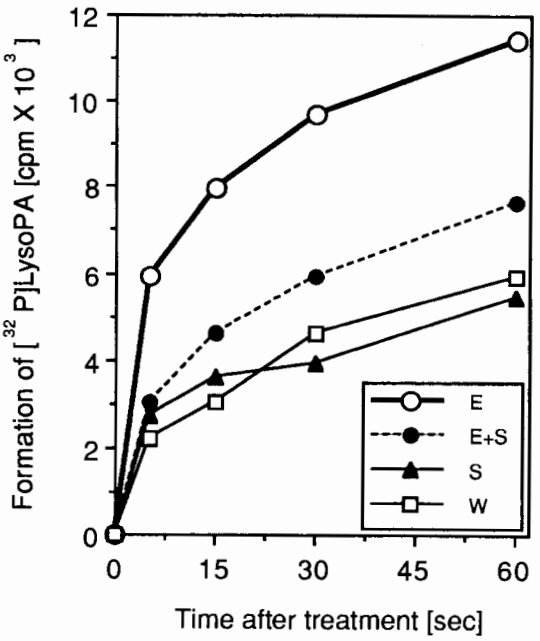

Fig. 2 Production of ^{32}P-labeled LysoPA in pea plasma membranes in response to fungal signals. Plasma membrane fractions were treated with the elicitor and/or suppressor, as described above, and the radioactivity of [^{32}P] LysoPA was determined, by a Bio-imaging scanner system, at 5, 15, 30 and 60 s after the start of incubation.

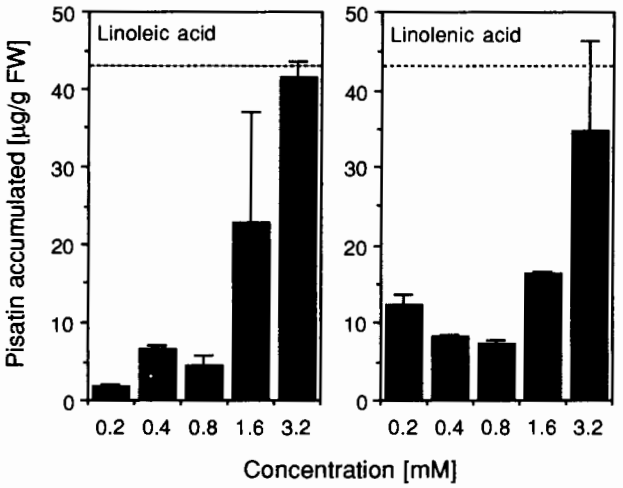

Fig. 3 Effects of exogenous fatty acids on the accumulation of pisatin in pea leaves. Detached leaves were treated with a solution of linoleic- (18:2) or linolenic acid (18:3) at the concentrations of 0.2 to 3.2 mM in the absence of fungal elicitor. Each fatty acid was suspended in 1% solution of dimethylformamide (DMF). Each of the data represents a mean with S.D. from three leaves. Broken line indicates the amount of pisatin accumulated in pea leaves treated with fungal elicitor (500 μg/ml). No accumulation of pisatin was observed in leaves that had been treated with 1% DMF (as solvent control).

tion of defense genes[7,8]. In slices of potato tuber inoculated with an incompatible race of *Phytophthora infestans*, activation of phospholipase A_2 was induced with subsequent activation of a membrane-bound lipooxygenase[4]. Furthermore, some peroxidized products of unsaturated fatty acids have been shown to induce accumulation of phytoalexins in potato[1]. Because exogenous phospholipids, such as PI, PIP and PA, did not induce accumulation of pisatin in pea tissues (not shown), it is likely that fatty acid(s) generated during activation of PI metabolism may participate in signal transduction, probably via activation of lipooxygenase (Fig. 4).

Our previous studies[21,24,30] have demonstrated that protein kinase and plasma membrane ATPase may play crucial roles in PI metabolism (see Fig. 4). Inhibitors of protein kinase and ATPase blocks elicitor-induced activation of PI metabolism in plasma membrane, thereby resulting in inhibition of phytoalexin accumulation in pea tissues[21,30]. Therefore, it is also probable that fatty acid(s) may function in regulating certain signaling pathways, since it can modulate several enzymes involved in signal transduction, such as plasma membrane NADH oxidase[2] responsible for production of O_2^-, protein kinase[20] and plasma membrane ATPase[20]. In this regard, further experiments are needed to elucidate whether the elicitor results in production of fatty acid(s)

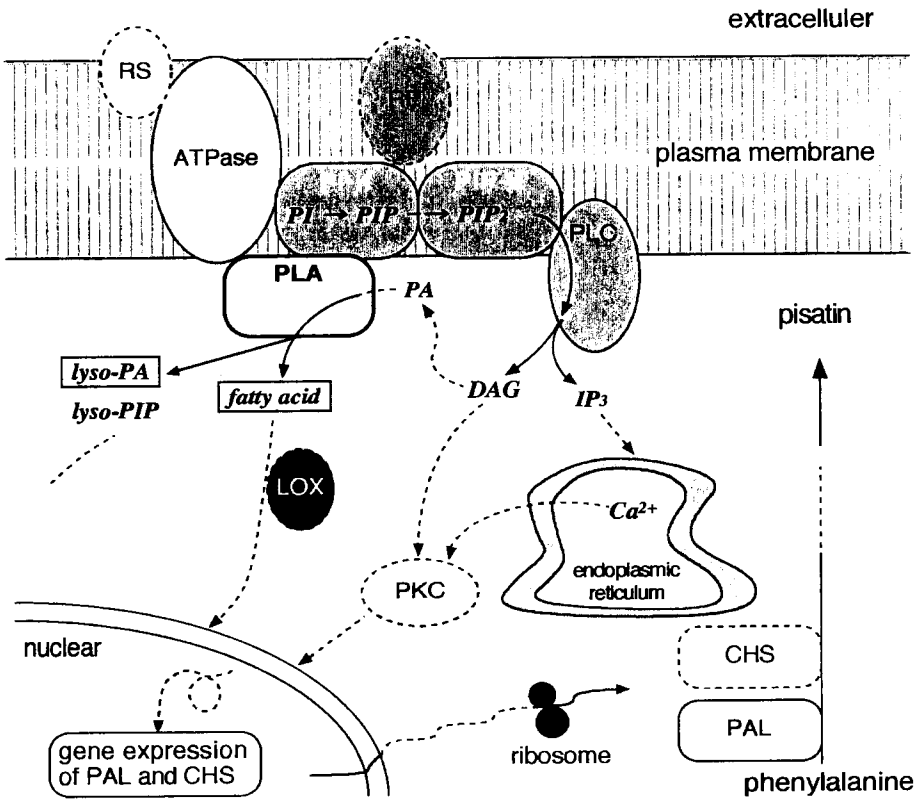

Fig. 4 A schematic diagram for regulation of signal transduction pathways by fungal signals from *M. pinodes* during induction of defense responses in pea. Enzymes and cascades, which are represented by solid lines, are regulated by the elicitor and suppressor, as described in the present study and previously [24-26]. Broken lines indicate putative proteins or cascades that might be affected by these fungal signals. CHS, chalcone synthase; LOX, lipooxygenase; PAL, phenylalanine ammonia-lyase; PI-K, phosphatidylinositol kinase; PIP-K, phosphatidylinositol 4-monophosphate kinase; PKC, protein kinase C; PLA, phospholipase A; PLC, phospholipase C; RE, receptor for elicitor; RS, receptor for suppressor; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PA, phosphatidic acid; lysoPA, lysophosphatidic acid; lysoPIP, lysophosphatidylinositol 4-monophosphate.

and in the consequent activation of enzymes, such as lipooxygenase, downstream from signal transduction.

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病原菌シグナルによるエンドウ原形質膜における ホスファチジルイノシトールリン脂質の リン酸化とリゾリン脂質生成の制御

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エンドウの上胚軸組織より分離した原形質膜画分を褐紋病菌の生産するエリシターで処理すると，ホスファチジルイノシトールリン脂質の急速なリン酸化とリゾリン脂質の生成が誘導されたが，同菌より調製したサプレッサーの共存下では双方とも著しく阻害された．本結果は，ポリホスホイノシチド代謝系と同調的に作動するホスホリパーゼA活性が存在すること，さらに，原形質膜における病原菌シグナルの受容・応答には複数の脂質代謝系が介在する可能性を示唆している。一方，ホスホリパーゼAの活性化の役割を調べる目的で、本酵素によって原形質膜から生成されると考えられる脂肪酸(リノール酸ならびにリノレン酸)をエンドウ葉に処理したところ，エリシターの非存在下においてもファイトアレキシンであるピサチンの生成が誘導されることが示された．以上から，ポリホスホイノシチド代謝系と同調的に働くホスホリパーゼAがエリシターシグナルの初期伝達に深く関連しているものと考えられた．