Elevation of Diacylglycerol during the Early Stage of Elicitor-signal Transduction in Pea (*Pisum sativum*)

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Key words : diacylglycerol, elicitor, pea (*Pisum sativum*), phospholipase C, R59022, signal transduction.

Plants respond to pathogen attack with a wide variety of defense responses, including transcriptional activation of defense response genes, accumulation of phytoalexins and pathogenesis-related (PR) proteins and the reinforcement of cell walls¹⁾. These active defenses are also induced in epicotyl tissues of pea by glycoprotein elicitors from germination fluid of *Mycosphaerella pinodes*, a fungal pathogen of pea^{2,6,13,14)}. The elicitor increased the levels of phosphatidylinositol 4,5-bisphosphate (PIP₂) and inositol 1,4,5-trisphosphate (IP₃) within a few minutes of the start of the elicitor treatment with pea epicotyls, then transcripts markedly accumulated for phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), key enzymes in the biosynthetic pathway to the pea phytoalexin pisatin^{11,13)}. However, the concomitant presence of suppressors from the same fungus specifically inhibited or delayed the elicitor-induced defenses, by inhibiting ATPase and polyphosphoinositide (PI) metabolism^{7,9-11,13,15)}. Moreover, neomycin, an inhibitor of phospholipase C (PLC), blocked the phytoalexin accumulation that is induced by the elicitor¹⁰⁾, and conditioned pea tissues to be susceptible¹¹⁾. These results suggest that activation of PI metabolism, especially PLC, is responsible for signal transduction leading to the pea defense response. In this study, to further confirm the importance of PI metabolism in elicitor signaling, we examine the changes in diacylglycerol (DAG) level in pea epicotyl tissues.

Seeds of *Pisum sativum* L. cv. Midoriusui were sown on moistened vermiculite in a plastic container and grown in darkness for 7 days. Excised epicotyl segments (1.5-cm length) were further sliced longitudinally into two parts with a razor blade as described previously¹⁰⁾. An elicitor and a suppressor were prepared from germination fluid of *Mycosphaerella pinodes* (Berk. et Blox.) Verstergren, strain OMP-1 (IFO-30342, ATCC-42741) as previously described^{6,15)}. Neomycin sulfate and R59022 were obtained from Sigma Chemicals Co., Ltd. (St. Louis, Mo, USA) and Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA), respectively.

To determine the DAG level in epicotyl tissues, five epicotyl segments (approximately 0.25 g) were treated with the elicitor (500 µg/ml) or distilled water, and incubated at 22°C for given times described in the legend for Fig. 1. The tissues were immediately transferred to a chilled mixture of chloroform and methanol (1/2, v/v) on dry ice to stop reaction. Lipids were then extracted from the frozen tissues as described previously¹¹⁾. DAG was determined using a DAG assay kit (RPN200) from Amersham, according to the manufacturer's instruction. This radioenzymatic assay employs an *Escherichia coli* sn-1,2-diacylglycerol kinase which quantitatively converts DAG to [³²P]phosphatidic acid (PA) in the presence of [γ -³²P]ATP. Following the enzyme-catalyzed phosphorylation of DAG, the ³²P-labeled PA formed was separated on a silica gel plate and the radioactivity attributable to [³²P]PA was quantitated with a Bio-imaging scanner system (Bas 2000 system; Fujix, Tokyo, Japan). In this study, a basal level of DAG in untreated tissues (control) varied from 2.9 to 10.2 nmol/g fresh weight, in respective experiments (5.43 ± 4.12 nmol/g fresh weight, $n=3$). To normalize such differences, respective data were expressed as a percentage of the control.

To measure phytoalexin accumulation, excised epicotyl segments (1.5-cm length) were treated with R59022 in the presence or absence of elicitor, and incubated at 22°C in darkness. R59022 was dissolved in dimethylformamide (DMF) as a stock solution. All test solutions thus included 0.5% (v/v) DMF (final conc.). The amount of pisatin in epicotyl tissues was determined 18 hr after the start of incubation as described previously¹⁰⁾.

When epicotyl tissues were treated with the elicitor from *M. pinodes*, it more rapidly stimulated the production of DAG in epicotyl tissues. DAG increased to 1.4-fold over the basal level in the first minute following the elicitor treatment, and usually attained a maximal level within 1 to 2 min, then decreased to the control level by 4 to 8 min (Fig. 1a). However, neomycin, an inhibitor of PLC¹²⁾ appreciably prevented the elicitor-induced increase in DAG (Fig. 1b), indicating that the initial in-

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Fig. 1. Changes in DAG level in elicitor-treated pea epicotyl tissues. a), Epicotyl tissues were treated with elicitor (500 $\mu\text{g}/\text{ml}$) or distilled water. DAG level was measured at 0.5, 1, 2, 4 and 8 min after the start of elicitor treatment, by a radio-enzymatic assay employing an *E. coli* sn-1,2-diacylglycerol kinase and [γ - ^{32}P]ATP as described in the text. Data were expressed relatively as percentages to that of the control, and the mean values with S.D. from three experiments were plotted. b-c), Effects of neomycin and the fungal suppressor on the production of DAG in epicotyl tissues induced by the elicitor. Epicotyl tissues were treated with neomycin (b) or suppressor (c) in the presence of elicitor. DAG level in the absence (■) or presence (▨) of the inhibitor was measured at times indicated in the figures. Data were expressed as percentages relative to the control. The concentrations of neomycin and the suppressor were 1 mM and 100 $\mu\text{g}/\text{ml}$, respectively. Figure 1b and c show typical data for measurement of DAG.

crease in DAG is associated with activation of inositol phospholipid-specific PLC. Furthermore, the suppressor also inhibited the increase in DAG that was induced by the elicitor (Fig. 1c). These results, consistent with our previous data¹¹⁾ quantifying intracellular IP_3 in elicitor-treated epicotyl tissues, further suggest that the activation of PLC participates in signal transduction of the elicitor-induced defense response in pea.

In a previous study¹¹⁾, we showed that intracellular IP_3 reached ~ 300 pmol/g fresh weight within 1 min after the start of elicitor-treatment of pea epicotyls and then decreased to a basal level (ca. 100 pmol/g fresh weight). However, the extent of the increase in DAG observed here was about 10-fold higher than that of IP_3 ¹¹⁾. One likely possibility is the presence of an alternative pathway(s) that releases DAG. Kamada and Muto³⁾ reported that adding the elicitor to suspension cultured cells of tobacco resulted in about a 15-fold increase in inositol 1,4-bisphosphate (IP_2), in parallel with the decrease in phosphatidyl-inositol 4-monophosphate (PIP), although IP_3 also increased to about 40% of the control. The elicitor may, therefore, cause a large increase in DAG, because DAG is also produced through the hydrolysis of PIP. Previously, we reported that the level of PIP rapid-

Fig. 2. Effect of DAG kinase inhibitor R59022 on induction of phytoalexin accumulation in pea epicotyl tissues. Epicotyl tissues were treated with R59022 in the presence (●) or absence (○) of 500 $\mu\text{g}/\text{ml}$ elicitor. The amount of pisatin in epicotyl tissues was determined 18 hr after the start of incubation. Mean values with S.D. from three epicotyl tissues were plotted.

ly decreased within 1 min of the elicitor treatment of pea epicotyls¹¹⁾. Together with these findings, PIP, as well as PIP_2 , is a potential source of DAG during signal transduction of the elicitor-induced defense response in pea.

To further define the role of DAG in the elicitor-signal transduction, the effect of a DAG kinase inhibitor (R59022), which increases intracellular DAG by inhibiting the conversion of DAG into PA^3 , on phytoalexin accumulation was examined. As shown in Fig. 2, adding R59022 to epicotyl tissues enhanced accumulation of pisatin in elicitor-treated tissues. Even the presence of 100 μM R59022 enhanced pisatin accumulation about 2.5-fold compared to that in the absence of the reagent (Fig. 2). Accordingly, R59022 may potentiate signal transduction by increasing or sustaining the endogenous DAG level in elicitor-treated tissues. However, R59022 did not show any effect on phytoalexin accumulation in tissues without the addition of the elicitor (Fig. 2). Moreover, the stimulatory effect of R59022 was not observed in the concomitant presence of neomycin that inhibits PLC (data not shown). These results probably suggest that, although intracellular DAG may play a key role in early signaling, other factor(s) must be stimulated by the elicitor for complete signal transduction.

Our previous studies⁹⁻¹¹⁾ showed that activation of lipid kinases may precede that of PLC during the early stage of elicitor signal transduction in pea. We also showed that a potent inhibitor of protein kinases K-252a remarkably decreased the elicitor-induced activation of lipid kinases in pea plasma membrane¹⁰⁾. Together with these findings, the initial process of elicitor signaling in pea may be composed as follows; i) fungal elicitors bind to cell surface receptor(s), ii) lipid kinases, such as phosphatidylinositol- and phosphatidylinositol 4-monophosphate kinases, are activated probably via protein

phosphorylation(s) to increase PIP and PIP₂¹⁰⁾, iii) PIP₂ is rapidly hydrolyzed by inositol phospholipid-specific PLC to produce IP₃¹¹⁾ and DAG, and iv) these products may act as second messengers that mediate intracellular signal transduction cascades leading to the activation of defense response genes, e.g. *PAL* and *CHS* genes. However, considering that inhibition of DAG kinase may result in enhanced or sustained induction of defense responses, DAG kinase is conceivably a component that negatively regulates the signal transduction.

Kurosaki *et al.*⁴⁾ reported that adding elicitor to carrot cells induced a rapid breakdown of phosphatidylinositols within a few minutes, in parallel with an increase in phospholipase activity. With the use of specific pharmacological agents, they further showed a potential role of protein kinase C (PKC)-like enzyme(s) in the induction of phytoalexin accumulation in carrot cells³⁾. More recently, Subramaniam *et al.*⁸⁾ demonstrated the presence and involvement of a homolog of mammalian PKC in the elicitor-induced defense response in potato. Further studies are now in progress to determine whether similar enzymes also participate in signal transduction in pea.

This research was supported in part by a grant from the Japan Society for Promotion of Sciences (JSPS-RFTF96L00603) and by a grant for the specific research "The study of the development of organisms effective to environmental conservation for human life" at Okayama University in 1998-1999). Financial support from Sankyo Co., Ltd., Tokyo, Japan is also acknowledged.

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和 文 摘 要

豊田和弘・河原智治・水越留美・小山昌史・一瀬勇規・山田哲治・白石友紀：エンドウのエリシターシグナルの伝達初期過程におけるジアシルグリセロール生成

エリシターの認識とそのシグナル伝達機構を解析する目的で、エンドウ上胚軸組織におけるジアシルグリセロール生成量を調べた。この結果、エリシター処理組織においては、処理後1分以内に急速なジアシルグリセロールの生成が認められたが、ホスホリパーゼCの阻害剤であるネオマイシンやエンドウ褐紋病菌のサプレッサーの共存下には、その生成は著しく阻害された。この結果は、細胞内におけるイノシトール3リン酸を定量した既報の結果と一致し、ホスホリパーゼC活性の一過的上昇がエリシターシグナルの伝達に必須であることを強く示唆する。このことは、細胞内のジアシルグリセロール濃度の上昇を引き起こすジアシルグリセロールキナーゼ阻害剤 (R59022) で処理された組織では、エリシターの処理によって誘導されるファイトアレキシンの生成量が約4倍に増加することからも支持された。

(Received April 13, 1998; Accepted July 6, 1998)