Regulation of Pisatin Biosynthesis in Pea Leaves by Elicitor and Suppressor Produced by *Mycosphaerella pinodes*

Motohiro HIRAMATSU**, Yuki ICHINOSE**, Tomonori SHIRAISHI**, Hachiro OKU** and Seiji OUCHI**

Abstract

Regulation of pisatin biosynthesis in naked mesophyll tissue of pea leaves by suppressor (F5) and elicitor isolated from *Mycosphaerella pinodes* was studied using a radioactive precursor of pisatin synthesis, 14C-phenylalanine. Incorporation of radioactivity became detectable 4.5-6 hr after elicitor treatment and increased thereafter. Concomitant presence of F5 inhibited the pisatin inducing activity of the elicitor completely. Treatment of pea leaves with F5 after elicitor-activation caused a reduction of pisatin synthesis and an increased accumulation of cinnamic acid, an intermediate of pisatin synthesis. F5 was found to inhibit in vitro the activity of phenylalanine ammonia-lyase and cinnamate 4-hydroxylase. The suppressing ability of F5 on pisatin synthesis seemed to be reversible.

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Key words: elicitor, suppressor, pisatin biosynthesis, enzyme inhibition, *Mycosphaerella pinodes*.

Introduction

Elicitation or suppression of defense reactions in higher plants by invading microorganisms may determine the fate of plants afterwards.

The mechanism of phytoalexin accumulation as one of the defense reactions have attracted much attention of many scientists and the experimental results have appeared in several reviews1,2,6-8,18,19).

In our laboratory, low molecular weight substances, F2 and F5, which suppress the accumulation of pisatin (a phytoalexin of pea plant), were isolated from the spore germination fluid of a pea pathogen, *Mycosphaerella pinodes*, and F5 was proved to play a key role as a determinant of host-specificity of this fungus12,14). In contrast to glycineollin20), a phytoalexin of soybean, accumulation of pisatin was attributed to the activa-
tion of the biosynthetic pathway of pisatin, but not to the inhibition of the biodegradation\(^{16}\).

This paper describes the experimental results on regulation of pisatin biosynthesis in pea leaves by elicitor and suppressor which are produced in the spore germination fluid of \(M. \text{pinodes}\), and discusses on the mechanism of pathogenicity of this fungus.

**Materials and Methods**

*Plant material.* Seeds of \(P. \text{sativum}\) L. cv. Alaska were soaked for overnight, sown on the vermiculite in plastic containers and grown in a growth chamber at 22 C under the artificial illumination (3,000 lux, 12 hr/day) for 2 weeks.

*Radioactive precursor.* \(L-(\text{U}^{14}\text{C})\)-phenylalanine (specific activity: 495 mCi/m mol) and \(\text{(side chain}-3^{14}\text{C})\)-cinnamic acid (specific activity: 57 mCi/m mol) were purchased from Radiochemical Center, Amersham.

*Preparation of suppressor and elicitor.* Elicitor and suppressor of pisatin accumulation were prepared from spore germination fluid of \(M. \text{pinodes}\) by the method described in the previous paper\(^{15}\) with several modifications.

Pycnospores produced on Czapek agar medium were collected, suspended in sterilized water (1-2 \(\times\) \(10^6\) spores/ml), and allowed to germinate for 24 hr at 25 C. The germination fluid was fractionated into low and high molecular weight fractions by ultrafiltration using Millipore filter (PTGC 142-05).

The filtrate (low molecular weight fraction) was concentrated under reduced pressure, spotted on TLC plate (Silica gel, Merck Art 5715) and developed with the solvent system of ethanol-acetic acid-water (4:1:1, v/v). After drying the plate, silica gel on the area correspond to the Rf value of suppressor, F5 (Rf, ca. 0.45, ninhydrin positive), was scratched off, was eluted with methanol at 4 C for 24 hr, and the eluate was concentrated. The concentrate was used as suppressor after diluting with distilled water to appropriate concentration. The concentration of the suppressor was determined by Lowry method\(^{10}\), bovine serum albumin as a standard. Unless otherwise stated, F5 was used at 50 ppm in this experiment.

The residue of the ultrafiltration (high molecular weight fraction) of the spore germination fluid was dialyzed at 4 C for 24 hr, and the inner dialysate was used as the elicitor for pisatin induction. The concentration of the elicitor was determined by phenol–sulfuric acid method\(^{31}\), using glucose as the standard.

*Determination of biosynthesis of pisatin and its intermediates.* Abaxial epidermis of pea leaves was eliminated and the naked leaves were cut into pieces of 8 \(\times\) 8 mm. The elicitor solution (500 ppm, 50 \(\mu\)l) was applied to the naked mesophyll tissue for an appropriate time at 22 C under light (3,000 lux) to activate the system of pisatin biosynthesis, and then the elicitor solution was replaced with 10 \(\mu\)l of \(1^{14}\text{C}-\text{phenylalanine} (25 \mu\text{Ci/ml}) and kept for 1 hr. The leaf section was extracted with 500 \(\mu\)l of ethanol at 80 C for 10 min, and the extract was concentrated under reduced pressure to dryness. The residue was dissolved in a small amount of ethanol and fractionated on TLC plate (Merck Art 5735) with the solvent system of toluene–ethylformate–formic acid (60:40:1, v/v). After drying the plate, fractions corresponding to pisatin and its intermediates
(Rf value of pisatin, cinnamic acid, p-coumaric acid, and naringenin was 0.60, 0.51, 0.37, and 0.41, respectively) were scanned under UV-light, cut, put into vials with 500 µl of ethanol and 3 ml of scintillator (ACS II), and the radioactivity incorporated into each compound was counted by liquid scintillation counter (Aloka LSC-653).

**Determination of phenylalanine ammonia-lyase and cinnamate 4-hydroxylase activity of elicitor-treated pea leaves and the effect of F5.** Pea leaf pieces (100 mg) treated with 500 ppm of elicitor for 3 hr was ground in 1 ml of 0.1 M Tris-Cl buffer (pH 8.5) with 10 mg of Polyclar AT and 10 µl of 0.1 M 2-mercaptoethanol using mortar and pestle at 4°C, filtered through 2 layers of Kimwipe, and the filtrate was centrifuged at 12,000 x g for 20 min. The supernatant was used as the enzyme solution.

Phenylalanine ammonia-lyase (PAL) activity was determined by the method of Dixon and Bendall. The reaction mixture was as follows: 100 µl of enzyme solution (700 µg/ml protein equivalent), 100 µl of 1 mM Tris-HCl buffer (pH 8.5), 1 µl of 1 mM dithiothreitol and 25 µl of 14C-phenylalanine (10 µCi/ml). The reaction mixture was added with 30 µl of F5 (400 µg/ml) or water and incubated at 30°C for 1 hr (final concentration of F5 in the reaction mixture was ca. 47 µg/ml). The reaction was stopped by addition with 500 µl of methanol. The solution was added with 3 ml of ethanol and kept at 4°C for 24 hr to precipitate proteinous substance. After centrifugation (3,500 rpm, 10 min) the supernatant was concentrated to dryness under the reduced pressure, dissolved in a small amount of ethanol, and fractionated on TLC plate. The zone corresponding to cinnamic acid was counted as described above.

Cinnamate 4-hydroxylase activity was determined by the method of Russel. The reaction mixture was as follows: 100 µl of enzyme solution (700 µg/ml protein equivalent), 100 µl of 1 mM phosphate buffer (pH 7.5), 1 µl of 0.1 M 1,4-dithiothreitol, 25 µl of 14C-cinnamic acid (10 µCi/ml), and 10 µl of NADPH generation system. As the NADPH generation system, 10 µl of 2 mM NADP, 10 µl of 4 mM glucose-6-phosphate, and 0.7 unit of glucose-6-phosphate dehydrogenase were incubated at 30°C for 30 min. The reaction mixture was added with 30 µl of water or F5 (400 µg/ml) and allowed to react at 30°C for 1 hr (concentration of F5 in the reaction mixture was ca. 45 µg/ml). The radioactivity incorporated into p-coumaric acid was determined.

**Results**

**Time required for activation of pisatin biosynthetic system by elicitor**

Pea leaf sections were treated with 500 ppm of the elicitor for 0, 3, 6, 9, 12, and 15 hr, then the elicitor solution was substituted with 14C-phenylalanine, and kept for 1 hr. The radioactivity incorporated into pisatin was determined to know the...
time required for the activation of pisatin biosynthetic system.

As shown in Fig. 1, 4.5-6 hr was needed for apparent activation of pisatin biosynthetic system by the elicitor under the experimental condition.

**Effect of suppressor on pisatin biosynthesis**

To know the effect of suppressor on the activation of pisatin biosynthetic system, pea leaf sections were treated with F5 for 3 hr before 500 ppm elicitor treatment (3 hr later, F5 was substituted with elicitor), or simultaneously with F5 plus elicitor. The leaf sections were incubated at 22 C for 9 hr after elicitor treatment, and then these solutions were substituted with 14C-phenylalanine. Effect of F5 on the activated pisatin biosynthetic system was also examined as follows: Leaf sections were treated with 500 ppm elicitor for 9 hr, and then, the elicitor solution was replaced by 14C-phenylalanine plus F5. One hour after incubation, the radioactivity of pisatin and its intermediates was determined.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity incorporated (DPM/mg fr. wt.) into</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cinnamic acid</td>
</tr>
<tr>
<td>Water</td>
<td>178± 25</td>
</tr>
<tr>
<td>Water</td>
<td>439±144</td>
</tr>
<tr>
<td>Water</td>
<td>119± 24</td>
</tr>
<tr>
<td>Water</td>
<td>964±214</td>
</tr>
<tr>
<td>F5</td>
<td>420±156</td>
</tr>
</tbody>
</table>

a) Time interval between 1st and 2nd treatment was 3 hr, 2nd and 3rd treatment was 9 hr.
b) PA : Phenylalanine.

d Table 1 shows the radioactivity incorporated into pisatin and its intermediates in pea leaves. A large amount of radioactivity was found in cinnamic acid fraction when F5 was applied 9 hr after the activation of the system, whereas the radioactivity found in each intermediate was nearly the same in the leaf section which was treated simultaneously with F5 and elicitor, as was found in unactivated leaf section.

The pretreatment of pea leaf tissues with F5 for 3 hr inhibited significantly the activation of pisatin biosynthetic pathway by elicitor, but not to the level of simultaneous treatment with elicitor.

**Effect of F5 on activities of phenylalanine ammonia-lyase and cinnamate 4-hydroxylase**

As was seen in Table 1, a large amount of radioactivity was found in cinnamic acid when F5 was added with 14C-phenylalanine on elicitor-activated pea leaves. This fact suggested that the conversion of cinnamic acid to p-coumaric acid in the pisatin biosynthetic pathway might be blocked by F5. Therefore, the effect of F5 on enzyme activities of PAL and cinnamate 4-hydroxylase was examined (Table 2, 3).

Enzyme activity of PAL was inhibited ca. 30%, and that of cinnamate 4-hydroxylase was inhibited ca. 50% in vitro by addition of ca. 45 ppm of F5.
Table 2. Effect of F5 on activity of phenylalanine ammonia-lyase prepared from elicitor-treated pea leaves

<table>
<thead>
<tr>
<th>Added with</th>
<th>Radioactivity incorporated into cinnamic acid from 14C-phenylalanine (DPM/100 µl of enz. sol.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>145774 (100)</td>
</tr>
<tr>
<td>F5 (ca. 45 ppm)</td>
<td>91109 (63)</td>
</tr>
</tbody>
</table>

Table 3. Effect of F5 on activity of cinnamate 4-hydroxylase prepared from elicitor-treated pea leaves

<table>
<thead>
<tr>
<th>Added with</th>
<th>Radioactivity incorporated into β-coumaric acid from 14C-cinnamic acid (DPM/100 µl of enz. sol.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>7845 (100)</td>
</tr>
<tr>
<td>F5 (ca. 47 ppm)</td>
<td>3543 (45)</td>
</tr>
</tbody>
</table>

Discussion

Suppression of defense reaction of host plants should be indispensable for pathogenic fungi to establish infection\(^{(5)}\).

In our laboratory, 2 kinds of oligopeptide isolated from spore germination fluid of a pea pathogen, *M. pinodes*, were proved to suppress pisatin accumulation in pea leaves inoculated with incompatible pathogens\(^{(12,15)}\). One of them, F5, allowed nonpathogen to infect the host plants of *M. pinodes* including pea plant\(^{(12)}\). On the other hand, an elicitor of pisatin accumulation was also found in spore germination fluid of *M. pinodes*\(^{(15,17)}\). The elicitor was proved to elicit pisatin in dose dependent manner\(^{(17)}\). F5 negated the activity of the elicitor\(^{(15)}\).

In the present work, the regulation of pisatin biosynthesis by elicitor and suppressor was studied. The time course study of pisatin synthesis using radioactive precursor after elicitor treatment of pea leaf sections indicated that pisatin biosynthesis became detectable 4.5-6 hr after elicitor treatment, and remarkable after 9 hr. Only small amount of pisatin was synthesized when treated with water. Addition of F5 to the elicitor solution suppressed the pisatin biosynthesis almost completely. Pretreatment of pea leaves with F5 before elicitor treatment suppressed considerably the pisatin biosynthetic activity of the elicitor, but not to the level when F5 was added at the same time with the elicitor. These results indicated that the suppressing ability of F5 against pisatin synthesis might be reversible.

Pisatin accumulates in host cells at a very early stage of infection by incompatible or nonpathogen of pea, but the accumulation delays when infected by compatible pathogen including *M. pinodes*\(^{(11,14)}\). Spores of *M. pinodes* secrete specific suppressor of pisatin synthesis, F5, in its spore germination fluid with elicitor, as described above. At this stage, the suppressor negates the activity of elicitor, hence pisatin may not be synthesized. The germ-tube of *M. pinodes* may penetrate pea leaf tissue during the absence of pisatin. After the penetration, invaded hyphae may continue to secrete elicitor, which induces pisatin accumulation at later stage of infection.
Application of F5 with 14C-phenylalanine 9 hr after elicitor treatment caused a marked reduction in the incorporation of radioactivity into pisatin, and conspicuous increase in the accumulation of 14C-cinnamic acid. The activity of cinnamate 4-hydroxylase, the enzyme which catalyzes the reaction from cinnamic acid to p-coumaric acid, was inhibited markedly by F5. In addition, PAL activity was also inhibited by F5.

Thus F5 has dual functions to pisatin accumulation in pea leaves, suppression of the activation of the pathway and inhibition of the enzymes involved in pisatin biosynthetic pathway.

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Literature cited