Suppression of the Activation of Chitinase and \(\beta\)-1,3-Glucanase in Pea Epicotyls by Orthovanadate and a Suppressor from *Mycosphaerella pinodes*

Hirofumi YOSHIOKA**, Tomonori SHIRAISHI**, Kimio NASU**, Tetsuji YAMADA**, Yuki ICHINOSE** and Hachiro OKU**

Abstract

A pea pathogen, *Mycosphaerella pinodes*, secretes both an elicitor and a suppressor in its pycnospore germination fluid. The effects of the elicitor, the suppressor and orthovanadate, an inhibitor of P-type ATPase, on the activation of pathogenesis-related proteins such as endochitinase and \(\beta\)-1,3-glucanase were examined. The elicitor induced the activation of these enzymes in pea, soybean and kidney bean. Such activation in 3 plant species tested was suppressed by the concomitant presence of 1 mM orthovanadate with the elicitor. The suppressor, however, suppressed the activation in only pea plants but did not in nonhost plants of *M. pinodes*. These results suggest that the inhibition of the ATPase in plasma membranes highly correlates to the suppression of the activation of endochitinase and \(\beta\)-1,3-glucanase.

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Key words: ATPase, chitinase, \(\beta\)-1,3-glucanase, orthovanadate, suppressor, *Mycosphaerella pinodes*.

INTRODUCTION

Pea has been found to synthesize several defensive products such as pisatin, a major phytoalexin of pea, and an unidentified infection-inhibitor by treatment with an elicitor from pea pathogen, *Mycosphaerella pinodes*\(^{1,15}\). However, these defense reactions are suppressed by a suppressor produced by the same fungus\(^{1,13}\), and pea plants become susceptible to nonpathogens of pea such as *Alternaria alternata*\(^{9,10}\). Recently, we reported that the suppressor inhibits ATPase activity in pea plasma membranes *in vitro*\(^{12,18}\) and *in situ*\(^{10}\). Orthovanadate, an inhibitor of P-type ATPase, also suppresses the accumulation of pisatin and mRNAs encoding phenylalanine ammonia-lyase and chalcone synthase in pea epicotyls induced by treatment with the fungal elicitor in a manner similar to that observed with the fungal suppressor\(^{14,17,18}\). Therefore, we presume that the primary action site of the suppressor might be the ATPase in pea plasma membranes.

In the last decade, much attention has been paid to pathogenesis-related proteins (PR-proteins) such as chitinase and \(\beta\)-1,3-glucanase produced by plants in response to pathogens and pathogen-derived elicitors\(^{4,6,16}\). Since chitinase and \(\beta\)-1,3-glucanase prepared from elicitor-treated pea plants show synergistic antifungal activity\(^{16}\), even pathogenic fungi may fail to invade and/or grow on the host where these PR-proteins highly accumulate.

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In the present paper, we report the effects of the suppressor and orthovanadate on the activations of chitinase and β-1,3-glucanase in the host and nonhost plants of *M. pinodes* induced by the elicitor from the same fungus and discuss the relationship between the inhibition of the P-type ATPase and the suppression of defense responses.

**MATERIALS AND METHODS**

**Plants.** Seeds of pea (*Pisum sativum* L. cv. Midoriusui), soybean (*Glycine max* Mer. cv. Greenhomer) or kidney bean (*Phaseolus vulgaris* L. cv. Hatsumidori) were soaked in tap water overnight, sown on vermiculite in a plastic container and grown in a growth chamber at 20-22°C in the dark for 6 to 10 days.

**Preparation of suppressor and elicitor from *M. pinodes.*** Throughout all experiments, we used a partially purified preparation of suppressor, which included the F2 and F5 fractions\(^{11}\), to examine the overall effects of the suppressor on the activation of the PR-proteins. A high-molecular-weight polysaccharide elicitor\(^{11,13}\) and a low-molecular-weight glycopeptide suppressor\(^{8,11}\) were prepared from spore germination fluid of *Mycosphaerella pinodes* (Berk. et Blox.) Vestergren, strain OMP-1 (IFO-30342, ATCC-42741) by the method as described previously\(^{18}\). The concentrations of the elicitor and the suppressor were adjusted to 1 mg/ml glucose equivalents by the method of Dubois *et al.*\(^{3}\) and 100 µg/ml bovine serum albumin (BSA) equiv. by the method of Lowry *et al.*\(^{7}\), respectively.

**Crude enzyme extraction.** The 1.5-cm length segments from seedlings of each plant were divided longitudinally into two parts with a razer blade and immediately treated with the elicitor (500 µg/ml, final concentration) or water in the absence or presence of the suppressor (50 µg/ml, final concentration) or 1 mM Na\(_3\)VO\(_4\) at 20°C in the dark for the time indicated in the legends to each figure. After incubation, one gram fresh weight of segments was frozen in liquid nitrogen and stored at -80°C until used. Crude enzyme was extracted from each sample by the method of Mauch *et al.*\(^{9}\) with slight modifications. Frozen segments were ground in liquid nitrogen with a mortar and pestle. The resulting powder was suspended in 0.1 M Tris-HCl (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride and β-mercaptoethanol at 4°C. The residue was removed by centrifugation at 20,000 × g for 20 min. Then, the

![Fig. 1. Time course of activation of endochitinase in elicitor-treated pea epicotyls in the presence or absence of orthovanadate or suppressor from *Mycosphaerella pinodes.* The activity of endochitinase (nmol of GluNac/mg protein/hr) was determined at 6-hr intervals after treatment with water alone (–△–), elicitor alone (500 µg/ml, glucose equiv.) (–●–), elicitor plus 1 mM orthovanadate (–○–) and elicitor plus suppressor (50 µg/ml, BSA equiv.) (–□–), respectively, by the method described by Boller *et al.*\(^{7}\). Each plotted value represents the mean of results of triplicate experiments, and the bar indicates the standard error.](image-url)
Fig. 2. Time course of activation of β-1,3-glucanase in elicitor-treated pea epicotyls in the presence or absence of orthovanadate or suppressor from Mycosphaerella pinodes. The activity of β-1,3-glucanase (nmol of glucose equiv./µg protein/hr) was determined at 6-hr intervals after treatment with the same solution as described in the legend to Fig. 1 by the method described by Abeles and Forrence. The plotted value and bar indicate the same items as described in the legend to Fig. 1.

<table>
<thead>
<tr>
<th>Addition of</th>
<th>Endochitinase activity(^a) (nmol of GluNAc/mg protein/hr)</th>
<th>Mean value as % of control</th>
<th>β-1,3-Glucanase activity(^b) (nmol of glucose equiv./µg protein/hr)</th>
<th>Mean value as % of control</th>
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<tbody>
<tr>
<td>Water</td>
<td>159.6±22.2</td>
<td>(100)</td>
<td>34.6±2.4</td>
<td>(100)</td>
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<tr>
<td>Suppressor</td>
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<tr>
<td>50 µg/ml</td>
<td>153.2±14.3</td>
<td>96</td>
<td>34.9±0.7</td>
<td>101</td>
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<tr>
<td>100 µg/ml</td>
<td>163.0±33.5</td>
<td>102</td>
<td>33.9±1.0</td>
<td>98</td>
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<tr>
<td>150 µg/ml</td>
<td>169.2±35.0</td>
<td>106</td>
<td>33.6±0.8</td>
<td>97</td>
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<tr>
<td>Orthovanadate</td>
<td></td>
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</tr>
<tr>
<td>100 µM</td>
<td>166.4±18.5</td>
<td>104</td>
<td>32.8±3.2</td>
<td>95</td>
</tr>
<tr>
<td>1 mM</td>
<td>146.6±21.6</td>
<td>92</td>
<td>35.2±0.8</td>
<td>102</td>
</tr>
</tbody>
</table>

\(^a\) The activities of endochitinase and β-1,3-glucanase were determined by the methods of Boller et al. and Abeles and Forrence, respectively. Each value represents the mean±SE in triplicate experiments. Note that no significant difference was observed among respective treatments (P<0.05).

\(^b\) Final concentrations.

Table 1. Direct effects of the fungal suppressor or orthovanadate on the activities of endochitinase and β-1,3-glucanase

Protein was precipitated with ammonium sulfate (80% saturation) at 0°C for 4 hr. The precipitate was collected by centrifugation at 20,000×g for 20 min and suspended in 30 mM sodium acetate buffer (pH 5.0). The protein content of each fraction was determined by the method of Lowry et al. with BSA as a standard.

**Enzyme assays.** The activities of chitinase and β-1,3-glucanase were determined by the colorimetric assay described by Boller et al. and by Abeles and Forrence, respectively. Chitinase activity was measured with the amount of N-acetylglucosamine (GluNAc) resulted from hydrolysis of chitin used as a substrate. For β-1,3-glucanase assay, laminarin was used as a substrate and the enzyme activity was estimated with glucose equiv. as the standard.
RESULTS AND DISCUSSION

The activity of exochitinase was scarcely detected in pea epicotyls for up to 24 hr after respective treatments (data not shown). This result coincides with the observation with pea pods reported by Mauch et al.5) Endochitinase and β-1,3-glucanase were slightly activated in pea epicotyls till 24 hr after treatment with water alone (Figs. 1 and 2). These activations in water-treated epicotyls may be due to the effect of wounding. By contrast, the treatment with the elicitor significantly enhanced the activities of endochitinase and β-1,3-glucanase within 6 and 12 hr, respectively, and the activities of both enzymes increased up to 24 hr. However, the concomitant presence of the suppressor or orthovanadate with the elicitor suppressed the increase of activities of these enzymes (Figs. 1 and 2). Such effects both of the compounds on PR-proteins seems to be similar to that on the biosynthesis of pisatin14,18). As shown in Table 1, the suppressor and orthovanadate did not directly inhibit the activities of these enzymes.
extracted from pea epicotyls 24 hr after the elicitor-treatment. Therefore, the compounds may act on the process from the signal transduction to synthesis of these PR-proteins.

The effects of the suppressor and orthovanadate on the activation of chitinase and β-1,3-glucanase in soybean and kidney bean were also examined. As shown in Figs. 3 and 4, orthovanadate suppressed the activation of these enzymes in all plant species tested. By contrast, the suppressor suppressed the activation in pea tissues but not in soybean and kidney bean that are nonhosts of M. pinodes. Interestingly, the treatment with the suppressor alone induces the activation of these enzymes in soybean and kidney bean (Figs. 3 and 4). These results show that the suppressor from M. pinodes acts as an elicitor to nonhosts of M. pinodes. Thus, the effect of the suppressor on the activation of PR-proteins seems to be specific likely to that on the phytoalexin productions\(^{19}\). We reported that the suppressor specifically inhibited the ATPase activity in plasma membranes of only pea cells out of 5 plant species tested, while orthovanadate inhibited all of them in vivo\(^{10}\). That is, the specific inhibition of P-type ATPase activity in situ seems to highly correlate to the specific suppression of the activation of these PR-proteins. Our findings may support the idea that P-type ATPase is also responsible for the defense responses as described previously\(^{10,16,18}\).

The roles of PR-proteins in fungal pathogenesis have been reported as follows; 1) β-1,3-glucanase of soybean releases the elicitor from mycelial walls of Phytophthora megasperma ver. sojae\(^{16}\) and 2) chitinase and glucanase from pea inhibit the growth of fungal pathogens in a synergistic manner\(^{3}\). These reports suggest that two PR-proteins participate in the defense responses of pea plants against the invasion or colony expansion by fungal pathogens in both ways as mentioned above. Together with our previous findings\(^{11,14,15}\), it is probable that M. pinodes may become a pea pathogen with the ability to suppress (to delay) the expression of pea defense responses including the production of these PR-proteins as well as the phytoalexin and an infection-inhibitor.

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**Literature cited**


和 文 摘 要

吉岡 博文・白石 友紀・那須 公雄・山田 哲治・一瀨 勇規・奥 八郎：エンドウ褐絹病菌サプレッサーとパナジン酸によるキチナーゼ、β-1,3-グルカナーゼの活性化抑制

エンドウ褐絹病菌 *Mycosphaerella pinodes* はその柄胞子発芽液中にエリシナーおよびサプレッサーを分泌する。本報告では、抵抗反応の一とと考えられているキチナーゼおよび β-1,3-グルカナーゼの活性化に及ぼすエリシナーとサプレッサーおよび P 型 ATPase の阻害剤であるパナジン酸の影響を調べた。エンドウ、ダイス、インゲンをエリシナーで処理すると、両酵素の活性増高が誘導された。しかし、1 mM パナジン酸の共存下では、いずれの植物においても活性の増高は抑制された。一方、サプレッサーは供試植物の中でエンドウに対してのみ抑制効果を示した。本結果は、原型質膜 ATPase の阻害とキチナーゼおよび β-1,3-グルカナーゼの抑制に強い関連のあることを示唆している。