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Plant Lectins Induce the Production of a Phytoalexin in *Pisum sativum*

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The effects of several plant lectins on the production of a pea phytoalexin, pisatin, were examined. Con A, PHA, PNA and PSA each induced the production of pisatin in pea epicotyl tissues, demonstrating that plant lectins can act as elicitors. The production of pisatin in response to PHA, PNA or PSA was not affected by the simultaneous presence of the respective hapten sugars, whereas haptens specific for Con A, such as α-D-mannose and methyl-α-D-mannoside, abolished the induction of pisatin by Con A. These results indicate that the elicitor effect of Con A is attributable to its ability to bind to specific carbohydrates in pea cells. Induction of the production of pisatin by Con A was markedly inhibited by the suppressor derived from a pea pathogen, *Mycosphaerella pinodes*, and by several inhibitors related to signal-transduction pathways. It is suggested, therefore, that the Con A-induced production of pisatin in pea tissues might be associated with activation of a signal-transduction pathway. An additive effect on the accumulation of pisatin was observed when Con A was present with a polysaccharide elicitor from *M. pinodes*, suggesting that exogenous Con A does not compete with the recognition site(s) for the fungal elicitor in pea cells. The present data also indicate that Con A may be useful for characterization of the signal-transduction system that leads to the synthesis of phytoalexin in pea epicotyl tissues.

Key words: Concanavalin A (Con A) — Defense response — Elicitor — Pea (*Pisum sativum* L.) — Phytoalexin — Signal transduction.

A pea pathogen, *Mycosphaerella pinodes*, secretes both a polysaccharide elicitor (mol wt = ca. 70 kDa) and a glycopeptide suppressor (mol wt < 5 kDa) of the production of phytoalexin in its pycnospore germination fluid (Oku et al. 1977, 1980, Shiraishi et al. 1978, 1994a, b, Thanutong et al. 1982). The elicitor induces the rapid activation of transcription of genes for PAL and CHS, with the subsequent production of pisatin, a major phytoalexin in pea, whereas the concomitant presence of the suppressor with the elicitor markedly inhibits or delays these defense responses (Yamada et al. 1989). Recently, we showed that the elicitor from *M. pinodes* rapidly activates enzymes such as PtdInsP kinase and PLC in pea epicotyl tissue, with the subsequent production of IP_3, whereas the suppressor, which inhibits the plasma membrane ATPase (Shiraishi et al. 1992, 1994a, b, Yoshioka et al. 1990, 1992), depresses the elicitor-mediated activation of PI metabolism, as well as the production of pisatin in pea tissues (Toyoda et al. 1992, 1993). That is, the rapid phosphorylation of phosphatidylinositols and the subsequent production of IP_3 occur in the elicitor-treated tissues, but these effects are negated by the suppressor (Toyoda et al. 1993). These findings indicate that putative receptors for both fungal signals are functionally linked with the plasma membrane ATPase and PI metabolism.

It has been reported that ion fluxes across the plasma membrane and the phosphorylation of proteins and lipids are probably associated with the signal-transduction that leads to the synthesis of phytoalexins in plants (Cosio et al. 1988, Dietrich et al. 1990, Farmer et al. 1989, Felix et al. 1991, Nürnberger et al. 1994, Renelt et al. 1993, Toyoda et

Abbreviations: αMG, methyl-α-D-glucoside; αMM, methyl-α-D-mannoside; BSA, bovine serum albumin; CHS, chalcone synthase; Con A, concanavalin A (lectin from *Canavalia ensiformis*); Glc, D-glucose; IP_3, inositol 1,4,5-trisphosphate; Man, α-D-mannose; PAL, phenylalanine ammonia-lyase; PHA, lectin from *Phaseolus vulgaris*; PI metabolism, polyphosphoinositide metabolism; PLC, phospholipase C; PNA, lectin from *Arachis hypogaea*; PSA, lectin from *Pisum sativum*; PtdIns kinase, phosphatidylinositol kinase; PtdInsP kinase, phosphatidylinositol 4-monophosphate kinase.

al. 1992, 1993). Although several studies have indicated that high-affinity binding sites for fungal elicitors are located in plasma membranes of plant cells (Cosio et al. 1988, 1990, 1992, Cheong and Hahn 1991, Cheong et al. 1993, Diekmann et al. 1994, Nürnberger et al. 1994, Schmidt and Ebel 1987, Shibuya et al. 1993, Yoshikawa et al. 1983, 1993), the mechanisms for recognition of fungal elicitors by plant cells and subsequent signal-transduction pathways are still obscure. Our major goals are, therefore, to isolate and identify the receptor molecule(s) responsible for regulation of the synthesis of phytoalexin and, also, to elucidate the functions of such a molecule(s) in the signal-transduction pathway.

In mammalian cells, several plant lectins, such as phytohemagglutinin (PHA) and Con A, evoke a wide variety of cell responses, for example, mitogenesis in lymphocytes, upon their binding to receptors on cell surfaces (Ferber and Resch 1973, Szamel et al. 1980). Lectin receptors responsible for activation of lymphocytes have been isolated by affinity chromatography with immobilized lectins (Allan and Crumpton 1973, Resch et al. 1978, Torti et al. 1991). Thus, plant lectins are suitable not only for immunological studies but also for elucidation of the signal-transduction pathway that leads to activation of lymphocytes (Ferber and Resch 1973, Szamel et al. 1980).

In this report, we describe the effects of exogenous lectins on plant defense responses, in particular on the synthesis of a phytoalexin, and the potential utility of lectins in investigations of the molecular mechanism of signal-transduction that leads to synthesis of phytoalexin is discussed.

Materials and Methods

Plant material—Seeds of pea (*Pisum sativum* L., cv. Midoriusui) were sown on moistened vermiculite in a plastic container and grown in darkness at 22±2°C for 6 days. Excised epicotyl tissues, unless otherwise stated, were subjected to the treatments described below.

Plant lectins—Highly purified, essentially salt-free preparations of plant lectins were used in all experiments. Con A (C-2010) was purchased from Sigma Chemical. Co. Ltd., St. Louis, MO. PHA (PHA-P), PNA and PSA were obtained from Seikagaku Kogyo Co. Ltd., Tokyo, Japan. Each lyophilized powder was dissolved in distilled water and tested for its ability to induce the production of pisatin, a major phytoalexin of pea, in epicotyl tissues.

Other chemicals—Neomycin sulfate, methyl-α-D-glucopyranoside (methyl-α-D-glucoside; αMG) and methyl-α-D-mannopyranoside (methyl-α-D-mannoside; αMM) were obtained from Sigma Chemical. Co. Ltd. Other chemicals were from Wako Pure Chemical Inc., Osaka, Japan.

Preparation of elicitor and suppressor from Mycosphaerella pinodes—Elicitor and suppressor were prepared from the germination fluid of pycnospores of *Mycosphaerella pinodes* (Berk. et Blox.) Verstergren, strain OMP-1 (IFO-30342, ATCC-42741) as described previously (Hiramatsu et al. 1986, Yoshioka et al. 1990). The concentrations in stock solution of elicitor and suppressor, which had previously been characterized as polysaccharides (Thauntong et al. 1982) and glycopeptides (Shiraishi et al. 1992), respectively, were adjusted to 1 mg ml^{-1} glucose equivalents (Dubois et al. 1956) and 1 mg ml^{-1} BSA equivalents (Lowry et al. 1951), respectively. In this study, we used a partially purified preparation of suppressor that included supprescins A and B (Kato et al. 1993, Shiraishi et al. 1992) in all experiments.

Determination of the amount of accumulated pisatin in pea epicotyl tissues—Six-day-old seedlings of pea epicotyl were cut into 1.5-cm lengths and the segments were divided longitudinally into two parts as described previously (Toyoda et al. 1992). The pieces were then placed on 40 μl of test solution or distilled water (as the control), with the cut surface in contact with each solution. After incubation at 20±2°C, the amount of accumulated pisatin was determined by HPLC (Masuda et al. 1983).

Results

Induction of the production of pisatin in pea epicotyl tissues by plant lectins—As shown in Figure 1, exogenous plant lectins induced the production of pisatin in pea epicotyl tissues. When lectins were applied at a concentration of 10 mg ml^{-1}, the level of induction of the production of pisatin varied from 4.4 to 20.4 μg (g fr wt)$^{-1}$. However, the production of pisatin was significantly induced by the various lectins at concentrations above 1 mg ml^{-1} and production was increased in a dose-dependent manner (Fig. 1). Among lectins tested, PHA and Con A strictly elicited the production of pisatin in pea tissues at 5 mg ml^{-1} (equivalent to 48 μM for Con A and 42 μM for PHA, respectively). These results clearly showed that certain plant lectins could act as elicitors in pea epicotyl tissues. However, the minimal concentrations required for elicitation in pea tissues was higher than those previously reported for other purified saccharide elicitors, such as hepta-β-glucosides (Sharp et al. 1984) and *N*-acetylchitoheptaose (Yamada et al. 1993).

Effect of hapten sugars on the production of pisatin in pea epicotyl tissues induced by lectins—To determine whether the elicitor effects of plant lectins were attributable to their ability to bind specific carbohydrates in pea cells, the effects of the respective hapten sugars on the induction by each lectin of the production of pisatin were examined. As shown in Table 1, the hapten sugars specific for PHA, PNA or PSA failed to hinder the induction of production of pisatin by these lectins. In PNA-treated epicotyl tissues, the concomitant presence of galactose tended even to enhance the accumulation of pisatin (Table 1). By constant,

Man, a specific hapten of Con A, significantly decreased the production of pisatin in pea tissues in response to Con A (Table 1). Further studies showed that Man, αMM and αMG could decrease the induction of pisatin by Con A in a dose-dependent manner (Fig. 2), and that the rate of inhibition by the respective haptens was consistent with the order in which each was recognized by Con A (Table 2). These results indicated that Con A recognized a certain molecule(s) with specific carbohydrates in pea cells, triggering the synthesis of pisatin.

Time course of the accumulation of pisatin in Con A-treated epicotyl tissues—Since the elicitor effect of Con A was apparently attributable to its activity as a lectin (Table 1, 2, Fig. 1. 2), the mode of action of Con A in the elicitation of the production of pisatin was analyzed in a comparison with that of a polysaccharide elicitor derived from *M. pinodes*. Figure 3 shows the time course of the accumulation of pisatin in pea epicotyl tissues. Pisatin was detected in pea tissues after 9 h of incubation with Con A and its level increased at least until 24 h, as in the case of the fungal elicitor (Fig. 3). However, in the concomitant presence of Man, the accumulation of pisatin was delayed by 6 h and the rate of increase in its level was significantly reduced (Fig. 3).

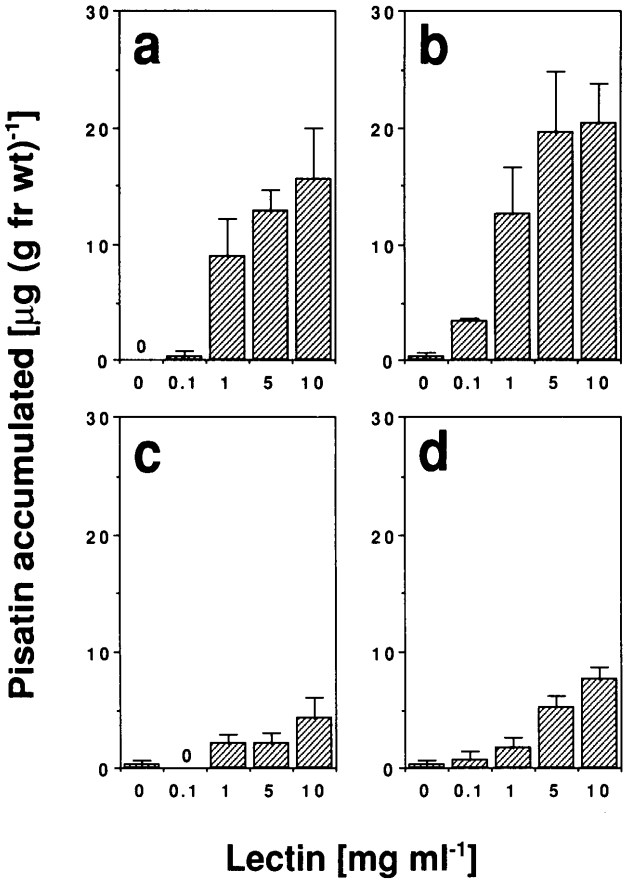

Fig. 1 Induction of the production of pisatin in pea epicotyl tissues by plant lectins. a, Con A; b, PHA; c, PNA; d, PSA. Pea epicotyl tissues were treated with the respective lectins or distilled water (as the control), and the amount of accumulated pisatin was determined 18 h after the start of each treatment as described in the text. Each value represents the mean with standard deviation (SD) of results from three epicotyls.

Table 1 Effects of hapten sugars on the lectin-induced production of pisatin in pea epicotyl tissues

Treatment[a]	Pisatin accumulated (μg (g fr wt)$^{-1}$)[b]
Con A alone	21.8±4.7
+D-mannose	5.4±1.2
PHA alone	20.8±2.2
+*N*-acetyl-D-galactosamine	22.7±5.5
PNA alone	4.7±0.5
+D-galactose	9.7±1.3
PSA alone	3.1±0.9
+D-mannose	3.0±1.1

[a] Pea epicotyl tissues were treated with respective lectins (5 mg ml^{-1}) in the presence or absence of respective hapten sugars at 10 mM.

[b] The amount of accumulated pisatin in epicotyl tissues was determined 18 h after the start of respective treatments. Each value represents the mean with standard deviation (SD) of results from three epicotyls.

Table 2 Effects of various sugars on the production of pisatin in pea epicotyl tissues induced by concanavalin A

Hapten sugar[a]	% of control[b]
None (control)	100.0
N-Acetyl-D-galactosamine	107.1
N-Acetyl-D-glucosamine	107.9
D-Galactose	98.5
D-Glucose[c]	81.9
D-Fucose	104.1
D-Lactose monohydrate	107.9
D-Mannose[c]	45.9
Methyl-α-D-glucoside[c]	73.9
Methyl-α-D-mannoside[c]	23.9

[a] Pea epicotyl tissues were treated with Con A (5 mg ml^{-1}) in the presence or absence of respective hapten sugars at 10 mM.

[b] Each value is expressed as a percentage of the amount of pisatin accumulated in tissues in the absence of sugars (27.3±4.6 μg (g fr wt)$^{-1}$).

[c] Specific haptens of Con A. Carbohydrate specificity is in the order: methyl-α-D-mannoside>D-mannose>D-glucose (=methyl-α-D-glucoside), according to the manufacture's instruction.

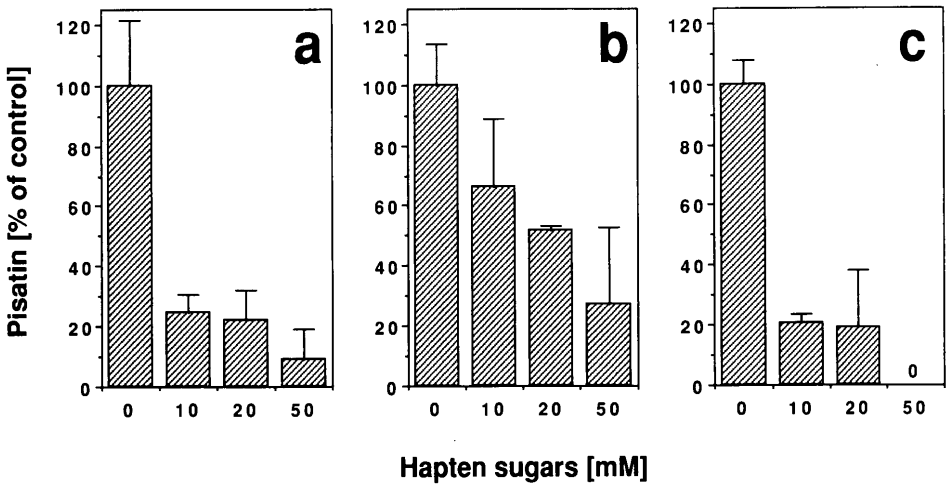

Fig. 2 Inhibition of the Con A-induced production of pisatin by several specific haptens. Pea epicotyl tissues were treated with Con A (5 mg ml^{-1}) in the simultaneous presence or absence of Man (a), αMG (b) and αMM (c). The amount of accumulated pisatin was determined 18 h after the start of treatment. Each value is expressed as a percentage of the amount of accumulated pisatin in epicotyls in the absence of hapten sugars (the amount of pisatin was 21.8±4.7 μg (g fr wt)$^{-1}$). Each result is the mean with standard deviation (SD) of the results obtained from three epicotyls.

Injury to epicotyl tissues is required for induction by Con A of the production of pisatin—When Con A was applied to uninjured (intact) tissues, no apparent production of pisatin was induced, as demonstrated in our earlier experiments with the fungal elicitor from *M. pinodes* (Table 3; Yamamoto et al. 1986).

Effect of the duration of treatment with Con A on the production of pisatin in pea epicotyl tissues—To determine the duration of treatment with Con A that is required for induction of the production of pisatin, pea epicotyl tissues were transferred from the solution of Con A to distilled water immediately (zero in Figure 4) or 15, 30, 60, 60, 90 and 120 min after the start of treatment with Con A, and they were further incubated for up to 18 h after the start of treatment with Con A. Accumulation of pisatin was detectable in tissues that had been in contact with Con A for a more 30 min, and the amount of pisatin increased in proportion to the duration of treatment with Con A (Fig. 4).

Inhibitory effects of the suppressor from M. pinodes and of several other inhibitors on the production of pisatin in pea epicotyl tissues induced by Con A—Induction of the production of pisatin by Con A was effectively inhibited by the concomitant presence of suppressor from *M. pinodes*, which inhibits the plasma membrane ATPase and PI metab-

Table 3 Production of pisatin in uninjured (intact) and injured pea epicotyl tissues treated with elicitor from *Mycosphaerella pinodes* and concanavalin A

Treatment with	Concentration	Injury	Pisatin accumulated (μg (g fr wt)$^{-1}$)[a]
Distilled water	—	−[c]	0.0±0.0
M. pinodes elicitor[b]	500 μg ml^{-1}	−	0.1±0.1
Con A	5 mg ml^{-1}	−	0.2±0.2
Distilled water	—	+[d]	0.8±0.3
M. pinodes elicitor[b]	500 μg ml^{-1}	+	6.4±2.1
Con A	5 mg ml^{-1}	+	6.9±0.9

[a] The amount of accumulated pisatin in epicotyl tissues was determined 18 h after the onset of respective treatments. Each value represents the mean with standard deviation (SD) of results from three epicotyls.

[b] Fungal elicitor was prepared from germination fluid of pycnospores of *M. pinodes* and tested at a concentration of 500 μg ml^{-1} (glucose equiv.).

[c] Forty microliters of solution were directly placed on the surface of pea epicotyl tissues.

[d] Excised pea epicotyl tissues (1.5 cm in length) were divided longitudinally into two parts with a razor blade and the pieces were placed on test solutions with the cut surface in contact with the solution.

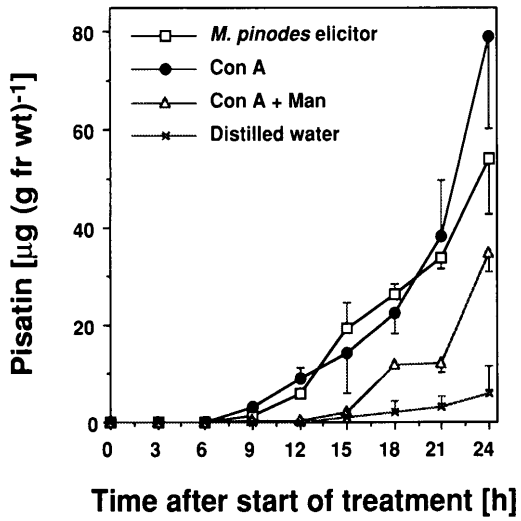

Fig. 3 Time course of the accumulation of pisatin in Con A-treated epicotyl tissues. The amount of accumulated pisatin was determined at 3-h intervals after the start of treatment with a fungal elicitor (500 μg ml^{-1}, glucose equiv.) derived from *M. pinodes*, with Con A (5 mg ml^{-1}) alone, with Con A plus 10 mM Man or with distilled water. Each value represents the mean with standard deviation (SD) of results from three epicotyls.

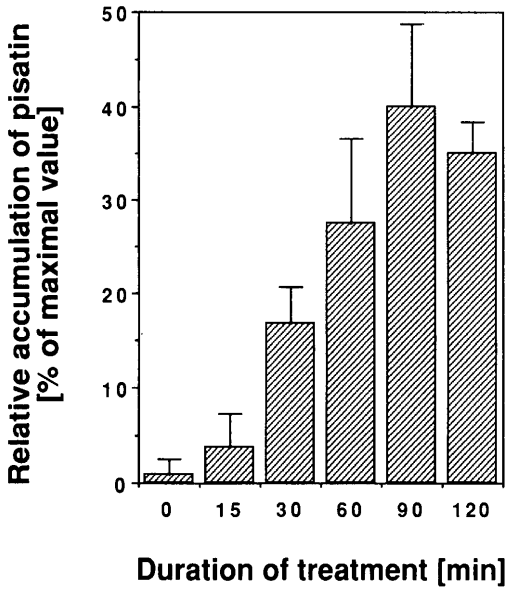

Fig. 4 Effect of the duration of treatment with Con A on the production of pisatin in pea epicotyl tissues. Pea epicotyl tissues were transferred from a solution of Con A (5 mg ml^{-1}) to distilled water, immediately (zero in the Figure) or 15, 30, 60, 90 and 120 min after the start of treatment with Con A. The amount of accumulated pisatin was determined 18 h after the start of treatment with Con A. Treatment of pea epicotyl tissues with Con A for 18 h induced production of 30.9±5.0 μg (g fr wt)$^{-1}$ of pisatin. Each value is expressed as a percentage of the amount of accumulated pisatin in pea epicotyl tissues that were treated with Con A for 18 h.

olism in pea tissues (Shiraishi et al. 1994a, b, Toyoda et al. 1992, 1993). The Con A-induced production of pisatin was also effectively inhibited by vanadate, verapamil and neomycin (Table 4). These observations resemble those obtained with the fungal elicitor from *M. pinodes* in the pres-

Table 4 Inhibitory effects of the suppressor from *Mycosphaerella pinodes*, vanadate, verapamil and neomycin on the production of pisatin in pea epicotyl tissues treated with concanavalin A

Treatment with	Concentration	Pisatin accumulated (μg (g fr wt)$^{-1}$)[a]	% of control[b]
Distilled water	—	0.8±0.3	11.1
Con A (5 mg ml^{-1}) alone	—	6.9±0.9	100.0
+*M. pinodes* suppressor[c]	50 μg ml^{-1}	1.3±0.9	19.3
	100 μg ml^{-1}	0.3±0.1	4.6
+vanadate	0.25 mM	3.9±0.9	56.4
	1 mM	0.2±0.2	3.5
+verapamil	0.25 mM	0.4±0.2	5.9
	1 mM	0.3±0.1	3.6
+neomycin	0.25 mM	0.7±0.3	9.5
	1 mM	0.1±0.1	1.6

[a] The amount of accumulated pisatin in epicotyl tissues was determined 18 h after the start of respective treatments. Each value represents the mean with standard deviation (SD) of results from three epicotyls.

[b] Each value is expressed as a percentage of the amount of pisatin accumulated in tissues treated with Con A alone.

[c] The suppressor was prepared from germination fluid of pycnospores of *M. pinodes* and the concentration was determined with BSA as the standard.

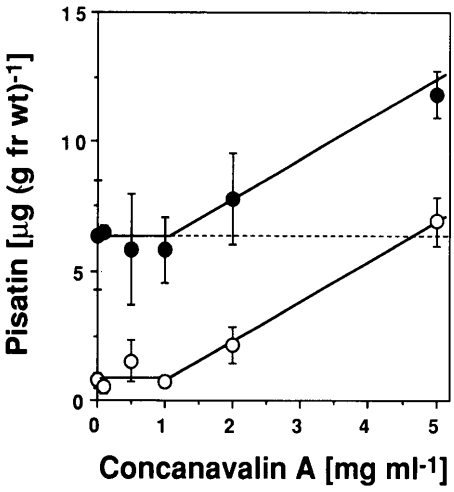

Fig. 5 The additive effect of Con A on the production of pisatin in pea epicotyl tissues that were treated with fungal elicitor from *M. pinodes*. Pea epicotyl tissues were treated with Con A at concentrations of 0.1 to 5 mg ml^{-1} in the presence (●) or absence (○) of the fungal elicitor (500 $\mu g\ ml^{-1}$, glucose equiv.). The accumulated pisatin was quantified after 18 h. Broken line indicates the amount of pisatin induced by the fungal elicitor alone. Each value represents the mean with standard deviation (SD) of results from three epicotyls.

ence of these inhibitors (Shiraishi et al. 1992, Toyoda et al. 1992, 1993, Yoshioka et al. 1990, 1992).

Additive effect of Con A on the fungal elicitor-induced production of pisatin in pea epicotyl tissues— Treatment with both fungal elicitor and Con A at various concentrations resulted in a marked elevation of the production of pisatin in pea epicotyl tissues, as compared to that achieved with each respective solution alone (Fig. 5). That is, the simultaneous presence of Con A and the elicitor had an additive effect on the accumulation of pisatin. This result indicated that exogenous Con A did not interfere with the induction by the elicitor from *M. pinodes* (Fig. 5).

Discussion

In this study, we found that exogenously applied plant lectins were able to induce the production of pisatin, which is one of defense responses in pea (Table 1, Fig. 1), as do fungal elicitors (Shiraishi et al. 1978, Thanutong et al. 1982). The extent of induction of the production of pisatin varied among the lectins tested. The effective concentrations of lectins on plant cells were, however, higher than those on mammalian cells (Fig. 1; Ferber and Resch 1973, Szamel et al. 1980). This result suggests that amounts of lectins that reach and bind to their respective target molecules are reduced as a consequence of their restricted translocation across certain plant-specific barriers, such as the cell walls. Alternatively, the sensitivity of plant cells to lectins might be constitutively different from that of mammalian cells. The former explanation seems plausible since treatment of uninjured pea tissues with Con A did not induce the production of pisatin (Table 3), and the production of pisatin was induced in injured tissues by treatment with Con A for at least 30 min. Detailed analysis is now needed to clarify the correlation between binding affinity and elicitor activity using isolated protoplasts or plasma membranes, as established elsewhere (Cheong and Hahn 1991, Diekmann et al. 1994).

Lectins are proteins or glycoproteins that recognize and bind to specific glycoproteins, glycolipids or polysaccharides with high affinity (Goldsten and Hayes 1978). Exogenous lectins induce diverse cellular responses in mammalian cells (Ferber and Resch 1973, Szamel et al. 1980). The elicitor effect of Con A was effectively inhibited by specific haptens, such as Man, αMM, Glc and αMG (Table 1, 2, Fig. 2), while the effects of PHA, PNA and PSA were not negated by the respective specific haptens (Table 1). Most plant lectins have been characterized with respected to their structures and binding properties, and a comparison of amino acid sequences reveals that lectins from leguminous plants, despite difference in sugar-binding specificities, contained conserved regions (Cunningham et al. 1979, Foriers et al. 1977, 1978, Miller et al. 1975). It is, therefore, possible that certain sequences and/or three-dimensional structures in PHA, PNA or PSA might be involved in the induction of production of pisatin in pea tissues. However, it is more likely that the effect of Con A is attributable to the lectin's ability to bind specific carbohydrates in pea cells. In other words, it is possible that a certain molecule with mannosyl and/or glucosyl residue(s) is crucially involved in the initial process of biosynthesis of pisatin that is induced by Con A.

In previous studies we showed that synthesis of pisatin is associated with activation of a signal-transduction pathway that is dependent on PI metabolism (Toyoda et al. 1992, 1993). We also demonstrated that both the suppressor and vanadate, which inhibit the plasma membrane ATPase, severely depress PI metabolism, suggesting a close, functional association between ATPase and PI metabolism (Shiraishi et al. 1994a, b). In our present study, we observed that the elicitor effect of Con A was also inhibited by the suppressor from *M. pinodes*, vanadate, verapamil and neomycin (Table 4), which have been reported to inhibit PI metabolism that is, in turn, responsible for the induction of the production of pisatin (Toyoda et al. 1992, 1993). In addition, the pattern of accumulation of pisatin induced by Con A was quite similar to that induced by a polysaccharide elicitor from *M. pinodes* (Fig. 3). These results indicate that exogenous Con A mimics the fungal elicitor and, moreover, that induction of the production of pisatin by Con A also involves the activa-

tion of PI metabolism. It is, thus, possible that a putative Con A-binding molecule(s) might be similar to a receptor molecule for fungal elicitor that is tightly linked to PI metabolism. However, it is important to remember that Con A did not interfere with the action of fungal elicitor since Con A had an additive effect on the accumulation of pisatin in pea tissues that was induced by the fungal elicitor (Fig. 5). The putative Con A-binding site may be different from that of the fungal elicitor, even if both sites exist on the same molecule. Characterization of the target molecule(s) of Con A will facilitate identification of the signal-transduction system that is linked with the receptor molecule(s) for the fungal elicitor.

Several studies have shown that plasma membranes or microsome fractions from plants contain certain proteinaceous molecules that recognize (bind) carbohydrate elicitors, such as hepta-β-glucosides (Cheong and Hahn 1991, Cheong et al. 1993), β-glucans (Cosio et al. 1988, 1990, 1992, Schmidt and Ebel 1987, Yoshikawa et al. 1983, 1993) and *N*-acetylchitooligosaccharides (Shibuya et al. 1993). However, the relationship between the corresponding binding proteins and signal-transduction systems is unknown. In mammalian cells, Con A receptors located on platelet membranes are associated with a GTP-binding protein (Torti et al. 1991). Our present data also indicate that a putative lectin receptor, which is recognized by Con A, may be located in plasma membranes rather than on the outer surface (cuticle) of epicotyl tissues since induction of pisatin by Con A was not observed when Con A was applied to intact tissues (Table 3), as mentioned above. Recently, we isolated a protein complex (mol wt=ca. 530 kDa) from detergent-solubilized plasma membranes of pea, that contained a vanadate-sensitive plasma membrane ATPase and the PtdIns kinase responsible for PI metabolism. The complex was isolated by HPLC on a Con A-affinity column (our unpublished data). Thus, Con A appears to be a very useful probe for investigation of molecular mechanisms involved in the synthesis of phytoalexin in pea plants.

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