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Suppression of Pisatin, Phenylalanine Ammonia-Lyase mRNA, and Chalcone Synthase mRNA Accumulation by a Putative Pathogenicity Factor from the Fungus *Mycosphaerella pinodes*

Tetsuji Yamada, Hisako Hashimoto, Tomonori Shiraishi, and Hachiro Oku

Laboratory of Plant Pathology, College of Agriculture, Okayama University, Tsushima, Okayama, 700, Japan.
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Effects of the fungal suppressor isolated from a pea pathogen, *Mycosphaerella pinodes*, were examined on pea defense reactions with respect to pisatin accumulation, phenylalanine ammonia-lyase (PAL) activity, and the corresponding PAL or chalcone synthase (CHS) mRNA level. Treatment of etiolated pea epicotyl tissues with elicitor activates the accumulation of PAL and CHS mRNAs within 1 hr, followed by an increase in PAL enzyme activity and in pisatin biosynthesis. Concomitant presence of suppressor with elicitor results in delays of these host defense reactions, and include a 3-hr delay in the accumulation of PAL and CHS mRNAs, a 6-hr delay in the increase of PAL enzyme activity, and a 6- to 9-hr suppression of pisatin accumulation. These results demonstrate that the fungal suppressor could play an important role in the host-parasite interaction by delaying the defense response of a plant.

Additional keyword: *Pisum sativum*.

Plants show natural disease resistance that involves inducible defense mechanisms, including the accumulation of phytoalexins, deposition of ligninlike materials, accumulation of cell wall hydroxyproline-rich glycoproteins (HRGPs), and increase in the activity of certain hydrolytic enzymes, such as chitinase and glucanase (Bell *et al.* 1986; Sequeira 1983). Inducible defense mechanisms are stimulated not only by microbial infections but also by biotic elicitors such as glycans, glycoproteins, or lipids isolated from fungal cell walls, or by abiotic elicitors such as UV light, some chemicals, or wounding (Darvill and Albersheim 1984; Dixon *et al.* 1983).The defense reaction is an active process that involves a series of defense gene expressions. Marked changes in the pattern of the specific RNA involving phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) genes, encoding the key enzymes leading to phytoalexin production, and the HRGP gene, have been shown in bean suspension-cultured cells or epicotyl tissues treated with *Colletotrichum lindemuthianum* (Saccardo *et* Magnus) Briosi *et* Cavara elicitor (Edwards *et al.* 1985; Ryder *et al.* 1984; Showalter *et al.* 1985).

A major question in relation to the early molecular events in the host-parasite interaction is what are the key factors that allow pathogens to escape the natural resistance mechanisms of the host. We have shown that phytoalexins are produced earlier during the infection of plants by incompatible fungi than in response to compatible fungi (Oku *et al.* 1975a; Oku *et al.* 1975b; Shiraishi *et al.* 1977). What determines host susceptibility may be largely dependent on whether a fungus can suppress phytoalexin production or not (Shiraishi *et al.* 1978). This was supported by the observation that the artificial administration of phytoalexin to the host plant early during the infection process significantly inhibits the infection by compatible fungi (Oku *et al.* 1975a; Oku *et al.* 1976). Thus, suppression of the defense responses of the plant could be an important event in pathogenesis. This possibility is also supported by the observation that induction of phytoalexin in barley leaves by an incompatible race of *Erysiphe graminis* DC. f. sp. *hordei* Marshal was suppressed by preliminary inoculation with a compatible race (Oku *et al.* 1975b; Oku *et al.* 1980).

Pycnospore germination fluid of *Mycosphaerella pinodes* (Berk. *et* Blox.) Stone, a fungus pathogenic on pea, contains both elicitors (high molecular mass glucans or glycoproteins [> 70,000 Da]) and suppressors (low molecular mass glycopeptides [< 5,000 Da]) of the accumulation of the pea phytoalexin, pisatin (Oku *et al.* 1977; Oku *et al.* 1987; Shiraishi *et al.* 1978). Purification of the suppressor from the low molecular mass fraction of the germination fluid by gel filtration and thin-layer chromatography gave two active components, F2 and F5 (Shiraishi *et al.* 1978). The concomitant presence of F5 with the elicitor negates the activity of the elicitor, that is, the induction of pisatin biosynthesis as well as PAL activity are suppressed in the mesophyll tissue of pea leaves (Hiramatsu *et al.* 1986). The suppressor could act by 1) inhibiting some of the enzyme activities leading to phytoalexin production, 2) blocking the signal transduction of the systematic defense reactions by the elicitor, or 3) repressing the transcription of the defense genes.

We began answering these questions by examining the accumulation of PAL and CHS mRNAs in etiolated pea epicotyls treated with elicitor in the presence or absence of the suppressor. Furthermore, PAL enzyme activity as well as pisatin accumulation were also determined.

MATERIALS AND METHODS

Plant material. Seeds of *Pisum sativum* L. cv. Alaska were soaked overnight, sown on vermiculite in a plastic container, and grown in an incubator at 22° C in the dark for 6–10 days. In all experiments epicotyls were cut into

Address correspondence to T. Yamada.

3-cm-long pieces (approximately 100 mg), and each piece was further sliced longitudinally in the middle along the epicotyl line with a razor blade.

Preparation of elicitor and suppressor. To explain the overall effects of suppressors on pea defense reactions, we used a crude suppressor fraction instead of an F2 or F5 fraction in this study. Crude elicitor and suppressor fractions were prepared from spore germination fluid of *M. pinodes*, OMP-1 (American Type Culture Collection [ATCC] 42741) using the method described by Hiramatsu *et al.* (1986) with a slight modification. Pycnospores formed on Czapek agar medium were collected by rubbing the colony surface with a platinum loop and suspending the spores in aseptic deionized water to give 2×10^6 spores per milliliter. The spores were allowed to germinate at 22–25° C for 24 hr and were filtered through Whatman No. 1 filter paper to eliminate spores. The filtrate was further filtered through a Millipore filter (PTGC 142-05) to separate molecules with a molecular mass smaller than 20,000 Da from the molecules with a larger molecular mass.

The upper residue (molecular mass > 20,000 Da), which contains the elicitor, was dialyzed exhaustively against aseptic deionized water to eliminate the low molecular mass substances, and the final concentration of the inner dialysate was adjusted to a 1 mg/ml of glucose equivalent. The filtrate (molecular mass < 20,000 Da), which contains suppressors, was concentrated in a freeze-dryer and dissolved in a minimum volume of aseptic deionized water. The solution was extracted with an equal volume of *n*-butanol several times. The butanol solution was air-dried at room temperature, and the residue was dissolved in aseptic deionized water to give a final protein concentration of 100 μg/ml (bovine serum albumin [BSA] equivalent). The concentration ratio of crude elicitor to crude suppressor from the original spore germination fluid was about 1:1 (Shiraishi *et al.* 1978). The concentration of the suppressor in the spore germination fluid was high enough to completely suppress the activity of the elicitor.

Determination of pisatin accumulation. Epicotyl pieces were soaked for 5 min. in the elicitor solution (500 μg/ml of glucose equivalent) in the presence or absence of suppressor (50 μg/ml of BSA equivalent) and extra solutions were wiped off. The treated epicotyl pieces were incubated at 22° C in the dark, and pisatin was extracted from approximately 100 mg of the epicotyl tissues at 3-hr intervals by the addition of 1 ml of ethanol and heating at 80° C for 10 min. The extract was evaporated under reduced pressure, and the residue was dissolved in 100 μl of high performance liquid chromatography (HPLC) solvent (*n*-hexane:tetrahydrofuran:acetic acid, 88:12:0.5). Ten microliters of the solution was analyzed by HPLC to determine the concentration of pisatin (Masuda *et al.* 1983).

Enzyme extraction and assay. Epicotyl tissues were treated with elicitor in the presence or absence of suppressor and incubated for 3, 6, 9, 12, 15, 18, 21, and 24 hr in the dark at 22° C. At each time point, approximately 100 mg of each sample was frozen in liquid nitrogen and stored at −80° C until used. After gently homogenizing the frozen epicotyl tissues in a cold (4° C) mortar, enzyme was extracted with 1.0 ml of 100 μM sodium borate buffer (pH 8.8). Cellular debris was removed by centrifugation at 4° C for 20 min at 12,000 × *g* and the supernatant was used as the crude enzyme solution. PAL activity was determined by the method described previously (Dixon and Fuller 1978; Hiramatsu *et al.* 1986).

Isolation of RNA. Twenty grams (fresh weight) of the epicotyl tissues treated with elicitor in the presence or absence of suppressor for 1, 2, 3, 4, 5, 6, 7, 8, and 9 hr (1–6 hr in the absence of suppressor) at 22° C in the dark was frozen in liquid nitrogen and stored at −80° C until used. Total cellular RNA was isolated from the tissue by a modification of the method of Chirgwin *et al.* (1979). The homogenized tissues were extracted with 100 ml of GIT buffer containing 4 M guanidine isothiocyanate, 75 mM sodium acetate (pH 6.0), and 75 mM 2-mercapto-ethanol. Sarkosyl solution (10%) was added to a final concentration of 0.5%, and the mixture was slowly shaken for 40 min at 60° C. Cellular debris was removed by centrifugation for 20 min at 12,000 × *g*. Three milliliters of the supernatant was layered on top of 1.7 ml of CsCl solution containing 5.7 M CsCl and 25 mM sodium acetate in a 5-ml ultracentrifugation tube and centrifuged at 44,000 rpm for 24 hr at 20° C in a Hitachi RPS50 rotor (210,000 × *g*). The pellet was dissolved in 100 μl of TESS (10 mM Tris-Cl [pH 7.5], 1 mM EDTA, 100 mM NaCl, and 10 mM 2-mercaptoethanol) and further purified as described previously (Ausubel *et al.* 1987).

Northern blot hybridization. Ten micrograms of RNA was denatured with formaldehyde and separated by electrophoresis on a 1.2% agarose gel (Maniatis *et al.* 1982). As molecular weight standards, *Escherichia coli* 23S and 16S rRNA (Pharmacia, Piscataway, NJ) and *Hin*dIII-digested λ-DNA denatured with formaldehyde were separated on the same agarose gel and were blotted onto a nylon membrane filter (Hybond-N, Amersham, Arlington Heights, IL). For the preparation of a probe, a bean PAL cDNA clone, pPAL 5 (Edwards *et al.* 1985), or a bean CHS cDNA clone, pCHS 1 (Ryder *et al.* 1984) (gifts from C. J. Lamb, Salk Institute, University of California, San Diego), were digested with the appropriate restriction endonucleases to separate the cDNA insert from the cloning vector.

The cDNA fragment was isolated from agarose gel using a NACS column (Bethesda Research Laboratories [BRL], Gaithersburg, MD) according to the manufacturer's specifications and labeled with a Multiprime DNA labeling system (Amersham). Hybridization was performed at 42° C in a buffer containing 50% formamide, 0.75 M NaCl, 0.075 M sodium citrate (pH 7.0), 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA, 5 mM EDTA, 0.1% sodium dodecyl sulfate, and 100 μg of denatured calf thymus DNA per milliliter. Filters were preincubated for 48 hr in buffer without a probe, followed by hybridization for 48 hr in the presence of a ^{32}P-labeled probe. To prepare an internal standard, *Eco*RI-digested total pea genomic DNA was shotgun-cloned in pUC19, and several clones were tested for hybridization with total pea RNA as described above. One of the genomic DNA clones, No. 24, that contains sequences complementary to an abundant transcript but not induced by the elicitor was used as an internal standard.

RESULTS

Time required for activation of pisatin biosynthetic

system by elicitor in the presence or absence of suppressor. Pea epicotyl segments were treated with the elicitor (500 μg/ml of glucose equivalent) alone or in the concomitant presence of the suppressor (50 μg/ml of BSA equivalent) (elicitor plus suppressor). As a control, epicotyl segments were treated with sterilized distilled water. The amount of accumulated pisatin in epicotyl tissues with respect to the time of incubation after the treatment was determined by HPLC (Fig. 1). The apparent activation of pisatin biosynthesis was initiated approximately 6 hr after treatment with the elicitor, and pisatin accumulation peaked at about 36 hr, followed by a gradual decline. In the presence of the suppressor, however, the apparent activation of pisatin biosynthesis was delayed by 6–9 hr compared to the treatment with elicitor alone, although the pattern of pisatin accumulation had very similar kinetics. On the other hand, only very little pisatin was synthesized when treated with water or suppressor alone, and possibly it was induced by the wounding of the epicotyls.

Time required for activation of PAL enzyme activity by elicitor in the presence or absence of suppressor. Relative PAL activity in the crude enzyme solution was determined by the rate of conversion of ^{14}C-phenylalanine into ^{14}C-cinnamic acid. Changes in PAL activity in elicitor-treated epicotyl tissues in the presence or absence of the suppressor with respect to time (in hours) of the treatment are shown in Figure 2. The activation of PAL activity had very similar kinetics in elicitor-treated and elicitor plus suppressor-treated tissues, except for a delay of about 6 hr as observed in the pattern of pisatin biosynthesis as shown in Figure 1. As in the case of pisatin accumulation, very little PAL activity was observed by treatment with water.

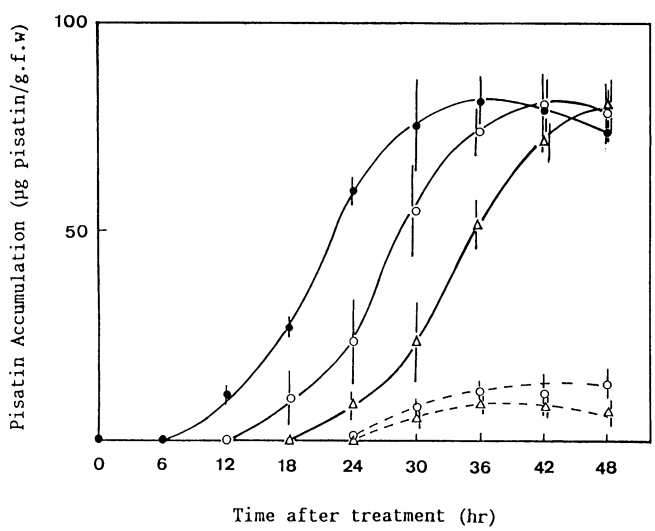

Fig. 1. Pisatin accumulation in elicitor-treated epicotyl tissues in the presence or absence of suppressor, and the effect of additional treatment with fresh suppressor. The concentrations of pisatin (μg pisatin per gram of fresh weight epicotyl tissues) were determined by high performance liquid chromatography (HPLC) every 3 hr after treatment with elicitor alone (—●—) and with elicitor plus suppressor (—○—) until 48 hr. Epicotyl segments were further treated with fresh suppressor 3 hr after the initial treatment with elicitor plus suppressor (—△—) and the amount of pisatin accumulation was also determined. As a control, the concentrations of pisatin were determined in water-treated (- - -△- - -) and suppressor-treated (- - -○- - -) epicotyl tissues as described in the text. Each plotted value represents the mean of triplicate experiments. The bar indicates the standard deviation.

Time required for transcriptional activation of PAL and CHS genes. To determine whether the suppressor affects the transcriptional activation of the genes leading to pisatin accumulation, both PAL and CHS mRNA levels in elicitor-treated tissues and elicitor plus suppressor-treated tissues were determined by northern blot hybridization with bean PAL or bean CHS cDNA as probes. For analyzing the PAL mRNA level, 2 μg of the PAL cDNA fragment was labeled with ^{32}P[dCTP] (approximately 1×10^8 cpm/μg of DNA) and a hybridization reaction was conducted. As shown in Figure 3, PAL cDNA hybridized to three RNA species of 2.8, 2.0, and 1.5 kilobases (kb) present in total cellular RNA isolated from elicitor-treated epicotyl tissues. In elicitor plus suppressor-treated tissues, the probe hybridized to two RNA species of 2.8 and 2.0 kb. According to the size of the purified pea PAL (81,000 Da) determined by Loschke and Hadwiger (1981), the 2.8-kb RNA species that hybridized strongly to bean PAL cDNA is considered to be a major pea PAL mRNA.

Elicitor treatment caused a marked and prolonged accumulation of the putative pea PAL mRNA as compared with relatively low basal levels in unelicited tissues (Fig. 3C). Dramatic increases in the 2.8-kb RNA species were first observed about 1 hr after elicitor treatment, after which the mRNA remained at high levels in the elicitor-treated tissues (Fig. 3A). The accumulation of the 2.0- and 1.5-kb RNA species followed a similar pattern, although at each time point these forms were considerably less abundant than the 2.8-kb RNA species. On the other hand, the dramatic increase in the 2.8-kb RNA species was first observed about 4 hr after elicitor plus suppressor treatment (Fig. 3B), and accumulation of only a 2.0-kb RNA followed a similar pattern. There was no appreciable increase in the level of the putative PAL mRNAs in the tissues treated with water (Fig. 3C). Furthermore, it is apparent that the increase in PAL mRNA accumulation shows at least two phases, one is approximately 1–2 hr after the initial

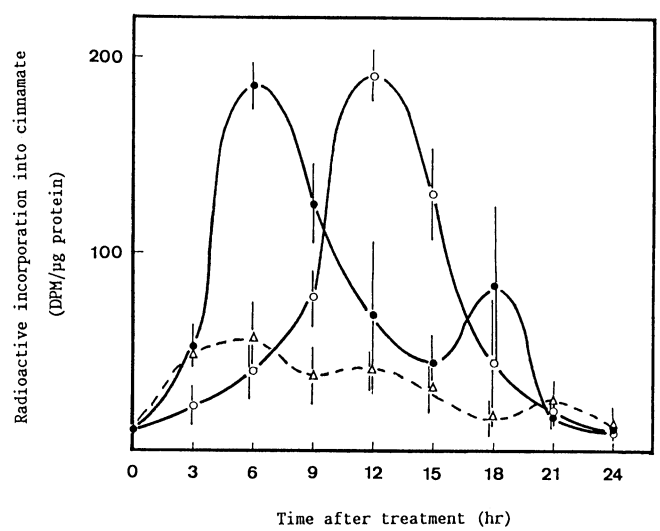

Fig. 2. Changes in phenylalanine ammonia-lyase (PAL) activity with respect to time after treatment with elicitor or elicitor plus suppressor. Incorporation of ^{14}C from L-phenylalanine into cinnamic acid was determined 3, 6, 9, 12, 15, 18, 21, and 24 hr after treatment with the elicitor (—●—) or with elicitor plus suppressor (—○—). As a control, PAL activity was also determined in water-treated epicotyl tissues (- - -△- - -). Each plotted value represents the mean of triplicate experiments. The bar indicates the standard deviation.

induction and the other is approximately 4–5 hr, although the exact mechanism of this event is unknown.

For the analysis of CHS mRNA accumulation, 2 μg of the ^{32}P-labeled bean CHS cDNA fragment was hybridized in the same way as in the analysis of PAL mRNA. Bean CHS cDNA hybridized to a single band of a 2.0-kb RNA species present in total cellular RNA isolated from both elicitor-treated tissues and elicitor plus suppressor-treated tissues. A dramatic increase in the 2.0-kb RNA species was first observed about 1 hr after elicitor treatment (Fig. 4A) and about 4 hr after elicitor plus suppressor treatment (Fig. 4B), which is a pattern very similar to that observed in PAL mRNA. There was no appreciable increase in the level of CHS mRNA in the cells treated with water (Fig. 4C) as a control. Furthermore, as observed in PAL mRNA accumulation, the increase in the CHS mRNA accumulation also has at least two phases similar to PAL mRNA.

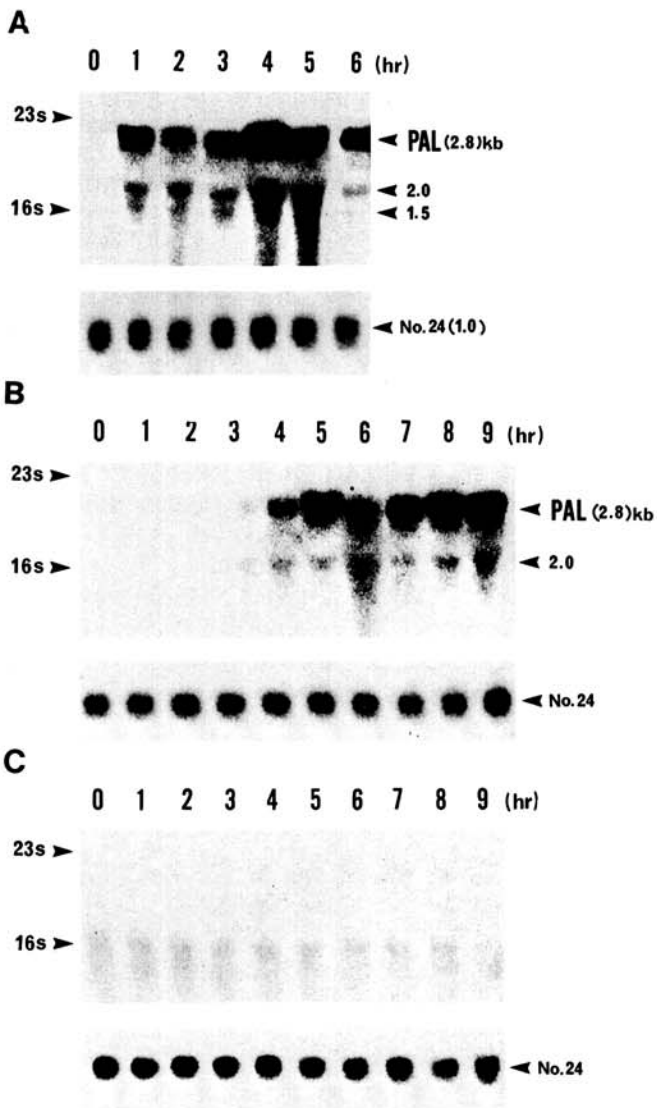

Fig. 3. Induction of phenylalanine ammonia-lyase (PAL) mRNA in elicitor- or elicitor plus suppressor-treated pea epicotyl tissues. Ten micrograms of total RNA isolated from epicotyl tissues treated with (**A**) elicitor, (**B**) elicitor plus suppressor, or (**C**) water was blot hybridized with a ^{32}P-labeled bean PAL cDNA fragment as described in the text. Numbers at the top indicate time (in hours) of incubation after treatment. The position of the putative pea mRNA is indicated with an arrow. The genomic DNA clone, No. 24, which contains sequences complementary to an abundant transcript (approximately 1.0 kb), was labeled with ^{32}P and hybridized as an internal standard.

Duration of suppressor activity. To rule out the possibility that the suppressor may be inactivated by the elicitor (for example, by enzyme degradation), we designed the following experiments.

According to the report by Wingate *et al.* (1988), the reduced form of glutathione (γ-L-glutamyl-L-cysteinyl-glycine) is elicitor-active on bean cells, and we found this compound to have an elicitor activity in pea with the optimum concentration of 50 mM (unpublished data). The amount of accumulated pisatin in epicotyl pieces treated with 50 mM glutathione in the presence or absence of the suppressor was determined every 3 hr after the treatment of the epicotyl tissues, as described above, until 48 hr (Fig. 5). The pattern of pisatin accumulation in glutathione-treated tissues and glutathione plus suppressor-treated tissues was very similar to the one observed in the fungal elicitor-treated or elicitor plus suppressor-treated tissues as shown in Figure 1.

To determine the duration of suppressor activity, epicotyl pieces treated with elicitor in the presence of the suppressor were further treated with fresh suppressor 3 hr after the initial elicitor plus suppressor treatment, and the amounts of accumulated pisatin in the treated epicotyl tissues were determined (Fig. 1.). Treatment with the subsequent addition of fresh suppressor results in prolonged delay in the activation of pisatin biosynthesis. This result indicates that the presence of fresh suppressor prolongs suppressive activity on the induction of pea defense reactions.

Our results demonstrate that the duration of suppressor activity was not affected by the factors present in the elicitor preparation but rather was affected by the factors present in the plant.

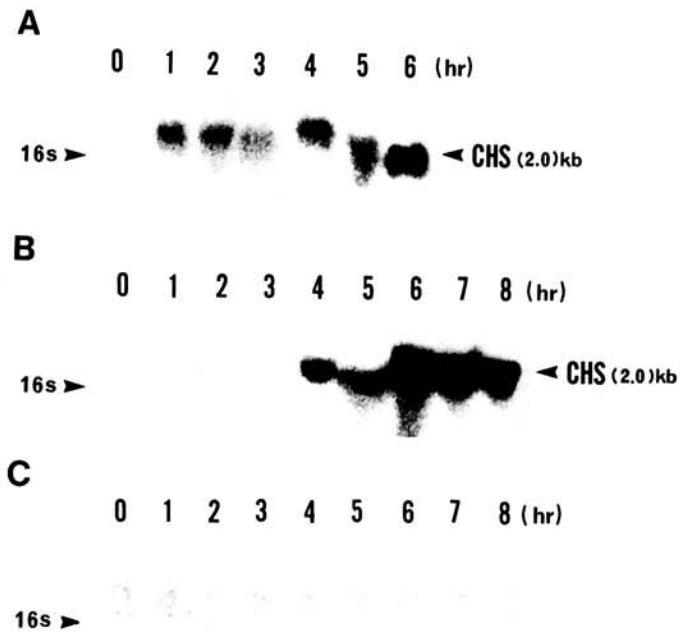

Fig. 4. Induction of chalcone synthase (CHS) mRNA in elicitor- or elicitor plus suppressor-treated pea epicotyl tissues. RNA was blot hybridized with a ^{32}P-labeled bean CHS cDNA fragment as described in Figure 3.

DISCUSSION

Our results with the fungal suppressor isolated from a pea pathogen, *M. pinodes*, on the response of the host defense reactions induced by the fungal elicitor suggest that the suppressor may be acting at or before the transcriptional level for the genes coding for the key enzymes, PAL and CHS, leading to phytoalexin production. Delayed induction of the defense reactions in elicitor plus suppressor-treated epicotyl tissues includes a 3-hr delay in the accumulation of PAL and CHS mRNAs, a 6-hr delay of PAL enzyme activity, and a 6-to 9-hr suppression of pisatin accumulation compared to elicitor-treated tissues. The limited time of the delay of the defense reactions may result from the degradation of suppressor by the host, because the treatment with glutathione instead of the elicitor preparation showed the same pattern of the shift on pisatin accumulation. The suppressor itself is not likely to contain factors that inactivate it, because the suppressor does not lose its activity after incubation at room temperature for several hours. That the subsequent addition of fresh suppressor 3 hr after the initial treatment with elicitor plus suppressor further delays the activation of pisatin biosynthesis supports the idea that the duration of suppressor activity is limited only to a few hours, and factors affecting the duration of suppressor activity may reside in the host cells rather than in the elicitor or suppressor preparation. Because proteinase K-treated suppressor loses its activity dramatically (unpublished results), host cells may have the proteolytic enzymes to inactivate the suppressor.

It should be noted that our results are in close agreement with the *in situ* hybridization study of the accumulation of potato PAL mRNA reported by Cuypers *et al.*(1988). They have demonstrated that the timing of PAL mRNA accumulation differed markedly between two types of interactions, compatible and incompatible races of *Phytophthora infestans* (Montagne) de Bary, against young potato leaves (*Solanum tuberosum* L.) cv. Datura. A marked increase in the accumulation of PAL mRNA was observed 3 hr after inoculation of the incompatible race at the infection sites, whereas, it was 6 hr after inoculation of the compatible race. This coincidence of a 3-hr delay in the accumulation of PAL mRNA in a compatible interaction further supports the idea that some factors such as fungal suppressors might determine host specificity.

An interesting feature of the northern blots probed with bean PAL cDNA is the presence of weakly hybridizing RNA species of 2.0 and 1.5 kb in addition to the strong hybridization of the 2.8-kb RNA species of the putative PAL mRNA, while the 1.5-kb RNA species was absent in elicitor plus suppressor-treated tissues. Because the minor RNA species were induced by the elicitor, they might be mRNA of minor PAL subunit(s) (Hanson and Havir 1981). It is possible that these minor RNAs are the degradation products of the major 2.8-kb RNA species, but if that is true, the 1.5-kb minor RNA species should also have been observed in elicitor plus suppressor-treated epicotyl tissues.

It appears that PAL and CHS gene expressions are coordinately regulated, because the induction of these gene expressions exhibits very similar kinetics after treatment with the elicitor in the presence or absence of the suppressor. Coordinate activation of PAL and CHS mRNA synthesis by fungal elicitor treatment in bean suspension culture as well as in bean hypocotyls has also been shown (Bell *et al.* 1986; Mehdy and Lamb 1987; Templeton and Lamb 1988).

To explain the role of the fungal suppressor on the host-parasite interaction, the suppressor gene(s) should be cloned and transformed into incompatible races of *M. pinodes* or nonpathogens of pea to test whether the transformant gains the ability to delay host defense reactions. Currently, we are establishing a transformation system for this fungus.

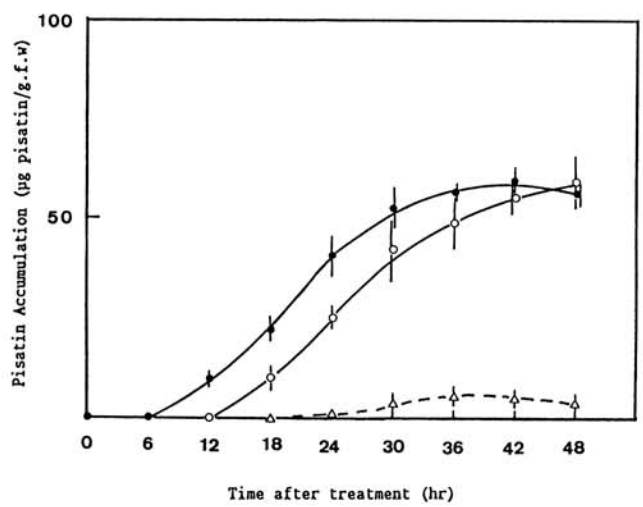

Fig. 5. Pisatin accumulation in glutathione-treated epicotyl tissues in the presence or absence of suppressor. The concentrations of pisatin (μg per gram of fresh weight epicotyl tissues) were determined by high performance liquid chromatography (HPLC) every 3 hr after treatment with glutathione (—●—), with glutathione plus suppressor (—○—), or water (- - -△- - -) until 48 hr. Each plotted value represents the mean of triplicate experiments. The bar indicates the standard deviation.

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