Suppression of Pisatin and Phenylalanine Ammonia Lyase-mRNA in a Compatible Interaction between *Pisum sativum* L. cv. Midoriusui and *Pseudomonas syringae* pv. *pisi*

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Abstract

The accumulation of pisatin in epicotyl tissues of *Pisum sativum* L. cv. Midoriusui inoculated with compatible races of *Pseudomonas syringae* pv. *pisi* (*P. pisi*) was significantly suppressed comparing to that by the inoculation with an incompatible race. This was reflected by the suppression of the accumulation of phenylalanine ammonia-lyase (PAL)-mRNA in the inoculated tissues. Low molecular mass (MW < ca. 10,000 Da) substances secreted in the culture filtrates from the compatible race of *P. pisi* exhibited the suppression of pisatin accumulation, whereas those from the incompatible race did not. The relationship between the suppression of host defense reactions and the specificity of race-cultivar in bacterial disease is discussed.

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Key words: *Mycosphaerella pinodes*, phenylalanine ammonia-lyase, *Pisum sativum* L. cv. Midoriusui, *Pseudomonas syringae* pv. *pisi*, suppressor, pisatin.

INTRODUCTION

Highly sophisticated and complexed biological programs underlie in the interaction between parasites and the host plants. A pycnosporer germination fluid of *Mycosphaerella pinodes* (Berk. et Blox.) Stone, a fungus pathogenic on pea, contains both elicitor (high molecular mass glucans or glycoproteins [$>70,000$ Da]) and suppressor (low molecular mass glycopeptides [$<5,000$ Da]) for the accumulation of the pea phytoalexin, pisatin^{8,9,12}. Concomitant presence of suppressor with elicitor results in delays of host defense reactions in pea epicotyl tissues that include a 3 hr delay in the accumulation of PAL- and chalcone synthase (CHS)-mRNAs, a 6 hr delay in the increase of PAL enzyme activity, and a 6 to 9 hr suppression of pisatin accumulation¹⁰. Yoshioka *et al.*¹⁸) and Shiraishi *et al.*¹¹) showed that *M. pinodes*-suppressor specifically inhibits pea plasma membrane (PM)-ATPase, and an inhibitor for PM-ATPase, orthovanadate, functions in a manner very similar to *M. pinodes*-suppressor. Currently, Toyoda *et al.*¹⁴) demonstrated that the polyphosphoinositide metabolism in pea plasma membrane plays a crucial role during the elicitation of defense responses. Recently, the structures of *M. pinodes*-suppressors, designated Suppressin A and B, have been determined as glycopeptides with mucin type, GalNAc-O-Ser-Ser-Gly and Gal(β 1-4)GalNAc-O-Ser-Ser-Gly-Asp-Glu-Thr¹³).

A number of suppressors from other phytopathogenic fungi have been reported, but very little studies have been performed on bacterial phytopathogens from the aspect of suppressor activity^{1,5}). Jakobek *et al.*³) reported that a compatible race of *P. syringae* pv. *phaseolicola* has an active mechanism to suppress the accumulation of phytoalexin and the transcripts encoding key enzymes in the biosynthesis of phaseolin such as PAL, CHS, CHI (chalcone isomerase) and CHT (chitinase) in bean.

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In this paper, we describe the suppression of the accumulation of pisatin and PAL-mRNA in epicotyl tissues of *Pisum sativum* L. cv. Midoriusui inoculated with a compatible race of *P. syringae* pv. *pisi*, and discuss the relationship between the suppression of host defense reactions and the specificity of race-cultivar.

MATERIALS AND METHODS

Bacterial strains and plant material. Strains of *Pseudomonas syringae* pv. *pisi* (*P. pisi*), 299A (race 1), 202 (race 2), 870A (race 3) and 895A (race 4) were obtained from Dr. J. D. Taylor, Institute of Horticulture Research, UK (Table 1). Bacteria were grown on a minimal medium (M9)⁽¹⁰⁾ or King's medium B⁽⁶⁾ at 27°C.

Seeds of *Pisum sativum* L. cv. Midoriusui (*P. sativum* cv. Midoriusui) were soaked in water overnight, sown on vermiculite in a plastic container, and grown in an incubator at 22°C for 6–10 days. In the experiments determining the level of pisatin accumulation, or the accumulation of PAL-mRNA, epicotyls were cut into 3-cm-long pieces, and each piece was further sliced longitudinally in the middle along the epicotyl line with a razor blade. To test for the pathogenicity, leaves from 2–3 month-old plant grown in a pot were used.

Pathogenicity test of different races of *P. pisi* to *Pisum sativum* L. cv. Midoriusui. Different race strains of *P. pisi* were grown in 10 ml of M9 medium at 27°C for a day, and harvested by centrifugation at $1,000 \times g$, at 4°C for 15 min. Pellets were resuspended in sterilized water at the concentration of $ca. 10^8$ cells/ml ($OD_{600} = 0.1$). Petioles of the primary and secondary leaves of *P. sativum* cv. Midoriusui were soaked in the bacterial suspension and incubated at 20–22°C for $ca. 10$ days under a daylight. As a control test, petioles were soaked in sterilized water. For each treatment, three to six plants were used and the experiments were repeated at least three times. Symptoms were recorded $ca. 10$ days after inoculation, and reactions exhibiting distinct water-soaking at leaves and/or petioles were regarded as susceptible (compatible) and the ones with no water-soaking or either localized necrosis were regarded as resistant (incompatible) (Table 1).

Preparation of the concentrated water-exudates from different race strains of *P. pisi*. Different race strains of *P. pisi* were inoculated in 500 ml of a minimal medium (M9), and bacterial cells were collected by centrifugation as described above. Pellets were resuspended in 300 ml of sterilized distilled water and recentrifuged to remove the ingredients in the medium. Pellets were resuspended in 400 ml of sterilized distilled water and incubated at 20–22°C for 2 days in a rotary shaker (100 rpm). After centrifugation, the supernatants were filter-sterilized with Steradisc 25 (Kurabo Inc., $0.45 \mu m$), freeze-dried, and dissolved in sterilized distilled water. A final concentration of the exudate was adjusted to 1 mg/ml of glucose equivalent as described⁽⁷⁾.

Fractionation of low molecular weight substances in the culture filtrates of *P. pisi*. Different race strains of *P. pisi* were inoculated in 500 ml of a minimal medium (M9), and bacterial cells were collected by centrifugation as described above. Culture-supernatant was filter-sterilized with Steradisc 25 (Kurabo Inc., $0.45 \mu m$). The filtrate was further filtered through a Millipore filter (PGTC 142-05) to separate molecules with a molecular mass smaller than $ca. 10,000$ Da from those with larger molecular mass. The filtrate (molecular mass $< 10,000$ Da) was freeze-dried, dissolved in sterilized distilled water, and exhaustively dialyzed against aseptic deionized water with a cellophane tube

Table 1. The relationship between *Pseudomonas syringae* pv. *pisi* races and *Pisum sativum* cv. Midoriusui

Cultivar	Strain			
	299A (race 1)	202 (race 2)	870A (race 3)	895A (race 4)
Midoriusui	+	–	+	+

+, compatible; –, incompatible.

Results from at least seven experiments.

(Spectra/Por molecularporous membrane tubing m.w. cutoff 1,000 Da, Spectrum medical Industries, Inc. Los Angeles, CA, USA) at 4°C to eliminate the low molecular mass substances such as the ingredients from the culture medium. The dialysate was freeze-dried and dissolved in a minimal volume of aseptic water. A final concentration of the culture filtrate was adjusted to 1 mg/ml of glucose equivalent (culture filtrate with LMW).

Preparation of elicitor from a fungus, *Mycosphaerella pinodes*. Crude elicitor was prepared from pycnosporer germination fluid of *M. pinodes*, OMP-1 (American Type Culture Collection [ATCC] 42741) using the method as described¹⁶⁾.

Determination of pisatin accumulation. Epicotyl pieces were soaked for 5 min in *M. pinodes*-elicitor (500 µg/ml of glucose equivalent) or in the concomitant presence of the various concentration of *P. pisi*-exudate or the culture filtrate with LMW, and extra solutions were wiped off. The treated epicotyl tissues were incubated at 22°C in the dark for the specified time and pisatin was extracted. The level of pisatin accumulation was determined using the high performance liquid chromatography (HPLC) as described¹⁶⁾.

Isolation of RNA and hybridization analysis to PAL-cDNA. Pea epicotyl tissues cut longitudinally in the middle of epicotyl line were inoculated with different race strains of *P. pisi*, and incubated at 20–22°C for the specified time. After the incubation, inoculated epicotyl tissues were chilled at –80°C, if not used immediately. Isolation of total RNA and Northern hybridization analysis were conducted as described previously^{16,17)}. Hybridization probes included the 1.7 kb *Hind*III-*Eco*RI fragment from pea PAL-cDNA⁴⁾ and 0.9 kb from pea histone H1-cDNA (a gift from Dr. S. Gantt²⁾), in which the specified sizes of ³²P-labeled DNA probes were synthesized by a random primer labeling method¹⁰⁾. Histone H1-cDNA probe was used as an internal standard that was constitutively expressed. Prehybridization and hybridization conditions were as specified¹⁰⁾. The specific radioactivity at the position of PAL-mRNA and histone H1-mRNA were counted and the relative value of PAL-mRNA against the constitutively expressed histone H1-mRNA was calculated by using Fuji Imaging Analyzer BAS2000 (Fuji Film Inc.).

RESULTS

Compatible race strains of *P. pisi* suppress the accumulation of pisatin

To examine the difference of the host defense responses by the inoculation with compatible and

Fig. 1. Changes of the accumulation of pisatin in pea (*Pisum sativum* L. cv. Midoriusui) epicotyl tissues inoculated with *P. s. pv. pisi* strain, 299A (race 1) and 202 (race 2), with respect to the time (hr) of incubation. The amount of pisatin was estimated as µg pisatin/g fresh weight tissues (g.f.w.). The bar indicates the standard deviation.

incompatible races, the accumulation of pisatin in epicotyl tissues of *P. sativum* cv. Midoriusui by the inoculation with different race strains were determined with respect to the time of incubation. As a control, epicotyl segments were treated with sterilized deionized water. The accumulation of pisatin initiated 16 hr after the inoculation with an incompatible race strain, 202 (race 2), and drastically increased (2-4 folds higher than that of water-treated as a control test) until *ca.* 48 hr after the inoculation, followed by the gradual decline, whereas the accumulation of pisatin by the inoculation with a compatible race strain, 299A (race 1), was initiated at 24 hr but the level was significantly lower than a control throughout the period tested (Fig. 1). The kinetics of the accumulation of pisatin by the inoculation with other compatible races, 870A (race 3) and 895A (race 4), were very similar to the one by the inoculation with race 1 (data not shown). The increase of the pisatin accumulation in water-treated epicotyls was possibly due to the induction by wounding.

These results indicate that the accumulation of pisatin is induced by the inoculation with an incompatible race, but it is rather suppressed by the inoculation with compatible races.

A compatible race strain, 299A, of *P. pisi* (race 1) suppresses the accumulation of PAL-mRNA

To further examine the difference of the host defense responses in the compatible and incompatible interactions between *Pisum sativum* L. cv. Midoriusui and *P. pisi*, the accumulation of PAL-mRNA in epicotyl tissues by the inoculation with different races of *P. pisi* with respect to the time of incubation

Fig. 2. Changes of the accumulation of Phenylalanine ammonia-lyase-mRNA in pea (*Pisum sativum* L. cv. Midoriusui) epicotyl tissues inoculated with *P. s. pv. pisi* strain, 299A (race 1) and 202 (race 2), with respect to the time (hr) of incubation. Ten micrograms of total RNA isolated from epicotyl tissues inoculated with race 1 strain, race 2 strain or water (control test) was blot hybridized with ^{32}P -labeled *Hind*III-*Eco*RI fragment (1.7 kb) from pea PAL-cDNA covering a part of exon $2^{4,17}$. Numbers on top indicate time (in hours) of incubation after the inoculation. Pea histone H1-cDNA 29 was labeled with ^{32}P and hybridized as an internal standard. Positions of PAL-mRNA (*ca.* 2.6 kb) and histone H1-mRNA (*ca.* 0.9 kb) are indicated with arrows. A graph below the autoradiogram denotes the relative value of the specific activity at the position of PAL-mRNA against that at the position of histone H1-mRNA.

were determined. The activation of the accumulation of PAL-mRNA initiated 6 hr and peaked 14 hr after the inoculation of incompatible race, 202, (race 2), followed by the gradual decline, whereas the accumulation of PAL-mRNA was suppressed throughout the period tested by the inoculation with a compatible race, 299A (Fig. 2). The level of the accumulation of PAL-mRNA was even lower than that by the treatment with water as a control.

These results demonstrate that the suppression of the accumulation of pisatin by the inoculation with a compatible race is reflected by the suppression of the expression of the gene encoding phenylalanine ammonia-lyase, a first key enzyme involved in phenylpropanoid pathway leading to pisatin biosynthesis.

Effects of the exudates from different races of *P. pisi* on the accumulation of pisatin in epicotyl tissues of *P. sativum* L. cv. Midoriusui

To test whether the suppressor-like element(s) could be secreted from compatible race strains of *P. pisi*, as in the case of *Mycosphaerella pinodes* in which suppressor secretes in pycnospore germination fluid of this fungus, the effect of the exudate from different race strains of *P. pisi* on the accumulation of pisatin in epicotyl tissues of *P. sativum* cv. Midoriusui was examined. The level of pisatin accumula-

Fig. 3. Changes of the accumulation of pisatin in pea epicotyl tissues treated with elicitor from *M. pinodes* in the absence or presence of the exudate from *P. s. pv. pisi* strain 895A (A) or 202 (B) with respect to the time (hr) of incubation. Epicotyl tissues were treated with *M. pinodes*-elicitor alone (Elicitor), or *M. pinodes*-elicitor in the concomitant presence of 500 μg/ml (glucose equivalent) of the exudate (500+E) or 100 μg/ml (glucose equivalent) (100+E). As a control, epicotyl tissues were treated with water (Water). The bar indicates the standard deviation.

Fig. 4. Changes of the accumulation of pisatin in pea epicotyl tissues treated with elicitor from *M. pinodes* in the absence or presence of the culture filtrate with LMW from *P. s. pv. pisi* strain, 299A (race 1) and 202 (race 2). Epicotyl tissues were treated with *M. pinodes*-elicitor alone (E), or *M. pinodes*-elicitor in the concomitant presence of various concentration of the culture filtrate (F+E) and the amount of pisatin ($\mu\text{g/g.f.w.}$) was determined 18 hr after the treatment. Numbers denote the concentration of the culture filtrate (F) as a unit of $\mu\text{g/ml}$ glucose equivalent (the concentration was expressed as ppm instead of $\mu\text{g/ml}$). As a control, epicotyl tissues were treated with water (W). The bar indicates the standard deviation.

tion was determined by the treatment with *M. pinodes*-elicitor (500 $\mu\text{g/ml}$ BSA equivalent) in the absence or presence of various concentrations of the exudates. The accumulation of pisatin was suppressed in epicotyl tissues treated with *M. pinodes*-elicitor in the concomitant presence of 500 $\mu\text{g/ml}$ or 100 $\mu\text{g/ml}$ (glucose equivalent) of the exudate from race 4 comparing to those in epicotyl tissues treated with elicitor alone (40–50% suppression at 24 and 30 hr after the treatment and 20–30% suppression at 36 and 42 hr after the treatment) (Fig. 3A). The effects of the exudates from other compatible race strains (race 1 and 3) were similar to race 4 (data not shown). Unexpectedly, however, 40–50% suppression of pisatin accumulation was observed throughout the period tested by the treatment with the exudate from an incompatible race strain, 202 (race 2) (Fig. 3B).

These results demonstrated that the suppressor activity did exist in the exudate of both compatible and incompatible race strains.

Effects of the culture filtrate with LMW from different races of *P. pisi* on the accumulation of pisatin in epicotyl tissues of *P. sativum* cv. Midoriusui

To test whether the specificity of suppressor activity in compatible and incompatible interaction reveals after the fractionation of the culture filtrates by molecular size, as in the case of *Mycosphaerella pinodes* in which suppressor activity is mainly present in the fraction of pycnosporer germination fluid with low molecular mass (<10,000 Da), the effect of the culture filtrate with LMW from different race strains of *P. pisi* on the accumulation of pisatin in *P. sativum* cv. Midoriusui epicotyl tissues was examined. The level of pisatin accumulation was determined by the treatment with *M. pinodes*-elicitor (500 $\mu\text{g/ml}$ BSA equivalent) in the absence or presence of various concentrations of the *P. pisi*-culture filtrate 24 hr after the treatment. The accumulation of pisatin was significantly suppressed in epicotyl tissues treated with *M. pinodes*-elicitor in the concomitant presence of 500–125 $\mu\text{g/ml}$ (glucose equivalent) of the filtrate from race 1 (ca. 60–70% suppression), however, the accumulation of pisatin was rather enhanced by the treatment with the filtrate from race 2 (Fig. 4).

DISCUSSION

We have shown that compatible races of *P. pisi* have a mechanism to suppress the accumulation of

pisatin and PAL-mRNA in *in vivo* interaction, while an incompatible race of *P. pisi* significantly induces these host defense responses. To examine the similarity of the suppressor activity in a bacterial pathogen, *P. pisi*, and a fungal pathogen, *M. pinodes*, culture filtrates from different races were fractionated by the molecular size and the suppressor activity in the fraction with low molecular mass were compared between the races. Surprisingly, the culture filtrate with LMW from race 1 significantly suppressed the accumulation of pisatin, whereas that from race 2 rather induced pisatin accumulation in *P. sativum* cv. Midoriusui. However, the total exudates collected from the water-suspension of different race strains of *P. pisi* all suppressed the accumulation of pisatin in *P. sativum* cv. Midoriusui. It is not clear, at present, why the total exudates collected from the water-suspension of different race strains of *P. pisi* nonspecifically suppress the accumulation of pisatin in *P. sativum* cv. Midoriusui.

To rule out the possibility that the suppression of the accumulation of pisatin and PAL-mRNA in a compatible interaction between *P. pisi* and *P. sativum* cv. Midoriusui is due to the toxic effect on the host, different concentrations of the exudates (1,000 and 200 µg/ml glucose equivalent) were placed on a leaf of cv. Midoriusui injured with a needle to check the toxicity. As a result, neither of the exudates from different races of *P. pisi* exhibited the toxic effect (data not shown).

Our results strongly suggest that a bacterial pathogen, *P. pisi*, also suppress the accumulation of pisatin and PAL-mRNA, whose corresponding enzyme is needed for phytoalexin synthesis and thus have a possible role in disease resistance. It is not clear yet whether correlation exists between the race-cultivar specificity and the activity of suppressor. Expanded examples from other race-cultivar lines are necessary to be examined. Currently, experiments are underway to determine if the quantity and/or quality of suppressor plays some role in the host-parasite specificity. We are also attempting to isolate Tn5 mutants of *P. pisi* race 1 that lack the suppressor activity to the host, *P. sativum* cv. Midoriusui, to facilitate the cloning of suppressor gene, and to check whether it is encoded by a *hrp* gene¹⁵.

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和 文 摘 要

山田哲治・林 昌治・中塚幸子・村谷恵子・加藤久晴・白石友紀：親和性関係にあるエンドウつる枯病細菌の宿主エンドウに対するピサチンとフェニールアラニンアンモニア・リアーゼ-mRNA の蓄積抑制

エンドウ品種ミドリウスイの胚軸にエンドウつる枯病細菌の親和性レースを接種した場合、非親和性レースを接種した場合に比べて、ピサチンの蓄積が著しく抑制された。この結果は、胚軸組織におけるフェニールアラニンアンモニア・リアーゼ-mRNA の蓄積抑制にも反映されていた。親和性関係にあるエンドウつる枯病細菌の培養液に分泌される低分子物質は品種ミドリウスイに対するピサチン蓄積抑制効果を発揮するが、非親和性レースではピサチン蓄積抑制効果が見られなかった。エンドウつる枯病細菌におけるレース・品種特異性と宿主の防御反応の抑制との関係を論ずる。