Host Responses in Peas to Challenge by Wall Components of *Pseudomonas syringae* pv. *pisi* Races 1, 2, and 3

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Scientific paper no. 7883, Washington State University, College of Agriculture and Home Economics Research Center.
Supported in part by Washington Sea Grant RX-13 and National Science Foundation grant DMB-8414870.
Accepted for publication 23 June 1988 (submitted for electronic processing).

ABSTRACT

Daniels, C. H., Cody, Y. S., and Hadwiger, L. A. 1988. Host responses in peas to challenge by wall components of *Pseudomonas syringae* pv. *pisi* races 1, 2, and 3. Phytopathology 78:1451-1453.

The induction potential of cell wall fractions of *Pseudomonas syringae* pv. *pisi* races 1, 2 and 3 were studied by using levels of pisatin and specific mRNA transcripts as a measure of host response. Neither the lipopolysaccharide, lipoprotein-peptidoglycan preparations, nor the entire outer membrane preparation of any of the races induced pisatin accumulation. The complete bacterial envelope, however, induced moderate accumulations of pisatin and enhanced accumulation of disease resistance response RNAs nonspecifically in almost all combinations of bacterial envelopes and plant cultivars.

Additional keywords: nonhost resistance, race-specific resistance, resistance genes.

One of the most intensively studied areas in host-pathogen interactions is that of the elicitation of a host-resistance response. It seems clear that there is no universal inducing component that will elicit a resistance response in all hosts. In fact, the origin of the elicitor(s) does not have to be exclusively from the pathogen (7,10,13,14), but can be endogenous to the host cell itself (16,18).

Much of the work focusing on pathogen components has been done with fungi, and a number of the elicitors are derived from the pathogen cell wall (1,2). Most such elicitors have been identified initially on the basis of their ability to induce phytoalexins. Cell wall based elicitors that have been isolated from phytopathogenic bacteria include cell envelope carbohydrate-protein moieties (5) and outer membrane lipopolysaccharides (3,9).

Races of *Pseudomonas syringae* pv. *pisi* differentially infect pea cultivars containing single dominant traits for disease resistance (19). In this system, using live *P. s. pisi* inocula, pisatin levels in incompatible interactions are higher than levels seen in compatible interactions (11). A number of pea disease resistance response genes (DRRGs) are also more active in incompatible *P. s. pisi* interactions than in compatible ones (6). These DRRGs were first identified as nonhost resistance response genes based on their differential mRNA expression during challenge by the bean pathogen *Fusarium solani* f. sp. *phaseoli* (17). Additional studies undertaken to identify other conditions under which these pea genes were specifically induced or suppressed indicated that they are also differentially active in the *P. s. pisi*/pea race-specific interaction.

In this study we ask: Are individual bacterial cell wall component(s) capable of eliciting a host response, or are live *P. s. pisi* cells required? We first evaluated the ability of the components to induce a host response by measuring pisatin accumulation. Preparations that were capable of eliciting pisatin were then assayed for their ability to activate DRRGs.

MATERIALS AND METHODS

Organisms and growth conditions. Five cultivars of *P. sativum* L. (Mars, Mini, Abador, Spring, and Ceras) were grown under greenhouse conditions for pod production (6). The incompatible fungal pathogen, *Fusarium solani* f. sp. *phaseoli* race W-8 (American Type Culture Collection 38135), was grown on plates of pea shoot amended potato-dextrose agar.

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Races 2 and 3 of the bacterial pathogen *P. s. pisi* were obtained from J. D. Taylor of the National Vegetable Research Station at Wellesbourne, England, and race 1 culture was obtained from David M. Webster, Asgrow Seed Company, Twin Falls, Idaho. *P. s. pisi* race 1 is virulent on the pea cultivars Mars and Spring, race 2 of *P. s. pisi* is virulent on Mini and Ceras, whereas race 3 is virulent on Mini, Ceras, Mars, and Abador. Bacterial strains were maintained on a nutrient broth-yeast extract agar medium (20). Lyophilized cultures served as reference stocks. For use in subsequent procedures, bacterial strains were grown to mid-log phase on a rotary shaker (250 rpm) in N minimal salts broth (20) at 25 C. Cells were harvested by centrifugation (10,000 *g* for 20 min at 4 C). For experiments utilizing dead cells, live cells were incubated at 70 C for 10 min. An aliquot was plated to confirm that this heat treatment had killed all cells.

Preparation of bacterial cell envelopes. Outer membrane portions of the cell walls were prepared by using a sodium dodecyl sulfate solubilization procedure (12). The lipoprotein-peptidoglycan complex was obtained from the cell walls of each race by using the method of Braun and Sieglin (5). Cells were disrupted with glass beads in the presence of DNase. Ethylenediamenetetraacetic acid was added to a final concentration of 0.01 M and the suspension centrifuged. The sediment was washed twice in ice-cold distilled water, then added dropwise with stirring into boiling 4% sodium dodecyl sulfate. The lipopolysaccharide portion of the bacterial cell wall was isolated by using the procedure of Westphal and Jann (6). Bacterial pellets were resuspended in distilled water and vigorously stirred with an equal volume of phenol at 68 C. Emulsions were incubated at 68 C for 15 min before cooling to 10 C in an ice bath. After centrifugation to break the emulsion, the aqueous phase was saved and the organic phase was reextracted. Aqueous phases were combined and dialysed in distilled water for 4 days. Bacterial cell envelopes were prepared by passing the cell paste in the presence of DNase repeatedly through an X-Press (Cable Biox Labs, Stockholm, Sweden) pressure cell to break the cells. Cell envelopes were washed four times with distilled water. The final suspension was given a 70 C heat treatment for 10 min to kill any remaining live cells.

Pisatin assay. The endocarp surface of 0.5-g lots of immature pods of the pea cultivars Mars, Ceras, Abador, Mini, and Spring were inoculated with bacterial whole cell envelope suspensions adjusted to A_{420} = 1.0, or dilutions of lipopolysaccharide and lipoprotein-peptidoglycan complex at 1 mg/ml concentration. Pisatin was extracted from the entire pod by submergence

overnight in 5 ml of hexane. The hexane residue was redissolved in 95% ethanol, and pisatin was quantitated at A_{309} (1.0 A unit = 43.8 μg of pisatin).

Preparation of plant material for RNA extraction. Immature pea pods approximately 1.5 cm long were harvested from cultivars Mars and Ceras for gene activation studies. Pods were weighed, split, and each gram inoculated with 0.5 ml of one of the following suspensions: sterile water; 1 A_{420} unit per milliliter of isolated cell envelopes of *P. s. pisi* races 1, 2, or 3 containing 1×10^6 dead cells per milliliter of the same race; 1 A_{420} unit per milliliter of isolated cell envelopes of *P. s. pisi* races 1, 2, or 3 containing 1×10^6 live cells per milliliter of the same race; 1×10^6 heat-killed cells per milliliter of each bacterial race. Treatments were then washed off pods with 500-fold excess sterile water after 10, 20, and 30 hr incubation. Tissue was frozen in liquid nitrogen and stored at −80 C. The RNA was extracted following previously published methods (21).

Northern analysis of DRRG activation. Twenty-five μg aliquots of pea total RNA were electrophoresed on 1.5% agarose gels (containing 10 mM NaH_2PO_4, pH 7.5, and 2.2 M formaldehyde) at 150 V for 7 hr with recirculation of the buffer. RNA was transferred to nitrocellulose filters (15). Five unique cDNA-containing plasmids (17) were ^{32}P-labeled by nick translation and used to probe northern blots by using hybridization conditions described previously (8). Autoradiograms for each filter were quantified by scanning densitometry on an LKB Ultroscan Densitometer. Raw data for each treatment were normalized by dividing area under the curve values by the appropriate 10-, 20-, or 30-hr water control values.

RESULTS

Elicitation of phytoalexin in pea pods treated for 24 hr with 1 A_{420} concentration of peptidoglycan complex, lipopolysaccharide, or outer membrane components yielded less than 10 μg of pisatin per gram of pod tissue (data not shown). Therefore, the whole cell envelope was utilized to investigate race-specific elicitation. With the exception of the cultivar Spring, all three cell envelope preparations nonspecifically induced moderate levels of pisatin (Table 1). Phytoalexin levels in Spring at 30 hr correlated with resistance or susceptibility of that cultivar to the intact bacterial race.

Because individual wall fractions were very weak pisatin elicitors, only whole cell envelope preparations were tested for their ability to induce DRRGs. When such preparations were tested as DRRG elicitors, it was evident that cell envelopes from all three *P. s. pisi* races could induce the accumulation of resistance response RNAs, but in a nonspecific manner (Table 2). Inocula containing only heat-treated cells could also induce accumulation of these RNAs (Table 2). This nonspecific elicitation was not limited to one cultivar, nor did there seem to be any specificity resident to the heat-treated cells alone. Earlier preparations of the cell envelope that were not heat treated contained traces of live bacterial cells (1×10^6 colony-forming units per milliliter). As a result, these preparations retained some of the specificity reported previously (6) for live cell (1×10^9 colony-forming units per milliliter) treatments (data not shown).

TABLE 1. Pisatin accumulation in pea pods treated with cell envelope preparations from races 1, 2, and 3 of *Pseudomonas syringae* pv. *pisi*

Pea cultivar	Cell envelope preparation of *P. s. pisi* race	Live inoc. reaction[a]	Pisatin accumulation in pea pods: μg/g of tissue at 8 hr	μg/g of tissue at 18 hr	μg/g of tissue at 30 hr
Mars	1	S	nd[b]	nd	nd
	2	R	nd	nd	11 ± 4[c]
	3	S	nd	nd	18 ± 11
Abador	1	R	nd	25 ± 7	31 ± 6
	2	R	nd	17 ± 1	37 ± 9
	3	S	nd	33 ± 3	49 ± 35
Ceras	1	R	2 ± 0	29 ± 4	36 ± 8
	2	S	11 ± 4	42 ± 8	44 ± 27
	3	S	17 ± 22	53 ± 20	84 ± 21
Mini	1	R	4 ± 1	68 ± 18	69 ± 21
	2	S	4 ± 5	40 ± 21	37 ± 12
	3	S	11 ± 1	62 ± 6	48 ± 1
Spring	1	S	nd	15 ± 2	5 ± 9
	2	R	nd	22 ± 1	86 ± 13
	3	R	nd	27 ± 2	33 ± 2

[a] Disease reaction type expected by using live *P. syringae* inoculum (11).
[b] nd = not detectable.
[c] ± standard deviation.

DISCUSSION

Races of live bacterial inocula of *P. s. pisi* have been shown to specifically induce greater accumulations of pisatin in pea cultivars during incompatible interactions than during compatible interactions (11) and to induce DRRG RNAs in a race-specific manner (6). To answer the question of whether *P. s. pisi* cell envelope components were capable of eliciting a host response, we

TABLE 2. Accumulation of RNA homologous with the disease-resistance response genes of peas after inoculation with isolated cell envelopes and heat-killed cells of *Pseudomonas syringae* pv. *pisi*

Cultivar	cDNA probe	Treatment[a]	Live inoc. reaction	Relative RNA accumulation[b]: 10 hr	20 hr	30 hr
Mars (resistant to race 2)	pI39	water		1	1	1
		CE-R2	R	1.15	3.28	2.58
		CD-R3	S	0.74	1.30	1.36
		deadR2	R	0.44	1.17	1.09
	pI49	water		1	1	1
		CE-R2	R	1.82	2.45	1.43
		CE-R3	S	1.60	3.85	2.58
		deadR2	R	1.25	4.30	1.01
	pI176	water		1	1	1
		CE-R2	R	1.78	2.41	2.79
		CE-R3	S	2.46	4.89	1.92
		deadR2	R	1.10	3.27	1.77
	pI204	water		1	1	1
		CE-R2	R	5.05	3.09	5.47
		CE-R3	S	8.49	3.69	3.55
		deadR2	R	6.14	1.58	1.46
	pI206	water		1	1	1
		CE-R2	R	1.17	3.71	3.51
		CE-R3	S	1.33	5.17	2.75
		deadR2	R	0.93	3	1.41
Ceras (resistant to race 1)	pI39	water		1	1	1
		CE-R1	R	2.09	11.33	3.75
		CE-R2	S	1	12.44	3.38
		deadR1	R	0.43	6.78	3.81
	pI49	water		1	1	1
		CE-R1	R	1.29	2.04	1.94
		CE-R2	S	2.02	2.99	0.99
		deadR1	R	1.18	1.98	1.07
	pI176	water		1	1	1
		CE-R1	R	1.26	1.50	0.57
		CE-R2	S	1.23	1.78	1.36
		deadR1	R	1.97	0.53	1.26
	pI204	water		1	1	1
		CE-R1	R	2.46	1.58	3.42
		CE-R2	S	1.69	3	2.67
		deadR1	R	1.31	1.32	2.63
	pI206	water		1	1	1
		CE-R1	R	1.46	2.46	3.11
		CE-R2	S	1.29	2.11	2.16
		deadR1	R	1.28	1.56	2.08

[a] Treatments consisted of sterile water, isolated cell envelopes (CE) (1 A_{420} units/ml) of *P. s. pisi* races 1, 2, or 3 containing trace dead cells (1×10^6/ml) contamination, or dead cells (1×10^6/ml) alone at equal amounts of cell wall inoculum.
[b] Data was normalized against the appropriate water control (see text).

examined the bacterial cell envelope and its fractions for easily measurable physical differences. Infrared spectraphotometry indicated no major differences in total envelope components between the three races (data not shown). Similarly, the lipopolysaccharide components showed no differences between races when separated by polyacrylamide gel electrophoresis (data not shown). The outer membrane protein profiles (not shown) of the three races were indistinguishable on polyacrylamide gels and closely resembled those reported earlier for *P. syringae* pv. *syringae* (12). We applied the cell envelope and its fractions to a more sensitive examination, the biological assay, and observed that individually, outer membrane proteins, lipopolysaccharide, and peptidoglycan fractions were ineffective elicitors of phytoalexin production. However, the whole bacterial envelope induced moderate levels of pisatin, indicating that the total cell envelope possesses much greater induction potential than do the individual components. We were unable to determine from the data whether this phenomenon indicated that the inducing component was destroyed upon wall fractionation or if there was a synergistic effect of several components in different fractions. The levels of pisatin induced in the tissue were still less than that seen with live cells, however (11). Thus, the inducing potential was both nonspecific and weaker in the absence of live inoculum.

Gene-activation (DRRG) studies were restricted to whole cell envelope inoculum as there was no pisatin induction with the isolated envelope components. Preparations of the bacterial envelope free of live cells were all capable of eliciting the RNAs but did not retain the specificity of live bacterial cells. Heat-treated cells (in only some interactions) were also capable of nonspecific elicitation, although they varied in their levels of gene induction. In general, the heat-treated cells alone were weaker inducers of RNA accumulation than were cell envelopes. The data here and in previous work (6) demonstrate that both live cells and whole cell envelopes of all three races have the potential to induce a host-resistance response.

The level of host response generated by challenge with *P. s. pisi* cell envelope is not equal to that of live bacterial cell-challenged tissue. Therefore, although we have identified the cell envelope as an inducer, it is probably not the only form of molecular communication between host and pathogen that determines reaction phenotype. Other possibilities include an (additional) specific inducer or a suppressor molecule generated by the bacterium once in contact with the pea that is actually responsible for race specificity.

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