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Bacteria Expressing Avirulence Gene D Produce a Specific Elicitor of the Soybean Hypersensitive Reaction

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Escherichia coli **cells carrying expression plasmids with the cloned avirulence (*avr*) gene D from *Pseudomonas syringae* pv. *tomato* produced substantial amounts of the 34-kDa protein product. Infiltration of these *E. coli* cells into soybean leaves also resulted in a hypersensitive reaction (HR) on soybean cultivars resistant to but not susceptible to *P. s.* pv. *glycinea* carrying the cloned *avrD* gene. However, lysed *E. coli* cells expressing the *avrD* gene elicited little or no HR. The partially purified *avrD*-encoded protein extracted from *E. coli* cells also appeared to be devoid of HR elicitor activity. However, *E. coli* cells expressing *avrD* secreted a low molecular weight factor into the culture medium that elicited the HR only on resistant soybean cultivars. The same elicitor activity was found in culture fluids of wild-type *P. s.* pv. *tomato* as well as several other *P. s.* pv. *glycinea* pathovars previously shown to contain DNA homologous to *avrD*. However, *P. s.* pv. *glycinea* did not produce significant *avrD* elicitor despite containing hybridizing DNA sequences. Several *P. syringae* pathovars that lacked hybridizing DNA and an *avrD* mutant strain of *P. s.* pv. *tomato* also did not produce detectable quantities of the *avrD* elicitor. However, introduction of the cloned *avrD* gene on a broad host range plasmid enabled these bacteria to produce the extracellular elicitor. Production of the *avrD* elicitor by *P. s.* pv. *glycinea* cells carrying the cloned *avrD* gene occurred independently of the *hrp* genes, considered important for pathogenicity and HR induction by certain *P. syringae* pathovars. The results indicated that expression of avirulence gene D in *P. syringae* pathovars and in *E. coli* causes them to produce a diffusible, elicitor-active molecule which initiates cultivar-specific induction of the HR.**

Additional keywords: plant defense, recognition, cell-cell signaling, gene-for-gene complementarity.

Gene-for-gene complementarity is a widely occurring phenomenon in which single dominant alleles in plants and their pathogens determine whether the plant will be resistant or susceptible to attack (Day 1974; Flor 1942; Ellingboe 1982). If the dominant, matching alleles of a pathogen avirulence (*avr*) gene and a plant disease resistance gene are present, recognition occurs and the plant responds to infection by invoking an active defense mechanism called the hypersensitive reaction (HR). However, if dominant alleles are not present in both partners, plant recognition does not occur and the pathogen develops extensively, causing disease.

While some aspects concerning expression of the HR are understood (for review, see Lamb *et al.* 1989), little is known about the molecular functions of avirulence genes and resistance genes, particularly in how they lead to specific recognition of incompatible races. The major hypothesis being tested in these systems, the elicitor-receptor model, holds that pathogen biotypes carrying a certain *avr* gene produce a discrete elicitor substance which is specifically recognized by a receptor present in plants carrying the matching resistance gene (Callow 1977; Day 1974; Gabriel *et al.* 1988; Keen 1985). This model therefore predicts that elicitor-receptor binding initiates the subsequent biochemical events associated with expression of the HR. While disease resistance genes have not been isolated from higher plants, several avirulence genes from bacterial and viral pathogens have been cloned and characterized (see Keen and Staskawicz 1988). Their availability permits more incisive tests of the elicitor-receptor model.

Kobayashi *et al.* (1989) cloned three avirulence genes from *Pseudomonas syringae* pv. *tomato* (Okabe) Young *et al.* that, when introduced into *P. s.* pv. *glycinea* (Coerper) Young *et al.* race 4, resulted in HRs only on certain soybean cultivars. One of these genes yielded a spectrum of resistant and susceptible responses on 10 soybean cultivars unlike that given by any known *P. s.* pv. *glycinea* race or any known *P. s.* pv. *glycinea* avirulence gene. This gene, designated *avrD*, was subsequently sequenced (Kobayashi *et al.* 1990a) and found to encode a 34-kDa protein product of 311 amino acids. Significantly, *avrD* was the first of five tandem open-reading frames (ORFs).

Kobayashi *et al.* (1990a, 1990b) also showed that four different races of *P. s.* pv. *glycinea* contained DNA sequences showing moderate hybridization to the *avrD* gene of *P. s.* pv. *tomato* as well as sequences with high homology to the other ORFs linked to the *avrD* gene of *P. s.* pv.

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Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Reference or source
Escherichia coli		
DH5α	*endA1 hsdR17* ($r_k^-m_k^+$) F$^-$ *supE44 thi-1*λ$^-$ *recA1 gyrA relA1* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169	Bethesda Research Laboratories, Gaithersburg, MD
JM109	*recA1* Δ(*lac pro*) *endA1 gyrA96 thi-1 hsdR17 supE44 relA1* F′ *traD36 proAB*$^+$ *lacI*^{q}ZΔM15	Vieira and Messing 1987
HB101	F$^-$ *hsdS20* (*hsdR hsdM*) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Str^r) *xyl-5 mtl-1 supE44* λ$^-$	Maniatis *et al.* 1982
Pseudomonas syringae pathovars		
pv. *glycinea*		
Race 1		Kobayashi *et al.* 1989
Race 4	*rif*^{r}, *ap*^{r}	Kobayashi *et al.* 1989
Race 5		Kobayashi *et al.* 1989
Race 6		Kobayashi *et al.* 1989
Race 0		Kobayashi *et al.* 1989
pv. *tomato*		
PT23		Bender and Cooksey 1986
PT23	*avrD*$^-$	This study
B120		T. Denny, Univ. of Georgia, Athens
DC3000		D. Cuppels, Univ. of Western Ontario, London
pv. *phaseolicola* 0285-1		Kobayashi *et al.* 1990a
pv. *phaseolicola* 14		N. Panopoulos, Univ. of California, Berkeley
pv. *phaseolicola* 3121		N. Panopoulos
pv. *pisi*		Kobayashi *et al.* 1990a
pv. *mori*		Kobayashi *et al.* 1990a
pv. *morsprunorum*		Kobayashi *et al.* 1990a
pv. *tabaci*		Kobayashi *et al.* 1990a
pv. *savastanoi*		Kobayashi *et al.* 1990a
pv. *lachrymans*		Kobayashi *et al.* 1990a
pv. *syringae* from bean		Kobayashi *et al.* 1990a
pv. *syringae* from tomato		Kobayashi *et al.* 1990a
pv. *atropurpurea*		Kobayashi *et al.* 1990a
Plasmids		
pBluescript KS	Cloning plasmid	Stratagene, La Jolla, CA
pUC118/pUC119	Cloning plasmids with a *lac* promoter	Vieira and Messing 1987
pUC128/pUC129	Cloning plasmids with a *lac* promoter	Keen *et al.* 1988
pRK415	Broad host range cloning plasmid	Keen *et al.* 1988
pRK2013	Helper plasmid	Ditta *et al.* 1980
pDSK519	Broad host range cloning plasmid	Keen *et al.* 1988
pINIII A-2	Expression plasmid for translational fusions	Masui *et al.* 1984
pINIIIOmp A-2	Expression plasmid for adding the *ompA* signal peptide sequence as a translational fusion to a cloned gene	Ghrayeb *et al.* 1984
pPT101	5.6-kb fragment of *P. s.* pv. *tomato* DNA containing the *avrD* gene cloned downstream of the vector *lac* promoter of pRK415	Kobayashi *et al.* 1990a
pPTD120	5.6-kb *Hin*dIII fragment of pPT101 cloned such that the *avrD* gene was downstream of the *lac* promoter of pUC119	Kobayashi *et al.* 1990a
pAVRD1	A20 from Kobayashi *et al.* 1990a; 1,218-bp deletion fragment from pPTD120 containing the *P. s.* pv. *tomato avrD* gene in PUC119; the *exo*III sequencing deletion occurred 132 bp 3′ to the TAG translational stop codon	This study
pAVRD3	Approximately 1.2-kb *Hin*dIII-*Eco*RI insert fragment from pAVRD1 inserted into the same sites of pBluescript KS	This study
pAVRD5	*Xho*I-*Bam*HI fragment from pAVRD3 containing the *avrD* coding region cloned into the same sites of pUC128 in the orientation opposite the vector *lac* promoter	This study
pAVRD5-0	Same as pAVRD5 except that an *Eco*RI site was introduced by oligonucleotide site-directed mutagenesis just ahead of the start codon of the *avrD* gene	Kobayashi *et al.* 1990b
pAVRD6	Same as pAVRD5 except that the *avrD* gene was cloned in the same orientation as the vector *lac* promoter of pUC129	This study

(*continued on next page*)

[a] Str, streptomycin; r, resistant; kb, kilobase; and bp, base pair.

Table 1 *continued*

Designations	Relevant characteristics[a]	Reference or source
Plasmids		
pAVRD7	Same as pAVRD8 except opposite insert orientation	This study
pAVRD8	Approximately 1.1-kb *Eco*RI fragment from pAVRD5-0 cloned into the same site of pINIIIOmp A-2 to form a translational fusion with the amino terminal signal peptide sequence of the *ompA*-encoded protein	This study
pAVRD10	Approximately 1.1-kb *Xho*I- *Bam*HI fragment from pAVRD5-0 cloned into pUC129 downstream of the vector *lac* promoter	This study
pAVRD11	Approximately 1.1-kb *Eco*RI fragment from pAVRD5-0 cloned into the same site of pINIII A-2; the coding region was reversed relative to the vector promoters; hypersensitive reaction inactive in *E. coli*	This study
pAVRD12	Same construction as pAVRD11 except that the 1.1-kb *Eco*RI fragment was oriented downstream of the vector *lac* promoters to afford an in-frame translational fusion with six extra N-terminal amino acids; *E. coli* cells carrying this construct caused a hypersensitive reaction in soybean leaves	This study
pAVRD33	Insert from pAVRD3 cloned ito the *Sal*I-*Bam*HI sites of pDSK519 such that the *avrD* gene was oriented downstreamof the vector *lac* promoter	This study
pAVRD34	1.2-kb *Hin*dIII-*Eco*RI insert of pAVRD3 cloned into the same sites of pRK415	This study
pPSG4000	5.6-kb *Hin*dIII fragment from *P. s.* pv. *glycinea* isolated by colony hybridization	Kobayashi *et al.* 1990b
pPSG4001	3.2-kb *Hin*dIII-*Bam*HI fragment from pPSG4000 cloned downstream of the *lac* promoter of pUC128	Kobayashi *et al.* 1990b
pPSG4005	(pPGR4:A11) ORF 1 of *avrD* from *P. s.* pv. *glycinea* cloned in pUC128; a 1,083-bp fragment created by a deletion subclone from pPSG4001; the deletion occurred 7 bp following the TAG translational stop site of the ORF	Kobayashi *et al.* 1990b
pPSGOR	pPSG4005 with an *Eco*RI site introduced immediately before the start codon of ORF 1 by oligonucleotide site-directed mutagenesis	Kobayashi *et al.* 1990b
pAVRDG-1	*Eco*RI-*Hin*dIII fragment from pPSGOR cloned into the same sites of pINIII A-2, resulting in a translational fusion with six extra amino acids at the N-terminal and including the entire *P. s.* pv. *glycinea* ORF 1 at the carboxyl end	This study

[a] ORF, open-reading frame

tomato. The *avrD* homologue in *P. s.* pv. *glycinea* was sequenced (Kobayashi *et al.* 1990b) and found to encode a colinear protein of identical size (311 amino acids) and 86% identical amino acids to the *avrD*-encoded protein of *P. s.* pv. *tomato*. Despite this similarity, the *P. s.* pv. *glycinea* homologue of *avrD* did not function as an avirulence gene when reintroduced into *P. s.* pv. *glycinea* on plasmid vectors.

To date, neither the biochemical functions of avirulence genes in the bacteria that harbor them nor the molecular mechanisms by which they elicit the plant HR are known. However, all *avr* genes thus far sequenced encode proteins devoid of either signal peptide secretion sequences or hydrophobic stretches of amino acids denoting membrane association (Keen and Staskawicz 1988). Tamaki *et al.* (1988) previously overexpressed the *avrC* gene from *P. s.* pv. *glycinea* in *Escherichia coli* cells and isolated the protein product. Under no circumstances, however, did the *avrC*-encoded protein function as an elicitor of the HR in soybean leaves or cotyledons. Such avirulence gene products might, therefore, not be elicitors themselves, but may possess enzymatic functions leading to bacterial metabolites that are the actual elicitors recognized by resistant plants.

To test this possibility, we expressed *avrD* at high levels in *E. coli* and introduced either these cells or the medium in which they were grown into primary soybean leaves. In this research article, we report evidence showing that expression of *avrD* causes *E. coli* and other bacteria to produce a cultivar-specific elicitor of the soybean HR.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. Bacterial strains and plasmids that were used in this study are listed in Table 1. *P. syringae* van Hall isolates were routinely grown on King's medium B agar plates (King *et al.* 1954) at 28° C, and *E. coli* strains were grown at 37° C on Luria-Bertani (LB) agar plates, all supplemented with appropriate antibiotics (ampicillin, 75 μg/ml; kanamycin, 25 μg/ml; rifampicin, 100 μg/ml; and tetracycline, 25 μg/ml). For overproduction of the *avrD*-encoded protein, *E. coli* strain DH5α or JM109 cells containing pAVRD10 were grown in LB liquid medium containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 hr at 28° C. In elicitor experiments, *P. syringae* or *E. coli* cells were grown in shaken flasks (15 ml in 50-ml Delong flasks or 100 ml

in 250-ml Erlenmeyer flasks) of M9 liquid medium, pH 7.4 (Maniatis *et al.* 1982), with glucose added at 2× the normal concentration for *P. syringae* isolates only. All *E. coli* M9 cultures were supplemented with thiamine at 4 μg/ml. *E. coli* cells carrying plasmids with *lac* promoters were induced at the time of culture initiation by adding IPTG to a concentration of 1 mM.

A *P. s.* pv. *tomato* PT23 *avrD* mutant strain was constructed by marker exchange mutagenesis (Staskawicz *et al.* 1987) using the cloned *avrD* gene with a Tn*5* insertion. Cosmid clone pPT112 (Kobayashi *et al.* 1989) was introduced into *P. s.* pv. *tomato* strain PT23 and, following several cycles of growth in medium without antibiotics, kanamycin-resistant but tetracycline-sensitive bacteria were identified. Occurrence of the desired replacement of *avrD* by the mutant gene was confirmed by Southern blot analysis following digestion with *Eco*RI or *Hin*dIII and probing with an intragenic *avrD* DNA fragment (Kobayashi *et al.* 1990a).

DNA manipulations and plasmid constructs. Total DNA isolations from bacteria were performed as previously described (Staskawicz *et al.* 1984). Plasmids were generally constructed by isolating desired fragments from soft agarose gels before ligation and transformation, usually of *E. coli* strain DH5α (Crouse *et al.* 1983).

The *avrD* gene of *P. s.* pv. *tomato* was initially obtained from a pUC119 sequencing deletion clone (A20) from D. Kobayashi and co-workers (Kobayashi *et al.* 1990a). The coding region was manipulated in various ways as summarized in Table 1 to generate expression constructs. An *Eco*RI site was introduced immediately ahead of the translational start codon of *avrD* as previously described (Kobayashi *et al.* 1990b) to generate pAVRD5-0. This restriction site was then used to construct a translational fusion with pINIIIOmp A-2, yielding pAVRD8, in which the *ompA* leader peptide sequence was fused to the amino terminus of *avrD*. After cleavage with signal peptidase, the secreted protein should have had an N-terminal sequence of Ala Glu Phe Met, where Met is the normal terminal amino acid. An analogous construct (pAVRD12) in which the *Eco*RI insert fragment of pAVRD5-0 was fused with pINIII A-2 yielded a fusion protein product with an amino terminus of Met Lys Gly Lys Glu Phe Met, where the seventh amino acid residue (Met) is the normal N-terminal amino acid of the *avrD*-encoded protein (Kobayashi *et al.* 1990a).

Thus, the *avrD* gene product encoded by pAVRD12 has six extra N-terminal amino acids and lacks a signal peptide sequence. An approximately 1.1-kilobase (kb) *Xho*I-*Bam*HI insert fragment from pAVRD5-0 was also cloned into pUC129, generating a transcriptional fusion (pAVRD10) in which the *avrD* gene with minimal *avrD* 5′ DNA was oriented downstream from the vector *lac* promoter (Table 1). The pINIII constructs were all verified by the sequencing of *Xba*I-*Hin*dIII fragments containing the *Eco*RI junctions. An analogous construct to pAVRD12 was constructed using the analogue of *avrD*, pPSGOR, cloned from *P. s.* pv. *glycinea* race 4 (Kobayashi *et al.* 1990b; Table 1). These constructs were used in attempts to show the production of elicitor activity by *E. coli* cells. For increased expression of *avrD* in *P. s.* pv. *tomato* and *P. s.* pv. *glycinea*, the approximately 1.2-kb insert from pAVRD3 was cloned into the broad host range vector pDSK519, yielding pAVRD33 (Table 1).

Partial purification of the *avrD*-encoded protein from *E. coli*. *E. coli* strain JM109 cells carrying pAVRD10 or pAVRD5 (negative control) were lysed in 10 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl (pH 8.0), and 1 mg of lysozyme per milliliter at 0° C for 30 min, and then at −70° C for 2 hr. The lysis reaction mixture was thawed on ice and centrifuged at 14,000 × *g* for 10 min. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis showed that the pellet contained little *avrD*-encoded protein but considerable amounts of other proteins. Three moles per liter sodium acetate (pH 7.0) was added to the supernatant to a final concentration of 10 mM, and the pH was reduced to approximately 5.5 using HCl. The resulting precipitate was removed by centrifugation at 6,000 × *g* for 8 min. Electrophoresis of fractions showed that the *avrD*-encoded protein was present in the pellet and constituted approximately 20% of the total protein, based on staining with Coomassie Brilliant Blue R 250. Many contaminant proteins remained in the supernatant. The sodium acetate pellet was redissolved in 10 mM NaCl, 25 mM Tris, pH 8.0, before plant assay.

Sep-Pak C18 desalting of culture fluids. Supernatant fluids from *E. coli* or *P. syringae* cells expressing *avrD* could be efficiently desalted and the *avrD* elicitor concentrated and partially purified by passage of 15–50 ml of M9 culture fluids through a single Sep-Pak C18 cartridge (Waters Associates, Milford, MA) that had been conditioned by washing with 5 ml of 50% aqueous acetonitrile followed by 5 ml of water. Following application of culture fluids to the Sep-Pak C18 column at approximately 20 ml per minute and washing with 5 ml of water, the elicitor activity was then eluted with 5 ml of 50% acetonitrile in water. This eluate was dried in a rotary evaporator at 50° C, and the residue was dissolved in water at final concentrations of 1× to 50× relative to the starting culture fluids before elicitor bioassays.

Large-scale fractionation of elicitor activity from *P. s.* pv. *tomato* or *E. coli* culture fluids. Cell-free supernatants (approximately 1 liter) were adjusted to pH 3.5 with HCl and passed through an Amberlite XAD-2 (Sigma, St. Louis, MO) column (8-cm diameter × 2-cm deep). After washing with water (100 ml), the column was eluted with 40 ml of 50% acetonitrile in water. The acetonitrile eluate was dried in a rotary evaporator at 50° C, dissolved in water, and passed through a 1.5-cm diameter × 10-cm deep Bio-Rad (Richmond, CA) AG1X8 anion exchange column (acetate form). The column was washed with 2 N acetic acid to remove impurities, and the bulk of the *avrD* elicitor activity was recovered by eluting with 5 N acetic acid. Eluates were neutralized using ammonium hydroxide and desalted by passage through a Sep-Pak C18 cartridge as described above. Further purification of the *avrD* elicitor was accomplished using high-pressure liquid chromatography (HPLC). A 250 × 4.6 mm phenyl HPLC column (Alltech Associates, Deerfield, IL) was eluted with a 0–50% methanol gradient, and fractions were collected for bioassay

in soybean leaves.

Elicitor assays. Fully expanded primary leaves of soybean plants were infiltrated with assay solutions or bacterial cell suspensions by appressing the blunt end of a disposable plastic 1-ml syringe to the leaf undersurface and applying pressure to the plunger. The injected preparations (approximately 20–50 μl per site) formed small water-soaked areas within and extending slightly outside the area contacted by the syringe. After infiltration, plants were placed in lighted growth chambers for a 16-hr photoperiod and maintained at 21° C and approximately 90% relative humidity.

The initial water-soaked areas disappeared after approximately 1 hr and leaves appeared normal. Depending on the activity of preparations and the soybean cultivar, visible necrosis of the infiltrated area and surrounding leaf tissue was observed at 12–24 hr after infiltration. Negative reactions were typified by the absence of visible necrotic responses, except for occasional slight mechanical damage where the syringe had been appressed to the leaf undersurface.

The soybean cotyledon bioassay was performed as previously described (Keen *et al.* 1983). This assay monitors phytoalexin production as an indicator of the hypersensitive defense response. Assay solutions were applied to wounded cotyledons of cultivars Harosoy or Merit, and the phytoalexin glyceollin was quantitated after incubation for 24 hr at room temperature. The glucan elicitor, mycolaminaran (Keen *et al.* 1983), was used as a positive control, and water was used as a negative control in all cotyledon assays. Soybean leaves infiltrated with preparations of the *avrD* elicitor were harvested after 24 or 48 hr, and glyceollin was extracted and quantitated as previously described (Keen 1978).

RESULTS

HRs in soybean leaves infiltrated with *E. coli* cells expressing *avrD*. Cells of *E. coli* harboring previously cloned avirulence genes other than *avrD* did not elicit an HR when infiltrated into primary soybean leaves (Keen and Staskawicz 1988; Tamaki *et al.* 1988). It was surprising, therefore, that *E. coli* DH5α, JM109, or HB101 cells carrying plasmid constructs pAVRD3, pAVRD6, pAVRD10, or pAVRD12 of *avrD* caused a pronounced HR when infiltrated into primary leaves of only those cultivars previously observed to react hypersensitively to *P. s.* pv. *glycinea* race 4 carrying *avrD* (Kobayashi *et al.* 1989; Table 2).

Leaves infiltrated with *E. coli* cells carrying the plasmid DNA inserts in reversed orientation to the vector *lac* promoters gave no reaction on any cultivar. When constructs pAVRD1 or pAVRD3 were used, high densities (approximately 10^9 per milliliter) of *E. coli* cells were required to obtain a readily visible HR. However, when constructs pAVRD10 or pAVRD12 were used (which resulted in more intense 34-kDa bands on SDS-polyacrylamide gels), a visible confluent HR was seen when 10^8 *E. coli* cells per milliliter were infiltrated. Generally, cells that were suspended in LB medium diluted 1:5 with water and supplemented with ampicillin at 100 μg/ml produced stronger plant reactions than did cells suspended in water with or without ampicillin. Infiltration of *E. coli* cells carrying the various constructs into cultivars that were susceptible to *P. s.* pv. *glycinea* race 4 with *avrD* yielded little or no visible reaction (Table 2).

Another unexpected feature was that high concentrations of *E. coli* cells carrying constructs pAVRD10 or pAVRD12, which produce relatively large amounts of the *avrD*-encoded protein, caused not only a hypersensitive necrosis in resistant leaves at the point of bacterial infiltration, but also a systemic, spreading necrosis that progressed outward from the inoculation site, particularly toward the leaf margin. Since few if any *E. coli* cells from leaf tissue showing such distal necrosis could be isolated on LB ampicillin-supplemented plates, the results suggested that the bacteria were elaborating a soluble elicitor that moved through the soybean leaves.

Attempts to show elicitor activity from the *avrD*-encoded protein product. Despite the fact that the *avrD*-encoded

Table 2. Reactions of various soybean cultivars to inoculation with race 4 of *Pseudomonas syringae* pv. *glycinea* carrying the cloned *avrD* gene or to *Escherichia coli* cells containing various constructs of the *avrD* gene

	Plant reaction to cells carrying plasmids[a]						
Cultivars	**pAVRD5**	**pAVRD6**	**pAVRD10**	**pAVRD11**	**pAVRD12**	**pAVRD33**	**pAVRD34**
Acme	0	0	0	0	0	C	C
Norchief	0	1	2	0	3	I	I
Flambeau	0	1	2	0	3	I	I
Merit	0	0	0	0	0	C	C
Harosoy	0	2	3	0	3	I	I
Peking	0	0	0	0	0	C	C
Hardee	0	0	0	0	0	C	C
Centennial	0	1	2	0	3	I	I
Lindarin	0	1	2	0	3	I	I
Chippewa	0	2	3	0	3	I	I

[a] pAVRD5, pAVRD6, pAVRD10, pAVRD11, and pAVRD12: Soybean leaves infiltrated with *E. coli* strain DH5α cells grown at 28° C on Luria-Bertani broth plus isopropyl-β-D-thiogalactopyranoside until stationary phase and suspended in L broth diluted 1:5 with water before infiltration. Cell densities were approximately 10^9 per milliliter. Results were read after 24–30 hr and scored according to the following index: 0 = no response for possible slight mechanical damage caused by infiltration; 1 = slight hypersensitive necrosis at the point of infiltration; 2 = strong hypersensitive necrosis of the area infiltrated and extending slightly outside; and 3 = severe hypersensitive necrosis extending considerably outside the infiltration area, frequently reaching the leaf margin. pAVRD33 and pAVRD34: Results of inoculation with *P. s.* pv. *glycinea* race 4 carrying the cloned *avrD* gene in plasmid pAVRD33 or pAVRD34. I = incompatible, hypersensitive reaction observed after 24–36 hr; C = compatible, water-soaked lesions observed after 48–72 hr.

protein lacks a signal peptide sequence and appears not to be significantly exported to the medium, it was possible that sufficient *avrD*-encoded protein might be liberated from *E. coli* cells in the soybean leaf such that the protein could elicit the soybean HR. However, all attempts to show elicitor activity from the *avrD*-encoded protein failed. First, heat (10 min at 55° C) or antibiotic (addition of 50 μg/ml rifampicin or 25 μg/ml streptomycin) treatment of HR-competent *E. coli* cells invariably negated their ability to elicit the HR (data not shown). Second, mild sonication of intact *E. coli* cells or spheroplasts carrying pAVRD10 or pAVRD12 resulted in extracts with little or no residual elicitor activity when they were infiltrated into soybean leaves.

To further test for possible elicitor activity by the *avrD*-encoded protein, the *avrD* gene was engineered to contain the *ompA* signal peptide sequence, thus facilitating its secretion to the periplasmic space of *E. coli* cells. This was done by constructing an *avrD* translational fusion, called pAVRD8, using pINIIIOmp A-2 as described previously in the text. *E. coli* cells carrying pAVRD8, but not the reversed insert control (pAVRD7), had the peculiar behavior of tenaciously sticking together and to the surface of LB agar plates supplemented with ampicillin. Thus, cells of even young colonies could not readily be removed with a transfer loop. Most noteworthy, however, was that cells carrying pAVRD7 or pAVRD8 caused little or no HR when infiltrated into soybean leaves of cultivars incompatible to *avrD*. These observations also suggested that the *avrD*-encoded protein itself may not function directly as an elicitor of the soybean HR.

Partially purified preparations of the *avrD*-encoded protein from lysed *E. coli* cells also were devoid of any detectable elicitor activity, even when solutions containing 1 mg/ml or more of the *avrD*-encoded protein were infiltrated into soybean leaves. SDS-polyacrylamide gel electrophoresis disclosed that the *avrD*-encoded protein constituted 20% or more of the total protein in these preparations (data not shown). However, none of the purification steps yielded protein preparations with detectable elicitor activity in soybean leaves or cotyledons. All of these results indicated that the *avrD*-encoded protein product was not the putative elicitor of the soybean HR.

An extracellular elicitor produced by bacteria expressing *avrD*. Culture fluids from *E. coli* cells expressing *avrD* that were grown in L broth did not yield detectable elicitor activity when directly assayed on soybean leaves. However, these fluids required an approximately 10-fold dilution with water before infiltration into leaves to avoid necrosis due to medium toxicity. *E. coli* cells carrying various constructs of *avrD* were therefore grown on M9 glucose minimal

Table 3. Reactions of soybean cultivars infiltrated with diluted M9 culture fluids of *Escherichia coli* strain DH5α cells containing various constructs of the *avrD* gene

Cultivars	Plant reaction to fluids carrying plasmids[a]					
	pAVRD3	pAVRD5	pAVRD6	pAVRD10	pAVRD11	pAVRD12
Acme	0	0	0	0	0	0
Norchief	1	0	1	3	0	3
Flambeau	1	0	2	2	0	3
Merit	0	0	0	0	0	0
Harosoy	2	0	2	3	0	3
Peking	0	0	0	0	0	0
Hardee	0	0	0	0	0	0
Centennial	1	0	1	2	0	3
Lindarin	1	0	1	1	0	3
Chippewa	2	0	2	3	0	3

[a] Cultures were grown at 28° C for 24 hr (approximately 12 hr of which was stationary phase) in M9 glucose medium (15 ml in 50 ml Delong flasks) supplemented with 100 μg/ml of ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside. Bacteria were removed by centrifugation, and the fluids were passed through 0.20-μm filters to ensure removal of bacteria. After diluting 1:3 with water, the fluids were infiltrated into primary soybean leaves. Reactions were rated after 24 hr according to the index in Table 2.

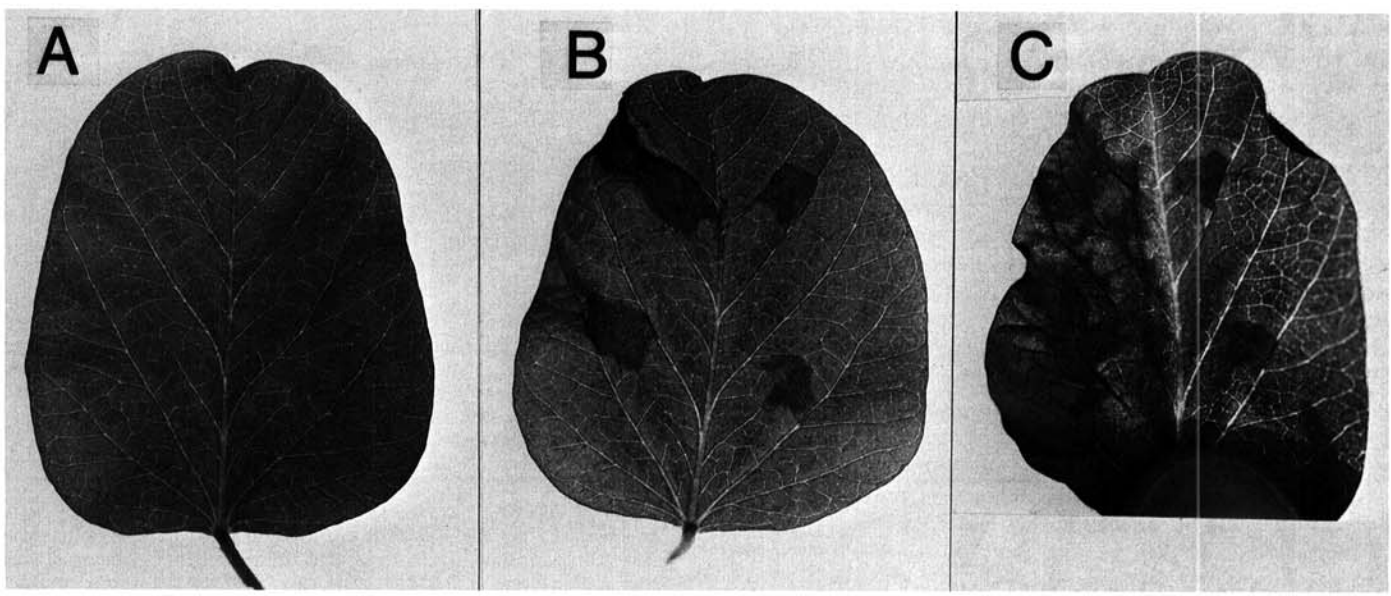

Fig. 1. Response of soybean leaves at 24 hr after infiltration with preparations of the *avrD* elicitor obtained after desalting and concentration of M9 culture fluids from PT23 (pAVRD33) with a Sep-Pak C18 cartridge. **A**, Cultivar Merit infiltrated at two points on the left side of the midvein with 50-fold concentrated elicitor and on the right side with 10-fold concentrated elicitor. **B**, Cultivar Harosoy infiltrated at two points on the left side of the midvein with 50-fold concentrated elicitor and on the right side with 10-fold concentrated elicitor. **C**, Cultivar Harosoy infiltrated with 100-fold concentrated elicitor at two points on the left side of the midvein and with 10-fold concentrated elicitor on the right side.

medium. These culture fluids also required twofold to fourfold dilution with water to avoid leaf toxicity. M9 fluids from DH5α cells carrying plasmids pAVRD3 or pAVRD6 caused necrosis of soybean leaf cells at 24 hr after infiltration (Table 3). Furthermore, these putative HRs were observed only on soybean cultivars that were resistant to *P. s.* pv. *glycinea* race 4 carrying the cloned *avrD* gene (Kobayashi *et al.* 1989, 1990a). As seen previously with *E. coli* cells, concentrated culture fluids of cells containing pAVRD10 or pAVRD12 produced systemic necrosis on leaves of sensitive cultivars. Controls in which the *avrD* insert was cloned in the reverse orientation to vector promoters (for example, pAVRD5) or when the gene fragment was reversed in the translational fusion vector pINIII A-2 (pAVRD11) yielded culture fluids with no detectable elicitor activity on any cultivar (Table 3).

The elicitor activity in M9 glucose culture fluids of *E. coli* cells expressing *avrD* was entirely removed by dialysis through 6,000–8,000 M_r dialysis tubing, but it was not significantly affected by boiling the culture fluids for 5 min in a water bath. *E. coli* cells carrying high expression constructs of *avrD*, such as pAVRD10 or pAVRD12, also yielded distinctly yellow-colored M9 culture fluids, but cells carrying control constructs (for example, pAVRD5 or pAVRD11) or the plasmids only did not produce significant pigment.

Sep-Pak C18 preparations from *E. coli* JM109 cells expressing pAVRD12 caused necrosis on sensitive soybean cultivars at the point of infiltration and slightly beyond when assayed at a concentration of 1× relative to the original culture fluids (Fig. 1). However, when 10× or greater relative concentrations of the Sep-Pak C18 desalted culture fluids were infiltrated into sensitive soybean primary leaves, spreading hypersensitive necrosis similar to or exceeding that seen earlier with intact *E. coli* cells occurred. No visible reactions were observed when elicitor concentrations of 100× were assayed on cultivars that did not yield a resistant reaction to *P. s.* pv. *glycinea* cells carrying *avrD*.

Leaves of sensitive soybean cultivars showing necrosis in response to the *avrD* elicitor also contained the phytoalexin glyceollin at quantities up to 500 μg/g fresh weight leaves (data not shown). Leaves of insensitive cultivars did not contain significant phytoalexin levels. Soybean cotyledons of the sensitive cultivar Harosoy but not the insensitive Merit also produced glyceollin in response to the *avrD* elicitor, but 10× or greater concentrations were required for activity as compared to leaves.

Production of the *avrD* elicitor by various *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* strains and other *P. syringae* pathovars. Culture fluids of *P. s.* pv. *glycinea* races 0, 1, 4, 5, and 6 grown on M9 glucose medium did not contain detectable *avrD* elicitor activity when concentrated to 1/50 the original medium volume by the Sep-Pak technique (Table 4). On the other hand, Sep-Pak C18 preparations from fluids of wild-type *P. s.* pv. *tomato* isolates PT23, B120, and DC3000 contained *avrD* elicitor activity on the predicted soybean cultivars when assayed at 5× or greater concentrations. A marker exchange *avrD* mutant strain of PT23 did not produce detectable *avrD* elicitor. However, the mutant strain carrying the *avrD* plasmid, pAVRD33, produced considerable elicitor activity, as did *P. s.* pv. *glycinea* race 4 cells carrying the same plasmid (Table 4).

Cells of PT23 harboring pAVRD33 produced approximately 10 times more *avrD* elicitor than did the wild-type cells as determined by assaying several dilutions of the respective Sep-Pak C18 preparations. All other *P. syringae* pathovars previously shown to contain DNA highly homologous to *avrD* (Kobayashi *et al.* 1990a) also produced the *avrD* elicitor, but bacteria that failed to hybridize did not produce the elicitor (Table 4). *P. s.* pv. *phaseolicola* (Burkholder) Young *et al.* isolate 0285-1 also produced *avrD* elicitor activity in culture fluids despite showing only a weakly hybridizing 5.6-kb *Hin*dIII band (Kobayashi *et al.* 1990a). Two other *P. s.* pv. *phaseolicola* isolates (14 and 3121, Table 1) also yielded *avrD* elicitor activity when M9 culture fluids were concentrated 25- to 50-fold (Table 4).

Table 4. Production of *avrD* elicitor activity by various *Pseudomonas syringae* isolates and occurrence of DNA homologous to *avrD*

Bacterial isolate	*avrD* homology[a]	Hypersensitive reaction to culture fluids[b]
P. s. pv. *atropurpurea*	No	No
P. s. pv. *syringae* tomato isolate	No	No
P. s. pv. *syringae* bean isolate	No	No
P. s. pv. *lachrymans*	Yes	Yes
P. s. pv. *glycinea* races 0, 1, 4, 5, or 6	Yes[a]	No
P. s. pv. *glycinea* race 4 with pPT101	—[a]	Yes
P. s. pv. *tomato* DC3000	Yes	Yes
P. s. pv. *tomato* B120	ND[c]	Yes
P. s. pv. *tomato* PT23	Yes	Yes
P. s. pv. *tomato* PT23 *avrD* mutant	—[a]	No
P. s. pv. *tomato* PT23 *avrD* mutant containing pAVRD33	—[a]	Yes
P. s. pv. *phaseolicola* 0285-1	Yes[a]	Yes
P. s. pv. *phaseolicola* 14	ND	Yes
P. s. pv. *phaseolicola* 3121	ND	Yes
P. s. pv. pisi	No	No
P. s. pv. *mori*	Yes	Yes
P. s. pv. *morsprunorum*	No	No
P. s. pv. *tabaci*	No	No
P. s. pv. *savastanoi*	No	No

[a] Hybridization data taken from Kobayashi *et al.* (1990a) using total genomic DNA from the various bacteria; all *P. s.* pv. *glycinea* races and the tested *P. s.* pv. *phaseolicola* isolate were observed to have less homology than the other *P. syringae* pathovers that tested positive (Kobayashi *et al.* 1990a); and (—) denotes homology irrelevant.

[b] Cultures were grown for 24 hr at 28° C on M9 medium (15 ml) with 2× glucose; bacterial cultures carring pAVRD33 were supplemented with kanamycin at 50 μg/ml; *P. s.* pv. *glycinea* race 4 carrying pPT101 was supplemented with tetracycline at 25 μg/ml; and cellfree fluids were processed by the Sep-Pak C18 method and redissolved in water at a final concentration of 20 times that in the original cultures. These preparations were infiltrated into at least six of the differential soybean cultivars shown in Table 2. Primary leaves were assessed after 24 hr for the occurrence of hypersensitive reactions, and preparations that elicited necrotic reactions only on cultivars which are incompatible to *P. s.* pv. *glycinea* race 4 cells expressing *avrD* were rated positive.

[c] ND = not determined.

Effect of *hrp* genes on expression of *avrD* and production of the *avrD* elicitor by *P. s.* pv. *glycinea*. The plasmid pAVRD34 carrying *avrD* was introduced into cells of wild-type *P. s.* pv. *glycinea* race 0 as well as six different mutants in the 20-kb *hrp* region of race 0 (mutants E2, E43, E19, E10-20, E10-2, and E10-18, Huynh *et al.* 1989) by conjugation with the helper plasmid pRK2013 (Ditta *et al.* 1980). Transconjugant bacteria were then tested for the ability to elicit the soybean HR and for production of the extracellular *avrD* elicitor in M9 culture medium.

P. s. pv. *glycinea* race 0 elicits an HR on all of the standard soybean differential cultivars except for cultivar Centennial because it carries *avrB* and *avrC* (Staskawicz *et al.* 1987). When *avrD* was introduced into wild-type race 0, these bacteria also caused an HR on cultivar Centennial. None of the six *hrp* mutants tested yielded an HR on cultivar Centennial or other soybean cultivars when they contained the *avrD* gene (data not shown). However, all of the *hrp* mutants as well as wild-type race 0 carrying *avrD* produced the *avrD* elicitor in culture medium. These results indicate that production of the *avrD* elicitor by *P. s.* pv. *glycinea* race 0 cells carrying *avrD* is not directly dependent on the *hrp* genes.

Production of the *avrD* elicitor by *E. coli* cells expressing the *avrD* homologue from *P. s.* pv. *glycinea*. *avrD* elicitor activity has not been detected in culture fluids of any *P. s.* pv. *glycinea* race (Table 4). However, the occurrence in *P. s.* pv. *glycinea* of a gene closely related to *avrD* (Kobayashi *et al.* 1990a) raised the question of whether the *P. s.* pv. *glycinea* gene might also lead to production of the *avrD* elicitor by bacterial hosts, albeit at reduced efficiency. To address this question, culture fluids from *E. coli* DH5α cells carrying the high expression constructs pPSGOR and pAVRDG-1 were analyzed for the presence of *avrD* elicitor activity. No activity was detected in soybean leaves from Sep-Pak C18 preparations concentrated up to 100× the starting concentration. However, when highly concentrated culture fluids were fractionated by HPLC, *avrD* elicitor activity was detected in soybean leaves of sensitive but not insensitive cultivars. Furthermore, the HPLC retention time of this activity was identical to that of the *avrD* elicitor produced by *E. coli* cells carrying pAVRD12 (data not shown). Quantities of the elicitor directed by the *P. s.* pv. *glycinea* gene were very low, however, since relative concentrations of 1,000× higher than the starting fluids were required to detect activity in soybean leaves of sensitive cultivars.

DISCUSSION

The *avrD* cultivar-specific elicitor described in this study has recently been isolated from *E. coli* and *P. s.* pv. *tomato* cells carrying the cloned *avrD* gene (S. Tamaki, M. Stayton, D. Gerhold, and N. T. Keen, unpublished). Although this elicitor has not yet been identified, it is of relatively low molecular weight and appears to be active in the nanomolar range. It is at least 10^3 times more active on soybean cultivars containing the putative complementary disease resistance gene. Similar specific HR phytoalexin elicitors from several pathogens have been previously reported (Bruegger and Keen 1979; De Wit *et al.* 1985; Keen and Legrand 1980; Mayama *et al.* 1986; Tepper and Anderson 1986). However, only one of them has been characterized, namely a small linear peptide isolated from tomato leaves inoculated with *Cladosporium fulvum* Cooke strains carrying an avirulence gene complementing the *Cf9* resistance gene (Schottens-Toma and De Wit 1988). Only *C. fulvum* races carrying the putative *Cf9* avirulence gene produce the peptide elicitor, but this elicitor has not yet been shown to directly result from avirulence gene activity. Our isolation of the *avrD* elicitor from bacteria carrying the cloned *avrD* gene is therefore significant since, for the first time, production of a cultivar-specific elicitor of the HR-phytoalexin mechanism has been causally linked to the action of a defined microbial avirulence gene.

The work described in this study on the detection and isolation of the *avrD* elicitor confirms the data of Bruegger and Keen (1979), indicating that race-specific elicitors may function in *P. syringae*-soybean interactions. Klement and Goodman (1967a, 1967b) also believed that bacterial pathogens produced substances which directly elicited the HR. It was not possible to isolate the active elicitors in the earlier investigations, but our work with the cloned *avrD* gene offers considerable insight into these failures.

First, availability of the cloned and overexpressed *avrD* gene in our study made it possible to isolate the *avrD* elicitor from culture fluids of *E. coli* and *P. s.* pv. *tomato*, but it would have been extremely difficult to isolate from wild-type cultures of *P. s.* pv. *tomato*. Further, other research has shown that *avrB*, *avrC*, and *avrD* are expressed at high levels only in the plant (Huynh *et al.* 1989; S. Tamaki and N. T. Keen, unpublished; D. Kobayashi, H. Shen, and N. T. Keen, unpublished). In the case of *avrD*, expression is low when *P. s.* pv. *tomato* cells are grown in culture medium but increases approximately 100 times when the bacteria are inoculated into soybean leaves (H. Shen, D. Kobayashi, and N. T. Keen, unpublished). Thus, as was also observed by De Wit *et al.* (1985), attempts to isolate specific elicitors from pathogens grown in culture may be difficult unless cloned and overexpressed avirulence genes are used.

Crosses of the soybean cultivars Flambeau × Merit have revealed the presence of a single dominant disease resistance gene, *Rpg4*, in cultivar Flambeau (N. T. Keen and R. I. Buzzell, unpublished). The results presented in this study indicate that the protein product of *avrD* does not directly interact with soybean plants carrying *Rpg4*. Instead, the *avrD*-encoded protein is presumed to possess an enzymatic function that converts a normal bacterial metabolite to the *avrD* elicitor which we have discovered.

These results consequently have several implications for our thinking on the mechanisms underlying recognition in gene-for-gene systems. For example, our observations concerning *avrD* rule out the function of the dimer model for gene-for-gene recognition (Ellingboe 1982) in which binding of the primary protein products of avirulence and resistance genes was proposed to account, per se, for the resistant state. Since production of the *avrD* elicitor by several *P. syringae* pathovars as well as *E. coli* cells solely requires the *avrD* gene and it encodes only a single

polypeptide, the evidence also rules out occurrence of the elicitor-suppressor model (Bushnell and Rowell 1981; Doke *et al.* 1979; Heath 1982; Ward and Stoessl 1976). On the other hand, the results with *avrD* are consistent with and directly prove one important prediction of the elicitor-receptor model for recognition in gene-for-gene systems, namely, that an avirulence gene leads directly or indirectly to the production of a metabolite which is specifically recognized by plant hosts carrying the matching disease resistance gene (Callow 1977; Day 1974; Gabriel *et al.* 1988; Keen 1985; Keen and Staskawicz 1988). It should be cautioned, however, that other *avr* genes, particularly from fungal and viral pathogens, may interact differently with plants. It is also possible that elicitors resulting from the activity of other avirulence genes may be relatively unstable or may have other properties which make their isolation difficult. The current availability of cloned avirulence genes, which permitted isolation of the *avrD* elicitor, should facilitate the search for other elicitors.

If the production of elicitors by proteins encoded by *avr* genes is a widespread phenomenon, it will explain several long-standing uncertainties about the interactions of bacterial pathogens with resistant plant hosts. First, certain antibiotic treatments or the killing of bacterial pathogens by mild means have long been known to destroy their ability to elicit the plant HR when infiltrated into leaves (Klement and Goodman 1967a, 1967b). Sasser (1978) also reported that antibiotics which block protein synthesis rapidly destroyed the ability of phytopathogenic bacteria to elicit the HR. In the case of *avrD* in *E. coli* or *P. s.* pv. *glycinea* cells, production of the *avrD* elicitor has also been observed to cease rapidly after heat-killing or antibiotic treatment and would appear to account for their loss of HR competency. This observation may also be related to the failure of *P. s.* pv. *glycinea hrp* mutants (Huyhn *et al.* 1989; Lindgren *et al.* 1986, 1988) carrying *avrD* to elicit the HR in appropriate plant hosts. If the bacteria are unable to grow in the host because of a *hrp* mutation, they would not be expected to produce elicitor, just as in the case of antibiotic inhibition. However, as our results have shown, *hrp* mutants carrying *avrD* produce the *avrD* elicitor in culture medium when the cells grow actively. Thus, expression of *avrD* and production of the *avrD* elicitor do not appear to directly require functional *hrp* genes.

While the limited number of isolates surveyed in this study do not permit general conclusions, they nevertheless showed that, except for *P. s.* pv. *glycinea*, all *P. syringae* pathovars which contained *avrD* as determined by Southern blot analysis also produced readily detectable quantities of the *avrD* elicitor (Table 4). Surprisingly, *P. s.* pv. *phaseolicola* isolate 0285-1 also produced the *avrD* elicitor despite the weak hybridization of its conserved 5.6-kb *Hin*dIII band to an *avrD* intragenic probe (Kobayashi *et al.* 1990a). Two other *P. s.* pv. *phaseolicola* isolates were also observed to produce the *avrD* elicitor.

E. coli cells carrying high-level expression constructs of *P. s.* pv. *glycinea* ORF 1 also produced low levels of the *avrD* elicitor. This observation is significant because it contends that the protein encoded by ORF 1 of *P. s.* pv. *glycinea* leads to formation of the *avrD* elicitor, albeit less efficiently than the protein encoded by *avrD* of *P. s.* pv. *tomato*. Although we have not observed production of the elicitor by *P. s.* pv. *glycinea* cells with or without the cloned *P. s.* pv. *glycinea* ORF 1 gene, the fact that ORF 1 does retain minimal *avrD* biochemical activity is of interest. Thus, while *P. s.* pv. *glycinea* race 4 cells containing the cloned *P. s.* pv. *glycinea* ORF 1 secrete insufficient quantities of the *avrD* elicitor to permit bacterial recognition by resistant soybean leaves, the level of production may be sufficient to meet the bacterial function of *avrD*.

A surprising observation made with *E. coli* cells carrying high-level expression plasmids of *avrD* as well as with relatively high concentrations of the *avrD* elicitor isolated from culture fluids was the production of a spreading, systemic HR (Fig. 1). Such a reaction has not previously been observed with plant pathogenic bacteria, and indeed *P. s.* pv. *glycinea* race 4 carrying pAVRD33 did not cause systemic hypersensitive necrosis, despite producing substantial amounts of the elicitor in culture fluids. It appears, therefore, that concentrations of the elicitor elaborated by bacteria carrying *avrD* are normally sufficient to elicit localized but not systemic hypersensitive necrosis. This is in keeping with the general observation that phytopathogenic bacteria do not produce systemic, massive hypersensitive necrosis. Indeed, a visible, confluent HR requires that relatively high bacterial concentrations (generally $\geq 5 \times 10^6$ cells per milliliter) are infiltrated into leaves. Otherwise, the HRs that occur are small and cannot be detected with the unaided eye (Essenberg *et al.* 1979; Holliday *et al.* 1981; Turner and Novacky 1974).

The systemic HRs that we observed appear to be a consequence of overproduction of the *avrD* elicitor by relatively high numbers of *E. coli* cells carrying high-level expression plasmids of *avrD* (Table 2). Infiltration of high amounts of the elicitor isolated from concentrated bacterial culture fluids also led to systemic necrosis. However, it has not been established whether the elicitor itself moves systemically or elicits the local production of a 'second messenger' that then moves through the leaf and produces additional hypersensitive necrosis.

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LITERATURE CITED

Bender, C. L., and Cooksey, D. A. 1986. Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: Conjugative transfer and role in copper resistance. J. Bacteriol. 165:534-541.

Bruegger, B. B., and Keen, N. T. 1979. Specific elicitors of glyceollin accumulation in the *Pseudomonas glycinea*-soybean host-parasite system. Physiol. Plant Pathol. 15:43-51.

Bushnell, W. R., and Rowell, J. B. 1981. Suppressors of defense reactions: A model for roles in specificity. Phytopathology 71:1012-1014.

Callow, J. A. 1977. Recognition, resistance and the role of plant lectins in host-parasite interactions. Adv. Bot. Res. 4:1-49.

Crouse, G. F., Frischauf, A., and Lehrach, H. 1983. An integrated and simplified approach to cloning into plasmids and single-stranded phages. Methods Enzymol. 101:78-89.

Day, P. 1974. Genetics of Host-Parasite Interactions. Freeman, San Francisco. 238 pp.

De Wit, P. J. G. M., Hofman, J. G. M., Velthuis, G. C. M., and Kuć, J. A. 1985. Isolation and characterization of an elicitor of necrosis isolated from intercellular fluids of compatible interactions of *Cladosporium fulvum* (syn. *Fulvia fulva*) and tomato. Plant Physiol. 77:642-647.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA 77:7347-7351.

Doke, N., Garas, N. A., and Kuć, J. 1979. Partial characterization and aspects of the mode of action of a hypersensitivity-inhibiting factor (HIF) isolated from *Phytophthora infestans*. Physiol. Plant Pathol. 15:127-140.

Ellingboe, A. H. 1982. Genetical aspects of active defence. Pages 179-192 in: Active Defense Mechanisms in Plants. R. K. S. Wood, ed. Plenum Press, New York.

Essenberg, M., Cason, E. T., Hamilton, B., Brinkerhoff, L. A., Gholson, R. K., and Richardson, P. E. 1979. Single cell colonies of *Xanthomonas malvacearum* in susceptible and immune cotton leaves and the local resistant response to colonies in immune leaves. Physiol. Plant Pathol. 15:53-68.

Flor, H. H. 1942. Inheritance of pathogenicity in *Melampsora lini*. Phytopathology 32:653-669.

Gabriel, D. W., Loschke, D. C., and Rolfe, B. G. 1988. Gene-for-gene recognition: The ion channel defense model. Pages 3-14 in: Molecular Genetics of Plant-Microbe Interactions, 1988. R. Palacios and D. P. S. Verma, eds. APS Press, St. Paul, MN.

Ghrayeb, J., Kimura, H., Takahara, M., Hsiung, H., Masui, Y., and Inouye, M. 1984. Secretion cloning vectors in *Escherichia coli*. EMBO J. 3:2437-2442.

Heath, M. C. 1982. The absence of active defense mechanisms in compatible host-pathogen interactions. Pages 143-156 in: Active Defense Mechanisms in Plants. R. K. S. Wood, ed. Plenum Press, New York.

Holliday, M. J., Keen, N. T., and Long, M. 1981. Cell death patterns and accumulation of fluorescent material in the hypersensitive response of soybean leaves to *Pseudomonas syringae* pv. *glycinea*. Physiol. Plant Pathol. 18:279-287.

Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: Regulation of a pathogen gene determining host cultivar specificity. Science 245:1374-1377.

Keen, N. T. 1978. Phytoalexins: Efficient extraction from leaves by a facilitated diffusion technique. Phytopathology 68:1237-1239.

Keen, N. T. 1985. Progress in understanding the biochemistry of race-specific interactions. Pages 85-101 in: Genetic Basis of Biochemical Mechanisms of Plant Disease. J. V. Groth and W. R. Bushnell, eds. APS Press, St. Paul, MN.

Keen, N. T., and Legrand, M. 1980. Surface glycoproteins: Evidence that they may function as the race-specific phytoalexin elicitors of *Phytophthora megasperma* f. sp. *glycinea*. Physiol. Plant Pathol. 17:175-192.

Keen, N. T., and Staskawicz, B. 1988. Host range determinants in plant pathogens and symbionts. Annu. Rev. Microbiol. 42:421-440.

Keen, N. T., Tamaki, S., Kobayashi, D., and Trollinger, D. 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene 70:191-197.

Keen, N. T., Yoshikawa, M., and Wang, M. C. 1983. Phytoalexin elicitor activity of carbohydrates from *Phytophthora megasperma* f. sp. *glycinea* and other sources. Plant Physiol. 71:466-471.

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of phycocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.

Klement, Z., and Goodman, R. N. 1967a. The hypersensitive reaction to infection by bacterial plant pathogens. Annu. Rev. Phytopathol. 5:17-44.

Klement, Z., and Goodman, R. N. 1967b. The role of the living bacterial cell and induction time in the hypersensitive reaction of the tobacco plant. Phytopathology 57:322-323.

Kobayashi, D. Y., Tamaki, S. J., and Keen, N. T. 1989. Cloned avirulence genes from the tomato pathogen *Pseudomonas syringae* pv. *tomato* confer cultivar specificity on soybean. Proc. Natl. Acad. Sci. USA 86:157-161.

Kobayashi, D. Y., Tamaki, S. J., and Keen, N. T. 1990a. Molecular characterization of avirulence gene D from *Pseudomonas syringae* pv. *tomato*. Mol. Plant-Microbe Interact. 3:94-102.

Kobayashi, D. Y., Tamaki, S. J., Trollinger, D. J., Gold, S., and Keen, N. T. 1990b. A gene from *Pseudomonas syringae* pv. *glycinea* with homology to avirulence gene D from *P. s.* pv. *tomato* but devoid of the avirulence phenotype. Mol. Plant-Microbe Interact. 3:103-111.

Lamb, C. J., Lawton, M. A., Dron, M., and Dixon, R. A. 1989. Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell 56:215-224.

Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. 1986. Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity on bean plants and hypersensitivity on nonhost plants. J. Bacteriol. 168:512-522.

Lindgren, P. B., Panopoulos, N. J., Staskawicz, B. J., and Dahlbeck, D. 1988. Genes required for pathogenicity and hypersensitivity are conserved and interchangeable among pathovars of *Pseudomonas syringae*. Mol. Gen. Genet. 211:499-506.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Masui, Y., Mizuno, T., and Inouye, M. 1984. Novel high-level expression cloning vehicles: 10^4-fold amplification of *Escherichia coli* minor protein. Bio/Technology 2:81-85.

Mayama, S., Tani, T., Ueno, T., Midland, S. L., Sims, J. J., and Keen, N. T. 1986. The purification of victorin and its phytoalexin elicitor activity in oat leaves. Physiol. Mol. Plant Pathol. 29:1-18.

Sasser, M. 1978. Involvement of bacterial protein synthesis in induction of the hypersensitive reaction in tobacco. Phytopathology 68:361-363.

Schottens-Toma, I. M. J., and De Wit, P. J. G. M. 1988. Purification and primary structure of a necrosis-inducing peptide from the apoplastic fluids of tomato infected with *Cladosporium fulvum* (syn. *Fulvia fulva*). Physiol. Mol. Plant Pathol. 33:59-67.

Staskawicz, B. J., Dahlbeck, D., and Keen, N. T. 1984. Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinea* determines race-specific incompatibility on *Glycine max* (L.) Merr. Proc. Natl. Acad. Sci. USA 81:6024-6028.

Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169:5789-5794.

Tamaki, S., Dahlbeck, D., Staskawicz, B., and Keen, N. T. 1988. Characterization and expression of two avirulence genes cloned from *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 170:4846-4854.

Tepper, C. S., and Anderson, A. J. 1986. Two cultivars of bean display a differential response to extracellular components from *Colletotrichum lindemuthianum*. Physiol. Mol. Plant Pathol. 29:411-420.

Turner, J. G., and Novacky, A. 1974. The quantitative relation between plant and bacterial cells involved in the hypersensitive reaction. Phytopathology 64:885-890.

Vieira, J., and Messing, J. 1987. Production of single-stranded plasmid DNA. Methods Enzymol. 153:3-11.

Ward, E. W. B., and Stoessl, A. 1976. On the question of 'elicitors' or 'inducers' in incompatible interactions between plants and fungal pathogens. Phytopathology 66:940-941.