Research Note

Avirulence Gene *avrPphC* from *Pseudomonas syringae* pv. *phaseolicola* 3121: A Plasmid-borne Homologue of *avrC* Closely Linked to an *avrD* Allele

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Cosmid clone pPsp01 from race 1 *Pseudomonas syringae* pv. *phaseolicola* isolate 3121 conferred a unique pattern of soybean cultivar reactions when expressed in *P. s.* pv. *glycinea* R4. The avirulence phenotype was shown to result from the presence in clone pPsp01 of an *avrD* allele as well as an additional avirulence gene located approximately 5-kb upstream. The new gene, called *avrPphC*, shows high identity to and is phenotypically identical to *avrC*, previously cloned from *P. s.* pv. *glycinea* race 0. *avrD* and *avrPphC* occur on an approximately 120-kb indigenous plasmid in *P. s.* pv. *phaseolicola* 3121. Although commonly observed in *Xanthomonas campestris*, this is the first noted occurrence of multiple avirulence genes on a single plasmid in *Pseudomonas syringae*. Unlike *avrD*, however, *avrPphC* does not appear to occur widely in pathovars of *Pseudomonas syringae*.

Additional key words: hypersensitive response, host range determinants.

Cosmid clone pPsp01, supplied by D. Dahlbeck and B. Staskawicz (Table 1), was isolated from a library of total DNA from *Pseudomonas syringae* pv. *phaseolicola* 3121 because it conferred the hypersensitive response (HR) on several soybean cultivars when introduced into *P. s.* pv. *glycinea* R4. Since the cultivar spectrum of hypersensitive reactions in response to *P. s.* pv. *glycinea* race 4 carrying pPsp01 was not exhibited by any previously cloned *P. syringae* avirulence gene, it was of interest to characterize genetic determinants of the avirulence phenotype. Yucel *et al.* (1994) previously showed that pPsp01 harbors an *avrD* allele as well as a second avirulence gene that elicits an HR on the soybean cv. Acme (Table 2). To identify this gene, several subclones of pPsp01 were constructed using methods described by Yucel *et al.* (1994) and introduced into *P. s.* pv. *glycinea* R4 (Table 1, Fig. 1). This permitted localization of the new avirulence gene to an approximately 1.4-kb *ClaI/HindIII* DNA fragment (pDAHR15, Fig. 1). The gene was designated *avrPphC*, in accordance with the nomenclature suggested by Vivian and Mansfield (1993).

The approximately 1.4-kb *ClaI/HindIII* DNA fragment of pDAHR15 carrying *avrPphC* was sequenced using double-strand methods (US Biochemicals, Cleveland, OH; Yucel *et al.* 1994). The nucleotide sequence is not shown here but has been entered in GenBank as accession no. U10377. The sequenced DNA contained a single long open reading frame directed from the *HindIII* end of the insert toward the *ClaI* restriction site (Fig. 1). Database searches revealed that the predicted protein product of *avrPphC* showed high homology only with AvrC, previously characterized from *P. s.* pv. *glycinea* R0 (Staskawicz *et al.* 1987; Tamaki *et al.* 1991). Only two amino acid substitutions were observed in AvrPphC relative to AvrC (Fig. 2), and the two genes shared 99.5% DNA sequence homology.

To test whether avrPphC behaved phenotypically the same as *avrC*, pDAHR15 (Table 1) was introduced into *P. s.* pv. *glycinea* race 4, and the bacteria were inoculated into primary leaves of several soybean cultivars. The soybean resistance gene *Rpg3* complements *avrC* (Keen and Buzzell 1991). Bacteria carrying pAVRC2 (Keen and Buzzell 1991) were accordingly inoculated as a control. The results showed that *P. s.* pv. *glycinea* R4 harboring *avrPphC* gave plant reactions that were indistinguishable from those carrying *avrC* in all tested cultivars. Hypersensitive reactions were observed in response to both genes in soybean cultivars Acme, Envy, Essex, Fayette, Flambeau, Gnome, Merrimax, 4R, and PI 290.136; susceptible plant responses were observed in cultivars BARC-2, Bonminori, Canatto, Centennial, Chapman, Chippewa, Columbia, Elgin, Enrei, Evans, Fiskeby V, Forrest, Grande, Hardee, Harosoy, Hidatsa, Higan, Hodgson, Hurrelbrink, Keburi, Lindarin, Manitoba Brown, Merit, Minsoy, Nezumisaya, Norin-2, Raiden, Peking, Vance, Williams, and 5S. Cultivar Norchief was generally scored as yielding a hypersensitive reaction to bacteria carrying either *avrPphC* or *avrC* but frequently showed signs of susceptibility. This cultivar is therefore considered somewhat intermediate in reactions to *avrPphC* and *avrC*.

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Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Source or reference
Bacteria		
Escherichia coli		
DH5α	F⁻*lacZ* M15 *endA1 hsdR17 supE44 thi-1 gyrA relA1*⁻	Bethesda Research Laboratories, Gaithersburg, MD
HB101	F⁻*hsdS20 (hsdR hsdM) recA13 ara-14 proA2 lacY1 gal K2 rpsL20* (Str^r) *xyl-5 mtl-1 supE44* λ⁻	Maniatis *et al.* 1982
Pseudomonas syringae		
pv. *glycinea* race 4	*rif*^{r}, *ap*^{r}	Kobayashi *et al.* 1989
Plasmids		
pUC128/pUC129	Ap^r cloning and sequencing vectors	Keen *et al.* 1988
pRK415	Tc^r broad host range vector, *mob*$^+$	Keen *et al.* 1988
pRK 2013	Km^r, Tra$^+$, helper plasmid	Ditta *et al.* 1980
pRK 2073	Sm^r, Tra$^+$, helper plasmid	Ditta *et al.* 1980
pDSK609	Sm^r broad host range vector	Murillo *et al.* 1994
pDSK600	Sm^r broad host range vector	Murillo *et al.* 1994
pPsp01	pLAFR3 cosmid clone containing an approx. 26-kb DNA fragment from *P. s.* pv. *phaseolicola* 3121	D. Dahlbeck and B. Staskawicz
pPLX14	Approx. 7.4-kb *Xba*I fragment from pPsp01 cloned into pUC129 in same orientation as Psp01	This study
pAHR14	Approx. 13-kb *Xba*I fragment from pPsp01 cloned into pRK415	This study
pPSX14	Approx. 5.6-kb *Xba*I fragment from Psp01 cloned into pUC129 in the same orientation as in Psp01	This study
pDAHR5	Approx. 5.8-kb *Apa*I-*Kpn*I subclone from pAHR1 cloned into the same sites in pUC128	This study
pDAHR7	Approx. 4.9-kb *Stu*I/*Kpn*I fragment from pAHR1 cloned into the *Kpn*I/*Hpa*I sites of pDSK609	This study
pDAHR11	Approx. 3.9-kb *Hin*dIII partial/*Sac*I(*Stu*I) fragment from pDAHR7 cloned into the *Sac*I/*Hin*dIII sites of pDSK600	This study
pDAHR14	Approx. 1.3-kb *Pvu*II/*Hin*dIII fragment from pDAHR11 cloned into the *Sma*I/*Hin*dIII sites of pUC129	This study
pDAHR15	Approx. 1.4-kb *Cla*I/*Bam*HI fragment from pDAHR11 cloned into the same sites of pUC129	This study
pDAHR16	Approx. 1.3-kb *Bgl*II/*Bam*HI fragment from pDAHR11 cloned into the *Bam*HI site of pUC129	This study
pDAHR17	Approx. 3-kb *Cla*I/*Kpn*I fragment from pDAHR5 cloned into the same sites of pUC129	This study

[a] Ap, ampicillin; Km, kanamycin; Rif, rifampicin; Sm, streptomycin; Tc, tetracycline; ^{r}, resistance; kb, kilobase. Subcloned fragments from the *avrPphC* region in pUC plasmids were re-cloned into pDSK600 or pDSK609 before introduction into *P. s.* pv. *glycinea* R4 cells.

Table 2. Reactions of several soybean cultivars to *Pseudomonas syringae* pv. *glycinea* R4 with cloned avirulence regions from *Pseudomonas syringae* pv. *phaseolicola*, *avrPphC*, *avrD*, or *avrC*

Cultivar	Construct		
	avrPphC	*avrD*	*avrC*
Acme	HR[a]	C[a]	HR
Centennial	HR	HR	C
Chippewa	HR	HR	C
Flambeau	HR	HR	HR
Hardee	C	C	C
Harosoy	HR	HR	C
Lindarin	HR	HR	C
Merit	C	C	C
Norchief	HR	HR	HR/I[a]
Peking	C	C	C

[a] HR, Hypersensitive reaction observed 24–36 hr after inoculation; C, compatible, water-soaked lesions observed 48–72 hr after inoculation; I, Intermediate reaction observed on cultivar Norchief in which reactions were initially scored as HR but appeared compatible after approximately 4 days. Constructs employed were pAVRD33 (*avrD*, Keen and Buzzell 1991); pAVRC2 (*avrC*, Keen and Buzzell 1991); and pDAHR15 (*avrPphC*, Table 1). The DNA inserts were recloned into pDSK609 before introduction in R4.

Previous studies with *avrD* established that this avirulence gene was present in several pathovars of *Pseudomonas syringae* on large indigeneous plasmids (Kobayashi *et al.* 1989, 1990; Murillo *et al.* 1994; Yucel and Keen 1994). Since *avrPphC* occurs on the same plasmid as *avrD* in *P. s.* pv. *phaseolicola* 3121, other *P. syringae* pathovars were surveyed for the presence of hybridizing DNA. Southern blot analyses (performed according to Murillo *et al.* 1994) of both indigenous plasmids and total DNA using approximately 600-bp *Bgl*II-*Xba*I or approximately 800 bp *Bgl*II-*Hin*dIII fragments internal to *avrPph3* (Fig. 1) as probes revealed that only *P. s.* pv. *phaseolicola* 3121 and *P. s.* pv. *glycinea* R6 and R0 showed strongly hybridizing bands (data not shown). This is consistent with previous observations (Staskawicz *et al.* 1987) in which internal fragments of the cloned *avrC* gene from *P. s.* pv. *glycinea* R0 also showed hybridization only to DNA from R6. No evidence for hybridizing DNA was observed with total DNA or plasmid DNA from *P. s.* pv. *tomato* PT23, the original source of *avrD* (Kobayashi *et al.* 1990). Total DNA restricted with *Eco*RI or *Hin*dIII of the following isolates also did not show significant hybridization to the *avrPphC* probes: *P. syringae* pvs. *aesculi* 2894, *apii* 1089-5, *atropurpurea* 2340, *ciccaronei* 2342, *cilantro* isolates 0-788-9 and 0790-2, *coronafaciens* 2216, *dendropanacis* 3226, *garcae* 1634, *glycinea* isolates R4 and 2214, *hibisci* 2895, *japonica* 2896, *lachrymans*, *mori* 0782-30, *morsprunorum* 2115, *phaseolicola* G50 (race 2), *savastanoi* 0485-9, *tomato* 10862, and *viburni* 1702 (all isolates are described in Yucel *et al.* 1994). Avirulence gene D, originally cloned from *P. s.* pv. *tomato* PT23 (Kobayashi *et al.* 1990), is located on an approximately 83-kb indigenous plasmid (Murillo *et al.* 1994). Although we have shown that *avrPphC* and *avrD* occur on an

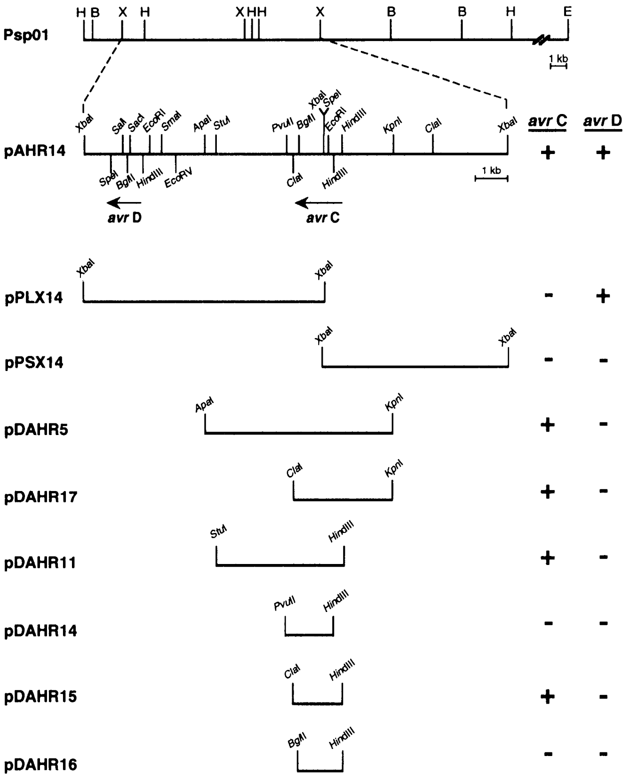

Fig. 1. Restriction map of the relevant portion of cosmid clone pPsp01 and subclones prepared to localize the *avrPphC* gene. The region between two *Xba*I sites in subclone pDAHR1 is shown expanded, and plant reactions for this and successive subclones for the *avrC* and *avrD* hypersensitive phenotypes are denoted by (+) and negative reactions shown by (–). Subclones are described more fully in Table 1. Since the terminal *Hin*dIII site in pDAHR11 is not unique, subsequent plasmid constructs were constructed using the adjacent polylinker *Bam*HI site. All subclones were recloned into pRK415, pDSK600, or pDSK609 using convenient polylinker restriction sites, introduced into *P. s.* pv. *glycinea* R4 by conjugation or electroporation, and the bacteria were inoculated into appropriate soybean cultivars for determining *avrC* and *avrD* phenotypes.

indigenous plasmid in *P. s.* pv. *phaseolicola* 3121, Southern blots disclosed that *avrPphC* is not present on the indigenous *P. s.* pv. *tomato* plasmid (pPT23B) carrying *avrD*. Whereas *avrD* is widely distributed in *P. syringae* pathovars (Yucel *et al.* 1994), *avrC* and *avrpPhC* have more limited distribution, thus far only observed in *P. s.* pv. *glycinea* R0 and R6 in addition to pv. *phaseolicola* 3121. It is noteworthy that the hybridizing DNA observed in R6 may represent a recessive *avrC* allele, because this bacterium does not exhibit the *avrC* phenotype.

Multiple members of the *avrBs3* gene family in *Xanthomonas campestris* pathovars have been shown to occur on indigenous plasmids (e.g., DeFeyter and Gabriel 1991), but multiple avirulence genes had not previously been identified on a single indigenous plasmid in *P. syringae*. Although it might be suspected from the close linkage of *avrPphC* and *avrD* in *P. s.* pv. *phaseolicola* 3121 that these genes could be functionally linked, there is currently no evidence for this. Furthermore, the avirulence phenotypes conferred by these genes do not require presence of the other gene.

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avrPphC  MGNVCFRPSRSHVSQEFSQSEFSAASPVRTSERPSDASLDAGLES 45
   avrC  -----------------------T--------------------- 45

avrPphC  SSACHRSGLRGPAKHSMLSLEEIGLVGAARWPDDAPGLNISNKSN 90
   avrC  --------------------D------------------------ 90

avrPphC  TQENKRYCESLYQAARIAGGSIASGRVTSFDGLWRNATKWRLSRI 135
   avrC  --------------------------------------------- 135

avrPphC  LSGDASKIDFATVRMPNTRFVTSLRRPYHSVIERVRNHSDANSEI 180
   avrC  --------------------------------------------- 180

avrPphC  YEGEYLGGIETKVYRQHGTISSTTIPMTIVSAVADDDDIHERLKS 225
   avrC  --------------------------------------------- 225

 avrPph  LPKNERRHLKDLMAASHPNMITHTDAVYLPMIKDHLESLYLQAID 270
   avrC  --------------------------------------------- 270

avrPphC  PSLEQHEALELIRRIPWWAASAAPDRRGSAAKAEFAARSIAFAHG 315
   avrC  --------------------------------------------- 315

avrPphC  IELPPFEHGAVPDIEAMLRSEEQFVEDYPNLFERPPQ 352
   avrC  ------------------------------------- 352
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Fig. 2. Predicted amino acid sequence of the protein product of *avrPphC* compared with that of the product from *avrC*. Different amino acids in AvrC are shown as their single-letter code, and identical amino acids are denoted by (–). Bold-type amino acids occur in the central region required for AvrC specificity, as determined by Tamaki *et al.* (1991).

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