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Cloned avirulence genes from the tomato pathogen *Pseudomonas syringae* pv. *tomato* confer cultivar specificity on soybean

(host–parasite interactions/hypersensitive response/disease resistance/incompatibility)

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ABSTRACT **Three different cosmid clones were isolated from a genomic library of the tomato pathogen *Pseudomonas syringae* pv. *tomato*, which, when introduced into the soybean pathogen *P. syringae* pv. *glycinea*, caused a defensive hypersensitive response (HR) in certain soybean cultivars. Each clone was distinguished by the specific cultivars that reacted hypersensitively and by the intensity of the HR elicited. Unlike wild-type *P. syringae* pv. *tomato* isolates, which elicit the HR on all soybean cultivars, all three clones exhibited cultivar specificities analogous to avirulence genes previously cloned from *P. syringae* pv. *glycinea*. However, the collective phenotypes of the three clones accounted for HRs on all tested soybean cultivars. One of the three *P. syringae* pv. *tomato* clones contained an avirulence gene homologous to *avrA*, which was previously cloned from *P. syringae* pv. *glycinea* race 6. The other two *P. syringae* pv. *tomato* clones expressed unique HR patterns on various soybean cultivars, which were unlike those caused by any known *P. syringae* pv. *glycinea* race or previously cloned *P. syringae* pv. *glycinea avr* gene. Further characterization of the second *P. syringae* pv. *tomato* clone indicated that the avirulence phenotype resided on a 5.6-kilobase *Hin*dIII fragment that, in Southern blot analyses, hybridized to an identical-size fragment in various *P. syringae* pathovars, including all tested *glycinea* races. These results demonstrate that avirulence genes may be distributed among several *P. syringae* pathovars but may be modified so that the HR is not elicited in a particular host plant. Furthermore, the data raise the possibility that avirulence genes may function in host-range determination at levels above race–cultivar specificity.**

Disease resistance in plants frequently results from a basic incompatibility between the host and invading pathogen that is established by specific genetic factors in both organisms (1). These factors appear to determine plant recognition of the pathogen during the early stages of infection, resulting in a series of biochemical events in the plant that constitute a localized defense mechanism called the hypersensitive response (HR) (2). The HR is characterized by rapid necrosis of plant cells in proximity to the invading pathogen, followed by the accumulation of antimicrobial compounds termed phytoalexins as well as other factors (3).

Genetic studies of both plants and pathogens have established a gene-for-gene relationship in which elicitation of the HR requires a single dominant allele for resistance in the host cultivar and a complementary dominant gene for avirulence in the infecting pathogen race (1, 4). If either dominant allele is absent from the genotypes of the interacting organisms, the defense response is not activated, and the plant is therefore susceptible to pathogen attack. Despite recent progress (for example, see ref. 5), disease-resistance genes have not yet been cloned and characterized from any higher plant. However, avirulence genes have been cloned recently from several bacterial pathogens.

Staskawicz *et al.* (6) first cloned a gene for avirulence from *Pseudomonas syringae* pv. *glycinea* race 6, the causal agent of bacterial blight of soybean. This gene, designated *avrA* (7), defines the *P. syringae* pv. *glycinea* race 6 phenotype and restricts the number of soybean cultivars that the pathogen can attack. Additional avirulence genes were subsequently identified in other *P. syringae* pv. *glycinea* races (8) as well as in pathovars of *Xanthomonas campestris* (9, 10). In all of these cases, avirulence genes have been shown to determine the race phenotype of the pathogens, defined according to their range of virulence on a standard set of host cultivars, and therefore result in cultivar level resistance.

In addition to race–cultivar specificity, higher orders of plant–pathogen specificity occur. Pathogen subdivisions such as pathovars or *formae speciales* are defined largely according to their specific plant-species host range. In the bacterial species *P. syringae*, there are >40 different pathovars specialized on a number of different hosts (11). Although the genetic factors conferring host-range determination within this group are currently unknown, many *P. syringae* pathovars as well as other plant pathogens frequently elicit the defensive HR on nonhost plant species (3). However, it has not been established whether these HRs causally limit the pathogen host range. In addition, the difficulty of intercrossing most plant species has hindered understanding the genetic basis of host species resistance to pathogens.

The demonstration that avirulence genes may determine race–cultivar specificity in several bacterial plant pathogens raised the possibility that higher levels of host–pathogen specificity such as pathovar–plant species interactions may, in some cases, also be determined by pathogen avirulence genes and corresponding plant disease-resistance genes. To test this hypothesis, *Pseudomonas syringae* pv. *tomato*, the causal agent of bacterial speck of tomato, was chosen to study the genetic factors involved in eliciting the HR on the nonhost plant, soybean. We report here, as in preliminary reports (12, 13), the cloning and partial characterization of three different avirulence genes from *P. syringae* pv. *tomato* that, when expressed in the soybean pathogen *P. syringae* pv. *glycinea*, elicit the HR on soybean in a race-specific manner.

MATERIALS AND METHODS

Bacterial Strains, Vectors, Culture Media, and Antibiotics. *P. syringae* strains, *Escherichia coli* strains, and plasmid vectors used in this study are listed in Table 1. *E. coli* cells were routinely maintained on LB agar (14) and grown at 37°C. *P. syringae* strains were maintained on KMB agar (19) at 28°C. Antibiotics purchased from Sigma were used at the following levels unless otherwise indicated: tetracycline at

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Abbreviation: HR, hypersensitive response.

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Table 1. Bacterial strains and plasmids

	Relevant characteristics or geographic origin	Source (ref.)
Strain		
E. coli		
HB101	*F⁻hsdS20 [hsdR hsdM recA13 ara-14 proA2 lacYI galK2 rpsL20* (Str^r) *xyl-5 mtl-1 supE44 λ⁻]*	(14)
DH5α	*F⁻lacZ* ΔM15 *endA1 recA1 hsdR17 supE44 thi-1 gyrA relA1 λ⁻*	Bethesda Research Laboratories
P. syringae		
pv. *glycinea* race 0		B. Staskawicz (8)
race 4	Rif^r	This lab
race 5	Rif^r	This lab
race 6	Rif^r	This lab
pv. *lachrymans*		This lab
pv. *mori* UCPPB 566		D. A. Cooksey
pv. *phaseolicola* 0285-1		D. A. Cooksey
pv. *maculicola* 1083-2		D. A. Cooksey
pv. *tomato* PT20	Imperial Valley, CA	D. A. Cooksey (15)
PT23	San Diego, CA	D. A. Cooksey (15)
P.T.F.	Michigan	D. A. Cooksey (15)
P144	New Jersey	D. A. Cooksey
PT178	North Carolina	D. A. Cooksey
B120	Delaware	T. Denny
Plasmid		
pLAFR3	Tc^r, cosmid derivative of RK2	(8)
pRK2013	Km^r, Tra, helper plasmid derivative from RK2	(16)
pRK415	Tc^r, RK2 plasmid derivative	(17)
pUC118	Ap^r, pUC18 derivative	(18)
pPSG6001	3.2-kb *Acc* I fragment containing *avrA* from *P. syringae* pv. *glycinea* race 6 in pUC8	(7)
pPT4D2	Class I cosmid clone	This work
pPT7H6	Class I cosmid clone	This work
pPT9A11	Class I cosmid clone	This work
pPT201	3.2-kb *Sal* I fragment subclone from pPT9A11 in pRK415	This work
pPT211	3.2-kb *Sal* I fragment subclone from pPT9A11 in pUC118	This work
pPT4E10	Class II cosmid clone	This work
pPT101	5.6-kb *Hin*dIII fragment from pPT4E10 in pRK415	This work
pPT112	pPT101 with Tn*5* insert mutating avirulence gene activity	This work
pPT10E9	Class III cosmid clone	This work

Ap, Ampicillin; Km, kanamycin; Rif, rifampicin; Tc, tetracycline; r, resistant.

12.5 μg/ml, kanamycin at 50 μg/ml, and rifampicin at 100 μg/ml.

Recombinant DNA Techniques and Cosmid Library Construction. Methods for plasmid DNA isolations, restriction digests, ligations, Southern DNA transfers, and nick-translations were performed as described by Maniatis *et al.* (14).

Isolation of total DNA from *P. syringae* pv. *tomato* PT23 was performed as described by Staskawicz *et al.* (6), and construction of a genomic library in the cosmid vector pLAFR3 was carried out as described (8). Cosmids were packaged in phage extracts purchased from Boehringer Mannheim.

For Southern blot analyses, 1 μg of plasmid DNA or 4 μg of total genomic DNA was digested with the appropriate restriction enzymes and electrophoresed in 0.7% agarose before transfer onto Zetabind nylon membranes (AMF). Hybridizations with ^{32}P-labeled probes were carried out at 42°C in 50% (wt/vol) formamide/0.75 M NaCl/0.075 M sodium citrate/0.02 M sodium phosphate, pH 6.7/0.1 mg of salmon sperm DNA per ml with gentle shaking for 12 hr. Blots were then washed in 0.3 M NaCl/0.03 M sodium citrate/0.1% $NaDodSO_4$ at room temperature for 30 min, followed by 0.015 M NaCl/0.001 M sodium citrate/0.1% $NaDodSO_4$ at 42°C for 2 hr prior to exposure to x-ray film.

Conjugations, Plant Inoculations, and Plant Growth Conditions. Triparental matings were performed as described by Ditta *et al.* (16). Matings were carried out at 28°C for 5–8 hr, followed by an incubation at 4°C for 24 hr before selection on KMB agar supplemented with rifampicin and tetracycline at 25 μg/ml. Single colonies were selected and successively transferred on selective medium to make up the inoculum, which was prepared to a final concentration of 10^7 cells per ml by suspending cells in sterile water.

Growth conditions for plants are described elsewhere (20). Cell suspensions were infiltrated into the primary leaves of 10-day-old soybeans by using a Hagborg device (21).

Tn*5* Mutagenesis of Cosmid Clones. Tn*5* mutagenesis of the cosmid clone pPT4E10 was conducted in *E. coli* DH5α by using phage λ::Tn*5* as described (22).

RESULTS

***P. syringae* pv. *tomato* Library Construction and Identification of Cosmid Clones.** Transduction of *E. coli* HB101 with packaged cosmid DNA yielded $>10^4$ tetracycline-resistant colonies per μg of DNA. One thousand colonies were selected to comprise the library, of which 25 clones were randomly selected for plasmid DNA analysis. Average insert size was determined to be *ca.* 20–30 kilobases (kb) (data not shown).

The *P. syringae* pv. *tomato* DNA library was initially conjugated into *P. syringae* pv. *glycinea* race 5, since it had previously been determined as the most efficient *P. syringae* pv. *glycinea* recipient of pLAFR plasmids (6). Stability of the cosmid clones in *P. syringae* pv. *glycinea* was determined by recovery of plasmid DNA from 25 randomly selected transconjugants and comparison of their restriction digest profiles to the original library clones maintained in *E. coli*. Less than 30% of the recovered clones exhibited detectable

alteration in the restriction profiles. However, to compensate for possible rearrangement of clones in *P. syringae* pv. *glycinea*, four transconjugants from each individual mating were selected for inoculation, and the entire library was conjugated and screened twice on the compatible soybean cultivar Harosoy for appearance of the HR.

Five cosmid clones were isolated that caused *P. syringae* pv. *glycinea* race 5 to elicit a HR on soybean cv. Harosoy. The five clones were further characterized by mobilizing each clone into *P. syringae* pv. *glycinea* race 4, which is compatible on nine soybean cultivars. Surprisingly, all of the clones yielded HRs on some but not all of the cultivars, and the clones fell into three classes according to the specific cultivars that reacted hypersensitively (Table 2). Three clones, pPT4D2, pPT7H6, and pPT9A11, were grouped into class I, while the remaining two clones, pPT4E10 and pPT10E9, comprised class II and class III, respectively. Appearance of the HR on all incompatible cultivars was typically observed within 24 hr after inoculation with *P. syringae* pv. *glycinea* race 4 transconjugants harboring class I and II clones. However, the HR elicited by the class III clone, pPT10E9, appeared at a slower rate, usually between 24 and 48 hr after inoculation, with an intensity of necrosis that was weaker than that observed with the other two classes.

The cultivar specificity of the HR elicited by the *P. syringae* pv. *tomato* cosmid clones indicated that they contained avirulence genes that behaved similarly to those previously cloned from various races of *P. syringae* pv. *glycinea*. In fact, inspection of the cultivar HR patterns revealed that the class I clones were identical to the cultivar HR pattern of *avrA*, previously cloned from *P. syringae* pv. *glycinea* race 6 (Table 2). The class II and class III clones did not exhibit cultivar HR patterns identical to any known *P. syringae* pv. *glycinea* races or *avr* genes.

Characterization of Class I Clones. Further characterization of the class I clones indicated the conservation of two *Eco*RI fragments of molecular sizes 0.95 kb and 0.56 kb, occurring in the open reading frame of *avrA* (7) (data not shown). To confirm the homology of these conserved fragments, the 0.95-kb *Eco*RI fragment of the *P. syringae* pv. *glycinea avrA* gene was probed to *Eco*RI-digested class I cosmid DNA in Southern blot analyses. Hybridization was observed to a conserved fragment of identical molecular weight (Fig. 1*A*).

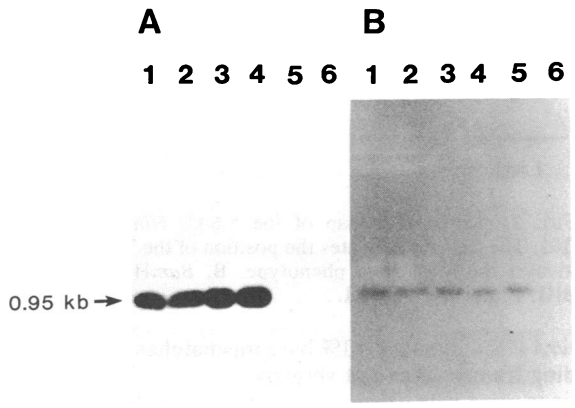

FIG. 1. Southern blot analysis of *P. syringae* pv. *tomato* DNA probed with a ^{32}P-labeled internal 0.95-kb *Eco*RI fragment of *avrA* conferring the *P. syringae* pv. *glycinea* race 6 phenotype. (*A*) *Eco*RI-digested *P. syringae* pv. *tomato* cosmid clone DNA. Lanes: 1, pPSG6001; 2, pPT4D2; 3, pPT7H6; 4, pPT9A11; 5, pPT4E10; 6, pPT10E9. (*B*) *Eco*RI-digested total DNA from various *P. syringae* pv. *tomato* isolates. Lanes: 1, PT23; 2, PT20; 3, P.F.T.; 4, Pt144; 5, PT178; 6, B120.

The indicated homology to *avrA* predicted that a conserved 3.2-kb *Sal* I fragment in pPT9A11 contained the avirulence gene phenotype. Therefore, this fragment was subcloned into pRK415 to form pPT201. As expected, *P. syringae* pv. *glycinea* race 4 transconjugants harboring pPT201 elicited the HR on precisely the same cultivars as did *P. syringae* pv. *glycinea* races 6 and 4 (harboring pPT9A11) (Table 2). To confirm the close relatedness of *avrA* to class I clones from *P. syringae* pv. *tomato*, as indicated by the similar cultivar HR patterns and homology in Southern blot experiments, the 3.2-kb *Sal* I fragment from pPT201 was subcloned into pUC118 to generate pPT211, and the two genes were further compared by restriction mapping and partial sequencing. Comparison of digests from pPT211 with pPSG6001, containing the *avrA* gene (7), indicated that various restriction sites, including four-base recognition enzymes, were conserved throughout both fragments (data not shown). The high degree of homology was further confirmed by comparing sequence data of >1 kb from the predicted 3′ end of pPT211

Table 2. Interactions of *P. syringae* strains and *P. syringae* pv. *glycinea* race 4 transconjugants containing cosmid clones from *P. syringae* pv. *tomato* on nine differential cultivars of soybean

Strain	Soybean cultivar								
	Acme	Chippewa	Flambeau	Hardee	Harosoy	Linderin	Merit	Norchief	Peking
P. syringae pv. *glycinea*									
Race 4	+	+	+	+	+	+	+	+	+
Race 5	−	−	−	+	+	+	+	−	+
Race 6	−	−	+	+	−	−	−	+	−
P. syringae pv. *tomato* PT23	−	−	−	−	−	−	−	−	−
Clones of *P. syringae* pv. *glycinea* race 4									
Class I									
pPT4D2	−	−	+	+	−	−	−	+	−
pPT7H6	−	−	+	+	−	−	−	+	−
pPT9A11	−	−	+	+	−	−	−	+	−
pPT201	−	−	+	+	−	−	−	+	−
Class II cosmid clone									
pPT4E10	+	−	−	+	−	−	+	−	+
pPT101	+	−	−	+	−	−	+	−	+
pPT112	+	+	+	+	+	+	+	+	+
Class III* cosmid clone									
pPT10E9	−	−	−	−	−	−	−	−	+

Plants were scored daily from 1 to 5 days after inoculation for compatible interactions (+) as visualized by a water-soaked lesion typically appearing 2–3 days after inoculation or incompatible interactions (−) as visualized by a hypersensitive reaction (HR) typically appearing within 24 hr after inoculation.

*Induced HR appeared slower (between 24 and 48 hr after inoculation) and weaker than a typical HR.

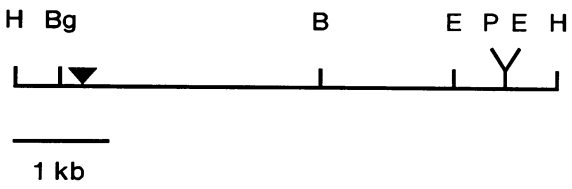

FIG. 2. Restriction map of the 5.6-kb *Hin*dIII fragment of pPT101. The triangle indicates the position of the Tn*5* insertion that inactivates the avirulence phenotype. B, *Bam*HI; Bg, *Bgl* II H, *Hin*dIII; P, *Pst* I; E, *Eco*RI.

to *avrA* (7), indicating <1% base mismatches within the open reading frames (data not shown).

Since *avrA* was previously detected in only one race of *P. syringae* pv. *glycinea* (6), it was of interest to determine if this gene existed in *P. syringae* pv. *tomato* isolates other than PT23. The 0.95-kb *Eco*RI fragment from pPT211 was accordingly probed to *Eco*RI-digested total DNA of 6 *P. syringae* pv. *tomato* isolates from various geographical locations. Hybridization of the conserved 0.95-kb fragment was observed in 5 of the 6 *P. syringae* pv. *tomato* isolates tested (Fig. 1*B*). Five additional *P. syringae* pv. *tomato* isolates showed similar results in Southern blot analyses (data not shown), indicating the presence of homologous sequences to *avrA* in 10 of 11 different *P. syringae* pv. *tomato* isolates tested.

Characterization of the Class II Clone. The cosmid clone pPT4E10 was subjected to Tn*5* mutagenesis, and recovered plasmids with kanamycin resistance were conjugated into *P. syringae* pv. *glycinea* race 4 and screened on the normally incompatible soybean cultivar Harosoy for loss of the avirulence phenotype. One Tn*5* mutant, pPT112, was identified that did not exhibit the avirulence phenotype on all normally incompatible cultivars (Table 2). The Tn*5* insertion in pPT112 mapped to a 5.6-kb *Hin*dIII fragment in pPT4E10 (Fig. 2) that was subsequently subcloned into pRK415 to form pPT101. *P. syringae* pv. *glycinea* race 4 transconjugants carrying pPT101 expressed the class II avirulence phenotype on the predicted soybean cultivars (Table 2).

The existence of *avrA* in both *P. syringae* pv. *glycinea* race 6 and *P. syringae* pv. *tomato* isolates encouraged us to determine the distribution of the 5.6-kb *Hin*dIII fragment of pPT101 in other *P. syringae* pathovars. The *Hin*dIII fragment from pPT101 was probed to *Hin*dIII-digested genomic DNA of various *P. syringae* pathovars, and homologous fragments with a conserved molecular weight of 5.6 kb were detected in several pathovars, including *P. syringae* pv. *lachrymans*, *P. syringae* pv. *mori*, *P. syringae* pv. *phaseolicola*, and *P. syringae* pv. *maculicola* (Fig. 3*A*). Additional hybridizing bands with molecular sizes of 4.3 kb and 2.8 kb were detected in *P. syringae* pv. *lachrymans*. Of considerable interest, all tested isolates of *P. syringae* pv. *glycinea* contained a hybridizing 5.6-kb *Hin*dIII fragment (Fig. 3*B*), although none of them expressed the class II avirulence phenotype.

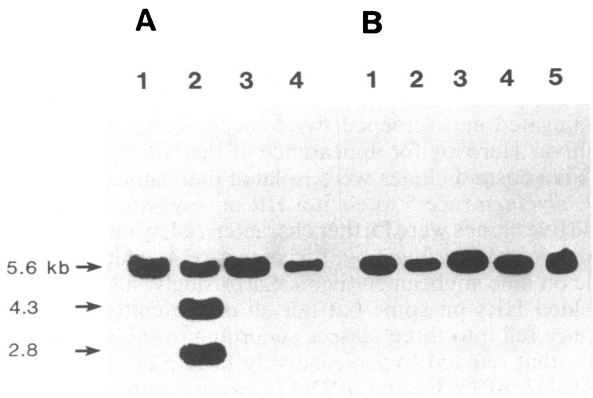

FIG. 3. Southern blot analysis of several *P. syringae* pathovars probed with a ^{32}P-labeled 5.6-kb *Hin*dIII fragment from class II (*A*) Lanes: 1, *P. syringae* pv. *maculicola*; 2, *P. syringae* pv. *lachrymans*; 3, *P. syringae* pv. *mori*; 4, *P. syringae* pv. *phaseolicola*. (*B*) *P. syringae* pv. *glycinea* races in lanes: 1, race 0; 2, race 4; 3, race 5; 4, race 6. Lane 5 shows analysis of *P. syringae* pv. *tomato* PT23.

DISCUSSION

Avirulence genes have been proven to be responsible for determining race phenotypes in interactions between *P. syringae* pv. *glycinea* and soybean plants (6, 8). We predicted that higher orders of specificity such as pathovar phenotypes in the *P. syringae* group might also be determined by single genetic factors, accounting in these cases for incompatibility to an entire plant species. However, our results with the tomato pathogen *P. syringae* pv. *tomato* indicated that this hypothesis is incorrect. Instead, it is apparent that *P. syringae* pv. *tomato* contains several different avirulence genes that, when expressed in *P. syringae* pv. *glycinea*, exhibit cultivar-specific incompatibility on soybean, similar to the *avr* genes previously cloned from *P. syringae* pv. *glycinea*. This observation is made evident by the homology of the class I *P. syringae* pv. *tomato* clone to *avrA*, originally cloned from *P. syringae* pv. *glycinea* race 6 (6). Although no *P. syringae* pv. *tomato* clone has been found to elicit the HR on all tested soybean cultivars, the identified *P. syringae* pv. *tomato avr* genes collectively account for the HR elicited in all cultivars (Table 2). Therefore, these results suggest that several cultivar-specific avirulence genes in *P. syringae* pv. *tomato* may be responsible for the restriction of its host range to exclude soybean.

Although the magnitude of the role for avirulence genes in determining pathovar specificity is still unclear, other mechanisms implicated in host-range determination have been reported with certain pathovars of *P. syringae* and other pathogens such as the related organism, *X. campestris*. For example, Lindgren *et al.* (23) defined a large cluster of HR-pathogenicity genes (*hrp*) in various *P. syringae* pathovars associated with pathogenicity on the respective host plant and HR elicitation on nonhost plants. However, it is uncertain whether differences exist in these genes that are directly responsible for pathovar specialization to specific plant species. More specific factors were reported by Mellano *et al.* (24) in mutagenesis studies that identified genetic elements associated with extending the host range of *X. campestris* pv. *translucens*. In addition, several genes have been identified that influence the host range of *Agrobacterium tumefaciens* and *Rhizobium* spp., some of which have positive-acting functions (reviewed in ref. 25).

Whalen *et al.* (26) have reported findings with an *avr* gene from *X. campestris* pv. *vesicatoria* that are analogous to ours. This *avr* gene functioned in *X. campestris* pv. *phaseoli* to elicit a HR in one but not another bean cultivar. Further, the *avr* gene also conditioned resistant reactions when introduced into several other *X. campestris* pathovars, and the resultant transconjugants were inoculated into their normally susceptible plant hosts. These results demonstrate that a single *avr* gene may determine pathogen host range.

The conservation of *avrA* between *P. syringae* pv. *glycinea* race 6 and several isolates of *P. syringae* pv. *tomato* shows that functional *avr* genes may occur in more than one *P. syringae* pathovar. The observed homology of pPT101 to DNA of several other *P. syringae* pathovars also indicates that avirulence genes associated with cultivar-specific resistance are widely distributed among pathovars in the *P. syringae* group. An important interpretation of this observa-

tion is the prediction that certain plants may contain disease-resistance genes directed to *P. syringae* pathovars specializing on other host plants and perhaps to other pathogen groups as well. Such interspecies resistance has previously been demonstrated by the transfer of functional resistance genes from one plant species to another. For example, certain resistance genes in wheat to diseases caused by *Puccinia* spp. were introduced via interspecific crosses with related plants (27). Although the genetic basis of resistance to pPT101 in soybean plants is currently undefined, the gene-for-gene relationship predicts that certain soybean cultivars contain a disease-resistance gene complementary to this *avr* gene. It is important to note that the putative disease-resistance gene could not have been previously detected in soybean because the avirulence phenotype of pPT101 has not been observed in any *P. syringae* pv. *glycinea* race thus far described.

The finding that all tested *P. syringae* pv. *glycinea* races contain conserved DNA sequences homologous to *P. syringae* pv. *tomato* clone pPT101 is suggestive of a recessive allele to this *P. syringae* pv. *tomato avr* gene, as opposed to a series of nonfunctional genes. Gabriel *et al.* (9) reported that similar recessive alleles for avirulence genes may exist in *X. campestris* pv. *malvacearum*. However, previously cloned avirulence genes from *P. syringae* pv. *glycinea* have not been observed to have conserved homologous sequences in races that do not express the respective Avr phenotypes (6, 8). Furthermore, the conserved homology of pPT101 sequences in all *P. syringae* pv. *glycinea* races may suggest that the conserved gene(s) has a function(s) of pleiotropic importance in the bacteria. Functional importance may also be implied with the conservation of *avrA* in several *P. syringae* pv. *tomato* isolates (Fig. 1*B*). Site-directed mutagenesis of these genes in the wild-type organisms may provide information to determine if such functions exist.

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