Amino Acid Residues Required for the Activity of *avr*D Alleles

Irem Yucel and Noel T. Keen

Department of Plant Pathology, University of California, Riverside 92521 U.S.A.
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Certain *Pseudomonas syringae* pathovars harbor *avr*D alleles belonging to two different homology classes. The nonfunctional *avr*D allele of *P. s.* pv. *glycinea* is highly homologous to active class II *avr*D alleles but has five unique amino acid substitutions. Three of these five amino acid changes were shown to be absolutely required for restoration of *avr*D activity to the *P. s.* pv. *glycinea* allele by oligonucleotide site-directed mutagenesis. They were cysteine 19 to arginine, alanine 280 to valine, and leucine 304 to serine. In addition, changing leucine 301 to phenylalanine was required for high activity. However, alteration of the leucine at position 245 of the *P. s.* pv. *glycinea* allele to serine, present in the active alleles, did not affect *avr*D activity. Results from recombinant gene constructs between the nonfunctional *P. s.* pv. *glycinea avr*D gene and the functional allele from *P. s.* pv. *phaseolicola* identified six other amino acid residues that may form contextual motifs important for AvrD function. These were a four amino acid stretch comprised of glutamate 41, alanine 42, asparagine 43 and arginine 44 in addition to aspartate 243 and phenylalanine 301. Some divergence is tolerated within the four amino acid motif, but phenylalanine 301 appears to be necessary for highly active class II *avr*D proteins.

Additional keywords: avirulence gene phenotype, oligomutagenesis, structure-function.

Pseudomonas syringae pv. *glycinea* harbors an allele of avirulence gene D (*avr*D) that does not confer the avirulence phenotype on soybean cultivars with the *Rpg*4 resistance gene (Keen and Buzzell 1991; Kobayashi *et al.* 1990a,b). However, it encodes a protein that is 97–98% identical at the amino acid level to those encoded by functional *avr*D alleles from *P. s.* pvs. *phaseolicola* and *lachrymans* (Yucel *et al.* 1994a). These genes were accordingly called class II alleles because of their high homology. The *avr*D allele from *P. s.* pv. *tomato* and *P. s.* pv. *lachrymans* allele 1 are 95% homologous to each other but less homologous to the class II alleles. They were therefore called class I alleles.

Proteins encoded by active *avr*D alleles are hypothesized to have an enzymatic activity that metabolizes bacterial compounds found in *Escherichia coli* and other gram-negative bacteria into substances, called syringolides, that elicit the cultivar-specific soybean hypersensitive response (HR) (Keen *et al.* 1990; Midland *et al.* 1993; Smith *et al.* 1993). Unlike active *avr*D alleles, the allele from *P. s.* pv. *glycinea* does not exhibit the avirulence phenotype when bacteria expressing the gene are infiltrated into soybean plants and directs little or no elicitor production (Kobayashi *et al.* 1990b; Keen *et al.* 1990; Yucel *et al.* 1994b).

Comparison of deduced amino acid sequences of three additional *avr*D alleles from *P. s.* pvs. *phaseolicola* and *lachrymans* identified four amino acid substitutions unique to the pv. *glycinea avr*D allele (Yucel *et al.* 1994a). Three of the four substitutions are clustered at the carboxyl end of the protein, consistent with previous findings indicating that the carboxyl terminus of AvrD may be involved in protein stability (Kobayashi *et al.* 1990b). The fourth substitution (arginine 19 to cysteine) in the nonfunctional pv. *glycinea* protein may cause a major change in charge and polarity. These substitutions in the pv. *glycinea avr*D allele could affect putative active sites and reduce protein stability, thereby diminishing enzymatic activity required for elicitor production. In this paper, oligonucleotide site-directed mutagenesis and recombinant gene constructions were used to determine the role of these and other amino acid substitutions in the dysfunction of *P. s.* pv. *glycinea* AvrD.

RESULTS

Oligomutagenesis of the *avr*D allele from *P. s.* pv. *glycinea*.

Figure 1 shows the location of the base changes introduced into the pv. *glycinea* allele through oligomutagenesis. To change the cysteine residue at position 19 to arginine, a thymine to cytosine change was introduced at nucleotide 195. An adenosine to guanine change at nucleotide 874 altered the leucine residue at position 245 to serine. Cytosine to thymine changes were introduced at nucleotides 979 and 1,051 to change alanine 280 to valine and leucine 304 to serine, respectively. These changes restored the amino acid residues to the consensus observed in the four functional *avr*D alleles that have been characterized (Yucel *et al.* 1994a).

Role of restored amino acids on *P. s.* pv. *glycinea avr*D protein function.

The importance of the various amino acid substitutions in the pv. *glycinea avr*D allele were assessed by determining their effects on the avirulence phenotype in *P. s.* pv. *glycinea*

Corresponding author: N. T. Keen.
Current address of I. Yucel: Bldg. 011A, Rm. 254, USDA, Beltsville, MD 20705 U.S.A.

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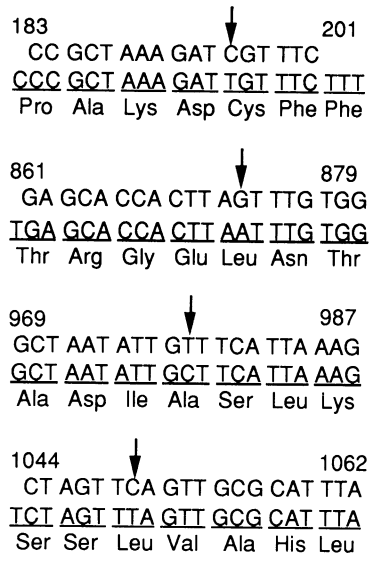
R4 as well as elicitor and protein production in *E. coli*. Table 1 summarizes the results from *P. s.* pv. *glycinea avr*D oligomutants with one, two, or three restored amino acids. Oligomutants with valine 280 and/or serine 304 directed neither elicitor production nor the avirulence phenotype (pVOM2, pSOM2, and pOMSV2). These mutations also did not appreciably increase the level of *avr*D protein production over the wild-type *P. s.* pv. *glycinea* allele, even though previous studies indicated that the carboxyl terminus of the *avr*D protein may be involved in protein stability (Kobayashi *et al.* 1990b). Oligomutant pRVS with arginine 19, valine 280, and serine 304 directed the production of low levels of elicitor activity in *E. coli* (Table 1; Fig. 2). However, *avr*D protein production was only slightly increased over wild-type levels as detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot) analysis in *E. coli* (data not shown), and *P. s.* pv. *glycinea* R4 carrying pRVS in pRK415 did not produce the avirulence phenotype on soybeans. The oligomutant with all four substitutions restored, pRSVS1, behaved similarly to pRVS, indicating that serine 245, in the context of arginine 19, valine 280, and serine 304, has little or no impact on protein function or elicitor production. These results indicated that arginine 19, valine 280, and serine 304 are essential but they do not, either alone or paired, fully restore *avr*D function or protein stability to the *P. s.* pv. *glycinea avr*D allele.

Recombinant gene constructions.

Results from recombinant constructions made with pPAD1 and pGAD1 are summarized in Figure 2 and further illustrate the importance of arginine 19, valine 280, and serine 304 for *avr*D activity. When the 5′ *Hin*dIII-*Bgl*II fragment of pPAD1, encoding 110 amino acids, was replaced by the analogous fragment from pGAD1 (Fig. 2, pCAD1), *avr*D activity was



Base		Amino Acid	
Position	Change	Position	Change
195	T to C	19	Cysteine to Arginine
874	A to G	245	Leucine to Serine
979	C to T	280	Alanine to Valine
1051	T to C	304	Leucine to Serine

Fig. 1. Amino acid and nucleotide sequences showing the location and types of changes introduced by oligomutagenesis to the *Pseudomonas syringae* pv. *glycinea avr*D allele. The top lines show the oligonucleotides with desired mutations, and the bottom, underscored lines illustrate the native nucleotide sequence of the coding strand. Three-letter designations for the amino acids are aligned under the indicated codons. Arrows indicate the base change introduced by each oligonucleotide.

Table 1. Avirulence phenotype, elicitor, and protein production of oligomutant *Pseudomonas syringae* pv. *glycinea* alleles

Construct[a]	Amino acid change[b]	Position of change	HR[c]	Elicitor[d]	Protein[e]
pGA11	NA	NA	–	–	+
pVOM2, pVOM3	V	280	–	–	+
pSOM2, pSOM3	S	304	–	–	+
pOMSV2, pOMSV3	V, S	280, 304	–	–	+
pRV2, pRV24	R, V	19, 280	–	–	++
pRS2, pRS24	R, S	19, 304	–	–	++
pRVS, pRVS24	R, V, S	19, 280, 304	–	+	++
pRSVS1, pRSVS14	R, V, S′, S	19, 245, 280, 304	–	+	++

[a] pGA11, Wild-type *avr*D allele from *P. s.* pv. *glycinea*; pVOM2 in pUC129, pVOM3 in pRK415; pSOM2 in pUC129, pSOM3 in pRK415; pOMSV2 in pUC129, pOMSV3 in pRK415; pRV2 in pUC128, pRV24 in pRK415; pRS2 in pUC128, pRS24 in pRK415; pRVS in pUC128, pRVS24 in pRK415; pRSVS1 in pUC129, pRSVS14 in pRK415.

[b] NA, not applicable; V, valine; S′, serine at 245; S, serine at 304; R, arginine.

[c] Hypersensitive response was scored in cv. Harosoy and Merit plants inoculated with *P. s.* pv. *glycinea* R4 carrying the various cloned genes in pRK415.

[d] Elicitor production was scored by assaying culture fluids from *Escherichia coli* cultures expressing the various genes in pUC plasmids.

[e] Protein production was assayed by electrophoresing *E. coli* cells expressing the various genes on sodium dodecyl sulfate-polyacrylamide gels and estimating intensity of the AvrD bands appearing at approximately 34 kDa.

completely abolished in elicitor and plant assays. The amount of *avr*D protein produced by pCAD1 was also dramatically decreased compared with the wild-type *P. s.* pv. *phaseolicola* *avr*D allele despite presence of the pv. *phaseolicola* valine 280 and serine 304 residues. The reciprocal switch (pCAD2) resulted in no detectable *avr*D activity, as expected, since the valine 280 and serine 304 residues were not present. The recombinant construction with arginine 19 alone (pCAD2) resulted in higher levels of protein production than the wild-type *avr*D allele from *P. s.* pv. *glycinea* (pGAD1) but no detectable elicitor activity. The importance of arginine 19, valine 280, and serine 304 was further illustrated with the recombinant constructs pCAD6 and pCAD7, in which the internal *Bgl*II-*Spe*I fragments of pPAD1 and pGAD1 were interchanged. Plasmid pCAD7 was inactive but pCAD6 was virtually indistinguishable from pPAD1 with respect to *avr*D activity and protein production. This shows that the internal amino acids of the pv. *glycinea* allele are correctly configured and further indicates that the serine to leucine substitution at position 245 does not play a role in activity, consistent with the site-directed oligomutagenesis results above.

Recombinant genes were constructed from the partially repaired *P. s.* pv. *glycinea* *avr*D oligomutants, pRVS (arginine 19, valine 280, and serine 304), pRSVS1 (pRVS with serine 245), and pPAD1 to determine the role of other amino acid substitutions found in but not unique to the pv. *glycinea* protein. The 5′ *Hin*d-*Bgl*II fragment of pPAD1 replaced the analogous fragments in pRVS and pRSVS1 to create pCAD3 and pCAD4, respectively. The data for pCAD4 are not shown because results were the same as for pCAD3 (Fig. 2). Surprisingly, instead of being fully functional, these constructs did not produce detectable elicitor, HR, or increased levels of protein, even though arginine 19 as well as valine 280 and serine 304 at the carboxyl terminus of the *P. s.* pv. *glycinea* portion were present. This indicated that the carboxyl portion of pRVS and pRSVS1 still contained at least one incorrect amino acid. By elimination, this residue must be phenylalanine 301 in the pv. *phaseolicola* allele, since all other amino acids are identical. Consistent with this prediction, pCAD5 (pPAD1 with the 5′ portion of pRVS), produced the same or even slightly stronger HR-inducing ability and elicitor production than the wild-type *avr*D allele from *P. s.* pv. *phaseolicola* (Fig. 2).

DISCUSSION

Comparison of the nonfunctional *avr*D protein of *P. s.* pv. *glycinea* with the products of four other functional alleles (from *P. s.* pvs. *tomato*, *phaseolicola*, and *lachrymans* alleles 1 and 2) identified several amino acids that were unique to the pv. *glycinea* protein. Figure 3 summarizes the different amino acid positions in the pv. *glycinea* R4 *avr*D protein relative to functional class II *avr*D proteins. The *avr*D protein of *P. s.* pv. *glycinea* was restored to partial function by oligomutagenesis to create arginine 19, valine 280, and serine 304 (pRVS, Figs. 2 and 3A). A fourth substitution, serine 245, did not affect protein function when in the context of the other three mutations. The valine 280 and serine 304 substi-

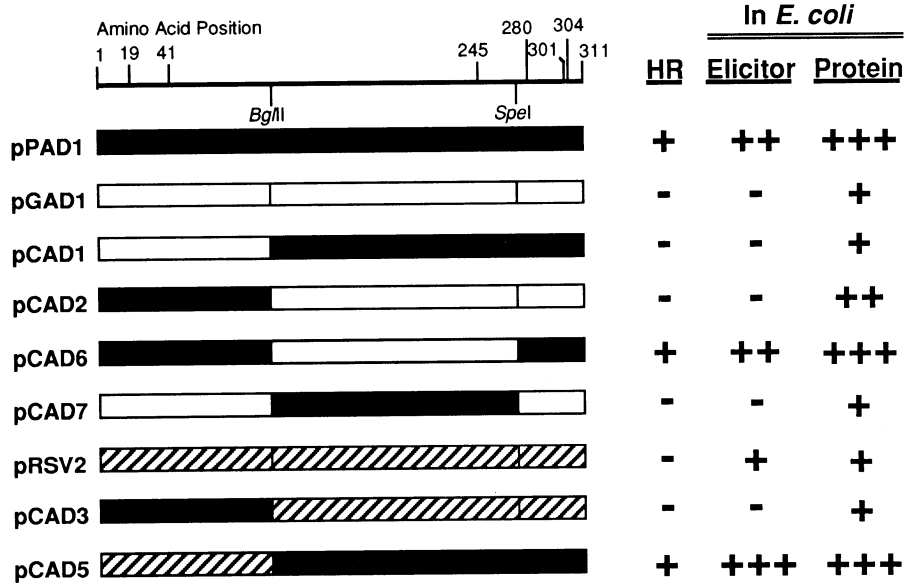
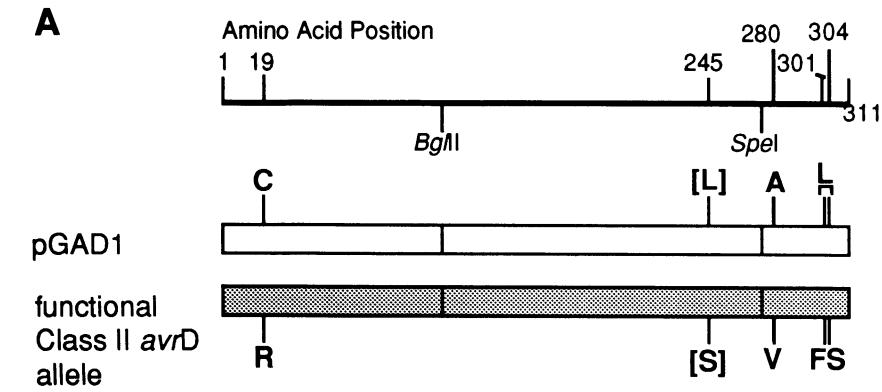


Fig. 2. Production of the hypersensitive response, *avr*D elicitor activity and protein by *Pseudomonas syringae* pv. *glycinea* R4 or *Escherichia coli* cells carrying *avr*D alleles from *P. s.* pvs. *phaseolicola* and *glycinea* or various recombinant genes constructed as denoted by the bars. Amino acid sequences from wild-type *P. s.* pvs. *phaseolicola* (pPAD1) and *glycinea* (pGAD1) are solid and open bars, respectively. The mutated, partially restored *P. s.* pv. *glycinea* (pRVS) allele is denoted by striped bars. Positions of relevant amino acids as well as the conserved *Bgl*II and *Spe*I restriction sites are shown in a line diagram at the top. Hypersensitive response (HR) was measured using *P. s.* pv. *glycinea* R4 cells harboring the various constructs on a broad host range plasmid, pRK415, that were infiltrated into leaves of appropriate soybean cultivars as described in Materials and Methods. The presence (+) or absence (–) of visible HR was determined 16-24 hr after infiltration. Elicitor preparations from *E. coli* carrying the different constructs were infiltrated into cultivar Harosoy soybean leaves, and the appearance of visible HR was scored: – indicates no HR (no elicitor activity) at 16–24 hr postinfiltration; + indicates visible HR in the area of infiltration; ++ denotes moderate necrosis extending beyond the infiltrated area; and +++ indicates near-complete hypersensitive collapse of the leaf. *avr*D protein levels were assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis of total protein from *E. coli* cells carrying the various constructs as described in Materials and Methods. Increasing relative levels of detectable protein are denoted by +, ++, and +++.

tutions did not restore any *avr*D activity by themselves but, in combination with arginine 19, enabled the protein to direct low but easily detectable levels of elicitor production. The arginine residue was absolutely required for *avr*D activity since solely altering this residue totally inactivated the *avr*D protein of *P. s.* pv. *phaseolicola* (pCAD1, Fig. 2). Conversely, changing only residue 19 from cysteine to arginine in the pv. *glycinea* protein (pCAD2) did not restore *avr*D activity but did increase protein levels in *E. coli*. Thus, arginine 19 is not only required for activity but may also contribute to protein stability in conjunction with valine 280 and serine 304 (Fig. 3A). Since these three amino acid changes did not totally restore the pv. *glycinea avr*D allele to full activity, however, we examined other amino acid residues.

The *avr*D alleles thus far studied fall into two distinct homology classes based on their deduced amino acid sequences (Yucel *et al.* 1994a) and the elicitors they direct in bacteria (Yucel *et al.* 1994b). The *P. s.* pv. *tomato* allele and *P. s.* pv. *lachrymans* allele 1 (95% identical) were grouped in class I, and the alleles from *P. s.* pvs. *phaseolicola* and *glycinea* and *P. s.* pv. *lachrymans* allele 2, (97–99% identical) were placed in class II. The two classes shared 88% identical amino acids, with divergent amino acids at single isolated positions or in short stretches dispersed throughout the proteins. The pv. *glycinea* amino acid sequence clearly belonged to class II because of its high level of identity with AvrD from pv. *phaseolicola* and pv. *lachrymans* allele 2 (97 and 98%, respectively). Realization that the nonfunctional pv. *glycinea*



B

***Avr*D allele**

	BglII 110	SpeI 270			
	19 41	243	280	301	304
Class I					
tomato	AKDRF..AVDANI..TRDESN..KRANIV..LSSS				
lachrymans 1	AKDRF..AVDANI..TRGESN..KRANIV..LSSS				
Class II					
lachrymans 2	AKDRF..EANRSI..TRGESN..KRANIV..FSSS				
phaseolicola	AKDRF..EANRSI..TRDESN..KRANIV..FSSS				
glycinea	AKDCF..AANASI..TRGELN..KRANIA..LSSL				
Recombinants					
pRSV2	AKDRF..AANASI..TRGELN..KRANIV..LSSS				
pCAD5	AKDRF..AANASI..TRDESN..KRANIV..FSSS				
pCAD3	AKDRF..EANRSI..TRGESN..KRANIV..LSSS				

Fig. 3. Amino acid sequences of contextual and invariant motifs found in class I and II alleles of *avr*D. **A,** Divergent amino acids found in the nonfunctional *Pseudomonas syringae* pv. *glycinea* R4 *avr*D protein (open bar) relative to functional class II proteins (gray bar). Amino acid positions and conserved *Bgl*II and *Spe*I restriction sites are shown in the line diagram above. A, alanine; C, cysteine; F, phenylalanine; L, leucine; R, arginine; S, serine; and V, valine. While amino acid 245 was serine in both active class II alleles and leucine in the pv. *glycinea avr*D allele, either amino acid is acceptable for activity, as denoted by the brackets. **B,** Hybrid nature of *P. s.* pv. *glycinea* and recombinant *avr*D alleles. Amino acid residues of the pv. *glycinea* and recombinant *avr*D alleles in common with class I alleles are denoted by asterisks (*), those in common with class II alleles are double-underscored, and amino acids that vary in both classes are underscored in bold. Location of conserved *Bgl*II and *Spe*I restriction sites used for recombinant gene constructions are indicated by arrows and amino acid number.

*avr*D allele most likely evolved from a functional class II allele permitted deduction of the remaining amino acid residue associated with its lack of activity.

In certain positions of divergence between class I and class II alleles, the pv. *glycinea avr*D allele contained amino acid residues that were hybrids of the motifs found in both classes. Figure 3B summarizes the deduced amino acid sequences from the five available *avr*D alleles to show amino acids in the pv. *glycinea* protein that differ from the class II pvs. *phaseolicola* and *lachrymans* allele 2 proteins. These residues were alanine 41, alanine 44, and leucine 301, replacing glutamate 41, arginine 44, and phenylalanine 301, respectively, in the functional proteins. Position 243 of the functional pv. *phaseolicola* protein is aspartate, but glycine in both the *lachrymans* 2 and *glycinea* proteins. Thus, this seems an acceptable alternative residue for functional class II alleles, as confirmed by the activity of pCAD6 (Fig. 2), which has the internal portion of the pv. *glycinea* gene. However, position 301 is leucine in the pv. *glycinea* protein but phenylalanine in both of the functional class II alleles from *P. s.* pvs. *phaseolicola* and *lachrymans* 2 (Fig. 3B). Since both functional class I *avr*D alleles have leucine 301, this position was not previously suspected to be important for activity. Because leucine 301 is the only remaining residue downstream of the *Spe*I site that differs between pRVS and the functional pv. *phaseolicola* and *lachrymans* 2 proteins, this amino acid must account for the lack of full activity in pRVS. The prediction was indeed confirmed when it was shown that pCAD5, with phenylalanine 301, expressed elicitor activity that appeared greater than the wild-type pv. *phaseolicola* gene (Fig. 2).

A stretch of four amino acids beginning at position 41 contained two alanine residues followed by an asparagine, and a third alanine residue in the pv. *glycinea* protein, but the other proteins varied considerably (Fig. 3B). Closer examination of these four amino acid positions as well as residue 301 in all five proteins showed that the pv. *glycinea* sequence was a hybrid of motifs shared by the *tomato* and *lachrymans* 1 class I alleles and *phaseolicola* and *lachrymans* 2 class II alleles (Fig. 3). The pv. *glycinea* protein contains alanine 42 and asparagine 43 of Class II *avr*D alleles as well as alanines 41 and 44 and leucine 301 of class I *avr*D alleles. The two single amino acid changes described above (glycine 243 and leucine 301), while not unique to the pv. *glycinea* protein, therefore, may not work efficiently in concert with the four-amino-acid sequence (positions 41–44) from the pv. *phaseolicola* protein. This may explain the inactivity of pCAD3 and pCAD4, which lacked only phenylalanine 301 to be completely converted to the functional pv. *lachrymans* allele 2 protein sequence.

It is not clear what structural changes are introduced into the pv. *glycinea avr*D protein by the four amino acids accounting for its lack of function (cysteine 19, alanine 280, leucine 301, and leucine 304). The cysteine 19 substitution in the pv. *glycinea* protein, for example, may affect protein structure through the formation of disulfide bonds with either of the two other cysteine residues (positions 94 and 167) in the protein. Furthermore, the hybrid nature of the pv. *glycinea* protein sequence at residues 41–44, 243, and 301 may result in contextual motifs that disrupt normal protein structure and function. Such contextual motifs, in concert with the four amino acids essential for activity, may determine the degree of function of a given *avr*D protein. The effect of mixing contextual motifs from the two classes of alleles on *avr*D activity is illustrated by the observed higher elicitor activity of pCAD5 (Figs. 2 and 3B), in which the 41–44 sequence hybrid from pv. *glycinea* was combined with aspartate 243 and phenylalanine 301 from the class II pv. *phaseolicola avr*D allele. The higher elicitor activity of pCAD5 relative to the wild-type pv. *phaseolicola* protein may result from greater compatibility of the pv. *glycinea* hybrid 41–44 motif with the aspartate 243 and phenylalanine 301 residues of the pv. *phaseolicola* protein. This interpretation is strengthened by the observation that the 41–44 motif from class II alleles coupled with the leucine 301 residue of class I alleles abolishes *avr*D activity in pCAD3 and pCAD4 (Fig. 2). These results suggest that alanines 41 and 44 (from class I) can work in concert with phenylalanine 301 (class II) for *avr*D protein activity, yet the reciprocal switch of glutamate 41 and arginine 44 (from class II) together with leucine 301 (from class I) results in nonfunctional proteins (pCAD3 and pCAD4). The glutamate 41, arginine 44, and phenylalanine 301 residues therefore may comprise an invariant motif required for active class II *avr*D proteins. This observation could be explained by the major change in charge and polarity at positions 41 and 44 of functional class II proteins and further supports the notion that contextual motifs, layered over arginine 19, valine 280, and serine 304, account for *avr*D functionality.

We have shown that class I and class II *avr*D alleles direct the production of different elicitor-active products (Yucel *et al.* 1994b). This raises the question of amino acid residues accounting for these distinct functions. The understanding of amino acid residues required to restore the nonfunctional pv. *glycinea avr*D class II allele to activity provides a basis on which to further explore the function of class I alleles.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media.

Bacterial strains and plasmids used or constructed in this paper are shown in Table 2. *E. coli* was grown at 37° C on Luria-Bertani (LB) medium (Maniatis *et al.* 1982) in liquid culture or on agar plates. *P. syringae* pv. *glycinea* R4 carrying various plasmids was grown at 28° C on King's medium B (KB) in liquid culture or on agar plates (King *et al.* 1954). Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; rifampicin, 100 μg/ml; and tetracycline, 15 μg/ml in LB and 30 μg/ml in KB. For elicitor studies, *E. coli* cells with plasmids of interest were grown in shaken 15-ml liquid cultures of M9 medium (Maniatis *et al.* 1982) amended with thiamine at 4 μg/ml and appropriate antibiotics; cells carrying plasmids with *lac* promoters were induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM at the time of culture initiation. Cultures were grown at 28° C for 16–24 hr prior to assays for elicitor production.

DNA manipulations.

Standard recombinant DNA methods were generally performed as described by Maniatis *et al.* (1982). A variation of the 10-min miniprep method of Zhou *et al.* (1990) was used for miniscreen analyses of recombinant plasmids in *E. coli.*

These plasmids were constructed by isolating desired DNA fragments from low-melting-point agarose gels (Crouse *et al.* 1983) for ligation into vectors and transformation of *E. coli* strain DH5α. Transformants harboring recombinant plasmids were selected on LB plates containing ampicillin, 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal) and IPTG. Table 2 lists the plasmids used in this study. Generally, DNA fragments were cloned into either pUC128 or pUC129 and recloned into the broad host range plasmid pRK415. These constructs were then introduced into *P. s.* pv. *glycinea* R4, which was infiltrated into primary leaves of the soybean cultivars Harosoy and Flambeau to test for occurrence of the HR.

Site-directed oligonucleotide mutagenesis.

Oligonucleotides used in this study (Table 2) were synthesized by the Biotechnology Instrumentation Facility (University of California, Riverside). Site-directed oligomutagenesis was performed using the polymerase chain reaction (PCR) as described by Mikaelin and Sergeant (1992). For the first round of PCR, two amplification reactions were prepared. The first included pGOR as template DNA and the pUC universal reverse (R) and mismatched forward (MF) primers (Heidecker *et al.* 1980; this study). The second reaction included the template DNA, very forward (VF) primer, and a primer with the desired mutation. After the first round of PCR, the amplified fragments were mixed and subjected to a second round of PCR using the R and VF primers. The first-round PCR reactions were carried out with Vent polymerase (New England Biolabs, Beverly, MA) and the buffer supplied by the manufacturer in the presence of 200 mM dNTPs, 4 μl of appropriate primers (34 ng/ml), 50 ng of template DNA, and 1 U of enzyme in a final volume of 50 μl for 25 cycles (94° C, 30 sec; 50° C, 45 sec; 72° C, 1 min). Amplified products were either isolated from a 1% agarose gel or diluted 1:30; 30–50 ng of each were mixed and used in the second PCR reaction as described above.

The resulting PCR fragments were treated with proteinase K to increase cloning efficiency (Crowe *et al.* 1991), digested with *Hin*dIII and cloned into pUC129 as described above. Transformant colonies were screened for introduced mutations by sequencing the entire length of the *avr*D gene to confirm that changes specified by the mutation primers were the only ones that occurred.

Construction of recombinant genes.

The *avr*D alleles from *P. s.* pvs. *phaseolicola* (pPAD1) and *glycinea* R4 (pGAD1) (Table 2) were used to construct the recombinant genes pCAD1, pCAD2, pCAD6, and pCAD7. Conserved *Hin*dIII, *Bgl*II, *Spe*I, and *Xba*I restriction sites were used to generate fragments that were reciprocally exchanged between the two genes. Recombinant genes pCAD3, pCAD4, and pCAD5 were constructed similarly with pPAD1, pRVS, and pRSVS1, respectively. Exchanges of restriction

Table 2. Bacterial strains, plasmids, and oligonucleotides used in this study

Designation	Relevant characteristics[a]	Source or reference[b]
Escherichia coli		
DH5α	F⁻ *lac*Z M15 *endA1 hsdR17 supE44 thi-1 gyrA relA1*⁻	BRL
Pseudomonas syringae pathovars		
glycinea race 4	Rif^r, Ap^r	Kobayashi *et al.* 1989
phaseolicola	Strain 3121	N. Panopoulos
Plasmids		
pUC128/pUC129	Ap^r cloning and sequencing vectors	Keen *et al.* 1988
pRK415	Tc^r broad range vector, *mob*$^+$	Keen *et al.* 1988
pRK2013	Km^r, Tra$^+$, helper plasmid	Ditta *et al.* 1980
pRK2073	Sm^r, Tra$^+$, helper plasmid	Ditta *et al.* 1980
pPAD1	1.8-kb *Hin*dIII-*Xba*I fragment subcloned from pMTLK4 with *avr*D positioned downstream of the *lac* promoter in pUC129	Yucel *et al.* 1994a
pPAD5	1.8-kb *Hin*dIII-*Xba*I fragment from pPAD1 with *avr*D positioned downstream of the *lac* promoter in pRK415	Yucel *et al.* 1994a
pPSG4000	5.6-kb *Hin*dIII fragment from *P. s.* pv. *glycinea* race 4 in pUC119	Kobayashi *et al.* 1990b
pGAD1	1.8-kb *Hin*dIII-*Xba*I fragment subcloned from pPSG4000 with *avr*D positioned downstream of the *lac* promoter in pUC129	This study
pGOR	~1.25-kb fragment harboring the *P. s.* pv. *glycinea avr*D allele opposite the *lac* promoter in pUC129 with an *Eco*RI site introduced immediately before the start codon	Kobayashi *et al.* 1990b
Oligonucleotides		
Universal forward M13	5′-GTA AAA CGA CGG CCA GT-3′	Heidecker *et al.* 1980, Sanger *et al.* 1983
Universal reverse M13	5′-AAC AGC TAT GAC CAT G-3′	Heidecker *et al.* 1980, Sanger *et al.* 1983
Mismatched forward	5′-TGC TGC AAG TAA AAC GAC GGC CAG T-3′	This study
Very forward	5′-GATGTGCTGCAAGGCGAT-3′	This study
Very reverse	5′-GTG GAA TTG TGA GCG GAT-3′	This study
Mismatched reverse	5′-CAC AGT TGA ACA GCT ATG ACC AT-3′	This study
Cysteine to arginine	5′-CCG CTA AAG ATC GTT TC-3′	This study
Alanine to valine	5′-GCT AAT ATT GTT TCA TTA AAG-3′	This study
Leucine to serine	5′-CTA GTT CAG TTG CGC ATT TA-3′, position 304	This study
Leucine to serine	5′-GA GCA CCA CTT AGT TTG TGG-3′, position 245	This study

[a] Ap = ampicillin; Km = kanamycin; Rif = rifampicin; Sm = streptomycin; Tc = tetracycline; Tra$^+$ = transfer-competent; ^{r} = resistance.
[b] BRL = Bethesda Research Laboratories, Gaithersburg, MD; N. Panopoulos, University of California, Berkeley.

fragments were confirmed through sequencing with *avr*D-specific primers. All constructs were recloned into the broad host-range plasmid pRK415 and introduced into *P. s.* pv. *glycinea* R4 for testing the avirulence D phenotype in soybean plants.

Bacterial conjugations, plant growth conditions, and inoculations.

Bacterial conjugations were based on the method described by Ditta *et al.* (1980). Overnight cultures were used to make a mating mixture containing donor and helper bacteria at approximately 10^6 cells of each per milliliter and the recipient, *P. s.* pv. *glycinea* R4, at approximately 10^8 cells per milliliter. The mating mixture was spotted onto nonselective KB plates, incubated at 28° C for 10–12 hr, streaked onto KB plates containing rifampicin and tetracycline, and incubated at 28° C. The resulting transconjugants were successively single-colony purified two or three times on selective KB medium before inoculation of soybean plants. Water suspensions containing *P. s.* pv. *glycinea* R4 transconjugants at approximately 5×10^7 cells per milliliter were prepared from overnight cultures grown on plates and used to inoculate fully expanded primary leaves as described previously (Keen *et al.* 1990). Soybean plants were grown from seed (Long *et al.* 1985) and maintained in a growth chamber (10,000 lux, 16-hr photoperiod) at 21° C with 90% relative humidity after inoculation. Inoculations were scored daily for the appearance of a visible HR or water-soaked lesions, which occurred typically after 24–30 or 48–72 hr, respectively.

Elicitor assays.

Supernatant M9 culture fluids from *E. coli* cells expressing wild-type or recombinant *avr*D genes were desalted with Sep-Pak C18 cartridges (Waters Associates, Milford, MA) and further processed according to Keen *et al.* (1990). Primary leaves of soybean cultivars Harosoy and Flambeau were infiltrated with the elicitor preparations and scored for appearance of the HR, which occurred 12–20 hr after infiltration with active preparations.

SDS-PAGE and Western blots.

E. coli cells containing desired plasmids were grown in 15-ml LB cultures amended with 50 μg/ml ampicillin and 1 mM IPTG at 28° C for 14–16 hr. Cells were pelleted in Eppendorf tubes and resuspended in 0.8 ml of 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 2 mM dithiothreitol, and lysozyme (1 mg/ml). After a 30-min incubation on ice, the samples were frozen for 30 min at –70° C or 2 hr at –20° C. Once thawed, the samples were microfuged for 20 min at 4° C. The loose pellets were removed by pipetting, and equal volumes of supernatant and 2× sample buffer were mixed and boiled for 5 min. Typically, 20–30 ml was loaded onto duplicate 12% polyacrylamide slab gels (1 mm thick) and run according to Laemmli (1970). One gel was reserved for staining with Coomassie Brilliant Blue R 250, and the other was used for Western blot analysis. Gels were electrophoretically blotted to nylon membranes (Micron Separations, Westboro, MA) to transfer the proteins. After transfer, blots were incubated at room temperature in 3% gelatin in Tris-buffered saline (TBS) for 45 min and subsequently incubated with anti-AvrD antibody (Kobayashi *et al.* 1990b) diluted 1:3,000 in 1% gelatin in TBS for 1 hr. Blots were then washed for 5 min with 0.05% Tween in TBS and incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase for 1 hr. After two 5-min washes, the blots were rinsed with water to remove the detergent and developed. Additional water rinses stopped development.

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