Theor Appl Genet (1991) 81:133–138

New disease resistance genes in soybean against *Pseudomonas syringae* pv *glycinea*: evidence that one of them interacts with a bacterial elicitor

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Received May 10, 1990; Accepted August 22, 1990
Communicated by A. R. Hallauer

Summary. Soybean [*Glycine max* (L.) Merr.] cultivars Flambeau and Merit differed in their resistance to *Pseudomonas syringae* pv *glycinea* (*Psg*) race 4, carrying each of four different avirulence (*avr*) genes cloned from *Psg* or the related bacterium, *Pseudomonas syringae* pv *tomato*. Segregation data for F_2 and F_3 progeny of Flambeau × Merit crosses indicated that single dominant and nonallelic genes account for resistance to *Psg* race 4, carrying avirulence genes *avrA*, *avrB*, *avrC*, or *avrD*. Segregants were also recovered that carried all four or none of the disease resistance genes. One of the disease resistance genes (*Rpg1*, complementing bacterial avirulence gene *B*) had been described previously, but the other three genes – designated *Rpg2*, *Rpg3*, and *Rpg4* – had not here to fore been defined. *Rpg3* and *Rpg4* are linked (40.5 ± 3.2 recombination units). *Rpg4* complements *avrD*, cloned from *Pseudomonas syringae* pv *tomato*, but a functional copy of this avirulence gene has not thus far been observed in *Pseudomonas syringae* pv *glycinea*. Resistance gene *Rpg4* therefore may account in part for the resistance of soybean to *Pseudomonas syringae* pv *tomato* and other pathogens harboring *avrD*.

Key words: Disease resistance genes – Soybean [*Glycine max* (L.) Merr.] – Avirulence genes – *Pseudomonas syringae* pathovars

Introduction

Classic work by Flor and others recognized the occurrence of single-gene variation within pathogen populations for virulence on certain host plant cultivars, and among cultivars of plant species for resistance to the pathogen biotypes (see Flor 1955, 1956). In such 'gene-for-gene' systems, the expression of resistance generally occurs only when the plant carries a dominant disease resistance gene and the pathogen harbors a complementary, dominant avirulence gene. It has been hypothesized (see Keen and Staskawicz 1988) that avirulence genes and their complementary resistance genes encode the elements of specific recognitional systems which, if activated, lead to an active plant defense reaction called the 'hypersensitive response' (HR).

The interaction of soybean plants with *Pseudomonas syringae* pv *glycinea* (*Psg*) is thought to be a gene-for-gene system, since several naturally occurring races of the bacterium have been described that cause disease on some, but not all, soybean cultivars (see Keen and Holliday 1982). Recently, avirulence genes have been molecularly cloned and characterized from three races of *Psg* that fully explain their race phenotypes (for review, see Keen and Staskawicz 1988). However, only one disease resistance gene against *Psg* has been described in soybean by analysis of cross progeny. This gene (*Rpg1*, Mukherjee et al. 1966) is a dominant allele that confers resistance only to bacterial isolates carrying avirulence (*avr*) gene *B* (Staskawicz et al. 1987). The identification of these plant and bacterial genes formally proves that the soybean-*Psg* interaction is a gene-for-gene system. It was of interest, however, to determine if soybean cultivars exhibiting resistance to *Psg* isolates harboring other avirulence genes also contain single Mendelian resistance genes.

It was recently observed that avirulence genes cloned from *P. syringae* pv *tomato* (*Pst*), a bacterium not normally pathogenic on soybean, also function in *Psg* and cause this bacterium to elicit hypersensitive, resistant reactions on some but not other soybean cultivars (Koba-

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yashi et al. 1989). One of these genes, *avrD*, encodes a 34-kDa protein product that causes bacterial hosts to elaborate a low-molecular-weight elicitor which, by itself, elicits the HR on only those soybean cultivars that are resistant to *Psg* race 4 carrying *avrD* (Keen et al. 1990). These results led to the suspicion that the resistant soybean cultivars contained a single Mendelian resistance gene complementing *avrD* from *Pseudomonas syringae* pv *tomato*. In order to experimentally test whether soybean in fact contains classical disease resistance genes complementing avirulence genes cloned from *Pst* and *Psg*, we analyzed segregating progeny from a cross of cultivars Flambeau and Merit for their reactions to *Pseudomonas syringae* pv *glycinea* race 4 carrying either of four different cloned avirulence genes. These parents were particularly suitable because both are susceptible to *Psg* race 4, but Flambeau is resistant to the bacterium carrying *avrC* or *avrD*, while Merit is resistant to *Psg* race 4 carrying either *avrA* or *avrB*.

Methods and materials

Bacteria and the preparation of inoculum

Pseudomonas syringae pv *glycinea* race 4 was used in all the experiments. Plasmid pDSK519 carrying the cloned genes *avrA*, *avrB*, *avrC*, or *avrD* (Table 1) was introduced into *Psg* race 4 by conjugation with the helper plasmid pRK2013 (Ditta et al. 1980). *Psg* cells carrying only the cloning plasmid, pDSK519, gave susceptible reactions on all tested soybean cultivars. Thus, the bacteria used as inoculum were highly isogenic, containing only one of the four different cloned avirulence genes. Furthermore, the DNA sequences of all four *avr* genes are known (Napoli and Staskawicz 1987; Tamaki et al. 1988; Kobayashi et al. 1990a). Therefore, it was known with certainty that only one unique gene had been introduced into *Psg* race 4 on each of the four plasmid constructs.

Psg race 4 cells carrying each of the four cloned avirulence genes were routinely maintained on petri plates of solid King's medium B containing 50 μg/ml kanamycin (King et al. 1954). For production of plant inoculum, single colony isolates were grown overnight at 28 °C in shaken 18-mm test-tubes containing 4 ml of King's medium B broth supplemented with kanamycin at 50 μg/ml (King et al. 1954). The resulting bacterial cells were directly diluted into distilled water to yield an absorbance of ca. 0.2 at 500 nm (ca. 10^8 cells/ml). These suspensions were utilized for plant inoculations within 2 h following preparation.

Plant culture and inoculation

Soybean plants were grown on greenhouse benches in small pots containing a mixture of peat moss and sandy loam soil. Primary leaves were inoculated by infiltrating small areas with bacterial suspensions. Generally, 1-ml plastic syringes without needles were employed, and the blunt end was appressed to the leaf undersurface. Gentle pressure on the plunger permitted infiltration of the immediately underlying and surrounding mesophyll intercellular space with the bacterial suspension. A single primary leaf was inoculated in duplicate with bacteria containing the four different avirulence genes. Inoculated plants were incubated at 21 °C and 90% RH in a plant growth chamber with a 16-h photoperiod. The water from inoculation dissipated after 1 h or less, and leaves were scored at 24 h after inoculation and on 5 subsequent days for their disease reactions. Leaves reacting with hypersensitive, resistant reactions typically exhibited substantial necrosis at the inoculation site at 24 h (in the case of bacteria carrying *avrA* or *avrB*) or at 36–48 h (in the case of *avrC* or *avrD*). Susceptible interactions exhibited no visible responses for ca. 48 h, after which water-congested disease symptoms, followed by necrosis of the lesion area and marginal chlorosis, appeared at 3–6 days after inoculation. None of these symptoms except hypersensitive necrosis was observed in resistant reactions. Plants were scored as hypersensitive resistant or susceptible to inoculation with *Psg* race 4 carrying each of the four avirulence genes. Thus, four reactions were obtained for each plant, except for occasional plants where inoculations/readings were missed.

Crosses

The cultivars Flambeau and Merit were used because Flambeau is resistant to *Psg* race 4 carrying either of two avirulence genes, *avrC* or *avrD*, and Merit is resistant to bacteria harboring either *avrA* or *avrB* (Table 2). This cross therefore permitted studies on the segregation of four putative disease resistance genes complementing the four different avirulence genes. Flambeau × Merit and Merit × Flambeau crosses were made in growth facilities. F_1 and F_2 plants were grown in a greenhouse to produce F_2 and F_3 seeds, respectively.

Reaction to the avirulence gene D elicitor

Bacteria harboring *avrD* produce a unique extracellular metabolite which, in the absence of bacteria, elicits an intense, necrotic hypersensitive reaction on only those soybean cultivars that are resistant to *Psg* race 4 carrying *avrD* (Keen et al. 1990). It therefore appeared probable that plants reacting to the *avrD* elicitor contained a putative disease resistance gene complementary to *avrD*. In order to test this rationale, leaves of F_2 and F_3 plants were assayed for their reactions to both the *avrD* elicitor and *Psg* race 4 cells carrying *avrD*.

For production of the *avrD* elicitor, *Pseudomonas syringae* pv *glycinea* race 4 cells carrying pAVRD33 (Table 1) were grown on 100 ml of liquid M9 minimal medium containing 0.4% glucose for ca. 24 h at 28 °C on a laboratory shaker (Keen et al. 1990). Bacteria were pelleted by centrifugation at 10,000 *g* for 5 min and the cell-free culture fluids were desalted and concentrated with a C18 Sep-pak, as described in Keen et al. (1990). Following drying at 50 °C in a rotary evaporator, Sep-pak preparations were redissolved in 4 ml of water (yielding preparations 25 × relative to the initial culture fluid concentration) and directly used for plant screening. The preparations could be stored at 4 °C for up to 1 week with little or no loss of biological activity. Elicitor preparations were infiltrated into primary soybean leaves with 1-ml syringes in exactly the same way as bacterial inoculum. The elicitor was infiltrated into different portions of the same leaves as were inoculated with *Psg* race 4 cells carrying pAVRD33. Plants that did not respond to the elicitor exhibited no visible effects, except for occasional slight mechanical damage caused by the syringe infiltration. Plants that responded to the elicitor exhibited substantial necrosis of leaf tissue within, and usually extending well outside, the area originally infiltrated (see Keen et al. 1990). Plants were accordingly screened visually for the presence or absence of hypersensitive necrosis in response to the elicitor.

Results

Segregation was observed in F_2 progeny for resistance to *Psg* carrying each of the four avirulence genes, and recip-

Table 1. Bacteria and plasmids utilized

Bacterial strain or plasmid	Description	Source or reference
E. coli		
DH5α	Cloning host	Bethesda Research Labs
Pseudomonas syringae pv *plycinea*		
race 4 *rif amp*[a]	Pathogenic strain, resistant to ampicillin and rifampicin	This laboratory
Plasmids		
pDSK519	Broad-host-range cloning plasmid	Keen et al. 1988
pRK2013	Helper plasmid for conjugations	Ditta et al. 1980
pAVR9A11	pLAFR3 cosmid clone containing the cloned *avrA* gene from *P.s. tomato*	Kobayashi et al. 1989
pAVRA20	3.2-kb *Pst*I/*Bam*HI insert fragment from pAVR9A11 cloned into the same sites of pDSK519 in the orientation placing the *avrA* gene downstream of the vector promoter	This paper
pAVRB1	ca. 1.3-kb *Bgl*I fragment containing the *avrB* gene of *P.s. glycinea* cloned into the *Sma*I site of pUC118 in the orientation opposite to the vector *lac* promoter	Tamaki et al. 1988
pAVRB7	ca. 1.3-kb *Kpn*I/*Xba*I insert fragment from pAVRB1 cloned into the same sites of pDSK519; the *avrB* gene was thus oriented downstream from the vector *lac* promoter	This paper
pAVRC2	ca. 1.2-kb DNA fragment containing the *avrC* gene of *P.s. glycinea* cloned into the *Sma*I site of pUC119 with the gene oriented downstream of the vector *lac* promoter	Tamaki et al. 1988
pAVRC22	*Pst*I/*Eco*RI fragment from pAVRC2 carrying the *avrC* gene cloned into the same sites of pDSK519 such that the gene was oriented downstream of the vector *lac* promoter	This paper
pAVRD33	ca. 1.2-kb DNA fragment carrying the *avrD* gene from *P.s.* pv *tomato* cloned in pDSK519 downstream from the vector *lac* promoter	Keen et al. 1990

[a] *rif* = rifampicin resistance; *amp* = ampicillin resistance

Table 2. Segregation of F_2 plants of Flambeau × Merit and Merit × Flambeau for reaction to *Psg* race 4 carrying each of four different cloned avirulence genes

	No. of plants			
	avrA	*avrB*	*avrC*	*avrD*
Parents				
Flambeau	Susceptible	Susceptible	Resistant	Resistant
Merit	Resistant	Resistant	Susceptible	Susceptible
Flambeau × Merit				
Resistant	211	220	205	210
Susceptible	79	68	74	72
Chi-square[a]	0.66	0.23	0.27	0.02
P[b]	0.5–0.3	0.7–0.5	0.7–0.5	0.9–0.8
Merit × Flambeau				
Resistant	78	89	81	78
Susceptible	30	18	27	28
Chi-square[a]	0.31	3.39	0.00	0.05
P	0.7–0.5	0.10–0.5	100	0.9–0.8

[a] Goodness of fit to a 3:1 ratio expected for the segregation of a dominant allele for resistance and a recessive allele for susceptibility

[b] Probability of a larger Chi-square value occurring

rocal crosses gave similar data, indicating that there were no maternal effects (Table 2). Each of the segregations gave a good fit ($P > 0.05$) to a 3 : 1 ratio expected from segregation of a dominant allele for resistance and a recessive allele for susceptibility. The inoculation of each plant with *Psg* race 4 carrying the four different cloned avirulence genes resulted in all possible combinations (RRRS, RRSR, RSRR, SRRR, etc.) of resistant and susceptible reactions on individual plants (results not shown); this indicated that the four putative resistance genes are nonallelic. $F_{2:3}$ segregations and F_3 segregations from segregating F_2 plants gave good fits to expected ratios and confirmed the presence of four resistance genes (Tables 3 and 4). In addition to *Rpg1*, which controls the reaction to *avrB*, we propose the designations *Rpg2*, *Rpg3*, and *Rpg4* for the soybean resistance alleles that complement *avrA*, *avrC*, and *avrD*, respectively. The alleles for susceptibility at these loci are *rpg2*, *rpg3*, and *rpg4*, respectively.

After most of the F_2 screening was completed, the *avrD* elicitor produced by bacteria expressing this gene was discovered (Keen et al. 1990). A few F_2 progeny and

Table 3. Segregation in a F_2 Flambeau × Merit cross determined by inoculation of F_3 plants with *Psg* race 4 carrying each of four different cloned avirulence genes

	No. of plants			
	avrA	*avrB*	*avrC*	*avrD*
Resistant	11	17	11	17
Segregating	31	27	30	30
Susceptible	20	12	17	18
Chi-square[a]	2.90	0.44	1.82	0.26
P[b]	0.3–0.2	0.9–0.8	0.5–0.3	0.9–0.8

[a] Goodness of fit to an expected 1.03:1.97:1.0 ratio (i.e., 1:2:1 ratio adjusted on the basis of an average of 12 F_3 plants for each F_2 plant)
[b] Probability of a larger Chi-square value

Table 4. F_3 segregation from heterozygous F_2 plants of Flambeau × Merit when inoculated with *Psg* race 4 carrying each of four different cloned avirulence genes

	No. of plants			
	avrA	*avrB*	*avrC*	*avrD*
Resistant	276	261	264	286
Susceptible	99	77	94	95
Chi-square[a]	0.32	0.77	0.24	0.00
P[b]	0.7–0.5	0.5–0.3	0.7–0.5	100

[a] Goodness of fit to a 3:1 ratio expected for the segregation of a dominant allele for resistance and a recessive allele for susceptibility
[b] Probability of a larger Chi-square value

Table 5. Reaction of F_2 and F_3 plants of Flambeau × Merit to *Psg* race 4 carrying *avrD* and to infiltration with the isolated *avrD* elicitor

	No. of plants							
	F_2[a]				F_3			
	avrD		Elicitor		*avrD*		Elicitor	
	R[b]	S[c]	+[d]	0[e]	R[b]	S[c]	+[d]	0[e]
Resistant	–	–	–	–	210	0	210	0
Segregating	143	52	143	52	286	95	286	95
Susceptible	–	–	–	–	0	211	0	211

[a] Includes plants of Merit × Flambeau
[b] R = Resistant, hypersensitive reaction to *Psg* race 4 carrying *avrD*
[c] S = Susceptible, compatible reaction to *Psg* race 4 carrying *avrD*
[d] + = Necrotic, hypersensitive reaction to the elicitor
[e] 0 = No reaction to the elicitor

extensive F_3 progeny were accordingly screened both by inoculation of *Psg* race 4 carrying *avrD* and by infiltration with the *avrD* elicitor isolated from these cells. Without exception, all plants that were hypersensitively resistant to inoculation with the bacteria also responded with a necrotic hypersensitive reaction to the elicitor; conversely, all plants that were susceptible to bacterial inoculation did not produce necrosis in response to the elicitor (Table 5). A few plants were suspected of being exceptions, but upon reinoculation and retreatment with the *avrD* elicitor, they yielded identical responses to the two treatments.

Rpg1 and *Rpg2* segregated independently of *Rpg3* and *Rpg4* (Table 6). *Rpg1* may be loosely linked with *Rpg2* but further work is needed to establish this. *Rpg3* is linked with *Rpg4* at 40.5 ± 3.2 recombination units. The genotype of Flambeau is *rpg1rpg1 rpg2rpg2 Rpg3Rpg3 Rpg4Rpg4*, and that of Merit is *Rpg1Rpg1 Rpg2Rpg2 rpg3rpg3 rpg4rpg4*. Several F_2 and F_3 segregants of the Flambeau × Merit cross appeared to contain all four of the putative resistance genes, and two F_2 segregants appeared to lack all four of these genes. These lines were advanced several generations and inoculated in each generation with *Psg* race 4 cells containing each of the four cloned avirulence genes. Single F_5 plants were eventually selected that had bred true for the presence of all or none of the resistance genes. F_6 seed from these plants was bulked and increased.

Discussion

Meaningful investigations on the genetics of avirulence in pathogens and disease resistance in plants are limited by the extent to which genetically well-defined partners are used. If the genetics of one partner is well understood, the gene-for-gene relationship permits prediction of the genetics of the other partner (Person 1967). Thus, if a certain plant cultivar is known to contain a defined single-disease resistance gene that is not present in a second cultivar, any pathogen strain that elicits a resistant reaction only on the first cultivar may be assumed to contain the matching or complementary avirulence gene. While this approach has generally been used when the genetics of plant resistance is known and pathogen genetics are not, we tested whether the opposite extrapolation was true. This was possible because the four cloned avirulence genes used in this work have been sequenced and characterized. Since only one protein-encoding open reading frame was present in each of these clones, we were confident that one and only one avirulence gene was present in the various plasmid constructs introduced into *Psg* race 4. Furthermore, since the different cloned avirulence genes were introduced into a single *Psg* isolate (race 4), the resultant bacteria were highly isogenic, eliminating the possibility of other, unidentified pathogen

Table 6. Segregation of pairs of *Rpg* genes from inoculation of soybean plants with *Psg* race 4 carrying each of four different cloned avirulence genes

Genes segregating	RR[b]	RS[c]	SR[d]	SS[e]	Sum	RU[f]	SE	Phase[g]
Rpg1 rpg1 Rpg2 rpg2	256	94	67	31	448	46.8	3.4	C
Rpg1 rpg1 Rpg3 rpg3	254	91	66	32	443	54.2	3.4	R
Rpg1 rpg1 Rpg4 rpg4	251	92	71	24	438	48.9	3.6	R
Rpg2 rpg2 Rpg3 rpg3	247	79	79	41	446	I		R
Rpg2 rpg2 Rpg4 rpg4	239	82	91	35	447	51.6	3.5	R
Rpg3 rpg3 Rpg4 rpg4	251	71	78	44	444	40.5	3.2	C

[a] Summary of F_2 and $F_{2:3}$ from Flambeau × Merit and F_2 from Merit × Flambeau
[b] RR = Resistant for both genes
[c] RS = Resistant for first gene, susceptible for second gene
[d] SR = Susceptible for first gene, resistant for second gene
[e] SS = Susceptible for both genes
[f] RU = Recombination units with standard error (SE)
[g] C = Coupling, R = repulsion

avirulence genes influencing the results. Thus, we were able to confidently predict the resistance genotypes of the various soybean differential cultivars from their reactions to *Psg* race 4 carrying each of the four avirulence genes. The segregation data from the Flambeau × Merit crosses confirmed the occurrence of these four different disease resistance genes in soybean. One of them, *Rpg1*, was previously described by Mukherjee et al. (1966) and has been shown to complement *avrB* (Staskawicz et al. 1987). The other three disease resistance genes, which had not been defined heretofore, are *Rpg2*, *Rpg3*, and *Rpg4*. These genes complement *avrA*, *avrC*, and *avrD*, respectively.

Until the cloning of *avrB*, which complements *Rpg1* (Staskawicz et al. 1987), the soybean-*Psg* system had not been proven to follow a gene-for-gene relationship (see Crute 1986). With the present demonstration of three additional Mendelian disease resistance genes in soybean, the system is firmly established as a gene-for-gene system. The new disease resistance genes, *Rpg2*, *Rpg3*, and *Rpg4* follow the general rule of being dominant, and no indications of incomplete dominance were observed. Screening with the *avrD* elicitor was expected to give the most sensitive indication of incomplete dominance in segregating populations, but no indications of such were observed. Thre was also no suggestion of maternal inheritance in results from reciprocal crosses. The indication of loose linkage between *Rpg1* and *Rpg2* and between *Rpg3* and *Rpg4* is interesting. Preliminary unpublished results indicate that *Rpg2* and *Rpg3* may each be loosely linked with T in linkage group 1. The possibility that all four *Rpg* alleles might be on the same chromosome will be studied further.

It is noteworthy that the newly described resistance genes differ in timing of the hypersensitive reactions they modulate, just as occurs with several other plant disease resistance genes (e.g. Knott 1989). The interaction of *avrB* and *Rpg1* leads to a relatively rapid hypersensitive reaction that is first visible at ca. 16 h after inoculation. The interaction of *avrA* and *Rpg2* is slightly delayed and inoculated plants exhibit less intense hypersensitive necrosis. The interaction of *avrD* and *Rpg4* results in an even slower appearing HR that is only visible between 36 and 48 h. The interaction of *avrC* and *Rpg3* leads to the slowest HR, which is generally not observed until ca. 48 h after inoculation (Staskawicz et al. 1987; Tamaki et al. 1988).

It is particularly noteworthy that soybean contains the gene *Rpg4* which complements the *Pst avrD* gene since a functional version of this avirulence gene has not been detected in any naturally occurring isolate of *Psg*. However, all *Psg* isolates thus far surveyed contain DNA that is highly homologous to *avrD*, despite the fact that an avirulence function is not expressed (Kobayashi et al. 1990b). It is therefore appealing to speculate that, through evolution, there has been a loss of *avrD* function in *Psg* so that it is compatible with the soybean *Rpg4* gene. Our observations also fuel the speculation that the function of *Rpg4* in soybean may precisely be to confer resistance to *Pseudomonas syringae* pv *tomato* and other *P. syringae* pathovars that carry a functional copy of *avrD*. Kobayashi et al. (1989) showed that *Pst* carries at least three different avirulence genes which elicit resistance in particular soybean cultivars when they are harbored by *Psg*. Similarly, Whalen et al. (1988) showed that an avirulence gene from a tomato strain of *Xanthomonas campestris* pv *vesicatoria* functioned to elicit defense reactions in several plant species following its introduction into *X. campestris* pathovars normally pathogenic on them. While as yet unproven, these results and our observations suggest that such avirulence genes and their complementary plant disease resistance genes may be involved with plant-pathogen specificity above the cultivar-race level.

The segregation data for *Rpg4* showed that the *avrD* elicitor affected only those progeny that were hypersensitively resistant to *Psg* race 4 carrying the same gene. This is an especially significant result, since it has profound implications concerning the mechanism of recognition of avirulent bacteria by soybean plants carrying *Rpg4*. The data strongly indicate that *avrD* causes bacteria harboring this gene to elaborate the extracellular elicitor which is, in turn, the agent recognized by *Rpg4* soybean plants to initiate hypersensitive resistance (Keen et al. 1990).

No exceptions were observed in the linkage of response to *Psg* race 4 cells carrying *avrD* and infiltration of leaves with the *avrD* elicitor among segregating F_2 and F_3 Merit × Flambeau progeny. While two different but closely linked resistance genes could account for these results, several factors support the view that both the inoculated bacteria and the *avrD* elicitor interact with the *Rpg4* resistance gene. First, the plant response to both treatments is the same, namely hypersensitive necrosis of leaf tissue. Second, *avrD* has been sequenced (Kobayashi et al. 1990a) and is known to encode only a single-34 kDa protein product that has been shown to mediate production of the *avrD* elicitor by bacterial hosts (Keen et al. 1990). Third, mutation of *avrD* negates both the ability of *Psg* race 4 cells harboring it to elicit the HR in soybean as well as to produce the *avrD* elicitor (Keen et al. 1990).

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