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GENETIC ANALYSIS OF RACE-SPECIFICITY OF *PSEUDOMONAS SYRINGAE* PV. *GLYCINEA*

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Abstract

The reactions of soybean cultivars to known races of *Pseudomonas syringae* pv. *glycinea* (*Psg*) in the light of known avirulence (*avr*) genes and their corresponding resistance genes were reevaluated to predict if new avr genes and corresponding resistance genes exist. The cloned *avrD* gene from tomato pathogen induced a unique pattern on these lines and elicits hypersensitive reaction on those lines which carry disease resistant gene *Rpg4*. None of the known races of *psg* carries *avrD* gene that elicit the resistant reaction on soybean carrying the disease resistant gene *Rpg4*.

Introduction

Pathogenic strains of *Pseudomonas syringae* (*Ps*) vary with respect to the plant species (or genus or family) that they infect. Some strains are pathogenic to soybean (pv. *glycineae*), others to tomato (pv. *tomato*), and so forth. Race designation for strains of *Psg* (Cross *et al*., 1966), and other pathovars (pv) reflect the variability in the ability of strains within a pv to infect different cultivars within a host species. Ten known races of *Psg* were distinguished by various workers (Cross *et al*., 1966; Thomas & Leary, 1980; Fett & Squeira, 1981; Gnanamanickam & Ward, 1982; Kucharek & Stall *et al*., 1985).

Avirulence (*avr*) genes in *Pseudomonas*: An *avr* gene is defined as a gene of a potentially virulent bacterium that renders the bacterium unable to cause disease in specific cultivars of the host plant. These *avr* genes can easily be determined by the response of the host to a specific race of the pathogen after inoculation. Different races of a bacterium carry different *avr* genes (Staskawicz *et al*., 1984; Staskawicz *et al*., 1987; Keen & Buzzell, 1991) that complement different resistance genes (Mukherjee *et al*., 1966; Keen & Buzzell, 1991) in the soybean host.

Although precise information on the corresponding host genotypes is still lacking for a number of other *avr* genes. However, since *avr* genes clearly are involved in recognition by the plant disease resistant genes, Kobayashi *et al*., (1989) introduced cosmid clones from a gene library of *Pst*, a bacterium not pathogenic on soybean, into *Psg* Race-4 and tested the transconjugants on a range of soybean cultivars. Some cultivars produced hypersensitive reaction, HR (Klement, 1963), while others did not. This gene was designated *avrD* (Kobayashi *et al*., 1990a). The *avrD* is the only bacterial gene for which information is available on how *avr* genes interact with

resistant plants. The cloned *avrD* gene functioned in *Psg* cells to elicit the HR in only those cultivars of soybean carrying the disease resistant gene *Rpg4* (Keen & Buzzell, 1991). The active *avrD* gene metabolizes bacterial compounds found in *E. coli* and other gram-negative bacteria into substances called syringolides 1 and 2, which are referred collectively as the specific elicitor or SE and act as a single molecule (Smith *et al.*, 1993; Midland *et al.*, 1993). The SE molecule elicits an HR only on those soybean cultivars that carry the disease resistance gene, *Rpg4* (Keen *et al.*, 1991; Smith *et al.*, 1993; Midland *et al.*, 1993). These reports also show that SE mediates the *avrD*/*Rpg4* gene-for-gene interaction.

Materials and Methods

These evaluations are based on the following information.

Molecular studies have identified three different avirulence (*avr*) genes (*avrA*, *avrB*, and *avrC*) in four different races (0, 1, 4 and 6) of *Psg* that have been examined (Table 1). The genetic analysis of Keen & Buzzell (1991) determined that soybean carries three resistance genes, *Rpg1*, *Rpg2*, and *Rpg3* that correspond to *avrB*, *avrA*, and *avrC* respectively. They also determined that soybean carries a resistance gene, *Rpg4*, that correspond to *avrD*, cloned from *Pst*,

A panel of 10 soybean cultivars has been screened with 10 known races of *Psg*. These 10 soybean cultivars were screened with the specific elicitor produced by *avrD* from *Pst*.

Specific elicitor (SE) preparation: The SE was isolated to a high degree of purity from *Psg* transformed with *avrD* by the method used by Keen *et al.*, (1990).

Specific elicitor preparation:

Specific elicitor (SE) was purified from *E. coli* cultures that express the *avrD* coding sequence under the control of the UV5 *lac* promoter (Midland *et al.*, 1993). The *E. coli* strain, MA1008 (*pavrD12*), was grown overnight with shaking at 37°C in rich medium (LB+ampicillin 0.1 mg ml^{-1} without IPTG). Cells were harvested by centrifugation and washed once with a minimal medium consisting of 1 x M9 salts, 0.1% glucose (w/v) and 50 µM IPTG. The cells were then resuspended in 0.5 volume of the same minimal medium and incubated at 28°C with shaking for 2 h. At this point the cells were collected by centrifugation and the cell pellet was discarded. The pale yellow culture supernatant was retained and adjusted to pH 6.0 with 1N HCl. After two extractions with 0.5 volumes of Ethyl acetate the residual water was removed by the following treatments:

a. The organic phase was allowed to stand for 10 min in a clean, dry glass beaker to permit any water to form beads on the glass.

b. The ethyl acetate was then poured off into a second dry, glass beaker containing anhydrous sodium sulfate. The suspension was gravity filtered through four layers of Whatman #1 paper. The solvent was removed from the crude SE extract under vacuum at room temperature, resulting in a brown oily residue.

Table 1. Reaction of soybean to different races of *Pseudomonas syringae* pv. *glycinea*.

Race	Avr	Acme	Centennial	Cippewa	Flambeau	Hardee	Harosoy	Lindarin	Merit	Norchief	Peking
0	B,C	R	S	U	R	U	R	U	U	R	R
1	C	S	S	R	S	R	R	R	R	R	S
2		S	U	R	S	U	S	S	S	S	U
3		S	U	R	R	U	S	S	I(R)	I(R)	U
4	d[1]	S	S	S	S	S	S	S	S	S	S
5		R	U	R	R	S	S	R	S	R	S
6		R	U	R	S	S	R	R	R	S	R
7		R	U	R	R	U	S	R	R	R	U
8		S	U	S	S	U	S	R	S	S	U
9		R	U	S	R	U	S	S	S	R	U
Pst	D	S	R	R	R	S	R	R	S	R	S

- Avr avirulence genes in respected race
- R= Resistant, S= Susceptible, I= Intermediate reaction characterized by more necrosis and less chlorosis than the compatible reaction (Cross *et al*., 1966)
- U= Unknown
- d[1]= Nonfunctional homologue of *avrD*

(Cross *et al*., 1966; Thomas & Leary, 1980; Fett & Squeira, 1981; Gnanamanickam & Ward, 1982; Staskawicz *et al*., 1984; Staskawicz *et al*., 1987; Kobayashi *et al*., 1989 and Keen *et al*., 1990)

For approximately 70% of the inoculations the crude SE was further purified by the following procedure according to Smith *et al.,* (1993). A silica gel (Merck, grad 60, 230-400 mesh, 60 Å) flash column was prepared in a glass syringe and equilibrated with 50-column volumes of HPLC-grade dichloromethane. The crude elicitor extract was dissolved in 5 ml of dichloromethane and applied to the column, which was then washed with 12 ml of dichloromethane. The elicitor activity was eluted with 12 ml of ethyl acetate. The ethyl acetate was removed from the solution at room temperature under vacuum. The residual elicitor was again dissolved in 5 ml of dichloromethane, applied to a new, identical flash column, and washed as previously described. The second column was eluted with HPLC-grade acetonitrile, followed by a methanol wash (4g L^{-1}) which was discarded. SE activity was found principally in the first 2 ml fraction of acetonitrile, which was saved, and in the final methanol wash. The elicitor thus produced from a 2-L culture was divided into 20 aliquots, taken to dryness under vacuum at room temperature, and stored in the dark under vacuum at 4°C. Alternatively, the mass of total elicitor produced was determined, and the preparation was stored as above in 10 mg aliquots.

Plant culture and inoculation: Soybean seed were germinated in a controlled environment under an 18 h day/6 h night photoperiod at a constant temperature of 25°C and 99% relative humidity. The fully expanded primary leaves of 15-d old soybean seedlings were used for inoculation by one of two methods:

1. Initially, an aliquot of SE was dissolved in 100% ethanol or methanol and diluted with water to a final concentration of 125 units ml^{-1} (where a unit is defined as the quantity of SE produced by 1 ml of *E. coli* MA1008 [*pavrD*]). This solution was then pressure-infiltrated into the bottom of each leaf with the blunt end of a disposable 1 ml syringe, sometimes with the aid of a Hagborg device as per Long *et al*., 1985 (Fig. 1).

In the later stages of these experiments, a more convenient method for SE inoculation was developed based on Whalen *et al*., (1991). Here the SE was dissolved in ethanol or methanol and then diluted at least 20-fold into an aqueous solution of 0.02% Silwet L-77 (Union Carbide), resulting in a SE concentration of 1 mg ml^{-1}. Leaves were inoculated by dipping them into the SE solution (Fig. 2).

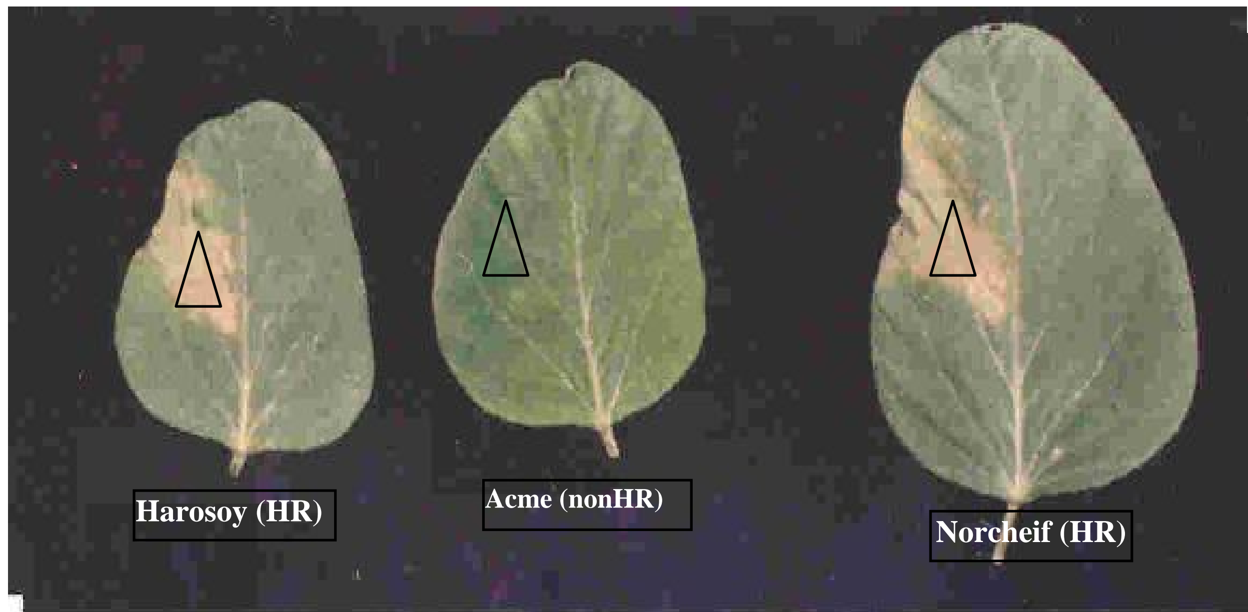

Fig. 1. Hypersensitive (HR) and nonHR response of soybean leaves to specific elicitor (SE) after 24-hr
△ = Site of SE infiltration

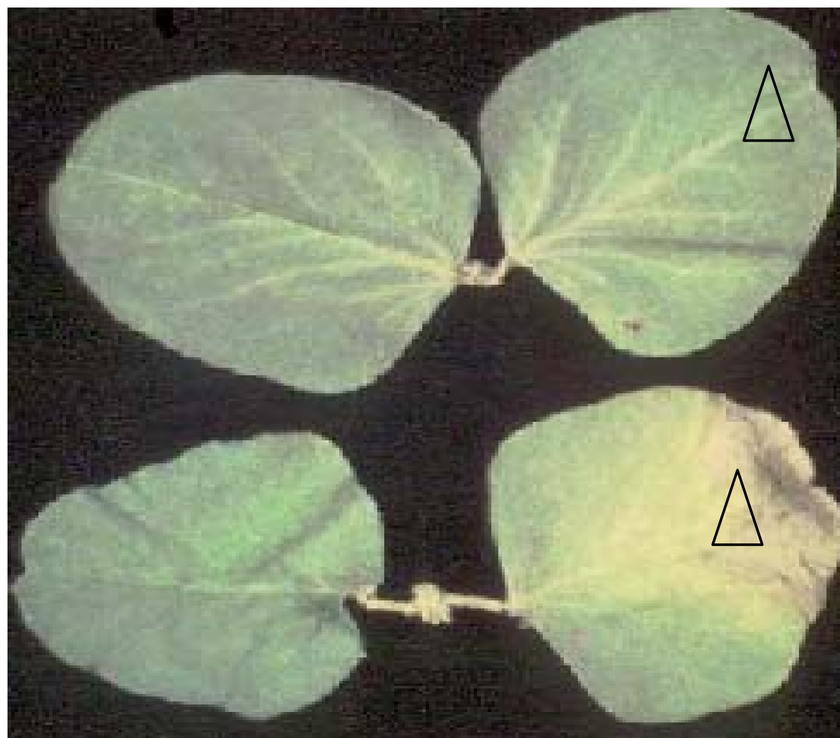

Fig. 2. HR and nonHR response of soybean leaves after 24-hr after dipping into SE.
△ = Site dipping into SE

Inoculated leaves were screened visually for the presence or absence of hypersensitive necrosis after 24-48 h. Visible necrosis (HR) of the inoculated area typified positive or resistant reactions. Negative or susceptible reactions displayed no HR. Occasionally; slight mechanical damage was produced by pressure infiltration, but could be readily distinguished from the HR.

The line Acme was used as a nonHR control throughout all of these experiments. HR controls were Harosoy or Norchief which typically exhibit a strong reaction to the SE.

Elicitor application:

Fully expanded primary leaves of soybean seedlings at the 2-leaf stage were infused with SE. After infusion, seedlings were placed in a controlled environment chamber for 18h light at a constant temperature of 25° C and 90% relative humidity for 24-48h (Keen *et al*., 1990).

Reaction of elicitor: Standard procedure (Keen *et al*., 1990) was used to read the reaction of leaves to SE. The inoculated leaves were screened visually for the presence of *Rpg4/-*(resistant to *Psg*) or *rpg4/rpg4* (susceptible to *Psg*) through hypersensitive necrosis, HR in response to the SE after 24-48h (Figs. 1 & 2).

Results

Importantly, each of the 10 races and *avrD* elicited a unique pattern of responses from the panel of 10 cultivars (Table 1). The two reactions that were reported as intermediate, cultivars Norchief and Merit with Race 3, are assumed to be resistant because there is possibility that weak HR may be due to either weak resistance gene(s) in the cultivars or weak *avr* gene(s) in the race 3, or due to the effects of genetic background in either organism that somehow modulate the intensity of the HR. As it turned out, however, neither of these reactions is critical to the conclusions of this analysis. For example, both reactions can be considered either resistant or susceptible and the set of reactions of race 3 is still unique.

The genotypes for all cultivars are assumed to be homozygous at all loci i.e., R1= *Rpg1/Rpg1*, r1= *rpg1/rpg1*, R2 = *Rpg2/Rpg2*, etc.

The conclusions were made on the basis of the simplest possible model, which is by postulating the least number of pairs of corresponding resistance and *avr* genes necessary to explain the observed interactions between soybean cultivars and *Psg* races (and *avrD*) according to gene-for-gene system.

The genotypes of all cultivars were worked out for all four known *Rpg* loci with the exception of the *Rpg3* locus in the cultivars Chippewa, Hardee, Harosoy, Lindarin and Norchief. All of them are resistant to race 0 which carries *avrB* and *avrC* but they are also resistant to race 1 which carries only *avrB*. Therefore, for these cultivars the resistant reaction to *avrB* masks their reaction to *avrC* in race 0.

For nearly every case with races 2, 3, 5, 7, 8, and 9, the presence of *avrA*, *avrB*, *avrC* and *avrD* could be ruled out except for races 7 and 9 which could carry *avrC* because Acme and Flambeau carry R3 and are resistant to these races. Importantly, the presence of *avrD* in any of the known races of *Psg* can be ruled out.

New postulated *avr* and their corresponding resistant genes:

Three new *avr* genes, *avrE*, *avrF* and *avrG*, were postulated and these would correspond to three postulated resistance genes R5, R6, and R7, respectively.

Explanation:

Race 2 does not carry any of the known *avr* genes, but Chippewa is resistant to this race. Therefore, race 2 must carry a new *avr* gene "*avrE*" that corresponds to a new resistant gene "R5" in Chippewa.

Race 3 carries another avr gene "*avrF*" because Flambeau (r1r2R3R4) is resistant to this race although it does not carry *avrC* or *avrD*. Although this race could carry *avrE* and Flambeau could not react to it because it carries r5 (and is susceptible to race 2). Thus, Flambeau must carry a resistance gene "R6" that corresponds to the new avr gene.

Race 8 carries another avr gene "*avrG*" because Lindarin is resistant to this race although it does not carry any of the known *avr* genes *avrA* through *avrD,* nor does it carry either of the previously postulated new *avr genes, avrE and avrF*. Therefore, Lindarin must carry a resistance gene "R7" that corresponds to the new avr gene.

Discussion

Several researchers determined the reactions of ten soybean cultivars to ten races of *Psg*, presented in Table 1 (Cross *et al*., 1966; Thomas & Leary, 1980; Gnanamanickam & Ward, 1982; Staskawicz *et al*., 1984; Staskawicz *et al*., 1987; Napoli & Staskawicz, 1987). The elicitor produced by avirulence gene *avrD* of *Pst* (Kobayashi *et al*., 1989; Keen *et al*., 1990) elicits hypersensitive reaction only on those cultivars carrying disease resistance gene *Rpg*4 (Keen & Buzzell, 1991). This unique pattern of response from *avrD* of *Pst* is the base of this evaluation.

This evaluation rather reevaluation indicates that at least seven avr genes are required in *Psg* to explain all of the reactions of the 10 cultivars to the 10 known races of the pathogen. Interestingly, seven *avr-Rpg* gene pairs could determine a total of $2^7 = 128$ different races of the pathogen and 128 corresponding homozygous genotypes of soybean.

Even more interesting, although none of the known races of *Psg* carries a functional *avrD*, there is still the possibility that *avrD* does exist in *Psg*. After all, race 4 carries a nonfunctional homologue of this gene (Kobayashi *et al*., 1990b) and a corresponding resistance gene, *Rpg4*, occurs in soybean (Keen & Buzzell, 1991; Keen *et al*., 1991). Therefore, if *avrD* does occur in *Psg*, then there would be a total of eight *avr-Rpg* gene pairs that could determine a total of $2^8 = 256$ different races of the pathogen and 256 corresponding homozygous genotypes of soybean.

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