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Race-specific molecules that protect soybeans from *Phytophthora megasperma* var. *sojae**

(host–pathogen interactions/specificity factors/extracellular glycoproteins/disease resistance/gene-for-gene relationship)

MARK WADE AND PETER ALBERSHEIM†

Department of Chemistry, University of Colorado, Boulder, Colorado 80309

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ABSTRACT **_Phytophthora megasperma_ var. _sojae_ (A. A. Hildebrand) is a fungal stem and root rot-causing pathogen of soybeans. Glycoproteins secreted into the medium of the aseptically cultured fungus have been partially purified by $(NH_4)_2SO_4$ precipitation and by column chromatography on norleucine-substituted Sepharose 4B and on DEAE-cellulose. Glycoprotein preparations from _P. megasperma_ var. _sojae_ races 1, 2, and 3 have been tested on four cultivars of soybeans. The partially purified glycoproteins from incompatible races of the pathogen (races that cannot successfully infect the plant), but not those from compatible races (races that can kill the plant), protect soybean seedlings from attack by compatible races. The seedlings are protected by introducing the glycoproteins into hypocotyl wounds of seedlings either 90 min prior to or at the time of inoculation of the wounds with mycelia of one of the pathogens. The glycoprotein preparations are poor nonspecific elicitors of phytoalexin accumulation; the glycoproteins have less than 1.0% of the elicitor activity of the glucans present in the mycelial walls of the pathogen.**

Plants are exposed to attack by an enormous variety of bacteria, fungi, viruses, insects, and other animals. Yet plants are not susceptible to serious injury by the vast majority of these potential pests. In most cases, the molecular details of the disease process and the mechanisms of plant resistance are not well understood. Many host-specific plant pathogen species exist as a number of races, each distinct from the others in its ability to attack the various cultivars (varieties) of its host plant species. In other words, race 1 of a pathogen of a particular crop may be able to infect cultivar A but not B, whereas race 2 may be able to infect cultivar B but not A.

The genetic relationship underlying many such race- and cultivar-specific host–pathogen systems was first recognized by Flor (1, 2) during his extensive studies of the flax rust fungus *Melampsora lini*. For each gene that governs resistance in the host, there is a corresponding gene in the pathogen that governs avirulence. Avirulence in this context is the inability of a race of a host-specific pathogen species to infect a particular cultivar of its host. Resistance in the host is usually dominant, as is avirulence in the pathogen. This relationship, which is the genetic basis governing the interaction of many plants and their pathogens, is called the gene-for-gene relationship (2).

Biochemical mechanisms that might underlie a gene-for-gene relationship have been suggested (3, 4). In these models, the avirulence genes of a pathogen encode or control the synthesis of molecules [specificity factors (5)] that can bind to receptors in the plant and thus activate defense mechanisms. Day (3) has proposed that the receptors are, themselves, the plant's resistance genes and that binding at these sites activates expression of other genes involved in resistance. Albersheim and Anderson-Prouty (4) have stressed the importance of cell surface interactions in pathogenesis and have proposed that genes for disease resistance in plants encode for receptors in the cell membrane that recognize the race-specific molecules of the pathogen. These authors have further suggested that the products of the pathogen's avirulence genes may be glycosyltransferases, which assemble the race-specific carbohydrate structures of the specificity factors. This suggestion is based in part on the fact that in yeast, species-specific cell surface structures are mannan-containing glycoproteins (6, 7). The species-specific differences in these glycoproteins reside in the carbohydrate portion of the molecules (6, 7). At least some of the enzymes secreted by yeast also contain these species-specific mannan structures (8).

We are studying the race- and cultivar-specific interaction of *Phytophthora megasperma* var. *sojae* (Pms) and soybeans. The fungus, Pms, is the causal agent of root and stem rot of soybeans. This interaction appears to be a typical gene-for-gene system; at least nine fungal races exist, and many differently susceptible cultivars of the host plant exist (9).

The carbohydrate portions of the extracellular invertases of Pms races 1, 2, and 3 have been shown by Ziegler and Albersheim (10) to possess race-specific differences in their glycosyl linkages. If the race-specific, carbohydrate structures of the extracellular glycoprotein population contain the specificity factors, the biological activity of these molecules should be demonstrable. We report here that the partially purified extracellular glycoproteins from avirulent races of Pms are capable of protecting soybean cultivars from infection by virulent races. These results are strong evidence that the specificity factors governing avirulence in the Pms–soybean system reside in glycoproteins secreted by these fungi.

MATERIALS AND METHODS

Organisms. The source and culture of the Pms races have been described (11). The extracellular glycoproteins were obtained from the culture filtrate of 14-day-old Pms cultures grown in an asparagine and sucrose medium. The mycelia used for inoculation of soybean (*Glycine max* L.) seedlings in the infectivity and protection experiments were harvested from 2-day-old cultures grown in liquid V8 medium (12). The inoculation cultures, 20 mg, were initiated with 2 plugs (1 cm diameter) of Pms cut from a V8/agar plate. The standing cultures were incubated at 24°C in the dark.

Soybean seed of cultivars Amsoy 71, Harosoy, Harosoy 63, and Wayne were Foundation quality and were obtained from

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Abbreviations: Pms, *Phytophthora megasperma* var. *sojae*; ECGP, extracellular glycoprotein.

* This paper is no. 16 of a series on host–pathogen interactions. Paper no. 15 is ref. 14.

† To whom reprint requests should be addressed.

commercial sources. Sanga seed was the gift of J. Paxton (University of Illinois) and of K. Athow (Purdue University).

All seeds were hand sorted for uniform size and soundness before planting. The seeds were surface treated by soaking in 0.75% sodium hypochlorite (15% Chlorox) for 5 min and then rinsed extensively. The surface-treated seeds were planted, at a density of 120 seeds per tray (48 × 31 × 9 cm), on a 3-cm layer of potting soil between two 2-cm layers of Vermiculite. Seedlings were grown in a Percival growth chamber with a regime of 14 hr at 2500 footcandles (1 footcandle = 1.076 lux) of illumination at 24°C and 10 hr of darkness at 19°C. The relative humidity was maintained at about 35%. Unhealthy or misshaped seedlings and ungerminated seeds were removed from the trays after 4–5 days. Seedlings were used at the first leaf stage (9–10 days after planting) both for the hypocotyl assay for protection against fungal infection and for the cotyledon elicitor assay. Seedlings used in the hypocotyl assay for elicitor activity were harvested 7–8 days after planting.

Assays for Elicitor Activity. The cotyledon and hypocotyl assays for elicitor activity have been described (11). Fraction I elicitor, isolated from acetic acid-dialyzed Pms mycelial walls, was used as a positive control (13). The presence of phytoalexins in tissue extracts was detected by bioassay as described (14).

Assay for Infectivity of Pms Races on Soybean Seedlings. Wounds were made in seedlings by impaling the midpoint of the hypocotyls on sharpened sterilized steel skewers (1.5 mm diameter). Only seedlings with hypocotyls equal to or greater than 2.5 mm in diameter were used. The size of the wound was kept to a minimum. Five plants were positioned 1 cm apart on each skewer. Racks of skewers (2.5 cm apart) holding the seedlings were placed in plastic trays (30 × 12 × 10 cm). Sufficient sterile distilled water was added to cover the roots. The plants were inoculated by placing about 1-mm-diameter pieces of mycelia in the hypoctyl wounds. The mycelia was taken from the growing edge of the 2-day-old mycelial mats. Care was taken to avoid further damage to the hypocotyl tissue during insertion of the mycelia. The plants were incubated for 48 hr in a growth chamber at 100% relative humidity with a regime of 16 hr light (2500 footcandles) at 27°C and 8 hr dark at 22°C. After 48 hr, the number of dead or dying plants was recorded. As a control in each experiment, five impaled plants were inoculated with sterile V8 medium (10 μl). The control plants remained healthy in every experiment.

Plants exposed to a compatible race of the fungus were usually killed (the part of the seedling above the skewer had fallen over) or made very sick within 48 hr. Plants inoculated with an incompatible Pms race remained healthy. Race 1 is compatible with Harasoy and Wayne but not with Harasoy 63, Amsoy 71, or Sanga. Race 2 is compatible with Harasoy, Wayne, and Sanga, but not with Harasoy 63 or Amsoy 71. Race 3 is compatible with Harasoy, Wayne, Harasoy 63, and Amsoy 71, but not with Sanga.

Protection Experiments. The plants were impaled on skewers as described above and the trays of plants were placed in the sterile air of a laminar-air-flow hood. Five 10-μl aliquots of an aqueous solution of the extracellular glycoproteins were applied to all surfaces of each wound over a period of 90 min (about one application every 20 min). Care was taken to ensure that all surfaces of the wound were thoroughly wetted with each application. The plants absorbed the solution before another aliquot was applied. The plants were inoculated with a Pms race 90 min after the first application of glycoprotein solution and incubated for 48 hr. Control plants were treated with five 10-μl aliquots of sterile distilled water instead of a glycoprotein solution. The control plants were then inoculated with a Pms race in order to check the cultivar specificity and virulence of the races at the time of each protection experiment.

Partial Purification of Extracellular Glycoproteins. All steps were performed at 0–5°C. The 14-day-old culture filtrates of 4–5 liters of culture filtrate (10–12 Fernbach flasks) were combined and separated from Pms mycelia and solid $CaCO_3$ by passage through Whatman GA glass-fiber discs.

Step 1: Ammonium sulfate precipitation. The filtered culture fluid was adjusted to 100% of saturation with respect to $(NH_4)_2SO_4$ and stirred gently overnight. The precipitate was collected by centrifugation at 10,000 × *g* for 25 min and washed twice with a saturated $(NH_4)_2SO_4$ solution in 0.1 M sodium acetate (pH 5.0). The pellet was dissolved in 200 ml of 0.1 M sodium acetate (pH 5.0). The solution was adjusted to 60% saturation with respect to $(NH_4)_2SO_4$ and stirred slowly for 30 min to precipitate non-carbohydrate-containing proteins. The precipitate was removed by centrifugation at 10,000 × *g* for 25 min. The supernatant solution was adjusted to 100% saturation with $(NH_4)_2SO_4$ and stirred for 30 min; the precipitate was collected and washed with a saturated solution of $(NH_4)_2SO_4$ in the sodium acetate buffer as before to free the glycoprotein preparation from nonproteinaceous carbohydrates. The precipitate was resuspended in a 60% saturated solution of $(NH_4)_2SO_4$ in the sodium acetate buffer and a small amount of $(NH_4)_2SO_4$-free buffer was added dropwise, with stirring, until the precipitate dissolved.

Step 2: Chromatography on a norleucine-substituted Sepharose 4B column. A norleucine-substituted Sepharose 4B column fractionates glycoproteins and neutral polysaccharides because of their different interactions with antichaotropic ions (15). The D,L-norleucine-substituted Sepharose was prepared from 4 g of cyanogen bromide-activated Sepharose 4B (Pharmacia) as described (10). The norleucine column (2 × 4.5 cm) was equilibrated with 60% saturated $(NH_4)_2SO_4$ in 0.1 M sodium acetate (pH 5.0). The sample from step 1 was applied to the column and the column was washed with 200 ml of the 60% saturated $(NH_4)_2SO_4$/sodium acetate buffer in an attempt to remove non-carbohydrate-containing proteins. The bound material was eluted in an 800-ml, linearly decreasing (60–0%) $(NH_4)_2SO_4$ gradient in 0.1 M sodium acetate (pH 5.0). Fractions of 10 ml each were collected and assayed for carbohydrate by the anthrone method, for protein by UV absorbance at 280 nm, and for invertase activity as described. All of the eluted material was pooled and dialyzed extensively against 10 mM potassium phosphate (pH 7.0).

Step 3: DEAE-cellulose chromatography. The dialyzed sample was applied to a 2.5 × 62-cm DEAE-cellulose (Whatman) column equilibrated with 10 mM potassium phosphate (pH 7.0). After sample application, the column was washed with 500 ml of the phosphate buffer to remove residual neutral polysaccharides not bound to the column and then with 800 ml of 250 mM NaCl in the same buffer to elute the bound glycoproteins. Fractions (10 ml) were collected during the salt wash.

Step 4: Lyophilization. The material in the first of the two peaks to elute in the salt wash of the DEAE-cellulose column was dialyzed extensively against deionized distilled water, sterilized by passing the solution through a 0.2-μm membrane filter (Nalgene, Sybron Co., Rochester, NY), and lyophilized. The material was stored dry at −20°C.

Analytical Methods. Invertase activity was assayed by the procedures of Ziegler and Albersheim (10). The hexose content of fractions was determined by the anthrone assay (16) with D-(+)-mannose (Sigma) as a standard. Protein concentrations were assayed by the method of Lowry *et al.* (17), with bovine serum albumin (Sigma) as a standard. Protein was detected in column fractions by UV absorbance at 280 nm. Neutral and amino sugar compositions were determined by the method of Jones and Albersheim (18) after an initial hydrolysis of the samples with 90% (vol/vol) formic acid at 100°C for 5 hr.

Table 1. Sugar composition* of the carbohydrate portion of the extracellular glycoproteins of Pms races 1, 2, and 3

Race	Mannose	Glucose	Glucosamine
Pms 1			
Exp. 1	63.4	23.3	13.3
Exp. 2	68.8	22.1	9.1
Exp. 3	66.8	21.0	12.2
Pms 2			
Exp. 1	78.4	5.6	16.0
Exp. 2	79.1	8.4	12.5
Pms 3			
Exp. 1	68.6	17.2	14.2
Exp. 2	70.6	17.0	13.4
Exp. 3	71.5	14.5	14.0

* Values are % by weight.

RESULTS

Two sharply defined protein- and carbohydrate-containing peaks elute from the DEAE-cellulose column in the salt wash. The first peak eluted in fractions 17–19 and contained about 25% of the protein, 50% of the carbohydrate, and more than 90% of the invertase activity applied to the column. The second peak eluted in fractions 26–29 and contained about 50% of the protein and 25% of the carbohydrate. We have limited our studies to the first of the two peaks to elute from the DEAE-cellulose column in the salt wash. The yield of the extracellular "glycoproteins" (ECGP) in this first peak is between 20 and 30 mg of lyophylized material per 5 liters of culture fluid.

The ECGP preparations from Pms races 1, 2, and 3 are composed, on an average, of 81.5% protein and 18.5% carbohydrate by weight. The ratio of protein to carbohydrate of numerous ECGP preparations from the same and from different Pms races varied between 4.2 and 4.8 to 1. The carbohydrate component of the ECGP preparations from the three races were quantitatively different in their compositions. Mannose, glucose, and glucosamine were the quantitatively predominant sugars present (Table 1). Galactose was found in trace amounts of all the ECGP preparations. No other sugars could be detected.

The ECGP preparations were tested for their ability to protect seedlings from attack by compatible races of the live fungus. In each combination, the ECGP preparation from an incompatible, but not from a compatible, race of Pms protected seedlings from infection by a compatible race of the pathogen (Tables 2–4). The level of protection afforded by the ECGP preparations from incompatible races was typically in the range of 80–90%. For example, the ECGP preparations from the incompatible races 1 and 2 protected, on average, 87% of the Harasoy 63 seedlings from the compatible race 3 fungus (Table 2). In contrast, only 10% of the race 3 ECGP-treated plants survived. This figure is the same as the number of untreated Harasoy 63 plants that survived inoculation with race 3 fungus in the absence of added ECGP (Table 2). Thus, the race 3 ECGP preparation was ineffective in protecting Harasoy 63 seedlings from race 3 fungus. The lowest amount of ECGP so far tested that protected Harasoy 63 seedlings from race 3 fungus was 20 μg per plant (Table 2).

Similar results were obtained with Amsoy 71 seedlings. This cultivar reacted in the same way as Harasoy 63 to the three Pms races (Tables 2 and 3). The ECGP preparations from races 1 or 2 protected, on average, 82% of the Amsoy 71 seedlings from race 3 fungus, whereas the race 3 ECGP preparation gave no protection (Table 3). Furthermore, the ECGP preparation from a compatible Pms race did not induce susceptibility in Amsoy 71 seedlings to an incompatible Pms race (Table 3).

Table 2. Protection of Harosoy 63 seedlings by race-specific molecules

Pms race	ECPG added from Pms race	Exp.* 1	2	3	4	5	6	7
1	None	100	100	100	100	100	100	100
2	None	100	100	100	100	100	100	100
3	None	0	0	30	10	30	30	0
3	1	100	100	100	70	60	100	100
3	2	90	80	100	70	40	100	100
3	3	0	10	30	0	0	30	0

Seedlings were inoculated with mycelia of the indicated Pms race. Nine- to ten-day-old seedlings were impaled on skewers 1.5 mm in diameter at the midpoint of the hypocotyls. Plugs of about 1 mm in diameter of Pms mycelia were inserted into the hypocotyl wounds. Values given are the percentage of plants surviving 48 hr after inoculation. Each data point is the average of survivors of 10 plants.

* In Exp. 1, each hypocotyl was treated with 20 μg of ECGP; in Exps. 2 and 3, with 50 μg; in Exps. 4, 5, and 6, with 100 μg; and in Exp. 7, with 150 μg. Exps. 3, 4, and 5 were performed double-blind (see text).

The ECGP preparations from the incompatible races 1 or 3 protected, on average, 97% of the Sanga seedlings from the compatible race 2 fungus (Table 4). The ECGP preparation from compatible race 2 failed to protect against the race 2 fungus. Protection of Sanga seedlings from Pms race 2 by the ECGP preparations from races 1 and 3 but not from race 2 can be seen in Fig. 1. Few experiments were conducted with Sanga because the supply of Sanga seed was limited and the quality of the seed was poor.

The ECGP preparations from the three Pms races did not protect Harasoy seedlings from the three Pms races, each of which is compatible with Harasoy (data not presented).

A series of double-blind experiments was performed to eliminate the possibility of bias. The identity of the ECGP preparations was unknown to the researcher who applied the preparations to the hypocotyl wounds. A second person, also unaware of which plants received which ECGP preparation, inoculated the plants with a Pms race. The results were assessed 48 hr later and the identity of the treatments was revealed. In three such experiments, an average of 73% protection against

Table 3. Protection of Amsoy 71 seedlings by race-specific molecules

Pms race	ECPG added from Pms race	Exp.* 1	2	3	4	5	6	7
1	None	100	100	100	100	100	100	100
2	None	100	100	100	100	100	100	100
3	None	0	30	0	10	0	10	0
3	1	90	100	60	100	90	—	—
3	2	80	90	40	90	—	—	—
3	3	0	30	0	30	0	—	—
1	3	—	—	—	—	—	100	100
2	3	—	—	—	—	—	100	100

See legend of Table 2 for experimental details. Values given are the percentage of plants surviving 48 hr after inoculation.

* Each hypocotyl was treated with 100 μg of ECGP. Exp. 2 was performed in a double-blind manner (see text).

Table 4. Protection of Sanga seedlings by race-specific molecules

Pms race	ECGP added from Pms race	Exp.* 1	Exp.* 2
1	None	100	100
2	None	11	20
3	None	100	100
2	1	88	100
2	2	0	0
2	3	100	100

See legend of Table 2 for experimental details. Values given are the percentage of plants surviving 48 hr after inoculation.

* Exp. 1, 50 μg of ECGP was used; Exp. 2, 150 μg was used. Exp. 1, nine plants were used without added ECGP and eight plants with added ECGP.

race 3 fungus on Harasoy 63 seedlings was achieved by application of the ECGP preparation from race 1 or 2. In a single such double-blind experiment on Amsoy 71, 95% of the seedlings were protected against race 3 fungus by the ECGP preparation from race 1 or 2. These figures can be compared, in these double-blind experiments, to only 10% survival of the Harasoy 63 seedlings and 30% survival of the Amsoy 71 seedlings when race 3 fungus was applied in the presence of the ECGP preparation from race 3.

The ECGP preparations from the three Pms races were tested in the hypocotyl assay for their ability to elicit the accumulation of the phytoalexin glyceollin in the four soybean cultivars used in the protection experiments. The concentration range tested, 20–300 μg per plant, included those concentrations effective in the protection experiments. Low levels of glyceollin accumulate in tissues of the four cultivars exposed to ECGP. The amounts of glyceollin that accumulate in hypocotyls in response to ECGP are 0–5% of the amounts of glyceollin in tissues treated with Pms elicitor (11). The levels of glyceollin accumulated in ECGP-treated tissue was independent of the amount of ECGP added and also of the race, compatible or incompatible, from which the ECGP had been prepared.

The ECGP preparation elicited limited glyceollin accumulation (Table 5) in the more sensitive cotyldeon assay (11). The level of phytoalexin accumulation induced was low, 25% or less of elicitor controls, even when the cotyledons were exposed to concentrations of ECGP equivalent to 150 times the total weight or 27.5 times the weight of carbohydrate required by the glucan elicitor to give maximum glyceollin accumulation. Furthermore, as in the hypocotyls, the ECGP preparation exhibited no race-specific elicitor activity in the cotyledons; the ECGP preparations resulted in the same level of phytoalexin accumulation regardless of whether the cotyledons were obtained from a compatible or an incompatible soybean cultivar. The cultivar Wayne was used instead of Sanga because of the poor and unreliable quality of the Sanga cotyledons.

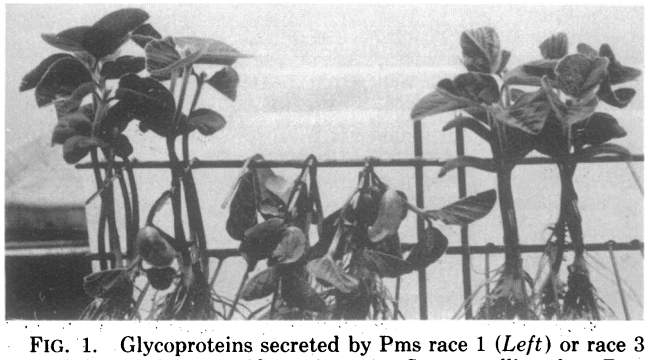

FIG. 1. Glycoproteins secreted by Pms race 1 (*Left*) or race 3 (*Right*), but not by race 2 (*Center*), protect Sanga seedlings from Pms race 2 fungus. Experimental details are given in the text.

Table 5. Ability of ECGP of Pms races to elicit glyceollin accumulation in soybean cotyledons

Sample	Sample applied, mg/ml Total	Carbohydrate†	% of maximum glyceollin content assayed by $A_{285\ nm}$* Wayne	Harosoy	Harosoy 63	Amsoy 71
Pms elicitor	2.0	2.0	100	100	100	100
ECGP from Pms race 1	50	9.3	0	3	0	13
	100	18.5	5	21	17	7
	150	27.8	3	23	16	9
	300	55.5	8	24	17	6
ECGP from Pms race 2	50	9.3	4	2	0	1
	100	18.5	16	17	16	0
	150	27.8	8	14	17	8
	300	55.5	19	14	18	4
ECGP from Pms race 3	50	9.3	0	0	1	10
	100	18.5	8	8	18	15
	150	27.8	8	8	18	9
	300	55.5	14	14	17	6

* Seventy-five microliters was applied to each of 10 cotyledons. Each data point is the average of two such sets of 10 cotyledons. The $A_{285\ nm}$ of the wound droplet is a measure of elicitor activity. The data are expressed as a % of maximum glyceollin content to compensate for differences in varietal response. Maximum $A_{285\ nm}$ values range from 0.20 to 0.28. All values were calculated after correction for the water blanks of 0.011 ± 0.003.

† Micrograms of ECGP carbohydrate was calculated from the fact that glycoproteins are, on average, 18.5% carbohydrate.

DISCUSSION

The results reported in this paper demonstrate race- and cultivar-specific protection against a fungal pathogen of soybean seedlings by a mixture of partially purified glycoproteins secreted by an incompatible race of the fungal pathogen. These results support the theory that gene-for-gene pathogens produce race-specific molecules that trigger a defensive reaction in incompatible, but not in compatible, cultivars of their hosts. Although the nature of the active components in the ECGP preparations responsible for race-specific protection has not been determined, the ECGP preparations do have the characteristics expected of the products of the avirulence genes of gene-for-gene pathogens or, rather, molecules whose synthesis is controlled by avirulence genes. Most significantly, the ECGP from incompatible, but not from compatible, races of Pms protect soybean cultivars from infection by compatible races of the fungus.

The ECGP preparation of an incompatible Pms race will protect a cultivar from infection by a compatible race. The same ECGP preparation will not protect a different cultivar against the compatible race if the race from which the ECGP was prepared is also compatible with the second cultivar. For example, the ECGP preparation from race 2 protects Amsoy 71 and Harasoy 63 against race 3 fungus, but cannot protect Harasoy from this race. Furthermore, the ECGP preparations from compatible races do not cause soybean cultivars resistant to (incompatible with) a Pms race to become susceptible to (compatible with) that race (Table 3). This is consistent with the concept in gene-for-gene systems that compatibility results from the absence of an interaction between an avirulence gene-produced specificity factor of the pathogen and a resistance gene-produced receptor in the plant. A positive interaction between a specificity factor of the pathogen and a resistance gene product of the plant is thought (4) to lead to the triggering of a defensive reaction in the plant and inhibition

of pathogen growth. We suggest that the ECGP preparation from an incompatible race triggers the defensive reaction in the plant.

We can only speculate as to how the specificity factors (ECGP) protect the plant. Perhaps the ECGP decreases the rate of pathogen development and allows inhibitory levels of phytoalexins, low molecular weight antimicrobial compounds (19–23), to accumulate. In the Pms–soybean system, phytoalexin accumulation is stimulated by a β-glucan present in the mycelial walls of the invading fungus (5, 24). Pathogen development may be slowed in incompatible cultivars by hypersensitive death of host tissue (25, 26). Hypersensitive death of invaded host tissue is a defense mechanism commonly associated with incompatibility (resistance) in gene-for-gene host–pathogen systems (3, 26).

The low, nonspecific ability of the ECGP preparations to elicit glyceollin accumulation in soybean tissues (see *Results* and Table 5) cannot account for the race-specific ability of the ECGP preparations to protect soybean seedlings. This fact argues that the mechanism of action of the specificity factors, themselves, is not directed towards elicitation of phytoalexin accumulation. However, this does not preclude a role for phytoalexins in the host's defense against gene-for-gene pathogens.

The ECGP preparations used in the experiments reported here are enriched in glycoproteins. However, because there are no criteria for purity other than the purification techniques used, other types of molecules could be present in the preparations. Thus, it is possible that the specific biological activity of the ECGP preparations obtained from the different Pms races resides in nonglycoprotein contaminants. We believe, however, that the evidence in this and in previous papers (6–8, 10) supports our hypothesis that the biological activity of the ECGP preparations resides in differences in the carbohydrate portions of the ECGP prepared from the races.

Race-specific protection by ECGP preparations (Tables 2–4) was reproducibly obtained only after several variables in the biological assay had been identified and controlled. The health and general vigor of the plant are very important for successful protection experiments. Weak, overcrowded, or etiolated seedlings could not be protected from compatible races of the fungus. It was important to keep the size of the hypocotyl wounds to a minimum and the amount of uninjured tissue surrounding the wound as large as possible by making the wound in the center of the hypocotyl. It was also important to ensure that no further injury to the ECGP-treated tissue occurred during insertion of mycelia into the wound. To minimize such effects, seedlings with hypocotyls with a diameter of less than 2.5 mm were not used in the protection experiments. The amount of inoculum was also important. An amount of mycelia (≈1 mm in diameter) just sufficient to kill untreated compatible seedlings in 2 days was best for the protection experiments.

Another important variable is the completeness with which the cells within the hypocotyl wound were exposed to the ECGP preparation. It seems essential that all wounded cells be coated with the ECGP preparation. Thus, five applications of a dilute solution of ECGP gave more uniform protection than one application of a more concentrated solution. However, fairly good results were obtained even with the single application procedure. An average of 60% protection was achieved in the three experiments in which this procedure was tried. In these cases, Harasoy 63 seedlings were inoculated with race 3 fungus simultaneously with application of the ECGP preparation from race 1.

A certain critical number of undamaged cells adjacent to the ECGP-treated wound area appears to be required if the plant is to be protected, for if a wound is made towards the edge of the hypocotyl and if infection occurs, the infection develops in the tissue adjacent to the wound that contains the fewest healthy cells. Perhaps the surrounding tissue must accumulate toxic levels of phytoalexins.

Our method of inoculating the plants with the pathogen, although convenient, is not natural. The normal vegetative infective organs of Pms are zoospores, not mycelia. We are attempting to develop a system using zoospores as the inoculum. Such a system would have the added advantage that the inoculum could be accurately quantitated.

We have proved the existence and demonstrated the biological activity of the postulated specificity factors in gene-for-gene host–pathogen systems (4, 5). Once the active constituents of the ECGP preparations have been isolated, these molecules should be powerful tools for further elucidation of the biochemistry of gene-for-gene host–pathogen interactions.

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