Involvement of Bacterial Protein Synthesis in Induction of the Hypersensitive Reaction in Tobacco

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ABSTRACT

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Pseudomonas pisi treated with 100 μg ml^{-1} streptomycin or 100 μg ml^{-1} chloramphenicol, which inhibit protein synthesis, did not induce the hypersensitive reaction (HR) in tobacco. In vitro, the bacteria respired at a normal rate for more than 8 hr after antibiotic treatments. Antibiotic-resistant mutants induced the HR in the presence of the antibiotics, indicating that the effect of inhibition of protein synthesis was on the bacteria, not on the plant. Auxotrophic mutants of *P. pisi* with protein synthesis inhibited by amino acid starvation did not induce the HR unless the required amino acid was added.

Additional key words: streptomycin, chloramphenicol, auxotrophs.

The hypersensitive reaction (HR) of plants to bacterial infection occurs in incompatible host-parasite relationships (7). Although generally the HR has been considered a plant defense mechanism (7), some workers interpret it as a consequence, not the cause, of plant disease resistance (4). A form of induced immunity against infection prevented the HR when, prior to inoculation, plants were treated with heat-killed bacteria (9), bacterial cell wall fractions (11, 13), or low numbers of living bacteria (12, 15). In a different manner, the HR was prevented by holding the plant at 36 C or higher temperatures (7), but the HR developed subsequently when the inoculated plants were moved to a lower temperature.

Streptomycin added to the inoculum or applied as a subsequent injection within 3-4 hr of the bacterial inoculation, prevented the HR (6). This effect was attributed to the killing of the bacteria by streptomycin and to the irreversible induction of the HR within 3-4 hr after inoculation. Ercolani (1) found that wild-type *Pseudomonas syringae* van Hall caused a localized necrosis (HR?) in tomato plants, but a glycine-requiring mutant did not cause such necrosis unless glycine was supplied to the bacterium. Klement and Goodman (8) reported that living bacteria were necessary to cause the HR, and that bacteria killed by heat, ultraviolet light, sonication, or streptomycin did not cause the HR when injected into plants.

The research presented here was designed to determine whether or not bacterial protein synthesis is involved in the induction of the HR.

MATERIALS AND METHODS

Chemicals.—Streptomycin sulfate, chloramphenicol {chloromycetin; D(−) threo-2,2-dichloro-N-[B-hydroxy-α-(hydroxymethyl)-p-nitrophenethyl acetamide]}, arginine, and histidine were obtained from Sigma Chemical Co., St. Louis, MO 63178.

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Bacteria.—An isolate of *Pseudomonas pisi* Sackett was obtained from R. N. Goodman, Department of Plant Pathology, University of Missouri. The stock culture was stored under oil. The inocula consisted of bacteria grown at 27 ± 0.5 C for 24 hr on nutrient agar slants. The cells were rinsed from the surface, washed once by centrifugation at 1,700 *g* and adjusted to 1×10^8 cells ml^{-1} by turbity measurements with a spectrophotometer. A tenfold dilution of this suspension was used as the inoculum. A streptomycin resistant (Str^r) *P. pisi* isolate was selected by plating a broth culture of the bacterium onto nutrient agar containing 100 μg ml^{-1} streptomycin. This mutant grew equally well on nutrient agar containing 100 μg ml^{-1} streptomycin as on nutrient agar. A chloramphenicol resistant (Cml^r) isolate was obtained similarly. An auxotroph for histidine (His$^-$) and one for arginine (Arg$^-$) were obtained by ultraviolet irradiation (2) and one-step auxanographic selection (3).

Plants.—Tobacco (*Nicotiana tabacum* L. 'Burley 2') plants were grown in the greenhouse until eight leaves were fully expanded. For 3 days prior to experimentation, the plants were placed in a controlled environment chamber with a 12-hr photoperiod, 25 ± 1 C, 20 ± 6% relative humidity, and light from high-intensity-discharge metal halide lamps at 1,000 ± 40 μeinstein m^{-2}sec^{-1} as measured by a quantum sensor (LI-COR LI-190S, Lambda Instruments, 4421 Superior St., Lincoln, NE 68504). Inoculations were made by the hypodermic syringe method of Klement (5) with bacterial inocula of about 10^7 cells ml^{-1} in all experiments. All treatments were replicated eight times except where noted.

RESULTS

Effects of antibiotics in vitro.—Streptomycin blocked incorporation of ^{14}C-leucine into protein within 5 min

after 100 $\mu g\ ml^{-1}$ streptomycin was added to a 1×10^7 cells ml^{-1} suspension of *P. pisi* (10). The effects of streptomycin on bacterial respiration were measured with a polarographic oxygen electrode. A 3.0-ml sample of a suspension of 1×10^7 cells ml^{-1} of *P. pisi* in minimal medium (glucose, 3 g; $NH_4H_2PO_4$, 0.5 g; KCl, 0.1 g; $MgSO_4{\cdot}7\ H_2O$, 0.2 g; and 1,000 ml H_2O) was placed in each of the four chambers of a constant-temperature stirrer-bath attached to a YSI polarographic oxygen monitor (Model 53, Yellow Springs Instruments Co., Yellow Springs, OH 45387). After establishing baselines for 1 hr, 0.1 ml H_2O was added to each of two chambers, and 0.1 ml of streptomycin solution (to make a final concentration of 100 $\mu g\ ml^{-1}$) was added to the other two. Additions were made with a micrometer syringe and capillary tubing via the air-vent slot. The experiment was performed three times. The O_2 consumption over an 8-hr period was monitored. The rate of respiration of the control bacteria and of the streptomycin-treated bacteria were not significantly different over the 8-hr period. Respiration rates prior to addition of the streptomycin were 0.39 ± 0.06 μliter $O_2\ ml^{-1}hr^{-1}$ for both the treated and the control bacteria. Over the period of the experiment, the respiration rates increased to 0.98 ± 0.08 μliters $O_2\ ml^{-1}hr^{-1}$ for the control and to 0.95 ± 0.11 μliters $O_2\ ml^{-1}hr^{-1}$ for the streptomycin-treated bacteria. Results of respiration studies with 100 $\mu g\ ml^{-1}$ chloramphenicol added to bacterial suspensions were similar to those obtained with streptomycin.

Effect of antibiotics on the HR.—Addition of 100 $\mu g\ ml^{-1}$ of streptomycin or 100 $\mu g\ ml^{-1}$ of chloramphenicol to the inoculum of *P. pisi* completely prevented the HR. Injection of tobacco leaves with Str^r *P. pisi* in 100 $\mu g\ ml^{-1}$ streptomycin, resulted in the HR, as did Cml^r *P. pisi* in 100 $\mu g\ ml^{-1}$ chloramphenicol. Thus, the inhibition of the HR is an effect of the antibiotics on the bacteria and not on the plant.

Auxotrophic bacteria.—Auxotrophic bacteria (His^- or Arg^-) were grown to the log phase in minimal broth to which either 100 $\mu g\ ml^{-1}$ histidine or 100 $\mu g\ ml^{-1}$ arginine, respectively, had been added. The bacteria were harvested by centrifugation, then suspended in minimal broth without added amino acids, and incubated with shaking for 6 hr. This period of incubation allowed for depletion of the internal pools of the amino acids for which the bacteria were auxotrophic. Cells were harvested by centrifugation, and then suspended in water. When injected into tobacco, neither the starved His^- nor the starved Arg^- bacteria induced visible symptoms. Addition of histidine at 100 $\mu g\ ml^{-1}$ to the His^- inoculum resulted in the typical HR (Table 1), as did injection of the amino acid up to 12 hr subsequent to the injection of the bacteria. Similar results were obtained with arginine and the Arg^- auxotroph. When injection of the amino acid followed injection of the bacteria, the time required to induce the HR following the addition of the amino acid was 6 hr, the same as if wild-type bacteria had been injected.

TABLE 1. The effect on the hypersensitive reaction in tobacco when *Pseudomonas pisi* protein synthesis was inhibited by antibiotics or by amino acid starvation

Bacteria[a]	Treatment[b]	HR
P. pisi		+
P. pisi	+ streptomycin	−
P. pisi, Str^r	+ streptomycin	+
P. pisi	+ chloramphenicol	−
P. pisi, Cml^r	+ chloramphenicol	+
P. pisi, Arg^-		−
P. pisi, Arg^-	+ arginine	+
P. pisi, His^-		−
P. pisi, His^-	+ histidine	+

[a]Suspensions were 1×10^7 cells ml^{-1}. Abbreviations: Str^r = streptomycin resistant; Cml^r = chloramphenicol resistant; Arg^- = arginine auxotroph; and His^- = histidine auxotroph.
[b]Antibiotics or amino acids were added at 100 $\mu g\ ml^{-1}$.

DISCUSSION

Inhibition of bacterial protein synthesis by streptomycin or by chloramphenicol totally prevents induction of the HR in tobacco. In vitro studies indicate that the bacteria are apparently still alive and respiring at about the same rate as without the antibiotic treatment. Similarly, the His^- and Arg^- amino acid auxotrophs of *P. pisi* do not cause the HR unless supplemented with the required amino acid. This is in agreement with the results of Ercolani (1) who used other auxotrophic bacteria in a similar manner. Auxotrophs are clearly living bacteria even when deprived of the amino acid for which they have a specific requirement. Although they carry out many other metabolic functions, synthesis of protein would be severely limited under these conditions. The fact that lack of bacterial protein synthesis prevents the HR is strong evidence that this synthesis is necessary to cause this reaction.

Although bacteria do not cause the HR if their protein synthesis is inhibited, it should be noted that peptide synthesis may be the important factor. Bacteria are known to produce many antibiotic peptides (e.g., syringomycin, 14) and their possible involvement in the HR is unknown. Also, I do not intend to suggest that the HR inducer is a protein (or peptide), because the effects of inhibition of bacterial protein synthesis on the HR may be indirect.

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