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Generalized Induction of Defense Responses in Bean Is Not Correlated with the Induction of the Hypersensitive Reaction

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Transcripts for phenylalanine ammonia-lyase, chalcone synthase, chalcone isomerase, and chitinase accumulated in common bean after infiltration with the *Pseudomonas syringae* pv *tabaci* Hrp⁻ mutant Pt11528::Hrp1, even though a hypersensitive reaction did not occur. The temporal pattern of this transcript accumulation was similar to that seen after infiltration with wild-type *P. s. tabaci* Pt11528, which resulted in a hypersensitive reaction. *Escherichia coli* DH5α, *P. fluorescens* Pf101, heat-killed Pt11528 cells, and Pt11528 cells treated with protein synthesis inhibitors also induced accumulation of defense transcripts but not a hypersensitive reaction. In contrast, these transcripts were not detected in plants infiltrated with water or *P. s.* pv *phaseolicola* NPS3121, a compatible pathogen that causes halo blight. Phytoalexins were produced in bean after infiltration with Pt11528, Pt11528::Hrp1, Pt11528 cells treated with neomycin, or Pf101, but not in plants infiltrated with NPS3121 or water. These results suggest that there are unique biochemical events associated with the expression of a hypersensitive reaction which are distinct from other plant defense responses such as phytoalexin biosynthesis. In addition, our results support the hypothesis that there is a general, nonspecific mechanism for the induction of defense transcripts and phytoalexins by pathogenic and saprophytic bacteria that is distinct from the more specific mechanism associated with the induction of the hypersensitive reaction.

INTRODUCTION

Plants respond to pathogen infection by activating certain responses that have been implicated as mechanisms of disease resistance (Bowles, 1990; Dixon and Harrison, 1990; Dixon and Lamb, 1990). These responses include the hypersensitive reaction; the production of phytoalexins, proteinase inhibitors, and hydrolytic enzymes, such as chitinase and glucanase; and deposition of hydroxyproline-rich glycoproteins, lignin, and callose into the plant cell wall. Although there are excellent correlative data supporting the role of these and other inducible responses in disease resistance, mechanistic analyses of their function have been complicated by the fact that they are usually activated coordinately during pathogen attack. Consequently, it has been difficult to determine how each of these responses contributes specifically to disease resistance.

The hypersensitive reaction is an inducible plant response that is considered to be an important component of disease resistance (Klement, 1982). This reaction is a rapid, localized necrosis of plant tissue at the infection site that is believed to limit the multiplication and spread of invading pathogens. The hypersensitive reaction is a response that occurs during resistant (or incompatible) plant–pathogen interactions and cannot be induced by organisms which are not plant pathogens. In this manuscript, the term "incompatible" will be used as an adjective to describe pathogens that induce a hypersensitive response and "compatible" will be used to describe pathogens that cause disease. Inhibitor studies have shown that the induction of the hypersensitive reaction is an active process dependent upon de novo host RNA and protein synthesis (Yoshikawa et al., 1978; Keen et al., 1981). Previous studies have begun to elucidate the physiological and ultrastructural changes that occur in plants during a hypersensitive reaction, but very little is known about the biochemical basis of this response. In addition, it has not been determined if other inducible plant responses, such as phytoalexin synthesis, contribute to the production of a hypersensitive reaction. Therefore, it is not known whether the hypersensitive reaction is the cause or merely a symptom of resistance.

The interactions of plants and incompatible pathovars of *Pseudomonas syringae* provide useful model systems for molecular and biochemical analyses of disease resistance. There are two known classes of genes present in *P. syringae* strains that contribute to their ability to elicit incompatible or resistant responses. The first class is the avirulence (*avr*) genes (Staskawicz et al., 1984; Keen and Staskawicz, 1988; Whalen et al., 1991). Genetic studies have shown that in gene-for-gene systems, a specific resistance gene (R gene) in the plant is paired with a specific *avr* gene in the pathogen, leading to resistance (Flor, 1971; Ellingboe, 1981; Keen, 1990). At this time, very little is known about how the products of *avr* genes interact with the products of R genes.

The second class of genes that enables *P. syringae* strains to elicit incompatible responses is the hypersensitive reaction

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and pathogenicity (*hrp*) genes (Lindgren et al., 1986; Willis et al., 1991). These genes control the ability of *P. syringae* strains to elicit a hypersensitive reaction on nonhost plants and resistant cultivars of susceptible plants, as well as the ability to produce disease symptoms on susceptible host plants. The *hrp* genes are essential for the growth and development of these bacteria within plant hosts but not for growth in vitro. Pathovars with mutations in *hrp* genes do not produce a hypersensitive reaction on nonhost plants or on resistant cultivars of host plants, nor do they produce disease symptoms on their normal host plants. Some pathovars also require a fully functional *hrp* region, in addition to *avr* gene function, to elicit an incompatible response on resistant cultivars (Lindgren et al., 1988; Huynh et al., 1989). As with *avr* genes, very little is known about how the products of *hrp* genes interact with the host plant.

In our laboratory, we conducted analyses of the induction of putative plant defense responses in relation to the development of a hypersensitive reaction during the incompatible interaction between bean and *P. s.* pv *tabaci*. Here we report that transcripts for phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), and chitinase (CHT) accumulated and phytoalexins were synthesized in bean after inoculation with the wild-type or Hrp$^-$ mutants of *P. s. tabaci*, *Escherichia coli*, or the saprophytic bacteria *P. fluorescens* irrespective of the development of a hypersensitive reaction. In addition, we present data that suggest that there are different signal transduction pathways for the activation of the hypersensitive reaction or phytoalexin biosynthesis by bacterial pathogens.

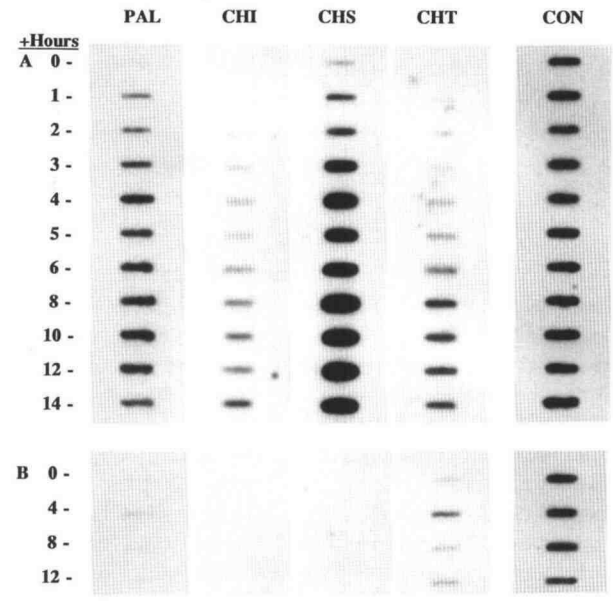

Figure 1. Transcript Accumulation after Infiltration with *P. s. tabaci* Pt11528.

RNA was isolated from bean leaf tissue at various times (0 to 14 hr) after infiltration. Total RNA (5 μg) from each time point was probed with PAL, CHI, CHS, CHT, and H1 (labeled as CON for control).
(A) Infiltrated with Pt11528 at 10^8 cells per mL.
(B) Infiltrated with sterile H_2O.

RESULTS

Defense Transcripts Accumulate in Bean in Response to *P. s. tabaci*

To determine whether putative defense transcripts accumulated in bean during the incompatible interaction with *P. s. tabaci*, bean plants were infiltrated with the wild-type *P. s. tabaci* Pt11528 using a suspension of ~10^8 cells per mL. This inoculum concentration causes a confluent hypersensitive reaction on leaves 12 to 16 hr after infiltration. Total RNA was isolated from leaf tissue harvested at various times after infiltration. Slot blot analyses were conducted with this RNA, using cDNA sequences for PAL, CHS, CHI, or CHT as hybridization probes. As shown in Figure 1A, hybridizable RNA corresponding to all four genes accumulated in bean leaf tissue after infiltration with Pt11528. When the RNA was probed with H1, a constitutively expressed gene, the hybridization signals were of equal intensity for all treatments, indicating that comparable amounts of RNA were analyzed for all treatments. The pattern of RNA accumulation varied for each defense transcript examined; however, in all cases, transcript accumulation occurred rapidly and before the onset of a visible hypersensitive reaction. Transcript levels for each gene remained high up to 14 hr after infiltration. The pattern of mRNA accumulation was essentially the same in all experiments, although there were slight variations from experiment to experiment in the timing of transcript accumulation. We did not attempt to isolate RNA beyond 14 hr after infiltration due to the tissue collapse associated with the hypersensitive reaction.

Transcripts for PAL, CHS, or CHI were not detected in control plants infiltrated with water (Figure 1B). Occasionally, small increases in CHT transcript levels were detected in plants after infiltration with water; however, these were insignificant when compared to the transcript levels observed in plants after infiltration with Pt11528.

Experiments were also conducted with two tabtoxin-minus mutants derived from Pt11528: Pt11528(Δ[*tbl*]1), a spontaneous deletion mutant, and Pt11528*tbl*-9::Tn*5*, a Tn*5* insertion mutant (T. G. Kinscherf and D. K. Willis, personal communication). Transcripts for PAL, CHS, CHI, and CHT accumulated in bean 6 hr after infiltration with either strain (data not shown). Previous studies have shown that tabtoxin is an important virulence factor during the *P. s. tabaci*–tobacco interaction (reviewed in Mitchell, 1984). These data indicate that tabtoxin was not responsible for the transcript accumulation that occurred after infiltration with Pt11528.

Defense Transcripts Accumulate in Bean in Response to *P. s. tabaci* Hrp⁻ Mutants

Analyses of defense transcript accumulation, similar to those described above, were conducted using two Hrp⁻ mutants of *P. s. tabaci.* As shown in Figure 2, Pt11528::Hrp1 and Pt11528::Hrp12 do not induce a hypersensitive reaction on bean, which is in distinct contrast to Pt11528, the wild-type strain. Figure 3 illustrates PAL, CHS, CHI, and CHT transcript accumulation in bean after infiltration with Pt11528::Hrp1 (using an inoculum of 10^8 cells per mL), even though a hypersensitive reaction did not occur. The temporal pattern of this transcript accumulation closely paralleled that detected after infiltration with Pt11528. We were able to measure RNA levels for 5 days (i.e., 120 hr) after infiltration because a hypersensitive reaction did not occur in response to Pt11528::Hrp1. Significant levels of CHI and CHS transcript were detected up to 14 and 48 hr after infiltration, respectively, while the PAL and CHT transcripts were evident up to 120 hr after infiltration. A second Hrp⁻ mutant, Pt11528::Hrp12, also induced all four transcripts in bean 8 hr after infiltration (data not shown).

Metabolically Inactive Pt11528 Elicits Transcript Accumulation

Previous studies have shown that bacterial plant pathogens must be living and metabolically active to elicit hypersensitive reactions on incompatible plant hosts (Klement, 1982). For instance, heat-killed bacteria will not induce a hypersensitive reaction (Lyon and Wood, 1976). In addition, the development

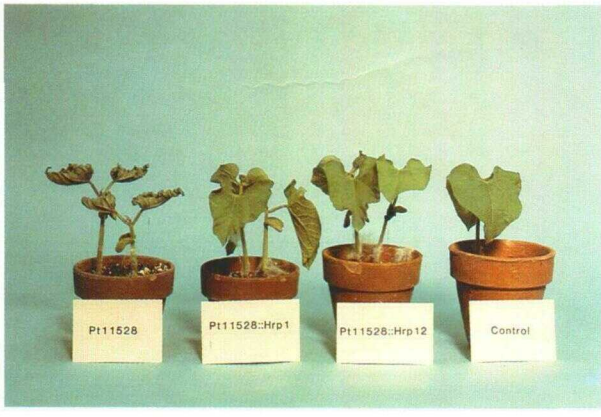

Figure 2. Phenotypes of Bean after Infiltration with Wild Type and Hrp⁻ Mutants.

Bean plants 24 hr after infiltration with *P. s. tabaci* Pt11528, and the Hrp⁻ mutants Pt11528::Hrp1 and Pt11528::Hrp12, and H_2O as a control. Plants were infiltrated by vacuum using an inoculum of 10^8 cells per mL.

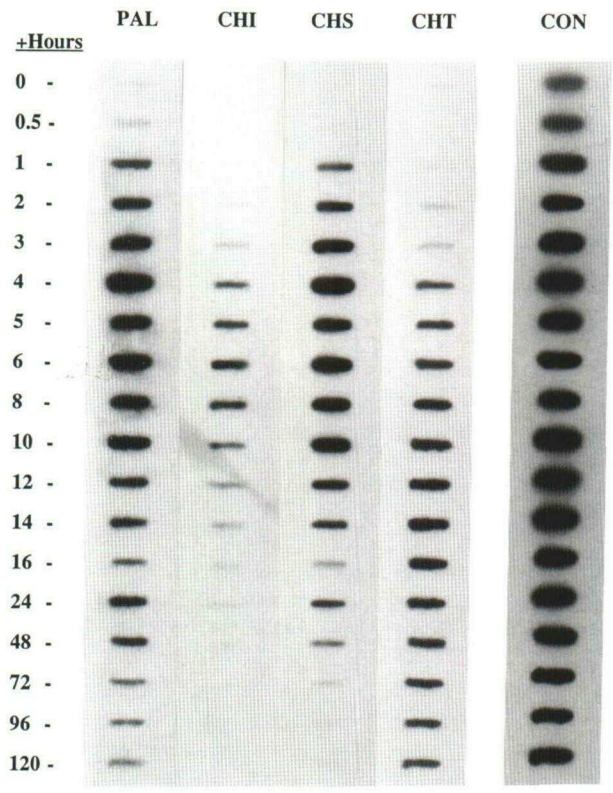

Figure 3. Transcript Accumulation after Infiltration with the Hrp⁻ Mutant Pt11528::Hrp1.

RNA was isolated from bean leaf tissue at various times (0 to 120 hr) after infiltration with 10^8 cells per mL of Pt11528::Hrp1. Total RNA (5 μg) from each time point was probed with PAL, CHI, CHS, CHT, and H1 (labeled as CON for control).

of a hypersensitive reaction is dependent upon de novo bacterial protein synthesis (Sasser, 1978, 1982). When bean was infiltrated with either heat-killed Pt11528 cells or Pt11528 cells that had been treated with the prokaryotic protein synthesis inhibitors streptomycin, neomycin, or kanamycin, a hypersensitive reaction did not occur. However, PAL, CHS, CHI, and CHT transcripts accumulated, as shown in Figure 4.

Defense Transcripts Accumulate in Response to Nonpathogenic Bacteria but Not to a Compatible Bacterium

Previously, it has been shown that saprophytic microorganisms will not elicit hypersensitive reactions on plants (Klement, 1982). *E. coli* DH5α and *P. fluorescens* Pf101 do not induce a hypersensitive reaction in our system. Slot blot analysis of total RNA isolated from bean after inoculation with these bacteria revealed that transcripts for PAL, CHS, CHI, and CHT

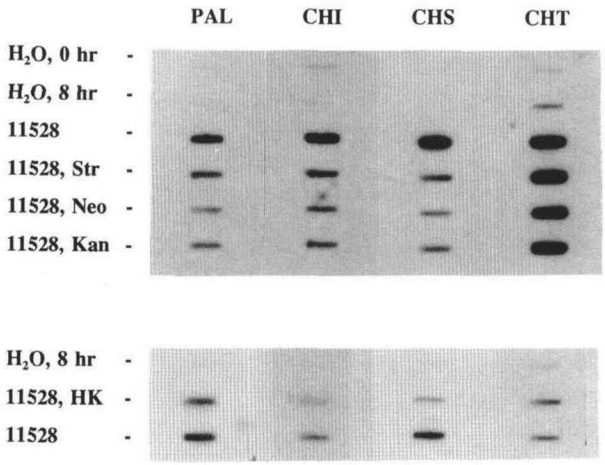

Figure 4. Transcript Accumulation in Response to Infiltration with Heat-Killed or Antibiotic-Treated *P. s. tabaci* Pt11528.

RNA was isolated from bean leaf tissue 8 hr after infiltration with Pt11528 (10^8 cells per mL) treated with streptomycin (Str), neomycin (Neo), or kanamycin (Kan), or heat-killed Pt11528 cells (HK). Total RNA (5 μg) from each treatment was probed with PAL, CHI, CHS, and CHT.

accumulated in bean 8 hr after inoculation with either bacteria (data not shown).

Similar experiments were also conducted with two compatible bacterial strains: *P. s.* pv *phaseolicola* NPS3121, the causal agent of halo blight of bean, and *P. s.* pv *syringae* 4076Br, the causal agent of brown spot. Slot blot experiments using RNA isolated 8 hr after infiltration revealed that PAL, CHS, CHI, and CHT transcripts did not accumulate in bean after infiltration with NPS3121; however, all four transcripts accumulated after infiltration with 4076Br (data not shown).

Phytoalexins Are Produced when PAL, CHS, and CHI Transcripts Accumulate

PAL, CHS, and CHI are key enzymes in the phytoalexin biosynthetic pathway. Therefore, we wanted to determine if phytoalexins were synthesized in those interactions described above when the corresponding transcripts accumulated. Ethyl acetate soluble fractions of ethanol extracts were prepared from leaf tissue (Keen, 1978) after infiltration with different bacterial treatments. The thin-layer chromatography (TLC)–*Cladosporium* bioassay was used to detect the presence of phytoalexins in these extracts (Keen et al., 1971). For our analysis, the area of the zones of spore inhibition on the TLC plates was calculated and used as a measure of phytoalexin production. As depicted in Figure 5, phytoalexins were detected in leaf tissue 8 hr after infiltration with Pt11528, Pt11528::Hrp1, Pt11528 cells treated with neomycin, and Pf101. Interestingly, the levels of phytoalexins produced in response to Pt11528 or Pf101 were not significantly different. In contrast, the levels of phytoalexins produced 8 hr after infiltration with Pt11528::Hrp1 and Pt11528 treated with neomycin were ~68 and 37%, respectively, of that produced after infiltration with Pt11528. Low levels of phytoalexins were also detected in plants after infiltration with NPS3121 or water; however, these amounts were not significantly different from the levels detected in control plants (i.e., plants harvested immediately after infiltration with H_2O). Phytoalexins were also detected in plants infiltrated with DH5α, heat-killed 11528 cells, or 11528 cells treated with streptomycin or kanamycin (data not shown).

Preliminary experiments indicated that one of the phytoalexins produced during the above plant–bacterium interactions was kievitone, based upon R_F value and UV adsorption spectra as compared with a kievitone standard (data not shown). We therefore concluded that phytoalexins were synthesized during the interactions where PAL, CHS, and CHI transcripts accumulated. Thus, it would appear that the genes encoding PAL, CHS, and CHI are not only transcribed during these interactions, but are also translated.

DISCUSSION

We have demonstrated that transcripts for PAL, CHS, CHI, and CHT accumulated and phytoalexins were produced in bean after infiltration with a wild-type isolate of the incompatible, hypersensitive reaction–inducing bacterium *P. s. tabaci*. Our results with *P. s. tabaci* are consistent with previous studies showing that incompatible bacteria induce the accumulation

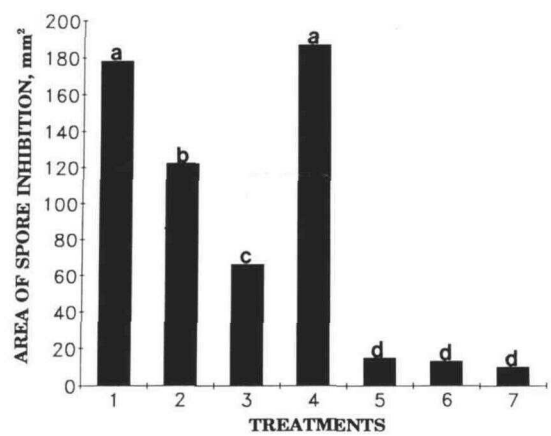

Figure 5. Accumulation of Phytoalexins in Bean after Infiltration with Bacteria.

Extracts were prepared from bean leaves 8 hr after infiltration and analyzed for phytoalexins using TLC and a *Cladosporium* bioassay, as described in Methods. Results are represented as mean values from two replicate experiments. Means designated with the same letter are not significantly different (P = 0.05) according to the LSD Test. Bar 1, Pt11528; bar 2, Pt11528::Hrp1; bar 3, Pt11528 treated with neomycin; bar 4, Pf101; bar 5, NPS3121; bar 6, H_20 control (i.e., harvested immediately after infiltration); bar 7, H_20 (at 8 hr after infiltration).

of putative defense transcripts and the production of phytoalexins on nonhost plants as well as on resistant cultivars of susceptible hosts (Gnanamanickam and Patil, 1977; Slusarenko and Longland, 1986; Voisey and Slusarenko, 1989; Godiard et al., 1990; Marco et al., 1990; Davis et al., 1991; Dong et al., 1991; Pautot et al., 1991; Yang et al., 1991). The slight variation, observed in this study, in the timing of transcript accumulation from experiment to experiment most likely reflects the fact that incompatible interactions are complex biological processes which are easily influenced by many physiological and environmental factors. Similar variations in timing of defense transcript accumulation were documented in a previous study (Pautot et al., 1991).

We have further demonstrated that *P. s. tabaci* Hrp$^-$ mutants, which do not induce the hypersensitive reaction on bean, induced the accumulation of the transcripts examined and phytoalexin production. The temporal pattern of transcript accumulation seen after infiltration with the Hrp$^-$ mutant Pt11528::Hrp1 was similar to that observed after infiltration with the wild-type strain. Thus, the induction of the transcript accumulation and phytoalexin production that we observed was not dependent upon a fully functional set of *hrp* genes in *P. s. tabaci.* Preliminary experiments have revealed that defense transcripts also accumulated in bean 6 hr after infiltration with two different Hrp$^-$ mutants of *P. s.* pv *glycinea* (data not shown). Therefore, the ability to induce these defense transcripts on nonhost plants may be a common characteristic of Hrp$^-$ mutants. The *hrp* genes are contained in large gene clusters in phytopathogenic bacteria and very little is known about the precise function of each *hrp* locus; therefore, it is difficult to predict the effect of specific Hrp$^-$ mutations on plant gene expression.

Interestingly, transcript accumulation and phytoalexin production also occurred in bean after infiltration with the nonpathogenic bacteria *P. fluorescens* Pf101 and *E. coli* DH5α. The fact that transcript accumulation and phytoalexin production are induced by *E. coli* and *P. fluorescens* is especially significant and suggests that these defense responses are general responses to bacteria, rather than specific responses to incompatible pathogens. Further experiments have revealed that Pf101 will induce the accumulation of transcripts for PAL and CHS on the bean cultivar Red Mexican, indicating that the response is not cultivar specific (data not shown). Nonpathogenic bacteria will not elicit hypersensitive reactions on plants; consequently, there have been few studies focusing upon defense gene activation by nonpathogens (Bowles, 1990; Dixon and Harrison, 1990; Dixon and Lamb, 1990). Our results indicate that nonpathogenic bacteria produce signals that are involved with the elicitation of defense transcript accumulation and phytoalexin biosynthesis on bean. The nature of these signals has not been determined, and it is not known whether these are the same signals produced by the incompatible *P. s. tabaci* Pt11528.

Phytoalexins were produced during those interactions in which transcripts for PAL, CHS, and CHI also accumulated. The fact that *P. fluorescens* induced comparable amounts of phytoalexins to wild-type *P. s. tabaci* suggests that phytoalexins are not sufficient for the production of a hypersensitive reaction in bean. Less phytoalexin was produced in response to Pt11528::Hrp1 than to Pt11528, probably because Hrp$^-$ mutants do not grow within host plants to the same extent as wild-type strains (Willis et al., 1991).

Experiments with protein synthesis inhibitors and heat-killed Pt11528 cells suggest that the bacterial compound(s) that functions to elicit transcript accumulation and phytoalexin production is constitutively expressed in Pt11528 and not destroyed by heat treatment. These observations are in contrast to the requirement for bacterial de novo protein synthesis prior to a hypersensitive reaction.

We did not detect transcripts for PAL, CHS, CHI, or CHT in H_20 controls and found no significant difference in the amount of phytoalexin produced at 0 or 8 hr after infiltration with H_20. Therefore, it would appear that the process of vacuum infiltration does not wound bean tissue or induce defense responses. These observation are significant because wounding may induce these responses (Lawton and Lamb, 1987; Mehdy and Lamb, 1987; Hedrick et al., 1988).

Hrp$^-$ mutants of *P. s. tabaci* provide a novel method to separate the hypersensitive reaction from the other coordinately expressed responses that are activated in bean during an incompatible response. Our data support the hypothesis that there is a general mechanism for the induction of defense transcripts and phytoalexin synthesis which is distinct from the more specific mechanism associated with induction of the hypersensitive reaction. Experiments are currently underway in our laboratory to identify genes that are specifically expressed during the hypersensitive reaction; cDNA libraries have been constructed using mRNA isolated from bean tissue after inoculation with *P. s. tabaci* Pt11528, and these libraries are being differentially screened with cDNA probes complementary to mRNA isolated from bean plants after inoculation with Pt11528 or Pt11528::Hrp1. Such studies should allow us to identify the unique biochemical events associated with the hypersensitive reaction.

P. s. phaseolicola was the only bacterium that we identified during our studies which did not activate defense responses of bean. We have completed additional experiments indicating that this bacterium does not induce PAL, CHS, or CHI transcript accumulation or phytoalexin production in bean up to 120 hr after infiltration, possibly due to the action of a suppressor of these defense responses (Jakobek et al., 1993).

METHODS

Bacterial Strains and Growth Conditions

Pseudomonas syringae pv *tabaci* Pt11528 and *P. s.* pv *phaseolicola* NPS3121 were kindly provided by N. J. Panopoulos (University of California, Berkeley); *P. s.* pv *syringae* 4076Br was kindly provided by S. Hirano (University of Wisconsin, Madison). The *hrp* region of *P. s. tabaci* shares considerable sequence homology with the *hrp* region of *P. s. phaseolicola*

(Lindgren et al., 1988). Therefore, transposon insertions within the *hrp* gene cluster of *P. s. phaseolicola* can be transferred into the genome of *P. s. tabaci* by marker exchange mutagenesis (Lindgren et al., 1988). This method was used to generate two Hrp$^-$ mutants of *P. s. tabaci* Pt11528 that were used in our studies: Pt11528::Hrp1 was generated by marker exchange mutagenesis using plasmid pPL1, as described previously (Lindgren et al., 1988), and presumably contains an insertion in a gene homologous to *P. s. phaseolicola hrpA*. Pt11528::Hrp12 was generated in a similar fashion using the plasmid pPL11-Tn*3*Spice 12 (Tn*3*Spice is a derivative of Tn*3* carrying a promoterless *P. syringae* ice nucleation gene; Lindgren et al., 1989) and presumably contains an insertion in a gene homologous to *P. s. phaseolicola hrpD*. (See Rahme et al., 1991, for a detailed genetic map of the *hrp* region of *P. s. phaseolicola*.) It is not known how *hrpA* or *hrpD* functions in the elicitation of hypersensitive reactions or pathogenicity. *P. fluorescens* Pf101 was kindly provided by A. G. Wollum (North Carolina State University, Raleigh). *Pseudomonas* strains were cultured at 28°C in King's medium B (King et al., 1954). *Escherichia coli* DH5α (Bethesda Research Laboratories) was cultured at 37°C in Luria-Bertani medium (Sambrook et al., 1989). Bacto agar (Difco) at 1.5% (w/v) was added to media for plate cultures. Antibiotics (Sigma) were used for selection at the following concentrations (μg/mL): rifampicin, 100; tetracycline, 15 or 20; kanamycin, 20; and spectinomycin, 20.

Growth and Inoculation of Plants

Phaseolus vulgaris cv Red Kidney was grown from seed in clay pots in Metro-Mix 220 planting mix in a greenhouse. Approximately 16 to 18 hr prior to inoculation, plants were moved to a growth chamber and incubated at 22°C with a 14-hr light/10-hr dark cycle.

Bacterial strains were grown overnight, pelleted by centrifugation, and washed in sterile distilled H_2O. Cell suspensions were adjusted to ~10^8 colony-forming units per mL with sterile distilled H_2O. Standard plate count procedures were used to verify inoculum concentrations. Primary leaves of 6- to 8-day-old Red Kidney plants were inoculated using vacuum infiltration (Young, 1974). Infiltrated plants were then returned to the growth chamber, and leaf tissue, for RNA or phytoalexin isolation, was harvested periodically. Leaf tissue for RNA isolation was immediately frozen in liquid N_2 and stored at −80°C. A hypersensitive reaction developed within 12 to 16 hr after infiltration with *P. s. tabaci* Pt11528; no hypersensitive reaction or any other visible response occurred after infiltration with Pt11528::Hrp1, Pt11528::Hrp12, Pf101, or DH5α. Bean leaves became necrotic between 24 and 48 hr after infiltration using an inoculum concentration of 10^8 cells per mL of *P. s. phaseolicola* NPS3121 or *P. s. syringae* 4076Br.

To test the effects of bacterial protein synthesis inhibitors on transcript accumulation and phytoalexin production, cultures of Pt11528 that had been grown overnight in King's medium B with rifampicin selection were harvested, washed with sterile distilled H_2O, and resuspended at a concentration of ~10^9 colony-forming units per mL in fresh King's B supplemented with 1 mg/mL streptomycin, neomycin, or kanamycin. The cells were incubated with shaking at 28°C for 1 hr, pelleted, washed, and resuspended in sterile water and infiltrated into plants, as described above. Pt11528 cells were heat killed by incubating a suspension of 10^8 cells per mL for 1 hr at 60°C. After antibiotic or heat treatment, cells were plated for viable counts; no colonies appeared for any treatments.

To decrease error due to plant-to-plant variation, four to six bean plants were used for each experimental time point or treatment. All experiments were repeated at least twice. Plants inoculated with water were included in all experiments as controls.

RNA Isolation and Blot Analysis

Total RNA was isolated from bean leaf tissue using the method of Wadsworth et al. (1988). This RNA was analyzed by established slot blot procedures (Sambrook et al., 1989; Ausubel et al., 1990); 5-μg samples were applied to a Nitroplus 2000 hybridization membrane (MSI, Westboro, MA) with a Schleicher & Schuell Minifold II slot blot apparatus. The filters were prehybridized and hybridized in 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 50% formamide, and 100 μg/mL salmon sperm DNA at 42°C. Hybridization probes included the 1.7-kb PstI fragment from pPAL5 (cDNA clone for bean phenylalanine ammonia-lyase [PAL]) (Edwards et al., 1985), the 1.4-kb EcoRI fragment from pCHS1 (cDNA clone for bean chalcone synthase [CHS]) (Ryder et al., 1984), the 865-bp EcoRI fragment from pCHI1 (cDNA clone for bean chalcone isomerase[CHI]) (Mehdy and Lamb, 1987), and the 650-bp BamHI-KpnI fragment from pCHT12 (cDNA clone for bean chitinase [CHT]) (Hedrick et al., 1988). These fragments were labeled with α-^{32}P-dCTP by random hexamer labeling (Feinberg and Vogelstein, 1983) and were added to the prehybridization buffer at a final concentration of 10^6 cpm per mL. The filters were hybridized for 18 hr and then washed at room temperature for 10 min in 2 × SSC, 0.1% SDS followed by a 30-min wash at 42°C in 0.1 × SSC, 0.1% SDS. Filters were exposed to Kodak XAR5 film for 24 hr with one Cronex Lightening Plus intensifying screen (Du Pont) at −80°C. All blots were subsequently hybridized with a bean cDNA clone, designated H1, that is complementary to a gene with an unknown function that is constitutively expressed (Lawton and Lamb, 1987). These control hybridizations verified that equal amounts of all RNA samples were loaded onto the hybridization membranes.

Phytoalexin Isolation and Analysis

Phytoalexins were isolated using the technique of facilitated diffusion (Keen, 1978). Briefly, leaves were removed from plants 8 hr after infiltration with various bacterial treatments, infiltrated with 40% ethanol, and incubated at room temperature on a platform shaker for 2 hr. The ethanol fractions were extracted with ethyl acetate, and the ethyl acetate fractions were dried using a rotary evaporator. The resulting samples were resuspended in ethyl acetate spotted onto silica gel 60A K6F thin-layer chromatography (TLC) plates (Whatman) and developed with acetone/chloroform (3:2, v/v). After air drying, the TLC plates were directly bioassayed for antifungal activity against *Cladosporium cucumerinum*, as described previously (Keen et al., 1971).

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