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Molecular Analysis of Plant Defense Responses to Plant Pathogens[1]

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Abstract: A number of inducible plant responses are believed to contribute to disease resistance. These responses include the hypersensitive reaction, phytoalexin synthesis, and the production of chitinase, glucanase, and hydroxyproline-rich glycoproteins. Because of the coordinate induction of these responses, it has been difficult to determine whether they are functional defense responses, and if they are, how they specifically contribute to disease resistance. Recent developments in molecular biology have provided experimental techniques that will reveal the specific contribution of each response to disease resistance. In this paper, we describe a strategy to determine if the hypersensitive reaction is a functional plant defense mechanism.

Key words: chalcone isomerase, chalcone synthase, chitinase, *hrp* genes, hypersensitive reaction, nematode, phenylalanine ammonia-lyase, phytoalexin, *Pseudomonas syringae*, resistance.

Plants respond to invasion by incompatible pathogens with the activation of a number of inducible responses that have been implicated as being mechanisms of disease resistance (3,6,29). These responses include the hypersensitive reaction, production of phytoalexins, hydrolytic enzymes such as chitinase and glucanase, pathogenesis-related proteins, peroxidases and proteinase inhibitors, and the deposition of callose, lignin and hydroxyproline-rich glycoproteins into the plant cell wall. Previous studies have shown that these responses are induced in plants after infection by incompatible pathogens and (or) in cell culture by elicitor obtained from incompatible pathogens. Gene transcripts and biosynthetic end products associated with these responses also accumulate at or near the infection site (3,6,29). Although there is excellent correlative evidence suggesting that these inducible responses have an active role in disease resistance, a causal relationship between their production and resistance has not been established. It should be pointed out, therefore, that the lack of pathogen adaptation to a plant host may be an important factor in determining whether disease will occur, rather than the induction of a specific plant response.

A major factor that has limited understanding of disease resistance has been the inability to determine the specific roles for each of these putative defense responses in resistance. Because defense responses are usually coordinately activated by pathogens or elicitors (6,29), it has been very difficult to design experiments that address the specific contribution of each component to disease resistance. Recent advances in molecular biology and genetics have provided new techniques and experimental strategies that will help researchers determine the specific function of each of these inducible responses in resistance. Techniques such as gene tagging via plant transposable elements (15), PCR-cDNA cloning procedures (1), genomic subtraction technologies (46), RAPD-PCR analysis (54), and anti-sense RNA strategies (5) provide powerful and sensitive methods to identify novel plant genes encoding products necessary for disease resistance. In addition, these techniques should also be useful for functional analyses of specific plant defense responses. The purposes of this review are to describe our molecular analyses of the hypersensitive reaction, a putative plant defense mechanism, and to relate our studies to the analysis of plant–nematode interactions.

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Plant–Nematode Interactions

Molecular studies of plant disease resistance have centered on fungal, viral, and bacterial systems (3,6,29). Therefore, very little is known about the molecular basis of host resistance to plant-parasitic nematodes (11,19,20,52). Studies of plant–nematode interactions have been hampered by the lack of not only general experimental techniques but also specific methods for genetic manipulation of the obligate nematode parasites. Consequently, most of the information concerning resistance to nematodes is descriptive and even speculative, and there is little functional or mechanistic data available that establishes the basis of resistance. A thorough review of the putative mechanisms by which plants defend themselves against nematode infection is beyond the scope of this paper; the reader is referred to other reviews for detailed information (11,19,20, 52). An examination of this literature indicates that molecular studies would be very useful in characterizing plant responses to nematode infection, particularly resistance mechanisms.

The hypersensitive reaction: A number of inducible responses have been implicated as plant resistance mechanisms against nematodes, including phytoalexin biosynthesis (17,50), the deposition of lignin as a structural barrier (11), and the hypersensitive reaction (19,20). Most pertinent to our discussion is the hypersensitive reaction, a rapid localized necrosis of plant tissue at the site of infection, which is believed to limit the multiplication and spread of invading bacteria or fungi (25,27). A significant feature of the hypersensitive reaction is that it is an active process dependent upon de novo host RNA and protein synthesis (23,47,57). It is a specific response to incompatible pathogens and cannot be elicited by saprophytic organisms or abiotic stress. The hypersensitive reaction can be induced by incompatible fungi (25), bacteria (27), viruses (10), or nematodes on resistant cultivars of host plants or nonhost species. In the case of plant-parasitic nematodes, the hypersensitive reaction may be associated with immobilization of the nematode or inhibition of nematode development (52). The hypersensitive reaction occurs during the expression of resistance in the following plant–nematode combinations: various species of *Meloidogyne* and tobacco (44); *M. incognita* and tomato (38), cowpea (51), and soybean (21); *Heterodera glycines* and soybean (8); *H. schachtii* and sugar beet (58); and *Tylenchulus semipenetrans* and citrus (49). Although the hypersensitive reaction may be an important component of host resistance to plant-parasitic nematodes and other plant pathogens, in no case has this reaction been causally linked to resistance (19,25,27).

Analysis of the Hypersensitive Reaction

As a first step towards a functional analysis of the hypersensitive reaction, we were interested in developing a method to separate the hypersensitive reaction from the other putative defense responses that are activated during an incompatible (or resistant) disease interaction. We chose an experimental system utilizing the phytopathogenic bacterium *Pseudomonas syringae,* because pathovars of this bacterium are host-specific, causing disease on distinct plant species (9). Consequently, each pathovar produces a characteristic response on host and nonhost plants. Susceptible responses vary depending on the specific plant–pathovar interaction (9); however, resistant responses are usually characterized by a hypersensitive reaction (9,27). Our studies of the hypersensitive reaction have focused on the interaction between common bean (*Phaseolus vulgaris*) and *Pseudomonas syringae* pv. *tabaci.* This bacterium is the causal agent of wildfire disease of tobacco but induces a hypersensitive reaction on bean.

P. syringae Hrp genes: Especially important for the development of our system was the inclusion of Hrp (hypersensitive reaction and pathogenicity) mutants of *P.*

s. pv. *tabaci* in our experiments (31,32,56). The *hrp* genes control the ability of *P. syringae* strains to elicit the hypersensitive response on nonhost plants and the ability to produce disease symptoms on susceptible host plants. These genes are essential for the growth and development of *P. syringae* pathovars in plants but are not required for growth in vitro. Thus, *P. s.* pv. *tabaci* strains with mutations in *hrp* genes do not produce a hypersensitive response, nor any other visible symptom on bean; these mutants are also nonpathogenic on their normal host, tobacco.

Much progress has been made towards understanding the molecular genetic organization of *hrp* genes (16,39,56); however, very little is known about the function of *hrp* genes in relation to eliciting a hypersensitive response or causing disease. In addition, it is also not known if, or how, the products of *hrp* genes specifically interact with the plant host. The best understood *hrp* gene is *hrpS* from *P. s.* pv. *phaseolicola;* this locus has been sequenced and the predicted amino acid sequence of the protein product shows much similarity to several procaryotic regulatory proteins (12).

RNA blot analysis: We have been studying the activation of four plant genes in bean after inoculation with various bacterial strains. These four genes encode proteins that could have important roles in plant disease resistance (6,29); they include genes for phenylalanine ammonia-lyase (PAL, the first enzyme of the pathway for lignin and isoflavonoid phytoalexin synthesis), chalcone synthase and chalcone isomerase (CHS and CHI, the first two enzymes of a branch pathway specific for flavonoids and isoflavonoid phytoalexins) and chitinase. Although each of these genes can be induced by biological stress, including wounding, treatment with heavy metals, and UV damage, as well as infection by pathogens, there is no direct evidence proving that PAL, CHS, CHI, or chitinase are involved with resistance to incompatible pathogens. There is, however, considerable correlative data to suggest a role in disease resistance; therefore, we will refer to the corresponding four genes as putative defense genes throughout the remainder of this article.

We first studied the interaction between bean and wild-type *P. s.* pv. *tabaci* strain Pt11528. Bean plants (cv. Red Kidney) were inoculated by vacuum infiltration with a cell suspension of approximately 10^8 Pt11528 cells/ml; this inoculum level results in a visible, confluent hypersensitive response on inoculated leaves. Total RNA was isolated from leaves at various times postinfiltration using a small-scale procedure (53). Slot-blot analyses were conducted with this RNA (42), using cDNA sequences for PAL (7), CHS (41), CHI (35), or chitinase (14) as hybridization probes. Hybridizable RNAs corresponding to PAL, CHS, CHI, and chitinase accumulated in bean leaf tissue after inoculation with Pt11528 (Table 1). The pattern of steady-state RNA accumulation varied for each gene but in all cases occurred rapidly and before the onset of a visible hypersensitive response. Transcript levels for each gene remained high until 14 hours after inoculation. In our system, hypersensitive cell collapse usually occurs 12 to 16 hours postinfiltration; therefore, RNA was not isolated beyond 14 hours. Bean plants infiltrated with water as a control had no change in steady-state RNA levels of the genes examined (Table 1).

Putative defense gene transcripts also accumulated in bean after inoculation with *P. s.* pv. *tabaci* Hrp mutants. Slot-blot analyses, similar to the experiments described above, were conducted with RNA isolated from bean leaves after inoculation with the Hrp mutant Pt11528::Hrp1, using a suspension of approximately 10^8 cells/ml. Pt11528::Hrp1 is a Tn*5*-insertion mutant that does not cause disease on tobacco or elicit a hypersensitive response on bean. Transcripts for PAL, CHS, CHI, and chitinase accumulated in bean after inoculation with Pt11528::Hrp1, even though a hypersensitive reaction did not occur (Table 1). The temporal pattern of this transcript accumulation is very similar to that observed after inoculation with the wild-type

TABLE 1. Summary of defense responses activated in bean (*Phaseolus vulgaris* cv. Red Kidney) after infiltration with *Pseudomonas syringae* pv. *tabaci* and other bacteria.

Treatment	Hypersensitive reaction†	Transcript accumulation‡	Phytoalexin production§
Pt11528	+	+	+
Pt11528::Hrp1	−	+	+
Pt11528::Hrp12	−	+	+
Pseudomonas fluorescens Pf101	−	+	+
Escherichia coli DH5α	−	+	+
P. s. pv. *phaseolicola NPS3121*	−	−	−
Control‖	−	−	−

+ indicates that the named response occurred; − indicates that the response did not occur.
† Determined approximately 12 hours after infiltration.
‡ Accumulation of transcripts for phenylalanine ammonia-lyase, chalcone synthase, chalcone isomerase, and chitinase determined by RNA blot analyses.
§ Measured by TLC bioassay against *Cladosporium cucumerinum.*
‖ Control plants were infiltrated with water.

strain. Because a hypersensitive reaction does not occur after inoculation with Pt11528::Hrp1, we were able to measure RNA levels for long periods of time postinfiltration. We found that significant levels of PAL and chitinase RNA were evident up to 120 hours postinfiltration. Pt11528::Hrp12, a second Hrp mutant of *P. s.* pv. *tabaci,* also caused transcripts for PAL, CHS, CHI, and chitinase to accumulate in bean by 8 hours postinfiltration (Table 1).

Analysis of phytoalexin biosynthesis: PAL, CHS, and CHI are key enzymes in the phytoalexin pathway. Because transcripts for these enzymes accumulated in bean after inoculation with Pt11528 or Pt11528::Hrp1, we were interested in determining if phytoalexins were also produced in plants after inoculation with either of these bacteria. Bean plants were infiltrated with Pt11528, Pt11528::Hrp1, or water; and crude phytoalexin extracts were isolated 8 hours after infiltration (22). The resulting crude extracts were separated by thin-layer chromatography (TLC), and the TLC plates were directly bioassayed for antifungal activity against *Cladosporium cucumerinum* (24). Briefly, the TLC plates were sprayed with spores of *C. cucumerinum;* inhibitory zones appeared on plates as white zones on a dark background, where inhibition of spore germination and fungal growth occurred. Phytoalexins were produced in bean leaves after inoculation with either of these bacteria but not in leaves infiltrated with water (Table 1). These data suggest that the genes encoding PAL, CHS, and CHI are not only transcribed but are also translated in bean after inoculation with either Pt11528 or Pt11528::Hrp1, irrespective of the development of a hypersensitive reaction.

We have completed additional experiments that further characterize the activation of these putative defense responses. Previously, it had been shown that the development of a hypersensitive reaction is dependent upon de novo protein synthesis by the bacterial pathogen (27,43). When we infiltrated bean plants with Pt11528 cells treated with procaryotic protein synthesis inhibitors (e.g., streptomycin, neomycin, or kanamycin), the hypersensitive reaction did not occur. However, PAL, CHI, CHS, and chitinase transcripts and phytoalexin were still detected. Other studies have shown that bacterial phytopathogens must be living and metabolically active in order to elicit a hypersensitive reaction (27). In our research, heat-killed Pt11528 cells did not elicit a hypersensitive reaction in bean but surprisingly did induce production of all four transcripts as well as phytoalexins.

Nonpathogenic bacteria such as *Escherichia coli* and *P. fluorescens* do not elicit a hypersensitive response on bean (27).

Therefore, we were interested in determining if they could elicit the putative defense responses that we had been investigating. Slot-blot experiments with total RNA isolated from bean after infiltration with either *E. coli* DH5α or *P. fluorescens* Pf101 revealed that RNAs for PAL, CHS, CHI, and chitinase accumulate in bean after inoculation with these bacteria, even though a hypersensitive reaction does not occur (Table 1). Also, the TLC bioassay revealed the production of phytoalexins during these interactions (Table 1). Preliminary experiments have also been conducted to determine if a compatible (i.e., pathogenic) bacterium will also activate these putative defense responses. The causal agent of halo blight of bean is *P. s.* pv. *phaseolicola* strain NPS3121. In slot-blot experiments 8 hours after infiltration of bean plants with NPS3121, PAL, CHI, CHS, and CHT, transcripts did not accumulate (Table 1). The TLC bioassay revealed that phytoalexins also were not produced during this interaction (Table 1).

A number of conclusions can be made from our experimental results. First, the induction of the putative defense responses we examined does not depend on a functional set of *hrp* genes in *P. s.* pv. *tabaci.* Second, in our system phytoalexin production can also occur without resulting in hypersensitive cell death. Third, because certain plant responses are activated by *E. coli* and *P. fluorescens,* it would appear that their activation is not a specific response to bacterial phytopathogens. Last, we have developed a novel experimental system for the study of plant disease resistance mechanisms. Our experimental data suggest that this method facilitates the separation of the hypersensitive reaction from other putative defense responses, including phytoalexin biosynthesis. We believe that our system provides a useful model for conducting functional analyses of the hypersensitive reaction.

Our studies support the hypothesis that there are unique biochemical events associated with the expression of a hypersensitive reaction that are distinct from other responses that occur during an incompatible interaction, such as phytoalexin biosynthesis. Therefore, it seems likely that there are plant genes that are specifically expressed during a hypersensitive reaction. We are currently attempting to identify such genes. We have generated cDNA libraries from mRNA isolated from bean tissue after inoculation with *P. s.* pv. *tabaci* strain Pt11528. By differentially screening these libraries with cDNA hybridization probes from bean plants inoculated with Pt11528 or Pt11528::Hrp1, we should be able to identify cDNA clones complementary to genes specifically expressed during a hypersensitive reaction.

Conclusions

Molecular and genetic approaches have proven to be very powerful for analyzing host–pathogen interactions. In the last few years, molecular studies have increased dramatically our understanding of the biology of *Rhizobium* (33), *Agrobacterium* (40,59), phytopathogenic pseudomonads, xanthomonads and *Erwinia* (4,28,56), phytopathogenic fungi (30,48), and gene-for-gene systems (18,37,45). Recent studies utilizing antisense RNA strategies (5), PCR (13), and RAPD analysis (34) indicate that molecular methodologies will continue to make important contributions towards the understanding of host–pathogen interactions. Our studies with Hrp mutants further underscore the value of using a molecular genetic approach for the study of host–pathogen interactions.

The study of plant–bacterial interactions would appear to be less complex than comparable studies of plant–nematode interactions. Genetic systems have been developed for many phytopathogenic bacteria, and cloning of bacterial pathogenicity and virulence genes is routine (4,28,56). In addition, the study of plant gene expression after bacterial infection is also relatively straightforward when compared to nematode infection. In contrast to plant and nematode mRNA, which is polyadenylated, bacterial mRNA is nonpolyadenyl-

ated and can be separated from plant mRNA using oligo(dT) cellulose columns. Hence, plant mRNA can be easily obtained, free of bacterial (i.e., pathogen) mRNA, for various experimental protocols such as cDNA cloning.

In contrast to bacterial systems, molecular analyses of plant–nematode interactions have presented difficult challenges. Plant-parasitic nematodes are obligate parasites; at present, there are no means for molecular transformation or creation of mutants. The lack of nematode mutants defined at the molecular level and differing in their ability to cause disease has hampered the understanding of pathogenicity and resistance mechanisms. Compatible–incompatible nematode–plant cultivar interactions do exist, and these would be useful for comparative molecular studies of plant resistance responses to nematode infection. Another major impediment to the study of plant–nematode interactions has been the inability to isolate plant genes that are expressed during nematode infection because of the limited amount of infected plant tissue available after nematode infection. Recently developed PCR-cDNA procedures have overcome this hindrance, however; cDNA clones were identified that appear to be specifically expressed in potato after infection by a compatible isolate of *Globodera rostochiensis* (13). By a similar strategy, it should be possible to identify plant genes specifically expressed during an incompatible plant–nematode interaction. Plant transposable elements might also provide a means to identify novel disease resistance or defense genes encoding products active against plant-parasitic nematodes (15). Finally, RFLP analysis has been used to map genes conferring resistance to plant-parasitic nematodes in a number of host plants (2, 26,36); this may aid the cloning of these genes by chromosome walking techniques.

Our studies should lead to the isolation of genes that specifically encode products needed for the hypersensitive reaction. Further studies should also eventually establish whether the hypersensitive reaction is a functional plant defense response. Long-term investigations into the molecular basis of the hypersensitive reaction will also provide valuable information about plant gene organization, structure, and regulation.

The information generated from our studies should have broad applications to the understanding of resistance to other phytopathogens. We anticipate that any hypersensitive-reaction-specific genes induced by *P. s.* pv. *tabaci* in bean will also be expressed during the hypersensitive reaction induced by other types of pathogens, including nematodes. This common expression will provide an opportunity to study the different signal transduction pathways and thus elucidate the differences in regulation associated with the activation of defense genes by phytopathogenic bacteria, fungi, or nematodes. It is also possible that any hypersensitive-reaction-specific gene identified in bean during our studies will share homology to genes in other plant species where hypersensitive reactions also occur; many of these plants may be important hosts for nematodes. Thus, it should be possible to identify these homologous clones in genomic and cDNA libraries and to conduct functional analyses of the hypersensitive reaction in plant–nematode interactions.

A thorough understanding of the mechanisms by which plants protect themselves against pathogen attack will be critical to develop genetically engineered plants with greater resistance to phytopathogens (55). As molecular techniques become more sensitive, researchers should be able to elucidate fully the contribution of inducible defense responses to disease resistance. The challenges will then be to determine which defense response functions in a given host–pathogen interaction and to exploit this knowledge in control of specific plant diseases.

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