Disease resistance in many plant-pathogen interactions results from the expression of a resistance (R) gene in the plant and a corresponding avirulence (avr) gene in the pathogen (1) and is often associated with the rapid, localized cell death of the hypersensitive response (HR). R genes that respond to specific bacterial, fungal, or viral pathogens have been isolated from a variety of plant species and several appear to encode cytoplasmic proteins (2–5). It has been unclear how such proteins could recognize an extracellular pathogen.

Sequence analysis of over 30 bacterial avr genes has generated little insight into the recognition process (6). Some bacterial pathogens of mammals use a protein secretion system, the type III pathway, to inject virulence proteins directly into the host cell (7). Components of a type III pathway are also encoded by the Hrp genes in many bacterial pathogens of plants, including Pseudomonas species (8). Thus phytopathogenic pathogens might directly introduce Avr proteins into plant cells. A report that avrB from Pseudomonas syringae pv. glycinea elicits an R gene–dependent HR when expressed within plant cells supports this model (9).

We examined the interaction between tomato and the bacterial pathogen Pseudomonas syringae pv. tomato. Research with this system has led to the isolation of the bacterial avrPto gene and the tomato Pto gene that, when expressed in the corresponding organisms, result in resistance to bacterial speck disease (2, 10, 11). Sequence analysis of avrPto has not revealed its function (11). The Pto gene encodes a serine-threonine kinase and is a member of a clustered gene family that also includes the Fen gene (2, 12, 13). The amino acid sequence of Fen kinase is 87% similar to Pto. Fen confers sensitivity to the insecticide fenthion (12). Other components of this signaling pathway in tomato include the Prf and Pti1 genes. Prf has similarities to a broad class of R gene products in that it contains leucine-rich repeats and a nucleotide binding site (14). Pti1 is a serine-threonine kinase probably acting downstream of Pto (15). We report here that the bacterial AvrPto protein directly interacts with the plant Pto kinase.

We used an Agrobacterium-mediated transient gene expression assay (16) to test if AvrPto protein could induce an HR when expressed inside plant cells (Fig. 1). Tobacco plants overexpressing the tomato Pto gene were used for this assay because they develop an enhanced HR specifically in response to Pseudomonas syringae pv. tabaci expressing avrPto (17). The avrPto gene under control of the cauliflower mosaic virus 35S promoter was introduced into Agrobacterium EHA105, which was subsequently infiltrated into fully expanded tobacco leaves (16). Ultraviolet-stimulated fluorescence was observed 32 hours after infiltration, indicating the accumulation of phenolic compounds associated with disease resistance (Fig. 1A). An HR appeared approximately 48 hours after injection (Fig. 1B). Neither fluorescence nor an HR occurred in leaves injected with either EHA105 containing pBl12 alone or with Agrobacterium strain A116 containing the 35S:avrPto construct but lacking the Ti plasmid (18). Thus, AvrPto protein induces a defense response when introduced directly into plant cells expressing the Pto gene (Fig. 1, A and B).

Because the Pto kinase confers recognition specificity in bacterial speck resistance, we tested whether Pto and AvrPto physically interact in the yeast two-hybrid system (19) (Fig. 2). Neither AvrPto nor Pto expressed individually activated the lacZ reporter gene in the two-hybrid system (Fig. 2). However, expression of both AvrPto and Pto in the same yeast cell activated the lacZ gene, demonstrating interaction of these two proteins (Fig. 2). Co-expression of AvrPto with kinases encoded by the recessive pto allele (20), the Fen gene, or with a mutant Pto protein that is unable to autophosphorylate (Pto[K69Q]; 13) did not activate lacZ (Fig. 2). The physical interaction of AvrPto with Pto suggests that Pto serves as a bacterial signal molecule and that Pto serves as the corresponding receptor.

To determine if particular regions of Pto are required for interaction with AvrPto, we constructed a series of chimeric proteins each consisting of different portions of Pto and Fen (Fig. 3A) (21). All Pto-Fen chimeric proteins possess kinase activity as determined by in vitro autophosphorylation assays [we were unable to express chimeric...
Pseudomonas syringae tabaci

Growth into tobacco leaves of determined at the specified time points (avrPto not overlap). Shown are inoculations with significant difference at probability level of 0.05. Means are different where error bars do not overlap. Shown are inoculations with P. syringae tabaci containing: no avrPto (white), CΔ12 (horizontal lines), CΔ25 (diagonal lines), CΔ12 (cross-hatched), or wild-type avrPto (black).

Fig. 3. (A) Interactions of Pto-Fen chimeric proteins with AvrPto. The diagram (left) depicts Pto (A) and Fen (B) and chimeric proteins (C through H) (21). The amino acids in Pto that demarcate the junction points between portions from Pto (in black) and portions from Fen (white) are shown at the bottom. EGY48 yeast cells containing AvrPto (in pJG4-5) and the various Pto-Fen chimeric proteins (in pEG202) were grown at 30°C for 2 days on galactose, X-Gal complete minimal medium (center). Similar expression of each protein in yeast was verified by protein immunoblots (Western) (32). I, Interaction assays in the two-hybrid system. D, disease responses of the corresponding transgenic Moneymaker plants inoculated with avirulent P. syringae tomato strain T1(pPtE6) (12). R, resistant; S, susceptible; and ND, not determined.

(B) Disease responses of transgenic tomato plants containing the Pto-Fen chimeric constructs. Leaves of primary transformants (24) were inoculated by dipping into a solution of avirulent P. syringae tomato strain T1(pPtE6) (4 × 10^7 cfu/ml). Photographs were taken 5 days after inoculation. The leaves shown are from plants containing the following transgenes under transcriptional control of the CaMV 35S promoter: (A) Pto, (B) Fen, and (C to G) chimeric constructs (C), (D), (E), (F), and (G). [Chimeric construct (H) was not transformed into Moneymaker]. A leaf from a nontransgenic Moneymaker plant is shown in (I).

Fig. 4. (A) Interactions of AvrPto deletion proteins with Pto. The diagram (left) depicts the wild-type AvrPto protein, and the series of deletion constructs of AvrPto: CΔ12, CΔ25, CΔ41, or CΔ74. EGY48 yeast cells containing Pto (in pEG202) and one of the AvrPto deletion constructs (in pJG4-5) were grown at 30°C for 2 days on galactose, X-Gal complete minimal medium. Similar expression of each protein in yeast was verified by Western blots (32). I, Interaction assays in the two-hybrid system. (B) Disease responses of primary transformants (24) were inoculated with a solution of avirulent P. syringae tomato strain T1(pPtE6) (4 × 10^7 cfu/ml). Disease resistance was quantified by measuring bacterial growth after inoculation of Pto-transgenic tobacco leaves with 10^8 cfu/ml (17) (Fig. 4B). Expression of CΔ41 in P. syringae tabaci did not affect bacterial growth in leaves, whereas expression of CΔ12 and CΔ25 reduced the final bacterial populations by 15- and 60-fold, respectively, compared to a P. syringae tabaci strain lacking AvrPto (Fig. 4B) (27). Therefore, the ability of AvrPto to interact with Pto in the two-hybrid system correlates with its ability to elicit disease resistance in plants.

Genetic analysis of many plant-pathogen associations has supported a model for direct interaction between R gene products and avr gene products (28). However, the inability to detect secretion of bacterial Avr...
proteins and the apparent cytoplasmic location of several R gene products seemed to preclude such a mechanism (2, 3, 29). Our results support such a model for bacterial speck resistance and suggest functional implications of the AvrPto-Pto interaction. The interaction of AvrPto with Pto, perhaps anchored to the plasma membrane by Pf, may stimulate Pto kinase activity and trigger a phosphorylation cascade. Alternatively, AvrPto may facilitate dimerization and cross-phosphorylation between Pto molecules. Finally, AvrPto might participate in a protein complex involving other proteins, including Pf, that activates the Pto signaling pathway.

How universal is this mode of recognition in plant-pathogen interactions? Gene products that confer resistance to Pseudomonas species (2, 3, 14), and to a fungal pathogen and an intracellular viral pathogen (4) appear to be cytoplasmic. Direct protein-protein interactions within the plant cell would be consistent with the gene-for-gene specificity seen in these associations. However, not all R gene products are alike. Pto, for example, is a cytoplasmic protein kinase (2). The other R genes, and Pf, encode proteins containing leucine-rich repeats and in some cases a nucleotide binding site (3–5, 14). Certain R gene products appear to have extracellular domains and may be involved in protein-protein interactions that are external to the plant cell (5).

Bacterial pathogens of plants and mammals share common components for the type III protein secretion pathway whereby virulence factors are delivered directly into host cells (7). In some Pseudomonas species, the same virulence factors are employed against both plants and animals (30). Yersinia pseudotuberculosis, a mammalian enteropathogen, disrupts host signal transduction by introducing a serine-threonine kinase and a phosphatase into the mammalian host cell (31). We have shown that a signal transduction pathway that leads to disease resistance in plants is also the target of a bacterial pathogen signal molecule; however, the result in this instance is recognition of the pathogen. Conservation of virulence mechanisms among plant and mammalian bacterial pathogens suggests that similar disease resistance mechanisms may have also evolved in these taxonomic kingdoms.

REFERENCES AND NOTES

16. The DNA sequence of the avrPto gene was ligated into the XbaI and SacI sites of pB121 and introduced into A. tumefaciens EHA105 and A136. Agrobacterium cells were inoculated into liquid AB medium supplemented with 50 μg/mL kanamycin and 0.2 mM acetylsyringone and grown at 30°C for 1 day. Cells were washed twice, resuspended in 10 mM MgCl2 to a final concentration of 107 cfu/mL and injected into tobacco leaves.
18. A136 strain is EHA105 lacking the Ti plasmid [E. Hood et al., Transgen. Res. 2, 206 (1993)].
21. Oligonucleotide primers containing restriction enzyme sites were designed from conserved regions of the Pto and Fen genes that allowed the three carboxy-terminal regions to be amplified by PCR (22). PCR products were cleaved with appropriate restriction enzymes and ligated together to create chimeric Pto-Fen constructs (22). A conserved Bgl II site in both Pto and Fen allowed for reciprocal amino terminal exchanges at amino acid 129. All constructs were verified by sequencing.
22. R. Frederick and G. Martin, unpublished results.
23. Chimeric Pto-Fen gene constructs were cloned into pEG202 with the use of either Eco RI (Fen carboxy-terminal region) or Eco RI-Bam HI (Pto carboxy terminal region) sites and introduced into yeast EGY48 containing the avrPto gene in pJG4-5 (19).
24. Chimeric Pto-Fen constructs were cloned into pB121 and the plasmids were introduced into A. tumefaciens EHA105. Chimeric constructs were transferred into tomato cultivar Moneymaker with the use of Agrobacterium-mediated transformation. Transgenic status of plants was verified by probing genomic DNA blots with a Pto gene probe and with the nptII gene from pB121 (22).
25. Full-length avrPto and the avrPto deletions were ligated into the Eco RI and Xho I sites of pJG4-5 (19). Constructs were introduced into the yeast strain EGY48 containing the Pto gene in pEG202 (19). All constructs were verified by sequencing.
26. avrPto deletions were cloned into pCP68 (11) and introduced into P. syringae tomato T1 or P. syringae tabaci 11528BR by triparental mating. Transconjugants were verified by DNA blot analysis.
27. The reduced avirulence activity of the mutant AvrPto proteins might be due to less efficient secretion of avrPto proteins. The ability to elicit the hypersensitive response was assayed by infiltrating 106 colony-forming units per milliliter into tomato or tobacco leaves. The ability of the Pseudomonas strains to cause disease symptoms was assayed by infiltrating tobacco leaves with 106 cfu/mL or dipping tobacco leaves into 106 cfu/mL. Tobacco lines analyzed were near isogenic cultivars Rio Grande-PtoR (Pto/Pto) and Rio Grande (pto/pto). Tobacco line was Wisconsin-38 containing a 35S::Pto transgene (17). † indicates a hypersensitive response or disease symptoms were observed; – indicates no hypersensitive response or disease symptoms were observed; N.D., not determined.
AvrPto from the Pseudomonas cell. To examine this, avrPto deletions ΔC25, ΔC41, and ΔC74 were placed into pBI121 and tested with the Agrobacterium tumefaciens transient assay. Agrobacterium EHA105 containing avrPto induced an HR in 2 days, whereas EHA105 containing the avrPto deletion ΔC25 induced an HR after 4 days; the other deletions did not elicit an HR (X. Tang and G. Martin, unpublished results). This suggests that the carboxy terminal 25 amino acids of AvrPto are not required for secretion from the bacterial cell; this portion of AvrPto may serve as an activation domain, interact with other components in the signaling pathway, or have a role in AvrPto stability.

32. Levels of protein expression were determined with the use of antibody to LexA (a gift from E. Golemis) for LexA fusion proteins (in pEG202) and antibody to the hemagglutinin (HA) epitope tag (Boehringer-Mannheim) for the AvrPto:HA fusion protein (in p304G).
33. We thank L. Dunkle, A. Friedman, S. Gelvin, and K. Perry for helpful comments on the manuscript. Supported, in part, by a Purdue Research Foundation Doctoral Fellowship (D.H.), National Science Foundation grant MCB-93-03559 (G.B.M.) and a David and Lucile Packard Foundation Fellowship (G.B.M.).

Molecular Basis of Gene-for-Gene Specificity in Bacterial Speck Disease of Tomato

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Transient expression of the Pseudomonas syringae avrPto gene in plant cells resulted in a Pto-dependent necrosis. The AvrPto avrPto genes were observed to interact directly with the Pto resistance protein in the yeast two-hybrid system. Mutations in the Pto and avrPto genes which reduce in vivo activity had parallel effects on association in the two-hybrid assay. These data suggest that during infection the pathogen delivers AvrPto into the plant host cell and that resistance is specified by direct interaction of Pto with AvrPto.

In plants, resistance to a variety of pathogens is determined by the action of complementary pairs of resistance (R) genes in the host and avirulence (avr) genes in the pathogen. These gene-for-gene interactions have been observed between plants and a diverse array of pathogens, including viruses, bacteria, fungi, nematodes, and insects (1). From genetic analysis it has been proposed that R genes recognize an elicitor produced directly or indirectly by the pathogen’s avr gene, which leads to a resistance response in the infected plant (2).

Bacterial speck disease of tomato is caused by Pseudomonas syringae pv. tomato (Pst). In tomato, resistance to strains of Pst that contain the avr gene avrPto is conferred by the Pto gene (3). The Pto locus encodes a family of related serine-threonine kinases. Among these, Fen is active in a parallel pathway that confers sensitivity to the insecticide fenitrothion (4).

R and avr proteins may interact directly, thereby activating plant defenses. For the protein product of Pto, this binding would likely occur intracellularly because of its predicted cytoplasmic localization. The activity of many avr genes, including avrPto, depends on an hsp60 secretion pathway which is similar to the type III secretory systems of Yersinia, Shigella, and Salmonella (5). These pathogens translocate a set of virulence proteins into host cells. Therefore, we considered the possibility that the bacterial AvrPto protein moves across the plant cell wall and plasma membrane where it directly interacts with the tomato Pto protein.

Evidence indicating that AvrPto acts inside the plant cell was obtained by transiently expressing avrPto in transgenic Nicotiana benthamiana plants transformed with Pto (6) (Fig. 1). This result in necrosis similar to the Pto-mediated HR elicited by P. syringae expressing avrPto and indicated that AvrPto was active within the plant cell. Deletion of 30 amino acids from the COOH-terminus of AvrPto did not eliminate this activity, whereas deletion of 59 amino acids destroyed activity. Activity of the deletion derivatives in the transient expression assays correlated with biological activity in P. syringae. These results suggested that the products of the avr and R genes may interact directly.

We employed the yeast two-hybrid system to directly test this hypothesis (7). Pto, Fen, and avrPto coding sequences were expressed as fusions to GAL4 DNA binding (BD) and transcriptional activating (AD) domains. Reciprocal combinations of BD and AD fusions were tested for β-galactosidase reporter gene activity in yeast. Interaction was only observed when the BD::Pto and AD::AvrPto fusions were coexpressed (Fig. 2). Controls did not show any interaction. Furthermore, no interaction was detected between BD::Fen and AD::AvrPto (Figs. 2 and 3A).

To test the biological relevance of the interaction, inactive alleles of Pto and avrPto were tested. Three inactive Pto alleles, pto6, pto7 and pto11, were previously identified through mutagenesis of resistant tomato plants (8). Sequence analysis revealed single amino acid changes in each mutant allele (Fig. 3B). The mutant Pto sequences showed no detectable interaction with AvrPto in yeast. Thus, mutant alleles that confer susceptibility to Pto also fail to interact with AvrPto in the two-hybrid system. The two deletions of AvrPto tested in
Initiation of Plant Disease Resistance by Physical Interaction of AvrPto and Pto Kinase
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