Initiation of Plant Disease Resistance by Physical Interaction of AvrPto and Pto Kinase

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Resistance to bacterial speck disease in tomato occurs when the Pto kinase in the plant responds to expression of the avirulence gene *avr*Pto in the *Pseudomonas* pathogen. Transient expression of an *avr*Pto transgene in plant cells containing *Pto* elicited a defense response. In the yeast two-hybrid system, the Pto kinase physically interacted with AvrPto. Alterations of AvrPto or Pto that disrupted the interaction in yeast also abolished disease resistance in plants. The physical interaction of AvrPto and Pto provides an explanation of gene-for-gene specificity in bacterial speck disease resistance.

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 pBI121 alone or with *Agrobacterium* strain A136 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 autophasphorylate (Pto[K69Q]; 13) did not activate lacZ (Fig. 2). The physical interaction of AvrPto with Pto suggests that AvrPto 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 autophasphorylation assays [we were unable to express chimeric...
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). Interaction assays in the two-hybrid system. D, disease responses of the corresponding transgenic Moneymaker plants inoculated with avirulent P. syringae tomato strain T1(pR6E6) (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 T10(pR6E6) (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: C142, CΔ25, C41, or C74. 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). Interaction assays in the two-hybrid system. (B) Growth in tobacco leaves of P. syringae tabaci expressing the avrPto deletion constructs. Pseudomonas syringae tabaci strain 11528R containing wild-type avrPto or one of the avrPto deletion constructs was injected into tobacco leaves. Bacterial populations were determined at the specified time points (17). Error bars equal one-half of the least 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), C41 (horizontal lines), CΔ25 (diagonal lines), C12 (cross-hatched), or wild-type avrPto (black).
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 Prf, may stimulate Pto kinase activity and trigger a phosphorylation cascade. Alternatively, AvrPto may facilitate dimerization and cross-phosphorylation between Pto proteins, including Prf, that activates the Pto signaling pathway.

How universal is this mode of recognition in plant pathogen interactions? \( R \) gene products confer resistance to \textit{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 Prf, 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 \textit{Pseudomonas} species, the same virulence factors are employed against both plants and animals (30). \textit{Veronica 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 also have evolved in these taxonomic kingdoms.

### REFERENCES AND NOTES

16. The DNA sequence of the \textit{avrPto} gene was ligated into the XbaI and SacI sites of \textit{pBl21} and introduced into \textit{A. tumefaciens} \textit{EHA105} and A136. Agrobacterium cells were inoculated into liquid AB medium supplemented with 50 mg/L kanamycin and 0.2 mM acetosyringone and grown at 30°C for 1 day. Cells were washed twice, resuspended in 10 mM MgCl2, to a final concentration of 10^7 cfu/ml, and injected into tobacco leaves.
18. A136 strain is \textit{EHA105} lacking the Ti plasmid (E. Hood et al., \textit{Transgen. Res.} 2, 206 (1993)).
21. Oligonucleotide primers containing restriction enzyme sites were designed from conserved regions of the \textit{Pto} and \textit{Fen} genes that allowed the three carbboxy-terminal regions to be amplified by PCR (22). PCR products were cleaved with appropriate restriction enzymes and ligated together to create chimeric \textit{Pto-Fen} constructs (22). A conserved Bgl II site in both \textit{Pto} and \textit{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 \textit{Pto-Fen} gene constructs were cloned into \textit{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 \textit{EGY48} containing the \textit{avrPto} gene in \textit{pJ4A-5} (19).
24. Chimeric \textit{Pto-Fen} gene constructs were cloned into \textit{pBl21} and the plasmids were introduced into \textit{A. tumefaciens} \textit{EHA105}. Chimeric constructs were transferred into tomato cultivar \textit{MoneyMaker} with the use of Agrobacterium-mediated transformation. Transgenic status of plants was verified by probing genomic DNA blots with a \textit{Pto} gene probe and with the \textit{ntfpl} gene from \textit{pBl21} (22).
25. Full-length \textit{avrPto} and the \textit{avrPto} deletions were ligated into the Eco RI and \textit{Xho I} sites of \textit{pJ4A-5} (19). Constructs were introduced into the yeast strain \textit{EGY48} containing the \textit{Pto} gene in \textit{pEG202} (19). All constructs were verified by sequencing.
26. \textit{avrPto} deletions were cloned into \textit{pPE6} (11) and introduced into \textit{P. syringae} tomato \textit{T1} or \textit{P. syringae} tabaci 11528R by triparental mating. Transconjugants were verified by DNA blot analysis.
27. The reduced avirulence activity of the mutant \textit{AvrPto} proteins might be due to less efficient secretion of
Molecular Basis of Gene-for-Gene Specificity in Bacterial Speck Disease of Tomato

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Transient expression of the Pseudomonas syringae avirulence gene avrPto in plant cells resulted in a Pto-dependent necrosis. The AvrPto avirulence protein was 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 hrp 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...
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