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 avrPto in the Pseudomonas pathogen. Transient expression of an avrPto 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 Prf1 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). Prf1 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 pBl121 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 autoprophosphorylate (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 autophosphorylation assays [we were unable to express chimeric proteins containing the Fen kinase domain, however]. We found that a chimeric Pto containing the first 213 amino acids of Pto and the last 142 amino acids of Fen (21) was necessary for interaction with AvrPto (Fig. 3B). Alterations of AvrPto and Pto that disrupted the interaction in yeast also abolished disease resistance in plants (Fig. 3C).

Fig. 1. Expression of avrPto in tobacco leaves. Agrobacterium tumefaciens strain EHA105 containing a 35S::GUS construct (left) or a 35S::avrPto construct (right) was injected into leaves of tobacco line W-38 expressing a 35S::Pto transgene (17). (A) Accumulation of ultraviolet-fluorescent compounds at 32 hours after infiltration. (B) Development of the HR at 48 hours after infiltration.

Fig. 2. Interaction of AvrPto with Pto. The LexA two-hybrid system (19) was used to test possible interaction of AvrPto with Pto and other closely related kinases. In all cases the avrPto gene was introduced on pUG4-S, and the other genes were introduced on pEG202. Yeast strains were grown at 30°C for 2 days on galactose, X-Gal complete minimal medium (19). Yeast strains contain: (A) AvrPto, (B) Pto, (C) AvrPto/Pto, (D) AvrPto/Pto, (E) AvrPto/Pto(K69Q), or (F) AvrPto/Fen.
construct C in Escherichia coli (13, 22)]. In the two-hybrid system, AvrPto specifically interacted with chimera G (23) (Fig. 3A). Comparison of chimera G with the other chimeras implicated a region in Pto from amino acids 129 to 224 that is required for interaction with AvrPto. We also generated stable transgenic tomato plants expressing each of the Pto-Fen constructs to examine the requirement of different regions of the Pto protein for disease resistance (24) (Fig. 3B). Only chimeric genes C and G conferred resistance in tomato to the avirulent pathogen whereas the other chimeric genes conferred sensitivity to fenthion (Fig. 3B) (22). Thus a 95–amino acid stretch of the Pto kinase is involved in pathogen recognition and also forms a part of the interaction site with the AvrPto protein.

Several carboxy-terminal deletions of AvrPto were assayed for interaction with Pto in the two-hybrid system (Fig. 4A) (25). Deletions CΔ12 and CΔ25, lacking 12 and 25 amino acids, respectively, from the carboxy terminus interacted with Pto whereas the others did not (Fig. 4A).

The avrPto deletions were also introduced into two P. syringae pathovars, tomato and tabaci, and inoculated onto tomato and tobacco plants to assess their effects on the HR and disease symptoms (26). Only the two AvrPto deletions that interacted with Pto in the two-hybrid system, CΔ12 and CΔ25, induced both an HR and disease resistance in a Pto-dependent manner (Table 1). 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 AvrPto
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 molecules. Finally, AvrPto might participate in a complex containing other proteins, including Prf, 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 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 Pseudomonas species, the same virulence factors are employed against both plants and animals (30).}

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 acetosyringone and grown at 30°C for 1 day. Cells were washed twice, resuspended in 10 mM MgCl2, to a final concentration of 106 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 carpoxyl-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 carboxyl-terminal region) or Eco RI-Bam HI (Pto carboxyl-terminal region) sites and introduced into yeast EGY48 containing the avrPto gene in pUG4-5 (19).
24. Chimeric Pto-Fen gene constructs were cloned into pBI121 and the plasmids were introduced into A. tumefaciens EHA105. Chimeric constructs were transferred into tomato cultivar Moneymaker by 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 pBI121 (22).
25. Full-length avrPto and the avrPto deletions were ligated into the Eco RI and XhoI 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 pP6E6 (11) and introduced into P. syringae tomato T1 and P. syringae tobacco 115258R 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

| Table 1. Analysis of avrPto deletions in P. syringae pv. tomato and P. syringae pv. tabaci inoculated on tomato or tobacco, respectively. 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 tomato leaves into 10⁵ 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.

<table>
<thead>
<tr>
<th>Plant Genotype</th>
<th>P. syringae pv. tomato</th>
<th>P. syringae pv. tabaci</th>
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<tr>
<td>Construct</td>
<td>Hypersensitive response*</td>
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<td>C974</td>
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<td>None</td>
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*Hypersensitive response appeared as tissue collapse of the infiltrated area and was scored 14 hours after infiltration.†Disease symptoms in tomato appeared as small necrotic lesions and in tobacco as dark, water-soaked regions. Symptoms were scored 5 days after inoculation.
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 genes 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...
Editor's Summary

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