

Recognition Specificity for the Bacterial Avirulence Protein AvrPto Is Determined by Thr-204 in the Activation Loop of the Tomato Pto Kinase

Reid D. Frederick,[†] Roger L. Thilmony,
Guido Sessa, and Gregory B. Martin*[‡]
Department of Agronomy
Lilly Hall of Life Sciences
Purdue University
West Lafayette, Indiana 47907-1150

Summary

The Pto kinase confers resistance in tomato to *P. syringae* pv. *tomato* strains expressing the AvrPto protein. Physical interaction of the Pto kinase and AvrPto protein in the plant cell initiates host defense responses. The recognition event between these two proteins is very specific; AvrPto does not interact with other closely related kinases, including the Fen kinase, which shares 80% amino acid identity with Pto. By using Pto-Fen chimeric proteins and site-directed mutagenesis, we found that Thr-204 is required for Pto interaction with AvrPto in a yeast two-hybrid system and for recognition specificity in a tobacco leaf transient assay. Substitution of Thr-204 into the Fen kinase allowed that kinase to interact with AvrPto and to confer an AvrPto-specific defense response in tobacco leaves. Thus, simple mutations appear capable of giving rise to new resistance gene specificities.

Introduction

Plant disease resistance is often initiated by a recognition event specified by a host resistance (*R*) gene and a corresponding pathogen avirulence (*avr*) gene (Keen, 1990; Dangl, 1994). Recognition of the invading pathogen by the host triggers a hypersensitive response (HR) typified by rapid, localized death of host cells (Goodman and Novacky, 1994). Over the past five years, *R* genes that confer such “gene-for-gene” resistance to specific strains of viruses, bacteria, or fungi have been isolated from several plant species (Bent, 1996; Hammond-Kosack and Jones, 1997). Most *R* genes that confer resistance to pathogenic bacteria encode proteins that lack a signal sequence or putative transmembrane domain that would indicate they are localized to the extracellular surface. Thus, it appears likely that these *R* proteins play a role in pathogen recognition by acting within the plant cell.

Recently, evidence has accumulated that certain bacterial pathogens of plants are capable of delivering avirulence proteins directly into the plant cell (Gopalan et al., 1996; Leister et al., 1996; Scofield et al., 1996; Tang et al., 1996; Van den Ackerveken et al., 1996). To effect

this delivery, it is likely that plant pathogens utilize a type III secretion pathway whose components are encoded by the *Hrp* genes (hypersensitive response and pathogenicity genes; Alfano and Collmer, 1996; Roine et al., 1997). Many *Hrp* genes have sequence similarity to genes that encode a type III pathway in *Yersinia* and other mammalian bacterial pathogens (Lee, 1997; Mudgett and Staskawicz, 1998). Transport of Avr proteins into the plant cell would permit an intracellular recognition event to occur involving an *R* protein and Avr protein. Evidence for such a protein–protein interaction has been obtained recently in the plant–pathogen interaction involving the tomato *Pto* disease resistance gene and the *Pseudomonas avrPto* gene (Scofield et al., 1996; Tang et al., 1996).

The *Pto* gene encodes a serine-threonine protein kinase that confers resistance in tomato to the causative agent of bacterial speck disease, *Pseudomonas syringae* pv. *tomato*. Resistance to bacterial speck occurs only when the pathogen expresses the *avrPto* gene, which encodes a small hydrophilic protein without similarity to other proteins in the data base (Ronald et al., 1992; Salmeron and Staskawicz, 1993). By using a yeast two-hybrid system, we (Tang et al., 1996) and others (Scofield et al., 1996) observed a physical interaction between Pto and AvrPto. The interaction is highly specific because the AvrPto protein does not interact with the closely related Fen kinase or with the product encoded by an allele of *Pto*, which is 87% identical to the Pto kinase (Martin et al., 1994; Jia et al., 1997). Three lines of evidence indicate that the Pto–AvrPto interaction occurs in vivo and plays a crucial role in disease resistance (Scofield et al., 1996; Tang et al., 1996). First, the AvrPto protein activates the HR when it is expressed transiently in plant cells using *Agrobacterium*-based DNA transfer. Second, deletion of portions of the AvrPto protein that are required for interaction with Pto render the truncated AvrPto nonfunctional when expressed in plant cells. However, deletions of carboxy-terminal sequences that are not essential for Pto interaction in the two-hybrid system do not interfere with pathogen recognition in the plant. Finally, domain-swapping analysis between Pto and the Fen kinase delineated a small region of Pto that is required for AvrPto interaction (Scofield et al., 1996; Tang et al., 1996).

We now report the development of additional Pto-Fen chimeric proteins and a series of site-directed mutant Pto and Fen proteins. The proteins were used in a yeast two-hybrid system and in a novel, two-gene transient assay to identify amino acid residues in the Pto kinase that are required for gene-for-gene recognition specificity of the AvrPto protein. Our experiments indicate that a single threonine residue in the activation loop of the Pto kinase plays a critical role in recognition specificity of AvrPto.

Results and Discussion

Previous studies using chimeric Pto-Fen proteins suggested that a region spanning kinase subdomain VIII is

*To whom correspondence should be addressed (e-mail: gmartin@dept.agry.purdue.edu).

[†]Present address: USDA-ARS-NAA, Foreign Disease-Weed Science Research Unit, 1301 Ditto Ave., Fort Detrick, Frederick, Maryland 21702-5023.

[‡]Present address: Boyce Thompson Institute at Cornell University, Ithaca, New York 14853-1801.

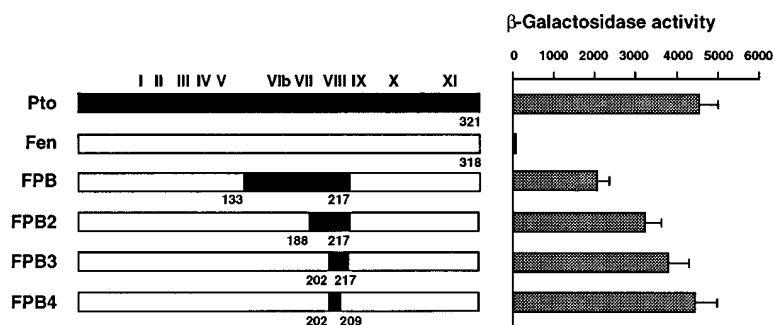


Figure 1. Pto-Fen Chimeric Proteins and Their Interactions with AvrPto in the Yeast Two-Hybrid System

The diagram (left) depicts Pto, Fen, and chimeric proteins FPB, FPB2, FPB3, and FPB4. The regions containing amino acids from Pto (closed bars) and from Fen (open bars) are shown. Numbers correspond to amino acid positions in Pto, and standard subdomains of protein kinases are indicated above Pto. Activation of the *lacZ* reporter gene was determined by measuring relative units of β -galactosidase activity in yeast strains expressing the Pto-Fen chimeric proteins and AvrPto. The β -galactosidase activity data are the means (shaded bars) and standard errors (error bars) of duplicate experiments, each with three independent colonies per construct.

required both for physical interaction of Pto with AvrPto in the yeast two-hybrid system (Scofield et al., 1996; Tang et al., 1996) and for *avrPto*-specific pathogen recognition in transgenic tomato plants (Tang et al., 1996). To identify amino acids in Pto that determine recognition specificity, we generated additional chimeric Pto-Fen proteins and tested them for interaction with AvrPto in a yeast two-hybrid assay using a *lacZ* reporter gene (Figure 1). Chimera FPB, which contains 85 residues from Pto that span the conserved kinase subdomains VIb through VIII, strongly interacted with AvrPto as indicated by high β -galactosidase activity in the two-hybrid assay (Figure 1). This 85 amino acid segment contains 18 residues that differ between Pto and Fen. A second chimera, FPB2, also interacted with AvrPto and further delimited the AvrPto recognition region to 30 Pto residues, of which 11 are polymorphic with Fen (Figure 1). Scofield et al. (1996) also placed AvrPto recognition specificity within this region. Finally, chimeras FPB3 and FPB4 were both found to interact with AvrPto, and chimera FPB4 further defined the region of recognition specificity to the 8 residues of Pto at positions 202–209 (Figure 1). There are just four amino acid differences between Pto and Fen among these 8 residues.

To determine which of the four Pto residues in chimera FPB4 are required for AvrPto recognition specificity, a PCR-based site-directed mutagenesis strategy was used to convert each of the residues individually to the corresponding Fen residue. Thus, mutants FPB3(K202R), FPB3(T204N), FPB3(L205I), and FPB3(D209A) are chimeric FPB3 proteins that each contain a substitution of a Fen residue for one of the four Pto residues (Figure 2). In the two-hybrid assay, FPB3(K202R), FPB3(L205I), and FPB3(D209A) each interacted with AvrPto, although the FPB3(L205I) substitution mutant showed substantially lower β -galactosidase activity than wild-type Pto or FPB3 chimera (Figure 2). However, the T204N substitution completely abolished the interaction of FPB3 (T204N) with AvrPto, indicating that this Pto residue is required for AvrPto recognition. Because changes in Pto residues T204 and L205 caused the greatest decrease in the interactions with AvrPto, further substitutions were made directly in the Pto kinase to examine the role of these two residues.

Pto constructs P(T204N) and P(L205I) were generated

by PCR site-directed mutagenesis and tested in the two-hybrid assay with AvrPto as the prey protein (Figure 2). P(L205I) interacted with AvrPto, whereas P(T204N) did not, demonstrating again the absolute requirement of T204 for Pto recognition of AvrPto. We next developed a Fen mutant protein that contained substitutions of just the T204 and L205 Pto residues (construct F(N204T, I205L)). Remarkably, these two substitutions alone were sufficient to allow the Fen protein to interact with AvrPto in the two-hybrid assay (Figure 2). Finally, mutant proteins F(N204T) and F(I205L) were developed to test the individual contribution of the Pto residues T204 and L205 to AvrPto recognition specificity. F(N204T) interacted with AvrPto, whereas F(I205L) did not interact. Similar expression levels of each Pto-Fen mutant protein were verified by Western blots (Figure 3).

We used a tobacco leaf transient assay to examine the role of Pto residues T204 and L205 in gene-for-gene recognition in plant cells. Previously, it was shown that *Agrobacterium tumefaciens* can be used to transiently express AvrPto in plant cells (Scofield et al., 1996; Tang et al., 1996). We refined this assay by using a binary plasmid vector (pBTEX) that permits simultaneous expression of both *avrPto* and *Pto* genes with each gene under the control of a separate cauliflower mosaic virus (CaMV) 35S promoter (see Experimental Procedures). This vector was used to express AvrPto with Pto, Fen, P(T204N), P(L205I), F(N204T), F(I205L), or F(N204T, I205L). The two-gene plasmid constructs were transformed into *A. tumefaciens* strain EHA105, which was then infiltrated into mature leaves of *Nicotiana benthamiana*. The strain expressing AvrPto/Pto, but not AvrPto/Fen, elicited an HR within the infiltrated area 3–5 days after treatment (Figure 4). *Agrobacterium* strains expressing AvrPto, Pto, or Fen alone did not elicit an HR (data not shown). Furthermore, an HR was not observed when a *vir*⁻ defective strain of *Agrobacterium* containing both AvrPto and Pto was infiltrated into *N. benthamiana*, indicating the absolute requirement for T-DNA transfer and subsequent expression of both *avrPto* and *Pto* within the plant cell. The strains expressing AvrPto along with P(L205I), F(N204T), F(I205L), or F(N204T, I205L) each elicited an HR (Figure 4). The significance of the HR with F(I205L) is unclear since the response was very weak and occurred in only 50% of the experiments. With the exception of F(I205L), we observed a strict correlation between

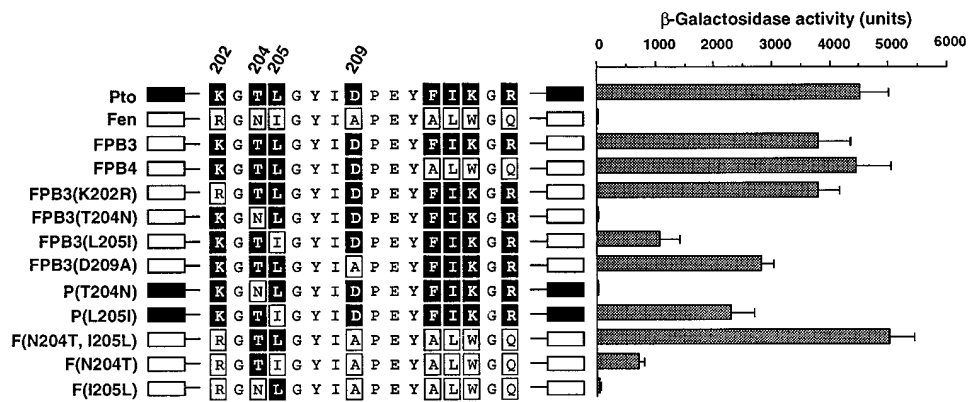


Figure 2. Effect of Amino Acid Substitutions in Subdomain VIII on the Interaction of Pto and Fen with AvrPto in the Yeast Two-Hybrid System. Portions of the proteins, and individual amino acids, derived from Pto (closed boxes) or Fen (open boxes) are shown (left). Unboxed amino acids are identical in both kinases. To simplify nomenclature, the numbering of amino acid positions and designation of amino acid substitutions correspond to the Pto sequence (Martin et al., 1993) for all proteins shown. Degree of *lacZ* reporter gene activation was determined by measuring relative units of β -galactosidase activity in yeast strains expressing the mutant proteins and AvrPto (right). The β -galactosidase activity data are the means (shaded bars) and standard errors (error bars) of duplicate experiments, each with three independent colonies per construct.

the ability of a Pto-Fen protein to interact with AvrPto in the two-hybrid system and its ability to cause an HR in tobacco leaves. The interaction of the Fen mutants F(N204T, I205L) and F(N204T) with AvrPto and their ability to elicit a strong HR in tobacco leaves raises the interesting possibility that these proteins might possess dual recognition specificity for AvrPto and the fenthion insecticide. Development of stable tomato transgenics will be required to test this possibility because tobacco

leaves, unlike tomato leaves, do not exhibit fenthion sensitivity.

Demonstration that T204 in subdomain VIII of the Pto kinase determines recognition specificity for AvrPto further supports a role for Pto as the intracellular receptor, or perhaps as part of a receptor complex, for a pathogen protein delivered by a type III secretion pathway. Recent reports indicate that several bacterial Avr proteins function within plant cells, and it is possible that other cytoplasmic R proteins are also directly involved in recognition events. Pto is unique among R proteins, however, in encoding an intracellular kinase, and it remains unclear if the majority of R proteins, which contain regions of leucine-rich repeats (LRR), participate directly or indirectly in recognition of pathogen Avr proteins. A recent

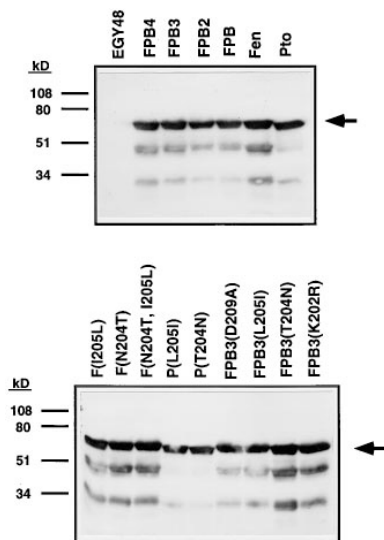


Figure 3. Similar Levels of Protein Expression for Each of the Chimeric and Mutant Proteins

The Pto-Fen proteins were expressed as LexA fusions from the bait plasmid (pEG202), and AvrPto was expressed from the prey plasmid (pJG4-5). LexA fusion proteins were detected by chemiluminescent visualization using polyclonal antibody to LexA. Lane MW contains molecular weight protein standards, and lane EGY contains protein extracts from yeast strain EGY48 containing the *lacZ* reporter plasmid.

Construct	HR
A. Pto	19/19
B. Fen	0/19
C. P(T204N)	3/19
D. P(L205I)	17/19
E. F(N204T)	17/19
F. F(I205L)	10/19
G. F(N204T, I205L)	14/19

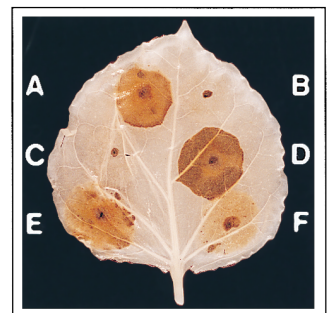


Figure 4. Transient Assay for the Hypersensitive Response in *N. benthamiana* Leaves

A. tumefaciens EHA105 strains carrying the pBTEx plasmid encoding AvrPto and the indicated protein (A-G) were infiltrated into mature leaves. The HR was observed as localized tissue collapse within 3-5 days. Development of the HR is sensitive to environmental conditions; therefore, we performed 19 independent experiments, and the frequency of leaves developing an HR is shown (HR column). On the right is a representative leaf showing the development of the HR 5 days after infiltration with constructs (A)-(F) ([G] is not shown). The leaf chlorophyll was removed with ethanol to aid the visualization of the response.

report of recognition specificity with the *Cf R* genes that encode membrane-bound extracytoplasmic proteins indicates that LRRs do play a role in recognition of some fungal pathogens (Thomas et al., 1997). Thus, it is possible that the Prf protein, which also contains LRRs and is required for Pto-mediated disease resistance, might participate in a recognition complex with Pto within the plant cell (Salmeron et al., 1996).

Taken together, our results indicate that T204 in Pto is required for specific recognition of AvrPto and that L205, although not required, plays a subsidiary role in this recognition event. A comparison of Pto with other protein kinases whose three-dimensional structures are known (e.g., Cdk2, cAMP-dependent protein kinase [PKA]) indicates that T204 is located in the Pto activation domain (De Bondt et al., 1993; Johnson et al., 1996). In PKA, phosphorylation of T197 in the activation domain is required for maximal kinase activity and substrate binding (Johnson et al., 1996). The activation domain in PKA is thought to be stabilized, and substrate binding facilitated, by the formation of hydrogen bonds between phosphate oxygens on phospho-T197 and the charged side chains of R165, K189, and the hydroxyl group of T195 (Johnson et al., 1996). A sequence alignment of Pto (and Fen) with PKA showed that corresponding arginine and lysine residues exist at Pto positions 163 and 187, respectively. Although Pto T204 does not directly align with PKA T197, we hypothesize that T204 in Pto might be phosphorylated, leading to a conformational change that stabilizes the kinase and facilitates binding of AvrPto. The importance of subdomain VIII for AvrPto recognition is further supported by analysis of the Pto kinase encoded by an allele from a bacterial speck susceptible tomato plant (Jia et al., 1997). This kinase, which does not interact with AvrPto in the two-hybrid assay, contains a phenylalanine at position L205 of Pto and contains a small deletion near T204/L205 at positions corresponding to 196–198 in Pto (Martin et al., 1993). Finally, it is interesting to note that several other kinases related to Pto have a threonine conserved at the position corresponding to Pto T204 (B. Riely and G. B. M., unpublished data). These kinases include the disease resistance protein Xa21 (GenBank accession number U37133), the *Arabidopsis* ERECTA (U47029), and maize CRINKLY4 (U67422) proteins, the *Drosophila* Pelle kinase (L08476), and the human interleukin receptor-associated kinase (IRAK; L76191). It is possible that phosphorylation of the threonine corresponding to Pto T204 in these kinases is also important for their function.

Our results open the way to understanding three-dimensional aspects of the recognition event between Pto and AvrPto and how interaction subsequently leads to activation of various defense responses associated with disease resistance. Investigation of Pto-related kinases in wild species of tomato, some of which are resistant to *avrPto*-expressing *Pseudomonas* strains (Tanksley et al., 1996), should assist in identifying region(s) of Pto that serve as contact points with AvrPto. From an agronomic perspective, gene-for-gene interactions play an important role in breeding for disease resistance in all major crop species. Further elucidation of the molecular basis of these interactions might allow for precise manipulation of recognition specificity of plant R

proteins and lead to new strategies for improving disease resistance in plants.

Experimental Procedures

Construction of Chimeric Proteins and Site-Directed Mutant Proteins

Chimeric constructs and site-directed mutations were generated by PCR with *Pwo* DNA polymerase (Boehringer Mannheim) and primers containing unique restriction sites for amplification of specific regions of the *Pto* and *Fen* genes as described previously (Tang et al., 1996). In addition to the unique internal restriction site, all Pto/Fen constructs contained flanking 5'-*EcoRI* and 3'-*BamHI* restriction sites for cloning into the yeast two-hybrid bait vector pEG202. A complete description of the primer sequences, DNA templates, and restriction enzymes used to generate the chimeric and mutant Pto/Fen constructs is available upon request from the corresponding author. The DNA sequence of each construct was verified prior to testing in yeast and in the *Agrobacterium*-mediated transient assay.

Yeast Two-Hybrid Interactions

Chimeric *Pto-Fen* and mutant genes (in pEG202) were introduced into yeast strain EGY48 and their proteins tested for interaction with AvrPto (in pJG4-5) as described (Tang et al., 1996). Equal levels of expression for all LexA fusion proteins in pEG202 were determined by Western blots using LexA antibody. Total proteins were extracted from yeast strains during mid-log phase (Ausubel et al., 1996). Equal amounts of protein were electrophoresed on 10% polyacrylamide gels and transferred to PVDF membrane (Millipore Immobilon P) by electroblotting according to the manufacturer's recommendations (BioRad). LexA fusion proteins were detected by chemiluminescent visualization (ECL kit) using polyclonal antibody to LexA (a gift from E. Golemis, Fox Chase Cancer Center, Philadelphia, PA).

Agrobacterium-Mediated Transient Assays in *N. benthamiana*

The *avrPto* DNA sequence was ligated into the *SmaI/SalI* sites downstream of the CaMV 35S promoter in the binary expression vector, pBTEx. Both the binary vector pBTEx and the shuttle vector pTEX[H] were generous gifts from D. Yun and R. A. Bressan (Dept. of Horticulture, Purdue University). The shuttle vector pTEX is a pUC19-derived plasmid that contains the CaMV 35S promoter in the *EcoRI/KpnI* sites and the polyadenylation signal from the octopine synthase gene in the *SphI/HindIII* sites. The derivative vector, pTEX[H], was constructed by digesting pTEX with *EcoRI*, blunting the ends with Klenow fragment, adding a *HindIII* linker, and self ligating. The binary vector pBTEx was created by replacing the *EcoRI/HindIII* 35S::GUS fragment from pBI121 (Clontech Laboratories, Palo Alto, California) with the *EcoRI/HindIII* 35S promoter Nos terminator fragment excised from pTEX. All *Pto*, *Fen*, and *Pto-Fen* chimeric constructs were first cloned into the shuttle vector pTEX[H] at *SmaI/BamHI* sites to form a 35S promoter fusion. This fusion was excised with *HindIII* and ligated into the pBTEx::*avrPto* plasmid at the *HindIII* site. The pBTEx constructs were introduced into *A. tumefaciens* strain EHA105 by electroporation. *Agrobacterium* strains for inoculation were grown in induction medium for approximately 10 hr at room temperature, diluted with induction medium to an $OD_{600} = 0.2$, and injected into leaves as described previously (Tang et al., 1996). The HR developed within 3–5 days.

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