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Constitutively active *Pto* induces a *Prf*-dependent hypersensitive response in the absence of *avrPto*

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Resistance in tomato to *Pseudomonas syringae* pv *tomato* (*avrPto*) is conferred by the gene *Pto* in a gene-for-gene relationship. A hypersensitive disease resistance response (HR) is elicited when *Pto* and *avrPto* are expressed experimentally within the same plant cell. The kinase capability of Pto was required for AvrPto-dependent HR induction. Systematic mutagenesis of the activation segment of Pto kinase confirmed the homologous P+1 loop as an AvrPto-binding determinant. Specific amino acid substitutions in this region led to constitutive induction of HR upon expression in the plant cell in the absence of AvrPto. Constitutively active Pto mutants required kinase capability for activity, and were unable to interact with proteins previously shown to bind to wild-type Pto. The constitutive gain-of-function phenotype was dependent on a functional *Prf* gene, demonstrating activation of the cognate disease resistance pathway and precluding a role for *Prf* upstream of *Pto*.

Keywords: disease resistance/epistasis/gain of function/ P+1 loop/tomato

Introduction

Plant resistance to pathogenic attack is often governed by single genes in the host. Such resistance (R) genes require the presence of a complementary avirulence (Avr) gene in the pathogen to specify recognition of the pathogen and induction of defense responses (Crute, 1986). This 'gene-for-gene' resistance has been the subject of intensive study owing to its biological and economic importance. Nearly 20 R genes have now been cloned and several classes recognized; the majority appear to encode components of signal transduction systems (Staskawicz *et al.*, 1995; Hammond-Kosack and Jones, 1996). However, little is known of recognition events that occur between the host and the pathogen, or subsequent signal transduction leading to the resistance response (Baker *et al.*, 1997; Yang *et al.*, 1997).

Most plant R genes are thought to encode receptors for their cognate avirulence determinants. Evidence for such direct interaction exists only in resistance to *Pseudomonas syringae* pv *tomato* (*Pst*) in tomato, specified by the *Pto* gene. A strong and specific binding event between Pto and the avirulence gene product AvrPto was observed in the yeast two-hybrid assay for detecting protein–protein interactions (Scofield *et al.*, 1996; Tang *et al.*, 1996). Mutants of *Pto* and *avrPto* that exhibited reduced activity *in vivo* showed impaired interaction when co-expressed in yeast. Although other complementary pairs of R and Avr genes have been cloned (Baker *et al.*, 1997), no evidence exists yet for physical interaction between the protein products of these genes. Most R proteins other than Pto include leucine-rich repeats (LRRs) which may mediate protein–protein interactions and constitute the receptor of the avirulence determinant of such R proteins (Bent, 1996; Jones and Jones, 1996; Parniske *et al.*, 1997).

Reception of the pathogen-derived signal leads to induction of signal transduction, culminating in the expression of a variety of host defenses. For *Pto*, which encodes a serine/threonine protein kinase (Martin *et al.*, 1993; Loh and Martin, 1995), and the *Xa21* gene of rice, which encodes both an LRR and a kinase domain (Song *et al.*, 1995), signaling may be a consequence of kinase activation leading to phosphorylation of a downstream target(s) (Zhou *et al.*, 1995, 1997). For other R genes, which possess combinations of an LRR motif, a putative nucleotide-binding site (NBS) and a toll/interleukin receptor homology domain (TIR), the potential mode of downstream signaling is less clear. However, an NBS/LRR gene, *Prf*, is required for resistance encoded by *Pto* (Salmeron *et al.*, 1994, 1996), and provides notional overlap between the disparate R signaling pathways.

Protein phosphorylation is a common theme in the control of metabolic and signaling pathways. Concerted effort to understand the structure and function of protein kinases has led to the solution of multiple three-dimensional crystal structures for these enzymes, of both serine/threonine and tyrosine specificity (reviewed in Morgan and de Bondt, 1994; Bossemeyer, 1995). Protein kinases share a catalytic core of 250–300 amino acid residues comprising 11 conserved subdomains, which fold into highly similar bilobal structures. The extent of spatial conservation of the catalytic core is such that individual crystal structures provide a useful template for kinases where only the amino acid sequence is known (Knighton *et al.*, 1992; Taylor *et al.*, 1993).

Control of protein kinase activity frequently is exerted by a structural element known as the activation segment (reviewed by Johnson *et al.*, 1996). This region lies between conserved sequence motifs and occupies the catalytic cleft of the enzyme. It comprises several smaller regulatory elements, including the T-loop, where activating phosphorylation events often occur, and the C-terminal P+1 loop, which plays a role in recognition and binding of protein substrates. The activation segment acts in

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disparate ways in different kinases, variously controlling ATP binding, alignment of key structural elements or protein substrate binding, hence suppressing kinase activity in the absence of specific stimulatory signals.

We have exploited the general conservation of protein kinase structure to conduct a mutational analysis of the *Pto* gene. *In vitro* generated mutants were characterized by analysis of ligand-binding properties in the yeast two-hybrid system, or by assay for induction of the hypersensitive disease resistance response (HR) in intact plants. We present molecular genetic evidence that the consequence of Pto–AvrPto interaction is activation of Pto kinase, and further map an AvrPto-binding determinant to the homologous P+1 loop of Pto. Mutations in the P+1 loop led to the constitutive induction of HR in the absence of AvrPto when expressed in *Nicotiana benthamiana* or tomato. Initiation of HR by *Pto* gain-of-function mutants was pathway dependent as judged by the requirement for a functional *Prf* gene. The generation of constitutive gain-of-function mutants of *Pto* provides insight into the consequence of AvrPto binding by Pto and the initiation of signal transduction subsequent to the binding event.

Results

Kinase activity of Pto is dispensable for AvrPto binding but required for AvrPto-dependent activation of HR

We previously showed that transient expression of *avrPto* in *N.benthamiana* tissue expressing a *Pto* transgene led to a *Pto*-dependent HR (Scofield *et al*., 1996). Pto is a protein kinase that autophosphorylates on serine and threonine residues *in vitro* (Loh and Martin, 1995). Previous mutational evidence suggested that the catalytic kinase activity of Pto is essential for binding AvrPto in the yeast two-hybrid system (Scofield *et al*., 1996). However, the dual deficiency of *in vitro* generated Pto mutants in autophosphorylation and AvrPto binding (Loh and Martin, 1995; Scofield *et al*., 1996) prevented investigation of whether formation of the AvrPto–Pto complex was sufficient in itself for activation of HR, or if the kinase activity of Pto was required further for signaling.

To examine the role of autophosphorylation in Pto function, we examined single amino acid mutations at Asp (D) 164. This residue is invariant in the protein kinase superfamily and is believed to act as the catalytic base for phosphoryl transfer based on comparisons with protein kinases of known structure (Bossemeyer, 1995). Asp164 was mutated individually to Ala (A), Glu (E) or Asn (N). Of these mutants, only pto^{D164N} was able to bind AvrPto in the yeast two-hybrid assay (Figure 1A). To test whether this mutant retained autophosphorylation activity, it was overexpressed in *Escherichia coli* as a maltose-binding protein (MBP) fusion. Wild-type Pto and the kinase-deficient mutant pto^{K69N} (Rommens *et al*., 1995) were included as positive and negative controls, respectively. Purified proteins were subjected to an autophosphorylation assay (Goring and Rothstein, 1992). Wild-type Pto was visualized as a band of ~77 kDa corresponding to the combined molecular mass of Pto plus the MBP (Figure 1B). No band corresponding to autophosphorylated Pto was observed either for pto^{K69N} or pto^{D164N}. Equivalent amounts of protein were loaded as assessed by visualiz-

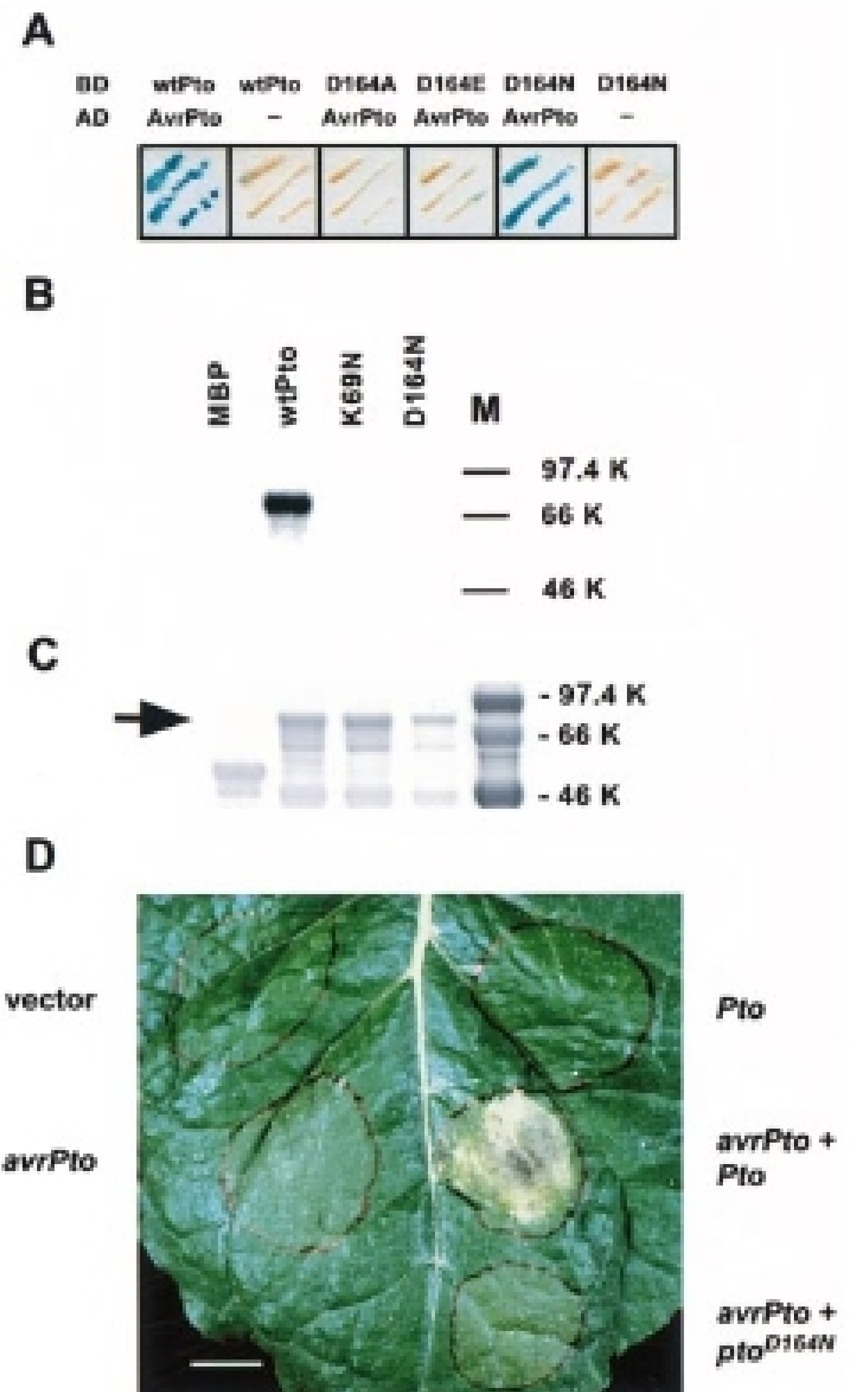

Fig. 1. Role of kinase activity in Pto function. (**A**) Yeast two-hybrid interactions between Pto Asp164 mutants [as GAL4-binding domain (BD) fusions in pAS2-1] and AvrPto [GAL4 activation domain (AD) fusion in pACT2]. wtPto indicates wild-type Pto; pto mutants are indicated using the single letter amino acid code. (–) represents empty activation domain plasmid pACT2. Representative yeast transformants were selected and streaked in triplicate on filter paper prior to further selective growth and assay for β-galactosidase activity. Blue color indicates a positive interaction between the fusion proteins. Pink coloration was associated with dense yeast growth. The strong interaction between Pto and AvrPto was associated with slow and inconsistent colony growth (compare with Figure 2B). Expression of all Pto–Gal4 BD fusion proteins in yeast used in this study was confirmed by Western analysis (data not shown). (**B**) The lack of auto-phosphorylation activity of Pto mutants. Mutations were introduced into *Pto* by site-directed mutagenesis and proteins expressed and purified as described in Materials and methods. Radiolabeled species were detected using a phosphoimager after 12% denaturing polyacrylamide gel electrophoresis. MBP indicates maltose-binding protein only control; M indicates molecular size standards obtained from Coomassie staining of the gel prior to autoradiography (Low Range; Bio-Rad, Hercules, CA). (**C**) Coomassie Blue-stained gel to show equivalent loading of fusion proteins tested for autophosphorylation activity. Lane order is as shown for (B). The position of the MBP–Pto fusion proteins is indicated by an arrow. (**D**) Requirement for Pto kinase capability for HR induction. Pto or its mutant derivatives were transiently expressed in *N.benthamiana* tissue using *A.tumefaciens* strain LBA4404 (~3×10^9 c.f.u./ml) as described in Materials and methods. Young expanding leaves of *N.benthamiana* were pressure infiltrated using a sterile 1 ml syringe, and the area of infiltration outlined using a black marker pen. Leaves were infiltrated as follows (binary plasmid identity in parentheses): (i) empty vector (pTFS-40); (ii) *Pto* (p40:35S:Ω:Pto); (iii) *avrPto* (pMD:35S:avrPto); (iv) *Pto* and *avrPto* (pMDA:35S:Ω:Pto); and (v) *pto*^{D164N} and *avrPto* (pMDA:35S:Ω:ptoD164N). HR development was photographed 4 days after infiltration. Scale bar = 1 cm.

ation of fusion proteins using Coomassie Blue (Figure 1D). Thus we conclude that pto^{D164N} is deficient in autophosphorylation, and that autophosphorylation is not required for Pto to bind AvrPto in the yeast two-hybrid system.

We next asked if the kinase activity of Pto is necessary for AvrPto-dependent induction of HR, using an *Agrobacterium tumefaciens*-mediated transient assay (Gopalan *et al.*, 1996; Scofield *et al.*, 1996). Infiltration of wild-type *N.benthamiana* leaves with suspensions of *A.tumefaciens* carrying an empty binary vector, or with *AvrPto* or *Pto* expressed individually from the T-DNA, did not result in a phenotypic change to the infiltrated plant tissue (Figure 1D). However, co-expression of *Pto* and *AvrPto* from the same T-DNA resulted in the tissue collapse and cell death that is characteristic of the HR and is a hallmark of *Pto*-mediated resistance (Scofield *et al.*, 1996). Initial tissue collapse was seen ~48 h after infiltration, and development of tissue necrosis was usually complete within 4 days. The area of HR development did not fill the entire area infiltrated with *Agrobacterium*. Co-expression of *pto*^{D164N} with *avrPto* did not result in development of an HR. Therefore, the kinase activity of Pto appeared to be required for induction of HR by AvrPto.

The activation segment of Pto has roles in AvrPto binding and Pto activation

We previously had mapped a determinant of AvrPto binding specificity in Pto to four variant amino acids with respect to the closely related Fen protein (Figure 2A; Scofield *et al.*, 1996). The amino acid residues responsible for AvrPto binding were K_{202}xTLxxxD_{209} (x indicates an invariant amino acid residue in the sequences of Pto and Fen). This sequence falls in the structural region known as the activation segment (Johnson *et al.*, 1996) residing between the conserved motifs $\underline{D}_{182}$FG and d_{209}P$\underline{E}$ (underlined residues are almost invariant in all serine/threonine and tyrosine protein kinase sequences known; lower case indicates a difference from the protein kinase consensus sequence; Hanks and Hunter, 1995). The activation segment has roles in regulation of kinase activity and positioning of the peptide substrate (Morgan and de Bondt, 1994; Bossemeyer, 1995), and activation of diverse protein kinases often occurs by regulatory phosphorylation events in this region (Morgan and de Bondt, 1994; Johnson *et al.*, 1996). The role of the activation segment in Pto function was analyzed using a mutational approach.

The activation segment of Pto (residues 182–209) contains seven phosphorylatable residues [Ser (S), Thr (T) or Tyr (Y)] approximately equally spaced throughout the region (Figure 2A). These were mutated individually to Ala and assayed for AvrPto binding activity in the yeast two-hybrid system. Five out of seven Pto Ala substitution mutants retained the ability to bind AvrPto in yeast (Figure 2B). The two mutants which lacked AvrPto binding activity had substitutions at the C-terminus of the activation segment, at Thr204 and Tyr207. Each of these residues lies in a substructure of the activation segment known as the P+1 loop, a region defined by analysis of crystal structures for which the consensus sequence G(T/S)xx(Y/F)xAPE can be written for protein serine/threonine kinases (Hanks *et al.*, 1988). The mutations that destroyed AvrPto binding activity correspond to the second and

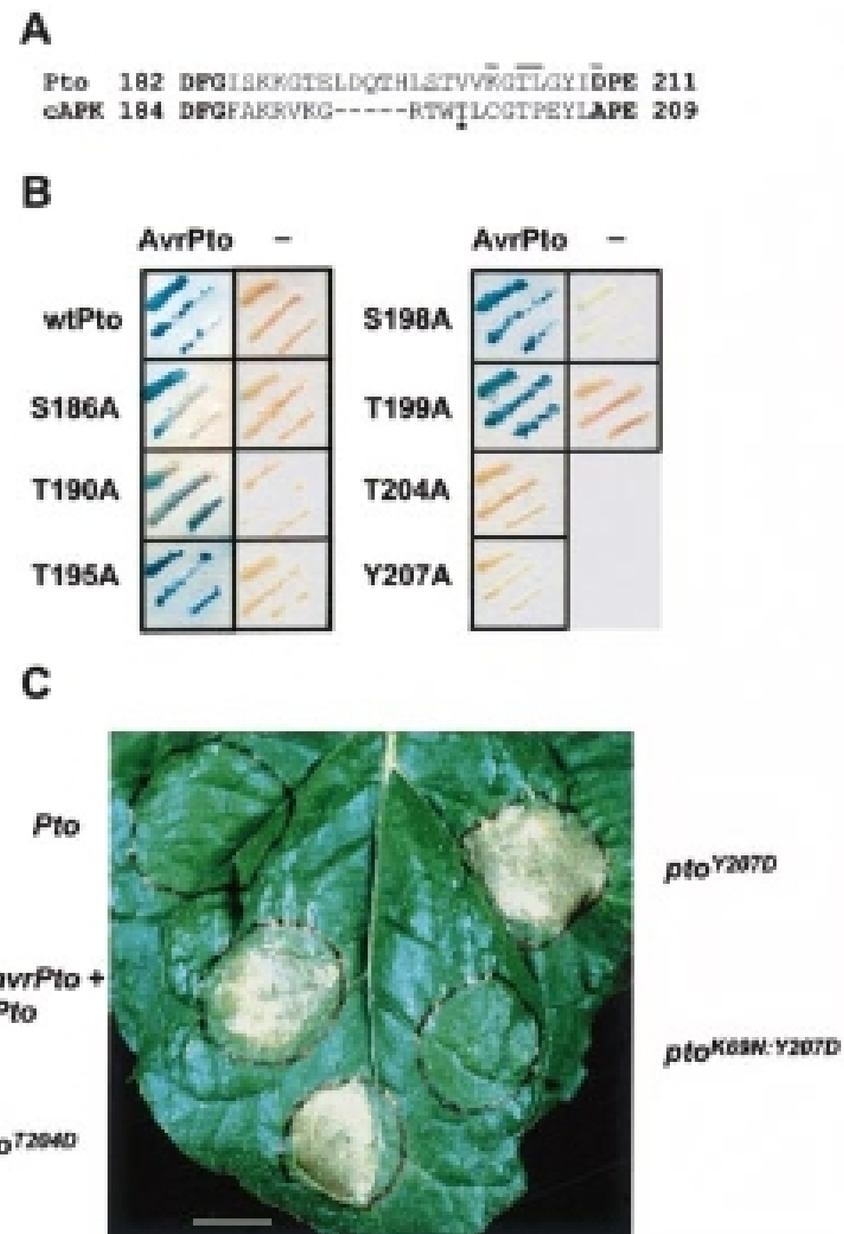

Fig. 2. Role of the activation segment in Pto function. (A) Sequence alignment of the activation segments of Pto and cAMP-dependent protein kinase (cAPK). The alignment follows Morgan and de Bondt (1994). Conserved sequence motifs bordering the segment are in bold. The activating phosphorylation site of cAPK (Thr197) is marked with an asterisk. The seven potentially phosphorylatable residues of Pto in this region are underlined, and the four residues contributing to AvrPto binding are overlined. (**B**) Activity of Ala substitution mutants in the Pto activation segment paired with AvrPto in the yeast two-hybrid assay. Pto and its mutant derivatives were expressed as described. wtPto and (–) indicate positive and negative controls, respectively (Figure 1A). The characteristic slow and erratic growth of Pto paired with AvrPto in yeast was seen for wild-type Pto and several of the mutants. (**C**) Mutations in the P+1 loop confer a constitutive gain-of-function phenotype that is dependent on kinase capability. *In vivo* assays were conducted as described for Figure 1. (i) *Pto* (p40:35S:Ω:Pto); (ii) *Pto* and *avrPto* (pMDA:35S:Ω:Pto); (iii) *pto*^{T204D} (p40:35S:Ω:ptoT204D); (iv) *pto*^{Y207D} (p40:35S:Ω:ptoY207D); and (v) *pto*$^{K69N;Y207D}$ (p40:35S:Ω:ptoK69N;Y207D). Scale bar = 1 cm.

fifth positions in the P+1 loop consensus sequence, respectively. The highly conserved nature of these residues indicates a role for this region in the tertiary structure of the Pto molecule. Thus, the P+1 loop of Pto was identified as an AvrPto-binding determinant both by amino acid differences defining a segment of Pto required for AvrPto binding relative to Fen (Scofield *et al.*, 1996; Frederick *et al.*, 1998) and by Ala substitution mutagenesis in the activation segment.

The role of the activation segment in Pto activity *in vivo* was tested using an Asp substitution strategy of the activation segment phosphorylatable residues. Acidic amino acids such as Asp or Glu can mimic the negative charge conferred by phosphorylation and may lead to partial constitutive activation of mutant animal kinases (e.g. Cowley *et al.*, 1994; Mansour *et al.*, 1994; Johnson