Constitutively active \textit{Pto} induces a \textit{Prf}-dependent hypersensitive response in the absence of \textit{avrPto}

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

\textit{Keywords}: disease resistance/epistasis/gain of function/ P\textsuperscript{+}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 \textit{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 \textit{et al}., 1997; Yang \textit{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 \textit{Pseudomonas syringae pv tomato} (\textit{Pst}) in tomato, specified by the \textit{Pto} gene. A strong and specific binding event between \textit{Pto} and the avirulence gene product \textit{AvrPto} was observed in the yeast two-hybrid assay for detecting protein–protein interactions (Scofield \textit{et al}., 1996; Tang \textit{et al}., 1996). Mutants of \textit{Pto} and \textit{avrPto} that exhibited reduced activity \textit{in vivo} showed impaired interaction when co-expressed in yeast. Although other complementary pairs of R and Avr genes have been cloned (Baker \textit{et al}., 1997), no evidence exists yet for physical interaction between the protein products of these genes. Most R proteins other than \textit{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; Parmiske \textit{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 \textit{Pto}, which encodes a serine/threonine protein kinase (Martin \textit{et al}., 1993; Loh and Martin, 1995), and the \textit{Xa21} gene of rice, which encodes both an LRR and a kinase domain (Song \textit{et al}., 1995), signaling may be a consequence of kinase activation leading to phosphorylation of a downstream target(s) (Zhou \textit{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, \textit{Prf}, is required for resistance encoded by \textit{Pto} (Salmeron \textit{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 \textit{et al}., 1992; Taylor \textit{et al}., 1993).

Control of protein kinase activity frequently is exerted by a structural element known as the activation segment (reviewed by Johnson \textit{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\textsuperscript{+}1 loop, which plays a role in recognition and binding of protein substrates. The activation segment acts in
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 ptoD164N 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 ptoK69N (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 ptoK69N or ptoD164N. 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 absence of activation domain plasmid pACT2. Representative yeast transformants that were selected and streaked in triplicate on nitrocellulose filter paper were assayed 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). (D) Requirement for Pto kinase capability for HR induction. Pto or its mutant derivatives were transiently expressed in N.benthamiana tissue using Agrobacterium strain LBA4404 (~3×10^7 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 (pMDA:35S:avrPto); (iv) Pto and avrPto (pMDA:35S:Ω:Pto); and (v) ptoD164N and avrPto (pMDA:35S:Ω:ptoD164N). HR development was photographed 4 days after infiltration. Scale bar = 1 cm.
The area of HR development did not fill the entire area of tissue necrosis was usually complete within 4 days. 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*Δ164N 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 K202XLxxxD209 (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 D182FG and d209PE (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)xxAPE can be written for protein serine/threonine kinases (Hanks *et al.*, 1988). The mutations that destroyed *AvrPto* binding activity correspond to the second and 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...
Expression of two mutants, product, consistent with the notion that AvrPto stimulates homologous P timing and extent to wild-type Pto stimulated by AvrPto. Two Ala substitution mutants (pto T204A and pto Y207A) that sensitive cell death in N.benthamiana signal transduction pathway by the mutant gene, we in either genetic background (Figure 3). Extensive HR Pto transient expression of wild-type Pst required for resistance to resistant tomato (et al., 1994). Individual Asp substitution mutants of Pto were tested for AvrPto-independent activity using the Agrobacterium-mediated transient assay. Expression of wild-type Pto (Figure 2C), or five of the seven Pto Asp substitution mutants (pto T190D, pto T195D, pto S186D and pto T199D), did not result in an HR (data not shown). Expression of two mutants, pto T204D and pto Y207D, in N.benthamiana resulted in tissue collapse and death similar to that resulting from co-expression of wild-type Pto and avrPto (Figure 2C). The phenotype of pto Y207D was the stronger of the two mutant genes and was visible 36–48 h after infiltration. Transient expression of this gene typically resulted in HR throughout the entire infiltrated area after the 4 day incubation period. Transient expression of pto T204D gave a weaker phenotype approximately equal in timing and extent to wild-type Pto stimulated by AvrPto. Strikingly, both activating mutations were located in the homologous P+1 loop region and corresponded to the two Ala substitution mutants (pto T204A and pto Y207A) that were unable to bind AvrPto in the yeast two-hybrid assay. Thus, the P+1 loop of Pto appears to play a major role in ligand binding and control of signal transduction. These two mutants represent constitutive gain-of-function variants of the Pto gene because they elicited hypersensitive cell death in the absence of the bacterial avirulence gene product. Furthermore, an interaction between AvrPto and Prf did not appear to be necessary for the HR response.

The role of kinase activity in the function of pto Y207D was investigated. We introduced a second mutation of this gene at the codon encoding Lys (K) 69, an invariant residue that frequently is mutated to abolish kinase activity in diverse kinases (Hanks and Hunter, 1995). Mutation of this residue abolished the autophosphorylation activity of Pto (Figure 1B; Loh and Martin, 1995; Rommens et al., 1995). Expression of the construct pto K69N / T207D did not induce the HR in N.benthamiana tissue (Figure 2C). Thus it appears that induction of HR by transient expression of pto Y207D was dependent on the kinase activity of the gene product, consistent with the notion that AvrPto stimulates Pto kinase activity.

pto Y207D activates a Prf-dependent HR in tomato

The results presented above indicated that in vivo expression of the mutant pto Y207D was sufficient to cause hypersensitive cell death in N.benthamiana. To establish whether this was due to activation of the cognate disease resistance signal transduction pathway by the mutant gene, we expressed wild-type Pto and pto Y207D in wild-type Pst-resistant tomato (Lycopersicon esculentum cv. Rio Grande 76R) and the isogenic fast neutron-induced mutant line that lacked a functional Prf gene (L.esculentum cv. Rio Grande 76R prf3/ prf3; Salmeron et al., 1994). Prf is required for resistance to Pst and sensitivity to the organophosphate insecticide Fenthion (Salmeron et al., 1994). Transient expression of wild-type Pto did not activate HR in either genetic background (Figure 3). Extensive HR was evident ~36 h after infiltration of wild-type tomato leaves with Agrobacterium expressing pto Y207D. In contrast, transient expression of pto Y207D in prf3/ prf3 tissue did not lead to an HR. Therefore, pto Y207D activates the cognate Pto–Prf signal transduction pathway and the mutant protein mimics the biologically active form of Pto. Moreover, these data demonstrate that Prf is epistatic to (and therefore not upstream of) Pto in the signal transduction pathway.

Tyro27 is a negative regulator of Pto activity and influences binding properties of Pto

Further analysis of the Pto constitutive gain-of-function phenotype focused on pto Y207D because of the strong HR induction by this gene. Tyr207 was changed to Trp (W), a chemically conservative change, or to Ala, a non-conservative change. For pto Y207W, no constitutive gain-of-function phenotype was observed in the normal time frame of the assay (4 days), although mild chlorotic symptoms could be seen ~7 days after infiltration (Figure 4A). Transient expression of pto Y207A caused a weak induction of HR, as evidenced by chlorosis and limited tissue collapse in the area of infiltration. Induction of hypersensitive cell death by the mutant pto Y207A indicates that disruption of the wild-type sequence at this point was sufficient to cause activation of Pto. Tyr207 is therefore a negative regulator of Pto activity. The constitutive gain-of-function phenotype of Pto genes mutated at codon 207 may not mimic the effect of a biological phosphorylation event at this residue because substitution of a non-phosphorylatable residue (Ala) still resulted in a constitutive gain-of-function phenotype. To investigate the role of phosphorylation at Tyr207 further, the mutant pto Y207W was co-expressed with the avrPto gene. This resulted in induction of sporadic HR and tissue chlorosis in the infiltrated area. Stimulation of the activity of pto Y207W by AvrPto further suggests that phosphorylation at Tyr207 is not a strict requirement for Pto activation. Similar experiments to examine the requirement for phosphorylation at Thr204 were not possible as we were unable to change this residue while retaining the ability of the mutant protein to interact with AvrPto.

We tested the ability of Pto constitutive gain-of-function mutants to bind AvrPto. Neither of the gain-of-function mutants pto Y207A (as shown above) or pto Y207D could bind AvrPto in the yeast two-hybrid system (Figure 4B). The mutant pto Y207W was able to bind AvrPto, consistent with
Mutants were assayed for activity in vivo mutations were made at Tyr207 to investigate the requirement for its stimulation of activity by AvrPto in vivo and the conservative nature of the amino acid change. A second Pto-binding protein, the serine/threonine kinase PtiI, had been identified in a yeast two-hybrid screen of a tomato cDNA library (Zhou et al., 1995). None of the three Pto Tyr207 mutants were able to interact with PtiI in the yeast assay, suggesting that the observed induction of HR was not dependent on an interaction between Pto and PtiI. Taken together, these data indicate that activation of Pto results in a change to the tertiary structure of the molecule, resulting in altered abilities to bind interacting proteins. The sequence integrity of the homologous P+1 loop of Pto appears to be necessary for correct regulation of signaling activity.

**Autophosphorylation activity of Pto is not required for HR induction by ptoY207D**

The Pto protein autophosphorylates in vitro when incubated in the presence of ATP (Loh and Martin, 1995). Although this activity does not appear to be required for the Pto–AvrPto interaction (see above), it may have a role in signal transduction in vivo. To test this, the mutants ptoY207D and ptoY207W were investigated for autophosphorylation activity. ptoY207W retained autophosphorylation capability, albeit at a lesser level than the wild-type fusion protein (Figure 4D). The strong constitutive gain-of-function mutant ptoY207D did not possess detectable autophosphorylation activity. Therefore, the ability of Pto to autophosphorylate is not necessary for induction of HR by the constitutive gain-of-function Pto mutants. The presence of approximately equal amounts of each fusion protein in the autophosphorylation assay was confirmed by staining with Coomassie Blue (Figure 1D). A biological role for the autophosphorylation event catalyzed by Pto kinase is not clear; however, loss of the autophosphorylation activity by ptoY207D does not necessarily imply that it is deficient in phosphorylation activity in trans.

**Discussion**

We describe here a molecular genetic analysis of the function of the plant disease resistance gene Pto. Reference to crystal structures of known protein kinases, particularly the ‘template’ cAMP-dependent protein kinase (CAPK; Knighton et al., 1991), allowed us to identify candidate residues in Pto for informative mutagenesis. The binding properties and in vivo activities of the mutant proteins permitted the dissection of avrPto-dependent signaling events specified by the Pto gene of tomato.

Several lines of evidence identified the homologous P+1 loop of Pto as an AvrPto-binding determinant. This structural feature binds and aligns the peptide substrate in diverse kinases (Taylor et al., 1995). The P+1 loop forms a hydrophobic pocket and contains several highly conserved residues. Individual mutation of two of these, Thr204 and Tyr207, to Ala deleted the ability of the mutant molecule to bind AvrPto in yeast. This finding is consistent with a role for the conserved residues in determining the three-dimensional structure of the Pto molecule. In addition, mutation of either of the residues to Asp conferred a constitutive gain-of-function phenotype to the mutants. Together, these data strongly suggest a role for the P+1 loop in AvrPto-dependent regulation of
the Pto molecule. Binding of AvrPto to Pto potentially leads to perturbation of the P+1 pocket, resulting in a modification of the tertiary structure of Pto into an active form. Activated Pto is structurally distinct from Pto unstimulated by AvrPto, as evidenced by the altered interaction of constitutive gain-of-function mutants with multiple binding proteins; the strong constitutively active mutant ptoY207D was unable to interact with either AvrPto or PtiL. Our data do not determine whether wild-type Pto is active as a monomer or an AvrPto–Pto heterodimer. It is also unclear whether ptoY207D is a molecular mimic as active kinase or PtiI. Our data do not determine whether wild-type Pto or PtiI. Our data do not determine whether wild-type Pto is active kinase or PtiI. Our data do not determine whether wild-type Pto is active kinase or PtiI. Our data do not determine whether wild-type Pto is active kinase or PtiI. Our data do not determine whether wild-type Pto is active kinase or PtiI. Our data do not determine whether wild-type Pto is active kinase or PtiI. The gain-of-function phenotype of ptoY207D in the absence of AvrPto has several implications. Activation of wild-type Pto by AvrPto is necessary and sufficient for induction of the resistance response. Binding of AvrPto to Prf is not required for induction of HR. Therefore, Pto is the receptor for AvrPto in vivo, as suggested by the interaction between these proteins in yeast (Scofield et al., 1996; Tang et al., 1996). Also, phosphorylation of AvrPto by Pto is not required for induction of hypersensitive cell death. Prf is necessary for induction of the HR, but there is no requirement for participation of Prf in recognition of the bacterial avirulence signal. Therefore, Prf lies coincident with or downstream of Pto in the signal transduction pathway. Recognition of a Pto–AvrPto complex by Prf was not sufficient to activate downstream signaling, because a kinase-deficient Pto mutant (ptoK69N) with AvrPto binding ability was unable to induce the HR when expressed with AvrPto in vivo. The role of AvrPto in initiation of HR is therefore limited to activation of Pto.

Activation of disease resistance signaling was assayed in these experiments as development of an HR in N. benthamiana or tomato. It is possible that the tissue collapse and death that we observed were due to a non-specific effect, such as a general stress response resulting from overexpression of signaling proteins. Tang et al. (1999) observed that overexpression of wild-type Pto in stable transgenic tomato lines activated defense responses and microscopic HR in high light conditions. However, we did not detect any phenotype from transient overexpression of wild-type Pto in planta. The constitutive gain-of-function phenotype of ptoY207D was dependent on the presence of a functional Prf gene. This implies that the Pto-dependent R pathway was being activated. Conditional expression of ptoY207D in the presence of a pathogen is necessary to determine if activation of Pto is sufficient for induction of the full disease resistance response.

Several lines of evidence suggest that AvrPto stimulates downstream phosphorylation by Pto. Co-expression of AvrPto and a kinase-deficient Pto mutant (ptoD164N) with AvrPto binding ability did not induce an HR. Therefore, the kinase ability of Pto was required for transduction of the AvrPto signal. Also, introduction of a second mutation (K69N), designed to destroy kinase activity in the constitutively active mutant ptoY207D, deleted the ability of the mutant to induce HR in vivo. Therefore, the ability of Pto to mediate the AvrPto signal seems dependent on its ability to phosphorylate a downstream target(s). Pto is an active kinase in vitro capable of autophosphorylation and transphosphorylation of PtiL (Loh and Martin, 1995; Zhou et al., 1995). However, the constitutively active mutant did not autophosphorylate. There appears to be a difference between the in vitro autophosphorylation activity of Pto and its ability to phosphorylate downstream component(s) in trans. The biological relevance of Pto autophosphorylation in vitro in the absence of AvrPto is therefore unclear, because autophosphorylation was not required for Pto to bind AvrPto, or for signal transduction by ptoY207D.

The Asp substitution strategy used here was designed to identify potential activating phosphorylation sites in Pto. Mutation of two sites (Thr204 and Tyr207) with residues to mimic the negative charge conferred by phosphorylation led to a constitutive gain-of-function phenotype for the mutant genes. However, further mutagenesis at Tyr207 showed that phosphorylation of this residue was not required for Pto activation. A regulatory phosphorylation event at Thr204 remains an open question, although this residue lies approximately five residues C-terminal to the canonical site of activating phosphorylation (Figure 2A). In addition, the HR-inducing activity of ptoY207D was equivalent to or in excess of that of wild-type Pto stimulated with AvrPto (Figure 2C), whereas Asp is a poor mimic of phosphoamino acids and leads to only partial activation of mutant kinases when substituted (Johnson et al., 1996). Mutational experiments at Tyr207 suggested that this residue, and by implication the P+1 loop, negatively regulates signaling by Pto. Consistent with this, we have found that substitution of non-phosphorylatable residues in the P+1 loop by Asp confers the constitutive gain-of-function phenotype (J.P. Rathjen and R.W. Michelmore, unpublished). The consequences of AvrPto binding or P+1 loop mutation on the conformation of Pto appear to be similar but await detailed structural characterization. Precedent for this type of negative autoregulation of kinase activity is given by titin kinase, which is inhibited by a P+1 loop tyrosine residue (Mayans et al., 1998). In addition, both ERK2 and the cyclin-dependent kinase CDK2 require substantial rearrangement of the P+1 loop prior to substrate binding and phosphorylation. This rearrangement is mediated either by activation segment phosphorylation or by binding of the regulatory (cyclin) subunit followed by phosphorylation in the activation segment, respectively (Russo et al., 1996; Canagarajah et al., 1997).

We propose the following model for Pto activation by AvrPto based on integration of the above data (Figure 5). Pto is present in the cytoplasm in an inactive form prior to interaction with AvrPto. The ability of Pto to bind AvrPto is dependent on the tertiary structure of the molecule, which may be contingent on the presence of ATP in the NBS, as evidenced by the inability of ptoK69N to interact with AvrPto in the yeast two-hybrid system (Hanks and Hunter, 1995; Scofield et al., 1996). Pto binds AvrPto, causing a distortion in the P+1 loop and a consequent change to the tertiary structure of Pto. Pto is now active and able to initiate signal transduction by phosphorylation of a downstream substrate(s). This model includes AvrPto and Prf dependence that were not aspects of previous models based on data available from interactions of native Pto observed ex planta (Zhou et al., 1995, 1997). A possible intermediate is an activated form of Pto stabilized by phosphorylation leading to release of AvrPto from the complex. If this occurred, the released AvrPto would be capable of activating further Pto.
molecules, resulting in amplification of the bacterial avirulence signal.

Confirmation that Pto rather than Prf is the receptor for AvrPto raises the question of the role of NBS/LRR genes in resistance signaling. Prf appears to have a primary role in signal transduction because its transgenic overexpression in resistance signaling. Prf appears to have a primary role in signal transduction because its transgenic overexpression raised the possibility that reception of the pathogen signal involved phosphorylation (possibly involving phosphorylation) in transduction of the AvrPto-derived signal. For other plant–pathogen interactions, the AvrPto-derived signal would not be detected. The AvrPto–Pto interaction is not detected using the AvrPto–Pto interaction.

\[ \text{AvrPto} \rightarrow \text{Pto} \rightarrow \text{Interaction} \]

**Materials and methods**

**Mutagenesis and generation of subclones for expression**

Standard techniques for manipulating plasmids in *E. coli* strain DH5α were used (Sambrook *et al.*, 1989). The *Pto* and *Fen* genes were cloned in the T/A site of the phagemid vector pCRII (Invitrogen, CA) and contain an engineered NcoI site at the 5′ ATG. Mutagenesis was performed by primer extension on single-stranded DNA templates following the method of Kunkel (1985). Primers for site-directed mutagenesis were as follows: PtoD164E, 5′ GCAATTTACATCGTTGAACTCTAGTCGCT3′; PtoD164A, 5′ GCAATTTACATCGTTGATGCTCTGACATGC3′; PtoD164N, 5′ GCAATTTACATCGTTGATGCTCTGACATGC3′; PtoD164N, 5′ GCAATTTACATCGTTGATGCTCTGACATGC3′; PtoL69N, 5′ GGGGCGGGCCAGTATGCGCTACCTCG3′. 

D and A mutants of phosphorylatable residues in the activation segment were constructed using degenerate primers, with selection of mutants by sequencing. Primer sequences were (M indicates A+C degeneracy): PtoSer186D/A, 5′ TTGGAGATGMAAGGAAAGGGACT3′; PtoThr190D/A, 5′ TCCAAGAAAGGAMTGGAGTGG3′; PtoThr195D/A, 5′ GCTGGTCAAGMCCACCTTACG3′; PtoSer198D/A, 5′ CCCATCTGGCAGACTGTTGAGG3′; PtoThr199D/A, 5′ CCCATCTGGCAGACTGTTGAGG3′; PtoThr204D/A, 5′ CTGGGAGGGAGMTCGCCGTACATT3′; and PtoY207D/A, 5′ ACTTCTGGGCGMCTTACGTCCAA3′.

All mutant genes were sequenced in their entirety using an automated DNA sequencer (Lico). For expression in *planta*, constructs were subcloned in the sense orientation into the vector pSPL4D4 (Jones *et al.*, 1992) using the *NcoI* and BanHI sites between the cauliflower mosaic virus (CaMV) 35S promoter–tobacco mosaic virus (TMV) Ω leader and the cos terminator. The entire cassette containing the gene flanked by the expression components was excised and cloned into the binary vector pTIPS-40 (British Sugar Corp, Norwich, UK) using the EcoRI and HindIII sites (for single expression; p40 series), or the binary vector pMD::AvrPto (derived from pbH121 by replacement of the GUS gene with *Pto*; Scofield *et al.*, 1996) using HindIII (for co-expression with avrPto; pMDA series). Recombinant binary plasmids were transferred to *A. tumefaciens* strain LBA4404 or GV2260 by the freeze–thaw technique. The presence of the plasmid in *A. tumefaciens* was confirmed by extraction and backtransformation of plasmid DNA into *E. coli*, after which the plasmid was amplified, purified and checked for identity by restriction digestion analysis. For yeast two-hybrid analysis, *Pto* and its mutant derivatives were cloned into the DNA-binding domain vector pAS2-1 (Clontech, CA) in-frame with the Gal4 fusion gene using *NcoI* and EcoRI. For expression in *E. coli*, *Pto* and its mutant derivatives were cloned in-frame with the MalE fusion gene in the plasmid vector pMalC2 (New England Biolabs, MA) using EcoRI.

**Cloning of Pth** Sequences homologous to Pth were obtained from RT–PCR or screening of a cDNA library. For PCR, primers corresponding to the 5′ *Pto* (5′ ATGAAGTGGCCTTACAGTTGTTG3′) and 3′ ends (Pth; 5′ CAAATT-CACGATCCTTGTGGA3′) of the Pth coding region were used to amplify from a *Marathon* cdNA library (Stratagene, CA) constructed from *L.esculentum* cv. Rio Grande 76R poly(A) RNA. Amplification products were cloned into pCR2.1 (Invitrogen, CA) using the T/A method according to the manufacturer’s instructions. Six independent clones were sequenced in their entirety and found to correspond exactly to the published sequence of Pth. The six genes were recovered; one identical to that obtained by RT–PCR, the other five mutant derivatives were cloned into the DNA-binding domain vector pAS2-1 (Clontech, CA) in-frame with the Gal4 fusion gene using *NcoI* and EcoRI. For expression in *E. coli*, *Pto* and its mutant derivatives were cloned in-frame with the MalE fusion gene in the plasmid vector pMalC2 (New England Biolabs, MA) using EcoRI.

**Transient Agrobacterium-mediated expression**

*Agrobacterium tumefaciens* strains LBA4404 (for *N. benthamiana*) or GV2260 (for tomato) containing the binary plasmid of interest were grown in 10 ml of LB media with appropriate antibiotics to stationary phase (~2 days) at 28°C. Cultures were diluted 1:10 into 10 ml of fresh LB plus antibiotics and grown overnight at 28°C. Cells were pelleted and washed once in 5 ml of infiltration media [0.1× MS salts, 0.1× B5 vitamins, 20 mM MOPS pH 5.4, 1% (w/v) glucose, 2% (w/v) sucrose; 200 uM acetylseryngone], then pelleted again and resuspended to an OD600 of 1.0 (for LBA4404) or 0.03 (for GV2260). Six- to 7-week-old
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**References**


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