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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*.

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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

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-

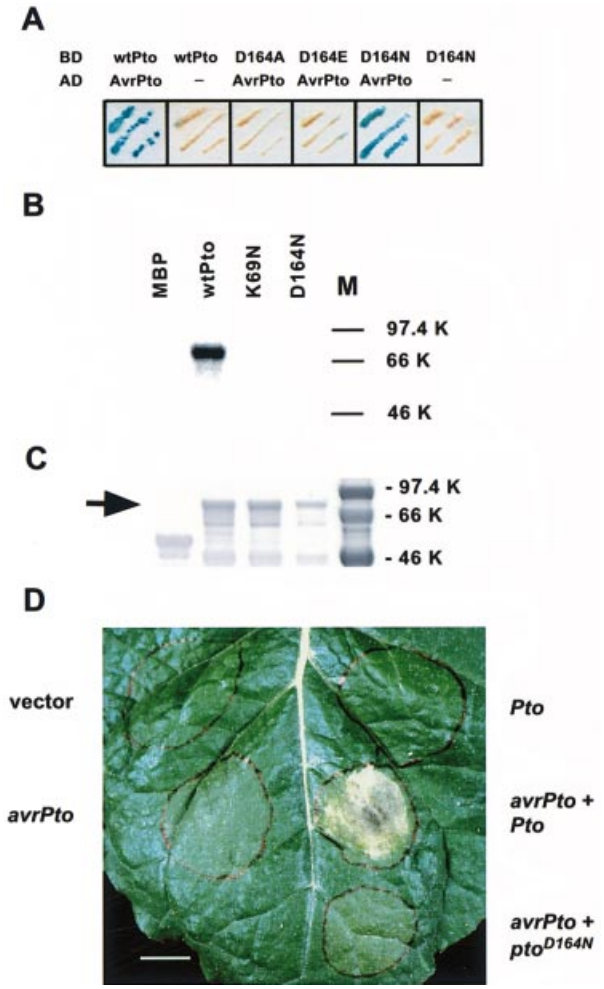

Fig. 1. Role of kinase activity in Pto function. (**A**) Yeast two-hybrid interactions between Pto Asp164 mutants [as GAL4-binding domain (BD) fusions in pAS2-1] and AvrPto [GAL4 activation domain (AD) fusion in pACT2]. wtPto indicates wild-type Pto; pto mutants are indicated using the single letter 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 $d_{209}\underline{PE}$ (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 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.

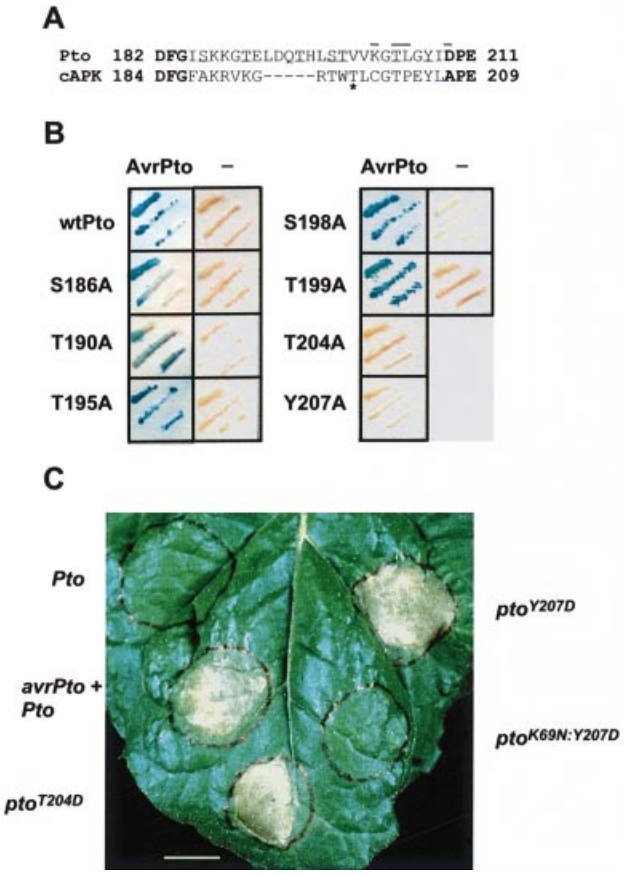

Fig. 2. Role of the activation segment in Pto function. (**A**) Sequence alignment of the activation segments of Pto and cAMP-dependent protein kinase (cAPK). The alignment follows Morgan and de Bondt (1994). Conserved sequence motifs bordering the segment are in bold. The activating phosphorylation 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.

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

et al., 1996). 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^{S186D}, pto^{T190D}, pto^{T195D}, pto^{S198D} 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 $pto^{K69N;Y207D}$ 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.

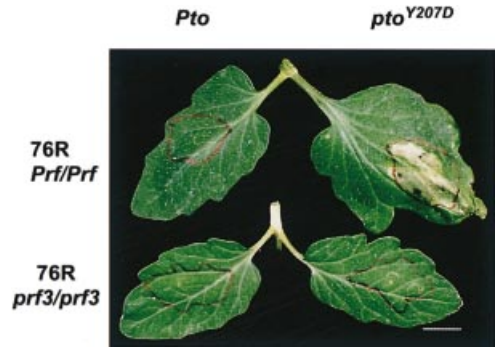

Fig. 3. A functional *Prf* gene is required for HR induction by Pto^{Y207D} in tomato. Constructs were expressed transiently in tomato similarly as for *N.benthamiana*, except that *A.tumefaciens* strain GV2260 (~1×10^8 c.f.u./ml) was used. Young tomato plants 3–3.5 weeks old (three-leaf stage) were infiltrated. (i) *Pto* (p40:35S:Ω:Pto) in resistant *L.esculentum* cv. Rio Grande 76R (*Pto*/*Pto Prf*/*Prf*); (ii) *Pto* [same as (i)] in *L.esculentum* cv. Rio Grande 76R *Pto*/*Pto prf3*/*prf3*; (iii) pto^{Y207D} (p40:35S:Ω:ptoY207D) in Rio Grande 76R; and (iv) pto^{Y207D} in Rio Grande 76R *Pto*/*Pto prf3*/*prf3*. Pto^{Y207D} also elicited an HR when expressed in Rio Grande 76S (*pto*/*pto Prf*/*Prf*) tissue (data not shown). Scale bar = 1 cm.

Tyr207 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

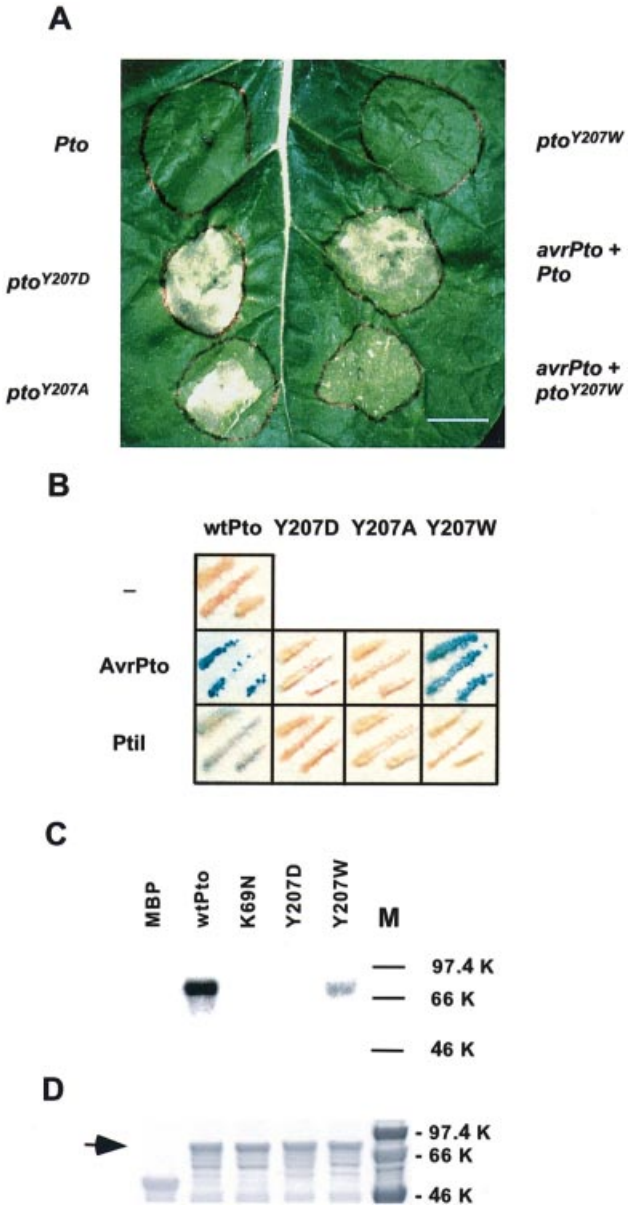

Fig. 4. Analysis of the role of Tyr207 in Pto regulation. (**A**) Further mutations were made at Tyr207 to investigate the requirement for phosphorylation at this site. Mutants were assayed for activity *in vivo* as described previously (Figure 1). (i) *Pto* (p40:35S:Ω:Pto); (ii) *pto*^{Y207D} (p40:35S:Ω:ptoY207D); (iii) *pto*^{Y207A} (p40:35S:Ω:ptoY207A); (iv) *pto*^{Y207W} (p40:35S:Ω:ptoY207W); (v) *Pto* and *avrPto* (pMDA:35S:Ω:Pto); and (vi) *pto*^{Y207W} and *avrPto* (pMDA:35S:Ω:ptoY207W). Scale bar = 1 cm. (**B**) Binding properties of Pto Tyr207 mutants. Pto, its mutant derivatives and AvrPto were expressed as described. PtiI was expressed as a GAL4 activating domain fusion in pACT2. Pto and its mutant derivatives (top line) were paired with binding partners as indicated in the left margin. '–' indicates empty activation domain plasmid pACT2. (**C**) Autophosphorylation activity of Tyr207 mutants. Mutant Pto proteins capable of inducing an HR *in vivo* were assayed for the ability to autophosphorylate *in vitro*. Expression, purification and assay for kinase activity of proteins was as described in Figure 1B. MBP indicates maltose-binding protein negative control. 'M' indicates molecular size standards obtained from Coomassie staining of the gel prior to autoradiography (Bio-Rad, Hercules, CA). (**D**) Coomassie Blue-stained gel to show equivalent loading of fusion proteins tested for autophosphorylation activity. Lane order is as shown for (C). The position of MBP–Pto is indicated by an arrow.

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 pto^{Y207D}

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 pto^{Y207D} and pto^{Y207W} were investigated for autophosphorylation activity. pto^{Y207W} retained autophosphorylation capability, albeit at a lesser level than the wild-type fusion protein (Figure 4D). The strong constitutive gain-of-function mutant pto^{Y207D} 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 pto^{Y207D} 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 pto^{Y207D} was unable to interact with either AvrPto or PtiI. Our data do not determine whether wild-type Pto is active as a monomer or an AvrPto–Pto heterodimer. It is also unclear whether pto^{Y207D} is a molecular mimic as well as a phenotypic mimic of Pto stimulated by AvrPto.

The gain-of-function phenotype of pto^{Y207D} 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 (pto^{D164N}) 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 *pto^{Y207D}* was dependent on the presence of a functional *Prf* gene. This implies that the Pto-dependent R pathway was being activated. Conditional expression of *pto^{Y207D}* 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 (pto^{D164N}) 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 pto^{Y207D}, 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 PtiI (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 pto^{Y207D}.

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 pto^{T204D} and pto^{Y207D} 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-phosphorylable 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 pto^{K69N} 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

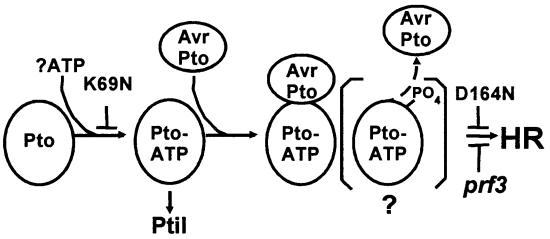

Fig. 5. Model of Pto activation by AvrPto. Mutation of Lys69, which anchors and orients the ATP moiety in other kinases (Hanks and Hunter, 1995), destroys the ability of Pto to interact with AvrPto (Scofield *et al*., 1996). Therefore, the AvrPto–Pto interaction is sensitive to Pto tertiary structure. Native Pto is able to interact with AvrPto (and PtiI; Zhou *et al*., 1995). Interaction with AvrPto causes activation of Pto, possibly mediated by displacement of the P+1 loop. A possible intermediate is activated Pto stabilized by phosphorylation, releasing AvrPto for activation of further Pto molecules. Activated Pto transduces the AvrPto signal, which is dependent on kinase capability and a functional *Prf* gene.

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 results in a constitutive systemic acquired resistance (SAR)-like phenotype (Oldryoyd and Staskawicz, 1998). There may be a physical interaction between Pto and Prf (possibly involving phosphorylation) in transduction of the AvrPto-derived signal. For other plant–pathogen interactions, NBS/LRR genes are thought to encode the receptors for the avirulence ligand because of their genetically defined roles as specificity determinants and the presence of the LRR motif in their predicted protein sequences. Certain regions of the LRRs encoded by several of these genes (e.g. *Cf9*, *Dm3*, *I2* homologs) appear to be under diversifying selection, suggesting that this region of these proteins has evolved in response to changes in pathogen populations (Parniske *et al.*, 1997; Botella *et al*., 1998; McDowell *et al*., 1998; Meyers *et al*., 1998). However, direct interaction between a pathogen-derived ligand and an NBS/LRR-containing protein has yet to be demonstrated. Prf does not seem to be the receptor for AvrPto, raising the possibility that reception of the pathogen signal is not mediated by an NBS/LRR protein in some other gene-for-gene interactions.

The constitutive gain-of-function Pto mutants described here overcome the ligand dependence of the wild-type protein. Expression of such mutants under the control of defined inducible promoters would be a useful strategy for expressing disease resistance or cell death in desired tissues and cell types. Pto homologs in tomato and other species might be made active by the same mutational strategy. This approach will be useful for evaluating the roles of protein kinases with unknown function in disease resistance. Furthermore, mutants such as pto^{Y207D} are potentially more pertinent in interaction cloning schemes than the wild-type protein, as the activated form is more likely to interact with downstream proteins.

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 *Nco*I 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′GCAATTATACATCGTGAAGTCAAGTCT3′; PtoD164A, 5′GCAATTATACATCGTGCTGTCAAGTCT3′; PtoD164N, 5′GCAATTATACATCGTAATGTCAAGTCT3′; PtoLys69N, 5′GGTGGCCCTGAATAGGCGTACACCTG3′.

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′TTTGGAATAGMCAAGAAAGGGACT3′; PtoThr190D/A, 5′TCCAAGAAAGGGGMTGAGCTTGAT3′; PtoThr195D/A, 5′GCTTGATCAAGMCCATCTTAGC3′; PtoSer198D/A, 5′CCCATCTTGMCACAGTAGTGAAAG3′; PtoThr199D/A, 5′CCCATCTTAGCGMCGTAGTGAAAG3′; PtoThr204D/A, 5′GTGAAAGGAGMTCTCGGCTACATT3′; and PtoY207D/A, 5′ACTCTCGGCGMCATTGACCCTGAA3′.

All mutant genes were sequenced in their entirety using an automated DNA sequencer (Licor). For expression *in planta*, constructs were subcloned in the sense orientation into the vector pSLJ4D4 (Jones *et al.*, 1992) using the *Nco*I and *Bam*HI sites between the cauliflower mosaic virus (CaMV) 35S promoter–tobacco mosaic virus (TMV) Ω leader and the ocs terminator. The entire cassette containing the gene flanked by the expression components was excised and cloned into the binary vector pTFS-40 (British Sugar Corp, Norwich, UK) using the *Eco*RI and *Hin*dIII sites (for single expression; p40 series), or the binary vector pMD::AvrPto (derived from pBI121 by replacement of the *GUS* gene with *avrPto*; Scofield *et al.*, 1996) using *Hin*dIII (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 *Nco*I and *Eco*RI. 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 *Eco*RI.

Cloning of PtiI

Sequences homologous to PtiI were obtained from RT–PCR or screening of a cDNA library. For PCR, primers corresponding to the 5′ (PtiF; 5′ATGAGGTGCTTCAGTTGTTGTG3′) and 3′ ends (PtiR; 5′CAAATTCACGATCCTCTTGTGA3′) of the PtiI 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 PtiI (DDBJ/EMBL/GenBank accession No. U28007; Zhou *et al.*, 1995) except for four insertions relative to the mRNA; a G between nucleotides 946 and 947; a T between nucleotides 957 and 958; a C between nucleotides 973 and 974; and a G between nucleotides 1124 and 1125. To determine if we had failed to detect the published sequence in the original PCR experiment, the PCR product was labeled with [^{32}P]dATP using nick translation (Stratagene kit, CA) and used to probe a *L.esculentum* cv. Rio Grande 76R cDNA library. Multiple clones were isolated and sequenced. Two classes of clones were recovered; one identical to that obtained by RT–PCR, the other evidently encoding a family member but containing all four insertions as outlined above (data not shown). The sequence obtained from RT–PCR was used in yeast two-hybrid analysis after subcloning into pACT2 (Clontech) using an engineered *Nco*I site at the 5′ ATG and the *Eco*RI site from the vector.

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 μM acetosyringone], then pelleted again and resuspended to an OD_{600} of 1.0 (for LBA4404) or 0.03 (for GV2260). Six- to 7-week-old

N.benthamiana plants, or 3-week-old tomato plants [*L.esculentum* cv. Rio Grande 76R or 76R *prf3/prf3* (Salmeron *et al.*, 1994)], both grown in the greenhouse, were inoculated by pressure infiltration using a 1 ml disposable syringe. The approximate area of infiltration was outlined immediately with a marker pen. Inoculated plants were kept in constant low light in laboratory conditions for 4 days to allow symptoms to develop.

Yeast two-hybrid analysis

Yeast two-hybrid analysis was performed using the MATCHMAKER GAL4 system (Clontech) following the manufacturer's protocols. *AvrPto* was expressed from the activating domain (AD) plasmid pACT2 (Scofield *et al.*, 1996), and *Pto* and its mutant derivatives were expressed from the DNA-binding domain (BD) plasmid pAS2-1. Yeast co-transformants containing GAL4 AD and BD plasmids were selected by growth on selective media and replica plated on filters (Whatman No. 1), before assay for activation of the β-galactosidase marker gene using the substrate X-gal following the manufacturer's directions.

Protein expression and autophosphorylation assays

Pto and its mutant derivatives were induced and expressed as fusion proteins with MBP (pMAL-c2; New England Biolabs) in *E.coli* strain DH5α according to the manufacturer's instructions. Overnight cultures of *E.coli* (200 ml) containing the desired clone were grown to stationary phase overnight at 37°C with shaking, then diluted 1:100 into fresh media (200 ml) and grown to an OD_{600} of 0.4 before induction with 1 mM isopropyl-β-D-thiogalactopyranside (IPTG). Induced cultures were incubated for a further 2 h at 37°C with shaking to express the protein, then pelleted, washed once in column buffer [20 mM Tris–HCl pH 7.4, 200 mM NaCl, 1 mM EDTA, containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin and 1 mM dithiothreitol (DTT)], pelleted again and resuspended in 10 ml of column buffer, and lysed by sonication. For autophosphorylation assays, 0.5 ml of the lysate was incubated with 50 μl of amylose resin (1:1 in column buffer), then washed twice in 200 vols of column buffer and once in kinase buffer [30 mM Tris–HCl pH 7.5, 20 mM HEPES pH 7.1, 10 mM $MgCl_2$, 2 mM $MnCl_2$ (Douville *et al.*, 1992) containing 1 mM PMSF and 1 mM DTT] in a batch purification procedure. Proteins retained on amylose beads were incubated in 50 μl of kinase buffer containing 25 μCi of [γ-^{32}P]ATP (6000 Ci/mmol) and 100 μM cold ATP, and incubated at room temperature for 30 min. The beads were pelleted, resuspended in 20 μl of 2× loading solution and boiled for 5 min, before electrophoresis through a 12% SDS–Tris/tricine gel. The gel was dried and radioactivity visualized using a PhosphorImager (Molecular Dynamics, CA). For estimation of protein loadings, aliquots were run on a parallel gel as described above except that the fusion proteins were not subjected to an autophosphorylation assay beforehand. The gel was stained with Coomassie Brilliant Blue R250 and dried prior to photography.

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References

Baker,B., Zambryski,P., Staskawicz,B. and Dinesh-Kumar,S.P. (1997) Signaling in plant–microbe interactions. *Science*, **276**, 726–733.

Bent,A.F. (1996) Plant disease resistance genes—function meets structure. *Plant Cell*, **8**, 1757–1771.

Bossemeyer,D. (1995) Protein kinases—structure and function. *FEBS Lett.*, **369**, 57–61.

Botella,M.A., Parker,J.E., Frost,L.N., Bittner-Eddy,P.D., Beynon,J.L., Daniels,M.J., Holub,E.B. and Jones,J.D. (1998) Three genes of the *Arabidopsis RPP1* complex resistance locus recognize distinct *Peronospora parasitica* avirulence determinants. *Plant Cell*, **10**, 1847–1860.

Canagarajah,B.J., Khokhlatchev,A., Cobb,M.H. and Goldsmith,E.J. (1997) Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. *Cell*, **90**, 859–869.

Cowley,S., Paterson,H., Kemp,P. and Marshall,C.J. (1994) Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell*, **77**, 841–852.

Crute,I.R. (1986) The genetic basis of relationships between microbial parasites and their hosts. In Fraser,R.S.S. (ed.), *Mechanisms of Resistance in Plant Diseases*. Martinus Nijhoff and W.Junk, Dordrecht, The Netherlands, pp. 80–142.

Douville,E.M., Afar,D.E.H., Howell,B.W., Letwin,K., Tannock,L., Ben-David,Y., Pawson,T. and Bell,J.C. (1992) Multiple cDNAs encoding the esk kinase predict transmembrane and intracellular enzyme isoforms. *Mol. Cell. Biol.*, **12**, 2681–2689.

Frederick,R.D., Thilmony,R.L., Sessa,G. and Martin,G.B. (1998) Recognition specificity for the bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase. *Mol. Cell*, **2**, 241–245.

Gopalan,S., Bauer,D.W., Alfano,J.R., Loniello,A.O., He.,S.-Y. and Collmer,A. (1996) Expression of the *Pseudomonas syringae* avirulence protein AvrB in plant cells alleviates its dependence on the hypersensitive response and pathogenicity (Hrp) secretion system in eliciting genotype-specific hypersensitive cell death. *Plant Cell*, **8**, 1095–1105.

Goring,D.R. and Rothstein,S.J. (1992) The S-locus receptor kinase gene in a self-incompatible *Brassica napus* line encodes a functional serine/threonine kinase. *Plant Cell*, **4**, 1273–1281.

Hammond-Kosack,K.E. and Jones,J.D. (1996) Resistance gene-dependent plant defense responses. *Plant Cell*, **8**, 1773–1791.

Hanks,S.K. and Hunter,T. (1995) The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.*, **9**, 576–596.

Hanks,S.K., Quinn,A.M. and Hunter,T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science*, **241**, 42–52.

Johnson,L.N., Noble,M.E.M. and Owen,D.J. (1996) Active and inactive protein kinases: structural basis for regulation. *Cell*, **85**, 149–158.

Jones,D.A. and Jones,J.D.G. (1996) The role of leucine-rich repeat proteins in plant defences. *Adv. Bot. Res.*, **24**, 89–167.

Jones,J.D.G., Shlumukov,L., Carland,C., English,J., Scofield,S.R., Bishop,G.J. and Harrison,K. (1992) Effective vectors for transformation, expression of heterologous genes and assaying transposon excision in transgenic plants. *Transgenic Res.*, **1**, 285–297.

Knighton,D.R., Zheng,J., Ten Eyck,L.F., Ashford,V.A., Xuong,N.-H., Taylor,S.S. and Sowadski,J.M. (1991) Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science*, **253**, 407–420.

Knighton,D.R., Pearson,R.B., Sowadski,J.M., Means,A.R., Ten Eyck,L.F., Taylor,S.S. and Kemp,B.E. (1992) Structural basis for intrasteric regulation of myosin light chain kinase. *Science*, **258**, 130–135.

Kunkel,T. (1985) Rapid and efficient site-directed mutagenesis without phenotypic selection. *Proc. Natl Acad. Sci. USA*, **82**, 488–492.

Loh,Y.-T. and Martin,G.B. (1995) The *Pto* bacterial resistance gene and the *Fen* insecticide sensitivity gene encode functional protein kinases with serine/threonine specificity. *Plant Physiol.*, **108**, 1735–1739.

Mansour,S.J., Matten,W.T., Hermann,A.S., Candia,J.M., Rong,S., Fukasawa,K., Vande Woude,G.F. and Ahn,N.G. (1994) Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science*, **265**, 966–970.

Martin,G.B., Brommonschenkel,S., Chunwongse,J., Frary,A., Ganal, M.W., Spivey,R., Wu,T., Earle,E.D. and Tanksley,S.D. (1993) Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science*, **262**, 1432–1436.

Mayans,O., van der Ven,P.F.M., Wilm,M., Mues,A., Young,P., Fürst,D.O., Willmanns,M. and Gautel,M. (1998) Structural basis for activation of the titin kinase domain during myofibrillogenesis. *Nature*, **395**, 863–869.

McDowell,J.M., Dhandayham,M., Long,T.A., Aarts,M.G.M., Goff,S., Holub,E.B. and Dangl,J.L. (1998) Gene conversion, positive selection and intergenic recombination contribute to the evolution of the *RPP8* pathogen resistance gene. *Plant Cell*, **10**, 1861–1874.

Meyers,B.C., Shen,K.A., Rohani,P., Gaut,B.S. and Michelmore,R.W. (1998) Receptor-like genes in the major resistance locus of lettuce are subject to divergent selection. *Plant Cell*, **11**, 1833–1846.

Morgan,D.O. and De Bondt,H.L. (1994) Protein kinase regulation: insights from crystal structure analysis. *Curr. Opin. Cell Biol.*, **6**, 239–246.

Oldroyd,G.E.D. and Staskawicz,B.J. (1998) Genetically engineered broad-spectrum disease resistance in tomato. *Proc. Natl Acad. Sci. USA*, **195**, 10300–10305.

Parniske,M., Hammond-Kosack,K.E., Golstein,C., Thomas,C.M., Jones, D.A., Harrison,K., Wulff,B.B.H. and Jones,J.D.G. (1997) Novel disease resistance specificities result from sequence exchange between tandemly repeated genes at the *Cf-4/9* locus of tomato. *Cell*, **91**, 821–832.

Rommens,C.M.T., Salmeron,J.M., Baulcombe,D.C. and Staskawicz,B.J. (1995) Use of a gene expression system based on potato virus X to rapidly identify and characterize a tomato *Pto* homolog that controls fenthion sensitivity. *Plant Cell*, **7**, 249–257.

Russo,A.A., Jeffrey P.D. and Pavletich,N.P. (1996) Structural basis of cyclin-dependent kinase activation by phosphorylation. *Nature Struct. Biol*., **3**, 696–700.

Salmeron,J.M., Barker,S.J., Carland,F.M., Mehta,A.Y. and Staskawicz, B.J. (1994) Tomato mutants altered in bacterial disease resistance provide evidence for a new locus controlling pathogen recognition. *Plant Cell*, **6**, 511–520.

Salmeron,J.M., Oldroyd,G.E.D., Rommens,C.M.T., Scofield,S.R., Kim,H.-S., Lavelle,D.T., Dahlbeck,D. and Staskawicz,B.J. (1996) Tomato *Prf* is a member of the leucine-rich-repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. *Cell*, **86**, 123–133.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scofield,S.R., Tobias,C.M., Rathjen,J.P., Chang,J.H., Lavelle,D.T., Michelmore,R.W. and Staskawicz,B.J. (1996) Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science*, **274**, 2063–2065.

Song,W.-Y. *et al*. (1995) A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. *Science*, **270**, 1804–1806.

Staskawicz,B.J., Ausubel,F.M., Baker,B.J., Ellis,J.G. and Jones,J.D. (1995) Molecular genetics of plant disease resistance. *Science*, **268**, 661–667.

Tang,X., Frederick,R.D., Zhou,J., Halterman,D.A., Jia,Y. and Martin,G.B. (1996) Initiation of plant disease resistance by physical interaction of AvrPto and the Pto kinase. *Science*, **274**, 2060–2063.

Tang,X., Xie,M., Kim,Y.J., Zhou,J., Klessig,D.F. and Martin,G.B. (1999) Overexpression of Pto activates defense responses and confers broad resistance. *Plant Cell*, **11**, 15–30.

Taylor,S.S., Knighton,D.R., Zheng,J., Sowadski,J.M., Gibbs,C.S. and Zoller,M.J. (1993) A template for the protein kinase family. *Trends Biochem. Sci.*, **18**, 84–89.

Taylor,S.S., Radzio-Andzelm,E. and Hunter,T. (1995) How do protein kinases discriminate between serine/threonine and tyrosine? Structural insights from the insulin receptor protein-tyrosine kinase. *FASEB J.*, **9**, 1255–1266.

Yang,Y., Shah,J. and Klessig,D.F. (1997) Signal perception and transduction in plant defense responses. *Genes Dev.*, **11**, 1621–1639.

Zhou,J., Loh,Y.-T., Bressan,R.A. and Martin,G.B. (1995) The tomato gene *PtiI* encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response. *Cell*, **83**, 925–935.

Zhou,J., Tang,X. and Martin,G.B. (1997) The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis*-element of pathogenesis-related genes. *EMBO J.*, **16**, 3207–3218.

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