A Patch of Surface-Exposed Residues Mediates Negative Regulation of Immune Signaling by Tomato Pto Kinase

Ai-Juan Wu, Vasilios M.E. Andriotis, Marcus C. Durrant, and John P. Rathjen

Tomato (*Lycopersicon esculentum*) Pto kinase specifically recognizes the *Pseudomonas* effector proteins AvrPto and AvrPtoB, leading to induction of defense responses and hypersensitive cell death. Structural modeling of Pto combined with site-directed mutagenesis identified a patch of surface-exposed residues required for native regulation of signaling. Mutations in this area resulted in constitutive gain-of-function (CGF) forms of Pto that activated AvrPto-independent cell death via the cognate signaling pathway. The patch overlaps the peptide binding region of the kinase catalytic cleft and is part of a broader region required for interaction with bacterial effectors. We propose that the negative regulatory patch is normally occupied by a peptide that represses Pto signaling. Furthermore, we found that Pto kinase activity was required for Avr-dependent activation but dispensable for signaling by CGF forms of Pto. This suggests that Pto signals by a conformational change rather than phosphorylation of downstream substrates in the defense signaling pathway.

INTRODUCTION

Plants possess innate defense responses against attack by pathogenic organisms, including viruses, microbes, and invertebrates (Hammond-Kosack and Jones, 1997). Specific disease resistance, in which the host responds to a narrow subset of pathogens, is associated with precise recognition events described in genetic terms by the gene-for-gene hypothesis (Flor, 1971). In such cases, plant resistance is inherited as a single dominant or semidominant gene. For the plant resistance (*R*) gene to be functional the pathogen must carry a complementary, dominant avirulence (*Avr*) gene. Host susceptibility results if one or the other of the complementary partners is absent.

Ellingboe (1981) first proposed that direct interaction between cognate *R* and *Avr* gene products might underlie gene-for-gene resistance. Most *R* genes encode proteins containing a central conserved nucleotide binding site (NBS) domain (i.e., an NBS typical of Apaf-1, R proteins, and CED4) and C-terminal leucinerich repeats (LRRs) (Dangl and Jones, 2001). Some data are consistent with a direct binding event between NBS-LRR-containing proteins and *Avrs* (Jia et al., 2000); however, the weight of evidence supports indirect perception (Dangl and Jones, 2001). Perception of *AvrRpm1*, *AvrB*, and *AvrRpt2* was coordinated by the RIN4 protein (Mackey et al., 2002), which interacted with both cognate *R* determinants RPM1 and RPS2 (Axtell and Staskawicz, 2003; Mackey et al., 2002, 2003). Another example is *Arabidopsis thaliana* PBS1 kinase, which was cleaved by the avirulence determinant AvrPphB (Shao et al., 2003). This event was necessary and possibly sufficient for elicitation of resistance but was upstream of cognate recognition specified by the NBS-LRR protein RPS5. Thus, there is evidence for the involvement of additional proteins in the specific recognition of avirulence determinants. However, it is likely that NBS-LRR proteins play a key proximal role in recognition because they act as specificity determinants particularly through their LRR domains (Rathjen and Moffett, 2003). The role of the NBS-LRR protein is sometimes described in terms of the guard hypothesis, in which each R protein is proposed to recognize a putative complex between the avirulence protein and its intracellular target to initiate downstream signaling (Dangl and Jones, 2001). Downstream events associated with *R* signaling appear to be similar for all pathways and frequently include a cell death phenotype known as the hypersensitive response (HR) (Hammond-Kosack and Jones, 1996).

The *Pto* gene of tomato conditions race-specific resistance to *Pseudomonas syringae* pathovar *tomato* strains carrying *avrPto* or *avrPtoB* (Pedley and Martin, 2003). *Pto* is a Ser-Thr protein kinase with no apparent receptor domain (Martin et al., 1993). *Pto* appears to interact directly with AvrPto and AvrPtoB, presumably after their delivery into host cells via the type III protein secretion system (Pedley and Martin, 2003). *Pto* binds to AvrPto and AvrPtoB in yeast two-hybrid assays, and mutations in either *Pto* or *avrPto* that disrupt disease resistance in planta also abolish the interaction in yeast. However, no physical interaction in planta between *Pto* and either Avr has been reported. Downstream signaling by *Pto* requires the *Prf* gene, which encodes a large NBS-LRR protein (Salmeron et al., 1996). The functional role of *Prf* is unknown, although it acts coincident with or downstream of *Pto* in the signal transduction pathway (Rathjen et al., 1999). Further potential effectors of *Pto* signaling are Pti,

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which encodes another protein kinase, and Pti 4/5/6, a family of transcription factor–like genes (Zhou et al., 1995, 1997). Both PtiI and Pti4 are substrates of Pto in vitro (Gu et al., 2000), although it is unknown whether these proteins interact with Pto in vivo.

Protein kinases are frequent points of control in diverse signaling pathways, and their structure and enzymology are well understood (Huse and Kuriyan, 2002). There are four kinase substructures that control ATP binding and orientation, binding of the peptide substrate, and catalytic phosphotransfer. These are folded within the bilobal kinase domain in precise molecular orientation to compose the functional enzyme. ATP binds to the smaller N-terminal lobe, whereas the substrate binding is controlled by the C-terminal lobe, with the catalytic loop resident in the interdomain cleft. Kinase activity can be repressed in various ways by altering the critical alignment of the key catalytic substructures or by preventing access of substrate molecules to their respective binding sites. Frequently, negative regulation is reversed by a phosphorylation event(s) in the activation segment, a loop region that lies within the catalytic cleft. The activation segment is composed of two recognizable regions: the T-loop, in which regulatory phosphorylation often occurs, and the P+1 loop, which forms the primary binding site for the substrate peptide. Regulatory phosphorylation causes the activation segment to flip out of the active site and assume a characteristic conformation, which may cause subdomain realignment and/or release of steric hindrance. The activated enzyme is then poised for catalysis.

Molecular analyses of the Pto protein showed that P+1 loop residues, rather than the activation segment per se, are important for the control of signal transduction. Firstly, Pto expressed and purified from Escherichia coli is active on several substrates (Sessa et al., 1998; Gu et al., 2000), which appears to exclude subdomain misalignment as an intrinsic mechanism of control. Secondly, structure–function studies revealed four residues within the P+1 loop necessary for AvrPto binding (Scofield et al., 1998); one of these (Thr-204) was sufficient to confer AvrPto binding to the Pto homolog Fen (Frederick et al., 1998). Scanning mutagenesis within the Pto T-loop region did not affect AvrPto binding, whereas mutation of the P+1 loop residues Thr-204 and Tyr-207 deleted the AvrPto interaction. Strikingly, Asp substitutions of Thr-204 and Tyr-207 conferred a CGF phenotype of AvrPto-independent HR to Pto (Rathjen et al., 1999). A functional Prf gene was required for this phenotype, indicating activation of the cognate signal transduction pathway. Therefore, residues required for AvrPto binding are also responsible for correct regulation of signaling activity.

In this study, a combination of site-directed mutagenesis and structural modeling revealed the involvement of most P+1 loop residues in Pto regulation. P+1 loop residues are part of a broader surface-exposed patch that mediates negative regulation of Pto signaling. This patch appears to be a shared docking site for both an unknown negative regulatory molecule and the specific Pto ligands AvrPto and AvrPtoB. The phosphorylation capability of Pto was dispensable for induction of the HR by Pto CGF mutants. Therefore, Pto does not phosphorylate the proximal protein in signal transduction and may act instead as an adaptor protein.

RESULTS

Mutations throughout the Pto P+1 Loop Confer CGF Activity

To explore if all P+1 loop residues are required for normal regulation of Pto, the amino acids between 201 and 210 (Rathjen et al., 1999; Moore et al., 2003) were mutated individually to Asp. Mutant genes were expressed transiently in Nicotiana benthamiana leaf tissue using Agrobacterium tumefaciens. Coexpression of wild-type Pto with avrPto resulted in the HR within 4 d, indicative of pathway activation, as did individual expression of ptoT204D or ptoG206D (Figure 1A). Transient expression of the mutants ptoV200D, ptoG206D, ptoI208D, ptoL205D, or ptoV201D also induced the HR, consistent with a role for these residues in control of Pto signaling. By contrast, expression of ptoT204D or ptoP210D, or wild-type Pto in the absence of avrPto, failed to elicit cell death. The requirement for Val-201 is unclear because the mutant protein did not accumulate (Figure 1B), whereas protein was detected from all other constructs using Pto-specific antisera. These data are consistent with involvement of most P+1 loop residues in control of Pto signaling with the further exception of Pro-210, which is the C-terminal residue in the sequence. Similarly, expression of ptoT199D (Rathjen et al., 1999) or ptoG206D (data not shown) did not induce the HR. Both of these residues are amino-proximal to the P+1 loop. Therefore, we conclude that the Pto P+1 loop is a negative regulator of Pto signaling leading to the HR.

We asked whether the cell death induced by P+1 loop mutants was attributable to specific elicitation of the cognate signal transduction pathway or to activation of a novel stress response. Signaling by Pto is dependent on the Prf gene, and virus-induced gene silencing (VIGS) of an N. benthamiana Prf homolog specifically compromises disease resistance to P. syringae pv tabaci expressing avrPto (Lu et al., 2003). Transient expression of the P+1 loop CGF mutants described above in N. benthamiana tissue silenced for Prf did not induce the HR (Figure 1C). Conversely, the HR induced by expression of these mutants was retained in control plants infected with the empty silencing vector. Thus, the loss of HR in Prf-silenced plants was attributable to the absence of Prf and not to secondary effects of VIGS. These data are consistent with specific activation of the Pto-Prf signal transduction pathway by the P+1 loop substitution mutants.

Surface-Exposed Amino Acids of the P+1 Loop Are Responsible for the CGF Phenotype

To explore the potential effect(s) of P+1 loop mutations on Pto kinase structure, a three-dimensional homology model was constructed using the crystal structures of the c-Abl and BTK Tyr kinases (Mao et al., 2001; Nagar et al., 2003). The BTK activation loop is in a noninhibitory conformation, whereas the c-Abl activation loop is oriented in essentially the opposite direction and obstructs the putative active site. We used these two structures to model alternative active and inactive structures for Pto. The models conform closely to the...
canonical bilobal kinase core but exclude the 20 N-terminal nonkinase amino acids for which a suitable template was not available (Figure 2). The junction of the two lobes forms the catalytic cleft, and the ATP molecule is shown docked onto the N-terminal lobe. The $P^{+1}$ loop and the presumed catalytic residue Asp-164 are located in close proximity in the C-terminal lobe. $P^{+1}$ loop residues were mapped onto the active Pto model. This revealed that the side chains of residues Val-201, Val-202, Gly-203, Thr-204, Leu-205, Gly-206, and Ile-208 are surface exposed, consistent with a role for these amino acids in substrate binding. The side chain of Tyr-207 is semi-buried; however, the partial exposure of the aromatic ring in our model is similar to crystal structures of cAPK, where it is thought to interact directly with substrate residues (Moore et al., 2003). Pro-210 is buried within the molecule. Thus, there is a correlation between the surface exposure of Pto $P^{+1}$ loop residues and their ability to specify cell death when mutated to Asp. The data are

![Figure 1. Acidic Mutations throughout the Pto $P^{+1}$ Loop Confer CGF Activity.](image)

(A) Expression of Pto $P^{+1}$ loop mutants in N. benthamiana leaves. Leaves were infiltrated with A. tumefaciens containing each construct as indicated and the area of infiltration outlined with a pen. Leaves are shown 4 d after infiltration.

(B) Accumulation of mutant proteins in planta. Total protein was extracted from leaves and blotted against polyclonal Pto antisera. Equivalent loadings were confirmed by Coomassie blue staining of the membrane (data not shown). Benth, noninfiltrated leaf tissue; EV, empty vector control.

(C) Prf is required for cell death induced by $P^{+1}$ loop mutants. Left, N. benthamiana leaves silenced for the Prf gene; right, leaves silenced with the empty VIGS vector. Top leaves were infiltrated with each Agrobacterium culture as indicated 4 weeks after inoculation with the tobacco rattle virus (TRV) silencing vector. Leaves were infiltrated identically on left and right.
consistent with a model in which the solvent-exposed residues mediate the regulation of Pto, possibly within the canonical role of P+1 loop residues in peptide binding.

**Kinase Activity Is Dispensable for Signaling by ptoL205D**

We showed previously that a pto double mutant containing a P+1 loop substitution and a kinase knockout mutation in Lys-69 (K69N) was unable to elicit cell death in planta (Rathjen et al., 1999). ptoK69N is unable to interact with AvrPto, whereas ptoD164N is kinase deficient but able to bind AvrPto in yeast, indicating minimal structural consequences of this mutation (Scofield et al., 1996; Tang et al., 1996). To test the role of kinase activity in CGF signaling, we introduced the mutation D164N to ptoL205D, chosen on the basis of its strong CGF phenotype. The AvrPto-dependent HR of Pto in *N. benthamiana* was abolished by the D164N mutation, consistent with previous data (Figure 3A; Rathjen et al., 1999). However, transient expression of ptoD164N:L205D in *N. benthamiana* tissue elicited a strong HR qualitatively similar to that of the single CGF mutant ptoL205D. To test the kinase activity of the CGF molecules, we established an in vitro assay based on phosphorylation of Pti1K96N by Pto (Figure 3B; Zhou et al., 1995). ptoD164N:L205D showed absent or greatly reduced autophosphorylation and transphosphorylation relative to wild-type Pto (cf. with Figure 6B), and these activities were completely absent in the double mutant ptoD164N:L205D. Staining of the radioactive gel confirmed equivalent protein loading in these assays. These data are consistent with the known role of the P+1 loop in kinase activity and dispensability of kinase activity for signaling once Pto has been made active by mutation. The data fundamentally contradict current models in which Pto acts at the apex of a phosphorylation cascade (Pedley and Martin, 2003) and dictate that P+1 loop mutations must act by some mechanism other than enhancement of kinase activity.

**Identification of Non-P+1 Loop Residues That Control Pto Signaling**

The data presented above require an alternative explanation for Pto signaling. We reasoned that the P+1 loop could represent a binding patch for a negative regulator, a dynamic loop that undergoes conformational change(s), or both. To investigate
these possibilities, we used our active homology model to identify representative buried and surface-exposed residues within 2 Å of the P-1 loop region as candidates for further mutagenesis. Buried residues could hold the P-1 loop in an inactive form before stimulation by AvrPto, whereas surface-exposed residues might form part of the binding site for a putative regulatory molecule. Buried residues were changed to Ala to delete side chain contacts while maintaining hydrophobicity, whereas surface-exposed residues were mutated to Asp according to our original strategy.

Mutant genes encoding the buried residue variants ptoL163A, ptoK166A, ptoL218A, ptoD223A, ptoY225A, ptoF227A, and ptoE233A accumulated in vivo but did not respond to AvrPto coexpression and therefore are potentially misfolded. The lack of positive data from this experiment means that role(s) for buried residues in Pto regulation cannot be identified.

For mutant genes encoding substitutions in surface residues, expression of wild-type Pto or any of ptoK123D, ptoD164A, ptoI185D, ptoL245D, and ptoY250D did not induce the HR. Transient expression of each of ptoF213D, ptoI214D, ptoN251D, and ptoA253D elicited strong AvrPto-independent cell death in N. benthamiana leaves similar to PtoL205D (Figure 4B). ptoY212D displayed CGF activity in ~50% of infiltrations. Signaling by ptoF213D, ptoI214D, ptoN251D, and ptoA253D was pathway dependent as judged by the inability to induce the HR in N. benthamiana silenced for Prf (Figure 4C). These CGF-conferring residues form a contiguous patch of surface-exposed residues in our three-dimensional model (Figure 5A) but are separated in the primary structure of Pto.

Mapping of a Surface-Exposed Patch That Controls Pto Signaling

The preceding experiments identified surface-exposed residues that control Pto signaling. To further define this patch, we used our model to find additional surface-exposed residues contiguous with those responsible for CGF activity. These included Phe-52, Ile-168, Asn-169, Lys-215, Leu-252, Glu-254, Trp-255, Glu-258, Leu-295, and Ser-296. Substitution of any of these new residues to Asp did not confer CGF activity upon expression of each mutant gene in N. benthamiana leaves (data not shown). These data, and the negative CGF data from the previous series of surface-exposed mutants, could be significant because protein accumulated from the expression of all genes except for ptoL252D (see Supplemental Figure 1B online). These mutants could represent functionally wild-type molecules that respond to AvrPto coexpression and would thus identify residues that do not control Pto signaling. To test this, the non-CGF genes comprising ptoF213D, ptoI123D, ptoD164A, ptoI185D, ptoN169D, ptoL245D, ptoY250D, ptoD252D, ptoE254A, ptoW255D, ptoE258A, ptoL252D, and ptoY250D were coexpressed in planta with AvrPto. ptoL168D, ptoK215D, ptoV250D, ptoW255D, ptoL295D, and ptoS296D were able to induce AvrPto-dependent cell death (Figure 4D). Thus, these mutants behave as wild-type Pto in this assay and define a partial border of the proposed regulatory patch (Figure 5A).

Most Surface Patch Residues Show Impaired Interaction with AvrPto and AvrPtoB

The P-1 loop is an important AvrPto and AvrPtoB binding determinant (Rathjen et al., 1999; Kim et al., 2002). To test the role of non-P-1 loop residues in ligand binding activity, surface patch mutants were assayed for the ability to interact with AvrPto and AvrPtoB in yeast. Most mutants lacked the ability to interact with either avirulence protein (Table 1). These data are significant.
because all mutant fusion proteins accumulated in yeast, with the exception of ptoN169D, ptoE254A, and ptoE258A (see Supplemental Figures 2A and 2B online). Thus, most of the mutated residues were required for interaction with ligands and define a second patch that substantially overlaps the regulatory patch defined above (Figures 5B and 5C). Seven residues (Ile-168, Ile-214, Leu-245, Val-250, Trp-255, Leu-295, and Ser-296) were dispensable for interaction with both AvrPto and AvrPtoB (Figure 5B). Strikingly, the mutant ptoI214D elicited AvrPto-independent HR; therefore, CGF activity and the ability to interact with Avr proteins are not mutually exclusive. ptoL245D, and the buried residue mutant ptoF227A, retained the ability to interact with AvrPto and AvrPtoB in yeast but were not activated by AvrPto coexpression in planta (Table 1). These mutants are presumably deficient in kinase activity or interaction with the downstream effector of signaling. A further point of interest is Lys-215, located on the extreme right edge of the proposed binding patch (Figures 5B and 5C). ptoK215D was able to interact with AvrPto but not AvrPtoB. Therefore, the binding requirements for these ligands can be functionally separated.

Figure 4. Surface-Exposed Residues Are Responsible for Native Regulation of Pto.
(A) Expression of Ala substitution mutants in planta. Identity of each mutant is indicated.
(B) Expression of Asp substitution mutants in planta. Identity of each mutant is indicated.
(C) CGF mutants require Prf for signaling. Left, leaves silenced for Prf; right, leaves silenced for empty vector only. Pto mutants were expressed as indicated.
(D) In planta recognition of AvrPto by non-CGF pto mutants. Mutants were expressed as indicated in N. benthamiana leaves carrying a dexamethasone-inducible avrPto transgene.
Kinase Activity Is Not Required for HR Signaling of Non-P+1 Loop CGF Mutants

Kinase activity is required for AvrPto-dependent activation of Pto but dispensable for the CGF variant pto\textsuperscript{L205D}. Despite this, we reasoned that some of the non-P+1 loop CGF molecules described here might mimic intermediate forms of Pto activation and hence require phosphorylation ability. To test this, we constructed double mutants incorporating the kinase knockout mutation D164N to genetic constructs containing CGF substitutions. Transient expression of pto\textsuperscript{D164N,F213D}, pto\textsuperscript{D164N,I214D}, pto\textsuperscript{D164N,N251D}, or pto\textsuperscript{D164N,A253D} in N. benthamiana induced the HR, similar to the positive control pto\textsuperscript{L205D} (Figure 6A). The HR induced by the double mutants was dependent on Prf as assessed by VIGS experiments (see Supplemental Figure 3 online). Thus, novel surface patch CGF mutants appeared to be similar to pto\textsuperscript{L205D} in mimicking an apparently terminally activated form of Pto. To test the phosphorylation potential of these mutants, pto\textsuperscript{F213D}, pto\textsuperscript{I214D}, pto\textsuperscript{N251D}, and pto\textsuperscript{A253D} were expressed and purified as glutathione S-transferase (GST) fusion proteins (Figure 6B) and subjected to an in vitro kinase assay. Autophosphorylated Pto was visualized as a band of \(\sim\)62 K, and a transphosphorylation band using pti1K96N as substrate migrated at \(\sim\)40 K. pto\textsuperscript{I214D} showed strong phosphorylation activity similar to the wild type, whereas the kinase activity of pto\textsuperscript{L205D} was strongly diminished. pto\textsuperscript{F213D}, pto\textsuperscript{N251D}, and pto\textsuperscript{A253D}, and the negative control pto\textsuperscript{D164N}, did not have detectable kinase activity in this assay. These data are consistent with the dispensability of kinase activity for signaling of CGF.
forms of Pto and with previous data showing that pto<sup>Y<sub>207D</sub></sup> lacked autophosphorylation activity (Rathjen et al., 1999).

**DISCUSSION**

The data presented here extend previous observations to firmly establish the P<sup>+</sup>1 loop as a key regulator of Pto signaling. Acidic substitutions of almost all P<sup>+</sup>1 loop residues conferred CGF activity to Pto. Most importantly, CGF-associated residues were predicted to be at least partially surface exposed, and we were able to use this criterion to identify further residues required for normal Pto regulation. The data suggest the existence of a surface patch that normally acts to repress signaling. We extended these observations to define a requirement for Pto

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<th>Residue Class</th>
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<th>AA Class</th>
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<th>Yeast Two-Hybrid</th>
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<td>CGF AvrPto Expression</td>
<td>AvrPto AvrPtoB Expression</td>
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<td>P&lt;sup&gt;+&lt;/sup&gt;1 loop residues</td>
<td>V201D</td>
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Solvent-exposed residues

| F52D | Hydrophobic | – | – | + | – | – |
| K123D | + | – | – | + | – | – |
| D164A | – | – | – | + | – | – |
| I168D | Hydrophobic | – | + | + | + |
| N169D | Polar | – | – | + | – | – |
| I185D | Hydrophobic | – | – | + | – | – |
| Y212D | Polar | + | N/A | – | – | + |
| F213D | Hydrophobic | + | N/A | – | – | + |
| I214D | Hydrophobic | + | N/A | + | + |
| K215D | + | – | + | + | – | – |

L245D | Hydrophobic | – | – | + | + | + |
| V250D | Hydrophobic | – | + | + | + |
| N251D | Polar | + | N/A | + | – | – |
| L252D | Hydrophobic | – | – | – | – |
| A253D | Hydrophobic | + | N/A | – | – | + |

E254A | – | – | – | + | – | – |
| W255D | Polar | – | + | + | + |
| E258A | – | – | – | + | – | – |
| L295D | Hydrophobic | – | + | + | + |
| S296D | Polar | – | + | + | + |

Buried residues

| R163A | + | – | – | + | – | – |
| K166A | + | – | – | + | – | – |
| L218A | Hydrophobic | – | – | + | – | – |
| D223A | Polar | – | – | – | – |
| Y225A | Polar | – | – | + | – | – |
| F227A | Hydrophobic | – | – | + | – | – |
| E233A | – | – | – | + | – | – |
| A256D | Hydrophobic | – | – | – | – |
| V257A | Hydrophobic | – | – | – | – |

D164N | – | – | + | + | + |
| Pto | None | – | + | + | + |

pto mutants are indicated using single letter amino acid (AA) codes. In planta assays: CGF, AvrPto-independent HR phenotype when expressed in planta, (+) indicates HR and (−) indicates no phenotype. AvrPto, AvrPto-dependent HR when coexpressed with each mutant in planta. N/A, not applicable because of CGF phenotype. Expression, accumulation of protein measured by protein gel blot analysis. Yeast two-hybrid interactions: AvrPto, interaction of each mutant with AvrPto in yeast, (+) represents a positive interaction and (−) designates a lack of interaction. AvrPtoB, interaction of each mutant with AvrPtoB in yeast.
arginine in stabilizing the substrate P
with the hydrophobic P
Pro-202, and Leu-205 form a hydrophobic pocket that interacts
1995). For the template kinase cAPK, the side chains of Leu-198,
carboxy-proximal to the phosphorylated residue; Taylor et al.,
interaction phenotypes of surface patch mutants leads to an
signaling. Finally, integration of the preceding data with the Avr
kinase activity in Avr-dependent activation but not downstream
activity in Avr-dependent activation and catalysis (Smith et al.,
expression of Pto CGF mutants containing a second-site kinase
knockout mutation in planta. Mutants were expressed as indicated.
Figure 6. Kinase Activity Is Dispensable for Signaling by Pto CGF
(A) Expression of Pto CGF mutants containing a second-site kinase
knockout mutation in planta. Mutants were expressed as indicated.
(B) Diminished kinase activity of most Pto CGF mutants. Mutant proteins
were expressed as GST fusions and assayed for the ability to autophos-
phorylate or transphosphorylate in vitro. Radiolabeled species were
detected by autoradiography after SDS-PAGE. The position of GST-Pto
fusion proteins and cleaved Ptk1(690) fusion proteins are indicated.
Bottom panel, Coomassie stain of the autoradiograph gel showing
equivalent loading.

The protein kinase P+1 loop was recognized originally for its
role in stabilizing the substrate P+1 residue (i.e., the residue
carboxy-proximal to the phosphorylated residue; Taylor et al.,
1995). For the template kinase cAPK, the side chains of Leu-198,
Pro-202, and Leu-205 form a hydrophobic pocket that interacts
with the hydrophobic P+1 residue of the substrate. These
residues correspond to Pto amino acids Val-201, Leu-205, and
Ile-208; both L205D and I208D mutants induced cell death in this
study, whereas V201D did not accumulate in planta. Subsequent
modeling work has shown that other cAPK P+1 loop residues
interact directly and indirectly with substrate residues (Moore
et al., 2003). Thus, cAPK Gly-200 (equivalent to Pto Gly-203)
forms a hydrogen bond with the backbone amide of the P+1
residue, and Glu-203 (Pto Gly-206) interacts with the P-6 Arg.
Tyr-204 (Pto Tyr-207) has profound effects on substrate affinity,
apparently because the aromatic ring is partially exposed and
interacts with the P-2 Arg. Moore et al. (2003) have proposed that
the term “peptide positioning” loop is a more accurate de-
scription of how this region of cAPK acts at several levels to
secure the peptide substrate in the correct orientation before
phosphorylation. Our results reveal that equivalent P+1 loop
residues control either peptide binding in cAPK or CGF activity in
Pto after mutation. Conservative extrapolation of these data
suggests that the Pto P+1 loop functions in a canonical sense to
bind a peptide, but the role of the proposed peptide is to repress
Pto signaling. We propose that the effect of Asp substitutions is
to delete the interaction with a negative regulator, leading to
derepression of signaling. Several protein kinases, including
cAPK, are known to be regulated in this manner by so-called
pseudosubstrate molecules (Taylor and Radzio-Andzelm, 1997).

Certain P+1 loop residues are known to interact with distal
residues during substrate recognition and catalysis (Smith et al.,
1999). We reasoned that mutation of some of these residues
might result in constitutively active molecules via indirect dis-
ruption of the P+1 loop. We analyzed our active model to identify
residues linked to the P+1 loop in three dimensions as candi-
dates for further mutagenesis. Experiments in which buried
residues were substituted for Ala were inconclusive, either
because of failure of the mutant protein to accumulate or the
absence of phenotypes associated with these mutations. Such
mutants may be misfolded or otherwise unstable. By contrast,
acidic substitutions of several surface-exposed residues re-
sulted in the CGF phenotype. We identified five residues that are
not resident in the P+1 loop; Tyr-212, Phe-213, Ile-214, Asn-251,
and Ala-253. These residues form an apparently contiguous
surface patch with the P+1 loop residues but are separated from
the P+1 loop in the primary sequence of Pto. Roles in substrate
binding for the corresponding residues in other protein kinases
have not been defined to our knowledge. We suggest that these
residues represent additional sites of contact that mediate
protein docking with the proposed inhibitory protein.

The CGF-responsive residues identified here appear to rep-
resent a concise patch. Several residues on the edge of the
studied region did not alter the wild-type characteristics of Pto
after substitution with Asp, including Ile-168, Lys-215, Val-250,
Trp-255, Leu-295, and Ser-296. Such residues evidently do not
mediate negative regulation of Pto or interaction with Avr
proteins and so define a boundary of the regulatory region. The
patch of amino acids that did confer CGF when substituted
consists mostly of hydrophobic residues, including Gly-203,
Leu-205, Gly-206, Ile-208, Phe-213, Ile-214, and Ala-253. Sites
of protein–protein interaction are frequently hydrophobic in
nature, although often include charged residues that form salt
bridges between the binding partners. However, we did not
identify any charged residues within the confines of the Pto
surface patch. The Asp substitution strategy used here is a contin-
uation of our earlier work and is slightly unorthodox in that the
small residue Ala is usually used for scanning mutagenesis
schemes. Incorporation of an acidic residue, such as Asp, should disrupt the presumed hydrophobic moment of the patch, potentially canceling a negative regulatory interaction leading to the observed CGF phenotype. By contrast, the effect of Ala substitutions is more subtle in that only the specific side chain interactions of the substituted amino acid would be deleted. Consistent with this reasoning, ptoY2027A conferred a qualitatively stronger CGF HR than ptoY2027A (Rathjen et al., 1999). Thus, the use of acidic substitutions may have been an important factor in our ability to detect the proposed surface patch.

In total, we identified 12 different CGF mutants that induced a Prf-dependent HR. The kinase dependence of these mutants provides insight into the possible effects of CGF mutations. We and others previously showed that the kinase activity of Pto is required for activation by AvrPto. This conclusion is supported most strongly by the mutant ptoD164N, which deletes kinase activity but is able to bind AvrPto and AvrPtoB in yeast (Rathjen et al., 1999; Table 1), whereas other kinase knockout mutations, such as K69N, destroy Avr interaction ability (Scofield et al., 1996; Tang et al., 1996). Lys-69 is an invariant residue that stabilizes ATP binding by interaction with the α and β phosphates through formation of a salt bridge with a conserved Glu in the αC helix (Huse and Kuriyan, 2002). Mutation of this residue would be expected to lead to significant conformational changes in the kinase structure. Surprisingly, we found that several CGF mutants containing the D164N mutation were able to induce the Pto activation mimicked by the CGF mutants. It follows that the surface patch. This would correspond to the terminal stage of activation because kinase activity is dispensable after this step. Pto appears to act as a conformation-sensitive molecular adaptor in signaling, rather than transphosphorylating a substrate. Similar roles have been described for the kinase-like signaling molecules KSR and interleukin-1 receptor-associated kinases -M and -2 (Michaud et al., 1997; Janssens and Beyaert, 2003). Pto phosphorylates several substrates in vitro, including the protein kinase Pti1 and the transcription factor–like protein Pti4. Our results suggest that these proposed phosphorylation events are not necessary for either the Prf-dependent HR, or disease resistance per se, because HR and disease resistance have not been separated upstream of Prf.

The data suggest that the role of kinase activity in Pto activation is to remove the proposed negative regulator from the surface patch. This would correspond to the terminal stage of Pto activation mimicked by the CGF mutants. It follows that removal of the negative regulator is probably sufficient for activation because kinase activity is dispensable after this step. The putative regulatory molecule is likely to be a peptide following the argument above, but its identity is unknown. Theoretically, it could be part of the Pto molecule itself or an unidentified member of the signaling pathway. Overexpression of Pto does not induce the HR in N. benthamiana (although quantitative phenotypes are evident in transgenic tomato plants; Tang et al., 1999); therefore, it seems that the repression cannot be overcome by titration. This is consistent with Pto itself mediating repression. Our inactive model of Pto shows an extensive interaction between the P-1 and T-loops, which is essentially absent in the active form (Figure 2) and could provide a structural basis for Pto self-regulation. Conversely, Pto signaling could be controlled by another molecule. To explain why Pto overexpression does not lead to the HR, we suggest that the regulatory molecule could also play a positive role in signaling. In this scenario, the regulatory protein would be limiting such that only a small number of Pto-regulator complexes competent for signaling would form, whereas overexpressed Pto would accumulate as nonfunctional monomers. This is consistent with the suggested role of Pto as an adaptor in a signaling complex. Avr-dependent conformational activation of Pto would result in a concomitant change in the proposed regulatory protein, thus triggering downstream signaling. Identification of the proposed regulatory partner(s) is likely to provide considerable insight to control of signaling by Pto.

Many of the surface residues mutated here were required for interaction with both AvrPto and AvrPtoB in yeast. There was considerable overlap in the sequence requirements for binding each Avr protein, with the exception of Lys-215, which was required only for AvrPtoB. Like the CGF area, the region controlling Avr binding appeared to be a concise patch because some peripheral residues (Ile-168, Ile-214, Leu-245, Val-250, Leu-295, and Ser-296) were dispensable for interaction with ligands. Thus, we have partially mapped a border of a second surface area that overlaps the previous patch responsible for CGF activity (Figure 5C). Avr proteins could bind to this area directly, or alternatively, mutations in this region could cause a conformational change that disrupts a binding site in some other part of the Pto molecule. Generally speaking, there is an inverse correlation between the CGF activity of each mutant and the ability to bind either Avr. However, the mutant protein ptoD140, which binds both Avr proteins but is constitutively active in vivo, shows that these activities are not mutually exclusive. This mutant appears to preclude a global conformational change in the Pto kinase domain upon activation because it retained both kinase activity and the ability to interact with ligands. If global rearrangement of Pto structure can indeed be ruled out, then the most likely explanation of our data is that Avr

![Figure 7. Model of Pto Activation by AvrPto and AvrPtoB.](image)

Native Pto (blue) is regulated by an inhibitory peptide (red) that represses kinase activity. Upon secretion into the plant cell, AvrPto or AvrPtoB (green) interacts with Pto, displacing the regulatory peptide from the catalytic cleft and derepressing kinase activity. Activated Pto catalyzes a phosphorylation event(s) leading to a specific conformational change. An unknown effector protein recognizes the altered Pto conformation, leading to activation of the resistance pathway. Although the Pto-effector complex is shown dissociated from the Avr and inhibitor proteins, this remains speculative.
molecules bind to the same region as the proposed negative regulatory molecule. This hypothesis is attractive because it suggests a mechanism for kinase-dependent activation of Pto by Avr proteins. We suggest the following model for activation of Pto by specific ligands (Figure 7). Before stimulation, Pto is held in the inactive state by a regulatory peptide that binds to the P1-1 loop in the kinase catalytic cleft. This peptide may repress kinase activity generally or simply suppress the specific phosphorylation event that leads to activation of signaling. The Avr protein binds to Pto, resulting in a phosphorylation event(s) that vacates the inhibitory peptide from the Pto peptide positioning loop. The overlap in negative regulatory and Avr interaction patches detected here suggests that these molecules might bind competitively to Pto. Thus, Avr binding might cause displacement of the inhibitory protein from the kinase catalytic cleft, allowing the regulatory phosphorylation event(s). The site of phosphorylation is unknown and could be on Pto itself or the proposed negative regulator. The outcome of phosphorylation is presumably a conformational change in Pto that is perceived by another protein. This scenario is reminiscent of the theoretical model proposed by Van der Biezen and Jones (1998), although the current data do not provide insight to the role of Prf in signaling by Pto. Another plant protein kinase that acts apparently as the primary receptor for a bacterial effector protein is Arabidopsis PBS1, which recognizes AvrPphB (Shao et al., 2003). However, this example appears to be different from Pto. AvrPphB is a protease that cleaves PBS1 in the T-loop region. This event is necessary for induction of resistance, but its consequence is unknown. However, it is known that the kinase activity of PBS1 is required for subsequent signaling. Thus, the next protein in the pathway could recognize a PBS1 phosphopeptide as suggested by Shao et al. (2003), or the cleavage event could stimulate the kinase activity of PBS1, resulting in phosphorylation of a substrate molecule. Either possibility is clearly different from our demonstration of kinase independence of Pto for downstream signaling. It may be necessary to define the roles of the respective NBS-LRR proteins of these pathways, Prf and RPSS, before these differences can be reconciled.

METHODS

Cloning and Expression of Pto Mutants

Standard techniques for manipulating plasmids in Escherichia coli strain DH5α were used (Sambrook et al., 1989). PCR-based site-directed mutagenesis was performed as described (Horton et al., 1989). All products contained 5’ BamHI and NcoI sites and 3’ XbaI and were cloned into pCRII (Invitrogen, Carlsbad, CA) before automated DNA sequencing (Applied Biosystems, Foster City, CA). For expression in planta, P1-1 loop mutants were cloned exactly as described (Rathjen et al., 1999). Non-P1-1 loop mutants were subcloned in the sense orientation into the vector pCB302-3 (Xiang et al., 1999) using BamHI and XbaI. All recombinant binary vectors were transferred to Agrobacterium tumefaciens strain GV2260 for expression in planta and the presence of the binary vector confirmed by PCR. For Y2H analysis, Pto and its mutant derivatives were cloned into the DNA binding domain vector pAS2-1 (Clontech, Palo Alto, CA) using Ncol and EcoRI. For expression in E. coli, Pto and its mutant derivatives were subcloned into pMalc2 (New England Biolabs, Beverly, MA) or pGEX-2TK (Amersham Biosciences, Buckinghamshire, UK).

Transient Agrobacterium-Mediated Expression

A. tumefaciens strain GV2260 containing the binary plasmid of interest was grown in LB media with appropriate antibiotics to stationary phase (~2 d) at 28°C. Cultures were diluted 1:10 into fresh LB plus antibiotics and grown overnight at 28°C. Cells were pelleted and washed once in infiltration media (10 mM Mes, pH 5.6, 10 mM MgCl2, and 200 μM acetoxyerigone) and then resuspended to an OD600 of 1.0. Six-week-old Nicotiana benthamiana plants grown in the greenhouse were inoculated by pressure infiltration using a disposable syringe, and the approximate area of infiltration was outlined with a marker pen. Infiltrated plants were kept in laboratory conditions for 4 d to allow symptoms to develop.

VIgs

VIgs was performed by infiltrating Agrobacterium strains carrying either TRV empty vector (pTV00; Ratcliffe et al., 2001) or TRV:PRF (Lu et al., 2003) into leaves of 2-week-old N. benthamiana seedlings. Systemic TRV infection developed 5 to 10 d postinoculation and systemic silencing after 3 to 4 weeks.

Construction of a Structural Model of Pto

A full description of the modeling procedure and PDB files containing coordinates of the active and inactive models can be found in the supplemental data online.

Yeast Two-Hybrid Analysis

Yeast two-hybrid analysis was performed using the MATCHMAKER GAL4 system (Clontech) following the manufacturer’s protocol. AvrPto and AvrPtoB were expressed from the plasmid pACT2 and transformed into yeast strain Y187 (MATα). Pto and its mutant derivatives were expressed from plasmid pAS2-1 and transformed into yeast strain AH109 (MATa). Pairwise matings were set up between AH109:ppto strains and either Y187:AvrPto or Y187:AvrPtoB. Yeast cotransformants were selected by growth on selective media. To assay for protein-protein interactions, cotransformants were replica plated onto separate selective media and growth was monitored based on activation of the ADE2 and HIS3 reporter genes. Growth on selective media was scored as a positive interaction between the fusion proteins, as presented in Table 1.

Protein Analysis

For the analysis of protein accumulation in planta, infiltrated leaf samples were extracted in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM DTT, 1% Triton X-100, and 1.5% polyvinylpyrrolidone supplemented with protease inhibitors (Sigma, St. Louis, MO). Fifty micrograms of total protein was loaded onto a 10% SDS-PAGE gel, and after electrophoresis, the gels were electroblotted onto a polyvinylidene difluoride (PVDF) membrane. Immunological reactions were performed with a Pto polyclonal antisera before detection of immunocomplexes with a horse-radish peroxidase–conjugated anti-rabbit secondary antibody, using the ECL detection reagent (Amersham Biosciences). To determine protein accumulation in yeast, yeast cultures were grown in selective SD medium overnight. The culture were diluted to an OD600 = 0.15 to 0.2 and monitored until OD600 = 0.4 to 0.6 was reached. The cells were pelleted and proteins extracted by boiling in 50 mM sodium phosphate, pH 7.0, 25 mM Mes, pH 7.0, 3 M urea, 1% SDS, 10% β-mercaptoethanol, and 0.1% bromophenol blue, supplemented with protease inhibitors (Roche,
Indianapolis, IN), OD units (0.5 total) per well were loaded onto an SDS-PAGE gel, and after electrophoresis, the gels were electroblotted onto PVDF membrane for Pto detection.

**Protein Expression in E. coli**

Pto and its mutant derivatives were expressed as fusion proteins with MBP (Rathjen et al., 1999) or GST in E. coli strain DH5α according to the manufacturer’s instructions. For GST-Pto expression, cultures in LB media were incubated at 16°C until OD₆₀₀ ~0.4, then induced with 0.2 mM isopropyl-thio-β-galactoside and incubated at 16°C with shaking overnight. Cells were harvested and resuspended in Tris-buffered saline containing 1 mM DTT, 0.1% (v/v) Triton X-100, and bacterial protease inhibitor cocktail (Sigma). Cells were lysed by sonication and the extract clarified by centrifugation at 13,000g for 30 min at 4°C. The clear supernatants were filtered through 0.45-μm filters and GST-fusion proteins pulled down with glutathione-Sepharose beads (Amersham Biosciences) according to the standard protocol. GST-PtiIK₉⁶₉ was expressed as described (Rathjen et al., 1999) and purified over GSTrap FF columns (Amersham Biosciences) according to the manufacturer’s instructions. GST-PtiIK₉⁶₉ was subsequently cleaved with thrombin at 22°C for 16 h. Thrombin was removed by benzamidine affinity chromatography. Cleaved PtiIK₉⁶₉ was concentrated by ultrafiltration (Centriprep-10 concentrators; Amicon, Beverly, MA) and used for Pto kinase activity assays.

**In Vitro Kinase Assay**

Pto autophosphorylation and transphosphorylation activities (against PtiIK₉⁶₉) were assayed in 50-μL reaction mixtures containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.5 mM MnCl₂, 20 μM ATP, 5 μCi [γ³²P]-ATP, 5 μg GST-PtiIK₉⁶₉, and immobilized GST-Pto equivalent to 5 μg of protein. For Figure 6B, PtiIK₉⁶₉ was cleaved from the GST moiety before the kinase assay reaction as described above. Reactions were held at 25°C for 30 min and terminated by adding SDS sample buffer and boiling for 5 min. Proteins were resolved on SDS-PAGE gels, then transferred onto PVDF membranes, visualized with Coomassie Brilliant Blue R 250, and subjected to autoradiography.

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A Patch of Surface-Exposed Residues Mediates Negative Regulation of Immune Signaling by Tomato Pto Kinase
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