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The N-terminal region of *Pseudomonas* type III effector AvrPtoB elicits Pto-dependent immunity and has two distinct virulence determinants

Fangming Xiao[1], Ping He[2], Robert B. Abramovitch[1,3], Jennifer E. Dawson[4], Linda K. Nicholson[4], Jen Sheen[2] and Gregory B. Martin[1,3,*]

[1]*Boyce Thompson Institute for Plant Research, Tower Road, Ithaca, NY 14853, USA,*

[2]*Department of Molecular Biology, Massachusetts General Hospital and Department of Genetics, Harvard Medical School, Boston, MA 02114, USA,*

[3]*Department of Plant Pathology, Cornell University, Ithaca, NY 14853, USA, and*

[4]*Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853, USA*

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*For correspondence (fax +1 607 255 6695; e-mail gbm7@cornell.edu).

Summary

Resistance to bacterial speck disease in tomato is activated by the physical interaction of the host Pto kinase with either of the sequence-dissimilar type III effector proteins AvrPto or AvrPtoB (HopAB2) from *Pseudomonas syringae* pv. *tomato*. Pto-mediated immunity requires Prf, a protein with a nucleotide-binding site and leucine-rich repeats. The N-terminal 307 amino acids of AvrPtoB were previously reported to interact with the Pto kinase, and we show here that this region ($AvrPtoB_{1-307}$) is sufficient for eliciting Pto/Prf-dependent immunity against *P. s.* pv. *tomato*. $AvrPtoB_{1-307}$ was also found to be sufficient for a virulence activity that enhances ethylene production and increases growth of *P. s.* pv. *tomato* and severity of speck disease on susceptible tomato lines lacking either Pto or Prf. Moreover, we found that residues 308–387 of AvrPtoB are required for the previously reported ability of AvrPtoB to suppress pathogen-associated molecular patterns-induced basal defenses in Arabidopsis. Thus, the N-terminal region of AvrPtoB has two structurally distinct domains involved in different virulence-promoting mechanisms. Random and targeted mutagenesis identified five tightly clustered residues in $AvrPtoB_{1-307}$ that are required for interaction with Pto and for elicitation of immunity to *P. s.* pv. *tomato*. Mutation of one of the five clustered residues abolished the ethylene-associated virulence activity of $AvrPtoB_{1-307}$. However, individual mutations of the other four residues, despite abolishing interaction with Pto and avirulence activity, had no effect on $AvrPtoB_{1-307}$ virulence activity. None of these mutations affected the basal defense-suppressing activity of $AvrPtoB_{1-387}$. Based on sequence alignments, estimates of helical propensity, and the previously reported structure of AvrPto, we hypothesize that the Pto-interacting domains of AvrPto and $AvrPtoB_{1-307}$ have structural similarity. Together, these data support a model in which $AvrPtoB_{1-307}$ promotes ethylene-associated virulence by interaction not with Pto but with another unknown host protein.

Keywords: immunity, Pto kinase, programmed cell death, guard hypothesis, type III effectors, structural biology.

Introduction

Pseudomonas syringae pv. *tomato*, the causative agent of bacterial speck disease in tomato (*Solanum lycopersicum*), enters the intercellular spaces of plant tissues via wounds or stomata (Preston, 2000). Recent evidence suggests that a key plant defense at this early stage of bacterial infection is the detection of pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors, which then activate basal defense responses (Abramovitch *et al.*, 2006a; Chisholm *et al.*, 2006). A counter-strategy of the pathogen appears to be the production and delivery via a type III

secretion system of a battery of 'effector' proteins, many of which act to suppress basal defenses (Alfano and Collmer, 2004; Buttner and Bonas, 2006; Chang *et al.*, 2004; He *et al.*, 2006; Kim *et al.*, 2005; Li *et al.*, 2005). *P. s.* pv. *tomato* DC3000, for example, expresses approximately 30 type III effector proteins that share little or no sequence similarity, although, based on mutagenesis studies, many of them appear to have redundant functions in promoting bacterial virulence (Badel *et al.*, 2003, 2006; Chang *et al.*, 2005; Lin and Martin, 2005; Mudgett, 2005; Petnicki-Ocwieja *et al.*, 2002; Schechter *et al.*, 2004). Plants have evolved resistance (R) proteins that either directly or indirectly detect the presence of certain effector proteins, and thereby activate a strong immune response typically associated with rapid localized cell death (the hypersensitive response, HR) (Greenberg and Yao, 2004; Lam *et al.*, 2001). As an apparent counter-attack to this defense response, some effectors have evolved to act either at or downstream of R protein recognition to impede signaling events leading to the HR (Abramovitch and Martin, 2004; Abramovitch *et al.*, 2003; Jamir *et al.*, 2004; Rosebrock *et al.*, 2007). This emerging view of plant–pathogen interactions highlights the importance of understanding the structural and functional aspects of virulence activities of pathogen effector proteins, the host targets of these proteins, and the mechanisms by which R proteins recognize avirulence effectors and activate defense responses.

The 'guard' hypothesis of plant disease resistance postulates that R proteins detect virulence activities of effectors via their structural features or enzymatic functions (Innes, 2004; Van der Biezen and Jones, 1998). If this is the case, then the avirulence determinants of effectors are expected to be identical to their virulence determinants. Otherwise, an effector could evolve to retain virulence activity while evading R protein detection and thereby defeat host defenses. This prediction is supported by studies that have relied upon either random or targeted mutagenesis to identify amino acid residues required for avirulence and to assess their contribution to virulence (Lim and Kunkel, 2004; Ong and Innes, 2006; Shan *et al.*, 2000a; Yang *et al.*, 2005). For example, mutant forms of the effector AvrB that are no longer recognized by the RPM1 or Rpg1-b proteins of Arabidopsis and soybean, respectively, generally lose virulence activity in ecotypes of those species that lack the cognate R gene (Ong and Innes, 2006). However, there are several reports of instances where avirulence and virulence determinants were found to be separable (Gassmann *et al.*, 2000; Lim and Kunkel, 2004; Yang *et al.*, 2005). For example, a naturally occurring mutant allele of *avrBs2* no longer elicits Bs2-mediated disease resistance in pepper and yet is unaffected in virulence activity (Gassmann *et al.*, 2000). Because the functionally important plant virulence targets of bacterial effectors are mostly unknown, and the mechanisms by which effectors are recognized by R proteins are unclear, it has been difficult to directly examine whether R proteins are detecting effector virulence determinants.

The interaction of tomato leaves with *P. s.* pv. *tomato* provides an excellent model for investigating these questions because much is known in this pathosystem about both the effector proteins and the host components that mediate recognition of these effectors (Pedley and Martin, 2003). Resistance in tomato to *P. s.* pv. *tomato* is conferred by the Pto serine/threonine protein kinase which is encoded by a member of a small, clustered family of five genes (Martin *et al.*, 1993; Pedley and Martin, 2003). Embedded within this *Pto* gene family is the *Prf* gene, which encodes a nucleotide-binding site/leucine rich-repeat (NBS-LRR) protein characteristic of the largest class of plant R proteins (Salmeron *et al.*, 1996). The Pto kinase physically interacts with the *P. s.* pv. *tomato* effector proteins AvrPto or AvrPtoB (also named HopAB2), and mutagenesis of Pto has revealed a critical requirement for residues in the kinase P loop for both of these interactions (Frederick *et al.*, 1998; Kim *et al.*, 2002; Scofield *et al.*, 1996; Tang *et al.*, 1996). The mechanism by which the Pto/AvrPto or Pto/AvrPtoB interaction triggers host defenses is unknown, but the region of Pto that binds these proteins also appears to serve as a negative regulator of Prf-mediated Pto defense activation and the effector proteins might interfere with this regulation in some way (Pedley and Martin, 2003; Wu *et al.*, 2004). Interestingly, there are a few differences in the Pto amino acid residues required for recognition of AvrPto or AvrPtoB, suggesting that some structural specificity may exist for these R protein–effector protein interactions (Bernal *et al.*, 2005; Wu *et al.*, 2004).

AvrPto is an intensively studied 18 kDa type III effector that, after delivery by *P. s.* pv. *tomato* into the plant cell cytoplasm, appears to be targeted to the plasma membrane by host-mediated myristylation of its N-terminus (Shan *et al.*, 2000b). In susceptible plants, AvrPto promotes bacterial virulence, as indicated by a 10- to 100-fold increase in growth compared with isogenic *P. s.* pv. *tomato* strains lacking the effector (Chang *et al.*, 2000; Shan *et al.*, 2000a). Over-expression of AvrPto in stable Arabidopsis transformants interferes with cell-wall-based basal defenses (Hauck *et al.*, 2003). Host-mediated phosphorylation of serine 149 in the AvrPto C-terminus contributes to this virulence activity and also affects AvrPto avirulence activity (Anderson *et al.*, 2006). Extensive random mutagenesis of AvrPto has revealed that a group of clustered amino acids play a key role in its interaction with the Pto kinase (Chang *et al.*, 2001; Shan *et al.*, 2000a). Determination of the NMR solution structure of AvrPto showed that most of these residues lie in a 19-residue Ω loop (referred to hereafter as the CD loop) lying between helices C and D of the four-helix structure that comprises the core of the protein (Wulf *et al.*, 2004). Mutation of some of the residues in this loop was previously reported

to disrupt avirulence but not the virulence activity of the effector (Shan *et al.*, 2000a).

AvrPtoB is a 59 kDa modular protein with a C-terminal region that functions to suppress R protein-associated HR (Abramovitch *et al.*, 2003). The three-dimensional structure of this C-terminal region was recently determined and found to contain a domain that structurally mimics host E3 ubiquitin ligases (Abramovitch *et al.*, 2006b; Janjusevic *et al.*, 2006). In contrast, two distinct avirulence domains are encoded by the N-terminus. One is a Pto-dependent recognition domain consisting of amino acids 1–307 [previously termed Δ7 (Abramovitch *et al.*, 2003), and now referred to as AvrPtoB$_{1\text{-}307}$]. The other is the Rsb-dependent recognition domain consisting of amino acids 308–387 (Rosebrock *et al.*, 2007) (the region comprising amino acids 1–387 was previously termed Δ6, and is now referred to as AvrPtoB$_{1\text{-}387}$). The AvrPtoB$_{1\text{-}387}$ region lacks sequence motifs to suggest function and its structure is unknown. The possible avirulence or virulence activity of AvrPtoB$_{1\text{-}307}$ or AvrPtoB$_{1\text{-}387}$ when expressed in *P. s.* pv. *tomato* has not been previously reported. An initial alignment found little overall similarity in amino acid sequences between AvrPtoB and AvrPto; however, four residues (GINP) that are present in the AvrPto CD loop are conserved in AvrPtoB (Kim *et al.*, 2002). Mutation of two of these residues (G325, I326) in full-length AvrPtoB decreased, but did not abolish, interaction with Pto (Kim *et al.*, 2002). Later work showed that AvrPtoB$_{1\text{-}307}$, which lacks the GINP motif, can still interact with Pto (Abramovitch *et al.*, 2003). Thus we have hypothesized that the GINP residues may contribute to Pto binding, along with other residues in AvrPtoB$_{1\text{-}307}$ (Abramovitch *et al.*, 2003).

Recently, AvrPtoB has been shown to have different virulence activities in different plant hosts. In susceptible tomato plants, AvrPtoB promotes disease-associated host cell death by enhancing ethylene production via up-regulation of ethylene biosynthesis genes (Cohn and Martin, 2005). In Arabidopsis, AvrPtoB suppresses basal defense by blocking PAMP-mediated early defense gene induction and MAP kinase signaling (He *et al.*, 2006; de Torres *et al.*, 2006). Finally, AvrPtoB has been found to suppress several other PAMP-elicited defense responses in *Nicotiana benthamiana* (Hann and Rathjen, 2007). Although the contribution of the various domains of AvrPtoB to these diverse activities is mostly unknown, it is clear that at least the basal defense suppression activity in Arabidopsis does not require the E3 ligase activity of AvrPtoB, suggesting this activity is encoded by the N-terminal region of AvrPtoB (He *et al.*, 2006).

Here we investigate recognition of the N-terminal region of AvrPtoB by the Pto kinase and the role of this region in promoting *P. s.* pv. *tomato* virulence. We present evidence that the AvrPtoB N-terminal region promotes virulence by two different mechanisms. One of them, involving the first 307 amino acids of AvrPtoB, promotes the production of ethylene, which has been previously reported to be required for enhancement of bacterial speck disease (Cohn and Martin, 2005). The other virulence activity, which requires amino acids 308–387, acts by suppressing PAMP-induced basal defenses. We show that Pto does not appear to be a virulence target of AvrPtoB as neither of these activities is dependent on Pto.

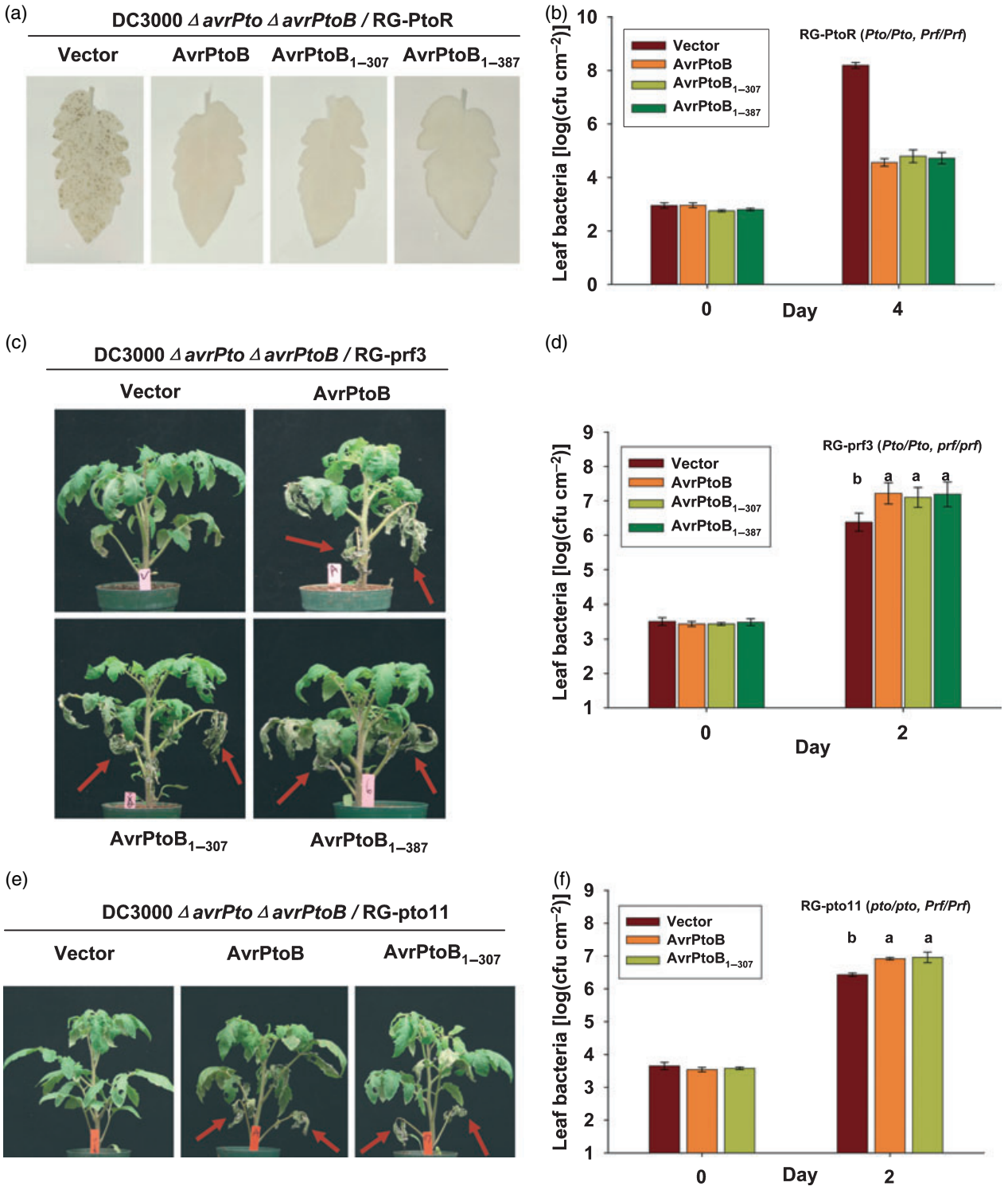
Results

An N-terminal region of AvrPtoB has both avirulence and virulence activity

We previously reported that the N-terminal 307 amino acids of AvrPtoB (previously called Δ7; hereafter AvrPtoB$_{1\text{-}307}$) is sufficient for the yeast two-hybrid interaction of AvrPtoB with Pto, as well as for elicitation of Pto-dependent cell death in an *Agrobacterium*-mediated transient assay (Abramovitch *et al.*, 2003). To determine whether AvrPtoB$_{1\text{-}307}$ is able to elicit Pto-mediated resistance when expressed by *P. s.* pv. *tomato*, we introduced *avrPtoB$_{1\text{-}307}$* under the control of the *avrPtoB* native *hrp* promoter into the strain DC3000Δ*avrPto*Δ*avrPtoB* (carrying deletions of both *avrPto* and *avrPtoB*; Lin and Martin, 2005). The resulting strain was infiltrated into RG-PtoR tomato leaves (*Pto*/*Pto*, *Prf*/*Prf*), which express a functional *Pto* pathway. DC3000Δ*avrPto*Δ*avrPtoB* strains carrying full-length *avrPtoB* or an empty vector served as positive and negative controls, respectively. A longer N-terminal fragment, AvrPtoB$_{1\text{-}387}$ (previously called Δ6; hereafter AvrPtoB$_{1\text{-}387}$), that elicits Rsb immunity was also tested. DC3000Δ*avrPto*Δ*avrPtoB* strains harboring *avrPtoB$_{1\text{-}307}$*, *avrPtoB$_{1\text{-}387}$* or full-length *avrPtoB* elicited resistance on RG-PtoR plants as evidenced by the lack of bacterial speck disease symptoms and limited bacterial populations 4 days after inoculation (Figure 1a,b). In contrast, DC3000Δ*avrPto*Δ*avrPtoB* carrying an empty vector caused typical symptoms of speck disease, and the bacterial population reached a high level (10^8 cfu cm^{-2}) after 4 days. AvrPtoB$_{1\text{-}307}$ is therefore a minimal N-terminal region that has full avirulence activity on *P. s.* pv. *tomato* in *Pto*-expressing tomato leaves.

To test whether the N-terminal region of AvrPtoB contributes to *P. s.* pv. *tomato* virulence, DC3000Δ*avrPto*Δ*avrPtoB* strains carrying *avrPtoB$_{1\text{-}307}$*, *avrPtoB$_{1\text{-}387}$*, full-length *avrPtoB* or an empty vector were infiltrated into RG-prf3 tomato leaves (*Pto*/*Pto prf*/*prf*). RG-prf3 plants have a 1 kb deletion in the *Prf* gene, rendering the plants susceptible to avirulent *P. s.* pv. *tomato* strains despite their having a functional *Pto* gene (Salmeron *et al.*, 1996). *P. s.* pv. *tomato* disease symptoms were monitored 4 days after infiltration. At the low inoculum level of 10^4 cfu ml^{-1}, which allows us to distinguish relatively subtle differences in disease severity, DC3000Δ*avrPto*Δ*avrPtoB* strains expressing AvrPtoB$_{1\text{-}307}$, AvrPtoB$_{1\text{-}387}$ or AvrPtoB caused

more severe disease symptoms on RG-prf3 plants compared with the empty vector control strain (Figure 1c). Specifically, on plants infiltrated with these strains, the lower four leaves developed extensive necrosis, and severe disease later occurred on the upper leaves (red arrows in Figure 1c). To assess whether this AvrPtoB N-terminus virulence activity is dependent on *Pto*, we inoculated a tomato line, RG-pto11 (*pto/pto Prf/Prf*), that lacks a functional Pto kinase, and visually assessed disease symptoms. As on the RG-prf3 plants, DC3000Δ*avrPto*Δ*avrPtoB* strains expressing either AvrPtoB or AvrPtoB$_{1\text{-}307}$ caused substantially worse disease symptoms than the



empty vector strain (Figure 1e) [note AvrPtoB$_{1\text{-}387}$ was not used in this experiment because it activates Rsb immunity in RG-pto11 (Abramovitch *et al.*, 2003)].

We assessed bacterial growth of the strains in leaves of RG-prf3 and RG-pto11 plants and found that, at 2 days after infiltration, the DC3000Δ*avrPto*Δ*avrPtoB* strains expressing AvrPtoB, AvrPtoB$_{1\text{-}387}$ or AvrPtoB$_{1\text{-}307}$ grew to statistically significantly higher levels than the empty vector control strain, demonstrating that AvrPtoB$_{1\text{-}307}$, like intact AvrPtoB, accelerates bacterial growth (Figure 1d,f). Taken together, these data show that AvrPtoB$_{1\text{-}307}$ is a minimal N-terminal region that has both full avirulence activity in *Pto/Prf*-expressing resistant tomato lines, and, significantly, also full virulence activity in susceptible lines independent of *Pto* or *Prf*. Based on this observation, we focused our initial attention on this region for further study of its avirulence and virulence activities.

Amino acids 121–200 of AvrPtoB are sufficient for interaction with Pto in yeast

To investigate the specificity underlying AvrPtoB/Pto-mediated resistance, we generated a series of *avrPtoB$_{1\text{-}307}$* truncations to determine the minimum region of AvrPtoB$_{1\text{-}307}$ sufficient for interaction with Pto in the yeast two-hybrid assay. These truncations express the N-terminal 280, 260, 244, 220, 200 or 190 amino acids, and are referred to as AvrPtoB$_{1\text{-}280}$, AvrPtoB$_{1\text{-}260}$, AvrPtoB$_{1\text{-}244}$, AvrPtoB$_{1\text{-}220}$, AvrPtoB$_{1\text{-}200}$ and AvrPtoB$_{1\text{-}190}$, respectively. Each of the truncations was expressed well as a prey fusion in yeast, and the yeast two-hybrid assays revealed that the region of 1–200 amino acids of AvrPtoB was sufficient for interaction with Pto (Figure 2a). Based on previous data showing that the N-terminal 120 amino acids of AvrPtoB are dispensable for interaction with Pto (Kim *et al.*, 2002), a clone expressing amino acids 121–200 (AvrPtoB$_{121\text{-}200}$) was generated for analysis. A yeast two-hybrid assay indicated that this 80 amino acid region interacts with Pto (Figure 2a). Deletion of ten additional amino acids from either end of this region abolished the interaction, even though these truncated proteins were expressed well in yeast (Figure 2a). Thus, this 80 amino acid region encompasses the minimal contact surface in AvrPtoB$_{1\text{-}307}$ for interaction with Pto in the yeast two-hybrid system.

Although AvrPtoB$_{121\text{-}200}$ interacts with Pto in yeast, *Agrobacterium*-mediated transient expression of this region in RG-PtoR tomato leaves did not elicit cell death (Figure 2b). Because we were not able to detect AvrPtoB$_{121\text{-}200}$ in *Agrobacterium*-transformed tomato leaves (data not shown), it is possible that this truncated protein is simply unstable. Further experiments established that AvrPtoB$_{1\text{-}200}$ elicits cell death on RG-PtoR leaves upon *Agrobacterium*-mediated transient expression (Figure 2b). We therefore examined whether AvrPtoB$_{1\text{-}200}$ elicits disease resistance on RG-PtoR when expressed in DC3000Δ*avrPto*Δ*avrPtoB* (note that type III-mediated secretion of AvrPtoB from *P. s.* pv. *tomato* requires some N-terminal residues of the effector). We found that AvrPtoB$_{1\text{-}200}$ did not elicit disease resistance or cause an HR (data not shown) on RG-PtoR, even though AvrPtoB$_{1\text{-}200}$ was expressed and secreted from *P. s.* pv. *tomato* at a similar level as AvrPtoB$_{1\text{-}307}$ (Figure 2c–e). The cell death caused by *Agrobacterium* delivery of AvrPtoB$_{1\text{-}200}$ might therefore be due to the higher protein expression levels associated with this system. Taken together, these data indicate that amino acids 121–200 encode specificity for interaction of AvrPtoB$_{1\text{-}307}$ with Pto, and suggest that amino acids that lie outside this region also contribute to avirulence activity upon delivery by *P. s.* pv. *tomato*.

F173 is a key amino acid mediating interaction of AvrPtoB$_{1\text{-}307}$ with Pto

We recently identified and characterized several AvrPtoB homologs from *P. s.* pv. *tomato*, *P. s.* pv. *syringae* and *P. s.* pv. *maculicola* (Lin *et al.*, 2006). Like AvrPtoB from *P. s.* pv. *tomato* DC3000, each of these AvrPtoB homologs interacts with Pto in yeast and elicits Pto-mediated host immunity in RG-PtoR tomato plants (Lin *et al.*, 2006). The homologs have 21 identical amino acids in the region between AvrPtoB amino acids 121–200, and we reasoned

Figure 1. The N-terminal region of AvrPtoB has both avirulence and virulence activities in *Pseudomonas syringae* pv. *tomato*.
(a) Disease symptoms or immunity on leaves from RG-PtoR tomato plants after inoculation with DC3000Δ*avrPto*Δ*avrPtoB* carrying either empty vector, or plasmids expressing AvrPtoB, AvrPtoB$_{1\text{-}307}$ or AvrPtoB$_{1\text{-}387}$. Leaves from a similar position on each plant were detached, de-pigmented, and photographed 4 days after inoculation.
(b) Bacterial populations of leaves as in (a). *P. s.* pv. *tomato* strains were vacuum-infiltrated into RG-PtoR tomato plants with an inoculum of 10^4 cfu ml^{-1}. Error bars represent the SE of three replicates. Experiments were repeated three times with similar results.
(c) Disease symptoms on RG-prf3 tomato plants 4 days after inoculation with the *P. s.* pv. *tomato* strains as in (a). The arrows indicate severe necrosis observed on lower leaves.
(d) *P. s.* pv. *tomato* populations in RG-prf3 leaves at 0 and 2 days after inoculation using a starting inoculum of 10^4 cfu ml^{-1}. Note that older leaves were sampled and that severe necrosis of these leaves prevented sampling later than day 2. Data from three independent experiments were used for statistical analysis using the SAS general linear model procedure. Values marked by the same letters were not significantly different from each other based on a least significant difference test ($P = 0.05$). Error bars indicate SE.
(e) Disease symptoms on RG-pto11 tomato plants 4 days after inoculation with the *P. s.* pv. *tomato* strains as in (a). AvrPtoB$_{1\text{-}387}$ was not used in this experiment because it activates Rsb immunity in RG-pto11 (Abramovitch *et al.*, 2003). The arrows indicate severe necrosis observed on lower leaves.
(f) *P. s.* pv. *tomato* populations and statistical analysis in RG-pto11 leaves as in (d).

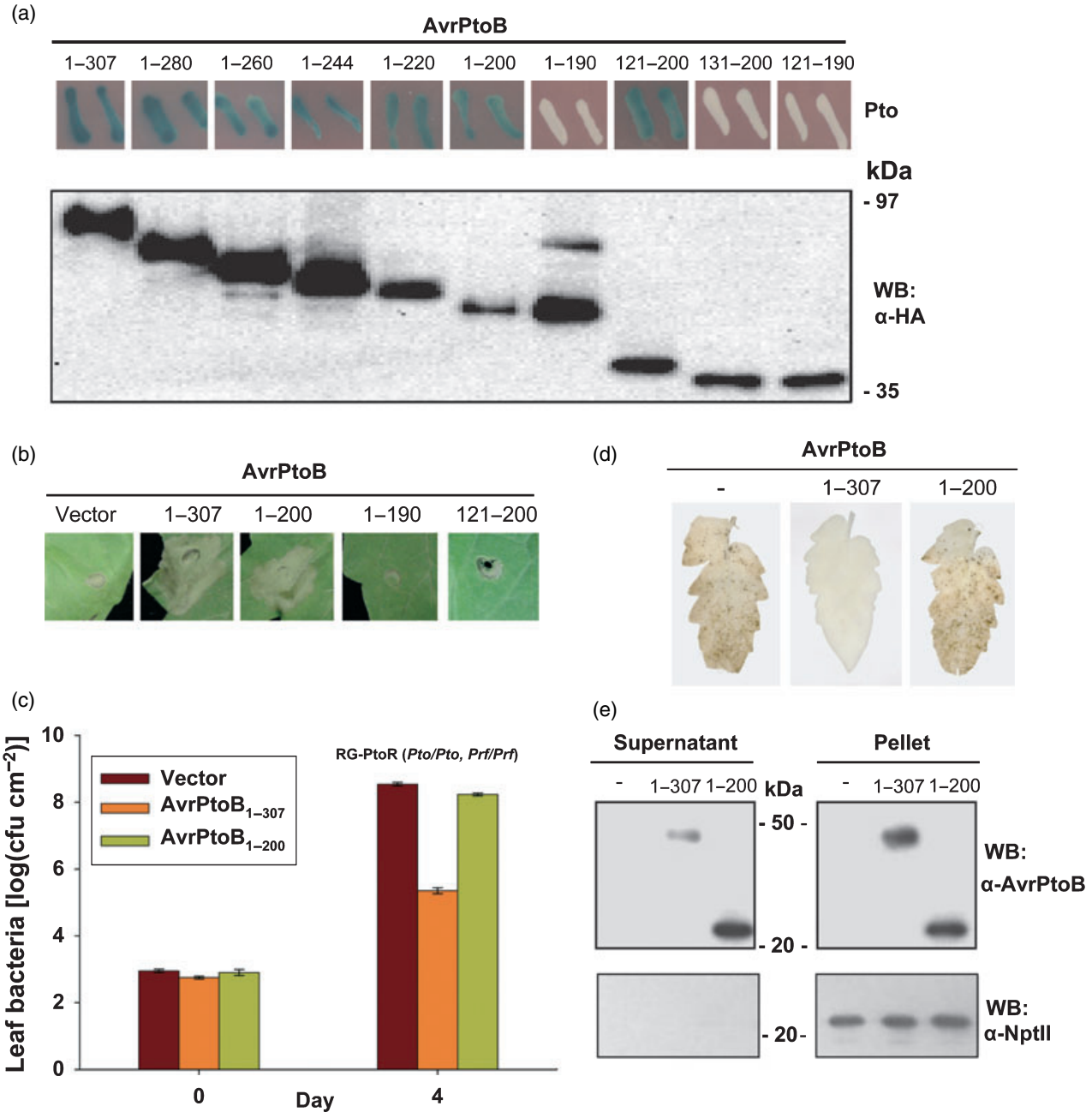


Figure 2. A small N-terminal region of AvrPtoB is sufficient for interaction with Pto kinase.
(a) Yeast two-hybrid interaction assays of AvrPtoB truncation mutants (N-terminally tagged with an hemagglutinin (HA) epitope) and Pto (upper panel). Pto was expressed as the bait and AvrPtoB truncation mutants as prey. Protein accumulation of the AvrPtoB proteins in yeast was detected by Western blotting using anti-HA antibody (lower panel). The origin of the higher molecular mass band cross-reacting with HA in the 1-190 lane is unknown. Positions of protein molecular mass markers are shown.
(b) Cell death assays of AvrPtoB truncation mutants using *Agrobacterium*-mediated transient expression. *Agrobacterium* containing individual truncation mutants were syringe-infiltrated into RG-PtoR tomato leaves at a concentration (OD_{600}) of 0.03. Photographs were taken 3 days after infiltration.
(c) Bacterial populations in RG-PtoR leaves 4 days after inoculation with DC3000Δ*avrPto*Δ*avrPtoB* strains harboring the empty vector or plasmids expressing $AvrPtoB_{1\text{-}307}$ or $AvrPtoB_{1\text{-}200}$ at inoculum of 10^4 cfu ml^{-1}. Error bars represent the SE of three replicates. Experiments were repeated three times with similar results.
(d) De-pigmented leaves from a similar position on each plant as used for the bacterial population measurements in (c).
(e) Expression and secretion of $AvrPtoB_{1\text{-}307}$ and $AvrPtoB_{1\text{-}200}$ from DC3000Δ*avrPto*Δ*avrPtoB* grown in minimal medium under *hrp* induction conditions (Lin *et al.*, 2006). Western blotting was performed using anti-AvrPtoB or anti-NptII antibody. Cytoplasmically localized NptII was used as a control for cell lysis. No NptII protein was detected in the culture medium using an anti-NptII antibody.

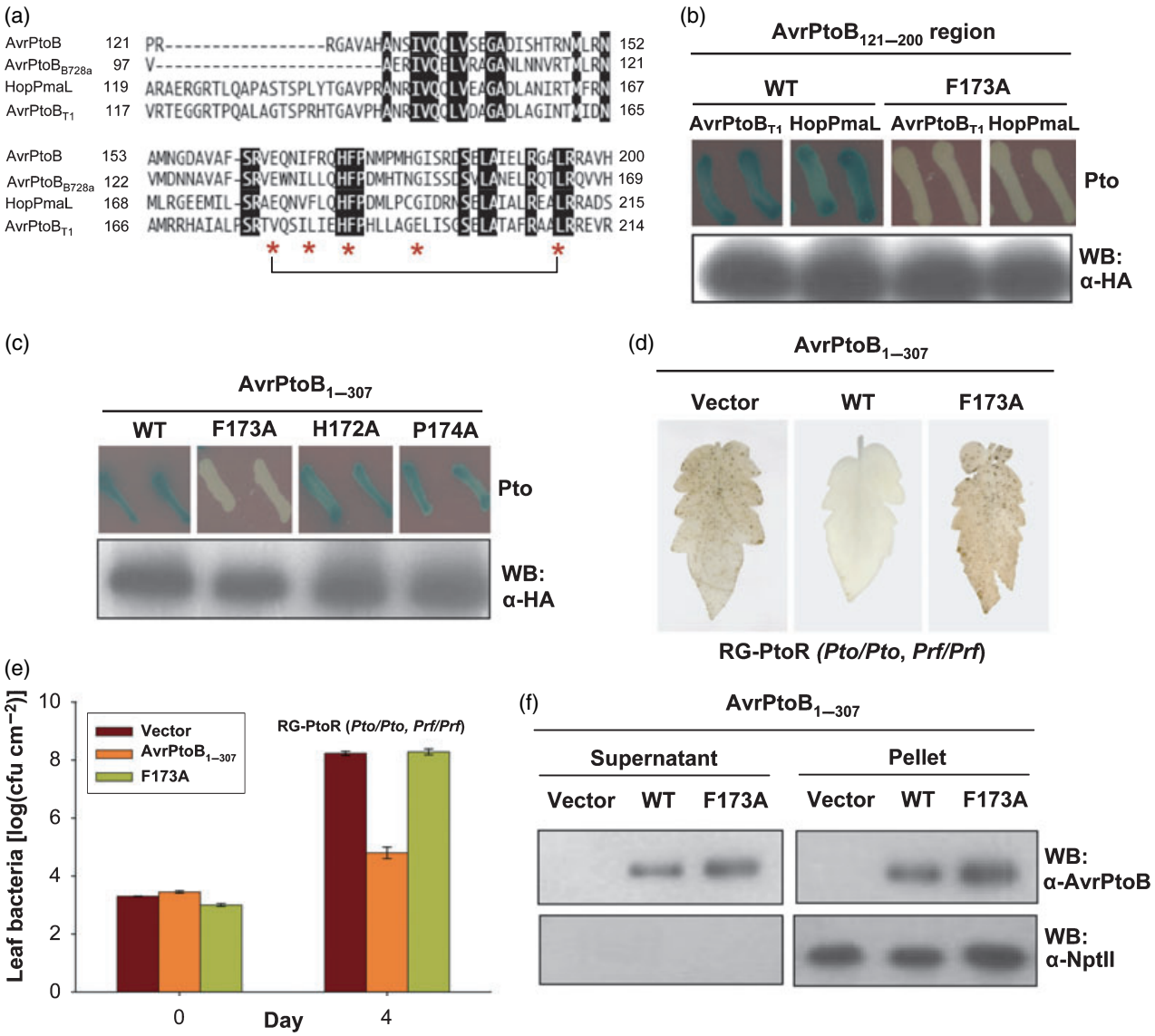


Figure 3. Phenylalanine 173 is required for interaction of AvrPtoB$_{1-307}$ with Pto.
(a) Alignment of the amino acids in the AvrPtoB$_{121-200}$ regions from AvrPtoB and three homologs. Identical amino acids are shaded in black. Positions of the AvrPtoB$_{1-307}$ amino acids that were later found to be required for interaction with Pto (see Figure 5) are indicated by asterisks.
(b) Yeast two-hybrid interaction between Pto and the 121–200 amino acid region from AvrPtoB$_{T1}$ and HopPmaL. Pto interacts with the wild-type 121–200 region from AvrPtoB$_{T1}$ and HopPmaL, but not F173A mutants of this region. Pto was expressed as the bait protein and the AvrPtoB$_{121-200}$ fragments were expressed as prey. Expression of the HA-tagged AvrPtoB proteins was confirmed by Western blotting using anti-HA antibody (lower panel).
(c) Yeast two-hybrid interaction between Pto and AvrPtoB$_{1-307}$ mutants F173A, H172A or P174A. Pto was expressed as the bait. The three mutants were expressed as prey. Western blotting using anti-HA antibody indicates that the HA-tagged AvrPtoB$_{1-307}$ and three mutants were expressed in yeast (lower panel).
(d) Disease symptoms or plant immunity of RG-PtoR tomato plants 4 days after inoculation with DC3000Δ*avrPto*Δ*avrPtoB* harboring the empty vector or plasmids expressing wild-type AvrPtoB$_{1-307}$ or the F173A mutant.
(e) Bacterial populations in leaves of plants as in (d). Each *P. s.* pv. *tomato* strain was vacuum-infiltrated into RG-PtoR tomato plants with an inoculum of 10^4 cfu ml^{-1}. Error bars represent the SE of three replicates. Experiments were repeated three times with similar results.
(f) Expression and secretion of AvrPtoB$_{1-307}$ and the AvrPtoB$_{1-307(F173A)}$ mutant from DC3000Δ*avrPto*Δ*avrPtoB* grown in minimal medium under *hrp* induction conditions. The *P. s.* pv. *tomato* secretion assay was carried out as described in Figure 2(e).

that they might therefore be useful in further defining the basis of Pto interaction specificity (Figure 3a). Fragments corresponding to AvrPtoB$_{121-200}$ (as aligned by Lin *et al.*, 2006) were PCR-amplified from two representative alleles, *avrPtoB*$_{T1}$ and *hopPmaL* (also named *HopAB3*), and examined in the yeast two-hybrid assay for interaction with Pto. The 121–200 amino acid regions from both AvrPtoB$_{T1}$ and HopPmaL interacted with Pto, suggesting that the Pto interaction capability of this small region is functionally conserved (Figure 3b).

To identify AvrPtoB residues contributing to the interaction with Pto, alanine substitution mutagenesis of the 18 non-alanine residues among the 21 conserved amino acids was carried out on the AvrPtoB$_{1\text{-}307}$ template, and the mutants were examined for interaction with Pto in the yeast two-hybrid assay. Of these mutants, only AvrPtoB$_{1\text{-}307(F173A)}$ (hereafter F173A) lost the ability to interact with Pto in yeast, whereas the others retained Pto interaction (Figure 3c and data not shown) (note we were unable to generate a AvrPtoB$_{1\text{-}307(L195A)}$ mutant; however, L195 was later found to be important for avirulence activity by random mutagenesis; see below). Western blot analysis indicated that F173A was expressed well in yeast (Figure 3c). Significantly, mutants with alanine substitutions at the two adjacent amino acids, H172A and P174A, retained the Pto interaction (Figure 3c), suggesting the loss of Pto interaction is unlikely to be due to a gross conformational change of AvrPtoB$_{1\text{-}307}$ by the F173A mutation.

We next tested whether the importance of F173 for AvrPtoB$_{1\text{-}307}$ interaction with Pto is functionally conserved among AvrPtoB homologs. The F173A mutation was introduced into the 121–200 amino acid region of AvrPtoB$_{T1}$ and HopPmaL, and interactions with Pto were examined. The F173A mutants were expressed in yeast at a similar level as the wild-type proteins but their interaction with Pto was abolished (Figure 3b), indicating that the role of F173 is conserved among these AvrPtoB homologs. Finally, we examined the specificity of F173 for interaction with Pto by analyzing the yeast two-hybrid interaction of the F173A mutant with an arbitrary AvrPtoB tomato-interacting (Bti) protein isolated previously (Bti6 is a putative lactate dehydrogenase; R.B.A. and G.B.M., unpubl. results). The F173A mutant interacted with Bti6 as strongly as wild-type AvrPtoB$_{1\text{-}307}$, further supporting the hypothesis that the F173A protein retains its overall conformation and that F173 mediates a specific interaction of AvrPtoB$_{1\text{-}307}$ with Pto (Supplementary Figure S1).

F173 is required for the avirulence activity of AvrPtoB$_{1\text{-}307}$

To determine whether abolishing AvrPtoB$_{1\text{-}307}$ interaction with Pto results in a loss of avirulence activity when the protein is expressed in *P. s.* pv. *tomato*, the *avrPtoB$_{1\text{-}307(F173A)}$* gene was introduced into DC3000Δ*avrPto*Δ*avrPtoB* and the resulting strain infiltrated into RG-PtoR plants. This strain caused similar disease symptoms and grew to the same population level in RG-PtoR tomato plants as the DC3000Δ*avrPto*Δ*avrPtoB* strain with an empty vector (Figure 3d,e). A *P. s.* pv. *tomato* secretion assay showed that the F173A protein was expressed and secreted at a similar level in the DC3000Δ*avrPto*Δ*avrPtoB* strain as wild-type AvrPtoB$_{1\text{-}307}$ (Figure 3f). Thus, these results indicate that F173 is required for AvrPtoB$_{1\text{-}307}$ avirulence activity.

Four additional amino acids in the 121–200 region are critical for the avirulence activity of AvrPtoB$_{1\text{-}307}$

In order to identify other amino acids essential for the avirulence activity of AvrPtoB$_{1\text{-}307}$, we performed a PCR-based random mutagenesis screen to identify AvrPtoB$_{1\text{-}307}$ mutants deficient in eliciting the HR in RG-PtoR tomato leaves (see Experimental procedures). Among 386 mutagenized clones examined in an *Agrobacterium*-mediated transient expression assay, 22 were found to be defective in HR elicitation. Of these clones, 18 were not studied further because they either had mutations within the first 200 N-terminal amino acids causing a stop codon or a frameshift or they had multiple point mutations (Supplementary Table S1). Four *avrPtoB$_{1\text{-}307}$* clones that had single point mutations in the 121–200 amino acid region (causing substitutions E165 K, F169S, G180 V or L195H) were found to be unable to elicit an HR in RG-PtoR leaves using the *Agrobacterium* infiltration assay and were therefore further characterized (Figure 4a). Interestingly, of these four residues, only L195 is conserved at the corresponding position of each homolog (Figure 3a). Analysis of the sequences, however, suggests some amino acid residue conservation at two of the other three positions. All of the AvrPtoB homologs have hydrophobic residues phenylalanine or leucine at position 169 (Lin *et al.*, 2006). The G180 residue is conserved in all the homologs except AvrPtoB$_{T1}$, where the glycine is substituted for a glutamate residue. However, in AvrPtoB$_{T1}$, there is a glycine in the position prior to the glutamate, and this might structurally accommodate the glutamate substitution (Lin *et al.*, 2006).

To further examine the loss of AvrPtoB avirulence activity due to these four mutations and to verify normal expression of these proteins, we tested them in a RG-PtoR tomato protoplast transformation assay. In this assay, transient expression of wild-type AvrPtoB$_{1\text{-}307}$ is recognized by the endogenous Pto protein, and immunity-associated cell death is monitored by Evans blue staining 24 h after transformation. We found that RG-PtoR tomato protoplasts transformed with each of the four mutants or with an empty vector control maintained reasonably high viability (<40% dead cells) at 24 h (Figure 4b). In contrast, 90% of the protoplasts transformed with wild-type AvrPtoB$_{1\text{-}307}$ died within 24 h due to immunity-associated cell death by Pto recognition (Figure 4b). Western blot analysis showed that all four mutants were expressed well at the 24 h time point, whereas wild-type protein was not detected, probably due to general protein degradation caused by cell death (Figure 4b).

We next tested whether the four mutations affected avirulence activity when expressed by *P. s.* pv. *tomato*. The mutant genes were introduced on plasmids into the DC3000Δ*avrPto*Δ*avrPtoB* strain, and the expression and secretion of their proteins was confirmed by a *P. s.* pv. *tomato* secretion assay (Figure 4e). Infiltration of these

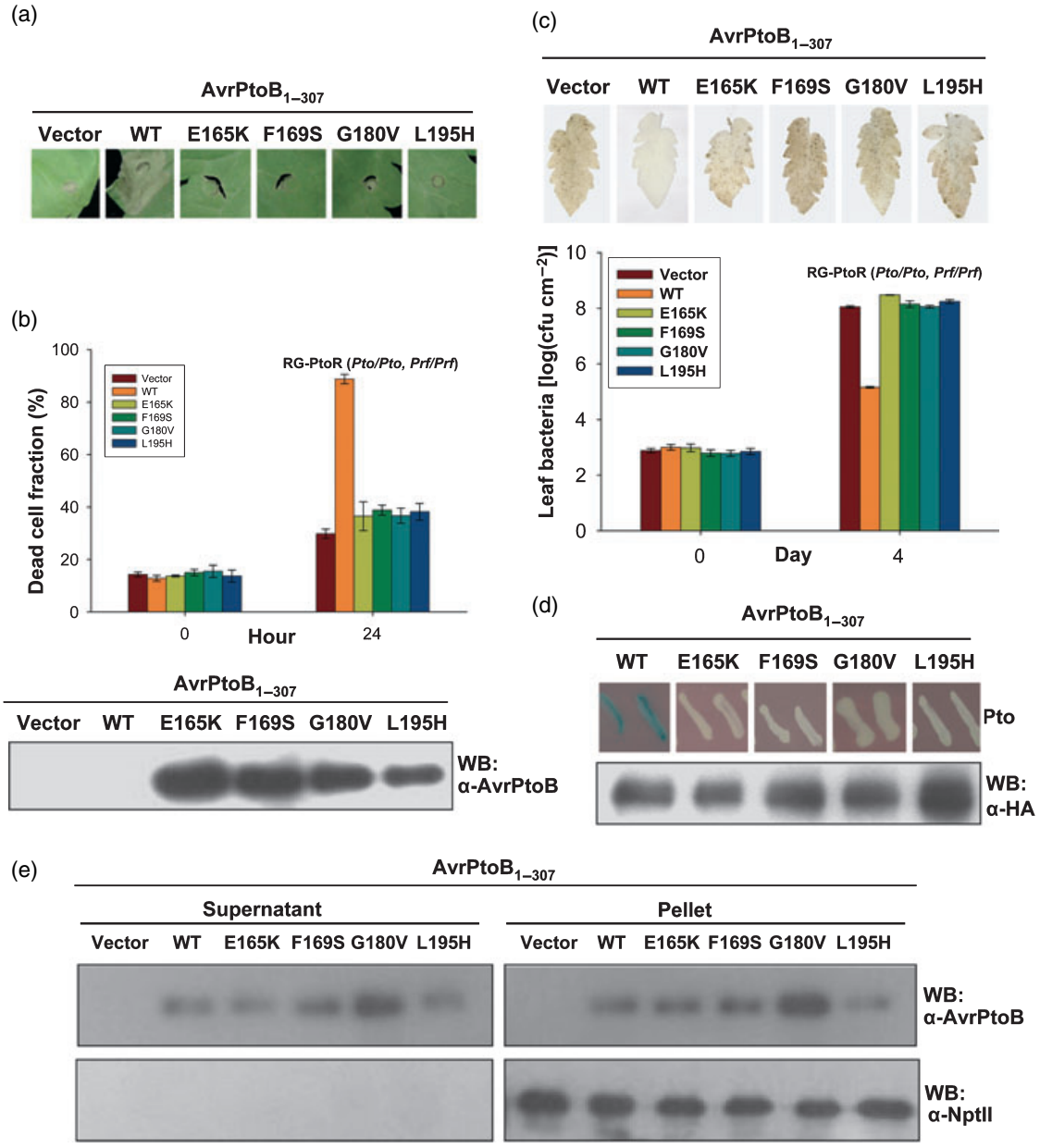


Figure 4. Four AvrPtoB$_{1\text{-}307}$ proteins with single amino acid substitutions are compromised in avirulence activity.
(a) HR assay of wild-type AvrPtoB$_{1\text{-}307}$ or E165 K, F169S, G180 V, L195H mutants using *Agrobacterium*-mediated transient expression in RG-PtoR tomato leaves. Photographs were taken 3 days after *Agrobacterium* infiltration.
(b) Transient expression of wild-type AvrPtoB$_{1\text{-}307}$, E165 K, F169S, G180 V or L195H mutants in RG-PtoR protoplasts. Plasmids expressing wild-type AvrPtoB$_{1\text{-}307}$ or the mutants were transformed into RG-PtoR protoplasts and cell death was monitored by Evans blue staining 24 h after transformation (upper panel). Approximately 200 randomly selected protoplast cells were counted for determination of the dead cell fraction. Protein expression was analyzed by Western blotting using an anti-AvrPtoB antibody (lower panel).
(c) *P. s.* pv. *tomato* assay of AvrPtoB$_{1\text{-}307}$, E165 K, F169S, G180 V or L195H mutants. Disease symptoms or plant immunity of RG-PtoR tomato leaves 4 days after inoculation with DC3000Δ*avrPto*Δ*avrPtoB* harboring the empty vector or plasmids expressing wild-type AvrPtoB$_{1\text{-}307}$, E165 K, F169S, G180 V or L195H (upper panel). Bacterial populations in leaves of plants inoculated with the indicated strains (lower panel). Each *P. s.* pv. *tomato* strain was vacuum-infiltrated into three RG-PtoR tomato plants with an inoculum of 10^4 cfu ml^{-1}. Error bars represent the SE of three replicates. Experiments were repeated three times with similar results.
(d) Yeast two-hybrid interaction between Pto and AvrPtoB$_{1\text{-}307}$ or the AvrPtoB$_{1\text{-}307}$ mutants. Wild-type AvrPtoB$_{1\text{-}307}$ or the four mutants were expressed as prey and Pto was expressed as the bait. Protein expression of the HA-tagged AvrPtoB proteins was confirmed by Western blot using an anti-HA antibody.
(e) Expression and secretion of AvrPtoB$_{1\text{-}307}$ mutants from DC3000Δ*avrPto*Δ*avrPtoB* grown in minimal medium under *hrp* induction conditions. The *P. s.* pv. *tomato* secretion assay was carried out as described in Figure 2(e).

strains into RG-PtoR tomato plants revealed that none of the four mutants elicited Pto-mediated immunity, as evidenced by formation of bacterial speck symptoms and final population levels that were not different to those with the control DC3000Δ*avrPto*Δ*avrPtoB* empty vector strain (Figure 4c).

These four mutations lie in the 121–200 amino acid region that is sufficient for AvrPtoB$_{1\text{-}307}$ interaction with Pto. As anticipated, based on their location in this region and their loss of avirulence activity, the yeast two-hybrid interaction between Pto and these four point mutants was abolished even though each of the proteins was expressed well in yeast (Figure 4d). Collectively, our mutagenesis data show that five clustered residues (E165, F169, F173, G180 and L195) residing in a small (approximately 30 amino acid) domain play an important role for AvrPtoB$_{1\text{-}307}$ interaction with Pto and correspondingly for avirulence activity of AvrPtoB$_{1\text{-}307}$.

Most AvrPtoB$_{1\text{-}307}$ avirulence-deficient mutants retain virulence activity

It has been hypothesized that R proteins might have evolved to recognize structural or enzymatic features of type III effectors that are required for their virulence activity (Dangl and Jones, 2001; Van der Biezen and Jones, 1998). We therefore tested whether the five avirulence-defective AvrPtoB$_{1\text{-}307}$ mutants were also defective in virulence activity. DC3000Δ*avrPto*Δ*avrPtoB* strains harboring the five AvrPtoB$_{1\text{-}307}$ mutants, wild-type AvrPtoB$_{1\text{-}307}$ or an empty vector were infiltrated into susceptible tomato RG-prf3 plants. Virulence activity was evaluated after infiltration, as measured by severity of disease symptoms and level of bacterial growth (Figure 5). DC3000Δ*avrPto*Δ*avrPtoB* strains carrying wild-type AvrPtoB$_{1\text{-}307}$ or the mutations E165 K, F169S, G180 V or L195H all caused severe disease symptoms and resulted in similar population levels (Figure 5a; note the four lowest leaves, which are necrotic and completely desiccated 4 days after inoculation). Interestingly, the DC3000Δ*avrPto*Δ*avrPtoB* strain expressing F173A (F173A was earlier shown to be expressed and secreted normally from *P. s.* pv. *tomato*; Figure 3f) caused much less severe disease symptoms and resulted in a bacterial population level at 2 days post-inoculation that was not statistically different from that of the empty vector control strain (Figure 5b). These results therefore indicate that, with one exception (F173), the AvrPtoB$_{1\text{-}307}$ residues involved in the interaction with Pto do not significantly contribute to AvrPtoB$_{1\text{-}307}$-mediated virulence.

The N-terminal region of AvrPtoB uses two distinct mechanisms to promote bacterial virulence

We reported previously that, in susceptible tomato plants, AvrPtoB induces the expression of a set of host genes associated with ethylene biosynthesis and promotes ethylene production during disease development (Cohn and Martin, 2005). To determine whether the N-terminus of AvrPtoB is responsible for this ethylene-dependent virulence activity, we tested whether AvrPtoB$_{1\text{-}307}$ is sufficient for the promotion of ethylene production and the induction of two ethylene biosynthesis genes, *S. lycopersicum ACC oxidase 1 and 2* (*SlAco1*and *SlAco2*). Using similar conditions as our previous experiments, we found by RT-PCR that expression of AvrPtoB$_{1\text{-}307}$ (or AvrPtoB$_{1\text{-}387}$) in *P. s.* pv. *tomato* was sufficient to induce *SlAco1* and *SlAco2* in RG-prf3 leaves as reported previously for the full-length AvrPtoB (Figure 6a) (Cohn and Martin, 2005)). The AvrPtoB$_{1\text{-}307(F173A)}$ mutant, which lacks virulence activity, did not induce expression of either of these two genes. We also examined the level of ethylene produced by susceptible tomato RG-prf3 plants infiltrated with DC3000Δ*avrPto*Δ*avrPtoB* strains harboring full-length AvrPtoB, AvrPtoB$_{1\text{-}387}$, AvrPtoB$_{1\text{-}307}$, AvrPtoB$_{1\text{-}307(F173A)}$ or an empty vector. The strains expressing AvrPtoB$_{1\text{-}307}$ or AvrPtoB$_{1\text{-}387}$ enhanced ethylene production, whereas the AvrPtoB$_{1\text{-}307(F173A)}$ mutant had no effect, like the empty vector control (Figure 6b). Expression of AvrPtoB in DC3000Δ*avrPto*Δ*avrPtoB* resulted in slightly higher ethylene production than expression of AvrPtoB$_{1\text{-}307}$ or AvrPtoB$_{1\text{-}387}$ (Figure 6b). Taken together, these data indicate that AvrPtoB$_{1\text{-}307}$, like full-length AvrPtoB, contributes to bacterial virulence by inducing ethylene biosynthesis genes and promoting ethylene production during a compatible disease interaction in tomato.

Recently, He *et al.* (2006) reported that AvrPtoB promotes *P. s.* pv. *tomato* virulence on Arabidopsis by targeting MAP kinase signaling to suppress various PAMP-mediated basal defenses. Importantly, this virulence activity appeared to be encoded by an N-terminal region of AvrPtoB as it was unaffected by disruption of the C-terminal E3 ligase activity. To extend this observation, we tested whether the two N-terminal regions, AvrPtoB$_{1\text{-}307}$ and AvrPtoB$_{1\text{-}387}$, can suppress activation of the basal defense marker gene *FRK1-LUC* by the PAMP elicitor flg22, a conserved 22 amino acid peptide from bacterial flagellin (Figure 7a). The *FRK1-LUC* reporter gene was co-transformed with AvrPtoB$_{1\text{-}307}$, AvrPtoB$_{1\text{-}387}$, full-length AvrPtoB or vector only, and Western blotting indicated that all AvrPtoB proteins were expressed in Arabidopsis protoplasts. As reported previously, full-length AvrPtoB strongly suppressed flg22 activation of *FRK1-LUC*. Interestingly, we found that AvrPtoB$_{1\text{-}307}$ did not suppress flg22 activation of *FRK1-LUC* whereas AvrPtoB$_{1\text{-}387}$ did (Figure 7a). Thus, basal defense suppression requires an additional domain of AvrPtoB encompassing amino acids 308–387. These results were further supported by real-time RT-PCR analysis on an additional six endogenous genes activated by flg22, including *WRKY30*, *WRKY53*, At5 g57220, chitinase, At2 g17740 and At2 g40180. The activation of all six genes by flg22 showed

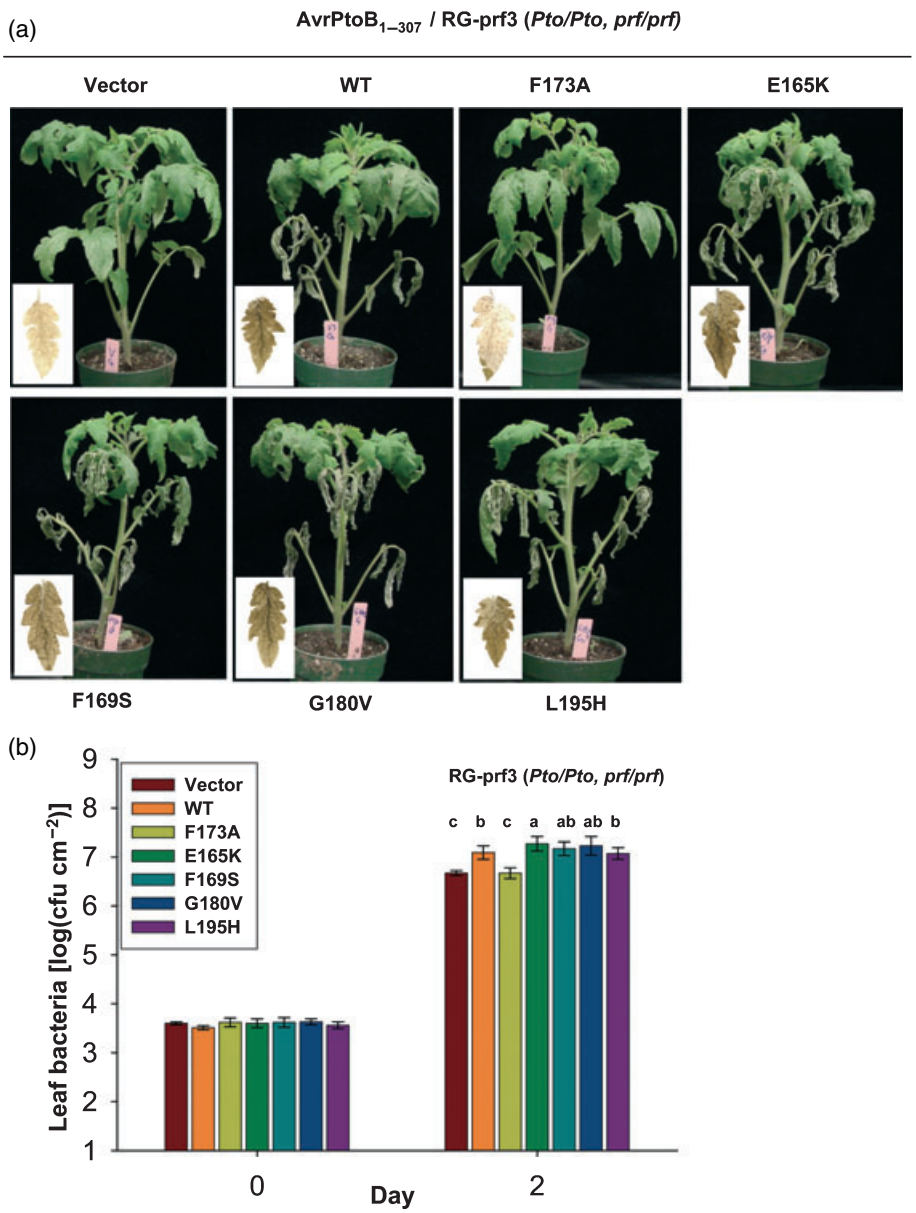


Figure 5. Virulence activities of AvrPtoB$_{1\text{-}307}$ mutants.
(a) Disease symptoms of tomato RG-prf3 plants inoculated with 10^4 cfu ml^{-1} of DC3000Δ*avrPto*Δ*avrPtoB* carrying the empty vector or plasmids expressing AvrPtoB$_{1\text{-}307}$, F173A, E165 K, F169S, G180 V or L195H. Representative de-pigmented leaves from each plant are shown in the bottom left corners. Plants were photographed 4 days after inoculation.
(b) Measurement of bacterial populations in leaves of the plants in (a) at 0 and 2 days after inoculation using a *P. s.* pv. *tomato* starting inoculum of 10^4 cfu ml^{-1}. Data pooled from three independent experiments (n = 9) were used for statistical analysis using the SAS general linear model procedure (see Experimental procedures). Values marked with the same letters were not significantly different from each other based on the least squares difference test (P = 0.05). Error bars indicate SE.

specific suppression by full-length AvrPtoB and AvrPtoB$_{1\text{-}387}$, but not by AvrPtoB$_{1\text{-}307}$ (Supplementary Figure S2).

To test whether suppression of PAMP-mediated basal defense by AvrPtoB$_{1\text{-}387}$ is associated with inhibition of MAP kinase signaling, we examined the flg22-induced activity of the MAP kinases MPK3 and MPK6, in the same assay used in our previous experiments (He *et al.*, 2006). Both full-length AvrPtoB and AvrPtoB$_{1\text{-}387}$, but not AvrPtoB$_{1\text{-}307}$, significantly blocked activation of MPK3 and MPK6 by flg22 in Arabidopsis protoplasts (Figure 7b), again indicating that amino acids 308–387 are required to interfere with MAP kinase signaling, and supporting a role for these residues in basal defense suppression in Arabidopsis.

It is possible that virulence-associated residues within the first 307 amino acids of AvrPtoB might contribute to the basal defense-suppressing activity associated with

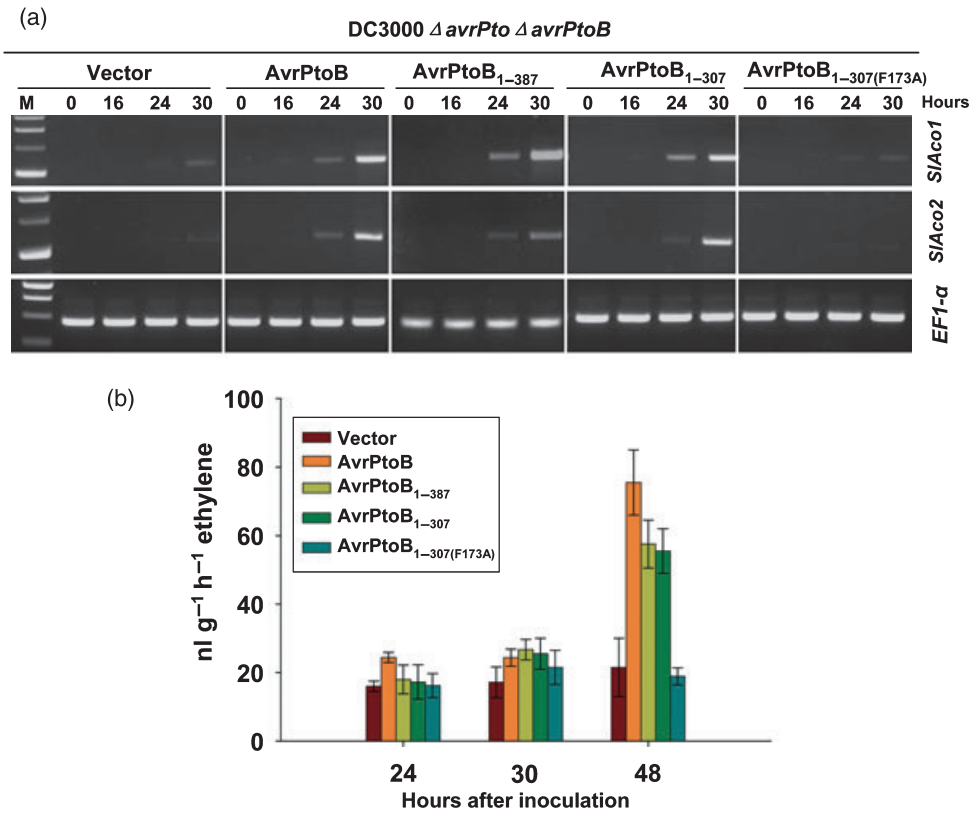


Figure 6. AvrPtoB$_{1\text{-}307}$ is sufficient to induce ethylene biosynthesis genes and promotes ethylene production during disease development in tomato plants.
(a) Tomato ACC oxidase 1/2 (*SlAco1/2*) gene expression was induced by AvrPtoB, AvrPtoB$_{1\text{-}387}$ and AvrPtoB$_{1\text{-}307}$, but not by the virulence-deficient AvrPtoB$_{1\text{-}307(F173A)}$ mutant. RG-prf3 plants were inoculated with 10^4 cfu ml^{-1} of DC3000Δ*avrPto*Δ*avrPtoB* carrying the empty vector, AvrPtoB, AvrPtoB$_{1\text{-}387}$, AvrPtoB$_{1\text{-}307}$ or AvrPtoB$_{1\text{-}307(F173A)}$. Total RNA was isolated at the indicated time points after infiltration, and 1 μg total RNA was used to generate first-strand cDNA. The induction of *SlAco1/2* was determined by 30 PCR cycles using *SlAco1/2* gene-specific primers, with *EF1-α* gene-specific primers as a control (Cohn and Martin, 2005; Lin and Martin, 2005). The data shown are representative of two experiments with independent biological replicates that gave similar results. Lane M, 1 kb DNA ladder.
(b) Ethylene production by susceptible tomato RG-prf3 plants infiltrated with 10^4 cfu ml^{-1} of DC3000Δ*avrPto*Δ*avrPtoB* carrying the empty vector or plasmids expressing full-length AvrPtoB, AvrPtoB$_{1\text{-}387}$, AvrPtoB$_{1\text{-}307}$ or AvrPtoB$_{1\text{-}307(F173A)}$. Ethylene was measured by gas chromatography at the time points indicated (Cohn and Martin, 2005; Lin and Martin, 2005). Error bars represent the SE of six replicates. The experiment was repeated twice with similar results.

AvrPtoB$_{1\text{-}387}$. We performed two experiments to test this hypothesis. Firstly, we considered the possibility that removal of 80 amino acids from the C-terminus of AvrPtoB$_{1\text{-}387}$ might impair a basal defense-suppressing activity encoded within the AvrPtoB$_{1\text{-}307}$ region. To examine this, we reduced the amount of flg22 from 1 μM to 100, 10 or 1 nM to induce weaker MPK6 activation, and tested whether AvrPtoB$_{1\text{-}307}$ might interfere with this lower activity (Figure 8a). However, we observed that AvrPtoB$_{1\text{-}307}$ was unable to suppress MPK6 activation induced by these lower flg22 levels, indicating it does not have any detectable activity towards this signaling pathway. Secondly, we assessed whether mutations in the AvrPtoB$_{1\text{-}307}$ Pto interaction domain might affect MPK6 suppression activity of AvrPtoB$_{1\text{-}387}$. Point mutations in AvrPtoB$_{1\text{-}387}$ of the ethylene-associated virulence residue F173 or four avirulence residues E165, F169, G180 or L195 caused no change in the ability of AvrPtoB$_{1\text{-}387}$ to suppress MPK6 activation (Figure 8b). Collectively, these data indicate that the 1-387 region of AvrPtoB has two autonomous virulence activities, one promoting ethylene and one suppressing basal defenses, and, significantly, these two virulence activities are structurally separable from AvrPtoB avirulence activity.

Discussion

A core domain of AvrPtoB$_{1\text{-}307}$ is required for Pto recognition and its activity is conserved in multiple AvrPtoB homologs

Homologs of *avrPtoB* are present in many *P. syringae* pathovars, and one of these, HopPmaL, encodes a truncated effector consisting just of a region corresponding to the N-terminal 354 amino acids of AvrPtoB (Guttman *et al.*, 2002). We reported previously that HopPmaL interacts with Pto and confers avirulence to *P. s.* pv. *tomato* on *Pto*-expressing tomato plants (Lin *et al.*, 2006). This observation suggested that the N-terminal region of AvrPtoB might be sufficient for avirulence if expressed in *P. s.* pv. *tomato*.

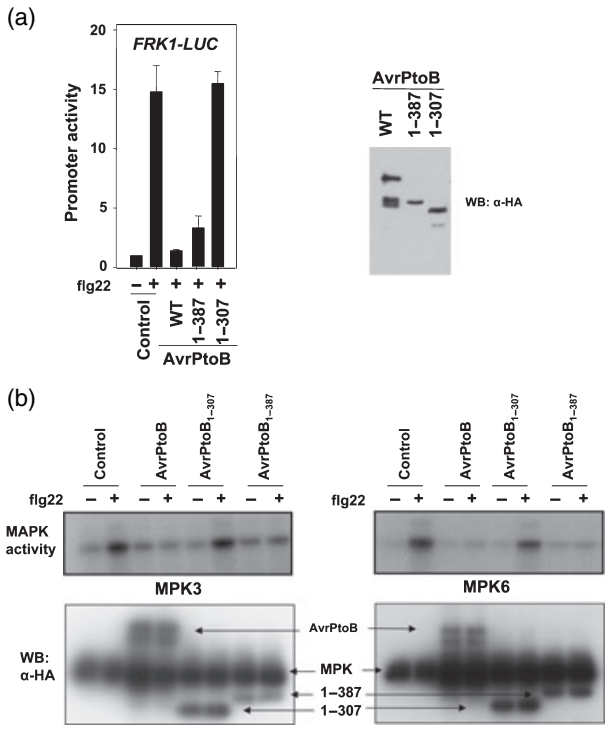


Figure 7. AvrPtoB$_{1\text{-}387}$ suppresses markers of pathogen-associated molecular patterns-induced basal defenses in Arabidopsis.
(a) AvrPtoB$_{1\text{-}387}$, but not AvrPtoB$_{1\text{-}307}$, suppresses flg22-induced *FRK1* promoter activity. Arabidopsis Col-0 protoplasts were co-transformed with AvrPtoB or derivatives and a *FRK1-LUC* reporter gene, incubated for 6 h, and then treated with 100 nM flg22 for 3 h. Luciferase activity was measured and is shown as relative promoter activity compared to a *UBQ10-GUS* control (He *et al.*, 2006). Protein expression of AvrPtoB and derivatives was analyzed by Western blotting using an anti-HA antibody (right panel).
(b) AvrPtoB and AvrPtoB$_{1\text{-}387}$, but not AvrPtoB$_{1\text{-}307}$, blocked flg22-induced MAP kinase activation. HA-tagged MPK3/6 and AvrPtoB or derivatives were co-expressed in Arabidopsis protoplasts for 6 h, and then 1 μM flg22 was added for 10 min. MAP kinases and effectors were immunoprecipitated with anti-HA antibody, followed by *in vitro* kinase assay (upper panel). Expression of MAP kinases and effectors were confirmed by Western blotting using an anti-HA antibody (bottom panel) (He *et al.*, 2006).

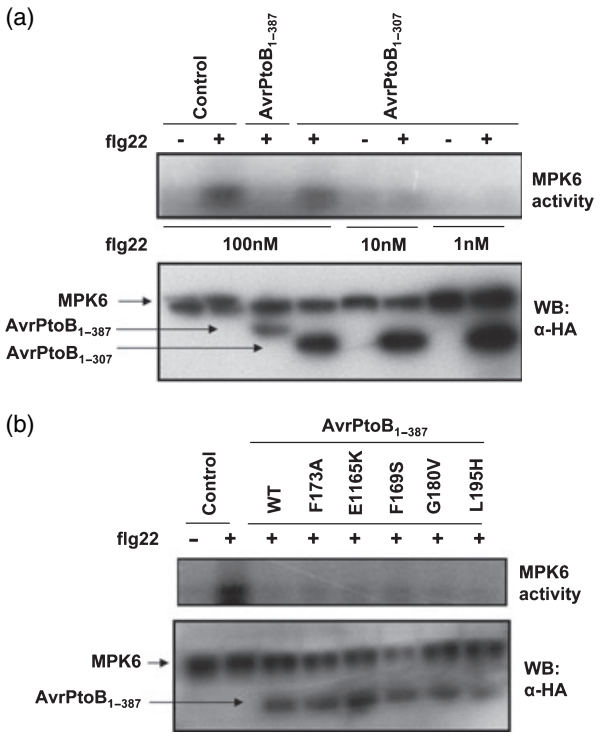


Figure 8. Amino acids 308–387 encode a determinant for suppression of pathogen-associated molecular patterns-induced basal defense in Arabidopsis.
(a) AvrPtoB$_{1\text{-}307}$ was unable to suppress even weak activation of MPK6 induced by one-tenth the amount (100 nM) of flg22 used in Figure 7, further supporting a requirement for amino acids 308–387. Note that flg22 at a concentration of 10 or 1 nM did not activate MPK6.
(b) Five mutations (E165 K, F169S, G180 V, L195H and F173A) that compromise the avirulence or ethylene-dependent virulence activities of AvrPtoB do not affect the suppression of flg22-induced MPK6 activation by AvrPtoB$_{1\text{-}387}$.

Indeed, in a study directed at understanding the cell death-suppression activity of AvrPtoB, it was found that AvrPtoB$_{1\text{-}307}$ is sufficient for interaction with Pto, and elicits *Pto/Prf*-dependent cell death in a transient *Agrobacterium* assay (Abramovitch *et al.*, 2003). Together, these observations suggested that *avrPtoB*$_{1\text{-}307}$ would be sufficient to elicit *Pto/Prf*-mediated resistance when introduced into DC3000Δ*avrPtoΔavrPtoB*. The data presented here confirmed this to be the case.

Random mutagenesis of AvrPtoB$_{1\text{-}307}$ indicated that a localized region is required for Pto interaction. Other random mutagenesis studies of effector proteins have also defined either localized stretches of amino acids or spatially clustered amino acids that are required for avirulence activity (Lim and Kunkel, 2004; Ong and Innes, 2006; Shan *et al.*, 2000b). For example, random mutagenesis of AvrRpt2 identified four residues required for avirulence associated with RPS2, with three lying within a 20 amino acid stretch and another one just 30 residues away (Lim and Kunkel, 2004). A three-dimensional structure is available for AvrB, and random mutagenesis of that effector revealed that residues required for its avirulence activity map to a spatially close upper lobe and pocket region of that protein (Ong and Innes, 2006). It is possible that these clustered avirulence determinants represent contact points for host proteins acting in concert with R proteins (and, in some cases, R proteins themselves), or catalytic residues if enzymatic activities are involved. Indeed, a recent report of the co-crystal structure of AvrB and a portion of RIN4, a host protein required for recognition of AvrB by RPM1, revealed that two AvrB residues, T125 and R266, required for avirulence serve as direct binding sites for RIN4 (Desveaux *et al.*, 2007).

The 80 amino acid region of AvrPtoB that is sufficient for interaction with Pto in yeast was not sufficient to elicit cell death upon *Agrobacterium*-mediated transient expression. Nor were the first 200 amino acids of AvrPtoB (which includes the N-terminal region needed for secretion)

sufficient to elicit disease resistance in *Pto*-expressing plants when delivered by *P. s.* pv. *tomato* DC3000, despite the fact that the AvrPtoB$_{1\text{-}200}$ truncated protein was secreted from the bacterium at normal levels. Together, these observations suggest that additional amino acids of AvrPtoB lying outside the Pto-interacting core region are required for avirulence activity in addition to being required for secretion from *P. s.* pv. *tomato*. In the plant cell, regions flanking the Pto-interacting core region could be acting to properly localize the effector, stabilize the interaction with Pto, or facilitate other protein–protein interactions that are required to elicit *Pto/Prf*-mediated immunity.

Our study focused mostly on amino acids 1–307 of AvrPtoB because we found that this region interacted with Pto, elicited avirulence, and promoted virulence in tomato in a fashion indistinguishable from full-length AvrPtoB. However, our data do not exclude the possibility that, in full-length AvrPtoB, other regions outside this region might also affect avirulence activity. For example, we reported previously that an alanine substitution at G325 weakened the interaction of full-length AvrPtoB with Pto and diminished its avirulence activity (Kim *et al.*, 2002). It is possible therefore that intramolecular interactions between the regions containing F173 and G325 influence interaction with Pto and other activities of full-length AvrPtoB. Interestingly, evidence for a possible intramolecular interaction was also observed with AvrPto. In that effector, a mutation at the C-terminal phosphorylation site (S149) weakened the avirulence activity mediated by I96 in the CD loop (Anderson *et al.*, 2006). Ultimately, it will be necessary to obtain a three-dimensional structure of full-length AvrPtoB to understand whether such intramolecular interactions play a role in AvrPtoB avirulence activity.

Two N-terminal virulence activities of AvrPtoB

We found that AvrPtoB$_{1\text{-}307}$ is sufficient to account for the increased disease symptoms and enhanced bacterial growth that were previously found to be associated with full-length AvrPtoB virulence activity when DC3000Δ*avrPto* is inoculated onto susceptible tomato plants (Cohn and Martin, 2005; Lin and Martin, 2005). We investigated the molecular basis underlying this virulence activity and found that AvrPtoB$_{1\text{-}307}$ induces expression of ethylene biosynthetic genes and promotes ethylene production associated with enhanced disease symptoms. The virulence-defective AvrPtoB$_{1\text{-}307(F173A)}$ mutant abolished induction of *SlAco1/2* genes and ethylene production (Figure 6). In addition to this ethylene-associated virulence in tomato, two recent reports show that AvrPtoB also enhances the virulence of *P. s.* pv. *tomato* DC3000 on Arabidopsis (He *et al.*, 2006; de Torres *et al.*, 2006). These reports showed that AvrPtoB suppresses basal defense-associated activation of MAP kinases and callose deposition, and this virulence activity did not require the C-terminal E3 ligase activity (He *et al.*, 2006; de Torres *et al.*, 2006). Significantly, our experiments now show that AvrPtoB residues 1–307 promote ethylene production associated with disease symptoms and bacterial growth, whereas residues 308–387 are required to suppress PAMP-mediated basal defenses. Thus these two virulence activities are encoded by distinct structural domains of AvrPtoB. We were unable to detect enhanced ethylene production from Arabidopsis plants infected by AvrPtoB-expressing *P. s.* pv. *tomato* (F.X. and G.M., unpubl. results). In addition, we are currently unable to investigate whether amino acids 308–387 suppress basal defenses in tomato as we lack a robust assay for this activity. Future studies will therefore be needed to clarify whether certain AvrPtoB virulence activities are general or specific to different plant species.

It is notable that we did not observe a significant difference in virulence activity conferred by AvrPtoB$_{1\text{-}307}$ or AvrPtoB$_{1\text{-}387}$ with regard to disease severity or bacterial growth rates. This could simply be due to a lack of sensitivity of our bacterial growth and disease assays, or alternatively it could be indicative of some functional redundancy of the two virulence activities. In this regard, AvrPtoB has been shown recently to act as suppressor of multiple plant defense responses in *N. benthamiana*, including ROS burst, callose deposition, calcium burst and MAP kinase activation (Hann and Rathjen, 2007). It will be interesting to determine which AvrPtoB domains are responsible for these various activities.

AvrPtoB$_{1\text{-}307}$ does not require Pto for virulence activity, suggesting alternative models for the guard hypothesis in Pto-mediated recognition

The guard model, which was originally inspired by the Pto/Prf system, postulated that Pto contributes to host basal defense, that the *P. s.* pv. *tomato* effectors AvrPto and AvrPtoB evolved to interact with Pto and thereby perturb its activity, and that Prf evolved to recognize ('guard') this perturbation of Pto and to activate host defenses (Dangl and Jones, 2001; Van der Biezen and Jones, 1998). Accordingly, avirulence and virulence activities of effector proteins are expected to be co-dependent. Several lines of evidence support tenets of the guard hypothesis for the Pto system. Firstly, Pto is a plausible virulence target for *Pseudomonas* effectors because its over-expression leads to enhanced resistance to virulent pathogens (and it therefore might play a role in basal defense) (Tang *et al.*, 1999). Secondly, Pto physically interacts with AvrPtoB and AvrPto (Kim *et al.*, 2002; Scofield *et al.*, 1996; Tang *et al.*, 1996). Thirdly, AvrPtoB and AvrPto have demonstrable virulence activity (Abramovitch *et al.*, 2003; Chang *et al.*, 2000; Lin and Martin, 2005; Shan *et al.*, 2000a). Finally, the activities of Pto and Prf are inter-dependent (Rathjen *et al.*, 1999; Xiao *et al.*, 2003).

However, we have previously discussed some observations involving AvrPto that are inconsistent with tenets of the guard model (Pedley and Martin, 2003). First, AvrPto exhibits similar virulence activity on tomato leaves whether or not they express *Pto* (Shan *et al.*, 2000a). Thus Pto does not appear to be an important virulence target of AvrPto. Second, certain mutations in AvrPto cause a loss of avirulence and yet the proteins still retain virulence activity (Shan *et al.*, 2000a). This observation indicates that there is a virulence target other than Pto for AvrPto. Similar results have been reported in the case of interaction between Rin4 and AvrRpt2 and AvrRpm1, in which Rin4, which is required for avirulence of AvrRpt2 and AvrRpm1, is not required for the virulence activity of AvrRpt2 or AvrRpm1 in Arabidopsis (Belkhadir *et al.*, 2004). Importantly, separable avirulence and virulence activities have also been observed with other type III effectors, including AvrXa7, AvrRpt2 and AvrBs2 (Gassmann *et al.*, 2000; Lim and Kunkel, 2004; Yang *et al.*, 2005).

Our present data extend these points to a second effector, AvrPtoB. First, four of five mutations that disrupt the avirulence activity of $AvrPtoB_{1\text{-}307}$ had no discernible effect on the ability of this region to promote early bacterial growth and exacerbate disease symptoms. We cannot rule out the possibility that some of these residues might contribute to bacterial fitness under field conditions. However, our observations strongly suggest that interaction of $AvrPtoB_{1\text{-}307}$ with Pto is not required for AvrPtoB virulence activity. Second, $AvrPtoB_{1\text{-}307}$ exhibited virulence activity on a tomato line that does not carry the *Pto* gene, further indicating that Pto is not a functionally important virulence target of this effector. Together, the previous data involving AvrPto and the present data regarding AvrPtoB are inconsistent with some of the central tenets of the guard hypothesis. Participation of Prf in a receptor complex with Pto is possible and even likely. However, our data suggest no reason why Pto should be viewed as a virulence target of AvrPtoB guarded by Prf.

Although recognizing that we still lack key details of this pathosystem (e.g. what the true virulence targets of $AvrPtoB_{1\text{-}307}$ are), we propose an alternative to the guard model to explain our results. In this alternative model, we postulate that the region encompassed by $AvrPtoB_{1\text{-}307}$ evolved to promote virulence by interaction with a host protein other than Pto. This virulence protein may share structural similarity with Pto, as suggested by the fact that one AvrPtoB residue (F173) is required for interaction with Pto and for virulence. However, other, presently unknown, residues of $AvrPtoB_{1\text{-}307}$ probably contribute to targeting of this other host protein. Why then does Pto interact with $AvrPtoB_{1\text{-}307}$? It is possible that Pto evolved to interact with AvrPtoB by mimicking certain structural or enzymatic features of the true virulence target of AvrPtoB (e.g. F173) and by utilizing other AvrPtoB residues to stabilize this interaction (e.g. E165, F169, G180 and L195). Alternatively, because the *Pto* gene is present in at least two diverse tomato species and appears to have arisen before *Solanum* speciation (Riely and Martin, 2001), it is possible that the similarities of Pto to the host virulence target were pre-existing and required for other functions of Pto. In this model, Pto (and other Pto family members) might have evolved to function in a signaling complex with Prf that arose to activate defense responses. In this scenario, Prf can be viewed as an important recognition and/or signaling component of the Pto pathway but one that evolved without any specific selection pressure exerted by AvrPtoB (or AvrPto). Future identification of the host virulence target(s) of AvrPtoB should help address many interesting questions that remain unanswered in this system, including what structural motif in the virulence target is required for interaction with AvrPtoB, and why there hasn't been selection pressure on AvrPtoB for mutation of the residues required for Pto recognition but dispensable for virulence.

Homology modeling suggests that $AvrPtoB_{121\text{-}200}$ may have structural similarity with the Pto-interacting domain of AvrPto

Our initial amino acid alignment of AvrPtoB with AvrPto showed the two effectors had little overall similarity although some regions had short stretches of conserved residues (Kim *et al.*, 2002). Additional mutagenesis of AvrPto and determination of its three-dimensional structure later revealed the importance of the CD loop for interaction with Pto (Chang *et al.*, 2001; Shan *et al.*, 2000a; Wulf *et al.*, 2004). The present mutagenesis of $AvrPtoB_{1\text{-}307}$ indicates a corresponding significance of the region around F173, and caused us to further scrutinize the $AvrPtoB_{1\text{-}307}$ sequence for similarity with the CD loop region of the AvrPto sequence. We focused on the functional core of AvrPto ($AvrPto_{33\text{-}128}$), the minimal Pto-binding 80-residue fragment of AvrPtoB ($AvrPtoB_{121\text{-}200}$), and for comparison the corresponding regions from HopPmaL and $AvrPtoB_{T1}$. These regions were found to have identical residues conserved at 10 positions, and residues with similar properties conserved at 13 positions (Figure 9a). This sequence similarity suggests that $AvrPtoB_{121\text{-}200}$ may have structural similarity to the three-helix bundle of $AvrPto_{33\text{-}128}$ (Wulf *et al.*, 2004). Indeed, secondary structure predictions are consistent with $AvrPtoB_{121\text{-}200}$ having multiple helices (Figure 9b). The sequence alignment and the AvrPto structure (pdb code 1R5E) were used to construct a homology model of $AvrPtoB_{121\text{-}200}$ (Figure 9c) using Swiss-Model (Peitsch, 1995; Schwede *et al.*, 2003). Overall, the level of sequence identity, helical propensities, and conserved structural features in the αC–CD loop–αD region provide high confidence in this region of the model, whereas the αA–AB loop–αB loop region is more speculative.

The homology model provides a reasonable framework from which to rationalize the AvrPtoB mutations that lead to

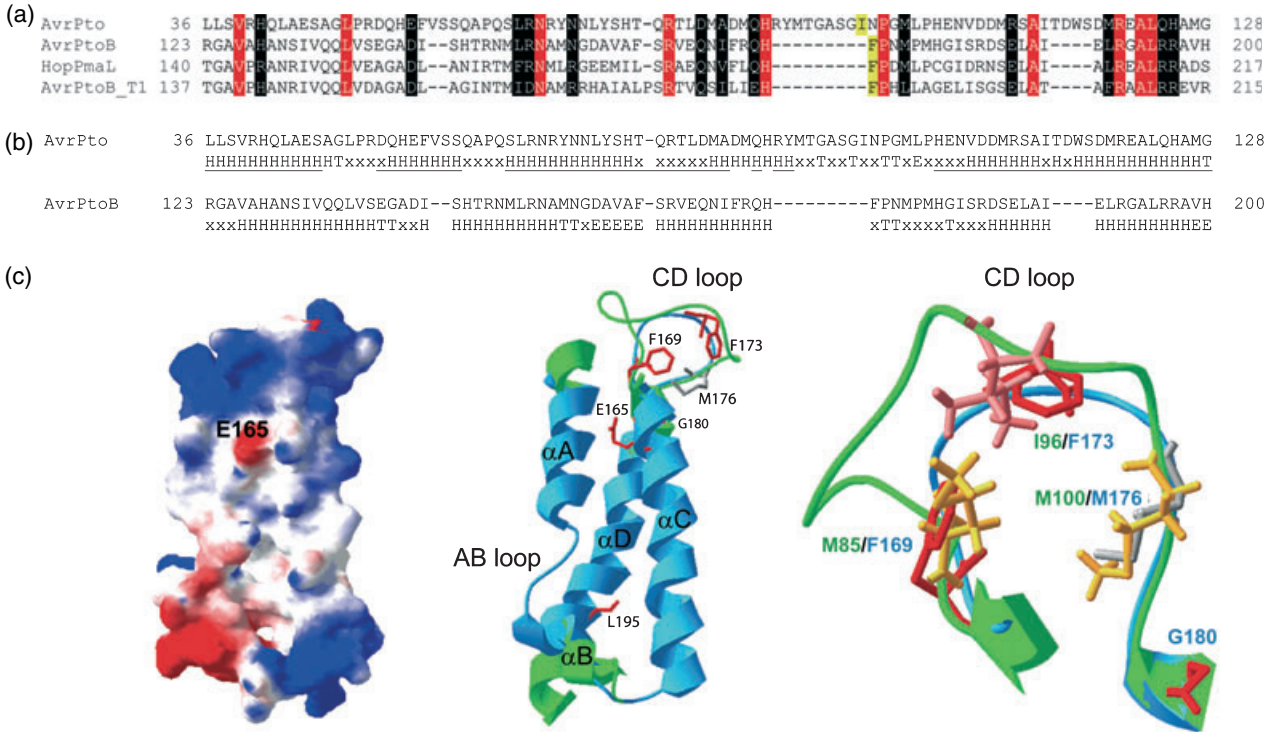


Figure 9. Sequence alignment and structural model for AvrPtoB$_{121\text{-}200}$.
(a) Sequence alignment of AvrPto$_{36\text{-}128}$, AvrPtoB$_{121\text{-}200}$ and the comparable region of AvrPtoB homologs from *P. s.* pv. *tomato* T1 and *P. s.* pv. *maculicola* ES4326. Identical residues (red) and similar residues (black) conserved across all four sequences are indicated. I96 in AvrPto and F173 in AvrPtoB are highlighted in yellow.
(b) Secondary structure prediction for AvrPto and AvrPtoB sequences. Categorization into α-helix (H), β-sheet (E), turn (T) or other (x) is based on the relative probabilities of six backbone conformational states as determined by HMMSTR-R (Bystroff *et al.*, 2000). AvrPto residues that actually adopt α-helical backbone conformation in the AvrPto NMR structure (Wulf *et al.*, 2004) are underlined.
(c) AvrPtoB homology model and conserved features of CD loop that may facilitate Pto binding. Ribbon overlay (center) of AvrPto (green) and AvrPtoB (blue), with essential residues (red) and conserved M176 (gray) shown in stick representation. The cluster of hydrophobic residues in the modeled AvrPtoB CD loop includes essential residues F169 and F173 and conserved residue M176 (right; AvrPtoB residues shown in blue). These residues overlie residues M85, I96 and M100, respectively, in the known AvrPto structure (AvrPto residues shown in green). The electrostatic surface potential of the homology model suggests an electrostatic role for essential residue E165 (left). Images were generated using DEEPVIEW (http://www.expasy.org/spdbv) (Guex and Peitsch, 1997).

loss of Pto binding (Figure 9c). A putative CD loop of the AvrPtoB model is shortened by nine residues relative to the corresponding 19-residue loop in the AvrPto structure, and consequently adopts a different shape. However, as in AvrPto, this abbreviated loop would form a pseudo-hydrophobic core that involves the conserved residues M176, F169 and F173. The latter two residues are required for interaction with Pto, and loss of either of them may disrupt the localized conformation. L195 is also required for Pto interaction and may influence fold stability, as it is predicted to be buried in the hydrophobic core. The essential role of E165 may be due to its electrostatic field contribution to the surface potential, as the E165 side chain is adjacent to a large positive surface region of the model (Figure 9c). Another residue identified by mutagenesis, G180, may fulfill additional conformational requirements of the fold. Based on these observations, we hypothesize that the putative CD loop of AvrPtoB and the known CD loop of AvrPto each play a central role as the contact surface for interaction with Pto, and that the kinase employs a common mode of binding to these two effector proteins. Efforts are underway to obtain sufficient amounts of AvrPtoB$_{121\text{-}200}$ for three-dimensional structural analysis to elucidate the actual features in this region that are involved in the interaction with Pto.

AvrPtoB is a multi-functional virulence protein

This study, and earlier work on the cell death-suppression activity of the C-terminus of AvrPtoB, reveals that this effector has at least three independent regions that make significant contributions to bacterial pathogenesis (Abramovitch *et al.*, 2003). Although these virulence activities are separable experimentally, it remains to be determined whether the three domains act independently or in concert during *P. s.* pv. *tomato* infection of leaves. Interestingly, processed species of AvrPtoB are detected *in vivo*, suggesting that the domains might be released and function separately (de Vries *et al.*, 2006; He *et al.*, 2006; R.B.A. and

G.B.M., unpubl. results). In addition, the existence in *P. s.* pv. *maculicola* of a C-terminus truncated homolog of AvrPtoB (HopPmaL) suggests that at least the N-terminal domains might act autonomously. In this way, AvrPtoB might be similar to viral polyproteins, where one large virulence protein is processed into multiple protein domains that act together in viral pathogenesis. As many type III effectors are large proteins (Desveaux *et al.*, 2006), it is likely that other type III effectors have multiple virulence and enzymatic activities. Careful structural and functional studies of these effectors are required to elucidate the relative contributions of these activities to bacterial virulence.

Experimental procedures

PCR-based random mutagenesis and Agrobacterium*-mediated transient assay*

Random mutagenesis of AvrPtoB$_{1\text{-}307}$ was carried out using a Gene Morph random mutagenesis kit (Stratagene, http://www.stratagene.com/) according to the manufacturer's instructions and using primers 5′-ACTCTAGAATGGCGGGTATCAATAGAGCGGGACCATCG-3′ and 5′-TTCTGCAGTCATACATGTCTTTCAAGGGCCGT-3′ (*Xba*I and *Pst*I sites underlined). The AvrPtoB$_{1\text{-}307}$ mutant alleles were digested with *Xba*I and *Pst*I, and cloned into the same sites of binary vector pBTEX, followed by electroporation into *Agrobacterium tumefaciens* strain GV2260. The *Agrobacterium*-mediated transient expression assay was performed as described by Sessa *et al.* (2000), with strains carrying AvrPtoB$_{1\text{-}307}$ or mutant alleles being syringe-infiltrated into tomato RG-PtoR leaves at a concentration (OD_{600}) of 0.03. Photographs were taken 3 days after infiltration.

P. s. *pv.* tomato *protein secretion and Western blotting assay*

A DNA fragment containing AvrPtoB$_{1\text{-}307}$ and its *hrp* promoter was amplified by PCR using primers 5′-GGTGTGATGGAACTCTTTCCTGCTC-3′ and 5′-TCATACATGTCTTTCAAGGGCCG-3′, and cloned into pCR2.1 (Invitrogen, http://www.invitrogen.com/). The resulting construct was also used as a template for creating AvrPtoB$_{1\text{-}307}$ mutants by site-directed mutagenesis with the following primer sets: E165 K, 5′-GCTTTTTCTCGAGTAAAACAGAACATATTTTCGC-3′ and 5′-GCGAAATATGTTCTGTTTTACTCGAGAAAAAGC-3′; F169S, 5′-GTAGAACAGAACATATCTCGCCAGCATTTCCCG-3′ and 5′-CGGGAAATGCTGGCGAGATATGTTCTGTTCTAC-3′; G180 V, 5′-ACATGCCCATGCATGTAATCAGCCGAGATTCGG-3′ and 5′-CCGAATCTCGGCTGATTACATGCATGGGCATGT-3′; L195H, 5′-GCTCCGTGGGGCGCATCGTCGAGCGGTTCA-3′ and 5-TGAACCGCTCGACGATGCGCCCCACGGAGC-3′. The *Eco*RI fragment from pCR2.1, containing AvrPtoB$_{1\text{-}307}$ or derived mutants plus the *hrp* promoter, was cloned into the broad-host-range vector pCPP45 (Lin and Martin, 2005). The resulting constructs were confirmed by sequencing, transformed into DC3000Δ*avrPto*Δ*avrPtoB* by electroporation, and analyzed using the *P. s.* pv. *tomato* secretion assay as described previously (Lin *et al.*, 2006) Western blotting was carried out using an anti-HA antibody (Roche Applied Science; http://www.roche.com), anti-NptII (US Biological; http://www.usbio.net) or anti-AvrPtoB antibody (Lin *et al.*, 2006). Detection of proteins was carried out using HRP-conjugated secondary antibodies and the ECL Plus detection system (Amersham-Pharmacia, http://www5.amershambiosciences.com/).

Tomato protoplast isolation and transient assay

Tomato protoplasts were isolated and transformed according to protocols described by Xing *et al.* (2001) and Sheen (http://genetics.mgh.harvard.edu/sheenweb/). Proteins were transiently expressed in protoplasts from the pTEX vector expression cassette. AvrPtoB$_{1\text{-}307}$ and derived mutants were released from the pBTEX vector using *Eco*RI and *Hin*dIII and cloned into the same sites of the pTEX vector. The resulting constructs were used for PEG-mediated transformation into protoplasts, and cell death was monitored by staining with 0.04% Evans blue 24 h after transformation (Asai *et al.*, 2000).

Yeast two-hybrid assays

A LexA yeast two-hybrid system was used to test protein interactions. Pto was expressed from the bait vector pEG202 (Tang *et al.*, 1996), and AvrPtoB$_{1\text{-}307}$, AvrPtoB homologs or the derived mutants were expressed from the prey vector pJG4-5. AvrPtoB truncation mutants and Ala substitution mutant constructs were produced by introduction of a stop codon or Ala codon at the appropriate sites by site-directed mutagenesis with pJG4-5::AvrPtoB$_{1\text{-}307}$ as the template. To create prey constructs containing the 121–200 amino acid region of AvrPtoB and homologs, the 121–200 fragments were PCR-amplified with the following primer pairs: AvrPtoB, 5′-ATGAATTCATGCCGCGTAGAGGGGCGGTTGCACACGCCAA-3′ and 5′-TTGTCGACGTGAACCGCTCGACGAAGCGCCCCA-3′; AvrPtoB$_{T1}$, 5′-ATGAATTCATGGTACGCACAGAGGGAGGACGCACA-3′ and 5′-TTGTCGACTCAGCGAACCTCCCGACGGAGAGCCGC-3′; AvrPtoB$_{B278a}$, 5′-ATGAATTCATGGTTGCAGAGCGCATCGTTCAGGAG-3′ and 5′-TTGTCGACTCAGTGAACCACCTGACGAAGCGTTTG-3′; HopPmaL, 5′-ATGAATTCGTGGCACGTGCAGAGAGAGGACGCACG-3′ and 5′-TTGTCGACTCATACATGTCTTTCAAGGGCCGTGCGC-3′ (*Eco*RI and *Sal*I sites underlined). The PCR products were digested with *Eco*RI/*Sal*I and cloned into the *Eco*RI and *Xho*I sites of pJG4-5. The resulting constructs were also used as the templates for creating the F173A mutants by site-directed mutagenesis using the following primer sets: AvrPtoB$_{T1}$, 5′-TTTTGATCGAGCATGCCCCTCACCTGCTAGCG-3′ and 5′-CGCTAGCAGGTGAGGGGCATGCTCGATCAAAA-3′; AvrPtoB$_{B278a}$, 5′-CTTGCTTCAGCATGCTCCTGACATGCATAC-3′ and 5′-GTATGCATGTCAGGAGCATGCTGAAGCAAGAT-3′; HopPmaL, 5′-GTTTTTCTCCAGCATGCTCCTGACATGCTACC-3′ and 5′-GGTAGCATGTCAGGAGCATGCTGGAGAAAAAC-3′. For interaction between Bti6 and AvrPtoB$_{1\text{-}307}$ or the F173A mutant, wild-type AvrPtoB$_{1\text{-}307}$ and the F173A mutant were PCR-amplified with primers 5′-GGGAATTCATGGCGGGTATCAATAGAGCGGGACCATCG-3′ and 5′-AACCATGGTCATACATGTCTTTCAAGGGCCGTGCGCAGGTGC-3′ (*Eco*RI and *Nco*I sites underlined) from pJG4-5 constructs and cloned into the same sites of pEG202 as the baits. Bti6 was expressed from pJG4-5 as prey. Yeast cells containing both prey and bait proteins were streaked on X-Gal plates with galactose to analyze the interactions, and photographed after 2 days after incubation at 30°C.

Measurement of bacterial populations on tomato

Three genotypes of Rio Grande tomato lines were used: RG-PtoR (*Pto*/*Pto*, *Prf*/*Prf*), RG-prf3 (*Pto/Pto*, *prf/prf*) and RG-pto11 (*pto/pto*, *Prf/Prf*). The method for preparation of *P. s.* pv. *tomato* inoculum has been described previously (Anderson *et al.*, 2006). Six-week-old greenhouse grown plants were vacuum-infiltrated with *P. s.* pv. *tomato* strains at an inoculum of 10^4 cfu ml^{-1}. For consistent results, after inoculation with *P. s.* pv. *tomato* bacteria, tomato plants were kept in a climate-controlled growth chamber under

optimized conditions (see Anderson *et al.*, 2006; for details). Bacterial populations were recovered from plant leaves and quantified at 2 and 4 days after infiltration following methods described by Anderson *et al.* (2006). To assess virulence activity, analysis of variance of bacterial populations on susceptible plants was based on the pooled data from two or three experiments (as indicated) using a general linear model procedure (SAS Institute Inc.; http://www.sas.com). The least significance difference (LSD) at a 0.05 probability level was used to test the difference between means.

Comparison of AvrPto and AvrPtoB structural similarities

The programs JNET (Cuff and Barton, 2000), a multiple-sequence alignment tool, and AGADIR (Lacroix *et al.*, 1998), which predicts α-helix propensities using the nucleation–condensation model of α-helix formation, were used to predict the secondary structure of AvrPto ($AvrPto_{36\text{-}128}$) and $AvrPtoB_{123\text{-}200}$. The AGADIR propensities were obtained for each protein at pH 6.1, 298 K, and 0.24 M ionic strength. The manual sequence alignment of $AvrPtoB_{123\text{-}200}$ and $AvrPto_{36\text{-}128}$ was used to construct a homology model using Swiss-Model (Peitsch, 1995; Schwede *et al.*, 2003).

Analysis of AvrPtoB virulence activities on tomato and Arabidopsis

Ethylene production by tomato leaves inoculated with various *P. s.* pv. *tomato* strains was measured at the indicated time points after vacuum infiltration of an inoculum of 10^4 cfu ml^{-1} as described previously (Cohn and Martin, 2005; Lin and Martin, 2005). Basal defense suppression assays and MAP kinase signaling interference by AvrPtoB in Arabidopsis protoplasts were performed as described previously (He *et al.*, 2006). *UBQ10-GUS* was co-transformed with *FRK1-LUC* as an internal control, and the promoter activity of *FRK1* is presented as LUC normalized to UBQ10-GUS activity. Detailed methods are described in the supplementary experimental procedures of a previous report (He *et al.*, 2006).

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Physical interaction of Bti6 with $AvrPtoB_{1\text{-}307}$ and $AvrPtoB_{1\text{-}307(F173A)}$.

Figure S2. $AvrPtoB_{1\text{-}387}$ suppresses PAMP marker gene activation by flg22.

Table S1. Details of clones isolated from the random mutagenesis screen for loss of avirulence activity on Pto-expressing tomato leaves.

This material is available a part of the online article from http://www.blackwell-synergy.com

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