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Two Distinct *Pseudomonas* Effector Proteins Interact with the Pto Kinase and Activate Plant Immunity

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Summary

The Pto serine/threonine kinase of tomato confers resistance to speck disease by recognizing strains of *Pseudomonas syringae* that express the protein AvrPto. Pto and AvrPto physically interact, and this interaction is required for activation of host resistance. We identified a second *Pseudomonas* protein, AvrPtoB, that interacts specifically with Pto and is widely distributed among plant pathogens. AvrPtoB is delivered into the plant cell by the bacterial type III secretion system, and it elicits *Pto*-specific defenses. AvrPtoB has little overall sequence similarity with AvrPto. However, AvrPto amino acids, which are required for interaction with Pto, are present in AvrPtoB and required for its interaction with Pto. Thus, two distinct bacterial effectors activate plant immunity by interacting with the same host protein kinase through a similar structural mechanism.

Introduction

Many bacterial pathogens of both mammals and plants rely on specific effector proteins to increase their virulence in host tissues (Galan and Collmer, 1999). These effectors are often introduced directly into the host cell via the type III secretion system (TTSS) where they may act to subvert host metabolism by mimicking key signaling proteins, suppressing defense responses, or possibly promoting release of nutrients required for growth of the bacterium (Galan and Collmer, 1999; Staskawicz et al., 2001). In bacterial pathogens of plants, the TTSS is encoded by the hypersensitive response and pathogenicity (*hrp*) genes (Lindgren, 1997). Mutations in key *hrp* genes prevent the secretion of effectors and inhibit pathogen growth and host defenses. A hallmark of effector genes is the presence of a "Hrp box" *cis* element in their promoter that is recognized by the HrpL ECF-like sigma factor (Innes et al., 1993; Xiao et al., 1994). A recent search for Hrp box-containing genes in the genome of *Pseudomonas syringae* pv. *tomato* strain DC3000 revealed over 20 putative effector genes (Fouts et al., 2002).

Effectors present in bacterial pathogens of plants were identified originally not by their promotion of virulence but rather by their "avirulence" activity (Collmer et al., 2000). In this role, effectors (Avr proteins) are recognized in a highly specific fashion by the products of host disease resistance (*R*) genes. A variety of defense responses are then activated including the hypersensitive response (HR), a localized form of programmed cell death (Hammond-Kosack and Jones, 2000). The recognition events involving many Avr and R proteins occur within the plant cell, although the molecular and structural basis of these events is mostly unknown (Dangl and Jones, 2001; Martin, 1999). Despite much effort, the simplest scenario of direct binding of the two proteins has been demonstrated for just two R-Avr pairs (Jia et al., 2000; Scofield et al., 1996; Tang et al., 1996). It is likely that R and Avr proteins participate in a complex with other proteins to activate host defense responses (Dangl and Jones, 2001; Nimchuk et al., 2001).

Bacterial effector proteins are highly diverse with little amino acid sequence similarity among them (one exception is the AvrBs3 family) (Lindgren, 1997; White et al., 2000). They have been identified from all four of the most common genera of plant bacterial pathogens (i.e., *Pseudomonas*, *Xanthomonas*, *Erwinia*, and *Ralstonia)*. In a still cryptic process, these pathogens utilize the TTSS to inject effectors across the plant cell wall into the cytoplasm (Galan and Collmer, 1999; Jin and He, 2001). Little is known of the fate of bacterial effectors once they are in the plant cell; although some members of the AvrBs3 family are localized to the nucleus, some effector proteins are targeted to the plasma membrane after being myristylated, and others are processed to smaller forms (Nimchuk et al., 2000; Shan et al., 2000b; Van den Ackerveken et al., 1996; Zhu et al., 1999).

The interaction of tomato with *P. s. tomato* is an excellent system to study the activity of effector proteins acting as either avirulence or virulence factors (Martin, 1999; Preston, 2000). In one gene-for-gene interaction in this pathosystem, a host *R* gene, *Pto*, has been isolated that encodes a serine/threonine protein kinase that specifically recognizes strains of *P. s. tomato* that express the effector protein AvrPto (Martin et al., 1993; Ronald et al., 1992). Interestingly, the Pto kinase shares sequence similarity with the human interleukin-1 receptor-associated kinase (IRAK) and with the *Drosophila* Pelle kinase, both of which, like Pto, play a role in immune responses (Cohn et al., 2001; Hoffman et al., 1999). The *Pto* gene belongs to a gene family of six members on tomato chromosome 5 (Martin et al., 1993; Michelmore and Meyers, 1998; Riely and Martin, 2001). One of these family members, *Fen*, encodes a kinase that confers sensitivity to an insecticide (fenthion), while the function of the others is unknown (Martin et al., 1994).

The AvrPto protein and the Pto kinase physically interact in a yeast two-hybrid system (Scofield et al., 1996; Tang et al., 1996). Coexpression of Pto and AvrPto as transgenes in a *pto* mutant leaf is sufficient to activate resistance. Mutations that disrupt this interaction also abolish the ability to elicit disease resistance in plant leaves (Chang et al., 2001; Frederick et al., 1998; Shan et al., 2000b). Resistance is dependent on the Prf protein, which bears striking similarity to the large NB-LRR class of R proteins (Salmeron et al., 1996). Pto-Fen chimeras were used to define the kinase activation loop as a

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key determinant of Pto interaction specificity for AvrPto (Frederick et al., 1998; Scofield et al., 1996; Tang et al., 1996). Pto kinase is phosphorylated on eight residues, and mutation of two of these residues (T38 and S198) abolishes its ability to elicit host resistance (Sessa et al., 2000). Recognition specificity of Pto for AvrPto appears to have evolved before *Lycopersicon* speciation because a Pto family member from a distantly related species, *L. hirsutum*, also recognizes AvrPto (Riely and Martin, 2001).

The AvrPto gene was originally isolated from *P. s. tomato* strain JL1065 based on its ability to confer avirulence to a virulent strain of *P. s. maculicola* (Ronald et al., 1992). AvrPto encodes an 18 kDa protein that bears little sequence similarity to proteins in current databases (Salmeron and Staskawicz, 1993). Its mechanism of activating resistance is unknown although it likely interacts with Pto inside the plant cell and possibly with certain "AvrPto-dependent Pto-interacting" (Adi) proteins as part of a complex (Bogdanove and Martin, 2000; Scofield et al., 1996; Tang et al., 1996). AvrPto acts as a virulence factor when Pto (or Prf) is absent from the plant cell and increases the growth of *P. s. tomato* about 10-fold as compared to a strain lacking the effector (Chang et al., 2000; Shan et al., 2000a). In common with several effectors, AvrPto has a myristylation motif at its N terminus that is required for both its avirulence and virulence activity (Nimchuk et al., 2000; Shan et al., 2000b). The amino acids of AvrPto that are required for its recognition by the Pto kinase have been examined by saturation mutagenesis (Chang et al., 2001; Shan et al., 2000a; Shan et al., 2000b). Mutation of three AvrPto residues—S94, I96, and G99—abolishes interaction with Pto and avirulence activity, but not virulence activity, in tomato (Shan et al., 2000a, 2000b). Along with the other observations (Chang et al., 2001), these results indicate that an internal region of AvrPto determines its binding specificity for Pto.

AvrPto-like DNA sequences are present in *Pseudomonas* strains that are known to be avirulent on *Pto* tomato plants (race 0 strains) and are absent from virulent ones (race 1 strains) (Ronald et al., 1992). Thus, a homolog of *avrPto* was identified in avirulent *P. s. tomato* strain DC3000 based on DNA blot hybridization (Ronald et al., 1992). Gene replacement strains in which the *avrPto* reading frame was deleted were constructed in strains JL1065 and DC3000. Surprisingly, both mutant strains were still recognized by *Pto*-expressing tomato leaves (Ronald et al., 1992). A later study found that a tomato line carrying a CaMV 35S::*Pto* transgene (and not a sibling line without *Pto*) is resistant to the *avrPto*ΔDC3000 deletion strain (G.B.M., unpublished). These results implied that strains DC3000 and JL1065 carry additional avirulence proteins that are recognized specifically by Pto.

Because the Pto kinase interacts with AvrPto, we hypothesized that the other avirulence protein in *P. s. tomato* DC3000 might also interact with Pto. Yeast two-hybrid screening of a DC3000 prey library with a Pto bait protein identified a gene that has the hallmarks of a typical Hrp-dependent effector. The protein is secreted via the TTSS and elicits Pto-specific defense responses in tomato leaves. Interestingly, it bears little resemblance to AvrPto except in certain regions, one of which is known to be required for interaction with Pto.

Results

Identification of *Pseudomonas* Proteins that Interact with the Pto Kinase

To identify potential effectors from *P. s. tomato* DC3000 that interact with the Pto kinase, we performed a yeast two-hybrid screen by using the tomato Pto kinase as the bait and a pool of DC3000 prey libraries (see Experimental Procedures). Based on the DNA sequences, ten classes of bacterial genes were identified in this screen (Y.J.K., unpublished; see Supplemental Table S1 at http://www.cell.com/cgi/contenct/full/109/5/589/DC1). For unknown reasons, AvrPto was not recovered. One Pto-interacting class, PtiDC1, which contained eight clones, shared sequence similarity with a previously described virulence-related protein (see below) and is the focus of this paper. The eight PtiDC1 clones did not autoactivate the reporter genes, and retransformation of them into the yeast expressing the Pto bait allowed growth on Leu medium and cleavage of X-gal (Figure 1A). Thus, the PtiDC1 clones encode a protein that interacts with Pto kinase in the yeast two-hybrid system.

PtiDC1 Sequence Is Similar to *virPphA* from *P. s. phaseolicola*

The nucleotide sequences were determined for the eight PtiDC1 clones and revealed they carried inserts truncated at three distinct 5′ ends but were otherwise identical (Figure 1B). Comparison of the nucleotide sequences of the PtiDC1 inserts to current databases showed similarity to the effector gene *virPphA* (GenBank accession number AF141883) from *P. s. phaseolicola* (Jackson et al., 1999). We designated this gene *avrPtoB* because the initial phenotype associated with the PtiDC1 sequence was avirulence (see below), and because we wish to denote its functional similarity with *avrPto* (i.e., *avrPto*, originally isolated from *P. s. tomato* strain JL1065, is formally $avrPtoA_{JL1065}$).

A cosmid was recovered from a DC3000 library by using a PtiDC1 probe, and a 6.0 kb *Pst*I fragment containing *avrPtoB* was subcloned and sequenced. The sequence revealed an open reading frame (ORF) spanning 1659 bp (Figure 1B; GenBank number AY074795) with 70% nucleotide identity to the *virPphA* gene. A putative Hrp box (GGAACT-N_{16}-CCAC) is located 85 nucleotides upstream of the predicted AUG initiation codon and conforms closely to a consensus Hrp box recently derived from a large set of effectors from DC3000 (Fouts et al., 2002). In accordance with this observation, we found that *avrPtoB* gene expression is induced in apoplast-mimicking medium and in planta in a *hrp*-dependent fashion (Fouts et al., 2002; N.-C.L. and G.B.M., unpublished data).

The *avrPtoB* ORF produces a predicted protein of 553 amino acids with a molecular mass of 59 kDa. Putative amino acid sequence of AvrPtoB is 52% identical to VirPphA (BLASTP e value equals e-140; see Supplemental Table S2 at http://www.cell.com/cgi/content/full/109/5/589/DC1). The truncation points in the PtiDC1 clones (Figure 1B) were found to remove the first 70, 112, or 121 amino acids of the AvrPtoB open reading frame. Database searches detected no sequence similarity between AvrPtoB and AvrPto. In addition, unlike

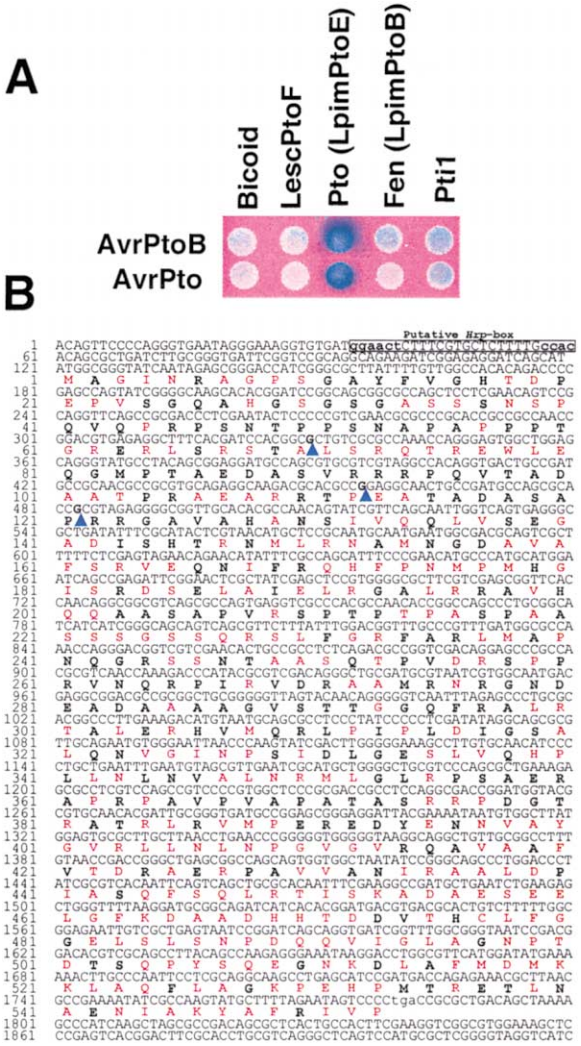

Figure 1. Interaction of *Pseudomonas* Effector Protein AvrPtoB with the Pto Kinase in the Yeast Two-Hybrid System and Features of the AvrPtoB Gene

(A) Test for specificity of AvrPtoB and AvrPto interaction with Pto family proteins and Pti1 kinase in the LexA yeast two-hybrid system. The *avrPtoB* (PtiDC1Δ70) and *avrPto* genes were cloned into the prey vector pJG4-5, and the Pto, Pti1, and Bicoid genes were cloned into the bait vector pEG202. The constructs were transformed into yeast strain EGY48 carrying a *lacZ* reporter gene, and the cells were plated onto medium containing X-gal. Dark blue color indicates interaction.

(B) DNA and encoded amino acid sequence of the *avrPtoB* gene (GenBank number AY074795). Blue arrows mark points at which truncations occurred in the *avrPtoB* clones retrieved from the two-hybrid screen. Shown is the region upstream of the putative start codon that contains the Hrp box *cis* element and the entire open reading frame of *avrPtoB*. The amino acids of the AvrPtoB protein are given in single letter code, and those residues that are identical with VirPphA are shown in red. (See Supplemental Table S1 at http://www.cell.com/cgi/content/full/109/5/589/DC1 for an alignment of AvrPtoB and VirPphA.)

AvrPto, the AvrPtoB protein has no myristylation motif immediately following the initiation methionine (Nimchuk et al., 2000; Shan et al., 2000b). However, pattern searching with PIR (protein information resource) detected a possible myristylation site near the N terminus (i.e., MAGINRAG; consensus motif is G-{not EDRKHPFYW}-x(2)-[STAGCN]–{not P}) and ten myristylation motifs within the protein.

Interaction Specificity of AvrPtoB Protein for the Pto Kinase

Interaction specificity between AvrPto and Pto has been characterized extensively (Scofield et al., 1996; Tang et al., 1996, Frederick et al., 1998). To initially compare the Pto interaction specificity of AvrPtoB with AvrPto, we introduced into the yeast two-hybrid system the AvrPtoB prey PtiDC1Δ70 with several bait plasmids expressing kinases closely related to Pto (Figure 1A). AvrPtoB did not interact with the Fen kinase (Martin et al., 1994), the Pti1 kinase (Zhou et al., 1995), or the LescPtoF kinase (Jia et al., 1997; Riely and Martin, 2001).

We next examined a series of chimeric Pto-Fen proteins and Pto mutants that were used previously to show that Thr-204 in the Pto activation loop is required for AvrPto-Pto interaction (Tang et al., 1996; Frederick et al., 1998). AvrPtoB specifically interacted with chimera G and not with other chimeric proteins (Figure 2A; Tang et al., 1996). Comparison of chimera G with the other chimeras implicated a region in Pto from amino acids 129 to 224 that is required for interaction with AvrPtoB. AvrPto also interacts with chimera G and elicits the HR in tomato plants expressing a chimeric G transgene (Tang et al., 1996). Additional Pto-Fen chimeras that subdivide the Pto region spanning amino acids 113 to 217 were all found to interact with AvrPtoB as they do with AvrPto (Figure 2B; Frederick et al., 1998). AvrPtoB also interacted in an identical fashion as AvrPto with a large series of Pto and Fen mutants that previously served to define recognition specificity of Pto for AvrPto (Figure 2C; Frederick et al., 1998). Taken together, AvrPtoB interacts with identical specificity as AvrPto with the Pto variants, and these interactions thus indicate that T204 also forms a key recognition determinant of Pto for the AvrPtoB protein.

Further indication of the interaction specificity of AvrPtoB for Pto was obtained by examining a series of Pto proteins that contain single amino acid substitutions for eight previously identified autophosphorylation sites (Sessa et al., 2000) and four Pto paralogs from the wild tomato species *L. hirsutum* (Riely and Martin, 2001). A mutation at Thr-38 of Pto, the main autophosphorylation site in this kinase, abolishes the interaction with AvrPtoB as it does with AvrPto; all other phosphorylation site mutants interact with both AvrPtoB and AvrPto (data not shown). Among the Pto kinases from *L. hirsutum*, only LhirPtoE interacts with the AvrPtoB and AvrPto proteins (data not shown). Together, these observations demonstrate remarkable, and biologically meaningful, interaction specificity of the AvrPtoB protein for the Pto kinase.

AvrPtoB Sequences Are Conserved in at least Three Genera of Bacterial Pathogens

To examine the distribution of *avrPtoB*-like sequences, we used the gene to probe DNA blots containing genomic DNA from many *Pseudomonas* pathovars and some *Xanthomonas* and *Erwinia* strains. We discovered that sequences with homology to *avrPtoB* are present in

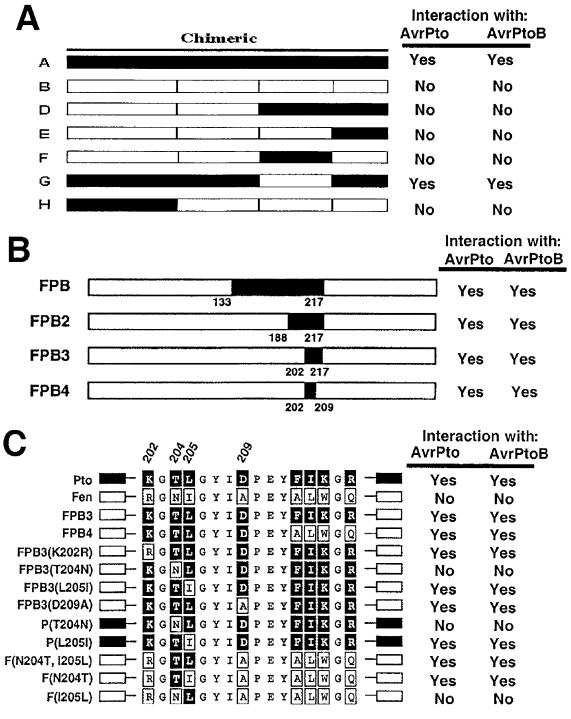

Figure 2. AvrPtoB and AvrPto Interact with the Same Variant Forms of the Pto Kinase

(A) Interactions of Pto-Fen chimeric proteins (Tang et al., 1996) with AvrPtoB and AvrPto in the LexA yeast two-hybrid system. The diagram depicts Pto (black regions) and Fen (white regions) chimeric proteins. EGY48 yeast cells containing the Pto-Fen chimeric proteins in bait vector pEG202, AvrPtoB or AvrPto in prey vector pJG4-5, and the *lacZ* reporter gene were grown on medium containing *X-gal*. Equal expression of each chimeric protein was verified by Western blot (Tang et al., 1996).

(B) Interaction of the internal region of Pto (Frederick et al., 1998) with AvrPtoB or AvrPto in the LexA yeast two-hybrid system. Chimeric proteins FPB, FPB2, FPB3, and FPB4 contain the amino acids from Pto (black regions) or from Fen (white regions). Numbers corresponding to amino acid positions in Pto are indicated. Equal expression of Pto-Fen chimeric proteins was verified by Western blot (Frederick et al., 1998).

(C) Effect of amino acid substitutions in Pto/Fen kinase subdomain VIII on the interaction with AvrPtoB and AvrPto in the yeast two-hybrid system. Portions of proteins and individual amino acids derived from Pto (in black) or Fen (in white) are shown. Unboxed amino acids are identical in both kinases. The numbering of amino acids and designation of substitutions correspond to the Pto sequence (Martin et al., 1993).

certain strains of each of these three genera (Figure 3). Because some of these strains (i.e., T1 and PT11) are virulent on *Pto*-expressing tomato leaves (Table 1), we conclude that not all of these *avrPtoB* sequences are recognized by Pto. We cloned several of these DNA fragments and by partial sequence analysis have confirmed their relatedness to *avrPtoB* (Y.J.K., N.-C.L., G.B.M., unpublished; R. Jackson et al., submitted).

Expression of *avrPtoB* in Several *P. s. tomato* Strains Elicits Resistance to Bacterial Speck Disease in Tomato

To determine if *P. s. tomato* strains carrying *avrPtoB* elicited *Pto*-specific disease resistance, we examined three race 1 (virulent) strains of *P. s. tomato* (T1, PT11,

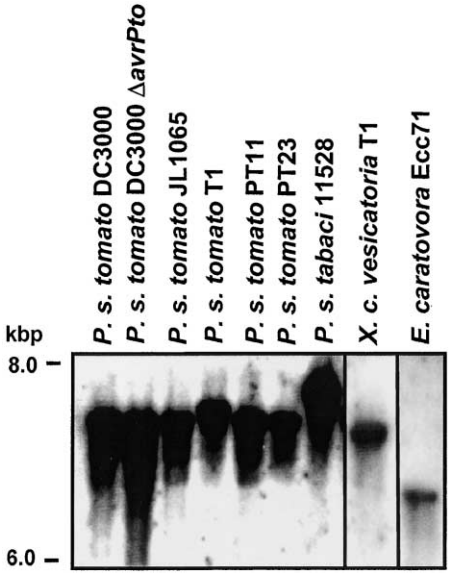

Figure 3. DNA Sequences with Similarity to *avrPtoB* Are Present in Diverse Bacterial Plant Pathogens

DNA was isolated from the *Pseudomonas*, *Xanthomonas*, or *Erwinia* strains indicated and analyzed on a gel blot using a radiolabeled *avrPtoB* gene probe. Stringency of the final wash was 0.1× SSC, 0.1% SDS.

and Bakersfield) that do not contain a functional *avrPto* gene. The pDSK519::*avrPtoB* clone (or pDSK519::*avrPto* as a control) was introduced into these strains, and a suspension of 10^4 cfu/ml was vacuum infiltrated into the leaves of resistant (RG-PtoR) or susceptible (RG-PtoS) tomato plants. As summarized in Table 1, no disease symptoms were observed on RG-PtoR plants inoculated with strains PT11 or Bakersfield expressing *avrPtoB*, while RG-PtoS plants were susceptible to these strains whether or not they carried *avrPtoB*. Identical results were observed for the *avrPto*-expressing strains. Interestingly, strain T1 elicited resistance in RG-PtoR only when expressing *avrPto*. Overall, these results con-

Table 1. Reaction of Tomato Leaves to Inoculation with *P. s.* pv. *tomato* Strains Expressing *avrPtoB*

	Disease Reaction	
P. s. pv. *tomato* Strain	RG-PtoR	RG-PtoS
T1	+	+
T1 (*avrPto*$_{JL1065}$)	−	+
T1 (*avrPtoB*)	+	+
PT11	+	+
PT11 (*avrPto*$_{JL1065}$)	−	+
PT11 (*avrPtoB*)	−	+
PT11 (*avrPtoB*^{J326T})	+	+
PT11 (*avrPtoB*^{G333A})	−	+
Bakersfield	+	+
Bakersfield (*avrPto*$_{JL1065}$)	−	+
Bakersfield (*avrPtoB*)	−	+

Plus sign (+) indicates >40 specks per leaflet; minus sign (−) indicates no specks observed.

Leaves of 6-week-old tomato plants RG-PtoR (*Pto*/*Pto*) or RG-PtoS (*pto*/*pto*) were vacuum infiltrated with 10^4 cfu/ml of the *Pseudomonas* strain indicated. Disease symptoms were recorded 5 days after inoculation.

firmed that when expressed in at least two virulent strains of the bacterial speck pathogen, *avrPtoB* triggers plant resistance responses in a *Pto*-specific manner.

AvrPtoB Is Translocated by the Type III Secretion System to Plant Cells

The interaction of AvrPtoB with Pto and the Hrp-dependent expression of the gene suggested that AvrPtoB is an effector that travels the TTSS to gain access to the plant cell cytoplasm. To test if AvrPtoB is secreted by the TTSS, we used a strain of *P. fluorescens* that carries the Hrp cluster from *P. s. syringae* strain 61. *P. fluorescens* was transformed with the pDSK519::*avrPtoB* plasmid. Infiltration of tomato leaves with this strain elicited a strong HR in the *Pto*-containing cultivar RG-PtoR but not in line RG-PtoS that lacks *Pto* (Figure 4A). Infiltrated leaves of two tomato lines that contain inactive alleles of *Pto* or *Prf* also did not show induction of the HR. A *P. fluorescens* strain carrying the Hrp cluster but lacking AvrPtoB did not elicit an HR in any of the tomato lines (data not shown). These results indicate that AvrPtoB is translocated into plant cells via the type III secretion system and that it is recognized specifically by the *Pto* locus in a *Prf*-dependent manner.

Expression of *avrPtoB* inside Tomato Leaf Cells Elicits a *Pto*- and *Prf*-Dependent HR

Expression of many Avr proteins directly in plant cells elicits *R* gene-specific defenses, indicating that they are the sole bacterial determinants of an intracellular recognition mechanism. We tested whether *avrPtoB* activates *R* gene-specific defense from within the plant cell by infiltrating *A. tumefaciens* strain GV2260 containing a CaMV *35S*::*avrPtoB* construct into tomato leaves with or without a functional Pto pathway (Figure 4B). Tomato leaves of line RG-PtoR exhibited an HR within 24 hr of infiltration, whereas the other leaves did not. *A. tumefaciens* carrying the empty binary vector elicited no responses in any of the leaves (data not shown).

To confirm that AvrPtoB is recognized in tomato leaves specifically by the Pto kinase (and not another member of the Pto family), two *A. tumefaciens* strains containing either a *35S::avrPtoB* construct or *35S::Pto* construct were prepared and infiltrated either separately or as a mixture into leaves of the susceptible *pto* mutant, RG-pto11. Tomato leaves infiltrated with *A. tumefaciens* carrying *35S::avrPtoB* alone exhibited no response in these leaves (this observation is in contrast to transient expression of *avrPto*, which causes necrosis in susceptible tomato leaves) (Chang et al., 2000). However, tomato leaves infiltrated with a mixture of the *35S::Pto* and *35S::avrPtoB* strains developed an HR within 24 hr (Figure 4C). Thus, AvrPtoB is specifically recognized in tomato leaves by the Pto kinase. An ancillary, but interesting, separate experiment revealed that infiltration of a mixture of *Agrobacterium* carrying *35S::avrPtoB* and *35S::Pto* into leaves of *Nicotiana benthamiana* or *N. tabacum* W38 did not elicit an HR. This is in contrast to similar experiments using AvrPto (Scofield et al., 1996; Frederick et al., 1998) and might indicate that AvrPtoB requires a distinct host component(s) for Pto-mediated HR that is lacking in these *Nicotiana* species.

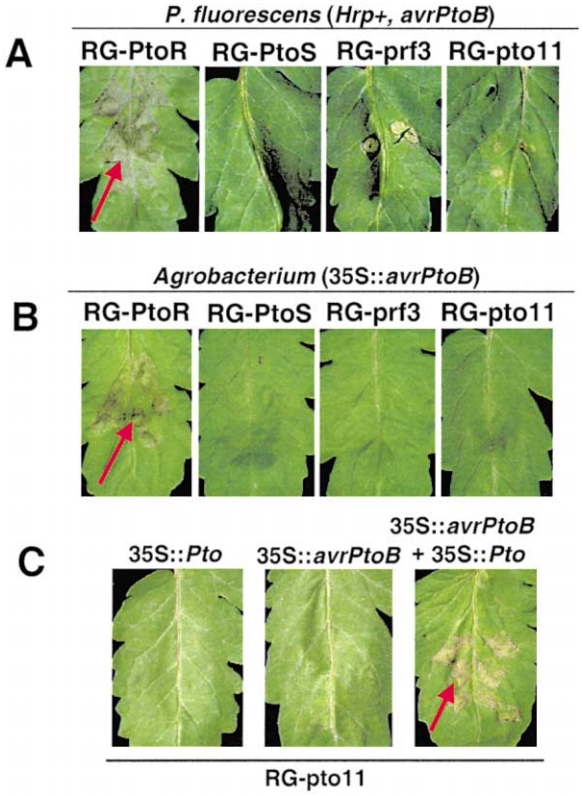

Figure 4. AvrPtoB Is Secreted via the *Pseudomonas* Type III Secretion System and Elicits a *Pto*- and *Prf*-Specific Hypersensitive Response (HR) in Tomato Leaves

(A) Elicitation of a *Pto*- and *Prf*-specific HR in tomato leaves by a *P. fluorescens* strain expressing a type III secretion system and *avrPtoB*. Tomato leaves of the indicated genotypes were syringe-infiltrated with 1×10^7 cfu/ml of *P. fluorescens* (pHIR11; Hrp+) carrying *avrPtoB* on the wide host range vector pDSK519. The HR appeared within 24 hr only in RG-PtoR leaves (see arrow; some necrosis due to wounding with the syringe is visible on other leaves). Infiltration of 1×10^7 cfu/ml of *P. fluorescens* (pHIR11; Hrp+) with pDSK519 alone elicited no response in any leaf genotypes. Photographs were taken 4 days after infiltration.

(B) Elicitation of a *Pto*- and *Prf*-specific HR in tomato leaves upon expression of an *avrPtoB* transgene directly in plant cells. A suspension of *Agrobacterium* strain GV2260 ($OD_{600} = 0.03$) carrying a binary vector with an *avrPtoB* transgene expressed by the CaMV 35S promoter was infiltrated into leaves of the indicated genotypes. The HR appeared within 18 hr only in RG-PtoR leaves (see arrow). Infiltration of *Agrobacterium* carrying an empty binary vector elicited no response in any leaf genotypes. Photographs were taken 4 days after infiltration.

(C) Coexpression of *Pto* and *avrPtoB* transgenes directly in *pto* mutant leaf cells elicits the HR. A mixture of suspensions of *Agrobacterium* strain GV2260 ($OD_{600} = 0.03$) carrying a binary vector with an *avrPtoB* or *Pto* transgene transcribed by the CaMV 35S promoter was infiltrated into leaves of RG-pto11. The HR appeared within 24 hr only in leaves expressing both *Pto* and *avrPtoB* (see arrow). Infiltration of *Agrobacterium* carrying an empty binary vector elicited no response in any leaf genotypes. Photographs were taken 4 days after infiltration.

AvrPtoB and AvrPto Proteins Are Similar in Several Dispersed Regions

Although searches of GenBank using BLASTN and BLASTX failed to reveal sequence similarity between AvrPtoB and AvrPto, an alignment of the two proteins using DNASTAR did reveal similarities in several dis-

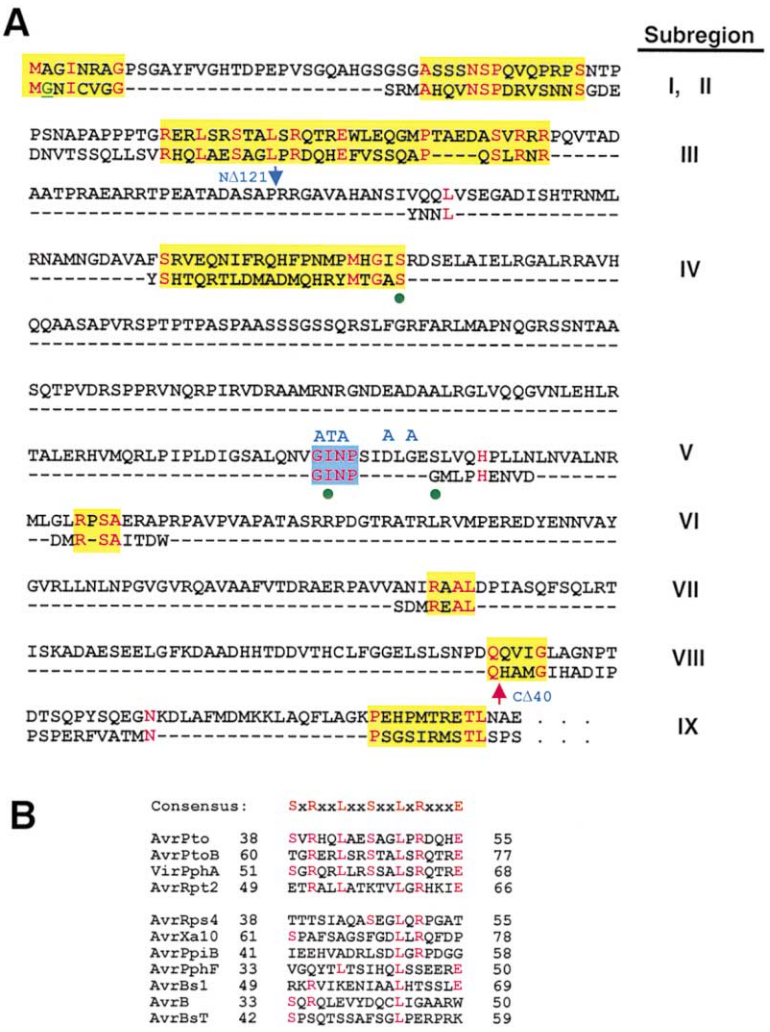

Figure 5. AvrPtoB and AvrPto Share Discrete Regions in Common and Subregion II Is Conserved among Diverse Bacterial Effector Proteins

(A) Amino acid sequences of AvrPtoB (top) and AvrPto (bottom) were aligned using DNAStar software and visually; dashes indicate gaps introduced to optimize the alignment. Nine subregions that contain identical amino acids (in red) are shown in yellow or blue boxes. The glycine residue present in the myristylation motif of AvrPto is underlined. Green dots indicate residues of AvrPto in which substitutions cause loss of Pto interaction in yeast two-hybrid system and HR in *Pto*-expressing tomato leaves (Shan et al., 2000b). The blue arrow indicates the most N-terminal truncated AvrPtoB protein (Δ121) that still interacts with Pto in the two-hybrid system. The red arrow indicates the most C-terminal truncated form of AvrPto (Δ40) that still interacts with Pto in the two-hybrid system (Chang et al., 2001). The GINP motif is boxed in blue and the substitutions that were made in this region are shown in blue letters above AvrPtoB.

(B) Alignment of part of subregion III in AvrPtoB and AvrPto that shares similar residues with diverse effector proteins from other bacterial phytopathogens. Amino acids in common are shown in red and a consensus is shown at the top. The amino acid position of the region in each effector protein is indicated. Origins of the effectors are *P. s. tomato* strain (AvrPto, AvrPtoB, AvrRpt2), *P. s. glycinea* (AvrB), *P. s. phaseolicola* (VirPphA, AvrPphF), *P. s. pisi* (AvrRps4, AvrPpiB), *Xanthomonas campestris* pv. *vesicatoria* (AvrBs1, AvrBsT), and *X. oryzae* pv. *oryzae* (AvrXa10).

persed regions (Figure 5A). The similarities between the two proteins have been used to designate nine subregions (I–IX) (Figure 5A).

Subregion I contains the putative myristylation site for AvrPto. This site is required for both avirulence and virulence activity of AvrPto but not for its physical interaction with Pto. As discussed above, AvrPtoB does not have a likely myristylation site (Nimchuk et al., 2000). Subregion III of both AvrPto and AvrPtoB contains the consensus sequences "RxxLxxSxxLxRxxxE" and "SxRxR." Interestingly, the first sequence is also found in a similar location in the protein sequences of VirPphA from *P. s. phaseolicola* race 7, AvrRpt2$_{JL1065}$ from *P. s. tomato*, and in less conserved form in several other Avr proteins (Figure 5B). In AvrRpt2, this sequence lies in an N terminus 7.5 kDa region that is essential for secretion and translocation, but not for in planta avirulence activity (Mudgett and Staskawicz, 1999). A substitution mutation (H54P) within this region, when introduced into AvrPto and expressed in *P. s. tomato* or *P. s. tabaci*, abolishes its HR-eliciting activity in *Pto*-expressing leaves (Chang et al., 2001). However, AvrPto(H54P) interacts with Pto in the yeast two-hybrid system and, when expressed directly within the plant cell, elicits an HR in *N. benthamiana* expressing *CaMV35S::Pto* (Chang et al., 2001). Therefore, this subregion might play a role in secretion or in translocating AvrPto and AvrPtoB (and possibly other proteins that have this sequence) into the plant cell. Finally, it should be noted that our retrieval from the two-hybrid screen of AvrPtoB proteins lacking the first 121 amino acids indicates that neither subregion I, II, nor III is necessary for Pto binding in yeast.

Subregion IV contains four shared residues and one of them, S94 of AvrPto, was previously found to be important for interaction of AvrPto with Pto and for recognition by Pto in tomato (but not tobacco) cells (Shan et al., 2000b). In AvrPto, this residue lies next to a sequence that constitutes subregion V in our alignment. Subregion V consists of four conserved residues, GINP. Shan et al. (2000b) reported that a substitution in AvrPto at I96 in this sequence or at the nearby G99 abolished recognition by Pto in yeast and tomato cells (G99, however, is not conserved in AvrPtoB). VirPphA from *P. s. phaseolicola* also has the GINP sequence (Jackson et al., 1999), and we have found that this protein both interacts with Pto in our yeast two-hybrid system and elicits an HR when expressed transiently in Pto-containing tomato leaves (Y.J.K., G.B.M., G. Tsiamis, and J. Mansfield, un-

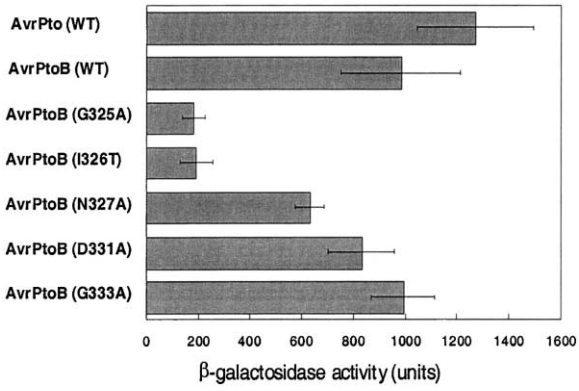

Figure 6. A Motif Shared by AvrPtoB and AvrPto Is Required for Interaction with the Pto Kinase

Amino acid substitutions in and near subregion V were created in AvrPtoB, and the mutant proteins were tested for interaction with the Pto kinase in the LexA yeast two-hybrid system. Degree of *lacZ* reporter gene activation was determined by measuring relative units of β-galactosidase activity in yeast strains expressing the mutant proteins and Pto (as in Frederick et al., 1998). The β-galactosidase activity data are the means (gray boxes) and standard errors (error bars) of duplicate experiments, each with three independent colonies per construct.

published data). Based on these observations, we speculated that residues in subregion V might be required for interaction of AvrPtoB with Pto (see below).

Finally, our alignment of AvrPto and AvrPtoB revealed four other discrete regions of shared amino acids in the C-terminal region. Deletion of the C-terminal 40 amino acids of AvrPto does not affect its interaction with Pto in yeast (Chang et al., 2001), and this suggests that subregions VIII and IX of AvrPtoB are not required for Pto interaction. Substitutions at N145, P146, S147, or S153 of AvrPto abolished its ability to elicit the HR in tobacco line W38, raising the possibility that another Pto-like R protein exists in that line (Shan et al., 2000b). N511 and P512 of AvrPtoB might serve a similar function, although AvrPtoB also has an NPSxxxxxS motif near subregion V (i.e., N327, P328, S329, S335). We have found that expression of AvrPtoB in W38 does not elicit the HR, but whether this is due to the different locations of this motif in the proteins or some other reason is not known.

Subregion V of AvrPtoB Contains Recognition Determinants for Interaction with Pto

We developed a series of point mutations in AvrPtoB to determine if subregion V, which is required for AvrPto interaction with Pto (Shan et al., 2000b), is also required for the AvrPtoB-Pto interaction (Figure 6). Each AvrPtoB point mutant was coexpressed with Pto in the yeast two-hybrid system, and activation of the *lacZ* reporter gene was measured. Expression of the mutant proteins was confirmed by Western blots (data not shown). Substitutions G325A, I326T, or N327A of AvrPtoB reduced the interaction with Pto as compared with wild-type AvrPtoB (Figure 6). Point mutations in nearby residues D331 and G333, which do not correspond to AvrPto residues, resulted in *lacZ* expression that was not statistically different from wild-type AvrPtoB. We transformed two of these mutated *avrPtoB* genes into the virulent *Pseudomonas* strain PTII and examined their avirulence activity on RG-PtoR and RG-PtoS tomato leaves. Consistent with the two-hybrid data, AvrPtoB(I326T) did not elicit disease resistance on *Pto*-expressing leaves, while AvrPtoB(G333A) elicited *Pto*-specific defense (Table 1). Thus, subregion V of AvrPto and AvrPtoB plays an important role in the Pto interaction and HR-eliciting activity of these effectors.

Discussion

We identified a second *Pseudomonas* protein, AvrPtoB, that interacts with the Pto kinase and elicits *Pto*-specific and *Prf*-dependent disease resistance in tomato leaves. Speculation that such a protein exists arose after it was found that deletion of AvrPto from *P. s. tomato* strains JL1065 or DC3000 did not alter the avirulence of these strains on *Pto*-expressing tomato leaves (Ronald et al., 1992). We hypothesized that, like AvrPto, this putative second effector might also interact directly with the Pto kinase in a yeast two-hybrid system. We employed a crosskingdom yeast two-hybrid screen, and it permitted rapid and efficient isolation of AvrPtoB. AvrPto and AvrPtoB proteins have exactly the same interaction specificity for Pto in the yeast two-hybrid system and despite many differences they share several small, discrete subregions in common. Subregion V plays a key role in the interaction with the Pto kinase, and it is possible that other subregions also have conserved roles. Our findings demonstrate that distinct bacterial effector proteins interact with the Pto kinase by using a common structural mechanism.

A yeast two-hybrid screen involving 12 *Pseudomonas* genomic prey libraries and a Pto bait construct was used to isolate AvrPtoB. The *Pseudomonas* genome is about 6.6 Mb, and therefore the screening of 5×10^7 random prey clones with an average insert size of 1 kb provides a >99.9% probability of testing every *Pseudomonas* genome sequence in the proper reading frame at least once for interaction with Pto. By using the DC3000 genome sequence (www.tigr.org), we examined each of the PtiDC clones recovered (see Supplemental Table S1 at http://www.cell.com/cgi/content/full/109/5/589/DC1) and, so far, have observed a Hrp box upstream of only the AvrPtoB open reading frame. Thus, unless the type III pathway also secretes non-Hrp-regulated proteins, it is likely that the interactions with Pto of the other proteins we identified are not biologically meaningful. We recovered eight AvrPtoB clones, including some that were missing up to 121 amino acids from the N terminus of AvrPtoB, but no clones that were missing anything downstream of this point. Because of the high probability that many subfragments of AvrPtoB are present in our *Pseudomonas* prey libraries, these results suggest that structural features spanning the C-terminal 432 amino acids of AvrPtoB are required for its interaction with Pto.

Several lines of evidence indicate that AvrPtoB is an effector that plays a role in restricting host range of *Pseudomonas*. First, in common with all previously identified *Avr* genes, the *avrPtoB* promoter contains a consensus Hrp box. As expected, expression of *avrPtoB* is

induced by growth medium that simulates the apoplastic fluid of plant leaves and is controlled by the Hrp regulon. Second, we showed that delivery of AvrPtoB from *P. fluorescens* to plant cells is strictly dependent upon the presence of the TTSS encoded by the Hrp cluster. Third, the delivery of AvrPtoB from two normally virulent *Pseudomonas* strains or by *Agrobacterium*-mediated expression in the plant cell is detectable based on the specific recognition of the protein by the Pto kinase. Because Pto is localized within the plant cell, this observation indicates that, as with many other Avr proteins (reviewed in Kjemtrup et al., 2000), AvrPtoB is active inside the plant cell. Finally, there is the similarity of AvrPtoB to the VirPphA protein. VirPphA was originally identified in a *P. s. phaseolicola* strain as a virulence factor because it promotes watersoaking by the pathogen in a bean pod assay. It was subsequently found to confer avirulence to *P. s. phaseolicola* bacteria infiltrated into soybean leaves (Jackson et al., 1999). In a related study, we found that AvrPtoB also promotes watersoaking in the bean pod assay and therefore has virulence activity, too (R. Jackson et al., submitted). VirPphA also interacts with Pto in the yeast two-hybrid system and elicits a *Pto*-specific HR in tomato leaves (Y.J.K., G.B.M., G. Tsiamis, and J. Mansfield, unpublished). Thus, the alignment of the two proteins (see Supplemental Table S2 at http://www.cell.com/cgi/content/full/109/5/589/DC1) should expedite the identification of key residues in each protein that play a role in avirulence and virulence.

We found that *avrPtoB* did not confer avirulence on all *P. s. tomato* strains tested (Table 1). This is consistent with the fact that AvrPtoB was not isolated previously by screening of DC3000 cosmids in a virulent strain of *Pseudomonas* (Ronald, et al., 1992). It is possible that another *Pseudomonas* protein (e.g., a chaperone) is required for the effective secretion or translocation of AvrPtoB from *Pseudomonas* and that this factor is not present in all *P. s. tomato* strains. It is also possible that expression of AvrPtoB in certain bacterial strains is "masked" as observed for some effectors in *P. s. phaseolicola* (Jackson et al., 1999).

AvrPto was previously found to interact with certain Pto variants, and these proteins were used to define residue T204 of Pto as a key determinant of recognition specificity for AvrPto (Frederick et al., 1998). Remarkably, AvrPtoB interacts with the same Pto variants as AvrPto, and thus T204 is also a key Pto determinant for interaction with AvrPtoB. AvrPtoB also interacts with the one AvrPto-interacting member of the Pto family isolated from a bacterial speck-resistant wild species of tomato, *Lycopersicon hirsutum*. These observations suggest that there has been selection in *Lycopersicon* spp. over a long period of time for Pto kinases that specifically recognize a conserved feature present in both the AvrPto and AvrPtoB proteins.

Dual recognition specificity previously has been reported for three other plant R proteins (i.e., RPM1, RPP8/HRT, Mi1) (for review see Dangl and Jones, 2001), although in none of these cases have the host and pathogen proteins been shown to interact directly. Thus, the dual (or perhaps even multiple) recognition specificity of R proteins may turn out to be a common feature of plant defense responses. This notion is consistent with the recent report that *Arabidopsis* contains only 150 putative *R* loci (of the NB-LRR class), yet is likely defending itself against many thousands of potential plant pathogens (Dangl and Jones, 2001). Although the pathogen proteins recognized by most of these R genes are unknown, our present work suggests that common structural motifs embedded within diverse pathogen proteins might play a role in their recognition. Finally, if we consider the possibility that the Pto kinase originally might have been an important target for several bacterial virulence proteins, then our data are also consistent with the "guard" hypothesis that postulates that NB-LRR proteins (e.g., Prf) have evolved to interact with a complex of Avr proteins and their virulence targets (Dangl and Jones, 2001).

A detailed structure-function analysis of both Avr proteins will be necessary to fully understand the importance of residues conserved between them. We began this analysis by examining subregion V (the "GINP motif") because it is perfectly conserved in both AvrPto and AvrPtoB, and previous work with AvrPto found that several residues within this subregion are required for interaction with Pto (Shan et al., 2000b). Substitutions in the three residues examined in subregion V significantly decreased interaction of AvrPtoB with Pto, while substitutions just outside subregion V did not. These results, along with the previous findings with AvrPto, suggest that the GINP motif may play a role as contact point between the Pto kinase and these two effector proteins. Alternatively, the GINP motif could affect the structure of another part of these proteins that interacts with Pto. We are currently determining the three-dimensional structure of the AvrPto protein, and this will allow us to further examine the role of the GINP motif in Pto recognition (L. Nicholson and G.B.M., unpublished).

Although *avrPto*-like sequences occur only in a subset of *Pseudomonas* strains that are known to be avirulent on *Pto*-expressing tomato plants (Ronald et al., 1992), *avrPtoB*-like sequences are present in at least three genera of bacterial phytopathogens (Figure 3). *AvrPtoB* is one of only a few known Avr genes to show this wide distribution (White et al., 2000). It might be anticipated that widely conserved effectors serve as virulence factors and this appears to be the case for AvrPtoB. We have cloned several *avrPtoB*-related sequences from selected *Pseudomonas*, *Erwinia*, and *Xanthomonas* strains, and from preliminary sequence analysis we find a high degree of similarity among them (unpublished data). Future study of the AvrPtoB/VirPphA family will reveal if it plays a conserved role in promoting virulence in these diverse phytopathogens.

Although our studies revealed many similarities between AvrPto and AvrPtoB, we also observed some striking and intriguing differences. First are the differences in the genes and corresponding proteins. *AvrPtoB*-like sequences are widely distributed, whereas *avrPto*-like sequences have not been observed outside of the *Pseudomonas* spp. The proteins encoded by each gene are very different with AvrPtoB, at 59 kDa, over three times the mass of AvrPto at 18 kDa. There are sequence similarities at both the N and C termini of the proteins, and the main additions of AvrPtoB lie within four large internal segments. We also found that, unlike AvrPto, the AvrPtoB protein lacks a myristylation motif at the penultimate position of the N terminus. The myris-

tylation motif of AvrPto is required for both its avirulence and virulence activity and also for association of AvrPto with the membrane fraction (Shan et al., 2000a). We cannot exclude the possibility that AvrPtoB protein might be processed to reveal an internal myristylation motif as is the AvrPphB protein (Nimchuk et al., 2000). However, in preliminary experiments using an AvrPtoB::GFP fusion, the protein does not appear to localize specifically to the cell periphery (B. Riely and G.B.M., unpublished).

The second major difference we observed between AvrPto and AvrPtoB is their apparent activity in plant cells. Unlike *avrPto*, the expression of *avrPtoB* in susceptible tomato or *N. benthamiana* leaves does not cause severe yellowing and necrosis that is dependent on the presence of Prf (Chang et al., 2000). It is not clear whether this AvrPto-mediated necrosis is a defense or susceptibility response, but the lack of the response in leaves expressing AvrPtoB might indicate that the two proteins target different host proteins as susceptibility targets when Pto is not present. In this regard, it will be interesting to see if host proteins that are known to interact with AvrPto or the AvrPto-Pto complex will also do so with AvrPtoB or AvrPtoB-Pto (Bogdanove and Martin, 2000). Finally, we were surprised to discover that coexpression of AvrPtoB and Pto in leaves of *N. benthamiana* did not lead to an HR as does coexpression of AvrPto and Pto. This suggests that although both effectors target the Pto kinase, they each may require additional and distinct host proteins for their avirulence activities.

Experimental Procedures

Bacterial Strains

The *E. coli* strains DH5α and DH10B (GIBCO-BRL, Grand Island, NY), *Agrobacterium tumefaciens* strains EH105 and GV2260, and *P. s. tomato* strains were used for plasmid maintenance, transgene delivery, and infection assays, respectively. Plasmids used were pBluescript SK(-) (Stratagene, La Jolla, CA), pCR2.1 (Invitrogen, Carlsbad, CA), and pDSK519. Isolates and transconjugants of *P. s. tomato* were grown on King's medium B (KB) agar at 30°C and *E. coli* strains on LB agar or in LB broth at 37°C.

Yeast Two-Hybrid Library Development and Screening

Plasmids (pEG202, pJG4-5, pSH18-34, pRFHM-1, and pJK101) and yeast strain EGY48 (*ura3*, *his3*, *trp1*, LexAop-LEU2) were provided by R. Brent (Massachusetts General Hospital, Boston, MA), and basic procedures for the yeast two-hybrid system were described previously (Zhou et al., 1995). The *Pseudomonas* prey library was generated in a modified vector series based on pJG4-5.

Insert DNA for the prey library was prepared by partial digestion of *P. s. tomato* DC3000 genomic DNA with the enzymes *Aci*I, *Msp*I, *HinP1*I, or *Taq*I. Ten micrograms of each digest was size fractionated and fragments of 500–3000 bp were recovered. The DNAs were used in 12 ligation reactions (three vectors $\times$ four enzyme digests). Each ligation was transformed into *E. coli* strain DH10B and yielded $>10^8$ transformants. An equal number of transformants derived from each of the twelve libraries was pooled and DNA extracted. The pooled DNA was transformed into *Saccharomyces cerevisiae* strain EGY48, which contained a LexA-Pto bait construct and the *lacZ* reporter plasmid pSH18-34 (Zhou et al., 1995).

Constructs for Expression of *avrPtoB* in *Pseudomonas* or Plant Cells

A DC3000 cosmid library from Alan Collmer (Cornell Univ.) was screened using an AvrPtoB probe. A clone, pDC101, carrying a 37 kb insert was identified, and a 6.0 kb *Pst*I fragment was found to have the entire AvrPtoB open reading frame and putative Hrp-box. A 2.1 kb fragment from this region was cloned into pCR2.1 and then into the broad host range vector pDSK519, creating pDSK519::*avrPtoB*. All *avrPtoB* constructs were verified by sequencing. pDSK519::*avrPtoB* was introduced by triparental mating into *P. s. tomato* strains. For expression in plant cells, the *avrPtoB* coding region was subcloned downstream of the CaMV 35S promoter in the vector pBTEX (Frederick et al., 1998). Site-directed mutagenesis of the *avrPtoB* sequence was performed in plasmid pJG4-5 or in pBTEX using the Quickchange kit from Stratagene (La Jolla, CA). The mutations were confirmed by sequencing.

Determination of Disease Symptoms on Plant Leaves

Tomato (*Lycopersicon esculentum*) plants of Rio Grande-PtoS (RG-PtoS; *pto*/*pto, Prf*/*Prf*), Rio Grande-PtoR (RG-PtoR; *Pto*/*Pto, Prf*/*Prf*), and the mutants RG-prf3 (*Pto*/*Pto, prf*/*prf*), and RG-pto11 (*pto*/*pto, Prf*/*Prf*) were grown in a greenhouse (24°C, 14 hr day). Tomato leaves on 7- or 8-week-old plants were infiltrated with *P. s. tomato* bacterial suspensions of 10^4 or 10^7 colony-forming units per milliliter (cfu/ml). In low-inoculum-level experiments, symptoms of bacterial speck disease developed over a 3 to 6 day period after inoculation. In high-inoculum-level experiments, the HR occurred within 30 hr.

Agrobacterium-Mediated Transient Expression in Plant Leaves

AvrPtoB expression constructs in pBTEX were introduced by electroporation into *Agrobacterium tumefaciens* strain GV2260 for tomato. *Agrobacterium* for inoculation was grown in LB medium overnight and diluted into induction medium (50 mM MES [pH 5.6], 0.5% (w/v) glucose, 1.7 mM NaH_2PO_4, 20 mM NH_4Cl, 1.2 mM $MgSO_4$, 2 mM KCl, 17 μM $FeSO_4$, 70 μM $CaCl_2$, and 200 μM acetosyringone) to an $OD_{600} = 0.03$. Bacterial suspensions were injected with a needleless syringe into leaves of 7- to 8-week-old tomato plants.

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References

Bogdanove, A.J., and Martin, G.B. (2000). AvrPto-dependent Pto-interacting proteins and AvrPto-interacting proteins in tomato. Proc. Natl. Acad. Sci. USA *97*, 8836–8840.

Chang, J.H., Rathjen, J.P., Bernal, A.J., Staskawicz, B.J., and Michelmore, R.W. (2000). *avrPto* enhances growth and necrosis caused by *Pseudomonas syringae* pv. *tomato* in tomato lines lacking either *Pto* and *Prf*. Mol. Plant Microbe Interact. *13*, 568–571.

Chang, J.H., Tobias, C.M., Staskawicz, B.J., and Michelmore, R.W. (2001). Functional studies of the bacterial avirulence protein AvrPto by mutational analysis. Mol. Plant Microbe Interact. *14*, 451–459.

Cohn, J., Sessa, G., and Martin, G.B. (2001). Innate immunity in plants. Curr. Opin. Immunol. *13*, 55–62.

Collmer, A., Badel, J.L., Charkowski, A.O., Deng, W.-L., Fouts, D.E., Ramos, A.R., Rehm, A.H., Anderson, D.M., Schneewind, O., van Dijk, K., and Alfano, J.R. (2000). *Pseudomonas syringae Hrp* type III secretion system and effector proteins. Proc. Natl. Acad. Sci. USA *97*, 8770–8777.

Dangl, J.L., and Jones, J.D.G. (2001). Plant pathogens and integrated defence responses to infection. Nature *411*, 826–833.

Fouts, D.E., Abramovitch, R.B., Alfano, J.R., Baldo, A.M., Buell, C.R., Cartinhour, S., Chatterjee, A.K., D'Ascenzo, M.D., Gwinn, M.L., Lazarowitz, S.G., et al. (2002). Genome-wide identification of *Pseu-*

domonas syringae pv. *tomato* DC3000 promoters controlled by the HrpL alternative sigma factor. Proc. Natl. Acad. Sci. USA *99*, 2275–2280.

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

Galan, J.E., and Collmer, A. (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. Science *284*, 1322–1328.

Hammond-Kosack, K., and Jones, J.D.G. (2000). Responses to plant pathogens. In Biochemistry and Molecular Biology of Plants, B. Buchanan, W. Gruissem, and R. Jones, eds. (Rockville, MD: American Society of Plant Physiologists), pp. 1102–1156.

Hoffman, J.A., Kafatos, F.C., Janeway, C.A., Jr., and Ezekowitz, R.A.B. (1999). Phylogenetic perspectives in innate immunity. Science *284*, 1313–1318.

Innes, R.W., Bent, A.F., Kunkel, B.N., Bisgrove, S.R., and Staskawicz, B.J. (1993). Molecular analysis of avirulence gene *avrRpt2* and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes. J. Bacteriol. *175*, 4859–4869.

Jackson, R.W., Athanassopoulos, E., Tsiamis, G., Mansfield, J.W., Sesma, A., Arnold, D.L., Gibbon, M.J., Murillo, J., Taylor, J.D., and Vivian, A. (1999). Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen *Pseudomonas syringae* pathovar *phaseolicola*. Proc. Natl. Acad. Sci. USA *96*, 10875–10880.

Jia, Y., Loh, Y.-T., Zhou, J., and Martin, G.B. (1997). Alleles of Pto and Fen occur in bacterial speck-susceptible and fenthion-insensitive tomato cultivars and encode active protein kinase. Plant Cell *9*, 61–73.

Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P., and Valent, B. (2000). Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. EMBO J. *19*, 4004–4014.

Jin, Q., and He, S.-H. (2001). Role of the Hrp pilus in type III protein secretion in *Pseudomonas syringae*. Science *294*, 2556–2558.

Kjemtrup, S., Nimchuk, Z., and Dangl, J.L. (2000). Effector proteins of phytopathogenic bacteria: bifunctional signals in virulence and host recognition. Curr. Opin. Microbiol. *3*, 73–78.

Lindgren, P.B. (1997). The role of *hrp* genes during plant-bacterial interactions. Annu. Rev. Phytopathol. *35*, 129–152.

Martin, G.B. (1999). Functional analysis of plant disease resistance genes and their downstream effectors. Curr. Opin. Plant Biol. *2*, 273–279.

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

Martin, G.B., Frary, A., Wu, T., Brommonschenkel, S., Chunwongse, J., Earle, E.D., and Tanksley, S.D. (1994). A member of tomato *Pto* gene family confers sensitivity to fenthion resulting in rapid cell death. Plant Cell *6*, 1543–1552.

Michelmore, R.W., and Meyers, B.C. (1998). Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Res. *8*, 1113–1130.

Mudgett, M.B., and Staskawicz, B.J. (1999). Characterization of the *Pseudomonas syringae* pv. *tomato* AvrRpt2 protein: demonstration of secretion and processing during bacterial pathogenesis. Mol. Microbiol. *32*, 927–941.

Nimchuk, Z., Marois, E., Kjemtrup, S., Leister, R.T., Katagiri, F., and Dangl, J.L. (2000). Eukaryotic fatty acylation drives plasma membrane targeting and enhances function of several type III effector proteins from *Pseudomonas syringae*. Cell *101*, 353–363.

Nimchuk, Z., Rohmer, L., Chang, J.H., and Dangl, J.L. (2001). Knowing the dancer from the dance: R-gene products and their interactions with other proteins from the host and pathogen. Curr. Opin. Plant Biol. *4*, 288–294.

Preston, G.M. (2000). *Pseudomonas syringae* pv. *tomato*: the right pathogen, of the right plant, at the right time. Mol. Plant Pathol. *1*, 263–275.

Riely, B.K., and Martin, G.B. (2001). Ancient origin of pathogen recognition specificity conferred by the tomato disease resistance gene *Pto*. Proc. Natl. Acad. Sci. USA *98*, 2059–2064.

Ronald, P.C., Salmeron, J.M., Carland, F.M., and Staskawicz, B.J. (1992). The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the Pto resistance gene. J. Bacteriol. *174*, 1604–1611.

Salmeron, J.M., and Staskawicz, B.J. (1993). Molecular characterization and *hrp* dependence of the avirulence gene *avrPto* from *Pseudomonas syringae* pv. *tomato*. Mol. Gen. Genet. *239*, 6–16.

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

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

Sessa, G., D'Ascenzo, M., and Martin, G.B. (2000). Thr38 and Ser198 are Pto autophosphorylation sites required for the AvrPto-Pto-mediated hypersensitive response. EMBO J. *19*, 2257–2269.

Shan, L., He, P., Zhou, J., and Tang, X. (2000a). A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto. Mol. Plant Microbe Interact. *13*, 592–598.

Shan, L., Thara, V.K., Martin, G.B., Zhou, J.M., and Tang, X. (2000b). The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane. Plant Cell *12*, 2323–2337.

Staskawicz, B.J., Mudgett, M.B., Dangl, J.L., and Galan, J.E. (2001). Common and contrasting themes of plant and animal diseases. Science *292*, 2285–2289.

Tang, X., Frederick, R.D., Zhou, J., Halterman, D.A., Jia, Y., and Martin, G.B. (1996). The avirulence protein AvrPto physically interacts with the Pto kinase. Science *274*, 2060–2063.

Van den Ackerveken, G., Marios, E., and Bonas, U. (1996). Recognition of the bacterial avirulence protein AvrBs3 occurs inside the host cell. Cell *87*, 1307–1316.

White, F.F., Yang, B., and Johnson, L.B. (2000). Prospects for understanding avirulence gene function. Curr. Opin. Plant Biol. *3*, 291–298.

Xiao, Y., Hey, S., Yi, J., Lu, Y., and Hutcheson, S.W. (1994). Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonase syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes. J. Bacteriol. *176*, 1025–1036.

Zhou, J., Loh, Y.T., Bressan, R.A., and Martin, G.B. (1995). The tomato gene Pti1 encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response. Cell *83*, 925–935.

Zhu, W., Yang, B., Wills, N., Johnson, L.B., and White, F.F. (1999). The C terminus of AvrXa10 can be replaced by the transcriptional activation domain of VP16 from the herpes simplex virus. Plant Cell *11*, 1665–1674.