Pto- and Prf-Mediated Recognition of AvrPto and AvrPtoB Restricts the Ability of Diverse *Pseudomonas syringae* Pathovars to Infect Tomato

Nai-Chun Lin^{1,2} and Gregory B. Martin^{1,3}

¹Boyce Thompson Institute for Plant Research, Tower Rd., Ithaca, NY 14853-1801, U.S.A.; ²Department of Agricultural Chemistry, National Taiwan University, Taipei 10617, Taiwan, Republic of China; ³Department of Plant Pathology, Cornell University, Ithaca, NY 14853-4203, U.S.A.

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The molecular basis underlying the ability of pathogens to infect certain plant species and not others is largely unknown. *Pseudomonas syringae* is a useful model species for investigating this phenomenon because it comprises more than 50 pathovars which have narrow host range specificities. Tomato (*Solanum lycopersicum*) is a host for *P. syringae* pv. *tomato*, the causative agent of bacterial speck disease, but is considered a nonhost for other *P. syringae* pathovars. Host resistance in tomato to bacterial speck disease is conferred by the Pto protein kinase which acts in concert with the Prf nucleotide-binding luciferin-rich repeat protein to recognize *P. syringae* pv. *tomato* strains expressing the type III effectors AvrPto or AvrPtoB (HopAB2). The *Pto* and *Prf* genes were isolated from the wild tomato species *S. pimpinellifolium* and functional alleles of both of these genes now are known to exist in many species of tomato and in other Solanaceous species. Here, we extend earlier reports that *avrPto* and *avrPtoB* genes are widely distributed among pathovars of *P. syringae* which are considered nonhost pathogens of tomato. This observation prompted us to examine the possibility that recognition of these type III effectors by *Pto* or *Prf* might contribute to the inability of many *P. syringae* pathovars to infect tomato species. We show that 10 strains from presumed nonhost *P. syringae* pathovars are able to grow and cause pathovar-unique disease symptoms in tomato leaves lacking *Pto* or *Prf*, although they did not reach the population levels or cause symptoms as severe as a control *P. syringae* pv. *tomato* strain. Seven of these strains were found to express *avrPto* or *avrPtoB*. The AvrPto- and AvrPtoB-expressing strains elicited disease resistance on tomato leaves expressing *Pto* and *Prf*. Thus, a gene-for-gene recognition event may contribute to host range restriction of many *P. syringae* pathovars on tomato species. Furthermore, we conclude that the diverse disease symptoms caused by different *Pseudomonas* pathogens on their normal plant hosts are due largely to the array of virulence factors expressed by each pathovar and not to specific molecular or morphological attributes of the plant host.

Additional keywords: nonhost resistance, *R* genes.

The reasons why specific pathogens infect certain plant species but are unable to infect others are mostly unknown (Keen 1988; Mysore and Ryu 2004; Thordal-Christensen 2003). In some cases, host range restriction appears to be due to the absence of a specific virulence factor in the pathogen that is required for colonization and growth in tissues of a certain species. One example of this scenario is the recent report that the HsvB type III effector protein is required for the ability of two pathovars of *Pantoea agglomerans* to grow on beet (Nissan et al. 2006). Other type III effectors also exhibit this ability to broaden host range (Chakrabarty and Gabriel 1997; Swarup et al. 1991). Alternatively, and not in a mutually exclusive manner, host range could be restricted by expression in the pathogen of a component that is recognized by the defense system of a nonhost plant species. A seminal observation supporting this possibility was the identification of three type III effector genes in the tomato pathogen *Pseudomonas syringae* pv. *tomato* which, when introduced into the soybean pathogen *P. syringae* pv. *glycinea*, caused a hypersensitive response (HR) in specific soybean cultivars (Kobayashi et al. 1989). Collectively, the three *P. syringae* pv. *tomato* genes accounted for all the HRs observed on different soybean cultivars. Therefore, this observation raised the possibility that the nonhost resistance of soybean to *P. syringae* pv. *tomato* might be due to recognition of one or more of these three type III effectors by widely conserved resistance (*R*) genes present in different soybean cultivars.

A recent review has proposed classifying nonhost resistance into two types (Mysore and Ryu 2004). In type I nonhost resistance, no symptoms of pathogen attack are observed because the bacterium, fungus, or oomycete is unable to counter preformed defenses such as the host cell wall and antimicrobial compounds, or early inducible defenses such as cell wall thickening and phytoalexins. In contrast, the more widely studied type II nonhost resistance, which also occurs in response to various bacterial, fungal, and oomycete pathogens, is associated with the development of rapid localized cell death (the HR) at the site of attempted attack. Consistent with the interpretation from the soybean HR work described above (Kobayashi et al. 1989), it has been proposed that type II nonhost resistance shares some mechanistic similarities to host resistance mediated by plant *R* genes (Abramovitch et al. 2006; Mysore and Ryu 2004).

Corresponding author: G. B. Martin; Telephone: 607-254-1208; Fax: 607-255-6695; E-mail: gbm7@cornell.edu

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Although no direct molecular evidence for a role of *R* genes in type II nonhost resistance has been reported, identification of *NH01* in *Arabidopsis thaliana* and *SGT1* in *Nicotiana benthamiana* provides strong evidence that nonhost and host resistance share similar mechanisms (Lu et al. 2001; Peart et al. 2002). *NH01*, which encodes a glycerol kinase, originally was identified as a key component for type I nonhost resistance to *P. syringae* pv. *phaseolicola* NPS3121 (Lu et al. 2001). It also was found to be required for resistance in *Arabidopsis*, mediated by the *R* genes *RPS2*, *RPS4*, *RPS5*, and *RPM1*. Similarly, the ubiquitin ligase-associated protein SGT1 is required for N-, Rx-, and Pto-mediated resistance and also for type II nonhost resistance of *N. benthamiana* against the Brassicaceae pathogen *P. syringae* pv. *maculicola* and pepper pathogen *Xanthomonas axonopodis* pv. *vesicatoria* (Peart et al. 2002).

Additional genetic evidence indicates that *R* gene-mediated recognition might be involved in nonhost resistance. For example, *avrPpiA1*, a type III effector from the pea pathogen *P. syringae* pv. *pisi* recognized by *RPM1* in pea, also elicits nonhost resistance in interactions with functional *RPM1* homologs in *Arabidopsis* and bean (Dangl et al. 1992). Similarly, the region of maize chromosome 6 carrying the *Rxol/Rbal* locus was found to mediate an HR to both the host pathogen *Burkholderia andropogonis* and the nonhost pathogen *X. oryzae* pv. *oryzicola*, although it is unknown whether this is due to the action of the *Rxol/Rbal* *R* genes (Zhao et al. 2004). The host range of a plant pathogen probably is determined in part by the stability of nonhost resistance directed against it. Therefore, it is likely that, if *R* proteins contribute significantly to nonhost resistance, they are targeted against widely distributed, conserved pathogen effectors or that *R* genes present in a given plant species allow recognition of multiple diverse effectors.

The species *P. syringae* is an excellent model system for investigating the molecular basis of bacterial pathogenesis and both host and nonhost resistance (Buell et al. 2003; Lindeberg et al. 2006; Preston 2000). Over 50 pathovars of *P. syringae* have been defined based on pathogenicity tests or on the plant host from which they were isolated and are thought to infect (Hirano and Upper 2000; Rudolph 1995; Sarkar and Guttman 2004). Thus, for example, *P. syringae* pv. *tomato* is a common pathogen of tomato but is considered a nonhost pathogen of bean, whereas the reverse is true for *P. syringae* pv. *phaseolicola*. Recently, the genomes have been sequenced of representative strains of both *P. syringae* pv. *tomato* and *P. syringae* pv. *phaseolicola*, and these pathogens have been shown to express approximately 30 and 20 confirmed type III effectors, respectively (Buell et al. 2003; Joardar et al. 2005; Lindeberg et al. 2006). Because type III effectors play a key role in bacterial virulence, it seems likely that they frequently make a fundamental contribution to host range specificity. The discovery that *P. syringae* pathovars express such a large number of type III effectors, combined with their host range specificity and experimental tractability, provides an ideal opportunity to use these organisms to investigate the molecular basis of nonhost resistance.

In tomato (*Solanum lycopersicum*), host resistance to *P. syringae* pv. *tomato*, the causative agent of bacterial speck disease, occurs upon recognition of the type III effectors AvrPto or AvrPtoB (*hopAB2*) by the *R* protein Pto, a serine/threonine protein kinase (Pedley and Martin 2003). Pto, which was isolated from the wild species *S. pimpinellifolium*, acts in concert with the nucleotide-binding luciferase-rich repeat protein Prf to activate a variety of defense responses, including the HR (Mucyn et al. 2006; Salmeron et al. 1996). Functional *Pto* genes recently were reported to occur in approximately 35% of a subset of accessions of tomato wild species, and *Pto*-like genes also exist in other Solanaceous species (He et al. 2004;

Riely and Martin 2001; Rose et al. 2005; Thilmony et al. 1995; Vleeshouwers et al. 2001). Interestingly, although there are more than 50 classes of type III effectors in *Pseudomonas* spp., tomato is known to target only two of them, AvrPto and AvrPtoB, for activation of resistance (via Pto/Prf) (Lin and Martin 2005; Pedley and Martin 2003). AvrPto and AvrPtoB interfere with basal defenses in *Arabidopsis* and promote bacterial growth and ethylene-dependent disease-associated cell death in tomato plants lacking Pto or Prf (Cohn and Martin 2005; He et al. 2006). The apparent broad occurrence of *avrPto* and *avrPtoB* (*hopAB*)-like genes in diverse *Pseudomonas* spp. suggests that their virulence activities contribute to bacterial fitness in nature (Jackson et al. 2002; Lin et al. 2006; Sarkar et al. 2006).

The wide distribution of *avrPto*- and *avrPtoB*-related sequences among *Pseudomonas* pathovars and similar conservation of *Pto* and *Prf* genes in wild tomato species raises the possibility that a recognition event involving these genes might play a role in nonhost resistance of tomato species to *P. syringae* pathovars. Here, we show that Pto- or Prf-expressing tomato lines mediate a type II-like (HR) nonhost resistance to diverse *P. syringae* pathovars which express AvrPto or AvrPtoB homologs. Interestingly, tomato leaves lacking a functional Pto/Prf pathway did not display a nonhost HR and, in fact, *P. syringae* pathovars were able to infect these plants and cause a variety of pathovar-distinct disease symptoms. Although populations of these *P. syringae* pathovars in leaves increased substantially during the infection period, they did not reach the high level of a control *P. syringae* pv. *tomato* strain. These observations suggest that the inability of many *Pseudomonas* pathovars to infect tomato species in the field is due to both a lack of certain tomato-specific type III effectors and, in many wild species of tomato, the presence of the Pto/Prf recognition system.

RESULTS

AvrPto and AvrPtoB homologs are present and expressed in many *P. syringae* pathovars.

We first examined whether *avrPto*- or *avrPtoB*-like sequences were present in 10 isolates of diverse *P. syringae* pathovars representing various clades of this species (Table 1) (Sarkar and Guttman 2004). We detected *avrPto*-like sequences in *P. syringae* pvs. *lachrymans*, *mori*, *phaseolicola*, and *syringae* (Table 1; Supplementary Figure S1). *AvrPtoB*-like sequences were observed in eight of the isolates (but note that, in *P. syringae* pv. *maculicola* ES4326, these are C-terminal truncated forms of AvrPtoB, termed HopPmaL and HopPmaN) (Guttman et al. 2002). Two of the 10 isolates we examined, *P. syringae* pv. *coronafaciens* B142 and *P. syringae* pv. *syringae* 61, did not contain either *avrPto*- or *avrPtoB*-like sequences (Table 1). We previously reported that the *P. syringae* pv. *tomato* T1 genome has an *avrPtoB*-related gene for which an mRNA transcript, but no protein, is detectable, leading to a compatible interaction with tomato lines expressing Pto/Prf (Lin et al. 2006). Therefore, we investigated by RNA and protein gel blot analyses whether *Pseudomonas* strains carrying *avrPto*- or *avrPtoB*-like sequences actually express these genes. Transcripts for *avrPto* were detected in all of the strains expected except for *P. syringae* pv. *phaseolicola* NPS3121, whereas *avrPtoB*-related transcripts were detected in all the strains expected except *P. syringae* pv. *glycinea* F117, *P. syringae* pv. *mori*, and *P. syringae* pv. *phaseolicola* NPS3121 (Table 1 and data not shown). However, these negative results likely are due to low abundance of the transcripts or lack of homology with our probe because AvrPto and AvrPtoB proteins were detected in all strains carrying *avrPto*- or *avrPtoB*-like sequences (Table 1 and Supplementary Figure S2). These data confirm and extend

earlier observations showing that the *avrPto*- and *avrPtoB*-related genes are present and expressed in many *P. syringae* pathovars which are not known to infect tomato (Lindeberg et al. 2006; Sarkar et al. 2006).

Elicitation of the HR by diverse *P. syringae* pathovars requires a functional *Pto*/*Prf* pathway in tomato plants.

P. syringae pathovars other than *P. syringae* pv. *tomato* (and, to a much lesser extent, *P. syringae* pv. *syringae*) (Jones 1991) are considered to be nonhost pathogens of tomato. Therefore, we investigated whether they would elicit a type II-like nonhost

HR on tomato plants. Electrolyte leakage from leaves often is used as an indicator of membrane damage that is associated with the HR (Goodman and Novacky 1994). Therefore, we infiltrated leaves of Rio Grande-PtoR (*Pto*/*Pto*, *Prf*/*Prf*) with a high titer (2×10^7 CFU/ml) of each strain, placed the disks in water, and at the timepoints shown, measured the conductivity (Fig. 1, Supplementary Table S1). We observed a relative conductivity of approximately 30 microSiemens (μ S) or more within 6 h in response to five of the seven strains expressing *avrPto* or *avrPtoB* (*P. syringae* pvs. *angulata* 9, *lachrymans* 3, *mori*, *phaseolicola* 3121, and *tabaci* 11528). Infiltration of *P. syringae* pvs.

Table 1. Characteristics of *Pseudomonas* isolates used in this study^x

<i>Pseudomonas syringae</i> isolate ^y	Host	DNA		RNA		Protein	
		<i>avrPto</i>	<i>avrPtoB</i>	<i>avrPto</i>	<i>avrPtoB</i>	AvrPto	AvrPtoB
<i>angulata</i> (Pan) Pan9	Tobacco	–	+	ND	+	ND	+
<i>coronafaciens</i> (Pco) B142	Oat	–	–	–	–	–	–
<i>glycinea</i> (Pgy) F117	Soybean	–	+	ND	–	ND	+
<i>lachrymans</i> (Pla) Pla3	Cucurbit	+	+	+	+	+	+
<i>mori</i> (Pmo) PDDCC4331	Mulberry	+	+	+	–	+	+
<i>maculicola</i> (Pma) ES4326	Crucifers	–	* ^z	ND	* ^z	ND	* ^z
<i>phaseolicola</i> (Pph) NPS3121	Bean	+	+	–	–	+	+
<i>syringae</i> (Psy) 61	Lilac, bean	–	–	–	–	–	–
<i>syringae</i> (Psy) B728a	Lilac, bean	+	+	+	+	+	+
<i>tabaci</i> (Pta) 11528	Tobacco	–	+	ND	+	ND	+
<i>tomato</i> DC3000	Tomato	+	+	+	+	+	+

^x Symbols: – = no detectable fragments, + = fragments detected using *avrPto* or *avrPtoB* as a probe in DNA or RNA gel blot assays or protein species detected using anti-AvrPto or anti-AvrPtoB antibodies, and ND = not done.

^y Proposed abbreviations are shown in parentheses (Sarkar et al. 2006; Vivian and Mansfield 1993).

^z *hopPmaL* and *holPmaN*, C-terminus truncated forms of *AvrPtoB* (Guttman et al. 2002).

Fig. 1. Ion leakage caused by infiltration of various *Pseudomonas syringae* pathovars into tomato leaves. Degree of ion leakage from leaf areas of RG-PtoR (black), RG-ptol1 (hatched), or RG-prf3 (gray) after infiltration with the *P. syringae* strains indicated at 2×10^7 CFU/ml. Ion leakage is shown as relative conductivity (in microSiemens; μ S). Timepoints in hours postinfiltration (hpi) are shown at which leaf tissues showed the greatest differential response between RG-PtoR, RG-ptol1, and RG-prf3 after infiltration with the indicated *P. syringae* isolate. Note that, at later timepoints, disease-associated cell death was observed with all *P. syringae* isolates. Bars indicate standard errors. Experiments presented in this figure were performed twice with similar results. The data from the two experiments were treated as replicates and were analyzed using Duncan's multiple range test. Means with the same letter assigned for each pathovar are not different at a significance level of 5%.

coronafaciens B142, *maculicola* ES4326, and *syringae* 61, which lack *avrPto* or *avrPtoB* homologs, resulted in lower conductivity at this timepoint (approximately 10 μ S), although *P. syringae* pv. *syringae* 61 developed cell death (with approximately 30 μ S of conductivity) at 12 h postinfiltration. The two other isolates expressing *avrPto* or *avrPtoB*, *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *glycinea* F117, caused extensive cell death in RG-PtoR leaves at 12 and 24 h postinfiltration which was associated with high conductivity levels (approximately 200 μ S). We previously have reported that infiltration of Rio Grande-PtoR leaves with *P. syringae* pv. *tomato* strains expressing AvrPto or AvrPtoB causes much greater cell death and high conductivity levels compared with an isogenic strain lacking these effectors (Lin and Martin 2005). Therefore, these data suggested that the conductivity levels above approximately 30 μ S were due to early host cell death associated with the HR and not due to disease (this is further supported below). In summary, all *P. syringae* pv. *tomato* strains that contained AvrPto or AvrPtoB homologs caused enhanced ion leakage from RG-PtoR leaves, whereas the strains lacking these effectors did not.

To test whether these early cell death responses were mediated in part by the Pto/Prf pathway, we examined the effect of these 10 *Pseudomonas* strains on two tomato lines with mutations disrupting *Pto* or *Prf* (RG-pto11 carries a point mutation in *Pto* and RG-prf3 carries a deletion in *Prf*) (Salmeron et al. 1994). Interestingly, whether they expressed *avrPto* or *avrPtoB* homologs or not, none of these 10 strains caused enhanced ion leakage when infiltrated into RG-pto11 or RG-prf3 leaves at the timepoints where we previously saw ion leakage from leaves of RG-PtoR (Fig. 1; at later timepoints, all leaves developed disease symptoms and showed enhanced ion leakage). Notably, statistically significantly lower conductivity levels were observed in RG-pto11 and RG-prf3 compared with RG-PtoR for those strains expressing *avrPto* or *avrPtoB* homologs (Fig. 1). In addition, there was no statistically significant difference among any of the three tomato lines in their response to *P. syringae* pvs. *coronafaciens* B142, *syringae* 61, or *macu-*

licola ES4326, which lack both *avrPto* and *avrPtoB* (Fig. 1; note that *P. syringae* pv. *maculicola* ES4326 carries truncated forms of *avrPtoB*). These observations indicated that a Pto/Prf-mediated gene-for-gene interaction is responsible for the rapid cell death (HR) caused by *avrPto*- or *avrPtoB*-expressing *P. syringae* pathovars in RG-PtoR.

Diverse *P. syringae* pathovars can infect tomato leaves, and Pto/Prf inhibit disease symptom formation.

Because these *P. syringae* pathovars are considered nonhost pathogens of tomato and we had used a high titer of bacteria in our earlier experiments, we were surprised at the lack of a non-host-like HR in RG-pto11 and RG-prf3 (Goodman and Novacky 1994). To further investigate the response of tomato lines with and without *Pto* or *Prf* to these *P. syringae* pathovars, we vacuum infiltrated tomato leaves with a low titer (2×10^4 CFU/ml) of the different strains and monitored the progress of disease development. Unexpectedly, disease symptoms developed on leaves of tomato lines RG-prf3 and RG-pto11 4 days after inoculation with each of the 10 strains (Fig. 2; Table 2; Supplementary Figure S3; data not shown for all RG-pto11 interactions). No symptoms occurred on leaves of RG-PtoR inoculated with the *avrPto*- or *avrPtoB*-expressing strains (Table 2). *P. syringae* pvs. *coronafaciens* B142, *maculicola* ES4326, and *syringae* 61, which did not trigger an HR on RG-PtoR, caused disease symptoms on leaves of RG-PtoR as well as on RG-pto11 and RG-prf3 (Table 2). It is possible that vacuum infiltration of leaves bypasses some plant defenses against these *P. syringae* pathovars (Melotto et al. 2006). To examine this possibility, we prepared suspensions of a subset of four of the pathovars (containing approximately 7×10^7 CFU/ml) and dip-inoculated RG-prf3 plants. Five days later, distinct disease symptoms developed on leaves of each of the inoculated plants (Supplementary Figure S4).

The lesions caused by any particular pathovar had virtually identical characteristics on RG-pto11 and RG-prf3, although the overall symptoms were often more severe on RG-prf3 than

Fig. 2. Disease symptoms caused by various *Pseudomonas syringae* pathovars on leaves of tomato line RG-prf3. Leaves were infiltrated with the *P. syringae* strains indicated at 2×10^4 CFU/ml. Photographs were taken 6 days after inoculation. Insets show close-up image of a single representative lesion taken with dissecting microscope. The black bar shown in the inset picture of *P. syringae* pv. *tomato* DC3000 represents 1 mm and the scale is the same for all photographs.

on RG-pto11, especially in cases where the pathovars expressed AvrPto or AvrPtoB (Table 2). Interestingly, the appearance of the lesions varied greatly among the different *P. syringae* pathovars. For example, *P. syringae* pv. *coronafaciens* B142 caused pinpoint necrotic lesions without chlorosis; *P. syringae* pv. *syringae* 61 caused pale yellow spots with a central, light-brown necrotic lesion; *P. syringae* pv. *glycinea* F117 caused necrotic lesions with a large chlorotic halo; and *P. syringae* pv. *angulata* 9 caused angular leaf spots reminiscent of the disease this pathovar causes on tobacco (Fig. 2). These observations indicate that many presumed nonhost *P. syringae* pathovars are capable of infecting tomato. Furthermore, they suggest that the broad array of disease symptoms caused by *P. syringae* pathovars on their normal hosts are due largely to the particular combination of virulence factors they express rather than to specific morphological or physiological characteristics of their plant hosts.

Populations of the *P. syringae* pathovars increase significantly in tomato leaves, and Pto/Prf can restrict this growth.

We next examined the extent to which the *P. syringae* pathovars are able to grow in leaves of the different tomato lines. Plants were vacuum infiltrated with a low-titer suspension (10^4 CFU/ml, corresponding to approximately 2×10^2 CFU/cm² of leaf area) of the individual *P. syringae* pathovars and bacterial populations were measured 4 days later (Fig. 3; Supplementary Table S2). In the tomato line lacking Prf, we observed that the strains attained an average population of approximately 10^5 CFU/cm² or higher at this timepoint. A nonpathogenic *hrp*⁻ and *hrc*⁻ mutant of DC3000 typically reaches no higher than 10^3 CFU/cm² in susceptible tomato leaves (Fouts et al. 2003) and an avirulent strain such as DC3000 on RG-PtoR grows to approximately 10^4 CFU/cm² (Fig. 3). Therefore, these data indicate that these pathovars can successfully multiply in tomato. Compared with RG-prf3, growth in RG-pto11 leaves was significantly less for 6 of the 10 pathovars (Fig. 3). The control strain, *P. syringae* pv. *tomato* DC3000, reached above 10^7 CFU/cm² in RG-prf3 at day 4, suggesting that this strain carries additional virulence factors associated with its specific adaptation on tomato (Fig. 3).

All but one of the pathovars (i.e., *P. syringae* pv. *phaseolicola* 3121) which express either an *avrPto* or *avrPtoB* gene

attained population levels significantly lower in the *Pto/Prf*-expressing line, RG-PtoR, than in RG-prf3 (Fig. 3). *P. syringae* pv. *maculicola* ES4326 also reached a lower population level on RG-PtoR compared with RG-prf3, although we had observed earlier that this strain did not trigger an HR on RG-PtoR and was able to cause disease symptoms on this line. *P. syringae* pv. *maculicola* ES4326 has two *avrPtoB*-related sequences, although they are both truncated (Guttman et al. 2002) and it is possible their proteins are either expressed in low abundance, unstable, or possibly partially masked by other effectors in *P. syringae* pv. *maculicola* ES4326. Interestingly, six of the eight pathovars which grew less well on RG-PtoR also reached a significantly lower population size on RG-pto11 compared with RG-prf3 (*P. syringae* pvs. *angulata* 9, *maculicola* ES4326, *lachrymans* 3, *syringae* B728a, *mori*, and *tabaci* 11528). *Pto* is a member of a small gene family and this difference might indicate that another *Pto* family member, whose activity is mediated through *Prf*, is able to recognize AvrPto or AvrPtoB homologs present in these six strains.

For *P. syringae* pv. *coronafaciens* B142 and *P. syringae* pv. *syringae* 61, which do not contain *avrPto* or *avrPtoB*, there was no significant difference in bacterial populations or disease symptoms among the tomato lines. This result is important because it demonstrates that these tomato lines do not differ in their general (basal) defense against these *P. syringae* pathovars.

We present a summary of our observations and define a “compatible” (C) interaction as one in which no HR is observed, disease symptoms form, and bacterial growth is higher compared with RG-PtoR (or when bacterial growth was similar in all three tomato genotypes) in Table 2. A “partially compatible” (pC) interaction refers to certain interactions in which no HR was observed and disease symptoms formed; however, bacterial growth was not statistically significantly different than growth in RG-PtoR. In an “incompatible” interaction, early cell death is observed, no disease forms, and there is less bacterial growth compared with RG-prf3.

Our data show a striking correlation between the presence of AvrPto or AvrPtoB in a strain and its incompatibility with RG-PtoR (Table 2). With one exception (*P. syringae* pv. *phaseolicola* NPS3121), all of these strains could form a compatible interaction with RG-prf3. The situation is less clear with the partially compatible interactions involving RG-pto11. In these cases, AvrPto- or AvrPtoB-expressing strains did not cause early cell

Table 2. Summary of the relationship between the gene-for-gene interaction and host–pathogen interaction for *Pseudomonas syringae* isolates

Isolate	<i>avrPto</i> or <i>avrPtoB</i>	Ion leakage ^v			Disease ^w			Growth ^x			Interaction ^y		
		PtoR	pto11	prf3	PtoR	pto11	prf3	PtoR	pto11	prf3	PtoR	pto11	prf3
<i>angulata</i> Pan9	<i>avrPtoB</i>	+	–	–	–	+	++	a	a	b	I	pC	C
<i>coronafaciens</i> B142	Neither	–	–	–	+	+	+	a	a	a	C	C	C
<i>glycinea</i> F117	<i>avrPtoB</i>	+	–	–	–	+	+	a	b	b	I	C	C
<i>lachrymans</i> Pla3	Both	+	–	–	–	+	++	a	a	b	I	pC	C
<i>mori</i> PDDCC4331	Both	+	–	–	–	+	+	a	a	b	I	pC	C
<i>maculicola</i> ES4326	Neither ^z	–	–	–	+	+	+	a	a	b	C	C	C
<i>phaseolicola</i> NPS3121	Both	+	–	–	–	+	+	a	a	a	I	pC	pC
<i>syringae</i> 61	Neither	–	–	–	+	+	+	a	a	a	C	C	C
<i>syringae</i> B728a	Both	+	–	–	–	+	++	a	b	c	I	C	C
<i>tabaci</i> 11528	<i>avrPtoB</i>	+	–	–	–	+	++	a	a	b	I	pC	C
<i>tomato</i> DC3000	Both	+	–	–	–	+	+	a	b	b	I	C	C

^v Symbols: + = enhanced electrolyte leakage from leaves associated with the hypersensitive response and – = no enhanced electrolyte leakage. PtoR = Rio Grande (RG) PtoR (*Pto/Pto*, *Prf/Prf*), pto11 = RG-pto11 (*pto11/pto11*, *Prf/Prf*), and prf3 = RG-prf3 = (*Pto/Pto*, *prf3/prf3*).

^w Symbols: ++ = severe disease symptoms observed, + = disease symptoms, and – = no disease symptoms observed.

^x Same letter assigned for tomato lines within a pathovar indicates no difference at a significance level of 5%.

^y I = incompatible interaction: enhanced ion leakage; no disease, and, with one exception (NPS3121), less growth compared with RG-prf3. pC = partially compatible interaction: no enhanced ion leakage and disease symptoms were observed; however, bacterial growth was not different than RG-PtoR. C = compatible interaction: for *P. syringae* pv. *coronafaciens* B142, *P. syringae* pv. *maculicola* ES4326, and *P. syringae* pv. *syringae* 61, similar results with all assays were observed with all tomato genotypes. For the other strains, on RG-pto11 and RG-prf3, C indicates no enhanced ion leakage and disease symptoms were observed; bacterial growth was statistically significantly higher than in RG-PtoR.

^z Pma ES4326 expresses *hopPmaL* and *holPmaN*, C-terminus truncated forms of AvrPtoB (Guttman et al. 2002).

death and disease symptoms formed; however, nevertheless, the strains were restricted in their growth to levels not different from that in RG-PtoR. This partial compatibility may be due to other *Pto* family members which recognize certain forms of AvrPto or AvrPtoB in a Prf-dependent manner and activate weaker defense responses (Abramovitch et al. 2003). In summary, our data suggest that a recognition event mediated by various *Pto* family members in concert with the Prf protein plays a role in restricting the ability of AvrPto- or AvrPtoB-expressing *P. syringae* pathovars from infecting tomato leaves.

Expression of AvrPto and AvrPtoB in *P. syringae* 61 restricts ability of this strain to cause disease in Pto/Prf-expressing tomato.

To further confirm that these *P. syringae* pathovars are growing in tomato leaves and that their growth can be restricted by Pto/Prf, we selected one pathovar, *P. syringae* pv. *syringae* 61, which lacks AvrPto and AvrPtoB, for further experiments. We cloned the *avrPto* genes from *P. syringae* pv. *tomato* JL1065 or the *avrPtoB* gene from *P. syringae* pv. *tomato* DC3000 into a broad host-range vector and introduced them individually into *P. syringae* pv. *syringae* 61. Inoculation of a control *P. syringae* pv. *syringae* 61 strain carrying an empty vector caused the formation of pale yellow spots on all of the tomato lines and the strain grew to approximately 10^5 CFU/cm² (Fig. 4). However, *P. syringae* pv. *syringae* 61 strains expressing either of the effectors elicited an HR (not shown) on RG-PtoR leaves, caused no disease symptoms, and grew to statistically significantly lower population levels on this tomato line (Fig. 4). All of the strains caused disease symptoms and grew to similar levels on RG-pto11 and RG-prf3, with the exception of *P. syringae* pv. *syringae* 61(*avrPtoB*), which appeared to be weakly recognized by RG-pto11, probably by another *Pto* family member expressed in this line. Therefore, these data further support the notion that *P. syringae* pv. *syringae* 61 can infect tomato and its growth can be restricted by the presence of the Pto/Prf-AvrPto/AvrPtoB gene-for-gene interaction.

DISCUSSION

The large number of pathovars of *P. syringae* provides an extraordinary example of host-range specificity in plant-pathogen interactions (Rudolph 1995). The molecular mechanisms underlying pathogen adaptation to a specific host and nonhost resistance are poorly understood and our data shed light on two forces that appear to contribute to these phenomena. First, we found that many presumed nonhost *P. syringae* pathovars can, in fact, cause disease symptoms on tomato and attain fairly high population levels. However, they do not reach the population levels of *P. syringae* pv. *tomato* strains. This suggests that these *P. syringae* pathovars may lack specific virulence factors, including perhaps key type III effectors, which are expressed by *P. syringae* pv. *tomato* and specially adapted for growth on tomato leaves. A second mechanism, which our work suggests limits growth of *P. syringae* pathovars in tomato, is recognition of the type III effectors AvrPto and AvrPtoB by *Pto* family members and Prf. The pathway activated by *Pto* family members acting through Prf confers strong host resistance to AvrPto- or AvrPtoB-expressing races of *P. syringae* pv. *tomato*, which limits pathogen growth and inhibits formation of bacterial speck symptoms. Because functional *Pto* and Prf genes are widespread among wild tomato species and other solanaceous species and *avrPto* or *avrPtoB* genes are present in many *P. syringae* pathovars, our data suggest that recognition mediated by *Pto* family members and Prf might play a broad role in restricting the ability of many *P. syringae* pathovars from infecting these plants in natural settings.

Nonhost resistance typically is defined as resistance in an entire plant species against all members of a given pathogen taxon (Goodman and Novacky 1994; Mysore and Ryu 2004; Thordal-Christensen 2003). In the case of *P. syringae*, the infrasubspecific classification “pathovar” is used to account for the specific host ranges which organisms in this species display (Agrios 1997; Young et al. 1992). Thus, tomato is considered to be a nonhost for *P. syringae* pathovars except for *P. syringae* pv. *tomato* and, to a lesser extent, *P. syringae* pv. *syringae* (Jones 1991). However, we have shown here that many *P. syringae* pathovars actually have the fundamental ability to infect tomato. These infections, though, can be severely restricted by the presence in the plant of a functional Pto or Prf pathway and in the pathovar of either AvrPto or AvrPtoB. Such gene-for-gene interactions originally were defined as determinants of pathogen host range at the pathovar-species and race-

Fig. 3. Growth of *Pseudomonas syringae* strains in tomato leaves. The *P. syringae* strains indicated were vacuum infiltrated (10^4 CFU/ml) into leaves of Rio Grande tomato lines RG-PtoR (black), RG-pto11 (hatched), or RG-prf3 (gray) and bacterial populations were measured 4 days later. Bars indicate standard errors. Experiments presented in this figure were performed twice with similar results. The data from the two experiments were treated as replicates and were analyzed using Duncan's multiple range test. Means with the same letter assigned for each pathovar are not different at a significance level of 5%.

cultivar levels (Ellingboe 1976; Lorang et al. 1994). However, our data indicate that *R*-gene-mediated resistance also might govern pathogen host range at the pathovar–cultivar level.

Pto is a member of a clustered gene family consisting of five or six members depending on the tomato species (Pedley and Martin 2003; Riely and Martin 2001). *Pto* itself is not present in any known accession of cultivated tomato (*S. lycopersicum*); however, paralogous members of the family reside at the same chromosomal region of this species. In this regard, it is interesting that RG-pto11, despite having a mutation creating a nonfunctional *Pto* kinase, still appeared to retain the ability to restrict growth of certain *P. syringae* pathovars expressing AvrPto or AvrPtoB (note that the RG lines all have the *S. pimpinellifolium* *Pto* haplotype). This observation suggests that other *Pto* family members are able to recognize forms of these type III effectors present in these pathovars. In this regard, we have reported previously that a Prf-dependent resistance referred to as Rsb can activate defenses in response to certain forms of AvrPtoB (Abramovitch et al. 2003). These considerations raise the possibility that cultivated tomato (and perhaps all species of tomato) may express *Pto*-like genes which confer some resistance to *avrPto*- and *avrPtoB*-expressing *P. syringae* pathovars. Critical testing of this potentially more general involvement of *Pto* family members in nonhost resistance would require an *S. lycopersicum* line lacking a functional *Prf* gene, and such a line is not presently available (note that the

RG-prf3 line we used carries the haplotype from *S. pimpinellifolium*).

Further support for gene-for-gene interactions playing an important role in nonhost resistance comes from recent work with *P. syringae* pv. *tomato* DC3000 and its nonhost *N. benthamiana*. In that system, it has been found that one DC3000 type III effector elicits an SGT1-mediated HR in *N. benthamiana* leaves (Wei et al. in press). Deletion of this effector from DC3000 allows the pathogen to grow and cause disease on *N. benthamiana* leaves. Interestingly, deletion of certain additional effectors from this now-virulent DC3000 strain mitigates its virulence, not only on *N. benthamiana* but also on tomato and *Arabidopsis*, suggesting that the activity of at least some effectors is not plant species-specific but, rather, broadly adapted to plant tissues (Wei et al. in press).

Although we have shown that certain *P. syringae* pathovars can infect tomato in an experimental setting, these pathovars apparently have not been observed to cause disease on tomato in agricultural settings and it is possible that such infections do not occur. Alternatively, these pathovars might infect tomato plants in the field but, because of the low population levels they attain in leaves compared with *P. syringae* pv. *tomato* strains, they may fail to produce noticeable disease symptoms. If such “low-grade” infections do occur, it is possible they could have subtle detrimental effects on tomato yields. This possibility could be testable, given that *P. syringae* pathovars

Fig. 4. Response of tomato leaves to *Pseudomonas syringae* pv. *syringae* 61 expressing *P. syringae* pv. *tomato* DC3000 alleles of *avrPto* or *avrPtoB*. **A**, Leaves were vacuum infiltrated with the strains indicated at 2×10^4 CFU/ml and photographs of 1-cm² areas were taken through a dissecting microscope 4 days later. **B**, *P. syringae* pv. *syringae* 61 strains expressing the genes indicated were vacuum infiltrated (2×10^4 CFU/ml) into leaves of Rio Grande tomato lines RG-PtoR, RG-pto11, or RG-prf3 and bacterial populations were measured 4 days later. Bars indicate the standard errors; ev = empty vector (pCPP45). Experiments presented in this figure were performed twice with similar results. The data from the two experiments were treated as replicates and were analyzed using Duncan's multiple range test. Means with the same letter assigned for each pathovar are not different at a significance level of 5%.

are widespread in the environment and the *Pto* gene is now deployed in 16 of the top 20 tomato processing cultivars and on approximately 60% of tomato acreage in California (Pedley and Martin 2003).

In the early days after *R* genes were molecularly cloned, it was hoped they might function in diverse unrelated plant species and, therefore, be broadly useful as a transgenic crop protection strategy (Martin et al. 2003). However, *R* genes have been found to work only among plants within the same family from which they were isolated (e.g., *Rxo1*) (Zhao et al. 2005)—possibly reflecting the requirement of other family-specific proteins as part of the recognition complex (Martin 1999). In addition, the recognition specificity of *R* proteins for effector molecules present only in pathogens adapted to the species from which the *R* gene was isolated may limit the usefulness of a transgenic application of *R* genes. However, because *avrPto* and *avrPtoB* are now known to be widely distributed among *P. syringae* pathovars, the transgenic expression of *Pto* in other solanaceous species might be worthwhile. For example, our work suggests that transgenic expression of *Pto* in tobacco might be an effective control for blackfire and wildfire disease in tobacco if *avrPto* and *avrPtoB* are found to be present in common field isolates species of *P. syringae* pvs. *angulata* and *tabaci*.

We found that, despite the ability of many *P. syringae* pathovars to grow in tomato leaves, they did not reach the high population levels of *P. syringae* pv. *tomato*. It is possible that certain type III effectors in these pathovars are weakly recognized by other *R* proteins in tomato, thus restricting their growth. Alternatively, it is possible that *P. syringae* pv. *tomato* strains have evolved specific effectors for colonization and growth in tomato tissues and these effectors are lacking in other *P. syringae* pathovars. A similar explanation was put forth recently for *P. syringae* pv. *syringae* B728a based on the activities of various type III effectors from that strain on different plant hosts and nonhosts (Vinatzer et al. 2006). There currently are few well-characterized examples of such host range-broadening type III effectors (e.g., HsvB and HsvG) (Nissan et al. 2006) and the availability of the complete set of approximately 30 type III effectors from *P. syringae* pv. *tomato* DC3000 now will allow a concerted strategy of transforming into a nonadapted *P. syringae* pathovar each of the DC3000 effectors to test for ones that promote growth specifically in tomato leaves.

Finally, the pathovars we examined cause distinct disease symptoms on their usual host plants, denoted by such names as angular leaf spot (*P. syringae* pvs. *angulata* and *lachrymans*), halo blight (*P. syringae* pv. *phaseolicola*), and wildfire (*P. syringae* pv. *tabaci*). It is formally possible that the physiological or structural features of the host leaf play an important role in the formation of the disease symptoms caused by these pathogens. However, we found that each of the pathovars we examined displayed distinct symptoms which, in many cases, were reminiscent of symptoms that occur in the normal host (e.g., *P. syringae* pv. *angulata* caused angular leaf spots on tomato). These observations suggest that the unique array of virulence factors (e.g., toxins and perhaps type III effectors) which each pathovar expresses plays a more important role in the appearance of disease symptoms than does the specific physiological or structural features attributes of the plant host.

MATERIALS AND METHODS

DNA, RNA, and protein gel blot analysis.

Genomic DNA (2 µg) digested with *Cla*I and total RNA (20 µg) isolated from bacteria grown in rich medium and *hrp*-minimal medium were used for DNA and RNA gel blot analysis, respectively, following procedures described previously (Fouts et al. 2002; Lin and Martin 2005). Expression of *AvrPto*

and *AvrPtoB* protein was detected using anti-*AvrPto* and anti-*AvrPtoB* antibodies following standard techniques for protein gel blot analysis (Sambrook et al. 1989).

A 0.7-kb fragment containing the *hrp*-box from *avrPto*, the open reading frame of *avrPto*, and the 200-bp downstream sequence of *avrPto* was polymerase chain reaction amplified using the forward primer 5'-GGTGTGATGGAAGCTTTTCCTGCT C-3' and reverse primer 5'-TCAG-GGGACTATTCTAAAGC-3', and cloned into pCR2.1 (Invitrogen Corp., Carlsbad, CA, U.S.A.). The *Kpn*I/*Xba*I fragment from pCR2.1 was cloned into the broad-host-range vector, pCPP45, to create *pavrPto*. *pavrPto* and previously constructed *pavrPtoB* (Lin and Martin 2005) were introduced into *P. syringae* pv. *syringae* strain 61 by triparental mating to obtain strains 61(*avrPto*) and 61(*avrPtoB*). Empty vector (ev; pCPP45) also was introduced into *P. syringae* pv. *syringae* 61 to generate 61(ev) as a control.

Ion leakage assays.

Leaf disks excised from the area infiltrated with the *Pseudomonas* strains (optical density at 600 nm = 0.05; approximately 2×10^7 CFU/ml) at the times indicated were incubated in water at room temperature for 1 h. This solution was used to measure sample conductivity (in microSiemens) with an Acorn series CON5 meter (Oakton Instruments, Vernon Hills, IL, U.S.A.). Leaf disks taken from areas without infiltration with *Pseudomonas* strains were used as a blank control to normalize sample conductivity. Ion leakage is represented as the conductivity difference between the samples and the blank control. Experiments were performed twice and the combined data were analyzed using Duncan's multiple range test.

Disease assays and *Pseudomonas* population measurements.

Tomato (*S. lycopersicum*) plants were grown as described previously (Kim et al. 2002). Three genotypes of tomato cv. Rio Grande (RG) were used in this study: RG-PtoR (*Pto/Pto*, *Prf/Prf*), RG-ptol1 (*ptol1/ptol1*, *Prf/Prf*), and RG-prf3 (*Pto/Pto*, *prf3/prf3*) (Salmeron et al. 1994). RG-PtoR carries the *Pto/Prf* region from *S. pimpinellifolium* introgressed into an *S. lycopersicum* background. RG-ptol1 and RG-prf3 were derived by mutagenesis of RG-PtoR (Salmeron et al. 1994).

P. syringae strains were grown in King's B (KB) medium (King et al. 1954) at 30°C. For pathogenicity assays, bacterial suspensions of 2×10^4 CFU/ml in 10 mM MgCl₂ and 0.002% Silwet L-77 (Crompton Co., Middlebury, CT, U.S.A.) were vacuum infiltrated into leaves of 4-week-old plants. Disease development and bacterial populations were monitored every other day after inoculation. Bacteria recovered from plants were plated on KB medium containing rifampicin (100 µg/ml). Fully expanded leaflets were used for recovery of bacteria (Lin and Martin 2005). Experiments were performed twice and the combined data were analyzed using Duncan's multiple range test. For the dip-inoculation experiments, approximately 7×10^7 CFU/ml in 10 mM MgCl₂ and 0.025% Silwet L-77 was used and the photographs were taken 5 days after dipping.

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