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Nonhost Resistance of Tomato to the Bean Pathogen *Pseudomonas syringae* pv. *syringae* B728a Is Due to a Defective E3 Ubiquitin Ligase Domain in $AvrPtoB_{B728a}$

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Abstract

The bean pathogen *Pseudomonas syringae* pv. *syringae* B728a expresses homologs of the type III effectors AvrPto and AvrPtoB, either of which can trigger resistance in tomato cultivars expressing *Pto* and *Prf* genes. We found that strain B728a also elicits nonhost resistance in tomato cultivars VFNT Cherry and Moneymaker that lack *Pto* but express other members of the *Pto* family (e.g., *SlFen* and *SlPtoC*). Here, we show that the AvrPtoB homolog from B728a, termed $AvrPtoB_{B728a}$ (also known as HopAB1), is recognized by 'VFNT Cherry' and 'Moneymaker' when the effector is expressed in *P. syringae* pv. *syringae* 61, a strain lacking the *avrPto* or *avrPtoB* homolog. Using a gene-silencing approach, this recognition was shown to involve one or more Pto family members and Prf. $AvrPtoB_{B728a}$ interacted with SlFen, SlPtoC, and SlPtoD, in addition to Pto, in a yeast two-hybrid assay. In *P. syringae* pv. *tomato* DC3000, the C-terminal domain of AvrPtoB is an E3 ubiquitin ligase that ubiquitinates Fen, causing its degradation and leading to disease susceptibility. Although the C-terminal domain of $AvrPtoB_{B728a}$ shares 69% amino acid identity with that of AvrPtoB, we found that it has greatly reduced E3 ligase activity and is unable to ubiquitinate Fen in an in vitro ubiquitination assay. Thus, the nonhost resistance of 'VFNT Cherry' and 'Moneymaker' to B728a appears to be due to recognition of $AvrPtoB_{B728}$ as a result of the effector's reduced E3 ligase activity, which prevents it from facilitating degradation of a Pto family member. We speculate that the primary plant host of B728a lacks a Fen-like protein and that, therefore, the E3 ligase of $AvrPtoB_{B728}$ was unnecessary for pathogenicity and has diverged and become ineffective.

Continuously encountering attempted pathogen attack, plants have evolved different weapons, including preexisting and induced biochemical or structural defense mechanisms, to block or inhibit pathogen infection (Agrios 2005). Pathogens that manage to surmount physical barriers and enter plant tissues may be recognized extracellularly when conserved microbial molecules, termed pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway 1997), are perceived by transmembrane pattern recognition receptors. This perception results in activation of mitogen-activated protein kinase signaling pathways,

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induction of the expression of pathogenesis-related genes, production of reactive oxygen species and nitric oxide, and callose deposition to reinforce cell wall structure. These responses, termed PAMP-triggered immunity (PTI), act to prevent further bacterial growth and disease development (Iriti and Faoro 2007; Nicaise et al. 2009; Zipfel 2009).

The efficacy and significance of PTI appear to have been a driving force for successful pathogens to evolve virulence factors to overcome PTI, leading to effector-triggered susceptibility (ETS) (Jones and Dangl 2006). In *Pseudomonas syringae* pv. *tomato* DC3000, type III effectors suppress innate immunity by using specific enzyme activities (Abramovitch et al. 2006; Bretz et al. 2003; Espinosa et al. 2003; Fu et al. 2007; Lopez-Solanilla et al. 2004; Wang et al. 2010) or by blocking early defense gene transcription and signaling pathways (Boudsocq et al. 2010; He et al. 2006; Wang et al. 2010). In addition to their roles as suppressors of immunity-associated programmed cell death, some virulence factors may enhance plant susceptibility via modulating hormone signaling pathways (Chen et al. 2007; Cohn and Martin 2005; de Torres-Zabala et al. 2007; Zhao et al. 2003) or by suppressing microRNA pathways involved in plant immunity (Navarro et al. 2008). This phytopathogen can also facilitate apoplast invasion by inducing the reopening of stomata via the action of the phytotoxin coronatine (Melotto et al. 2006; Zhang et al. 2008).

To counter the effects of ETS caused by pathogens, some plants have evolved resistance (R) proteins to recognize type III effectors, by which a robust defense system, termed effector-triggered immunity (ETI), is initiated (Jones and Dangl 2006). ETI is also known as gene-for-gene resistance or cultivar-specific resistance, in which plant R proteins interact with one or more host proteins, which act as a guardee or a decoy to detect the presence of an effector or a perturbation in the host cell caused by an effector (van der Biezen and Jones 1998; van der Hoorn and Kamoun 2008).

One of the best studied gene-for-gene interactions, tomato resistance against *P. syringae* pv. *tomato*, is determined by the interaction between the Pto kinase in tomato and AvrPto or AvrPtoB from *P. syringae* pv. *tomato* (Kim et al. 2002; Lin et al. 2006; Martin et al. 1993; Ronald et al. 1992; Salmeron and Staskawicz 1993). *P. syringae*, a γ-proteobacterium, is notorious for its ability to cause destructive diseases on a wide variety of plants. The impact of *P. syringae* in agriculture and its wide host range have made it one of the most important model pathosystems for understanding the molecular mechanisms underlying disease development and plant immunity (Cunnac et al. 2009; Lindeberg et al. 2008, 2009; Mansfield 2009; Staskawicz 2009; van der Biezen and Jones 1998). Like many other phytopathogenic bacteria, for successful infection of host tissues, *P. syringae* relies on a type III secretion system (T3SS) which translocates certain proteins, termed type III effectors, into the host cell (Cunnac et al. 2009; Greenberg and Vinatzer 2003; Hueck 1998; Tang et al. 2006).

The availability of genome sequences of several representative strains of *P. syringae*, as well as the development of new experimental methods, is accelerating the progress in the search for functions of type III effectors (Almeida et al. 2009; Feil et al. 2005; Joardar et al. 2005; Lindeberg et al. 2008; Studholme et al. 2009; Vinatzer and Greenberg 2007; Zwiesler-Vollick et al. 2002). It is now known that certain *P. syringae* effector proteins act to enhance pathogen growth and promote disease development by co-opting host processes to suppress the plant immune response (Dodds and Rathjen 2010).

Upon translocation into the host cell by the T3SS, AvrPto and AvrPtoB physically interact with Pto and trigger Prf-mediated resistance in tomato. Mutational studies revealed that interaction between Pto and AvrPto/AvrPtoB is required for this tomato resistance because disruption of the specific interaction between Pto and AvrPto or AvrPtoB abolishes their

ability to elicit Pto-dependent tomato resistance against *P. syringae* pv. *tomato* expressing AvrPto or AvrPtoB (Kim et al. 2002; Scofield et al. 1996; Tang et al. 1996). In addition to their ability to elicit Pto-dependent resistance in tomato, AvrPto and AvrPtoB also have virulence activity that enhances plant susceptibility to *P. syringae* pv. *tomato* (Gimenez-Ibanez et al. 2009; He et al. 2006; Lin et al. 2006; Rosebrock et al. 2007; Xiang et al. 2008; Xiao et al. 2007; Yeam et al. 2010; Zong et al. 2008).

Pto was originally identified in a wild relative of tomato, *Solanum pimpinellifolium*, and is a member of a multigene family that is clustered within a 60-kb region containing *SpimPtoA*, *Prf*, *Fen*, *SpimPtoC*, *SpimPtoD*, *Pto*, and the pseudogene *SpimPtoF* (Chang et al. 2002; Pedley and Martin 2003; Riely and Martin 2001). The Fen protein not only confers sensitivity to an organophosphorous insecticide, fenthion, but also accounts for the tomato resistance suppressed by AvrPtoB C-terminus (Rsb) phenotype. It is now known that the Fen protein is ubiquitinated by the E3 ligase domain present at the C terminus of AvrPtoB, leading to degradation of Fen and suppression of ETI triggered by the interactions between the N-terminal domain of AvrPtoB and Fen (Abramovitch et al. 2003, 2006; Rosebrock et al. 2007). Moreover, avoidance of Fen degradation via Pto phosphorylation of AvrPtoB within the E3 ligase domain has been reported to be responsible for the reinitiation of specific tomato resistance (Ntoukakis et al. 2009). These data suggest that an “arms race” between tomato expressing Fen and *P. syringae* resulted in the acquisition of the E3 ligase domain at the C terminus of AvrPtoB to overcome the Fen-mediated resistance and, subsequently, in the duplication of a Fen-like sequence with the ability to counteract the E3 ligase activity during the co-evolutionary process (Rosebrock et al. 2007).

Both Pto- and Fen-mediated resistances require Prf, a nucleotide-binding leucine-rich-repeat protein (Mucyn et al. 2006; Salmeron et al. 1996). Heterocomplexes containing Prf and Pto or other Pto-like kinases were identified using stable or transient expression of these genes in *Nicotiana benthamiana* (Gutierrez et al. 2010), suggesting that, in tomato and *N. benthamiana*, Prf may form a regulatory switch with Pto-type kinases in the presence of AvrPto or AvrPtoB, controlling a specific immune signaling in an incompatible interaction (Gutierrez et al. 2010; Mucyn et al. 2006; Ntoukakis et al. 2009; Salmeron et al. 1994).

The fact that Pto- and Prf-dependent recognition of AvrPto and AvrPtoB homologs can restrict the ability of diverse *P. syringae* pathovars to infect tomato suggests that these “nonhost resistance” phenotypes are actually due to gene-for-gene recognition events (Lin and Martin 2007). It also implies that Pto- and Prf-mediated resistance might be a common defense mechanism in protecting tomato plants from attack by a wide array of bacterial pathogens (Lin and Martin 2007). Although functional *Pto* and *Prf* genes are widespread among wild tomato and other Solanaceous species, the *Pto* allele has not been found in any accessions of cultivated tomato (*S. lycopersicum*) (Chang et al. 2002; Martin et al. 1993; Riely and Martin 2001; Rose et al. 2005, 2007).

Here, we report that tomato cultivars VFNT Cherry and Moneymaker exhibit nonhost resistance to the bean pathogen *P. syringae* pv. *syringae* B728a but not to *P. syringae* pv. *syringae* 61. To investigate the basis of this resistance in tomato, putative effector genes of B728a (Feil et al. 2005; Greenberg and Vinatzer 2003; Guttman et al. 2002; Vinatzer et al. 2006) were individually expressed in strain 61 and screened for their ability to elicit defense on ‘VFNT Cherry’ and Moneymaker. Surprisingly, only *avrPtoB* from B728a was found to convert strain 61 to an avirulent strain on ‘VFNT Cherry’ and ‘Moneymaker’ tomato, neither of which expresses *Pto*. This resistance required Prf, and a Pto family member in tomato. Further investigation revealed that the C-terminal E3 ligase domain of $AvrPtoB_{B728a}$ has very weak E3 ligase activity and is unable to ubiquitinate Fen in an in vitro assay. This diminished E3 ligase activity likely hinders the ability of $AvrPtoB_{B728a}$ to

cause degradation of a Pto family member in 'VFNT Cherry' and Moneymaker, thereby explaining the nonhost resistance to B728a in these tomato lines.

RESULTS

Tomato cultivars that do not express *Pto* are resistant to *P. syringae* pv. *syringae* B728a but not to *P. syringae* pv. *syringae* 61

We discovered that 'VFNT Cherry' and 'Moneymaker' tomato, which do not have *Pto*, are resistant to the bean pathogen *P. syringae* pv. *syringae* B728a but not to strain 61 (Supplementary Fig. S1). To investigate the basis of this resistance in tomato, we examined putative effector genes of B728a that had been identified by bioinformatic analysis or functional screens (Feil et al. 2005; Greenberg and Vinatzer 2003; Guttman et al. 2002; Lin and Martin 2007; Vinatzer et al. 2006). This analysis revealed that B728a expresses homologs of *avrPto* and *avrPtoB* whereas strain 61 does not have either of these effector genes.

Ectopic expression of $avrPtoB_{B728a}$ but not $avrPto_{B728a}$ allows strain 61 to be recognized by 'VFNT Cherry' and 'Moneymaker' tomato

AvrPto and AvrPtoB are the only two type III effectors known to elicit resistance to *P. syringae* pv. *tomato* in tomato cultivars, such as 'Rio-Grande PtoR' (RG-PtoR), which express Pto and Prf (Kim et al. 2002; Ronald et al. 1992). Homologs of these two effector proteins, which occur in many other *P. syringae* pathovars, also elicit resistance in *Pto*- and *Prf*-expressing tomato cultivars (Lin and Martin 2007; Lin et al. 2006; Nguyen et al. 2010). To examine whether these effectors elicit resistance in VFNT Cherry, a broad-host-range vector expressing each effector was introduced into strain 61, and the transformants were used in an inoculation assay. As expected, the *avrPto* and *avrPtoB* (*hopAB*) homologs from strain B728a (called $avrPto_{B728a}$ and $avrPtoB_{B728a}$ hereafter) allowed strain 61 to be recognized in leaves of RG-PtoR expressing the *Pto* and *Fen* genes, and this resistance was *Prf* dependent (Fig. 1A). However, unexpectedly, strain 61 expressing $avrPtoB_{B728a}$ was also recognized by 'VFNT Cherry' and 'Moneymaker' (Fig. 1A). The bacterial numbers in leaves of RG-PtoR, VFNT Cherry, and 'Moneymaker' 4 days after infiltration with designated *Psy*61($avrPtoB_{B728a}$) were approximately 10^4 CFU/cm^2 but reached 10^7 to 10^8 CFU/cm^2 in the susceptible RG-prf3 line (Fig. 1B). Thus, although both $avrPto_{B728a}$ and $avrPtoB_{B728a}$ elicit Pto- and Prf-dependent resistance in RG-PtoR, only $avrPtoB_{B728a}$ elicits resistance in 'VFNT Cherry' and 'Moneymaker'.

Deletion of *avrPtoB* but not *avrPto* from strain B728a abolishes its ability to elicit resistance on VFNT Cherry

To confirm that $avrPtoB_{B728a}$ is the only effector in *P. syringae* pv. *syringae* B728a that elicits resistance in VFNT Cherry, marker-exchange mutagenesis was performed to replace $avrPto_{B728a}$ and $avrPtoB_{B728a}$ with the *neo* gene and ΩSp/Sm, respectively (Supplementary Fig. S2). The single mutants were designated *Psy*B728aΔ*avrPto* and *Psy*B728aΔ*avrPtoB* while the double mutant is referred to as *Psy*B728aΔ*avrPto*Δ*avrPtoB*. The deletions of *avrPto* or *avrPtoB* in B728a in these mutants were confirmed by DNA gel blotting. The growth rates of *Psy*B728aΔ*avrPto* and *Psy*B728aΔ*avrPtoB* in liquid King's B (KB) medium containing rifampicin were the same as the wild type (data not shown).

Next, the mutants *Psy*B728aΔ*avrPto*, *Psy*B728aΔ*avrPtoB*, or *Psy*B728aΔ*avrPto*Δ*avrPtoB* at 2×10^4 CFU/ml were individually inoculated into leaves of 'VFNT Cherry' by vacuum infiltration. Four days after inoculation, no symptoms were observed on leaves of 'VFNT Cherry' infiltrated with *Psy*B728a Δ*avrPto* or wild-type B728a. In contrast, *Psy*B728aΔ*avrPto* Δ*avrPtoB* and *Psy*B728aΔ*avrPtoB* caused bacterial speck-like symptoms

on leaves of 'VFNT Cherry' (Fig. 2A) (the same result was observed with Moneymaker; data not shown). Bacterial populations in the tomato leaves were measured at 4 days postinoculation to confirm the loss of recognition of *Psy*B728aΔ*avrPtoB* and *Psy*B728aΔ*avrPto*Δ*avrPtoB*. The bacterial population in the leaves of 'VFNT Cherry' infiltrated with the wild type or *Psy*B728aΔ*avrPto* were close to 100 CFU/cm^2 but increased to 10^5 to 10^6 CFU/cm^2 in tomato leaves infiltrated with *Psy*B728aΔ*avrPtoB* or *Psy*B728aΔ*avrPto*Δ*avrPtoB* (Fig. 2B). We also observed that AvrPto$_{B728a}$ contributes to bacterial virulence in tomato because *Psy*B728aΔ*avrPto* Δ*avrPtoB* grew less than *Psy*B728aΔ*avrPtoB* (Fig. 2B). The reduced bacterial populations of *Psy*B728aΔ*avrPto*Δ*avrPtoB* in susceptible tomato leaves could be restored when a broad-host-range vector carrying *avrPto$_{B728a}$* under its native promoter was transformed into this double mutant (Supplementary Fig. S3). Moreover, disruption of the avirulence activity of B728a due to deletion in *avrPtoB$_{B728a}$* was complemented when *avrPtoB$_{B728a}$* was expressed from a broad-host-range vector under its native promoter and this strain was inoculated on tomato. These results indicate that *avrPtoB$_{B728a}$* is responsible for eliciting resistance to B728a in 'VFNT Cherry' as well as Moneymaker.

It has been reported that strain B728a can also elicit Pto- and Prf-dependent resistance (Lin and Martin 2007). Hence, this tomato resistance was also evaluated, and only *Psy*B728a Δ*avrPto*Δ*avrPtoB* caused disease symptoms and showed increased bacterial numbers in leaves of RG-PtoR when bacteria were infiltrated at a level of 2×10^4 CFU/ml, implying that *avrPto$_{B728a}$* and *avrPtoB$_{B728a}$* function redundantly in B728a to elicit Pto-dependent resistance (Supplementary Fig. S4).

Resistance to B728a requires *Prf* in 'VFNT Cherry' and Moneymaker

The Pto and Fen kinases, originally identified in *S. pimpinellifolium*, are two well-known R proteins required for AvrPto/AvrPtoB-triggered immunity (Kim et al. 2002; Ronald et al. 1992) and the Rsb phenotype (Abramovitch et al. 2003; Rosebrock et al. 2007), respectively. Resistance conferred by each of these proteins requires Prf. Although there is no *Pto* allele in 'VFNT Cherry' or Moneymaker, these cultivars do have other members of the *Pto* family, referred to as *SlPtoA*, *SlFen (SlPtoB)*, *SlPtoC*, *SlPtoD*, and *SlPtoF*, as well as *Prf* (Chang et al. 2002). Therefore, we used virus-induced gene silencing (VIGS) to investigate the possibility that the resistance of 'VFNT Cherry' to B728a depends on a member of the *Pto* family or *Prf*.

Due to high DNA sequence similarity of *Prf* in tomato lines and the similarity among *Pto* family members, *Tobacco rattle virus* (TRV) constructs generated in previous studies were effective in silencing these genes in 'VFNT Cherry' (Ekengren et al. 2003). Four days after inoculation with B728a, speck-like symptoms started to develop on leaves of 'VFNT Cherry' silenced for either *Prf* (Fig. 3A) or members of the *Pto* family (Supplementary Fig. S5A), and bacterial populations increased 5- to 10-fold in these plants (Fig. 3B). The abundance of *Prf* transcripts was reduced in leaves showing symptoms upon infection with B728a (Fig. 3C). In addition, transcript abundance of *SlFen* (*SlPtoB*) and *SlPtoF* was decreased in leaves silenced for the *Pto* family members but transcript abundance of *SlPtoC* and *SlPtoD* was unaffected (note that *SlPtoA* is not detectably expressed in leaves). Inoculation of a 'Moneymaker' line that has a mutation in *Prf* also indicated a requirement of this gene for recognition of B728a (Fig. 3D and E). These data support the hypothesis that *Prf* and one or more *Pto* family members (*SlFen*, *SlPtoF*, or both) are involved in 'VFNT Cherry' resistance against B728a.

AvrPtoB$_{B728a}$ interacts with SlFen, SlPtoC, and SlPtoD of 'VFNT Cherry' in a yeast two-hybrid assay

'VFNT Cherry' tomato has five members of the *Pto* family (*SlPtoA*, *SlFen/SlPtoB*, *SlPtoC*, *SlPtoD*, and *SlPtoF;* it lacks *PtoE* [*Pto*]), each with 80 to 90% sequence homology to its ortholog in the *Pto* family present in *S. pimpinellifolium* (Fig. 4A). Moreover, each of the *Pto* family members except *SlPtoA* is expressed in leaves of 'VFNT Cherry' (Chang et al. 2002). It is known that AvrPto and AvrPtoB both physically interact with Pto in a yeast two-hybrid system, and this interaction is correlated with their ability to trigger resistance in tomato. Therefore, we tested possible interactions between the AvrPto or AvrPtoB homologs from B728a and each Pto family member from 'VFNT Cherry' to find clues as to which Pto family member is involved in AvrPtoB$_{B728a}$-triggered immunity.

AvrPtoB$_{B728a}$ interacted weakly with SlFen (SlPtoB) and SlPtoD and more strongly with SlPtoC (Fig. 4B). It also interacted with Pto and Fen from *S. pimpinellifolium*. It is noteworthy that, in contrast to AvrPtoB, AvrPtoB$_{B728a}$ was unable to mask its interaction with Fen, suggesting that it is unable to ubiquitinate this protein efficiently (Rosebrock et al. 2007). As expected, AvrPtoB and AvrPto$_{B728a}$ only interacted with Pto in a yeast two-hybrid assay. Western blot analysis indicated that all bait or prey proteins were expressed in the yeast cells (Fig. 4C and D). Therefore, these data suggested that AvrPtoB$_{B728a}$ might be recognized by SlFen, SlPtoC, or SlPtoD in 'VFNT Cherry' to elicit *R*-gene-mediated resistance.

AvrPtoB$_{B728a}$ has weaker E3 ligase activity than AvrPtoB

AvrPtoB$_{B728a}$ shares 52% amino acid sequence identity overall with AvrPtoB and, notably, has reasonably high sequence similarity to the E3 ligase domain of AvrPtoB (the amino acid sequences share 69% identity with and 73% similarity to that of AvrPtoB). It also has residues known in AvrPtoB to be required for ubiquitination activity (Supplementary Fig. S6A). Based on this similarity, it might be expected that the C-terminal domain of AvrPtoB$_{B728a}$ is an active E3 ligase, allowing AvrPtoB$_{B728a}$ to facilitate degradation of the tomato resistance protein Fen (Rosebrock et al. 2007). At the same time, the fact that 'VFNT Cherry' and 'Moneymaker' both recognize AvrPtoB$_{B728a}$ in a Prf-dependent manner might suggest that its E3 ligase activity is ineffective, thereby allowing a Pto family member in these cultivars to recognize B728a.

To assess AvrPtoB$_{B728a}$ E3 ligase activity, we purified AvrPtoB$_{B728a}$ and AvrPtoB-GST fusion proteins and used them in an in vitro ubiquitination assay in the presence or absence of E2 conjugating enzyme. AvrPtoB rapidly synthesized large amounts of high molecular weight polyubiquitin species (Fig. 5A). In contrast, AvrPtoB$_{B728a}$ showed greatly reduced E3 ligase activity. Although polyubiquitin accumulation reached a maximum after 5 min in the presence of AvrPtoB, AvrPtoB$_{B728a}$ synthesized much less even after 15 min of incubation.

To test whether our AvrPtoB$_{B728a}$ protein preparation contained possible inhibitors that might interfere specifically with the E3 ligase activity in this assay, we conducted a titration experiment. Mixing of AvrPtoB$_{B728a}$ with AvrPtoB protein in our assay conditions showed no decrease in AvrPtoB E3 ligase activity, ruling out the presence of an inhibitor in the AvrPtoB$_{B728a}$ preparation. Thus, the E3 ligase activity of the AvrPtoB$_{B728a}$ C-terminus was reduced compared with that of AvrPtoB. In conjunction with the yeast-two-hybrid analysis, this suggests that AvrPtoB$_{B728a}$ might be unable to degrade a Pto family member, possibly SlFen, efficiently enough, resulting in robust resistance in VFNT Cherry.

To test this hypothesis, we next subjected a purified maltose-binding-protein–Fen fusion protein (MBP-Fen) to a cross-ubiquitination assay using either AvrPtoB or AvrPtoB$_{B728a}$. Only AvrPtoB from *P. syringae* pv. *tomato* DC3000 was able to efficiently polyubiquitinate Fen in the presence of E2. In contrast, no ubiquitinated forms of MBP-Fen were detectable in the presence of AvrPtoB$_{B728a}$ (Fig. 5B). These observations support the hypothesis that the greatly reduced general E3 ligase activity of AvrPtoB$_{B728a}$ results in an inability to degrade cellular targets and, consequently, to suppress resistance mediated by a Pto family member in conjunction with Prf. The abundance of AvrPtoB proteins and Fen used in the above experiments is shown in Figure 5C.

DISCUSSION

The bean pathogen *P. syringae* pv. *syringae* can cause “syringae disease” on tomato (Jones 1991), and our observation that *S. lycopersicum* cultivars Moneymaker and VFNT Cherry recognize only one of two *P. syringae* pv. *syringae* strains suggested a race specificity for tomato resistance against this pathogen. By means of heterologous expression of strain B728a effectors in strain 61 and mutation analysis in B728a, we identified AvrPtoB$_{B728a}$ as the sole effector protein that is responsible for eliciting nonhost disease resistance to B728a in tomato. In addition, results from our yeast two-hybrid and VIGS experiments indicated that one or more members of the *Pto* gene family in ‘VFNT Cherry’ and ‘Moneymaker’ are responsible for this resistance to B728a. Importantly, we found that AvrPtoB$_{B728a}$ has weaker E3 ligase activity compared with AvrPtoB. This lower E3 ligase activity was found to be incapable of ubiquitinating the Fen kinase and, therefore, likely hinders the ability of AvrPtoB$_{B728a}$ to cause degradation of the responsible Pto family member, resulting in disease resistance.

Why do these two AvrPtoB homologs have such different ubiquitination activities despite the relatively high amino acid sequence similarities in their E3 ligase domains? One possibility is that they differ for certain amino acids that play a specific role in E3 ligase activity. In this regard, it is interesting that one of the three E2-binding sites (located at position 476) is a histidine in AvrPtoB$_{B728a}$ whereas, in AvrPtoB, it is phenylalanine. We used site-directed mutagenesis to modify this site in AvrPtoB$_{B728a}$ and observed that the AvrPtoB$_{B728a}$ (H476F) variant protein still elicited resistance in ‘VFNT Cherry’ against B728a (C.-F. Chien and N.-C. Lin, *unpublished data*). Thus, this single polymorphism in AvrPtoB$_{B728a}$ is not solely responsible for the reduced E3 ligase activity we observed. The second, more likely possibility is that the 36 residues that are different in the E3 ligase domains of the two proteins alter the overall conformation of the enzyme, interfering with protein folding or leading to gross changes hindering interaction with the E2 conjugating enzyme.

The presence of other Pto family kinases linked to Prf in ‘VFNT Cherry’ and ‘Moneymaker’ suggests that, through recruitment of different kinases, the functions of Prf can be expanded to detect several type III effectors to trigger specific defense responses (Gutierrez et al. 2010). In addition to SlPto, AvrPtoB$_{B728a}$ also interacts with Fen, suggesting that AvrPtoB$_{B728a}$ probably elicits both Fen- and Pto-dependent resistance in the resistant tomato line RG-PtoR. This is supported by the observation that more severe symptoms develop on tomato line RG-prf3 (a *Prf*-deficient line) than on RG-*pto11* (a *Pto*-deficient line) when they are inoculated with B728a (Lin and Martin 2007). The fact that AvrPtoB$_{B728a}$ triggers Pto-independent resistance provides additional evidence that the existence of multiple Pto-like genes is likely the result of evolution for recognition of a continuously evolving effector (AvrPtoB homolog in this case) to activate robust tomato immunity.

It is possible that $AvrPtoB_{B728a}$ is a transitional version of AvrPtoB during its evolution when it had acquired the C-terminal domain but had not yet developed E3 ligase activity. However, it is perhaps more likely that $AvrPtoB_{B728a}$ is a version of AvrPtoB that has lost its E3 ligase activity because its primary host (bean) lacks a Pto family member (e.g., Fen) that recognizes it and maintenance of this activity carries a metabolic cost (Martin 2012). This possibility is further supported by the existence of HopPmaL, a C-terminal truncated version of AvrPtoB that occurs in *P. syringae* pv. *maculicola* ES4326. This variant has no E3 ligase domain and yet retains its full virulence activity (Guttman et al. 2002; Lin et al. 2006).

Our data provide further support for the hypothesis that *R*-gene-mediated ETI is the mechanism that underlies nonhost resistance in many plant interactions with bacterial pathogens and possibly those involving other pathogens. We reported earlier that a broad array of *P. syringae* pathovars that are nonhost pathogens of tomato are, in fact, able to infect tomato if the *Prf* gene is mutated (Lin and Martin 2007). The basis for these nonhost interactions was found to be recognition of either an AvrPto or AvrPtoB protein in the diverse *P. syringae* pathovars. Another compelling example is the finding that mutation of a single effector gene, *hopQ1-1*, in the *P. syringae* pv. *tomato* DC3000 allows it to become a pathogen on the nonhost *N. benthamiana* (Wei et al. 2007). More recently, it has been reported that a single effector, HopAS1, in the *P. syringae* pv. *tomato* T1 is recognized by *Arabidopsis*, giving rise to the apparent nonhost resistance to this tomato pathogen (Sohn et al. 2012). As additional effector repertoires of bacterial pathogens are investigated, we predict that many more examples of such gene-for-gene interactions underlying nonhost resistance will be identified.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Strains of *P. syringae* were grown in KB medium at 28°C with appropriate antibiotics. *Escherichia coli* DH5α used for DNA maintenance and triparental mating was grown in Luria-Bertani (LB) medium at 37°C. *Agrobacterium tumefaciens* GV3101 was grown in LB medium at 28°C, unless indicated otherwise. The concentrations of antibiotics used in each selective medium were as follows: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; rifampicin, 100 μg/ml; spectinomycin (Sp) and streptomycin (Sm), 25 μg/ml; and tetracycline, 25 μg/ml.

DNA manipulation

Plasmid DNA was isolated using a High-Speed Plasmid DNA Purification Mini Kit (Geneaid Biotech Ltd., Taiwan). The Gel Extraction/PCR Clean-up Kit (Geneaid Biotech Ltd.) was utilized to recover DNA fragments from the agarose gel or the reaction mixture after polymerase chain reaction (PCR). Oligonucleotide primers (Supplementary Table S1) were synthesized at Genomics Biosci & Tech (Taipei, Taiwan) and sequencing was performed at Genomics Biosci & Tech with an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). DNA sequences were analyzed using the Vector NTI (Invitrogen Co, Carlsbad, CA, U.S.A.).

A broad-host-range vector expressing $avrPto_{B728a}$, p$avrPto_{B728a}$, was obtained by cloning a 0.7-kb *Kpn*I/*Xba*I fragment containing the *hrp*-box, open reading frame (ORF), and 0.2-kb downstream sequences of $avrPto_{B728a}$ into pCPP45. p$avrPto_{B728a}$ or $pAvrPtoB_{B728a}$ constructed previously (Lin et al. 2006) was then introduced into *P. syringae* pv. *syringae* 61 by triparental mating to generate *Psy*61($avrPto_{B728a}$) or *Psy*61($avrPtoB_{B728a}$) for further analysis.

Construction of pΔavrPto$_{B728a}$ and pΔAvrPtoB$_{B728a}$ for marker exchange mutagenesis

To delete *avrPto* in *Psy*B728, a 2.5-kb fragment containing the upstream region and a 2-kb fragment containing the downstream region of *avrPto* were PCR amplified using primer pairs B728amut1-F/B728mut1-R and B728mut2-F/B728amut2-R, respectively. A DNA fragment of the *nptII* gene without terminator was released from pCPP2988 (Alfano et al. 1996) digested with *Hin*dIII and *Xho*I. These three fragments were sequentially cloned into pBluescript II SK(−), and the *Kpn*I-*Xba*I fragment was subcloned into pRK415 to generate pΔavrPto$_{B728a}$, which was then transformed into B728a to screen for a *Psy*B728aΔ*avrPto*$_{B728a}$ mutant whose *avrPto* was exchanged with the *nptII* selectable marker. *pΔavrPtoB*$_{B728a}$ was constructed as pΔavrPto$_{B728a}$, because the *Kpn*I-*Bam*HI fragment containing an ΩSp fragment from pHP45ΩSp and the flanking regions of *avrPtoB*$_{B728a}$ amplified with primer pairs B728a-mt1/B728a-mt2 and B728a-mt3/B728a-mt4 was subcloned into pRK415. pΔavrPtoB$_{B728a}$ was then introduced into B728a and *Psy*B728aΔ*avrPto*$_{B728a}$ to generate *Psy*B728aΔ*avrPto*$_{B728a}$ and *Psy*B728aΔ*avrPto*$_{B728a}$Δ*avrPtoB*$_{B728a}$, whose *avrPtoB* loci were replaced by a ΩSp fragment which confers spectinomycin and streptomycin resistance to the marker-exchanged mutants. Marker-exchanged mutants *Psy*B728aΔ*avrPto*$_{B728a}$, *Psy*B728aΔ*avrPtoB*$_{B728a}$, and *Psy*B728aΔ*avrPto*$_{B728a}$Δ*avrPtoB*$_{B728a}$ were all confirmed using Southern blot analysis.

Pathogenicity assay

Tomato (*S. lycopersicum*) cultivars Rio-Grande, VFNT Cherry, and Moneymaker were grown in a growth chamber setting of 22°C, a 16-h photoperiod, and 70% relative humidity. Tomato inoculation and determination of bacterial populations in tomato leaves was performed using the procedure described previously (Lin and Martin 2005). Disease development was monitored every day and symptoms were photographed 7 days after inoculation. For clear observation of specks on leaves of 'VFNT Cherry' tomato, leaves were treated with Carnoy's solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid) overnight to remove chlorophylls, and preserved in storage buffer containing 5% acetic acid, 20% ethanol, and 20% glycerol before photographing.

VIGS

VIGS using strains of *A. tumefaciens* GV3101 containing pTRV2:*Pto* and pTRV2:*Prf* obtained previously was done by procedures described before (Ekengren et al. 2003). Plants were kept in the growth chamber for 2 to 3 weeks until the pathogenicity assay was implemented. To monitor bacterial population and silencing efficiency, each leaf disc collected with a cork borer was cut in half because one part was used for bacterial recovery and the other part was kept in liquid nitrogen for semi-quantitative reverse-transcription (RT)-PCR. RNA was extracted using the Trizol reagent (Invitrogen) and cDNA was synthesized with a Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). PCR was performed using primers specific to each *Pto* ortholog or all *Pto*-like sequences (designated as '*Pto*') and *Prf* (Ekengren et al. 2003), and expression of *EF-1α* was used as an internal control.

Yeast two-hybrid analysis

A LexA yeast two-hybrid system was used to test interactions between Pto family members and AvrPtoB, AvrPto$_{B728a}$, and AvrPtoB$_{B728a}$. A DNA fragment containing ORF of the *Pto* family members, including *SlPtoA*, *SlFen*, *SlPtoC*, *SlPtoD*, or *SlPtoF*, was PCR amplified from genomic DNA of 'VFNT Cherry' tomato with each gene-specific primer pair, digested with *Bam*HI, and cloned into *Bam*HI-polished pJG4-5 vector to generate prey constructs. A DNA fragment containing ORF of *avrPtoB*, *avrPto*$_{B728a}$, and *avrPtoB*$_{B728a}$ was cloned into the *Eco*RI/*Bam*HI site of pGilda (Clontech, Mountain View, CA, U.S.A.) to create a bait

construct. The bait and prey constructs subsequently were introduced into *Saccharomyces cerevisiae* EYG48 containing a reporter vector, and yeast cells containing both bait and prey constructs were streaked on complete medium dropout media containing raffinose/galactose and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside to investigate interactions between prey and bait proteins. pJG4-5::Pto and pJG4-5::Fen were used as positive controls.

In vitro ubiquitination assay

In vitro ubiquitination assays were performed as described (Andersen et al. 2004), with minor modifications. In brief, 10 μl of protein master mix containing 1 μg of purified glutathione-*S*-transferase–fusion protein (and, in the case of the SlFen cross-ubiquitination assays, 200 ng of purified MBP:Fen) (Rosebrock et al. 2007) was added to the reaction mixture containing 50 nM E1 (UBE1; BostonBiochem, Cambridge, MA, U.S.A.), 5 μM E2 (UbcH5a; BostonBiochem), and 10 μg of Ubiquitin (BostonBiochem) in reaction buffer (50 mM Tris [pH 7.5], 2 mM dithiothreitol, 5 mM ATP, and 5 mM $MgCl_2$) to a total of 30 μl and incubated at 30°C for the time indicated. The reaction was terminated by the addition of 30 μl of 2× Laemmli buffer and boiling at 98°C for 5 min. Proteins were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels (5 or 10%), followed by Western blotting onto polyvinylidene diflouride membranes. Proteins were visualized either by infrared imaging on an Odyssey system (LI-COR, Lincoln, NE, U.S.A.) using primary anti-Ubiquitin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) and secondary anti-mouse-680 (LI-COR) antibodies or using horseradish peroxidase-coupled secondary anti-mouse and anti-rabbit antibodies (Santa Cruz Biotechnology Inc.,) and Amersham ECL plus (GE Healthcare, Piscataway, NJ, U.S.A.) detection reagent. Primary rabbit anti-MBP antibody was purchased from Sigma-Aldrich (St Louis). Because of high signal intensities, protein samples had to be diluted 1/20 in Laemmli buffer prior to SDS-polyacrylamide gel electrophoresis [spelled “PAGE”] in the case of horseradish peroxidase detection. To show equal loading, the remaining master mix for each sample mix was resolved on a separate gel and stained with Coomassie Brilliant Blue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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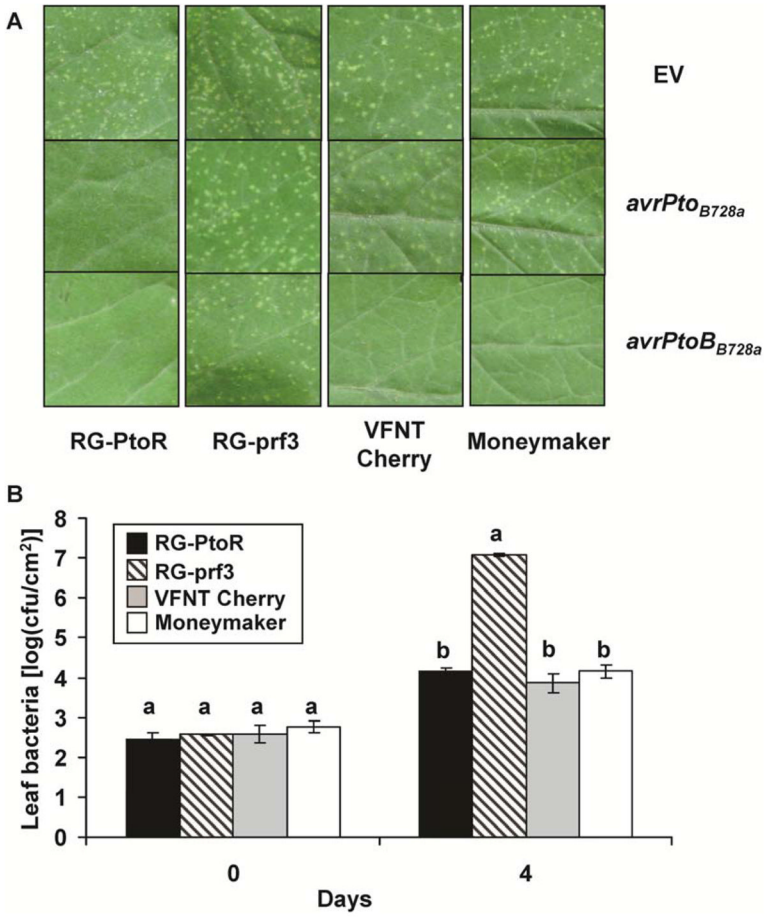

Fig. 1.

$AvrPtoB_{B728a}$ elicits resistance on tomato cultivars that do not express the *Pto* gene. **A,** Disease symptoms on leaves of tomato 'Rio-Grande' (RG)-PtoR (*Pto/Pto* and *Prf/Prf*), RG-*prf3* (*Pto/Pto* and *prf3/prf3*), VFNT Cherry, and Moneymaker (both have *Prf* but not *Pto*) after vacuum infiltration with strain 61 carrying pCPP45 (empty vector [EV]), pCPP45::$avrPto_{B728}$, or pCPP45::$avrPtoB_{B728a}$ at 2×10^4 CFU/ml. All photographs were taken 7 days after inoculation. **B,** Bacterial populations were measured at day 0 and 4 after inoculation with strain 61 expressing $avrPtoB_{B728a}$. Each value represents the mean of the log of CFU/cm^2 obtained from three plants, and error bars represent the standard deviation. Data analysis was performed using Duncan's multiple range test. Means with the same letter above the bars indicate no difference at a significance level of 5%. Each experiment was performed at least three times with similar results.

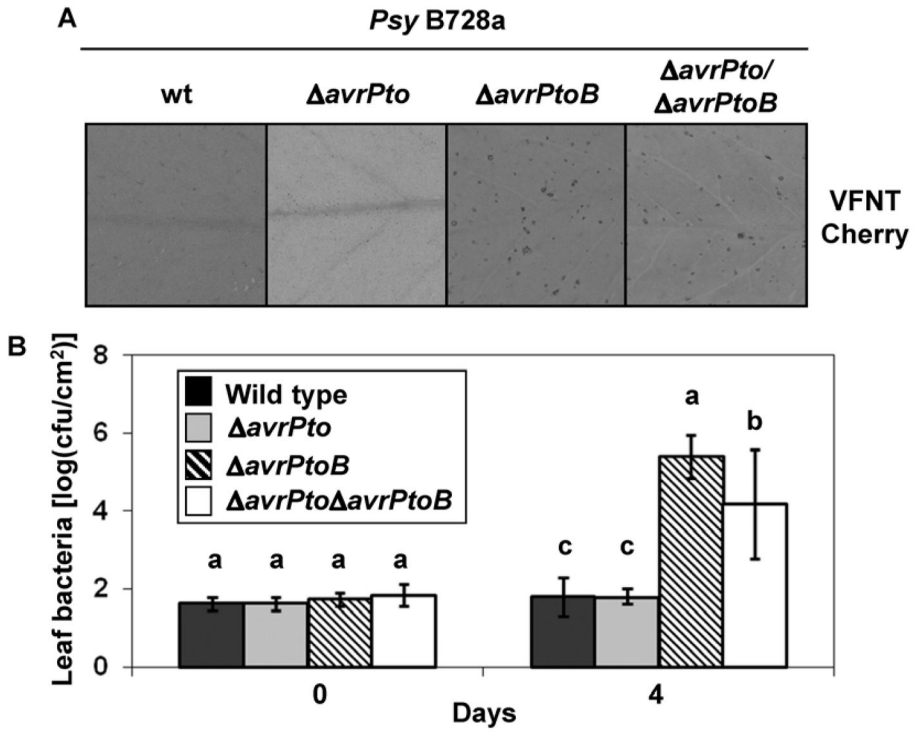

Fig. 2.

Deletion of *avrPtoB* but not *avrPto* from *Pseudomonas syringae* pv. *syringae* strain B728a abolishes its ability to elicit resistance on VFNT Cherry. **A,** Elicitation of Pto-independent resistance by infiltration of tomato leaves of VFNT Cherry with B728a and its derivative mutants at 2×10^4 CFU/ml. Photographs were taken 7 days after inoculation, and specks were observed 3 days after vacuum infiltration with *Psy*B728aΔ*avrPtoB* or *Psy*B728aΔ*avrPto*Δ*avrPtoB*. **B,** Bacterial populations in tomato leaves infiltrated with B728a and its derivative mutants. Error bars indicate standard deviation for three replicates. Data analysis was performed using Duncan's multiple range test. Means with the same letter above the bars indicate no difference at a significance level of 5%. Each experiment was performed three times with similar results.

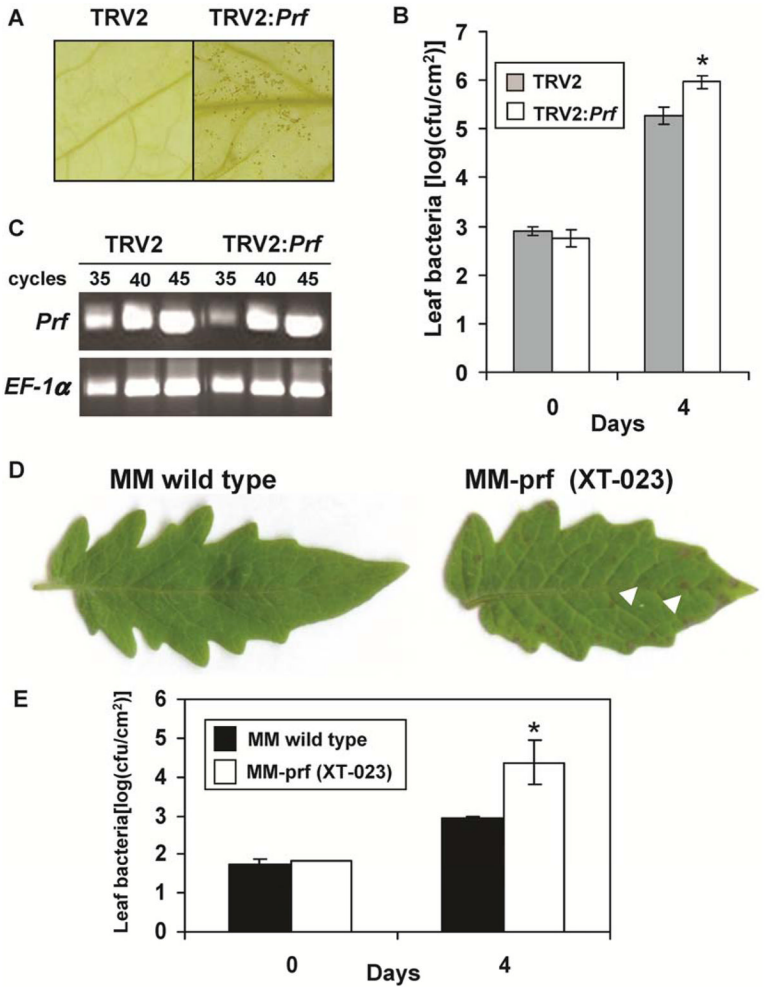

Fig. 3.

Resistance elicited by *avrPtoB*$_{B728a}$ in VFNT Cherry is Prf-dependent. **A,** *Tobacco rattle virus* (TRV2) was used to silence *Prf* in VFNT Cherry. The silenced plants were compromised in their resistance to *Pseudomonas syringae* pv. *syringae* strain B728a and specks were observed 4 days after inoculation. At 7 days postinoculation (dpi), chlorophyll was removed from the leaves with ethanol before photographs were taken. **B,** Bacterial populations in VFNT Cherry leaves silenced for *Prf* and infiltrated with B728a. **C,** Silencing of *Prf* in VFNT Cherry leaves was evaluated using semi-quantitative reverse-transcription polymerase chain reaction. **D,** Symptoms on leaves after vacuum infiltration of B728a into wild-type Moneymaker (MM) and a *prf* mutant of Moneymaker (XT-023). Disease symptoms started to develop 3 dpi and photographs were taken 7 days after inoculation. **E,** Bacterial populations in MM and XT-023 leaves at 0 and 4 dpi. Error bars indicate the standard deviations for three replicates. Data analysis was performed using Student's *t* test, and the asterisk (*) indicates significant difference at a level of 5%. Each experiment was performed three times with similar results.

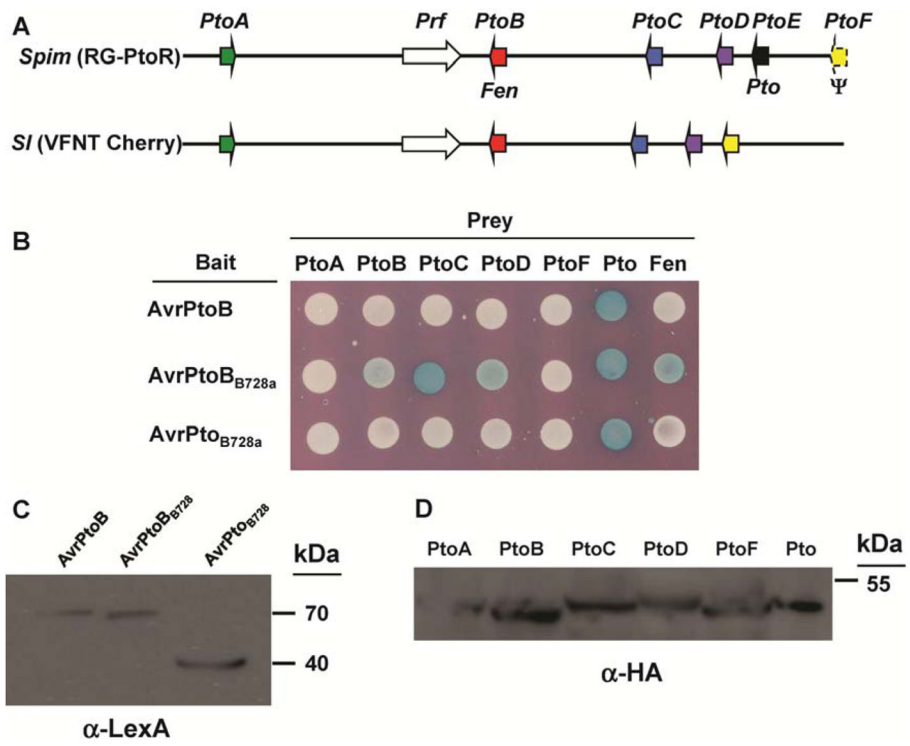

Fig. 4.

Interactions in a yeast two-hybrid system between AvrPtoB$_{B728a}$ and Pto family members. **A,** Genome organization of *Pto* region in 'Rio-Grande' (RG)-PtoR (from *Solanum pimpinellifolium* or *Spim*) (Pedley and Martin 2003) and 'VFNT Cherry' (*S. lycopersicum*, *Sl*). Arrows indicate the location and the orientation of genes in these regions, and open reading frames with the same color are orthologs. **B,** Yeast two-hybrid analysis. AvrPtoB, AvrPto$_{B728a}$, and AvrPtoB$_{B728a}$ all interact with Pto but differentially interact with SlFen, SlPtoC, and SlPtoD (labeled as PtoB, PtoC, and PtoD, respectively). Expression of the bait and prey proteins was confirmed by Western blotting using an **C,** anti-LexA or **D,** anti-HA antibodies, respectively.

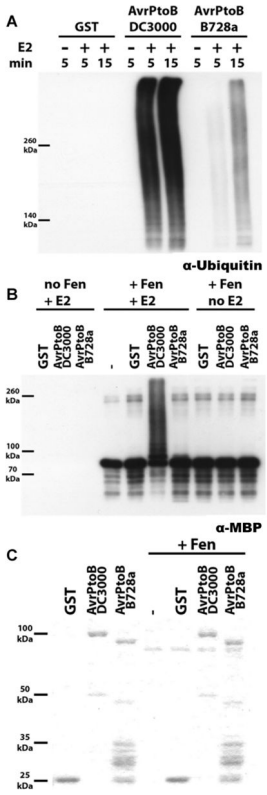

Fig. 5.

AvrPtoB$_{B728a}$ has reduced E3 ligase activity compared with AvrPtoB. **A,** N-terminal glutathione-*S*-transferase (GST) fusions of AvrPtoB and AvrPtoB$_{B728a}$ were purified from *Escherichia coli* and subjected to an in vitro ubiquitination assay with or without an E2 conjugating enzyme for 5 min and with E2 for 15 min. **B,** Purified maltose-binding-protein (MBP):Fen protein was used as a substrate for AvrPtoB and AvrPtoB$_{B728a}$ E3 ligases. Only AvrPtoB efficiently ubiquitinated SlFen. **C,** The remaining protein master-mixes used in the experiments presented in A and 5B were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue to show equal abundance of the different proteins. Both experiments were conducted in parallel using the same master mixes.

Table 1

Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics[a]	Source or reference
Bacterial strains		
Escherichia coli DH5α	…	Invitrogen
E. coli BL21(DE3)	…	Invitrogen
Pseudomonas syringae pv. *syringae*	Rifampicin derivative of field-isolated strain	
B728a	Rifampicin derivative of field-isolated strain	Loper and Lindow 1987
B728aΔ*avrPto*	Δ*avrPto::nptII*, Rif ^{r} Kan^{r}	This study
B728aΔ*avrPtoB*	Δ*avrPtoB*::Ω Sp^{r}/Sm^{r}, Rif ^{r} Sp^{r}/Sm^{r}	This study
B728aΔ*avrPto*Δ*avrPtoB*	Δ*avrPto::nptII*, Δ*avrPtoB*::Ω Sp^{r}/Sm^{r}, Rif ^{r}, Kan^{r}, Sp^{r}/Sm^{r}	This study
Agrobacterium tumefaciens GV3101	Rif ^{r}	Holsters et al. 1980
Plasmids		
pGilda	*HIS3*, Amp^{r}, under *GAL1* promoter for Y2H with LexA	Clontech Laboratories
pJG4-5	*TRP1*, Amp^{r}, under *GAL1* promoter for Y2H with HA epitope tag	Invitrogen
pCPP45	Broad-host-range vector with RP4 *par* region, Tc^{r}	Charkowski et al. 1997

[a] Rif ^{r}, Kan^{r}, Sp^{r}, Sm^{r}, Amp^{r}, and Tc^{r} indicate resistant to rifampicin, kanamycin, spectinomycin, streptomycin, ampicillin, and tetracycline, respectively.