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A bacterial E3 ubiquitin ligase targets a host protein kinase to disrupt plant immunity

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Abstract

Many bacterial pathogens of plants and animals use a type III secretion system to deliver diverse virulence-associated ‘effector’ proteins into the host cell¹. The mechanisms by which these effectors act are mostly unknown; however, they often promote disease by suppressing host immunity². One type III effector, AvrPtoB, expressed by the plant pathogen *Pseudomonas syringae* pv. *tomato*, has a carboxy-terminal domain that is an E3 ubiquitin ligase³. Deletion of this domain allows an amino-terminal region of AvrPtoB (AvrPtoB_{1–387}) to be detected by certain tomato varieties leading to immunity-associated programmed cell death⁴. Here we show that a host kinase, Fen, physically interacts with AvrPtoB_{1–387} and is responsible for activating the plant immune response. The AvrPtoB E3 ligase specifically ubiquitinates Fen and promotes its degradation in a proteasome-dependent manner. This degradation leads to disease susceptibility in Fen-expressing tomato lines. Various wild species of tomato were found to exhibit immunity in response to AvrPtoB_{1–387} and not to full-length AvrPtoB. Thus, by acquiring an E3 ligase domain, AvrPtoB has thwarted a highly conserved host resistance mechanism.

Pseudomonas syringae pv. *tomato* (*Pst*) causes bacterial speck disease of tomato (*Solanum lycopersicum*) by using its type III secretion system to deliver about 30 effectors into the plant cell⁵. Tomato varieties that are immune to speck disease express the Pto kinase that detects either of two *Pst* effector proteins, AvrPto or AvrPtoB. This detection involves the physical interaction of Pto with AvrPto or AvrPtoB and results in the rapid activation of an array of host defence responses including localized programmed cell death (PCD)⁶. The *Pto* gene was isolated from the wild tomato species *S. pimpinellifolium* and is a member of a small clustered gene family^{6,7} (Fig. 1a). Embedded within this gene cluster is the *Prf* gene that encodes a leucine-rich repeat-containing protein that physically interacts with Pto and is required for Pto-mediated immunity^{6,8}. Another member of the *Pto* gene family encodes the Fen kinase, which shares 80% amino acid identity with Pto but does not recognize AvrPto or AvrPtoB^{9,10}.

AvrPtoB is a modular protein⁴. An N-terminal region (AvrPtoB_{1–307}) is sufficient to elicit Pto/Prf-mediated PCD, whereas a C-terminal region (AvrPtoB_{388–553}) contains a domain that is an E3 ubiquitin ligase^{3,11}. Two truncations of AvrPtoB (AvrPtoB_{1–509} and AvrPtoB_{1–387}) lacking the E3 ligase domain, or AvrPtoB point mutants compromised in E3 ligase activity,

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Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

are detected by tomato varieties lacking the Pto kinase and by a tobacco species, *Nicotiana benthamiana*^{3,4}. This Pto-independent detection leads to PCD, is dependent on Prf and is referred to as Rsb (for 'resistance suppressed by AvrPtoB C terminus')⁴. AvrPtoB₁₋₃₀₇ does not elicit Rsb immunity, indicating that a domain between amino acid residues 308 and 387 is required for Rsb-associated recognition (Supplementary Fig. 1). Here we identify the host protein responsible for Rsb and explain the mechanism by which AvrPtoB E3 ligase activity suppresses this recognition.

Dependence on Prf indicated that Rsb might involve a member of the Pto family. Four members of the *Pto* gene family in *S. pimpinellifolium* are transcribed in leaves (*Fen*, *PtoC*, *PtoD* and *Pto*), of which only two, *Pto* and *Fen*, encode active kinases¹². The kinase activity of Pto is necessary for immunity, leading us to propose that the protein responsible for Rsb would also be an active kinase⁶. To determine whether a *Pto* family member is involved in Rsb immunity, we examined a tomato line, RG-PtoR(hpPto), that is a stable transformant knocked down for expression of the *Pto* gene family by RNA-mediated interference¹³. RG-PtoR(hpPto) plants were inoculated with *Pst* DC3000 strains delivering either AvrPtoB or AvrPtoB₁₋₅₀₉ (Fig. 1b). In leaves of RG-PtoR(hpPto) plants, both of these strains reached populations similar to that of the control susceptible line, RG-prf3, indicating a complete loss of Pto-mediated immunity and the Rsb phenotype. As expected, RG-PtoR plants were resistant to both *Pst* strains, whereas RG-ptol1, which lacks Pto, showed it had the Rsb phenotype by being resistant to only the strain delivering AvrPtoB₁₋₅₀₉. By using virus-induced gene silencing in *N. benthamiana*, we found that knocked-down expression of the *Pto* gene family, *Prf*, or previously described components of the Pto-mediated PCD-associated signalling pathway compromised the Rsb phenotype¹⁴ (Fig. 1c, and Supplementary Fig. 2). These results implicated one or more members of the *Pto* family in Rsb immunity.

There is a strict correlation between immunity conferred by Pto and the ability of this kinase to interact with AvrPtoB in the yeast two-hybrid system¹⁰. We proposed that the host protein responsible for Rsb would interact with AvrPtoB₁₋₃₈₇ and not with the non-Rsb-eliciting fragment, AvrPtoB₁₋₃₀₇. Using the yeast two-hybrid system we tested the Pto family members for interaction with AvrPtoB, AvrPtoB₁₋₃₈₇ or AvrPtoB₁₋₃₀₇ (Fig. 1d). Of these, only *Fen* interacted exclusively with AvrPtoB₁₋₃₈₇. PtoC interacted with AvrPtoB₁₋₃₈₇ but also with AvrPtoB₁₋₃₀₇. Pto, as expected, interacted with AvrPtoB and both AvrPtoB truncations, whereas PtoA and PtoD showed no interactions. Western blots confirmed the expression of each of the Pto family proteins and AvrPtoB proteins (Fig. 1d, and Supplementary Fig. 3).

To determine whether *Fen* or PtoC activates immunity, we expressed each of these proteins with AvrPtoB, AvrPtoB₁₋₃₈₇ or AvrPtoB₁₋₃₀₇ in protoplasts of tomato RG-PtoS or *N. benthamiana* (Fig. 1e, Supplementary Fig. 4A). *Fen* activated PCD when expressed with AvrPtoB₁₋₃₈₇ but not with AvrPtoB or AvrPtoB₁₋₃₀₇, whereas PtoC was unable to initiate PCD when expressed with any of these AvrPtoB proteins. Western blots confirmed expression of the Pto family proteins (Fig. 1e, and Supplementary Fig. 4B). As expected, Pto activated PCD in tomato when expressed with AvrPtoB, AvrPtoB₁₋₃₈₇ or AvrPtoB₁₋₃₀₇. The inability of PtoC to activate effector-elicited PCD, its interaction with the non-Rsb eliciting fragment AvrPtoB₁₋₃₀₇ and its lack of kinase activity¹² excluded a function for this protein in Rsb. Taken together, these data indicate that *Fen* is responsible for Rsb immunity.

It was possible that the lack of interaction between *Fen* and AvrPtoB in yeast (Fig. 1d) involved the E3 ligase activity of AvrPtoB. We therefore tested whether *Fen* interacted with E3 ligase-deficient AvrPtoB mutants. The AvrPtoB-Quad protein (Quad) contains four mutations in critical lysine residues¹¹, and a second mutant, E2BS, has three point mutations at predicted E2-binding sites³. Both AvrPtoB mutants interacted with *Fen* and PtoC, indicating that the E3

ligase activity interferes with certain protein interactions (Fig. 2a). As expected, the altered proteins interacted with Pto and were unable to interact with PtoA or PtoD.

We proposed that the AvrPtoB E3 ligase might ubiquitinate Fen to disrupt recognition of the AvrPtoB N-terminal region. To test this possibility, ubiquitination assays were performed *in vitro* with Fen and AvrPtoB and a series of controls (Fig. 2b). Among five kinases tested, only Fen was ubiquitinated in the presence of AvrPtoB, as indicated by the appearance of high-molecular-mass Fen proteins (Fig. 2b). Absence of E1, E2 or AvrPtoB proteins in the assay abolished the high-molecular-mass forms of Fen (Fig. 2b). In particular, Pto was not ubiquitinated by AvrPtoB in this assay. Fen ubiquitination might be due to unique ubiquitination sites (namely lysine residues) in this kinase. There are only five lysine residues that are present in Fen but absent from Pto and PtoC (Lys 70, Lys 72, Lys 155, Lys 253 and Lys 290; Supplementary Fig. 5A). Of these, Lys 70 and Lys 72 are close to the ATP-binding site (Lys 69) of Fen, raising the possibility that this region might be targeted for ubiquitination. However, arginine substitutions at any one of the five lysine residues had no effect on Fen ubiquitination (Supplementary Fig. 5B), suggesting that either multiple lysine residues are ubiquitinated or other structural differences between Pto and Fen account for the differential ubiquitination.

Ubiquitination of Fen by AvrPtoB raised the possibility that, in the plant cell, AvrPtoB E3 ligase activity might target Fen for degradation. To test this, we expressed Fen, PtoC or Pto with AvrPtoB or Quad proteins in RG-prf3 tomato protoplasts and assessed protein abundance. Fen accumulated poorly in the presence of AvrPtoB, reaching only about 35% of the abundance with the Quad protein (Fig. 3a). In contrast, Pto and PtoC accumulated in the presence of AvrPtoB to levels comparable to their abundance with the Quad protein (Fig. 3a).

Ubiquitination often marks a protein for degradation by means of the 26S proteasome. If AvrPtoB targets Fen for degradation, then inhibition of the proteasome should allow Fen to accumulate in the presence of AvrPtoB. In RG-prf3 protoplasts, we expressed Fen or Pto with AvrPtoB or Quad in the presence of MG132, a proteasome inhibitor. Treatment with MG132 resulted in a roughly 80% increase in Fen accumulation when expressed with AvrPtoB (Fig. 3b). MG132 had no effect on Fen coexpressed with Quad or with Pto coexpressed with AvrPtoB or Quad. Similar results were seen with a second proteasome inhibitor, MG115 (data not shown). We tested the effect of a general plant protease inhibitor cocktail on Fen abundance and found no effect on Fen accumulation (Fig. 3c). These results support a function for the AvrPtoB E3 ligase in proteasome-dependent degradation of the Fen kinase.

The specific targeting of Fen by AvrPtoB to suppress Rsb immunity suggested a selective advantage for the N-terminal region of AvrPtoB to acquire and maintain the E3 ligase domain. We therefore examined whether Rsb immunity is conserved among cultivated and wild species of tomato. Cultivars MoneyMaker (MM), VFNT Cherry (VFNT-C), and Ailsa Craig (AC) were tested for the presence of Rsb by infiltrating their leaves with two Rsb-eliciting *Pst* strains (DC3000 Δ avrPtoB plus AvrPtoB₁₋₃₈₇, or DC3000 Δ avrPtoB plus Quad) or two non-Rsb-eliciting strains (DC3000 Δ avrPtoB plus AvrPtoB, or DC3000 Δ avrPtoB plus AvrPtoB₁₋₃₀₇). RG-PtoR, RG-ptol1 and RG-prf3 lines were included as controls. Rsb immunity was activated in MM, VFNT-C and AC and, as expected, in RG-ptol1 (Fig. 4a).

To test whether Rsb immunity was activated by a *S. lycopersicum* orthologue of Fen, we examined a previously described Fen allele from AC (AC-fen)¹⁵. As assessed by ion leakage, expression in *N. benthamiana* leaves of AC-fen with AvrPtoB₁₋₃₈₇, but not with AvrPtoB₁₋₃₀₇, caused PCD comparable to the expression of Pto or Fen with AvrPtoB₁₋₃₈₇ (Fig. 4b). As expected, only Pto initiated PCD when expressed with AvrPtoB₁₋₃₀₇. Orthologues of Fen can therefore confer Rsb immunity. A screen of 37 accessions of wild species of tomato

revealed that 21 express Rsb immunity but only 5 seem to have Pto-mediated immunity (Supplementary Table 1; Fig. 4c shows three examples). The wide occurrence of Rsb is consistent with phylogenetic analyses indicating that the *Fen* gene arose before the *Pto* gene in the *Solanum* species¹⁶ and suggests that the E3 ligase activity of AvrPtoB evolved to circumvent a broadly conserved host resistance mechanism.

The interaction of AvrPtoB_{1–387} with Fen kinases might indicate that the effector evolved to interfere with a possible function of these kinases in basal defence. In fact, E3 ligase-deficient AvrPtoB proteins are able to suppress certain basal defences^{17,18}. We therefore compared the growth of a type III secretion-defective *Pst* strain (DC3000Δ*hrcQ-U* (ref. 19)) in wild-type tomato plants and in a line overexpressing Fen (Supplementary Fig. 6). Similar overexpression experiments have demonstrated a function for the *Arabidopsis* protein RIN4 as a negative regulator of basal defence²⁰. However, we found no effect of Fen overexpression on growth of the DC3000Δ*hrcQ-U* strain (Supplementary Fig. 6).

Molecular mimicry of host proteins by bacterial pathogens is common; however, so far only a handful of bacterial proteins are known to manipulate the host ubiquitination system^{3,21–23}. Of these, AvrPtoB, *Salmonella* SopA, the *Shigella* IpaH family and *Salmonella* SspH1 have intrinsic E3 ubiquitin ligase activity, potentially releasing them from dependence on host proteins to secure substrate specificity^{22,23}. Interestingly, IpaH9.8 and SspH1 also ubiquitinate protein kinases, although the relevance of this activity to the host–pathogen interaction has not been established²⁴. Overexpression of the AvrPtoB E3 ligase in leaves suppresses PCD induced by the pro-apoptotic protein Bax and by many, but not all, plant resistance proteins^{4,17,18,25}. AvrPtoB also suppresses PCD in yeast⁴. Our present data do not explain these general PCD suppression activities; they may be due to a second, highly conserved target of AvrPtoB or to overexpression of the effector. However, our data fully account for the immunity suppression observed with AvrPtoB-expressing *Pseudomonas* strains on infection of tomato leaves lacking Pto^{3,4,11}. Furthermore, our results suggest that Pto evolved not only to recognize AvrPto and AvrPtoB but also to be invulnerable to AvrPtoB-mediated ubiquitination and subsequent degradation.

Our results can be viewed in the context of the evolutionary processes that may have shaped this pathogen–host interaction (Supplementary Fig. 7). It is known that the N-terminal region of AvrPtoB is able to suppress host basal defences^{17,18}. This region promotes pathogen virulence and therefore an N-terminal-only form of AvrPtoB might have existed independently of the C-terminal E3 ligase domain. We now know that Fen binds the AvrPtoB_{1–387} region to negate basal defence suppression, activate defence signalling and confer immunity on the host. Immunity mediated by Fen and by Pto requires Prf, suggesting that Prf evolved to function with a progenitor of the Pto family. It is possible this progenitor, unlike Fen, had a function in basal defence. An alternative is that members of the Pto family evolved together with Prf solely for effector-triggered immunity. To counter recognition by Fen, disrupt immunity-associated PCD and restore basal defence suppression activity, the N-terminal region of AvrPtoB may have acquired an E3 ligase domain to mediate the degradation of Fen and Fen orthologues. A similar ability to disrupt immunity has been reported for AvrRpt2, which disrupts signalling by the resistance protein RPM1 in *Arabidopsis* by cleaving an RPM1-interacting protein, RIN4 (ref. 26). It is possible that some AvrPtoB truncated proteins evolved to evade Fen recognition. For example, *P. syringae* pv. *maculicola* expresses an AvrPtoB homologue (HopPmaL27) that lacks both the E3 ligase domain and part of the Fen recognition determinant (residues 307–387). HopPmaL elicits immunity in response to Pto but not Fen²⁸. Pto seems to have evolved to counter both of these strategies. It remains to be determined how Pto evades AvrPtoB-mediated ubiquitination.

METHODS SUMMARY

Plant inoculations

Tomato plants were vacuum infiltrated with *Pst* (about 5.5×10^4 colony-forming units per ml) suspended in 10 mM MgCl₂ and 0.00002% Silwet. Bacterial leaf populations were measured from three plants per treatment, three days after infiltration.

Virus-induced gene silencing (VIGS) and *Agrobacterium*-mediated transient expression

VIGS was induced by using the tobacco rattle virus vector delivered by *Agrobacterium tumefaciens*¹⁴. For transient gene expression, *A. tumefaciens* was used to deliver a 35S cauliflower mosaic virus expression cassette (pTEX)⁴. Ion leakage was measured two days after infiltration with *A. tumefaciens*.

Yeast two-hybrid assay

A LexA-based two-hybrid system was used to test for interactions between Pto family members in the bait vector and AvrPtoB or AvrPtoB mutants or truncations in the prey vector.

In vitro ubiquitination assay

The *in vitro* ubiquitination reactions were performed with recombinant maltose-binding protein (MPB)-tagged Pto family proteins, His₆-tagged E1, His₆-tagged E2, ubiquitin and glutathione *S*-transferase (GST)-tagged AvrPtoB.

Protoplast bioassays

Protoplasts were isolated from seedling leaves and transformed with pTEX by using polyethylene glycol. Protoplast viability was determined by staining with Evans Blue.

Immunoblotting quantification

After detection of immunolabelled proteins by chemiluminescence, signal intensities were quantified by the Storm blot imaging system and quantified using ImageQuant TL software.

Plant material

Accessions of wild species of tomato were obtained from the Tomato Genetics Resource Center (<http://tgrc.ucdavis.edu/>).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The Fen kinase is responsible for the Rsb phenotype

a, Genome organization of the *Pto* family and *Prf* from *Solanum pimpinellifolium*. Rio Grande-PtoR (RG-PtoR) plants are wild type⁶, RG-pto11 plants have a deleterious point mutation (yellow dot) in *Pto*, and RG-prf3 plants have a deletion in *Prf*⁶. **b**, Growth of *Pst* strains delivering AvrPtoB (white) or AvrPtoB₁₋₃₈₇ (grey) in RG-PtoR, RG-pto11, RG-prf3 and RG-PtoR(hpPto) leaves. Error bars represent s.e.m. ($n = 6$). c.f.u., colony-forming units. **c**, Response after transient expression of Pto with AvrPto, AvrPtoB₁₋₃₈₇, mitogen-activated protein kinase kinase kinase (MAPKKK) α or Bax in *N. benthamiana* leaves silenced for genes in the Pto pathway¹⁴ (listed at the left) (see Supplementary Fig. 2). MEK, MAP kinase/ERK kinase; SIPK, salicylic-acid-induced protein kinase; WIPK, wound-induced protein kinase; dashes indicate no PCD. **d**, Pto family members or empty vector (ev) tested for interaction with forms of AvrPtoB in the yeast two-hybrid system. Blue patches show positive interactions. **e**, Response of RG-PtoS protoplasts after coexpression of AvrPtoB₁₋₃₈₇ with Pto (red columns), Fen (orange columns), PtoC (green columns) or empty vector (white columns). Data are presented as percentage cell death in experimental samples after subtraction of cell death percentages occurring with empty vector. Error bars represent s.e.m. ($n = 3$).

Figure 2. Fen is ubiquitinated in the presence of AvrPtoB

a, Pto family members or empty vector (ev) tested for interaction with AvrPtoB, or AvrPtoB proteins with compromised E3 ligase activity (Quad and E2BS) in the yeast two-hybrid system. Blue patches show positive interactions. **b**, Immunoblots of an *in vitro* ubiquitination assay with recombinant E1, E2, AvrPtoB, ubiquitin (Ub) and MBP fusions of the kinases shown. Polyubiquitination of Fen is indicated by (Fen-(Ub)_n). Polyubiquitinated forms of AvrPtoB (AvrPtoB-(Ub)_n) confirmed E3 ligase activity. An anti-Ub antibody detected the incorporation of ubiquitin in each assay.

Figure 3. In the presence of AvrPtoB, Fen is degraded in a proteasome-dependent manner
a, Immunoblot to detect accumulation of Fen, Pto or PtoC in RG-prf3 protoplasts in the presence of AvrPtoB or Quad. Visualization of ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco) subunits by Coomassie blue confirmed equal loading. The Fen panel shows a roughly 20-s exposure, in contrast with about 10 s for the others; however, the images are from the same experiment. HA, haemagglutinin. **b**, As in **a** after treatment with MG132 (minus, dimethylsulphoxide (DMSO) only; plus, with 50 μ M MG132). **c**, As in **a** with the addition of general plant protease inhibitor (PPI) cocktail (minus, DMSO only; plus, 0.2% PPI).

Figure 4. Rsb is present in many cultivated and wild species of tomato
a, Inoculation of cultivated tomato varieties MM, VFNT-C or AC with *Pst* strains. R and S indicate resistant or susceptible plants, respectively. **b**, Electrolyte leakage after coexpression of AC-fen, Fen or Pto with AvrPtoB₁₋₃₈₇ (orange), AvrPtoB₁₋₃₀₇ (green) or AvrPtoB (grey). The ratio of conductivity of samples coexpressing AvrPtoB₁₋₃₀₇, AvrPtoB₁₋₃₈₇ or AvrPtoB versus empty vector control is shown. Error bars represent s.e.m. ($n = 3$). **c**, Leaves from 3 of 21 wild tomato species shown to exhibit Rsb immunity in response to inoculation with *Pst* delivering AvrPtoB₁₋₃₈₇ (see Supplementary Table 1): left, disease; right, Rsb immunity.