Type III effector AvrPtoB requires intrinsic E3 ubiquitin ligase activity to suppress plant cell death and immunity

Robert B. Abramovitch*†, Radmila Janjusevic‡, C. Erec Stebbins‡, and Gregory B. Martin*†§

*Boyce Thompson Institute for Plant Research, ‡Department of Plant Pathology, Cornell University, Ithaca, NY 14853; and §Laboratory of Structural Microbiology, The Rockefeller University, New York, NY 10021

Edited by Steven E. Lindow, University of California, Berkeley, CA, and approved January 3, 2006 (received for review September 9, 2005)

Microbial pathogens of both plants and animals employ virulence factors that suppress the host immune response. The tomato pathogen Pseudomonas syringae injects the AvrPtoB type III effector protein into the plant cell to suppress programmed cell death (PCD) associated with plant immunity. AvrPtoB also inhibits PCD in yeast, indicating that AvrPtoB manipulates a conserved component of eukaryotic PCD. To identify host targets of AvrPtoB, we performed a yeast two-hybrid screen and identified tomato ubiquitin (Ub) as a strong AvrPtoB interactor. AvrPtoB is ubiquitinated in vitro and exhibits Ub ligase activity in the presence of recombinant E1 activating enzyme and specific E2 Ub-conjugating enzymes. The C terminus of AvrPtoB is sufficient for both anti-PCD and Ub ligase activities, suggesting the two functions are associated. Indeed, mutation of AvrPtoB lysine residues in the C terminus, between K512 and K529, disrupts AvrPtoB–Ub interactions, decreases AvrPtoB–mediated anti-PCD activity, and abrogates P. syringae pathogenesis of susceptible tomato plants. Remarkably, quantitative decreases in AvrPtoB anti-PCD activity are correlated with decreases in AvrPtoB ubiquitination and Ub ligase activity. Overall, these data reveal a unique bacterial pathogenesis strategy, where AvrPtoB manipulates the host Ub system and requires intrinsic E3 Ub ligase activity to suppress plant immunity.

pathogenesis | plant disease | programmed cell death | ubiquitin | Pseudomonas

Bacteriologists have long observed that efficient plant pathogens include pathogenicity factors that suppress the host immune response. The tomato pathogen P. syringae Pto suppresses the plant immune response by inhibiting HR-based PCD (6). In the absence of Pto, however, both AvrPtoB and AvrPtoB function to promote bacterial growth by suppressing plant defenses (6, 7).

AvrPtoB suppresses plant immunity by inhibiting HR-based PCD (6). In susceptible plants, AvrPtoB suppresses PCD-induced by diverse R proteins and the proteopptic mouse protein Bax. Moreover, AvrPtoB also suppresses PCD in yeast, indicating that AvrPtoB acts as a general eukaryotic cell death suppressor. AvrPtoB is a 59-kDa protein with a modular architecture, where recognition by the Pto R protein is localized on the N-terminal region (NTR) (comprising amino acids 1–387), and anti-PCD activity is localized on the C-terminal region (CTR) (comprising amino acids 308–553). Therefore, a host target of AvrPtoB anti-PCD activity is predicted to be conserved among plants and yeast, and interaction with a host target will depend on the AvrPtoB CTR.

The biochemical functions of several type III effectors have been reported. For animal pathogen type III effectors, at least nine enzymatic activities have been discovered (8). However, enzymatic activities for plant pathogenic type III effectors have been difficult to identify, because many effectors have no sequence or structural similarity to known proteins. To date, demonstrated enzymatic activities of plant pathogenic effectors are limited to cysteine protease, ubiquitin (Ub)-like protein protease, and tyrosine phosphatase activities (4, 8). Here, we report that AvrPtoB manipulates the host Ub machinery and employs intrinsic E3 Ub ligase activity to suppress HR-based PCD and plant immunity.

Results and Discussion

AvrPtoB Interacts with Ub in a Yeast Two-Hybrid (Y2H) System and Is Ubiquitinated in Vitro. To identify potential host targets of AvrPtoB, we performed a Y2H screen using an AvrPtoB bait fusion and a tomato cDNA prey library. The strongest interactor isolated was Ub (Fig. 1A). Ub also interacts with the related effector VirPshA, which shares 51% identity with AvrPtoB (9) (Fig. 1A) and AvrPtoB homologues from P. syringae pv. syringae B728a and Prf T1 (data not shown), indicating that interaction with Ub is a conserved property of AvrPtoB-like effectors. Ub does not interact with the AvrPtoB CTR NTR bait, demonstrating that the AvrPtoB–Ub interaction depends on the CTR. Ub is conserved among all eukaryotes (10) and plays an important role in plant cell death and immunity (11) and, therefore, was considered an excellent candidate target of AvrPtoB anti-PCD activity.

Ub is an 8.5-kDa protein that is conjugated to target proteins by an isopeptide linkage of Ub to substrate lysine residues. Ubiquitination is carried out by an ATP-dependent cascade involving an E1 Ub activating enzyme (E1), an E2 Ub conjugating enzyme (E2), and an E3 Ub ligase (E3) (10). Proteins also interact noncovalently with Ub through Ub interaction motifs (UIMs) (12), and we identified a UIM that is conserved in diverse AvrPtoB proteins (see Fig. 6, which is published as supporting information on the PNAS web site). Therefore, if the observed AvrPtoB–Ub Y2H interaction was a conserved property of AvrPtoB-like effectors. Ub does not interact with the AvrPtoB CTR NTR bait, demonstrating that the AvrPtoB–Ub interaction depends on the CTR. Ub is conserved among all eukaryotes (10) and plays an important role in plant cell death and immunity (11) and, therefore, was considered an excellent candidate target of AvrPtoB anti-PCD activity.

Ub is an 8.5-kDa protein that is conjugated to target proteins by an isopeptide linkage of Ub to substrate lysine residues. Ubiquitination is carried out by an ATP-dependent cascade involving an E1 Ub activating enzyme (E1), an E2 Ub conjugating enzyme (E2), and an E3 Ub ligase (E3) (10). Proteins also interact noncovalently with Ub through Ub interaction motifs (UIMs) (12), and we identified a UIM that is conserved in diverse AvrPtoB proteins (see Fig. 6, which is published as supporting information on the PNAS web site). Therefore, if the observed AvrPtoB–Ub Y2H interaction was a conserved property of AvrPtoB-like effectors. Ub does not interact with the AvrPtoB CTR NTR bait, demonstrating that the AvrPtoB–Ub interaction depends on the CTR. Ub is conserved among all eukaryotes (10) and plays an important role in plant cell death and immunity (11) and, therefore, was considered an excellent candidate target of AvrPtoB anti-PCD activity.

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: CTR, C-terminal region; DC3000, P. syringae pv. tomato DC3000; E1, E1 ubiquitin activating enzyme; E2, E2 ubiquitin conjugating enzyme; E3, E3 ubiquitin ligase; HR, hypersensitive response; NTR, N-terminal region; PCD, programmed cell death; R, resistance; RRL, rabbit reticulocyte lysates; Ub, ubiquitin; Y2H, yeast two-hybrid.

§To whom correspondence should be addressed. E-mail: gbm7@cornell.edu.

© 2006 by The National Academy of Sciences of the USA
is a direct interaction, it could be the result of ubiquitination of AvrPtoB or a noncovalent interaction between AvrPtoB and Ub. By using standard in vitro approaches, noncovalent interactions of recombinant AvrPtoB with mono- or polyubiquitin were not observed (Fig. 6) (12). When expressed in plants and yeast, however, AvrPtoB is detected as multiple bands separated by ~8 kDa (Fig. 1B, data not shown) and, with longer exposures, as a high-molecular-weight smear (data not shown); these observations are consistent with AvrPtoB ubiquitination. Interestingly, when Western blots of AvrPtoB-expressing plant tissue were probed with anti-Ub antibody, we also observe an enhanced Ub smear. This Ub smear may be the result of ubiquitinated AvrPtoB or enhanced ubiquitination of host proteins.

To determine whether AvrPtoB can be ubiquitinated, we performed an in vitro ubiquitination assay using rabbit reticulocyte lysates (RRL) as the source of E1, E2, and E3 enzymes, Flag-Ub, an ATP regeneration system, and GST-AvrPtoB (13, 14). GST-AvrPtoB was isolated from the reaction, and ubiquitinated GST-AvrPtoB was detected by Western blot (Fig. 2A). We tested the GST-AvrPtoB1–387 NTR and GST-AvrPtoB308–553 CTR proteins in the in vitro ubiquitination assay and observed that the CTR was necessary and sufficient as a substrate for ubiquitination by an unknown E3 Ub ligase (Fig. 2A).

In this ubiquitination assay, Ub may be conjugated to lysine residues on AvrPtoB or the GST fusion protein. To determine whether AvrPtoB itself was ubiquitinated, we performed the same ubiquitination assay; however, after purifying GST-AvrPtoB from the reaction, we cleaved AvrPtoB from GST by using the TEV protease. AvrPtoB without the GST tag was ubiquitinated (Fig. 2B). Ubiquitination of AvrPtoB is further supported by the detection of RRL- and Ub-dependent higher-molecular-weight bands of AvrPtoB and GST-AvrPtoB308–553 CTR on Western blots probed with anti-AvrPtoB antibody (Fig. 2A and B).

**AvrPtoB Exhibits E3 Ub Ligase Activity.** Ubiquitination of AvrPtoB in RRLs reveals that the AvrPtoB CTR is an efficient substrate for Ub attachment. Interestingly, GST and GST-AvrPtoB1–387 NTR are not ubiquitinated in RRLs, even though GST has several lysine residues that can act as ubiquitination substrates, suggesting that AvrPtoB may specifically manipulate a component of the host ubiquitination system. E3 Ub ligases bind E2 Ub-conjugating enzymes and often become autoubiquitinated as part of their normal function (10). Therefore, the observed AvrPtoB ubiquitination is suggestive of an intrinsic AvrPtoB E3 activity. To test this hypothesis, we performed E3 assays using recombinant E1, a panel of E2s, Flag-Ub, an ATP regeneration system, and GST-AvrPtoB or GST alone. AvrPtoB became strongly ubiquitinated specifically in the presence of the E2s: UbcH5a, UbcH5c, or UbcH6 (Figs. 2C and 6); ubiquitination was not observed in assays with GST alone (Fig. 6). Ubiquitination in the presence of specific E2s is a hallmark of E3 Ub ligase activity. The observed AvrPtoB E3 activity is substantiated by the crystal structure of the AvrPtoB336–553 C-terminal domain, which is homologous to the eukaryotic RING finger/U-box E3 Ub ligase fold (15). To our knowledge, AvrPtoB is the first example of a prokaryotic protein mimicking a eukaryotic E3 Ub ligase. Interestingly, viral proteins with E3 activity have been identified, demonstrating that this virulence mechanism is widespread (16, 17).

**Identification of a Domain in the AvrPtoB C Terminus Required for Anti-PCD Activity and AvrPtoB–Ub Interactions.** The AvrPtoB308–553 CTR is necessary and sufficient for both E3 activity and cell-death suppression, demonstrating that these two activities are associated. To further explore the connection between anti-PCD and E3
activities, we set out to identify specific amino acid residues of AvrPtoB that are necessary for both activities. The AvrPtoB amino acid sequence, however, shows no similarities to known E3s, and, at the time of this study, the AvrPtoB1–387 NTR C-terminal domain crystal structure had not yet been solved. Remarkably, all seven AvrPtoB lysine residues are clustered in the AvrPtoB C terminus between amino acids 453 and 546. We mutated each lysine to arginine to determine whether these residues are involved in AvrPtoB–Ub interactions and anti-PCD activity. In the Y2H system, all of the K-to-R mutants expressed well as bait fusions, as determined by strong interactions with the Pto prey (Fig. 3A) and by Western blot (data not shown). Three K-to-R mutants (K512R, K520R, and K529R) showed 5- to 50-fold weaker Ub interactions as compared with wild type AvrPtoB (Fig. 3A), revealing that a region encompassing lysines K512 to K529 is important for AvrPtoB–Ub interactions.

We expressed the K-to-R mutants in tomato to determine whether AvrPtoB–Ub interactions are associated with anti-PCD activity. Intact AvrPtoB elicits immunity-associated PCD on Pto-expressing tomato plants (RG–PtoR); however, AvrPtoB does not elicit PCD on the susceptible RG–pto11 or RG–prf3 tomato lines (18) (Fig. 3B and see Fig. 7, which is published as supporting information on the PNAS web site). On RG–pto11, AvrPtoB suppresses PCD elicited by a second R protein that recognizes the AvrPtoB1–387 NTR (6) (Fig. 3B). Loss of AvrPtoB anti-PCD activity reveals this normally hidden PCD-eliciting phenotype. As such, RG-pto11 can be used to assay for the presence or absence of AvrPtoB anti-PCD activity. All the K-to-R mutants elicited PCD on RG–PtoR, indicating that the proteins are expressed (Fig. 7). The K512R mutant elicited PCD on RG–pto11 (Fig. 3B), and coexpression with wild-type AvrPtoB suppressed the K512R-induced PCD (Fig. 7), demonstrating that K512R has decreased cell-death-suppression activity in tomato. K529R also elicited weak PCD on RG–pto11, suggesting that it, too, has decreased anti-PCD activity in tomato.

AvrPtoB–Ub Interactions Are Required for DC3000 Pathogenesis of Tomato. To test the importance of AvrPtoB–Ub interactions in P. syringae pathogenesis, we cloned the K-to-R mutants under control of the native AvrPtoB promoter and transformed these constructs into DC3000 and the DC3000::ΔavrPtoB and DC3000::ΔavrPtoB/ΔavrPtoB mutants (19). All the altered proteins were properly translocated into the plant cell, because they elicited resistance on RG–PtoR when delivered by the DC3000::ΔavrPtoB/ΔavrPtoB double mutant (Fig. 7). When the altered proteins are delivered by DC3000::ΔavrPtoB, all the strains caused disease on susceptible RG–prf3 and elicited immunity on resistant RG–PtoR (Fig. 3C). Strains expressing AvrPtoB and the K453R, K520R/K521R, and K546R mutant proteins caused disease on susceptible RG–pto11 because they suppress cell death and host immunity; however, like the AvrPtoB1–387 NTR, the K512R and K529R mutants elicited immunity on normally susceptible RG–pto11. Wild-type AvrPtoB, delivered at physiological levels, can suppress the immunity elicited by K512R and K529R, because these constructs do not elicit resistance when delivered by DC3000 (Fig. 7). Therefore, observations of K-to-R mutants when overexpressed in tomato are relevant when delivered at physiological levels by the pathogen. Remarkably, the K512R and K529R proteins had the greatest decrease of interaction with Ub in the Y2H system and also had decreased ubiquitination in vitro (Fig. 4C), demonstrating that a region between K512 and K529 plays a key role in AvrPtoB ubiquitination, anti-PCD activity, and P. syringae pathogenesis.

AvrPtoB-Mediated Cell Death Suppression Depends on AvrPtoB Auto-ubiquitination and E3 Ub Ligase Activity. Cell-death-suppression assays in the wild tobacco plant Nicotiana benthamiana were undertaken to further examine anti-PCD activity of the K-to-R mutants. Cell death in N. benthamiana develops more slowly than in tomato and allows measurement of incremental decreases in anti-PCD activity. Furthermore, unlike in tomato, in N. benthamiana leaves, AvrPtoB can suppress Pto-mediated PCD elicited by coexpression of AvrPto and Pto (6) (Fig. 4A and B; and see Fig. 8, which is published as supporting information on the PNAS web site). Like wild-type AvrPtoB, the K453R, K464R, and K546R mutants exhibited full anti-PCD activity in N. benthamiana (Fig. 4A and B). The K512R, K520R/K521R, and K529R mutants also suppress PCD up to 5 days postinoculation, after which AvrPto/Pto-dependent PCD is observed (Figs. 4A and B and 8), demon-
Substitution of AvrPtoB Lysine Residues Disrupts AvrPtoB E3 Ub Ligase Activity. Our data raise multiple models for the role of lysine residues between K512 and K529 in AvrPtoB ubiquitination, E3 Ub ligase activity, and cell death suppression. The mechanism by which AvrPtoB K-to-R substitutions alters AvrPtoB virulence function is ambiguous, however, because the K-to-R mutants may be (i) disrupting AvrPtoB E3 activity or (ii) decreasing sites for Ub attachment, which may be essential for AvrPtoB function.

To test the hypothesis that the K-to-R substitutions disrupt AvrPtoB E3 activity, we examined whether AvrPtoB K-to-R mutants are impaired in the ability to promote formation of Ub conjugates. In E3 Ub ligase assays, E3s often generate unassembled Ub chains (20) or ubiquitinate nonspecific substrate proteins present in the E3 Ub ligase assay (e.g., trace Escherichia coli proteins that copurify with the reaction components). Formation of these Ub conjugates can be detected by examining the supernatant of an AvrPtoB E3 ligase assay (Fig. 5 A). The ubiquitinated proteins are not AvrPtoB,

The K512R, K529R double mutant, and quadruple mutant showed the most substantial decreases in ubiquitination and anti-PCD activity. Using gel filtration and circular dichroism, we did not detect substantial structural changes in the altered proteins (see Fig. 9, which is published as supporting information on the PNAS web site). We tested these mutants in the Y2H system and found they had a complete loss of AvrPtoB–Ub interactions (Fig. 3 A). These mutants also elicited PCD when expressed in DC3000::avrPtoB (Fig. 3 C). Therefore, data from Y2H assays, transient expression assays in tomato and N. benthamiana, pathogenesis assays in tomato, and in vitro ubiquitination assays, all support a model where AvrPtoB targets the host Ub system and requires E3 Ub ligase activity to suppress PCD and immunity.

The different levels of anti-PCD activity observed in the K-to-R mutants represent an allelic series that allowed us to test whether anti-PCD activity depends on AvrPtoB ubiquitination. In the in vitro ubiquitination assay, we observed that the mutants with partial reductions of anti-PCD activity (K512R, K529R double mutant, the triple mutants, and the quadruple mutant all lost the ability to suppress AvrPto/Pto-dependent PCD (Fig. 4 A and B). In N. benthamiana, as in tomato, complete loss of AvrPtoB anti-PCD activity reveals a normally hidden PCD-eliciting phenotype that depends on the AvrPtoB1–387 NTR. Interestingly, like the AvrPtoB1–387 NTR, only the quadruple mutant elicits PCD when expressed alone (Fig. 4 C), demonstrating that the quadruple mutant exhibits the greatest decrease in anti-PCD activity. All of the K-to-R mutants expressed well as determined by Western blot (Fig. 8), indicating that differences in anti-PCD activity are the result of changes in protein function.

The K512R, K529R double mutant, and quadruple mutant showed the most substantial decreases in ubiquitination and anti-PCD activity. Using gel filtration and circular dichroism, we did not detect substantial structural changes in the altered proteins (see Fig. 9, which is published as supporting information on the PNAS web site). We tested these mutants in the Y2H system and found they had a complete loss of AvrPtoB–Ub interactions (Fig. 3 A). These mutants also elicited PCD when expressed in DC3000::avrPtoB (Fig. 3 C). Therefore, data from Y2H assays, transient expression assays in tomato and N. benthamiana, pathogenesis assays in tomato, and in vitro ubiquitination assays, all support a model where AvrPtoB targets the host Ub system and requires E3 Ub ligase activity to suppress PCD and immunity.

The K512R, K529R double mutant, and quadruple mutant showed the most substantial decreases in ubiquitination and anti-PCD activity. Using gel filtration and circular dichroism, we did not detect substantial structural changes in the altered proteins (see Fig. 9, which is published as supporting information on the PNAS web site). We tested these mutants in the Y2H system and found they had a complete loss of AvrPtoB–Ub interactions (Fig. 3 A). These mutants also elicited PCD when expressed in DC3000::avrPtoB (Fig. 3 C). Therefore, data from Y2H assays, transient expression assays in tomato and N. benthamiana, pathogenesis assays in tomato, and in vitro ubiquitination assays, all support a model where AvrPtoB targets the host Ub system and requires E3 Ub ligase activity to suppress PCD and immunity.
AvrPtoB functions as an E3 Ub ligase and has a structure homologous to RING-finger/U-box E3 Ub ligases. This is a unique example of a bacterial E3 Ub ligase, although other prokaryotic virulence proteins have been described that manipulate the host Ub–proteasome system using other mechanisms (21–23). As an E3 Ub ligase, AvrPtoB may be ubiquitinating host proteins and targeting them for degradation. This mechanism is supported by the enhanced smear of ubiquitinated proteins observed in plant extracts expressing AvrPtoB (Fig. 1B). In a simple model of AvrPtoB anti-PCD activity, AvrPtoB binds and ubiquitinates a positive regulator of plant cell death, targeting it for degradation. Alternatively, AvrPtoB may disrupt normal pro tease function of the plant cell to interfere with protein degradation associated with disease resistance. Identifying additional interacting proteins of the AvrPtoB isoforms might enable identification of AvrPtoB substrates and other host components that are essential for cell death and immunity.

### Materials and Methods

#### Y2H Screen

AvrPtoB was cloned into the pEG202 bait vector and used to screen a tomato cDNA prey library (24). Transformants (~2 x 10^9) were plated on −Leucine, −Galactose plates, and 1,500 transformants displaying leucine prototrophy were transferred to − Leucine, + X-Gal, +Galactose plates. Of these, several blue colonies were observed after 72 h of incubation. Interacting prey constructs were isolated from yeast and sequenced, and two independent clones were identified as Ub. To improve protein expression, the K-to-R mutants and wild-type AvrPtoB were cloned as bait fusions in the galactose-inducible pGILDA vector. Quantitative Y2H β-galactosidase assays were performed as described by Reynolds and Lundblad (25).

#### Protein Expression

GST-AvrPtoB protein was cloned, expressed, and purified as described in ref. 26. GST-AvrPtoB was NTR and GST-AvrPtoB CTR were generated by Gateway cloning PCR products into the pDEST15 Gateway-compatible expression vector (Invitrogen). Proteins were expressed in BL21 Star (DE3) E. coli (Invitrogen) and purified by using standard protein expression methods. A TEV protease site was engineered upstream of the AvrPtoB start codon, allowing the GST tag to be released from the protein by using the AcTEV protease (Invitrogen). Circular-dichroism experiments, examining the K512R, K529R, and quadruple mutants (Fig. 9) can be found in Supporting Materials and Methods, which is published as supporting information on thePNAS web site.

#### In Vitro Ubiquitination Assays

In vitro ubiquitination assays were performed as described by Paolini et al. (13) and Matsuda et al. (14), with the following modifications. Assays were performed in a final volume of 150 μl of ubiquitination buffer containing 50 mM Tris-HCl, pH 7.5, 0.5 mM MgCl2, 0.1 mM DTT, 25 μM MG132, 1 mM ATP, 1 mM creatine phosphate, 10 units of creatine phosphokinase (Sigma), 10 μg of Flag-ubiquitin (Sigma), and 3%/vol/vol of RRL (Promega). Individual GST-fusion proteins (75 μg) or GST alone attached to glutathione (GSH) Sepharose 4 Fast Flow resin (Amersham Pharmacia) was added to each assay. GST fusion proteins were quantified by using a Bradford assay (Bio-Rad). Reactions were incubated at 30°C for 3 h. Proteins were purified from the reaction by spinning down the GSH Sepharose and washed five times in a buffer composed of 50 mM potassium phosphate, pH 7.5, 150 mM KCl, 1 mM MgCl2, 10% glycerol, and 1% Triton X-100. For experiments presented in Fig. 2A, proteins were boiled in SDS sample buffer and Western blotted. For experiments presented in Figs. 2B and 4C, after purifying proteins from the ubiquitination assay, proteins were cleaved from the GSH Sepharose by incubation with 10 units of AcTEV protease (Invitrogen) for 3 h at 30°C following the manufacturers protocol. Ubiquitination was detected by using Anti-Flag M2 Peroxidase.

Because AvrPtoB was not detected in the supernatant by Western blot (see Fig. 10, which is published as supporting information on the PNAS web site). Only a weak Ub smear was observed with the K512R, K529R double mutant, and the quadruple mutant, comparable to that observed with GST alone, suggesting that these mutants have decreased E3 activity.

The conclusion that the K-to-R substitutions disrupt E3 activity is strongly supported by insights from the AvrPtoB crystal structure, given the close structural proximity of the tested lysine residues to the predicted Ub binding site (Fig. 5B). For example, lysines K520/K521 are located adjacent to predicted Ub-binding residues F525 and F479. Lysine K529 is located in a flexible loop adjacent to the conserved Ub-binding sites, above residues F525 and P533. Finally, K512 is located in the disordered loop proximal to residues F479 and P533 (Fig. 5B). Arginine residues are larger than lysines and have two additional atoms capable of forming hydrogen-bond contacts, and these differences may disrupt normal AvrPtoB–E2 interactions. Although these substitutions did not cause any gross structural changes that could be detected by circular dichroism (Fig. 9), specific structural interference with Ub binding may lead to observed reduction in AvrPtoB E3 activity. This model is further supported by studies of the E2-binding mutants, F479A, F525A, and P533A (15). Substitutions in the E2-binding domain resulted in a loss of E3 Ub ligase activity, anti-PCD activity in tomato, and loss of DC3000 virulence on RG–pto11 tomato. These results are identical to the observations of the K512R, K529R double mutant, and quadruple mutant.

Although, the K-to-R mutants have decreased E3 activity, this does not preclude the possibility that ubiquitination of AvrPtoB may also be essential for its function. For example, this post-translational modification may play a role in AvrPtoB E3 activity or be required independent of its E3 activity, perhaps by altering AvrPtoB stability or localization. Therefore, our data reveal that AvrPtoB stability or localization. Therefore, our data reveal that AvrPtoB E3 activity is required for suppression of plant immunity, although further experiments will be required to determine whether the virulence mechanism of AvrPtoB is ubiquitination of AvrPtoB itself, or ubiquitination of an unknown host substrate.

---

**Fig. 5.** AvrPtoB K-to-R mutants have decreased E3 Ub ligase activity. (A) To determine whether AvrPtoB K-to-R mutants have altered levels of E3 activity, the supernatants of E3 Ub ligase assays were examined for the presence of high-molecular-weight Ub conjugates. In this assay, the K-to-R mutants K512R, K529R, and the quadruple mutant have decreased E3 Ub ligase activity as compared with wild-type AvrPtoB. (B) Crystal structure of the AvrPtoB436–553 C-terminal domain reveals that the K512, K520, K521, and K529 are all spatially clustered near the conserved E2-binding-site residues F479, F525, and P533, suggesting that the K-to-R substitutions may be interfering with E3 activity by disrupting the E2-binding site.
(horseradish peroxidase)-conjugated primary antibody (Sigma) or polyclonal anti-AvrPtoB primary antibody. In these assays, it is possible that AvrPtoB is binding and copurifying with Flag-ubiquitin. Therefore, we performed the E3 Ub ligase assay described above, with the specific E2 enzyme (Boston Biochem, Cambridge, MA), 2 μg of Flag-UB, and 75 μg of GST-AvrPtoB or GST alone attached to GSH Sepharose were performed in a reaction buffer containing 25 mM Tris-HCl, pH 7.5, 1 mM MgCl$_2$, 120 mM NaCl, 0.3 mM DTT, 2 mM ATP, 1 mM creatine phosphate, and 1 unit of creatine phosphokinase. The assay was incubated for 2 h at 30°C. GST-AvrPtoB was purified from the reaction mixture and washed five times with wash buffer as described for the in vitro ubiquitination assays above. AvrPtoB was cleaved from the TEV protease. Proteins were separated on 10% SDS/PAGE and Western blotted.

AvrPtoB-mediated synthesis of Ub conjugates was assayed by performing the E3 Ub ligase assay described above, with the following modifications. Approximately 1 μg of GST-AvrPtoB, GST-AvrPtoB mutants, or GST alone attached to GSH-Sepharose, was incubated in a E3 Ub ligase assay containing 100 ng of E2 GST-UbcH5c. After the reaction, two rounds of purification were undertaken to ensure no residual E2 or AvrPtoB were present in the supernatant. First, GSH-Sepharose was added to the reaction and purified from the supernatant to remove the GST-UbcH5c from the supernatant. Second, anti-AvrPtoB polyclonal antibody was used to immunoprecipitate any residual GST-AvrPtoB from the cleared supernatant. No GST-AvrPtoB or GST-UbcH5c was detected in the supernatant by using Anti-GST horseradish peroxidase conjugate and anti-AvrPtoB primary antibodies (see Fig. 10).

**Generation of K-to-R Mutants.** AvrPtoB K-to-R mutants were generated by site-directed mutagenesis using the QuickChange mutagenesis protocol supplied by the manufacturer (Stratagene). Mutagenesis was performed on three templates, pCR2.1::AvrPtoB, pCR2.1::hrpAvrPtoB, and pDEST15::GST-AvrPtoB or GST alone attached to GSH-Sepharose were cleaved from the beads with the TEV protease. Proteins were separated on 10% SDS/PAGE and Western blotted from

**Pathogenesis Assays in Tomato.** Tomato plants were inoculated by dipping plants for 30 seconds in a solution containing 2 × 10$^8$ colony-forming units per ml of bacteria in 10 mM MgCl$_2$ and 0.025% Silwet L-77. Plants were incubated in a greenhouse, and disease symptoms and bacterial growth were examined and quantitated 5 days postinoculation. For evaluating tomato sensitivity to DC3000 strains presented in Fig. 7, three tomato plants for each treatment were inoculated by dipping. The experiments were repeated twice for all the tested strains. For counting bacterial growth, three plants of each genotype were inoculated with each strain tested, and two samples were taken from each plant (for a total of six samples per treatment). For each sample, three 1-cm$^2$ leaf disks were floated on 1 ml of 10 mM MgCl$_2$/100 mM sucrose solution in a 12-well plate and shaken at 120 rpm at room temperature for 3 h. Leaf disks were removed from the water, and conductivity was measured by using an Acorn series CONS meter (Oakton Instruments, Vernon Hills, IL).

We thank Jeff Anderson, Phil Bronstein, Lisa Schechter, and Haiyang Wang for critical reading of this manuscript and Nai-Chun Lin and Pete Paszucci for providing important insights about DC3000 pathogenesis assays on tomato. R.B.A. was supported by a fellowship from the Natural Sciences and Engineering Research Council of Canada. Research in the G.B.M. laboratory was supported, in part, by grants from the Triad foundation and the U.S. Department of Agriculture–National Research Initiative. Research in the C.E.S. laboratory was supported, in part, by a Burroughs–Welcome Investigators in Pathogenesis of Infectious Disease award and funds from The Rockefeller University.
Supplemental Figure 1

A

Consensus UIM \( \Phi \; X \; X \; A \; X \; X \; X \; S \)
AvrPtoB(DC3000) \( I \; S \; K \; A \; D \; A \; E \; S \)
VirPphA \( I \; S \; K \; A \; D \; A \; E \; S \)
AvrPtoB (JL1065) \( V \; S \; K \; A \; D \; A \; A \; S \)
AvrPtoB (PT23) \( V \; S \; K \; A \; D \; A \; A \; S \)
AvrPtoB (B728a) \( V \; S \; K \; A \; D \; A \; A \; S \)
AvrPtoB (T1) \( V \; S \; K \; A \; D \; A \; A \; S \)
VirPphA_{gly} \( I \; S \; K \; A \; D \; A \; E \; S \)
VirPphA_{sv} \( I \; S \; K \; A \; D \; A \; E \; S \)

B

<table>
<thead>
<tr>
<th></th>
<th>L</th>
<th>MonoUb</th>
<th>PolyUb</th>
<th>L</th>
<th>10% Input</th>
</tr>
</thead>
<tbody>
<tr>
<td>AvrPtoB</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>M-Ub</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P-Ub</td>
</tr>
</tbody>
</table>

WB: \( \alpha \)-ubiquitin

C

<table>
<thead>
<tr>
<th>UbcH2</th>
<th>UbcH3</th>
<th>UbcH5a</th>
<th>UbcH5b</th>
<th>UbcH6</th>
<th>UbcH7</th>
<th>UbcH10</th>
</tr>
</thead>
</table>

WB: \( \alpha \)-Flag

WB: \( \alpha \)-AvrPtoB

D

<table>
<thead>
<tr>
<th>UbcH2</th>
<th>UbcH3</th>
<th>UbcH5a</th>
<th>UbcH5b</th>
<th>UbcH5c</th>
<th>UbcH6</th>
<th>UbcH7</th>
<th>UbcH10</th>
</tr>
</thead>
</table>

WB: \( \alpha \)-AvrPtoB

E

NaCl (150 mM-1M)

Detergent (0.5% SDS/2% DO-0.75% SDS/4% DO)

PD: GST

WB: \( \alpha \)-Flag

Coomassie
### Supplemental Figure 2

#### A

![Images showing RG-PtoR and RG-prf3 constructs with various mutants and AvrPtoB](image)

#### B

![Image showing RG-pto11 with K512R and K512R + AvrPtoB](image)

#### C

<table>
<thead>
<tr>
<th></th>
<th>DC3000 RG-PtoR</th>
<th>DC3000 RG-pto11</th>
<th>DC3000 RG-prf3</th>
<th>DC3000::ΔavrPtoB RG-PtoR</th>
<th>DC3000::ΔavrPtoB RG-pto11</th>
<th>DC3000::ΔavrPtoB RG-prf3</th>
</tr>
</thead>
<tbody>
<tr>
<td>K453R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>K512R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>K520R/K521R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>K529R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>K546R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>AvrPtoB(WT)</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>Vector</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

*Legend: R = resistant, S = susceptible*
AvrPto + Pto

Empty Vector


AvrPto + Pto

Empty Vector

AvrPtoB (308-553)  K512R, K520R, K521R  K529R  Quad  AvrPtoB (1-387)  Vector

B

WB: α-AvrPtoB

Coomassie

D

Spearman rank rs = -0.854, p = 0.0016

Supplemental Figure 3
Supplemental Figure 5