# *Pseudomonas syringae* Type III Effector AvrPtoB Is Phosphorylated in Plant Cells on Serine 258, Promoting Its Virulence Activity[*,S]

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## Abstract

The *Pseudomonas syringae* pv. *tomato* protein AvrPtoB is translocated into plant cells via the bacterial type III secretion system. In resistant tomato leaves, AvrPtoB acts as an avirulence protein by interacting with the host Pto kinase and eliciting the host immune response. Pto-mediated immunity requires Prf, a Pto-interacting protein with a putative nucleotide-binding site and a region of leucine-rich repeats. In susceptible tomato plants, which lack either Pto or Prf, AvrPtoB acts as a virulence protein by promoting *P. syringae* pv. *tomato* growth and enhancing symptoms associated with bacterial speck disease. The N-terminal 307 amino acids of AvrPtoB (designated AvrPtoB$_{1-307}$) are sufficient for these virulence activities and for Pto-mediated avirulence. We report that AvrPtoB is phosphorylated by a Pto- and Prf-independent kinase activity that is conserved in several plant species, including tomato (*Solanum lycopersicum*), *Nicotiana benthamiana*, and *Arabidopsis thaliana*. AvrPtoB$_{1-307}$ was phosphorylated in tomato protoplasts, and mass spectrometry identified serine 258 as the major *in vivo* phosphorylation site of this protein. An alanine substitution of Ser$^{258}$ resulted in the loss of virulence and the diminution of avirulence activity of AvrPtoB$_{1-307}$, whereas a phosphomimetic S258D mutant had activities similar to wild type AvrPtoB$_{1-307}$. These observations suggest that AvrPtoB has evolved to mimic a substrate of a conserved plant kinase, leading to enhancement of its virulence and avirulence activities in the host cell.

Pathogens of plants have evolved strategies to evade or suppress both basal and R (resistance) gene-mediated host defenses (1). For example, *Pseudomonas syringae* pv. *tomato* (*Pst*),[2] the causative organism of bacterial speck disease in tomato, uses its type III secretion system to deliver ~30 "effector" proteins into the plant cell (1–3). These sequence-diverse proteins are collectively essential for pathogenesis, and many appear to have functionally redundant roles in promoting bacterial virulence (4). Mounting evidence suggests that a major function of type III effectors is the suppression of the plant basal defenses that are induced following detection of pathogen-associated molecular patterns by host pattern recognition receptors (5–8). Pathogen-associated molecular patterns are structurally conserved and functionally

indispensable in both pathogenic and nonpathogenic bacteria and reveal the presence of a potential danger to the plant upon recognition by pattern recognition receptors (9).

To overcome the suppression of basal defense by type III effectors, plants have evolved R genes that encode proteins that directly or indirectly recognize certain effectors thereby activating a strong plant immune response (10). R gene-mediated defense is usually associated with rapid localized cell death known as the hypersensitive response (11). Effectors which are detected by plant R proteins are referred to as avirulence (Avr) proteins. In response to this host surveillance mechanism, bacteria have evolved additional effectors to interfere with hypersensitive response-associated plant immunity (12–15). Type III effector proteins therefore play a central role in plant-pathogen interactions, and it is important to understand the molecular mechanisms they employ to manipulate host defense responses (1,3,16).

Type III effector proteins function within the plant cell, and interestingly, many of them appear to be post-translationally modified by host enzymes. For example, effectors have been reported to be acylated, phosphorylated, or proteolytically cleaved after their delivery into plant cells (8,16,17). In some cases, these post-translational modifications have been shown to be important for the function of effectors in the host cell. For example, effectors AvrPto, AvrRpm1, and AvrB are myristylated, and this modification promotes their localization to the plasma membrane, which is critical for the avirulence activity of all three effectors and for the virulence activity of AvrPto and AvrRpm1 (18,19). *Pseudomonas* effectors AvrPto and AvrB are both phosphorylated by host kinases, and this alteration is required for their full avirulence and virulence activities (20,21). Recently, two type III effectors from *Rhizobium* strain NGR234, NopL and NopP, have been shown to be phosphorylated by plant extracts (22–24). Both NopL and NopP are required for the nodulation ability of NGR234 on certain legumes, but it is unknown yet whether phosphorylation is involved in this function.

In the interaction between *Pst* strain DC3000 and tomato plants, resistance is conferred by the host R protein Pto, a serine/threonine kinase (25,26). Although molecular details of the recognition mechanism are unknown, Pto physically interacts with either of two *Pst* DC3000 effectors, AvrPto or AvrPtoB, and activates immunity by interacting with the host protein Prf, an NB-ARC LRR protein (26–29). When either Pto or Prf is absent, the plant is susceptible to *Pst* DC3000. Moreover, in this case AvrPto and AvrPtoB act as virulence factors promoting bacterial growth and inducing ethylene biosynthesis, which promotes disease-associated host cell death (4,30–33).

We have found previously that AvrPto is both phosphorylated and myristylated after it is delivered into the plant cell by *Pst* (21). The N terminus-dependent myristylation is critical for plasma membrane localization of AvrPto and indispensable for virulence activity and for eliciting Pto/Prf-mediated immunity (18,21). Phosphorylation of AvrPto occurs at serine 149 located in the C-terminal region of AvrPto (21). An alanine substitution at Ser$^{149}$ significantly decreased AvrPto virulence activity and partially compromised its ability to elicit Pto/Prf-mediated immunity, suggesting that phosphorylation of this residue plays an important role in AvrPto function (21).

AvrPtoB was originally identified as an avirulence protein based on its ability to trigger immunity on resistant tomato plants expressing Pto and Prf (34). AvrPtoB was later found to have two distinct avirulence determinants, both of which are in the N-terminal region of AvrPtoB. One, contained within the first 307 amino acids of the protein (AvrPtoB$_{1–307}$), is recognized by the Pto kinase (13). The other requires the additional amino acids 308–387 (AvrPtoB$_{1–387}$) and is recognized by the Fen kinase (13,15).

AvrPtoB is a modular protein with both pathogenicity and virulence activities. The C-terminal region is an E3 ligase that acts as a pathogenicity factor by ubiquitinating the host R protein

Fen, thereby promoting its degradation and causing disease susceptibility (15,35,36). The N-terminal region has two distinct virulence activities (33). In susceptible tomato plants (lacking either Pto or Prf), AvrPtoB$_{1-307}$ is sufficient for promoting bacterial growth and enhancing disease symptoms associated with increased ethylene production (33). This ethylene-associated virulence is dependent on phenylalanine 173 in AvrPtoB. Substitution of an alanine for this residue abolishes the virulence activity of AvrPtoB$_{1-307}$ (33). AvrPtoB residues 308–387 are required for a second virulence activity that involves the suppression of pathogen-associated molecular pattern-triggered immunity in *Arabidopsis* (33).

In susceptible tomato plants, the virulence activities of AvrPtoB$_{1-307}$, AvrPtoB$_{1-387}$, or the full-length AvrPtoB are indistinguishable (33), indicating that amino acids 308–553 either lack virulence activity in tomato or have an activity that is phenotypically redundant. Thus, AvrPtoB$_{1-307}$ is sufficient both for eliciting Pto/Prf-dependent immunity in resistant tomato plants and for promoting bacterial virulence in susceptible tomato plants. Our primary interest in this work was the AvrPtoB virulence activity in tomato, and we therefore focused initially on AvrPtoB$_{1-307}$.

Here we report that AvrPtoB is phosphorylated by a Pto- and Prf-independent protein kinase activity that is conserved in a diverse array of plant species. We used mass spectrometry to identify the *in vivo* phosphorylated residue and demonstrate that this residue plays an important role in AvrPtoB$_{1-307}$ virulence activity. Our data support the emerging theme that type III effector proteins have evolved to mimic substrates of certain host enzymes to manipulate host defense signaling.

## EXPERIMENTAL PROCEDURES

### AvrPtoB Cloning and Mutagenesis

AvrPtoB$_{1-307}$ tagged at the C terminus with a hemagglutinin (HA) epitope was generated previously (33). AvrPtoB$_{1-307}$ S258A and S258D mutants were generated using a site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the following primers: 5′-AGACGCCGGTCGACAGGGCCCCGCCACGCGTCAACCAAAG-3′; 5′-CTTTGGTTGACGCGTGGCGGGGCCCTGTCGACCGGCGTCT, and 5′-AGACGCCGGTCGACAGGGACCCGCCACGCGTCAACCAAAG-3′; and 5′-CTTTGGTTGACGCGTGGCGGGTCCCTGTCGACCGGCGTCT-3′.

### In Vitro Phosphorylation Assay of AvrPtoB

AvrPtoB was expressed in bacteria as a fusion protein in-frame with the C terminus of glutathione *S*-transferase (GST) and purified as described previously using a glutathione-Sepharose resin (21). Five leaf discs (~100 mg) from each plant species were ground in 1 ml of lysis buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 10 mM EDTA, 0.1% Triton X-100, and 10 $\mu$l/ml plant protease inhibitor mixture (Product 9599; Sigma-Aldrich)). Plant extracts were centrifuged at $8000 \times g$ for 3 min, and 10 $\mu$g of the protein extract was used for an *in vitro* phosphorylation assay, which was performed with 10 $\mu$g of AvrPtoB-GST protein in the presence of 10 $\mu$Ci of [$\gamma$-$^{32}$P]ATP for 15 min at room temperature, followed by SDS-PAGE separation and autoradiography.

### In Vivo $^{32}$P Labeling of AvrPtoB

HA-tagged AvrPtoB$_{1-307}$ or its derivatives were transiently expressed in RG-prf3 tomato protoplasts by polyethylene glycol-mediated transformation (33). [$^{32}$P]Orthophosphate (0.1mCi/ml) was added to the protoplasts 1 h after polyethylene glycol treatment and incubation continued for another 7 h in the dark. Protoplasts were collected and lysed with 1 ml of protoplast lysis buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 10 mM EDTA, 1% Triton X-100,

and 10 $\mu$l/ml plant protease and phosphatase inhibitor I cocktails (Product 2850; Sigma-Aldrich)). AvrPtoB$_{1-307}$-HA and derivatives were immunoprecipitated with anti-HA affinity matrix (Roche Applied Science) and subjected to SDS-PAGE, followed by vacuum drying of the gel and autoradiography.

## Mass Spectrometry Analysis

100 $\mu$g of plasmid expressing HA-tagged AvrPtoB$_{1-307}$ was transformed into 1 ml of RG-prf3 protoplasts (about $2 \times 10^5$ cells) and incubated for 9 h in the dark. Transformed protoplasts were collected and lysed with 1 ml of protoplast lysis buffer. The lysates were centrifuged at $12,000 \times g$ for 20 min, and the supernatant was incubated with 100 $\mu$l of anti-HA affinity matrix at 4 °C for 4 h. The immunoprecipitation-complex was separated on 10% SDS-PAGE and stained with Coomassie Brilliant Blue G250. AvrPtoB$_{1-307}$-HA was excised from the gel and sent for mass spectrometry analysis at the Proteomics and Mass Spectrometry Facility (Donald Danforth Plant Science Center, St. Louis, MO). The nano-ESI-MS/MS analysis was performed exactly as described previously (21).

## Pseudomonas Protein Secretion and Western Blotting Assays

pCPP45 plasmids harboring *avrPtoB$_{1-307}$* or its derivatives were transformed into the DC3000Δ*avrPto*Δ*avrPtoB* strain by electroporation. The resulting strains were grown in minimal medium for 24 h at 18 °C, and secreted proteins were detected by standard Western blotting as described previously (37). The antibodies used for Western blotting were anti-HA (Roche Applied Science), anti-NptII (U.S. Biological Corp.), or anti-AvrPtoB (37). Detection of proteins was carried out using horseradish peroxidase-conjugated secondary antibodies and the ECL Plus detection system (Amersham Biosciences).

## Measurement of Bacterial Populations in Tomato Leaves

Two genotypes of Rio Grande (RG) tomato lines were used: RG-PtoR (*Pto*/*Pto*, *Prf*/*Prf*) and RG-prf3 (*Pto*/*Pto*, *prf*/*prf*) (28). The method used to prepare *P. syringae* pv. *tomato* inoculum has been described previously (21). Five- to six-week-old greenhouse grown plants were vacuum-infiltrated with different *P. syringae* pv. *tomato* strains at an inoculum level of $10^4$ colony-forming units/ml and maintained in a climate-controlled growth chamber under optimized conditions (see Ref. 21 for details). Bacterial populations in tomato leaves were measured at 2 or 4 days after infiltration. For consistency, day 2 samples were collected from the third and fourth leaves from the bottom of the plants. To assess the effect of Ser$^{258}$ substitutions on the virulence and avirulence activities of AvrPtoB$_{1-307}$ and mutants, analysis of variance of bacterial populations on plants was based on the pooled data from three experiments using the general linear model procedure of a statistical analysis system (SAS Institute Inc., Cary, NC). The least significance difference at a 0.05 probability level was used to test the differences between means.

# RESULTS

To examine whether AvrPtoB is phosphorylated, we first adopted the *in vitro* phosphorylation assay, previously used for AvrPto, to test whether AvrPtoB could be phosphorylated by tomato leaf extracts (21). Recombinant AvrPtoB fused to GST was expressed in *Escherichia coli* and purified with a glutathione-Sepharose resin. The *in vitro* phosphorylation assay of AvrPtoB was performed in the presence of leaf extracts from resistant tomato RG-PtoR leaves (*Pto*/*Pto*, *Prf*/*Prf*) that express a functional Pto kinase. We observed incorporation of $^{32}$P into GST-AvrPtoB and not GST alone in the presence of leaf extracts (Fig. 1 and supplemental Fig. S1). GST-AvrPtoB without leaf extracts or RG-PtoR leaf extracts without AvrPtoB showed no evidence of $^{32}$P incorporation in this assay (Fig. 1). These observations indicated that AvrPtoB can be phosphorylated by a kinase activity present in RG-PtoR tomato leaves.

To assess whether phosphorylation of AvrPtoB is dependent on the Pto or Prf proteins that function to recognize the effector, we used leaf extracts from RG-pto11 (*pto/pto Prf/Prf*) or RG-prf3 (*Pto/Pto prf/prf*) plants for the *in vitro* phosphorylation analysis of AvrPtoB. RG-pto11 plants lack a functional Pto kinase because of a point mutation in the *Pto* gene, whereas RG-prf3 plants have a 1-kb deletion in the *Prf* gene rendering the plants susceptible to avirulent *Pst* strains despite their having a functional *Pto* gene (28). Leaf extracts from both RG-pto11 and RG-prf3 plants phosphorylated AvrPtoB as well as RG-PtoR leaf extracts did (Fig. 1). We further tested whether the kinase activity responsible for AvrPtoB phosphorylation is conserved in other plant species. Leaf extracts from the wild tobacco species *Nicotiana benthamiana* or *Arabidopsis thaliana* were tested, and both were found able to phosphorylate AvrPtoB (Fig. 1). Thus, AvrPtoB is phosphorylated by a Pto- or Prf-independent kinase activity that is conserved in several plant species.

We narrowed our initial focus to $AvrPtoB_{1-307}$ because this region is recognized by Pto/Prf and is the minimal fragment in tomato with virulence activity. To test whether this region is phosphorylated *in vivo*, we developed a construct expressing $AvrPtoB_{1-307}$ with a C-terminal HA tag and transiently transformed it into RG-prf3 protoplasts in the presence of $[^{32}P]$ orthophosphate. $AvrPtoB_{1-307}$-HA was then immunoprecipitated with an anti-HA antibody and subjected to SDS-PAGE to monitor the phosphorylation of $AvrPtoB_{1-307}$ as indicated by incorporation of $^{32}P$. $AvrPtoB_{1-307}$ appeared as single $^{32}P$-labeled band (Fig. 2), indicating that the N-terminal domain of AvrPtoB is phosphorylated in plant cells.

To characterize the *in vivo* phosphorylation site of $AvrPtoB_{1-307}$, we performed nano-ESI-MS/MS on the phosphorylated protein. $AvrPtoB_{1-307}$-HA or an empty vector control were expressed in RG-prf3 tomato protoplasts, the proteins were immunoprecipitated with an anti-HA antibody and resolved by SDS-PAGE. Staining of this gel revealed a putative $AvrPtoB_{1-307}$-HA band that was absent from the empty vector control (Fig. 3*A*). This band was excised from the gel for tryptic in-gel digestion and subjected to nano-ESI-MS/MS analysis. The resulting MS and MS/MS spectra were searched against the NCBInr data base using the Mascot software tool and revealed a clear identification of AvrPtoB as the protein present in this gel band (Fig. 3*B*). The protein was represented by several peptides providing an overall sequence coverage of ~65% (Fig. 3*B*). Among the identified peptides one was selected for MS/MS because it represented a single triply charged precursor with a mass-to-charge (*m/z*) ratio of 617.57, which matches the *m/z* of a singly phosphorylated peptide from AvrPtoB (namely amino acids 245–261; SSNTAASQTPVDRSPPR). A series of *y*-ions and several *b*-ion fragments revealed serine 258 to be the phosphorylated amino acid residue in this peptide (Fig. 3, *C* and *D*).

We next sought to confirm that $Ser^{258}$ is a phosphorylation site of $AvrPtoB_{1-307}$ in the plant cell. We generated a construct expressing an $AvrPtoB_{1-307}$(S258A) mutant and repeated the *in vivo* labeling assay to evaluate the level of $[^{32}P]$orthophosphate incorporation of the mutant compared with the wild type $AvrPtoB_{1-307}$ protein. The level of phosphate incorporation by each protein was evaluated by autoradiography after immunoprecipitation with anti-HA antibody and SDS-PAGE. The alanine substitution at $Ser^{258}$ reduced the level of $^{32}P$ incorporation by ~80% compared with wild type $AvrPtoB_{1-307}$ (Fig. 4). Western blotting indicated that both $AvrPtoB_{1-307}$ and the $AvrPtoB_{1-307}$(S258A) mutant proteins were equally expressed in tomato protoplasts (Fig. 4*A*). Therefore, we conclude that $Ser^{258}$ is the major *in vivo* phosphorylation site of $AvrPtoB_{1-307}$. Because a low level of $^{32}P$ incorporation of $AvrPtoB_{1-307}$(S258A) mutant was detected, it is possible there are other weakly phosphorylated residues in $AvrPtoB_{1-307}$ that were not identified by mass spectrometry. Alternatively, blocking of $Ser^{258}$ might result in other residues being phosphorylated.

To gain insight into a possible role of $Ser^{258}$ phosphorylation in the virulence activity of $AvrPtoB_{1-307}$, we used the $DC3000\Delta avrPto\Delta avrPtoB$ strain, which has deletions in both *avrPto* and *avrPtoB* genes and is less virulent on susceptible tomato than the wild type DC3000 strain and no longer avirulent on resistant tomato plants (4). We transformed into this strain a broad host range plasmid expressing either wild type $AvrPtoB_{1-307}$, $AvrPtoB_{1-307}(S258A)$, $AvrPtoB_{1-307}(S258D)$, or an empty vector control. The S258D substitution is a potential "phosphomimetic" mutation, mimicking the negative charge of a phosphorylated residue. The virulence activity of each of these strains was first evaluated by vacuum infiltrating them into susceptible RG-prf3 tomato plants and assessing their ability to promote more severe disease symptoms (Fig. 5*A*). At 4 days after infiltration, we observed that the strain expressing wild type $AvrPtoB_{1-307}$ caused severe necrosis of the lower leaves, whereas the empty vector control strain did not cause this phenotype (Fig. 5*A*, *red arrows*). Significantly, the S258A substitution abolished this disease promoting activity of $AvrPtoB_{1-307}$, whereas the possible phosphomimetic S258D variant retained its ability to cause enhanced disease of the lower leaves (Fig. 5*A*).

As another measure of virulence activity, we assessed bacterial growth of each strain in susceptible tomato leaves 2 days after infiltration (leaf necrosis at later time points interfered with growth measurements). The $DC3000\Delta avrPto\Delta avrPtoB$ strains expressing wild type $AvrPtoB_{1-307}$ or the $AvrPtoB_{1-307}(S258D)$ variant grew to a statistically significantly higher level than the strains carrying $AvrPtoB_{1-307}(S258A)$ or the empty vector control (Fig. 5*B*). To exclude the possibility that the disease and growth phenotypes we observed are due to unequal expression and/or secretion of the proteins from *Pst*, type III secretion assays were performed. Each of the three $AvrPtoB_{1-307}$ proteins was expressed and secreted by *Pst* (Fig. 5*C*). Taken together, these assays indicate that the phosphorylation of $Ser^{258}$ is required for the full virulence activity of $AvrPtoB_{1-307}$.

We recently identified phenylalanine 173 as a key virulence determinant of $AvrPtoB_{1-307}$ (33). To investigate the relationship between $Phe^{173}$ and the phosphorylated residue $Ser^{258}$, we generated F173A/S258A and F173A/S258D mutants in $AvrPtoB_{1-307}$ and tested the virulence activities of these proteins (Fig. 6). If $Phe^{173}$ and $Ser^{258}$ act independently, they would be expected to contribute additively to the virulence of $AvrPtoB_{1-307}$. Specifically, F173A/S258A and F173A/S258D may exhibit distinct degrees of virulence compared with F173A or S258A single mutants. However, we found that both F173A/S258A and F173A/S258D double mutants had virulence activity similar to that of the F173A or S258A single mutants, all of which led to less severe disease and lower bacterial populations than wild type $AvrPtoB_{1-307}$ or, significantly, than the $AvrPtoB_{1-307}(S258D)$ protein (Fig. 6).

Finally, we investigated whether the phosphorylation of $Ser^{258}$ might affect the avirulence activity of $AvrPtoB_{1-307}$. The same $DC3000\Delta avrPto\Delta avrPtoB$ strains used above were vacuum-infiltrated into resistant RG-PtoR plants. Avirulence activity was evaluated 4 days after inoculation using the presence of visible specks and the level of bacterial growth. As expected, *Pst* strains carrying an empty vector caused speck disease on RG-PtoR tomato plants, whereas *Pst* expressing $AvrPtoB_{1-307}$ did not exhibit any speck disease (data not shown). Interestingly, two to three specks/leaflet were observed on plants infiltrated with *Pst* expressing $AvrPtoB_{1-307}(S258A)$, whereas no specks were detected on the plants infiltrated with the *Pst* strain expressing $AvrPtoB_{1-307}(S258D)$ mutant (data not shown). Bacterial populations in leaves were measured at 4 days after infiltration. *Pst* strains expressing wild type $AvrPtoB_{1-307}$ or $AvrPtoB_{1-307}(S258D)$ attained a population of ~$10^5 cfu/cm^2$, whereas the *Pst* strain carrying empty vector reached $10^8 cfu/cm^2$ (Fig. 7). Notably, the strain expressing $AvrPtoB_{1-307}(S258A)$ reached a population about 10-fold greater than *Pst* strains expressing either $AvrPtoB_{1-307}$ or $AvrPtoB_{1-307}(S258D)$ (Fig. 7). These results indicate that the S258A

substitution partially compromises the avirulence activity of AvrPtoB$_{1-307}$ and suggest that phosphorylation of Ser$^{258}$ contributes to the full avirulence activity of AvrPtoB.

## DISCUSSION

We have identified a phosphorylation site in the type III effector AvrPtoB that enhances the ability of the protein to promote bacterial growth and enhance disease on tomato. The identity of the kinase(s) responsible for this phosphorylation is unknown, but it acts independently of both Pto and Prf, host proteins involved in recognition of AvrPtoB and subsequent immune signaling. Two other *P. syringae* effectors, AvrPto and AvrB, have been reported to be phosphorylated by host proteins (20,21). In both of those cases, the kinase identity is also unknown but was found to be independent of host proteins that are involved in effector recognition (Pto/Prf for AvrPto and RIN4/RPM1 for AvrB (20,21)). Several type III effectors of bacterial pathogens of plants have been shown previously to be acylated after delivery into plant cells, and our present data firmly establish that phosphorylation is another common post-translational mechanism involved in activation of type III effectors.

Substitution of a negatively charged amino acid into a protein can often mimic a phosphorylated residue. This appeared to be the case for S258D as AvrPtoB$_{1-307}$(S258D) exhibited full virulence and avirulence activity (in contrast to AvrPtoB$_{1-307}$(S258A), which was compromised for both of these activities). We took advantage of the phosphomimetic S258D substitution to examine the relationship between a previously identified virulence determinant, Phe$^{173}$, and the phosphorylated residue Ser$^{258}$. Significantly, introduction of F173A into the AvrPtoB$_{1-307}$(S258D) protein completely abolished the ability of the protein to enhance disease symptoms and increase *Pst* growth. This result suggests that Phe$^{173}$ may be the "primary" virulence determinant of AvrPtoB$_{1-307}$ and that phosphorylation of Ser$^{258}$ facilitates the function of Phe$^{173}$. This facilitation could be based on either an intramolecular interaction between the two regions carrying these residues or possibly on their interaction with a host protein. These observations differ from what we have found previously for AvrPto. AvrPto has two virulence determinants, the CD loop and a phosphorylated residue Ser$^{149}$, and they appear to act additively and not synergistically (21).

In earlier work, we found that an alanine substitution at Phe$^{173}$ of AvrPtoB$_{1-307}$ also abolishes Pto-mediated recognition of this protein (*i.e.* avirulence activity of AvrPtoB$_{1-307}$ is disrupted (33)). Here we found that the S258A substitution partially compromised AvrPtoB$_{1-307}$ avirulence activity. This is again consistent with the possibility that the regions containing these amino acids are involved in either an intramolecular interaction or that they are both involved in an interaction with a host protein (in this case possibly Pto or Prf). Interestingly, this type of cooperativity between distinct parts of an effector for avirulence was also observed with AvrPto (21). In that case, we found that an alanine substitution of phosphorylated residue Ser$^{149}$ compromised avirulence mediated by the CD loop. A fuller understanding of possible avirulence and virulence-associated interactions between Phe$^{173}$ and Ser$^{258}$ will probably require a crystal structure of AvrPtoB$_{1-307}$. Although a three-dimensional structure for AvrPto exists (38), it unfortunately does not encompass Ser$^{149}$ and therefore has shed no light on the interaction between that residue and the CD loop.

It remains unknown whether the same kinase activity is involved in the phosphorylation of both AvrPto and AvrPtoB. For both proteins, the phosphorylation involves a Pto- and Prf-independent kinase activity that is conserved in diverse plant species (note that even protein extracts from a monocot, rice, are able to specifically phosphorylate AvrPto).[3] However, there does not appear to be any sequence similarity between the phosphorylation sites of AvrPto

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[3]J. Anderson and G. B. Martin, unpublished observations.

($^{145}$NPSGpSIRMS$^{153}$) and AvrPtoB ($^{254}$PVDRpSPPRV$^{262}$). It is possible, of course, that these two regions are located in a similar three-dimensional structure. We are currently using genomics and biochemical approaches to isolate the AvrPto kinase, and if this is successful, we will be able to directly test whether that kinase can phosphorylate AvrPtoB.

Although we do not yet have an estimate of the mass of the AvrPtoB kinase, it is intriguing that the Ser$^{258}$ phosphorylation site bears some similarity to a consensus MAPK site (P$X$(pS/T)P (39)). It was reported recently that phosphorylation of the *Rhizobium* type III effector NopL was partially suppressed by the MAPK kinase inhibitor PD 98059, possibly suggesting involvement of a MAPK in that phosphorylation event (24). We have directly tested one MAPK, wound-induced protein kinase, with a well characterized role in defense signaling for the ability to phosphorylate AvrPtoB$_{1-307}$ *in vitro*, but no phosphorylation was observed.[4] However, plants have a large number of MAPKs (~20 in *Arabidopsis*), and a more systematic effort will be required to thoroughly examine this possibility.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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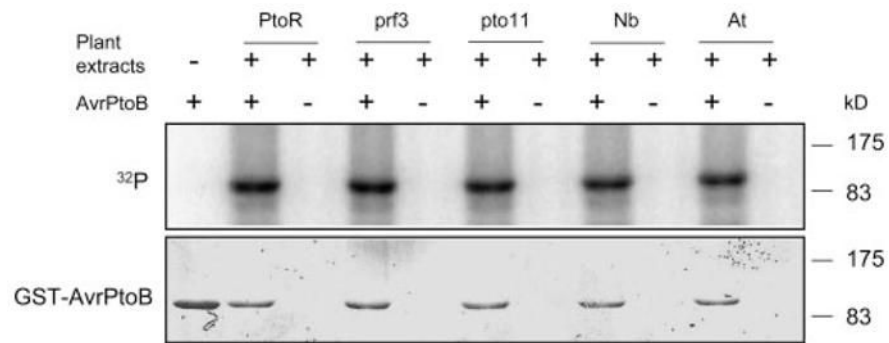39. Gonzalez FA, Raden DL, Davis RJ. J Biol Chem 1991;266:22159–22163. [PubMed: 1939237]

**FIGURE 1.** *In vitro* **phosphorylation of AvrPtoB by leaf extracts from different tomato lines and other plant species**
Recombinant GST-AvrPtoB protein was expressed in and purified from *E. coli* with glutathione-Sepharose resin. GST-AvrPtoB was incubated with leaf extracts in the presence of [$\gamma$-$^{32}$P]ATP and separated by 10% SDS-PAGE, followed by autoradiography. Incorporation of $^{32}$P into GST-AvrPtoB (*top panel*) occurred in the presence of leaf extracts from tomato lines RG-PtoR (*PtoR*), RG-prf3 (*prf3*), RG-pto11 (*pto11*), *N. benthamiana* (*Nb*), and *A. thaliana* Columbia-0 (*At*). Equal loading of GST-AvrPtoB protein was verified by Coomassie Blue staining (*bottom panel*).
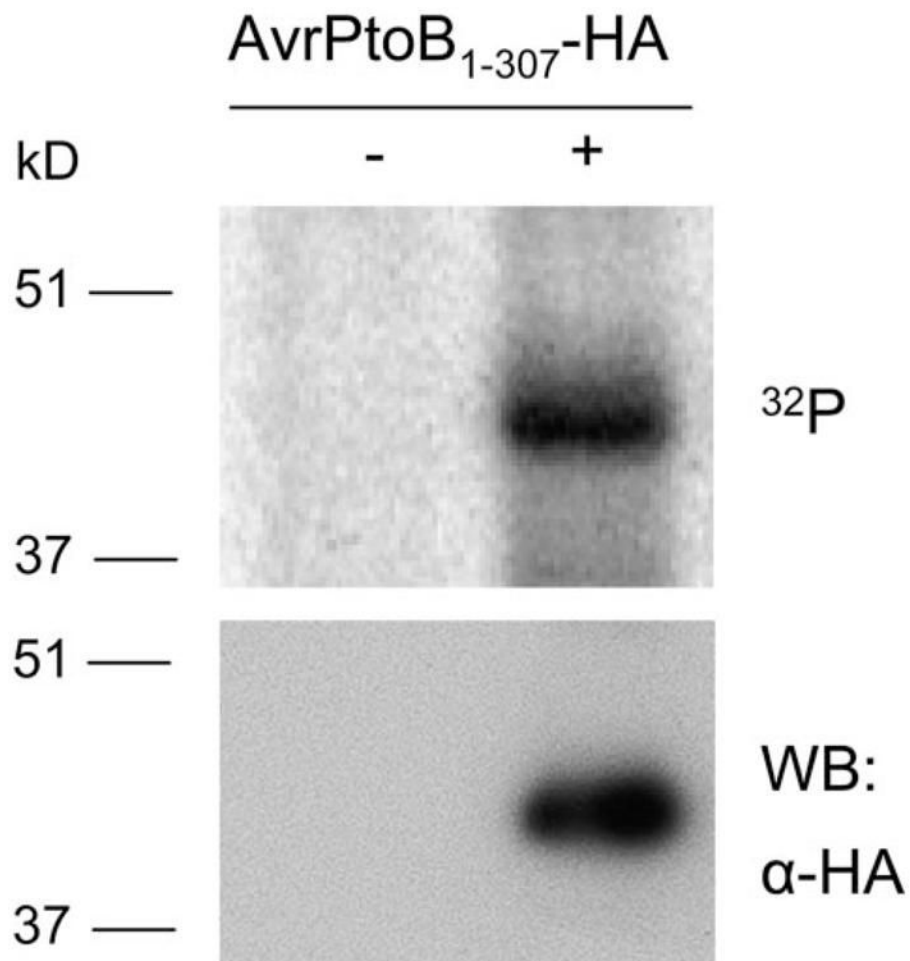
**FIGURE 2.** *In vivo* **phosphorylation of AvrPtoB$_{1-307}$ in plant cells**
AvrPtoB$_{1-307}$ with a C-terminal HA epitope tag was expressed in tomato RG-prf3 protoplasts by polyethylene glycol-mediated transformation in the presence of [$^{32}$P]orthophosphate. AvrPtoB$_{1-307}$-HA was immunoprecipitated with anti-HA affinity matrix, followed by SDS-PAGE separation and autoradiography. AvrPtoB$_{1-307}$-HA incorporated [$^{32}$P]phosphate (*top panel*). The identity of AvrPtoB$_{1-307}$-HA band was confirmed by Western blotting (*WB*) with an anti-HA antibody using a small amount of lysate before immunoprecipitation (*bottom panel*).
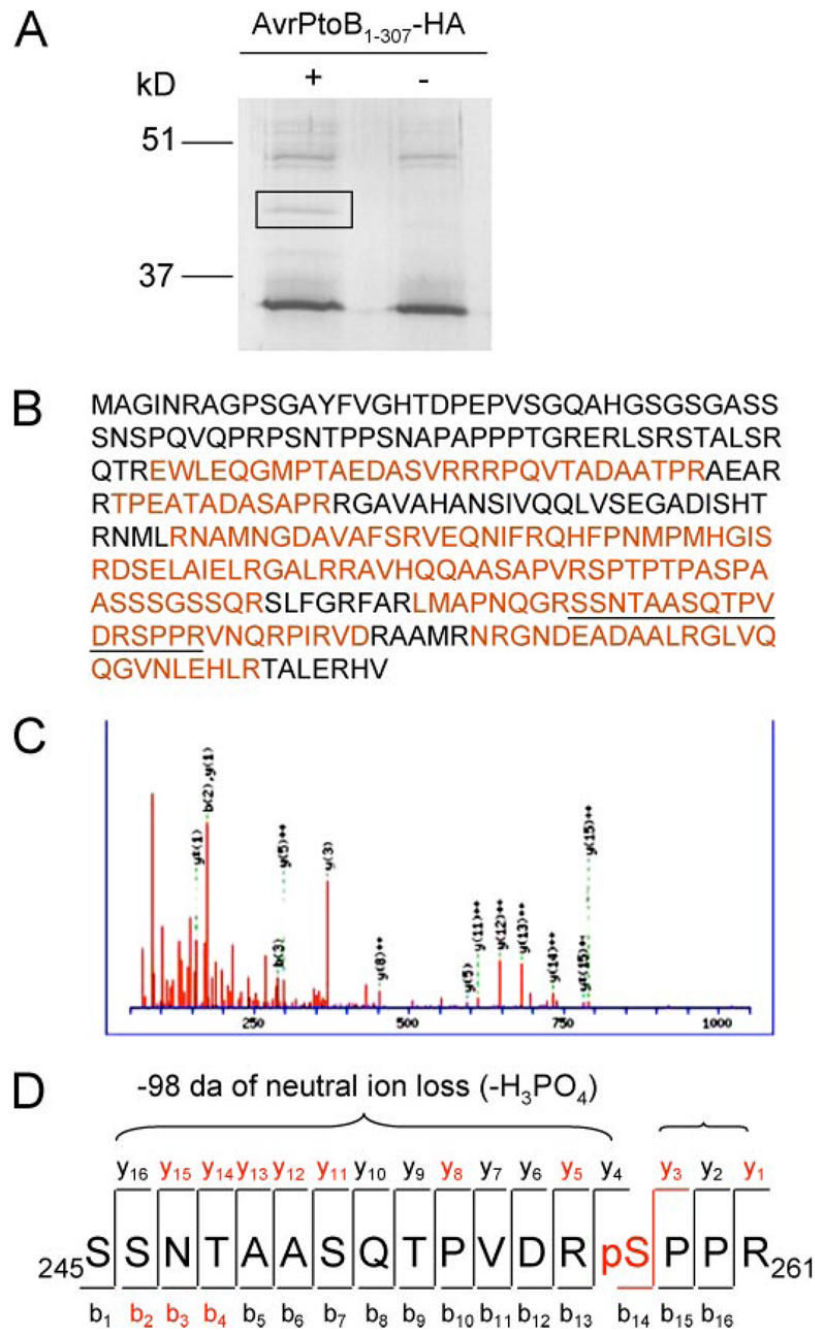
**FIGURE 3. Identification of the *in vivo* phosphorylation site of AvrPtoB$_{1-307}$ by mass spectrometry**
*A*, purification of AvrPtoB$_{1-307}$ protein after expression in plant cells. AvrPtoB$_{1-307}$-HA was transiently expressed in tomato RG-prf3 protoplasts, immunoprecipitated with anti-HA affinity matrix, and separated by SDS-PAGE. AvrPtoB$_{1-307}$-HA appeared as a unique band in the Coomassie Blue-stained gel (marked by *rectangle*), and this band was excised for trypsin digestion. *B*, coverage of AvrPtoB$_{1-307}$ sequence by MS/MS. Sequence recovered from ESI-MS/MS is highlighted in *red*. The peptide [245]SSNTAASQTPVDRSPPR[261] (*underlined*) with mass-to-charge (*m/z*) value of 617.57 was selected for collision-induced dissociation fragmentation. *C*, MS/MS spectrum of precursor [245]SSNTAASQTPVDRSPPR[261] indicates the phosphorylation on Ser[258]. *D*, diagram of the series of *y* fragment ions and several *b*

fragment ions observed in *C*. Several *y*-ions featured loss of 98 Da because of neutral loss of phosphoric acid ($-H_3PO_4$). *Red letters* indicate ions present in the MS/MS spectrum.
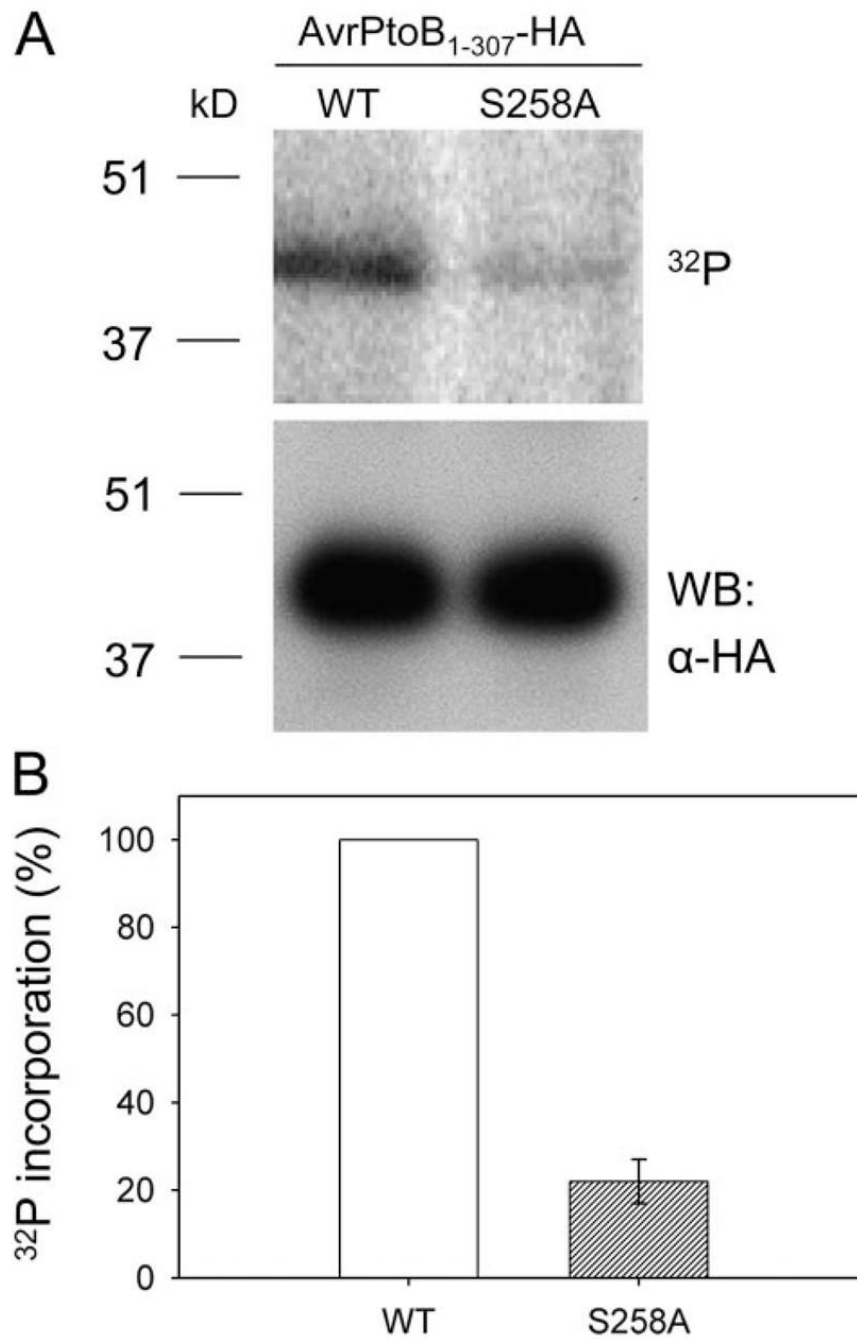
**FIGURE 4. Serine 258 is the major *in vivo* phosphorylation site of AvrPtoB$_{1-307}$**
*A*, phosphorylation assay of wild type (*WT*) AvrPtoB$_{1-307}$ and the AvrPtoB$_{1-307}$(S258A) mutant in RG-prf3 tomato protoplasts. *In vivo* $^{32}$P labeling was performed as described in the legend to Fig. 2. Incorporation of $^{32}$P into AvrPtoB$_{1-307}$ and AvrPtoB$_{1-307}$(S258A) mutant is shown by autoradiography after SDS-PAGE separation (*top panel*). Equal expression of WT AvrPtoB$_{1-307}$ and AvrPtoB$_{1-307}$(S258A) mutant in plant cells was confirmed by Western blotting with an anti-HA antibody using a small amount of lysate before immunoprecipitation (*bottom panel*). *B*, relative $^{32}$P incorporation level of WT AvrPtoB$_{1-307}$ and AvrPtoB$_{1-307}$(S258A) mutant. The data are shown as percentages of $^{32}$P incorporation with WT set to 100%. The *bar* represents standard error from three biological replicates.
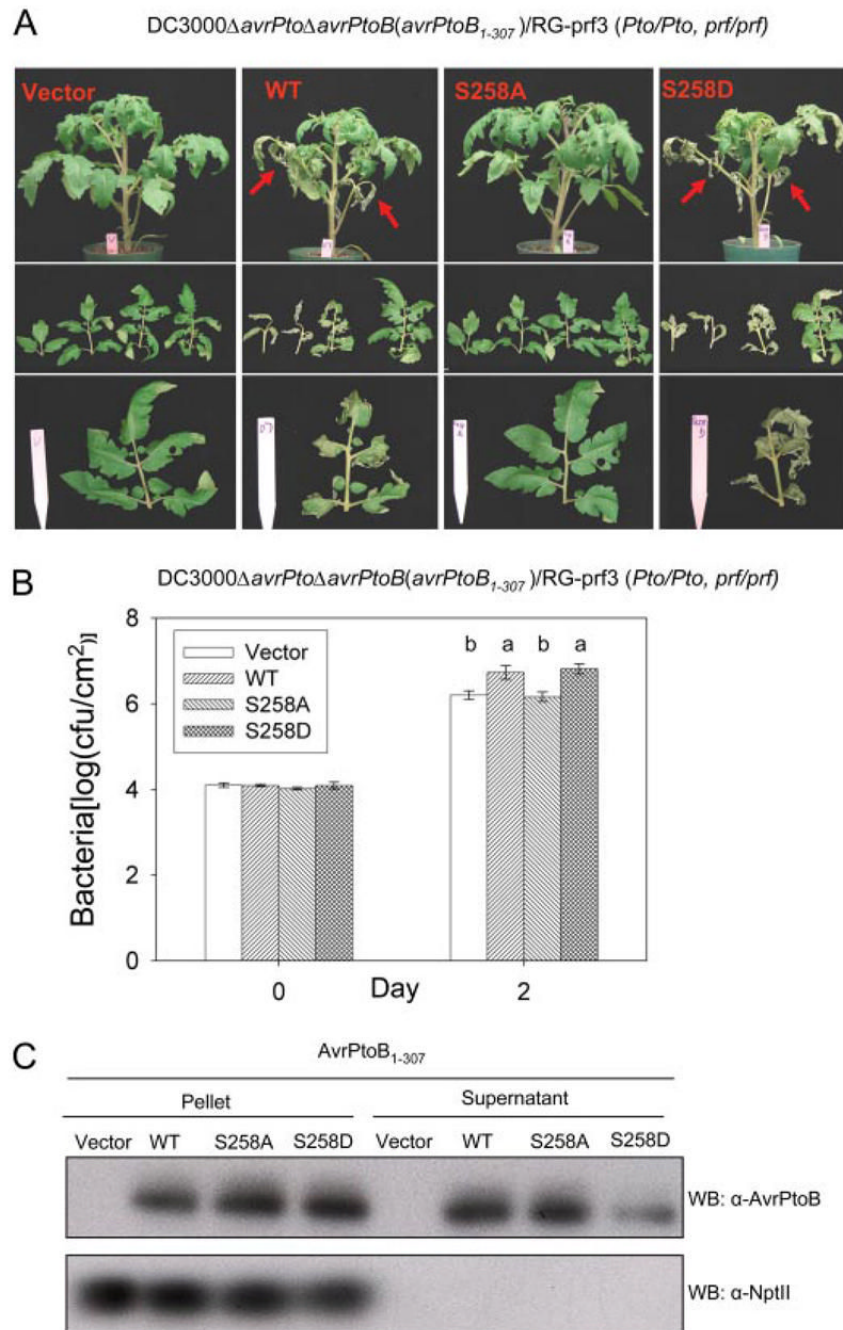
**FIGURE 5. Contribution of phosphorylation of Ser[258] to the virulence activity of AvrPtoB$_{1-307}$**
*Pst* DC3000Δ*avrPto*Δ*avrPtoB* strains carrying either empty vector, or plasmids expressing wild type (*WT*) AvrPtoB$_{1-307}$, AvrPtoB$_{1-307}$(S258A), or AvrPtoB$_{1-307}$(S258D) mutants were vacuum-infiltrated into susceptible RG-prf3 tomato plants using an inoculum level of $10^4$ cfu/ml. *A*, disease symptoms of plants 4 days after infiltration. Severe necrosis observed on lower leaves is indicated by *red arrows* in the *top panel*. The *middle panel* shows four diseased bottom branch leaves, and the *bottom panel* shows comparison of the third branch leaves among plants infiltrated with the different bacterial strains. *B*, *Pst* populations in RG-prf3 leaves shown in *A* at 2 days after inoculation. The data from three independent experiments were used for statistical analysis using the general linear model procedure of a statistical analysis system.

The means shown with the same letters were not significantly different based on least significant difference test ($p = 0.05$). The *bars* indicate standard errors. *C*, expression and secretion of and derivatives from AvrPtoB$_{1–307}$ DC3000Δ*avrPto*Δ*avrPtoB* grown in minimal medium under *hrp* induction conditions (37). Western blotting was performed using anti-AvrPtoB or anti-NptII antibody. Cytoplasmically localized NptII was used as a control for cell lysis. No NptII protein was detected in the culture medium with an anti-NptII antibody.
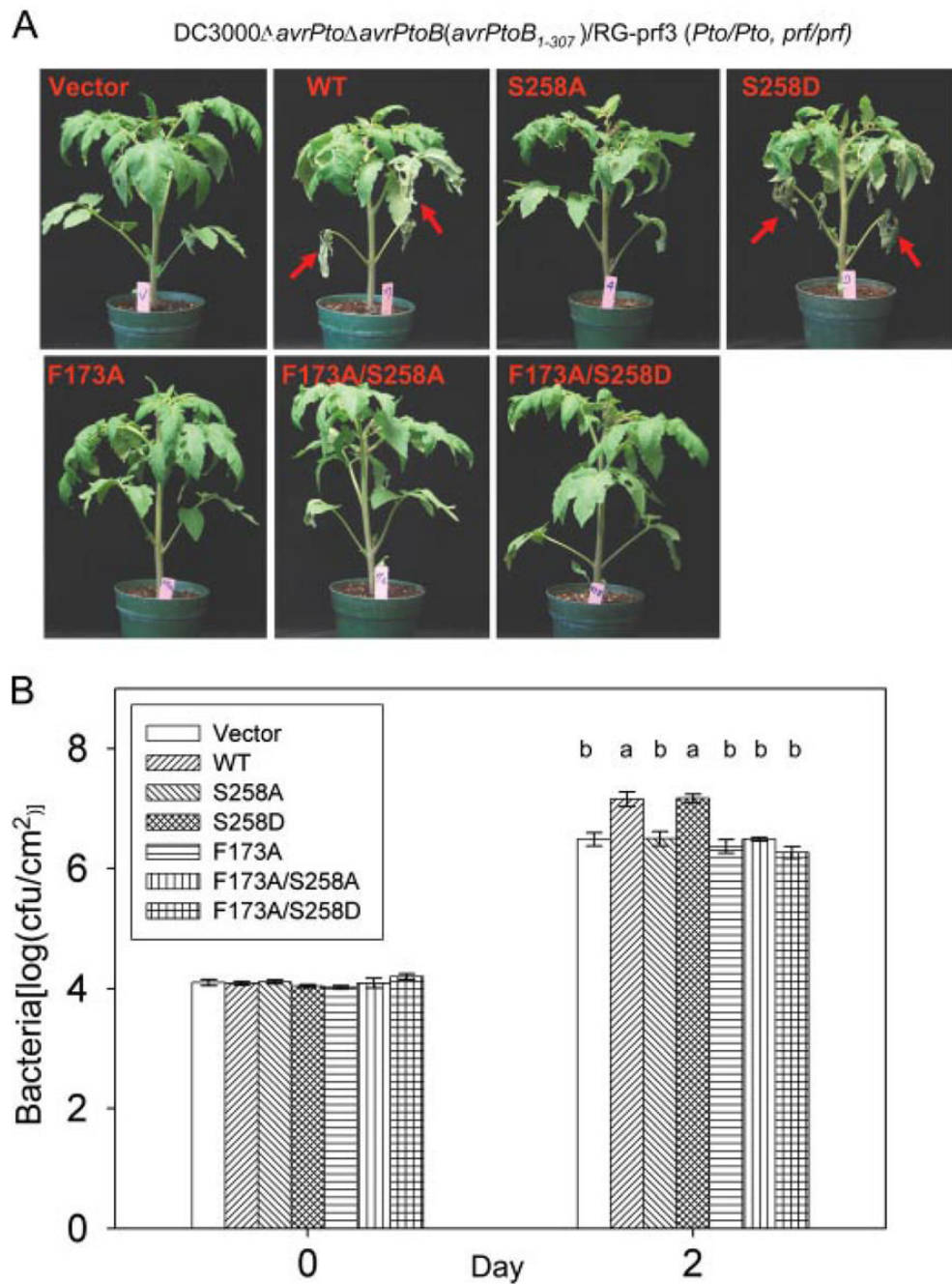
**FIGURE 6. AvrPtoB virulence activity associated with Ser[258] phosphorylation is dependent on Phe[173]-mediated virulence activity**

Disease assay with DC3000Δ*avrPto*Δ*avrPtoB* on susceptible RG-prf3 tomato plants was performed as described in the legend to Fig. 5. *A*, disease symptoms of plants 4 days after infiltration. Severe necrosis observed on lower leaves is indicated by *red arrows*. *B*, bacterial populations in RG-prf3 leaves at 2 days post-inoculation. The data from two independent experiments were used for statistical analysis using the general linear model procedure of a statistical analysis system. The means shown with the same letters were not significantly different from each other based on least significant difference test ($p = 0.05$). The *bars* indicate standard errors. *WT*, wild type.
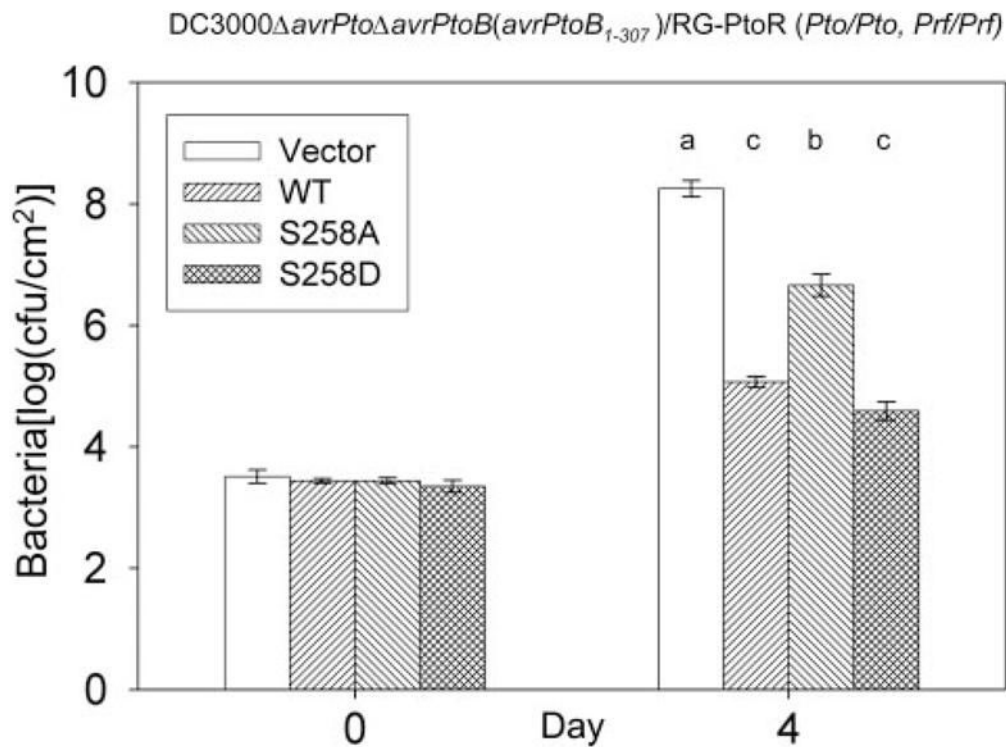
DC3000$\Delta$avrPto$\Delta$avrPtoB(avrPtoB$_{1-307}$)/RG-PtoR (*Pto/Pto, Prf/Prf*)



**FIGURE 7. Requirement of phosphorylation of Ser$^{258}$ for the full avirulence activity of AvrPtoB$_{1-307}$**

The experiments were performed as described in the legend to Fig. 5 except the resistant tomato line, RG-PtoR, was used. Bacterial populations at 4 days after inoculation of leaves. The means shown with the same letters were not significantly different based on least significant difference test ($p = 0.05$). The *bars* indicate standard errors. *WT*, wild type.