

***Pseudomonas* type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death**

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The AvrPtoB type III effector protein is conserved among diverse genera of plant pathogens suggesting it plays an important role in pathogenesis. Here we report that *Pseudomonas* AvrPtoB acts inside the plant cell to inhibit programmed cell death (PCD) initiated by the Pto and Cf9 disease resistance proteins and, remarkably, the pro-apoptotic mouse protein Bax. AvrPtoB also suppressed PCD in yeast, demonstrating that AvrPtoB functions as a cell death inhibitor across kingdoms. Using truncated AvrPtoB proteins, we identified distinct N- and C-terminal domains of AvrPtoB that are sufficient for host recognition and PCD inhibition, respectively. We also identified a novel resistance phenotype, *Rsb*, that is triggered by an AvrPtoB truncation disrupted in the anti-PCD domain. A *Pseudomonas syringae* pv. *tomato* DC3000 strain with a chromosomal mutation in the AvrPtoB C-terminus elicited *Rsb*-mediated immunity in previously susceptible tomato plants and disease was restored when full-length AvrPtoB was expressed *in trans*. Thus, our results indicate that a type III effector can induce plant susceptibility to bacterial infection by inhibiting host PCD.

Keywords: bacterial pathogenesis/effector proteins/plant disease susceptibility/programmed cell death/type III secretion

Introduction

Pseudomonas syringae pv. *tomato* DC3000 is a widely studied model plant pathogen that causes disease on tomato and *Arabidopsis*. DC3000 uses a type III secretion system (TTSS) to directly deliver bacterial effector proteins into the host cell (Galan and Collmer, 1999). Loss-of-function mutations in the TTSS completely abrogate *P. syringae* disease formation, indicating that effectors are essential agents of *P. syringae* pathogenesis (Collmer *et al.*, 2000). Genome-wide analyses of *P. syringae* have defined an inventory of type III effectors (Boch *et al.*, 2002; Fouts *et al.*, 2002; Guttman *et al.*, 2002; Petnicki-Ocwieja *et al.*, 2002; Zwiesler-Vollick *et al.*, 2002) and the functions of these effectors are now being

examined. Although the role of effector proteins in pathogen virulence is poorly understood, many effectors have been isolated based on their ability to trigger host immunity.

In the ‘gene-for-gene’ model of plant immunity, disease resistance is initiated by recognition of a pathogen avirulence (Avr) effector protein by a plant resistance (R) protein. The tomato R protein Pto, a serine/threonine protein kinase, recognizes and directly interacts with DC3000 effector proteins AvrPto and AvrPtoB, and initiates immunity in tomato by characterized and uncharacterized signaling mechanisms (Scofield *et al.*, 1996; Tang *et al.*, 1999; Sessa and Martin, 2000; Kim *et al.*, 2002). In this paper, recognition will be defined as the physical interaction of Pto with an effector protein. Recognition alone, however, is not sufficient to elicit immunity. For example, Pto-dependent immunity requires the *Prf* gene (Salmeron *et al.*, 1996) and presumably other additional factors to signal host immunity (Shirasu and Schulze-Lefert, 2000; Martin *et al.*, 2003).

The R gene-mediated plant immune response is characterized by a series of physiological changes in the plant cell, including the formation of reactive oxygen species, induction of defense genes and the hypersensitive response (HR). The HR is defined as a defense response involving rapid, localized cell death that functions to limit pathogen growth (Goodman and Novacky, 1994). The cell death associated with the HR is a genetically controlled and regulated process and an example of programmed cell death (PCD) in plants (Greenberg, 1997; Heath, 2000). As such, PCD is a hallmark of HR-based immunity in plants, and cell death phenotypes are often used in laboratory experiments to discover and dissect plant immune responses.

AvrPtoB was isolated in a cross kingdom yeast two-hybrid screen of *P. syringae* pv. *tomato* DC3000 prey libraries using a Pto bait construct (Kim *et al.*, 2002). The AvrPtoB protein has a molecular mass of ~59 kDa, is secreted via the TTSS, and triggers the HR and immunity in Pto-expressing tomato plants. AvrPtoB has limited similarity to AvrPto; however, it shares 52% amino acid identity with the *P. syringae* pv. *phaseolicola* effector VirPphA (Jackson *et al.*, 1999). Studies in our lab and by others (Guttman *et al.*, 2002; Jackson *et al.*, 2002; Kim *et al.*, 2002) reveal that, unlike many effector proteins, AvrPtoB is widely conserved among diverse genera of plant pathogens including *Xanthomonas*, *Erwinia* and many strains of *Pseudomonas*. This broad conservation in plant pathogens suggests AvrPtoB plays an important role in promoting bacterial virulence and causing plant disease.

In recent years, evidence has accumulated that effector proteins can interfere with host defense responses. In a breakthrough study, Jackson *et al.* (1999) demonstrated

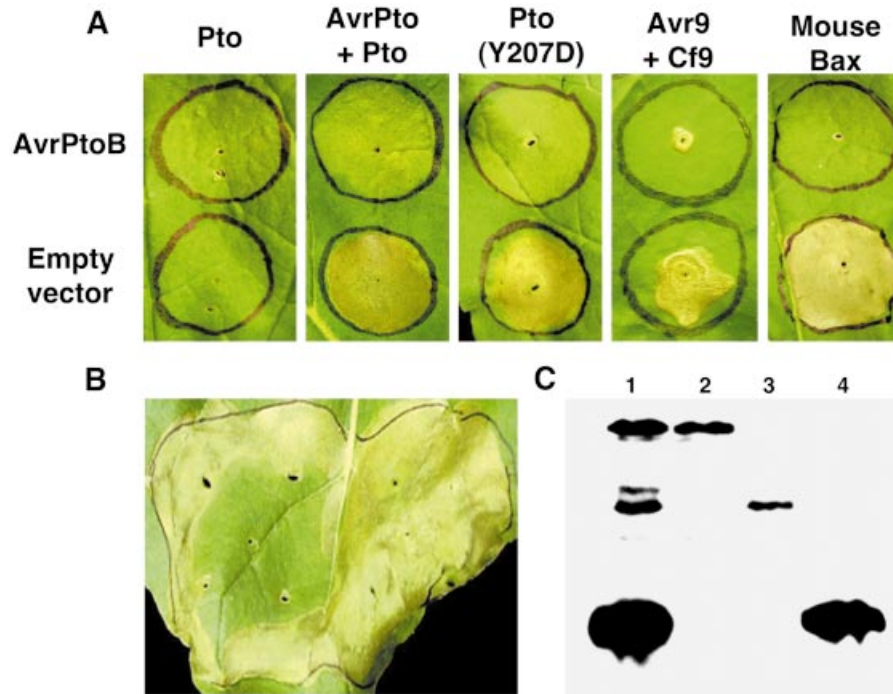


Fig. 1. AvrPtoB-mediated inhibition of PCD in *N.benthamiana* leaves. (A) The proteins indicated were co-expressed in *N.benthamiana* using *Agrobacterium*-mediated transient expression. Leaves were agroinfiltrated within the marked circles and photos were taken 7 days after agroinfiltration. (B) AvrPtoB suppresses cell death initiated by AvrPto/Pto recognition in *N.benthamiana*. The *N.benthamiana* leaf was agroinfiltrated with *avrPto* and *Pto* and left to dry. On the left hand side, *avrPtoB* was then agroinfiltrated, and on the right hand side, an empty vector control was agroinfiltrated. After 7 days, an island of cell death suppressed tissue was observed in AvrPtoB-expressing cells. (C) Immunoblot analysis of AvrPto-HA, AvrPtoB-HA and Pto-HA co-expression in *N.benthamiana*. Lane 1: AvrPto, AvrPtoB, Pto; lane 2, AvrPtoB; lane 3, Pto; lane 4, AvrPto.

that VirPphA allows *P.syringae* pv. *phaseolicola* to evade HR-based immunity in bean. Other *P.syringae* pv. *phaseolicola* effectors also allow the pathogen to avoid triggering host immunity, including AvrPphC and AvrPphF (Tsiamis *et al.*, 2000). Additionally, in the *P.syringae* pv. *maculicola*-*Arabidopsis* pathosystem, interference has been observed with the effector proteins AvrRpt2 and AvrRpm1 and the HR initiated by the R proteins RPS2 and RPM1, respectively (Reuber and Ausubel, 1996; Ritter and Dangl, 1996). These findings suggest that for some effector proteins virulence activity can be dominant over avirulence activity. Although the phenomenon of effector-mediated evasion of plant immunity has been well documented, the molecular basis of this activity has remained a mystery. Several hypotheses have been proposed to explain how some effector proteins (such as VirPphA, AvrPphC and AvrPphF) prevent a host from detecting a pathogen, including: (i) inhibition of *avr* gene expression; (ii) blocking of Avr protein secretion or translocation; (iii) interference with Avr/R protein recognition inside the plant cell; or (iv) suppression of HR or disease resistance signaling downstream of Avr recognition (Jackson *et al.*, 1999; Tsiamis *et al.*, 2000). Specific support, however, for any one of these hypotheses has not been reported. In this study, we report that AvrPtoB is a pathogenicity factor that acts inside the plant cell to suppress PCD and HR-based immunity. Our data reveal that AvrPtoB functions generally as a cell death inhibitor in both plants and yeast and suggest that PCD is an essential component of gene-for-gene immunity in plants.

Results

AvrPtoB broadly suppresses PCD in *Nicotiana benthamiana* leaves

The signaling components necessary for Pto-mediated PCD are conserved in the wild tobacco species *Nicotiana benthamiana*, because *Agrobacterium*-mediated transient co-expression of AvrPto and Pto in *N.benthamiana* leaves causes HR-related cell death (Figure 1A) (Scofield *et al.*, 1996; Sessa *et al.*, 2000). AvrPtoB, however, does not trigger cell death when co-expressed with Pto in *N.benthamiana* (Figure 1A). This observation was unexpected because from yeast two-hybrid interactions and expression in tomato, it is known that AvrPtoB can interact with Pto and initiate PCD. We hypothesized that AvrPtoB, although likely to bind to Pto in *N.benthamiana*, may also block downstream signaling events that lead to PCD.

To test whether AvrPtoB could suppress AvrPto/Pto-mediated PCD, we co-expressed AvrPto, AvrPtoB and Pto in *N.benthamiana* leaves and found that AvrPto/Pto-dependent cell death was suppressed (Figure 1A and B). Cell death suppression was stable and was observed for as long as two weeks after inoculation. We verified expression of the three proteins in plant leaves by using hemagglutinin (HA) epitope-tagged constructs of AvrPto, AvrPtoB and Pto; all three proteins were detected together and separately by immunoblot (Figure 1C). A possible explanation for the observed cell death suppression was that AvrPtoB out-competed AvrPto for interaction with the Pto kinase. To examine this possibility, we co-expressed AvrPtoB and Pto(Y207D). Pto(Y207D) is a mutant kinase

that, independent of effector recognition, initiates PCD (Rathjen *et al.*, 1999). Expression of AvrPtoB suppressed Pto(Y207D)-initiated cell death (Figure 1A). This observation suggests AvrPtoB acts downstream of Pto recognition when suppressing cell death.

We further investigated the activity of AvrPtoB by examining Avr9/Cf9-initiated PCD. The Avr9 avirulence protein is produced by the fungus *Cladosporium fulvum* and elicits immunity in tomato plants expressing the Cf9 R protein (Van Kan *et al.*, 1991; Jones *et al.*, 1994). Avr9 and Cf9 also cause HR-related cell death when they are transiently co-expressed in *N.benthamiana* (Van der Hoorn *et al.*, 2000). Cf9- and Pto-dependent PCD differ in several ways. First, Pto requires the *Prf* gene to signal PCD whereas Cf9-dependent PCD does not require *Prf* (O.del Pozo and G.B.Martin, unpublished data). Also, in the transient assay, Cf9-initiated cell death is substantially delayed compared with Pto-initiated cell death. Given the apparent differences in Cf9- and Pto-dependent signaling, we were interested to see whether AvrPtoB activity was sufficiently broad to interfere with this divergent pathway. Co-expression of AvrPtoB with Avr9 and Cf9 inhibited Avr9/Cf9-dependent cell death in *N.benthamiana* (Figure 1A). This finding suggests that AvrPtoB-mediated suppression of PCD acts on a target downstream of a point where these two R gene signaling pathways converge.

Given its surprisingly broad inhibitory activity, we examined if AvrPtoB acts generally on the process of PCD in *N.benthamiana*. The mouse protein Bax is a member of the Bcl-2 family of pro-apoptotic proteins and initiates PCD by disrupting the mitochondrion and causing the release of cytochrome *c* and other pro-apoptotic factors (Jurgensmeier *et al.*, 1998). Expression of the Bax protein in plants has been found to initiate a rapid cell death response that closely resembles the HR (Lacomme and Santa Cruz, 1999; Kawai-Yamada *et al.*, 2001). In both plants and yeast, Bax-induced cell death is dependent on a C-terminal mitochondrion-targeting domain (Lacomme and Santa Cruz, 1999), suggesting a common PCD-initiating mechanism across kingdoms. The mouse Bax protein was transiently expressed in *N.benthamiana* under control of a promoter that is inducible by the glucocorticoid hormone dexamethasone (Dex) (Aoyama and Chua, 1997). The *Bax* gene by itself or constitutive *avrPtoB* and inducible *Bax* genes were co-transformed into *N.benthamiana* leaves and *Bax* expression was induced 48 h after agroinfiltration by spraying leaves daily with 30 μ M Dex. After five days of Dex induction, cell death was observed in leaves expressing Bax alone, while cell death was not observed in leaves expressing Bax and AvrPtoB (Figure 1A). The ability of AvrPtoB to broadly suppress PCD initiated by two distinct R proteins as well as the pro-apoptotic mouse protein Bax, suggests that AvrPtoB acts generally as an inhibitor of PCD in *N.benthamiana*.

AvrPtoB suppresses PCD in yeast

Because AvrPtoB broadly suppressed PCD in *N.benthamiana*, we hypothesized that AvrPtoB may act on general components of eukaryotic cell death execution and perhaps AvrPtoB anti-PCD activity would be conserved in yeast. In *Saccharomyces cerevisiae*, PCD induced by oxidative stress or mammalian pro-apoptotic factors such

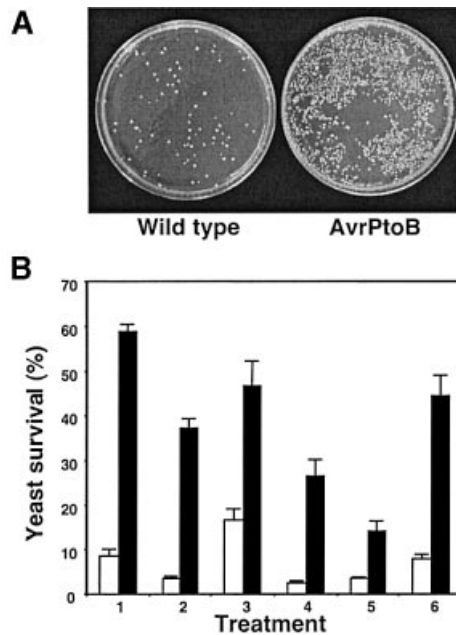


Fig. 2. AvrPtoB suppresses oxidative and heat stress-induced cell death in yeast. (A) AvrPtoB protects *S.cerevisiae* strain EGY48 from 3 mM H₂O₂-induced PCD. The agar plates show increased survival of yeast cells expressing AvrPtoB compared with the wild type after treatment with 3 mM H₂O₂. (B) AvrPtoB protects yeast from cell death triggered by: lane 1, 3 mM H₂O₂; lane 2, 5 mM H₂O₂; lane 3, 5 mM menadione; lane 4, 10 mM menadione; lane 5, heat shock at 50°C and lane 6, heat shock at 50°C with a 37°C pre-treatment. White bars represent wild-type yeast and black bars represent AvrPtoB-expressing yeast. Error bars show the standard deviation about the mean for three trials.

as Bax, exhibits many of the hallmarks of metazoan apoptosis, including cytochrome *c* release, DNA fragmentation and chromatin condensation (Madeo *et al.*, 2002). As with mammalian apoptosis, oxidative stress is an important regulator of yeast PCD, and apoptotic responses can be induced by addition of low concentrations of hydrogen peroxide (Madeo *et al.*, 1999). AvrPtoB was expressed in the yeast strain EGY48 and yeast cells were treated with H₂O₂. Strikingly, we observed that AvrPtoB protected yeast from PCD induced by 3 mM H₂O₂ (Figure 2) and 5 mM H₂O₂ (Figure 2B). We also found that AvrPtoB protected yeast from cell death induced by menadione and heat shock (Figure 2B). AvrPtoB, however, did not suppress Bax-induced cell death in yeast (data not shown), suggesting that differences exist between Bax and AvrPtoB functions in *N.benthamiana* and yeast. The capacity of AvrPtoB to suppress PCD in plants and protect yeast from stress-induced PCD, clearly establishes AvrPtoB as a eukaryotic cell death inhibitor.

AvrPtoB has a modular structure with distinct recognition and anti-PCD domains

To better understand the basis of AvrPtoB recognition and anti-PCD functions, we constructed a series of AvrPtoB N- and C-terminal truncations. Each of the truncations discussed in this study leads to an observable phenotype when expressed in plant leaves, thus establishing protein expression *in vivo*. We hypothesized that if AvrPtoB suppresses PCD but is still recognized by Pto, then an

AvrPtoB mutant could be developed such that the anti-PCD function was eliminated while the Pto recognition domain was maintained. In such a case, AvrPtoB/Pto-mediated cell death might be observed in *N.benthamiana*.

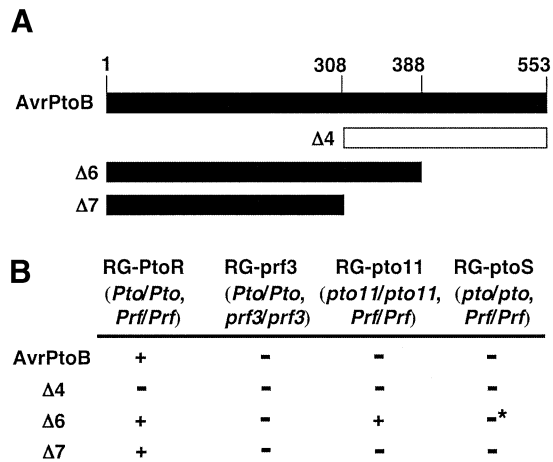


Fig. 3. Structural analysis of AvrPtoB recognition and anti-PCD activity. (A) A schematic representation of AvrPtoB truncations examined in this study and yeast two-hybrid analysis of physical interactions between AvrPtoB truncations and the Pto R protein. AvrPtoB truncations were cloned as bait fusions and tested against a Pto prey fusion. Constructs shaded black interacted strongly with Pto. (B) *In planta* transient expression of AvrPtoB truncations in tomato. RG-PtoR, RG-ptol1 and RG-prf3 are isogenic tomato lines with the *L.pimpinellifolium* Pto haplotype and genotypes as indicated. RG-ptoS is a near-isogenic line with the *L.esculentum* Pto haplotype. *Note: a late-onset weak cell death phenotype was observed with Δ6 expression in RG-ptoS. +, cell death, -, no response.

To map domains involved in AvrPtoB/Pto recognition, several deletion mutants were cloned as bait fusions and tested for interaction with a Pto prey fusion in a yeast two-hybrid system. Δ6 and Δ7 interacted strongly with Pto (Figure 3A). Therefore, an AvrPtoB fragment from amino acids 1–308 is sufficient for strong interaction with Pto in yeast.

The Pto-interacting AvrPtoB truncations were expressed in tomato and *N.benthamiana* to test for Pto-dependent cell death. As predicted from the yeast two-hybrid interaction, Δ7 triggered cell death in a *Pto*- and *Prf*-dependent manner in tomato (Figure 3B). In *N.benthamiana*, co-expression of Δ7 and Pto also resulted in cell death (Figure 4A). This gain of Δ7/Pto-initiated PCD demonstrates that the AvrPtoB N-terminus is sufficient for *in vivo* Pto-mediated recognition and suggests that the C-terminus is necessary for the observed PCD suppression. Significantly, intact AvrPtoB suppressed Δ7/Pto-initiated cell death when these three proteins were co-expressed (Figure 4B). Given that (i) AvrPtoB was shown to act downstream of recognition for PCD suppression, and (ii) full-length AvrPtoB dominantly suppresses Δ7/Pto-initiated PCD, we propose that the N-terminal domain of AvrPtoB is recognized by the Pto kinase in *N.benthamiana*, but that the C-terminus of the same protein suppresses PCD signaled by this recognition event.

The newly observed Δ7/Pto-initiated PCD suggested that anti-PCD activity may reside in the AvrPtoB C-terminus. Several N-terminal deletions were tested for anti-PCD activity in *N.benthamiana*. We found that Δ4 was capable of inhibiting cell death initiated by AvrPto/Pto (Figure 4A), Pto(Y207D) (data not shown) and Avr9/Cf9 (data not shown) in *N.benthamiana*. However, Δ4 PCD suppression was not as stable as full-length AvrPtoB, often

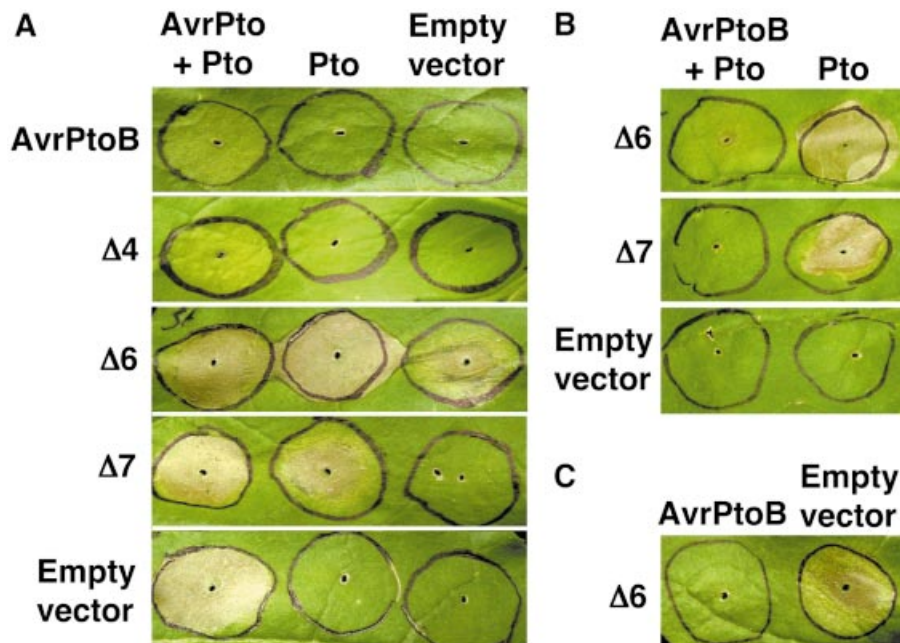


Fig. 4. Recognition and anti-PCD activity of AvrPtoB truncations in *N.benthamiana*. (A) Full-length and truncated AvrPtoB constructs were transiently expressed: with AvrPto + Pto to test for anti-PCD activity (left); with Pto to test for Pto-mediated PCD (middle) and alone to test for *Rsb*-mediated PCD (right). Protein expression of each truncation is established by an observable phenotype. (B) Epistasis experiments examining the molecular basis of Δ6/Pto- and Δ7/Pto-initiated PCD and (C) Δ6-initiated PCD. Intact AvrPtoB suppressed PCD initiated by Δ6/Pto, Δ7/Pto and Δ6, suggesting an intermolecular mechanism of anti-PCD activity. Photos were taken 7 days after agroinfiltration.

breaking down after seven days. Also, $\Delta 4$ did not suppress Bax-induced cell death, which is the most rapid and severe of the cell death phenotypes examined. The weaker anti-PCD function may be the result of altered localization, decreased protein stability or lower expression of the truncated form. Nevertheless, these data show that the C-terminus of AvrPtoB is sufficient for PCD inhibition. As such, recognition and anti-PCD functions could be separated into two non-overlapping AvrPtoB regions. Therefore, AvrPtoB has a modular structure with Pto-recognition in the N-terminal module and anti-PCD function in the C-terminal module.

Truncated AvrPtoB elicits a novel resistance phenotype, *Rsb*

When testing $\Delta 6$ for recognition activity in tomato and *N.benthamiana*, we unexpectedly discovered that this truncation triggered PCD in the absence of Pto. In tomato plants that have a mutant *pto* gene, RG-pto11 (Salmeron *et al.*, 1994), expression of $\Delta 6$ initiated rapid cell death (Figure 3B); however, in the absence of the *Prf* gene, $\Delta 6$ did not initiate cell death (Figure 3B), indicating that $\Delta 6$ -mediated cell death is not the result of cytotoxicity. We have designated this new tomato resistance phenotype *Rsb* (Resistance Suppressed by AvrPtoB C-terminus). Similarly, $\Delta 6$ initiated cell death when expressed by itself in *N.benthamiana* (Figure 4A), demonstrating the conservation of the *Rsb* phenotype; *Rsb*-mediated cell death is also *Prf*-dependent in *N.benthamiana* (R.B.Abramovitch, O.del Pozo and G.B.Martin, unpublished data). Interestingly, when $\Delta 6$ and Pto were co-expressed, a faster and more severe cell death phenotype was observed compared with $\Delta 6$ - or $\Delta 7$ /Pto-initiated cell death (Figure 4A and B). This enhanced cell death phenotype may be indicative of multiple recognition events. Because $\Delta 7$ does not elicit Pto-independent cell death, we have mapped a domain that triggers *Rsb*-mediated PCD between amino acids 308–388.

Several explanations exist for the observed $\Delta 6$ /*Rsb*-mediated PCD. One possibility is that the C-terminus of full-length AvrPtoB physically hides the recognition domain, thus making it inaccessible to *Rsb*. Alternatively, full-length AvrPtoB may normally suppress *Rsb*-initiated PCD downstream of *Rsb* recognition. We co-expressed AvrPtoB and $\Delta 6$ in *N.benthamiana* and tomato *pto* null mutants and in both cases PCD was not observed (Figure 4C; data not shown), indicating that suppression of *Rsb*-dependent cell death occurs by an intermolecular mechanism. Moreover, intact AvrPtoB also suppressed the more severe $\Delta 6$ /Pto-initiated PCD (Figure 4B). Given our evidence that AvrPtoB can act downstream of recognition for PCD suppression, we propose that intact AvrPtoB is recognized by a determinant of the *Rsb* resistance phenotype in tomato and *N.benthamiana*, but that the C-terminal module normally suppresses subsequent downstream events leading to PCD.

AvrPtoB is a pathogenicity factor that induces plant susceptibility to *P.syringae* pv. *tomato* DC3000 infection

The discovery of *Rsb*-mediated PCD presented an opportunity to examine the role of PCD suppression in DC3000 pathogenesis. Since wild-type DC3000 causes disease in

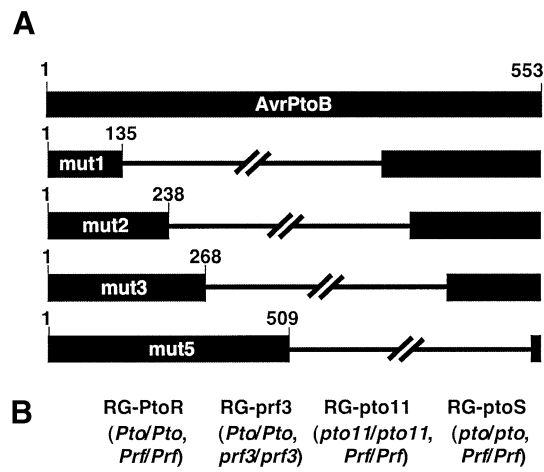


Fig. 5. *P.syringae* pv. *tomato* DC3000 chromosomal mutants of *avrPtoB* and disease responses of inoculated tomato plants. (A) A schematic representation of *avrPtoB* chromosomal mutations in *P.syringae* pv. *tomato* DC3000, generated by insertion of the 6 kb pKnockout plasmid. Amino acid numbers correspond to the amino acid residue where the expressed mutant protein is interrupted by the insertion. (B) Disease responses of tomato plants inoculated with DC3000::mutants. Note that only DC3000::mut5 triggers immunity in RG-pto11 plants and this is the only mutant that expresses AvrPtoB with the *Rsb* triggering domain described in the text. The immunity observed in RG-PtoR plants is likely to be the result of AvrPto recognition. I, immunity; D, disease.

RG-pto11 plants, we hypothesized that intact AvrPtoB normally inhibits *Rsb*-mediated immunity in RG-pto11 tomato plants. Therefore, plant immunity might be elicited by a DC3000 mutant expressing an AvrPtoB C-terminal truncation where the anti-PCD function was destroyed but *Rsb* recognition was maintained. In parallel to this study, we had constructed a series of C-terminal AvrPtoB truncations on the DC3000 chromosome by means of recombination with a plasmid by a single crossover event (Figure 5A). One of the mutants, DC3000::mut5, expressed an AvrPtoB fragment from amino acids 1–509 (Figure 5A). Like wild-type DC3000, DC3000::mut5 triggered immunity on RG-PtoR plants and caused disease on RG-prf3 plants (Figures 5B and 6). However, like $\Delta 6$ in the transient assay, DC3000::mut5 triggered immunity when inoculated on RG-pto11 plants (Figures 5B and 6). Wild-type and mutant DC3000 strains with several other AvrPtoB chromosomal truncations did not trigger immunity on RG-pto11 (Figure 5B), demonstrating that the observed immunity is likely to be the result of the *Rsb* phenotype discovered in the transient assay.

To confirm that AvrPtoB acted as a pathogenicity determinant, we transformed DC3000::mut5 with the pDSK519 broad host range plasmid (Keen *et al.*, 1988) expressing full-length AvrPtoB from its native promoter. Expression of intact AvrPtoB *in trans* enabled DC3000::mut5 to cause disease in RG-pto11 (Figure 6). The observed DC3000::mut5-pDSK519::AvrPtoB disease

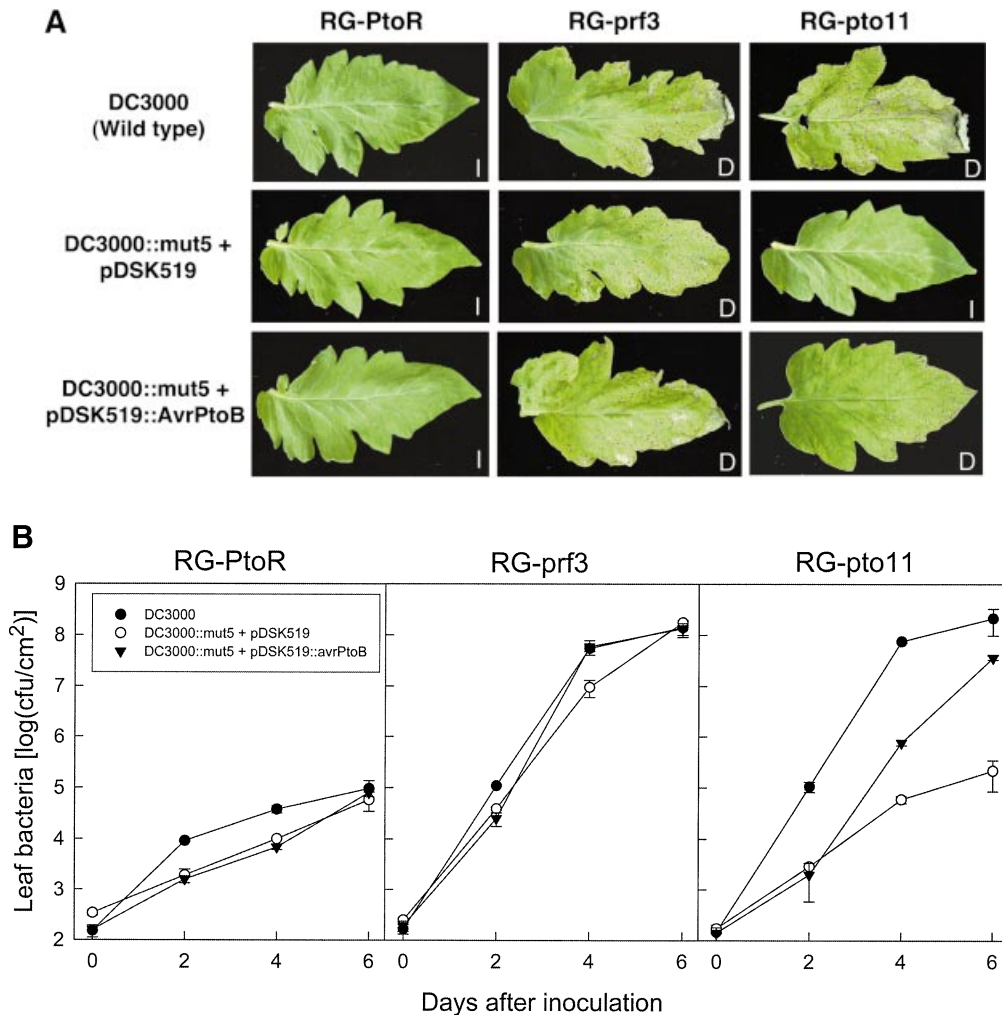


Fig. 6. AvrPtoB induces plant susceptibility to *P.syringae* pv. *tomato* DC3000 infection. (A) Disease symptoms or host immunity on tomato leaves 6 days after inoculation with indicated bacterial strains. Mutant DC3000::mut5 triggers immunity in RG-ptol1 and expression of AvrPtoB *in trans* restores DC3000::mut5 pathogenicity. pDSK519 is a broad host range plasmid. I, immunity; D, disease. (B) Bacterial growth in leaves over a period of 6 days as measured by the number of c.f.u. per cm² of leaf tissue. Errors bars represent the standard deviation of bacterial counts.

symptoms were less severe than wild-type DC3000, with ~10-fold less growth and smaller specks on the leaves. These slightly reduced disease symptoms are consistent with reported observations in *P.syringae* pv. *maculicola* that effectors are sometimes better expressed from the chromosome than from a plasmid (Guttman and Greenberg, 2001). Nevertheless, addition of AvrPtoB was sufficient to shift the DC3000::mut5/RG-ptol1 interaction from immunity to disease, demonstrating that AvrPtoB is a pathogenicity factor and that the final 44 amino acids of AvrPtoB are necessary to inhibit *Rsb*-mediated immunity. Interestingly, at four days after inoculation, DC3000::mut5 grew ~10-fold less on diseased RG-prf3 plants and caused less severe disease symptoms, when compared with wild type or DC3000::mut5 expressing intact AvrPtoB *in trans*. This observation hints that intact AvrPtoB may also act quantitatively as a virulence factor, perhaps by suppressing cell death. Because immunity was triggered by DC3000::mut5 and disease was regained with AvrPtoB expression *in trans*, and taken together with our findings that AvrPtoB acts downstream of recognition to inhibit PCD, these data

suggest that AvrPtoB induces plant susceptibility to bacterial infection by inhibiting host PCD. Therefore, we propose that effector-mediated inhibition of PCD is an important novel bacterial pathogenesis strategy. Moreover, these data suggest that PCD is a necessary component of HR-based immunity in plants.

The discovery of *Rsb*-mediated immunity was an unexpected but useful tool to explore the role of AvrPtoB in plant disease. Although the *Rsb*-phenotype remains mostly uncharacterized, several clues point towards the basis of this immune response. First, the response was shown to be *Prf*-dependent, indicating it is likely to be a classical gene-for-gene resistance response. Given the observed $\Delta 6$ -initiated HR in RG-PtoR and RG-ptol1, and the absence of $\Delta 6$ -initiated HR in RG-prf3 tomato plants, it was possible that *Prf* was the *Rsb* determinant. To examine this possibility, we expressed $\Delta 6$ in RG-ptoS tomato plants and also inoculated DC3000::mut5 on RG-ptoS plants. RG-ptoS is a near isogenic line with RG-PtoR and differs mainly at the introgressed Pto region, where RG-ptoS and RG-PtoR have the *Lycopersicon esculentum* and *L.pimpinellifolium* Pto

haplotypes, respectively (Martin *et al.*, 1993). RG-ptoS has a functional *Prf* gene, since ectopic expression of Pto in RG-ptoS plants leads to AvrPto-dependent cell death. Transient expression of $\Delta 6$ in RG-ptoS did not lead to an HR (Figure 3B) and DC3000::mut5 caused disease in RG-ptoS (Figure 5B). Together, these findings exclude *Prf* as the sole determinant of the *Rsb* phenotype and strongly indicate that the *Rsb* phenotype is governed by another gene (or genes) residing in the *L.pimpinellifolium* Pto region.

Discussion

We have shown that the *P.syringae* pv. *tomato* DC3000 type III effector AvrPtoB is a pathogenicity factor that can suppress HR-based plant immunity. By means of transient expression of individual proteins, we identified inhibition of plant PCD as the pathogenic mechanism of action of AvrPtoB. Given the presumed importance of PCD in HR-based plant defense, it is logical that a type III effector would target this process to induce host susceptibility. It is possible that other type III effectors that have been implicated in allowing plant pathogens to evade HR-based resistance (e.g. VirPphA, AvrPphC and AvrPphF) also function using a similar mechanism. Previous to this work, several hypotheses had been proposed for the molecular basis of effector-mediated evasion of the HR. Our data present a conceptual stride forward in understanding the role of type III effectors in facilitating bacterial pathogenicity, and offer several new and interesting insights into the molecular basis of plant susceptibility and immunity.

AvrPtoB suppresses PCD in *N.benthamiana* triggered by two distinct R proteins and the pro-apoptotic mouse protein Bax and also suppresses cell death in yeast triggered by hydrogen peroxide, menadione and heat shock. Given its broad anti-PCD activity, AvrPtoB probably acts on a target far downstream in the process of HR and PCD signaling. AvrPtoB may act to suppress PCD by directly interfering with a host component necessary for PCD or by altering host gene expression or cell physiology to stimulate a PCD suppressive cellular environment. The molecular basis of plant PCD is still poorly characterized and few components that are known to control metazoan PCD have been characterized for plant PCD. Suppressors of plant PCD, however, have been identified, including pharmacological agents such as caspase inhibitors (del Pozo and Lam, 1998; Lam and del Pozo, 2000) and in *Arabidopsis*, the At-BI1 protein, that was identified as a general suppressor of Bax-triggered PCD in both yeast and *Arabidopsis* (Kawai *et al.*, 1999; Kawai-Yamada *et al.*, 2001). These observations indicate that, although still uncharacterized, targets for PCD inhibition exist in plants. It will be interesting to use AvrPtoB as a tool to investigate PCD in plants and yeast and possibly in other eukaryotic systems, such as insect and mammalian cells. Lesser and Miller (2001) have demonstrated that yeast can be a powerful tool to study the virulence activity of bacterial effector proteins of mammalian pathogens. Given that little is known about plant PCD, a yeast model should accelerate further study of the genetics, cell biology and biochemistry of AvrPtoB cell death inhibition in both yeast and plants.

Plant immunity is a multifaceted phenomenon associated with an array of physiological responses including defense gene induction, phytoalexin production, reactive oxygen species formation and HR-related PCD. Although PCD is widely believed to play a role in limiting pathogen growth, the importance of PCD in plant immunity is the subject of debate, and gene-for-gene based immunity without HR-like PCD has been proposed (Yu *et al.*, 1998; Clough *et al.*, 2000). Our finding that AvrPtoB functions to suppress both HR-based immunity and PCD strongly suggests that PCD is an essential and perhaps key component of HR-based immunity to *P.syringae* pv. *tomato* DC3000. Further study, however, of how AvrPtoB affects plant physiology and gene expression will be necessary to explore this hypothesis.

To our knowledge, suppression of PCD by a bacterial type III effector is a novel pathogenesis strategy. Modulation of host PCD, however, is clearly important for bacterial pathogenesis as it has been observed in numerous model systems. For example, induction of PCD by type III effectors has been associated with disease formation of animal pathogens, including *Yersinia* (Juris *et al.*, 2002) and *Salmonella* (Knodler and Finlay, 2001). Although not experimentally associated with type III effectors, inhibition of PCD has been described for animal pathogens including *Chlamydia* (Geng *et al.*, 2000), *Neisseria* (Massari *et al.*, 2000) and *Rickettsia* (Clifton *et al.*, 1998). Interestingly, *Chlamydia* has a TTSS and it is therefore possible that effector-mediated PCD suppression is a common bacterial pathogenesis strategy in both plant and animal disease.

Plant pathogen effectors were initially isolated as avirulence proteins based on their ability to elicit the HR and plant immunity. Given the strong selective pressure for a pathogen to lose a factor that triggers immunity, it is widely assumed that type III effectors must also play an important role in disease formation. This assumption is supported by the observation that the TTSS is required for disease formation and experimental evidence that effector proteins can improve pathogen growth on plants (Chang *et al.*, 2000; Chen *et al.*, 2000; Shan *et al.*, 2000). One of the longstanding questions of plant pathogen effector research has been if avirulence and virulence functions of an effector could be physically separated. We have identified distinct N- and C-terminal domains of AvrPtoB that are sufficient for recognition and anti-PCD activity, respectively. The modular nature of AvrPtoB raises several important questions about AvrPtoB evolution and function. For example, given its modular nature, it is possible that AvrPtoB evolved from a fusion of two ancestral proteins. Supporting this observation, truncated homologs of AvrPtoB that only contain the N-terminal module have been identified occurring naturally, including *P.syringae* pv. *maculicola* effectors HopPmaL and HopPmaN (Guttman *et al.*, 2002) and the *P.syringae* pv. *tomato* JL1065 AvrPtoB homolog (Y.J.Kim, N.Lin, and G.B.Martin, unpublished data). Intriguingly, the conservation of the recognized N-terminal domain by itself or with the anti-PCD domain, suggests that this domain may also serve a function in virulence, otherwise it would not be maintained in the pathogen. In fact, preliminary evidence using the DC3000:mut mutants described in this paper, suggests that the recognized N-terminal domain of

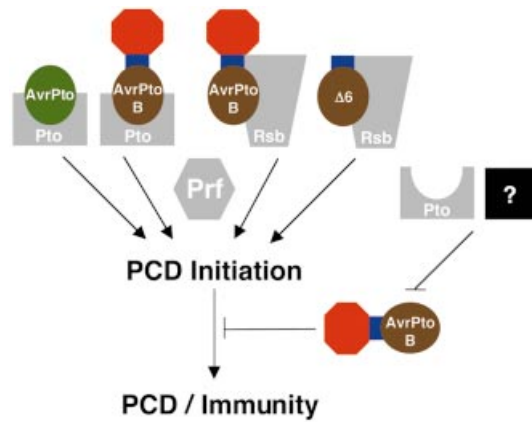


Fig. 7. A model for AvrPtoB recognition and PCD inhibition in tomato. The modular structure of AvrPtoB is depicted with the Pto-recognized N-terminal module shown as a brown circle, the anti-PCD C-terminal module shown as a red octagon, and the region recognized by *Rsb* shown as a blue connecting line. The black box represents an unknown factor predicted to act with Pto to suppress AvrPtoB anti-PCD function. *Rsb*-mediated cell death and immunity only occurs in the presence of the $\Delta 6$ truncation and the absence of Pto and intact AvrPtoB. Note: the gene(s) controlling the *Rsb* phenotype has not been identified; therefore, *Rsb* is presented in this model as a hypothetical R protein.

AvrPtoB does play a role in *P.syringae* pv. *tomato* DC3000 virulence (Y.J.Kim and G.B.Martin, unpublished data).

It is noteworthy that AvrPtoB inhibits Pto-initiated PCD in *N.benthamiana*, but not in tomato. This observation reveals that tomato has evolved a novel resistance response that acts to suppress AvrPtoB anti-PCD activity. Tomato, however, is not completely recalcitrant to AvrPtoB PCD inhibition, because *Rsb*-mediated PCD and immunity can be suppressed in RG-pto11. Because RG-PtoR and RG-pto11 plants are isogenic, except at *pto*, this implicates the Pto R protein as a candidate factor that acts to suppress anti-PCD activity, perhaps by binding and sequestering AvrPtoB. However, Pto alone is not sufficient, since AvrPtoB can suppress Pto-dependent PCD in *N.benthamiana*. Therefore, in tomato, we predict that other factors act in conjunction with Pto to inhibit AvrPtoB anti-PCD function (Figure 7). Overall, our model suggests that a chimeric effector can function at multiple points in a plant immune response and can either elicit or suppress plant immunity depending on the host genetic background. Such host-specific mechanisms are likely to be widespread, given observations from the *P.syringae* pv. *phaseolicola*-bean pathosystem, where the effector AvrPphF inhibits HR-based resistance in bean cv. Tendergreen but triggers immunity in bean cv. Canadian Wonder (Tsiamis *et al.*, 2000). Isolating factors that suppress the anti-PCD activity of AvrPtoB may reveal new signaling components of plant disease resistance and offer novel strategies for crop protection.

We reported previously that the AvrPtoB GINP motif, from amino acids 325–328, was involved in AvrPtoB/Pto-mediated recognition (Kim *et al.*, 2002). This result was based on the observations that: (i) point mutations in the GINP motif weakened the interaction of AvrPtoB with Pto in a yeast two-hybrid system; (ii) *P.syringae* pv. *tomato* PT11 expressing AvrPtoB with a mutation in the GINP

motif did not elicit an HR or immunity on Pto-expressing tomato plants; and (iii) the GINP motif is conserved in the AvrPto effector and is required for AvrPto/Pto interaction (Shan *et al.*, 2000). In this study, however, we found that $\Delta 7$, an AvrPtoB truncation that does not contain the GINP motif, still interacted strongly with Pto and triggered Pto-dependent PCD in plants. These seemingly contradictory data may offer insight into structural aspects of AvrPtoB. Since an AvrPtoB truncation missing the GINP motif is sufficient for Pto recognition, but intact AvrPtoB requires the GINP motif for Pto recognition, we suspect that the GINP motif plays a key role in maintaining the structure of full-length AvrPtoB. Interestingly, when mutations are introduced into the AvrPto GINP motif, the virulence function of AvrPto is maintained, indicating that GINP mutations do not necessarily destabilize the global structure of an effector. Rather, the GINP motif may act to ‘present’ a contact surface to the Pto kinase. Data reported in this paper indicate that the AvrPtoB/Pto contact surface resides between amino acids 1–308.

The unusually broad conservation of the AvrPtoB type III effector in many plant pathogens suggests AvrPtoB-mediated suppression of PCD and immunity plays an important role in bacterial pathogenesis. Certainly, AvrPtoB will be a useful tool to dissect the molecular basis of plant R protein PCD signaling, which presently is poorly understood. AvrPtoB anti-PCD activity may also have biotechnological applications; for example, AvrPtoB may allow efficient transgenic expression of proteins that otherwise elicit host PCD or may function to alter PCD-dependent plant developmental processes, such as senescence. Further study of AvrPtoB structure and function should lead to new insights into the basis of effector-mediated PCD inhibition and host mechanisms that guard against PCD inhibition.

Materials and methods

Agrobacterium-mediated transient expression

Agrobacterium-mediated transient expression was performed as described in Sessa *et al.* (2000). Unless indicated otherwise, *A.tumefaciens* strain GV2260 was used to syringe-infiltrate tomato and *N.benthamiana* leaves at a final density of 0.1 and 0.4 OD₆₀₀, respectively. All genes were expressed from the constitutive 35S CaMV promoter, except for the mouse Bax protein that was expressed from a Dex-inducible promoter (Aoyama and Chua, 1997). Avr9 and Cf9 constructs and strains are as described in Van der Hoorn *et al.* (2000). Co-expression experiments were performed by mixing *A.tumefaciens* cultures at equal ratios. For controls and to test responses in the absence of individual genes, *A.tumefaciens* carrying the appropriate empty vector replaced the missing component in the mixtures.

Plasmid and strain construction

All AvrPtoB truncations were generated by PCR using the following primer sets: $\Delta 4$, 2–26 5'-GTAATGCAGCGCCTCCCTATC-3' and R5 5'-TCAGGGGACTATTCTAAAAGC-3'; $\Delta 6$, F1 5'-ATGGCGGGTATCAATAGAGCG-3' and R4 5'-TCACACCCGCAATCGTGTGCAC-3'; $\Delta 7$, F1 and R3 5'-TCATACATGTCTTTCAAGGGCCG-3'. Truncations were cloned into pCR2.1 (Invitrogen, Carlsbad, CA) and sequenced. For yeast two-hybrid bait constructs, the truncations were excised from pCR2.1 using *EcoRI* and subcloned into the *EcoRI* site of the pEG202 vector (provided by R.Brent, Massachusetts General Hospital, Boston, MA). Yeast two-hybrid analysis was performed as described by Kim *et al.* (2002). For transient expression, the cloned truncations were excised from pCR2.1 using *XbaI* and *SpeI* enzymes and cloned into the *XbaI* site of the pBTEx binary vector (provided by R.Bressan, Purdue University, West Lafayette, IN).

DC3000 chromosomal truncations of AvrPtoB were generated using the pKnockout vector and methods as described in Windgassen *et al.* (2000). Using an AvrPtoB template, 400–500 bp PCR products were generated using the following primer sets: Mut1, A2MUT1F: 5'-GTATCAATAGAGCGGGACCATC-3' and A2MUT1R: 5'-CAC-TGACCACTTGTGAACG-3'; Mut2, A2MUT2F: 5'-TGTCGCGCC-AAACCAGGCGTG-3' and A2MUT2R: 5'-CCATCACCAAGGG-CAAACC-3'; Mut3, A2MUT3F: 5'-GTATCGTTCAGCAATTGGT-CAGTG-3' and A2MUT3R: 5'-ACGCGTATGGGTCTTTGGTTG-3'; Mut 5, A2MUT5F: 5'-ACGATTGCGGGTGATGC-3' and A2MUT5R: 5'-CCTCTGGCTGTAAGGCTGC-3'. Each PCR product was cloned into pCR2.1, subcloned into pKnockout-G (provided by K.Jaeger, Bochum, Germany) and introduced into DC3000 by triparental mating. After primary selection, plasmid insertion into the chromosome was verified by: (i) PCR using T7 and 2–30 (5'-ATGGCGGGTATCAATA-GAGCGG-3') primers, and (ii) Southern blot analysis using *Pst*I-digested genomic DNA and the *avrPtoB* open reading frame as a probe.

Immunoblotting

Detection of proteins expressed in the *Agrobacterium*-mediated transient assay was performed using standard immunoblotting procedures. Briefly, 48 h after agroinfiltration, two 1 cm² leaf discs were ground in 400 µl of protein extraction buffer, composed of phosphate-buffered saline amended with 1% Triton-X and plant protease inhibitor cocktail (Sigma, St Louis, MS). Protein extracts were denatured and equal amounts of protein were electrophoresed on 12% polyacrylamide gels and transferred to PVDF membrane (Millipore Immobilon P, Bedford, MA) by electroblotting according to the manufacturer's recommendation (Bio-Rad, Hercules, CA). HA-tagged proteins were detected using rat anti-HA primary antibody (Boehringer-Mannheim, Indianapolis, IN), HRP-conjugated anti-Rat Ig secondary antibody (Amersham-Pharmacia, Piscataway, NJ) and a chemiluminescent visualization kit (ECL Plus, Amersham-Pharmacia).

Yeast cell death assays

The *S.cerevisiae* strain EGY48 [MATa, *ura3*, *his3*, *trp1*, *lexA_{op}*(×6)-*LEU2*] was obtained from Clontech (Palo Alto, CA) and the growth, transformation and expression of genes was performed essentially as described by Kampranis *et al.* (2000). The EGY48 cells were grown in YPD medium containing 1% yeast extract, 2% Difco peptone and 2% glucose. AvrPtoB was cloned under the control of a galactose inducible plasmid in the high-copy yeast expression vector p423 (Mumberg *et al.*, 1994) and the plasmid was transformed into EGY48. Cells were grown in synthetic dropout (SD) medium with 2% glucose lacking histidine (SD/*glu*-*his*) to select for the presence of the plasmid. EGY48 cells containing AvrPtoB were grown overnight in SD/*glu*-*his*. The cells were pelleted, washed and resuspended in SD medium containing 2% galactose and 1% raffinose as carbon sources (SD/*gal*/*raff*-*his*), to induce expression of the fusion protein from the GAL1 promoter. After 6 h of induction, cells were diluted to 0.05 OD₆₀₀ and treated in one of the following ways. For chemical treatments, H₂O₂ or menadione were added at selected final concentrations in the medium and cultures were incubated at 30°C with vigorous shaking for 6 h. For heat stress, yeast cells were incubated at 37°C for 30 min with vigorous shaking, transferred to a water bath at 50°C for 30 min and then returned to 30°C with vigorous shaking for 6 h. Following these treatments, viability was determined by plate counting. Treated and untreated cells were sampled and spread onto YPD medium with 2% agar, then incubated at 30°C for 48 h. The number of colony forming units (c.f.u.) from treated cells (both EGY48 and EGY48 carrying AvrPtoB) were compared with the c.f.u. of untreated cells. All experiments were repeated in triplicate.

Tomato infection and measurement of pathogen growth in leaves

Rio Grande (RG) tomato lines with the following genotypes were used in this study: RG-PtoR (*Pto/Pto*, *Prf/Prf*), RG-pr3 (*Pto/Pto*, *prf3/prf3*), RG-pt11 (*pto11/pto11*, *Prf/Prf*) and RG-ptoS (*pto/pto*, *Prf/Prf*). Bacterial growth measurements from tomato leaves were performed as described by Tang *et al.* (1999). Briefly, *P.syringae* pv. *tomato* DC3000 strains were grown overnight in King's B (KB) medium with appropriate antibiotics. Cultures were washed twice with 10 mM MgCl₂ and resuspended in 10 mM MgCl₂. Washed cultures were prepared for inoculation by diluting cultures to 10⁴ cells/ml in 10 mM MgCl₂ and 0.04% Silwet L-77 (Osi, Danbury, CT). Six-week-old tomato plants were inoculated by vacuum infiltration and kept in a greenhouse during the course of infection. Bacterial growth was measured by grinding two 1 cm²

leaf discs in 10 mM MgCl₂, and tissue samples were serially diluted and plated on solid KB medium with antibiotics.

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Note added in proof

We have identified predicted structural features of AvrPtoB that show similarity to domains present in the Bcl-2 family of cell death regulators. This finding suggests that AvrPtoB and Bcl-2 family members might share common mechanisms of PCD inhibition.