

# Characterization of the *Pseudomonas syringae* pv. *tomato* AvrRpt2 protein: demonstration of secretion and processing during bacterial pathogenesis

Mary Beth Mudgett and Brian J. Staskawicz\*

Department of Plant and Microbial Biology, 111 Koshland Hall, University of California, Berkeley, CA 94720-3102, USA.

## Summary

*Pseudomonas syringae* pv. *tomato* strain DC3000 (Pst DC3000) expressing *avrRpt2* is specifically recognized by plant cells expressing RPS2 activity, resulting in localized cell death and plant resistance. Furthermore, transient expression of this bacterial *avrRpt2* gene in plant cells results in RPS2-dependent cell death. This indicates that the AvrRpt2 protein is recognized inside RPS2 plant cells and is sufficient for the activation of disease resistance-mediated cell death *in planta*. We explored the possibility that Pst DC3000 delivers AvrRpt2 protein to plant cells via the *hrp* (type III) secretion pathway. We now provide direct evidence that mature AvrRpt2 protein is secreted from Pst DC3000 and that secretion is *hrp* dependent. We also show that AvrRpt2 is N-terminally processed when *Arabidopsis thaliana* plants are infected with Pst DC3000 expressing *avrRpt2*. Similar N-terminal processing of AvrRpt2 occurred when *avrRpt2* was stably expressed in *A. thaliana*. No cleavage of AvrRpt2 was detected in bacteria expressing *avrRpt2* in culture or in the plant extracellular fluids. The N-terminus of AvrRpt2 was not required for RPS2 recognition *in planta*. However, this region of AvrRpt2 was essential for Pst DC3000-mediated elicitation of RPS2-dependent cell death in *A. thaliana* leaves.

## Introduction

Plant pathogenic bacteria enter leaves through stomatal openings and wounds to colonize the intercellular spaces. For bacteria in the genera *Erwinia*, *Pseudomonas*, *Ralstonia* and *Xanthomonas*, pathogenesis requires the gene products of the *hrp* locus (Alfano and Collmer, 1997). Several of the Hrp proteins of plant pathogenic bacteria have

been found to be related to proteins encoding virulence systems used by several animal pathogens (Van Gijsegem *et al.*, 1993). This indicates that the Hrp proteins encoded by the *hrp* loci of plant pathogenic bacteria may comprise similar type III protein secretion systems, each capable of delivering effector proteins to plant cells. Evidence to support the existence of a functional type III secretion pathway in phytopathogenic bacteria was first revealed when harpin proteins were found to be secreted from *Erwinia amylovora* (Wei *et al.*, 1992; Bogdanove *et al.*, 1996), *R. solanacearum* (Arlat *et al.*, 1994) and *Pseudomonas syringae* (He *et al.*, 1993). Additional proteins have subsequently been shown to travel the *hrp* secretion pathway. *P. syringae* secretes a pilin HrpA (Roine *et al.*, 1997), two novel extracellular proteins, EXP-22 and EXP-43 (Yuan and He, 1996), and a class III pectate lyase, HrpW (Charkowski *et al.*, 1998). *E. amylovora* secretes an avirulence protein DspE(DspA) (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998) and HrpW (Kim *et al.*, 1998). Interestingly, none of these secreted proteins share homology with effector proteins secreted by mammalian pathogens. Thus, although the mechanism to establish pathogenicity is conserved in Gram-negative pathogenic bacteria, the putative virulence determinants for each host–pathogen interaction appear to be more specialized.

Intriguingly, most plants can resist bacterial pathogenesis. Evolution has equipped plants with precise defence pathways to recognize and impede invading bacteria actively. Plant strategies used to inhibit the growth of pathogens include the activation of localized cell death (classically referred to as the hypersensitive response, HR) (Greenberg, 1997), the release of reactive oxygen intermediates (Lamb and Dixon, 1997) and nitric oxide (Delledonne *et al.*, 1998; Durner *et al.*, 1998), the fortification of plant cell walls and the production of numerous antimicrobial secondary metabolites and defence-related proteins (Hammond-Kosack and Jones, 1997). Despite this diverse arsenal, we still do not understand the molecular mechanism(s) for the inhibition of pathogen growth.

Many instances of plant disease resistance follow the gene-for-gene model (Flor, 1971). In this genetic model, resistance genes in the plant encode proteins that recognize effector proteins encoded by specific avirulence genes in the pathogen (Hammond-Kosack and Jones,

Received 14 December, 1998; revised 15 February, 1999; accepted 19 February, 1999. \*For correspondence. E-mail stask@nature.berkeley.edu; Tel. (+1) 510 642 3721; Fax (+1) 510 642 9017.

1997). Recognition of an avirulence determinant results in a resistance gene-activated plant defence response. This mechanism of plant resistance is overcome when the pathogen acquires mutations at avirulence gene loci (Dangl, 1994). The pathogen now avoids plant detection and is virulent on the host because it has maintained its pathogenicity determinants. Thus, the outcome of plant-pathogen interactions is defined at the molecular level by the presence or absence of functional alleles of avirulence and resistance gene pairs.

The biochemical explanation for gene-for-gene resistance is not known. However, mounting indirect evidence suggests that molecular recognition of some avirulence determinants occurs inside plant cells by resistance proteins, either directly or indirectly (Mudgett and Staskawicz, 1998). This hypothesis is based primarily on research showing that several avirulence proteins function *in planta* to induce resistance gene-dependent cell death (Gopalan *et al.*, 1996; Leister *et al.*, 1996; Scofield *et al.*, 1996; Tang *et al.*, 1996; Van den Ackerveken *et al.*, 1996). Plant resistance proteins are thus expected to function as the receptors for bacterial avirulence effectors. However, a direct physical interaction has been demonstrated in only one case, namely Pto with AvrPto (Scofield *et al.*, 1996; Tang *et al.*, 1996).

The pivotal observation that some avirulence genes and *hrp* genes are co-regulated (Huynh *et al.*, 1989; Gopalan *et al.*, 1996; Pirhonen *et al.*, 1996) suggested that avirulence proteins are delivered to plant cells by the *hrp*-encoded type III secretion pathway. The delivery of effector proteins to host cells by the type III pathway requires co-ordinate regulation of bacterial secretion and bacterial translocation. However, type III-dependent secretion of some effector proteins can be detected in cultures of induced mammalian bacterial pathogens in the absence of the host (Hueck, 1998). Conversely, the secretion of avirulence proteins from plant bacterial pathogens has been difficult to detect. To date, only *E. amylovora* has been shown to secrete an avirulence protein, DspE(DspA), into bacterial cultures in a *hrp*-dependent manner (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998). This may simply reflect a protein detection problem. Only small amounts of avirulence protein are hypothesized to be secreted from plant pathogens. Alternatively, this may suggest that bacterial cell and plant cell contact is required for type III secretion of some avirulence proteins from plant pathogenic bacteria (Alfano and Collmer, 1997).

The failure to detect plant pathogen avirulence proteins in culture media has consequently limited our understanding regarding the mechanisms of *hrp*-dependent delivery of effector proteins to plant cells. The recent development of a heterologous *E. coli* *hrp* secretion system capable of secreting copious amounts of avirulence protein into

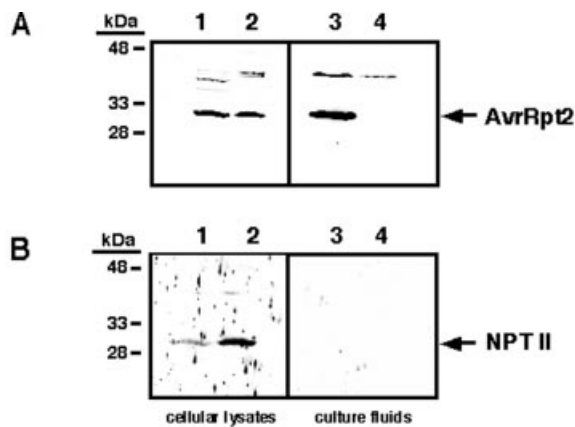
bacterial cultures, however, has provided an alternative means for the study of the *hrp* secretion pathway (Ham *et al.*, 1998). Ham *et al.* (1998) have engineered *E. coli* with the *hrp* gene cluster from *E. chrysanthemi* ( $E_{ch}$  *hrp*). Using this strain, they were able to detect the efficient secretion of two *P. syringae* proteins, AvrB and AvrPto. Previous attempts to detect AvrB and AvrPto in *P. syringae* culture fluids had been unsuccessful (Ham *et al.*, 1998). Importantly, this study shows that avirulence proteins can be secreted indiscriminately by other *hrp* machineries. Furthermore, this heterologous system can now be used to test the secretion of any protein potentially targeted through a *hrp* pathway by plant pathogens.

Our primary interest is to elucidate the mechanism for AvrRpt2-mediated signal transduction in *Arabidopsis thaliana*. *A. thaliana* plants carrying the *RPS2* gene (resistance to *P. syringae*) (Bent *et al.*, 1994; Mindrinos *et al.*, 1994) are resistant to Pst DC3000 that express the *avrRpt2* avirulence gene (Whalen *et al.*, 1991; Innes *et al.*, 1993). The virulence function of AvrRpt2 is not known. However, AvrRpt2 is sufficient *in planta* to activate *RPS2*-mediated resistance (Leister *et al.*, 1996; McNellis *et al.*, 1998). We predict that Pst DC3000 delivers the AvrRpt2 elicitor to the plant cytoplasm. The goal of this work was to obtain direct evidence that Pst DC3000 secretes AvrRpt2. Here, we demonstrate that the mature AvrRpt2 polypeptide is secreted by non-pathogenic *E. coli* carrying the  $E_{ch}$  *hrp* gene cluster and by pathogenic Pst DC3000. In both strains, secretion of AvrRpt2 was *hrp* dependent. We also report that N-terminal cleavage of AvrRpt2 accompanies Pst DC3000 infections in *A. thaliana*. Moreover, proteolytic processing of AvrRpt2 requires a functional *hrp* apparatus as well as contact with the plant host. This work demonstrates that *hrp* secretion of AvrRpt2 from Pst DC3000 is one bacterial process required for the delivery of this effector protein to host plant cells. Furthermore, these data suggest that proteolytic cleavage of AvrRpt2 occurs once the protein is translocated to the plant cell.

## Results

### *Secretion of AvrRpt2 protein from E. coli containing E<sub>ch</sub> hrp cluster*

To demonstrate that Pst DC3000 ultimately delivers AvrRpt2 to plant cells, we first needed to determine whether AvrRpt2 protein is secreted by the *hrp* pathway. An *E. coli* strain carrying the  $E_{ch}$  *hrp* locus was used previously to show indiscriminate, but efficient, secretion of two *P. syringae* proteins, AvrB and AvrPto (Ham *et al.*, 1998). We used this heterologous *E. coli* strain to test *hrp*-dependent secretion of AvrRpt2. *E. coli* cells carrying a wild-type (pCPP2156) and a mutated (pCPP2368)  $E_{ch}$



**Fig. 1.** Secretion of AvrRpt2 from *E. coli* containing the  $E_{ch}$  *hrp* gene cluster. Immunoblot analysis of (A) AvrRpt2 and (B) NPT II protein in cellular lysate protein fractions (lanes 1 and 2) and concentrated culture fluid fractions (lanes 3 and 4) isolated from *E. coli* strains expressing *avrRpt2* (pDSK519(*navrRpt2*)) and the wild-type  $E_{ch}$  *hrp* gene cluster (pCPP2156) (lanes 1 and 3) or the mutated  $E_{ch}$  *hrp* gene cluster (pCPP2368) (lanes 2 and 4). Soluble cellular protein (5  $\mu$ g) and 40  $\mu$ g of concentrated culture fluid protein were loaded. AvrRpt2 (28 kDa) and NPT II (29 kDa) were detected using AvrRpt2 and NPT II antibodies respectively. Bands appearing above 33 kDa are non-specific proteins recognized by the antisera. Protein standards (28, 33 and 48 kDa; Bio-Rad) were used to standardize protein migration.

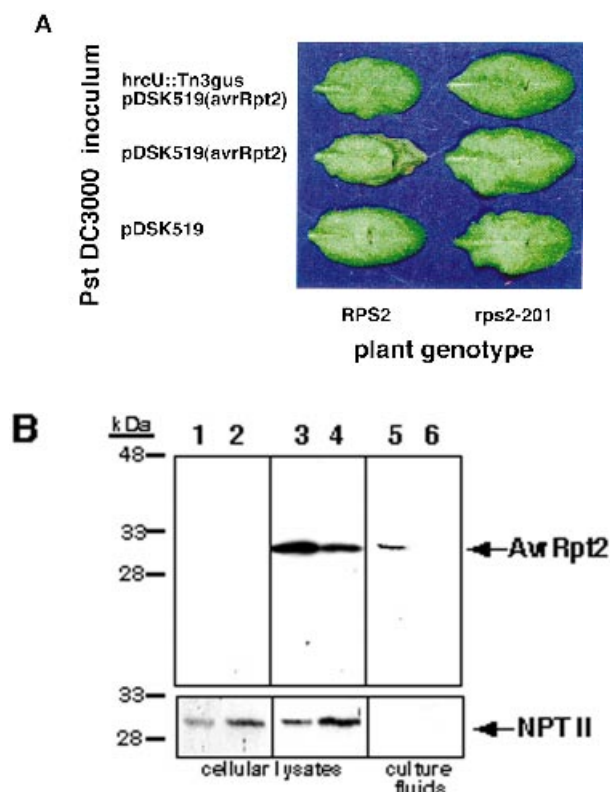
*hrp* gene cluster were used to express *avrRpt2* constitutively from pDSK519(*navrRpt2*). The mutated  $E_{ch}$  *hrp* gene cluster (pCPP2368) is secretion defective, because it contains the transposon Tn5Cm in the intergenic region between *hrpJ* and *hrcV* in the *hrpJ* operon (Ham *et al.*, 1998). To analyse secreted protein, fluids from bacterial cultures in late logarithmic phase growth were collected by centrifugation, filtered and then plated to check for bacterial contamination. Cell-free culture fluids concentrated  $\approx$  200-fold were separated by SDS-PAGE and then analysed by immunoblot analysis using AvrRpt2-specific antibodies (McNellis *et al.*, 1998). Both  $E_{ch}$  *hrp*-containing *E. coli* strains expressed similar levels of cellular AvrRpt2 protein (Fig. 1A, lanes 1 and 2). However, only the *E. coli* strain containing a functional  $E_{ch}$  *hrp* gene cluster secreted mature AvrRpt2 protein into the culture fluid (Fig. 1A, lanes 3 and 4). To confirm that the presence of AvrRpt2 in the culture fluids of *E. coli* carrying both pCPP2156 and pDSK519(*navrRpt2*) was not caused by cell lysis, the immunoblot was reprobed for neomycin phosphotransferase (NPT II) protein expressed by pDSK519(*avrRpt2*) using NPT II-specific antisera. NPT II protein was only detected in cellular protein fractions (Fig. 1B, lanes 1 and 2) and not in concentrated culture fluid fractions (Fig. 1B, lanes 3 and 4), demonstrating that the AvrRpt2 secretion occurred without cell lysis. These data show that AvrRpt2 was secreted by the  $E_{ch}$  *hrp* pathway in *E. coli* and that secretion was *hrp* dependent.

The heterologous *E. coli*  $E_{ch}$  *hrp* system has been used to show  $E_{ch}$  *hrp*-dependent delivery of AvrB and AvrPto effectors to resistant *Nicotiana clelandii* and *Lycopersicon esculentum* cv. Rio Grande, respectively, both solanaceous hosts for *E. chrysanthemi* (Ham *et al.*, 1998). The secretion of AvrRpt2 from the  $E_{ch}$  *hrp* pathway suggested that the AvrRpt2 effector could be delivered similarly to resistant host cells. To test this, *N. clelandii* possessing *RPS2* activity was inoculated with wild-type and mutant *E. coli*  $E_{ch}$  *hrp* strains expressing *avrRpt2*. *E. coli* expressing wild-type  $E_{ch}$  *hrp* (pCPP2156) and pDSK519(*navrRpt2*) induced localized cell death in *N. clelandii* only at high inoculum ( $2-3 \times 10^9$  cells  $ml^{-1}$ ; data not shown). No symptoms were observed in leaves inoculated with *E. coli* expressing mutated  $E_{ch}$  *hrp* (pCPP2368) and pDSK519(*navrRpt2*) (data not shown). These data indicate that the  $E_{ch}$  *hrp* cluster can deliver AvrRpt2 to a host of *E. chrysanthemi*.

#### Secretion of AvrRpt2 protein from Pst DC3000

Next, we wanted to determine whether AvrRpt2 is secreted from the pathogen Pst DC3000. Secretion was tested using wild-type and secretion mutant strains of Pst DC3000. In *P. syringae* pv. *syringae*, HrcU and HrcC are inner and outer membrane components, respectively, of the *hrp* secretion machinery required for the secretion of HrpZ (Huang *et al.*, 1992; Charkowski *et al.*, 1997). Sequence analysis of the *hrp* locus of Pst DC3000 has identified the corresponding HrcU and HrcC homologues (C. Tobias and B. Staskawicz, unpublished; Deng *et al.*, 1998). Tn3gus transposon mutagenesis of the Pst DC3000 *hrp* gene cluster was performed to generate mutations in *hrcU* and *hrcC* (C. Boucher, D. Dahlbeck and B. Staskawicz, unpublished). As *hrcU* lies at the end of the *hrpU* operon, it is assumed that the Tn3gus mutation is non-polar. Conversely, *hrcC* lies in the middle of the *hrpC* operon (Deng *et al.*, 1998). Thus, the Tn3gus mutation in *hrcC* may be polar. Resistant *A. thaliana* *RPS2* leaves infected with wild-type Pst DC3000 expressing *avrRpt2* induce *RPS2*-dependent localized cell death (Fig. 2A). No symptoms were observed in mutant *A. thaliana* *rps2-201* leaves lacking a functional *RPS2* allele. Conversely, cell death was not induced in *A. thaliana* *RPS2* leaves infected with Pst DC3000 *hrcU::Tn3gus* bacteria (Fig. 2A). Similarly, *avrRpt2*-specific symptoms were not observed in *RPS2* leaves inoculated with the Pst DC3000 *hrcC::Tn3gus* expressing *avrRpt2* (data not shown). This demonstrates that a functional Pst DC3000 *hrp* secretion apparatus is required for *avrRpt2*-mediated cell death in resistant *RPS2* plant cells.

To test for AvrRpt2 secretion, Pst DC3000 strains were first grown in rich media and then transferred to minimal media to co-ordinately induce the expression of the *hrp*



**Fig. 2.** Secretion of AvrRpt2 from Pst DC3000.  
**A.** HrcU-dependent localized cell death in resistant *A. thaliana* *RPS2* leaves. Resistant *RPS2* and susceptible *rps2-201* *A. thaliana* leaves were inoculated with a  $2 \times 10^7$  cells  $\text{ml}^{-1}$  suspension of wild-type Pst DC3000 carrying pDSK519 or pDSK519(*avrRpt2*) and mutant *hrcU::Tn3gus* Pst DC3000 bacteria carrying pDSK519(*avrRpt2*). Symptoms were photographed 24 h after inoculation.  
**B.** Immunoblot analysis of AvrRpt2 and NPT II protein in cellular lysate and concentrated culture fluid protein fractions isolated from Pst DC3000 strains expressing *avrRpt2* from plasmid pDSK519(*avrRpt2*). Lanes 1, 3 and 5, wild-type Pst DC3000. Lanes 2, 4 and 6, secretion mutant *hrcU::Tn3gus* Pst DC3000. Lanes 1 and 2, cellular lysate protein isolated from strains grown in rich media. Lanes 3 and 4, cellular lysate protein isolated from strains grown in minimal media. Lanes 5 and 6, concentrated minimal media culture fluid protein. Five micrograms of cellular lysate protein and 4  $\mu\text{g}$  of concentrated culture fluid protein were loaded. As the *hrcU::Tn3gus* mutant overexpresses NPT II compared with wild-type cells (because of a chromosomal and plasmid copy of *nptII*), 0.75  $\mu\text{g}$  of protein was loaded in lanes 2 and 4. AvrRpt2 (28 kDa) and NPT II (29 kDa) were detected using AvrRpt2 and NPT II antibodies respectively. Protein standards (28, 33 and 48 kDa; Bio-Rad) were used to standardize protein migration.

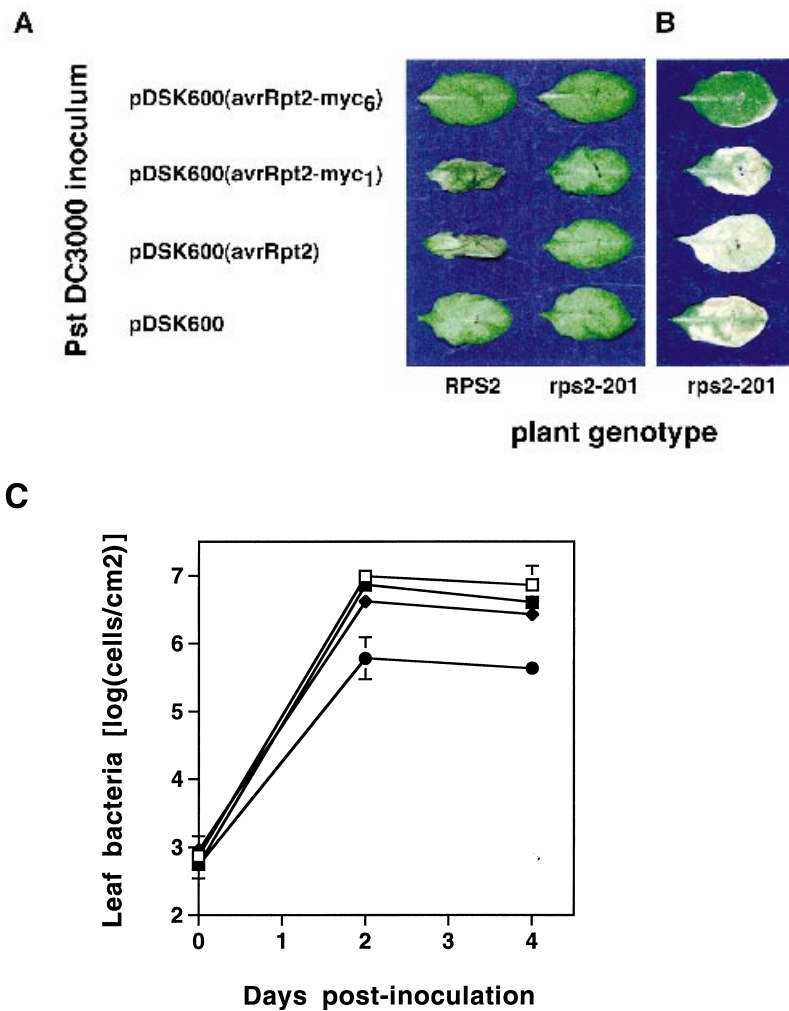
locus and *avrRpt2* (Innes *et al.*, 1993). After 14 h of induction, culture fluids were collected by centrifugation, filtered and then plated to ensure no bacterial contamination. Cell-free culture fluids were concentrated 400-fold, and equal amounts of total protein were analysed. Immunoblot analysis shows that wild-type and mutant *hrcU::Tn3gus* Pst DC3000 strains carrying pDSK519(*avrRpt2*) did not express AvrRpt2 protein when cells were grown in rich media (Fig. 2B, lanes 1 and 2). However, strains expressed

AvrRpt2 protein when they were cultured subsequently in minimal media (Fig. 2B, lanes 3 and 4). Mature AvrRpt2 protein was only detected in the culture fluids of wild-type Pst DC3000 cells carrying pDSK519(*avrRpt2*) (Fig. 2B, lanes 5 and 6). To confirm that the presence of AvrRpt2 protein in the culture fluids of wild-type cells was not caused by cell lysis, immunoblot analysis was repeated using antisera for NPT II. Wild-type Pst DC3000 cells contain one *nptII* gene encoded by pDSK519(*avrRpt2*). *hrcU::Tn3gus* Pst DC3000 cells contain two *nptII* genes, one encoded by pDSK519(*avrRpt2*) and one encoded by the transposon inserted into the chromosome. NPT II protein was only detected in cellular protein fractions (Fig. 2B, lanes 1–4) and not in concentrated culture fluid fractions (Fig. 2B, lanes 5 and 6). These data demonstrate that AvrRpt2 is secreted in a type III-dependent manner from Pst DC3000.

#### Modification of AvrRpt2 alters Pst DC3000-dependent phenotypes in *A. thaliana*

Secretion of AvrRpt2 protein into Pst DC3000 culture fluids indicated that type III secretion and translocation of AvrRpt2 into plant cells is probable. In order to detect AvrRpt2 protein trafficking in Pst DC3000-infected plant cells, we constructed several epitope-tagged AvrRpt2 fusion proteins. These included AvrRpt2-his6, AvrRpt2-myc1, AvrRpt2-myc6, AvrRpt2-gfp, AvrRpt2-cyA' and N-flag-AvrRpt2. Interestingly, we found that several of the protein epitopes interfered directly with AvrRpt2 function. Small peptides fused to the C-terminus of AvrRpt2 did not affect *avrRpt2*-dependent phenotypes in *RPS2* plants. Both AvrRpt2-his6 (data not shown) and AvrRpt2-myc1 (Fig. 3A) caused localized cell death in resistant *A. thaliana* *RPS2* leaves inoculated with Pst DC3000 expressing *avrRpt2-his6* or *avrRpt2-myc1*. No symptoms were observed in mutant *A. thaliana* *rps2-201* leaves lacking a functional *RPS2* allele (Fig. 3A). Conversely, larger polypeptides fused to the C-terminus of AvrRpt2, including AvrRpt2-myc6 (Fig. 3A), AvrRpt2-gfp and AvrRpt2-cyA' (data not shown) were unable to induce localized cell death in Pst DC3000-infected *A. thaliana* *RPS2* leaves. Similarly, N-terminal modification of AvrRpt2 (N-flag-AvrRpt2) prevented Pst DC3000-dependent cellular collapse in *A. thaliana* *RPS2* leaves (data not shown).

Surprisingly, we found that some AvrRpt2 modifications also inhibited disease phenotypes associated with *P. syringae* pathogenesis. Chlorotic disease symptoms develop when susceptible *A. thaliana* *rps2-201* plants are infected with Pst DC3000 or Pst DC3000 carrying *avrRpt2* (Kunkel *et al.*, 1993). Disease symptoms resulting from infection with large populations ( $2 \times 10^7$  cells  $\text{ml}^{-1}$ ) of pathogenic bacteria were reduced significantly in



**Fig. 3.** Phenotypes of AvrRpt2 and AvrRpt2 epitope-tagged proteins in plants.

**A.** Localized cell death in resistant *A. thaliana* *RPS2* leaves induced by Pst DC3000 strains expressing *avrRpt2* and *avrRpt2-myc1*. Resistant *RPS2* and susceptible *rps2-201* *A. thaliana* leaves were inoculated with a  $2 \times 10^7$  cells  $\text{ml}^{-1}$  suspension of bacteria. Bacteria carried the pDSK600 vector or pDSK600 containing *avrRpt2*, *avrRpt2-myc1* or *avrRpt2-myc6*. Symptoms were photographed 24 h after inoculation.

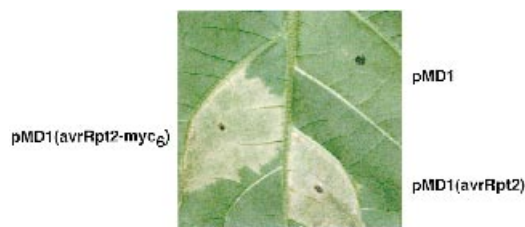
**B.** Inhibition of disease phenotype in *A. thaliana* *rps2-201* leaves inoculated with Pst DC3000 expressing *avrRpt2-myc6*. Susceptible *rps2-201* *A. thaliana* leaves were inoculated with a  $2 \times 10^7$  cells  $\text{ml}^{-1}$  suspension of bacteria. Symptoms were photographed 4 days after inoculation.

**C.** Reduced virulence of Pst DC3000 expressing *avrRpt2-myc6*. The growth of Pst DC3000 alone (open squares) and Pst DC3000 carrying pDSK600 (filled squares), pDSK600(*avrRpt2*) (filled diamonds) or pDSK600(*avrRpt2-myc6*) (filled circles) was assayed in susceptible *rps2-201* *A. thaliana* plants. Plants were inoculated by vacuum infiltration with a  $5 \times 10^4$  cells  $\text{ml}^{-1}$  bacterial suspension. Data points represent mean  $\log_{10}$  (cells  $\text{cm}^{-2}$ )  $\pm$  sample SD.

susceptible *A. thaliana* *rps2-201* leaf tissue after inoculation with Pst DC3000 expressing AvrRpt2-myc6 (Fig. 3B), AvrRpt2-gfp or AvrRpt2-cyA' (data not shown). However, disease symptoms were clearly visible in susceptible leaves inoculated with Pst DC3000 alone or with bacteria expressing AvrRpt2 and AvrRpt2-myc1 (Fig. 3B). These results suggested that large protein modifications of AvrRpt2 may compromise Pst DC3000 in virulence. To test this, we quantified the growth of Pst DC3000 expressing AvrRpt2-myc6 in susceptible *rps2-201* *A. thaliana* plants infected with  $5 \times 10^4$  cells  $\text{ml}^{-1}$ , a concentration of bacteria typically used for disease assays (Fig. 3C). The growth of Pst DC3000 expressing *avrRpt2-myc6* in *A. thaliana* *rps2-201* leaves was typically one order of magnitude lower than bacteria expressing wild-type *avrRpt2* (Fig. 3C). Thus, Pst DC3000 bacteria expressing AvrRpt2-myc6 protein were less virulent in susceptible *A. thaliana* *rps2-201* plants.

#### Expression of AvrRpt2 fusion proteins in planta cause RPS2-mediated localized cell death

To determine whether the myc6 epitope was affecting AvrRpt2 specificity directly, *avrRpt2-myc6* was expressed *in planta* using the *Agrobacterium*-mediated transient transformation assay (Scofield *et al.*, 1996; Tang *et al.*, 1996). Acetosyringone-induced *A. tumefaciens* GV3101 strains carrying the vector pMD1, pMD1(*avrRpt2*) and pMD1(*avrRpt2-myc6*) were infiltrated into *N. tabacum* cv. Xanthi *RPS2* plants. Transient expression of *avrRpt2-myc6* in resistant *N. tabacum* cv. Xanthi *RPS2* plants caused localized cell death similar to that observed for the wild-type *avrRpt2* gene (Fig. 4). Similar symptoms were observed when *flag-avrRpt2*, *avrRpt2-gfp* and *avrRpt2-cyA'* were transiently expressed in *N. tabacum* *RPS2* leaves (data not shown). This demonstrates that, when delivered to the host cell by transient transformation,



**Fig. 4.** *avrRpt2*- and *avrRpt2-myc6*-dependent cell death in *N. tabacum* cv. Xanthi *RPS2* leaves. *avrRpt2* and *avrRpt2-myc6* were expressed in tobacco using *Agrobacterium*-mediated transient transformation. Tobacco plants were inoculated with a  $1 \times 10^9$  cells  $\text{ml}^{-1}$  suspension of *A. tumefaciens* strain GV3101 carrying the pMD1 vector or pMD1 containing *avrRpt2* or *avrRpt2-myc6*. Symptoms were photographed 48 h after inoculation.

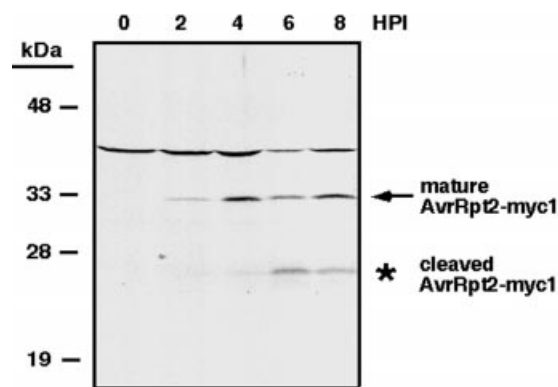
these N-terminal and C-terminal protein epitopes did not affect *in planta* recognition of AvrRpt2 by *RPS2*. These data suggest that the addition of these peptides to AvrRpt2 may have impaired type III secretion of AvrRpt2. Furthermore, the reduction in disease symptoms on susceptible plants (Fig. 3B and C) indicates that AvrRpt2-myc6, as well as AvrRpt2-gfp and AvrRpt2-cyA', may have impaired or blocked general type III secretion in Pst DC3000.

#### *N-terminal processing of AvrRpt2 in A. thaliana after Pst DC3000 infection*

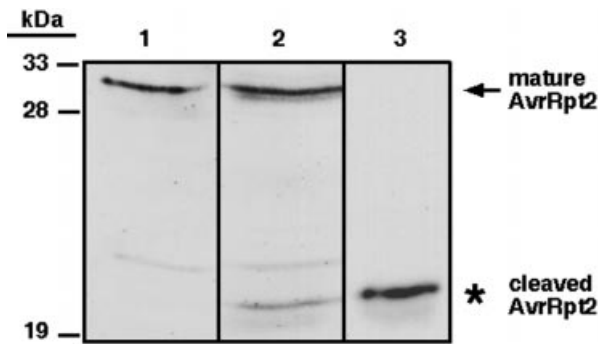
To follow AvrRpt2 delivery during Pst DC3000 pathogenesis, we monitored the time course of AvrRpt2 protein expression in resistant *A. thaliana RPS2* leaves inoculated with Pst DC3000 carrying *avrRpt2-myc1*. Leaves infiltrated with a bacterial suspension of  $1 \times 10^9$  cells  $\text{ml}^{-1}$  were collected up to 8 h after inoculation, and total protein was analysed by immunoblot analysis using c-myc(Ab-1) antibodies. The AvrRpt2-myc1 fusion protein migrates at  $\approx 31$  kDa. The native, mature AvrRpt2 protein is 28.2 kDa (Innes *et al.*, 1993) and the myc1 peptide tag is  $\approx 3$  kDa. *A. thaliana RPS2* leaves infiltrated with Pst DC3000 expressing *avrRpt2-myc1* accumulated the mature 31 kDa AvrRpt2-myc1 protein after 2 h of infection (Fig. 5). Expression of the mature AvrRpt2-myc1 protein increased by 4 h after inoculation; however, the protein did not accumulate. Instead a processed  $\approx 24$  kDa AvrRpt2-myc1-specific polypeptide appeared after 4 h of infection and continued to accumulate by 6–8 h after infection (Fig. 5). Detection of the truncated, C-terminally tagged AvrRpt2-myc1 protein with the c-myc(Ab-1) antisera indicated that proteolysis occurred at the N-terminus of AvrRpt2. N-terminal cleavage of AvrRpt2-myc1 was also observed in resistant *N. tabacum* cv. Xanthi *RPS2* leaves and susceptible *A. thaliana rps-201* leaves infected with Pst DC3000 expressing *avrRpt2-myc1* or *avrRpt2* (data not shown). This indicates that AvrRpt2 proteolysis was not *A. thaliana* specific or *RPS2* specific.

#### *Proteolysis of AvrRpt2 during bacterial infection is similar to the proteolysis of AvrRpt2 in planta*

N-terminal processing of AvrRpt2 has also been observed in transgenic *avrRpt2(RPS2)* *A. thaliana* seedlings that conditionally express native AvrRpt2 protein (McNellis *et al.*, 1998). In this transgenic line, *avrRpt2* expression results in the accumulation of a 21 kDa AvrRpt2-specific polypeptide. Interestingly, the mature 28 kDa AvrRpt2 protein was not detected. The N-terminal cleavage of AvrRpt2-myc1 in bacterially infected leaves also generated a 21 kDa AvrRpt2-specific polypeptide (Fig. 5; 24 kDa including the myc-1 epitope). To determine whether similar N-terminal AvrRpt2 proteolysis was occurring *in planta*, we analysed protein from *avrRpt2(RPS2)* transgenic plants induced for *avrRpt2* expression and from *A. thaliana* plants infected with Pst DC3000 expressing *avrRpt2*. Immunoblot analysis shows that Pst DC3000 cultured in minimal media in the absence of the host only accumulated mature AvrRpt2 protein (Fig. 6, lane 1). However, a cleaved AvrRpt2-specific polypeptide accumulated in protein extracts from *A. thaliana* leaves infected with Pst DC3000 expressing *avrRpt2* for 4 h (Fig. 6, lane 2) and in protein extracts from *avrRpt2(RPS2)* *A. thaliana* leaves expressing *avrRpt2* (Fig. 6, lane 3). The AvrRpt2-specific polypeptides co-migrated as 21 kDa species (Fig. 6, lanes 2 and 3). This demonstrates that the proteolytic cleavage of AvrRpt2 observed *in planta* is similar to that observed in bacterially infected leaves. Furthermore, these data show that N-terminal processing of native AvrRpt2



**Fig. 5.** N-terminal cleavage of AvrRpt2 protein in *A. thaliana RPS2* leaves after inoculation with Pst DC3000 expressing *avrRpt2-myc1*. The presence of bacterially produced AvrRpt2-myc1 protein in leaves was determined by immunoblot analysis using c-myc(Ab-1) antibodies. Leaves were inoculated with a  $1 \times 10^9$  cells  $\text{ml}^{-1}$  suspension of bacteria and then collected at 0, 2, 4, 6 and 8 h after inoculation (HPI). Fifty micrograms of total protein was loaded in each lane. Protein standards (19, 28, 33 and 48 kDa; Bio-Rad) were used to standardize protein migration. Mature and N-terminally processed AvrRpt2-myc1 proteins migrate at 31 and 24 kDa species respectively. The  $\approx 40$  kDa band appearing in all lanes is a non-specific protein recognized by the c-myc(Ab-1) antibody.



**Fig. 6.** AvrRpt2 protein cleaved in bacterially inoculated plant tissue co-migrates with AvrRpt2 protein stably expressed *in planta*. The presence of AvrRpt2 protein in cells was determined by immunoblot analysis using AvrRpt2-specific antibodies. Lane 1 shows the expression of AvrRpt2 protein in induced Pst DC3000 cells carrying pDSK600(*avrRpt2*). Lane 2 shows the accumulation of AvrRpt2 protein in *A. thaliana* RPS2 leaves infected with Pst DC3000 carrying pDSK600(*avrRpt2*) for 4 h. The experiment was performed as described in the legend to Fig. 5. Lane 3 shows the accumulation of AvrRpt2 protein in transgenic *avrRpt2*(RPS2) *A. thaliana* seedlings 48 h after treatment with 0.1  $\mu$ M dexamethasone. Protein standards (28, 33 and 48 kDa; Bio-Rad) were used to standardize protein migration. The mature 28 kDa and N-terminally cleaved 21 kDa AvrRpt2 proteins are denoted. The  $\approx$  23 kDa band in lanes 1 and 2 is a non-specific bacterial protein recognized by the AvrRpt2 antisera. Protein samples were analysed in the same gel and then realigned for clarity in this figure.

occurred only when bacteria were in contact with plant cells.

#### Processing of AvrRpt2 is *hrp* dependent

To determine whether proteolysis of AvrRpt2 is *hrp* dependent, we analysed the processing of native AvrRpt2 protein in wild-type and mutant *hrcC::Tn3gus* strains of Pst DC3000 in a parallel immunoblot experiment using AvrRpt2-specific antibodies. Although the mature AvrRpt2 protein accumulated in bacterially infected leaves with both wild-type and mutant *hrcC::Tn3gus* Pst DC3000 strains, the cleaved 21 kDa polypeptide was only detected in leaves infected with wild-type Pst DC3000 (Fig. 7). Thus, *hrp*-dependent secretion of AvrRpt2 is required for N-terminal processing of the mature AvrRpt2 polypeptide in infected plant leaves.

#### Host-dependent processing of AvrRpt2

The *hrp*-dependent processing of AvrRpt2 in infected leaf tissue indicated that cleavage of the polypeptide is likely to occur after its secretion, in between plant cells (the apoplast) or within plant cells. To identify the location of the protease, extracellular fluids and crude homogenate were isolated from *A. thaliana* leaves and then incubated with purified N-his6-AvrRpt2 protein. The 21 kDa AvrRpt2 polypeptide appeared only when crude *A. thaliana* homogenate

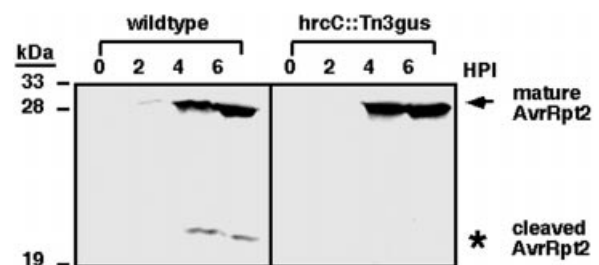
was incubated with purified N-his6-AvrRpt2 protein (Fig. 8A, lanes 1 and 2). *A. thaliana* extracellular fluids did not cleave purified N-his6-AvrRpt2 (data not shown). Furthermore, processing of N-his6-AvrRpt2 did not occur when protein was infiltrated directly into the extracellular spaces of leaves and then recovered later (data not shown). We hypothesize that N-terminal processing of AvrRpt2 occurs by a plant protease once the mature polypeptide is translocated to the host cell.

#### Host-specific protease cleavage site in AvrRpt2

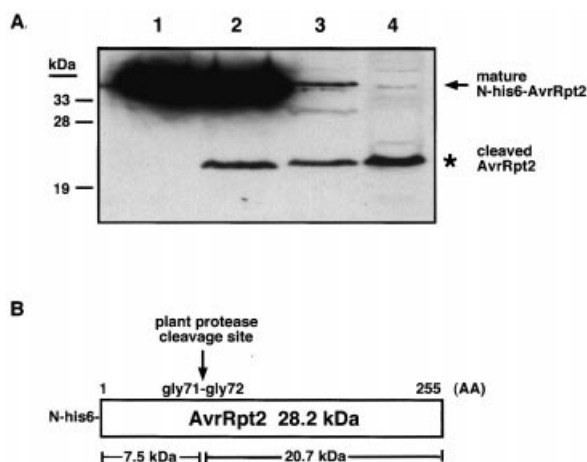
*In vitro* N-terminal processing of purified N-his6-AvrRpt2 protein by *A. thaliana* crude extract enabled us to gel purify the 21 kDa polypeptide for N-terminal Edman sequencing (Fig. 8A, lane 2). Sequencing revealed that the N-terminal amino acid of the isolated 21 kDa protein was glycine at position 72 in the mature AvrRpt2 protein (Fig. 8B). Proteolysis between glycine-71 and glycine-72 would generate a 20.7-kDa polypeptide. Co-migration of the *in vitro* cleaved N-his6-AvrRpt2 polypeptide with N-terminally truncated AvrRpt2 protein (amino acids 70–255) expressed in Pst DC3000 (Fig. 8A, lane 3) and the 21 kDa polypeptide detected in transgenic *avrRpt2*(RPS2) *A. thaliana* plants (Fig. 8A, lane 4) suggests that proteolysis most probably occurred after glycine-71 in all of the N-terminally cleaved AvrRpt2 polypeptides examined.

#### Deletion analysis of AvrRpt2

Considering that the N-terminus of proteins targeted through the type III secretion pathway in mammalian pathogens is essential for secretion, we were interested next in determining whether the N-terminus of AvrRpt2 is required for the *avrRpt2* *hrp*-dependent phenotype in Pst DC3000. AvrRpt2 N-terminal deletions were constructed using the polymerase chain reaction (PCR) to



**Fig. 7.** HrcC-dependent N-terminal processing of AvrRpt2 protein. Immunoblot analysis showing AvrRpt2 protein present in *A. thaliana* RPS2 leaves after inoculation with wild-type and mutant *hrcC::Tn3gus* Pst DC3000 strains carrying pDSK600(*avrRpt2*). The experiment was performed as described in the legend to Fig. 5. AvrRpt2 protein was detected using AvrRpt2-specific antibodies. Protein standards (28, 33 and 48 kDa; Bio-Rad) were used to standardize protein migration. Mature and N-terminally processed AvrRpt2 protein migrate as 28 and 21 kDa species respectively.



**Fig. 8.** *In vitro* proteolysis of AvrRpt2.

A. Immunoblot analysis showing the accumulation of N-terminally cleaved N-his6-AvrRpt2 after incubation with *A. thaliana* crude homogenate. Mature N-his6-AvrRpt2 and N-terminally processed protein migrate as 33 and 21 kDa species respectively. Lane 1 shows purified N-his6-AvrRpt2 incubated with plant extraction buffer (1.5  $\mu$ g of protein). Lane 2 shows purified N-his6-AvrRpt2 incubated with *A. thaliana* crude homogenate (1.5  $\mu$ g of protein). Lane 3 shows amino acids 70–255 of AvrRpt2 expressed in Pst DC3000 (10  $\mu$ g of total protein). Lane 4 shows AvrRpt2 protein present in transgenic *avrRpt2(RPS2)* *A. thaliana* seedlings 48 h after 0.1  $\mu$ M dexamethasone treatment (30  $\mu$ g of total protein). AvrRpt2 protein was detected using AvrRpt2 antibodies. Proteins running between 28 and 33 kDa in lanes 3 and 4 are non-specific bacterial and plant proteins recognized by the antisera.

B. Schematic diagram depicting protease cleavage site in AvrRpt2. *A. thaliana* protease cleaves 28.2 kDa AvrRpt2 between glycine-71 and glycine-72, generating a 7.5 kDa N-terminal polypeptide (not resolved in Fig. 8A) and a 20.7 kDa C-terminal polypeptide (Fig. 8A).

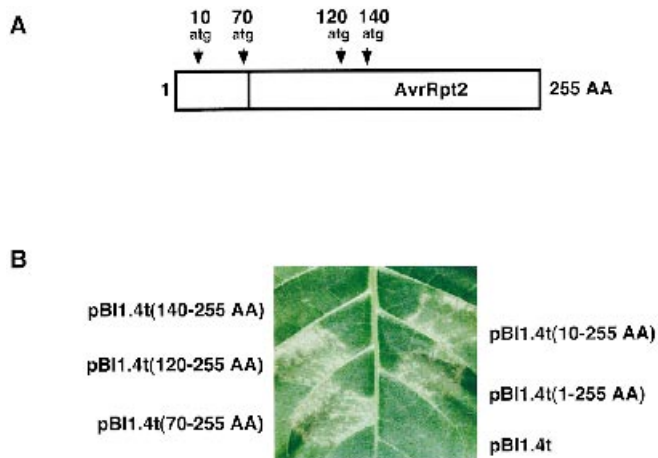
introduce an ATG codon at various positions at the N-terminus of the AvrRpt2 protein (Table 1, Fig. 9A). The *avrRpt2* 5' deletion constructs were then subcloned into pDSK519(*ngfp*) for constitutive expression by the *nptII* promoter. Interestingly, none of the N-terminally deleted AvrRpt2 polypeptides expressed in Pst DC3000

**Table 1.** Phenotypes of AvrRpt2 deletion polypeptides expressed by Pst DC3000 in infected *A. thaliana* *RPS2* leaves and by *Agrobacterium*-mediated transient transformation in *N. tabacum* *RPS2* leaves.

AvrRpt2 protein	Pst DC3000 expression		<i>A. tumefaciens</i> expression <i>in planta</i>	
	Phenotype <sup>a</sup>	Protein	Phenotype <sup>a</sup>	Protein
Wild-type protein				
1–255	HR	Yes	HR	Yes
N-terminal deletions				
10–255	NS	Yes	HR	Yes
20–255	NS	Yes	HR	
30–255	NS	Yes	HR	
40–255	NS	Yes	HR	
50–255	NS	Yes	HR	
60–255	NS	Yes	HR	
70–255	NS	Yes	HR	Yes
80–255	NS	Yes	HR	
90–255	NS	Yes	HR	
100–255	NS	Yes	HR	
120–255	NS	Yes	HR	Yes
140–255	NS	No	NS	No

a. HR, hypersensitive cell death response; NS, no symptoms.

induced localized cell death in infected *A. thaliana* *RPS2* leaves (Table 1). However, most of the N-terminally deleted polypeptides induced cell death when the corresponding genes were expressed directly *in planta* using *Agrobacterium*-mediated transient transformation (Table 1, Fig. 9A). Amino acid residues 120–255 of AvrRpt2 were sufficient to induce *in planta* cell death in resistant *N. tabacum* *RPS2* (Fig. 9B). However, no AvrRpt2 activity was detected for amino acids 140–255 (Table 1, Fig. 9B). We were unable to determine precisely the region of AvrRpt2 required for *in planta* recognition by *RPS2* because polypeptides deleted beyond amino acid 120 were not detected by the AvrRpt2 polyclonal antisera (Table 1). Failure to detect these polypeptides may reflect antibody specificity and/or protein stability. These results demonstrate that, although the N-terminus of AvrRpt2 is



**Fig. 9.** Phenotypes of N-terminally deleted AvrRpt2 polypeptides in tobacco.

A. Schematic diagram depicting some of the N-terminally deleted AvrRpt2 polypeptides analysed. PCR was used to introduce start codons (atg) before the *avrRpt2* nucleotides encoding amino acids 10, 70, 120 and 140. These 5' *avrRpt2* deletion constructs were cloned into pBI1.4t.

B. *Agrobacterium*-mediated transient expression of the N-terminally deleted AvrRpt2 proteins in *N. tabacum* cv. Xanthi *RPS2* leaves. Plants were inoculated with a  $1 \times 10^9$  cells  $\text{ml}^{-1}$  suspension of *A. tumefaciens* strain GV3101 carrying pBI1.4t, pBI1.4(1–255 amino acids), pBI1.4t(10–255 amino acids), pBI1.4t(70–255 amino acids), pBI1.4t(120–255 amino acids) or pBI1.4t(140–255 amino acids). Symptoms were photographed 48 h after inoculation.

not required for AvrRpt2 recognition in *RPS2* plants, this region is absolutely essential for AvrRpt2 signalling when expressed in Pst DC3000. We were unable to assay C-terminally deleted polypeptides for AvrRpt2 activity because they were unstable in minimal media-induced Pst DC3000 cells (data not shown).

## Discussion

In this study, we have characterized AvrRpt2 protein expression in Pst DC3000 and in Pst DC3000-infected *RPS2* plants to address the role of this effector molecule in *RPS2*-mediated programmed cell death. We provide evidence supporting the *hrp*-dependent secretion of AvrRpt2. We show that AvrRpt2 can be secreted efficiently from *E. coli* through the *hrp* secretion apparatus of the necrotrophic phytopathogenic bacterium, *E. chrysanthemi*. Expression of AvrRpt2 in *E. coli* carrying the  $E_{ch}$  *hrp* gene cluster also caused localized cell death in *N. clevelandii* bearing *RPS2* activity. These data demonstrate that the  $E_{ch}$  *hrp* cluster can secrete AvrRpt2 and deliver this effector to solanaceous hosts of *E. chrysanthemi*.

We also show that the pathogen Pst DC3000 secretes mature AvrRpt2 protein into culture fluids under *hrp*-inducing growth conditions. The secretion of AvrRpt2 from Pst DC3000 requires a functional *hrp* secretion system. Mutation of *hrcU*, an inner membrane structural component of the *hrp* apparatus, prevented the secretion of AvrRpt2 from Pst DC3000 and the induction of localized cell death in resistant *A. thaliana RPS2* plants. These results indicate that AvrRpt2 is targeted through the Pst DC3000 *hrp* apparatus and that secretion is one event required for the molecular recognition of this effector protein by *RPS2* plants. However, the AvrRpt2 secretion from Pst DC3000 was inefficient and more difficult to detect compared with the *E. coli* system. So far, only mammalian pathogens are known to secrete copious amounts of type III-specific effector proteins into culture fluids (Hueck, 1998). Thus, the Pst DC3000 *hrp* secretion pathway may be tightly regulated, enabling the detection of only a small amount of secreted protein in the absence of the host cell. This supports the hypothesis that secretion and translocation of avirulence proteins from *P. syringae* strains to plant cells may be co-ordinately regulated (Alfano and Collmer, 1997). Although YopE can be detected in culture fluids, its targeting from *Yersinia enterocolitica* into the cytosol of HeLa cells has been shown to occur only by one translocation step, requiring the SycE chaperone and host cell contact (Lee *et al.*, 1998).

Interestingly, we found that N-terminal processing of the 28 kDa AvrRpt2 protein also accompanies *P. syringae* plant pathogenesis. Plants infected with Pst DC3000 expressing *avrRpt2* accumulated an N-terminally cleaved

21 kDa AvrRpt2 polypeptide, in a *hrp*-dependent manner. Moreover, proteolysis of AvrRpt2 appeared to be plant specific. The AvrRpt2 protease was present in both resistant and susceptible plants, demonstrating that cleavage occurred independently of the *RPS2* resistance pathway. Because no AvrRpt2-specific protease activity was detected in plant intercellular fluids, we hypothesize that AvrRpt2 cleavage occurred inside the plant cell. This would corroborate the observed N-terminal processing of AvrRpt2 in *avrRpt2(RPS2)* transgenic *A. thaliana* plants (McNellis *et al.*, 1998). N-terminal sequence analysis of the 21 kDa species verified that proteolysis occurred between glycine-71 and glycine-72 in the mature AvrRpt2 polypeptide. Computer-assisted protease mapping of AvrRpt2 did not reveal a processing site after amino acid 71, indicating that the AvrRpt2 protease may be novel. To our knowledge, this is the first avirulence protein shown to undergo specific degradation during plant infection. However, it is interesting to note that the avirulence protein AvrPphB is N-terminally cleaved in *hrp*-induced *P. syringae* pv. *phaseolicola* cells in the absence of the host (Puri *et al.*, 1997).

N-terminal cleavage of the AvrRpt2 polypeptide questioned the identity of the *avrRpt2*-specific elicitor recognized by *RPS2* plants. Protein deletion studies clearly demonstrated that the C-terminal 21 kDa polypeptide had AvrRpt2 activity when it was expressed inside *RPS2* plants. The N-terminal 7 kDa AvrRpt2 polypeptide was unstable in Pst DC3000; therefore, we could not assay its activity. However, the N-terminal amino acids were found to be essential for Pst DC3000-dependent AvrRpt2 phenotypes in *RPS2* plants. It is known that protein trafficking through the type III secretion system in *Yersinia* spp. requires signals residing in the 5' region of the mRNA (Anderson and Schneewind, 1997), as well as the N-terminal amino acids encoding individual Yop effectors (Michiels and Cornelis, 1991; Sory *et al.*, 1995; Schesser *et al.*, 1996). We are now interested in determining whether similar motifs exist for the secretion and translocation of the *avrRpt2*-encoded elicitor.

What then is the function of AvrRpt2 processing? The processing of other type III effector proteins has not been shown to accompany their translocation to host cells. We know that AvrRpt2 proteolysis does not interfere with the molecular recognition of the *avrRpt2* elicitor by *RPS2* inside plant cells. Thus, the avirulence function of AvrRpt2 seems to be unaffected by the removal of its N-terminal 71 amino acid residues. It is possible that proteolysis may instead affect some virulence function of AvrRpt2. Removal of the N-terminus could activate AvrRpt2 as a virulence factor and/or target it to another location in the cell, as shown for the Shiga toxin in mammalian cell cultures (Garred *et al.*, 1995). The virulence role of AvrRpt2 is currently not known. However, it

is interesting to note that transgenic susceptible *avrRpt2(rps2-101C)* *A. thaliana* seedlings induced for *avrRpt2* expression accumulate N-terminally processed AvrRpt2 protein, which is lethal at high levels (McNellis *et al.*, 1998).

Pst DC3000 type III-dependent secretion of AvrRpt2 and host-specific proteolysis of AvrRpt2 support the delivery of this effector protein to plant cells. However, in order to elucidate specific AvrRpt2 function(s), our next challenge is to acquire direct evidence for the delivery of AvrRpt2 to plant cells. Localization of AvrRpt2 inside plant cells will help to identify potential virulence and avirulence targets. The availability of transgenic *avrRpt2* *A. thaliana* plant lines (McNellis *et al.*, 1998) is enabling us to study AvrRpt2 localization and function inside plant cells in the absence of the pathogen. We will also explore the exciting possibility that host-specific proteolysis of AvrRpt2 may activate it as a virulence factor. Finally, further characterization of the *hrp* pathway in Pst DC3000 will probably identify structural features of AvrRpt2 and potential interacting proteins required for type III delivery of the AvrRpt2 effector. Progress in understanding AvrRpt2 function(s) will strengthen our understanding of *Pseudomonas hrp* secretion machineries and the specific mechanisms controlling plant pathogenesis and plant disease resistance.

## Experimental procedures

### Bacterial strains, media and plasmids

Bacterial strains and plasmids used in this study are described in Table 2. *E. coli* and *A. tumefaciens* strains were grown in LB medium (Sambrook *et al.*, 1989) at 37°C and 28°C respectively. Pst DC3000 was grown in NYGB liquid medium (Turner *et al.*, 1984) and on *Pseudomonas* agar F plates (PA) (Difco) at 28°C. The plasmid pRK2013 (Table 2) was used in triparental matings (Figurski and Helinski, 1979) to mobilize broad-host-range vectors from *E. coli* into *P. syringae* and *A. tumefaciens*. Antibiotics were used for plate selection at the following concentrations (mg l<sup>-1</sup>): nalidixic acid (Nal), 50; gentamicin (Gm), 50; kanamycin (Km), 50; rifampicin (Rif), 100; streptomycin (Sm), 25; spectinomycin (Sp), 50. The concentration of antibiotics used in liquid cultures was decreased by half.

Pst DC3000 *hrp* mutants containing Tn3gus transposon insertions were constructed and characterized by C. Boucher, D. Dahlbeck and B. J. Staskawicz (unpublished results). *hrcC::Tn3gus* and *hrcU::Tn3gus* mutants were identified by sequencing the DNA 5' of the Tn3gus insertion using the primer 5'-TGACACCATTAAGAGGCGT-3', specific for the 5' end of the transposon. Sequencing was performed by cycle sequencing using the Prism ready reaction dye deoxy terminator cycle sequencing kit and the ABI Prism model 377 DNA sequencer (Applied Biosystems) according to the manufacturer's instructions. Mutations in *hrcC* and *hrcU* were then identified using the sequence of the Pst DC3000 *hrp* locus generated by C. Tobias (unpublished results).

### Secretion assays

*E. coli* DH5 $\alpha$  cells containing pDSK519(*navrRpt2*) were transformed individually with pCPP2156 and pCPP2368, generating the secretion-competent strain *E. coli* [pDSK519(*navrRpt2*), pCPP2156] and the secretion mutant strain *E. coli* [pDSK519(*navrRpt2*), pCPP2368]. Glycerol stocks were streaked on LM plates (Hanahan, 1983) and grown overnight at 37°C. Cells were then restreaked on LM plates and grown again at 37°C. Fresh cells were resuspended and washed twice in LM broth. Cells were diluted to 2  $\times$  10<sup>8</sup> cells ml<sup>-1</sup> in 40 ml of LM broth containing antibiotics and cultured at 28°C in a rotary shaker at 220 r.p.m. until the densities reached 7–8  $\times$  10<sup>8</sup> cells ml<sup>-1</sup>. After centrifugation at 2900  $\times$  g, supernatants (20 ml) were filtered through low-protein-binding 0.2  $\mu$ m membranes (Schleicher & Schuell) and then precipitated with TCA (10% final concentration) at 4°C for 3 h. Filtered supernatants (100  $\mu$ l) were also plated on LM selection plates to control for bacterial contamination. Precipitates were collected by centrifugation at 17 900  $\times$  g for 40 min, washed with 5 ml of cold acetone and then recollected. Precipitates were resuspended with sample buffer (see below), typically 100  $\mu$ l, and then identified as concentrated culture fluid fractions. Protein was isolated from the bacterial cell pellets as described below.

Pst DC3000 strains grown overnight on PA plates at 28°C were resuspended and washed in NYGB. Cells were diluted to 1  $\times$  10<sup>8</sup> cells ml<sup>-1</sup> in 200 ml of fresh NYGB containing antibiotics. Cultures were grown overnight at 28°C in a rotary shaker until late log-phase growth. Cells were collected by centrifugation at 3000  $\times$  g and washed with minimal media containing 50 mM K-PO<sub>4</sub>, pH 5.7, 7.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.7 mM MgCl<sub>2</sub>, 1.7 mM NaCl, 10 mM fructose and 10 mM mannitol (Huynh *et al.*, 1989). Cells were then diluted to 2  $\times$  10<sup>8</sup> cells ml<sup>-1</sup> in 200 ml of minimal media lacking antibiotics and grown for 14 h at 21°C in a rotary shaker. Cultures typically grew to cell densities of 6–7  $\times$  10<sup>8</sup> cells ml<sup>-1</sup>. After centrifugation at 9000  $\times$  g, supernatants were filtered through 0.2  $\mu$ m filtering units (Nalgene), stored on ice and mixed with a solution containing 1 mM phenylmethylsulphonyl fluoride (PMSF), 1  $\mu$ M leupeptin, 1 mM dithiothreitol and 1 mM sodium EDTA. Filtered supernatants (100  $\mu$ l) were also plated on PA selection plates to control for bacterial contamination. Centriprep-10 concentrators (Amicon) were then used to concentrate supernatants to 0.5 ml. This sample was identified as the concentrated culture fluid. Soluble protein was isolated from the bacterial cell pellets as described below.

### Protein extraction, protein gels and immunoblot analysis

Protein was isolated from bacteria by lysing cell pellets in buffer containing 8 M urea, 0.1 M NaPO<sub>4</sub>, 0.01 M Tris-HCl, pH 8.0. After incubation for 30 min with rocking, the cell lysates were centrifuged at 14 000  $\times$  g for 15 min to obtain the supernatant. This protein fraction was then identified as the cellular lysate fraction. Protein from bacterially infected leaves and transgenic seedlings was extracted from liquid nitrogen-frozen tissue by homogenization using a Kontes pestle (Fisher) in a microcentrifuge tube. Ground tissue was then resuspended with sample buffer (180 mM Tris-HCl, pH 6.8, 6% SDS, 2.1 M 2-mercaptoethanol, 35.5% glycerol

**Table 2.** Bacteria and plasmids used in this work.

Strain/plasmid	Characteristics	Reference/source
<b>Bacteria</b>		
<i>Pseudomonas syringae</i> pv. <i>syringae</i>		
Wild-type strain DC3000	Virulent, Rif <sup>r</sup>	D. Cuppels
<i>hrp</i> mutants derived from Tn3Gus insertional mutagenesis		
<i>hrcC</i> ::Tn3Gus7	<i>hrcC</i> polar mutant	C. Boucher, D. Dahlbeck, B. Staskawicz (unpublished)
<i>hrcU</i> ::Tn3Gus175	<i>hrcU</i> non-polar mutant	
<i>Agrobacterium tumefaciens</i>		
GV3101(pMP90)		Koncz and Schell (1986)
<i>Escherichia coli</i>		
DH5 $\alpha$	F <sup>-</sup> <i>lacZ</i> M15 <i>endA1</i> <i>recA1</i> <i>hsdR17</i> <i>supE44</i> <i>thi-1</i> <i>gyrA</i> <i>relA1</i> I <sup>-</sup> Nal <sup>r</sup>	Life Technologies
<b>Plasmids</b>		
pCR2.1	Km <sup>r</sup> , ColE1 replicon, T-overhang vector for cloning PCR products	Invitrogen
pBS( <i>myc6</i> stop)	Ap <sup>r</sup> , 288 bp <i>Apal</i> – <i>EcoRI</i> fragment containing six repeats of <i>c-myc</i> epitope in pBluescript-II KS+, in frame stop codon at <i>SpeI</i> site	C. Tobias
p519 <i>ngfp</i>	Km <sup>r</sup> , IncQ replicon, <i>nptII</i> promoter cloned directly in front of <i>gfp</i>	Matthysse <i>et al.</i> (1996)
pDSK600	Sp <sup>r</sup> , Sm <sup>r</sup> , IncQ replicon, 3 <i>xlacUV5</i> promoter	Murillo <i>et al.</i> (1994)
pRK2013	Km <sup>r</sup> , ColE1 replicon, Tra <sup>+</sup> , Mob <sup>+</sup> , helper plasmid	Figurski and Helinski (1979)
pMD1	Km <sup>r</sup> , RK2 replicon, replicates in <i>A. tumefaciens</i> , CaMV 35S promoter	M. Dixon
pBI1.4t	Km <sup>r</sup> , RK2 replicon, replicates in <i>A. tumefaciens</i> , modified CaMV *35S promoter for lower expression in bacteria	Mindrinos <i>et al.</i> (1994)
pRSR0	Ap <sup>r</sup> , 1.4 kb <i>SalI</i> fragment containing <i>avrRpt2</i> in pUC119	Innes <i>et al.</i> (1993)
pCPP2156	Sp <sup>r</sup> , pCPP19 carrying <i>E. chrysanthemi</i> <i>hrp</i> cluster	Ham <i>et al.</i> (1998)
pCPP2368	Sp <sup>r</sup> , Cm <sup>r</sup> , pCPP2156::Tn5 <i>Cm</i> that has HR <sup>-</sup> phenotype	Ham <i>et al.</i> (1998)
pBS( <i>avrRpt2-myc6</i> )	947 bp <i>XhoI</i> – <i>XhoI</i> fragment containing <i>avrRpt2</i> promoter and orf in pBS( <i>myc6</i> stop)	This work
pBS( <i>avrRpt2-myc1</i> )	Five <i>c-myc</i> repeats removed by <i>Clal</i> – <i>NcoI</i> digest of pBS( <i>avrRpt2-myc6</i> )	This work
pDSK519( <i>navrRpt2</i> )	777 bp <i>NdeI</i> – <i>EcoRI</i> fragment containing <i>avrRpt2</i> in p519 <i>ngfp</i> , <i>nptII</i> promoter expressing <i>avrRpt2</i>	This work
pDSK600( <i>avrRpt2</i> )	1.8 kb <i>HindIII</i> fragment containing <i>avrRpt2</i> promoter and orf in pDSK600	C. Tobias
pDSK519( <i>avrRpt2</i> )	2.2 kb <i>KpnI</i> fragment from pDSK600( <i>avrRpt2</i> ) in pDSK519	This work
pDSK600( <i>avrRpt2-myc1</i> )	1.0 kb <i>XbaI</i> fragment from pBS( <i>avrRpt2-myc1</i> ) containing <i>avrRpt2-myc1</i> in pDSK600	This work
pDSK600( <i>avrRpt2-myc6</i> )	1.25 kb <i>XbaI</i> fragment from pBS( <i>avrRpt2-myc6</i> ) containing <i>avrRpt2-myc6</i> in pDSK600	This work
pMD1( <i>avrRpt2</i> )	793 bp <i>BamHI</i> – <i>SalI</i> fragment containing <i>avrRpt2</i> in pMD1	This work
pMD1( <i>avrRpt2-myc1</i> )	1.1 kb fragment containing <i>avrRpt2-myc1</i> in pMD1	This work
pMD1( <i>avrRpt2-myc6</i> )	871 bp fragment containing <i>avrRpt2-myc6</i> in pMD1	This work
pBI1.4t( <i>avrRpt2</i> )	782 bp <i>XbaI</i> – <i>EcoRI</i> fragment containing <i>avrRpt2</i> in pBI1.4t	This work

and 0.004% bromophenol blue). Soluble protein was collected after centrifugation at 14 000  $\times$  *g* at 4°C for 15 min. A modification of the Lowry procedure (Bailey, 1967) was used to determine the concentration of protein after precipitation with 1 ml of 10% trichloroacetic acid. Protein samples were analysed by SDS–polyacrylamide slab gel electrophoresis using the buffer system described by Laemmli (1970). Protein fractions were mixed in a ratio of 2:1 (v/v) with sample buffer and boiled for 4 min. These fractions were electrophoresed in a 12.5% (w/v) acrylamide–0.43% (w/v) *N,N*-methylenebisacrylamide separating gel. Gels were stained in Coomassie brilliant blue or silver (Morrisey, 1981).

For immunoblot analysis, proteins were transferred from gels to MSI nitrocellulose membranes (Micron Separations) by electroblotting in transfer buffer containing 25 mM Tris-HCl, pH 8.3, 192 mM glycine and 28% (v/v) methanol at 0.5 A for 1 h. AvrRpt2 and AvrRpt2-myc1 protein was detected using rabbit polyclonal anti-N-His6-AvrRpt2 sera (1:1000 dilution) (McNellis *et al.*, 1998) and mouse monoclonal anti-c-myc(Ab-1)

sera (1  $\mu$ g ml<sup>-1</sup>) (Oncogene Research Products), respectively, in buffer containing 3% non-fat milk, 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween-20. NPT II was detected using rabbit polyclonal NPT II sera (1:1000 dilution) (5 Prime to 3 Prime). Goat anti-rabbit and goat anti-mouse IgG second antibodies conjugated to horseradish peroxidase (Bio-Rad) were used to detect primary antibodies. All detections of immunoblots were carried out with enhanced chemiluminescence as described by the manufacturer (Amersham).

#### Polymerase chain reaction (PCR) and plasmid construction

PCR was used to engineer *avrRpt2* constructs containing gene fusions and restriction endonuclease sites for cloning. All *avrRpt2*-specific primers used for PCR will be available upon request. The *avrRpt2* promoter and/or coding region

was PCR amplified using *Pfu* polymerase (Stratagene) and the pRSR0 plasmid containing *avrRpt2* (Innes *et al.*, 1993). PCR reactions (50  $\mu$ l) typically contained 10 pmol of each primer, 200  $\mu$ M dNTPs, 10 ng of pRSR0 template DNA and 1 unit of *Pfu* polymerase. Reactions were heated to 95°C for 1 min, and then 15 amplification cycles were performed at 95°C for 30 s (denaturation), 55°C for 30 s (annealing) and 72°C for 30 s (extension). PCR reactions were incubated with 1 unit of *Taq* polymerase (Promega) at 72°C for 10 min to add a single deoxyadenosine to the 3' end of the PCR products. Amplified DNA was cloned directly into pCR2.1, containing a single deoxythymidine residue, as described by the manufacturer (Invitrogen). All PCR-generated *avrRpt2* constructs were sequenced.

Primer pair MB1 and MB2 was used to amplify the *avrRpt2* coding region containing a 5' *Bam*HI site and a 3' *Sal*I site. Once cloned into pCR2.1, the *Bam*HI–*Sal*I PCR-amplified *avrRpt2* fragment was isolated and cloned into the corresponding sites in pMD1, creating pMD1(*avrRpt2*).

Primer pair MB3 and MB4 was used to amplify the promoter and coding region of *avrRpt2* containing 5' *Xho*I–*Xba*I sites and a 3' *Xho*I site. The PCR products were digested with *Xho*I and cloned in pBS(*myc6* stop), creating pBS(*avrRpt2-myc6*). An in frame stop codon was introduced into pBS(*myc6*) (gift from N. Raikhel), creating pBS(*myc6* stop) by digesting pBS(*myc6*) with *Pst*I, filling in cleaved DNA with Klenow DNA polymerase (New England Biolabs) and then religating the plasmid. The *Xba*I fragment containing *avrRpt2-myc6* was removed from this plasmid and subcloned into the *Xba*I site of pDSK600 creating pDSK600(*avrRpt2-myc6*). To construct an *avrRpt2-myc1* gene fusion, pBS(*avrRpt2-myc6*) was digested with *Cla*I and *Nco*I removing the five *c-myc* internal repeats. The restricted plasmid DNA was filled in with DNA polymerase and then ligated to generate pBS(*avrRpt2-myc1*). To clone the *avrRpt2-myc* fusions into pMD1, pBS(*avrRpt2-myc1*) and pBS(*avrRpt2-myc6*) were digested with *Sac*I, cutting *avrRpt2* at nucleotide 605 (location relative to GenBank submission Z21715) and the vector 3' of *c-myc* sequence. The *Sac*I fragment was then cloned into pMD1(*avrRpt2*) restricted with *Sac*I, creating pMD1(*avrRpt2-myc1*) and pMD1(*avrRpt2-myc6*).

Primer pair MB133 and MB81 was used to amplify the *avrRpt2* coding region containing 5' *Xba*I–*Nde*I sites and a 3' *Eco*RI site. Once cloned into pCR2.1, the *Nde*I–*Eco*RI-amplified *avrRpt2* fragment was isolated and cloned into p519(*ngfp*) digested with *Nde*I–*Eco*RI to remove *gfp*. The resulting plasmid, pDSK519(*navrRpt2*), constitutively expresses *avrRpt2* using the *npt2* promoter. The original *Xba*I–*Eco*RI-amplified *avrRpt2* fragment was then cloned into pB11.4t, creating pB11.4t(*avrRpt2*).

For brevity, the description of the following *AvrRpt2* fusion constructs will be made available upon request: pDSK600(*flag-avrRpt2*), pDSK600(*avrRpt2-his6*), pDSK600(*avrRpt2-gfp*), pDSK600(*avrRpt2-cyA'*), pMD1(*flag-avrRpt2*), pMD1(*avrRpt2-his6*), pMD1(*avrRpt2-gfp*) and pMD1(*avrRpt2-cyA'*).

For *AvrRpt2* N-terminal deletion analysis, all PCR primers contained 5' *Xba*I–*Nde*I sites and a 3' *Eco*RI site to facilitate the cloning of *avrRpt2*-amplified fragments into p519(*ngfp*) and pB11.4t as described above.

### Plant growth and bacterial inoculations

*A. thaliana* ecotype Col-0 wild-type *RPS2* and mutant *rps2-201* (Kunkel *et al.*, 1993) plants were grown in growth chambers at 22°C under an 8 h photoperiod to promote leaf expansion and delay flowering. Fully expanded leaves of 3- or 4 week-old plants were used for bacterial inoculations. *N. tabacum* cv. Xanthi and *N. clevelandii* plants were grown under greenhouse conditions. Tobacco plants with two to four leaves were used for bacterial inoculations. Bacterial suspension in 10 mM MgCl<sub>2</sub> was hand-infiltrated into the extracellular spaces of a plant leaf through a small wound site using a 1 cc syringe. For localized cell death assays, *Pst* DC3000 inoculum was  $2 \times 10^7$  cells ml<sup>-1</sup>, and *E. coli* E<sub>ch</sub> *hrp* inoculum was  $2-3 \times 10^9$  cells ml<sup>-1</sup>. Reactions were typically scored 24 h after inoculation. For immunoblot analyses, the inoculum was  $1 \times 10^9$  cells ml<sup>-1</sup>.

### In vivo *Pst* DC3000 growth curve

To determine *Pst* DC3000 growth in *A. thaliana* leaves, plants were vacuum infiltrated with bacterial suspensions of  $5 \times 10^4$  cells ml<sup>-1</sup>. Bacteria in the leaves were sampled by grinding four leaf discs (No 1. cork borer, 0.4 cm diameter) in 10 mM MgCl<sub>2</sub> and plating appropriate dilutions on PA containing appropriate antibiotics and cyclohexamide (50  $\mu$ g ml<sup>-1</sup>). Bacterial growth was assayed 0, 2 and 4 days after inoculation. Three replicates were taken for each sampling.

### Agrobacterium-mediated transient transformation assays

*A. tumefaciens* GV3101 strains were grown in LB at 28°C until late log phase. Cells were recovered by centrifugation at  $3000 \times g$  for 10 min, washed and then resuspended in fresh media to a suspension of  $1 \times 10^8$  cells ml<sup>-1</sup>. Acetosyringone (3',5' dimethoxy-4'-hydroxyacetophenone; Aldrich) was added to cultures (25  $\mu$ M) for *A. tumefaciens* virulence induction. Cells were grown again until late log phase, recovered by centrifugation, washed and then resuspended with 10 mM MgCl<sub>2</sub>. Bacteria suspensions of  $1 \times 10^9$  cells ml<sup>-1</sup> were used to inoculate (see above) tobacco leaves. Inoculated plants were incubated in a growth chamber at 22°C under an 8 h photoperiod. Reactions were scored 24–48 h after inoculation.

### *AvrRpt2* protein expression in transgenic *avrRpt2*(*RPS2*) *A. thaliana* plants

Transgenic *avrRpt2*(*RPS2*) plants were used to analyse the size of the *AvrRpt2* polypeptide that accumulates *in planta*. To induce *AvrRpt2* protein expression, *avrRpt2*(*RPS2*) seedlings were treated with 0.1  $\mu$ M dexamethasone for 48 h as described by McNellis *et al.* (1998). Protein was extracted from the tissue with sample buffer as described above.

### In vitro cleavage of *AvrRpt2* and protein sequencing

N-his6-*AvrRpt2* protein was purified as described by McNellis *et al.* (1998). Crude homogenate was isolated from *A. thaliana* leaves as described by Mudgett and Clarke (1994). N-his6-*AvrRpt2* protein (20  $\mu$ g) was incubated with plant extraction buffer or 20  $\mu$ g of *A. thaliana* crude

homogenate for 2 h at room temperature. Then, 1.5 µg of these protein samples was analysed by immunoblot analysis. For N-terminal Edman sequencing, protein samples were separated by SDS–polyacrylamide slab gel electrophoresis and then transferred to nitrocellulose as described above. The 21 kDa AvrRpt2 polypeptide was excised and sequenced (≈ 100 pmol of protein) by Dr Audree Fowler at the UCLA Protein Microsequencing Facility.

*In vitro* cleavage of N-his6-AvrRpt2 protein was tested similarly using protein isolated from leaf extracellular fluids. Entire *A. thaliana* leaves were hand infiltrated with 10 mM MgCl<sub>2</sub>. After incubation for 0 and 3.5 h, the leaves were put on top of a circular plastic screen placed inside a conical centrifuge tube. The leaves were centrifuged at 2300 × *g* for 15 min. The liquid at the bottom of the centrifuge tube was collected and identified as the extracellular fluid. Typically, 0.1–0.2 mg ml<sup>-1</sup> protein was isolated from leaf extracellular fluids. Purified N-his6-AvrRpt2 protein (20 µg) was incubated with 2.5 µg of extracellular protein or 2.5 µg of crude homogenate from *A. thaliana* for 2 h and then analysed by immunoblot analysis.

### Acknowledgements

We are especially grateful to Doug Dahlbeck for technical assistance and helpful discussion. We also thank Alan Collmer for the gift of the heterologous *E. coli* E<sub>ch</sub> hrp system. We are grateful to Dr Audree Fowler for protein sequence analysis performed at the UCLA Protein Microsequencing Facility aided by a Cancer Center Support Grant (CA 16042) from the NCI. We appreciate comments given by Walter Gassmann, Peter Repetti, Manuel Sainz and Maureen Whalen regarding this manuscript. This work was supported by the Department of Energy grant DE-FG03-88ER13917 given to B.J.S. M.B.M. was supported by the NIH NRSA post-doctoral training grant 1 F32 GM18414-02.

### References

Alfano, J.R., and Collmer, A. (1997) The type III (hrp) secretion pathway of plant pathogenic bacteria: trafficking harpins, avr proteins, and death. *J Bacteriol* **179**: 5655–5662.

Anderson, D.M., and Schneewind, O. (1997) A mRNA signal for the type III secretion of Yop proteins by *Yersinia enterocolitica*. *Science* **278**: 1140–1143.

Arlat, M., Van Gijsegem, F., Huet, J.C., Pernollet, J.C., and Boucher, C.A. (1994) PopA1, a protein which induces a hypersensitivity like response on specific *Petunia* genotypes, is secreted via the Hrp pathway of *Pseudomonas solanacearum*. *EMBO J* **13**: 543–553.

Bailey, J.L. (1967) *Techniques in Protein Chemistry*. New York: Elsevier.

Bent, A.F., Kunkel, B.N., Dahlbeck, D., Brown, K.L., Schmidt, R., Giraudat, J., *et al.* (1994) RPS2 of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. *Science* **265**: 1856–1860.

Bogdanove, A.J., Wei, Z.-M., Zhao, L., and Beer, S.V. (1996) *Erwinia amylovora* secretes harpin via a type III pathway and contains a homolog of yopN of *Yersinia* spp. *J Bacteriol* **178**: 1720–1730.

Bogdanove, A.J., Bauer, D.W., and Beer, S.V. (1998) *Erwinia amylovora* secretes DspE, a pathogenicity factor and functional AvrE homolog, through the hrp (type III secretion) pathway. *J Bacteriol* **180**: 2244–2247.

Charkowski, A.O., Huang, H.-C., and Collmer, A. (1997) Altered localization of hrpZ in *Pseudomonas syringae* pv. *syringae* hrp mutants suggests that different components of the type III secretion pathway control protein translocation across the inner and outer membranes of Gram-negative bacteria. *J Bacteriol* **179**: 3866–3874.

Charkowski, A.O., Alfano, J.R., Preston, G., Yuan, J., He, S.Y., and Collmer, A. (1998) The *Pseudomonas syringae* pv. *tomato* HrpW protein has domains similar to harpins and pectate lyases and can elicit the plant hypersensitive response and bind to pectate. *J Bacteriol* **180**: 5211–5217.

Dangl, J.L. (1994) The enigmatic avirulence genes of phytopathogenic bacteria. In *Bacterial Pathogenesis of Plants and Animals*, Vol. 192. Dangl, J.L. (ed.), pp. 99–118. Berlin: Springer-Verlag.

Delledonne, M., Xia, Y., Dixon, R.A., and Lamb, C. (1998) Nitric oxide functions as a signal in plant disease resistance. *Nature* **394**: 585–588.

Deng, W.-L., Preston, G., Collmer, A., Chang, C.-J., and Huang, H.-C. (1998) Characterization of the hrpC and hrpRS operons of *Pseudomonas syringae* pathovars syringae, tomato, and glycinea and analysis of the ability of hrpF, hrpG, hrcC, hrpT, and hrpV mutants to elicit the hypersensitive response and disease in plants. *J Bacteriol* **180**: 4523–4531.

Durner, J., Wendehenne, D., and Klessig, D.F. (1998) Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc Natl Acad Sci USA* **95**: 10328–10333.

Figurski, D., and Helinski, D.R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. *Proc Natl Acad Sci USA* **76**: 1648–1652.

Flor, H.H. (1971) Current status of the gene-for-gene concept. *Annu Rev Phytopathol* **9**: 275–296.

Garred, O., Dubinina, E., Holm, P.K., Olsnes, S., van Deurs, B., Kozlov, J.V., *et al.* (1995) Role of processing and intracellular transport for optimal toxicity of Shiga toxin and toxin mutants. *Exp Cell Res* **218**: 39–49.

Gaudriault, S., Malandrin, L., Paulin, J.-P., and Barny, M.-A. (1997) DspA, an essential pathogenicity factor of *Erwinia amylovora* showing homology with AvrE of *Pseudomonas syringae*, is secreted via the Hrp secretion pathway in a DspB-dependent way. *Mol Microbiol* **26**: 1057–1069.

Gopalan, S., Bauer, D.W., Alfano, J.R., Loniello, A.O., He, S.Y., and Collmer, A. (1996) Expression of the *Pseudomonas syringae* avirulence protein AvrB in plant cells alleviates its dependence on the hypersensitive response and pathogenicity (hrp) secretion system in eliciting genotype-specific hypersensitive cell death. *Plant Cell* **8**: 1095–1105.

Greenberg, J.T. (1997) Programmed cell death in plant–pathogen interactions. *Annu Rev Plant Physiol Plant Mol Biol* **48**: 525–545.

Ham, J.H., Bauer, D.W., Fouts, D.E., and Collmer, A. (1998) A cloned *Erwinia chrysanthemi* Hrp (type III protein secretion) system functions in *Escherichia coli* to deliver *Pseudomonas syringae* Avr signals to plant cells and to

- secrete Avr proteins in culture. *Proc Natl Acad Sci USA* **95**: 10206–10211.
- Hammond-Kosack, K.E., and Jones, J.D.G. (1997) Plant disease resistance genes. *Annu Rev Plant Physiol Plant Mol Biol* **48**: 575–607.
- Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**: 557–580.
- He, S.Y., Huang, H.-C., and Collmer, A. (1993) *Pseudomonas syringae* pv. *syringae* harpin<sub>PSS</sub>: a protein that is secreted via the hrp pathway and elicits the hypersensitive response in plants. *Cell* **73**: 1255–1266.
- Huang, H.-C., He, S.Y., Bauer, D.W., and Collmer, A. (1992) The *Pseudomonas syringae* pv. *syringae* 61 hrpH product, an envelope protein required for elicitation of the hypersensitive response in plants. *J Bacteriol* **174**: 6878–6885.
- Hueck, C.J. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* **62**: 379–433.
- Huynh, T., Dahlbeck, D., and Staskawicz, B.J. (1989) Bacterial blight of soybeans: regulation of a pathogen gene determining host cultivar specificity. *Science* **245**: 1374–1377.
- Innes, R.W., Bent, A.F., Kunkel, B.N., Bisgrove, S.R., and Staskawicz, B.J. (1993) Molecular analysis of avirulence gene *avrRpt2* and identification of a putative regulatory sequence common to all known *Pseudomonas syringae*. *J Bacteriol* **175**: 4859–4869.
- Kim, J.F., Zumoff, C.H., and Beer, S.V. (1998) HrpW of *Erwinia amylovora*, a new harpin that contains a domain homologous to pectate lyases of a distinct class. *J Bacteriol* **180**: 5203–5210.
- Koncz, C., and Schell, J. (1986) The promoter of T<sub>1</sub>-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol Gen Genet* **204**: 383–396.
- Kunkel, B.N., Bent, A.F., Dahlbeck, D., Innes, R.W., and Staskawicz, B.J. (1993) *RPS2*, an Arabidopsis disease resistance locus specifying recognition of *Pseudomonas syringae* strains expressing the avirulence gene *avrRpt2*. *Plant Cell* **5**: 865–875.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
- Lamb, C., and Dixon, R.A. (1997) The oxidative burst in plant disease resistance. *Annu Rev Plant Physiol Plant Mol Biol* **48**: 251–275.
- Lee, V.T., Anderson, D.M., and Schneewind, O. (1998) Targeting of *Yersinia* Yop proteins into the cytosol of HeLa cells: one-step translocation of YopE across bacterial and eukaryotic membranes is dependent on SycE chaperone. *Mol Microbiol* **28**: 593–601.
- Leister, R.T., Ausubel, F.M., and Katagiri, F. (1996) Molecular recognition of pathogen attack occurs inside of plant cells in plant disease resistance specified by the Arabidopsis genes *RPS2* and *RPM1*. *Proc Natl Acad Sci USA* **93**: 15497–15502.
- McNellis, T.W., Mudgett, M.B., Li, K., Aoyama, T., Horvath, D., Chua, N.-H., et al. (1998) Glucocorticoid-inducible expression of a bacterial avirulence gene in transgenic Arabidopsis induces hypersensitive cell death. *Plant J* **14**: 247–257.
- Matthysse, A.G., Stretton, S., Dandie, C., McClure, N.C., and Goodman, A.E. (1996) Construction of GFP vectors for use in Gram-negative bacteria other than *Escherichia coli*. *FEMS Microbiol Lett* **145**: 87–94.
- Michiels, T., and Cornelis, G.R. (1991) Secretion of hybrid proteins by the *Yersinia* Yop export system. *J Bacteriol* **173**: 1677–1685.
- Mindrinos, M., Katagiri, F., Yu, G.-L., and Ausubel, F.M. (1994) The *A. thaliana* disease resistance gene *RPS2* encodes a protein containing a nucleotide-binding site and leucine-rich repeats. *Cell* **78**: 1089–1099.
- Morrissey, J.H. (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal Biochem* **117**: 307–310.
- Mudgett, M.B., and Clarke, S. (1994) Hormonal and environmental responsiveness of a developmentally regulated protein repair L-isoaspartyl methyltransferase in wheat. *J Biol Chem* **269**: 25605–25612.
- Mudgett, M.B., and Staskawicz, B.J. (1998) Protein signaling via type III secretion pathways in phytopathogenic bacteria. *Curr Opin Microbiol* **1**: 109–114.
- Murillo, J., Shen, H., Gerhold, D., Sharma, A., Cooksey, D.A., and Keen, N.T. (1994) Characterization of pPT23B, the plasmid involved in syringolide production by *Pseudomonas syringae* pv. *tomato* PT23. *Plasmid* **31**: 275–287.
- Pirhonen, M.U., Lidell, M.C., Rowley, D.L., Lee, S.W., Jin, S., Liang, Y., et al. (1996) Phenotypic expression of *Pseudomonas syringae* *avr* genes in *E. coli* is linked to the activities of the hrp-encoded secretion system. *Mol Plant-Microbe Interact* **9**: 252–260.
- Puri, N., Jenner, C., Bennett, M., Stewart, R., Mansfield, J., Lyons, N., et al. (1997) Expression of *avrPphB*, an avirulence gene from *Pseudomonas syringae* pv. *phaseolicola*, and the delivery of signals causing the hypersensitive reaction in bean. *Mol Plant-Microbe Interact* **10**: 247–256.
- Roine, E., Wei, W., Yuan, J., Nurmiho-Lassila, E.-L., Kalkkinen, N., Romantschuk, M., et al. (1997) Hrp pilus: an hrp-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000. *Proc Natl Acad Sci USA* **94**: 3459–3464.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Schesser, K., Frithz-Lindsten, E., and Wolf-Watz, H. (1996) Delineation and mutational analysis of the *Yersinia pseudotuberculosis* YopE domains which mediate translocation across bacterial and eukaryotic cellular membranes. *J Bacteriol* **178**: 7227–7233.
- Scofield, S.R., Tobias, C.M., Rathjen, J.P., Chang, J.H., Lavelle, D.T., Michelmore, R.W., et al. (1996) Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science* **274**: 2063–2065.
- Sory, M.-P., Boland, A., Lambermont, I., and Cornelis, G.R. (1995) Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the *cyaA* gene fusion approach. *Proc Natl Acad Sci USA* **92**: 11998–12002.
- Tang, X., Frederick, R.D., Zhou, J., Halterman, D.A., Jia, Y., and Martin, G.B. (1996) Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. *Science* **274**: 2060–2063.

- Turner, P., Barber, C., and Daniels, M. (1984) Behaviour of the transposons Tn5 and Tn7 in *Xanthomonas campestris* pv. *campestris*. *Mol Gen Genet* **195**: 101–107.
- Van den Ackerveken, G., Marois, E., and Bonas, U. (1996) Recognition of the bacterial avirulence protein AvrBs3 occurs inside the host plant cell. *Cell* **87**: 1307–1316.
- Van Gijsegem, F., Genin, S., and Boucher, C. (1993) Conservation of secretion pathways for pathogenicity determinants of plant and animal bacteria. *Trends Microbiol* **1**: 175–180.
- Wei, Z.-M., Laby, R.J., Zumoff, C.H., Bauer, D.W., He, S.Y., Collmer, A., *et al.* (1992) Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* **257**: 85–88.
- Whalen, M.C., Innes, R.W., Bent, A.F., and Staskawicz, B.J. (1991) Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *Plant Cell* **3**: 49–59.
- Yuan, J., and He, S.Y. (1996) The *Pseudomonas syringae* Hrp regulation and secretion system controls the production and secretion of multiple extracellular proteins. *J Bacteriol* **178**: 6399–6402.