Molecular signals required for type III secretion and translocation of the *Xanthomonas campestris* AvrBs2 protein to pepper plants

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Strains of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) carrying *avrBs2* are specifically recognized by *Bs2* pepper plants, resulting in localized cell death and plant resistance. *Agrobacterium*-mediated transient expression of the *Xcv avrBs2* gene in plant cells results in *Bs2*-dependent cell death, indicating that the AvrBs2 protein alone is sufficient for the activation of disease resistance-mediated cell death *in planta*. We now provide evidence that AvrBs2 is secreted from *Xcv* and that secretion is type III (*hrp*) dependent. N- and C-terminal deletion analysis of AvrBs2 has identified the effector domain of AvrBs2 recognized by *Bs2* pepper plants. By using a truncated *Pseudomonas syringae* AvrRpt2 effector reporter devoid of type III signal sequences, we have localized the minimal region of AvrBs2 required for type III secretion in *Xcv*. Furthermore, we have identified the region of AvrBs2 required for both type III secretion and translocation to host plants. The mapping of AvrBs2 sequences sufficient for type III delivery also revealed the presence of a potential mRNA secretion signal.

Bacterial spot disease of tomato and pepper is caused by *Xanthomonas campestris* pathovar *vesicatoria* (*Xcv*). Most *Xc* pathovars infecting a wide host range contain *avrBs2* in the chromosome (1). Such prevalence suggests that the conservation of *avrBs2* in *Xc* may be crucial for the maintenance of pathogen fitness. In fact, *Xcv* strains possessing natural or introduced mutations at the *avrBs2* locus are less virulent on susceptible hosts (1–3). Susceptibility to *Xcv* often leads to devastating losses in commercial production of crop plants in regions with high humidity and heavy rainfall. Fortunately, resistance to strains of *Xcv* carrying *avrBs2* has been identified in a wild species of pepper (*Capsicum chacoense*) and introduced into cultivated pepper (*Capsicum annuum*) by traditional breeding.

Molecular studies show that the *Bs2* gene from *C. chacoense* specifically recognizes and confers resistance to strains of *Xcv* that contain *avrBs2* (4, 5). The *Bs2* gene (4) is a member of the nucleotide-binding site-leucine-rich repeat class of plant disease resistance genes (6, 7) and is predicted to reside in the plant cytoplasm. The successful engineering of *Bs2* resistance in transgenic tomatoes provides a source of protection to *Xcv* strains containing *avrBs2* (4). However, durability of *Bs2* resistance in the field is challenged by the emergence of *Xcv* strains that are able to cause disease in previously resistant pepper plants (8, 9). The characterization of natural field isolates overcoming *Bs2* resistance has revealed that *Xcv* is evolving under selection pressure at the *avrBs2* locus to evade recognition and to maintain *avrBs2*-dependent virulence in its host (2). Although the virulence function of *avrBs2* is not known, transient expression of *avrBs2* in plant cells has confirmed that AvrBs2 protein is recognized in *Bs2* plants and that this effector is sufficient for the activation of *Bs2*-dependent disease resistance *in planta* (4). The predicted AvrBs2 protein shares homology with agrocinopine synthase of *Agrobacterium tumefaciens* and the glycerophosphoryl diester phosphodiesterase UgpQ of *Escherichia coli* (3), suggesting that AvrBs2 may function in plant cells as an enzyme to synthesize or hydrolyze phosphodiester linkages.

The *hrp* locus encoding the type III pathway is essential for *Xcv* to induce *avrBs2*-dependent localized cell death in resistant *Bs2* pepper plants and to cause disease in susceptible *bs2* pepper plants (10). This suggested to us that *Xcv* delivers AvrBs2 to the plant cytoplasm via the type III secretion pathway. Type III secretion systems function to target virulence proteins to host cells (11, 12). The conservation of the type III pathway in distantly related Gram-negative pathogenic bacteria (12, 13) has revealed a shared mechanism used by pathogens for the delivery of specialized virulence factors. Type III effector proteins are structurally unrelated and do not share a conserved signal peptide for export. However, extensive analysis of type III effector proteins, Yops, from *Yersinia*, has identified two modular domains at the N terminus of Yops sufficient for secretion and translocation (14). Secretion signals in some Yops are confined to the N-terminal 15–17 codons (15, 16), whereas translocation signals are located in the first 50–100 codons (15, 17). A specific chaperone binds within the translocation domain and facilitates the secretion and translocation of the respective Yop (18). Frameshift mutagenesis within some Yop secretion domains can be tolerated, suggesting that the secretion signal is the mRNA (16).

Significantly less is known about type III signals in effectors from phytopathogenic bacteria. Only recently have methods been established to study *in vitro* secretion from *Xcv* (19) and *Pseudomonas syringae* (20, 21). The translocation of a plant pathogen effector into a host cell has not yet been formally demonstrated. Technical limitations stem from the inability to target some reporters through the type III apparatus (20) and to identify reporters sensitive enough for detection *in planta.* However, indirect evidence does exist for the delivery of effectors to plant cells (11). What is known about plant pathogen type III signals is that the signals are similarly localized to N termini (20, 22), secretion signals can be encoded in the mRNA (22), and chaperones may exist (23). Moreover, the type III secretion systems of *Xcv* (19) and *Erwinia chrysanthemi* (20, 22, 24) secrete proteins from both plant and animal pathogens, implying that the recognition of type III secretion signals may be functionally conserved among type III pathogens.

Herein we have explored the mechanism for *Xcv* delivery of AvrBs2 to pepper plants. We have taken advantage of *Xcv* for studying type III trafficking because *in vitro* secretion of protein is robust (19). Moreover, the promiscuous secretion of proteins through the type III apparatus in *Xcv* (19) suggested to us that

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AvrRpt2, a *P. syringae* type III effector, would be a novel, sensitive reporter for detecting type III delivery of AvrBs2 to plants. Some *P. syringae* type III effector proteins are secreted by the type III pathways of unrelated animal and plant bacterial pathogens, including *Xcv* (19, 20, 22, 24). In some cases, the *P. syringae* effectors can induce localized cell death in resistant plants, inferring that the effectors are being translocated to the host cell by the heterologous type III apparatus (20, 24). This indicated to us that effectors devoid of type III targeting signals would be sensitive protein reporters to elucidate not only type III secretion signals but also type III translocation signals. We explicitly chose the *P. syringae* AvrRpt2 protein as a reporter because *Xcv* cannot secrete or translocate this effector. Furthermore, the C terminus of AvrRpt2, devoid of type III signals, is specifically recognized by *RPS2* and is sufficient to induce cell death *in planta* (20). Thus, we predicted that the N terminus of AvrBs2 fused to the C-terminal domain of AvrRpt2 would be sufficient to secrete and translocate the fusion protein, providing us with a tool to further map AvrBs2 type III signals.

We now report that *Xcv* secretes AvrBs2 by the type III pathway. N-terminal and C-terminal deletion analysis of AvrBs2 has identified the effector domain of AvrBs2 specifically recognized by *Bs2* in pepper plants. By using chimeric AvrBs2-AvrRpt2 fusion proteins, we have localized the minimal region of AvrBs2 required for *in vitro* type III secretion in *Xcv*. Furthermore, we have identified the region of AvrBs2 required for both type III secretion and translocation to plants. We also show that an mRNA signal within the first 18 codons of AvrBs2 is recognized by *Xcv* and required for the induction of *Bs2* resistance in pepper.

Materials and Methods

Strains and Growth. Strains used in this study were *E. coli* DH5α, *Xcv* strain 85–10 hrpG* (85*) and 85–10 hrpG* (85*) Δ*hrcV* (19, 25), *Xcv* strain GM98–38 mutant at the *avrBs2* locus (2), *Xc* pv. *campestris* (*Xcc*) strain 8004 (M. Daniels, Sainsbury Laboratory, Norwich, U.K.), and *A. tumefaciens* C58C1 (pCH32) (4). *E. coli* and *A. tumefaciens* strains were grown on Luria agar medium (26) at 37°C and 28°C, respectively. *Xcv* and *Xcc* strains were grown on NYGA (27) at 28°C. Vectors were mobilized from *E. coli* into *Xcv*, *Xcc*, and *A. tumefaciens* by triparental matings by using standard methods.

Plasmid Construction. PCR was used to construct gene fusions, deletions, and frameshifts. Numbering herein refers to the codon of the gene described. For AvrBs2, codon 1 represents the second AUG in the predicted ORF1 (3). PCR-generated DNA fragments were cloned into pCR4Blunt-TOPO (Invitrogen). Primers and conditions used for PCR will be available on request. The sequence of DNA constructs was verified by cycle sequencing.

For expression in *Xc*, constructs were cloned into pVSP61 (DNA Plant Technology, Oakland, CA) or a derivative, pDD62. For pDD62, the *Hin*dIII-*Eco*RI fragment of pVSP61 was replaced with a linker containing a *Bam*HI site, a *Xho*I site, and stop codons in all three reading frames downstream of the *lac* promoter. *Hin*dIII and *Eco*RI sites were eliminated in this step. To construct pDD62 (*avrBs2*), the *Bam*HI fragment from p81533b (B. Kearney and B.J.S., unpublished) containing the *avrBs2* promoter and ORF was cloned into the *Bam*HI site in pDD62. To construct pDD62(*avrBs2*-HA), PCR was used to amplify the DNA region 3′ of the *avrBs2* *Cla*I site in p81533 to introduce a HA epitope, an in-frame stop codon, and a *Bam*HI site followed by a *Sal*I site. This *Cla*I-*Sal*I fragment replaced the *Cla*I-*Sal*I fragment in p81533 (3) creating p81533(*avrBs2*-HA). The *Hin*dIII-*Sal*I fragment of p81533(*avrBs2*-HA) was then used to replace the *Hin*dIII-*Xho*I fragment in pDD62(*avrBs2*) creating pDD62(*avrBs2*-HA). The *Hin*dIII-*Bam*HI fragment of p81533(*avrBs2*-HA) was cloned into pVSP61 creating pVSP61(promoterless $avrBs2_{97-714}$-HA). 3′ *avrBs2* deletions were made by digesting p81533b with *Sca*I, *Pvu*II, *Nco*I, or *Stu*I, adding a *Bam*HI linker to the cleaved DNA, and cloning the subsequent *Bam*HI fragment into pDD62 to create pDD62($avrBs2_{1-417}$), pDD62($avrBs2_{1-497}$), pDD62($avrBs2_{1-519}$), and pDD62($avrBs2_{1-574}$), respectively. To engineer 5′ *avrBs2* deletions, the promoter and coding region of interest (codon 1–97 at the *Hin*dIII site) were amplified separately and used as DNA template for a subsequent overlap PCR reaction. Once cloned into pCR4Blunt-TOPO, *Hin*dIII fragments were cloned into the *Hin*dIII site of pVSP61(promoterless $avrBs2_{97-714}$-HA), creating pVSP61($avrBs2_{19-714}$-HA), pVSP61($avrBs2_{51-714}$-HA), pVSP61($avrBs2_{62-714}$-HA), and pVSP61($avrBs2_{97-714}$-HA), all possessing the *avrBs2* promoter. For *avrBs2-avrRpt2* fusions, the 3′ end of $avrRpt2_{80-255}$ was amplified by using pRSR0 (28) as DNA template and cloned into pDD62(*avrBs2*) as a *Hin*dIII-*Xho*I fragment replacing the 3′ *avrBs2* sequence creating pDD62-($avrBs2_{1-97}$+$avrRpt2_{80-255}$). Smaller regions of *avrBs2* were amplified and cloned into the *Bam*HI-*Hin*dIII site of pDD62-($avrBs2_{1-97}$+ $avrRpt2_{80-255}$), creating pDD62($avrBs2_{1-58}$+ $avrRpt2_{80-255}$), pDD62($avrBs2_{1-41}$+ $avrRpt2_{80-255}$), pDD62-($avrBs2_{1-28}$+ $avrRpt2_{80-255}$), and pDD62($avrBs2_1$+$avrRpt2_{80-255}$), all possessing the *avrBs2* promoter. By using overlap PCR, frameshifts (fs) were constructed by inserting a G nucleotide(s) (+1 and +2) after the AUG start codon of *avrBs2*. Reciprocal changes after *avrBs2* codon 18 were made creating pVSP61-($avrBs2_{+1fs}$-HA) and pVSP61($avrBs2_{+2fs}$-HA).

For transient expression by using *A. tumefaciens*, constructs were made in pMD1 (4) or a derivative pMDD1, where the *Xba*I-*Sac*I fragment was replaced with a *Xba*I-*Sac*I linker containing a *Bam*HI site and a *Xho*I site followed by stop codons in all three reading frames. *Bam*HI fragments in pDD62(*avrBs2*) and pDD62(*avrBs2*-HA) were cloned into the *Bam*HI site of pMD1 creating pMD1(*avrBs2*) and pMD1(*avrBs2*-HA), respectively. *Xba*I-*Bam*HI fragments in pVSP61($avrBs2_{19-714}$-HA), pVSP61($avrBs2_{51-714}$-HA), pVSP61($avrBs2_{62-714}$-HA), pVSP61($avrBs2_{97-714}$-HA), pVSP61($avrBs2_{+1fs}$HA), and pVSP61($avrBs2_{+2\ fs}$HA) were cloned into the corresponding sites in pMD1 creating pMD1($avrBs2_{19-714}$-HA), pMD1($avrBs2_{51-714}$-HA), pMD1($avrBs2_{62-714}$-HA), pMD1($avrBs2_{97-714}$-HA), pMD1($avrBs2_{+1fs}$-HA), and pMD1($avrBs2_{+2fs}$-HA), respectively. *Bam*HI fragments in pDD62($avrBs2_{1-417}$), pDD62($avrBs2_{1-497}$), pDD62($avrBs2_{1-519}$), and pDD62-($avrBs2_{1-574}$) were cloned into the *Bam*HI site of pMDD1, creating pMDD1($avrBs2_{1-417}$), pMDD1($avrBs2_{1-497}$), pMDD1-($avrBs2_{1-519}$), and pMDD1($avrBs2_{1-574}$), respectively.

Secretion Assay. *Xcv* 85* and 85* Δ*hrcV* strains grown overnight at 28°C on NYGA were suspended in secretion media, pH 7 (19), containing rif 5 μg/ml and kan 12 μg/ml. Ten-milliliter cultures (1×10^8 cells ml^{-1}) were shaken for 12 h at 28°C and collected at 2,500 $\times$ *g* for 5 min at room temperature. Cells were washed in 1 mM $MgCl_2$, repelleted, and resuspended in $MgCl_2$. Bacteria were diluted to 4×10^8 cells ml^{-1} in 4 ml of secretion media, pH 5.4, containing rif 5 μg/ml, and 50 μg/ml BSA. Cultures were shaken for 4.5 h at 28°C. Cellular lysate fractions were obtained by precipitating 125 μl of each culture with 10% trichloroacetic acid on ice for 30 min. Protein was collected by centrifugation at 14,000 $\times$ *g* for 30 min, washed with 100% ethanol, and resuspended with 40 μl of sample buffer (20). Culture fluid fractions were obtained by removing cells from the remaining culture by centrifugation and then directly filtering supernatants through a 0.45-μm filter (HT Tuffryn, Gelman). Filtrates were precipitated and resuspended in 60 μl of sample buffer. Twenty microliters of the cellular lysate fraction and 30 μl of the culture fluid fraction were analyzed.

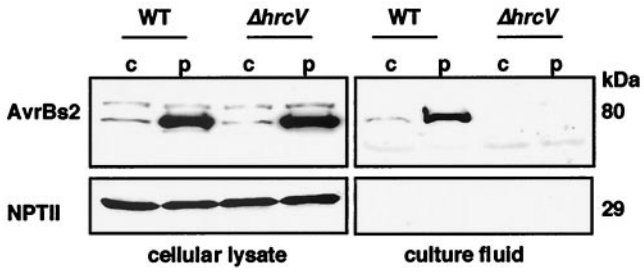

Fig. 1. Type III-dependent secretion of AvrBs2 from *Xcv*. Immunoblot analysis of AvrBs2 and NPT II protein in cellular lysate and culture fluid isolated from *Xcv* strain 85* pDD62(*avrBs2*) expressing AvrBs2 (80 kDa) and NPT II (29 kDa). wt; wild-type secretion strain. Δ*hrcV*; secretion mutant. c; chromosomal *avrBs2*. p; plasmid *avrBs2*.

Protein Gels and Immunoblot Analysis. Protein from *Xc* strains and *A. tumefaciens*-infected leaves was extracted from frozen samples by homogenization by using a Kontes pestle (Fisher) in a microfuge tube. Samples were suspended with buffer (8 M urea/0.1 M $NaPO_4$/0.01 M Tris·HCl, pH 8.0), rocked for 30 min at room temperature, and then centrifuged at 14,000 × *g* for 15 min to obtain the supernatant identified as the total cellular protein. Protein fractions were mixed with sample buffer, boiled for 5 min, and then analyzed in a 10% gel by SDS/PAGE. For immunoblot analysis, proteins were transferred from gels to nitropure nitrocellulose (Osmonics) by electroblotting at 0.3 amps for 1 h in transfer buffer containing 10 mM 3-[cyclohexylamino]-1-propane sulfonic acid, pH 11.0, 10% (vol/vol) methanol. AvrBs2, AvrRpt2, and NPT II were detected by using rabbit polyclonal antisera at 1:2,000 (2), 1:2,000 (29), and 1:1,000 (5 Prime→3 Prime), respectively, in 20 mM Tris·HCl, pH 7.5/0.5 M NaCl/0.05% Tween 20 buffer containing 5% nonfat milk, followed by horseradish peroxidase-conjugated secondary antibodies and chemiluminescence.

Plant Growth and Bacterial Inoculation. Pepper cultivars (cv.) Early Calwonder (ECW; *bs2,bs2*) and the near-isogenic cv. ECW-20R (*Bs2,Bs2*) were used for *Xcv* and *A. tumefaciens* inoculations. *Arabidopsis thaliana* ecotype Col-0 wild-type (*RPS2,RPS2*) and *rps2–201* mutant (*rps2, rps2*) plants (30) were used for *Xcc* inoculations. *A. thaliana* plants were grown in chambers at 22°C under 8 h photoperiod. Pepper plants were grown under greenhouse conditions. Bacteria were hand infiltrated into plant leaves through a small wound by using a 1-cc syringe.

Agrobacterium-Mediated Transient Expression Assay. *A. tumefaciens* was grown overnight at 28°C on Luria agar medium containing rif 100 μg/ml, tet 5 μg/ml, and kan 35 μg/ml. Bacteria were collected and incubated in inducing media (10 mM Mes, pH 5.6/10 mM $MgCl_2$/and 150 μM acetosyringone) for 2 h before inoculation.

Results

Type III-Dependent Secretion of AvrBs2 Protein from *Xcv*. To dissect the targeting of AvrBs2 protein to host cells, we first determined whether AvrBs2 is secreted by the type III pathway. We used the *Xcv* strain 85* that contains a chromosomal copy of *avrBs2* and constitutively expresses *hrp* genes (locus encoding the type III apparatus) because of a mutation in the regulatory gene *hrpG* (25). We also used *Xcv* strain 85* Δ*hrcV*, a secretion defective strain, to test *hrp*-dependent secretion of AvrBs2. Conditions previously established to assay for *Xcv* secretion (19) were used to detect AvrBs2 secretion. Immunoblot analysis shows that AvrBs2 protein is present in cellular lysate from *Xcv* 85* and *Xcv* 85* Δ*hrcV* strains when *avrBs2* is expressed chromosomally or by pDD62 (Fig. 1). Importantly, the secretion competent *Xcv* strains released AvrBs2 into the culture fluid (Fig. 1). Robust AvrBs2 expression and secretion was observed only when *avrBs2* was expressed from pDD62. To confirm that AvrBs2 protein in the *Xcv* 85* culture fluids was not because of cell lysis, immunoblot analysis was repeated by using antisera for NPT II, a cytoplasmic protein encoded by the plasmid pDD62. NPTII was detected only in the cellular lysate and not in the culture fluid (Fig. 1.). These data demonstrate that *Xcv* secretes AvrBs2 by the type III pathway and that secretion is *hrp* dependent. Considering that secretion of AvrBs2 from *Xcv* strain 85* pDD62(*avrBs2*) was more robust, we subsequently expressed all *avrBs2* constructs from pDD62.

Table 1. Phenotypes of AvrBs2 deletion polypeptides expressed by *Xcv* and by *Agrobacterium*-mediated transformation in pepper *Bs2* leaves

	Xcv expression		*A. tumefaciens* expression *in planta*	
AvrBs2 protein*	Phenotype†	Protein	Phenotype†	Protein
Mature protein				
1-714	HR	Yes	HR	Yes
1-714-HA‡	HR	Yes	HR	Yes
N-terminal deletions				
19-714-HA	NS	Yes	HR	Yes
51-714-HA	NS	Yes	HR	Yes
62-714-HA	NS	Yes	HR	Yes
97-714-HA	NS	Yes	NS	Yes
C-terminal deletions				
1-417	NS	No	NS	No
1-497	w-HR	Yes	HR	Yes
1-519	HR	Yes	HR	Yes

*Codon numbering is based on the predicted protein for ORF1 (3).
†HR, hypersensitive cell death response; NS, no symptoms, w-HR, weak HR.
‡HA, hemagglutinin epitope.

Identification of AvrBs2's *in Planta* Effector Domain by Deletion Analysis. We next performed mutagenesis of *avrBs2* to determine the effector domain of AvrBs2 required for *avrBs2*-dependent localized cell death in *Bs2* pepper plants. AvrBs2 N-terminal deletions were constructed introducing an ATG before AvrBs2 codon 19, 51, 62, and 97 (3). *Xcv* GM98–38 strains carrying pDD62(*avrBs2*-HA), pVSP61($avrBs2_{19-714}$-HA), pVSP61($avrBs2_{51-714}$-HA), pVSP61($avrBs2_{62-714}$-HA), or pVSP61($avrBs2_{97-714}$-HA) all expressed the expected AvrBs2-specific polypeptides (Table 1). However, when hand inoculated into *Bs2* pepper leaves, only *Xcv* expressing AvrBs2-HA was able to induce *avrBs2*-dependent cell death (Table 1). To determine whether the N-terminal deletions were affecting the delivery of AvrBs2 or *Bs2*-dependent recognition (25) directly, all of the N-terminal AvrBs2 deletions were cloned into pMD1 and then expressed *in planta* by using the *Agrobacterium*-mediated transient expression assay. AvrBs2-specific cell death was induced by all of the N-terminal deletion constructs, except $AvrBs2_{97-714}$-HA (Table 1). These data clearly show that the N terminus of AvrBs2 is not required to trigger a *Bs2*-dependent response *in planta*, suggesting that these truncated proteins are instead defective in type III targeting. Moreover, *in planta Bs2*-dependent recognition requires the N-terminal AvrBs2 domain between codon 62 and 97.

We also deleted the C-terminal region of AvrBs2 to define the effector domain required for *avrBs2*-dependent localized cell death in *Bs2* pepper plants. *Xcv* GM98–38 carrying pDD62(*avrBs2*), pDD62($avrBs2_{1-417}$), pDD62($avrBs2_{1-497}$),

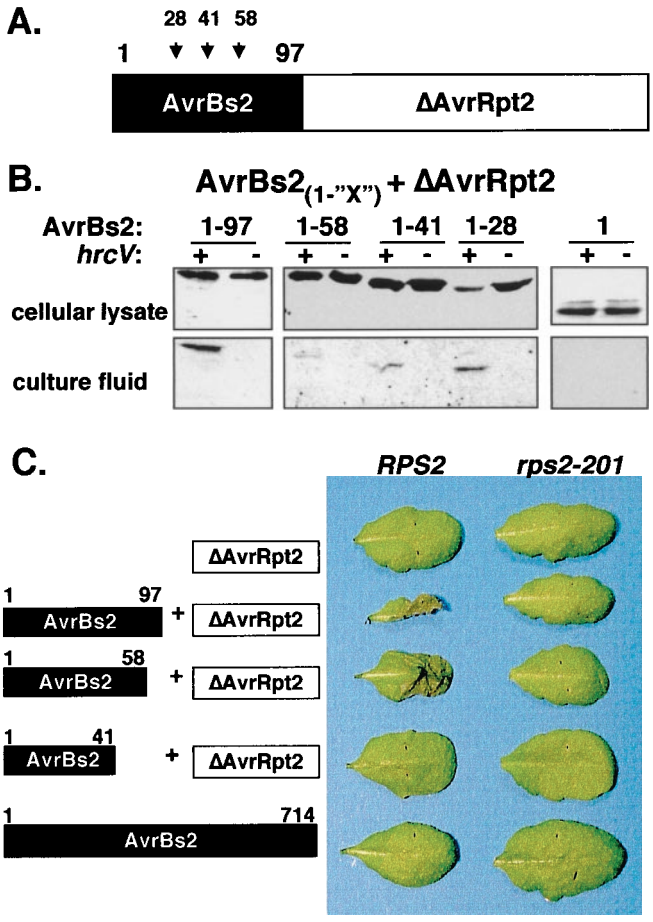

Fig. 2. (*A*) Schematic of chimeric AvrBs2-AvrRpt2 fusion proteins. AvrBs2 codons 1–28, 1–41, 1–58, and 1–97 were independently fused to AvrRpt2 codons 80–255 (ΔAvrRpt2). (*B*) AvrBs2 N-terminal codons secrete the AvrRpt2$_{80–255}$ effector via the *Xcv* type III pathway. Immunoblot analysis of cellular lysate and culture fluid isolated from *Xcv* strain 85* expressing AvrBs2$_{1-"X"}$+AvrRpt2$_{80–255}$ in pDD62 by using AvrRpt2 antisera. Numbers refer to the respective codon positions of AvrBs2 fused to the reporter AvrRpt2$_{80–255}$ protein. +, wild-type secretion strain. −, Δ*hrcV* secretion mutant. (*C*) AvrRpt2-dependent cell death in resistant *A. thaliana RPS2* leaves inoculated with *Xcc* strains expressing the AvrBs2-AvrRpt2 fusion proteins. Resistant *RPS2* and susceptible *rps2–201 A. thaliana* leaves were inoculated with a 2.5×10^8 cells ml^{-1} suspension of bacteria and then photographed 24 h later. Schematic of protein expressed in *Xcc* shown on left. Symptoms of *Xcc* in leaves shown on right.

pDD62(*avrBs2*$_{1–519}$), or pDD62(*avrBs2*$_{1–574}$) were inoculated into *Bs2* pepper leaves. All of the C-terminal deleted AvrBs2 polypeptides induced cell death except for AvrBs2$_{1–417}$ (Table 1). However, AvrBs2$_{1–417}$ protein could not be detected in *Xcv*. Failure to detect this polypeptide may reflect antibody specificity and/or protein stability. These results demonstrate that the C-terminal 217 codons of AvrBs2 are dispensable for *in planta* *Bs2*-dependent recognition of AvrBs2.

Identification of AvrBs2 Type III Secretion Signals By Using a AvrRpt2 Reporter. To identify the type III signals in AvrBs2 required for secretion and translocation to host plants, we fused the N-terminal coding region of AvrBs2 (codons 1–97) to the C-terminal coding region of AvrRpt2 (codons 80–255), a *P. syringae* type III effector (20). We predicted that the N-terminal 97 codons of AvrBs2 fused to AvrRpt2$_{80–255}$ (devoid of type signals, Fig. 2*A*) would be sufficient to secrete the fusion protein through the *Xcv* type III apparatus. Immunoblot analysis shows that *Xcv* strain 85* carrying pDD62(*avrBs2*$_{1–97}$+*avrRpt*$_{80–255}$) expressed the predicted 30-kDa fusion protein in cell lysate and then secreted it into the culture fluid (Fig. 2*B*). No AvrBs2$_{1–97}$+AvrRpt2$_{80–255}$ protein was detected in the culture fluid of *Xcv* strain 85* Δ*hrcV* carrying pDD62(*avrBs2*$_{1–97}$+*avrRpt*$_{80–255}$) demonstrating that secretion was *hrp* dependent. The intracellular NPT II marker was not detected in the culture fluid of any strain presented in Fig. 2*B* (data not shown), confirming that the presence of all detected fusion proteins in the culture fluid was not a result of *Xcv* lysis. These data show that the first 97 codons of AvrBs2 contain the AvrBs2 type III secretion signal, which is sufficient to target the *P. syringae* truncated AvrRpt2$_{80–255}$ protein through the *Xcv* type III apparatus.

Next, we deleted codons within the AvrBs2 domain of the AvrBs2$_{1–97}$+AvrRpt2$_{80–255}$ fusion protein to identify the minimal region of AvrBs2 required for *in vitro* type III secretion in *Xcv*. *Xcv* 85* and 85* Δ*hrcV* strains expressing AvrBs2 codons 1–58, 1–41, and 1–28 fused to AvrRpt2$_{80–255}$ (Fig. 2*A*) were tested for secretion into culture fluid. Immunoblot analysis shows that all of the fusion proteins were expressed in *Xcv* cellular lysate and secreted into the culture fluid in a *hrp*-dependent manner (Fig. 2*B*.). A fusion protein containing the first 16 codons of AvrBs2 was poorly expressed in *Xcv*; therefore, its secretion could not be confirmed (data not shown). Thus, we have defined the first 28 codons of AvrBs2 to be the minimal signal sufficient for detectable *in vitro* secretion of AvrRpt2$_{80–255}$ into *Xcv* culture fluid.

Identification of AvrBs2 Type III Signals Required for Translocation to a Host By Using a AvrRpt2 Reporter. To determine whether codons 1–97 of AvrBs2 are sufficient for directing the secretion and translocation of the AvrRpt2 reporter to host cells, we inoculated *Xc* pv. *campestris* (*Xcc*) strains expressing the AvrBs2$_{1–97}$+AvrRpt2$_{80–255}$ fusion protein into *A. thaliana* leaves. We chose *Xcc* to express the fusion protein because *Xcc* does not inherently induce localized cell death in *A. thaliana* (data not shown). Furthermore, resistant *RPS2 A. thaliana* plants specifically recognize AvrRpt2$_{80–255}$ when it is expressed *in planta* (20). Thus, if codons 1–97 of AvrBs2 are sufficient for type III translocation of the AvrRpt2 reporter, then *Xcc* expressing the AvrBs2$_{1–97}$+AvrRpt2$_{80–255}$ fusion protein should induce AvrRpt2-dependent localized cell death on resistant *RPS2 A. thaliana* plants. Resistant *RPS2 A. thaliana* leaves infected with *Xcc* expressing AvrBs2$_{1–97}$+AvrRpt2$_{80–255}$ induced AvrRpt2-dependent cell death (Fig. 2*C*). No symptoms were observed on *rps2–201* leaves, an *A. thaliana* mutant lacking a functional *RPS2* allele. Conversely, *avrRpt2*-dependent cell death was not induced in *RPS2 A. thaliana* leaves infected with *Xcc* expressing AvrRpt2$_{80–255}$ or mature AvrBs2 (Fig. 2*C*). This data demonstrates that the first 97 codons of AvrBs2 are required for *Xanthomonas* to translocate AvrRpt2$_{80–255}$ to *RPS2 A. thaliana* plants.

To identify the minimal region of AvrBs2 required for type III translocation, the AvrBs2-AvrRpt2 fusion proteins used to dissect AvrBs2-specific type III secretion signals (Fig. 2*B*) were expressed in *Xcc*. The resulting *Xcc* strains were used to inoculate *A. thaliana* plants. Only resistant *RPS2 A. thaliana* plants infected with *Xcc* expressing AvrBs2$_{1–97}$+AvrRpt2$_{80–255}$ and AvrBs2$_{1–58}$+AvrRpt2$_{80–255}$ induced *avrRpt2*-dependent cell death in *RPS2 A. thaliana* plants (Fig. 2*C*). No symptoms were observed in *rps2–201 A. thaliana* plants. Conversely, *Xcc* expressing AvrBs2$_{1–41}$+AvrRpt2$_{80–255}$ was unable to translocate AvrRpt2$_{80–255}$ to *RPS2* plants (Fig. 2*C*), even though it is targeted for secretion (Fig. 2*B*). All fusion proteins were equally expressed in *Xcc* (data not shown); therefore, type III translocation deficiency was not because of protein instability. We have thus found that AvrBs2 codons 1–58 are required for *Xanthomonas* to translocate the AvrRpt2 reporter protein to *A. thaliana*.

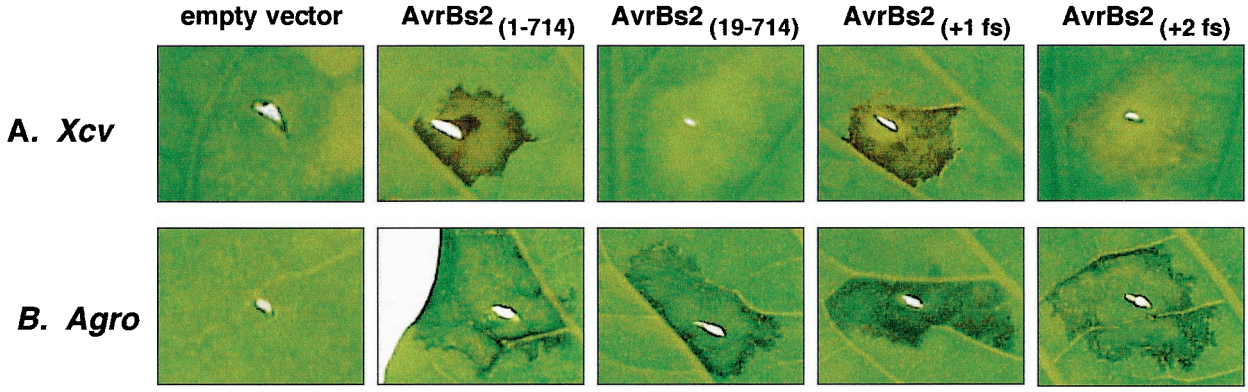

Fig. 3. *avrBs2*-dependent localized cell death in resistant *Bs2* pepper is mediated via an mRNA signal. (*A*) *Xcv* symptoms on resistant *Bs2* pepper leaves. Plants were inoculated with a 2×10^8 cells ml^{-1} suspension of *Xcv* strain GM98–38 carrying pDD62 (empty vector), pDD62(*avrBs2*$_{1–714}$), pVSP61(*avrBs2*$_{19–714}$-HA), pVSP61(*avrBs2*$_{+1fs}$-HA), or pVSP61(*avrBs2*$_{+2fs}$-HA). Symptoms were photographed 48 h after inoculation. (*B*) *Agrobacterium*-mediated transient expression of *avrBs2* genes in resistant *Bs2* pepper leaves. Plants were inoculated with a 6×10^8 cells ml^{-1} suspension of *A. tumefaciens* (*Agro*) strain C58C1 (pCH32) carrying pMD1 (empty vector), pMD1(*avrBs2*$_{1–714}$), pMD1(*avrBs2*$_{19–714}$-HA), pMD1(*avrBs2*$_{+1fs}$-HA), or pMD1(*avrBs2*$_{+2fs}$-HA). Symptoms were photographed 72 h after inoculation.

We next explored the possibility that the type III apparatus in *P. syringae* might recognize the AvrBs2 type III signals used in *Xanthomonas*. *P. syringae* pv. *tomato* DC3000 strains expressing the AvrBs2$_{1–97}$+AvrRpt2$_{80–255}$ fusion protein were inoculated onto *A. thaliana* plants. No symptoms were observed in these plants (data not shown). This indicates that the type III apparatus in *P. syringae* pv. *tomato* cannot recognize AvrBs2-specific type III signal sequences for the delivery of the *Pseudomonas*-specific AvrRpt2 reporter.

Identification of an mRNA Secretion Signal in AvrBs2. Some bacterial effectors possess mRNA secretion signals (16, 22), suggesting that type III apparatuses are capable of recognizing signals that couple mRNA translation to polypeptide secretion. To determine whether AvrBs2 possesses an mRNA secretion signal, we constructed frameshift mutations in *avrBs2* by inserting nucleotides immediately after the AUG start codon. The correct reading frame was restored after codon 18 by reciprocal nucleotide changes. We chose to introduce the frameshifts within the first 28 codons of AvrBs2 because this is the region required for the secretion of AvrRpt2$_{80–255}$ in *Xcv* (Fig. 2*B*). Because changes made within the first 28 codons of AvrBs2 affected protein stability in *Xcv* (data not shown), the AvrBs2 frameshift proteins, AvrBs2$_{+1fs}$-HA and AvrBs2$_{+2fs}$-HA, were not assayed for *in vitro* type III secretion. Instead, we analyzed the frameshift proteins for biological activity in sensitive *Xcv* infection assays.

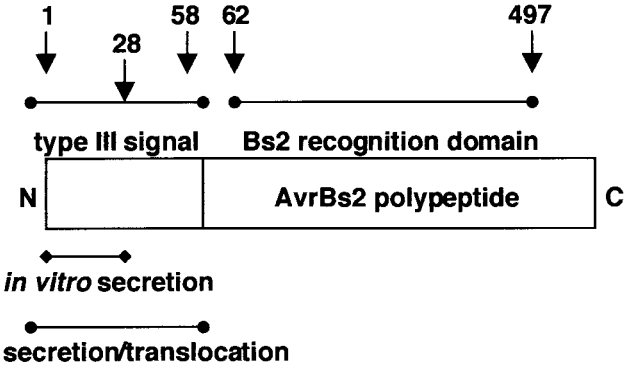

Fig. 4. Schematic of AvrBs2 type III signals and *Bs2 in planta* recognition domain. Numbers refer to the codons in AvrBs2.

Resistant *Bs2* pepper plants infected with *Xcv* GM98–38 expressing wild-type AvrBs2 induced *Bs2*-dependent localized cell death (Fig. 3*A*), whereas *Xcv* GM98–38 alone displayed no symptoms (Fig. 3*A*). Leaves infected with *Xcv* GM98–38 expressing AvrBs2$_{19–714}$ also displayed no symptoms (Fig. 3*A*), indicating that the first 18 codons of AvrBs2 are required for the effective delivery of the AvrBs2 signal to *Bs2* pepper plants. However, if AvrBs2$_{19–714}$ is expressed transiently *in planta*, it is recognized by *Bs2* plants and cell death is induced (Fig. 3*B*). These data support our type III secretion and translocation assays demonstrating that the region within the first 28 codons of AvrBs2 is essential for type III delivery to the host (Fig. 2 *B* and *C*). Interestingly, resistant *Bs2* pepper plants infected with *Xcv* GM98–38 strains expressing either AvrBs2$_{+1fs}$-HA or AvrBs2$_{+2fs}$-HA induce *Bs2*-dependent cell death (Fig. 3*A*). Although the AvrBs2$_{+1fs}$-HA effector protein was more potent in inducing *Bs2*-dependent cell death in *Xcv* infections, both frameshift proteins were equally effective in inducing cell death when transiently expressed *in planta* compared with wild type AvrBs2 (Fig. 3*B*). The reading frames (+1 and +2) resulting from mutagenesis did not encode peptides with physical properties distinctly different from that encoded by the correct AvrBs2 reading frame. Considering that the frameshift proteins retain AvrBs2-specific biological activity when expressed by *Xcv*, we have identified a possible mRNA signal within the first 18 codons of AvrBs2.

Discussion

In this study, we provide evidence supporting type III-dependent secretion of AvrBs2. We show that the phytopathogenic bacterium *Xcv* secretes mature AvrBs2 protein into culture fluid, and that secretion requires a functional type III secretion system. Mutation of *hrcV*, a predicted inner membrane structural component of the type III apparatus in *Xcv* (31, 32), prevented the secretion of AvrBs2 from *Xcv* and the induction of localized cell death in resistant *Bs2* pepper plants (data not shown). These results show that secretion of AvrBs2 through the type III apparatus is one event essential for the molecular recognition of this effector *in planta*.

In addition, we have characterized the molecular signals required for the type III secretion and translocation of AvrBs2 from *Xcv* to pepper plants (summarized in Fig. 4). The use of AvrRpt2, a *P. syringae* type III effector protein, as a reporter in *Xcv* enabled us to localize key regions in AvrBs2 required for type III-dependent secretion and translocation. We show that

the truncated AvrRpt2$_{80-255}$ protein, a region devoid of type III signals, was an ideal reporter for the mapping of AvrBs2 type III signals in *Xcv*. Consistent with our hypothesis, the N-terminal 97 codons of AvrBs2 fused to AvrRpt2$_{80-255}$ was sufficient to target AvrRpt2$_{80-255}$ through the *Xcv* type III pathway. AvrBs2$_{1-97}$+AvrRpt2$_{80-255}$ was secreted into *Xcv* culture fluid in a type III-dependent manner. Moreover, the same fusion protein was able to induce *avrRpt2*-dependent cell death in *Xcc* infected *RPS2 A. thaliana* plants, indirectly demonstrating translocation of the AvrRpt2$_{80-255}$ protein. By using the AvrRpt2$_{80-255}$ reporter, the minimal region of AvrBs2 required for efficient type III secretion into *Xcv* culture fluids was mapped to the first 28 codons of AvrBs2. Yet the first 58 codons of AvrBs2 were required for phenotypic expression of the AvrRpt2$_{80-255}$ reporter *in planta*. We have thus identified two modular domains required for type III delivery, one for *in vitro* secretion and another for secretion and translocation. The spatial distribution of AvrBs2's secretion and translocation domains resembles those identified for YopE and YopH (15, 17, 33). Curiously, the translocation domain of YopE has been recently shown to be involved in the inhibition of YopE type III targeting in the absence of its SycE chaperone (34). We are further investigating the functional role of the secretion and translocation domains encompassing AvrBs2's type III signal, as well as the possibility of a chaperone.

Type III effectors from *Yersinia* and *P. syringae* have been recently shown to possess mRNA targeting signals, which are recognized by the type III secretion apparatus in *Yersinia enterocolitica* (16, 22). The existence of mRNA signals in type III effectors suggests a chaperone-independent mechanism for protein targeting via this pathway. We explored the possibility that an mRNA targeting signal may be present within the defined type III secretion domain (codons 1–28) identified for AvrBs2. Sensitive *Xcv* infection assays in *Bs2* pepper plants revealed that frameshift mutations within the first 18 codons of AvrBs2 are tolerated and still enable phenotypic expression of *avrBs2* in the host. This suggests that an mRNA targeting domain resides within first 18 codons of this effector, and that the type III secretion pathway in *Xcv* is also capable of recognizing an mRNA signal sequence. Our work suggests that type III secretion in *Xcv*, as in *Y. enterocolitica* (16), is governed by signals that couple mRNA translation to polypeptide targeting.

We have also identified the effector domain of AvrBs2 that is sufficient to initiate *Bs2*-specific disease resistance-mediated cell death *in planta*. Our localized cell death assays have mapped the recognition domain of AvrBs2 to codons 62 and 497 of the mature polypeptide. Although the biological function of AvrBs2 remains unclear, it shares homology to agrocinopine synthase (ACS) of *A. tumefaciens* and the glycerophosphoryl diester phosphodiesterase UgpQ of *E. coli* (3). The effector domain of AvrBs2 mapped herein resides within the region of highest homology to ACS and UgpQ (2, 3). Interestingly, all natural field strains of *Xcv* overcoming *Bs2* plant resistance possess molecular lesions within codons 62 and 497 of the *avrBs2* gene (2).

In conclusion, this work has identified the structural domains in AvrBs2 required for *Xcv* type III delivery to *Bs2* pepper plants. We are using this information to design experiments to formally demonstrate the direct translocation of AvrBs2 into plant cells. Phytopathogenic bacteria residing in the apoplastic fluid of plant mesophyll cells have to traverse the cell wall to deliver effector proteins. Thus, although plant and animal pathogens may share similar type III secretion signals, we expect plant pathogens to use unique strategies to invade the cell wall barrier. Progress in understanding how *Xcv* presents this key virulence determinant to host cells will provide new insight in to strategies used by the pathogen to avoid host recognition and to cause disease.

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