

A genetic screen to isolate type III effectors translocated into pepper cells during *Xanthomonas* infection

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The bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) uses a type III secretion system (TTSS) to translocate effector proteins into host plant cells. The TTSS is required for *Xcv* colonization, yet the identity of many proteins translocated through this apparatus is not known. We used a genetic screen to functionally identify *Xcv* TTSS effectors. A transposon 5 (Tn5)-based transposon construct including the coding sequence for the *Xcv* AvrBs2 effector devoid of its TTSS signal was randomly inserted into the *Xcv* genome. Insertion of the *avrBs2* reporter gene into *Xcv* genes coding for proteins containing a functional TTSS signal peptide resulted in the creation of chimeric TTSS effector::AvrBs2 fusion proteins. *Xcv* strains containing these fusions translocated the AvrBs2 reporter in a TTSS-dependent manner into resistant *BS2* pepper cells during infection, activating the *avrBs2*-dependent hypersensitive response (HR). We isolated seven chimeric fusion proteins and designated the identified TTSS effectors as *Xanthomonas* outer proteins (Xops). Translocation of each Xop was confirmed by using the calmodulin-dependent adenylate cyclase reporter assay. Three *xop* genes are *Xanthomonas* spp.-specific, whereas homologs for the rest are found in other phytopathogenic bacteria. XopF1 and XopF2 define an effector gene family in *Xcv*. XopN contains a eukaryotic protein fold repeat and is required for full *Xcv* pathogenicity in pepper and tomato. The translocated effectors identified in this work expand our knowledge of the diversity of proteins that *Xcv* uses to manipulate its hosts.

bacterial plant pathogenesis | virulence proteins

Many phytopathogenic bacteria use a conserved type III secretion system (TTSS) to infect plant cells (1). The structural components of the TTSS apparatus are encoded by the hypersensitive response and pathogenicity (*hrp*) gene cluster, a pathogenicity island required for these bacteria to colonize susceptible plants and to elicit the hypersensitive response (HR) in resistant plants (2). TTSSs facilitate the translocation of a group of proteins, called type III or TTSS effectors, into plant and animal cells (1). The precise function of phytopathogenic TTSS effectors is not known; however, their actions within the plant cell enable bacteria to grow outside plant cells (3).

We are interested in elucidating how *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) uses TTSS effectors to colonize its pepper and tomato hosts. *Xcv* is a Gram-negative bacterium that infects leaves and fruit, causing necrotic lesions and chlorosis, resulting in leaf abscission and fruit loss (4). Several *Xcv* effectors have been identified (5), and molecular analysis has revealed new insight into the role of these proteins in *Xcv* pathogenesis. AvrBs2, AvrBs3, HpaA, and XopA are required for full virulence of *Xcv* (6–9). XopD and AvrXv4 encode cysteine proteases that cleave the small ubiquitin-like modifier (SUMO) from plant SUMO-protein conjugates (10, 11), potentially disrupting eukaryotic processes regulated by SUMO modification. AvrBs3 is translocated into the plant nucleus and binds DNA, inducing transcription of several genes, including auxin-induced proteins, α -expansins, and pectate lyases (9).

These studies provide only a glimpse of the role of TTSS effectors in *Xcv* pathogenesis because this pathogen may contain many more effectors. Computer-based analyses of *Pseudomonas syringae* pv. *tomato* (*Pst*) and *Ralstonia solanacearum* have identified 40–50 effectors in each organism by using two criteria: (i) proximity to *hrp*-dependent promoter elements, suggesting co-regulation with the TTSS, and (ii) N-terminal export-associated signal peptides defined by known TTSS effectors (12–14). In *Xcv*, the expression of *hrp* genes and some TTSS effectors is under the control of the response regulator HrpG (15). Several *Xcv* effectors, including XopA, XopB, XopC, XopD, and XopJ were discovered by using cDNA-amplified fragment length polymorphism (AFLP) analysis of *Xcv* 85*, which contains a constitutively active HrpG (16). The existence of effectors regulated independently of HrpG indicates that alternative searches for *Xcv* effectors are necessary.

In this study, we performed a genetic screen to functionally identify *Xcv* TTSS effectors by exploiting the modular nature of these proteins. Generally, the N terminus of effectors is sufficient to target protein reporters into plant cells, whereas the C terminus is sufficient to activate HR in resistant plants (17, 18). Using this information, Guttman *et al.* (19) randomly integrated a gene reporter encoding the C terminus of the AvrRpt2 effector lacking its TTSS signal sequence throughout the *Pseudomonas* pv. *maculicola* (*Psm*) genome (19). By creating TTSS effector::AvrRpt2 chimeric fusion proteins that activate an AvrRpt2-dependent HR in plants, they functionally identified 13 new effectors and predicted another 38 effectors by means of bioinformatics (19).

Using a similar strategy, we show that the *Xcv* AvrBs2 effector can be used as a reporter protein to identify *Xcv* effectors. AvrBs2 was selected because its TTSS signal has been identified (20) and it elicits a strong HR in resistant *BS2* pepper cells (6, 20). We have defined a minimal domain of AvrBs2 capable of triggering HR in resistant *BS2* pepper. This domain, devoid of known TTSS signal sequences, was used as a reporter and randomly inserted into the *Xcv* genome by means of transposon 5 (Tn5). We have identified seven translocated effectors by looking for *Xcv* insertion strains that induce an *avrBs2*-dependent HR in *BS2* pepper. The identified TTSS effectors are designated as *Xanthomonas* outer proteins (Xops). Here, we describe the initial characterization of these proteins and discuss their potential roles in pathogenesis.

Abbreviations: *Xcv*, *Xanthomonas campestris* pv. *vesicatoria*; TTSS, type III secretion system; HR, hypersensitive response; Xop, *Xanthomonas* outer protein; *hrp*, HR and pathogenicity; *Pst*, *Pseudomonas syringae* pv. *tomato*; *Xac*, *Xanthomonas axonopodis* pv. *citri*; *Xcc*, *Xanthomonas campestris* pv. *campestris*; *Xoo*, *Xanthomonas oryzae* pv. *oryzae*; *Cya*, calmodulin-dependent adenylate cyclase; CFU, colony-forming unit; IR, inverted repeat.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AY756267 (XopN/XopF2), AY756268 (XopQ), AY756269 (XopO), AY756270 (XopP), and BK005592 (XopF1)].

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Materials and Methods

Bacterial Strains, Growth, and Matings. *Escherichia coli*, *Agrobacterium tumefaciens*, and *Xcv* strains were grown as described (10). Bacterial strains used in this study are listed in Table 3, which is published as supporting information on the PNAS web site.

Gene Manipulation and Plasmid Construction. Gene constructs generated by PCR were verified by restriction analysis and sequencing. For expression in *Xcv*, genes were subcloned into the broad host range vector pVSP61 and expressed under the control of native promoters. For transient expression using *A. tumefaciens*, genes were subcloned into the binary vector pMDD1 and expressed by a CaMV 35S promoter. Relevant plasmids are listed in Table 3. Information regarding constructs is available on request.

Construction of AvrBs2 Reporters. *avrBs2*₆₂₋₅₇₄::*HA* was amplified from pMDD1(*avrBs2*₁₋₅₇₄::*HA*) (courtesy of D. Dahlbeck, University of California, Berkeley) and subcloned into pMDD1. To make *avrXv4*₁₋₁₀₀::*avrBs2*₆₂₋₅₇₄::*HA*, the *avrXv4*₁₋₁₀₀ *Bgl*II fragment from pMS107 (10) was ligated to pBS(*Bgl*II::*avrBs2*₆₂₋₅₇₄::*HA*), creating pMS107(*avrXv4*₁₋₁₀₀::*avrBs2*₆₂₋₅₇₄::*HA*). This gene was moved into pVSP61 and pMDD1. BS2 peppers were inoculated with 6×10^8 colony-forming units (CFU)/ml *A. tumefaciens* carrying pMDD1 clones for transient expression or 2×10^8 CFU/ml *Xcv* carrying pVSP61 clones as described (10).

Construction of pTn5(*avrBs2*₆₂₋₅₇₄::*HA*) Transposon. A pTn5*cat* plasmid derivative was constructed by exchanging the *cat* gene with the *avrBs2*₆₂₋₅₇₄::*HA* reporter gene inserted in frame adjacent to the left inverted repeat (IR) of Tn5 (21). Digestion of pUIRM504 by *Eco*RI and *Sca*I removed the *cat* gene and the left IR. A *Spe*I linker was ligated into this vector to create a unique site at the 5' end of the Tn5 transposon. PCR amplification and subcloning of the N terminus of *avrBs2* added a *Spe*I site and a 27-bp Tn5 IR upstream of the *avrBs2*₆₂₋₅₇₄ coding sequence, forming pBS(*Spe*I::IR::*avrBs2*₆₂₋₅₇₄::*HA*). This chimeric gene was inserted into the *Spe*I site of the modified pTn5 vector to create pTn5(*avrBs2*₆₂₋₅₇₄::*HA*), which is referred to as pTn5(*avrBs2*) hereafter.

Construction of *Xcv* 85-10 Δ *avrBs2* Strain. p815*avrBs2*::*Gm*, a cosmid encompassing a 20-kb genomic region surrounding *avrBs2*, contains a replacement of the *Nde*I/*Cla*I fragment of the *avrBs2* coding sequence with a gentamycin resistance cassette (22). p815*avrBs2*::*Gm* was introduced into *Xcv* 85-10 by triparental mating to exchange the *Gm* cassette with the chromosomal *avrBs2* gene by homologous recombination.

***Xcv* Effector Screen.** pTn5(*avrBs2*) was introduced into *Xcv* 85-10 Δ *avrBs2* by triparental mating. Mutagenized strains were replica plated onto NYGA (0.5% peptone/0.3% yeast extract/2% glycerol/1.5% agar) rifampicin/kanamycin/tetracycline to identify tetracycline-sensitive strains, which contain a true Tn5(*avrBs2*) insertion rather than genomic integration of the plasmid. These strains were grown overnight in NYGB (0.5% peptone/0.3% yeast extract/2% glycerol) rifampicin at 28°C. Pools of eight strains, containing $\approx 1 \times 10^8$ CFU per strain, were combined, centrifuged, and resuspended in 1 mM MgCl₂ to 1×10^9 CFU/ml per pool. Pools were hand-inoculated into BS2 pepper leaves and kept under continuous light for 2–3 days. Each *Xcv* strain from a pool that showed an *avrBs2*-dependent HR was independently inoculated at 2×10^8 CFU/ml to identify the HR-positive insertion strain. Genes disrupted by the transposon were identified by plasmid rescue and sequencing. National Center for Biotechnology Information BLAST algorithms were used to identify homologs (23).

Southern Blot Analysis. We transferred 5 μ g of genomic DNA digested with *Bam*HI or *Eco*RV to nylon membranes, and they were visualized by using the CDP-Star chemiluminescent detection system (Amersham Pharmacia). A 1,000-bp fragment of *avrBs2* was used to confirm single Tn5(*avrBs2*) insertions. Whole or partial *xop* ORFs were hybridized to DNA isolated from *Xanthomonas* spp. to probe for *xop* homologs. *avrBs2* and *xop* hybridizations were done at 65°C and 55°C, respectively.

Xop::Calmodulin-Dependent Adenylate Cyclase (Cya) Assays. The promoter and coding regions of each *xop* gene 5' to the Tn5 insertion site were amplified by PCR and subcloned into the *Bgl*II site in pMS107 (24) to create translational Cya fusions. Each *xop*::*cya* construct was subcloned into pVSP61. *E. coli* strains carrying the pVSP61(*xop*::*cya*) plasmids were triparentally mated into *Xcv* 85-10 *hrpG** (25) and *Xcv* 85-10 *hrpG** Δ *hrpF* (26). The expression and activity of Xop::Cya proteins in bacteria and plants were assessed by immunoblot analysis and cAMP ELISA as described (10).

Signal Sequence Analysis. The first 55 aa of each protein and the remaining C-terminal residues were compared for amino acid composition. The statistical significance of amino acid biases between these groups was measured by using the Kolmogorov–Smirnov test. Analyzed effectors included Xops, identified here, and the following, with GenBank accession numbers given in parentheses: *Xcv* 81-23 effector AvrBs1 (P19520); *Xcv* 75-3 effectors AvrBs2 (AAD11434), AvrBsT (AAD39255), and AvrRvx (Q08678); *Xcv* 71-21 effector AvrBs3 (P14727); *Xcv* 85-10 effectors XopA (AAL78294), XopB (AAK72487), XopC (AAR23832), XopD (AAL78292), XopJ (AAK72486), HpaA (AAD21323), and XopX (AAT39020); and *Xcv* 91-118 effectors AvrXv3 (AAG18480) and AvrXv4 (AAG39033).

In Planta Growth of *Xcv* Δ *xop* Strains. To create *xop* knockouts, 300-bp fragments internal to each *xop* gene were subcloned into the suicide vector pLVC18-RfC (courtesy of C. Morales, University of California, Berkeley). pLVC18(*xop*) plasmids were moved into *Xcv* 85-10 by triparental mating, and selected by growth on NYGA rifampicin/tetracycline. Sequencing confirmed homologous recombination within the gene of interest. Then, 1×10^5 CFU/ml suspensions of *Xcv* 85-10 and *Xcv* Δ *xop* strains were hand-

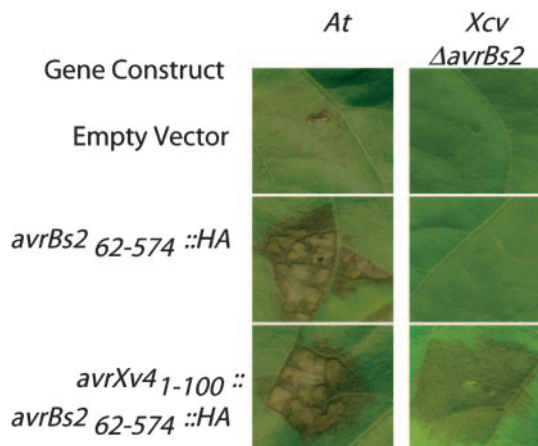


Fig. 1. *avrBs2*-dependent HR activity in BS2-resistant pepper. *A. tumefaciens* (*At*) strains carrying the empty vector pMDD1, pMDD1(*avrBs2*₆₂₋₅₇₄::*HA*), or pMDD1(*avrXv4*₁₋₁₀₀::*avrBs2*₆₂₋₅₇₄::*HA*) were inoculated into resistant BS2 pepper leaves at 6×10^8 CFU/ml. *Xcv* Δ *avrBs2* strains carrying the empty vector pDD62, pDD62(*avrBs2*₆₂₋₅₇₄::*HA*), or pDD62(*avrXv4*₁₋₁₀₀::*avrBs2*₆₂₋₅₇₄::*HA*) were inoculated into BS2 leaves at 2×10^8 CFU/ml. Symptoms were recorded 24–48 h postinoculation.

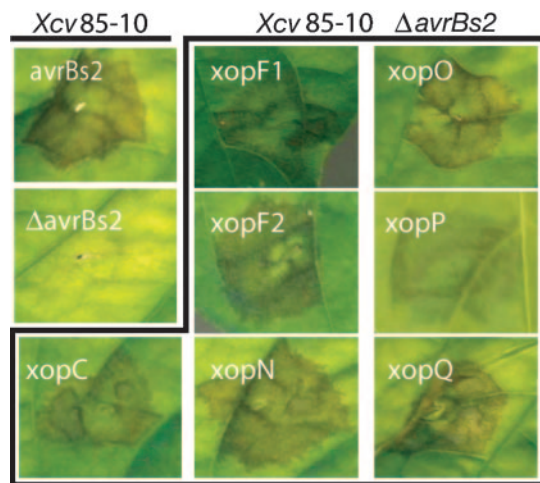


Fig. 2. *Xcv* Xop::AvrBs2 proteins induce *avrBs2*-dependent HR during infection. *BS2*-resistant pepper leaves were independently inoculated with a 2×10^8 CFU/ml suspension of *Xcv* 85-10, *Xcv* 85-10 Δ *avrBs2*, or seven *Xcv* 85-10 Δ *avrBs2* strains each containing an *xop::avrBs2*₆₂₋₅₇₄::HA gene fusion. Symptoms were recorded 75 h postinoculation.

inoculated into multiple *bs2* pepper or VF36 tomato leaves. Plants were grown under 10 h of light per day at 22°C. Leaf disks (total area, 1 cm²) were ground in 1 ml of MgCl₂ and dilution-plated onto selective NYGA in triplicate to determine bacterial load.

Results

AvrBs2 as a Translocation Reporter. To use AvrBs2 as a TTSS reporter, we defined the minimal AvrBs2 peptide capable of activating *BS2*-dependent resistance in pepper. AvrBs2 is composed of 714 aa, of which amino acids 1–58 contain the TTSS secretion and translocation signals and amino acids 62–714 activate *BS2* disease resistance (17). C-terminal deletion analysis of AvrBs2 shows that amino acids 575–714 are not required for *BS2* recognition (D. Dahlbeck and B. Staskawicz, unpublished results). Therefore, the minimal region required for AvrBs2-specific HR activity *in planta* was predicted to be amino acids 62–574. Transient expression of AvrBs2₆₂₋₅₇₄::HA in *BS2* pepper leaves induced a strong HR response (Fig. 1), similar to that observed with the mature polypeptide (data not shown). This finding shows that AvrBs2₆₂₋₅₇₄::HA is a functional protein reporter for *avrBs2*-dependent HR activity in plant cells.

We tested TTSS delivery of AvrBs2₆₂₋₅₇₄::HA to ascertain whether the protein can serve as a reporter to identify effectors. A useful reporter must accumulate in *Xcv* but cannot traverse the TTSS. To create an *Xcv* strain suitable for screening, we replaced the chromosomal copy of the *avrBs2* gene in *Xcv* 85-10 with a

gentamycin resistance cassette. *Xcv* 85-10 Δ *avrBs2* did not trigger an *avrBs2*-dependent HR in *BS2* pepper (Fig. 1) or produce AvrBs2 protein (data not shown). Moreover, *Xcv* 85-10 Δ *avrBs2* exhibited reduced growth on susceptible plants as compared with *Xcv* 85-10, consistent with published results (data not shown and ref. 6). *Xcv* 85-10 Δ *avrBs2* pVSP61(*avrBs2*₆₂₋₅₇₄::HA) expressed AvrBs2₆₂₋₅₇₄::HA protein (data not shown) but failed to produce an *avrBs2*-dependent HR in pepper (Fig. 1). However, *Xcv* 85-10 Δ *avrBs2* expressing the fusion protein AvrXv₄₁₋₁₀₀::AvrBs2₆₂₋₅₇₄::HA triggered an *avrBs2*-dependent HR (Fig. 1). This result demonstrated that the AvrBs2 reporter fused to a functional TTSS signal peptide could be targeted through the *Xcv* TTSS. Furthermore, transient expression of AvrXv₄₁₋₁₀₀::AvrBs2₆₂₋₅₇₄::HA in pepper elicited the same phenotype as AvrBs2₆₂₋₅₇₄::HA, indicating that modification of the reporter does not affect its recognition by *BS2* (Fig. 1). These studies show that AvrBs2₆₂₋₅₇₄::HA can be used as a sensitive TTSS reporter in the *Xcv* Δ *avrBs2* strain.

A Genetic Screen to Identify TTSS Effectors. The AvrBs2₆₂₋₅₇₄::HA reporter was randomly integrated into the *Xcv* Δ *avrBs2* genome to create protein fusions, an approach previously used by Guttman *et al.* (19). To do this, we modified a Tn5-based transposon, pTn5*cat*, that randomly integrates into *Xanthomonas* spp. (21). In pTn5(*avrBs2*), the *avrBs2*₆₂₋₅₇₄::HA gene fragment is downstream of the left IR of Tn5. Approximately 14,000 *Xcv* Tn5 insertion strains were assayed for AvrBs2 activity on pepper. We identified seven strains that triggered HR when inoculated into *BS2* pepper leaves (Fig. 2). The timing and strength of the HR elicited by each strain varied, suggesting that the stability and/or quantity of protein translocated to the host was effector-dependent. We confirmed that the HR was *avrBs2*-specific by inoculating both resistant *BS2* and susceptible *bs2* pepper leaves. All strains elicited a *BS2*-dependent HR (data not shown). Southern blot analysis verified that only one Tn5(*avrBs2*) insertion was responsible for each phenotype (data not shown).

Identity of Xop Effectors. To identify the site of transposon integration, we determined the DNA sequence adjacent to Tn5 for each HR-inducing *Xcv* strain. Each strain contained a single insertion in a unique gene, generally near the 5' end (Table 1). We have designated these genes as *xops* in accordance with current effector nomenclature. BLAST analysis showed that the seven Xop proteins are found only in phytopathogenic bacteria (Table 1). Three Xops are similar to known or predicted TTSS effectors. XopC is an effector previously discovered in *Xcv* strain 85-10 (27). XopQ shares homology with the *R. solanacearum* effector RipB (14) and the *Pst* DC3000 effector HolPtoQ (19). XopO is homologous to the *Pseudomonas* pv. *pisi* effector AvrRps4 (28). XopN and XopP have homology to hypothetical proteins from *Xanthomonas* spp. (29). Fragments of the *xopF1* gene are located within the *hrp* cluster of many *Xanthomonas* spp., although a complete ORF is present only

Table 1. Identity of Xop effectors

| Effector | Homology (GenBank accession no.) | BLASTP,* bits/e-value | Predicted size, aa | Insertion site, aa | Homologs in sequenced species |
|----------|---|--------------------------|-----------------------|-----------------------|--|
| XopC | Type III effector XopC (AAR23832) | 575/e-163 | 834 | 708 | <i>Rs</i> |
| XopF1 | Xoo conserved hypothetical protein ORF1 (BAD30000) | 879/0.0 | 670 | 151 | <i>Xoo</i> , <i>Xcc</i> [†] |
| XopF2 | Xoo conserved hypothetical protein ORF1 (BAD30000) | 517/e-145 | 667 | 108 | <i>Xoo</i> , <i>Xcc</i> [†] |
| XopN | <i>Xac</i> conserved hypothetical protein XAC2786 (NP_643095) | 1059/0.0 | 733 | 197 | <i>Xac</i> , <i>Xcc</i> |
| XopO | <i>Psp</i> avirulence protein AvrRps4 (AAB51082) | 119/4e-26 | 220 | 93 | <i>Psp</i> <i>Pst</i> DC3000 |
| XopP | <i>Xac</i> conserved hypothetical protein XAC1208 (NP_641544) | 865/0.0 | 658 | 321 | <i>Xac</i> , <i>Xcc</i> , <i>Rs</i> |
| XopQ | <i>Xac</i> conserved hypothetical protein XAC4333 (NP_644627) | 704/0.0 | 464 | 81 | <i>Xac</i> , <i>Pst</i> DC3000, <i>Xcc</i> , <i>Rs</i> |

*BLASTP queries were done by using databases at the National Center for Biotechnology (23).

[†]BLASTN of nucleotide sequence also yields homology with *Xac*, *Xag*, and *Xcc*.

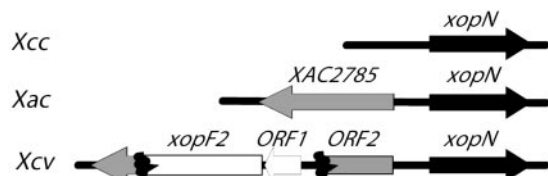


Fig. 3. Organization of the *xopN/xopF2* genomic region. *ORF1* and *xopF2* are depicted as white arrows. *XopN* homologs are shown as black arrows. *XAC2785* and its disrupted *Xcv* homolog *ORF2* are depicted as gray arrows. Arrowheads indicate the predicted direction of transcription.

in the *Xcv* and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) *hrp* clusters. *XopF2* is 59% identical and 68% similar to *XopF1* when analyzed with the pairwise BLAST algorithm (23) although it is located adjacent to *xopN* (Fig. 3). Comparison of this genomic region with syntenic regions in *Xanthomonas axonopodis* pv. *citri* (*Xac*) and *Xanthomonas campestris* pv. *campestris* (*Xcc*) showed that *xopF2* was inserted into *ORF2*, which is homologous to *XAC2785* (29). *Xcc* does not contain orthologs of *xopF2* or *XAC2785*. *xopF2* seems to be cotranscribed with *ORF1*, a small gene of 148 aa with a predicted pI of 4.93 (Fig. 3). Structural analysis of *ORF1* using the PROTEAN program (DNASTAR, Madison, WI) indicated that the protein may contain amphipathic α -helices. These characteristics are shared by type III chaperones (30), suggesting that *ORF1* may encode an *Xcv* chaperone.

To gain insight into effector function, we examined the predicted secondary structure of each Xop using a three-dimensional position-specific scoring matrix (3D-PSSM, www.sbg.bio.ic.ac.uk/~3dpssm) and identified two Xop proteins with structural homologs (31). The most similar fold to *XopN* is found in the constant regulatory domain of protein phosphatase 2a (pr65/A) with an e-value of 0.0701. This protein contains HEAT (*huntingtin*, elongation factor 3, PR65/A, TOR1) repeats, which are tandem anti-parallel α -helices that stack on each other to form a solenoid and are structurally superimposable with the Armadillo (ARM) repeat family (32). 3D-PSSM analysis of *XopQ* shows a significant match with an e-value of 1.66×10^{-8} to the crystal structure of the inosineuridine nucleoside *N*-ribohydrolase, IU-NH, from the protozoan parasite *Crithidia fasciculata* (33).

Xop::Cya Assays Confirm TTSS-Dependent Translocation. Although we removed known TTSS signals from the *AvrBs2*₆₂₋₅₇₄ reporter, this peptide may contain additional translocation signals. Therefore, we used the Cya domain of *Bordetella pertussis* cyclolysin as a biochemical reporter (24, 26) to confirm that the identified Xops are sufficient to target non-TTSS proteins to the plant cell. *Xop::Cya* proteins were made by fusing each *xop* promoter

region and coding sequence upstream of the transposon insertion to the *cya* reporter. The *xopF2::cya* gene construct also contained the proposed chaperone, *ORF1*. Constructs were expressed in the isogenic strains *Xcv hrpG**, which contains a point mutation in the HrpG response regulator leading to constitutive expression of the TTSS (15), and *Xcv hrpG* ΔhrpF*, which lacks the putative TTSS translocon HrpF and does not translocate proteins into plant cells (34).

All fusion proteins were equally expressed in the translocation-competent (*Xcv hrpG**) and translocation-deficient (*Xcv hrpG* ΔhrpF*) strains (Fig. 6, which is published as supporting information on the PNAS web site). Surprisingly, *xopF1::cya* and *xopP::cya* encoded proteins with larger molecular weights than expected given the predicted Met start codons. We identified GTG start codons with Shine–Delgarno sites (35) upstream of the predicted start that would yield the appropriate size fusion proteins. Protein extracts were then isolated from *Xcv hrpG** and *Xcv hrpG* ΔhrpF* expressing each *Xop::Cya* fusion to test for endogenous Cya enzyme activity. Each *Xop::Cya* protein had little endogenous Cya activity, but addition of calmodulin to protein extracts from both strains restored enzyme activity (data not shown). To assess Xop-dependent TTSS translocation of the Cya reporter into plant cells, we inoculated all strains into susceptible pepper leaves and measured cAMP levels in tissue samples at 0, 8, and 27 h after inoculation. Leaves infected with *Xcv hrpG** strains expressing each *Xop::Cya* protein accumulated 10- to 1,000-fold higher levels of cAMP compared with mock infections, in an HrpF- and time-dependent manner (Fig. 4). This finding confirms that all Xop proteins are targeted to the plant by means of the *Xcv* TTSS in the absence of the *AvrBs2* reporter.

Analysis of Xcv TTSS Signals. We compiled the sequence of our six new Xop proteins with 14 known *Xcv* effectors (see *Materials and Methods*) to determine whether the N termini of these proteins exhibit an amino acid bias similar to that reported for effectors from other phytopathogens. In *Pst* DC3000 and *R. solanacearum*, the N-terminal 50 aa of effectors have significantly higher levels of serine and proline and decreased levels of leucine as compared with proteins not associated with the TTSS (12, 14, 19). Because the genome sequence of *Xcv* is not available, we used the C termini of our effectors as a control for amino acid composition. We found that the levels of Ser (14.8%), Pro (11.7%), and Leu (6.9%) in the first 55 aa of 20 *Xcv* TTSS effectors are statistically different from levels of Ser (8.3%), Pro (5.4%), and Leu (9.9%) in the C-terminal portions of these proteins. Other rules defined for *Pst* signal sequences are less conserved in *Xcv* and *R. solanacearum* (12, 14). Hydrophobic residues at position 5 are rare in *Pst*, whereas 9 of 20 of the *Xcv* effectors have a Met, Ile, Leu, Val, Phe, Tyr, or Trp at this

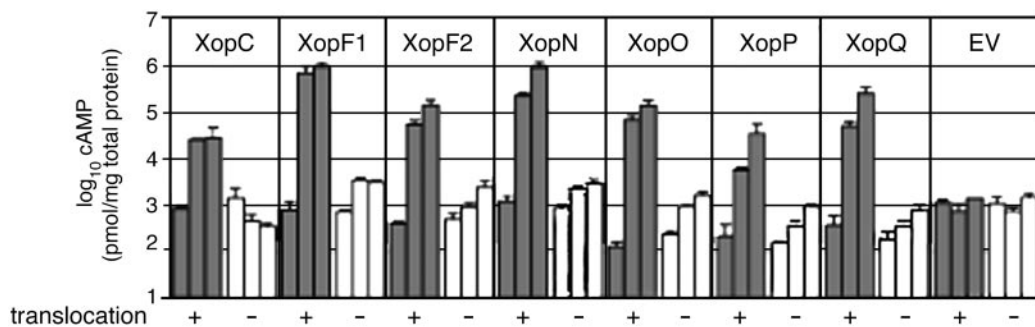


Fig. 4. TTSS-dependent translocation of *Xop::Cya* proteins into plant cells. Susceptible *bs2* pepper leaves were inoculated with a 5×10^8 CFU/ml suspension of *Xcv* expressing each *Xop::Cya* protein. *Xcv* 85-10 *hrpG** strains are translocation competent (+, gray bars) and *Xcv* 85-10 *hrpG* ΔhrpF* strains are translocation deficient (-, white bars). cAMP levels were measured as the \log_{10} (pmol cAMP per mg total protein). Tissue samples were taken at 0, 8, or 27 h postinoculation. Time points are shown by sequential bars.

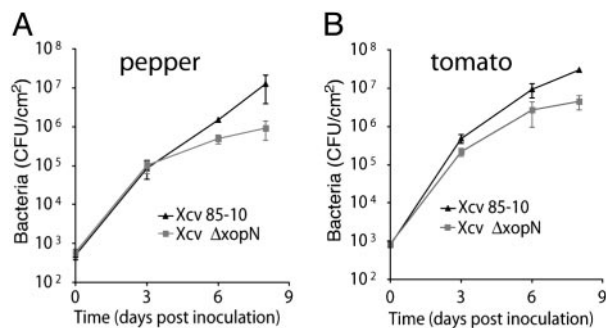


Fig. 5. Growth of *Xcv* $\Delta xopN$ in susceptible hosts. Susceptible *bs2* pepper plants (A) and VF36 tomato plants (B) were hand-inoculated with a 1×10^5 CFU/ml suspension of *Xcv* 85-10 or the mutant strain *Xcv* 85-10 $\Delta xopN$. Data points represent the mean \log_{10} cells per cm^2 of leaf tissue \pm SD of a representative growth assay. This analysis was performed three times per plant host.

position. Similar to *R. solanacearum*, 8 of 20 of the *Xcv* effectors have an Arg or Lys at position 2, and a majority (18 of 20) contain at least one basic residue in the first 7 aa. In contrast to both *Pst* and *R. solanacearum* effectors, where acidic residues are not found in the first 12 aa, half of the *Xcv* effectors incorporate at least one Asp or Glu in this region.

Contribution of Xops to *Xcv* Pathogenicity. We disrupted each gene in *Xcv* 85-10 by homologous recombination and tested mutant strains for a reduction in pathogenicity in susceptible plants. None of the *Xcv* Δxop strains exhibited growth defects when grown in a rich NYGB medium or minimal media that mimics the conditions in the apoplast (data not shown). Each *Xcv* Δxop strain was inoculated into susceptible pepper and tomato leaves by hand infiltration, and *in planta* bacterial growth was measured. By day 8, *Xcv* $\Delta xopN$ bacteria grew to only 1/10 to 1/100 the level of *Xcv* 85-10 in pepper and tomato (Fig. 5). Thus, XopN is required for maximal *Xcv* growth in both pepper and tomato plants. No other *Xcv* Δxop mutant exhibited significant growth defects in either host (data not shown).

Distribution of Xops among *Xanthomonas* spp. We determined the presence of each *xop* gene in a diverse group of *Xanthomonas* spp. using Southern hybridization (Table 2). We hybridized *xop*-specific probes to *Xanthomonas* strains with different characteristics. *Xanthomonas* spp. have been grouped according to the following traits: phenotype on different plant cultivars (36), amylolytic activity designated as serovars A–D (37), and 16S rRNA nucleotide homology (38). *Xcv* 75-3 and *Xcv* 81-18 are closely related to *Xcv* 85-10, because all three strains are pepper race 2, tomato race 1, and serovar A. *Xcv* 81-23, *Xcv* 81-18, *Xcv*

597, and *Xcv* 82-8 are also serovar A strains, but they are different pepper or tomato races. *Xcv* 71-4 and *Xcv* 81-6 are both in serovar B, *Xv* 938 in serovar C, and *Xv* 444 in serovar D (38). *X. axonopodis* pv. *glycines* (*Xag*), *Xac*, *Xanthomonas campestris* pv. *armoraciae* (*Xca*), *Xcc*, and *X. oryzae* pv. *oryzae* (*Xoo*) are pathogenic on different host plants.

All seven *xop* genes tested hybridized to DNA from each *Xcv* serovar A strain (Table 2). Consistent with BLAST results (Table 1), *xopF1*, *xopN*, *xopP*, and *xopQ* are conserved across most *Xanthomonas* spp., whereas *xopC*, *xopF2*, and *xopO* are present only in closely related *Xcv* strains. Protein homologs of XopO and XopQ are present in *Pseudomonas* spp.; however, we did not observe hybridization of *xopO* and *xopQ* probes to *Pseudomonas* spp. DNA (data not shown). This result was not unexpected, because nucleotide homology for the respective genes is low (50% identity for *Xcv* *xopQ* and *Pst* *holPtoQ*) or undetectable (*Xcv* *xopO* and *Psp* *avrRps4*).

Discussion

In this work, we used AvrBs2 as a sensitive reporter (19) to isolate new *Xcv* effectors. The AvrBs2/Bs2 reporter system is a tool that can be used to identify translocated effectors in bacterial pathogens that infect other naturally occurring or transgenic BS2 plant lines. We identified four TTSS effectors of unknown function (XopN, XopF1, XopP, and XopF2), one previously identified *Xcv* effector (XopC), one effector with homology to a known *P. syringae* effector (XopO = AvrRps4), and one that is widely conserved among phytopathogens (XopQ = RipB, HolPtoQ). The fact that we did not detect all known *Xcv* 85-10 effectors is not surprising. We screened 14,000 insertion strains, but only one of six permutations of transposon insertion in an effector gene will be detected because recognition of AvrBs2 relies on the creation of a translational fusion. Many more insertion strains must be tested for this screen to reach saturation.

Understanding acquisition and evolution of type III effectors in *Xcv* may help us deduce the roles of these proteins in pathogenicity. As a first step, we examined Xop acquisition by using the three criteria used for analysis of *P. syringae* effectors: GC content, strain distribution, and genomic context (39). *xopN*, *xopQ*, *xopP*, and the C terminus of *xopF1* are conserved across *Xanthomonas* spp. (Table 2), and their GC content is comparable with that of the *Xcv* *hrp* cluster (8). These *xop* genes may be members of a “core” group of *Xanthomonas* spp. effectors. *xopC* and *xopO* did not hybridize to many distant *Xanthomonas* spp., but have homologs in two tomato phytopathogens, *Pst* and *R. solanacearum* (Tables 1 and 2), suggesting that these genes may have been introduced into *Xcv* by horizontal gene transfer. In fact, the *xopC* gene is flanked by 62-bp IRs and multiple ORFs predicted to encode transposases and integrases (8). A peptide fragment with homology to the *Xcc* IS1479 transposase (29) was identified upstream of *xopO*. Both *xopC* and *xopO* have GC

Table 2. Distribution of *xop* genes in phytopathogenic bacteria

| | Serovar A | | | | | | B | | C | | D | | <i>Xanthomonas</i> spp. | | | | |
|-------|-----------|------|-------|-------|-----|------|------|------|---------------|---------------|------------|------------|-------------------------|------------|------------|--|--|
| | 85-10 | 75-3 | 81-23 | 81-18 | 597 | 82-8 | 71-4 | 81-6 | <i>Xv</i> 938 | <i>Xv</i> 444 | <i>Xcg</i> | <i>Xac</i> | <i>Xca</i> | <i>Xcc</i> | <i>Xoo</i> | | |
| XopC | X | X | X | X | X | X | | | | | X | | | | | | |
| XopF1 | X | X | X | X | X | X | X | W | | X | X | X | X | W | X | | |
| XopF2 | X | X | X | X | X | X | X | W | | X | | | | | | | |
| XopN | X | X | X | X | X | X | X | | | X | X | X | X | X | X | | |
| XopO | X | X | X | X | X | X | | | | | | | | | | | |
| XopP | X | X | X | X | X | X | | X | X | | X | X | X | | X | | |
| XopQ | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | | |

Genomic DNA was probed with each *xop* gene by Southern hybridization. X, strong hybridization; W, weak hybridization; blank, no hybridization. All numbered strains are different serovars of *Xcv*. Other strain abbreviations are as follows: *Xv*, *X. vesicatoria*; *Xcg*, *X. campestris* pv. *glycines*; *Xac*, *X. axonopodis* pv. *citri*; *Xca*, *X. c. pv. armoraciae*; *Xcc*, *X. c. pv. campestris*; *Xoo*, *X. oryzae* pv. *oryzae*.

contents 10–15% lower than the *Xcv hrp* cluster (8). These genes may be recent additions to the *Xanthomonas* spp. effector cadre considering their uneven species distribution, low GC content, and association with mobile elements. As Xop homologs are identified in other bacterial species, we can more fully explore the evolution of these effector genes among diverse pathogens.

Recent articles report a bias in the amino acid composition of TTSS signal sequences from *P. syringae* spp. and *R. solanacearum* as compared with N termini of nonsecreted proteins (12, 14, 19). We found that *Xcv* TTSS signal sequences share attributes with both *R. solanacearum* and *Pst*, but *Xcv* elements defining these signals are more similar to *R. solanacearum*. This finding is consistent with the phylogenetic distribution of the TTSS machinery: *Xanthomonas* and *Ralstonia* TTSS genes are similar, whereas *Pseudomonas* sequences are more divergent (40). Thus, it is likely that subtle differences in the TTSS signal sequences of each organism may provide specificity to the structures they traverse. However, the fact that *Xcv* can secrete *R. solanacearum* PopA, *Pseudomonas* pv. *glycinea* AvrB, and *Yersinia pseudotuberculosis* YopE effectors (25) suggests that there may be redundancy or promiscuity for effector targeting. The field has yet to discover the precise combination of factors that defines a type III signal peptide.

Most of the Xops that we isolated were not required for *Xcv* growth on pepper and tomato hosts. This finding is not surprising considering that the loss of a single TTSS effector does not typically affect pathogenicity. This result may reflect effector functional redundancy or simply the lack of sensitive assays available to assess effector roles in bacterial–plant interactions. However, the growth of *Xcv xopN* mutants was significantly impaired in both pepper and tomato (Fig. 5). *xopN* is present in many *Xanthomonas* spp. but not found in the published genomes of other bacterial pathogens containing TTSS (Tables 1 and 2). This finding suggests that XopN may be an important *Xanthomonas*-specific effector. XopN shares structural homology with a superfamily of proteins containing tandemly repeated α -helices, known as ARM/HEAT repeats, that are found in almost all eukaryotes but are rare in bacteria (32). XopN is the first

pathogen molecule thought to mimic this eukaryotic fold. The *Arabidopsis* genome contains many proteins with HEAT repeats including protein phosphatase 2A PR65/A regulatory subunits (41); several homologs of the nuclear transport protein importin- β (42); and AtCAND1, which is involved in the regulation of auxin signaling (43). We speculate that XopN may use HEAT repeat structures to modulate plant signal transduction by mimicking or interfering with proteins containing these repeats. The identification of XopN plant interactors will provide clues to its role in *Xcv* pathogenicity.

XopQ is conserved among many plant pathogens (Tables 1 and 2). These proteins have structural homology to an inosine-uridine nucleoside *N*-ribohydrolase enzyme, a protein implicated in the ability of many organisms to salvage nucleotides from their environment (44). Like other homologs, XopQ contains conserved aspartate residues found in the active site of the enzyme (44). Although nucleoside hydrolase enzymes are present in *Xac* and *Xcc* and likely present in *Xcv* as well, these genes do not share sequence homology with XopQ (29). It is possible that XopQ functions as a scavenging hydrolase *in planta*, or may bind and sequester nucleosides important for plant signaling and/or metabolism.

In summary, we have identified seven TTSS effectors used by *Xcv* during pathogenesis of pepper and tomato. Predictive homology of two effectors points the way toward elucidating their role in disease. Additional Xops may continue to be identified, defining a repertoire of effectors used by *Xcv* during infection. Probing the biochemical function of the Xops would give us clues as to plant processes modified by these effectors. Moreover, it would allow us to better understand the diversity of mechanisms used by this phytopathogen to modify its host during pathogenesis.

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