

Direct biochemical evidence for type III secretion-dependent translocation of the AvrBs2 effector protein into plant cells

Catharina Casper-Lindley[†], Douglas Dahlbeck[†], Eszter T. Clark, and Brian J. Staskawicz[‡]

Department of Plant and Microbial Biology, 111 Koshland Hall, University of California, Berkeley, CA 94720-3120

Contributed by Brian J. Staskawicz, April 11, 2002

The calmodulin-dependent adenylate cyclase domain (Cya) of the *Bordetella pertussis* cyclolysin was used as a reporter protein to study the direct translocation of the *Xanthomonas* effector protein, AvrBs2, into the plant host cell. Adenylate cyclase activity (production of cAMP) depends on the presence of eukaryotic plant calmodulin and is only active after translocation from the prokaryotic cell into the eukaryotic plant cell. Here, we show that infection of pepper plants by *Xanthomonas campestris* pv. *vesicatoria* strains expressing the AvrBs2:Cya fusion protein results in detectable increases of cAMP levels in plant cells as early as 3 h after inoculation. Adenylate cyclase activity was shown to be type III secretion-dependent as the *Xanthomonas hrp* mutations, *hrpV* or *hrpF*, failed to produce detectable levels of cAMP in infected pepper plants. Furthermore, the N-terminal secretion and translocation signals of AvrBs2 were shown to be required for activity of the fusion protein in the plant. A single genomic copy of the *avrBs2:cya* fusion gene expressed under the control of the wild-type *avrBs2* promoter was used to compare the effect of a susceptible and resistant plant interaction on the kinetics of effector protein delivery. Implications of these results and additional applications of this reporter construct are discussed.

The type III secretion system (TTSS) is a protein translocation system conserved in many Gram-negative pathogenic bacteria that infect plants and animals (1–3). The main function of the TTSS is to deliver bacterial effector proteins into the eukaryotic host cell to mimic, suppress, or modulate host defense signaling pathways and to cause disease. In the case of plants, a surveillance system encoded in part by plant resistance genes has evolved to specifically recognize these virulence effector proteins and to trigger host defense signaling pathways that result in pathogen inhibition. However, definitive evidence that effector proteins are translocated from phytopathogenic bacteria into the plant cell is currently lacking. Indirect evidence that effector proteins are translocated to the plant host is supported by the data that transient expression of these genes in host cells is sufficient to elicit a genetically defined hypersensitive cell death-resistance response (4–9). In addition, initiation of plant disease or plant resistance depends on a functional (TTSS) mechanism in these bacteria (10, 11), suggesting that bacterial effector proteins are translocated from the pathogen into the plant cytosol. Finally, that the majority of NBS/LRR disease-resistance proteins are thought to be cytosolic or associated with the plasma membrane is consistent with this hypothesis.

In plant pathogens, the TTSS is encoded by the clustered *hrp* (hypersensitive reaction and pathogenicity) genes (12). The homologies between the TTSS of mammalian and plant pathogens suggest that phytopathogenic bacteria translocate proteins into host cells by a process similar to that of animal pathogens. Indeed, the phytopathogen *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) is able to secrete type III effector proteins of the mammalian pathogen *Yersinia enterocolitica* (13). Plant cells, unlike animal cells, have a cell wall, however, and one might expect specific differences between pathogen secretion systems. Along these lines, a recent report discusses the role of pili in the

in vitro protein secretion of phytopathogenic bacterial effector proteins (14).

In vitro evidence exists for type III effector protein secretion by the phytopathogenic bacteria *Xcv*, (13, 15), *Pseudomonas syringae* (16, 17), *Ralstonia solanacearum* (18), and *Erwinia amylovora* (19). Furthermore, the *P. syringae* AvrRpt2 effector protein was shown to be cleaved at the N terminus during bacterial infection and only occurred in bacteria that contained a functional TTSS. The proteolytic processing was demonstrated not to occur outside the host, suggesting that cleavage most likely occurred after translocation into the cytosol of the plant cell (16). Direct evidence for translocation into plant cells is lacking (10, 15), as it has been problematic to target reporter proteins through the TTSS (16, 20). In contrast to plant pathogens, type III-secreted effector proteins of the mammalian pathogens *Y. enterocolitica* (21, 22) and *Salmonella dublin* (23) have been detected inside host cells by immunofluorescence (24) as well as by detection of reporter fusions (21, 25).

In this article, we present biochemical evidence that supports the notion that phytopathogenic bacteria can deliver effector proteins directly into the plant host cell. We constructed a chimeric protein between the calmodulin-dependent adenylate cyclase domain (*cya*) of *Bordetella pertussis* cyclolysin (21) and the *Xcv* effector protein AvrBs2. Adenylate cyclase fusions have been used to demonstrate translocation of type III-secreted bacterial effector proteins of *Yersinia*, *Salmonella*, and *Chlamydia* into mammalian cells (21, 23, 26). Activity of this reporter enzyme depends on eukaryotic calmodulin and therefore the Cya fusion is only active after translocation from the bacteria into eukaryotic host cells (21). The use of this reporter strategy will also allow us to analyze further the TTSS in phytopathogenic bacteria in a more sensitive and precise manner.

Materials and Methods

Bacterial Strains. The strains used in this study were *Escherichia coli* DH5 α , *Xcv* strain 85-10 (contains *avrBs2* and *avrBs1*) (27), 85-10 *hrpG*^{*} (85^{*}) (85-10 with a mutation for constitutive *hrp* gene expression) (13), 85^{*} Δ *hrpV* (85^{*} with a type III secretion-defective mutation) (15, 28), 85^{*} Δ *hrpF* (85^{*} with a mutation in *hrpF*) (29), and *Xcv* strain GM98-38 (lacks *avrBs2* activity) (30). *Xcv* strains were grown at 28°C on nutrient yeast glucose agar containing 100 μ g/ml of rifampicin. *E. coli* strain DH5 α was grown at 37°C on Luria agar.

Genetic Constructs. For protein expression experiments, the broad host range vector pDD62 was used (15). Plasmids were mobilized from *E. coli* into *Xcv* by triparental mating with standard methods (15). The plasmid pDD62(*avrBs2:HA*) (15) has wild-type *avrBs2* activity and was used as a positive control strain. To

Abbreviations: TTSS, the type III secretion system; HR, hypersensitive cell death response; p.i., post inoculation.

[†]C.C.-L. and D.D. contributed equally to this work.

[‡]To whom reprint requests should be addressed. E-mail: stask@nature.berkeley.edu.

construct pDD62 (*avrBs2:cya*), plasmid 81533B (pUC118 with *avrBs2*) (31) was modified by replacing the translational stop site at codon 714 with a *Bam*HI site. *AvrBs2* was then cloned as a *Bam*HI fragment into the *Bgl*III site of pMS107 (*cya*) (21), in-frame with *cya*. The *avrBs2:cya* fusion was then cloned as an *Xho*I/*Sac*I fragment into the *Xho*I/*Sac*I sites of pDD62, yielding pDD62 (*avrBs2:cya*). To make the C-terminal deletion constructs pDD62 (*avrBs2*₁₋₉₇:*cya*), pDD62 (*avrBs2*₁₋₅₈:*cya*), pDD62 (*avrBs2*₁₋₄₁:*cya*), pDD62 (*avrBs2*₁₋₂₈:*cya*), and pDD62 (*avrBs2*₁₋₁₈:*cya*), we recloned *cya* from pMS107 (*cya*) cut with *Bgl*III and *Sac*I, and cloned into the *Bam*HI/*Sac*I sites of pUC19. A *Hind*III/*Sac*I *cya* fragment from pUC19(*cya*) was used to replace the *avrRpt2*₈₀₋₂₅₅ *Hind*III/*Sac*I fragments of pDD62 (*avrBs2*₁₋₉₇+*avrRpt2*₈₀₋₂₅₅), pDD62 (*avrBs2*₁₋₅₈+*avrRpt2*₈₀₋₂₅₅), pDD62 (*avrBs2*₁₋₄₁+*avrRpt2*₈₀₋₂₅₅), pDD62 (*avrBs2*₁₋₂₈+*avrRpt2*₈₀₋₂₅₅), and pDD62 (*avrBs2*₁₋₁₈+*avrRpt2*₈₀₋₂₅₅) (10). pDD62 (*avrBs2*₁₈₋₇₁₄:*cya*) was constructed by replacing the N-terminal *Bam*HI and *Hind*III fragment of pDD62 (*avrBs2:cya*) with the N-terminal *Bam*HI and *Hind*III fragments of pDD62 (*avrBs2*₁₈₋₇₁₄+*avrRpt2*₈₀₋₂₅₅) (15).

For chromosomal gene exchanges, vector pLVC18 (DNA Plant Technology, Oakland, CA) was used. pLVC18 is a derivative of pBR322 with a 1.8-Kb *mob*+ fragment of pRSF1010 cloned into the *Pvu*II site. To fuse *Cya* to the chromosomal copy of *avrBs2*, we cloned the *Eco*RI fragment from pDD62 (*avrBs2:cya*) (codons 74–714) fused to *cya*, into the *Eco*RI site of pLVC18, and the vector was conjugated into *Xcv* strains 85-10 and 85* by triparental mating. Single crossover events were selected on 10 µg/ml of tetracycline. Immunoblot analysis verified that strains 85-10(*avrBs2:cya*) and 85*(*avrBs2:cya*) expressed the expected 126-kDa fusion protein (data not shown).

To construct pTOPO(*hrpF*), a 2,565-bp fragment (beginning 140 bp N-terminal to the start site) was PCR-amplified from *Xcv* 85-10 genomic DNA with *Xba*I restriction sites on each end and cloned into pCR 2.1-Topo (Invitrogen). pSVP61 (*hrpF*) was made by cloning the 2,565-Kb *Xba*I *hrpF* fragment (C,T partially filled in) into the pSVP61 *Hind*III site (G,A partially filled in) behind the *Lac* promoter.

To construct the 85* Δ *hrpF* mutant, a 700-bp *Eco*RI-*Xho*I fragment of the *hrpF* ORF was cloned from pSVP61(*hrpF*) into the *Eco*RI and *Xho*I sites of the pBlueKS(+) vector pBKS+*hrpF*(0.7). The *hrpF*(0.7) fragment was cut back out with *Kpn*I and *Xba*I, and cloned into the *Kpn*I and *Xba*I sites of pLVC18L to make pLVC18L*hrpF*(0.7). Vector pLVC18L is vector pLVC18 with an additional polylinker from the puc19-polylinker (sites *Eco*RI to *Pst*I). pLVC18L*hrpF*(0.7) was conjugated into strain 85*. Single crossover events between chromosomal DNA and vector *hrpF* 700-bp *Eco*RI-*Xho*I piece of DNA resulted in disruption of the chromosomal *hrpF* gene and were selected by tetracycline resistance. The selected colonies were confirmed to have a *hrp*(–) phenotype. To confirm that the *hrp*(–) phenotype resulted from the disruption of *hrpF*, pSVP61 (*hrpF*) was conjugated into the 85* Δ *hrpF* mutant and tested for complementation to wild-type activity.

Plant Growth and Inoculation. Pepper cultivars ECW-0 (*bs2/bs2*) and the nearly isogenic line ECW-20R (*Bs2/Bs2*) (32) were grown in a greenhouse. The upper fully expanded leaves of 2-month-old plants were hand-infiltrated with a 10⁸ colony-forming unit per ml bacterial suspension with a 1-ml syringe. After infiltrations, plants were kept in continuous low light, and leaf samples were harvested at various times and stored at –80°C for cAMP and protein analyses.

Cya Activity Assay and cAMP Extraction. *Xcv* cells were grown on nutrient yeast glucose agar plates, resuspended to an OD₆₀₀ of 2.0 in sonication buffer (3.3 mM Tris·HCl, pH 7.4; 0.75 M sucrose), and sonicated on ice 3 times for 30 sec (Branson

sonifier at 50% power). An aliquot of 20 µl was suspended in 79 µl of reaction buffer (final concentrations: 50 mM Tris·HCl, pH 8; 2 mM ATP; 6 mM MgCl₂; 100 µg/ml BSA; 0.12 mM CaCl) (21). As indicated, 1 µl of bovine calmodulin (Sigma, 100 mM stock) or water was added to the reaction mix. The reaction was allowed to proceed for 5 min at 30°C and was stopped by the addition of 650 µl of HClO₄ (1.1 M). After centrifugation, a 670-µl aliquot of the supernatant was neutralized with 90 µl of K₂CO₃ (6 M). The salt was removed by pelleting in an Eppendorf microfuge for 10 min. Two 100-µl aliquots of the resulting supernatant were transferred to new tubes and dried in a heated speed-vac. Samples were stored frozen at –80°C until assayed for cAMP. The cAMP levels were measured with a cAMP enzyme immunoassay kit (Biotrak, Amersham Pharmacia Biotech) according to the manufacturer's instruction and expressed as nmol of cAMP per mg of total protein.

For the isolation of cAMP from plants (modified after ref. 33), two leaf discs (0.5 cm² each) were collected and ground in liquid nitrogen. After evaporation of the liquid nitrogen, leaves were further ground in 325 µl of HClO₄ (1.1 M). After centrifugation, the pellet was saved for protein concentration determination [a modified Lowry procedure (34)]. Then 280 µl of the supernatant was neutralized in 37.3 µl of K₂CO₃ (6 M). The salt was removed by pelleting in an Eppendorf microfuge for 10 min. Two 100-µl aliquots of the resulting supernatant were transferred into new tubes and dried in a heated speed-vac. Samples were stored frozen at –80°C until assayed for cAMP.

Secretion Assay. The secretion of AvrBs2:Cya fusion protein by *Xcv* strains 85* pDD62 (*avrBs2:cya*), 85* Δ *hrpF* pDD62 (*avrBs2:cya*), and 85* Δ *hrpF* pDD62 (*avrBs2:cya*) into culture media was detected by immunoblot analysis of trichloroacetic acid (TCA) protein-precipitated culture filtrates as described (15).

SDS/PAGE Gels and Immunoblot Analysis. *Xcv* cells were grown on nutrient yeast glucose agar plates and resuspended to an OD₆₀₀ of 2.0 in 100 mM MgCl₂. A 50-µl aliquot was mixed with 50 µl 3 times Laemmli buffer (35), boiled for 5 min, and centrifuged at 14,000 × g for 10 min. Fifteen microliter of the supernatant was loaded on 8% SDS/PAGE gels. Proteins were transferred onto nitrocellulose membranes and detected with polyclonal rabbit antiserum raised against AvrBs2 (30) and NPT II (obtained from 5 Prime → 3 Prime), and monoclonal mouse antiserum against adenylate cyclase (courtesy of N. Giuso, Institut Pasteur, Paris). Membranes were incubated with peroxidase-conjugated secondary Ab and proteins were visualized by chemiluminescence.

Results

Avirulence Activity of the AvrBs2:Cya Fusion. To determine whether *Cya* could be used as reporter protein without altering the avirulence activity of *avrBs2*, a translational C-terminal fusion of the *Cya* protein to the full-length AvrBs2 protein was constructed. The plasmid pDD62(*avrBs2:cya*), which encodes this chimeric protein, was conjugated into *Xcv* strain GM98-38 and was tested for *avrBs2* activity (30). The phenotype of this exconjugant and GM98-38 pDD62 (*avrBs2:HA*) showed a *Bs2*-specific hypersensitive cell death response (HR) when inoculated on the *Bs2*-resistance pepper cultivar (ECW-20R) (Fig. 1A). These results indicated that the AvrBs2:Cya fusion protein fully complemented *avrBs2* activity (Fig. 1B).

Immunoblot analysis of proteins from whole-cell lysates from the two *Xcv* strains, 85* and GM98-38 expressing AvrBs2:Cya, were probed with either the AvrBs2 polyclonal Ab or monoclonal adenylate cyclase antisera (Fig. 1C). As shown in Fig. 1C, only the *Xcv* strains that express *avrBs2:cya* contain the expected 126-kDa hybridizing band with both antisera (lanes 2 and 4). The *Xcv* strain 85* (lane 1) has a genomic wild-type copy of *avrBs2* and has the appropriate 80-kDa hybridizing band when probed

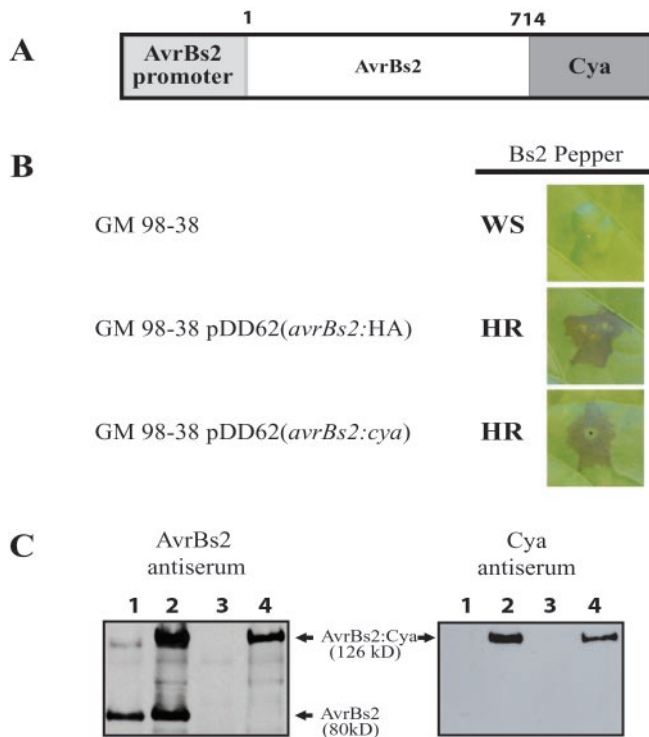


Fig. 1. Biological activity of the AvrBs2:Cya fusion protein. (A) Schematic of chimeric AvrBs2:Cya fusion protein construct shows the 180-bp promoter region of *avrBs2* with the full-length codons (1–714) fused to Cya as a translational fusion. (B) *Xcv* strain GM98-38 (no *avrBs2* activity) with *avrBs2* fusion constructs showing *avrBs2*-dependent cell death on Bs2-resistant pepper (ECW-20R). Leaves were inoculated with a 5×10^8 colony-forming units per ml suspension of bacteria and then photographed 48 h p.i. WS, water-soaked lesions; HR, hypersensitive resistance. (C) Immunoblot analysis of cellular lysate from *Xcv* expressing AvrBs2:Cya with either α -AvrBs2 polyclonal Ab or monoclonal mouse antiserum against adenylate cyclase. Lane 1, 85* (*hrpG* mutant with constitutive *hrp* expression and a wild-type copy of *avrBs2*); lane 2, 85* pDD62(*avrBs2:cya*) (85* also expressing AvrBs2:Cya); lane 3, GM98-38 (mutant for *avrBs2*); lane 4, GM98-38 pDD62(*avrBs2:cya*).

with the AvrBs2 polyclonal Ab. The 80-kD band is missing in strain GM 98-38 (lane 3), which lacks the genomic copy of *avrBs2*.

Type III-Dependent Secretion of AvrBs2:Cya Fusion Protein. Once it was established that the AvrBs2:Cya fusions were capable of eliciting a Bs2-specific resistance reaction, we tested whether these proteins were secreted *in vitro* in a TTSS-dependent manner. To this end, we used the *Xcv* 85* strain containing the AvrBs2:Cya fusion and the previously characterized *hrp* mutation, *hrcV*. Immunoblot analyses revealed that AvrBs2:Cya fusion protein was present in whole-cell lysates from strains of *Xcv* 85* and 85* Δ *hrcV* (Fig. 2A). As predicted, *Xcv* 85* secreted the AvrBs2:Cya fusion protein into the culture media, whereas the negative control strain, 85* Δ *hrcV*, failed to secrete the protein into the culture medium (Fig. 2A).

Furthermore, we constructed an additional control strain 85* Δ *hrpF*, which contained the *avrBs2:cya* fusion. The inclusion of this strain served as an important control as it is unable to elicit a Bs2-specific HR response when inoculated into pepper plants (data not shown), but is still capable of secretion (Fig. 2A). A plasmid-borne copy of the wild-type *hrpF* gene pSVP61(*hrpF*) was introduced into 85* Δ *hrpF* and the strain was complemented for HR-inducing activity (data not shown).

Calmodulin-Dependent Adenylate Cyclase Activity of the AvrBs2:Cya Fusion Protein Expressed in Bacteria. Because the AvrBs2:Cya fusion should only be active in the presence of plant calmodulin,

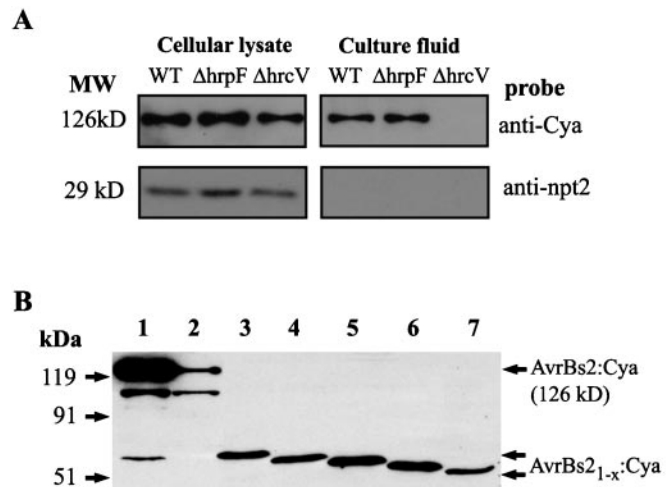


Fig. 2. Type III-dependent secretion of AvrBs2:Cya protein from *Xcv*. (A) Immunoblot analysis of AvrBs2:Cya protein and NPT II protein in cellular lysates and proteins secreted into culture fluids isolated from *Xcv* strains expressing AvrBs2:Cya protein (126 kD) and NPT II protein (29-kDa nonsecreted control). Lane WT, wild-type 85* secretion strain; lane Δ *hrpF*, strain 85* with an *hrpF* mutation; lane Δ *hrcV*, strain 85* with a TTSS mutation. (B) Immunoblot analysis of AvrBs2 translocation domains with Cya fusion reporter. Equally loaded bacterial lysates of *Xcv* strain 85* expressing AvrBs2 or various AvrBs2 deletions as translational fusions with Cya. Lane 1, AvrBs2₁₋₇₁₄:Cya; lane 2, AvrBs2₁₈₋₇₁₄:Cya; lane 3, AvrBs2₁₋₉₈:Cya; lane 4, AvrBs2₁₋₅₈:Cya; lane 5, AvrBs2₁₋₄₁:Cya; lane 6, AvrBs2₁₋₂₈:Cya; lane 7, AvrBs2₁₋₁₈:Cya. Immunoblot was probed with monoclonal mouse antiserum against adenylate cyclase.

we determined whether the AvrBs2:Cya fusion protein was biochemically active with respect to production of cAMP when expressed in bacteria. Adenylate cyclase activity in total bacterial protein extracts was assayed to test the enzymatic activity of the AvrBs2:Cya fusion protein and to demonstrate that it was possible to activate the enzyme by the addition of eukaryotic calmodulin *in vitro*. Bacterial extracts containing the AvrBs2:Cya fusion protein had negligible cAMP activity in the absence of calmodulin, whereas addition of exogenous calmodulin resulted in significant increases in cAMP levels (Table 1). The calmodulin-dependent increase in *in vitro* Cya activity was comparable to that reported in *Yersinia* strains expressing a YopE:Cya fusion (21), although the total amount of *in vitro* Cya activity in *Xcv* was slightly lower. These experiments confirmed the eukaryotic calmodulin-dependent nature of the activity of the fusion protein expressed in *Xcv* and the measurement of the *in planta* translocation of the AvrBs2:Cya fusion protein.

Translocation of the AvrBs2:Cya Fusion Protein into the Plant Cell. To determine whether AvrBs2:Cya is translocated into the plant cytosol and reacts with plant-produced calmodulin, we inoculated pepper plants with *Xcv* strains expressing the AvrBs2:Cya fusion protein. We predicted that if the fusion protein was translocated into the plant cytosol, Cya would be active in the presence of plant-produced calmodulin and increased levels of adenylate cyclase activity could be measured. Table 1 depicts a large increase in cAMP levels in plants (adenylate cyclase activity *in planta*) 24 h after inoculation with *Xcv* strain 85* carrying pDD62 (*avrBs2:cya*) compared with a control strain with no AvrBs2:Cya. In addition, there was no increase in adenylate cyclase activity *in planta* with either *Xcv* strain 85* Δ *hrcV*, a secretion-defective strain, or 85* Δ *hrpF*, a translocation-defective mutant. The lack of Cya activity in 85* Δ *hrpF* is especially significant as this result rules out the possibility that

Table 1. Calmodulin-dependent adenylate cyclase activity of AvrBs2:Cya fusion protein expressed *in vitro* and *in planta*

Xcv strain	AvrBs2 protein [†]	<i>in vitro</i> Cya activity cAMP [§] (nmol/mg total proteins)		<i>in planta</i> Cya activity cAMP [§] (nmol/mg total proteins)
		– Calmodulin	+ Calmodulin	Time (24 h p.i.)
	Mature protein			
85*	1-714-HA [‡]	0.6 ± 0.6 [¶]	0.6 ± 0.4	0.02 ± 0.006
85*	1-714-Cya	0.9 ± 1.1	917 ± 196	38.0 ± 0.9
85*ΔhrpF	1-714-Cya	0.6 ± 0.6	1,340 ± 60	0.02 ± 0.002
85*ΔhrcV	1-714-Cya	0.9 ± 1.0	941 ± 23	0.03 ± 0.002
	N-terminal deletions			
85*	18-714-Cya		340	0.4 ± 0.2
	C-terminal deletions			
85*	1-98-Cya		580	43.9 ± 1.6
85*	1-58-Cya		529	38.6 ± 1.2
85*	1-41-Cya		850	5.7 ± 3.2
85*	1-28-Cya		910	0.9 ± 0.19
85*	1-18-Cya		334	0.3 ± 0.17

[†]Codon numbering is based on predicted protein for ORF1 (30).

[‡]HA, hemagglutinin epitope.

[§]Adenylate cyclase activity *in vitro* (nmol cAMP per mg total protein) in *Xcv*-sonicated cellular lysates with and without exogenous calmodulin. Adenylate cyclase activity *in planta* (nmol cAMP per mg total protein) pepper (ECW) inoculated with *Xcv* and sampled 24 h p.i. All AvrBs2 fusion proteins are expressed in plasmid pDD62.

[¶]Each measurement has three replications that are averaged with SDs represented.

the reporter protein can be first secreted and then delivered to the plant cell.

Analysis of the AvrBs2 Translocation Domains on the Delivery of Cya to Plant Cells. To compare the quantitative sensitivity of the Cya reporter to the published AvrRpt2 reporter protein (15), we exchanged the AvrRpt2 reporter with Cya on a number of AvrBs2 deletions that define the *in planta* secretion and translocation domains of AvrBs2 (15). Immunoblot analyses of whole-cell lysates from these *Xcv* strains showed the expected sizes of these fusion proteins (Fig. 2B). As shown in Table 1, the calmodulin-dependent *in vitro* Cya activity measurements of bacterial extracts expressing these fusion proteins was reduced compared with the expression of full-length AvrBs2:Cya fusion protein.

To measure increases of *in planta* Cya activity of the various constructs, leaf samples were taken 24 h after inoculation and assayed for cAMP levels. Table 1 shows the results of the inoculation of pepper plants with *Xcv* strains expressing AvrBs2₁₋₉₈:Cya and AvrBs2₁₋₅₈:Cya fusions. These constructs resulted in increased *in planta* cAMP accumulation (similar to the expression level of full-length AvrBs2:Cya) in plants and were comparable to previously reported experiments for the translocation of the AvrRpt2 reporter. Infections of pepper plants with *Xcv* strains expressing progressively smaller truncated proteins, AvrBs2₁₋₄₁:Cya, AvrBs2₁₋₂₈:Cya, and AvrBs2₁₋₁₈:Cya resulted in significantly less cAMP accumulation in plants. The AvrBs2₁₋₁₈:Cya and AvrBs2₁₋₂₈:Cya fusions were inactive *in planta*, but the AvrBs2₁₋₄₁:Cya fusion had low activity (Table 1). As reported (15), these three C-terminal deletions, when fused to the AvrRpt2₂₈₀₋₂₅₅ reporter, were not able to translocate high enough levels to elicit the AvrRpt2-dependent cell death. Thus, the AvrBs2:Cya reporter protein seems to be a more sensitive assay in the case of AvrBs2₁₋₄₁:Cya fusion.

Quantitative Changes in Early Type III Translocation Kinetics. The development of a sensitive biochemical reporter to assay translocation of bacterial effector proteins to plant cells prompted us to investigate the early translocation kinetics of AvrBs2:Cya. To carry out these experiments, we initially characterized *Xcv*

strains expressing different levels of AvrBs2:Cya fusion protein based on whether the gene was expressed in an HrpG* or wild-type background and whether the gene was expressed from a plasmid or as a single chromosomal copy. Immunoblot analyses of three *Xcv* strains with different expression levels of the AvrBs2:Cya fusion protein all contained the predicted 126-kDa-size fusion protein (data not shown). *Xcv* 85* pDD62(*avrBs2:cya*) contained the highest level of protein expression and *Xcv* 85*(*avrBs2:cya*) expressed an intermediate level, whereas 85-10(*avrBs2:cya*) expressed the lowest level (data not shown). Furthermore, the levels of protein expression correlated with the level of adenylate cyclase activity of these strains in the *in vitro* cAMP assay (Table 2).

To determine the kinetics of AvrBs2:Cya translocation in the early stages of plant infection, Cya activity was assayed over an 8-h period at 0, 3, 6, and 8 h post inoculation (p.i.). The kinetic analysis was limited to 8 h p.i. to minimize the effects of *in planta* bacterial growth and to precede the visual symptoms associated with hypersensitive cell death. At the 8-h time point there was no significant increase in bacterial growth in any strains used. A comparison between the 85*(*avrBs2:cya*) and 85*pDD62(*avrBs2:cya*) strains revealed that the *in planta* Cya activity can be detected as early as 3 h p.i. and rapidly increases to a high level within 8 h (Fig. 3). Although there is a significant difference in

Table 2. Effect of gene copy no. on adenylate cyclase activity *in vitro* (nmol cAMP per mg total proteins) in *Xcv*-sonicated cellular lysates with and without exogenous calmodulin

Xcv strain	Plasmid construct	<i>in vitro</i> Cya activity cAMP (nmol/mg total proteins)	
		– Calmodulin	+ Calmodulin
85*	pDD62 (<i>avrBs2</i> :HA)	0.6 ± 0.6 [†]	0.6 ± 0.4
85*	pDD62(<i>avrBs2:cya</i>)	0.9 ± 1.1	917 ± 196
85*(<i>avrBs2:cya</i>)	—	0.7 ± 1.0	120 ± 27
85-10(<i>avrBs2:cya</i>)	—	0.9 ± 1.0	11.4 ± 4.3

HA, hemagglutinin.

[†]Each measurement has three replications that are averaged with SDs represented.

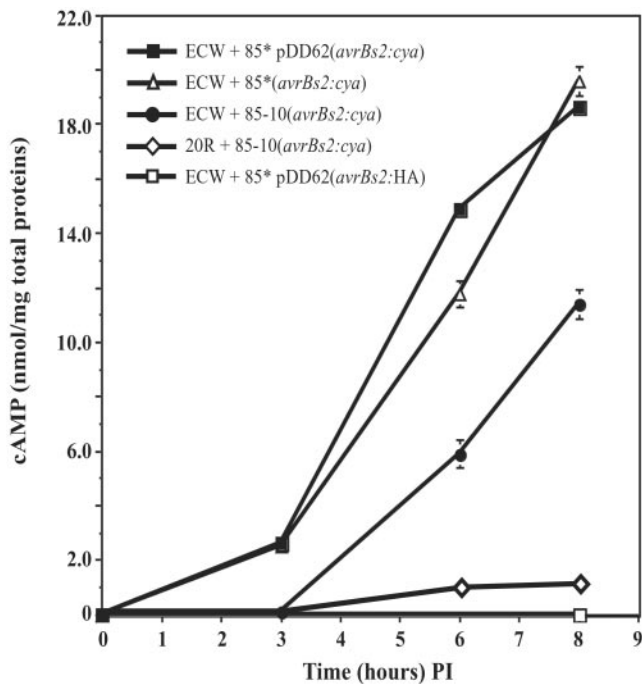


Fig. 3. Kinetic analysis of *in planta* adenylate cyclase activity for different strains of *Xcv* inoculated into pepper plants. Leaf samples (1 cm²) were taken at times 0, 3, 6, and 8 h p.i. with a 5×10^8 cell per ml suspension of bacteria. Each point represents three replications that are averaged with SDs represented by error bars.

the calmodulin-reconstituted *in vitro* activity between these two strains, there seems to be little effect on whether the reporter protein is expressed from a single chromosomal copy [85*(*avrBs2:cya*)] or a from a multiple-copy plasmid [85*pDD62(*avrBs2:cya*)]. Finally, it is also apparent that the 85-10(*avrBs2:cya*) strain, which lacks the HrpG* mutation, is delayed in its ability to translocate the reporter protein, as *in planta* activity can only be slightly detected after 6 h p.i. However, these levels may more closely reflect natural infections and the time it takes to synthesize the secretion apparatus encoded by the Hrp loci. Thus, we used this strain to compare the effect of resistant host genotype on effector protein delivery.

Xcv strain 85-10(*avrBs2:cya*) was inoculated on the resistant pepper ECW-20R (Bs2/Bs2) to observe the effect of an incompatible host-pathogen interaction on the delivery of AvrBs2:Cya to plant cells. Interestingly, the translocation of AvrBs2:Cya is significantly inhibited in the ECW-20R pepper plants at 8 h p.i. (Fig. 3).

Discussion

The work described in this article provides biochemical evidence for TTSS-dependent translocation of a bacterial Avr effector protein from a phytopathogenic bacterium into the cytosol of plant cells. We fused the calmodulin-activated adenylate cyclase domain (Cya) of *B. pertussis* cyclolysin (21) to AvrBs2 and expressed this construct either from the bacterial chromosome or *in trans* from a broad host-range vector. Inside bacteria, no calmodulin is available and Cya has little activity (Table 1). However, after translocation of AvrBs2:Cya into plant cells, the eukaryotic calmodulin activates the adenylate cyclase, and cAMP levels inside the plants increase significantly.

Translocation of AvrBs2 depends on the TTSS of *Xcv*. A mutant deficient in *hrcV*, an inner membrane protein of the type III secretion protein complex (29), did not secrete AvrBs2:Cya, and pepper infections with this strain did not result in increased cAMP levels in the plant. In accordance with this result,

an N-terminal truncation of AvrBs2:Cya lacking the previously identified type III translocation signal (15) of AvrBs2 (AvrBs2₁₈₋₇₁₄:Cya) did not result in increased cAMP levels when assayed *in planta* (Table 1).

To ensure that accumulation of cAMP in plants depends not only on secretion of AvrBs2:cya into the plant apoplast but also on translocation into plant cells, we used a bacterial mutant that lacks *hrpF*, a putative translocator of the type III export mechanism (29). The mutant bacteria secreted the fusion protein into induction medium but failed to increase cAMP levels in plant cells, suggesting that it was inhibited in type III translocation.

In addition, we fused the adenylate cyclase reporter to C-terminal truncations of AvrBs2 that lacked the defined translocation signal sequence between amino acids 41 and 58 (15). The AvrBs2₁₋₄₁:Cya fusion construct resulted in reduced adenylate cyclase activity in plant cells, whereas the AvrBs2₁₋₅₈:Cya fusion was similar to a full-length AvrBs2 fusion (Table 1). These results suggest that AvrBs2 has two N-terminal secretion and translocation signals: one within the first 18 amino acids, and a second signal between amino acids 41 and 58. The first signal is absolutely required for secretion (15) and the second signal enhances translocation. The existence of two translocation signals has also been observed in the type III-secreted *Yersinia* effector YopE. The first YopE signal is located in the 15 N-terminal amino acids. However, there is controversy as to whether it resides in the mRNA (36) or in the protein (37). The second signal is located between amino acid residues 15–100 and requires binding of the chaperone SycE for protein stability and type III secretion (38).

Our experiments showed that cAMP accumulation was significantly lower in plants that are resistant to AvrBs2-expressing *Xcv* strains compared with susceptible cultivars. There was no difference in bacterial growth on resistant or susceptible plants during the duration of the experiment. An HR-related general protein breakdown may reduce the amount of the fusion protein, although Boyes *et al.* (39) have shown that a general protein breakdown only occurs well after the visible HR-related tissue collapse. Indeed, preliminary results suggest that the decrease in the adenylate cyclase activity of AvrBs2:Cya is specifically related to the resistance response mediated by the AvrBs2 effector protein and the Bs2 plant-resistance gene product. This decrease does not seem to involve a specific AvrBs2:Cya breakdown, and it is therefore possible that AvrBs2-induced plant resistance mechanisms interfere directly with type III-dependent translocation of effector proteins into plant cells. As our results indicate, such interference seems to occur within hours of infection of resistant pepper cultivars. Alternatively, it is also possible that calmodulin levels may rapidly decrease in a Bs2-specified resistance response or that the AvrBs2:Cya fusion protein may be sequestered into complexes that are not mutually accessible.

The adenylate cyclase reporter protein will have wide utility to establish further early events after pathogen infection. Preliminary results in our laboratory have demonstrated that chimeric fusions of Cya with the translocation signals of either AvrRpt2 and AvrRpm1 result in accumulation of cAMP *in planta* when delivered from *P. syringae* (M. Mudgett, D.D., and B.J.S., unpublished observations). Finally, fusions to other effector proteins will be instrumental to dissect specific and common responses of the numerous resistance pathways that are mediated by bacterial Avr genes and the corresponding plant-resistance genes.

We thank Christina Morales for technical assistance in the PCR-cloning of *hrpF*. We are thankful to Dr. Nicole Giuso for the generous gift of adenylate cyclase antibody. We also thank Dr. Ulla Bonas for use of the various published *hrp* strains of *Xcv*. We thank Mary Beth Mudgett, Stephen Chisholm, Mike Axtell, Brad Day, Todd Leister, and other members of the Staskawicz lab for helpful suggestions and critical reading of the manuscript. This work was supported by the Department of Energy and the Torrey Mesa Research Institute, Syngenta Research and Technology, San Diego.

1. Anderson, D. M., Derrick, E. F., Collmer, A. & Schneewind, O. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 12839–12843.
2. Galán, J. E. & Collmer, A. (1999) *Science* **284**, 1322–1327.
3. Cornelis, G. R. & Van Gijsegem, F. (2000) *Annu. Rev. Microbiol.* **54**, 735–774.
4. Gopalan, S., Bauer, D. W., Alfano, J. R., Loniello, A. O., He, S. Y. & Collmer, A. (1996) *Plant Cell* **8**, 1095–1105.
5. Leister, R. T., Ausubel, F. M. & Katagiri, F. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 15497–15502.
6. Tang, X., Frederick, R. D., Zhou, J., Halterman, D. A., Jia, Y. & Martin, G. B. (1996) *Science* **274**, 2060–2063.
7. Scofield, S. R., Tobias, C. M., Rathjen, J. P., Chang, J. H., Lavelle, D. T., Micheltore, R. W. & Staskawicz, B. J. (1996) *Science* **274**, 2063–2065.
8. Bonas, U. & Van den Ackerveken, G. (1997) *Plant J.* **12**, 1–7.
9. McNellis, T. W., Mudgett, M. B., Li, K., Aoyama, T., Horvath, D., Chua, N.-H. & Staskawicz, B. J. (1998) *Plant J.* **14**, 247–257.
10. Kjemtrup, S., Nimchuk, Z. & Dangl, J. L. (2000) *Curr. Opin. Microbiol.* **3**, 73–78.
11. Mudgett, M. B. & Staskawicz, B. J. (1998) *Curr. Opin. Microbiol.* **1**, 109–114.
12. Bonas, U., Schulte, R., Fenselau, S., Minsavage, G. V. & Staskawicz, B. (1991) *Mol. Plant–Microbe Interact.* **4**, 81–88.
13. Rossier, O., Wengelnik, K., Hahn, K. & Bonas, U. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 9368–9373.
14. Jin, Q. & He, S.-Y. (2001) *Science* **294**, 2556–2558.
15. Mudgett, M. B., Chesnokova, O., Dahlbeck, D., Clark, E. T., Rossier, O., Bonas, U. & Staskawicz, B. J. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 13324–13329.
16. Mudgett, M. B. & Staskawicz, B. J. (1999) *Mol. Microbiol.* **32**, 927–941.
17. Van Dijk, K., Fouts, D. E., Rehm, D. E., Hill, A. H., Collmer, A. & Alfano, J. R. (1999) *J. Bacteriol.* **181**, 4790–4797.
18. Van Gijsegem, F., Vasse, J., Camus, J.-C., Marendra, M. & Boucher, C. (2000) *Mol. Microbiol.* **36**, 249–260.
19. Wei, Z., Kim, J. F. & Beer, S. V. (2000) *Mol. Plant–Microbe Interact.* **13**, 1251–1262.
20. Collmer, A., Badel, J. L., Charkowski, A. O., Deng, W.-D., Fouts, D. E., Ramos, A. R., Rehm, A. H., Anderson, D. M., Schneewind, O., van Dijk, K. & Alfano, J. R. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 8770–8777.
21. Sory, M. P. & Cornelis, G. R. (1994) *Mol. Microbiol.* **14**, 583–594.
22. Sory, M.-P., Boland, A., Lambermont, I. & Cornelis, G. R. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 11998–12002.
23. Jones, M. A., Wood, M. W., Mullan, P. B., Watson, P. R., Wallis, T. S. & Galyov, E. E. (1998) *Infect. Immun.* **66**, 5799–5804.
24. Rosqvist, R., Magnusson, K.-E. & Wolf-Watz, H. (1994) *EMBO J.* **13**, 964–972.
25. Jacobi, C. A., Roggenkamp, A., Rakin, A., Zumbihl, R., Leitritz, L. & Heesemann, J. (1998) *Mol. Microbiol.* **30**, 865–882.
26. Subtil, A., Parsot, C. & Dautry-Varsat, A. (2001) *Mol. Microbiol.* **39**, 792–800.
27. Bonas, U., Stall, R. E. & Staskawicz, B. (1989) *Mol. Gen. Genet.* **218**, 127–136.
28. Wengelnik, K., Rossier, O. & Bonas, U. (1999) *J. Bacteriol.* **181**, 6828–6831.
29. Rossier, O., Van den Ackerveken, G. & Bonas, U. (2000) *Mol. Microbiol.* **38**, 828–838.
30. Gassmann, W., Dahlbeck, D., Chesnokova, O., Minsavage, G. V., Jones, J. B. & Staskawicz, B. J. (2000) *J. Bacteriol.* **182**, 7053–7059.
31. Swords, K. M. M., Dahlbeck, D., Kearney, B., Roy, M. & Staskawicz, B. J. (1996) *J. Bacteriol.* **178**, 4661–4669.
32. Minsavage, G. V., Dahlbeck, D., Whalen, M. C. & Kearney, B. (1990) *Mol. Plant–Microbe Interact.* **3**, 41–47.
33. Legendre, L., Derckel, J. P., Wrisez, F., Correze, C., Audran, J. C., Haye, B. & Lambert, B. (1997) *Phytochemistry* **44**, 769–774.
34. Bailey, J. L. (1967) *Techniques in Protein Chemistry* (Elsevier, New York).
35. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
36. Cheng, L. W. & Schneewind, O. (1999) *J. Biol. Chem.* **274**, 22102–22108.
37. Lloyd, S. A., Norman, M., Rosqvist, R. & Wolf-Watz, H. (2001) *Mol. Microbiol.* **39**, 520–531.
38. Cheng, L. W., Anderson, D. M. & Schneewind, O. (1997) *Mol. Microbiol.* **24**, 757–765.
39. Boyes, D. C., Nam, J. & Dangl, J. L. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 15849–15854.