Pseudomonas syringae Type III Secretion System Targeting Signals and Novel Effectors Studied with a Cya Translocation Reporter

Lisa M. Schechter,[1] Kathy A. Roberts,[1] Yashitola Jamir,[2] James R. Alfano,[2] and Alan Collmer[1]*

Department of Plant Pathology, Cornell University, Ithaca, New York 14853-4203,[1] and The Plant Science Initiative and Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska 68588-0660[2]

Pseudomonas syringae pv. tomato strain DC3000 is a pathogen of tomato and *Arabidopsis*. The *hrp-hrc*-encoded type III secretion system (TTSS), which injects bacterial effector proteins (primarily called Hop or Avr proteins) into plant cells, is required for pathogenicity. In addition to being regulated by the HrpL alternative sigma factor, most *avr* or *hop* genes encode proteins with N termini that have several characteristic features, including (i) a high percentage of Ser residues, (ii) an aliphatic amino acid (Ile, Leu, or Val) or Pro at the third or fourth position, and (iii) a lack of negatively charged amino acids within the first 12 residues. Here, the well-studied effector AvrPto was used to optimize a calmodulin-dependent adenylate cyclase (Cya) reporter system for Hrp-mediated translocation of *P. syringae* TTSS effectors into plant cells. This system includes a cloned *P. syringae hrp* gene cluster and the model plant *Nicotiana benthamiana*. Analyses of truncated AvrPto proteins fused to Cya revealed that the N-terminal 16 amino acids and/or codons of AvrPto are sufficient to direct weak translocation into plant cells and that longer N-terminal fragments direct progressively stronger translocation. AvrB, tested because it is poorly secreted in cultures by the *P. syringae* Hrp system, was translocated into plant cells as effectively as AvrPto. The translocation of several DC3000 candidate Hop proteins was also examined by using Cya as a reporter, which led to identification of three new intact Hop proteins, designated HopPtoQ, HopPtoT1, and HopPtoV, as well as two truncated Hop proteins encoded by the naturally disrupted genes *hopPtoS4::tnpA* and *hopPtoAG::tnpA*. We also confirmed that HopPtoK, HopPtoC, and AvrPphE_{Pto} are translocated into plant cells. These results increased the number of Hrp system-secreted proteins in DC3000 to 40. Although most of the newly identified Hop proteins possess N termini that have the same features as the N termini of previously described Hop proteins, HopPtoV has none of these characteristics. Our results indicate that Cya should be a useful reporter for exploring multiple aspects of the Hrp system in *P. syringae*.

Pseudomonas syringae is a host-specific bacterial pathogen of plants whose various pathovars cause necrotic lesions on leaves or fruits of susceptible plants and elicit the hypersensitive response (HR), a defense-associated localized programmed cell death, in resistant plants. *P. syringae* interactions with plants depend on the *hrp* (for "hypersensitive response and pathogenicity") gene cluster, which encodes a type III secretion system (TTSS) or Hrp system that injects bacterial proteins into plant cells (1). Similar TTSSs exist in a variety of other gram-negative plant and animal pathogens (13, 28). The *P. syringae* proteins injected into host cells by the Hrp system are primarily known as effectors or Avr (avirulence) or Hop (Hrp-dependent outer protein) proteins. Hop proteins may contribute to *P. syringae* virulence by suppressing plant immunity. For example, AvrB, AvrRpm1, and AvrRpt2 all interfere with the function of RIN4, a plant protein implicated in the regulation of plant defense responses (3, 36, 37). However, certain plants are able to resist *P. syringae* infection through the actions of R proteins, which detect effectors or their activities (45). For example, the *Arabidopsis* R protein RPM1 triggers the HR when AvrB or AvrRpm1 modifies RIN4 (37).

Like the effectors found in other plant and animal pathogens, *P. syringae* Hop proteins are targeted to the Hrp secretion apparatus by N-terminal sequences (2, 39, 40, 47). The N termini of most Hop proteins do not exhibit significant amino acid homology, and the mechanism by which Hops are targeted to the Hrp system has not been elucidated. Although recent studies of the *Yersinia* effector protein YopE suggest that amphipathic N-terminal amino acid sequences are important for directing this protein to the *Yersinia* TTSS, there is evidence that mRNA sequences additionally contribute to effector targeting (34, 35, 42). Type III chaperones, which are small, acidic proteins that bind to and stabilize specific effectors in the bacterial cytoplasm, also play a role in targeting some effector proteins to the type III secretion apparatus (18).

P. syringae Hop proteins have been identified in a variety of different pathovars and strains, and the complete inventory of these proteins in any single strain is not yet known. However, the recent completion of the genome sequence of *P. syringae* pv. tomato strain DC3000 has facilitated bioinformatic and genetic studies that have led to identification of many Hops in this organism (9, 12, 22). In several recent reports, new Hops were identified on the basis that they have several common characteristics. First, most *hop* genes appear to be regulated by HrpL, an alternative sigma factor that activates the Hrp regulon (4, 30, 46, 50, 51). Because HrpL interacts with *hrp* box (5'-GGAACC-N_{16}-CCACNNA-3') sequences or variants of these sequences, searches were conducted to identify all of the

* Corresponding author. Mailing address: Department of Plant Pathology, Cornell University, 334 Plant Science Building, Ithaca, NY 14853-4203. Phone: (607) 255-7843. Fax: (607) 255-4471. E-mail: arc2@cornell.edu.

potential *hrp* promoters in the DC3000 genome (19, 52). Several open reading frames (ORFs) with homology to known *hop* genes were identified downstream of such promoter sequences, and many of the encoded proteins were confirmed to be secreted by the DC3000 Hrp system (19, 24, 41, 52). Second, the N-terminal regions of most Hops appear to have common characteristics. For example, the first 50 amino acids of known effector proteins have a high serine content compared to the serine contents of a set of random housekeeping proteins in *P. syringae* (24). In addition to the high serine content, the N termini of most Hops are amphipathic, have an aliphatic amino acid (Ile, Val, or Leu) or Pro at the third or fourth position, and have no acidic amino acids (Asp or Glu) in the first 12 amino acids (41). Genomic searches for ORFs with these characteristics have yielded many potential *hop* genes (24, 41). Although some of these ORFs were confirmed to encode Hops in secretion or translocation assays, many of the *hop* candidates have yet to be tested.

Several methods have been used to show that Hop candidate proteins are secreted or translocated by the *P. syringae* Hrp system. Immunoblot analyses have revealed that certain Hop proteins are present in the culture supernatants of *P. syringae* grown in *hrp*-inducing media (40, 49). The drawback of this assay is that it does not distinguish translocated effectors from other proteins that may function as extracellular components of the Hrp secretion apparatus. In addition, certain known Hops, such as AvrB, are secreted poorly by *P. syringae* in culture (11, 49).

P. syringae Hop proteins have also been identified by using the reporter protein ′AvrRpt2. ′AvrRpt2 is the C-terminal functional domain of AvrRpt2, an effector protein that elicits the HR in plants containing the *RPS2* R gene. Although ′AvrRpt2 itself cannot be translocated into plants, the N-terminal secretion signals of other effectors can substitute for the native AvrRpt2 secretion signal (23, 39). Thus, new Hops have been discovered by searching for proteins that, when fused to ′AvrRpt2, allow translocation of the hybrids into plant cells in an *hrp*-dependent manner (24, 52). Two potential problems with this method are that it is not quantitative and that it is formally possible that residual sequences in the ′AvrRpt2 reporter could contribute to translocation and yield false-positive results.

Finally, the calmodulin-dependent adenylate cyclase (Cya) domain of the cyclolysin toxin from *Bordetella pertussis* has also been exploited as a reporter for translocation of effector proteins (32). The Cya reporter was initially used to demonstrate the translocation of YopE into animal cells by *Yersinia enterocolitica* and subsequently was utilized to show that AvrBs2 is delivered into pepper plants by *Xanthomonas campestris* pv. vesicatoria (10, 48). Cya possesses two features that allow it to be a reporter for type III translocation: (i) it is not active in bacterial cytoplasm because bacteria do not possess calmodulin, and (ii) it is not secreted or translocated by the TTSS. However, when the N-terminal portion of an effector is fused to Cya, bacteria can deliver the resulting hybrid protein into the cytosol of host cells, where it can bind to calmodulin and produce cyclic AMP (cAMP) from ATP.

We are interested in identifying the complete set of Hop proteins translocated into plants by *P. syringae* pv. tomato strain DC3000, as well as in gaining a better understanding of

Hop targeting signals and the translocation process. To that end, we optimized Cya as a reporter for *P. syringae* Hrp-mediated translocation using the known Hop proteins AvrPto and AvrB and truncated derivatives of these proteins. We then used Cya to test whether several HrpL-regulated *hop* candidate genes encode proteins that are translocated by the Hrp system into plant cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* TOP10 and DH5α were used as hosts for all cloned plasmids except pCPP3234, which was maintained in *E. coli* DB3.1. *E. coli* was grown in Luria-Bertani or terrific broth at 37°C unless otherwise specified, and *P. syringae* and *Pseudomonas fluorescens* were grown in King's B medium (KB) or *hrp*-derepressing fructose minimal medium (hrpMM) at 30°C (29, 31, 43). For *E. coli* and *P. syringae*, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; rifampin, 50 μg/ml; spectinomycin, 50 μg/ml; and tetracycline, 10 μg/ml. Because *P. fluorescens* 55 is resistant to spectinomycin and low levels of tetracycline, plasmids were maintained in this strain by using 100 μg of streptomycin per ml and 40 μg of tetracycline per ml.

DNA manipulations. Plasmid DNA was isolated and manipulated by using standard methods (43). Restriction enzymes were obtained from New England Biolabs. PCR was performed with either ExTaq (Takara) or Vent (New England Biolabs) polymerase used according to the manufacturer's instructions. DNA sequencing was performed at the Cornell Biotechnology Center with an automated 3700 DNA analyzer (Applied Biosystems).

Construction of plasmids. Plasmids expressing AvrPto-Cya fusion proteins were created in two steps. First, the template pCPP2329 DNA (25), which contained *avrPto* from *P. syringae* pv. tomato JL1065, and the *avrPto* primer pairs listed in Table 2 were used to generate PCR products that were digested with *Xba*I and *Ssp*I and cloned into the *Xba*I and *Sma*I sites of pMJH20. The reulting plasmids were then digested with *Xba*I and *Hin*dIII, and the *avrPto-cya* fragments were cloned into pVLT35.

Other *cya* fusions were created by using the Gateway cloning technology (Invitrogen). In this system, an effector gene contained in an entry clone can be transferred by recombination into a *cya* destination vector, creating an in-frame *cya* gene fusion. Entry clones were constructed by cloning the PCR products obtained with the primer pairs listed in Table 2 (excluding the *avrPto* primers) into the pENTR/SD/D-TOPO vector (Invitrogen) according to the manufacturer's directions. pCPP2327 (21) was used as the template for the *avrB* PCRs, and DC3000 genomic DNA was used as the template for all other reactions. The *cya* destination vector, pCPP3234, was constructed in two steps. First, pMJH20 was digested with *Sac*I and *Hin*dIII, and the *cya*-containing fragment was cloned into pVLT35 to create pCPP3214. The Gateway reading frame B cassette was then cloned into the *Sma*I site of pCPP3214 to create pCPP3234. Plasmids expressing *cya* gene fusions were created by site-specific recombination reactions (or LR reactions) between entry clones and pCPP3234. DNA sequencing confirmed that the effector or effector candidate sequences in all of the *cya* fusion plasmids did not contain any mutations in the 5′ region (~first 100 codons) important for targeting proteins to the *hrp* secretion system.

The *hrcC* deletion in pCPP3297 was constructed by crossover PCR as described by Link et al. (33) by using pLN18 as the template DNA, the upstream primers 5′-CACTCGAGGAAGCCCTGGCATTGATTG-3′ and 5′-GGCAAC CGGGAGTTTGAAGAGAGCCGGCCATCACTG-3′, and the downstream primers 5′-CAGTGATGGCCGGCTCTCTTCAAACTCCCGGTTGCC-3′ and 5′-CACTCGAGACATCGCCAACAGTTTGCTG-3′. The ~2.4-kb Δ*hrcC* crossover PCR fragment was digested with *Xho*I and cloned into pLD55. The resulting plasmid was then digested with *Pst*I, and the Δ*hrcC* fragment was cloned into the temperature-sensitive plasmid pMAK705 (26) to create pCPP3296. pCPP3296 was transformed into *E. coli* MC4100 containing pLN18, and replacement of the wild-type *hrcC* gene with the Δ*hrcC* allele was carried out by sequential temperature shifts as described by Hamilton et al. (26). pLN18 mutants that acquired the Δ*hrcC* mutation were confirmed by PCR and restriction enzyme analysis.

Preparation of protein samples. DC3000 strains carrying plasmids expressing AvrPto-Cya fusions and neomycin phosphotransferase II (NptII) were scraped off KB plates, washed twice in hrpMM, and resuspended in 43 ml of hrpMM containing 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) and appropriate antibiotics. Wild-type and Δ*hrp* mutant bacteria were resuspended at optical

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant phenotype	Source or reference
E. coli strains		
DH5α	F⁻ φ80*lacZ*Δ*M15* Δ*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17 phoA supE44 thi-1 gyrA96 relA1* λ⁻	Invitrogen
TOP10	F⁻ *mcrA* Δ*mrr-hsdRMS-mcrBC*) φ80*lacZ*Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)7679 *galU galK rpsL endA1 nupG*	Invitrogen
DB3.1	F⁻ *gyrA462 endA1* Δ(*sr1-recA*) *mcrB mrr hsdS20 supE44 ara-14 galK2 lacY1 proA2 rpsL20 xyl-5* λ⁻ *leu mtl-1*	Invitrogen
P. syringae pv. tomato strains		
DC3000	Wild type, Rif^r	15
CUCPB5114	DC3000 Δ*hrpK-hrpR*::ΩCm, Rif^r Cm^r	20
P. fluorescens 55	Wild type, Nx^r	27
Plasmids		
pLN18	pLAFR3 derivative containing 25-kb *P. syringae* pv. syringae 61 *hrc-hrp* cluster with *shcA* and *hopPsyA* replaced by an *nptII* cassette, Tc^r Km^r	30a
pCPP3297	pLN18 containing an unmarked deletion in *hrcC*	This study
pUFRO34	Broad-host-range cloning vector expressing NptII, Km^r	16
pVLT35	Broad-host-range cloning vector containing *tac* promoter and *lacI*^{q}, Sp^r/Str^r	17
pMJH20	pWSK29 containing codons 2 to 406 of *cya*, Amp^r	38
pCPP3214	pVLT35 containing codons 2 to 406 of *cya*, Sp^r/Str^r	This study
pCPP3234	pVLT35 containing Gateway reading frame B cassette and codons 2 to 406 of *cya*, Sp^r/Str^r Cm^r	This study
pCPP3221	pVLT35 expressing AvrPto$_{(1-164)}$-Cya	This study
pCPP3222	pVLT35 expressing AvrPto$_{(1-135)}$-Cya	This study
pCPP3223	pVLT35 expressing AvrPto$_{(1-100)}$-Cya	This study
pCPP3224	pVLT35 expressing AvrPto$_{(1-50)}$-Cya	This study
pCPP3225	pVLT35 expressing AvrPto$_{(1-16)}$-Cya	This study
pCPP3236	pVLT35 expressing HopPtoC-Cya	This study
pCPP3271	pVLT35 expressing ShcV-Cya	This study
pCPP3288	pVLT35 expressing AvrB$_{(1-30)}$-Cya	This study
pCPP3290	pVLT35 expressing AvrB$_{(1-321)}$-Cya	This study
pCPP3298	pVLT35 expressing PSPTO4589 and PSPTO4588-Cya	This study
pCPP3299	pVLT35 expressing PSPTOB003-Cya	This study
pCPP3300	pVLT35 expressing PSPTO0524-Cya	This study
pCPP3302	pVLT35 expressing ShcV and HopPtoV-Cya	This study
pCPP3303	pVLT35 expressing HopPtoQ-Cya	This study
pCPP3304	pVLT35 expressing PSPTO0834-Cya	This study
pCPP3305	pVLT35 expressing PSPTO0836-Cya	This study
pCPP3306	pVLT35 expressing PSPTO0837-Cya	This study
pCPP3307	pVLT35 expressing PSPTO2695-Cya	This study
pCPP3308	pVLT35 expressing AvrPphE$_{Pto}$-Cya	This study
pCPP3309	pVLT35 expressing HopPtoK-Cya	This study
pCPP3310	pVLT35 expressing HopPtoT1-Cya	This study
pCPP3311	pVLT35 expressing PSPTO4781-Cya	This study
pCPP3312	pVLT35 expressing PSPTO0835-Cya	This study
pCPP3313	pVLT35 expressing HopPtoW′-Cya	This study
pLN401	pVLT35 expressing ShcS4 and HopPtoS4′-Cya	This study

densities at 600 nm (OD$_{600}$) of 0.15 and 0.12, respectively. These cultures were grown at 22°C with shaking until the OD$_{600}$ were ~0.5. Cellular protein fractions were collected by pelleting 1 ml of a culture and resuspending the bacteria in 100 μl of protein sample buffer (43). To collect extracellular protein fractions, 40-ml portions of cultures were centrifuged at 21,000 × *g* for 1 h, and the upper 30 ml of each supernatant was removed and filtered through a 0.45-μm-pore-size polysulfone syringe filter (Pall Life Sciences). Three milliliters of trichloroacetic acid was added to each of the supernatant fractions, and proteins were precipitated at 4°C overnight. Samples were then centrifuged at 21,000 × *g* for 1 h, and the pellets were washed twice with 1 ml of ice-cold acetone. Each pellet was resuspended in 105 μl of protein sample buffer. After boiling for 5 min, 15-μl portions of cellular protein samples and 45-μl portions of supernatant protein samples were loaded onto a sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel electrophoresis (PAGE) gel.

P. fluorescens 55 isolates expressing Cya fusion proteins were grown on KB plates for 2 days. Bacteria were scraped off the plates, resuspended at an OD$_{600}$ of 0.1 in liquid KB supplemented with streptomycin and 200 μM IPTG, and grown at 28°C for 7 h. After the OD$_{600}$ of each culture was recorded, 1.5 ml was centrifuged with a microcentrifuge, and the pellet was suspended in protein

sample buffer. Equal amounts of cells, based on OD$_{600}$, were loaded onto SDS-PAGE gels.

Immunoblot analysis. Protein samples were separated by electrophoresis on SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore) by using a Semiphor semidry transfer system (Hoefer). Western blot analysis was carried out by using a Western-Light Plus kit (Tropix) according to the manufacturer's instructions. Cya fusion proteins were detected by using primary anti-Cya (3D1) mouse monoclonal immunoglobulin G (IgG) antibodies (Santa Cruz Biotechnology) at a dilution of 1:5,000 and secondary anti-mouse IgG-alkaline phosphatase conjugate antibodies (Sigma) at a dilution of 1:30,000. NptII was detected by using primary anti-NptII rabbit polyclonal IgG antibodies (United States Biological, Swampscott, Mass.) at a dilution of 1:5,000 and secondary anti-rabbit IgG-alkaline phosphatase conjugate antibodies (Sigma) at a dilution of 1:30,000.

Plant growth and bacterial inoculation. Tomato (*Lycopersicon esculentum* cv. Money Maker) and wild tobacco (*Nicotiana benthamiana*) plants were grown under greenhouse conditions and transferred to the laboratory 1 day prior to inoculation. During experiments, plants were maintained in the laboratory at room temperature (24°C) with illumination. Bacteria grown on KB plates for

TABLE 2. Primers used to construct the *cya* gene fusions in this study

cya fusion gene	5′ (upstream) primer	3′ (downstream) primer
avrPto$_{(1-164)}$	5′-GTTCTAGAGTGAGCGGATAACAATTTCAC-3′	5′-GTCCATAATATTGACTTGCCAGTTACGGTACG-3′
avrPto$_{(1-135)}$	5′-GTTCTAGAGTGAGCGGATAACAATTTCAC-3′	5′-TGGTTTAATATTCGGTGGGATGTCGGCATG-3′
avrPto$_{(1-100)}$	5′-GTTCTAGAGTGAGCGGATAACAATTTCAC-3′	5′-CTCACAAATATTCATTCCCGGATTGATTCCTG-3′
avrPto$_{(1-50)}$	5′-GTTCTAGAGTGAGCGGATAACAATTTCAC-3′	5′-AGTTCAATATTTGGTAGACCAGCAGACTC-3′
avrPto$_{(1-16)}$	5′-GTTCTAGAGTGAGCGGATAACAATTTCAC-3′	5′-GTCTGCAATATTCACCTGATGGGCCATC-3′
avrB$_{(1-321)}$	5′-CACCATGGGCTGCGTCTCGTCAAAAAG-3′	5′-AAAGCAATCAGAATCTAGCAAGCT-3′
avrB$_{(1-30)}$	5′-CACCATGGGCTGCGTCTCGTCAAAAAG-3′	5′-GGGGAGTGCTCTGAAAGACGTAC-3′
hopPtoT1	5′-CACCATGAAAACAGTCAGCAA-3′	5′-TGACTTTTGAGCCGCCT-3′
hopPtoQ	5′-CACCATGCATCGTCCTATCACCG-3′	5′-ATCTGGGGCTACCGTCGA-3′
hopPtoS4′	5′-CACCTTGCTAGGTATTAATGTCATG-3′	5′-GACCTTCCCAAGCTCTGG-3′
hopPtoAG′	5′-CACCGCGCAGGAGTGCTCAACAATG-3′	5′-CTTCAGACCTTCCTTAACTACCCG-3′
hopPtoV	5′-CACCATGAGCTTATCGCCGACG-3′	5′-CTTTCTATCCGGAATAACTTTTTTCC-3′
shcV	5′-CACCATGAGCTTATCGCCGACG-3′	5′-GTTAAAACGAAAATGTGTCGCTG-3′
PSPTO4588	5′-CACCATGATCGCGTTCGCAACCGG-3′	5′-GCCAGATGAGCTCGCCACAG-3′
PSPTO2695	5′-CACCATGACTCAGACCGCCCTG-3′	5′-GGGAATCAGCCGATCACGAC-3′
PSPTO4781	5′-CACCGTGCTCGCTTTTGCA-3′	5′-AGGCCGTCCTTCCATCCTC-3′
PSPTOB003	5′-CACCATGAACAGGCTTCACAAGACC-3′	5′-GTCCTCGTTTAGCCGCAGC-3′
PSPTO0834	5′-CACCATGAAAGCACTGGGCTTAATG-3′	5′-AAGATAAGCGAGATTTGGTGTGA-3′
PSPTO0835	5′-CACCAGGAATAAAGGAGTAACAATGAAAG-3′	5′-GTACGGGCTCCGATTGTTCGA-3′
PSPTO0836	5′-CACCATGCAGGCGATAGGGGC-3′	5′-TTTTTCTGCAAACCATTGCTTG-3′
PSPTO0837	5′-CACCATGAAAAAACTCAATTTTTCCGAGC-3′	5′-GAAACGGGAGGAGGTATGC-3′
PSPTO0524	5′-CACCATGAAAAAATGTATTGCTCTGCTCC-3′	5′-CTGGTTACCATATAGCGGCTCAG-3′

~24 h were prepared for inoculation by suspension in 5 mM morpholinoethane-sulfonic acid (MES) (pH 5.5) at an OD_{600} of 0.3 (1×10^8 CFU/ml) for adenylate cyclase assays, at an OD_{600} of 0.4 for AvrB-Cya HR assays, or at an OD_{600} of 0.8 for all other HR assays. Unless otherwise noted, each bacterial inoculum also contained 100 µM IPTG to induce expression of *cya* fusions. Bacteria were infiltrated into the fully expanded upper leaves of plants as previously described (27). The area of infiltration was marked to ensure that the leaf tissue subsequently collected for cAMP assays contained bacterial inoculum.

Adenylate cyclase assays. To assay adenylate cyclase activity in plant tissue, leaf disks were collected with a 1-cm-diameter cork borer, frozen in liquid nitrogen, ground to a powder, and suspended in 300 µl of 0.1 M HCl. For the time course experiments, samples were collected in an identical manner except that a 0.8-cm-diameter cork borer was used and the ground tissue was suspended in 250 µl of 0.1 M HCl.

To assay the adenylate cyclase activity of Cya fusion proteins expressed in *E. coli*, strains were grown in 5 ml of Luria-Bertani medium containing 100 µM IPTG to an OD_{600} of 0.6 to 0.8. The cultures were centrifuged, and the pellets were washed and resuspended in sonication buffer (20 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$). The bacteria were disrupted by sonication with a microtip for 2 min, and the cellular debris was pelleted by centrifugation with a microcentrifuge. Adenylate cyclase activity was determined in the presence or absence of calmodulin by using 5 µl of each lysate, as previously described (48).

cAMP was quantified in bacteria or leaf samples by using a Correlate-EIA cAMP immunoassay kit (Assay Designs) according to the manufacturer's directions. The amount of each sample used for quantification was adjusted so that it was in the detection range of the assay. The protein content of each sample was determined by the Bio-Rad protein assay (Bio-Rad).

RESULTS

Characterization of AvrPto-Cya and AvrB-Cya hybrid proteins. To determine whether Cya can be used as a reporter to study *P. syringae* type III-mediated protein translocation into plants, we constructed C-terminal translational Cya fusions to the well-characterized *P. syringae* effector proteins AvrPto (164 amino acids) and AvrB (322 amino acids). The *avrPto*$_{(1-X)}$-*cya* gene fusions, which contained the first 164, 135, 100, 50, or 16 codons of *avrPto* fused to codons 2 to 406 of *B. pertussis cya*, were cloned downstream from the *tac* promoter in the broad-host-range plasmid pVLT35 (Table 1). The resulting plasmids were conjugated into *P. syringae* pv. tomato DC3000 and

CUCPB5114 (DC3000 Δ*hrpK-hrpR*::ΩCm), and Hrp-mediated secretion of the AvrPto$_{(1-X)}$-Cya hybrid proteins into the culture supernatant was examined after bacteria were grown in hrpMM (Fig. 1). As a control for cellular lysis, each strain also contained plasmid pUFRO34, which constitutively expresses the cytoplasmic NptII protein. Cellular and supernatant fractions were separated by SDS-PAGE, and the AvrPto$_{(1-X)}$-Cya and NptII proteins were detected by Western analysis by using antibodies to Cya and NptII. AvrPto$_{(1-164)}$-Cya, AvrPto$_{(1-135)}$-Cya, AvrPto$_{(1-100)}$-Cya, and AvrPto$_{(1-50)}$-Cya were all secreted into the culture supernatant in an Hrp-dependent manner, indicating that Cya does not inhibit secretion through the *P. syringae* Hrp system. AvrPto$_{(1-16)}$-Cya secretion was not detected even though this protein was expressed at levels comparable to those of all of the other hybrid proteins. In some cases, the Cya antibodies detected multiple protein species on the immunoblot (Fig. 1). The additional AvrPto-Cya species could have been degradation products or proteins with alternative translation start sites. The additional AvrPto-Cya species did not appear to affect secretion of the full-length AvrPto$_{(1-X)}$-Cya proteins.

Two *avrB*$_{(1-X)}$-*cya* gene fusions, *avrB*$_{(1-321)}$-*cya* and *avrB*$_{(1-30)}$-*cya*, were also constructed in pVLT35. The plasmid expressing AvrB$_{(1-321)}$-Cya was toxic to DC3000 and caused cellular lysis in liquid culture (data not shown). Therefore, we expressed AvrB$_{(1-321)}$-Cya and AvrB$_{(1-30)}$-Cya in *P. fluorescens* containing the cosmid pLN18, which encodes the complete Hrp system of *P. syringae* pv. syringae 61 (Fig. 2A). pLN18 is a Δ*shcAhopPsyA*::ΩKm derivative of pHIR11, which allows *P. fluorescens* to deliver Avr-Hop proteins into plants (27, 30a), *P. fluorescens*(pLN18) expressing AvrB$_{(1-321)}$-Cya induced an HR in *N. benthamiana*, showing that Cya does not inhibit the translocation or activity of the full-length AvrB protein (Fig. 2B). AvrB$_{(1-321)}$-Cya delivery was Hrp dependent, as this protein did not cause the HR when it was expressed in *P. fluorescens* containing pCPP3297, a Δ*hrcC* derivative of pLN18. The HR
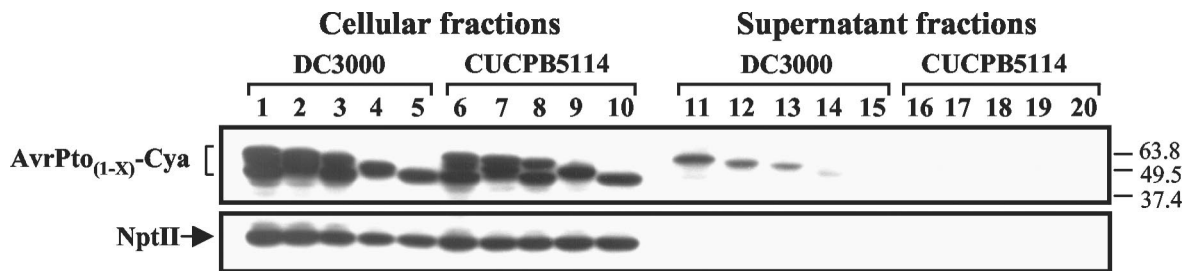
FIG. 1. Secretion of AvrPto-Cya hybrid proteins from *P. syringae* pv. tomato DC3000. DC3000 and CUCPB5114 (DC3000 Δ*hrpK-hrpR*::ΩCm) strains containing plasmids that express AvrPto$_{(1-X)}$-Cya fusion proteins (where X is 164, 135, 100, 50, or 16) were grown in culture under conditions that induce *hrp*-mediated protein secretion, as described in Materials and Methods. These strains also contained pUFRO34, a plasmid that expresses the cytoplasmic NptII protein. Cultures were separated into cellular and supernatant fractions by centrifugation, and an immunoblot analysis was performed with protein samples electrophoresed on an SDS–12.5% PAGE gel. The supernatant samples loaded on the gel were 86-fold more concentrated than the cellular samples. The AvrPto$_{(1-X)}$-Cya and NptII (29.1 kDa) proteins were detected by using antibodies to Cya and NptII, respectively. Lanes 1, 6, 11, and 16, AvrPto$_{(1-164)}$-Cya (62.1 kDa); lanes 2, 7, 12, and 17, AvrPto$_{(1-135)}$-Cya (58.6 kDa); lanes 3, 8, 13, and 18, AvrPto$_{(1-100)}$-Cya (54.8 kDa); lanes 4, 9, 14, and 19, AvrPto$_{(1-50)}$-Cya (49.0 kDa); lanes 5, 10, 15, and 20, AvrPto$_{(1-16)}$-Cya (45.3 kDa). The positions of prestained protein standards on the gel are indicated on the right.

caused by AvrB$_{(1-321)}$-Cya was not due to its Cya domain because AvrPto$_{(1-100)}$-Cya, which was translocated into *N. benthamiana* (as shown below), did not cause the HR in *N. benthamiana*. AvrB$_{(1-30)}$-Cya also did not induce the HR in *N. benthamiana*.

Optimization of AvrPto$_{(1-164)}$-Cya delivery into plants. To determine the optimal conditions for studying *P. syringae* translocation of Cya hybrid proteins into plant cells, we analyzed AvrPto$_{(1-164)}$-Cya translocation with regard to the kinetics of cAMP accumulation, the delivery system, and the test plant. The kinetics of cAMP accumulation in plant leaves were examined following inoculation with DC3000 or CUCPB5114 (DC3000 Δ*hrpK-hrpR*::ΩCm) strains expressing AvrPto$_{(1-164)}$-Cya (Fig. 3A). As an additional control, plants were inoculated with DC3000 containing pCPP3214, a pVLT35-based plasmid that includes codons 2 to 406 of *cya* and does not express Cya due lack of a translation start site. cAMP was extracted from leaf samples taken 1, 3, 5, 7, and 9 h postinoculation and quantified. In tomato (*L. esculentum* cv. Money Maker), a

susceptible host for DC3000, cAMP levels increased over time only in leaf samples inoculated with DC3000 expressing a wild-type Hrp system and AvrPto$_{(1-164)}$-Cya (Fig. 3A). Similar results were observed in *N. benthamiana*, even though DC3000 induces the HR in this plant (Fig. 3A). However, we did not observe any increases in cAMP levels that were indicative of AvrPto$_{(1-164)}$-Cya translocation into *Nicotiana tabacum* cv. Xanthi leaves (H.-S. Oh and A. Collmer, unpublished data). To rule out the possibility that AvrPto$_{(1-164)}$-Cya present in the plant apoplast could have caused cAMP accumulation, plants were inoculated with a lysate prepared from an *E. coli* culture expressing AvrPto$_{(1-164)}$-Cya. This lysate did not cause cAMP accumulation in tomato or *N. benthamiana* leaves (Fig. 3A), supporting the conclusion that AvrPto$_{(1-164)}$-Cya is translocated into plant cells via the DC3000 Hrp secretion system. Plants inoculated with bacteria expressing AvrPto containing a C-terminal FLAG tag instead of Cya also did not induce cAMP accumulation, indicating that the Cya domain of AvrPto$_{(1-164)}$-
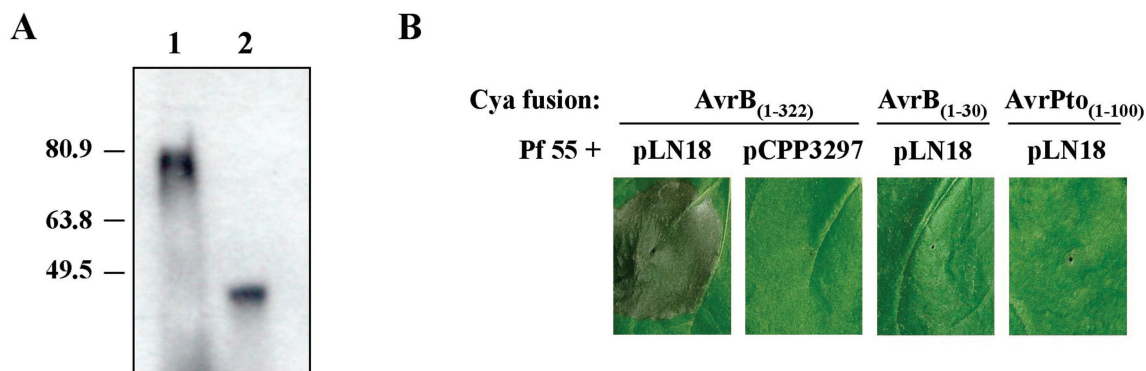

FIG. 2. HRs elicited by AvrB-Cya fusion proteins in *N. benthamiana* after delivery by *P. fluorescens* 55 expressing the *P. syringae* pv. syringae 61 Hrp system. (A) Expression of AvrB-Cya hybrid proteins in *P. fluorescens*. *P. fluorescens* 55 strains containing plasmids that express the Hrp system from *P. syringae* pv. syringae 61 (pLN18) and AvrB$_{(1-321)}$-Cya (pCPP3290) or AvrB$_{(1-30)}$-Cya (pCPP3288) were grown in culture as described in Materials and Methods. Protein samples were separated on an SDS–12.5% PAGE gel, and an immunoblot analysis was performed by using antibodies to Cya. Lane 1, AvrB$_{(1-321)}$-Cya (79.5 kDa); lane 2, AvrB$_{(1-30)}$-Cya (46.8 kDa). The positions of prestained protein standards on the gel are indicated on the left. (B) AvrB$_{(1-321)}$-Cya induces an HR in *N. benthamiana*. *N. benthamiana* leaves were infiltrated with suspensions (OD$_{600}$, 0.4) of *P. fluorescens* 55 (Pf 55) strains expressing AvrB$_{(1-321)}$-Cya, AvrB$_{(1-30)}$-Cya, or AvrPto$_{(1-100)}$-Cya and a wild-type Hrp system from pLN18 or a Δ*hrcC* mutant Hrp system from pCPP3297. Photographs were taken 48 h after inoculation.
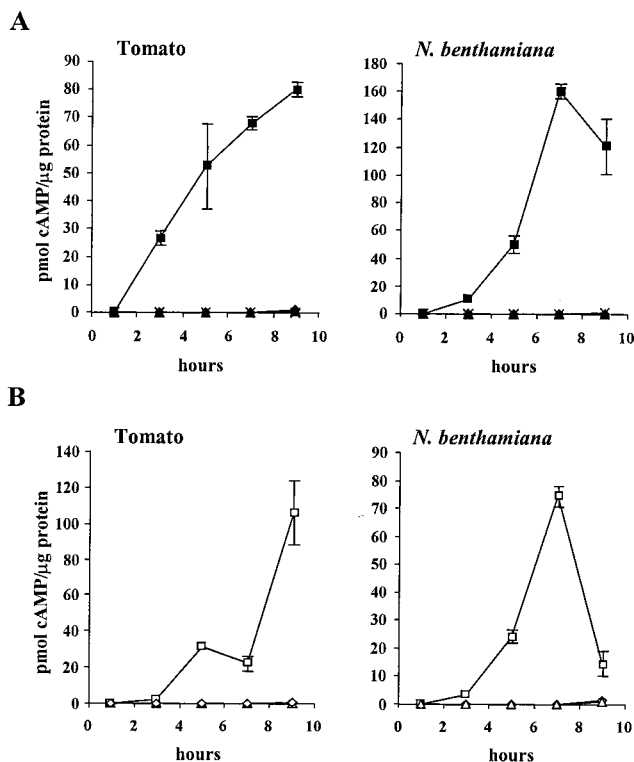
A

B

FIG. 3. Time course of cAMP accumulation in plants after inoculation with *Pseudomonas* or *E. coli* cells expressing AvrPto$_{(1-164)}$-Cya. (A) Tomato (*L. esculentum* cv. Money Maker) and *N. benthamiana* plants were inoculated with DC3000 bacteria (OD$_{600}$, 0.3) containing pCPP3214 [Cya$_{(2-406)}$] (◆) or pCPP3221 [AvrPto$_{(1-164)}$-Cya] (■) or with CUCPB5114 (DC3000 Δ*hrpK-hrpR*::ΩCm) containing pCPP3221 (▲). Plants were also infiltrated with a cleared lysate prepared from a culture of *E. coli* containing pCPP3221 (✕). (B) Tomato (*L. esculentum* cv. Money Maker) and *N. benthamiana* plants were inoculated with *P. fluorescens* 55 cells (OD$_{600}$, 0.3) containing pLN18 and pCPP3214 (◇), pLN18 and pCPP3221 (□), or pCPP3297 and pCPP3221 (△). pLN18 and pCPP3297 contain the wild-type and Δ*hrcC* hrp system genes from *P. syringae* pv. syringae 61, respectively. Leaf samples were collected with a 0.8-cm-diameter cork borer 1, 3, 5, 7, and 9 h postinoculation. cAMP was quantified in triplicate for each sample, and the standard deviations are indicated by error bars. The graphs are based on data from one representative experiment. Repeated experiments on different days yielded similar results, although the cAMP levels varied by up to 50% for each strain.

Cya was responsible for the production of cAMP (data not shown).

Because plasmids expressing certain Cya fusion proteins were toxic to DC3000, we examined whether *P. fluorescens* containing the cosmid pLN18 could be used as an alternative delivery system for translocation of Cya fusion proteins into plant cells. *P. fluorescens* expressing AvrPto$_{(1-164)}$-Cya increased cAMP levels in tomato leaves when bacteria also contained pLN18 but not when bacteria also contained the Δ*hrcC* mutant pCPP3297 (Fig. 3B). In addition, *P. fluorescens*(pLN18) containing pCPP3214 (pVLT35::*cya*) did not cause cAMP accumulation in tomato leaves. These results indicate that like DC3000, *P. fluorescens*(pLN18) translocates AvrPto$_{(1-164)}$-Cya into tomato in an Hrp-dependent manner. Similar results were observed in *N. benthamiana* (Fig. 3B). *N.*

benthamiana was used as the host plant in subsequent experiments due to the ease of infiltrating its large leaves. Because *P. fluorescens*(pLN18) does not cause the HR in *N. benthamiana* and because plasmids expressing Cya fusion proteins did not appear to be as toxic to this strain, this delivery system was used in the experiments described below unless otherwise indicated.

Characterization of the translocation domains of AvrPto-Cya and AvrB-Cya hybrid proteins. To determine the minimal N-terminal region needed for AvrPto-Cya translocation into plant cells, cAMP accumulation was analyzed in *N. benthamiana* after infiltration with DC3000 or *P. fluorescens*(pLN18) strains expressing AvrPto$_{(1-X)}$-Cya hybrid proteins. AvrPto$_{(1-16)}$-Cya caused a weak increase in cAMP production in *N. benthamiana*, and AvrPto$_{(1-50)}$-Cya and AvrPto$_{(1-100)}$-Cya caused progressively stronger cAMP production (Table 3). However, AvrPto$_{(1-135)}$-Cya and AvrPto$_{(1-164)}$-Cya did not appear to be translocated better than AvrPto$_{(1-100)}$-Cya, which suggests that all of the targeting information is present in the first 100 amino acids of AvrPto, with the minimal signal being in the first 16 amino acids and/or codons. AvrB$_{(1-30)}$-Cya and AvrB$_{(1-321)}$-Cya resulted in a similar trend in cAMP production in *N. benthamiana*. In *E. coli* lysates supplemented with calmodulin, AvrPto$_{(1-16)}$-Cya and AvrB$_{(1-30)}$-Cya were able to produce cAMP levels comparable to those produced by Cya fusions with larger N-terminal fragments of these effectors (Table 3). Thus, the nominal signal produced by these fusions in *N. benthamiana* was presumably the result of weak translocation. These observations indicate that N-terminal signals are both sufficient and required for the Hrp-mediated translocation of AvrPto-Cya and AvrB-Cya into plants. Furthermore, although the minimal information for AvrPto translocation is in the first 16 amino acids, information in the first 100 residues contributes cumulatively to translocation efficacy.

Analysis of the production and stability of candidate Hop-Cya fusions. Because Cya was successfully used as a reporter for AvrPto and AvrB translocation into plant cells, we sought to use it as a tool to identify novel Hop proteins in DC3000. We compiled a list of potential *hop* genes by analyzing the coding regions downstream of HrpL-regulated promoters identified in previous reports (19, 41). Eighteen genes were chosen for further study, and in-frame *cya* fusions to each entire ORF were constructed in the broad-host-range plasmid pVLT35 (Table 4). Three of the *hop* candidate genes (PSPTO4597, PSPTO4588, and PSPTO4720) were located downstream of ORFs encoding small, acidic proteins that may be dedicated type III chaperones (PSPTO4599, PSPTO4589, and PSPTO4721, respectively). Because these potential chaperones might be required for secretion of their putative target Hops, they were expressed along with the appropriate Cya fusion proteins. Every Cya hybrid protein exhibited calmodulin-dependent adenylate cyclase activity in *E. coli* lysates (data not shown), indicating that all of them had the potential to induce cAMP accumulation in planta. Western blot analysis also confirmed that each Cya hybrid protein was expressed in *P. fluorescens* (Fig. 4). Although a hybrid protein of the expected size was detected in each lane of the immunoblot, the protein levels varied, and multiple bands were present in some cases. These results may have been due to protein degradation or translation from alternative start sites.

TABLE 3. Calmodulin-dependent adenylate cyclase activity of AvrPto-Cya and AvrB-Cya hybrid proteins in vitro and in planta

AvrPto or AvrB-Cya fusion protein	In vitro activity in *E. coli* lysates (nmol of cAMP/μg of protein)[a,d]		In planta activity in *N. benthamiana*[d]			
			Translocation by DC3000 (pmol of cAMP/μg of protein)[b]		Translocation by *P. fluorescens* (pmol of cAMP/μg of protein)[c]	
	With calmodulin	Without calmodulin	Wild-type	Δ*hrp*	pLN18	pCPP3297
AvrPto$_{(1-164)}$	140.6 ± 22.0	0.6 ± 0.1	157.1 ± 7.7	0.0 ± 0.0	103.6 ± 12.9	0.0 ± 0.0
AvrPto$_{(1-135)}$	179.5 ± 14.9	1.0 ± 0.3	101.6 ± 14.9	0.0 ± 0.0	114.3 ± 33.2	0.1 ± 0.0
AvrPto$_{(1-100)}$	186.1 ± 26.2	1.9 ± 0.0	122.3 ± 0.0	0.0 ± 0.0	56.6 ± 2.8	0.0 ± 0.0
AvrPto$_{(1-50)}$	145.0 ± 16.5	1.1 ± 0.2	66.7 ± 10.9	0.0 ± 0.0	12.3 ± 2.9	0.0 ± 0.0
AvrPto$_{(1-16)}$	134.2 ± 1.8	0.6 ± 0.2	2.0 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
AvrB$_{(1-321)}$	114.3 ± 16.6	0.3 ± 0.0	ND[e]	ND	73.1 ± 12.7	0.0 ± 0.0
AvrB$_{(1-30)}$	275.7 ± 8.1	0.7 ± 0.1	ND	ND	3.4 ± 0.3	0.0 ± 0.0

[a] cAMP was quantified in sonicated cellular lysates of *E. coli* in the presence or absence of exogenous calmodulin as described in Materials and Methods.
[b] cAMP was quantified in *N. benthamiana* leaf samples 7 h after infiltration with wild-type or Δ*hrp* (CUCPB5114) DC3000 at an OD$_{600}$ of 0.3.
[c] cAMP was quantified in *N. benthamiana* leaf samples 7 h after infiltration with *P. fluorescens* (OD$_{600}$, 0.3) containing a cosmid expressing either a wild-type (pLN18) or Δ*hrcC* mutant (pCPP3297) Hrp system from *P. syringae* pv. syringae 61.
[d] cAMP was quantified in triplicate for each sample, and the values are means ± standard deviations. Repeated experiments yielded similar results.
[e] ND, not determined.

Analysis of the translocation of class I Hop candidate Cya fusions. The Hop candidate proteins were divided into three classes based on whether they had the following common characteristics of type III secreted proteins in *P. syringae*: (i) an aliphatic amino acid (Ile, Leu, or Val) or Pro at the third or fourth position, (ii) a relatively high serine content (more than 10%) in the first 50 amino acids, and (iii) no negatively charged amino acids (Asp or Glu) in the first 12 amino acids. Class I, II,

TABLE 4. Hop candidates tested in Cya translocation assay

Effector or candidate[a]	Alternative designation		Homolog(s) and/or likely function (BLASTP E value; GenBank accession no.)
	Petnicki-Ocwieja et al.[b]	Guttman et al.[c]	
HopPtoK (PSPTO0044)	HopPtoK	HolPtoAB	AvrRps4 of *P. syringae* pv. pisi (2e-44; AAB51082)
HopPtoC (PSPTO0590)	HopPtoC	AvrPpiC2$_{Pto}$	AvrPpiC2 of *P. syringae* pv. pisi (1e-148; CAC16701)
AvrPphE$_{Pto}$ (PSPTOA0012)	AvrPphE$_{Pto}$	AvrPphE$_{Pto}$	AvrPphE of *P. syringae* pv. phaseolicola (1e-117; AAA67930)
PSPTO0877 (HopPtoQ)	Orf19	HolPtoQ	Hypothetical protein of *X. campestris* pv. campestris (1e-134; NP_636447), hypothetical protein of *X. axonopodis* pv. citri (1e-128; NP_644627), hypothetical protein of *R. solanacearum* (3e-92; CAD13773)
PSPTOA0019 (HopPtoT1)	Orf16	HolPtoU	HolPtoU2 of *P. syringae* pv. tomato (1e-156; AAL84264)
PSPTO0901 (HopPtoAG′)	Orf28	NA[d]	HolPsyAG of *P. syringae* pv. syringae B728A (1e-58; DAA00387)
PSPTO4599 + PSPTO4597 (ShcS4 + HopPtoS4′)	Orf25 + Orf26	ChpPtoZ + HolPtoZ	PSPTO4588 (6e-6)
PSPTO 4589 + PSPTO4588	Orf14 + Orf15	NA	HopPtoS4′ (6e-6)
PSPTO2695	NA	NA	Hypothetical protein of *X. campestris* pv. campestris (1e-99; NP_637485), hypothetical protein of *X. axonopodis* pv. citri (2e-99; NP_642404), *Arabidopsis thaliana* wound-induced protein (9e-60; AAM64873)
PSPTOB003	Orf18	NA	Probable exported protein YPO3534 of *Y. pestis* (2e-6; AG0429)
PSPTO4721 (ShcV)	Orf6	NA	Chaperone for HopPtoV?
PSPTO4721 + PSPTO4720 (ShcV + HopPtoV)	Orf6 + Orf17	NA	None
PSPTO0834	Orf22	NA	Mannitol dehydrogenase of *Leuconostoc mesenteroides* (3e-22; AAM09029)
PSPTO0835	Orf23	NA	Riboflavin-specific deaminase of *Aquifex aeolicus* (1e-37; G70339)
PSPTO0836	Orf24	NA	Predicted phosphatase of *Clostridium acetobutylicum* (2e-40; AAK78609)
PSPTO0837	NA	NA	Conserved hypothetical protein of *Vibrio cholerae* (5e-41; A82470)
PSPTO4781	Orf8	NA	Esterase of *Pyrobaculum calidifontis* (1e-16; BAC06606)
PSPTO0524	Orf27	NA	Carboxypeptidase G2 of *Bacillus halodurans* (1e-28; BAB04071)

[a] Coding sequences are indicated by the name and/or *P. syringae* pv. tomato ORF (PSPTO) number in the complete genome sequence of DC3000 (GenBank accession number AE016853).
[b] See reference 41.
[c] See reference 24.
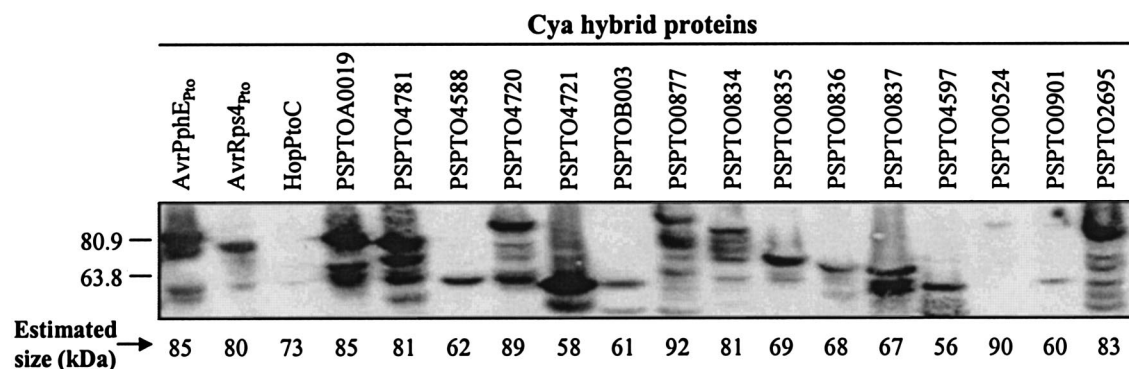[d] NA, not applicable.

Cya hybrid proteins

FIG. 4. Expression of DC3000 effector-Cya or effector candidate-Cya fusion proteins in *P. fluorescens* 55. *P. fluorescens* 55 strains containing plasmids that express the Hrp system from *P. syringae* pv. syringae 61 (pLN18) and the different Cya fusion proteins were grown in culture as described in Materials and Methods. Protein samples were separated on an SDS–7% PAGE gel, and an immunoblot analysis was performed by using antibodies to Cya. The estimated molecular masses of the hybrid proteins and the positions of prestained protein standards on the gel are indicated.

and III Hop candidate proteins were defined as proteins that had all three, two, or less than two of these characteristics, respectively. To determine whether the Hop candidates were translocated into plants, cAMP was quantified in *N. benthamiana* leaf samples infiltrated with *P. fluorescens* 55 strains expressing one Cya hybrid protein and either a wild-type or nonfunctional Hrp system. We predicted that proteins in class I (HopPtoK-Cya, HopPtoC-Cya, AvrPphE$_{Pto}$-Cya, PSPTO0877-Cya, PSPTOA0019-Cya, PSPTO0901-Cya, and PSPTO4597-Cya)

would be translocated into plants because they had all three of the characteristics of *P. syringae* Hops listed above (Table 5).

All seven of the strains expressing class I hybrid proteins generated cAMP in *N. benthamiana* in an *hrp*-dependent manner, suggesting that they were translocated into plant cells by the *P. syringae* Hrp system (Table 5). The lower levels of cAMP induced by HopPtoC-Cya may have been due to the low levels of this protein present in *P. fluorescens* (Fig. 4). As indicated in previous reports, two of the proteins in class I, HopPtoK and

TABLE 5. In planta adenylate cyclase activity in *N. benthamiana* of DC3000 Hop candidate-Cya fusion proteins

Cya fusion protein[a]	First 12 residues[b]	% of Ser[c]	In planta activity (pmol of cAMP/µg of protein) after translocation by[d]:	
			P. fluorescens (pLN18)	*P. fluorescens* (pCPP3297)
Class I				
HopPtoK (PSPTO0044)	MNRISTSSVNSS	18	771.9 ± 27.5	0.7 ± 0.2
HopPtoC (PSPTO0590)	MT**I**VSGHIGKHP	16	21.5 ± 2.3	0.6 ± 0.6
AvrPphE$_{Pto}$ (PSPTOA0012)	MK**I**HNAGLTPPL	14	1,109.5 ± 109.0	1.9 ± 0.3
HopPtoQ (PSPTO0877)	MH**R**PITAGHTTS	24	1,928.6 ± 94.0	0.3 ± 0.2
HopPtoT1 (PSPTOA0019)	MKT**V**SNHSIPST	14	84.0 ± 13.7	0.2 ± 0.1
HopPtoAG' (PSPTO0901)	MNP**I**THSFSHLG	12	151.0 ± 10.6	1.9 ± 0.4
ShcS4 + HopPtoS4 (PSPTO4599 + PSPTO4597)	MK**I**SGSTSPAHT	24	944.7 ± 77.5	0.9 ± 0.1
Class II				
PSPTO4589 + PSPTO4588	MKKSGAGTQAYA	14	0.2 ± 0.1	0.2 ± 0.1
PSPTO2695	MTQTALVVGASG	10	0.0 ± 0.0	0.6 ± 0.1
PSPTOB003	MNR**L**HKTSLLAA	8	0.0 ± 0.0	0.1 ± 0.1
ShcV (PSPTO4721)	MS**L**SPTLQKLTN	8	0.0 ± 0.0	0.1 ± 0.0
Class III				
ShcV + HopPtoV (PSPTO4721 + PSPTO4720)	ML<u>D</u>KVGNSARV<u>D</u>[e]	6	378.2 ± 27.1	0.6 ± 0.3
PSPTO0834	MKA**L**GLM<u>D</u>NQRL[e]	4	0.6 ± 0.4	0.4 ± 0.3
PSPTO0835	MK**V**TVFSQISI<u>D</u>	8	0.0 ± 0.0	0.4 ± 0.1
PSPTO0836	MQA**I**GAVIF<u>D</u>M<u>D</u>[e]	8	0.3 ± 0.1	0.2 ± 0.1
PSPTO0837	MKK**L**NFSELNWI	6	0.0 ± 0.0	0.2 ± 0.1
PSPTO4781	M**L**AFAYVSLIR<u>E</u>	6	0.0 ± 0.0	0.7 ± 0.4
PSPTO0524	MKKCIALLLTLV	4	1.7 ± 0.7	1.5 ± 0.9

[a] Coding sequences fused to *cya* are indicated by the name and/or *P. syringae* pv. tomato ORF (PSPTO) number in the complete genome sequence of DC3000 (GenBank accession number AE016853). In cases where two ORFs are listed, *cya* was fused to the second ORF. Plasmids expressing these hybrid proteins also contained the preceding ORFs, which encoded small (14- to 17-kDa), acidic proteins that may have acted as type III chaperones.

[b] Acidic residues are underlined. The Ile, Leu, Val, or Pro residues located at the third or fourth position of the proteins are in bold type.

[c] Percentage of Ser residues in the first 50 amino acids of the predicted protein.

[d] cAMP measurement experiments were performed in triplicate with *N. benthamiana* leaf samples collected 7 h after infiltration with *P. fluorescens* strains (OD$_{600}$, 0.3) containing pLN18 or pCPP3297. The values are means ± standard deviations, and repeated experiments yielded similar results.

[e] The sequence had more than one potential translation start site, and the most N-terminal methionine is shown.

A

P_hrp

shcS4   hopPtoS4'   tnpA   'hopPtoS4   hopPtoS3

1 kb

B

P_hrp

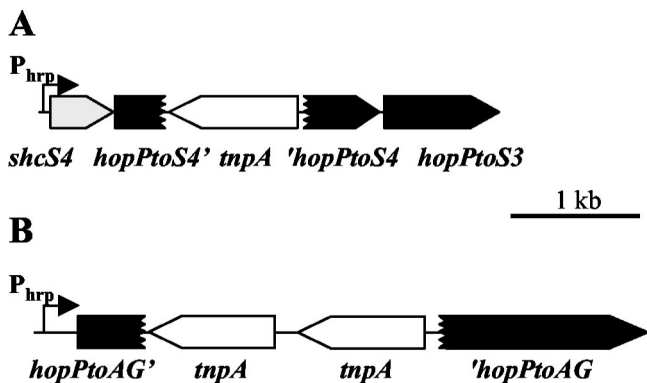*hopPtoAG'   tnpA   tnpA   'hopPtoAG*

FIG. 5. The *hopPtoS4* and *hopPtoAG* genes in *P. syringae* pv. tomato DC3000 are interrupted by transposon insertions. Schematic diagrams of the *hopPtoS4* (A) and *hopPtoAG* (B) regions of the DC3000 chromosome show the positions of coding sequences for effectors (solid arrows and boxes), transposases (open arrows), and a predicted chaperone (gray arrow). The *hopPtoS4*::*tnpA* and *hopPtoAG*::*tnpA* genes encode truncated products containing 118 and 152 amino acids, respectively. The bent arrows indicate the positions of promoters containing *hrp* boxes (P_{hrp}).

HopPtoC, travel the Hrp pathway, as does an AvrPhE_{Pto} homolog in another *P. syringae* strain. Specifically, a HopPtoK-'AvrRpt2 fusion is translocated by DC3000, HopPtoC is secreted in culture by DC3000, and an AvrPhE_{Pma}-'AvrRpt2 fusion is translocated by *P. syringae* pv. maculicola (24, 41). All of the remaining Hrp system-translocated proteins in class I were not tested previously for secretion in culture or translocation into plants and therefore were given Hop designations. The proteins encoded by PSPTO0877 and PSPTOA0019 were designated HopPtoQ and HopPtoT1, respectively.

When the sequences surrounding PSPTO4597 and PSPTO0901 on the DC3000 chromosome were analyzed, transposon insertions were found following both of these ORFs (Fig. 5). In the PSPTO4597 region, the ORF following the transposon insertion encodes a predicted protein with similarity to the C-terminal portion of HopPtoS3. Therefore, we propose that PSPTO4597 should be designated *hopPtoS4*::*tnpA* and that its product should be designated HopPtoS4' to indicate that it most likely is the N-terminal portion of a truncated effector protein. We also designated the protein encoded by PSPTO4599 ShcS4 to reflect its potential role as a chaperone for HopPtoS4. Two transposase genes follow PSPTO0901. PSPTO0901 and the ORF following the transposase gene insertions encode predicted proteins with high levels of homology to the N and C termini, respectively, of the same protein encoded by *P. syringae* pv. syringae B728A. Greenberg and Vinatzer recently designated this B728A ORF *holPsyAG* (ho̲p-li̲ke), and our results further suggest that this gene encodes a Hop (22). PSPTO0901 was designated *hopPtoAG*::*tnpA* and its product was designated HopPtoAG' to indicate that the gene is a truncated effector gene in DC3000 and the protein is a homolog of HolPsyAG.

Analysis of the translocation of class II Hop candidate Cya fusions. The proteins in class II (PSPTO4588-Cya, PSPTO2695-Cya, PSPTOB003-Cya, and PSPTO4721-Cya) had two of the three characteristics of *P. syringae* Hop proteins described above. Although the protein encoded by PSPTO4588 had a low level of similarity to HopPtoS4' and was encoded downstream from a potential chaperone (encoded by PSPTO4589), this hybrid protein did not induce cAMP accumulation in *N. benthamiana*. In addition, Cya fusions to the proteins encoded by PSPTO2695 and PSPTOB003, which are similar to a wound-induced protein in *Arabidopsis* and a protein annotated as a probable exported protein in the genome of *Y. pestis*, respectively (Table 4), did not induce accumulation of cAMP in *N. benthamiana* (Table 5). Therefore, proteins having only two of three characteristics of Hops may not be targeted to the Hrp system for translocation into plants. PSPTO4721-Cya also did not induce cAMP in planta, suggesting that this protein remains in the bacterial cytoplasm. This result was not surprising, as the PSPTO4721 gene (ORF6) encodes a small (14.7-kDa), negatively charged protein that has the characteristics of type III chaperones.

Analysis of the translocation of class III Hop candidate Cya fusions. We predicted that proteins in class III (PSPTO4720-Cya, PSPTO834-Cya, PSPTO835-Cya, PSPTO836-Cya, PSPTO837-Cya, PSPTO4781-Cya, and PSPTO0524-Cya) would not be translocated into plants because they have none or only one of the three characteristics of *P. syringae* Hops. Although six of these proteins were apparently not translocated into plant cells, PSPTO4720-Cya generated cAMP in *N. benthamiana* in an Hrp-dependent manner. Thus, PSPTO4720 was designated *hopPtoV*, and the ORF directly upstream (PSPTO4721), encoding a potential chaperone for HopPtoV, was designated *shcV*. These results demonstrate that most, but not all, Hops have the characteristics listed above.

Elicitation of the HR in *N. benthamiana* by HopPtoK-Cya and HopPtoQ-Cya. None of translocated Cya hybrid proteins caused the macroscopic HR, which is manifested by visible tissue collapse, in *N. benthamiana* at the cell density (OD_{600}, 0.3) used for the adenylate cyclase experiments (data not shown). However, when bacteria were inoculated at a higher cell density (OD_{600}, 0.8), HopPtoK-Cya and HopPtoQ-Cya induced the *hrp*-dependent HR in *N. benthamiana* (Fig. 6). These results confirmed that high levels of cAMP can accumulate at 7 h postinoculation in *N. benthamiana* even when the Cya hybrid protein is capable of eliciting the HR, and they provided further evidence that Cya fusions do not disrupt the biological activity of these particular effectors. None of the other Cya fusion proteins consistently caused the HR in *N. benthamiana* after delivery by *P. fluorescens* containing pLN18 (Fig. 6 and data not shown).

DISCUSSION

Cya has been used as a reporter to study TTSS-mediated translocation of effector proteins by *Yersinia*, *Salmonella*, enteropathogenic *E. coli*, and *Xanthomonas* (10, 14, 38, 48). In this study, the Cya reporter system was optimized in *P. syringae* by using the well-studied effector protein AvrPto. AvrPto-Cya induced cAMP accumulation in tomato or *N. benthamiana* when it was delivered by either DC3000 or *P. fluorescens* expressing a *P. syringae* Hrp system. This system permitted demonstration that the well-studied effector AvrB is translocated into plants despite its poor secretion in culture. Similar to translocation of other effector proteins,
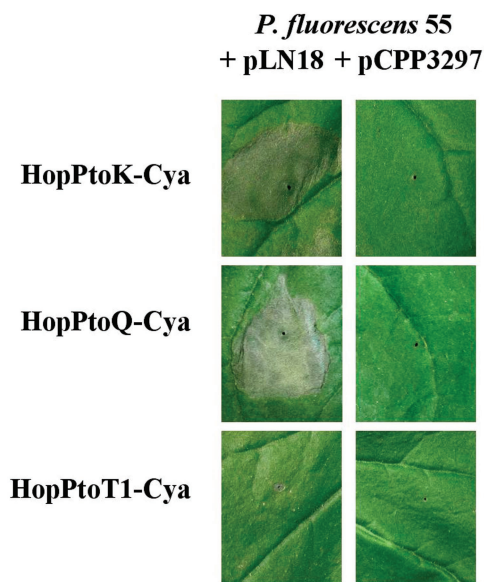
P. fluorescens 55
+ pLN18 + pCPP3297

FIG. 6. HopPtoK-Cya and HopPtoQ-Cya induce HRs in *N. benthamiana*. *N. benthamiana* leaves were infiltrated with suspensions (OD$_{600}$, 0.8) of *P. fluorescens* 55 strains expressing HopPtoK-Cya, HopPtoQ-Cya, or HopPtoT1-Cya and a wild-type *P. syringae* pv. syringae 61 Hrp system from pLN18 or a mutant *P. syringae* pv. syringae 61 Hrp system from pCPP3297. The photographs were taken 48 h after inoculation.

translocation of both AvrPto-Cya and AvrB-Cya was dependent upon N-terminal sequences. The Cya reporter also enabled further exploration of the targeting signals in DC3000 TTSS substrates and identification of several new effector proteins that are translocated into plant cells, two of which are truncated due to natural transposon insertions.

Cya as a reporter for *P. syringae* Hrp-mediated translocation into plants. Cya was developed several years ago as a reporter for TTSS translocation of *Yersinia* effector proteins into animal cells, yet this enzyme was not used to investigate Hrp-mediated translocation of phytopathogen effector proteins until recently. *X. campestris* pv. vesicatoria expressing AvrBs2-Cya causes a substantial increase in cAMP levels in susceptible pepper plants as soon as 3 h postinoculation (10). Our results show that AvrPto$_{(1-164)}$-Cya produces comparable results in susceptible tomato plants and *N. benthamiana* when it is expressed in *P. syringae* pv. tomato DC3000. *P. fluorescens* 55 containing pLN18 was also tested as a delivery system for effectors because some Cya fusion proteins were toxic to DC3000 and because the use of pLN18 permits translocation to be studied in the absence of other DC3000 proteins. *P. fluorescens* (pLN18), which expresses the Hrp system of *P. syringae* pv. syringae 61, translocates AvrPto$_{(1-164)}$-Cya into tomato and *N. benthamiana* almost as well as DC3000. Although *P. fluorescens*(pLN18) is slightly less efficient than DC3000 at delivering truncated AvrPto-Cya fusions [AvrPto$_{(1-100)}$-Cya, AvrPto$_{(1-50)}$-Cya, and particularly AvrPto$_{(1-16)}$-Cya] into *N. benthamiana*, it may be more suitable for testing candidate effectors because of its ability to tolerate overexpression of effectors and because translocation of individual effectors can be studied in the ab-

sence of potential competition from other effectors, which may complicate analysis of some TTSS effectors (8).

Casper-Lindley et al. reported that AvrBs2-Cya induction of cAMP accumulation is significantly reduced when bacteria are inoculated into a resistant pepper cultivar (10). We observed similar results in a resistant tomato cultivar infected with DC3000 expressing AvrPto$_{(1-164)}$-Cya (data not shown). However, cAMP accumulation induced by AvrPto$_{(1-164)}$-Cya was not inhibited in *N. benthamiana* even though DC3000 elicited the HR in this plant. In addition, AvrB-Cya, HopPtoK-Cya, and HopPtoQ-Cya each induced cAMP accumulation in *N. benthamiana* when it was delivered by *P. fluorescens*(pLN18) despite eliciting the HR. Thus, *N. benthamiana*, which is easily infiltrated and widely used in studies of plant-microbe interactions (5), appears to be useful as a universal host for Cya reporter studies of Hops that may cause the HR in plants. It is possible that induction of the HR occurs more slowly in *N. benthamiana*, allowing cAMP accumulation induced by Cya hybrid proteins to be quantified before resistance signaling pathways are activated. Alternatively, the resistance response mechanisms in *N. benthamiana* may be different from those present in tomato or pepper plants.

'AvrRpt2 has also been utilized as a reporter fusion to identify *P. syringae* proteins that are translocated into plant cells by the Hrp system (23, 52), but our results demonstrate that Cya has two advantages. First, as previously noted (10), adenylate cyclase assays appear to be more sensitive than HR assays. In order to observe the HRs caused by AvrB-Cya, HopPtoK-Cya, and HopPtoQ-Cya, it was necessary to inoculate plants with more bacteria than were used for Cya assays. Second, adenylate cyclase assays are at least semiquantitative, whereas HR assays are qualitative. Thus, unlike HR assays, Cya assays can be used to compare the amounts of effectors that are translocated into plants. However, the Cya reporter has limitations. In our studies, the amount of cAMP produced by a particular Cya fusion protein could vary as much as 50% in different experiments, although the relative cAMP levels produced by different fusion proteins in the same experiment were consistent. Therefore, quantitative comparisons can only be made with data from the same experiment.

Targeting signals of type III secreted proteins. Many studies have shown that N-terminal sequences are required for effectors to be secreted by TTSSs (2, 7, 39, 44, 47). We have previously shown that the first 10 codons of AvrPto are required for Hrp-mediated secretion in culture (2). Here, we found that the first 16 codons of AvrPto are sufficient to direct weak Hrp-mediated translocation into plant cells. Similarly, the first 15 codons of AvrB were shown to be necessary for Hrp-mediated secretion in culture (as determined by using a cloned *Erwinia chrysanthemi* Hrp system that appears to secrete *P. syringae* effectors more permissively than the *P. syringae* Hrp system) (2), and here we found that the first 30 codons of AvrB (the smallest fragment tested) are sufficient to direct translocation of a Cya fusion protein into plant cells.

Thus, consistent with findings for effectors such as the *Y. enterocolitica* YopE and enteropathogenic *E. coli* Tir proteins, residues and/or codons 1 to 15 of at least one plant pathogen effector are sufficient to direct translocation of the Cya reporter into eukaryotic cells, although translocation

directed by this minimal TTSS targeting signal can be very weak (8, 14). Previous work with translocation of AvrBs2-Cya fusions into pepper leaf cells by *X. campestris* pv. vesicatoria suggested that the first 41 residues of AvrBs2 were required for translocation, and no translocation was directed by smaller N-terminal fragments (10). However, it is possible that the weaker translocation of smaller fragments was below the threshold of detection in the apparently less sensitive pepper system.

The N termini of most known Hop proteins have a high serine content, an aliphatic amino acid (Ile, Val, or Leu) or Pro at the third or fourth position, and no acidic amino acids (Asp or Glu) in the first 12 amino acids (24, 41). To determine whether newly identified Hop proteins have these characteristics, we used the Cya reporter to test translocation of 18 Hop candidates that are encoded downstream of *hrp* promoter sequences. Seven Hop candidates had N termini that have all of the characteristics of Hop targeting signals, and all of these Cya hybrid proteins were translocated into *N. benthamiana*. In contrast, the Hop candidates that did not have all three of the characteristics of Hop N termini (with the exception of HopPtoV) were not translocated. Thus, the previously observed characteristics of known Hops also generally apply to new Hops. However, one newly identified Hop, HopPtoV, had none of the characteristics of Hop N termini (even when potential alternative start sites were considered), suggesting that some Hop proteins may have unique targeting signals that do not conform to any previously observed pattern. The ORF upstream of *hopPtoV*, which was designated *shcV*, appears to encode a putative type III chaperone. It is possible that ShcV binds and targets HopPtoV to the Hrp system, which may explain why HopPtoV has an unusual targeting signal, although other Hops with apparent chaperones have N termini that are typical of most Hops.

Interestingly, the PSPTO4588-Cya fusion protein (a class II candidate lacking an aliphatic amino acid at the third or fourth position) was not translocated into *N. benthamiana* even though the PSPTO4588-encoded protein has a low level of similarity to HopPtoS4′ and contains a high percentage of serine in its first 50 residues. Like *hopPtoV*, the PSPTO4588 gene is located downstream from a potential type III chaperone gene. Although it is possible that Cya may have prevented translocation of the PSPTO4588 protein or that the PSPTO4588 protein may have inhibited the adenylate cyclase activity of Cya, we favor and are currently testing the idea that the N-terminal region of the PSPTO4588 protein may have acquired mutations that eliminated the ability of this protein to be targeted to the type III secretion apparatus. In addition, we are exploring the possibility that sequences in other regions of the PSPTO4588 protein may block translocation despite the presence of otherwise functional targeting signals at the N terminus.

Expansion of the effector inventory in DC3000. One of the goals of our laboratory is to determine the complete inventory of Hop proteins in DC3000. In a previous study, genetic and bioinformatic methods were used to identify *hrp* promoter sequences in the DC3000 genome, which led to the discovery of many candidate *hop* genes (19). A subsequent study revealed that the Hrp system of DC3000 secretes several of the Hops into the culture supernatant, but many Hop candidates

were not tested (41). In this study, the Cya reporter system was used to test whether several of the candidate *hop* genes encode TTSS substrates. Our results confirm the previous conclusions or assumptions that HopPtoK, HopPtoC, and AvrPphE_{Pto} are translocated into plant cells. We also identified five new Hops in DC3000 that are translocated into plant cells, which increases the number of confirmed Hrp system-secreted proteins in this organism to 40.

HopPtoQ is of particular interest because it may be an important virulence factor in many phytopathogens; homologs are present in *X. campestris* pv. campestris, *Xanthomonas axonopodis* pv. citri, and *Ralstonia solanacearum* (Table 4). Furthermore, HopPtoQ-Cya produced far more cAMP in *N. benthamiana* than any other Cya fusion protein tested produced, despite the fact that HopPtoQ-Cya also elicited the HR in *N. benthamiana*. In contrast to HopPtoQ, HopPtoT1 and HopPtoV are unique to DC3000 according to the current databases, although at least two other copies of *hopPtoT1*-like genes are present in the DC3000 genome.

Two of the new *hop* genes identified in this study, *hopPtoS4::tnpA* and *hopPtoAG::tnpA*, encode proteins that are truncated due to natural transposon insertions. Seven percent of the genes in DC3000 are predicted to be mobile genetic elements, which may account for the large number of inactivated and duplicate *hop* genes present in this organism (9). Our results indicate that the naturally truncated proteins HopPtoS4′ and HopPtoAG′ are still targeted for translocation by the *P. syringae* Hrp system. Furthermore, the HopPtoS4′ gene is one of the DC3000 genes previously identified as being both induced during infection and activated by HrpL in culture (6). Hence, it seems likely that genetic rearrangements involving the 5′ regions of *hop* genes could lead to generation of new effector genes in DC3000. The genome of *P. syringae* pv. tomato DC3000 contains several regions that are enriched for genes encoding additional complete and disrupted effector candidates. The Cya reporter, used in conjunction with the cloned *P. syringae* Hrp system and the experimentally tractable *N. benthamiana* system that we describe here, should provide a useful resource for functional testing of these genes in DC3000 and other *P. syringae* genomes.

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