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Bioinformatics-Enabled Identification of the HrpL Regulon and Type III Secretion System Effector Proteins of *Pseudomonas syringae* pv. *phaseolicola* 1448A

Monica Vencato,[1] Fang Tian,[2] James R. Alfano,[2] C. Robin Buell,[3] Samuel Cartinhour,[4] Genevieve A. DeClerck,[4] David S. Guttman,[5] John Stavrinides,[5] Vinita Joardar,[3] Magdalen Lindeberg,[1] Philip A. Bronstein,[4] John W. Mansfield,[6] Christopher R. Myers,[7] Alan Collmer,[1] and David J. Schneider[4]

[1]Department of Plant Pathology, Cornell University, Ithaca, NY 14853, U.S.A.; [2]The Plant Science Initiative and the Department of Plant Pathology, University of Nebraska-Lincoln, 68588, U.S.A.; [3]The Institute for Genomic Research, 9712 Medical Center Dr., Rockville, MD 20850, U.S.A.; [4]United States Department of Agriculture–Agricultural Research Service, Ithaca, NY 14853, U.S.A.; [5]Department of Botany, University of Toronto, 25 Willcocks St., Toronto, ON M5S 3B2 Canada; [6]Agricultural Sciences Department, Imperial College at Wye, Ashford, Kent TN25 5AH, United Kingdom; [7]Cornell Theory Center, Cornell University, Ithaca, NY 14853, U.S.A.

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The ability of *Pseudomonas syringae* pv. *phaseolicola* to cause halo blight of bean is dependent on its ability to translocate effector proteins into host cells via the hypersensitive response and pathogenicity (Hrp) type III secretion system (T3SS). To identify genes encoding type III effectors and other potential virulence factors that are regulated by the HrpL alternative sigma factor, we used a hidden Markov model, weight matrix model, and type III targeting-associated patterns to search the genome of *P. syringae* pv. *phaseolicola* 1448A, which recently was sequenced to completion. We identified 44 high-probability putative Hrp promoters upstream of genes encoding the core T3SS machinery, 27 candidate effectors and related T3SS substrates, and 10 factors unrelated to the Hrp system. The expression of 13 of these candidate HrpL regulon genes was analyzed by real-time polymerase chain reaction, and all were found to be upregulated by HrpL. Six of the candidate type III effectors were assayed for T3SS-dependent translocation into plant cells using the *Bordetella pertussis* calmodulin-dependent adenylate cyclase (Cya) translocation reporter, and all were translocated. PSPPH1855 (ApbE-family protein) and PSPPH3759 (alcohol dehydrogenase) have no apparent T3SS-related function; however, they do have homologs in the model strain *P. syringae* pv. *tomato* DC3000 (PSPTO2105 and PSPTO0834, respectively) that are similarly upregulated by HrpL. Mutations were constructed in the DC3000 homologs and found to reduce bacterial growth in host *Arabidopsis* leaves. These results establish the utility of the bioinformatic or candidate gene approach to identifying effectors and other genes relevant to pathogenesis in *P. syringae* genomes.

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Corresponding author: David J. Schneider; E-mail: djs30@cornell.edu

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The HrpL alternative sigma factor activates the expression of multiple genes that are essential to the plant pathogenicity of *Pseudomonas syringae* (Willis and Kinscherf 2004). The most important of these are genes encoding the type III secretion system (T3SS) and effector proteins that are injected by the T3SS into host cells. The T3SS is encoded by hypersensitive response and pathogenicity (*hrp*) and *hrp* conserved (*hrc*) genes. Effector proteins are encoded by avirulence (*avr*) and Hrp outer protein (*hop*) genes, whose different names reflect the phenotype used to discover them (Lindeberg et al. 2005). *P. syringae* is a host-specific pathogen divided into more than 50 pathovars based largely on host range, and *hrp* mutants lose the ability to elicit the defense-associated hypersensitive response (HR) in nonhosts or to be pathogenic in hosts (Lindgren et al. 1986).

HrpL is a member of the extracytoplasmic family (ECF) of alternative sigma factors and activates promoters that have the canonical sequence GGAACC-N_{16}-CCACNNA as well as many variants of this sequence (Innes et al. 1993; Shen and Keen 1993; Xiao and Hutcheson 1994). A hidden Markov model (HMM) and weight matrix (WM) analysis was used to identify putative Hrp promoters in the genome of *P. syringae* pv. *tomato* DC3000 during the draft phase of a now-completed genome project (Buell et al. 2003; Fouts et al. 2002). The set of putative Hrp promoters enabled efficient identification of candidate effector genes based on several additional features, most notably N-terminal amino-acid patterns typical of T3SS substrates (Guttman et al. 2002; Petnicki-Ocwieja et al. 2002; Schechter et al. 2004). Because virtually all type III effector genes are associated with Hrp promoters and T3SS targeting patterns in DC3000, this bioinformatic approach is predicted to provide an efficient and potentially comprehensive means to identify novel candidates in newly sequenced *P. syringae* strains.

In addition, our analysis of the DC3000 HrpL regulon revealed several genes whose products are unlikely to have a T3SS-related function (Ferreira et al. 2006; Fouts et al. 2002). These include indoleacetate-lysine ligase, a syringomycin phytotoxin biosynthesis homolog, and a regulator of the biosynthesis of the coronatine phytotoxin. These observations suggest that HrpL coordinates the expression of multiple classes of factors promoting virulence and growth within plants. Comparison of the Hrp promoter inventories of phylogenetically divergent strains of *P. syringae* should reveal which components of the HrpL regulon are conserved and potentially involved in basic interactions with all plants and which components are variable and potentially control host specificity.

P. syringae pv. *phaseolicola* 1448A recently has been sequenced and is the focus of this study (Joardar et al. 2005). Phylogenetic analyses have revealed that most *P. syringae* pathovars group into three separate clusters, and pvs. *tomato* and *phaseolicola* represent two of these (Sarkar and Guttman 2004; Sawada et al. 1999). *P. syringae* pv. *phaseolicola* can be a devastating pathogen and is a useful experimental model for several reasons. *P. syringae* pv. *phaseolicola* strains have been assigned to nine races based on interactions with various bean cultivars, and several type III effector genes have been cloned from these races based on their gain-of-function avirulence phenotypes in tests with bean cultivars carrying different resistance (*R*) genes (Arnold et al. 2001; Jenner et al. 1991; Puri et al. 1997). Race 6 strains, such as 1448A, are compatible with all differential bean cultivars and, therefore, have been particularly useful for identifying effector genes from other races (Tsiamis et al. 2000). Studies of the *P. syringae* pv. *phaseolicola* Hrp system have been seminal. The *hrp* genes were discovered in *P. syringae* pv. *phaseolicola*, and experiments with this pathovar provided the first evidence that some effectors can suppress defense responses triggered by other effectors in the same bacterium (Jackson et al. 1999; Lindgren et al. 1986). Recently, quantitative real-time polymerase chain reaction (PCR) was used to determine the expression of each of the promoters expressing the T3SS and four substrates (HrpZ1, HopX1, HopF1, and HopAB1) in *P. syringae* pv. *phaseolicola* under inducing conditions in culture and in planta (Thwaites et al. 2004). A key finding was that the HrpL regulon is expressed much more strongly in planta than in culture.

In this report, bioinformatic analysis of the HrpL regulon of *P. syringae* pv. *phaseolicola* 1448A was performed using an HMM that was developed and validated with *P. syringae* pv. *tomato* DC3000 (Ferreira et al. 2006; Fouts et al. 2002). A preliminary analysis was performed on a draft genome sequence of 1448A, which aided annotation of virulence-related genes in the published genome (Joardar et al. 2005). The analysis was repeated on the completed genome and candidate HrpL-regulated genes were experimentally profiled, as reported here. The assays included real-time PCR and T3SS translocation tests involving both the *Bordetella pertussis* calmodulin-dependent adenylate cyclase (Cya) and AvrRpt2 translocation reporters. The results of these experiments enabled us to compare the HrpL regulons and type III effector inventories of 1448A and DC3000. Two genes that are activated by HrpL in both strains but have no apparent T3SS-related function were mutated and tested for virulence in the model *P. syringae* strain DC3000. Importantly, while this work was in preparation, a report of a near-saturating screen for HrpL-responsive promoters and type III effector genes in DC3000 and 1448A was published (Chang et al. 2005). Comparison of the datasets resulting from the genetic screen and the bioinformatic approach confirmed the comprehensiveness of the latter. Furthermore, the bioinformatic approach yielded three more 1448A T3SS substrates that had not been reported before.

RESULTS

Identification of candidate Hrp promoters in the genome of *P. syringae* pv. *phaseolicola* 1448A using an HMM trained on *P. syringae* pv. *tomato* DC3000.

The HMM analysis reported here was applied to the complete and annotated sequence (Joardar et al. 2005). The bioinformatic methods used are based on the refined HMM2 and WM2 analyses that were developed in conjunction with microarray verification of HrpL-responsive promoters in DC3000 (Ferreira et al. 2006). These methods are summarized in Figure 1. Our analysis revealed 44 candidate *hrp* promoters with a high likelihood of being active because they had an HMM2 score greater than 10.0, a WM2 score greater than 4.0, and a distance between the promoter and apparent start site of less than 220 bp. We exploited the reciprocal best hit BLAST analysis reported by Joardar and associates (2005) to determine whether the DC3000 orthologs of open reading frames (ORFs) in the predicted 1448A HrpL regulon had been shown by microarray analysis to be in the DC3000 HrpL regulon (Ferreira et al. 2006). A subset of these candidates (Table 1) are of particular interest because downstream genes encode Avr/Hops or have HrpL-activated orthologs in *P. syringae* pv. *tomato* DC3000. A complete list of candidate *hrp* promoters, including those with weaker scores, is presented in Supplementary Table 1 along with genome coordinates, operon predictions, and other information. Instructions and files that support viewing of the candidate Hrp promoters in the annotated 1448A genome sequence using Artemis software are available at the *Pseudomonas*–Plant Interaction website. In total, 31 of the high-probability candidate Hrp promoters in 1448A are found on the chromosome and 14 on the larger of the two plasmids.

Homologs of known *hop* genes were identified by BLAST queries of the 1448A genome with a comprehensive set of known effectors from all *P. syringae* pathovars (Lindeberg et al. 2005). Novel *hop* gene candidates were identified based on their possession of at least two of the three major T3SS targeting-associated patterns (Schechter et al. 2004,2006) (Fig. 1). For clarity, the *hop* candidates in Table 1 are given the Hop names that were designated upon experimental confirmation. Table 1 presents four groups of genes: i) *avr*/*hop* genes with HrpL-activated DC3000 homologs, ii) *avr*/*hop* genes without DC3000 homologs, iii) genes with no apparent role in the T3SS that have a HrpL-activated homolog in DC3000, and iv) genes with promoters that are below the HMM2 cutoff but above the WM2 cutoff. Within each group, genes are ranked by their HMM2 scores. The differential fluorescence induction (DFI) screen and subsequent translocation tests of Chang and associates (2005) show that a majority of the genes found by our bioinformatic criteria are activated by HrpL and encode Avr/Hop proteins (Table 1). However, the results we present below demonstrate that the bioinformatic approach yields at least one additional member for each of the first three classes presented in Table 1.

Real-time PCR analysis of candidate genes for regulation by the HrpL alternative sigma factor.

Real-time PCR analysis was used to determine whether 13 Hrp promoters of particular interest were activated by HrpL and to determine the relative expression of those activated by HrpL (Table 2). The downstream ORFs included genes encoding the T3SS, several Avr/Hop proteins, and proteins with no obvious T3SS-related function. Primers were designed to specifically amplify 100-bp regions in each ORF, and real-time PCR with SYBR Green I technology was used to determine HrpL-dependent expression in either wild-type strains carrying an empty plasmid (pML122) or a plasmid constitutively expressing *hrpL* (pCPP2308). Analyses were normalized to the

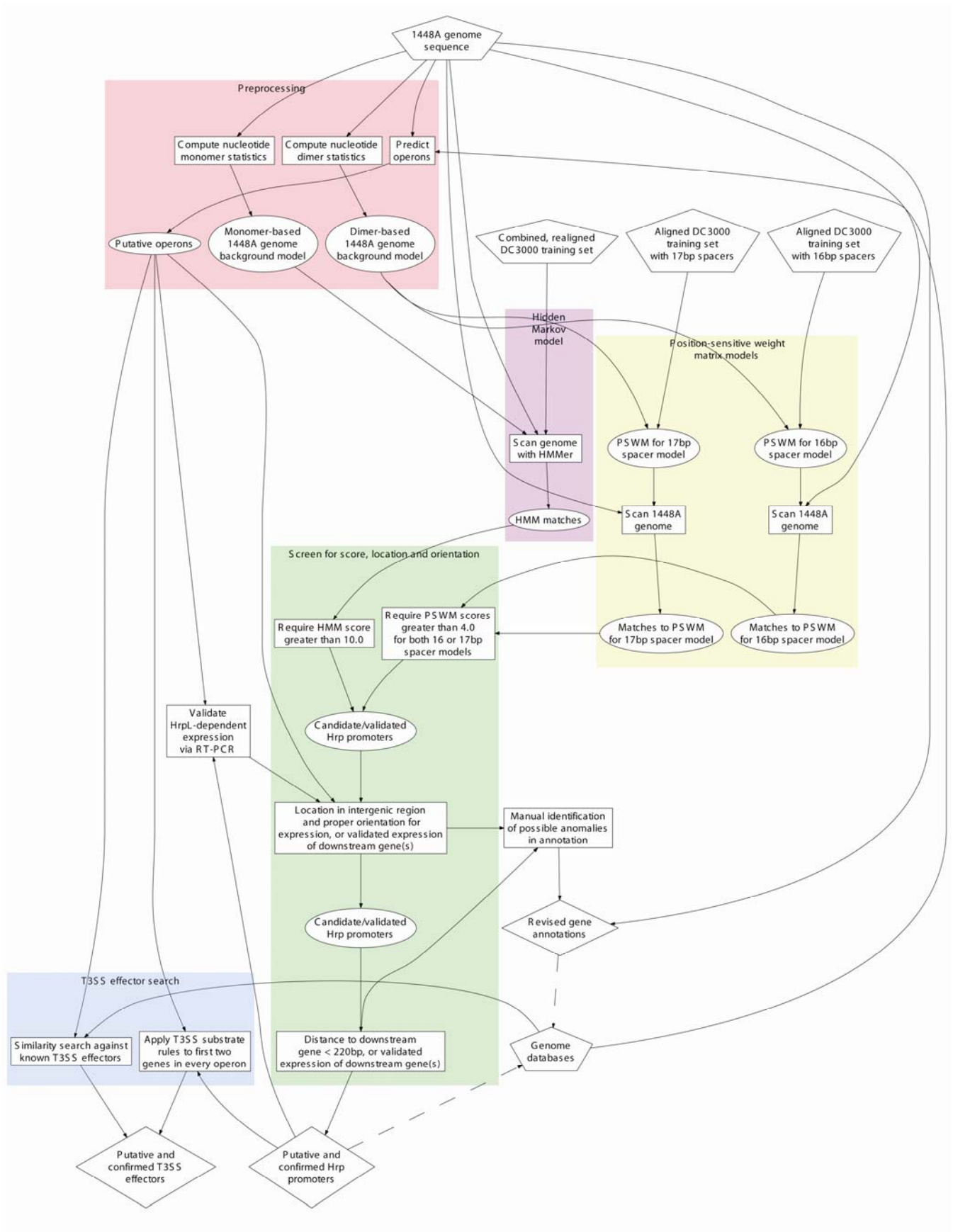

Fig. 1. Bioinformatic workflow represented as a directed graph. The major processing modules are grouped and highlighted by color. The nodes representing primary inputs, primary outputs, processing steps, and intermediate datasets are denoted with pentagons, diamonds, squares, and ellipses, respectively. Feedback pathways that connect primary outputs to primary inputs are indicated by dashed arrows.

housekeeping gene *gyrA* (PSPPH3667), which is expressed at a constant level regardless of the *P. syringae* pv. *phaseolicola* strain and is not regulated by HrpL. This corrected for any inherent differences in the samples, such as initial starting concentration and PCR efficiency.

Four *avr/hop* genes, PSPPH3028 (*avrB4-1*), PSPPH4736 (*hopAS1*), PSPPHA0031 (*hopAU1*), and PSPPHA0113 (*avrD1*), were shown to be strongly activated by HrpL. Interestingly, ORFs PSPPH1855 and PSPPH3759, which encode an ApbE-family protein and a putative alcohol dehydrogenase, respectively, also were activated by HrpL. PSPPH1519 (SlyA/MarR homolog) and PSPPH1525 (shikimate kinase-like) showed weaker activation, and results varied among biological replicates. Negative controls included PSPPH3960 (putative regulator), PSPPH1381 (putative deoxycytidine triphosphate deaminase), and PSPPH1127 (putative lipoprotein), which are

Table 1. Representative *Pseudomonas syringae* pv. *phaseolicola* 1448A genes that are predicted to be in the HrpL regulon by hidden Markov model (HMM) analysis and either encode Hops or have HrpL-activated orthologs in *P. syringae* pv. *tomato* DC3000

				Evidence		*P. syringae* pv. *tomato*[a]	
Locus[b]	Name (function)[c]	HMM2	WM2[d]	Expression[e]	Translocation	Ortholog	Name
avr/hop with DC3000 homolog[f]							
A0012-A0011	*hopQ1*	23.3	6.01	C	C	0877	*hopQ1-1 hopQ1-2*
	…	…	…	…	…	4732	…
0171	*hopR1*	20.7	6.24	C	C	0883	…
1443	*hopAF1*	20.5	6.05	C	C	1568	…
A0010	*hopD1*	20.4	6.22	C	C	0876, 4724, 4726	*hopD*
1263	*hopAA1′* (frameshift)	19.6	6.03	–		1372, 4718	*hopAA1-1 hopAA1-2*
2351	*hopV1*	17.6	5.37	C	…	4720	…
1296	*hopX1*	17.5	6.16	CR	C	A0012	…
1268	*avrE1*	17.0	4.85	C	…	1377	…
1424	*hopAK1*	16.6	5.08	C	…	4101	…
3498	*hopF3*[g]	16.2	6.06	C	C	0502	*hopF2*
4736	*hopAS1*	15.9	5.79	R	Table 3	0474	*hopAS1′*
0763	*hopAJ1*	15.5	6.15	C	…	0852	…
	…	…	…	…	…	4817	*hopAJ1*
4366	*hopI1*	15.4	6.02	C	C	4776	…
1265-1266	*hopM1′* (frameshift)[h]	14.6	4.97	C	…	1374-1375	…
1264	*hrpW1*	13.9	6.17	C	…	1373	…
2294	*hopAB3′* (frameshift)	13.6	5.75	–	–	3087	*hopAB2*
0767	*hopG1*	11	5.02	C	C	4727	…
avr/hop without DC3000 homolog[i]							
A0113	*avrD1*	23	6.8	CR	Table 3	…	…
A0122	*hopAW1*	19	5.84	C	C	…	…
3028	*avrB4-1*[j]	18.8	5.47	CR	Table 3	…	…
4326	*hopAE1*	17.2	5.62	R	Table 3	…	…
A0120	*avrB2*[k]	16.1	5.75	CR	Table 3	…	…
A0031	*hopAU1*	12.3	5.44	CR	C, Table 3	…	…
A0075	*hopW1-2*	12.1	5.02	C	…	…	…
A0009	*hopW1-1*	12.1	5.02	C	C	…	…
A0056	*hopAV1*	11.6	5.26	–	C	…	…
A0087	*avrRps4*[l]	11.4	4.74	C	…	…	…
Unrelated to T3SS[m]							
1855	ApbE-family protein	22.7	6.25	R	…	2105	…
3759	alcohol dehydrogenase (oxidoreductase, zinc-binding)	0.6	6.3	CR	…	0834	…
1519	SlyA/MarR family transcription regulator	12.5	4.68	R	…	1645	…
Confirmed *hop* with atypical promoter[n]							
5225	*hopAT1*	7.5	4.79	C	C		…
A0127	*hopAB1*[o]	–	4.84	–	C	3087	*hopAB2*

[a] Orthologs PSPTO number or numbers and name, if different.
[b] Genes are ranked within categories by the HMM2 scores. PSPPH locus numbers beginning with "A" indicate genes on the larger plasmid.
[c] Name or predicted function.
[d] Weight matrix analysis.
[e] Evidence for expression or translocation is from C = Chang and associates. (2005), R = real-time polymerase chain reaction, or Table 3, as indicated.
[f] Avirulence (*avr*)/*hop* genes with HrpL-activated DC3000 homologs.
[g] Referred to as *hopF2* by Chang et al, 2005, phylogenetic comparison with the *hopF* family indicates that this gene should be assigned to a new subgroup, *hopF3*.
[h] Analysis of the region downstream indicates that the similarity with other members of the HopM1 family is prematurely truncated by an internal frameshift.
[i] Genes without homologs in DC3000 effector families (Lindeberg et al. 2005).
[j] Referred to as *avrB3* by Chang et al. (2005), phylogenetic comparison with the *avrB* family indicates that this gene should be assigned to a new subgroup, *avrB4-1*, with its non-HrpL regulated homolog (PSPPH0784) assigned the name *avrB4-2*.
[k] Referred to as *avrB2-3* by Chang et al. (2005) in an apparent typo in Table 1 of that work; the actual name is *avrB2*, as indicated in Chang et al. (2005) (Table 2).
[l] Referred to as *hopK1* by Chang et al. (2005), this is in fact *avrRps4* (*hopK1* being a chimeric protein with similarity to *avrRps4* at its N-terminus).
[m] Genes with no apparent role in the type III secretion system (T3SS) that have HrpL-activated homologs in DC3000.
[n] Confirmed *hop* genes with HMM2 scores falling below the cutoff.
[o] Referred to as *hopAB2* by Chang et al. (2005), phylogenetic comparison with the *hopAB* family indicates that this is a member of the *hopAB1* (*virPphA*) subgroup rather than the *hopAB2* (*avrPtoB*) subgroup.

three ORFs that do not have an apparent Hrp promoter preceding them, as well as the housekeeping gene *gap-1* (PSPPH1176). Two genes associated with the *hrp/hrc* cluster, PSPPH1273 (*hrpZ1*) and PSPPH1295 (*hrpK1*), provided positive controls. These genes have been shown in *P. syringae* pv. *phaseolicola* and other *P. syringae* pathovars to be regulated in an HrpL-dependent manner (Alfano et al. 2000; Guttman et al. 2002; Tamaki et al. 1988; Thwaites et al. 2004). All controls behaved as expected, and our data extend the findings of Chang and associates (2005) in establishing the predictive value of the HMM2 analysis.

Translocation of Hop candidates using the Cya and AvrRpt2 reporter systems.

We chose to test several HrpL-activated genes that appeared to encode Hops based on N-terminal T3SS targeting-associated patterns (Guttman et al. 2002; Petnicki-Ocwieja et al. 2002; Schechter et al. 2004). Those tested included candidate Avr/Hops encoded by PSPPH3028 (AvrB4-1), PSPPH4326 (HopAE1), PSPPH4736 (HopAS1), PSPPHA0031 (HopAU1), PSPPHA0113 (AvrD1), and PSPPHA0120 (AvrB2). AvrD1 was chosen for testing because it has been shown to produce syringolide elicitors of a cultivar-specific HR in soybean and, therefore, may not be translocated into plant cells like other typical Avr or effector proteins (Keen et al. 1990). Translational fusions at the C-terminus to Cya enabled testing for translocation based on a calmodulin-dependent increase of cyclic AMP (cAMP) levels in plant cells (Casper-Lindley et al. 2002; Sory and Cornelis 1994). Quantification of cAMP was performed by infiltrating *Nicotiana benthamiana* leaves with *P. fluorescens* 55 containing a plasmid encoding either a wild-type or a nonfunctional T3SS (pLN18 and pCPP3297) and a second plasmid expressing the full-length test protein fused to Cya (pCPP3234) (Schechter et al. 2004).

All six 1448A Avr/Hop proteins, as well as known effector AvrPto1 from *P. syringae* pv. *tomato* (Schechter et al. 2004) produced an increased level of cAMP in leaves when they were expressed with a functional T3SS (Table 3), indicating that these proteins are translocated. Furthermore, PSPPH3028 (*avrB4-1*) and PSPPH4326 (*hopAE1*) are translocated at a level comparable with the well-characterized type III secretion substrate AvrPto1. PSPPHA0113 (AvrD1) and PSPPHA0031 (HopAU1) also can be translocated into plant cells, but at a much lower level. Two proteins unlikely to be Hops also were included in these studies as negative controls; PSPPH1127 is a protein with similarity to a lipoprotein in *Ralstonia solanacearum* and does not have a Hrp promoter preceding the coding sequence, and PSPPH3960 is similar to a predicted signal transduction protein and also lacks a Hrp promoter or target-

Table 3. Translocation tests based on calmodulin-dependent adenylate cyclase activity of *Pseudomonas syringae* pv. *phaseolicola* candidate proteins in planta

	Translocation by *P. fluorescens* (pmol of cAMP per microgram of protein)[a]	
Protein fused to Cya	**pLN18**	**pCPP3297**
PSPPH3028 (AvrB4-1)	170.7 ± 14.2	0.5 ± 0.0
PSPPH4326 (HopAE1)[b]	332.9 ± 8.3	3.8 ± 0.7
PSPPH4736 (HopAS1)	33.8 ± 3.6	0.7 ± 0.1
PSPPHA0031 (HopAU1)	3.6 ± 0.8	0.5 ± 0.1
PSPPHA0113 (AvrD1)	5.3 ± 1.7	1.2 ± 0.6
PSPPHA0120 (AvrB2)	68.1 ± 20.0	0.3 ± 0.0
PSPPH1127 (putative lipoprotein)	0.7 ± 0.3	0.5 ± 0.2
PSPPH3960 (putative regulator)	0.3 ± 0.1	0.3 ± 0.2
AvrPto1	172.3 ± 22.7	–

[a] Cyclic AMP (cAMP) levels in *Nicotiana benthamiana* leaf samples were quantified in triplicate at 7 h postinoculation with *P. fluorescens* (optical density at 600 nm = 0.3) containing a cosmid expressing either a wild-type (pLN18) or Δ*hrcC* mutant (pCPP3287) hypersensitive response and pathogenicity (Hrp) system from *P. syringae* pv. *syringae* 61. A plasmid expressing an AvrPto1-Cya fusion from *P. syringae* pv. *tomato* was used as a positive control. The Cya fusion protein corresponds to the first open reading frame downstream of the respective Hrp promoter. The values presented are the means and standard deviation from three samples. Repeated experiments yielded similar results.

[b] HopAE1-Cya was assayed in a separate experiment in which the AvrPto1-Cya reference yielded 198.3 ± 113.0 pmol of cAMP per microgram of protein.

Table 2. HrpL-dependent expression in rich media of 14 *Pseudomonas syringae* pv. *phaseolicola* 1448A genes downstream of hypersensitive response and pathogenicity (Hrp) promoters, as measured by real-time polymerase chain reaction

PSPPH no.[a]	Name or predicted function	HMM2[b]	Δ(Log concentration)[c]	Fold induction[d]	Induced[e]
1269	Lytic murein transglycosylase	18.1	0.33 ± 0.21	2.14	+
1273	*hrpZ1*	15.9	0.95 ± 0.27	8.99	+
1295	*hrpK1*	17.5	0.68 ± 0.29	4.80	+
1519	SlyA/MarR family	12.5	0.1 ± 0.26	1.25	±
1525	ARM protein	22.6	0.17 ± 0.23	1.49	+
1855	ApbE family	22.7	0.38 ± 0.29	2.41	+
3028	*avrB4-1*	18.8	0.42 ± 0.21	2.60	+
3759	Alcohol dehydrogenase	20.6	0.44 ± 0.18	2.73	+
4326	*hopAE1*	17.2	0.65 ± 0.21	4.42	+
4736	*hopAS1*	15.9	0.23 ± 0.1	1.68	+
A0031	*hopAU1*	12.3	0.31 ± 0.15	2.04	+
A0113	*avrD1*	23	0.53 ± 0.4	3.40	+
A0120	*avrB2*	16.1	0.21 ± 0.1	1.64	+
3960	Putative regulator	–	0.03 ± 0.07	1.07	–
1381	Deoxycytidine triphosphate deaminase	–	0.02 ± 0.09	1.04	–
1127	Lipoprotein	–	0.02 ± 0.03	1.05	–
1176	*gap-1*	–	0.04 ± 0.19	1.09	–

[a] Gene designation corresponds to the PSPPH number of the open reading frame presented in ascending order except for the three negative controls at the bottom.

[b] HMM = hidden Markov model.

[c] Log(Δconcentration) is the difference of the logarithm of relative mRNA concentrations between 1448A strains carrying the plasmid pML122 and pCPP2308, respectively. A housekeeping gene, *gap-1* (PSPPH1176), as well as two genes that are not preceded by an Hrp promoter, (PSPPH1381 and PSPPH1127), were used as negative controls. The values represent the mean and standard deviation from four independent analyses with two biological replicates for each sample. All genes were normalized to the expression of the housekeeping gene *gyrA* (PSPPH3667).

[d] Fold induction was determined by reversing the log transformation: $10^{\Delta \log \text{ concentration}}$.

[e] Symbols: + indicates significant HrpL regulation, – indicates no HrpL-dependent regulation, and ± indicates that evidence of regulation was variable.

ing-associated patterns. As expected, these proteins did not elevate the levels of cAMP in plant cells.

To further test whether PSPPHA0031 (HopAU1) and PSPPHA0113 (AvrD1) are translocated into plant cells, a second reporter system was used. AvrRpt2$_{(81-255)}$ fusions were constructed with full-length PSPPHA0031 and PSPPH0113 proteins as previously described (Petnicki-Ocwieja et al. 2002) and inoculated into *Arabidopsis* accession Columbia (Col-0) wild-type *RPS2* or mutant *rps2* plants. AvrRpt2 is an effector that elicits the HR when delivered into *Arabidopsis* carrying the *RPS2* resistance gene (Mudgett and Staskawicz 1999). Previous studies have shown that a hybrid protein consisting of the biologically active C-terminal portion of the AvrRpt2 protein and a type III N-terminal targeting signal from another Hop can elicit the HR in *Arabidopsis* in a T3SS-dependent manner in wild-type *RPS2* but not in mutant *rps2* plants (Guttman and Greenberg 2001; Mudgett et al. 2000). We found that strains expressing PSPPH0031 and PSPPH0113 fused with AvrRpt2$_{(81-255)}$ elicited the HR in *RPS2 Arabidopsis* plants but not in *rps2 Arabidopsis* plants (Fig. 2). Positive controls in this experiment included a plasmid encoding a full-length AvrRpt2 construct as well as a PSPPH4736(HopAS1)-AvrRpt2$_{(81-255)}$ fusion. PSPPH4736 was chosen as a positive control because it gave conclusive results in the Cya translocation assay (Table 3). Negative controls included a plasmid encoding a hybrid protein of full-length PSPPH1127 (a putative lipoprotein that is not produced in a HrpL-dependent manner and does not have type III targeting signal patterns) as well as AvrRpt2$_{(81-255)}$, which is biologically active in planta but lacks targeting signals for translocation by the T3SS (Mudgett et al. 2000). Designations for all of the plasmids used are listed in Table 4. Because RPS2 protein mediates AvrRpt2 recognition within plant cells (Axtell and Staskawicz 2003; Mackey et al. 2003), the *rps2* mutant provides further control for T3SS-dependent translocation of the test proteins.

Analysis of *P. syringae* pv. *tomato* DC3000 mutants involving genes that are activated by HrpL in both DC3000 and 1448A but do not appear to have a function related to the T3SS.

Two genes in the 1448A HrpL regulon that have homologs in DC3000 that were experimentally confirmed to be activated by HrpL in DC3000 are identified in Table 1. PSPPH1855 (ApbE-family protein) and PSPPH3759 (alcohol dehydrogenase) appear to have no function related to the T3SS and they have predicted enzymatic activities that may provide clues to metabolic adaptations that bacteria make during T3SS-mediated pathogenesis. The association of the two genes with Hrp promoters in two phylogenetically divergent pathovars suggests that they direct responses that are broadly important in *P. syringae* infection. As a first step in the exploration of these genes, we used pKnockout-Ω (Windgassen et al. 2000) to construct mutations in the model strain DC3000, which ablated PSPTO2105 (PSPPH1855 ApbE-family protein) and PSPTO0834 (PSPPH3759 alcohol dehydrogenase homolog), to produce mutants CUCPB5390 and CUCPB5391, respectively. We chose to use DC3000 here because more is known about this strain and we were able to assay for subtle virulence effects in the model plant *Arabidopsis*. PSPTO2105 appears to be in a monocistronic operon and is separated from the downstream PSPTO2106 by 139 nucleotides, a distance that exceeds that of predicted members of the same operon (Moreno-Hagelsieb and Collado-Vides 2002). In contrast, PSPTO0834 is the first gene in what appears to be an operon comprising five genes. The orientation of the pKnockout-Ω insertion in PSPTO0834, as determined by sequencing, would permit transcription of downstream genes from

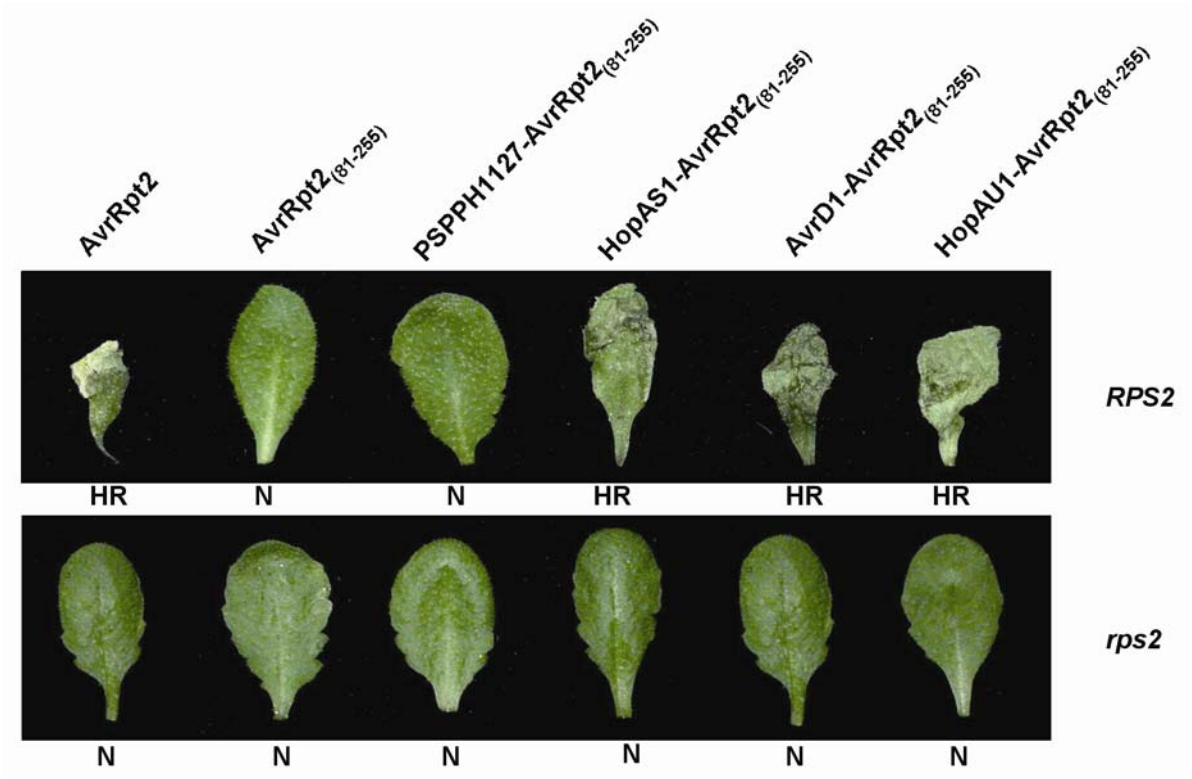

Fig. 2. Assay for hypersensitive response and pathogenicity (Hrp)-dependent translocation of candidate effector proteins. *Pseudomonas syringae* pv. *phaseolicola* 1448A cells carrying the tested proteins fused to AvrRpt2 were infiltrated into *Arabidopsis* Col-0 (*RPS2*) and Col-0-201 (*rps2*) plants. Strains expressing the full-length AvrRpt2 and AvrRpt2$_{(81-255)}$ were used as positive and negative controls, respectively. Plant responses were scored 24 hrs after inoculation for hypersensitive response (HR) or no visible response (N).

an outreading *lac* promoter (Windgassen et al. 2000). *Arabidopsis* plants were inoculated by vacuum infiltration with DC3000, CUCPB5390, and CUCPB5391 at 1×10^6 CFU/ml. PSPTO2105 (ApbE homolog) mutant CUCPB5390 produced noticeably reduced symptoms at 4 and 8 days postinoculation, and both mutants showed reduced growth in planta (Fig. 3).

DISCUSSION

We have comprehensively identified *P. syringae* pv. *phaseolicola* 1448A Hrp promoters and type III effectors using two bioinformatic tools that were developed through previous analysis of the model pathogen *P. syringae* pv. *tomato* DC3000. The HMM tool enabled us to identify 44 high-probability Hrp promoters, and T3SS targeting signal patterns enabled us to identify 27 candidate effectors and related T3SS substrates. The inventory of predicted promoters and effectors generally is congruent with that found through a near-saturation DFI-based screen for type III effectors in *P. syringae* pv. *phaseolicola* (Chang et al. 2005). However, we have gained evidence for three additional 1448A proteins that can be translocated into plant cells by the T3SS. The majority of the Hrp promoters in

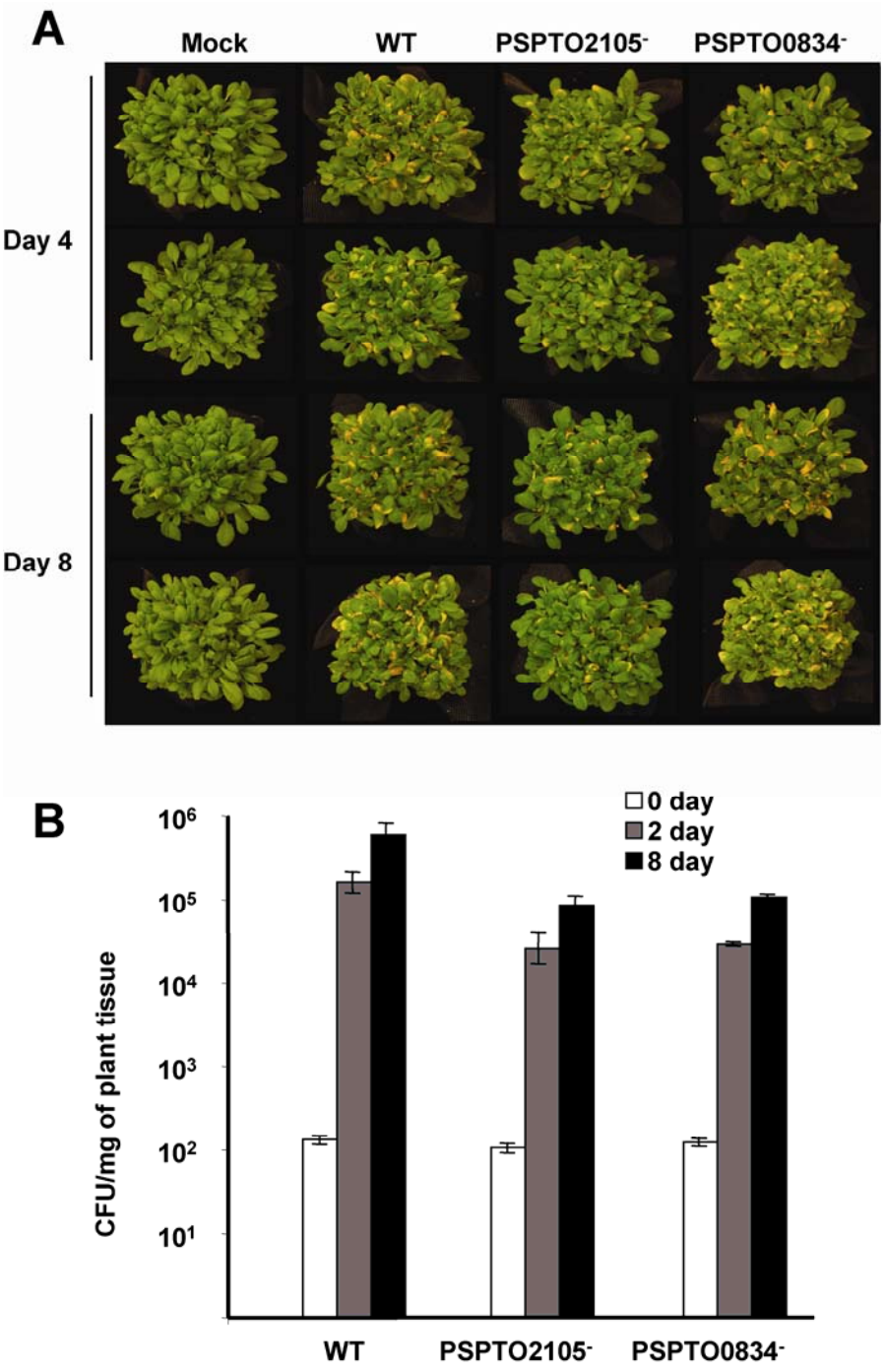

Fig. 3. Assay for reduced virulence in *Arabidopsis* of *Pseudomonas syringae* pv. *tomato* DC3000 mutants deficient in PSPTO2105 (PSPPH1855 ApbE homolog) and PSPTO0834 (PSPPH3759 alcohol dehydrogenase homolog). Arabidopsis Col-0 plants were inoculated by vacuum infiltration of wild-type DC3000, CUCPB5390 (PSPTO2105::pKnockout-Ω), and CUCPB5391 (PSPTO0834::pKnockout-Ω) at 1×10^6 CFU/ml. **A,** Representative symptoms 4 and 8 days after inoculation. **B,** Bacterial growth at 0, 2, and 8 days after inoculation. Results are the mean and standard deviation of six samples from three plants. The experiment was repeated four times with similar results.

DC3000 activate genes directing the T3SS, effectors, and related substrates, and only 10 appear to have functions not directly related to the T3SS. Genes that function independently of the T3SS and have been experimentally confirmed to be in the HrpL regulons of both DC3000 and 1448A were of particular interest. Our data, along with the microarray analysis of DC3000 (Ferreira et al. 2006) and the DFI screens of both DC3000 and 1448A (Chang et al. 2005), permit comprehensive comparison of the HrpL regulons and effector inventories of these two strains.

The three novel effectors we identified in 1448A were PSPPH4326 (HopAE1), PSPPH4736 (HopAS1), and PSPPHA0113 (AvrD1). The relatively low induction ratio observed for the *hopAS1* promoter is consistent with it being missed in the DFI screen of Chang and associates (2005). In contrast, *avrD1* was detected in the DFI screen, but an AvrD1-AvrRpt2$_{(81-255)}$ fusion driven by its native promoter failed to elicit an HR in *Arabidopsis* that would be indicative of translocation (Chang et al. 2005). Here, we gained evidence that AvrD1 can be translocated by using assays that employ a *tac* promoter and both the Cya and AvrRpt2$_{(81-255)}$ translocation reporters. *hopAE1* was not detected by the DFI screen but is strongly induced by HrpL, and its product is strongly translocated. Furthermore, its homolog in *P. syringae* pv. *syringae* B728a was shown to be translocated (Vinatzer et al. 2005); therefore, HopAE1 represents an effector that is lacking from DC3000 but present in two divergent pathovars that are pathogens of bean.

AvrD1 warrants further discussion because it is unlike other known *P. syringae* Avr proteins in its production of a low molecular weight cultivar-specific elicitor of the HR (Keen et al. 1990). AvrD1 directs the production of syringolides that can be isolated from culture fluids of *Escherichia coli* or *P. syringae* cells expressing *avrD1*, and these syringolides elicit the HR in cultivars of soybean that are resistant to races of *P. syringae* pv. *glycinea* that naturally carry *avrD1* (Keen et al. 1990; Midland et al. 1995; Smith et al. 1993). Bacteria expressing *avrD1* do not need the Hrp T3SS to produce active elicitor in their culture fluids but, interestingly, *P. syringae* pv. *glycinea* needs the Hrp T3SS to elicit an *avrD1*-dependent HR when bacterial cells, rather than culture fluids, are introduced into sensitive soybean cultivars (Keen et al. 1990). In contrast, *E. coli* cells expressing *avrD1* without the Hrp T3SS could elicit the HR in test soybean cultivars, apparently because of stronger expression of *avrD1* in *E. coli*. These results raise the possibility that AvrD1 can produce syringolides from common metabolites in either the bacterium or the plant; and, in natural infections involving T3SS-proficient *P. syringae* pv. *glycinea,* it is the syringolide produced in plant cells by translocated AvrD1 that is biologically relevant. Our data do not demonstrate whether AvrD1 can produce syringolides within plant cells, but they do demonstrate, with two different reporters, that AvrD1 can be translocated into plant cells by the T3SS. Regarding the AvrRpt2$_{(81-255)}$ reporter, it is important to note that the failure of the AvrD1-AvrRpt2$_{(81-255)}$ hybrid to elicit the

Table 4. Strains and plasmids used

Strain or plasmid	Genotype or relevant phenotype[a]	Source or reference
Escherichia coli strains		
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL endA1 nup*G	Invitrogen
E. coli DH5α	*supE44 DlacU169(ϕ80lacZDM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*, Nx^r	Life Technologies; Hanahan 1983
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
Pseudomonas syringae		
pv. *phaseolicola* 1448A	Wild type, Rif^r	Joardar et al. 2005
pv. *tomato* DC3000	Wild type, Rif^r	Cuppels 1986
CUCPB5390	PSPTO2105::pKnockout-Ω derivative of DC3000 (using 768-bp internal fragment) Sp^r, St^r	This work
CUCPB5391	PSPTO0834::pKnockout-Ω derivative of DC3000 (using 536-bp internal fragment) Sp^r, St^r	This work
P. fluorescens 55	Wild type, Nx^r, Sp^r	Huang et al. 1988
Plasmids		
pLN18	pLAFR3 derivative containing *P. syringae* 61 *hrc-hrp* cluster with *shcA* and *hopPsyA* replaced by an *nptII* cassette, Tc^r, Km^r	Jamir et al. 2004
pLN921	pBBR1-MCS5 derivative containing '*avrRpt2* lacking N-terminal 80 residues, Gm^r	Petnicki-Ocwiega et al. 2005
pAvrRpt2-600	pDSK600 derivative containing full-length *avrRpt2*, Sp^r/Sm^r	Pirhonen et al. 1996
pML122	RSF1010-derived broad-host-range expression vector, Gm^r	Labes et al. 1990
pCPP2308	pML122 carrying *hrpL* from *P. syringae* pv. *syringae* 61, Gm^r	David Bauer, Cornell University
pCPP3297	pLN18 containing an unmarked deletion in *hrcC,* Tc^r, Km^r	Schechter et al. 2004
pCPP3234	pVLT35 containing Gateway reading frame B cassette and codons 2 to 406 of *cya*, Sp^r/St^r Cm^r	Schechter et al. 2004
pCPP5186	pCPP3234 expressing PSPPH3960-Cya, Sp^r/St^r Cm^r	This study
pCPP5187	pCPP3234 expressing PSPPH3028-Cya, Sp^r/St^r Cm^r	This study
pCPP5188	pCPP3234 expressing PSPPH1127-Cya, Sp^r/St^r Cm^r	This study
pCPP5189	pCPP3234 expressing PSPPH4736-Cya, Sp^r/St^r Cm^r	This study
pCPP5191	pCPP3234 expressing PSPPHA0113-Cya, Sp^r/St^r Cm^r	This study
pCPP5192	pCPP3234 expressing PSPPHA0120-Cya, Sp^r/St^r Cm^r	This study
pCPP5193	pCPP3234 expressing PSPPHA0031-Cya, Sp^r/St^r Cm^r	This study
pCPP3221	pCPP3234 expressing AvrPto(1-164)-Cya, Sp^r/St^r Cm^r	This study
pLN1364	pLN921 expressing PSPPH1127-AvrRpt2$_{(81-255)}$, Gm^r	This study
pLN1403	pLN921 expressing PSPPH4736-AvrRpt2$_{(81-255)}$, Gm^r	This study
pLN1365	pLN921 expressing PSPPHA0113-AvrRpt2$_{(81-255)}$, Gm^r	This study
pLN1366	pLN921 expressing PSPPHA0031-AvrRpt2$_{(81-255)}$, Gm^r	This study
pKnockout-Ω	Sp^r, St^r *mob* in T-vector	Windgassen et al. 2000
pKnockout-Ω::PSPTO0834	Carries 539-bp internal fragment of PSPTO0834; Sp^r, St^r	This study
pKnockout-Ω::PSPTO2105	Carries 768-bp internal fragment of PSPTO2105; Sp^r, St^r	This study

[a] Nx^r, Rif^r, Gm^r, Km^r, Sp^r, Tc^r, Str^r, Cm^r, and Ap^r = nalidixic acid, rifampicin, gentamicin, kanamycin, spectinomycin, tetracycline, streptomycin, chloramphenicol, and ampicillin resistant, respectively.

HR in *rps2 Arabidopsis* plants eliminates the possibility that the HR observed in *Arabidopsis* by the hybrid is the result of AvrD1-dependent syringolide production in the bacterium. Future work will be required to determine whether the apparently low level of AvrD1 that can be translocated into plant cells is physiologically significant.

The "shikimate kinase-like" proteins encoded by PSPPH1525 and PSPPHA0133 also warrant discussion. Although both are similar to the mlr6331 and mlr6361 proteins of *Mesorhizobium loti* (MAFF303099), which contain AroK-like domains (COG0703) at the extreme C-terminus, this domain clearly is absent from PSPPH1525 and PSPPHA0133. However, the PSPPH1525 and PSPPHA0133 proteins contain an ARM-like domain (InterPro IPR011989), as do highly similar proteins from *Xanthomonas* and *Ralstonia* spp. The presence of upstream Hrp promoters and of two of the three major targeting-associated patterns suggests that these two ARM-like proteins in 1448A could be T3SS substrates. *Ralstonia solanacearum* proteins containing ARM-like domains are similarly believed to be T3SS effectors (Gabriel et al. 2006). However, because of the large size of PSPPH1525 and PSPPHA0133, we were unable to construct full-length Cya fusions and did not test the ability of these proteins to travel the T3SS.

Our current knowledge of the composition and function of the type III effector inventories of *P. syringae* strains indicates a mix of conserved and polymorphic effectors and a primary role of effectors in suppressing basal resistance and manipulating cell death pathways in plants (Alfano and Collmer 2004; Nomura et al. 2005). However, we have little knowledge of other factors that may have a role in either basic parasitism or host specificity. We looked for clues in other genes in the HrpL regulon that appear to function independently of the T3SS. The HrpL regulons of DC3000 and 1448A revealed several interesting differences regarding toxins, phytohormone metabolism, and plant cell-wall-degrading enzymes. Coronatine biosynthesis genes are activated by HrpL in DC3000, but there is no evidence that genes associated with phaseolotoxin or any other phytotoxin are activated by the HrpL regulon of 1448A. Similarly, *iaaL* is in the HrpL regulon of DC3000 but not 1448A (Chang et al. 2005; Fouts et al. 2002). On the other hand, we have found that two polygalacturonases are associated with Hrp promoters in 1448A. Although coronatine has an established role in the virulence of DC3000 (Brooks et al. 2004; Mittal and Davis 1995), there is no evidence that coronatine or any of these factors are involved in the host specificity of any *P. syringae* pathovars.

Genes unrelated to the T3SS that nevertheless are common to the HrpL regulons of divergent pathovars may have a role in basic parasitism in all plants. We explored two pairs of such genes: PSPPH1855/PSPTO2105 (ApbE-family) and PSPPH3759/PSPTO0834 (alcohol dehydrogenase). PSPPH1855 was missed by the DFI screen (Chang et al. 2005), but it is preceded by a Hrp promoter and was confirmed to be HrpL activated by real-time PCR. Furthermore, the DC3000 homolog was identified in an in vivo expression technology (IVET) screen for genes expressed during infection of *Arabidopsis* (Boch et al. 2002), and it was also shown to be coregulated with *hrp* genes in culture (Zwiesler-Vollick et al. 2002). Furthermore, a similar ApbE-family protein gene in *P. syringae* pv. *syringae* B728a was found to be induced during epiphytic growth on healthy leaves (Marco et al. 2005), which suggests that these proteins may be broadly important in *P. syringae*-plant interactions.

The PSPPH1855/PSPTO2105 ApbE-family proteins may play a role in protecting the bacteria from oxidative stress or other stresses found in the apoplastic environment. The *Salmonella enterica* serovar typhimurium homolog, ApbE, is a periplasmic protein that is conditionally required for thiamine biosynthesis (Beck and Downs 1998, 1999), and there is evidence that the protein plays a role in ThiH Fe-S cluster repair to protect the bacterial cell from oxidative stress (Gralnick et al. 2000; Martinez-Gomez et al. 2004; Skovran and Downs 2003). The ApbE protein from *P. syringae* has a lipoprotein signal peptide detectable by LipoP. The predicted cleavage site is between residues 23 and 24. By analogy to ApbE, it is reasonable to presume that the mature PSPTO2105 gene product is localized to the periplasmic side of the inner membrane. However, the metabolic role of ApbE-family proteins in *P. syringae* and *S. typhimurium* appears to be quite different: the *apbE* mutant in DC3000 grows well without thiamine (data not shown), whereas the *apbE* mutant in *S. typhimurium* does not grow in the absence of thiamine. Interestingly, homologs of PSPPH1855/PSPTO2105 are found in *P. aeruginosa* and *P. fluorescens*, but they are not preceded by Hrp promoters.

The putative alcohol dehydrogenase encoded by PSPPH3759/PSPTO0834 does not have a signal peptide detected by SignalP or LipoP. However, consensus methods such as Meta suggest that it assumes a three-dimensional structure resembling proteins from both eukaryotic and prokaryotic sources. The structure for a closely related protein from *P. aeruginosa* is available. Members of this class of proteins have a GroES-like N-terminal domain and an NAD(P)-binding Rossmann-fold domain at the C-terminus. These proteins catalyze redox reactions involving a broad spectrum of alcohols. The structural predictions are insufficient to make detailed predictions regarding substrate specificity. PSPPH3759 and PSPTO0834 are located at the head of putative polycistronic operons containing four genes, but the existing annotation of the remaining genes does not provide any insight into their individual or collective roles. Orthologs of these four genes are missing from *P. syringae* pv. *syringae* B728a, *P. aeruginosa*, and *P. fluorescens*; interestingly, these genes are flanked by different mobile genetic elements in 1448A and DC3000.

We observed no reduction relative to wild-type DC3000 in the growth in King's B (KB) or Hrp minimal medium of the PSPTO2105::pKnockout-Ω mutant or the PSPTO0834::pKnockout-Ω mutant (data not shown); however, both mutants were reduced in their ability to grow in *Arabidopsis*, and the PSPTO2105::pKnockout-Ω mutant produced symptoms that were discernibly milder upon repeated testing. Because of the relatively small reductions in growth and virulence, it is unlikely that these mutants would be detected in a screen for reduced virulence mutants. Thus, they demonstrate the power of using bioinformatic methods to identity candidate genes worthy of close scrutiny.

The data presented here for 1448A suggest that the bioinformatic methods honed with DC3000 are robust enough to predict accurately most of the effectors in any *P. syringae* strain and that this approach is more comprehensive and efficient than genetic screens, such as the DFI screen of Chang and associates (2005). The HMM2 analysis identified 38 of the 40 Hrp promoters in 1448A that were identified by DFI, plus 3 that were not detected by DFI but were experimentally confirmed here: an ApbE-domain protein, HopAE1, and HopAS1. The two promoters we missed, which are upstream of *hopAB1* and *hopAT1*, are preceded by atypical Hrp boxes, whose HMM2 scores are below the cutoff that captures all of the other effector genes. However, the WM2 scores for these promoters are above the cutoff that was developed based on our microarray-based analysis of the DC3000 HrpL regulon (Ferreira et al. 2006). Thus, they also can be identified by a comprehensive bioinformatic approach.

It also is important to note limitations in the bioinformatics approach that resulted in us missing three of the effectors

found by Chang and associates (2005) during our analysis of the draft genome of 1448A: GLIMMER failed to identify the HopAW1 and HopAV1 ORFs, and the HopAT1 size was below the cutoff we used to identify candidate effectors. Importantly, the correct identification of start codons is a limiting factor in analyzing potential N-terminal targeting-associated patterns. Type III effector genes are particularly problematic for gene-finding programs because these genes often reside in genomic islands and islets with atypical percent GC content and codon usage. However, one useful lesson that we have learned from our analyses of the HrpL regulons of DC3000 and 1448A is that almost all high-probability Hrp promoters are within 200 bp of the start of an ORF. Thus, these promoters provide useful reference points for manual annotation of ORFs following automated gene finding. In summary, the bioinformatic protocol that we developed with the model *P. syringae* strain DC3000 and then tested here with 1448A should enable efficient identification of almost all type III effector genes and other interesting HrpL-regulated virulence genes in any strain of *P. syringae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The strains and plasmids used are listed in Table 4. All *E. coli* strains were grown in Luria-Bertani (LB) (Sambrook et al. 1989) or LM media (Hanahan 1983) at 37°C. All *Pseudomonas* strains were grown in KB (King et al. 1954) or *hrp*-derepressing fructose minimal medium (Hrp MM) (Huynh et al. 1989). For the real-time PCR and Cya translocation assays, *Pseudomonas* strains were grown at 30°C and, for the AvrRpt2 translocation assay, *Pseudomonas* strains were grown at 28°C. For the real-time PCR and Cya translocation assays, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; rifampicin, 50 μg/ml; and tetracycline, 10 μg/ml. Because *P. fluorescens* 55 is resistant to spectinomycin, plasmids were maintained in this strain by using streptomycin at 100 μg/ml and tetracycline at 40 μg/ml. For the AvrRpt2 translocation assay, antibiotics were used at the following concentrations: kanamycin, 100 μg/ml; chloramphenicol, 20 μg/ml; gentamicin, 10 μg/ml; rifampicin, 100 μg/ml; and spectinomycin, 20 μg/ml.

Plant material.

N. benthamiana plants were grown under greenhouse conditions and transferred to the laboratory 1 day prior to inoculation. During Cya translocation assays, *N. benthamiana* plants were maintained at 24°C with 10 h of light per day. *Arabidopsis thaliana* ecotype Columbia (Col-0) plants were grown in a growth chamber at 24°C with 10 h of light per day.

Bioinformatic methods: Predicting operons.

Preliminary operon predictions were made by using simple heuristic rules based on intergenic distances (Salgado et al. 2000) from the initial genome annotation (Joardar et al. 2005) and locations of REP sequences identified by HMMer (Tobes and Pareja 2005) and rho-independent transcription terminator sequences identified by TransTerm (Ermolaeva et al. 2000). Annotated genes on the same strand were assumed to be in the same operon if they were separated by less than 65 bp with no intervening transcription terminator or REP sequence (G. Moreno-Hagelsieb, *personal communication*). Genes on opposite strands or separated by larger distances or intervening transcription terminator or REP sequences were assigned to separate operons. In most cases, these rules are consistent with more sophisticated prediction methods applied to the *P. syringae* pv. *tomato* DC3000 genome (Karp et al. 2005; Price et al. 2005). In a small number of instances, there is experimental data for operon structure in DC3000 and strong sequence conservation between the corresponding regions of 1448A and DC3000; it was assumed that the corresponding operons in 1448A and DC3000 had the same structure.

Bioinformatic methods: Hrp promoter searches using HMM and WM models.

The level 2 training set from the accompanying article by Ferreira and associates (2006) was used to identify putative Hrp promoters in the *P. syringae* pv. *phaseolicola* 1448A genome sequence (Joardar et al. 2005). The methods of Fouts and associates (2002) and Fereirra and associates (2006) were modified as necessary. The overall bioinformatics workflow is schematically represented in Figure 1. Very briefly, the separately aligned subsets with 16- and 17-bp spacers between the nominal "–35 box" and "–10 box" were used to construct position-sensitive weight matrices (PSWM) using a whole-genome nucleotide dimer-based statistical background model and a suite of custom Python/BioPython scripts. PSWM scores are reported in relative units—the deviation from the mean divided by the standard deviation. The combined, aligned level 2 training set of Ferreira and associates (2006) and a whole-genome nucleotide monomer-based statistical background model were used to construct a calibrated HMM for scanning the 1448A genome sequence.

The orientation and distance to the nearest downstream gene were determined by examining the context in the annotated genome sequence. In some cases, putative Hrp promoters were identified upstream of anomalously long 5′ untranslated regions or regions containing transposable elements. In these cases, very careful comparative analyses were conducted using *P. syringae* pv. *tomato* DC3000, *P. syringae* pv. *syringae* B728a, and other strains of *P. syringae* as well as *Yersinia*, *Xanthomonas,* and *Ralstonia* spp. to identify inconsistencies or omissions in the 1448A genome annotation. New gene calls were made where substantial sequence conservation was detected with respect to putative genes in other species, strains, or elsewhere in the 1448A genome. The majority of changes could be traced to the association of Hrp promoters with regions of atypical nucleotide composition and proximity to transposable elements. The unusual nucleotide composition causes problems with most de novo gene-finding methods, and the appearance of small gene fragments created by transposition events presents severe difficulties for even expert annotators. Finally, the first examples of T3SS effector gene families are difficult to identify by sequence similarity searches because T3SS effectors tend to have unusual primary structure. In view of these facts, it is likely that the annotation of the regions containing Hrp promoters and genes encoding T3SS effectors will continue to be revised as genomes of related plant pathogens become available.

Putative Hrp promoters were required to have HMM scores greater than 10.0 or at least one PSWM score greater than 4.0, be located in an intergenic region, have the correct orientation with respect to the nearest downstream gene, and be within 220 bp of the translation start codon for that gene. The putative and experimentally validated Hrp promoters have been submitted to National Center for Biotechnology Information as updates to the genome annotation.

Bioinformatic methods: Identifying T3SS substrates via structure or composition rules.

Novel *hop* genes were identified by analyzing the first two ORFs downstream of Hrp promoters for at least two of the following targeting-associated patterns in the N-terminal regions of the predicted proteins: i) lack of acidic amino acids in the first 12 residues; ii) an Ile, Leu, Val, or Pro (or sometimes Ala) residue in position 3 or 4 but not in both, and often preceded

by a Pro, polar, or basic amino acid; and iii) at least 10% Ser in the first 50 amino acids. Additional evidence for novel *hop* genes was sought through identification of associated class 1A chaperones based on the presence of such a gene in the same operon as the candidate *hop* gene and on characteristics of the candidate chaperone: small size (10 to 20 kDa), acidic isoelectric point, and C-terminal amphipathic region (Parsot et al. 2003).

Bioinformatic methods: Identifying T3SS effectors via protein-based similarity searching.

Homologs in the *P. syringae* pv. *phaseolicola* 1448A genome of known effector genes in other strains of *P. syringae* were identified through tblastx of the 1448A genome using a single representative from each phylogenetically defined effector family and, where applicable, from each subfamily. An ORF was defined for each significant hit (*e* value < 10^{-5}), and a comparison performed with the original query to identify truncated and degenerate loci. The designations for *avr*/*hop* genes and proteins in this report employ the recently adopted nomenclature system of Lindeberg and associates (2005), as maintained at the *Pseudomonas*–Plant Interaction website. Note that the 1448A HrpA pilus protein is now designated HrpA2 to reflect groupings revealed by a recent phylogenomic analysis of *hrpA* genes in multiple pathovars of *P. syringae* (Inoue and Takikawa 2006).

RNA preparation.

All RNA extractions were performed using RNase-free tubes, filter tips, and equipment treated with RNAzap (Ambion, Austin, TX, U.S.A.). Real-time PCR grade water (Ambion) also was used for all procedures. A 2-ml overnight culture of each strain was grown in KB supplemented with rifampicin at 50 μg/ml and kanamycin at 50 μg/ml. The cultures were re-inoculated into 10 ml of KB media supplemented with rifampicin at 50 μg/ml and kanamycin at 50 μg/ml to an optical density at 600 nm (OD_{600}) of 0.1 and allowed to grow to an OD_{600} of 0.2. One milliliter of culture then was centrifuged at 13,000 × *g* for 5 min, the supernatant was removed, and the cell pellet was flash frozen in liquid nitrogen. Total RNA was prepared from cell pellets using the SV Total RNA Isolation System from Promega according to the manufacturer's instructions. An additional DNase I digestion step was performed with DNA-*free* (Ambion) according to the rigorous DNase treatment included in the manufacturer's instructions. This additional DNase step was necessary to remove trace DNA contamination in the RNA preparation. RNA was further purified using the RNeasy Minikit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Quantification and sample integrity of RNA samples was assessed using spectrophotometer readings of absorbance at 260 nm (A_{260}) and the $A_{260/A280}$ ratio, respectively.

Real-time PCR.

Real-time PCR was performed by using the ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.) and iTaq SYBR Green Supermix with Rox (Bio-Rad, Richmond, CA, U.S.A.) following the manufacturer's protocols. Total RNA (100 ng) extracted from strains of *P. syringae* pv. *phaseolicola* 1448A was reverse transcribed in a thermocycler using a cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. The resulting total cDNA population (1 μl) was mixed with 0.3 μM concentrations of each primer (Table 5) and 12.5 μl of master mix in a 25-μl final volume. The PCR assay was carried out with one cycle at 95°C for 2.5 min followed by 35 cycles of 95°C for 15 s and 60°C for 30 s. The amount of fluorescence that resulted from the incorporation of the SYBR Green dye into double-stranded DNA was measured at the end of each cycle to determine the kinetics of PCR for each sample. DNA contamination and the formation of primer dimers were assessed by using controls lacking reverse transcriptase and template, respectively. The

Table 5. Primers for SYBR Green real time polymerase chain reaction (PCR) experiments (sequences are 5′ to 3′)

Target	Forward (upstream) primer	Reverse (downstream) primer
For real-time PCR		
PSPPH1269	GCCAATGATCGAAAGCTCCTAC	AAAGATTGAAGCTGCGCCC
PSPPH1273	ACCGGACAACACGACTTGATG	TCGGAAAAACGAGTTCCGTC
PSPPH1295	CCCGATGTTCAGGAGTACCTCA	TTTGAACCTGCTCAGTCACGG
PSPPH1525	GCTCAAGGCTTACAGCATGGT	TCAACCCATTTCCCCAACTC
PSPPH1855	ATGCATTGCCTGCCAATAGC	TCAGGTCGAAAGCACCATCAC
PSPPH3028	TCTCCCCAGTGGCATAATTCA	TTCCGGTGATCCAGACATAGC
PSPPH3759	TTGCGAACCTTGCCGTCTT	CGCTCGGCAAAAGTTCCAT
PSPPH3960	GAAGATCGCGACGGTTTTGA	CGATCAGTGCCAACAGCAGTAA
PSPPH4326	TACGGCATGGGTTTCGATG	GGCACTTGGCAAATCACCA
PSPPH4736	CTGTCGCAGGTGACCTTAAAA	CCGGCTTATCCCATATCTGTGA
PSPPHA0031	AGACCTCAGCCAATTGCAGAC	CGTCGTGTACCCGAAAAGTTT
PSPPHA0113	AGTTTATCATTCCCGCCGCT	CGTCGATGAATTGAGTGCGA
PSPPHA0120	AATTGCTGGTGGCTCCATAGC	GCATCGCCCGAAAGAATTCT
PSPPH3667 (*gyrA*)	GGTCGTGGACGCATTTACATG	TAAGCTGGTACGGCAGTTCGGT
PSPPH1176 (*gap1*)	GTGCCCGCAAGGTCATTATCTC	AGATGATCTGGTGCGACTGACG
PSPPH1381	CGCAGACGAATTCAAGGTGTT	GCATACGTCGCTCTTGATATCG
PSPPH1127	CCAATGCCAACGATATTCAGG	ACTGGTGCCGTATTGCTTGAC
PSPPH1127	CCAATGCCAACGATATTCAGG	ACTGGTGCCGTATTGCTTGAC
For cloning into pENTR/SD/D-TOPO		
PSPPH3028	CACCATGGGATGCATTACTTCA	ATCATCAAACATTGACGGATACTG
PSPPH4326	CACCATGCGTGAAGATAAACACCC	TGCCGACTCCTCCGAGGTAG
PSPPH4736	CACCATGACCTTAAGAATCAATACTC	CGGCGGCGGAAACTGCG
PSPPHA0031	CACCGTGATTTTCGAACACTCATTGG	TTCCTGCTGCGTGCGGGCAC
PSPPHA0113	CACCATGCAAGACCTTAGCTT	GGGATTCTCGGCGATAA
PSPPHA0120	CACCATGGGAAATGTTTGTTTCCG	CTGAGGGGGCCGCTCAAAAA
PSPPH1127	CACCATGAACCTGCGTTATCTGAA	GTCAGTACCGTACTTCGGCG
For mutagenesis using pKnockout-Ω		
PSPTO0834	ACACCTACAAAAAGGGATGCGGGT	ACAATTCTCCCACCGCGACC
PSPTO2105	GTCGATCAACAGATGTCGCT	GCTTATCGGCACGAATCACA

production of nonspecific products was determined by the dissociation protocol included in the software provided with the ABI 7000 real-time PCR machine. The resulting cycle threshold (Ct) values were calculated by the ABI 7000 software and analyzed using the relative standard curve method (separate tubes) described in ABI User Bulletin no. 2. In each strain, the Ct values of each gene tested were normalized to the Ct values of two housekeeping genes *gyrA* (PSPPH3667), and *gap1*(PSPPH1176) separately, and these values then were averaged to obtain relative expression data for each gene.

Adenylate cyclase assay.

Plasmids expressing C-terminal fusions of *P. syringae* pv. *phaseolicola* 1448A proteins with Cya were constructed and verified according to the methods described by Schechter and associates (2004). Briefly, candidate genes were cloned into the pENTR/SD/D-TOPO vector (Invitrogen, Carlsbad, CA, U.S.A.) following amplification off of genomic DNA using the primer pairs in Table 5. Plasmids containing candidate genes fused in frame to *cya* were constructed by recombining entry vectors with pCPP3234 using Gateway cloning technology (Invitrogen). The plasmids expressing the *cya* fusions were mated into *P. fluorescens* 55 isolates containing either pLN18 or pCPP3297. The *P. fluorescens* 55 strains expressing the Cya fusion proteins were grown on KB plates containing tetracycline at 40 μg/ml and streptomycin at 100 μg/ml and grown at 30°C for 2 days. In preparation for inoculation, bacterial cultures then were scraped and resuspended in 5 mM morpholinoethanesulfonic acid (MES), pH 5.5, supplemented with tetracycline at 40 μg/ml and streptomycin at 100 μg/ml with 100 μM IPTG, to an OD_{600} of 0.3. Inoculation of 1-month-old *N. benthamiana* plants and subsequent assay of adenylate cyclase activity both in plants at 7 h postinoculation and in *E. coli* strains during plasmid construction was performed according to the methods described by Schechter and associates (2004) using the Correlate-EIA cAMP immunoassay kit (Assay Designs, Ann Arbor, MI, U.S.A.) according to the manufacturer's instructions.

AvrRpt2 translocation assays.

The candidates tested were cloned into pLN921, which expresses AvrRpt2 missing the first 80 residues. These constructs were electroporated into *P. syringae* pv. *phaseolicola* 1448A. The strains were infiltrated into *Arabidopsis* Col-0 and *rps2* plants at an OD_{600} of 0.4 in 5 mM MES, pH 5.6, by using a blunt syringe. Plant responses were scored 24 h after infiltration.

Construction of mutant strains.

Genes were disrupted in *P. syringae* pv. *tomato* DC3000 using pKnockout-Ω (Windgassen et al. 2000). Internal fragments of PSPTO0834 and PSPTO2105 were amplified using Extaq polymerase (Takara, Tokyo) using 1 μl of each forward and reverse primer (Table 5), 12.5 μl of Extaq, and 1 μl of DC3000 genomic DNA in a 25-μl reaction volume. An annealing temperature of 58 and 52°C was used for PSPTO0834 and PSPTO2105, respectively, and a 1-min extension at 72°C was used to amplify both DNA fragments. The resulting PCR products were resolved on a 0.8% agarose gel, excised, and cleaned using the Qiagen gel extraction kit (Qiagen). Each DNA fragment (60 fmol) then was ligated to 20 fmol of *Xcm*I-digested pKnockout-Ω plasmid using T4 ligase (Invitrogen) for 1 h at room temperature according to the manufacturer's instructions. The resulting ligation products were electroporated into *E. coli* DH5α cells, which were plated on LB media supplemented with spectinomycin and X-gal to select for the desired transformants. Putative transformants were checked by colony PCR for recombinant plasmids using the initial insert-specific primers. Plasmids were extracted from overnight cultures of *E. coli* DH5α using the QIAprep Spin Miniprep Kit (Qiagen). Plasmid DNA (1 μg) was electroporated into 50 μl of DC3000 electrocompetent cells with 2 μl of boiled salmon sperm DNA. After a 2-h recovery period in liquid KB media, the cells were plated on KB plates supplemented with rifampicin and streptomycin to select for transformants. pKnockout plasmids are not able to replicate in DC3000; therefore, any colonies that grow on these plates have undergone a single crossover into the genome at the point of homology, thereby disrupting the target gene. Putative mutants were checked for disruption in the genome using PCR and primers specific for an area within the genome and the pKnockout-Ω plasmid.

Virulence assay.

Wild-type DC3000 and mutant strains were grown for 2 days at 30°C on KB plates supplemented with rifampicin (50 μg/ml). The cells were resuspended to approximately 10^6 CFU/ml in water containing 0.01% Silwet and vacuum-infiltrated into 3-week-old *Arabidopsis* Col-0 plants. The infected plants were kept in a humid growth chamber at 25°C with a 14-h light cycle and were watered every 2 days. The plants were observed daily to follow the development of disease symptoms. In addition, leaf-associated bacteria were counted on days 0 (immediately after infection), 2, and 8 by homogenizing the leaves in 10 mM $MgCl_2$ buffer and spreading on KB plates supplemented with rifampicin (50 μg/ml) and cycloheximide (2 μg/ml). Mock infection without bacteria was included as a negative control.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The BioInfoBank Meta server: bioinfo.pl/Meta
Center for Biological Sequence Analysis (CBS) LipoP server: www.cbs.dtu.dk/services/LipoP
National Center for Biotechnology Information (NCBI) LipoP webpage: www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=pubmed&dopt=Abstract&list_uids=12876315&itool=iconabstr
Protein Data Bank (PDB) database: pdbbeta.rcsb.org/pdb/explore.do?structureId=1llu
Pseudomonas–Plant Interaction website: pseudomonas-syringae.org