***Pseudomonas syringae* pv. *tomato* DC3000 Type III Effector HopAA1-1 Functions Redundantly with Chlorosis-Promoting Factor PSPTO4723 to Produce Bacterial Speck Lesions in Host Tomato**

Kathy R. Munkvold, Alistair B. Russell, Brian H. Kvitko, and Alan Collmer

Department of Plant Pathology and Plant-Microbe Biology, Cornell University, Ithaca NY 14853, U.S.A.

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The ability of *Pseudomonas syringae* pv. *tomato* DC3000 to cause bacterial speck disease in tomato is dependent on the injection, via the type III secretion system, of approximately 28 Avr/Hop effector proteins. HopAA1-1 is encoded in the conserved effector locus (CEL) of the *P. syringae* Hrp pathogenicity island. Transiently expressed HopAA1-1 acts inside *Saccharomyces cerevisiae* and plant cells to elicit cell death. *hopAA1* homologs were cloned and sequenced from the CEL of seven *P. syringae* strains representing diverse pathovars. Analysis of the sequences revealed that HopAA1-1 carries a potential GTPase-activating protein (GAP) domain, GALRA, which is polymorphic (FEN instead of LRA) in HopAA1-2, a paralogous DC3000 effector. Deleting *hopAA1-1* from DC3000 reduces the formation of necrotic speck lesions in dip-inoculated tomato leaves if effector-gene cluster IX or just PSPTO4723 within this region has been deleted. A HopAA1-1 mutant in which the putative catalytic arginine in the GAP-like domain has been replaced with alanine retains its ability to kill yeast and promote the formation of speck lesions by the Δ *hopAA1-1* Δ IX mutant, but a HopAA1-1 mutant carrying the FEN polymorphism loses both of these abilities. Unexpectedly, PSPTO4723 does not appear to encode an effector and its deletion also reduces disease-associated chlorosis.

Pseudomonas syringae pv. *tomato* DC3000 is a pathogen of tomato and *Arabidopsis* that has become a model for functional genomics and the study of plant-pathogen interactions (Buell et al. 2003; Lindeberg et al. 2008). DC3000 virulence is dependent upon the injection by the type III secretion system (T3SS) of approximately 28 effector proteins that collectively suppress defenses and promote bacterial growth and the production of ‘bacterial speck’ disease lesions in hosts (Chang et al. 2005; Kvitko et al. 2009; Lindeberg et al. 2006; Schechter et al. 2006). Although there has been exciting progress in identifying the activities and targets of several *P. syringae* effectors inside plant cells, the functions of most of the DC3000 effectors await elucidation (Block et al. 2008; Cunnac et al. 2009; Gohre and Robatzek 2008). Common obstacles to the study of *P. syringae* effectors include the lack of any biological phenotype following mutation or heterologous expression of the respective genes and the lack of clues to biochemical activity.

K. R. Munkvold and A. B. Russell contributed equally to this study.

Corresponding author: Alan Collmer; Telephone: +1.607.255.7843; Fax: +1.607.255.4471; E-mail: arc2@cornell.edu

One approach we have taken to these problems is to comprehensively identify the subset of DC3000 effectors that are toxic when expressed in the model eukaryote *Saccharomyces cerevisiae* (Munkvold et al. 2008). Another approach is to construct combinatorial deletions in DC3000 effector genes to eliminate redundant effector groups and produce stronger phenotypes for single effector genes in virulence tests (Kvitko et al. 2009; Wei et al. 2007). The HopAA1 effector family is of particular interest to us because the encoding genes are present in diverse pathovars of *P. syringae* (Alfano et al. 2000; Badel et al. 2002), and HopAA1-1 is one of seven DC3000 effectors that are toxic when expressed in yeast cells (Munkvold et al. 2008). Furthermore, *hopAA1-1* also induces cell death when expressed within plant cells (Munkvold et al. 2008). However, *hopAA1* mutations in *P. syringae* reported to date have only subtle phenotypes (Badel et al. 2002; Vinatzer et al. 2006), and we have no clue to the biochemical activity of this effector family.

HopAA1-1 is considered to be among the ancient *P. syringae* effectors that were acquired before the radiation of *P. syringae* into the current pathovars (Rohmer et al. 2004), and this concept is supported by the location of the *hopAA1* gene in the conserved effector locus (CEL), which is part of the tripartite Hrp pathogenicity island that also carries the *hrp/hrc* (hypersensitive response and pathogenicity/conserved) genes encoding the T3SS (Alfano et al. 2000). The DC3000 gene is denoted *hopAA1-1* because it has a paralog, *hopAA1-2*, in a cluster of four effector genes (‘cluster IX’) that is located elsewhere in the genome (Badel et al. 2002; Wei et al. 2007). The DC3000 gene will sometimes be denoted *hopAA1-1*_{Pto DC3000} in this report for clarity when being compared with *hopAA1* genes from other *P. syringae* pathovars (Lindeberg et al. 2005).

Most of the *P. syringae* pathovars fall into three major phylogroups based on phylogenetic analyses (Sarkar and Guttman 2004; Sawada et al. 1999). Strains in pathovars representing each of these major phylogroups have been sequenced. These are *P. syringae* pv. *tomato* DC3000 (group 1) (Buell et al. 2003), *P. syringae* pv. *syringae* B728a (group 2) (Feil et al. 2005), and *P. syringae* pv. *phaseolicola* 1448A (group 3) (Joardar et al. 2005). The CEL in each of these sequenced strains carries three effector genes—*avrE*, *hopM1*, and *hopAA1*—although *hopM1* and *hopAA1* appear nonfunctional in 1448A (Lindeberg et al. 2006). The DC3000 CEL contains a fourth effector gene, *hopN1* (López-Solanilla et al. 2004).

Among the CEL effectors, the AvrE effector family is the most widespread among plant pathogenic bacteria and appears universal to pathogens with the Hrp1 class of T3SS (plant pathogens in the Pseudomonadaceae and Enterobacteriaceae)

(Bogdanove et al. 1998b; Cornelis 2006; Ham et al. 2006). Mutation of *dspA/E*, the *avrE* homolog in *Erwinia amylovora*, leads to a complete loss of pathogenicity (Bogdanove et al. 1998a; Gaudriault et al. 1997).

The DC3000 AvrE and HopM1 effectors have been studied extensively (Badel et al. 2003, 2006; DebRoy et al. 2004; Nomura et al. 2006). Deletion of the DC3000 CEL region carrying *avrE*, *hopM1*, and *hopAA1-1* results in a loss of virulence (Alfano et al. 2000). Virulence can be restored by expression in trans of either AvrE or HopM1 but not by HopAA1-1 (DebRoy et al. 2004). Evidence for a strong virulence function in plants for HopAA1 family members is lacking, although previous work has shown that mutation of both *hopAA1-1* and *hopAA1-2* in DC3000 produces the subtle phenotype of an increased frequency of small colonies in *Arabidopsis* without an associated decrease in symptom development or bacterial multiplication in planta (Badel et al. 2002).

To further characterize the HopAA1 effector family, we cloned and sequenced the *hopAA1* homologs from seven different pathovars of *P. syringae* and determined the effects of expressing these genes in yeast, thus revealing the apparent inactivity of many phylogroup 3 *hopAA1* genes. We exploited the multiple *hopAA1* sequences to discern a putative GTPase-activating protein (GAP) domain in these proteins, which was represented by three strongly divergent amino acids in HopAA1-2. Introduction of this GAP domain polymorphism into HopAA1-1 abolished the ability of HopAA1-1 to inhibit yeast growth. In search of a more useful virulence phenotype for *hopAA1-1* mutations, we discovered that a mutant deleted for both *hopAA1-1* and effector-gene cluster IX was significantly reduced in the number of bacterial speck lesions it produced in dip-inoculated tomato leaves. Importantly, the virulence defect could be restored by expression in trans of native *hopAA1-1* but not by *hopAA1-1* carrying the *hopAA1-2* GAP domain polymorphism or any other *hopAA1-1* derivative that had lost the ability to inhibit yeast growth. We noted that deletion of cluster IX also reduced the diffuse chlorosis in tomato leaves that accompanies DC3000 infections. Surprisingly, deletion of PSPT04723, one of two open reading frames (ORF) within cluster IX encoding hypothetical proteins with no properties of type III effectors, also reduced diffuse chlorosis and reduced lesion numbers if *hopAA1-1* was deleted.

RESULTS

hopAA1 family members cloned from representative *P. syringae* strains reveal the prevalence of a truncated form in the pathovar phylogroup containing *P. syringae* pv. *phaseolicola*.

To find regions of conserved amino-acid sequence within the HopAA1 family that might provide a clue to the molecular

Fig. 1. Strategy for cloning *hopAA1* family members from *Pseudomonas syringae* pathovars. The diagram depicts the genomic region of *P. syringae* pv. *tomato* DC3000 that contains *hrpW1*, *hopAA1-1*, and PSPT01371. Primer sets P1 and P2 and P3 and P4 were used to amplify regions containing *hopAA1* and flanking sequences. Primer sets P5 and P6 and P5 and P7 correspond to the predicted start and stop codons of the newly sequenced *hopAA1* family members. The bent arrow designates the location and direction of the *hopAA1-1*_{Pto DC3000} native promoter.

function of HopAA1-1, we cloned seven *hopAA1* family members from different pathovars of *P. syringae* that were selected based on the presence of *hopAA1*-like sequences observed via Southern hybridization (Badel et al. 2002). The cloning strategy employed the targeting of sequences within and flanking *hopAA1* in the CEL of each pathovar (Fig. 1). Therefore, the potential presence of *hopAA1*-like sequences outside this region of the genome, for example, *hopAA1-2*_{Pto DC3000}, was not addressed in this work. A primer set corresponding to conserved regions of *hrpW1* and *hopAA1* was used to amplify the 3' region of *hopAA1*-like sequences. Another primer set was designed to conserved regions of *hopAA1* and PSPT01371 for amplification of the 5' region of *hopAA1*-like sequences (Alfano et al. 2000). Upon determining the sequence of these regions, primers were designed to amplify the full-length *hopAA1* sequence from each pathovar. Amplification products were cloned and consensus sequences were derived from sequencing data of three independent clones.

hopAA1 homologs from *P. syringae* pvs. *tabaci* and *angulata* were found to be 100% identical (Fig. 2), and in subsequent work, we used only *hopAA1*_{Pta}. Another highly similar pair of homologs that share 99% identity comes from *P. syringae* pvs. *glycinea* and *phaseolicola*. These pathovars are closely related and have been shown to cluster together in a phylogenetic study of *P. syringae* pathovars based on the core genome (Sarkar and Guttman 2004). A third set of closely related *hopAA1* homologs (99% identical) includes those from strains T1 and DC3000, both of *P. syringae* pv. *tomato*. A recent, next-generation sequencing analysis of the T1 genome provides an independent and identical sequence of the T1 *hopAA1* gene (Almeida et al. 2009).

The observation that similarity among particular *hopAA1* family members was correlated with overall pathovar relatedness provoked an investigation of whether this finding held true for the entire *hopAA1* family. Using CLUSTAL X (Thompson et al. 1997), the DNA sequences of the newly identified *hopAA1* family members were aligned along with six other *hopAA1*-like sequences already present in GenBank. The topology of the phylogenetic tree generated from *hopAA1* family sequences (Fig. 2) was similar to the topology of the phylogenetic tree for the core genome of *P. syringae* (Sarkar and Guttman 2004). This result supports previous evidence that *hopAA1* is among the ancient and stably maintained effector genes in *P. syringae* (Rohmer et al. 2004). Therefore, it is likely that *P. syringae* acquired *hopAA1* prior to the diversification of different pathovars, which is also consistent with its presence in the CEL (Alfano et al. 2000). Interestingly, the *hopAA1* homologs from *P. syringae* pvs. *tabaci*, *angulata*, *phaseolicola*, and *glycinea* contain a frameshift leading to a premature stop codon directly after codon 189 (Fig. 2). The frameshift occurs due to a single insertion of an A after position 562 of the DNA sequence. However, *hopAA1*_{Pta} does possess a start codon and is predicted to encode a truncated form of HopAA1. Thus, with the exception of *hopAA1*_{Pta}, all of the *hopAA1* genes in members of *P. syringae* phylogroup 3 are either truncated, not expressed, or both.

A consensus sequence that was generated by CLUSTAL X multiple alignment of the predicted amino-acid sequences for all HopAA1 family members was used in several bioinformatic analyses. As for many effectors, BLAST, InterProScan, SMART, and Phyre did not return significant hits to proteins of known function for the HopAA1 consensus sequence (data not shown).

All full-length HopAA1 homologs inhibit the growth of yeast.

Our previous work on HopAA1-1_{Pto DC3000} showed that expression of this effector in yeast leads to the strong gain-of-

function phenotype of cell death (Munkvold et al. 2008). Therefore, we tested the ability of each HopAA1 homolog to inhibit the growth of yeast. Expression of homologs that were predicted to encode a full-length protein resulted in strong growth inhibition in yeast (Fig. 3). In contrast, expression of *hopAA1*_{Pta}, which is predicted to encode a truncated form of the protein, did not affect yeast growth, although immunoblot analysis confirmed that the predicted protein was produced in yeast (data not shown). This observation provides further support of the finding that deletion of 100 or more amino acids from the C terminus of HopAA1-1_{Pto DC3000} abolishes growth-inhibiting activity in yeast (Munkvold et al. 2008).

A putative GTPase activation domain is located in the N-proximal region of HopAA1-1_{Pto DC3000} and homologs from other *P. syringae* strains but is significantly polymorphic in HopAA1-2_{Pto DC3000}.

Examination of the less significant hits obtained from our Phyre analysis revealed weak similarity to the N-terminal domain of the *P. aeruginosa* effector ExoS. ExoS has a bipartite structure in which the N-terminal domain functions as a GAP for Rho GTPases and the C-terminal domain has ADP-ribosyltransferase activity (Goehring et al. 1999). GAP activity has been observed in effectors from animal pathogenic bacteria. For example, the *Yersinia* effector YopE and the *Salmonella* effector SptP both possess GAP activity (Fu and Galán 1999; Von Pawel-Rammingen et al. 2000). Alignment of the HopAA1-1_{Pto DC3000} amino-acid sequence with ExoS, YopE, and SptP revealed that HopAA1-1_{Pto DC3000} maintains the conserved arginine finger motif required for GAP activity (Fig. 4A) (Scheffzek et al. 1997).

Because HopAA1_{Pto DC3000} is conserved among strains of *P. syringae*, we wondered if the putative GAP domain was present in all HopAA1 family members. Alignment of all known HopAA1 homologs from *P. syringae* revealed that the conserved arginine was present in all HopAA1 family members except HopAA1-2_{Pto DC3000}. The GXLRL sequence that is found in all bacterial effectors with GAP activity is present only in the strains from *P. syringae* phylogroup 1, i.e., HopAA1-1_{Pto DC3000}, HopAA1_{Pto T1}, and HopAA1_{Pma ES4326} (Fig. 4B). In contrast, the glycine in this region was replaced with a threonine in phylogroup 2 and a serine in phylogroup 3.

The ability of HopAA1-1_{Pto DC3000} to inhibit yeast growth is unaffected by site-directed mutation of the putative GAP domain catalytic arginine but is diminished by substitution of the HopAA1-2_{Pto DC3000} GAP domain polymorphism.

To test whether HopAA1-1_{Pto DC3000}-associated growth inhibition in yeast required the putative GAP activity, the conserved arginine at amino acid 121 was changed to alanine by site-directed mutagenesis to produce HopAA1-1_{R121A}. As expected (Munkvold et al. 2008), HopAA1-2_{Pto DC3000} did not inhibit yeast growth (Fig. 4C). Therefore, we also constructed a mutant of HopAA1-1 that carried the sequence of

Fig. 3. Expression in yeast of HopAA1 family members that are not truncated results in inhibition of growth. Yeast strains (BY4741a) harboring the high-copy plasmid pYES-DEST52 encoding LacZ as a control or HopAA1 family members with a C-terminal V5-epitope tag were grown overnight in selective, noninducing, raffinose media. The cultures were normalized to an optical density at 600 nm of 0.45, and serial 10-fold dilutions were spotted onto selective media containing galactose to induce the expression of the protein. Photographs were taken after 2 days of incubation. Pto = *P. syringae* pv. *tomato*; Pma = *P. syringae* pv. *maculicola*; Psy = *P. syringae* pv. *syringae*; Ppi = *P. syringae* pv. *pisi*; Pla = *P. syringae* pv. *lachrymans*; Pta = *P. syringae* pv. *tabaci*.

Fig. 2. Comparisons of DNA and predicted amino-acid sequences for *hopAA1* family members. Multiple-sequence alignment was performed for *hopAA1* family member DNA sequences using the CLUSTAL X method (Thompson et al. 1997). All *hopAA1* sequences aligned were equivalent in length to *hopAA1-1*_{Pto DC3000}. A neighbor-joining tree was constructed using PAUP* 4.0, rooted with *hopAA1*_{Pv} as a monophyletic outgroup, and was visualized with TreeViewX version 0.5.0. Nucleotide substitution rates are indicated as substitutions per site in bold along the branches, and bootstrap values (designated in percentages) are displayed in gray italics at branch nodes. Numbers next to braces (far right) indicate the phylogroup as determined for the *Pseudomonas syringae* core genome within which each strain clusters (Sarkar and Guttman 2004). HopAA1 proteins that are truncated to 189 amino acids are denoted with '<', and those without a start codon, as compared with the *hopAA1-1*_{Pto DC3000} sequence, are denoted with 'x'. Pto = *P. syringae* pv. *tomato*; Pag = *P. syringae* pv. *angulata*; Pta = *P. syringae* pv. *tabaci*; Pla = *P. syringae* pv. *lachrymans*; Pgy = *P. syringae* pv. *glycinea*; Pph = *P. syringae* pv. *phaseolicola*; Pma = *P. syringae* pv. *maculicola*; Ppi = *P. syringae* pv. *pisi*; Ppp = *P. syringae* pv. *papulans*; Psy = *P. syringae* pv. *syringae*. Pv = *P. viridiflava*.

HopAA1-2 in this region (HopAA1-1_{LRA-FEN}). The HopAA1-1 mutants were expressed in yeast and were assayed for their ability to inhibit growth. The single arginine to alanine substitution did not alleviate growth inhibition induced by HopAA1-1 in yeast (Fig. 4C). However, the mutation of amino acids 120 to 122 from LRA to FEN did alleviate most of the growth inhibition associated with HopAA1-1_{Pto DC3000} expression in yeast (Fig. 4C). Immunoblot analysis confirmed that the HopAA1-1_{LRA-FEN} protein was produced in yeast (Fig. 4D). Thus, the polymorphism in this region of

HopAA1-2_{Pto DC3000} may account for the failure of this protein to inhibit yeast growth.

Analysis of *P. syringae* pv. *tomato* DC3000 mutants reveals that HopAA1-1 significantly contributes to lesion formation in host tomato when DC3000 derivatives lacking effector-gene cluster IX are inoculated by dipping.

To facilitate exploration of any activity of HopAA1-1 in promoting DC3000 virulence in tomato, we sought conditions that would yield a workable virulence phenotype for

Fig. 4. Analysis of the putative GTPase-activating protein (GAP) domain within HopAA1-1_{Pto DC3000}. **A**, Alignment of bacterial effectors with known GAP activity and HopAA1-1_{Pto DC3000}. Numbers designate the region of the protein aligned. Amino acids shown in red indicate conserved residues in bacterial effectors with GAP activity. The underlined residues show the location of the arginine finger motif. **B**, Alignment of HopAA1 family members using AlignX within the Vector NTI software suite. Boxed regions indicate the conserved residues shown in red in A. Black arrow indicates R121. Pto = *P. syringae* pv. *tomato*; Pma = *P. syringae* pv. *maculicola*; Pag = *P. syringae* pv. *angulata*; Pta = *P. syringae* pv. *tabaci*; Pgy = *P. syringae* pv. *glycinea*; Pla = *P. syringae* pv. *lachrymans*; Pph = *P. syringae* pv. *phaseolicola*; Ppi = *P. syringae* pv. *pisi*; Ppp = *P. syringae* pv. *papulans*; Psy = *P. syringae* pv. *syringae*. **C**, HopAA1-1_{Pto DC3000}, HopAA1-2_{Pto DC3000}, HopAA1-1_{R121A}, and HopAA1-1_{LRA-FEN} were expressed in *Saccharomyces cerevisiae* BY4741a under the control of the *GAL1* galactose-inducible promoter. Yeast cultures were grown overnight at 30°C in raffinose-containing media. Cultures were resuspended to an optical density at 600 nm of 0.45, were serially diluted 10-fold in raffinose media, and were spotted onto galactose-containing media to induce expression of the protein. Photographs were taken after 2 days of incubation. **D**, Immunoblot analysis using anti-V5 antibody to detect expression of HopAA1-1_{R121A}-V5 and HopAA1-1_{LRA-FEN}-V5 fusion proteins after induction for 3 h in galactose-containing media. Predicted protein size is 55.7 kDa for both fusion proteins.

hopAA1-1 mutations. The variables we addressed were genetic background (particularly composition of the effector repertoire), method of inoculation, and phenotype measured. We had previously observed an increase in the number of small ('failed') colonies relative to wild-type DC3000 when a green fluorescent protein-labeled *hopAA1-1::nptII/hopAA1-2::aadA1* double mutant was infiltrated with a blunt syringe into *Arabidopsis* leaves (Badel et al. 2002). However, this mutant and inoculation method did not reveal any defect in the final growth or symptom production in *Arabidopsis* or tomato, and assays involving confocal microscopy are time consuming.

To facilitate future construction of unmarked combinatorial mutants, we used pK18*mobsacB* to construct a complete deletion of the *hopAA1-1* ORF, with the spectinomycin-resistance cassette marking the deletion flanked by FRT sites, as previously described (Wei et al. 2007). We also exploited previously constructed intermediates for marker-exchange mutagenesis (Wei et al. 2007) to construct combinatorial deletions involving *hopAA1-1* and effector-gene cluster IX, which includes *hopAA1-2*. Dip-inoculation of the leaves of whole tomato plants with these mutants enabled us to observe significant differences in the numbers and appearance of the resulting speck lesions.

Although we observed no major difference in the number of necrotic specks produced by DC3000 and the Δ IX or Δ *hopAA1-1* mutants, the Δ IX Δ *hopAA1-1* double mutant was strongly reduced in lesion formation, typically producing only $\frac{1}{3}$ the number of necrotic specks per plant (Fig. 5A and B). The Δ IX mutant was interestingly altered in its virulence rela-

Table 1. Competitive index assays of bacterial growth in tomato leaves and in culture

Strains	Competitive index		
	Blunt-syringe inoculation ^a	Dip inoculation ^b	King's B medium ^c
DC3000 vs. Δ <i>hopAA1-1</i>	1.14 $\pm$ 0.45	ND	ND
Δ IX vs. Δ IX Δ <i>hopAA1-1</i>	0.71 $\pm$ 0.24	0.75 $\pm$ 0.53	0.95 $\pm$ 0.23

^a Leaves were sampled at 3 days postinoculation (dpi); data represent mean and standard deviation (SD) for four samples from each of two plants in three independent experiments.

^b Leaves were sampled at 5 dpi; data represent mean and SD for four samples from each of two plants in three independent experiments.

^c Bacterial populations were determined in cultures grown overnight; data represent mean and SD for three samples from each of three independent experiments.

Fig. 5. Dip-inoculated *Pseudomonas syringae* pv. *tomato* DC3000 mutants lacking *hopAA1-1* and effector-gene cluster IX have a reduced ability to produce lesions in host tomato leaves. **A** and **B**, Tomato plants were inoculated by dipping in inoculum containing the indicated strains at 1×10^5 CFU/ml, and the numbers of lesions on entire plants were counted 5 days later. The dotted line separates two sets of independent experiments. Data represent the mean and standard deviation for six samples involving two plants in three independent experiments. Values marked with an asterisk are significantly different ($P < 0.01$) from DC3000 (A) or the Δ IX mutant (B) based on Student's *t*-test. **C**, Photographs show characteristic differences in symptoms produced in tomato leaves by DC3000, Δ *hopAA1-1*, Δ IX, Δ IX Δ *hopAA1-1*, and Δ *hrcQ-U* (type III secretion system-minus CUCPB5113) mutants 5 days after inoculation as above.

tive to wild-type DC3000, in that chlorosis was more focused around speck lesions (Fig. 5C). Consequently, the ΔIX mutant infection produced leaves that are comparatively greener overall, and the speck-associated chlorotic halos show more contrast against this background. The symptoms produced by the $\Delta IX \Delta hopAA1-1$ mutant also have this appearance of reduced diffuse chlorosis (Fig. 5C).

To address the possibility that the $\Delta IX \Delta hopAA1-1$ mutant produced fewer speck lesions because of a mutation elsewhere in the DC3000 genome, we used recombination in yeast to assemble, from two polymerase chain reaction (PCR)-cloned DNA fragments, the entire cluster IX region in broad-host range vector pCPP5296. The introduction of this plasmid, pCPP5957, into the $\Delta IX \Delta hopAA1-1$ mutant restored parental levels of speck production (Fig. 5B). To determine whether the $\Delta hopAA1-1$ or $\Delta IX \Delta hopAA1-1$ mutants were reduced in their ability to grow in tomato, we used highly sensitive, competitive index assays (Macho et al. 2007). Interestingly, we did not detect a significant reduction in growth of the $\Delta IX \Delta hopAA1-1$ mutant in tissue coinoculated with its ΔIX parent, when inoculated by either infiltration or dipping (Table 1). Thus, HopAA1-1 has an important role in enabling a ΔIX mutant to produce lesions, but it appears less important in enabling the ΔIX mutant to grow in tomato leaves.

Wild-type $hopAA1-1_{P_{to DC3000}}$ expressed in trans can promote virulence in the $\Delta IX \Delta hopAA1-1$ mutant, but $hopAA1-2$ and all $hopAA1-1_{P_{to DC3000}}$ mutants that have lost the ability to inhibit yeast fail to promote virulence.

To determine if the loss of $hopAA1-2$ (as a consequence of deleting effector cluster IX) was responsible for the reduced ability of the $\Delta IX \Delta hopAA1-1$ double mutant to produce speck lesions, we tested the previously constructed $hopAA1-1::nptIII/hopAA1-2::aadA1$ double mutant for its ability to produce lesions in dip-inoculated tomato. The lesion counts produced by this mutant were not significantly reduced relative to the ΔIX mutant, whereas, as expected from results shown in Figure 5, lesion counts produced by the $\Delta IX \Delta hopAA1-1$ mutant were significantly lower (Fig. 6A). To determine the ability of HopAA1 variants to promote lesion formation by the $\Delta IX \Delta hopAA1-1$ mutant, we expressed these proteins with C-terminal hemagglutinin (HA) tags from HrpL-responsive promoters in pCPP5372. Immunoblot analysis confirmed that most of the proteins were produced at the same levels as HopAA1-1 (Fig. 6B). However, HopAA1-2 accumulation was weak and, therefore, we could not meaningfully test the ability of HopAA1-2 to complement the $\Delta IX \Delta hopAA1-1$ mutant in the experiment shown in Figure 6A. Nevertheless, the wild-type

Fig. 6. Wild-type HopAA1-1_{P_{to DC3000}} expressed in trans can restore lesion formation activity to the $\Delta IX \Delta hopAA1-1$ mutant, but HopAA1-2 and all HopAA1-1_{P_{to DC3000}} mutants that have lost the ability to inhibit yeast growth fail to restore bacterial virulence. **A**, Tomato leaves were inoculated by dipping and the numbers of resulting lesions per plant were determined. Values marked with an asterisk are significantly different ($P < 0.01$) from the ΔIX mutant based on Student's t -test. HopAA1 variants were expressed from HrpL-responsive promoters and produced with a C-terminal hemagglutinin (HA) tag provided by vector pCPP5372. HopAA1 variants shown to inhibit yeast growth are boxed. **B**, Production in the $\Delta IX \Delta hopAA1-1$ mutant of HopAA1-1 and the derivatives analyzed in panel A. Bacteria were grown in Hrp minimal medium. Protein samples were separated on a 7% sodium dodecyl sulfate polyacrylamide gel, and immunoblot analysis was performed using antibodies to the HA tag at the C terminus of each test protein.

behavior of the *hopAA1-1::nptIIIhopAA1-2::aadA1* double mutant suggests that the reduced lesion-number phenotype of the $\Delta IX\Delta hopAA1-1$ mutant involves some factor in cluster IX other than HopAA1-2.

The primary goal of constructing combinatorial deletions involving *hopAA1-1* and other effector genes in DC3000 was to produce a mutant with a virulence defect that could be complemented by production of the native HopAA1-1, thus facilitating investigation of features of HopAA1-1 associated with virulence function. Importantly, the expression of HopAA1-1 restored parental levels of lesion formation to the $\Delta IX\Delta hopAA1-1$ mutant (Fig. 6A). With native HopAA1-1 available as a benchmark, we were able to test the various HopAA1-1 derivatives for their ability to promote virulence, including truncated derivatives that were previously constructed and tested for their toxicity when expressed in yeast (Munkvold et al. 2008). The two mutants that retained their ability to inhibit yeast growth (HopAA1-1₁₋₄₃₆ and HopAA1-1_{R121A}) also restored parental levels of lesion formation to the $\Delta IX\Delta hopAA1-1$ mutant, whereas the two mutants that lost the ability to inhibit yeast growth (HopAA1-1₁₋₃₈₆ and HopAA1-1_{LRA-FEN}) similarly lost the ability to promote lesion formation. Thus, HopAA1-1 appears to have the same structural requirements for biological activity in yeast and plants.

PSPTO4723 is responsible

for the ΔIX speck lesion and chlorosis phenotypes.

The DC3000 genomic region encompassed by the cluster IX deletion includes four active effector genes, one effector pseudogene with an associated insertion element, and two ORF of unknown function (PSPTO4719 and PSPTO4723) (Fig. 7A). To determine which gene or genes were responsible for the phenotypes associated with deleting cluster IX, we first tested each of the effectors, aside from HopAA1-2, for ability to restore higher levels of lesion formation to the $\Delta IX\Delta hopAA1-1$ mutant. We used the same expression vector employed above with HopAA1-1, but we observed no significant restoration of the lesion formation phenotype by any of these effectors (Fig. 8A). This lack of complementation did not appear to be due to a failure to produce these effectors, as immunoblot analysis confirmed that each was produced, although HopAO1 levels were lower in repeated analyses (Fig. 8B). To address the possibility that a combination of cluster IX effectors was needed to restore wild-type levels of speck lesions and diffuse chlorosis, we deleted the central 'inter-effector' region of pCPP5957 (Fig. 7). The resulting plasmid retained all four full-length effector genes but, nevertheless, failed to complement either the HopAA1-1-dependent lesion phenotype or the chlorosis phenotype associated with cluster IX (data not shown). These

Fig. 7. Physical map of the effector-gene cluster IX region, including PSPTO4723 and fragmentary sequences with homology to genes directing coronatine production. **A**, The DC3000 chromosomal region that was deleted to remove effector-gene cluster IX encompasses *hopAA1-2* through *hopG1*. Hrp promoters (black arrows) are located upstream of all four active *hop* genes, as well as the *hopD1::IS52* pseudogene, which is disrupted by the PSPTO4725 *IS52* transposase (the unexpressed region downstream is annotated as PSPTO4726 and is homologous to the 3' sequence of an active *hopD1* gene located in effector-gene cluster IV). The *SacI* sites used to delete the central 'inter-effector' region of pCPP5957 are shown. **B**, The region between *hopAO1* and *hopD1::IS52* is expanded to show PSPTO4723, restriction sites used to clone PSPTO4723 for complementation experiments, and regions with similarity to the coronatine regulatory region in DC3000 upstream of PSPTO4723. The percent identity between each region is shown. **C**, The region downstream of PSPTO4723 contains sequences with similarity to a portion of *cmaU* that is present in *Pseudomonas syringae* pv. *glycinea* but lacking in the DC3000 *cmaU*. The percent identity between each region is shown.

observations pointed to PSPTO4723 as being responsible for the cluster IX phenotypes.

PSPTO4723 displays no characteristics of an effector gene. It is not preceded by a Hrp promoter, and the predicted N-terminal sequence shows none of the amino-acid patterns associated with type III substrates (Lindeberg et al. 2006; Vinatzer et al. 2005). Analyses with WebACT (Abbott et al. 2005) and BLASTN revealed that PSPTO4723 is flanked by apparent pseudogene fragments of *cmaU*, *corP*, and *corS* (Fig. 7BC). Active copies of these genes occur in DC3000 in the coronatine regulatory region and the *cma* gene cluster, which directs the biosynthesis of coronamic acid. Conjugation of coronafacic acid with coronamic acid produces coronatine, which is responsible for the chlorosis associated with DC3000 infections of tomato. Altered production of coronatine would be more consistent with the reduced chlorosis that is associated with the cluster IX deletion than loss of an effector.

To definitively test the role of PSPTO4723, we subcloned a 579-bp fragment containing PSPTO4723, and we precisely deleted the PSPTO4723 ORF from the DC3000 genome. We first attempted to PCR-clone the PSPTO4723 ORF under control of the *nptII* promoter in pBS46, but the resulting plasmid was unstable in DC3000. Subsequently, we used flanking *BsaAI* and

EcoRV sites to subclone from pCPP5956 the PSPTO4723 ORF along with 200 bp upstream and 50 bp downstream, which permitted expression of the gene without a vector promoter (Fig. 7B). A marked deletion of the PSPTO4723 ORF in both wild-type DC3000 and a $\Delta hopAA1-1$ mutant was also constructed. The addition of the plasmid carrying the 579-bp fragment containing PSPTO4723 to a $\Delta IX \Delta hopAA1-1$ strain fully complemented lesion formation, and the $\Delta PSPTO4723$ mutants behaved in the same manner as ΔIX strains in exhibiting reduced chlorosis and a dependence upon HopAA1-1 for wild-type lesion formation (Fig. 9). Thus, PSPTO4723 appears to be responsible for the phenotypes associated with the deletion of the cluster IX region that we observed in this work.

DISCUSSION

By cloning and sequencing the *hopAA1* locus in the CEL of several *P. syringae* strains, we were able to discern a GAP-like domain in the N-proximal half of the predicted proteins. Introduction into HopAA1-1 of a polymorphism in this region found naturally in the DC3000 paralog, HopAA1-2, abolishes the ability of HopAA1-1 to be toxic in yeast and to promote

Fig. 8. The effectors HopV1, HopAO1, and HopG1 do not complement the HopAA1-1-associated lesion phenotype when expressed in trans. **A**, Number of lesions on tomato plants 5 days after being infected with the indicated strains in a dip inoculation at 1×10^5 CFU/ml. The $\Delta IX \Delta hopAA1-1$ strains tested for complementation all have effectors expressed under control of a HrpL-dependent promoter and have a C-terminal hemagglutinin (HA) tag; this is abbreviated as *phop* with effectors further indicated. HopV1 is expressed with its chaperone, ShcV. The $\Delta hrcQ-U$ mutant is type III secretion system-minus CUCPB5113. Data values represent averages of three independent trials of two samples each, and error bars represent standard deviation. Asterisks mark strains that formed lesions at significantly different numbers ($P < 0.05$) than in an infection by the ΔIX mutants as measured by a Student's *t*-test. **B**, Immunoblot confirming production of the indicated effector protein in complemented $\Delta IX \Delta hopAA1-1$ strains. HrpL-dependent expression of the proteins was induced by growing cultures overnight in Hrp minimal media. Pellets of these overnight cultures were then resolved on a 7% sodium dodecyl sulfate polyacrylamide gel, after which an immunoblot was performed using a primary antibody against the C-terminal HA tags. Sizes determined by a protein ladder are marked in kDa.

Fig. 9. PSPTO4723 is the major determinant of the cluster IX-associated speck and chlorosis symptoms in tomato. **A**, Speck lesion counts on tomato plants 5 days postinoculation from a dip inoculation with 1×10^5 CFU of the indicated strains per milliliter. Data represent averages from three independent trials of two samples each. Error bars represent standard deviations. Asterisks mark strains that produced lesions in tomato that were significantly different from those produced by wild-type DC3000, as measured by a Student's *t*-test ($P < 0.01$). **B**, Photographs of symptoms caused by selected strains at 5 days postinfection. Photographs show typical symptom formation for the selected strain.

DC3000 virulence in host tomato. As is typical of *P. syringae* effectors, the contribution of HopAA1-1 to DC3000 virulence is best observed in a mutant background that reduces redundancy in virulence factors. Unexpectedly, the gene acting redundantly with *hopAA1-1* is PSPTO4723, which encodes a conserved hypothetical protein and is necessary for the diffuse chlorosis that DC3000 infection produces in tomato leaves. Our discussion will address the function of HopAA1-1 in the context of current knowledge of *P. syringae* effector functions, the location of *hopAA1-1* in the CEL, HopAA1-1 structural features, the functional interplay with PSPTO4723, and the possible relationship of coronatine production with HopAA1-1 function.

The parasitic success of *P. syringae* is thought to depend on the ability of type III effectors to suppress pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), while evading detection by (or suppressing) *R* gene-mediated effector-triggered immunity (ETI), which is typically manifested in the hypersensitive response (Jones and Dangl 2006). The molecular functions of AvrPto and HopM1 provide examples suggesting that effectors interfere with at least two important processes in PTI, namely, PAMP perception and cell wall defense-associated vesicle trafficking, respectively (Nomura et al. 2006; Shan et al. 2008; Xiang et al. 2008). In addition, AvrPtoB provides an example of an effector that has multiple domains that may function to suppress PTI and to both elicit and suppress ETI (Abramovitch et al. 2003; Rosebrock et al. 2007; Xiao et al. 2007).

HopM1 is the best understood effector in the CEL and has been shown to suppress callose deposition, a cell wall-based PTI defense, and to target to the proteasome an ADP ribosylation factor guanine nucleotide exchange factor protein that is associated with vesicle trafficking (DeBRoy et al. 2004; Nomura et al. 2006). Less is known about the molecular function of AvrE, but interestingly, observations based on mutant phenotypes and heterologous expression assays reveal that the three CEL effectors AvrE, HopM1, and HopAA1-1 make a larger contribution to lesion formation than they do to growth in planta and can cause cell death in host cells (Badel et al. 2003, 2006; Wei et al. 2007). Furthermore, whereas PAMP perception involves specific pattern-recognition receptor kinases and signaling components not likely to be found in yeast, defense-associated vesicle trafficking and associated GTPases are likely universal among eukaryotes. Thus, the broad toxicity of transiently expressed HopAA1-1 to yeast and plant cells would be consistent with a primary role for this and other CEL effectors in disrupting fundamental cellular processes like vesicle trafficking.

It is also important to note that the *P. syringae* pv. *syringae* B728a *hopAA1* appears to have weak avirulence (ETI-eliciting) activity in *Nicotiana benthamiana* but not bean, as indicated by the lethality of transiently expressed *hopAA1* in *N. benthamiana* but not bean, and by the observation that a *hopAA1* mutant is better able to grow in spray-inoculated *N. benthamiana* than wild-type B728a (Vinatzer et al. 2006). However, the toxicity of HopAA1_{Psy B728a} to yeast that we observed here cannot easily be attributed to ETI, which raises the possibility that HopAA1 family members may promote cell death in plants by multiple means. It should also be noted that the DC3000 HopAA1-1 expressed in *Arabidopsis* protoplasts was able to suppress flagellin-induced accumulation of *NONHOST1* (*NHO1*) transcripts, a marker for PTI-associated gene expression (Li et al. 2005). This observation suggests that HopAA1-1 can block PAMP perception, although it is possible that secondary effects of our proposed disruption of a basic cellular process like vesicle trafficking by HopAA1-1 contributed to this observation.

We presently have no evidence that HopAA1-1 has GAP activity or that such activity is required for biological function. The HopAA1-1_{R121A} mutation affecting the putative catalytic arginine had no effect on toxicity in yeast or virulence in tomato. Similarly, full-length HopAA1 family members from other phylogroups were still toxic to yeast despite the substitutions for the glycine in the GCLR motif that is found in type III effectors known to have GAP activity (Scheffzek and Ahmadian 2005). In contrast, the HopAA1-1_{LRA-FEN} mutation abolished both the yeast and virulence phenotypes. This polymorphism, naturally occurring in HopAA1-2, involves the substitution of three amino acids with strongly different properties, which would be expected to significantly alter HopAA1-1 structure in this region, although it had no effect on the stability of the protein in yeast or *P. syringae*. We did not observe the FEN polymorphism in any of the CEL-associated HopAA1 family members we cloned from other strains. Interestingly, the region containing *hopAA1-2* as well as the rest of the cluster IX effectors and the coronatine biosynthesis genes is missing from *P. syringae* pv. *tomato* T1 (Almeida et al. 2009), and *hopAA1-2* is also missing from the sequenced strains *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *phaseolicola* 1448A (Lindeberg et al. 2006).

Two aspects of the virulence phenotype resulting from the deletion of *hopAA1-1* in a Δ IX background warrant discussion. First, observing a Δ *hopAA1-1* phenotype required deleting PSPTO4723. Importantly, PSPTO4723 does not appear to encode an effector and deleting this gene alone from DC3000 caused a striking reduction in the diffuse chlorosis associated with DC3000 infections in tomato. BLASTP analysis revealed PSPTO4723 to be in the DUF1330 family of proteins, which are widespread in members of phylum Proteobacteria but are of unknown function. PSPTO4723 is flanked by fragmentary sequences with similarity to genes associated with the regulation and production of coronatine. PSPTO4723 and the rest of the cluster IX region are part of variable region 107, an apparent genomic island that also contains the *cfa* and *cma* genes directing coronatine biosynthesis (Lindeberg et al. 2008). We are now pursuing the hypothesis that PSPTO4723 alters the production of coronatine during infection. The notion that altered coronatine production could enhance the Δ *hopAA1-1* lesion phenotype is consistent with several observations. DC3000 mutants altered in defined steps in coronatine biosynthesis are altered in lesion formation in tomato (Uppalapati et al. 2007), the regulation of coronatine and the T3SS in DC3000 are interconnected (Boch et al. 2002; Fouts et al. 2002; Peñaloza-Vázquez et al. 2000; Sreedharan et al. 2006), coronatine may promote programmed cell death in plants (Mach et al. 2001), and coronatine has been proposed to be a factor in triggering the necrogenic phase of the hemibiotrophic interaction of DC3000 with plants (Uppalapati et al. 2007). However, it is important to note that we have no evidence at this point of altered coronatine production or any causal relationship between the chlorosis and lesion phenotypes of mutants lacking PSPTO4723.

The second noteworthy aspect of the virulence phenotype resulting from the deletion of *hopAA1-1* in a Δ IX background is the significant reduction in the numbers of lesions produced in tomato leaves without a commensurate reduction in bacterial growth. As noted above, we have also observed that mutations in either *avrE* or *hopM1* in DC3000 have a similar phenotype. In the future, we would like to learn if relatively subtle reductions in growth can have a disproportionate effect on lesion formation or whether specific effectors in the DC3000 repertoire directly control lesion formation. The observation that various *P. syringae* pathovars that become virulent on normally nonhost tomato (because of genetic manipulations blocking Pto/Prf-mediated

ETI) produce symptoms characteristic of the diseases on their normal hosts indicates the importance of pathogen factors in directing symptom development (Lin and Martin 2007). Current models of effector function focus on the suppression of PTI and the evasion of ETI (Gohre and Robatzek 2008), which are considered to be early events in the infection. In contrast, lesion formation occurs late in the infection and is accompanied by the expression of defense genes associated with ETI and the cessation of bacterial growth (Tao et al. 2003). Although integrating these observations remains a challenge, the reduced speck phenotype we observed provides a useful bioassay for guiding exploration of the function of HopAA1-1 in pathogenesis. Additional challenges include determining the significance (if any, given the retention of biological activity by mutants altered in the putative catalytic arginine) of the GAP-like domain in HopAA1-1 and the basis for loss of activity in the associated LRA-FEN polymorphism. Another important challenge will be to understand how PSPTO4723 controls the production of diffuse chlorosis in infected leaves and how this apparent noneffector overlaps functionally with HopAA1-1 in promoting bacterial speck symptoms.

MATERIALS AND METHODS

Plasmids, strains, and media.

All strains and plasmids used in this study are presented in Table 2. Plasmids were maintained in *Escherichia coli* DH5 α

except for pYES-DEST52, pCPP5447, pCPP5372, pCPP5296, and pBS46, all of which carry the *ccdB* gene for counter-selection during Gateway cloning and, thus, were maintained in *E. coli* DB3.1. *E. coli* strains were grown at 37°C in Luria-Bertani medium, and *Pseudomonas* strains were grown at 30°C or room temperature in King's B (KB) medium or at room temperature in *hrp* minimal media (Huynh et al. 1989; King et al. 1954; Sambrook and Russel 2001). The following concentrations of antibiotics were used for all bacterial strains: ampicillin, 100; chloramphenicol, 20; gentamicin, 10; kanamycin, 50; rifampicin, 50; spectinomycin, 50; and cycloheximide, 4.

Drop out base (DOB) media (Qbiogene and Sunrise Science Products, Morgan Irvine and San Diego, CA, U.S.A., respectively) containing 2% glucose (Glc), 2% raffinose (Raf), or 1% raffinose and 2% galactose (Gal) as carbon sources was used for the growth of all yeast strains except for the RFY206 strain used in the creation of an effector-gene cluster IX clone; this strain was grown on YPAD media (Gietz et al. 1992), but after being transformed with the cluster IX clone, synthetic defined medium lacking uracil was used to maintain the plasmid. Plasmids were maintained in other strains by supplementing the media with complete supplement mixture lacking uracil (–Ura). Yeast strains were grown at 30°C in all cases.

DNA manipulations.

Standard molecular cloning procedures were used for creation, maintenance, and transformation of plasmid constructs

Table 2. Strains and plasmids

Strain or plasmid	Genotype ^a	Source or reference
<i>Escherichia coli</i>		
DH5 α	F ⁺ Φ80lacZΔM15 ΔlacZYA-argF)U169 deoR recA1 endA1 hsdR17 phoA supE44 thi-1 gyrA96 relA1 λ [–]	Invitrogen
DB3.1	F ⁺ gyrA462 endA1Δ (sr1-recA) mcrB mrr hsdS20 supE44 ara-14 galK2 lacY1 proA2 rpsL20 xyl-5 λ [–] leu mtl-1	Invitrogen
XL1-Blue S17-1	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F ⁺ proAB lacIqZΔM15 Tn10 (Tet ^r)] thi pro hsdR [–] hsdM ⁺ recA (chr::RP4-2-Tc::Mu- Km::Tn7)	Stratagene
<i>Saccharomyces cerevisiae</i>		
BY4741a	MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0	Brachmann et al. 1998
RFY206	MATa his3 Δ200 leu2-3 lys2 Δ201 ura3-52 trp1Δ::hisG	Finley and Brent 1994
<i>Pseudomonas syringae</i> pv. <i>tomato</i>		
DC3000	Wild type Rif ^r	Buell et al. 2003
CUCPB5429	effector cluster IX (<i>hopAA1-2/hopV1/hopAO1/hopG1</i>) deletion mutant containing FRT scar	Wei et al. 2007
CUCPB5134	<i>hopAA1-1::nptII</i> , <i>hopAA1-2::aadA1</i> Km ^r Sp ^r	Badel et al. 2002
CUCPB5113	Δ <i>hrcQbRSTU</i> ::Ω <i>aadA1</i> Sp ^r	Badel et al. 2006
CUCPB5524	<i>hopAA1-1</i> deletion mutant containing a FRT-flanked cassette carrying <i>aadA1</i> Sp ^r	This study
CUCPB5526	<i>hopAA1-2/hopV1/hopAO1/hopG1</i> deletion containing FRT scar, <i>hopAA1-1</i> deletion containing a FRT flanked cassette carrying <i>aadA1</i> , Sp ^r	This study
CUCPB5563	PSPTO4723 deletion containing a FRT-flanked cassette carrying <i>aadA1</i> , Sp ^r	This study
CUCPB5564	<i>hopAA1-1</i> deletion marked with FRT scar; PSPTO4723 deletion containing a FRT-flanked cassette carrying <i>aadA1</i> , Sp ^r	This study
Plasmids		
pENTR/SD/D- TOPO	Gateway entry vector, Km ^r	Invitrogen
pCR2.1-TOPO	TA cloning vector; Km ^r and Ap ^r	Invitrogen
pCPP5107	pENTR/SD/D-TOPO:: <i>hopAA1-1</i> _{Pto DC3000}	Munkvold et al. 2008
pCPP5108	pENTR/SD/D-TOPO:: <i>hopAA1-2</i>	Munkvold et al. 2008
pCPP5409	pENTR/SD/D-TOPO:: <i>hopAA1</i> _{Pto TI}	This study
pCPP5348	pENTR/SD/D-TOPO:: <i>hopAA1</i> _{Pma ES4326}	This study
pCPP5294	pENTR/SD/D-TOPO:: <i>hopAA1</i> _{Psy B728a}	This study
pCPP5639	pENTR/SD/D-TOPO:: <i>hopAA1</i> _{Ppi}	This study
pCPP5640	pENTR/SD/D-TOPO:: <i>hopAA1</i> _{Pta}	This study
pCPP5430	pENTR/SD/D-TOPO:: <i>hopAA1</i> _{Pta ATCC11528}	This study
pCPP5351	pENTR/SD/D-TOPO:: <i>P_{hrp}hopAA1-1</i> _{Pto DC3000}	This study
pCPP5470	pENTR/SD/D-TOPO:: <i>hopAA1-1</i> ₁₋₄₃₆	Munkvold et al. 2008
pCPP5471	pENTR/SD/D-TOPO:: <i>hopAA1-1</i> ₁₋₃₈₆	Munkvold et al. 2008
pCPP5994	pENTR/SD/D-TOPO:: <i>hopAA1-1</i> _{R121A}	This study
pCPP5995	pENTR/SD/D-TOPO:: <i>hopAA1-1</i> _{LRA-FEN}	This study
pCPP5559	pENTR/SD/D-TOPO:: <i>P_{hrp}hopAA1-1</i> _{R121A}	This study
pCPP5560	pENTR/SD/D-TOPO:: <i>P_{hrp}hopAA1-1</i> _{LRA-FEN}	This study

(continued on the next page)

^a Ap = ampicillin, Km = kanamycin, Sp = spectinomycin, and Rif = rifampicin.

(Sambrook and Russel 2001). All primers used in this study were purchased from Integrated DNA Technologies (Skokie, IL, U.S.A.). DNA amplification was carried out with either Deep Vent DNA polymerase (New England Biolabs, Ipswich, MA, U.S.A.), cloned Pfu DNA polymerase (Stratagene, La Jolla, CA, U.S.A.), PrimeSTAR HS DNA polymerase, or ExTaq (Takara Bio Inc., Madison, WI, U.S.A.). DNA sequencing was performed on an Applied Biosystems Automated 3730 DNA Analyzer at the Cornell University Biotechnology Resource Center. Sequence analysis was carried out using Sequencher 4.2.2 software (Gene Codes Corporation, Ann Arbor, MI, U.S.A.).

Cloning of *hopAA1* family members.

Genomic DNA was isolated from various *P. syringae* strains with the Wizard genomic DNA purification kit (Promega, Madison, WI, U.S.A.). Primers sets P1 and P2 and P3 and P4 were used to amplify the 3' and 5' regions of *hopAA1* homologs, respectively, from genomic DNA (Fig. 1; Table 3). Amplification products were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, U.S.A.) via TA cloning. For each product, three individual clones were sequenced using primers complementary to the M13 priming sites present on the vector. For those assayed in subsequent experiments, new primer sets (P5 and P6) were designed to regions containing the predicted start and stop codons for each *hopAA1* homolog as compared with *hopAA1-I*_{Pto DC3000} (Fig. 1; Table 3). Forward primers were designed to contain a 5'

overhang of CACC- followed by 15 to 25 nucleotides beginning with the predicted start codon of the gene. Reverse primers were designed to the 3' 17 to 25 nucleotides of the gene but lacking the stop codon, to allow for expression of C-terminal fusion proteins. The amplification products were TOPO (Invitrogen) cloned into pENTR/SD/D-TOPO. Again, three individual clones for each *hopAA1* homolog were sequenced, using M13 primers. Sequencing of *hopAA1*_{Pta} and *hopAA1*_{Pag} revealed the presence of a frameshift leading to a premature stop codon within the predicted coding sequence. For these homologs, a second reverse primer (P7) was designed to the new predicted stop codon (Fig. 1; Table 3). Amplification, cloning, and sequencing were carried out as described above.

In order to clone *hopAA1* homologs for which sequences previously had been deposited in GenBank (*hopAA1*_{Pma ES4326} and *hopAA1*_{Psy B728a}), TOPO cloning primers were designed as described above to the regions containing the start and stop codons. Amplification, cloning, and sequencing were performed in the same manner as described above.

Other plasmid and mutant constructions.

DC3000 *hopAA1-I* with its native promoter was cloned into pENTR/SD/D-TOPO. Amplification of 216 basepairs upstream of the *hopAA1-I* start codon to the 3' end of the gene lacking the stop codon was carried out with primer pair P1829 and P1998. The amplification product was cloned via the directional

Table 2. (continued from previous page)

Strain or plasmid	Genotype ^a	Source or reference
pCPP5372	pBBR1MCS containing <i>avrPto</i> promoter (<i>P_{avrPto}</i>), Gateway reading frame B cassette, and C-terminal HA tag; Gm ^r Cm ^r	Oh et al. 2007
pCPP5581	pCPP5372:: <i>hopAA1-I</i> _{Pto DC3000}	This study
pCPP5582	pCPP5372:: <i>hopAA1-2</i>	This study
pCPP5928	pCPP5372: <i>hopAA1-I</i> ₁₋₄₃₆	This study
pCPP5927	pCPP5372:: <i>hopAA1-I</i> ₁₋₃₈₆	This study
pCPP5932	pCPP5372:: <i>P_{hrp}</i> <i>hopAA1-I</i> _{R121A}	This study
pCPP5933	pCPP5372:: <i>P_{hrp}</i> <i>hopAA1-I</i> _{LRA-FEN}	This study
pCPP5394	pENTR/D carrying 1-kb flanks for <i>hopAA1-2-hopG1</i> deletion marked with FRT-flanked <i>aadA1</i> , Sp ^r Km ^r	Wei et al. 2007
pBS46	pBBR1MCS5 containing Gateway reading frame B cassette for expression of HA-tagged proteins from <i>nptII</i> promoter; Gm	Wei et al. 2007
pENTRgus	Gateway entry vector containing <i>gus</i>	Invitrogen
pENTR11	Gateway entry vector, MCS Km ^r	Invitrogen
pCPP5929	pBS46:: <i>gus</i> ; used as an empty vector control	This study
pYES-DEST52	Gatewaydestination vector 2μ, <i>P_{GAL1}</i> , ApR, <i>URA3</i> , adds a C-terminal V5-6His tag	Invitrogen
pYES2/CT/LacZ	LacZ-V5-6his expressed from <i>P_{GAL1}</i> , 2μ, Ap ^r	Invitrogen
pCPP5229	pYES-DEST52 expressing HopAA1-I _{Pto DC3000} -V5-6his	Munkvold et al. 2008
pCPP5165	pYES-DEST52 expressing HopAA1-2-V5-6his	Munkvold et al. 2008
pCPP5417	pYES-DEST52 expressing HopAA1 _{Pto T1} -V5-6his	This study
pCPP3286	pYES-DEST52 expressing HopAA1 _{Pma ES4326} -V5-6his	This study
pCPP5349	pYES-DEST52 expressing HopAA1 _{Psy B728a} -V5-6his	This study
pCPP3287	pYES-DEST52 expressing HopAA1 _{Ppi} -V5-6his	This study
pCPP5425	pYES-DEST52 expressing HopAA1 _{Pta} -V5-6his	This study
pCPP5457	pYES-DEST52 expressing HopAA1 _{Pta ATCC11528} -V5-6his	This study
pCPP5492	pYES-DEST52 expressing HopAA1-I _{R121A} -V5-6his	This study
pCPP5493	pYES-DEST52 expressing HopAA1-I _{LRA-FEN} -V5-6his	This study
pCPP5950	LR recombinant between pYESDEST52 and pCPP5394; recipient vector for yeast recombination cloning of entire cluster IX, Sp ^r Ap ^r	This study
pCPP5447	pENTR/SD/D-TOPO:: <i>attP</i> Gateway cassette Km ^r	This study
pCPP5956	pCPP5447:: <i>hopAA1-2-hopG1</i> Km ^r	This study
pCPP5296	pBBR1MCS containing Gateway reading frame B cassette and C-terminal HA tag; Gm ^r Cm ^r	This study
pCPP5957	pCPP5296:: <i>hopAA1-2-hopG1</i> Gm ^r	This study
pK18mobsacB	Small mobilizable vector, Km ^r , sucrose-sensitive (<i>sacB</i>)	Schafer et al. 1994
pCPP5242	pKD4 FRT cassette plasmid with <i>nptII</i> replaced by <i>aadA1</i> , Sp ^r	Wei et al. 2007
pCPP5924	pK18mobsacB carrying the <i>hopAA1-I</i> deletion flanks, Km ^r	This study
pCPP5926	pCPP5924 with a FRT-flanked <i>aadA1</i> cassette in the deletion construct's central <i>EcoRI</i> site, Km ^r Sp ^r	This study
pCPP6000	pENTR11::PSPTO4723 with promoter region	This study
pCPP6001	pCPP5296::PSPTO4723 with promoter region (=PSPTO4723)	This study
pCPP5998	pK18mobsacB carrying the PSPTO4723 deletion construct. Km ^r	This study
pCPP5999	pCPP5998 with a FRT-flanked cassette carrying <i>aadA1</i> cloned into the deletion construct's central <i>SpeI</i> site.	This study

TOPO cloning reaction (Invitrogen). The resulting plasmid pCPP5351 was confirmed by DNA sequence analysis.

Site-directed mutagenesis of *hopAAI-I*_{Pto DC3000} and *P_{hrp} hopAAI-I*_{Pto DC3000} was carried out using the QuikChange kit (Stratagene) on the entry vectors pCPP5107 and pCPP5351, respectively. For each mutation (R121A and LRA to FEN), complementary primers containing the mutated sequence were flanked by a minimum of 15 nucleotides of complementary sequence (P2646, P2647, P2648, P2649). Mutated plasmids were maintained in *E. coli* XL1-Blue, and mutations were confirmed by sequencing.

The Gateway destination vector pYES-DEST52 was recombined in an LR recombination reaction (Invitrogen) with the pENTR/SD-TOPO vectors carrying promoterless *hopAAI* homologs and *hopAAI-I*_{Pto DC3000} substitution mutants, generating vectors for yeast expression. Similarly, for expression in *P. syringae* for complementation experiments, the destination vector pCPP5372 was recombined in LR recombination reactions with pENTR/SD/D-TOPO vectors carrying *hopAAI-I* and derivatives (pCPP5107, pCPP5471, pCPP5470, pCPP5559, and pCPP5560). The vector control pCPP5929 was obtained from an LR recombination between pBS46 and pENTR-gus from Invitrogen. The destination vector pBS46 is of similar origin to

pCPP5372, save for having an *nptII* promoter rather than a HrpL-activated promoter, and so, pBS46::gus was used as a vector control for complementation experiments.

For cluster IX complementation, the broad-host range Gateway destination vector pCPP5296 was created first by removing the Lac promoter from pBBR1MCS5 by *SphI* and *KpnI* restriction digestion followed by gel purification (Kovach et al. 1995). Next, the Gateway cassette and HA tag from pCPP5040 was amplified with primer pair P1817 and P1818 and was cloned into pBBR1MCS5 via its *SphI* and *KpnI* sites (López-Solanilla et al. 2004).

To delete the *hopAAI-I* ORF from DC3000, flanking sequence was amplified with P2642 and P2643 and P2644 and P2645 and was ligated together using primer-introduced *EcoRI* sites. The ligated fragment was then cloned into pK18mobsacB via *HindIII* and *BamHI* sites, yielding pCPP5924. An FRT-flanked cassette containing a gene conferring spectinomycin resistance was then amplified using P2640 and P2641, primers that added *EcoRI* sites to both ends of the cassette. This PCR product was then cloned into the central *EcoRI* site of pCPP5924 to create pCPP5926.

The PSPTO4723 deletion construct was made by amplifying a 1.5-kb region upstream of the ORF with P2661 and P2662

Table 3. Primers used

Primer	Sequence ^a	Target gene/restriction site
P1828	5' TCATGTCATCAAACTGCTT 3'	P1 (<i>hrpW</i>)
P1816	5' AGTGATATTTTTCGTCGCCAT 3'	P2 (<i>hopAAI-I</i>)
P1815	5' GCAGCCTGCCATCAACA 3'	P3 (<i>hopAAI-I</i>)
P1827	5' TTGCGAAAGCTGATCAG 3'	P4 (PSPTO1371)
P1834	5' CACCATGCACATCAACCGACCCG 3'	P5 (<i>hopAAI</i> _{Pto T1})
P1998	5' CGACCGCATAGGCCGAAAC 3'	P6 (<i>hopAAI</i> _{Pto DC3000/T1})
P1811	5' CACCATGTACATCAACCGTCCATC 3'	P5 (<i>hopAAI</i> _{Pma ES4326})
P1812	5' TGGCCGCATTGCCTCGAAAG 3'	P6 (<i>hopAAI</i> _{Pma ES4326})
P1813	5' CACCATGCACATCAACCGAACCG 3'	P5 (<i>hopAAI</i> _{psy B728a})
P1990	5' CACCATGCACATCAACCGAACCGG 3'	P5 (<i>hopAAI</i> _{Ppi})
P1814	5' TGAGCGCCCTGGCTGGAA 3'	P6 (<i>hopAAI</i> _{psyl/Ppi})
P1838	5' CACCATGCACATCCACCGATC 3'	P5 (<i>hopAAI</i> _{Pta})
P1991	5' CGACCGCCCAGGCTGAA 3'	P6 (<i>hopAAI</i> _{Pta})
P1995	5' CACCATGCACATCAACCGATCCG 3'	P5 (<i>hopAAI</i> _{Pta/Pag})
P1992	5' CGACACCCAGGCTGAAACG 3'	P6 (<i>hopAAI</i> _{Pta/Pag})
P1988	5' TGGATGCCTGTCCGGCG 3'	P7 (<i>hopAAI</i> _{Pta/Pag})
P1829	5' CACCTGGGCGTACGAGATTATTC 3'	<i>P_{hrp}hopAAI-I</i>
P2646	5' CTACATCAGCGCGTGTGCGCGACGCCCTTTGCC 3'	<i>hopAAI-I</i> _{R121A}
P2647	5' GGCAAAGGGCGTCGCGCCAACGCGCCTGATGTAG 3'	<i>hopAAI-I</i> _{R121A complement}
P2648	5' CGACTACATCAGGCGCGTTTGAGAACACGCCCTTTGCC ATGGCC 3'	<i>hopAAI-I</i> _{LRA-FEN}
P2649	5' GGCCATGGCAAAGGGCGGTGTTCTCAACACGCGCTGATG TAGTCG 3'	<i>hopAAI-I</i> _{LRA-FEN complement}
P1817	5' TGAGCATGCACAAGTTTGTACAAAAAAGCTG 3'	<i>SphI</i>
P1818	5' AGTGGTACCACTCACTATAGGGAGTCC 3'	<i>KpnI</i>
P2642	5' TCTAAGCTTACGTAGGGGTAGGGCAGTGG 3'	<i>HindIII</i>
P2643	5' TAGAATTCGCGTTGTTGGACGCGTCG 3'	<i>EcoRI</i>
P2644	5' ATGAATTCGAAATACCGTTTCGGCCTAT 3'	<i>EcoRI</i>
P2645	5' ACAGGATCCATCATGCTGTTGCTGCAGATG 3'	<i>BamHI</i>
P2640	5' TAGAATTCGCTGGAGCTGCTTCGAA 3'	<i>EcoRI</i>
P2641	5' ATGAATTCATGGGAATTAGCCATGGT 3'	<i>EcoRI</i>
P1627	5' TATTAGATCTGGACATAAGCCTGTTTCGGTT 3'	<i>BglII</i>
P1628	5' TATTAGATCTTATTGCGGACTACCTTGG 3'	<i>BglII</i>
P2512	5' CTAATCCACACCACCTGACG 3'	—
P2513	5' CAAAGCGCTCACCTAGCTCT 3'	—
P2514	5' AGTTGAAAGGTGATCTAGAGC 3'	—
P2515	5' AGCTAGTTGGATTAATGCGG 3'	—
P504	5' ATTGAATTCACATCAACCGACGC 3'	<i>EcoRI</i>
P505	5' ACAGGATCCATTACGACCGCATAG 3'	<i>EcoRI</i>
P2253	5' ATTACCTAGGGTGTAGGCTGGAGCTGCTTC 3'	<i>AvrII</i>
P2254	5' ATTACCTAGGCATATGAATATCCTCCTTA 3'	<i>AvrII</i>
P2661	5' TAATGAATTCAGTTCGTGAATCATGGCA 3'	<i>EcoRI</i>
P2662	5' GCATACTAGTAACTAGAATTTACGCCATGA 3'	<i>SpeI</i>
P2663	5' TAATACTAGTCTCCACACATCAAGCAAAA 3'	<i>SpeI</i>
P2664	5' TAATAAGCTTAATTGAAGTGTGCCATCCTG 3'	<i>HindIII</i>
P2667	5' GCCACGCGGCGATAGGT 3'	—
P2668	5' AGCGTTTCGGTTCTCACGGTATCG 3'	—

^a Underlined sequence designates restriction sites; bold sequence designates a mutation.

and a 1-kb region downstream of PSPTO4723 with P2663 and P2664 and ligating the two together after a *SpeI* digest. The resulting 2.5-kb fragment was then cloned into pK18mobsacB with an *EcoRI* and *HindIII* digest. To mark the deletion the FRTSp^r cassette was amplified from pCPP5242 using P2253 and P2254 to add *AvrII* sites to both ends. This PCR product was then digested with *AvrII* and cloned into the central *SpeI* site of the deletion construct to create pCPP5999.

The PSPTO4723 expression vector was created by obtaining a 579-bp fragment from a *BsaAI/EcoRV* digest of pCPP5956 and cloning it into pENTR11 with an *EcoRV/XmnI* digest to create pCPP6000. Orientation was confirmed such that, when pCPP6000 was LR-cloned into pCPP5296 to create pCPP6001, the transcriptional terminator can provide robust termination for the transcript.

Yeast growth inhibition assay.

Saccharomyces cerevisiae BY4741a was transformed with pYES-DEST52 expression vectors carrying *hopAAI* homologs according to the lithium acetate transformation method (Gietz et al. 1992). Strains were inoculated into DOB Glc –Ura liquid culture and were grown overnight with shaking. The cultures were subinoculated into DOB Raf –Ura and, again, grown overnight with shaking. The following day, cultures were normalized to an optical density at 600 nm (OD₆₀₀) of 0.45 in DOB Raf –Ura media using a Spectronic 20 (Bausch & Lomb, Rochester, NY, U.S.A.). Cultures were serially diluted by 10-fold four times in the same media. Each dilution was dropped onto DOB Gal –Ura plates, was incubated for two days, and was photographed.

Construction of deletion mutations.

Suicide vectors containing deletion constructs were electrotransformed into *E. coli* S17-1 and delivered into *P. syringae* strains by conjugation. Transconjugants were plated on KB supplemented with kanamycin to select for integrants and both ampicillin and rifampicin to select against the donor strain. Resulting integrants were then plated on 10% sucrose plates supplemented with spectinomycin to counterselect the integrated vector while concurrently selecting for the deletion construct. Subsequent clones were also confirmed to be kanamycin-sensitive to ascertain that the counterselection was successful. Confirmation of the deletion of *hopAAI-1* in CUCPB5526 came from Southern hybridization using the FRT-flanked cassette amplified by P1627 and P1628 from pCPP5242 as a probe, with an *MfeI* digest of genomic DNA, with a 4.2-kb band indicative of correct marker exchange and deletion of *hopAAI-1*. The *hopAAI-1::nptIII/hopAAI-2::aadA1* double mutant was created in a previous study (Badel et al. 2002), as was the cluster IX deletion. (Wei et al. 2007). Confirmation of the PSPTO4723 deletion in CUCPB5563 and CUCPB5564 came from a PCR check using P2667 and P2668. In order to create the marked PSPTO4723 deletion in the already marked Δ *hopAAI-1* background, the FRT-flanked cassette had to first be removed from the *hopAAI-1* mutant. To do this, the strain was transformed with pCPP5264, an easily cured plasmid expressing the FLP recombinase. After this transformation, the plasmid was cured and the removal of the FRT cassette was confirmed both by spectinomycin sensitivity and a PCR check using P504 and P505.

Complementation of the effector-gene cluster IX deletion.

pCPP5394, an entry vector containing a deletion construct for effector cluster IX of *P. syringae* DC3000 was LR recombined into pYESDEST52 to create pCPP5950. A 5.7-kb fragment of cluster IX amplified by P2512 and P2513 and a 7.3-kb fragment of cluster IX amplified by P2514 and P2515, using

HS Primestar DNA polymerase, were cotransformed into the yeast strain RFY206 along with *BclI*-linearized pCPP5950, using the procedure outlined in the Yeastmaker yeast transformation system 2 user manual from Clontech (Palo Alto, CA, U.S.A.). Fragments were designed such that they shared 35-bp regions of perfect homology with both one another and the vector backbone to allow for recombination-based assembly of the plasmid. Colonies were then plated on defined synthetic media lacking uracil to select for a successfully circularized plasmid. At this point, plasmids were obtained from a pool of yeast and were transformed into DH5 α , after which they were screened for a correctly assembled recombinant plasmid. The resulting plasmid was BP cloned into pCPP5447, the product of recombination between pENTR/SD/D-TOPO and a Gateway Destination vector containing reading frame A, to create the entry vector, pCPP5956. pCPP5956 was then LR cloned into pCPP5296 to create the cluster IX expression vector pCPP5957, which was then electrotransformed into CUCPB5526 and was maintained through gentamicin selection to create the complemented strain.

Plant materials and bacterial virulence assays.

Tomato (*Solanum lycopersicum* cv. Moneymaker) plants, 3.5 to 4 weeks postgermination, were used in virulence growth and symptom assays. All symptom assays were performed by dip inoculation, whereas growth assays were performed both by blunt-syringe infiltration and dip inoculation. Plants were grown under greenhouse conditions. For dipping assays, plants were transferred to a growth chamber with 50% relative humidity and a 16-h day and were covered with autoclave bags to increase humidity and open stomata 24 h before infiltration. No such steps were taken for syringe-infiltration assays. Syringe infiltrations were performed as previously described (Alfano et al. 1996), infiltrating the youngest fully expanded leaves that were large enough to take leaf tissue from. For syringe-inoculation assays, 2-day-old lawns were harvested off solid KB media and were resuspended in 100 mM sucrose, 10 mM MgCl₂ to an OD₆₀₀ of 0.3. The resulting suspension was diluted in 10 mM sucrose for a final bacterial count of 1×10^4 CFU per milliliter. This suspension was then inoculated into plant leaves using a blunt syringe. For competitive growth indices, equal amounts of the 1×10^4 suspensions of each strain were mixed for the final inoculum. Plants were then kept at 80% relative humidity with a 12-h day at 25°C for 3 days postinfection, at which point, tissue was harvested for growth counts.

Dip assays were performed as previously described (Wei et al. 2007), with bacteria also being resuspended from 2-day-old plates in 100 mM sucrose, 10 mM MgCl₂ to an OD₆₀₀ of 0.3. This suspension was diluted in 1 liter of water with 0.02% Silwet L77 to 1×10^5 CFU/ml. Plants were dipped into the bacterial suspension and were swirled for 30 s and were allowed to air dry. Plants were then kept in a growth chamber with a 16-h day at 24°C during the day and 20°C at night with 50% relative humidity for either 3 days for growth assays or 5 days for growth and symptom assays.

For growth assays, counts were obtained from initial inoculum by diluting and plating on KB media and also, in order to determine the input ratio in competitive indices, on KB supplemented with spectinomycin. At either day 3 or 5 after inoculation, leaf tissue was harvested and ground in 100 mM sucrose, 10 mM MgCl₂ and was plated as a series of dilutions on KB agar supplemented with cycloheximide. For competitive indices, dilutions were also plated on KB agar supplemented with spectinomycin and cycloheximide to determine final growth ratios. For syringe infiltrations, 1.5 cm² of tissue was harvested, and for dip assays, 2 cm² of tissue was harvested. Competitive indices and relative increase ratios were calculated as previ-

ously described (Macho et al. 2007). Symptom assays were completed 5 days after inoculation. Specks were visually scored as chlorotic lesions with necrotic centers. The entire plant was scored, and the data represent an average number of specks per plant. All datapoints represent three independent trials of two plants apiece.

Immunoblot analyses.

In order to confirm production of HopAA1-1 variants in yeast and *P. syringae*, immunoblot analyses were performed. Yeast samples were prepared as previously described (Munkvold et al. 2008). *P. syringae* strains were grown in Hrp-minimal media overnight, to induce HrpL-dependent expression of vectors. Bacteria in these overnight broths were harvested by centrifugation, and proteins in the pellet fraction resolved with a 7% sodium dodecyl sulfate-polyacrylamide gel. Yeast and bacterial proteins were blotted onto an Immobilon P transfer membrane from Millipore (Bedford, MA, U.S.A.) using a Hoefer Semi-Phor semidry transfer unit. The membrane was blotted using a high-affinity rat monoclonal anti-HA antibody from Roche Diagnostics (Mannheim, Germany) and a secondary anti-rat antibody conjugated to alkaline phosphatase from Sigma-Aldrich (Tokyo). The membrane was then incubated with chemiluminescent substrate before being exposed on X-ray film.

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