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Analysis of the Role of the Type III Effector Inventory of *Pseudomonas syringae* pv. phaseolicola 1448a in Interaction with the Plant[▽]

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In *Pseudomonas syringae*, the type III secretion system (T3SS) is essential for disease in compatible hosts and for eliciting the hypersensitive response in incompatible hosts. *P. syringae* pathovars secrete a variable number of type III effectors that form their secretomes. The secretome of *Pseudomonas syringae* pv. phaseolicola 1448a (*Pph*1448a) currently includes 22 experimentally validated effectors, one HrpL-regulated candidate for which translocation results have been inconsistent, two translocated candidates for which *in planta* expression has not been established, one bioinformatically identified candidate, and six candidates that have been experimentally discarded. We analyzed the translocation and/or expression of these and other candidates to complete the *Pph*1448a effector inventory, bringing this inventory to 27 bona fide effectors, including a new one that does not belong to any of the previously described effector families. We developed a simple process for rapidly making single and double knockout mutants and apply it to the generation of an effector mutant collection that includes single knockouts for the majority of the *Pph*1448a effector inventory. We also generated two double mutant strains containing effectors with potentially redundant functions and analyzed the virulence of the single and double mutant strains as well as strains expressing each of the effectors from a plasmid. We demonstrate that AvrB4-1 and AvrB4-2, as well as HopW1-1 and HopW1-2, are fully redundant and contribute to virulence in bean plants, thus validating this approach for dissecting the contribution of the *Pph*1448a type III effector inventory to virulence. We also analyzed the effect that the expression of these four effectors from *Pseudomonas syringae* pv. tomato DC3000 (*Pto*DC3000) has during its interaction with *Arabidopsis thaliana*, establishing that AvrB4-1, but not the others, determines a restriction of bacterial growth that takes place mostly independently of the salicylic acid (SA)-signaling pathway.

Type III secretion systems (T3SS) are complex and specialized machineries that inject effector proteins directly into the host cell cytosol (2). In *Pseudomonas syringae*, T3SS-mediated secretion is essential for disease in compatible hosts and for eliciting the hypersensitive response (HR) in incompatible hosts (1). *P. syringae* pathovars secrete a variable number of type III effectors that form their so-called secretomes and are expressed within the plant under the control of the alternative sigma factor HrpL (47). Understanding how the T3SS determines pathogenicity requires the functional characterization of the complete type III effector inventory. However, this characterization has been partially hindered by the fact that mutation of individual effectors, usually the most straightforward approach, rarely causes virulence attenuation (14). Thus, reports showing the contribution of the type III effector to virulence in *P. syringae* pathovars have resorted to ectopic expression in homolog-lacking related strains (40), plasmid-cured derivatives (21), double mutants (6, 28), or polymutants (3, 26). In relation to this, we have previously established the use of the competitive index (CI) in mixed infections (13, 42) as a more sensitive virulence assay for *P. syringae* pathovars than traditional assays (31). Using CIs, we demonstrated for the first time the individual contribution of AvrPto, an otherwise thoroughly characterized type III effector from *Pseudomonas syringae* pv. tomato (9, 17, 18, 27, 36, 39, 40, 46), to pathogen growth within its natural host (31). Therefore, analysis of effector mutants by use of the CI may provide the means to establish the quantitative contribution of the members of *P. syringae* T3SS secretomes to virulence. In addition, genetic analysis of the effects of combinations of effector mutations on virulence has already proven a useful approach to establishing the contribution of the members of the *P. syringae* pv. tomato DC3000 secretome to virulence by revealing a functional overlap (6, 26, 28). Thus, generation of knockouts in all individual effector genes of a given secretome, achieved in such a manner as to allow for easy combination of these strains into double or multiple mutant strains, is a desirable task, albeit a cumbersome one, considering the size of most secretomes.

The secretome of the fully sequenced wild-type (wt) representative of the *Pseudomonas syringae* pv. phaseolicola 1448a strain (*Pph*1448a) has previously been analyzed, using a differential fluorescence induction screen (7) and bioinformatics (44), to identify effector genes. Our laboratory contributed to establishing this secretome through the development and application of a very sensitive assay for T3SS-mediated translo-

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cation based on CI assays (30). This assay represents an improvement over the sensitivity of the commonly used AvrRpt2 reporter assay. When fused to a T3SS-secreted protein, AvrRpt2$^{81-255}$ is translocated inside the host cell, eliciting a hypersensitive response (HR), dependent on the resistance protein RPS2 (32). By using CIs to measure the bacterial growth reduction associated with the AvrRpt2-RPS-mediated defense response, we detected translocation for two out of four *Pph*1448a effector candidates previously discarded by other assays, HopAJ1 and HopAK1 (30), and demonstrated translocation for two out of five previously untested candidates, HopAH2 and A0129. However, although *in planta* expression has been shown to take place in an HrpL-dependent manner for HopAJ1 and HopAK1 (7), it has not been established for HopAH2 and A0129. Effector nomenclature guidelines recommend that the abbreviation for the pathovars as well as the name of the strain should be included within the effector name (29). For simplicity, we include this indication only when effectors from other pathovars are mentioned. In summary, to date, 22 effectors in *Pph*1448a have been experimentally validated (7, 30, 44), one HrpL-regulated candidate has given inconsistent translocation results (AvrE1) (7), two translocated candidates have not been analyzed for expression *in planta* (HopAH2 and A0129) (30), one bioinformatically identified candidate has not been experimentally tested (AvrB4-2) (23), and six additional candidates have been proposed but experimentally ruled out (PSPPH3757, HopAN1, HopAJ2, HopW1-2, HopV1, and HopJ1) (7, 30).

In this work, we analyzed the translocation and/or expression of these and other candidate effectors to close the type III effector inventory of *Pph*1448a. Our results indicate that the *Pph*1448a complete type III secretome is formed by 27 validated effectors, including a new one, HopAY1, which does not belong to any of the previously described effector families. The work includes the development of a simplified process for quick generation of single and double knockout mutants and its application to constructing a collection of single mutants for almost all members of the *Pph*1448a type III secretome. Additionally, we generated two double mutant strains containing effectors with potentially redundant functions and analyzed the virulence of the four single and two double mutant strains as well as the double mutants expressing each of the effectors from a plasmid. We demonstrate that AvrB4-1 and AvrB4-2, as well as HopW1-1 and HopW1-2, are fully redundant and contribute to the virulence of *Pph*1448a. The tools and approach used in this work set the groundwork for dissecting the contribution of the entire *Pph*1448a type III secretome to virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this work are listed in Table 1. *Escherichia coli* and *Pph*1448a derivative strains were grown at 37°C and 28°C, respectively, with aeration in Luria-Bertani (LB) medium. Antibiotics were used at the following concentrations: for ampicillin, 100 μg/ml for *E. coli* DH5α and 100 μg/ml for liquid cultures and 300 μg/ml for plates for *Pph*1448a derivative strains; for kanamycin (Km), 50 μg/ml for *E. coli* DH5α and 15 μg/ml for *Pph*1448a derivative strains; and for cycloheximide, 2 μg/ml.

Plasmids and cloning procedures. All plasmids used in this work are listed in Table 2. To obtain pAME9.18, pAME9.19, pAME9.20, and pAME9.21, the open reading frames (ORFs) of *avrB4-2*, *hopW1-2*, PSPPH1154, and *avrE1* without their Stop codons and with their putative ribosome-binding sites were PCR amplified and cloned into pAME9 (30), using *Pph*1448a as a template and the corresponding primers (primers and cloning sites are listed in Table 3). To clone *avrE1*, only the first 978 bp of the ORF were used. Primers were designed to generate translational fusions of the proteins encoded by these genes to the sequence encoding amino acids 81 to 255 of AvrRpt2, to be expressed under the control of the P_{nptII} promoter. Attention was paid during primer designing to avoid cross amplification of highly homologous genes.

To obtain pGEM-T-*nptII*-EcoRI, pGEM-T-*nptII*-BamHI, or pGEM-T-*nptII*-XhoI, a fragment containing the *nptII* kanamycin resistance gene, flanked by FRT (flippase recognition target) sites, was PCR amplified using the Expand high-fidelity PCR system (Roche, Germany), pKD4 (11) as a template, and primers P1 and P2 (introducing either EcoRI, BamHI, or XhoI sites at both ends of the fragment) (Table 3). The products were A/T cloned into pGEM-T (Promega, Madison, WI) and confirmed by restriction analysis.

To generate each knockout vector, we carried out two PCR amplifications of approximately 500 bp (unless otherwise indicated) of the 5′ and 3′ regions flanking the ORF to be deleted, using *Pph*1448a genomic DNA as a template, and primers including an EcoRI site (or BamHI or XhoI, when an EcoRI site was present on the flanking sequences) and the T7 primer sequence in such a manner as to provide homology and a cloning site between the fragments (see Fig. 2A). Each reaction was carried out at 94°C for 3 min, followed by 20 cycles at 94°C for 20s, 55°C for 30s, and 72°C for 50s, followed by 7 min at 72°C, and the reaction mixture contained 0.64 mM deoxynucleoside triphosphate (dNTP) mix, 1× buffer 2 (Expand high-fidelity PCR system [Roche]), 5% dimethyl sulfoxide (DMSO), 0.4 μM corresponding primers, 10 ng genomic DNA, and double-distilled water (H_2O_{dd}) (Nalgene, Rochester, NY). Five microliters of each gel-purified PCR product was used for a PCR, with eight cycles of polymerization at 94°C for 30 min, 52°C for 1 min, and 72°C for 1 min, finishing with 7 min at 72°C without primers or a template. The product from the previous step was used as a template in a reaction mixture (5 μl per reaction mixture) that also contained 0.64 mM dNTP mix, 1× buffer 2 (Expand high-fidelity PCR system [Roche]), 5% DMSO, 0.4 μM corresponding primers (Table 3), and H_2O_{dd} (Nalgene). The mixture was incubated at 94°C for 3 min, followed by 20 cycles at 94°C for 20 s, 53°C for 30 s, and 72°C for 1 min, finishing with 7 min at 72°C. The resulting products, the deletion alleles, were A/T cloned into pGEM-T and fully sequenced to discard mutations on flanking genes. As appropriate, EcoRI, BamHI, or XhoI fragments containing the FRT-flanked kanamycin resistance gene were obtained from pGEM-T-*nptII*-EcoRI, pGEM-T-*nptII*-BamHI, or pGEM-T-*nptII*-XhoI, respectively, and ligated into the EcoRI, BamHI, or XhoI fragments of the corresponding pGEM-T derivatives carrying the deletion alleles. Ligations were transformed into *E. coli* DH5α (16) and transformants selected directly in LB-kanamycin plates, rendering a collection of effector knockout vectors for *Pph*1448a (Table 2).

To generate the plasmids used in complementation experiments (Table 2), the full ORFs from *avrB4-2* and *hopW1-1/2* (sequences from their ORFs are 100% identical) were PCR amplified and cloned into pBBR1MCS4 (to obtain expression from a P_{lacZ} promoter) or pAMEX (to obtain expression from a P_{nptII} promoter). The full *avrB4-1* gene, including its promoter region, was cloned into pBBR1MCS4 (to obtain expression from its native promoter) or pAMEX (to obtain expression from a P_{nptII} promoter). The primers used, as well as the restriction sites, are listed in Table 3.

Recombinant DNA techniques were performed in accordance with standard methods (37). Genomic DNA was extracted using a Jet Flex extraction kit (Genomed, Germany), and plasmid DNA was extracted using a FavorPrep plasmid DNA extraction minikit (Favorgen Biotech Corporation, Taiwan). Routine clone analysis was carried out by quick boiling extraction (20). DNA gel purification was performed using a FavorPrep gel/PCR purification minikit (Favorgen Biotech Corporation).

Generation of knockout strains. Allelic exchange vectors (Table 2) were transformed by electroporation into the *Pph*1448a strain by use of a modification of an electroporation protocol previously described for *P. aeruginosa* (8), using SOB medium (16) to grow *Pph*1448a. Transformants were plated into LB plates supplemented with kanamycin. Replica plates of the resulting colonies were carried out using LB plates supplemented with ampicillin (300 μg/ml) to determine whether each transformant was the result of plasmid integration (a single recombination event) or allelic exchange (a double recombination event). Since ampicillin selection is typically a problem in *P. syringae*, prospective clones were further tested for growth in liquid LB medium containing 100 μg/ml of ampicillin, with 50 μg/ml of nitrofurantoin added to avoid cross-contamination. Southern blot analysis, using *nptII*-FRT as a probe (a 1,495-bp fragment amplified with primers P1 and P2 from pKD4), was used to confirm that allelic exchange occurred at a single and correct position within the genome.

Double mutant strains (Table 1) were generated as follows. Plasmid pFLP2, expressing the flipase enzyme, was transformed into the corresponding single

TABLE 1. Bacterial strains used and effector mutant collection generated in this work

Bacterial strain	Description	Antibiotic resistance	Source or reference
E. coli DH5α	F$^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ*lacU189* ϕ80 Δ*lacZD*M15	None	16
P. syringae pv. tomato DC3000	Wild-type strain	Rif^r	10
P. syringae pv. phaseolicola			
1448a	Race 6; wild-type strain	Nal^r	43
IOM7	Δ*hrpL*	Nal^r Km^r	33
AZJ11	Δ*hopW1-1*::*nptII*	Nal^r Km^r	This work
AZJ12	Δ*hopW1-1*	Nal^r	This work
AZJ13	Δ*hopW1-2*::*nptII*	Nal^r Km^r	This work
AZJ14	Δ*hopW1-1* Δ*hopW1-2*::*nptII*	Nal^r Km^r	This work
AZJ15	Δ*avrB4-1*::*nptII*	Nal^r Km^r	This work
AZJ16	Δ*avrB4-2*::*nptII*	Nal^r Km^r	This work
AZJ17	Δ*avrB4-2*	Nal^r	This work
AZJ18	Δ*avrB4-1*::*nptII* Δ*avrB4-2*	Nal^r Km^r	This work
AZJ19	Δ*hopAB1*::*nptII*	Nal^r Km^r	This work
AZJ20	Δ*hopX1*::*nptII*	Nal^r Km^r	This work
AZJ21	Δ*avrB2*::*nptII*	Nal^r Km^r	This work
AZJ22	Δ*hopR1*::*nptII*	Nal^r Km^r	This work
AZJ23	Δ*hopAY1*::*nptII*	Nal^r Km^r	This work
AZJ24	Δ*hopAW1*::*nptII*	Nal^r Km^r	This work
AZJ25	Δ*hopAS1*::*nptII*	Nal^r Km^r	This work
AZJ26	Δ*hopAJ1*::*nptII*	Nal^r Km^r	This work
AZJ27	Δ*hopAU1*::*nptII*	Nal^r Km^r	This work
AZJ28	Δ*hopAK1*::*nptII*	Nal^r Km^r	This work
AZJ29	Δ*hopAE1*::*nptII*	Nal^r Km^r	This work
AZJ30	Δ*hopD1*::*nptII*	Nal^r Km^r	This work
AZJ31	Δ*hopQ1*::*nptII*	Nal^r Km^r	This work
AZJ32	Δ*hopG1*::*nptII*	Nal^r Km^r	This work
AZJ33	Δ*hopI1*::*nptII*	Nal^r Km^r	This work
AZJ34	Δ*avrD1*::*nptII*	Nal^r Km^r	This work
JRP1	Δ*avrB4-1* Δ*avrB4-2*	Nal^r	This work

mutant strain to promote removal of the *nptII* gene by flipase-mediated site-specific recombination. Transformants were tested in LB plates with kanamycin to identify clones in which the kanamycin gene had been removed. Kanamycin-sensitive isolates were then grown in LB plates supplemented with 5% sucrose to select those that had lost the pFLP2 vector. A second allelic exchange vector was then transformed into the resulting kanamycin-sensitive single knockout strain, and transformants were selected and analyzed as described above.

Competitive index and disease scoring in bean plants. CI assays with bean plants (*Phaseolus vulgaris* cv. Canadian wonder) were carried out as previously described (31). Eight-day-old bean plants, grown at 22°C to 28°C with a photoperiod consisting of a 16-h-light–8-h-dark cycle, were inoculated with 200 μl of a 5×10^4-CFU/ml mixed-bacterial suspension, containing equal numbers of CFU of the wild type and the mutant or gene-expressing strain, using a 2-ml syringe without a needle. Serial dilutions of the inoculum were plated onto LB agar or LB agar with the appropriate antibiotic to confirm the relative proportion of bacterial CFU between the strains, which should be close to 1. At different days postinoculation (dpi), bacteria were recovered from the infected leaves. Bacterial recovery was carried out by taking five 10-mm-diameter discs with a cork borer, which were homogenized by mechanical disruption into 1 ml of 10 mM $MgCl_2$. Bacterial enumeration was performed by serial dilution and plating of the samples onto agar plates with cycloheximide and the appropriate antibiotic to differentiate the strains within the mixed infection. The CI is defined as the mutant-to-wild-type ratio within the output sample divided by the mutant-to-wild-type ratio within the input (inoculum) (13, 42). Competitive indices presented are the means for three replicates showing typical results from three independent experiments. Error bars represent standard errors. Each CI was analyzed using a homoscedastic 2-tailed Student *t* test and the null hypothesis, which states that the mean index is not significantly different from 1 or from another CI value when specified ($P < 0.05$).

For scoring of disease symptoms, 8-day-old bean plants were inoculated with bacterial suspensions at either 5×10^6 or 5×10^5 CFU/ml in 10 mM $MgCl_2$, using a 2-ml syringe without a needle. Symptoms were documented at a different dpi.

Virulence and translocation assays with *Arabidopsis thaliana*. Seeds of *Arabidopsis* ecotype Col-0 (RPS2/RPS2), an *rps2* mutant derivative (*rps2*/*rps2*) (SALK_087581; European *Arabidopsis* Stock Center), or plants carrying the *NahG* transgene (12) were germinated and grown in growth chambers with 8-h-light–16-h-dark cycles at 21°C. For symptom visualization, 3- to 4-week old plants were sprayed with a bacterial suspension containing 5×10^7 CFU/ml in 10 mM $MgCl_2$ and 0.02% Silwet L-77. Plants were then covered to keep humidity high, and symptoms were photographed at 7 dpi.

Competitive index assays for measuring growth attenuation, as well as translocation assays, were performed with *Arabidopsis* as previously described (30, 31). Briefly, 4- to 5-week-old plants were inoculated with a 5×10^4-CFU/ml mixed bacterial suspension, containing equal numbers of CFU of wild-type and effector-expressing strains, using a blunt syringe. Serial dilutions of the inoculum were plated onto LB agar or LB agar with kanamycin to confirm the relative proportion of bacterial CFU between the strains, which should be close to 1. At 4 days postinoculation (dpi), three 10-mm-diameter leaf discs were homogenized by mechanical disruption into 1 ml of 10 mM $MgCl_2$. Bacterial enumeration was carried out as described for CI virulence assays with bean plants. A CI in a translocation assay is defined as the effector-expressing-strain-to-wt output ratio divided by the ratio of these strains within the inoculum. CI translocation indices were treated and presented as described for CI virulence assays.

Quantitative real-time PCR (qRT-PCR) expression analysis. RNA was isolated from bacterial cell lysates or infected leaves by use of an RNeasy bacterial minikit (Qiagen, Germany) in accordance with the instructions provided by the manufacturer.

For extractions from infected leaves, each leaf was inoculated with 2×10^8 CFU/ml bacterial suspensions, using a blunt syringe. Two discs of 10 mm were cut from inoculation sites at 4 h postinoculation (hpi) and frozen immediately in liquid nitrogen. After macerating in 500 μl 1× RNAprotect reagent (Qiagen,

TABLE 2. Plasmids used and effector knockout vector collection generated in this work

Plasmid	Description	Antibiotic resistance	Source or reference
pGEM-T	Cloning vector	Amp^r	Promega
pKD4	pANTSγ derivative containing an FRT-flanked kanamycin resistance gene	Amp^r Km^r	11
pFLP2	Contains a flipase gene	Amp^r	19
pGEM-T-KmFRT EcoRI	pGEM-T derivative containing a Km resistance gene flanked by FRT and EcoRI sites	Amp^r Km^r	This work
pGEM-T-KmFRT BamHI	pGEM-T derivative containing a Km resistance gene flanked by FRT and BamHI sites	Amp^r Km^r	This work
pGEM-T-KmFRT XhoI	pGEM-T derivative containing a Km resistance gene flanked by FRT and XhoI sites	Amp^r Km^r	This work
pAZJ6	pGEM-T derivative carrying the Δ*avrB2*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ7	pGEM-T derivative carrying the Δ*hopR1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ8	pGEM-T derivative carrying the Δ*avrB4-1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ9	pGEM-T derivative carrying the Δ*hopW1-1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ10	pGEM-T derivative carrying the Δ*hopX1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ11	pGEM-T derivative carrying the Δ*hopAE1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ12	pGEM-T derivative carrying the Δ*hopAB1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ13	pGEM-T derivative carrying the Δ*hopAW1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ14	pGEM-T derivative carrying the Δ*avrRps4*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ15	pGEM-T derivative carrying the Δ*hopG1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ16	pGEM-T derivative carrying the Δ*hopQ1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ17	pGEM-T derivative carrying the Δ*hopD1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ18	pGEM-T derivative carrying the Δ*hopI1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ19	pGEM-T derivative carrying the Δ*hopAV1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ20	pGEM-T derivative carrying the Δ*hopAT1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ21	pGEM-T derivative carrying the Δ*hopAF1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ22	pGEM-T derivative carrying the Δ*hopAS1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ23	pGEM-T derivative carrying the Δ*hopW1-2*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ24	pGEM-T derivative carrying the Δ*avrD1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ25	pGEM-T derivative carrying the Δ*hopAU1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ26	pGEM-T derivative carrying the Δ*hopAK1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ27	pGEM-T derivative carrying the Δ*avrB4-2*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ28	pGEM-T derivative carrying the Δ*hopAJ1*::KmFRT knockout allele	Amp^r Km^r	This work
pAZJ29	pGEM-T derivative carrying the Δ*hopF3*::KmFRT knockout allele	Amp^r Km^r	This work
pAME8	pP_{nptII}::*avrRpt2*	Amp^r Km^r	30
pAMEX	Vector for expression of N-terminal protein fusions to $AvrRpt^{281-255}$ under a P_{nptII} promoter	Amp^r Km^r	30
pAME9.18	pAMEX derivative expressing the HopW1-1::′AvrRpt2 protein fusion	Amp^r Km^r	This work
pAME9.19	pAMEX derivative expressing the AvrB4-2::′AvrRpt2 protein fusion	Amp^r Km^r	This work
pAME9.20	pAMEX derivative expressing the PSPPH1154::′AvrRpt2 protein fusion	Amp^r Km^r	This work
pAME9.21	pAMEX derivative expressing the avrE1::′AvrRpt2 protein fusion	Amp^r Km^r	This work
pJRU1	pP_{lac}::*hopW1-1/2*	Amp^r Km^r	This work
pJRU2	pP_{lac}::*avrB4-2*	Amp^r Km^r	This work
pJRU3	pP_{nptII}::*avrB4-1*	Amp^r Km^r	This work
pJRU4	pP_{nptII}::*avrB4-2*	Amp^r Km^r	This work
pJRU5	p*avrB4-1*	Amp^r Km^r	This work
pJRU6	pP_{nptII}::*hopW1-1/2*	Amp^r Km^r	This work

Germany), samples were incubated at room temperature and centrifuged at 5,000 × *g* for 10 min. Pellets were resuspended in 100 μl Tris-EDTA (TE) containing 100 μg of lysozyme (Sigma) and incubated at room temperature for 10 min. DNase treatment was carried out twice, during and after the extraction process. An aliquot of RNA was tested to ensure that no trace of contaminant genomic DNA was detectable. cDNA was synthesized using the SuperScript II reverse transcriptase system (Invitrogen, CA) with random primers (Promega). After synthesis, cDNA was used as a template for quantitative real time-PCR (qRT-PCR). qRT-PCR was carried out in a RotorGene thermocycler (Qiagen) using Sybr green reagent (Qiagen) in accordance with the instructions from the manufacturer. The primers used for amplifications are detailed in Table 3.

RESULTS

Completing the *Pph*1448a type III effector inventory. The genome of 1448a contains the genes *hopW1-2* and *avrB4-2*, which are highly similar to those encoding the effectors HopW1-1 and AvrB4-1, respectively (7, 44). Although its HrpL-dependent expression had been established, HopW1-2 was previously discarded as not translocated (7). AvrB4-2 had not previously been tested for either T3SS-mediated translocation or HrpL-dependent expression. Nevertheless, sequence alignments showed that the predicted protein sequences of HopW1-1 and HopW1-2 are identical but that those of AvrB4-1 and AvrB4-2 differ by just 3 amino acids, thus raising the possibility of translocation for these two candidate effectors. We tested translocation of AvrB4-2 and HopW1-2, using the more sensitive AvrRpt2 CI-based translocation assay recently developed in our laboratory (30). With the use of these previously described experimental settings, interference between the HR triggered by a translocated candidate fusion to AvrRpt2 and growth of DC3000 is completely avoided, allowing detection of previously discarded candidate effectors due to the increased sensitivity of the assay (30). We generated plasmids encoding translational fusions of *avrB4-2* and *hopW1-2* to the sequence encoding the C-terminal domain of AvrRpt2 ($AvrRpt2^{81-255}$), expressed under the control of the P_{nptII} promoter (Table 2). Particular attention was paid to primer design to avoid cross-amplification of *avrB4-1* and *hopW1-1* by taking

TABLE 3. Primers used in this work

Primer	Sequence	Localization in genome	Restriction site[a]
A1-*hopW1-1*	5′CCCTATAGTGAGTCGAATTCCACTGGAGTCAAGCTTACG3′	6121-6139c	EcoRI
A2-*hopW1-1*	5′GAATTCGACTCACTATAGGGTCTCAGCGGTGTAGCTTTCC3′	6711-6730	EcoRI
B1-*hopW1-1*	5′GTTCTGCTAATGTGGACC3′	5607-5624	NA
B2-*hopW1-1*	5′TAGGATGGGATTGCTGGG3′	7232-7249c	NA
A1-*hopW1-2*	5′CCCTATAGTGAGTCGGATCCGCCTCTATTTGCAACAGC3′	63160-63177c	BamHI
A2-*hopW1-2*	5′GGATCCGACTCACTATAGGGCTATTCCTTCTCTCAGCG3′	63471-63488	BamHI
B1-*hopW1-2*	5′TCCAGGTCGATCATTTGC3′	62568-62585	NA
B2-*hopW1-2*	5′CCGTGATTTCTCTTAGCC3′	64178-64195c	NA
A1-*avrB4-1*	5′CCCTATAGTGAGTCGAATTCGAGCCTTAGTGGTAGTAACC3′	3515337-3515356c	EcoRI
A2-*avrB4-1*	5′GAATTCGACTCACTATAGGGACTAGGTTCATGCATAGGC3′	3516388-3516406	EcoRI
B1-*avrB4-1*	5′CGTGACGAGTGATCATCTGC3′	3514781-3514800	NA
B2-*avrB4-1*	5′ATCCTTTTGCACACCGATCC3′	3516888-3516907c	NA
A1-*avrB4-2*	5′CCCTATAGTGAGTCGGATCCAGAAGCCCGCAGAATTCG3′	927554-927571c	BamHI
A2-*avrB4-2*	5′GGATCCGACTCACTATAGGGTAGATGACGAGCCTTAGG3′	928565-928582	BamHI
B1-*avrB4-2*	5′TTCCAGCACGAAGTATCC3′	926946-926963	NA
B2-*avrB4-2*	5′AAGCGCGAAGAACACGCTG3′	929015-929033c	NA
A1-*hopAB1*	5′CCCTATAGTGAGTCGAATTCAGACAAAGGCTTGATGCC3′	107146-107163c	EcoRI
A2-*hopAB1*	5′GAATTCGACTCACTATAGGGTCCTGATCTCATGGTTGC3′	108740-108757	EcoRI
B1-*hopAB1*	5′TCCATCACCTGTTGAAGC3′	106559-106576	NA
B2-*hopAB1*	5′ATCCGCAACCTCATAGAG3′	109299-109316c	NA
A1-*hopAE1*	5′CCCTATAGTGAGTCGAATTCTCTTGCCTGGCTTACCAC3′	4943896-4943913c	EcoRI
A2-*hopAE1*	5′GAATTCGACTCACTATAGGGTACTGTCGGTGGATATGG3′	4946706-4946723	EcoRI
B1-*hopAE1*	5′AAGCCATGCAGATCCTCG3′	4943302-4943319	NA
B2-*hopAE1*	5′TAATCAGCGCCGAACAGC3′	4947287-4947304c	NA
A1-*avrB2*	5′CCCTATAGTGAGTCGAATTCCGTTCTTACGATCGCGTAGC3′	102383-102402c	EcoRI
A2-*avrB2*	5′GAATTCGACTCACTATAGGGATTCTAGGTGGCATTGCAGG3′	103535-103554	EcoRI
B1-*avrB2*	5′GCCGATAACCTGATCTACG3′	101280-101298	NA
B2-*avrB2*	5′CTTAACTGAGACATCAACGGC3′	104481-104501c	NA
A1-*avrD1*	5′CCCTATAGTGAGTCGGATCCAAACAGCTGCTGATTCCC3′	96818-96835c	BamHI
A2-*avrD1*	5′GGATCCGACTCACTATAGGGTGCTGAAGCTATACAGCC3′	97836-97853	BamHI
B1-*avrD1*	5′TCCGGATGGGGTATACTC3′	96266-96283	NA
B2-*avrD1*	5′TGGCGGCTTTGATCTGTG3′	98300-98317c	NA
A1-*hopD1*	5′CCCTATAGTGAGTCGAATTCCAATGTGTGGTCGCAAGG3′	7093-7110c	EcoRI
A2-*hopD1*	5′GAATTCGACTCACTATAGGGATCATAGTCGACGCGACCT3′	9280-9298	EcoRI
B1-*hopD1*	5′GCATTCAGTTCACCAGACG3′	6554-6572	NA
B2-*hopD1*	5′CGTATAGTGACAAAGGAGG3′	9859-9877c	NA
A1-*hopF3*	5′CCCTATAGTGAGTCGAATTCAGCTTGATGCTGTGCTCC3′	4039619-4039636c	EcoRI
A2-*hopF3*	5′GAATTCGACTCACTATAGGGATGCCCATGGAAGATTCC3′	4040252-4040269	EcoRI
B1-*hopF3*	5′TGCAACTGAACCAGCACC3′	4038961-4038978	NA
B2-*hopF3*	5′ATCTGGCGAGCATTCTCG3′	4040823-4040840c	NA
A1-*hopG1*	5′CCCTATAGTGAGTCGAATTCAGGCAGCATGCGTATTTGC3′	897388-897406c	EcoRI
A2-*hopG1*	5′GAATTCGACTCACTATAGGGTACGTGGTCATCACAAAGC3′	899013-899031	EcoRI
B1-*hopG1*	5′CCTTCATGCCTTGATGACTG3′	896841-896860	NA
B2-*hopG1*	5′ACAGACCGTTGTTAGCCAG3′	899582-899600c	NA
A1-*hopI1*	5′CCCTATAGTGAGTCGAATTCTTTTCGGCGTGAGGAGAC3′	4988642-4988659c	EcoRI
A2-*hopI1*	5′GAATTCGACTCACTATAGGGATGCTTCTCCGGGAACTC3′	4989672-4989689	EcoRI
B1-*hopI1*	5′AGATCACCGGATACATCG3′	4988059-4988076	NA
B2-*hopI1*	5′TTCTGTAGAGCAACACGG3′	4990274-4990291c	NA
A1-*hopQ1*	5′CCCTATAGTGAGTCGAATTCATCTGCGCTTGTCCAGTC3′	9414-9431c	EcoRI
A2-*hopQ1*	5′GAATTCGACTCACTATAGGGCTGGATAGATGAACCTGC3′	10751-10768	EcoRI
B1-*hopQ1*	5′TGTCGGAACAGATACTGC3′	8876-8893	NA
B2-*hopQ1*	5′CGAGACATGGATGTGTGG3′	11381-11398c	NA
A1-*hopR1*	5′CCCTATAGTGAGTCGAATTCAACAAAGGAAGCGACCTG3′	198648-198665c	EcoRI
A2-*hopR1*	5′GAATTCGACTCACTATAGGGGCATCATGCCGAATAGG3′	204628-204644	EcoRI
B1-*hopR1*	5′GCATTCTCAGGTTGTAGATGG3′	198090-198110	NA
B2-*hopR1*	5′CGTCGAGTATCTGTATCGTTGG3′	205178-205199c	NA
A1-*hopX1*	5′CCCTATAGTGAGTCGAATTCTGTTCCTCACTGATTGCG3′	1508676-1508693c	EcoRI
A2-*hopX1*	5′GAATTCGACTCACTATAGGGACAGGTCATAGAGTTCGG3′	1509903-1509920	EcoRI
B1-*hopX1*	5′TGAGCGTGAGAGTTACACG3′	1508038-1508056	NA
B2-*hopX1*	5′GGTCTGACATAATCTGCG3′	1510496-1510513c	NA
A1-*hopAJ1*	5′CCCTATAGTGAGTCGGATCCCTTCATAGGTTTAGCTCGC3′	892773-892791c	BamHI
A2-*hopAJ1*	5′GGATCCGACTCACTATAGGGTGTGAAGTGAAAGTGGCC3′	894080-894097	BamHI
B1-*hopAJ1*	5′TGACAAGCATGCCAAAGC3′	892244-892261	NA
B2-*hopAJ1*	5′CAATCACGGAAGAAGCAC3′	894731-894748c	NA
A1-*hopAK1*	5′CCCTATAGTGAGTCGAATTCAAAAGGGCGACCGAAGTC3′	1652010-1652027c	EcoRI
A2-*hopAK1*	5′GAATTCGACTCACTATAGGGTGAACGATTCGTGATCCG3′	1653668-1653685	EcoRI
B1-*hopAK1*	5′TTGCGTGAAGAGCAACAG3′	1651461-1651478	NA

Continued on following page

TABLE 3—*Continued*

Primer	Sequence	Localization in genome	Restriction site[a]
B2-*hopAK1*	5′CGCCAGCTATAGCAAGAC3′	1654216-1654233c	NA
A1-*hopAS1*	5′CCCTATAGTGAGTCGAATTCGATCAATACAGGTGGTGG3′	5376014-5376031c	EcoRI
A2-*hopAS1*	5′GAATTCGACTCACTATAGGGACGCTGGTCATTCAACTG3′	5380168-5380185	EcoRI
B1-*hopAS1*	5′TGGACGCGTCTTGAATGG3′	5375449-5375466	NA
B2-*hopAS1*	5′TGTCGGATTAGTTCAGGG3′	5380661-5380678c	NA
A1-*hopAT1*	5′CCCTATAGTGAGTCGGATCCACGCTTCTGGATCTTCGG3′	894814-894831c	BamHI
A2-*hopAT1*	5′GGATCCGACTCACTATAGGGTTGAGGATCACAAAGCGC3′	895177-895194	BamHI
B1-*hopAT1*	5′TACATATCCTGTGCGCTG3′	894188-894205	NA
B2-*hopAT1*	5′GGATCTGAATTCCATCGC3′	895766-895783c	NA
A1-*hopAU1*	5′CCCTATAGTGAGTCCTCGAGGGAGGGTTCCAAAGACAG3′	26320-26339c	XhoI
A2-*hopAU1*	5′CTCGAGGACTCACTATAGGGAGCCTGGAGACATTCATGC3′	28431-28449	XhoI
B1-*hopAU1*	5′AGTACCGCAGTCCTTCAC3′	25800-25817	NA
B2-*hopAU1*	5′CTCTGGTGATGTTTTCCG3′	28993-29010c	NA
A1-*hopAV1*	5′CCCTATAGTGAGTCGGATCCCTGCTCCAACTATTAGCC3′	45681-45698c	BamHI
A2-*hopAV1*	5′GGATCCGACTCACTATAGGGATCTCGTCGATGAAGGAC3′	48004-48021	BamHI
B1-*hopAV1*	5′TGTTCAACTACGACCGTC3′	45126-45143	NA
B2-*hopAV1*	5′AACATCCGGCTACTTCAG3′	48631-48648c	NA
A1-*hopAW1*	5′CCCTATAGTGAGTCGAATTCTATGCGTAGTGAACAGGG3′	104780-104797c	EcoRI
A2-*hopAW1*	5′GAATTCGACTCACTATAGGGAGAAACACTGAGTGGTCG3′	105491-105508	EcoRI
B1-*hopAW1*	5′TTCACGTACCTCATCTGC3′	104148-104165	NA
B2-*hopAW1*	5′ATCCAACTGCACATCAGC3′	106029-106046c	NA
A1-*hopAF1*	5′CCCTATAGTGAGTCGGATCCCCGTTGGTTATGTGATGC3′	1685066-1685083c	BamHI
A2-*hopAF1*	5′GGATCCGACTCACTATAGGGCAGCGCTTCGATTTTGCC3′	1686077-1686094	BamHI
B1-*hopAF1*	5′CACTCAAGCCGATCTACC3′	1684461-1684478	NA
B2-*hopAF1*	5′GCTTCATCCCCGATATCC3′	1686634-1686651c	NA
A1-*avrE1*	5′CCCTATAGTGAGTCGAATTCCTGTAGAAATGCGCGAGC3′	1477655-1477672c	EcoRI
A2-*avrE1*	5′GAATTCGACTCACTATAGGGATGTGCCACTGATGGCAG3′	1482889-1482906	EcoRI
B1-*avrE1*	5′GAAGCTCGCGTGTCCGTC3′	1477068-1477085	NA
B2-*avrE1*	5′CGATCATTGGCAACAGC3′	1483518-1483534c	NA
A1-*avrRps4*	5′CCCTATAGTGAGTCGGATCCAACACATCATAGCCCCTG3′	75967-75984c	BamHI
A2-*avrRps4*	5′GGATCCGACTCACTATAGCCTAATGCGCATGAGCAGGC3′	76681-76698	BamHI
B1-*avrRps4*	5′TTTGCCTTCGCCGTACAG3′	74923-74940	NA
B2-*avrRps4*	5′CGTCAAGACGACGGTCAG3′	77748-77765c	NA
A1-*hopAY1*	5′CCCTATAGTGAGTCGAATTCTCTCCGCTTACTGGCTTGC3′	110642-110660c	EcoRI
A2-*hopAY1*	5′GAATTCGACTCACTATAGGGCACCTGCAATTGGAGAGC3′	111601-111618	EcoRI
B1-*hopAY1*	5′CGGTAATAATTGGCATGG3′	110198-110215	NA
B2-*hopAY1*	5′TGCGCAACATCAACGAGG3′	112117-112134c	NA
P1-EcoRI	5′TCAGAATTCGTGTAGGCTGGAGCTGCTTC3′		EcoRI
P2-EcoRI	5′TCAGAATTCCATATGAATATCCTCCTTAG3′		EcoRI
P1-BamHI	5′TCAGGATCCGTGTAGGCTGGAGCTGCTTC3′		BamHI
P2-BamHI	5′TCAGGATCCCATATGAATATCCTCCTTAG3′		BamHI
P1-XhoI	5′TCACTCGAGGTGTAGGCTGGAGCTGCTTC3′		XhoI
P2-XhoI	5′TCACTCGAGCATATGAATATCCTCCTTAG3′		XhoI
qRT-*avrB4-1/2* F	5′GGCGATGTTCAATGGCTAAT3′	3515731-3515732; 928198-928217	
qRT-*avrB4-1/2* R	5′TTTTGCAAGCTCCCATCAG3′	3515842-3515860c; 928070-928088c	
qRT-*hopW1-1/2* F	5′AATCGTCGCAGCAGGTTC3′	63361-63378c; 6591-6608c	
qRT-*hopW1-1/2* R	5′CTCTCCAACTCATGCTGAAGG3′	63246-63266; 6476-6496	
qRT-*hopAY1* F	5′CGCTAGACCCTCGAACAGTC3′	110760-110779	
qRT-*hopAY1* R	5′CCCTGACCTGACCCTTGTT3′	110937-110955c	
qRT-*hopAH2* F	5′TTGCTTGCCAGTCAACAGAC3′	3525934-3525953	
qRT-*hopAH2* R	5′GCGATGTCATCAGGATTGG3′	3526118-3526136c	
qRT-*hrpA* F	5′AGGGTATCAACAGCGTCAAG3′	1487676-1487695	
qRT-*hrpA* R	5′TCAGAACTGGACGACCGAGT3′	1487916-1487935c	
qRT-*16S* F	5′CAATGGGCGAAAGCCTGAT3′	804368-804386	
qRT-*16S* R	5′TGCTGGCACAGAGTTAGC3′	804502-804519c	
hopW1-1/2 F	5′GTCAAGCTTTTATGGAAAGCTACACCGC3′	6716-6734c; 63486-63504c	HindIII
hopW1-1/2 R	5′GTCGAATTCCGATTCATTTTGCTGTTGC3′	6379-6397; 63149-63167	EcoRI
avrB4-1 F	5′GTCCTCGAGGAGCGGAACCGAATAAGAGG3′	3515319-3515338	XhoI
avrB4-2 F	5′GTCCTCGAGAGTGCTGAGGACTCGGTAGC3′	928612-928631c	XhoI
avrB4-1/2 R	5′GTCTCTAGATTTGTTACGAATTCTGCGGG3′	3516364-3516383c; 927547-927566	XbaI

[a] NA, not applicable.

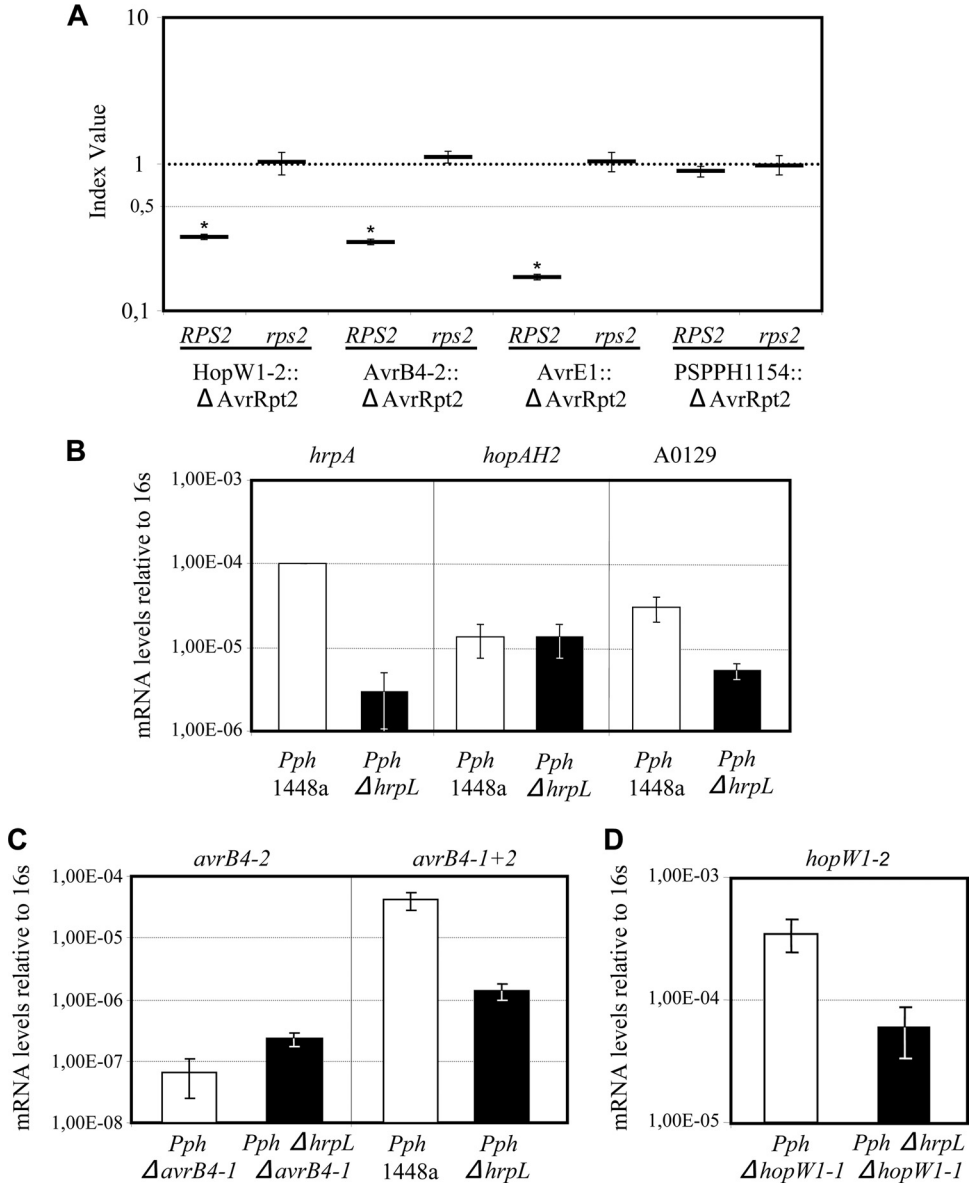

FIG. 1. (A) Graphical representation of competitive index translocation assays. Wild-type DC3000 bacteria were coinoculated into either wild-type *Arabidopsis* Col-0 or *rps2* plants, together with DC3000 strains expressing translational fusions of candidate effector genes to Δ*avrRpt2* (AvrB4-2::AvrRpt2$^{81\text{-}255}$, HopW1-1::AvrRpt2$^{81\text{-}255}$, AvrE1$^{1\text{–}326}$::AvrRpt2$^{81\text{-}255}$, or PSPPH1154::AvrRpt2$^{81\text{-}255}$). Four- to five-week-old *Arabidopsis* plants were inoculated with a 5×10^4-CFU/ml mixed bacterial suspension, containing equal numbers of CFU of wild-type and fusion-expressing bacteria. Competitive indices correspond to the means for three samples. A dashed line, corresponding to a CI of 0.5, is included for reference. Error bars represent the standard errors. Experiments were repeated twice, with similar results. Asterisks indicate results significantly different from 1, as established by Student's *t* test ($P < 0.05$). (B, C, and D) qRT-PCR expression analysis of A0129, *hopAH2*, and *avrB4-2*, carried out with *Pph*1448a and the Δ*hrpL* mutant, and expression analysis of *avrB4-1* and *hopW1-2*, carried out with the Δ*avrB4-1* and Δ*avrB4-1* Δ*hrpL* strains and the Δ*hopW1-1* and Δ*hopW1-1* Δ*hrpL* strains, respectively, growing *in planta*. The expression levels of 16S and *hrpA* are included as controls.

advantage of the differences between their flanking regions. *Pto*DC3000 expressing either AvrB4-2::AvrRpt2$^{81\text{-}255}$ or the HopW1-2::AvrRpt2$^{81\text{-}255}$ protein fusion was coinoculated with *Pto*DC3000 into *Arabidopsis* Col-0 and *rps2* plants. Figure 1A shows that both CIs were significantly different from 1 in the wild type in Col-0 but not *rps2* plants. Therefore, both AvrB4-2 and HopW1-2 have functional T3SS translocation signals that allow the fusion proteins to be translocated into the plant cell, thus triggering AvrRpt2-RPS2 defense responses that determine bacterial growth restriction as detected using the CI. Considering the similarities between the pair of effectors comprising HopW1-1 and HopW1-2 and that comprising AvrB4-1 and AvrB4-2, these results raised the possibility of functional redundancy between HopW1-1 and HopW1-2 and provided that AvrB4-2 was expressed within the plant, between AvrB4-1 and AvrB4-2.

We also tested the translocation of the effector candidate AvrE1, previously analyzed by Chang and colleagues (7).

These authors found AvrE1 to be expressed in an HrpL-dependent manner but were not able to conclude as to its translocation, since it produced inconsistent results in their translocation assays (7). Technical difficulties in the generation of the translational fusion of the complete ORF of *avrE1* to AvrRpt2$^{81-255}$ (5,142 bp) led us to use the first 978 bp to generate the protein fusion. Figure 1A shows that the CI of DC3000 expressing AvrE1$^{1-978}$::AvrRpt2$^{81-255}$ in mixed infection with DC3000 is significantly different from 1, thus demonstrating that AvrE1 also carries type III translocation signals.

In order to establish the complete type III secretome of *Pph*1448a, we also analyzed the translocation of the protein encoded by another gene, PSPPH1154, which raised our interest, as this protein was previously identified in a bioinformatics analysis as containing a eukaryotic F-box domain (4). The F-box is a short domain characteristic of the eukaryotic F-box proteins, which allows them to participate in the forming of SCF-type E3 ubiquitin (Ub) ligase complexes, thus controlling specific protein ubiquitinylation (38, 45). This pathway is essential to many developmental processes in plants, ranging from hormone signaling and flower development to stress responses. Recently, several bacterial effectors from plant pathogenic bacteria have been shown to mimic host E3 Ub ligases, possibly to alter plant defenses (5), thus raising the interesting possibility that PSPPH1154 encodes a type III effector with a similar function. However, we could not establish type III-dependent translocation for this candidate, since the value for growth of DC3000 expressing PSPPH1154::AvrRpt2$^{81-255}$ in mixed infection with DC3000 was not significantly different from 1 (Fig. 1A).

To fully establish these translocated candidates as effectors, we also analyzed *in planta* expression of AvrB4-2, A0129, and HopAH2, which had not previously been determined. We extracted RNA from bean plants 4 h after infiltration with 2×10^8 CFU/ml of either *Pph*1448a or a Δ*hrpL* mutant derivative (Table 1) and carried out qRT-PCR to detect the expression of these genes. Primers for the *hrpA* gene were included as a control for HrpL-dependent expression, and primers for 16S amplification (Table 3) were used for comparison purposes. As expected, expression of *hrpA* was induced *in planta* in an HrpL-dependent manner (Fig. 1B). Expression of A0129 was also found to be dependent on HrpL, although the differences between its expression in plants infected with the wild type and that in plants infected with the Δ*hrpL* mutant derivative were notably smaller than the differences established for *hrpA* (Fig. 1B). However, *in planta* expression of *hopAH2* was found to be HrpL independent since expression in the Δ*hrpL* mutant was comparable to that detected in the wild-type strain (Fig. 1B).

Surprisingly, expression of *avrB4-2*, which lacks an *hrp* box (23), was found to be HrpL dependent since its expression was clearly detectable in plants infected with *Pph*1448a but just barely detectable in those infected with its Δ*hrpL* derivative (Fig. 1C). Given the strong sequence similarity between the *avrB4-1* and *avrB4-2* ORFs, we also tested the expression of *avrB4-2* in a Δ*avrB4-1* background to avoid cross-amplification. Expression of *avrB4-2* in this background was found to be HrpL independent (Fig. 1C), in keeping with the absence of an *hrp* box in its promoter region, thus indicating that the HrpL-dependent expression detected in the wild type was due to cross-amplification of *avrB4-1*. We also analyzed the expression of *hopW1-2*, previously reported as HrpL regulated (7), in a Δ*hopW1-1* background. Expression of this gene was found to be HrpL dependent, although its expression in the absence of a functional HrpL protein was not strongly reduced (Fig. 1D).

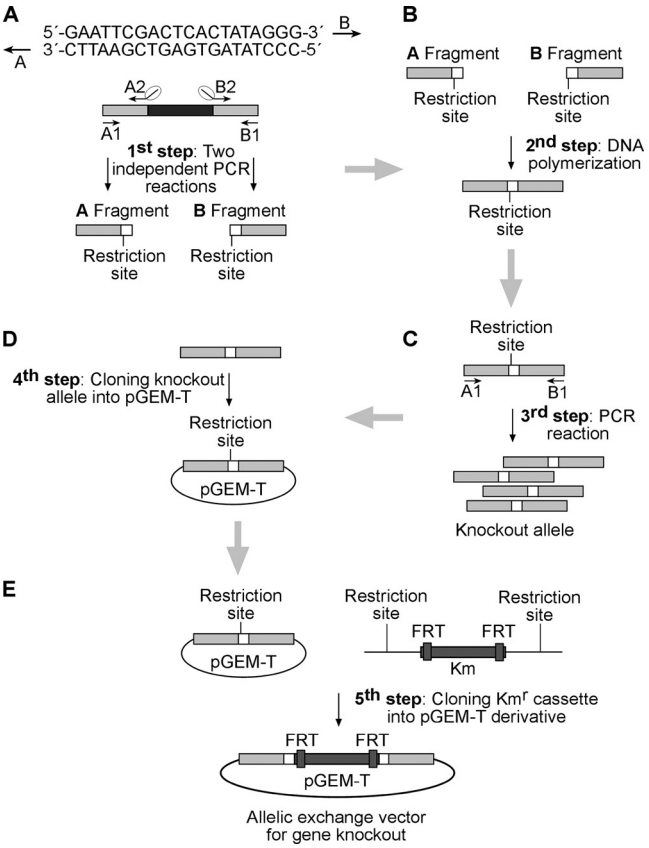

FIG. 2. Generating allelic exchange vectors for gene knockout. (A) The flanking regions (0.5 kb) of the target gene are amplified independently. Primers A and B share a 20-nucleotide-long homologous sequence at their 5′ ends. (B) With the resulting PCR products used as both the primers and the template, a polymerization is carried out, resulting in a joint 1-kb fragment, the knockout allele. (C) Amplification of the knockout allele by use of primers A1 and B1. (D) The knockout allele is A/T cloned into pGEM-T. (E) With the use of the restriction site incorporated into primers A2 and B2, a FRT-flanked *nptII* fragment from the appropriate plasmid is cloned into the knockout allele, thus generating the effector knockout vector.

Simplifying the process of generating single and double knockout mutants. In order to simplify and accelerate the process of generating knockout mutations in *Pph*1488a, we applied a series of sequential PCRs and a selectable cloning step to generate allelic exchange vectors that could be directly introduced into the target strain to produce the mutants. Each knockout vector is generated by a 5-step procedure shown in Fig. 2. Briefly, approximately 500 bp flanking the target ORF is amplified from *Pph*1448a genomic DNA, using primers that include a restriction site not present on the flanking sequences and the T7 primer sequence, in such a manner as to provide homology and a cloning site between the resulting fragments (Fig. 2A). These fragments are then joined through the homology provided by the T7 primer sequence in a PCR without

additional primers or a template, thus generating the deletion allele (Fig. 2B). Once amplified, the deletion alleles are A/T cloned (Fig. 2C and D) and fully sequenced. Importantly, since effector genes can be carried as part of an operon or located close to other genes, accumulation of PCR-associated mutations must be kept to a minimum. For that purpose, we used a high-fidelity polymerase, DMSO, commercial H_2O_{dd}, and very few PCR cycles (see the corresponding section in Materials and Methods for details).

The knockout alleles generated are marked with an *nptII* kanamycin resistance gene flanked by FTR sites (Fig. 2D), which can be easily eliminated by flipase-mediated site-specific recombination (19). This allows the introduction of a second analogue vector for generation of a double mutant when required.

Allelic exchange is typically carried out by conjugation to increase the chances that the necessary recombination events take place before the nonreplicative vector is lost. However, to save the additional cloning steps required to clone the knockout allele into a conjugative vector, we used a modification of an electroporation protocol previously described for *P. aeruginosa* (8). In this manner, we achieved transformation efficiencies (10^8 to 10^9 CFU/μg DNA) high enough as to allow not only detection of clones having undergone plasmid integration events (single crossover) but also detection of those having undergone allelic exchange events (double crossover) in the transformation plate.

Generating knockout mutants for the *Pph*1448a type III effector inventory. We set out to generate a mutant collection including single knockout strains for each of the 27 members of the complete type III effector inventory of *Pph*1448a. With the exception of *avrE1*, for which we met with technical problems when amplifying its flanking regions, we generated knockout vectors for all type III effectors and transformed these vectors into *Pph*1448a. On average, we obtained a frequency of transformants having undergone recombination (Km^r) that ranged between 1 and 10^2 CFU/μg DNA. Interestingly, when the transformants (Km^r) were tested to identify clones having undergone a double recombination event (Amp^s) (see Materials and Methods), the frequency of clones having sustained allelic exchange (Km^r Amp^s) was found to be surprisingly high (5 to 50%). However, if Km-resistant clones with the plasmid integrated (Amp^r) were further grown without selective pressure, the frequency of allelic exchange was lower than the frequency of Km-resistant transformants on the initial transformation. It is possible that during transformation, the presence of the integrated plasmid determines a local increase in recombination frequencies.

The recombination frequencies achieved with this method allowed us to recover most mutants, those resulting from a double recombination event leading to allelic exchange, directly from the transformation plate. The mutants that could not be recovered this way could be obtained either after a few rounds of transformation or by doubling the bacterial concentration used in the electroporation. In one case, that of *avrB2*, allelic exchange was achieved only after the length of the flanking regions was increased to 1,000 bp in order to boost recombination frequencies. Four out of the 27 effector genes, *hopAT1*, *hopAV1*, *hopAF1*, and *hopF3*, have not yet been knocked out, due to the low frequency of recombination found for their loci (data not shown). A fifth effector gene, *avrRps4*, was not mutated either, since Southern analyses of all the transformants isolated indicate that recombination was recurrently taking place at an incorrect location within the *Pph*1448a genome (data not shown). Deletion of these five effector genes is currently being attempted by increasing the sizes of their flanking regions.

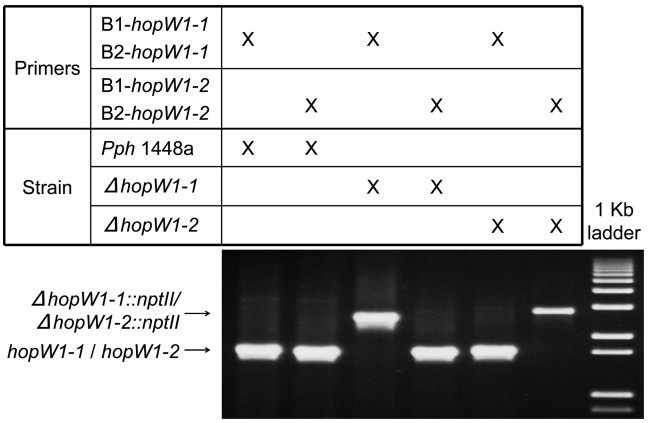

FIG. 3. Genotyping analysis carried out with single knockout mutants for plasmid-located effector genes *hopW1-1* and *hopW1-2*. PCR analyses were carried out using primers to amplify each of the two genes plus approximately 600 bp of their 5′ and 3′ flanking regions. Genomic DNA from either *Pph*1448a or its Δ*hopW1-1* and Δ*hopW1-2* mutant derivatives was used as templates. Arrows indicate the bands corresponding to the wild-type (*hopW1-1*/*hopW1-2*) or mutant (Δ*hopW1-1*::*nptII*/Δ*hopW1-2*::*nptII*) alleles.

Many type III effectors are encoded within native plasmids. In *Pph*1448a, this is the case for 12 effectors, including HopW1-1 and HopW1-2 (23). Generation of knockout mutations in these genes poses potential problems since, although native plasmids are maintained at a very low copy number, they can still be present in more than one copy, raising the possibility of having a given strain carrying both the functional and the deletion alleles. To test this possibility, we carried out genotyping experiments using PCR to detect the presence of both types of alleles in all the selected and Southern analysis-confirmed knockout mutants of plasmid-located effector genes. Figure 3 shows the results of genotyping strains AZJ11 (Δ*hopW1-1*::*nptII*) and AZJ13 (Δ*hopW1-2*::*nptII*). Both strains carry only the knockout alleles and can therefore be used for further virulence analysis. Similar tests carried out with the rest of the mutants did, however, detect one case (Δ*hopQ1*), in which both alleles were present in the putative mutant strain (data not shown). Two rounds of growth of this strain, merodiploid for the *hopQ1* effector gene, in LB medium supplemented with kanamycin were sufficient to obtain the final mutant strain where the wild-type allele was no longer detectable (data not shown). Transformants selected as knockout mutants of the plasmid-located *avrB2* gene gave results by Southern analysis consistent with the carrying of the deletion but gave inconclusive results when genotype analyses were carried out, and therefore, these transformants were disregarded.

In summary, to date, we have generated single knockout mutants for 20 of the 27 members of the *Pph*1448a secretome (Table 1).

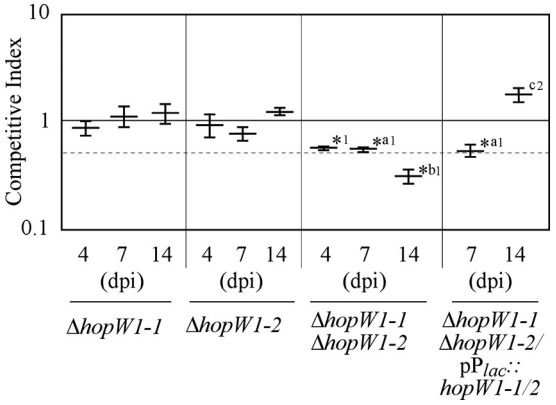

FIG. 4. Competitive index virulence analysis of the Δ*hopW1-1* or Δ*hopW1-2* single mutant, the Δ*hopW1-1* Δ*hopW1-2* double mutant, or the Δ*hopW1-1/2* Δ*hopW1-2* double mutant carrying a plasmid expressing the *hopW1-1/2* ORF from a P_{lac} promoter in mixed infections with *Pph*1448a. Eight-day-old bean plants were inoculated with 200 μl of a 5×10^4-CFU/ml mixed bacterial suspension, containing equal numbers of CFU of wild-type and mutant bacteria, and leaves were sampled at 4, 7, and 14 days postinoculation (dpi). For virulence assays, the CI is defined as the mutant-to-wt output ratio divided by the mutant-to-wt input ratio. CI values correspond to the means for three samples. A dashed line, corresponding to a CI of 0.5, is included for reference. Error bars represent the standard errors. Asterisks indicate results significantly different from 1 as established by Student's *t* test ($P < 0.05$). Mean values marked with the same letter (a, b, or c) indicate results not significantly different from each other, as established by Student's *t* test ($P < 0.05$). Mean values marked with 1 indicate results significantly different from the CI for *Pph*1448a carrying the empty vector (pAMEX), as established by Student's *t* test ($P < 0.05$). Mean values marked with 2 indicate a result not significantly different from the CI for *Pph*1448a carrying the empty vector (pAMEX), as established by a Mann-Whitney test ($P < 0.05$). A different test was used for this value in accordance with the specific recommendation provided by the statistics software program used.

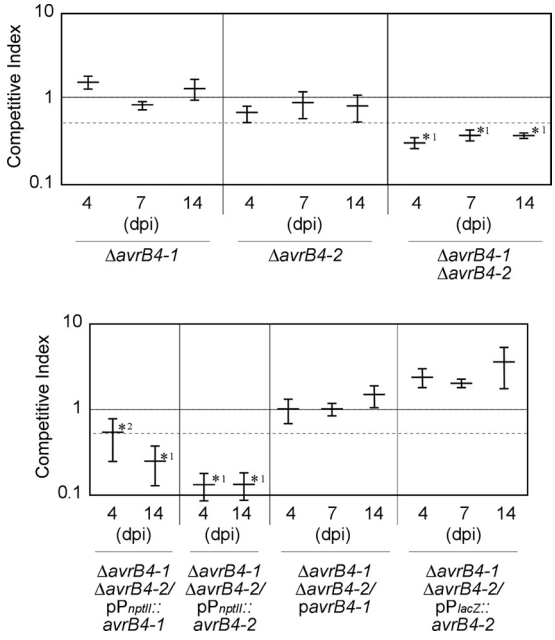

FIG. 5. Competitive index virulence analysis of the Δ*avrB4-1* or Δ*avrB4-2* single mutant, the Δ*avrB4-1* Δ*avrB4-2* double mutant, or the Δ*avrB4-1* Δ*avrB4-2* double mutant carrying plasmids expressing either AvrB4-1 or AvrB4-2 from either its own promoter, a P_{lac} promoter, or a P_{nptII} promoter in mixed infections with *Pph*1448a. Eight-day-old bean plants were inoculated with 200 μl of a 5×10^4-CFU/ml mixed bacterial suspension, containing equal numbers of CFU of wild-type and mutant bacteria, and leaves were sampled at 4, 7, and 14 days postinoculation (dpi). The CI values correspond to the means for three samples. A dashed line, corresponding to a CI of 0.5, is included for reference. Error bars represent the standard errors. Asterisks indicate results significantly different from 1 as established by Student's *t* test ($P < 0.05$). Mean values marked with 1 indicate results significantly different from the CI obtained for *Pph*1448a carrying the empty vector (pAMEX), as established by Student's *t* test ($P < 0.05$), whereas those marked with 2 indicate results not significantly different.

Establishing functional redundancy within the *Pph*1448a secretome. To prove the validity of our approach to the analysis of the role in virulence of the members of the *Pph*1448a type III secretome, we set out to establish whether the pair of effectors comprising AvrB4-1 and AvrB4-2 and that comprising HopW1-1 and HopW1-2 were functionally redundant. We generated single knockout mutations in the genes encoding all four proteins (Table 1) and analyzed the virulence of each mutant in bean plants on different days postinoculation (dpi), using CI assays carried out as described above, with inoculation doses shown to avoid complementation between coinoculated strains (31). None of the single knockout mutants showed any attenuation in bacterial growth, since the CI values obtained were not significantly different from 1 (Fig. 4 and 5).

To generate the corresponding double mutants, the kanamycin resistance gene from the Δ*avrB4-2* and Δ*hopW1-1* single mutant strains (AZJ16 and AZJ11) (Table 1) was removed by flipase-mediated site-specific recombination on their FRT sites (19), and the resulting strains (AZJ17 and AZJ12) (Table 1) were used as recipients for transformation with the vectors previously used in the generation of the *avrB4-1* or *hopW1-2* single knockout mutant. We carried out a Southern analysis of the single and double mutant strains, as well as that of the single mutants from which the kanamycin resistance gene had been removed, confirming the positions and number of insertions in each strain (data not shown). The double mutant strains thus obtained, AZJ18 (Δ*avrB4-1* Δ*avrB4-2*) and AZJ14 (Δ*hopW1-1* Δ*hopW1-2*) (Table 1), were analyzed for disease symptom induction and bacterial growth. A slight delay was observed in the induction of disease symptoms by the double mutant strains (Fig. 6); however, these differences were hard to establish confidently and were not always reproducible. However, when virulence in the bean was analyzed using the CI, a statistically significant attenuation was detected for both double mutant strains at all time points analyzed, as indicated by CI values significantly different from 1, with the CI values for Δ*avrB4-1* Δ*avrB4-2* ranging between 0.31 and 0.37 and those for Δ*hopW1-1* Δ*hopW1-2* ranging between 0.32 to 0.57 (Fig. 4 and 5). These results demonstrate that these pairs of effectors, that comprising HopW1-1 and HopW1-2 and that comprising AvrB4-1 and AvrB4-2, are functionally redundant and make a quantitative contribution to *Pph*1448a virulence.

Complementing virulence attenuation of the Δ*avrB4-1* Δ*avrB4-2* and Δ*hopW1-1* Δ*hopW1-2* double mutant strains. Given the functional redundancy of the above-mentioned pairs of effectors, the virulence attenuation detected using the CI for each of the double mutants should be complemented by ex-

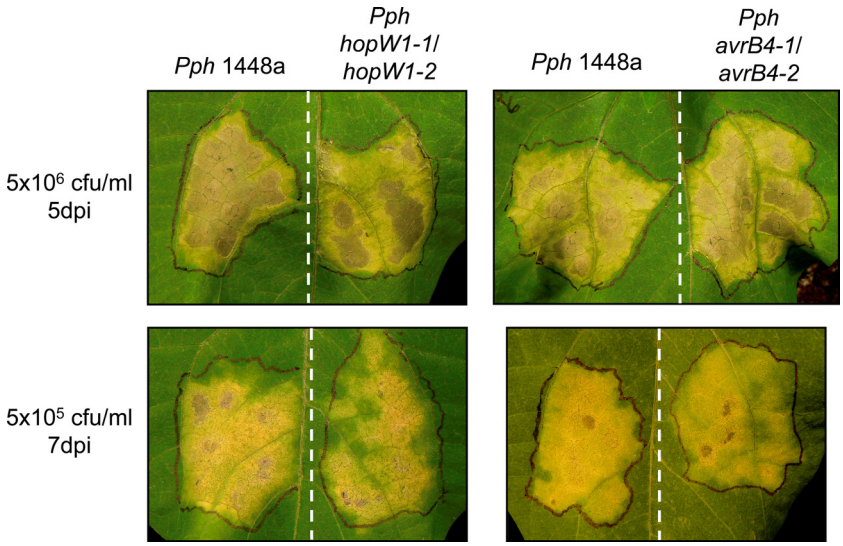

FIG. 6. Disease symptoms in bean plants. Plants were infiltrated with the indicated doses of either the *Pph*1448a, the *hopW1-1 hopW1-2* double mutant, or the *avrB4-1 avrB4-2* double mutant strain, and the symptoms induced were documented at the indicated days postinoculation (dpi). The experiment was carried out twice, with similar results. The images show representative results.

pression of just one of the effector genes. This should be the case in particular for the *hopW1-1 hopW1-2* double mutant strain since the nucleotide sequences of these two genes, including their promoter regions, are 100% identical. Indeed, when a plasmid carrying the common ORF region of either *hopW1-1* or *hopW1-2* (*hopW1-1*/2), expressed under the control of the medium-to-low-expression-level promoter P_{lac}, was introduced into the Δ*hopW1-1* Δ*hopW1-2* double mutant strain, growth in bean plants was fully restored to wild-type levels by 14 dpi, as the CI values for the complemented strain coinoculated with the wild type were no different from 1 and were significantly different from the CI obtained for the double mutant strain without the plasmid (Fig. 4). Similarly, plasmids expressing either *avrB4-1* from its own promoter or *avrB4-2* from the P_{lac} promoter were capable of fully complementing the growth of the Δ*avrB4-1* Δ*avrB4-2* double mutant strain at all time points analyzed, since the CIs obtained for these strains in mixed infection with the wild type were not significantly different from 1 (Fig. 5). Interestingly, if expression of either *avrB4-1* or *avrB4-2* was driven from the stronger P_{nptII} promoter, no complementation of growth was detected (Fig. 5). Furthermore, the presence of the plasmid expressing *avrB4-2* from the P_{nptII} promoter resulted in a stronger attenuation of growth in the double mutant strain, since the CIs obtained for this strain were significantly smaller that the CI obtained for the double mutant strain (Fig. 5). The stability of the vector used in these assays was confirmed by establishing that the CI for the wild type expressing the empty vector in mixed infection with the wild-type strain (CI = 0.99 ± 0.09) was not significantly different from 1 and was significantly different from all significant CIs shown in Fig. 4 and 5.

Analyzing the defense responses triggered by AvrB4-1, AvrB4-2, HopW1-1, and HopW2-2 in *Arabidopsis*. We also analyzed the capability of the AvrB4-1, AvrB4-2, HopW1-1, and HopW2-2 effectors to protect *Arabidopsis* against infection by *Pto*DC3000. Plasmids expressing HopW1-1/2, AvrB4-1, and AvrB4-2 from the P_{nptII} promoter were transformed into *Pto*DC3000, and the resulting transformants were sprayed over Col-0 plants. Symptoms were observed and documented at different time points after inoculation. As controls, Col-0 plants were also sprayed with *Pto*DC3000 expressing AvrRpt2. The AvrRpt2-RPS2-triggered defense response determines the resistance against *Pto*DC3000, thus preventing the development of disease symptoms in Col-0 plants inoculated with *Pto*DC3000 expressing AvrRpt2 (25). Figure 7A shows that whereas plants sprayed with *Pto*DC3000 expressing AvrRpt2 displayed no symptoms, plants expressing any of the four *Pph* effectors showed necrotic leaves. The intensity of the symptoms developed by plants sprayed with *Pto*DC3000 expressing AvrB4-1 in different experiments seemed lower than that developed by plants sprayed with *Pto*DC3000 expressing AvrB4-2 or HopW1-1/2 (Fig. 7A and data not shown), suggesting that this effector could be triggering a defense response in *Arabidopsis*. However, this type of assay does not allow quantification of subtle differences. In order to quantify such a difference, we carried out CI assays to determine the difference in growth of *Pto*DC3000 that the expression of any of these effectors may have through a putative activation of defense responses. Although expression of HopW1/2 or AvrB4-2 did not affect growth of *Pto*DC3000 (with CIs not significantly different from 1), expression of AvrB4-1 caused a clear attenuation of growth (with a CI significantly different from 1), suggesting that this effector may trigger a defense response in *Arabidopsis* (Fig. 7B), although at a lower intensity than that triggered by AvrRpt2. Interestingly, when a similar experiment was carried out with plants carrying the *NahG* transgene, in which, as expected, the restriction of growth determined by AvrRpt2 in Col-0 was abolished, the CI for *Pto*DC3000 expressing AvrB4-1 was also significantly different from 1 (Fig. 7B). The *NahG* transgene encodes a salicylate hydroxylase that converts salicylic acid (SA) to catechol. *NahG* transgenic plants accumulate extremely low levels of SA, thus hindering AvrRpt2-

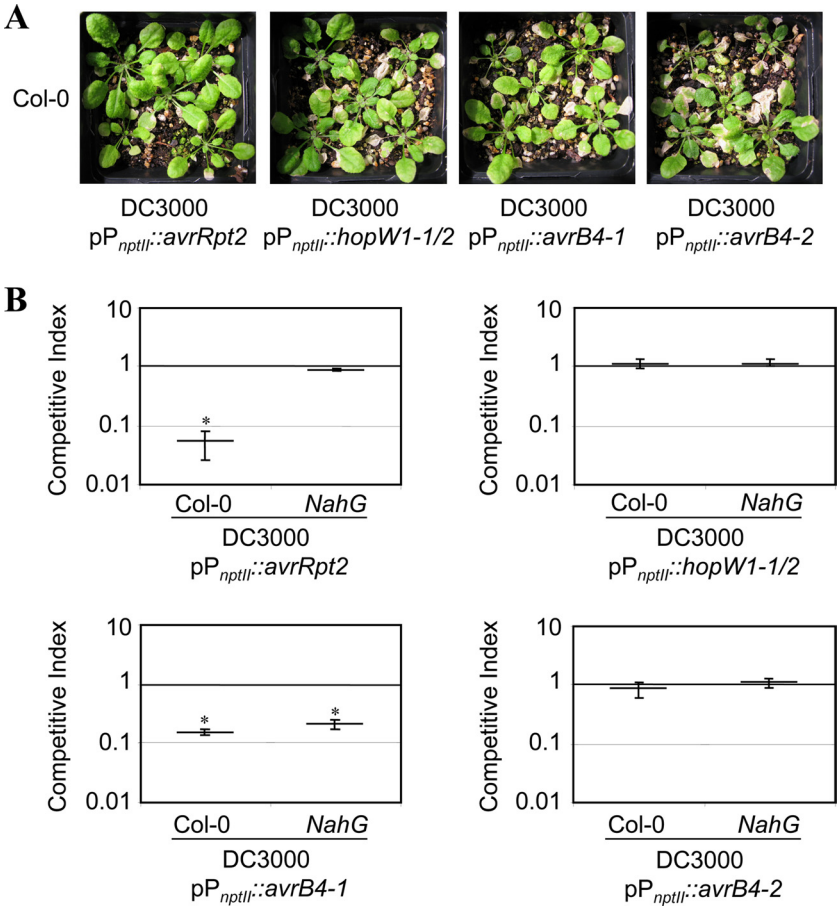

FIG. 7. (A) Disease symptoms caused by *Pto*DC3000 expressing *Pph*1448a effectors in *Arabidopsis thaliana*. Plants were sprayed with a bacterial suspension of *Pto*DC3000 or *Pto* expressing different effectors from the *Pph*1448a secretome, containing 5×10^7 CFU/ml in 10 mM $MgCl_2$ and 0.02% Silwet L-77. After inoculation, plants were covered to keep humidity high, and symptoms were documented by 7 dpi. (B) Competitive index assays of *Pto*DC3000 bacteria coinoculated into either *Arabidopsis* Col-0 or *NahG* plants, together with DC3000 strains expressing different effector genes. Four- to five-week-old *Arabidopsis* plants were inoculated with a 5×10^4-CFU/ml mixed bacterial suspension, containing equal numbers of CFU of wild-type and effector-expressing bacteria, and leaves were sampled at 7 dpi. Competitive indices correspond to the means for three samples. Error bars represent the standard errors. Asterisks indicate results significantly different from 1, as established by Student's *t* test ($P < 0.05$).

triggered resistance, and display enhanced susceptibility to bacterial pathogen infection (12). The fact that growth restriction determined by expression of AvrB4-1 is not abolished in *NahG* plants could indicate that AvrB4-1 may trigger a defense response in *Arabidopsis* mostly independent from SA-signaling pathways. Although the CI values for *Pto*DC3000 (*avrB4-1*) were always slightly closer to 1 in *NahG* than in Col-0 plants, which could indicate a small contribution of SA-signaling pathways in AvrB4-1-triggered resistance, this strain could not be confidently established, since only in some of the experiments could we detect it in a statistically significant manner (data not shown). Bacterial growth in LB was not affected by the expression of AvrB4-1 (data not shown); however, further work would be necessary to rule out an unspecific toxic effect that could affect bacterial fitness and confirm this putative SA-independent defense response.

DISCUSSION

We applied a highly sensitive CI-AvrRpt2-based translocation assay, recently developed in our laboratory (30), to analyze the translocation of four effector candidates from *Pph*1448a, two previously untested (AvrB4-2 and PSPPH1154) and two either discarded or found to be unclear for type III-mediated translocation in other assays (HopW1-2 and AvrE1) (7). Three of them were found to be translocated, including HopW1-2, the only previously discarded candidate effector not yet tested with our translocation assay. Expression within the plant was also confirmed for these candidates, which should therefore be included as validated members of the *Pph*1448a secretome. *In planta* expression was established for the translocated candidate HopAH2, although this expression was established as taking place in an HrpL-independent manner, in keeping with the absence of an *hrp* box in its promoter region. Interestingly, although *avrB4-2* does not have a consensus *hrp* box upstream of its promoter sequence, we found that the expression of *avrB4-2* was HrpL regulated. Chang and colleagues also found two effector-encoding operons in which HrpL regulation was experimentally demonstrated in the absence of *hrp* boxes (7). However, the absence of HrpL regula-

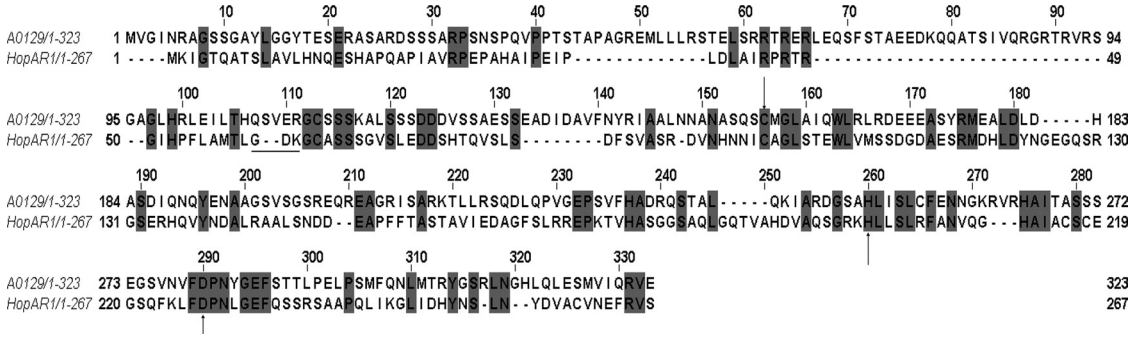

FIG. 8. Amino acid alignment of A0129 and HopAR1 (*Pph* race 3). Squares represent residues identical between the two proteins. Arrows point to the residues that form the putative catalytic triad (cysteine, histidine, and aspartic acid).

tion detected when a Δ*avrB4-1* background was used for the analysis indicates that this apparent regulation is the result of cross-amplification of HrpL-regulated *avrB4-1*. In the case of *hopW1-2*, our results show a difference in expression between wild-type and Δ*hrpL* mutant strains, in keeping with previous reports classifying *hopW1-2* as HrpL regulated; however, this difference is small. Expression of A0129 was also found to depend on HrpL, although the level of regulation for A0129 was also lower than that measured for the *hrpA* gene. Our results are in keeping with A0129 being initially reported as fulfilling the three criteria for bioinformatic identification of a putative *hrp* box (30). Although the correlation between predicted *hrp* boxes and HrpL regulation is high, there are occasional examples in which genes with *hrp* boxes do not exhibit HrpL-dependent regulation (7, 44). Regulation by HrpL is a hallmark for most type III effector genes, but there are also exceptions to be found (e.g., $hopO1\text{-}2_{Pto\mathrm{DC3000}}$) (7). However, the level of expression required for an effector to be functional would largely depend on its function and will most likely be below the level required for the function of HrpA as a major component of the secretion pilus. Thus, this level of regulation is likely to be biologically meaningful, particularly if these effectors require a high level of expression to carry out the function of HrpA. Alternatively, these effectors may be expressed to functional levels in the absence of HrpL and respond to the presence of the activator by modulating its expression.

A0129 has a predicted catalytic triad and functional domain characteristic of cysteine proteases (http://www.sbg.bio.ic.ac.uk/~phyre/) (24), which makes it a putative member of the C58 family of papain-like proteases (41), according to the MEROPS database for peptidases (http://merops.sanger.ac.uk/) (35). Another well-characterized member of this family, HopAR1 (previously AvrPphB and AvrPph3), defines race 3 of *Pph* (22, 34). However, sequence similarity between HopAR1 and the predicted protein sequence of A0129 is very limited (Fig. 8). Thus, by following the guidelines for a unified nomenclature proposed by Lindeberg and colleagues (29), we have renamed A0129 as $HopAY1_{Pph1448a}$.

We have developed and applied a simplified method for generating knockout mutants to the task of deleting each of the 27 members of the complete type III effector inventory of *Pph*1448a. We have successfully generated an effector knockout vector collection and an effector mutant collection, which already include 20 single effector mutant strains and their corresponding vectors. To our knowledge, this is the first collection of single effector mutants available for such a large type III secretome in *P. syringae* and represents a valuable tool for future work on the role of the type III effector in virulence. We are currently applying CI assays to this mutant collection to characterize the individual contributions of the members of the *Pph*1448a type III secretome to virulence.

The mutant and knockout vector collections generated in this work present the additional advantage of allowing for easy generation of any double mutant combination. We have validated this use and its application to determining functional relationships between effectors by establishing functional redundancy between two pairs of effectors, with one pair comprising AvrB4-1 and AvrB4-2 and one comprising HopW1-1 and HopW1-2, through analysis of single and double mutant strains, as well as double mutant strains expressing each of the individual effectors, using CI assays.

One interesting result, obtained while the double mutant strains were being characterized, was that the complementation of their growth defect by plasmid-based expression of one of the deleted effectors strongly depended on the promoter driving its expression. These results suggest that strong expression of some effectors, such as AvrB4-2, may result in growth attenuation within the plant. Considering the interplay between defense activation and suppression already shown to take place among effectors of a given secretome (21), increasing the expression of one of these effectors may affect bacterial growth by either favoring defense activation versus defense suppression or altering the hierarchy of translocation. In support of the relevance of such a fine equilibrium between the effectors acting within a given secretome, the best complementation results were obtained when the native promoter was used to express AvrB4-1.

The contribution of the effectors HopW1-1 and HopW1-2 to virulence has interesting implications for effector evolution since these two effectors are both 94 amino acids long, whereas their closest homologues, HopW1-1 and HopW1-2 from *Pseudomonas syringae* pv. maculicola ES4326, are 774 and 240 amino acids long, respectively (15, 29). Since $HopW1\text{-}1_{Pma\mathrm{ES4326}}$ and $HopW1\text{-}2_{Pma\mathrm{ES4326}}$ are both functional effectors, $HopW1\text{-}1_{Pph1448a}$ and$HopW1\text{-}2_{Pph1448a}$ could have easily been dismissed as inactive truncated versions, when in fact they do contribute to *Pph*1448a virulence.

The presence of two functionally redundant pairs of genes expressing T3SS effectors in the genome of 1448a illustrates a common evolutionary strategy for generating diversity based on gene duplication and divergent evolution. This can be an essential step both in the generation of T3SS effector families and in the adaptation of the pathogen to new hosts. In this regard, the fact that the expression of AvrB4-1 but not that of AvrB4-2 determines a reduction of bacterial growth in *Arabidopsis*, despite their being functionally redundant in bean plants and their differing by only 3 amino acids, supports such an evolutionary strategy.

Most effector-triggered immunities depend on SA-signaling pathways and are therefore abolished in plants expressing the *NahG* transgene, where the concentration of SA is kept to very low levels (12). Thus, the analysis of the plant response to AvrB4-1 carries considerable interest and could lead to a better understanding of the plant responses that shape the *P. syringae*-plant interaction.

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