Bioinformatics Correctly Identifies Many Type III Secretion Substrates in the Plant Pathogen *Pseudomonas syringae* and the Biocontrol Isolate *P. fluorescens* SBW25

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The plant pathogen *Pseudomonas syringae* causes disease by secreting a potentially large set of virulence proteins called effectors directly into host cells, their environment, or both, using a type III secretion system (T3SS). Most *P. syringae* effectors have a common upstream element called the *hrp* box, and their N-terminal regions have amino acids biases, features that permit their bioinformatic prediction. One of the most prominent biases is a positive serine bias. We previously used the truncated AvrRpt2⁸¹⁻²⁵⁵ effector containing a serine-rich stretch from amino acids 81 to 100 as a T3SS reporter. Region 81 to 100 of this reporter does not contribute to the secretion or translocation of AvrRpt2 or to putative effector protein chimeras. Rather, the serine-rich region from the N-terminus of AvrRpt2 is important for protein accumulation in bacteria. Most of the N-terminal region (amino acids 15 to 100) is not essential for secretion in culture or delivery to plants. However, portions of this sequence may increase the efficiency of AvrRpt2 secretion, delivery to plants, or both. Two effectors previously identified with the AvrRpt2⁸¹⁻²⁵⁵ reporter were secreted in culture independently of AvrRpt2, validating the use of the C terminus of AvrRpt2 as a T3SS reporter. Finally, using the reduced AvrRpt2¹⁰¹⁻²⁵⁵ reporter, we confirmed seven predicted effectors from *P. syringae* pv. *tomato* DC3000, four from *P. syringae* pv. *syringae* B728a, and two from *P. fluorescens* SBW25.

Additional keywords: *Arabidopsis thaliana*, *RPS2*, hypersensitive response.

Animals and plants readily detect many invading pathogens and respond with a wide array of defenses. Successful pathogens have developed virulence factors that can efficiently interfere with host defenses. In the case of gram-negative pathogens, an important group of such virulence factors are proteins called effectors, secreted by a type III secretion system (T3SS) (Blocker et al. 2003). Effectors are secreted into the extracellular host environment and are injected directly into the cyto-

plasm of host cells, where they alter host signaling, metabolism, and homeostasis (Buttner and Bonas 2003). Since pathogens deficient in type III secretion are usually nonpathogenic, these effector-induced changes must be essential to create conditions in the host that permit bacterial growth.

Individual effector loss-of-function mutants of plant pathogens usually have quantitative alterations in fitness, showing only slight decreases or increases in growth and symptom production. An exception is the group of avirulence (Avr) effectors that elicits strong host defense responses and programmed cell death on some plants upon their direct or indirect recognition by plant resistance (R) proteins (Martin et al. 2003). Programmed cell death, also called the hypersensitive response (HR) (Greenberg and Yao 2004) is macroscopically visible as total leaf collapse when a high enough dose of the pathogen is infiltrated into leaves. Since the defense-inducing activity of these Avr effectors is usually dominant, it is possible to identify many of these effectors by screening for genomic clones from a HR-inducing strain that confer, on a virulent strain, the ability to induce a HR (Staskawicz et al. 1984). Avr effectors are often virulence factors on plants missing the cognate R gene (Alfano and Collmer 2004).

We previously developed functional and bioinformatics approaches to identify effectors in *Pseudomonas syringae* independently of an avirulence phenotype. Our functional approach was based on the observation that effectors are modular. The Avr effector AvrRpt2 has an N-terminal secretion region that is distinct from the region harboring its HR-eliciting avirulence activity, the so-called effector region (Guttman and Greenberg 2001; Mudgett and Staskawicz 1999). This effector region (amino acids 81 to 255) is delivered to plants when fused to the N-terminal secretion region of a heterologous effector (Guttman and Greenberg 2001). We performed a large-scale screen using the AvrRpt2⁸¹⁻²⁵⁵ effector region as a reporter to capture N-terminal secretion signals of previously unknown effector genes. We identified thirteen genes called *hops* (for *hrp* outer protein) (Alfano and Collmer 1997) in *P. syringae* pv. *maculicola* ES4326 (Guttman et al. 2002).

Of the 13 genes found in the functional screen, 12 were preceded by an *hrp* box, an upstream element known to precede *P. syringae* *avr* genes and *hrp/hrc* genes that encode the structural T3SS components (Innes et al. 1993). All 13 *P. syringae* pv. *maculicola* ES4326 effectors and most other known *P. syringae* Avr proteins had a positive serine or proline bias and a negative aspartate bias in their N-terminal

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regions, as compared with the rest of these proteins (Greenberg and Vinatzer 2003; Guttman et al. 2002). These factors (the presence of upstream *hrp* boxes and amino acid biases) in combination with comparative genomics enabled us to predict many *P. syringae* and *P. fluorescens* effectors from draft genome sequences (Guttman et al. 2002; Greenberg and Vinatzer 2003). Petnicki-Ocwieja and associates (2002) also used a bioinformatics approach based on amino acid features in the N termini of proteins to predict effectors. Based on these studies, *P. syringae* pv. *tomato* DC3000 has 58 predicted effectors (Collmer et al. 2002; Greenberg and Vinatzer 2003), *P. syringae* pv. *syringae* B728a has 29 predicted effectors, and the biocontrol isolate *P. fluorescens* SBW25 has seven predicted effectors (Greenberg and Vinatzer 2003). To date, 40 of the predicted effectors in *P. syringae* pv. *tomato* DC3000 (Schechter et al. 2004), three in *P. syringae* pv. *syringae* B728a (Deng et al. 2003), and none in *P. fluorescens* SBW25 have been confirmed.

To understand the mechanism of *P. syringae* virulence, it is essential to have a full inventory of its effectors. The main goal of this work was to test the validity of our previous bioinformatic effector predictions. We sought to use the AvrRpt2⁸¹⁻²⁵⁵ T3SS reporter previously developed for this purpose. However, it seemed possible that the N-terminal portion of this reporter might contribute a secretion or translocation signal to protein chimeras. In particular, the 20-amino acid long region AvrRpt2⁸¹⁻¹⁰⁰ is highly serine-rich, a feature commonly found in the N-terminal region of effectors. Here, we show that the serine-rich N-terminal portion of AvrRpt2 strongly contributes to the steady-state level of this protein in bacteria. This region is not essential for either secretion in culture or delivery to plant cells. However, the region spanning amino acids 34 to 79 may contribute to the efficiency of these processes. We confirm that amino acids 81 to 100 in the AvrRpt2⁸¹⁻²⁵⁵ reporter specifically do not contribute a secretion or translocation signal, or both, to protein chimeras. We also show that the reduced AvrRpt2¹⁰¹⁻²⁵⁵ reporter can be used instead of AvrRpt2⁸¹⁻²⁵⁵ to

eliminate any remaining doubt that AvrRpt2⁸¹⁻¹⁰⁰ could contribute to the delivery of protein::AvrRpt2⁸¹⁻²⁵⁵ chimeras to plants. Using the reduced AvrRpt2¹⁰¹⁻²⁵⁵ reporter, we confirm seven effectors in *P. syringae* pv. *tomato* DC3000, four in *P. syringae* pv. *syringae* B728a, and the first two T3SS effectors in *P. fluorescens*.

RESULTS

Characterization of the N terminus of AvrRpt2.

We previously used AvrRpt2⁸¹⁻²⁵⁵ as a reporter for the delivery of heterologous effectors to plants. However, it seemed possible that the high serine content of amino acids 81 to 100 (Fig. 1) might contribute to the ability of effectors to be targeted to plants. Therefore, we sought to characterize the role of these amino acids, other serine-rich portions of the AvrRpt2 N terminus, or both in the context of both the native AvrRpt2 protein and in effector chimeras. A total of 17 of the 100 most N-terminal amino acids of AvrRpt2 are serines, whereas the rest of the protein contains less than 4% serines (Fig. 1). We first deleted serine-rich subregions from the 100 most N-terminal amino acids and assayed the steady level of AvrRpt2 derivatives and their ability to be secreted in culture and to elicit a HR on *Arabidopsis*. For these experiments, constructs with *avrRpt2* derivatives encoding various deletion versions of the protein were integrated at the *recA* locus of *P. syringae* pv. *maculicola* ES426 (Guttman and Greenberg 2001).

Figure 2 shows that amino acids 81 to 100 did not affect the abundance, secretion, or HR strength of AvrRpt2. Deletion of a larger portion of the N terminus between amino acids 19 and 100 also had very little effect on total AvrRpt2 abundance, as seen in two independent experiments (Fig. 2A and B). However, the ability to induce a HR was reduced with strains expressing AvrRpt2^{1-19, 101-255} and AvrRpt2^{1-33, 101-255} (Fig. 2C). Secretion of AvrRpt2^{1-19, 101-255} and AvrRpt2^{1-33, 101-255} was somewhat reduced in one of the two experiments. This suggests the region between amino acids 34 and 79 may contribute to the efficiency of secretion, targeting to plants, or both of AvrRpt2.

Because N termini of *P. syringae* effectors are typically poor in aspartate, we tested the effect of substituting serine 19 for aspartate within the AvrRpt2^{1-19, 101-255} derivative. This S19D substitution dramatically reduced the accumulation of AvrRpt2, as did further deletions to give AvrRpt2^{1-15, 101-255} or AvrRpt2^{1-14, 101-255}. Figure 2B and C shows that derivatives AvrRpt2^{1-15, 101-255}, AvrRpt2^{1-14, 101-255}, and AvrRpt2^{1-18, S19D, 101-255} can be secreted and elicit a HR, suggesting there is no obligatory role for amino acids 15 to 100 in secretion or delivery to plants. Figure 2A shows that we could not detect the secretion of all derivatives in all experiments, probably due to the low abundance of the total protein and, thus, the low level of secreted protein. Note that in Figure 2B, the blot of secreted protein was exposed for a longer period than that shown in Figure 2A, allowing detection of AvrRpt2 derivatives in concentrated culture supernatants.

Surprisingly, two AvrRpt2 derivatives affected secretion and HR elicitation differently. While the difference in the ability of AvrRpt2^{1-19, 101-255} and AvrRpt2^{1-18, S19D, 101-255} to induce the HR was modest (Fig. 2C), the amount of these proteins that was found in culture supernatants was very reduced. In addition, derivative AvrRpt2^{1-14, 101-255} induced a reduced HR relative to AvrRpt2^{1-18, S19D, 101-255} but accumulated to similar amounts in culture supernatants. These data suggest that there are different requirements for efficient secretion versus HR elicitation. There may be specific amino acids in the region of 15 to 19 that are particularly permissive for eliciting the HR, possibly due to translocation efficiency, protein stability, or targeting once inside the plant.

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1  MKIAPVAINH 10
11 SPLS      14  AvrRpt21-14
15 R         15  AvrRpt21-15
16 EVPS     19  AvrRpt21-19
20           H 20
21 AAPTQAKQTN 30
31 LQS      33  AvrRpt21-33
34   EAGDLDA 40
41 RKSSASSPET 50
51 RALLATKTVL 60
61 GRHKIEVPAF 70
71 GGWFKKKSSK 80  AvrRpt21-80

81 HETGGSSANA 90
91 DSSSVASDST 100

101EKPLFRLTHVPYVSQGNERMGCWYAC
ARMVGHSVEAGPRLGLPELYEGREAPAGL
QDFSDVERFIHNEGLTRVDLPDNERFTHE
ELGALLYKHGPIIFGWKTPNDSWHMSVLT
GVDKETSSITFHDPRQGPDLAMPLDYFNQ
RLAWQVPHAMLYR 255

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Fig. 1. The AvrRpt2 effector. The AvrRpt2 effector is shown here divided in different regions that were deleted to determine their contribution to steady-state levels of protein in bacteria, secretion in vitro, and delivery to plant cells. Serines (S) are in bold and aspartates (D) are italicized. The S in position 19 was changed to D in the construct AvrRpt2^{1-18, S19D, 101-255}.

The AvrRpt2 reporter for type III secretion can be reduced from amino acids 81 to 255 to amino acids 101 to 255.

The finding that amino acids 81 to 100 of AvrRpt2 were not important for protein accumulation, secretion, or HR strength suggested these amino acids would not contribute to translocation in effector chimeras. This is important, since we seek to use the C terminus of AvrRpt2 to faithfully report whether heterologous proteins are T3SS substrates. To directly test the role of amino acids 81 to 100 in the AvrRpt2 reporter, we deleted these amino acids from protein chimeras we previously studied in *P. syringae* pv. *maculicola* ES4326 (Guttman et al. 2002). We examined four chimeras with various amounts of N-terminal sequence from different heterologous proteins: HopPmaG¹⁻¹⁴, HopPmaK¹⁻⁵⁶, HopPtoP¹⁻¹¹¹, and HopPmaH¹⁻²²⁶. Since these proteins are expressed from constructs that we previously published, we retained their original names for easier reference. We used the new unified nomenclature (Lindeberg et al. 2005) for all effectors that we confirmed here for the first time (discussed below). New and old names for all effectors used in this paper are listed in Table 1. Figure 3A shows that reducing the AvrRpt2⁸¹⁻²⁵⁵ reporter to AvrRpt2¹⁰¹⁻²⁵⁵ did not affect protein accumulation of the effector::AvrRpt2 fusions of HopPmaH, HopPtoP, and HopPmaK in *P. syringae* pv. *maculicola* ES4326 bacteria grown in *hrp*-inducing medium. The ability to elicit a HR on *Arabidopsis* by these fusions was the same as that seen with the original fusions (Fig. 3B).

HopPmaG¹⁻¹⁴::AvrRpt2¹⁰¹⁻²⁵⁵ accumulated to lower levels than HopPmaG¹⁻¹⁴::AvrRpt2⁸¹⁻²⁵⁵ in bacteria grown in *hrp*-inducing medium (Fig. 3A) and caused a barely visible HR (Fig. 3B). We confirmed that HopPmaG¹⁻¹⁴::AvrRpt2¹⁰¹⁻²⁵⁵ was secreted in culture by monitoring its appearance in concentrated culture supernatants (Fig. 3C). In the original HopPmaG¹⁻¹⁴::AvrRpt2⁸¹⁻²⁵⁵ fusion, amino acids 81 to 100 of the AvrRpt2⁸¹⁻²⁵⁵ reporter likely increased protein stability sufficiently to allow a strongly visible HR.

HopPmaG has a predicted sec signal and a predicted cleavage site between amino acid 26 and 27, identified by SignalP 3.0 using hidden Markov models (Bendtsen et al. 2004). In the HopPmaG¹⁻¹⁴ fusions to portions of AvrRpt2, this secretion signal was destroyed and the cleavage site was absent. We fused HopPmaG to AvrRpt2¹⁰¹⁻²⁵⁵, in an attempt to determine whether full-length HopPmaG shows T3SS-dependent secretion and translocation. We were unable to detect secretion or translocation of HopPmaG::AvrRpt2¹⁰¹⁻²⁵⁵ (data not shown). However, the expression level of this fusion protein was low, similar

Fig. 2. Analysis of the role serine-rich regions play in in vitro steady-state levels, secretion, and in planta translocation of AvrRpt2. **A** and **B**, Steady-state levels of AvrRpt2 deletion derivatives expressed in *Pseudomonas syringae* pv. *maculicola* ES4326 grown in *hrp*-inducing medium and their secretion in vitro are shown. Protein was extracted from bacteria and concentrated from culture supernatants. Protein was analyzed by Western blot, using α -AvrRpt2 antibody to detect AvrRpt2 and using α -Npt2 to detect cytoplasmic Npt2 protein to control for bacterial lysis. **A**, The percentage of serines and aspartates in the first 50 amino acids of each deletion mutant is also indicated. **A** and **B** show two independent experiments. **B**, The blot of proteins was exposed for a longer time period than the blots in **A**. Individual lanes in **A** and **B** are from the same blots exposed for the same amount of time but have been rearranged for clarity using Power Point. **C**, Assay of the hypersensitive response (HR)-eliciting ability of the AvrRpt2 deletion mutants. Strains were infiltrated in *RPS2*⁺ *Arabidopsis* leaves at three different concentrations (optical density at 600 nm of 0.3, 0.1, and 0.03, respectively). Seven leaves for each strain and concentration were infiltrated in two independent experiments, and the HR was scored double-blind. The numbers shown are the sum of both experiments. Variation between experiments was negligible. The same strains were also infiltrated in leaves of congenic *rps2*⁻ *Arabidopsis*, in which none of the strains elicited a HR.

to that seen with HopPmaG¹⁻¹⁴::AvrRpt2¹⁰¹⁻²⁵⁵, whose secretion and translocation occurred at the limit of detection of our assays. Therefore, we were unable to distinguish whether full-length HopPmaG is not a type III substrate or its expression level is below the detection limit of our assays.

HopPmaA and HopPmaI have functional secretion signals for type III-dependent in culture secretion independent of the AvrRpt2 reporter.

Since the in vivo screen for type III effectors described by Guttman and associates (2002) relied exclusively on the AvrRpt2⁸¹⁻²⁵⁵ reporter, we wanted to confirm type III-dependent secretion of a few of the effectors identified in that screen independently of this reporter. We optimized the in culture secretion assay previously described (Mudgett and Staskawicz 1999; van Dijk et al. 1999), allowing the use of volumes of culture supernatant as small as 1.5 ml. We used full-length AvrRpt2 expressed at the *recA* locus in *P. syringae* pv. *maculicola* ES4326 on plasmid pDSG6 (Guttman and Greenberg 2001) as a positive control for this assay (Fig. 4A). We confirmed secretion of C-terminally HA-tev-Myc-His-tagged full-length HopPmaI expressed from its native promoter in *P. syringae* pv. *maculicola* ES4326 after Campbell integration of

plasmid pJJ19 (Fig. 4B). We also confirmed secretion of a HopPmaA¹⁻¹⁸::Npt2 fusion protein expressed from the HopPmaA promoter after Campbell integration of plasmid pBAV41 at the *hopPmaA* locus (Fig. 4C). Npt2 has been widely used as a reporter for T3SS-dependent secretion in *Yersinia enterocolitica* (Anderson and Schneewind 1997). We were unable to detect the secretion of full-length HA-tagged HopPmaA (data not shown), possibly due to its instability in culture supernatants.

Confirmation of effectors predicted in *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *syringae* B728a, using Gateway-based *P. syringae* expression vectors.

To increase the number of confirmed effectors in *P. syringae*, we constructed two Gateway vectors to express effectors as fusions to the reduced AvrRpt2¹⁰¹⁻²⁵⁵ reporter. The first, vector pBAV124, has no origin of replication for *P. syringae* and allows the Campbell integration (Guttman and Greenberg 2001) of effectors as fusions to the DNA coding for AvrRpt2¹⁰¹⁻²⁵⁵ reporter at their native loci in their native strains and expression from their native promoters. The second, vector pBAV178, has an *npt2* promoter and a high-copy-number ori-

Fig. 3. Comparison of the AvrRpt2⁸¹⁻²⁵⁵ reporter with the reduced AvrRpt2¹⁰¹⁻²⁵⁵ reporter. DNA coding for amino acids 81 to 100 of the AvrRpt2⁸¹⁻²⁵⁵ reporter was deleted from HopPmaG¹⁻¹⁴::AvrRpt2⁸¹⁻²⁵⁵, HopPmaK¹⁻⁵⁶::AvrRpt2⁸¹⁻²⁵⁵, HopPtoP¹⁻¹¹¹::AvrRpt2⁸¹⁻²⁵⁵, and HopPmaH¹⁻²²⁶::AvrRpt2⁸¹⁻²⁵⁵. **A** Steady-state levels of effector::AvrRpt2⁸¹⁻²⁵⁵ and effector::AvrRpt2¹⁰¹⁻²⁵⁵ fusions in *Pseudomonas syringae* pv. *maculicola* ES4326 grown in *hrp*-inducing medium. Western blots loaded with equal amount of sample and probed with α -AvrRpt2 antibody are shown. Lanes 1, 3, 5, and 7 are the effector::AvrRpt2⁸¹⁻²⁵⁵ fusions and lanes 2, 4, 6, and 8 are the effector::AvrRpt2¹⁰¹⁻²⁵⁵ fusions. Note that HopPmaG¹⁻¹⁴::AvrRpt2¹⁰¹⁻²⁵⁵ comigrates with a cross-hybridizing band and that expression of HopPmaG¹⁻¹⁴::AvrRpt2¹⁰¹⁻²⁵⁵ is lower than expression of HopPmaG¹⁻¹⁴::AvrRpt2⁸¹⁻²⁵⁵. Expression of all other effector::AvrRpt2 fusions was not affected by deletion of amino acids 81 to 100. **B**, Hypersensitive response (HR)-inducing ability of effector::AvrRpt2⁸¹⁻²⁵⁵ and effector::AvrRpt2¹⁰¹⁻²⁵⁵ fusions. *P. syringae* pv. *maculicola* ES4326 either expressing the effector::AvrRpt2 fusions from their native loci, containing the empty vector pCKTR integrated at the *recA* locus or expressing AvrRpt2 from the pDSG6 plasmid integrated at the *recA* locus were infiltrated in leaves of *RPS2*⁺ *Arabidopsis* at an optical density at 600 nm of 0.3 and were photographed 18 h later. Note that the effector::AvrRpt2¹⁰¹⁻²⁵⁵ fusions had the same ability to induce a HR as did the effector::AvrRpt2⁸¹⁻²⁵⁵ fusions, with the exception of HopPmaG¹⁻¹⁴::AvrRpt2¹⁰¹⁻²⁵⁵ that elicited a much weaker HR than HopPmaG¹⁻¹⁴::AvrRpt2⁸¹⁻²⁵⁵. None of the strains elicited a HR on *rps2*⁻ *Arabidopsis* (data not shown). **C**, In vitro secretion of HopPmaG¹⁻¹⁴::AvrRpt2¹⁰¹⁻²⁵⁵ from *P. syringae* pv. *maculicola* ES4326 grown in *hrp*-inducing medium. Western blots probed with α -AvrRpt2 antibody are shown. Sample 1 contains the empty vector pCKTR, integrated at the *recA* locus, while sample 2 expresses HopPmaG¹⁻¹⁴::AvrRpt2¹⁰¹⁻²⁵⁵ from pBAV18 at its native locus. *b* = protein extracted from bacteria, *sn* = protein concentrated from culture supernatants. Note that HopPmaG¹⁻¹⁴::AvrRpt2¹⁰¹⁻²⁵⁵ was found inside bacteria and in the supernatant, while the cytoplasmic Npt2 protein was only found inside bacteria, indicating that the presence of HopPmaG¹⁻¹⁴::AvrRpt2¹⁰¹⁻²⁵⁵ in the supernatant was due to secretion and not due to bacterial lysis. In A and C, bands corresponding to effector::AvrRpt2 fusions and Npt2 are distinguished from cross-hybridizing bands by an asterisk.

gin of replication derived from pVS1, functional in *P. syringae* (Heeb et al. 2000), to express effectors in any *P. syringae* strain. Using the confirmed effectors HopQ1-1_{PtoDC3000}, HopT1-1_{PtoDC3000}, and HopS1_{PtoDC3000} (Table 1 lists previously used names), we obtained much stronger HRs when expressing their full-length fusions to AvrRpt2¹⁰¹⁻²⁵⁵ from the *npt2* promoter on pBAV178 than when expressing them from their native promoters using pBAV124 (data not shown). Nevertheless, we saw clear reproducible T3SS- and RPS2- dependent, albeit modest, HRs using expression from the native effector loci.

Based on our pilot experiments, we chose pBAV178 to express 11 predicted effectors fused to AvrRpt2¹⁰¹⁻²⁵⁵: HopAE1_{PsyB728a}, HopAG1_{PsyB728a}, HopAH1_{PsyB728a}, HopAH1_{PtoDC3000}, HopAH2_{PtoDC3000}, HopAI1_{PtoDC3000}, HopAQ1_{PtoDC3000}, HopI1_{PsyB728a}, HopR1_{PtoDC3000}, HopT1-2_{PtoDC3000}, and HopY1_{PtoDC3000} (Table 1). We expressed the fusions in *P. syringae* pv. *tomato* DC3000 and in the T3SS-deficient strain *P. syringae* pv. *tomato* DC3000 *hrcC*⁻ and infiltrated the resulting 22 strains on *RPS2*⁺ *Arabidopsis* and an *rps2*⁻ mutant that gives no HR in response to AvrRpt2. We used full-length fusions for all effectors except HopR1, for which we used HopR1¹⁻³²⁷, due to its large size (1,957 amino acids).

Figure 5A shows that all 11 predicted effectors showed HRs that were dependent on an intact T3SS and on *RPS2*. We sought to establish that all fusions were expressed in the *P. syringae* pv. *tomato* DC3000 *hrcC*⁻ strain, to ensure that a HR⁻ phenotype was not due to a failure to accumulate effector chimeras. Figure 5B shows the expression of eight of the effector::AvrRpt2 fusions by Western blot analysis in *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *tomato* DC3000 *hrcC*⁻. The expression of the HopAG1_{PsyB728a}, HopAH2_{PtoDC3000}, and HopAI1_{PtoDC3000} fusions to AvrRpt2¹⁰¹⁻²⁵⁵ could not be detected in either *P. syringae* pv. *tomato* DC3000 or *P. syringae* pv. *tomato* DC3000 *hrcC*⁻ bacteria, although they elicited a strong *RPS2*-dependent HR when expressed in *P. syringae* pv. *tomato* DC3000 in repeated experiments. Type III-dependent secretion for these three effectors can, therefore, be inferred but could not be shown directly. No other known secretion system able

to translocate proteins inside plant cells has been found to be encoded in the *P. syringae* pv. *tomato* DC3000 genome sequence. Furthermore, the HR caused by these effector::AvrRpt2¹⁰¹⁻²⁵⁵ chimeras was *hrcC*-dependent. Thus, it is very likely that the translocation of these three effector-reporter fusions is T3SS dependent.

Two predicted effectors in *P. fluorescens* are secreted from *P. syringae* pv. *tomato* DC3000 into plant cells.

The genome of the plant-growth-promoting *P. fluorescens* strain SBW25 (Sanger Institute) harbors genes coding for a T3SS and several putative effector genes (Greenberg and

Table 1. Published names^a

Name ^b	Previous name	Accession number
AvrRpt2 _{PtoJL1065}	AvrRpt2	L11355
HopAJ1 _{PmaES4326}	HopPmaG	AAL84245
HopAK1 _{PmaES4326}	HopPmaH	AAL84246
HopAL1 _{PmaES4326}	HopPmaK	AAL84251
HopI1 _{PmaES4326}	HopPmaI	AAL84247
HopW1-1 _{PmaES4326}	HopPmaA	AAL84239
HopAE1 _{PsyB728a}	HolPsyAE	DAA00391
HopAG1 _{PsyB728a}	HolPsyAG	DAA00387
HopAH1 _{PsyB728a}	HolPsyAH	DAA00388
HopI1 _{PsyB728a}	HopPmaI _{psy}	ZP_00126156
HopAH1 _{PtoDC3000}	HolPtoAH	AAO54439
HopAH2 _{PtoDC3000}	HolPtoAH2	AAO56770
HopAI1 _{PtoDC3000}	HopPtoAI	AAO54440
HopAQ1 _{PtoDC3000}	HolPtoAA	AAO58143
HopP1 _{PtoDC3000}	HopPtoP	AAO56180
HopQ1-1 _{PtoDC3000}	HolPtoQ, HopPtoQ	AAO54411
HopR1 _{PtoDC3000}	HolPtoR	AAO54417
HopS1 _{PtoDC3000}	HolPtoZ, HopPtoS1	AAO58043
HopT1-1 _{PtoDC3000}	HolPtoU, HopPtoT1	AAO59044
HopT1-2 _{PtoDC3000}	HolPtoU2	AAO58039
HopY1 _{PtoDC3000}	HolPtoY	AAO53615
—	AvrA _{PfSBW25}	PFLU4868
—	ExoY _{PfSBW25}	PFLU1622

^a Names are based on new unified nomenclature (Lindeberg et al. 2005) and GenBank accession numbers (Sanger Institute unique identifiers in the case of *Pseudomonas fluorescens* SBW25) of effectors used in this paper.

^b Based on new unified nomenclature.

Fig. 4. In vitro secretion assay of AvrRpt2, HopPmaA, and HopPmaI. Native AvrRpt2, C-terminally HA-tagged HopPmaI, and the 18 most N-terminal amino acids of HopPmaA fused to the cytoplasmic Npt2 protein were expressed from their native promoters in *Pseudomonas syringae* pv. *maculicola* ES4326 (indicated by +) and in type III secretion system (T3SS)-deficient *P. syringae* pv. *maculicola* ES4326 *hrcC*⁻ (indicated by -). Strains were grown in hrp-inducing medium. Protein was extracted from bacteria (b) and concentrated from culture supernatants (sn). Western blot analysis using the indicated antibodies shows that all three effectors were found in the supernatant of *P. syringae* pv. *maculicola* ES4326 but not in the supernatant of *P. syringae* pv. *maculicola* ES4326 *hrcC*⁻ strains, indicating T3SS-dependent in vitro secretion. The cytoplasmic Npt2 protein was not present in the supernatant, showing that no bacterial lysis occurred.

A

B

Vinatzer 2003; Preston et al. 2001). We cloned the putative effectors with homology to *avrA* of *P. syringae* pv. *glycinea*, *ospC2* and *ipaH* of *Shigella* spp., *popC* of *Ralstonia solanacearum*, and *exoY* of *P. aeruginosa*, recombined them into the pBAV178 vector, and transformed *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *tomato* DC3000 *hrcC*⁻ with the resulting plasmids, to determine T3SS-dependent delivery to plants. We tested whether *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *tomato* DC3000 *hrcC*⁻ both expressed these effector::AvrRpt2 fusions by Western blot analysis. Only the homologs of ExoY and AvrA fused to AvrRpt2¹⁰¹⁻²⁵⁵ were detectable (Fig. 6A). Upon infection of *RPS2*⁺ and *rps2*⁻ plants with *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *tomato* DC3000 *hrcC*⁻ strains expressing AvrA::AvrRpt2¹⁰¹⁻²⁵⁵ and ExoY::AvrRpt2¹⁰¹⁻²⁵⁵, *RPS2*⁺ plants infected with *P. syringae* pv. *tomato* DC3000 strains showed a HR, while all other combinations did not (Fig. 6B). Thus, these two effectors have type III secretion signals that are functional in *P. syringae*. Fusions of the *OspC2*, *IpaH*, and *PopC* homologs to AvrRpt2¹⁰¹⁻²⁵⁵ were not expressed to high enough levels to be detected by Western blot and gave no HR (data not shown). Therefore, we cannot exclude the possibility that these predicted effectors are also T3SS substrates; their fusions to AvrRpt2¹⁰¹⁻²⁵⁵ may not have accumulated to high enough levels in *P. syringae* pv. *tomato* DC3000 to cause a HR.

DISCUSSION

A high serine content is a hallmark of effectors in *P. syringae* and other gram-negative plant and animal pathogens (Greenberg and Vinatzer 2003; Guttman et al. 2002). We were concerned here that a serine-rich portion (amino acids 81 to 100) of the AvrRpt2⁸¹⁻²⁵⁵ T3SS reporter might contribute to secretion or translocation of heterologous proteins. However, we find these amino acids to be dispensable for secretion and elicitation of the HR, an indirect measure of translocation. In one case (HopPmaG¹⁻¹⁴), we found these amino acids did contribute to the stability of a protein chimera. Within AvrRpt2, the serine-rich region from amino acid 15 to 99 does not play a major role in secretion in culture or translocation to plant cells but is key for high-level protein accumulation in bacteria. The serine in position 19 is particularly important for steady-state levels of protein in bacteria. A minimal N-terminal secretion signal of AvrRpt2 spanning amino acids 1 to 14 is sufficient for secretion in culture of the AvrRpt2¹⁰¹⁻²⁵⁵ effector region and of its delivery to plant cells. Finally, the region between amino acid 34 and 80 may increase the efficiency of secretion, translocation to plant cells, or both. However, conclusions about the efficiency of translocation based on the HR assays presented herein have important caveats. HR strength only indirectly measures translocation. Factors in addition to translocation may affect HR strength. These include the in planta protein stability, localization, and

Fig. 6. In planta assays for type III secretion system (T3SS)-dependent delivery to plants of predicted *Pseudomonas fluorescens* SBW25 effectors, using the AvrRpt2¹⁰¹⁻²⁵⁵ reporter. Full-length *P. fluorescens* SBW25 homologs of *P. syringae* AvrA and *P. aeruginosa* ExoY were fused to the AvrRpt2¹⁰¹⁻²⁵⁵ reporter and were expressed from the *npt2* promoter on the high-copy-number plasmid pBAV178 in *P. syringae* pv. *tomato* DC3000. **A**, Western blot analysis of effector::AvrRpt2¹⁰¹⁻²⁵⁵ fusions expressed in *P. syringae* pv. *tomato* DC3000 (indicated with +) and *P. syringae* pv. *tomato* DC3000 *hrcC*⁻ (indicated with -). Note that both fusions were expressed to the same level in *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *tomato* DC3000 *hrcC*⁻ showing that the inability of the *P. syringae* pv. *tomato* DC3000 *hrcC*⁻ strains to elicit a hypersensitive response (HR) was due to T3SS-deficiency in these strains and not due to the absence of expression of the effector::AvrRpt2¹⁰¹⁻²⁵⁵ fusions. **B**, HR assay for T3SS-dependent translocation. The indicated constructs were expressed in *P. syringae* pv. *tomato* DC3000 (indicated with +) and *P. syringae* pv. *tomato* DC3000 *hrcC*⁻ (indicated with -), infiltrated at an optical density at 600 nm of 0.3 in *RPS2*⁺ and of *rps2*⁻ *Arabidopsis* leaves. Photographs were taken 18 h later.

Fig. 5. In planta assays for type III secretion system (T3SS)-dependent delivery to plants of predicted effectors using the AvrRpt2¹⁰¹⁻²⁵⁵ reporter. Full-length predicted effectors fused to the AvrRpt2¹⁰¹⁻²⁵⁵ reporter were expressed from the *npt2* promoter on the high-copy-number plasmid pBAV178 in *Pseudomonas syringae* pv. *tomato* DC3000. *P. syringae* pv. *tomato* DC3000 containing the empty vector pME6012, the parent vector of pBAV178, was used as negative control. *P. syringae* pv. *tomato* DC3000 containing pBAV204 expressing full-length AvrRpt2 from the *npt2* promoter on the high-copy-number plasmid pBAV179 was used as positive control. **A**, Hypersensitive response (HR) assay for T3SS-dependent delivery to plants of effector::AvrRpt2¹⁰¹⁻²⁵⁵ fusions. The indicated constructs were expressed in *P. syringae* pv. *tomato* DC3000 (indicated with +) and *P. syringae* pv. *tomato* DC3000 *hrcC*⁻ (indicated with -), infiltrated at an optical density at 600 nm of 0.3 in *RPS2*⁺ and of *rps2*⁻ *Arabidopsis* leaves. Photographs were taken 18 h later. HR elicited only on *RPS2*⁺ plants by the *P. syringae* pv. *tomato* DC3000 strains but not on *rps2*⁻ plants and not by *P. syringae* pv. *tomato* DC3000 *hrcC*⁻ on *RPS2*⁺ plants indicates T3SS-dependent delivery to plants of the effector::AvrRpt2¹⁰¹⁻²⁵⁵ fusions. **B**, Western blot analysis with α -AvrRpt2 antibody of effector::AvrRpt2¹⁰¹⁻²⁵⁵ fusions expressed in *P. syringae* pv. *tomato* DC3000 (indicated with +) and *P. syringae* pv. *tomato* DC3000 *hrcC*⁻ (indicated with -). Bands corresponding to effector::AvrRpt2¹⁰¹⁻²⁵⁵ fusions are distinguished from cross-hybridizing bands by an asterisk. Note that all shown fusions are expressed to similar levels in *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *tomato* DC3000 *hrcC*⁻, showing that the inability of the *P. syringae* pv. *tomato* DC3000 *hrcC*⁻ strains to elicit a HR was due to T3SS-deficiency in these strains and not due to the absence of expression of the effector::AvrRpt2¹⁰¹⁻²⁵⁵ fusions.

interactions with plant targets, parameters that have not been directly measured in these experiments.

What might account for the lower levels of AvrRpt2 derivatives lacking the serine-rich region of the N terminus? We don't know whether lower levels of AvrRpt2 derivatives are due to reduced synthesis of the proteins or increased turnover. However, it seems likely that increased turnover is responsible for the overall reduction in protein levels, since it has been reported that *P. syringae* effectors are subject to degradation by the Lon protease (Bretz et al. 2002; Losada and Hutcheson 2005). The N-terminal region of AvrRpt2 on its own is predicted to be unstructured due to its low sequence complexity. We speculate that *P. syringae* harbors a chaperone that binds to this low-complexity protein region and helps protect it from proteolysis by Lon protease (Kabisch et al. 2005; Losada and Hutcheson 2005).

Deleting the 20 most N-terminal amino acids of the AvrRpt2⁸¹⁻²⁵⁵ reporter in several previously identified effector::AvrRpt2⁸¹⁻²⁵⁵ chimeras and assaying secretion of two effectors that were originally identified with the AvrRpt2⁸¹⁻²⁵⁵ reporter in the absence of AvrRpt2, we further conclude that i) using the AvrRpt2⁸¹⁻²⁵⁵ reporter in our previous effector screen likely did not lead to false positives and ii) that a reduced AvrRpt2¹⁰¹⁻²⁵⁵ reporter can be used instead of AvrRpt2⁸¹⁻²⁵⁵ to further reduce any possible remaining doubts that the AvrRpt2 reporter could affect the final destination of effector::AvrRpt2 fusions.

The use of AvrRpt2⁸¹⁻²⁵⁵ in some situations may enhance the ability to detect secretion, translocation, or both, due to stabilization of fusion partners. For example, HopPmaG¹⁻¹⁴::AvrRpt2¹⁰¹⁻²⁵⁵ is secreted in culture but has reduced steady-state levels in bacteria and elicits a barely visible HR compared with that of HopPmaG¹⁻¹⁴::AvrRpt2⁸¹⁻²⁵⁵. This may be due to an increased degradation of HopPmaG¹⁻¹⁴::AvrRpt2¹⁰¹⁻²⁵⁵ as compared with HopPmaG¹⁻¹⁴::AvrRpt2⁸¹⁻²⁵⁵ by Lon protease (Losada and Hutcheson 2005). Full-length HopPmaG fused to AvrRpt2¹⁰¹⁻²⁵⁵ also has a low steady-state level. We could not detect secretion or translocation of this fusion. However, HopPmaG is expressed in an hrp-dependent manner, its 50 most N-terminal amino acids contain features of effectors such as 16% serines, the fusion of its first 14 amino acids to AvrRpt2⁸¹⁻²⁵⁵ is translocated into plant cells in a T3SS-dependent manner (Guttman et al. 2002), and HopPmaG¹⁻¹⁴::AvrRpt2¹⁰¹⁻²⁵⁵ is secreted in culture. Based on these results, we interpret the absence of HR elicitation and secretion of full-length HopPmaG fused to AvrRpt2 as a consequence of its low steady-state levels and not because it is not a T3SS substrate. More sensitive methods for detecting secretion and translocation will be required to resolve whether HopPmaG is secreted, translocated, or both.

Using the reduced AvrRpt2¹⁰¹⁻²⁵⁵ reporter, we expanded the known repertoire of proteins encoded in the *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *syringae* B728a genomes with functional T3SS signals to 47 and seven proteins, respectively. Proteins encoded by alleles of an additional 16 predicted *P. syringae* pv. *syringae* B728a effectors in other *P. syringae* strains are also known to be secreted. This brings the total number of probable effectors in *P. syringae* pv. *syringae* B728a to 23.

In most cases, we confirmed predicted T3SS substrates by expressing effector::AvrRpt2¹⁰¹⁻²⁵⁵ fusions from the constitutive *npt2* promoter on a high-copy-number plasmid. This system allows us only to determine if a protein has a functional T3SS signal capable of delivering to plants the AvrRpt2¹⁰¹⁻²⁵⁵ reporter. However, it does not tell us if that protein is expressed and translocated to plants from *P. syringae* pv. *tomato* DC3000 during infection. We accepted this disadvantage, since it allowed for higher sensitivity of the assay than expressing effectors from their native promoters on the chromosome. However, we

note that in the three cases in which it was tested in this study, effector::AvrRpt2¹⁰¹⁻²⁵⁵ fusions were delivered to plants when expressed from the native promoters in the normal chromosomal context. This suggests that use of the *npt2* promoter to drive expression can reveal whether a protein is a T3SS substrate.

In contrast to our findings, Chang and associates (2005) reported that the fusion of several full-length proteins to AvrRpt2⁸⁰⁻²⁵⁵ that we confirm in this report to be effectors did not elicit a HR when expressed from their native promoters. Several possibilities could account for this discrepancy. First, the extrachromosomal plasmid used by Chang and associates (2005) may not lead to expression levels sufficient for a detectable HR. Second, the fusions reported by Chang and associates (2005) may not have resulted in the production of protein. Since the production of protein chimeras with AvrRpt2⁸⁰⁻²⁵⁵ was not validated, it is unclear whether the proteins tested are not T3SS substrates or if the problem lay in the expression of protein. Third, some effectors may not be expressed during the infection conditions used or may be expressed only at low levels during infection in *P. syringae* pv. *tomato* DC3000. Homologs of these effectors may be expressed at a higher level in other strains or in the context of the native chromosomal loci. Since we observed a HR elicited by a full-length fusion of HopT1-1_{PtoDC3000} fused to AvrRpt2¹⁰¹⁻²⁵⁵ when expressed from its native promoter in the *P. syringae* pv. *tomato* DC3000 chromosome, whereas Chang and associates (2005) did not, the sensitivity of the HR assays may differ between laboratories. Moreover, we have found that AvrRpt2 elicits a much stronger HR when expressed from *P. syringae* pv. *maculicola* ES4326 than when expressed from *P. syringae* pv. *tomato* DC3000 (data not shown); another reason why some effectors identified in *P. syringae* pv. *maculicola* ES4326 may not have been confirmed by Chang and associates (2005) in *P. syringae* pv. *tomato* DC3000.

Three effectors we characterized here are encoded by the *hopAG1-hopAH1-hopAII* operon that is disrupted in different ways in strains DC3000 and B728a. In *P. syringae* pv. *tomato* DC3000, *hopAG1* is interrupted by a transposon insertion (Greenberg and Vinatzer 2003; Schechter et al. 2004) that is predicted to truncate the HopAG1 protein and may abolish or reduce expression of HopAH1 and HopAII. In *P. syringae* pv. *syringae* B728a, the operon harbors an early STOP codon in *hopAII* (Greenberg and Vinatzer 2003). It will be interesting to compare expression of the members of this operon in the two strains and to determine if this operon is intact in any *P. syringae* isolates. The loss of effectors from *P. syringae* due to insertions and point mutations could indicate that there are as-yet-undiscovered host mechanisms to detect and defend against these effectors. In this scenario, effectors in the operon are selected against to increase pathogen fitness on hosts able to recognize and respond to the presence of effectors by activating defenses.

Expression during infection of the 47 proteins with functional T3SS signals in *P. syringae* pv. *tomato* DC3000 has not been well-documented. Our unpublished data show that detection of expression of effectors during infection is not straightforward. We were unable to detect HA-tagged HopPmaI by Western blot expressed during infection (J. Jelenska, and J. T. Greenberg, *unpublished observations*), although we know that it is expressed during infection, since it gives a strong HR when fused to AvrRpt2⁸¹⁻²⁵⁵ and is driven by its own promoter (Guttman et al. 2002). Differential effector expression may be one way *P. syringae* uses to regulate the activity of the many members of its large effector repertoire. It is also possible that effectors have different intrinsic stabilities inside plant cells, something that could be tied to their function. Optimization of in planta detection of effectors is therefore important for further elucidation of *P. syringae* pathogenesis.

P. fluorescens SBW25 is a biocontrol strain isolated from the roots of beet. This particular strain and several other *P. fluorescens* strains have been found to contain a gene cluster coding for a T3SS (Preston et al. 2001). Mutations in this cluster do not affect the ability of *P. fluorescens* SBW25 to colonize beet roots (Preston et al. 2001). *P. fluorescens* SBW25 does not induce a HR on tobacco and is unable to efficiently translocate *P. syringae* effectors into plant cells. However, the T3SS of *P. fluorescens* SBW25 can function to deliver proteins into plant cells (Preston et al. 2001). When the T3SS is overexpressed, *P. fluorescens* SBW25 ectopically expressing the *P. syringae* effector AvrB causes cell death that resembles a HR on *Arabidopsis*. When the T3SS is overexpressed, *P. fluorescens* SBW25 also induces rapid cell death on tobacco, indicating that it likely has one or more effectors with HR-eliciting activity. Interestingly *P. fluorescens* SBW25 contains several genes with homology to effectors of other plant and animal effectors in its genome sequence (Greenberg and Vinatzer 2003). We showed here that two of these genes, a homolog of the *P. syringae* effector gene *avrA* and a homolog of the *P. aeruginosa* effector gene *exoY*, encode proteins that deliver the AvrRpt2¹⁰¹⁻²⁵⁵ reporter to plant cells. Humans with Crohn's disease produce antibodies against

the PflT protein of *P. fluorescens*, although *P. fluorescens* could not be detected in the feci of these patients (Wei et al. 2002). *P. fluorescens* was also recently reported to cause meningitis in humans (Picot et al. 2003). Since the *P. fluorescens* SBW25 T3SS does not have any role in root colonization (Preston et al. 2001), we hypothesize that it may have a role in aiding its survival in animal hosts or in association with specific plant species not yet assayed.

In summary, after validating the C-terminus of AvrRpt2 as a reliable reporter for T3SS-dependent translocation of putative effectors to plant cells, we further expanded the confirmed effector repertoire in the genus *Pseudomonas*. This is an important step towards a more comprehensive understanding of the biology and evolution of *P. syringae* and *P. fluorescens*.

MATERIALS AND METHODS

Bacterial strains, media, and growth.

Pseudomonas strains used in this study are listed in Table 2. *Escherichia coli* DH5 α and DB3.1 (Invitrogen, Carlsbad, CA, U.S.A.) were grown at 37°C in Luria Bertani medium (Sambrook et al. 1989), and *P. syringae* and *P. fluorescens* strains

Table 2. *Pseudomonas* strains

Strain or plasmid	Description	Reference or source
<i>P. syringae</i> pv. <i>maculicola</i> ES4326	Wild-type pathovar <i>maculicola</i>	F. M. Ausubel
<i>P. syringae</i> pv. <i>maculicola</i> ES4326 <i>hrcC</i>	Type III secretion deficient mutant	Guttman et al. 2002
<i>P. syringae</i> pv. <i>tomato</i> PtoDC3000	Wild-type pathovar <i>tomato</i>	Cuppels 1986
<i>P. syringae</i> pv. <i>tomato</i> DC3000 <i>hrcC</i>	Type III secretion deficient mutant	A. Collmer
<i>P. syringae</i> pv. <i>syringae</i> B728a	Wild-type pathovar <i>syringae</i>	Willis et al. 1988
<i>P. fluorescens</i> SBW25	Wild type	Preston et al. 2001
<i>P. syringae</i> pv. <i>maculicola</i> ES4326 hopPmaG ¹⁻¹⁴ ::avrRpt2 ⁸¹⁻²⁵⁵	Expresses HopPmaG ¹⁻¹⁴ ::AvrRpt2 ⁸¹⁻²⁵⁵	Guttman et al. 2002
<i>P. syringae</i> pv. <i>maculicola</i> ES4326 hopPmaH ¹⁻²⁶⁶ ::avrRpt2 ⁸¹⁻²⁵⁵	Expresses HopPmaH ¹⁻²⁶⁶ ::AvrRpt2 ⁸¹⁻²⁵⁵	Guttman et al. 2002
<i>P. syringae</i> pv. <i>maculicola</i> ES4326 hopPmaK ¹⁻⁵⁶ ::avrRpt2 ⁸¹⁻²⁵⁵	Expresses HopPmaK ¹⁻⁵⁶ ::AvrRpt2 ⁸¹⁻²⁵⁵	Guttman et al. 2002
<i>P. syringae</i> pv. <i>maculicola</i> ES4326 hopPtoP ¹⁻¹¹¹ ::avrRpt2 ⁸¹⁻²⁵⁵	Expresses HopPtoP ¹⁻¹¹¹ ::AvrRpt2 ⁸¹⁻²⁵⁵	Guttman et al. 2002

Table 3. Plasmids

Plasmid name	Parent vector	Promoter	Expressed effector/protein	Tag/reporter	Resistance	Reference
CKTR	BC SK+	None	Multiple cloning site	None	Kan	Guttman and Greenberg 2001
DSG6	CKTR	Native	AvrRpt2	None	Kan	Guttman and Greenberg 2001
JJ19	CKTR	Native	HopPmaI	HA-tev-Myc-His	Kan	This study
pLH12	LAFR3	Native	AvrRpt2	None	Tet	Whalen et al. 1991
ME6012	VS1	None	Multiple cloning site	None	Tet	Heeb et al. 2000
BAV15	DSG6	Native	AvrRpt2 ^{1-14, 101-255}	None	Kan	This study
BAV18	DSG6	Native	HopPmaG ¹⁻¹⁴	AvrRpt2 ¹⁰¹⁻²⁵⁵	Kan	This study
BAV20	DSG6	Native	AvrRpt2 ^{1-15, 101-255}	None	Kan	This study
BAV22	DSG6	Native	AvrRpt2 ^{1-80, 101-255}	None	Kan	This study
BAV23	DSG6	Native	HopPmaH ¹⁻²⁶⁶	AvrRpt2 ¹⁰¹⁻²⁵⁵	Kan	This study
BAV25	DSG6	Native	HopPmaK ¹⁻⁵⁶	AvrRpt2 ¹⁰¹⁻²⁵⁵	Kan	This study
BAV26	DSG6	Native	HopPtoP ¹⁻¹¹¹	AvrRpt2 ¹⁰¹⁻²⁵⁵	Kan	This study
BAV41	CKTR	Native	HopPmaA ¹⁻¹⁸	Npt2	Kan	This study
BAV124	CKTR	None	Gateway cassette	AvrRpt2 ¹⁰¹⁻²⁵⁵	Kan	This study
BAV178	ME6012	Npt2	Gateway cassette	AvrRpt2 ¹⁰¹⁻²⁵⁵	Tet	This study
BAV178-144	BAV178	Npt2	HopY1 _{PtoDC3000}	AvrRpt2 ¹⁰¹⁻²⁵⁵	Tet	This study
BAV178-1A06	BAV178	Npt2	HopAG1 _{PsyB728a}	AvrRpt2 ¹⁰¹⁻²⁵⁵	Tet	This study
BAV178-1A07	BAV178	Npt2	HopAH1 _{PsyB728a}	AvrRpt2 ¹⁰¹⁻²⁵⁵	Tet	This study
BAV178-1B01	BAV178	Npt2	HopI1 _{PsyB728a}	AvrRpt2 ¹⁰¹⁻²⁵⁵	Tet	This study
BAV178-1C12	BAV178	Npt2	HopAH1 _{PtoDC3000}	AvrRpt2 ¹⁰¹⁻²⁵⁵	Tet	This study
BAV178-1D01	BAV178	Npt2	HopAI1 _{PtoDC3000}	AvrRpt2 ¹⁰¹⁻²⁵⁵	Tet	This study
BAV178-1D04	BAV178	Npt2	HopT1-2 _{PtoDC3000}	AvrRpt2 ¹⁰¹⁻²⁵⁵	Tet	This study
BAV178-2B06	BAV178	Npt2	AvrA _{PflSBW25}	AvrRpt2 ¹⁰¹⁻²⁵⁵	Tet	This study
BAV178-2B08	BAV178	Npt2	ExoY _{PflSBW25}	AvrRpt2 ¹⁰¹⁻²⁵⁵	Tet	This study
BAV178-2D05	BAV178	Npt2	HopAH2 _{PtoDC3000}	AvrRpt2 ¹⁰¹⁻²⁵⁵	Tet	This study
BAV178-2E05	BAV178	Npt2	HopAE1 _{PsyB728a}	AvrRpt2 ¹⁰¹⁻²⁵⁵	Tet	This study
BAV178-2E07	BAV178	Npt2	HopR1 ¹⁻³²⁷ _{PtoDC3000}	AvrRpt2 ¹⁰¹⁻²⁵⁵	Tet	This study
BAV178-2G01	BAV178	Npt2	HopAQ1 _{PtoDC3000}	AvrRpt2 ¹⁰¹⁻²⁵⁵	Tet	This study
BAV179	ME6012	Npt2	Gateway cassette	HA	Tet	This study
BAV204	BAV179	Npt2	AvrRpt2	HA	Tet	This study
BAV217	DSG6	Native	AvrRpt2 ^{1-33, 101-255}	None	Kan	This study
BAV219	DSG6	Native	AvrRpt2 ^{1-19, 101-255}	None	Kan	This study
BAV220	DSG6	Native	AvrRpt2 ^{1-18, S19D, 101-255}	None	Kan	This study

were grown at 30°C in King's B (KB) medium (King et al. 1954) or at 17°C in *hrp*-inducing medium (Huynh et al. 1989). Bacteria were grown in the presence of antibiotics to maintain purity of strains and to maintain plasmids as indicated in Tables 2 and 3. Antibiotics were used at the following concentrations: rifampicin, 35 µg/ml; streptomycin, 75 µg/ml; kanamycin, 50 µg/ml; tetracycline, 13 µg/ml; gentamycin, 15 µg/ml.

Plant lines and growth conditions.

Plants were grown as described by Greenberg and associates (2000). All assays were performed with either *Arabidopsis thaliana* accession 'Columbia' or the *Arabidopsis thaliana*

rps2-101c mutant (accession 'Columbia') provided by F. M. Ausubel (Harvard University and Massachusetts General Hospital, Boston).

Effector cloning and mutational analysis.

To make deletions in effector genes or effector::*avrRpt2* fusions that were cloned previously, we used 'round the vector' cloning as described previously (Guttman and Greenberg 2001). Primers are listed in Table 4. All genes in which deletions were introduced were sequenced. All PCR reactions for cloning were performed with Pfu (Stratagene, La Jolla, CA, U.S.A.).

Table 4. Primers used to clone, tag, or mutate effectors^a

Name	Sequence
AvrA _{PfSBW25} F	<u>AAAAAGCAGGCT</u> ATTTGAGGTGATGTATGCGAGA
AvrA _{PfSBW25} R	<u>AGAAAGCTGGGT</u> ATGTCTGTGGCAAAGGCGTGTCCAACAGAGACTTG
AvrRpt2 101aa F	GAAAAACCTTTGTTCCGTCTCA
AvrRpt2 14aa R	GCTGAGCGGGCTGTGATTTA
AvrRpt2 15aa R	CCTGCTGAGCGGGCTGT
AvrRpt2 18aaS19D R	GTCCGGGACCTCCCTGTGAG
AvrRpt2 19aa R	TGACGGGACCTCCCTGCT
AvrRpt2 255aa R	TTAGCGGTAGAGCATTGCGTGT
AvrRpt2 33aa R	AGATTGAAGGTTGGTTTGCTTT
AvrRpt2 80aa R	AGATGATTCTTTTGAACAC
EcoRI-HA-NheI-I	AATTCGTACCCATACGACGTCCCAGACTACG
EcoRI-HA-NheI-II	CTAGCGTAGTCTGGGACGTGCTATGGGTACG
ExoY _{PfSBW25} F	<u>AAAAAGCAGGCT</u> CGACGTATCGGTAAACCA
ExoY _{PfSBW25} R	<u>AGAAAGCTGGGT</u> AACCTTGACGCAAGGCTGAC
Gate F	ACAAGTTTGTACAAAAAGCTGAACGAGAAACG
Gate F no att	GCTGAACGAGAAACGTAAATG
Gate R	AACCACTTTGTACAAGAAAGC
HopAE1 _{PsyB728a} F	<u>AAAAAGCAGGCT</u> TAGGTAAAAACGCCATGATG
HopAE1 _{PsyB728a} R	<u>AGAAAGCTGGGT</u> CTGGCTCGCCAGCCGCT
HopAG1 _{PsyB728a} F	<u>AAAAAGCAGGCT</u> TATTCCAAGGACGAATGTTCA
HopAG1 _{PsyB728a} R	<u>AGAAAGCTGGGT</u> CTTTGAAAGGGCCCTTTAGATAAGCC
HopAH1 _{PsyB728a} F	<u>AAAAAGCAGGCT</u> TAAAGGAGTAATGAAATGAGTATGA
HopAH1 _{PsyB728a} R	<u>AGAAAGCTGGGT</u> CGGAAAACTGATCGCCTTTCGAG
HopAH1 _{PtoDC3000} F	<u>AAAAAGCAGGCT</u> TAAAGGAGTAATGAAATGAGTATGA
HopAH1 _{PtoDC3000} R	<u>AGAAAGCTGGGT</u> AGGAGAATTGATCCCCCTTTCG
HopAH2 _{PtoDC3000} F	<u>AAAAAGCAGGCT</u> TAAAGAGACGCTCGATGAAT
HopAH2 _{PtoDC3000} R	<u>AGAAAGCTGGGT</u> AGTATTGATCTGACTTTGAAAAATCCGACA
HopAI1 _{PtoDC3000} F	<u>AAAAAGCAGGCT</u> TGGAAAAACAACGTATATGCC
HopAI1 _{PtoDC3000} R	<u>AGAAAGCTGGGT</u> AGCGAGTCCAGGGCGGTG
HopAQ1 _{PtoDC3000} F	<u>AAAAAGCAGGCT</u> GTCAAAGAGGTTTTCATGAATC
HopAQ1 _{PtoDC3000} R	<u>AGAAAGCTGGGT</u> ATGCACTGCCACCAGCAAT
HopI1 _{PsyB728a} F	<u>AAAAAGCAGGCT</u> TTCCGGAGAAGCAGATGAT
HopI1 _{PsyB728a} R	<u>AGAAAGCTGGGT</u> CGGTCTTGAACCTGTTACCGT
HopPmaA 18aa R	CCGGAATTTCGTAAAACTTGGCGGGAATGA
HopPmaA F	ATGAACCGGGGTTAATTGC
HopPmaG 14aa R	CATAATGACTACCAGCGATGT
HopPmaH 226 aa R	TGCAGGTTTGGCACTTTTT
HopPmaI F	CCGCTCAGGGGTCATGCACCTGACCATC
HopPmaI R	GTACGAATTCACATACTGACCCTTTTC
HopPmaK 56 aa R	GACGCGAGGCGGTGCTAT
HopPtoP 111 aa R	CGTTGACGAATCAGTCTGA
HopR1 _{PtoDC3000} F	<u>AAAAAGCAGGCT</u> GGAGTTTAATAATAACGATGGTCAA
HopR1 _{PtoDC3000} R	<u>AGAAAGCTGGGT</u> TGTATCAAGGATCAGTGCAGC
HopT1-2 _{PtoDC3000} F	<u>AAAAAGCAGGCT</u> GACGAGTGACTCATATGATCAAAACAGTCAG GATCGGGTAGGCT
HopT1-2 _{PtoDC3000} R	<u>AGAAAGCTGGGT</u> AGCCTACCCGATCAGCGGCTGAATGCTCGAC
HopY1 _{PtoDC3000} F	<u>AAAAAGCAGGCT</u> CAGCGATAAAGGGAGATTA
HopY1 _{PtoDC3000} R	<u>AGAAAGCTGGGT</u> CTGGTAGTTGATGCCCGTG
Myc-His R	TAGTGGATCCAATGATGATGATG
NheI-tev-ApaI	GCTAGCGAAAACCTGTATTTTCAGGGGCC
Npt2 ORF F	ACCGGAATTCGATTGAACAAGATGGATT
Npt2 ORF R	TAGTGGATCCGCGAATCGGGAGCG
Npt2 p F	TCATGAGATTATCAAAAAGGATCTTC
Npt2 p R	TTTTTTGTACAACTTGTCTCTCTTGATCAGAGCTT
Npt2 Xho F	TATCTCGAGTCATGAGATTATCAAAAAGGATCTTC
Ori R	CAACGGTGGTATATCCAGTGA
TrpA F	AAAGGGAACAAAAGCTGGAG
TrpA <i>Hind</i> III R	AAAAAAGCTTAAAGGGAACAAAAGCTGGAG

^a Gateway sequences are underlined. F = forward primer, R = reverse primer.

Plasmid pBAV41, which expresses HopPmaA¹⁻¹⁸ fused to Npt2, was derived from pDSG6 (Guttman and Greenberg 2001) by replacing *avrRpt2* with the *hopPmaA* promoter, the coding sequence corresponding to the first 18 N-terminal amino acids of HopPmaA, and the *npt2* gene. The *hopPmaA* sequences were amplified from *P. syringae* pv. *maculicola* ES4326 with the primers 'HopPmaA F' and 'HopPmaA 18aa R.' The *npt2* gene was amplified with 'Npt2 ORF F' and 'Npt2 ORF R' from pCKTR (Guttman and Greenberg 2001).

Plasmid pJJ19 expressing HA-tev-Myc-His tagged HopPmaA was constructed by assembling into *Xho*I- and *Bam*HI-digested pCKTR the following fragments: *hopPmaA* amplified from clone 3C11 of a *P. syringae* pv. *maculicola* ES4326 cosmid library with the primers 'HopPmaA F' and 'HopPmaA R,' linker *Eco*RI-HA-NheI made of oligos *Eco*RI-HA-NheI-I and *Eco*RI-HA-NheI-II, an NheI-tev-ApaI fragment, and the Myc-His fragment amplified from the picZ-A plasmid (Invitrogen) with primer '5'AOX' (Invitrogen) and primer 'Myc-His R.'

The Gateway expression vector pBAV124 for Campbell integration of effectors at their native loci in *P. syringae* was constructed in a series of blunt-end ligations combining the following PCR fragments: the *trpA* terminator sequence, the origin of replication and the *npt2* kanamycin resistance gene amplified from pCKTR with the primer 'trpA F' upstream of the *trpA* terminator sequence and the 'ori R' primer downstream of the origin of replication, the Gateway cassette amplified with the primers 'Gate F' and 'Gate R' from a Gateway destination vector, the AvrRpt2¹⁰¹⁻²⁵⁵ reporter amplified with the primers 'AvrRpt2 101aa F' and 'AvrRpt2 255aa R' from pLH12.

Vector pBAV178 was constructed similarly. First, the following PCR products were ligated to each other in a series of sequential blunt-end ligations leading to a plasmid without an origin of replication for *P. syringae*: the *trpA* terminator sequence, the origin of replication and the *npt2* gene amplified from pCKTR as described above for pBAV124, the *npt2* promoter amplified from pCKTR with 'Npt2 p F,' and 'Npt2 p R' (a primer also containing the attR1 sequence), the Gateway cassette amplified with the primers 'Gate F no att' (a primer 3' of the attR1 sequence) and 'Gate R,' and the AvrRpt2¹⁰¹⁻²⁵⁵ reporter amplified with 'AvrRpt2 101aa F' and 'AvrRpt2 255aa R' from pLH12. To obtain pBAV178, the '*npt2* promoter-Gateway cassette-avrRpt2¹⁰¹⁻²⁵⁵ reporter-*trpA* terminator' fragment was amplified from this plasmid with 'Npt2 *Xho* F' (a forward primer on the *npt2* promoter, with an overhang corresponding to the *Xho*I restriction site) and 'TrpA *Hind*III R' (a reverse primer downstream of the *trpA* terminator sequence of pCKTR, with an overhang corresponding to the *Hind*III restriction site) and was ligated into pME6012 (Heeb et al. 2000), which was digested with *Xho*I and *Hind*III. The pME6012 vector was provided by D. Haas (Université de Lausanne, Switzerland).

Effectors were cloned into the Gateway pDONR207 (Invitrogen) vector following the attB-adaptor PCR protocol described in the Invitrogen Gateway Technology instruction manual, using primers listed in Table 4. Clones were sequenced, and correct clones were recombined into the expression vectors, using the LR recombination reaction also described in the Invitrogen Gateway Technology instruction manual.

Effector expression and secretion assays.

For effector expression analysis, 1 ml of cultures of *P. syringae* grown at 17°C in liquid *hrp*-inducing medium were centrifuged at 16,000 × *g* at room temperature and were resuspended in 100 µl of alkaline lysis solution 1 (Sambrook et al. 1989). After vortexing, 100 µl of denaturing sample buffer (125 mM Tris, pH 6.8; 20% glycerol; 4% sodium dodecyl sulfate [SDS]; 5 M urea; 0.01% bromophenol blue) and 10 µl of 1 M dithio-

threitol were added, and the samples were heated for 5 min at 95°C. For effector secretion assays, individual *P. syringae* strains were grown for 20 h on KB plates without antibiotics. Half a plate was scraped from the plate using a sterile spatula and was resuspended in 2 ml of *hrp*-inducing medium at room temperature. Suspensions were vortexed at medium speed until all clumps were dissolved. The suspension was diluted to an optical density at 600 nm of 0.3 in *hrp*-inducing medium in a total volume of 6 ml. Bacteria were grown at 17°C for approximately 14 h with vigorous shaking. Two 1.5-ml microcentrifuge tubes were filled with each culture and were centrifuged at 16,000 × *g* at 4°C for 10 min. The upper 1 ml of supernatants from both tubes were combined in one 2-ml microcentrifuge tube, pipetting very slowly. A total of 10 µl of 10mM phenylmethylsulphonyl fluoride was added, and the 2-ml microcentrifuge tubes were centrifuged for an additional 20 min at 4°C at 16,000 × *g*. The remaining supernatant from one 1.5-ml tube was aspirated, and the pellet was processed as described above for effector expression analysis. After centrifuging the 2-ml microcentrifuge tubes, the upper 1.5 ml of supernatant was transferred to a 1.5-ml microcentrifuge tube containing 150 µl of ice cold 100% trichloroacetic acid supplemented with 1% sodium deoxycolic acid (adapted from Tantin and associates [1996]). Tubes were vortexed at high speed for 30 s and were put on ice for at least 1 h. Tubes were centrifuged for 20 min at 4°C at 16,000 × *g*, and the supernatant was poured off. Acetone (1 ml) was added, and pellets were left sitting on the bench for 10 min before resuspending nondissolved pellets by inverting several times. Tubes were centrifuged for 5 min at 16,000 × *g* at room temperature, and the supernatant was poured off. Then another 1 ml of acetone was added and centrifuged 5 min, and the pellet was allowed to dry for a few minutes. A total of 30 µl of a 1:1 mixture of loading buffer and alkaline lysis solution 1 (Sambrook et al. 1989) was added, heated for 5 min at 95°C and was stored on ice. Concentrated supernatant (10 µl) and resuspended pellet (5 µl) were loaded on denaturing 12% SDS-polyacrylamide gel electrophoresis gels, were blotted, and were probed with antibodies as described by Guttman and Greenberg (2001). Effector AvrRpt2 fusions were detected using an anti-AvrRpt2 antibody provided by B. Kunkel (Washington University, St. Louis) at a 1:10,000 dilution. HA-tagged effectors were detected using a monoclonal HA antibody, HA.11 (Covance, Berkeley, CA, U.S.A.), at a 1:1,000 dilution, and Npt2 was detected, using anti-Npt2 antibody (US Biologicals, Swampscott, MA, U.S.A.) at a 1:50,000 dilution. Secondary antibodies (Pierce, Rockford, IL, U.S.A.) were used at a 1:20,000 dilution.

HR assays.

Plants were grown and assayed for HR as described by Guttman and Greenberg (2001).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Wellcome Trust Sanger Institute *Pseudomonas fluorescens* SBW25 webpage: www.sanger.ac.uk/Projects/P_fluorescens