the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2009) doi: 10.1111/j.1365-313X.2009.03998.x

TECHNICAL ADVANCE

Recombineering and stable integration of the *Pseudomonas syringae* pv. *syringae* 61 *hrp/hrc* cluster into the genome of the soil bacterium *Pseudomonas fluorescens* Pf0-1

William J. Thomas[1,2,†], Caitlin A. Thireault[1,†], Jeffrey A. Kimbrel[1,2] and Jeff H. Chang[1,2,3,*]

[1]*Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331, USA,*

[2]*Molecular and Cellular Biology Program, Oregon State University, Corvallis, OR 97331, USA, and*

[3]*Center for Genome Research and Biocomputing, Oregon State University, Corvallis, OR 97331, USA*

Received 7 June 2009; revised 5 August 2009; accepted 6 August 2009.

[*]For correspondence (fax 541 737 3573; e-mail changj@science.oregonstate.edu).

[†]These authors contributed equally to this work.

SUMMARY

Many Gram-negative bacteria use a type III secretion system (T3SS) to establish associations with their hosts. The T3SS is a conduit for direct injection of type-III effector proteins into host cells, where they manipulate the host for the benefit of the infecting bacterium. For plant-associated pathogens, the variations in number and amino acid sequences of type-III effectors, as well as their functional redundancy, make studying type-III effectors challenging. To mitigate this challenge, we developed a stable delivery system for individual or defined sets of type-III effectors into plant cells. We used recombineering and Tn5-mediated transposition to clone and stably integrate, respectively, the complete *hrp/hrc* region from *Pseudomonas syringae* pv. *syringae* 61 into the genome of the soil bacterium *Pseudomonas fluorescens* Pf0-1. We describe our development of Effector-to-Host Analyzer (EtHAn), and demonstrate its utility for studying effectors for their *in planta* functions.

Keywords: type-III secretion system, *hrp*/*hrc,* type-III effectors, plant defense, *Pseudomonas syringae*, virulence.

INTRODUCTION

Pathogens must overcome plant defenses in order to successfully infect their hosts. One defense mechanism that plant pathogens encounter is basal defense, or PAMP-triggered immunity (PTI). PTI relies on pattern recognition receptors (PRRs) to perceive microbes by their conserved molecular patterns (pathogen- or microbial-associated molecular patterns, PAMPs or MAMPs, respectively; Ausubel, 2005; Jones and Dangl, 2006; Schwessinger and Zipfel, 2008). Perception results in the induction of plant responses that include the deposition of callose into the cell walls (Schwessinger and Zipfel, 2008). Hereafter, for the sake of simplicity, we use the terms PAMPs in reference to both PAMPs and MAMPs, and PTI in reference to basal defense, regardless of the source of the molecular pattern being perceived by the plant.

Many Gram-negative phytopathogenic bacteria deliver type-III effectors into host cells through a type-III secretion system (T3SS) as countermeasures against PTI (Cunnac *et al.*, 2009). The more than 20 genes encoding the regulatory elements and structural components of the T3SS are often clustered together in bacterial genomes (Galan and Wolf-Watz, 2006). In *Pseudomonas syringae*, the T3SS-encoding genes of the *hrp*/*hrc* region are located in a 26-kb pathogenicity island (Huang *et al.*, 1988; Alfano *et al.*, 2000; Oh *et al.*, 2007). Central to its expression is the alternative sigma factor HrpL (Xiao *et al.*, 1994). HrpL is a member of the extracytoplasmic function (ECF) family of sigma factors, and activates the expression of T3SS-encoding genes and type-III effector genes by recognizing a *cis*-regulatory element, the *hrp*-box (Innes *et al.*, 1993; Fouts *et al.*, 2002). Their expression is usually low or repressed when cells are grown in rich media, but are induced to high levels when grown *in planta* or *in vitro* in *hrp*-inducing media (Huynh *et al.*, 1989; Rahme *et al.*, 1992; Xiao *et al.*, 1992).

Type-III effectors of phytopathogens are collectively necessary for the bacteria to cause disease. A T3SS-deficient mutant is incapable of causing disease on its normally compatible host (Niepold *et al.*, 1985; Lindgren *et al.*, 1986). Moreover, studies of transgenic plants expressing individual type-III effectors provide strong evidence that their functions are to dampen host PTI (Hauck *et al.*, 2003; Nomura *et al.*, 2006; Fu *et al.*, 2007; Underwood *et al.*, 2007; Shan *et al.*, 2008; Xiang *et al.*, 2008).

Some type-III effectors can also betray the presence of the infecting bacterium to the host. Plants encode disease resistance proteins (R) that can perceive a single or limited number of cognate type-III effector proteins (DeYoung and Innes, 2006; Jones and Dangl, 2006). Perception triggers a defense response that can be viewed as amplified PTI. This effector-triggered immunity (ETI) is frequently associated with visual evidence of a programmed cell death response, called the hypersensitive response (HR; Greenberg and Yao, 2004).

Germane to the work described herein are three ETI-elicitors: AvrRpt2, AvrRpm1 and HopQ1-1. AvrRpt2 and AvrRpm1 are type-III effectors of *P. syringae* that perturb the Arabidopsis protein RIN4. The R proteins RPS2 and RPM1, respectively, perceive their corresponding modifications to RIN4 to elicit ETI (Mackey *et al.*, 2002, 2003; Axtell and Staskawicz, 2003). HopQ1-1 of *P. syringae* pv. *tomato* DC3000 (*Pto*DC3000) elicits ETI in tobacco, and is a negative host range determinant; its deletion from the genome enables *Pto*DC3000 to grow significantly more than the wild-type strain in tobacco (Wei *et al.*, 2007).

Type-III effectors are challenging to study for many reasons. There is a great diversity of effectors even between strains of the same species. A draft genome sequence of the pathogen *P. syringae* pv. *tomato* T1 (*Pto*T1) was completed and compared with the completed genome sequence of *Pto*DC3000 (Almeida *et al.*, 2009). Both are pathogens of tomato, and their inventories of genes had a high degree of conservation. In striking contrast, the number of homologous type-III effector genes common to both strains was much lower. This diversity in type-III effector collections is likely a response to the selective pressures host defenses impose on pathogens (Jones and Dangl, 2006).

Deletion or overexpression of type-III effector genes in pathogens does not often result in changes in phenotype (Chang *et al.*, 2004). This reflects the observation that pathogens have collections of type-III effectors with overlapping functions (Kvitko *et al.*, 2009). The overlap in functions of homologous and especially non-homologous type-III effectors is thus a significant impediment in genetic-based studies of their functions.

Pseudomonas fluorescens 55, carrying the pHIR11 cosmid or its derivatives, has been a workhorse heterologous delivery system and a valuable resource for studying type-III effectors (Kim *et al.*, 2002; Jamir *et al.*, 2004; Schechter *et al.*, 2004; Fujikawa *et al.*, 2006). This cosmid clone has the entire T3SS-encoding region of *P. syringae* pv. *syringae* 61 (*Psy*61), and also a chaperone and its cognate type-III effector gene, *shcA* and *hopA1* (aka *hopPsyA*), as well as 1.6 kb of the type-III effector gene *avrE1* (Huang *et al.*, 1988). HopA1 elicits ETI in tobacco and the Ws-0 cultivar of Arabidopsis (Jamir *et al.*, 2004; Gassmann, 2005). The presence of *hopA1* complicates the characterization of defined sets of type-III effectors, and it has been disrupted via marker-exchange mutagenesis (Fouts *et al.*, 2003; Jamir *et al.*, 2004).

One limitation to pHIR11, or its variants, is that bacterial cells do not stably maintain the cosmids in the absence of antibiotic selection. After 24 h of growth *in planta*, only 50% of a derivative of *Pto*DC3000 still carried pCPP2071, a derivative of pHIR11 (Fouts *et al.*, 2003). By 4 days, only 4% of the cells still had the cosmid. We have observed approximately 90% loss from *P. fluorescens* grown overnight in culture in the absence of selection (K.A. Unrath and JHC, unpublished data). This rapid loss of the T3SS-encoding locus potentially limits the utility of pHIR11 and its derivatives for studying type-III effectors *in planta*.

To address the limitations in stability and potential conflict with antibiotic resistance markers, we have developed a new system to deliver individual or defined sets of type-III effector proteins directly into host cells. We used recombineering to transfer the *hrp*/*hrc* region from *Psy*61, cloned in a cosmid vector, into a mini-*Tn5* Tc plasmid (de Lorenzo *et al.*, 1990; Court *et al.*, 2002; Jamir *et al.*, 2004; Oh *et al.*, 2007). Recombineering is catalyzed by bacteriophage-encoded systems that inhibit bacteria RecBCD nucleases from degrading double-stranded linear DNA fragments, and can be used to precisely delete, insert or alter a DNA sequence (Court *et al.*, 2002; Sharan *et al.*, 2009). We then stably integrated the *hrp*/*hrc* region directly into the genome of *P. fluorescens* Pf0-1 to ensure a robust delivery system (Silby *et al.*, 2009). We present our development of Effector to Host Analyzer (EtHAn), and validate its use for characterizing type-III effectors.

RESULTS

Development of a stable type-III effector delivery system

We used a variety of PCR methods and recombineering to clone the *hrp/hrc* cluster from cosmid clone pLN18 into a mini-*Tn5* Tc vector (Figure 1; de Lorenzo *et al.*, 1990; Court *et al.*, 2002; Jamir *et al.*, 2004). The genes we cloned spanned *hrpK* to *hrpH*, and we will refer to this as the T3SS-encoding region. Despite evidence that HrpH may be injected directly into host cells, we purposefully included *hrpH* because its product is necessary for the efficient translocation of other type-III effectors (Oh *et al.*, 2007). Our design avoided the inclusion of genes encoding

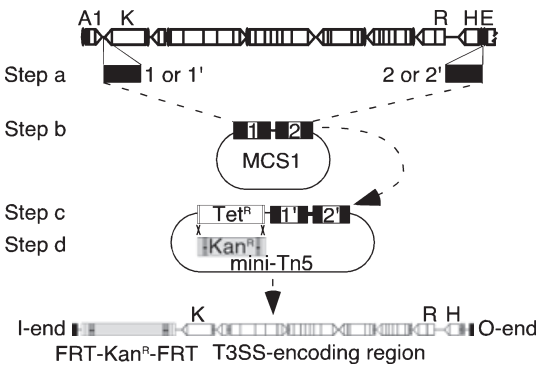

Figure 1. Construction of the effector-to-host analyzer (EtHAn). The upper bar represents the cloned insert of pLN18. Boxes represent protein-coding regions, and triangles indicate their direction of transcription. Black vertical discs represent *hrp* boxes. Some protein-coding regions are labeled for landmarks; A1, *shcA1-hopA1*; K, *hrpK*; R, *hrpR*; H, *hrpH*; E, *avrE1*. The *avrE1* gene is truncated in pLN18 (broken line).

Step a: Two 0.5 kb fragments (black boxes; 1, 1′, 2 and 2′) flanking the T3SS-encoding region were amplified and recombined. The 1 + 2 and 1′ + 2′ recombined products were cloned into pBBR1-MCS1 (MCS1) or mini-*Tn5* Tc, respectively.

Steps b and c: Vectors containing recombined flanking DNA fragments were linearized by restriction digestion, and used to sequentially capture the T3SS-encoding region by recombineering, first into pBBR1-MCS1 (step b) then into mini-*Tn5* Tc (step c).

Step d: Recombineering was used to replace the *Tet*^{R} gene with the *Kan*^{R} gene flanked by FRT sites (darker gray bars with a horizontal dashed line). The resulting DNA fragment between the *Tn5*-repeats (I and O ends) is shown at the bottom. The orientation of the T3SS-encoding region relative to the *Tn5* repeats is unknown and arbitrarily presented in one direction. The entire fragment was stably integrated into the genome of *P. fluorescens* Pf0-1 via *Tn5* transposition, and the *Kan*^{R} gene was excised using the site-specific recombinase FLP (not shown).

shcA-hopA1, and included <300 bp of the *avrE* coding region. We further modified the mini-*Tn5* Tc by replacing the *Tet*^{R} gene with a *Kan*^{R} gene, flanked by site-specific recombinase recognition sequences (FRT), to facilitate the excision of the antibiotic marker from the genome.

We used Tn5 to mediate the direct and stable integration of the T3SS-encoding region into the genome of the soil bacterium *P. fluorescens* Pf0-1. We selected this strain for two reasons. The genome sequence of Pf0-1 has been completed, and searches have failed to confidently identify a T3SS-encoding region or candidate type-III effector genes (Ma *et al.*, 2003; Grant *et al.*, 2006; Silby *et al.*, 2009). Secondly, Pf0-1 is a soil bacterium not adapted for survival within plants, and is therefore expected to elicit PTI, and to be incapable of dampening PTI or eliciting ETI when injected directly into plants (Compeau *et al.*, 1988).

We also used the site-specific recombinase FLP to excise the *Kan*^{R} gene from the genome of Pf0-1. Through these manipulations, we developed EtHAn, an unmarked, PTI-eliciting bacterium capable of delivering individual or defined sets of type-III effector proteins directly into the cells of plants.

Pseudomonas fluorescens Pf0-1 and the modified EtHAn cannot grow *in planta*

To validate our selection of Pf0-1 as the recipient strain for the T3SS-encoding region, we first determined its growth behavior *in planta* (Figure 2). We infiltrated 1.0×10^6 cfu ml^{-1} of wild-type Pf0-1 and EtHAn into leaves of Arabidopsis and enumerated their growth. The compatible pathogen *Pto*DC3000 grew extensively *in planta*, and its T3SS mutant (Δ*hrcC*) failed to grow significantly. Pf0-1 and EtHAn failed to grow, even to levels comparable with the Δ*hrcC* mutant. EtHAn expressing the T3SS appears to grow even less than Pf0-1, but we note that this is more likely to be a reflection of differences in infiltrated bacterial concentrations (at day 0, EtHAn had lower cfu levels than Pf0-1). We also enumerated growth of EtHAn following inoculation with higher concentrations of bacteria, and had similar results to those presented (data not shown). These results validated our selection of Pf0-1 as the recipient for the T3SS-encoding region, and show that the T3SS is, by itself, insufficient to confer virulence.

EtHAn carries a functional T3SS

To demonstrate that EtHAn encodes a functional T3SS, we tested its ability to deliver type-III effectors *in planta*. Delivery is easily detected through the elicitation of ETI, where perception of a type-III effector protein by a corresponding R protein leads to a rapid HR. We tested whether EtHAn carrying either *avrRpm1* or *avrRpt2* would elicit an HR in Arabidopsis Col-0. Their protein products are perceived by corresponding R proteins, RPM1 and RPS2, leading to a visible HR at approximately 6 h post-inoculation (6 hpi) and 20 hpi, respectively (Kunkel *et al.*, 1993; Yu *et al.*, 1993; Bisgrove *et al.*, 1994).

We infiltrated 1.0×10^8 cfu ml^{-1} of EtHAn carrying *avrRpm1* or *avrRpt2* into leaves of Arabidopsis (Figure 3a).

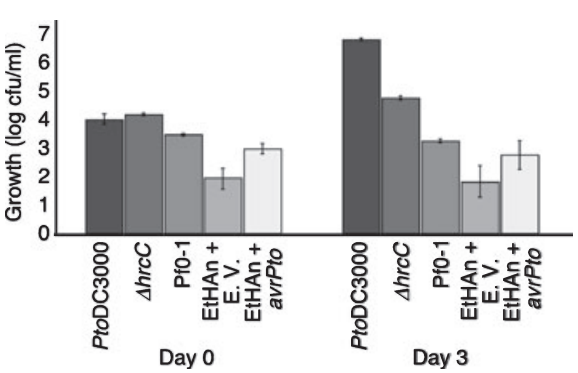

Figure 2. Enumeration of bacterial growth *in planta*. Wild-type *Pto*DC3000, a Δ*hrcC* mutant, Pf0-1, EtHAn carrying an empty vector (E.V.) and EtHAn carrying *avrPto* were assessed for growth in Arabidopsis over a period of 3 days post-inoculation. Each time point was measured in triplicate; standard errors are presented. Experiments were repeated at least three times with similar results.

At 6 hpi, the majority of leaves infected with *Pto*DC3000 or EtHAn carrying *avrRpm1*, showed visible HR phenotypes. By 20 hpi, the majority of the leaves infected with *Pto*DC3000 or EtHAn carrying *avrRpt2* had collapsed. By 28 hpi, with the exception of EtHAn carrying an empty vector, all infected leaves had collapsed. This latter observation emphasizes one additional advantage of using EtHAn as opposed to wild-type *Pto*DC3000 for studying ETI. Because Pf0-1 is not adapted for survival within a plant, it is unable to elicit any visible symptoms in Arabidopsis, unlike wild-type *Pto*DC3000, which causes massive tissue collapse because of disease.

We next asked whether EtHAn carrying a single type-III effector gene would gain virulence. The type-III effector AvrPto has global effects on perturbing host PTI, because it interacts with PAMP receptors to dampen plant defense responses (Shan *et al.*, 2008; Xiang *et al.*, 2008). Transgenic plants overexpressing AvrPto support more growth of the Δ*hrcC* mutant of *Pto*DC3000 (Hauck *et al.*, 2003). These transgenics are also severely compromised in responding to PAMPs, as assayed by enumerating callose deposition (Hauck *et al.*, 2003). Finally, analysis of microarray data for host transcriptional changes suggested that the changes caused by AvrPto account for the majority of changes caused by wild-type *Pto*DC3000 delivering its entire set of type-III effectors (Hauck *et al.*, 2003). We therefore determined if expression of AvrPto in EtHAn would be sufficient to confer virulence to a soil bacterium (Figure 2). EtHAn carrying *avrPto* did not have any significant increase in growth relative to EtHAn carrying an empty vector. Thus, our growth enumeration studies not surprisingly indicated that a single type-III effector protein was not sufficient to confer virulence to a soil bacterium.

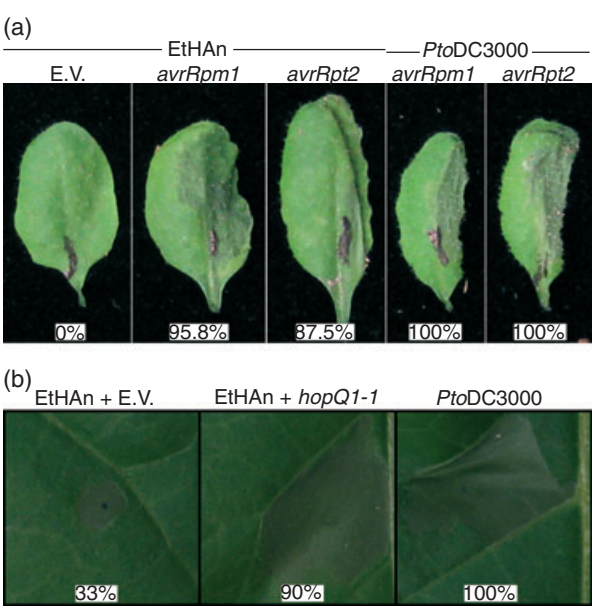

Figure 3. Effector-to-host analyzer (EtHAn) has a functional type-III secretion system.
(a) EtHAn and *Pto*DC3000 carrying *avrRpm1* and *avrRpt2* elicited a strong hypersensitive response (HR) in Arabidopsis Col-0. Twenty-four leaves were infiltrated for each strain, and the percentages of responding leaves are presented at the bottom of each panel. Experiments were repeated at least three times with similar results. Black lines denote infiltrated leaves.
(b) EtHAn carrying full-length *hopQ1-1* elicited an HR in *N. tabacum*. EtHAn by itself elicited a spotty and inconsistent HR. Eighteen leaves were infiltrated for each strain, and the percentages of responding leaves are shown. This experiment was repeated three times with similar results.

EtHAn expresses the T3SS and type-III effectors to high levels

Our design of EtHAn relied on the HrpL-controlled expression of T3SS and its type-III effector genes. Because proteins encoded by Pf0-1 are necessary to activate proteins upstream of HrpL, there is potential that EtHAn will not express type-III effector genes to native levels, as in *P. syringae*. We used quantitative real-time PCR (qRT-PCR) to measure the relative expression levels of a T3SS-encoding gene, *hrcV*, and the type-III effector gene *avrPto*. To ensure that expression levels were reflective of transcriptional regulation, and not copy number of the type-III effector gene, we used Tn7 to integrate a single copy of full-length *avrPto* into an intergenic region of the genome of Pf0-1 (Peters and Craig, 2001; Chang *et al.*, 2005).

As expected, *hrcV* and *avrPto* were expressed to high levels in *Pto*DC3000 7 h after the shift to *hrp*-inducing media, but were not significantly expressed in the Δ*hrpL* mutant (Figure 4a). EtHAn and EtHAn + *avrPto* expressed *hrcV* and *avrPto* in the latter strain, to higher levels than wild-type *Pto*DC3000 (Figure 4b). Note that expression is presented as relative to *Pto*DC3000: i.e. we would have expected a normalized -fold expression value of 1 if EtHAn expressed the HrpL-regulated genes to levels similar to *Pto*DC3000. By 24 h after shift, expression of both genes in *Pto*DC3000 was still detectable and significant, but far less than their levels at 7 h after shift to *hrp*-inducing media. In contrast, expression of both genes continued to increase in EtHAn and EtHAn + *avrPto*, respectively. We also noticed that expression of both genes was higher in EtHAn grown in KB media than in *Pto*DC3000 grown in KB media (data not shown). Together, these results indicate that HrpL-regulated genes are not under the same negative control as they are in *P. syringae*. Regardless of this observation, results indicate that EtHAn expresses HrpL-regulated genes to sufficient levels, compared with *P. syringae*.

Type-III effectors delivered by EtHAn are sufficient to dampen PTI

Next, we determined whether EtHAn is suitable for studying the virulence functions of type-III effector proteins. Several type-III effectors, including AvrPto and HopM1, have demonstrable roles in significantly suppressing the deposition of callose when expressed directly in transgenic plants (Hauck *et al.*, 2003; Nomura *et al.*, 2006; Fu *et al.*, 2007;

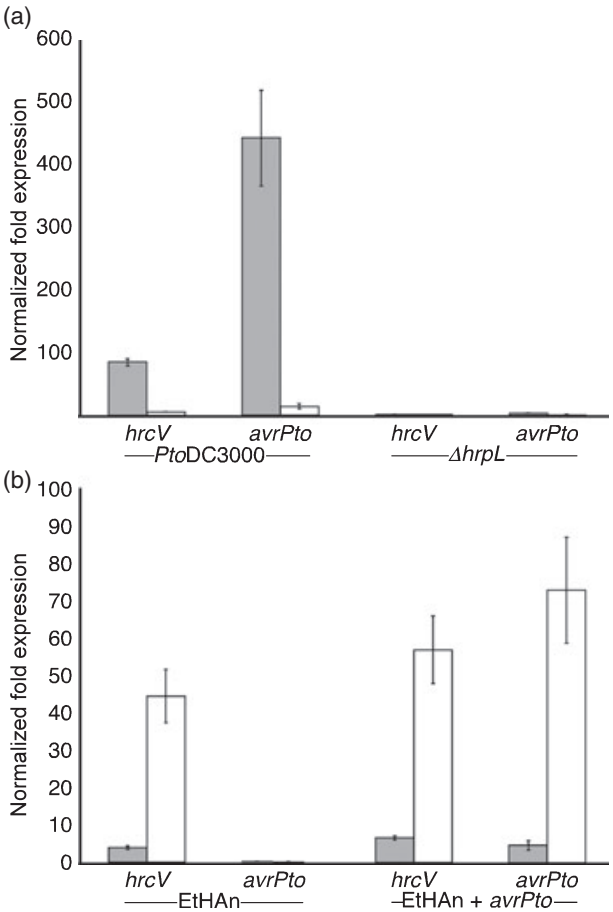

Figure 4. Expression of HrpL-regulated genes in effector-to-host analyzer (EtHAn).
(a) Normalized -fold expression of *hrcV* and *avrPto* in *Pto*DC3000 and Δ*hrpL* were measured using the efficiency-corrected $2^{-\Delta\Delta Ct}$ qRT-PCR method. Gene expression was normalized to 23S rRNA, and calculated relative to corresponding genes of cells grown in KB media. Expression was assessed at 7 (gray bars) and 24 h (white bars) after the shift to *hrp*-inducing media.
(b) The normalized -fold expressions of *hrcV* and *avrPto* in EtHAn or EtHAn carrying *avrPto* were measured at 7 (gray bars) and 24 h (white bars) after the shift to *hrp*-inducing media. Expression was normalized to 23S rRNA, and calculated relative to corresponding genes in *Pto*DC3000 grown in *hrp*-inducing media (a). All genes were measured in triplicate. The standard errors are shown.

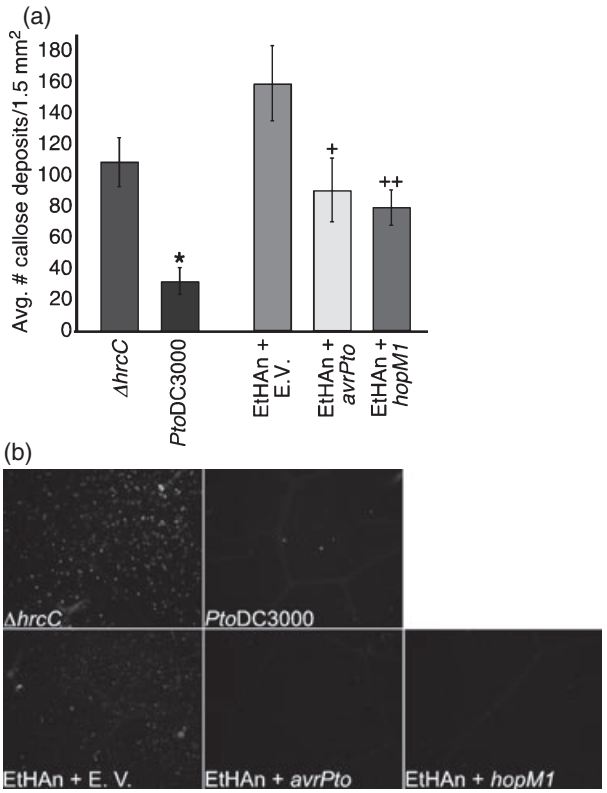

Figure 5. Effector-to-host analyzer (EtHAn) carrying *avrRpm1* or *hopM1* dampens the callose response.
(a) Average number of callose deposits per field of view (1.5 mm^2). Fifteen leaves were photographed per treatment, with 10 fields photographed per leaf. The significance of differences between averages were determined using a Student's *t*-test (*Pto*DC3000 versus *hrcC*, or EtHAn carrying *avrPto* or *hopM1* versus empty vector control); $^{*,+}P < 0.05$; $^{++}P < 0.01$. This experiment was repeated multiple times with similar results.
(b) Representative gray-scaled images of Arabidopsis leaves stained with aniline blue for callose deposition.

Underwood *et al.*, 2007). We therefore infiltrated leaves of Arabidopsis with EtHAn carrying *avrPto* or *hopM1*, and enumerated the number of callose deposits (Figure 5). We also enumerated the number of callose deposits in leaves infiltrated with *Pto*DC3000, its Δ*hrcC* mutant, and EtHAn carrying an empty vector, as controls. Representative leaf pictures are presented in Figure 5b.

As expected, the Δ*hrcC* mutant that is incapable of delivering type-III effectors was unable to suppress callose deposition, whereas *Pto*DC3000 significantly suppressed callose deposition. Infiltration of leaves with EtHAn without any type-III effector genes resulted in even more callose deposits than the Δ*hrcC* mutant. In contrast, EtHAn carrying *avrPto* or *hopM1* significantly and reproducibly suppressed the deposition of callose, as compared with EtHAn carrying an empty vector. These results indicate that EtHAn by itself elicits PTI, and that type-III effectors delivered by EtHAn are sufficient to dampen PTI. Thus, EtHAn is a suitable system for studying the virulence functions of type-III effector proteins *in planta*.

Delivery of type-III effectors can be generalized

The possibility that Pf0-1 cannot deliver all *P. syringae* type-III effectors is unlikely, given the observation that *P. fluorescens* 55 carrying pLN18 was sufficient to deliver all tested type-III effectors (Jamir *et al.*, 2004; Schechter *et al.*, 2004). Regardless, we tested approximately one half of the type-III effectors from *Pto*DC3000 for delivery by EtHAn. The type-III effectors were expressed as single-copy genes integrated via Tn7, and from their native promoters as translational fusions to *Δ79AvrRpt2* (Guttman *et al.*, 2002; Chang *et al.*,

2005). The tested type-III effectors AvrE1, AvrPto1, HopAF1, HopAB2, HopAM1-1, HopC1, HopE1, HopI1, HopP1, HopQ1-1, HopX1, HopY1, (ShcA1)-HopA1, (SchF2)-HopF2 and (SchO1)-HopO1 were all sufficient for EtHAn to elicit an HR in Arabidopsis, encoding the corresponding *R* gene, *Rps2*. For type-III effectors expressed from operons, we also included their upstream genes (included in parentheses). We therefore concluded that EtHAn can deliver most, if not all, type-III effectors of *P. syringae*.

EtHAn by itself elicits a defense response in species other than Arabidopsis

Because wild-type Pf0-1 does not encode any of its own type-III effector genes, we reasoned that EtHAn has potential applications for studying effectors in hosts other than Arabidopsis. We assayed the effects of EtHAn in leaves of tomato and *Nicotiana tabacum*. EtHAn by itself elicited a response in leaves of tomato (data not shown) and a spotty, inconsistent response in leaves of *N. tabacum* (Figure 3b). This response was specific to EtHAn; tomato or tobacco infiltrated with *P. fluorescens* Pf0-1 had no responses (data not shown). These results suggest the T3SS-encoding locus encodes a protein that may elicit a defense response in tomato or tobacco.

Nevertheless, we asked if EtHAn could deliver type-III effectors into leaves of *N. tabacum*. *Pto*DC3000 is not compatible with tobacco, because it delivers the perceived type-III effector HopQ1-1 and elicits ETI (Wei *et al.*, 2007). We therefore mobilized *hopQ1-1* into EtHAn and infiltrated the strain into leaves of tobacco (Figure 3b). After approximately 24 hpi, *Pto*DC3000 elicited a robust HR. The compatible pathogen *P. syringae* pv. *tabaci* 11528, in contrast, elicited strong tissue collapse because of disease, whereas its corresponding T3SS mutant (*ΔhrcV*) did not elicit a phenotype (data not shown). EtHAn carrying *hopQ1-1* elicited a strong HR similar to that of *Pto*DC3000, and more robust compared with that of EtHAn alone.

DISCUSSION

Our goal is to understand the mechanisms by which a single pathogen uses its entire collection of type-III effector proteins to dampen host defenses. One difficulty is that pathogens can deliver more than thirty different type-III effectors, of which many share overlapping functions (Kvitko *et al.*, 2009). The redundancy within a collection of type-III effectors is likely to be a consequence of the need to ensure robustness, so that the loss of single type-III effectors will not compromise virulence. However, this redundancy is clearly a significant challenge that we must overcome in order to understand the contributions of each type-III effector during host-association.

To address this hurdle, we developed a stable system for delivering type-III effector proteins into host cells. We moved a defined fragment of approximately 26 kb, spanning a region necessary to regulate and assemble the T3SS, into a modified mini-*Tn5* Tc vector. We used Tn5-mediated transposition to stably integrate the T3SS-encoding region directly into the genome of the soil bacterium, *P. fluorescens* Pf0-1, to make EtHAn. We show that when lacking type-III effector genes, EtHAn elicits PAMP-triggered immunity, and is non-pathogenic on Arabidopsis.

Potentially, EtHAn may be less efficient in delivering type-III effector proteins into host cells than pathogenic *Pto*DC3000, as was the case for *P. fluorescens* 55 carrying pLN18 (Schechter *et al.*, 2004). We did observe variability in the robustness of the HR between different type-III effector-Δ79AvrRpt2 fusions (data not shown). This is, however, consistent with previous results using *Pto*DC3000 to deliver fusions with Δ79AvrRpt2 or *P. fluorescens* carrying pLN18 to deliver type-III effector fusions to adenylate cyclase (Schechter *et al.*, 2004; Chang *et al.*, 2005). Furthermore, our reliance on native promoters may have contributed to variable expression. Nevertheless, when carrying type-III effector genes, EtHAn expressed and translocated sufficient quantites of their proteins into host cells to elicit ETI or dampen the PTI-associated deposition of callose. Therefore, EtHAn is a sufficient elicitor of PAMP-triggered immunity that can subsequently be engineered to study the roles of delivered type-III effectors in perturbing host defenses.

EtHAn offers several advantages over the currently used methods for studying plant–pathogen interactions. Because EtHAn is decorated with molecular patterns, and can be injected into the apoplastic space of plants, it can be used directly to study host defenses without the need to introduce additional PAMPs. We selected *P. fluorescens* Pf0-1 for modification, because it appears to be devoid of any endogenous type-III effector genes, as determined by surveying its completed genome sequence for homologous DNA sequences (Grant *et al.*, 2006). Furthermore, Pf0-1 appears to lack most, if not all, necessary virulence factors required for growth *in planta*. Thus, any observed phenotypes can be attributed to the delivered type-III effector protein of interest. Additionally, because the T3SS-encoding region is stably integrated, and because the type-III effector genes can also be stably integrated, EtHAn can be used to characterize type-III effector proteins *in planta* over the course of days.

EtHAn has applications beyond characterizing the type-III effector proteins of *P. syringae*. Fusions between oomycete effectors and the N-terminal domain of AvrRps4 or AvrRpm1 are delivered by *Pto*DC3000 directly into plant cells via its T3SS (Sohn *et al.*, 2007; Rentel *et al.*, 2008). However, in most cases the virulence functions of the oomyete effector will be masked by the functions conferred by the collection of type-III effectors normally delivered by *Pto*DC3000 (Sohn *et al.*, 2007). In contrast, as EtHAn is apparently devoid of virulence factors, the functions for effectors are more apt to be observed. EtHAn has been successfully used to deliver

the *Hyaloperonospora parasitica* effectors ATR1 and ATR13, fused to AvrRpm1, directly into Arabidopsis (B. Staskawicz, personal communication). Furthermore, *P. fluorescens* 55 carrying pHIR11 can deliver type-III effectors from *Xanthomonas* spp. (Fujikawa *et al.*, 2006). Although we did not explicitly reconfirm this finding, we expect EtHAn should behave similarly. EtHAn therefore has potential applications for characterizing virulence functions of other bacterial, as well as fungal and oomycete, effectors.

EtHAn has strong potential for studying type-III effector functions in Arabidopsis. EtHAn by itself does not elicit any symptoms in 88 different accessions of Arabidopsis (Q. Lu, M. Nishimura and J. Dangl, personal communication). Most notably, EtHAn does not elicit an HR in the Ws-0 accession, which is further evidence that our recombineering of the T3SS-encoding locus avoided inclusion of the type-III effector gene *hopA1* (Gassmann, 2005).

In contrast, however, in its current form, EtHAn may have limited use in other plant species. Our results suggest that a factor encoded by the T3SS-encoding locus elicits a weaker non-host defense response in tomato or tobacco. Alternatively, but less likely, is the possibility that the phenotype elicited by EtHAn is a consequence of the *Tn*5-mediated integration event into the genome of Pf0-1. Regardless of the cause, our observations are not in agreement with published reports showing that *P. fluorescens* Pf-55 carrying pHIR11 elicits an HR in tobacco, whereas the same strain carrying pLN18 (disruption in *hopA1*) does not (Jamir *et al.*, 2004; Schechter *et al.*, 2004). Therefore, we speculate that EtHAn elicits a defense response in tobacco because the T3SS is stably integrated and expressed at higher levels than in pathogenic *P. syringae*.

Our results highlight the utility of recombineering for manipulating DNA fragments. This method has been used to delete genes directly from the genome of the plant pathogen *Erwinia amylovora*, but we have not had success with recombineering directly in *P. syringae* (Zhao *et al.*, 2009; JHC, unpublished data). Nevertheless, we demonstrate that recombineering can be used to alter large and otherwise challenging to manipulate fragments of DNA in *Escherichia coli*. Other potential applications include modifying large genomic clones or plasmids, such as binary vectors (Rozwadowski *et al.*, 2008). To request EtHAn, please visit our webpage at: http://changlab.cgrb.oregonstate.edu/.

EXPERIMENTAL PROCEDURES

Bacterial strains, plant lines and growth conditions

The bacterial strains used in this study are *Pto*DC3000, its T3SS structural mutant Δ*hrcC* (Yuan and He, 1996), its T3SS regulatory mutant Δ*hrpL* (Zwiesler-Vollick *et al.*, 2002) and *P. fluorescens* Pf0-1 (Silby *et al.*, 2009), *E. coli* DH5α and HB101λpir. Pseudomonads were grown at 28°C in King's B (KB) liquid media with shaking, or on KB agar plates. For *in vitro* induction of HrpL-regulated genes, Pseudomonads were grown overnight in KB media, washed and resuspended at OD_{600} = 0.1 in *hrp*-inducing media, and were then grown for 7 h or 24 h (Huynh *et al.*, 1989). *E. coli* were grown at 37°C in Luriae-Bertani (LB) liquid media with shaking, or on LB agar plates. Antibiotics were used at the final concentrations of: 25 μg ml^{-1} rifampicin, 30 μg ml^{-1} kanamycin (100 μg ml^{-1} for Pf0-1), 30 μg ml^{-1} chloramphenicol, 5 μg ml^{-1} tetracycline (50 μg ml^{-1} for Pf0-1), 25 μg ml^{-1} gentamycin (100 μg ml^{-1} for Pf0-1) and 100 μg ml^{-1} ampicillin. Concentrations listed were for *P. syringae* and *E. coli*, unless otherwise noted.

Arabidopsis thaliana accession Col-0 and *N. tabacum* were grown in a controlled-environment growth chamber (9-h day, at 22°C; 15-h night, at 20°C) for 5 to 6 weeks.

Plasmid constructions

The plasmids used were pBBR1-MCS1, -MCS2 and -MCS5 (Kovach *et al.*, 1994, 1995), pRK2013 (Figurski and Helinski, 1979), mini-*Tn5* Tc (de Lorenzo *et al.*, 1990), pKD4 and pKD46 (Datsenko and Wanner, 2000), pBH474 (House *et al.*, 2004), pME3280a (Zuber *et al.*, 2003), pUX-BF13 (Bao *et al.*, 1991), and pLN18 (Jamir *et al.*, 2004). The oligonucleotides used are listed in Table S1. All restriction enzymes were purchased from New England Biolabs (NEB, http://www.neb.com).

We first used recombinant and sticky-end PCR to fuse together two 0.5 kb-sized fragments flanking the T3SS-encoding region carried on pLN18 (Jamir *et al.*, 2004). A 0.5 kb fragment immediately downstream of *hrpK* was amplified in two separate reactions using Pfu and primer pairs JHC124 + JHC125 or JHC148 + JHC150 (fragments 1 and 1′ of Figure 1, respectively). A 0.5 kb fragment immediately upstream of *hrpH* (aka ORF1 of conserved effector locus; Alfano *et al.*, 2000) was amplified in two separate reactions with primer pairs JHC126 + JHC128 or JHC151 + JHC128 (fragments 2 and 2′, respectively). The products were gel-purified. Fragments 1 + 2 and 1′ + 2′ were mixed in approximately equal ratios, and each were amplified in two separate reactions using Pfu and primer pairs JHC123 + JHC125 and JHC124 + JHC127, or JHC148 + JHC128 and JHC149 + JHC152, respectively. Recombined products carried an inserted unique *Xba*I or *Acc*65I site, respectively. Corresponding approximately 1.0 kb products were mixed in approximately equal ratios, incubated at 95°C for 5 min and were then slowly cooled to room temperature, leading to a proportion of the PCR products with overhanging ends compatible with recipient vectors.

Sticky-end products derived from the 1 + 2 fusion were gel-purified and cloned into pBBR1-MCS5 cleaved with *Eco*RI + *Xho*I, and subsequently subcloned into pBBR1-MCS1 cleaved with *Sac*I + *Xho*I (Kovach *et al.*, 1994, 1995). Sticky-end products from the 1′ + 2′ fusion were gel-purified and cloned into mini-*Tn5* Tc cleaved with *Not*I, and treated with calf-intestinal phosphatase (CIP).

Recombineering

We digested pBBR1-MCS1 carrying the 1 + 2 fusion with *Xba*I and transformed the gel-purified, linear fragment into electrocompetent cells made from arabinose-induced (10 mM) DH5α cells carrying pKD46 and pLN18 (de Lorenzo *et al.*, 1990; Jamir *et al.*, 2004). Transformants were selected on chloramphenicol. Successful transfer of the entire ~26 kb T3SS-encoding region was confirmed by restriction digestion and PCR with pairs of primers that amplified different fragments along the length of the T3SS-encoding region.

We next cleaved mini-*Tn5* Tc carrying the 1′ + 2′ fusion with *Acc*65I, and transformed the gel-purified, linear fragment into electrocompetent cells made from arabinose-induced (10 mM) HB101λpir cells carrying pKD46 and pBBR1-MCS1 with the T3SS-encoding region. Transformants were selected on tetracycline. Successful transfer of the T3SS-encoding region was confirmed by

restriction digestion and PCR, with pairs of primers that amplified different fragments along the length of the T3SS-encoding region.

We also used recombineering to replace the *Tet*^{R} gene of mini-*Tn5* Tc vector with *Kan*^{R}, flanked by site-specific recombinase FRT sequences. We used a two-step PCR to amplify *Kan*^{R} flanked by FRT sites from pKD4 with CAT0001 and CAT0002, and subsequently with CAT0003 and CAT0004, to include 50 bp of homology to either side of the *Tet*^{R} gene of mini-*Tn5* Tc (de Lorenzo *et al.*, 1990). Recombineering was performed as described above. Transformants were selected on kanamycin, and successful recombineering was confirmed via PCR and lack of growth on LB + Tet.

Plasmid mobilization

Plasmids were mobilized into recipients, as previously described (Chang *et al.*, 2005). For mobilizing *Tn*7-based vectors, cells were mixed at a ratio of 10:1:1:1, with the latter being *E. coli* carrying pUX-BF13 (Bao *et al.*, 1991). Integration of genes into the genome of *P. fluorescens* Pf0-1 was confirmed using PCR. Eviction of *Kan*^{R} was mediated by pBH474, which encodes the FLP site-specific recombinase, and was confirmed by replica plating on KB agar plates with and without kanamycin (House *et al.*, 2004). Finally, cells resistant to 5% sucrose were selected to identify those that lost pBH474.

Quantitative real-time PCR (qRT-PCR)

Cells were collected and immediately suspended in RNAprotect (Qiagen, http://www.qiagen.com), and then stored at −80°C. RNA was extracted using RNeasy following the instructions of the manufacturer (Qiagen), and was subsequently treated with Dnase I (NEB). Qualities of each RNA preparations were assessed on 1.0 × FA, 1.2% agarose gels. We measured the quantity of RNA using a Nanodrop ND-1000 (Thermo Scientific, http://www.thermo.com).

Total RNA (1 μg) from each sample was used to synthesize single-stranded cDNA according to the instructions of the manufacturer (Superscript III; Invitrogen, http://www.invitrogen.com). We used reverse transcriptase PCR to determine the quality of the cDNA. We used oligonucleotides 23S-T and 23S-B to amplify 23S rRNA from 1.0 ng of each sample. We also used hrpEF-T and hrpEF-B oligonucleotides in separate reactions to confirm the complete removal of DNA from each sample. These oligonucleotides span the intergenic region of separately transcribed genes *hrpE* and *hrpF* (Rahme *et al.*, 1991).

Single-stranded cDNA (1 ng) was used in SYBR-Green qRT-PCR on a CFX96 real-time machine (Bio-Rad, http://www.bio-rad.com). Oligonucleotides hrcV-T and hrcV-B, as well as avrPto-T and avrPto-B, were used to amplify portions of *hrcV* and *avrPto*, respectively. All reactions were performed in triplicate and normalized to 23S rRNA. To calculate efficiencies for each primer pair, we carried out qRT-PCR on 1.0, 0.1 and 0.01 ng of single-stranded cDNA made from *Pto*DC3000 grown in *hrp*-inducing media for 24 h. Efficiency-corrected $2^{-\Delta\Delta Ct}$ values were determined using the CFX MANAGER software (Bio-Rad; Pfaffl, 2001).

In planta assay

Bacterial cells were grown overnight in KB with appropriate antibiotics, washed and resuspended in 10 mM $MgCl_2$. For the *in planta* growth assay, we resuspended *P. syringae* and *P. fluorescens* to an OD_{600} of 0.002 (1.0×10^6 cfu ml^{-1}). We used 1.0-ml syringes lacking needles to infiltrate bacterial suspensions into the abaxial side of leaves of 5 to 6-week-old plants. We used a number-2 cork borer to core four discs for each triplicate of each treatment at 0 and 3 days post inoculation (dpi). Leaf discs were ground to homogeneity in 10 mM $MgCl_2$, serially diluted and plated on KB with appropriate antibiotics. Colonies were enumerated once visible. Experiments were repeated at least three times. For HR assays, cells were infiltrated in leaves at an OD_{600} of 0.2 (equivalent to 1.0×10^8 cfu ml^{-1}). Phenotypes were scored starting at 6 hpi, and were examined up until disease symptoms were visible.

Callose staining

We collected leaves at 7 hpi, and cleared them of chlorophyll by heating at 65°C in 70% EtOH. Leaves were then rinsed and stained in aniline blue staining solution (150 mM K_2HPO_4, 0.1% aniline blue, pH 8.4) overnight. Leaves were placed in 70% glycerol containing 1.0% of the aniline blue staining solution, and were then mounted onto microscope slides. We viewed the leaves using a light microscope (BX40; Olympus, http://www.olympus-global.com), with a filter under ultraviolet 10 × magnification. Digital images of 10 fields per leaf were taken (Magnafire; Optronics, http://www.optronics.com), and 15 leaves were imaged per treatment. We used a custom PERL script to enumerate callose deposits.

ACKNOWLEDGEMENTS

We thank Mark Silby and Stuart Levy for *P. fluorescens* Pf0-1, Brenda Schroeder and Michael Kahn for pBH454, Dieter Haas for pME3280a and pUX-BF13, Sheng Yang He for Δ*hrcC*, Brian Staskawicz for Δ*hrpL*, and Alan Collmer for pLN18 and the Δ*hrcV* mutant of *Pta* 11528. We thank Jeffery Dangl, Jason Cumbie, Rebecca Pankow and Philip Hillebrand for their assistance and critical reading of this manuscript. We gratefully acknowledge Jim Carrington for the use of his light microscope. Finally, we thank Ethan S. Chang for his assistance and inspiration in the naming of the type-III effector delivery system. This research was supported in part by start-up funds from Oregon State University to JHC, and by a grant from the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service (grant 2008-35600-18783).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Oligonucleotides used in this study.

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