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

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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 \textit{hrp}/\textit{hrc} region from \textit{Pseudomonas syringae} pv. \textit{syringae} 61 into the genome of the soil bacterium \textit{Pseudomonas fluorescens} Pf0-1. We describe our development of Effector-to-Host Analyzer (EtHAn), and demonstrate its utility for studying effectors for their \textit{in planta} functions.

Keywords: type-III secretion system, \textit{hrp}/\textit{hrc}, type-III effectors, plant defense, \textit{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 \textit{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 \textit{Pseudomonas syringae}, the T3SS-encoding genes of the \textit{hrp}/\textit{hrc} region are located in a 26-kb pathogenicity island (Huang \textit{et al}., 1988; Alfano \textit{et al}., 2000; Oh \textit{et al}., 2007). Central to its expression is the alternative sigma factor HrpL (Xiao \textit{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 \textit{hrp}-box (Linnes \textit{et al}., 1993; Fouts \textit{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 \textit{in planta} or \textit{in vitro} in \textit{hrp}-inducing media (Huynh \textit{et al}., 1989; Rahme \textit{et al}., 1992; Xiao \textit{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).

Germame 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 (ProDC3000) elicits ETI in tobacco, and is a negative host range determinant; its deletion from the genome enables PtoDC3000 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 (PtoT1) was completed and compared with the completed genome sequence of PtoDC3000 (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 S5, 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 (Psy61), 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 PtoDC3000 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 Psy61, 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
We also used the site-specific recombinase FLP to excise the \textit{hrpK} gene from the genome of \textit{Pf0-1}. Through these modifications, 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 \textit{avrRpm1} or \textit{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 \textit{et al.}, 1993; Yu \textit{et al.}, 1993; Biggrove \textit{et al.}, 1994).

We infiltrated 1.0 \texttimes 10^6 cfu ml\(^{-1}\) of EtHAn carrying \textit{avrRpm1} or \textit{avrRpt2} into leaves of Arabidopsis (Figure 3a).

\textit{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 \texttimes 10^6 cfu ml\(^{-1}\) of wild-type Pf0-1 and EtHAn into leaves of Arabidopsis and enumerated their growth. The compatible pathogen \textit{PtoDC3000} grew extensively in planta, and its T3SS mutant (\textit{ΔhrpC}) failed to grow significantly. Pf0-1 and EtHAn failed to grow, even to levels comparable with the \textit{ΔhrpC} 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.

\textbf{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 \textit{avrRpm1} or \textit{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 \textit{et al.}, 1993; Yu \textit{et al.}, 1993; Biggrove \textit{et al.}, 1994).

We infiltrated 1.0 \texttimes 10^6 cfu ml\(^{-1}\) of EtHAn carrying \textit{avrRpm1} or \textit{avrRpt2} into leaves of Arabidopsis (Figure 3a).

\begin{figure}[h]
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\caption{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 \textit{hpl} boxes. Some protein-coding regions are labeled for landmarks: A1, \textit{shcA1-hopA1}; K, \textit{hpK}; R, \textit{hpR}; H, \textit{hpH}; E, \textit{avrE1}. The \textit{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, 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 \textit{TetR} gene with the \textit{Kan}\(^R\) gene flanked by FRT sites (darker gray bars with a horizontal dashed line). The resulting DNA fragment between the \textit{Tn5}-repeats (I and O ends) is shown at the bottom. The orientation of the T3SS-encoding region relative to the \textit{Tn5} repeats is unknown and arbitrarily presented in one direction. The entire fragment was stably integrated into the genome of \textit{P. fluorescens} Pf0-1 via \textit{Tn5} transposition, and the \textit{Kan}\(^R\) gene was excised using the site-specific recombinase FLP (not shown).}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Enumeration of bacterial growth in planta. Wild-type \textit{PtoDC3000}, a \textit{ΔhrpC} mutant, Pf0-1, EtHAn carrying an empty vector (E.V.) and EtHAn carrying \textit{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.}
\end{figure}
At 6 hpi, the majority of leaves infected with PtoDC3000 or EthAn carrying avrRpm1, showed visible HR phenotypes. By 20 hpi, the majority of the leaves infected with PtoDC3000 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 PtoDC3000 for studying ETI. Because Pto-1 is not adapted for survival within a plant, it is unable to elicit any visible symptoms in Arabidopsis, unlike wild-type PtoDC3000, 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 ΔhrcC mutant of PtoDC3000 (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 PtoDC3000 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.

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 Pto-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 Pto-1 (Peters and Craig, 2001; Chang et al., 2005).

As expected, hrcV and avrPto were expressed to high levels in PtoDC3000 7 h after the shift to hrp-inducing media, but were not significantly expressed in the Δhrlp mutant (Figure 4a). EthAn and EthAn + avrPto expressed hrcV and avrPto in the latter strain, to higher levels than wild-type PtoDC3000 (Figure 4b). Note that expression is presented as relative to PtoDC3000: i.e. we would have expected a normalized -fold expression value of 1 if EthAn expressed the HrpL-regulated genes to levels similar to PtoDC3000. By 24 h after shift, expression of both genes in PtoDC3000 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 PtoDC3000 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;
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 _DhrcC_ mutant, and EtHAn carrying an empty vector, as controls. Representative leaf pictures are presented in Figure 5b.

As expected, the _DhrcC_ 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 _DhrcC_ 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_ S5 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, (SchF3)-HopA1, (SchF2)-HopF2 and (SchO1)-HopD1 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*. *PtoDC3000* 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, *PtoDC3000* 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 *PtoDC3000*, 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 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 *PtoDC3000*, 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 *PtoDC3000* 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 quantities 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 *PtoDC3000* directly into plant cells via its T3SS (Sohn *et al.*, 2007; Rentel *et al.*, 2008). However, in most cases the virulence functions of the oomycete effector will be masked by the functions conferred by the collection of type-III effectors normally delivered by *PtoDC3000* (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 confirm this finding, we expect EhAn should behave similarly. EhAn therefore has potential applications for characterizing virulence functions of other bacterial, as well as fungal and oomycete, effectors.

EhAn has strong potential for studying type-III effector functions in Arabidopsis. EhAn by itself does not elicit any symptoms in 88 different accessions of Arabidopsis (Q. Lu, M. Nishimura and J. Dangl, personal communication). Most notably, EhAn 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, EhAn 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 EhAn is a consequence of the Tn5-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 EhAn 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 EhAn, 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 PtoDC3000, its T3SS structural mutant JhrcC (Yuan and He, 1996), its T3SS regulatory mutant JhrlFl (Qwiesler-Vollick et al., 2002) and P. fluorescens Pf0-1 (Silby et al., 2009). E. coli DH5α and HB101(ppir). 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 OD600 = 0.1 in hrp-inducing media, and were then grown for 7 h or 24 h (Huyhn et al., 1989). E. coli were grown at 37°C in Luria-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 (Datensko and Waner, 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 + JHC151 (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 XbaI or Acc65I 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 EcoRI and XhoI, and subsequently subcloned into pBBR1-MCS1 cleaved with SalI and XhoI (Kovach et al., 1994, 1995). Sticky-end products from the 1’ + 2’ fusion were gel-purified and cloned into mini-Tn5 Tc cleaved with NotI, and treated with calf-intestinal phosphatase (CIP).

Recombineering

We digested pBBR1-MCS1 carrying the 1 + 2 fusion with XbaI and transformed the gel-purified, linear fragment into electrocompetent cells made from arabinoose-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 Acc65I, and transformed the gel-purified, linear fragment into electrocompetent cells made from arabinoose-induced (10 mM) HB101(ppir) 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 TSSS-encoding region.

We also used recombineering to replace the Tet<sup>+</sup> gene of mini-Tn5 Tc vector with Kan<sup>R</sup>, flanked by site-specific recombinase FRT sequences. We used a two-step PCR to amplify Kan<sup>R</sup> 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<sup>+</sup> 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., 2006). For mobilizing Tn7-based vectors, cells were mixed at a ratio of 10:1:1, 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<sup>R</sup> 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 preparation 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 hrfEF-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 hreP 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 hrvc-T and hrvc-B, as well as avrPto-T and avrPto-B, were used to amplify portions of hrvc 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 PtoDC3000 grown in hrp-inducing media for 24 h. Efficiency-corrected 2<sup>−ΔΔCT</sup> 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<sub>2</sub>. For the in planta growth assay, we resuspended P. syringae Pov<sub>B</sub> and P. fluorescens to an OD<sub>600</sub> of 0.002 (1.0 × 10<sup>8</sup> cfu ml<sup>−1</sup>). 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 replicate of each treatment at 0 and 3 days post inoculation (dpi). Leaf discs were ground to homogeneity in 10 mM MgCl<sub>2</sub> 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<sub>600</sub> of 0.2 (equivalent to 1.0 × 10<sup>8</sup> cfu ml<sup>−1</sup>). 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<sub>2</sub>HPO<sub>4</sub>, 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.

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**SUPPORTING INFORMATION**

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

Table S1. Oligonucleotides used in this study. Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

**REFERENCES**


Stable integration of a T3SS-locus into Pf0-1


