Detection and Functional Characterization of a 215 Amino Acid N-Terminal Extension in the *Xanthomonas* Type III Effector XopD

Joanne Canonne¹, Daniel Marino¹, Laurent D. Noël¹, Ignacio Arechaga³, Carole Pichereaux², Michel Rossignol², Dominique Roby¹, Susana Rivas¹*

¹Laboratoire des Interactions Plantes-Micro-organismes (LIPM), UMR CNRS-INRA 2594/441, Castanet Tolosan, France, ²Institut Fédératif de Recherche (IFR40), Plateforme protéomique Génopole Toulouse Midi-Pyrénées, Institut de Pharmacologie et Biologie Structurale, Université de Toulouse, Toulouse, France, ³Departamento de Biología Molecular, Universidad de Cantabria (UC) and Instituto de Biomedicina y Biotecnología de Cantabria, IBBTEC (CSIC-UC-IDICAN), Santander, Spain

Abstract

During evolution, pathogens have developed a variety of strategies to suppress plant-triggered immunity and promote successful infection. In Gram-negative phytopathogenic bacteria, the so-called type III protein secretion system works as a molecular syringe to inject type III effectors (T3Es) into plant cells. The XopD T3E from the strain 85-10 of *Xanthomonas campestris* pathovar vesicatoria (Xcv) delays the onset of symptom development and alters basal defence responses to promote pathogen growth in infected tomato leaves. XopD was previously described as a modular protein that contains (i) an N-terminal DNA-binding domain (DBD), (ii) two tandemly repeated EAR (ERF-associated amphiphilic repression) motifs involved in transcriptional repression, and (iii) a C-terminal cysteine protease domain, involved in release of SUMO (small ubiquitin-like modifier) from SUMO-modified proteins. Here, we show that the XopD protein that is produced and secreted by *Xcv* presents an additional N-terminal extension of 215 amino acids. Closer analysis of this newly identified N-terminal domain shows a low complexity region rich in lysine, alanine and glutamic acid residues (KAE-rich) with high propensity to form coiled-coil structures that confers to XopD the ability to form dimers when expressed in *E. coli*. The full length XopD protein identified in this study (XopD¹⁻⁷⁶⁰) displays stronger repression of the XopD plant target promoter *PR1*, as compared to the XopD version annotated in the public databases (XopD²¹⁶⁻⁷⁶⁰). Furthermore, the N-terminal extension of XopD, which is absent in XopD²¹⁶⁻⁷⁶⁰, is essential for XopD type III-dependent secretion and, therefore, for complementation of an *Xcv* mutant strain deleted from XopD in its ability to delay symptom development in tomato susceptible cultivars. The identification of the complete sequence of XopD opens new perspectives for future studies on the XopD protein and its virulence-associated functions in planta.

Introduction

Plants have developed a complex defence network to fight off invading pathogens. The first layer of plant defence involves recognition of PAMPs (Pathogen-Associated Molecular Patterns), defined as invariant epitopes within molecules that are fundamental to pathogen fitness and widely distributed among different microbes [1]. This recognition, previously known as basal defence, is now referred to as PAMP-triggered immunity (PTI) [2]. PTI is associated to the production of reactive oxygen species and antimicrobial compounds, the induction of mitogen-activated protein kinase (MAPK) cascades, the modulation of host gene transcription and the deposition of lignin and callose at the plant cell wall [3–6]. Some bacterial pathogens evolved to suppress PTI and promote successful infection by injecting T3Es (type III effectors) into plant cells using the type III protein secretion system (T3SS) [7–9]. In turn, plants acquired the ability to recognize directly or indirectly effectors through resistance (R) proteins. This recognition response is associated with the long-standing gene-for-gene hypothesis, and more recently with the guard hypothesis [10], and is now known as effector-triggered immunity (ETI). Several T3Es from Gram-negative phytopathogenic bacteria have also been shown to suppress ETI [11], suggesting that T3Es may have multiple targets or that they target shared components between PTI and ETI.

The coevolutionary arms race between plants and pathogens has generated highly polymorphic repertoires of R proteins and T3Es. A significant number of T3Es from different bacteria has been identified during the last few years. Known biochemical activities of T3Es include manipulation of host protein turnover, either through protease activity [12–14] or protein degradation via the 26S proteasome [15–17], modification of host transcription or RNA stability [18–21] and alteration of the phosphorylation state of plant proteins [22–27].
Gram-negative pathogenic bacteria of the genus Xanthomonas infect a wide range of host plants and are responsible for important crop plant diseases. Xanthomonas campestris pathovar vesicatoria (Xcv, also known as Xanthomonas axonopodis pathovar vesicatoria or Xanthomonas euvesicatoria [28,29]) is the causal agent of bacterial spot on tomato (Solanum lycopersicum) and pepper (Capsicum annum) [30]. The T3SS of Xcv is encoded by the chromosomal hrp (HR and pathogenicity) gene cluster, which contains 25 genes [31,32]. hrp gene expression is activated during plant infection or when bacteria are incubated in special minimal media by two regulatory proteins, HrpG and HrpX. HrpG is a member of the OmpR family of two-component system response regulators and controls the expression of a genome-wide regulon including hrpX [33,34]. HrpX is an AraC-type transcriptional activator that binds to a conserved DNA motif [plant-inducible promoter (PIP) box; consensus: TTGCG-N15-TTTCGC], which is present in the promoter regions of most hrpX-regulated genes [33,35].

T3SS-dependent secretion of a protein is mediated by an N-terminal secretion signal within its first 15–20 residues. The presence of hydrophilic amino acids, absence of acidic residues in the first 12 amino acids, amphipathicity and a bias towards serine and glutamine in the first 50 residues are common features of the N-terminal sequence of T3Es [36–39]. In addition to the secretion signal, effector proteins contain a 1–50 to 1–100 amino acid region at their N-terminus that is required for translocation across the eukaryotic plasma membrane [40].

The presence of a variety of putative structural motifs in the primary sequence of Xanthomonas T3Es has provided insights into their putative biochemical function [41]. The XopD T3E from Xcv 85-10 has been classified as a member of the C48 protease family, and it has been shown to release SUMO (small ubiquitin-like modifier) from SUMO-modified plant proteins [42,43]. XopD is a modular protein that contains (i) an N-terminal DNA-binding domain (DBD), (ii) two tandemly repeated EAR (ERF-associated amphiphilic repression) motifs [L/FDLNL/F(X)P] [44], previously described in plant transcriptional repressors that negatively regulate gene transcription during stress and defence responses [45], and (iii) a C-terminal cysteine protease domain with structural similarity with the yeast ubiquitin-like protease 1 (ULP1) [42]. XopD is localized in nuclear foci indicating that host targets are likely nuclear SUMOylated proteins [43]. Intriguingly, consistent with its protein structure, XopD has been reported to be a non-specific DNA-binding protein that represses plant gene transcription, delaying the onset of symptom development and altering basal defence and cell death responses, which in turn promotes pathogen growth in infected leaves [46]. However, as for many of the known bacterial effectors, XopD direct plant targets remain unknown.

XopD was previously predicted to code for a protein of 612 amino acids [47]. However, secretion assays and Western blot analysis, following expression of c-myc-tagged XopD within its native Xcv chromosomal environment, allowed the detection of a unique band of a molecular mass close to 100 kDa [47]. Importantly, this protein was undetectable in culture supernatants of T3SS mutant bacteria cultured in secretion medium, demonstrating that this protein is secreted in a T3SS-dependent manner. In contrast, the XopD protein sequence annotated in the public databases predicts a protein of 345 amino acids with an expected molecular mass of 61 kDa. Noel and co-workers could not detect a (consensus) PIP box in the xopD promoter, but rather a putative hp box, which is found in all hrp/-dependent promoters in Pseudomonas syringae and Erwinia spp [47–49]. Later inspection of the xopD promoter region identified the presence of a PIP box (ATTCGCG-N15-TTTCGC), bound by HrpX, and a −10 putative sequence (TAATT), which are situated 747 and 690 bp upstream the annotated start, respectively (Figure 1A) [55]. In agreement with these observations, the HrpX-dependent expression of XopD has been confirmed experimentally [35,47].

In the view of these conflicting data, we set out to identify the starting amino acid of XopD and thereby determine the sequence of the XopD protein that is produced and secreted from Xcv. Our work confirms that the XopD protein sequence previously annotated in the databases is incomplete. We demonstrate that the functional protein expressed by Xcv presents a previously overlooked N-terminal extension of 215 amino acids that is essential for its T3SS-dependent secretion and virulence-associated functions in the plant cell.

**Results**

**Sequence analysis of the xopD locus**

In bacteria, the most frequently used codon for the initiation of translation is the triplet AUG, although GUG and, occasionally, UUG may also be used. The meaning of the GUG, or the rarely used UUG, codon depends on their context. When present within a gene, they provide valine (V) or leucine (L), as specified by the genetic code. However, present as first codons, they mediate the incorporation of methionine (M). Close inspection of the Xcv 85-10 genomic sequence neighbouring the annotated xopD gene shows that upstream of the annotated starting codon AUG, there are 6 AUG, 1 GUG and 5 UUG additional codons that are in frame and may potentially be used to start translation of the protein (Figure 1C). In addition, since HrpX-dependent expression of XopD was previously demonstrated, the presence of a PIP box and a −10 sequence upstream of the first putative starting codon suggested that translation of XopD may start immediately downstream these regulatory elements (Figure 1A).

BlastP analysis using the Xcv 85-10 XopD sequence starting at the first possible translation start (UUG, encoding or not a M residue depending on whether it is or not the translation start) identified three major hits corresponding to (i) a hypothetical, not yet characterized, protein from the strain B100 of Xanthomonas campestris pathovar campestris (Xcc) [50], (ii) a protein from Acidovorax avenue, annotated as peptidase C48 SUMO/Sentrin/Ub11, and (iii) two virulence proteins that correspond to a shorter version of XopD deleted from its DBD, from the Xcv strains 8004 [51] and ATCC 33913 [52] (Figure 1B). In addition, sequencing of xopD using genomic DNA from the Xcv 147 strain [53] showed that this XopD protein is very similar to the one present in Xcv strains 8004 and ATCC 39913 (Figure 1B,C). It is noteworthy that the Met residue previously annotated as XopD starting amino acid is not conserved in Xcv B100 (Figure 1C; red arrow), suggesting that this may not be a starting amino acid for XopD. In contrast, the putative N-terminal extension of XopD is well conserved in both A. avenue and Xcv B100, indicating that it may be important for XopD function (Figure 1C).

**T3SS-dependent secretion of an 85 kDa XopD protein from Xcv**

To allow detection of the XopD protein that is produced by Xcv from its native promoter, Xcv genomic DNA was tagged with an HA epitope at the 3′ end of xopD coding sequence using a suicide vector. XopD protein expression was analyzed in strains 85*, which allows constitutive expression of all hrp genes [54], and 85* ΔhrpV, which carries a deletion in a conserved component of the T3SS [55]. Western blot analysis of bacterial total protein extracts with an anti-HA antibody did not allow detection of any band at ~61 kDa, which is the predicted molecular mass of the annotated...
Figure 1. In silico analysis of the xopD locus. (A) The xopD locus in Xcv 85-10 is shown (the genome interval for the Xcv 85-10 shown sequence and the xopD gene are respectively: 486209–489200 and 486544–488826). Open arrows indicate ORFs and filled arrows show promoter elements, such as the PIP box and the −10 sequence. The position of XopD translation start annotated in the public databases is indicated by an asterisk. (B) Schematic representation of XopD functional domains in Xcv 85-10, in comparison to its known protein homologues. The N-terminal extension, essential V and L residues in the DBD, tandemly repeated EAR motifs, conserved catalytic residues in the cysteine protease domain, and NLS motif are shown. (C) Sequence alignment of XopD from Xcv 85-10, hypothetical protein from Xcc B100 (YP_001902662), virulence protein from Xcc 8004 (AYA48282) or Xcc ATCC 33913 (AAM42168), peptidase C48 SUMO from Acidovorax avenae (EFA39722) and XopD from Xcc 147. The figure shows the longest ORF possible for all proteins. For XopD from Xcv, the first shown M residue corresponds to a UUG codon, not conserved among Xcc B100 and A. avenae coding sequences. The M residue previously annotated as the starting amino acid of the XopD protein is indicated by a red arrowhead. Putative translation starts situated in frame and upstream the previously annotated starting M are also indicated as follows: translation starts conserved among Xcv 85-10, Xcc B100 and Aea are indicated with a full red dot, otherwise, they appear indicated by an empty red dot. Yellow box: putative N-terminal extension; green box: DNA-binding domain (V and L residues in the helix-loop-helix domain essential for maximal DNA-binding [46] are indicated by an empty green dot); red boxes: tandemly-repeated EAR motifs [L/FDNLL/F(X)P] [44]; black box: SUMO protease domain (H, D and C catalytic core residues are indicated by a black empty dot); blue box: NLS. Gray and black highlighting of amino acids indicates, respectively, 70–80% and 90–100% of similarity.

doi:10.1371/journal.pone.0015773.g001
XopD protein (Figure 2). In contrast, a unique band of ~85 kDa was detected (Figure 2, lane 1), strongly suggesting that (i) there is a unique start for XopD translation and that (ii) the protein that is produced by Xcv presents a significant extension at the N-terminus of the annotated protein. No HA-tagged protein could be detected in the untransformed strain 85* (Figure 2, lane 2).

T3SS-dependent secretion of XopD was next tested after incubation of bacteria in secretion medium. A single band of ~85 kDa was detected in culture supernatants of the 85* strain (Figure 2, lane 1). XopD was not detectable in culture supernatants of T3SS mutant 85* Δhrv (Figure 2, lane 3), reflecting the presence of a functional T3SS secretion signal in the ~85 kDa form of XopD. No protein could be detected in the supernatant fractions with an antibody against the intracellular chaperone GroEL, demonstrating that detection of proteins in the supernatant was not due to bacterial lysis (Figure 2).

Since the expected molecular mass for the longest possible ORF (UUG codon; Figure 1C) is 86 kDa, this analysis shows that the molecular mass of the XopD protein produced and secreted by Xcv 85* in a T3SS-dependent manner is consistent with the protein starting at one of its very first putative translation starts.

Identification of a 215 amino acid N-terminal extension in XopD using mass spectrometry

A mass spectrometry analysis was thus conducted to determine the starting amino acid of the XopD protein produced by Xcv. HA-tagged XopD was immunopurified from Xcv 85* cultures and subjected to electrophoresis. After Coomasie staining of the gel, a ~85 kDa band corresponding to XopD was excised, digested with trypsin and analyzed by mass spectrometry (Nano LC/ESI MS/MS). Trypsin cleaves at the C-terminal side of arginine (R) and lysine (K) residues, which are well distributed throughout the XopD sequence. In total, 28 peptides corresponding to XopD were detected (Figure 2, lane 1). XopD was not detectable in culture supernatants of the 85* strain (Figure 2, lane 2). The N-terminal domain of XopD 216-760 was previously shown to be required for targeting the effector to subnuclear foci [43]. To show that bacterial lysis had not occurred, doi:10.1371/journal.pone.0015773.g002

To gain insight into the putative function of XopD N-terminal extension, XopD amino acid sequence (full length XopD 1-760) was analyzed for its biochemical properties. Interestingly, we found a region rich in lysine, alanine and glutamic residues (KAE-rich; residues 168–202) with high propensity to form coiled-coil structures (Figure 3A). The presence of this type of coiled-coil structures generally indicates that the protein may interact with other amino acid chains of the same polypeptide to form oligomers or other polypeptides to form complexes. Further analysis predicted that this coiled-coil, KAE-rich region is likely to be involved in dimer formation, rather than trimer or other oligomeric structures (Figure 3A).

In the view of these observations, we first tested the oligomerization state of HA-tagged XopD produced by Xcv 85*. Size exclusion chromatography of bacterial total protein extracts, followed by Western blot analysis of the collected fractions, showed that the peak of elution of HA-tagged XopD corresponds to an estimated molecular mass of approximately 86 kDa, which coincides with the predicted molecular mass of an HA-tagged XopD monomer. This result (i) suggests that, despite the presence of a predicted coiled-coil structure, XopD is not able to dimerize in Xcv 85* and (ii) is consistent with the idea that, before translocation into the plant cell, T3E are unfolded or associated to T3S chaperones to facilitate their passage through the secretion apparatus [56,57].

The oligomerization state of XopD1-760 and XopD216-760 was next studied following overexpression of His-tagged protein versions in E. coli cells. The expected molecular mass for His-tagged XopD1-760 and XopD216-760 monomers is 89 and 65 kDa, respectively. After protein chromatography, aliquots from collected fractions were analyzed for the presence of XopD by immunoblot analysis. The estimated molecular mass of XopD1-760 ranged between 46 and 70 kDa whereas XopD216-760 elution ranged between 128 and 190 kDa (Figure 4B). These results strongly suggest that, consistent with the presence of a predicted coiled-coil structure, XopD1-760, but not XopD216-760, is able to dimerize in E. coli.

Subcellular localization and functions of XopD1-760 in N. benthamiana

The N-terminal domain of XopD216-760 was previously shown to be required for targeting the effector to subnuclear foci [43]. To determine the effect of the newly identified N-terminal extension...
of XopD on its subcellular localization in planta, XopD1-760 was fused to the Yellow Fluorescent Protein venus (YFPv) and transiently expressed, under the control of the 35S promoter, in *Nicotiana benthamiana* leaf epidermal cells. As XopD 216-760, XopD1-760 was also localized in subnuclear foci (Figure 5A). Both proteins were detected by Western blot using an anti-GFP antibody (Figure 5B).

Agrobacterium-mediated transient expression of XopD216-760 in *N. benthamiana* leaves results in tissue necrosis by 4 to 7 days after agroinfiltration, likely reflecting cell death due to XopD accumulation and cytotoxicity (Figure 5C; [46]). Interestingly, up to 5 days after agroinfiltration, *N. benthamiana* leaves expressing XopD1-760 showed a significant delay in the development of the necrotic phenotype observed after transient expression of XopD216-760 (Figure 5C), despite similar levels of protein accumulation (Figure 5B). These results were confirmed by ion leakage measurements in leaf disk assays, which showed significantly lower conductivity values in leaves expressing YFP-tagged XopD1-760, compared to leaves that expressed XopD216-760 (Figure 5D). These observations indicate that the N-terminal extension of XopD may negatively regulate the previously described cytotoxic effect induced by XopD216-760 expression. From 7 days after agroinfiltration, a similar cell death phenotype was observed with both YFP-tagged XopD216-760 and XopD1-760 (Figure 5C).

**Figure 3. Analysis of the XopD protein sequence by mass spectrometry.** XopD216-760 protein sequence is shadowed. All possible translation starts situated in frame and upstream the annotated M216 are underlined. Peptides identified by Nano LC/ESI MS/MS analysis following trypsin (A) or V8 protease (B) digestion of the purified XopD protein are shown in bold. The 30 amino acid low complexity KAE-rich region is indicated in red. doi:10.1371/journal.pone.0015773.g003
Finally, the SUMO protease activity of YFP-tagged XopD<sup>1-760</sup> was investigated after co-expression with an HA-tagged LzSUMO construct [43] in N. benthamiana leaves. Western blot analysis of HA-SUMO conjugates showed that, as in the case of XopD<sup>216-760</sup>, expression of XopD<sup>1-760</sup> led to significant reduction in the detection of SUMO-modified proteins (Figure 5E). These data demonstrate that, like XopD<sup>216-760</sup>, mutated in the conserved Cys residue in XopD catalytic core, was not able to hydrolyze the SUMO substrates [43] (Figure 5E). These data were confirmed by inoculation of tomato leaves with the susceptible cultivar Pearson inoculated with XopD<sup>216-760</sup> mutant strain. In agreement with previously published data, tomato leaves developed cell death by 10 days post inoculation (dpi) whereas leaves inoculated with Xcv<sup>85*</sup> were relatively healthy at the same time point (Figure 6C). These data were confirmed by inoculation of susceptible tomato plants of the Moneymaker cultivar, which showed identical results (data not shown). Western blot analysis with an anti-HA antibody showed expression of both XopD<sup>216-760</sup> and XopD<sup>1-760</sup> in bacterial total protein extracts (Figure 6D). As expected, the HA-tagged GUS control protein expressed by Xcv<sup>85*</sup> was detected in the GUS reporter gene after salicylic acid (SA) treatment [46]. Consistent with previous results, SA treatment induced PR1p transcriptional activation whereas, in the presence of XopD<sup>216-760</sup>, PR1p activation was significantly reduced (Figure 6A). Interestingly, co-expression of XopD<sup>1-760</sup> in these assays led to a stronger repression of PR1p transcriptional activation, suggesting that the N-terminal extension of XopD is necessary to modulate XopD function in the host (Figure 6A).

Second, XopD was previously reported to delay the onset of symptom development in susceptible tomato leaves after inoculation with Xcv [46]. In order to assess the role of XopD<sup>1-760</sup> and XopD<sup>216-760</sup> in Xcv symptom development in tomato, we first engineered a XopD null mutant in Xcv strain 85*. The sequence encoding the entire XopD ORF (XopD<sup>1-760</sup>) was deleted by homologous recombination to generate an Xcv 85* ΔxopD mutant strain. In agreement with previously published data, tomato leaves of the susceptible cultivar Pearson inoculated with Xcv ΔxopD developed cell death by 10 days post inoculation (dpi) whereas leaves inoculated with Xcv<sup>85*</sup> were relatively healthy at the same time point (Figure 6C). Interestingly, the mutant strain Xcv 85* ΔxopD expressing wild-type HA-tagged XopD<sup>1-760</sup> from a constitutive lac promoter in a broad host range plasmid was complemented for symptom development, whereas transformation of the same strain with an equivalent construct to express XopD<sup>216-760</sup> did not allow complementation and inoculated leaves became necrotic (Figure 6D). These data were confirmed by inoculation of susceptible tomato plants of the Moneymaker cultivar, which showed identical results (data not shown). Western blot analysis with an anti-HA antibody showed expression of both XopD<sup>216-760</sup> and XopD<sup>1-760</sup> in bacterial total protein extracts (Figure 6D). XopD<sup>216-760</sup> lacks the first 215 amino acids of XopD, which contain the T3S-dependent secretion signal. As a result, XopD<sup>1-760</sup>, but not XopD<sup>216-760</sup>, could be detected in culture supernatants from bacteria incubated in secretion medium (Figure 6D; lanes 3,4). As expected, the HA-tagged GUS control protein expressed by Xcv<sup>85*</sup> or Xcv 85* ΔxopD was detected in the GUS reporter gene after salicylic acid (SA) treatment [46]. Consistent with previous results, SA treatment induced PR1p transcriptional activation whereas, in the presence of XopD<sup>216-760</sup>, PR1p activation was significantly reduced (Figure 6A). Interestingly, co-expression of XopD<sup>1-760</sup> in these assays led to a stronger repression of PR1p transcriptional activation, suggesting that the N-terminal extension of XopD is necessary to modulate XopD function in the host (Figure 6A).
total bacterial extracts but not in culture supernatants (Figure 6D; lanes 1,2). These results demonstrate that XopD\textsuperscript{1\textendash}760 contains all necessary elements for functional N-terminal secretion and virulence function \textit{in planta}. Together, our data \cite{[47]} are consistent with XopD being a 760 amino acid protein and (ii) stress the biological significance of the newly identified N-terminal extension of XopD.

**Discussion**

Prediction of the translation start in bacterial proteins is particularly difficult, rendering systematic annotation of effector proteins a challenging task. Here, we took a mass spectrometry approach to determine the protein sequence of XopD following immunopurification from \textit{Xcv}. After trypsin digestion, 13 peptides were identified upstream the formerly annotated starting M residue, confirming that the XopD protein sequence annotated in the public databases is not complete. Moreover, trypsin digestion of purified XopD led to detection of the peptide IFNFDYK (starting after R\textsuperscript{3} in Figure 3), showing that XopD may start either at the first (UGG codon) or the second (AUG codon) M residue shown in Figures 1C and 3. Detection of the peptide MDRIFNFD, after V8 protease treatment, confirmed that the newly identified N-terminal domain present in XopD produced and secreted by \textit{Xcv} comprises 215 amino acids and that XopD is thus a 760 amino acid protein. This finding is consistent with the observation that initiation of translation of 84% of the predicted Xcv ORFs is predicted to start at an AUG codon, whereas only 4% of the predicted Xcv ORFs are predicted to start at a UUG triplet. In agreement with this assumption, out of a total of 28 inspected Xcv T3Es effectors, none is predicted to be translated starting from a UUG codon. Interestingly, the first predicted UUG codon in XopD from \textit{Xcv} is not present in the XopD coding regions from either \textit{Xcv} B100 or \textit{A. avenae}, whereas the AUG codon, which is used to initiate XopD translation, is well conserved. Finally, inoculation of tomato susceptible plants with \textit{Xcv} expressing XopD\textsuperscript{1\textendash}760 provided further confirmation that XopD from \textit{Xcv} is a functional 760 amino acid secreted protein (see below).

In the case of T3Es, the existence of the T3S N-terminal secretion and translocation signals could theoretically help prediction of their starting amino acid. However, secretion signals are not conserved at the amino acid level, even among related or conserved effectors \cite{[36,38]} and, in some cases, they are highly tolerant of substitutions \cite{[38,59]}. Moreover, in many cases, the presence of several in frame putative initiation codons makes it difficult to determine the starting amino acid for a given effector. Several programs have been developed for prediction of T3S signals in secreted proteins from Gram-negative bacteria \cite{[60–62]}. In the case of XopD, two different recent algorithms \cite{[60,61]} assigned the highest probability of secretion to XopD\textsuperscript{51\textendash}760, which, as shown in this study, does not correspond to the XopD protein secreted by \textit{Xcv}, whereas the assigned probability of secretion for XopD\textsuperscript{1\textendash}760 was very low. This is in agreement with previous reports estimating that algorithm-based gene prediction may lead to up to 40% of wrongly assigned start codons \cite{[63]}. Indeed, prediction of T3Es secretion is not straightforward, since real translation starts of T3Es have only occasionally been determined experimentally. In a few cases, manual changes of predicted translational start positions have been reported, particularly in the case of myristoylated T3Es, for which the presence of the conserved myristoylation motif facilitates determination of the translation start \cite{[64]}. Several reasons may explain why the newly identified N-terminal extension of XopD was previously overlooked. First, XopD N-terminal extension presents a low coding probability using standard codon usage matrices. Indeed, the G+C content in this genomic region is lower (47%) than in the rest of the \textit{xopD} gene (54%) and much lower than in the rest of the \textit{Xcv} genome (65%), suggesting its acquisition from a different organism with a different codon usage. For instance, the ACUR0 matrix (alternative codon usage regions \cite{[65]}), developed for systematic annotation of \textit{Ralstonia} T3Es, assigns higher coding probability to this region, although, using this matrix, M\textsuperscript{AE} is predicted to be XopD starting amino acid. Indeed, when analyzed for base composition, most ACURs differ significantly from the average 67% G+C content found in the entire genome, with variations ranging from 50\textendash}70% G+C content. Furthermore, ACURs are often associated with mobile genetic elements, suggesting that ACURs may have been acquired through horizontal transfer \cite{[65]}. Interestingly, xopD acquisition by horizontal transfer during evolution was previously proposed \cite{[47]}.

T3SS-dependent translocation of XopD into plant cells was previously demonstrated using a translational C-terminal fusion
with the calmodulin-dependent adenylate cyclase domain (Cya) of Bordetella pertussis cycloysin [66]. In these assays, 815 bp corresponding to what was previously annotated as the xopD promoter region and the annotated XopD coding region (XopD216-760) were fused to the Cya sequence and detection of the Cya activity in pepper leaves reflected XopD translocation [43]. However, considering our present findings, the construct used for the Cya assays comprised the N-terminal extension identified here (XopD1-760) preceded by a promoter xopD region of only 171 bp. Interestingly, this small promoter region appears to contain all necessary elements to allow XopD expression and wrong annotation of XopD was thus not suspected. Likewise, previously reported complementation studies of a Xcv strain deleted from XopD were performed with a construct that contained the xopD promoter and the complete XopD coding sequence [46]. Although the authors did not describe the length of the xopD promoter sequence used for complementation of the Xcv ΔxopD mutant strain, it must have contained at least the N-terminal extension described in the present work and a promoter region that is long enough to allow complementation.

The present study demonstrates that the newly identified N-terminal extension in XopD is essential for its secretion and promotes XopD virulence function(s) in planta. First, XopD N-terminal domain appears to negatively regulate XopD-induced cytotoxicity. In addition, XopD1-760 is more efficient than XopD216-760 in repressing transcription of PR1 after SA treatment, further confirming the presence of important regulatory elements in the N-terminal stretch of XopD. Finally, inoculation of tomato plants showed that XopD216-760 is not secreted from Xcv due to the lack of its N-terminal T3S-dependent secretion signal and is thus

Figure 5. Characterization of XopD1-760 expression and function in N. benthamiana. (A) Confocal images of epidermal cells of N. benthamiana leaves expressing YFPv-tagged XopD216-760 and XopD1-760 36 hours after agroinfiltration. Bright field images are shown on the right. Bars = 15 μm. (B) Western blot analysis using an anti-GFP antibody shows expression of YFPv-tagged XopD216-760 and XopD1-760 constructs, 36 hours after agroinfiltration. Ponceau S staining of the membrane illustrates equal loading. (C) Cell death development 4 (upper panel) and 7 (lower panel) days after agroinfiltration of YFPv-tagged XopD216-760 (left) and XopD1-760 (right) constructs. (D) Cell death was quantified by measuring electrolyte leakage in N. benthamiana leaves expressing YFPv-tagged XopD216-760 (open circles) and XopD1-760 (filled circles) at the indicated time points after agroinfiltration. Mean and SEM values are calculated from 3 independent experiments (8 replicates/experiment). The statistical significance in mean conductivity values obtained with leaves expressing XopD216-760 or XopD1-760 was assessed by using a Student’s t test (P value <10^-5). (E) SUMO-conjugates detected by Western blot analysis using an anti-HA antibody 36 hours after agroinfiltration of the indicated constructs. Expression of XopD proteins was revealed using an anti-GFP antibody. Ponceau S staining of the membrane illustrates equal loading.

doi:10.1371/journal.pone.0015773.g005
N-Terminal Extension in the Xcv T3E Effector XopD

not able to complement an Xcv ΔxopD mutant strain. In contrast, XopD1-760 is detected in supernatants from bacterial cultures and complements the Xcv ΔxopD mutant. This observation is consistent with the fact that translation of XopD is initiated at the first AUG codon (XopD1-760), and that all necessary elements for functional T3S-dependent secretion and in planta translocation are thus present in XopD1-760.

XopD was formerly described as a modular protein comprising a DNA-binding domain, two transcriptional repression motifs of the EAR type and a SUMO-protease domain [46]. Here, we describe a previously non-identified protein domain in XopD. Although determining the biological function of this protein domain is clearly beyond the scope of this study, our findings provide intriguing clues to the putative role of XopD N-terminal extension. For example, the fact that XopD KAE-rich domain presents a high probability to form coiled-coil structures, together with gel filtration analysis of XopD1-760 expressed in E. coli, strongly suggest that the KAE-rich domain is indeed involved in the formation of XopD dimers. Interestingly, detection of XopD dimers was not possible in Xcv, perhaps indicating that XopD is unfolded or associated to T3S chaperones prior to its injection in the plant cell. This idea is consistent with previous reports indicating that efficient effector translocation requires the assistance of specialized chaperones that promote the stability and/or secretion of their corresponding interaction partners, keeping them in a partially unfolded and, thus, secretion-competent conformation and guiding them to the secretion apparatus [56,57]. In protein dimers, cooperativity between the two proteins that form the dimer may increase the binding affinity for DNA [67]. Dimerization can also enhance the specificity of DNA binding by doubling the length of the DNA region bound by a protein dimer [67]. XopD1-760 was previously described as a transcriptional repressor of plant target genes that displays non-specific DNA binding activity [46]. Therefore, our finding that the XopD N-terminal extension is involved in dimer formation opens new perspectives regarding the study of XopD specificity of DNA binding as well as the search of its host targets.

BLAST analysis of the KAE-rich region with a high propensity to form coiled-coil dimers (residues 177-202; Figure 4A) identified a number of hits corresponding to protein families with a high degree of amino acid sequence homology. Particularly interesting was the homology of this region of XopD with the TolA protein family. TolA are membrane proteins involved in colicin uptake [68]. Interestingly, these proteins also contain a region rich in K, A, E residues that has been shown to form long α-helical structures [69]. The high degree of similarity observed after sequence alignment of this KAE-rich region of TolA with XopD1-760 amino acid residues (Figure S3A) suggests that this region of XopD may adopt a similar structural conformation.

Further analysis of the sequence upstream of the KAE-rich region in XopD allowed us to identify a sequence (residues 165–175) with homology to the MarR (Multiple antibiotic resistance)
family of proteins (Figure S3B). This family of transcriptional regulators is named after E. coli MarR, a repressor of genes that activate multiple antibiotic resistance and oxidative stress regions [70]. MarR homologs are homodimers that bind sequence-specific palindromic or pseudopalindromic DNA via a winged HTH (helix-turn-helix) motif. The crystal structures of several members of the MarR family show that the winged HTH DNA-binding core is flanked by helices involved in protein dimerization [71,72]. Interestingly, the XopD sequence containing residues 165–175 shows homology with the DNA recognition helix H5 (x4), which confers specificity of DNA binding to MarR family members [73]. It is noteworthy that XopD 165–175 sequence, as its homologous sequence in MarR proteins, is located next to a domain (KAE-rich) involved in protein dimerization that may adopt a α-helical structure, based on its homology with the TolA protein family. The DNA recognition helix x4 in MarR proteins contains conserved arginine (R) residues that are also present in XopD (Figure S3B). Mutations in this recognition helix and, in particular, in conserved R73 and R77 residues in XopD demonstrate that amino acids 165–175 of XopD may confer specificity for DNA recognition. Further studies are required to confirm the putative structural and functional similarities between this region of XopD and E. coli MarR proteins. Future work will determine whether this newly identified region in XopD and/or the KAE-rich domain, related to dimer formation, determine the specificity of the DNA binding activity displayed by the XopD protein.

Together, our data stress the difficulties associated to correct annotation of T3Es and open new perspectives for future studies on the XopD protein and its virulence-associated functions in planta.

Materials and Methods

Bacterial strains and plasmids

Xce 85* [54] and Xce 85* ΔavrV, strains carrying the hpg* mutation which confers constitutive hpg gene expression [54] were cultivated overnight at 28°C in MOKA rich medium [74] or in secretion medium (MA) [75]. Plasmids were introduced into E. coli by electroporation and into Xcv by triparental mating using pRK2073 as helper plasmid [76,77]. Oligonucleotide primers used for PCR amplification will be provided upon request.

Unless otherwise indicated, plasmids used in this study were constructed by Gateway technology (GW; Invitrogen) following the instructions of the manufacturer. PCR products flanked by the attB sites were recombined into the pDONR207 vector (Invitrogen) via a BP reaction to create the corresponding entry clones with attL sites. Inserts cloned into the entry clones were subsequently recombined into the destination vectors via an LR reaction to create the expression constructs.

XopD1-760 and XopD216-760 were amplified from Xce 85-10 genomic DNA. XopD 216-760 carrying a mutation in the conserved catalytic Cys residue was amplified from a pGEX-XopD1-760 -C470A vector, kindly provided by Mary Beth Mudgett (Stanford University, USA) [43]. 6xHis-, HA-, and YFP-tagged constructs were generated by recombinant of the corresponding entry vectors with pTH119 [78], pBin19-33S-GW-3HA, or pBin19-33S-GW-YFPv destination vectors, respectively (YFPv for YFPvenus, an enhanced form of the YFP; [79–80]). For complementation of the Xce 85* ΔxopD mutant strain, XopD1-760 and XopD216-760 were recombined into a pLAFR6-GW-3HA vector (kind gift of Laurent Deslandes, LIPM, Castanet-Tolosan, France) that allows expression of HA-tagged XopD proteins under the control of a constitutive lac promoter.

For GUS reporter assays in N. benthamiana, a 1-kb fragment of the PR1 promoter (PR1p) was amplified from Arabidopsis Col-0 genomic DNA. The PCR product was cloned into the entry vector pDONR207 and subsequently recombined into the pKGWF57 destination vector [81], resulting in a plant expression vector that contains a transcriptional fusion between the PR1p and the GUS reporter gene.

Epitope tagging of XopD and secretion assays

A 307 bp fragment containing the C-terminal end of XopD fused to an HA epitope was amplified by PCR. The amplified fragment was digested with BsmHI and XbaI and cloned into the suicide plasmid pVO155 [82]. This construct was introduced into the Xce 85* and Xce 85* ΔavrV strains. Secretion experiments were performed as described previously [73] and XopD was detected by Western blot analysis.

Expression and gel separation of HA-tagged XopD

500 ml of bacteria Xce strain 85* expressing HA-tagged XopD were cultivated overnight at 28°C in MOKA rich medium. The bacterial pellet was washed, resuspended in protein extraction buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10% glycerol (v/v), 1 mM DTT, 1 mM PMSF] and lysed using a French Press. Total protein extracts were ultracentrifuged at 100,000 g for 30 min at 4°C and the supernatant was subjected to immunoprecipitation using anti-HA affinity matrix (clone 3F10; Roche). Immunoprecipitated proteins were separated on a NuPage 4–12% Bis-Tris gel (Invitrogen) according to the manufacturer’s instructions. The proteins were stained using a commercial solution (PageBlue, Fermentas) and the band corresponding to XopD was excised from the gel for subsequent analysis by mass spectrometry.

Determination of XopD starting amino acid by mass spectrometry

The gel slice containing the XopD protein was digested by incubating with trypsin (Promega, Madison, WI, USA) or V8 protease (Roche) and the resulting peptides were extracted following established protocols [83]. The trypsin digest was then reconstituted in 18 μl 5% acetonitrile, 0.05% trifluoroacetic acid. 5 μL were analysed by nanolC-MS/MS using an Ultimate 3000 system (Dionex, Amsterdam, The Netherlands) coupled to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The peptide mixture was loaded on a C18 precolumn (300 μm ID x 15 cm PepMap C18, Dionex) equilibrated in 95% solvent A (5% acetonitrile, 0.2% formic acid) and 5% solvent B (80% acetonitrile, 0.2% formic acid). Peptides were eluted using a 5 to 50% gradient of solvent B during 80 min at 300 nl/min flow rate. Data were acquired with Xcalibur (LTQ Orbitrap Software version 2.2, Thermo Fisher Scientific). The mass spectrometer was operated in the data-dependent mode and was externally calibrated. Survey MS scans were acquired in the Orbitrap on the 300–2000 m/z range with the resolution set to a value of 60,000 at m/z 400. Up to 5 most intense multiply charged ions (2+, 3+ or 4+) per scan were CID fragmented in the linear ion trap. A dynamic exclusion window was applied within 60 sec. All tandem mass spectra were collected using normalized collision energy of 35%, an isolation window of 4 m/z, and 1 μs/can. Other instrumental parameters included maximum injection times and
automatic gain control targets of 250 ms and 500,000 ions for the FTMS, and 100 ms and 10,000 ions for LTQ MS/MS, respectively. Data were analyzed using Xcalibur software (version 2.0.6, Thermo Fisher Scientific) and MS/MS centroid peak lists were generated using the extract_msn.exe executable (Thermo Fisher Scientific) integrated into the Mascot Daemon software (Mascot version 2.2.05, Matrix Sciences). Dynamic exclusion was employed within 600 seconds to prevent repetitive selection of the same peptide. The following parameters were set to create peak lists: parent ions in the mass range 400–4,500, no grouping of MS/MS scans, and threshold at 1,000. The data were searched against the protein database of Xanthomonas campestris pv. vesicatoria 85-10 (NCBI) containing 4,411 sequences to which the longest possible ORF for XopD was added (Figure 1C). Mass tolerances in MS and MS/MS were set to 5 ppm and 0.8 Da, respectively, and the instrument setting was specified as “ESI Trap”. Tryptsin (specificity set for cleavage after K or R) and V8 (specificity set for cleavage after D or E) were designated as proteases, and one missing cleavage was allowed. Oxidation of methionine was searched as variable modification and carbamidomethylation of cysteine was set as fixed modification. All fragmentation spectra of peptides were manually checked as shown in Figure S1 and S2.

Protein expression in N. benthamiana

Agrobacterium-mediated transient expression in N. benthamiana leaves was performed as described [84].

Fluorescence Microscopy

YFPv fluorescence in N. benthamiana leaves was analyzed with a confocal laser scanning microscope (TCS SP2-SE; Leica) using a 63x water immersion objective lens (numerical aperture 1.20; PL APO). YFP fluorescence was excited with the 514 nm line ray of the argon laser and detected in the range between 520 and 575 nm. Images were acquired in the sequential mode (20 Z plains per stack of images; 0.5 μm per Z plain) using Leica LCS software (version 2.61).

XopD expression in E. coli

Expression vectors containing 6xHis-tagged XopD1-760 and XopD216-760 were transformed in E. coli Rosetta cells. For expression of recombinant proteins, cells were grown in Luria-Bertani (LB) medium at 28°C to OD600 = 0.6 to 0.8 and then induced with 0.2 mM isopropyl-β-D-galactoside (Roche) for 4 h at 28°C. Cells were lysed in PBS, pH 8.0 and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) using a French Press.

Gel Filtration Analysis

Gel filtration was performed using a fast protein liquid chromatography system (Pharmacia) with HR 10/30 Superdex S-200 high-resolution columns (Pharmacia). Prior to chromatography, protein extracts were ultracentrifuged to remove any aggregates. All gel filtration assays were performed at 4°C with pre-filtered protein extracts. Column equilibration and chromatography were performed in PBS buffer. Fractions were collected every 0.4 ml and analyzed by Western blot.

Protein Gel Blot Analysis

Proteins were separated on NuPage 4%–12% Bis-Tris gels (Invitrogen) following the manufacturer’s instructions and transferred onto Protran BA85 nitrocellulose membranes (Schleicher & Schuell) by wet electroblotting (Mini-Protean II system; Bio-Rad). For detection of HA-, YFPv- and His-tagged proteins, blots were respectively incubated with rat monoclonal anti-HA [clone EF10 (Roche); 1:5,000] and mouse monoclonal anti-His6-peroxidase [clone-His-2 (Roche); 1:50,000] antibodies, linked to horseradish peroxidase. Anti-GroEL rabbit polyclonal (Stressgen Biotechnologies Corporation) and mouse monoclonal anti-GFP IgG1 K [clones 7.1 and 13.1 (Roche)] were used at 1:10,000. Proteins were visualized using the Immobilon kit (Millipore) under standard conditions.

Fluorimetric GUS Assays

For GUS reporter assays, the indicated constructs were transiently expressed in N. benthamiana leaves using Agrobacterium. Leaves were sprayed with 2 mM salicylic acid (SA; SIGMA-Aldrich) 18 hours after agroinfiltration. 12 hours later, leaf discs were collected, frozen in liquid nitrogen and stored at -80°C until processing. GUS activity was measured using the substrate 4-methylumbelliferyl-β-D-glucuronide as described previously [80]. After protein extraction, 1 μg of total protein was used in replicates to measure enzymatic GUS activity of individual samples.

Construction of a Xcv 85* ΔxopD deletion mutant strain

An Xcv 85* xopD deletion mutant strain was constructed by using the sacB system [85]. Briefly, 830 bp upstream and 850 bp downstream regions of full-length xopD were amplified by PCR using Xcv 85-10 gDNA as template. PCR products were subsequently cloned in a GoldenGate-compatible pK18 plasmid (L. Noel, unpublished). GoldenGate is a cloning method based on the use of Type II restriction enzymes, BsoI in our study [86]. This plasmid was then introduced into Xcv 85* by triparental mating and deletion of xopD was verified by PCR.

For complementation of the Xcv 85* ΔxopD mutant strain, pLAFR6 vectors carrying either an HA-tagged GUS control [kind gift of Laurent Deslandes, LIPM, Castanet-Tolosan, France], XopD216-760 or XopD1-760 were introduced into Xcv 85* ΔxopD by triparental mating.

Inoculation of susceptible tomato cultivars

Whole leaves of Solanum lycopersicum cv Moneymaker or cv Pearson were inoculated with a 1×10^7 cfu/mL suspension of bacteria in 10 mM MgCl2 using a needleless syringe. Leaves of the same age on the same branch were used for each experimental test. Plants were kept under 16 h light/day at 28°C. Symptoms were analyzed 10 days after plant inoculation.

Quantification of cell death using electrolyte leakage

For electrolyte leakage measurements, 8 N. benthamiana leaf discs (6 mm diameter) were harvested 24 hours after agroinfiltration, washed and incubated at room temperature in 10 ml of distilled water before measuring conductivity.

Supporting Information

Figure S1 MS/MS spectra of peptides issued from tryptic hydrolysis of the XopD protein. Peptides were analyzed by LC-MS/MS using a capillary LC system coupled directly to an LTQ-Orbitrap mass spectrometer. Each MS/MS spectrum is a collection of ions produced by collision-induced dissociation of the intact peptide in the linear ion trap. The predominant b and y product ion peaks are labeled accordingly with the subscripts denoting their position in the identified peptide and 2+ or 3+ indicating doubly or triply protonated ions respectively. y and b ions that were detected on the graph are shown in bold.
Figure S2  MS/MS spectra of peptides issued from V8 hydrolysis of the XopD protein. Peptides were analyzed by LC-ESI-MS using a capillary LC system coupled directly to an LTQ-Orbitrap mass spectrometer. Each MS/MS spectrum is a collection of ions produced by collision-induced dissociation of the intact peptide in the linear ion trap. The predominant b and y product ion peaks are labeled accordingly with the subscripts intact peptide in the linear ion trap. The predominant b and y hydrolysis of the XopD protein.

Acknowledgments

We are grateful to Mary Beth Mudgett for the kind gift of the Agrobacterium strain expressing HA-ZSUMO as well as the pGEX-XopD-C470A vector, and to Laurent Deslandes for the pLAFR6 vectors. We also thank Stéphane Genin and Laurent Deslandes for helpful discussions and critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: JC DM SR. Performed the experiments: JC DM CP SR. Analyzed the data: JC DM LDN IA MR DR SR. Contributed reagents/materials/analysis tools: LDN. Wrote the paper: JC DM SR.

References

1. Zipfel C (2008) Pattern-recognition receptors in plant innate immunity. Curr Opin Immunol 20: 10–16.
2. Jones JD, Dang JL (2006) The plant immune system. Nature 444: 323–329.
3. Leake G, Grant M (2007) Salicylic acid in plant defense: the players and protagonists. Curr Opin Plant Biol 10: 466–472.
4. Tao Y, Xie Z, Chen W, Glazebrook J, Chang HS, et al. (2003) Quantitative Nature of Arabidopsis Responses during Compatible and Incompatible Interactions with the Bacterial Pathogen Pseudomonas syringae. Plant Cell 15: 317–330.
5. Torres MA, Dangl JL, Jones JD (2002) Arabidopsis gp91phox homologues are required for accumulation of reactive oxygen intermediates in the plant defense response. Proceedings of The National Academy of Sciences of USA 99: 517–522.
6. Asai T, Tena G, Collmer A (2004) Type III secretion system effector proteins: double agents in bacterial plant disease and plant defense. Annu Rev Phytopathol 42: 289–309.
7. Alfano JR, Collmer A (2006) Type III secretion system effector proteins: double agents in bacterial disease and plant defense. Annu Rev Phytopathol 42: 309–324.
8. Chisholm ST, Coaker G, Day B, Staakwicz BJ (2006) Host-microbe interactions: shaping the evolution of the plant immune response. Cell 124: 803–814.
9. Hauck P, Thilmann R, He SY (2003) A Pseudomonas syringae type III effector suppresses cell wall-based extracellular defense in susceptible Arabidopsis plants. Proc Natl Acad Sci U S A 100: 8277–8282.
10. van der Beien EA, Jones JDG (1998) Plant disease resistance proteins and the gene for gene concept. Trends in Biochemical Sciences 23: 454–456.
11. Gohre V, Robazek S (2000) Breaking the barriers: microbial effector molecules subvert plant immunity. Annu Rev Phytopathol 48: 189–215.
12. Shao F, Golstein C, Ade J, Stoutemyer M, Dixon JE, et al. (2003) Cleavage of Arabidopsis PBS1 by a bacterial type III effector. Science 301: 1230–1233.
13. Aktell MJ, Staakwicz BJ (2003) Activation of NPR1-specified disease resistance in Arabidopsis is coupled to the avrRpt2-directed accumulation of RIN4. Cell 114: 369–377.
14. Mackey D, Belkadih Y, Alonso JM, Ecker JR, Dang JL (2003) Arabidopsis RIN4 is a target of the type III virulence Effector avrRpt2 and mediates RPS2-mediated resistance. Cell 112: 379–389.
15. Abramowitch RB, Anderson JC, Martin GB (2006) Bacterial elicitation and evasion of plant innate immunity. Nat Rev Mol Cell Biol 7: 601–611.
16. Rosebrook TR, Zeng L, Brady JJ, Abramowitch RB, Xiao F, et al. (2007) A bacterial E3 ubiquitin ligase targets a host protein kinase to disrupt plant immunity. Nature 448: 370–374.
17. Nomura K, Dehroy S, Lee YH, Pumpin P, Jones J, et al. (2006) A bacterial virulence protein suppresses host innate immunity to cause plant disease. Science 313: 220–223.
18. Kay S, Hahn S, Marois E, Haeuse G, Bonas G (2007) A bacterial effector acts as a plant transcription factor and induces a cell size regulator. Science 318: 644–651.
19. Romer P, Hahn S, Jordán T, Strauss T, Bonas U, et al. (2007) Plant pathogen recognition mediated by promoter activation of the pepper Bs3 resistance gene. Science 318: 645–648.
49. Xiao Y, Hutcheson SW (1994) A single promoter sequence recognized by a functional screen for the type III (Hrp) secretome of the plant pathogen *Pseudomonas syringae*. Science 259: 1722–1726.

50. Petričkiová-Cwiejta T, Schneider D, Tam VG, Chancey ST, Shan L, et al. (2002) Genome-wide identification of proteins secreted by the Hrp type III secretion system of *Pseudomonas syringae pv. tomato* DC3000. Proc Natl Acad Sci U S A 99: 7662–7657.

51. Ghosh P (2004) Process of protein transport by the type III secretion system. Microbiol Mol Biol Rev 68: 771–795.

52. White FF, Potnis N, Jones JB, Koebnik R (2009) The type III effectors of *Xanthomonas*. Molecular Plant Pathology 10: 749–766.

53. Chosed R, Tomchick DR, Brautigam CA, Mukherjee S, Negi VS, et al. (2007) *Xanthomonas* type III effector XoPD targets SUMO-conjugated proteins in planta. Mol Microbiol 50: 377–389.

54. Ohta M, Matsui K, Hirasu T, Shimasu H, Ohane-Takagi M (2001) Repression of class II ERF transcriptional repressors share an essential motif for active repression. Plant Cell 13: 1599–1608.

55. Kazan K (2006) Negative regulation of defense and stress genes by EAR-motif-containing repressors. Trends Plant Sci 11: 109–112.

56. Kim JG, Taylor KW, Hotson A, Keegan M, Schmelz EA, et al. (2008) XopD SUMO protease affects host transcription, promotes pathogen growth, and delays symptom development in *Xanthomonas*-infected tomato leaves. Plant Cell 20: 1915–1929.

57. Noël I, Thieme F, Neremst D, Bonas U (2002) Two novel type III-secreted proteins of *Xanthomonas campestris pv. vesicatoria* are encoded within the *hrp* pathogenicity island. J Bacteriol 184: 1340–1341.

58. Innes RW, Bent AF, Kunkel BN, Bisgrove SR, Staskawicz BJ (1993) Molecular characterization of type III secretion systems. PLoS Pathog 5: e1000375.

59. Arnold R, Brandmaier S, Kleine F, Tischler P, Heinz E, et al. (2009) Sequence-based prediction of type III secretion signals in genomes. PLoS Pathog 5: e1000375.

60. Akeda Y, Galan JE (2005) Chaperone release and unfolding of substrates in type III secretion of *Yersinia*. J Bacteriol 185: 6747–6755.

61. Arnal L, Sandhu GS, van den Ackerveken G, Bonas U (2000) HrpB2 and HrpF from *Xanthomonas* are type III-secreted proteins and essential for pathogenicity and recognition by the host plant. Mol Microbiol 36: 828–838.

62. Akeda Y, Galan JE (2003) Chaperone release and unfolding of substrates in type III secretion. Nature 437: 911–915.

63. Gauthier A, Findlay BB (2005) Translocated intimin receptor and its chaperone interact with ATPase of the type III secretion apparatus of enteropathogenic *Escherichia coli*. J Bacteriol 187: 6747–6763.

64. Russmann H, Kubori T, Sauer J, Galan JE (2002) Molecular and functional analysis of the type III secretion signal of the *Salmonella enterica* invB protein. Mol Microbiol 46: 769–779.

65. Lloyd SA, Stjernmå M, Andersson S, Wolf-Watz H (2002) Molecular characterization of the type III secretion signal sequence analysis of synthetic N-terminal amino acid sequences. Mol Microbiol 43: 51–59.

66. Lower M, Schneider G (2009) Prediction of type III secretion signals in genomes of gram-negative bacteria. PLoS One 4: e6917.

67. Arnold R, Brandmaier S, Kleine F, Tischler P, Heinz E, et al. (2009) Sequence-based prediction of type III secreted proteins. PLoS Pathog 5: e1000376.

68. Samudrala R, Heffron F, McDermott JE (2009) Accurate prediction of secreted proteins of *Yersinia enterocolitica* using a hybrid *YopE*-adenylate cyclase from *E. coli enterocolitica* in HeLa Cells. Molecular Microbiology 14: 583–594.

69. Mariyangayam NJ, Sunde M, Matthews JM (2004) The power of two: protein dimerization in biology. Trends Biochem Sci 29: 618–625.

70. Bourdinaudeau JP, Howard SP, Lazdunski C (1989) Localization and assembly into the *Escherichia coli* envelope of a protein required for entry of colicin A. J Bacteriol 171: 2459–2465.

71. Levensoug BD, Beyer WF, Webster R (1991) Tol A: a membrane protein involved in colicin uptake contains an extended helical region. Proc Natl Acad Sci USA 88: 5939–5943.

72. Alekshun MN, Levy SB (1999) The mar regulon: multiple resistance to antibiotics and other toxic chemicals. Trends Microbiol. pp 410–413.

73. Alekshun MN, Levy SB, Mealy TR, Seaton BA, Head JF (2001) The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 Å resolution. Nat Struct Biol 8: 710–714.

74. Chin KH, Tu ZL, Li JN, Chou CC, Wang AHJ, et al. (2006) The Crystal Structure of XCI739: A Putative Multiple Antibiotic-Resistance Repressor (MarR) from *Xanthomonas campestris* at 1.8 Å Resolution. PROTEINS: Structure, Function, and Bioinformatics 65: 239–242.

75. Alekshun MN, Kim YS, Levy SB (2000) Mutational analysis of MarR, the negative regulator of marXAR expression in *Escherichia coli*, suggests the presence of two regions required for DNA binding. Mol Microbiol 35: 1394–1404.

76. Blanvillain S, Meyer D, Boulanger A, Lautier G, Guyon G, Derancé N, Yasse J, Lauber E, Aclat M (2007) Plant carbohydrate scavenging through tonymbond-dependent receptors: a feature shared by phytopathogenic and aquatic bacteria. PLoS One 2: e224.

77. Rossier O, Wengelin K, Hahn K, Bonas U (1999) The *Xanthomonas* Hrp type III system secretes proteins from plant and mammalian pathogens. Proc Natl Acad Sci USA 96: 9368–9373.

78. Murray NE, Braemglin K, Murray K (1977) Lambdoid plasmids that stabilize the recovery of in vitro recombinants. Mol Gen Genet 150: 53–61.

79. Finan TM, Kunkel B, De Vos GF, Signer ER (1986) Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopoly saccharides and thiamine synthase genes. J Bacteriol 167: 66–72.

80. Pemeyroino M, Cunanan S, Barberis P, Deslandes L, Peeters N, Cazale-Noel AC, Bocher C, Genin S (2009) Two Type III Secretion System Effectors from *Ralstonia solanacearum* GM100 Determine Host-Range Specificity on Tobacco. Mol Plant Microbe Interaction 22: 530–550.

81. Nagai T, Ibata K, Park ES, Kubota M, Mikoshita K, et al. (2002) A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat Biotechnol 20: 87–90.

82. Veyron C, Canonne J, Daniel X, Jauneau A, Briere C, et al. (2010) AtsPLA2-N-Terminal Extension in the Xcv T3E Effector XoPD. PLoS ONE | www.plosone.org 13 December 2010 | Volume 5 | Issue 12 | e15773