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The *Pseudomonas syringae* HrpJ protein controls the secretion of type III translocator proteins and has a virulence role inside plant cells

Emerson Crabill,[1,2†] Andrew Karpisek[1,3†‡] and James R. Alfano[1,3*]

[1]*Center for Plant Science Innovation, University of Nebraska, Lincoln, NE 68588-0660, USA.*
[2]*School of Biological Sciences, University of Nebraska, Lincoln, NE 68588-0118, USA.*
[3]*Department of Plant Pathology, University of Nebraska, Lincoln, NE 68588-0722, USA.*

Summary

The bacterial plant pathogen *Pseudomonas syringae* injects effector proteins into plant cells via a type III secretion system (T3SS), which is required for pathogenesis. The protein HrpJ is secreted by *P. syringae* and is required for a fully functional T3SS. A *hrpJ* mutant is non-pathogenic and cannot inject effectors into plant cells or secrete the harpin HrpZ1. Here we show that the *hrpJ* mutant also cannot secrete the harpins HrpW1 and HopAK1 or the translocator HrpK1, suggesting that these proteins are required in the translocation (injection) of effectors into plant cells. Complementation of the *hrpJ* mutant with secretion incompetent HrpJ derivatives restores the secretion of HrpZ1 and HrpW1 and the ability to elicit a hypersensitive response, a measure of translocation. However, growth *in planta* and disease symptom production is only partially restored, suggesting that secreted HrpJ may have a direct role in virulence. Transgenic *Arabidopsis* plants expressing HrpJ-HA complemented the virulence phenotype of the *hrpJ* mutant expressing a secretion incompetent HrpJ derivative and were reduced in their immune responses. Collectively, these data indicate that HrpJ has a dual role in *P. syringae*: inside bacterial cells HrpJ controls the secretion of translocator proteins and inside plant cells it suppresses plant immunity.

Accepted 11 May, 2012. *For correspondence. E-mail jalfano2@unl.edu; Tel. (+1) 402 472 0395; Fax (+1) 402 472 3139. †These authors contributed equally to this work. ‡Present address. Biology Department, Creighton University, 2500 California Plaza, Omaha, NE 68178, USA.

Introduction

Numerous Gram-negative bacterial pathogens and eukaryote-associated bacteria use type III protein secretion systems (T3SSs) to inject or translocate effector proteins into animal or plant cells (Galán and Collmer, 1999; Cornelis, 2010). There are several prerequisites before a bacterium possessing a T3SS can successfully inject effectors into host cells: (i) the basal body of the T3SS apparatus, which spans both bacterial membranes needs to be assembled, (ii) the proteins that make up the extracellular conduit (a long pilus in plant-associated bacteria and a short needle in animal pathogens) are secreted and assembled, (iii) translocator proteins are secreted and these somehow aid in the formation of a pore in the eukaryotic plasma membrane and finally (iv) type III effectors are delivered across the host's plasma membrane gaining entrance into the eukaryotic cell (Cornelis, 2006; Galán and Wolf-Watz, 2006).These prerequisites necessitate that the construction of a type III apparatus and type III secretion is a highly regulated and ordered process. For example, it is logical to expect that the pilus or needle proteins would be secreted prior to translocators or type III effectors. There appear to be multiple strategies used by bacteria to insure that type III secretion is carried out in a temporal and hierarchical manner (Deane *et al.*, 2010; Osborne and Coombes, 2011).

One protein family that plays an important role in type III secretion control and hierarchy is the YopN-TyeA/InvE/SepL family (Pallen *et al.*, 2005; Botteaux *et al.*, 2009). The prototype for this family is from *Yersinia* spp. where it is actually two different proteins, YopN and TyeA, which interact with each other in a complex to regulate the secretion of Yop proteins, which include effectors and other type III-secreted substrates such as translocators (Pallen *et al.*, 2005; Joseph and Plano, 2007). In most other bacteria YopN and TyeA homologues are fused and are encoded by one gene (Pallen *et al.*, 2005). *Yersinia* spp. mutants of either *yopN* or *tyeA* constitutively secrete Yop proteins in the presence of calcium and prior to host cell contact, conditions that normally inhibit their secretion (Forsberg *et al.*, 1991; Boland *et al.*, 1996; Iriarte *et al.*, 1998). The TyeA protein has been implicated in the

translocation of effectors (Iriarte *et al*., 1998; Day *et al*., 2003). *Salmonella enterica* mutants lacking InvE or SsaL, YopN-TyeA/InvE/SepL family members of the two T3SSs of *S. enterica*, do not secrete type III translocator proteins (Kubori and Galán, 2002; Coombes *et al*., 2004). SepL from enteropathogenic *Escherichia coli* is required for secretion of translocator proteins in culture and the translocation of type III effectors (O'Connell *et al*., 2004). *Shigella flexneri* mutants lacking MxiC, another YopN-TyeA/InvE/SepL family member, exhibit increased secretion of type III effectors (Botteaux *et al*., 2009) but also secrete reduced amounts of translocators (Martinez-Argudo and Blocker, 2010). Most of the proteins belonging to this family are themselves type III-secreted proteins. The exception seems to be InvE, which has been reported to remain inside the bacterial cell (Kubori and Galán, 2002) and TyeA is not secreted (Cheng and Schneewind, 2000; Ferracci *et al*., 2004). Thus, the picture that has emerged from studies on members of this protein family from animal pathogens is that they control the secretion of type III-secreted substrates and are often associated with controlling the secretion of type III translocators.

There are three conserved proteins that are involved in the translocation of type III effectors into animal cells (Cornelis, 2006). In the prototypical *Yersinia* spp. T3SS, these are YopB, YopD and LcrV. YopB and YopD are translocator proteins and they can form pores in the host plasma membrane (Hakansson *et al*., 1996; Neyt and Cornelis, 1999; Montagner *et al*., 2011). These proteins are thought to be situated at the tip of the type III needle by the LcrV tip protein (Mueller *et al*., 2005). In plant pathogens the proteins involved in type III translocation appear quite different perhaps because they have to deliver proteins across the plant cell wall as well as the eukaryotic plasma membrane (Buttner and Bonas, 2002). The *Pseudomonas syringae* HrpK1 protein, *Xanthomonas campestris* HrpF, and *Ralstonia solanacearum* PopF1 and PopF2 share similarity with each other and share biochemical characteristics with the YopB family of translocators from animal pathogens (Buttner *et al*., 2002; Petnicki-Ocwieja *et al*., 2005; Meyer *et al*., 2006). However, plant pathogens have not been reported to possess the YopD translocator or the LcrV tip protein family members. Instead another family of proteins called harpins, which are unique to plant-associated bacteria, have long been implicated in type III translocation (Alfano and Collmer, 1997). Harpins were originally identified because when purified and infiltrated into plant tissue they can elicit an immunity-associated programmed cell death response in plants called the hypersensitive response (HR) (Wei *et al*., 1992; He *et al*., 1993). They share common biochemical characteristics including being glycine-rich and lacking in cysteines. The genome of *P. syringae* pv. *tomato* DC3000 encodes four harpins, *hrpZ1*, *hrpW1*, *hopAK1* and *hopP1* and all except *hopP1* encode proteins that appear to contribute to translocation (Kvitko *et al*., 2007). However, it is currently unknown how harpins interact with HrpK1/HrpF family members to translocate type III effectors into plant cells.

Pseudomonas syringae is a phytopathogen that uses its T3SS to inject type III effectors into host plant cells to subvert plant immunity (Block *et al*., 2008; Zhou and Chai, 2008). Its T3SS is encoded by the *hrp-hrc* (HR and pathogenicity and HR conserved) gene cluster. One gene (*hrpJ*) carried within the *P. syringae hrp-hrc* cluster encodes HrpJ, a member of the YopN-TyeA/InvE/SepL protein family (Alfano and Collmer, 1997; Pallen *et al*., 2005; Fu *et al*., 2006). A *P. syringae* pv. *tomato* DC3000 *hrpJ* mutant cannot secrete the HrpZ1 harpin in culture and is greatly reduced in virulence and in its ability to translocate effectors into plant cells (Fu *et al*., 2006). Similar phenotypes are also associated with an *Erwinia amylovora hrpJ* mutant (Nissinen *et al*., 2007). The implication from these results is that HrpZ1 is a translocator that cannot participate in translocation in the absence of HrpJ because it is not secreted. However, the severity of the phenotypes associated with the *P. syringae hrpJ* mutant suggests that it controls the secretion of a large suite of proteins in addition to HrpZ1 because *P. syringae hrpZ1* mutants exhibit only subtle phenotypes (Alfano *et al*., 1996). Coupled with the observation that many other members of the YopN-TyeA/InvE/SepL protein family are unable to secrete translocators these data imply that HrpZ1 is a translocator. Identifying the complete inventory of proteins that are dependent on HrpJ for their secretion may be a viable strategy to better define the *P. syringae* translocator class.

Here, we show that HrpJ is required for the secretion of the HrpK1 translocator and the HrpZ1, HrpW1 and HopAK1 harpins, but not the HopP1 harpin or other classes of type III-secreted substrates. Interestingly, elevated amounts of HrpA1, the major component of the type III pilus, were secreted by the *hrpJ* mutant. Secretion incompetent HrpJ derivatives can restore the ability of a *hrpJ* mutant to secrete HrpZ1 and HrpW1 in culture indicating that HrpJ controls their secretion from within the bacterial cell. Additionally, we show that a C-terminal HrpZ1 deletion derivative can be secreted in the absence of HrpJ suggesting that HrpJ exerts its secretion control by interacting either directly or indirectly with this region of HrpZ1. HrpJ is itself translocated into plant cells and *in planta* expression of HrpJ can partially restore virulence to a *hrpJ* mutant expressing a secretion incompetent HrpJ derivative and results in reduced plant immune responses. Taken together, these data indicate that HrpJ acts inside the bacterial cell as a control protein that regulates the temporal secretion of translocators and it also acts inside the plant cell to suppress plant immunity.

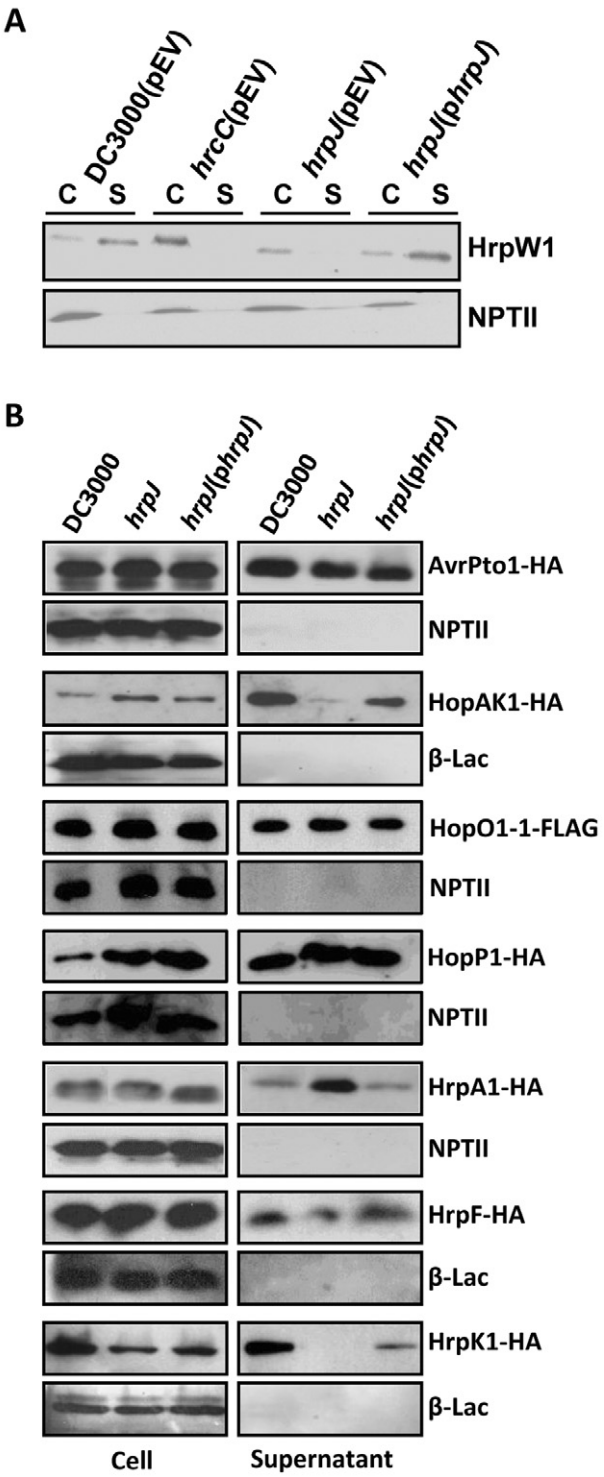

Fig. 1. The *hrpJ* mutant is impaired in its ability to secrete HrpW1, HrpK1 and HopAK1, but not HopP1, HrpF, HrpA1, HopO1-1 or AvrPto1.
A. Wild-type DC3000, a type III defective mutant *hrcC*, and a *hrpJ* mutant were grown in type III-inducing conditions and then separated into cell (C) and supernatant (S) fractions by centrifugation. Proteins were resolved with SDS-PAGE and immuno-stained with anti-HrpW1 antibodies.
B. Wild-type DC3000 and a *hrpJ* mutant carrying a plasmid that encoded one of several type III-secreted substrates fused at their C-termini to an HA or FLAG epitope were grown in type III-inducing conditions and separated into cell and supernatant fractions. Type III-secreted proteins were detected with anti-HA antibodies.
A and B. Bacteria also expressed NPTII or β-lactamase as negative controls because these remain cell-bound unless non-specific cell leakage occurred. All experiments were repeated at least three times with similar results.

Results

The hrpJ mutant is unable to secrete harpins and HrpK1 but retains the ability to secrete HrpA1 (the Hrp pilus), effector proteins, and other type III-secreted proteins encoded by the hrp/hrc cluster

We reported earlier that the *P. syringae* pv. *tomato* DC3000 *hrpJ* mutant was unable to secrete HrpZ1 in culture (Fu *et al*., 2006). DC3000 *hrpZ1* mutants have a subtler virulence phenotype than the DC3000 *hrpJ* mutant (Alfano *et al*., 1996), which suggests that other proteins cannot be secreted from the *hrpJ* mutant in addition to HrpZ1. Because HrpZ1 is a candidate translocator, the *hrpJ* mutant may be defective in the secretion of translocators and by identifying proteins that are not secreted from the *hrpJ* mutant we may better define the group of proteins that make up the DC3000 translocon. To test this, we first determined the extent that the HrpW1 harpin was secreted from the *hrpJ* mutant. We performed in culture secretion assays by growing DC3000 cultures in a medium that induces the T3SS and separated the cultures into cell-bound and supernatant fractions. HrpW1 was found in the supernatant fraction from wild-type DC3000 but only in the cell fraction of the *hrpJ* mutant (Fig. 1A) indicating that HrpW1 cannot be secreted from cells lacking HrpJ. The ability to secrete HrpW1 was restored to the *hrpJ* mutant when *hrpJ* was provided *in trans* (Fig. 1A). The inability of the *hrpJ* mutant to secrete HrpW1 further suggests that HrpJ may be required for the secretion of a larger group of proteins that need to be secreted early in the type III secretion hierarchy.

In order to identify other proteins that cannot be secreted by the *hrpJ* mutant and therefore, possibly linked in function to HrpZ1 and HrpW1 we screened a wide array of type III-secreted substrates for their inability to be secreted by the *hrpJ* mutant. Included in these experiments were HrpA1 (the major protein component of the pilus), type III effectors, other harpin proteins and other type III-secreted proteins encoded by the *hrp-hrc* cluster. Because the overexpression of harpins can have aberrant effects on type III secretion (Alfano and Collmer, 1996; Charkowski *et al*., 1997), harpin and *hrpK1* genes were expressed from a type III promoter using a Tn7 expression system (see *Experimental procedures*). DC3000 and

hrpJ mutant strains containing different genes that encoded type III-secreted substrates fused to a haemagglutinin (HA) or a FLAG epitope were grown in type III-inducible medium and separated into cell and supernatant fractions. Interestingly, the two additional putative translocator proteins, the HopAK1 harpin and HrpK1, were not detectable in the supernatant fraction of the mutant indicating that HrpJ is required for their secretion (Fig. 1B). The HopP1 harpin was secreted by the *hrpJ* mutant (Fig. 1B) indicating that it likely has a different role in the T3SS than the other harpins tested. The secretion of both HopAK1 and HrpK1 was restored when *hrpJ* was provided *in trans* to the *hrpJ* mutant (Fig. 1B). The type III effectors AvrPto1 and HopO1-1, the HrpA1 pilus protein, and HrpF, a type III-secreted protein encoded by the *hrp-hrc* cluster (Ramos *et al*., 2007), were all secreted by the *hrpJ* mutant (Fig. 1B). We reported previously that HrpA1 was secreted by the *hrpJ* mutant (Fu *et al*., 2006). Further experimentation suggests that HrpA1 is actually secreted in higher amounts by the *hrpJ* mutant as shown in Fig. 1B. Thus, the harpins HrpZ1, HrpW1 and HopAK1, and the translocator HrpK1 all require HrpJ to be secreted via the T3SS. This result suggests that the type III secretion of these proteins is coordinated by the HrpJ control protein and that they likely all perform related translocation functions. Furthermore, the increased secretion of HrpA1 by the *hrpJ* mutant suggests that HrpJ may aid in the transition from production of the pilus to the translocon.

Cell-bound HrpJ restores HrpZ1 and HrpW1 secretion from the hrpJ mutant

HrpJ is a type III-secreted protein (Fu *et al*., 2006). Because a DC3000 mutant lacking HrpJ does not secrete HrpZ1, we wanted to determine whether HrpJ secretion was needed for the secretion of HrpZ1 or HrpW1 or whether their secretion required HrpJ to be present inside the bacterial cell. The type III secretion signal for HrpJ is present on its N-terminus (Fu *et al*., 2006). N-terminal GST fusions have been made with type III-secreted substrates to render them impassable to the T3SS (Riordan *et al*., 2008). We made a *hrpJ* construct that produces a HrpJ derivative containing GST fused to the N-terminus of HrpJ. This HrpJ derivative was not secreted by the *hrpJ* mutant (Fig. 2). We carried out in culture secretion assays to determine the extent that HrpZ1, HrpW1 and HrpA1 could be secreted from the *hrpJ* mutant complemented with the secretion incompetent derivative of HrpJ. Both HrpZ1 and HrpW1 were secreted from the *hrpJ* mutant producing the secretion incompetent HrpJ derivative (Fig. 2). We also found that the enhanced secretion of HrpA1 by the *hrpJ* mutant was reduced back to wild-type levels when GST-HrpJ was introduced into the *hrpJ* mutant (Fig. 2). These results suggest that HrpJ is needed

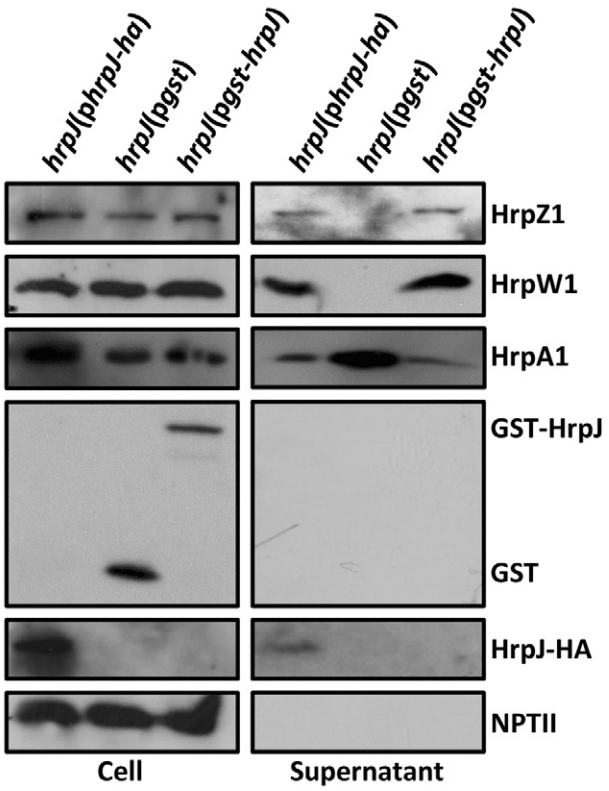

Fig. 2. A secretion incompetent HrpJ fusion protein restores the ability to secrete HrpZ1 and HrpW1 to a *hrpJ* mutant. The DC3000 *hrpJ* mutant carrying a construct that encoded HrpJ-HA, GST or a GST-HrpJ N-terminal fusion were grown in type III-inducing conditions and separated into cell and supernatant fractions by centrifugation. Proteins were resolved with SDS-PAGE and immuno-stained with anti-HrpZ1, anti-HrpW1, anti-HrpA1, anti-GST, anti-HA or anti-NPTII antibodies. NPTII was used as a lysis control. The experiment was repeated two times with similar results.

inside the bacterial cell in order to allow for the secretion of HrpZ1 and HrpW1 and likely the other translocators and, perhaps, to act as a substrate switch from the secretion of HrpA1 pilus protein to translocator secretion. Furthermore, the purpose of HrpJ's own secretion appears to be independent of its function in controlling the secretion of HrpZ1 and other translocators.

Expression of a secretion incompetent HrpJ derivative in the hrpJ mutant complements HrpZ1 secretion, elicitation of an HR in tobacco, and partially restores virulence in Arabidopsis

In order to confirm that cell-bound HrpJ is sufficient to restore the secretion of DC3000 translocators to the *hrpJ* mutant, several additional *hrpJ* constructs were made that produced HrpJ derivatives lacking either its type III secretion signal ($HrpJ_{\Delta 2-75}$), an N-terminal half region ($HrpJ_{\Delta 2-185}$), or a large C-terminal region ($HrpJ_{\Delta 161-368}$), each fused to an HA epitope. These constructs were confirmed

by sequencing and produced stable HrpJ derivatives (data not shown). The HrpJ$_{\Delta 2-75}$ and HrpJ$_{\Delta 2-185}$ derivatives could not be detectably secreted or translocated in culture secretion assays and translocation assays respectively, whereas the HrpJ$_{\Delta 161-368}$ derivative was detectably secreted and translocated (data not shown). In culture secretion assays were performed with *hrpJ* strains separately containing these constructs to determine if any could restore HrpZ1 secretion. The HrpJ$_{\Delta 2-75}$ derivative restored the secretion of HrpZ1 from the *hrpJ* mutant (Fig. 3A). Both HrpJ$_{\Delta 2-185}$ and HrpJ$_{\Delta 161-368}$ did not restore HrpZ1 secretion (Fig. 3A). We performed similar experiments with the YopN and TyeA moieties of HrpJ and found that neither could restore the secretion of HrpZ1, and, therefore, HrpJ's function in translocator secretion requires both (Fig. S1). Our results confirm the data shown in Fig. 2 indicating that secretion incompetent HrpJ is sufficient to restore the secretion of HrpZ1 to the *hrpJ* mutant. Additionally, it also suggests that the amino acids deleted from HrpJ$_{\Delta 2-185}$ and HrpJ$_{\Delta 161-368}$ derivatives are required for HrpJ's ability to control HrpZ1 secretion.

The ability of DC3000 to elicit an HR in tobacco is dependent upon its ability to inject type III effectors into the plant cells. Therefore, the HR is a measure of translocation. In order for type III effectors to be injected, HrpJ must be present to allow for the secretion of HrpK1 and the harpin proteins (Fig. 1), which collectively are necessary for translocation (Petnicki-Ocwieja *et al*., 2005; Kvitko *et al*., 2007). The *hrpJ* mutant cannot elicit an HR in tobacco because it cannot inject type III effectors, but this phenotype was complemented by expression of *hrpJ in trans* (Fu *et al*., 2006; Fig. 3B). The secretion incompetent HrpJ$_{\Delta 2-75}$ was also capable of restoring HR elicitation while the other HrpJ deletions tested did not restore the ability to elicit an HR to the *hrpJ* mutant (Fig. 3B). These results support the hypothesis that cell-bound HrpJ is required for the secretion of translocators.

As has previously been shown (Fu *et al*., 2006), a *hrpJ* mutant was severely reduced in its ability to grow *in planta* and cause disease symptoms in *Arabidopsis* (Fig. 3C and D). The production of HrpJ$_{\Delta 2-185}$ and HrpJ$_{\Delta 161-368}$ was unable to complement the virulence phenotype exhibited by the *hrpJ* mutant (Fig. 3C and D). It is important to note that full-length HrpJ was unable to fully complement the virulence phenotype of the *hrpJ* mutant (Fig. 3C and D). HrpJ$_{\Delta 2-75}$ was able to partially restore virulence to the *hrpJ* mutant, but could not restore virulence to levels exhibited by the *hrpJ* mutant complemented with full-length HrpJ (Fig. 3C and D). Because HrpJ$_{\Delta 2-75}$ was able to fully restore secretion of HrpZ1 and HR elicitation, the difference in growth of the *hrpJ* mutant complemented with full-length *hrpJ* or *hrpJ*$_{\Delta 2-75}$ may be attributable to the function of secreted HrpJ rather than the function of its cell-bound form.

A HrpZ1 C-terminal deletion derivative can be secreted in culture by the hrpJ mutant

The requirement of cell-bound HrpJ for HrpZ1 secretion suggests that HrpZ1 may interact with HrpJ or a HrpJ complex near the pore of the T3SS apparatus. However, we were unable to demonstrate an interaction between HrpJ and HrpZ1 in yeast two hybrid interaction assays or in GST-HrpJ pull-down assays (data not shown). We also included HrpK1, HrpW1 and HopAK1 in these yeast two hybrid experiments and were unable to detect any interactions with these proteins and HrpJ (data not shown). In spite of this apparent lack of interaction experimentally between these proteins, which may be due to the transient nature of these interactions or that these interactions may require a protein complex, we wanted to test the extent that any HrpZ1 deletion derivatives could be secreted by the *hrpJ* mutant. The rationale for this experiment was that if a region within the HrpZ1 protein was required to interact with HrpJ or a HrpJ complex in order for it to be secreted, then it is possible that the *hrpJ* mutant may be able to secrete a HrpZ1 deletion derivative lacking this region. To test this, a series of *hrpZ1* gene constructs were made that when introduced into DC3000 produced HrpZ1 deletion derivatives lacking 50 amino acid portions in different regions of this protein. Interestingly, the HrpZ1$_{\Delta 271-320}$-HA derivative, which lacked amino acids 271–320 was secreted in culture from the *hrpJ* mutant (Fig. 4A). Only low amounts of HrpZ1$_{\Delta 271-320}$-HA were secreted; however, this experiment was repeated several times with similar results. All of the other HrpZ1 derivatives were not secreted from the *hrpJ* mutant. Each *hrpZ1* gene construct produced a stable HrpZ1 derivative and all except for the most N-terminal deletion derivative (HrpZ1$_{\Delta 21-70}$-HA), which likely lacked part of the type III secretion signal, were detectably secreted from the *hrpJ* mutant expressing *hrpJ in trans* (Fig. 4B). The implication of this result is that a C-terminal region of HrpZ1 is required for HrpJ-dependency, and therefore, may interact with HrpJ allowing HrpJ to control the secretion of HrpZ1.

The reduced virulence phenotype exhibited by the hrpJ mutant expressing the cell-bound HrpJ is complemented by in planta*-expressed HrpJ-HA*

The *hrpJ* mutant complemented with a secretion incompetent HrpJ derivative was less virulent than when it was complemented with full-length HrpJ (Fig. 3C). Because HrpJ is itself a secreted protein we determined the extent that *in planta*-expressed HrpJ could complement the observed reduced virulence phenotype. To test this we made transgenic *Arabidopsis* plants that constitutively expressed HrpJ-HA and performed pathogenicity assays using these plants. The transgenic *Arabidopsis* plants were

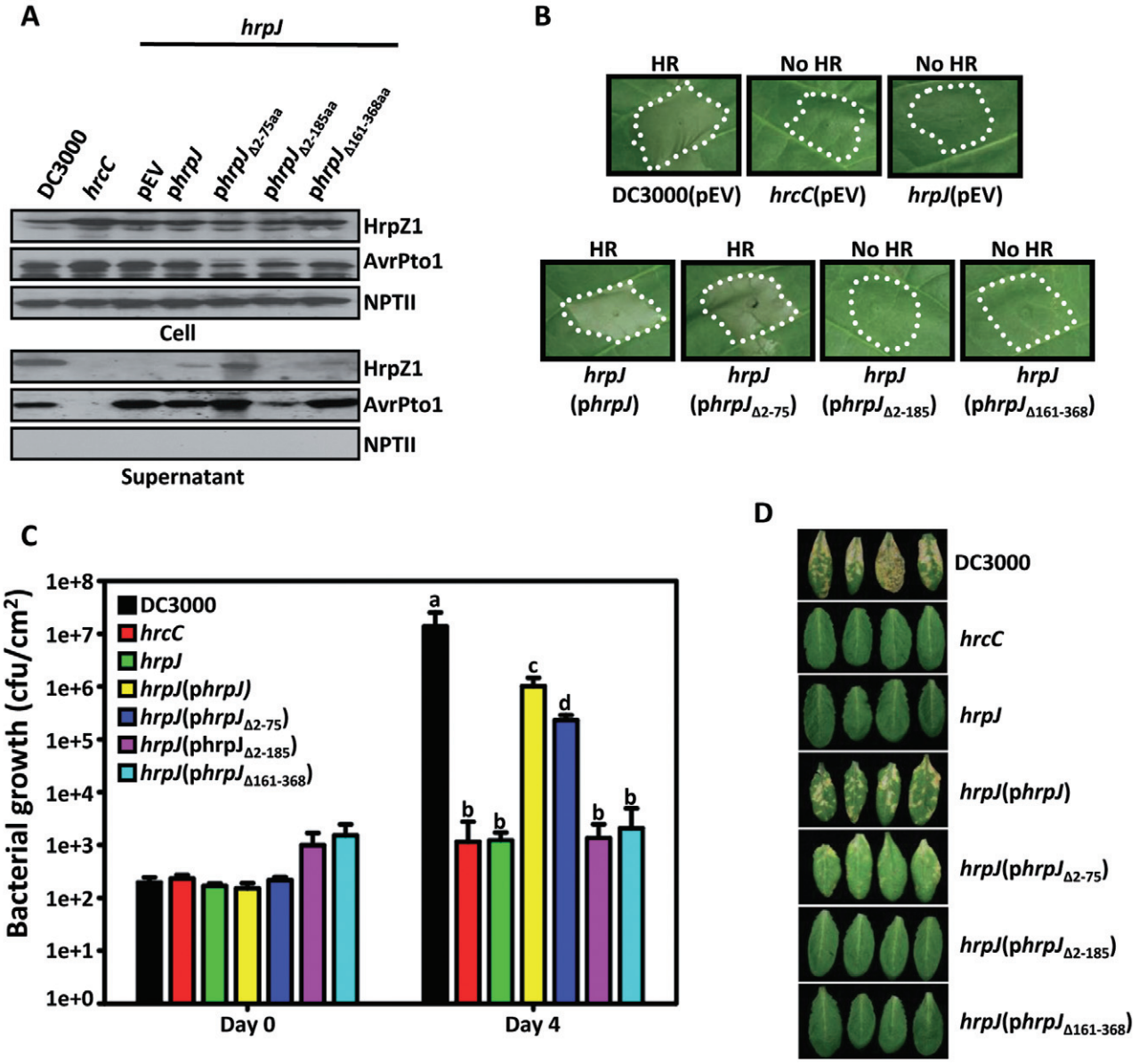

Fig. 3. A HrpJ derivative lacking its secretion signal restores secretion of HrpZ1 and the ability to elicit an HR to a *hrpJ* mutant but only partially complements pathogenicity.

A. DC3000 strains were grown in type III-inducing conditions and separated into cell and supernatant fractions by centrifugation and assessed for the secretion of HrpZ1 or AvrPto1 with immunoblot anaylses. NPTII was used as a cell lysis control. pML123 was used as an empty vector (pEV) control. The experiment was repeated three times with similar results.

B. The DC3000 *hrpJ* mutant strains expressing either the full-length HrpJ or $HrpJ_{\Delta 2-75}$ were capable of eliciting an HR in tobacco indicating that these strains were capable of injecting type III effectors. Bacteria were infiltrated at 1×10^8 cells ml^{-1} and the HR was observed within 24 h after infiltration. The experiment was repeated four times with similar results.

C. Growth of the *hrpJ* mutant on *Arabidopsis thaliana* Col-0 was partially restored when full-length *hrpJ or* $hrpJ_{\Delta 2-75}$ *was provided in trans.* Lower case letters indicate whether growth of the different strains were statistically different based on *t*-tests ($P < 0.1$) and error bars indicate standard deviation.

D. Photos of disease symptoms on *Arabidopsis* leaves were taken 4 days after infection.

C and D. The experiments were repeated twice with similar results.

confirmed to constitutively express HrpJ-HA (Fig. 5A). Consistent with the results presented in Fig. 3C, the *hrpJ* mutant grew poorly likely due to the failure of this mutant to inject type III effectors because it cannot secrete translocators (Fig. 5C). However, the *hrpJ* mutant expressing the secretion incompetent HrpJ ($HrpJ_{\Delta 2-75}$) grew similarly and caused similar disease symptoms in transgenic *Arabidopsis* plants expressing HrpJ-HA to the *hrpJ* mutant express-

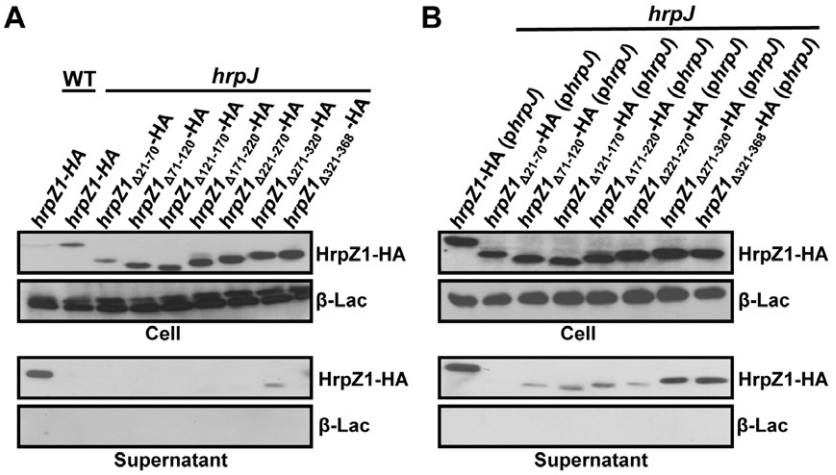

Fig. 4. A C-terminal HrpZ1 deletion derivative can be secreted in culture by a *hrpJ* mutant.
A. Wild-type DC3000 (WT) and *hrpJ* mutant strains expressing HrpZ1 and HrpZ1 derivatives C-terminally fused to a haemagglutinin (HA) tag were grown in type III-inducing conditions and separated into cell and supernatant fractions by centrifugation and assessed for HrpZ1 secretion by immunoblot analysis. The HrpZ1 derivatives were not secreted by the *hrpJ* mutant except for a HrpZ1 derivative that lacked amino acids 271–320 (HrpZ1$_{\Delta 271-320}$).
B. The *hrpJ* mutant strains expressing HrpZ1 and HrpZ1 derivatives and complemented with full-length *hrpJ* were grown in type III-inducing conditions and separated into cell and supernatant fractions to determine the extent that the HrpZ1 derivatives could be secreted in the presence of HrpJ. With the exception of *hrpZ1*$_{\Delta 21-70}$, which lacks its secretion signal, all of the HrpZ1 derivatives were secreted from the *hrpJ* mutant when *hrpJ* was provided *in trans*.
A and B. HrpZ1 and HrpZ1 derivatives were expressed from a type III promoter using a Tn7 expression system. HrpZ1-HA and derivatives were detected with anti-HA antibodies. β-Lactamase was used as a lysis control and detected with anti-β-lactamase antibodies. These experiments were done four times with similar results.

ing full-length HrpJ (Fig. 5B and C). Thus, the growth difference observed between these two strains in wild-type *Arabidopsis* plants (Fig. 3C) was not detectable on transgenic *Arabidopsis* plants expressing HrpJ-HA indicating that *in planta*-expressed HrpJ contributed to virulence by acting inside plant cells.

Expression of hrpJ in transgenic plants suppresses pathogen-associated molecular pattern-triggered immunity

Because the primary role of type III effectors injected by *P. syringae* appears to be to suppress the plant's innate immune system (Guo *et al.*, 2009), we sought to determine if *Arabidopsis* plants expressing HrpJ-HA were altered in their innate immune responses relative to wild-type plants. We made several independent lines of transgenic *Arabidopsis* plants that constitutively expressed HrpJ-HA. Pathogen-associated molecular patterns (PAMPs) can be recognized by plants and animals resulting in the induction of PAMP-triggered immunity (PTI) (Segonzac and Zipfel, 2011). We used two commonly used assays to evaluate PTI in *Arabidopsis* plants expressing HrpJ-HA: The ability of a type III defective *P. syringae* strain (*hrcC*), which is a de facto-PTI inducing strain, to grow in *Arabidopsis* plants expressing HrpJ-HA compared to wild-type plants and callose (a β-1,3-glucan) deposition in the cell wall in response to flg22, a peptide derived from the flagellin PAMP. A DC3000 *hrcC* mutant, defective in the T3SS, was spray-inoculated onto wild-type *Arabidopsis* and *Arabidopsis* plants expressing HrpJ-HA and bacterial cells were enumerated at 0 and 4 days after infection. Interestingly, the *hrcC* strain exhibited significantly better survival on plants expressing HrpJ-HA compared to wild-type plants (Fig. 6A). We next measured the ability of transgenic *Arabidopsis* plants expressing HrpJ-HA to deposit callose compared to wild-type plants in response to flg22. The number of callose deposits was more than twofold higher in wild-type plants than in plants expressing HrpJ-HA (Fig. 6B) indicating that HrpJ-HA can suppress flg22-induced callose deposition. We observed similar results with other plant lines expressing HrpJ-HA (data not shown). These results suggest that HrpJ-HA can suppress PTI. Collectively, these experiments suggest that HrpJ acts as a virulence factor inside plant cells and can suppress PTI.

Discussion

The YopN-TyeA/InvE/SepL protein family members function as control proteins for type III-secreted substrates and are particularly important for the secretion of translocator proteins. In most T3SS-containing bacteria these translocators are easily identified because they have high

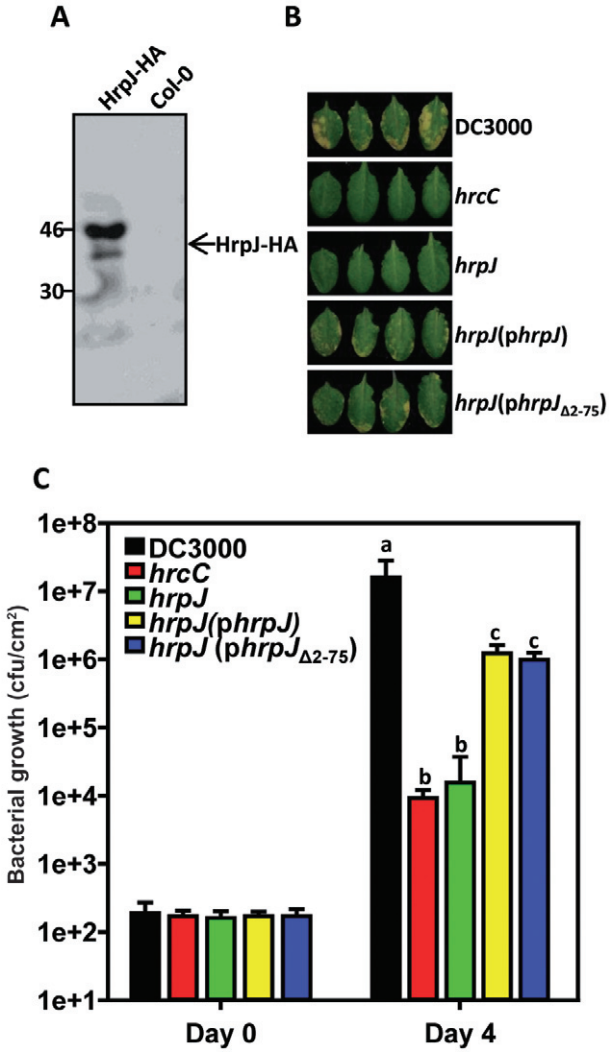

Fig. 5. *In planta* HrpJ-HA expression complements the reduced growth phenotype associated with the *hrpJ* mutant complemented with cell-bound HrpJ.
A. Transgenic *Arabidopsis* plants express detectable amounts of HrpJ-HA. Total protein extracts from wild-type *A. thaliana* Col-0 (right) and a representative HrpJ-HA transgenic plant that constitutively expresses HrpJ-HA (left) were subjected to immunoblot analysis using anti-HA antibodies.
B. Photos of disease symptoms on transgenic HrpJ-HA *Arabidopsis* leaves were taken 4 days after infection with the *Pseudomonas syringae* strains indicated. This experiment was done twice with similar results.
C. Transgenic HrpJ-HA *Arabidopsis* plants were spray-inoculated with 2×10^8 cells ml^{-1} with wild-type DC3000, the type III defective *hrcC* mutant, the *hrpJ* mutant, *hrpJ* mutant complemented with full-length *hrpJ*, or the *hrpJ* mutant complemented with a *hrpJ* derivative ($hrpJ_{2-75}$) that encodes a secretion incompetent form of HrpJ. Lower case letters indicate whether growth of the different strains were statistically different based on *t*-tests ($P < 0.05$), and error bars indicating standard deviation are shown.

sequence identity with the YopB and YopD translocators and the LcrV tip protein from *Yersinia* spp. (Hueck, 1998). Bacterial plant pathogens appear to have a substantially different translocon than animal pathogens due probably to the need for the T3SS apparatus to cross the plant cell wall (Buttner and He, 2009). Putative tranlsocators in the *P. syringae* T3SS are the HrpZ1 harpin and HrpK1 (Lee *et al*., 2001; Petnicki-Ocwieja *et al*., 2005), but the relationship between these proteins is unclear. The observation that HrpZ1 was not secreted from a *P. syringae hrpJ* mutant (Fu *et al*., 2006) revealed a strategy to better identify the *P. syringae* translocator class by screening type III-secreted substrates for HrpJ-dependent secretion. Interestingly, we found that HrpK1 and the harpins HrpW1 and HopAK1, but not the HopP1 harpin, are required HrpJ for their secretion into culture supernatants (Fig. 1). An earlier study found these same proteins were capable of restoring to differing degrees the ability to elicit an HR to a *P. syringae* mutant lacking the harpins and HrpK1 (Kvitko *et al*., 2007). The fact that HrpZ1, HrpW1, HopAK1 and HrpK1 all require HrpJ for their secretion further links these proteins in the translocation process and provides an explanation for the greatly reduced virulence and HR phenotypes exhibited by the *hrpJ* mutant (Fu *et al*., 2006; Fig. 3).

YopN-TyeA/InvE/SepL protein family members are considered 'switch proteins' because bacterial mutants lacking them are generally defective in the secretion of translocators and secrete increased amounts of type III effectors (Deng *et al*., 2005; Wang *et al*., 2008). Presently, there is no evidence to suggest that HrpJ is acting as a switch protein to shift from the secretion of translocators to effectors because the *P. syringae hrpJ* mutant secretes similar amounts of type III effectors as the wild-type strain (Fig. 1). Interestingly, however, the *hrpJ* mutant did secrete increased amounts of the HrpA1 pilus protein (Fig. 1) suggesting that HrpJ negatively controls the secretion of HrpA1, perhaps, acting as a switch protein between pilus assembly and translocation. This result is in contrast to the secretion phenotype exhibited by a *Shigella mxiC* mutant, which secreted wild-type levels of the type III needle protein (enhanced amounts of effectors, and delayed and weak secretion of translocators) after induction with congo red (Martinez-Argudo and Blocker, 2010). Another report describing the phenotype of a *Shigella mxiC* mutant found that it was enhanced for type III effector secretion but that it secreted translocators at wild-type levels when grown in cultures in the absence of any activation signal such as congo red (Botteaux *et al*., 2009). This highlights an important point to consider – comparisons between the phenotypes exhibited by mutants defective in YopN-TyeA/InvE/SepL family members can be problematic because bacterial secretion and translocation assays are done differently by different researchers and in different bacterial systems. The involvement of HrpJ in the control of translocator secretions appears undeniable because of its strong virulence and translocation phenotypes (Fu *et al*., 2006 and Fig. 2) and because of its inability to secrete the

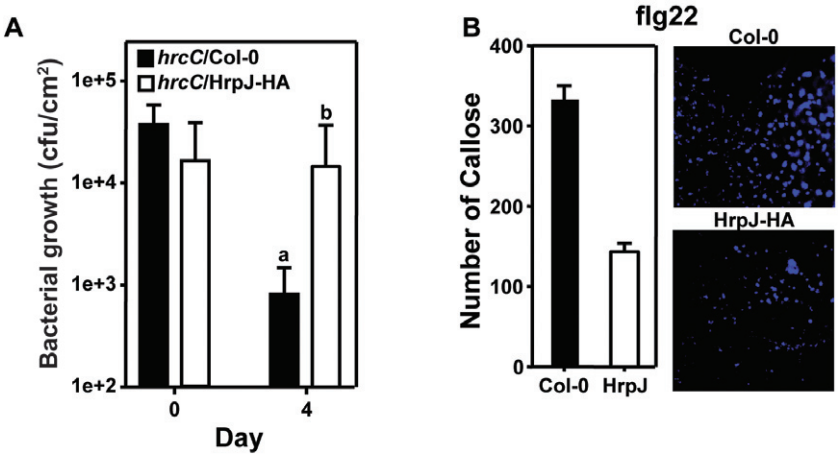

Fig. 6. Transgenic HrpJ-HA *Arabidopsis* plants exhibit reduced plant innate immune responses.
A. Bacterial growth assays of a type III defective *hrcC* mutant spray-inoculated at 2×10^8 cells ml^{-1} onto wild-type *Arabidopsis* (Col-0) and transgenic *Arabidopsis* plants expressing HrpJ-HA. The *hrcC* mutant persisted at higher numbers in HrpJ-HA plants than it did in wild-type plants at 4 days post infection.
B. Wild-type and HrpJ-HA plants were infiltrated with 1 μM flg22 and after 16 h the leaves were stained with aniline blue and examined by fluorescence microscopy for callose deposition. *Arabidopsis* plants expressing HrpJ-HA showed fewer callose foci than wild-type plants (bar graph) as depicted in a representative micrographs (right panels). Numbers are the average of 120 images taken from 12 leaves of two individual plants. Representative micrographs are shown in the panels on the right. These experiments were done at least twice with similar results.

harpins and HrpK1 translocators. The extent that HrpJ acts as a switch protein between the secretion of different classes of type III-secreted substrates will be a focus of future studies.

We found that a secretion incompetent HrpJ derivative was able to restore in culture secretion of HrpZ1 and HrpW1 to a *P. syringae hrpJ* mutant (Figs 2 and 3A). This is consistent with similar experiments done with the *Salmonella invE*, *Yersinia pestis yopN* and *Shilgella mxiC* mutants (Kubori and Galán, 2002; Ferracci *et al*., 2005; Botteaux *et al*., 2009), and also with the finding that the *Shigella* MxiC interacts with the Spa47 ATPase, an ATPase associated with the cytoplasmic side of the *Shigella* T3SS (Botteaux *et al*., 2009). Interestingly, introduction of the secretion incompetent GST-HrpJ fusion into the *hrpJ* mutant also restored the reduced levels of HrpA1 secretion observed from the wild-type strain consistent with HrpJ acting as a substrate switch from pilus assembly and translocation (Fig. 2). Thus, it is clear that HrpJ functions inside the bacterial cell to control translocator secretion. The model for HrpJ function is that it binds to the inner face of the *P. syringae* T3SS and facilitates the secretion of the HrpK1 and harpin translocators. Importantly, in the absence of HrpJ, translocators are not secreted and because a HrpZ1 C-terminal deletion derivative regained its ability to be secreted from a *hrpJ* mutant (Fig. 4), it appears that translocators may have domains that make them dependent on HrpJ for their secretion. However, we have thus far been unable to demonstrate interactions between HrpJ and T3SS apparatus proteins or between HrpJ and HrpK1 or the harpins using yeast two hybrid screens and co-immunopreciptation experiments (A. Karpisek and J.R. Alfano, unpublished). In spite of this, it remains likely that these interactions are occurring but may be too transitory or weak to be detected, or require additional proteins.

What remains less clear is why the majority of YopN-TyeA/InvE/SepL family members, including HrpJ, are secreted. Do they function extracellularly or inside eukaryotic cells? There are several plausible scenarios that are not mutually exclusive, which could explain the need for these proteins to be secreted. (i) To act as switch proteins they need to be released from the cell. These proteins may not have a function outside of the bacterial cell per se, but in order to act as switch proteins they need to be absent from the bacterial cell and this is facilitated by their secretion. (ii) These proteins may have an extracellular accessory function in the T3SS. While there is little evidence to support this, it remains possible that these proteins act in this manner. And finally, (iii) the secreted YopN-TyeA/InvE/SepL family members are translocated into eukaryotic cells where they function as effectors. Our results with HrpJ are supportive of this last scenario in that HrpJ is translocated into plant cells (Fu *et al*., 2006) and *in planta* expressed HrpJ can suppress innate immune responses (Fig. 6).

The T3SSs of bacterial plant pathogens can be divided into two groups based on the possession of similar genes, operon structures, and regulatory systems. Group 1 includes the *P. syringae* T3SS and group 2 includes the

well-studied T3SS of *X. campestris* (Alfano and Collmer, 1997; Cornelis, 2006). Group 2 T3SSs do not use a YopN-TyeA/InvE/SepL family member. Instead, based on research on the *X. campestris* T3SS, they use the HpaC protein, which is not present in group 1 T3SSs and appears to serve an analogous secretion control function as HrpJ. HpaC is known to control the secretion of early and late type III-secreted substrates from the *X. campestris* T3SS (Buttner *et al*., 2006). A *hpaC* mutant is deficient in secretion of several type III effectors as well as the translocators HrpF and XopA but retains the ability to secrete the HrpE pilus protein (Buttner *et al*., 2006; Schulz and Buttner, 2011). Additionally, HpaC interacts with and prevents the secretion of HrpB2, which is secreted early and is known to be essential for the assembly of the pilus (Lorenz *et al*., 2008). Thus, it appears that HpaC is acting as a substrate specificity switch protein in the *X. campestris* T3SS shifting the secretion from HrpB2 to the secretion of translocators and effector proteins. The differences in the secretion control proteins used by groups 1 and 2 T3SSs illustrate how plant pathogenic T3SSs apparently evolved different strategies to control type III secretion hierarchy.

In our review of the literature, we were unable to find many reports indicating that YopN-TyeA/InvE/SepL family members were translocated into eukaryotic cells and/or had effects in eukaryotic cells. There are differing reports on whether the *Yersinia* YopN is translocated into animal cells (Boland *et al*., 1996; Lee *et al*., 1998; Day *et al*., 2003). *Escherichia coli* SepL is secreted in culture and, even though it has not been reported to be translocated, Younis *et al*. suggested that it resembles a type III effector because it utilizes a class I type III chaperone, accessory proteins required by many type III effectors for their secretion (Page and Parsot, 2002; Younis *et al*., 2010). The only published evidence that a YopN-TyeA/InvE/SepL family member can act as an effector inside host cells is with the *Chlamydia pneumoniae* CopN protein (Huang *et al*., 2008; Archuleta *et al*., 2011). Expression of *C. pneumoniae* CopN in yeast or animal cells caused cell cycle arrest and disruption of microtubules (Huang *et al*., 2008). Further studies found that CopN directly binds αβ-tubulins and inhibits tubulin polymerization (Archuleta *et al*., 2011). Because genetic manipulations are not possible in *Chlamydia* the contribution of CopN to virulence could not be conventionally established using bacterial mutants. However, Huang *et al*. identified small molecules that inhibited CopN-induced growth inhibition in yeast and found that these compounds reduced *C. pneumoniae* replication in animal cells consistent with CopN contributing to virulence (Huang *et al*., 2008).

A *P. syringae hrpJ* mutant is severely debilitated in its ability to infect plants (Fu *et al*., 2006; Fig. 3). A large part of the observed reduction in virulence is due to the role HrpJ plays inside bacterial cells in translocator secretion. We know this because when the *hrpJ* mutant is complemented with a construct that produces a secretion incompetent HrpJ derivative virulence is substantially but not completely restored (Fig. 3C and D). However, we found that the *hrpJ* mutant producing a secretion incompetent HrpJ derivative could restore virulence to the same extent as a *hrpJ* mutant producing full-length HrpJ if these strains were inoculated into *Arabidopsis* plants expressing HrpJ-HA (Fig. 5). This clearly shows that HrpJ can also contribute to virulence by acting inside plant cells. Together with the finding that a *P. syringae* type III defective mutant grows to higher levels in *Arabidopsis* plants expressing HrpJ-HA compared to wild-type *Arabidopsis* and that these plants produce reduced amounts of callose deposition suggest that HrpJ contributes to virulence by suppressing innate immune responses. Our future experiments will seek to determine the extent that HrpJ produces CopN-like phenotypes in eukaryotic cells and on the identification of targets and activities of HrpJ inside plant cells.

Other future studies will be focused on the identification of *P. syringae* proteins that interact with HrpJ. Even though we have been unable to identify HrpJ interactors, there has been some success at identifying interactors for other TyeA/InvE/SepL family members (Kresse *et al*., 2000; O'Connell *et al*., 2004; Yang *et al*., 2007; Wang *et al*., 2008; Younis *et al*., 2010; Yu *et al*., 2010). The *Salmonella* SsaL family member was relatively recently found to be part of a pH-sensing complex that withholds effector secretion in the low pH conditions found inside host vacuoles (Yu *et al*., 2010). Interestingly, the plant apoplast has long been known to be acidic and it is possible that HrpJ participates in such a pH-sensing control mechanism. Elucidating the molecular roles that HrpJ plays inside bacterial cells and plant cells will likely shed light on both the timing and hierarchy of type III secretion and strategies *P. syringae* uses to disable the plant's immune system.

Experimental procedures

Bacterial strains and media

Bacterial strains and plasmids used in this work are listed in Supporting Information Table S1. *Escherichia coli* strain DH5α was used for general cloning and was grown in Luria-Bertani broth at 37°C. *Pseudomonas syringae* pv. *tomato* DC3000 was grown in King's B (KB) broth at 30°C or in type III-inducing fructose minimal medium at 22°C (Huynh *et al*., 1989). Antibiotics were used at the following concentrations (micrograms per millilitre): ampicillin, 100; chloramphenicol, 20; gentamicin, 10; kanamycin, 50; rifampin, 100; spectinomycin, 50; and tetracycline, 20.

General DNA manipulation

Restriction enzymes, T4 ligase, and DNA polymerase were purchased from New England Biolabs (Beverly, MA). Thermo-

stable DNA polymerase used in the polymerase chain reaction (PCR) was *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Primers were made by Integrated DNA Technologies (Coralville, IA). A list of the primers, their oligonucleotide sequences, and additional information are shown in Supporting Information Table S2. For cloning using Gateway technology, we amplified genes with PCR and recombined them into the pENTR/D TOPO. QuikChange Site-Directed Mutagenesis Kit was used to make site-directed mutations in *hrpJ* or *hrpZ1* following the manufacturer's instructions (Stratagene, La Jolla, CA). Constructs were introduced into *P. syringae* strains by electroporation.

Type III secretion assays

Bacterial strains were grown on KB media with appropriate antibiotics for 16 h in a 30°C incubator. Cells were harvested from plates, resuspended in 100 ml of type III-inducing fructose minimal media, and adjusted to a concentration of 4×10^8 cells ml^{-1} ($OD_{600} = 0.4$) with the appropriate antibiotics. Cultures were incubated in a 22°C shaker at 220 r.p.m. for 6 h. Cultures were then separated into cell and supernatant fractions by centrifugation. Protein precipitation of the supernatant fraction was performed by adding 25% trichloroacetic acid (Sigma Aldrich, St Louis, MO) to the supernatant, mixing and incubating at 4°C for 15 h. Supernatant fractions were centrifuged and excess supernatant was discarded. Precipitated protein was washed briefly with acetone and air-dried. The protein pellet was then resuspended in SDS buffer containing dithiothreitol (DTT) (New England BioLabs). Cell pellets were resuspended in type III-inducing fructose minimal media containing SDS and DTT. Proteins were separated by 12% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Billerica, MA) for immunoblot analyses. β-Lactamase or NPTII was used as lysis control.

Tn7 expression system

A transposon 7 (Tn7) expression system was used to express certain genes in *P. syringae* (Choi *et al*., 2005). Briefly, a Tn7 Gateway compatible entry vector was made with left and right flanking sequences, Tn7R and Tn7L, the transposase complex, an *avrPto1* promoter sequence, and a gentamycin resistant *FRT* cassette. The final gene product contained an in-frame 3′ HA tag. Genes of interest were amplified by PCR using *Pfu* polymerase with Gateway compatible, gene specific primers. Upon completion of Gateway cloning into the pLN2992 destination vector, plasmids were confirmed by PCR. The positively confirmed constructs were then transformed by electroporation into wild-type or the *P. syringae hrpJ* mutant. Transformants were checked for expression by growing them in type III-inducing condition for 6 h at 22°C. Proteins were detected with commercially available HA (Roche Diagnostics, Basel, Switzerland) or CyaA antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Plant bioassays

Hypersensitive response assays were done in *Nicotiana tabacum* cv. Xanthi. DC3000 and DC3000 mutant strains were grown for 16 h on KB media with appropriate antibiotics at 30°C. Bacteria were resuspended in 5 mM 2-(*N*-morpholino)ethanesulfonic (pH 5.6) at a cell density of 1×10^8 cells ml^{-1} and serially diluted. Leaves were infiltrated with a blunt syringe and the HR was evaluated after 24 h. The growth and disease symptoms caused by DC3000 and mutant strains were assessed in *Arabidopsis thaliana* Col-0 plants or transgenic Col-0 plants constitutively expressing HrpJ-HA. Transgenic *Arabidopsis* plants were made by introducing the *hrpJ* gene fused at its 3′ with nucleotides encoding an HA tag into pLN462, a Gateway version of the binary vector pPZP212, downstream of a CaMV 35S promoter. The resulting construct (pLN4501) was electroporated into *Agrobacterium* and *hrpJ-ha* was introduced into the plant's genome using the *Agrobacterium*-mediated floral dip method (Bechtold *et al*., 1993). T2 generation plants were confirmed to express HrpJ-HA with immunoblots using anti-HA antibodies prior to their use in experiments. To infect plants, *P. syringae* strains were grown for 16 h on KB media with antibiotics at 30°C. The strains were resuspended in 10 mM $MgCl_2$ containing 0.02% Silwet L-77 (Lehle Seeds, Round Rock, TX) and spray-inoculated at a concentration of 2×10^8 cells m^{-1}. Four leaf discs were taken for each strain at 0 and 4 days with a 0.4 cm^2 cork borer. The samples were ground in 250 ml of autoclaved water and serially diluted aliquots were grown on KB plates with the appropriate antibiotics and enumerated. Disease symptoms were assessed and documented 4 days after inoculation.

Callose deposition assays

Callose deposits were measured in leaves of *A. thaliana* Col-0 or transgenic Col-0 plants constitutively expressing HrpJ-HA. Callose depositions were induced by infiltration of 1 μM flg22. Leaves were harvested 16 h after infiltration and evacuated in alcoholic lactophenol (1:1:1:1:2 phenol : glycerol : lactic acid : water : ethanol) for 15 m and then incubated in alcoholic lactophenol at 65°C until cleared. Leaves were stained with the fluorescent dye aniline blue (0.01%) in a solution of 150 mM K_2HPO_4 (pH 9.5) for 30 m as previously described (Adam and Somerville, 1996) then mounted on slides in 50% glycerol. The aniline blue-stained callose was visualized on a fluorescence microscope (Zeiss Axionplan 2, Carl Zeiss, Oberkochen, Germany), and the number of callose deposits was quantified using Quantity One (Bio-Rad).

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Author contributions

A. K. and E. C. did the experiments; A. K., E. C. and J. R. A. analysed the data; E. C. and J. R. A. wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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