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# THE *PSEUDOMONAS SYRINGAE* TYPE III SECRETION SYSTEM: THE TRANSLOCATOR PROTEINS, THEIR SECRETION, AND THE RESTRICTION OF TRANSLOCATION BY THE PLANT IMMUNE SYSTEM

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By

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# THE *PSEUDOMONAS SYRINGAE* TYPE III SECRETION SYSTEM: THE TRANSLOCATOR PROTEINS, THEIR SECRETION, AND THE RESTRICTION OF TRANSLOCATION BY THE PLANT IMMUNE SYSTEM

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*Pseudomonas syringae* is a Gram-negative plant pathogen whose virulence is dependent upon its type III secretion system (T3SS), a nanosyringe that facilitates translocation, or injection, of type III effector (T3E) proteins into eukaryotic cells. The primary function of *P. syringae* T3E proteins is suppression of plant immunity. Bacterial proteins called translocators form a translocon that forms a pore in the host plasma membrane which is traversed by T3Es. HrpK1, a putative *P. syringae* translocator, is a type III-secreted protein important for virulence and T3E injection, but not secretion of T3Es. Harpins are a group of proteins specific to plant pathogens that are also important for T3E translocation. *P. syringae* pv. *tomato* DC3000 has 4 harpins – HrpZ1, HrpW1, HopAK1, and HopP1. Here, HrpK1 is confirmed to be a translocator. HrpK1 had a greater impact on T3E translocation than the harpins. HrpK1 and HrpZ1 disrupted liposomes. Both proteins interacted with phosphatidic acid which interfered with T3E translocation. HrpJ, a type III-secreted protein required for HrpZ1 secretion, was also required for secretion of HrpK1, HrpW1, and HopAK1. A *hrpJ* mutant

secreted elevated levels of the Hrp pilus protein HrpA1. HrpJ appears to control transition from Hrp pilus secretion to translocator secretion. Secretion was complemented by secretion incompetent HrpJ derivatives indicating that HrpJ controls secretion from inside the bacteria. The *hrpJ* mutant expressing secretion incompetent HrpJ was reduced in virulence but was complemented by HrpJ expressed inside plant cells. Additionally, transgenic Arabidopsis plants expressing HrpJ were reduced in their immune responses indicating that HrpJ can suppress plant immunity. Plants pretreated with an inducer of pathogen-associated molecular pattern-triggered immunity are unable to produce an HR. Plants, as an immune response, have evolved the ability to block T3E translocation when plant immunity has been induced prior to bacterial inoculation. This is especially true in non-host interactions whereas virulent bacteria appear to be able to attenuate injection restriction in host plants via T3E activity.

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## Table of Contents

# List of Figures and Tables

Chapter 1



Chapter 2

Chapter 4

# Chapter 1

Background

**Background on *Pseudomonas syringae***

*Pseudomonas syringae* is a Gram-negative hemi-biotrophic bacterial plant pathogen comprised of more than 50 pathovars capable of infecting a wide variety of plant species in a host-pathovar specific manner (5, 147). *P. syringae* causes a number of different diseases, often the symptoms include, but are not limited to, chlorotic and necrotic lesions on aerial plant tissues (129). In addition to its endophytic lifestyle as a pathogen growing within a plant, *P. syringae* can also exist as an epiphyte, that is it can grow on the outer surface of a plant (78).

A γ-proteobacteria, *P. syringae* is rod-shaped with polar flagella and is an obligate aerobe that produces fluorescent pigments (63). The fluorescence can be observed when grown on King's B media and is attributable to the production of a pyoverdin siderophore that functions in iron acquisition (37, 99). *P. syringae* strains are members of the order Pseudomonadales (150) and are in the family of Pseudomonadaceae; the common bacteria associated with this family are water bacteria or plant pathogens (191). The genus *Pseudomonas* was first described by the German botanist Walter Migula at the end of the 19th Century (131).

Individual strains within the *P. syringae* species are generally able to cause disease in a narrow host range and are given the pathovar epithet to distinguish among them at the infrasubspecific level. For example, the first identified strain of *P. syringae* was isolated from lilac (*Syringa vulgaris*) and was ultimately given the name *P. syringae* pv. *syringae* where the pathovar name is indicative of the host plant (195). Some pathovars are further divided into races

on the basis of host range within a plant cultivar (38). In non-host plants *P.* syringae induces a programmed cell death response known as the hypersensitive response (HR) that is a plant immune response associated with resistance (100). While *P. syringae* is primarily thought of as a pathogen, there is evidence that some strains may not be pathogenic (78, 134). It remains possible that these strains are pathogens but simply have not been tested on the proper host plant; however, in certain cases, strains are lacking genes thought to be essential for the type III secretion system (T3SS), which is generally considered to be necessary for pathogenesis (36).

One of the model *P. syringae* pathovars and the most extensively studied is *P. syringae* pv. *tomato* DC3000 which causes bacterial speck on its hosts tomato (*Solanum lycopersicum*) and the model plant *Arabidopsis thaliana* (93, 189). DC3000 has a 6.4 megabase genome and 2 plasmids and is an ideal strain for research as it was the first pathovar of *P. syringae* to be sequenced and it infects Arabidopsis (29). *A. thaliana* Col-0 has also been fully sequenced and is considered amenable to laboratory research due to its small size, relatively rapid growth, and abundant production of seeds (13). Additionally, DC3000 is an economically relevant pathogen as it causes bacterial speck in tomato, which results in necrotic lesions on the leaves and, more importantly, on the fruit which reduces yield and marketability (87). DC3000 serves as a model strain for a species that collectively can cause disease in a number of economically important plant species.

**Type III Secretion Systems**

A necessary component of the virulence of *P. syringae* is the presence of a functional T3SS (4, 38). Often the term injectisome or non-flagellar T3SS is used to specify virulence associated T3SSs rather than flagellar T3SSs (52, 192). Here, the term T3SS will refer to virulence-associated T3SS unless otherwise specified. T3SSs are used by numerous Gram-negative bacteria to translocate, or inject, type III effector (T3E) proteins into the cells of eukaryotes (39, 62). More than 25 species of bacteria are known to employ T3SSs as a mechanism of interaction with their respective eukaryotic hosts (40). T3SSs are found in animal pathogens such as *Yersinia, Salmonella,* and *Shigella*, phytopathogens such as *Xanthomonas, Erwinia,* and *Ralstonia*, but are also found in bacteria that use their T3SS to develop symbiotic relationships with eukaryotes such as *Rhizobium* (39).

At least 25 proteins are used to construct the T3SS apparatus spanning both the inner and outer bacterial membranes and possessing an extracellular needle or pilus (Fig. 1) (46, 192, 194). Many of these proteins are similar in either sequence or function to proteins of bacterial flagella and export of the component proteins is analogous between the two systems (22, 132). T3SSs differ from flagella in that they inject T3E proteins into host cells and do so in a manner that requires host cell contact (21, 137, 188).

Bacterial plant pathogens gain access to their hosts by entering through wounds or natural openings such as stomata (20). While many animal bacterial pathogens are intracellular, bacterial plant pathogens exclusively attack their

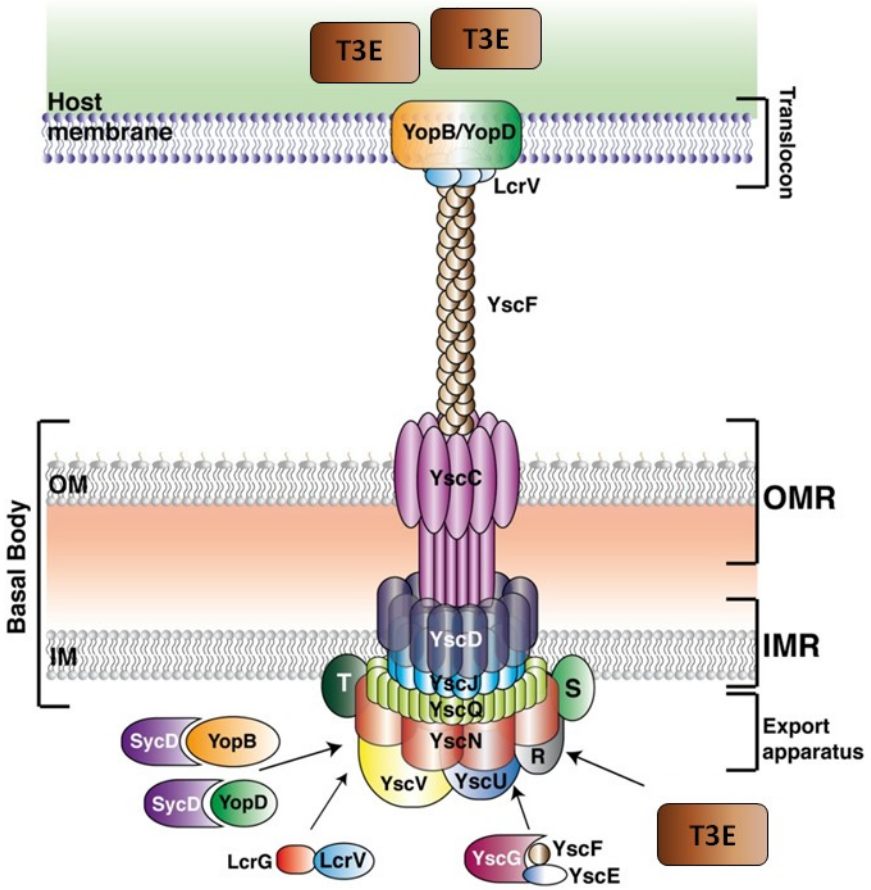


**Fig. 1. Schematic diagram of the T3SS.** The protein names used here are from the *Yersinia* spp. T3SS. The basal body consists of an inner membrane ring (IMR) and an outer membrane ring (OMR) spanning both the inner and outer bacterial membranes (IM and OM). The needle proteins (YscF) followed by the translocators and tip protein (YopB, YopD, and LcrV) travel through the basal body (YscC, YscD, and YscJ) and are thought to self-assemble. The type III effectors (T3E) then travel through the needle and translocon in the host membrane directly into the cytoplasm of the host cell. The export apparatus proteins are YscN, YscV, YscU, YscQ, YscR (R), YscS (S), and YscT (T). Many of the secreted proteins have congnate chaperones such as SycD for the translocators, LcrG for the tip protein, YscG and YscE for the needle, as well as assorted chaperones for T3E (not depicted). This figure is taken from Izore *et al*. (83).

hosts extracellularly from the apoplastic environment which necessitates penetration of the cell wall prior to injecting T3E proteins into the plant cell cytoplasm. In order to accomplish this, the injectisome used by plant pathogens is a long thin structure of variable length termed the Hrp pilus, in contrast to the shorter needle structures typically found in animal pathogens (86). The similarities and differences between the T3SSs of plant and animal pathogens are illustrated in Figure 2.

**Type III Effectors**

Ultimately the function of a T3SS is to inject T3E proteins into the host cells. In plant pathogenic bacteria that make use of T3SSs, the T3E proteins are responsible for suppressing immune responses and ultimately allow bacterial colonization and disease (3, 7). T3E proteins are known to function in an assortment of ways, having very diverse host targets, disrupting various signal transduction pathways, cell trafficking and gene expression, and localizing to different organelles (Fig. 2). Often the T3E proteins will mimic host proteins, work together in concert, or even have their function under strict temporal regulation (61, 164).

Most individual T3Es in *P. syringae* appear to suppress plant immune responses, which will be discussed in more detail later (26, 69, 84). The majority of T3E targets and biochemical functions are unknown and this remains a popular area of research (Fig. 3) (14, 60, 64, 65, 112, 123, 124, 144, 165, 193, 197). The number of T3E proteins in plant pathogens tends to be larger than in human pathogens; DC3000 itself has at least 30 T3E genes (29, 177). Couple

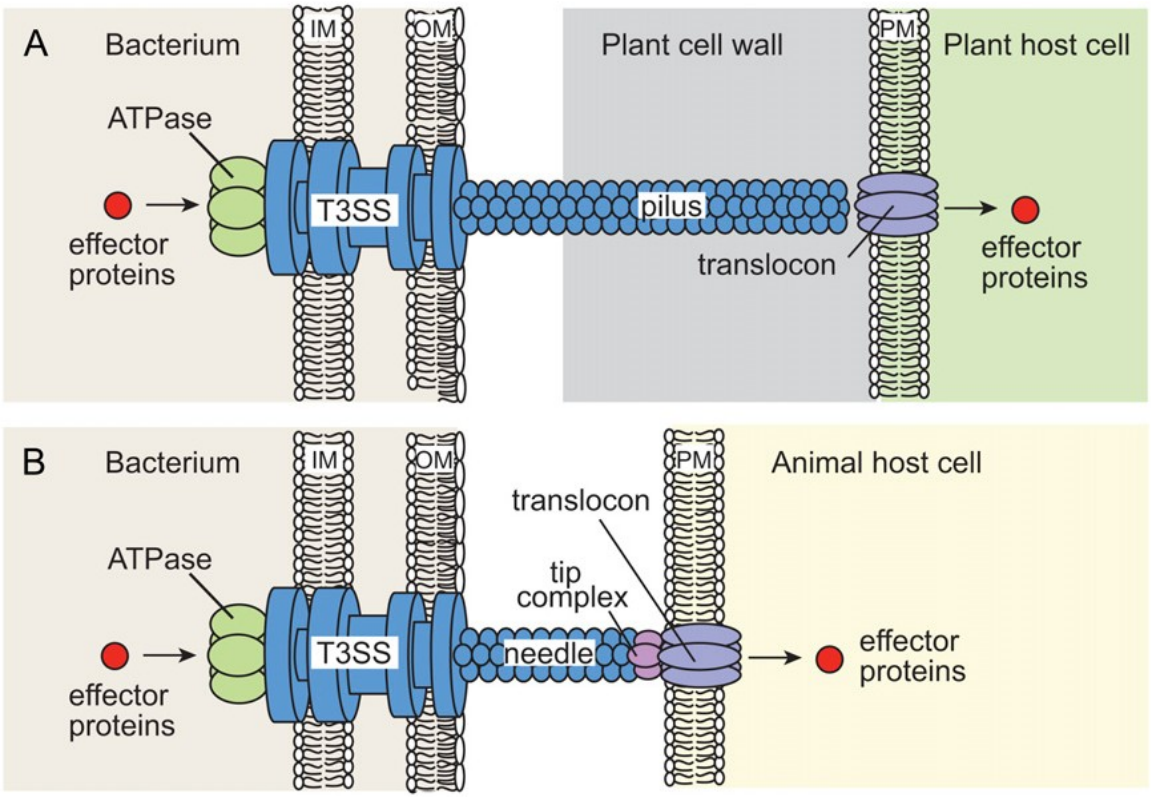


**Fig. 2. Schematic diagram highlighting the differences and similarities between the T3SSs of plant and animal pathogens.** A) Representative T3SS from a plant pathogen. B) Representative T3SS from animal pathogens. The secretion apparatus spans both bacterial membranes and is associated with a cytoplasmic ATPase. The T3SS from plant pathogenic bacteria is connected to an extracellular pilus that presumably spans the plant cell wall. The T3SS system from animal pathogenic bacteria is associated with a short extracellular needle, which serves as a transport channel for secreted proteins. The needle is linked via the so-called tip complex to the translocon, which forms a proteinaceous channel in the host plasma membrane and allows transport of effector proteins into the host cell cytosol. Evidence for the presence of a tip complex in plant pathogenic bacteria is still missing. IM, Inner membrane; OM, outer membrane; PM, plasma membrane. This figure is taken from Buttner and He (30).

that with the diversity of T3E genes in the pangenome of *P. syringae* and the highly divergent nature of T3E repertoires of individual strains and it seems likely that studying T3E function will continue to be a popular topic of research and likely will yield a number of new and exciting discoveries (15, 117).

One might ponder the benefit of *P. syringae* maintaining so many T3E genes in its T3E inventory. To compound this issue further is the fact that single mutants lacking individual *P. syringae* T3E genes have very little effect on virulence. The reason for this appears to be effector redundancy (185). This becomes an important point when experimenting with mutants that may have incremental or masked virulence phenotypes. Because of these issues, strategies have been employed to make large-scale reductions in the T3E repertoires of *P. syringae* (77, 185). These strategies have been used to identify minimal sets of T3E genes for virulence, identify T3E genes responsible for host range, group similar functioning T3E genes, and create intermediately virulent strains (42, 107, 139, 185).

All known T3E proteins function inside the host cells after having been translocated by the T3SS. Understanding how the pathogens are able to get bacterial derived proteins across both membranes, through a pilus or needle, across host cell walls, through host plasma membranes and into the cytoplasm is therefore very important. Additionally, the T3SS apparatus and translocator proteins, those that form the opening in the host plasma membrane, form a literal bottleneck for bacterial pathogens as all type III-secreted proteins must traverse

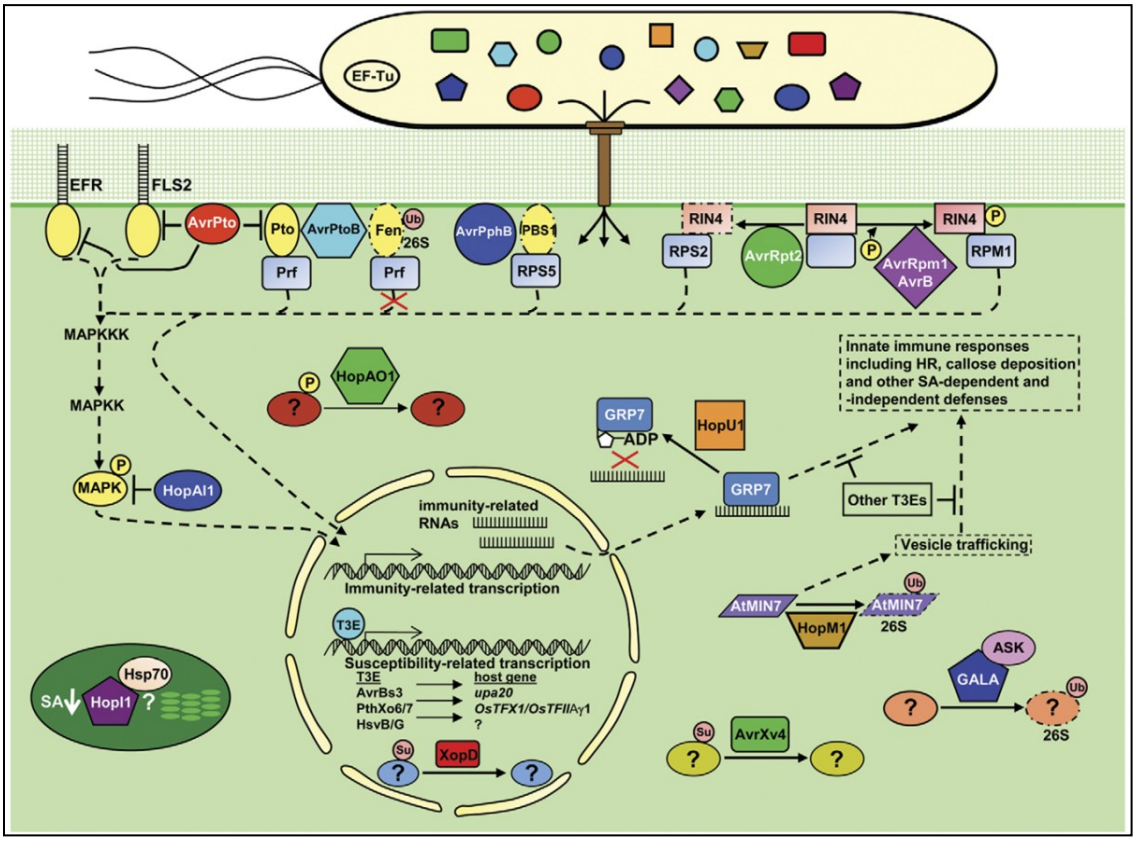


**Fig. 3. Plant targets and activities of type III effectors from phytobacterial pathogens.** Bacterial plant pathogens inject many different T3Es into plant cells via the T3SS. The activities of T3Es can be recognized by plant R proteins inducing ETI. The R protein RPM1 causes ETI by recognizing the phosphorylation (P) of RIN4 by the T3Es AvrRpm1 and AvrB, while RPS2 causes ETI upon the cleavage of RIN4 by the T3E AvrRpt2. The T3E AvrPphB degrades the PBS1 kinase inducing RPS5-dependent ETI. The R protein Prf recognizes the interaction of the Pto kinase with AvrPto or AvrPtoB to elicit ETI; however, AvrPtoB ubiquinates (Ub) the Fen kinase targeting it for degradation and preventing recognition by Prf. Plants can also use receptor kinases such as EFR or FLS2 to detect PAMPs. This leads to PTI. AvrPto inhibits the kinase activity of Pto, FLS2, and EFR. The HopAI1 T3E is a phosphothreonine lyase that suppresses MAPKs. The HopAO1 T3E is a protein tyrosine phosphatase whose target is unknown. The HopU1 T3E is a mono-ADP-ribosyltransferase that modifies GRP7 glycine-rich RNA-binding protein and probably prevents it from binding to RNA. The HopM1 T3E causes the ubiquination and degradation via the 26S proteasome of AtMIN7, which may be involved in vesicle trafficking. The GALA T3Es contain F-box domains and can interact with plant ASK proteins (part of an SCF-type E3 ubiquitin ligase complex). GALAs are predicted to change the ubiquitination status of host proteins. The T3Es XopD and AvrXv4, which function in different locations in the plant cell, are isopeptidases that remove SUMO (Su) from host proteins. The chloroplast localized, J domain-containing T3E HopI1, suppresses salicylic acid (SA) production and may associate with Hsp70. T3Es AvrBs3, PthXo6/7, and HsvG/B bind to specific promoters in the nucleus inducing the transcription of genes favoring pathogenesis. Broken lines indicate plant responses and solid lines T3E activities. This figure is taken from Block *et al*. (20).

this system in order to subvert host immunity and cause disease. This point of confluence for T3E protein delivery could be a target for both host immune systems as well as for therapeutic or preventative measures to reduce the effects of the resulting diseases. The latter can only be accomplished by more fully understanding the mechanisms involved in T3E translocation.

**Secretion vs. Translocation**

Secretion by definition occurs when the bacterium moves any of its own proteins from inside the cell to outside the cell. Translocation takes place when a bacterial protein is injected into the cytoplasm of a eukaryotic cell. It is important to differentiate between the two. All translocated proteins by definition are secreted but a protein can be secreted without being translocated. All T3E proteins act inside the plant cell and therefore must be both secreted and translocated. Surprisingly it seems that most secreted substrates are also translocated even in instances where the primary function appears to take place extracellularly (59, 108, 152, 156). It is unknown whether the translocation of all type III-secreted substrates predicted to act outside the plant cell is biologically relevant. Potentially the translocation observed for some of the proteins may be an artifact of the assays themselves and they are not translocated in nature.

Importantly though, through genetic manipulation, the difference between secretion and translocation can be exploited for research. Whereas mutants that block both secretion and translocation are considered T3SS-deficient, mutants that are positive for secretion of T3E proteins but severely reduced or completely unable to inject T3E proteins have been observed. In these cases it is presumed

that these genes encode proteins that function in translocation, either directly as with translocator proteins, or indirectly such as the regulation of translocator protein secretion (23, 31, 59, 108, 152).

**Hrp/Hrc T3SSs**

The T3SS of *P. syringae* is encoded by the *hrp* (*HR* and *p*athogenicity) and *hrc* (*HR* and *c*onserved) genes which are contained in the *hrp*/*hrc* cluster (6). The Hrp T3SSs are conserved among, but not limited to, bacterial plant pathogens (41, 119, 173). *hrp* genes were first described in *P. syringae* pv. *phaseolicola* (120) and then in other strains of *P. syringae* (9, 16, 43, 91, 92) as well as other phytopathogenic bacteria such as *Xanthomonas* (9, 91, 92), *Erwinia* (16, 17), and *Ralstonia* (28) among others (119). The nomenclature was established based on the discovery that mutations made in these genes in *P. syringae* pv. *phaseolicola* resulted in reduced pathogenicity in bean plants and the inability to elicit the HR in tobacco (120).

Hrp T3SSs can be divided into two groups with *P. syringae* belonging to Group 1 along with *E. amylovora* and the human pathogen *Vibrio parahaemolyticus* all of which are γ-proteobacteria. The other group of Hrp T3SSs, Group 2, includes *X. campestris*, *R. solanacearum*, and the human pathogen *Burkholderia pseudomallei*, the latter two being β-proteobacteria (6, 39). The two groups are divided based on the presence of similar genes, regulatory elements, and arrangement of the genes within their respective *hrp*/*hrc* clusters (4). When one compares the two groups based on Hrp systems

versus their phylogenic groupings there are obvious discrepancies, suggesting horizontal acquisition (6).

Included in the approximately 26 kb *hrp*/*hrc* locus are 9 genes that share homology with animal T3SSs called *hrc* genes, and 17 *hrp* genes that do not have clear homologs in other T3SSs (147). The 9 widely conserved *hrc* genes are named with the final letter of the gene name corresponding to the gene name used in the *Yersinia* spp. homolog (24). Most T3E genes are known as *avr* (*avi*rulence) and *hop* (*h*rp-dependent *o*uter *p*roteins) in the *hrp* T3SS (38). The difference in nomenclature is that *avr* genes were discovered initially because the induce immunity in resistant plants, rendering the bacteria avirulent. But in essence all T3Es are thought to possess a virulence function even though for many T3Es these remain to be explored.

The *hrp/hrc* gene cluster present in *P. syringae* strains is one-third of a three part pathogenicity island (pai). Pais are sequences of DNA, often large, that contain virulence genes and are frequently found in pathogenic strains but are absent from non-pathogenic strains of the same or related species (73). Often pais are thought to have been acquired through horizontal gene transfer (73). The *hrp/hrc* pai contains the conserved effector locus (CEL) and exchangeable effector locus (EEL), which flank the *hrp*/*hrc* cluster on either side. The EEL encodes diverse genes including effectors and contains mobile genetic element sequences. The CEL, as its name implies, is more conserved, and contains effectors shown to be important to virulence (4). In addition to this pai, *P. syringae* contains at least 5 additional chromosomal pais and a pai on one of its

plasmids that contain T3E genes (68, 77, 107, 185). These pais were identified on the basis of having multiple virulence genes, a unique G + C content relative to the rest of the genome, occupying large regions, often having multiple repeat sequences at the borders, being unstable, and flanked by mobile genetic elements (4, 73).

**Structure of the T3SS apparatus**

The biogenesis of the supramolecular complex that is the T3SS requires highly regulated and precise interactions among the proteins that comprise it. Production of this complex necessitates the coordinated construction of cytosolic, periplasmic, membrane-bound, and extracellular proteins (83). Based on its structure and mode of function, the T3SS is considered a nanosyringe as it is able to deliver T3E proteins through a narrow channel in an ATP-dependent manner (127). Assembly of the T3SS is thought to take place in a concerted stepwise fashion. First, the basal body spanning both the inner and outer membranes is constructed. Second, the needle/pilus structure is assembled. Third, the translocator proteins are secreted and ultimately inserted in the host plasma membrane, at which point T3Es are injected (83).

The basal body begins to form the opening through which type III secreted substrates will be secreted (127). Three proteins make up the stable core of the type III basal body (98, 103). Based on the relatively small size of these proteins (all are <70 kDa) and the necessarily large size of the basal body structure (>300 angstroms), the proteins must form highly symmetric oligomers (127). In confirmation of this, electron microscopy experiments have shown that the basal

body is a ring-like structure composed of high-order radial symmetry (79, 125, 126). These rings span both the inner and outer bacterial membranes. The inner membrane-spanning ring (IMR), which is the larger of the two membrane-spanning portions, is itself made up of two sets of rings (135). PrgH and PrgK are the constituent proteins of the *Salmonella enterica* IMR in which the seminal and most elegant research has been conducted (30, 103, 162). The corresponding IMR genes in *P. syringae* are *hrcJ* (75) and *hrcQ*. BLAST searches on NCBI identify *hrcQ* as encodes a *yscD* domain (*Yersinia spp.* IMR protein) though this similarity has never been published. The *hrcQ* gene is encoded on the *hrp/hrc* cluster and has been annotated as a T3SS apparatus protein. The outer membrane ring (OMR) of the T3SS is composed of proteins belonging to the secretin family of proteins (135). Secretins are proteins that form multimeric ring structures in the outer membranes for a variety of different specialized multiprotein secretion systems including type II and type IV secretion (102). The secretin making up the OMR in *P. syringae* T3SSs is HrcC (47). Mutants in the *hrcC* gene are deficient in type III secretion and are often used as negative controls.

As conserved as the basal body is among T3SSs, the Hrp pili and needles of T3SSs are very divergent. This is likely due to the different hosts, environments, and modes of infection of the different bacteria that employ them. The main component of the Hrp pilus is the HrpA1 protein. This protein is secreted via the T3SS through the basal body and polymerizes to form the pilus (86, 115). The Hrp pilus appears to be much longer than the T3S needles of

*Yersinia* spp. which are consistently only 60 nm in length (80, 104). The indefinite length of the Hrp pilus is probably necessitated by the pathogen's need to bridge the variably thick cell wall of the host plant. This is analogous to the filament found in enteropathogenic *E. coli* where the short needle, composed of EspF and comparable in length to the *Yersinia* needle, is extended by a long flexible filamentous structure composed of the protein EspA. This supramolecular sheath is thought to be necessary for the pathogen to deliver type III substrates across the mucus layer of enterocytes in the intestine (45, 163). In addition to the variability caused by mechanical necessity due to infection types, the pili, needles, and filaments may have further variability imposed on them because they come in direct contact with host cells. This contact makes pilus/needle proteins poteintial targets of host defense responses, likely adding selective pressures via co-evolution. Indeed HrpA1 seems to be under strong diversifying selection which enables maintenance of genetic diversity (71).

**Translocon and harpins**

In terms of molecular host-pathogen interactions nothing epitomizes the literal term more than the translocon. It is the exact point where phytopathogenic bacteria and plant cells make contact. The translocon proteins have been implicated in recognizing host cell contact (1). The translocators imbed themselves within the eukaryotic plasma membrane (128), making them the intimate interface between the plant cell and the bacterial cell. T3Es cannot be efficiently translocated without translocators, effectively rendering the bacteria non-pathogenic; therefore, translocators are of the utmost importance to

pathogens employing T3SSs (182). Translocator proteins, being essential and entrenched in the host-pathogen interface, are also likely targets of host immune responses (12, 168).

The translocon complexes for some strains of animal pathogens have been more extensively characterized than in plant pathogens. They are made of two hydrophobic translocator proteins that physically form the pore in the host plasma membrane and one hydrophilic protein known as the tip protein that connects the needle to the translocators (138). The translocon of plant pathogens seems to be quite different. In *P. syringae* HrpK1 is a putative translocator protein. It is hydrophobic, has a transmembrane domain and shares certain biochemical properties with the YopB family of translocators from *Yersinia* spp. (152). There is no protein similar to the other hydrophobic translocator, typified by YopD; nor is there a hydrophilic tip protein that can be identified (30).

Coupled with the absence of the canonical components of the translocon, is the presence of a unique class of proteins conserved among bacterial plant pathogens but absent in their animal counterparts. These are the harpin proteins. Harpins have long been implicated in translocation of T3E proteins (6). Rich in glycine and lacking cysteine, harpins are able to elicit an HR when purified and infiltrated into plant tissue (74, 187). While highly conserved among plant pathogenic bacteria, the number of harpins present in each strain is variable. DC3000 has a suite of four harpin genes, *hrpZ1, hrpW1, hopAK1,* and *hopP1* (108). HrpZ1 only contains a harpin domain, while the others have additional

domains. HrpW1 and HopAK1 both have pectate lyase domains, and HopP1 has a lytic transglycosylase domain (32, 108, 148).

**Regulation of T3SS**

Regulation of the expression of the T3SS is obviously important because the bacterial cells using a T3SS need to make sure that the apparatus is constructed prior to contact with host cells. The *hrp* genes are not constitutively expressed. For example under nutrient rich conditions they are turned off. The *hrp* genes are only turned on when necessary, that is, in the plant apoplast at the concentration where pathogenicity is most beneficial to the bacteria. When T3SS genes are turned on there is a concomitant down-regulation of many housekeeping genes suggesting that the T3SS comes at a cost to the basic bacterial lifestyle (109). Expression of the Hrp T3SS and most of the T3E genes is activated by the alternative sigma factor HrpL, which recognizes type III-related promoters (54). By using a reporter transposon to enlarge the set of known Hrp promoters, a hidden Markov model was defined to search for additional Hrp promoters (57). This helped further identify T3E genes, as well as virulence genes unrelated to the T3SS such as the toxin coronatine. A whole-genome microarray was constructed for DC3000 that identified nearly 200 genes regulated by HrpL (54). Like DC3000, the T3SSs of other *P. syringae* strains are also under the control of HrpL (116).

HrpL is a 54 kDa alternative sigma factor (sigma$^{54}$). Regulation of sigma$^{54}$-RNA polymerase activity is achieved by the action of specific enhancer-binding proteins. In phytopathogenic species of *P. syringae hrpL* expression is regulated

by HrpR and HrpS (81, 90). HrpR and HrpS, two atypical two component response regulators, are encoded by the *hrpRS* operon and form a heterohexamer (HrpRS) that binds the *hrpL* promoter to activate its transcription (90).

HrpR and HrpS are controlled by HrpV and HrpG (90). *hrpV* and *hrpG* are both encoded on the *hrpC* operon (48). HrpV interacts with and negatively regulates HrpS (90, 153). HrpG interacts with and suppresses HrpV which frees HrpS allowing HrpRS to activate downstream *hrp/hrc* genes (184). In addition to HrpG and HrpV, *hrpR* and *hrpS* expression is effected by the GacS/GacA two component system (33). This two component system is found in numerous Gram-negative bacteria and often plays a role in regulating pathogenicity (76). GacS is the sensory histidine kinase and GacA is the response regulator (174). Also, the HrpA1 protein, the main component of the Hrp pilus, is important for *hrpR* and *hrpS* expression, as a *hrpA1* mutant exhibits reduced transcription of *hrpR* and *hrpS* (186).

As mentioned the T3SS is only turned on under certain environmental conditions. This can be accomplished in the lab using certain T3SS-inducing minimal media that simulate the plant apoplastic environment. T3SS-inducing media are acidic, nutritionally poor, and contain specific sugars, often strain-dependent, as the lone carbon source. Expression of T3SS genes is highest when *P. syringae* bacteria are grown at about 22°C (181). Induction of the *P. syringae* pv. *phaseolicola hrpL* gene *in planta* is much greater compared to induction in T3SS-inducing media, suggesting that there are signals in the plant

that contribute to T3SS gene induction (155, 174). Indeed, the expression of *hrpA1* was shown to be induced to higher levels in the presence of small, water-soluble compounds from plant exudates (72).

**Secretion hierarchy**

Secretion hierarchy, that is the order in which T3SS substrates are secreted, is an important area of research. This process is thought to be tightly regulated such that certain classes of proteins are secreted together in a temporal manner. For example, all the pilus components would be secreted together early and then turned off prior to translocator secretion (Fig. 4) (160). A hierarchy likely exists even within classes of proteins, as there are probably T3E proteins that are secreted early or late relative to one another. This would prevent interference among their activities or potentially allow for certain T3E proteins to modify the activity of other T3E proteins (164). This likely all takes place within the bacterial cell, in *Salmonella* T3SSs there is a sorting platform where the order of secretion is determined (110).

Control of secretion hierarchy may be controlled in part by HrpL. Induction kinetics of the HrpL regulon identified eight genes whose products are rapidly expressed, including the pilus protein HrpA1, the translocator HrpK1, and the harpins HrpW1 and HrpZ1 (54). It makes intuitive sense that these proteins would necessarily be among the first proteins secreted as T3E translocation would not be possible without them.

Beyond gene expression there are other proteins known to be involved in secretion hierarchy. The YopN/TyeA family of proteins is important in controlling

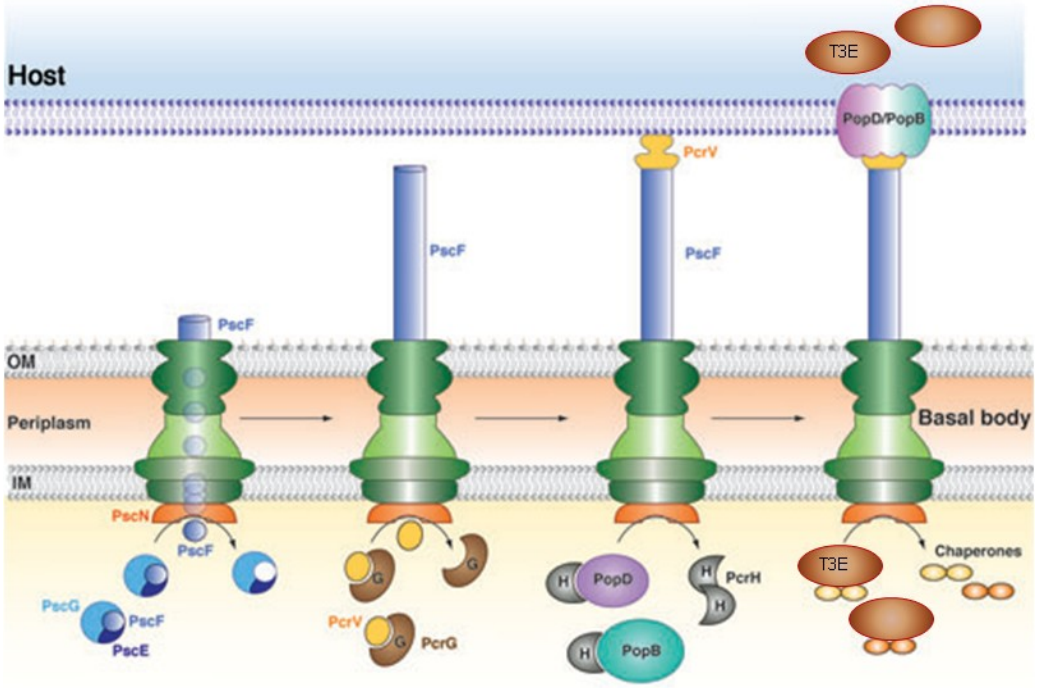


**Fig. 4. Schematic diagram showing the hierarchical nature of type III secretion in the T3SS of *P. aeruginosa*.** Figure depicted sequentially moving from left to right. Upon formation of the base rings (green), PscF is released from its chaperones (PscG and PscE) and polymerizes to form the T3SS needle. The tip protein PcrV is released from its cytoplasmic partner (PscG) and forms the tip of the PscF needle after being secreted through it. Translocator proteins PopB and PopD release PcrH and are secreted. Upon formation of the Pop translocon on the eukaryotic membrane, type III effectors (T3Es) produced in the bacterial cytoplasm release their cognate chaperones and are injected through the translocon pore and into the target cytoplasm. IM, inner membrane; OM, outer membrane. This figure is taken from Mattei *et al.* (128).

the secretion of translocator proteins (27, 151). The family member from *P. syringae* is HrpJ which is necessary for the secretion of the harpin HrpZ1 but does not control T3E secretion (59). A *hrpJ* mutant is severely reduced in virulence and T3E translocation in both *P. syringae* and *E. amylovora* (59, 143). Because *hrpZ1* mutants have subtle virulence phenotypes and the *hrpJ* mutant phenotype is so severe, HrpJ likely controls the secretion of more proteins than just HrpZ1. Further investigation of this protein will help identify *P. syringae* translocators as well as provide insight into the control of secretion hierarchy.

Another component of the regulation of secretion hierarchy may be proteins containing a feature known as the type III secretion substrate specificity switch (T3S4). YscP is the *Yersinia* protein with a T3S4 domain and is referred to as a molecular ruler as it is thought to control the length of the needle (2). YscP function is dependent on its interaction with YscU, a component of the T3SS basal body (89). The *yscP* mutant secretes the needle protein in uncontrolled abundance (137). A mutant lacking the protein proposed to be the molecular ruler in *P. syringae*, HrpP, actually secretes less pilus subunits (i.e., the HrpA1 protein) than wild type DC3000, the opposite phenotype of the *yscP* mutant (136). Therefore, the transition of secretion of HrpA1 to translocator secretion is likely controlled by a different protein.

### *P. syringae* toxins and hormones

In addition to the T3SS, there are a number of other pathogenicity factors employed by *P. syringae* including toxins and hormones that are important contributors to interactions with hosts. *P. syringae* strains produce a large array

of pathovar-specific phytotoxins including coronatine, syringomycin, syringopeptin, tabtoxin, syringolin, and phaseolotoxin (18). It has been reported that there may be a correlation between the size of the T3E repertoire of a given strain and the number of toxins it produces suggesting a possible compensatory relationship between the two (15).

DC3000 contains the phytotoxin coronatine which is a non-host specific toxin that contributes to the chlorosis associated with disease symptoms. Coronatine appears to function by mimicking the plant hormone methyl jasmonate (55). Additionally, coronatine plays a role in opening stomata, enabling the bacteria to enter the leaf by overcoming pre-invasive immunity which normally causes stomata to temporarily close (130). Syringomycin is produced by most strains of *P. syringae* pv. *syringae* and functions to induce necrosis in infected plants. It functions by inserting itself in lipid membranes allowing for cation leakages that are deadly to the plant cells (18). A toxin with a similar function to syringomycin is syringopeptin. It too forms pores in membranes contributing to necrosis but the structure is much different (82). Like syringomycin, syringopeptin was initially identified in *P. syringae* pv. *syringae*. All strains of *P. syringae* pv. *syringae* that have been analyzed produce both syringomycin and syringopeptin suggesting interrelated roles for the toxins in the plant pathogen interaction; however, the reason for keeping two toxins with such similar function remains unclear (18). Tabtoxin is a monocyclic β-lactam produced by *P. syringae* pv. *tabaci* that irreversibly inhibits glutamine synthetase. This mode of action prevents glutamine synthetase from detoxifying ammonia.

Phenotypically the toxin manifests itself by inducing chlorosis in plants (175). Syringolin A (SylA) inhibits the 26S proteasome (67). This toxin has been shown to help *P. syringae* pv. *syringae* B728a open stomata in bean plants similar to the function of coronatine (161). Additionally, SylA has recently been implicated in distant colonization (101). Phaseolotoxin is produced by *P. syringae* pv. *phaseolicola* the causative agent of halo blight on legumes. Phaseolotoxin competitively inhibits ornithine carbamoyl transferase resulting in a deficiency in arginine which contributes to chlorosis or the characteristic “halo” of the disease (133). This is not a comprehensive list of *P. syringae* toxins. Some are more conserved than others while many are strain specific. (15).

In addition to producing toxins and T3E proteins, many *P. syringae* strains produce the plant hormone indole-3-acetic acid (IAA) (158, 169). The *iaaL* gene, which is involved in IAA biosynthesis, is preceded by a *hrp* promoter suggesting a role in pathogenicity but the function in colonization remains unclear. *P. syringae* may also induce IAA responses in plant cells through transformation of plant-derived IAA precursors. *P. syringae* pv. *syringae* B728a has been shown to produce a nitrilase and an aldoxime dehydratase; together these enzymes can transform indole-3-acetaldoxime (IAOx) to IAA. During infection tryptophan catabolism is activated resulting in accumulation of IAOx, which suggests these genes may play an important role in manipulating plant metabolism to promote infection (158).

**Plant immunity**

The interactions between pathogens and plants lead to a constant struggle in which plants evolve to avoid disease and the pathogens evolve to subvert detection. This has inevitably led to a back-and-forth genetic battle between the plant immune system and bacterial virulence factors, a so-called molecular arms race. Plants evolve genes whose products can recognize pathogens while the genes corresponding to the recognized bacterial proteins are modified or discarded to prevent recognition (10, 105). Plants have developed a two-pronged defense strategy against pathogens. First, plants likely evolved extracellular receptors to detect the presence of microbial organisms. Epitopes from highly conserved microbial structural elements termed pathogen (microbe)-associated molecular patterns (PAMPs/MAMPs) are recognized by these receptors called pattern recognition receptors (PRRs) (88). Recognition of PAMPs leads to PAMP-triggered immunity (PTI). Second, plants have intracellular receptors (R proteins) that recognize the presence of translocated bacterial effectors, historically referred to as avirulence (Avr) proteins (51). Originally proposed to be a direct interaction (96), it is now widely accepted based on experimental evidence that the recognition of bacterial Avr proteins is indirect (44, 180). This second line of defense is known as effector-triggered immunity (ETI).

Differences between PTI and ETI are clear and defined in terms of the recognition events that lead to the activation of each. Similarly the elicitors themselves are also usually clearly different. PAMPs are generally conserved

throughout microbes and contribute to their fitness in general ways. For example, both flagellin and peptidoglycan are PAMPs where flagellin contributes to motility and peptidoglycan contributes rigidity and structure to the bacterial cell (35, 70) Other PAMPs include, elongation factor Tu (EF-Tu) and lipopolysaccharides from bacteria, oomycete glucans, and fungal chitins (176). Effectors instead are very specific to certain pathogens, sometimes only found in a single pathovar, and play a role in virulence. However, at this level the lines between PTI and ETI begin to be blurred as many effectors are highly conserved among pathogens and some PAMPs are narrowly conserved (146, 176).

While the mode of recognition is clearly different, the responses from both PTI and ETI are very similar. The fact that pathogens present both PAMPs and effectors simultaneously further obscures differences between PTI and ETI (94, 154, 176). Both lines of defense are complex and highly coordinated having been driven by eons of co-evolution (118). In spite of the overlap between PTI and ETI, in this dissertation the responses will be treated as separate and each will be further characterized individually to highlight differences in the biology.

**PAMP-Triggered Immunity**

PTI is the frontline of immunity, where the plant recognizes the presence of microbes. PAMPs are recognized by PRRs. The PRR that recognizes flagellin is FLAGELLIN SENSITIVE 2 (FLS2). It is able to recognize a 22 amino acid peptide within flagellin known as flg22 (172). FLS2, like many PRRs, is a leucine-rich repeat receptor kinase (LRR-RK) belonging to the subfamily XII of LRR-RK. FLS2 has an extracellular domain with 28 LRR motifs, a transmembrane domain,

and a cytoplasmic serine/threonine kinase domain. FLS2, which was first identified in Arabidopsis (66), has since been found in a number of other plants (142). FLS2 has been shown biochemically to directly bind flg22 (34).

EFR is a receptor kinase homologous to FLS2 (196). It recognizes elf18, an 18 amino acid peptide from EF-Tu that is sufficient to induce PTI (106, 198). EFR is structurally similar to FLS2, belongs to the same subfamily of LRR-RK, and has 21 LRR motifs. Mutants in either of these receptors exhibit increased susceptibility to pathogens (142, 199).

In Arabidopsis the fungal PAMP chitin is recognized by CERK1 which has three extracellular LysM domains and an intracellular Ser/Thr kinase domain (196). Interestingly, CERK1 has been shown to be involved in bacterial recognition as Arabidopsis mutants were more susceptible to *P. syringae* suggesting that PRRs recognize multiple PAMPs (64). Indeed, CERK1 together with LYM1 and LYM3, two LysM domain proteins, recognizes peptidoglycan in Arabidopsis (190). Given that the majority of PRRs interact with unknown PAMPs the number of recognized PAMPs and therefore complexity of PTI could be significantly more multifaceted than already hypothesized.

The PTI response to a PAMP is a complex network of reactions most of which have a characteristic time frame in which they occur. Typically, it has been shown that PAMPs induce a stereotypical response suggesting that unique initial signals converge to a common multilayered response (25).

Almost immediately following PAMP treatment (< 2 minutes) there is an influx of $Ca^{2+}$ and $H^{+}$ into plant cells and concomitant $K^{+}$ and anion efflux into the

plant apoplast leading to an alkalinization (146). The elevated levels of cytoplasmic $Ca^{2+}$, or calcium burst, may serve as signaling molecules to open membrane channels (111, 157) or activate calcium-dependent kinases (122). Concurrent with or immediately following the calcium burst is the oxidative burst, another early PTI response. The reactive oxygen species produced, such as $H_2O_2$, may act directly as antimicrobial agents, be used as signaling molecules, or strengthen the cell wall by inducing cross-linking (8). Another early response involved in PTI is the activation of mitogen-associated protein kinases (MAPK) signal transduction pathways which peak 5-10 minutes after PAMP treatment (145). In an Arabidopsis leaf cell system flg22 was found to activate a cascade of MEKK1, MKK4/MKK5 and MPK3/MPK6 sequentially, which activated WRKY22 and WRKY29 transcription factors (11).

The aforementioned responses happen almost immediately and all are thought to be involved in downstream signaling events. Other responses happen on the order of minutes and are likely turned on by the cellular signals described above. Ethylene biosynthesis takes place early in PTI (170). Shortly after activation by flg22 (10-20 minutes), FLS2 undergoes ligand-induced endocytosis (159). This is likely involved in further PTI signaling (114) as well as the degradation of the receptor so that PTI is not turned on indefinitely (121). Activation of nearly 1,000 genes also occurs relatively quickly following PTI activation and the genes activated appear to be similar regardless of the PAMP used for elicitation (25).

While many responses happen quickly following PAMP recognition, other responses take place over several hours or days. Callose deposition is the quintessential late PTI response. Callose is the main component of extracellular papillae that form in response to PAMPs. These callose-based papillae also contain phenolic compounds and can be seen microscopically in the cell wall at the area where T3SS-deficient mutants are located which is attributable to PTI-induction. Fully pathogenic bacteria are able to suppress PTI and are not associated with large papillae (97). Another response that takes a long time to observe is seedling growth inhibition induced by PAMP treatments. The significance of this response is only beginning to be understood (113).

The multitude of different and reliable responses associated with PTI has lent itself well to use in molecular plant-microbe interaction research. The oxidative burst can be measured using luminol which fluoresces when it interacts with hydrogen peroxide. Callose deposition can be easily visualized and quantified microscopically by staining with aniline blue. Expression of different genes can also be used to monitor PTI. These tests and others can be used not only qualitatively to look at induction or suppression of PTI but in some instances can be used quantitatively to differentiate between assorted responses based on the intensity of the response evoked.

**Effector-Triggered Immunity**

With plants likely having evolved PTI as the first active layer of plant immunity, pathogens probably responded by evolving ways to suppress PTI such as T3SS-injection of T3Es. In response, the plant evolved a second line of

defense in order to recognize the presence of T3Es and did so by evolving intracellular receptors known as R proteins. This evolved intracellular recognition by the plant represents ETI. Presumably the pathogen would continue to acquire new T3Es to suppress ETI while the plant would evolve new R proteins (53). This two-layered plant immunity model can be illustrated within the context of the zigzag model in which ETI and PTI are described as similar responses with ETI being a more prolonged and robust response such that it goes above a certain threshold until it induces an HR (Fig. 5) (88). Lending credence to this idea of a sequential evolution with PTI developing prior to ETI is the discovery that PRRs appear to have evolved very early while R proteins are evolutionarily much younger (176).

**Hypersensitive Response**

The hypersensitive response (HR) is a programmed cell death response that is often associated with ETI, which is triggered when a plant R protein recognizes a cognate bacterial effector. The HR was first observed in wheat in response to the fungal pathogen *Puccinia glumarum* in 1902 and the term was coined in 1915 to describe pathogen-triggered cell death associated with disease resistance to *P. graminis* (171, 183). Zoltan Klement was the first to show that bacteria could elicit an HR and established it as a valuable and frequently used tool in the laboratory. The need for both an *R* gene from the plant as well as the *avr* effector gene from the pathogen led Harold Flor to develop the gene-for-gene hypothesis. Flor wrote, “for each gene that conditions resistance in the host there is a corresponding gene that conditions pathogenicity in the parasite” (56).

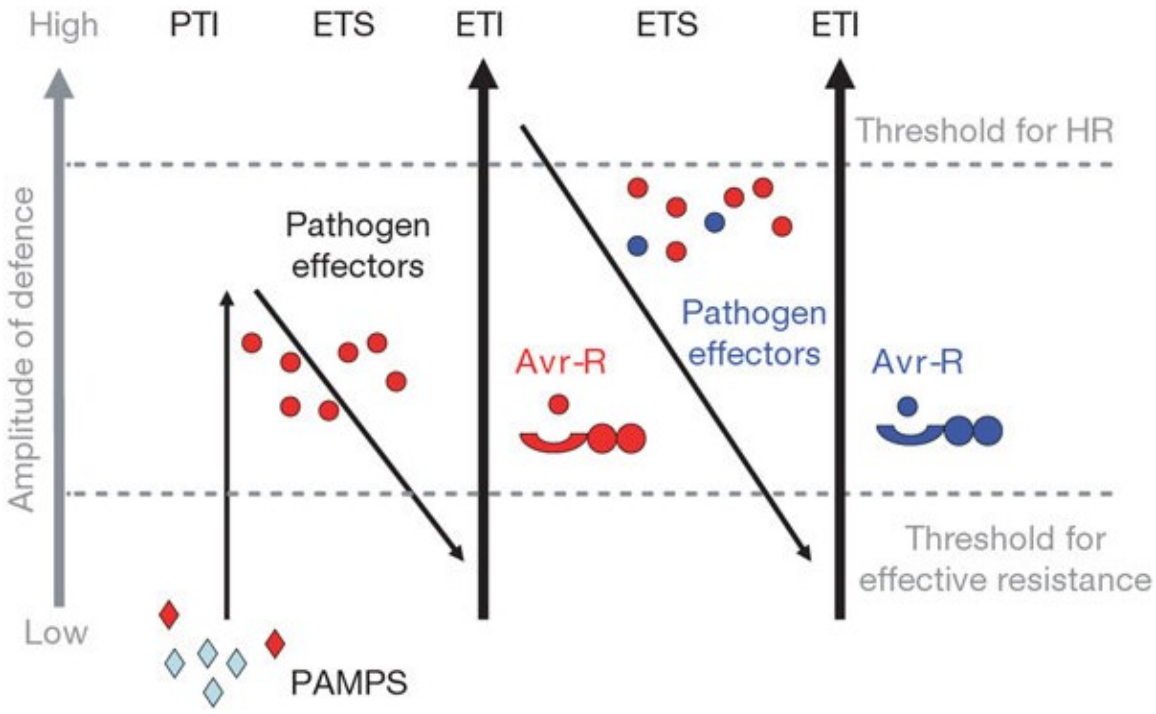


**Fig. 5. A zigzag model illustrates the quantitative output of the plant immune system.** Plants detect microbial/pathogen-associated molecular patterns (MAMPs/PAMPs, red diamonds) via PRRs to trigger PAMP-triggered immunity (PTI). Successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS). When one effector (indicated in red) is recognized by an NB-LRR protein, effector-triggered immunity (ETI) is activated. ETI often passes a threshold for induction of hypersensitive cell death (HR). Pathogen isolates are selected that have lost the recognized red effector, and perhaps gained new effectors through horizontal gene transfer (in blue)—these may help pathogens suppress ETI. Selection favours new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI. This figure is taken from Jones and Dangl (88).

The HR is often compared to animal apoptosis but in some ways it is different. Many of the cellular responses, such as cytoplasmic shrinkage, chromatin condensation, and mitochondrial swelling, are similar. Other responses are plant specific, for example vacuolization and chloroplast disruption. The HR lacks some hallmarks of apoptosis, such as the production of apoptotic bodies (140).

**Guard and decoy hypotheses**

The gene-for-gene hypothesis now sits on firm ground on the basis of the number of *avr*/*R* gene combinations that have been discovered. However, the model for perception and interaction between the two encoded proteins remains in flux. It is now widely accepted that R proteins recognize T3E by an indirect detection. This came about because so many Avr/R protein sets did not directly interact with one another, although in a few cases direct interaction has been shown (49, 50, 85, 178).

Initially, it was discovered that the T3E AvrPto1, which interacts with the Ser/Thr kinase Pto, required a third protein, Prf, to initiate ETI (58, 149). Pto interacts with several known defense-related transcription factors suggesting a role in immunity. However, Prf is an NB-LRR protein signifying that it, rather that Pto, is the key recognition component for immunity (i.e. the R protein) in response to AvrPto1. To explain this it was proposed that Pto was the actual virulence target and that Prf evolved to ‘guard’ Pto such that when Pto is targeted by AvrPto1 Prf initiates ETI (44, 179).

Other examples have lent further support to the guard hypothesis such as RIN4 and PBS1. RIN4 is targeted by at least three T3Es, AvrRpm1, AvrB1, and AvrRpt2, and guarded by two R-proteins, RPM1 and RPS2, which are activated based on the T3E modifications to RIN4 (88). PBS1 is proteolytically cleaved by AvrPphB and guarded by the R protein RPS5 (167). In these cases the R proteins mediate resistance by recognizing the modifications to target proteins by T3Es.

A variation of the Guard hypothesis has been proposed in which the plant has evolved decoy proteins that specialize in interacting with T3E proteins by mimicking the virulence target to initiate R protein mediated defense by 'tricking' the effector into binding with it. (19). The decoy protein is itself not directly involved in pathogen fitness but its modification by the Avr protein is recognized by the R protein resulting in ETI.

There are several examples of T3Es and plant protein targets that support this hypothesis. Again the example of AvrPto1 and Pto is relevant. Pto closely resembles the kinase domain of PRR proteins involved in PAMP perception such as FLS2 and EFR. AvrPto1 has been shown to bind both EFR and FLS2 and block their plant immune responses in protoplasts (193). So, in this scenario, Pto may have evolved as the decoy, mimicking the PRR proteins such that the R protein Prf can recognize AvrPto1 when it activates Pto. Under the decoy model, the PRRs are the true virulence targets of AvrPto1 and Pto evolved as a decoy that the pathogen does not benefit by targeting (200). This is supported by noting

that Pto is not required for the virulence function of AvrPto1 based on the fact that tomato *Pto* mutants are not hypersusceptible to *P. syringae* (166).

A more unique example that supports the decoy hypothesis comes from *Xanthomonas campestris* pv. *vesicatoria* and its T3E AvrBs3, a transcription factor that binds to the promoter of the master regulator Upa20 which controls cell size (95). Resistant plants carry an *R* gene *Bs3* which is expressed by AvrBs3 but *Bs3* does not appear to be expressed under any other conditions, suggesting its sole purpose is for recognition of AvrBs3 (180).

The guard and decoy hypotheses can be thought of as unique models of R gene mediated immune responses, or the decoy model as a specific off-shoot of the original guard model. These models both highlight the complex nature of gene-for-gene resistance, which is an indirect method of detecting T3E proteins involving multiple host proteins that detect an individual T3E making that plant completely resistant to an otherwise pathogenic strain.

**Summary of thesis objectives**

In order to more fully understand *P. syringae* translocation of T3E proteins, defining its translocon was chosen as a primary focus for the research presented in this dissertation. In order to accomplish this, the putative translocator HrpK1 was selected for further investigation (152). In doing so HrpK1 has been confirmed to be a translocator based on disruption of liposomes, a hallmark of translocator proteins. Further investigation showed with which phospholipids HrpK1 interacts, the strongest of which phosphatidic acid matches

another putative translocator HrpZ1. The impact that HrpK1 has on T3E translocation has been determined by testing its efficacy in multiple systems.

Along with HrpK1 three harpins, HrpZ1, HrpW1, and HopAK1, have been shown to contribute to T3E translocation (108). Experiments were conducted and are presented in this dissertation that characterize to what extent each of these proteins affects translocation and identify which are most important for translocation.

HrpJ, a protein previously shown to control the secretion of HrpZ1 in culture, was used to further confirm the suite of translocator proteins in *P. syringae*. This was done by screening other type III-secreted substrates to determine which were dependent on HrpJ for them to be secreted in culture. Additionally, it was discovered that HrpJ may act as a switch protein, controlling the transition from Hrp pilus secretion to translocator secretion.

HrpJ is injected into plant cells, which appears to be independent of its primary function. Another objective was to determine if this has a biological function for the pathogen. Here, data is presented that suggests that HrpJ functions within plant cells to suppress PTI.

Plant immune systems can be pre-activated allowing for the blockage of the HR (141). Why PTI preactivation inhibits the HR has been a long-standing question. A final objective of my dissertation project was to determine how this happens. Several mechanisms were possible including bacterial death, PTI biochemically preventing ETI induced cell death, or PTI responses somehow blocking the T3SS. Here, data are presented that show that PTI-induced plants

accomplish this by blocking T3E injection. Bacteria seem to be able to combat this in certain cases by the suppressive activity of T3E proteins.

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# Chapter 2

## The *Pseudomonas syringae* HrpK1 protein - a translocator that exhibits pore-forming activity and is a primary translocator for the type III secretion system

**Abstract**

The bacterial plant pathogen *Pseudomonas syringae* uses a type III secretion system (T3SS) to translocate, or inject, type III effector (T3E) proteins into plant cells. The T3SS is required for pathogenicity. In order for T3Es to be translocated, *P. syringae* must create an opening in the plant plasma membrane, using a group of proteins known as a translocon, through which the T3E proteins can be delivered. The translocon is composed of membrane proteins called translocators that span the plant plasma membrane. HrpK1 has many of the characteristics of a translocator: it is a type III-secreted protein, has a transmembrane domain, and is important for pathogenicity. Most importantly, *hrpK1* mutants are secretion competent but defective in T3E translocation. Here, we confirm that HrpK1 can act as a translocator for multiple T3Es and find that it is capable of interacting with liposomes, which is consistent with properties of other established translocators. HrpK1 is more important to T3E translocation than other *P. syringae* proteins known as harpins, which have been implicated in translocation. Both HrpK1 and HrpZ1, one of the harpins, interact with phosphatidic acid and this compound can block T3E translocation in a HrpK1 and HrpZ1 specific manner. These experiments support that HrpK1 is a primary translocator for the *P. syringae* T3SS and that it may function by binding phospholipids in the plant plasma membrane.

## Introduction

*Pseudomonas syringae* is a Gram negative bacterial pathogen that is capable of causing diseases on a wide variety of plants (8, 47). A specialized secretion system known as the type III secretion system (T3SS), sometimes referred to as a molecular syringe, is essential to the virulence of the pathogen (9, 50). The *P. syringae T*3SS is encoded by the *hrp* (*HR* and *p*athogenicity) and *hrc* (*HR* and *c*onserved) genes. The T3SS allows *P. syringae* to translocate, or inject, bacterial type III effector (T3E) proteins directly into plant cells in order to manipulate the cellular activities of the host to allow for growth of the pathogen (3). The main function of *P. syringae* T3E proteins appears to be suppression of the plant innate immune response (37). T3SSs are also necessary for elicitation of the hypersensitive response (HR); an immune-associated programmed cell death response that is associated with resistant plants (20).

T3SSs can be divided into at least five major families based on phylogenetic analyses (64, 71, 80). The T3SSs of bacterial plant pathogens belong to one of two groups known as group 1 or group 2 Hrp T3SSs. *P. syringae* belongs to group 1, along with *Erwinia amylovora* and others. *Ralstonia solanacearum* and *Xanthomonas campestris* are representative members of group 2 (4, 21, 2). In *P. syringae* the T3SSs and T3E genes, as well as many other virulence-associated genes, are regulated by the alternative sigma factor HrpL (31, 33).

The T3SS is a molecular syringe that allows for the translocation of T3E proteins directly into the cytoplasm of eukaryotic cells (22). Found in a wide

variety of Gram negative bacteria, T3SSs are relatively conserved in structure and constructed from more than 20 proteins (21, 34, 49). T3SSs are composed of three components: a basal body spanning both bacterial membranes, a needle (animal pathogens) or pilus (plant pathogens) which serves as a conduit through which partially unfolded T3E proteins are proposed to travel, and a translocon complex which forms a channel, or pore, in the eukaryotic plasma membrane through which the T3E proteins are delivered into the host cell (11).

The translocon complex is better understood in animal pathogens. It is composed of three proteins typically encoded on a single operon (64). Two of these proteins are hydrophobic translocators that form an integrated pore in the host plasma membrane (10, 41, 61, 67). A third, hydrophilic protein, or tip protein, is present at the end of the needle; it is thought to help integrate the hydrophobic translocators into the membrane and act like a bridge connecting the translocators to the needle (32, 35, 56, 74). The translocator class in the prototypical *Yersinia* T3SS contains YopB, a larger hydrophobic translocator and YopD, a smaller hydrophobic translocator (23). YopB and YopD are attached at the tip of the needle by the tip protein LcrV, presumably forming a continuous conduit (12, 35, 65). These three proteins are conserved in animal pathogens at the sequence level within T3SS families and at the functional level among families (64).

While the translocators of animal pathogens are highly conserved, there are typically no clear homologs in phytopathogens. No tip protein or YopD homolog has been identified for any phytopathogen T3SS (14). Because plants

have the additional barrier of the cell wall that the bacteria have to penetrate, it is likely that additional proteins are required. In spite of the additional complexities that exist, progress has been made in phytopathogen translocator research. *P. syringae* encodes a type III-secreted protein thought to be a translocator called HrpK1 (73). HrpK1 shares weak similarity with YopB and two other putative translocators, HrpF and PopF, found in the group 2 Hrp T3SSs of *X. campestris* and *R. solanacearum,* respectively (15, 60, 73). However, HrpK1 has many features that suggest it is a translocator protein. For example, HrpK1 contains a predicted transmembrane domain, which is pervasive among translocators (73). Expression of *hrpK1* is regulated in a HrpL-dependent manner, is activated rapidly, and, along with the pilus protein HrpA1, is involved in the early deployment of type III secretion machinery (31). A *hrpK1* mutant causes severely reduced disease symptoms and is significantly reduced in growth in host plants. Additionally, a *hrpK1* mutant has a reduced capacity to elicit the HR in tobacco in spite of the retained ability to secrete T3E proteins (73). This suggests that although the T3E proteins are secreted, they fail to be translocated inside of the plant cells; this is one hallmark of translocator proteins. Another hallmark of translocator proteins is that they often interact with lipid membranes (30).

Along with HrpK1, *P. syringae* has an additional family of proteins called harpins that have been reported to aid in T3E translocation. Harpins are a group of proteins unique to plant pathogens that are rich in glycine but lacking cysteine that, when purified and infiltrated into plant tissue, elicit an HR-like cell death (45). *P. syringae* pv. *tomato* DC3000 has four harpins: HrpZ1, HrpW1, HopP1,

and HopAK1. Three of these, HrpZ1, HrpW1, and HopAK1 play a role in T3E translocation (52). HrpZ1 has previously been shown to interact with lipid membranes, consistent with it being a translocator (29, 55). Because harpins are only found in plant pathogens, it is likely that some contribute to T3E translocation in ways that are specific to infection of their plant hosts. In support of this HrpW1 targets plant cell walls and, both it and HopAK1 possess C-terminal domains that resemble pectate lyases, enzymes that act on plant cells walls. This suggests that HrpW1 and HopAK1 function by allowing the Hrp pilus to traverse through the plant cell wall (18, 52). Conversely, HopP1, a harpin that resembles a lytic transglycosylase does not appear to function in translocation of T3E proteins but is important for T3SS-dependent infection (68). The particular role that each individual harpin plays in translocation needs to be further explored, but the variety of their potential functions illustrates the complexity of plant pathogen T3E translocation.

Secretion hierarchy is thought to be very important for the successful deployment of T3SSs (70). Different types of proteins are thought to be secreted in an ordered fashion. For example the pilus protein, HrpA1, is assumed to be secreted prior to translocators. Recent research identified a *P. syringae* protein, HrpJ, which appears to function as a molecular switch that controls the transition of secretion from pilus to translocators. HrpK1, HrpZ1, HopAK1, and HrpW1 are not secreted in a *hrpJ* mutant while HrpA1 has elevated secretion (25). That the secretion of these proteins is similarly regulated further suggests that they are linked as translocators. Additionally, a poly-harpin *hrpK1* mutant, which is unable

to elicit an HR in tobacco, is partially complemented by individually expressing *hrpK1*, *hrpZ1*, *hopAK1*, or *hrpW1* (52). While these types of experiments solidify that these proteins constitute a functional class, they do little to explain how each protein functions individually in terms of T3E translocation.

Here, we confirm that HrpK1 from DC3000 is a translocator protein. Deletion of *hrpK1* results in reduced translocation of T3Es. I found that HrpK1 plays a more important role in translocation than the harpins. Furthermore, complementation of poly-harpin *hrpK1* mutants with the harpins *hrpZ1*, *hrpW1*, or *hopAK1* is only partial and dependent on high expression levels, which in itself is problematic (1, 18) and illustrates that HrpK1 contributes more significantly to translocation than any of the harpins. HrpK1 disrupts liposomes, which is consistent with the activities of other translocators. HrpZ1 is the only harpin to interact in a similar manner suggesting HrpK1 and HrpZ1 are the translocators that act on the plant plasma membrane. HrpK1 and HrpZ1 both interact with phosphatidic acid (PA) and when PA is co-infiltrated with bacteria, translocation of T3E proteins is blocked. These data suggest that HrpK1 and HrpZ1 may interact with PA and other phospholipids to translocate T3Es.

**Results**

**A *hrpK1* mutant is reduced in its ability to translocate T3E proteins and harpins into *N. benthamiana* cells.** Since *hrpK1* mutants are not affected in type III secretion of T3E proteins in culture but are significantly reduced in HR elicitation (73), an indicator of T3E injection, we wanted to determine the extent that the *hrpK1* mutant is affected in T3E translocation. To do this, I made T3E-

adenylate cyclase (CyaA) fusions. CyaA is an enzyme from *Bordetella pertussis* that converts AMP to cAMP but only in the presence of calmodulin. Since bacteria do not produce calmodulin the only way for cAMP concentrations to significantly increase in infected tissue samples is if the T3E-CyaA protein fusion is translocated into the plant cell. The amount of cAMP is quantified and this is an indicator of T3E translocation (76, 78). A large subset of T3E proteins was chosen to test the translocation from both DC3000 and its *hrpK1* mutant derivative. Eight different T3E-CyaA gene constructs were made, *avrB-cyaA*, *avrPto1-cyaA, avrRpt2-cyaA*, *hopC1-cyaA, hopR1-cyaA*, *hopX1-cyaA*, *hopAI1-cyaA,* and *hopAM1-1-cyaA*. These were each expressed individually in both DC3000 and the *hrpK1* mutant and translocation of each T3E-CyaA was quantified 7 hours after infiltration into *Nicotiana benthamiana* leaves. In all eight cases the translocation of the T3E-CyaA fusion was reduced from the *hrpK1* mutant strain compared to DC3000 (Fig. 1), consistent with HrpK1 operating as a translocator. Interestingly, *P. syringae* still appears able to inject small amounts of T3E proteins in the absence of HrpK1.

**HrpK1 is translocated into plant cells but truncation derivatives are not translocated in the absence of HrpK1; HrpK1 truncation derivatives with intact transmembrane domains can restore T3E translocation in a *hrpK1* mutant.** HrpK1 has a predicted transmembrane domain between amino acids 698 and 750 that has been shown to be functionally important based on HR elicitation in tobacco (73). We wanted to determine the extent that the translocation of HrpK1 was linked to its ability to assist in T3E translocation.

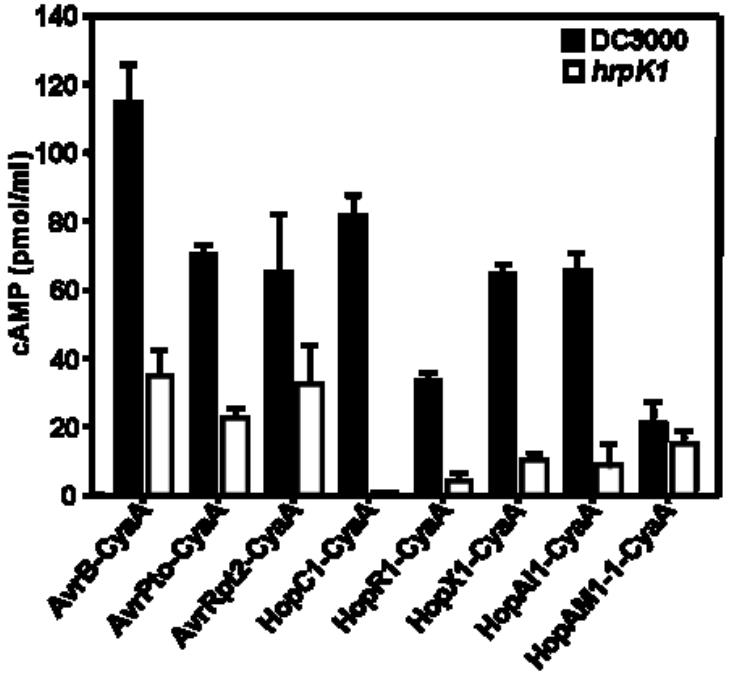



**Fig. 1. A *hrpK1* mutant is reduced in its ability to translocate type III effector (T3E) proteins.** Eight different T3E-CyaA fusions, AvrB-CyaA, AvrPto1-CyaA, AvrRpt2-CyaA, HopC1-CyaA, HopR1-CyaA, HopX1-CyaA, HopAI1-CyaA, and HopAM1-1-CyaA, were individually tested to measure translocationof T3E-CyaA from DC3000 and a *hrpK1* mutant in *N. benthamiana* leaves. The bacteria were infiltrated at 4 x $10^8$ cells/ ml 7 hours before measuring cAMP.

When various *hrpK1-cyaA* truncations were expressed in wild type bacteria all were translocated into the leaves of *N. benthamiana* plants at 1 x $10^8$ cells/ml, albeit at reduced levels. When the same constructs were expressed in a *hrpK1* mutant, however, none except for full-length HrpK1 were translocated (Fig. 2A). This suggests that translocation of any HrpK1 protein derivative requires the bacterial cell to have a full-length HrpK1.

To determine if T3E translocation was affected in a similar manner, the same truncations were expressed, this time with a hemagglutinin (HA) tag, in the *hrpK1* mutant to see if any could restore the ability to translocate the T3E HopU1-CyaA. The truncations that contained the transmembrane domain were able to almost fully complement the mutant. The strains expressing constructs lacking the transmembrane domain behaved similar to the mutants (Fig. 2B). The putative transmembrane domain of HrpK1 is likely required for HrpK1 to function as a translocator.

**Translocation experiments indicate that HrpK1 is more important than the harpins for T3E translocation.** Because knocking out *hrpK1* alone does not completely stop T3E translocation, and because harpins contribute to T3E translocation (52) we wanted to see to what extent T3E translocation was reduced in mutants lacking different combinations of HrpK1 and the harpins. It has previously been shown that when all harpin genes are knocked out, translocation of AvrPto1 is not reduced at high inoculums, but when *hrpK1* is additionally knocked out a significant reduction in translocation is observed (52).

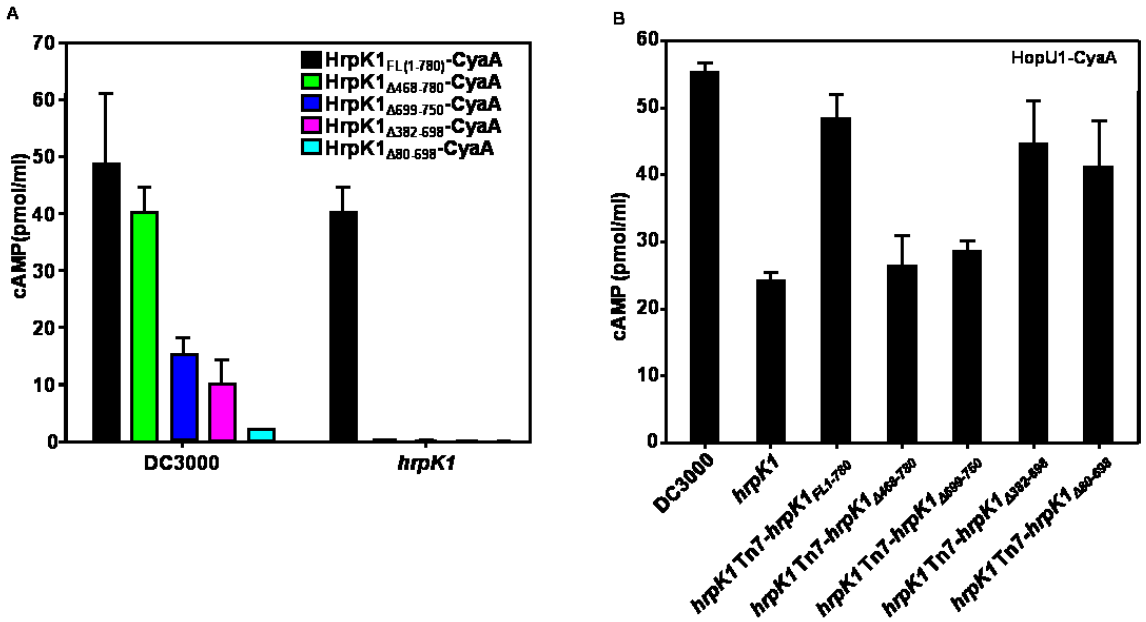



**Fig. 2. HrpK1 truncations are not translocated from *hrpK1* mutants but HrpK1 derivatives containing transmembrane domains can partially complement T3E translocation.** A) HrpK1 and derivative truncations were expressed in wild type bacteria and *hrpK1* mutants using the Tn7 expression system and carrying CyaA tags. Only when the full length *hrpK1* was expressed was translocation observed. None of the truncated HrpK1-CyaA proteins were injected from the *hrpK1* mutant. Translocation was measured in *N. benthamiana* 8 h after infiltration with 2 x $10^8$ cells/ml of bacteria. B)Translocation of plasmid encoded HopU1-CyaA was measured from DC3000, UNL111, and UNL111 complemented by *hrpK1* truncations expressed using the Tn7 system carrying HA tags. The Tn7-*hrpK1* derivatives containing transmembrane domain region, amino acids 698-750, markedly increased translocation of HopU1-CyaA from *hrpK1.*

Here, we were interested in the effect of translocation of T3E proteins at a high bacterial concentration when different combinations of harpin genes were deleted by themselves or with *hrpK1*.

As expected, deletion of harpins alone had only a moderate effect on translocation of AvrPto1-CyaA and a large reduction in translocation was only seen when *hrpK1* was additionally deleted (Fig. 3A). A similar trend was also observed for translocation of AvrB1-CyaA (Fig. S1). While both *hrpW1* and *hrpZ1* mutants reduced translocation neither was as strong as *hrpK1*. In fact loss of all four harpins had less of a reduction in translocation than did deletion of *hrpK1* by itself (Figs. 3A & S1).

To confirm the results observed using the CyaA reporter a direct measure of translocation was employed. AvrRpt2 has been shown to be processed upon entry into plant cells leading to a detectable band with a reduced molecular mass on immunoblots that is only present when AvrRpt2 is translocated (63). This assay was consistent with the CyaA translocation assay in that deletion of harpin genes negligibly reduced the presence of the processed AvrRpt2-HA band compared to wild type bacteria, while deletion of *hrpK1* either alone or in combination with harpins consistently showed a more substantial reduction in AvrRpt2-HA translocation (Fig. 3B). Clearly, from these results, HrpK1 plays a more important role than the harpins in T3E translocation.

In addition to T3E proteins, other type III-secreted substrates such as the harpins are translocated into the plant cell (52, 75). As with T3E proteins, translocation of harpins is T3SS-dependent; therefore, harpins would also be

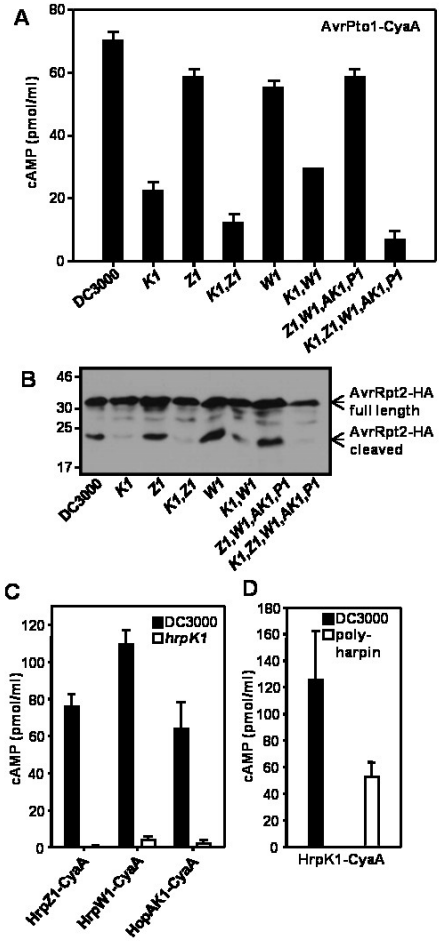



**Fig. 3. HrpK1 is more important for type III translocation than the harpins.** (A) To determine AvrPto-Cya translocation, cAMP was measured from samples of *N. benthamiana* leaves infiltrated with a construct expressing AvrPto-CyaA in DC3000 and mutants lacking *hrpZ1*, *hrpW1*, or all four harpins as well as each strain with additional loss of *hrpK1*. cAMP was measured from samples of the leaves of *N. benthamiana* infiltrated with each strain at 4 x $10^8$ cells/ml for 7 h to determine translocation of AvrPto-CyaA. (B) A construct expressing AvrRpt2-HA was electroporated into the same strains. Translocation of AvrRpt2-CyaA was observed on immunoblots after samples were taken from *N. benthamiana* leaves 7 h after infiltration at 1 x $10^9$ cells/ml. Equal amounts of each sample in total protein were separated on an SDS-PAGE gel and subjected to immunoblot analysis using anti-HA antibodies. The smaller band represents translocation as it is the processed protein after it has been cleaved in the plant cell. (C) Levels of translocation of three harpins (HrpZ1, HrpW1, and HopAK1) expressed from Tn7 constructs fused at their C-terminal ends to CyaA were also individually measured from both DC3000 and *hrpK1* in *N. benthamiana* leaves. (D) Translocation of HrpK1-CyaA, expressed by Tn7 system, was measured from both DC3000 and the poly-harpin mutant in *N. benthamiana* leaves. (A-D) the bacteria was infiltrated at 4 x $10^8$ cells/ml for 7 h before measuring cAMP.

impacted by aberrant formation of the translocon. As before, gene constructs were made such that each gene product was fused to a C-terminal CyaA reporter. This time, only the three harpin proteins (HrpZ1, HrpW1, and HopAK1) known to be involved in translocation were used. The harpin proteins were expressed using a Tn7 system. This system has the advantage that it introduces the gene in single copy and expresses it from a type III-related promoter, which allows the gene to be expressed at a biologically relevant level. As expected, the translocation of harpins was severely reduced from the *hrpK1* mutant relative to DC3000 (Fig. 3C).

HrpK1 has also been shown to be translocated (Fig. 2) (73). HrpK1-CyaA translocation was measured from both DC3000 and a mutant lacking all harpin genes (52). There was a reduction in translocation from the poly-harpin mutant (CUCPB 5401) compared to DC3000 (Fig. 3D); however, this reduction was not as severe as for translocation of harpins from the *hrpK1* mutant (Fig. 3C), suggesting again that HrpK1contributes more significantly to translocation than the harpins.

Because the previous experiments were done in a DC3000 background, which has evolved a large repertoire of genes that contribute to pathogenicity, we wanted to conduct similar experiments in a reductionist system. pHIR11 is a cosmid that contains a 31 kb DNA fragment of the *P. syringae* pv. *syringae* 61 genome that encodes a functional T3SS along with a single T3E, HopA1 (48). This cosmid can be introduced into a non-pathogen such as *P. fluorescens* 55 and confer the ability of this bacterium to inject T3Es via T3SS when infiltrated in

leaf tissue (5). In these experiments I used derivatives of pHIR11 that are defective in *hrpK1* (pLN468) (73) or *hrpZ1* (pCPP5024) (1). I made an additional pHIR11 derivative lacking both *hrpK1* and *hrpZ1* (pLN4495). A construct expressing AvrRpt2 fused at its C-terminus to CyaA was electroporated into all strains. Levels of cAMP were assessed 7 h after infiltration at 4 x $10^8$ cells/ml in *N. benthamiana* to determine the amount of translocation. The concentration of cAMP, and, therefore, the amount of AvrRpt2-CyaA translocated from the mutant strains, was much lower in all mutants than from *P. fluorescens*(pHIR11) (Fig. S1). *P. fluorescens*(pLN468), the pHIR11 *hrpK1* derivative, was completely devoid of T3E translocation, *P. fluorescens*(pCPP5024), the pHIR11 *hrpZ1* derivative, maintained a low level of T3E translocation. Not surprisingly, the double mutant, *P. fluorescens*(pLN4495) also failed to inject the T3E reporter (Fig. S1).

**Complementation of the poly-harpin *hrpK1* mutant with single harpin genes or *hrpK1* is dependent on expression levels.** When all four harpin genes and *hrpK1* are deleted from DC3000, the mutant is unable to elicit an HR in tobacco leaves even at concentrations of 3 x $10^8$ cells/ml. When the individual genes are reintroduced on multi-copy plasmids and constitutively expressed, *hrpZ1, hrpW1*, or *hopAK1* can restore HR elicitation to the poly-harpin *hrpZ1* mutant when infiltrated at 3 x $10^8$ cells/ml while only *hrpK1* is able restore the HR phenotype to the poly-harpin *hrpZ1* mutant when infiltrated at 1 x $10^7$ cells/ml (Fig. 4A) (52). It has already been shown that overexpression of the harpin proteins HrpZ1 and HrpW1 can affect HR elicitation (1, 18). Because of these aberrant phenotypes

involving expression levels of harpins we decided to repeat these experiments complementing this mutant with genes in single copy and natively expressed, which may be less problematic and more biologically relevant than complementation experiments using muti-copy plasmids and constitutive expression.

In order to accomplish this, a Tn7 expression system was adapted to be Gateway compatible (19). Because Tn7 integrates into a specific region of the bacterial chromosome the introduced gene is single copy. Our Tn7 construct was designed with an *avrPto1* promoter such that it was induced in conditions that express the T3SS. We tested the effect of expression of *hrpZ1*, *hrpW1*, *hopAK1* and *hrpK1* using the Tn7 expression system and none of the strains were altered in their ability to elicit the HR in tobacco plants (data not shown).

The results we observed using the multi-copy plasmids were consistent with Kvitko *et al.* (52) in that *hrpZ1, hrpW1, and hopAK1* in addition to *hrpK1* were able to restore the ability to elicit an HR to the poly-harpin *hrpK1* mutant when infiltrated at 3 x $10^8$ cells/ml (Fig. 4A, upper panel), while only *hrpK1* was able to restore the HR when infiltrated at the 1 x $10^7$ cells/ml (Fig. 4A, lower panel). However, when we expressed the same genes using the Tn7 expression system, none of the harpins were able to restore the ability to elicit the HR to the poly-harpin *hrpK1* mutant at any of the cell densities tested (Fig. 4B). In spite of this, *hrpK1* was still able to restore the HR to this mutant in every cell density tested (Fig 4B). These data, along with the results from Kvitko *et al.* (52), once again confirm that HrpK1 makes a more important contribution to T3E translocation

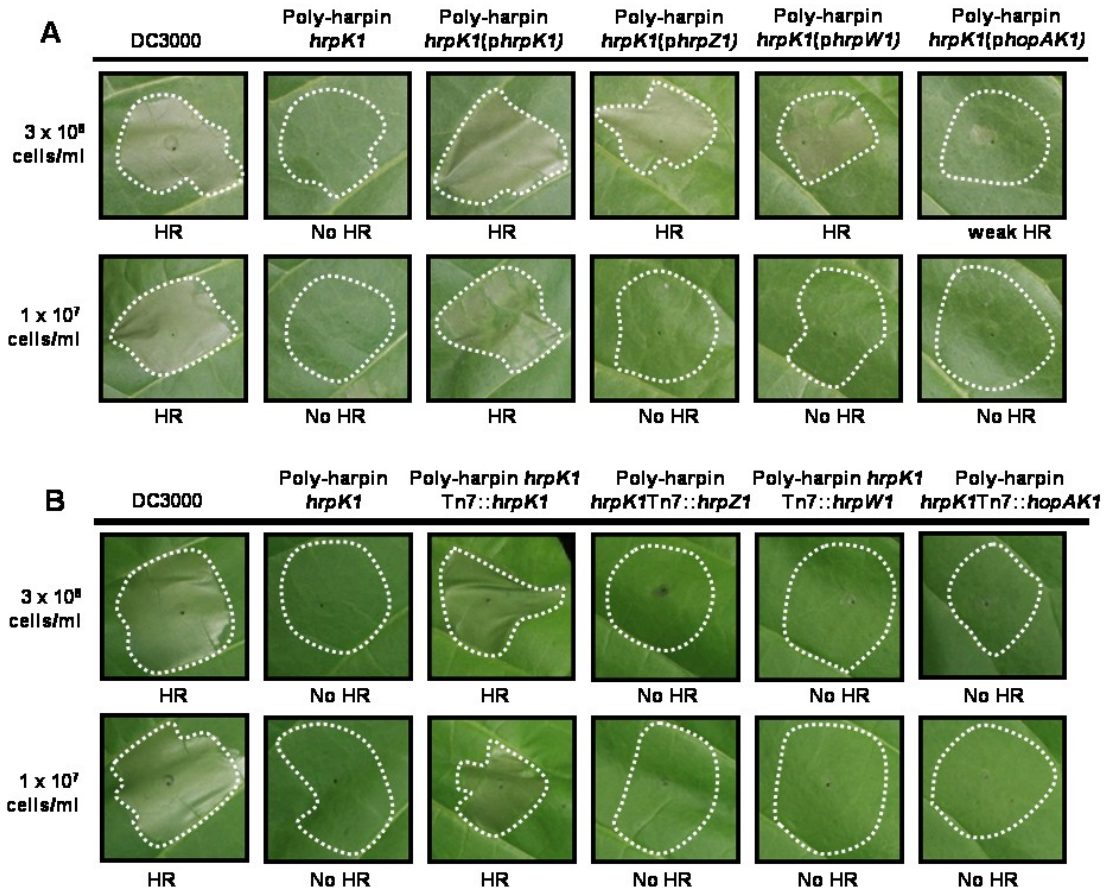



**Fig. 4. Restoration of the poly-harpin *hrpK1* mutant HR phenotype with individual harpins is expression dependant, while HrpK1 fully restores the ability to elicit an HR.** Tobacco leaves were infiltrated with a blunt syringe with either 3 x $10^8$ cells/ml (upper panels) or 1 x $10^7$ cells/ml (lower panels) of bacteria which resulted in elicitation of an HR from DC3000 but not from the poly-harpin *hrpK1* mutant. Restoration of the HR phenotype of the mutant was attempted by individually expressing the deleted genes either by a plasmid (A) or using a Tn7 single copy, suicide vector with an *avrPto* promoter (B). Restoration of the HR was observed in both systems by *hrpK1* at both bacterial concentrations. No other gene could restore the HR phenotype using the Tn7 expression system or at 1 x $10^7$ cells/ml by plasmid.

than any of the harpins and, along with Fig. 3, suggests that HrpK1 is the only translocator sufficient to restore the HR and moderate translocation to the poly-harpin *hrpK1* mutant.

**HrpK1 and the HrpZ1 harpin disrupt liposomes *in vitro*; other harpins lack this activity.** Translocator proteins must insert themselves into the host plasma membrane in order to form a pore (21). The propensity for translocator proteins to insert into liposomes to allow small molecular release has been documented in the *Shigella* T3SS (27, 74). A similar experiment has already been done with HrpZ1, one of the harpins in DC3000, showing membrane interaction (29). In this study HrpK1 and all four harpin proteins were tested for interactions with liposomes. Purified recombinant protein was made for HrpK1-GST, HrpZ1-His, HopP1-His, HopAK1-His, and the harpin domain of HrpW1 ($HrpW1_{HD}$-His), because the full-length HrpW1 protein is lethal in *Escherichia coli* (Fig. S2) (18). The known *Shigella* translocator protein IpaB was used as a positive control for the release of sulforhodamine-B (SRB) from liposomes. The *Shigella* type III secretion apparatus needle tip protein IpaD was used as a negative control since it does not insert into phospholipid membranes under physiological conditions. As shown in Fig. 5, both HrpZ1-His and HrpK1-GST resulted in a rapid and significant release of SRB from liposomes within 5 min (Fig. 5). These are similar to the positive control IpaB, which caused a rapid release of SRB with approximately 70% of the fluorophore released after 5 min (Fig. 5). In contrast, the amount of fluorophore released by liposomes treated with $HrpW1_{HD}$-His, HopAK1-His, and HopP1-His was similar to the amount detected from liposomes

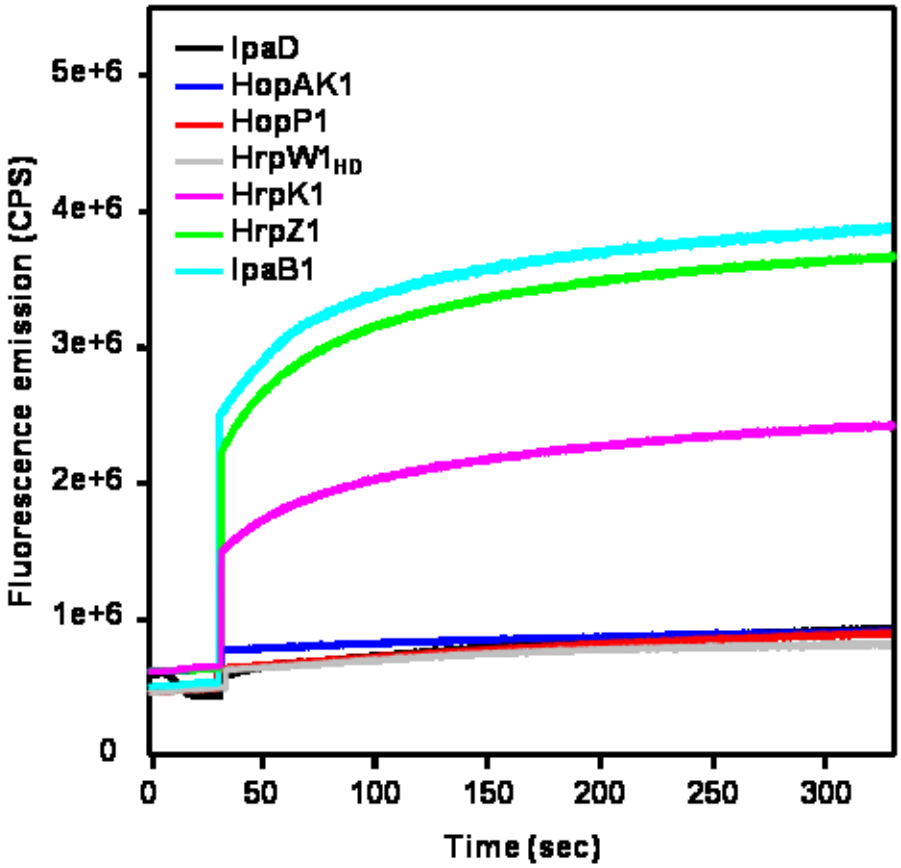



**Fig. 5. HrpK1-GST disrupts liposomes.** Protein-mediated release of sulforhodamine-B (SRB) from liposomes. SRB-containing liposomes were incubated for 30 sec in PBS. 7.5 µg/ml of protein was added and the relief of SRB auto-quenching was monitored for 300 sec more.

treated with the negative control, IpaD (Fig. 5). This evidence further confirms that HrpK1 is a translocator protein. HrpZ1 likely also plays a role in formation of the translocon because of its interaction with liposomes and other lipid membranes (Fig. 5) (29, 55) as well as the previous reports that are consistent with it acting as a translocator (1, 52). Because none of the other harpins tested showed the ability to significantly disrupt liposomes above the level of the negative control (Fig. 5), their function in translocation may not be in the formation of a pore in the plant plasma membrane.

**HrpK1 interacts with phosphatidic acid.** Because HrpK1 is able to disrupt liposomes we wanted to test if it interacted with any phospholipids. Commercially available PIP strips (Invitrogen) that carry an assortment of membrane lipids were used to determine if HrpK1 interacted with any phospholipids. We confirmed that HrpZ1 could bind only to phosphatidic acid (PA) (Fig. 6A) (40). Similarly, HrpK1-GST showed its strongest interaction with PA. However, in contrast to HrpZ1, HrpK1 was more promiscuous in its interactions with phospholipids, exhibiting a positive interaction with 10 of the 15 membrane lipids tested, although none as strongly as with PA (Fig. 6A). To ensure that the binding was with HrpK1, purified GST was used in the same assay and was unable to bind any of the spots (data not shown).

**Co-infiltration with phosphatidic acid prevents translocation of T3Es.** *In planta* growth is significantly reduced when wild type bacteria are co-infiltrated with 1 mM PA compared to infiltration of bacteria alone in *Arabidopsis thaliana* (Fig. 6B). Co-infiltration with phosphatidylcholine (PC), a phospholipid that does

not interact with HrpK1 or HrpZ1, had no effect on bacterial growth (Fig. 6B). The growth of the type III-deficient mutant *hrcC* was unaltered by the presence of PA suggesting that the reduction in growth is T3SS-specific, likely due to the interaction between translocators and PA. To test this, the *in planta* growth of *hrpK1* and *hrpZ1* single mutants and a *hrpK1 hrpZ1* double mutant was measured when co-infiltrated with PA in Arabidopsis. In both single mutants there was a significant decrease in growth in the strains co-infiltrated with PA but growth of the double mutant was not affected by PA (Fig. 6C). This suggests that both proteins can bind PA independently and the significant growth defect occurs when either HrpK1 or HrpZ1 is present, but in the absence of both proteins PA has no impact on bacterial growth *in planta*.

Since the reduced growth of P. syringae in the presence of PA may be due to the interaction between translocators and PA, it is possible this interaction prevents translocation of T3E proteins into plant cells. This may be because the PA infiltrated in the apoplast is binding the translocon in such a manner that prevents it from making productive contact with the plasma membrane blocking the injection of T3Es into plant cells. To test this we measured the ability of DC3000 to inject the T3E-CyaA fusion HopU1-CyaA when co-infiltrated into *A. thaliana* leaves in buffer containing 1 mM concentrations of specific phospholipids. In the presence of PA there was a severe reduction in the amount of HopU1-CyaA translocated by DC3000 (Fig. 6D). Neither PC, which did not interact with HrpK1 or HrpZ1 in our binding assays (Fig. 6A), nor PS, which did interact with HrpK1, had a significant effect on HopU1-CyaA translocation. To

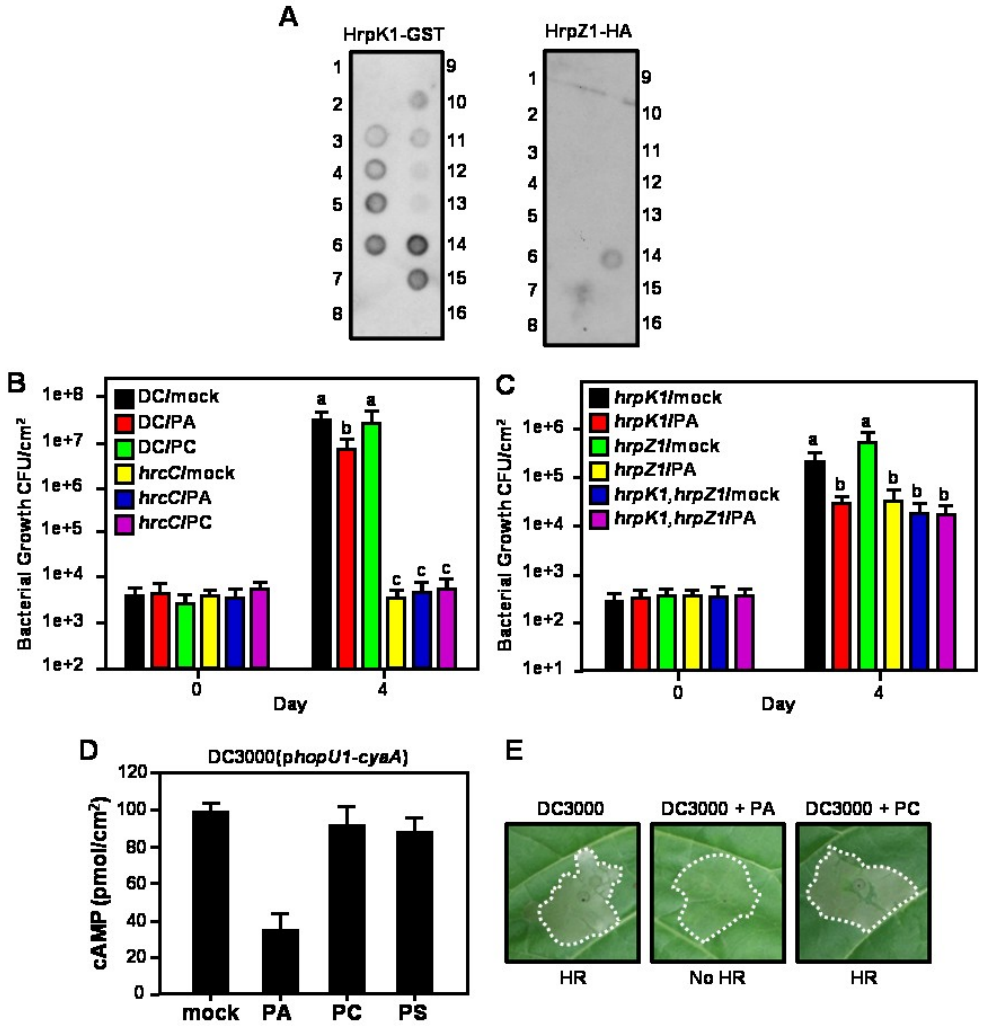



**Fig. 6. HrpK1-GST and HrpZ1-HA bind to phosphatidic acid which can block T3E translocation.** (A) Both HrpK1-GST and HrpZ1-HA had a higher affinity for PA than the other phospholipids present on the membrane. These phospholipids are lysophosphatidic acid (1), lysophosphatidylcholine (2) phosphatidylinositol (PI) (3) PI(3)P (4) PI(4)P (5) PI(5)P (6) phosphatidylethanolamine(7) phosphatdiylcholine (PC) (8) sphingosine 1-phosphate (9) PI(3,4)$P_2$ (10) PI(3,5)$P_2$ (11) PI(4,5)$P_2$ (12) PI(3,4,5)$P_3$ (13) PA (14) phosphatidylserine (PS) (15) Blank (16). (B) DC3000 or the hrcC mutant were infiltrated at 1 x $10^5$ cells/ml into Arabidopsis leaves in 5 mM MES buffer alone or containing 1 mM PA or PC and bacterial growth was measured by direct colony counts. (C) *hrpK1*, *hrpZ1,* or *hrpK1,hrpZ1* double mutant were infiltrated at 1 x $10^5$ cells/ml into Arabidopsis leaves in 5 mM MES buffer alone or containing 1 mM PA and bacterial growth was measured by direct colony counts. (C & D) Lower case letters indicate whether growth of the different strains was statistically different based on t-tests ($P < 0.05$) and error bars indicate standard deviation. (D) Translocation of the T3E HopU1-CyaA was measured in Arabidopsis leaves 8 h after being infiltrated with 2 x $10^8$ cells/ml of DC3000(p*hopU1-cyaA*) either in buffer alone (mock) or with 1 mM PA, PC, or PS. (D) Tobacco leaves were infiltrated with DC3000 at 2 x $10^6$ cells/ml either alone or with 1 mM PA or PC and elicitation of the HR was assessed after 48 hours.

confirm that PA does not specifically inhibit CyaA enzyme activity, a direct translocation assay using AvrRpt2 was again employed. There was noticeably less cleaved AvrRpt2, which represents the *in planta* form of AvrRpt2, in the PA-infiltrated samples than buffer alone or with PC, confirming the results with the CyaA reporter (Fig. S3). The HR elicited by DC3000 is dependent upon the translocation of T3Es. Thus, the HR is a measure of the ability of DC3000 to translocate T3Es. DC3000 was unable to elicit an HR when it was infiltrated at 2 x $10^6$ cells/ml in the presence of 1 mM PA even though this cell density was sufficient for DC3000 to elicit an HR when it was infiltrated in buffer alone or with 1 mM PC (Fig. 6E).

**Discussion**

One of the most interesting aspects of studying the T3SSs of plant bacterial pathogens is that the translocator proteins are so divergent from animal translocator proteins. Plant bacterial pathogens lack specific proteins present in the protypical animal translocon, such as a YopD or tip protein family members, and encode additional proteins called harpins that are unique to plant pathogens and play roles in translocation. Even HrpK1 is only weakly similar to YopB, sharing 26% identity across 105 amino acid residues of this 780 amino acid long protein (14). In spite of a lack of sequence similarity it is assumed that plant pathogens like *P. syringae* inject proteins in a similar manner, and, therefore, these bacteria must form a translocon that allows the T3SS apparatus to traverse the plant cell wall and plasma membrane (10, 64). We have confirmed that HrpK1 disrupts liposomes in a manner similar to the *Shigella* translocator IpaB as

well as the HrpZ1 harpin (Fig. 1), both of which have been previously shown to have pore-forming activities in similar assays (10, 29). Disruption of lipid membranes is a characteristic of a functional translocator and it has been used to determine if a protein is a translocator. The other harpin proteins from DC3000 do not share the ability to interact with liposomes (Fig. 1). This suggests then that, like animal pathogens, *P. syringae* uses two proteins to form pores in the plant plasma membrane; however, these proteins are highly divergent from the animal translocators, particularly HrpZ1; and, unlike animal pathogens are not encoded on the same operon along with a type III chaperone gene (59).

Since HrpW1 and HopAK1 are known to be involved in T3E translocation their inability to interact with liposomes suggests that they function outside of the plasma membrane pore. This is consistent with their predicted pectate lyase activity which would implicate their function at the cell wall (18, 52). Additionally, since animal pathogens do not have harpins it makes sense that some harpins target a plant specific structure. It is worth noting that different strains of *P. syringae* contain different sets of harpins suggesting certain harpins may be host specific. There also seems to be a certain level of functional redundancy among harpins (39, 52). In one case, *P. syringae* pv. *tabaci*, the *hrpZ1* gene has an internal deletion and a frame-shift (81). This suggests that another protein, possibly another harpin fills the functional void left by HrpZ1, or that *P. syringae* pv. *tabaci* can still be a successful pathogen without it. This is supported by noting that the *hrpZ1* mutant phenotype is only moderately reduced in virulence and T3E injection (44, 46). HrpZ1 is known to trigger immune responses,

perhaps explaining why there would be environmental selection for this mutation in *P. syringae* pv. *tabaci*. The pilus protein, HrpA1, which like translocators is exposed to the plant and, therefore, may be vulnerable to recognition, is known to be under positive evolutionary selection (38). This may mean that translocators are under selective pressures to evade detection or even suppress detection. It would be reasonable to assume that type III secretion substrates that are translocated early, which would include translocators, may have evolved to suppress plant immunity.

The specific function of individual harpins in the infection process remains to be elucidated; however, they (except for HopP1) are involved in T3E translocation and like HrpZ1 and HrpK1 their secretion is dependent upon HrpJ. HrpJ is a T3SS control protein that controls translocator secretion, suggesting that these proteins comprise the DC3000 translocator set and are deployed by the T3SS at the same stage of infection (25). No tip protein has been identified in a plant pathogen but one possible strategy to find the protein that functions in this capacity is to find another protein similarly regulated by HrpJ, since it would likely be secreted at a similar stage as the other translocators. Alternatively, phytopathogens may not have a traditional tip protein. Perhaps the harpins that act at the cell wall, such as HrpW1 and HopAK1, help bring the needle into close proximity or physically connect it to the pore, but this needs to be determined experimentally.

Like HrpZ1, HrpK1 interacts strongly with the membrane phospholipid PA (Fig. 2A). Infiltration of bacteria in buffer containing PA resulted in reduced *in*

*planta* growth that was dependent on HrpK1 and HrpZ1 (Fig. 2B & C). This reduced growth is likely due to a decreased ability of the bacteria to deliver T3Es when co-infiltrated with PA (Fig. 2D). The interaction between the translocators and PA presumably is important to *P. syringae.* Perhaps PA is used as a docking station for the translocon. PA molecules may occupy the translocators and lead to less efficient T3E translocation. Another possibility for the PA blockage of translocation is that the plant uses PA to block translocation. It has previously been shown that expression of *avr* genes *in planta* leads to accumulation of PA (6, 28). PA has also been shown to be a second messenger involved in plant immunity such as the oxidative burst (54, 58). PA blocking translocation by acting as a second messenger to trigger different signal transduction pathways seems less likely than direct blockage for a couple reasons. First, HrpK1 and HrpZ1 both directly interact with PA and growth of *hrcC* and the *hrpK1 hrpZ1* double mutant was not affected by PA. Second, PAMPs, such as flg22, which strongly and rapidly induce the oxidative burst, are able to block translocation but only when treated in advance of bacterial inoculation (24, 69). Simultaneous co-infiltration with flg22 caused no reduction in translocation unlike PA (Fig. S4). Outputs of PTI such as callose deposition and reactive oxygen species production were not induced by PA (Fig. S5A & B). Cell death, which has been reported to be induced by PA in Arabidopsis (72) was not observed in this study. It is important to note that the PA used in our study had different acyl chains than in Park *et al.* (72), which may have caused the discrepancy between the two studies (Fig. S5C).

A *hrpK1* mutant has a drastic reduction in its ability to translocate T3E proteins both in *P. syringae* and in a heterologous system in which *P. fluorescens* possesses a functional T3SS (Figs. 1 & S1). Type III-secreted proteins would not be able to enter the plant cell in the absence of a translocon; because *hrpK1* mutants are so severely impaired in their ability to translocate these proteins it is likely that HrpK1 is a translocator, however, animal pathogen translocators mutants such as *yopB* have been reported to be completely devoid of T3E translocation, however, differences in translocation levels may be attributable to differences in the assays used to measure translocation in the different systems. There are several possible explanations for the remaining low levels of translocation observed in the *hrpK1* mutant. First, it may be attributable to other proteins, such as HrpZ1, which interact with the plant plasma membrane potentially forming a partially functional translocon. Second, DC3000 T3SS may be more complicated and the need to carry multiple harpins may indicate multiple mechanisms for T3E translocation. Perhaps, HrpK1 is a part of a conventional translocon and that is the most common or efficient way to inject T3Es. Finally, *hrpK1* and *hrpZ1* are not as intimately linked and therefore may function more independently of one another than translocators in animal systems. Whatever the explanation, this highlights another distinction from animal pathogens where deletion of any single translocator completely blocks T3E translocation (57).

The *hrpK1* mutant was much more reduced in T3E translocation than any single harpin mutant or the poly-harpin mutant lacking all of the known harpins (Figs. 3 & S1). This clearly confirms that HrpK1 is more important for

translocation than the harpins, including HrpZ1 which also shares pore-forming activities. There is a precedent for translocators playing unequal roles in phytopathogenic bacteria. For example in *Ralstonia solanacearum* the putative translocator PopF1 is of greater importance to pathogenicity than its homologous partner PopF2 (60). Additionally, the YopB family member which is sometimes referred to as the major translocator may have additional regulatory functions in animal pathogens (57, 77). An undiscovered regulatory function may be another explanation for why HrpK1 seems to be more important than the harpins in translocation.

One interesting facet of *P. syringae* infection is that HrpK1 is itself translocated inside of the plant cell. If it were only functioning as part of the translocon, intuitively, it seems inefficient for the pathogen to inject HrpK1 in high amounts into the plant cytoplasm (Figs. 2 & 3). There are numerous examples where translocators have functions in addition to their translocon forming functions. In addition to its role in forming the translocon SipC, a hydrophobic translocator from *Salmonella,* can nucleate actin and bundle F-actin (16, 17, 43, 66). Similarly, the *Shigella* translocator IpaC has been shown to induce ruffles through the activation of Cdc42, recruitment of Src kinase, and activation of Abl kinase (13, 62, 79). The other *Shigella* translocator IpaB is necessary for induction of apoptosis in macrophage cells by binding to and activating the cysteine protease caspase-1 (36, 83). YopB has been shown to suppress TNF-α production, which significantly contributes to evasion of host defenses (7).

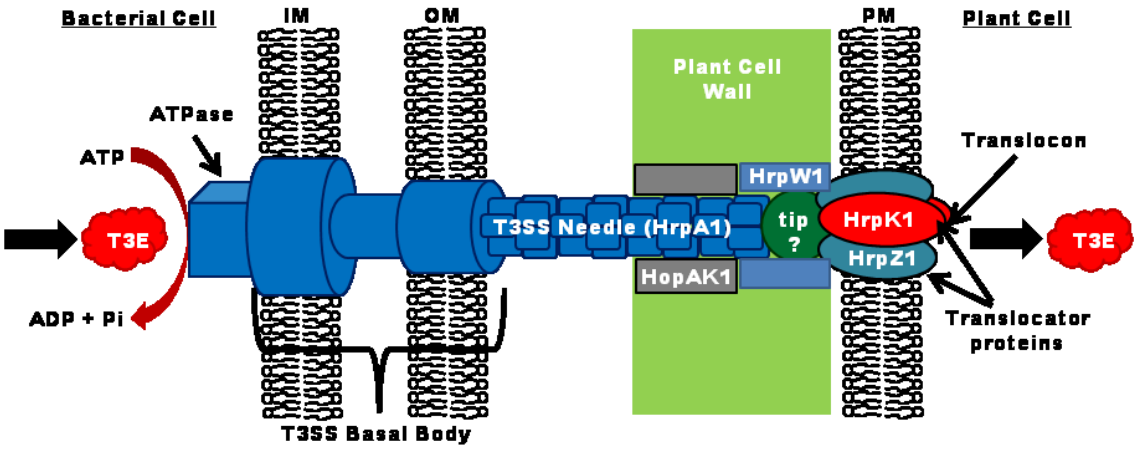



**Fig. 7. Model of the *P. syringae* HrpK1/harpin translocon.** The T3SS forms a continuous conduit spanning both inner (IM) and outer membranes (OM). The T3SS injects T3Es into the plant cytoplasm using energy supplied by the associated ATPase. The T3Es travel through the needle, eventually going through the pore which is formed by HrpK1 and HrpZ1. Other harpins, such as HrpW1 and HopAK1 are type III secreted proteins and likely function to aid in translocation by physically allowing the needle to penetrate the plant cell wall. A tip protein may or may not be present in plant pathogenic bacteria.

Identifying whether HrpK1 has an additional function inside plant cells will be a focus of future studies.

Based on our results, we propose a model in which HrpK1 and HrpZ1 together form the plasma membrane pore, following the animal model where it is formed by a multimer of two proteins (Fig. 7). In this model, the HrpW1 and HopAK1 harpins aid in translocation while functioning within the cell wall likely allowing the pilus access to the plant plasma membrane (Fig. 7). This model needs to be confirmed by further studies identifying interactions among the proteins involved in translocation and also by identifying additional functional characteristics that may show how they operate together to promote translocation. In any event, elucidating the mechanisms of type III translocation for plant pathogens and their divergence from animal T3SS remains an exciting endeavor.

**Experimental Procedures**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and DNA constructs used are listed in Table S1. Plasmids were typically maintained in DH5α. *Pseudomonas syringae* and *P. fluorescens* strains were grown in King's B (KB) medium at 30° with appropriate antibiotics. Induction of type III-regulated genes was done by growing them in Hrp minimal media (HrpMM) supplemented with fructose at 22°. *Escherichia coli* strains were grown at 37° in Luria-Bertani medium with appropriate antibiotics. The antibiotics used were at the following concentrations (μg/ml): ampicillin, 100; rifampicin, 100; kanamycin, 50; spectinomycin, 50; and gentamicin, 10 or 1.

**Construction of plasmids.** Restriction enzymes, T4 DNA ligase, and *Taq* polymerase were purchased from New England Biolabs (Beverly, MA, USA). Amplification of DNA fragments for cloning was done using thermostable *Pfu* polymerase from Stratagene (La Jolla, CA, USA). The primers used were ordered from Integrated DNA Technologies (Coralville, IA, USA). All Gateway cloning was done by amplifying desired gene fragments by PCR using *Pfu* polymerase and cloning those products into PENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA) using the protocol provided by the manufacturer. LR reactions were carried out to recombine the pENTR constructs into appropriate Gateway destination vectors using LR clonase from Invitrogen.

**Purification of proteins.** For purification of the 6x-Histidine containing proteins, HrpZ1, HopP1, HrpW1-harpin domain, and HopAK1 were expressed from pET21 and purified from *E. coli* BL21 DE3. Proteins were induced at an optical density at 600nm (OD600) of 0.4 at 37° for 4 h with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation, resuspended in 50 mM $NaH_2PO_4$, 300 mM NaCl solution, and broken by sonication. After removal of the cell debris the supernatant was mixed with Ni-nitrilotriacetic acid-agarose (Qiagen, Valencia, CA, U.S.A.) and was purified from a gravity-flow column using an elution buffer containing 50 mM $NaH_2PO_4$, 300 mM NaCl, and 500 mM Imidazole.

HrpK1 was tagged with glutathione S-transferase (GST) and was expressed on pGEX-5X-1 and purified from *E. coli* DH5α. 4 L of LB media was inoculated with 40 ml of bacterial culture and grown overnight. The inoculum was

allowed to grow in a 37° shaker for 8 h. Cells were harvested and broken by sonication. Upon centrifugation, the cell debris was resuspended in 1x PBS containing 8 M Urea. The supernatant from another centrifugation was dialyzed in 1x PBS overnight at 4°C. After dialysis the supernatant was mixed with Glutathione Sepharose 4B (Amersham Biosciences, Piscataway, NJ, USA) and was purified using a gravity-flow column. After multiple washes with PBS, HrpK1 was eluted using 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0.

The purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining and Western blotting after transferring to a membrane for protein confirmation using affinity-tag specific antibodies. Conjugated anti-His C-terminal-AP antibodies (Invitrogen, Carlsbad, CA, USA) were used to detect 6x-His labeled proteins. Anti-GST primary antibodies (Amersham Biosciences, Piscataway, NJ, USA) and anti-goat IgG (whole molecule)–alkaline phosphatase secondary antibodies produced in rabbit (Sigma, St. Louis, MO, USA) were used for confirmation of HrpK1-GST. Buffers for all proteins were exchanged for PBS using a Microcon centrifugal filter device (Millipore, Billerica, MA, USA) before use in liposome disruption assays. Protein concentrations were determined using the Bio-Rad (Hercules, CA, USA) protein assay.

**Protein interaction with phospholipid membranes.** To determine the ability of different purified proteins to interact with phospholipids, a liposome release assay was used. At high concentrations within liposomes, encapsulated sulforhodamine-B is auto-quenched, but this is relieved by disruption of the

liposomes due to dilution of the escaping SRB. In these experiments, liposomes comprised of 80% 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (PG) and 20% 1,2-dioleoyl-sn-glycero-3-phosphotidylcholine (PC) were prepared at 1 mg/ml to contain 100 mM SRB by mixing these components, sonicating them, and then extruding them through a 100 nm pore to ensure uniformly sized unilamellar vesicles. The SRB was excited at 558 nm with emission detected at 565 nm using 2.5 nm slits.

The liposome preparation (10 µl) was mixed with 575 µl of 10 mM phosphate containing 150 mM NaCl (PBS) in a quartz cuvette and scanned using time-based acquisition mode on a FluoroMax Spectrofluorometer (Horiba-Jobin-Yvon) for 30 s. The scan was then paused and 15 µl of protein was added from a stock solution prepared at 300 µg/ml. Scanning was resumed for 5 m, after which 60 µl of 1% Triton X-100 in PBS was added and the sample scanned for an additional 30 s. The last step allowed determination of the total amount of fluorescence that could be released if all liposomes were disrupted.

**Lipid Binding Assays.** The PIP strips used in this study were purchased from Invitrogen (San Diego, CA, USA). Each strip contains 15 different lipids and one negative control. The strips are incubated in TBS-T buffer, 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, containing 0.1% (v/v) Tween 20 plus 3% BSA, for 1 h. Then purified protein was added at a concentration of 1 µg/ml in TBS-T. The strip was then washed with TBS-T twice for 10 m. Protein was detected using the antibodies corresponding to the affinity tag listed above and then using standard immunoblotting techniques.

**Plant HR assays.** All HR assays were done on *N. tabacum* cv. Xanthi leaves. The HR was assessed at 24 h. To test for restoration of the HR by harpins and HrpK1, constructs encoding these proteins were electroporated into the poly-harpin *hrpK1* mutant (CUCPB5483). Construction of this mutant was described previously (52). Leaves were infiltrated with bacteria at $OD_{600}$ of 0.3 (3 x $10^8$ cells/ml), 0.1 (1 x $10^8$ cells/ml), 0.05 (5 x $10^7$ cells/ml) and 0.01 (1 x $10^7$ cells/ml) in 5 mM MES (pH=5.6) using a blunt syringe.

**Adenylate Cyclase translocation assays.** All constructs used in translocation assays were made using the Gateway system (Invitrogen, Carlsbad, CA, USA). The procedure was described in Schechter *et al.* (76). Briefly, the tested strains were infiltrated in the leaves of *N. benthamiana* or *A. thaliana* plants at an $OD_{600}$ of 0.4 (4 x $10^8$ cells/ml) in 5 mM MES and samples were taken after 7 h using a 0.8 cm diameter cork borer. The leaf discs were ground in liquid nitrogen and resuspended in 300 µl of 0.1 M HCl. Protein concentrations were determined using the Bio-Rad (Hercules, CA, USA) protein assay and were normalized to ensure equal protein loading. The level of cAMP from the tissue was quantified using Correlate-EIA Direct cAMP Enzyme Immunoassay kit (Assay Designs, Ann Arbor, MI, USA) using the directions provided by the manufacturer.

In order to create the CyaA tagged constructs expressed from the Tn7 expression system, pUC18-mini-Tn7-Gm was altered to create a destination vector giving the gene of interest an *avrPto1* promoter and a C-terminal CyaA tag in a suicide vector. In order to make this, pLN2190 was digested with XhoI, blunt ended with T4 DNA polymerase, and then digested with ClaI to have one blunt

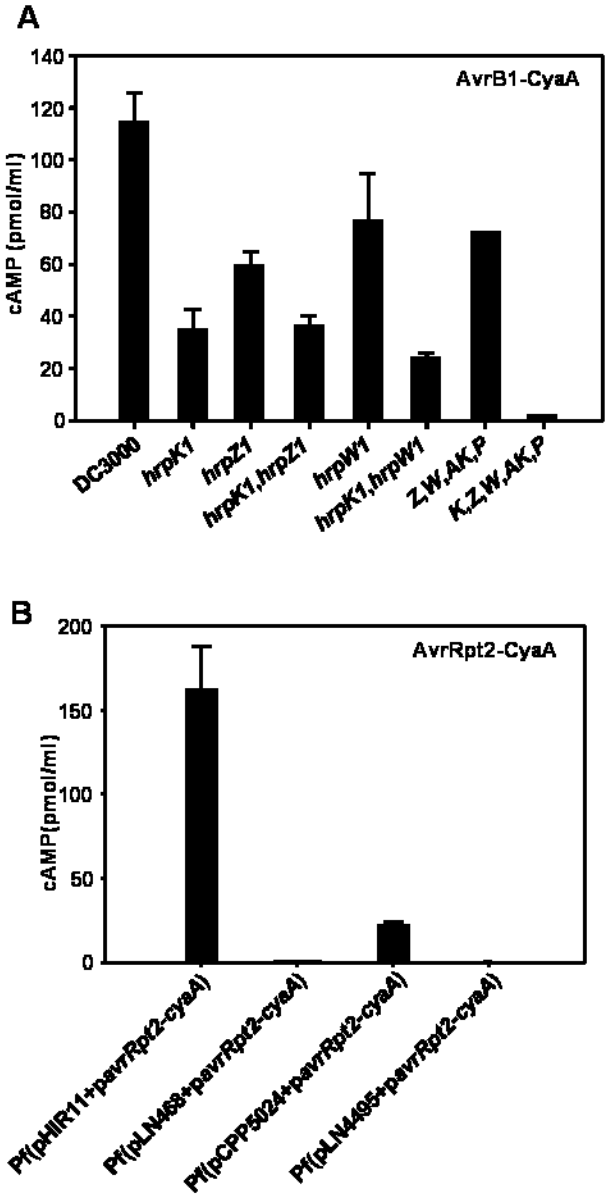
end and a recessed end. The a*vrPto1* promoter was PCR amplified from DC3000 genomic DNA. The PCR fragment was digested with ClaI. pLN2190 (XhoI,T4,ClaI) was ligated to the *avrPto1* promoter(ClaI) to produce pLN4047. pLN4047 and pUC18-mini-Tn7-Gm were digested with SacI and ligated with T4 DNA polymerase to make plasmid pLN4048. Desired entry clones were then used in an LR reaction with pLN4048 destination vectors, confirmed by PCR, and electroporated into wild type or mutant *P. syringae* strains. pTNS2 helper plasmid was used in these transformations. Protein expression in the transformants was confirmed by inducing the bacteria in HrpMM for 6 h. Proteins were then detected via Western blot with CyaA antibodies (3D1) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) with anti-mouse IgG (whole molecule)–alkaline phosphatase secondary antibodies (Sigma, St. Louis, MO, USA).

**AvrRpt2 translocation assay.** This assay was modified from Mudgett and Staskawicz (63). pLN2637 was transformed by electroporation into DC3000 and the poly-harpin *hrpK1* mutant derivatives. The bacteria were grown overnight on KB media containing appropriate antibiotics, collected, and resuspended in 10 mM $MgCl_2$ at a concentration of 1 x $10^9$ cells/ml. Each strain was then infiltrated into the leaves of tobacco plants with a blunt syringe. Six h later leaf samples were taken with a cork borer, ground in liquid nitrogen using a pestle and microcentrifuge tube, and then resuspended in phosphate buffered saline (PBS) containing Complete protease inhibitor cocktail (Roche, Basel, Switzerland). Soluble protein was collected upon centrifugation at 13,000 x g for 5 m at 4°C. Protein concentration was determined using Bradford assay (BioRad) and all

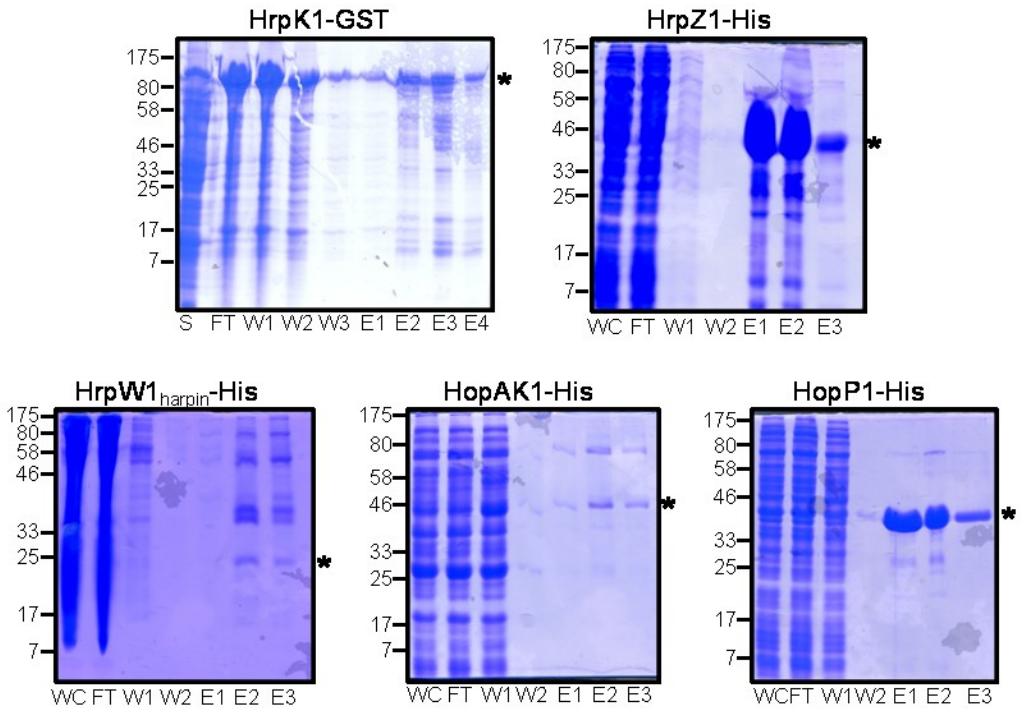
samples were normalized to 1 µg/µl total protein. Protein fractions were mixed 1:1 with 2x Laemmli sample buffer, boiled for 5 m, centrifuged at 13,000 x g for 3 m, and analyzed 25 µg of total protein by immunoblotting using high affinity HA antibodies (Roche) and anti-rat IgG (whole molecule)–alkaline phosphatase secondary antibodies (Sigma, St. Louis, MO, USA).

**Tn7 protein expression system.** The construction of the Tn7 expression vectors has been previously described (19). Briefly, the Tn7 Gateway compatible entry vector was made with left and right flanking sequences, Tn7R and Tn7L, the transposase complex, an *avrPto1* promoter sequence, a Gm resistant *FRT* cassette, and the final gene product contains a C-terminal HA tag. Genes of interest were amplified by PCR using Pfu polymerase with Gateway compatible, gene specific primers. Upon completion of a pENTR reaction, the construct was transformed via heat-shock into DH5α cells and selected for using Ap and Gm. The plasmid was isolated and then was electroporated into DC3000 or mutant derivatives along with the helper plasmid pTNS2 (19). The positive colonies were selected using 1 µg/ml Gm along with strain appropriate antibiotics and colonies were confirmed by PCR. Expression of the proteins was confirmed by Western blot after cells were grown for 7 h at 22°C in HrpMM to induce expression.

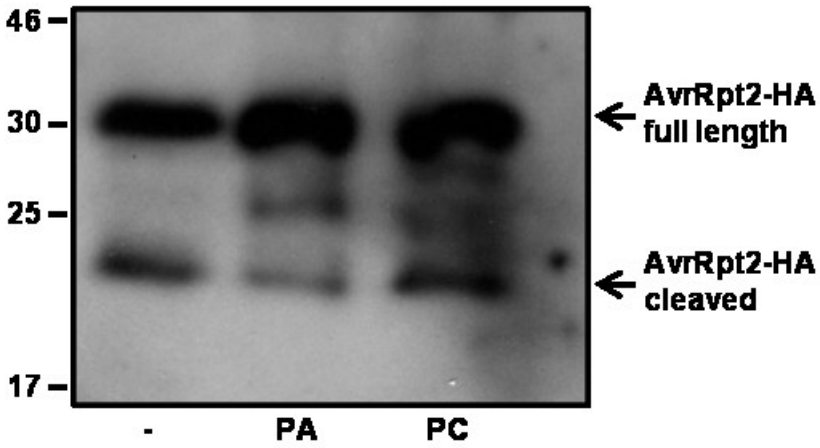
## Supporting Figures and Tables



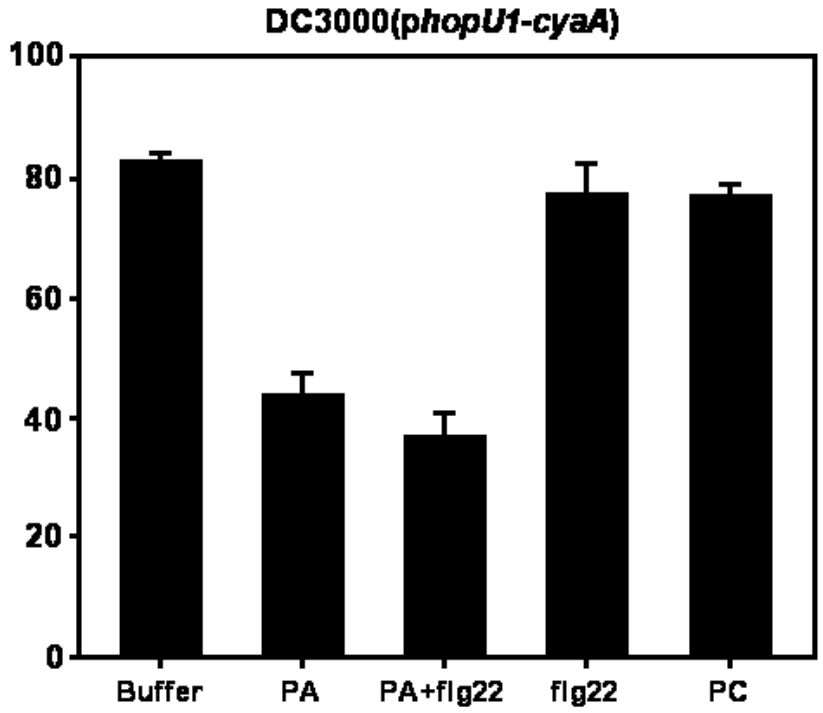

**Supporting Figure 1. Deletion of hrpK1 has a stronger effect on T3E translocation than the harpin proteins in DC3000 and a heterologous *Pseudomonas fluorescens* system.** (A) A construct expressing AvrPto-CyaA was electroporated into DC3000 and mutants lacking *hrpZ1*, *hrpW1*, or all four harpins as well as each strain with additional loss of *hrpK1*. cAMP was measured from samples of the leaves of *N. benthamiana* infiltrated with each strain at 4 x $10^8$ cells/ml for 7 h to determine translocation of AvrPto-CyaA. (B) *hrpK1*, *hrpZ1*, and a *hrpK1,hrpZ1* double mutant were made in the cosmid pHIR11. pHIR11 and the mutant derivatives were electroporated along with a plasmid expressing AvrRpt2-CyaA. Leaves were infiltrated at 4 x $10^8$ cells/ml and cAMP was measured after 7 h.



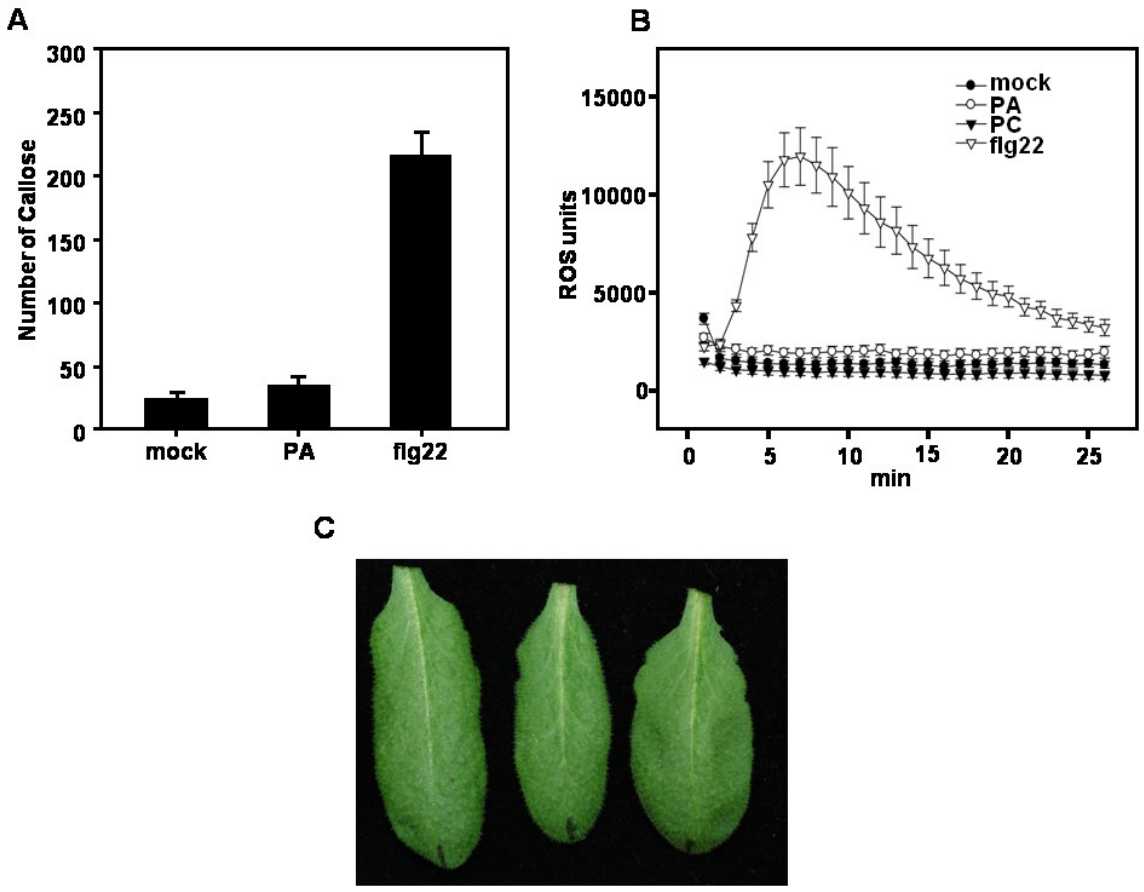

**Supporting Figure 2. Purification of HrpK1-GST and Harpin proteins.** Coomassie blue stained gels containing the purified recombinant protein samples used for the liposome assay in Fig. 1. The band corresponding to the protein of interest is labeled to the right of the gel with an *. All proteins were confirmed by Western blot with antibodies specific to the epitopes used for purification. S = Supernatant, WC = whole cell lysate, FT = Flow through, W = washes, E = elutions.




**Supporting Figure 3. AvrRpt2 translocation from DC3000 is reduced when co-infiltrated with phosphatidic acid.** AvrRpt2-HA is cleaved only when present inside plant cells. DC3000 containing a construct that encodes AvrRpt2-HA was infiltrated in buffer alone (-) or with 1 mM of phosphatidic acid (PA) or phosphatidylcholine (PC) into the leaves of Arabidopsis plants. Samples were taken after 6 h and analyzed by immunoblot using HA antibody. A reduced amount of the cleaved product can be seen in the PA sample. Molecular mass markers in kilodaltons are indicated at the left.




**Supporting Figure 4. Injection blockage by PA does not mimic PTI-induced injection restriction.** Arabidopsis plants were infiltrated with DC3000(p*hopU1-cyaA*) in 5 mM MES buffer either alone (Buffer) or with 1mM phosphatidic acid (PA), 1 µM flg22 (flg22), both flg22 and PA (PA+flg22), or 1 mM phosphatidylcholine (PC). Only with the addition of PA was there a reduction in HopU1-CyaA injection.




**Supporting Figure 5. Phosphatidic acid does not induce PTI and does not cause cell death.** In all experiments 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphate was used a phosphatidic acid (PA) containing mixed acyl groups. A) Infiltration with 1 mM PA does not induce a significantly higher amount of callose deposition compared to the buffer alone unlike the PAMP flg22 which induced much higher levels when infiltrated at1 μM B) Leaf discs floated on buffer alone or with 1 mM PA or PC did not induce a spike in ROS which was observed with flg22 C) Photographs of representative leaves 72 hours after having been infiltrated with 1 mM PA  more than 20 leaves from more than 7 plants grown at 3 different times were infiltrated and 0 leaves had visible cell death even after 5 days.

**Supporting Table 1. Strains and Plasmids used in this study.**

| Strain or Plasmid | Relevant Characteristics | Reference or Source |
|---|---|---|
| *E. coli* DH5α | *supE44 ΔlacU169(Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1relA1, Nal*$^r$ | (42); Life Technologies |
| *E. coli* BL21 DE3 | *F⁻ ompT hsdS20(rB⁻ mB⁻) gal* | Stratagene |
| *Pseudomonas syringae* pv. *tomato* DC3000 | Wild type, Rf$^r$ | (26) |
| DC3000 *hrcC* | *hrcC* mutant defective in T3SS, Rf$^r$ Cm$^r$ | (82) |
| UNL111 | DC3000 *hrpK1* mutant, Rf$^r$ Km$^r$ | (73) |
| UNL234 | DC3000 *hrpK1* mutant Km$^r$ *hrpW1*::ΩSp$^r$/Sm$^r$ | This work |
| CUCPB5094 | DC3000 Δ*hrpZ1::nptII hrpW1*::ΩSp$^r$/Sm$^r$ | (52) |
| CUCPB5096 | DC3000 *hrpW1*::ΩSp$^r$/Sm$^r$ | (52) |
| CUCPB5401 | DC3000 Δ*hrpZ1::nptII hrpW1*::ΩSp$^r$/Sm$^r$ Δ*hopAK1*Δ*hopP1* | (52) |
| CUCPB5482 | DC3000 Δ*hrpK1* Δ*hrpZ1*::*nptII* | (52) |
| CUCPB5483 | DC3000 Δ*hrpK1*Δ*hrpZ1::nptII hrpW1*::ΩSp$^r$/Sm$^r$ Δ*hopAK1* Δ*hopP1* | (52) |
| *P.fluorescens* 55 | Wild type Nalr$^r$ | |
| pHIR11 | Cosmid pLAFR3 derivative with T3SS DNA from genome of *P.syringae* pv. *syringae* 61, Tc$^r$ | (48) |
| pML123 | Broad host range cloning vector, Gm$^r$ Km$^r$ | (53) |
| pBBR1mcs1 | Broad host range cloning vector, Cm$^r$ | (51) |
| pGEX-5X-1 | N-terminal GST expression vector, Ap$^r$ | Pharmacia |
| pET21 | N-terminal 6x-His expression vector, T7 promoter, Ap$^r$ | Novagen |
| pTNS2 | pTNS1 derivative helper plasmid, Ap$^r$ | (19) |
| pCPP3234 | pVLT35 derivative Gateway destination containing the *adenylate cyclase* (*cyaA*) gene for C-terminal fusions, Sp$^r$/Sm$^r$ Cm$^r$ | (76) |
| pCPP5024 | pHIR11 derivative with a *hrpZ1* mutation, Tc$^r$ Km$^r$ | (1) |
| pLN174 | pRG930 derivative that contains *hrpK1* with its native promoter and a Gm$^r$ cassette, Sp$^r$/Sm$^r$ Gm$^r$ | (73) |
| pLN293 | entry vector carrying *hrpK1*, Km$^r$ | (73) |
| pLN468 | pHIR11 derivative with a *hrpK1* mutation, Tc$^r$ Km$^r$ | (73) |
| pLN615 | pML123 derivative gateway destination vector containing a HA tag for C-terminal fusions, Gm$^r$ Cm$^r$ | (37) |
| pLN1682 | entry vector carrying *hrpZ1*, Km$^r$ | (25) |
| pLN1696 | pLN615 derivative containing *hrpZ1-ha*, Gm$^r$ | This work |
| pLN1985 | pCPP3234 derivative containing *avrPto-cyaA* Sp$^r$/Sm$^r$ | This work |
| pLN2193 | pML123-derivative Gateway destination vector containing *cyaA* gene for C-terminal fusions, Gm$^r$ | This work |
| pLN2254 | pLN2193 derivative carrying *hopU1-cyaA*, Gm$^r$ | |
| pLN2583 | entry vector carrying *hopAK1*, Km$^r$ | (25) |
| pLN2590 | entry vector carrying *hopP1*, Km$^r$ | (25) |
| pLN2637 | pML123 derivative containing *avrRpt2-ha*, Gm$^r$ | (24) |
| pLN2635 | pLN615 derivative containing *hopAK1-ha*, Gm$^r$ | This work |
| pLN2742 | entry vector carrying *hrpW1*, Km$^r$ | (25) |
| pLN2759 | pLN2193 derivative carrying *hopX1-cyaA*, Gmr | This work |
| pLN2760 | pLN2193 derivative carrying *hopAI1-cyaA*, Gm$^r$ | This work |
| pLN2761 | pLN2193 derivative carrying *hopAM1-1-cyaA*, Gm$^r$ | This work |

| | | |
|---|---|---|
| pLN2786 | pLN2193 derivative carrying *hopR1-cyaA*, $Gm^r$ | This work |
| pLN2790 | pLN2193 derivative carrying *hopC1-cyaA*, $Gm^r$ | This work |
| pLN3066 | pLN2992 derivative carrying *hrpZ1*, $Gm^r$ | (25) |
| pLN3067 | pLN2992 derivative carrying *hrpW1*, $Gm^r$ | This work |
| pLN3069 | pML123 derivative containing *hrpW1-ha*, $Gm^r$ | This work |
| pLN3121 | pLN2992 derivative carrying *hopAK1*, $Gm^r$ | This work |
| pLN3149 | pLN2992 derivative carrying *hrpK1*, $Gm^r$ | This work |
| pLN3161 | pGEX-5X-1 expressing *hrpK1-gst*, $Ap^r$ | This work |
| pLN3224 | pET21 expressing *hrpZ1-his*, $Ap^r$ | This work |
| pLN3252 | pLN2193 derivative carrying *avrB-cyaA*, $Gm^r$ | This work |
| pLN3253 | pLN2193 derivative carrying *avrRpt2-cyaA*, $Gm^r$ | This work |
| pLN3305 | pLN615 derivative carrying *hrpK1-ha*, $Gm^r$ | This work |
| pLN3415 | pLN2193 derivative carrying *avrPto1-cyaA*, $Gm^r$ | This work |
| pLN3661 | pET21 expressing *hopP1-His*, $Ap^r$ | This work |
| pLN3662 | pET21 expressing *hopAK1-His*, $Ap^r$ | This work |
| pLN4048 | puc18mini-Tn7-$Gm^r$::*avrPto*$_{prom}$/Gateway-*cyaA* | This work |
| pLN4086 | pLN4048 derivative carrying *hrpZ1-cyaA*, $Gm^r$ | This work |
| pLN4199 | pLN4048 derivative carrying *hrpK1-cyaA*, $Gm^r$ | This work |
| pLN4200 | pLN4048 derivative carrying *hrpW1-cyaA*, $Gm^r$ | This work |
| pLN4201 | pLN4048 derivative carrying *hopAK1-cyaA*, $Gm^r$ | This work |
| pLN4253 | pET21 expressing the harpin domain of *hrpW1*, $Ap^r$ | This work |
| pLN4495 | pHIR11 derivative with a *hrpZ1* and *hrpK1* mutation, $Tc^r$, $Km^r$ | This work |

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# Chapter 3

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

**Abstract**

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 nonpathogenic and cannot inject type III 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.

## 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 (20, 30). 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 (19, 31). 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 (23, 57).

One protein family that plays an important role in type III secretion control and hierarchy is the YopN-TyeA/InvE/SepL family (9, 59). 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 (43, 59). In most other bacteria YopN and TyeA homologs are fused and are encoded by one gene (59). *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 (8, 27, 41). The TyeA protein has been implicated in the translocation of effectors (22, 41). *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 (18, 45). SepL from enteropathogenic *Escherichia coli* is required for secretion of translocator proteins in culture and the translocation of type III effectors (56). *Shigella flexneri* mutants lacking MxiC, another YopN-TyeA/InvE/SepL family member, exhibit increased secretion of type III effectors (9) but also secrete reduced amounts of translocators (50). 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 (45) and TyeA is not secreted (15, 25). 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 (19). 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 (35, 52, 54). These proteins are thought to be situated at the tip of the type III needle by the LcrV tip protein

(53). 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 (10). 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 (13, 51, 60). 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 (4). 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) (37, 67). 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 (46). However, it is currently unknown how harpins interact with HrpK1/HrpF family members to translocate type III effectors into plant cells.

*P. syringae* is a phytopathogen that uses its T3SS to inject type III effectors into host plant cells to subvert plant immunity (7, 72). 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 (4, 28, 59). 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 (28). Similar phenotypes are also associated with an *Erwinia amylovora hrpJ* mutant (55). 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 (2). 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.

**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 (28). DC3000 *hrpZ1* mutants have a subtler virulence phenotype than the DC3000 *hrpJ* mutant (2), 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 (3, 14), 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 hemagglutinin (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 had 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

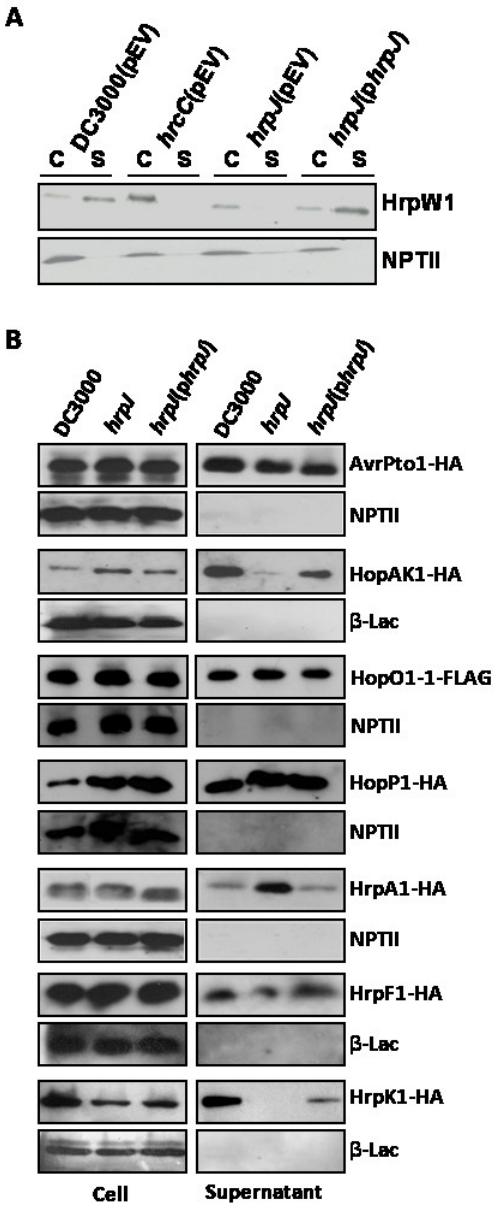


**Fig. 1. The hrpJ mutant is impaired in its ability to secrete HrpW1, HrpK1, and HopAK1, but not HopP1, HrpF, HrpA1, HopO1-1, or AvrPto1.** (A) Wild type DC3000, a type III defective mutant *hrcC*, and a *hrpJ* mutant were grown in type III-inducing conditions and then separated into cell (C) and supernatant (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-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.

effectors AvrPto1 and HopO1-1, the HrpA1 pilus protein, and HrpF, a type III-secreted protein encoded by the *hrp-hrc* cluster (62), were all secreted by the *hrpJ* mutant (Fig. 1B). We reported previously that HrpA1 was secreted by the *hrpJ* mutant (28). 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 (28). 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 (28). N-terminal GST fusions have been made with type III-secreted substrates to render them impassable to the T3SS (63). 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

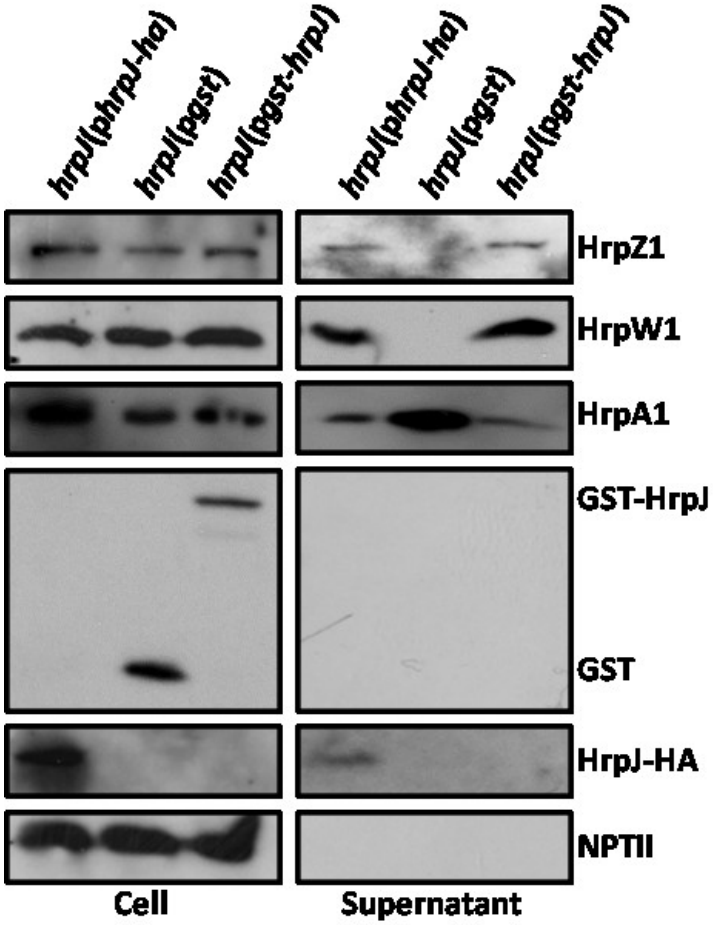


**Fig. 2. A secretion incompetent HrpJ fusion protein restores the ability to secrete HrpZ1 to a *hrpJ* mutant.** The DC3000 *hrpJ* mutants carrying a construct that encoded HrpJ-HA C-terminal fusion, GST alone, or a GST-HrpJ N-terminal fusion were grown in type III-inducing conditions and separated 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 additional times with similar results.

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 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 ($HrpJ_{\Delta 2\text{-}75}$), an N-terminal half region ($HrpJ_{\Delta 2\text{-}185}$), or a large C-terminal region ($HrpJ_{\Delta 161\text{-}368}$), each fused to an HA epitope. These constructs were confirmed by sequencing and produced stable HrpJ derivatives (Data not shown). The $HrpJ_{\Delta 2\text{-}75}$ and $HrpJ_{\Delta 2\text{-}185}$ derivatives could not be detectably secreted or translocated in in culture secretion assays and translocation assays, respectively, whereas the $HrpJ_{\Delta 161\text{-}368}$ 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 $HrpJ_{\Delta 2\text{-}75}$ derivative restored the secretion of HrpZ1 from the *hrpJ* mutant (Fig. 3A). Both $HrpJ_{\Delta 2\text{-}185}$ and $HrpJ_{\Delta 161\text{-}368}$ 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 $HrpJ_{\Delta 2\text{-}185}$ and $HrpJ_{\Delta 161\text{-}368}$ 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 (46, 60). 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* ((28); Fig. 3B). The secretion incompetent $HrpJ_{\Delta 2\text{-}75}$ 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 (28), a *hrpJ* mutant was severely reduced in its ability to grow *in planta* and cause disease symptoms in *Arabidopsis* (Fig.

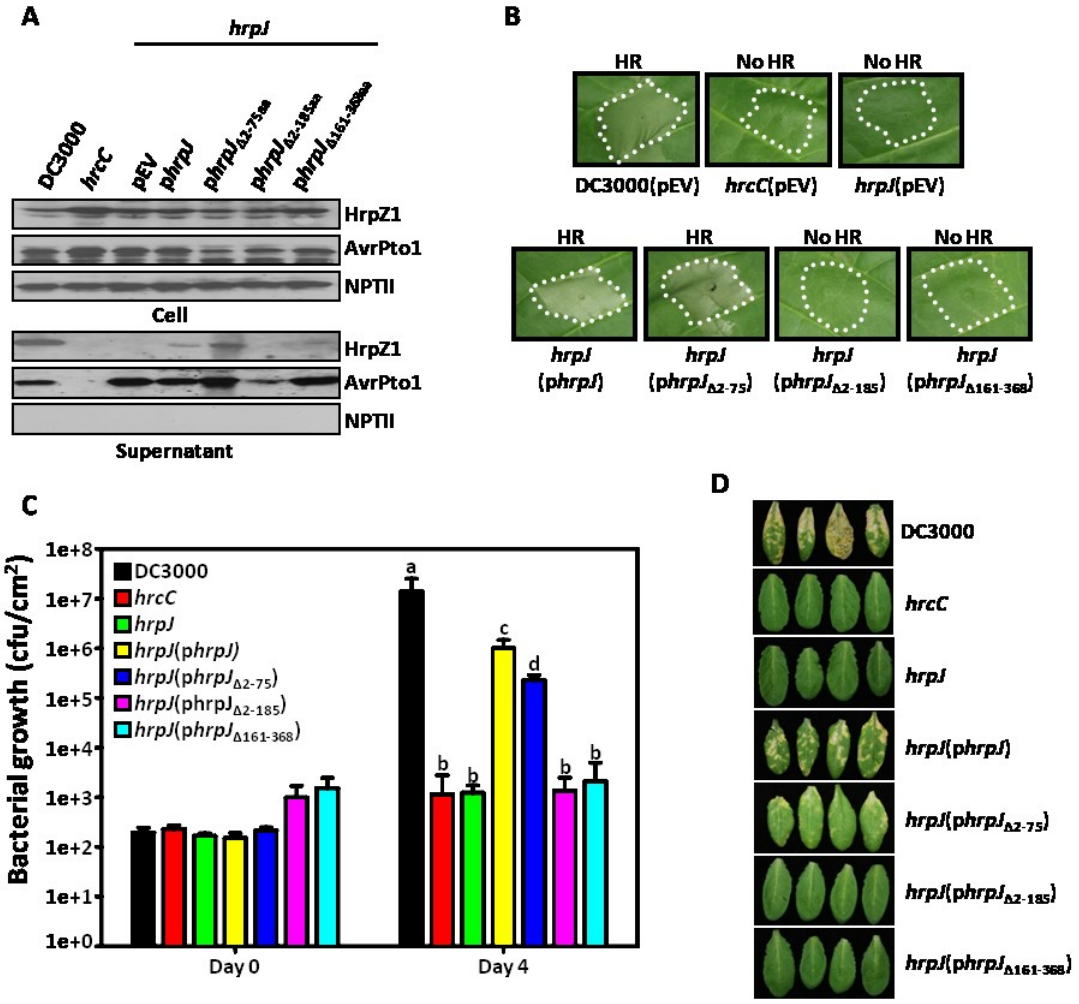


**Fig. 3. A HrpJ derivative lacking its secretion signal restores secretion of HrpZ1 and the ability to elicit an HR to a *hrpJ* mutant but only partially complements pathogenicity.** (A) DC3000 strains were grown in type III-inducing conditions and separated into cell and supernatant fractions by centrifugation 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 $HrpJ_{\Delta 2\text{-}75}$ were capable of eliciting an HR in tobacco indicating that these strains were capable of injecting type III effectors. Bacteria were infiltrated at 1 x $10^8$ cells/ml and the HR was observed within 24 hours 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* $hrpJ_{\Delta 2\text{-}75}$ *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-D) The experiments were repeated twice with similar results.

3C and D). The production of $HrpJ_{\Delta 2\text{-}185}$ and $HrpJ_{\Delta 161\text{-}368}$ 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). $HrpJ_{\Delta 2\text{-}75}$ 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 $HrpJ_{\Delta 2\text{-}75}$ 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 $hrpJ_{\Delta 2\text{-}75}$ 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$_{\Delta 271\text{-}320}$-HA derivative, which lacked amino acids 271-320 was secreted in culture from the *hrpJ* mutant (Fig.4A). Only low amounts of HrpZ1$_{\Delta 271\text{-}320}$-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$_{\Delta 21\text{-}70}$-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

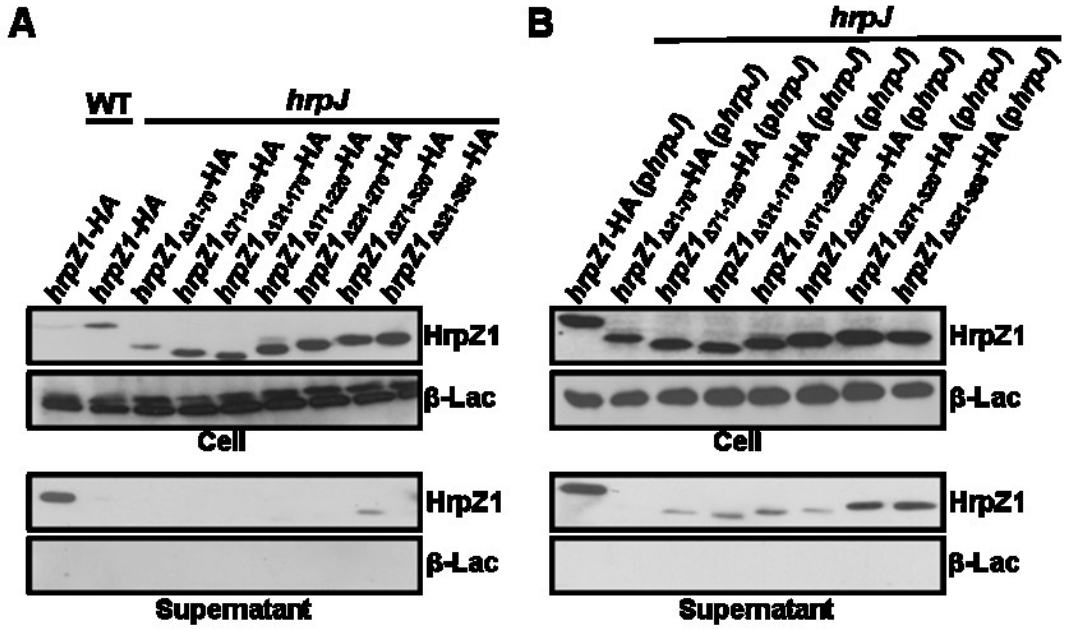


**Fig. 4. A C-terminal HrpZ1 deletion derivative can be secreted in culture by a *hrpJ* mutant.** (A) Wild type DC3000 (WT) and *hrpJ* mutant strains expressing HrpZ1 and HrpZ1 derivatives C-terminally fused to a hemagglutinin (HA) tag were grown in type III-inducing conditions and separated into cell 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 (HrpZ1D271-320). (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*_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-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 4 times with similar results.

transgenic Arabidopsis plants were 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 ($HrpJ_{\Delta 2\text{-}75}$) grew similarly and caused similar disease symptoms in transgenic Arabidopsis plants expressing HrpJ-HA to the *hrpJ* mutant expressing 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 PAMP-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 (34), 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) (65). 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

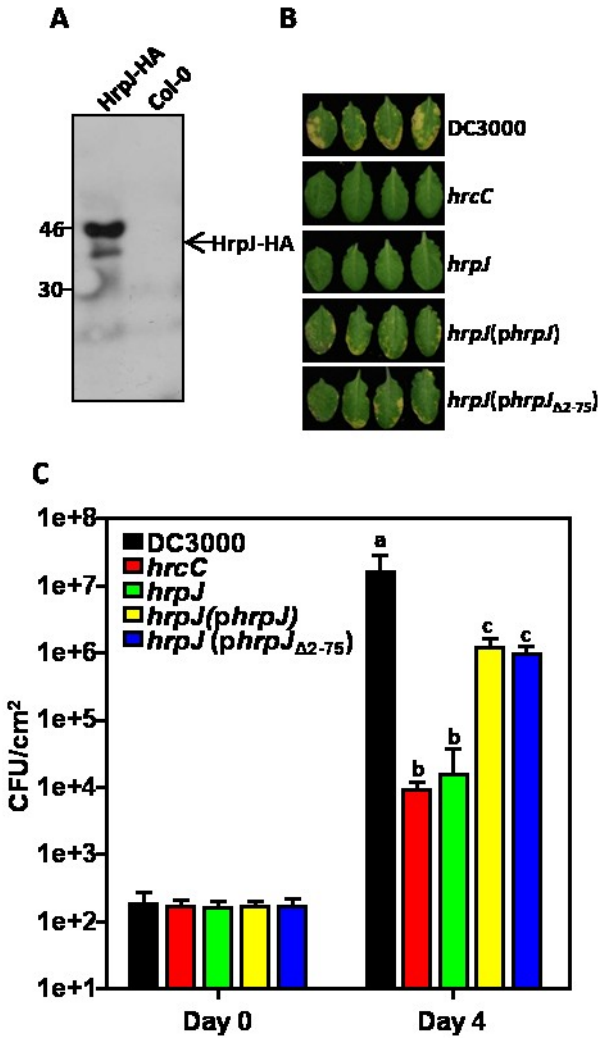


**Fig. 5. *In planta* HrpJ-HA expression complements the reduced growth phenotype associated with the *hrpJ* mutant complemented with cell-bound HrpJ.** (A) Transgenic Arabidopsis plants express detectable amounts of HrpJ-HA. Total protein extracts from wild type *A. thaliana* Col-0 (right) and a representative 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 *P. syringae* strains indicated. This experiment was done twice with similar results. (C) Transgenic HrpJ-HA Arabidopsis plants were spray-inoculated with 2 x $10^8$ cells/ ml 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 ($hrpJ_{\Delta 2\text{-}75}$) 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.

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 two-fold 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 sequence identity with the YopB and YopD translocators and the LcrV tip protein from *Yersinia* spp. (39). 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 (11). Putative translocators in the *P. syringae* T3SS are the HrpZ1 harpin and HrpK1 (47, 60), but the relationship between these proteins is unclear. The observation that HrpZ1 was not secreted from a *P. syringae hrpJ*

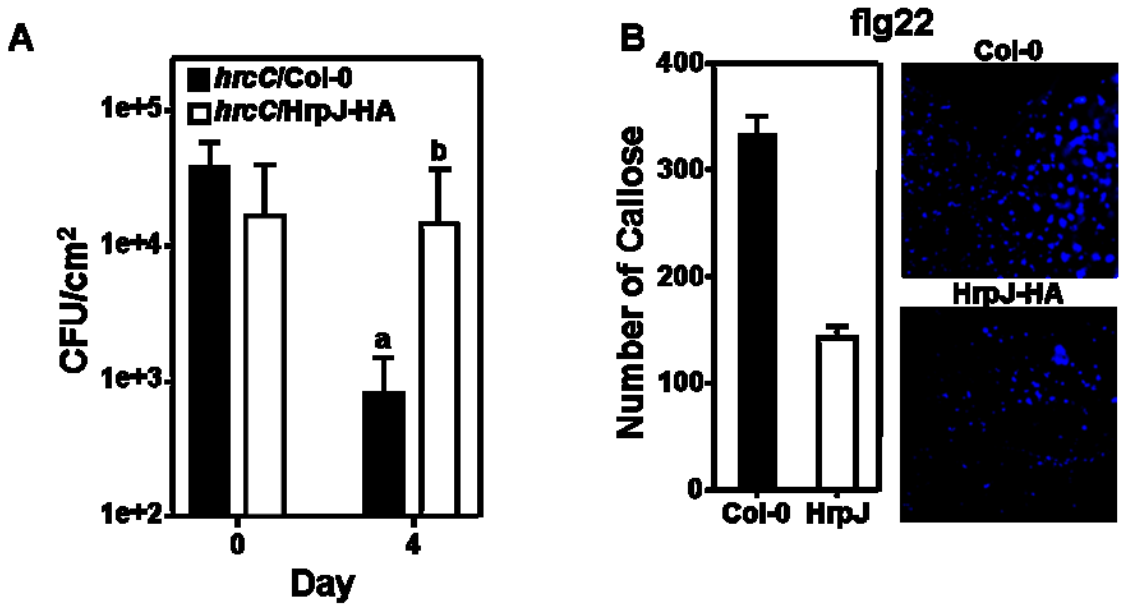


**Fig. 6. Transgenic HrpJ-HA Arabidopsis plants exhibit reduced plant innate immune responses.** (A) Bacterial growth assays of a type III defective *hrcC* mutant spray-inoculated at 2 x $10^8$ cells/ml onto wild type Arabidopsis (Col-0) and transgenic Arabidopsis plants expressing HrpJ-HA. The *hrcC* 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 2 individual plants. Representative micrographs are shown in the panels on the right. These experiments were done at least twice with similar results.

mutant (28) 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 (46). 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 ((28), 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 (24, 66). 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 (50). 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 (9). 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 secretion appears undeniable because of its strong virulence and translocation phenotypes ((28) and Fig. 2) and because of its inability to secrete the 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 *Shigella mxiC* mutants (9, 26, 45) 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 (9). 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-immunoprecipitation 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 (28) 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 *Xanthomonas campestris* (4, 19). 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 (12). 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 (12, 64). 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 (49). 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 group 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 (8, 22, 48). *E. 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 (58, 69). 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 (5, 38). Expression of *C. pneumonia* CopN in yeast or animal cells caused cell cycle arrest and disruption of microtubules (38). Further studies found that CopN directly binds αβ-tubulins and inhibits tubulin polymerization (5). 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. pneumonia* replication in animal cells consistent with CopN contributing to virulence (38).

A *P. syringae hrpJ* mutant is severely debilitated in its ability to infect plants ((28); 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 (44, 56, 66, 68-70). 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 (70). 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 (40). Antibiotics were used at the following concentrations (micrograms per milliliter): Ampicillin, 100; chloramphenicol, 20; gentamicin, 10; kanamycin, 50; rifampicin, 100; spectinomycin, 50; and tetracycline, 20.

**General DNA manipulation.** Restriction enzymes, T4 ligase, and DNA polymerase were purchased from New England Biolabs (Beverly, MA). Thermostable 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 vector (Invitrogen, Carlsbad, CA). 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 type III-inducing fructose minimal media, and adjusted to a concentration of 4 x $10^8$ cells/ml ($OD_{600}$ = 0.4) with the appropriate antibiotics. Cultures were incubated in a 22°C shaker at 220 rpm 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* (16). 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 gentamicin 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.** HR 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 x $10^8$ cells/ml 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 *A. 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 (6). 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 x $10^8$ cells/m. 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 (1) 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).

**Supporting Figures and Tables**

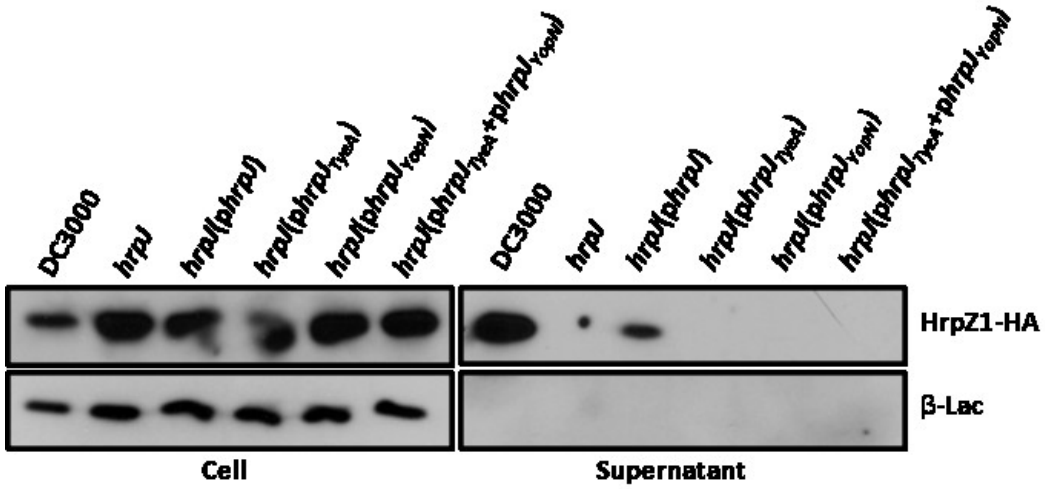


**Fig. S1. The YopN and TyeA moieties of HrpJ do not restore the secretion of HrpZ1 from the *hrpJ* mutant.** Plasmids expressing the YopN and/or TyeA moieties of HrpJ, pLN4101 and pLN4109, respectively, were expressed in the *hrpJ* mutant and the secretion of HrpZ1-HA was determined. When either the YopN or TyeA moiety or both were expressed, the ability to secrete HrpZ1 was not restored to the *hrpJ* mutant. Only when full length HrpJ was expressed was secretion of HrpZ1-HA restored to the *hrpJ* mutant. Secretion assays were performed as described in the associated paper. To PCR clone the nucleotides corresponding to the YopN and TyeA moieties primers P3654 and P3655 and P3652 and P3653 were used, respectively. These DNAs were cloned into Gateway entry vectors and recombined into Gateway destination plasmid pLN615 or pLN705.

**Table1. Strains and Plasmids used in this study**

| Strain or plasmid | Relevant Characteristics | Source or Reference |
|---|---|---|
| *E. coli* DH5α | *supE44 ΔlacU169(Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA*<br>*relA1, Nal*$^r$ | (36); Life Technologies |
| *Pseudomonas syringae* pv. *tomato* DC3000 | Wild type, Rf$^r$ | (21) |
| DC3000 *hrcC* | *hrcC* mutant defective in T3SS, Rf$^r$ Cm$^r$ | (71) |
| UNL140 | Non-polar *hrpJ* mutant, Rf$^r$, Km$^r$ | (29) |
| pENTR/D-TOPO | Gateway entry vector, Km$^r$ | Invitrogen |
| pLN293 | entry vector carrying *hrpK1*, Km$^r$ | (61) |
| pLN307 | entry vector carrying *avrPto1*, Km$^r$ | (29) |
| pLN335 | pBBR1MCS-1 derivative gateway destination vector containing a FLAG tag for C-terminal fusions, Cm$^r$ | This work |
| pLN375 | entry vector carrying *hrpJ*, Km$^r$ | (29) |
| pLN462 | pPZP212 derivative gateway destination binary vector containing 35S promoter and HA tag for C-terminal fusions, Sp$^r$ | (42) |
| pLN615 | pML123 derivative gateway destination vector containing a HA tag for C-terminal fusions, Gm$^r$ Cm$^r$ | (33) |
| pLN705 | pBBR1MCS-1 derivative gateway destination vector containing a HA tag for C-terminal fusions, Gm$^r$ | (32) |
| pLN736 | pLN705 derivative carrying *hrpJ*, Gm$^r$ | (29) |
| pLN814 | entry vector carrying *hopO1-1*, Km$^r$ | (32) |
| pLN1327 | pLN615 derivative carrying *avrPto1*, Gm$^r$ | (33) |
| pLN1622 | pLN615 derivative carrying *hopO1-1*, Gm$^r$ | This work |
| pLN1681 | entry vector carrying *hrpA1*, Km$^r$ | This work |
| pLN1682 | entry vector carrying *hrpZ1*, Km$^r$ | This work |
| pLN1695 | pLN615 derivative carrying *hrpA1*, Gm$^r$ | This work |
| pLN2590 | entry vector carrying *hopP1*, Km$^r$ | This work |
| pLN2583 | entry vector carrying *hopAK1*, Km$^r$ | This work |
| pLN2647 | pLN615 derivative carrying *hrpJ*, Gm$^r$ | This work |
| pLN2648 | pLN615 derivative carrying $hrpJ_{\Delta 186\text{-}368\ a.a.}$, Gm$^r$ | This work |
| pLN2653 | entry vector carrying $hrpJ_{\Delta 186\text{-}368\ a.a.}$, Km$^r$ | This work |
| pLN2742 | entry vector carrying *hrpW1*, Km$^r$ | This work |
| pLN2913 | entry vector carrying *hrpF1*, Km$^r$ | This work |
| pLN2992 | pUCP18T-mini-Tn7 derivative destination vector containing a HA tag for C-terminal fusions, $P_{avrPto}$, Gm$^r$ | This work |
| pLN3066 | pLN2992 derivative carrying *hrpZ1*, Gm$^r$ | This work |
| pLN3075 | entry vector carrying $hrpJ_{\Delta 2\text{-}185\ a.a.}$, Km$^r$ | This work |
| pLN3079 | pLN615 derivative carrying $hrpJ_{\Delta 2\text{-}185\ a.a.}$, Gm$^r$ | This work |
| pLN3109 | entry vector carrying $hrpZ1_{\Delta 2\text{-}70}$, Km$^r$ | This work |
| pLN3110 | entry vector carrying $hrpZ1_{\Delta 71\text{-}120}$, Km$^r$ | This work |
| pLN3111 | entry vector carrying $hrpZ1_{\Delta 121\text{-}170}$, Km$^r$ | This work |
| pLN3112 | entry vector carrying $hrpZ1_{\Delta 171\text{-}220}$, Km$^r$ | This work |
| pLN3113 | entry vector carrying $hrpZ1_{\Delta 221\text{-}270}$, Km$^r$ | This work |
| pLN3114 | entry vector carrying $hrpZ1_{\Delta 271\text{-}320}$, Km$^r$ | This work |

| | | |
|---|---|---|
| pLN3115 | entry vector carrying *hrpZ1*$_{\Delta 321\text{-}368}$, Km$^r$ | This work |
| pLN3116 | pLN2992 derivative carrying *hrpZ1*$_{\Delta 2\text{-}70}$, Gm$^r$ | This work |
| pLN3117 | pLN2992 derivative carrying *hrpZ1*$_{\Delta 71\text{-}120}$, Gm$^r$ | This work |
| pLN3118 | pLN2992 derivative carrying *hrpZ1*$_{\Delta 121\text{-}170}$, Gm$^r$ | This work |
| pLN3119 | pLN2992 derivative carrying *hrpZ1*$_{\Delta 221\text{-}270}$, Gm$^r$ | This work |
| pLN3120 | pLN2992 derivative carrying *hrpZ1*$_{\Delta 271\text{-}320}$, Gm$^r$ | This work |
| pLN3123 | pLN615 derivative carrying *hopP1*, Gm$^r$ | This work |
| pLN3133 | pLN2992 derivative carrying *hrpZ1*$_{\Delta 171\text{-}220}$, Gm$^r$ | This work |
| pLN3134 | pLN2992 derivative carrying *hrpZ1*$_{\Delta 321\text{-}368}$, Gm$^r$ | This work |
| pLN3231 | entry vector carrying *gst*, Km$^r$ | This work |
| pLN3232 | entry vector carrying *gst-hrpJ*, Km$^r$ | This work |
| pLN3233 | pLN615 derivative carrying *gst*, Gm$^r$ | This work |
| pLN3234 | pLN615 derivative carrying *gst-hrpJ*, Gm$^r$ | This work |
| pLN3258 | entry vector carrying *hrpJ*$_{\Delta 2\text{-}75\ a.a.}$, Km$^r$ | This work |
| pLN3260 | pLN615 derivative carrying *hrpJ*$_{\Delta 2\text{-}75\ a.a.}$, Gm$^r$ | This work |
| pLN3478 | pLN335 derivative carrying *hrpJ*, Cm$^r$ | This work |
| pLN4084 | entry vector carrying hrpJ$_{YopN}$, Km$^r$ | This work |
| pLN4085 | entry vector carrying hrpJ$_{TyeA}$, Km$^r$ | This work |
| pLN4101 | pLN615 derivative carrying hrpJ$_{YopN}$, Gm$^r$ | This work |
| pLN4109 | pLN615 derivative carrying hrpJ$_{TyeA}$, Gm$^r$ | This work |
| pLN4501 | pLN462 derivative carrying *hrpJ*, Sp$^r$ | This work |
| pLN4607 | pLN615 derivative carrying *hrpF1*, Gm$^r$ | This work |
| pLN5037 | entry vector carrying *hrpJ*$_{YopN}$*-stop*, Km$^r$ | This work |
| pLN5038 | pLN615 derivative carrying *hrpJ*$_{YopN}$*-stop*, Gm$^r$ | This work |
| pTNS2 | pTNS1 derivative helper plasmid, Ap$^r$ | (17) |

**Table 2. Primers used in this study.**

| Gene name | Primer number | Primer sequence |
|---|---|---|
| *hrpK1* | P0638 | 5'-CACCACTCTCGGAAGGCAACTAACAATGCGT-3' |
| | P0639 | 5'-ATGCGCCTTCGCAATCCCGAA-3' |
| *avrPto1* | P0689 | 5'-CACCTGTACTAAAGAGGGTATAAGAATGGGA-3' |
| | P0690 | 5'-TTGCCAGTTACGGTACGGGCT-3' |
| *hrpJ* | P0759 | 5'-CACCGGCTTAGAACTGAACAATGAAAATCG-3' |
| | P0760 | 5'-GACGAGCCCCCCGTAGAGCTGC-3' |
| *hopO1-1* | P0814 | 5'- CTCGTCAGAGCTCTCTGCGAT-3' |
| | P1120 | 5'-CACCCGAGTGATTTTAACAATGGGTAATATTTGTGG-3' |
| *hrpA1* | P1752 | 5'-CACCCACGCTGGTAAATCTTAA-3' |
| | P1753 | 5'-GTAACTGATACCTTTAGC-3' |
| *hrpZ1* | P1754 | 5'-CACCGGCCGCTACCTTGGGATG-3' |
| | P1755 | 5'-GGCCACAGCCTGGTTAGT-3' |
| $hrpJ_{\Delta 2\text{-}185}$ | P2301 | 5'-GCTTAGAACTGCCCTATGAACAGCGCCAAGGCATTCG-3' |
| | P2302 | 5'-CGAATGCCTTGGCGCTGTTCATAGGGCAGTTCTAAGC-3' |
| $hrpJ_{\Delta 186\text{-}368}$ | P2303 | 5'-CGTGCTCGCGCCGGGATCATGAAGGGTGGGCGCGC-3' |
| | P2304 | 5'-GCGCGCCCACCCTTCATGATCCCGGCGCGAGCACG-3' |
| *hopP1* | P2327 | 5'-CACCGTGAGGTAACTGGCTATG-3' |
| | P2328 | 5'- AGCGGGTAAATTGCCCTGCC-3' |
| *hopAK1* | P2329 | 5'-CACCCCACAGGCGGGGAACTCAACAATG-3' |
| | P2330 | 5'-TTTCACGACCTGTGCACCCGC-3' |
| *hrpW1* | P2582 | 5'-CACCTTAGCGAGGTAACGCAGCATGAGCATCGGC-3' |
| | P2583 | 5'-AAGCTCGGTGTGTTGGGT-3' |
| *hrpF1* | P2654 | 5'-CACCAAGCTTCTGCTGGAGCACCAGGACATG-3' |
| | P2655 | 5'-TCTAGACTGAATTCCATCGATGACTG-3' |
| $hrpZ1_{\Delta 2\text{-}70}$ | P2776 | 5'-TCATTGTTCCCCGTGTCGGGCAAGTCGGCTAACAGC-3' |
| | P2777 | 5'-GCTGTTAGCCGACTTGCCCGACACGGGGAACAATGA-3' |
| $hrpZ1_{\Delta 71\text{-}120}$ | P2778 | 5'-CAAGGCCATGGCTGCGGATGGTGGCGGTCTGAGCAGC-3' |
| | P2779 | 5'-GCTGCTCAGACCGCCACCATCCGCAGCCATGGCCTTG-3' |
| $hrpZ1_{\Delta 121\text{-}170}$ | P2780 | 5'-GTTCTGGTTCGGGTGTCGAAAAAGTCGCCCAGTTC-3' |
| | P2781 | 5'-GAACTGGGCGACTTTTTCGACACCCGAACCAGAAC-3' |
| $hrpZ1_{\Delta 171\text{-}220}$ | P2782 | 5'-GATGACATGCCGACCCTGCTCGGCCAGCAACAAGGTG-3' |
| | P2783 | 5'-CACCTTGTTGCTGGCCGAGCAGGGTCGGCATGTCATC-3' |
| $hrpZ1_{\Delta 221\text{-}270}$ | P2784 | 5'-GACGTCATTGGTCAACAGATCGGTCAACTCATCGAC-3' |
| | P2785 | 5'-GTCGATGAGTTGACCGATCTGTTGACCAATGACGTC-3' |
| $hrpZ1_{\Delta 271\text{-}320}$ | P2786 | 5'-GTCGACGTAGGTCAACTGAGCGGCTTGCTGCAACGCG-3' |
| | P2787 | 5'-CGCGTTGCAGCAAGCCGCTCAGTTGACCTACGTCGAC-3' |
| $hrpZ1_{\Delta 321\text{-}368}$ | P2788 | 5'-GACCTGGGTCAACTGCTGAAGGGTGGGCGCGCCG-3' |
| | P2789 | 5'-CGGCGCGCCCACCCTTCAGCAGTTGACCCAGGTC-3' |
| *gst-hrpJ* | P2814 | 5'-CACCAGGAAACAGTATTCATGTCCC-3' |
| | P2857 | 5'-TCAGACGAGCCCCCCGTAGAGCTGC-3' |
| *gst* | P2814 | 5'-CACCAGGAAACAGTATTCATGTCCC-3' |
| | P2883 | 5'-TCAGAATTCGGGGATCCCAC-3' |
| $hrpJ_{\Delta 2\text{-}75}$ | P2942 | 5'-GCTTAGAACTGCCCTATGGAGCTTTATCAACTGCTG-3' |
| | P2945 | 5'-CAGCAGTTGATAAAGCTCCATAGGGCAGTTCTAAGC-3' |

| | | |
|---|---|---|
| *hrpJ*$_{TyeA}$ | P3652 | 5'-GCTTAGAACTGCCCTATGAACACGGCACGCCACGTG-3' |
| | P3653 | 5'-CACGTGGCGTGCCGTGTTCATAGGGCAGTTCTAAGC-3' |
| *hrpJ*$_{YopN}$ | P3654 | 5'-ACACTGATGCACGGCCTCAAGGGTGGGCGCGCCGAC-3' |
| | P3655 | 5'-GTCGGCGCGCCCACCCTTGAGGCCGTGCATCAGTGT-3' |
| *hrpJ*$_{YopN}$*-stop* | P4475 | 5'-TCAGAGGCCGTGCATCAGTGT-3' |

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# Chapter 4

## Plant immunity directly or indirectly restricts the injection of type III effectors by the *Pseudomonas syringae* type III secretion system

**Abstract**

Plants perceive microorganisms by recognizing microbial molecules known as pathogen-associated molecular patterns (PAMPs) inducing PAMP-triggered immunity (PTI) or by recognizing pathogen effectors inducing effector-triggered immunity (ETI). The hypersensitive response (HR), a programmed cell death response associated with ETI, is known to be inhibited by PTI. Here we show that PTI-induced HR inhibition is due to direct or indirect restriction of the type III protein secretion system's (T3SS) ability to inject type III effectors (T3Es). We found that the *Pseudomonas syringae* T3SS was restricted in its ability to inject a T3E-adenylate cyclase (CyaA) injection reporter into PTI-induced *Nicotiana tabacum* (tobacco) cells. We confirmed this restriction with a direct injection assay that monitored the *in planta* processing of the AvrRpt2 T3E. Virulent *P. syringae* strains were able to overcome a PAMP pretreatment in tobacco or *Arabidopsis thaliana* and continue to inject a T3E-CyaA reporter into host cells. In contrast, ETI-inducing *P. syringae* strains were unable to overcome PTI-induced injection restriction. A *P. syringae* pv. *tomato* DC3000 mutant lacking about one-third of its T3E inventory was less capable of injecting into PTI-induced *A. thaliana* plant cells, grew poorly *in planta*, and did not cause disease symptoms. PTI-induced transgenic *A. thaliana* expressing the T3Es HopAO1 or HopF2 allowed higher amounts of the T3E-CyaA reporter to be injected into plant cells compared to wild type plants. Our results show that PTI-induced HR inhibition is due to direct or indirect restriction of T3E injection and that T3Es can relieve this restriction by suppressing PTI.

## Introduction

Plants come into contact with a myriad of microorganisms and rely on their innate immune systems to perceive potential microbial infections and induce immune responses. Plant innate immunity can be broadly portrayed as consisting of two major branches, distinguished primarily by their mode of microbe detection. The first branch is activated by extracellular pattern recognition receptors (PRRs) (7, 43) that perceive broadly conserved molecules called pathogen (microbe)-associated molecular patterns (PAMPs or MAMPs) (4, 37). The response induced by this recognition is termed PAMP-triggered immunity (PTI) (31). A well characterized example of PTI in plants is the recognition of and subsequent immune response to a small N-terminal region of bacterial flagellin by the FLS2 receptor kinase of *Arabidopsis thaliana* (16, 56). Plant resistance (R) proteins activate the second branch of the plant innate immune system by recognizing specific pathogen effector proteins. The response induced by this recognition is termed effector-triggered immunity (ETI) (31). ETI and PTI induce similar innate immune responses including ion fluxes, reactive oxygen species (ROS), and callose (β-1,3- glucan) deposition in the cell wall (7, 52), however, ETI generally also includes the induction of a programmed cell death called the hypersensitive response (HR) (25).

The induction of ETI in response to a bacterial plant pathogen is generally due to the recognition of bacterial type III effector (T3E) proteins injected into the plant cell by the pathogen's type III protein secretion system (T3SS)(2, 9). These recognized T3Es were classically known as avirulence (Avr) proteins because

they induced ETI responses sufficient to prevent a normally virulent pathogen from causing disease thereby rendering it ‘avirulent’ (33). However, it has become increasingly apparent that many T3Es benefit their bacteria by suppressing PTI and ETI (6, 12, 20). Under the current model plants first developed PTI to reduce microbial colonization of the apoplast. Successful bacterial pathogens countered this by acquiring a T3SS and PTI-suppressing T3Es (10, 15, 31).

The bacterial pathogen *Pseudomonas syringae* infects the aerial parts of many plant species. It displays host-specificity and its strains have been separated into greater than 50 pathovars based on the host plants that they infect. For example, *P. syringae* pv. *tabaci* is virulent in *Nicotiana tabacum* (tobacco), but it triggers non-host resistance in *A. thaliana*, a plant-microbe interaction referred to as a non-host interaction. Non-host resistance describes the resistance observed when all members of a plant species are resistant to a specific pathogen (40, 51). While not well understood, both PTI (34) and ETI (44, 54) have been shown to play a role in non-host resistance to bacterial pathogens. In some cases, *P. syringae* strains display race-cultivar resistance. This is generally due to the resistant cultivar possessing an R protein that can recognize a T3E from the pathogen inducing ETI (5). One well-studied *P. syringae* strain is *P. syringae* pv. *tomato* DC3000, which causes bacterial speck disease on specific tomato (*Lycopersicon esculentum*) cultivars and disease on all ecotypes of *A. thaliana* tested. These interactions have been classically

referred to as compatible interactions. However, DC3000 triggers non-host resistance in tobacco and many other plants.

DC3000 contains greater than thirty T3Es (12, 13, 35). These are encoded by genes contained within the Hrp pathogenicity island, which also encodes the T3SS apparatus (1), other pathogenicity islands, or as individual genes throughout the genome of DC3000 (8, 54). One molecular tool that has been useful in studying the effect individual T3Es have on plants is the cosmid pHIR11 (28). This cosmid encodes a functional T3SS from *P. syringae* pv. *syringae* 61 and the T3E HopA1. It confers upon non-pathogenic bacteria such as *P. fluorescens* the ability to inject HopA1 into plant cells. In tobacco and other plants, injected HopA1 induces ETI including an HR (3, 28). The expression of other T3Es in *P. fluorescens*(pHIR11) enabled them to be screened for the ability to suppress HopA1-induced ETI (20, 30). Bacterial strains carrying the pHIR11 derivatives pLN18 or pLN1965 both of which lack *hopA1* and so no longer induce ETI were used to determine which T3Es could suppress PTI (20, 45). Collectively, these experiments demonstrated that many *P. syringae* T3Es possessed the ability to suppress both ETI and PTI.

One PTI suppression assay using *P. fluorescens*(pLN18) employed by Oh and Collmer (45) took advantage of earlier observations indicating that PTI could inhibit the ability of the plant to mount an HR (i.e., ETI) in response to avirulent or non-host bacteria (32, 42). In this assay, the PTI inducers *P. fluorescens*(pLN18) or a 22 amino acid peptide from flagellin (flg22) are infiltrated into *N. benthamiana*. Six h later the ETI inducer DC3000 is infiltrated in a region of the

leaf that overlaps with the earlier infiltration. The HR is typically inhibited in the overlapping region that was pretreated with a PTI inducer. Several T3Es suppressed this inhibition when they were separately delivered at time of pretreatment (45). It has been speculated that the probable mechanisms for inhibition of the HR caused by PTI include impairment of delivery of T3Es that induce the HR, modification of the events downstream of T3E recognition, or a shutdown of programmed cell death (42).

Here we show that PTI inhibits the HR on tobacco because it restricts the ability of *P. fluorescens*(pLN1965) or DC3000 to inject T3Es based on injection (translocation) assays using T3E-adenylate cyclase (CyaA) fusions. This was confirmed using an independent injection assay that monitored the amount of the cleaved *in planta* form of the T3E AvrRpt2. Interestingly, this injection restriction was not observed in the compatible interactions between DC3000 and *A. thaliana* or between *P. syringae* pv. *tabaci* 11258 and tobacco. A DC3000 mutant lacking four clusters of T3E genes, which corresponds to eleven T3Es, was less able to inject a T3E-CyaA fusion into PTI-induced *A. thaliana* suggesting that the PTI suppressing activities of the T3E inventory of DC3000 it to overcome the injection restriction. Transgenic *A. thaliana* plants separately expressing specific T3Es known to be capable of PTI suppression increased the ability of *P. fluorescens*(pLN1965) to inject a T3E-CyaA fusion into PTI-induced plant cells. Collectively, these data suggest that PTI can directly or indirectly restrict type III injection and PTI suppression by T3Es can relieve this restriction in susceptible plant cells but not plant cells undergoing ETI.

## Results

**PTI inhibits the *P. fluorescens*(pHIR11)-dependent HR on tobacco.** To explore the mechanism underlying PTI inhibition of the HR (32, 42, 45) we first needed to define the conditions necessary for it to occur in our system. To cause PTI we used two well established PTI-inducers – one was the nonpathogenic bacterial strain *P. fluorescens*(pLN1965) (20, 24), and the other was a 21 amino acid peptide (flg21) from the N-terminus of flagellin (16). To cause an HR we used *P. fluorescens*(pHIR11), which encodes a functional T3SS and the T3E HopA1 that elicits an HR on tobacco (28). To test HR inhibition the PTI-inducer (pretreatment) was infiltrated into a *N. tabacum* cv. Xanthi (tobacco) leaf. After the specified time interval the HR-inducer was infiltrated into a partially overlapping region of the same leaf. Presence or absence of HR was scored in the overlapping region 48 h after the second infiltration. PTI-induction by infiltration of *P. fluorescens*(pLN1965) at a cell density of 3 X $10^8$ cells/ml at 4 h but not at 2 h before infiltration of *P. fluorescens*(pHIR11) was sufficient to inhibit the HR (Fig. 1A). Similarly, infiltration with 1 μM flg21 at 2 h but not at 1 h to induce PTI was sufficient to inhibit the pHIR11-dependent HR (Fig. 1B). In addition a 2 h pretreatment with 0.1 μM flg21 but not with 0.01 μM flg21 could inhibit *P. fluorescens*(pHIR11)-dependant HR (Fig. 1C). We used the pretreatment times, bacterial cell densities, and flg21 concentrations established in these experiments for the experiments described below.

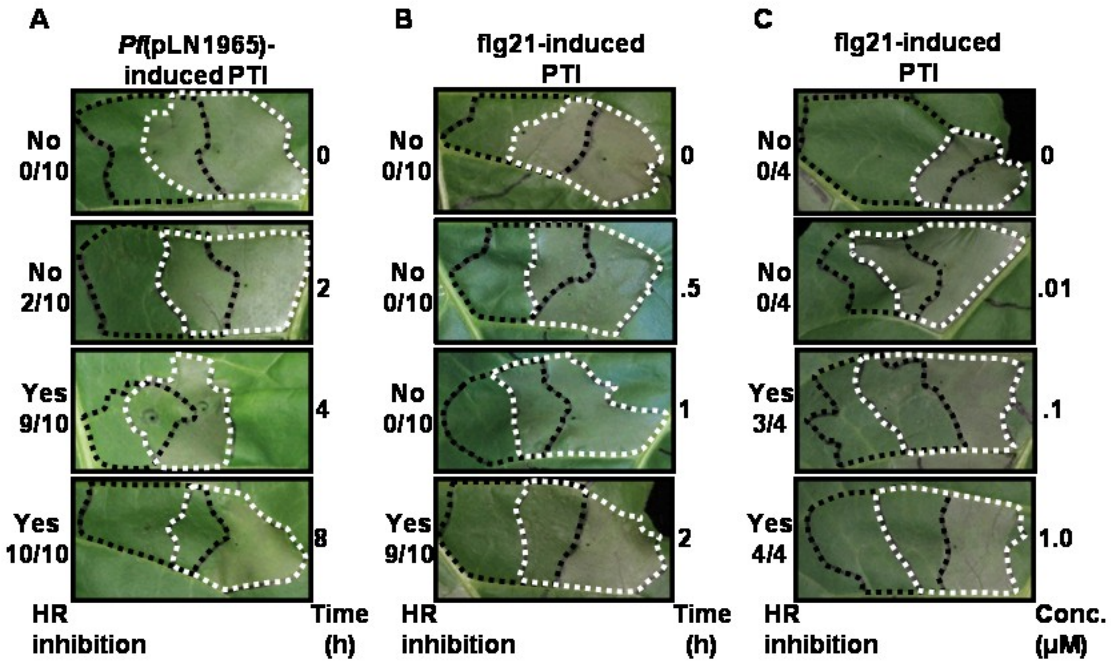



**Figure 1. PTI-induction times and flagellin concentrations that inhibit the *P. fluorescens*(pHIR11)-dependent HR in tobacco.** PTI was induced by infiltration with 3 x $10^8$ cells/ml of *P. fluorescens* (pLN1965) or various flg21 concentrations (indicated by black dashed lines). After the specified period an overlapping infiltration of 3 x $10^8$ cells/ml of the HR-inducing strain *P. fluorescens*(pHIR11) (indicated by white dashed lines) was performed. The presence or absence of HR in the overlapping region (bordered by white and black dashed lines) was evaluated 48 h after *P. fluorescens*(pHIR11) infiltration. (A) Tobacco leaves were infiltrated with *P. fluorescens*(pHIR11) 0, 2, 4, or 8 h after having been infiltrated with *P. fluorescens*(pLN1965). (B) Tobacco leaves were infiltrated with *P. fluorescens*(pHIR11) 0, 0.5, 1, or 2 h after having been infiltrated with 1µM flg21. (C) Tobacco leaves were infiltrated with 0, 0.01, 0.1 or 1 µM of flg21, 2 h prior to infiltration with *P. fluorescens*(pHIR11). The fraction to the left of each image indicates the number of times that the HR was inhibited over the total number of times the assay was performed.

**PTI restricts T3E injection.** One possible reason why the HR is inhibited by PTI is that the T3Es causing ETI are not entering the plant cells. This could be due to a direct block of the process or an indirect effect on bacterial physiology such that they can no longer inject T3Es. To test whether T3Es were injected into PTI-induced plant cells, we used an adenylate cyclase (CyaA) injection (translocation) assay (49). This assay determines whether a CyaA fusion protein is injected into eukaryotic cells because CyaA's ability to produce cAMP is dependent on calmodulin, a protein only present in significant amounts in eukaryotic cells. If a substantial amount of cAMP is detected, it indicates that the CyaA fusion protein was injected. At defined time points after infiltration of the PTI inducer (flg21 or *P. fluorescens*(pLN1965)) into tobacco, we infiltrated *P. fluorescens*(pLN1965) carrying an additional construct that encoded the AvrPtoB or HopU1 T3E fused to CyaA. Construct pLN1965 encodes a functional *P. syringae* T3SS and enables *P. fluorescens* to inject T3Es. As shown in Fig. 2, pretreatment with either PTI inducer greatly restricted the injection of AvrPtoB-CyaA or HopU1-CyaA into tobacco cells as demonstrated by the low levels of cAMP. Importantly, we were unable to detect changes in cAMP levels in plants transiently expressing *cyaA* after PAMP treatment indicating that PTI does not directly affect CyaA activity (Supplemental Fig. 1). Moreover, the decrease in cAMP levels in PTI-induced tobacco occurs in the same time frame as HR inhibition consistent with HR inhibition being due to direct or indirect restriction of T3E injection.

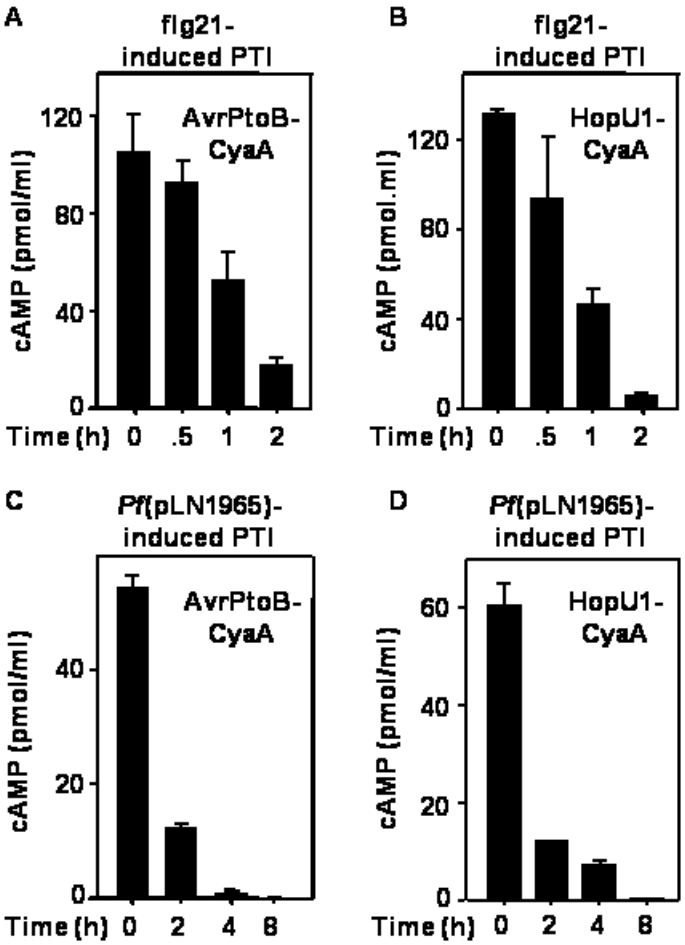



**Figure 2. PTI restricts injection of T3E-adenylate cyclase (CyaA) fusion proteins into tobacco cells.** PTI was induced by infiltration with 3 x $10^8$ cells/ml *P. fluorescens*(pLN1965) or 1μM flg21 at the indicated times prior to an overlapping infiltration of 3 x $10^8$ cells/ml *P. fluorescens*(pLN1965) expressing AvrPtoB-CyaA or HopU1-CyaA. The level of T3E injection was determined by quantifying cAMP in the overlapping infiltration area 16 h later. cAMP levels in tobacco leaves infiltrated with (A) *P. fluorescens*(pLN1965 + p*avrPtoB-cyaA*) pretreated with flg21, (B) *P. fluorescens*(pLN1965 + p*hopU1-cyaA*) pretreated with flg21, (C) *P. fluorescens*(pLN1965 + p*avrPtoB-cyaA*) pretreated with *P. fluorescens*(pLN1965), or (d) *P. fluorescens*(pLN1965 + p*hopU1-cyaA*) pretreated with *P. fluorescens*(pLN1965). Reduced cAMP levels in PTI-induced plant cells shows that PTI restricts T3E injection. Standard error bars are shown and each experiment was repeated three times with similar results.

The ability of the non-pathogenic bacterial strain *P. fluorescens*(pLN1965) to inject T3E-CyaA fusions in tobacco was clearly restricted by the induction of PTI. We next sought to determine the extent that the pathogenic bacterial strain *P. syringae* pv. *tomato* DC3000 was blocked in its ability to elicit an HR and inject a T3E-CyaA fusion into PTI-induced tobacco plant cells. The PTI-inducers used were flg21 and a DC3000 *hrcC* mutant, which is defective in type III secretion (20, 24). DC3000 induces non-host resistance on tobacco and normally causes an HR within 24 h. Using HR inhibition assays we found that a 2 h pretreatment with flg21 or a 4 h pretreatment with the *hrcC* mutant were sufficient to prevent the DC3000-induced HR on tobacco (Fig. 3A & B). In conjunction with this, the ability of DC3000 to inject HopU1-CyaA was also strongly inhibited by a 2 h pretreatment of flg21 or the *hrcC* strain (Fig. 3C). Thus, PTI also directly or indirectly blocks the ability of DC3000 to inject T3Es into tobacco cells indicating that this phenotype is not limited to the *P. fluorescens*(pLN1965) injection system. One explanation for this result is that PTI kills the bacterial cells in the apoplast. To test this, bacterial test strains were infiltrated into tobacco leaves at various time points and cell densities after PTI-induction. We were unable to detect any reduction in the number of bacterial cells in PTI-induced plants compared to control plants suggesting that PTI was not inhibiting the HR or restricting injection simply by causing bacterial cell death (Supplemental Fig. 2). These data suggest that a PTI-induced restriction of injection is responsible for the HR inhibition phenotype. One could envision that reduced T3E injection into tobacco cells would lead to insufficient delivery of recognized T3Es and,

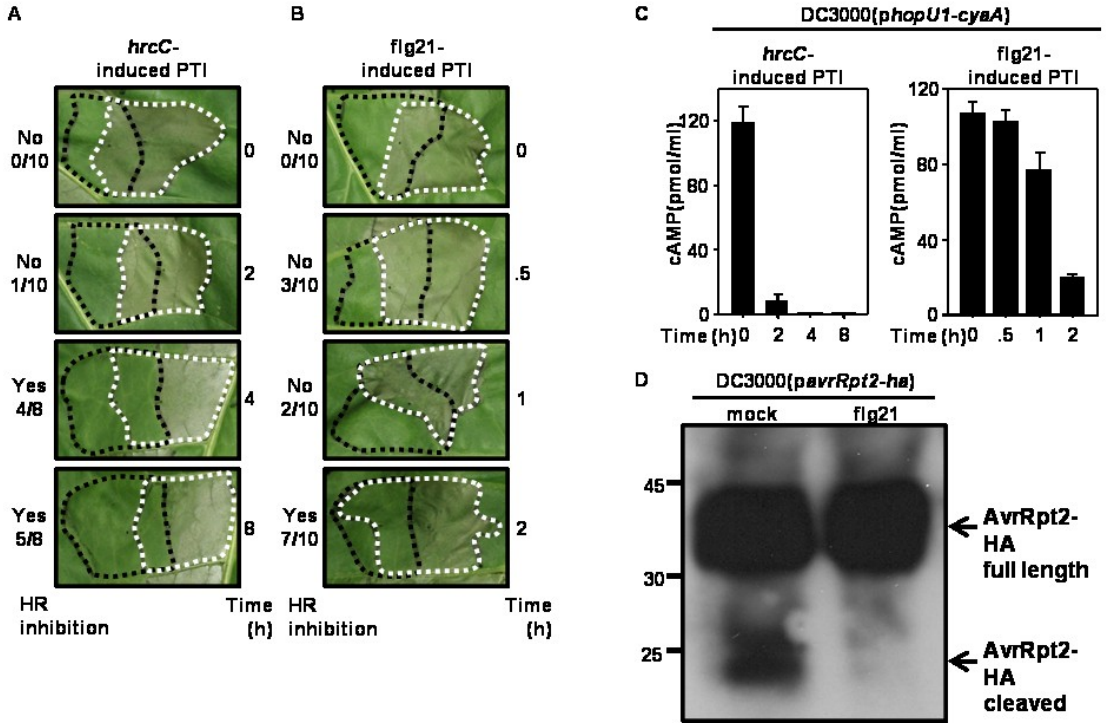



**Figure 3. The ability of DC3000 to elicit an HR and inject T3E-CyaA fusions in tobacco is inhibited by PTI.** (A) PTI was induced in tobacco leaves by infiltration with 3 x $10^8$ cells/ml of the DC3000 *hrcC* mutant 0, 2, 4, or 8 h prior to an overlapping infiltration with 2 x $10^7$ cells/ml of the wild type DC3000 and scored for HR inhibition. The fraction to the left of each image indicates the number of times that the HR was inhibited over the total number of times the assay was performed. (B) PTI was induced in tobacco leaves by infiltration of 1μM flg21 0, 0.5, 1, or 2 h prior to infiltration with 2 x $10^7$ cells/ml DC3000 and scored for elicitation of HR inhibition. (C) The level of injection was determined by measuring cAMP in tobacco 7 h after infiltration with 3 x $10^8$ cells/ml of DC3000(p*hopU1-cyaA*) in plants pretreated with *hrcC* or flg21 at the times indicated. (D) AvrRpt2-HA is cleaved only when present inside plant cells. PTI was induced in tobacco leaves with a 1μM flg21 treatment prior to infiltration of DC3000 containing a construct that encodes AvrRpt2-HA. In a water (mock) treatment control cleavage of AvrRpt2-HA can be detected with anti-HA antibodies but no or reduced amounts of cleaved AvrRpt2-HA can be detected in PTI-induced tobacco tissue. Molecular mass markers in kilodaltons are indicated at the left. PTI induction inhibited the HR and the ability of DC3000 to inject HopU1-CyaA or AvrRpt2-HA into tobacco cells. Each experiment was repeated at least three times with similar results. Standard error bars are shown when appropriate.

therefore, prevent induction of ETI responses including the HR.

**PTI restricts the injection of AvrRpt2 as PAMP pretreatment greatly reduced the amount of *in planta* AvrRpt2 cleavage.** To confirm that PTI restricted T3E injection using an independent assay, we developed a direct assay to monitor the injection of a T3E after PTI induction. The *P. syringae* T3E AvrRpt2 has been shown to be processed once it is injected into plant cells by the *P. syringae* T3SS (39). Wild type DC3000 was transformed with a construct that encoded AvrRpt2 fused to a hemagglutinin (HA) tag at its C-terminus. This strain was infiltrated into tobacco leaf tissue pretreated with flg22 or a mock control. After 6 h crude plant samples were isolated and subjected to SDS-PAGE and immunoblot analysis. Cleaved AvrRpt2-HA was detected in the mock treated leaf tissue samples and greatly reduced amounts of cleaved AvrRpt2-HA were detected in the flg21 pretreated samples infiltrated with DC3000 (Fig. 3D). These results indicate that AvrRpt2 is not injected into PTI-induced plant cells and confirm the findings of the CyaA injection assay. Because the CyaA injection assay data are more easily quantifiable we chose to use this assay to further define this phenomenon.

**DC3000 but not *P. fluorescens*(pLN1965) can overcome PTI-induced injection restriction in *A. thaliana*.** We next sought to determine if this injection restriction also occurred in *A. thaliana*. To do this we tested if the PTI-inducers flg21 and *hrcC* could prevent *P. fluorescens*(pLN1965) or DC3000 injecting the HopU1-CyaA fusion into *A. thaliana* cells. As in tobacco, pretreatment with flg21 or the *hrcC* mutant reduced *P. fluorescens*(pLN1965) injection of the HopU1-

CyaA fusion into *A. thaliana* cells (Fig. 4A). This result was somewhat expected because tobacco and *A. thaliana* are known to induce PTI in response to PAMP treatments (16) and these plants respond similarly to infiltration of the *P. fluorescens*(pLN1965) injection system. Interestingly, when we performed similar experiments using DC3000 to inject HopU1-CyaA into *A. thaliana* cells, plant leaf tissue contained high amounts of cAMP at all time points indicating that the HopU1-CyaA fusion continued to be injected by DC3000 after PTI induction (Fig. 4B). These data suggest that in compatible interactions such as that of DC3000 with *A. thaliana,* but not in incompatible or non-host interactions, the bacteria can overcome PTI-induced injection restriction.

**PTI-induction restricts injection by *P. syringae* into non-host plant cells.** To determine the extent that host or non-host interactions influenced the PTI-induced restriction of T3E injection we electroporated the construct encoding HopU1-CyaA into *P. syringae* pv. *tabaci* 11258, which has a non-host interaction with *A. thaliana* and a compatible interaction with its host tobacco. In stark contrast to DC3000, this strain was capable of injecting HopU1-CyaA into PTI-induced tobacco cells (Fig. 4C) but not into PTI-induced *A. thaliana* cells (Fig. 4D). Normalization of the cAMP levels to the growth of these bacterial strains in *A. thaliana* and tobacco within the 16 h time frame of the cAMP measurements did not significantly change these conclusions (Supplemental Fig. 3). Collectively, these data suggest that in compatible interactions, the initial low amount of T3Es injected is capable of suppressing PTI such that normal levels of T3E injection can be achieved. In non-host interactions, the bacterial T3Es are incapable of

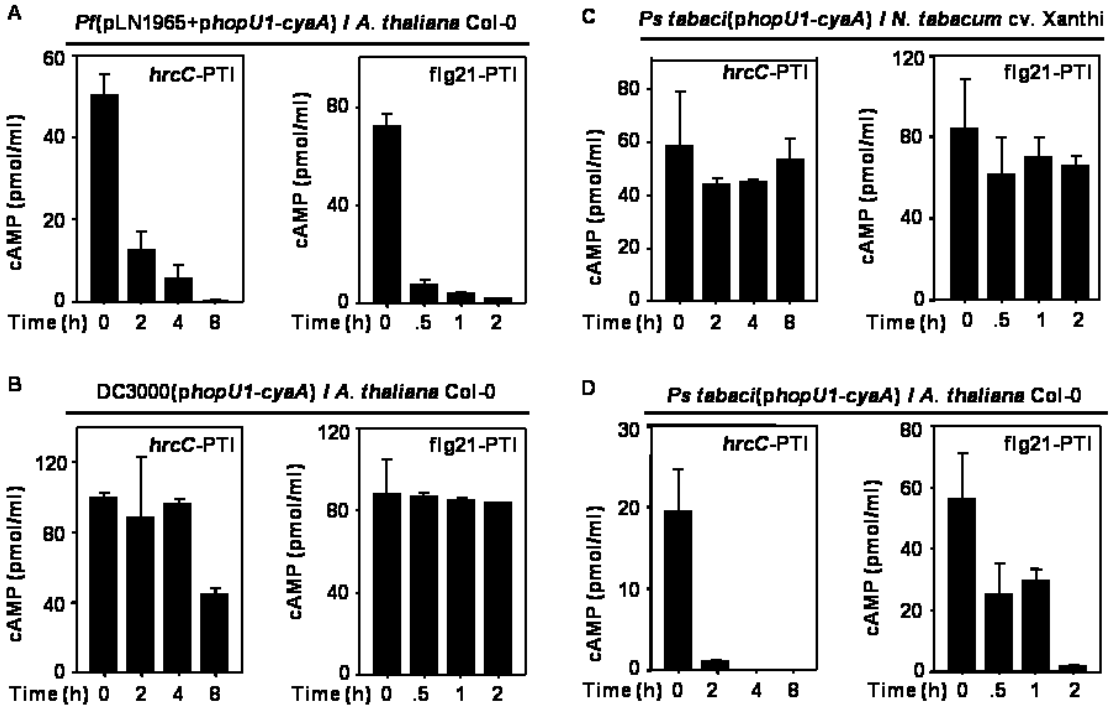



**Figure 4. PTI-induced injection restriction occurs in tested *P. syringae* non-host interactions but not in compatible interactions.** PTI was induced with 3 x $10^8$ cells/ml of the *hrcC* mutant (left panels) or 1$\mu$M flg21 (right panels). At the times indicated 3 x $10^8$ cells/ml of bacterial strains expressing HopU1-CyaA were infiltrated into an overlapping area and cAMP amounts were determined. The following bacteria/plant combinations were used to assess injection capability: (A) *P. fluorescens*(pLN1965 + p*hopU1-cyaA*) / *A. thaliana* Col-0; (B) DC3000(p*hopU1-cyaA*) / *A. thaliana* Col-0, a compatible interaction; (C) *P. syringae* pv. *tabaci* 11258(p*hopU1-cyaA*) / *N. tabacum* cv. Xanthi, a compatible interaction; and (D) *P. syringae* pv. *tabaci* 11258(p*hopU1-cyaA*) / *A. thaliana* Col-0, a non-host interaction. PTI induction severely restricted the ability of bacterial strains in non-host plants to inject the HopU1-CyaA fusion into plant cells based on the reduced levels of cAMP. However, bacterial strains in host plants were capable of relatively high levels of HopU1-CyaA injection irrespective of PTI induction. Each experiment was repeated at least three times with similar results and standard error bars are shown.

suppressing PTI sufficiently to allow normal injection, perhaps because T3Es can be recognized by R proteins and induce ETI reinforcing the injection restriction or at least preventing T3Es from suppressing PTI. In support of this, several DC3000 T3Es when expressed transiently in tobacco using *Agrobacterium* induce ETI (Supplemental Fig. 4).

**A DC3000 poly-T3E mutant loses the ability to inject T3Es into PTI-induced *A. thaliana* and does not grow well or cause disease symptoms *in planta*.**

To determine whether T3Es were allowing DC3000 to continue to inject into its susceptible host *A. thaliana* despite PTI induction we made a series of DC3000 mutants lacking different subsets of T3Es. We deleted DNA clusters of T3E genes from DC3000 using a homologous recombination approach that relies on yeast Flp recombinase to act on introduced Flp recombinase target sequences resulting in unmarked mutations (11, 27). One DNA cluster we deleted was the exchangeable effector locus (EEL) from the Hrp pathogenicity island (1) resulting in DC3000 mutant UNL155. This mutant lacks the T3E gene, *hopB1* (47). The other T3E gene-containing DNA clusters were pathogenicity islands distributed around the DC3000 genome (8), which we gave the following temporary names: Effector pathogenicity island 1 (EPai1) that contains *hopD1, hopQ1-1*, and *hopR1*; EPai2 that contains *hopAA1-2, hopV1, hopAO1, hopG1,* and *hopQ1-2;* and EPai3 that contains *hopF2,* and *hopU1.* DC3000 mutants lacking EPai1, EPai2, or EPai3 were named UNL158, UNL159, and UNL184, respectively. Additionally, we made a DC3000 quadruple mutant lacking the EEL, EPai1, EPai2, and EPai3, which was named UNL227. All of the mutants were confirmed

using PCR using primers adjacent to the deleted DNA clusters (Supplemental Fig. 5).

We tested the extent that the DC3000 T3E mutants were altered in their ability to inject a HopU1-CyaA fusion into PTI-induced *A. thaliana* plants compared to wild type DC3000. The DC3000 mutants lacking different T3E gene (UNL155, UNL158, UNL159, and UNL184) were subtly affected in T3E injection, but all retained the ability to inject HopU1-CyaA (Fig. 5A). However, the DC3000 quadruple mutant UNL227 was greatly reduced in its ability to inject HopU1-CyaA into PTI-induced *A. thaliana* plants based on low cAMP levels in infiltrated *A. thaliana* tissue (Fig. 5A). The growth differences between the DC3000 and UNL227 could not account for the different cAMP levels (Supplemental Fig. 3). UNL227 is lacking eleven T3Es normally present in DC3000, which accounts for about one-third of its T3E inventory. This clearly prevented UNL227 from being able to suppress PTI sufficiently to inject T3Es in PTI-induced *A. thaliana*. We performed *in planta* growth assays with the different DC3000 mutants and found that the DC3000 single cluster mutants were only slightly reduced in disease symptoms and in their ability to grow *in planta* (Fig. 5B-C). Surprisingly, the DC3000 quadruple mutant UNL227 was dramatically affected in its ability to cause disease symptoms and grow in plants (Fig. 5B-C). It is remarkable considering the number of extant T3Es that the *in planta* growth of UNL227 is reduced to a level similar to a DC3000 *hrcC* mutant, which has a defective T3SS and grows poorly in plants. Additionally, we performed *in planta* growth assays using wild type DC3000, a DC3000 *hrcC* mutant, and the UNL227 mutant on *A.*

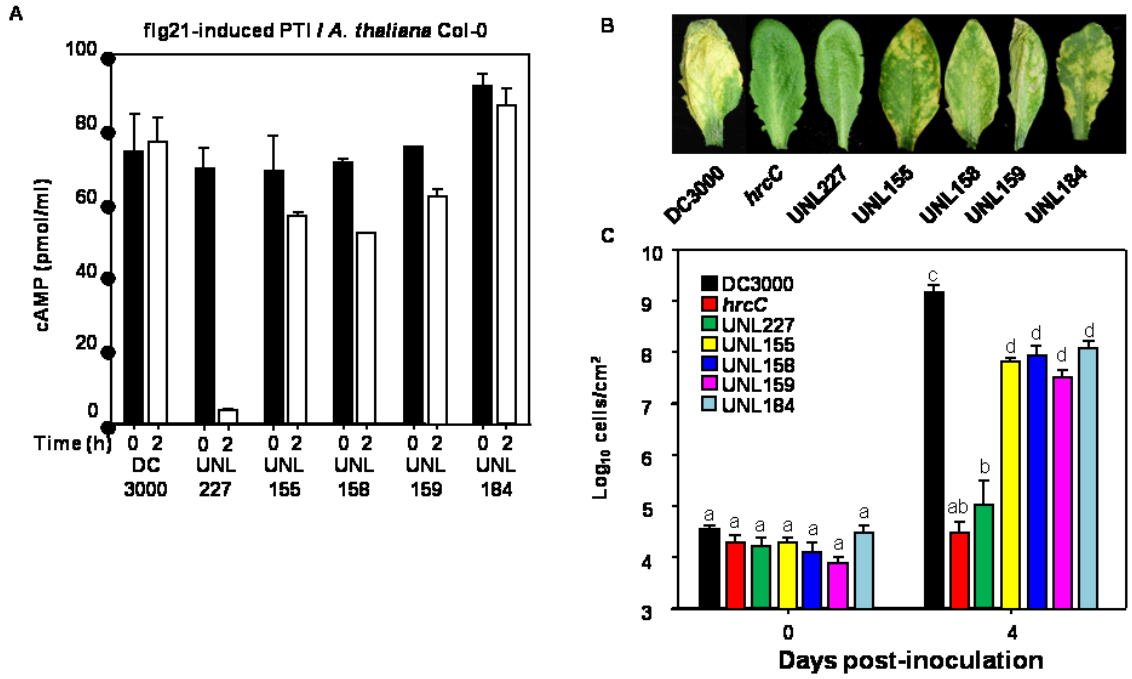



**Figure 5. A DC3000 poly-effector mutant loses its ability to inject T3Es into PTI-induced *A. thaliana*.** (A) Infiltrations of 3 x $10^8$ cells/ml of wild type DC3000, DC3000 EEL mutant (UNL155), DC3000 effector pathogenicity island (EPai) mutants (UNL158, UNL159, and UNL184) and a DC3000 quadruple mutant (UNL227) each expressing HopU1-CyaA were perfomed into *A. thaliana* after a 0 or 2 h pretreatment with 1μM flg21 and the production of cAMP was determined. DC3000 single mutants were differentially affected, but retained their ability to inject the HopU1-CyaA fusion, however, the DC3000 quadruple mutant was restricted in its T3E injection after PTI induction based on low cAMP levels. (B) The virulence of wild type DC3000, the DC3000 *hrcC* mutant, the DC3000 single EPai mutants, and the DC3000 quadruple mutant were compared by spray-inoculating them at 2 x $10^7$ cells/ml onto untreated *A. thaliana.* Virulence was assessed by disease symptoms at 4 days post-inoculation (B) and enumeration of bacteria at 0 and 4 days post-infiltration (C). Letters a-d are statistically different ($p<0.05$) and standard error bars are shown. These data indicate that the quadruple mutant loses the ability to inject T3Es in PTI-induced *A. thaliana* and cannot produce disease symptoms and grows poorly in uninduced plant tissue.

*thaliana* plants pretreated with flg21 for 2 h or 24 h to determine if the flg21 pretreatments had an effect on the ability of these strains to grow *in planta*. The 2 h flg21 pretreatment did not alter the ability of these strains to grow in *A. thaliana* plants (Supplemental Fig. 6). In contrast, a 24 h pretreatment altered the ability of DC3000 to grow in *A. thaliana* plants (Supplemental Fig. 6), consistent with earlier reports (20, 23, 34, 56). These data support the hypothesis that T3Es of virulent bacterial pathogens are responsible for mitigating the PTI-induced injection restriction.

**PTI restricts T3E injection into *A. thaliana* cells by an ETI-causing *P. syringae*.** The normally virulent DC3000 strain on *A. thaliana* can be converted to an ETI-inducing strain (i.e., 'avirulent' strain) by the introduction of a T3E that is recognized by an *A. thaliana* R protein. The T3E AvrRpm1 is recognized by the RPM1 R protein present in *A. thaliana* Col-0 (18) resulting in ETI induction. DC3000 carrying *avrRpm1* cannot cause disease on *A. thaliana* Col-0 due to this race-cultivar resistance. We wanted to determine the effect that ETI induction had on the ability of DC3000 to suppress PTI-induced injection restriction. *A. thaliana* Col-0 leaves were pretreated with flg21 and at different time points DC3000(p*hopU1-cyaA*) with or without an *avrRpm1* construct were infiltrated into the same leaf regions. DC3000 expressing AvrRpm1 was less able to inject HopU1-CyaA then the DC3000 strain indicating that AvrRpm1-dependent ETI contributed to the PTI-induced T3E injection restriction (Fig. 6).

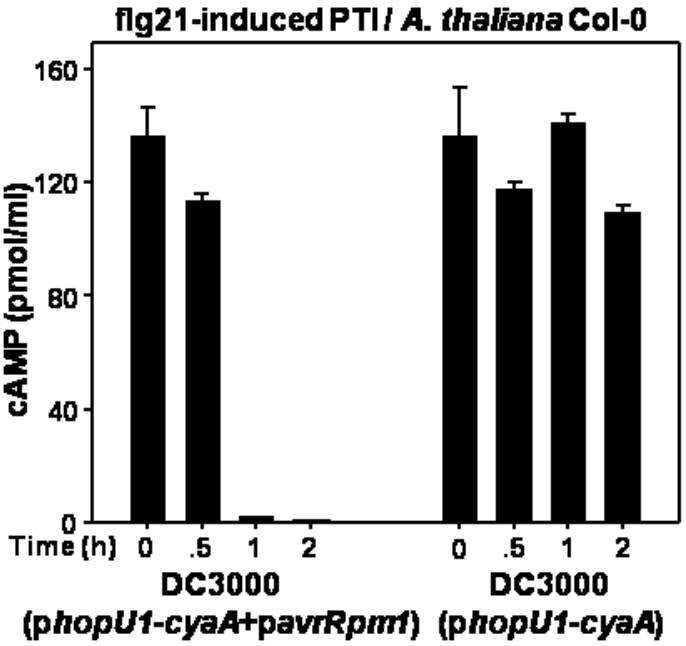



**Figure 6. PTI-induced injection restriction occurs in Arabidopsis when DC3000 carries the avirulence gene *avrRpm1*.** PTI was induced with 1 μM flg21. At the times indicated, 3 x $10^8$ cells/mL of DC3000 strains expressing HopU1-CyaA and with or without AvrRpm1 were infiltrated into Arabidopsis Col-0 leaves, and cAMP amounts were determined. T3E injection was severely restricted when DC3000 expressed AvrRpm1, suggesting that ETI contributed to T3E injection restriction. The experiment was repeated three times with similar results, and SE bars are shown.

**Transgenic expression of the T3Es HopAO1-HA or HopF2-HA in *A. thaliana* relieves PTI-induced injection restriction.** We used the *P. fluorescens*(pLN1965) injection system to determine the amount of the HopU1-CyaA reporter injected into transgenic *A. thaliana* plants expressing HopAO1-HA or HopF2-HA, two T3Es that have been shown to suppress PTI (20, 45, 53). After 1 h or 2 h flg21 pretreatments both transgenic lines allowed increased amounts of HopU1-CyaA to be injected compared to wild type controls (Fig. 7). These results show that the PTI suppression activities of at least two T3Es from DC3000 are independently sufficient to partially relieve PTI-induced injection restriction in *A. thaliana.* These data in conjunction with the mutant studies above suggest that the combined PTI suppression activity in *A. thaliana* of multiple T3Es is required for the complete removal of the injection restriction that is observed in DC3000.

**flg21-induced PTI inhibits the HR and restricts type III injection for a shorter time period than *hrcC*-induced PTI.** To characterize the persistence of the injection restriction and HR inhibition following PTI-induction in tobacco, we performed flg21 and *hrcC* pretreatments at defined times during a 2 week period before HR induction with DC3000 or injection monitoring with DC3000(p*hopU1-cyaA*). Tobacco pretreated with flg21 inhibited the HR and restricted injection through two days. Somewhat surprisingly the *hrcC* mutant inhibited the HR and restricted injection through the entire two week period (Fig. 8). It is likely that the flg21 PAMP is cleared from the apoplast by endocytosis with FLS2 (48) and suggests that this process requires two days to complete when flg21 is present in

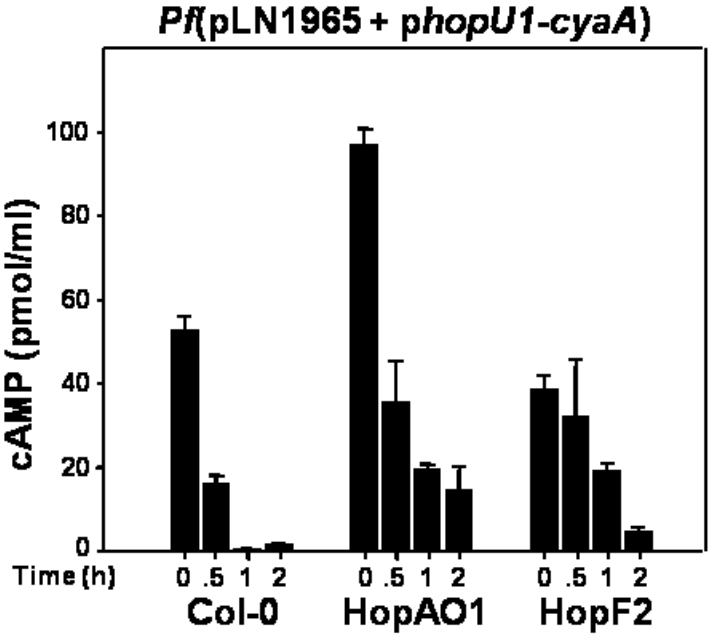



**Figure 7. Transgenic expression of PTI-suppressing T3Es in Arabidopsis relieves PTI-induced injection restriction.** Arabidopsis wild type plants and Arabidopsis transgenic plants expressing the DC3000 T3Es HopAO1-HA or HopF2-HA were pretreated with 1 µM flg21 for indicated time periods and infiltrated with 3 x $10^8$ cells/mL of *P. fluorescens*(pLN1965) expressing HopU1-CyaA. The higher amounts of cAMP in PTI-induced transgenic plants compared to the wild type control indicated that these T3Es can relieve PTI-induced injection restriction. This experiment was repeated at least three times with similar results, and SE bars are shown.

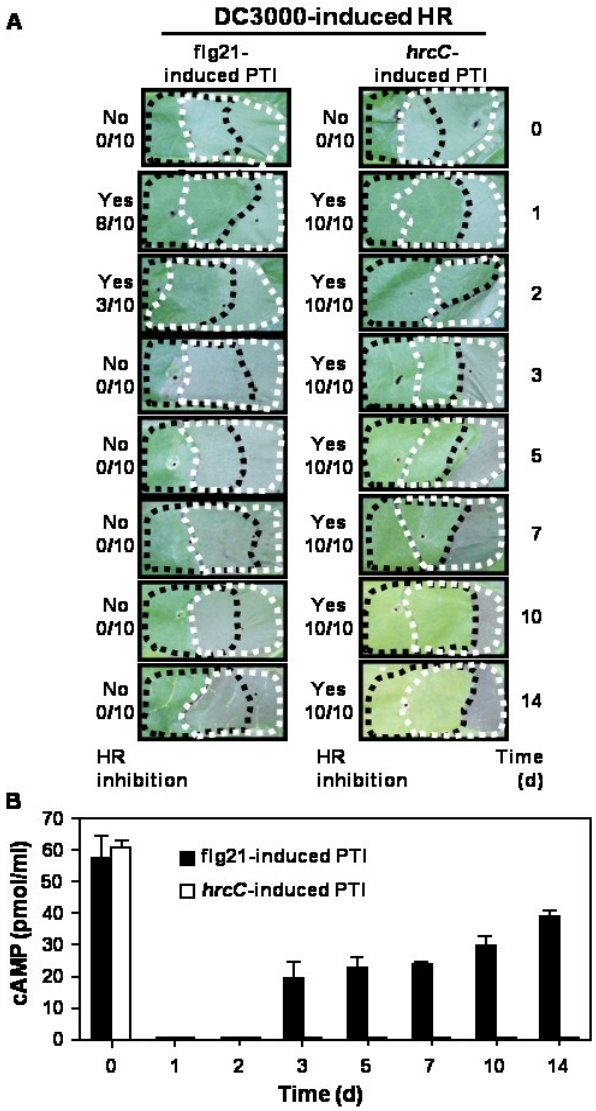



**Figure 8. PTI-induced by *hrcC* inhibits the HR and restricts the injection longer than PTI-induced by flg21.** (A) PTI was induced in tobacco leaves by infiltration with 1μM flg21 or 3 x $10^8$ cells/ml of *hrcC* 0, 1, 2, 3, 5, 7, 10 and 14 d prior to an overlapping infiltration with 2 x $10^7$ cells/ml of DC3000. Pictures were taken 48 h post DC3000 infiltration. The fraction to the left of each image indicates the number of times that the HR was inhibited over the total number of times the assay was performed. (B) PTI was induced in tobacco leaves by infiltration with 1μM flg21 or 3 x $10^8$ cells/ml of *hrcC* 0, 1, 2, 3, 5, 7, 10 and 14 days prior to an overlapping infiltration with 3 x $10^8$ cells/ml of DC3000(*hopU1-cyaA*). cAMP levels were measured 7 h post DC3000(*hopU1-cyaA*) infiltration. PTI induced by flg21 no longer inhibited the HR or restricted T3E injection after 2 d, whereas PTI induced by the *hrcC* mutant inhibited the HR and restricted injection throughout the 14 d experiment. These experiments were repeated twice with similar results and standard error bars are shown.

the apoplast at 1μM. The persistent inhibition caused by the *hrcC*-induced PTI is probably due to the continual lysis of *hrcC* bacterial cells and release of PAMPs during the two week period, preventing the PAMPs from being effectively cleared from the apoplast.

**Discussion**

We show here that the PTI-induced inhibition of the HR occurs within two h of PAMP pretreatment (Fig. 1). This a reasonably quick response relative to the twelve to twenty-four hours generally required for production of an HR. However, PTI-induced inhibition of the HR appears to require *de novo* transcription and translation as it was previously reported that the protein synthesis inhibitor cycloheximide restores the HR (32). Interestingly, HR inhibition induced by flg21 lasted through two days while HR inhibition induced by intact bacteria persisted over a two week period. This difference probably reflects the clearing of flg21 peptide from the apoplast by endocytosis of its receptor FLS2 (48). In contrast, when bacteria are used to induce PTI, PAMPs continue to be released over an extended time period. Plants often cannot perceive PAMPs while they are part of the bacterial cell. For example, flagellin cannot be detected by the FLS2 receptor kinase while the flagellum filament is intact because the recognized part of the protein is on the inner side of the filament tube and not surface exposed (55). Researchers sometimes use PTI inducers interchangeably. Our results indicate that the choice between PTI-inducers flg21 (and likely other purified PAMPs) and intact bacteria may be an important consideration depending on the experimental design.

We show that the PTI-induced inhibition of the HR is due to direct or indirect restriction of T3E injection. The reduced amount of T3Es, a subset of which trigger ETI, entering the plant cell is then insufficient to cause the macroscopic HR. It remains unclear if PTI is directly blocking the T3SS apparatus from injecting T3Es or whether it has a more general effect on the bacteria in the apoplast that prevents them from injecting T3Es. For example, it is intriguing to speculate that the cell wall defenses induced by PTI may strengthen the plant cell wall such that the T3SS pilus can no longer traverse it to inject T3Es. However, the time limit on the effectiveness of at least flg21-induced injection restriction indicates that it is of a transitory rather than permanent nature. The early and spatially limited character of the PTI-induced injection restriction suggests that it may involve ROS, perhaps directly killing bacteria in the apoplast. Indeed, a related study showed that PTI-induced tissue infiltrated with green fluorescent protein (GFP)-labeled bacteria had dramatically less fluorescence than untreated plant tissue, which the authors concluded was due to the inhibition of bacterial growth caused by PTI (46). However, we were unable to detect any significant reduction of bacterial populations in leaf tissue by direct bacterial counting after PTI induction (Supplemental Fig. 2). We think this discrepancy may be due to reduced GFP production in PTI-induced tissue instead of PTI causing bacterial growth inhibition. It remains possible that a PTI response such as ROS production may affect bacterial transcription or translation preventing the T3SS apparatus from being assembled. In support of this, there has been a report that the expression of *Erwinia amylovora* T3SS-related genes

is altered after treatment with bacterial lipopolysaccharide (38). We are currently testing whether PTI effects the expression of the T3SS apparatus genes in the *P. syringae-A. thaliana* pathosystem.

The PTI-induced inhibition of the HR has previously only been reported in the solanaceous plants tobacco, *N. benthamiana*, and *Capsicum annuum* (pepper) (32, 42, 45). PTI-induced inhibition of the HR can also be recapitulated in *A. thaliana* leaves (Supplemental Fig. 7), although *A. thaliana* small leaf size makes overlapping infiltrations difficult. We show here that *A. thaliana* PTI restricts the T3E injection. Both assays should be added to the limited collection of assays that are currently being used to assess PTI.

PTI in tobacco and *A. thaliana* was more effective at restricting T3E injection by *P. syringae* strains in non-host than in compatible interactions (Fig. 4). This difference was attributed to the PTI-suppressing activity of T3Es since a DC3000 poly-effector mutant lacking about one third of its T3E inventory was less able to overcome PTI-induced injection restriction on *A. thaliana* compared to wild type DC3000 (Fig. 5A). PTI-induced transgenic *A. thaliana* plants separately expressing two DC3000 T3Es known to suppress PTI relieved injection restriction. Collectively, these results suggest that in compatible interactions T3Es are apparently injected at a low level during PTI-induced injection restriction. This allows the injected T3Es to suppress PTI in susceptible hosts relieving restriction. Importantly, longer PAMP pretreatments beginning at about sixteen h begin to severely restrict T3E injection even in the compatible interaction between DC3000 and *A. thaliana* (Supplemental Fig. 8). In addition, a

twenty-four but not a two hour PAMP pretreatment allows *A. thaliana* to restrict the growth of normally virulent DC3000 (Supplemental Fig. 2). These data indicate that whatever mechanism is causing this phenotype is so substantial by twenty-four hour that it cannot be overcome by virulent *P. syringae* strains.

We found that PTI-induced injection restriction was severe in non-host plants tobacco and *A. thaliana* (Fig. 4). It is possible that in non-host plants the T3Es have an insufficient ability to suppress PTI, however, the severity of the restriction may be due to the induction of ETI. We did find that DC3000 expressing AvrRpm1 was clearly less able to inject the T3E-CyaA reporter than DC3000 not expressing it (Fig. 6). This suggests that ETI induction prevents DC3000 from delaying T3E injection restriction. The induction of ETI may prevent T3Es from acting to suppress PTI and relieving injection restriction. Alternatively, it is possible that ETI may also evoke or enhance injection restriction. We favor the latter explanation because of the extensive overlap between ETI and PTI responses (41, 52). Additionally, when we compared PTI-induced injection restriction initiated by *P. fluorescens*(pLN1965) (which induces PTI) and *P. fluorescens*(pLN1965 + p*avrRpm1*) (which induces PTI and ETI) we found that injection restriction induced by *P. fluorescens*(pLN1965 + p*avrRpm1*) occurred more quickly than when it was induced by *P. fluorescens*(pLN1965) (Supplemental Fig. 9). One important caveat is that we only investigated specific *P. syringae* interactions and other *P. syringae* strains may fail to grow on non-host plants for reasons other than T3E recognition. For example, flagellin from certain *P. syringae* pathovars induce an HR on non-host tobacco (50). We are

currently taking several approaches to determine the extent that ETI, in the absence of PTI, can induce injection restriction.

The PTI-induced inhibition of the HR and injection restriction assays should provide a unique perspective on how PTI affects bacteria in the apoplastic environment. This should facilitate the dissection of the plant immune response in such a manner as to potentially identify the mechanism behind these phenomena. For example, do certain plant mutants defective in specific immune responses relieve PTI-induced injection restriction? If this approach is successful it may help us better understand one of the long-standing questions in molecular phytobacteriology – what plant immune responses are important for successfully defending against bacterial pathogens.

**Experimental Procedures**

**Bacterial strains, plasmids, and growth conditions.** Bacterial strains, plasmids, and primers are listed in Supplemental Table 1 and Supplemental Table 2. *Pseudomonas syringae* and *Pseudomonas fluorescens* strains were grown in King's B (KB) medium at 30°C with appropriate antibiotics. The antibiotics were used at the following concentrations (µg/ml): rifampicin (Rf), 100; gentamicin (Gm), 10; tetracycline (Tc), 10; kanamycin (Km), 50; naldixic acid (Nx), 20; and spectinomycin (Sp), 50. Plasmids used in unmarked mutagenesis were: pRK2013 and pRK2073, mobilizing helper plasmids; pBH474, a Flp recombinase encoding plasmid; pMK2016 and pMK2017, both containing FRT cassettes (26). Plasmids used for plant bioassays were pHIR11 (29), a cosmid containing the genes for a functional T3SS and the T3E *hopA1*, and pLN1965

(19), a pHIR11 derivative lacking the *hopA1* T3E gene. For translocation assays constructs encoding AvrPtoB or HopU1 C-terminal CyaA fusions were made by LR reactions between pENTR/D-TOPO (Invitrogen, Carlsbad, CA, U.S.A.) constructs containing each gene and the Gateway vector pLN2193 creating the plasmids pLN2250 and pLN2254 respectively.

**Plant Bioassays.** Wild type *Arabidopsis thaliana* (Col-0) and transgenic plants were grown in a growth chamber at 24°C on 10 h days. *Nicotiana tabacum* cv. Xanthi (tobacco) plants were grown in greenhouse conditions. HR inhibition assays were done with infiltrations of fresh overnight cultures resuspended in 5 mM morpholineethanesulfonic acid (MES) at pH 5.5 to 3 x $10^8$ cells/ml for *P. fluorescens*(pHIR11), *P. fluorescens*(pLN1965), and *hrcC* and at 2 x $10^7$ cells/ml for DC3000. flg21 was used at 1 µM concentrations in $H_20$ unless otherwise indicated. Fully expanded leaves of 4 week old *A. thaliana* or 6 week old tobacco plants were PTI induced by infiltration with flg21, *P. fluorescens*(pLN1965), or *hrcC* using a blunt-ended syringe. Then the HR elicitor, either DC3000 or *P. fluorescens*(pHIR11), was infiltrated in an overlapping region of the same leaf 0, 0.5, 1 or 2 h after infiltration with flg21 or 0, 2, 4 or 8 h after infiltration with *P. fluorescens*(pLN1965) or *hrcC*. Infiltration outlines were marked with a felt-tipped pen and the overlapping area was assessed 48 h after HR elicitor infiltration for the presence or absence of an HR.

Plant growth assays were carried out using fresh bacteria grown on KB plates overnight and resuspended in 5 mM MES. PTI induction and bacterial infiltration of tobacco were carried out as in HR inhibition assays. Bacterial

growth in *A. thaliana* was done by spray-inoculation with 2 x $10^8$ cells/ml. Four leaf disks were harvested for each strain at each time point with a 0.8 $cm^2$ cork borer for tobacco and a 0.4 $cm^2$ cork borer for *A. thaliana*. The samples were ground in 250 µl of sterile water and serial dilutions were plated on KB with appropriate antibiotics, and colonies were counted to determine bacterial growth. Statistical differences were calculated using single factor anova.

**Adenylate cyclase (CyaA) injection assay.** pLN2250 and pLN2254 were transformed via electroporation into *P. syringae* pv. *tomato* DC3000, *P. syringae* pv. *tabaci* 11528, *P. fluorescens* 55, or DC3000 mutant derivatives and T3E protein expression was confirmed with immunoblots. The CyaA injection assays were performed following a previously published protocol (Schechter *et al*., 2004). Briefly, tobacco or *A. thaliana* were initially challenged to induce PTI in the same manner as in other plant bioassays and were subsequently infiltrated with the strain carrying the *T3E-cyaA* gene fusion suspended at 3 x $10^8$ cells/ml in 5 mM MES in an area overlapping the initial infiltration using a blunt-ended syringe. Leaf discs (0.9 $cm^2$) were taken from the area of overlapping infiltration 16 h (7 h for DC3000 in tobacco) after the second infiltration. The samples were ground in liquid nitrogen and resuspended in 0.1M HCl. Samples were adjusted to 10 ng/µl after quantification of total protein using Bradford assay (BioRad, Hercules, CA). A direct cyclic AMP (cAMP) immunoassay kit (Assay Design, Ann Arbor, MI) was used to measure cAMP levels following the manufacturer's instructions.

**AvrRpt2 *in planta* processing injection assay.** This assay was modified from Mudgett and Staskawicz (39) to fit the experimental conditions. pLN2637 was

transformed by electroporation into DC3000. The bacteria were grown overnight on KB media containing appropriate antibiotics, collected, and resuspended in 10 mM $MgCl_2$ at a concentration of 1 x $10^9$ cells/ml. Tobacco leaves were infiltrated with either 1 µM flg21 or $H_20$ (mock) using a blunt-ended syringe and 2 h later were infiltrated in the same tissue with the bacteria. Six h after bacterial infiltration, leaf samples were taken with a cork borer, ground in liquid $N_2$ using a pestle and microcentrifuge tube, and then resuspended in 1x phosphate buffered saline (PBS) containing complete protease inhibitor cocktail (Roche, Basel, Switzerland). Soluble protein was collected upon centrifugation at 13,000 x g for 5 min at 4°C. Protein concentrations were determined using the Bradford assay (BioRad, Hercules, CA) and all samples were normalized to 800 ng/µl total protein. Protein fractions were mixed 1:1 with 2x sample buffer, boiled for 5 min, centrifuged at 13,000 x g for 3 min, and 20 µg of total protein was analyzed by immunoblotting using anti-HA antibodies (Roche, Basel, Switzerland).

**Unmarked mutagenesis.** The construction of the DNA cluster deletion mutants was done by unmarked mutagenesis (House *et al*., 2004). Plasmids and primers are listed in Supplemental Table 1 and Supplemental Table 2, respectively. DNA clusters containing T3E genes were identified in *Pseudomonas syringae* pv. tomato DC3000 based on the presence of type III promoters using the Artemis Genome Viewer (Sanger Institute, Hinxton, Cambridge, UK). For each DNA cluster, the 2.5 Kb upstream (US) DNA sequence and 2.0 Kb downstream (DS) DNA sequence were amplified by PCR using the following Gateway compatible primer sequences: for the EEL the primers P2443/P2444 and P2445/P2446 were

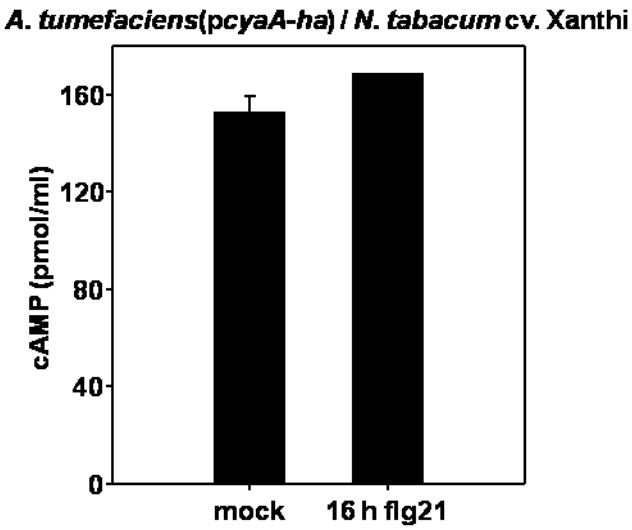
used, for EPai3 the primers P2404/P2405 and P2406/P2407 were used, for EPai1 the primers P2091/P2092 and P2093/P2094 were used, and primers P2097/2098 and P2099/P2100 were used for EPai2. The purified PCR products were cloned into the pENTR/D-TOPO vector (Invitrogen). The entry vectors were then recombined by LR reactions into pMK2017 for US sequences and pMK2016 for DS sequences.

The plasmids containing the US flanking sequences were integrated into DC3000 chromosome using bi-parental mating with selection on KB plates containing Rf and Tc. Positive colonies were then confirmed for proper integration of the plasmid with PCR using P1790 and the relevant US reverse primer and checked for UV fluorescence, Sp sensitivity, and lack of growth at 37°C. The confirmed single integrant DC3000 mutants were subsequently tri-parental mated with DH5α containing the DS sequence plasmid and HB101(pRK2013). Colonies were selected on KB plates containing Rf, Sp, and Tc and tested for UV fluorescence and lack of growth at 37°C. Proper integration of both plasmids flanking the targeted DNA cluster was confirmed with PCR using P1790 with the relevant right border reverse primer and P1789 with the relevant left border reverse primer.

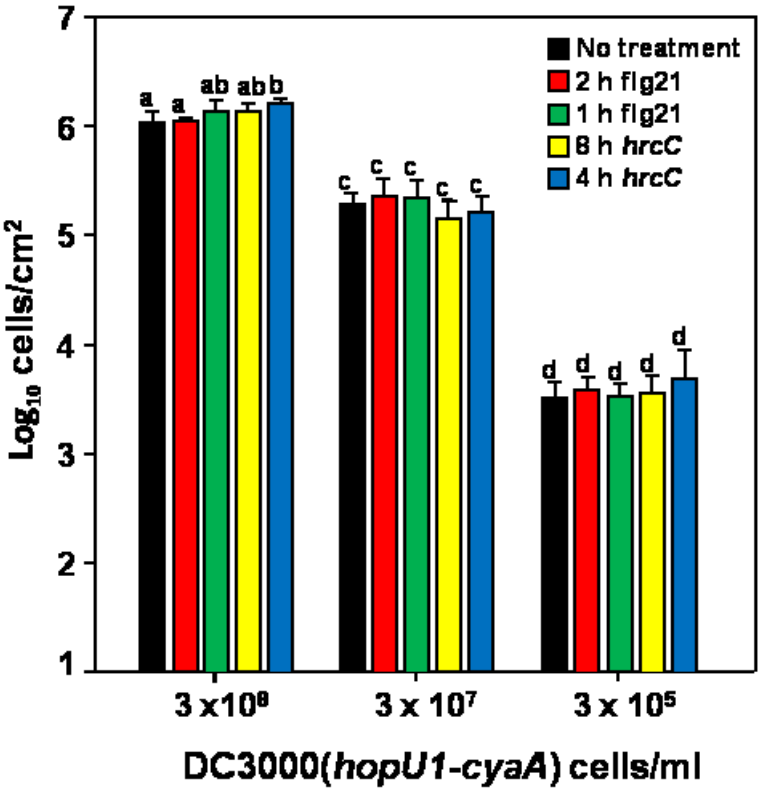
Upon confirmation of double integrants, excision of the integrated plasmids at FRT sites was conducted. Triparental mating of the DC3000 double integrant mutants, DB3.1(pBH474), and DB3.1(pRK2073) was performed and colonies selected on KB plates containing Rf and Gm. Excision of the integrated plasmids was confirmed by testing for sensitivity to Sp and Tc. Isolated colonies

were streaked onto KB plates containing Rf and 5% sucrose to select against retention of pBH474. After deletion by the Flp recombinase enzyme a 0.2 kb FRT scar remained in place of the targeted DNA cluster. As a final measure of proper excision, the DC3000 mutants were confirmed with PCR using primers designed to anneal 0.5 kb within the border region, resulting in a 1.2 kb PCR product.

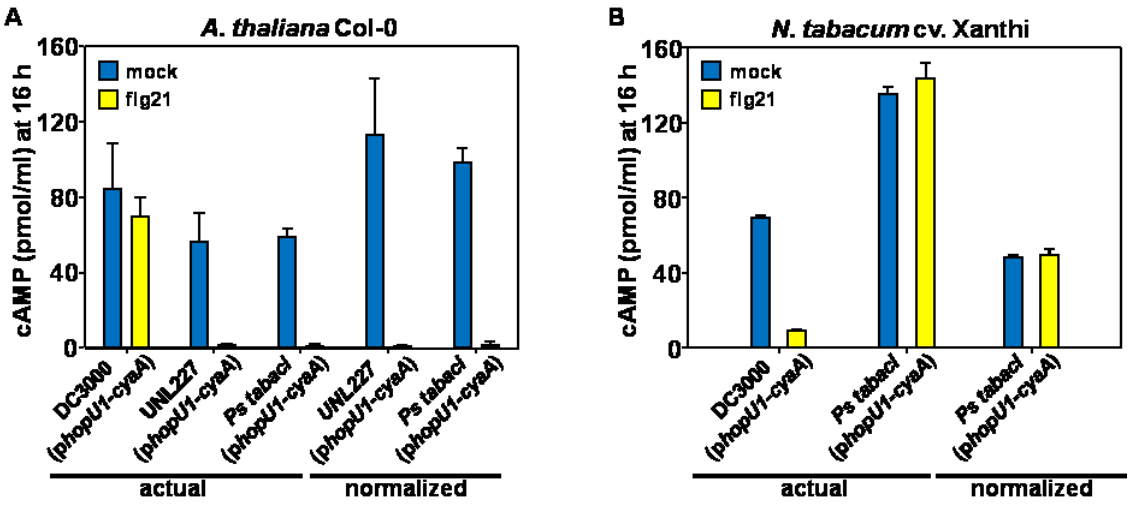
## Supplemental Figures and Tables



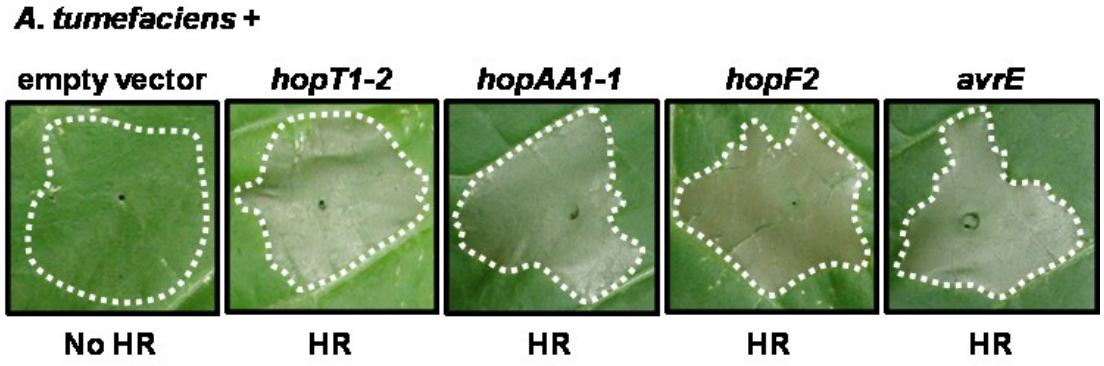

**Supplemental Figure 1. CyaA remains functional in PTI-induced tissue.** Samples were taken from *N. tabacum* cv. Xanthi leaves that were infiltrated with *A. tumefaciens* transiently expressing *cyaA-ha*. 24 h post infiltration the leaves were infiltrated again with either water (mock) or a 1 µM flg21 solution. Samples were taken 16 h after the second infiltration and a CyaA assay was performed to measure cAMP levels.




**Supplemental Figure 2. Growth of DC3000(p*hopU1-cyaA*) was not affected by pre-treatment with either flg21 or *hrcC*.** DC3000(p*hopU1-cyaA*) was infiltrated into tobacco at 3 x $10^8$, 3 x $10^7$, or 3 x $10^5$ cells/ml either alone, 1 or 2 h after 1 µM flg21 infiltration, or 4 or 8 h after infiltration of 3 x $10^8$ cells/ml of *hrcC*. Bacteria were enumerated in the overlapping area 7 h after DC3000(p*hopU1-cyaA*) infiltration. PTI-induction does not alter the growth of DC3000(p*hopU1-cyaA*) in tobacco within 7 h. These experiments were repeated three times with similar results and standard error bars are shown.



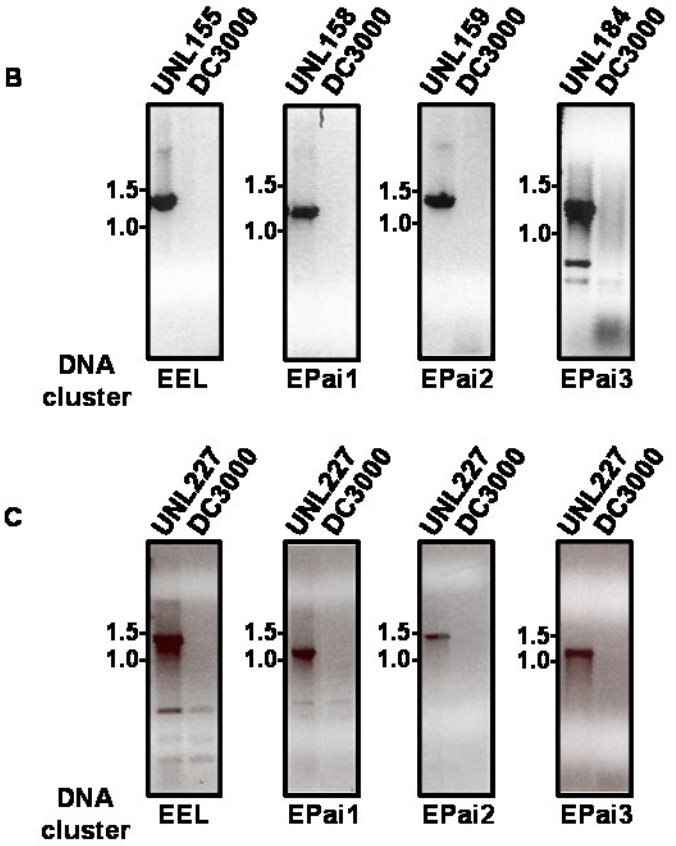

**Supplemental Figure 3. cAMP levels were not affected by differences in growth.** The growth of DC3000(p*hopU1-cyaA*), UNL227(p*hopU1-cyaA*), and *P. syringae* pv. *tabaci*(p*hopU1-cyaA*) was determined at 0 and 16 h after infiltration in *A. thaliana* Col-0 or *N. tabacum* cv. Xanthi leaves pretreated for 2 h with 1 µM flg21 or water (mock). The levels of cAMP were determined at 16 h in *A. thaliana* Col-0 (A) and *N. tabacum* cv. Xanthi (B) plant tissue. The cAMP levels were normalized with respect to the differences in growth between DC3000 and the other strains. The cAMP amounts are shown before (actual) and after normalization (normalized). While there were significant changes in cAMP levels in UNL227(p*hopU1-cyaA*) and *P. syringae* pv. *tabaci*(p*hopU1-cyaA*) after normalization, there was little change in the differences in cAMP amounts between flg21-induced and mock samples. Therefore, the growth differences between the *P. syringae* strains are not responsible for the dramatic differences in cAMP levels that occur after PTI induction. These experiments were repeated twice with similar results and standard error bars are shown.



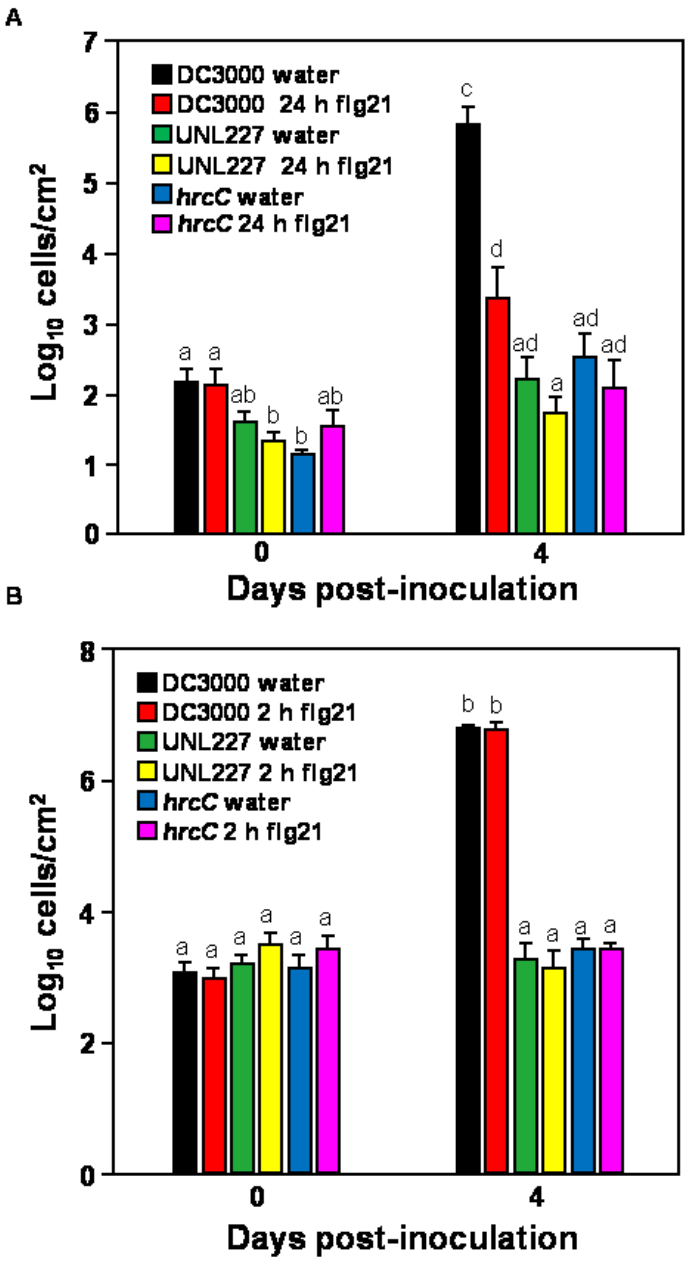

**Supplemental Figure 4. Tobacco leaves were infiltrated with *Agrobacterium* carrying binary constructs containing each of the indicated T3E genes.** After 24-48 h HR-like responses developed within the infiltrated zone for these T3E genes suggesting that they encode T3Es that induce ETI. Pictures were taken 48 h after infiltration.

A

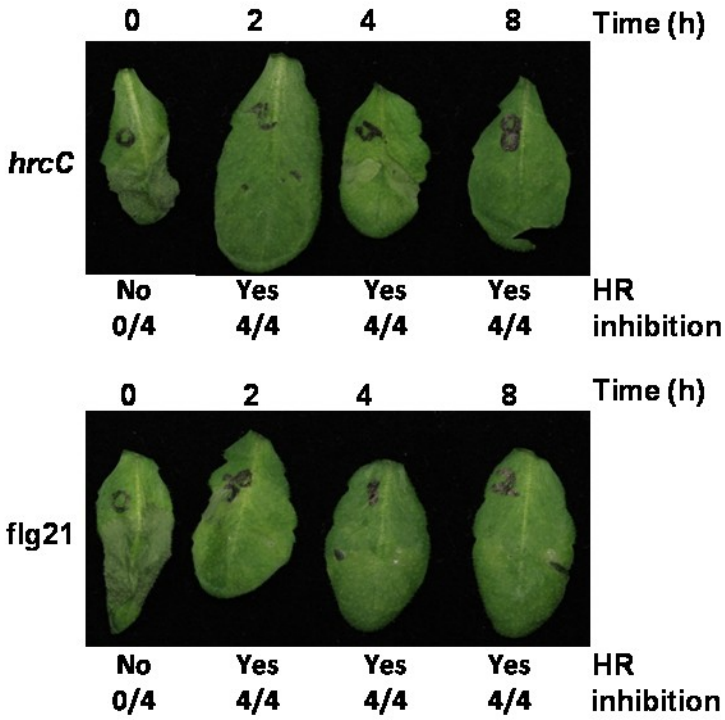
| DNA cluster | Primer set | Predicted band length in DC3000 | Predicted band length in mutant | Observed band length in mutant |
|---|---|---|---|---|
| EEL | P2087/P2088 | 7 kb | 1.2 kb | 1.2 kb |
| EPai1 | P2095/P2096 | 26 kb | 1.3 kb | 1.3 kb |
| EPai2 | P2101/P2102 | 20 kb | 1.2 kb | 1.2 kb |
| EPai3 | P2447/P2448 | 4 kb | 1.3 kb | 1.3 kb |



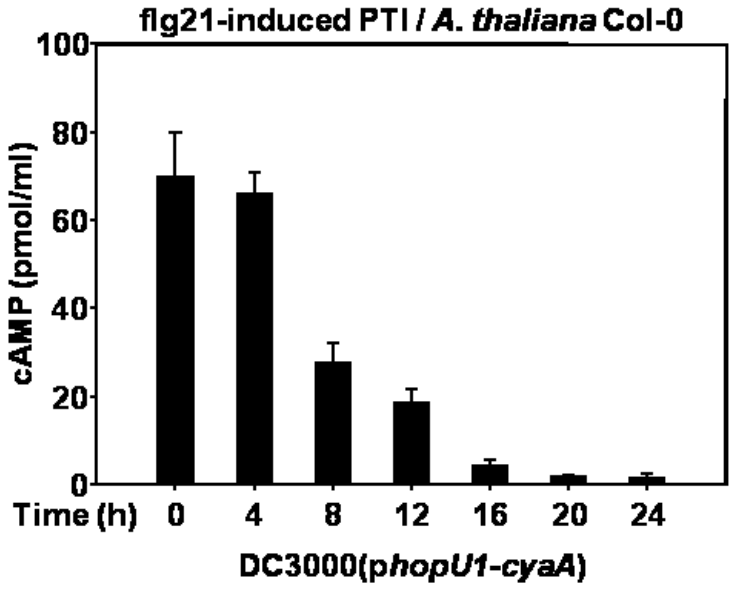

**Supplemental Figure 5. Confirmation of DC3000 DNA cluster mutants.** Colony PCR was done for each of the mutants and compared to wild type bacterial samples to confirm that a T3E-related DNA cluster was deleted from the different bacterial mutants. (A) The DNA cluster names, primer sets used, and expected and observed PCR band lengths are indicated. Primers were made approximately 600 base pairs upstream and downstream of the DNA cluster, which would result in a PCR product of about 1.2 kb if the putative mutant carried the correct mutation. (B) DNA agarose gels of PCR reactions from the single T3E-related DNA clusters. (C) DNA agarose gels of PCR reactions from the poly DNA cluster mutant UNL227. In DC3000 the DNA clusters were too big to be amplified in the PCR conditions used and resulted in the absence of bands of the predicted length.



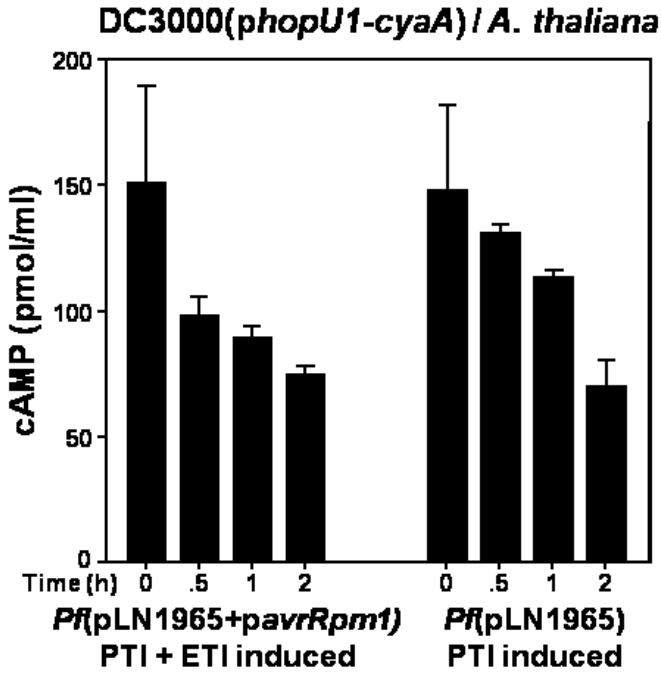

**Supplemental Figure 6. The induction of PTI by flg21 at 24 h but not 2 h before inoculation with DC3000 inhibits bacterial growth in *A. thaliana*.** *A. thaliana* Col-0 plants were infiltrated with 1 µM flg21 or a water control at 24 h (A) or 2 h (B) prior to spray-inoculation with 2 x $10^8$ cells/ml of DC3000, UNL227 or *hrcC*. Bacteria were enumerated at 0 and 4 d post-inoculation. Letters a-d are statistically different ($p<0.05$) and standard error bars are shown. These experiments were repeated at least three times with similar results.




**Supplemental Figure 7. PTI-induction inhibits AvrRpm1 induced HR in *A. thaliana* by *hrcC* and flg21 PTI-inducers.** Infiltration of the leaves of *A. thaliana* with 1 x $10^7$ cells/ml of *P. fluorescens*(pLN1965 + p*avrRpm1*) was unable to cause an HR in PTI-induced leaves pretreated with the *hrcC* mutant or flg21. With the exception of the zero time point, the HRs were inhibited in leaves at the remaining time points. The fraction below each leaf indicates the number of times that the HR was inhibited over the total number of times the assay was performed. Photos were taken after 48 h after *P. fluorescens*(pLN1965 + p*avrRpm1*) infiltration.




**Supplemental Figure 8. Time course of the restriction of HopU1-CyaA injection into PTI-induced *A. thaliana* plant cells.** PTI was induced with 1 µM flg21 and at the indicated times DC3000(p*hopU1-cyaA*) at a cell density of 3 x $10^8$ cells/ml was infiltrated into the pretreated leaf regions. After 16 h cAMP levels were determined. This experiment was repeated three times with similar results.




**Supplemental Figure 9. PTI- and ETI-induced *A. thaliana* plants appear to restrict T3E injection more quickly than when PTI only is induced in *A. thaliana* plants.** *A. thaliana* plants were pretreated with 3 x $10^8$ cells/ml *P. fluorescens*(pLN1965), which induces PTI, or *P. fluorescens*(pLN1965 + p*avrRpm1*), which induces PTI and ETI. At the indicated times DC3000(p*hopU1-cyaA*) at a cell density of 3 x $10^8$ cells/ml was infiltrated into the pretreated leaves and the level of T3E injection was determined by quantifying cAMP in the overlapping infiltration area 16 h later. These experiments were repeated at least three times with similar results.

## Supplemental Table 1. Strains and plasmids used in this study

| Strain or plasmid | Characteristics | Reference or source |
|---|---|---|
| *E. coli* DH5α | *supE44 ΔlacU169(Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1,* Nalr | (22); Life Technologies, Gaithersburg, MD, USA |
| *E. coli* DB3.1 | *F-gyrA462 endA1 Δ(sr1-recA) mcrB mrr hsdS20 (rB-, rB-) supE44 ara-14 galK2 lacY1 proA2 rpsL20* (Smr) *xyl-5 λ-leu mtl-1* | Invitrogen, Carlsbad, CA, USA |
| *E. coli* HB101 | *supE44 hsdS20* (rB-, mB-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 Δleu mtl-1* | New England Biolabs, Beverly, MA, USA |
| *Pseudomonas fluorescens* 55 | $Nal^r$ | M. Sasser |
| *Pseudomonas syringae* pv. *tomato* DC3000 | Wild type, $Rf^r$ | (14) |
| DC3000 *hrcC* | *hrcC* mutant defective in T3SS, $Rf^r$ $Cm^r$ | (26) |
| *Pseudomonas syringae* pv. *tabaci* 11528 | Wild type, $Nal^r$ | American Type Culture Collection |
| UNL155 | DC3000 EEL mutant lacking T3E *hopB1*, $Rf^r$ | This work |
| UNL158 | DC3000 EPai1 mutant lacking T3E genes *hopD1*, *hopQ1-1*, and *hopR1*, $Rf^r$ | This work |
| UNL159 | DC3000 EPai2 mutant lacking T3E genes *hopAA1-2*, *hopV1/shcV*, *hopAO1*, *hopG1*, and *hopQ1-2*, $Rf^r$ | This work |
| UNL184 | DC3000 EPai3 mutant lacking T3E genes *hopF2/shcF* and *hopU1*, $Rf^r$ | This work |
| UNL227 | DC3000 EPai1, EPai2, EPai3, EEL mutant, $Rf^r$ | This work |
| pBH474 | Sucs derivative of pTH474 | (26) |
| pHIR11 | Cosmid pLAFR3 derivative carrying T3SS DNA from *P. syringae* pv. *syringae* 61, $Tc^r$ | (29) |
| pENTR/D-TOPO | Gateway system entry vector, $Km^r$ | Invitrogen, Carlsbad, CA, U.S.A. |
| pLN525 | pPZP212 derivative carrying *hopF2*, $Km^r$ | This work |
| pLN953 | pPZP212 derivative carrying *hopT1-2*, $Km^r$ | This work |
| pLN956 | pPZP212 derivative carrying *avrE1*, $Km^r$ | This work |
| pLN958 | pPZP212 derivative carrying *hopAA1-2*, $Km^r$ | This work |
| pLN1965 | pHIR11 derivative containing a deletion of *shcA/hopA1* operon replaced by a sp resistant cassette, $Tc^r$ $Sp^r$ | (19) |
| pLN2193 | pML123 derivative gateway destination vector containing a CyaA tag for C-terminal fusions, $Gm^r$ | (17) |
| pLN2194 | pMK2017 derivative carrying 2.5 kb sequence US of EPai1, $Tc^r$, $Sp^r$ | This work |
| pLN2195 | pMK2017 derivative carrying 2.5 kb sequence US of EPai2, $Tc^r$, $Sp^r$ | This work |
| pLN2204 | pMK2016 derivative carrying 2.0 kb sequence DS of EPai1, $Sp^r$ | This work |
| pLN2205 | pMK2016 derivative carrying 2.0 kb sequence DS of EPai2, $Sp^r$ | This work |
| pLN2250 | pLN2193 derivative carrying *avrPtoB*, $Gm^r$ | This work |
| pLN2254 | pLN2193 derivative carrying *hopU1*, $Gm^r$ | This work |
| pLN2616 | pMK2017 derivative carrying 1.5 kb sequence US of EPai3, $Tc^r$, $Sp^r$ | This work |

| | | |
|---|---|---|
| pLN2617 | pMK2016 derivative carrying 2.0 kb sequence DS of EPai3, $Sp^r$ | This work |
| pLN2637 | pML123 derivative carrying *avrRpt2-ha*, $Gm^r$ | This work |
| pLN2665 | pMK2017 derivative carrying 2.4 kb sequence US of EEL, $Tc^r$, Spr | This work |
| pLN2666 | pMK2016 derivative carrying 2.5 kb sequence DS of EEL, $Sp^r$ | This work |
| pMK2016 | $Sp^r$ $St^r$ *oriV oriT*ColE1 with FRT cassette from pMK2014 | (26) |
| pMK2017 | $Tc^r$ *oriV*R6K *oriT*RP4 with FRT cassette from pMK2015 | (26) |
| pRK2013 | $Km^r$ mobilization helper plasmid | |
| pRK2073 | $Sp^r$ mobilization helper plasmid | |
| pVSP61::*avrRpm1* | pVSP61 derivative containing *avrRpm1*, $Km^r$ | (36) |
| pPZP212 | *Agrobacterium tumefaciens* binary vector, $Km^r$ | (21) |

## Supplemental Table 2. Primers used in this study

| Primer | Sequence | Use[a] |
|---|---|---|
| P1789 | 5'-GAACTTCAAGATCCCCTGATTCCCTT-3' | pMK2016 insert |
| P1790 | 5'-GAGCGCTTTTGAAGCTGATGTGC-3' | pMK2017 insert |
| P2087 | 5'-GCACGTTGGGTACGCTGCAAG-3' | confirm EEL |
| P2088 | 5'-CGCCGCCGCCATCGATC-3' | confirm EEL |
| P2091 | 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGCGCTTCTCCCTGGC-3' | pLN2194 |
| P2092 | 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGCCTGCGGGCTGGATG-3' | pLN2194 |
| P2093 | 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCTCCTGGGCATTCTTCAGACG-3' | pLN2204 |
| P2094 | 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGGCGTTGACACCTACGTCATAC-3' | pLN2204 |
| P2095 | 5'-CCGTCCGCAGTTCAGGCG-3' | confirm EPai1 |
| P2096 | 5'-CCGGCAAGCGGGTATGC-3' | confirm EPai1 |
| P2097 | 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGCTCTATTATCGCAGCCCCCTG-3' | pLN2195 |
| P2098 | 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGCTACTCAGCGTATGGGGCGAG-3' | pLN2195 |
| P2099 | 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGACAACCCCCAAGACAAACTCC-3' | pLN2205 |
| P2100 | 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGCAACTGGGGTTTGCGGAGC-3' | pLN2205 |
| P2101 | 5'-GAAGAGTTTTCCCAGGGGCTGC-3' | confirm EPai2 |
| P2102 | 5'-CGGTGAAACTGCTTCCCCTATTCC-3' | confirm EPai2 |
| P2404 | 5'-CACCGTTTTTCATAATGCATCTCCTCAT-3' | pLN2617 |
| P2405 | 5'-CCCTCCTACCTGGCATCGAAATG-3' | pLN2617 |
| P2406 | 5'-CACCGTCATTCGTTCCAGGATTCATCAG-3' | pLN2616 |
| P2407 | 5'-CAGATTTGAGTCCATGAAGGAGGCC-3' | pLN2616 |
| P2443 | 5'-CACCCTCAATGGTGGTGCCCCGAG-3' | pLN2665 |
| P2444 | 5'-GTATAAAAAGCAGGAAAAACTCGTTC-3' | pLN2665 |
| P2445 | 5'-CACCCGATCTCGATCATTTTTTCTGG-3' | pLN2666 |
| P2446 | 5'-CGCGGAGATTCAATCATG-3' | pLN2666 |
| P2447 | 5'-GAACAAGGAATGGGGCGAGC-3' | confirm EPai3 |
| P2448 | 5'-GGCGATGTTGCTGACGACCAAATAC-3' | confirm EPai3 |

[a]Primers were used to make a construct, confirm a deletion, or confirm a sequence insertion.

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# Chapter 5

Summary

*Pseudomonas syringae* requires the use of a type III secretion system (T3SS) for pathogenicity. T3SSs allow pathogenic bacteria to inject type III effector (T3E) proteins into the eukaryotic cells of hosts which lead to disease. Understanding how the bacteria inject T3Es is paramount to understanding plant/bacterial pathogen interactions at a molecular level. Here we have sought to more fully characterize the hypersensitive response and pathogenicity (Hrp) T3SS translocon by identifying which proteins it is composed of, which putative translocators function away from the translocon, and what impact these proteins have on T3E injection. Comparing the differences between the *P.* syringae translocon and those of animal pathogens provides insight into how these pathogens evolved to colonize such different hosts and what selective pressures and physical obstacles they have overcome to accomplish T3E injection. The similarities between the translocons may point out elements that have remained conserved out of necessity in spite of eons of divergent co-evolution, for example the presence of transmembrane domains or that the translocon pore appears to always be composed of two different proteins.

The advances in understanding the control of secretion of translocators by HrpJ presented here were beneficial for at least two reasons. First, they help further demonstrate that type III-secreted substrates are regulated based on functional classes of proteins and are secreted in a hierarchical manner. Second, it helped independently show that the harpins and HrpK1 constitute a functional group of proteins that aid in T3E translocation and are similarly regulated. This study also established that HrpJ not only controls secretion of translocators but

may function as a molecular switch, controlling the transition from pilus to translocon formation (8).

Normally different classes of T3SS proteins have been described as having distinct functions but the HrpJ data shows that functions overlap among different classes of proteins (3, 18, 28, 35, 39, 48, 50). T3E proteins were presented as the only type III-secreted proteins to function within the plant cell to suppress immunity and ultimately allow the bacteria to grow and cause disease (21, 26). Other regulatory and structural proteins have likewise been thought to singularly function in those capacities. Here, we have shown that a T3SS-injected protein with a defined regulatory function, has a secondary function within the plant cell contributing to the suppression of plant immunity. While it has been shown that *P. syringae* T3SS extracellular proteins, such as the pilus protein HrpA1, are under selection to avoid detection by plant immunity, no other structural or regulatory protein has been shown to suppress plant immunity (17). However, the same selective pressures would likely favor a structural protein that after secretion evaded detection or better yet suppressed plant immunity. It is likely that other type III-secreted proteins with established functions will be found to similarly suppress pathogen associate molecular pattern (PAMP)-triggered immunity (PTI).

As important as it is to the pathogen to evade and suppress plant immunity, it is equally important to the plant to identify and mount a strong immune response to prevent the pathogen from growing and causing disease. One facet of a successful immune response, which we established here, is

prevention of the pathogen from injecting T3E proteins. We have shown that plants are capable of blocking T3E injection when PTI (or effector-triggered immunity (ETI)) is pre-induced. That response is much stronger in non-host interactions as the pathogen suppresses immunity most efficiently in its host plant. Importantly, this research answers the question of why a normally HR eliciting strain no longer induces a hypersensitive response (HR) in areas pre-treated with a PAMP or PTI-inducing bacteria (32). A number of possible explanations existed but now we know that it is due to the inability of the pathogen to inject T3Es precluding intracellular R proteins from detecting their presence.

**HrpK1 and harpin proteins contribute to T3E translocation.** The work presented in Chapter 2 demonstrates that HrpK1 as well as HrpZ1 interact with liposomes. The HopAK1 and HrpW1 harpins, which contribute to translocation (2, 23), do not interact with liposomes. Because both HopAK1 and HrpW1 contain pectate lyase domains their contribution to T3E translocation probably occurs at the cell wall (6, 23). In order for these proteins to function efficiently they likely form protein-protein interactions. We have tried to show interactions among these proteins with no success. Yeast-2-hybrid (Y2H) assays, pull-down assays, and co-immunoprecipitation experiments have all been negative or problematic. For example HrpZ1 has auto-activation activity in Y2H experiments, and the difficulty to purify HrpK1 without denaturing it complicates *in vitro* interaction assays.

In the future further experiments need to be done to show protein-protein interactions among the translocator proteins. In order to do this non-traditional assays may need to be employed. For example it may be necessary to look for interactions in ways that consider the hydrophobic environment in which the proteins would bind one another. Perhaps phosphatidic acid (PA) would need to be added to buffers for pull-down assays to work. Alternatively, microscopy techniques such as bimolecular fluorescence complementation may need to be employed to see if any *in vivo* interaction can be detected either *in planta* or in cell culture.

The importance of understanding which proteins interact and where they interact is very important in establishing how plant bacterial pathogens inject T3E proteins. Because the proteins involved are unique compared to animal pathogens figuring out which proteins form complexes and where those complexes exist may lead to new and exciting discoveries that highlight the differences among these pathogens. A more detailed understanding of the interactions may provide mechanistic insights to *P. syringae* T3E translocation.

Another related area of future study is the identification of a *P. syringae* tip protein. As mentioned, in all animal systems there is a third translocator, a hydrophilic protein that is thought to connect pore forming translocators to the needle that is also important for identifying host cell contact (31, 43). *P. syringae* strains lack similar proteins (5). It seems likely that there must be a protein serving the same function but due to different hosts and evolutionary divergence the phytopathogenic tip protein is dissimilar to the animal tip proteins. Whether

there is a yet unidentified protein that serves this function or a protein known to aid in translocation, such as a harpin, that serves to connect the pilus to the translocon via a novel mechanism needs to be investigated. Because it has been established that different classes of proteins have overlapping functions it is possible that a protein with an established function may have a second function to connect the pilus to the translocon. Recently, the tip protein from *Vibrio parahaemolyticus* was the first to be shown to be translocated (50). This discovery shows how, when searching for the tip protein in DC3000, one cannot solely rely on the rules established in other systems to identify it. Defining protein interactions among the known translocator proteins, which could piece together how the translocon is assembled, could also help in the identification of a tip protein.

It has long been thought that the T3SS functions as an injectisome, that is that the T3SS acts like syringe, injecting T3Es directly into eukaryotic cells through a conduit in a unidirectional one-step manner. Recent studies have results suggesting that T3E translocation may be much more complicated. Akopyan *et al.* (1) showed that type III substrates are visible outside of the bacteria prior to translocation and that purified YopH protein could be translocated into infected host cells in a T3SS-dependent manner. This discovery along with the fact that no one has shown that T3Es are injected directly into eukaryotic cells, led to the suggestion that the T3SS translocation mechanism is more similar to AB toxin delivery than needle-like injection (14). This proposed toxin-like mechanism is not mutually exclusive to the injection model and both

may occur. Certain systems or specific T3Es may employ different translocation mechanisms. That these discrepancies exist in the well-characterized *Yersinia* system, the system in which T3SS were discovered (29), exemplifies how much research is needed before it can be said with high confidence that T3SSs mechanistic function is fully understood. Additionally, multiple translocation mechanisms may help explain why, for example, *hrpK1* mutants are not fully devoid of T3E injection. Or, why different phytopathogen bacterial strains have different harpin repertoires (36, 42).

**HrpJ controls the secretion of the translocators and may function as a molecular switch controlling the transition from Hrp pilus formation to translocator secretion.** Secretion hierarchy as it relates to T3SSs is an active and important area of research but overall relatively little is known about how secretion is controlled. Type III substrates are thought to be secreted in an orderly fashion, for example pilus proteins, followed by translocators, followed by T3E proteins (12, 28, 45). In *P. syringae* the expression of all of these proteins is regulated by HrpL (15, 16, 24, 44). Since HrpL controls the expression of all type III-secreted substrates, regulatory proteins must control the timing of secretion from within the bacterial cell. Here, HrpJ has been shown to regulate the secretion of the translocator proteins. In *hrpJ* mutants the translocators are no longer secreted while the pilus protein HrpA1 is secreted at elevated levels. Additionally, we have confirmed that HrpJ regulates secretion from within the cell.

HrpJ is a member of a conserved family of type III secretion regulators. This family of proteins operates by different mechanisms in different pathogens.

For example, the YopN-TyeA proteins of *Yersinia spp.* are two separate proteins that correspond to two domains present in HrpJ (19). InvE, the representative protein from *S. Typhimurium* appears to be the lone family member that is not secreted (22). Most regulatory proteins, including MxiC and SepL from *Shigella* and enteropathogenic *Escherichia coli*, respectively, also have an effect on T3E secretion while HrpJ does not (27, 34). In some cases the differences among members of this family of proteins may be due to the different methods used to study them, but in other cases it may highlight important differences among the pathogens in terms of host interactions or pathogen evolution.

It is not known with which proteins HrpJ interacts. Because it regulates the secretion of translocators, it is logical to assume that HrpJ interacts with them. We have been unable to confirm interactions directly but showed that deletion of a C-terminal region of HrpZ1 could relieve the control by HrpJ, suggesting that this region is required for HrpJ interaction. More research needs to be done to establish how HrpJ interacts with the proteins it regulates.

Many of the YopN-TyeA/SepL/InvE family members interact with chaperones (7, 10, 48). Chaperones are typically grouped based on substrate class with class I chaperones binding effectors, class II binding translocators, and class III binding needle or pilus components (3). Since HrpJ has a T3E-like function, in addition to its regulatory function, it will be interesting to see what chaperone class it may bind. The only other family member shown to have T3E-like function is CopN from *Chlamydia* which binds a class II chaperone (3). Interestingly, SepL, which has not been shown to function like a T3E, binds a

class I chaperone which normally only interact with T3E proteins (48). Proteins and their associated chaperones are usually encoded in proximity to one another on the chromosome. The closest chaperone-like gene to *hrpJ* is *hrpG*, although present on a different operon (13). The tertiary structure of HrpG shares similarity to the class I T3E chaperones SicP, SycE, and CesT which are chaperones of T3SS effectors for SptP in *Salmonella*, YopE in *Yersinia* and Tir in *E. coli* (46). It would be very interesting if HrpG is the chaperone for HrpJ and would give added credence to HrpJ functioning as a T3E protein in plant cells. Identifying a HrpJ chaperone and any other bacterial proteins it interacts with will be an area of future study.

That HrpA1 is secreted more abundantly from the *hrpJ* mutant adds an additional layer of complexity to the role of HrpJ that is not evident in its animal pathogen counterparts. In animal pathogens the length of the needle is controlled by a protein that has the type III secretion substrate specificity switch, which in *Yersinia* is found in YscP (20). Homologues of this protein, such as InvJ and Spa32, have been studied in *Salmonella* and *Shigella* respectively and control needle length. Mutants in these genes secrete elevated levels of their respective needle protein (11). HrpP is the most similar protein from DC3000 (5). It contains the substrate specificity switch, but the mutant does not function in the same manner; instead, it reduces HrpA1 secretion and blocks secretion of all other T3SS substrates tested (30). It appears as if HrpJ may function as a substrate switch and may control the length of the Hrp pilus. The most likely explanation is

that it is mediated by recognition of cell contact although further studies are needed to confirm this (47).

In order to confirm that HrpJ controls the regulation of HrpA1secretion and pilus length, it should be tested whether HrpA1 and HrpJ interact, but this may be difficult as interaction experiments with HrpJ have not been successful, as mentioned. Next, it should be examined whether the increased HrpA1 secretion results in longer Hrp pili on the surface of the bacteria using electron microscopy techniques. A potential problem with these experiments is that the Hrp pili appear to grow indefinitely in these types of experiments and that may preclude the ability to observe differences in pilus length between wild-type and mutant strains (4, 37). However, experiments showing differences in the ability of *hrpA1* mutants to polymerize have been successful in spite of the delicate nature of the techniques (25).

**HrpJ suppresses plant innate immunity.** HrpJ is an injected protein that plays an important role in the T3SS-mediated injection of T3E proteins. Because *hrpJ* mutants cause such strong phenotypic changes in the ability of DC3000 to inject T3Es, it was a challenge to study if the injection of HrpJ had any benefit to the pathogen. There were logical reasons to explain why HrpJ would need to be injected. For example secretion may have been required in order to allow for the translocators to be secreted, to give just one example. However, we showed that HrpJ controls secretion from within the bacterial cell. With this in mind we made transgenic plants expressing HrpJ and tested different outputs of PTI. Somewhat surprisingly HrpJ suppressed the plant immune response.

Based on this discovery *hrpJ* should be studied as a bona fide T3E gene. To this end the same series of tests used to identify T3E target and function should be applied HrpJ. The *in planta* subcellular localization should be determined; likewise, yeast-2 hybrid assays should be conducted to look for Arabidopsis proteins that interact with HrpJ in hopes of identifying a target. Identifying the *in planta* target may provide a way to identify the *in planta* activity of HrpJ as has been done with other T3E proteins.

**PTI or ETI induced plants can restrict T3E injection.** This work helps establish a defense mechanism employed by plants likely to prevent infection. Additionally, this work offers a new output that can be tested, the injection restriction assay. It can be used to test mutant strains of bacteria that are more or less able to overcome injection restriction. Alternatively, it can be used to test if genes expressed *in planta* can affect injection restriction. These types of experiments would indicate whether the gene of interest suppresses or enhances PTI.

It is interesting to speculate on the biological significance of injection restriction. Is it merely a lab manipulated phenomenon, or does it have implications in natural settings? It could be that PTI has many outputs that try to stymie bacterial pathogens and giving it a head start simply allows it to block T3E injection indirectly. But perhaps it is an evolved defense mechanism where environmental conditions that allow for the exposure to an abundance of PAMPs may often be accompanied by interactions with many potential pathogens. In this scenario, some event like a heavy rain induces wounding allowing for many non-

pathogenic microbes to present PAMPs. It is beneficial to induce PTI, or more specifically the branch of PTI responsible for injection restriction, so that pathogenic bacteria that may later gain access to the newly acquired wounds are less able to inject T3E proteins and therefore less able to cause disease.

That PTI and ETI can both cause injection restriction exemplifies the overlapping nature of the two immune responses. ETI appears to trigger a stronger injection restriction which matches the established paradigm that it is the more robust of the two plant immunity responses.

**How does the plant mechanistically restrict injection?** The most interesting unresolved issue about injection restriction is establishing how the plants accomplish it. The most straightforward way to begin to answer this question is to screen different mutant plants deficient in specific outputs of immunity to see which genes or outputs contribute to injection restriction. This could be done by screening mutants deficient in callose deposition, ROS outputs, or other genes specifically involved in PTI or ETI (9, 33, 38, 40, 41).

A second line of study would be to investigate what impact injection restriction has on the bacteria. First, one could investigate whether the bacteria under these conditions have any changes in gene expression. If this is the case it would be interesting to know whether the plant can target T3SS genes specifically and down-regulate them, or whether it affects gene expression in general. Alternatively, if the expression is altered it could be because the environment in the leaf may have changed to make T3SS less favorable, for example pH is known to be important for T3SSs (49).

It would also be possible to look for bacterial mutants in genes unrelated to T3SS that may be important in overcoming injection restriction. For example, mutants less able to deal with oxidative stress, pH changes, or even motility may be hypersensitive to injection restriction even when looking at a pathogen in its host. Experiments like these could help establish mechanistic functions responsible for injection restriction. More importantly, this could help solve a long standing question in molecular plant pathology – what are the plant immune responses that specifically target bacterial pathogens in the plant apoplast.

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