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Sruti Bandyopadhyay

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TYPE III VIRULENCE EFFECTORS OF *PSEUDOMONAS SYRINGAE* PV. *TOMATO* STRAIN DC3000

By

Sruti Bandyopadhyay

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ABSTRACT

TYPE III VIRULENCE EFFECTORS OF *PSEUDOMONAS SYRINGAE* PV. *TOMATO* STRAIN DC3000

By

Sruti Bandyopadhyay

As a group, plant pathogenic bacteria infect virtually all crop plants and cause significant damage to crop production worldwide. *Pseudomonas syringae* pathovar *tomato* strain DC3000 (*Pst* DC3000) is a virulent pathogen of both tomato and *Arabidopsis thaliana*. The *Arabidopsis thaliana-Pst*DC3000 pathosystem has proven to be a very useful model system to study plant-pathogen interactions and results are generally correlated with observations from other systems. During infection in *Arabidopsis*, the pathogen multiplies vigorously for two days before the onset of disease symptoms, which are characterized by water-soaking in the apoplast followed by tissue necrosis and chlorosis. The *hrp* gene-encoded type III protein secretion system (TTSS) is essential for *Pst* DC3000 pathogenesis. The TTSS is highly conserved and secretes bacterial proteins, collectively referred to as effectors, directly into the host cytoplasm. Once translocated into the plant host cell, the effectors promote disease presumably by suppressing host defense responses and promoting the release of nutrients for utilization by bacteria. My research has identified new effectors and revealed their contribution to *Pst* DC3000 virulence on *Arabidopsis*.

A mutant carrying a deletion of six genes in the Conserved Effector Locus (CEL) of *Pst* DC3000 shows a drastic reduction in growth and symptom development on *Arabidopsis* plants. HopPtoM (ORF3) and ShcM (ORF4), two of the deleted ORFs, were

found to be sufficient to restore virulence to the ΔCEL mutant. HopPtoM is translocated into plant cells and translocation of HopPtoM is dependent on its chaperone, ShcM. The first 200 amino acids of HopPtoM are required for interaction with ShcM. The ΔCEL mutant was compromised in its ability to suppress basal host immunity characterized by the deposition of callose-containing papillae in the cell wall in response to pathogen attack. Activation of papilla formation by the ΔCEL mutant is dependent on salicylic acid, whereas papilla formation activated by the *hrp* mutant is independent of salicylic acid. The ΔCEL mutant can be restored to suppress papilla formation by HopPtoM and ShcM, suggesting that HopPtoM is a suppressor of an SA-dependent cell wall defense in *Arabidopsis*.

Using a gene homology-based method, I identified three new effectors in *Pst* DC3000. These were *avrPtoB*, $avrPphE_{Pto}$, and $avrPpiB_{Pto}$. All three effectors were demonstrated to be translocated by the TTSS. Using bacterial mutagenesis method, no contribution to virulence was detected for either $avrPphE_{Pto}$ or $avrPpiB_{Pto}$. On the other hand, even though I detected a significant virulence effect for *avrPtoB* in both tomato and *Arabidopsis*, I was unable to complement this mutant. Transgenic expression of $\mathrm{AvrPphE_{Pto}}$ in *Arabidopsis* resulted in symptoms that mirror those seen during *Pst* DC3000 infection. $\mathrm{AvrPphE_{Pto}}$ plants promoted the growth of the non-pathogenic *hrpH* mutant, which does not deliver any type III effectors. In addition, microarray experiments showed that the expression profile of the TTSS-dependent host gene cluster of *Arabidopsis* was 90% similar between plants expressing $\mathrm{AvrPphE_{Pto}}$ and plants infected with *Pst* DC3000, further supporting a virulence role of $\mathrm{AvrPphE_{Pto}}$ in disease promotion.

To
My Parents

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Chapter 1

Introduction and Literature Review

Virulence in phytopathogenic Pseudomonads

Introduction

Plants are the primary carbon source for nearly all terrestrial non-photosynthetic organisms, including ourselves. Many species seek to tap this phytosynthate, and plants are continuously resisting their strenuous and intimate advances. Plants are constantly being challenged by a wide variety of pathogenic organisms including bacteria, viruses, fungi, nematodes and protozoa. As a group, they infect virtually all crop plants and cause significant damage to crop production worldwide. The worldwide loss in major crops due to pathogens was estimated at $76.9 billion for 1988-1990 (Baker et al., 1997). Prevalent practices of pathogen control involve widespread use of pesticides. Not only is such extensive usage of these control agents a large expense, but also a source of environmental damage and toxicity. Breeding and cultivation of disease resistant and high-yield varieties of crop plants have helped combat pathogens and increase productivity. However, large-scale genetic uniformity serves as an evolutionary pressure for adaptive changes in pathogens, which can render enormous areas susceptible to devastation. An understanding of the molecular mechanisms of pathogen infection and plant defense response could be instrumental in developing novel technologies to battle these epidemics.

Bacterial diseases of plants are difficult to control and require a combination of approaches. The most devastating bacterial phytopathogens belong to the gram-negative genera of *Agrobacterium*, *Erwinia*, *Xanthomonas*, *Ralstonia* and *Pseudomonas*. Lately, another gram-negative bacteria, *Xylella fastidiosa*, has become a major cause for concern for grape vines (Hopkins, 1981) and citrus fruits (Simpson et al., 2001). The gram-positive genera of plant pathogens include *Clavibacter* and *Streptomyces*. Members of each of these genera cause a wide variety of spots, blights, cankers, scabs, wilts, soft rots and other diseases on a wide variety of host plants. Each symptom can be caused by members of different genera and each genera may contain pathogens that can cause different types of diseases. Members belonging to different genera have also been developed as model systems to further our understanding of the molecular mechanisms that promote pathogenesis in divergent hosts.

Virulence mechanisms in phytopathogenic bacteria

Despite the wide and diverse nature of bacterial phytopathogens and the diseases they cause, they have maintained striking similarities in the mechanisms they utilize to overcome their respective hosts to cause parasitism and pathogenesis. One common feature shared by most bacteria is the extracellular localization of the pathogen during infection. Thus, pathogens cause the movement of water and nutrients from the host cells into the apoplastic space. The apoplast is considered to be a nutritionally poor environment that requires enrichment during pathogenesis in order to support the large population levels that are typically attained by invading bacteria. This is also thought to cause dilution of antimicrobial compounds that may be present in the apoplast.

Bacterial plant pathogens have several methods to subvert their hosts including secretion of enzymes, production of toxins, and injection of bacterial proteins into the host. The genomes of many bacterial phytopathogens have been sequenced and their analysis is providing new insight into the mechanisms of pathogenesis. In this chapter, I will discuss the virulence mechanisms employed by pathogenic bacteria of the genus *Pseudomonas* with a greater emphasis on the type III protein secretion system.

***P. syringae* as a pathogen**

Pseudomonas belongs to the gamma subgroup of proteobacteria that contains animal pathogens such as *Yersinia*, *Salmonella*, *Shigella* and *Escherichia* and plant pathogens such as *Erwinia*, *Xanthomonas*, *Pantoea* and *Xyllela*. The various strains of *P. syringae* are noted for their diverse and host-specific interactions and can be classified into at least 40 pathovars based on host specificity (Gardan et al., 1999). Individual *P. syringae* strains often exhibit a high degree of host specificity towards a few plant species or only a few cultivars within a plant species. In nature, *P. syringae* often lives initially on the surface of leaves as an epiphyte, and later in the intercellular spaces of the plant as a pathogenic endophyte. As a pathogen, *P. syringae* generally gains access into the leaf tissue of plants through stomata, multiplies vigorously in the apoplastic space, and eventually produces necrotic lesions that are surrounded by chlorotic halos (Hirano and Upper, 2000).

P. syringae pv. *tomato* (*Pst*) strain DC3000 has emerged as an important model organism in molecular plant pathology because of its genetic tractability, its pathogenicity on both tomato and *Arabidopsis thaliana*, and its ability to deliver virulence proteins into host cells via the type III secretion system.

The type III secretion system (TTSS)

Bacterial pathogens of plants, animals and humans cause very different diseases, ranging from bubonic plague to leaf blights and cankers. One of the amazing discoveries made in the last two decades is the presence of a protein secretion system in both plant and animal bacterial pathogens. This system was classified as type III. The unique property of this system is its remarkable ability to transport bacterial proteins directly into the cytoplasm of the eukaryotic host cell. This system is broadly conserved among phytopathogenic bacteria like *Erwinia*, *Xanthomonas, Ralstonia* and *Pseudomonas* and enteric bacterial pathogens of animals like *Yersinia*, *Shigella, Escherichia* and *Salmonella.*

Among phytopathogenic bacteria, the type III secretion system was first identified in *P. syringae* pv. *phaseolicola* (Lindgren et al., 1986) and *P. syringae* pv. *syringae* (Niepold et al., 1985). Mutants of *P. s. phaseolicola* were isolated that were unable to elicit either the hypersensitive response in the non-host tobacco or disease in the normal host, bean. These were named *hrp* mutants (for hypersensitive response and pathogenicity). These *hrp* genes were later found to encode the TTSS. Since then, *hrp* genes have been found in other pathovars of *Pseudomonas,* and in *Erwinia, Xanthomonas* and *Ralstonia* species (Bonas et al., 1991; Beer et al., 1991; Gough et al., 1991).

Interestingly, even though the TTSS was initially identified as a major determinant of pathogenicity, the TTSS is not restricted to pathogenic bacteria. The TTSS has been detected in several nitrogen-fixing symbiotic bacterium including *Rhizobium* species NGR234, *Bradyrhizobium japonicum*, *Sinorhizobium fredii* USDA257 and *Mesorhizobium loti* MAFF303099. The TTSS in these bacteria is involved in the secretion of proteins called Nops (nodulation outer proteins) (Krause et al., 2002; Krishnan et al., 2003; Marie et al., 2003). *Sodalis glossinidius* is a maternally transmitted secondary endosymbiont residing intracellularly in tissues of the tsetse flies. It was found to harbour homologues of the *inv/spa* genes that encode the *Salmonella/Shigella* TTSS and to utilize them to parasitize their host (Dale et al., 2001). The function of the *inv/spa* genes in maintaining symbiosis has also been demonstrated in *Sitophilus zeamais*, a mutualistic bacterial endosymbiont of grain weevils (Dale et al., 2002).

Gene organization

In plant pathogenic bacteria, the TTSS (also called the Hrp secretion pathway or system) is encoded by *hrp* genes (He, 1998; Lindgren , 1997; Lindgren et al., 1986). The *P. syringae hrp* genes are encoded by a single locus that spans a 25 Kb chromosomal region that forms part of a pathogenicity island. The *hrp* cluster contains 27 genes that are arranged into six operons. There seems to be at least three kinds of genes in the *hrp* cluster.

The largest group of *hrp* genes encode various components of the Type III secretion apparatus. The core apparatus is likely composed of 13 proteins. Nine of these *hrp* genes have been renamed *hrc* (for *hrp* genes conserved) because of their broad conservation among all bacteria that harbor type III protein secretion systems, and the flagellar assembly system. The second class consists of *hrp* genes that are responsible for the expression of all type III-associated genes *in planta* or in *hrp*-inducing minimal medium. The third class encodes proteins that are secreted by the TTSS, including some extracellular components of the type III secretion apparatus.

The *hrp* clusters from the four major phytopathogens have been sequenced and characterized. Based on gene organization, sequence relatedness and regulatory systems, the *hrp* clusters have been divided into two groups. *Pseudomonas* and *Erwinia* belong to Group I, whereas *Xanthomonas* and *Ralstonia* belong to Group II.

Recent studies conducted in three different *P. syringae* strains suggest that the *P. syringae* pv. *syringae* strain 61 and *P. syringae* pv. tomato DC3000 *hrp* clusters and flanking sequences constitute a pathogenicity island (PAI). Pathogenicity islands are defined as gene clusters that include many virulence genes, are present selectively in pathogenic strains, have a different G+C content compared to the host bacterial DNA, occupy large chromosomal regions, are often flanked by direct repeats, are bordered by tRNA genes and/or cryptic mobile genetic elements, and are unstable (Alfano et al., 2000). The *hrp* PAI has a tripartite mosaic structure consisting of the *hrp/hrc* cluster, an exchangeable effector locus (EEL) and a conserved effector locus (CEL). The EEL begins downstream of *hrpK* and contains four ORFs, two of which have the *hrp* box and are putative *hrp*-regulated genes. The *Pst* DC3000 EEL also contains mobile genetic elements. The CEL contains ten ORFs, three of which have been previously identified as *avrE*, *avrF* (Lorang and Keen, 1995) and *hrpW* (Charkoswki et al., 1998). The CEL has no known mobile genetic elements. (Alfano et al., 2000).

Regulation

The expression of *P. syringae hrc/hrp* genes is under tight transcriptional control. In nature, the expression of *hrc/hrp* genes is induced only when the bacteria enter the host apoplast. Most *hrp* genes are expressed at a very low level in standard, nutrient-rich medium, but high levels of *hrp* gene expression are observed in infected plant tissues or in artificial *hrp*-inducing minimal media (Rahme et al., 1992; Xiao et al., 1992). The *hrp*-inducing medium is acidic, lacks complex nitrogen sources and somehow mimics the conditions that *P. syringae* experiences in the plant apoplast. Transcriptional regulation of the TTSS involves three positive regulators – HrpR, HrpS and HrpL. *hrpR* and *hrpS* are transcribed from a single operon and belong to the NtrC family of response regulators which are σ 54-dependent enhancer-binding proteins (Grimm et al., 1995; Hutcheson et al., 2001). These proteins are members of two-component regulatory systems which control transcription in bacterial systems (Stock et al., 2000). *hrpL* encodes an alternative sigma factor which shares homology with the ECF family of sigma factors (Xiao et al., 1994). It is believed that HrpR and HrpS are involved in the activation of *hrpL* expression in response to a signal in host tissue or in *hrp*-inducing minimal medium. HrpL then recognizes a conserved sequence motif called the *hrp* box, which is present in the promoter region of all TTSS-associated genes (Xiao and Hutcheson, 1994).

Although the transcriptional regulation of the *hrpRS* operon is not well understood, recent studies showed that the HrpR protein is posttranscriptionally regulated via the Lon protease (Bretz et al., 2002). The HrpR protein of *Pst* DC3000 has a shorter half life when the bacteria are cultured in rich medium than in *hrp*-inducing medium, but, the half life is unaffected by the kind of medium in *lon*$^-$ mutants. The increased stability of HrpR in *hrp*-inducing conditions would favour the expression of the TTSS-associated genes. How the Lon protease affects the stability of HrpR is not yet known. Two *hrp* genes have been shown to function as negative regulators of the TTSS in *P. syringae.* In *hrp*-inducing minimal medium, overexpression of the *hrpV* gene down-regulates *hrp*/*hrc* gene expression, whereas a *hrpV* mutant is elevated in *hrp*/*hrc* gene expression (Preston et al., 1998). Overexpression of both *hrpV* and *hrpRS* results in normal levels of *hrp* gene transcripts. In addition to *hrpV*, the *hrpA* gene also plays a key role in secretion of TTSS-associated proteins. Deletion of *hrpA*, the major component of the *hrp* pilus, was found to down regulate the expression of all examined TTSS-associated genes. Again, overexpression of *hrpRS* can compensate for the lack of *hrpA*. Further study is required for a complete understanding of this regulatory circuit.

Unlike Group I, the Group II *hrp* operons are activated by a member of the AraC family, which is designated HrpB in *R. solanacearum* and HrpX in *X. campestris* (Wengelnik and Bonas, 1996; Oku et al, 1995; Genin et el, 1992)

The type III apparatus and the Hrp pilus

Structurally, the type III apparatus consists of an intracellular membrane-embedded basal body and an extracellular appendage. The core apparatus was initially predicted to be similar in structure to the flagellum based on the high level of sequence similarity between eight *hrc* genes and the flagellar assembly genes. The type III basal body has been isolated from several mammalian pathogens. It consists of an inner membrane ring structure and an outer membrane ring structure with a periplasmic rod-like component connecting them. The type III basal body has not been isolated from any of the plant pathogenic bacteria yet.

The extracellular appendage in mammalian pathogens is very short and has been called the needle. It is about 8 nm in diameter and < 100 nm in length (Kubori et al., 1998). Plant pathogenic bacteria assemble a type III-dependent appendage known as the Hrp pilus. Hrp pili are much longer (several micrometers long) than the needle, but have the same diameter (~8nm). The Hrp pilus has been reported in *Erwinia amylovora* (Jin et al., 2001) and *Ralstonia solanacearum* (Van Gijsegem et al., 2000). In *Pst* DC3000 the major structural constituent of this pilus is HrpA. Non-polar *hrpA* mutants do not assemble the pilus, do not secrete effector proteins in culture, and are unable to cause disease in host plants or elicit the HR in non-host plants (Roine et al., 1997; Wei et al., 2000). This suggests that the Hrp pilus plays an important role in the type III protein secretion process. An *in situ* immunogold labeling procedure has been used to demonstrate that secretion of type III effectors occurs only at the site of the Hrp pilus assembly and the secreted proteins are localized specifically along the Hrp pilus, but not along the flagellum or randomly in the intercellular space (Jin et al., 2001; Brown et al., 2001). Further immunogold-labeling experiments were used to visualize the extrusion of newly synthesized effector protein from the tip of the Hrp pilus, providing direct evidence that the Hrp pilus functions as a conduit for protein delivery (Jin and He, 2001; Li et al., 2002). The assembly mechanism of the Hrp pilus in *Pst* DC3000 has also been established. The Hrp pilus has been shown to elongate by the addition of the new HrpA subunit at the distal end, further supporting the mechanistic similarity between the type III system and the flagellar system (Li et al., 2002; Emerson et al., 1970).

Secreted proteins

There are two kinds of proteins that are secreted in a *hrp*-dependent manner. They are classified based on their localization in plants. The first class of proteins, exemplified by the harpins, are secreted in the medium when bacteria are cultured under *hrp*-inducing conditions. The other class of proteins consist of the type III effectors. This group of proteins travel through the *hrp* system and are delivered directly into the cytoplasm of the plant cell.

Harpins

Harpins are a family of type III secreted proteins that are unique to plant pathogenic bacteria. The first harpin, HrpN, was identified in *Erwinia amylovora* (Wei et al., 1992). The Hrp secretion system of *Pseudomonas syringae* has been shown to secrete two harpins - HrpZ and HrpW (He et al., 1993; Yuan and He, 1996; Charkowski et al, 1998). These are heat stable proteins that are rich in glycine and proline, lack cysteine, and elicit an HR-like response when injected into the intercellular space of plant leaves. Their precise function in pathogenesis remains elusive. It has been observed that under certain conditions, HrpZ is required for *avr* genes to trigger an R gene-dependent HR. This suggests that HrpZ assists in the delivery of Avr proteins (Gopalan et al., 1996; Alfano et al., 1996). Recently, purified HrpZ has been demonstrated to associate with lipid bilayers to form cation-conducting pores *in vitro* and bind to plant protoplast membranes (Lee et al., 2001; Lee et al., 2001). HrpW also associates with the plant cell wall. HrpW has a C-terminal pectate lyase domain that binds to calcium pectate, a major plant cell wall component (Charkowski et al, 1998). These proteins could be involved in assisting the penetration of the TTSS pilus through the plant cell wall even though there is no direct evidence of such a function yet. Analysis of the recently completed *Pst* DC3000 genome revealed the presence of other HrpW-like proteins in *P. syringae* and *Ralstonia* (Petnicki-Ocwieja et al., 2002 ; Guttman et al., 2002 ; Zwiesler-Vollick et al., 2002 ; Salanoubat et al., 2002).

Type III virulence effectors

This group of type III secreted proteins is by far the most interesting and is currently the focus of intense research. Although type III effectors are very diverse, as a group, these proteins have several characteristics: i) unlike harpins, their site of action has been demonstrated to be inside the plant cell (Gopalan et al., 1996); ii) genes encoding type III effectors in a given pathogen are scattered within the genome, being encoded on the chromosome as well as on plasmids. While some are within or physically associated with the *hrp* cluster, others are known to be located elsewhere in the genome but tend to be in clusters (Buell et al., 2003); iii) they are hydrophilic proteins and often do not share sequence similarities with any genes of known function; iv) infiltration of purified effectors into leaf apoplast does not elicit any response; v) distribution of these genes within different pathovars of a single species is scattered; vi) the virulence contribution of a given effector is often specific to certain pathogen/plant genotypes and the same effector has been demonstrated to function differently in different cultivars of the same host; vii) deletion of a single effector often has subtle or no impact on virulence as measured by attenuation of disease symptoms and bacterial growth; viii) they are often flanked by mobile genetic elements indicating horizontal movement among pathovars and possibly across species; and ix) they are the prime targets for host recognition to activate plant defense responses.

Historically, a large number of type III effectors have been identified by their ability to elicit host defense responses, including the HR, in plant genotypes that carry cognate disease resistance genes. These type III effectors have been named avirulence (*avr*) genes. While *avr* genes are naturally present in avirulent strains, cloned *avr* genes can convert virulent strains into avirulent strains if the test host contains the corresponding R gene (Leach and White, 1996). This gain-of-function property has been used to identify several *avr* genes (Staskawicz et al., 1984). Analyses of many bacterial pathovar-plant species combinations has established the idea that *avr* gene activity can play a major role in restricting the host range of plant pathogenic bacteria (Dangl et al., 1992; Debener et al., 1991; Innes et al., 1993a ,b; Jenner et al., 1991; Ritter and Dangl, 1994; Vivian et al., 1989; Whalen et al., 1991). It was later found that all *avr* genes require a functional *hrp* cluster for phenotypic expression of race-specific resistance and that the site of action of *avr* gene products is inside the plant cell (Gopalan et al., 1996; Keen et al 1990; Pirhonen et al., 1996; He , 1997; Kjemtrup et al., 2000; Huynh et al., 1989; Huang et al., 1988; Leister et al., 1996.; Tang et al., 1996; Van der Ackerveken et al., 1996). It has long been an enigma why bacterial populations should maintain factors that have a negative effect on pathogen fitness (Dangl, 1994). Certain *avr* genes, although characterized by their ability to induce HR, have been shown to play a role in virulence in the absence of the interacting R gene These include *avrBs2* of *Xanthomonas campestris* pv. *vesicatoria* (Kearney and Staskawicz, 1990), the *pthA* gene of *X. citri* (Swarup et al., 1991; Swarup et al., 1992), *avrBs6* from *X. campestris* pv. *maculicola* (Yang et al., 1996), and *avrXa7* from *X. oryzae* pv. *oryzae* (Choi, 1993). Among *P. syringae* , *avrRpm1* from *P. syringae* pv. *maculicola* (Dangl, 1994; Ritter and Dangl, 1995), and *avrA* and *avrE* from *P. syringae* pv. *tomato* strain PT23 (Lorang et al., 1994) have been demonstrated to contribute to pathogen aggressiveness or fitness.

This has led to the following question: how many effectors are present in a single bacterium? Several studies have aimed at addressing this question using different approaches. One of the earliest attempts to identify effectors utilized the fact that type III effectors were secreted in *hrp*-inducing minimal medium. This approach has been used to identify many effectors from animal pathogens. However, the only phytobacterial proteins identified in this manner have been HrpZ, HrpW, and HrpA (Yuan and He, 1996). The main limitation of this method is that only abundantly secreted proteins can be detected. *P. syringae* and other plant pathogens produce very low quantities of effectors that are below the level of detection on SDS-PAGE gels by coomassie-staining.

Boch et al. (2002) used a modified *in vivo* expression technology (IVET) approach to identify type III effectors of *Pst* DC3000. This methodology was based on the fact that all known effector genes are co-ordinately regulated by the *hrp* system, which is induced *in planta*. This study identified several known and potential virulence genes, including *hrp/hrc, avr* and coronatine biosynthesis genes as well as several genes with unknown function that may encode novel virulence factors.

The recent release of the sequence of the *Pst* DC3000 genome has facilitated several independent surveys for type III effectors. These studies were based on the presence of the *hrp* box motif in the promoter, induction of gene expression in *hrp*-inducing medium, and secretion and translocation assays (Fouts et al., 2002; Guttman et al., 2002; Petnicki-Ocwieja et al., 2002; Zwiesler-Vollick et al., 2002). Proteins secreted by the TTSS were designated Hops for *hrp* outer proteins. From these studies, it was concluded that *Pst* DC3000 has more than 30 putative effector genes. These studies have also revealed specific biophysical properties of the N-terminal region of type III effectors that might be a secretion signal that dictates these proteins to travel the TTSS (Guttman et al., 2002; Petnicki-Ocwieja et al., 2002). This signal may be similar to that found in type III effectors of bacterial pathogens of animals (Anderson et al., 1997, 1999; Lloyd et al., 2001).

Virulence role of effector proteins

Recent studies show that a single virulence factor often shows virulence and avirulence functions in different host cultivars. This phenomenon is described most extensively in the *P. syringae* pv *phaseolicola*–bean pathosystem, where two effectors have been shown to have cultivar-specific avirulence and virulence functions. These are *virPphA* and *avrPphC*. VirPphA is the first virulence factor from *P. s. phaseolicola*. *P. syringae* pv. *phaseolicola* (*Pph*) strains harbor a large plasmid which contains several known *avr* genes. When cured of this plasmid, strains lose virulence and cause HR in previously susceptible cultivars of bean. Virulence was restored by complementing these strains with the region of the cured plasmid encoding *virPphA* (Jackson et al., 1999). Thus, VirPphA functions as a suppressor of HR. In a subsequent study, another effector AvrPphC was found to suppress the HR triggered by AvrPphF. Both *virPphA* and *avrPphC* are plasmid-borne and function as *avr* genes in soybean (Tsiasmis et al., 2000). Thus, some type III effectors seem to have evolved to mask the presence of endogenous *avr* genes that have been previously selected as recognition determinants by the same plant. Orthologs of *virPphA* are present in many strains of *P. syringae* (Jackson et al., 2002). In *Pst* DC3000, the ortholog of *virPphA* is *avrPtoB*. AvrPtoB has also been shown to inhibit programmed cell death initiated by *Pto* and *Cf9* resistance genes (Kim et al., 2002; Abramovitch et al., 2003). The presence of *virPphA*-like genes in diverse pathovars and similarity in their function indicate that it might play a conserved role in suppressing *avr* gene-mediated HR.

Another type III effector, HopPtoD2, suppresses programmed cell death in plants. The N-terminal domain of HopPtoD2 shares sequence homology with AvrPphD, but the

C-terminal domain is similar to protein tyrosine phosphatases. Purified HopPtD2 has tyrosine phosphatase activity and can suppress HR elicited by an avirulent strain of *P. syringae* on *Nicotiana benthamaina*. The enzymatic activity as well as the HR-suppressing ability of HopPtoD2 requires a conserved cysteine residue in the catalytic site. Using *Agrobacterium*-mediated gene co-delivery, it was shown that HopPtoD2 acts by modulating the MAP kinase pathway in tobacco that involves *NtMEK2*. *Pst* DC3000 mutants lacking HopPtoD2 were slightly reduced in their ability to multiply *in planta* and had enhanced ability to elicit an HR, supporting the role of HopPtoD2 as a suppressor of HR (Espinosa et al., 2003; Bretz et al., 2003).

Transgenic expression of effector proteins in susceptible plants has been utilized to demonstrate the virulence contribution of these proteins. Ectopic expression of *avrRpt2* in *Pst* DC3000, results in enhanced growth of *Pst* DC3000 in *Arabidopsis* plants lacking *RPS2*, the corresponding R gene (Chen et al., 2003). Interestingly, expression of AvrRpt2 in *rps2*$^-$ plants also suppresses the HR elicited by another effector, *avrRpm1* (Ritter and Dangl, 1996). This interference is apparently mutual, since *avrRpm1* prevents *avrRpt2*-elicited host responses (Reuber et al., 1996). AvrRpt2 interferes with *avrRpm1*-mediated HR by causing the disappearance of RIN4. RIN4 interacts physically with RPM1. Disappearance of RIN4 prevents RPM1-mediated signal transduction, which normally leads to the activation of defense responses (Axtell et al., 2003; Mackey et al., 2003; Mackey et al., 2002).

While type III effectors show no similarity to any proteins of known function, motif searches reveal the presence of enzymatic motifs in some effectors. AvrPphB is similar to a *Yersinia* type III effector, YopT. Both YopT and AvrPphB belong to a family of cysteine proteases that are involved in bacterial pathogenesis. YopT cleaves the posttranslationally modified RhoGTPases leading to the disruption of the actin cytoskeleton in host cells (Shao and Dixon, 2003; Shao et al., 2003). The enzymatic activity of AvrPphB is required for the autoproteolytic activity of the AvrPphB precursor in the plant as well as for eliciting the hypersensitive response (Shao et al., 2002). In *Arabidopsis*, resistance to *P. syringae* strains containing AvrPphB requires *RPS5*, its cognate resistance gene and PBS1, a protein kinase. AvrPphB proteolytically cleaves PBS1 and this activity is required for RPS5-mediated resistance (Shao et al., 2003).

The biochemical function of another Avr protein had been defined previously. AvrD directs the synthesis of low molecular weight elicitors called syringolides, which elicit an HR on soybean (Midland et al., 1993). Different alleles of *avrD* are involved in the synthesis of different host-specific syringolides (Yucel et al., 1994a,b). Homologs of *avrD* are widespread among Pseudomonads (Keith et al., 1997). Strains of *Pst* PT23 that lack *avrD*, however, are not impaired in virulence on tomato (Lorang et al., 1994; Murillo et al., 1994). Ji et al. (1997, 1998), identified a syringolide-binding protein, Rpg4, in soybean (Ji et al., 1997, 1998). Although *avrD* is co-regulated with the *hrp* system, it is still not known whether AvrD is translocated into the host cells by the TTSS, or what its biological function is in *P. s.* pv *tomato* (Shen and Keen, 1993).

Testing for the ability of an effector protein to suppress the hypersensitive response in resistant host plants has provided information about the function of several *P. syringae* type III effectors. Previous microscopic studies suggested that in susceptible plants, the TTSS of plant-pathogenic bacteria also transports suppressors of an HR-independent cell wall-based plant defense that is activated by the TTSS-defective *hrp* mutant (Bestwick et.al, 1995; Brown and Bonas, 1995; Brown et al., 1998). However, the identity of such suppressors remained elusive for many years. It was recently found that the *P. syringae* TTSS down-regulates the expression of a set of *Arabidopsis* genes encoding putatively secreted cell wall and defense proteins in a salicylic acid-independent manner. Transgenic expression of AvrPto, a type III effector of *Pst* DC3000, represses a similar set of host genes, compromises defense-related callose deposition in the host cell wall, and permits substantial multiplication of a *hrp* mutant. AvrPto seems to be one of the long-postulated suppressors of a salicylic acid-independent, cell wall-based defense (Hauck et al., 2003).

Another type III effector, HopPtoA1, contributes to the efficient formation of *Pst* DC3000 bacterial colonies *in planta*. The gene encoding HopPtoA1 is located in the Conserved Effector Locus and has a functionally redundant partner, *hopPtoA2*, which is located elsewhere in the genome. Confocal laser-scanning microsocopy of GFP-labelled bacteria in *Arabidopsis* leaves show a higher frequency of undeveloped individual colonies in the *hopPtoA1* mutant and an even higher frequency in the *hopPtoA1/hopPtoA2* double mutant (Badel et al., 2002).

Type III Chaperones

Studies conducted mainly with mammalian pathogens show that efficient type III secretion requires chaperones. Type III chaperones are usually co-regulated with the *hrp* system and are typically small, acidic proteins ($pI<5.5$) that often contain an amphipathic α-helix near the C-terminus. The first type III chaperone, SycE, discovered in *Yersinia*, is required for the secretion of its cognate effector, YopE (Wattiau and Cornelis, 1993).

Since then, more than 30 chaperones have been found in various plant and animal pathogenic bacteria and more are predicted from genome-wide analyses (Buell et al., 2003; van Dijk et al. 2002). In plant pathogens, type III chaperones have been designated Shc for specific *hrp* chaperone. ShcA was the first chaperone in *P. syringae* pv. *syringae* that was required for the secretion of HopPsyA and for the ability of HopPsyA to cause an HR in the non-host tobacco (Van Dijk et al., 2002). AvrPphF$_{\mathrm{Pto}}$ also requires ShcF for efficient secretion into culture media (Shan et al., APS, 2003). Chaperones are thought to maintain their effectors in a secretion-competent state within the bacterial cytosol and even prioritize their secretion (Boyd et al., 2000). Chaperones have also been demonstrated to function to maintain the stability of their cognate effectors within the bacterium (Cheng and Schneewind, 1999; Frithz-Lindsten et al., 1995, Menard et al., 1994; Neyt and Cornelis, 1999). In the plant pathogen *Erwinia amylovora*, DspF is required for the stability and secretion of DspE (Gaudriault et al., 1997; 2002).

Phytotoxins

Toxins have long been known to be virulence factors in *P. syringae*. For example, *P. s.* pv. *tomato* and *P. s.* pv. *maculicola* produce the phytotoxin coronatine (COR). It is also produced by *P. syringae* pv. *morsprunorum, atropurpurea*, and *glycinea*. Coronatine is the only known phytotoxin produced by *Pst* DC3000 (Bender et al., 1999). Coronatine is a polyketide toxin consisting of coronofacic acid (cfa) and coronamic acid (cma) joined by an amide linkage (Bender et al., 1999). The enzymes are required for the biosynthesis of CFA and CMA are encoded by the *cfa* and *cma* genes, respectively, and are collectively referred to as COR genes. Unlike many *P. syringae* pathovars where the

COR genes are encoded on plasmids, in *Pst* DC3000 the COR genes are chromosomally encoded. In *Pst* DC3000, the *corRS* operon, which encodes a two-component regulatory system, regulates the expression of the *cfa* and *cma* genes. Two of the *cfa* genes, *cfa1* and *cfa6*, have been shown to be expressed in a *hrp*-dependent manner, which implies that the synthesis of coronatine might be co-ordinately regulated with the *hrp* system (Fouts et al., 2002). Recently, RpoN, which encodes a sigma 54 factor, was shown to be required for coronatine synthesis in *P. s.* pv. *glycinea* (Alarcon-Chaidez et al., 2003). The primary symptom associated with coronatine production is chlorosis. Coronatine causes the development of chlorotic symptoms when applied to tomato leaves (Gnanamanickam et al., 1982). However, the precise virulence role of coronatine remains unclear. Mutation of COR genes renders *Pst* DC3000 less virulent on tomato. There is a reduction in bacterial multiplication and leaves show smaller chlorotic lesions (Bender et al., 1987). A different COR mutant of *Pst* DC3000 (DC3661) was found to grow to lower levels than wildtype bacteria following surface inoculation of *Arabidopsis*, but no difference in multiplication was observed when bacteria were infiltrated into leaf intercellular spaces directly (Mittal and Davis, 1995)

Other phytotoxins produced by *P. syringae* pathovars include tabtoxin, phaseolotoxin, syringomycin, and syringopeptin. Tabtoxin is produced by *P. syringae* pvs. *tabaci, coronofaciens* and *garcae.* It is a monocyclic β-lactam that elicits chlorosis after hydrolytic release of tabtoxinine-β-lactam (Levi and Durban,1986). Tabtoxinine-β-lactam inhibits the plant enzyme glutamine synthetase, causing accumulation of ammonia, which is thought to cause chlorosis (Thomas et al., 1983). Mutant strains of pathogens that lack tabtoxin remain pathogenic but do not cause chlorosis (Kinscherf et al., 1991; Willis et al., 1991).

Phaseolotoxin is primarily produced by *P. s. phaseolicola*. A phaseolotoxin-like substance has also been characterized from some *P. s. tomato* strains (Bagdache et al., 1990). Mutants of *P. s. phaseolicola* that do not produce phaseolotoxin are reduced in virulence (Patil, 1974). This toxin consists of ornithine, arginine, homoarginine, and N'-sulfodiaminophosphinyl (Moore et al., 1984). It inhibits the plant ornithine carbamoyltransferase resulting in lower levels of arginine, which has been implicated in the development of chlorosis and inhibition of plant growth (Patil, 1974; Mitchell and Bielski, 1977).

Syringomycin and syringopeptin are synthesized in *P. s. syringae*. Mutants of *P. s. syringae* that are impaired in the synthesis of both the toxins are reduced in virulence (Scholz-Schroeder et al., 2001). Syringomycin and syringopeptin are lipopeptide toxins that trigger necrosis when applied exogenously on plants. They cause pore formation in plant plasma membranes (Hutchinson and Gross, 1997). Pore formation is thought to cause electrolyte leakage, cell death and tissue necrosis.

Additional virulence factors

In addition to the TTSS and toxin biosynthesis, several other virulence factors have been implicated in Pseudomonad pathogenesis.

Several factors have been identified that are linked to epiphytic survival of *P. syringae*. These include ice nucleation, protease synthesis, and utilization of citric and malic acid. Epiphytic growth is a key part of the *P. syringae* life cycle in nature and could be influenced by a range of traits including chemotaxis, attachment, microcolony formation, nutrient acquisition, antibiosis and resistance to UV stress (Beattie and Lindow, 1995; Beattie and Lindow, 1999; Hirano and Upper, 1990). Type IV pili of *P. s.* pv. *tomato* have been implicated in adhesion and UV tolerance (Roine et al., 1998b). Recent studies from our laboratory have identified a *uvrD* mutant of *Pst* DC3000 that shows increased sensitivity to UV light. The *uvrD* gene encodes a type II helicase that is involved in DNA repair and replication. *Pst* DC3000 strains lacking *uvrD* show a 1000-fold reduction in bacterial multiplication *in planta*. The *rulA* mutants also exhibit a reduced tolerance to UV light and are not as competitive as the wildtype bacteria in the phyllosphere (Sundin et al., 2000). As the bacteria face an onslaught of UV light during the epiphytic phase of growth (Sundin and Jacobs, 1999), it is possible that these genes are required for epiphytic fitness.

Extracellular polysaccharides (EPS) also play an important role in the virulence of many bacterial phytopathogens including *Ralstonia solonacearum, Erwinia amylovora, Erwinia stewartii, Xanthomonas campestris* and *Xanthomonas oryzae* (Buddenhagen and Kelman, 1964; Denny and Baek, 1991; Kao et al., 1992; Poetter and Coplin, 1991; Geier and Geider, 1993; Denny, 1995; Rajeshwari and Sonti, 2000). In vascular pathogens, EPSs cause blockage of the host xylem resulting in wilt. EPSs in *R. solanacearum* have been implicated in assisting attachment to the host (Sequira, 1985). Secreted EPS is also thought to form a layer around the bacteria that provides protection against desiccation, antimicrobial compounds from the plant, and impedes recognition by preventing contact with plant cells (Denny, 1995; Braun, 1990). Alginate is an EPS that is an important virulence factor in *P. s.* pv. *syringae*. Alginate has also been linked to epiphytic fitness and production of water-soaking lesions (Jing et al., 1999). *P. s. tomato* produces alginate (Gross and Rudolph, 1987), but its contribution to virulence has not been investigated.

Cell wall degrading enzymes (CWDE) are also important virulence factors, particularly in members of the *Erwinia* genera of plant bacterial pathogens, which are collectively referred to as soft-rot bacteria. These bacteria macerate plant tissues via destruction of pectins and cellulose, which are important components of the plant cell wall. The enzymes responsible for maceration are pectate lyases, pectin methylesterases and polygalacturonases, collectively called CWDEs (Barras et al., 1994). In *Erwinia chrysanthemi*, there are at least eight pectate lyases. They are encoded by the *pel* genes, which contribute to the soft rot ability of *E. chrysanthemi* on potato tubers (Ried and Collmer, 1987; Jafra et al., 1999). These enzymes are secreted by the type II secretion system (Bouley et al., 2001; He et al., 1991). Pectic enzymes and cellulases influence the development of final symptoms in *P. s.* pv. *lachrymans* (Bauer and Collmer, 1997). The recent completion of the sequence of the *Pst* DC3000 genome has revealed that *Pst* DC3000 contains a pectin lyase, a polygalacturonase and three enzymes with predicted cellulolytic activity (Buell et al., 2003). At least one pectate lyase gene may be co-regulated with the *hrp* system (Fouts et al., 2002).

Pst DC3000 also contains genes required for the synthesis of pyoverdin, a siderophore that is found in fluorescent Pseudomonads (Buell et al., 2003; Bultreys and Gheysen, 2000). Siderophores are low molecular weight, high affinity iron chelators that function as important virulence factors in many bacterial pathogens because of their role in sustaining growth in iron-limiting host environments. A cluster of genes that are homologous to those required for biosynthesis of the siderophore pyochelinin in *P.*

aeroginosa was found in *Pst* DC3000 (Reimmann et al., 2001). Three genes predicted to encode FHA-like proteins were identified in *Pst* DC3000 (Buell et al., 2003). Filamentous haemagglutinin (FHA) has been shown to be an adhesin and a virulence factor in animal pathogenic bacteria and the plant pathogen *Erwinia* (Rojas et al., 2002).

Project Summary

Tremendous progress has been made in the last two decades in understanding the virulence mechanisms utilized by bacterial phytopathogens. This field has witnessed some exciting discoveries in microbiology and molecular plant pathology. These include the discovery of the *hrp* system and the Hrp pilus, demonstration of the action of Avr proteins within plant cells, finding a large number of effectors in a single bacterial strain and preliminary evidence for effector function in suppressing host defense responses, and completion of the *Pst* DC3000 genome sequence.

In contrast to their mammalian counterparts, plant pathogens have an unusually large number of effectors. Efforts to understand the virulence role of these effectors face several challenges, the most obvious being the functional redundancy among type III effectors. This results in weak loss-of-virulence phenotypes of strains inactivated for single effectors. This field is also in need of a better understanding of pathogenesis at the cellular level. A plethora of cellular markers, such as actin, in mammalian cells have resulted in an avalanche of knowledge about the role of effectors in animal bacterial pathogens. Identification and development of similar markers in plants would assist in understanding the role of effector proteins. *In planta* visualization of infection, functional genomics, and proteomics should also help to elucidate mechanisms by which effectors contribute to plant pathogenesis.

At the time this project was initiated, only two type III effectors were known to be present in *Pst* DC3000. My aim was to identify new type III effectors and to understand their contribution to virulence of *Pst* DC3000. I chose to use the *Pst* DC3000-*Arabidopsis thaliana* pathosystem, which is an important model system in molecular plant pathology for several reasons. It is currently the only pathosystem in which the genomes of both organisms are completely sequenced. *Pst* DC3000 is genetically tractable, and has an extensively studied TTSS. Additionally, it is also a pathogen of the economically important plant, tomato. This aspect will help in extending the knowledge acquired through the *Arabidopsis-Pst* DC3000 pathosystem to design better disease control techniques for economically important crops. Genomic resources are also available for the host *Arabidopsis*, including a large number of mutants, knockouts, and transgenics that can facilitate the dissection of host pathways that are targeted by the pathogen during infection.

Chapter 2 describes my research on the Conserved Effector Locus of *Pst* DC3000 and its contribution to virulence of *Pst* DC3000 on *Arabidopsis*. A mutant carrying a deletion of six genes in the CEL region shows a drastic reduction in growth and symptom development on *Arabidopsis* plants. Two ORFs within the deleted region, ORF3 and ORF4, are sufficient to complement the CEL deletion mutant. ORF3 is a translocated type III effector which requires ORF4 for secretion and translocation. ORF4, which is a chaperone of ORF3, is neither secreted nor translocated, but physically interacts with ORF3 in a yeast two-hybrid system. ORF3 was designated HopPtoM and ORF4, ShcM.

Expression of *hopPtoM* in *Arabidopsis* plants causes the development of water soaking, chlorosis, and necrosis that are very similar to those induced by *Pst* DC3000 during infection. Expression of HopPtoM *in planta* also complements the growth and symptom development defects of the ΔCEL mutant, further confirming that HopPtoM functions inside the plant cell. Unlike *Pst* DC3000, the ΔCEL mutant is impaired in suppressing host cell wall-based papilla defenses. HopPtoM and ShcM restores the ability of the ΔCEL mutant to suppress papilla formation. Examination of the response of salicylic acid (SA)-deficient NahG plants and *eds5* plants (Delaney et al., 1994; Gaffney et al., 1993; Nawrath and Metraux, 1999) to the ΔCEL mutant and a non-pathogenic *hrp* mutant showed that activation of papilla formation was partly dependent on SA. Thus, HopPtoM is involved in the suppression of SA-dependent activation of plant cell wall-based host immunity.

Chapter 3 reports my research on the identification of several type III effector genes in *Pst* DC3000. A set of ten known *avr* genes from different pathovars of *P. syringae* were used to identify homologs in *Pst* DC3000 based on Southern hybridization. Three orthologues were identified. An AvrRpt2 based translocation system was used to show that all three identified effectors were secreted by the TTSS. Strains lacking *avrPphE*$_{Pto}$ or *avrPpiB*$_{Pto}$ were still able to infect *Arabidopsis thaliana* and tomato, whereas an *avrPtoB* mutant was reduced in virulence. However, I was unable to complement the *avrPtoB* mutant. To further investigate the role that AvrPphE$_{\mathrm{Pto}}$ may play in virulence, I expressed the *avrPphE*$_{Pto}$ gene in *A. thaliana* under the control of a dexamethasone-(DEX) inducible promoter. Transgenic plants which express AvrPphE$_{\mathrm{Pto}}$ develop water soaking, chlorosis, and necrosis when maintained under high humidity.

These symptoms mirror those seen with *Pst* DC3000 infection. These plants promoted the growth of the *hrpH* mutant. In addition, microarray experiments showed that the expression profile of the TTSS-dependent host gene cluster of *Arabidopsis* was 90% similar between plants expressing AvrPphE$_{Pto}$ and plants infected with *Pst* DC3000. These experiments provided evidence for a virulence role of AvrPphE$_{Pto}$ in disease promotion.

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Chapter 2

HopPtoM is a type III virulence protein of *Pseudomonas syringae* pv. *tomato* DC3000 that suppresses salicylic acid-dependent activation of cell wall-based extracellular host defenses in *Arabidopsis thaliana*

I would like to acknowledge Kinya Nomura for contribution of figures 2-5, 2-6 and 2-7.

Abstract

Pseudomonas syringae pv. *tomato* DC3000 (*Pst* DC3000) is a pathogen of tomato and *Arabidopsis* that injects virulence effector proteins into host cells via a type III secretion system (TTSS). TTSS-deficient mutants have a Hrp$^-$ phenotype, that is, they cannot elicit the hypersensitive response (HR) in nonhost plants or pathogenesis in host plants. On the other hand, mutations in individual effector genes typically have weak virulence phenotypes. A very singular case is that of the CEL deletion mutant (ΔCEL mutant) of *Pst* DC3000: deletion of six ORFs in the *Pst* DC3000 conserved effector locus (CEL) reduces parasitic growth and abolishes disease symptoms without affecting the function of the TTSS. The inability of the ΔCEL mutant to multiply and cause disease symptoms in *Arabidopsis* was restored by a clone expressing two of the six deleted ORFs: CEL ORF3 (HopPtoM) and ORF4 (ShcM). ShcM was found to be required for HopPtoM to restore virulence to the ΔCEL mutant. HopPtoM was secreted in culture and translocated into *Arabidopsis* cells by the TTSS during infection. Secretion and translocation of HopPtoM were dependent on ShcM, which itself, was neither secreted nor translocated but, like typical TTSS chaperones, could be shown to interact with HopPtoM, its cognate effector, in yeast two-hybrid experiments. Expression of HopPtoM in *Arabidopsis* promoted the growth of the ΔCEL mutant but not of the TTSS-deficient *hrpH* mutant. The inability of the ΔCEL mutant to cause disease was linked to activation of cell wall-based host defense in wildtype *Arabidopsis* plants. HopPtoM and ShcM restored the ability of the ΔCEL mutant to suppress extracellular cell wall-based host defenses. The ΔCEL mutant triggered significantly fewer number of callose deposits and

grew better in salicylic acid (SA)-impaired NahG and *eds5* plants, than in wildtype plants, suggesting that HopPtoM is a suppressor of SA-dependent cell wall-based defenses.

Introduction

Bacterial pathogens cause numerous diseases in animals, plants and humans. These include members from diverse genera such as *Yersinia*, *Salmonella*, *Shigella*, *Escherichia* and *Pseudomonas*, *Xanthomonas*, and *Ralstonia*. All these pathogens harbour a unique secretion system classified as the type III (Galan and Collmer, 1999). This secretion apparatus is unique in its ability to deliver proteins from the bacterial cytoplasm directly into host cells. The TTSS is integral to pathogenicity, which is evident from an often complete loss of pathogenicity when inactivated. In plant pathogens, the type III system is encoded by *hrp* and *hrc* genes. The *hrp* genes govern the ability of bacteria to cause disease in susceptible plants and elicit the hypersensitive response (HR) in non-host plants (Alfano and Collmer, 1997; Lindgren, 1997; He, 1998). During pathogenesis, the TTSS secretes numerous virulence effector proteins into the host that synergistically function to modulate host responses to favour pathogenesis. Recent completion of the *P. syringae* pv. tomato DC3000 genome sequence suggests the presence of at least 31 different type III effectors in this pathogen (Buell et al., 2003). Historically, most of the type III effectors were identified by their ability to cause an HR in the presence of their cognate resistance gene in the resistant host genotype and were named avirulence (*avr*) genes (Leach and White, 1996; Collmer et al., 2001). Many of the putative effectors identified from bioinformatic analysis of the *Pst* DC3000 genome have been demonstrated to be secreted by the TTSS and have been renamed Hop for *hrp* outer protein.

Another category of proteins that have been shown to be required for efficient secretion of effectors are the type III chaperones. Chaperones have been found in both plant and animal pathogens and are typically small, acidic proteins (pI<5.5) with an amphipathic α-helix near the C-terminus (Parsot et al., 2003; Feldman and Cornelis, 2003; Gaudriault et al., 2002; Van Dijk et al., 2002). In most cases, they bind to the N-terminus of their cognate effectors and may stabilize their substrates and maintain them in a secretion-competent conformation (Stebbins et al., 2001). Chaperones have also been implicated to prioritize the secretion of their cognate effectors in the presence of other effectors in the bacterial cytosol (Boyd et al., 2000). Prior to my study, HopPsyA was the only *P. syringae* type III effector that had been demonstrated to have a chaperone, ShcA. ShcA was required for the secretion of HopPsyA (van Dijk *et al.*, 2002). ShcA binds to a site located within the N-terminal 166 amino acids of HopPsyA (van Dijk *et al.*, 2002).

The most puzzling aspect of type III effectors of plant pathogens is their virulence function in the host. In comparison, a lot is known about the biochemical activities of type III effectors injected by mammalian pathogens. Although there has been an ongoing effort to achieve a better understanding of the functions of type III effectors produced by plant pathogenic bacteria, these efforts have been thwarted by the typically weak phenotypes resulting from inactivation of single and sometimes multiple effectors, compared to the strong loss-in-virulence phenotypes observed when *hrp* genes are inactivated. This has been attributed to redundancy among the large number of type III effectors that are present in a single pathogen. Despite these hindrances the functions of several effectors have been identified. Five have been shown to function as suppressors of cell death (Jackson et al., 1999; Tsiasmis et al., 2000; Abramovitch et al., 2003; Espinosa et al., 2003; Bretz et al., 2003; Chen et al., 2003), and one was shown to suppress cell wall-based defense (Hauck et al., 2003).

In *P. syringae*, genes encoding the TTSS are clustered on a pathogenicity island (Pai), which also contains two loci that encode putative effector proteins (Alfano *et al.*, 2000). An exchangeable effector locus (EEL), whose number of ORFs and their nucleotide sequences vary between closely related strains of the same pathovar, is located downstream of *hrpK*. A conserved effector locus (CEL), encoding at least seven ORFs that are conserved between the divergent strains *Pss* B728a and *Pst* DC3000, is located upstream of *hrpR*. Deletion of the *Pst* DC3000 EEL causes a slight reduction of bacterial growth in tomato, whereas deletion of six ORFs of the CEL drastically reduces bacterial multiplication in tomato (Alfano et al., 2000). The deleted region contains *avrE*, *ORF2*, *ORF3*, *ORF4*, *hrpW* and *hopPtoA1*. However, single mutations in *avrE* (Lorang and Keen, 1995), *hrpW* (Charkowski et al., 1998), and *hopPtoA1* (Badel et al., 2002) do not cause a reduction in virulence. This is consistent with the idea of functional subtlety and/or redundancy among effectors in *Pst* DC3000.

In this study, we found that 1) the ΔCEL mutant had a drastically reduced virulence phenotype in *Arabidopsis*, 2) CEL ORF3 and ORF4 completely restored the virulence of the ΔCEL mutant in *Arabidopsis*, And 3) the CEL ORF3 protein was secreted in culture and translocated into the plant cell by the TTSS and that translocation was dependent on a chaperone encoded by CEL ORF4. Accordingly, we designated CEL ORF3 and CEL ORF4, HopPtoM and ShcM respectively. Transgenic expression of

HopPtoM *in planta* caused symptoms that are similar to those caused by *Pst* DC3000 and allowed the ΔCEL mutant to grow and cause disease symptoms. Finally, we show that HopPtoM is a suppressor of salicylic acid (SA)-dependent host cell wall-based immunity.

Materials and Methods

Bacterial strains and media

E. coli was grown in low salt (5g/L) Luria Bertani (LB) medium (Sambrook et al. 1989) at 37°C. *P. syringae* were grown in low salt LB or *hrp*-inducing minimal medium (Huynh et al., 1989) at 30°C or 20°C. Antibiotics were used at the following concentrations unless otherwise specified – rifampicin 100mg/L, kanamycin 50 mg/L, ampicillin 200mg/L, tetracycline 10mg/L, spectinomycin 50mg/L.

Recombinant DNA techniques

All DNA manipulations including polymerase chain reaction (PCR) were performed using standard protocols (Sambrook *et al.*, 1989). Oligonucleotide primers for sequencing or PCR were synthesized at Integrated DNA Technology (Coralville, IA). PCR was performed using HIFI polymerase (Invitrogen, Carlsbad, CA). DNA sequencing was done at the Michigan State University Genomic Technology Support Facility with Automated DNA sequencers model 373A (Applied Biosystems, Foster City, CA). DNA sequences were analyzed with Assemblylign®, MacVector® and the Sequence Manipulation Suite (http://www.bioinformatics.org/sms/index.html). Database searches were performed using gapped BLASTN, BLASTP, and BLASTX (Altschul *et al.*, 1997) (http://www.ncbi.nlm.nih.gov/BLAST/).

Construction of complementation plasmids

Various fragments within the CEL were either subcloned from a cosmid clone, pCEL (for p*ORF2-5*, p*ORF234*, p(*hrpHRPW-ORF5*) and p(*ORFf6-10*) or amplified by PCR (for p*ORF2*, p*ORF23*, p*ORF24*, p*ORF43*, p*ORF3*, and p*ORF4*) and cloned into appropriate vectors. The following primers were used to amplify *ORF43* (for construction of p*ORF43*): sense primer, 5'-GTGAATTCGCTAAGTGGGCAATTGGAC-3' and antisense primer, 5'-CAGGATCCTTTAAGGTTAAAACAGCAT-3'; *ORF4* (for construction of p*ORF4*): sense primer, 5'-GTGAATTCGCTAAGTGGGCAATTGGAC-3' and antisense primer, 5'-CGGGATCCGATCATTGGAATCTCCCAG-3'; and *ORF3* (for construction of p*ORF3*): sense primer, 5'-CAGGATCCAAACGCGAGAGCCTTTCGG-3' and antisense primer, 5'-CTTCTAGATTAAAACAGCATGAAGCATGC-3'. p*ORF3* also contains the *ORF4* promoter upstream of *ORF3*. The *ORF4* promoter for the above constructs was amplified using sense primer 5'-GTGAATTCGCTAAGTGGGCAATTGGAC-3' and antisense primer 5'-CAGGATCCGTTGATAAGGGTGTGGTAC-3'. The following primers were used to amplify *ORF2* (for construction of p*ORF2*): sense primer, 5'-TATCTAGACGCTTTGAATAACATCCGT-3', and antisense primer, 5'-GGGGATCCAACTGAAGAGCTAATAACG-3'; and *ORF23* (for construction of p*ORF23*); sense primer, 5'-ACTCTAGAGCCTTTCGGCTCCTGGGAG-3' and antisense primer, 5'-GGGGATCCAACTGAAGAGCTAATAACG-3'. For constructs p*ORF2* and p*ORF23*, the *ORF4* promoter was amplified using sense primer 5'-GTGAATTCGCTAAGTGGGCAATTGGAC-3' and antisense primer 5'-GTTCTAGACATTGTTGGTCATTTCAAG-3'. For construction of p*ORF24*, *ORF2* was amplified using sense primer, 5'-TATCTAGACGCTTTGAATAACATCCGT-3' and antisense primer, 5'-GGGGATCCAACTGAAGAGCTAATAACG-3'and *ORF4* was amplified with its own promoter using sense primer 5'- GTGAATTCGCTAAGTG GGCAATTGGAC-3' and antisense primer 5'-ATTCTAGAACTGATCATTGGAAT CTCC-3'. Plasmids were introduced into bacteria by electroporation.

Plant growth and bacteria enumeration

Wildtype *Arabidopsis* accession Columbia *glabrous1* (Col *gl1*) and transgenic plants were grown in soil in growth chambers with a day/night cycle of 12h/12h, a light intensity of 100 µE, and a constant temperature of 20 °C. Four- to five-week-old plants were used for experiments. Bacteria were grown in low-salt LB to the mid-logarithmic phase at 30°C. Bacterial cultures were centrifuged to recover bacterial cells, which were resuspended in sterile water to a final OD_{600} of 0.002 (equivalent to $1x10^6$ CFU/ml). Fully expanded leaves were either vacuum-infiltrated or syringe-infiltrated with bacterial suspensions, and bacteria were enumerated as described by Katagiri *et al.* (2002). The mean values of the bacterial populations were plotted with the standard deviation displayed as error. Plants analyzed in Figure 2-9 were sprayed daily with a 0.003 µM dexamethasone (DEX) solution to induce the *hopPtoM* transgene. Bacterial suspensions were infiltrated into leaves one day after the first DEX treatment.

Secretion assays

Bacteria were grown in low-salt LB broth until $OD_{600} = 0.6$. Bacteria were collected by centrifugation and resuspended in *hrp*-inducing minimal medium or *hrp*-repressing LB and incubated with shaking at 20°C for 12 h. Cultures were separated into cell and supernatant fractions by centrifugation at 15,000*g*. The cell and supernatant fractions were concentrated 5 and 50 times, respectively. Proteins were separated on SDS-PAGE gels and transferred to Immobilin-P membrane (Millipore Corp., Bedford, MA). Immunoblot analyses were performed using rabbit and chicken antibodies raised against *E. coli*-expressed HopPtoM and ShcM, respectively, at Cocalico Biologicals, Inc., Reamstown, PA.

Type III translocation analysis

The truncated *avrRpt2*$_{80\text{-}255}$ gene, which encodes type III secretion/translocation-incompetent, but biologically active, AvrRpt2 (Mudgett *et al.*, 2000) was cloned into the *Xba*I-*Hin*dIII sites of pUCP19 (Schweizer, 1991). Full-length *ORF3* or *ORF4* genes were amplified by PCR and fused to the 5' end of *avrRpt2*$_{80\text{-}255}$. The recombinant plasmids were introduced into ΔCEL mutant by electroporation. The transformants were grown in low-salt LB to $OD_{600} = 0.6$. Bacteria were collected by centrifugation and resuspended in sterile water to $OD_{600} = 0.2$. The bacterial suspensions were infiltrated into leaves of 6-week-old *RPS2*$^{+}$ *Arabidopsis* ecotype Col-0 plants or *rps2* mutant plants (Kunkel et al., 1993). HR was monitored over a 48-h period at room temperature.

The following primers were used in the construction of *avrRpt2*$_{80\text{-}255}$ gene fusions. *ORF4*::*avrRpt2*$_{80\text{-}255}$: sense primer, 5'-GTGAATTCGCTAAGTGGGCAATTGGAC-3' antisense primer, 5'-ACTCTAGATTGGAATCTCCCAGGAG-3'; *ORF4+ORF3::avrRpt2*$_{80\text{-}255}$: sense primer, 5'-GTGAATTCGCTAAGTGGGCAATTGGAC-3' and antisense primer, 5'-GTTCTAGAAAGCGTCTCGGTACGGTCC-3', using genomic DNA as a template; *ORF3::avrRpt2*$_{80\text{-}255}$: sense primer, 5'-GTGAATTCGCTAAGTGGGCAATTGGAC-3' and antisense primer, 5'-GTTCTAGAAAGCGTCTCGGTACGGTCC-3', using p*ORF3* as a template.

Yeast two-hybrid analysis

The LexA-based yeast two-hybrid system (Clontech Laboratories Inc.) was used. *ORF4* and *ORF3* fragments were amplified by PCR and cloned into pB42AD or pGILDA. The following primers were used to amplify full-length *ORF4*: sense primer, 5'-CGAATTCATGACCAACAATGACCAGTAC-3' and antisense primer, 5'-GATCCTCGAGCTGATCATTGGAATCTCC-3'; full-length *ORF3*: sense primer, 5'-GGAATTCATGATCAGTTCGCGGATCGGC-3' and antisense primer, 5'-CCTGCTCGAGTGACGGATGTTATTCAAAG-3'; sequence corresponding to the first 100 amino acids of ORF3: sense primer, 5'-GGAATTCATGATCAGTTCGCGGATCGGC-3' and antisense primer, 5'-CCTGCTCGAGACTAACCGATCAACAACGC-3'; and sequence corresponding to the first 200 amino acids of ORF3: sense primer, 5'-GGAATTCATGATCAGTTCGCGGATCGGC-3' and antisense primer, 5'-CTTGCTCGAGCGGCCTATTCGCCAAGGGC-3'. The constructs were transformed into the EGY48 yeast strain carrying the *lacZ* reporter plasmid. Activation of the LacZ reporter was determined colorimetrically using X-gal as the substrate.

Generation of transgenic plants

Pst DC3000 genomic DNA was extracted as described by Chen and Kuo (1993). PCR was used to amplify *hopPtoM* using HIFI Taq polymerase and the following primers :

sense primer 5'GGCTCGAGACCATGGGGCATCATCATCATCATCATATCAGTTC GCGGATCGGC-3'

antisense primer 5'-GCACTAGTTCATAGTCCTTTAAGGTTAAAACAG-3'.

The *hopPtoM* gene was cloned into the pTA7002 vector (Aoyama and Chua, 1997; McNellis *et al.*, 1998). pTA7002 allows for inducible expression of transgenes after application of the animal glucocorticoid hormone, DEX. Electroporation was used to transform *Agrobacterium tumefaciens* strain GV3850 with the recombinant plasmid (Keen et al., 1990). Four pots of *Arabidopsis thaliana* Col *gl1* plants were transformed with *A. tumefaciens* carrying pTA7002-*hopPtoM* using the floral dip method (Clough and Bent, 1998). Seeds collected from each pot were kept separate to ensure that independently transformed lines could be isolated. T1 seeds were vapor-sterilized in a dessicator for 4 hours with 80ml of bleach mixed with 3ml of concentrated HCl. Seeds were placed on Murashige-Skoog (MS) (Gibco BRL, #11117-074) plates supplemented with 1x vitamins (SIGMA, #M7150) and 40 units/ml hygromycin B (hyg) (Calbiochem Cat # 400051), kept at 4°C for three days, and then moved to growth chambers (see previous section). Transformants were selected on the basis of hygromycin resistance.

Dexamethasone-induction of transgene expression

Dexamethasone (Sigma Aldrich Cat # D1756) (DEX) was dissolved in 100% ethanol to the concentration of 30mM. This stock solution was diluted in water to required concentrations (see Results section). Plants were sprayed with the DEX solution to induce the transgene.

Generation of antibody against HopPtoM and ShcM

The *hopPtoM* and *shcM* genes were amplified by PCR from *Pst* DC3000 genomic DNA using the following primers :

hopPtoM : sense primer 5'-GGAGATTCATATGATCAGTTCGCGGATC-3'
antisense primer 5'-GGAATTCGGATGTTATTCAAAGCGTCTC-3'

shcM : sense primer 5'-AGGCCTTCATATGACCAACAATGACCAG-3'
antisense primer 5'-CGAATTCATCATTGGAATCTCCCAGGAG-3'

The genes were individually cloned into the pET28(a) vector (Invitrogen, Carlsbad, CA) and transformed into *E. coli* BL21(DE3) cells by electroporation (Sambrook *et al.*, 1989). Protein was induced by addition of 1mM IPTG to a mid-log culture and incubated for 4 hours at 37°C. HopPtoM and ShcM protein was extracted from *E. coli* cells using standard protocol (Qiagen, Valencia, CA) and purified using the Ni-NTA column. Purified protein was analyzed by SDS-PAGE and was used to raise antibodies in rabbit at Cocalico Biologicals, Inc., Reamstown, PA. Pre and post immune sera was obtained and checked for the ability to recognize the antigen using immunoblot analysis (see below).

Immunoblotting

Arabidopsis Col *gl1* and *hopPtoM* plants were sprayed with an appropriate concentration (see results) of DEX and maintained under high humidity for 24 hours. Two cm^2 tissue was collected using a #5 cork borer (Boekel, #1601BD 1-10), homogenized in 100µl 2X treatment buffer (0.125M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol) and denatured at 100°C for 10 min. An equal volume of each sample was separated on a 10% SDS-polyacrylamide gel (Sambrook *et al.*, 1989) and proteins were transferred onto Immobilin P membrane (Millipore, #IPVH00010) using a semi-dry apparatus (SEMI PHOR, Hoefer Scientific Instruments). Immunoblotting was carried out using HopPtoM antiserum and anti-rabbit alkaline phosphatase conjugate. HopPtoM protein bands were visualized by a standard colour reaction using SIGMA FAST (B5655).

Northern blot analysis

Arabidopsis Col *gl1* and transgenic plants were sprayed with 0.003 µM DEX and plants were kept under humidity domes. Tissue was harvested 24 hours later and snap-frozen in liquid nitrogen. Total RNA was isolated using the Promega RNAgents kit (Cat # Z5110). Ten µg RNA was denatured with an equal volume of loading buffer (500 µl formamide, 170 µl formaldehyde, 100 µl 10X MOPS buffer, and 10 µl of 1 mg/ml ethidium bromide) for 15 minutes at 65°C, separated on a 1.2% formaldehyde agarose gel and transferred to nylon membrane (HybondN; Amersham Pharmacia Biotech #RPN303B) (Sambrook *et al.*, 1989). Approximately 100 ng of probe DNA was labeled with ^{32}P and purified using BIORAD columns (Cat #732-6223) according to the manufacturer's instructions. Membranes were hybridized overnight at 65°C in PerfectHyb Plus (SIGMA, #H7033). Membranes were washed to a stringency of 0.1X SSC (20 mins; 65°C) and exposed to film (Kodak Scientific Imaging Film X-OMAT AR, #1651454).

Callose assay

Arabidopsis Col *gl1* leaves were vacuum-infiltrated with a bacterial suspension of $OD_{600} = 0.2$ ($1x10^8$ cfu/ml) as described in Katagiri *et* al. (2002). Leaves were harvested 7 hours after infiltration, cleared with alcoholic lactophenol (1:1:1:1:2 phenol:glycerol:lactic acid:water:ethanol), and stained with aniline blue (0.01% aniline blue, SIGMA #M6900, in 150mM K_2HPO_4 pH 9.5) for callose as described by Adam and Somerville (1996). Leaves were examined with a Zeiss Axiophot D-7082 Photomicroscope with an A3 fluorescence cube (ex.: 535 ± 25, DC: 560, EM; 590 long pass). The number of callose depositions was determined with ImagePro Plus software. More than 10 adjacent fields of view along the length of the leaf were analyzed and averaged. The values in Figures 2-12 and 2-13 are the average and standard deviation of 4 or more independent leaves for each treatment.

Results

The *Pst* DC3000 ΔCEL mutant can be restored by a fragment containing ORF3 and ORF4

A large deletion mutant was generated in the conserved effector locus (CEL) of the *Pst* DC3000 Hrp pathogenicity island (Pai) and was demonstrated to be required for bacterial virulence on tomato (Alfano et al., 2000). We assessed the ΔCEL mutant for its ability to infect *Arabidopsis* and found that it was severely compromised in its ability to cause disease symptoms and multiply in the host tissue. The ΔCEL mutant exhibited 100 to 500 fold reduced growth compared to *Pst* DC3000 when infiltrated at a concentration of 10^6 cfu/ml and elicited no disease symptoms (Figure 2-1). A cosmid, pCEL, was isolated from a cosmid library of *Pst* DC3000 genomic DNA by screening for *avrE*, one of the genes in the deleted region. This cosmid, which carries *ORF1* through *ORF10*, fully complemented the ΔCEL mutant (Figure 2-2).

Since the deleted region contained several putative effector genes, we conducted complementation analysis to identify key players in the ΔCEL mutant phenotype. We introduced plasmids carrying different fragments from the deleted region into the ΔCEL mutant and tested for restoration of disease symptoms and bacterial multiplication in *Arabidopsis* plants. The smallest fragment tested that could complement the ΔCEL mutant phenotype contained three genes, *ORF2, ORF3* and *ORF4* (Figure 2-2). These three genes constitute an operon and have a *hrp* box upstream of *ORF4* implying regulation by the Hrp system. Of these genes, *ORF2* is thought to encode a chaperone to AvrE.

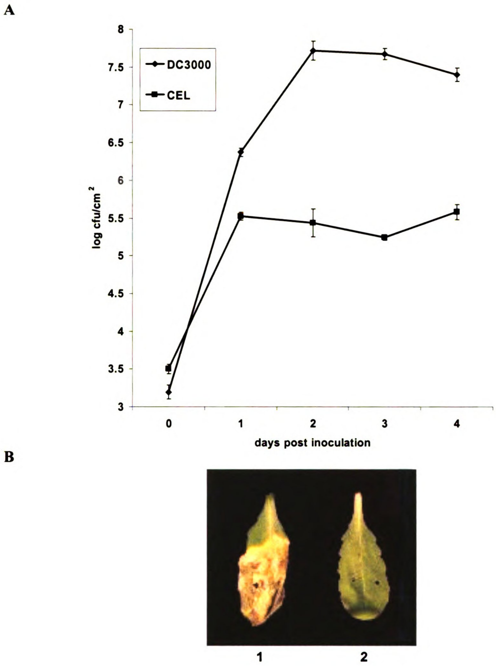


Figure 2-1: Disease symptoms and growth *in planta* of *Pst* DC3000 and the ΔCEL mutant following vacuum-infiltration with an inoculum of 10^6 cfu/ml. (A). Bacterial multiplication in *Arabidopsis* Col *gl1* of DC3000 (solid diamonds) and ΔCEL mutant. Each datum point reflects the average bacterial population recovered from nine 0.5 cm^2 leaf discs. Vertical lines indicate standard deviation. (B). Symptoms in *Arabidopsis* Col *gl1* leaves four days after inoculation with 10^6 cfu/ml DC3000 (leaf 1) and ΔCEL mutant (leaf 2).

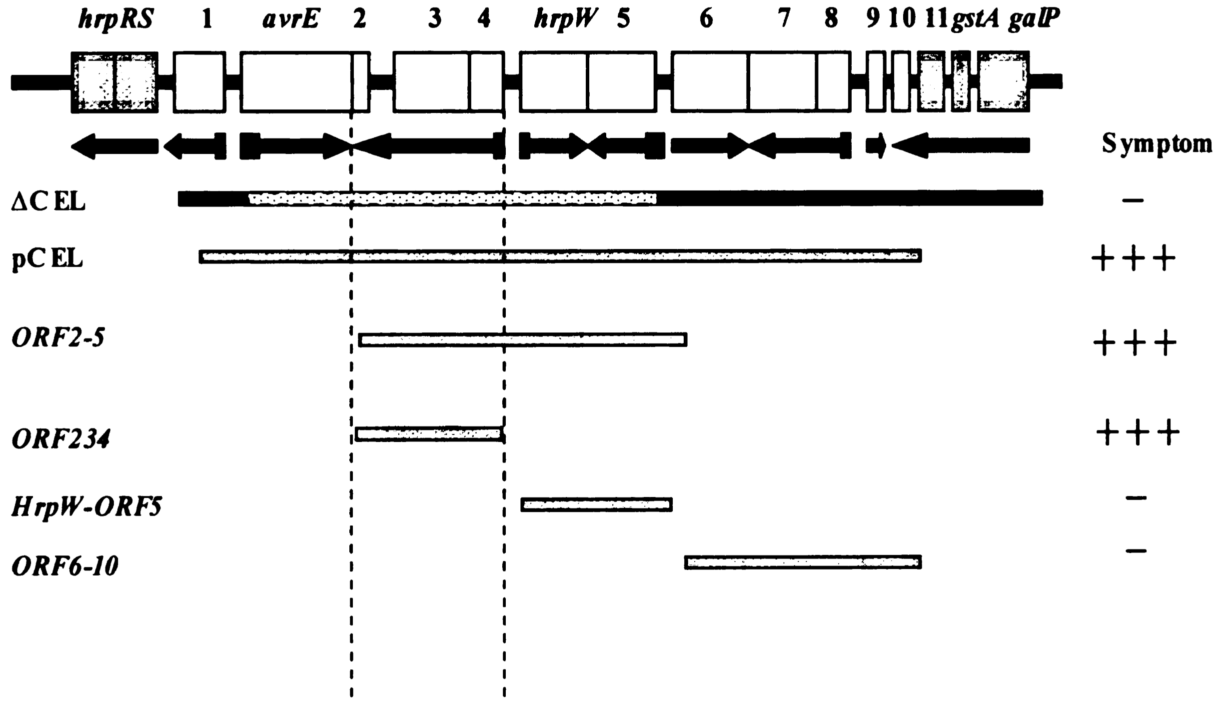


Figure 2-2: Schematic representation of the complementation of the ΔCEL mutant. Col *gl1* plants were syringe-infiltrated with 10^6 cfu/ml of the ΔCEL mutant carrying different regions of the conserved effector locus. Plants were scored for symptom development and bacterial multiplication four days after inoculation. A minus sign indicates no disease symptom. Three plus signs indicate typical disease symptoms: extensive water-soaking followed by necrosis and chlorosis. Appearance of disease symptoms was correlated with high levels of bacterial multiplication.

Further complementation studies were conducted within the delineated fragment. Complementation of the ΔCEL mutant with *ORF2, ORF23* or *ORF24* did not restore the virulence defect (Figure 2-3). The adjacent location of the large ORF3 (712 amino acids) and the small ORF4 (164 amino acids) in the same operon is suggestive of an effector-chaperone relationship. To determine if ORF4 is required for the ability of ORF3 to restore virulence to the ΔCEL mutant, *Arabidopsis* plants were inoculated with the ΔCEL mutant containing either *ORF3*, *ORF4* or *ORF43* on a plasmid. Symptom development and *in planta* bacterial growth were monitored. We found that *ORF4* alone did not complement the ΔCEL mutant. The ΔCEL mutant carrying *ORF3* alone was partially restored in growth, but caused little or no symptom development. However, the presence of both *ORF3* and *ORF4* in the ΔCEL mutant restored *in planta* growth and symptom development completely (Figure 2-4). Based on these results, we concluded that ORF3 and ORF4 were sufficient to restore virulence to the ΔCEL mutant in *Arabidopsis*.

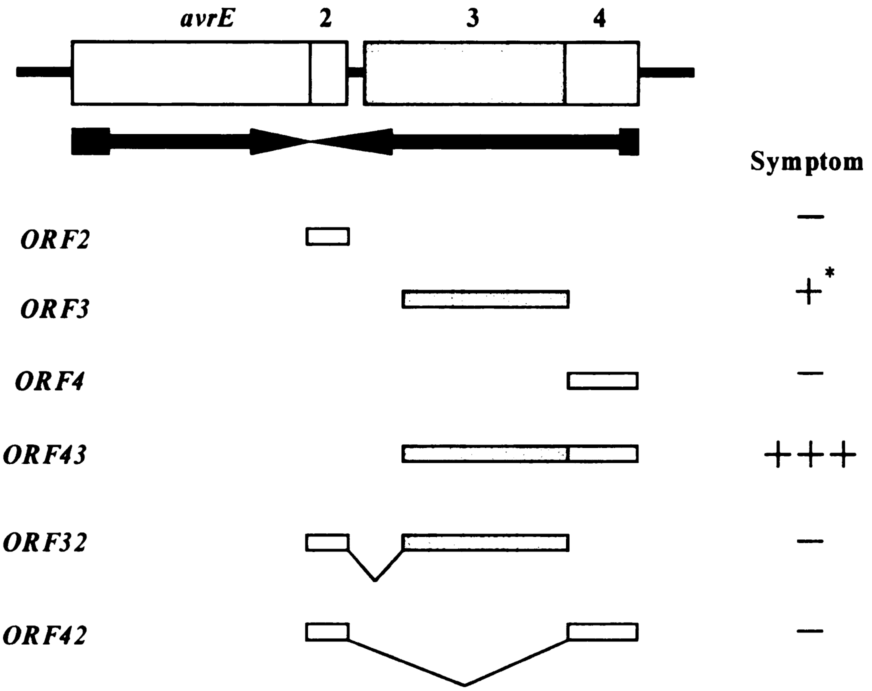
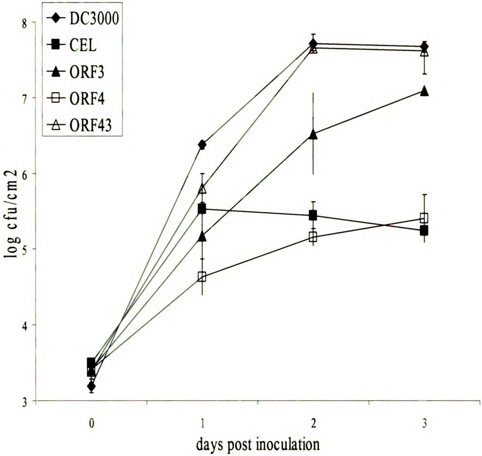


Figure 2-3: Schematic representation of further complementation of the ΔCEL mutant. Col *gl1* plants were syringe-infiltrated with 10^6 cfu/ml of the ΔCEL mutant carrying different *ORF*s from the delineated fragment. Plants were scored for symptom development and bacterial multiplication (not shown) four days after inoculation. A minus sign indicates no disease symptom. A plus sign with an asterisk indicates infrequent yellow spots in only some of the inoculated leaves. No typical water-soaking or necrosis symptoms were observed. Three plus signs indicate typical disease symptoms: extensive water-soaking followed by necrosis and chlorosis. Appearance of disease symptoms was correlated with high levels of bacterial multiplication.

A



B

Figure 2-4: Restoration of the ability of the ΔCEL mutant to multiply and elicit disease symptoms in *Arabidopsis*. (A). Bacterial growth curves. Col *gl1* plants were vacuum-infiltrated with 10^6 cfu/ml of *Pst* DC3000 (solid diamonds), ΔCEL (solid squares), ΔCEL mutant containing *ORF3* (solid triangles), *ORF4* (empty squares) or *ORF43* (empty triangles). Each time point reflects the mean of nine 0.5 cm^2 leaf discs. Error bars indicate standard deviations. (B). Disease symptoms in Col *gl1* leaves were scored 4 days after inoculation with 10^6cfu/ml of DC3000 (leafl), ΔCEL mutant (leaf 2), ΔCEL mutant containing *ORF43* (leaf 3), *ORF4* (leaf 4) or *ORF3* (leaf 5).

ORF3, but not ORF4, is secreted in culture by the Hrp system

The results from the complementation studies prompted us to investigate whether ORF3 and ORF4 are secreted through the TTSS. For this, *ORF*3 and *ORF*4 under the control of their native promoter (p*ORF43)*, were introduced on a plasmid into the wildtype *Pst* DC3000, the ΔCEL mutant, and the *hrcC* mutant strain (Yuan and He, 1996). The parental and transformed strains were tested for secretion of ORF3 and ORF4 in both LB and *hrp*-inducing liquid media. The *hrcC* mutant is incapable of assembling the type III apparatus and does not secrete any type III effectors. Both ORF3 and ORF4 proteins were observed in the cell-bound fractions of all strains tested, except the ΔCEL mutant, when grown in *hrp*-inducing minimal medium but not when grown in LB medium. This suggests that *ORF3* and *ORF4* are induced under conditions that favor *hrp* gene expression. As expected, the protein bands observed were stronger in all strains expressing *ORF3* and *ORF4 in trans*. However, only ORF3 was detected in the supernatant fractions of the wild-type *Pst* DC3000, and *ORF43* transformants of *Pst* DC3000 and the ΔCEL mutant, when grown in *hrp*-inducing minimal medium. This indicated that only ORF3, but not ORF4, was secreted. ORF3 was not detected in the supernatant fractions of the *hrcC* mutant grown in minimal medium, indicating that its secretion is TTSS-dependent.

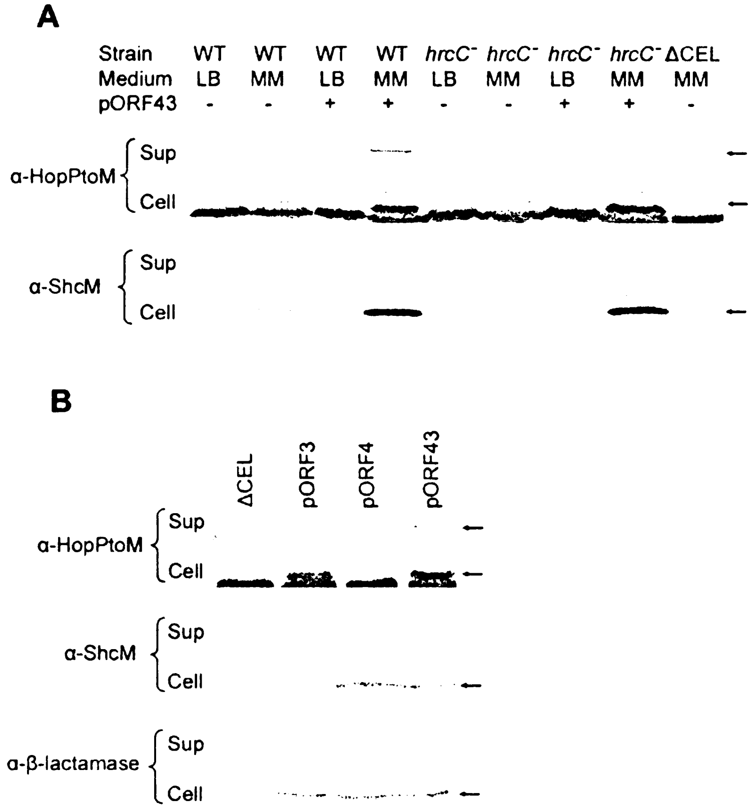


Figure 2-5: (A). Analysis of type III-dependent secretion of ORF3 (HopPtoM) and ORF4 (ShcM). *Pst* DC3000 (WT) or *hrcC* mutant derivatives carrying (+) or lacking (-) p*ORF43* were grown in rich media (LB) or *hrp*-inducing media (MM). Cultures were separated into supernatant (sup) and cell (cell) fractions by centrifugation and the presence of ORF3 or ORF4 in each fraction was detected by immunoblot analysis using an antibody against HopPtoM (α-HopPtoM) or ShcM (α-ShcM). WT: wild-type DC3000. p*ORF43* expresses *ORF3* and *ORF4* under the native promoter to enhance the expression of both genes in *hrp*-inducing medium. In the cell fraction of the α-HopPtoM blot, a cross-reacting protein present in all lanes migrated slightly faster than ORF3.

(B) Dependence of ORF3 secretion on ORF4. .The ΔCEL mutant and its derivative carrying p*ORF3*, p*ORF4*, or p*ORF43* were grown in *hrp*-inducing medium (MM). Other conditions were the same as described in panel A.

ORF3 is translocated into plant cells by the TTSS in an ORF4-dependent manner

We decided to further investigate the requirement of ORF4 for the function of ORF3 during infection by determining whether ORF3 and ORF4 are translocated into plant cells. We fused full-length ORF3 (712 amino acids) and ORF4 (164 amino acids) proteins to a truncated AvrRpt2 protein (80-255 amino acids, AvrRpt2$_{80-255}$). We then introduced various constructs expressing wildtype AvrRpt2, ORF3 + ORF4, ORF4::AvrRpt2$_{80-255}$, ORF3::AvrRpt2$_{80-255}$, ORF4 + ORF3::AvrRpt2$_{80-255}$ or ORF4::AvrRpt2$_{80-255}$ + ORF3 into the ΔCEL mutant and inoculated *RPS2* and *rps2* *Arabidopsis* plants with these strains. No HR developed in leaves inoculated with bacteria expressing ORF3 and ORF4. A robust AvrRpt2-dependent HR was observed at 9 hours after infiltration for the wildtype AvrRpt2 and at 15 hours for the ORF3::AvrRpt2$_{80-255}$ fusion protein expressed together with ORF4 (Figure 2-6). Leaves inoculated with ORF3::AvrRpt2$_{80-255}$ alone were reduced in turgidity, but no typical HR occurred. ORF4::AvrRpt2$_{80-255}$ alone or when expressed with ORF3 did not give an HR. Therefore, ORF3 is a translocated type III effector of *Pst* DC3000. Translocation of ORF3 requires the presence of ORF4, which itself is not translocated.

12/14	1/14	1/12	0/12	11/14	0/14	Col-0
0/14	0/14	0/12	0/12	1/14	1/14	*rps2*

Figure 2-6: Type III translocation analysis of ORF3 and ORF4 in *Arabidopsis*. Full-length ORF3 and ORF4 proteins were fused to a truncated AvrRpt2 protein (80-255 amino acids, $AvrRpt2_{80\text{-}255}$). Plasmids were introduced into the ΔCEL mutant. *Arabidopsis* Col 0 (*RPS2*) or *rps2* mutant leaves were infiltrated with bacterial suspensions at OD_{600}=0.2 and evaluated for HR elicitation. 1: ΔCEL (pAvrRpt2), 2: ΔCEL (pORF43), 3: ΔCEL (pORF3:: $AvrRpt2_{80\text{-}255}$) , 4: ΔCEL (pORF4:: $AvrRpt2_{80\text{-}255}$), 5: ΔCEL (pORF4 + pORF3:: $AvrRpt2_{80\text{-}255}$), 6: ΔCEL (pORF4:: $AvrRpt2_{80\text{-}255}$ + pORF3). Col-0 leaves (1 and 5) showing HR collapse appear wrinkled. Top: Number of leaves showing HR/number of leaves infiltrated for Col-0 and *rps2* plants. Picture was taken 18 h after inoculation. Leaves representing the majority of each treatment are shown.

ORF4 physically interacts with ORF3 in the yeast two-hybrid system

The results from complementation, virulence, secretion, and translocation analyses suggested that ORF4 may act as a chaperone for ORF3. To obtain more conclusive evidence for this possibility, we fused the full-length ORF4 protein (164 amino acids) to the DNA binding domain (BD) and the full-length ORF3 protein (712 amino acids) to the activation domain (AD) of the LexA-based two-hybrid system and tested for physical interaction of the two proteins. Yeast strains carrying the above constructs individually, did not turn blue, indicating that these genes were not auto-activators. A blue color developed in positive control yeast strains, in which BD was fused to the SV40 activation domain and AD was fused to the p53 binding domain (Figure 2-6). No color developed when yeast strains carried empty AD or BD, regardless of the nature of the other partner. A blue color developed in strains carrying AD fused to ORF3 and BD fused to ORF4, demonstrating physical interaction of these proteins in yeast.

To determine the portion of ORF3 that interacts with ORF4, we constructed fusions of the LexA AD with the N-terminal 100 and 200 amino acids of ORF3 ($ORF3_{100}$ and $ORF3_{200}$, respectively) and tested for interaction in yeast as described above. Yeast strains carrying the N-terminal 200 amino acids of ORF3 turned blue after two days of growth in galactose X-gal minimal medium. In contrast, yeast strains carrying the N-terminal 100 amino acids of ORF3 remained white (Figure2-7). These results indicate that the first 200 amino acids of ORF3 are required for interaction with ORF4.

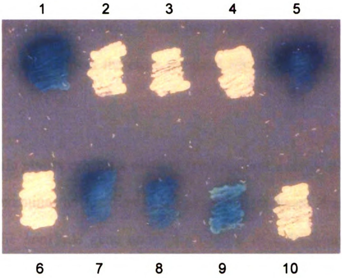


Figure 2-7: Physical interaction between ORF3 and ORF4 in the LexA two-hybrid system. Full-length ORF4 protein was fused to the DNA binding domain (BD) in pGILDA and a series of truncated ORF3 proteins were fused to the transcriptional activation domain (AD) in pB42AD. Yeast strains were grown at 30°C for 2 days on galactose X-gal complete minimal medium. Blue color indicates interaction, whereas white color indicates no interaction. 1 = BD::SV40/AD::p53, 2 = BD-empty/AD-empty, 3 = BD-empty/AD::ORF4, 4 = BD::ORF3 (full-length)/AD-empty, 5 = BD::ORF3 /AD::ORF4, 6 = BD::ORF4/AD::ORF3$_{1\text{-}100}$ (1-100 amino acids), 7 = BD::ORF4/AD::ORF3$_{1\text{-}200}$ (1-200 amino acids), 8 = BD::ORF4/AD::ORF3$_{201\text{-}712}$ (201-712 amino acids), 9 = BD::ORF4/AD::ORF3$_{301\text{-}712}$ (301-712 amino acids), 10 = BD::ORF4/AD::ORF3$_{401\text{-}712}$ (401-712 amino acids). Yeast colonies containing AD::ORF3$_{1\text{-}200}$ alone, AD::ORF3$_{201\text{-}712}$ alone, and AD::ORF3$_{301\text{-}712}$ alone were white (data not shown).

The results described so far, indicate that ORF3 is a translocated type III effector of *Pst* DC3000 and was therefore renamed HopPtoM (Hop for *hrp* outer protein). Sequence analysis indicated that ORF4 had numerous characteristics of a chaperone protein. It is a small protein, 18 kDa, with an acidic pI of 5.3 containing an amphipathic α-helix in the C-terminal portion. These properties, in combination with the complementation, secretion, translocation and interaction data, suggest that ORF4 is a chaperone for HopPtoM and was therefore renamed ShcM (Specific hrp chaperone of HopPtoM).

HopPtoM transgenic plants exhibit a distinct seedling and growth phenotype

To further examine the function of HopPtoM, I produced *Arabidopsis* Col *gl1* plants expressing the *hopPtoM* gene under the control of a DEX-inducible promoter system. A 6X His tag was added at the N-terminus of the HopPtoM protein. Homozygous lines were chosen on the basis of heritable resistance to hygromycin. Three independent lines were carried to homozygosity. Western analysis using anti-HopPtoM and anti His-tag antibodies confirmed the presence of the protein when plants were induced with DEX. Data presented here are from two lines, 11 and 44.

These lines were found to have several unique morphological and developmental features. Transgenic *hopPtoM* seeds were delayed in germination compared to wildtype Col *gl1* seeds. When germinated on MS media, *hopPtoM* seeds showed a 2-3 day delay. However, this delay was more marked when seeds were directly sown in soil, being at least 5-7 days late compared to wildtype seeds. When grown in MS medium and soil, *hopPtoM* seeds also exhibited an inability to achieve synchronous germination, even after stratification for three days at 4°C. Usually, three days of incubation at 4°C causes wildtype plants to achieve synchronized germination.

***Arabidopsis thaliana* plants expressing HopPtoM exhibit chlorosis and necrosis upon induction**

The *hopPtoM* plants exhibited a distinct phenotype in response to DEX induction. When sprayed with a high level of DEX (30µM) and maintained under high humidity, *hopPtoM* plants developed water soaking symptoms after 6 to 12 hours. By 24-36 hours, severe necrosis and chlorosis developed (Figure 2-8). While this phenotype was similar to the symptoms caused by *Pst* DC3000 infection of *Arabidopsis*, HopPtoM-caused symptoms were faster compared to an infection. At lower levels of DEX (0.3µM), the HopPtoM-induced symptoms were comparable to those of an infection.

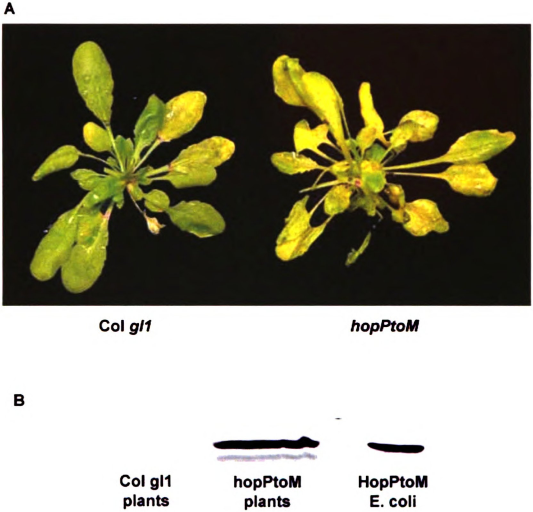


Figure 2-8: (A) Expression of HopPtoM *in planta* causes disease-like symptoms. Six-week-old *Arabidopsis* Col *gl1* and *hopPtoM* plants were sprayed with 30 µM DEX and kept under high humidity. The upper two leaves of Col *gl1* were inoculated with 10^6 cfu/ml *Pst* DC3000 and pictures were taken four days later. Induction of the *hopPtoM* transgene caused development of chlorosis and necrosis that very closely resembled the disease symptoms caused by *Pst* DC3000 on *Arabidopsis* Col *gl1*.

(B) Western blot analysis of HopPtoM expression in leaves of wild-type *Arabidopsis* Col *gl1* plants and HopPtoM transgenic plants (*hopPtoM*) 24 hours after spraying with 30 µM DEX. Recombinant HopPtoM from *E. coli* was used as control.

Expression of HopPtoM *in planta* allows the growth of the CEL deletion mutant

We demonstrated that the ΔCEL mutant is fully complemented by *hopPtoM* and *shcM*. We also showed that ShcM is required for the secretion and translocation of HopPtoM. Based on these results, we hypothesized that the ΔCEL mutant may be able to grow and cause symptoms in plants that express *hopPtoM*. In order to investigate this, I identified a concentration of inducer (0.003 µM DEX), which by itself caused no macroscopic symptom development in *hopPtoM* plants. I was unable to detect the HopPtoM protein at this level of DEX by Western blotting. However, Northern blot analysis confirmed the expression of the transgene at this concentration of DEX (data not shown).

Arabidopsis Col *gl1* and *hopPtoM* plants were sprayed with 0.003 µM DEX, 6 hours prior to bacterial inoculation and daily thereafter. Both plant genotypes were inoculated with *Pst* DC3000, ΔCEL mutant, and the *hrpH* mutant. The *hrpH* mutant is unable to assemble a functional type III secretion apparatus and is thus incapable of secreting any type III effector proteins (Yuan and He, 1996). Bacterial population was estimated 3 days later. *Pst* DC3000 showed similar growth in both wildtype and transgenic plants. The *hrpH* mutant grew 5-fold in *hopPtoM* plants as compared to Col *gl1* plants. While the ΔCEL mutant grew a 100-fold in Col *gl1* plants, it multiplied 5000-fold in the *hopPtoM* plants, representing a 50-fold increase. In fact, the ΔCEL mutant multiplied only 5-fold less than *Pst* DC3000 in *hopPtoM* plants. We concluded that the ΔCEL mutant can be complemented by *in planta* expression of *hopPtoM*. The *hopPtoM* plants inoculated with the ΔCEL mutant also developed chlorosis and necrosis. Uninoculated leaves remained asymptomatic during the experiment.

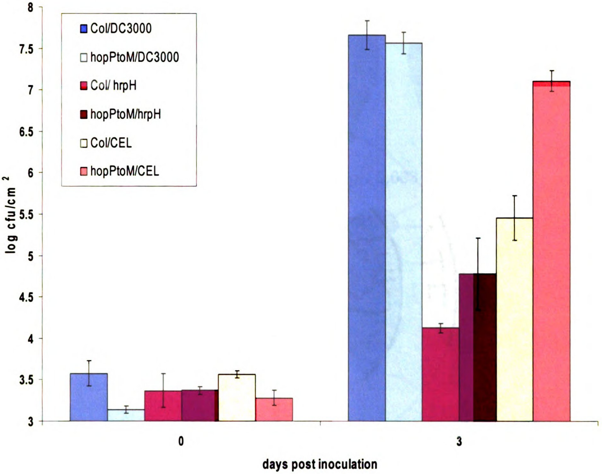


Figure 2-9: Complementation of the ΔCEL mutant multiplication in plants expressing HopPtoM. *Arabidopsis* Col *gl1* (Col) plants and *hopPtoM* transgenic plants (hopPtoM) were sprayed with 0.003 μM DEX, 6 hours prior to bacterial infiltration and daily during the course of the experiment. Bacteria were syringe-infiltrated into plants at 10^6 cfu/ml. Bacterial growth was monitored after three days. Bacterial numbers are the average of 12 leaf discs from three individual leaves. Error bars indicate standard deviation. DC3000 represents *Pst* DC3000. hrpH represents the *hrpH* mutant and CEL represents the ΔCEL mutant. The *hopPtoM* transgenic plants support a 50-fold higher growth of the ΔCEL mutant compared to wildtype Col *gl1*.

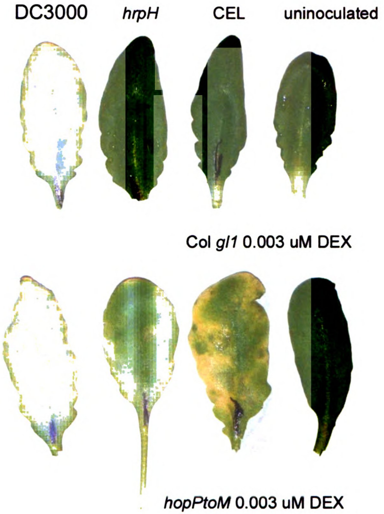


Figure 2-10: Growth of the ΔCEL mutant in *hopPtoM* transgenic plants is accompanied by the development of symptoms similar to those of *Pst* DC3000 infection. Wildtype Col *gl1* and *hopPtoM* transgenic plants were induced with 0.003 μM DEX, 6 hours prior to inoculation and daily during the course of the experiment. Bacterial inoculum of 10^6 cfu/ml was syringe-infiltrated. Symptoms were recorded three days later. DC3000 represents *Pst* DC3000, hrpH represents the *hrpH* mutant and ΔCEL represents the ΔCEL mutant. Leaves labeled "uninoculated" were treated with DEX but were not infiltrated with bacteria.

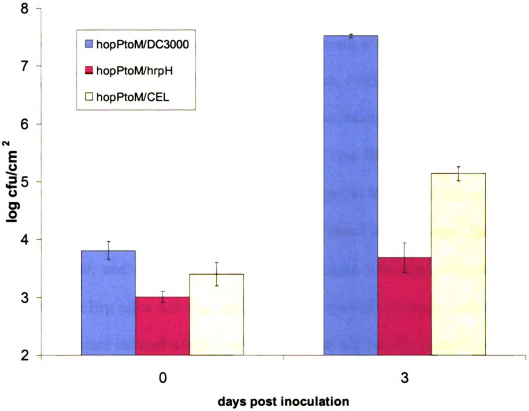


Figure 2-11: The multiplication of the ΔCEL mutant is not complemented in uninduced *hopPtoM* transgenic plants. Bacteria were syringe-infiltrated into plants at 10^6 cfu/ml. Bacterial growth was monitored after three days. Each bar represents the mean titer of 12 leaf discs from three individual leaves. Error bars indicate standard deviation. hopPtoM represents *hopPtoM* transgenic plants, DC3000 represents *Pst* DC3000, hrpH represents the *hrpH* mutant, and CEL represents the ΔCEL mutant.

The ΔCEL mutant is impaired in suppressing cell wall-based defenses

As a successful pathogen of *Arabidopsis*, *Pst* DC3000 is capable of suppressing defense responses mounted by the host in response to bacterial attack. One type of defense elicited by non-pathogenic bacteria, such as *hrp* mutants, are extracellular cell wall-based defense including the formation of papillae. Papillae are characterized by highly localized depositions of callose, phenolic compounds, and hydroxyproline-rich glycoproteins into paramural deposits directly beneath sites of bacterial interaction with plant cells (Bestwick et.al, 1995; Brown and Bonas, 1995; Brown et al., 1998). Staining for callose, as a marker for papilla, has revealed that wildtype *Pst* DC3000 has the ability to suppress papilla deposition through the action of type III effector proteins.

To investigate if the ΔCEL mutant was impaired in suppressing extracellular host defenses, I analyzed callose deposition in Col *gl1* plants in response to *Pst* DC3000, the ΔCEL mutant, and the non-pathogenic *hrpA* mutant. The *hrpA* mutant is unable to assemble the Hrp pilus and does not secrete any type III effectors (Roine et al., 1997). The *hrpA* mutant induced a high number of callose deposits. In contrast, leaves infected with *Pst* DC3000 showed very low numbers of callose deposits, clearly demonstrating that the TTSS of *Pst* DC3000 is involved in the suppression of callose-associated cell wall modifications in *Arabidopsis*. The ΔCEL mutant induced a large number of callose deposits in wildtype leaves as compared to *Pst* DC3000. The number of callose deposits were about 50% of that caused by the *hrpA* mutant. Because HopPtoM and ShcM are sufficient to restore virulence to the ΔCEL mutant, I analyzed the callose response to the ΔCEL mutant containing *hopPtoM* and *shcM*, to see if these genes are sufficient to suppress callose deposition. I found that *hopPtoM* and *shcM* restored the ability of ΔCEL mutant to suppress callose accumulation to a level comparable to that of *Pst* DC3000 (Figure 2-12).

The ΔCEL mutant is compromised in eliciting callose formation in NahG and *eds5* plants

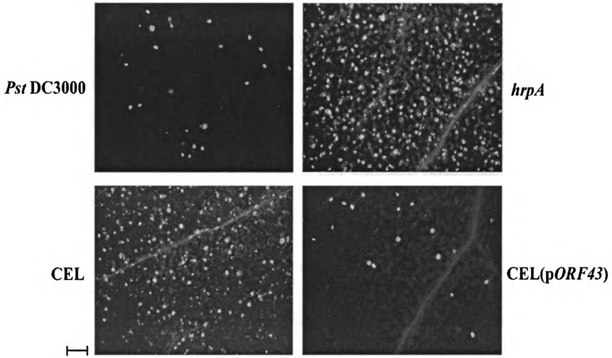
I also analyzed the callose response of *Pst* DC3000, ΔCEL mutant and *hrpA* mutant in NahG plants. NahG plants express the salicylate hydroxylase gene from *Pseudomonas putida*. Salicylate hydroxylase converts SA to catechol and these plants are therefore incapable of accumulating SA and are compromised in the induction of SA-dependent defenses (Delaney et al., 1994; Gaffney et al., 1993). The *hrpA* mutant induced similar numbers of callose deposits in NahG leaves and wildtype Col *gl1* leaves (Figure 2-13). This result also confirmed the earlier study which showed largely SA-independent regulation of *Arabidopsis* genes by the *Pst* DC3000 *hrpH* mutant. In contrast, the ΔCEL mutant failed to trigger a high level of callose deposits in NahG leaves. The number of callose deposits in NahG leaves infected with the ΔCEL mutant was very similar to those induced by *Pst* DC3000 in wildtype leaves. As expected, *Pst* DC3000 did not induce a significant number of callose deposits in NahG leaves. This result indicated that the ΔCEL mutant activated SA-dependent host cell wall-based defense. To further assess the SA-dependency of the ΔCEL mutant-activated cell wall defenses, we analyzed the ability of the ΔCEL mutant to induce callose deposition in *eds5* plants. The *eds5* mutant of *Arabidopsis*, accumulate little or no SA after pathogen inoculation and are hypersusceptible to pathogens (Nawrath and Metraux, 1999). Leaves of *eds5* plants inoculated with the ΔCEL mutant exhibited a similar number of callose deposits as that in

NahG leaves. The response of *eds5* plants to *Pst* DC3000 and *hrpA* mutant was similar to that of wildtype Col *gl1* i.e. very few callose deposits in response to *Pst* DC3000 and a high number of callose deposits to the *hrpA* mutant. These results confirmed that the ΔCEL mutant activates SA-dependent papilla formation in *Arabidopsis*.

NahG plants support growth of the ΔCEL mutant

The near absence of papillae formation in NahG leaves in response to the ΔCEL mutant suggested that the ΔCEL mutant might be able to multiply better in NahG plants compared to wildtype plants. *Arabidopsis* Col *gl1* and NahG plants were infiltrated with *Pst* DC3000, the *hrpH* mutant, and the ΔCEL mutant. Growth and symptom development were monitored over a period of 4 days. We found that the growth of the ΔCEL mutant and symptom development on the NahG plants were partially restored to those of *Pst* DC3000 on Col *gl1*. *Pst* DC3000 and the *hrpH* mutant grew slightly more in NahG plants (about 5-fold) compared to that in Col *gl1* plants. The ΔCEL mutant multiplied 200-fold more at day 3, and 400-fold more at day 4 in NahG plants than in Col *gl1* plants. These results further demonstrated that the effector genes in the CEL region interfere with SA-mediated defenses in the host.

A

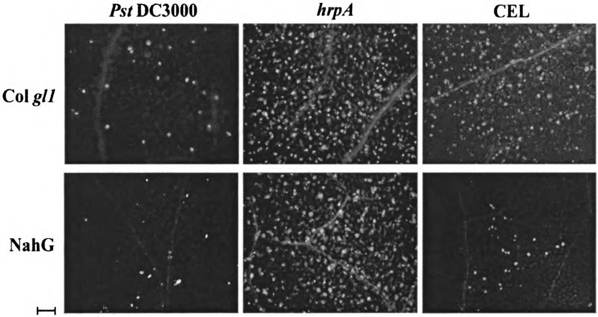


B

	Pst DC3000	*hrpA* mutant	ΔCEL mutant	ΔCEL(p*orf43*)
Col *gl1*	26 ± 8	438 ± 48	202 ± 36	23 ± 2

Figure 2-12: The ΔCEL mutant triggers the callose response in *Arabidopsis*. (A) Portions of wildtype *Arabidopsis* Col *gl1* leaves stained with Aniline blue for callose (white dots in these images) after inoculation with *Pst* DC3000, the *hrpA* mutant, the ΔCEL mutant, and the ΔCEL mutant containing p*ORF43*. The ability of the ΔCEL mutant to suppress callose deposition was restored by *ORF3* and *ORF4*. Scale bar, 100 µm. (B) Average number of callose depositions per field of view (0.9 mm^2) with standard deviation displayed as error.

A



B

	Pst DC3000	*hrpA* mutant	ΔCEL mutant
Col *gl1*	30 ± 7	491 ± 49	223 ± 29
NahG	29 ± 5	473 ± 32	31 ± 3

C

	Pst DC3000	ΔCEL mutant	*hrpA* mutant
Col *gl1*	31 ± 6	232 ± 37	353 ± 47
eds5	21 ± 7	26 ± 4	378 ± 45

Figure 2-13: The ΔCEL mutant is compromised in the ability to activate callose response in leaves of the SA-deficient NahG plant. (A) Portions of wild-type *Arabidopsis* Col *gl1* and NahG leaves stained with aniline blue for callose (white dots in these images) after inoculation with *Pst* DC3000, the *hrpA* mutant, and the ΔCEL mutant. Scale bar, 100 μm. (B) Average number of callose depositions per field of view (0.9 mm^2) with standard deviation displayed as error in Col *gl1* and NahG plants. (C) Average number of callose depositions per field of view (0.9 mm^2) with standard deviation displayed as error in Col *gl1* and *eds5* plants.

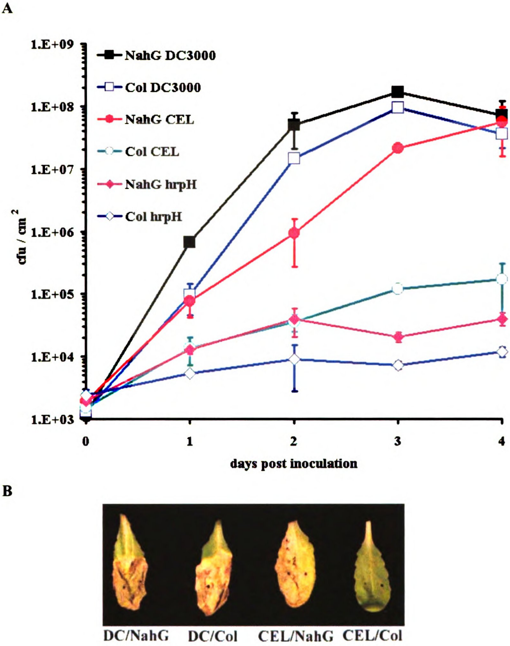


Figure 2-14: The ΔCEL mutant proliferates and causes symptoms more aggressively in NahG plants than in Col *gl1* plants. (A) Bacterial growth curves: Col *gl1* and NahG plants were vacuum-infiltrated with 10^6 cfu/ml of *Pst* DC3000, the ΔCEL,mutant and the *hrpH* mutant. Each time point reflects the mean of nine 0.5 cm^2 leaf discs. Error bars indicate standard deviations. (B) Disease symptoms in Col *gl1* and NahG leaves were scored 4 days after inoculation.

Discussion

During infection, *Pst* DC3000 utilizes the TTSS to inject at least 31 different effector proteins into the host. These effectors are believed to collectively promote the development of disease. Attempts to understand the function of individual effectors by gene inactivation in bacteria has proven to be challenging because of the typically weak contributions that individual effectors make to virulence. This is in sharp contrast to the strong loss-of-virulence phenotype observed when *hrp* genes are inactivated. Deletion of six ORFs in the CEL of *Pst* DC3000 created a mutant that was exceptional, in that it possessed a strong reduced-virulence phenotype in tomato (Alfano et al., 2000). This deletion however, did not affect its ability to cause HR in tobacco plants indicating that the TTSS was not aberrant in this mutant.

In this chapter I have demonstrated that the ΔCEL mutant also has a strong reduced-virulence phenotype in *Arabidopsis thaliana* and that this loss in virulence can be fully complemented by two genes from the deleted region, CEL *ORF3* (*hopPtoM*) and CEL *ORF4* (*shcM*). Moreover, we have found that HopPtoM is secreted in culture and is translocated into plant cells in a TTSS-dependent manner. *hopPtoM* is conserved and linked to the Hrp TTSS genes in divergent *P. syringae* pathovars, suggesting that this effector has played a significant function in the evolution of the species *P. syringae* as an aggressive pathogen of diverse plants.

In this study, we demonstrated that ShcM is a chaperone for HopPtoM. The demonstration of the requirement of ShcM for the efficient translocation and function of HopPtoM in the plant cell is consistent with the presence of customized chaperones in plant pathogenic bacteria and supports recent findings with *Pss* ShcA and *E. amylovora*

DspB/F (Gaudriault *et al.*, 2002; van Dijk *et al.*, 2002). Bacterial chaperones have been suggested to maintain their cognate effectors in a state that is competent for secretion, thereby conferring a competitive advantage over other non-chaperoned effectors for traffic through the TTSS.

Although HopPtoM can be translocated efficiently in the presence of ShcM, our results suggest that some HopPtoM may still be injected into the plant cell in the absence of its chaperone. This conclusion is supported by two observations. First, the ΔCEL mutant carrying the HopPtoM::AvrRpt2$_{80\text{-}255}$ fusion without simultaneous expression of ShcM is able to cause loss of turgidity in *Arabidopsis* leaves. Second, the ΔCEL mutant complemented with only *hopPtoM* shows partial restoration of bacterial growth even though symptom development is not augmented substantially. Both complementation and translocation analyses suggest that secretion and/or translocation of HopPtoM in the absence of its chaperone is probably an inefficient process. Consistent with our interpretation, an *E. amylovora dspB/F* mutant retains some virulence to pear seedlings, suggesting that some DspA/E still travels the TTSS in the absence of the chaperone (Gaudriault *et al.*, 2002). In addition, it has been shown that chaperones are not absolutely required for translocation of some effectors of animal pathogenic bacteria. For instance, deletion of the binding site for the chaperone SycE in the *Yersinia enterocolitica* YopE effector does not prevent its translocation into the eukaryotic cell owing to the presence of the N-terminal secretion signal that is present in all type III effectors (Boyd *et al.*, 2000).

Based on a study of the *Yersinia* type III effector YopE, and its chaperone, SycE, Boyd et al. (2000) suggested that chaperoned effectors may be secreted by the bacterium either more efficiently or at an early stage during the interaction with the eukaryotic cell (Boyd et al., 2000). HopPtoM is the only effector of the *P. syringae* CEL locus explored thus far whose virulence function is chaperone-dependent. Thus, it is possible that HopPtoM is translocated early into the plant cell. Being probably one of the first effectors to encounter the host cytoplasm, HopPtoM could be expected to target early host responses that may promote the establishment of an infection.

I demonstrated the ability of HopPtoM expressed *in planta* to complement the ΔCEL mutant to multiply and cause symptoms. This is especially noteworthy since such complementation has not been demonstrated for any *P. syringae* type III effectors. It is also worth noting that this complementation was observed at very low levels of the DEX. It is known that during infection, the pathogen injects minute quantities of effectors into the host. I speculate that the very low level of DEX that allows *hopPtoM* plants to support growth of the ΔCEL mutant must have resulted in a HopPtoM concentration that is comparable to that delivered by *Pst* DC3000. These results also suggest that HopPtoM can correctly fold into its functional conformation in the host cytoplasm without assistance from its cognate chaperone or other bacterial factors.

Immunoblotting analyses sometimes revealed the presence of a second band below that of HopPtoM in *hopPtoM* transgenic plants. This band was not present in uninduced transgenic plants or in Col *gl1* plants treated with DEX. Thus, this band is specific to expression of the transgene. This band could result from a non-specific degradation of HopPtoM. Alternatively, this band was generated from specific processing of the HopPtoM protein within the plant cytoplasm. Because this extra band was not observed consistently, it might be the result of non-specific protein degradation rather than specific host-dependent processing.

Several years ago, electron microscopic studies revealed that pepper and lettuce leaf cells deposit papilla on the inner side of the cell wall at the site of attempted infection by non-pathogenic bacteria including *hrp* mutants (Bestwick et al., 1995; Brown *et al.*, 1995, 1998). This highly localized cell wall thickening response could be a critical part of the poorly defined basal resistance that prevents multiplication of the vast majority of non-pathogenic bacteria to which plants are exposed in nature. Since the cell wall is the first host barrier that pathogens encounter, strengthening the cell by forming papilla would exert a local inhibitory effect on bacterial infection. Such fortification could theoretically reduce the penetration capacity of the type III pilus as well as reduce the movement of nutrients into the apoplastic space that would otherwise promote bacterial multiplication. In a previous study, another effector, AvrPto, was demonstrated to function in reducing the deposition of callose in the *Arabidopsis* cell walls during infection by the non-pathogenic *hrpH* mutant. It was also noticed that the transcriptional profile of plants expressing AvrPto showed repression of a large percentage of genes that are putatively predicted to be localized to the cell wall. It was therefore suggested that AvrPto is involved in overcoming the cell wall-based defense of the host (Hauck et al., 2003).

I tested the ability of the ΔCEL mutant to suppress the deposition of callose in the host cell wall. I found that unlike the wildtype *Pst* DC3000 bacteria, the ΔCEL mutant was severely compromised in suppressing callose deposition in Col *gl1* plants.

Furthermore, HopPtoM and ShcM were able to completely restore the papilla-suppressing ability of the ΔCEL mutant.

We have therefore identified a second type III effector in *Pst* DC3000 that thwarts plant cell wall-based defense. The role of HopPtoM in the suppression of one of the first lines of host defense further strengthens the concept that this effector is one of the first type III effectors to be translocated into the host during infection.

I also showed that while the *hrpA* mutant elicited callose deposition in a SA-independent manner, the ΔCEL mutant activated the cell wall response in a SA-dependent manner. The elicitation of callose response by the ΔCEL mutant was not as dramatic as the non-pathogenic *hrp* mutant, suggesting that effectors (e.g. AvrPto) other than HopPtoM function in the ΔCEL mutant to suppress callose deposition. Specifically, the ΔCEL mutant does not activate high levels of papilla response in NahG or *eds5* plants. In contrast, the non-pathogenic *hrpA* mutant triggered callose deposition in wildtype *Arabidopsis*, NahG, and *eds5* leaves. Thus, there appears to be two pathways leading to callose response. While AvrPto seems to suppress the SA-independent callose response, HopPtoM inactivates the SA-independent pathway. One of the major inducers of the SA-independent pathway seems to be the flagellin protein that is the major constituent of the flagella (Underwood and He, unpublished data).

A previous study had also reported a SA-dependent reduction in the amount of callose deposits formed around the fungal haustoria during infection of *Arabidopsis thaliana* by *Pernospora parasitica.* Callose encasements formed in response to fungal penetration have been suggested to function as defense structures that decrease the efficiency of the haustorium to obtain nutrients from host cells (Allen and Friend, 1983).

NahG plants, which permit higher growth of the pathogen, had a smaller number of haustoria surrounded by thick callose encasements, and in most haustoria callose was limited to thin collars around haustorial necks (Donofrio and Delaney 2001).

We have demonstrated that the ΔCEL mutant can multiply in NahG plants to levels close to those achieved by the wildtype *Pst* DC3000. This result, coupled with the papilla suppression data, supports a role for papilla to function as a defense structure that reduces the efficiency of bacterial multiplication in the apoplast perhaps by affecting type III secretion and/or nutrient movement across the host cell wall.

At this point, we do not know which bacterial factors trigger the SA-dependent papilla deposition. However, this factor must not be present in the *hrp* mutant, therefore must either be the components of type III secretion system or effectors injected into the host cell (Figure 2-15).

Overall, this study has led to the identification of an effector-chaperone system in *Pst* DC3000 that is a key player in pathogenesis. We show here that HopPtoM contributes to *Pst* DC3000 virulence by suppression of SA-dependent host cell wall-based immunity.

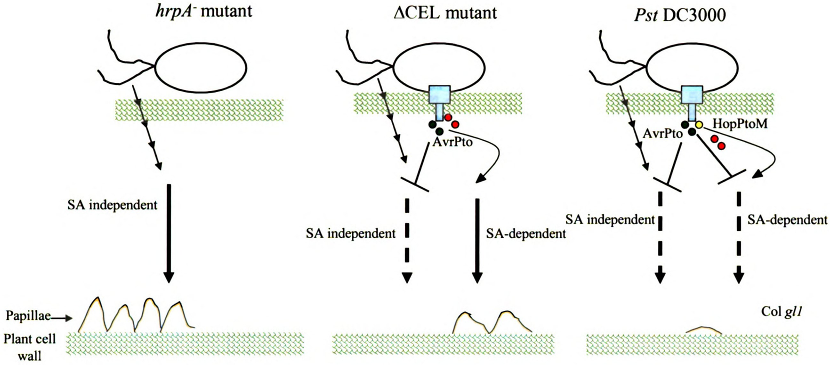


Figure 2-15: Schematic representation of the pathways that trigger callose deposition in *Arabidopsis* plants in response to the wildtype *Pst* DC3000, the ΔCEL mutant and the non-pathogenic *hrpA* mutant. This study provides evidence that suggests the existence of a SA-dependent and a SA-independent pathway that contributes to the expression of this form of extracellular cell wall-based defense.

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Chapter 3

Homology-based identification of type III effectors in *Pseudomonas syringae* pv. *tomato* DC3000 and characterization of transgenic *Arabidopsis thaliana* plants expressing *avrPphE*$_{Pto}$.

Abstract

Pseudomonas syringae pv. *tomato* strain DC3000 (*Pst* DC3000) is a virulent pathogen of *Arabidopsis thaliana* and tomato. It utilizes the type III secretion system (TTSS) to deliver into host cells, effector proteins that function to promote pathogenesis. These effector proteins were initially identified as mediators of gene-for-gene resistance responses and thereby named Avr (avirulence) proteins. Increasing evidence supports dual functions for Avr proteins as recognition determinants in resistant hosts and virulence determinants in susceptible hosts. Screening a *Pst* DC3000 cosmid library using ten known *P. syringae avr* genes as probes, led to the identification of three new effector genes. These are *avrPtoB*, $avrPphE_{Pto}$, and $avrPpiB_{Pto}$ and are homologous to *virPphA* and *avrPphE* from *P. syringae* pv. *phaseolicola*, and *avrPpiB* from *P. syringae* pv. *pisi*, respectively. All three genes encode bona fide effectors that are translocated in a type III dependent manner. Insertional inactivation of *avrPtoB* caused a dramatic loss of virulence on tomato but not on *Arabidopsis*. The lack of $avrPpiB_{Pto}$ or $avrPphE_{Pto}$ or both, did not result in a loss of virulence on *Arabidopsis* or tomato. However, expression of $avrPphE_{Pto}$ in *Arabidopsis* caused development of symptoms that closely resembled those caused by *Pst* DC3000 during infection. Microarray analysis revealed that transgenic expression of $avrPphE_{Pto}$ in *Arabidopsis* was capable of regulating host genes in a manner that closely resembled that of the *Pst* DC3000 TTSS. These transgenic plants allowed the non-pathogenic *hrpH* mutant to grow like *Pst* DC3000, supporting the role for $avrPphE_{Pto}$ in promoting pathogenesis.

Introduction

Pseudomonas syringae infects numerous plant species and can be classified into more than 40 pathovars on the basis of host specificity (Gardan *et al.*, 1999). *Pst* DC3000 is a virulent pathogen of both tomato and the model plant *Arabidopsis thaliana.* Like most plant pathogenic bacteria, *Pst* DC3000 is a noninvasive, extracellular pathogen that colonizes the host intercellular space outside the plant cell wall. During infection in *Arabidopsis*, the pathogen multiplies vigorously for two days before the onset of disease symptoms, which is characterized by water-soaking in the apoplast, followed by tissue necrosis and chlorosis (Whalen *et al.*, 1991; Katagiri *et al.*, 2002). The TTSS is encoded by *hrp* (for hypersensitive reaction and pathogenicity) and *hrc* (hrp gene conserved) genes (Van Gijsegem et al., 1993; Bonas, 1994; Alfano and Collmer, 1997; Lindgren, 1997; He, 1998; Hutcheson, 1999; Mudgett and Staskawicz, 1998). The TTSS is crucial to pathogenesis because *hrp* mutants (e.g., *hrpS* and *hrcC* [formerly *hrpH*] mutants) of *Pst* DC3000 are unable to multiply or cause disease in susceptible hosts (Yuan and He, 1996; Roine et al., 1997). This system, which is also utilized by pathogens of mammalian hosts , encodes a protein secretion machinery that is believed to translocate proteins from the bacteria directly into the eukaryotic host cell. *Pst* DC3000 secretes and translocates numerous effector proteins into the plant host cell, which collectively function to modulate host cellular processes and cause disease development. Although the primary function of type III effectors is to promote plant susceptibility, in the event that the pathogen attempts to infect a resistant cultivar or a non-host plant, some of these effectors may be recognized by the corresponding plant disease resistance proteins and trigger the hypersensitive response (HR) (Keen, 1990). HR is a defense-associated programmed cell death at the site of attempted infection. In such situations, these virulence effector proteins have been named avirulence (Avr) proteins (Leach and White, 1996; Collmer et al., 2001). In fact, most of the *avr* genes known so far were identified by screening for altered host response to transformants of a virulent race of *P. syringae* carrying a genomic library of an avirulent strain (Staskawicz et. al., 1984; Ronald et al., 1992; Lorang and Keen, 1995). The presence of an *avr* gene would result in HR instead of pathogenesis. Although *avr* genes were traditionally considered to be negative determinants of host specificity at the race-cultivar level, there is evidence that many of these *avr* genes actually contribute to the virulence of the pathogen in susceptible hosts. Among these are *avrBs2* of *Xanthomonas campestris* pv. *vesicatoria*, *pthA* of *X. citri*, *avrRpm1* from *P. syringae* pv. *maculicola*, *avrA* and *avrE* from *P. syringae* pv. *tomato* strain PT23, *avrBs6* from *X. campestris* pv. *maculicola* and *avrXa7* from *X. oryzae* pv. *oryzae* (Kearney and Staskawicz, 1990; Swarup et al., 1991; Swarup et al., 1992; Dangl, 1994; Lorang et al., 1994; Ritter and Dangl, 1995; Yang et al., 1996; Choi, 1993).

Several effectors have also been identified based on approaches that do not depend on plant reactions. Surveys of secreted proteins found in culture led to the identification of HrpA, HrpZ and HrpW (Yuan and He, 1996). In strains where the pathogenicity island (PAI) has been sequenced (Alfano et al., 2000), some putative effectors have been identified by their inclusion in the Conserved Effector Locus (CEL) or Exchangeable Effector Locus (EEL) associated with the *hrp* PAI (Badel et al., 2002). Potential effectors have also been identified either by their location downstream of a putative *hrpL*-dependent promoter or by the presence of a potential type III secretion signal (Fouts et al., 1999; Petnicki-Ocwieja et al., 2002; Zwiesler-Vollick et al., 2002).

Another group used a modified *in vivo* expression technology (IVET) to identify genes from the plant pathogen that are induced upon infection of *Arabidopsis thaliana* (Boch et al., 2002). Type III effectors have also been predicted based on a genetic screen that identified effectors based on their ability to be translocated into the host (Guttman et al., 2002). Combined, these studies, estimated *Pst* DC3000 to have at least 31 type III effectors.

Despite the identification of a number of new effectors, demonstration of a virulence function/contribution of single effectors remains a major challenge in the field. While *P. syringae* mutants blocked in the *hrp* secretion pathway are deficient in their interactions with plants, phenotypes of mutants deficient in individual effector genes are too subtle to be observed by traditional methods of virulence assessment such as determining bacterial populations isolated from infected plant tissues (Kjemtrup et al. 2000). The lack of an effect on virulence has been attributed primarily to functional redundancy amongst effectors harbored in a single bacterium.

At the time this study was initiated, *Pst* DC3000 was known to harbor only two type III effector genes – *avrE* and *avrPto*. To identify unknown effector genes in *Pst* DC3000, I screened for the presence of orthologs of known *avr* genes from various *P. syringae* pathovars. Three type III effectors were identified and were demonstrated to be translocated by the *hrp* secretion system. To understand the virulence contribution of effectors, I expressed *avrPphE*$_{Pto}$ in susceptible *Arabidopsis* using a dexamethasone-inducible promoter (Aoyoma and Chua, 1997). *avrPphE*$_{Pto}$ transgenic *Arabidopsis* exhibited symptoms that mimic those caused by wildtype *Pst* DC3000 during infection. Global host gene profiling analysis showed that AvrPphE$_{\mathrm{Pto}}$ modulated host gene expression in a fashion similar to that caused by the *Pst* DC3000 TTSS. The *avrPphE$_{Pto}$* transgenic plants also supported significant growth of the non-pathogenic *hrpH* mutant. These results strongly support that AvrPphE$_{Pto}$ contributes to *Pst* DC3000 virulence.

Materials and Methods

Bacterial strains and media

Bacterial cultures of *Escherichia coli* (*E. coli*) were grown in low salt (5g/L NaCl) Luria Bertani (LB) medium (Sambrook et al. 1989) at 37°C. *P. syringae* cultures were grown in low salt LB or *hrp*-inducing minimal medium (Huynh et al., 1989) at 30°C or 20°C. Antibiotics were used at the following concentrations unless otherwise specified – rifampicin 100mg/L, kanamycin 50 mg/L, ampicillin 200mg/L, tetracycline 10mg/L, spectinomycin 50mg/L.

Recombinant DNA techniques

DNA manipulations and polymerase chain reaction (PCR) were performed using standard protocols (Sambrook et al., 1989). Oligonucleotide primers for sequencing or PCR were synthesized at Integrated DNA Technology (Coralville, IA). PCR was performed using HIFI DNA polymerase (Stratagene, Madison, WI). DNA sequencing was done at the Michigan State University Genomic Technology Support Facility with Automated DNA sequencers, model 373A (Applied Biosystems, Foster City, CA). DNA sequences were analyzed with the Assemblylign®, MacVector®, and the Sequence Manipulation Suite (http://www.bioinformatics.org/sms/index.html). Database searches were performed using gapped BLASTN, BLASTP, and BLASTX (Altschul et al., 1997) (http://www.ncbi.nlm.nih.gov/BLAST/).

DNA gel blots

Pst DC300 genomic DNA was extracted (Chen and Kuo, 1993) and digested overnight at 37°C with appropriate restriction enzymes (New England Biolabs, Beverly, MA). The digested DNA was electrophoretically separated on a 0.7 % agarose gel. The gel was treated with 0.25 M HCl for 5 min and rinsed with distilled water. DNA was transferred onto nylon membranes (HybondN; Amersham Pharmacia Biotech #RPN303B) in 0.4M sodium hydroxide (Sambrook *et al.*, 1989). Approximately 100 ng of probe DNA was labeled with ^{32}P ATP and purified using BIORAD columns (Cat #732-6223) according to the manufacturer's instructions. Membranes were hybridized overnight at 65°C in hybridization buffer (6X SSC, 1% SDS, 5X Denhardt's solution, 0.1mg/ml ssDNA, 0.05M Tris pH 8.0, 0.0125M EDTA). Membranes were washed to a stringency of 0.1X SSC (20 mins; 65°C) and exposed to X-ray film (Kodak Scientific Imaging Film X-OMAT AR, #1651454).

Cosmid library screening for detection of effector orthologs

A *Pst* DC3000 cosmid library was plated on LB plates containing tetracycline. After overnight growth at 37°C, plates were chilled for 30 min at 4°C. Colonies were lifted onto nylon membranes (MagnaLift; Micron Separation Inc., #NL4HY08250) and both the plates and the membrane were marked with ink for orientation. The membranes were treated twice with 0.5N sodium hydroxide for 2 min each, followed by two treatments with 1M Tris for 5 min each. Membranes were finally treated with 1.5M sodium chloride, 0.5 M Tris for 5 min and then air dried. The membranes were UV-crosslinked in UV Stratalinker 1800 (Stratagene, Madison, WI). Bacterial debris sticking to the membranes were removed by washing the membranes with hybridization buffer, prior to hybridization. Hybridization was performed as described in the previous section. Positive colonies for each probe were identified by comparing the films and plates from which they had been lifted. These colonies were subjected to a second hybridization screen.

The following primer pairs were used in PCR reactions to amplify full-length *avr* genes for use as probes :

avrPma1: sense 5'CCGCTCGAGATGGGCTGTGTATCGAGCACTTCA3'
antisense 5'GGACTAGTTTAAAAGTCATCTTCTGAGTCAGAC3'
avrPphB : sense 5'CCGCTCGAGATGAAAATAGGTACGCAGGCCACC3'
antisense 5'GGACTAGTTTACGAAACTCTAAACTCGTTTACGCA3'
avrRpt2: sense 5'CCGCTCGAGATGAAAATTGCTCCAGTTGCCATAAAT3'
antisense 5'GGACTAGTTTAGCGGTAGAGCATTGCGTG3'
avrPphE: sense 5'CCGCTCGAGATGAGAATTCACAGTGCTGGTCACAGC3'
antisense 5'GGACTAGTTTATCTTCGTGGAGGCATGCCTTT3'
avrPpiB: sense 5'CCGCTCGAGATGCACGCAAATCCTTTAAGCTCTTTCAAC3'
antisense 5'GGACTAGTTTAGTCGCCTAGGAAATTATTTAGTTC3'
ORF4: sense 5'AGCATATGGGTATCAACAGAGCAGGGTCA3'
antisense 5'CCACTAGTTCACTCCACACGCTGAATAACCATGCTCTC3'
virPphA: sense 5'CTATATGCCGGGTATCAACGGAGCAGGACCA3'

antisense 5’CCACTAGTTCATGGAACAATTTTAAAAGCGTACTT3’

avrPpiA: sense 5’CCGCTCGAGATGGGCTGTGTATCGAGCACTTCAAGA3’

antisense 5’GGACTAGTTTAAAAGTCATCTTCTGAGTCAGACTG3’

avrPphF

ORF1: sense 5’CCGCTCGAGATGAAGAATTCGTTCGACCGCTTAATCGAT3’

antisense 5’GGACTAGTGACCGAACTCTCAGACAAATCCAA3’

avrPphF

ORF2: sense 5’CCGCTCGAGATGGGTAATATCTGCAATTGCGGGGGCGTC3’

antisense 5’GGACTAGTATCGAGGATATTGACCGGTACTTT3’

Cloning and sequencing full-length effector genes

Cosmid DNA was isolated from the positive colonies by a modified alkaline lysis method (Sambrook *et al.*, 1989), digested with different restriction enzymes, and separated on 0.7% agarose gels. Southern hybridization was used to identify the single, smallest fragments that hybridized to their respective probes. Appropriate DNA bands were excised from agarose gels and cloned into pBS SK (Invitrogen, Carlsbad, CA). These constructs were sequenced with the M13 forward and reverse primers. The DNA sequence obtained was identified by BLASTN. Using the initial sequence, further primers were designed to obtain the complete sequence of the ORF.

Type III translocation analysis using an AvrRpt2 fusion

The truncated *avrRpt2*$_{80\text{-}255}$ gene, which encodes type III secretion/translocation-incompetent, but biologically active AvrRpt2 (Mudgett *et al.*, 2000), was cloned into the

XbaI-HindIII sites of pUCP19 (Schweizer, 1991). Candidate type III effector genes were amplified using appropriate primers (see below) and cloned into the *Eco*RI- *Xba*I sites in pUCP19::*avrRpt2*$_{80\text{-}255}$ to create in-frame fusions (5'-effector gene-*avrRpt2*$_{80\text{-}255}$-3'). All gene fusions contained the full-length putative effector genes. The recombinant plasmids were then electroporated into *Pst* DC3000. The transformants were grown in LB to OD_{600} = 0.6. Bacteria were collected by centrifugation and resuspended in sterile water to an OD_{600} = 0.2. The bacterial suspensions were infiltrated into leaves of six-week-old *RPS2*$^+$ *Arabidopsis* accession Columbia *glaborous 1* (Col *gl1)* or *rps2* mutant (Kunkel et al., 1993) plants. Tissue collapse was monitored over a 48 hour period at room temperature.

The following primers were used for making *avrRpt2* fusions:

avrPtoB: sense 5'GCGAATTCGGGCATGGAAAAATCCTCTTC3'

antisense 5'GCTCTAGAGCTGTCAGCGCGGTCAGGGGA3'

avrPpiB$_{Pto}$: sense 5'GCG GAT CCG GCT CGA ATC GAT CTT CCC AA3'

antisense 5'GCT CTA GAG TCG CCT AGG AAA TTA TTT AGT TC3'

avrPphE$_{Pto}$: sense 5' GCG AAT TCG CAA CTG CTG GAG GTG AGA GC3'

antisense 5'GCT CTA GAG ACA TCT CGT CTC GCC AAG CC3'

Transposon mutagenesis and marker exchange

Cosmid clones containing the full-length ORFs of the identified genes and their flanking sequences were cloned into the broad host range vector pRK415 (Keen et al., 1988) and transformed into *E. coli* DH5α. Two transposons were used for generating the mutants – mini-*Tn5 Sp* and mini-*Tn5 Km* (Lorenzo et al., 1990). *E. coli* strains containing the target cosmids and the transposon were grown to log phase in LB medium. Bacteria were then centrifuged and the pellet resuspended in 10mM $MgCl_2$. The donor and recipient strains were mixed and plated onto LB plates and incubated overnight at 30°C. Colonies were scraped off the LB plate, resuspended in sterile water and plated onto selective plates containing nalidixic acid (10mg/L), tetracycline and spectinomycin or kanamycin. Plasmid DNA was extracted from the transconjugants and the presence of transposon insertions was confirmed by restriction enzyme digestion and Southern blotting. Plasmids were then introduced into *Pst* DC3000 by electroporation. The inactivated copy of the gene was introduced into the *Pst* DC3000 genomic DNA by allelic exchange. Genomic DNA was isolated from the mutant strain and was used to confirm a successful marker exchange by Southern hybridization.

Plant growth and bacteria enumeration

Arabidopsis Col *gl1* and transgenic plants were grown in soil or in growth chambers with a day/night cycle of 12h/12h, a light intensity of 100 μE, and a constant temperature of 20 °C. Four- to five-week-old plants were used for experiments. Tomato (*Lycopersicon esculentum*) cv Castlemart was used for all tomato experiments. Tomato seedlings were grown in Jiffy peat pots (Hummert International, Earth City, MO) in a growth chamber maintained under 17 h of light (200 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 28°C and 7 h of dark at 18°C. Six- to eight-week-old plants were used for experiments.

Bacteria were grown in low-salt LB to the mid-log phase at 30°C. Bacterial cultures were centrifuged to recover bacteria, which were resuspended in sterile water to the required level of inoculum (see Results section). Fully expanded leaves were either vacuum-infiltrated or syringe-infiltrated with bacterial suspensions, and bacteria were enumerated as described by Katagiri *et al.* (2002). The mean values of the bacterial populations were plotted with the standard deviation displayed as error. Plants analyzed in Figure 3-9 and 3-11 were sprayed daily with 30 μM and 0.1 μM dexamethasone (DEX) solution respectively, to induce the transgene. Bacterial suspensions were infiltrated into leaves one day after the first DEX treatment

Generation of transgenic plants

Pst DC3000 genomic DNA was extracted as described by Chen and Kuo (1993). PCR was used to amplify *avrPphE*$_{Pto}$ using HIFI Taq polymerase and the following primers :

avrPphE$_{Pto}$ sense primer 5'-GCACTAGTACCATGAAAATACATAACGCTGGC-3'

avrPphE$_{Pto}$ antisense primer 5'-GGACTAGTTTATCTTCGTGGAGGCATGCCTTT-3'

The *avrPphE*$_{Pto}$ gene was cloned into the pTA7002 vector (Aoyama and Chua, 1997; McNellis *et al.*, 1998). pTA7002 allows for inducible expression of transgenes after application of the animal glucocorticoid hormone, dexamethasone (DEX). Electroporation was used to transform *Agrobacterium tumefaciens* strain GV3850 with the recombinant plasmid (Keen et al., 1990). Four pots of *Arabidopsis thaliana* Col *gl1* plants were transformed with *A. tumefaciens* carrying pTA7002-*avrPphE*$_{Pto}$ using the floral dip method (Clough and Bent, 1998). Seeds collected from each pot were kept separate to ensure that independently transformed lines could be isolated. T1 seeds were vapor-sterilized in a dessicator for 4 hours with 80ml of bleach mixed with 3ml of concentrated HCl. Seeds were placed on Murashige-Skoog (MS) (Gibco BRL, #11117-074) plates supplemented with 1x vitamins (SIGMA, #M7150) and 40 units/ml hygromycin B (hyg) (Calbiochem Cat # 400051), kept at 4°C for three days, and then moved to growth chambers (see previous section).Transformants were selected on the basis of hygromycin resistance.

Production of antibody

The *avrPphE$_{Pto}$* gene was amplified by PCR from *Pst* DC3000 genomic DNA using the following primers :

sense primer 5'-GCCATATGAAAATACATAACGCTGGCC -3'

antisense primer 5'- GCCTCGAGGACATCTCGTCTCGCCAAGCC -3'.

The gene was cloned into the pET28(a) vector (Invitrogen, Carlsbad, CA) and transformed into *E. coli* BL21(DE3) cells by electroporation (Sambrook *et al.*, 1989). Protein was induced by addition of 1mM IPTG to a mid-log culture and incubated for 4 hours at 37°C. AvrPphE$_{Pto}$ protein was extracted from *E. coli* cells using a standard protocol (Qiagen, Valencia, CA) and purified using the Ni-NTA column. Purified protein was analyzed by SDS-PAGE and used to raise antibodies in rabbit at Cocalico Biologicals, Inc., Reamstown, PA. Pre and post immune sera were obtained and checked for the ability to recognize the antigen using immunoblot analysis (see below).

Immunoblotting

Arabidopsis Col *gl1* and *avrPphE$_{Pto}$* plants were sprayed with the appropriate concentration of DEX and maintained under high humidity for 24 hours. Leaf disks of 2cm^2 tissue were collected using a #5 cork borer (Boekel, #1601BD 1-10), homogenized in 100μl 2X treatment buffer (0.125M Tris Cl pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol)and denatured at 100°C for 10 mins. Protein samples were separated on a 10% SDS-polyacrylamide gel (Sambrook *et al.*, 1989) and transferred onto Immobilin-P membrane (Millipore, #IPVH00010) using a semi-dry transfer apparatus (SEMI PHOR, Hoefer Scientific Instruments). AvrPphE$_{Pto}$ was visualized using the AvrPphE$_{Pto}$ antiserum, followed by anti-rabbit alkaline phosphatase conjugate and colour reaction using SIGMA FAST (B5655) .

DEX-induction of transgene expression

A 30mM stock of dexamethasone (Sigma Aldrich Cat # D1756) (DEX) was made in 100% ethanol. This stock solution was diluted in water to required concentrations (see Results section) and sprayed onto the plants to induce the transgene.

Microarray analysis

Four- to five-week-old *Arabidopsis thaliana* Col *gl1* leaves were vacuum-infiltrated with bacterial suspensions containing 1x10^6 cfu/ml bacteria (Katagiri et al., 2002). Infiltrated leaves were collected at 12, 24, and 36 h post-inoculation, before the appearance of water-soaking symptoms (at about 48 h) and necrosis and chlorosis (at about 72 h). Total RNA was isolated from each leaf sample and equal amounts of RNA from different time points were pooled for DNA microarray analysis according to the protocol described previously (Schaffer et al., 2001). The microarray experiments were performed using a 864-element subarray enriched for *Pst* DC3000-regulated genes (R. Thilmony and S. Y. He, unpublished). The subarray was derived from the Arabidopsis Functional Genomic Consortium's microarray slides, each containing about 7,200 unique genes (Schaffer et al., 2001). For microarray analysis of transgenic plants, five to six week-old *avrPphE*$_{Pto}$ transgenic plants and Col *gl1* plants were sprayed with appropriate concentration of DEX (see Results section) and kept under humidity domes. Tissue was collected at 24 h post induction and total RNA was isolated from each sample for microarray analysis.

Supporting Information Table B-1 and B-2 (Appendix B) lists genes that are regulated in a TTSS-dependent manner in at least two of the three biological replicates for the DC3000/*hrpS* mutant comparison in Col *gl1* plants and in both biological replicates for the *avrPphE*$_{Pto}$/Col *gl1* comparison. Gene clustering analysis was performed using the Cluster and TreeView programs (Eisen et al., 1998).

Results

Identification of putative effectors genes in *Pst* DC3000 that are homologous to known *avr* genes

At the time this study was initiated, there were only two effector genes known to be present in *Pst* DC3000 – *avrE* and *avrPto.* To discover additional effector genes in *Pst* DC3000, ten known *avr* genes from four different pathovars were chosen (Table 3-1). The genes were amplified from genomic DNA by PCR using gene-specific primers (see Materials and Methods). *Pst* DC3000 genomic DNA was digested with restriction enzymes, electrophoretically separated, transferred to nylon membranes and hybridized with the amplified *avr* gene probes. This analysis revealed the presence of three genes in *Pst* DC3000 that were putative orthologs of *avrPphE* (Stevens et al., 1998; Mansfield et al., 1994) and *virPphA* (Jackson et al., 1999) from *P. s.* pv. *phaseolicola* and *avrPpiB* (Arnold et al., 2001; Cournoyer et al., 1995) from *P. s.* pv. *pisi* respectively. These genes were named *avrPphE*$_{Pto}$, *avrPtoB,* and *avrPpiB*$_{Pto}$.

Table 3-1: List of *avr* genes used for identification of orthologs in *Pst* DC3000

Avirulence/virulence gene	***Pseudomodnas syringae* pathovar**
avrPma1	*maculicola*
avrPph3	*phaseolicola*
avrPphE	*phaseolicola*
avrPphF-ORF1	*phaseolicola*
avrPphF-ORF2	*phaseolicola*
virPphA	*phaseolicola*
ORF4	*phaseolicola*
avrPpiA	*pisi*
avrPpiB	*pisi*
avrRpt2	*tomato*

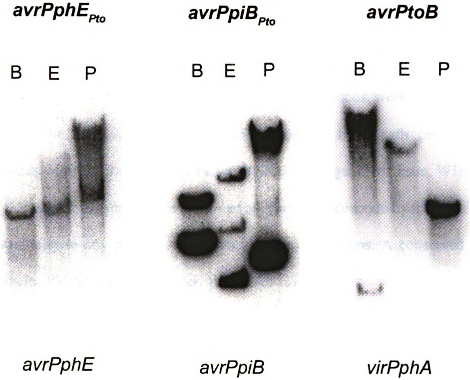

Figure 3-1: Southern blot analysis of genomic DNA from *Pst* DC3000 for the presence of putative orthologs of known *avr* genes. Ten μg of genomic DNA was digested with restriction enzymes (B: *BamH*I; E: *EcoR*I; P: *Pst*I) and hybridized with ^{32}P-labelled probe of the *avr* gene listed below each set of lanes. The ortholog of *avrPpiB* has two copies in *Pst* DC3000, while orthologs of *avrPphE* and *virPphA* are single-copy genes. The name designated to each ortholog is indicated on top.

Isolation of full-length clones of the three putative effector genes

In order to isolate full-length genes of the orthologs identified in *Pst* DC3000 by Southern hybridization, *avrPphE, avrPpiB* and *virPphA* were used as probes to screen a genomic library of *Pst* DC3000 constructed in the cosmid pCPP47 (Bauer and Collmer, 1997) using colony hybridization. Three independent cosmids were isolated for *avrPtoB*, fourteen cosmids for *avrPpiB$_{Pto}$*, and three cosmids for *avrPphE$_{Pto}$*. These cosmid clones were subjected to digestion with three different restriction enzymes and the single, smallest fragments that hybridized to their respective probes, were identified by Southern blotting. The identified fragment could contain the full length gene The respective fragments were subcloned into pBluescript SKII (Strategene, Madison, WI).

Sequencing analysis revealed that all three genes encode hydrophilic proteins with no similarity to any proteins of known biological functions. A conserved *hrp* box was found in the putative promoter region of *avrPtoB* and *avrPpiB$_{Pto}$,* implying the requirement of the *hrp* regulatory circuit for expression and secretion. *avrPphE$_{Pto}$* however, had a modified *hrp* box in its promoter region. The ORF of *avrPtoB* was 1,652 nt long and encoded a protein of 551 amino acids. AvrPtoB shares 58% similarity with VirPphA at the amino acid level. The *avrPpiB$_{Pto}$* ORF was 828 nt long and encoded a protein of 276 amino acids, identical to that of AvrPpiB. The *avrPphE$_{Pto}$* ORF was 1,156 nt long and shared 83% identity with *avrPphE* at the nucleotide level and 67% identity at the amino acid level. Recent analysis of the *Pst* DC3000 genome reveals that *avrPpiB$_{Pto}$* and *avrPphE$_{Pto}$* are located on one of the two plasmids, pDC3000A. A second copy of *avrPpiB$_{Pto}$* and *avrPtoB* are located on the single circular chromosome (Buell et al., 2003). The G+C contents of *avrPtoB* (59%), *avrPphE$_{Pto}$* (54%) and *avrPpiB$_{Pto}$* (39%) are lower than the G+C content of the *Pst* DC3000 genome (about 60%; Alfano *et al.*, 2000). No specific plant organelle-targeting sequences are present in these proteins. None of the identified effectors have any known functional domains or structural motifs.

Translocation analysis of identified orthologs

The presence of the *hrp* box motif in the promoter region of all three orthologs implies that they are possibly secreted and translocated into the host cytoplasm in a *hrp*-dependent fashion. To investigate this, we utilized a translocation assay that is based on the properties of another *avr* gene, *avrRpt2*, from *P. s.* pv. *tomato* JL1065. It has been established that AvrRpt2 has a modular structure and the N-terminally localized secretion signal can be physically separated from the C-terminal HR-inducing domain. When type III secretion/translocation-incompetent, but biologically active, AvrRpt2$_{80\text{-}255}$ (80-255 amino acids; C-terminal portion) is fused to a protein carrying a type III secretion signal, the fusion protein can be translocated into the plant cell and elicits a hypersensitive response (HR) in *Arabidopsis* plants carrying a functional copy of the corresponding resistance gene, *RPS2* (Mudgett et al., 2000; Guttman and Greenberg, 2001).

We fused full-length AvrPphE$_{\mathrm{Pto}}$, AvrPtoB, and AvrPpiB$_{\mathrm{Pto}}$ proteins to the HR inducing domain of AvrRpt2. These expression constructs were electroporated into *Pst* DC3000. The ability of *Pst* DC3000 carrying the recombinant fusions to elicit an HR in leaves of *RPS2* and *rps2 Arabidopsis* plants (ecotype Col-0) was examined. The negative control, wildtype *Pst* DC3000 carrying the empty vector, caused *RPS2*-independent, slow disease necrosis visible only after 24 h post-inoculation. *Pst* DC3000 expressing the wild-type AvrRpt2 elicited an *RPS2*-dependent HR at about 9 hours post-inoculation.

Arabidopsis plants exhibited a *RPS2*-dependent HR for all three effectors at about 14 to 16 hrs after inoculation. These results confirmed that all three identified orthologs were bona fide type III effectors that travel the *hrp* pathway and enter the host cell.

0/14	14/14	14/14	13/14	11/14	Col *gl1* (*RPS2*)
0/14	0/14	0/14	1/14	1/14	*rps2*

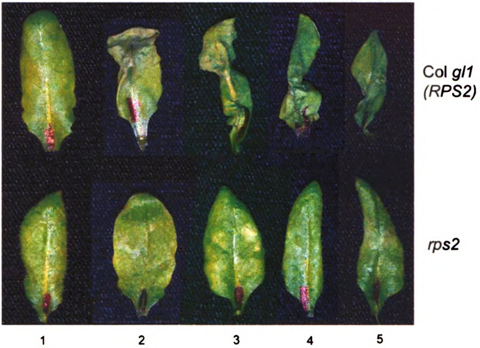

Figure 3-2: Type III translocation analysis of identified effectors in *Arabidopsis*. Full-length proteins were fused to a truncated AvrRpt2 protein (80-255 amino acids, AvrRpt2$_{80\text{-}255}$). Plasmids were introduced into *Pst* DC3000. *Arabidopsis* Col *gl1* (*RPS2+*) or *rps2* mutant leaves were infiltrated with bacterial suspensions at OD_{600}=0.2 and evaluated for HR elicitation. 1: DC3000, 2: DC3000 (pAVRRPT2), 3: DC3000 (pAvrPtoB::AvrRpt2$_{80\text{-}255}$), 4: DC3000 (pAvrPpiB$_{Pto}$::AvrRpt2$_{80\text{-}255}$), 5: DC3000(pAvrPphE$_{Pto}$::AvrRpt2$_{80\text{-}255}$). Col *gl1* leaves (2 - 5) showing HR collapse appear wrinkled. Top: Number of leaves showing HR/number of leaves infiltrated for Col *gl1* and *rps2* plants. Picture was taken 18 h after inoculation. Leaves representing the majority of each treatment are shown.

Generation of mutants by transposon mutagenesis

All three effector genes were cloned into the broad host range vector pRK415 (Keen et al., 1988) and subjected to transposon mutagenesis. *avrPtoB* and *avrPpiB*$_{Pto}$ were mutagenized using the mini-*Tn5 Sp* transposon while the mini-*Tn5 Km* (Lorenzo et al., 1990) was used to create insertion mutants of *avrPphE*$_{Pto}$. Transposons conferring resistance to different antibiotics were used in order to facilitate the generation of double mutants of the effector genes.

The insertion events were confirmed by restriction digestion and Southern blot analyses. The inactivated genes were then introduced into the *Pst* DC3000 genome by marker exchange. Genomic DNA isolated from the mutants was analyzed by Southern blotting to confirm successful marker exchange. Double mutants were generated by sequential marker exchanges with two effector genes and were also confirmed by Southern blot analysis.

Analysis of the mutants on *Arabidopsis* and tomato plants

To determine if the inactivation of one or more of these three genes affects *Pst* DC3000 virulence in *Arabidopsis*, mutant strains were infiltrated into *Arabidopsis* Col *gl1* and the plants were scored for both symptom development and bacterial multiplication for a period of 4 days. Using an inoculum of 10^5 cfu/ml, a small, but insignificant reduction in bacterial growth (about 1-fold) was observed for the *avrPtoB* mutant. The double mutant of *avrPtoB* and *avrPphE*$_{Pto}$ had a more significant growth reduction, about 10-fold, as compared to that of the wild-type strain. However, symptom development was not dramatically affected for either the single or the double mutant. The other effectors, AvrPpiB$_{\mathrm{Pto}}$ and AvrPphE$_{\mathrm{Pto}}$ did not have a demonstrable virulence function in this pathosystem, although a double mutant of *avrPpiB*$_{Pto}$ and *avrPphE*$_{Pto}$ did exhibit a slight reduction of about 1-2 fold in bacterial multiplication.

The virulence function of these three effectors was also assayed in tomato plants. Eight-week-old Castlemart II plants were vacuum infiltrated at 5 X 10^5 cfu/ml and disease symptoms and bacterial multiplication were scored 5 days post inoculation. While *avrPpiB*$_{Pto}$ and *avrPphE*$_{Pto}$ mutants showed no significant changes in either symptom development or bacterial multiplication, the *avrPtoB* mutant exhibited a significant reduction in virulence. Tomato plants infiltrated with the *avrPtoB* mutant showed reduced chlorosis with a near absence of necrotic specks as compared to plants infiltrated with *Pst* DC3000. There was a slight, further reduction in both chlorosis and necrosis in plants infiltrated with the *avrPtoB/avrPphE*$_{Pto}$ double mutant. The reduction in disease symptoms was also accompanied by a 50-fold reduction in bacterial multiplication in this host.

Although the *avrPtoB* mutant showed a reduction in virulence on tomato, I was unable to complement this mutant with a plasmid-borne copy of the gene. It is possible, that the observed phenotype may be an artifact of secondary mutations that might have occurred during allelic exchange.

To investigate the possibility of ecotype-specific virulence functions of the three effector, I also analyzed the response of seventeen *Arabidopsis* ecotypes to all single and double effector mutants. None of the ecotypes tested showed any significant alteration in symptom development for any of the mutants.

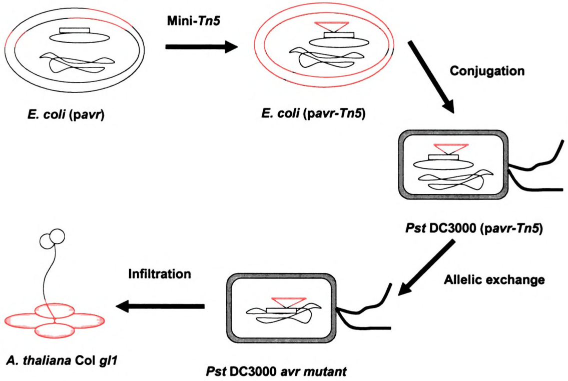

Figure 3-3: Schematic representation of the strategy employed to obtain insertion mutants of the identified *Pst* DC3000 effectors genes. *E. coli* strains carrying full-length clones of a given effector gene was mutagenized with mini-*Tn5 Sp* or mini-*Tn5 Km* which confers Sp and Km resistance respectively. The insertion event was confirmed by restriction analysis and Southern blotting. Plasmids were then introduced into *Pst* DC3000 by conjugation and the inactivated copy of the gene was introduced into the *Pst* DC3000 genomic DNA by allelic exchange. Genomic DNA from the mutant strain was used to confirm successful marker exchange by Southern hybridization.

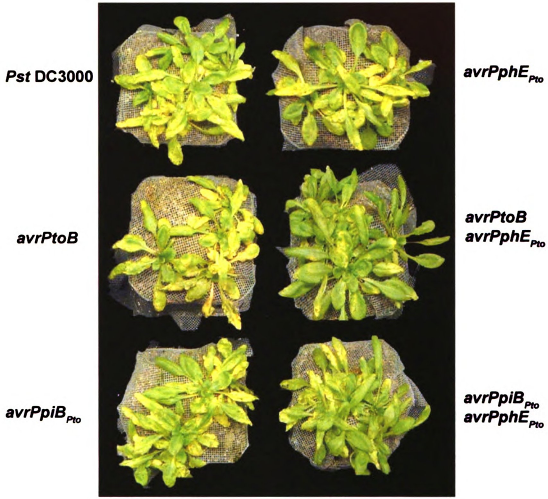

Figure 3-4: Symptoms of *Pst* DC3000 mutants in *A. thaliana* Col *gl1* plants. Bacteria were vacuum-infiltrated into Col *gl1* leaves at 10^5 cfu/ml. Pictures were taken four days after infection.

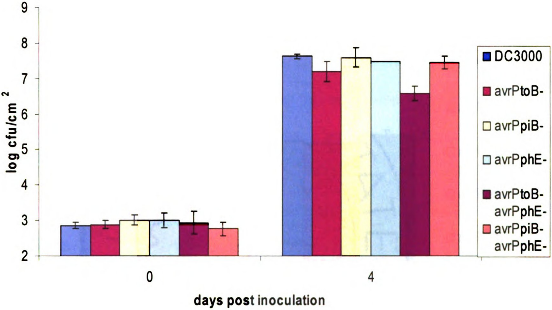

Figure 3-5: Bacterial proliferation in *A. thaliana* Col *gl1* plants. Bacteria were vacuum-infiltrated into Col *gl1* plants at 10^5 cfu/ml. Bacterial growth was monitored after four days. Each bar represents the mean titer of 12 leaf discs from three individual leaves. Error bars represent standard deviation. DC3000 represents *Pst* DC3000, avrPpiB represents $avrPpiB_{Pto}$, and avrPphE represents $avrPphE_{Pto}$.

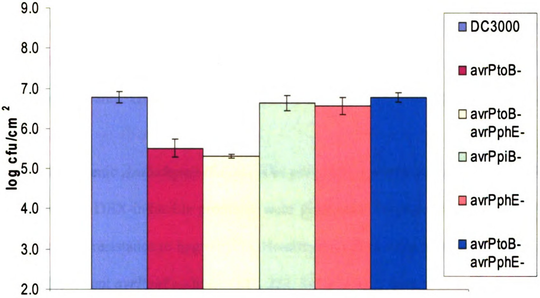

Figure 3-6: Bacterial proliferation in tomato Castlemart II plants. Bacteria were syringe-infiltrated into tomato plants at 5 X 10^5 cfu/ml. Bacterial growth was monitored after four days. Each bar represents the mean titer of 9 leaf discs from three individual leaves. Error bars represent standard deviation. DC3000 represents *Pst* DC3000, avrPpiB represents $avrPpiB_{Pto}$ and avrPphE represents $avrPphE_{Pto}$.

In planta expression of AvrPphE$_{Pto}$

Because of the presumed redundancy among type III effectors, inactivation of one or more genes often does not result in a measurable difference in the ability of the mutant strain to cause disease symptom development or reduce bacterial multiplication, as indicated in my work with *avrPpiB*$_{Pto}$ and *avrPphE*$_{Pto}$. This redundancy is probably caused by the presence of a large number of effector genes in a single strain. For example, analysis of the recently sequenced *Pst* DC3000 genome suggests the presence of at least 31 different effectors in *Pst* DC3000. In order to study the effect of a single effector, I have adopted a new approach that involves the expression of a single effector in *Arabidopsis* plants. Of the three genes that were identified, *avrPphE*$_{Pto}$ was chosen for further study.

Transgenic *Arabidopsis thaliana* Col *gl1* plants that express *avrPphE*$_{Pto}$ under the control of the DEX-inducible promoter were generated. Transgenic lines were selected based on their resistance to hygromycin. Homozygous lines were used for all experiments. Four independent *avrPphE*$_{Pto}$ lines, (141, 222, 342 and 422) were chosen for initial study. None of these lines showed any gross morphological or developmental abnormalities in comparison to wildtype Col *gl1* plants. All four lines were sprayed with 30µM DEX to induce the transgene. Leaf tissue samples collected 24 hours later were analyzed for protein expression by immunoblot analysis. Two of these lines, 141 and 222, expressed high levels of AvrPphE$_{Pto}$ upon induction, whereas lines 342 and 422 expressed lower levels of the protein (Figure 3-7).

Induction of the *avrPphE*$_{Pto}$ transgene led to the development of a distinct phenotype. When the *avrPphE*$_{Pto}$ plants were maintained under high humidity, they showed water-soaking 24 hours after DEX exposure. Chlorosis and necrotic spots started developing 36 to 48 hours post induction. The induced phenotype in these lines mirrored the phenotype of *Pst* DC3000 infection in *Arabidopsis*. There was a close correlation between the severity of the induced phenotype and the amount of protein produced upon induction. The lines that expressed higher amounts of AvrPphE$_{Pto}$, 141 and 222, developed extensive necrosis and chlorosis, rendering these lines unfit for further characterization using bacterial multiplication and gene expression profiling. The 342 and 422 lines showed low and moderate levels of AvrPphE$_{Pto}$ induction and developed relatively less chlorosis and necrosis. These two lines were used for further characterization. Protein expression was also analyzed at 24 hours post induction using different levels of DEX. Decreasing levels of the inducer was found to cause decreasing amounts of protein production in these lines and decreasing disease-like symptoms (data not shown).

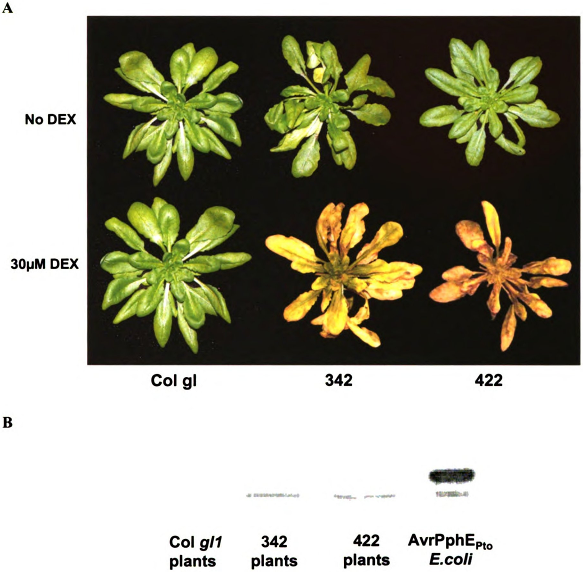

Figure 3-7: (A) Phenotype of *avrPphE*$_{Pto}$ transgenic plants, lines 342 and 422. Six-week-old plants were used. Uninduced plants, labeled "No DEX" looked similar to wildtype *A. thaliana* Col *gl1* plants. *avrPphE*$_{Pto}$ transgenic plants labeled "30 µM DEX" were sprayed with 30 µM DEX and kept under high humidity. Pictures were taken four days later. Induction of the *avrPphE*$_{Pto}$ transgene caused the development of chlorosis and necrosis.

(B) Western blot analysis of AvrPphE$_{Pto}$ expression in leaves of wild-type *A. thaliana* Col *gl1* plants and AvrPphE$_{Pto}$ transgenic plants 24 hours after spraying with 30 µM DEX.

Growth of non-pathogenic *hrpH* mutant in *avrPphE*$_{Pto}$ plants

The striking similarity between the induced phenotype of *avrPphE*$_{Pto}$ plants and that of *Pst* DC3000 infection on *Arabidopsis* plants prompted us to examine the virulence contribution of AvrPphE$_{Pto}$ by assessing the susceptibility of the *avrPphE*$_{Pto}$ transgenic plants to the *hrpH* mutant. The *hrpH* mutant is unable to assemble a functional type III secretion apparatus and is thus incapable of secreting any type III effector proteins (Yuan and He, 1996). Wildtype Col *gl1* and transgenic *avrPphE*$_{Pto}$ plants were sprayed with 30μM DEX, 24 hours prior to inoculation with bacteria and daily thereafter during the course of the experiment. While the *hrpH* mutant was unable to grow beyond the inoculation level in DEX treated Col *gl1* plants, DEX treated *avrPphE*$_{Pto}$ plants allowed the *hrpH* mutant to grow to levels very similar to that of *Pst* DC3000. Transgenic expression of *avrPphE*$_{Pto}$ did not significantly affect *Pst* DC3000 multiplication because *Pst* DC3000 multiplied similarly in *avrPphE*$_{Pto}$ plants and wild-type Col *gl1* plants. In the absence of DEX induction, the transgenic *avrPphE*$_{Pto}$ plants did not allow *hrpH* to grow beyond the inoculation level, indicating that the increased susceptibility observed was due to the presence of the effector protein. The ability of *avrPphE*$_{Pto}$ plants to support the growth of the *hrpH* mutant bacteria could be due to extensive cellular damage caused by overexpression of *avrPphE*$_{Pto}$, resulting in non-specific nutrient leakage. To determine if the multiplication of the *hrpH* mutant bacteria can occur in the absence of tissue damage, I identified a level of induction which did not promote any visible macroscopic symptoms during the course of the experiment. When induced with 0.1μM DEX, *avrPphE*$_{Pto}$ plants did not exhibit any chlorosis or necrosis in response to DEX. Immunoblotting confirmed the presence of AvrPphE$_{Pto}$ in these plants after induction with 1μM DEX (data not shown). However, I was unable to detect protein in plants treated with 0.1μM DEX. Transgenic *avrPphE*$_{Pto}$ plants and Col *gl1* plants were sprayed with 0.1 μM DEX, 24 hours prior to bacterial inoculation and daily during the experiment. I found that expression of *avrPphE*$_{Pto}$ allowed 10 to 50-fold increase in the *hrpH* mutant population in line 342 and 100-fold in line 422. At this DEX concentration, leaves inoculated with the *hrpH* mutant showed some chlorosis in line 342. In line 422 multiplication of the *hrpH* mutant led to the development of chlorosis and few necrotic spots. Thus, AvrPphE$_{Pto}$ could promote multiplication of and symptom production by the *hrpH* mutant in the absence of extensive tissue damages.

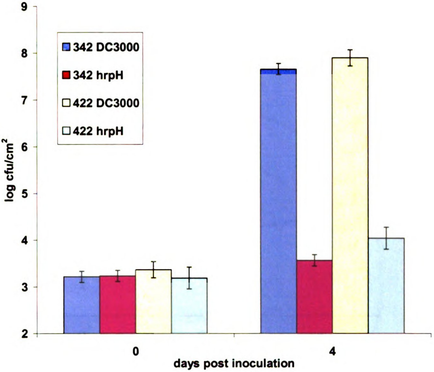

Figure 3-8: Bacterial proliferation in uninduced *avrPphE$_{Pto}$* transgenic plants. Bacteria were syringe-infiltrated into plants at 10^6 cfu/ml. Bacterial growth was monitored after four days. Each bar represents the mean titer of 12 leaf discs from three individual leaves. Error bars indicate standard deviation. "342" and "422" represent two independent lines of *avrPphE$_{Pto}$* transgenic plants. "DC3000" represents *Pst* DC3000. "hrpH" represents the *hrpH* mutant.

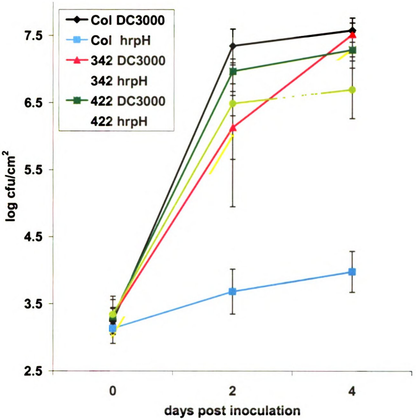

Figure 3-9: Multiplication of the *hrpH* mutant in *avrPphE$_{Pto}$* transgenic plants. Two independent lines of *avrPphE$_{Pto}$* plants were analysed – 342 and 422. Plants were sprayed with 30 µM DEX, 24 hours prior to bacterial infiltration and daily during the course of the experiment. Bacteria were syringe-infiltrated into plants at 10^6 cfu/ml. Bacterial growth was monitored over four days. Bacterial numbers are the average of 12 leaf discs from three individual leaves. Error bars indicate standard deviation. Col represents Col *gl1* plants. "DC3000" represents *Pst* DC3000. "hrpH" represents the *hrpH* mutant.

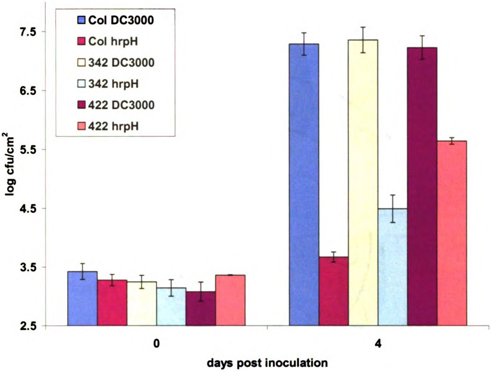

Figure 3-10: Growth of the *hrpH* in *A. thaliana* Col *gl1* and *avrPphE*$_{Pto}$ plants. Plants were sprayed with 0.1 μM DEX, 24 hours prior to bacterial infiltration and daily during the course of the experiment. Bacteria were syringe-infiltrated into plants at 10^6 cfu/ml. Bacterial growth was monitored after four days. Bacterial numbers are the average of 12 leaf discs from three individual leaves. Error bars indicate standard deviations. Col represents Col *gl1* plants. “342” and “422” represent the two lines of *avrPphE*$_{Pto}$ transgenic plants. “DC3000” represents *Pst* DC3000. “hrpH” represents the *hrpH* mutant.

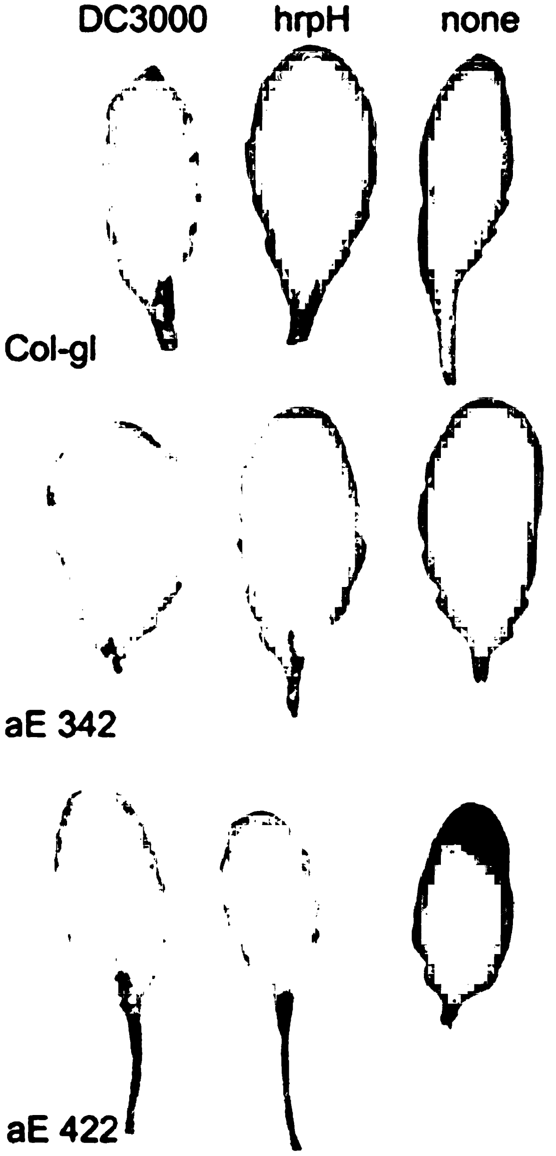

Figure 3-11: Growth of the *hrpH* mutant in *avrPphE*$_{Pto}$ transgenic plants is accompanied by the development of symptoms similar to those of *Pst* DC3000 infection. Wildtype Col *gl1* and *avrPphE*$_{Pto}$ transgenic plants were induced with 0.1 uM DEX, 24 hours prior to inoculation and daily during the course of the experiment.. Bacterial inoculum of 10^6 cfu/ml was syringe-infiltrated. Symptoms were recorded four days later. "aE 342" and "aE 422" represent two independent lines of *avrPphE*$_{Pto}$ transgenic plants, "DC3000" represents *Pst* DC3000 and "hrpH" represents the *hrpH* mutant. Leaves labeled "none" were treated with DEX but were not infiltrated with bacteria.

Expression profiling of plants expressing AvrPphE$_{Pto}$

The ability of transgenic *avrPphE$_{Pto}$* plants to support high levels of the non-pathogenic *hrpH* mutant, prompted us to examine the host gene expression profile in response to the expression of *avrPphE$_{Pto}$* in *Arabidopsis* plants. For this, we used a cDNA microarray that had been developed in our laboratory and essentially constitutes a gene expression signature for *Pst* DC3000 infection of *Arabidopsis* – PAGES (Pseudomonas Arabidopsis Gene Expression Signature). The PAGES array was developed by examining the expression of about 7,200 randomly chosen *Arabidopsis* genes in pre-symptomatic leaves inoculated with *Pst* DC3000 or *hrp* mutants. A set of 864 genes that were identified as being reproducibly regulated during the infection of *Arabidopsis thaliana* Col *gl1* plants with *Pst* DC3000 constitutes the PAGES array. The expression of a subset of 117 genes on the array was found to be associated with the functions of the *Pst* DC3000 TTSS. Of the 117 genes, 53 were repressed and 64 were induced in a TTSS-dependent manner.

In order to determine the virulence contribution of *avrPphE$_{Pto}$*, transgenic plants were sprayed with 30 μM DEX and kept under high humidity conditions. Leaf tissue was harvested 24 hours later. At this time, the plants develop small amounts of water soaking, but do not develop any chlorosis or necrosis. Total RNA from this tissue sample was used to hybridize the microarray. Wildtype Col *gl1* plants sprayed with DEX was used as control. Both *avrPphE$_{Pto}$* lines were assessed in a similar manner and two independent biological replicates were performed for each line. *In planta* expression of AvrPphE$_{Pto}$ was found to regulate approximately 50% of the *Arabidopsis* genes known to be regulated by *Pst* DC3000 infection. We also found that 90% of the TTSS-specific gene cluster was regulated similarly by *Pst* DC3000 and AvrPphE$_{\mathrm{Pto}}$ (Table B-1).

Similar microarray analysis was also performed with *avrPphE*$_{Pto}$ plants that had been sprayed with 0.1µM DEX. At this lower level of induction, which causes neither chlorosis nor necrosis, the transcriptional profile resembled that observed at the higher level of DEX but appeared to be dampened (Table B-2). The striking similarity between the *Pst* DC3000 TTSS- and AvrPphE$_{\mathrm{Pto}}$-regulated host gene expression profiles demonstrates that *avrPphE*$_{Pto}$ expression in transgenic *Arabidopsis* globally mimicked the *Pst* DC3000 TTSS functions at the molecular level.

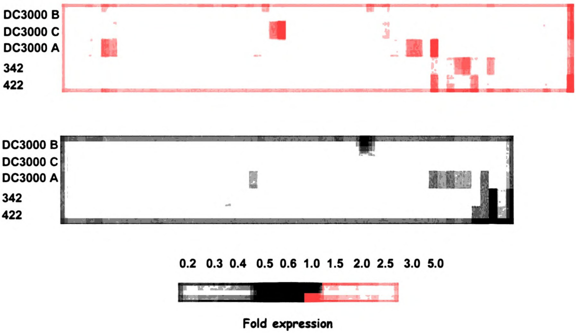

Figure 3-12: Cluster analysis of the expression profiles of 117 TTSS-regulated genes (colored bars) after *Pst* DC3000 infection and transgenic expression of AvrPphE$_{\mathrm{Pto}}$ with 30 μM DEX. Rows DC3000 A, DC3000 B and DC3000 C represent *Pst* DC3000 TTSS-regulated genes from three independent biological replicates (Table B-1). Rows 342 and 422 represent two independent biological replicates in two independent *avrPphE*$_{Pto}$ transgenic lines 342 and 422, respectively, 24 hours after induction with 30 μM DEX (Table B-1).

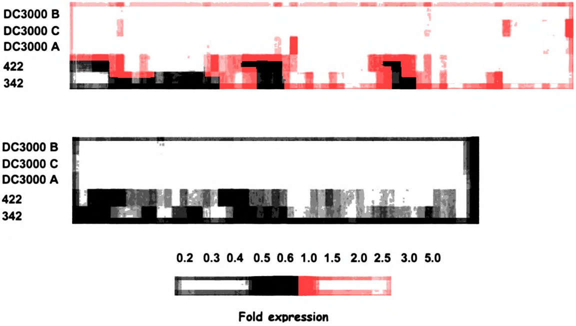

Figure 3-13: Cluster analysis of the expression profiles of 117 TTSS-regulated genes (colored bars) after *Pst* DC3000 infection and transgenic expression of AvrPphE$_{\mathrm{Pto}}$ with 0.1 μM DEX Rows DC3000 A, DC3000 B and DC3000 C represent *Pst* DC3000 TTSS-regulated genes from three independent biological replicates (Table B-2). Rows 342 and 422 represent two independent biological replicates in two independent *avrPphE*$_{Pto}$ transgenic lines 342 and 422, respectively, 24 hours after induction with 0.1 μM DEX (Table B-2).

Discussion

The aim of this study was to identify type III effectors of *Pst* DC3000 and to investigate their contribution towards the development of disease. At the time this project was initiated, the *Pst* DC3000 genome had not been sequenced and *Pst* DC3000 was known to harbour only two effector genes – *avrE* and *avrPto*. While *avrE* is required for full virulence of *Pst* strain PT23 on tomato (Lorang et al., 1995; Lorang et al., 1994), and the homolog of *avrE* in *Erwinia amylovora*, *dspE*, is required for pathogenicity on apple and pear (Bogdanove *et al.*, 1998; Gaudriault *et al.*, 1997; Tharaud *et al.*, 1994), the role of *avrE* in *Pst* DC3000 virulence is not very clear. Characterization of transgenic plants conditionally expressing *ssavrE* (the *avrE* gene tagged with the PR-1 signal sequence) suggested that *avrE* has a role in virulence in the *Pst* DC3000-*Arabidopsis* pathosystem (Zwiesler-Vollick et al., unpublished data). Bacterial mutants lacking *avrE*, however, are not affected in their ability to infect *Arabidopsis* (S. Y. He, unpublished results). Inactivation of *avrPto* also does not affect virulence of *Pst* DC3000 on *Arabidopsis* (Q. L. Jin, unpublished results), but the expression of *avrPto* in a less virulent strain, T1, that lacks the gene, gives it a growth advantage on tomato, indicating that *avrPto* contributes to virulence (Shan et al., 2000; Chang et al., 2000). In *Pst* DC3000, *avrPto* has recently been shown to function as a suppressor of the extracellular cell wall-based defenses of its host, *Arabidopsis* (Hauck et al., 2003)

In order to identify other putative type III effectors in *Pst* DC3000, I searched for putative orthologs of ten known *avr* genes that had been identified in other pathovars of *P. syringae* (Table 3-1). I was able to detect the presence of orthologs of three of the genes examined using Southern hybridization. These were putative orthologs of *virPphA* and *avrPphE* from *P. s.* pv. *phaseolicola* and *avrPpiB* from *P. s.* pv. *pisi.* Of these, only VirPphA has previously been demonstrated to function as a virulence factor. *Pph* race 7 strain 1449B harbors *virPphA* on a 154 Kb plasmid. When cured of this plasmid, strains lose virulence in bean and instead cause an HR defense response in previously susceptible cultivars. Virulence was restored when *virPphA* was re-introduced on a plasmid. Although *virPphA* has a HR-suppressing function in bean, it was shown to function like an *avr* gene in soybean (Jackson et al., 1999). The ortholog of *virPphA* in *Pst* DC3000, *avrPtoB,* has been shown to be widely conserved among different genera of plant pathogens including *Xanthomonas*, *Erwinia* and many strains of *Pseudomonas* (Jackson et al., 1999; Kim et al., 2002; Guttman et al., 2002). AvrPtoB has been shown to play a role in suppressing programmed cell death and HR-based immunity in tomato plants (Abramovich et. al, 2003).

avrPphE was initially cloned from *Pph* race 4 strain 1302A and confers resistance on bean cultivars harboring the R2 resistance gene. *avrPphE*, like all other *avr* genes, is *hrp*-regulated and shares no similarity with any protein of known function. It is near the left border of the *Pph hrp* cluster and is linked to *hrpK.* Orthologs have been identified in all eight races of *Pph* and *P. s. tabaci.* Disruption of *avrPphE* by transposon mutagenesis blocks induction of HR but does not seem to affect virulence. An interesting aspect of this gene, however, is that orthologs have also been found in races that are virulent on cultivars with the R2 resistance locus (Stevens et al., 1998; Mansfield et al., 1994).

avrPpiB confers race-specific resistance to pea cultivars harboring the R3 resistance locus. It contains the *hrp* box and has homologs in three other races of *P. s. pisi* and several other *P. syringae* pathovars. PCR-RFLP patterns of *avrPpiB* and its homologs are identical, suggesting conservation of gene structure among *P. syringae* strains (Arnold et al., 2001; Cournoyer et al., 1995).

Analysis of the recently sequenced *Pst* DC3000 genome revealed that our approach had failed to identify the putative orthologs of two *avr* genes that were used for the screen. These were orthologs of *avrPphF* ORF1 and ORF2. *Pst* DC3000 has a single copy of *avrPphF* ORF1 and ORF2 (Buell et al., 2003; Fouts et al., 2002). These two genes constitute an operon and have been named $avrPphF_{Pto}$. Sequence analysis shows that $avrPphF_{Pto}$ is only 59% identical at the nucleotide level to *avrPphF* from *P. s.* pv. *phaseolicola* (Tsiasmis et al., 2000), probably explaining our inability to identify this gene. Consistent with this reasoning, all the orthologs that I identified had high levels of identity at the nucleotide level to their respective probes: *avrPtoB* (79%), $avrPphE_{Pto}$ (72%), and $avrPpiB_{Pto}$ (99%).

The fact that $avrPphE_{Pto}$ and $avrPpiB_{Pto}$ mutants had no detectable reduction in virulence is not surprising. Bacterial mutagenesis approaches have often proved to be unsuccessful in detecting subtle virulence phenotypes because of presumed functional redundancy among the set of effectors harbored by a single pathogen. As a result, inactivation of one or more genes often does not have a measurable impact on virulence as assessed by symptom development and bacterial multiplication. The relatively few effectors that have been shown to have virulence phenotypes were largely demonstrated to do so by heterologous expression in weakly virulent strains or pathovars (Chang *et al.*, 2000; Shan *et al.*, 2000; Chen et al., 2000; Guttman and Greenberg, 2001). To date, in *P. s.* pv. *tomato*, only *avrA* and *avrE* in *P. syringae* pv. *tomato* PT23 (Lorang *et al.*, 1994) and *avrRpm1* in *P. syringae* pv. *maculicola Psm* M2 (Ritter and Dangl, 1995) have been shown to contribute visibly to the symptom development and growth of native strains on their susceptible hosts.

The *avrPtoB* mutant was found to be reduced in its ability to multiply in both *Arabidopsis* and tomato with a significant reduction in symptom development in tomato (data not shown). Although this mutation was confirmed by Southern analysis, I was unable to complement the mutant. I provided the *avrPtoB* gene *in trans* on several different plasmids that range from high to single copy number, as well as the original cosmid that had been isolated from the *Pst* DC3000 genomic library. I cloned *avrPtoB* both with its own promoter as well as the promoter of the plasmid. None of these attempts were successful in complementing the mutant. It is possible that the mutant phenotype observed is not due to inactivation of *avrPtoB*, but due to secondary mutations that occurred during the allelic exchange. Plasmid instability could also be an explanation. Perhaps a wildtype copy integrated into the genome would function better. Interestingly, an independent study in Dr. Greg Martin's lab (Cornell University) also showed a reduced virulence phenotype for *avrPtoB*. Complementation of the mutant with *avrPtoB* was found to be only partial in that case as well (Abramovitch et al., 2003).

Given the limitation of bacterial genetics, we adopted the approach of generating transgenic *Arabidopsis* plants that express the desired effector in an effort to study the virulence function of a single effector in susceptible plants In this chapter, I have demonstrated that expression of $avrPphE_{Pto}$ in plants caused the development of macroscopic symptoms such as water soaking, chlorosis and necrosis, that closely resemble those caused by *Pst* DC3000 during infection. *Arabidopsis* plants expressing $avrPphE_{Pto}$ support growth of the non-pathogenic *hrpH* mutant.

The macroscopic necrosis caused by the high level of AvrPphE$_{Pto}$ protein complicates the interpretation of the growth promotion by AvrPphE$_{Pto}$. The aggressive growth of the *hrpH* mutant could be due to massive cellular damage in host cells. To address this issue, I analyzed *avrPphE$_{Pto}$* plants at a level of DEX that does not cause the development of any symptoms during the experiment. It was found that even at this low level of induction, the transgenic lines still allowed the *hrpH* mutant to multiply substantially. Surprisingly, line 342, which allowed 10-fold increase in *hrpH* mutant growth, also developed patchy chlorosis. In line 422, the *hrpH* mutant attained population levels close to 5.5 log cfu/cm^2 leaf tissue. These leaves developed even more significant symptoms including chlorosis and necrotic spots. Generally, when *Pst* DC3000 is present in infected tissue at levels near 5.5 log cfu/cm^2 leaf tissue, macroscopic disease symptoms are not observed. For example, plants infected with the CEL deletion mutant (Chapter 2), which multiplied to ~ 5.5 log cfu/cm^2 leaf tissue, are completely symptom-free. Therefore, even though low levels of DEX did not elicit macroscopic symptoms in *avrPphE$_{Pto}$* plants, these plants are potentiated to cellular damages in response to bacterial inoculation.

A low level of expression of AvrPphE$_{Pto}$ appears to cause microscopic changes in the host physiology, making the plant more fit for supporting bacterial growth. Such a function of *avrPphE$_{Pto}$* is further supported by the microarray results. At 30μM DEX, expression of AvrPphE$_{Pto}$ generated a host gene expression profile that is very similar to that seen in wildtype plants infected with *Pst* DC3000, with more than 90% of the TTSS-dependent gene cluster being regulated in a manner similar to that of the *Pst* DC3000. At the lower level of DEX induction, the gene expression profile of the transgenic lines is qualitatively similar to that observed at the high level of the inducer. However, the overall expression is dampened. Therefore, expression of the effector at this low level brings about molecular changes in the host that occur during disease progression and renders the host susceptible to the non-pathogenic *hrpH* mutant, which is not able to inject any type III effectors into the host. Apparently, the expression of AvrPphE$_{\mathrm{Pto}}$ compensated for the lack of the effector translocated by the *hrp* mutant. Our results strongly support a role for AvrPphE$_{\mathrm{Pto}}$ in *Pst* DC3000 virulence since it renders the host a better niche for the *hrp* mutant despite its inability to inject type III effectors into the host cell.

While we have evidence that supports a role for AvrPphE$_{\mathrm{Pto}}$ in *Pst* DC3000 virulence, we do not know how this effector brings about changes that promote the diseased state. *Pst* DC3000 is an extracellular pathogen and colonizes the intercellular space in the leaf tissue. It is thought that the apoplast maybe limited in nutrients. As a major component of the *Pst* DC3000 arsenal, the type III effectors are believed to cause leakage of water and nutrients into the apoplastic space to allow for high levels of bacterial multiplication. Water soaking that occurs during the early stage of infection is believed to reflect the leakage of water/nutrients into the apoplast. Since we observed water-soaking after DEX-induced expression of AvrPphE$_{\mathrm{Pto}}$ in transgenic plants, it is possible that this effector is involved in nutrient/water release. However, the fact that we did not detect microscopic water soaking at the low level of DEX treatment, but still observed significant growth of the *hrpH* mutant suggests that this is not the primary or the only function of AvrPphE$_{\mathrm{Pto}}$.

Overall, this study identified three type III effectors that were not previously known to be present in *Pst* DC3000. The mutagenesis approach failed to demonstrate a virulence contribution of two effectors in *Pst* DC3000. Although we did observe a reduced-virulence phenotype for the *avrPtoB* mutant, we were unable to draw any conclusions due to the inability to achieve successful complementation. However, the transgenic approach coupled with microarray analysis has been helpful in showing that *avrPphE*$_{Pto}$ contributes to *Pst* DC3000 virulence. Given the difficulty in uncovering functions of individual effectors due to redundancy, this approach might provide a way of determining the functions of type III effectors in disease development

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Chapter 4

Conclusions and Future Perspectives

The long term objective of this study is to elucidate mechanisms of bacterial pathogenesis in plants, which should help in the development of improved methods of pathogen control and reduction of losses in crop production. Bacterial plant pathogens infect a wide range of crop plants and cause severe losses in crop production worldwide. Current practices of pathogen control involve the use of large quantities of pesticides and antibiotics. These methods are costly, non-specific, and a major source of environmental toxicity. An understanding of the process by which a pathogen triggers disease in a host plant will enable us to design strategies that target key steps in the process of pathogenesis, leading to the development of specific, environment-friendly and durable methods to combat pathogens.

Pseudomonas syringae pathovars cause diseases in important crop plants such as beans, peas, cucumber, tomatoes and tobacco. Although much research has been conducted on crop plants, many of them are not very amenable to genetic manipulations. The model plant *Arabidopsis thaliana* has many advantages in this respect. These include a rapid generation time, small genome size, established methods for transformation, completely sequenced genome, large collection of tagged mutants, and availability of microarray and other genomic resources. A tomato pathogen, *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*DC3000), was found to be pathogenic on most tested ecotypes of *Arabidopsis thaliana* in the early 1990s (Whalen et al., 1991). Since then, the *Arabidopsis thaliana-Pst*DC3000 pathosystem has proven to be a useful model system to study plant-pathogen interactions and results are generally correlated with observations from other systems (Baker et al., 1997). *Pst* DC3000 utilizes two known virulence systems. One is the phytotoxin coronatine. Although coronatine is thought to be primarily responsible for causing chlorosis (Bender, 1987), the mechanism by which this toxin promotes disease is not yet known. *Pst* DC3000 also utilizes a type III protein secretion system (TTSS) during pathogenesis. The TTSS secretes bacterial proteins, collectively referred to as effectors, directly into the plant host cytoplasm. Once they are translocated into the plant host cell, the effectors promote disease presumably by suppressing host defense responses and promoting the release of nutrients for utilization by bacteria. My thesis research focused on understanding the virulence function of type III effectors of *Pst* DC3000.

When this study was initiated, only two effectors, AvrE and AvrPto, were known to be present in *Pst* DC3000. However, bacterial mutants of *avrE* and *avrPto* do not show any reduction in virulence (Lorang and Keen, 1995). The lack of phenotype of individual effector mutations has been attributed to functional redundancy. The ΔCEL mutant was generated by the deletion of six ORFs in the Conserved Effector Locus of *Pst* DC3000 (Alfano et al., 2000). This mutant is unique in that it has a dramatic reduced-virulence phenotype. Typically, mutation of single and even multiple type III effectors results in weak virulence phenotypes.

Chapter 2 summarized my research to identify the effector gene(s) that restores the virulence of the ΔCEL mutant, and elucidating the mechanism that it plays in promoting pathogenesis. HopPtoM (ORF3) and ShcM (ORF4), two of the deleted ORFs, were found to be sufficient to restore the ΔCEL mutant phenotype to wildtype. Translocation of HopPtoM is dependent on ShcM, and the first 200 amino acids of HopPtoM are required for interaction with ShcM. The ΔCEL mutant was found to be compromised in its ability to suppress basal host immunity that is characterized by the deposition of callose-containing papillae in the cell wall in response to pathogen attack. Wildtype *Pst* DC3000 suppresses this defense response. Furthermore, I found that the ΔCEL mutant-activated papillae formation was dependent on salicylic acid, whereas the *hrp* mutant-activated papilla formation was independent of salicylic acid. The inability of the ΔCEL mutant to suppress papilla formation can also be restored by providing HopPtoM and ShcM *in trans*, suggesting that HopPtoM is a suppressor of an SA-dependent cell wall defense in *Arabidopsis*.

Clearly, the next step is to identify *Arabidopsis* proteins that are targeted by HopPtoM to suppress the SA-dependent cell wall based defense. In a yeast two hybrid screen, I identified a putative aminocyclopropane carboxylic acid (ACC) synthase, ACS10, as the only *Arabidopsis* protein that interacts with HopPtoM. The first 100 amino acids of HopPtoM were found to be sufficient to interact with ACS10. However, HopPtoM with a 100-amino acid C-terminal truncation was unable to interact with ACS10 (Figure A-1). ACC synthase catalyzes the first committed step in ethylene biosynthesis, and converts S-adenosyl-L-methionine to ACC (Bleeker and Kende, 2000). Ethylene has been implicated in symptom development during the compatible interaction. *Pst* DC3000 causes fewer symptoms in the ethylene insensitive *ein2* mutant, with no reduction of bacterial growth (Bent et al., 1992).

It is possible that the interaction of ACS10 with HopPtoM alters ACS10 in a positive or negative fashion, modulating ethylene levels in the plant. In other words, inability of the ΔCEL mutant to grow *in planta* could be associated with its inability to elevate or repress ethylene levels in the host. To test this hypothesis, I sprayed *Arabidopsis* plants with either ACC, which is the substrate for ethylene, or with AVG, which is an inhibitor of ACC synthase, followed by bacterial inoculation. Neither treatment affected the virulence of ΔCEL mutant or *Pst* DC3000. I also monitored the ability of ΔCEL to infect *ein2* mutant plants, which had been pre-treated with either ACC or AVG or untreated. However, I did not observe any alterations in ΔCEL mutant or *Pst* DC3000 virulence under any of the experimental conditions (Figure A-2). I also adopted a genetic approach to examine whether ACS10 is involved in *Pst* DC3000 pathogenesis. I obtained two knockout lines for *ACS10* from the SALK collection (http://signal.salk.edu/tdna) and assessed them for differences in their ability to support/enhance the growth of the ΔCEL mutant and/or the wildtype pathogen. Although I confirmed the T DNA insertion by PCR, the mutant plants behaved like wildtype plants in their responses to both the wildtype pathogen and the ΔCEL mutant (Figure A-3 and A-4). The T-DNA insertions in both SALK lines are upstream of the start codon of the *ACS10* gene. It is possible that insertion of the T-DNA in the 5' UTR did not eliminate the transcription and translation of this gene. To further investigate whether ACS10 is involved in HopPtoM action and *Pst* DC3000 pathogenesis, it would be helpful to generate transgenic plants that overexpress the *ACS10* gene, and to produce antisense lines, or new T-DNA insertion lines to provide us new clues about the function of HopPtoM.

Because high level DEX-induced expession of HopPtoM in *Arabidopsis* results in a necrosis phenotype, I also initiated a genetic approach to find putative host proteins that are targeted/required by HopPtoM function in plants. I have generated a population of EMS–mutagenized *hopPtoM* transgenic seed. Screening for suppressor mutants from this population is currently underway. We hope to obtain at least two groups of mutants from this screen: i) those that carry lesions in an *Arabidopsis* protein that is required by HopPtoM to cause necrosis and ii) mutants carrying lesions within HopPtoM itself that affect its function. This screen can yield mutants from both of the above mentioned categories. Western blotting will be used to determine if the mutant still synthesizes HopPtoM. Only those that synthesize HopPtoM, but do not develop any symptoms upon 30 μM DEX induction, will be characterized on the basis of their ability to support the growth of the ΔCEL mutant when the transgene is induced with low dose of DEX (3 nM) and the ability to suppress callose deposition activated by the ΔCEL mutant. We can determine if the phenotype results from an alteration in the *hopPtoM* gene or a host gene by testing for the ability of ΔCEL mutant containing HopPtoM and ShcM *in trans* to cause disease and suppress callose deposits in the mutant plants. Mutation in a host gene may block the action of HopPtoM delivered from bacteria and therefore make bacteria less virulent, whereas mutation within the *hopPtoM* transgene should not affect the function of bacteria-delivered HopPtoM, and therefore these mutant plants should remain susceptible to the bacteria. In summary, we may get mutants that will now enable us to dissect symptom development, growth promotion and callose suppression by HopPtoM.

Chapter 3 summarized my research to identify more effectors in *Pst* DC30000 by using a gene homology-based method. I was successful in identifying three new effectors in *Pst* DC3000. These were orthologs of *virPphA* and *avrPphE* from *P. s.* pv. *phaseolicola* and *avrPpiB* from *P. s.* pv. *pisi*, respectively (Stevens et al., 1998; Mansfield et al., 1994; Jackson et al., 1999; Arnold et al., 2001; Cournoyer et al., 1995). Using the traditional bacterial mutagenesis method, I did not detect a virulence effect for either *avrPphE*$_{Pto}$ or *avrPpiB*$_{Pto}$. On the other hand, even though I detected a significant virulence effect for *avrPtoB* in both tomato and *Arabidopsis*, I was unable to complement this mutant and hence the virulence function of *avrPtoB* was not conclusive. Transgenic expression of AvrPphE$_{Pto}$ in *Arabidopsis* revealed its ability to support growth of the non-pathogenic *hrpH* mutant. Microarray analysis of transgenic *avrPphE$_{Pto}$* plants show mimicry of *Pst* DC3000 infection in terms of host gene expression profile by AvrPphE$_{Pto}$.

Although, my study suggests that transgenic expression of type III effectors in *Arabidopsis* can be used to assess the virulence contribution of a single effector, such results should be interpreted carefully. During an infection, *Pst* DC3000 injects effectors into its host in minute quantities. These quantities are so small, that they are not detectable using any currently available detection techniques. Thus, we have no estimate of the quantity of effector that the host experiences during pathogenesis. In a transgenic plant, even low doses of the inducer probably results in effector quantities that are larger compared to that during an infection. Hence, our observations could be to an extent, non-physiological. Therefore, great care should be taken in interpreting data and drawing conclusions when transgenic plants are used.

How AvrPphE$_{Pto}$ promotes disease remains to be determined. I have performed experiments that provide clues for future elucidation of the function of AvrPphE$_{Pto}$. I conducted a yeast two-hybrid screen to identify *Arabidopsis* proteins that interact with AvrPphE$_{Pto.}$ I identified three interactors (Table B-3). Of these, one was a receptor protein kinase-like protein (At5g20480). Sequence analysis revealed the presence of 13 leucine rich repeats (LRR). It is predicted to be a transmembrane protein with two short membrane-spanning domains. The LRRs and kinase domain are probably extracytoplasmic (Figure B-1). The other interactors were a putative phosphoprotein phosphatase (At2g27210) and a putative protein (At3g51650). Interestingly, the putative phosphoprotein phosphatase transcript accumulated in *Arabidopsis* plants in response to infection with the *hrpRS* mutant. The *hrpRS* mutant does not assemble the TTSS and therefore does not secrete any effectors. The At2g27210 transcript was strongly induced by the *hrpRS* mutant, only mildly induced by *Pst* DC3000, and almost not induced by the ΔCEL mutant (Figure B-2). It would be interesting to detect whether $\text{AvrPphE}_{\text{Pto}}$ or other effectors mediate suppression of this transcript.

SALK lines that contain T-DNA insertions in the receptor-like protein and the putative phosphoprotein phosphatase are available (Table B-3). These SALK lines can be used to assess if the presence or absence of the identified interactors is required for *Pst* DC3000 to establish a successful infection. This could be done by infecting these plants with *Pst* DC3000 as well as $avrPphE_{Pto}$ mutants and observing them for possible alterations in bacterial multiplication and symptom development. Transgenic plants overexpressing these interactors can also be useful in studying the impact these host proteins have on the ability of *Pst* DC3000 to cause infection. Transgenic plants that overexpress these interactors can also be crossed with the $avrPphE_{Pto}$ plants. Analysis of progeny could determine whether overexpression of these host proteins has an inhibitory effect on the necrosis phenotype induced by transgene expression of $\text{AvrPphE}_{\text{Pto}}$.

The field of molecular plant pathology was initially focused on deciphering gene-for-gene resistance that restricts an avirulent pathogen from infecting its host. Clearly, a new trend is to understand how compatible interactions proceed in the absence of gene-for-gene resistance. A better understanding of the compatible interaction can also help us better understand resistance because type III effectors have been shown to target defense pathways. The recent completion of the *Pst* DC3000 genome and its subsequent analysis by several independent studies has estimated the presence of at least 30 different type III effectors in *Pst* DC3000. An understanding of how these effectors modulate the host can yield a lot of information about the molecular basis of pathogenesis.

My research has provided two important pieces of information in bacterial pathogenesis. First, I have shown that the CEL of *Pst* DC3000 encodes effectors that suppress extracellular cell wall-based defense. The presence of papilla during interaction of wildtype *Arabidopsis* plants with non-pathogenic bacteria, including the *hrp* mutant, and the ΔCEL mutant is correlated with their inability to multiply in plants. The cell wall-based defense could therefore be a critical part of basal plant immunity that keeps myriads of non-pathogens from establishing themselves in the plants. Second, I have demonstrated that this basal immunity is partly dependent on salicylic acid. The ΔCEL mutant is impaired in suppressing this SA-dependent triggering of callose deposition in wildtype leaves. SA has been demonstrated to play a major role in mediating defenses, especially those related to gene-for-gene related resistance (Hunt et al., 1996; Shirasu et al., 1996). This study demonstrates that SA is also involved in mediating basal defenses that function even against the successful pathogen. This study provides new insight into the evolution of a successful pathogen. Acquiring the CEL might have been an important step in the evolution of the successful pathogen and that is why it is so highly conserved among the strains in which it has been studied so far.

Finally, I also identified three type III effectors that were not known to be present in *Pst* DC3000 at the time this research was initiated. My research suggests that transgenic expression is a useful tool to study the function of type III effectors and to demonstrate their contribution to disease development.

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Appendix A

This Appendix contains supplementary information for Chapter 2

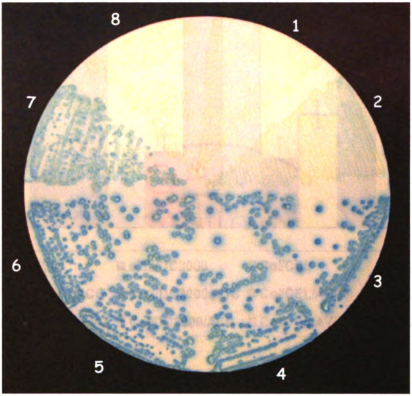

Figure A-1: Physical interaction between ACS10 and HopPtoM in the LexA two-hybrid system. ACS10 was identified as the only *Arabidopsis* host protein that interacts with HopPtoM. The isolated ACS10 interactor was fused to the DNA binding domain (BD) in pGILDA and a series of truncated HopPtoM proteins were fused to the transcriptional activation domain (AD) in pB42AD. Yeast strains were grown at 30°C for 5 days on galactose X-gal complete minimal medium. Blue color indicates interaction, whereas white color indicates no interaction. ACS10 interacted with all fragments of HopPtoM, but there was a significant decrease in the strength of interaction between ACS10 and HopPtoM lacking 124 amino acids at the C-terminal. 1: BD::HopPtoM/AD::empty; 2: BD::HopPtoM/AD::ACS10; 3:BD::HopPtoM100 (first 100 aa)/AD::ACS10; 4:BD::HopPtoM200/AD::ACS10;5:BD::HopPtoM300/AD::ACS10;6:BD::HopPtoM400/ AD::ACS10;7: BD::HopPtoM500/AD::ACS10; 8: BD::HopPtoM600/AD::ACS10.

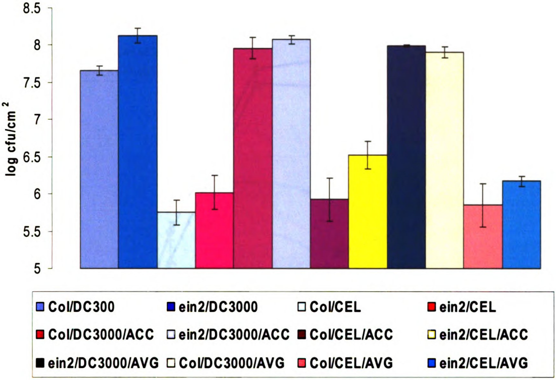

Figure A-2 : Multiplication of *Pst* DC3000, and the ΔCEL mutant in wildtype *Arabidopsis* and *ein2* mutant plants after treatment with ACC or AVG. The interaction of ACS10 with HopPtoM raised the possibility that alteration of ethylene levels in plants might alter the interaction with wild-type pathogen or the ΔCEL mutant. Plants were sprayed with ACC (ethylene precursor) or AVG (ACS inhibitor) and vacuum-infiltrated with 10^6 cfu/ml bacteria. Each bar represents the mean titer of 12 leaf discs from three individual leaves. Error bars represent standard deviation. "DC3000" represents *Pst* DC3000, "CEL" represents the ΔCEL mutant, "Col" represents wild-type *Arabidopsis*, and "ein2" represents the ethylene insensitive *ein2* mutant plant.

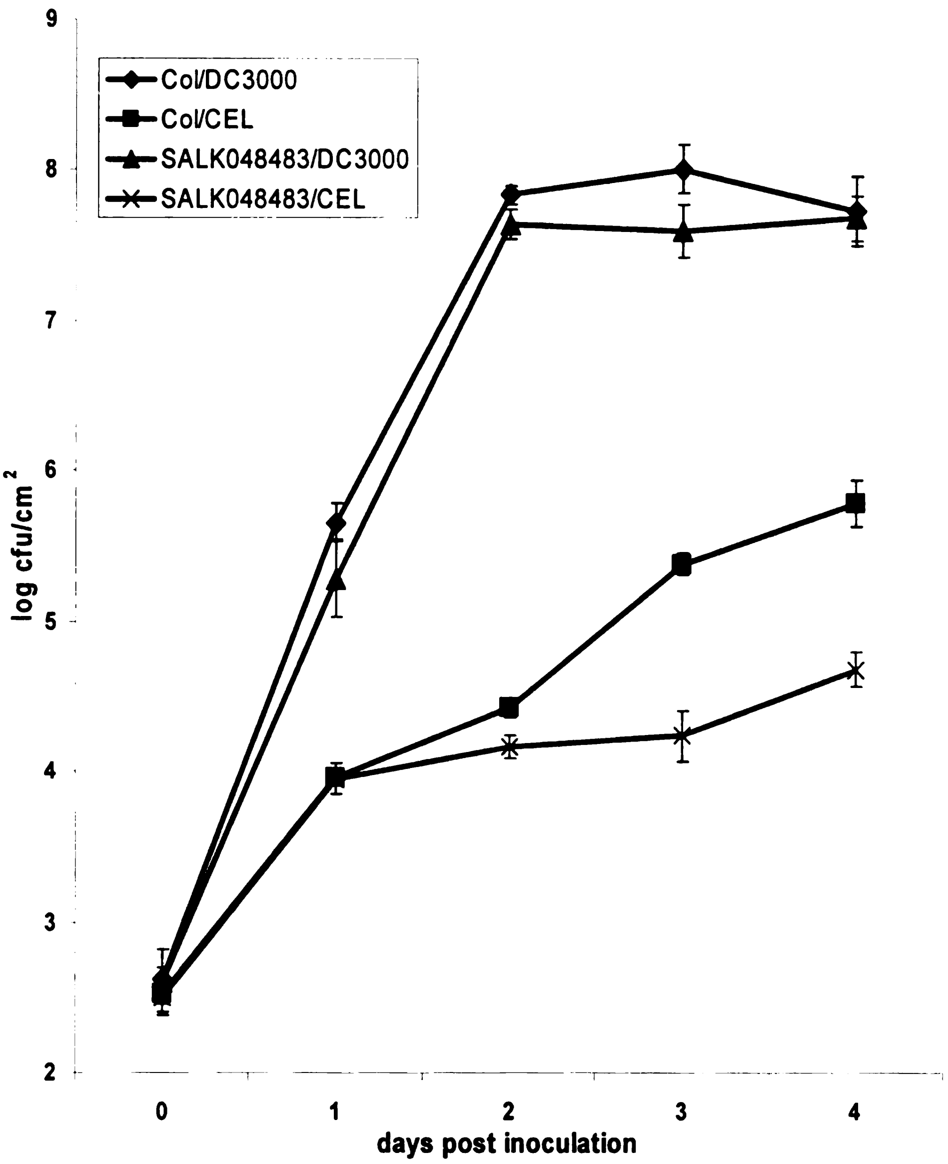

Figure A-3: Growth of *Pst* DC3000 and the ΔCEL mutant in *Arabidopsis* Col *gl1* and *ACS10* knockout lines. *Arabidopsis* plants containing T-DNA insertions in *ACS10* were obtained from the SALK collection. Wild-type and homozygous mutant plants were vacuum-infiltrated with 10^6 cfu/ml bacteria. Bacterial growth was monitored over four days. Bacterial numbers are the average of 12 leaf discs from three individual leaves. Error bars indicate standard deviation. "Col" represents Col *gl1* plants. "SALK048483" represents the T-DNA insertion mutant of ACS10. "DC3000" represents *Pst* DC3000. "CEL" represents the ΔCEL mutant.

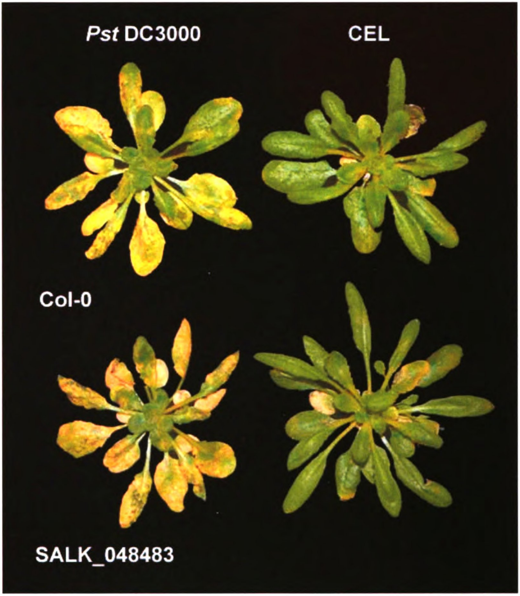

Figure A-4: Symptom development on wild-type *Arabidopsis* and *ACS10* knockout plants. Plants were inoculated with 10^6 cfu/ml bacteria. Pictures were taken four days later. The knockout line was not altered in response to either bacterium.

Appendix B

This appendix contains supplementary information for Chapter 3

Table B-1. *Arabidopsis* **genes repressed or induced in a type III secretion-dependent manner by** ***Pst*** **DC3000 infection and AvrPphEPto expression (30 µM DEX)**

Locus	Description	I	II	IIIa	IIIb	IIIc
Repressed by the TTSS of DC3000:						
At2g38540	LTP1 non-specific lipid transfer protein 1, cell wall-localized	**0.06**	**0.04**	0.31	0.25	0.23
At1g72610	GLP1 germin-like protein 1, cell wall-localized	**0.58**	**0.14**	0.23	0.16	0.33
At1g12090	Extensin-like protein	**0.03**	**0.02**	0.37	0.19	0.28
At2g10940	Proline-rich protein, extensin-like protein	**0.02**	**0.01**	0.24	0.15	0.26
At1g03870	Putative arabinogalactan-protein, fasciclin-like	**0.24**	**0.26**	0.24	0.19	0.29
At3g45970	At-EXPL1, expansin-like protein 1	**0.37**	**0.76**	0.29	0.07	0.41
At1g29660	Proline-rich protein APG-like, extracellular GDSL-motif lipase/hydrolase-like	**0.16**	**0.17**	0.50	0.24	0.32
At3g16370	Proline-rich protein APG-like, extracellular GDSL-motif lipase/hydrolase-like	**0.08**	**0.10**	0.19	0.13	0.23
At5g45950	Proline-rich protein APG-like, extracellular GDSL-motif lipase/hydrolase-like	**0.18**	**0.17**	0.33	0.35	0.42
At1g29670	Proline-rich protein APG-like, extracellular GDSL-motif lipase/hydrolase-like	**0.06**	**0.04**	0.32	0.19	0.28
At1g68560	XYL1, alpha-xylosidase 1	**0.18**	**0.17**	0.73	0.32	0.37
At4g23820	Putative polygalacturonase	**0.15**	**0.10**	0.26	0.15	0.28
At1g04040	Putative acid phosphatase	**0.07**	**0.08**	0.42	0.16	0.23
At5g44020	Putative acid phosphatase	**0.06**	**0.05**	0.23	0.18	0.26
At2g37450	MtN21 nodulin-like	**0.71**	**0.72**	0.72	0.35	0.34
At4g08950	Phosphate-induced protein 1 (phi-1)-like	**0.28**	**0.31**	0.65	0.22	0.43
At4g34260	Putative large secreted protein	**0.59**	**0.57**	0.26	0.24	0.25
At5g15350	Putative blue copper-binding protein	**0.88**	**1.14**	0.27	0.25	0.26
At3g07460	Hypothetical protein	**0.26**	**0.29**	0.35	0.24	0.34
At4g17340	Putative water channel protein	**0.17**	**0.17**	0.26	0.17	0.34
At3g16240	Putative tonoplast water channel protein	**0.05**	**0.02**	0.25	0.12	0.22
At2g19860	AtHXK2, hexokinase 2	**0.20**	**0.24**	0.19	0.21	0.25
At5g02160	Hypothetical protein	**0.12**	**0.15**	0.38	0.18	0.33
At5g56500	CPN-60 BETA 60kDa RuBisCO chaperonin	**0.18**	**0.14**	0.22	0.16	0.33

Table B-1 contd.

At4g01310	Plastid ribosomal proteins, L5P family	**0.21**	**0.22**	0.48	0.35	0.34
At1g33290	Putative ATPase	**0.12**	**0.11**	0.43	0.46	0.25
At1g14150	Putative oxygen-evolving enhancer protein 3-2 of photosystem II	**0.22**	**0.24**	0.37	0.33	0.29
At3g26060	Peroxiredoxin Q-like protein	**0.15**	**0.22**	0.49	0.32	0.35
At5g58260	Hypothetical protein	**0.16**	**0.19**	0.37	0.55	0.29
At1g20020	Putative FNR ferredoxin--NADP+ reductase	**0.13**	**0.23**	0.30	0.30	0.32
At3g63140	Putative mRNA-binding protein	**0.15**	**0.19**	0.30	0.32	0.28
At5g23060	Unknown protein containing a rhodanese homology domain	**0.25**	**0.27**	0.34	0.33	0.32
At2g29630	Putative THIC thiamine biosynthesis protein	**0.24**	**0.24**	0.28	0.24	0.32
At3g61870	Hypothetical protein	**0.36**	**0.43**	0.43	0.42	0.34
At5g13630	CHL H Mg chelatase subunit, cobalamin biosynthesis protein	**0.36**	**0.40**	0.72	0.40	0.39
At2g36290	Hypothetical protein	**0.38**	**0.38**	0.37	0.38	0.31
At5g44680	3-methyladenine-DNA glycosylase 1-like	**0.21**	**0.15**	0.33	0.21	0.22
At1g67700	Hypothetical protein	**0.25**	**0.24**	0.44	0.40	0.34
At3g62550	ER6 protein-like	**0.41**	**0.27**	0.42	0.33	0.23
At1g09310	RHD1-like, rice susceptibility homeodomain transcription factor	**0.07**	**0.06**	0.32	0.24	0.18
At1g49210	RING zinc finger protein	**0.18**	**0.27**	0.63	0.16	0.23
At5g48250	Zinc finger protein constans-like 14	**0.27**	**0.26**	0.23	0.21	0.29
At5g35480	Hypothetical protein	**0.26**	**0.18**	0.32	0.33	0.25
At2g15970	Cold acclimation protein WCOR413-like	**0.22**	**0.21**	0.37	0.18	0.42
At5g43270	SQUAMOSA promoter-binding protein-like 2	**0.49**	**0.58**	0.28	0.22	0.35
At4g16985	Probable resistance protein	**0.33**	**0.34**	0.36	0.23	0.46
At1g01430	Hypothetical protein	**0.24**	**0.22**	0.45	0.31	0.35
At5g16030	Hypothetical protein with poly glutamic acid stretch	**0.17**	**0.13**	0.36	0.12	0.20
At1g18620	Hypothetical protein	**0.18**	**0.15**	0.28	0.18	0.22
At2g28900	Putative membrane channel protein	**0.05**	**0.03**	0.67	0.30	0.34
At5g09660	Microbody NAD-dependent malate dehydrogenase-like	**0.21**	**0.30**	0.26	0.22	0.26

Table B-1 contd.

Induced by the TTSS of DC3000:

At3g11340	UDP-glucuronosyltransferase-like, salicylate-induced	**10.69**	**24.80**	1.89	15.32	4.75
At3g57260	PR-2 endo beta 1,3 glucanase, BGL2	**8.63**	**6.57**	2.53	8.24	3.47
At5g19230	Hypothetical protein	**2.22**	**3.33**	3.20	6.02	3.23
At3g50930	AAA-type ATPase-like protein	**6.05**	**6.20**	2.68	3.84	2.57
At5g39670	Calcium-binding calmodulin-like protein	**13.42**	**14.10**	3.09	4.55	3.13
At2g43590	PR-3-like endochitinase	**7.86**	**12.66**	5.45	7.71	4.13
At1g63840	RING-H2 finger protein	**7.59**	**9.08**	2.29	5.55	2.58
At3g49120	prxCb peroxidase	**10.41**	**12.04**	1.50	6.95	3.46
At3g29030	Expansin-like	**9.18**	**5.50**	2.26	3.35	1.13
At2g14610	PR-1, pathogenesis-related protein 1	**14.78**	**19.03**	2.52	11.71	2.38
At1g29330	Erd2 ER lumen protein-retaining HDEL receptor	**2.11**	**2.42**	3.66	4.78	3.91
At5g60900	S-receptor kinase homolog 2	**3.25**	**3.03**	1.43	2.78	1.80
At2g17500	Hypothetical protein	**7.89**	**10.67**	8.56	10.50	8.18
At5g02020	Hypothetical protein	**1.93**	**1.21**	2.59	1.77	1.58
At4g34200	D-3-phosphoglycerate dehydrogenase	**2.93**	**4.58**	2.22	3.55	2.20
At1g17745	D-3-phosphoglycerate dehydrogenase	**14.56**	**17.45**	2.57	5.02	1.49
At1g72890	TIR-NBS-LRR disease resistance-like protein	**3.65**	**4.29**	2.62	2.96	2.55
At3g22231	Hypothetical protein	**5.44**	**5.66**	3.10	2.34	1.74
At2g42890	Putative RNA-binding protein, AtMEI2	**2.36**	**2.37**	1.35	2.01	2.19
At5g05730	ASA1 Anthranilate synthase alpha subunit-like	**4.65**	**6.75**	2.57	2.78	3.18
At3g44720	Chloroplast prephenate dehydratase-like	**5.67**	**4.42**	2.67	4.00	3.98
At1g74710	ICS1 isochorismate synthase 1, Salicylic Acid (SA) biosynthesis	**10.32**	**12.73**	4.25	5.55	4.60
At3g26840	Hypothetical protein	**9.04**	**9.83**	3.59	4.95	3.53
At5g10630	FPF1 flower promotion factor-like	**4.51**	**7.63**	6.63	17.62	7.94
At1g61820	Beta-glucosidase-like	**8.10**	**24.09**	5.71	24.82	13.74
At5g54810	trpB tryptophan synthase beta subunit (TSB)-like	**2.50**	**3.75**	1.75	2.84	2.08

Table B-1 contd.

At5g40530	Hypothetical protein	**1.65**	**2.33**	3.96	2.88	2.56
At3g14050	RSH2 RelA/SpoT homolog; interacts with RPP5	**5.99**	**4.61**	2.60	2.46	2.87
At2g30770	Putative cytochrome P450	**17.00**	**33.64**	2.19	11.45	6.93
At4g37010	CALTRACTIN (CENTRIN)-like, cytoskeletal protein	**13.33**	**24.06**	3.01	7.05	4.16
At5g17760	AAA-type ATPase-like protein-like	**6.16**	**6.94**	5.49	7.58	2.89
At1g13340	Hypothetical protein	**9.89**	**11.87**	7.06	3.40	5.83
At5g07440	Glutamate dehydrogenase-like	**9.07**	**10.35**	6.79	7.92	8.26
At5g57900	Hypothetical protein	**3.20**	**3.43**	4.82	5.92	4.89
At2g47130	Putative alcohol dehydrogenase	**11.15**	**8.80**	2.70	1.97	2.00
At3g60450	Similar to pRIB5 black-currant-ripening induced	**3.99**	**3.97**	2.56	1.94	2.05
At5g25250	Nodulin-like protein	**8.68**	**12.76**	1.92	3.22	2.12
At4g36990	Heat shock transcription factor (HSF4)-like	**12.53**	**14.63**	2.42	2.57	2.25
At4g12720	Growth-factor-like protein containing a mutT domain	**5.40**	**6.38**	4.10	2.73	2.84
At3g60440	Similar to pRIB5 black-currant-ripening induced	**5.23**	**6.73**	4.23	3.75	3.44
At1g27730	zat10; salt-tolerance zinc finger protein STZ/ZAT10	**1.84**	**1.40**	1.02	2.70	2.58
At2g04430	Antisense basic fibroblast growth factor-like	**6.31**	**7.80**	3.15	2.71	2.59
At4g39670	Hypothetical protein	**13.92**	**17.71**	1.50	7.07	3.22
At3g52430	PAD4, phytoalexin deficient 4	**5.67**	**4.70**	5.15	3.85	3.82
At1g68620	PrMC3-like	**20.84**	**20.76**	3.44	9.05	5.54
At2g35940	Homeotic BEL1-like protein	**2.51**	**2.44**	2.40	3.96	2.37
At4g17500	AtERF-1, ethylene response factor 1	**3.18**	**3.62**	3.09	4.24	3.42
At4g39950	Putative cytochrome P450	**11.28**	**15.45**	2.82	4.63	3.64
At5g13330	AP2 domain transcription factor-like	**5.03**	**5.66**	3.65	7.52	5.52
At1g23850	Hypothetical protein	**2.19**	**1.51**	7.16	4.11	4.73
At3g06500	Putative neutral invertase	**1.59**	**2.02**	6.26	10.27	6.91
At4g21830	Transcription regulator Ycl033cp-like	**5.22**	**2.93**	13.94	92.03	18.89
At1g52890	Non apical meristem-like transcription factor (NAM)-like	**6.22**	**6.20**	11.38	21.98	16.48

Table B-1 contd.

At1g19180	Similar to PnFL-2 and Prg1	**2.40**	**3.06**	6.34	9.06	9.52
At3g44870	BcCJS1-like	**1.47**	**1.83**	12.09	39.32	21.38
At1g02450	NIMIN-1, NPR1 interacting protein 1	**9.32**	**7.12**	3.08	4.47	2.57
At5g26340	Putative monosaccharide/H+ symporter (STP13)	**8.41**	**7.14**	3.60	3.17	5.16
At5g60800	ATFP3-like, putative metal-binding protein	**5.13**	**3.75**	13.00	9.70	6.43
At1g33960	AIG1, avirulence induced gene 1	**25.47**	**39.75**	6.28	16.90	8.22
At3g60420	SIR4-like, Peronospora-induced gene	**21.94**	**25.56**	9.15	19.90	8.40
At4g02380	Late embryogenesis abundant protein homolog	**5.86**	**5.80**	4.48	5.86	3.93
At2g24850	Tyrosine transaminase-like	**15.96**	**20.71**	24.19	107.53	28.06
At1g77760	NR1, nitrate reductase 1	**2.01**	**1.63**	4.55	18.01	4.59
At5g08790	ATAF2 NAC domain tramscription factor	**3.47**	**4.62**	4.66	12.00	4.53

Each data column in bold text lists the average ratios from two biological replicates. Other data columns list the average ratios from two technical replicates of the same biological sample (a, b or c). Experiments: I: AvrPphEPto-342 plants/Col gl1 plants 24 h after spraying with 30 μM dexamethasone; II: AvrPphEPto-422 plants/Col gl1 plants 24 h after spraying with 30 μM dexamethasone; IV: DC3000/hrpS mutant in Col-0 plants, 12, 24 and 36 h pooled.

Table B-2. *Arabidopsis* **genes repressed or induced in a type III secretion-dependent manner by** *Pst* **DC3000 infection and AvrPphEPto expression (0.1 µM DEX)**

Locus	Description	I	II	IIIa	IIIb	IIIc
Repressed by the TTSS of DC3000:						
At2g38540	LTP1 non-specific lipid transfer protein 1, cell wall-localized	**0.92**	**0.73**	0.25	0.22	0.24
At1g72610	GLP1 germin-like protein 1, cell wall-localized	**1.10**	**0.75**	0.23	0.16	0.33
At1g12090	Extensin-like protein	**0.50**	**0.50**	0.37	0.19	0.28
At2g10940	Proline-rich protein, extensin-like protein	**0.52**	**0.57**	0.24	0.15	0.26
At1g03870	Putative arabinogalactan-protein, fasciclin-like	**0.61**	**0.58**	0.24	0.19	0.29
At3g45970	At-EXPL1, expansin-like protein 1	**0.44**	**0.65**	0.29	0.07	0.41
At1g29660	Proline-rich protein APG-like, extracellular GDSL-motif lipase/hydrolase-like	**0.79**	**0.74**	0.50	0.24	0.32
At3g16370	Proline-rich protein APG-like, extracellular GDSL-motif lipase/hydrolase-like	**0.89**	**0.69**	0.19	0.13	0.23
At5g45950	Proline-rich protein APG-like, extracellular GDSL-motif lipase/hydrolase-like	**0.71**	**0.80**	0.33	0.35	0.42
At1g29670	Proline-rich protein APG-like, extracellular GDSL-motif lipase/hydrolase-like	**0.47**	**0.47**	0.32	0.19	0.28
At1g68560	XYL1, alpha-xylosidase 1	**0.88**	**0.48**	0.47	0.32	0.37
At4g23820	Putative polygalacturonase	**0.77**	**0.50**	0.26	0.15	0.28
At1g04040	Putative acid phosphatase	**0.70**	**0.71**	0.42	0.16	0.23
At5g44020	Putative acid phosphatase	**0.69**	**0.53**	0.23	0.18	0.26
At2g37450	MtN21 nodulin-like	**0.70**	**0.68**	0.45	0.35	0.34
At4g08950	Phosphate-induced protein 1 (phi-1)-like	**1.61**	**0.75**	0.30	0.22	0.43
At4g34260	Putative large secreted protein	**0.59**	**0.61**	0.26	0.24	0.25
At5g15350	Putative blue copper-binding protein	**0.81**	**0.79**	0.27	0.25	0.26
At3g07460	Hypothetical protein	**0.72**	**0.61**	0.35	0.24	0.34
At4g17340	Putative water channel protein	**0.70**	**0.38**	0.25	0.18	0.29
At3g16240	Putative tonoplast water channel protein	**0.67**	**0.46**	0.26	0.13	0.20
At2g19860	AtHXK2, hexokinase 2	**0.70**	**0.51**	0.19	0.21	0.25
At5g02160	Hypothetical protein	**0.77**	**0.75**	0.38	0.18	0.33
At5g56500	CPN-60 BETA 60kDa RuBisCO chaperonin	**0.98**	**1.02**	0.22	0.16	0.33

Table B-2 contd.

At4g01310	Plastid ribosomal proteins, L5P family	**0.81**	**0.64**	0.48	0.35	0.34
At1g33290	Putative ATPase	**0.97**	**0.67**	0.43	0.46	0.25
At1g14150	Putative oxygen-evolving enhancer protein 3-2 of photosystem II	**0.65**	**0.58**	0.37	0.33	0.29
At3g26060	Peroxiredoxin Q-like protein	**0.60**	**1.05**	0.49	0.32	0.35
At5g58260	Hypothetical protein	**0.58**	**0.67**	0.37	0.55	0.29
At1g20020	Putative FNR ferredoxin--NADP+ reductase	**1.00**	**1.00**	0.69		
At3g63140	Putative mRNA-binding protein	**0.66**	**0.74**	0.30	0.32	0.28
At5g23060	Unknown protein containing a rhodanese homology domain	**0.92**	**0.80**	0.22	0.28	0.26
At2g29630	Putative THIC thiamine biosynthesis protein	**0.69**	**0.66**	0.28	0.24	0.32
At3g61870	Hypothetical protein	**0.80**	**0.92**	0.43	0.42	0.34
At5g13630	CHL H Mg chelatase subunit, cobalamin biosynthesis protein	**1.05**	**1.01**	0.44	0.40	0.39
At2g36290	Hypothetical protein	**0.78**	**0.63**	0.37	0.38	0.31
At5g44680	3-methyladenine-DNA glycosylase 1-like	**1.19**	**0.79**	0.35	0.14	0.22
At1g67700	Hypothetical protein	**0.69**	**0.79**	0.44	0.40	0.34
At3g62550	ER6 protein-like	**1.02**	**1.14**	0.32	0.26	0.18
At1g09310	RHD1-like, rice susceptibility homeodomain transcription factor	**0.96**	**0.59**	0.32	0.24	0.18
At1g49210	RING zinc finger protein	**0.81**	**0.75**	0.26	0.16	0.23
At5g48250	Zinc finger protein constans-like 14	**0.69**	**0.49**	0.23	0.21	0.29
At5g35480	Hypothetical protein	**1.02**	**0.93**	0.32	0.33	0.25
At2g15970	Cold acclimation protein WCOR413-like	**0.98**	**0.65**	0.37	0.18	0.42
At5g43270	SQUAMOSA promoter-binding protein-like 2	**1.01**	**0.96**	0.28	0.22	0.35
At4g16985	Probable resistance protein	**0.95**	**0.67**	0.36	0.23	0.46
At1g01430	Hypothetical protein	**1.49**	**0.77**	0.45	0.31	0.35
At5g16030	Hypothetical protein with poly glutamic acid stretch	**1.22**	**0.54**	0.36	0.12	0.20
At1g18620	Hypothetical protein	**0.75**	**0.61**	0.28	0.18	0.22
At5g06690	Thioredoxin-like 5, M-type	**0.72**	**0.98**	0.42	0.40	0.35
At1g09340	mRNA-binding protein CSP41 precursor-like	**0.86**	**0.71**	0.24	0.33	0.25

Table B-2 contd.

At2g28900	Putative membrane channel protein	**0.69**	**0.72**	0.34	0.30	0.34
At5g09660	Microbody NAD-dependent malate dehydrogenase-like	**0.77**	**0.95**	0.48	0.33	0.30
Induced by the TTSS of DC3000:						
At3g11340	UDP-glucuronosyltransferase-like, salicylate-induced	**1.77**	**4.56**	2.79	15.32	4.75
At3g57260	PR-2 endo beta 1,3 glucanase, BGL2	**3.33**	**5.06**	2.17	4.91	3.27
At5g19230	Hypothetical protein	**0.54**	**0.73**	3.20	6.02	3.23
At3g50930	AAA-type ATPase-like protein	**1.05**	**1.68**	2.68	3.84	2.57
At5g39670	Calcium-binding calmodulin-like protein	**1.98**	**3.54**	3.09	4.55	3.13
At2g43590	PR-3-like endochitinase	**1.50**	**1.50**	5.45	7.71	4.13
At1g63840	RING-H2 finger protein	**1.83**	**2.53**	2.29	5.55	2.58
At3g49120	prxCb peroxidase	**1.37**	**3.67**	2.01	6.95	3.46
At3g29030	Expansin-like	**4.63**	**3.37**	2.26	3.35	1.13
At2g14610	PR-1, pathogenesis-related protein 1	**4.89**	**11.18**	2.52	11.71	2.38
At1g29330	Erd2 ER lumen protein-retaining HDEL receptor	**1.01**	**1.75**	3.66	4.78	3.91
At5g60900	S-receptor kinase homolog 2	**2.35**	**3.38**	1.85	2.78	1.80
At2g17500	Hypothetical protein	**0.66**	**1.42**	8.56	10.50	8.18
At5g02020	Hypothetical protein	**1.15**	**0.88**	2.59	1.77	1.58
At4g34200	D-3-phosphoglycerate dehydrogenase	**1.09**	**1.51**	2.03	4.23	2.19
At1g17745	D-3-phosphoglycerate dehydrogenase	**2.74**	**3.98**	2.57	5.02	1.49
At1g72890	TIR-NBS-LRR disease resistance-like protein	**1.34**	**1.31**	2.62	2.96	2.55
At3g22231	Hypothetical protein	**5.98**	**4.40**	3.10	2.34	1.74
At2g42890	Putative RNA-binding protein, AtMEI2	**1.02**	**1.02**	1.70	2.01	2.19
At5g05730	ASA1 Anthranilate synthase alpha subunit-like	**2.30**	**1.31**	2.57	2.78	3.18
At3g44720	Chloroplast prephenate dehydratase-like	**1.22**	**1.46**	2.67	4.00	3.98
At1g74710	ICS1 isochorismate synthase 1, Salicylic Acid (SA) biosynthesis	**3.38**	**2.84**	4.25	5.55	4.60
At3g26840	Hypothetical protein	**1.36**	**3.08**	3.59	4.95	3.53

Table B-2 contd.

At5g10630	FPF1 flower promotion factor-like	**1.14**	**1.78**	7.74	13.33	7.32
At1g61820	Beta-glucosidase-like	**0.99**	**1.63**	5.71	24.82	13.74
At5g54810	trpB tryptophan synthase beta subunit (TSB)-like	**1.22**	**1.49**	1.75	2.84	2.08
At5g40530	Hypothetical protein	**1.07**	**1.41**	3.96	2.88	2.56
At3g14050	RSH2 RelA/SpoT homolog; interacts with RPP5	**0.81**	**0.71**	2.60	2.46	2.87
At2g30770	Putative cytochrome P450	**2.59**	**4.14**	2.19	11.45	6.93
At4g37010	CALTRACTIN (CENTRIN)-like, cytoskeletal protein	**3.12**	**8.62**	3.01	7.05	4.16
At5g17760	AAA-type ATPase-like protein-like	**1.76**	**2.52**	5.49	7.58	2.89
At1g13340	Hypothetical protein	**1.45**	**2.10**	7.06	3.40	5.83
At5g07440	Glutamate dehydrogenase-like	**0.82**	**1.04**	3.25	5.63	6.48
At5g57900	Hypothetical protein	**1.25**	**1.73**	4.82	5.92	4.89
At2g47130	Putative alcohol dehydrogenase	**3.30**	**2.52**	2.70	1.97	2.00
At3g60450	Similar to pRIB5 black-currant-ripening induced	**1.52**	**1.50**	2.56	1.94	2.05
At5g25250	Nodulin-like protein	**1.42**	**2.73**	1.92	3.22	2.12
At4g36990	Heat shock transcription factor (HSF4)-like	**2.62**	**1.89**	2.42	2.57	2.25
At4g12720	Growth-factor-like protein containing a mutT domain	**1.20**	**1.17**	4.10	2.73	2.84
At3g60440	Similar to pRIB5 black-currant-ripening induced	**1.42**	**1.38**	4.23	3.75	3.44
At1g27730	zat10; salt-tolerance zinc finger protein STZ/ZAT10	**0.32**	**0.56**	1.03	2.70	2.58
At2g04430	Antisense basic fibroblast growth factor-like	**1.05**	**2.09**	3.15	2.71	2.59
At4g39670	Hypothetical protein	**1.01**	**1.74**	1.99	7.07	3.22
At3g52430	PAD4, phytoalexin deficient 4	**1.54**	**1.83**	5.15	3.85	3.82
At1g68620	PrMC3-like	**2.24**	**2.53**	3.44	9.05	5.54
At2g35940	Homeotic BEL1-like protein	**1.42**	**1.09**	2.40	3.96	2.37
At4g17500	AtERF-1, ethylene response factor 1	**1.31**	**0.83**	3.09	4.24	3.42
At4g39950	Putative cytochrome P450	**1.85**	**1.93**	2.82	4.63	3.64
At5g13330	AP2 domain transcription factor-like	**1.12**	**2.06**	3.65	7.52	5.52
At1g23850	Hypothetical protein	**1.44**	**1.89**	6.80	18.02	5.98

Table B-2 contd.

At3g06500	Putative neutral invertase	**0.40**	**1.09**	6.26	10.27	6.91
At4g21830	Transcription regulator Ycl033cp-like	**1.86**	**1.48**	13.94	92.03	18.89
At1g52890	Non apical meristem-like transcription factor (NAM)-like	**0.39**	**0.69**	8.40	20.22	17.34
At1g19180	Similar to PnFL-2 and Prg1	**0.33**	**0.89**	6.99	11.96	10.14
At3g44870	BcCJS1-like	**1.43**	**2.07**	12.09	39.32	21.38
At1g02450	NIMIN-1, NPR1 interacting protein 1	**4.32**	**7.85**	3.08	4.47	2.57
At5g26340	Putative monosaccharide/H+ symporter (STP13)	**1.49**	**1.30**	3.60	3.17	5.16
At5g60800	ATFP3-like, putative metal-binding protein	**2.51**	**2.92**	13.00	9.70	6.43
At1g33960	AIG1, avirulence induced gene 1	**5.33**	**4.22**	6.28	16.90	8.22
At3g60420	SIR4-like, Peronospora-induced gene	**4.30**	**4.93**	9.15	19.90	8.40
At4g02380	Late embryogenesis abundant protein homolog	**1.40**	**1.19**	2.90	4.61	3.83
At2g24850	Tyrosine transaminase-like	**5.22**	**10.93**	24.19	107.53	28.06
At1g77760	NR1, nitrate reductase 1	**0.62**	**1.25**	5.15	11.95	3.53
At5g08790	ATAF2 NAC domain tramscription factor	**0.27**	**0.48**	4.85	9.13	3.94

Each data column in bold text lists the average ratios from two biological replicates. Other data columns list the average ratios from two technical replicates of the same biological sample (a, b or c). Experiments: I: AvrPphEPto-342 plants/Col gl1 plants 24 h after spraying with 0.1 μM dexamethasone; II: AvrPphEPto-422 plants/Col gl1 plants 24 h after spray with 0.1 μM dexamethasone; IV: DC3000/hrpS mutant in Col-0 plants, 12, 24 and 36 h pooled.

Table B-3: *Arabidopsis* proteins that interact with AvrPphE$_{Pto}$ in yeast

Interactor ID	**At locus**	**Description**	**SALK knockout lines**
EIP 1	At5g20480 (3,096 bp)	Receptor protein kinase-like	SALK_044334, SALK_044305, SALK_044338, SALK_068675;
EIP 2	At3g51650 (2,480 bp)	Putative protein	SALK_037943, SALK_037935, SALK_079737, SALK_085968
EIP 3	At2g27210 (2,148 bp)	Putative phosphoprotein phosphatase	SALK_071689, SALK_072431, SALK_072437

Interactors were identified in a yeast two-hybrid screen using a LexA-based system (Clontech, Palo Alto, CA). Two independent libraries (kindly provided by Jonathan Jones) made using infected and uninfected *Landsberg erecta* plants were screened. None of these proteins autoactivated reporters in the absence of AvrPphE$_{Pto}$.

MKLSFSLVFNALTLLLQVCIFAQARFSNETDMQALLEFKSQVSENNKREVLAS
WNHSSPFCNWIGVTCGRRRERVISLNLGGFKLTGVISPSIGNLSFLRLLNLADNSF
GSTIPQKVGRLFRLQYLNMSYNLLEGRIPSSLSNCSRLSTVDLSSNHLGHGVPSEL
GSLSKLAILDLSKNNLTGNFPASLGNLTSLQKLDFAYNQMRGEIPDEVARLTQM
VFFQIALNSFSGGFPPALYNISSLESLSLADNSFSGNLRADFGYLLPNLRRLLLGTN
QFTGAIPKTLANISSLERFDISSNYLSGSIPLSFGKLRNLWWLGIRNNSLGNNSSSG
LEFIGAVANCTQLEYLDVGYNRLGGELPASIANLSTTLTSLFLGQNLISGTIPHDIG
NLVSLQELSLETNMLSGELPVSFGKLLNLQVVDLYSNAISGEIPSYFGNMTRLQK
LHLNSNSFHGRIPQSLGRCRYLLDLWMDTNRLNGTIPQEILQIPSLAYIDLSNNFL
TGHFPEEVGKLELLVGLGASYNKLSGKMPQAIGGCLSMEFLFMQGNSFDGAIPDI
SRLVSLKNVDFSNNNLSGRIPRYLASLPSLRNLNLSMNKFEGRVPTTGVFRNATA
VSVFGNTNICGGVREMQLKPCIVQASPRKRKPLSVRKKVVSGICIGIASLLLIIIV
ASLCWFMKRKKKNNASDGNPSDSTTLGMFHEKVSYEELHSATSRFSSTNLIGSG
NFGNVFKGLLGPENKLVAVKVLNLLKHGATKSFMAECETFKGIRHRNLVKLITV
CSSLDSEGNDFRALVYEFMPKGSLDMWLQLEDLERVNDHSRSLTPAEKLNIAID
VASALEYLHVHCHDPVAHCDIKPSNILLDDDLTAHVSDFGLAQLLYKYDRESFL
NQFSSAGVRGTIGYAAPEYGMGGQPSIQGDVYSFGILLLEMFSGKEPTDESFAGD
YNLHSYTKSILSGCTSSGGSNAIDEGLRLVLQVGIKCSEEYPRDRMRTDEAVRELI
SIRSKFFSSKTTITESPRDAPQSSPQEWMLNTDMHTM

Figure B-1: Predicted structural domains of At5g20480, the receptor-like protein kinase that interacts with AvrPphE$_{Pto}$. Sequence colored in red denote transmembrane regions; sequence in blue denote LRR repeats; sequence highlighted in green indicates the protein kinase ATP binding region signature, and sequence highlighted in yellow indicates –the Serine/Threonine protein kinases active-site signature. Predictions for the kinase domain and signatures were obtained using ScanProsite (http://us.expasy.org/cgi-bin/scanprosite).

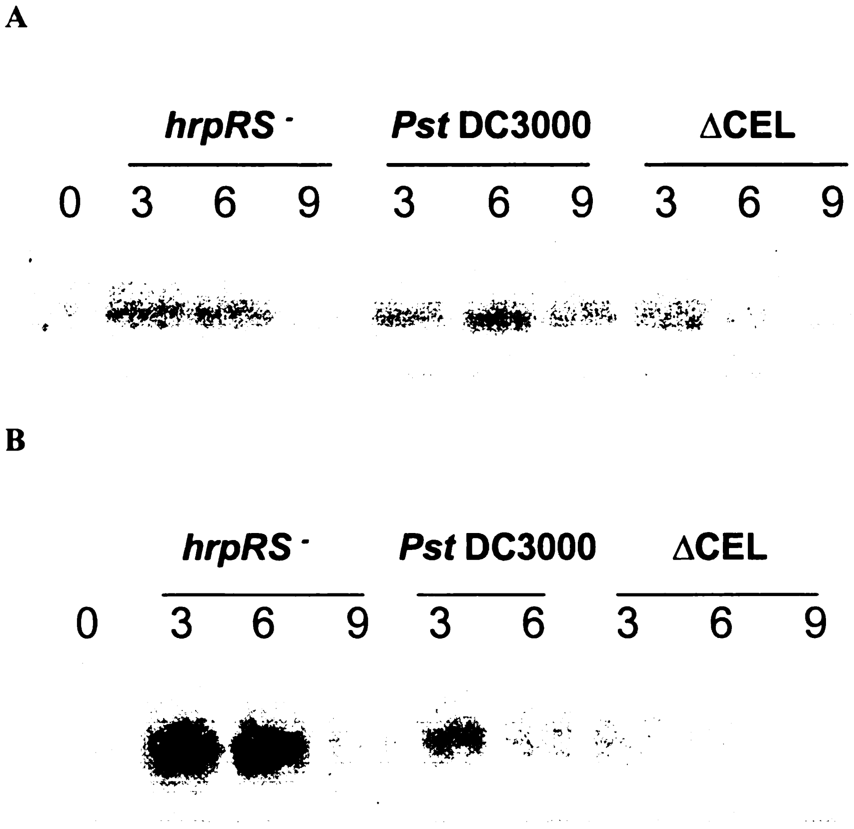

Figure B-2: Northern blot analysis of total RNA isolated from *Arabidopsis* plants infected with *Pst* DC3000, the non-pathogenic *hrpRS* mutant, and the ΔCEL mutant with (A) At3g51650 and (B) At2g27210 as probes. The transcript for At3g51650, encoding a hypothetical protein, showed early and transient induction during infection by *hrpRS* and ΔCEL mutants but retained elevated transcript levels during *Pst* DC3000 infection. The putative phosphoprotein phosphatase transcript was strongly induced by the *hrpRS* mutant, mildly induced by *Pst* DC3000, and almost not induced by the ΔCEL mutant.