STUDY
OF THE VIRULENCE PROTEIN AVRE
IN *PSEUDOMONAS SYRINGAE* PATHOGENESIS

By

Francisco José Uribe Romeo

A DISSERTATION

Submitted to Michigan State University
in partial requirements
for the degree of

DOCTOR OF PHILOSOPHY

Biochemistry and Molecular Biology

2011

ABSTRACT

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AvrE is an effector protein injected into the plant cell by the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 through the type three secretion system to promote disease. AvrE and its orthologs in other species of pathogenic bacteria, such as DspE in *Erwinia amylovora* and WtsE in *Pantoea stewartii*, are major virulence factors. Deletion of genes encoding AvrE-family effectors often greatly reduces the virulence of bacterial pathogens. Despite their importance in bacterial pathogenesis, the virulence functions of AvrE-family effectors remain enigmatic.

This dissertation describes a detailed sequence-function study of the AvrE protein, which does not share any significant overall sequence similarities with other proteins, except for other members of the AvrE family. Several sequence motifs and conserved amino acid residues were identified and shown to be important for AvrE function. Most intriguing was the finding that the N-terminal and central regions of AvrE contain two WxxxE motifs, which are also found in some mammalian pathogen effectors that function as novel guanine nucleotide exchange factors (GEFs). Site-directed mutagenesis and pathogenesis assays were performed, yielding information about the essential amino acid features required for the function of the WxxxE motif in AvrE. In the C-terminus of AvrE, another conserved motif, LKKxGxE, was found. Results

from site-directed mutagenesis and pathogenesis assays suggest that a pair of lysine residues in this motif is essential for the virulence function of AvrE.

Transgenic expression of AvrE was found to be toxic to plant and yeast cells, suggesting that the host targets of AvrE may be conserved among eukaryotes. A large-scale screen was performed to identify Arabidopsis proteins that were capable of suppressing AvrE toxicity in yeast, by cotransforming *avrE* and an Arabidopsis cDNA library into yeast cells. In addition, non-toxic, truncated AvrE derivatives were used in yeast two-hybrid screens to identify Arabidopsis proteins that could interact with AvrE. Although several candidate host proteins were isolated, further studies to determine their relevance to the AvrE function during bacterial infection gave no definitive answers. Nonetheless, an AvrE fragment spanning the first 522 amino acids interacted with the Arabidopsis protein Rad23-A, a known interactor of another *Pst* DC3000 effector, HopM1. Previous studies have shown that AvrE and HopM1, although dissimilar in protein sequences, are functionally redundant, presumably because they affect the same host cellular processes. Rad23-A was found to be degraded during infection of Arabidopsis plants with *Pst* DC3000, but degradation was not observed when plants were infected with the ΔCEL mutant bacteria, in which both *avrE* and *hopM1* are deleted. The ΔCEL mutant complemented with either the *avrE* or *hopM1* gene induced degradation of Rad23-A during infection. Rad23-A may be used as a molecular marker for the virulence function of AvrE and HopM1 in *Pst* DC3000 infection of Arabidopsis.

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DEDICATION

"We are like dwarfs standing upon the shoulders of giants,

and so able to see more and see farther than the ancients."

Bernardus Carnotensis
XII Century

a Marcela…

a mis padres…
a mi abuelo…

Gracias!

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ARF	ADP ribosylation factor
AtMIN	*Arabidopsis thaliana* HopM1 interactor
avr	avirulence gene
BFA	Brefeldin A
CEL	Conserved effector locus
DC3000	Pseudomonas syringae *pv.* tomato
ΔCEL	*Pst* DC3000 mutant with a partial deletion mutation in the CEL.
EEL	Exchangeable effector locus
EFR1	Plant receptor for EF-TU
EF-TU	Bacterial translation elongation factor TU
ETI	Effector triggered immunity
FLS2	Plant receptor for flagellin
GEF	Guanine exchange factor
HR	Hypersensitive response
hrp	Hypersensitive response and pathogenicity
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
NB-LRR	Nucleotide binding-leucine-rich repeats
NMR	Nuclear magnetic resonance
ORF	Open reading frame
PAGE	Polyacrilamide gel electrophoresis
PBS1	*avrPphB* susceptible 1. An Arabidopsis R gene.
PCR	Polymerase chain reaction
***Pst* DC3000**	*Pseudomonas syringae* pv. *tomato*
R	Plant disease resistance gene
ROS	Reactive oxygen species
TCA	Trichloroacetic acid
TTSS	Type three secretion system
Y2H	Yeast two-hybrid

CHAPTER 1

Literature Review

Introduction

Historically, the driving force behind the study of plant pathogenesis has been the losses produced by plant diseases in economically important crops. The attitude of modern plant pathogenicity researchers is best illustrated by the words of J.Z. Zadoks: "We want to be relevant" (Zadoks, 1985).

The Irish Potato Famine of the 19th century provides us with an extreme situation of how a plant pathogen is not only capable of severe decimation of a plant crop, but also to produce strong economical and social impact along with it (Fraser 2003). Dramatic examples like this one are frequently used to show the relevance of the study of plant diseases. Nonetheless, and not withstanding the occasional epidemic, plant pathogens are mostly recurrent factors that have a strong negative impact in crop plants, reducing their production quality and volume (Savary et al. 2006). Worldwide assessments of crop yield losses have been performed in the past decades (Cramer, 1967; Oerke et al. 1994) but due to the continuous changes in farming and control technologies they require continuous updates. Yield losses, due to microbial pathogens, for eight major food and cash crops (wheat, rice, maize, barley, potatoes, soybean, sugar beet and cotton) were recently estimated (Oerke and Dehne 2004) to be from 11% to 22%. Understanding how plant diseases occur and, more importantly, how we could prevent and/or manage plant diseases is therefore critical for meeting the increasing worldwide demand for food and energy.

Plant-pathogen interactions are often described according to the pathogen's ability to produce disease in a given host. When a plant is susceptible to infection, the pathogen is said to

be virulent and the interaction compatible. In an incompatible interaction, the plant is resistant and the pathogen avirulent.

Why are some plants susceptible to disease, whereas others remain resistant? Even more specifically, why are some crop cultivars resistant to a particular pathogen, when related cultivars of the same plant species are not? These are fundamental questions that have attracted the attention of generations of plant pathologists. A major milestone in our understanding into the nature of plant-pathogen interactions was achieved with the advancement of the gene-for-gene disease resistance theory (Flor, 1971). This elegant and simple theory describes the basis of the variable resistance observed among different cultivars of the same plant species to a particular pathogen. According to Flor, some plants carried resistance (*R*) genes that were capable of recognizing corresponding pathogen avirulence (*avr*) genes, resulting in an incompatible plant pathogen interaction. Often, an incompatible reaction is characterized by a fast and localized death of the infected plant cell, known as the hypersensitive response (HR). The HR may be involved in preventing the spread of infection to the rest of the plant. Flor's theory provided a conceptual framework for the observations and work that farmers, and later, plant breeders have been making or doing for centuries: crossing varieties of crops to incorporate desired disease resistance traits (i.e., *R* genes). The theory also guided the mapping and characterization of specific gene loci responsible for resistance to specific pathogens and paved the way for the understanding of plant-pathogen interactions at a molecular level.

The *Arabidopsis thaliana-Pseudomonas syringae* interaction is a good model for studying plant-pathogen interactions

The study of plant-pathogen interactions at the molecular level was made possible by the use of plant-pathogen models that can be studied under controlled laboratory conditions. The Gram-negative bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000) causes bacterial speck on tomato and *Arabidopsis thaliana*. This bacterium represents an important model in molecular plant pathology. On the other hand, the genetic and genomic tractability of Arabidopsis makes this plant a widely accepted model for plant-pathogen studies despite the initial discussion that Arabidopsis is not a natural host of *P. syringae* (Katagiri et al. 2002). Moreover, the *Arabidopsis thaliana-Pseudomonas syringae* interaction behaves according to the conceptual framework of Flor's gene-for-gene hypothesis. Also, sequence and annotation of both the bacterial and plant genomes have been brought to completion and made readily available to the scientific community. Both organisms are easy to grow and manipulate within the laboratory confines, facilitating functional and genetic studies. The advantages of this experimental model outweigh its imperfections, and as such it has allowed answering some of the most important questions regarding plant-pathogen interactions. This is attested by a proven record of high impact research using this model in the past two decades (Block et al. 2008; Cunnac et al. 2009; Mansfield 2009).

Plant-pathogen interactions: An evolutionary arms race

Current knowledge of plant-pathogen interactions may be best understood in terms of an "evolutionary arms race" (Boller and He, 2009). In other words, during the course of millions of years of evolution, a repetitive cycle is believed to have taken place. Plants evolved defenses

against microbes, which in turn evolved ways to overcome these defenses. Plants can evolve new defenses to counter pathogen virulence and pathogens can develop new virulence factors to defeat such defenses (Chisholm et al. 2006; Jones and Dangl 2006; Bent and Mackey 2007). Such an arms race is believed to have played a defining role in shaping the arsenals of both plant defense and pathogen virulence.

In general, it can be said that plants are able to defend themselves from most microbes by recognizing highly conserved microbial molecules upon contact. For detection of microbial molecules (commonly known as microbe-associated molecular patterns or MAMPs) plants use specific cell surface receptors which trigger transduction signal cascades in the plant cell. These signaling cascades in turn result in downstream immune responses that ultimately limit the microbial growth. This MAMP-triggered immunity is regarded as the critical component of the plant immunity. Currently, it is generally accepted that successful plant pathogens evolved strategies to overcome basal plant immunity. In the case of bacterial pathogens, many have developed specialized secretion systems to deliver a variety of virulence factors, such as toxins, effector proteins and DNA that effectively suppress the basal immunity response. In turn, plants have evolved a secondary layer of defense, which is activated only when plants are exposed to pathogens, more specifically, pathogen effector proteins. Recognition of pathogen effector proteins, or their biological activities, is mediated by intracellular receptors encoded by disease resistance (*R*) genes. This effector-triggered immunity is accompanied by immune responses that are faster and stronger than those of basal immunity. In the following pages, I will examine in more detail each of the aforementioned points. Afterwards, I will describe the bacterial effector protein AvrE, which is the focus of this dissertation.

Plant basal immunity: The first layer of defense

Basal immunity is generally regarded as the first line of plant defense. Passive elements of this line of defense include physical barriers to microbial penetration, such as the cell walls or the waxy cuticle of epidermal cells. There are also active components to this line of defense that allow the plant to respond to invading microbes. Lipopolysaccharide (LPS), flagellar filament proteins (flagellin), bacterial transcription elongation factor TU (EF-TU) and chitin are but a few well-known examples of MAMPs that are able to elicit the plant basal immune response. These elicitors are highly conserved among different microbes. The plant receptors for bacterial flagellin and EF-TU (FLS2 and EFR1, respectively) have been identified (Gómez-Gómez and Boller 2000; Zipfel et al. 2006). These receptors are type I transmembrane proteins that display extracellular leucine-rich-repeats (LRR), which are known to mediate protein-protein interactions (Kobe and Deisenhofer, 1994; Kobe and Kajava, 2001). The intracellular kinase domains are involved in activating intracellular signaling, including the MAP kinase signal transduction cascades, leading to modification of gene transcription, and production of defense proteins, reactive oxygen species (ROS), and other antimicrobial compounds (Hückelhoven 2007). These defense responses are accompanied by reorganization of intracellular transport to deliver the newly formed defense molecules into the focal point of infection (Hückelhoven 2007). One microscopically visible response to infection is the formation of structures known as papillae at the point of microbial contact with the plant cell. Papillae represent structures where the plant cell wall has been highly modified by the deposition of callose, phenolic compounds, and ROS that promote cross-linking of cell wall components (Hückelhoven 2007). In the guard cells of the stomata, binding of MAMPs to their receptors translates into closure of the stomatal

aperture. This creates a physical barrier for bacterial infection, which effectively adds to the basal immunity response (Melotto et al. 2006).

Pseudomonas syringae as a model plant pathogen

The plant pathogen *Pseudomonas syringae* is an example of a Gram-negative plant pathogenic proteobacterium. Various *P. syringae* isolates are classified into over 50 different pathovar groups based on their host ranges. Each pathovar represents a compatible interaction with one or more specific plant species. Collectively, *P. syringae* infects virtually all crop species. Disease symptoms often include foliage blight and necrotic spots on plant fruits. Commonly, *P. syringae* enters the plant apoplast through wounds or natural openings such as stomata. The bacterial toxin coronatine, which is produced by five pathovars (Ma et al. 1991), has been demonstrated to induce the opening of stomata to facilitate bacterial invasion (Melotto et al. 2006). Once inside the plant, *P. syringae* injects a battery of bacterial effector proteins, through the type three secretion system (TTSS), into the plant cell to promote disease development (Büttner and He, 2009). The proteins that make up the TTSS are encoded by genes located in a ~25-kb pathogenicity island in the *P. syringae* genome. This pathogenicity island has also been called the *hrp* (hypersensitive response and pathogenicity) gene cluster, since it is required for bacterial elicitation of the HR in resistant plants and causation of disease in susceptible plants (Lindgren et al. 1986). Minimal medium, which is thought to resemble the plant apoplastic environment, induces the expression of *hrp* genes and the formation of the TTSS-associated Hrp pilus on the bacterial surface (Roine et al. 1997). The Hrp pilus functions as a conduit that allows the pathogen to inject effector proteins into the plant cell (Jin and He, 2001). Flanking the *hrp* gene cluster are genes coding for several bacterial effector proteins.

Comparison among all the *P. syringae* isolates studied to date has shown that several effector genes localized next to one side of the *hrp* gene cluster are conserved among most, if not all, *P. syringae* strains. The genomic region that encodes these conserved effectors has been named the Conserved Effector Locus (CEL) (Alfano et al. 2000). On the other side of the *hrp* gene cluster lies a group of effector genes that vary among different *P. syringae* strains. This genomic region is known as the Exchangeable Effector Locus (EEL) (Alfano et al. 2000). In addition to effector genes near the *hrp* gene cluster, there are many effector genes located throughout the *P. syringae* genome. To date, 28 effectors have been confirmed in *Pst* DC3000 (Schechter et al. 2006) and several research groups are working toward elucidating their functions. In general, despite the ongoing efforts, the specific roles in disease of most bacterial effectors remain unclear.

Bacterial effector functions: Suppressing basal immunity and promoting disease

As a general statement, it can be said that bacterial effectors are to fulfill two goals inside the host plant cell. On the one hand, they suppress the immune responses that the host mounts against invading pathogens. On the other hand, they may subvert plant metabolism to promote the nutrition of the pathogen. Current literature is abundant with examples of different molecular mechanisms used by bacterial effectors to suppress plant immunity. In contrast, the literature is rather poor in information regarding how these effectors subvert plant metabolism in order to promote the conditions required for better nutrition and finally promote disease symptoms in the host. Below, I will review some of the strategies used by some of the plant effectors that have been characterized.

AvrPto and AvrPtoB are *P. syringae* DC3000 effectors that have been demonstrated to redundantly block the plant basal immune response. An Arabidopsis line that expresses AvrPto is known to be deficient in basal immunity that is associated with reduced callose deposition in the cell wall (Hauck et al. 2003). The *P. syringae hrp* mutant, which cannot deliver TTSS effectors into the host cell or multiply inside wild-type Arabidopsis plants, was able to multiply significantly in AvrPto-expressing transgenic plants (Hauck et al 2003). Direct expression of AvrPto in protoplasts demonstrated that this effector is capable of suppressing the induction of flagellin-activated plant defense genes (Li et al 2005), which depends on the blockage of MAPK cascade activation immediately after the perception of bacterial flagellin (He et al 2006). Moreover, it was demonstrated that AvrPto binds FLS2 and EFR1, inhibiting the autophosphorylation of the receptor kinases (Xiang et al. 2008). In turn, this results in the inactivation of the MAPK signal transduction cascade and the suppression of all downstream immune responses initiated by FLS2, such as induced gene expression, generation of ROS and callose deposition on the cell wall (Xiang et al. 2008). The other effector, AvrPtoB, can also target the signal cascade triggered by flagellin recognition, using distinctive virulence functions housed in different domains of its protein structure. For example, residues 308 to 387 are required (Xiao et al. 2007) for the effector to suppress the FLS2 triggered MAP kinase signaling (He et al. 2006). Moreover, the C-terminal half of AvrPtoB is structurally similar to eukaryotic E3 ligases and was demonstrated to be capable of acting as an ubiquitin ligase. This activity was demonstrated to be required for bacterial effector function (Janjusevic et al, 2006, Abramovitch et al. 2006). In Arabidopsis, AvrPtoB ubiquitinates the flagellin receptor FLS2, promoting its degradation through the proteasome (Göhre et al. 2008). AvrPtoB has similar effects on other plant protein kinases, such as CERK1 that has been involved in chitin-elicited responses

(Gimenez-Ibañez et al. 2009). These two effectors exemplify a bacterial strategy that targets protein kinases, which are common mediators of signal transduction.

AvrPphB is another example of an effector that disrupts signal transduction immediately after perception of MAMPs. This effector is a papain-like cysteine protease (Zhu et al. 2003) that cleaves the Arabidopsis PBS1 kinase (Shao et al. 2003). It was recently reported that AvrPphB inhibits MAMP-triggered immunity when it is directly expressed in plants. Moreover, AvrPphB is capable of cleaving the cytoplasmic kinase BIK1 and PBS1-like proteins (PBL) (Zhang et al. 2010). BIK1 is a protein that associates with the FLS2/BAK1 receptor complex. Upon flagellin stimulation of FLS2, BIK1 is phosphorylated in a FLS2/BAK dependant manner (Lu et al. 2009). Seedlings from a *bik1* knock-out transgenic line are much less sensitive to flagellin. They also lost flagellin peptide flg22-induced resistance to infection by *Pst* DC3000. In addition, *bik1* seedlings are significantly more susceptible to the non-pathogenic DC3000 *hrc* mutant than wild type plants (Zhang et al. 2010).

The effector HopAI1 provides an example of directly blocking the MAP kinase cascade. This effector binds to MAPK3 and MAPK6, inactivating the kinases through removal of phosphate groups used for their activation. Biochemically, HopAI1 has a novel phosphothreonine lyase activity, and this activity is required for the effector virulence function (Zhang et al. 2007).

The Arabidopsis protein RIN4 is a negative regulator of disease resistance. It prevents the inappropriate activation of immune responses (Belkhadir et al. 2004). This protein is the target of

at least 3 different bacterial effectors: AvrRpt2, AvrRpm1 and AvrB. AvrRpt2 is a cysteine protease (Axtell et al. 2003) that promotes destabilization of RIN4 during infection (Axtell and Staskawicz 2003). RIN4 is normally bound to the plasma membrane through C-terminal acylation or prenylation. AvrRpt2 cleaves the C-terminus of RIN4, releasing it into the cytoplasm where it is subjected to ubiquitination followed by proteasomal degradation (Takemoto and Jones 2005). The effectors AvrRpm1 and AvrB are myristoylated after translocation into the plant cell. This modification is required for the effectors to localize to the membrane and for virulence function (Nimchuk el al. 2000). Both effectors can independently induce the phosphorylation of RIN4 (Mackey et al. 2002). It is not yet known how effector-mediated modifications of RIN4 lead to disease promotion.

HopM1 and AvrE are two *P. syringae* DC3000 effectors involved in the suppression of basal plant immunity (DebRoy et al. 2004). The molecular details for AvrE's virulence function remain obscure. However, HopM1 targets several Arabidopsis proteins for proteasomal degradation (Nomura et al. 2006). One of them, AtMIN7 belongs to the adenosine diphosphate ribosylation factor (ARF) family of guanine exchange factor (GEF) protein (Grebe et al. 2000). ARFs are small GTPases responsible for the budding of intracellular vesicles at the membranes. ARF GTPases are activated when their bound GDP is exchanged by GTP. This exchange triggers the recruitment of structural proteins necessary for vesicle formation. The activation of ARFs is catalyzed by ARF-GEF proteins, such as AtMIN7, that actively promote the dissociation of GDP from and binding of GTP to ARF GTPases (Anders and Jurgens 2008). HopM1 is an example of how an effector can disrupt the plant immune responses at levels other than pathogen perception and the consequent signal transduction cascades. Instead, immune

suppression can be achieved by interfering with vesicular trafficking that is presumably involved in the implementation of the immune response.

Recognition of bacterial effectors: The second layer of plant defense

Some plants have evolved *R* genes that confer resistance to specific pathogens. Most proteins coded by the *R* genes belong to a large family of nucleotide binding-leucine-rich repeat (NB-LRR) proteins, which act as intracellular receptors of pathogen effectors (Dangl and Jones, 2001). There are approximately 125 NB-LRR receptor proteins encoded in the Arabidopsis genome (Jones and Dangl, 2006). Bacterial effectors can be recognized, directly or indirectly through effector actions on host targets, by NB-LRR proteins, resulting in effector-triggered immunity (ETI). ETI is thought to be an accelerated and amplified form of basal immunity that results in disease resistance and HR at the infection site (Jones and Dangl 2006).

Nonetheless, genes coding for NB-LRR proteins are not the only type of *R* genes that plants possess. One notable exception is the example of the *BS3* gene, which encodes a protein similar to a flavin-dependent monooxygenase in some cultivars of pepper (Römer et al. 2007). The transcription of this gene is activated by the binding of the *Xanthomonas campestris* AvrBs3 effector onto its promoter sequence. In addition to binding to the promoter of the *BS3* gene, AvrBs3 binds to the promoter of the *upa20* pepper gene, which acts as a master regulator of plant cell size (Kay et al. 2007). During infection, the transcriptional activation of *upa20* by AvrBs3 results in pepper cell hypertrophy and disease development in susceptible pepper cultivars. However, activation of the *BS3* gene promoter, which is present only in resistance pepper cultivars, triggers ETI (Römer et al. 2007).

Tomato *PTO* is an example of another type of *R* gene. This gene encodes a serine-threonine kinase (Martin et al. 1993) that is able to interact with the bacterial effectors AvrPto (Scofield et al. 1996; Tang et al. 1996) and AvrPtoB (Kim et al. 2002). Pto is able to engage the plant defense responses upon recognition of either effector (Ronald et al. 1992; Kim et al. 2002). This requires the accessory protein Prf, encoded in a locus tightly linked to Pto (Salmeron et al. 1994). Unlike Pto, Prf is a member of the NB-LRR protein family (Salmeron et al. 1996). Upon translocation into the plant cell, AvrPto binds to Pto forming a protein complex (Xing et al. 2007). Protein crystallography and biochemical studies of the AvrPto-Pto complex determined that AvrPto inhibits the kinase activity of Pto. Normal kinase function of Pto is required to inhibit Prf activity, thus keeping the immune response repressed. The formation of the AvrPto-Pto complex frees Prf to initiate downstream signaling which results in ETI, and therefore, in plant immunity (Xing et al. 2007).

RIN4 is another well documented example of an Arabidopsis protein associated with *R* genes. As mentioned above, RIN4 is targeted by three different bacterial effectors that promote the polypeptide's cleavage or phosphorylation (Axtell et al. 2003; Mackey et al. 2002). The Arabidopsis *R* genes *RPS2* and *RPM1* code for proteins that can detect the modifications induced to RIN4 by bacterial effectors, which in turn activate ETI (Kim et al. 2005). In its natural state, RIN4 keeps the resistance protein RPM1 in an inactive state. Phosphorylation of RIN4 triggers RPM1 signaling, as this event abolishes the suppression of RPM1 exerted by RIN4 (Mackey et al. 2003). RIN4 also keeps RPS2 in a repressed state (Axtell and Staskawicz 2003) through physical interaction. Proteolytic cleavage of RIN4, catalyzed by the AvrRpt2 effector (Axtell et

al. 2003) promotes the RIN4-RPS2 complex dissociation, leading to signaling activation by RPS2 (Day et al. 2005).

Initially it was thought that *R* and *avr* gene products would recognize each other based upon a ligand-receptor model. The physical interaction between the polypeptides would trigger plant defenses. This idea was reinforced by the fact that most of the *R* genes code for proteins that have LRR domains and that several examples of direct interaction between pairs of *R* and *avr* gene products were found (Van de Hoorn and Kamoun, 2008). Rice Pi-ta and *Magnaporthe grisea* AvrPi-ta (Jia et al. 2000) or Arabidopsis RRS1-R and *Ralstonia solanacearum* PopP2 (Deslandes et al. 2003) are some examples of direct physical interaction that supported the ligand-receptor model. The major drawback of this model is that for most pairs of *R* and *avr* gene products no direct interaction could be shown and perception was believed to be indirect (Van de Hoorn and Kamoun, 2008). The Guard model was originally proposed to explain the perception of AvrPto by Pto and Prf (Van der Biezen and Jones 1998). Further evidence supporting this model came from the study of protein RIN4 and its interaction with the products of the *R* genes *RPM1* and *RPS2*. In the Guard model, R proteins monitor the function/stability of a host protein (e.g., Pto or Rin4) that is a target of a bacterial effector, triggering ETI when an effector-induced change is perceived by the cognate R protein (Dangl and Jones 2001). For example, Prf monitors the normal kinase function of Pto, which is inhibited by AvrPto, initiating ETI (Xing et al. 2007), whereas RPM1 and RPS2 detect effector-induced phosphorylation or cleavage of RIN4 (Kim et al. 2005; Day et al. 2005). In recent years, the Guard model has shown certain inconsistencies with experimental results. For example, many effectors have multiple host targets and many of the guarded proteins are dispensable for the virulence function of effectors in plants lacking the

corresponding R protein. These observations have brought about the Decoy model (Van de Hoorn and Kamoun, 2008). This new model proposes that plants have evolved proteins that mimic some properties of the true host targets (i.e., important for pathogen virulence) of effectors, but that, unlike the true host targets of effectors, these decoys have no role in pathogen virulence. Nevertheless, NB-LRR proteins would monitor the stability of such decoys, initiating ETI upon effector interaction (Van de Hoorn and Kamoun, 2008). As mentioned above, the *X. campestris* AvrBs3 effector binds to specific DNA sequence elements on host genes promoters, and functions as a transcription factor (Kay et al. 2007). Certain pepper cultivars have acquired the *R* gene *BS3*, which codes for a monooxygenase. The promoter of this gene has the DNA sequence elements recognizable by AvrBS3. Thus, the promoter of *BS3* is a decoy to initiate AvrBS3-mediated ETI (Römer et al. 2007).

AvrE, a conserved effector in *P. syringae*

AvrE is encoded within the Conserved Effector Locus (CEL) of *P. syringae.* As mentioned before, this locus contains a number of genes that encode effector proteins conserved between the divergent *P. syringae* strains studied so far (Alfano et al. 2000). A *Pst* DC3000 mutant, named ΔCEL, lacking part of the CEL, has been created and characterized (Alfano et al. 2000). This strain lacks six genes in the CEL: *avrE, avrF* (encoding the chaperone protein for AvrE secretion), *hopM1, shcM1* (encoding the chaperone for HopM1 secretion), *hrpW* and *hopA1*. This mutant displays greatly reduced pathogenicity in tomato (Alfano et al. 2000) and in Arabidopsis (DebRoy et al. 2004). It has been demonstrated that independent mutations in *hrpW* (Charkowski et al. 1998) and *hopA1* (Badel et al. 2002) do not have a significant impact on *Pst* DC3000 virulence. It was demonstrated in tomato plants infected with *Pst* DC3000, that only

when both *avrE* and *hopM1* are mutated, there is a serious compromise of bacterial virulence (Badel et al. 2006). Furthermore, in Arabidopsis, either *avrE* or *hopM1* can complement the ΔCEL mutant (DebRoy et al. 2004). These results suggest that AvrE and HopM1, despite not sharing sequence similarities, are functionally redundant.

AvrE orthologs are found in other bacterial pathogen species such as WstE in *Pantoea stewartii*, the causal agent of Stewart's Wilt of corn, and DspE in *Erwinia spp*., which cause fire blight of rosaceous plants and soft rot of a wide range of crops. Loss-of-function mutants of *wtsE* and *dspE* show greatly decreased virulence (Bogdanove et al. 1998, Frederick et al. 2001). DspE from *E. amylovora* not only shares sequence identity with AvrE (~26% overall), the two effectors also seem to have a similar function, since AvrE of *Pst* DC3000 is able to partially restore pathogenicity to the *dspE* mutant of *E. amylovora* (Bogdanove et al. 1998).

Translocation of AvrE has been indirectly demonstrated using a type III translocation reporter, as part of screens for TTSS effectors in *P. syringae* (Guttman et al. 2002; Chang et al. 2005). Specifically, AvrE was fused to a secretion-incompetent fragment of the effector AvrRpt2 ($AvrRpt2_{280-255}$). $AvrRpt_{280-255}$ is able to trigger the HR inside Arabidopsis cells expressing the *RPS2* resistance gene (Mudgett and Staskawicz, 1999). Although $AvrRpt2_{280-255}$ is not competent for translocation by itself when expressed in *P. syringae* bacteria, when expressed in *P. syringae* the AvrE-$AvrRpt_{280-255}$ fusion caused an HR, indicating that AvrE carries the type III translocation signal and the AvrE-$AvrRpt_{280-255}$ fusion is delivered into the plant cell (Guttman et al. 2002 and Chang et al. 2005). In *E. amylovora*, type III translocation of DspE has

also been demonstrated, and shown to be facilitated by the DspF chaperone (Gaudriault et al. 1997). It was determined that the first 51 amino acids of DspE were sufficient for minimal type III translocation and that the first 150 amino acids are required for optimal translocation (Tripplet et al. 2009). In *P. stewartii*, the stability of the protein WtsE requires the WtsF chaperone (Ham et al. 2006). In *Pst* DC3000, co-expression of the chaperone AvrF with AvrE is required to restore the virulence of the ΔCEL mutant (DebRoy et al. 2004). In general, TTSS chaperones bind to the N-termini of the nascent effector polypeptides, increasing stability and aiding in effector translocation (Ghosh 2004). TTSS chaperones have hydrophobic surface patches formed by broadly conserved amino acid residues, which act as effector-binding sites. An analysis of AvrF sequence reveals that this chaperone protein displays this type of structural motif (Bogdanove et al. 1998), an observation that is consistent with the idea that AvrF is required for translocation of the AvrE effector into the plant cell.

To date, there are no reports that have identified plant *R* genes associated with a defense response to the bacterial effector proteins HopM1 or AvrE.

The WxxxE family of effector proteins

In this dissertation I will present evidence that links the virulence function of DC3000 AvrE to that of a recently characterized family of animal pathogen effectors. For better understanding of the results I have generated for this dissertation, I summarize the current knowledge available for the aforementioned effector family in animal pathogens.

Sequence alignment of effector sequences of several enteropathogenic bacteria demonstrated that they share a common amino acid sequence motif with the sequence WxxxE,

where xxx represents any three residues (Alto et al. 2006). This effector family has been characterized as modulators of the host cytoskeletal structure in several enteropathogens, such as *Escherichia coli*, *Salmonella typhimurium*, *Citrobacter rodentium*, and *Shigella flexneri*. First attempts to characterize the biochemical function of the WxxxE effectors indicated that they acted as mimics of activated Rho GTPases (Alto el al. 2006). Further studies of the WxxxE motif of the *C. rodentium* EspM2 and EspM3 effectors demonstrated that mutation of the conserved W or E residues abolish their virulence function (Alto et al. 2006). On the other hand, conserved substitutions of Y for W or D for E do not affect their ability to induce changes in the host cell cytoskeleton (Arbeloa et al. 2008). Similarly, substitutions in the xxx sequence do not have any effect on effector function (Arbeloa et al. 2008).

Cytoskeleton disruption by effectors had been previously described in enteropathogenic bacteria. The *S. typhimurium* SopE effector, which is not a WxxxE-family effector, is capable of inducing membrane ruffling indicative of actin cytoskeleton rearrangement. SopE interacts with Rac-1 and CDC42 and promotes the GDP/GTP exchange of these Rho-family GTPases (Hardt et al. 1998). The crystal structure of CDC42 in conjunction with SopE (Buchwald et al 2002) or SopE2 (Williams et al. 2004) showed they belong to a distinctive family of RhoGTPase GEFs. Although they have a conformation that is strikingly similar to that of Rac1 in complex with the eukaryotic Dbl-like GEF Tiam1, the catalytic domain of SopE and SopE2 share an architecture that is completely different from that of Tiam1. The *S. typhimurium* SifA effector belongs to the WxxxE protein family. SifA is capable of interacting with GDP-bound RhoA, promoting actin cytoskeleton rearrangements; it loses its virulence function when W or E is mutated to A (Ohlson et al. 2008). Remarkably, despite sharing no sequence similarity, the crystal structure of the SifA

C-terminus shares a similar fold with *S. typhimurium* SopE, including the spatial disposition of the catalytic loop crucial for GDP/GTP exchange. Interestingly, the WxxxE motif is not part of the catalytic loop (Ohlson et al. 2008). However, Ohlson and collaborators suggested that the WxxxE motif may be a structural feature responsible for three-dimensional structure stabilization. The crystal structure of the Map effector (also belonging to the WxxxE effector family) in conjunction with CDC42 also shows a similar fold (Huang et al. 2009). Furthermore, Huang and coworkers provided biochemical evidence for the GEF activity of Map and showed that this activity is affected by the mutations in the WxxxE motif (Huang et al. 2009). All these results suggest that WxxxE effectors in animal pathogens are GEFs.

The work presented in this dissertation is divided in two chapters. The first chapter presents the results of research aimed at determining the sequence-function relationship in the AvrE effector. In this chapter I show the identification of specific conserved amino acids residues and amino acid motifs that are required for the virulence function of AvrE. The second chapter presents the results of research aimed at identifying putative host targets of the effector AvrE, by screening for Arabidopsis proteins that interact with AvrE. Each chapter is followed by a discussion of the results obtained, and of implications for future research on this particular *P. syringae* DC3000 effector.

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CHAPTER 2

Sequence-Function relationship of the AvrE protein

ABSTRACT

AvrE is an effector protein injected into the plant cell by the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst* DC3000) through the type three secretion system to promote disease. In *Pst* DC3000, AvrE is functionally redundant to another effector protein, HopM1. Other plant pathogens, such as *Erwinia spp.* and *Pantoea stewartii*, have *avrE* orthologues (*dspE* and *wtsE*, respectively). Although AvrE-family effectors are important virulence factors, the biochemical basis of their virulence function remains unknown.

Here I present the results of a sequence-function study for the AvrE protein. I found that the function of AvrE depends on the presence of two WxxxE motifs, which are present in some mammalian pathogen effectors. These two motifs are functionally redundant. Recently, the WxxxE motif in mammalian pathogen effectors has been demonstrated to be a required moiety for effector function as guanine nucleotide exchange factors (GEFs). I found that the spacer sequence of the WxxxE motif (i.e., xxx) can be shortened or expanded to a certain extent without affecting the virulence function of AvrE. At the C-terminus, AvrE contains an LKKxGxE motif. I determined that a pair of lysine residues in this motif is required for virulence function. I will discuss the implications of the requirement of these amino acid motifs as well as other conserved amino acid residues for AvrE function, and how these residues relate to computational predictions of the three-dimensional structure and putative functions of the AvrE protein.

Note:

Dr. Kinya Nomura and Dr. Christy Mecey were involved in producing Figure 2-5 and Figure 2-6.

For Figure 2-5 (page 52) I performed mutagenesis of single amino acids derivatives of AvrE. Dr. Christy Mecey performed mutagenesis that generated the double amino acid AvrE mutants. Afterwards, I performed bacterial transformation and detection of AvrE expression in the ΔCEL mutant. Bacterial growth curves for all single amino acids derivatives of AvrE were performed by me, while Dr. Kinya Nomura performed bacterial growth curves for the double amino acid AvrE mutants. The secretion assays for the double amino acid AvrE mutants, shown in Figure 2-6 (page 54), were done by Dr. Kinya Nomura.

Part of the results presented in Figures 2-5 and 2-6 have been published in collaboration with the laboratories of Dr. David Coplin and Dr. David Mackey at Ohio State University (Ham et al. 2009).

INTRODUCTION

Pseudomonas syringae pv. *tomato* (*Pst* DC3000) relies on the type three secretion system (TTSS) to cause disease in plants. The TTSS delivers bacterial proteins known as effectors into the host cell to overtake plant signaling and/or metabolism (Boller and He, 2009). *Pst* DC3000 harbors a large pathogenicity island where the TTSS-encoding genes (i.e., *hrp* genes) are located (Alfano et al. 2000). This pathogenicity island can be divided into three regions: At the center is a cluster of *hrp* genes that code for the TTSS, and flanking the *hrp* cluster are two clusters of effector genes known as the exchangeable effector locus (EEL) and the conserved effector locus (CEL), respectively. These two loci code for bacterial effectors injected into the host cell. The genes in the CEL are conserved among different strains of *P. syringae*, whereas the genes in the EEL are not (Alfano et al. 2000). It has been observed that the loss of individual effectors does not significantly affect bacterial virulence. This observation has led to the popular belief that either there is extensive effector redundancy or each effector has small, additive contributions to virulence. Further large deletion studies of the DC3000 EEL and CEL demonstrated that deletion of the EEL (ΔEEL) does not hinder the ability of DC3000 to promote disease symptoms in host tomato, but slightly affects bacterial multiplication (Alfano et al. 2000). On the other hand, deletion of the CEL locus (ΔCEL) drastically affects the virulence of DC3000 in tomato. The ΔCEL mutant is not able to produce disease symptoms and multiplies up to 1000 times less than that of wild-type DC3000 (Alfano et al. 2000).

In the ΔCEL mutant, six genes--*avrE, avrF* (coding for the chaperone for AvrE secretion), *hopM1, shcM1* (coding for the chaperone for HopM1 secretion), *hrpW* and *hopA1*--

are replaced by a spectinomycin-resistance-gene cassette (Alfano et al. 2000). Deletion of the effector genes *hrpW* and *hopA1* alone does not impact DC3000 virulence (Charkowski et al. 1998, Badel et al. 2002). In contrast, the ΔCEL mutation can be complemented either by expression of *avrE* plus *avrF* or *hopM1* plus *shcM* from a plasmid. This result indicates that AvrE and HopM1 are functionally redundant and together they are responsible for the reduced virulence of ΔCEL mutant bacterium (DebRoy et al. 2004). Consistent with this observation, individual mutations in *avrE* and *hopM1* had little impact on DC3000 virulence, but double mutations in *avrE* and *hopM1* phenocopied the ΔCEL mutation (Badel et al. 2006).

At the cellular level, both HopM1 and AvrE suppress plant cell wall-based defenses, such as callose (β-1,3 glucan) deposition in response to bacteria (DebRoy et al. 2004). Callose is part of a cell wall structure known as the papilla, which is the focal accumulation point of callose, phenolic compounds and other molecules thought to restrict pathogen fitness and growth (Bestwick et al. 1995), although direct evidence for such a role is lacking. Nevertheless, there is a close relationship between plant defense activation and callose deposition in the cell wall in diverse plant-pathogen interactions. Conversely, virulent pathogens, such as *Pst* DC3000, efficiently suppress callose deposition in a TTSS-dependent manner (Hauck et al. 2003; DebRoy et al. 2004). The ΔCEL mutant is reduced in its ability to suppress callose deposition. These findings suggest that one function of the HopM1 and AvrE effectors is to suppress the plant immune response.

The virulence function of HopM1 has been studied and characterized (Nomura et al. 2006). A yeast two-hybrid (Y2H) screen of an Arabidopsis cDNA library yielded several

proteins that interact with the N-terminal portion of HopM1. The proteins have been dubbed AtMINs (*Arabidopsis thaliana* HopM1 interactors). The disease susceptibility of Arabidopsis knock-out lines for each of the *AtMIN* genes was tested. Only the *atmin7* knock-out plant was more susceptible to the ΔCEL bacterium. This result suggests that HopM1 promotes disease in part by targeting AtMIN7. HopM1 not only interacts with AtMIN7, but also induces the degradation of AtMIN7 through the proteasome (Nomura et al. 2006). AtMIN7 is a member of the ADP ribosylation factor (ARF) family of guanine nucleotide exchange factors (GEFs), which are regulators of the vesicle trafficking system (Grebe et al. 2000; Geldner et al. 2003). ARF-GEFs are inhibited by the well characterized fungal compound Brefeldin A (BFA). BFA is able to physically block the GDP for GTP exchange promoted by GEFs in ARFs proteins (Renault et al. 2003). Interestingly, the growth of the ΔCEL mutant could be almost completely restored in wild type plants treated with BFA. Moreover, callose deposition in response to the ΔCEL mutant in *atmin7* knock-out plants was also impaired (Nomura et al. 2006). This work reinforces the relationship between the modulation of host vesicle trafficking and the promotion of an environment that is amenable to pathogen survival and multiplication (Nomura 2006).

The host targets and virulence mechanism of AvrE remain unknown. AvrE is so far the largest bacterial effector discovered in any pathogen (1,795 aa; ~200 kDa), and has orthologs in other plant pathogens, such as DspE in *Erwinia amylovora* (Gaudrialt et al. 1997) and WtsE in *Pantoea stewartii* (Frederick et al. 2001). However, these other bacterial pathogens do not have effectors that are functionally redundant to AvrE. Thus, mutations in the *dspE* and *wtsE* genes alone are sufficient to render those plant pathogens non-virulent in their host plants (e.g., apple and corn) (Bogdanove et al. 1998, Frederick et al. 2001).

The primary structure of AvrE does not bear significant overall similarities to any other protein, beyond its orthologs. AvrE resembles neither HopM1 nor other effectors of *Pst* DC3000. Moreover, there is no x-ray crystallography or nuclear magnetic resonance (NMR) data that would allow for comparison with the three-dimensional folds of proteins of known function. Very little is known about what parts of the protein are required for the function of AvrE-family effectors. *E. amylovora* DspE is rendered non-functional by in-frame deletions between amino acids G203 to G720 or from T1064 to V1570 (DspE has 1,838 amino acids). Remarkably, the N-terminal deletions (i.e., from G203 to G720) can be complemented in trans by the 5' terminal half of *dspE*. This finding suggests that the N-terminal half of the protein could potentially form a stable functional domain required for bacterial virulence (Bogdanove et al. 1998). The last dozen amino acids in the C-terminus of WtsE have been demonstrated to be important for function. More specifically, a mini-Tn*5gus* transposon insertion replacing the last 12 amino acids renders WtsE non-functional (Ham et al. 2006). Also, removal of only the last 4 amino acids of WtsE has the same effect (Ham et al. 2008).

A new family of animal bacterial effectors has been recently characterized (Alto et al. 2006). Using the sequence of the *Escherichia coli* effector Map as index for BLAST analysis, a family of 24 animal pathogen effectors was identified. The members of this family are between 180 to 360 kDa in size and do not share overall amino acid sequence similarity. However, they do share regions with similar secondary structures. Further analysis of the secondary structures showed that they could share a common protein fold. Only two amino acids were found to be invariable among this protein family, one being a tryptophan (W) and the other a glutamate (E)

(Alto et al. 2006). These conserved amino acids are contained within a larger amino acid motif, with the WxxxE sequence, where "x" represents any amino acids. Members of the WxxxE effector family, such as *E. coli* Map and *Shigella flexneri* IpgB1 and IpgB2, can reorganize cytoskeleton structures through activation of Rho GTPase signaling (Alto et al. 2006). This ability is lost after the conserved W or E is mutated to alanine (A) (Alto et al. 2006). However, W and E residues may be changed to residues of similar properties without significantly abolishing effector function. For example, *Citrobacter rodentium* effectors EspM2 and EspM3, which belong to the WxxxE family, are able to induce actin stress-fiber formation in mammalian culture cells (Arbeloa et al. 2008). Substitution of W with Y or of E with D does not significantly reduced stress-fibers formation, but in the case of EspM3 it did change the overall configuration of the fibers architecture. Mutagenesis of the three spacer amino acids of the EspM3 WxxxE motif caused only minor effects on the conformation of the stress fiber, but no effect on the stress fiber quantity (Arbeloa et al. 2008).

Comparison between the structures of crystallized proteins established a link between the family of WxxxE effectors and known GEF proteins. The *Salmonella typhimurium* effector SifA belongs to the WxxxE protein family and its C-terminus has a similar fold to that of *S. typhimurium* SopE (Ohlson et al. 2008). The SopE effector had been previously identified as a GEF for Rho GTPase (Rudolph el at. 1999). Moreover, SifA is capable to interact with GDP-bound RhoA (Ohlson et al. 2008). GEF activity was confirmed for the *E. coli* effector Map, and this activity disappeared in derivatives harboring mutations in the WxxxE motif (W or E for A) (Huang 2009). These results provide the proof that, in the case of animal pathogens, the WxxxE effector family acts as GEFs.

In this chapter I will describe evidence showing that AvrE contains two functionally redundant WxxxE motifs. In addition, computational analysis of all AvrE orthologues allowed me to define a large number of conserved amino acids along the length of the AvrE effector family, as well as potentially novel amino acid motifs that could be related to AvrE function. I used site-directed mutagenesis to change many of these amino acids to A and then tested the functionality of each AvrE derivative for the ability to complement the ΔCEL mutant bacterium during infection.

MATERIALS AND METHODS

Site-directed mutagenesis of AvrE

The template used for mutagenesis was a functional *avrE* construct previously made and tested in our laboratory (Ham et al. 2009). Specifically, a ~5.5-kb fragment containing the *avrE* open reading frame (ORF) with its native promoter was amplified by polymerase chain reaction (PCR) from a cosmid clone containing a fragment of the DC3000 CEL (Sruti DebRoy and Sheng Yang He, unpublished) using Pfu Turbo DNA polymerase from Stratagene. The *avrE* gene was then cloned into the *Kpn*I and *Xba*I restriction enzymes sites of the broad host-range plasmid pUCP19, resulting in pAVRE. The *avrF* ORF with its native promoter was also PCR-amplified from the same template using Pfu Turbo DNA polymerase from Stratagene, and then cloned into the *EcoR*I and *Bam*HI restriction sites of the broad host range plasmid pDSK512, resulting in pAVRF. The two plasmids were transformed into the ΔCEL mutant bacterium and tested for complementation of the ΔCEL mutation. Full complementation was observed (Ham et al. 2009).

Site-directed mutagenesis was performed using the Stratagene™ QuikChange® XL Site-Directed Mutagenesis Kit (Catalog #200516 and #200517; Agilent Technologies™), as per vendor's instructions. Oligonucleotide design was performed using the QuikChange® Primer Design software available through the Agilent Technologies™ website (http://www.genomics.agilent.com/).

After mutagenesis was performed the immediate vicinity of the mutated bases was sequenced to confirm the successful introduction of base changes. DNA fragments containing

the engineered mutations were cut from the mutated vector and cloned back into pAVRE, to eliminate possible secondary mutations elsewhere in the *avrE* gene.

hrp gene induction and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Pst DC3000 from a single isolated colony was introduced into 10 ml low-salt Luria Bertani medium (Katagiri et al. 2002, Sambrook et al. 1989) with proper antibiotics. Cultures were grown overnight (12-15 hours) in 18x150mm borosilicate glass tubes to OD_{600nm}=0.7 to 1.0. Cells were then gently separated from culture medium by centrifugation (2,000xg for 15 minutes at 25°C) and resuspended in 10 ml sterile water. Centrifugation was repeated and cells were suspended in *hrp* gene-inducing medium (Huynh et al. 1989) to OD_{600nm}=0.6 without antibiotics. Fifteen-ml of the bacterial suspension in 18x150 mm borosilicate glass tubes were placed in an incubator (20°C and 150 rpm) for 20 to 24 hours.

Total cell lysates were prepared by separating the cell fraction from the supernatant in an Eppendorf centrifuge (20,817xg for 3 minutes at 25°C). One-ml of cell culture was concentrated 10 times in SDS-PAGE loading buffer (100 µl total) and boiled for 10 minutes prior to SDS-PAGE.

To detect AvrE secreted into the culture, cell and supernatant fractions were separated. Twelve-ml of bacterial suspension were centrifuged at 10732.8xg for 10 min at 4°C. Ten-ml of supernatant was recovered using a pipette without disturbing the cell pellet. In a 1.5 ml Eppendorf tube, 1.2 ml of supernatant was mixed with 300 µl of trichloroacetic acid (TCA) using a vortex, and then incubated for one hour at -20°C. Aliquots were centrifuged at 20,817xg for 10 minutes at 4°C in a refrigerated Eppendorf centrifuge. Supernatants were carefully discarded by

aspiration without disturbing the pellet. Each Eppendorf tube was washed twice with 200 μl acetone and centrifuged in the same conditions. Eppendorf tubes were then placed in a heat block (95°-100°C) for five minutes to evaporate acetone. The pellet in each Eppendorf tube was resuspended in 10 μl water using a pipette. Two-μl of 5x SDS loading buffer was added and the mixture was boiled for 10 minutes. Aliquots may be pooled or resuspended in less water. The resulting samples should have been concentrated by ~100 fold from the original cell culture supernatant.

Western blotting of AvrE proteins.

Detection of AvrE protein derivatives in *P. syringae* was performed using SDS-PAGE, followed by western blot. Gel preparations were conducted according to Sambrook et al. (1989). For bacterial total lysates and supernatants, 10 to 20 μl of samples were used for SDS-PAGE. Transfer of the proteins from the gel to nitrocellulose membrane was performed using a Hoefer TE-70 semi-dry transfer unit, using a continuous current of 60 mA. Time of transfer varied depending on the molecular weights of the protein to be visualized. One hour was used for smaller proteins (<70 kDa) and two hours for bigger proteins (>70 kDa).

Growth of Arabidopsis plants.

Arabidopsis plants were grown in mesh-covered square pots. A total of four plants were grown in each pot. Fifteen pots (3 rows by 5 columns) were put in each tray. Trays were partially covered with a plastic lid to maintain humidity. Watering was performed weekly by allowing one-inch deep water sit in the tray from one evening to the next morning, when excess water was

drained out. All plants were grown in a day/night cycle of 12 h/12 h, under a light intensity of 100 $\mu E/m^2s^2$, and at a constant temperature of 20°C.

Pathogenesis assays.

Procedures for pathogenesis assay followed those described by Hauck et al. (2003), with some modifications. *P. syringae* from a single colony was inoculated into 10 ml low-salt Luria Bertani medium with proper antibiotics. Cultures were grown overnight (12-15 hours) in 18x150mm borosilicate glass tubes to OD_{600nm}=0.7 to 1.0. Cells were then gently separated from medium by centrifugation (2,000xg for 15 minutes at 25°C) and resuspended in 10 ml water. This suspension was then diluted with water to OD_{600nm}=0.2 (equivalent to $1x10^8$ cfu/ml). Immediately before plant infiltration, a 1:100 dilution was performed (100 μl cell suspension in 10 ml water) to make up a $1x10^6$ cfu/ml suspension.

Four- to five-week-old plants were used for the experiments. Plants were hand-infiltrated with the bacterial suspension using a needleless syringe on the abaxial side of fully expanded leaves. Bacterial populations were enumerated as described by Katagiri et al. (2002) at day 0 and day 3 after infiltration. Infiltration experiments were technically repeated at least three times, using four biological replicas in each occasion. This dissertation presents enumeration data for one representative experiment, presented in bar charts as the average bacterial population of four biological replicas, with corresponding standard deviation. For some experiments, plants were dip-inoculated with OD_{600nm}=0.2 (10^8 cfu/ml) bacterial suspension containing 0.025% Silwet L-77. The inoculated plants were immediately covered with a plastic dome and kept in high humidity for 4 days.

RESULTS

The phylogenic relation of AvrE-family effectors

A comparative analysis of the members of the AvrE protein family was performed. Only complete protein sequences of AvrE-family members from different bacterial species and strains were subjected to sequence alignment analysis using the ClustalW2 algorithm (Chenna et al. 2003). Alignment of the AvrE-family sequences shows that the C-terminal halves are slightly more conserved than the N-terminal halves. Nonetheless, overall the amino acid conservation is scattered along the entire length of the protein family. No long stretches of highly conserved amino acid sequences, which may indicate conserved protein domains, are found. Amino acid sequence identity in the AvrE family can be as low as 25% between the most divergent members (Figure 2-1). Phylogeny analysis using *Pst* DC3000 AvrE sequence as index shows that bacterial strains group in three branches (Figure 2-2). The first branch contains *Pectobacterium carotovorum, Erwinia spp.*, *Pantoea stewartii* and *Pectobacterium agglomerans* sequences. These sequences are most divergent from *Pst* DC3000 AvrE (~26% identity). The second branch contains a clade of *Pseudomonas viridiflava* strains with an identity score of ~40% when compared to *Pst* DC3000 AvrE. The third branch includes a clade of *P. viridiflava* strains and *Pseudomonas spp.* strains that are most closely related to *Pst* DC3000 AvrE, with identity higher than 65%. Overall, the AvrE sequence-based tree is consistent with bacterial species classification, suggesting that AvrE-family effector genes are ancient and have not undergone any significant horizontal gene transfer after separation of these bacterial species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Pv SP8.1a	100	100	100	100	97	93	93	94	94	93	54	42	42	39	40	40	40	40	24	24	25	25	25	27	27
Pv RMX23.1a	100	100	100	100	97	93	93	93	93	93	54	42	41	39	40	40	40	40	24	24	25	25	25	27	27
Pv ME3.1b	100	100	100	100	97	93	93	94	93	93	54	42	42	39	40	40	40	40	24	24	25	25	25	27	27
Pv SP5.3c	100	100	100	100	97	93	93	93	93	93	54	42	42	39	40	40	40	40	24	24	25	25	25	27	27
Pv KY1.1a	97	97	97	97	100	93	93	93	93	93	53	41	41	39	40	40	40	40	25	24	25	25	25	28	28
Pv DUS2.2a	93	93	93	93	93	100	100	100	99	99	53	41	41	39	40	40	40	40	24	24	25	25	25	27	27
Pv DUD4.5a	93	93	93	93	93	100	100	100	99	99	53	41	41	39	40	40	40	40	24	24	25	25	25	27	27
Pv DUD2.5a	94	93	94	93	93	100	100	100	100	99	53	41	41	39	40	40	40	40	24	24	25	25	25	27	27
Pv RMX3.1b	94	93	93	93	93	99	99	100	100	99	53	41	41	40	40	40	40	40	24	24	25	25	25	27	27
Pv KY2.1e	93	93	93	93	93	99	99	99	99	100	53	41	41	40	40	40	40	40	24	24	25	25	25	27	27
Pci 83-1	54	54	54	54	53	53	53	53	53	53	100	41	42	40	40	40	40	40	26	24	26	26	26	28	28
Pst DC3000	42	42	42	42	41	41	41	41	41	41	41	100	88	68	65	65	65	65	26	25	26	26	26	26	26
Psp	42	41	42	42	41	41	41	41	41	41	42	88	100	68	66	66	66	66	26	25	26	26	26	26	26
Pss B728a	39	39	39	39	39	39	39	39	40	40	40	68	68	100	63	63	63	63	25	25	26	26	26	26	26
Pv LU13.1a	40	40	40	40	40	40	40	40	40	40	40	65	66	63	100	100	100	99	26	25	26	26	26	26	26
Pv LP23.1a	40	40	40	40	40	40	40	40	40	40	40	65	66	63	100	100	100	99	26	25	26	26	26	26	26
Pv SP3.1a	40	40	40	40	40	40	40	40	40	40	40	65	66	63	100	100	100	99	26	25	26	26	26	26	26
Pv LU5.1a	40	40	40	40	40	40	40	40	40	40	40	65	66	63	99	99	99	100	26	25	26	26	26	26	26
Ps	24	24	24	24	25	24	24	24	24	24	26	26	26	25	26	26	26	26	100	70	60	60	60	39	39
Pag	24	24	24	24	24	24	24	24	24	24	24	25	25	25	25	25	25	25	70	100	60	60	61	39	39
Ea CFBP1430	25	25	25	25	25	25	25	25	25	25	26	26	26	26	26	26	26	26	60	60	100	100	90	40	40
Ea 321	25	25	25	25	25	25	25	25	25	25	26	26	26	26	26	26	26	26	60	60	100	100	90	40	40
Ep WT3	25	25	25	25	25	25	25	25	25	25	26	26	26	26	26	26	26	26	60	61	90	90	100	41	41
Pca SCRI1043	27	27	27	27	28	27	27	27	27	27	28	26	26	26	26	26	26	26	39	39	40	40	41	100	100
Pca SCRI1039	27	27	27	27	28	27	27	27	27	27	28	26	26	26	26	26	26	26	39	39	40	40	41	100	100

Figure 2-1 Identity matrix of the AvrE protein family.

A total of 25 complete protein sequences representing members of the AvrE protein family from different bacterial species/strains were analyzed using the ClustalW2 algorithm. Presented are percentages of overall sequence identity among different AvrE family members. Bacterial names abbreviations: Pv (*Pseudomonas viridiflava*), Pci (*Pseudomonas cichorri*), Pst DC3000 (*Pseudomonas syringae* pv. *tomato*), Psp (*Pseudomonas syringae* pv *phaseolicola*) Pss (*Pseudomonas syringae* pv *syringae*), Ps (*Pantoea stewartii*), Pag (*Pectobacterium agglomerans* pv. *gypsophilae*), Ea (*Erwinia amylovora*), Ep (*Erwinia pyrifoliae*), Pca (*Pectobacterium carotovorum subsp. atroseptica*). The magenta diagonal line (■) corresponds to comparison with self. The yellow row and column (■) show comparisons of *Pst* DC3000 AvrE to other AvrE-family members. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

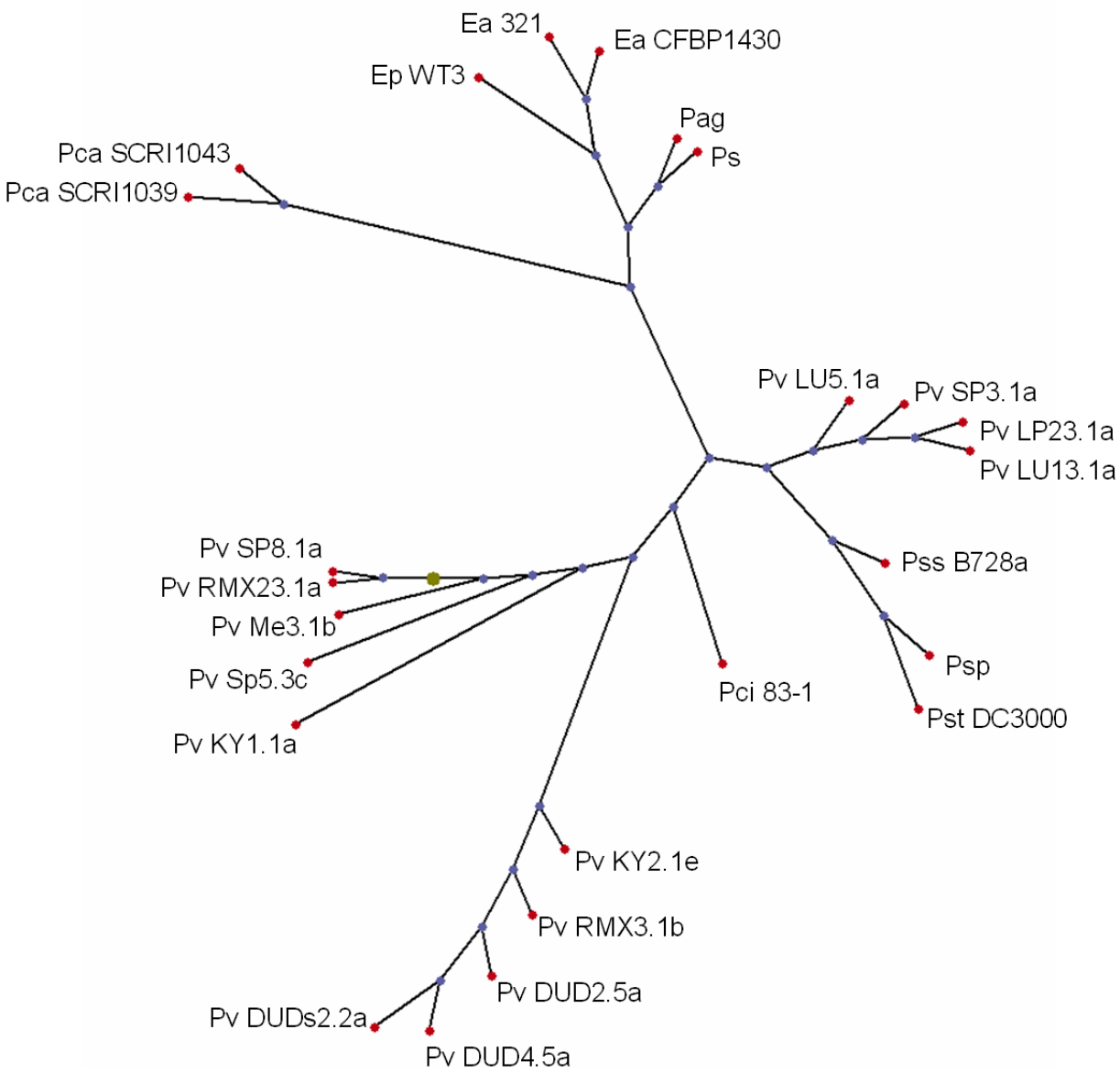

Figure 2-2 A phylogenetic tree of the AvrE protein family.

A phylogenetic tree was constructed based on the ClustalW2 analysis of 25 complete protein sequences representing members of the AvrE protein family from different bacterial species/strains. Bacterial names abbreviations: Pv (*Pseudomonas viridiflava*), Pci (*Pseudomonas cichorri*), Pst DC3000 (*Pseudomonas syringae* pv. *tomato*), Psp (*Pseudomonas syringae* pv *phaseolicola*) Pss (*Pseudomonas syringae* pv *syringae*), Ps (*Pantoea stewartii*), Pag (*Pectobacterium agglomerans* pv. *gypsophilae*), Ea (*Erwinia amylovora*), Ep (*Erwinia pyrifoliae*), Pca (*Pectobacterium carotovorum subsp. atroseptica*).

A putative sequence motif at the C-termini among the AvrE protein family

The C-terminal region of AvrE is of special interest since deletion of the last 4 to 12 amino acids of WstE has been shown to render *P. stewartii* avirulent (Ham et al. 2006; Ham et al. 2008). There is a high degree of sequence conservation in the last few amino acid residues, with the exception of DspE proteins from *P. carotovorum* strains, which contain the most divergent sequences in this region (Figure 2-3). In many family members, there are two lysine (KK) residues at -8 and -9 positions. Some transmembrane type I proteins contain double KK at amino acids positions -3 and -4 of their cytosolic C-terminal tails (Jackson et al. 1990). Such a KK motif acts as an endoplasmic reticulum membrane retrieval signal (ERMRS) that prevents the diffusion of these proteins into other intracellular compartment (Nilsson et al. 1989). It has been speculated that the KK-containing C-terminus of AvrE may mimic the ERMRS (Ham et al. 2006). Moreover, because the KK residues of AvrE are located in positions -8 to -9, it is also believed that a possible proteolytic cleavage of the C-terminus may be required for the ERMRS to function (Ham et al. 2006).

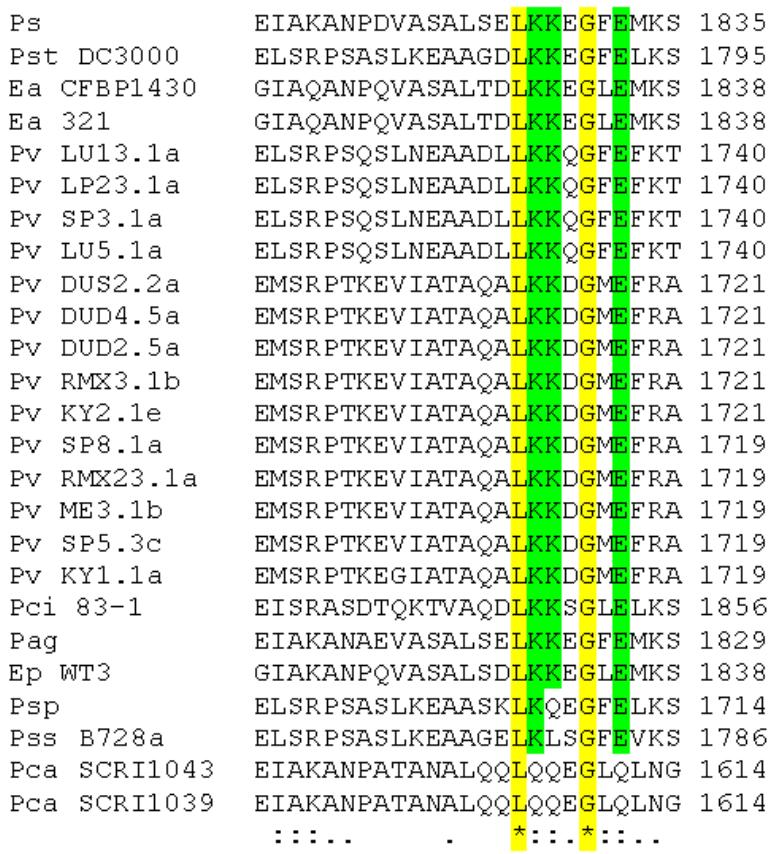

Figure 2-3 C-termini sequences of the AvrE protein family display a conserved LKKxGxE sequence.

A ClustalW2 alignment of the C-termini ends of 25 members of the AvrE protein family. At the bottom of the alignment, ClustalW2 grades the conservation of specific residues using * (asterisk) for absolute conservation, : (colon) for a highly conserved residue, and . (period) for a conserved residue. Note that the conservation of highlighted amino acids in the form of an LKKxGxE motif. Bacterial names abbreviations from top to bottom; Pv (*Pseudomonas viridiflava*), Pci (*Pseudomonas cichorri*) Pst DC3000 (*Pseudomonas syringae* pv. *tomato*), Psp (*Pseudomonas syringae* pv *phaseolicola*) Pss (*Pseudomonas syringae* pv *syringae*), Ps (*Pantoea stewartii*), Pag (*Pectobacterium agglomerans* pv. *gypsophilae*), Ea (*Erwinia amylovora*), Ep (*Erwinia pyrifoliae*), Pca (*Pectobacterium carotovorum subsp. atroseptica*).

The WxxxE motif in the AvrE effector family

Visual sequence inspection of DC3000 AvrE also shows that the protein harbors two WxxxE motifs in the N-terminal half. The WxxxE motif has been shown to be critical for the function of a recently characterized family of animal pathogen effectors acting as GEFs (Alto et al. 2006; Huang 2009). In this dissertation I have named the two WxxxE motifs W_1xxxE_1 (W393 → E397) and W_2xxxE_2 (W829 → E833), respectively.

Further sequence inspection of AvrE-family members shows that most of them have two WxxxE motifs (Figure 2-4). However, the relative positions of the WxxxE motifs in each protein sequence may be substantially different. In total, seven different positions of the WxxxE motif can be identified along the aligned sequences of the AvrE protein family. W is highly conserved in five of the seven positions of all family members, but an E may or may not be present (Figure 2-4). In the other two positions, although W is not conserved in all family members, bulky amino acids (e.g., Y, I, F, or H) are found (Figure 2-4). I have named the seven WxxxE locations as W_1, W_2, W_3, W_4, W_5, W_6 and W_7. In general, AvrE-family members closely related to each other in the phylogenetic tree (Figure 2-2) use similar pairs of conserved Ws for positioning their WxxxE motifs (Figure 2-4). Family members closer to the nodes of the phylogenetic tree seem to use combinations of WxxxE locations used by members of the outward branches. Interestingly, *P. viridiflava* Sp5.3c, *P. syringae* pv. *syringae* B728a and *P. agglomerans* have only one WxxxE motif at positions W_1, W_2 and W_4, respectively. However, each of these bacteria has an additional WxxxD motif at the W_2, W_1 and W_2 positions, respectively. In a similar fashion, *P. carotovorum* strains do not have any WxxxE motifs. However I find a WxxxD motif at the W_3

position (WNLSD) (Figure 2-4). It has been shown that in EspM2 and EspM3 of *C. rodentium*, conservative amino acid changes from WxxxE to WxxxD do not affect effector function (Arbeloa et al. 2008). This suggests that the WxxxD motif found in the aforementioned AvrE orthologues may be functional and that the WxxxD motif may be an accepted evolutionary variant of WxxxE.

Figure 2-4 Conserved positions of WxxxE motifs in the AvrE protein family (next page).

The top diagram represents the ClustalW2 alignment of 25 complete protein sequences of AvrE-family members. The red bars (■) represent highly conserved W residues in the sequences aligned. These residues are designated W_1 to W_7. Below, detailed sequences surrounding each conserved W are presented. Residues in the WxxxE motifs are highlighted in turquoise (■). Note that each WxxxE motif is identified based on a conserved starting W residue. At the bottom of the alignment, ClustalW2 grades the conservation of specific residues using * (asterisk) for absolute conservation, : (colon) for a highly conserved residue, and . (period) for a conserved residue. At the very bottom, the specific position of each conserved residue W is provided, as it corresponds to in the amino acid sequence of *Pst* DC3000 AvrE Aligned sequences names 1) *P. viridiflava* LU13.1a, 2) *P. viridiflava* Lp23.1a, 3) *P. viridiflava* SP3.1a, 4) *P. viridiflava* LU5.1a, 5) *P. syringae* DC3000, 6) *P. syringae* pv *phaseolicola*, 7) *P. syringae* pv *syringae* B728a, 8) *Pseudomonas cichorri* 83-1 9) *P. viridiflava* DUS2.2a, 10) *P. viridiflava* DUD4.5a, 11) *P. viridiflava* DUD2.5a, 12) *P. viridiflava* RMX3.1b, 13) *P. viridiflava* KY2.1e, 14) *P. viridiflava* SP81.a, 15) *P. viridiflava* RMX23.1a, 16) *P. viridiflava* ME3.1b, 17) *P. viridiflava* SP5.3c, 18) *P. viridiflava* KY1.1a, 19) *P. carotovorum* SCRI104, 20) *P. carotovorum* SCRI1039, 21) *Pantoea. stewartii*, 22) *P. agglomerans* pv. *gypsophilae* , 23) *E. amylovora* CFBP1430, 24) *E. amylovora* 321, 25) *Erwinia pyrifoliae*.

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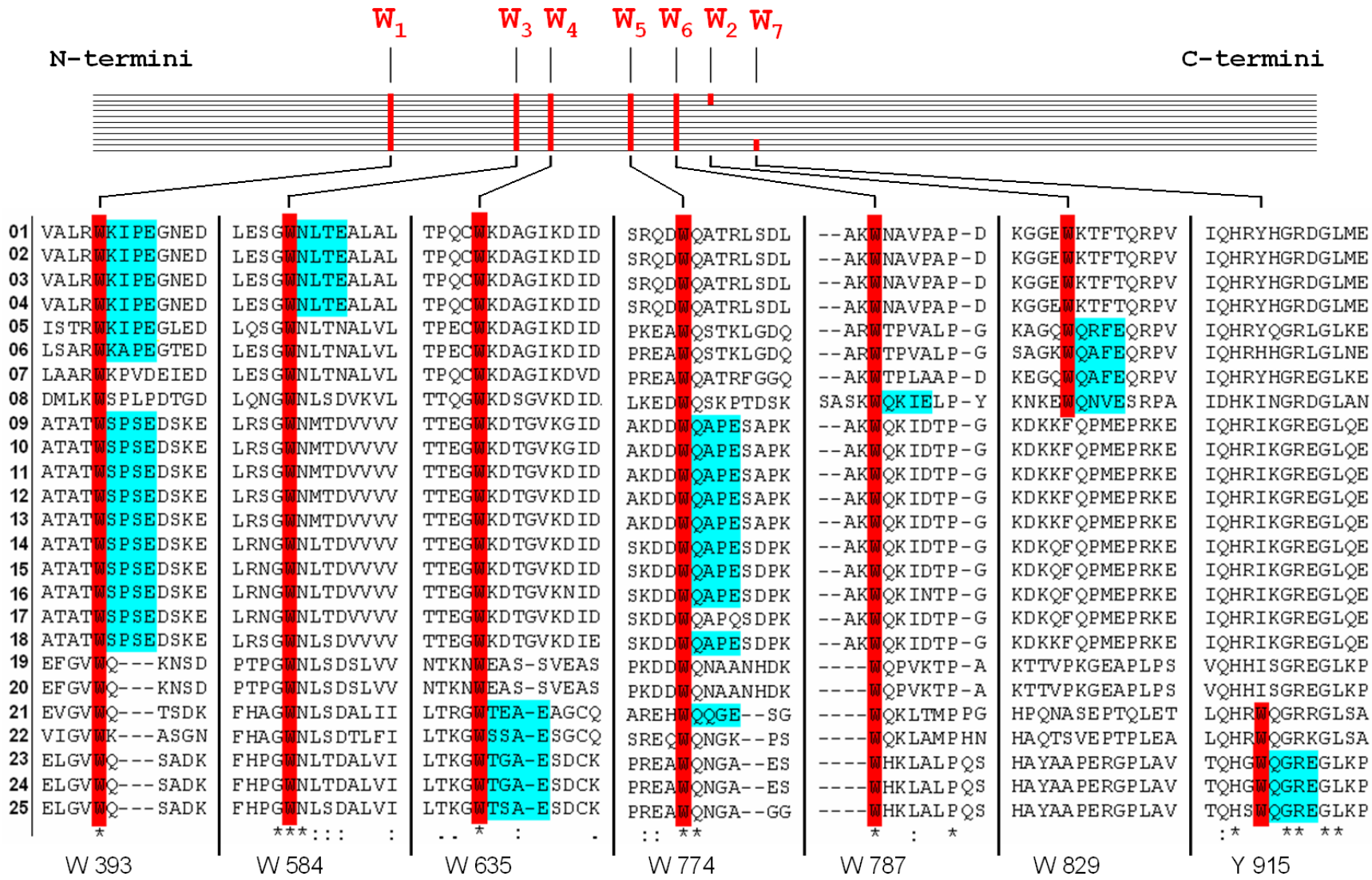

W1
W3 W4
W5 W6 W2 W7
N-termini
C-termini
01 VALRWKIPEGNED LESGWNLTEALAL TPQCWKDAGIKDID SRQDWQATRLSDL --AKWNAVPAP-D KGGEWKTFTQRPV IQHRYHGRDGLME
02 VALRWKIPEGNED LESGWNLTEALAL TPQCWKDAGIKDID SRQDWQATRLSDL --AKWNAVPAP-D KGGEWKTFTQRPV IQHRYHGRDGLME
03 VALRWKIPEGNED LESGWNLTEALAL TPQCWKDAGIKDID SRQDWQATRLSDL --AKWNAVPAP-D KGGEWKTFTQRPV IQHRYHGRDGLME
04 VALRWKIPEGNED LESGWNLTEALAL TPQCWKDAGIKDID SRQDWQATRLSDL --AKWNAVPAP-D KGGEWKTFTQRPV IQHRYHGRDGLME
05 ISTRWKIPEGLED LQSGWNLTNALVL TPECWKDAGIKDID PKEAWQSTKLGDQ --ARWTPVALP-G KAGQWQRFEQRPV IQHRYQGRLGLKE
06 LSARWKAPEGTED LESGWNLTNALVL TPECWKDAGIKDID PREAWQSTKLGDQ --ARWTPVALP-G SAGKWQAFEQRPV IQHRHHGRLGLNE
07 LAARWKPVDEIED LESGWNLTNALVL TPQCWKDAGIKDVD PREAWQATRFGGQ --AKWTPLAAP-D KEGQWQAFEQRPV IQHRYHGREGLKE
08 DMLKWSPLPDTGD LQNGWNLSDVKVL TTQGWKDSGVKDID LKEDWQSKPTDSK SASKWQKIELP-Y KNKEWQNVESRPA IDHKINGRDGLAN
09 ATATWSPSEDSKE LRSGWNMTDVVVV TTEGWKDTGVKGID AKDDWQAPESAPK --AKWQKIDTP-G KDKKFQPMEPRKE IQHRIKGREGLQE
10 ATATWSPSEDSKE LRSGWNMTDVVVV TTEGWKDTGVKGID AKDDWQAPESAPK --AKWQKIDTP-G KDKKFQPMEPRKE IQHRIKGREGLQE
11 ATATWSPSEDSKE LRSGWNMTDVVVV TTEGWKDTGVKDID AKDDWQAPESAPK --AKWQKIDTP-G KDKKFQPMEPRKE IQHRIKGREGLQE
12 ATATWSPSEDSKE LRSGWNMTDVVVV TTEGWKDTGVKDID AKDDWQAPESAPK --AKWQKIDTP-G KDKKFQPMEPRKE IQHRIKGREGLQE
13 ATATWSPSEDSKE LRSGWNMTDVVVV TTEGWKDTGVKDID AKDDWQAPESAPK --AKWQKIDTP-G KDKKFQPMEPRKE IQHRIKGREGLQE
14 ATATWSPSEDSKE LRNGWNLTDVVVV TTEGWKDTGVKDID SKDDWQAPESDPK --AKWQKIDTP-G KDKQFQPMEPRKE IQHRIKGREGLQE
15 ATATWSPSEDSKE LRNGWNLTDVVVV TTEGWKDTGVKDID SKDDWQAPESDPK --AKWQKIDTP-G KDKQFQPMEPRKE IQHRIKGREGLQE
16 ATATWSPSEDSKE LRNGWNLTDVVVV TTEGWKDTGVKNID SKDDWQAPESDPK --AKWQKINTP-G KDKQFQPMEPRKE IQHRIKGREGLQE
17 ATATWSPSEDSKE LRNGWNLTDVVVV TTEGWKDTGVKDID SKDDWQAPQSDPK --AKWQKIDTP-G KDKQFQPMEPRKE IQHRIKGREGLQE
18 ATATWSPSEDSKE LRSGWNLSDVVVV TTEGWKDTGVKDIE SKDDWQAPESDPK --AKWQKIDTP-G KDKKFQPMEPRKE IQHRIKGREGLQE
19 EFGVWQ---KNSD PTPGWNLSDSLVV NTKNWEAS-SVEAS PKDDWQNAANHDK ----WQPVKTP-A KTTVPKGEAPLPS VQHHISGREGLKP
20 EFGVWQ---KNSD PTPGWNLSDSLVV NTKNWEAS-SVEAS PKDDWQNAANHDK ----WQPVKTP-A KTTVPKGEAPLPS VQHHISGREGLKP
21 EVGVWQ---TSDK FHAGWNLSDALII LTRGWTEA-EAGCQ AREHWQQGE--SG ----WQKLTMPPG HPQNASEPTQLET LQHRWQGRRGLSA
22 VIGVWK---ASGN FHAGWNLSDTLFI LTKGWSSA-ESGCQ SREQWQNGK--PS ----WQKLAMPHN HAQTSVEPTPLEA LQHRWQGRKGLSA
23 ELGVWQ---SADK FHPGWNLTDALVI LTKGWTGA-ESDCK PREAWQNGA--ES ----WHKLALPQS HAYAAPERGPLAV TQHGWQGREGLKP
24 ELGVWQ---SADK FHPGWNLTDALVI LTKGWTGA-ESDCK PREAWQNGA--ES ----WHKLALPQS HAYAAPERGPLAV TQHGWQGREGLKP
25 ELGVWQ---SADK FHPGWNLSDALVI LTKGWTSA-ESDCK PREAWQNGA--GG ----WHKLALPQS HAYAAPERGPLAV TQHSWQGREGLKP
W 393
W 584
W 635
W 774
W 787
W 829
Y 915

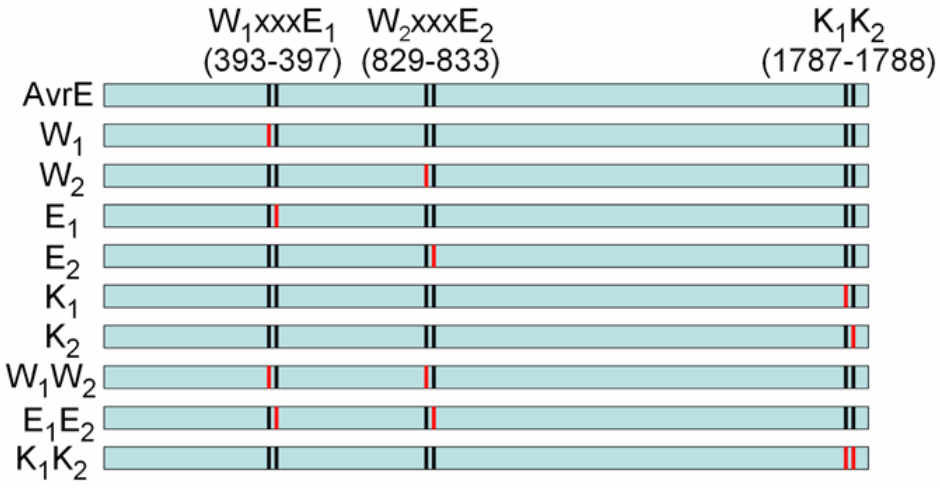
The WxxxE and LKKxGxE motifs are required for the virulence function of AvrE.

To determine if the WxxxE and LKKxGxE motifs are required for AvrE function, a large collection of single (i.e., from W, E, K, to A) or double (i.e., from W_1W_2, E_1E_2, and K_1K_2 to A_11A_2) substitutions of W, E and K in each motif was produced. The biological activity of these AvrE derivatives was then evaluated by their ability to complement the ΔCEL mutant bacterium during bacterial infection of Arabidopsis (next page). These experiments involved a concerted effort by Dr. Christy Mecey, Dr. Kinya Nomura and I (see page 31).

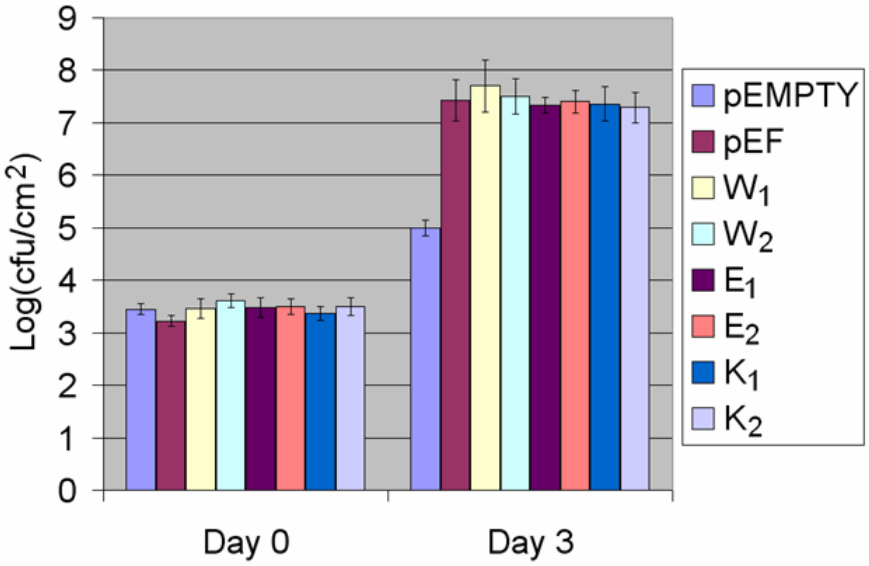
Figure 2-5 Effects of mutagenesis of WxxxE and KK motifs on the function of AvrE (next page).

A) A diagram showing the positions of the WxxxE and KK motifs in the sequence of AvrE. Native amino acids are denoted by black bars (■). Residues mutated to A are denoted by red bars (■). **B)** A chart showing the population growth of the ΔCEL mutant complemented with AvrE derivatives carrying single mutations (W_1, W_2, E_1, E_2, K_1 and K_2). **C)** A chart showing bacterial populations of the ΔCEL mutant complemented AvrE derivatives carrying double mutations (W_1W_2, E_1E_2, or K_1K_2). Arabidopsis leaves were infiltrated with bacterial suspensions ($1x10^6$ cfu/ml). Bacterial population enumeration was performed at day 0 and day 3. The ΔCEL mutant complemented with wild-type *avrE* (pAVRE + pAVRF = pEF) was used as a positive control. The ΔCEL mutant carrying pUCP19 + pAVRF (pEMPTY) was used as a negative control. Each column represents the average of four biological replicas, with standard deviations displayed. This figure was made in collaboration with Dr. Christy Mecey and Dr. Kinya Nomura (see page 31).

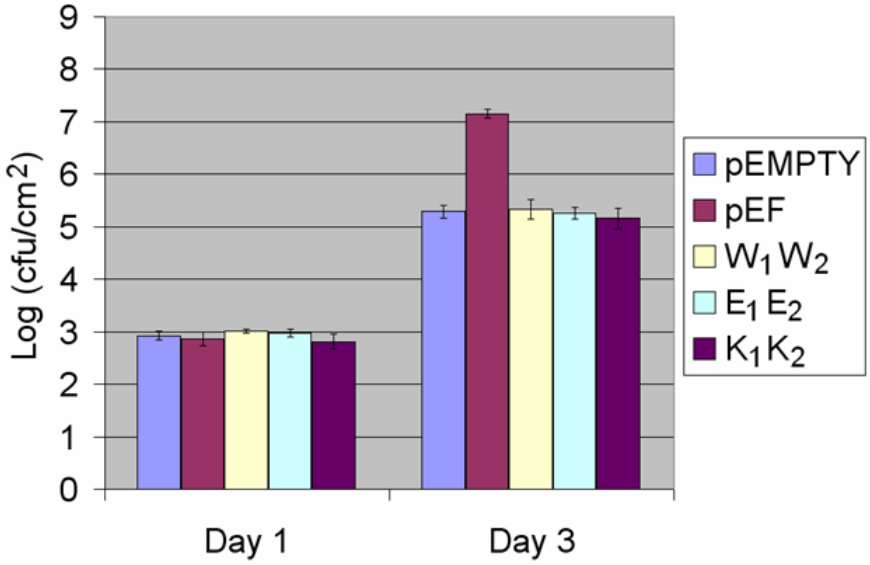
A)


W1xxxE1 (393-397)
W2xxxE2 (829-833)
K1K2 (1787-1788)
AvrE
W1
W2
E1
E2
K1
K2
W1W2
E1E2
K1K2

B)


Log(cfu/cm2)
0
1
2
3
4
5
6
7
8
9
Day 0
Day 3
pEMPTY
pEF
W1
W2
E1
E2
K1
K2

C)


Log (cfu/cm2)
0
1
2
3
4
5
6
7
8
9
Day 1
Day 3
pEMPTY
pEF
W1 W2
E1 E2
K1 K2

Substitution of any single amino acid to A in either the WxxxE motif or the KK motif had no effect on the virulence function of AvrE (Figure 2-5). On the other hand, mutations that disrupt both Ws or Es of the two WxxxE motifs abolished the function of AvrE (Figure 2-5). This result suggests that the two WxxxE motifs are functionally redundant. The same strong effect was observed for the KK double mutations in the C-terminal KK motif (Figure 2-5), suggesting that the two KK residues are also functionally redundant.

AvrE mutants are secreted through the TTSS

The inability of AvrE-W_1W_2, -E_1E_2 or -K_1K_2 to complement the ΔCEL mutant could be because these mutants are not expressed or not secreted through the TTSS. This possibility was examined by performing western blots of total bacterial lysates and culture supernatants (Figure 2-6). As a negative control the DC3000 *hrcC* secretion deficient mutant was used (Yuan and He, 1996). The AvrE derivatives W_1W_2, E_1E_2 and K_1K_2 were found in the bacterial culture supernatant, suggesting that these AvrE mutant proteins are expressed and secreted normally.

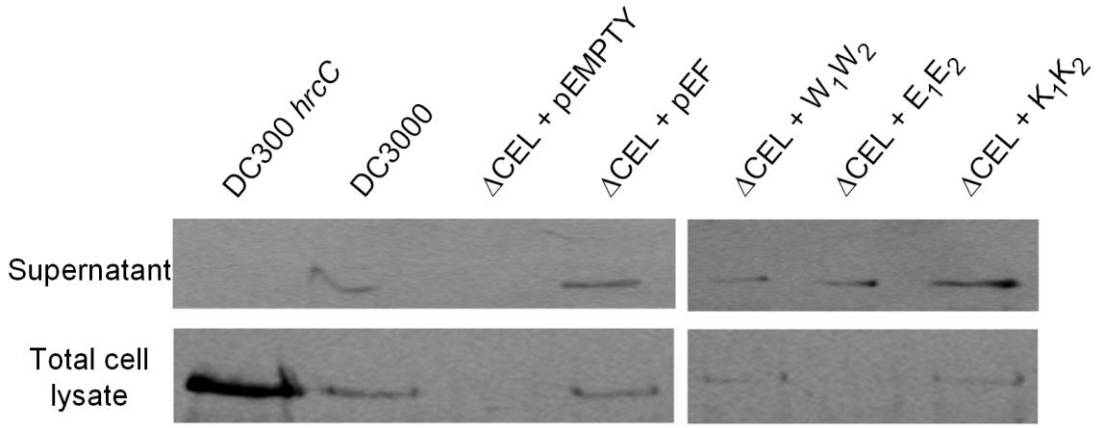


Figure 2-6 The AvrE-W_1W_2, -E_1E_2 and -K_1K_2 mutants are expressed and secreted to the supernatant of bacterial cultures.

Western blot using a rabbit AvrE antiserum shows that expression of AvrE and its derivatives in total cell lysates and secretion to the supernatants of bacterial cultures. This figure was made in collaboration with Dr. Kinya Nomura (see page 31).

WxxxE vs. WxxxD.

Although the majority of the AvrE family members possess two WxxxE motifs in their sequences, some members (e.g., in *P. viridiflava* Sp5.3c, *P. syringae* pv. *syringae* B728a, *P. agglomerans*, and *P. carotovorum*) have a WxxxD motif in replacement of a WxxxE motif. In *C. rodentium* effectors EspM2 and EspM3, changing WxxxE to WxxxD does not affect effector function (Arbeloa et al. 2008). I wanted to determine whether this would also be true for AvrE-family effectors.

Since AvrE has two WxxxE motifs, I decided to perform the A to D mutagenesis in the non-functional AvrE-E_1E_2 mutant, in which E_1 and E_2 were replaced with A residues. I then tested if reverting A to D was capable of restoring AvrE function (next page).

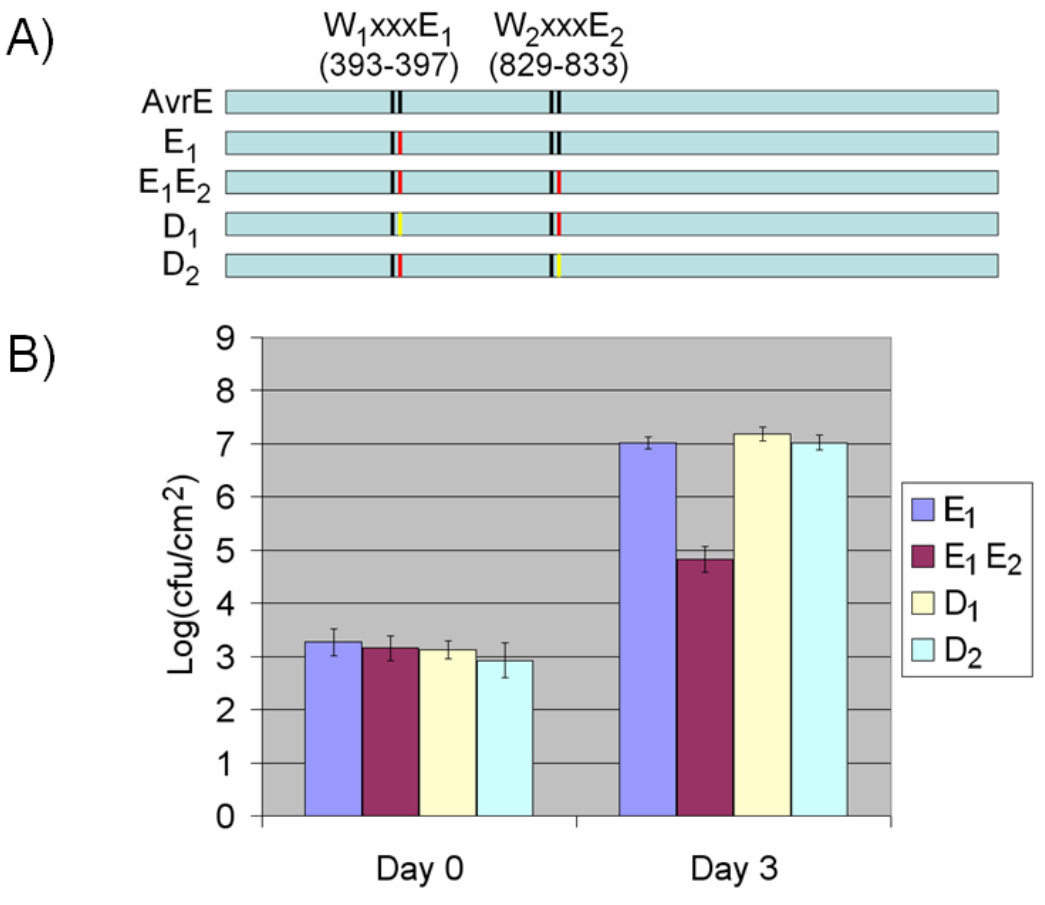


Figure 2-7 WxxxD is a functional variant of the WxxxE motif.

A) A diagram showing the positions of the WxxxE and KK motifs in the AvrE sequence. Native amino acids are denoted by black bars (■). Amino acids mutated to A are denoted using red bars (■), whereas amino acids mutated to D are denoted by yellow bars (■). **B)** A chart showing population growth of the ΔCEL mutant expressing the E_1 mutant (functional) as positive control, the E_1E_2 mutant (non-functional) as negative control, as well as the D_1 and D_2 mutants. Arabidopsis leaves were infiltrated with bacterial suspensions ($1x10^6$ cfu/ml). Bacterial population enumeration was performed at day 0 and day 3. Each column represents the average of four biological replicas, with standard deviations displayed.

Changing either W_1xxxA_1 to W_1xxxD_1 or W_2xxxA_2 to W_2xxxD_2 in the AvrE-E_1E_2 mutant background recovered the function of AvrE (Figure 2-7). According to these results, I conclude that the substitution of D for E does not affect effector function. The function recovery

of the AvrE-E_1E_2 mutant reinforces the notion that the two WxxxE motifs are redundant (Figure 2-5).

Functional WxxxE motifs can be created *de novo* in the AvrE sequence at the conserved W locations.

There are seven identifiable locations (W_1 through W_7) where the WxxxE motifs are found among different AvrE family members (Figure 2-4), even though each member has more than seven W residues in its sequence and different members seem to use different Ws to position their WxxxE motifs. This observation suggests either that any of the seven W positions can be used by any AvrE-family effector to position the WxxxE motif, or that each member has to use specific Ws because of the constraints from the different overall backbone sequences. To distinguish these possibilities I created *de novo* WxxxE/D motifs in the conserved W locations (positions W_3 to W_6) that are not used by DC3000 AvrE (Figure 2-4). Note that in position W_7 AvrE does not have a W in its sequence; instead it harbors a Y in that location. All mutations were created in the non-functional AvrE-E_1E_2 mutant background (next page).

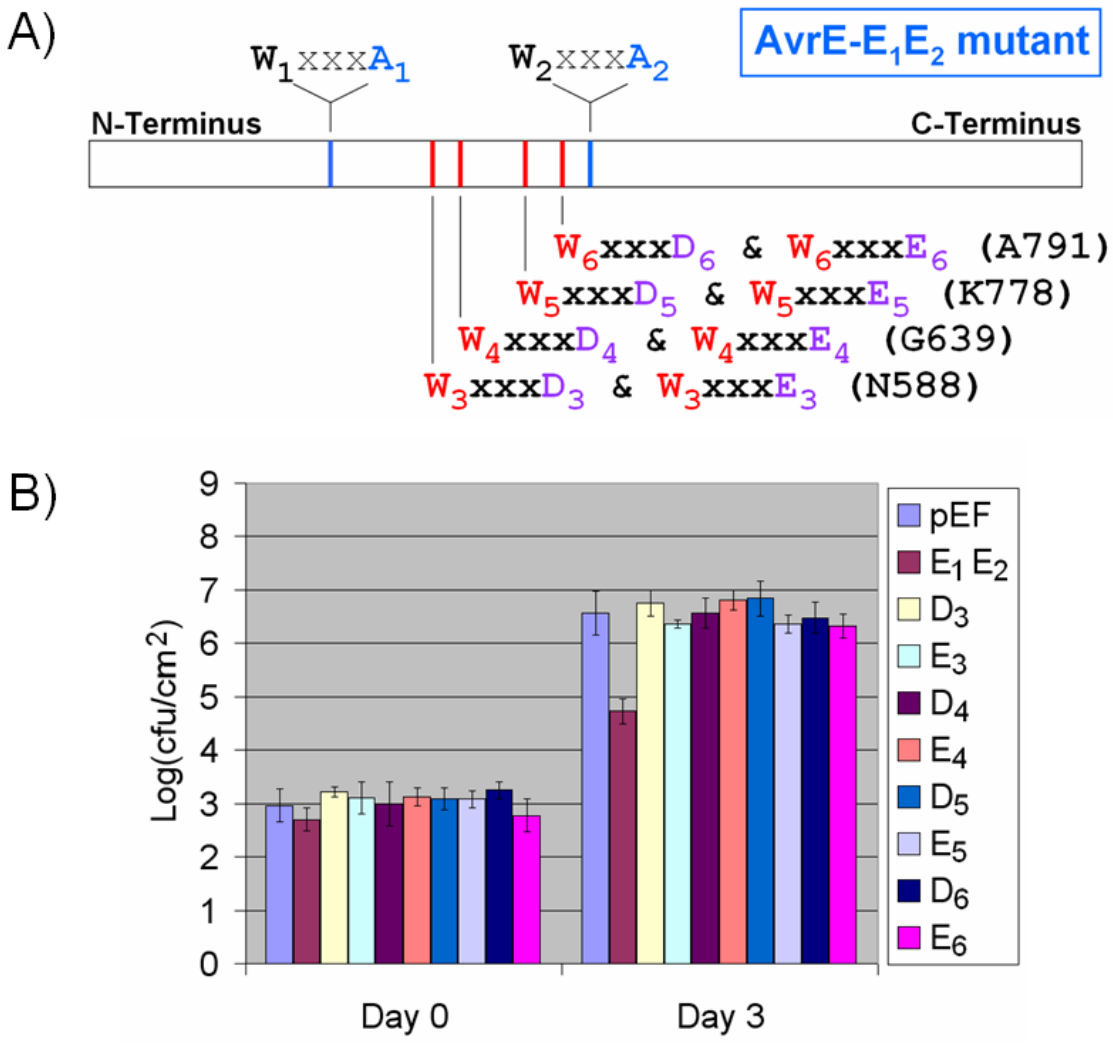


Figure 2-8 *De novo* WxxxE motifs can be created in AvrE *Pst* DC3000 at the positions marked by the conserved W residues.

A) A diagram showing the positions of the two WxxxE motifs in AvrE and of four other W residues that are highly conserved among AvrE-family effctors. The fifth amino acids at the W_3, W_4, W_5 and W_6 locations are also shown. D or E substitution of the fifth amino acids are named D_3 to D_6 and E_3 to E_6, respectively. All mutations were performed in the non-functional *avrE-E1E2* mutant background. **B)** A chart showing the population growth of the ΔCEL mutant complemented with wild-type *avrE* (pAVRE + pAVRF = pEF) as positive control, ΔCEL expressing *avrE-E1E2* (non-functional) as negative control, and ΔCEL expressing *avrE* carying *de novo* WxxxD and WxxxE motifs. Arabidopsis leaves were infiltrated with bacterial suspensions ($1x10^6$ cfu/ml). Bacterial population enumeration was performed at day 0 and day 3. Each column represents the average of four biological replicas, with standard deviations displayed.

The AvrE-E_1E_2 mutant carrying a *de novo* WxxxE motif at the W_3 through W_6 locations became functional and complemented the ΔCEL mutant (Figure 2-8). The *de novo* WxxxD variant was also capable of restoring the function of the AvrE-E_1E_2 mutant. These results demonstrate that any of the six conserved Ws can be used to position a functional WxxxE motif in AvrE.

Length pliability of the spacer sequence of the WxxxE motif

It has been reported that substitutions with A of the three spacer amino acids in the WxxxE motif do not affect the virulence function of the EspM3 effector (Arbeloa et al. 2008). However, it is not known whether a spacer of exactly three amino acids is required. To address this question, I introduced amino acid insertions or deletions in the spacer sequence and examined the ability of the resulting AvrE mutants to complement the ΔCEL mutant. Because AvrE possesses two WxxxE motifs, I made the sequential alterations of the amino acid sequence in the second WxxxE motif of the AvrE-W_1 mutant, to determine how much the spacer length could be changed before AvrE function would be lost.

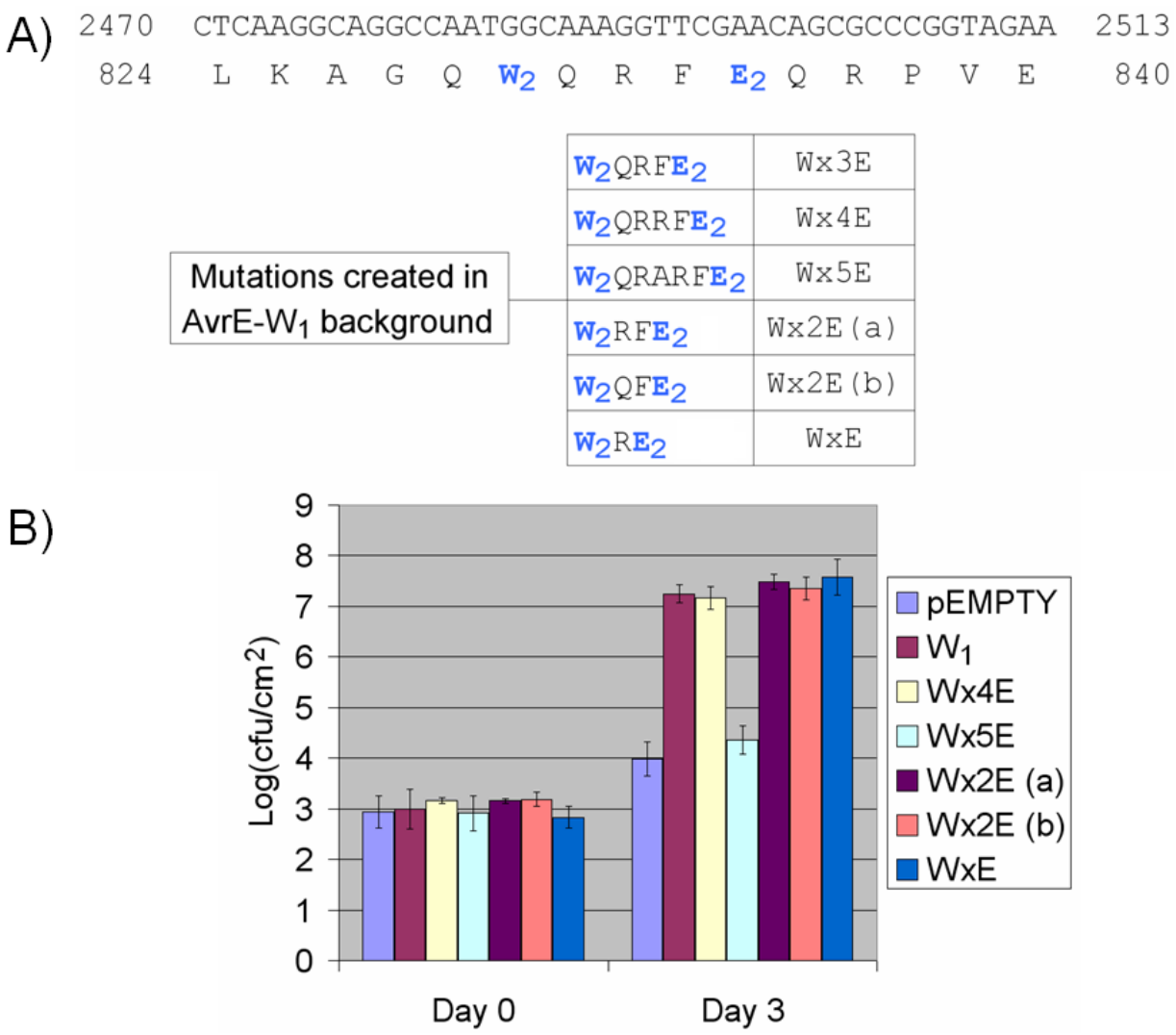


Figure 2-9 Alteration of the spacer sequence of the WxxxE motif.

A) The original sequence surrounding the second W_2xxxE_2 motif is shown on top. Below are W_2xxxE_2 motif mutants created in *avrE-W*$_1$ background (left) with corresponding names (right). **B)** A chart showing population growth of the ΔCEL mutant containing pUCP19 + pAVRF (pEMPTY) as a negative control, ΔCEL complemented with pAVRF + the *avrE-W*$_1$ plasmid as a positive control, and ΔCEL containing pAVRF + pAVRE derivative carrying mutations in the spacer sequence of the second WxxxE motif. Arabidopsis leaves were infiltrated with bacterial suspensions ($1x10^6$ cfu/ml). Bacterial population enumeration was performed at day 0 and day 3. Each column represents the average of four biological replicas, with standard deviations displayed.

Addition of a bulky and charged residue (W4xE) had no effect on the virulence function of AvrE (Figure 2-9). Increasing the spacing using an additional A in between two Rs (W5xE),

however, abolished AvrE function. On the other hand, removal of an existing amino acid (W2xEa and W2xEb variants) does not have an effect on the function of AvrE. Even deletion of two amino acids (WxE) does not have an impact on the effector function. Thus, the spacer sequence in the WxxxE motif can be from one to four amino acids.

Analysis of the LKKxGxE motif at the C-terminus of AvrE.

Having determined the importance of the C-terminal double lysine motif of AvrE, I wanted to determine if other highly conserved amino acids on the C-terminus are also important for AvrE function (Figure 2-3; selected sequences are shown in Figure 2-10). In addition, some *P. syringae* strains have KQ or KL variations. The QQ variation in *P. carotovorum* is of particular interest because it is the most divergent one. I wanted to test if the recreation of these naturally occurring variations in AvrE would disrupt the function of AvrE (next page).

A)

```
Pst DC3000      ELSRPSASLKEAAGDLKKEGFELKS 1795
Ps              EIAKANPDVASALSELKKEGFEMKS 1835
Ea 321          GIAQANPQVASALTDLKKEGLEMKS 1838
Pv SP5.3c       EMSRPTKEVIATAQALKKDGMEFRA 1719
Psp             ELSRPSASLKEAASKLKQEGFELKS 1714
Pss B728a       ELSRPSASLKEAAGELKLSGFEVKS 1786
Pca SCRI1043    EIAKANPATANALQQLQQEGLQLNG 1614
                 :::..     .   *: .*::..
```

B)

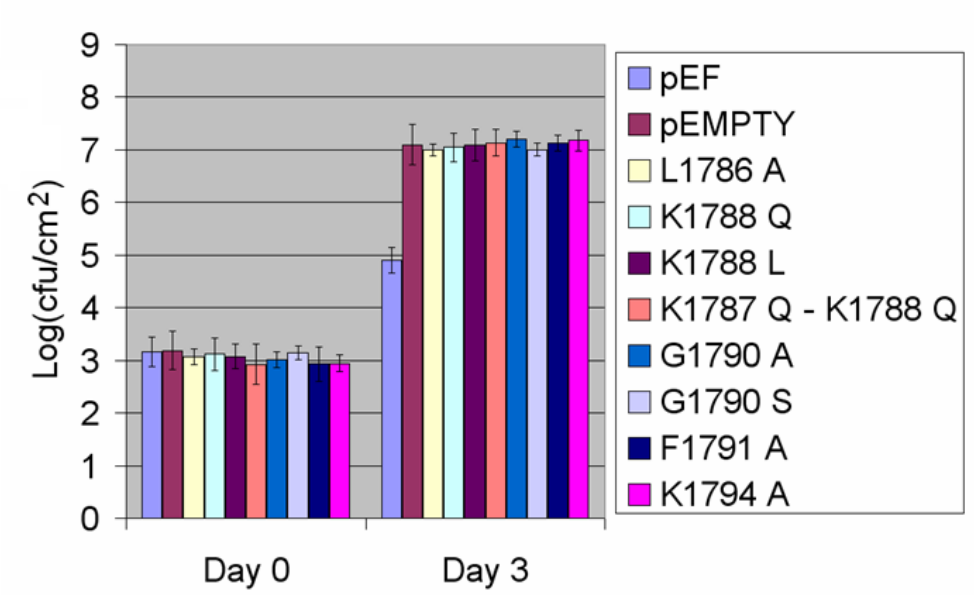


Figure 2-10 Further mutagenesis of the conserved C-terminal LKKxGxE sequence.
A) A ClustalW2 alignment showing C-terminal sequences of selected members of the AvrE-family. Abbreviations for bacterial strains/species; Pst DC3000 (*P. syringae* pv. *tomato*), Ps (*P. stewartii*), Ea (*E. amylovora*), Pv (*P. viridiflava*), Psp (*P .syringae* pv *phaseolicola*) Pss (*P. syringae* pv *syringae*), Pac (*P. carotovorum subsp. atroseptica*). At the bottom of the alignment, ClustalW2 grades the conservation of specific residues using * (asterisk) for absolute conservation, : (colon) for a highly conserved residue, and . (period) for a conserved residue. **B)** Population growth of the ΔCEL mutant complemented with wild-type *avrE* (pAVRE + pAVRF = pEF) as a positive control, ΔCEL containing pUCP19 + pAVRF (pEMPTY) as a negative control, and ΔCEL containing pAVRF + each of the *avrE* mutant plasmids indicated. Arabidopsis leaves were infiltrated with bacterial suspensions ($1x10^6$ cfu/ml). Bacterial population enumeration was performed at day 0 and day 3. Each column represents the average of four biological replicas, with standard deviations displayed.

Mutations of K_1K_2 to K_1L_2, K_1Q_2 or Q_1Q_2 did not affect the function of AvrE. This suggests that these variations, in contrast to the K_1K_2 to A_1A_2 substitutions (Figure 2-5), are all

functional (Figure 2-3). Permutations in the more highly conserved L1786 and G1790 residues also had no effect on AvrE function (Figure 2-10). Since deletion of the last four amino acids of WstE abolish effector function (Ham et al. 2008), I wanted to determine if substitutions of residues at the very C-terminus would affect AvrE function. I was able to mutate F1791 and K1794 to A. These single mutations did not impact AvrE function (Figure 2-10). All together, these results suggest that, with the exception of the K_1K_2 to A_1A_2 double mutations, none of the tested single amino acid makes a critical contribution to the biological function of AvrE.

Repetition of a C-terminal FELK sequence in the N-terminal part of AvrE.

The last five amino acids of AvrE harbor the sequence FELKS. I noticed that this sequence was repeated in the N-terminal half of AvrE. Also, both C-terminal and N-terminal FELK sequences are somewhat conserved among the different strains of *P. syringae* and *P. viridiflava*, but not in all members of the AvrE-family. As mentioned before (page 45), the last four amino acids of WtsE are important for biological function (Ham et al. 2008). In addition, it had been previously hypothesized that these C-terminal residues may be required for protease recognition and cleavage to emulate the canonical ERMRS signal (Ham et al. 2006). I hypothesized that the N-terminal FELK repetition may have some biological function in *Pseudomonas* AvrE orthologues (next page).

Figure 2-11 Analysis of the FELK sequence in the N-terminus of AvrE (next page).
A) A ClustalW2 alignment showing C-terminal sequences of selected members of the AvrE-family. The red box highlights the FELKS sequence. The last four amino acids of Ps WtsE are required for effector function (Ham et al. 2008). **B)** The FELK sequence (in blue box) is also present in the N-terminus of *Pst* DC3000 AvrE and variations of it are found in some other AvrE-family members. Abbreviations for bacterial strains/species; Pst DC3000 (*P. syringae* pv. *tomato*), Ps (*P. stewartii*), Ea (*E. amylovora*), Pv (*P. viridiflava*), Psp (*P .syringae* pv *phaseolicola*) Pss (*P. syringae* pv *syringae*), Pac (*P. carotovorum subsp. atroseptica*). At the bottom of the alignment, ClustalW2 grades the conservation of specific residues using * (asterisk) for absolute conservation, : (colon) for a highly conserved residue, and . (period) for a conserved residue. **C)** Population growth of the ΔCEL mutant containing pUCP19 + pAVRF (pEMPTY) as a negative control, the ΔCEL mutant containing pAVRE + pAVRF (pEF) as a positive control, and the ΔCEL mutant containing each of the *avrE* mutant plasmids indicated. Arabidopsis leaves were infiltrated with bacterial suspensions ($1x10^6$ cfu/ml). Bacterial population enumeration was performed at day 0 and day 3. Each column represents the average of four biological replicas, with standard deviations displayed.

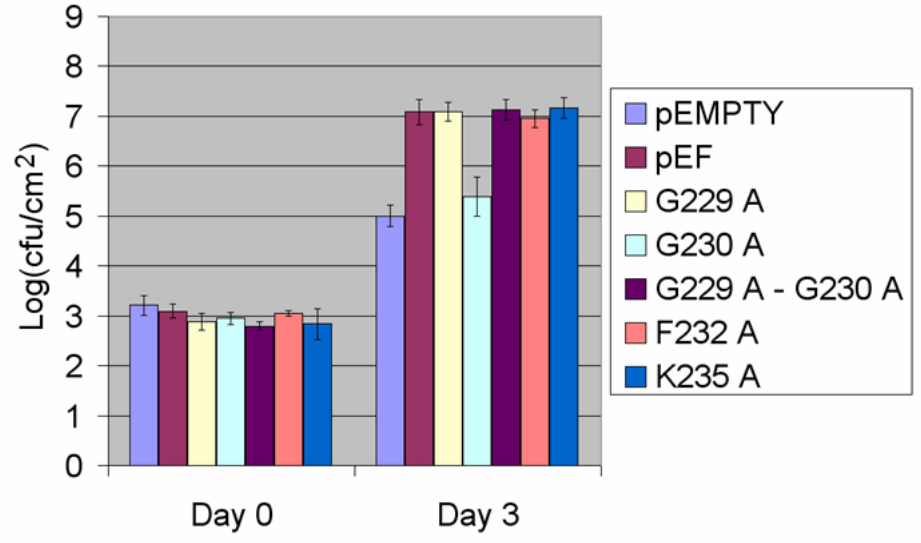
A)

```
Pst DC3000    ELSRPSASLKEAAGDLKKEGFELKS 1795
Ps            EIAKANPDVASALSELKKEGFEMKS 1835
Ea 321        GIAQANPQVASALTDLKKEGLEMKS 1838
Pv SP5.3c     EMSRPTKEVIATAQALKKDGMEFRA 1719
Psp           ELSRPSASLKEAASKLKQEGFELKS 1714
Pss B728a     ELSRPSASLKEAAGELKLSGFEVKS 1786
Pca SCRI1043 EIAKANPATANALQQLQQEGLQLNG 1614
                :::..     .   *: .*:..
```

B)

```
Pst DC3000    ---------GGRFELKDEKLVRNSE---PQGSIQLDAKGK 256
Ps            IAENTAE-AEANRRIQPLQTPPPLENTPVSPLSLTLDKGK 302
Ea 321        VAESVLEGTDTTQSPLKPQSMLKGSGAGVTPLAVTLDKGK 315
Pv SP5.3c     ---------AGTFRMENGNLERNTP---SINIISLDAKGR 202
Psp           ---------GGRFELKDEKLVRNSE---PQGSIQLDAKGK 177
Pss           ---------GGSSELRSEQLIRDTR---PQATIRLSADGK 258
Pca SCRI1043 ------------------------------------HGK 121
                                                  .*:
```

C)



G229 and G230 (Figure 2-11) were also selected for mutagenesis based on the fact that the serine protease Thrombin hydrolyzes peptide bonds immediately after a GR sequence (Castillo et al. 1983). The G229A mutation does not have effect on AvrE function. On the other hand, the G230A impairs AvrE function. Surprisingly, the G229A/G230A double mutation does not disrupt AvrE function (Figure 2-11). Neither of these G residues have a high conservation among the AvrE family members.

Other amino acids conserved along the length of AvrE

In addition to identifiable motifs such as WxxxE and LKKxGxE in the AvrE protein, there are over one hundred other residues that are conserved in all AvrE-family members. I hypothesized that some of these residues would be required for AvrE function. Because of the large number of conserved residues, I further narrowed the list of these residues to those that are commonly found in the active sites of enzymes, acting as proton acceptors/donors or at the sites of common posttranslational modifications, such as acylation, methylation, glycosylation, phosphorylation, prenylation and carboxylation, amounting to 42 residues. The ΔCEL mutant was transformed with each of the resulting AvrE mutants, and the transformants were tested in pathogenesis assays. All 42 AvrE mutants were tested for their ability to promote disease, evaluated by symptoms after bacterial infiltration of Arabidopsis plants. Only two of the mutants tested (K1171A and Y1756A) were incapable of promoting disease symptoms. Bacterial population growth assays were performed with the ΔCEL mutant carrying either of these two non-functional AvrE derivatives and severe bacterial multiplication defects were found for both (Figure 2-12). I conducted further analysis of sequences surrounding K1171 and &1171. However, neither K1171 nor Y1756 is surrounded by local sequences of particularly high

conservation among the AvrE family. An algorithm used to identify putative protein motifs (Pagni et al. 2007) also did not show either of these two residues as being part of a possible functional motif (next page).

A)

S318 A	Y655 A	Y925 A	E1164 A	R1252 A	N1420 A	E1678 A
R377 A	D752 A	E982 A	Y1167 A	Y1254 A	N1444 A	K1680 A
S428 A	R848 A	S1058 A	K1171 A	R1260 A	R1445 A	D1681 A
D504 A	R902 A	K1100 A	K1205 A	R1314 A	N1450 A	R1712 A
H569 A	H913 A	R1102 A	R1222 A	H1364 A	T1502 A	Y1756 A
N585 A	R918 A	Y1144 A	Y1224 A	R1410 A	D1534 A	D1759 A

B)

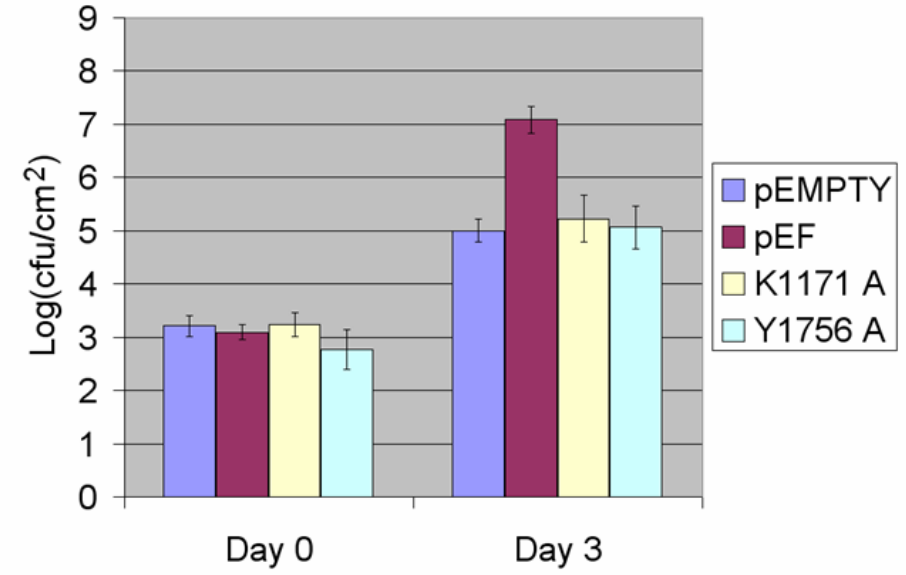


Figure 2-12 Population growth of the ΔCEL mutant complemented with AvrE mutants K1171 A and Y1756.

A) A list of the 42 conserved amino acids along the sequence of AvrE that were selected for site-directed mutagenesis. All amino acids were mutated to alanine (A) and the resultant AvrE mutants were tested for their capability to complement the ΔCEL bacterium, as determined by their ability to allow the ΔCEL mutant to produce symptoms in infected plants. Only mutations in amino acids K1171 and Y1756 (highlighted in yellow ■) abolished disease symptom development. **B)** Bacterial population enumeration was then performed only for the K1171A and the Y1756A mutants. The ΔCEL mutant containing pUCP19 + pAVRF (pEMPTY) was used as a negative control. The ΔCEL mutant containing wild-type *avrE* (pAVRE + pAVRF = pEF) was used as a positive control. Arabidopsis leaves were infiltrated with bacterial suspensions ($1x10^6$ cfu/ml). Bacterial population enumeration was performed at day 0 and day 3. Each column represents the average of four biological replicas, with standard deviations displayed.

AvrE-K1171A, -Y1756A, -G230 and AvrE–W_1W_2x5E are produced and secreted

To determine whether the loss-of-function of AvrE-K1171A, -Y1756A, -G230 and -W_2x5E mutants was due to protein expression problem or defect in secretion through the TTSS, I performed western blot analysis, as described previously for other AvrE mutants (see Figure 2-6).

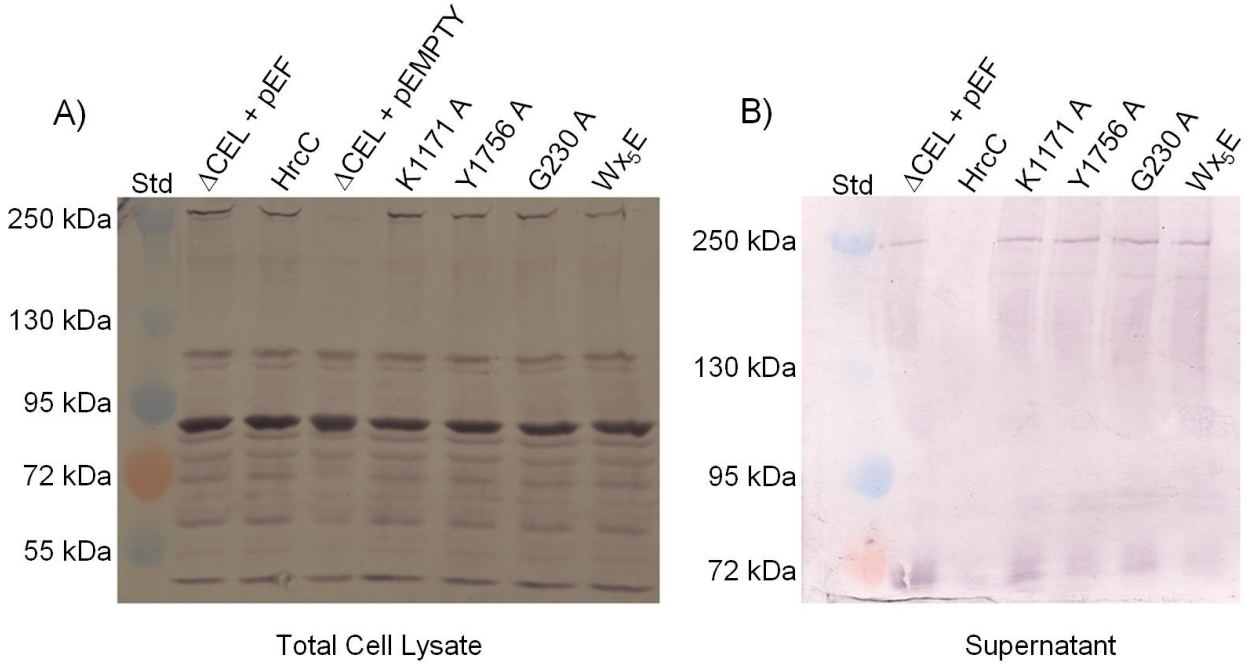


Figure 2-13 The AvrE mutants K1171A, Y1756A, G230A and Wx5E are expressed and secreted to the supernatant of bacterial cultures.
A) Western blot showing detection of AvrE and AvrE mutants in total cell lysate. **B)** Western blot showing detection of AvrE and AvrE mutants in culture supernatants. The ΔCEL mutant complemented with *avrE* (pAVRE + pAVRF = pEF) was used as a positive control in both blots. The ΔCEL mutant with pUCP19 + pAVRF (pEMPTY) was used as a negative control for detection of AvrE in total lysate samples (A). The secretion-defective *hrc* mutant was used as a negative control for the secretion assay (B). Western blots were performed using an AvrE antiserum produced in rabbit.

Like wild type AvrE, all mutant AvrE proteins can be detected in the culture supernatant (Figure 2-13). Thus, the loss of effector function is not due to problems of protein expression or secretion.

The predicted three-dimensional structure of AvrE contains β-propeller and ubiquitin-associated UBA folds

To gain further insights into the structure-function relationship of AvrE, I used structure prediction algorithms to perform analysis of potential three-dimensional folds of the AvrE-family. I used the HHpred (Söding et al. 2005) and Modeller (Sali and Blundell 1993) programs (http://toolkit.tuebingen.mpg.de/sections/tertstruct) for this analysis. These algorithms can be used to predict putative secondary structures based upon protein family alignments. The predicted secondary structure can then be fed into an algorithm that calculates the three-dimensional folding of the protein, which is then compared to known structural data of other proteins (Figure 2-14).

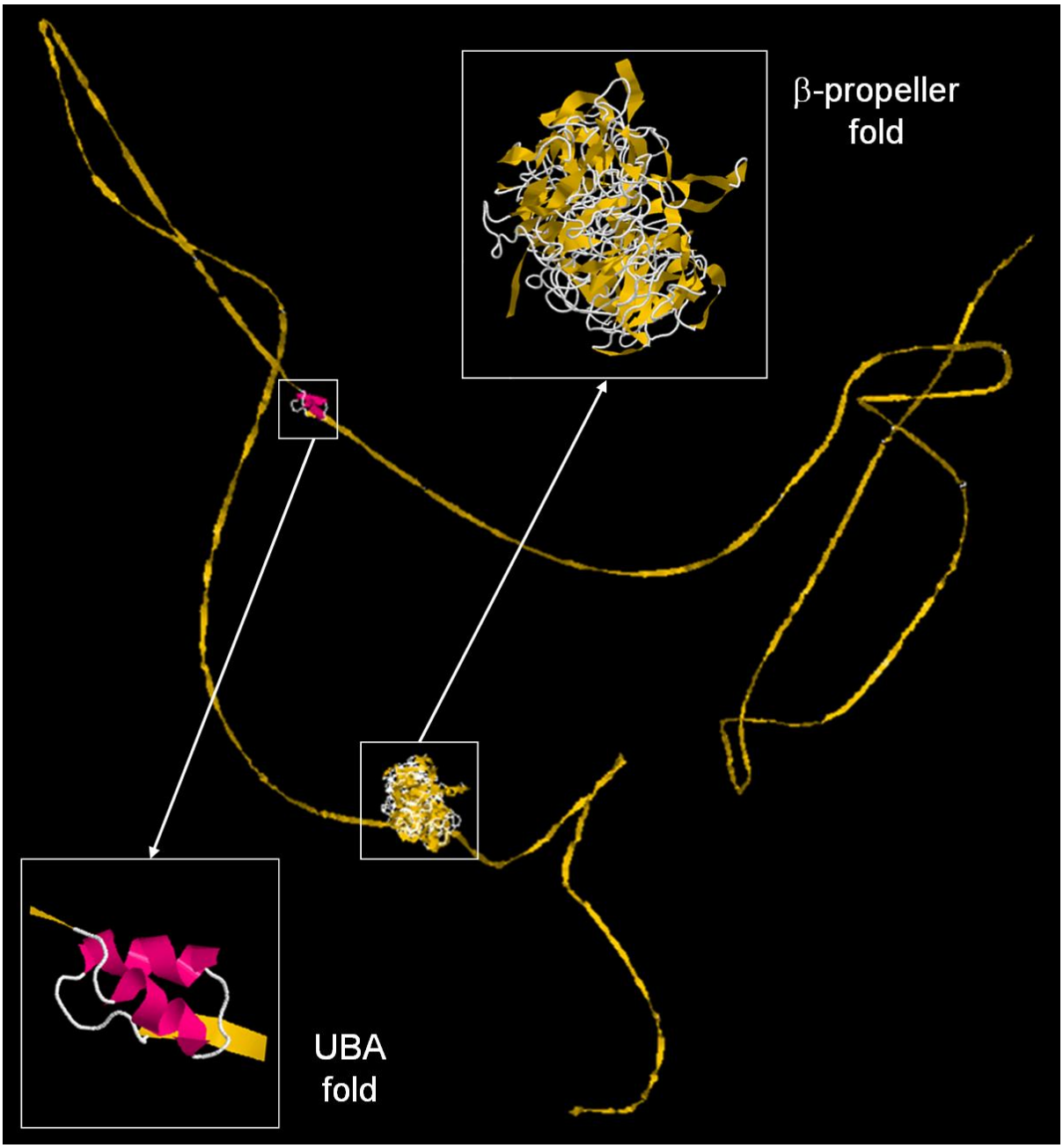
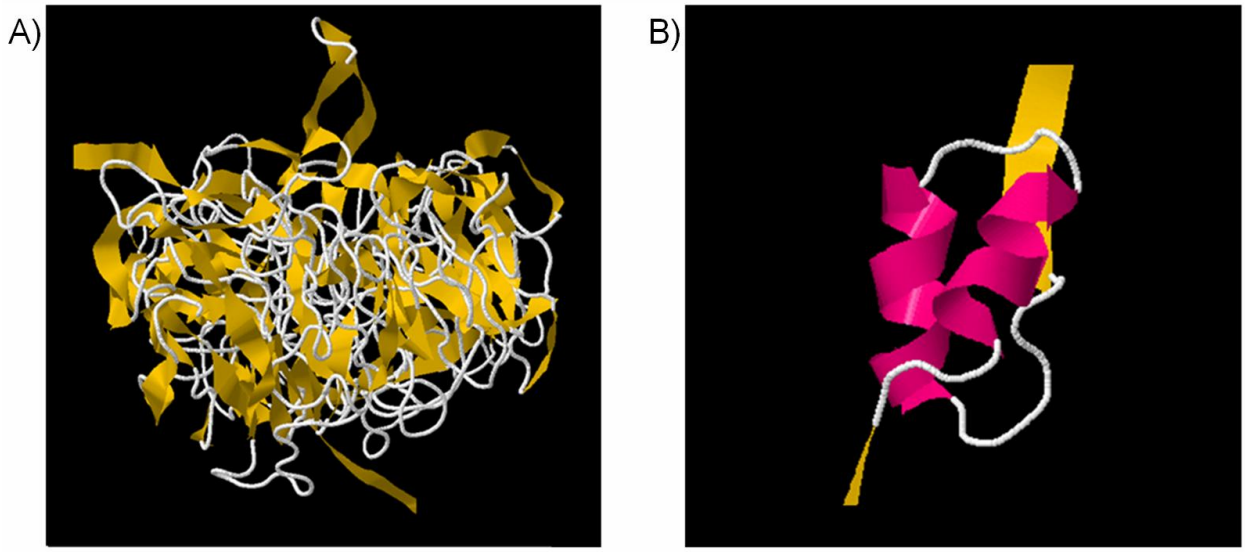


Figure 2-14 Predicted three-dimensional folding of AvrE-family.
Sequences for AvrE (*Pst* DC300), DspE (*E. amylovora*) and WstE (*P. stewartii*) were fed into the HHpred and Modeller algorithms which provide a prediction of a protein tertiary structure. Prediction is based upon multiple sequence alignments that provide a consensus of the primary structure, then predicts a consensus secondary structure that is compared to the secondary structures of proteins with known three-dimensional folds. Most of the AvrE family protein sequence bears no similarity to known structural folding patterns, with the exception of the β-propeller and UBA folds. Output results are visualized using Jmol (http://www.jmol.org).

Figure 2-14 shows the overall three-dimensional folding result based on the protein sequences of *Pst* DC3000 AvrE, *E. amylovora* DspE and P. *stewartii* WstE. Sequences were analyzed separately and as a group with similar prediction outcomes.



V171 → E887
(AvrE *Pst* DC3000)

G1145 → K1175
(AvrE *Pst* DC3000)

G230	W829 (W_2)	G639 (W_4)
W393 (W_1)	E833 (E_2)	K778 (W_5)
E397 (E_1)	N588 (W_3)	A791 (W_6)

K1171

Figure 2-15 Detailed views of predicted β-propeller and UBA folds of the AvrE protein family.

A) The β-propeller fold, similar to that of RCC1, is found between V171 and E887 of *Pst* DC3000 AvrE. **B)** The UBA fold, similar to that of HUWE1, is found between G1145 and K1175 of *Pst* DC300 AvrE. Beneath each predicted structure, there is a list of the amino acids identified in this dissertation that are required for AvrE function and that fall within the boundaries of each predicted fold. Images are visualized using Jmol.

Although the folds of most regions of the AvrE protein cannot be predicted (Figure 2-14), the AvrE family shares a β-propeller folding pattern (Figure 2-15) with RCC1 (Regulator of Chromosome Condensation 1; Renault et al. 2001) (E-value=37, P-value=0.0018 and Score=37.8). RCC1 is a GEF for Ran, a small GTPase. Another predicted fold is an ubiquitin-

associated (UBA) domain (Figure 2-15) similar to that of the E3 ubiquitin ligase HUWE1 (Mueller and Feigon 2002) (E-value=1.2, E-value=5.8E-05 and Score=37.7). UBA is known to directly bind to ubiquitin (Bertolaet et al. 2001, Chen et al. 2001 and Wilkinson et. al 2001). In *Pst* DC3000 the β-propeller fold is located between amino acids V171 and E887, whereas the UBA folds lies between G1145 and K1175.

Interestingly, of the 13 amino acid mutations that affected the function of AvrE, nine are within the predicted β-propeller fold (G230, W393, E397, W829, E833, N588, G639, K778 and A791). A single amino acid important for AvrE function was identified within the UBA fold (K1171). The remainder three amino acids (Y1756, K1787 and K1788) are located near the C-terminus of AvrE, the structure of which cannot be predicted by the HHpred and Modeller algorithms.

DISCUSSION

In this study, I demonstrated that AvrE possesses two functionally redundant WxxxE motifs that are required for effector function. These motifs are characteristic of and required for the function of a family of animal pathogen effectors acting as GEFs (Alto et al. 2006). I found that the locations of these WxxxE motifs in the AvrE-family proteins can vary greatly within the N-terminal half of the protein. Most interestingly, I showed that functional WxxxE motifs can be created *de novo* in the AvrE protein in at least six of these specific conserved locations. I found that, just like in animal pathogen effectors, conservative substitution of the WxxxE motif to WxxxD retains effector function. Despite the invariable 3-aa spacer sequence in the motif, insertion of one spacer amino acid or deletion of up to two spacer amino acids can be tolerated, without impacting the function of the WxxxE motif. Although there are several highly conserved amino acids at the C-terminus of AvrE-family effectors, I found that only the double KK residues are required for AvrE function. Finally, I mutagenized 42 other amino acid residues that are located along the length of AvrE and conserved in all other AvrE-family effectors. Two of these residues, K1171 and Y1756, located at the C-terminal half of the protein, were found to be required for AvrE function. In total, my dissertation research represents, to date, the most comprehensive mutagenesis study of AvrE-family effectors, which are arguably among the most important virulence factors of bacterial plant pathogens. Below, I will discuss the experimental results in the context of the structure-function relationship.

Sequencing of loss-of-function AvrE mutants showed that all of them carry two additional common amino acid changes at the N-terminus: K91E and S311G. Sequencing of the template DNA used for mutagenesis showed that it also carried these two mutations. These random mutations must have been incorporated during the original PCR amplification and cloning of AvrE into pUCP19 to produce pAVRE. However, these amino acids by themselves are not associated with changes in the virulence function of AvrE because pAVRE is fully capable of complementing the CEL mutant of DC3000 (Ham et al. 2009). It could be argued that these extra mutations may produce synergistic effects with some of the subsequent mutations to mask or accentuate the effect of intended mutations. Although possible, this probability may not be very high for two reasons. First, residues at these two positions are not conserved among AvrE-family members; indeed, amino acids of very different properties are present in these two positions among different AvrE-family effectors. Second, the nonfunctional AvrE-E_1E_2 mutant, which carried these two extra changes, could be restored to full virulence function by tertiary mutations that create *de novo* WxxxE/D motifs elsewhere in the AvrE protein.

The most interesting results came from the mutagenesis of the WxxxE motifs. I have shown that, like in animal effectors, WxxxE motifs are required for the virulence function of AvrE. Also, I have demonstrated that conservative substitutions of E for D in this motif do not affect function; just as reported for animal pathogen effectors. I found that some AvrE family members naturally harbor the WxxxD variant. In animal pathogens WxxxE effectors restructure the host cytoskeleton by inducing the formation of stress fibers, filopodia, and lamellipodia/ruffles (Bulgin et al 2010), which promote bacterial growth and disease development. Based on my findings and on previous reports (DebRoy et al. 2004), a similar

function may be hypothesized for AvrE; since this effector interferes with cell wall/extracellular defenses, including callose deposition (DebRoy et al. 2004). Callose deposition in response to bacteria is associated with papilla formation and other extracellular defenses, which likely rely on vesicular trafficking (Bestwick et al. 1995; Hückelhoven, 2007). Because the cytoskeleton is critical for vesicle traffic, AvrE may exert its virulence function by modulating the function of cytoskeleton and vesicle traffic. This possibility may provide an explanation for the functional redundancy between HopM1 and AvrE (Deb Roy et al. 2004). HopM1 targets an ARF-GEF protein for proteasomal degradation (Nomura et al. 2006). ARF-GEFs are activators of small GTPases and are critical regulators of vesicle trafficking in eukaryotic cells (Geldner et al. 2003; Tanaka et al. 2009). WxxxE-family effectors in animal pathogens are GEF proteins (Huang et al. 2009). It is therefore possible that AvrE may mimic plant GEFs. However, more work is required to substantiate this idea. For example, studies are needed to determine whether vesicular trafficking is affected by AvrE, where AvrE is localized in the host cell, and if this bacterial effector also affects the cytoskeleton. Final confirmation of AvrE as a GEF would require the identification of specific Arabidopsis small GTPase(s) targeted by AvrE.

Recent crystal structure analyses of animal pathogen WxxxE effectors suggest a structural, instead of catalytic, role for the WxxxE motif (Ohlson et al. 2008, Huang et al. 2009). In the case of effector SifA, the WxxxE motif is thought to stabilize the catalytic loop responsible for promoting the GDP to GTP exchange (Ohlson et al. 2008). For the effector Map, WxxxE is believed to act as the vertex of a V-shaped structure that stabilizes two structural domains of this small protein, allowing for the physical interaction of Map with the small GTPase Cdc42, as well as for the proper positioning of the catalytic loop of Map (Huang et. al

2009). At this point, it is difficult to extrapolate such structure information to AvrE for several reasons. First, animal pathogen effectors are much smaller proteins than AvrE-family proteins. Map has 203 residues and SifA has 336, whereas AvrE has 1,795. Second, the overall primary and secondary structures of Map and SifA are very different to those predicted for AvrE. Third, AvrE has two functionally redundant WxxxE motifs that are located in two positions where the surrounding sequence contexts are completely different. Thus, unlike the WxxxE effector in animal pathogens, the function of the WxxxE motif in AvrE may be independent of adjacent protein sequences/domains. Moreover, I found that the length of the spacer sequence of the WxxxE motif in AvrE can be altered without affecting the virulence function (Figure 2-9). This finding suggests that the WxxxE variant in the W_1 location of *P. carotovorum* (W_1QKNSD) may be functional (Figure 2-4). It would be interesting to determine in the future whether this spacer length flexibility applies to animal pathogen effectors. Considering these differences and uncertainties, it remains to be determined whether the WxxxE motif plays a similar structural stabilization function in AvrE.

The *de novo* creation of just one WxxxE/D motif at any of the four conserved W locations in the AvrE sequence is enough to restore the virulence function of the AvrE-E_1E_2 mutant. This result further suggests that the WxxxE motif may have a function independent of surrounding sequences/domains, as I could not find similar sequences near the seven conserved W residues. If we were to assume that somehow part (i.e., near one of the WxxxE motifs) of AvrE has a fold similar to that of the animal pathogen WxxxE effectors, it seems highly unlikely that the recreation of a WxxxE motif in another conserved W site would reconstitute that particular fold, unless AvrE has multiple GEF catalytic loops made of very different sequences.

Thus, it seems possible that the WxxxE motifs may perform a function in AvrE that is different or in addition to playing a structural role shown for the WxxxE motif of animal pathogen effectors.

A precise evaluation of the function of the WxxxE motif may only be achieved by x-ray crystallography of AvrE. However, I believe that computational predictions may offer some insight. For example, the conserved W_1 to W_6 locations are contained within the predicted β-propeller fold of the AvrE-family. If the WxxxE motif indeed has a structural stabilization function, it could be hypothesized that WxxxE motifs may be required for maintaining the overall structure and proper folding of the predicted β-propeller. Moreover, it may be possible that only one WxxxE motif is enough to provide minimal overall stabilization of the β-propeller fold, whereas two motifs generate greater stabilization. This could explain not only why WxxxE motifs are functionally redundant, but also why they often show up in pairs in effector proteins. It is also possible that this β-propeller fold can be stabilized at different points of its structure, as marked by seven conserved Ws. This could explain why WxxxE motifs are found in different conserved W locations in different AvrE-family members and why it is possible to recover the function of the AvrE-E_1E_2 mutant by recreating WxxxE motifs at only one of the remainder conserved Ws ($W_3 \rightarrow W_6$).

Interestingly, residue G230 is also located within the boundaries of the predicted β-propeller. It is however difficult to render a specific hypothesis about why the G230A mutation causes the loss of AvrE function. Perhaps this mutation produces major destabilization of the β-

propeller scaffold. However, the effect of G230A mutation is puzzling because double G229-G230 mutations do not affect AvrE function. Again, the three-dimensional structure of AvrE is required to properly understand this puzzle.

Even if the predicted β-propeller structure in AvrE-family is not accurate, the conserved W_1 through W_6 locations do not seem to be randomly located along the length of the AvrE. There are no WxxxE motifs in the C-terminal half of the AvrE-family, nor is there any WxxxE motifs close to the very N-terminus. It is therefore likely that this portion of the AvrE-family sequence defines a protein domain that is important for the function of all the members of the AvrE protein family.

Another three-dimensional structural feature predicted for the AvrE-family by the HHpred and the Modeller algorithms is an UBA domain (Mueller and Feigon 2002). The UBA domain is capable of directly binding to ubiquitin (Bertolaet et al. 2001, Chen et al. 2001 and Wilkinson et. al 2001). In AvrE, the predicted UBA domain spans between residues G1145 to K1175. Mutation of K1171, a residue located inside the putative UBA domain causes the loss of AvrE function (Figure 2-12). The UBA domain relies on a hydrophobic surface patch to bind ubiquitin (Wilkinson et. al 2001). Further mutagenesis could test if changes on the hydrophobic surface of the predicted UBA domain disrupt AvrE function. Direct interaction of AvrE and ubiquitin or ubiquitinated proteins could be tested by performing immuno-precipitation. Some proteins containing a UBA domain targets ubiquitinated proteins for degradation. One such example is Rad23, which shuttles ubiquitinated misfolded proteins to the proteasome for degradation (Kim et al. 2006). Interestingly, targeting plant proteins for proteasomal degradation

is a well documented strategy used by *Pst* DC3000 to promote disease and to avoid the plant immune response. For example, the bacterial effector AvrPtoB is an E3 ligase that targets the host proteins Fen (Rosebrock et al. 2007) and FLS2 (Göhre et al. 2008) for proteasomal degradation. The effector HopM1, functionally redundant to AvrE, targets several host proteins for proteasomal degradation (Nomura et al. 2006). Thus, it is possible that AvrE may engage proteasomal degradation of ubiquitinated host proteins through its predicted UBA domain, and this activity may be part of its virulence function.

If the structural predictions are further supported by future experimental evidence, there is a possibility that AvrE may have more than one biochemical activity--a GEF activity and an ubiquitin-binding activity. Such a possibility is not unprecedented. For example, the *S. typhimurium* effector SopB is involved in the internalization of the pathogen into the host cell by regulating the bacterial vacuolar biogenesis during infection (Bakowski et al. 2008). SopB is an inositol phosphate phosphatase (Norris et al. 1998) that is also capable of binding Cdc42 (Rogers et al. 2008). It is believed that interaction between Cdc42 and SopB directs the effector to membrane patches areas of high Cdc42 concentration, where the effector can then remove phosphate groups from membrane lipids, inducing localized effects (Rogers et al. 2008). Another example of an effector with multiple biochemical activities is *E. coli* EspF (Holmes et al. 2010). EspF contains proline-rich repeats that mimicking SH3-binding domains (McNamara and Donnenberg, 1998) that allows for interaction with eukaryotic proteins containing the SH3 domain (Mayer, 2001). Moreover, EspF modular structure includes two functional subcellular localization signals. In the mitochondria, this allows EspF to initiate caspase-dependant

programmed cell death (Nougayrede and Donnenberg, 2004). In the nucleolus, EspF promotes the complete removal of nucleolin and other nucleolar factors (Dean et al. 2010).

Single mutations of the highly conserved residues (e.g., L1786 and G1790) at the C-terminus of AvrE do not affect the effector function (Figure 2-10). When two double mutations were tested, only one of them (K_1K_2 to AA) negatively impacted the effector virulence function (Figure 2-5). The other double mutation corresponds to a K_1K_2 motif variation (QQ) found naturally in *P. carotovorum*, suggesting that the QQ variation is functional (Figure 2-10). We had expected otherwise since TTSS effectors are believed not to play an important contribution in the virulence of this bacterium (Alfano and Collmer 1996; Holeva et al. 2004), given that *P. carotovorum* relies heavily on the production of cell-wall degrading enzymes for virulence (Barras et al. 1994). Other naturally occurring variations of this motif (KL and KQ) are also functional when introduced into AvrE. Thus, the function of AvrE does not require a double lysine motif and strongly implies that the LKKxGxE motif found in AvrE-family effectors is not a mimic of the ERMRS first hypothesized by Ham and co-workers (2006). It is possible that the LKKxGxE motif represents a novel subcellular targeting signal. Future subcellular localization studies of wild-type AvrE and AvrE-K_1K_2 could shed light on this question.

The Y1756A mutation affected the virulence function of AvrE. This amino acid is not in the putative β-propeller fold, where the WxxxE motifs are located, nor at the extreme C-terminus, where the LKKxGxE motif is found. Thus, the requirement of Y1756 for AvrE

function is unlikely related to the two motifs. Again, a three-dimensional structure of AvrE is required to properly understand the role of this residue.

In summary, through this study I have identified several motifs, conserved amino acids, and potential structural domains in AvrE that are associated with effector function. I believe that these results provide a valuable foundation for future study of the virulence function of AvrE-family effectors. I anticipate that the full impact and usefulness of this study will become obvious once the host targets of AvrE are identified and the three-dimensional structure of AvrE becomes available.

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CHAPTER 3

Screen for potential host targets of the AvrE protein

ABSTRACT

The plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 *(Pst* DC3000) is a Gram-negative bacterium that causes the bacterial speck disease in tomato and Arabidopsis. It has been previously demonstrated that deletion of the Conserved Effector Locus (CEL) in the *Pst* DC3000 genome strongly reduces bacterial virulence in tomato and Arabidopsis. The virulence of the ΔCEL mutant can be restored by complementation with CEL effector gene *avrE* or *hopM1*, which indicates that *avrE* and *hopM1* are functionally redundant. Unlike *hopM1*, which is restricted mainly in *P. syringae* strains, *avrE* has orthologs in other pathogenic bacterial species, such as *dspE* in *Erwinia amylovora* and *wtsE* in *Pantoea stewartii*. Deletion of *dspE* or *wtsE* also greatly reduced the virulence of the corresponding pathogens. The plant host targets of AvrE-family effectors remain unknown. Transgenic expression of *avrE* is toxic in plant and yeast, suggesting that the host targets of AvrE may be widely conserved in eukaryotes.

In my dissertation research, I took two approaches to identify a pool of potential host targets for AvrE. First, I co-expressed *avrE* with an Arabidopsis cDNA library in yeast, with the goal of identifying plant proteins capable of suppressing AvrE toxicity. Secondly, I produced C-terminal deletion mutants of AvrE, which are no longer toxic to yeast, and used them to identify Arabidopsis proteins that interact with AvrE. Each approach yielded several host proteins that either suppress AvrE toxicity or interact with truncated AvrE in yeast. I conducted a number of follow-up studies, attempting to determine the biological relevance of some of the identified host proteins to the AvrE function. Unfortunately, no definitive conclusion can be reached for any of the host proteins identified. However, I found that an AvrE fragment spanning the first 522

amino acids interacted with the Arabidopsis protein Rad23, which is a known interactor of HopM1. Although Rad23 does not suppress the toxicity of the full-length AvrE protein in yeast, it is degraded when Arabidopsis plants are infected with DC3000, but not when infected with the ΔCEL mutant. Furthermore, Rad23 degradation occurs when plants are infected with ΔCEL mutants that are complemented with either *avrE* or *hopM1*, indicating that Rad23 may be a common target for HopM1 and AvrE.

Note:

Figure 3-7 was made in collaboration with Dr. Kinya Nomura. Dr. Nomura ordered T-DNA insertion lines (SALK institute) and performed genotyping to verify that they were homozygous for the T-DNA insertions in the genes of interest. Then, Dr. Nomura proceeded to infect the different T-DNA insertion lines with bacteria and performed bacterial multiplication assays (page 119).

INTRODUCTION

The Gram-negative bacterium *Pseudomonas syringae* relies on the type three secretion system (TTSS) to deliver dozens of effectors into the plant cell (Chang et al. 2005; Schechter et al. 2006). Once inside the plant host, the concerted actions of these effector proteins overcome the plant immune response and promote disease (Boller and He 2009). The TTSS is encoded by *hrp* genes in the chromosome of *P. syringae*. The *hrp* gene cluster is flanked by two additional clusters of genes encoding bacterial effector proteins (Alfano et al. 2000). Effector genes at one side of the *hrp* cluster are variably present among different *P. syringae* strains. This locus is named the exchangeable effector locus (EEL). On the other side of the *hrp* cluster, effector genes are highly conserved among different *P. syringae* strains. This locus is called the conserved effector locus (CEL) (Alfano et al 2000). Deletion of the EEL (ΔEEL) affects only slightly the overall virulence of *Pst* DC3000 (Alfano et al. 2000). In contrast, deletion of the CEL (ΔCEL) has a very strong negative impact on the virulence of *Pst* DC3000. The ΔCEL mutant can no longer produce disease symptoms. Moreover, the bacterial population growth is greatly diminished (Alfano et al. 2000).

In the ΔCEL mutant, six CEL genes —*avrE, avrF* (coding for the chaperone for AvrE secretion), *hopM1, shcM1* (coding for the chaperone for HopM1 secretion), *hrpW* and *hopA1*— are replaced by a spectinomycin-resistance-gene cassette. *hrpW* and *hopA1* do not make significant contributions to the virulence of *Pst DC3000* (Charkowski et al. 1998; Badel et al. 2002). However, the ΔCEL mutation can be independently complemented by *avrE* plus *avrF* or

hopM1 plus *shcM* (DebRoy et al. 2004). This result indicates that AvrE and HopM1 are functionally redundant and that they make an important contribution to the virulence of *Pst* DC3000 (DebRoy et al. 2004). A later report confirmed these conclusions (Badel et al. 2006). In particular, deletion of *avrE* or *hopM1* alone does not have any effect in *Pst* DC3000 virulence. However, an *avrE* and *hopM1* double mutant exhibits the phenotype originally observed for the ΔCEL mutant (Badel et al. 2006). Although *hopM1* seems to be restricted to *P. syringae*, *avrE*-family effector genes are found in many other bacterial plant pathogens, such as *dspE* in *Erwinia amylovora* (Gaudriault et al. 1997) and *wtsE* in *Pantoea stewartii* (Frederick et al. 2001). Loss of function *wtsE* or *dspE* mutants alone exhibit strong virulence defects (Frederick et al. 2001, Bogdanove et al 1998). These results suggest that *E. amylovora* and *P. stewartii* do not have other effectors that are functionally redundant to *dspE* or *wtsE*, as is the case of *hopM1* for *avrE* in *Pst* DC3000.

An in-frame deletion of *dspE* (between amino acids G203 to G720) in *E. amylovora* can be complemented by the 5' terminal half of *dspE*. This suggests that the N-terminal half of the protein may form a stable functional domain that is required for effector function (Bogdanove et al. 1998). Furthermore, the same *E. amylovora dspE* mutant can be weakly complemented by full-length *avrE* from *Pst* DC3000 (Bogdanove et al. 1998), suggesting that AvrE-family effectors may have a similar function in diverse bacteria.

Plant cells react to microbes by thickening the cell wall at the point of contact, forming microscopically visible structures known as papillae. Papillae contain callose (β-1,3 glucan), phenolic compounds and other molecules and are thought to restrict the growth of invading

microbes (Bestwick et al. 1995), although direct evidence is lacking. It has been shown that *Pst* DC3000 uses the TTSS to suppress cell wall-based defense responses, such as callose deposition, during infection (Hauck et al. 2003). Interestingly, the ΔCEL mutant is no longer able to inhibit callose deposition in the cell wall (DebRoy et al. 2004). However, the ability to suppress callose deposition can be restored by complementing the ΔCEL mutant with either *hopM1* or *avrE*. These results suggest that AvrE and HopM1 are suppressors of cell wall-based defense (DebRoy et al. 2004). Both HopM1 and AvrE also have a potent cell death-promoting function (DebRoy et al. 2004).

Little is known about how AvrE suppresses host cell wall-associated defense or promotes host cell death. On the other hand, a host target of HopM1 has been identified (Nomura et al. 2006). In these experiments, the N-terminal portion of HopM1 was used to screen an Arabidopsis yeast two-hybrid (Y2H) cDNA library. Several Arabidopsis proteins that interact with this HopM1 fragment were identified and called AtMINs (*Arabidopsis thaliana* HopM1 interactors). Importantly, *atmin7* knock-out mutants showed significantly increased susceptibility to the ΔCEL bacterium, suggesting that HopM1 degrades AtMIN7 to promote host susceptibility. Moreover, the *atmin7* knock-out mutant is reduced in callose deposition in response to the ΔCEL bacterium (Nomura et al. 2006). AtMIN7 is a member of the ADP ribosylation factor (ARF) family of guanine nucleotide exchange factors (GEFs), which are regulators of the vesicle trafficking system (Grebe et al. 2000; Geldner et al. 2003). It was noted that the multiplication of the ΔCEL bacterium in the *atmin7* mutant plant was not as good as that of *Pst* DC3000 in wild-type plants, suggesting AtMIN7 is likely an important, but not the sole target of HopM1 in

Arabidopsis (Nomura et al. 2006). Presumably, some of the other AtMIN proteins identified are additional host targets of HopM1.

Few plant proteins have been identified as putative interactors of DspE. A Y2H screen of an apple cDNA library using the N-terminal 967 amino acids of *E. amylovora* DspE identified several putative leucine-rich repeat (LRR) receptor-like serine/threonine kinases (RLK) (Meng et al. 2002). Silencing of the genes coding for these LRR-RLKs increases the resistance of apple to *E. amylovora* infection (Borejsza-Wysocka et al. 2004). However, it not clear whether these host proteins are biologically relevant to the AvrE virulence function because it was not shown whether the virulence of the *dspE* mutant is restored in these apple plants. In this chapter I describe a series of experiments aimed at identifying Arabidopsis proteins that interact with AvrE and assessing their involvement in the virulence function of AvrE.

MATERIALS AND METHODS

Construction of AvrE deletion mutants for Y2H experiments

For studies in yeast, full length and truncated *avrE* constructs corresponding to the first 522, 600, 900 and 1000 amino acids from the N-terminal end were created by PCR using *Pst* DC3000 genomic DNA as template and Platinum^{TM} Pfx DNA polymerase (Invitrogen), according to the manufacturer's instructions. The constructs were then cloned into the *Bam*HI and *Xho*I restriction sites of plasmid pGilda. *avrF* was similarly PCR-amplified using *Pst* DC3000 genomic DNA and cloned in the pB42AD plasmid at the *Eco*RI and *Xho*I restriction sites.

Yeast growth and manipulation

Transformation of yeast cells with Arabidopsis cDNA library were performed according the Clonetech yeast protocol handbook and the Matchmaker Gal4 Two-Hybrid System 3 & Libraries User Manual. Minimal SD base medium (non-inducing medium) was used to grow yeast cells (Clontech^{TM} catalog #630411). Minimal SD base medium supplemented with a mixture of galactose/rafinose was used to induce the expression of the relevant genes (Clontech^{TM} catalog #630420).

Screen for Arabidopsis proteins that could suppress AvrE toxicity in yeast

An Arabidopsis cDNA (kindly provided Dr. Jonathan Jones, The Sainsbury Laboratory at Norwich, England) in pB42AD was screened to search for clones that were able to suppress the toxic effect of AvrE expression in yeast. About 157.5 million clones, representing approximately 25 times genome coverage were screened. Yeast clones were plated on solid inducing medium in 15x150 mm Petri plates at densities of $1x10^5$ to $5x10^6$ cfu per plate. Surviving yeast clones were collected, cultured in non-inducing medium, and stored at -80°C in 15% glycerol. To verify that survival was due to suppression of the AvrE toxic effect and not due to spontaneous AvrE mutations, the library plasmids (pB42AD) were cured from each of the surviving yeast clones to determine if AvrE (in pGilda) was still toxic. Yeast clones growing in selective non-inducing liquid medium were subcultured every 24 hours. After five days of subculturing, individual colonies were isolated for each clone by plating on non-inducing media. Ten colonies of each clone were then patched on media with selection markers for each plasmid. Plasmid loss, as well as sustained AvrE toxicity, was assessed according to the ability of yeast clones to grow in different culture media. Yeast plasmid DNA minipreps were performed for those library clones that were positive for sustained toxicity suppression. cDNA inserts in the pB42AD plasmid were then amplified by PCR. PCR products were sequenced and compared to Genbank sequence databases using the BLAST algorithm for gene identification (Altschul et al. 1997). To verify toxicity suppression and physical interaction with AvrE, the library plasmids containing the clones of interest were transformed into *Escherichia coli* KC8 cells. Plasmid DNA was purified from KC8 transformants and was transformed back into yeast. Toxicity suppression was tested again.

Evaluation of AvrE toxicity and protein-protein interaction in yeast

To evaluate the toxicity of AvrE and AvrE derivatives in yeast, as well as AvrE-toxicity suppression by Arabidopsis cDNA library clones, the following two procedures were used.

A) Direct plating: From a single isolated colony, yeast cells were grown overnight in 3 ml liquid culture (30°C at 250 rpm) using 18x150 mm borosilicate glass tubes. One-ml aliquots were washed three times, each time with one volume of water and centrifuged in 1.5 ml Eppendorf tubes (3 minutes at 10,621xg). After final resuspension, 15 μl aliquots were spotted onto non-inducing and inducing media. Toxicity was evaluated by comparing yeast growth between inducing and non-inducing media at 48 or 72 hours. When necessary, X-gal and BU salts were added to the inducing media to test for bait-prey interaction.

B) Serial dilutions: From a single isolated colony, yeast cells were grown overnight with agitation in 3 ml liquid culture (30°C at 250 rpm) using 18x150 mm borosilicate glass tubes. One-ml aliquots were washed three times, as described above. Ten-fold serial dilutions of yeast cell suspensions were performed and 15 μl droplets of each dilution were plated on inducing and non-inducing media. Plates were incubated at 30°C for 48 hours. Growth was compared at 48 to 72 hours after plating.

Growth of Arabidopsis plants

Arabidopsis plants were grown in mesh-covered square pots. A total of four plants were grown in each pot. Fifteen pots (3 rows by 5 columns) were put in a tray. Trays were partially covered with a plastic lid to maintain humidity. Watering was performed weekly by allowing one-inch deep water to sit in the tray from one evening to the next morning, when excess water

was drained out. All plants were grown in a day/night cycle of 12 h/12 h, under a light intensity of 100 μE/m^2s^2, and at a constant temperature of 20°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Evaluation of protein expression in yeast and plants was performed by SDS-PAGE followed by western blot. Gel preparations were conducted according to Sambrook et al. (1989). Yeast cultures (5 ml) were grown from isolated colonies in non-inducing media overnight (12-15 hours, 30°C, 250 rpm) in 18x150mm borosilicate glass tubes until OD_{600nm} reached 0.7 to 1.0. Cells were then gently separated from culture medium by centrifugation (2,000xg for 15 minutes at 25°C) and resuspended in an equal volume of inducing medium prior to incubation for another 8-10 hours (30°C at 250 rpm). After induction, total cell lysates were prepared by separating yeast cells from the supernatant by centrifugation (3 minutes at 20,817xg). One-ml of culture was concentrated 10 times in SDS-PAGE loading buffer (100 μl total) and boiled for 10 minutes prior to loading gels. Plant tissue sampling was performed by taking 5-mm-diameter discs from fully open leaves of 5 to 6 week-old Arabidopsis plants. Two leaf discs were ground in 50 μl SDS loading buffer and boiled for 10 minutes before loading onto gels.

Western blotting

Detection of protein of interest was performed using SDS-PAGE, followed by western blot. Gel preparations were conducted according to Sambrook et al. (1989). For yeast total lysates and for plant tissue, 10 to 20 μl of samples were used for SDS-PAGE. Transfer of the proteins from the gel to nitrocellulose membrane was performed using a Hoefer TE-70 semi-dry transfer unit, using a continuous current of 60 mA. Time of transfer varied depending on the

molecular weight of the protein to be visualized. One hour was used for smaller proteins (<70 kDa) and two hours for bigger proteins (>70 kDa).

Pathogenesis assays

Procedures for pathogenesis assay followed those described by Hauck et al. (2003), with some modifications. *P. syringae* from a single isolated colony was inoculated into 10 ml low-salt Luria Bertani medium with proper antibiotics. Cultures were grown overnight (12-15 hours) in 18x150mm borosilicate glass tubes to OD_{600nm}=0.7 to 1.0. Cells were then gently separated from medium by centrifugation (2,000xg for 15 minutes at 25°C) and resuspended in 10 ml water. This suspension was then diluted with water to OD_{600nm}=0.2 (equivalent to $1x10^8$ cfu/ml). Immediately before plant infiltration, a 1:100 dilution was performed (100 μl cell suspension in 10 ml water) to make up a $1x10^6$ cfu/ml suspension.

Four- to five-week-old plants were used for the experiments. Plants were hand-infiltrated with the bacterial suspension using a needleless syringe on the abaxial side of fully expanded leaves. Bacterial populations were enumerated as described by Katagiri et al. (2002) at day 0 and day 3 after infiltration. Infiltration experiments were repeated at least three times, using four biological replicas in each occasion. This dissertation presents enumeration data for one representative experiment, presented in bar charts as the average bacterial population of four biological replicas, with standard deviations displayed. For some experiments, plants were dip-inoculated with OD_{600nm}=0.2 (10^8 cfu/ml) bacterial suspension containing 0.25% Silwet L-77. The inoculated plants were immediately covered with a plastic dome and kept in high humidity for 4 days.

RESULTS

AvrE induces cell death in eukaryotes.

A commonly used approach to understand the virulence functions of effectors is to identify host proteins that interact with effectors. This would produce a list of putative targets of effectors, whose biological functions can then be evaluated by subsequent biological assays. Y2H screen is a widely used approach to identify interacting proteins. In preliminary experiments, it was found that expression of AvrE in both plants (Zwiesler-Vollick and Sheng Yang He, unpublished) and yeast (Qiaoling Jin and Sheng Yang He, unpublished) led to cell death.

Figure 3-1 (next page) shows the effect of AvrE expression in yeast cells. Yeast cells carrying *avrE* were not able to grow in inducing media. Moreover, AvrE toxicity prevented the detection of expected protein-protein interactions between AvrE and its cognate secretion chaperone, AvrF (Figure 3-1). Note that yeast cells expressing AvrF alone grew normally in inducing medium. The strong toxicity of AvrE, however, made it impossible to perform Y2H analyses.

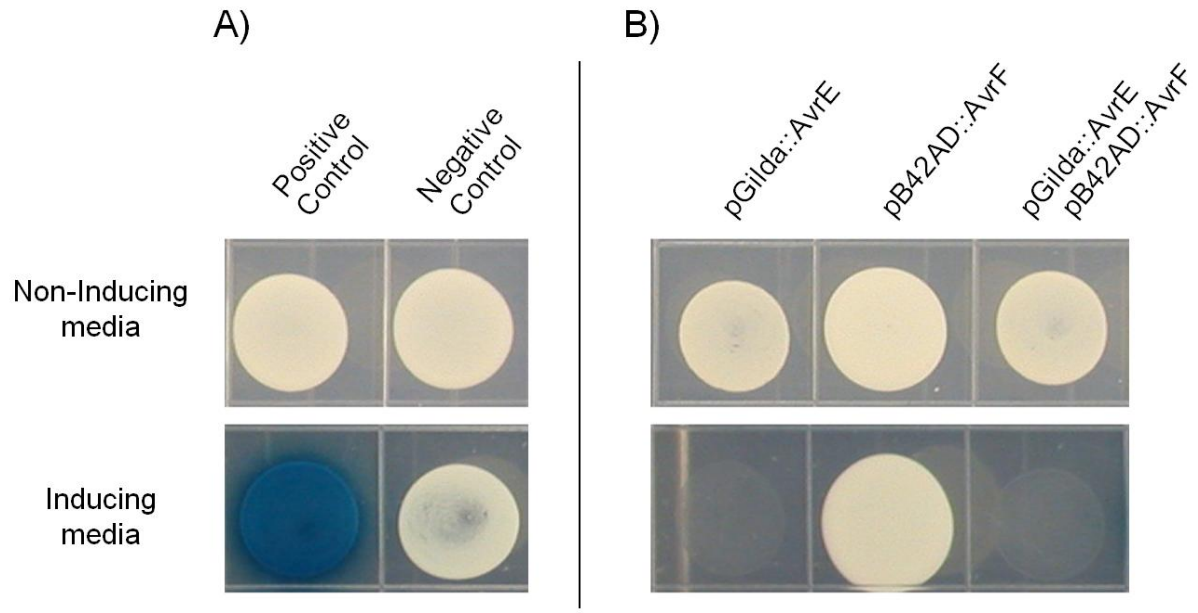


Figure 3-1 AvrE is toxic to yeast.

One-ml aliquots from overnight yeast cultures were washed three times with equal volumes of water. Fifteen-µl of washed yeast culture were spotted on inducing and non-inducing solid media. Yeast growth was evaluated 48 hours later. **A)** Positive control: Yeast containing commercial plasmids pGBKT7-53 and pGADT7-T. Negative control: Yeast containing commercial plasmids pGBKT7-53 and pGADT7-Lam. Blue coloration indicates positive protein-protein interaction. **B)** Yeast colonies expressing either AvrE (pGilda:AvrE), AvrF (pB42AD::AvrF), or co-expressing AvrE and AvrF. While yeast expressing AvrE died when spotted onto inducing media, cells expressing AvrF can grew normally. Yeast cells co-expressing both bacterial proteins died due to AvrE toxicity, which prevents the detection of expected interaction between AvrE and AvrF.

Y2H Screen of an Arabidopsis cDNA library for suppressors of AvrE toxicity.

I hypothesized that the inherent toxicity of AvrE in plants and in yeast cells may be caused by the action of AvrE on a conserved eukaryotic cellular process. I reasoned that it might be possible to perform an Y2H-based screen for Arabidopsis proteins that would be able to suppress AvrE toxicity in yeast. Furthermore, the identification of proteins capable of AvrE-toxicity suppression may suggest the virulence function of AvrE *in planta*. In particular, if AvrE destroys the function of its host target (and the yeast orthologue), it may be possible that overexpression of the host target could alleviate AvrE-induced toxicity by compensating for the

loss of the endogenous yeast protein, or by physical interfering with AvrE interactions with yeast targets, or by restoring the downstream steps of the AvrE-affected pathway in yeast.

From an initial screen of 157.5 million clones, representing approximately 25 times genome coverage, I obtained 19 cDNA clones that reproducibly suppressed AvrE toxicity in yeast (Table 3-1).

Table 3-1 Arabidopsis proteins identified as AvrE-toxicity suppressors in yeast.

Locus	Description	# of Clones
At1g20340	Putative plastocyanin/recombination and DNA-damage resistance protein (DRT112)	1
At1g29920	Photosystem II type I chlorophyll a/b binding putative protein	1
At1g54040	Kelch repeat-containing protein / Jasmonate inducible protein	1
At1g55330	Arabinogalactan-protein (AGP2) / biological process unknown, anchored to membrane	1
At1g60950	Ferrodoxin precursor / Chloroplast stroma, iron ion binding, photosynthetic electron transport	1
At1g67090	RuBisCO small subunit	1
At2g36830	Putative tonoplast intrinsic protein gamma aquaporin	1
At3g21055	Photosystem II 5 kD putative protein	1
At3g26070	Plastid-lipid associated protein PAP / fibrillin family protein. Chloroplast structural molecule activity	1
At3g49910	Putative 60S ribosomal protein	1
At5g14200	3-isopropylmalate dehydrogenase, chloroplast putative	1
At5g18380	Putative 40S ribosomal protein S16	1
At5g38410	RuBisCO small subunit 3b	1
At5g59840	GTP-binding protein ara-3 / Ras-related GTP-binding family protein/ RabE1.b	1
At5g64040	Photosystem I reaction centre subunit psaN precursor	1
At2g23090	Expressed protein	1
At3g47070	Expressed protein	1
At5g16030	Unknown protein	1
At5g23040	Expressed protein	1

Yeast cells containing the AvrE-toxicity suppressors identified in Table 3-1 were plated in inducing media with X-gal and BU salts to determine if there would be physical interactions between these suppressors and AvrE. None of them tested positive for AvrE interaction (data not shown).

After this initial screen, I decided to conduct another Y2H screen for simultaneous toxicity suppression and interaction with AvrE. For this screen, yeast clones containing the cDNA library and the AvrE plasmid were plated on inducing medium containing X-Gal and BU salts. Almost all of the surviving colonies were white (indicating toxicity suppression, but no physical interaction) and only a few colonies were blue (indicative of toxicity suppression and possible physical interaction). Only blue yeast clones were further analyzed. Table 3-2 presents a summary of the Arabidopsis cDNA clones characterized for both toxicity suppression and AvrE interaction.

Table 3-2 Arabidopsis proteins identified both as AvrE-toxicity suppressors and AvrE interactors.

Locus	Description	# of Clones
At3g08580	ADP, ATP carrier protein 1, mitochondrial / ADP/ATP translocase 1	3
At2g30620	Histone H1.2	3
At5g38420	RuBisCO small subunit 2B (RBCS-2B)	2
At1g78630	Arabidopsis thaliana putative ribosomal protein L13	1
At1g03130	Photosystem I 20 kDa subunit, putative / PSI-D	1
At1g79040	Photosystem II 10 kDa polypeptide	1
At1g42970	Glyceraldehyde-3-phosphate dehydrogenase subunit B, chloroplast NADP-	1

As shown in Tables 3-1 and 3-2, most of the identified loci encode highly abundant proteins, such as histones, ribosomal or Rubisco subunits, or Photosystem proteins. Isolation of these types of proteins is frequent in Y2H screens. They typically reflect non-specific interactions that are not considered to have real biological meaning. In almost all cases, no clear connection can be made between the isolated cDNA fragments and their possible relationship to the plant processes that AvrE is known to interfere with, such as cell wall-based defense or cell death induction.

RabG is a suppressor of AvrE toxicity in yeast.

My initial toxicity suppressor screen yielded a cDNA coding for RabE1.b (Table 3-1). RabE1.b has been previously identified as an interactor of the *Pst* DC3000 effector AvrPto (Speth et al 2009). Other small GTPases, such as Api2 and Api3 in tomato are also putative targets of AvrPto (Bogdanove and Martin, 2000). RabE1.b, Api2 and Api3 are similar to mammalian Rab8 (Huber et al. 1993), Ypt2 from *Schizosaccharomyces pombe* (Craighead et al. 1993) and Sec4p from *Saccharomyces cerevisiae* (Goud et al. 1988). These small GTPases have been shown to regulate the polarized vesicle transport from the Golgi network to specific regions of the plasma membrane. Based upon these reports, I decided to further study RabE1.b. The DNA sequence of the RabE1.b clone isolated from the cDNA library displays a stop-codon mutation at position 175. This mutation results in a C-terminal truncated protein 42 amino acids shorter than the original RabE1.b (216 amino acids). Because the function of Rab proteins depends on association with membranes through prenylation of a double cysteine motif on their C-terminal ends, I wanted to determine if full-length, functional RabE1.b was able to suppress AvrE toxicity and if other Rab family members have such activity. Our laboratory had previously made a Rab family protein library for Y2H assays (Dr. Paula Hauck, unpublished). The library includes a full length clone of RabE1.b. I cotransformed yeast cells with plasmids coding for AvrE and a Rab protein. A total of 11 Rab proteins representing one or more members of the seven Rab subfamilies (RabA to RabG) were tested.

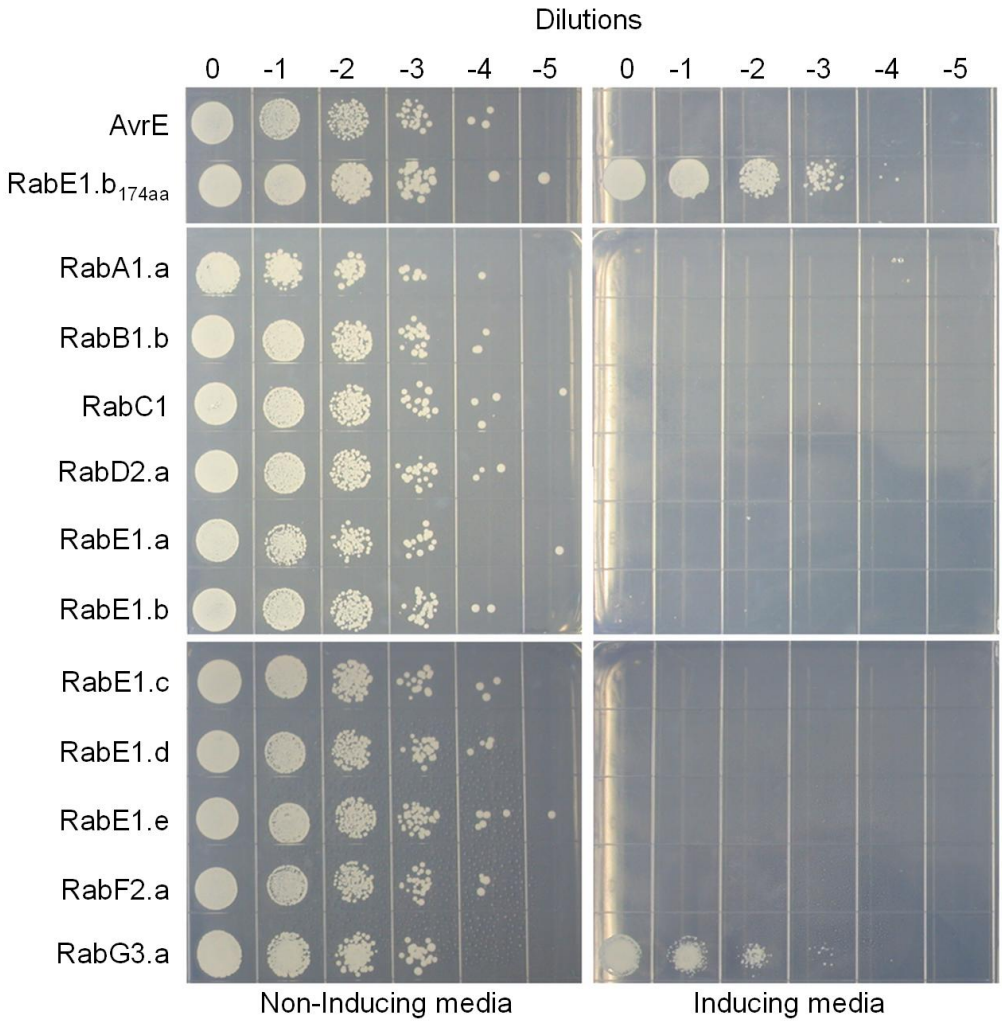


Figure 3-2 RabG3.a expression suppresses AvrE toxicity in yeast.

Arabidopsis genes representative of the Rab family of small GTPases were cotransformed with *avrE* in yeast. Yeast cultures were grown in non-inducing media overnight. One-ml aliquots from yeast cultures were washed three times with an equal volume of water. Fifteen-µl of ten-fold serial dilutions of each yeast culture were spotted on inducing and non-inducing solid media (dilutions $1:10^0$ to $1:10^5$ represented using the numbers 0 to -5). Yeast growth was evaluated 48 hours later to determine toxicity suppression. The first row shows toxicity of AvrE expression in yeast. The second row shows AvrE-toxicity suppression by a clone identified from the Arabidopsis cDNA library (isolated from Y2H screen, Table 3-1) that codes for only the first 174 amino acids of RabE1.b ($RabE1.b_{174aa}$). However, full-length RabE1.b (216aa) does not have any suppression activity, whereas full-length RabG3.a does.

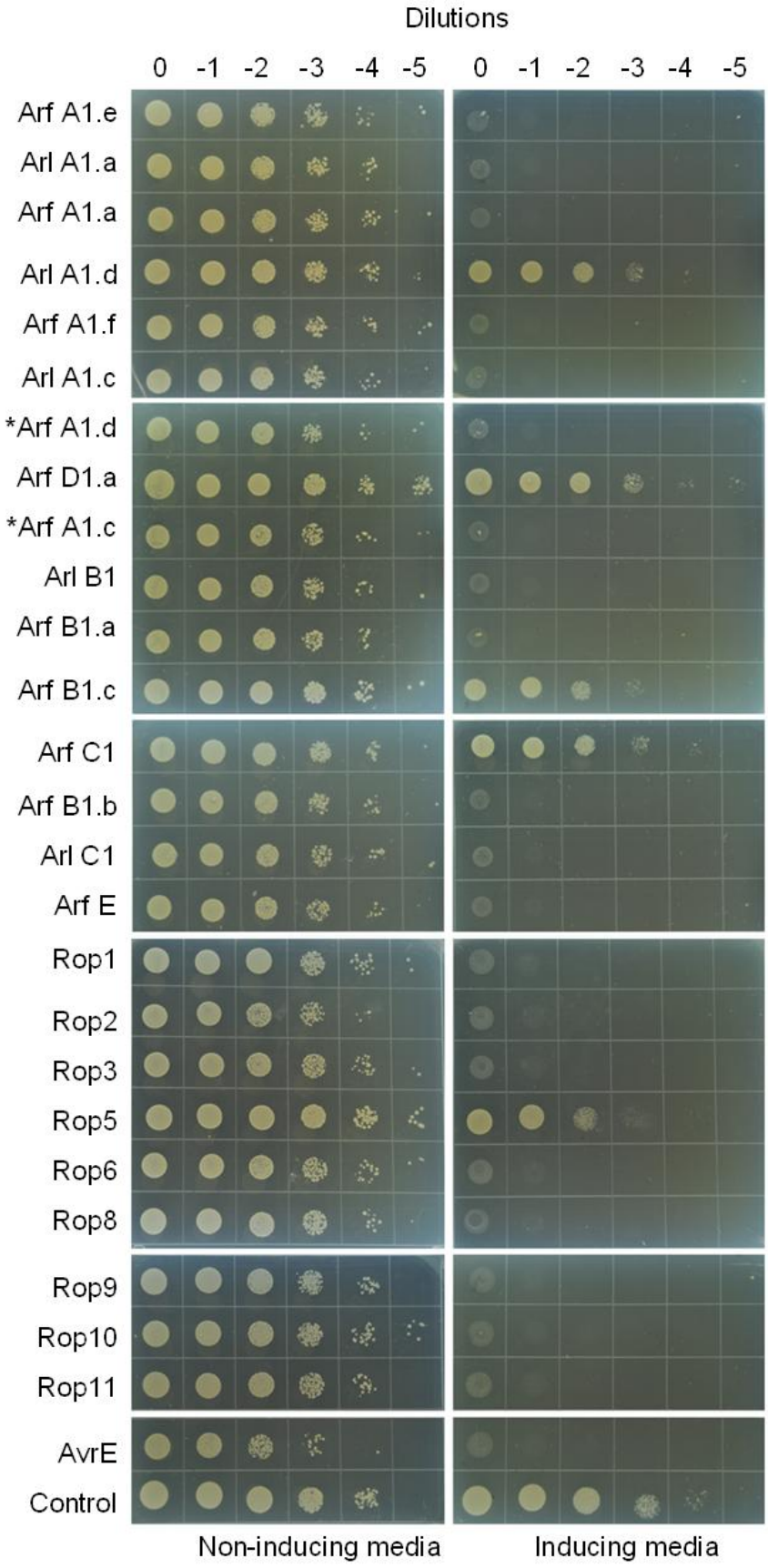
Unlike the RabE1.b$_{174aa}$-encoding cDNA isolated in the original Y2H screen (Table 3-1), the full length *RABE1.b* gene was unable to suppress the toxicity of AvrE in yeast (Figure 3-2). Among the other Rab proteins tested, only RabG3.a was able to suppress AvrE toxicity.

AvrE-toxicity suppression by other Arabidopsis Small GTPases families

In addition to Rab proteins, Arabidopsis has several other families of small GTPases, including Arf/Arl (vesicle trafficking), Rop (actin dynamics) and Ran (nuclear transport) protein families (Vernoud et al. 2003). To determine if members of other small GTPases families could also suppress AvrE toxicity in yeast, I cotransformed yeast cells with *avrE* and a collection of *ARF/ARL* and *ROP* genes. The *ARF/ARL* genes were made available for use in Y2H screens by Lori Imboden, a graduate student in our laboratory. *ROP* genes had been cloned, and kindly provided by Dr. Masaki Shimono (Dr. Brad Day's laboratory, Plant Pathology Department, Michigan State University). I performed further subcloning of the *ROP* genes into the Y2H vector pB42AD. Toxicity suppression results are presented in Figure 3-3 (next page).

Figure 3-3 AvrE-toxicity suppression by the Arf/Arl and the Rop families of small GTPases in yeast (next page).

Arabidopsis genes representative of the Arf/Arl and Rop families of small GTPases were cotransformed with *avrE* in yeast. One-ml aliquots from yeast cultures were washed three times with an equal volume of water. Fifteen-μl of ten-fold serial dilutions of each yeast culture were spotted on inducing and non-inducing solid media (dilutions $1{:}10^{0}$ to $1{:}10^{5}$ represented using the numbers 0 to -5). Yeast growth was evaluated 48 hours later to determine toxicity suppression. Only ArlA1.d, ArfD1.a, ArfB1.c, ArfC1 and Rop5 suppressed AvrE toxicity. In the bottom row there are yeast colonies containing empty pGilda vector (no *avrE*), which grew normally on inducing and non-inducing media.


Dilutions
0 -1 -2 -3 -4 -5 0 -1 -2 -3 -4 -5
Arf A1.e
Arl A1.a
Arf A1.a
Arl A1.d
Arf A1.f
Arl A1.c
*Arf A1.d
Arf D1.a
*Arf A1.c
Arl B1
Arf B1.a
Arf B1.c
Arf C1
Arf B1.b
Arl C1
Arf E
Rop1
Rop2
Rop3
Rop5
Rop6
Rop8
Rop9
Rop10
Rop11
AvrE
Control
Non-inducing media
Inducing media

Three Arf and one ARL proteins were capable of suppressing AvrE toxicity: ArlA1.d, ArfD1.a, ArfB1.c, and ArfC1 (Figure 3-3). In addition, Rop5 was the only member of the Rop protein family capable of AvrE-toxicity suppression (Figure 3-3). Physical interaction with AvrE was tested for each of the AvrE-toxicity-suppressing small GTPases. As shown in Figure 3-4, none of these small GTPases displayed positive physical interaction with AvrE. Thus, in these cases, suppression of AvrE toxicity is not associated with physical interaction.

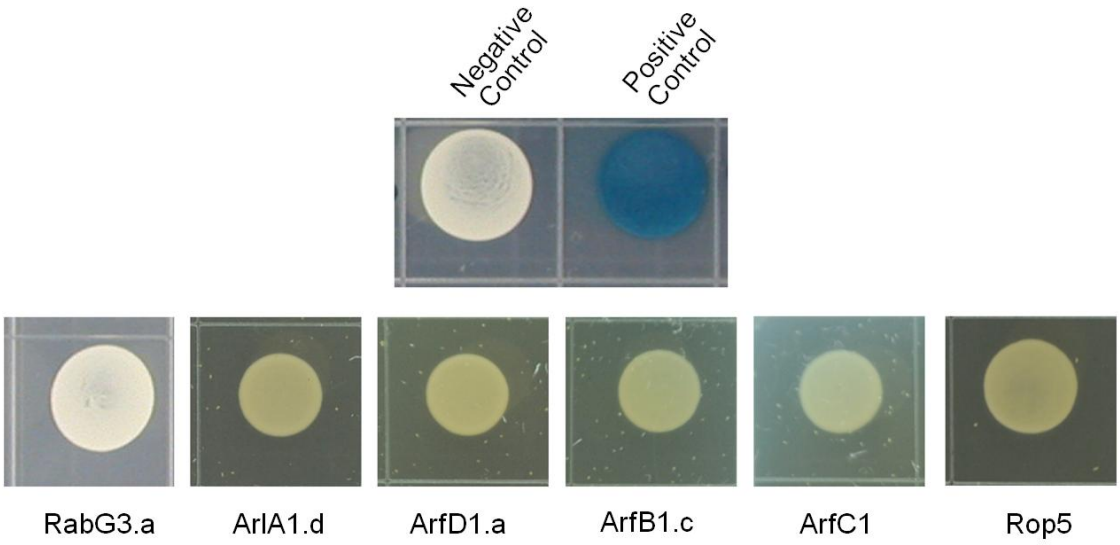


Figure 3-4 AvrE does not physically interact with the small GTPases capable of AvrE-toxicity suppression.
Yeast cells co-expressing AvrE and each of the small GTPases capable of AvrE-toxicity suppression (Figures 3-2 and 3-3) were plated on inducing media with X-gal and BU salts. One-ml aliquots from overnight yeast cultures were washed three times with an equal volume of water. Fifteen-µl of washed yeast culture were spotted on inducing and non-inducing solid media. Yeast growth and coloration was evaluated 48 hours later. Positive control: Yeast containing plasmids pGBKT7-53 and pGADT7-T. Negative control: yeast containing pGBKT7-53 and pGADT7-Lam.

Functional studies of RabG overexpression *in planta*.

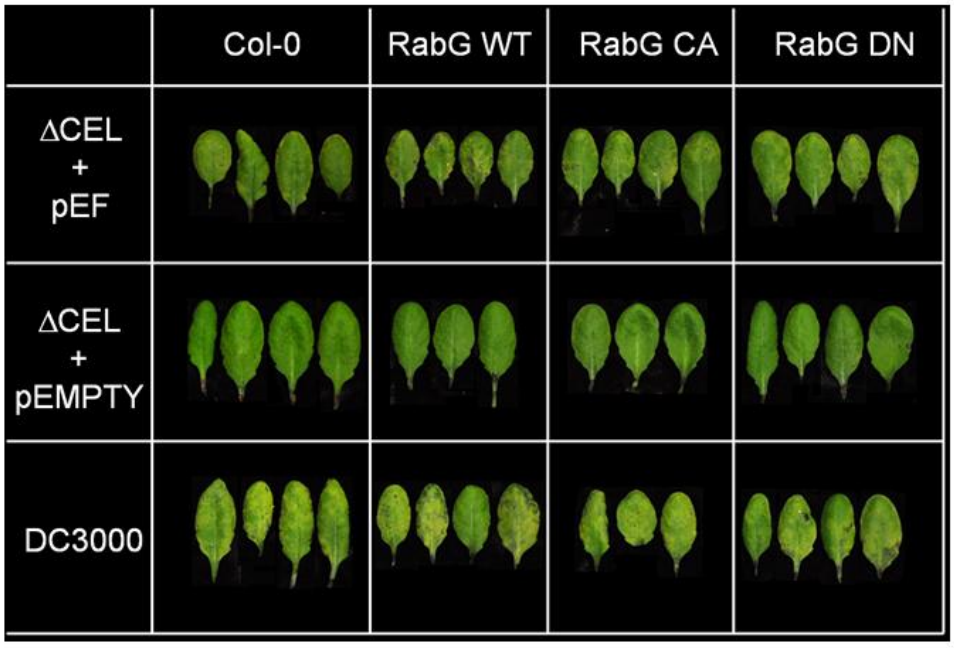
Next, I wanted to determine if small GTPase suppression of AvrE toxicity observed in yeast could be extrapolated to plants. A previous report had identified RabG3.b as a modulator of plant cell death during fungal infection (Kwon et al. 2009). In that study Arabidopsis transgenic

lines overexpressing wild-type, constitutively-active, and dominant-negative forms of RabG3.b were produced under the control of the CaMV 35S promoter. These transgenic plants were kindly provided to us by Dr. Ohkmae Park (School of Life Sciences and Biotechnology, Korea University, Korea). Although related, RabG3.a and RabG3.b are not identical. RabG3.b is 217 amino acids long, whereas RabG3.b is only 203. The two RabG family members are 72% identical at the amino acid level. Arabidopsis transgenic lines overexpressing wild-type, constitutively-active, and dominant-negative forms of RabG3.b was infiltrated with *Pst* DC3000, the ΔCEL mutant, or the ΔCEL mutant complemented with *avrE*. Disease symptom development and bacterial population growth was studied (next page).

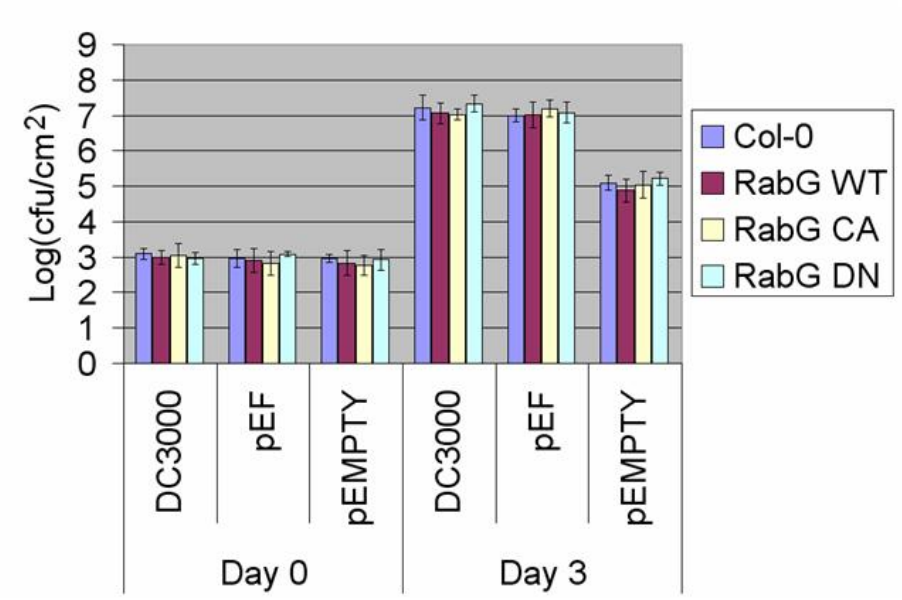
Figure 3-5 Arabidopsis transgenic plants expressing RabG are not hypersensitive nor hyper-resistant to bacterial infection than wild type Col-0 plants (next page).

Arabidopsis leaves were infiltrated with bacterial suspensions (10^6 cfu/ml). Symptom development and bacterial population growth was evaluated three days after bacterial infiltration. **A)** Symptom development three days after bacterial infiltration. No differences can be observed among wild-type Columbia (Col-0) and transgenics plants overexpressing wild-type (RabG WT), constitutively active (RabG CA), or dominant-negative (RabG DN) forms of RabG3.b. **B)** Population growth of *Pst* DC3000, ΔCEL, and ΔCEL mutant containing pAVRE + pAVRF (pEF) or pUCP19+ pAVRF (pEMPTY) in Col-0 and RabG3.b transgenic lines. Each column represents the average of four biological replicas, with standard deviations displayed.

A)


Col-0
RabG WT
RabG CA
RabG DN
ΔCEL + pEF
ΔCEL + pEMPTY
DC3000

B)


Log(cfu/cm2)
0
1
2
3
4
5
6
7
8
9
DC3000
pEF
pEMPTY
DC3000
pEF
pEMPTY
Day 0
Day 3
Col-0
RabG WT
RabG CA
RabG DN

As shown in Figure 3-5, after three days the wild-type *Pst* DC3000 bacterium increased its population up to ten thousand times. By comparison the virulence-defective ΔCEL mutant increases its population only about 100-fold. The ΔCEL mutant with *avrE* is fully restored in its growth *in planta*. Overexpression of none of the RabG3.b forms had any effect on disease symptom development or bacterial population growth of all strains tested (Figure 3-5).

C-terminal AvrE deletion mutants are non-toxic in yeast

Considering the technical problems posed by the toxicity of AvrE to yeast, I hypothesized that nontoxic AvrE deletion derivatives may be useful as a bait for Y2H screens. To determine what portion of AvrE may be better suited for this purpose I took in account published information as well as preliminary data produced in our laboratory.

As described in the introduction of this chapter, a non-virulent *E. amylovora dspE* mutant can be restored by transforming the bacterium with a plasmid that expresses the N-terminal half of DspE (Bogdanove et al. 1998). This result suggests that the N-terminal portion of DspE may constitute a discrete domain and perhaps could bind to some host targets. Moreover, a previous Y2H screen of an apple cDNA library using the first N-terminal 967 amino acids of *E. amylovora* DspE identified several putative plant LRR-RLK (Meng et al. 2002). However, silencing of these LRR-RLKs increased the resistance of apple to *E. amylovora* infection, a result that is difficult to interpret in terms of DspE virulence function (Borejsza-Wysocka et al. 2004). Preliminary studies done in our laboratory suggested that the first 600 amino acids of *Erwinia amylovora* DspE, when expressed transiently in *Nicotiana benthamiana*, had a dominant-negative effect on wild-type DspE delivered from bacteria in causing plant cell death (Yulia

Trukhina and Sheng Yang He, unpublished). This result suggested the possibility that this portion of the polypeptide may act as an independent domain *in vivo*, since it interferes with the function of full-length DspE. Frequently, the dominant negative effect on a cellular process reflects some non-productive protein-protein interactions (Shpak et al. 2003; Wang et al 2005). Taken altogether, I hypothesized that an N-terminal fragment of AvrE may be a candidate to use as bait in Y2H screens.

ClustalW2 alignment showed that the first 522 amino acids of AvrE are equivalent to the first 600 amino acids of DspE. I created a series of AvrE C-terminal deletion mutants, resulting truncated AvrE proteins with the first 522, 600, 900 and 1000 N-terminal amino acids ($AvrE_{522aa}$, $AvrE_{600aa}$, $AvrE_{900aa}$ and $AvrE_{1000aa}$ respectively). To determine if the expression of these AvrE derivatives was still toxic to yeast, each of the deletion mutants was cotransformed with *avrF* into yeast cells. Transformants were tested for AvrE-dependant toxicity and for AvrE-AvrF interaction.

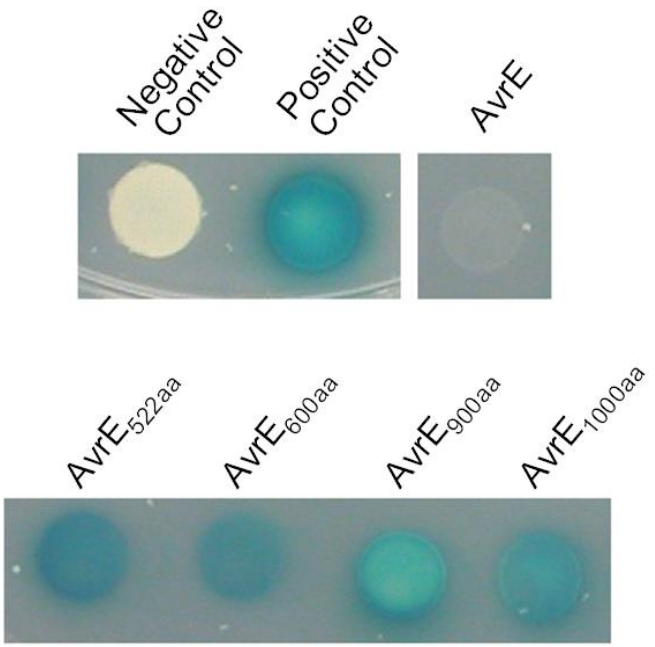


Figure 3-6 C-Terminally truncated AvrE derivatives are not toxic to yeast and they interact with the AvrF chaperone.

avrE fragments coding for the first 522, 600, 900 and 1000 amino acids of AvrE were cloned into pGilda and cotransformed with pB42AD::AvrF into yeast cells. One-ml aliquots from overnight yeast cultures were washed 3 times with an equal volume of water. Fifteen-μl of washed yeast culture were spotted on inducing media with X-gal and BU salts. Yeast growth and coloration was evaluated 48 hours later. Positive control: Yeast containing plasmids pGBKT7-53 and pGADT7-T. Negative control: Yeast containing pGBKT7-53 and pGADT7-Lam. Unlike yeast expressing full-length AvrE, which did not grow, all the deletion mutants ($AvrE_{522aa}$, $AvrE_{600aa}$, $AvrE_{900aa}$ and $AvrE_{1000aa}$) allowed yeast to grow and they also interact with the cognate chaperone AvrF.

None of the AvrE derivatives showed any signs of AvrE toxicity as evidenced by the normal growth of yeast transformants (Figure 3-6). Moreover, all of the AvrE derivatives tested positive for interaction with AvrF, the secretion chaperone of AvrE. In contrast, expression of full-length AvrE prevented yeast growth and detection of the AvrE-AvrF interaction.

Screening of an Arabidopsis cDNA library using a non-toxic, N-terminal fragment of AvrE.

I decided to screen the Arabidopsis cDNA library using $AvrE_{522aa}$. The cDNA library was cotransformed with pGilda harboring *$avrE_{522aa}$* into yeast. Transformants (representing 10x genome coverage) were plated in inducing media containing with X-gal and BU salts. Blue colonies, indicative of positive protein-protein interaction, were isolated. Plasmid containing cDNAs were purified and sequenced. The sequences were used to identify Arabidopsis genes using the BLAST algorithm (Altschul et al. 1997). Table 3-3 (next page) summarizes the results of this screening. Notably, the interactors isolated in this screen were very different from those isolated in toxicity suppression screens using full-length AvrE. In particular, protein phosphatases (At2G29400, AT5G59160), a receptor kinase (AT2G26730), and an F-box protein (AT1G10780) were among the interactors (next page).

Table 3-3 Arabidopsis proteins identified as $AvrE_{522aa}$ interactors

Locus	Description	# of clones
AT2G29400.1	TOPP1 Type 1 protein phosphatase, expressed in roots, rosettes and flowers	23
AT2G26730.1	Leucine-rich repeat transmembrane protein kinase, putative	15
AT5G14200.3	Similar to 3-isopropylmalate dehydrogenase, chloroplast, putative [Arabidopsis thaliana] (TAIR:At1g31180.1); similar to putative dehydrogenase, 3'-partial [Oryza sativa (japonica cultivar-group)] (GB:AAT78826.1); similar to putative 3-isopropylmalate dehydrogenase [Oryza sativa (japonica cultivar-group)] (GB:AAP50991.1); contains InterPro domain Isocitrate/isopropylmalate dehydrogenase (InterPro:IPR001804)	5
AT5G54430.1	Universal stress protein (USP) family protein, low similarity to early nodulin ENOD18 (Vicia faba) GI:11602747, ER6 protein (Lycopersicon esculentum) GI:5669654; contains Pfam profile PF00582: universal stress protein family	4
AT1G10780.1	F-box family protein, similar to SKP1 interacting partner 2 (SKIP2) TIGR_Ath1:At5g67250	3
AT1G10740.2	Expressed protein 2	2
AT2G41680.1	Thioredoxin reductase, putative / NADPH-dependent thioredoxin reductase, putative, The last 2 exons encode thioredoxin. There is an EST match to exons 5-7, and the distance between exon 7 and exon 8 is only 90bp. It is unlikely this is two separate genes, but more likely a hybrid protein.	2
AT1G62780.1	Expressed protein 3	1
AT2G20010.1	Expressed protein 1	1
AT2G25140.1	heat shock protein 100, putative / HSP100, putative / heat shock protein clpB, putative / HSP100/ClpB, putative, similar to HSP100/ClpB GI:9651530 (Phaseolus lunatus)	1
AT2G28630.1	Beta-ketoacyl-CoA synthase family protein	1
AT3G25480.1	rhodanese-like domain-containing protein, contains Rhodanese-like domain PF:00581	1
AT3G53870.1	40S ribosomal protein S3 (RPS3B)	1
AT5G05110.1	Cysteine protease inhibitor, putative / cystatin, putative, similar to cysteine proteinase inhibitor (Glycine max) GI:1944342; contains Pfam profile PF00031: Cystatin domain	1
AT5G36160.1	aminotransferase-related, similar to nicotianamine aminotransferase B GI:6469087 from (Hordeum vulgare subsp. vulgare)	1
AT5G58070.1	lipocalin, putative, similar to temperature stress-induced lipocalin (Triticum aestivum) GI:18650668	1
AT5G59160.3	TOPP2. Encodes the catalytic subunit of a Type 1 phosphoprotein Ser/Thr phosphatase, expressed in roots, shoots and flowers.	1

Preliminary functional studies of $AvrE_{522aa}$ interactors

It has been demonstrated that HopM1 promotes disease by initiating degradation of its host targets, thus a knock-out plant of AtMIN2 is hypersusceptible to the ΔCEL bacterium (Nomura et al. 2006). Considering the functional redundancy between HopM and AvrE, I

hypothesized AvrE may also promote infection by destroying its putative host targets. In collaboration with Dr. Kinya Nomura, preliminary examination of Arabidopsis T-DNA insertion lines for some $AvrE_{522aa}$ interactors was performed (read note page 90). Population growth of the ΔCEL mutant was evaluated in confirmed Arabidopsis homozygous knock-out lines corresponding to some of the $AvrE_{522aa}$ interactors identified. These included phosphatases (At2G29400, AT5G59160), a receptor kinase (AT2G26730), and an F-box protein (AT1G10780). For some genes, more than one independent homozygous T-DNA insertion line was available and studied. Also, it must be noted that all of the candidate genes tested belong to gene families with multiple members that share high degrees of identity.

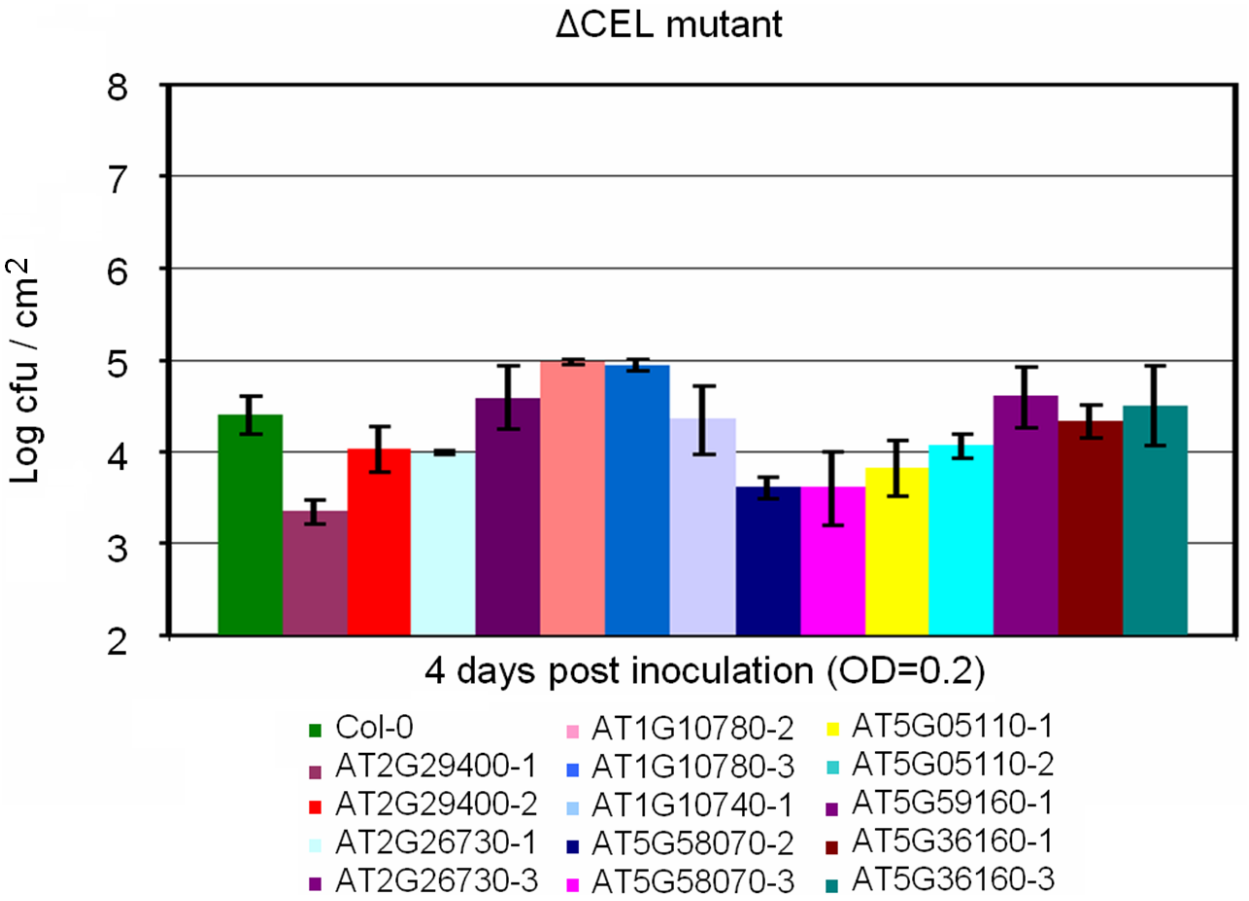


Figure 3-7 Population growth of the ΔCEL mutant in homozygous Arabidopsis T-DNA insertion lines for selected $AvrE_{522aa}$-interactors.

Confirmed homozygous T-DNA insertion lines of selected $AvrE_{522aa}$ interactors were dip-inoculated with ΔCEL bacterial suspension (10^8 cfu/ml). Plants were then kept in high humidity conditions for 4 days. Whenever available, more than one T-DNA insertion line was tested for each $AvrE_{522aa}$ interactor. Bacterial population growth is compared to wild type Columbia (Col-0) plants. Each column represents the average of four biological replicas, with standard deviations displayed. This experiment was performed only once. This figure was made in collaboration with Dr. Kinya Nomura (see page 90).

No significantly increased susceptibility to the ΔCEL mutant was found for any of the homozygous T-DNA insertion lines tested (Figure 3-7).

AvrE$_{522aa}$ interacts with Rad23, a previously identified interactor of HopM1.

Several Arabidopsis proteins have been identified as HopM1 interactors in a Y2H screen (Nomura et al. 2006). These Arabidopsis proteins have been named AtMINs (Arabidopsis thaliana HopM1 interactors). One of them, AtMIN7, has been demonstrated to be a biologically relevant target of HopM1. Since AvrE and HopM1 are functionally redundant effectors (DebRoy et al. 2004), there may be an overlap between host targets of HopM1 and AvrE. Because I found that the N-terminus of AvrE is not toxic and can be used for Y2H assay, I cotransformed yeast cells with *avrE$_{522aa}$* and each of the *AtMIN* genes to test this hypothesis.

Figure 3-8 AvrE$_{522aa}$ interacts with AtMIN2 (Rad23a).

Yeast cells were cotransformed with *avrE$_{522aa}$* and each of the *AtMIN* genes. One-ml aliquots from overnight yeast cultures were washed 3 times with an equal volume of water. Fifteen-µl of washed yeast culture were spotted on inducing media with X-gal and BU salts. Yeast growth and coloration was evaluated 48 hours later. Each number identifies a particular AtMIN protein as described by Nomura and collaborators (2006). Blue color indicates positive protein-protein interaction. Yeast containing plasmids pGBKT7-53 and pGADT7-Lam (Neg) was used as a negative control.

Only AtMIN2 interacted with $AvrE_{522aa}$ (Figure 3-7). AtMIN2 is Rad23-a (At1g16190), one of the four members of the Rad23 protein family (Watkins et al. 1993; Masutani et al. 1994). The other members of the Rad23 family are Rad23b (At1g79650), Rad23c (At3g02540) and Rad23d (At5G38470). Rad23 functions as a shuttle protein that binds ubiquitinated proteins and delivers them to the proteasome for degradation (Kim et al. 2006). However, in contrast to *atmin7* knockout plants, *atmin2/rad23-a* knock-out plants were not more susceptible to infection by the ΔCEL bacterium (Nomura et al. 2006). It is not clear whether Rab23 is not a biologically relevant host target or that members of the Rad23 protein family are functionally redundant. It was recently reported that single knock-out Arabidopsis lines for each *rad23* gene do not exhibit strong phenotypes. Only *rad23-b* mutants manifested some minor changes, which include altered leaf arrangement around the stem, slower root growth, and partial sterility (Farmer et al. 2010).

Bacterial infection promotes destabilization of Rad23 in a HopM1 and AvrE-dependent manner

It was previously shown that during infection of Arabidopsis leaves, ΔCEL mutant bacteria complemented with *hopM1*, promotes destabilization of the host protein AtMIN7 (Nomura et al. 2006). However, it is not known whether such HopM1-dependent destabilization of AtMIN2/Rad23-A occurs in Arabidopsis during bacterial infection and whether AvrE may also destabilize AtMIN2/Rad23-A. To address this question, I infiltrated Arabidopsis leaves with different bacterial strains and collected plant tissue samples 10 hours post infiltration. The time point for sample collection was chosen to provide enough time for the bacterial TTSS to translocate bacterial effectors before the onset of symptomatic plant cell death. Plant tissue homogenates were loaded into 12% SDS-PAGE, followed by western blot analyses using a

polyclonal Rad23 antibody. Additionally, tissue samples for ΔCEL- and *Pst* DC3000-infected plants were subject to two-dimensional gel electrophoresis prior to western blotting.

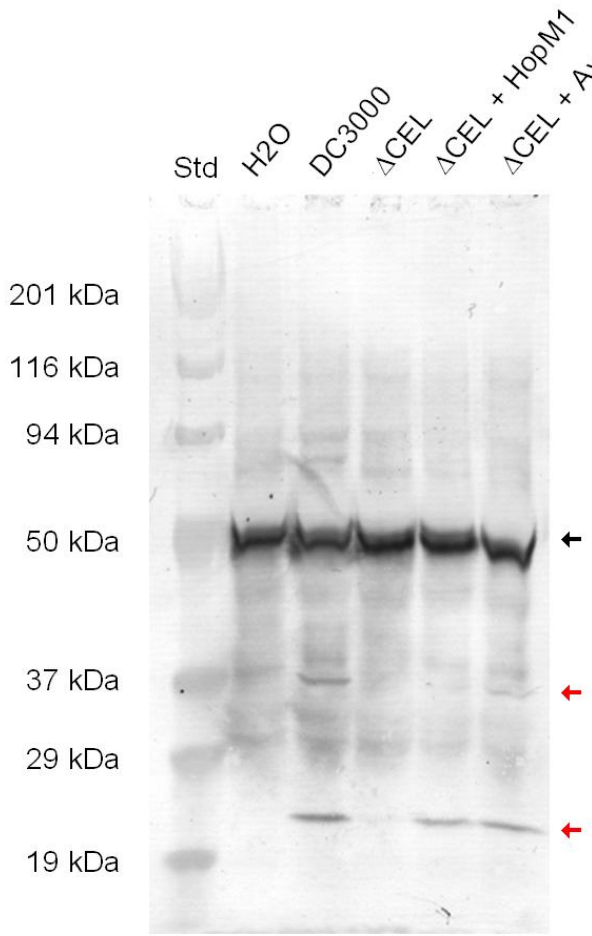


Figure 3-9 Western blot of Rad23 proteins showing possible destabilization of Rad23 in Arabidopsis plants infected with bacteria.

Plant leaves were infiltrated with water or suspensions of *Pst* DC3000, ΔCEL or ΔCEL complemented either with *hopM1* or *avrE* (10^8 cfu/ml). Plant tissue samples were collected 10 hours after infiltration. Plant tissue homogenates were loaded into a 12% SDS-PAGE gel. Western blotting was performed using a polyclonal Rad23 rabbit antiserum. Rad23 proteins have an apparent molecular weight of ~50 kDa (black arrow). The red arrows mark possible degradation products of Rad23 in the plants treated with DC3000 and ΔCEL complemented with either *hopM1* or *avrE,* but not in the plants infiltrated with water or ΔCEL.

The predicted molecular sizes of Arabidopsis Rad23 proteins are about 40 kDa. Nonetheless, in SDS-PAGE the apparent molecular weights were much higher, ranging from 55 to 60 kDa. Similar observations have been made for yeast (Watkins et al. 1993) and human

(Masutani et al. 1994) Rad23 proteins. The overall amount of Rad23 protein seems to remain constant during bacterial infection (Figure 3-9). However, two distinctive bands of about 20 kDa and 35kDa, respectively, appeared visible upon infection with DC3000 or the ΔCEL complemented with either *hopM1* or *avrE* (arrows on Figure 3-9). Preliminary two-dimensional electrophoresis for the same infected plant tissue samples shows that DC3000 treatment changes the isoelectric point of Rad23 proteins to a lower pH and appearance of smaller protein fragments that correlate with those observed in one-dimensional SDS-PAGE (data not shown). Taken together, these results suggest that HopM1 and AvrE may independently induce degradation of a fraction of the total Rad23 protein in the plant cell.

The four members in the Rad23 protein family have been named Rad23a (At1g16190), Rad23b (At1g79650), Rad23c (At3g02540) and Rad23d (At5G38470). I wanted to determine if all members of the Rad23 protein family are destabilized upon bacterial infection. Homozygous *rad23* knockout plants for each family member were kindly made available by Dr. Richard Vierstra from the University of Wisconsin, Madison (Farmer et al. 2010). I hypothesized that, if Rad23 degradation is restricted to a single family member, upon bacterial infection smaller Rad23 fragments should not be detected in the corresponding *rad23* knock-out line. Moreover, if the degradation of a particular Rad23 family member is a determinant of AvrE virulence function, then the corresponding *rad23* knock-out plant line should be more susceptible to the ΔCEL mutant bacterium (next page).

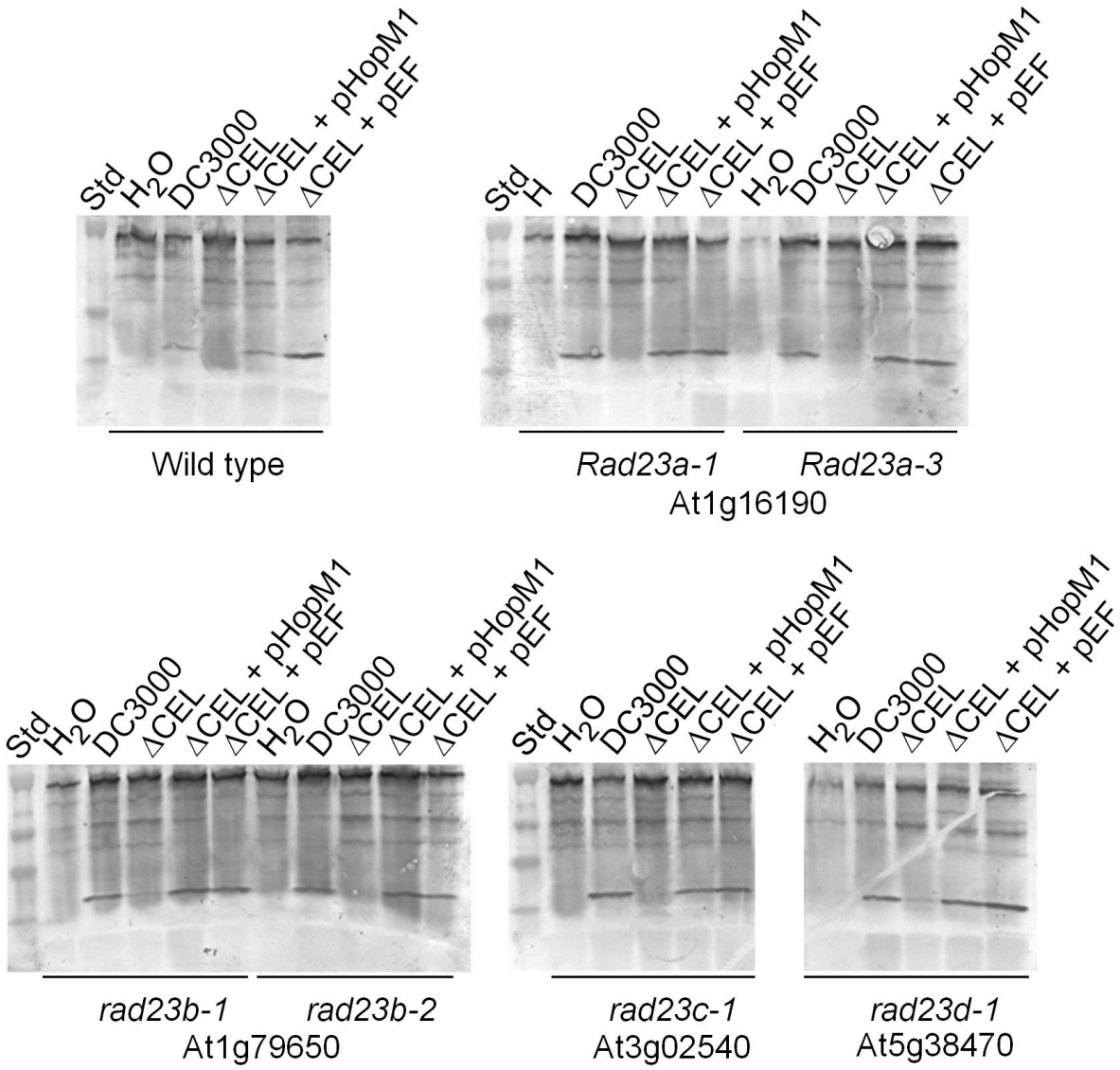


Figure 3-10 AvrE and HopM1 induces destabilization of all members of the Rad23 family. Knock-out plant lines for each *Rad23* family member (*rad23a*, *rad23b*, *rad23c* and *rad23d*) were tested for potential Rad23 destabilization. When available, more than one knock-out line was tested. Wild-type and knock-out plants were infiltrated with the following bacteria (10^8 cfu/ml): *Pst* DC3000, ΔCEL or ΔCEL complemented with *hopM1* or *avrE*. A mock inoculation control with water was included. Plant tissue samples were collected and processed 10 hours after infiltration. Plant tissue homogenates were loaded into 12% SDS-PAGE gels. Western blotting was performed using a Rad23 rabbit antiserum. Degradation products similar to those observed in Figure 3-9 could be observed in all plants, upon infection with *Pst* DC3000 or ΔCEL complemented with *hopM1* or *avrE*. This suggests that Rad23 degradation may not be limited to Rad23a or to any other individual members of the Rad23 family.

Figure 3-10 shows the appearance of a ~20 kDa protein band in plants infected with *Pst* DC3000, ΔCEL and ΔCEL complemented with *hopM1* or *avrE*. This band appeared in infected Col-0 (control) and in each *rad23* knock-out line (Figure 3-10). This result suggests that all members of the Rad23 family is subjected to degradation during *Pst* 3000 infection.

To determine if the loss of any of the four *RAD23* genes had any impact on the susceptibility of the knock-out lines to infection, I inoculated *rad23* knock-out plants with ΔCEL (to test for increased susceptibility) or ΔCEL complemented with *avrE* (to test for reduced susceptibility). Disease symptoms and bacterial population growth were determined 3 days after initial infection (Figure 3-11).

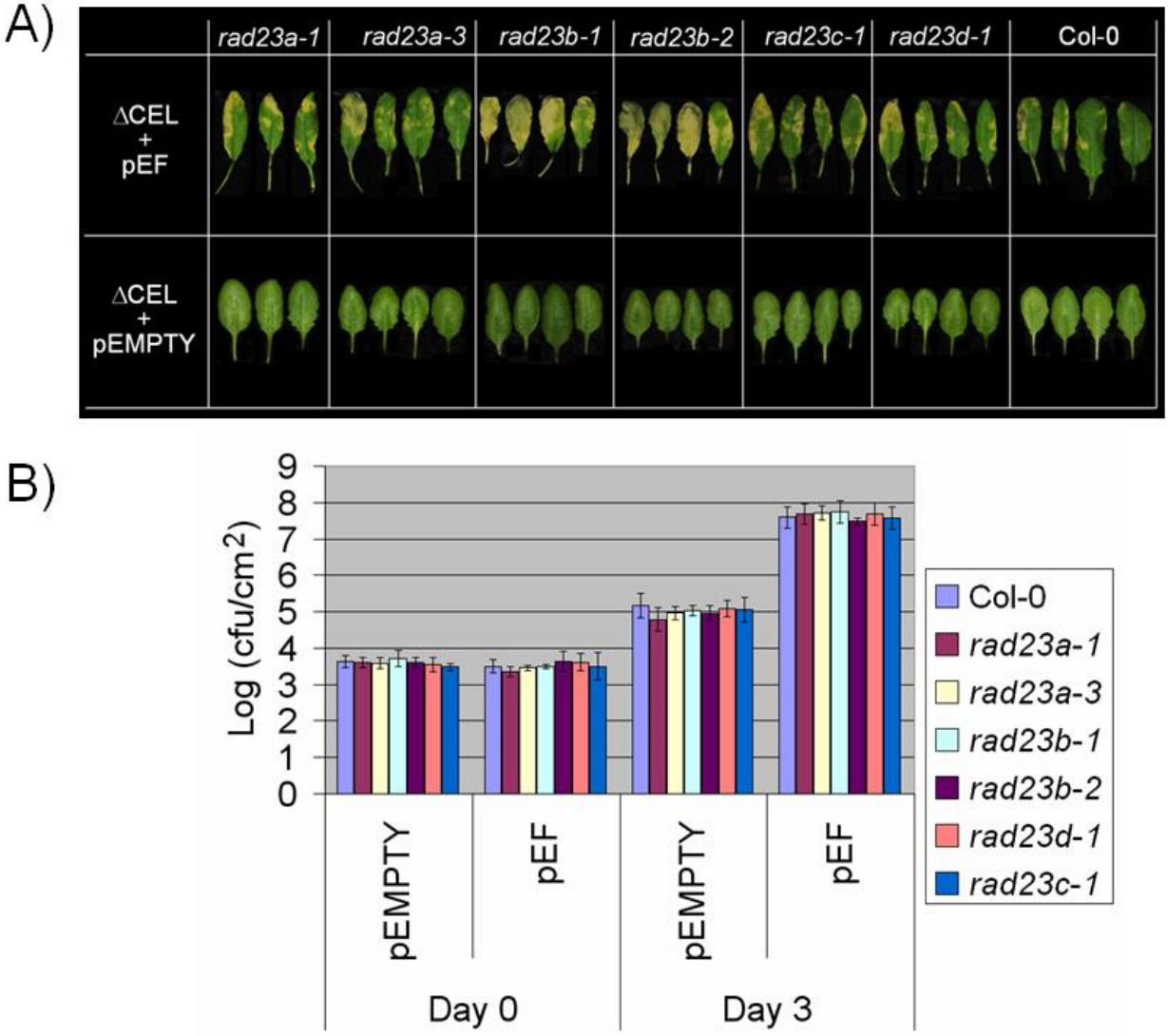


Figure 3-11 *rad23* knock-out plants do not display altered susceptibility to bacterial infection.

Rad23 knockout plants were infiltrated with bacteria to determine if they were more resistant or susceptible to infection. Disease symptoms evaluation and bacterial population enumeration were performed 3 days after initial infiltration with bacterial suspensions (10^6 cfu/ml). **A)** Symptom development (necrosis and chlorosis). **B)** Bacterial population enumeration. Each column represents the average of four biological replicas, with standard deviations displayed.

Like wild type Arabidopsis (Col-0), all of the *rad23* knock-out lines developed normal disease when infected with ΔCEL complemented with *avrE* (Figure 3-11). Moreover, none of *rad23* knock-out lines was more susceptible to infection with the ΔCEL mutant (Figure 3-11).

DISCUSSION

The AvrE-family effectors are among the most important virulence factors in bacterial pathogens that cause plant diseases. Despite their important virulence role, the inherent toxicity of AvrE in yeast and plant cells has prevented rapid progress in understanding the molecular action of AvrE and orthologues in host plants. In this study, I attempted to identify host proteins that could either suppress AvrE toxicity in yeast or interact with AvrE. I also performed a series of follow-up experiments to assess the biological relevance of these host proteins to the virulence function of AvrE. Despite my efforts, I have not been able to make a definitive conclusion regarding the role of the host proteins identified. Nevertheless, I have made a number of observations which I believe will be useful for further studies of this important effector.

For AvrE-toxicity suppressor screens, I was hoping to identify Arabidopsis proteins that are associated with cell death suppression or, because of the functional redundancy to HopM1, modulators of vesicle trafficking. As shown in Tables 3-1 and 3-2, no cell death regulators were recovered. However, a truncated version of RabE1.b (174aa) was identified as an AvrE toxicity suppressor. RabE1.b is a known interactor of the bacterial effector AvrPto. Also, the Rab GTPase family is involved in intracellular trafficking, controlling vesicle formation, motility and tethering to its target compartments (Molendijk et al, 2004). These processes could be directly related to the polarized vesicular transport necessary for cell wall-based or extracellular defenses However, further experiments showed that full length RabE1.b did not suppress AvrE toxicity in yeast. This difference may be because the truncated version of RabE1b cannot be C-terminally

isoprenylated, a modification thought to be required for proper association with cell membranes (Joberty et al. 1993). Furthermore, a previous study failed to establish a clear role of RabE proteins in the Arabidopsis-*Pst* DC3000 interaction (Speth et al. 2009). Therefore, it seems unlikely that RabE1.b would be a biologically relevant host target of AvrE.

The identification of the truncated RabE1.b as an AvrE-toxicity suppressor brought my attention to the idea that other Arabidopsis Small GTPases may also suppress AvrE toxicity in yeast. Indeed, I identified one Rab-family (RabG3.a), four ARF/ARL-family (ArlA1.d, ArfD1.a, ArfB1.c, and ArfC1) and one Rop-family GTPase (Rop5) GTPases that show strong suppressor activity in yeast cells. In general, small GTPases regulate processes that are required for vesicle trafficking, cytoskeleton organization, receptor recycling, autophagy, and nuclear transport. Many of these processes can be envisioned to be related to plant defense and/or cell death and could be a target of AvrE. I found that although these small GTPases suppress AvrE toxicity in yeast, they do not interact with AvrE. This result suggests that AvrE toxicity suppression by these GTPases is probably mediated by an indirect mechanism.

It is possible that AvrE targets a cellular pathway that may be linked to vesicle trafficking and cytoskeleton dynamics regulated by RabG3.a, ArlA1.d, ArfD1.a, ArfB1.c, ArfC1 and Rop5. For example, If AvrE is a negative regulator of an intracellular vesicle traffic pathway, overexpression of the small GTPases identified as AvrE toxicity suppressors could be acting as positive regulators, which may compensate for the deleterious effect of AvrE. Overexpression of RabG, which is involved in vacuolar targeting (Schimmöller and Riezman, 1993), and ARF/ARLs, which are involved in vesicle formation (Vernoud et al. 2003), could collectively

increase the traffic flow to the vacuole to alleviate the putative traffic jam caused by AvrE action. Overexpression of Rop5 could promote certain arrangement of the cytoskeleton to counter the process impaired by AvrE. However, a more detailed study would be required to determine the nature of the compensation effect by these GTPases.

I had hypothesized that AvrE toxicity in yeast and plants may be due to evolutionary conservation of host targets of AvrE. If so, transgenic overexpression in Arabidopsis of AvrE-toxicity suppressor identified in yeast may counter AvrE toxicity and function in plants. I tested this hypothesis with a RabG GTPase. However, I found that RabG3,b overexpression has neither negative nor positive impact in plant susceptibility to ΔCEL complemented with *avrE* (Figure 3-5). Thus, at least for RabG3.b, my hypothesis does not hold. However, the failure to observe an effect of RabG3.b overexpression on the AvrE function does not rule out the possibility that overexpression of other AvrE-toxicity-suppressing GTPases would affect AvrE function in Arabidopsis. Indeed, there have been successful examples of using yeast toxicity as a surrogate system to identify host targets for mammalian pathogen effectors (Alto et al. 2006).

Because the toxic full-length AvrE cannot be used in standard Y2H screen, I attempted a Y2H screen using a truncated, nontoxic AvrE fragment, $AvrE_{522aa}$. A potential drawback of this approach is that the selected fragment may not account for all the interactors that the full-length protein could. Thus it is important to have a rationale upon the selection of what portion of a protein to select for use as bait in Y2H screens. The use of a protein fragment as bait was successfully used to identify an Arabidopsis protein targeted by the effector HopM1 (Nomura et al. 2006). I selected the AvrE522aa fragment in consideration of evidence suggesting that the N-

terminal half of the AvrE may contain a domain responsible for effector function (Bogdanove et al. 1998) and preliminary data in our laboratory suggesting that the first 600 amino acids of DspE may have a dominant negative effect over the full-length effector (Yulia Trukhina and Sheng Yang He, unpublished). Some of the identified $AvrE_{522aa}$ interactors are putative intermediates of signal transduction cascades, such as phosphatases, LRR-RLK and F-box proteins. However, preliminary studies showed that none of the homozygous T-DNA insertion lines were hypersusceptible to ΔCEL infection (Figure 3-7), suggesting that none of the $AvrE_{522aa}$ interactors identified is target of AvrE function. Nonetheless, because these genes belong to gene families with high degree sequence identities; it is possible that redundancy may mask any possible phenotypes. In such scenario, study of polymutants knock-out lines or gene silencing of the gene families will be required.

The preliminary results suggest that some knock-out lines, such as At2G29400-1, At5G58070-2, At5G58070-3 and At5G05110-1, may be hyper-resistant to ΔCEL mutant bacteria (Figure 3-7). More experimental replicas are required to determine if the difference observed holds true. Also, T-DNA insertion lines should be tested for possible hyper-resistance to *Pst* DC3000 and ΔCEL complemented with *avrE*. Interestingly, a Y2H screen of an apple cDNA library using *E. amylovora* DspE as bait (Meng et al. 2002) also identified several similar LRR-RLKs. It was later reported that silencing of DspE-interacting apple-LRR-RLKs, increases apple resistance to *E. amylovora* infection (Borejsza-Wysocka et al. 2004). This suggests that AvrE may promote virulence through positive modulation of the function of these LRR-RLK proteins, which may explain why I did not observe increased susceptibility of the Arabidopsis knock-out lines (Figure 3-7). In this scenario, definitive proof that AvrE-interacting proteins are targets of

AvrE-family effectors requires demonstrating increased susceptibility to the ΔCEL mutant in plants (Arabidopsis or apple) overexpressing AvrE/DspE-interacting proteins.

Given the functional redundancy between HopM1 and AvrE (DebRoy et al. 2004), I had hypothesized that these two effectors may share some common host targets or affect the same pathway inside the host cell. It is therefore of great interest that $AvrE_{522aa}$ interacts with AtMIN2 (Rad23a). Moreover I have shown that Rad23 proteins appear to be degraded in a HopM1- or AvrE-dependent manner during infection (Fig. 3-10). However, I did not observed changes in disease susceptibility of knock-out lines for each member of the Rad23 family. The negative results suggest that Rad23 proteins are not biologically relevant host targets of HopM1 or AvrE, or that *rad23* polymutants are needed to show significantly altered disease susceptibility due to functional redundancy of Rad23 family members.

In summary, through this study I have identified several Arabidopsis proteins that either suppress the toxicity of full-length AvrE or interact with the N-terminus of AvrE in yeast. I was able to analyze the role of several of these host proteins in AvrE function using single knockout mutants or transgenic overexpression. Unfortunately, the biological importance of these Arabidopsis proteins in AvrE function or *Pst* DC3000 pathogenesis remains unclear. It was challenging to find the balance between the desire to assess the biological relevance of all candidate host proteins and the need to analyze all members of a given host protein family. Further analyses, including construction of polymutants and transgenic overexpression of all members of a gene family, are needed to critically assess the involvement of the host proteins I identified in AvrE function.

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CHAPTER 4

Future Work

FUTURE WORK

AvrE-family effectors are among the most important virulence factors in plant pathogenic bacteria. In the previous chapters, I described a detailed sequence-function study of the AvrE protein, including characterization of several important sequence motifs and conserved amino acid residues with respect to AvrE function during *Pst* DC3000 infection of Arabidopsis. I also attempted to identify putative host targets of AvrE by Y2H-based protein-protein interaction screens and AvrE-toxicity suppressor screens. I believe that these results provide a valuable foundation for future study of the virulence function of AvrE-family effectors. Here I discuss future work that is needed to further advance our understanding of AvrE-family effectors in plant pathogens.

In my research, I was not able to produce alanine substitution mutants of several conserved amino acid residues. For example, amino acids Y416, K658, R1090, D1106 and N1147 are conserved among AvrE-family effectors and these residues are frequently found at the catalytic sites of enzymes or at the sites where posttranslational modifications occur (Figure 2-12). It is not clear why site-directed mutagenesis of these residues did not work in my experiments. I suggest that new mutagenesis experiments be attempted to determine whether one or more of these residues may be important for AvrE function. Also, my analysis of the spacer sequence of the WxxxE motif showed that deletion of two residues (i.e., WxE) does not affect the function of AvrE (Figure 2-9). However I did not determine the shortest possible spacer sequence. An additional deletion of the spacer sequence to produce WE will address this question. Moreover, the analysis of the spacer sequence was done for just one of the two WxxxE

motifs in AvrE. It would be informative to determine whether both motifs can be modified in similar fashions, without affecting the effector function. Finally, it will be of interest to determine whether the flexibility of the spacer sequence discovered in AvrE is applicable to WxxxE effectors in animal pathogens, for which this question has not been addressed.

My research shows that WxxxE motifs can be created *de novo* at least in four different locations in the sequence of *Pst* DC3000 AvrE. These locations are marked by four W residues that are highly conserved among AvrE-family effectors. In the AvrE sequence, there are other Ws that are not conserved among the AvrE family. An interesting question to be addressed is whether a functional WxxxE motif can be created only at the conserved W sites or if they can also be created at non-conserved W sites. Thus, mutagenesis should be attempted at one or more non-conserved W sites to create WxxxE motifs and the resulting AvrE derivatives tested for the ability to complement the ΔCEL mutant, as described in previous chapters.

Computer predictions of the tertiary structure of AvrE, using the HHpred and Modeller algorithms, show that the AvrE have a tertiary structure similar to the fold of RCC1, a well known small GTPase, and to a UBA fold (Figures 2-14 and 2-15). These predictions suggest specific biological functions that could be empirically tested. For example, RCC1 relies on a catalytic loop known as the β-wedge to promote GDP for GTP exchange. Further computer analysis of the β-wedge structure of RCC1 and the predicted β-wedge of AvrE could guide mutagenesis of specific amino acids that are responsible for the catalytic activity in RCC1. If such amino acids are found to be responsible for the biological function of AvrE, the idea that AvrE may function as a GEF would be strengthened. A similar analysis should be done with the

predicted UBA domain. The ubiquitin-binding activity depends on a hydrophobic patch in the UBA domain (Wilkinson et. al 2001). Systematic mutagenesis of residues predicted in the UBA-associated hydrophobic patch in AvrE can be performed to determine whether the residues in such a patch are important for the function of AvrE. Potential binding of ubiquitin could be performed by *in vitro* protein pull down of purified AvrE and ubiquitin and/or *in vivo* co-immunoprecipitation using AvrE transgenic Arabidopsis or by transient expression of AvrE and ubiquitin proteins in *Nicotiana benthamiana*.

An unresolved outstanding question is the identity of the host target(s) of AvrE. In this dissertation, I used suppression of AvrE toxicity in yeast as an approach to identify potential Arabidopsis proteins involved in AvrE function. This screen assumed that AvrE would work as a negative regulator of the host target/pathway, so that overexpression of such target (or components of the AvrE-targeted host pathway) might suppress AvrE toxicity. This approach will not work if AvrE is instead a positive regulator of its intended target/pathway, because AvrE toxicity may be increased in yeast cells that overexpress the targeted protein. Future research may incorporate an alternative yeast-based screen that has been used with success to identify host proteins involved in the function of WxxxE-family effector IpgB2, which is also toxic to yeast (Alto et al. 2006). In that study, a yeast library of ~5000 viable deletion mutants was cotransformed with IpgB2. Yeast mutants that survived expression of IpgB2 are defective in Rho1p signaling pathway (Alto et al. 2006). A similar approach may be used for the study of AvrE. If AvrE toxicity is caused by activation of the yeast target/pathway, the lack of the yeast target/pathway, or a downstream signaling component, should prevent the toxic effect of AvrE.

Further experiments can then be performed to determine whether an analogous Arabidopsis protein or pathway is required for AvrE function in plants.

In Y2H screens, I used the non-toxic N-terminal 522 amino acids fragment of AvrE ($AvrE_{522aa}$). However, the whole AvrE protein is 1795 amino acids long. In particular, $AvrE_{522aa}$ does not contain the entire predicted β-barrel fold nor the UBA fold, which were discovered at a late stage of my research. It could be argued that use of the N-terminal 522-aa fragment may have precluded the identification of biologically meaningful interactions between AvrE and Arabidopsis proteins. Further Y2H screens could be performed using larger N-terminal fragments that, for example, contain the predicted β-barrel and/or UBA folds. These predicted folds may be necessary for host target interactions. Alternatively C-terminal fragments could be used to identify Arabidopsis proteins that interact with the C-terminal end of AvrE. I was not able to explore these possibilities, because of time constraints.

Nevertheless, I identified several Arabidopsis proteins that interact with $AvrE_{522aa}$ in yeast (Table 3-3). The biological relevance of these Arabidopsis proteins to AvrE function was evaluated using homozygous T-DNA insertion lines. (Figure 3-7). I presupposed that AvrE might negatively regulate the function of its intended target, as has been demonstrated for the effector HopM1, which is functionally redundant to AvrE (Nomura et al. 2006). If so, I expected that knock-out lines for these putative interactors would be hypersusceptible to infection with the ΔCEL mutant bacteria. However, No hypersusceptibility was observed in any of the lines tested (Figure 3-7). As mentioned in Chapter 3, these lines have not been checked for gene transcripts to determine whether they are true knockouts. Also, the identified $AvrE_{522aa}$ interactors belong to

protein families. There is a possibility that their contributions to AvrE function may be masked by gene redundancy, even if the analyzed T-DNA insertion lines turn out to be true knock-out lines. Construction of polymutant lines would be necessary to further test the involvement of these interactors in AvrE function. An alternative approach would be to silence several related gene family members to determine whether a more obvious disease phenotype can be observed. In addition, preliminary data (Figure 3-7) suggests that some of the T-DNA lines may be more resistant to bacterial infection than wild-type Col-0. This would be consistent with the idea that AvrE may be a positive regulator of its host target/pathway. However, further testing of confirmed knock-out lines is required to determine if these differences are indeed statistically significant. Moreover, infection with *Pst* DC3000 and ΔCEL complemented with *avrE* should be performed to determine whether the knockout plants are hyper-resistant (i.e., insensitive to AvrE).

Given the functional redundancy between AvrE and HopM1 (DebRoy et al. 2004), my finding of AvrE interaction with Arabidopsis Rad23 is of special interest. Moreover I have presented evidence that a fraction of the total Rad23 protein is degraded during bacterial infection in HopM1- and AvrE-dependant manners (Figure 3-9). This degradation seems to be common to all four isoforms of Rad23 (Figure 3-10). However, some concerns remain regarding the degradation of Rad23. The 37 and 25 kDa protein bands (Figure 3-9) suspected to be Rad23 degradation products may be plant proteins, other than Rad23, that are expressed in response to AvrE- or HopM1-containing bacteria and that cross-react with the Rad23 antiserum. One way to address this problem would be to preincubate the antiserum with nitrocellulose membranes blotted with purified Rad23 to remove Rad23-specific antibodies. If the bands observed in the

western blot are indeed Rad23, they should disappear or should be greatly reduced in western blots. Alternatively, the Rad23-specific antibody could be affinity-purified using purified Rad23 protein before being used in western blot analysis. Another issue with Rad23 proteins is that no alteration in disease phenotype was observed in individual *rad23* knock-out lines for any of the four members of the Rad23 family (Figure 3-11). It is likely that Rad23 members are functionally redundant. Thus, detection of pathogenesis-related phenotypes in single gene knock-outs may be impossible. Future work should focus on construction of multiple *rad23* gene knock-out lines.

Finally, an important question that needs to be addressed is whether AvrE has GEF activity on host GTPases. Since I found several small GTPases that could suppress AvrE toxicity in yeast (Figures 3-2 and 3-3), GEF assays could be performed with these small GTPases, following the protocol used for WxxxE family members from mammalian pathogens (Huang et al. 2009). If no GEF activity is detectable on these particular GTPases, a more systematic approach may be attempted to perform GDP/GTP exchange assays on all Arabidopsis GTPases. However, this approach would involve considerable effort of gene cloning, protein purification, and GEF assays of several dozens of small GTPases.

Discovery of the plant cell pathways affected by AvrE could open a window to unexplored areas of plant metabolism and physiology. Many interesting possibilities exist. For example, it is clear that AvrE is capable of inducing plant cell death. Does AvrE hijack the mechanisms of programmed cell death? If so, would it be possible to find novel regulators of plant cell death targeted by AvrE? AvrE also suppresses cell wall-based defenses, which appears

to be dependent on vesicle trafficking. Considering the focal nature of cell-wall based defenses, a better understanding of the AvrE mechanism could provide sharper insights to the elusive mechanism underlying polarized cargo delivery in plant cells.

From a purely practical application standpoint, the eventual understanding of the host targets and action mechanisms of AvrE could have significant implications. For example, bacterial virulence may be disrupted by engineering plants that express a mutated version of the targeted protein that is no longer sensitive to the action of AvrE. Defining the biochemical function of AvrE could open a door to identifying chemical compounds that interfere with AvrE action. Such compounds may be used in bacterial disease control.

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