Identification and functional characterization of RXLR effector proteins that are conserved between downy mildew pathogens and *Phytophthora* species

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ABSTRACT

Diverse pathogens secrete effector proteins into plant cells to manipulate host cellular processes. The genome of *Hyaloperonospora arabidopsidis* (*Hpa*), the causative agent of downy mildew of *Arabidopsis*, contains at least 134 candidate RXLR effector genes. These genes contain an RXLR motif required for effector entry into host cells. Only a small subset of these candidate effectors is conserved in related oomycetes. Here, we describe a comparative functional characterization of the *Hpa* RXLR effector *HaRxL96* and a homologous gene, *PsAvh163,* from the soybean pathogen *Phytophthora sojae*. *HaRxL96* and *PsAvh163* are induced during early stages of infection and carry a functional RXLR motif that is sufficient for protein uptake into plant cells. Both effectors can suppress or activate immune responses in soybean, *Nicotiana*, and *Arabidopsis*. Several SA-responsive defense genes are suppressed in *Arabidopsis* Col:*HaRxL96* and Col:*PsAvh163* during an incompatible interaction with *Hpa* Emoy2. Both effectors are localized to the nucleus and cytoplasm of plant cells. Nuclear localization of both effectors is required for proper virulence functions, including suppression of basal resistance and RPP4-mediated immunity to virulent and avirulent *Hpa,* respectively. In addition, both effectors interact with plant U-box (PUB) proteins that are conserved

between diverse plant species. The targeted PUB proteins are negative regulators of plant immunity in *Arabidopsis*. These experiments demonstrate that evolutionarily-conserved effectors from different oomycete species can suppress immunity in plant species that are divergent from the source pathogen's `primary host.

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Abbreviations

AAD- acidic transcription activation domain

ATTA- *Agrobacterium*-mediated transient assays in *N. benthamiana*

CEL- conserved effector locus

CLSM – confocal laser scanning microscopy

DEX- dexamethasone

DPI- days post inoculation

EHM- extrahaustorial matrix

ETI- effector-triggered immunity

ETS- effector triggered susceptibility

gDNA- genomic DNA

GFP- green fluorescent protein

HA- hemagglutinin

HMM- hidden markov model

Hpa– *Hyaloperonospora arabidopsidis*

HR- hypersensitive response

HTS- host targeting signal

ICD- INF1-induced cell death

LRR- leucine rich repeat

MAMP- microbe associated molecular pattern

MAPK- mitogen activated protein kinsae

MLA- mildew A

NB- nucleotide binding site

NES- functional nuclear export signal

nes- non-functional nuclear export signal

NLS- nuclear localization signal

PAMP- pathogen associated molecular patterns

PCD- programmed cell death

PRR- pathogen recognition receptor

PTI- pattern triggered immunity

qPCR- quantitative real-time polymerase chain reaction

R17- race 17

R2- race 2

R7- race 7

RFP- red fluorescent protein

ROS- reactive oxygen species

RPP4- Recognition of *Peronosporap parasitica 4*

SP- signal peptide

SV40- simian virus 40

TTSS- type III secretion system

TAL- transcriptional activator-like

TE- transposable elements

TMV- tobacco mosaic virus

Y2H- yeast-two hybrid

YFP- yellow florescent protein

Attributions

Several colleagues have contributed to both the research and writing in the chapters of this dissertation. A brief description of each of their contributions is described here.

John M. McDowell, PhD. is the primary advisor and committee chair for this project. He assisted in manuscript preparation, editing, project inception and advising.

Chapter 2: Initial identification and characterization of homologous RXLR effectors from divergent oomycete species

Rachel A. Fee was an undergraduate student in the McDowell laboratory for two years. She assisted with the original project and contributed to the generation and breeding of the following transgenic lines: HaRxL96, PsAvh163, HaRxL89 and HaRxLL108. She also contributed to data in Figure 2.7 A and Figure 2.8.

Megan S. Casady was an undergraduate student in the McDowell laboratory for two years. She assisted with the original project and contributed to the generation and breeding of *Arabidopsis* transgenic lines. She contribute data to Figure 2.7 B, C

Daniel Deegan was an undergraduate student in the McDowell laboratory for two years. He supported daily research efforts at the bench and contributed to many aspects of this project.

Shiv D. Kale, Ph.D. is a former Ph.D. student of Dr. Brett Tyler. He developed the double barrel gene gun and cell death suppression assays. He also contributed to

HaRxL96 and PsAvh163 Bax suppression data, provided Avr4/6 clones and supplied technical assistance.

Brett M. Tyler, Ph.D. is a professor at the Virginia Bioinformatics Institute at Virginia Tech. Brett Tyler contributed bioinformatic analysis and to the oversight of the project.

Chapter 3: Homologous RXLR effectors from *Hyaloperonospora arabidopsidis* and *Phytophthora sojae* suppress immunity in distantly related plants

Devdutta Deb is a Ph.D. student in the McDowell laboratory. She contributed RNA extractions and cDNA for quantitative PCR experiments.

Rachel A. Fee was an undergraduate student in the McDowell laboratory for two years. She assisted with the original project and contributed to the generation and breeding of transgenic *Arabidopsis* lines. In addition she assisted with daily bench work.

Megan S. Casady was an undergraduate student in the McDowell laboratory for two years. She assisted with the original project and contributed to the generation and breeding of *Arabidopsis* transgenic lines. She contributed data on INF1 suppression.

Brett M. Tyler, Ph.D. is a professor at the Virginia Bioinformatics Institute at Virginia Tech. Brett Tyler contributed bioinfomatic analysis and project oversight.

Chapter 4: The conserved oomycete RXLR effectors HaRxL96 and PsAvh163 exert their virulence functions within the plant cell nucleus

Regina W. Hanlon is a research specialist with Brett Tyler's laboratory at the Virginia Bioinformatics Institute at Virginia Tech. She is responsible for the cloning of HaRxL96 and PsAvh163 into yeast 2-hybrid vectors and testing protein interactions between PUBs and effectors.

Megan S. Casady was an undergraduate student in the McDowell laboratory for two years. She assisted with the original project and contributed to the generation and breeding of *Arabidopsis* transgenic lines. She contributed data on INF1 suppression.

Martin Stegmann is a Ph.D. student at Leibniz Institute of Plant Biochemistry in Halle Germany. He is responsible for the cloning of PUB22, PUB23, PUB24, identifying PUB interacting proteins and generating transgenic *Arabidopsis* PUB plants.

Brett M. Tyler, Ph.D. is a professor at the Virginia Bioinformatics Institute at Virginia Tech. Brett Tyler contributed bioinfomatic analysis and project oversight.

Marco Trujillo, Ph.D. is a professor at Leibniz Institute of Plant Biochemistry in Halle, Germany and the Ph.D. advisor to Martin Stegmann. He is responsible for providing transgenic *Arabidopsis* seed, constructs and project advice.

Keri Cavanaugh, is a laboratory technician for Dr. Richard W. Michelmore at the University of California, Davis. She is responsible for identifying interactions between HaRxL96 and NbCMPG1.

Richard W. Michelmore, Ph.D. is a professor at the University of California, Davis and is responsible for advising the protein interactions screen.

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Chapter 1

Literature review

Abbreviations: acidic transcription activation domain (AAD), extrahaustorial matrix (EHM), effector triggered immunity (ETI), effector-triggered susceptibility (ETS), host-targeting signal (HTS), hypersensitive response (HR), INF1-induced cell death (ICD), leucine rich repeat (LRR), mitogen activated protein kinsae (MAPK), microbe-associated molecular patterns (MAMP), mildew A (*MLA*), nuclear localization signal (NLS) nonexpressor of PR genes 1 (NPR1), pattern triggered immunity (PTI), programmed cell death (PCD), red fluorescent protein (RFP), reactive oxygen species (ROS) signal peptide (SP), type III secretion system (T3SS), transcriptional activator-like (TAL), transposable elements (TEs), tobacco mosaic virus (TMV)

The plant immune system

Molecular basis of plant-pathogen interactions

Phytopathogens must overcome multiple layers of constitutive and inducible defenses to successfully exploit their host and cause disease. Plant-pathogen interactions can be classified into broad categories, described as "compatible" or "incompatible" (Zipfel et al., 2005). During compatible interactions the plant is unable to detect the pathogen. Therefore, the pathogen can proliferate in the plant and cause disease. In contrast, in an incompatible interaction, the pathogen is unable to proliferate in host tissue. Incompatibly often results from the plant detecting the presence of the pathogen and activating inducible defenses to halt pathogen growth (Zipfel et al., 2005).

Much research in the field of plant-pathogen interactions has been invested in learning the molecular mechanisms that underlie these different outcomes. It is now understood that plant innate immune responses can be triggered by two types of receptors, which recognize different classes of pathogen-derived signals (Zipfel et al., 2005). These two classes of receptors are termed and pattern recognition receptors (PRR) and resistance (R) proteins.

Pattern recognition receptors recognize conserved microbial molecules

Pattern recognition receptors (PRRs) recognize conserved microbial molecules required for overall fitness and/or pathogenicity, and are classified by the type of

molecules that they bind. The conserved pathogen molecules recognized by PRRs are termed microbe-associated molecular patterns (MAMPs) (Jones & Dangl, 2006, Zipfel & Felix, 2005). Examples of MAMPs include fungal chitin; the Pep-13 peptide from a conserved *Phytophthora* transglutaminase (Brunner et al, 2002); the bacterial flagellin subunit, flg22 (Zipfel et al., 2004); and the elf18 peptide from bacterial Elongation Factor-Tu (Zipfel et al., 2006). When a MAMP activates a PRR, the downstream responses include activation of MAP kinase cascades, reprogramming of thousands of genes and microRNAs (Navarro et al., 2004), production of reactive oxygen species, and callose formation at the site of invasion (Zipfel et al., 2006). These responses comprise "pattern-triggered immunity" (PTI) that halts further pathogen colonization (Jones & Dangl, 2006, Chisholm et al., 2006, Katagiri & Tsuda, 2010)

Two PRRs have been identified and studied in detail. The first, *Flagellin Sensing 2* (*FLS2*) is a LRR receptor like-kinase (RLK) that recognizes and binds the flagellin peptide *flg22* at the plasma membrane (Zipfel et al., 2004, Chinchilla et al., 2006). *FLS2* confers robust PTI; when deleted from the *Arabidopsis* genome, mutant *fls2* plants are more susceptible to bacterial pathogens (Zipfel et al., 2004). The prominent role of FLS2 in recognizing bacterial pathogens has made it a target of bacterial effector proteins including AvrPtoB from the bacterial pathogen *Pseudomonas syringae* (*Psy*) (Robatzek et al., 2008).

The second well-characterized PRR is the LRR receptor-like kinase, *EF-Tu Receptor* (*EFR*), which was identified and cloned from *Arabidopsis* (Zipfel et al., 2006).

Expression of *EFR* in *Nicotiana benthamiana*, which naturally lacks *EFR*, was sufficient to confer responsiveness to EF-Tu and restrict bacterial growth. Notably, EFR and FLS2 responses overlap and activate similar signaling pathways (Zipfel et al., 2006).

PTI responses are regulated with ligand-inducible protein complexes

The overlap in EFR and FLS2 signaling is attributable to common ligand-induced protein complex formation with BRI1-associated receptor kinase (BAK1) (Chinchilla et al., 2007). Previously, BAK1 was shown to regulate brassinosteroid signaling in *Arabidopsis* (Li & Nam, 2002), but it is now known to have a pivotal role in PTI. The central role of BAK1 in PTI has made it a target of virulence proteins of bacterial pathogens. Two sequence-unrelated effector proteins, AvrPto and AvrPtoB, bind BAK1 and inhibit broad PTI responses (Shan et al., 2008). The role of BAK1 in mediating PTI responses to non-bacterial pathogens has also been established. It is now understood that BAK1 and its interacting partners from the *Somatic-Embryogenesis Receptor-Like Kinase* (*SERK*) family mediate the PTI response to hemibiotrophic and biotrophic pathogens including oomycetes (Roux et al., 2011).

U-box proteins can promote or repress plant immunity via proteolysis

The FLS/BAK1 protein complex is large and involves another interacting partner, BIK1, that is phosphorylated to trigger FLS2-dependent endocytosis for the full *flg22*-dependent PTI (Chinchilla et al., 2010, Shan et al., 2010). During this response, BAK1

phosphorylates two U-box proteins that are part of the FLS2/BAK1 complex, PLANT U BOX PROTEIN 12 (PUB12) AND PUB13. The phosphorylation of both PUB proteins activates their E3 ubiquitin ligase activity targeting FLS2 for degradation and damping the *flg22*- PTI response (He et al., 2011). Mutant *pub12/pub13* plants display enhanced resistance to *Psy* infection, consistent with their proposed role as negative regulators of plant immunity (He et al., 2011). There are 37 predicted *PUB* genes in the *Arabidopsis* genome that are implicated in regulating biotic and abiotic stresses. They have been divided into five classes based on their gene structure (Azevedo et al., 2001). Both *PUB12* and *PUB13* are Class II *PUB* genes with U-box domains and Armadillo repeats that mediate protein-protein interactions.

Class III PUBs maintain an N-terminal U-box domain followed by a C-terminal leucine-rich domain with unknown functions (Azevedo et al., 2001). Twelve gene members comprise Class III PUBs, and only a handful of which have been studied. These include PUB20, 21, 22, 23, and 24. Of the Class III *PUB* genes, *PUB22, PUB23, PUB24* are implicated in drought response and plant innate immunity (Shirasu et al., 2008, Kim et al., 2008). Mutant *pub22/23/24* plants displayed enhanced resistance to a bacterial and an oomycete pathogen, suggesting that they are negative regulators of plant immunity analogous to *PUB12* and *PUB13.* U-box proteins are conserved between plant species and a homolog of PUB20 exists in *N. benthamiana* and tomato. The *N. benthamiana* and tomato homolog, *CMPG1,* is required for full resistance during *Cladosporium fulvum* Avr9 elicitation of its cognate immune receptor Cf-9 (Gonzalez-Lamothe et al., 2006). Furthermore, the *N. benthamiana* CMPG1 gene is required for activation of cell death in

response to a variety of elicitor/resistance gene combinations including Cf-9/Avr9, Pto/AvrPto, cellulose-binding elicitor lectin (CBEL) and INF1 (Gilroy et al., 2011). Thus, by genetic criteria, CMPG1 is a positive regulator of defense.

In sum, plants maintain a surveillance network comprised of extracellular receptors that perceive conserved pathogen molecules and activate downstream immune signaling networks. It conceivable that this branch of the plant immune system is sufficient to render most plants resistant to most pathogens (Nurnberger et al., 2004). How then are disease-causing pathogens able to circumvent this branch of the immune system to cause disease? In part, this is accomplished by effectors, which are pathogen proteins that are secreted to the inside or outside of plants cells.

Effectors are deployed by every major class of plant pathogen.

All successful plant pathogens have evolved effector proteins that suppress PTI by interfering with MAMP-induced signaling and response (He et al., 2006). This subversion of PTI often leads to successful pathogen colonization and is termed effector-triggered susceptibility (ETS) (Jones & Dangl, 2006, Chisholm et al., 2006). It has been proposed that effectors evolved to suppress PTI and thus the second branch of the plant immune system likely evolved to perceive effectors as signals of invasion.

Resistance proteins recognize effectors as signals of invasion

The second class of plant immune receptors is encoded by *R* genes, which produce proteins that directly or indirectly detect specific pathogen effectors. Recognition of a pathogen effector by an R protein triggers a multicomponent signaling cascade that rapidly induces plant defenses and often leads to programmed cell death (PCD) at the infection site (termed the hypersensitive response or HR) (Dangl & McDowell, 2006). The HR is exceptionally successful in prohibiting progression of pathogens that extract nutrients from living plant cells ("biotrophs." see below). This outcome is referred to as effector-triggered immunity (ETI) (Jones & Dangl, 2006, Chisholm et al., 2006). Effectors that elicit ETI are referred to as avirulence (*avr*) proteins based on the immunity they elicit if they are detected by an R protein. This gene-for-gene concept was developed in the 1950's by H.H. Flor, a pioneer in the genetics of plant immunity (Flor, 1955). We now understand that avirulence proteins can benefit the pathogen but can also betray the pathogen's presence if the plant host contains a cognate *R* gene.

The *Arabidopsis* genome contains approximately 150 *R* genes (Meyers et al., 2003). This is a small number in comparison to the diversity of pathogens, and pathogen derived molecules, that are associated with plants. There are species-specific limits to the number *R* genes maintained within a genome (Guo et al., 2011). A comparison of *A. thaliana* (a selfing species) to its close relative *A. lyrata* (an outcrossing species) demonstrated similar numbers of predicted *R* genes and the loci with a single *R* gene are

less variable than *R* genes located in a tandem array (Guo et al., 2011). Owing to the limited number of *R* genes and the extreme diversity of pathogen molecules, *R* genes are under considerable pressure to evolve and sustain recognition diversity. Comparative studies have shown that *R* gene diversity is created through NB-LRR gene duplication, deletions, sequence exchanges and diversifying selection (McDowell and Simon, 2008). *R* gene diversity concomitantly drives selection of pathogen effector genes to elude R protein recognition (Abramovitch et al., 2006). This co-evolutionary dynamic is often referred to as an "arms race" (McDowell and Simon, 2008).

Many cloned *R* genes encode proteins containing a nucleotide-binding site, followed by leucine-rich repeat motifs (NB-LRR). The NB and LRR domains are described as being involved in downstream defense signaling and effector recognition, respectively, although this is an overly simplistic generalization. (Martin et al., 2003, DeYoung & Innes, 2006, Jones & Dangl, 2006). In general, NB-LRR genes exist in two forms, either with a coil-coil (CC) domain or an unrelated "TIR" domain with sequence similarity to the cytoplasmic signaling domains of animal Toll and Interleukin-1 immune receptor proteins. In uninfected plants, CC-NB-LRR and TIR-NB-LRR proteins are maintained in a resting state, in which the domains are folded in such a way as to inhibit downstream signaling. This lessens the likelihood of spurious defense signaling which is detrimental to the plant. However, these domains adopt a different conformation to quickly activate immune responses upon perception of cognate pathogen effector (avirulence) proteins (Takken & Tameling, 2009).

R proteins can recognize pathogen proteins directly or indirectly

R proteins detect effectors through one of two different mechanisms. The first is best described as a direct receptor-ligand interaction between the R protein and the specific effector that it detects. Direct interactions have been demonstrated in several cases, including the flax rust fungus (*Melampsora lini*) effector proteins, AvrL567 and cognate flax(*Linum usitatissimum*) R proteins (Dodds et al., 2006). The second mechanism of effector detection is indirect, whereby R proteins guard specific plant proteins that are targeted by effectors (Van der Biezen & Jones, 1998). Resistance is then activated when the effector perturbs its target. This mode of detection need not involve direct interaction between the R protein and the cognate effector. For example, the *Arabidopsis* R protein RPM1 detects specific modifications of an *Arabidopsis* defense regulator, called RIN4, that is targeted by effectors from the bacterial pathogen, *Psy*. RPM1 does not bind directly to these effectors, but is activated when the effectors modify RIN4 (Kim et al., 2005, Mackey et al., 2003).

Proteins implicated in plant immunity exhibit dynamic relocalization

Many cellular processes are dynamic, meaning that proteins are modified, activated and/or can rapidly relocalize to different cellular compartments to accomplish their assigned task. In the case of immunity related proteins, NONEXPRESSOR OF PR GENES 1 (NPR1) provides a notable example of a dynamic protein. In uninfected cells, NPR1 exists as an oligomer linked by disulfide bonds. Upon immune activation, cellular

redox changes reduce these bonds and release NPR1 monomers, which relocalize to the nucleus and activate immune signaling (Mou et al., 2003). Another example is provided by the tobacco gene, *N receptor-interacting protein (NRIP1*). The NRIP1 protein interacts with the resistance protein, N which is a TIR-NB-LRR protein that recognizes the viral protein p50 from *Tobacco mosaic virus* (TMV). The interaction of NRIP1 and N is specifically induced in the presence of p50 and this recognition event relocalizes NRIP1 from the chloroplast to the cytosol of TMV-infected cells (Caplan, Mamillapalli et al. 2008). This relocalization is necessary for efficient activation of immunity by the N immune receptor.

Not all immune related proteins relocalize upon activation. Proteins encoded by the *R* genes mildew A (MLA) from barley and Resistance to *Pseudomonas syringae* pv. maculicola 1 (RPM1) from *Arabidopsis* are localized to unique subcellular sites. The localization and functions of MLA10 lie within the nucleus. Recognition of barley powdery mildew effector, A10, by MLA10 induces alterations to a WRKY transcription factor, thereby de-repressing defense responses (Shen et al., 2007). On the other hand, RPM1 is localized to the plasma membrane (PM) where it is activated and functions to regulate immunity (Gao et al., 2011). As highlighted previously, RPM1 senses alterations to RIN4 that are induced by bacterial effectors. Detailed analysis of RPM1 localization established that RPM1 resides at the PM before and during activation, indicating that it does not relocalize in response to pathogens (Gao et al., 2011).

In sum, the interaction between plant and pathogen is determined by the plant's ability to recognize pathogen-derived molecules as signals of invasion. It appears that this paradigm is applicable to many combinations of plant and pathogen species. Knowledge on the inner workings of plant immunity and the tools that pathogens use to sabotage these complex signaling pathways holds the key to engineering durable resistance to a broad range of pathogens.

Oomycete biology and impact on agriculture

Oomycetes are an important agricultural threat

Oomycetes are characterized by cellulose-based walls and non-septate hyphae (Slusarenko & Schlaich, 2003). Despite physical features similar to true fungi, molecular evidence indicates oomycetes are phylogenetically distinct and are classified in the kingdom Chromalveolata, which include diatoms and brown algae (Sogin & Silberman, 1998). This suggests that oomycetes and fungi have evolved independently to colonize land plants (Baldauf et al., 2000). Annually, billions of dollars are spent controlling oomycetes with inconsistent success. The most common control measure in agricultural settings is the use of fungicides. However, fungicides provide an unreliable means of oomycete control due to rapidly evolving fungicide resistance and inability of the fungicide to reach the site of infection (in most cases underground). Moreover, the modes of action for many fungicides are specific for true fungi (Tyler 2007).

Downy mildew pathogens are oomycetes and have a significant impact on agriculture

Downy mildew pathogens infect many monocot and dicot plants. Infamous examples include *Plasmopara viticola*, the causative agent of grape downy mildew (Wong et al., 2001) and *Bremia lactucae*, the causative agent of lettuce downy mildew (Hulbert et al., 1988). Downy mildews affect the marketability and yield of crops; in some cases downy mildews have caused complete yield loss in individual fields (Raid and Datnoff, 1992). Two downy mildew pathogens of *Maize*, *Peronosclerospora philippines* and *Sclerophthora rayssiae*, are highly virulent and are considered as biosecurity threats to US crops. Below, I introduce a downy mildew pathogen of *Arabidopsis thaliana* that I am using as a model system to understand plant-oomycete interactions. I expect that my research will be applicable to related, agronomically-important downy mildews and thereby facilitate translational science that will improve yields and resistance and aid in maintaining critical food security in an increasingly hungry world

Introduction to Hyaloperonospora arabidopsidis

Hyaloperonospora arabidopsidis (*Hpa*) is the causative agent of *Arabidopsis* downy mildew and one of the few naturally-occurring pathogens of the *Arabidopsis* plant species. *Hpa* is a biotrophic organism that obtains nutrients only from living plant tissue. Consequently, *Hpa* cannot be cultured separately from its host. *Hpa* was originally isolated in the 1990's from wild *Arabidopsis* plants in Switzerland (Koch & Slusarenko,

1990). Since then, *Hpa* has proven to be an excellent pathosystem to study plant resistance mechanisms owing to its well-characterized host and its relatedness to the *Phytophthora* genus of agronomically-important plant pathogens (see below). Due to its biotrophic lifestyle, *Hpa* is not amenable to genetic engineering. Despite these shortcomings, *Hpa* has been adopted as a model pathogen because it is one of only a handful of eukaryotic microbes that can cause disease on the model plant *Arabidopsis*. Thus, an ever-growing community of scientists focuses on this interaction and important experimental resources have been developed for *Hpa*, including a sequenced genome (Baxter et al., 2010). Many *Arabidopsis* NB-LRR genes that recognize specific *Hpa* isolates have been isolated. The predicted intracellular localization of the encoded proteins indicates that the corresponding pathogen effector proteins are recognized inside the host cell (Rehmany et al., 2005, Slusarenko & Schlaich, 2003, Allen et al., 2004).

Haustorium formation and structure

After spore germination and penetration through the leaf epidermal cell layer, *Hpa* hyphae grow inside the leaf (**Figures 1.1 and 1.2**). Short branches from the hyphae produce morphologically distinct haustoria that take on a pear shape (Mims et al., 2004). The haustorium contains a full complement of cellular organelles including a vacuole, mitochondria, Golgi bodies and nucleus. In the initial stages of haustorium penetration, a penetration matrix is formed between the host cell wall and the plasma membrane of the plant cell (Mims et al., 2004). This matrix spreads around the developing haustorium as it beings to invaginate the host cell plasma membrane. Host cell wall material at the site of

haustorium penetration forms a small, constricted collar that contains callose, a 1-3 β-glucan polysaccharide. Between the collar and the pathogen, the penetration matrix is thought to seal the space between the extrahaustorial matrix (EHM) and the apoplast, thus preventing leakage (Mims et al., 2004). The EHM resides between the interface of the haustorium cell wall and the extrahaustorial membrane, a modified host cell plasma membrane. The EHM is an electron dense, amophorous mixture of carbohydrates and other molecules (Mims et al, 2004, Szabo and Bushnell, 2001). The *Hpa* EHM appears highly convoluted and more electron dense than the EHM from other fungus-plant interactions. At the extrahaustorial membrane, heavy vesicle trafficking was observed as well as finger-like protrusions of the EHM into the host cell. This is the likely site for nutrient uptake and effector delivery as suggested by previous studies on similar fungal interactions.(Mims et al., 2004, Szabo & Bushnell, 2001).

The Phytophthora genus of plant pathogens infects a broad range of crops

Downy mildew pathogens are related to the *Phytophthora* genus of plant pathogens that infect a broad range of economically significant crops essential to global food security (Sogin & Silberman, 1998). Thus, it is of great importance to examine the mechanisms of *Phytophthora* pathogenesis to assist development of enhanced control methods. *Phytophthora* is an oomycete pathogen and the genus has over 80 species that cause rot of roots, leaves, and fruits. Each *Phytophthora* species exhibits various degrees of host specificity that varies from exceedingly broad to extremely specific (Tyler, 2007). The *Phytophthora* genus and the downy mildew species are closely related.

Despite the relatedness of *Phytophthora* to downy mildew pathogens, the life cycle of *Phytophthora* species is different in some respects from those of downy mildew pathogens. *Phytophthora* species are hemi-biotrophic, marked by an initial biotrophic stage, later switching to a nectrophic life style characterized by exploitation of dead host tissue. Unlike *Hpa*, *Phythophthora* species are non-obligate and can be cultured in the absence of its host. Thus, techniques for genetic manipulation exist for *Phytophthora*.

Phytophthora sojae is the causative agent of "damping off" and root rot in soybean, costing growers worldwide between one to two billion dollars in crop losses annually (Tyler, 2007). *P. sojae* is a tractable model for oomycete pathogenicity. Numerous molecular tools have been developed for this oomycete, including a sequenced genome (Tyler et al., 2006). *P. sojae* can be cultured and transformed (e.g., targeted gene silencing and gene overexpression) which is not possible with *Hpa*. Thus, *P. sojae* is a complementary experimental system to the *Hpa-Arabidopsis* interaction. My dissertation project is designed to exploit the power of *Arabidopsis* genetics with the *Hpa* pathosystem and also utilize the genetic tractability of *P. sojae*. This relationship can be leveraged to study conserved effectors between the two species.

Penetration resistance mediates early defense against oomycete pathogens

Non-adapted fungal and oomycete pathogens are often unable to enter plant cells beyond their host range. For example, *P. sojae* is unable to penetrate *Arabidopsis*

epidermal cells (Takemoto et al., 2003). This is due at least in part to "penetration resistance" wherein pathogen growth is halted before the formation of haustoria. This layer of immunity is considered to be a critical component of non-host resistance, in which an entire species of plants is resistant to an entire pathogen species. Non-host immunity is conditioned by transcriptional responses quantitatively similar to a PTI response, but the underling mechanisms are poorly understood (Zellerhoff et al., 2010). Penetration resistance has been genetically defined using a collection of *Arabidopsis* mutants that are susceptible to non-host fungal pathogens, including *Phytophthora infestans* (Lipka et al., 2005, Assaad et al., 2004, Collins et al., 2003). These mutant plants are deficient in their ability to form cell wall appositions and papillae at the site of infection and are collectively termed *pen* mutants (Assaad et al., 2004). Adapted *Arabidopsis* pathogens such as *Hpa* are able to suppress defense-associated gene expression, enter the mesophyll, and establish haustoria during the first 12 hours of interaction (Eulgem et al., 2007). Thus, *Hpa* is able to evade or suppress penetration resistance, but the mechanisms through which this is accomplished are not known.

Insights from the genomes of haustoria-producing plant pathogens

The last several years have yielded genome sequences of several plant pathogens that utilize haustoria to parasitize their host. Interestingly, the haustoria-associated biotrophic lifestyle has evolved independently in three unrelated plant pathogen lineages: oomycetes (downy mildews and *Phytophthora* species), ascomycetes (powdery mildews) and basidiomycetes (rust and smut fungi) (Dodds, 2010). The genomes represented by

each lineages include *Hpa* (Baxter et al., 2010), *P. sojae*, *P. ramorum* (Tyler et al., 2006), and *P. infestans* (Haas et al., 2009) for the oomycetes; *Blumeria graminis* and *B .hordei* for the ascomycetes (Spanu et al., 2010); and *Sporisorium reilianum*, *Ustilago maydis* (Schirawski et al., 2010), *Puccinia graminis* and *Melampsora larici* (Duplessis et al., 2011) for the basidiomycetes.

A long-standing question in plant pathology has been the molecular basis that led to obligate biotrophy for pathogens that include downy mildews, powdery mildews, and rusts. For each of these pathogens, sequenced genomes suggest gene loss is commonplace. Genes that encode proteins involved in cell wall degradation and elicitors are missing in *Hpa, Blumeria* species, and rust fungi, suggesting that these genomes are optimized for stealth to avoid host immunity and thereby prolong the intimate associations between host and pathogen (Baxter et al., 2010, Spanu et al., 2010, McDowell, 2011, Duplessis et al., 2011). In addition, the *Hpa, Blumeria*, and *Puccinia* genomes lack genes for key enzymes involved in nitrate and sulfate assimilation. These key losses reveal the grounds for obligate parasitism and represent interesting convergent evolution where similar pathways have been discarded.

Another theme conserved in most haustorial pathogens is the fluidity of the genomes. For example, the genomes of the *P. infestans* lineage displays uneven evolutionary rates and have been described as "two-speed" genomes, in which core orthologs that comprise "housekeeping" genes evolve much more slow rate than genes predicted to be involved in pathogenesis of their host (Raffaele et al., 2010). Many of the

virulence-associated genes are located in gene-sparse regions and are distributed unevenly throughout the *P. infestans* genome (Raffaele et al., 2010). Effector proteins from *P. sojae* and *P. ramorum* are encoded by large, rapidly-evolving gene superfamilies. Effector genes are often located near synteny breakpoints (Jiang et al., 2008). Taken together, virulence-associated genes of oomycetes are dynamic and diverse. A fascinating comparison of the genomes of two fungal pathogens that colonize the same host captured the power of comparative genomics and revealed very little conservation of predicted virulence genes despite both pathogens having to overcome the same obstacles to colonize their host (Schirawski et al., 2010).

P. infestans has the largest sequenced genome among the chromalveolates. At almost 240 megabases (Mb), this is much larger than genomes in related oomycetes such as *P. sojae* and *P. ramorum* (95 Mb and 65 Mb, respectively). This expansion was like driven by proliferation of repetitive DNA, including transposable elements (TEs), gene duplication and subsequent divergence that likely facilitated evolution of novel effector genes and explains the location of these genes that are located in disperse, gene poor regions of the genome (Haas et al., 2009). The genomes of rust and powdery mildew fungi exhibit a similar proliferation of TEs (McDowell, 2011,Spanu et al., 2010, Duplessis et al., 2011), suggesting that their effector genes may exploit a similar method for effector evolution.

In sum, research over the last five years has produced many sequenced and annotated genomes of fungal and oomycete plant pathogens. Consequently, comparative

genomics and bioinformatics have aided our understanding of plant pathogens and their evolutionary histories. Genome fluidity is prevalent amongst plant pathogen genomes. However, it appears that pathogen genomes also contain sets of "core" effectors that are relatively conserved (see below).

Effectors as tools for pathogen invasion and evasion

Functions of pathogen effector proteins

Effectors are pathogen-derived proteins that are secreted to manipulate host cell structure and function. Both animal and plant pathogens utilize effectors to suppress host defenses and metabolism (Mudgett, 2005, Kamoun, 2006, Haldar et al., 2006). Effector gene structure and function have been well studied in bacterial pathogens of plants and animals.

Bacterial type III secretion systems translocate bacterial effectors into host cells

Effectors from gram-negative bacteria such as *Psy* and *Xanthomonas campestris* are translocated to host cells via the Type III Secretion system (TTSS) (Galan & Collmer, 1999). The TTSS structure resembles a syringe and is composed of over 20 proteins related to bacterial flagellar machinery (Galan & Collmer, 1999). After effector proteins are translocated to the inside of plant cells they interfere with host immunity by altering host proteins (Espinosa & Alfano, 2004, Abramovitch et al., 2006). The collection of

effectors maintained by pathogens vary from strain to strain and may dictate the pathogen's host specificity (Vinatzer et al., 2006).

Pseudomonas bacteria attack plant proteins that regulate defense responses

Several effectors from *Psy* have been characterized in detail. A predominant theme is that these effectors target plant proteins that regulate immune responses. For example, *AvrPto* and *AvrPtoB* are effectors of *Psy* DC3000 that function as suppressors of mitogen-activated protein kinsae (MAPK) activity in early MAMP-induced signaling (He et al., 2006). By suppressing specific early MAPK signaling events, AvrPto and AvrPtoB effectively suppress host basal defenses thereby contributing to virulence. Additionally, AvrPtoB functions as an E3 ubiquitin ligase *in planta* that targets other host proteins including the kinase, *Fen,* that is responsible for inducing host defenses. AvrPtoB-mediated degradation of Fen by the host proteasome interferes with activation of host defense signaling (Rosebrock et al., 2007). A third AvrPtoB target has been identified as the pattern recognition receptor FLS2, for which AvrPtoB utilizes it E3 ligase activity to direct FLS2 for degradation via the 26S proteasome (Robatzek et al., 2008). The multiplicity of AvrPtoB targets implies that effector proteins can be multifunctional or promiscuous.

Another *Psy* DC3000 effector, HopM1, interferes with host defenses by reducing callose deposition during pathogen attack. Callose deposition near the site of infection is an inducible defense that is thought to limit pathogen virulence. Suppression of callose

formation by HopM1 is accomplished through posttranscriptional events that destabilize AtMIN proteins involved in vesicle trafficking/callose formation (Nomura et al., 2006).

Xanthomonas bacteria attack the host cell nucleus

Xanthomonas campestris pv. vesicatoria is an adapted bacterial pathogen of pepper plants that utilizes a number of DNA-binding effectors called TALs (transcriptional activator-like), named for their ability to bind host DNA and activate transcription in the plant cell nucleus (Boch et al., 2009). This family of effector proteins is not found in the *Pseudomonas* genus. TAL effectors maintain a nuclear localization signal (NLS) and an acidic transcription activation domain (AAD). In the middle are 33-34 amino acids repeats; the number of repeats varies between TAL effectors and contributes to DNA-binding specificity (Bogdanove et al., 2010). A simple and elegant code within the 34 amino acid repeats (Lahaye et al., 2006) dictates the DNA sequences that are bound by the TAL effector (Bogdanove & Moscou, 2009, Boch et al., 2009).

The *X. c. pv. vesicatoria* TAL effector, AvrBs3, triggers a strong HR on pepper plants that have the cognate *R* gene, *Bs3* (Romer et al., 2007). An intact T3SS must be present for *AvrBs3-Bs3* dependent HR, suggesting that AvrBs3 is secreted and recognized in the host cell (Van den Ackerveken et al., 1996). *AvrBs3* contains recognizable motifs, including a NLS and AAD required for avirulence activity, and dimerization is necessary for full virulence function and nuclear localization (Van den Ackerveken et al., 1996, Gurlebeck et al., 2005). Once inside the host, AvrBs3 directly alters host gene expression

to induce hypertrophy of mesophyll cells in susceptible plants (Marois et al., 2002). This is accomplished by binding the promoter of the pepper gene, *up20,* and subsequent transcriptional activation. (Kay et al., 2007). *up20* is a master regulator of cell size and regulates various aspects of cell metabolism and auxin-related genes (Kay et al., 2007). Thus, misregulation of *up20* by AvrBs3 induces the hypertrophy phenotype.

The decoding of TAL repeats has enormous potential to identify the DNA targets of TAL effectors by decoding the predicted DNA binding site of the TAL effector and searching the host genome. Furthermore, these repeats can be altered to target specific DNA sequences and have strong biotechnological potential for genome editing (Voytas et al., 2010). Thus, the narrative of TAL effectors provides an excellent example (along with *Agrobacterium tumefaciens* and *A. rhizogenes*) of how basic research on plant-microbe interactions can lead to novel tools for biotechnology.

Influencing host gene expression is not restricted to phytopathogenic bacteria alone. *Shigella flexneri,* the causative agent of bacillary dysentery in humans, exploits an effector that alters chromatin access for NF-kB and in so doing suppresses host immunity (Arbibe et al., 2007). Thus, pathogens of animals and plants exploit similar host processes to cause disease.

Despite the ever-growing knowledge of bacterial effectors, much is still unknown about their evolutionary origins, representation in natural populations, targets, and biochemical modes of action. Even less is understood about effectors from oomycete

pathogens. Oomycete effectors were first identified as *Avr* genes that triggered gene-for-gene resistance in their respective hosts (Rehmany et al., 2005). The nature of cognate *R* genes suggests that oomycete effectors are perceived inside the host cell.

Oomycete effectors

Oomycete effectors contain conserved host-targeting signals

Oomycete effectors are known to be translocated into the host cytosol (Morgan & Kamoun, 2007), but oomycetes do not produce a structure equivalent to the bacterial TTSS. Oomycete effectors targeted to the host cell contain an endoplasmic reticulum - targeted signal sequence (SP), indicating they are secreted to the exterior of the pathogen where they act in the apoplast or host cyotsol (Birch et al., 2006). Cytosol-targeted effectors contain a conserved host-targeting signal (HTS) that mediates uptake into plant cells (Rehmany et al., 2005). The HTS is approximately 25 to 30 amino acids in length and contains the core conserved motif RxLR, where "x" is most commonly glutamic acid (E), aspartic acid (D) or glutamine (Q) residues. Although the RxLR consensus appears to be strongly conserved, some degeneracy is apparently permissible (Dou et al., 2008b). The RxLR is followed by a group of acidic residues (E or D) that comprise the dEER motif (Rehmany et al., 2005, Dou et al., 2008b, Whisson et al., 2007a). Both the RXLR and DEER motifs are required for proper host targeting in most effectors, although RXLR alone is sufficient in some cases **(Figure 1.3)**. The RXLR is physically located within 60 amino acids downstream of the SP. RXLR motifs are enriched in *Phytophthora* proteins predicted to be secreted, compared to non-secreted proteins. An RXLR motif has

been identified in all known oomycete *Avr* genes, including *Avr1b* of *P. sojae*, *Avr3a* of *P. infestans*, and *ATR13* and *ATR1* of *Hpa*.

The RXLR is required for effector translocation into the host cells, and the translocation appears to be independent of pathogen-encoded machinery (Whisson et al., 2007b, Dou et al., 2008b). The inability of the RXLR alone to activate host defenses infers the HTS to be a distinct entity from its effector cargo (Bos et al., 2006). A molecular mechanism that underpins RXLR-mediated host entry has been proposed, through which the RXLR motif binds to the phospholipid, phosphatidylinositol-3-phosphate (PI3P), on the plasma membrane of the host cell and is taken into the cell through lipid raft-mediated endocytosis (Kale et al., 2010).

Conserved C-terminal motifs named after the core conserved amino acid residues, tryptophan (W), tyrosine (Y), and leucine (L), were identified in a large subset of oomycete effectors (Jiang et al., 2008). In general, W, Y, and L motifs are arranged as repeating modules. Mutagenesis experiments have shown that these motifs participate in defense suppression and recognition by R proteins (Dou et al., 2008a, Jiang et al., 2008). As an example, the W motifs of the *P. infestans* effector Avr4 mediate recognition by the cognate R protein, R4 (Poppel et al., 2009).

Insights from structural biology

Boutemy and colleagues resolved the structure of *P. capsici* Avr3a11 revealing core α-helical folds with spacer loops (Boutemy et al., 2011). The structure is amenable to modifications including insertions/deletions and amino acid substitutions at surface residues. Additionally, extensions to N or C termini of core α-helical folds may add additional functionality (Boutemy et al., 2011). A second crystal structure of the *P. infestans* effector, PexRD2, revealed a similar α -helical fold that maps to W/Y motifs and, analogous to the ATR1 structure, polymorphic residues are located on the effector surface (Chou et al., 2011, Boutemy et al., 2011).

These studies highlight the structural adaptability of oomycete effectors, whereby surface residues are exchangeable while maintaining overall structure and function. Notably, the core α-helical folds map to the W/Y motifs that were described earlier. W/Y motifs are enriched in RXLR effectors and they can contain up to 11 W/Y motifs; 26% of the *Hpa* effectors contain W/Y motifs while 44% of the *Phytophthora* effectors have such motifs (Boutemy et al., 2011). With the resolved structure of Avr3a11 and PexRD2, it may be possible to infer predicted protein structures of W/Y-containing effector proteins that would aid in solving the function of the effector.

Proliferation of RXLR effector genes in oomycete genomes

Genomic mining of candidate effector genes using the SP and RXLR as signature motifs has led to the identification of hundreds of candidate genes in the genomes of oomycete pathogens. The *P. sojae* and *P. ramorum* genomes are predicted to contain 396 and 374 effector genes, respectively (Tyler et al., 2006). There are 563 predicted effector genes in the genome of *P. infestans* (Haas et al., 2009) and the *Hpa* genome is predicted to contain 134 effector genes (Baxter et al., 2010).These potential oomycete effectors display high sequence divergence, and computational analysis suggest they have likely evolved from a single ancestor by duplication events and subsequent divergence (Jiang et al., 2008).

Consistent with selection pressure applied by host defenses, the large effector families constitute the most rapidly-evolving portions of the genome, with most effectors occupying gene sparse-regions of the genome (Jiang et al., 2008, Raffaele et al., 2010). Analysis of RXLR gene expression during *P. infestans* infection revealed specific host-induction of effector genes, signifying effector participation during plant colonization (Whisson et al., 2007b). Interestingly, RXLR effector genes are not present in all oomycete genomes and are notably absent from genomes representing the *Pythium*, *Albugo,* and *Aphanomyces* genera (Schornack et al., 2010) (**Figure 1.4).** It has been postulated that RXLR containing effectors represent a recently-evolved class of effectors that are associated with haustoria-forming oomycete pathogens (Schornack et al., 2010).

The RXLR effector, HpaATR1

HpaATR1 was identified by map-based cloning using the avirulence that *ATR1* confers on *Arabidopsis* plants with the resistance gene *RPP1*. *ATR1* is differentially recognized by *RPP1* genes from two *Arabidopsis* ecotypes. Other than the SP and RxLR, *ATR1* has no identifiable functional motifs (Rehmany et al., 2005). *ATR1* alleles that confer virulence are highly divergent in comparison to avirulence alleles and signify a high level of genetic diversity at the *ATR1* locus. Several alleles of *ATR1* support increased growth of transgenic *Psy* engineered to deliver ATR1 via the TTSS. This is the only documented virulence role of ATR1 (Sohn et al., 2007). Recent structural analysis of the ATR1 protein revealed an effector with elongated, all-helical, two-domain, seahorse-like structure with no structural homology to other known proteins (Chou et al., 2011). The ATR1 structure lends itself to rapid adaptability, likely to avoid recognition by RPP1. The residues required for RPP1 recognition map to the surface of the protein and gain-of-recognition mutants demonstrate that multiple residues are additive and enhance recognition by RPP1 (Chou et al., 2011). Unfortunately, resolving the structure of ATR1 did not provide any clues to its virulence-promoting function due to the lack of homology to known protein structures.

The RXLR effector, HpaAtr13

The *Hpa* effector *ATR13* shares no similarity with known proteins based on BLAST searches, with the exception of the predicted SP and RXLR motifs (Allen et al., 2004). Sequence analysis of *ATR13* alleles indicates a high level of polymorphisms between alleles, suggesting selective host pressure. In accordance with the high level of allelic diversity at the *ATR13* loci, the cognate *Arabidopsis R* gene *RPP13* also displays a high level of genetic diversity (Allen et al., 2004). The delivery of ATR13 by *Psy* into *Arabidopsis* suppressed callose elicited by *Psy* (Sohn et al., 2007). Other than its ability to suppress callose, little is known about the virulence functions of ATR13.

The RXLR effector, PiAvr3a

The *P. infestans* effector, Avr3a, is the only oomycete effector for which a target has been identified to date. Avr3a is recognized specifically by the cognate potato *R* gene, *R3a* (Armstrong et al., 2005, Bos et al., 2009). Two *P. infestans* alleles with three amino acid substitutions have been identified as Avr3a^{KI} and Avr3a ^{EM} (Armstrong et al., 2005). The lack of null mutations and genetic diversity at the *Avr3a* locus suggest that this effector is important for virulence. Accordingly, gene knockdown experiments indicate that Avr3a makes an important contribution during colonization of potato (Bos et al., 2010). Avr3a is expressed during infection and, in a clever experiment, transgenic *P. infestans* expressing an Avr3a fusion to monomeric red fluorescent protein (RFP) revealed Avr3a-RFP accumulation at the haustorium during host colonization. Moreover,

host cell entry of Avr3a was evaluated with a fusion to GUS. Expression of the Avr3a-GUS construct by transgenic *P. infestans* revealed GUS positive cells specifically in contact with *P. infestans* haustoria. Mutational analysis demonstrated the requirement of an intact RXLR motif for proper delivery of the Avr3a-GUS fusion (Whisson et al., 2007b).

A virulence mechanism for Avr3a has been determined and thus far is the only oomycete effector with a known function. Avr3a interacts with the *N. benthamiana* U-box protein, CMPG1 that regulate immune responses (Gonzalez-Lamothe et al., 2006). During positive immune signaling, CMPG1 is degraded in a proteasome-dependent manner. However, during colonization with virulent *P. infestans*, Avr3a interacts with and stabilizes CMPG1 in the host nucleus, suppressing immune signaling (Bos et al., 2010). The ability of Avr3a to interact with and stabilize CMPG1 is required for suppression of INF1-induced cell death (ICD).

Analysis of the *Avr3a* protein structure been reported by two groups. Yaeno *et al*. 2011, resolved the structure of *P. capsici Avr3a4*, a homolog of *Avr3a*. A positively charged patch of amino acids in a conserved region of the effector domain was implicated in binding to phosphatidylinositol monophosphates (PIPs). Mutations in the PIP-binding region of Avr3a4 did not abolish recognition by the resistance protein R3a but eliminated ICD suppression and reduced Avr3a4 protein stability. These experiments validated the biological significance of PIP binding. It is now thought that Avr3a interacts with PIPs to target CMPG1, thus releasing CMPG1 from vesicles and trafficking the complex to the

nucleus where CMPG1 is stabilized in an Avr3a-dependent manner. This hypothesis is not farfetched given the presence of CMPG1 in slow-moving vesicles and the nuclear localization of Avr3a-CMPG1 interaction (Bos et al., 2010).

The RXLR effector, PsAvr1b

The *Avr1b* locus isolated from *P. sojae* confers avirulence on soybean plants with the genotype *Rps1b* (Shan et al., 2004). Sequencing of the *Avr1b* locus indicated strong divergence among *P. sojae* isolates. Virulent races possess highly divergent alleles of *Avr1b-1* with many amino acid substitutions. Two *Avr1b* paralogs have been identified at the Avr1b locus including *Avr1b-1,* whose mRNA accumulation during infection is dependent on the presence of *Avr1b-2* (Shan et al., 2004). *Avr1b-1* mRNA was detected in close proximity to the site of infection in wound-inoculated soybean hypocotyls and roots infected with zoospores, indicating expression during host colonization (Valer et al., 2006).

Oomycetes may exercise loss of effector transcription to evade host recognition. In several *P. sojae* races, the *Avr1b* allele that confers avirulence in Rps1b plants was present but *P. sojae* failed to accumulate *Avr1b* transcript during infection (Shan et al., 2004). Furthermore, effector copy number affects pathogen fitness and represents a virulence strategy in which effectors may be fine tuned for successful host invasion/evasion (Qutob et al., 2009).

Like many other studied effectors, Avr1b boasts the ability to suppress cell death. Yeast expressing *Avr1b* as a transgene display an enhanced tolerance to reactive oxygen species (ROS) thus increasing the proportion of living yeast cells (Dou et al., 2008a). Additionally, Avr1b suppresses cell death elicited by the pro-apoptotic Bax protein in soybean and *Nicotiana* (Dou et al., 2008a). The ability of Avr1b to suppress diverse cell death elicitors in a cross-kingdom manner indicates that this protein targets a universal mechanism for cell death suppression.

Conclusions

Hundreds of oomycete effectors have been identified using bioinformatic approaches. Yet the mechanisms through which these effectors coordinate defense suppression and (possibly) alter host cell structure and metabolism remain poorly understood. Homology searches using NCBI BLAST against the non-redundant database offer few clues for effector targets or function, impeding our understanding of the complex interaction between oomycetes and plants. Several broad questions remain to be addressed. Why do oomycetes maintain large collections of effectors? How do these effectors function on the molecular level? And are there conserved effector mechanisms amongst the oomycetes?

Avr3a represents our best understanding of the molecular mechanisms that effectors employ to evade host immunity. Yet, we have much to learn. The biology of bacterial effectors that were cloned decades ago, are still inadequately understood,

underscoring the effort necessary to understand how effectors work. Additionally, many effectors have multiple host targets, complicating our ability to determine the biological relevance of effector targets. It has become obvious, from protein structure and genome analysis that a conserved virulence strategy of plant pathogens is the designed fluidity of the virulence apparatus that includes the plasticity of effector proteins and the genome competency to rapidly evolve new effector genes.

Oomycetes are of profound agronomic importance. With increasing food demand worldwide, it is of the utmost of importance to maintain food supply security and increase productivity to meet these demands. My dissertation research is designed to increase understanding of molecular mechanisms that enable oomycete pathogens to cause destructive diseases and reveal novel host processes that regulate plant immunity. I focused on effectors that are conserved between *Hpa* and *P. sojae*. I anticipate that the analysis of conserved effectors will reveal virulence functions that are important for all oomycete plant pathogens that aid in the design of novel and durable resistances.

FIGURES

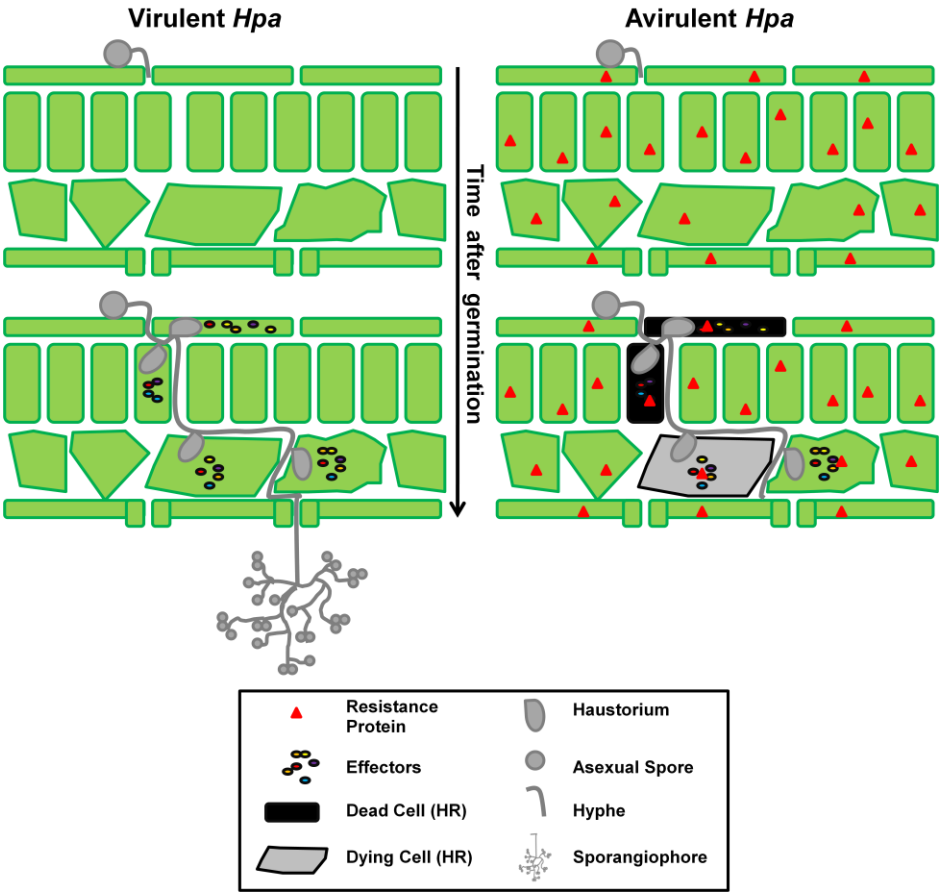

Figure 1.1. *Life cycle of Hyaloperonospora arabidopsidis*. The life cycle begins when a spore germinates on the surface of the leaf. An appressorium forms and a penetration hypha penetrates between epidermal cells. Threadlike hyphae then elongate through intracellular spaces in the mesophyll layer of the leaf. Haustoria extend from hyphae and penetrate plant cell walls interacting with the plant cell plasma membrane and secreting effector proteins that enter the host cell. Nutrient uptake presumably occurs as at the haustorium. The *Hpa* life cycle is completed when sporangiophores grow out of stomata and release their spores. Avirulent *Hpa* triggers HR in cells that have one or more R proteins that recognize specific effector proteins. This response effectively halts pathogen growth.

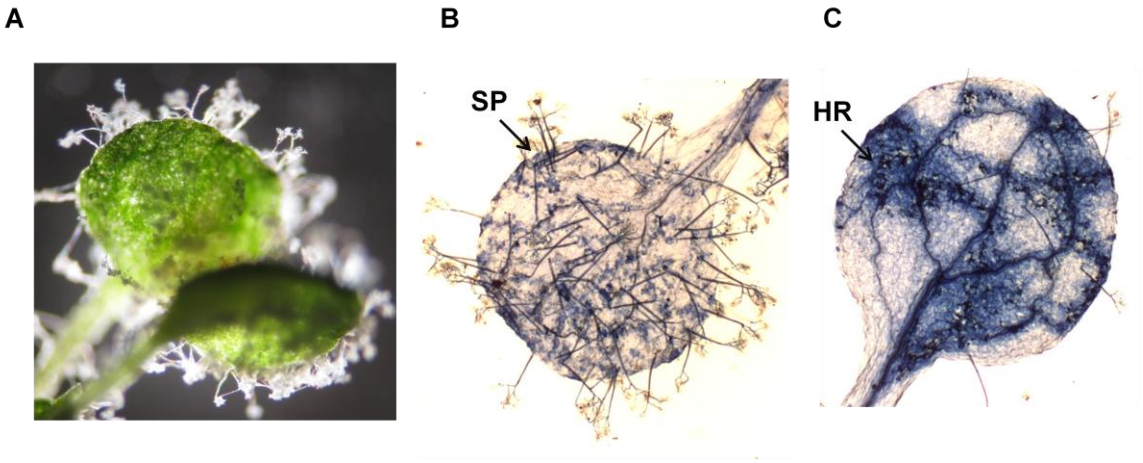

Figure 1.2. *Images of Hyaloperonospora arabidopsidis infection*. **A)** *Arabidopsis* seedling infected with virulent *Hpa*. White sporangiophores are growing from the cotyledons. **B)** A heavily colonized *Arabidopsis* seedling, stained with trypan blue to highlight hyphal growth and the production of sporangiophores (SP). **C)** A resistant seedling, in which **t**rypan blue staining highlights host cell death, from the HR, around the hyphae.

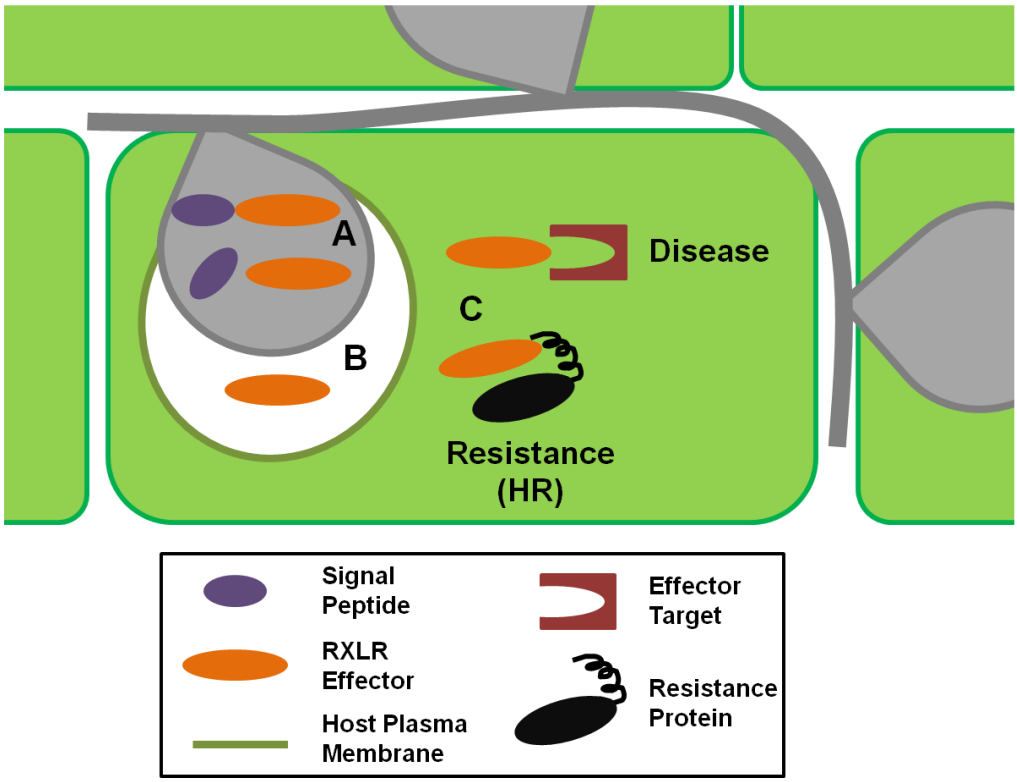

Figure 1.3. *Oomycete effectors are translocated into the host cytosol*. **A)** The signal peptide (blue) targets the effector to the ER, the SP is cleaved, and the RXLR effector (orange) is secreted into the extrahaustorial matrix. **B)** The RXLR motif directs the secreted oomycete protein into the host cytoplasm by interacting with the phospholipid, phosphatidylinosital-3phosphate (PI3P) on the plasma membrane of the host cell. The protein is taken into the cell by endocytosis. **C)** The effector protein interacts with its plant target (red) and promotes disease. In incompatible interactions, the RXLR effector can be recognized, directly or indirectly, by host R protein (black) triggering immunity.

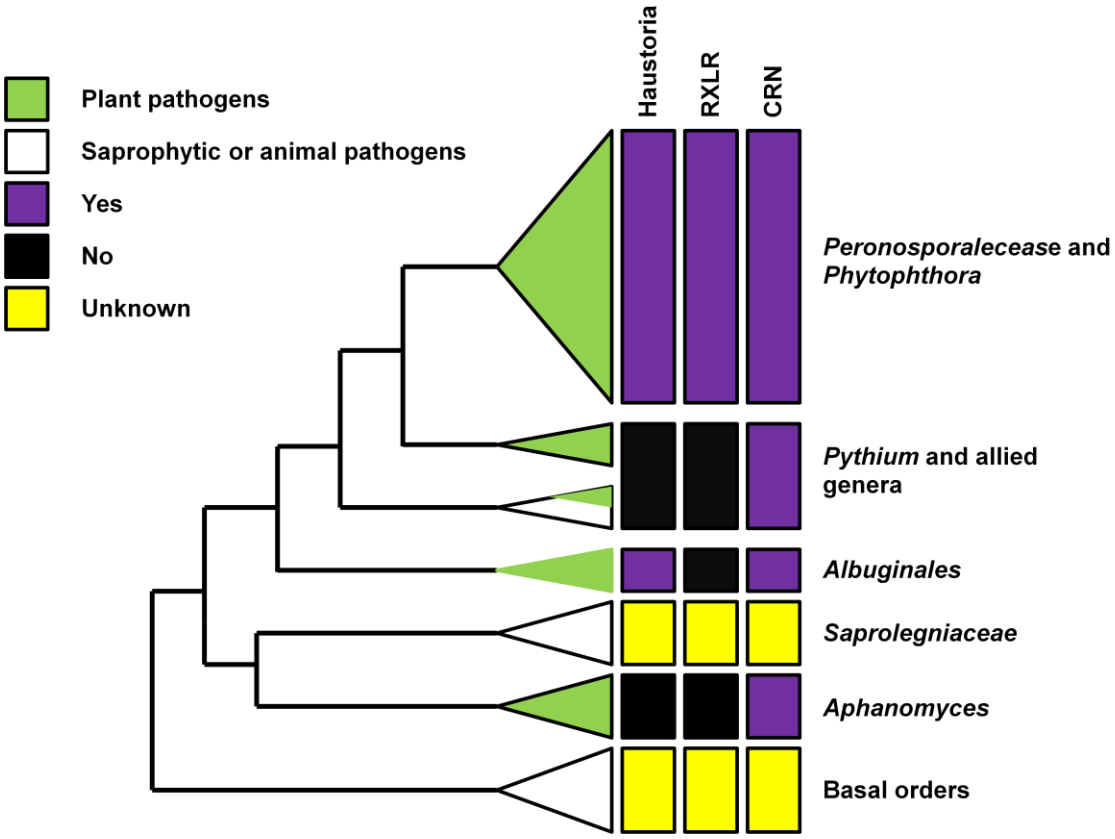

Figure 1.4 *Relationships of oomycetes, the occurrence of haustoria, and RXLR or CRN effectors*. The presence of haustoria correlates with plant pathogens that lead biotrophic lifestyle or have an initial biotrophic phase to establish infection. RXLR effectors are confined to the haustoria bearing pathogens. CRN effector proteins represent an ancient class of effector that are independent of haustoria. Adapted from Schornack et al., (2010).

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Chapter 2

Initial identification and characterization of homologous RXLR effectors from divergent oomycete species

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Keywords: Oomycete, effector, plant immunity, RXLR

Abbreviations: 4',6-diamidino-2-phenylindole (DAPI), days post inoculation (DPI), effector detector vector (EDV), effector triggered immunity (ETI), green florescent protein (GFP), hypersensitive response (HR), microbe associated molecular pattern (MAMP), methylenetetrahydrofolate dehydrogenase (MTD), MAMP triggered immunity (MTI), nuclear localization signal (NLS), programmed cell death (PCD), signal peptide (SP).

ABSTRACT

Diverse pathogens secrete effector proteins that enter plant cells to manipulate host cellular processes. Bioinformatic analysis suggests that the genome of *Hyaloperonospora arabidopsidis* (*Hpa*), the downy mildew pathogen of *Arabidopsis thaliana*, encodes at least 134 RXLR effector proteins. The majority of predicted *Hpa* effector genes have no homologs in sequenced genomes of *Phytophthora* species. However, a subset of predicted *Hpa* effectors is conserved within the *Phytophthora* genus. Here, we describe functional characterization of six candidate *Hpa* effector genes. All six genes are induced during infection, consistent with a role during infection. Five of the six *Hpa* effectors carry a predicted nuclear localization signal. Accordingly, fusions of the effectors to green florescent protein exhibit nuclear localization when transiently expressed in onion epithelial cells. Two effectors, HaRxL89 and HaRxL108, are able to suppress cell death elicited by the *Phytophthora infestans* protein INF1. Stably transformed *Arabidopsis* plants expressing *HaRxL89* suppress basal resistance to virulent *Hpa* Emco5. Candidate *Arabidopsis* target proteins were identified for HaRxL89 and HaRxL108 in the recently-published plant-pathogen interaction database (PPIN1). These experiments demonstrate that homologous effectors from related oomycetes suppress defense mechanisms in distantly-related plant species and their primary action may take place within the plant nucleus.

INTRODUCTION

Phytopathogens must overcome multiple layers of constitutive and inducible defenses to successfully colonize their hosts and cause disease. Plant-pathogen interactions can be classified into broad categories, described as "compatible" or "incompatible." During compatible interactions the plant is unable to detect the pathogen and disease occurs. This stands in contrast to an incompatible interaction in which the activation of the host immune system prevents pathogen proliferation. (Zipfel and Felix 2005).

Research in the field of plant-pathogen interactions has been directed towards learning the molecular mechanisms that underlie these different outcomes. It is now understood that plant immune responses can be triggered by two types of receptors that recognize different classes of pathogen-derived signals (Jones and Dangl 2006). These two classes of receptors, termed resistance (R) proteins and pattern recognition receptors (PRR) comprise the surveillance components of the plant immune system. PRRs at the surface of the plasma membrane recognize conserved microbial molecules known as microbe-associated molecular patterns (MAMPs). Upon recognition, PRRs activate signaling cascades that trigger the production of reactive oxygen species, antimicrobial compounds, and deposition of callose at infection sites (Jones and Dangl 2006). This response is called pattern-triggered immunity (PTI) and is sufficient to confer resistance to many different types of pathogens (Katagiri and Tsuda 2010).

Resistance proteins recognize specific pathogen effector proteins in a direct or indicted manner, typically inside the plant cell, and elicit effector-triggered immunity (ETI) that culminates in programmed cell termed the hypersensitive response (HR) (Beers and McDowell 2001). This powerful HR response provides potent immunity against a broad spectrum of pathogens.

In turn, plant pathogens have evolved the ability to deliver effector proteins to the interior of plant cells that sabotage the host immune system and alter metabolism, (Dodds and Rathjen 2010). Effectors from the bacterial plant pathogen *Pseudomonas syringae* have been studied with the most detail and have provided insights to the molecular mechanism that bacteria employ to evade host detection. Bacterial effectors can directly influence host transcription, suppress cell death, alter hormone signaling, suppress vascular trafficking or direct host proteins to the proteasome for premature degradation (Hann, Gimenez-Ibanez et al. 2010). These methods of "molecular mimicry" in which bacterial proteins imitate the functions of host proteins, collectively function to create a comfortable niche for the pathogen.

Oomycete pathogens can be devastating to monocot and dicot crops alike. Morphologically, oomycetes appear fungus-like but have evolved independently to colonize land plants (Baldauf, Roger et al. 2000). The genome of the downy mildew pathogen of *Arabidopsis thaliana*, *Hyaloperonospora arabidopsis* (*Hpa*), has been sequenced (Baxter, Tripathy et al. 2010). Bioinformatic surveys of the *Hpa* genome revealed 134 effector candidates that contain a predicted signal peptide followed by an

RxLR motif. The latter motif is thought to facilitate entry into the host cell through interactions with phosphatidylinositol 3-phosphate and subsequent endocytosis (Dou, Kale et al. 2008; Kale, Gu et al. 2010).

Cross-species comparison of the effector collections maintained by oomycetes reveals little evolutionary conservation (Jiang, Tripathy et al. 2008; Baxter, Tripathy et al. 2010; Raffaele, Farrer et al. 2010). This likely reflects strong host pressure and adaptation. Even between closely related *Hpa* isolates, effector pools may differ (Cabral, Stassen et al. 2011). Despite the high level of diversity amongst oomycete effectors, a small subset is conserved between species (Baxter, Tripathy et al. 2010). These conserved oomycete effectors may have an important role in establishing the host-pathogen interaction. Studies of these effectors may provide insights to the mechanisms of effector evolution and reveal host processes that are targeted by all oomycetes.

From the candidate *Hpa* effector genes, six were selected for detailed characterization based on several criteria: an identifiable homologue in *P. sojae*, a putative nuclear localization signal (NLS), expression during infection, and the presence of W, Y and L motifs (Dou, Kale et al. 2008; Jiang, Tripathy et al. 2008). In this chapter, We provide evidence that all six computationally predicted RxLR genes encode *bona fide* effectors proteins. We confirmed that all six candidate effectors are expressed during infection. Transiently expressed effector-GFP fusions exhibited nuclear localization. Furthermore, several effectors are able to suppress programmed cell death triggered by divergent elicitors. A recently-published study (Mukhtar, Carvunis et al. 2011) identifies

candidate target proteins for the effectors HaRxL89 and HaRxL108. These experiments demonstrate that homologous effectors from related oomycetes suppress defense mechanisms in distantly-related plant species and that their functions may lie within the plant nucleus.

RESULTS

Bioinformatic prediction of conserved oomycete effector genes

Candidate *Hpa* effector genes were identified with specific search criteria and manually curated during a genome annotation jamboree in Blacksburg, VA, in June 2007, as described in Baxter et al. (2010). Briefly, predicted proteins from a six frame translation of the entire genome were scanned with SignalP 3.0 (Emanuelsson, Brunak et al. 2007) to identify proteins with a N-terminal endoplasmic reticulum signal peptide (SP). Coding potential, BLAST searches, and hidden markov models (HMMs) to score RxLR motifs were utilized to assess each candidate.

After generating a master list of candidate effector genes from the *Hpa* genome a handful of promising effector candidates were selected for preliminary analysis based on several criteria: 1. an identifiable homolog in the soybean pathogen, *Phytophthora sojae* suggesting conservation between distantly-related oomycetes; 2. a putative nuclear localization signal (NLS), with the exception of HaRxL96; and 3. the presence of W, Y

and L motifs. Six candidate effectors genes met these criteria and are summarized in **Table 2.1**.

Candidate effectors are expressed during infection

Authentic effectors, by definition, are expressed during the process of host colonization. Expression of candidate effector genes was monitored during infection of *Arabidopsis* Oy-1 by the *Hpa* isolateEmoy2 (a compatible interaction), by reverse transcriptase-polymerase chain reaction (RT-PCR). This assay confirmed expression of all six candidate effectors during the course of infection (**Table 2.1 and Figure 2.1**). Collectively the effectors displayed unique expression patterns. HaRxL89 and HaRxLL108 displayed strong expression that is detectable by four DPI while HaRxLL120 had early and strong expression that tapers off late in the *Hpa* interaction. Ha330 maintained weak expression that was barely detectable.

Candidate effectors are localized to the nucleus and cytosol

Localization of each candidate effector inside plant cells was determined by transiently expressing fusions of green fluorescent protein (GFP) to the C-terminus of each effector without the predicted signal peptide (SP) (**Figure 2.2**). Omitting the SP will mimic the presumed structure of the protein in plant cells, following cleavage of the signal peptide during translation in *Hpa*. Each construct was expressed transiently in onion epidermal cells via ballistics and nuclear localization was confirmed for the five

candidate effectors with a predicted NLS (**Table 2.1, Figure 2.3**). Localization of HaRxL96 is described in Chapter 4. Each effector-GFP fusion localized to the nucleus and weakly to the cytoplasm while HaRxL89 demonstrated stronger cytoplasmic abundance.

Using Pseudomonas syringae as a surrogate for delivering oomycete effectors into Arabidopsis cells

P. syringae utilizes a type three secretion system (TTSS) to deliver its effectors to the interior of plant cells, where they interact with host proteins (Cunnac, Lindeberg et al. 2009). This secretion system has been exploited to deliver oomycete effectors into *Arabidopsis* cells using *P. syringe* transformed with an "effector detector vector" (EDV) designed to express oomycete effector fused to a leader that is addressed to the TTSS (Sohn, Lei et al. 2007). The system is described in **Figure 2.4**. Cultures of *P. syringae* DC3000 (*Psy* DC3000) expressing oomycete effectors were infiltrated into four-week-old *Arabidopsis* plants and *in planta* growth of *Psy* was assayed. In these experiments growth of *Psy* D3000 was not enhanced or repressed by the addition of any candidate effector gene in either the Ws or Col-0 accessions of *Arabidopsis* (**Figure 2.5**).

HaRxL96 and PsAvh163 suppress Bax-induced cell death in soybean

Many bacterial effectors have demonstrated an ability to suppress Bax-induced cell death in plants (Abramovitch, Kim et al. 2003). The mouse Bax protein is a well-

characterized elicitor of cell death in plants and mammals and elicits an HR-like PCD response in plants by disrupting the mitochondrial membrane (Lacomme and Cruz 1999; Epand, Martinou et al. 2002). We quantified suppression of Bax-induced PCD in soybean using a double barrel device retrofitted to BioRad PDS-1000 Gene Gun. This arrangement allows us to bombard the control and experimental samples together, reducing the between sample variability (**Figure 2.6 A**) (Dou, Kale et al. 2008). Co-transforming via ballistics, Bax with *B*-glucuronidase (Gus) into soybean leaves reduced the amount of *Gus* expressing cells up to 85%, relative to the control sample (empty vector and *Gus*). Concurrently, co-transformation with a third gene that suppresses PCD restored cell viability, manifested by higher numbers of blue spots (Dou et al. 2008). This assay allows for quantitative assessments of a candidate effector's ability to suppress Bax-elicited PCD.

Co-transformation of *Bax*, *Gus* and *HaRxL96* increases the number of cells expressing *Gus* relative to the empty vector (EV) control. The number of living cells increased 3.6 fold in comparison to the EV control, suggesting that HaRxL96 is able to suppress Bax-induced PCD (**Figure 2. 6B**). The homolog of *HaRxL96* in *P. sojae*, *PsAvh163*, was also able to suppress Bax-induced cell death in soybean, increasing the number of living cells 1.6 fold (**Figure 2.6 B**). The functions of these proteins is further described in Chapters 3 and 4. HaRxL108 and HaRxL89 were not able to suppress Bax-induced cell death in soybean. The other three candidate effectors were not tested (**Figure 2.7 A)**

HaRxL89 and HaRxLL108 suppress INF1-induced cell death in N. benthamiana

HaRxLL108 and HaRxL89 were expressed in *N. benthamiana* using *Agrobacterium* transient expression. Neither effector elicited a macroscopic PCD phenotype six days post inoculation (DPI) (Data not shown). We then tested whether either effector could suppress cell death elicited by the *P. infestans* effector INF1. *Agrobacterium* containing binary plasmid vectors with HaRxL108, HaRxL89, or YFP as a control were infiltrated into *N. benthamiana*. Two days later, the same sites were infiltrated with INF1 and the appearance of cell death was visually monitored over a period of seven days. Both effectors were able to partially suppress INF1 elicited PCD. Only 40% or less of the challenged sites demonstrated any significant cell death in contrast to the YFP control or INF1 alone, which exhibited 80% or greater sites with cell death (**Figure 2.7 B and A**). Similar experiments with HaRxL96 are described in Chapter 3. The other four effectors were not tested with this assay.

HaRxL89 expressed in transgenic Arabidopsis enhances susceptibility to virulent Hpa

To test whether *HaRxL89* or *HaRxLL108* affects resistance to pathogens of *Arabidopsis*, we generated stably transformed *Arabidopsis* plants expressing *HaRxL89* or *HaRxLL108*. Transgene expression was confirmed by RT-PCR (**Figure 2.8 C**). The stably transformed plants exhibited no visible morphological phenotypes. We tested for alterations to PTI in the effector-expressing lines by challenging them with non-

pathogenic *Psy* or virulent *Hpa*. When challenged with virulent *Hpa* Emco5, HaRxL89 enhanced the growth of *Hpa.* No enhancement was observed in plants expressing *HaRxLL108* (**Figure 2.8 A**). Both effector lines were challenged with the non-pathogen, *P. syrinage* DC3000*(*Δ*hrc)*, that lacks a functional TTSS and thus is unable to deliver effectors to suppress immunity. In contrast to the results from the *Hpa* infection, neither transgenic line was able to support enhanced bacterial growth in comparison to wild-type plants (**Figure 2.8 B**).

Searches for Hpa effector targets

Screens for protein interactions have revealed putative effector targets for a number of bacterial effectors (Abramovitch, Janjusevic et al. 2006; Nomura, Debroy et al. 2006). Identifying effector targets will offer clues to the molecular and virulence function(s) of each candidate effector. To identify putative effector targets, the candidate effectors were provided to several collaborating groups, including a consortium that screened an immunity-related enriched collection of *Arabidopsis* proteins using a yeast two-hybrid system. Results from this set of pairwise screens were recently published (Mukhtar et al. 2011) and can be queried through an internet-accessible search tool (Plant-Pathogen Interactome version 1 or PPIN1, (signal.salk.edu/interactome/PPIN1.html). We screened the dataset to determine whether our effector proteins interacted with *Arabidopsis* proteins. Only two of the candidate *Hpa* effectors had positive interaction partners in the screen (**Table 2.2**). One effector, HaRxL108, interact with *Arabidopsis* CSN5A (At1g22920), a central hub in the

interactome that also includes three predicted R proteins, 11 *Hpa* effectors, and 11 *Psy* effectors (**Figure 2.10**). Additionally, HaRxL89 interacts with a protein annotated as a mitochondrial glycoprotein (At5g02050) (Mukhtar et al. 2011). These proteins are thus putative targets of the respective *Hpa* effectors.

DISCUSSION

The sequencing of several oomycete genomes has facilitated the identification of hundreds of oomycete effectors. The *P. sojae* and *P. ramorum* genomes contain 396 and 374 predicted effector genes respectively, (Tyler et al. 2006) while the *Hpa* genome contains 134 effectors (Baxter et al. 2010) and the *P. infestans* genome contains 563 effectors (Haas et al. 2009). We understand very little about how these effectors are utilized by oomycete pathogens to suppress host immunity and coordinate infection. With numerous putative effector genes, the bottleneck now lies in characterizing and understanding the functions of oomycete effectors in a methodical manner. Two questions arise. First, how do we select biologically interesting effectors and second, what are the best approaches to study them? Cross-species examination of effector genes revealed very little conservation amongst the pools of effectors maintained by oomycete pathogens. However, a core group of conserved effectors exist amongst oomycetes and we hypothesized that they have important virulence functions. To address this hypothesis, we chose to focus on a relatively small number of effectors with identifiable homologs in other species. Our long-term plan is to compare the functions of homologous effectors

from different oomycete species to determine if they are indeed functionally similar (e.g., Chapters 3 and 4).

As a first step towards experimental validation of each effector candidate, expression was confirmed during infection with RT-PCR. Expression was detectable for most effectors by four DPI (**Figure 2.1**). It is likely that some or all of these genes are expressed at earlier stages of infection, but transcript abundance was below the threshold for detection under the experimental conditions that we used. Together, the effector candidates displayed differing transcriptional profiles during infection, akin to the diverse transcriptional profiles of *P. infestans* and *P. sojae* effectors (Whisson, Boevink et al. 2007). This hints at a carefully coordinated effector deployment in oomycete pathogens.

Querying the NCBI BLAST non-redundant database with the candidate effector sequences yielded no discernable motifs, with the exception of one effector. BLAST homology searches with *Ha330* denoted sequence similarity to a Tudor motif that is present in mammals and plants. Proteins with Tudror motifs have known chromatin modifying functions and in some cases specificity to methylated histones (Maurer-Stroh, Dickens et al. 2003; Saleh, Alvarez-Venegas et al. 2008). The functionality of the Ha330 tudor motif is unknown, but the presence of functional NLS in conjunction, may motivate the efforts for further studies. It is plausible that Ha330 enters the nucleus to sabotage host transcription. Further experimentation is necessary to confirm these hypotheses.

Approximately 17% of the *Hpa* effectors have a predicted NLS (unpublished observations). Previous studies of nuclear localized bacterial effectors demonstrated an ability to hijack the nucleus and alter host transcription. For example, AvrBs3 is an effector of the bacterial pepper pathogen, *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) that functions as a molecular mimic, acting as a transcription factor and inducing expression of the cell size regulator of *upa20* (Kay, Hahn et al. 2007). It is feasible that the conserved, nuclear-localized effectors from oomycetes may function in a comparable manner, as molecular mimics of transcriptional regulatory proteins. Several of the effectors that we selected for this study have a functional NLS, suggesting that their virulence functions may lie within the nucleus. A strong cytoplasmic fluorescent signal was observed for HaRxL89 in addition to the fluorescent signal in the nucleus. A weak cytoplasmic fluorescent signal was observed the other candidate effectors (**Figure 2.3**).

A caveat that must be considered in these localization studies is the lack of pathogen stimuli which may influence effector localization. Cellular components are dynamic and protein shuttling between the nucleus and other subceullular components are important for proper function and output (Shen, Saijo et al. 2007; Caplan, Mamillapalli et al. 2008). Future experiments may address effector localization during *Hpa* colonization and exploit protein mis-localization to establish the biological relevance of nuclear localization (Schornack, Fuchs et al. 2009; Schornack, van Damme et al. 2010). We used this approach in the study described in Chapter 4 to assess the relevance of nuclear localization of the effectors HaRxL96 and PsAvh163.

Since *Hpa* is not amenable to genetic manipulation, we could not assess the virulence contribution of each effector by deleting them from the *Hpa* genome or by downregulating their expression with RNAi. Thus, we assessed the contribution of each effector by individually expressing them in plant cells. Our first test exploited the EDV system that delivers oomycete effectors via the *Psy* TTSS (**Figure 2.4**). This system has been used successfully to study the function of the *Hpa* effectors ATR1 and ATR13. (Sohn, Lei et al. 2007; Rentel, Leonelli et al. 2008). This delivery method provides the advantage of delivering low levels of effector protein that would reflect a natural infection. Additionally, growth of the *P. syringae* strain itself can be measured to determine whether the effector activates or suppresses immunity. Neither HaRxL96 nor HaRxL89 enhanced the growth of *Psy* DC3000 in two different accessions of *Arabidopsis* (**Figure 2.5**). These negative results could be explained by three different hypotheses. First, the oomycete effectors failed to be properly delivered to the interior of host cells or were improperly folded. Second, the functions of HaRxL96 or HaRxL89 were masked by the functions of the *Psy* DC3000 effectors. Third, the effectors were delivered to the host cell, but target host components that only confer advantages to *Hpa* and not to *Psy*.

Our next test measured the ability of the candidate effectors to suppress cell death in two different transient assays that provide sufficient resolution to detect weak virulence activities. We exploited two cell death elicitors that likely activate the HR in different manners. Our first screen for suppression of cell death relied on the pro-apoptotic protein, Bax. Using a transient system in soybean, we are able to co-deliver

Bax and a candidate effector to assess the quantity of living cells. One *Hpa* effector HaRxL96, and its homolog in *P. sojae,* PsAvh163 interfered with the ability of Bax to elicit PCD and increased the proportion of living cells (Dou, Kale et al. 2008). Neither HaRxL89 nor HaRxLL108 were able to do so (**Figure 2.6, Figure 2.7**). The Bax PCD-suppressive function of HaRxL96 and PsAvh163 are similar to other *P. sojae* and bacterial effectors including PsAvh331, Avr1b and AvrPtoB (Jamir, Guo et al. 2004; Dou, Kale et al. 2008; Liu, Ye et al. 2011). The absence of a *Bax* homolog in plants and the dissimilarity in cell death pathways between plants and animals, suggests that these effectors may act as general suppressors of cell death.

The second functional screen was for suppression of cell death elicited by *P. infestans* INF1 in *Nicotiana spp*. (Gonzalez-Lamothe, Tsitsigiannis et al. 2006; Gilroy, Taylor et al. 2011). Transient effector expression via *Agrobacterium* delivery in *N. benthamiana* indicated that HaRxL89 and HaRxLL108 were able to suppress INF1-induced PCD (**Figure 2.7**). This suggests that these effectors have a virulence function in *N. benthamiana*, analogous to the *P. infestans* effector Avr3a that is a strong suppressor of INF1 (Bos, Armstrong et al. 2010). Whether HaRxL89 and HaRxLL108 operate in a similar manner to Avr3a requires further experimentation.

Transgenic plants expressing pathogen effectors have provided useful tools for studying a number of bacterial effectors including HopM1, ArvPtoB, and AvrPto (He, Hauck et al. 2003; de Torres, Mansfield et al. 2006; Nomura, Debroy et al. 2006). Given the encouraging results from our transient functional screens, we created stably

transformed *Arabidopsis* plants that express *HaRxL89 or HaRxLL108*. We challenged the transgenic effector lines with virulent *Hpa* Emco5 to examine effector-mediated alterations in basal immunity. *Arabidopsis* plants expressing *HaRxL89* displayed enhanced susceptibility to *Hpa* Emco5, however HaRxLL108 did not (**Figure 2.8**). Additionally, we examined callose formation during *Hpa* infection. No alterations in callose are evident; callose depositions appear wild type with analine blue fluorescence surrounding the haustoria similar to documented cases of *P. sojae* haustoria in soybean (Enkerli, Hahn et al. 1997) (**Figure 2.9**). These lines will be of great value for future experiments to understand the mechanisms of these effectors in more detail.

The failure of HaRxLL108 to enhance susceptibility to *Hpa* Emco5 may be attributable to transgene expression levels that are insufficient to confer enhanced pathogen susceptibility. Conversely, the phenotype of the HaRxL89 plants may be a result of a neomorphic mutation created as an artifact of overexpression. Arguing against this hypothesis, *HaRxL89* transgene expression is low. Furthermore, there are no documented cases of misleading virulence functions that were identified during overexpression studies (He, Hauck et al. 2003; de Torres, Mansfield et al. 2006).

Recently, a protein interaction network comprised of over 8000 *Arabidopsis* proteins and *Hpa* and *Psy* effector proteins was created (Mukhtar, Carvunis et al. 2011). The protein interaction network, "plant-pathogen immune network version 1" (PPN1) has a searchable online browser in which protein interactions can be queried. We probed the network with our effectors to identify host targets. The putative target of HaRxL89 is

member of the mitochondrial glycoprotein family protein/MAM33 (At5g02050) with unknown functions (Kiba, Naitou et al. 2005). Given the importance of the mitochondria it is unsurprising that bacteria and oomycetes alike, target the organelle. (Alfano, Block et al. 2010).

Interestingly, the localization of HaRxL89 is nuclear-cytoplasmic and the presence of HaRxL89 has not been observed in the mitochondria (**Figure 2.3**). It appears that the interaction pair may operate in different cellular compartments, however, this disconnect could be reconciled by two hypotheses. The first is founded on the assumption that the glycoprotein localizes to the mitochondria. Second, the mitochondrial glycoprotein may re-localize in the presence of HaRxL89, analagous to a chloroplast protein re-localizing in the presence of the viral effector, p50. (Caplan, Mamillapalli et al. 2008). A substantial amount of experimentation will be required to address the biological relevance of this interaction.

The second identified protein interaction belongs to HaRxL108 which is predicted to interact with CSN5a, a component of the COP9 signalosome complex that is implicated in regulating signaling and developmental processes via ubiquitination (Gusmaroli, Feng et al. 2004). The CSN5a protein interaction network is highly connected to many cellular processes and is targeted by effectors from diverse pathogens including, bacteria, oomycetes, and viruses (Lozano-Duran, Rosas-Diaz et al. 2011; Mukhtar, Carvunis et al. 2011) (**Figure 2.10**). The highly targeted nature of CSN5a

suggests an important role in plant immunity. The nature of these effector interactions will require further experimental attention.

The initial bioinformatic identification of six *Hpa* effectors and their subsequent functional analysis allowed us to validate computational predictions of candidate effector genes. Some of the tested effectors did not demonstrate immunosuppressive activity in our assays. It's possible that these effectors have no function. However, our initial screens were limited. Considering most *Psy* DC3000 effectors retain the ability to suppress plant immunity (Alfano, Guo et al. 2009) further experimentation will be required to demonstrate that the effectors with no putative functions are truly non-functional effectors.

In summary, our candidate effector genes have provided a starting point in which to explore the molecular interactions between host and microbe. The conserved nature of these effector genes suggests an essential role in establishing the host-pathogen interaction. Future studies will reveal insights in host processes that are manipulated by oomycetes. In the next two chapters, will provide detailed characterization of the homologous pair of effectors *Hpa HaRxL96* and *P. sojae PsAvh163* that were identified in this study.

FIGURES AND TABLES

Gene	ORF (bp)	AA	Transcribed? *Hpa/Ps*	Identity		Motifs			Nuclear Localized?
				Ps	Pr	NLS	W	Y	
HaRxL89	**479**	**160**	**Yes/Yes**	**36**	**33**	**Y**	**0**	**0**	**Yes**
HaRxL96	**1245**	**415**	**Yes/Yes**	**27**	**25**	**N**	**3**	**1**	**Yes**
Ha99	**1038**	**346**	**Yes/Yes**	**32**	**31**	**Y**	**2**	**2**	**Yes**
HaRxLL108	**338**	**113**	**Yes/Yes**	**36**	**37**	**Y**	**0**	**0**	**Yes**
HaRxLL120	**944**	**331**	**Yes/Yes**	**31**		**Y**	**1**	**0**	**Yes**
Ha330	**944**	**331**	**Yes/Yes**	**57**	**58**	**Y**	**1**	**0**	**Yes**

Table 2.1. *Summary of candidate effectors in this study.* Gene names are indicated on the left hand side of the table. OFR (bp) indicates the open reading frame in base pairs. The size of the full length including the signal peptide is indicated in amino acids (AA). Gene expression was also verified in *Hpa* and *Ps* using a variety of methods including RT-PCR, microarray experiments, and representation in a cDNA library. Identifiable homologs in *P. sojae (Ps)* and *P. ramorum (Pr)* are indicated by their percent amino acid identity. The presence of a nuclear localization signal (NLS) is indicated by Yes (Y) or No (N) and the numbers of W or Y motifs are also indicated. Nuclear localization was determined by transient assays.

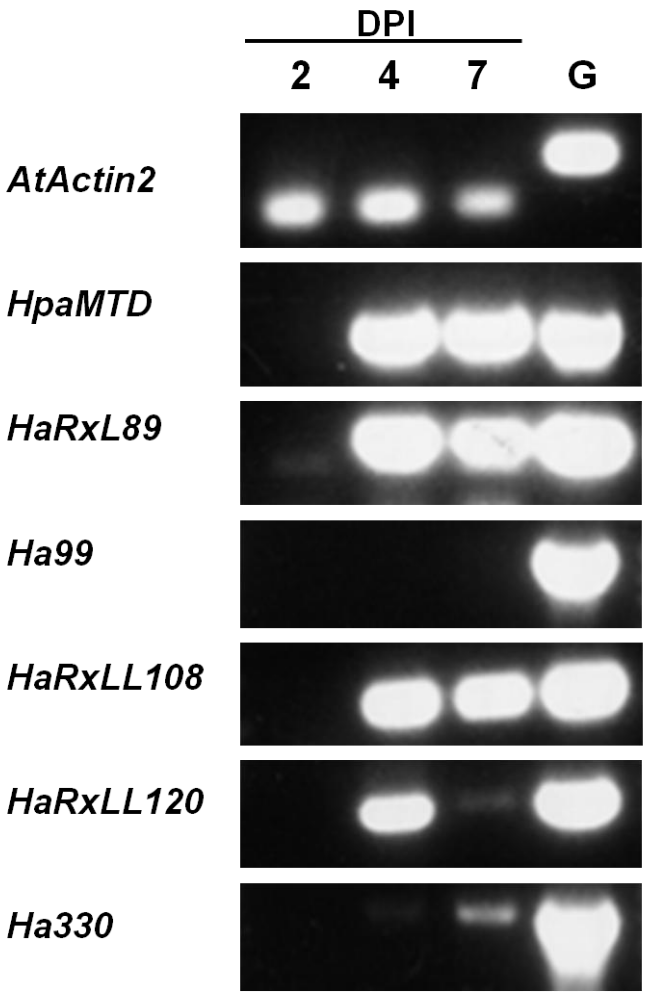

Figure 2.1. *Candidate effectors are differentially expressed during infection.* RT-PCR assays of *Hpa* effector gene expression in susceptible *Arabidopsis* tissue, infected with *Hpa* Emoy2. cDNA was prepared from tissue sampled at 2, 4, and 7 days post inoculation (DPI). Transcripts from the indicated *Hpa* effector genes, *Arabidopsis Actin 2, Hpa Methylenetetrahydrofolate dehydrogenase* (*MTD*) were assayed with gene-specific primers. A positive genomic DNA control (G) is included.

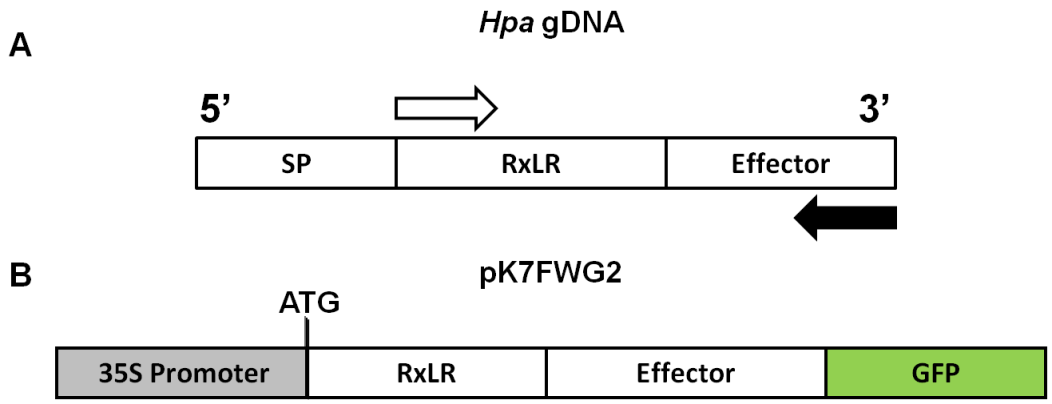

Figure 2.2. *Cloning candidate effector genes*. **A)** Candidate effectors excluding the predicted SP were amplified from genomic DNA. The 5' primer (white arrow) was designed to amplify downstream of the predicted cleavage point. The black arrow indicates 3' primer that includes either a stop codon or no stop codon. **B)** Genes were cloned into a Gateway™ compatible plasmid, pK7FWG2 and fused to GFP (green box) under the control of the 35S promoter (grey box). Green fluorescent protein (GFP), signal peptide (SP) genomic DNA (gDNA).

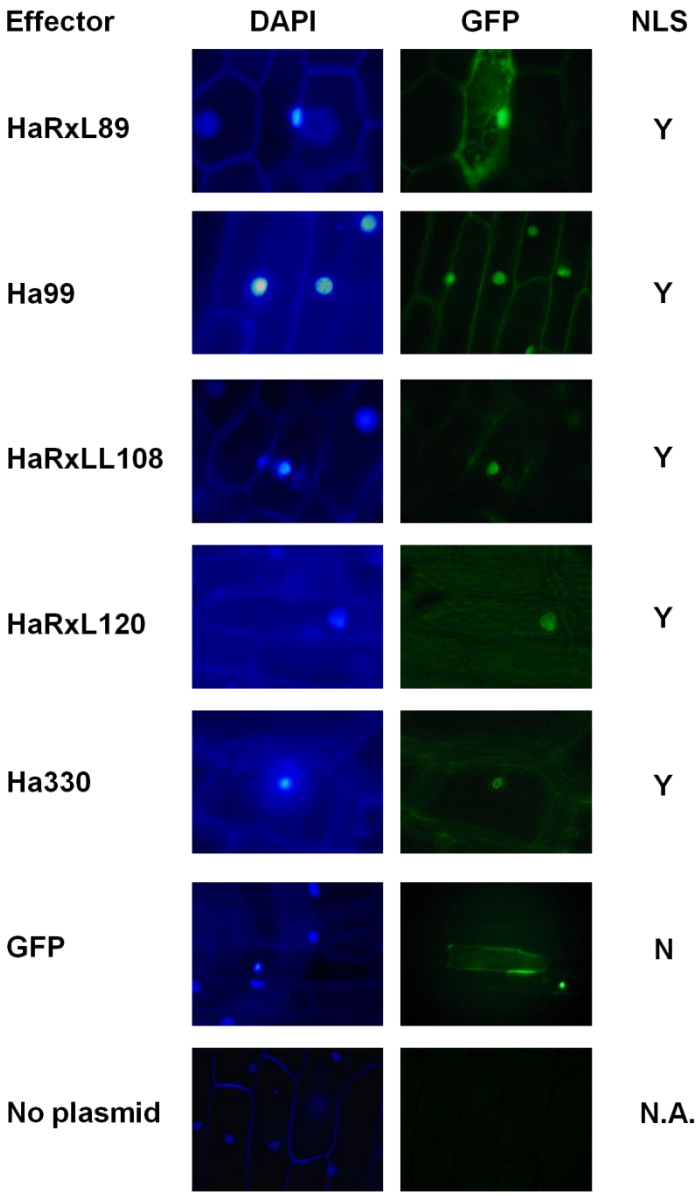

Figure 2.3. *Candidate effectors are localized to the nucleus and cytosol*. The indicated candidate effectors were cloned as C-terminal GFP fusions and transiently expressed in onion epithelial cells via ballistic delivery. 4',6-diamidino-2-phenylindole (DAPI) was used to visualize nuclei. Green fluorescent protein (GFP) panels indicate subcellular localization of the effector protein. The presence of a predicted nuclear localization signal (NLS) is indicated with yes (Y), no, (N) or not applicable (N.A.).

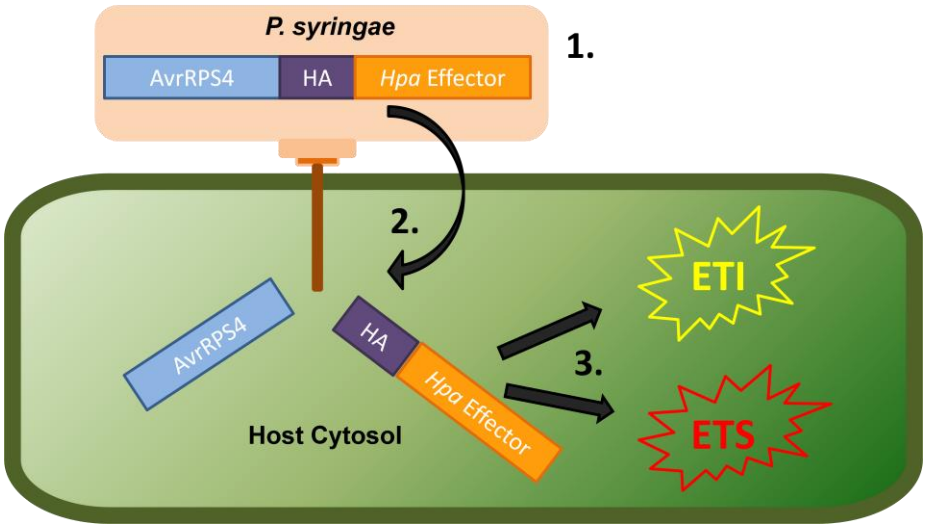

Figure 2.4. *Overview of the EDV system.* **1.** The *Hpa* effector of interest is cloned into pEDV6 by means of Gateway technology and delivered to *P. syringae* by triparental mating. **2.** The transgenic *P. syringae* is infiltrated into an *Arabidopsis* leaf by a needleless syringe. **3.** The AvrRps4 leader directs the *Hpa* effector through the T3SS into the host cytosol and is cleaved by an unknown cellular mechanism (Sohn, Lei et al. 2007). The effector protein targets host proteins to suppress defenses, reprogram host metabolism, etc. Effector triggered susceptibility (ETS). Effector triggered immunity (ETI) can be initiated if the effector is recognized by a host resistance protein.

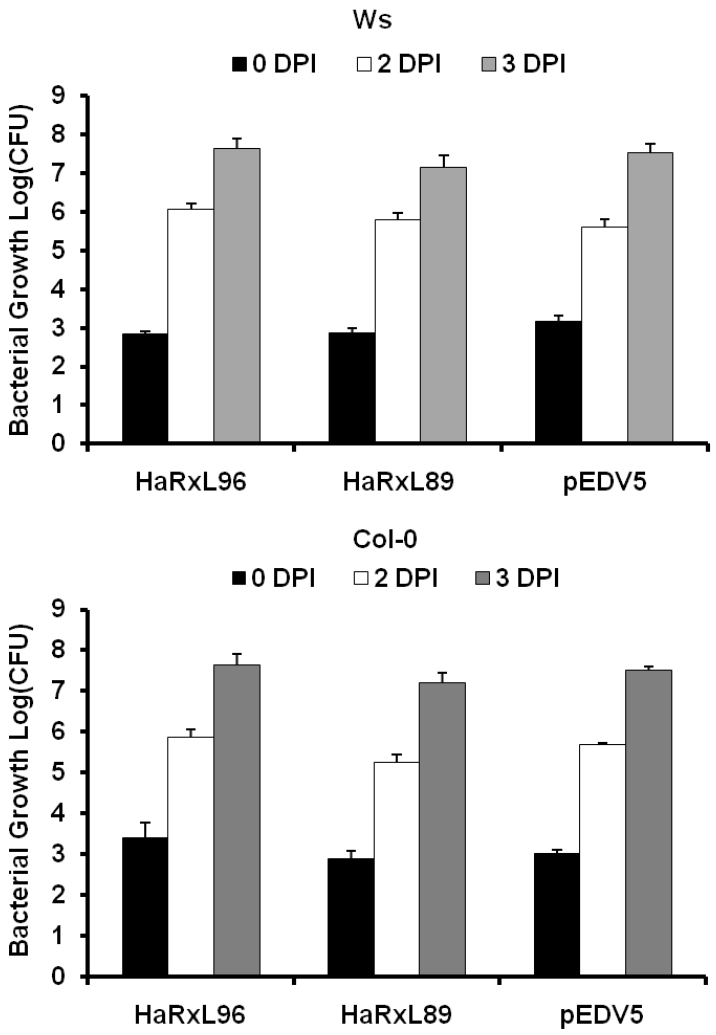

Figure 2.5. *Neither HaRxL96 nor HaRx89 enhance bacterial growth in Arabidopsis***.** Effector genes were cloned into pEDV6 and transformed into *P. syringae* DC3000. Transformed strains harboring the *Hpa* effector *HaRxL96*, *HaRxL89* or the empty vector control pEDV5 were infiltrated into *Arabidopsis* and their growth was subsequently monitored. Days post inoculation (DPI), colony forming units (CFU). Error bars represent standard error.

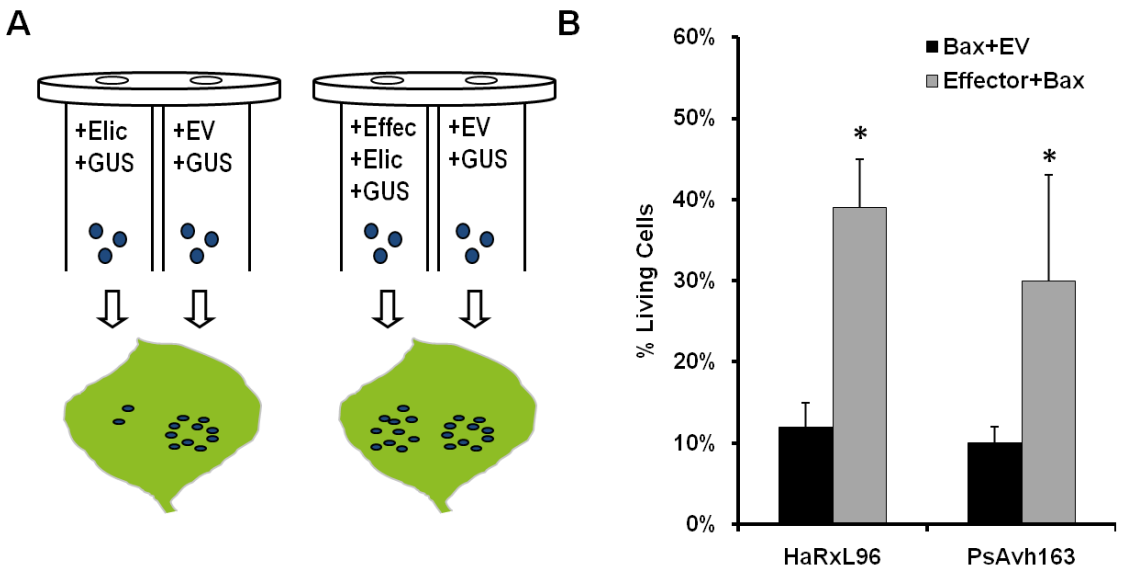

Figure 2.6. *HaRxL96 and PsAvh163 suppresses PCD elicited by Bax*. **A)** Cartoon depicting co-bombardment of experimental and control tungsten particles with the plasmids carrying Gus, Effector, and the elicitor Bax to soybean leaves with the double barrel addition to the gene gun. A double-barreled bombardment device allows for control and experimental samples to be bombarded on the same leaf. **B)** Both HaRxL96 and the *P. sojae* homolog PsAvh163 suppress Bax induced cell death. Cell viability is measured by counting GUS-expressing sectors. Percent living cells were was calculated relative to co-bombarded controls. * designates a statistically significant difference in cell viability in samples that receive HaRxL96 or PsAvh163, compared to an empty vector control ($p<0.05$, Wilcockson Rank Sum test) (Dou and Kale, *et*. al, 2008).

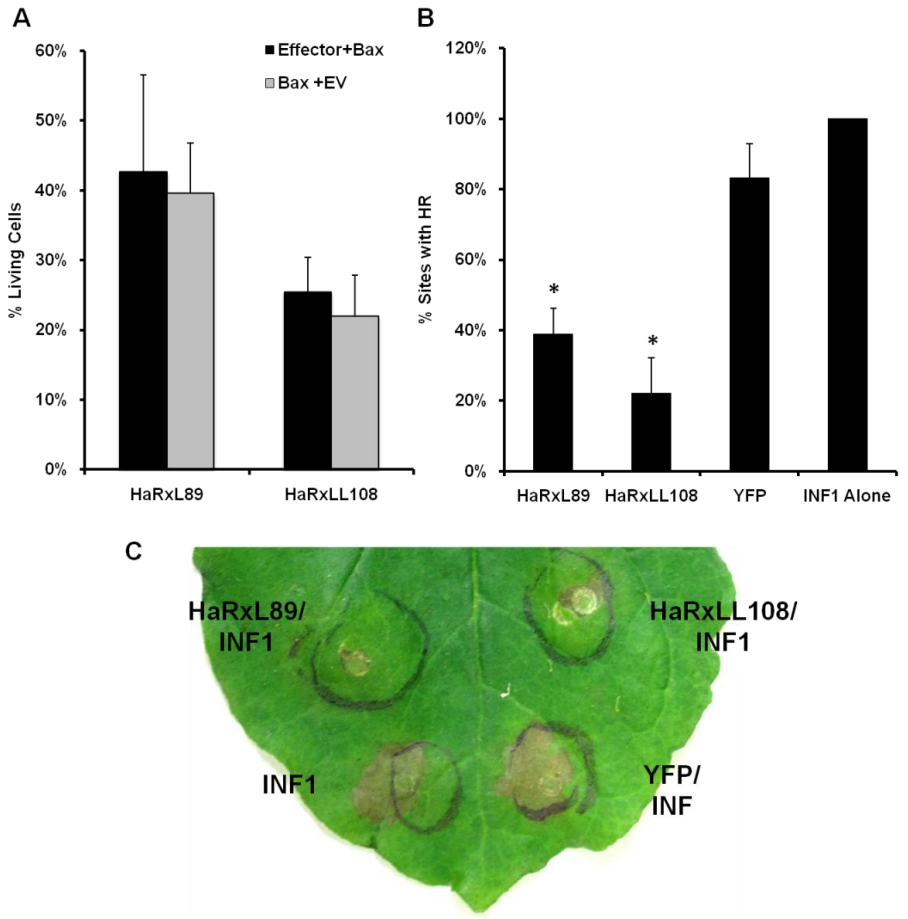

Figure 2.7 *Transient assays to test whether HaRxL89 and HaRxLL108 suppress PCD in soybean or N. benthamiana.* **A)** Co-bombardment of *Bax* and either *HaRxL89* or *HaRxL108* into soybean leaves does not suppress Bax induced cell death, relative to the empty vector (EV) control. **B)** Agrobacterium-mediated transient expression assay (ATTA) of HaRxL89, HaRxLL108, yellow fluorescent protein (YFP), or INF alone. Effectors and the YFP control were infiltrated into *N. benthamiana* leaves, 2 days later the same sites were challenged with INF1 and cell death was monitored visually for six days. Sites with visible cell death were scored as percentage of total sites score. **C)** Visual representation of a *N. benthamiana* leaf from B. * p= <0.05Error bars represent standard error.

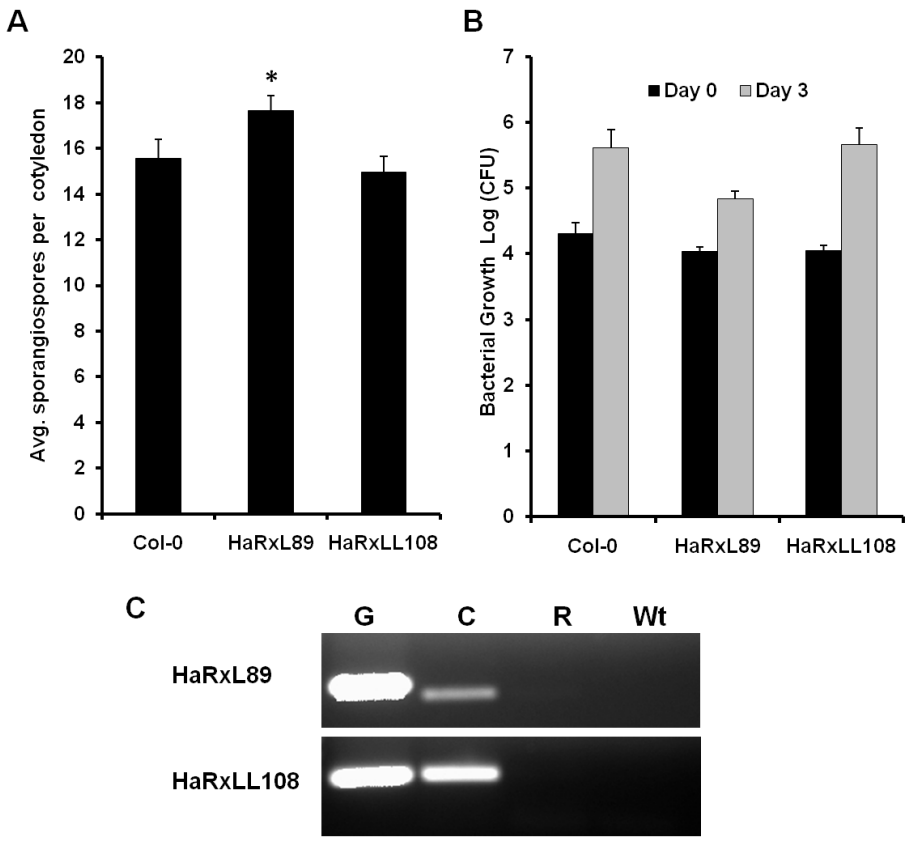

Figure 2.8 *Transgenic Arabidopsis expressing HaRxL89 are more susceptible to virulent Hpa Emco5.* **A)** Transgenic plants expressing either *HaRxL89* or *HaRxLL108* were challenged with virulent *Hpa* Emco5. Disease was quantified as average sporangiophore per cotyledon. **B)** The non-pathogen *P. syringae* DC3000(Δ*Hrc*) was inoculated into four-week-old transgenic plants and pathogen growth was measured at zero and three days post-infection (DPI). *Psy* DC300 growth is expressed as colony forming units (CFU). **C)** Reverse-transcription PCR (RT-PCR) with effector-specific primers and cDNA purified from *Arabidopsis* transformed with HaRxL89 or HaRxLL108, to assay transgene expression. Genomic DNA (G) serves as a positive control, cDNA (C) extracted from transgenic plants, RNA prior to cDNA synthesis (R) demonstrates DNA free RNA extraction. Wild type cDNA (Wt) serves as a non-transgenic control. * p= <0.05 Error bars represent standard error.

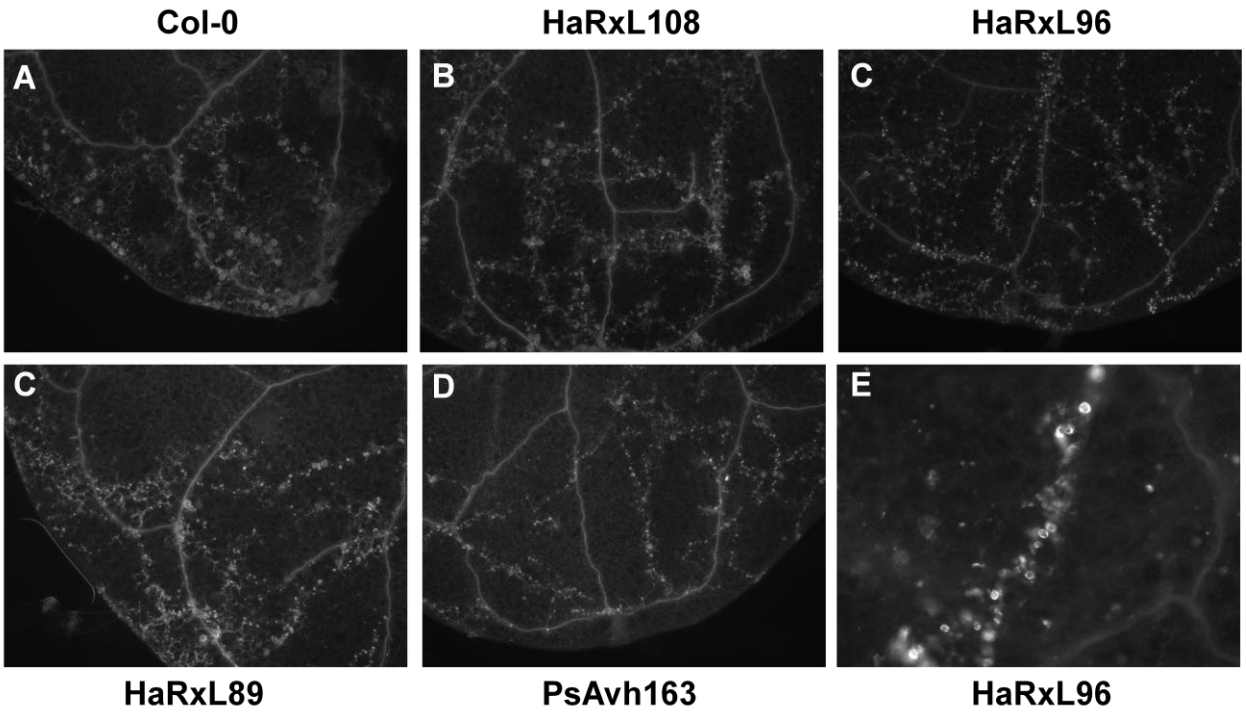

Figure 2.9 *Callose formations during Hpa Emco5 infection is not altered in transgenic plants expressing select Hpa effectors.* Aniline blue stain was used to visualize callose in *Hpa* infected transgenic seedlings. 4X objective in panels A-D show profuse mycelial growth and haustoria-associated structures that fluoresce when stained with aniline blue. 10X objective shows magnified haustoria in panel E and is representative of haustoria in panels A-D.

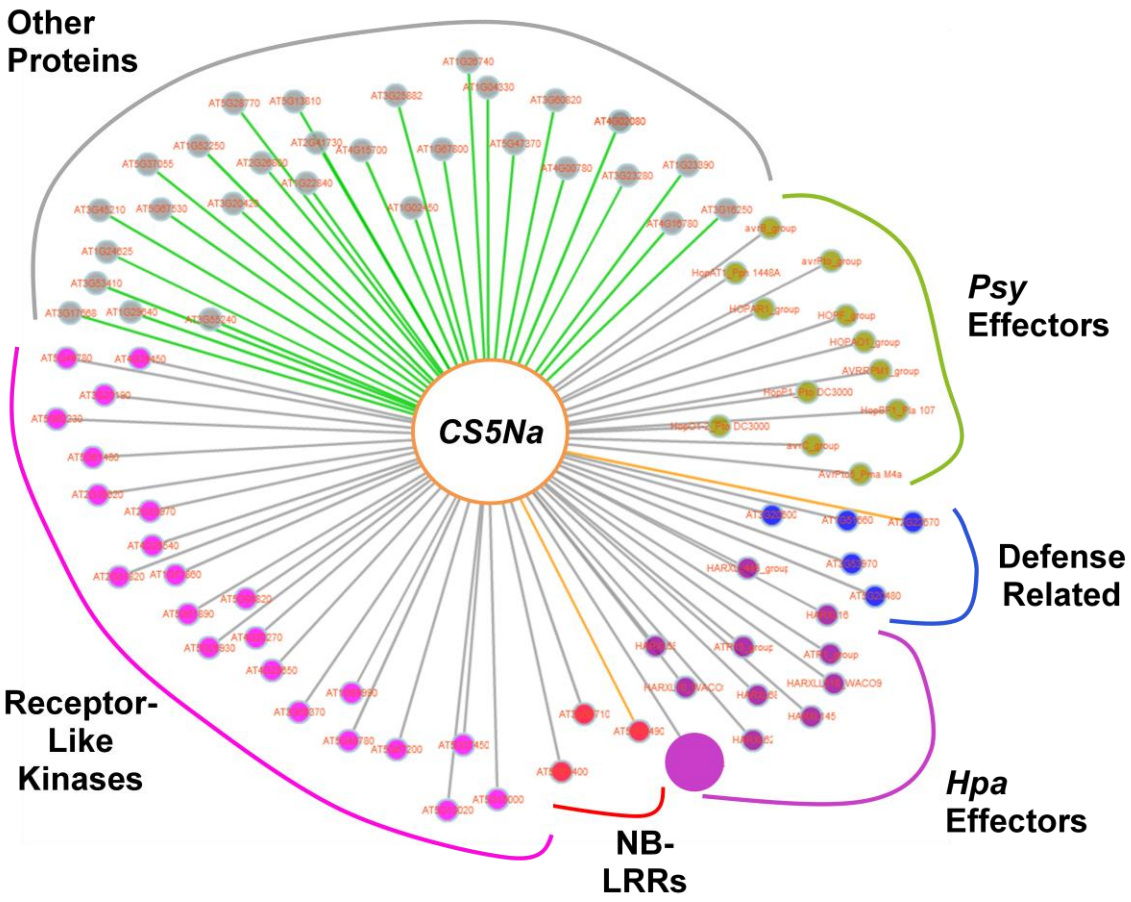

Figure 2.10 *Interaction network for HaRxLL108.* The CS5Na hub is highly connected hub. CS5Na interacts with 11 *Hpa* effectors, 3predicted R proteins, 11 *Psy* effectors interact with CSN5a. HaRxLL108 is depicted as a large purple circle. CS5Na interacting proteins are grouped by their function depicted by coloring. Adapted from Mukhtar et al.

Hpa **Effector**	**PPN1** ***Arabidopsis*** **Target**
HaRxL89	Mitochondrial Glycoprotein (At5g02050), which interacts with another mitochondrial protein that is a high-degree hub (At2g17670)
Ha330	None detected
HaRxLL108	CSN5a, highly connected hub.
HaRxL120	None detected
HaRxL96	None detected

Table 2.2. *Hpa candidate effectors and their host targets.* Hpa effectors and their candidate targets as identified in the PPN1 interactome network as described in Mukhtar et al.

MATERIALS AND METHODS

Construction of expression plasmids

Effector protein-coding sequences were amplified from genomic DNA. For *Hpa* effectors, genomic DNA was isolated from *Arabidopsis* Oy-1 tissue colonized by *Hpa* Emoy2 and used as a template for PCR with primer sets that amplified from the predicted cleaveage point of the effector. For *Ps163*, genomic DNA was isolated from the *P. sojae* isolate P6954 (Race 2) and used as a template for PCR using the primer sets Ps163 NOSP and Ps163 S (with stop codon) or Ps163 NS (without stop codon). The Ps163 Race 2 allele was used for all experiments described in this manuscript. PCR amplicons with and without a stop codon were cloned in pENTR D/TOPO and shuttled into expression plasmids using the LR recombinase (Invitrogen). For *Agrobacterium* mediated transient expression studies, effectors were shuttled from pENTR D/TOPO into pB2GW7. All PCR products and resultant clones were confirmed by sequencing.

Plant growth, maintenance of Hyaloperonospora arabidopsidis, and generation of transgenic Arabidopsis

Arabidopsis, soybean, and *N. benthamiana* plants were grown in Sunshine Mix #1 at 16h light, 8h dark, 22^{o}C. *Arabidopsis* plants for pathogen assays were grown under 8 hours light at 22^{o}C, 16 hours dark at 20^{o}C. The *Hpa* isolates Emoy2 and Emco5 were propagated and maintained respectively on Oy-1 and Ws-0 *Arabidopsis* plants, as described (McDowell, Hoff et al. 2011). Conidial suspensions of 5x10^{4} spores/ml were

applied with a Preval spray unit and the plants were then kept under short day conditions. *Agrobacterium tumifaciens* strain GV3101 was transformed via electroporation and maintained on LB media with Rifampin (100 μg/ml), Gentamicin (25 μg/ml), Spectinomycin (50 μg/ml) at 28c. Transgenic *Arabidopsis* Col-0 were generated by the floral dipping (Clough and Bent 1998). Transgenic plants with resistance to BASTA were selected, presence of the transgene was confirmed by PCR from genomic DNA, and transgene transcription was verified by reverse-transcriptase PCR. Lines with single transgene loci were identified by segregation in the T2 generation and homozygous lines were identified by progeny testing in the T3 generation. All *Arabidopsis* experiments described in this study were performed on non-segregating T3 or T4 populations.

RNA isolation, reverse-transcriptase PCR and real-time PCR

Tissue infected with *Hpa* Emoy2 was harvested at the indicated time points and RNA was extracted with TriSure reagent (Bioline). cDNA synthesis was performed using OmniScript cDNA synthesis kit (Qiagen). 40 PCR cycles were used to amplify effector transcripts from cDNA templates.

Transient assays in soybean

Two-week old detached soybean leaves were transformed using a modified BioRad PDS1000 gene gun (BioRad) as described (Kale and Tyler 2011). Plasmid DNA mixtures were created for the effector, control, and elicitor samples. The effector samples

were prepared as in Dou et al. For Bax suppression assays; 115 ug of the effector, 15 ug of Bax and 50 ug of GUS were combined. The control GUS sample contained 115 ug of empty vector and 50 ug of GUS plasmid DNA. The elicitor samples were mixed as follows. Avr4/6: 30ng of Avr4/6, 50 ug GUS plasmid, 70 ug empty vector. Bax; 15 ug Bax, 50 of Gus, 85 ug empty vector. Tungsten preparations were prepared according to (Dou, Kale et al. 2008). After bombardment, detached soybean leaves were incubated in Petri dishes with moistened Whatman filter paper at 22C/20C (8 hours light, 16 hours dark). Leaves were stained with X-Gluc and cleared with 70% ethanol for two days. GUS-expressing cells were visually quantified with a dissecting microscope. Wilcoxon Rank Sum method was used to assess statistical significance.

Transient assays in N. benthamiana

Agrobacterium tumifaciens GV3101 strains were grown overnight in LB with the appropriate antibiotics. Liquid cultures were harvested by centrifugation. Pellets were resuspended to the desired OD_{600} in MMA buffer (10 mM MgCl, 10 mM MES, 200 mM Acetosyringone), incubated at room temperature for 1-3 hours, and infiltrated using needless syringes on the abaxial side of 3 to 5 week old leaves**.** *Agrobacterium* harboring effectors were infiltrated into *N. benthamiana* at OD_{600} 0.5. INF was infiltrated 2 days later at an OD_{600} 0.1. Cell death was visually quantified 7 days later.

Assays for in planta growth of Pseudomonas syringae

An overnight culture of the pathogen *Psy* DC3000(*ΔHrcC)* or *Psy* DC3000 harboring EDV plasmids were grown for 20 hours at 37˚C at 200 RPM, and the culture was centrifuged at 1500×g for 10 minutes at 4˚C. The bacterial pellet was resuspended in 10 mM magnesium sulfate buffer to an OD_{600} of 0.1. The culture was then diluted to give a final concentration of 0.01. Four-week-old transgenic plants or wild-type Col-0 plants were infiltrated with the appropriate strains. Leaf disks with a diameter of 8 mm were obtained from six leaves of each plant line using a hole puncher. The leaf disks were soaked in 70% ethanol and then dried. Each leaf disk was added to 200 µl of 10 mM magnesium sulfate buffer. The tissue-buffer mixture was ground until a homogenous mixture appeared. Serial dilutions were performed for these mixtures. The diluted and undiluted cultures were plated and were placed in the 28˚C incubator until visible colonies were present (Kim & Mackey, 2008).

Subceullular localization of effector proteins

Plasmids harboring effector genes with N-terminal fusions to GFP were mini-prepped. The plasmids were used in tungsten preparations according the manufactures protocol (BioRad) and delivered to onion epithelial peels on ½ MS plates with 1100 PSI rupture disk. The bombarded plates were incubated 48 hours in a growth chamber and imaged with an inverted Zeiss Axiovert 200 with the DAPI and FITC filter sets. Images were acquired with a Cannon Powershot A620.

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Chapter 3

Homologous RXLR effectors from *Hyaloperonospora arabidopsidis* and *Phytophthora sojae* suppress immunity in distantly related plants

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Contributions: Megan S.Casady contributed to INF1 suppression assays, Rachel A.Fee contribute to the generation of transgenic plants, Devdutta Deb contributed RNA and cDNA for quantitative PCR. Brett M. Tyler contributed bioinformatic analysis and data, John M. McDowell incepted the project, is the primary advisor, contributed to manuscript preparation, editing, and gene alignments.

Keywords: effector, oomycete, resistance, pathogenesis, Arabidopsis

Abbreviations, effector triggered immunity (ETI), effector-triggered susceptibility (ETS), host-targeting signal (HTS), hypersensitive response (HR), INF1-induced cell death (ICD), leucine rich repeat (LRR), mitogen activated protein kinsae (MAPK), microbe-associated molecular patterns (MAMP), mildew A (*MLA*), nuclear localization signal (NLS) nonexpressor of PR genes 1 (NPR1), pattern triggered immunity (PTI), programmed cell death (PCD), reactive oxygen species (ROS) signal peptide (SP), Tobacco Rattle Virus (TRV), type III secretion system (TTSS), virus-induced gene silencing (VIGS)

ABSTRACT

Diverse pathogens secrete effector proteins into plant cells to manipulate host cellular processes. The genome of *Hyaloperonospora arabidopsidis* (*Hpa*, downy mildew of *Arabidopsis*) contains at least 134 candidate RXLR effector genes. Only a small subset of these is conserved in related oomycetes. Here, we describe a comparative functional characterization of the *Hpa* RXLR effector *HaRxL96* and a homologous gene, *PsAvh163,* from the soybean pathogen *Phytophthora sojae*. *HaRxL96* and *PsAvh163* are induced during early stages of infection and carry a functional RXLR motif that is sufficient for protein uptake into plant cells. Both effectors can suppress or activate immune responses in soybean, *Nicotiana*, and *Arabidopsis*. Several SA-responsive defense genes are suppressed in Arabidopsis Col:*HaRxL96* and Col:*PsAvh163* during an incompatible interaction with *Hpa* Emoy2. Together, these experiments demonstrate that evolutionarily conserved effectors from different oomycete species can suppress immunity in plant species that are divergent from the source pathogen's host.

INTRODUCTION

Plant pathogens have evolved from diverse kingdoms of life and collectively pose a perennial threat to agriculture. Some of the most destructive pathogens are oomycetes, which include over 800 species of downy mildew pathogens in 17 genera (*Peronosporaceae*), along with over 100 species in the *Phytophthora* genus. *Hyaloperonospora arabidopsidis* (*Hpa*) is a naturally occurring downy mildew pathogen of *Arabidopsis thaliana* (Koch & Slusarenko, 1990). *Hpa* and other downy mildews are obligate biotrophs, which obtain nutrients exclusively from living plant cells and cannot be cultured apart from their hosts (Coates & Beynon, 2010). Contrastingly, *Phytophthora* species employ a hemi-biotrophic lifestyle, in which an initial phase of biotrophic growth is followed by a transition to necrotrophy, in which host tissue is destroyed (Judelson & Blanco, 2005). Oomycete diseases are difficult to control because of the pathogens' high evolutionary potential to nullify plant immunity and resist fungicides (Tyler, 2007).

Successful pathogens must evade or suppress multiple layers of innate immune surveillance. The first level is comprised of extracellular receptors that recognize microbe-associated molecular patterns (MAMPs) and activate defenses responses that include production of reactive oxygen species (ROS), induction of defense genes, and deposition of papillae containing callose at sites of infection (Jones & Dangl, 2006). This suite of responses comprises pattern-triggered immunity (PTI) (Katagiri & Tsuda, 2010) The second level is comprised of resistance (R) proteins that directly or indirectly recognize pathogen effector proteins inside the host cell. This recognition triggers a rapid and robust immune response that often includes programmed cell death (PCD), termed

the hypersensitive response (HR) (Dodds & Rathjen, 2010). This suite of responses, which shares considerable overlap with PTI, is termed effector-triggered immunity (ETI) (Chisholm et al., 2006). Together, PTI and ETI enable most plants to resist most pathogens.

Many plant pathogens utilize effector proteins to subvert plant immunity and promote disease. Bacterial species secrete approximately 20-40 effectors to the interior of plant cells through a Type III secretion system (TTSS) (Grant et al., 2006). Bacterial effectors have been studied in detail, and have been shown to suppress PTI and/or ETI through a variety of mechanisms (Hann et al., 2010). In contrast, understanding of effector functions from oomycetes and fungi is in its infancy.

Bioinformatic analyses of sequenced genomes indicate that oomycete pathogens maintain large collections of effector genes, which fall into several distinct families based on sequence motifs (Kamoun, 2006, Stassen & Van den Ackerveken, 2011). The most extensively studied oomycete effector family is defined by an N-terminal signal peptide that directs the effector for secretion to the outside of the pathogen, followed by RXLR and EER motifs that are required for targeting to the interior of host cells (Rehmany et al., 2005, Whisson et al., 2007). Uptake of RXLR effectors into host cells likely involves binding to host membrane phospholipids, followed by endocytosis (Kale et al., 2010). Almost every oomycete avirulence protein cloned to date falls within the RXLR family. *Phytophthora* species contain large families of predicted RXLR proteins, ranging in size from 370 to over 550 (Haas et al., 2009, Tyler et al., 2006). Functional surveys of 169 *P.*

sojae candidate RXRL effectors, using *Agrobacterium*-mediated transient expression in *N. benthamiana*, indicated that the majority can suppress immune responses triggered by diverse elicitors (Wang et al., 2011).

Only a handful of RXLR effectors have been characterized in detail. For example, the *Phytophthora sojae* effector Avr1b increases the virulence of *P. sojae* when overexpressed and suppresses cell death, triggered by multiple elicitors, in soybean and *N. benthamiana* (Dou et al., 2008*)*. The virulence and avirulence functions of *Avr1b* are dependent on so-called W, Y, and L motifs that are also found in a large proportion of RXLR effectors (Jiang et al., 2008). Mutations in these motifs abolished its ability to be recognized by the cognate R gene (*Rps1b*) and abolished the ability to suppress Bax-induced cell death (Dou et al., 2008).

The genome of *Hpa* isolate Emoy2 was recently sequenced (Baxter et al., 2010). Bioinformatic surveys revealed at least 134 candidate RXLR proteins, including the avirulence genes *Arabidopsis thaliana Recognized 1* (*ATR1*) and *ATR13* (Rehmany et al., 2003, Allen et al., 2004). At least 42 *Hpa* effectors are confirmed to be expressed during host colonization (Cabral et al., 2011). Very little conservation exists between pools of effector genes maintained by oomycete pathogens (Baxter et al., 2010), furthermore effector repertoires may differ between *Hpa* isolates, (Cabral et al., 2011). These large differences between collections of effector genes may reflect the host selection pressure imposed on these pathogens as they co-evolve with their host (Raffaele et al., 2010). Only 30% of the *Hpa* effectors have greater than 20% identity with their best

Phytophthora match and a smaller subset of 5% share greater than 40% identity with their best match. This small subset of effectors may represent core pathogenicity genes that target cellular processes conserved between diverse plant species. Thus, we are focusing on functional analysis of a subset of relatively conserved effectors.

Here, we describe a functional comparison of a pair of computationally predicted, homologous effectors: *HaRxL96* from *Hpa*, and its homolog *PsAvh163* from the soybean pathogen *Phytophthora sojae*. These genes are expressed early in infection and have functional RXLR motifs, confirming that they are *bona fide* effectors. Both effectors suppress or induce plant immunity in diverse plant species, suggestive of interaction with conserved host target(s).

RESULTS

HaRxL96 and PsAvh163 share conserved functional domains

HaRxL96 and *PsAvh163* encode predicted proteins of 415 and 504 amino acids, respectively, that share 26% identity and 43% similarity with each other. Both proteins contain a predicted signal peptide at the N-terminus, followed by a predicted host-targeting sequence (HTS) consisting of an RXLR-like motif and a short stretch of acidic amino acids (**Figure 3.1; Supplemental Figure 3.1**). Finally, both effectors contain multiple copies of degenerate W, Y, and L motifs (Dou et al., 2008). No other functional

domains or subcellular localization signals were apparent in the predicted sequences of these effectors.

To further examine the conservation of these genes, we searched for homologs within and outside the *Phytophthora* genus. Genomes of *P. infestans* and *P. ramorum* contain homologs in which the SP, HTS, and W, Y, and L motifs are apparent at conserved positions (e.g. PITG_10341 from *P. infestans*, **Figure 3.1B; Supplemental Figure 3.1**). No homologs were evident in the genomes of *Pythium irregulare*, *Saprolegnia parasitica*, or Albugo indicating that the *HaRxL96*/*PsAvh163* lineage evolved after the divergence of the *Peronosporales* (i.e., the common ancestor of *Phytophthora* and genera containing downy mildew pathogens (Thines & Kamoun, 2010)).

HaRxL96 is conserved but PsAvh163 is under diversifying selection

RXLR effectors with known avirulence functions often display a high degree of allelic divergence (Allen et al., 2004). We amplified and sequenced alleles of *HaRxL96* from five *Hpa* isolates (Emoy2, Cala2, Emco5, Hiks1, Noco2). Alleles of *PsAvh163* were obtained from genome sequences of four *P. sojae* races Races 2, 7, 17,and 19 that comprise a survey of genetic diversity of *P. sojae* (Wang et al., 2011). The *HaRxL96* nucleotide sequences were identical in all of the *Hpa* isolates. Contrastingly, the *PsAvh163* alleles exhibit a high degree of allelic diversity (**Supplemental Figure 3.2**). The alleles from Race 2 and Race 4 are identical, while the nucleotide sequences from

Race 7 and Race 17 differ from the Race 2/4 reference sequence by 75 and 43 non-synonymous substitutions and only 2 and 3 synonymous substitutions, respectively. The amino acid substitutions are unevenly distributed over the length of the protein. For example, only one substitution occurs in the N-terminal 60 amino acids that span the signal peptide and RXLR-EER regions. Contrastingly, at least one substitution occurs at 7 of the 18 positions in the first W domain. The dN/dS ratios over the entire length of the Race 7 and Race 17 allele, compared to the Race 2 allele, are 6.4 and 3.8, indicative of diversifying selection ($p<1.0E-08$, 6.6E-04, respectively). This diversity contrasts strongly with the absence of variability among *HaRxL96* alleles.

HaRxL96 and PsAvh163 are induced during infection

Effector genes are often induced during early stages of infection. We monitored *HaRxL96* transcript levels *in planta* following infection of susceptible *Arabidopsis* ecotype Oystese (Oy-0) by virulent *Hpa* isolate Emoy2 using quantitative PCR. *HaRxL96* is upregulated during the first 12 hours after inoculation. Its expression declines during subsequent stages of the interaction (**Figure 3.2**). Wang et al. described a similar pattern of induction for *PsAvh163* during the interaction between *P. sojae* and soybean, and termed this pattern "immediate early, low". (Wang et al., 2011). Thus, both effectors display similar patterns of expression suggestive of a function during the initial stages of infection.

HaRxL96 and PsAvh163 contain functional host targeting motifs

To further validate that *HaRxL96* and *PsAvh163* encode functional effector proteins, we tested whether their predicted host targeting sequences (HTS) could deliver a chimeric fusion to the interior of soybean cells, using a quantitative bombardment assay that employs a double-barreled gene gun (Kale & Tyler, 2011). We constructed chimeric genes encoding proteins with the signal peptide from soybean Pathogenesis-Related 1a protein, followed by the predicted HTS of HaRxL96 or PsAvh163 and the avirulence domain of *P. sojae* Avr1b (**Supplemental Figure 3.3A**). We co-bombarded soybean leaves with each chimeric construct, along with a plasmid containing the Beta-glucuronidase (*Gus*) gene to measure cell viability (**Supplemental Figure 3.3B, 3.3C**). Bombardments with either HaRxL96HT-Avr1b or PsAvh163HT-Avr1b trigger a strong HR response in *Rps1b* soybean but not in *rps1b* soybean (**Figure 3.3**). Furthermore, mutating the RXLR motif to AAAA abolished cell entry of HaRxL96HT-Avr1bCTD and PsAvh163HT-Avr1bCTD fusions. These results suggest that the HTSs of both HaRxL96 and PsAvh163 can mediate uptake by plant cells, further validating that the *HaRxL96* and *PsAvh163* genes encode functional effector proteins.

PsAvh163 triggers a hypersensitive response in N. benthamiana

To test for potential avirulence activity of both effectors, we used transient assays to express each gene inside plant cells, and then screened for a macroscopic cell death response. For screens of *Arabidopsis* ecotypes, we used *Pseudomonas syringae*

DC3000(Δ*CEL*) to deliver HaRxL96 or PsAvh163(Race 2) via the Type III secretion system, as described in (Sohn et al., 2007). DC3000*(ΔCEL)* was transformed with "effector detector vector" expression constructs in which the leader from *P. syringae* AvrRps4 is fused to HaRxL96 or PsAvh163, beginning from the predicted signal peptide cleavage site. The AvrRPS4 leader directs the fusion through the T3SS and is then removed by an endogenous plant protease (Sohn et al., 2007). The processed effector could then induce an HR response if it is recognized by the plant. The Δ*CEL* mutant was chosen because it produces minimal disease symptoms that could be mistaken for a weak HR. We screened 48 *Arabidopsis* ecotypes for a macroscopic HR in response to *P. syringae* with pEDV-Ha96 or pEDV-PsAvh163, injected at a concentration of OD600 0.01. We did not observe a robust HR in any of the ecotypes (**Supplemental Figure 3.4**). Similarly, transient assays in soybean using particle bombardment indicated that neither HaRxL96 nor PsAvh163 (Race 2) triggered HR in the soybean cultivars L-77 and Willams-82.

In addition, we screened *Nicotiana benthamiana* for an HR response using *Agrobacterium tumefaciens* mediated transient expression (ATTA). *HaRxL96* did not trigger cell death, but the *PsAvh163* alleles from Race 2, Race 7, and Race 17 triggered a rapid cell death response within 24 hours (**Figure 3.4A**). Our observations are consistent with those of Wang et al (Wang et al., 2011). The three alleles also triggered strong cell death in *Nicotiana tabaccum* (**Figure 3.4B**).

To learn more about the nature of the cell death triggered by PsAvh163 in *N. benthamiana*, we used virus-induced gene silencing (VIGS) with the Tobacco Rattle Virus (TRV) system to knock down expression of genes that have been previously associated with *R* gene signaling. PsAvh163-dependent cell death was abolished in plants that were silenced for Hsp90.1 and SGT1 but not Hsp90.2, Hsp90.3 or RAR1 (**Figure 3.4C**). The requirement for Hsp90.1 and SGT1 suggests that PsAvh163-induced cell death is a defense response resulting from a recognition event (e.g., effector-triggered immunity), rather than non-specific cell toxicity.

HaRxL96 suppresses programmed cell death in N. benthamiana

Effectors from bacterial and oomycete pathogens are often able to suppress programmed cell death (PCD) triggered by avirulence proteins, cell death regulators, and chemical treatments (Alfano et al., 2009, Wang et al., 2011, Cabral et al., 2011). Suppression of PCD is a potentially important virulence mechanism for biotrophic pathogens that extract nutrients from living plant cells. Previous examinations of oomycete effector function have utilized an assay for suppression of cell death in *N. benthamiana* triggered by the elicitor Infestans 1 (INF1) from *Phytophthora infestans* (Bos et al., 2006). We transiently expressed *HaRxL96* using ATTA, and then delivered INF1 using ATTA 48 hours later. We then scored infiltration sites for macroscopic cell death 5 days later. These experiments indicate that HaRxL96 can suppress INF1-induced cell death (**Supplemental Figure 3.5**). We then tested whether HaRxL96 can suppress the cell death triggered by PsAvh163, using ATTA as described above. HaRxL96 also

suppressed this cell death (**Supplemental Figure 3.5**), indicative of a general capacity to suppress PCD in *N. benthamiana*.

HaRxL96 and PsAvh163 suppress programmed cell death in soybean

Previously, we demonstrated that HaRxL96 and PsAvh163 can suppress programmed cell death in soybean, triggered by mouse Bax in a transient assay (Dou et al., 2008). To further investigate the PCD suppression capacity of HaRxL96 and PsAvh163 in soybean, we tested for suppression of cell death triggered by the avirulence protein Avr4/6 from *P. sojae*. Avr4/6 triggers ETI in soybean in the presence of the cognate R gene *RPS4* or *RPS6* (Dou et al., 2010). The double barreled particle bombardment assay was employed to test whether cell death was reduced in soybean co-bombarded with a mixture that contains HaRxL96 or PsAvh163, *Avr4/6*, and *Gus*, compared to a mixture with equimolar amounts of empty vector, *Avr4/6* and *Gus*. We observed a 50% decrease of *Gus*-expressing cells in tissue bombarded with Avr4/6 + EV, relative to control samples bombarded with Gus but not Avr4/6, similar to reports by (Dou et al., 2010). When *HaRxL96* or *PsAvh163* was included with *Avr4/6* we observed a significant increase in cell viability after bombardment, indicating that HaRxL96 and PsAvh163 suppress Avr4/6 cell death (**Figure 3.5**). This suggests that PsAvh163 and HaRxL96 share the ability to suppress ETI in soybean.

HaRxL96 and PsAvh163 suppress effector-triggered immunity and basal resistance against H. arabidopsidis in Arabidopsis

Stably transformed plants that express pathogen effector genes have provided important insights into the functions of bacterial effectors (Nomura et al., 2006, Munkvold & Martin, 2009). We generated stably transformed *Arabidopsis* Col-0 lines that express either *HaRxL96* or *PsAvh163* under the control of the CaMV 35S promoter. We bred multiple, independent lines to homozygosity and determined transgene mRNA abundance with quantitative, real-time PCR (**Supplementary Figure 3.6**). As expected, independent lines exhibited a range of transcript abundance. We then tested lines expressing each transgene at different levels for alterations in their responses to adapted and non-adapted pathogens.

Our first set of experiments tested whether Col expressing *HaRxL96* or *PsAvh163* exhibited altered resistance against avirulent *Hpa* isolate Emoy2. Resistance to Emoy2 in Col-0 is provided by RPP4, a Toll/interleukin-1 receptor, nucleotide binding, leucine-rich repeat protein (TIR-NB-LRR) (van der Biezen et al., 2002). We tested multiple transgenic lines expressing each effector for reduced resistance to *Hpa* Emoy2, as measured by counting sporangiophores at seven days after inoculation. This assay indicated that seedlings expressing either *HaRxL96* or *PsAvh163* are more susceptible to Emoy2, compared to wild type plants. The degree of suppression varied between independent lines and was roughly proportional to the abundance of transcript from the transgene. We also tested transgenic seedlings for increased susceptibility to the virulent

Hpa isolate Emco5. *HaRxL96* and *PsAvh163* transgenic plants supported increased pathogen growth, compared to wild-type, as measured by sporangiophore counts. These results indicate that both HaRxL96 and PsAvh163 can suppress ETI and basal resistance against *Hpa*.

HaRxL96 and PsAvh163 transgenes suppress callose deposition induced by non-pathogenic P. syringae in Arabidopsis

One well-studied component of the *Arabidopsis* PTI response is formation of callose at the site of infection (Luna et al., 2011). Non-pathogenic strains of *P. syringae* trigger abundant callose deposition that is easily quantified, while virulent *P. syringe* secretes effectors that interfere with pattern-triggered immunity and thereby suppress callose deposition. We tested whether HaRxL96 and PsAvh163 can suppress the callose response to *Pseudomonas syringe* DC3000(Δ*H*rcC), which cannot form a functional type three secretion system and is thus unable to secrete its natural complement of effectors. Using a needless syringe, plants were infiltrated $1x10^7$ CFU/mL of *P. syringe DC3000* (Δ*HrcC)* into transgenic and control lines, and stained with analine blue at 16 hours post inoculation to visualize callose formation. Col:*HaRxL96* and Col:*PsAvh163* transgenic plants had an attenuated response, exhibiting up to 50% less callose production compared to wild type plants treated with the Δ*HRC* mutant (**Figure 3.7**). These results taken together suggest that HaRxL96 and PsAvh163 interfere with *Arabidopsis* PTI deployed against *P. syringae*.

HaRxL96 and PsAvh163 transgenes suppress induction of defense genes in response to avirulent Hpa.

To further study the molecular basis for suppression of immune responses in Col:HaRxL96 and Col:PsAvh163, we used quantitative real time PCR (qPCR) to measure transcript abundance of four defense marker genes that were previously shown to be upregulated during RPP4 resistance to *Hpa*: *Arabidopsis thaliana mitogen activated protein kinsae 3* (*AtMPK3*), *Pathogenesis-Related 1* (*PR1*), *Accelerated cell death 6* (*ACD6*), and *Wall associated kinase 1* (*WAK1*) (Eulgem et al., 2007). A previous study demonstrated that these genes are transiently suppressed in a compatible interaction between *Hpa* and *Arabidopsis*, suggesting that they are direct or indirect targets of *Hpa* effectors (Eulgem et al., 2007). Both HaRxL96 and PsAvh163 suppressed RPP4-mediated defense gene induction in response to the incompatible isolate Emoy2. In some cases as with ACD6 and MPK3, suppression was greater than the compatible interaction between Oy-1 and *Hpa* Emoy2 (**Figure 3.8**). These results suggest that *HaRxL96* and *PsAvh163* interfere with activation of defense genes, either directly or indirectly.

DISCUSSION

It is increasingly clear that RXLR effector proteins play a major role in the outcome of plant-oomycete interactions. Every oomycete avirulence gene cloned to date belongs to the RXLR superfamily (Stassen & Van den Ackerveken, 2011), and a recent high-throughput survey of RXLR proteins indicated a substantial capacity to suppress

plant immune responses (Wang et al., 2011). Thus, if we are to fully understand how oomycete pathogens cause disease, it is essential to understand the collective functions of the RXLR secretome in detail. We are focusing on a small group of predicted RXLR genes that are conserved between *Hpa* and pathogens in the *Phytophthora* genus. Analysis of RXLR candidate genes in the *Hpa* genome revealed that the majority have no recognizable homologs in *Phytophthora* genomes, suggesting that oomycetes are under strong selective pressure to invent or re-invent their RXLR weaponry. When considered in this context, it is plausible that the conserved RXLR genes might play particularly important, "core" roles in pathogenicity.

This rationale is supported by studies of conserved effectors in other species, for example the bacterial genes in the conserved effector locus or the LysM effector, Ecp6, in fungi (Collmer et al., 2000, de Jonge et al., 2010). Ecp6 from the tomato pathogen C*ladosporium fulvum* provides a particularly relevant example of insights to be gained from studies of conserved effectors. *Ecp6* contains *LysM* motifs that sequester chitin oligosaccharides produced as a byproduct of host invasion (de Jonge et al., 2010). By sequestering chitin, the pathogen conceals an important elicitor of microbe-associated molecular pattern (MAMP) triggered immunity. Homologs of *Ecp6* exist in multi-gene families in divergent fungi and functional equivalents such as *C. fulvum* Avr4 exists, demonstrating the conserved nature of this virulence mechanism (Thomma & de Jonge, 2009, de Wit et al., 2006). These effectors represent an important virulence strategy conserved in diverse fungal pathogens that encounter similar obstacles during host colonization.

Another rationale for focusing on conserved effectors is that comparative studies of homologous effectors across species can provide insight into effector gene evolution. Finally, conserved effectors can be utilized as probes in "effector-directed" screens for new resistance genes that could be introduced by breeding or transgenic means (e.g. Avrblb2/Rpi-blb2, (Oh et al., 2009, Vleeshouwers et al., 2010)).

HaRxL96 and *PsAvh163* are one of several pairs of homologous effectors from *Hpa* and *P. sojae* under investigation in our group. Although the proteins are not highly conserved, signature motifs in the important functional domains are apparent. Interestingly, HaRxL96 and PsAvh163 appear to be evolving under very different modes in *Hpa* and *P. sojae*. The lack of divergence among *HaRxL96* alleles implies a conservative mode, or perhaps a recent selective sweep, while *PsAvh163* is one of the most divergent RXLR effector loci in *P. sojae* (Wang et al., 2011). The divergence of *PsAvh163* may reflect selection pressure to avoid a recently evolved resistance gene. In this regard, it is intriguing that PsAvh163 induces an HR-like response in *N. benthamiana*. This response requires components that have also been associated with ETI (SGT1 and HSP90). The requirements for SGT1 and Hsp90.1 is similar to the *Solanum lycopersicum* pest resistance gene *Mi-1* (Kaloshian et al., 2007). Incomplete silencing may have led to a false negative result with the other members of Hsp90 family or RAR1, thus it is possible they too are required for PsAvh163 cell death. These attributes suggest that *N. benthamiana* contains a resistance gene that recognizes multiple alleles of *PsAvh163*, perhaps by guarding their virulence target or a decoy thereof. The cell death induced in *Nicotiana* by PsAvh163 will be very useful for future studies of

these effectors' structure-function and interaction with host targets, which can exploit ATTA and other tools in *Nicotiana* (Goodin et al., 2008).

An experimental challenge, relevant to any effector predicted from bioinformatic criteria, is to confirm that the predicted effector is a *bona fide* effector. To this end, we confirmed that both genes are induced *in planta*, with an "immediate-early, low" pattern that suggests a role in preparation of host tissue for colonization. For further validation of effector function, we confirmed that HTS from both proteins is necessary and sufficient for translocation of the Avr1b avirulence domain into soybean cells. The ability of the HaRxL96 HTS to enter soybean cells is not surprising, given previous demonstrations that RXLR-mediated entry is host non-specific. In sum, both proteins are capable of entering plant cells and are transcribed in a pattern consistent with a role in early infection, suggesting that both genes encode *bona fide* effectors.

Additionally, we demonstrated with multiple assays that the proteins can suppress or induce plant immunity in diverse species. Our approach in these experiments was to express each effector individually inside plant cells. This approach has provided important insights into the function of effectors from bacteria and oomycetes (Munkvold & Martin, 2009, Cabral et al., 2011). We used a combination of transient assays and stably transformed plants to determine whether these genes function in plant species that are divergent from the host of the source pathogen. Our experiments indicate that both effectors can function in plant species that are divergent from those of the source pathogen's host. For example, the HaRxL96 effector can suppress programmed cell

death triggered by oomycete elicitors in soybean, and PsAvh163 can suppress PTI and ETI responses in *Arabidopsis*. The abilities of HaRxL96 and PsAvh163 to suppress basal immunity in *Arabidopsis* are similar to ATR1, ATR13 and HaRxL29 (Cabral et al., 2011, Sohn et al., 2007). In the case of *Arabidopsis* plants expressing the HaRxL29 transgene, the effector-mediated reduction of callose during a bacterial challenge is similar to HaRx96 and PsAvh163 transgenes with an approximant 50% reduction in callose. Suppression of cell wall defenses by oomycete effector may represent an important virulence strategy for *Hpa*.

A caveat to these studies is the use of a strong constitutive promoter to drive transgene expression. This may result in neomorph in which the observed phenotypes are artifacts of overexpression rather than a biologically relevant effect. However, expression levels in our transgenic plants are low. Moreover, no documented cases of over expressing an effector gene have given misleading virulence functions (Hauck et al., 2003, de Torres et al., 2006).

The ability of both effectors to suppress cell death triggered by a variety of elicitors, in diverse plant species, suggests that HaRxL96 and PsAvh163 may be targeting conserved processes that mediate early responses to PCD-inducing stimuli. Given their conservation, it is conceivable that HaRxL96 and PsAvh163 are targeting the same or similar proteins in their respective hosts. To challenge this hypothesis, we are currently attempting to identify the respective targets of HaRxL96 and PsAvh163 in *Arabidopsis* and soybean. The ETI triggered by PsAvh163 may provide a new source of useful

resistance to target conserved effectors of oomycete pathogens. We attempted ATTA transient expression of *PsAvh163* in other plant species including two cultivars of tomato and failed to provoke a cell death response; thus. PsAvh163 HR appears to be species specific (Data not shown). Future studies may include a larger screen with PsAvh163 to identify novel resistances that could be applicable to soybean breeding programs.

Neither effector has a strong effect on plant immunity, partial but significant effects were observed in many of the functional assays. In comparison, effectors such as Avr3a possess a strong ability to suppress INF1 induced cell death or the *Psy* effector AvrPtoB's ability to suppress cell death elicited by Bax and several R proteins (Abramovitch et al., 2003, Bos et al., 2006). This may be part of a strategy where by oomycetes secrete large collections of effectors that have small quantitative effects on plant immunity that results, *in toto*, immune suppression and disease. This may allow for flexibility in the pools of effectors in which detrimental effector genes may be disposed of with limited impact to the oomycete effector toolbox. This comparative approach of two homolog effector proteins provides insight into effector evolution and leverages complementary experimental tools of two pathosystems revealing functional conservation of two homologous effectors from divergent oomycetes.

FIGURES

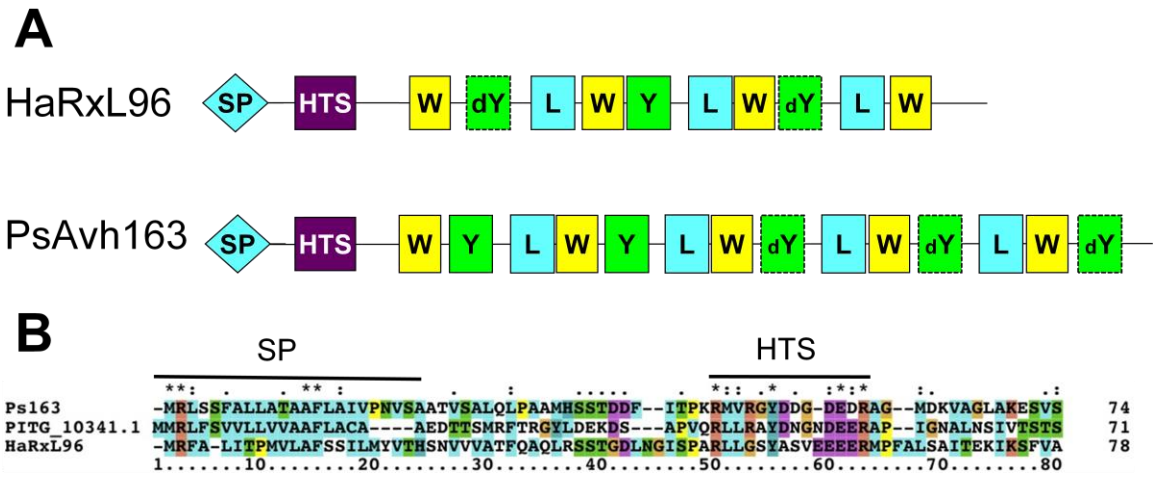


Figure 3.1. *HaRxL96 and PsAvh163 share conserved functional domains***.** (a) Schematic of HaRxL96 and PsAvh163 proteins in which functional domains are highlighted: Signal peptide (SP), RXLR and EER host targeting motifs (HTS), and W, Y, L motifs. (b) N-terminal sequences encompassing the signal peptide and HTS of HaRxL96, PsAvh163, and the homologous gene from *Phytophthora infestans* (PITG10341.1).

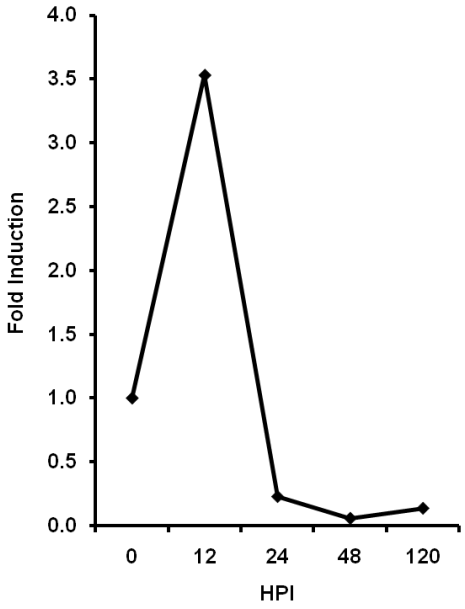


Figure 3.2. *HaRxL96 is induced during early stages of infection by Hpa.* Expression of HaRxL96 was determined by quantitative real-time PCR, using cDNA derived from Arabidopsis Oy-1 seedlings inoculated with virulent *Hpa* Emoy2 and harvested at the indicated time points. The X-axis depicts transcript abundance of *HaRxL96* relative to HpaActin, normalized to expression at T=0.

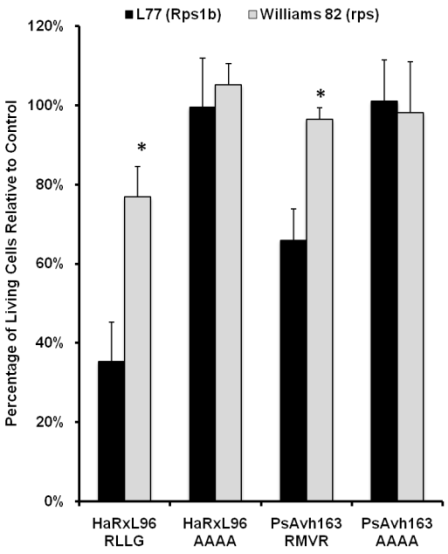


Figure 3.3. *HaRxL96 and PsAvh163 contain functional host targeting signals (HTS).* This assay employs particle bombardment of chimeric genes consisting of a signal peptide from soybean PR-1a, followed by the HTS of HaRxL96 or PsAvh163, fused to the C-terminal avirulence domain of Avr1b. Proteins expressed from these transgenes are secreted via the plant endoplasmic reticulum to the apoplast. Then, the effector is translocated back inside host cells if the HTS is functional. This can be visually assayed through activation of Avr1b-dependent hypersensitive response, using co-bombarded 35S-Gus as a marker for soybean cell viability. Fusions were transiently expressed in soybean genotypes *Rps1b* or *rps1b*. A double-barreled bombardment device allows for control and experimental samples to be bombarded on the same leaf. Percent living cells were was calculated relative to co-bombarded controls. * The reduction in *Gus* expressing cells between *Rps1b* and *rps1b* is statistically significant ($p<0.05$, using the Wilcockson Rank Sum test). Replicated three times with similar results.

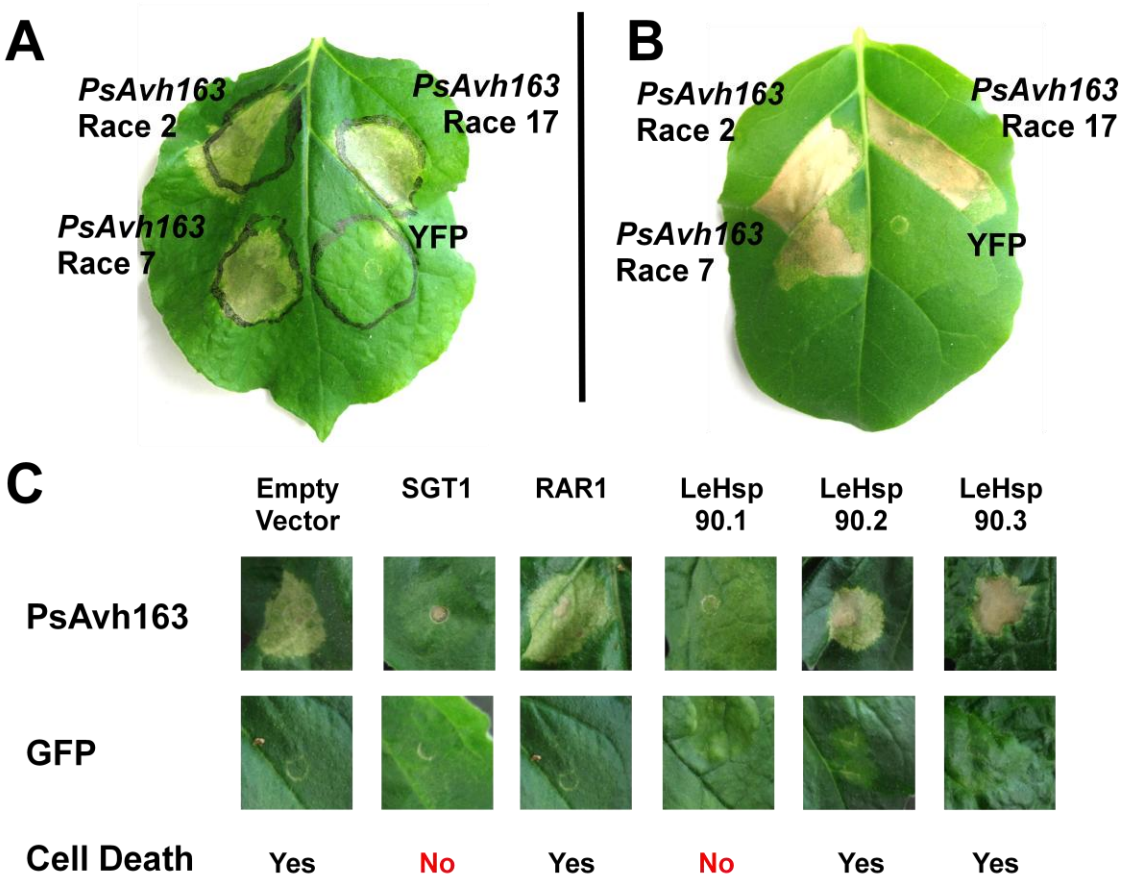


Figure 3.4. *PsAvh163 elicits an allele-dependent cell death response in Nicotiana spp.* that requires SGT1 and Hsp90.2. *A. tumefaciens* GV3101 containing alleles of *PsAvh163* from *P. sojae* Race 2, Race7 or Race 17 were transiently expressed in (a) *N. benthamiana or (b) N. tabacum,* (variety Turk). Cell death is evident after 24 hours in response to PsAvh163. No cell death was observed in response to the YFP control. (c) Virus induced gene silencing was used to reduce expression of the indicated genes. *A. tumifaciens* containing *PsAvh163* (Race 2) and controls were infiltrated two weeks after initiation of silencing and cell death was scored 7 days later. Replicated at least three times with similar results.

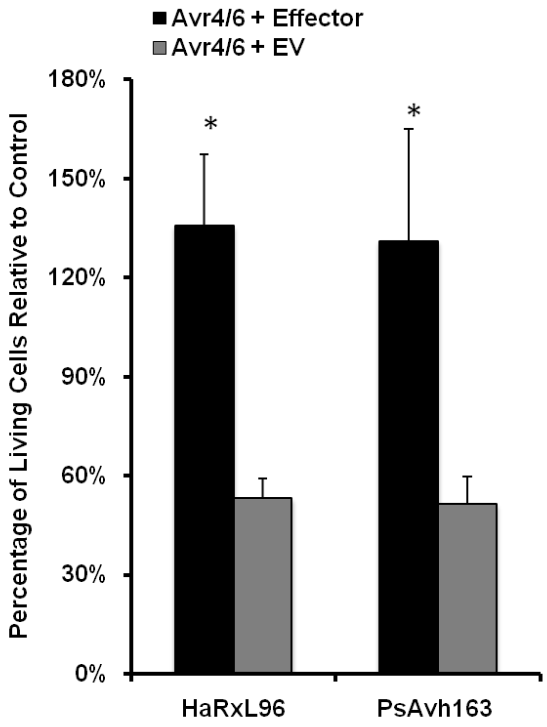


Figure 3.5. *HaRxL96 and PsAvh163 suppress cell death triggered by Avr4/6 in soybean.* A plant expression vector containing CaMv35S-Avr4/6 is co-bombarded with vectors expressing the effector (or control empty vector, EV) onto soybean expressing the RPS4 resistance gene. Note that the signal peptide is not included in the effector constructs, thus, they are localized inside plant cells. A *Gus* construct is also included to measure cell viability. Following bombardment, cell viability is measured by counting GUS-expressing sectors. A double-barreled bombardment device allows for control and experimental samples to be bombarded on the same leaf. Percent living cells were was calculated relative to co-bombarded controls. * designates a statistically significant difference in cell viability in samples that receive HaRxL96 or PsAvh163, compared to an empty vector control ($p<0.05$, Wilcockson Rank Sum test). Replicated four times with similar results.

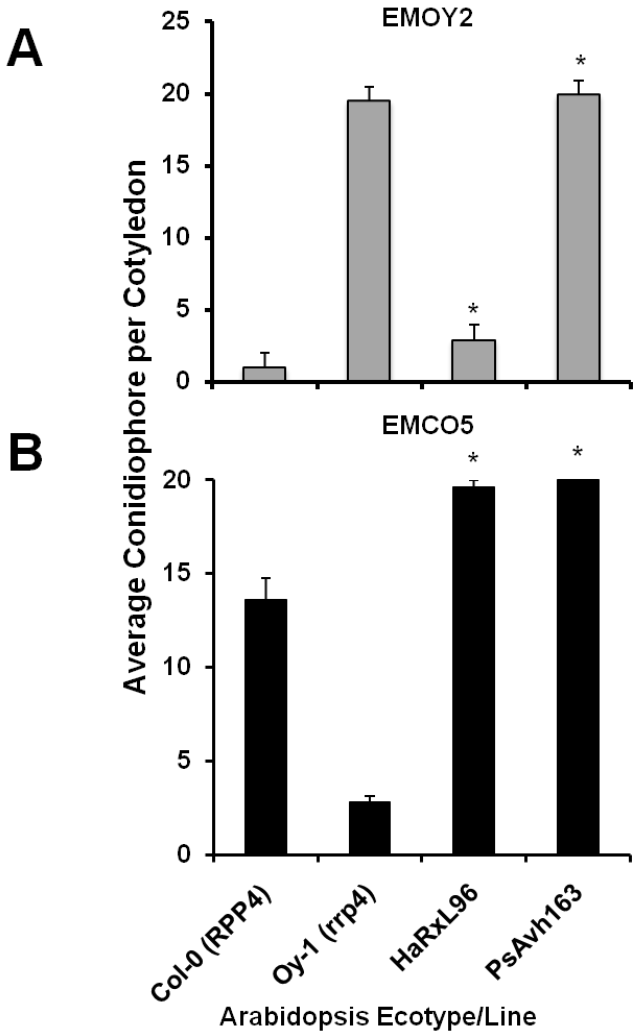


Figure 3.6. *HaRxL96 and PsAvh163 partially suppress RPP4 resistance to avirluent Hpa (Emoy2) and enhance susceptibility to virulent Hpa (Emco5).* 10-12 day old *Arabidopsis* Col-0 seedlings constitutively expressing either HaRxL96 or PsAvh163 were challenged with 5 X 10^4 spores/ml of (a) Emoy2 (b) or Emco5. Pathogen reproduction was quantified 7 days post inoculation as conidiophores per cotyledon. Wild type Col is resistant to Emoy2 because of the *RPP4* NB-LRR gene. Col seedlings are susceptible to Emco5. Oy-1 is susceptible to Emoy2 and resistant to Emco5. * $p < 0.05$ Replicated three times with similar results.

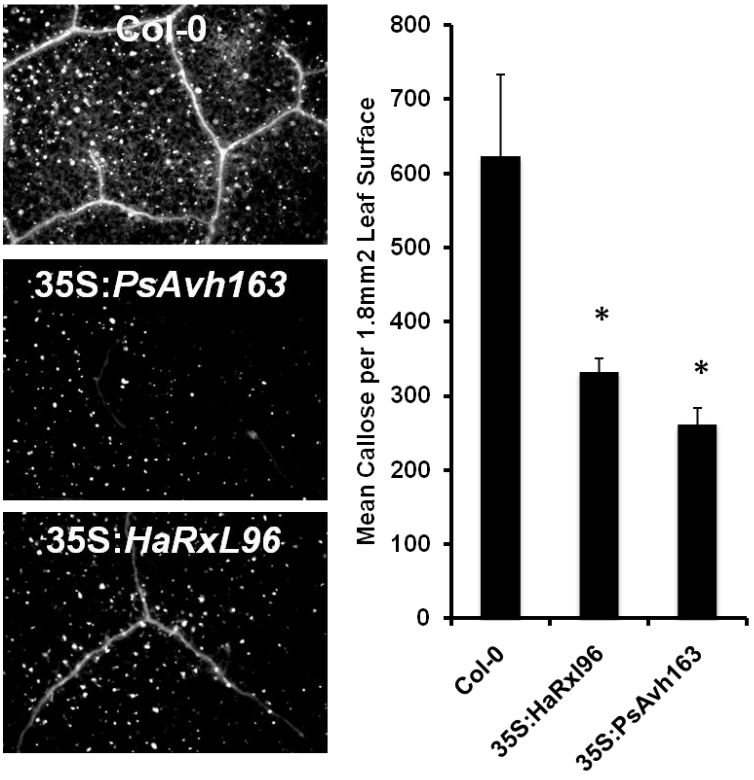


Figure 3.7. *HaRxL96 and PsAvh163 partially suppress callose formation triggered by the non-pathogenic mutant Pseudomonas syringae DC3000(ΔHrc).* Transgenic plants constitutively expressing either *HaRxL96* or *PsAvh163* were infiltrated with *P. syringae.* Callose was visualized by staining with aniline blue and quantified with QuantityOne (BioRad) (* $p < 0.05$, Student's T-test). Replicated three times with similar results.

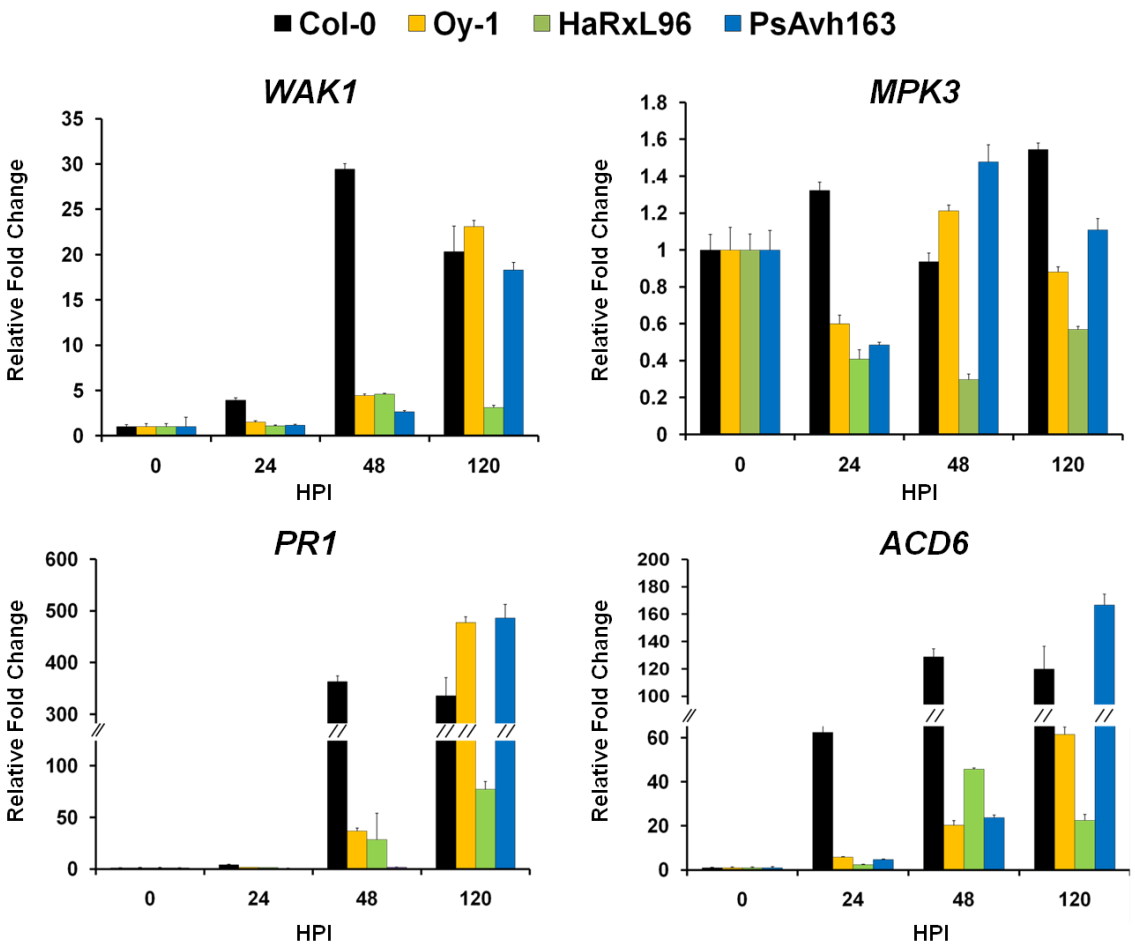


Figure 3.8. *HaRxL96 and PsAvh163 suppress defense gene induction in response to avirulent Hpa Emoy 2.* 10-12 day-old Col-0 plants constitutively expressing *HaRxL96* or *PsAvh163* were challenged with 5 X 10^4 spores/ml Emoy 2 and tissue was collected at 0, 12, 24, 48, and 120 hours post inoculation. Transcript abundance of the indicated gene was assayed by quantitative RT-PCR and normalized to AtActin. Fold change in reference to 0 HPI. Error bars represent SE. Replicated four times with similar results.

MATERIALS AND METHODS

Construction of expression plasmids

HaRxL96 and *PsAvh163* protein-coding sequences were amplified from genomic DNA. For *HaRxL96*, genomic DNA was isolated from Arabidopsis Oy-1 tissue colonized by *Hpa* Emoy2. This DNA was used as a template for PCR using the primer sets Ha96 NOSP and Ha96 S (with stop codon) or Ha96 NS (without stop codon). The *HaRxL96* allele from *Hpa* Emoy2 was used for all experiments described in this manuscript. For *PsAvh163*, genomic DNA was isolated from the *P. sojae* isolate P6954 (Race 2) and used as a template for PCR using the primer sets Ps163 NOSP and Ps163 S (with stop codon) or Ps163 NS (without stop codon). The PsAvh163 Race 2 allele was used for all experiments described in this manuscript except as depicted in Figure 4. Alleles of PsAvh163 were cloned from genomic DNA extracted from P7064 (race7), P7074 (race 17), or P7076 (race 19). For all cloned ORFs, the 5' primer began with the codon immediately downstream of the signal peptide cleavage site predicted by SignalP. PCR amplicons with and without a stop codon were cloned in pENTR D/TOPO and shuttled into expression plasmids using the LR recombinase (Invitrogen). For *Agrobacterium* mediated transient expression studies, *HaRxL96* and *PsAvh163* Race2, Race7, and Race17 were shuttled from pENTR D/TOPO into pB2GW7. For host targeting analysis, clones were created by overlapping PCR extension. The soybean PR1a signal peptide was cloned with Pr1a F and Pr1a R from soybean genomic DNA. The Avr1b-1 C-Terminal domain was cloned from genomic DNA with the primer set Avr1b CTD F and Avr1b CTD R. The RXLR host targeting region included sequence from the predicted

signal peptide cleavage site to 60bp beyond the last codon in the predicted –EER motif. The host targeting region was amplified from HaRxL96 using the primer set Ha96 OPE F and Ha96 OPE R and for Ps163 the primer set Ps163 OPE F and Ps163 OPE R was used. Three PCR products, including the Pr1A signal peptide, effector host targeting region and the Avr1B CTD were used as a template to generate a fusion product using the Pr1A F and Avr1b CTD primers. The fusion products were digested and subsequently ligated in Xma1 and Kpn1 sites of a modified puc19 plasmid with a 35S promoter (Dou et al., 2008). All PCR products and resultant clones were confirmed by sequencing.

Plant growth, maintenance of Hyaloperonospora arabidopsidis, and generation of transgenic Arabidopsis

Arabidopsis, soybean, and *N. benthamiana* plants were grown in Sunshine Mix #1 at 16h light, 8h dark, 22^{o}C. *Arabidopsis* plants for pathogen assays were grown under 8 hours light at 22^{o}C, 16 hours dark at 20^{o}C. The *Hpa* isolates Emoy2 and Emco5 were propagated and maintained respectively on Oy-1 and Ws-0 *Arabidopsis* plants, as described (McDowell et al., 2011). Conidial suspensions of 5x10^{4} spores/ml were applied with a Preval spray unit and the plants were then kept under short day conditions. *Agrobacterium tumifaciens* strain GV3101 was transformed via electroporation and maintained on LB media with Rifampin (100 μg/ml), Gentamicin (25 μg/ml), Spectinomycin (50 μg/ml) at 28c. Transgenic *Arabidopsis* Col-0 were generated by the floral dipping (Clough & Bent, 1998). Transgenic plants with resistance to BASTA were selected, the presence of the transgene was confirmed by genomic DNA PCR, and

transgene transcripts were verified by reverse-transcriptase PCR. Lines with single transgene loci were identified by segregation in the T2 generation and homozygous lines were identified by progeny testing in the T3 generation. All *Arabidopsis* experiments described in this study were performed on non-segregating T3 or T4 populations.

RNA isolation, reverse-transcriptase PCR and real-time PCR

Tissue infected with *Hpa* Emoy2 was harvested at the indicated time points and RNA was extracted with TriSure reagent (Bioline). cDNA synthesis was performed using OmniScript cDNA synthesis kit (Qiagen). 40 PCR cycles were used to amplify effector targets from cDNA templates. For real-time PCR, 25uL samples were prepared by mixing 1uL of cDNA template with 12.5uL of Sybr Green Mastermix with the appropriate primers and water. Real-time PCR reactions were preformed on an ABI 7300 device and fold change was calculated relative to 0 DPI time point.

Transient assays in soybean

Two week old detached soybean leaves were transformed using a modified BioRad PDS1000 gene gun (BioRad) as described (Kale & Tyler, 2011). Plasmid DNA mixtures were created for the effector, control, and elicitor samples. The effector samples were prepared as in Dou et al (Dou et al., 2008). 115 ug of the effector, 50 ug of Avr4/6 and 50 ug of GUS were combined for Avr4/6 suppression assays. For Bax suppression assays; 115 ug of the effector, 15 ug of Bax and 50 ug of GUS were combined. The

control GUS sample contained 115 ug of empty vector and 50 ug of GUS plasmid DNA. The elicitor samples were mixed as follows. Avr4/6: 30ng of Avr4/6, 50 ug GUS plasmid, 70 ug empty vector. Bax; 15 ug Bax, 50 of Gus, 85 ug empty vector. Tungsten preparations were prepared according to (Dou et al., 2008). After bombardment, detached soybean leaves were incubated in Petri dishes with moistened Whatman filter paper at 22c/20c (8 hours light, 16 hours dark). Leaves were stained with X-Gluc and cleared with 70% ethanol for two days. GUS-expressing cells were visually quantified with a dissecting microscope. Wilcoxon Rank Sum method was used to assess statistical significance.

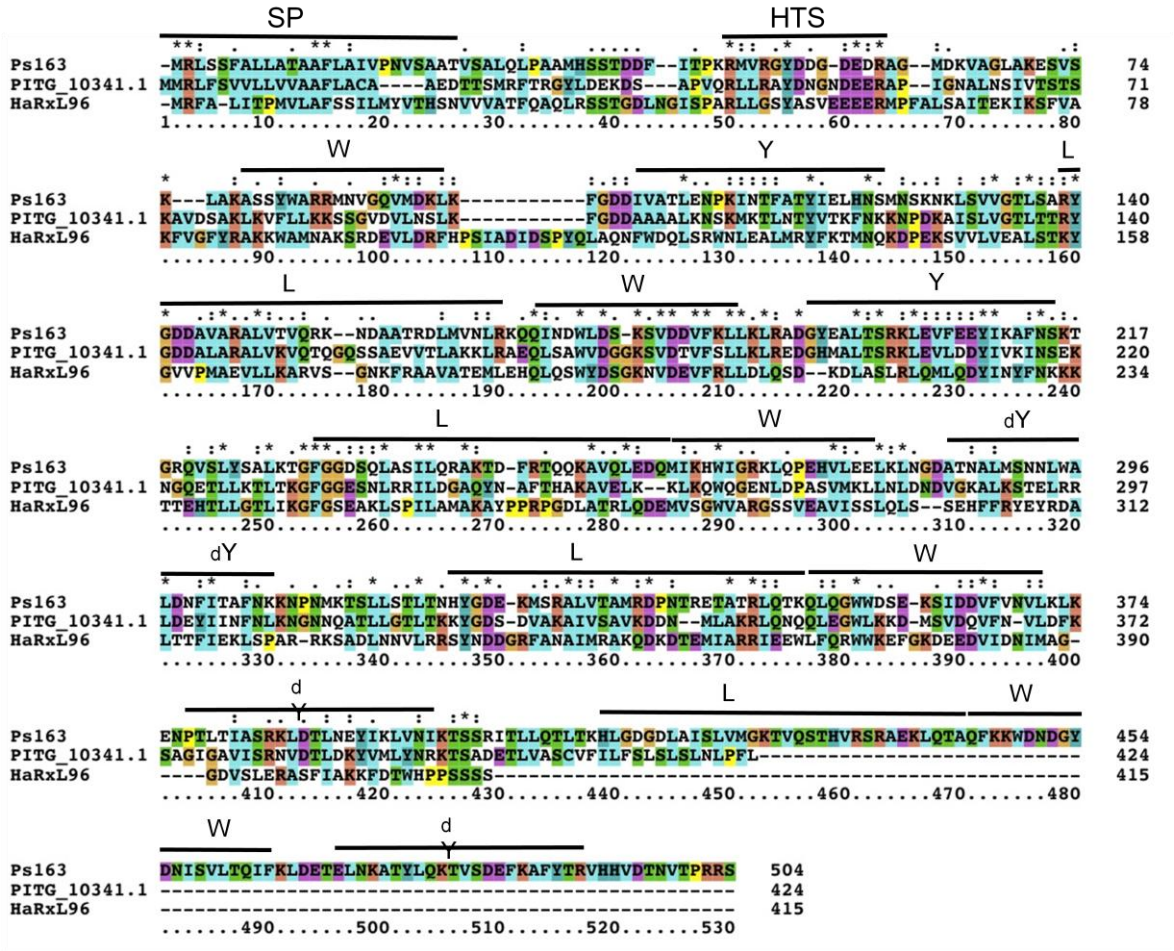
Transient assays and VIGS in N. benthamiana

Agrobacterium tumifaciens GV3101 strains with the appropriate constructs were grown overnight in LB with the appropriate antibiotics. Liquid cultures were harvested by centrifugation. Pellets were resuspended to the desired OD_{600} in MMA buffer (10 mM MgCl, 10 mM MES, 200 mM Acetosyringone), incubated at room temperature for 1-3 hours, and infiltrated using needless syringes on the abaxial side of 3 to 5 week old leaves. VIGS was preformed as described (Liu et al., 2004)**.** Plants that were silenced for the target genes were challenged in the upper leaves with *A. tumifaciens* GV3101 containing PsAvh163 Race 2, two weeks after the initial inoculation with the VIGS constructs. Cell death was visually quantified 5 days later.

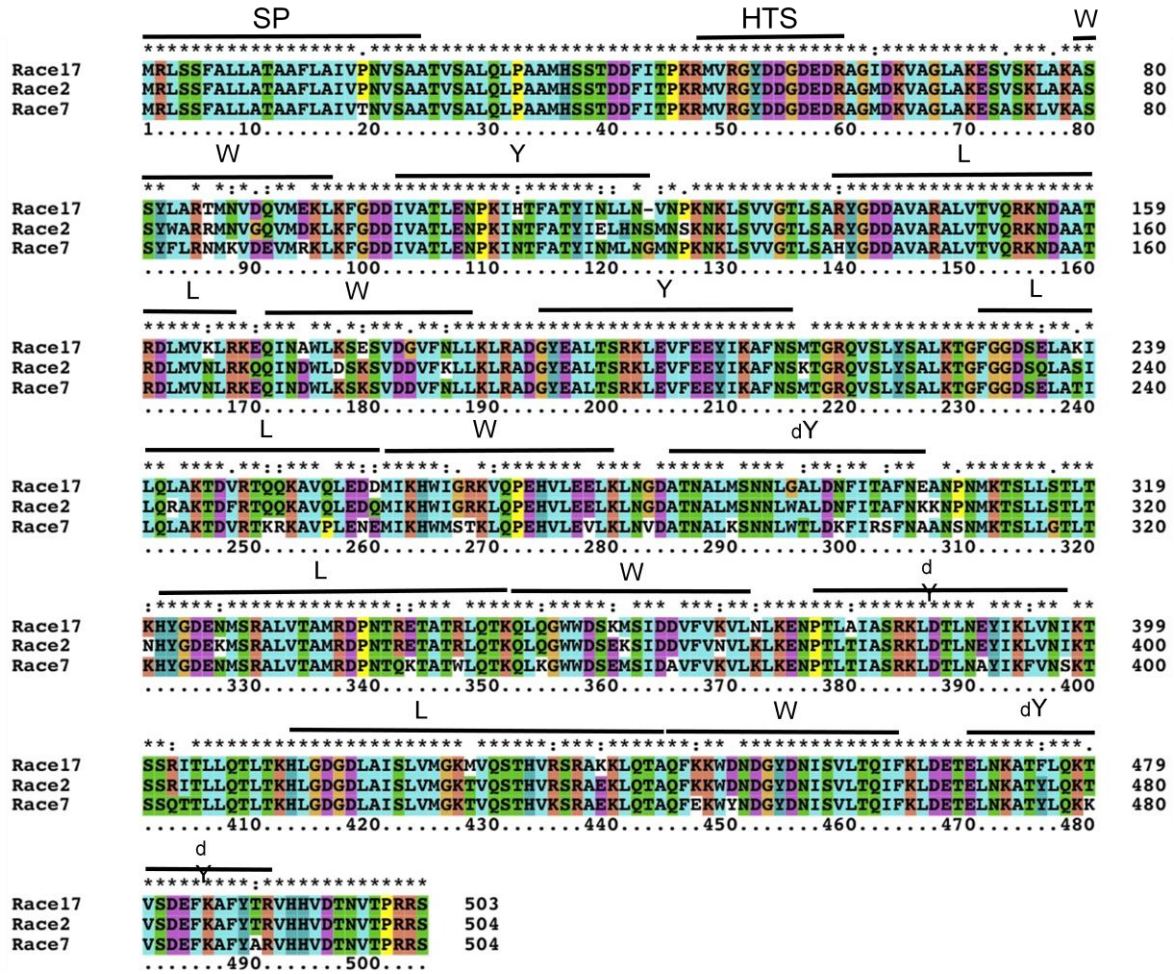
Callose suppression

Liquid cultures of *P. syringae* were grown in LB overnight at 28˚C, 200 RPM. Cultures were centrifuged at 1500×g for 10 minutes at 4˚C. The bacterial pellet was resuspended in 10 mM magnesium sulfate to an OD_{600} of 0.1. Four-week-old transgenic and wild-type plants were infiltrated using needless syringes. Challenged leaf tissue was harvested 16 hours post inoculation, stained with analine blue, and cleared as described (Sohn et al., 2007). Leaves were imaged with a Zeiss Axio Imager.M1 and DAPI filterset. Callose was quantified with Quantify One (BioRad).

SUPPORTING INFORMATION



Supplemental Figure 3.1. *Amino acid alignment of PsAvh163, HaRxL96 and PITG_10341.1 in which functional domains are highlighted*. Signal peptide (SP), RXLR and EER host targeting motifs (HTS), and W, Y, L motifs.



Supplemental Figure 3.2. *Amino acid alignment of PsAvh163 alleles in which functional domains are highlighted.* Signal peptide (SP), RXLR and EER host targeting motifs (HTS), and W, Y, L motifs.

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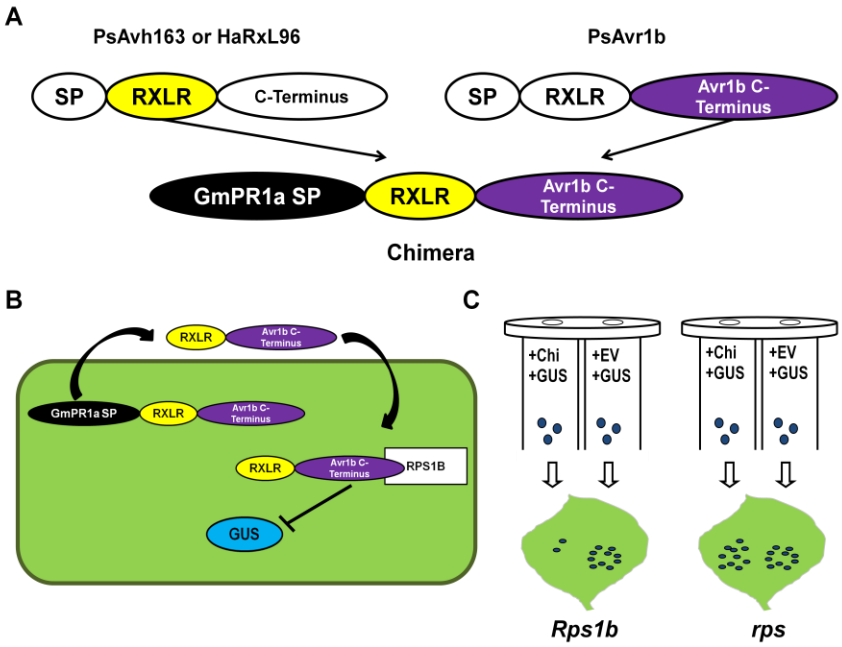
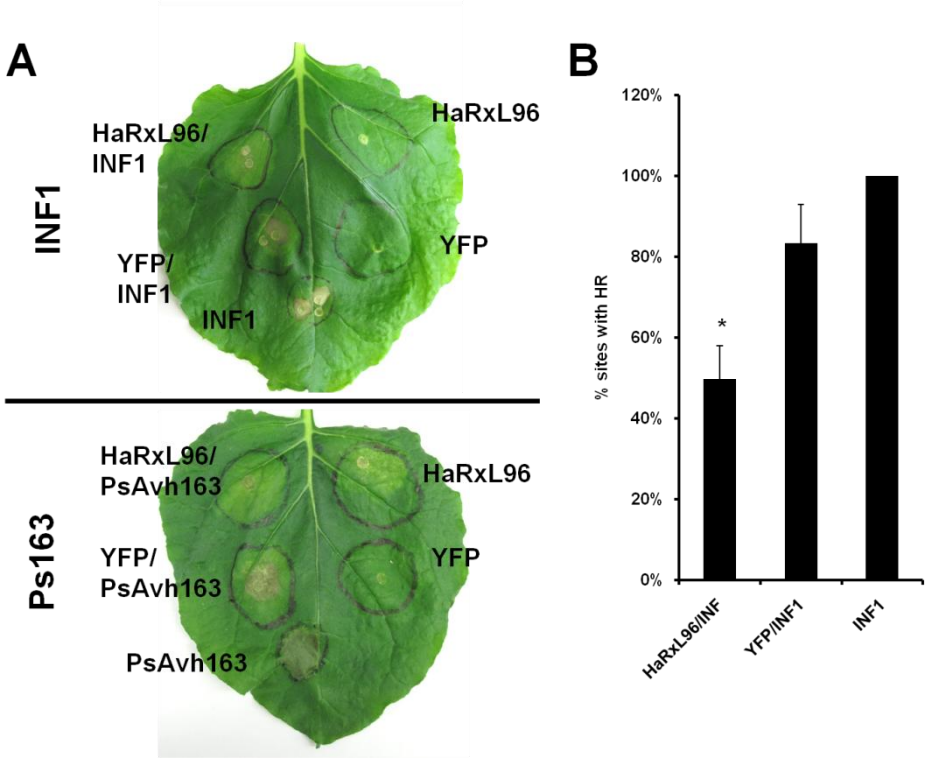
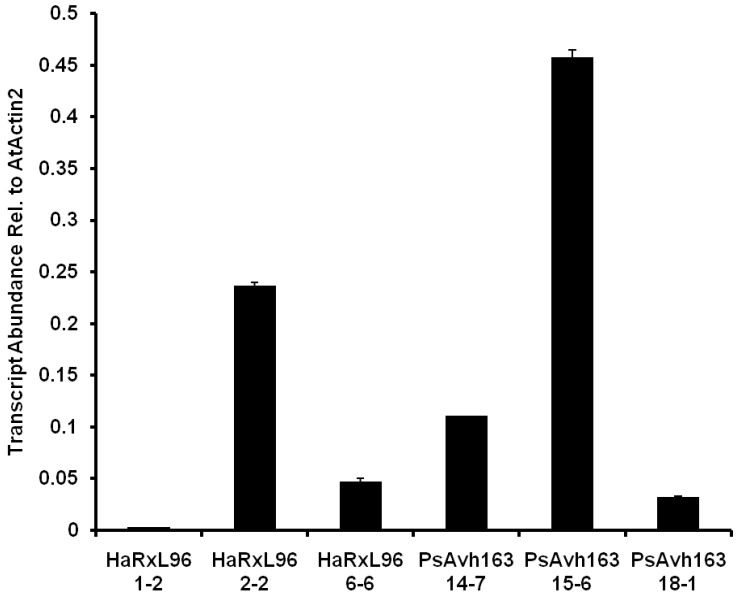


Supplemental Figure 3.3. *Cloning and delivery strategy for HTS functional assay.* a) Chimeric genes were produced by fusing the HTS (Yellow) from the target effector between the soybean PR1a signal peptide (Black) and the Avr1B C-terminus (Purple) b) The chimeric protein is produced in soybean cells and directed for secretion by the SP which is cleaved in the endoplasmic reticulum. If the HTS is functional, then the protein will translocated from the apoplast back to the cytosol where it is recognized by RPS1b. This recognition will trigger the HR and block *Gus* expression. c) Schematic depicting the double barrel gene gun. Tungsten particles with the chimeric gene (Chi) and Gus were co-bombarded along with a control shot of empty vector (EV) and Gus. Functional HTSs delivered the secreted, chimeric protein back into the cell triggering HR in Rps1b soybean, reducing the amount of cells expressing Gus relative to the control bombardment. Percent living cells were calculated relative to co-bombarded controls. Adapted from Dou et al. 2008.



Supplemental Figure 3.4. *HaRxL96 suppresses cell death in N. benthamiana*. *A. tumefaciens* GV3101 containing HaRxL96 was transiently expressed in *N. benthamiana.* 2 days later the same sites were challenged with INF1 or PsAvh163 and cell death was monitored visually over a period of 7 days. b) Quantification of HaRxL96 suppression of INF1. % HR, represents the percentage of HR positive sites scored of total scored sites. Error bars represent standard error. Replicated three times. * p=<.05



Supplemental Figure 3.5. *Quantification of transgene transcript levels in independently transformed lines, using quantitative PCR.* Transcript accumulation is expressed as a fold change relative to AtActin. Error bars represent standard deviation.

		HR Triggered	
Ecotype	Origin	PsAvh163	HaRxL96
An-1	Antwerpen, Belgium	NO	NO
Be-0	Bensheim/Bergstr., Germany	NO	NO
Bs-5	Basel, Switzerland	NO	Very Weak
Co-4	Coimbra, Portugal	NO	NO
Condara	Unknown	NO	NO
Cvi-0	Cape Verdi Islands	NO	NO
Di-0	Dijon, France	NO	NO
Dra-0	Drahonin, Czechoslovakia	NO	NO
Dra-2	Drahonin, Czechoslovakia	NO	NO
Ei-4	Eifel, Germany	NO	NO
Ei-5	Eifel, Germany	NO	NO
En-1	Enkheim/Frankfurt, Germany	NO	NO
Est-0	Estonia	NO	NO
Fl-1	Finland	NO	NO
Ga-0, 3O1	Gabelstein, Germany	NO	NO
Gr-1	Graz, Austria	NO	NO
Gy-0	La Miniere, France	NO	NO
Hodja	Tadjikistan	Weak	NO
Jm-0	Jamolice, Czechoslovakia	NO	NO
Le-0	Leiden, Netherlands	NO	NO
Ms-0	Moscow, Russia	NO	NO
Nd-0	Niederzenz, Germany	NO	YES
Np-0	Nieps/Salzwedel, Germany	NO	NO
Ob-0	Oberursel/Hasen, Germany	NO	NO
Oy-0	Oystese, Norway	NO	NO
Per-1	Perm, Russia	NO	YES
Petergof, 3L1	Petergof, Russia	NO	NO
Pla-1	Playa de Aro, Spain	NO	NO
Sah-0	Sierra Alhambra, Spain	NO	NO
Sf-2	San Feliu, Spain	NO	NO
Sorbo	Tadjikistan	NO	NO
Sp-0	Berlin/Spandau, Germany	NO	NO
Stu-0	Unknown	NO	NO
Ta-0	Tabor, Czechoslovakia	NO	NO
Ts-7	Tossa del Mar, Spain	NO	NO
Tsu-0	Tsu, Japan	NO	NO
Tsu-1	Tsu, Japan	NO	N/A
Uk-1	Umkirch, Germany	NO	NO
Ux-1	Unknown	NO	NO
Van-0	University of British Columbia, Canada	NO	NO
Wa-1	Warsaw, Poland	NO	NO
Wil-2	Wilna/Litvanian, Russia	NO	NO
Wt-2	Wietze, Germany	NO	NO
Yo-0	Yosemite Nat. Park, USA	NO	NO

Supplemental Table 3.1. *Table summarizing HaRxL96 and PsAvh163 HR screens in Arabidopsis ecotypes.*

Ha96 NOPS	CACCATGGTGGTGGTAGCCACCTTCCAGGCT
Ha96 NS	CGATGACGATGAGGGCGGGTGCCA
Ha96 S	TCACGATGACGATGAGGGCGGGTGCCA
Ps163 NOSP	CACCATGGTATCAGCACTCCAGCTCCCCG
Ps163 NS	AGAGCGCCGAGGGGTCACA
Ps163 S	CTAAGAGCGCCGAGGGGTCACAT
Avr1b CTD F	ACCTTCAGCGTGACTGACCTGTGG
Avr1b CTD R	GGTACCTCAGCTATACCGGTGAAAGG
Pr1A F	AAACCCGGGGGGCAATGAGATATGGGGTACATCTGCATTAAGATTTCGTTT
Pr1A R	AGCGTAGGCAACATCACCCACGA
163 OPE F	GTGGGTGATGTTGCCTACGCTGTATCAGCACTCCAG
163 OPE R	CCACCTTGTTCCACAGGTCAGTCACGCTGAAGGTGCCCACATTCATGCGCCGA
96 OPE F	GTGGGTGATGTTGCCTACGCTGTAGCCACCTTCCAGGCTCA
96 OPE R	CCACCTTGTTCCACAGGTCAGTCACGCTGAAGGTCCACAGGTCAGTCACGCTGAAGGTAAC GAACTT

Supplemental Table3. 2. *Table of primers used in this study*

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Chapter 4

The conserved oomycete RXLR effectors HaRxL96 and PsAvh163 exert virulence functions within the plant cell nucleus

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Contributions: Regina W. Hanlon is responsible for protein interactions screens, Megan S. Casady assisted with INF1 suppressions assays, Martin Stegmann generated mutant *pub/pip Arabidopsis* and provided *PUB* clones; Keri Cavanaugh preformed protein interaction screens.

Keywords: oomycete, *Hyaloperonospora arabidopsidis*, RXLR, HaRxL96, PsAvh163, plant immunity, effector, *Arabidopsis*

Abbreviations: confocal laser scanning microscopy (CSLM), days post inoculation (DPI), effector-triggered immunity (ETI), *Hyaloperonospora arabidopsis* (*Hpa*), hypersensitive response (HR), functional nuclear export signal (NES), non-functional nuclear export signal (nes), nuclear localization signal (NLS), pattern recognition receptor (PRR), microbe associated molecular pattern (MAMP), pattern-triggered immunity (PTI), race 2 (R2), race 7(R7), race 17 (R17), Recognition of *Peronospora Parasitica 4* (RPP4), simian virus 40 (SV40), yellow florescent protein (YFP). *Agrobacterium*-mediated transient assays in *N. benthamiana* (ATTA)

ABSTRACT

Diverse pathogens secrete effector proteins into plant cells to manipulate host cellular processes. The oomycete *Hyaloperonospora arabidopsidis* (*Hpa*, downy mildew of *Arabidopsis*) contains at least 134 candidate RXLR effector genes. Only a small subset of these is conserved in related oomycetes. The *Hpa* RXLR effector, *HaRxL96,* and a homologous effector, *PsAvh163,* from the soybean pathogen *Phytophthora sojae,* are moderately conserved genes with immunosuppressive activities in soybean, *Nicotiana,* and *Arabidopsis*. In this study, we demonstrate that both effectors target the host nucleus. Nuclear localization of both effectors is required for virulence functions, including suppression of basal and RPP4-mediated resistance to virulent and avirulent *Hpa,* respectively. In addition, both effectors interact with plant U-box (PUB) proteins that are conserved between diverse plant species. The targeted PUB proteins are negative regulators of plant immunity. The identification and investigation of homologous effector protein targets suggests that highly conserved plant processes are targeted by distantly related oomycetes.

INTRODUCTION

Oomycete pathogens pose a significant worldwide agricultural threat, costing billions of dollars and affecting the marketability and yield of agricultural products. Despite similar physical features to true fungi, oomycetes are phylogenetically distinct from fungi and evolved independently to parasitize land plants (Baldauf et al., 2000). *Hyaloperonospora arabidopsidis* (*Hpa*) is the downy mildew of *Arabidopsis thaliana* (Koch & Slusarenko, 1990). The *Arabidopsis-Hpa* interaction occurs frequently in nature, with a high level of genetic diversity (Coates & Beynon, 2010).

The genomes of several oomycete pathogens have been sequenced including *Hpa* and three pathogens from the *Phytophthora* genus (Baxter et al., 2010, Tyler et al., 2006, Haas et al., 2009). Bioinformatic screens have revealed that these genomes potentially encode hundreds of putative effector proteins that carry a signal peptide for secretion from the pathogen, followed by an RXLR motif that mediates entry into plant cells (Kale et al., 2010, Whisson et al., 2007, Dou et al., 2008). Oomycete effectors are highly divergent (Raffaele et al., 2010, Jiang et al., 2008, Win et al., 2007). Very little conservation of effector proteins exists between oomycete species, likely reflecting selection to avoid recognition by the host surveillance system and/or to adapt to new targets in the plant host cells (Raffaele et al., 2010). However, a small subset of effectors are conserved between oomycete species, suggesting that they may play an important role by targeting conserved plant processes (Baxter et al., 2010).

Plant immunity is comprised of two inducible systems. The first relies on pattern recognition receptors (PRRs) that recognize conserved microbial molecules called microbe associated molecular patterns (MAPMs) (Jones & Dangl, 2006). MAMPs such as flg22, a subunit of bacterial falgellin are recognized by the PRR FLS2 triggering a suite of defenses that comprise basal immunity or pattern-triggered immunity (PIT)(Katagiri & Tsuda, 2010). The second level of inducible defenses relies on a class of genes that maintain a nucleotide binding site (NB) and a leucine rich repeat (LRR). Theses NB-LLR proteins recognize pathogen proteins, either directly or indirectly, triggering programmed cell death known as the hypersensitive response (HR)(Dodds & Rathjen, 2010). The HR response is effective, preventing further pathogen colonization. This is called effector triggered immunity (ETI) (Jones & Dangl, 2006).

Plant pathogens have evolved effector proteins to co-opt plant immunity and alter host metabolism to complete their life cycle (Hann et al., 2010). Bacterial effectors are known suppressors of plant immunity (Zhou & Chai, 2008). Recent advances in our knowledge of oomycete effectors demonstrate that they too maintain the capacity to sabotage the plant immune network and interact with similar host targets (Mukhtar et al., 2011, Kamoun, 2006). This suggests that divergent pathogens have independently evolved effector proteins to target similar host proteins. Two homologous effectors, HaRxL96 from *Hpa* and its homolog from the soybean pathogen *Phytophthora sojae*, PsAvh163 have known virulence functions in diverse plants species. Both effectors are able to suppress PTI and ETI responses to a variety of elicitors in *Arabidopsis*, *N.*

benthamiana and soybean. Additionally, PsAvh163 has three identified alleles that trigger a RAR1/HSP90.1 dependent cell death in *N. benthamiana.*

The molecular mechanisms that underline the virulence functions of HaRxL96 and PsAvh163 are unclear. In this study, we demonstrate that both effectors are localized to the host nucleus and that this localization is required for proper virulence functions. In addition, both effectors interact with plant U-box (PUB) proteins that are conserved between diverse plant species (Azevedo et al., 2001). The targeted PUB proteins are negative regulators of plant immunity (He et al., 2011, Trujillo et al., 2008), suggesting that HaRxL96 and PsAvh163 interact with these proteins to enhance their immune-suppressive function.

RESULTS

HaRxL96 and PsAvh163 localize to the nucleus and cytoplasm of plant cells

To examine where HaRxL96 and PsAvh163 localize inside plant cells, we created plant expression plasmids encoding yellow fluorescent protein (YFP) fused to the N-terminus of each effector, beginning at the predicted signal peptide cleavage site. The YFP-effector fusions were transiently expressed via *Agrobacterium*-mediated transient assays in *N. benthamiana* (ATTA) and imaged with confocal laser scanning microscopy (CSLM). YFP-HaRxL96 localizes strongly to the cytoplasm with weak accumulation in the nucleus (**Figure 4.1**). This is similar to untagged YFP that accumulates in the nucleus

and cytoplasm, although the nuclear signal from YFP-HaRxL96 is much weaker than the signal from YFP alone. YFP-PsAvh163 accumulated weakly in the cytoplasm and strongly in the nucleus; the nuclear signal was stronger than the nuclear signal from YFP-HaRxL96. Additionally, YFP-PsAvh163 was visible in the nucleolus, while YFP-Ha96 was not (**Figure 4.1**). Western blots of extracts from *N. benthamiana* infiltrated with either YFP fusion demonstrated that the effector-YFP fusions were not cleaved *in planta* (Data not shown).

The weak nuclear signal from YFP-HaRxL96 led us to utilize subcellular fractionation as a second, more sensitive assay to resolve whether HaRxL96 accumulates in the nucleus. *YFP- HaRxL96* was transiently expressed in *N. benthamiana*, and tissue was collected for fractionation. Nuclear depleted (ND) fractions show barely detectable levels of histone H3 (a nuclear protein) while nuclear enriched (NE) fractions had abundant levels of histone H3, exhibiting positive enrichment. Anti-GFP antibodies were used to detect pools of YFP-HaRxL96. Western blot analysis revealed pools of YFP-HaRxL96 in NE fractions with barely detectable levels in ND fractions. This establishes the presence of HaRxL96 in the nucleus (**Figure 4.2**).

Three divergent alleles of *PsAvh163* were previously identified by genome sequencing of *P. sojae* Race 2 (R2), Race 7 (R7), and Race 17 (R17) (Chapter 3 and (Wang et al., 2011)). We previously showed that all three alleles trigger cell death when transiently expressed in *N. benthamiana* or *N. tabacum*, most likely due to activation of effector-triggered immunity (Chapter 3). We investigated possible localization

polymorphisms by imaging YFP fusions to all three alleles. The reference allele (R2) is described in the previous paragraph. YFP fusions to PsAvh163 Race 7 and Race 17 localized in a similar manner to PsAvh163 R2 (**Figure 4.3**).

Confining PsAvh163 to the nucleus reduces the cell death response in N. benthamiana.

The assays above indicate that HaRxL96 and PsAvh163 reside in the nucleus and the cytoplasm. Thus, these proteins could exert their virulence function in either subcellular compartment, or both. Protein mis-localization has been used successfully to establish biological significance of subcellular localization (Gao et al., 2011, Shen et al., 2007, Wirthmueller et al., 2007, Garcia et al., 2010). Our first experiment was to fuse both effectors to a strong nuclear localization signal (NLS), thereby depleting the cytoplasmic pools of each effector, and test whether this alteration interferes with previously documented functions of the effector proteins. To this end, we fused the simian virus 40 (SV40) nuclear localization sequence (NLS) to the C-terminus of HaRxL96 and PsAvh163 and assayed for altered effector functions in *N. benthamiana*. Previously, we showed that PsAvh163 elicited cell death in *N. benthamiana* when delivered via ATTA (Chapter 3). Transient expression of PsAvh163-NLS in *N. benthamiana* triggered reduced cell death symptoms in comparison to native PsAvh163 (**Figure 4.4**). This result suggests that cytoplasmic localization of PsAvh163 is necessary for induction of cell death in *N. benthamiana*.

Confining HaRxL96 to the nucleus does not interfere with its virulence function in N. benthamiana.

We showed previously that HaRxL96 can suppress cell death triggered in *N. benthamiana* by the unrelated elicitors *P. infestans* INF1 and PsAvh163 (**Figure S3.7**). We tested whether these functions are compromised by addition of the NLS to HaRxL96, thereby reducing the cytoplasmic pool of HaRxL96. As shown (**Figure 4.5**), the HaRxL96-NLS fusion can suppress both types of cell death as efficiently as untagged HaRxL96. This indicates HaRxL96 cell death suppression activity takes place within the nucleus.

Reducing nuclear accumulation of HaRxL96 and PsAvh163 abolishes virulence activity in soybean

To test for virulence functions in the nucleus, we fused HaRxL96 and PsAvh163 with to a nuclear export signal (NES) from the rabbit protein kinase inhibitor (PKI) of cAPK (Wen et al., 1995). We also made control fusions with non-functional nuclear export signal (nes) that differs from the functional version in 3 out of 14 residues. This approach has been used previously to test the effect of nuclear exclusion on the function of several plant R proteins (Shen et al., 2007, Garcia et al., 2010, Gao et al., 2011, Burch-Smith et al., 2007, Schornack et al., 2008, Wirthmueller et al., 2007). First, we confirmed the functionality of NES and nes sequences by constructing YFP-effector-NES/nes fusions and tracking their localization following ATTA in *N. benthamiana.*

YFP-HaRxL96-NES and YFP-PsAvh163-NES localized predominantly to the cytoplasm with very weak fluorescent signal around the periphery of the nucleus, similar to previous reports with this system (**Figure 4.4**). Contrastingly, HaRxL96-nes and PsAvh163-nes retained their native nucleo-cytoplasmic localization, with no obvious differences in protein abundance compared to YFP-HaRxL96 and YFP-Ps163 (**Figure 4.4**). These experiments confirmed that additions of a functional NES substantially reduced the nuclear pool of both effectors, while fusions to the non-functional nes displayed the same localization as fusions without a C-terminal tag. ATTA in *N. benthamiana* of PsAvh163-NES and PsAvh163-nes did not reveal any alterations in the cell death phenotype, compared to untagged PsAvh163, indicating that cell death activity is retained by these fusions and thus they are functional (**Figure 4.6**). Additionally, this result suggests that the nuclear pool of PsAvh163 is not necessary for recognition in *N. benthamiana*.

In soybean, HaRxL96 and PsAvh163 are suppressors of Avr4/6 ETI and the pro-apoptotic protein Bax (Chapter 3). We evaluated the requirement of nuclear localization for cell death suppression in transient soybean assays via co-bombardment with a double barreled gene gun device, as described in Chapter 3. PsAvh163-NES fusions were unable to suppress the strong cell death response elicited by Bax. On the other hand, PsAvh163-nes retained the ability to suppress Bax-induced cell death in soybean (**Figure 4.7**). Similarly, HaRxL96-NES fusions were unable to suppress RPS6-dependent PCD, while HaRxL96-nes could suppress RPS6-mediated PCD (**Figure 4.8**). Similar to HaRxL96-NES, PsAvh163-NES did not suppress PCD triggered by RPS6 while PsAvh163-nes did suppress RPS6 PCD. In sum, reducing the abundance of either

effector in the nuclear compartment inhibited their ability to properly suppress cell death triggered by Bax or Avr4/6. These experiments suggest that the virulence-promoting activities of HaRxL96 and PsAvh163 occur within in the nucleus in soybean.

Reducing nuclear accumulation of HaRxL96 and PsAvh163 abolishes virulence activity in stably transformed Arabidopsis

Previously, we demonstrated that expressing *HaRxL96* or *PsAvh163* as transgenes in *Arabidopsis* suppressed basal defense and effector-triggered immunity against *Hpa* (Chapter 3). We therefore evaluated the requirement for nuclear localization in *Arabidopsis* and its link to the immunosuppressive activity of HaRxL96 and PsAvh163. Transgenic plants were created that express *HaRxL96-NES, HaRxL96-nes*, *PsAvh163-NES,* or *PsAvh163-nes* as transgenes. Fusions of HaRxL96 and PsAvh163 to a non-functional nuclear export signal suppressed basal immunity to virulent *Hpa* Emco5, increasing pathogen reproduction by approximately 10% (**Figure 4.9**). In addition, the *HaRxL96-nes* and *PsAvh163-nes* transgenes partially suppressed RPP4 resistance to avirulent *Hpa* Emoy2, increasing pathogen reproduction by approximately 50% (**Figure 4.9**). This is consistent with the abilities of untagged HaRxL96 and PsAvh163 to suppress basal and RPP4 mediated immunity in *Arabidopsis*. Contrastingly, the HaRxL96-NES and PsAvh163-NES transgenes were unable to suppress basal immunity in response to virulent *Hpa* Emco5 or effector-triggered immunity to avirulent *Hpa* Emoy2. These results suggest that HaRxLR96 and PsAvh163 suppress *Hpa* immunity from within the nucleus in *Arabidopsis* (**Figure 4.9**).

HaRxL96 and PsAvh163 interact with conserved U-box proteins from divergent plant species

To identify candidate effector targets, clones of HaRxL96 and PsAvh163 were given to two independent groups for directed yeast two hybrid (Y2H) screens. The first group (Jeff Dangl, University of North Carolina, Chapel Hill) screened an immunity-related enriched collection of *Arabidopsis* proteins using a yeast two-hybrid system. Results from this set of pairwise screens were recently published (Mukhtar et al., 2011) and can be queried through an internet-accessible search tool (Plant-Pathogen Interactome version 1 or PPIN1, (signal.salk.edu/interactome/PPIN1.html). We searched the dataset to determine whether our effector proteins interacted with *Arabidopsis* proteins. However, we did not identify positive protein interactions. The second screen for effector targets was conducted in Richard Michelmore's group (University of California, Davis). This directed Y2H screen included a wider range of defense related genes from plant species other than *Arabidopsis*. This screen uncovered a weak interaction between HaRxL96 and the *N. benthamiana* U-box protein, CMPG1. NbCMPG1 is a member of a highly conserved gene family with homologs in soybean and in *Arabidopsis.* PUB proteins utilize E3 ligase activity to modulate plant immunity and stress responses (Azevedo et al., 2001). Several PUB proteins function as negative regulators of plant immunity (He et al., 2011, Trujillo et al., 2008) and in some case act as a positive regulator (Gonzalez-Lamothe et al., 2006). The previously documented roles

of these proteins in immunity suggest that they are plausible targets for the immune-suppressive effectors HaRxL96 and PsAvh163.

CMPG1 homologs in *Arabidopsis* include *PUB22, PUB 23, PUB24*, which have been previously documented as negative regulators of PTI in Arabidopsis (Trujillo et al., 2008). We tested the interactions of HaRxL96, PsAvh163 and homologs of CMPG1 from soybean with yeast 2-hybrid screens (Y2H). Both HaRxL96 and PsAvh163 positively interacted with a number of soybean PUB homologs including Glyma13g38890, Glyma19g38740 and Glyma14g39300. HaRxL96 did not interact with the *Arabidopsis* PUBs while PsAvh163 interacted with AtPUB23 and AtPUB24 (**Figure 4.10**).

We used the bimolecular fluorescence complementation (BIFC) assay to test for interaction between HaRxL96, PsAvh163, and *PUB22, PUB 23,* and *PUB24*. BIFC has provided a useful tool to study the nature of protein interactions in plant cells (Walter et al., 2004). BIFC relies on the principle that fluorescent proteins such as YFP can be split into N and C terminal halves and fused to proteins of interest. If the proteins of interest interact, the two halves of YFP will be close enough to recapitulate the fluorescent capacity of intact YFP. The C-terminal half of YFP (C) was fused to the N-terminus of the three PUB proteins, while the N-terminal half of YFP (N) was fused to the N-terminus of the effectors. Following co-infiltration of *Agrobacterium* strains with N-HaRxL96 or C-PUB22/24, we observed weak fluorescence that localized predominantly to the cytoplasm (**Figure 4.11**). The control infiltrations between N-YFP and C-YFP did

not provide an appreciable signal, nor did the co-infiltrations between N-HaRxL96 and C-YFP, demonstrating the specificity of the interaction (**Figure 4.11**).

BIFC with N-PsAvh163 and the three PUB genes produced fluorescence in all three interactions (**Figure 4.12**). The weakest fluorescence of the PUB interactions was between N-PsAvh163 and C-PUB24. In all three tested interactions between N-PsAvh163 and C-PUBs with BIFC, the fluorescence signal was detected in the cytoplasm with CSLM. Together, the Y2H interaction and BIFC data, indicate that HaRxL96 and PsAvh163 interact with homologous PUB proteins from diverse plant species.

A plant target of PUB22 is necessary for basal resistance to Hpa

The *PUB22*, *PUB23*, *PUB24* genes in *Arabidopsis* are redundant negative regulators of plant immunity (Trujillo et al., 2008). To examine which aspects of plant immunity are being regulated by PUB22/23/24, PUB targets were identified by Martin Stegmann, led by Marco Trujillo. The protein interaction screens identified a PUB interacting protein (PIP) that binds to PUB22 (personal communication; Note that Dr. Trujillo requested that we keep the identity of this protein confidential, until publication). Epistasis analysis of *pub22/23/24/* and *pip* during *Hpa* infection revealed that *pub22/23/24* displayed enhanced resistance to *Hpa* Emco5 consistent with their role as negative regulators of basal immunity (**Figure 4.13A**). A combinatorial mutant, pub22/23/24/*pip* partially restored *Hpa* levels to wild type, while the *pip* mutant alone displays enhanced susceptibility to *Hpa* Emco5, consistent with PIP's role in basal

immunity (**Figure 4.13A**). We correlated the levels of cell death to *Hpa* pathogen growth by trypan blue staining and subsequent visual inspection. *pub22/23/24* and *pub22/23/24*/*pip* mutant have increased levels of cell death consistent with pathogen growth (**Figure 4.13B**).

DISCUSSION

The virulence-promoting activity of oomycete effectors is undoubtedly a function of their subcellular localization (Schornack et al., 2009, Shen et al., 2007, Schornack et al., 2008). One objective of this study was to determine the subcellular addresses of two homologous effector genes *HaRxL96* and *PsAvh163* from *Hpa*, and *P. sojae*. The sequences of *HaRxL96* and *PsAvh163* do not have any recognizable subcellular targeting motifs. YFP fusions to both effectors revealed a nuclear-cytoplasmic localization. Additionally, three divergent alleles of *PsAvh163* displayed similar localization patterns for all three alleles despite their sequence divergence, suggesting that nuclear localization is important for PsAvh163 function and therefore conserved.

Because these proteins lack a discernable nuclear localization signal, it is unclear how they are transported to the nucleus or how the relative proportion of the nuclear and cytoplasmic pools might be regulated. One possibility is that these proteins might be "piggybacked" into the nucleus by association with a nuclear-localized plant protein. Due to their relatively small size, it is also possible that the nuclear entry of the effectors is a result of passive diffusion. YFP-HaRxL96 and YFP-PsAvh163are 67kDa and 78

kDa, respectively. Although it is commonly stated that the nuclear size exclusion limit is approximately 60 kiloDaltons (kDa) (Goldfarb et al., 2004), a recent report provided evidence that proteins larger than 60 kDa can diffuse into the nucleus (Brattain & Wang, 2007). Thus, passive diffusion of these proteins cannot be excluded. However, it is important to consider the possibility that passive diffusion might still be a biologically relevant mode of effector entry into the nucleus.

This led us to study the biological significance of localization in the nucleus and cytoplasm for both effectors. To address this we mis-localized the effector proteins and studied the effect on the virulence activities of HaRxL96 and PsAvh163 in soybean, *Nicotiana*, and *Arabidopsis*. This type of mis-localization study has proven informative for many proteins including the effector AvrHah1, the resistance proteins RPS4, MLA10, N, RPM1 and the *Arabidopsis* immune regulator EDS (Shen et al., 2007, Garcia et al., 2010, Gao et al., 2011, Burch-Smith et al., 2007, Schornack et al., 2008, Wirthmueller et al., 2007) . We previously demonstrated that HaRxL96 and PsAvh163 have the ability to suppress RPS6 ETI in soybean. Fusions to a functional NES inhibited the ability of both effectors to suppress cell death, while fusions to the control (nes) maintained the ability to inhibit RPS6 mediated HR. This suggests that both effectors act at similar sites within the soybean cell.

We extended our mis-localization studies to *Arabidopsis* by expressing both effectors with a functional and non-functional NES as stably integrated transgenes, driven by the CaMV35 promoter. Both effectors, when fused to the non-functional nes,

suppressed RPP4-mediated resistance to *Hpa* Emoy2 and enhanced the growth of virulent *Hpa* Emco5, consistent with the data from Chapter 3. The addition of functional NES ameliorated the virulence-promoting effects of HaRxL96 and PsAvh163. These results are consistent with the soybean transient assays that signify the virulence functions of both effectors lie within the nucleus of soybean and *Arabidopsis*.

Paradoxically, our data suggest that PsAvh163 is recognized in the cytoplasm of *N. benthamiana* cells. We had previously shown that PsAvh163 triggers an HR-like response in N. benthamiana, most likely from ETI (Chapter 3). Interestingly, a PsAvh163-NLS fusion triggered an attenuated cell death response, compared to untagged. PsAvh163. Addition of the NLS likely forces much of the protein into the nucleus and thereby reduces the nuclear pool of PsAvh163. Contrastingly, Ps163-NES fusions are capable of triggering a full PCD response. This suggests that PsAvh163 is recognized in the cytoplasm of cell. Thus, the virulence and avirulence functions of PsAvh163 occur in different subcellular compartments. Nuclear-cytoplasmic effector proteins have been reported previously, including the tobacco mosaic virus (TMV) p50, but their sites of action are not well defined. In these cases, the cognate R protein required nuclear localization to mount a proper immune response (Burch-Smith et al., 2007, Caplan et al., 2008).

Given the cellular processes governed by the nucleus, it is to be expected that pathogens might target this organelle. This is indeed the case as nuclear localized effectors have been described previously in bacteria. The *Xanthomonas campestris pv.*

vesicatoria bacterial TALE AvrBs3 acts inside the host nucleus as a transcription factor inducing host genes (Kay et al., 2007). Additionally, the *Ralstonia solanacearum* effector PopP2 has a functional NLS, localizing PopP2 to the nucleus along with its cognate R protein RRS1-R (Deslandes et al., 2003). Many of the crinkler (CRN) class of oomycete effectors maintain a functional nuclear localization signal and target the nucleus, where they like exert their virulence function (Schornack et al., 2010). However, the functions of many nuclear localized bacterial and oomycete effectors are inadequately understood. This study presents the first detailed analysis of RXLR effector localization and its effect on virulence functions. In oomycetes, several effectors are dependent on α-importin for nuclear localization (Schornack et al., 2010). However, these effectors have a predicted NLS unlike *HaRxL96* and *PsAvh163*. Both HaRxL96 and PsAvh163 maintain homologs in other oomycete species and the localization patterns for HaRxL96 and PsAvh163 are similar. Even amongst divergent alleles of *PsAvh163*, subcellular localization is maintained, emphasizing the functional importance of both effectors and their associated nuclear localization.

Of equal importance to understanding effector localization is identifying the effectors' putative targets inside plant cells. Recently, an interactome that includes effectors from *Pseudomonas syringae* and *Hpa* revealed common targets for divergent species. Many effectors in the interactome primarily targeted a limited set of "molecular machines" that are implicated in host immunity (Mukhtar et al., 2011). Detailed analysis of effector interactions and their relevance is also available. For example, the *Psy* effector HopM1 interacts with AtMIN7 and targets it to the proteasome for destruction. AtMIN7

is a component of the vesicle trafficking system that may play a role in cell wall associated defense (Nomura et al., 2006). Additionally, *Psy* AvrPtoB interacts with the *Arabidopsis* defense-related proteins CERK1, BAK1 and FLS2 (Shan et al., 2008, Robatzek et al., 2008, Gimenez-Ibanez et al., 2009) all three of which play an important role in PTI responses.

Both HaRxL96 and PsAvh163 were included in the interactome screens, but these screens yielded no positive interactions. This can be explained by the absence of PUB genes in the tested interactome collection of Y2H constructs of *Arabidopsis* genes. Additional protein interaction screens revealed both HaRxL96 and PsAvh163 interact with conserved plant U-box proteins that are implicated as negative regulators of plant immunity (He et al., 2011, Trujillo et al., 2008). PUB proteins are conserved amongst diverse plants species and utilize their E3 ligase activity to modulate cellular processes including responses to biotic and abiotic stresses (Azevedo et al., 2001). The *Arabidopsis* PUB triplet PUB22, PUB23, and PUB24 function redundantly to negatively regulate basal immunity. Consistent with their role in negative regulation of PTI responses, pub22/23/24 mutants display enhanced resistance to normally virulent *Hpa* (Trujillo et al., 2008). In addition, other PUB proteins such as PUB12 and PUB13 modulate FLS2 regulation through their E3 ligase activity (He et al., 2011). PUB genes in other species also play an important role in host immunity, including *N. benthamiana* CMPG1. CMPG1 is required for proper HR response to a variety of elicitors (Gilroy et al., 2011, Gonzalez-Lamothe et al., 2006).

Despite identifying candidate effector targets, we do not understand the mechanism in which HaRxL96 or PsAvh163 act on PUB proteins. PUB genes are conserved amongst plants (Azevedo et al., 2001) thus making them attractive targets for pathogens to manipulate. The *P. infestans* effector Avr3a interacts with and stabilizes the U-box protein CMPG1. This interaction appears to be mediated by phosphatidylinositol monophosphate binding (Yaeno et al., 2011, Bos et al., 2010). It is postulated Avr3a interacts with phosphatidylinositol monophosphate to target CMPG1 that is normally localized to slow moving vesicles. This releases CMPG1 and Avr3a traffics its target to the nucleus where it is stabilized, sabotaging CMPG1-dependent responses and enhancing *P. infestans* virulence. It is plausible that both HaRxL96 and PsAvh16 act in a similar manner, regulating U-box protein stability and consequently corrupting immune signaling by damping PTI responses. The *Arabidopsis* PUB triplet, PUB22/23/24 modulates PTI by regulating a protein involved in cellular trafficking, PUB interacting protein (PIP). Deleting PIP compromises basal immunity linking the PUB target to plant immunity. Thus, HaRxL96 and PsAvh163 target a protein complex that functions in regulating plant immunity.

The molecular mechanisms that underpin the functions of HaRxL96 and PsAvh163 are unclear. More so, the localization PUB proteins (cytoplasmic) and effector proteins require reconciliation. Do HaRxL96 and PsAvh163 adjust subcellular localization of PUB proteins? Do HaRxL96 and PsAvh163 interact with other host targets? (**Figure 4.14**) Despite these uncertainties, it's clear that homologous effectors from divergent oomycetes have maintained conserved molecular functions, requiring

nuclear localization and target highly conserved U-box proteins from plants. Both HaRxL96 and PsAvh163 represent effectors utilized by divergent oomycetes that maintained the ability to target important regulators of plant immunity.

FIGURES

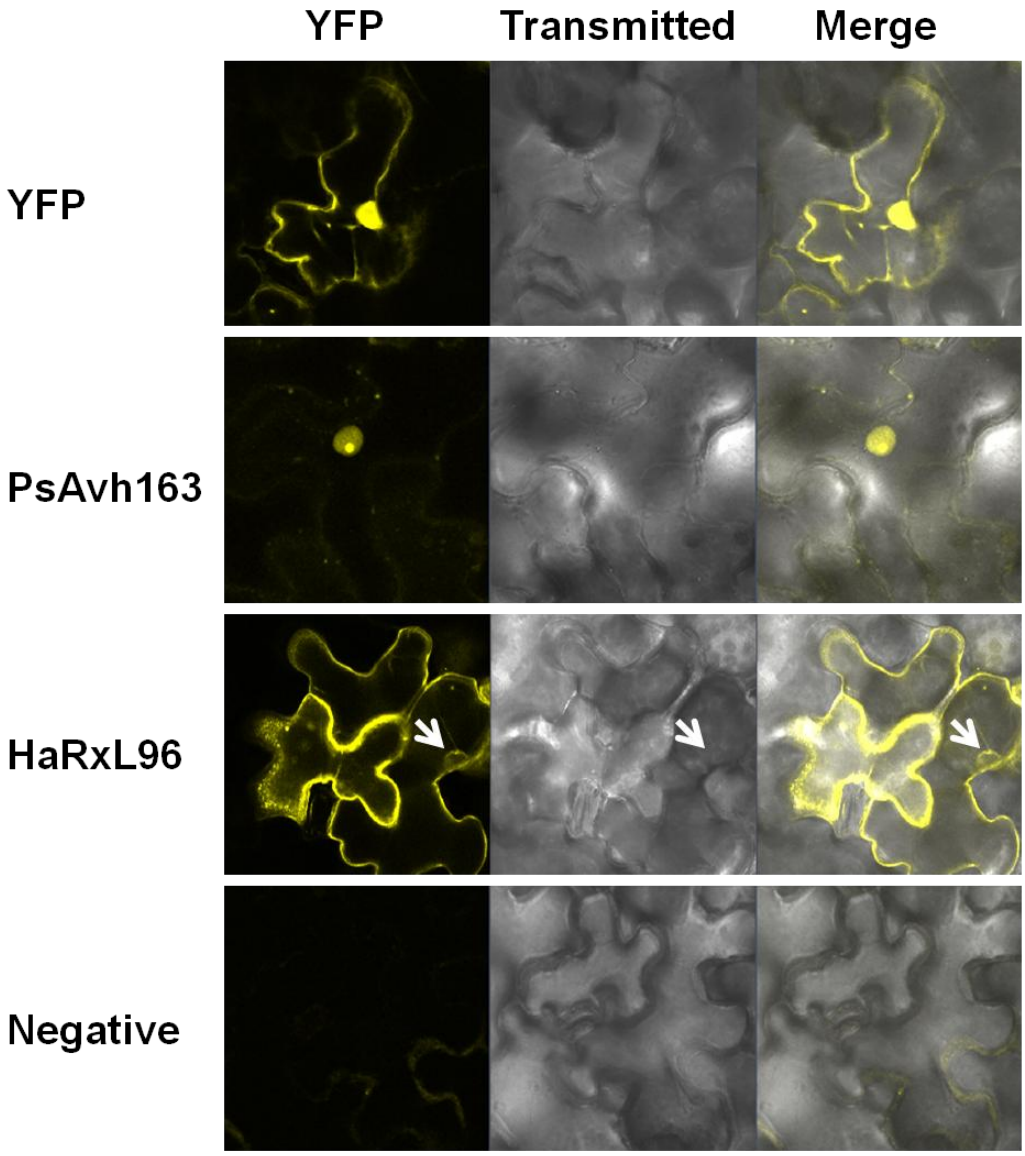


Figure 4.1 *HaRxL96 and PsAvh163 are nuclear cytoplasmic localized proteins*. The indicated effectors with N-terminal fusions to YFP were transiently expressed in *N. benthamiana* and imaged with confocal laser-scanning microscopy (CLSM). The YFP, transmitted, and merged channels are shown. Arrows designate nuclei.

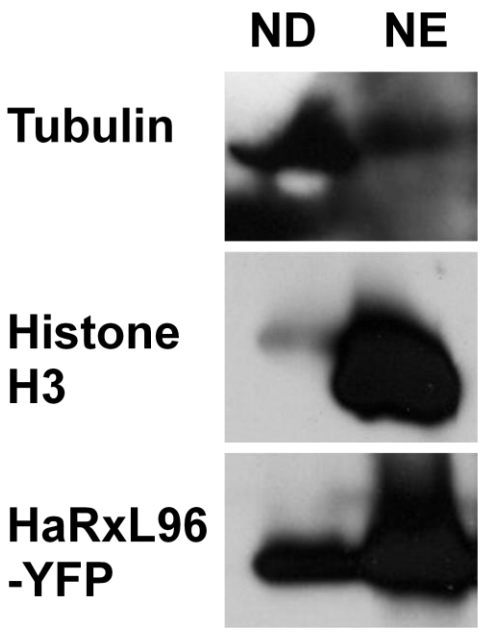


Figure 4.2 *HaRxL96 is detectable in the nucleus and cytoplasm.* Sub-cellular fractionation with ATTA extracts derived from *N. benthamiana* expressing YFP-HaRxL96, demonstrates the presence of HaRxL96 in the nuclear depleted (ND) and 20-fold (v/v) nuclear enriched (NE) fractions. A western blot with anti-Histone H3 and anti-Tubulin antibodies were used as purity markers for nuclear and cytoplasmic fractions, respectively.

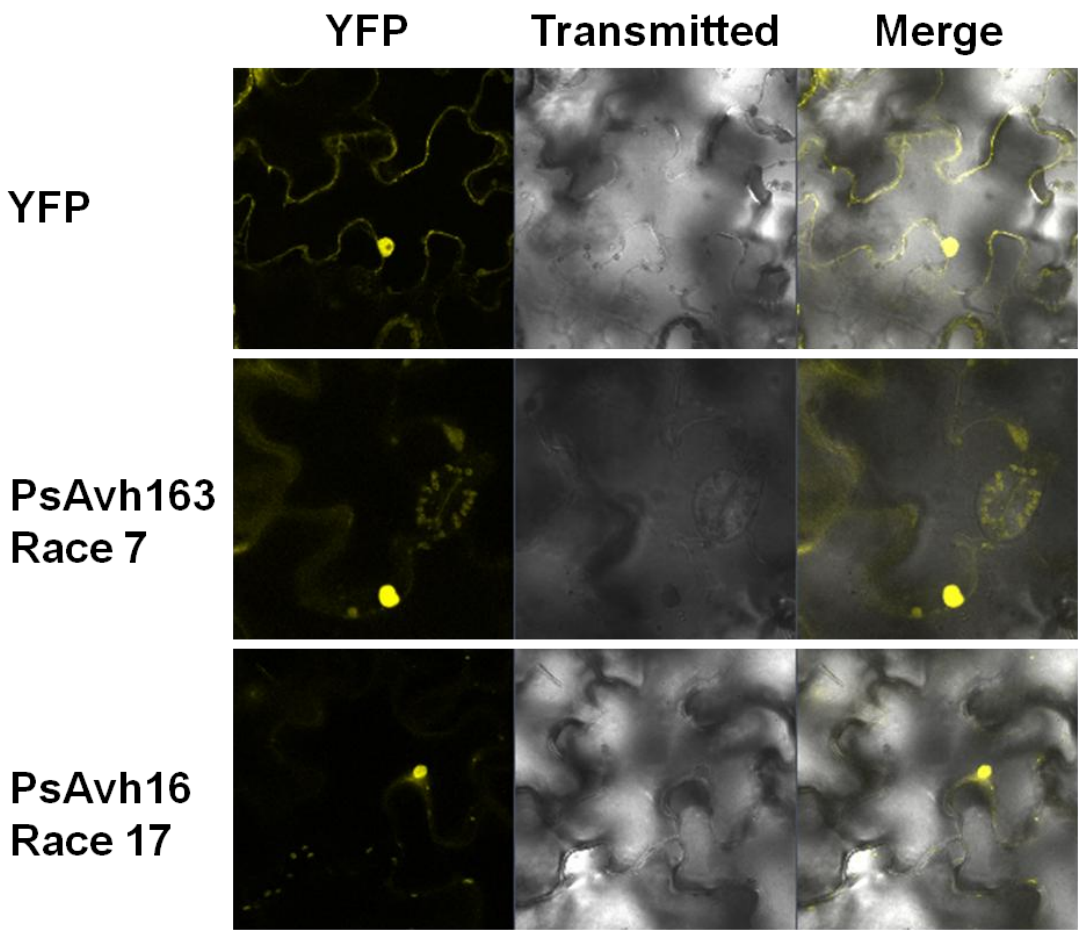


Figure 4.3 *Divergent alleles of PsAvh163 localize in a similar fashion to the PsAvh163 Race2 allele*. The indicated effectors with N-terminal fusions to YFP were transiently expressed in *N. benthamiana* and imaged with CLSM. The YFP, transmitted, and merged channels are shown.

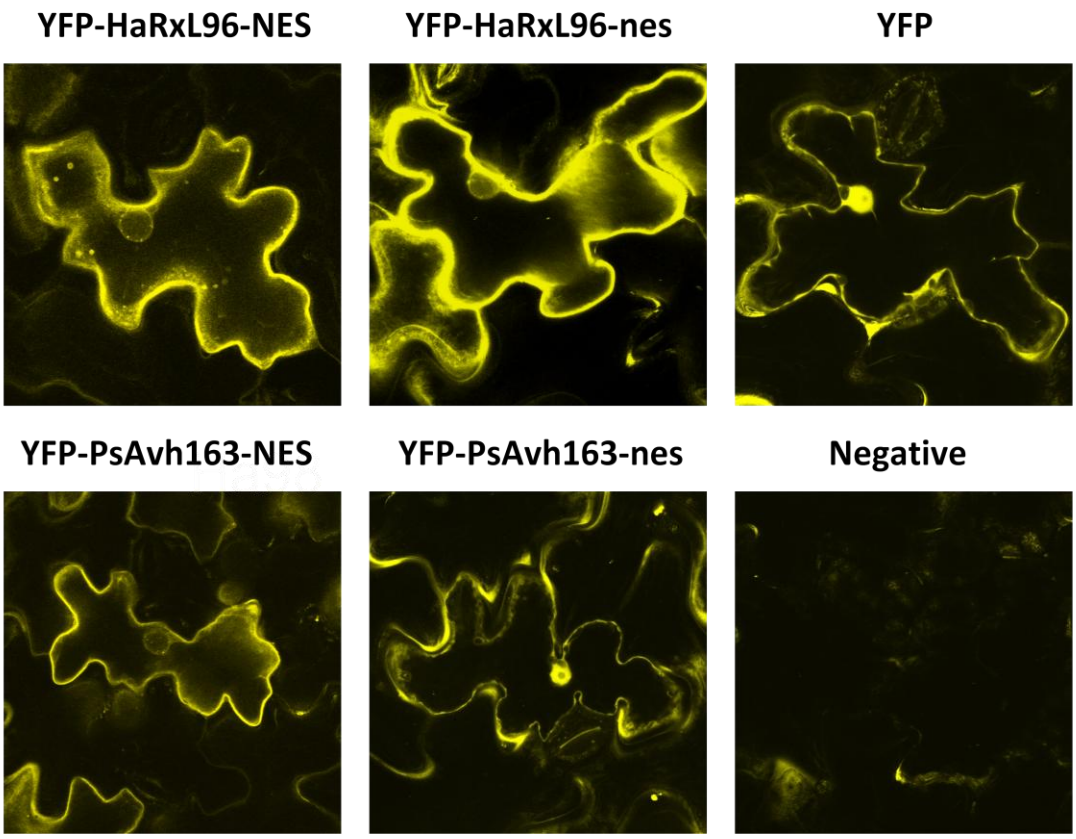


Figure 4.4 *Fusions to a functional nuclear export depletes the pool of HaRxL96 and PsAvh163 in the nucleus.* Effectors were cloned with a N-terminal YFP fusion and a C-terminal fusion to a functional nuclear export signal (NES) or a non-functional nuclear export signal (nes). The clones were transiently expressed in *N. benthamiana* via ATTA and imaged with CLSM.

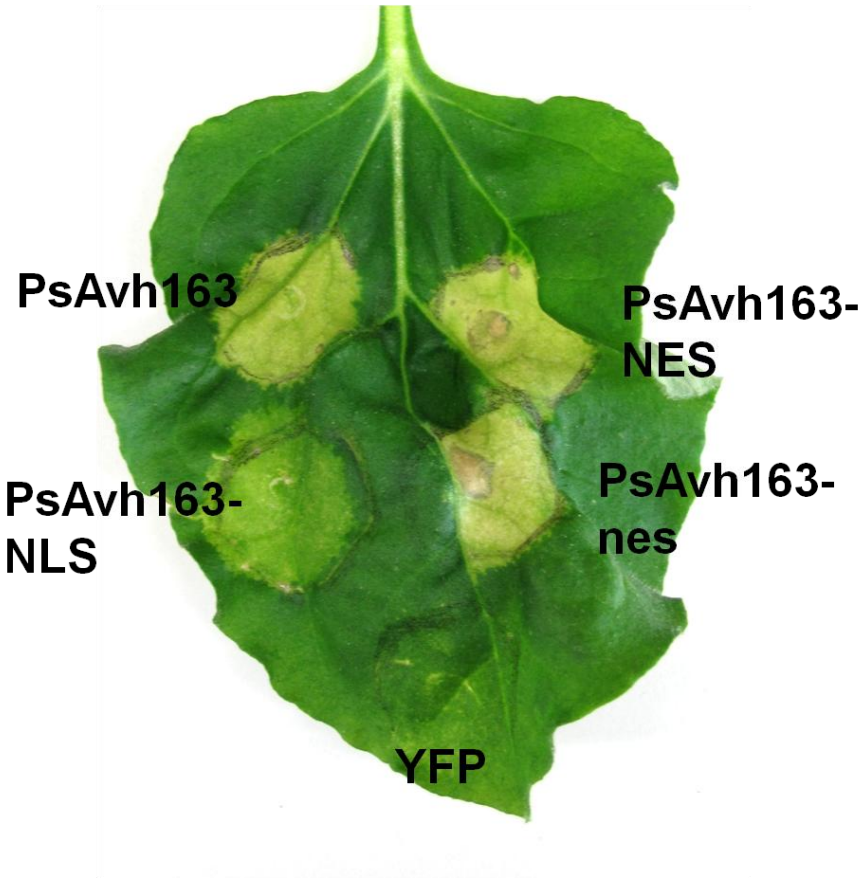


Figure 4.5 *Confining PsAvh163 to the nucleus reduces the cell death symptoms in N. benthamiana. PsAvh163* was cloned with a nuclear localization signal (NLS), functional nuclear export signal (NES) or a non-functional nuclear export signal (nes) and transiently expressed in *N. benthamiana* via *Agrobacterium*. Cell death symptoms were visually monitored over a period of 7 days. Untagged PsAvh163 and YFP serve as positive and negative controls respectively.

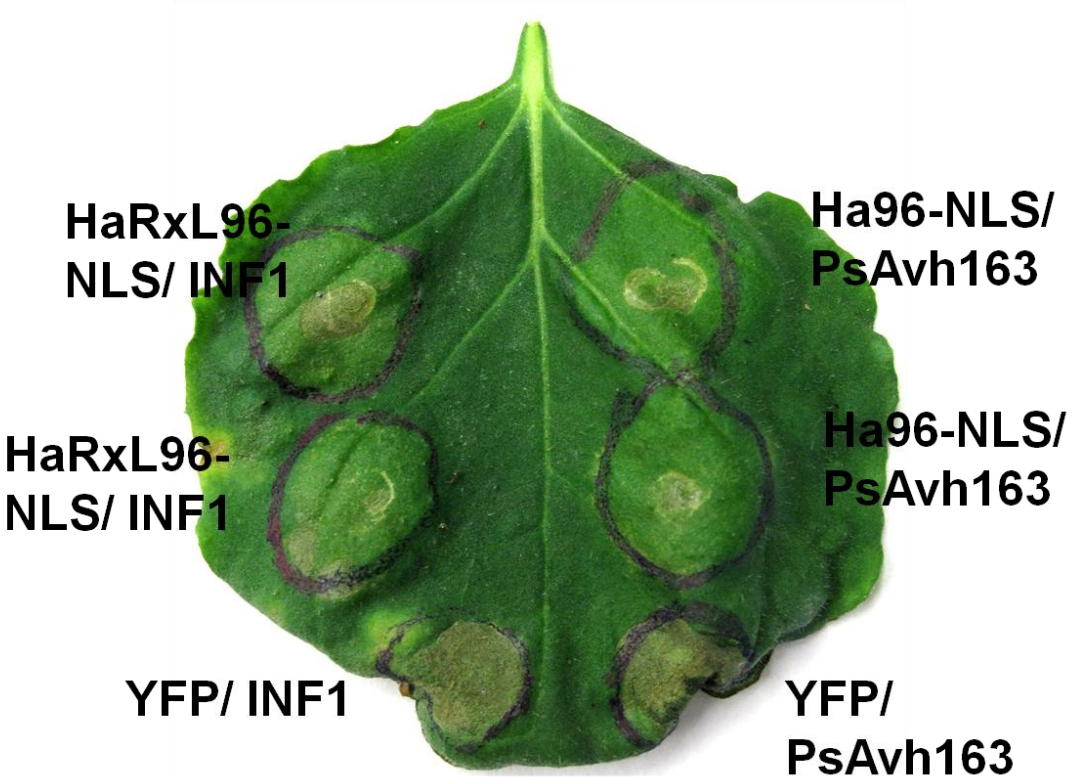


Figure 4.6 *Confining HaRxL96 to the nucleus does not interfere with cell death suppression in N. benthamiana. HaRxL96* was cloned with a nuclear localization signal (NLS) and transiently expressed in *N. benthamiana* via *Agrobacterium*. 2 days later, the same sites were challenged with the elicitors INF1 or PsAvh163. Cell death symptoms were visually monitored over a period of 7 days. YFP serves as a negative control.

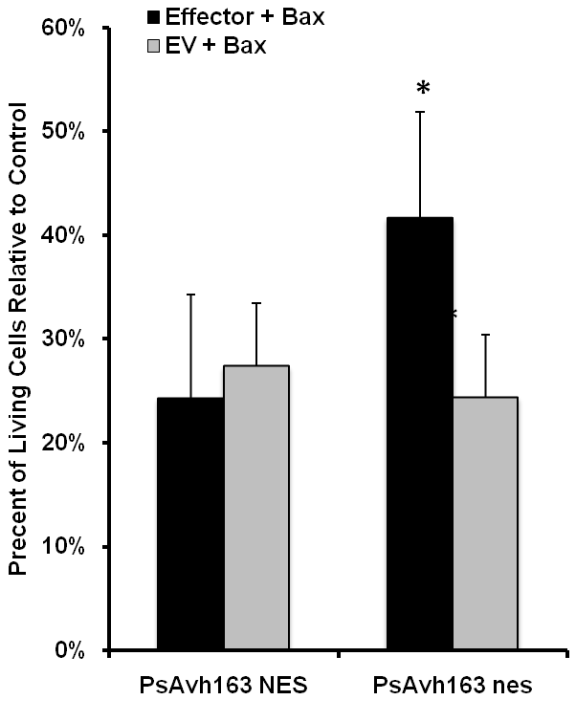


Figure 4.7 *Excluding PsAvh163 from the nucleus interferes with suppression of Bax-induced cell death in soybean.* PsAvh163 was cloned with a functional nuclear export signal (NES) or a non-functional export signals (nes) and co-bombarded with beta-glucuronidase (Gus) and Bax into detached soybean leaves. The control bombardment included empty vector (EV), Gus and Bax. Living cells were scored 3 days post bombardment by staining for Gus activity and subsequent visual quantification. Error bars represent standard error. * $p = < 0.05$

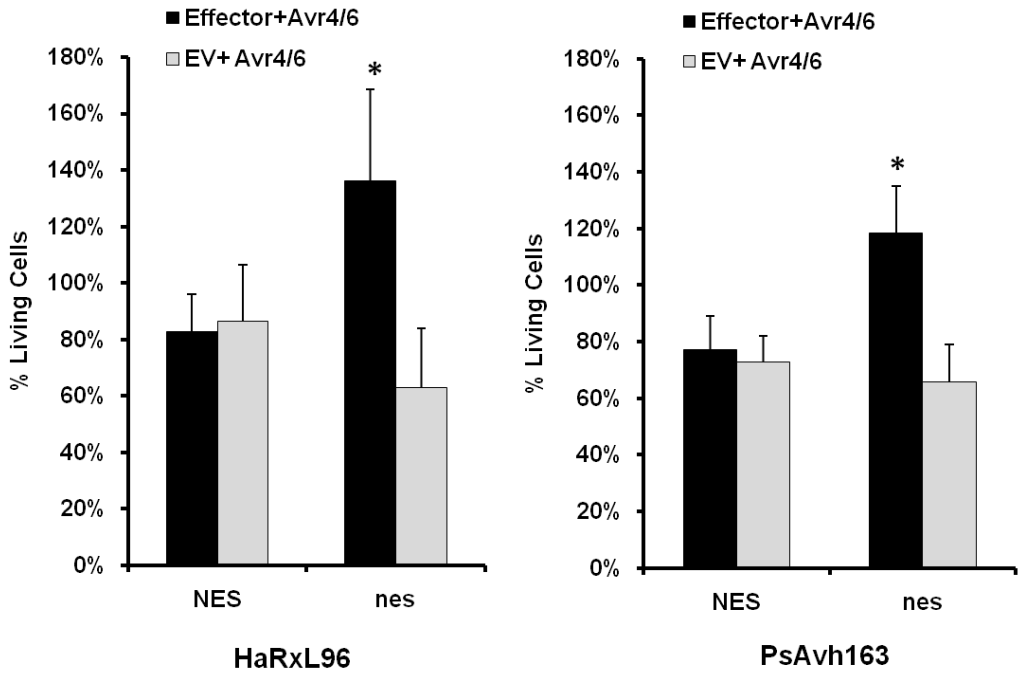


Figure 4.8 *Excluding PsAvh163 and HaRxL96 from the nucleus interferes with suppression of Avr4/6-induced PCD in soybean. PsAvh163* was cloned with a functional nuclear export signal (NES) or a non-functional export signal (nes) and co-bombarded with Gus and Avr4/6 into detached soybean leaves. The control bombardment included empty vector (EV), beta-glucuronidase (Gus) and Bax. Living cells were scored 3 days post bombardment by Gus staining and subsequent visual quantification. Error bars represent standard error. $p = < 0.05$

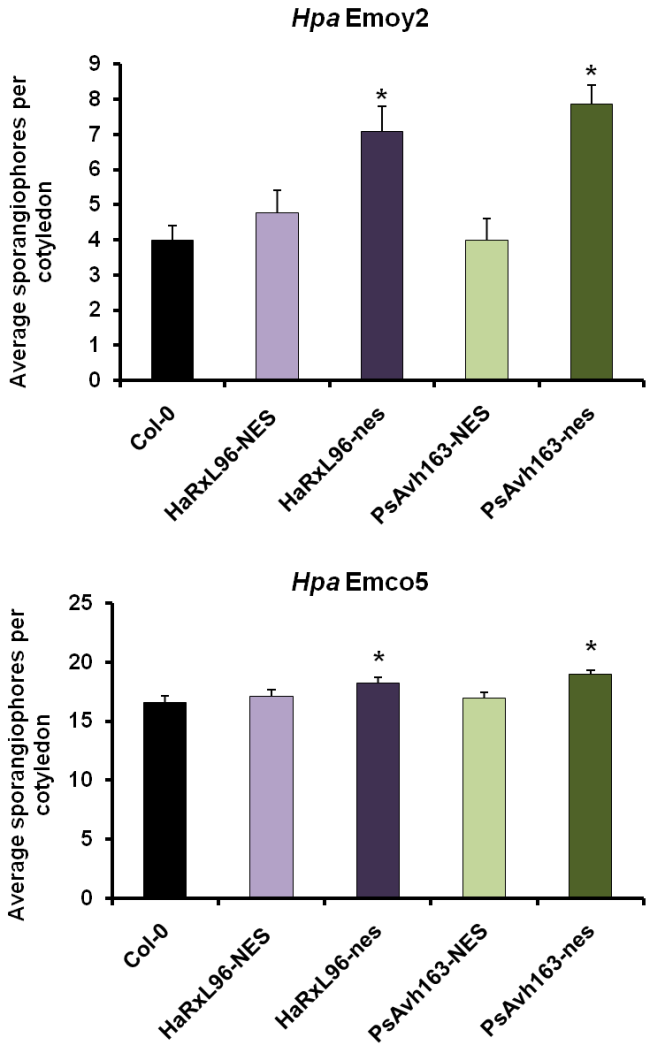


Figure 4.9 *Excluding PsAvh163 and HaRxL96 from the nucleus interferes with suppression of basal and RPP4 mediated resistance to Hpa.* Stably transformed *Arabidopsis* expressing *PsAvh163* or *HaRxL96* with a functional nuclear export signal (NES) or a non-functional export signal (nes) where challenged with *Hpa*. Growth of avirulent *Hpa* Emoy2 and virulent *Hpa* Emco5 is enhanced in effector expressing lines with a non-functional NES while growth of both pathogens occurs at wild-type levels in transgenic plants expressing effectors with a functional NES. Error bars represent standard error. $p = < 0.05$

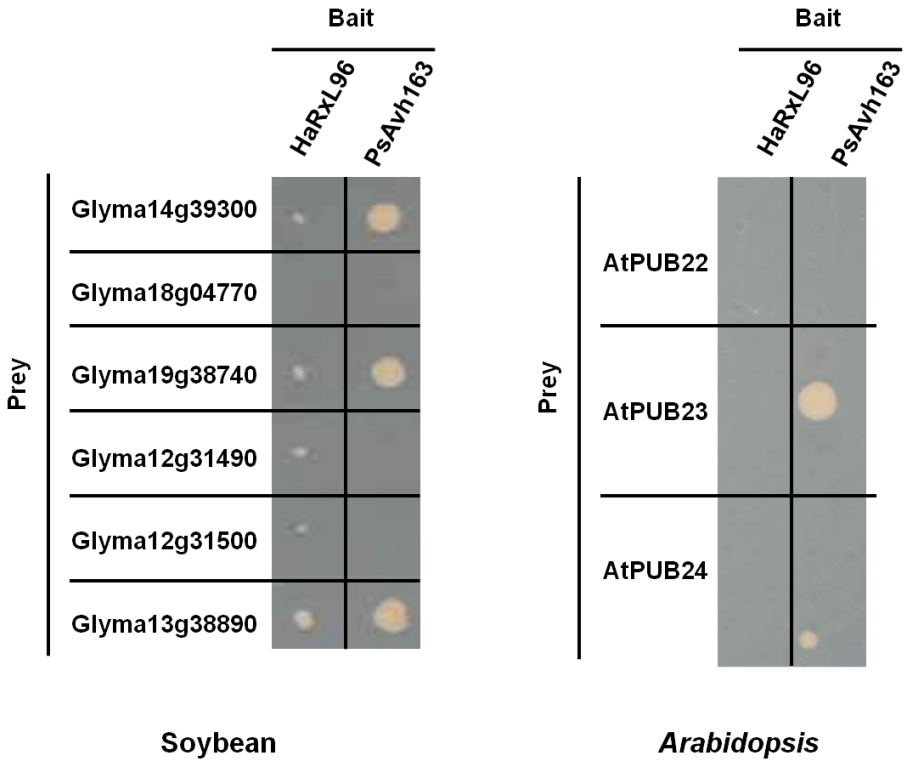


Figure 4.10 *HaRxL96 and PsAvh163 interact with PUB proteins from soybean and Arabidopsis in a yeast two-hybrid assay*. HaRxL96 and PsAvh163 were cloned into bait vectors while soybean PUB genes were cloned into prey constructs. Bait and prey strains were mated and subsequently plated on selective media. PsAvh163 interacts with 3 soybean PUB genes while HaRxL96 weakly interacts with all but one. PsAvh163 interacts with *Arabidopsis* PUB23 and PUB24, while Ha96 does not interact with any of the three AtPUB proteins. Images and Y2H screens were provided by Regina Hanlon, VBI.

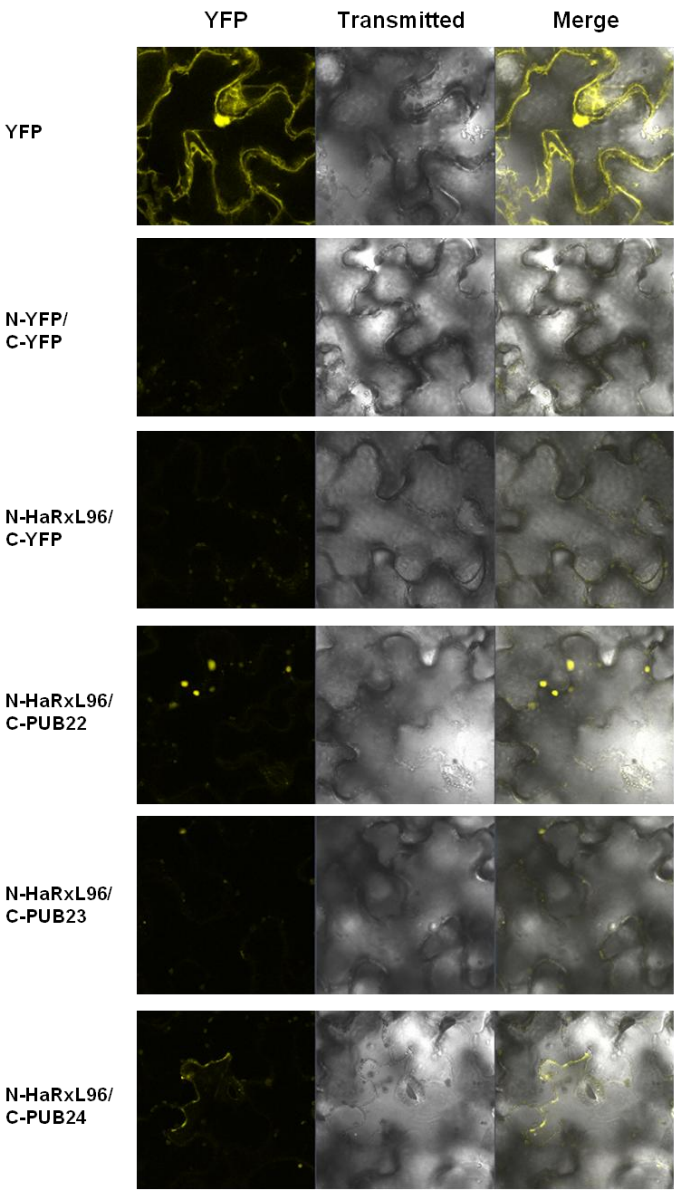


Figure 4.11. *HaRxL96 interacts with AtPUB22 and AtPUB24 in N. benthamiana.* Bimolecular fluorescence complementation (BIFC) was performed in *N. benthamiana* via ATTA. The indicated constructs were co-infiltrated and imaged 6 days post inoculation by confocal laser-scanning microscopy (CSLM). N-YFP/C-YFP serves as a negative control. Yellow florescent protein (YFP), transmitted and merged channels are shown. N terminal half of YFP (N), C terminal half of YFP (C).

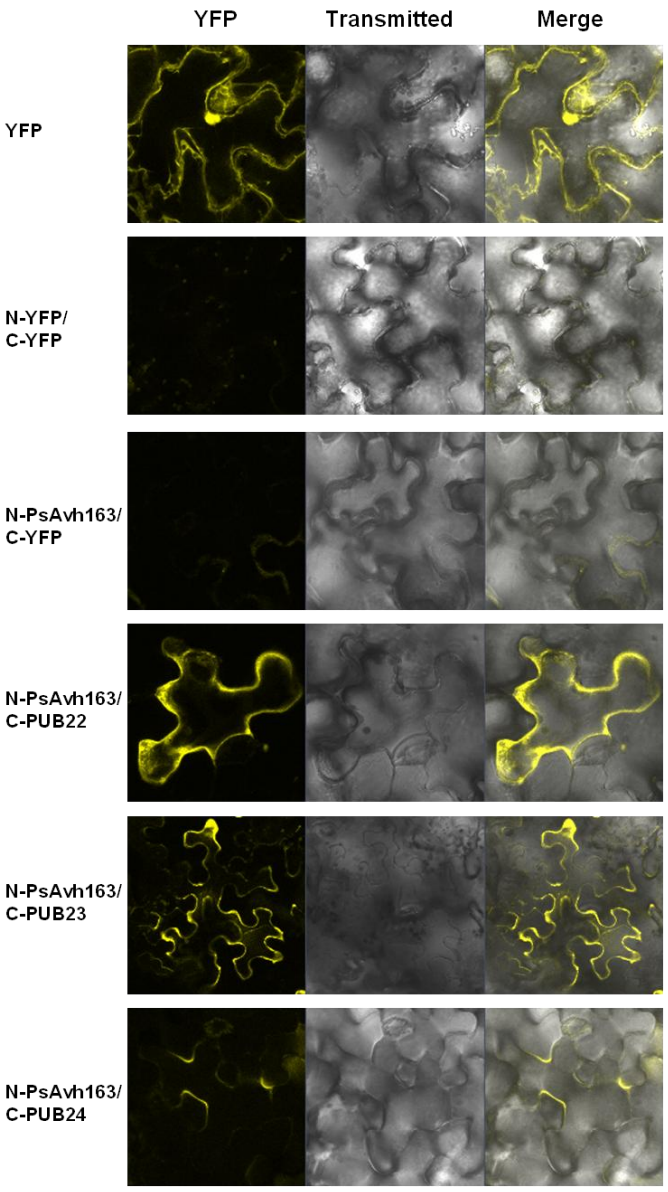


Figure 4.12. *PsAvh163 interacts with AtPUB22, AtPUB23 and AtPUB24 in N. benthamiana.* Bimolecular fluorescence complementation (BIFC) was performed in *N. benthamiana* via ATTA. The indicated constructs were co-infiltrated via ATTA and imaged 6 days post inoculation by confocal laser-scanning microscopy (CSLM). N-YFP/C-YFP serves as a negative control. Yellow florescent protein (YFP), transmitted and merged channels are shown. N terminal half of YFP (N), C terminal half of YFP (C). YFP and N-YFP/C-YFP panel are the same as in Figure 4.11

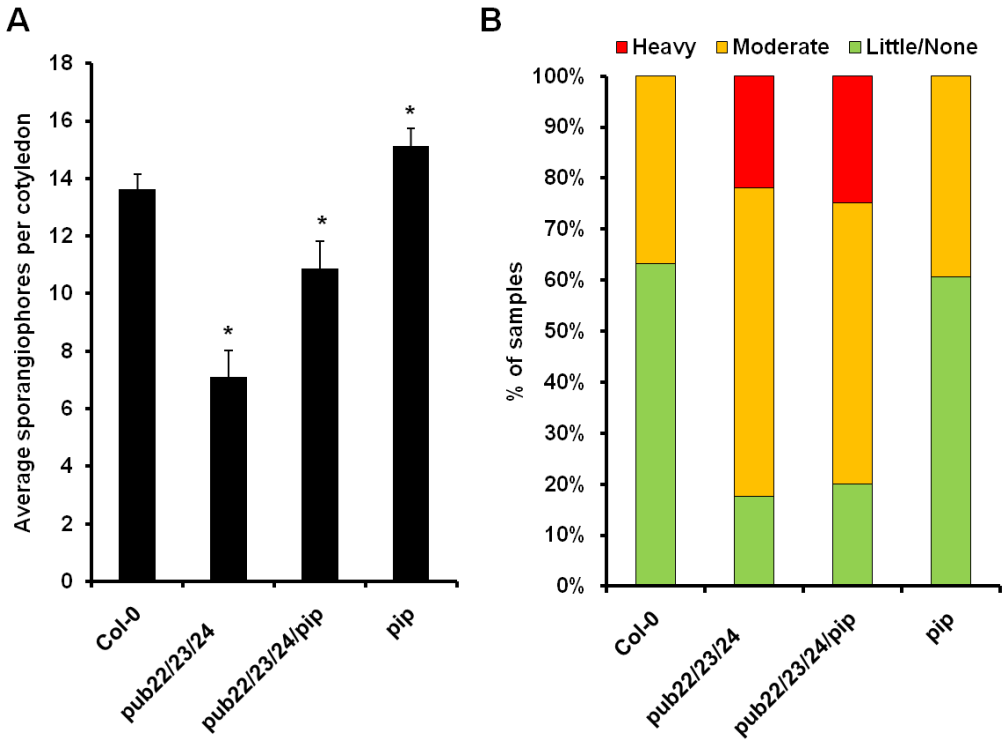


Figure 4.13 *PUBs regulate plant basal immunity.* **A)** Knock-out lines of *pub22/23/24*, *pub22/23/24/pip* and the *pip* mutant alone were challenged with virulent *Hpa* Emco5. Growth of *Hpa* Emco5 was reduced in the triple PUB knock-out while *pip* displayed enhanced growth of *Hpa* Emco5. **B)** Cotyledons of *Hpa* Emco5 infected plants were stained with trypan blue, assessed visually and scored for the level of cell death ranging from little/none, moderate, and heavy. % of samples indicates the percentage of cotyledons that were scored within the specified category. Error bars represent standard error. Significance determined by t-test. * p = < 0.05.

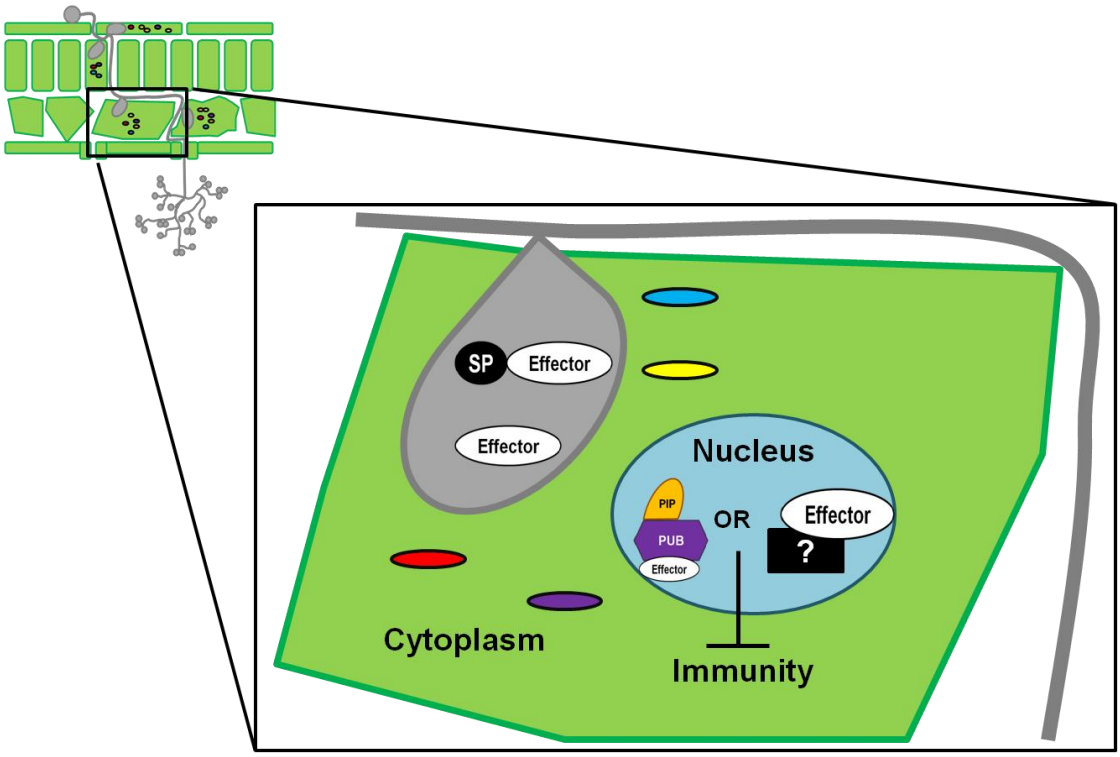


Figure 4.14 *Working model.* The signal peptide (Black, SP) of HaRxL96 and PsAvh163 (White, Effector) are cleaved off and direct the effectors for secretion. Effectors enter the cell and traffic to the nucleus where they interact with PUB proteins that in turn regulate PIP to suppress basal immunity. Alternatively, HaRxL96 and PsAvh163 interact with unknown host proteins (Black box,?) to suppress immunity from the nucleus.

MATERIALS AND METHODS

Construction of expression plasmids

HaRxL96 and PsAvh163 protein-coding sequences were amplified from genomic DNA. For *HaRxL96,* genomic DNA was isolated from Arabidopsis Oy-1 tissue colonized by *Hpa* Emoy2 and used as a template for PCR using the primer sets Ha96 NOSP and Ha96 S (with stop codon) or Ha96 NS (without stop codon) (Chapter 3). The *HaRxL96* allele from *Hpa* Emoy2 was used for all experiments described in this manuscript. For *PsAvh163,* genomic DNA was isolated from the *P. sojae* isolate P6954 (Race 2) and used as a template for PCR using the primer sets Ps163 NOSP and Ps163 S (with stop codon) or Ps163 NS (without stop codon). The PsAvh163 Race 2 allele was used for all experiments described in this manuscript. For all cloned ORFs, the 5' primer began with the codon immediately downstream of the signal peptide cleavage site predicted by SignalP. PCR amplicons with and without a stop codon were cloned in pENTR D/TOPO and shuttled into expression plasmids using the LR recombinase (Invitrogen). For *Agrobacterium* mediated transient expression studies, *HaRxL96* and *PsAvh163* Race2, Race7, and Race17 were shuttled from pENTR D/TOPO into pB2GW7. For subceullular localization, N-terminal YFP fusions were made by shuttling effector candidates into *pEarleyGate 104* (Earley et al., 2006) using LR recombinase (Invitrogen). All PCR products and resultant clones were confirmed by sequencing.

Plant growth, maintenance of Hyaloperonospora arabidopsidis, and generation of transgenic Arabidopsis

Arabidopsis, soybean, and *N. benthamiana* plants were grown in Sunshine Mix #1 at 16h light, 8h dark, 22^{o}C. *Arabidopsis* plants for pathogen assays were grown under 8 hours light at 22^{o}C, 16 hours dark at 20^{o}C. The *Hpa* isolates Emoy2 and Emco5 were maintained on Oy-1 and Ws-0 *Arabidopsis* plants, respectively (McDowell et al., 2011). Conidial suspensions of $5x10^{4}$ spores/ml were applied with a Preval spray unit and the plants were then kept under short day conditions. *Agrobacterium tumifaciens* strain GV3101 was transformed via electroporation and maintained on LB media with rifampin (100 μg/ml), gentamicin (25 μg/ml), spectinomycin (50 μg/ml) at 28C. Transgenic *Arabidopsis* Col-0 were generated by the floral dipping (Clough & Bent, 1998). Transgenic plants with resistance to were selected for BASTA resistance, the presence of the transgene was confirmed by PCR, and transgene, and transcription was verified by reverse-transcriptase PCR. Lines with single transgene loci were identified by segregation in the T2 generation and homozygous lines were identified by progeny testing in the T3 generation. All *Arabidopsis* experiments described in this study were performed on non-segregating T3 or T4 populations.

Transient assays in soybean

Two week old, detached soybean leaves were transformed using a modified BioRad PDS1000 gene gun (BioRad) as described (Kale & Tyler, 2011). Plasmid DNA mixtures were created for the effector, control, and elicitor samples. The effector samples were prepared as in Dou et al. 115 ug of the effector, 50 ug of Avr4/6 and 50 ug of GUS were combined for Avr4/6 suppression assays. For Bax suppression assays; 115 ug of the effector, 15 ug of Bax and 50 ug of GUS were combined. The control GUS sample contained 115 ug of empty vector and 50 ug of GUS plasmid DNA. The elicitor samples were mixed as follows. Avr4/6: 30ng of Avr4/6, 50 ug GUS plasmid, 70 ug empty vector. Bax; 15 ug Bax, 50 of Gus, 85 ug empty vector. Tungsten preparations were prepared according to Dou et al 2008. After bombardment, detached soybean leaves were incubated in Petri dishes with moistened Whatman filter paper at 22C/20C (8 hours light, 16 hours dark). Leaves were stained with X-Gluc and cleared with 70% ethanol for two days. GUS-expressing cells were visually quantified with a dissecting microscope. Wilcoxon Rank Sum method was used to assess statistical significance.

Transient assays in N. benthamiana and subcellular localization

Agrobacterium tumifaciens GV3101 strains with the appropriate constructs were grown overnight in LB with the appropriate antibiotics. Liquid cultures were harvested by centrifugation. Pellets were resuspended to the desired OD_{600} in MMA buffer (10 mM MgCl, 10 mM MES, 200 mM acetosyringone), incubated at room temperature for 1-3

hours, and infiltrated using needless syringes on the abaxial side of 3 to 5 week old leaves. Subceullular localization was preformed 2 days post infiltration using *Agrobacterium* at an OD600 between 0.7 and 1.0. The viral suppressor p19 was co-infiltrated with *Agrobacterium* strains harboring YFP constructs. Images were taken with confocal microsopy using a Zeiss Z.1, 25x water imersion objective, 488 HeNe laser line. Images were processed with Zeiss Zen software. For cell death suppression assays, *Agrobacterium* strains harboring effectors were infiltrated at an OD_{600}=0.5. Two days later the same sites were challenged with INF1 (OD_{600}=0.1) or PsAvh163 (OD_{600}=0.5) Cell death was monitored visually over a period of 7 days.

Trypan stain and cell death quantification

Trypan blue staining was used to visualize the pathogen growth and regions of cell death (McDowell et al., 2011). Tissue harvested from infect plants were incubated in alcoholic trypan blue solution (50 g phenol, 50 ml lactic acid, 50 ml glycerin, 50 ml water, and 100 mg trypan blue) that had been diluted with two parts 95% ethanol. The samples were incubated in solution for 3 minutes at 90˚C and an additional 5 minutes at room temperature. Samples were destained in a solution of chloral hydrate (2.5g/mL) for 48 hours to remove excess trypan blue. Samples were mounted 70% glycerol and imaged with a Ziess Primo Star light microscope and a 4x objective.

Nuclear enrichment of HaRxL96

Enriched nuceli were prepared from *N. benthamiana* as described (Fiil et al., 2008). Agrobacterium strains containing expression plasmids for YFP-HaRxL96 and the viral gene silencing suppressor p19 were coinfiltrated. After 2 days, tissue was harvested and frozen in liquid nitrogen. 5 grams of tissue was ground with morter and pestle into 30ml of NEB1 (2mM EDTA, 2.5mM DTT, v10 mM HEPES, pH 8, 0.4M sucrose and protease inhibitor). The suspension was filtered through a double layer of Miracloth into a 50mL conical tube. The filtrate was spun at 4c, 3000 x g for 10 min. The supernatant was decanted and the pellet resuspended in NEB2 (10 mM $MgCl_2$, protease inhibitor cocktail, 0.25M sucrose, 0.5 or 1.0% v/v Triton X-100) The mixture was resuspended gently and centrifuged at 10,000 x g for 10 min, at 4c. This was repeated with NEB2, 0.5% Triton X-100 until the pellet was white, 4 to 8 times. The resulting pellet was resuspended in 300uL of NEB3 (10mM HEPES, 2mM $MgCl_2$, 1.7M sucrose, 0.15% Triton X-100) and overlayed on 500uL NEB3 in a 1.5mL tube and centrifuged at 16,000 x g for 45 min. The resulting pellet was resuspended in 1X loading buffer, boiled for 20 minutes and centrifuged 15,000 x g for 15 min. The supernatant was considered nuculi enriched (NE). The supernatant from NEB2 spins are considered nuclear depleted (ND). HaRxL96 was detected by western blot. Transfers were blocked overnight, 4c, 4% non-fat dry milk and blotted with anti GFP antibodies (Covance) 1:2,000 overnight at 4c. For Histone H31:2,000 3 hours, and ant- tubulin (Sigma) 1:1,000 overnight at 4c.

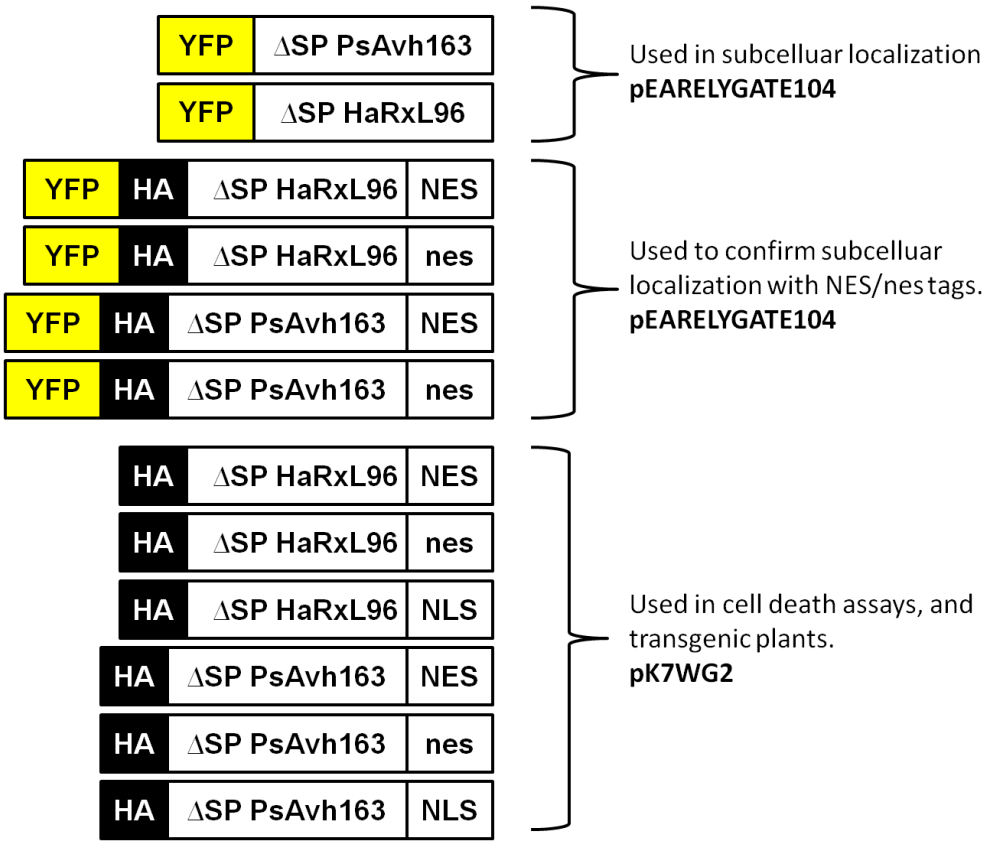
Yeast-2 hybrid screens

Yeast (Y8930) was transformed with a bait vector encoding the effector gene without the signal peptide. Concurrently, prey strain yeast (Y8800) was transformed with prey vectors containing host genes. Transformation was accomplished with lithium acetate/ polyethylene glycol. Positive transformants were selected on the appropriate drop-out media. For *pLAW10* (Trp) and *pLAW11* (Leu) we select on (-Trp) for the bait and on (-Leu) for the prey. T-streak plates (-Leu or -Trp) were created from a single colony from the transformation plate. Mating was accomplished with a loop of the haploid strains in YEPD +Ade overnight and plated 40 to 100uL of the 2mL overnight culture on (-LT). Diploid colonies were selected on (-LT). Colonies were overnight in -LT liquid and diluted to OD_{600}= 0.400 . Cultures were plated in duplicate on:-LT, -LTH, -LTA, -LTH, and +3AT. Preformed by Regain Hanlon, VBI.

SUPPORTING INFORMATION

Localization Tag	Protein Sequence	DNA Sequence
NES	**NELALKLAGLDINK**	**AACGAGCTTGCTCTTAAGTTGGCTGGACTTGATATTAACAAG**
nes	**NELALKAAGADANK**	**AACGAGCTTGCTCTTAAGGCAGCTGGAGCAGATGCTAACAAG**
NLS	**GAPKKKRKVK**	**GGAGCTCCGAAGAAGAAACGAAAGGTAAAGTGA**

Supplemental Table 4.1 Summary of localization tags used in this study. Functional nuclear export sequence (NES), non-functional export sequence (nes), nuclear localization sequence (NLS)



Supplemental Figure 4.1 *Constructs used and cloning strategy.* Functional nuclear export sequence (NES), non-functional export sequence (nes), nuclear localization sequence (NLS), yellow flurocent protein (YFP), hemagglutinin (HA) epitote tag, signal peptide (SP). Plasmids used are highlighted in bold.

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Chapter 5

Quantitative PCR monitoring of *Hyaloperonospora arabidopsidis* reveals differential growth dynamics

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Abbreviations: days post inoculation (DPI), *Hyaloperonospora arabidopsidis* (*Hpa*), quantitative real-time PCR (qPCR), genomic DNA (gDNA), recognition of *peronospora parasitica 4* (RPP4), hypersensitive response (HR), polymerase chain reaction (PCR)

ABSTRACT

Accurate quantification of disease severity is important for assessing host-pathogen interactions in laboratory or field settings. Current methods for measuring disease severity in *Arabidopsis thaliana* infected by the oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) rely on measurements at the terminal stage of pathogen development; namely, visual counts of spore-producing structures or measuring spore production with a hemocytometer. These methods are useful, but do not offer sensitivity for robustly quantifying small changes in virulence or accurate quantification at earlier stages of the interaction that are not amenable to visual assays. Here, we describe a quantitative PCR assay for monitoring *Hpa* growth *in planta*. This assay is highly specific and can monitor pathogen development at biologically important early stages of the plant-pathogen interaction. We infiltrated the defense compromised *Arabidopsis* mutant, *eds1-1* with fungicide and effectively monitored a significant growth reduction. This approach provides a method for identifying novel oomycete growth inhibitors. Our protocol is rapid, inexpensive, can robustly distinguish small changes in virulence, and provides insight into the dynamics of *Hpa* mycelial growth.

INTRODUCTION

Plant pathogens have evolved from diverse lineages including bacteria, fungi, viruses, and oomycetes. These pathogens pose a significant worldwide threat to growers, costing billions of dollars and affecting the marketability and yield of agricultural products. Oomycetes are characterized by cellulose based walls and non-septate hyphae. Despite similar physical features to true fungi including haustoria, spore bearing structures, and filamentous growth (Latijnhouwers et al., 2003), oomycetes are phylogenetically distinct and are classified in the kingdom *Stramenophila* which include diatoms and brown algae (Baldauf et al., 2000). Thus, oomycetes and fungi have evolved independently to infect land plants. Oomycetes include the orders *Peronosporales and Albuginales*, and the genera *Pythium* and *Phytophthora* (Cooke et al., 2000), all of which have tens or hundreds of species that cause important plant diseases.

Hyaloperonospora arabidopsidis (*Hpa*) is the downy mildew of *Arabidopsis thaliana* and is characterized by formation of aerial sporangiophores (asexual fruiting bodies) during the late stages of infection (Holub, 2008, Koch & Slusarenko, 1990). The *Arabidopsis-Hpa* interaction occurs frequently in nature, with a high level of genetic diversity (Coates & Beynon, 2010). A large number of *Arabidopsis* ecotypes have been documented as resistant or susceptible to various isolates of *Hpa* collected from field populations of *Arabidopsis* (Holub, 2008). In most cases, resistance is conditioned by genes encoding NB-LRR immune surveillance proteins, which recognize cognate effectors that are secreted from the pathogen during infection (Jones & Dangl, 2006).

Moreover, many induced mutants with altered responses to *Hpa* have been described (Coates & Beynon, 2010, Slusarenko & Schlaich, 2003). For example, loss-of-function mutations in several immune signaling genes produce an "enhanced disease susceptibility" phenotype, in which growth of already-virulent isolates is enhanced, compared to the wild-type host.

Hpa is one of only a few eukaryotic microbes that are specifically adapted to *Arabidopsis*. Thus, the *Hpa-Arabidopsis* interaction has proven to be a useful pathosystem to exploit the experimental advantages of *Arabidopsis* to understand the molecular basis of plant-pathogen interactions. Additionally, *Hpa* serves as a model to understand the molecular basis and evolution of obligate biotrophy, in which the pathogen extracts nutrients exclusively from living cells and cannot be cultured apart from its host (Baxter et al., 2010). Typically, *Hpa* growth is quantified at terminal stages of pathogen growth after the pathogen has completed its lifecycle. This is done through visual quantification of sporangiophores (asexual fruiting bodies) or by counting the spores with a hemocytometer and normalizing to tissue weight (McDowell et al., 2011). Both techniques offer limited precision when determining small changes in resistance or susceptibility. Furthermore, pathogen growth cannot be monitored with these techniques at the early stages of the host-pathogen interaction.

Quantitative PCR (qPCR) is increasingly adopted for plant pathogen genotyping, diagnostics, and for quantifying pathogen growth i*n planta*. Previously, several groups have published methods for real-time PCR monitoring of plant pathogens including

Phytophthora infestans (Alonso et al., 2010), the downy mildews *Peronospora arborescens, Peronospora sparsa* (Landa et al., 2011, Kokko et al., 2006), the fungi *Alternaria brassicicola, Botrytis cinerea* (Gachon & Saindrenan, 2004), *Hpa* and *Pseudomonas syringae* (Brouwer et al., 2003). Here, we describe a protocol utilizing real time PCR for *Hpa* monitoring that employs standard primers and crude genomic DNA preparations, with low cost and high throughput. Our procedure also includes a real-time PCR assay with *Arabidopsis* actin, to enable normalization to host biomass and provide accurate resolution of small differences in susceptibility. In addition, we describe a practical application of this method by monitoring *Hpa* growth in a defense compromised *Arabidopsis* plants treated with the systemic benzenoid fungicide metalaxyl (Kerkenaar & Sijpesteijn, 1981).

RESULTS

Assay sensitivity and specificity

Quantitative real-time PCR (qPCR) is a sensitive assay and requires robust primers that amplify the target sequence with high efficiency. Our target genes are actin in *Hpa* (Actin, CL32Contig1, gene_id_807716) and in *Arabidopsis* (AtActin2, At1g49240). The use of internal host controls for normalization and pathogen quantification has been utilized by other groups (Kokko et al., 2006, Alonso et al., 2010). To determine primer efficiency, a standard curve of known genomic DNA (gDNA) concentrations from infected tissue was used a template for qPCR (**Figure 5.1**). The input of gDNA ranged from 5ng to 1000ng of total template per qPCR reaction, which

encompasses and extends beyond normal operating conditions for this assay. qPCR assays for both *AtActin* and *HpaActin* generate ideal curves and efficient amplification over a large range of template concentrations (**Figure 5.1A**). A standard curve of Ct values plotted against the Log value of gDNA yielded slope values (M) of -2.322 and -2.895 for the AtActin and HaActin respectively (**Figure 5.1B**). The primer sets for AtActin and HaActin yielded highly linear amplification over the range of template concentration with a correlation coefficient of R^2>0.99 with similar slopes, suggesting matching efficiencies for both primer sets (**Figure 5.1B, Supplemental Figure 5.1**) (Livak & Schmittgen, 2001).

Because actin is a highly conserved gene (Mitchison, 1995), it is important to validate the specificity and lack of cross reactivity of each primer set in a mixed sample of genomic DNA from the plant and the pathogen. To confirm primer specificity, a dissociation curve analysis was performed, comparing the products amplified from infected with *Hpa* to uninfected tissue. Dissociation curve analysis of the final qPCR amplicons from uninfected tissue revealed that HaActin primers did not amplify any products, as expected. Conversely, a single peak was produced by HaActin primers from tissue infected with *Hpa* (**Figure 5.2**). The Tm of the HaActin amplicon is 82.93^{o}C. The AtActin primer set produced a single peak with a Tm of 79.77^{o}C from both infected and uninfected templates. The strong single peak of AtActin in samples with infected tissue demonstrates that AtActin does not amplify from the *Hpa* Actin gene.

In planta growth assays on three Arabidopsis genotypes that vary in their susceptibility to Hpa Emoy2

Seedlings of three *Arabidopsis* genotypes (Col-0, Oy-1 and the mutant *eds1-1*) were inoculated with the *Hpa* isolate Emoy2, these genotypes are resistant, moderately susceptible, and highly susceptible to *Hpa* Emoy2, respectively (Aarts et al., 1998, van der Biezen et al., 2002, Holub et al., 1994). Tissue homogenization was accomplished with a bead beater that allows us to process a large number of samples and reducing between sample variability in the level of tissue disruption. *Hpa* growth was monitored over the course of infection up to six days post inoculation, just prior to the onset of sporulation, using the primer sets for HaActin and AtActin (**Figure 5.3**). Additionally, we stained plants with trypan blue, which highlights pathogen hyphae and host cell death. These samples were used to visually correlate hyphal growth at each time point with the values from the qPCR assay.

The *RPP4*-mediated HR was macroscopically visible after three DPI in cotyledons stained with trypan blue (**Figure 5.4**) and became very apparent by four DPI with profuse HR by seven DPI. Correspondingly, the growth rate of *Hpa* Emoy2, as measured by qPCR, in Col-0 slows at three DPI although the reduction in growth rate is apparent at two DPI (**Figure 5.2**). qPCR indicated that *Hpa* Emoy2 growth was insignificant by 7 DPI. qPCR confirmed the strong Col-0 resistance to *Hpa* Emoy2 owing to the *RPP4* gene, which encodes a Toll/interleukin-1 receptor, nucleotide binding, leucine rich repeat (TIR-NB-LRR) (van der Biezen et al., 2002).

Oy-1 is a naturally occurring ecotype of *Arabidopsis* that is susceptible to *Hpa* Emoy2 (Holub et al., 1994). Growth was steady over the seven-day period (**Figure 5.2**) and little to no HR cell death was visible in samples stained with trypan blue. Oospore (sexual spore) production was apparent by four DPI. Growth measured by qPCR correlated to the levels of hyphal growth observed in samples stained with trypan blue. As expected, *Hpa* biomass was much higher in Oy-1 than in the resistant ecotype Col-0.

The third genotype that we tested was the immuno-compromised mutant *eds1-1* (enhanced disease susceptibility 1). EDS1 is an important regulator of plant immunity. Loss-of-function mutations in this gene therefore cause a phenotype of enhanced susceptibility, relative to wild-type, susceptible Arabidopsis genotypes (Parker et al., 1996). We included this genotype to test whether the qPCR assay could accurately resolve small differences in virulence. The *eds1-1* mutant supported higher levels of *Hpa* Emoy2 and the difference in the rate of growth was clear by two DPI. At six DPI the overall levels of *Hpa* Emoy2 were approximately three-fold higher than the naturally susceptible Oy-1 ecotype. As with Col-0 and Oy-1, hyphal growth in leaves stained with trypan blue visually correlated with qPCR measurements. Abundant hyphal growth was observed at two DPI and is clearly higher in comparison to Oy-1 and Col-0 at the same time (**Figure 5.2**). Oospores were visible at four DPI, similar to Col-0. These results indicate that we successfully resolved small changes in virulence at early time points of the interaction.

Correlation between qPCR measurements and traditional sporangiophore counts

To further validate the qPCR assay, we compared the measurements acquired by qPCR with estimates of growth based on traditional sporangiophore counts. We counted sporangiophores per cotyledon at seven DPI, from the same experiments used for the qPCR assays. The qPCR asssay of *Hpa* growth at six DPI correlated with previous time points, with little biomass in Col-0, moderate biomass in Oy-1 and enhanced growth in *eds1-1* (**Figure 5.5B**). As expected, production of sporangiophores per cotyledon on Col-0 was very low, and higher on Oy-0 and *eds1-1* (**Figure 5.5A**). The measurements derived from qPCR and sporangiophore counts correlated well when the values were plotted against each other. A correlation coefficient of 0.99 was obtained (**Figure 5.5C**) suggesting that the qPCR assay is a valid method for measuring *Hpa* growth.

Potential applications for qPCR measurements of Hpa growth

Identifying novel compounds that can inhibit pathogen growth is valuable for disease control. qPCR is a potentially valuable approach for accurate quantification of the effects of growth inhibitors. As a proof-of-concept, we infiltrated four-week old *eds1-1* plants with the systemic benzenoid fungicide metalaxyl, or water as a control. The leaves were allowed to dry for one hour and were subsequently infected with *Hpa* Emoy2. Leaf punches were collected at six DPI and gDNA was extracted and used as the template for qPCR. Metalaxyl is a known inhibitor of oomycete growth and is used regularly as a seed treatment for control of downy mildew disease. qPCR verified that metalaxyl inhibited

Hpa growth on the immune compromised *eds1-1* plants, compared to *eds1-1* plants that were infiltrated with water alone (**Figure 5.6**). This demonstrates that infiltrating compounds and subsequent infection, measured by qPCR, will be useful for identifying novel growth inhibitors.

DISCUSSION

Hpa is an obligate biotroph and has proven to be a useful pathosystem in which to study the interaction between plant hosts and oomycete pathogens. The recent sequencing of the *Hpa* genome has created opportunities to understand this interaction in further detail by revealing many effector gene candidates that are predicted to mediate the interaction (Baxter et al., 2010). Recently, a large scale survey of the plant-pathogen interactome, using yeast two hybrid technology, revealed many putative plant targets of *Hpa* effectors (Mukhtar et al., 2011). Many of the knockout lines of *Hpa* targets yielded small changes in *Hpa* growth (Mukhtar et al., 2011). These and other advances have created a need for new assays that accurately measure small differences in *Hpa* growth *in planta*. The small changes in susceptibility will require increased resolution beyond the current methods of quantifying terminal points of *Hpa* growth. For example, sporangiophores are difficult to count accurately when they grow densely from the surface of the leaves of heavily infected plants. As an alternative to sporangiophore counts, spores can be harvested, counted in suspension using a hemocytometer, and normalized to fresh tissue weight. In our hands, this method has to potential for high variability introduced during spore collection as *Hpa* conidia are easily dispersed. Thus,

this method is best for observing large changes in host resistance or pathogen virulence. In the last several years, protocols detailing methods to quantify pathogen growth with qPCR have been developed and shown to offer sensitivity, precision, and accuracy. qPCR procedures offer the added advantage of measuring all pathogen structure including asexual conidia, sexual oospores and hyphae and thus is a better measure for overall pathogen fitness.

For these reasons, we sought to develop an inexpensive, high-throughput procedure for qPCR quantification of *Hpa* growth during infection of *Arabidopsis*. Our protocol complements and extends a previously published qPCR method for *Hpa* quantification (Brouwer et al., 2003). Our protocol differs from the previously published version in three respects: First, our protocol includes an assay for AtActin, which enables accurate normalization of pathogen biomass to plant biomass and eliminates the need for normalization based on tissue weight or other measures. Second, we use a very simple method for gDNA extraction that uses common, low-cost reagents. Additionally, we optimized a high-hroughput procedure for DNA extraction using a leaf punch to collect tissue and a bead-beater for tissue disruption, with subsequent steps performed in microtiter plates. Finally, we demonstrate the feasibility of developing an assay to identify novel *Hpa* growth inhibitors. In sum, this protocol enables large numbers of samples to be processed simultaneously with minimal expense.

Our optimization experiments demonstrate that the AtActin and HaActin primers are specific for their targets and provide linear amplification through a large

concentration range of gDNA that represent the upper limits and lower limits of the normal operating range of this assay. Importantly, the estimates of *Hpa* biomass from the qPCR method correlated well with hypal growth observed with trypan blue and with sporangiophore production. These correlations held true in resistant, moderately susceptible, and highly susceptible interactions. This indicates that amplification from the single copy HaActin target serves as an unbiased and faithful proxy for *Hpa* growth *in planta*. The qPCR method is also sensitive, as demonstrated during the early time points of the *Hpa-Arabidopsis* interaction. RPP4 resistance to *Hpa* Emoy2 is manifested through HR and is visible with typan blue staining by three DPI. However, differences in *Hpa* growth rate were clear as early as two DPI with qPCR, suggesting that RPP4 resistance is established earlier than can be visually detected.

Our time course experiment comparing *Hpa* growth rates in *Arabidopsis* lines with moderate and extreme susceptibility provide a previously unattainable quantification of *Hpa* growth from the beginning to the end of the infection cycle. Interestingly, the rate of growth in the enhanced disease susceptibility mutant was the same as in the wild-type, moderately susceptible Oy-1 accession during the first day after inoculation. During this interval, the *Hpa* spores germinate and hyphae emerge to penetrate between epidermal cells into the mesophyll layer. The first haustoria are formed during this time interval. At two DPI, hyphae have extended further into the mesophyll. This was the time point at which differences in colonization became apparent between Oy-1 and the *eds1-1* mutant, suggesting that EDS1-dependent basal defenses are activated after the initial penetration steps. It should be noted that *eds1-1* is in a different genetic background (Ws-0) and is

thus not isogenic with Oy-1. Thus, relative rates of *Hpa* Emoy2 growth in the two host backgrounds could be affected by other genetic differences in addition to the *eds1-1* mutation. However, this experiment still provides proof-of-concept that the qPCR assay can enable accurate measurements of growth rates *in planta*.

Measuring pathogen growth from gDNA extracted from adult leaves allows the possibility of infiltrating compounds and observing their effect on *Hpa* growth and disease progression. As a proof of concept, we infiltrated metalaxyl, a systemic benzenoid fungicide that inhibits nucleic acid synthesis. A large reduction of growth was observed even in the extremely susceptible mutant genotype *eds1-1,* confirming that metalaxyl's inhibitory effects are due entirely to its inherent toxicity rather than activation of plant immunity. Infiltration of compounds allows the flexibility of testing not only chemicals but recombinant proteins or extracts that could not be applied with a foliar spray. In addition, our protocol now allows the possibility for testing early generation transgenic *Arabidopsis* plants without breeding them to a homozygous state for traditional seedling infection assays, which require large, genetically uniform populations for accurate quantification of sporangiophores. Thus, our procedure will increase the throughput and accuracy at which we can investigate the contribution of host and pathogen genes to the *Hpa-Arabidopsis* interaction.

FIGURES

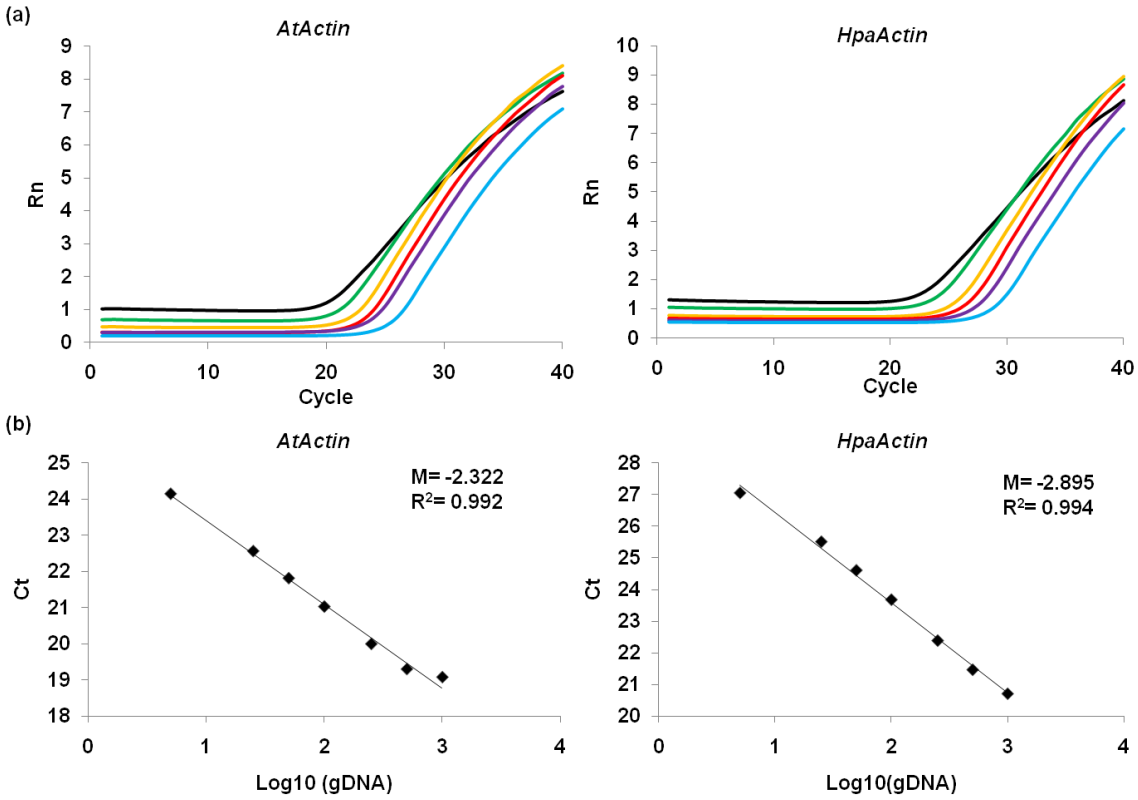

Figure 5.1: *Quantification of HpaActin and AtActin genes in genomic DNA extracted from Arabidopsis infected with Hyaloperonospora arabidopsidis.* (a) Quantitative, real-time PCR amplification profiles of normalized florescence (Rn) plotted against cycle number with AtActin and HpaActin primers. (Black 500ng, Green 250ng, Orange 100 ng, Red 50ng, Purple 25 ng, Blue 5ng) (b) Standard curve assays to measure the efficiency of AtActin and HpActin primers fron dilution series of genomic DNA extracted from *Arabidopsis* infected with *Hyaloperonospora arabidopsis*. M: slope, R^2: correlation coefficient.

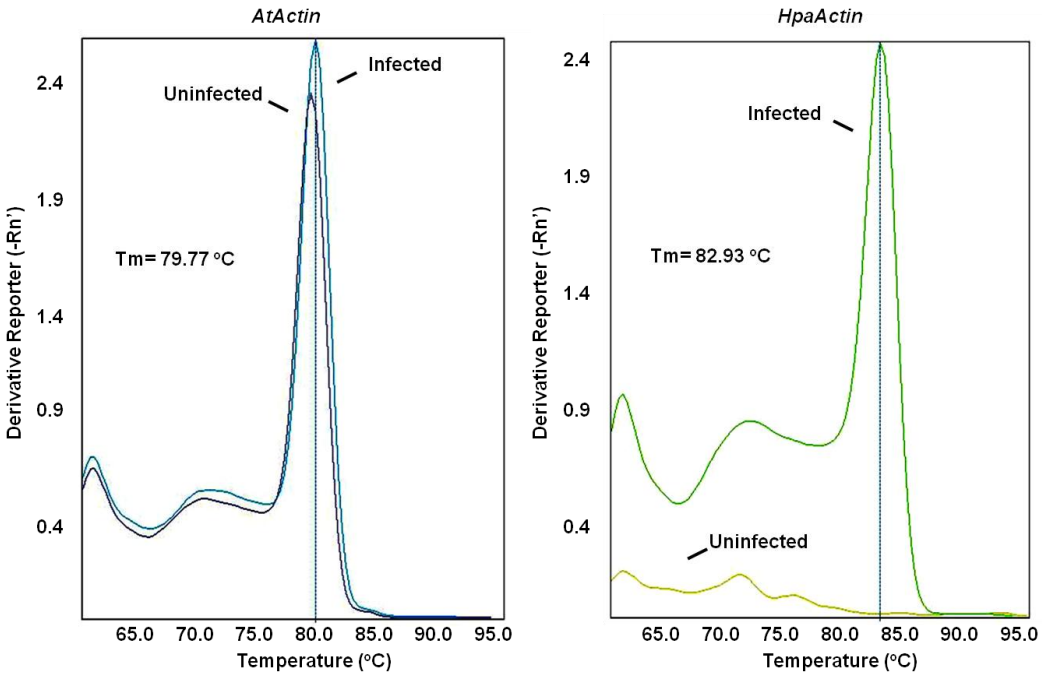

Figure 5.2: *Dissociation curve analysis of PCR amplicons generated by the AtActin and HpaActin primer demonstrate target specificity.* Inverse fluorescence (Derative reporter) plotted against temperature. (Light blue: uninfected *Arabidopsis*, Dark blue: infected *Arabidopsis*, Yellow: uninfected *Arabidopsis*, Green: infected *Arabidopsis*)

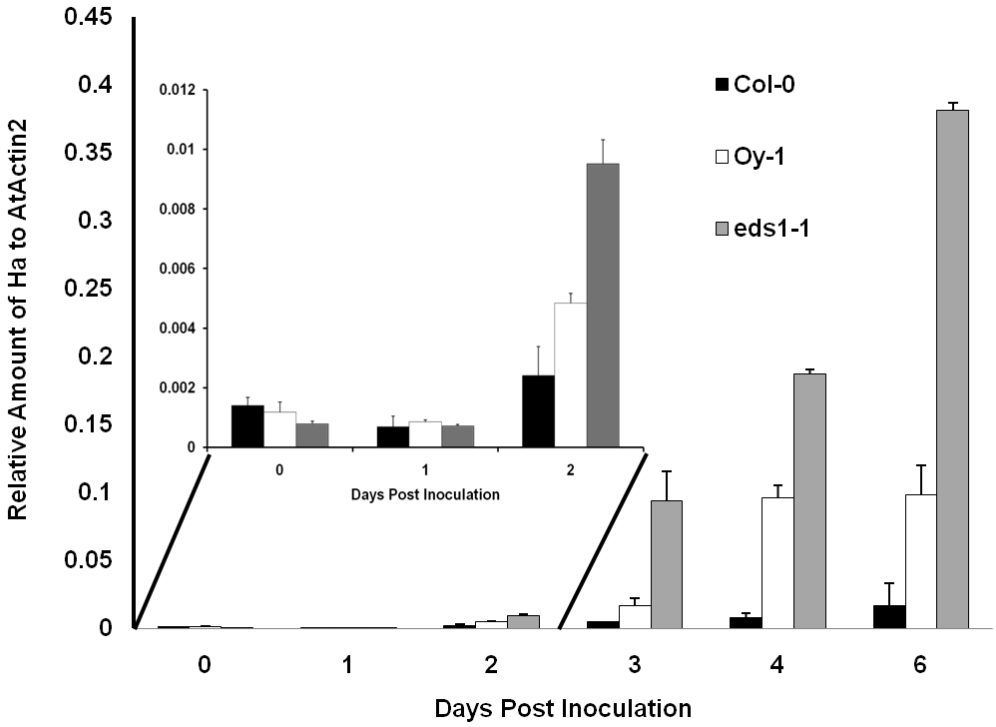

Figure 5.3: *Time course of Hyaloperonospora arabidopsidis Emoy2 growth during infection and colonization of resistant (Col-0), moderately susceptible (Oy-1), and highly susceptible (eds1-1) Arabidopsis. Hpa* growth was monitored over a 6 day time course on three different genotypes using real-time PCR. Growth is plotted as the relative quantity of HpaActin to AtActin (2^(dCt)). (Black: Col-0, White: Oy-1, Grey: *eds1-1*) Error bars represent standard deviation.

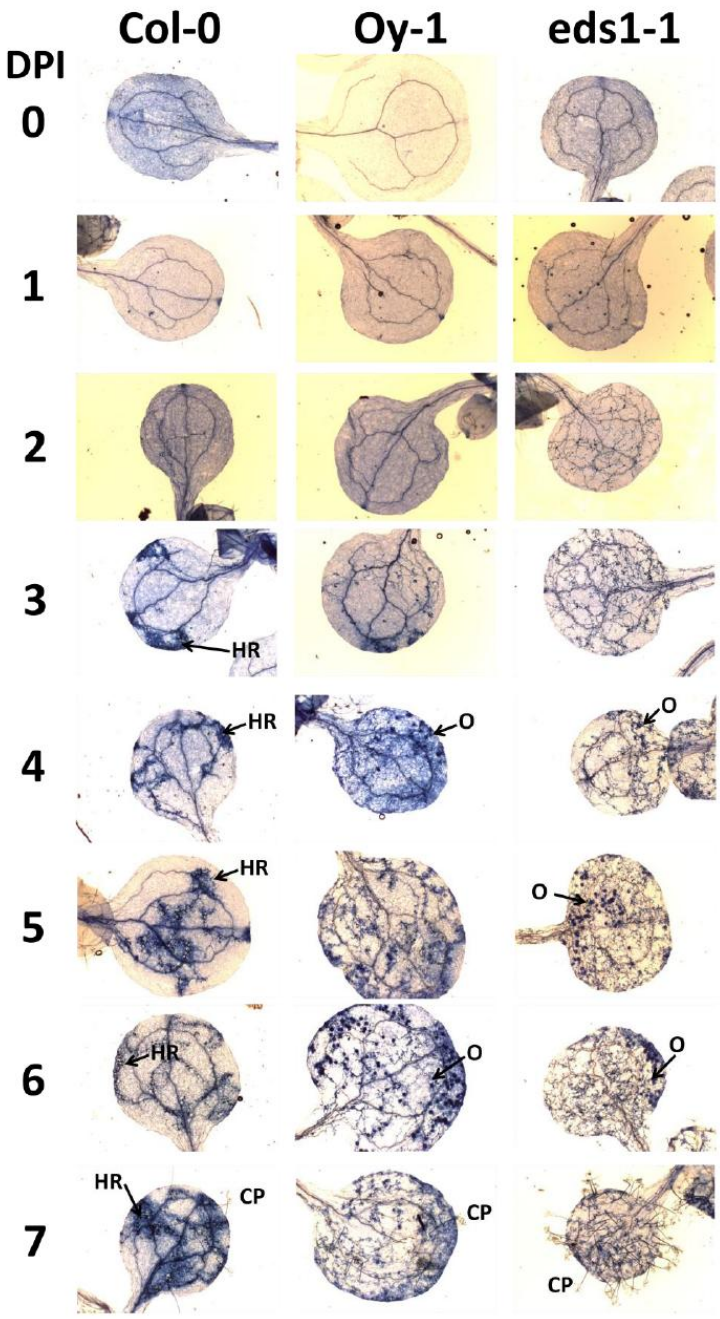

Figure 5.4: *Hpa Emoy2 growth correlates with real-time results. Hpa* infected tissue was stained with trypan blue and visualized with a light microscope during a time course of 7 days. (HR: hypersensitive response, CP: conidiophore, O: oospore DPI: days post inoculation)

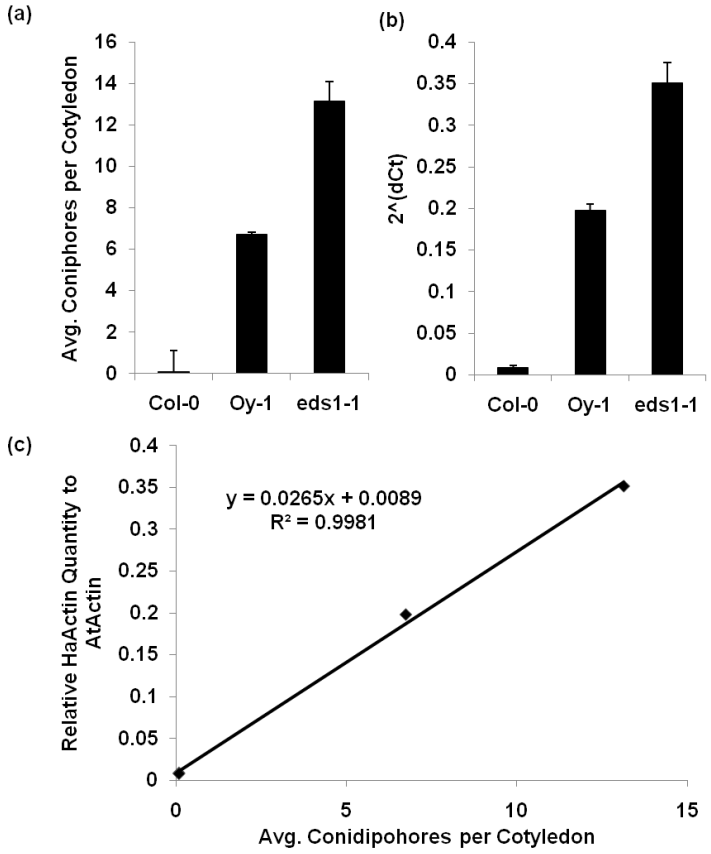

Figure 5.5. *Hpa sporulation correlates with PCR-based quantification of hyphal growth in infected tissue.* (a) Visual spore counts 7 days post inoculation (DPI). (b) Relative abundance of *HpaActin* to *AtActin* of infected plants 6 DPI. (c) Results of qPCR quantification depicted in B, plotted against the spore counts depicted in A. (R^2: correlation coefficient) Error bars represent standard error in A and standard deviation in B.

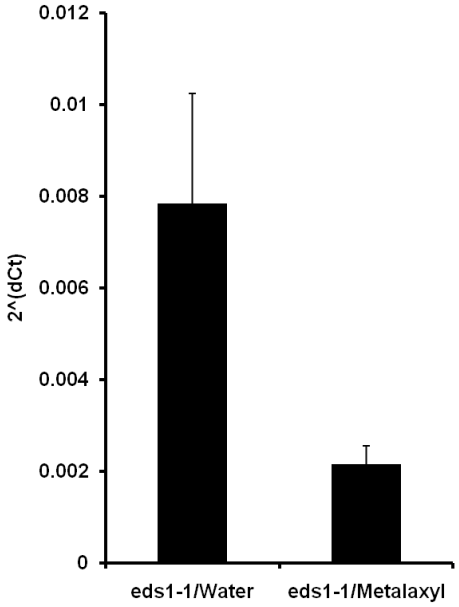

Figure 5.6. *PCR-based quantification of the extent to which metalaxyl inhibits Hpa growth. Arabidopsis eds1-1* plants were infiltrated with water or the fungicide Metalaxyl and subsequently inoculated with *Hpa* Emoy2. Growth was quantified at 5 DPI with qPCR Error bars represent standard deviation.

MATERIALS AND METHODS

Plant growth and maintenance of Hyaloperonospora arabidopsidis

Arabidopsis plants for pathogen assays were grown under short day conditions (8 hours light 16 hours dark) at 22c/20c in Sunshine Mix #1. The *Hyaloperonospora arabidopsidis* isolate Emoy2 was propagated and maintained on Oy-1 and *eds1-1 Arabidopsis* plants. Conidial suspensions of 5x10^4 spores/ml were prepared from sporulating plants and applied with a Preval^{TM} spray unit to 10-12 day old seedlings. Inoculated seedlings were covered for 24 hours, then uncovered and kept under short day conditions as described above. At six DPI, infected seedlings were covered and the relative humidity was raised to 100% to trigger sporulation.

Sample collection and genomic DNA extraction

Five seedlings were collected at random and pooled in a 1.2 ml library tube (VWR 83009-678). This constituted one sample. Three samples were collected per treatment and this represented one biological replicate. Three 2mm glass balls were added to each library tube with 50uL of extraction buffer (200mM Tris pH 7.5, 25mM EDTA, pH 7.5, 250mM NaCl, 0.5% SDS). Samples were placed into the corresponding library tube rack and homogenized for 2 minutes in a Mini Beadbeater 96+ (BioSpec, Bartlesville, OK). Samples were subjected to a second round of homogenization if needed, based on visual inspection. After homogenization, samples were spun at 14,000 RPM for 1 minute.

Library caps were removed and discarded. 400 uL of extraction buffer was added to each sample, new library caps were added and samples were briefly mixed. Samples were then spun at 14,000 RPM for three minutes to pellet cell debris. 200 uL of the supernatant was added to equal parts of 100% isopropanol in a 1.5mL plastic tube, mixed gently and incubated at room temperature for greater than two minutes. Samples were then spun down at 14,000 RPM for five minutes and the supernatant discarded. Pellets were air dried for five minutes and resuspended overnight at 4c in 30uL of sterile water. gDNA samples were quantified and diluted to 10ng/uL final concentration for real time PCR and stored at -20c.

Real time PCR

25uL samples were prepared by mixing 5uL of gDNA sample with 12.5uL of Sybr Green Mastermix (ABI, Carlsbad, California) and with the appropriate primers and water. PCR reactions were preformed on an ABI 7500 device. Ct values were determined using ABI software.

Trypan blue staining

Trypan blue staining was used to visualize hyphal growth and regions of cell death as described(McDowell et al., 2011) . Briefly, tissue harvested from infect plants was incubated in alcoholic trypan blue solution (50 g phenol, 50 ml lactic acid, 50 ml glycerin, 50 ml water, and 100 mg trypan blue that had been diluted with two parts 95%

ethanol). The samples were incubated in solution for three minutes at 90˚C and an additional five minutes at room temperature. Samples were destained in a solution of chloral hydrate (2.5g/mL) for 48 hours to remove excess trypan blue. Samples were mounted 70% glycerol and imaged with Zeiss Axio Imager M1.

Metalaxyl treatment

Four week old *Arabidopsis eds1-1* plants, grown under 8hr light/16 hour dark, were infiltrated with water or metalaxyl (0.1grams/L) using needless syringes. Infiltrated plants were subsequently infected with condical suspensions of *Hpa* Emoy2. Tissue was collected 6 DPI using a hole punch. Two hole punches were collected per leaf from 6 leaves total and pooled into three samples. Genomic DNA was extracted for qPCR analysis.

ACKNOWLEDGEMENTS

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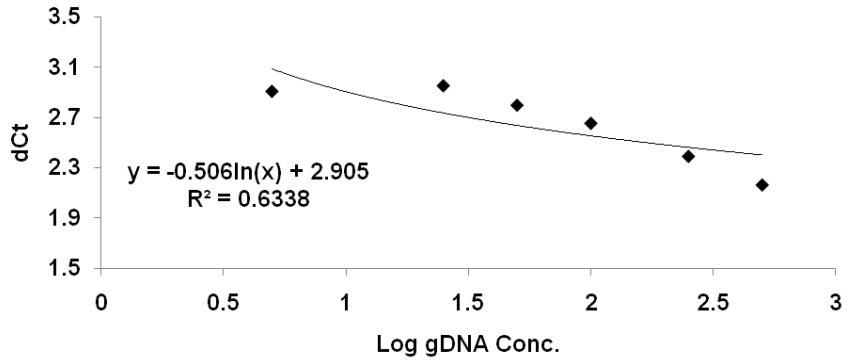
SUPPORTING INFORMATION



Supplemental Figure 5.1. *Primer efficiencies are similar.* dCt values were plotted againt the Log of gDNA concentrations. A slope close to 0 indicates matching efficiencies. Genomic DNA (gDNA).

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Chapter 6

Conclusions, Future Directions, and Perspective

Abbreviations: effector triggered immunity (ETI), hypersensitive response (HR), INF induced cell death (ICD), leucine rich repeat (LRR) microbe associated molecular patterns (MAMP), MAMP triggered immunity (MTI), nuclear localization signal (NLS), nuclear export signal (NES) programmed cell death (PCD), programmed cell death (PCD), Pseudomonas syringae (*Psy*), Type III Secretion System (T3SS), yellow fluorescent protein (YFP), yeast0two hybrid (Y2H)

CONCLUSIONS

Oomycetes have profound importance for agriculture. With the increasing demand for food products, it is of the utmost importance to maintain the security of our food supply and increase agricultural productivity. Basic research into the molecular mechanisms of oomycete pathogenesis and host resistance holds great promise for new approaches to control oomycete diseases. A substantial advance has been provided recently through the identification of hundreds of candidate oomycete effector genes by means of genome sequencing and subsequent bioinformatic screens. Yet, the mechanisms through which these effectors coordinate infection, suppression of defense, and alteration of host cell structure and metabolism remain poorly understood. Homology searches offer few clues for effector targets or function, impeding our understanding of the complex interaction between oomycetes and plants. Several broad questions remain to be addressed. Why do oomycetes maintain large collections of effectors? How do these effectors function on the molecular level and are there conserved effector functions amongst oomycetes? Lastly, can we use this information to develop durable resistance strategies to oomycete pathogens?

My dissertation research was designed to increase understanding of molecular mechanisms that enable oomycete pathogens to cause destructive diseases. The results of this work have advanced our comprehension of oomycete effector function and may facilitate the development of innovative and effective control strategies. The majority of predicted effector genes are rapidly evolving and highly divergent. Consequently,

relatively few conserved effector genes have been identified in oomycete genomes. I have focused on effectors that are conserved between the *Arabidopsis* downy mildew, *Hyaloperonospora arabidopsidis* (*Hpa*) and the soybean pathogen, *Phytophthora sojae.* My focus on conserved effectors may identify universal mechanisms required for oomycete pathogenesis.

Bioinformatic surveys of the *Hpa* genome created a list of predicted effector genes (Baxter et al., 2010). The first step in my project was to select genes for preliminary characterization. From a master list of approximately 134 genes, we identified several candidate effectors with a conserved RXLR motif required for cell entry, a nuclear localization signal (NLS) and an identifiable homolog in *P. sojae.* We confirmed expression of the selected genes, and subcellular localization studies validated their predicted NLS. The next step was to select a pair of biologically interesting homologs for detailed characterization. To this end, we used two different functional screens to identify immunosuppressive capabilities of conserved candidate effectors.

The first assay relied on delivering oomycete effectors via the type three secretion system (T3SS) using *Pseudomonas syringae* (*Psy*) as a surrogate for delivery into *Arabidopsis* cells (Sohn et al., 2007). This screen did not provide any informative results. This is attributed to several possible causes, including the failure to properly deliver the foreign effector. In the second assay, effectors were transiently expressed in soybean cells, delivered by ballistics. Co-bombardments of effectors with the pro-apoptotic mammalian protein Bax allowed quantitative cell death measurements. Only one of our

Hpa effectors suppressed Bax in a significant manner and its homolog from *P. sojae* displayed the same function.

These results motivated us to study the *Hpa* effector *HaRxL96* and its *P. sojae* homolog, *PsAvh163,* in further detail. My research proposal outlined experiments that utilize transient assays and stably transformed plants to elucidate defense suppression activities and the *in planta* molecular targets of *HaRxL96* and *PsAvh163.* Below, I discuss the success and failures of the original research proposal and the future directions of the project.

The original research plan outlined three specific aims that were designed to functionally-characterize *HaRxL96* and *PsAvh163*. The three specific aims were 1) determining the functions of HaRxL96 and PsAvh163 through the use of transient assays, 2) analysis of stable *Arabidopsis* transformants expressing *HaRxL96* or *PsAvh163*, and 3) determining effector targets and specific functions.

Due to the lack of genetic tractability in *Hpa*, specific aim one relied on additional transient assays in divergent plant species including onion, *Arabidopsis, Nicotiana benthamiana, N. tabacum*, and soybean. Subcellular localization studies of HaRxL96 and PsAvh163 with fusions to yellow fluorescent protein (YFP) indicate that both proteins exhibit nuclear-cytoplasmic localization in onion and *N. benthamiana*. Interestingly, PsAvh163 triggers cell death in *N. benthamiana* and *N. tabacum* that is dependent on Hsp90 and SGT1, suggesting that the cell death is an immune-related

response. This hindered our abilities to test virulence functions of PsAvh163 in *N. benthamiana*. However, we showed that HaRxL96 can suppress cell death triggered by the INF1 elicitor and by PsAvh163 itself. In soybean, we extended our studies with Bax to include a relevant elicitor of cell death, the *P. sojae* effector Avr4/6 that triggers RPS4 or RPS6 immunity in soybean (Dou et al., 2010). Both effectors suppressed RPS4- and RPS6-mediated immunity. The successful experiments in specific aim one provided strategic functional data and motivated the efforts of generating stably transformed *Arabidopsis* expressing effectors as transgenes.

Expressing effectors as transgenes has proven informative for elucidating the functions of bacterial effectors. The knowledge derived from these previously published studies created the justification and framework for specific aim two. Transgenic *Arabidopsis* plants expressing *HaRxL96* and *PsAvh163* allowed us to study different aspects of defense suppression that were unattainable with transient assays in specific aim one, including suppression of basal immunity against *Psy* and *Hpa*, as well as defense gene expression. The abilities of HaRxL96 and PsAvh163 to suppress callose elicited by bacteria suggest that both effectors have conserved functionality. Furthermore, the effector-mediated suppression of RPP4 immunity in response to avirulent *Hpa* Emoy2 indicates that both effectors have a broad capacity to suppress both PTI and effector triggered immunity (ETI) in *Arabidopsis*. PTI and ETI are known to overlap in their regulatory pathways; perhaps these effectors target a protein that acts in both types of immunity.

The last major objective of my research project was to identify the target(s) of both HaRxL96 and PsAvh163 and to evaluate the biological relevance of candidate effector targets. At the inception of this project in the fall of 2005, no host targets had been identified for an oomycete effector and thus this aim provided a great deal of scientific merit. Specific aim three proved to be the most challenging. Protein interaction screens were performed by three different collaborators. The first screen (Dangl Lab, UNC) did not reveal candidate interactors, while the second protein interaction screen (Michelmore Lab, UC Davis) uncovered a weak interaction between HaRxL96 and the *N. benthamiana* U-box protein CMPG1. This led the effort in a third screen (Tyler Lab, VBI) to evaluate interactions between U-box homologs of CMPG1 and both HaRxL96 and PsAvh163. Both effectors were found to interact with several closely-related U-box proteins from soybean. The *P. infestans* effector, Avr3a, interacts with and stabilizes CMPG1 in a phosphatidylinositol monophosphate dependent manner to suppress cell death triggered by INF1 (ICD) in *N. benthamiana* (Yaeno et al., 2011, Bos et al., 2010). The weak interaction between HaRxL96 and CMPG1 may explain the weak suppression of ICD by HaRxL96 in *N. benthamiana*.

The knowledge of PUB proteins is limited, but information about the mechanisms of their regulation and their functions in plant immunity is beginning to emerge. Some PUB proteins function as negative regulators of plant immunity including the CMPG1 homologs in *Arabidopsis*, PUB22, PUB23, and PUB24 (Trujillo et al., 2008). In contrast, CMPG1 is a positive regulator of immunity (Gonzalez-Lamothe et al., 2006). Other *Arabidopsis* PUBs, including PUB12 and PUB13, function in mediating PTI responses

through the pathogen recognition receptor (PRR), FLS2, by regulating FLS2 turnover (He et al., 2011). Thus, PUB proteins represent potentially-important targets of pathogen effectors. It will be of great interest to explore this interaction further and to use the effectors as probes to better understand how PUBs regulate immunity in plants.

We spent a large effort validating and studying the relationship of subcellular localization and functions for HaRxL96 and PsAvh163. To accomplish this, we mis-localized both effectors by inhibiting their ability to gain access to the nucleus. Both effectors demonstrated a requirement for nuclear localization for proper virulence functions. Confining HaRxL96 to the nucleus did not compromise its ability to suppress INF1 induced cell death, further suggesting activities inside the nucleus. With virulence functions inside the nucleus and the lack of an identifiable NLS, several questions arise including the mechanisms of nuclear import and the functions/targets within the nucleus.

My dissertation research was launched with a spreadsheet of predicted *Hpa* effectors and has matured into a comprehensive analysis of the virulence activities of two homologous effectors in a number of plant species. Furthermore, relevance of nuclear localization has been addressed, and putative host targets have been identified for each effector. In addition, this project has contributed preliminary analysis of several additional effectors that lays the groundwork for future investigations. Other contributions include the development of a quantitative PCR method for monitoring *Hpa* growth and an initial report suggesting *Hpa* manipulation of antagonistic hormone networks to promote virulence. The goals of this project have been fulfilled, but we lack

an extensive understanding of the functions of HaRxL96 and PsAvh163. Below, I discuss knowledge gaps that exist for the effector pair and how these gaps might be bridged in future projects.

FUTURE DIRECTIONS

Establishing the biological relevance of PUB-effector interactions

Despite identifying protein interactions between HaRxL96/PsAvh163 and PUB proteins, we still understand very little about the nature of these interactions. In particular, we do not understand how these interactions contribute to the fitness of *Hpa*. Additionally, we do not know if there are other host targets of these effectors, and if these effectors interact with PUB proteins, *in vivo*, during infection. To address whether or not the interactions between PUBs and HaRxL96/PsAvh163 contribute to the fitness of *Hpa*, will require working knowledge of the PUB proteins. It is clear the PUB triplet of PUB22, PUB23 and PUB24, functions as partially redundant, negative regulators of plant immunity and thus triple *pub22/23/34* mutants display enhanced resistance to virulent *Hpa* (Trujillo et al., 2008). Furthermore the PUB22/23/24 triplet regulates basal immunity through the PUB interacting protein (PIP). Thus, we can propose that if HaRxL96 and PsAvh163 enhance virulence, they may do so by regulating the stability of the PUB proteins and their PIP target during active immune signaling. This hypothesis predicts, the levels of PUB proteins should be lower in *Arabidopsis* plants infected with virulent *Hpa* and higher in plants challenged with avirulent *Hpa*. To assess this, stably

transformed plants with tagged PUB proteins will be infected and immuno-blots will be used to quantify the levels of PUB22/23/24. Additionally, we can exploit transient assays in *N. benthamiana* to study PUB protein stabilization in the presence of either HaRxL96 or PsAvh163. *Agrobacterium tumefaciens* harboring effector genes with N terminal HA fusions will be co-infiltrated with *Agrobacterium* carrying PUB genes with YFP fusions. Two days post-inoculation, the infiltrated regions will be challenged with or without avirulent *Pseudomonas syringae*, and fluorescence will be quantified by confocal laser scanning microscopy. In parallel, immuno-blots will be used to quantify PUB protein levels.

As a validating experiment to address the turnover of PUB proteins and their relevance in immune signaling, we will also exploit the proteasome inhibitor. MG132. Using the assays described above, co-infiltrations with MG132, PUB-YFP, and either effector should have similar proteins levels of the PUB targets as measured by western blots. These experiments would indicate that the effector pair is regulating the stability of their PUB targets via proteasomal degradation. Additionally, these experiments will lay the groundwork for mutational analyses to identify key regions required for PUB interaction and stabilization.

Identifying additional effector targets

Effector proteins can be promiscuous, therefore we were surprised to find few positive interactions from yeast two-hybrid screens. To circumvent the pitfalls of Y2H

screens, I recommend co-immunoprecipitation to enrich effector targeted protein complexes and to identify interacting proteins. This method provides an unbiased approach for identifying protein interactions. Transgenic *Arabidopsis* plants that express either effector with fusion tags would provide starting material. Owing to the dynamic changes that occur during plant immune responses, transgenic plants would be challenged with *Hpa* and tissue harvested six days after inoculation to provide the best chances of identifying *bona fide* targets. However, it is important to emphasize that the technique is fraught with potential pitfalls. In addition to the challenge of successfully purifying complexes, it is likely that we will identify artifactual targets and will need to prioritize candidate targets for further analysis. Owing to the Y2H interactions identified between PUB22/23/24 and the effectors, we also expect to detect these interactions with this approach.

Further studies to validate biological relevance of nuclear localization

Subcellular localization studies place both effectors in the nucleus and cytoplasm. Further analysis established the biological relevance of nuclear location, required for cell death suppression. Should the effectors stabilize PUB proteins, we can exploit the PUB stabilization assay in *N. benthamiana* co-infiltrated with various fusions to a nuclear localization signal (NLS), or a nuclear export signal (NES), to study requirements for either nuclear or cytoplasmic localization in relation to PUB stabilization. Similar to the experiment described previously, PUB stabilization can be monitored with CSLM and immuno-blots.

Investigating the role of the W and Y motifs

Both effectors maintain a number of W and Y motifs that have been implicated in mediating the virulence and avirulence function of the *P. sojae* effector, Avr1b, and the *P. infestans* effector, Avr4 (Poppel et al., 2009, Dou et al., 2008a). We can perform random or directed mutagenesis screens with both effectors. As a high throughput screen in *N. benthamiana*, we will use the loss of PsAvh163 HR as a proxy to identify mutants that lose recognition capacity. To study the functions of W and Y motifs in HaRxL96, we will use directed mutagenesis to screen for mutants that lose the ability to suppress INF1 induced cell death. It may be possible to make informed mutations with a homology modeling to known protein structures of oomycete W /Y effectors (Boutemy et al., 2011). As a low risk experiment with high value, we can perform random mutagenesis of HaRxL96 and screen for gain of avirulence. This may provide insight to the evolution of these conserved effectors. Lastly we can address possible links of PsAvh163 cell death in *N. benthamiana* to its virulence function. To accomplish this, biologically interesting PsAvh163 mutants (loss of cell death phenotype, etc) can be tested for their ability to interact with their PUB targets to examine links between HR induction and PUB stabilization.

Protein structure analysis

Longer term experiments may entail solving the crystal structures of both effectors with collaborators. This would aid our understanding of both effectors and complement mutational data that was generated. Also, we anticipate that this may help resolve the activities of each effector, as in the case of AvrPtoB and its E3 ligase activity (Janjusevic et al., 2006). This would support further experimental framework.

PERSPECTIVE

Translational value of the current study

The molecular and genomic tools now available to the scientific community will help develop new technologies and methods to prevent widespread plant diseases. The results derived from this study may not contribute directly or immediately to agriculture, but the information resulting from this study could be applied to a variety of purposes. For example, it is now possible to screen effectors against a collection of relevant crop plants to identify unique resistances for field use. This is already underway with effectors from *P. infestans* that were expressed transiently via *Agrobacterium* to screen wild potato plants for new resistance genes (Vleeshouwers et al., 2008, Vleeshouwers et al., 2010). In addition, lower sequencing costs will make it possible to monitor effector alleles from field isolates of oomycete pathogens in such a way that R gene deployment would reflect the current effector population (Vleeshouwers et al., 2010). This genomic forecasting

would bolster R gene deployment analogous to the preemptive flu vaccine. These approaches in combination should help lower disease severity and increase yields. The HR elicited by PsAvh163 inspired us to screen crop plants for recognition of PsAvh163. We have attempted screens in two varieties of tomato, pumpkin, grape and 24 cultivars of lettuce. Interestingly, PsAvh163 triggers a strong cell death response in the lettuce cultivar, LSE18, that possesses the temperature-sensitive DMR16 resistance gene (**Figure 6.1, Table 6.1**) (Judelson & Michelmore, 1992). This provides a possible probe for the identification of novel resistances against downy mildew of lettuce, which causes significant damage.

Additionally, the knowledge derived from this study could be used to alter host targets to prevent manipulation by effectors. Specifically, we could alter the surface structures of targeted PUB proteins so that their function is retained, while their ability to dock with HaRxL96 or PsAvh163 is abolished. However, should this approach be successful, it may contribute little disease resistance owing to the effect on pathogen growth in the *pub22/23/24* mutants and the effector expressing *Arabidopsis* plants which were significant but lack substantial impact on *Hpa* growth. Finally, with the knowledge of the interactome network, we may be able to identify resistance proteins that could be engineered to "guard" host process targeted by oomycete effectors. This would be high risk and difficult, but could lead to new avenues of resistance.

Emerging technologies for preventing oomycete plant diseases

The oomycete community has made great strides in the last several years. Promising new strategies are on the horizon that pledge durable disease resistance. The ability to detect specific MAMPs and the downstream PTI responses are species specific. For example, the *Arabidopsis* pattern recognition receptor (PRR) EFR recognizes the MAMP Ef-Tu from bacteria and consequently restricts *Agrobacterium* growth in *Arabidopsis* (Zipfel et al., 2006). *N. benthamiana* naturally lacks *EFR*, hence the ease of *Agrobacterium* transient expression in this species. It has recently been demonstrated that expressing *EFR* as a transgene in *N. benthamiana* confers broad spectrum disease resistance to bacteria, suggesting that EFR is functional in other plant species (Lacombe et al., 2010). This opens possibilities of transferring PTI between crop species to confer broad spectrum disease resistance. Another promising approach is founded on the recently solved cell entry mechanisms of RXLR containing effectors that bind external lipids for cell entry (Kale et al., 2010). Attempts are currently being made to block host cell entry by restricting effector access to specific external lipids.

Substantial and remarkable progress has been made in our understanding oomycete diseases

Seven years ago, our understanding of oomycete effectors and the mechanisms they use to parasitize their host and evade immunity were poorly understood. A handful of known effectors from *P. sojae*, *Hpa*, and *P. infestans* had been identified based on

avirulence functions and map-based cloning (Shan et al., 2004, Rehmany et al., 2003, Allen et al., 2004, Armstrong et al., 2005), but their specific functions and contribution to virulence were not known. A conserved RXLR motif had been identified but its functions were unknown (Rehmany et al., 2005). Fast-forward seven years and the oomycete community has made scientific progress by leaps and bounds. The community now boasts several oomycete genomes (Baxter et al., 2010, Tyler et al., 2006, Haas et al., 2009) and genomes other fungal obligate biotrophs have been sequenced (McDowell, 2011) . We now know that *Hpa* has lost genes required for sulfate and nitrate assimilation similar to other obligate biotrophic parasites. In addition, it has been shown the genomes of oomycete pathogens appear to maintain large collections of virulence genes reliant on an RXLR motif for cell entry in a pathogen-independent manner (Dou et al., 2008b, Whisson et al., 2007). Moreover, now we know that the RXLR motif binds external phosphatidylinositol-3-phosphate (PI3P) and subsequent endocytosis facilitates host cell entry (Kale et al., 2010). Analysis of effector collections has led to the identification of W and Y motifs implicated in effector function and these genes make up the most rapidly-evolving portions of the genome with the C-terminal portions targeted for evolution (Jiang et al., 2008, Dou et al., 2008a, Poppel et al., 2009, Win et al., 2007).

Even virulence functions have now been worked out for a handful of effectors (Dou et al., 2008a, Sohn et al., 2007, Bos et al., 2010). The best understood effector is arguably *P. infestans* Avr3a, where resistance genes, virulence functions, and evolutionary history have been established (Bos et al., 2010, Qutob et al., 2009, Bos et al., 2009, Kamoun et al., 2006, Armstrong et al., 2005, Yaeno et al., 2011). Several

effectors have had their crystal structures resolved including a homolog of Avr3a and the *Hpa* effector ATR1 (Yaeno et al., 2011, Chou et al., 2011) . Interestingly, the W and Y motifs underpin the functional diversity of effector proteins based on the Avr3a structure (Boutemy et al., 2011).

With lower sequencing costs, comparative genomics have become an important tool to study host adaptation and virulence mechanisms. This approach has already proven to be valuable by contributing to our knowledge of effector evolution and revealing genome plasticity that enhances pathogen fitness (Jiang et al., 2008, Raffaele et al., 2010). The future lies in "omics" that provide data layers to build working computational models that will generate testable hypotheses in parallel to functional characterization of select genes. The past seven years have seen tremendous growth in the oomycete field, with knowledge that is applicable for diverse plant pathogens. As a community, we will not eradicate plant pathogens, but we must become as adaptable as they are and our resistance schemes must be as novel as are their virulence mechanisms. The studies described in this dissertation filled knowledge gaps in oomycete effector function and one day may implicitly facilitate and aid the design of novel resistance strategies employable by agriculture.

FIGURES AND TABLES

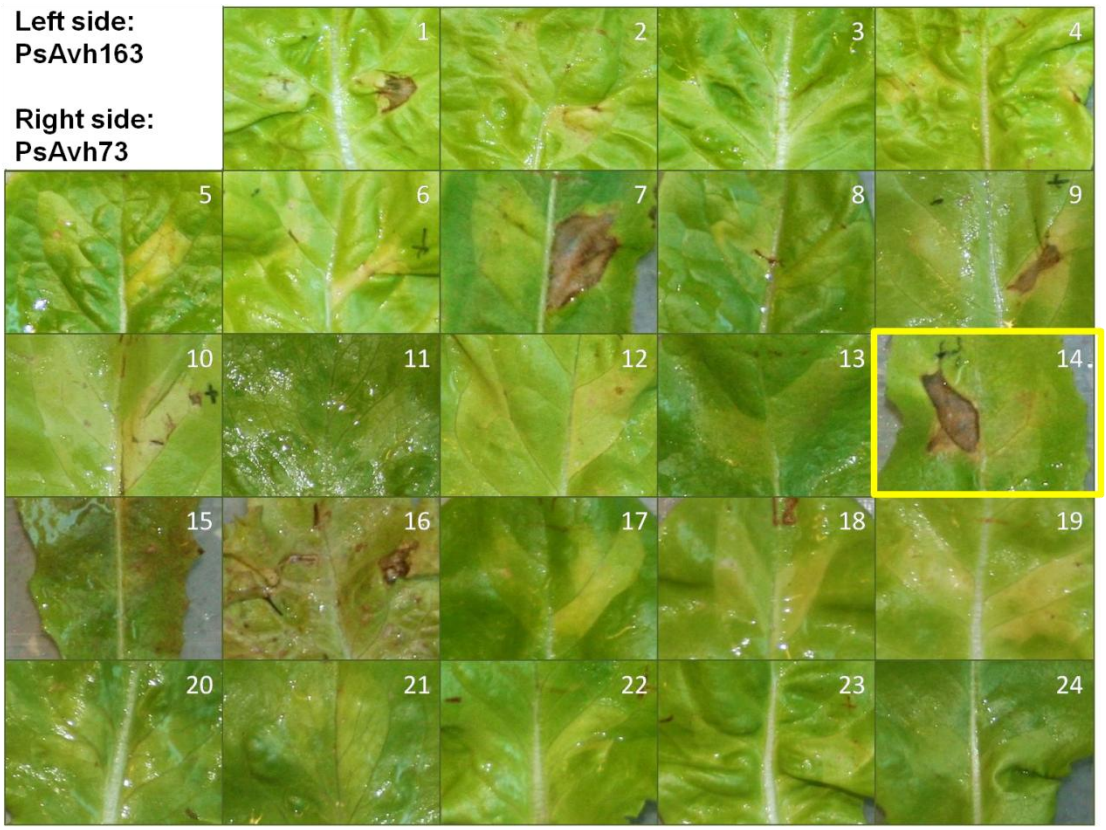

Figure 6.1 *HR screens with PsAvh163 in lettuce cultivars*. *Agrobacterium* strains harboring *PsAvh163* were infiltrated into 24 lettuce cultivars with an associated *Dmr* resistance gene (Left side) and cell death progression was monitor visually for a period of seven days. PsAvh163 triggers cell death in #14 (Yellow Box), the lettuce cultivar LES18 (*Dmr16*). Numbers correspond to the lettuce differential series. (**Table 6.1**) Experiments credited to Joan Wong, UC Davis.

	Lettuce Differential Series	
#	**Genotype**	**Resistance (*Dm*)**
1	Lednicky	1
2	UCDM2	2
3	Dandie	3
4	R4 T57	4
5	Valmaine	5/8
6	Sabine	6
7	LSE57/15	7
8	UCDM10	10
9	Capitan	11
10	Hilde	12
11	Pennlake	13
12	UCDM14	14
13	PIVT1309	15
14	LSE18	16
15	LS102	17
16	Colorado	18
17	Ninja	R36
18	Discovery	R37
19	Mariska	R18
20	Eldorado	R18
21	R32	R18
22	Argeles	R38
23	Amplus	2,4,?
24	Cobham Green	0

Table 6.1. *Lettuce series screened for PsAvh163 HR.* Numbers correspond to Figure 7.1. Cultivar names are indicated with their associated *Dmr* gene. Table provide by Joan Wong, UC Davis.

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