ELUCIDATING ESSENTIAL ROLES OF OOMYCETE EFFECTOR PROTEINS IN IMMUNE SUPPRESSION AND IN TARGETING HORMONAL PATHWAYS

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ABSTRACT

Effector proteins are exported to the interior of host cells by numerous plant pathogens. Effector proteins have been well characterized in bacteria. However, the mechanisms through which these effectors promote virulence are largely unknown. Bioinformatic analysis of genome sequences from oomycete pathogens *Phytophthora sojae*, *P. ramorum*, *P. infestans* and *Hyaloperonospora arabidopsidis* (*Hpa*) have led to the identification of a large number of candidate effector genes. These effector genes have characteristic motifs (signal peptide, RxLR and dEER) that target the effectors into plant cells. Although these effector genes are very diverse, certain genes are conserved between *P. sojae* and *H. arabidopsidis*, suggesting that they play important roles in pathogenicity. The goal of my first project was to characterize a pair of conserved effector candidates from *Hpa* and *P. sojae*. We hypothesized that these effectors have important conserved roles with regard to infection. We found that the *Hpa* effector was expressed early during the course of infection of *Arabidopsis* and triggered an ecotype-specific defense response in *Arabidopsis*, suggesting that it was recognized by host surveillance proteins. Both the effectors from *Hpa* and *P. sojae* respectively could suppress immunity triggered by pathogen associated molecular patterns (PTI) and by effectors (ETI) *in planta*. They also enhanced bacterial virulence in *Arabidopsis* when delivered by the Type III secretion system. Similar results were seen with experiments with transgenic *Arabidopsis* expressing the effectors.

My second project showed that a different *Hpa* effector protein, HaRxL10, targets the Jasmonate-Zim Domain (JAZ) proteins that repressed responses to the phytohormone jasmonic acid (JA). This manipulation activates a regulatory cascade that reduces accumulation of a second phytohormone, salicylic acid (SA) and thereby attenuates immunity. This virulence mechanism is functionally equivalent to but mechanistically distinct from activation of JA-SA crosstalk by the bacterial JA mimic coronatine. These results reveal a new mechanism underpinning oomycete virulence and demonstrate that the JA-SA crosstalk is an Achilles' heel that is manipulated by unrelated pathogens through distinct mechanisms.

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I would like to take this opportunity to acknowledge professors, colleagues, friends and family as their support has played a major role in the shaping of this dissertation. I would like to begin by thanking Dr. John M. McDowell for giving me with the opportunity to work in his laboratory and providing me with his support, guidance and independence in directing this research. John has not only supported my academic development in the past few years, but has played a major role in shaping it. His teaching and mentoring philosophies will help me lay the foundation for all the endeavors I undertake in the future. Secondly, I would like to acknowledge the Molecular Plant Science (MPS) program of Virginia Tech and especially Dr. Brenda Winkel and Dr. Glenda Gillaspy (MPS Graduate co-ordinator 2008) for my recruitment into this excellent inter-disciplinary program. Next I would like to thank my Ph.D. advisory committee members, Dr. Boris Vinatzer, Dr. Bingyu Zhao and Dr. Glenda Gillaspy for their guidance and support during the course of this research. Dr. Ryan G. Anderson needs a special mention, as he was involved in the shaping of many aspects of this research. I also thank Dr. Bhadra Gunesekera, my fellow graduate students and the undergraduates in the laboratory for their support. I would like to say a special thanks to my father, mother and brother for their support, encouragement, motivation and love for all these years. Finally, an extremely special thanks to my husband Pritish without whose insistence and support, I would not have undertaken Virginia Tech as my Graduate institution.

Abbreviations

AAD - acidic transcription activation domain

ABA - abscisic acid

AtPPIN - *Arabidopsis* plant-pathogen interactome (AtPPIN)

ATR - *Arabidopsis thaliana* responsive

BiFC - bimolecular fluorescence complementation

BSMT1 - SA methyltransferse

CDS - coding sequence

CEL - conserved effector locus

COR - coronatine

CRN - crinkling and necrosis

DEX - dexamethasone

DPI - days post inoculation

EDV - effector detector vector

EPS - exo-polysaccharide

ER-MRS - endoplasmic reticulum membrane retention/retrieval signal

ER-SS - endoplasmic reticulum type signal sequence

EtHAn - effector to host analyzer

ETI - effector-triggered immunity

ETS - effector triggered susceptibility

EV - empty vector

gDNA - genomic DNA

GFP - green fluorescent protein

GUS - β glucoronidase

HA - hemagglutinin

HGT - horizontal gene transfer

HMM - hidden markov model

Hpa - *Hyaloperonospora arabidopsidis*

HR - hypersensitive response

HT - host targeting

ICS1 - isochorismate synthase 1

JA - jasmonic acid

JAZ - jasmonate-ZIM domain

LRR - leucine rich repeat

MAMP - microbe associated molecular pattern

MAPK - mitogen activated protein kinsae

NAC - petunia NAM and *Arabidopsis* ATAF1, ATAF2, CUC2

NB - nucleotide binding site

NLP - necrosis and ethylene inducing peptide-like proteins

NLS - nuclear localization signal

NOD - nucleotide binding and oligimerization

PAMP - pathogen associated molecular patterns

PcaH - protocatechuate, 3, 4-dioxygenase β-subunit

PI3P - phosphatidylinositol-3-phosphate

PCD - programmed cell death

PLS - partial least square

PR - pathogenesis-related

PRR - pattern recognition receptor

Pph - *Pseudomonas phaseolicola*

Pst - *Pseudomonas syringae* pv. *tomato*

Psy - *Pseudomonas syringae*

PTI - PAMP-triggered immunity

qPCR - quantitative real-time polymerase chain reaction

R - resistance protein

REG - redundant effector group

RLK - receptor-like kinase

RLP - receptor-like protein

ROS - reactive oxygen species

RPP4 - Recognition of *Peronospora parasitica 4*

SA - salicylic acid

SAGT1 - SA glucosyl transferase gene 1

SCF^{COI1} - Skp/Cullin/F box-coronatine 1

SP - signal peptide

T3 - type III

TBSV - tomato bushy stunt virus

TE - transposable elements

TTSS - type III secretion system

Y2H - yeast-two hybrid

YFP - yellow florescent protein

Contributions

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

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

Chapter 2: Conserved RxLR effectors from oomycetes *Hyaloperonospora arabidopsidis* and *Phytophthora sojae* suppress PAMP and Effector-triggered immunity in plants

Theresa H. Y. Kin was an undergraduate student in the McDowell laboratory for two years. She contributed to the generation and breeding of the following *Arabidopsis* overexpression lines: 35S::HaRxL23, HaRxL23::pDEXHA, 35S::PsAvh73 and PsAvh73::pDEXHA. She also contributed to data involving INF1 suppression assay by PsAvh73 in *N. benthamiana* (Figure 2.4 A). Apart from her responsibilities towards her own project, she contributed immensely to daily bench-work involving media preparation, bacterial culture maintenance, weekly planting and transplanting, taking care of plants etc.

Ryan G. Anderson, PhD. was a former graduate student and post doc in the McDowell laboratory. He assisted with the project and contributed significantly towards performing qPCR experiments and data analysis for Figure 2.8

Brett M. Tyler, Ph.D. is a professor at the Oregon State University and he contributed to bioinformatic analysis.

Chapter 3: Functional similarity between the *Hyaloperonospora arabidopsidis* effector protein HaRxL23 and *Pseudomonas syringae* AvrE

Stephen O. Opiyo, PhD. is a research scientist at Molecular and Cellular Imaging Center-South, Ohio Agricultural Research and Development Center, Ohio State University. He contributed to the project by performing bioinformatics-driven structural prediction and analysis of effectors from *Hyaloperonospora arabidopsidis* and *Pseudomonas syringae*, HaRxL23 and AvrE respectively using I-TASSER (Supplemental Figure 3.2).

David Mackey, PhD. is an associate professor at the Department of Horticulture and Crop Science, Ohio State University. He is one of the principal investigators who initiated the project, along with John M. McDowell from Virginia Tech. He is responsible for providing bacterial constructs and project advice.

Chapter 4: An oomycete RxLR effector triggers antagonistic plant hormone crosstalk to suppress host immunity

John Withers, PhD. was a former graduate student in Sheng Yang He's laboratory at Michigan State University. He is responsible for the cloning of HaRxL10 and the 12 *Arabidopsis* JAZ proteins into yeast 2-hybrid vectors and testing protein interactions (Figure 4.1B). He also generated deletion derivatives of JAZ9 and performed yeast-2-hybrid experiments using them and HaRxL10 (Supplemental Figure 4.5). He is also responsible for providing transgenic *Arabidopsis* seed, constructs and project advice.

Ryan G. Anderson, PhD. was a former graduate student and post doc in the McDowell laboratory. He contributed to the project by performing *Hyaloperonospora arabidopsidis* inoculation experiments with *Arabidopsis* jaz3 knock out mutant seedlings (Figure 4.1A) and *Arabidopsis* JA signaling mutant seedlings (Supplemental Figure 4.2B-D). He also performed qPCR experiment and analysis that contributed to Supplemental Figure 4.2A.

Sheng Yang He, PhD. is a Distinguished Professor at the Howard Hughes Medical Institute, DOE Plant Research Lab at Michigan State University. He is one of the principal investigators who initiated the project, along with John M. McDowell from Virginia Tech. He is responsible for providing project advice and oversight.

Table of contents

Summary of figures

Chapter 2

Summary of tables

Chapter 2

Chapter 3

Chapter 4

Chapter 1

Literature review

Manuscript in preparation for: American Phytopathological Society, Teaching Article

Abbreviations: acidic transcription activation domain (AAD), abscisic acid (ABA), coronatine (COR), crinkling and necrosis (CRN), exo-polysaccharide (EPS), endoplasmic reticulum-type signal sequence (ER-SS), Effector-triggered immunity (ETI), effector-triggered susceptibility (ETS), horizontal gene transfer (HGT), *Hyaloperonospora arabidopsidis* (*Hpa*), hypersensitive response (HR), host targeting (HT), jasmonic acid (JA), leucine rich repeat (LRR), microbe-associated molecular patterns (MAMP), nucleotide binding (NB), necrosis and ethylene inducing peptide-like proteins (NLP), nuclear localization signal (NLS), nucleotide binding and oligomerization (NOD), pathogen-associated molecular patterns (PAMP), protocatechuate, 3, 4-dioxygenase β-subunit (PcaH), phosphatidylinositol-3-phosphate (PI3P), pathogenesis-related (PR), pattern recognition receptor (PRR), *Pseudomonas syringae* (*Psy*), PAMP-triggered immunity (PTI), resistance protein (R), receptor-like kinase (RLK), receptor-like protein (RLP), reactive oxygen species (ROS), salicylic acid (SA), transposable elements (TE), type III secretion system (TTSS).

Summary

Whole genome sequences of several oomycete phytopathogens have revealed both common and unique features associated with oomycete biology and evolution and have answered several questions regarding the diversity of oomycete lifestyles, gene composition, and horizontal gene transfer from bacteria and fungi. We now know that genomes of most of the oomycete phytopathogens are made up of regions of repetitive DNA that are rapidly evolving and harbor effector genes that are involved in virulence. Secondly, reduction of the number of pathogenicity genes seems to be one of the important reasons for adaptation to obligate parasitism. We now also know oomycete genomes maintain large number of RXLR effectors that are modular in nature. The conserved host-targeting RXLR motif is involved in cell entry in a pathogen-independent manner. Finally, we now have new evidences regarding the expression patterns, localization sites, structural details and virulence functions of a few of the oomycete effectors. Hence, the field of oomycete research has and continues to make remarkable progress due to the advancement made in the recent years in the areas of genome sequencing, bioinformatics screening and structural studies.

Plants maintain a robust immune system

Oomycetes comprise a highly destructive class of plant pathogenic microbes. In order to be successful in establishing contact with the host and causing disease, oomycete plant pathogens must overcome multiple layers of defenses in the plant host. Plant-pathogen interactions can be placed broadly under two categories namely, "compatible" or "incompatible" (Zipfel et al., 2006) . During a compatible interaction, the plant is unable to recognize the pathogen which leads to pathogen contact, penetration, growth and finally, the plant is rendered susceptible to disease. On the other hand, during an incompatible interaction, the pathogen is unable to grow and survive within the host tissue which is due to the activation of inducible defenses by the plants (Zipfel et al., 2006). Much of the research in the field of plant-oomycete interactions has been directed towards understanding what molecular mechanisms and strategies plants and microbes utilize that finally lead to these two very different outcomes.

This article is designed to provide an update of recent insights into oomycete pathogenesis from genomic analysis, which in turn has catalyzed functional characterization and structural elucidation of oomycete proteins known as "effectors". By definition, effectors are secreted from the pathogen and promote virulence from either the exterior or interior of host plant cells. A major task of these effectors is to subvert host immunity. Thus, I begin this article by summarizing the general mechanisms that underpin plant defense against pathogen infection. This section will be followed by a summary of the mechanisms used by pathogens to avoid plant defenses, based on insights from studies of bacterial pathogens. Then the focus will shift to oomycetes, beginning

with a summary of the recent development in oomycete biology and pathogenesis from genomic studies followed by information regarding developments in oomycete effector proteins starting from how and when they were identified, to their characteristic features and the functional characterization of some of the widely studied effectors. Finally, I will be giving a brief description regarding the rationale, focus and relevance of my dissertation project.

Plant defenses are multilayered

Plants maintain a complex system of pre-formed and inducible defenses. For example, surface layers like cutin, suberin, and waxes provide a physical barrier to pathogens. The cell wall is another example of a "pre-formed" defense structure that functions as an effective barrier, and serves as source of chemical components that trigger inducible defenses when the cell wall is breached (Zipfel & Felix, 2005).

For pathogens that breach the first layer of defense, plants deploy a second layer of inducible defenses that are activated only when the plant detects a pathogen. Examples of such defenses include production of reactive oxygen molecules, and secondary metabolites such as phytoalexins that are directly toxic to the pathogen. Plants can also synthesize physical barriers to infection, such as papillae which are composed primarily of callose, a β-1, 3 glucan, that is deposited between the cell wall and cell membrane near the invading pathogen. One of the last defenses the plant deploys to prevent the spread of infection is the hypersensitive response (HR) (Dodds & Rathjen, 2010), characterized by

dying cells or characteristic necrotic lesions surrounding an infection site. The HR restricts the growth and spread of pathogens to other parts of the plant trapping the pathogen within dead cells. Additionally, the HR serves as a source of signals that activates defenses in distal tissues (Dodds & Rathjen, 2010).

Taken together, these defenses render most plants resistant to most pathogens. However, it is critically important for these defenses to be induced in a timely manner, and to be robust to pathogen co-evolution. Thus plants have evolved two distinct, but inter-connected, surveillance systems. First, plants can recognize conserved, pathogen-associated molecular patterns (PAMPs), also termed microbe-associated molecular patterns or (MAMPs), as broad signatures of pathogen species. This is termed PAMP-triggered immunity (PTI) (Chisholm, Coaker, Day, & Staskawicz, 2006; Katagiri & Tsuda, 2010; Zipfel & Robatzek, 2010). Second, plants maintain a genetically complex system for recognizing effectors as signals of invasion, termed effector-triggered immunity or ETI (Chisholm et al., 2006; Jones & Dangl, 2006; van der Hoorn & Kamoun, 2008). I summarize the molecular logic of these systems in the following sections.

PAMP- triggered immunity (PTI)

PTI is activated by so called pattern recognition receptors (PRRs) (Jones & Dangl, 2006; Zipfel & Robatzek, 2010) in the host. PRRs- are trans-membrane proteins and belong to either the receptor-like kinase (RLK) or the receptor-like protein (RLP)

families. Most of the PRRs have extracellular leucine-rich repeats (LRRs) (Dardick, Schwessinger, & Ronald, 2012; Ronald & Beutler, 2010) and therefore resemble the Toll-like receptors in animals. Some PRRs have intracellular kinase domains whereas others lacking such domains are known to interact with signaling proteins via adaptor proteins. A few of the PRRs characterized so far include FLS2 and EFR in *Arabidopsis*, Xa21 in rice, and Cf and Ve in tomato (Kawchuk et al., 2001; W. Y. Song et al., 1995; Wulff, Chakrabarti, & Jones, 2009; Zipfel et al., 2004). PAMPs are best-studied in bacteria; Examples of bacterial PAMPs include the cell wall component peptidoglycan; the flagellin subunit, flg22 (Zipfel et al., 2004); and the Elongation Factor-Tu-, subunit elf18 (Zipfel et al., 2006). Oomycete PAMPs include; Pep-13, a subunit of a *Phytophthora* transglutaminase protein (Brunner et al., 2002); *P. parasitica* var. *nicotianae* call wall elicitor protein, cellulose binding elicitor lectin (CBEL) (Gaulin et al., 2006); the cell wall-associated necrosis-inducing protein NPP1 of *P. parasitica* (Qutob et al., 2006) and the *P. infestans* elicitin, infestin 1 or INF1 (Kamoun, 2006). PTI is associated with specific defense responses including the production of reactive oxygen species (ROS), callose deposition, lignin production and the induction of pathogenesis-related (PR) gene expression (Chisholm et al., 2006; Gomez-Gomez, Felix, & Boller, 1999; Jones & Dangl, 2006; Navarro et al., 2004; Zipfel & Robatzek, 2010). The HR is typically not activated during PTI.

Pathogen effector proteins interdict PTI

PTI is often sufficient to contain most pathogens. However "adapted" pathogens, by definition, are successful pathogens are obviously successful in overcoming these basal defenses (Nurnberger, Brunner, Kemmerling, & Piater, 2004). To a large extent, this is due to the combined action of afore-mentioned "effector" proteins that are secreted into the interior of host cells and suppress PTI by interfering with the PAMP-induced signaling mechanism (He et al., 2006; van der Hoorn & Kamoun, 2008). Hogenhout broadly defined effector proteins as "all pathogen proteins and small molecules that alter host cell structure and function" (Hogenhout, Van der Hoorn, Terauchi, & Kamoun, 2009). These changes can either result in successful infection and consequent development of disease symptoms or result in robust immune responses or both (Huitema et al., 2004; Kamoun, 2006, 2007; van der Hoorn & Kamoun, 2008). Many phytopathogens maintain a large effector repertoire for successful pathogenesis. Their collective, virulence-promoting activities in cells comprise "effector triggered susceptibility" or ETS (Chisholm et al., 2006; Jones & Dangl, 2006; van der Hoorn & Kamoun, 2008). Below are a few examples of effectors from bacteria and fungi for which the mechanism of ETS is well-understood.

Effector proteins from bacteria

Gram-negative bacterial pathogens like utilize a type III secretion system (TTSS) that enable direct delivery of effector proteins into plant cells to suppress PTI (Galan & Collmer, 1999). Others produce toxins (e.g. coronatine), which are able to overcome PTI (Block & Alfano, 2011). Plant pathogens such as *Pseudomonas syringae* can secrete

around 20 to 30 effector proteins during infection (Abramovitch, Anderson, & Martin, 2006; Chang et al., 2005; Gohre & Robatzek, 2008; Lindeberg, Cunnac, & Collmer, 2009, 2012). Effectors are known to promote pathogenicity, and the TTSS is indispensable not only for effector delivery but also for enhancement of bacterial virulence (Staskawicz, Mudgett, Dangl, & Galan, 2001). After effector proteins are translocated to the inside of the plant cells they interfere with host defenses in susceptible plants (Abramovitch et al., 2006; Block, Li, Fu, & Alfano, 2008; Cunnac, Lindeberg, & Collmer, 2009; Espinosa & Alfano, 2004; Xin & He, 2013). The effector repertoires maintained by pathogens vary from strain to strain and may be involved in determining the host specificity (Vinatzer et al., 2006).

An example of an effector that can enhance bacterial virulence by altering the host's ubiquitination system is AvrPtoB of *Pseudomonas syringae* (*Psy*) DC3000. AvrPtoB is multi-functional. In tomato, AvrPtoB acts as an E3 ubiquitin ligase that targets a host kinase called *Fen* that is responsible for inducing host defenses. The degradation of the host kinase, *Fen* interferes with host defense signaling mechanism (Rosebrock et al., 2007b). Interestingly, AvrPtoB also targets *Arabidopsis* FLS2, a pattern recognition receptor (PRR) for degradation via the 26S proteasome complex (Rosebrock et al., 2007a). Another *Psy* DC3000 effector, HopM1, interferes with host defenses by suppressing callose deposits from accumulating around the infection site during pathogen attack. Reducing callose deposition by HopM1 is achieved through a post-transcriptional event that induces poly-ubiquitination of AtMIN proteins (especially AtMIN7) (Nomura et al., 2006; Nomura et al., 2011).

The members of the HopX1 (AvrPphE) family from *P. syringae* and *Xanthomonas campestris* contain a conserved N-terminal domain and a set of three amino acid residues which are common to cysteine proteases (Nimchuk, Fisher, Desveaux, Chang, & Dangl, 2007). HopX1 is thought to be involved in the degradation of host proteins thereby conferring its virulence activity. *P. syringae* effectors, HopAR1 and AvrRpt2, are also known to function as cysteine proteases (Axtell & Staskawicz, 2003; Shao et al., 2003). HopAR1 is known to promote virulence by cleaving the *Arabidopsis* protein PBS1 whereas contribution to the virulence by AvrRpt2 is thought to be through an SA-independent mechanism (Chen et al., 2007) that includes suppression of MAMP signaling (Kim et al., 2005). AvrRpt2 is thought to suppress PTI by cleaving RIN4. RIN4, itself, is a negative regulator of PTI, hence AvrRpt2 is able to disrupt the RIN4-associated protein complex (Kim et al., 2005). The *P. syringae* effector HopI1 that is located in the chloroplast is known to suppress accumulation of salicylic acid (Jelenska et al., 2007). It is predicted that HopI1 interacts with Hsp70 chaperones in the chloroplast. This is probably the only evidence of a chloroplast-targeting bacterial effector protein to date.

The *Xanthomonas campestris pv. vesicatoria* effector AvrBs3 triggers a strong HR on pepper plants carrying the corresponding R-gene, *Bs3* (Romer et al., 2007). *AvrBs3* contains recognizable motifs including a functional nuclear localization signal (NLS) and an acidic transcription activation domain (AAD) required for avirulence activity and gets secreted and recognized in the host cytosol (Van den Ackerveken, Marois, & Bonas, 1996). Dimerization of the effector is necessary for full virulence and

nuclear localization (Gurlebeck, Szurek, & Bonas, 2005). The effector is involved in directly altering host gene expression which leads to the induction of hypertrophy of mesophyll cells in susceptible plants (Marois, Van den Ackerveken, & Bonas, 2002).

A variety of plant hormones, including salicylic acid (SA), jasmonic acid (JA), ethylene, auxin, and abscisic acid (ABA), have been shown to be involved in plant defenses. These pathways can be targeted by effectors. For example, the *P.syringae* effector, AvrRpt2 can cause *Arabidopsis* to produce more auxin which in turn suppresses immunity (Chen et al., 2007). Coronatine (COR) from *P.syringae* is also known to induce the expression of genes that are involved in auxin metabolism (Uppalapati et al., 2005). Coronatine is a functional analog of jasmonic acid and is a commonly known phytotoxin. Very recently, it has been shown that COR promotes bacterial virulence by suppressing SA-dependent defenses *in planta* (Zheng et al., 2012). *Ralstonia* species are known to directly synthesize ethylene (Valls, Genin, & Boucher, 2006). Several bacterial effectors are known to induce expression of genes involved in ethylene and JA biosynthesis and signaling (Cohn & Martin, 2005; He et al., 2004; Thilmony, Underwood, & He, 2006; Uppalapati et al., 2005).

There has been no report to date of any secretion system(s) that deliver effector proteins from fungal pathogens into host plant tissues. However, it has been speculated that effectors may be delivered from the haustoria into the apoplast of plants. The activities of most fungal effectors are yet to be determined but some information is beginning to emerge. For example Avr2 and Avr4 have been characterized from

Cladosporium fulvum, the leaf-mold fungus. Avr2 is known to encode a cysteine-rich protein that binds and inhibits the tomato cysteine protease Rcr3 (De Wit, Mehrabi, Van den Burg, & Stergiopoulos, 2009; Rooney et al., 2005). The Avr4 effector, on the other hand, contains a chitin binding domain that binds chitin (van den Burg, Harrison, Joosten, Vervoort, & de Wit, 2006), a major component of fungal cell wall. The mechanism is mirrored in the *Magnaporthe oryzae* effector LysM protein 1 (Slp1) was found to bind to chitin oligosaccharides thereby preventing recognition (Mentlak et al., 2012).

These examples illustrate that effectors can be powerful weapons to suppress host immunity. Thus, it is commonly believed that pathogens evolved effectors at least in part to combat host PTI. In turn, host plants have evolved a surveillance system to detect effectors. This is described in the following section.

Effector-triggered immunity (ETI)

ETI is a robust and prolonged deployment of immunity triggered by effector proteins that encounters a corresponding surveillance protein in the host (Chisholm et al., 2006; Jones & Dangl, 2006; van der Hoorn & Kamoun, 2008). These receptors are encoded by characteristic disease resistance ("*R*") genes that were first defined by Flor (Flor 1955) in the 1950s. Flor postulated the classic ''gene-for-gene'' model, predicting that resistance is activated when a plant *R* gene allele recognizes the product of a corresponding "avirulence" allele from the pathogen. It has become quite clear now that avirulence proteins are typically effector proteins that are recognized by the host R

proteins. The majority of *R* genes encode proteins that are located inside the cell and are called ‘‘NB-LRR’’ (nucleotide binding, leucine-rich repeat) proteins. These proteins contain two signature domains: a nucleotide-binding and oligomerization domain (NOD), followed by leucine rich repeats (LRRs) (DeYoung & Innes, 2006). In general, NB-LRR genes are known to exist in two distinct forms, either with an N-terminal coil-coil (CC) domain or a “TIR” domain with sequence similarity to the cytoplasmic signaling domains of animal Toll and Interleukin-1 immune receptor proteins. The NB and the LRR domains have been known to be involved in both defense signaling and effector recognition events (DeYoung & Innes, 2006; Jones & Dangl, 2006; Martin, Bogdanove, & Sessa, 2003; Yue, Meyers, Chen, Tian, & Yang, 2012; Zhang et al., 2010) whereby the receptor activates a complex signaling network that controls the final responses. This is extremely important due to the fact that signaling components are potential targets whereby effectors can suppress defenses.

R proteins recognize effectors *in planta* through one of several mechanisms. The most intuitive mechanism is that R proteins detect specific effector proteins by direct interaction; once the effector protein gets translocated from the pathogen to the host cell, it makes physical contact with the corresponding R protein. Evidence for direct interactions have been provided in several instances, for example the flax rust fungus (*Melampsora lini*) *AvrL567* genes and corresponding *R* genes in flax (*Linum usitatissimum*) (Dodds et al., 2006) and the recognition of NB-LRR protein RPP1 in *Arabidopsis* by the oomycete effector protein ATR1 (Krasileva, Dahlbeck, & Staskawicz, 2010). The second mechanism of effector recognition occurs indirectly, through which R

proteins *"guard"* specific plant proteins that are targeted by effectors (Van der Biezen & Jones, 1998). This mode of recognition explains how several effectors can be recognized by a single R protein (Dangl & Jones, 2001). This mechanism suggests that the guardee or the effector target is required for the virulence function of the effector protein. For example, the *Arabidopsis* R protein RPM1 is known to associate with some forms of RIN4. RIN4, on the other hand is targeted by several *Pseudomonas syringae* effectors. Once the effectors alter or modify RIN4, RPM1 is activated (Kim et al., 2005; Mackey, Belkhadir, Alonso, Ecker, & Dangl, 2003). Recently, some effector targets were categorized as decoy (van der Hoorn & Kamoun, 2008). A target is called a "decoy" if it plays no role in host defense processes in absence of the corresponding R-protein.

The sections above have hopefully provided a foundation from which I can now focus on mechanisms through which oomycetes subvert plant immunity to cause diseases.

Oomycetes are destructive plant pathogens

Oomycetes comprise a large group of filamentous microbes that include both saprophytes and parasites of plants, animals, and insects. Oomycetes, due to their filamentous growth and feeding habits, were once classified as true fungi. However, oomycetes are now commonly classified along with the diatoms and brown algae in a group broadly called the stramenopiles. Oomycetes are commonly known as "water molds", and are non-photosynthetic organisms and found worldwide in fresh and salt

water habitats. Most oomycetes produce the characteristic filamentous vegetative structures called the hyphae. Although, oomycetes and true fungi have superficially similar morphologies, they are different in many aspects. For example, the cell walls of oomycetes are contain beta-1,3- and beta-1,6-glucans and cellulose as major components rather than chitin as in the case of fungi. Most oomycetes are filamentous and lack septa except where reproductive cells are produced (Slusarenko & Schlaich, 2003) and all oomycetes have a diploid vegetative phase. Fungi, on the other are mostly haploid in nature.

Oomycetes are a major agricultural threat

This section focusses on a general introduction of two classes of plant-pathogenic oomycetes, *Phytophthora spp*. and the downy mildews. The section will focus on how these pathogens are a threat to agriculture, their mode of lifestyle and their disease cycle.

The *Phytophthora* genus has over 80 species that cause diseases involving rot of roots, leaves, and fruits. *Phytophthora* species show a range of host specificity varying from exceedingly broad to extremely specific (Tyler, 2007a). *Phytophthora* species maintain a hemi-biotrophic lifestyle, whereby an initial biotrophic phase is followed by a necrotrophic mode of nutrition. During the initial biotrophic stage, they proliferate in living host tissue and later, during the necrotrophic phase, feed on dead and decaying tissue. *Phytophthora* species are pathogens of dicotyledonous plants. Several species of *Phytophthora* cause enormous damage to crop plants (Tyler, 2007b). The best example to

illustrate this is the notorious *Phytophthora infestans,* which was the causative agent of the potato late blight disease that caused the Great Irish Famine during 1845-1849. It is noteworthy, that to date, it still remains to be the most destructive pathogen of potato crops. Another important example is the soybean root and stem rot pathogen, *Phytophthora sojae*, which continues to be a recurring problem (Sogin & Silberman, 1998). Overall, it is quite difficult to control plant diseases caused by *Phytophthora* using fungicides or other chemicals, as they are distinct from true fungi and may differ in their responsiveness to fungicide modes of action. Moreover, application of fungicidies can be expensive, time-consuming, and environmentally unfriendly. Hence, as with many other pathogens, host genetic resistance is considered to be the most desirable control strategy. However, oomycetes are also very adept at co-evolving to overcome host immunity. Hence, it becomes extremely important for us to examine and understand the mechanisms of pathogenesis of this destructive plant pathogen, to inform breeding strategies for durable resistance.

Downy mildew pathogens comprise another class of destructive oomycete pathogens of monocot and dicot plants, consisting of greater than 800 species. Downy mildew pathogens belong to the family Peronosporaceae which comprises the closest relative to the Phytophthora clade. Some examples include *Plasmopara viticola* causing downy mildew of grape (Wong, Burr, & Wilcox, 2001), *Bremia lactucae* causing downy mildew of lettuce (Hulbert et al., 1988), the hop downy mildew pathogen *Pseudoperonospora humuli* (Morel, G. 1944), and *Pseudoperonospora cubensis* (Palti et.al 1980) which cause diseases on numerous cucurbits (e.g., cantaloupe, cucumber,

pumpkin, squash, watermelon). Downy mildews pathogenss affect yield of many crops and in some cases have led to 100% yield loss in individual fields (Raid and Datnoff, 1990). 20% of the 4.7 billion dollar fungicide market is devoted to the control of downy mildews, which vary in effectiveness for the reasons described above. Two downy mildew pathogens of maize, *Peronosclerospora philippines* and *Sclerophthora rayssiae* are highly virulent and are on the list of the USDA select agents.

Hyaloperonospora arabidopsidis (*Hpa*) is the causative agent of *Arabidopsis* downy mildew and one of a handful of naturally occurring pathogens of this reference plant species. Like other downy mildews, *Hpa* is an obligate biotroph and hence requires living tissue for its survival. *Hpa* was first isolated in the 1990's from wild *Arabidopsis* plants in Switzerland (E. Koch & A. J. Slusarenko, 1990). *Hpa* has proven to be an excellent pathosystem to study plant resistance mechanisms due to the massive research done on its host and its similarity with the agronomically-important *Phytophthora* species. Despite the experimental inconvenience of being an obligate biotroph, many resources are available for research on *Hpa* including a fully sequenced genome (Baxter et al., 2010). As a result of many years of *Hpa* research, many *Arabidopsis* NBS-LRR "*R*" genes have been isolated that recognize specific *Hpa* isolates (Allen, Bittner-Eddy, Grenvitte-Briggs, et al., 2004; Krasileva et al., 2010; Rehmany et al., 2005; Slusarenko & Schlaich, 2003).

Phytophthora and downy mildew life cycle, haustorium structure and function

Disease by oomycetes in the field is typically most prevalent during high humidity and at relatively cool temperatures (e.g., between 10 and 15°C). Sporulation is known to occur primarily at night and the spores are dispersed during the morning by the characteristic flinging action of dried sporangiophores. After that, successful infection occurs only after a conidium germinates either directly producing an appressorium or after making a short germ tube. This phenomenon occurs within six hours of contact with the leaf. Leaf penetration can only occur after the formation of a penetration hypha which grows from the lower portion of the appressorium (E. Koch & A. Slusarenko, 1990). Hyphae often branch off into the epidermal cells, as the penetration hypha grows further down, and later pyriform haustoria are produced and directly enter the mesophyll cells as the hyphae grow through the intercellular spaces (eg: Mims et al., 2004). At the end of the asexual life cycle, conidiophore initials grow out of the stomates, develop into branched conidiophores, and release their spores (E. Koch & A. Slusarenko, 1990). The haustorium is a very fascinating structure of oomycetes and is regarded as a major site for the occurrence of interesting molecular and cellular biology. Haustoria are thought to be the "feeding structure", i.e., the main sites for carbohydrate and amino acid uptake by the pathogen. In addition to nutrient uptake, haustoria are considered to be the primary site for the secretion of effector proteins (Whisson et al., 2007)

Recent developments in oomycete genomics

Whole genome sequences of several oomycetes are now available and have underpinned the revelation of novel features associated with oomycete biology and

evolution. Answers to several questions regarding the diversity of oomycete lifestyles, gene composition, horizontal gene transfer etc. have emerged from genome analysis and comparisons. These are summarized in the following sections.

Genome size and architecture

Over the past seven years, genomes of over 10 phytopathogenic oomycetes have been described in the literature. One of the first revelations of genome analysis has been the variation of repetitive DNA, which largely explains the variability in genome sizes of oomycetes ranging from as small as 37 and 43Mb for *Albugo laibachii* and *Pythium sylvaticum* respectively (Levesque et al., 2010; Links et al., 2011) to as large as 240 Mb in *Phytophthora infestans* (Haas et al., 2009; Raffaele et al., 2010). Most of the sequenced genomes encode similar number of genes (14,000 to 19,000), while the major difference lies in the repeat content. For instance, more than 75% of the *A. laibachii* genome is non-repeated whereas 75% of *P. infestans* genome is made up of repeated elements (Haas et al., 2009; Kemen et al., 2011). Most of the repetitive DNA in these genomes is in the form of transposable elements (TE). These regions of repetitive DNA comprise rapidly evolving, plastic regions.

Genome-wide ortholog analysis has led to the identification of a common proteome of about 8,000 to 9,500 genes that is conserved across phylogenetically divergent oomycete species (Haas et al., 2009; Seidl, Van den Ackerveken, Govers, & Snel, 2012; Tyler et al., 2006). These genes are predicted to be involved in core cellular

processes including DNA replication, transcription and protein translation. However, genes involved in pathogenesis are reduced in this core proteome region. Rather, genes involved in host interactions typically are located in regions of the genome that are depleted in core genes but enriched in repetitive elements. In other words, the genome is partitioned into "gene-dense" and "gene-sparse" regions. This partitioning suggests that genes which co-evolve with the host are found in the dynamic, rapidly evolving regions of the genome, whereas the genes that control essential function are located in stable regions that are relatively depleted in repetitive DNA. For example, in the genome of *P. infestans*, around 80% of the genes are found in tight clusters whereas the rest of the genes are separated from each other, sometimes, at a distance of >2kb. The repeat-dense regions harbor effectors and genes involved in epigenetic processes, implying the importance of evolution of gene regulation in order to adapt to new hosts. Studying gene-sparse regions gives an insight into the diversity of pathogenicity gene arsenals of these pathogens, as the majority of rapidly evolving effector genes are harbored in this region (Haas et al., 2009; Raffaele et al., 2010).

Genome reduction for adaptation to obligate parasitism

Two lineages of oomycetes have evolved an obligate lifestyle: The downy mildew pathogens and the *Albugo* (white blister rust) genus. Representatives of each lineage have been sequenced: *H. arabidopsidis* and *Albugo laibachii* with genome sizes of 100Mb and 37Mb respectively. This provides the opportunity to identify genomic signatures of biotrophy. These studies have revealed dramatic gene reductions in gene

families that encode pathogenicity proteis such the cell wall-degrading hydrolytic enzymes, PAMPs, and RXLR effectors. These reductions probably reflect an infection strategy of these pathogens to minimize host damage and thereby avoid the triggering of host immunity (Baxter et al 2010). Another common theme is mutations in genes for uptake and processing of inorganic nitrogen (nitrate and nitrite reductases, nitrate transporters) and sulphite metabolism (sulphite reductase). Interestingly, these features are also commonly observed in the case of biotrophic fungi (powdery mildew) (Spanu et al., 2010), rust fungi (Duplessis et al., 2011) and *Plasmodium* (Gardner et al., 2002), suggestive of convergent evolution. Another interesting fact is that, all the genes associated with motility or flagellum and zoospore adhesion are lost in the *H. arabidopsidis* genome (Kemen et al., 2011), suggestive of adaptation to a terrestrial lifestyle.

Horizontal gene transfer

Because fungi and oomycetes are known to have evolved independently to occupy common hosts, it is speculated that the phenomenon of horizontal gene transfer had taken place between them (Richards, Dacks, Jenkinson, Thornton, & Talbot, 2006). This hypothesis is supported by studies revealing that 7.6% of secreted proteins in the genome of *P. ramorum* are acquired through HGT from fungi (Richards et al., 2011). The most striking example of this event is the transfer of genes that encode necrosis and ethylene inducing peptide-like proteins (NLP) toxins from fungi as an elicitor of immune responses in several plants (Richards et al., 2011). Acquisition of PAMPs such as

transglutaminases is also thought to have occurred through this phenomenon from bacteria to oomycetes. Another example of a gene that is thought to have been transferred from fungi to the oomycetes, which encodes a putative protocatechuate, 3, 4-dioxygenase β-subunit (PcaH), a key functional component of the β - ketoadipate pathway, which would provide a means to use aromatic compounds from the environment. Acquisitions and loss of such genes are thought to be one of the key events in the origin of the oomycete plant pathogens.

Oomycete effectors and their functions

Effectors have characteristic signature motifs

Both the apoplastic and cytoplasmic oomycete effectors are proteins that contain characteristic signature motifs (Kamoun, 2006, 2007). Apoplastic effectors are characterized by the presence of N-terminal signal peptides utilized for secretion which is followed by the C-terminal effector domain(s) (Damasceno et al., 2008; Tian, Benedetti, & Kamoun, 2005; Tian, Huitema, Da Cunha, Torto-Alalibo, & Kamoun, 2004; Tian et al., 2007). The signal peptide is usually composed of a short stretch of amino acids that helps in the transport of the effector protein to the endoplasmic reticulum and finally through the secretory pathway. On the other hand, cytoplasmic effectors or the "RxLR effectors" contain the endoplasmic-reticulum type signal sequence (ER-SS) in the N-terminal region that is involved in secretion and translocation inside host cells which is then followed by a C-terminal domain carrying the effector activity (Kamoun, 2006,

2007; Morgan & Kamoun, 2007). The presence of the N-terminus signal peptide in effectors suggests that they are secreted to the outside of the pathogen (Birch, Rehmany, Pritchard, Kamoun, & Beynon, 2006). Cytoplasmic effectors also include a conserved host-targeting (HT) motif (Rehmany et al., 2005). This HT motif is around 30 amino acids in length and has a conserved domain termed RxLR within it. The RxLR effectors are termed so due to their amino acid sequence Arg-x-Leu-Arg (where "x" is commonly aspartic acid (D), glutamine (Q) or glutamic acid (E)) (Dou, Kale, Wang, Chen, Wang, Wang, et al., 2008; Rehmany et al., 2005; Whisson et al., 2007). Downstream of the RxLR within the HT motif are negative residues, E or D which make up the dEER motif (Bhattacharjee et al., 2006). Both the RxLR and dEER motif are required for proper host targeting (Bhattacharjee et al., 2006). The C-terminal half of RxLR effectors is highly polymorphic and it is thought that this region is responsible for the effector activity inside plant cells (Allen, Bittner-Eddy, Grenville-Briggs, et al., 2004; Allen et al., 2008; Rehmany et al., 2005). Many effectors also contain conserved C-terminal motifs namely tryptophan (W), tyrosine (Y) and leucine (L). These motifs are generally arranged as repeats and have been shown to be required for defense suppression activity and recognition by R proteins (Dou, Kale, Wang, Chen, Wang, Jiang, et al., 2008; Jiang, Tripathy, Govers, & Tyler, 2008). It is interesting to note that RxLR effector proteins are not present in all oomycete genomes, for example, *Pythium*, *Albugo*, and *Aphanomyces spp.* (Schornack et al., 2010). It has been hypothesized that RxLR effectors have recently evolved and are characteristic to haustoria-forming oomycete pathogens (Schornack et al., 2010).

Hundreds of candidate effector genes occur in Phytophthora and Hyaloperonospora genomes

Catalogues of effector genes from *Hyaloperonospora arabidopsidis*, *P. infestans*, *P. ramorum* and *P. sojae* were identified from bioinformatic screens based on the presence of conserved domains and motifs in the N-terminus of the RxLR effectors (Haas et al., 2009; Jiang et al., 2008; Tyler et al., 2006). *Phytophthora* species contain a large number of candidate RxLR genes, with 563 in *P. infestans*, 396 in *P. sojae* (396), and 374 in *P. ramorum*. The *Hpa* genome is predicted to harbor 134 effector proteins (Baxter et al., 2010). Often the RxLR genes are found in distinct clusters in the *Phytophthora* genomes (Haas et al., 2009; Jiang et al., 2008; Tyler et al., 2006). Hence, non-allelic homologous recombination and gene duplication event has been fairly common in the diversification of these effectors (Haas et al., 2009). Numerous oomycete effector genes have been cloned to date from *P. sojae* (PsAvr1a, PsAvr1b, PsAvr1k, PsAvr3a/5, PsAvr3b, PsAvr3c, PsAvr4/6, and PsAvh73), *P. infestans* (PiAvr1, PiAvr2, PiAvr3a, PiAvr3b, PiAvrBlb1, PiAvrBlb2 and PiAvrVnt1) and *H. arabidopsidis* (HaATR1, HaATR13, HaATR39, HaRxL17, and HaRxL96).

Amino acid polymorphism, a common occurrence among most effectors

Because effectors are major virulence determinants that can be recognized by plant *R* proteins, effector ranges are shaped through co-evolutionary arms races (Hogenhout et al., 2009; Kamoun, 2007). Hence effector genes undergo rapid sequence

alterations and show high rates of amino acid polymorphisms, particularly non-synonymous substitutions with positive selection (Allen *et al.*, 2004; Liu *et al.*, 2005; Rehmany *et al.*, 2005; Win *et al.*, 2007). The characterization of some of these effector genes has shown that positive selection has targeted the C-terminal effector domain regions of the effectors (Allen, Bittner-Eddy, Grenville-Briggs, et al., 2004; Z. Liu et al., 2005; Rehmany et al., 2005). Positive selection is more prevalent in the C-terminal effector domain regions as it contains repeats of conserved tryptophan (W), tyrosine (Y) and leucine (L) residues required for defense suppression activity and recognition by R proteins (Dou, Kale, Wang, Chen, Wang, Jiang, et al., 2008; Jiang et al., 2008).

Import of RXLR proteins into host cell

It is known that bacterial pathogens use specialized secretion machineries to directly inject effectors into host cells (Tseng, Tyler, & Setubal, 2009). (Lafont, Abrami, & van der Goot, 2004) showed that many bacterial toxins can enter host cells by endocytosis after binding glycolipid receptors. Parasites such as *Plasmodium* have a host targeting signal (HTS), that includes the Pexel motif, which enables translocation of secreted effectors across the vacuolar membrane (Bhattacharjee et al., 2006; Hiller et al., 2004; Martin et al., 2003). It is known from previous studies that effectors from oomycetes have the N-terminus HTS which contain the RxLR and dEER motifs (Jiang et al., 2008; Rehmany et al., 2005; Tyler et al., 2006), which have been found in some studies to be necessary and sufficient for effector translocation into host cells in the absence of the pathogen (Dou, Kale, Wang, Jiang, et al., 2008; Whisson et al., 2007). The

mechanism of translocation has been under active investigation. Further research suggests that RXLR effectors could bind with high affinity to the cell-surface phospholipid, phosphatidylinositol-3-phosphate (PI3P) and/or phosphatidylinositol-4-phosphate (PI4P), via RxLR motifs, but not to other PI polyphosphates or any anionic phospholipids, and mediate effector translocation into the host cytoplasm (Kale et al., 2010). However the mechanism of effector entry is still under debate and is an area of active research due to the lack of reproducibility of the PI3P binding experiment results (Ellis & Dodds, 2011; Gan et al., 2010; Yaeno et al., 2011). Gan et al 2010 have shown that the C-terminal domain of the effector AvrM and AvrL567 from the flax rust pathogen was involved in PI3P binding and more recently, Yaeno et al., 2011 showed that the effector domain and not the RxLR domain of Avr3a was required for PI3P binding and this was essential for the stabilization of its target CMPG1 in order to effectively suppress cell death induced by INF1. For the oomycete fish pathogen, *Saprolegnia parasitica*, it was found that that binding to tyrosine-o-sulphate and not PI3P was necessary for the translocation of the SpHtp1 effector (Wawra et al., 2012). It is highly possible that there are multiple mechanisms of cell entry by oomycete effectors like the ones that have been proposed for the malarial parasite, *Plasmodium falciparum* having the characteristic PEXEL host-targeting motif. This is an area of oomycete biology that needs much more study in the short term.

Effectors are differentially expressed during infection

Effector genes from *Phytopthora spp*. show diverse patterns of mRNA expression during the infection of host plants. Many of the known *P. infestans* RxLR effectors get induced during the stages of pre-infection and early stages of infection of potato (Wang et al., 2011; Whisson et al., 2007). Other effectors like *Avr3a*, *Avr4* and *AVRblb1* are upregulated during the biotrophic phase of infection, which is until 2-3 days post inoculation (Haas et al., 2009). Finally, there are some like the NPP1 toxin that get expressed at only the late necrotrophic phase (Qutob, Kamoun, & Gijzen, 2002). Regulation of these effector genes results in distinct stage-specific expression patterns that reflect the elaborate processes of cellular control urged by *Phytophthora* as effectors get deployed during host colonization (Wang et al., 2011). In most cases, this stage-specific expression pattern is utilized by the pathogen for its own benefit. The most strongly expressed effector genes of *P. sojae* showed two distinct expression patterns, namely, the "immediate early" and the "early" genes. Immediate early genes are very strongly expressed at the very beginning of the infection whereas the early genes are strongly induced within 6 to 12 hours post infection which corresponds to the appearance and development of numerous haustoria in the infected host (Wang et al., 2011), hence, underlying the importance of the initial biotrophic phase. It has been suggested that the immediate early effectors are successful in ETI suppression, thereby paving the way for early effectors to successfully suppress PTI responses (Wang et al., 2011).

Effectors have distinct localization sites in the host tissue

In several cases, oomycete RXLR effector proteins are known to have different locations and targets in the host (Kamoun, 2006, 2007). In a very recently conducted study, out of the 49 HaRxL effector proteins tested, 16 of them localized to the host nucleus and cytoplasm, 3 strictly to the host cytoplasm and 1 to the vacuole (Caillaud et al., 2012). It is interesting to note that some of the effectors that were strictly localized to the nucleus did not have the characteristic nuclear localization signal (NLS) motif (Caillaud et al., 2012). A second class of effectors was found to be targeting the plant membrane trafficking network including the regions of endoplasmic reticulum (Caillaud et al., 2012).

Structure of some RXLR effector proteins

Recent structural studies of some of the RXLR effectors have provided additional information regarding the function and evolution of these proteins (Boutemy et al., 2011; Chou et al., 2011; Leonelli et al., 2011; Yaeno et al., 2011). The structures generated using nuclear magnetic resonance (NMR) for ATR1 and crystallography for others revealed that the C-terminal domains of some of these proteins had a conserved fold comprising three helices that span conserved trypyophan (W) and tyrosine (Y) residues in the hydrophobic core of the fold; this was termed the WY fold region (Win et al., 2012). A fourth helix forming a bundle was identified in some other effector proteins that corresponded to a positive charged lysine (K) residue (Boutemy et al., 2011; Yaeno et al., 2011). It is predicted that the WY fold played an important role in forming a scaffold that supported the RXLR effectors from their increased changes in primary sequence and

structural architecture (Win et al., 2012). This insight is a valuable foundation for future studies to understand how this fold evolves to support interaction with different host proteins.

Several effectors suppress plant immunity

Other than the host targeting motifs and the WY fold, there are essentially no other recognizable motifs in the sequences of RxLR effectors. In other words, the primary sequences of these proteins provide no clues to their molecular functions. Furthermore, the catalogs of candidate effectors were constructed based on only on short motifs. Thus, much of the initial efforts towards understanding the virulence activities of oomycete effectors have been focused on generic assays to obtain evidence that the candidate effectors are bona fide effectors that can suppress plant immunity. In terms of effectors from *P. sojae*, overexpression of the Avr1b protein causes increase in pathogen growth on soybean plants (Dou, Kale, Wang, Jiang, et al., 2008) and also suppress cell death induced by the pro-apoptotic BAX protein in both yeast and plants (Dou, Kale, Wang, Jiang, et al., 2008). Similarly, using agro-infiltration assay in *Nicotiana benthamiana*, Wang et al. 2011 found a vast majority of *P. sojae* effectors could suppress cell death induced by BAX and/or INF1. For *Phytophthora infestans,* the Avr3a, PexRD8 and $PexRD36_{45\text{-}1}$ suppress hypersensitive cell death induced by INF1 (Armstrong et al., 2005; Bos, Chaparro-Garcia, Quesada-Ocampo, McSpadden Gardener, & Kamoun, 2009; Bos et al., 2006; Oh et al., 2009). For *Hyaloperonospora arabidopsidis,* the ATR13 protein is known to suppress callose deposition triggered by *Pseudomonas syringae*

(Sohn, Lei, Nemri, & Jones, 2007). In a large-scale callose deposition screen, 35 out of 62 *Hpa* effectors tested were found to suppress callose deposition (Fabro et al., 2011). In addition, (Sohn et al., 2007) have shown that both the *H. arabidopsidis* effectors ATR1 and ATR13 enhance the virulence *P. syringae* when delivered through the effector detector vector method and also suppress the production of Reactive Oxygen Species (ROS).

For a handful of effectors, there is now evidence supporting probable mechanisms of virulence. For example, it has been shown that Avr3a interacts with the *N. benthamiana* U-box protein, CMPG1 and this interaction is thought to promote virulence by stabilizing CMPG1 (Bos et al., 2010; Gonzalez-Lamothe et al., 2006). Also, another *P. infestans* effector SNE1 was recently found to suppress plant cell death and cell death triggered by NLP toxins (Kelley et al., 2010). AvrBlb1 effector protein from *P. infestans* is thought to disrupt the RGD-motif-mediated adhesions between the plant cell wall and the plasma membrane (Gouget et al., 2006; Senchou et al., 2004). Interestingly, AvrBlb2 from the same oomycete inhibits the secretion of host defense cysteine protease, C14 by accumulating on the inner face of the host plasma-membrane (Bozkurt et al., 2011). It has been recently shown that another *P. sojae* effector Avr3b, which is a nudix hydrolase having the ability to destroy NADP and ADP-ribose, can suppress ROS accumulation (Dong et al., 2011). Further studies to understand the mechanisms through which RxLR effectors promote virulence will be a major area of emphasis in the years to come.

Non-RXLR effectors from oomycetes

Genomic and molecular studies involving oomycetes have identified additional protein families that are involved in oomycete virulence. For example, the second major class of widely distributed cytoplasmic effector proteins that oomycetes such as those of the orders *Saprolegniales*, *Pythiales*, *Albuginales* and *Peronosporales* produce are termed as the crinklers (CRN) (Gaulin et al., 2008; Levesque et al., 2010; T. Liu et al., 2011). They are named as crinklers because many trigger a crinkling and necrosis phenotype when transiently overexpressed in plants (Torto et al., 2003). Similar to RXLR proteins, crinklers are diverse, rapidly evolving and have characteristic motifs with a conserved N-terminal LXLFLAK motif or the "crinkler domain" for host-cell entry followed by diverse C-terminal domains (Haas et al., 2009; Schornack et al., 2010). Most of the crinklers identified to date localize to the host nucleus (T. Liu et al., 2011; Schornack et al., 2010) and one crinkler, CRN8, encodes a kinase that triggers HR by a mechanism dependent on its nuclear localization (Schornack et al., 2010). Several crinklers have also been found to be important for virulence; one of those achieves this by ETI suppression in the host (Links et al., 2011).

A number of other effector superfamilies await detailed functional characterization. Recent genome studies of the white rust obligate biotrophic plant pathogen, *Albugo laibachi* identified candidate effector proteins having the novel CHXC motif (cysteine, histidine, x, cycteine) (Kemen et al., 2011). These include the extracellular toxins, hydrolytic enzymes and several enzyme inhibitors (Tseng et al., 2009). Specific examples include apoplastic effector proteins that inhibit plant hydrolytic proteins (i.e., the plant glucanase and protease inhibitors) which protect the pathogen

from damage by these host degradative enzymes (Kamoun, 2006). Oomycetes also secrete proteinase inhibitors to degrade the several serine and cysteine-rich proteases the host plant produces as a means of its natural defense mechanism. For example, *P. infestans* produces two serine protease inhibitors, EPI1 and EPI10, which bind to and inhibit P69B, a tomato apoplastic protease (Tian et al., 2005; Tian et al., 2004). Two extracellular cycteine protease inhibitors EPIC1 and EPIC2 from *P. infestans* inactivate C14, Pip1 and Rcr3 proteases in the apoplast thereby protecting the intracellular hyphae (J. Song et al., 2009; Tian et al., 2007). The best studied of these is a papain-like cysteine protease, C14 which is essential in plant immunity (Bozkurt et al., 2011). However not a lot is known about these candidate effectors and functional characterization of these still needs to be established.

Examples of extracellular toxins are provided by the necrosis and ethylene inducing peptide-like proteins (NLP) (Qutob et al., 2006), the *Phytophthora cactorum* factor (PcF) and the secreted cysteine-rich (Scr) toxin (Z. Liu et al., 2005; Orsomando, Brunetti, Pucci, Ruggeri, & Ruggieri, 2011; Orsomando et al., 2001). Of these, NLP families of proteins are best studied. They are highly toxic in nature and are known to trigger strong programmed cell death in a number of host plants (Gijzen & Nurnberger, 2006; Qutob et al., 2006). They are found extensively in large copies in all *Phytophthora spp* (Haas et al., 2009; Tyler et al., 2006), along with some fungal and bacterial plant pathogens. As mentioned above, it is hypothesized that horizontal transfer of these NLP genes from fungi facilitated the emergence of oomycetes as plant pathogens (Reiss et al., 2011). Other families, called PcF and Scr, show greater heterogeneity among some

oomycetes and are found extensively in the genomes of *Phytophthora spp* (Haas et al., 2009; Orsomando et al., 2011) and *Pythium* (Levesque et al., 2010).

Oomycetes also produce several complex carbohydrate-degrading enzymes such as lipases, proteases, lyases, glucanases, cellulases and pectin esterases that presumably aid in pathogen penetration (Haas et al., 2009; Tyler et al., 2006). Obligate biotrophs such as *H. arabidopsisdis* and *Albugo* have reduced numbers of genes encoding these enzymes as it is believed that the carbohydrate fragments generated as a result of enzyme activity are putative PTI triggers and this gene reduction in obligate biotrophs suggest a genomic adaptation for stealth (Baxter et al., 2010; Kemen et al., 2011; Links et al., 2011).

Conclusions

When the collections of effectors are compared between *Hpa* and *Phytophthora*, there is a large amount of divergence. Only 30% of these effectors have greater than 20% identify with their best *P. sojae* match. Only 5% shared greater than 40% identity. These are the ones that have become the most interesting effectors for us and I plan to highlight this in the next two chapters of my dissertation. There is a possibility that these effectors were selected during the evolution of oomycetes and are maintained because of their importance in establishing or maintaining the interaction with their host.

One advantage of *Phytophthora* that helps in understanding pathogenesis is that, it can be genetically modified. Hence we use it as a parallel model system along with *Arabidopsis-Hpa* interaction for our purposes. Numerous *Phytophthora* tools including

sequenced genomes are available to study this model system. Since *Phytophthora* are heterotrophic, it is possible to culture and transform them (e.g. gene silencing and overexpression) which is not possible with *Hpa*. We have exploited the power of *Arabidopsis* genetics with the *Hpa* pathosystem and utilized the genetic tractability of *P. sojae* for our experimental purposes. Our overall goal is to use this relationship to study conserved effectors between the two related species. All-together, examining conserved effectors gives us some insight to how these large effector families have evolved.

Despite the substantial progress in recent years, there is much that we still do not know about oomycete effectors. A mechanistic understanding of how oomycete effectors traffic inside host cells, modify host targets and alter plant processes remain poorly understood. Homology searches to known proteins offer little or no clues for effector targets or function, delaying our understanding of the complex interaction between oomycetes and plants. The identification of effector molecules from various eukaryotic pathogens enables us to draw parallels between prokaryotic and eukaryotic pathogens and helps us to investigate the extent to which these diverse pathogens share virulence strategies and target similar pathways of plant immunity.

My dissertation research is designed to increase understanding of the molecular mechanisms that enable oomycete pathogens to cause diseases on plants. I focus on effectors that are conserved between *Hpa* and *P. sojae*. I show that analysis of conserved effectors reveal virulence functions that are important for all oomycete plant pathogens. In the next two chapters, I summarize identification and functional analysis of a one effector from *H. arabidopsidis* that has an identifiable homolog in *Phytophthora sojae*.

My dissertation research focuses first on a detailed functional characterization of *HaRxL23* from *H. arabidopsidis* and *PsAvh73* from *Phytopthora sojae*. The second part of my dissertation focuses on the interaction between an effector protein from *H. arabidopsidis* and its target in the host.

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Chapter 2

Conserved RxLR effectors from oomycetes *Hyaloperonospora arabidopsidis* and *Phytophthora sojae* suppress PAMP- and effector-triggered immunity in diverse plants

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Abbreviations: *Arabidopsis thaliana* responsive (ATR), conserved effector locus (CEL), crinkling and necrosis 2 (CRN2), effector detector vector (EDV), effector to host analyzer (EtHAn), Effector-triggered immunity (ETI), empty vector (EV), β-glucoronidase (GUS), *Hyaloperonospora arabidopsidis* (*Hpa*), hypersensitive response (HR), host targeting (HT), microbe-associated molecular patterns (MAMP), pathogen-associated molecular patterns (PAMP), programmed cell death (PCD), pattern recognition receptor (PRR), *Pseudomonas syringae* pv. *tomato* (*Pst*),PAMP-triggered immunity (PTI), resistance protein (R), signal peptide (SP), Type 3 secretion system (T3S).

Abstract

Effector proteins are exported to the interior of host cells by diverse plant pathogens. Effector proteins have been well characterized in bacteria. Contrastingly, their functions and targets in oomycete pathogenicity are poorly understood. Bioinformatic analysis of genome sequences from oomycete pathogens *Phytophthora sojae*, *P. ramorum*, *P. infestans* and *Hyaloperonospora arabidopsidis* (*Hpa*) have led to the identification of a large number of candidate RxLR effector genes, encoding proteins with host cell targeting motifs. Although most of these genes are very divergent between oomycete species, several genes are conserved between *Phytophthora* species and *H. arabidopsidis*, suggesting that they play important roles in pathogenicity. This research aims to characterize a pair of conserved effector candidates, *HaRxL23* and *PsAvh73*, from *Hpa* and *P.sojae* respectively. We show that *HaRxL23* is expressed early during the course of *Hpa* infection of *Arabidopsis*. *HaRxL23* triggers an ecotype-specific defense response in *Arabidopsis*, suggesting that it is recognized by a host surveillance protein. *HaRxL23* and *PsAvh73* can suppress immunity triggered by pathogen associated molecular patterns (PTI) and by effectors (ETI) *in planta*. Both effectors enhance bacterial virulence in *Arabidopsis* when delivered by the Type III secretion system. Experiments with transgenic *Arabidopsis* constitutively expressing *HaRxL23* and *PsAvh73* also suggest suppression of immunity triggered by pathogen associated molecular patterns, enhancement of bacterial and oomycete virulence and suppression of defense gene induction. Hence, these conserved oomycete RxLR effectors suppress PAMP- and Effector-triggered immunity across diverse plants.

Introduction

Oomycetes pathogens have evolved sophisticated mechanisms to overcome host recognition by suppressing host defenses (Bos et al., 2006; Fabro et al., 2011; Oliver & Ipcho, 2004; van der Hoorn & Kamoun, 2008). One of the most destructive group oomycete genera is *Phytophthora*, which is comprised of over 80 species (Tyler, 2007). For example, *Phytophthora infestans,* the causative agent of the potato late-blight disease, caused the Great Irish Famine during 1845-1849 and it remains to be the most destructive pathogen of potato and tomato to date. Downy mildews pathogens comprise a sister group to the *Phytophthora* genus. They are obligate parasites on plants and cause diseases on grape, lettuce, hop and cucurbits. They have affected yield of many crops and in some cases have led to 100% yield loss in individual fields (Raid and Datnoff, 1990). Overall, it is quite difficult to control oomycete diseases because they rapidly evolve to overcome fungicides or genetic resistance in the host.

Plants maintain a robust, multilayered immune system that enables them to resist most pathogens (Jones & Dangl, 2006). The primary layer of plant immunity is activated by the recognition of conserved microbial molecules termed as pathogen (or microbe) - associated molecular patterns (PAMPs or MAMPs) by the plant pattern recognition receptors (PRRs) (Jones and Dangl, 2006; Zipfel and Robatzek, 2010). This is termed PAMP-triggered immunity (PTI). Outputs of PTI include production of reactive oxygen species, callose deposition, activation of MAP kinase signaling cascades and induction of defense genes (Jones & Dangl, 2006; Zipfel & Robatzek, 2010).

In response to PTI, successful pathogens deliver effector proteins to the host cytoplasm to interfere with PTI responses (Bos, Chaparro-Garcia, Quesada-Ocampo,

McSpadden Gardener, & Kamoun, 2009; Bos et al., 2006; Fabro et al., 2011; van der Hoorn & Kamoun, 2008). Effectors have been extensively studied in bacterial phytopathogens such as *Pseudomonas syringae* pv. *tomato* (*Pst*), which secrete around 20 to 30 effector proteins during infection (Abramovitch, Anderson, & Martin, 2006; Chang et al., 2005; Gohre & Robatzek, 2008; Lindeberg, Cunnac, & Collmer, 2009, 2012). Some pathogen effectors are recognized by host resistance (R) proteins directly or indirectly, activating a second line of plant immunity (effector-triggered immunity or ETI) (Chisholm, Coaker, Day, & Staskawicz, 2006; Jones & Dangl, 2006; van der Hoorn & Kamoun, 2008). The culmination of ETI is the production of localized cell death or hypersensitive response (HR) that stops the pathogen in its tracks (Dodds & Rathjen, 2010). Pathogens, on the other hand, have evolved more effectors to counteract ETI by either avoiding R protein recognition or suppressing downstream signaling events (Jones & Dangl, 2006; Links et al., 2011; Wang et al., 2011).

Phytophthora species and downy mildew pathogens are thought to secrete effectors from intracellular feeding structures called haustoria (Torto et al., 2003; Whisson et al., 2007). Based on their targets in the host, effectors are broadly categorized as apoplastic or cytoplasmic (Birch, Rehmany, Pritchard, Kamoun, & Beynon, 2006; Kamoun, 2006; Qutob et al., 2006). Bioinformatic approaches have led to the identification of many effector candidates in the genomes of oomycetes (Baxter et al., 2010; Kamoun, 2006; Schirawski et al., 2010; Spanu et al., 2010). Some of the most widely studied oomycete effector proteins defined by the presence of signal peptide (SP) in its N-terminal followed by host targeting (HT) region comprised of the amino acid motifs RxLR (arginine (Arg)-any amino acid-leucine (Leu), (Arg)) and EER (glutamine

(Glu)-Glu-Arg)) motifs (Rehmany et al., 2005; Whisson et al., 2007). The SP enables secretion of the effectors outside the pathogen and the host-targeting motifs are required for targeting the effectors to the interior of host cells (Bhattacharjee et al., 2006; Dou et al., 2010; Dou, Kale, Wang, Chen, Wang, Wang, et al., 2008; Dou, Kale, Wang, Jiang, et al., 2008; Grouffaud, van West, Avrova, Birch, & Whisson, 2008; Kamoun, 2006; Rehmany et al., 2005; Whisson et al., 2007). These signals are typically followed by a C-terminal domain that mediates the effector protein's specific function inside the host cell (eg: interaction with host protein). Many effectors also contain conserved C-terminal motifs containing tryptophan (W), tyrosine (Y) and leucine (L) residues. These motifs are generally arranged as repeats and have been shown to be required for defense suppression activity and recognition by R proteins (Dou, Kale, Wang, Chen, Wang, Jiang, et al., 2008; Jiang, Tripathy, Govers, & Tyler, 2008).

Phytophthora spp. and downy mildew pathogens maintain large repertoires of predicted RxLR proteins (Haas et al., 2009; Tyler et al., 2006) but functional analysis of only a handful have occurred to date. For example, cytosolic effectors of *Phytophthora infestans* Avr3a, PexRD8 and PexRD36$_{45\text{-}1}$ suppress hypersensitive cell death induced by another *P. infestans* protein, INF1 (Armstrong et al., 2005; Bos et al., 2009; Bos et al., 2006; Oh et al., 2009). More recently, it has been shown that Avr3a interacts with the *N. benthamiana* U-box protein, CMPG1 and this interaction is thought to promote virulence by stabilizing CMPG1 (Bos et al., 2010; Gonzalez-Lamothe et al., 2006). Another *P. infestans* effector SNE1 was recently found to suppress plant cell death and cell death triggered by NLP toxins (Kelley et al., 2010). The AvrBlb1 effector protein from *P. infestans* is thought to disrupt the RGD-motif-mediated adhesions between the plant cell

wall and the plasma membrane (Gouget et al., 2006; Senchou et al., 2004). More recently, it has been shown that AvrBlb2 inhibits the secretion of host defense cysteine protease, C14 by accumulating on the inner face of the host plasma-membrane (Bozkurt et al., 2011).

Hyaloperonospora arabidopsidis (*Hpa*) is an obligate biotroph and is the causative agent of *Arabidopsis* downy mildew (Slusarenko & Schlaich, 2003). Recently, sequencing the genome of the *Hpa* isolate *Emoy2* revealed at least 134 candidate effectors (HaRxLs) (Baxter et al., 2010). Of these, at least 42 have been found to be expressed during infection (Cabral et al., 2011). To date, only a few *Hpa* effector genes including *Arabidopsis thaliana* recognized 1 (ATR1) and ATR13 have been confirmed as *bona fide* effectors (Allen et al., 2004; Anderson et al., 2012; Fabro et al., 2011; Rehmany et al., 2003). Very few of the *Hpa* effectors have recognizable homologs in *Phytophthora* species (Baxter et al., 2010). However, some are conserved, and we are characterizing these to determine whether they are maintained because of particular importance in establishing or maintaining the interaction with their host (eg: (Anderson et al., 2012)). We anticipate that analysis of conserved effectors will reveal virulence functions that are important for all oomycete plant pathogens.

In this study we describe a homologous pair of effectors from *H. arabidopsidis* and *Phytophthora sojae, HaRxL23* and *PsAvh73*. We show that *HaRxL23* and *PsAvh73* are expressed early during the course of infection. *HaRxL23* and not *PsAvh73* trigger an ecotype-specific defense response in *Arabidopsis*, suggesting that it is recognized by a host surveillance protein. Both the effectors are able to suppress immunity triggered by PAMPs and by effectors in diverse plants and can also enhance bacterial virulence in

Arabidopsis. Experiments with transgenic *Arabidopsis* constitutively expressing *HaRxL23* and *PsAvh73* confirm the above results with regard to PTI suppression, and enhancement of bacterial virulence. Finally, transgenic plants also show enhancement of oomycete virulence and suppression of defense gene induction.

Results

HaRxL23 and *PsAvh73* share conserved functional domains and are syntenic between *Phytophthora spp.* and *H. arabidopsidis*.

Effector *HaRxL23* is one of four high-confidence *Hpa* RxLR candidate effector genes for which a homolog is present at syntenic loci in *Phytophthora* genomes (Baxter et al., 2010).

HaRxL23 and *PsAvh73* contain a predicted N-terminal signal peptide (SP). The SP is followed by the host-targeting (HT) region comprising the RxLR (RLLR and RALR) motif in *HaRxL23* and *PsAvh73 respectively*. Both also contain a short acidic dEER-like motif in their host-targeting (HT) region (**Figure 2.1**). Both *HaRxL23* and *PsAvh73* effectors contain multiple copies of the degenerate W, Y, and L motifs (Dou, Kale, Wang, Chen, Wang, Jiang, et al., 2008). There are no other discernible motifs or subcellular localization signals in the sequences of these effectors.

HaRxL23 and *PsAvh73* are induced early during pathogen infection.

Since *HaRxL23* and *PsAvh73* were selected solely on sequence motifs, we needed experimental evidence to confirm whether these genes encode *bona-fide* effector proteins. As a first step, we tested whether *HaRxL23* was induced during infection by

virulent *Hpa*. Four weeks old, short-day grown *Arabidopsis* ecotype Oystese (Oy-1) plants were inoculated with conidial suspension of spores from the virulent *Hpa* isolate Emoy2. RNA was isolated from infected plant tissue harvested at different time points, and cDNA was generated. Effector expression was assayed using quantitative real-time PCR (qRT-PCR) with primers specific for *HaRxL23*. Abundance of *HaRxL23* transcript peaked at around 12 hours post infection (**Figure 2.2**) and declined during the later time points. A similar pattern of "early" expression was observed for *PsAvh73* in a compatible interaction study between *P. sojae* and soybean (Wang et al., 2011). These assays together demonstrate that both genes are expressed early during infection, consistent with a function as effectors.

***HaRxL23* is recognized in the host in an ecotype-specific manner.**

As another test of effector function for *HaRxL23*, we determined whether it induces effector-triggered immunity (ETI) in *Arabidopsis*. We used the "effector detector vector (EDV)" system, in which *Pseudomonas* bacteria delivered *HaRxL23* or *PsAvh73* via type III secretion system (T3S) to the interior of *Arabidopsis* cells (Sohn, Lei, Nemri, & Jones, 2007). We used *Pseudomonas fluorescens* EtHAn (Effector to Host Analyzer), which is a non-pathogen of *Arabidopsis* and does not encode any effector proteins of its own (Thomas, Thireault, Kimbrel, & Chang, 2009). This strain was genetically recombineered to have a functional T3SS similar to that of *P.syringae.* This ensured the exclusive delivery of our effector in the host, without complications from endogenous bacterial effectors. Equally important is that EtHAn does not trigger disease symptoms, making it easier to visually discern an HR without background from disease symptoms.

As a standard of comparison for a typical hypersensitive response (HR), we used *Pst* DC3000 carrying AvrRpt2 in the resistant *Arabidopsis* ecotype Col-0 **(Figure 2.3A-B)** (Sohn et al., 2007). We also used the *Hpa* avirulence effector, Atr13, which has been previously shown to trigger HR due to recognition of this effector by the RPP13 resistance protein. When delivered via the *Pseudomonas* system, Atr13 produced a typical leaf collapse phenotype **(Figure 2.3A-B)** (Sohn et al., 2007). We inoculated Pfo EtHAn expressing *HaRxL23* onto 48 *Arabidopsis* ecotypes. We observed leaf collapse symptoms comparable to the two controls in the *Arabidopsis* ecotype Ei-4 **(Figure 2.3A-B)**. Two other *Arabidopsis* ecotypes, Ob-0 and Pla-1, also showed partial leaf collapse phenotypes in response to EtHAn (*HaRxL23*), indicative of a weak HR (**Supplemental Figure 2.1**). When *PsAvh73* was delivered to Ei-4, a weak leaf collapse phenotype was observed. This experiment indicates that *HaRxL23* and not *PsAvh73* is recognized by the *Arabidopsis* immune system in an ecotype-specific manner, suggestive of gene-for-gene resistance.

To further confirm our results we quantified bacterial growth *in planta.* We predicted that if *HaRxL23* is triggering the cell death response in Ei-4 then there should be less growth of bacteria in these plants compared to control bacteria that did not contain the effector. Hence, we compared the growth, in Ei-4, of virulent *Pst* DC3000 expressing *HaRxL23* to *Pst* DC3000. Accordingly, we observed a four-fold reduction in bacterial growth in DC3000(*HaRxL23*) compared to DC3000 **(Figure 2.3C)** in Ei-4. This is consistent with the *HaRxL23*-dependent HR response and further supports that *HaRxL23* is triggering ecotype-specific resistance, consistent with effector functionality.

***HaRxL23* and *PsAvh73* suppress programmed cell death in *Nicotiana benthamiana*.**

As a first test of whether *HaRxL23* and *PsAvh73* contribute to virulence, we assayed whether; these effectors could suppress programmed cell death (PCD) when transiently expressed, via *Agrobacterium*-mediated delivery, in *N. benthamiana*. Neither of the effectors could induce cell death in *N. benthamiana*. For these assays, we induced PCD by delivering the *P. infestans* elicitin, infestin 1 (INF1) or *P. sojae* PsAvh163, which cause a strong HR in leaves of *N. benthamiana* (Anderson et al., 2012; Bos et al., 2006; Wang et al., 2011). *Agrobacterium tumefaciens* GV3101 strain expressing either *HaRxL23* or *PsAvh73* was infiltrated into the leaves of *N. benthamiana*. 24 or 48 hours later, these leaves were challenged with INF1 or *PsAvh163* via *Agrobacterium*-mediated delivery. Five to seven days later, we visually scored each infiltration site for cell death. We observed that *HaRxL23* does not suppress the programmed cell death caused by INF1 (**Supplemental Figure 2.2**), but *PsAvh73* does so successfully (**Figure 2.4A**). Both *HaRxL23* and *PsAvh73* were able to suppress PsAvh163-induced cell death (**Figure 2.4B**). An *Agrobacterium* strain expressing YFP served as the control for this experiment and did not suppress PCD either by INF1 or PsAvh163. These results suggested that, like many bacterial and oomycete effectors (Alfano, 2009; Cabral et al., 2011; Wang et al., 2011), these two effectors were also capable of suppressing PCD in *N. benthamiana*.

***HaRxL23* and *PsAvh73* suppress programmed cell death in soybean.**

Our next experiment was to test for suppression of immunity in soybean leaves. *Avr4/6* is an RxLR effector from *P. sojae* that triggers an HR in soybean cultivars with the *RPS4* or *RPS6* resistance genes (Kale & Tyler, 2011). When *Avr4/6* is co-transformed

with *β*-glucuronidase (GUS) into leaves with *RPS4* or *RPS6*, programmed cell death (PCD) from a hypersensitive response elicited by *Avr4/6* reduces the amount of blue spots visualized. Co-transformation with a third gene that suppresses PCD will enhance plant cell viability, which manifests itself as a larger number of GUS-positive spots in the leaves bombarded with the effector (Dou, Kale, Wang, Chen, Wang, Jiang, et al., 2008). This assay allows for quantitative assessments of candidate effector's ability to suppress PCD. A second elicitor from *P. infestans*, CRN2, was also used in this assay. CRN2 is a necrosis-inducing extracellular protein (crinkling and necrosis 2) and its ectopic expression results in cell death response in *N. benthamiana* leaves and also induce the expression of defense responses in tomato (Torto et al., 2003).

Avr4/6 was cloned into pUC19 plasmid driven by a dual *CaMV35s* promoter. Candidate effectors were cloned into a Gateway compatible binary vector with a CmV35S promoter. Using a double barrel apparatus retrofitted to BioRad PDS-1000 Gene Gun, control and experimental samples are bombarded together reducing variability and assisting in shooting a defined area (**Supplemental Figure 2.4**) (Dou et al., 2010; Dou, Kale, Wang, Chen, Wang, Jiang, et al., 2008). Transiently expressing Gus and *Avr4/6* in soybean using the double barrel gene gun as the delivery method reduced the amount of *Gus* expressing cells up to 60% compared to the empty vector control (Dou et al., 2010). However, a combination of *HaRxL23* or *PsAvh73, Gus* and *Avr4/6* (**Figure 2.5A**) increased the amount of *Gus* expressing viable cells. Similarly, we observed a 65% decrease of *Gus*-expressing viable cells in tissue bombarded with CRN2 + EV, relative to control samples bombarded with *Gus* but not CRN2 and there was a significant increase in cell viability when *HaRxL23* or *PsAvh73* was included in the setup (**Figure 2.5B**). This

experiment indicates that both *HaRxL23* and *PsAvh73* are able to suppress Avr4/6 and CRN2-induced PCD in soybean.

HaRxL23 and *PsAvh73* enhance susceptibility to virulent *Hpa*.

Another approach to test effectors in suppressing PAMP-triggered and Effector-triggered immunity is to stably express the effector genes in transgenic *Arabidopsis* plants. This method has been previously used in the case of several bacterial and oomycete effectors (Fabro et al., 2011; Munkvold & Martin, 2009; Nomura et al., 2006). We generated stably transformed *Arabidopsis* Col-0 transgenic lines under the control of the constitutive CaMV35S promoter. We obtained several, independent, single insertion-locus lines that showed variable transcript abundance and these were bred to homozygosity. mRNA abundance of the transgene in each experimental line was verified by semi-quantitative and quantitative RT-PCR. Representative lines expressing variable transcript abundance were selected for all subsequent experiments (**Supplemental Figure 2.5**).

We first tested whether *in planta* overexpression of the two effectors resulted in alteration in pathogen virulence. Wild type and transgenic seedlings were inoculated with virulent *Hpa* Emco5 spores and pathogen growth was quantified by counting sporangiophores at seven days after inoculation. Representative *Arabidopsis* lines constitutively expressing either *HaRxL23* or *PsAvh73* showed enhanced susceptibility to virulent *Hpa* isolate Emco5 compared to wild type Col-0 plants (**Figure 2.6**). This result suggests that both effectors are capable of suppressing basal resistance to virulent *Hpa* in *Arabidopsis*.

***HaRxL23* and *PsAvh73* suppress callose formation in stably transformed *Arabidopsis* in response to *Pseudomonas syringae DC3000(ΔCEL)* mutant.**

Because *H. arabidopsidis* makes intimate contact with host cell walls during pathogenesis, we hypothesized that one important function of *Hpa* effectors might be the suppression of cell wall-based defenses like callose. Callose are a β-1, 3 glucans that get deposited between the cell wall and cell membrane near the invading pathogen, and hence are key indicators of PTI response.

The EDV system was again used to determine whether the effectors can suppress callose deposition *in planta* (Sohn et al., 2007). The non-pathogenic *Pst* DC3000(ΔCEL) mutant contains deletions of at least four effector genes that are conserved among *P. syringae* DC3000. Deletion of the effectors result in dramatically reduced virulence in tomato and *Arabidopsis* and extensive callose deposits in the host plant, because this strain is significantly compromised in its ability to suppress PTI (DebRoy, Thilmony, Kwack, Nomura, & He, 2004; Sohn et al., 2007).

Accordingly, we observed extensive callose deposition in wild type *Arabidopsis* Col-0 plants when syringe-infiltrated with the ΔCEL mutant (**Figure 2.7A**). Contrastingly, a reduction of 30-50% in callose deposits is observed in multiple lines of *Arabidopsis* plants constitutively expressing either *HaRxL23* or *PsAvh73* (**Figure 2.7**). This degree of suppression is similar to other *Hpa* effectors assayed elsewhere (Fabro et al., 2011; Sohn et al., 2007). This result indicates that both the effectors interfere with cell wall-based defenses in *Arabidopsis*.

The previous experiment proved that HaRxL23 and PsAvh73 were capable of suppressing callose deposits *in planta*, hence it was important to determine whether the

effectors were also capable of enhancing virulence of the *Pst* DC3000(ΔCEL) mutant. Wild type and transgenic plants were syringe infiltrated with the non-pathogenic *Pst* DC3000(ΔCEL) strain. Bacterial growth was assayed zero and three days post infiltration. Representative transgenic lines constitutively expressing either *HaRxL23* or *PsAvh73* showed enhanced susceptibility to *Pst* DC3000(ΔCEL) (**Supplemental Figure 2.3**) three days post inoculation, hence demonstrating that both *HaRxL23* and *PsAvh73* disable immunity in *Arabidopsis.*

Stably transformed *HaRxL23* and *PsAvh73* suppress defense gene induction.

We next tested whether *HaRxL23* was capable of suppressing induction of defense genes in response to *Hpa.* For this, a quantitative real time RT-PCR approach was taken where the transcript abundance of four defense marker genes were measured in pathogen infected Col:35S-HaRxL23 seedlings. The four marker genes selected based on their up-regulation profile during *Hpa* infection were *Accelerated cell death 6 (ACD6), Pathogenesis-related 1 (PR-1), Arabidopsis thaliana mitogen activated protein kinase 3 (AtMPK3) and Wall associated kinase 1 (WAK1)* (Anderson et al., 2012; Eulgem et al., 2007). *HaRxL23* (**Figure 2.8**) suppressed defense gene induction in response to avirulent *Hpa* isolate Emoy2. However, this suppression was not evident in the case of *WAK1*, indicating a stage- and defense gene-specific suppression. Together these results suggest that *HaRxL23* intervene early in the activation of defense thereby providing further evidence of immune suppression.

Discussion

Recently published genome sequences of several oomycete plant pathogens have led to the identification of large collections of candidate RXLR genes, predicted to encode effector proteins that manipulate host cells (Baxter et al., 2010; Fabro et al., 2011; Haas et al., 2009; Jiang et al., 2008; Tyler et al., 2006; Win et al., 2012). 134 high-confidence HaRxL candidate genes have been identified in *Hpa* genome (Baxter et al., 2010; Win et al., 2012), but only a small sub-set of these have been functionally characterized to date. Characterization of RxLR effectors in detail is important to understand the molecular mechanism of pathogenesis and host adaptation.

In this study, we are characterizing a pair of conserved RxLR effectors from *Hpa* and its identifiable homolog in *P. sojae*.

There are several reasons to study conserved effector proteins of plant pathogens. To begin with, such genes represent potential opportunities for new insights into important mechanisms of virulence. This is exemplified by previous studies of genes present in the conserved effector locus (CEL) of gram negative plant pathogenic bacterium, *Pseudomonas spp*. (Collmer et al., 2000), the SIX4 effector from *Fusarium oxysporum* (Thatcher, Gardiner, Kazan, & Manners, 2012) and the Ecp6 effector from *Cladosporium fulvum* (de Jonge et al., 2010). The secreted-in-xylem (SIX) proteins of *Fusarium oxysporum* f. sp. *lycopersici* are secreted during infection that causes wilting of the tomato vascular system. It has been shown that the SIX genes are highly conserved in nature and are also found in other formae speciales of *F. oxysporum* namely *lilii*. *melonis*, *vasinfectum* and *radices-cucumerinum* (Lievens, Houterman, & Rep, 2009). Also, there are four identifiable homologs of the SIX genes in *Arabidopsis* infecting *F. oxysporum* isolate Fo5176 (Louise et al., 2011). The highly conserved SIX4 gene homolog was

shown to be required for full virulence in *Arabidopsis* suggesting a common origin and mechanistically-related conserved function of the gene in diverse (tomato and *Arabidopsis*) hosts (Thatcher et al., 2012).

Another reason for studying conserved effectors is that they represent potential targets for developing durable resistance in plants. Deployment of resistance (R) proteins in the field is one of the widely used strategies for providing disease resistance in crops. However there have been several cases where the resistance was defeated rapidly because the pathogen could discard the Avirulence effector with no loss of virulence (Fu et al., 2009; Kunkeaw, Tan, & Coaker, 2010). Hence, identification and breeding of resistance gene(s) against conserved effector proteins can be utilized as an effective strategy for long-term durable resistance (Jacobs et al., 2010; Oh et al., 2009).

In anticipation that analysis of conserved effectors will reveal virulence functions that are important, we investigated a pair of homologous and fairly conserved effectors from *Hpa* (*HaRxL23*) and *P. sojae* (*PsAvh73*). *HaRxL23* and its orthologs in *Phytophthora* are syntenic, which implies importance for oomycete pathogenicity. Our first set of experiments was directed at confirming that these bioinformatically-predicted effector genes encode *bona fide* effectors, capable of promoting oomycete virulence. We achieved this through expression studies during pathogen infection where both genes were found to be expressed early during infection *in planta.* Secondly, a large scale screen in the *Hpa* host *Arabidopsis*, demonstrated recognition of *Hpa* in an ecotype specific manner. Ecotype specificity will help to identify candidate resistance genes that recognize HaRxL23 and which could be potentially used to breed resistance (e.g., against oomycete pathogens of crop Brassicas).

We next hypothesized that these two effector proteins acts as suppressor of defense pathways in divergent hosts and we tested this hypothesis with studies involving transient assays and stably transformed plants. For instance, in *Arabidopsis*, both effectors were successful in suppressing *Pst*-ΔCEL- induced callose deposition and showed small, but significant enhanced virulence when delivered transiently using bacteria as a delivery vehicle (EDV). We predict that these two effectors enhance virulence through additional PTI suppression like callose deposition. Further validation was provided with multiple, variably expressing overexpression lines showing similar results. Moreover, overexpression lines of the effectors showed increased susceptibility to virulent *Hpa* isolate *Emco5*, similar to other *Hpa* effectors (Cabral et al., 2011; Fabro et al., 2011; Sohn et al., 2007). Additionally, in soybean and *N. benthamiana*, both the effectors could suppress ETI triggered by various oomycete elicitors, Avr4/6, CRN2 and PsAvh163. Hence our data support that the function of both these effector proteins, like some others, is to inhibit plant immunity.

As with many screening protocols, our transient assays, especially using the EDV system has some limitations. Since it is a heterologous system, the co-delivery of *Hpa* effector proteins with those of *Pst* DC3000(ΔCEL) might change the outcome, if some interactions were to exist among them. Hence we confirmed all our assays in *Arabidopsis* with stably transformed transgenic lines.

Homology searches to known proteins offer little or no clues for effector targets or function, delaying our understanding of the complex interaction between oomycetes and plants. Targets of very few plant pathogenic oomycete effectors have been identified to date. Some of the reasons for this include complex lifestyles, large genome sizes,

enormous effector collection and reduced ability for genetic approaches such as transformation and gene knockout strategies. The identification of effector molecules from various eukaryotic pathogens enables us to draw parallels between prokaryotic and eukaryotic pathogens and helps us to investigate the extent to which these diverse pathogens share virulence strategies and target similar pathways of plant immunity. Hence, our current approach is to identify targets of *HaRxL23* and *PsAvh73* with the hypothesis that given their conserved nature, it is probable that they may be targeting common proteins and or processes in the host. In conclusion, further detailed investigation of these two effectors is necessary to help reveal how *Hpa* modifies and alters host cellular processes and mechanisms to promote its growth, survival and reproduction.

Figures

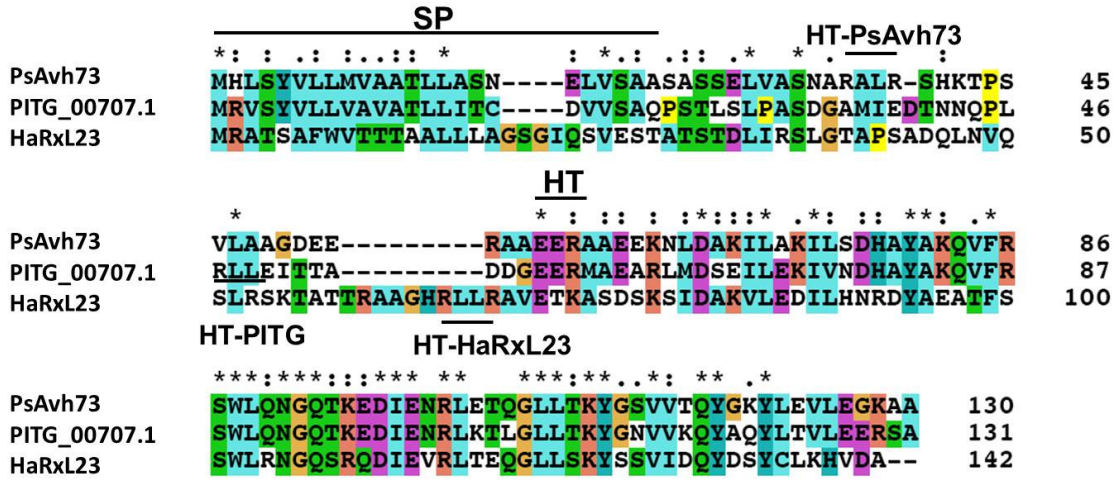


Figure 2.1 *Alignments of amino acid sequences from HaRxL23, PsAvh73 and Phytophthora infestans (PITG_00707.1).* The predicted signal peptide (SP) and the host targeting (HT) regions RxLR and EER are highlighted. The HT RxLR regions are designated as HT-PsAvh73 (RALR), HT-PITG (RLLE) and HT-HaRxL23 (RLLR). The HT EER region is designated as HT.

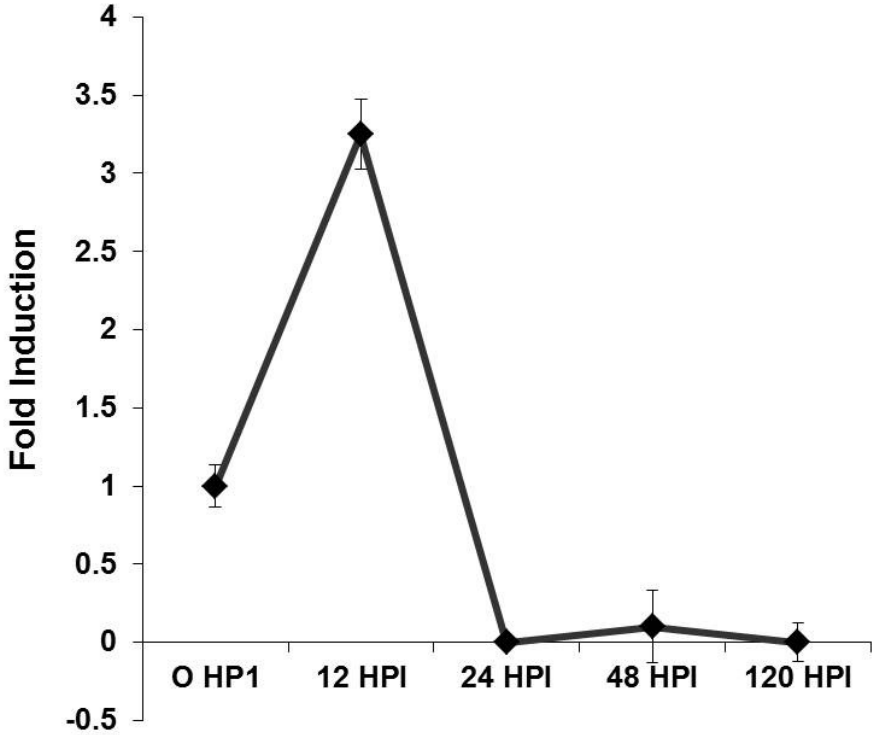


Figure 2.2 *HaRxL23 is induced at an early time point in Hpa* infection. *Arabidopsis* Oy-1 plants were challenged with 5 X 10^4 spores/ml of the virulent *Hpa* isolate Emoy2. *HaRxL23* expression was assayed using quantitative, real-time PCR with primers specific for *HaRxL23,* over a time course using the cDNA obtained at different time points (hours post infection or HPI; X-axis). Transcript abundance of *HaRxL23* was measured relative to *HpaActin*, and is shown normalized to its expression at zero hour time point.

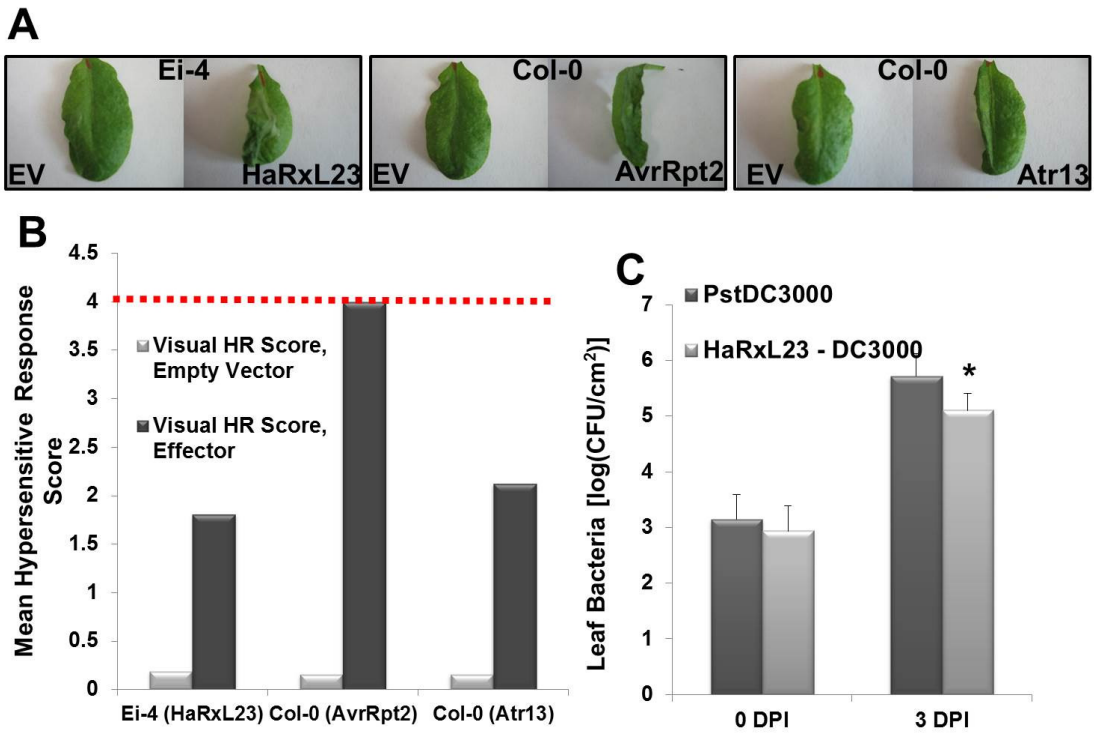


Figure 2.3 *HaRxL23 is recognized in the host in an ecotype-specific manner when delivered by bacteria*. (a) Images from leaves of *Arabidopsis* Ei-4 or Col-0, infiltrated with 1 X 10^8 cfu/ml *Pseudomonas fluorescens* (EtHAn) suspension delivering empty vector, *HaRxL23*, *Psedomonas syringae* effector, *AvrRpt2* or *Hpa* effector, *Atr13*. HR symptoms were visually monitored over a period of 24 hours and images were taken 24 hours after inoculation (b) Hypersensitive response score. A score of "4" designates complete leaf collapse, "3" designates partial leaf collapse, "2" designates leaf curling, "1" designates partial leaf curling and "0" designates no change compared to the empty vector control. The experiment was repeated four times with similar results. (c) PstDC3000 (*HaRxL23)* multiplication in leaves of *Arabidopsis* ecotype Ei-4 plants syringe-infiltrated with a suspension of 5 X 10^5 cfu/ml. Bacterial populations were determined at day zero and day three after inoculation. * $P < 0.1$; t-test comparisons

representing significant differences with *Pst* DC3000. Error bars indicate Standard Error of six independent leaf samples tested at the same time. This experiment was repeated three times with similar results**.**

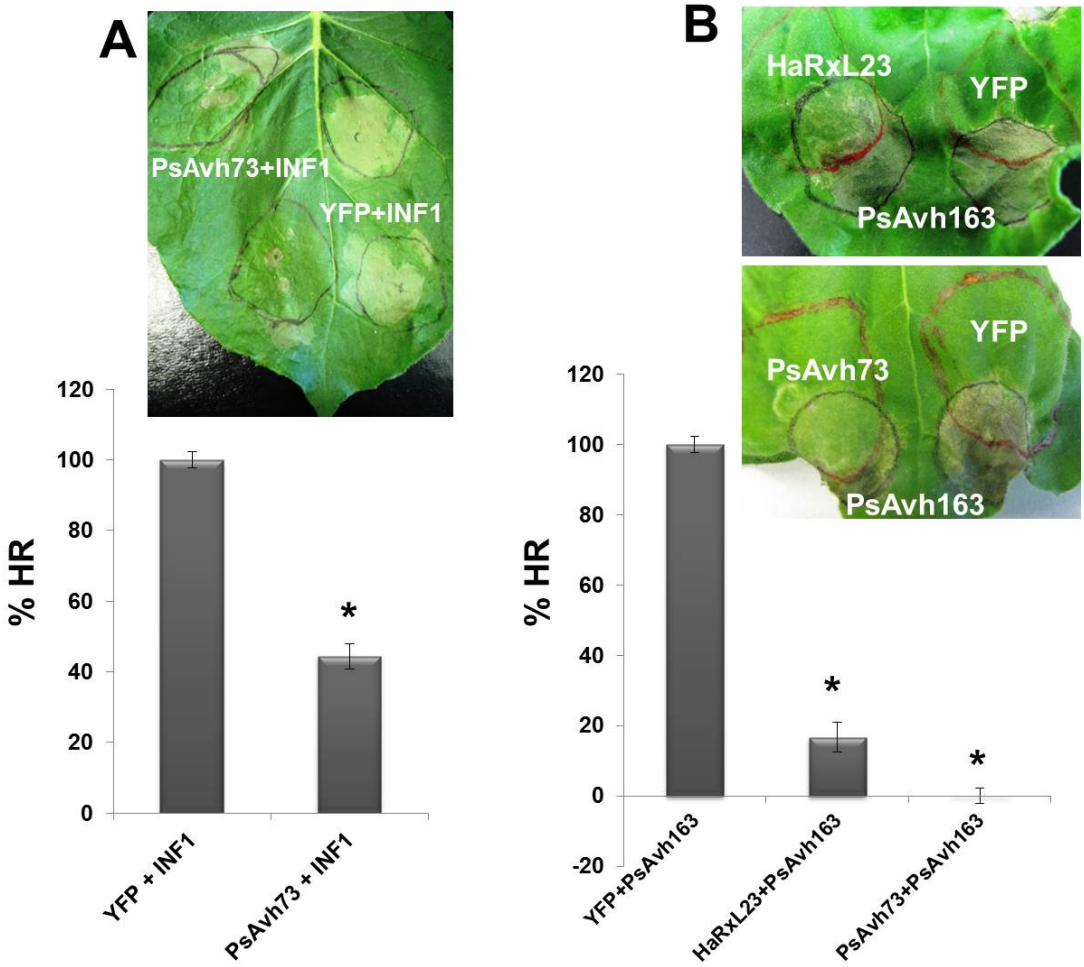


Figure 2.4 *HaRxL23 and PsAvh73 suppress programmed cell death in Nicotiana benthamiana*. *N. benthamiana* leaves were infiltrated with *A. tumefaciens* GV3101 containing *HaRxL23*, *PsAvh73,* or YFP and challenged two days post infiltration with *A. tumefaciens* GV3101 carrying either (a) *INF1* or (b) *PsAvh163*. Cell death symptoms were visually monitored over a period of five to seven days. Graphs show percentage of infiltration sites with macroscopic cell death. **P*<0.05, t-test comparisons representing

statistically significant differences with YFP. Error bars indicate Standard Error from four independent biological replicates**.**

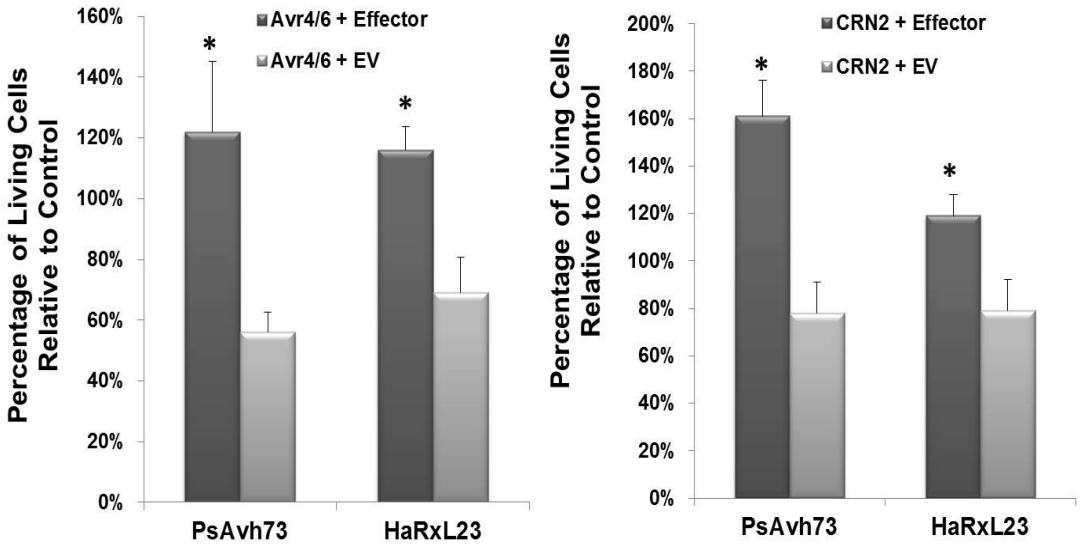


Figure 2.5 *HaRxL23 and PsAvh73 suppress programmed cell death in soybean.* A plant plasmid expressing (a) CaMv35S-Avr4/6 or (b) CaMv35S-CRN2 was co-bombarded, with plasmids containing the effectors *HaRxL23,* or *PsAvh73,* or the empty vector along with a vector expressing CaMv35S- GUS reporter gene onto soybean. Leaves were then stained for GUS activity, and cell viability was then estimated by counting blue spots under a dissecting microscope. The percentage of surviving cells was quantified relative to the co-bombarded empty vector control. *P<0.05, Wilcoxon Rank Sum test comparisons representing significant differences with the empty vector. Error bars indicate Standard Error from technical replicates. This experiment was repeated at-least three times with similar results.

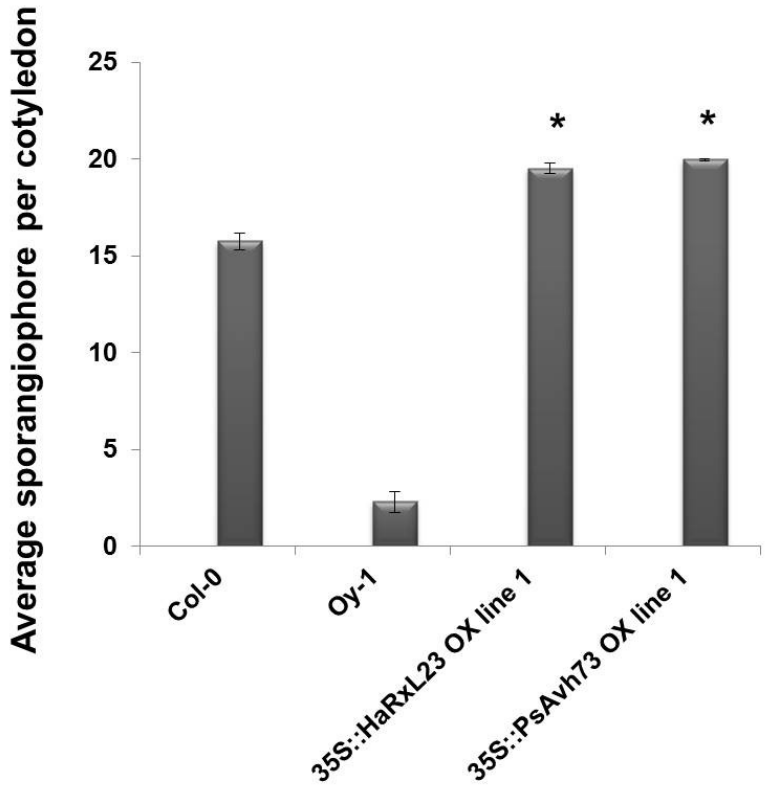


Figure 2.6 *HaRxL23 and PsAvh73 enhance susceptibility to virulent Hpa (Emco5)*. 10-12 day old transgenic *Arabidopsis* Columbia seedlings stably transformed with *CaMV35-HaRxL23* or *CaMV35-PsAvh73* were challenged with 5 X 10^4 spores of the virulent *Hpa* isolate Emco5. Infection was quantified seven days post inoculation by counting sporangiophores per cotyledon. *Arabidopsis* Col-0 and Oy-1 are controls for susceptibility and resistance to *Hpa* Emco5, respectively. * $P < 0.01$; t-test comparisons representing significant differences with Col-0. Error bars indicate Standard Error from

technical replicates. This experiment was repeated three times with similar results.

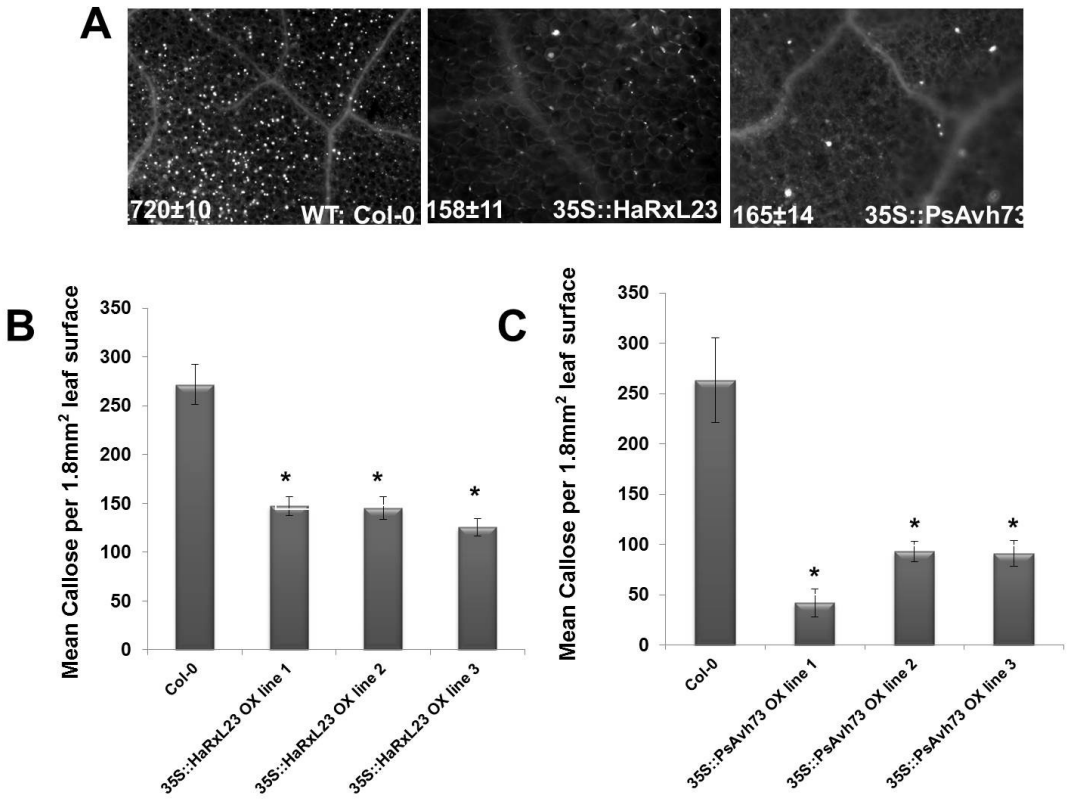


Figure 2.7 *HaRxL23 and PsAvh73 suppress callose formation in stably transformed Arabidopsis in response to Pseudomonas syringae DC3000(ΔCEL) mutant.* Four-week old transgenic *Arabidopsis* Columbia plants stably transformed with *CaMV35-HaRxL23* or *CaMV35-PsAvh73* were infiltrated with 5 X 10^7 cfu/ml *P. syringae* ΔCEL mutant. Callose deposits were visualized by staining with aniline blue and callose was quantified using Autospots software program (Cumbie et al., 2010). Six leaves, four pictures per leaf were analyzed per transgenic and control lines. * *P-value* < 0.01; t-test comparisons representing significant differences with Col-0. Error bars represent Standard Error of six independent leaf samples tested at the same time. This experiment was repeated three times with similar results.

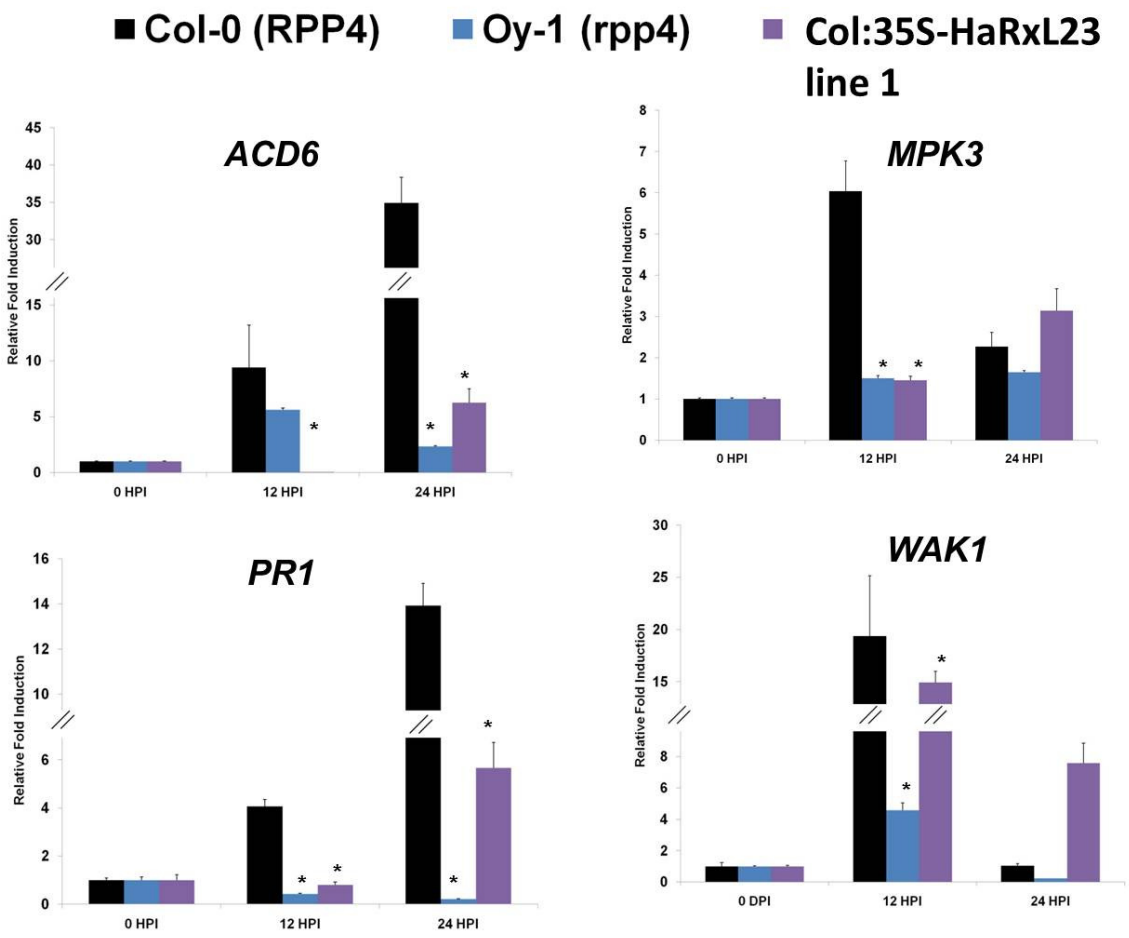


Figure 2.8 *Stably transformed HaRxL23 suppress defense gene induction.* 10-12 day-old *Arabidopsis* Col-0 and transgenic plants constitutively expressing *HaRxL23* were inoculated with 5 X 10^4 spores/ml of the avirulent *Hpa* isolate Emoy2. Transcript abundance was measured using quantitative, real-time PCR with primers specific for the indicated genes over a time course using the cDNA obtained at different time points (0, 12 and 24 hours post inoculation). Transcript abundance was normalized to *AtActin2*. * ddCt values representing statistically significant (*$P < 0.05$) differences with Col-0. This experiment was repeated four times. The induction profiles of individual genes varied to some degree between replicates, but we consistently observed decreased induction of

defense marker genes (except for WAK1) in transgenic lines compared to the control plants.

Materials and methods

Construction of expression plasmids

HaRxL23 was amplified from genomic DNA extracted from *Arabidopsis* Oy-1 plants infected with *Hpa* isolate *Emoy2,* using proofreading polymerase (Pfu, Invitrogen). Forward and reverse primers were designed to amplify from the signal peptide cleavage site (HaRxL23 NOSP) with (HaRxL23 S) or without the stop codon (HaRxL23 NS) depending on the type of fusion. Similarly, *PsAvh73* was amplified from *P. sojae* genomic DNA and forward (PsAvh73 NOSP) and reverse (PsAvh73 S or PsAvh73 NS) primers were designed.

For cloning into Gateway destination vectors, the sequence CACC was added at the 5' end of the forward primer and PCR was performed using the genomic DNA as template. PCR products were gel purified (Qiagen) and finally recombined into pENTR-D-TOPO gateway entry vector following the manufacture's protocol (Invitrogen). This step was followed by transformation into *Escherichia coli* DH5α competent cells. Kanamycin resistant colonies were selected on agarose plates followed by colony PCR with plasmid specific M13 F and M13R primers. Colonies having the correct size insert were selected for plasmid purification and confirmed by sequencing. The pENTR clone generated was then used to create Gateway expression plasmids using LR recombination (Invitrogen). For *Agrobacterium* and *Pseudomonas*-mediated transient studies, the

pENTR clones of *HaRxL23* and *PsAvh73* were shuttled into pB2GW7 and pEDV6 by LR recombination (Invitrogen, Carlsbad, CA).

Plant materials and growth conditions

Arabidopsis, soybean, and *Nicotiana benthamiana* were grown in Sunshine Pro-mix 1. For experiments involving *Hpa* and *Pseudomonas spp.*, *Arabidopsis* were grown in controlled growth chambers under short day cycles (8h/16h light/dark and 150-200 $\mu E/m^2s$) at 22°C and 60% relative humidity. For all other experiments, *Arabidopsis*, soybean and *N. benthamiana* were grown under long day cycles (16h/8h light/dark, 90-100 $\mu E/m^2s$) at 22°C and 60% relative humidity.

Generation of transgenic *Arabidopsis* plants

Plants expressing effectors were generated by recombining ORF's cloned in pENTR in the gateway destination binary vector pB2GW7 (Karimi, Inze, & Depicker, 2002) under the control of the CaMV 35S promoter. The constructs were transferred to *Agrobacterium tumefaciens* GV3101 strain (Koncz et al., 1986) by electroporation and transformed into *Arabidopsis* Col-0 by floral dip method (Clough & Bent, 1998). T_1 generation was selected using BASTA. For the T_2 generation, 3:1 (BASTA-resistant / BASTA-susceptible) segregating lines were tested for homozygosity in the T_3 and T_4 generation. Presence of the transgene was confirmed by genomic DNA PCR and transcript abundance was quantified by reverse-transcriptase PCR. Three to five independent non-segregating transgenic lines (T_3 or T_4) displaying varying mRNA expression patterns were used in all of the experiments.

Hyaloperonospora arabidopsidis inoculations

The *Hpa* isolates Emoy2 and Emco5 were propagated and maintained in compatible *Arabidopsis* ecotypes Oy-1 and Ws-0, respectively (McDowell, Hoff, Anderson, & Deegan, 2011). Conidial suspensions of $5x10^4$ spores/ml were applied with a Preval spray unit and the plants were kept under short day conditions. *Hpa* disease assays were performed as described (McDowell et al., 2011).

Bacterial strains

The following bacterial strains were used in this study: *Escherichia coli* DH5α, *Pseudomonas syringae* pv *tomato* DC3000 wild type and ΔCEL (Alfano et al., 2000; Yuan & He, 1996), *Pseudomonas fluorescens* Pf0-1 carrying functional TTSS and EtHAn (Thomas et al., 2009) and *Agrobacterium tumefasciens* GV3101. *E. coli* and *Agrobacterium* were grown in Luria-Bertani medium at 37°C and 28°C respectively in liquid media or petri dishes with appropriate antibiotic selections. *Pseudomonas* strains were grown in King's B medium at 28°C. Plasmids were introduced from *E. coli* DH5α to wild type or mutant Pst DC3000 and Pf0-1 strains by standard triparental matings using *E. coli* RK600 as a helper strain.

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

Total RNA was extracted from *Arabidopsis* tissue with TriSure reagent (Bioline). 2 µg DNAse-treated RNA was reverse transcribed using the OmniScript cDNA synthesis kit (Qiagen) and oligo(dT) primer. For semi-quantitative RT-PCR analysis, 1 µl of cDNA was used per reaction and 40 and 35 PCR cycles were used to amplify effector targets

and At Actin respectively. PCR products were separated on 1% agarose gel in TAE buffer. For real-time PCR analysis, samples were prepared by mixing cDNA template with SYBR Green Mastermix (Applied Biosystems) with the appropriate amount of primers and water. Real-time PCR reactions were performed on an ABI 7300 device and the fold change was calculated relative to the 0 DPI time point.

Assays involving HR, bacterial virulence and callose suppression in *Arabidopsis*

For HR, bacterial growth assays and callose suppression assays involving *Pseudomonas spp.*, 4-5 week old *Arabidopsis* plants were syringe-infiltrated with $1x10^5$ cfu/ml (bacterial growth assays) or $1x10^8$ cfu/ml (callose suppression assays) in 10mM $MgSO_4$.

For HR assays, five-week-old *Arabidopsis* leaves were syringe-infiltrated with needle less syringe with 1 x 10^8 cfu/mL suspensions. A total of 6 plants, 3 leaves each were infiltrated and visual scoring was performed 16 hours later.

For growth curve assays, leaf discs were cored at zero and three dpi, surface sterilized with 70% ethanol and homogenized using a mini-bead beater (Biospec products). Serial dilutions were performed to count colony forming units. For each sample, three leaf discs were pooled three times per data point. Bacterial growth was measured as described previously.

For callose suppression assays, whole leaves were harvested 16 hpi, treated with alcoholic lactophenol and stained with 0.01% (w/v) Aniline blue stain in K_2HPO_4 buffer as described previously (Sohn et al., 2007). Aniline blue stained leaves were mounted on glass slides using 50% glycerol and imaged with a Zeiss Axio Imager.M1 using the filter

settings for DAPI. Quantification of callose spots was performed using the Autospots software (Cumbie et al., 2010).

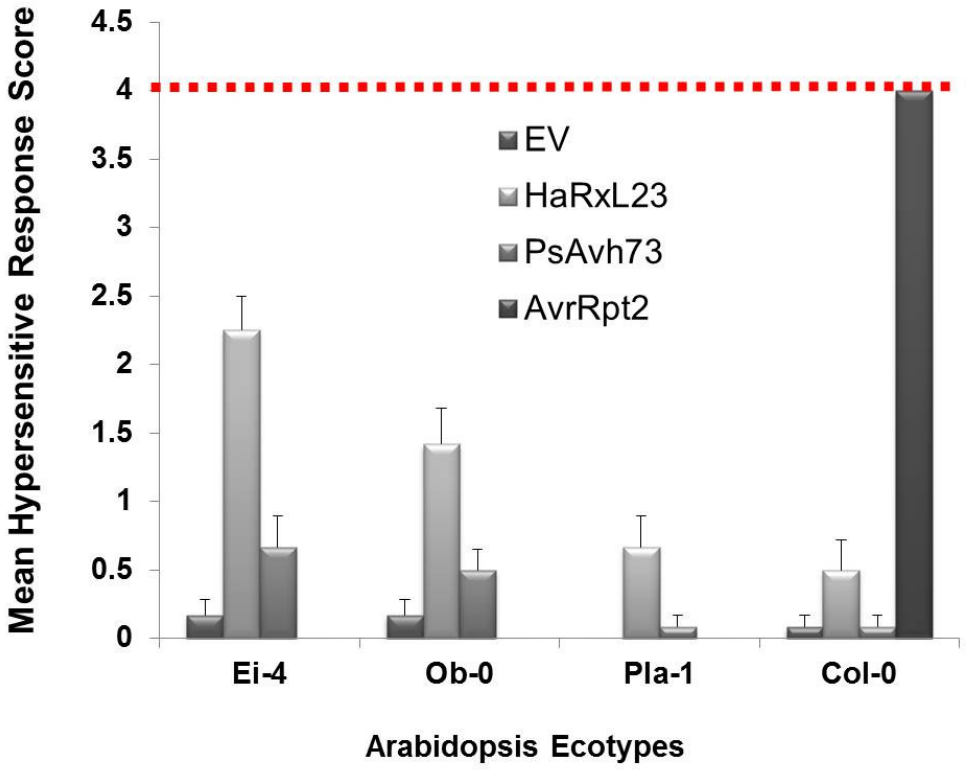
Transient assays in soybean

For transient assays in soybean, 2 week old leaves were transformed with a mixture of plasmid DNA following the modified BioRad PDS1000 gene gun (Borad) protocol as described (Kale & Tyler, 2011). The plasmid DNA mixtures were comprised of the effector, elicitor and control setups. The effector setup comprised of 115µg of the effector, 50µg of the elicitor and 50µg of GUS were mixed as described previously (Dou, Kale, Wang, Chen, Wang, Wang, et al., 2008). The elicitor setup comprised of 70µg of the empty vector, 50µg of the Avr4/6 or CRN2 and 50µg of GUS. Finally the control setup comprised of 115µg of the empty vector and 50µg of GUS plasmid DNA. Tungsten was prepared with the above mixtures as described earlier (Dou, Kale, Wang, Chen, Wang, Wang, et al., 2008). Individual detached soybean leaves were transformed using particle bombardment and the tungsten preparation. After bombardment, the leaves were placed under controlled conditions of high humidity conditions in petri-dishes at 8h/16h light/dark at 22°C for 2-3 days. Next, the leaves were stained with the x-gluc solution and de-stained with 70% ethanol for 3-4 days. The number of living cells was determined by counting the GUS-expressing blue-colored cells under a dissecting microscope. The percentage of surviving cells was quantified relative to the co-bombarded empty vector control using the Autospots software program (Cumbie et al., 2010). Statistical analyses were performed on means using Wilcoxon Rank sum method.

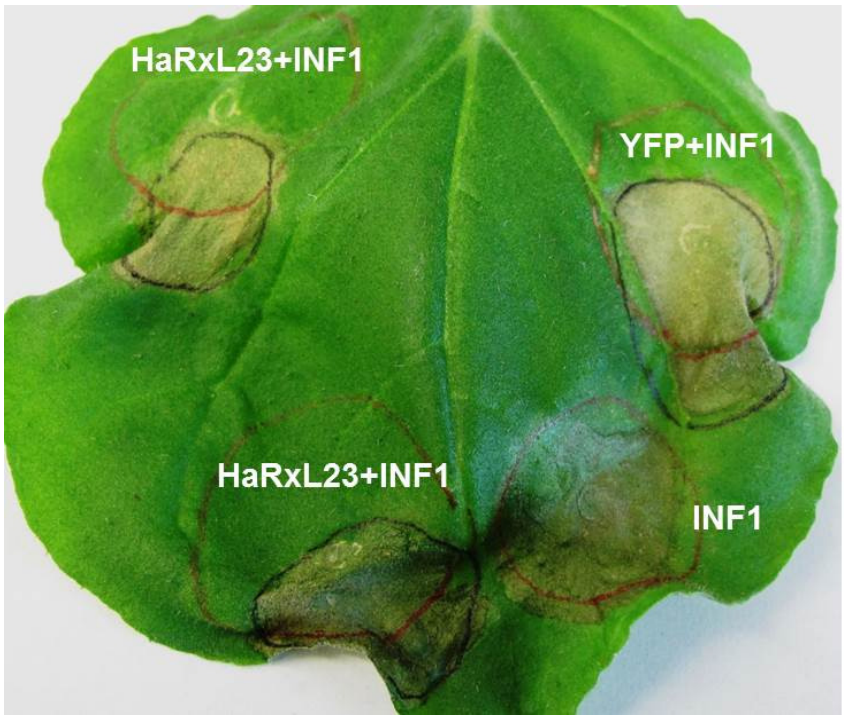
Transient assays in *N. benthamiana*

Recombinant *Agrobacterium tumefaciens* were grown as described previously (Van der Hoorn, Laurent, Roth, & De Wit, 2000) with the appropriate antibiotics. Overnight grown *Agrobacterium* liquid cultures were centrifuged, and the pellets were resuspended in MMA induction buffer (10mM $MgCl_2$, 10mM MES, 200mM Acetosyringone). The bacterial suspensions were incubated at room temperature for 1-3 hours and agro-infiltration using needleless syringe was performed on the abaxial side of 3-5 weeks old, *N. benthamiana* leaves at a final OD_{600} of 0.3 - 0.5. Cell death or suppression of cell death were monitored for 4-5 days and visually quantified after 5 days.

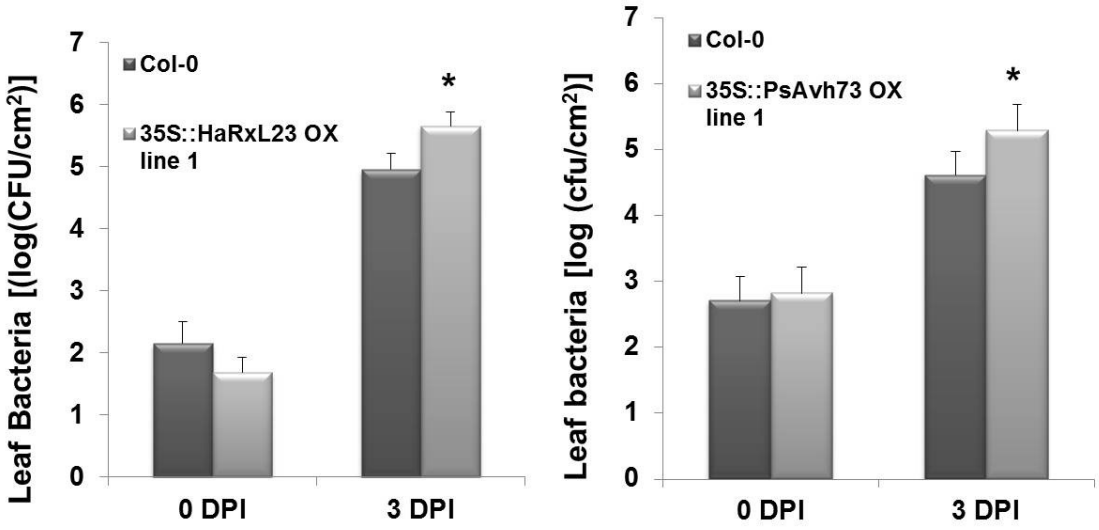
Supporting information



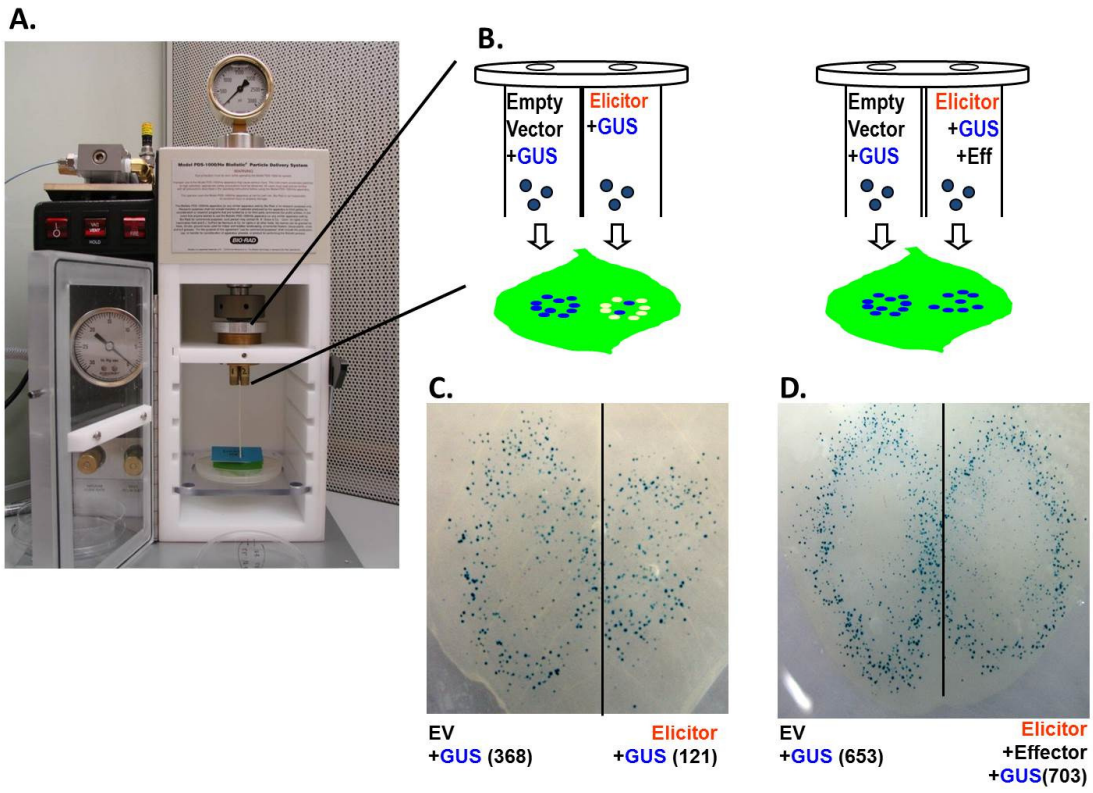
Supplemental Figure 2.1 *HaRxL23 but not PsAvh73 is recognized in the host in an ecotype-specific manner when delivered by Pfo EtHAn.* Hypersensitive response score. A score of "4" designates complete leaf collapse, "3" designates partial leaf collapse, "2" designates leaf curling, "1" designates partial leaf curling and "0" designates no change compared to the empty vector control. Leaves from *Arabidopsis* ecotypes Ei-4, Ob-0, Pla-1 and Col-0 were infiltrated with 1 X 10^8 cfu/ml *Pseudomonas fluorescens* (EtHAn) suspension delivering empty vector, *HaRxL23*, *PsAvh73*, or *Pseudomonas syringae AvrRpt2*. HR symptoms were visually monitored over a period of 24 hours and images were taken 24 hours after inoculation. Error bars represent Standard Error from at-least three independent biological replicates.



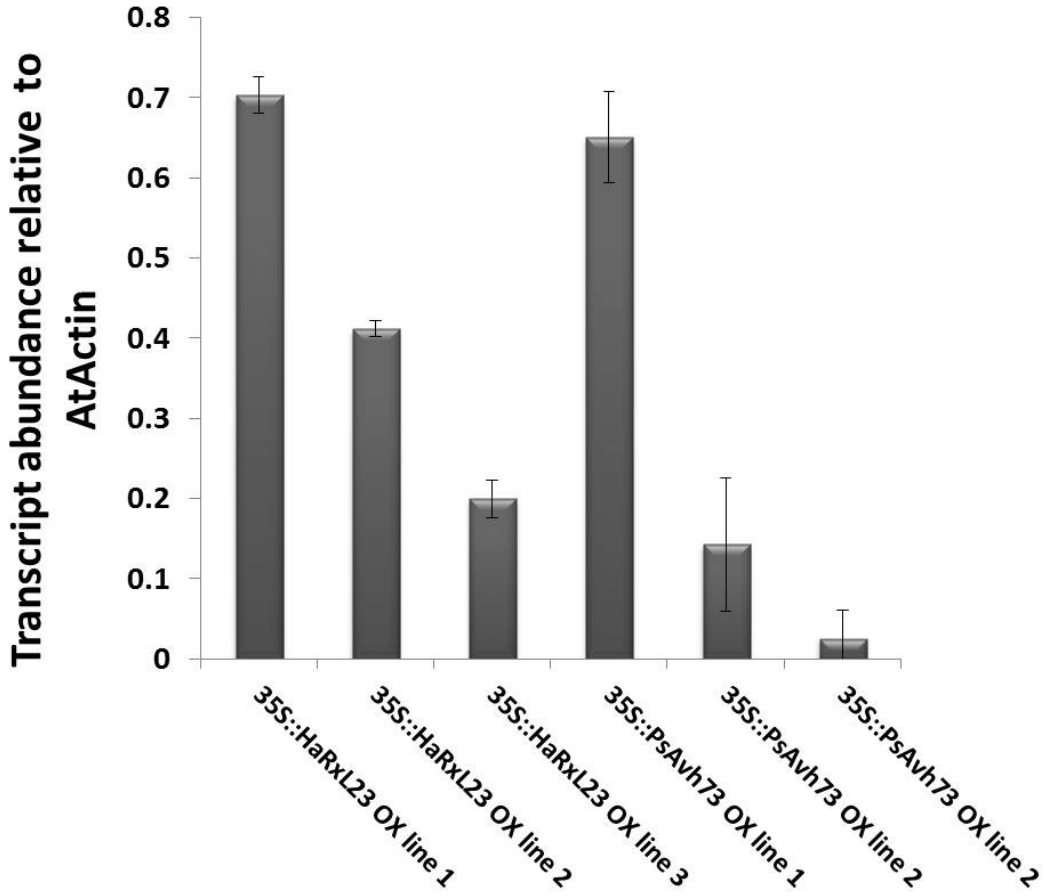
Supplemental Figure 2.2 *HaRxL23 does not suppress INF1-cell death in Nicotiana benthamiana*. Specific sites in *N. benthamiana* were infiltrated with *A. tumefaciens* GV3101 containing *HaRxL23* or YFP and challenged two days post infiltration with *INF1*. Cell death symptoms were monitored over a period of five to seven days. This experiment was repeated at-least four times with similar results.



Supplemental Figure 2.3 *HaRxL23 and PsAvh73 enhance Pseudomonas syringae virulence in stably transformed Arabidopsis. B*acterial multiplication in three-week old transgenic plants stably transformed with *CaMV35-HaRxL23* or *CaMV35-PsAvh73* Plants were syringe-infiltrated with a bacterial suspension of 1 X 10^5 cfu/ml and bacterial populations were determined at day zero and day three after inoculation. * $P < 0.1$; t-test comparisons representing significant differences with Col-0. Error bars indicate Standard Error of six independent leaf samples tested at the same time. This experiment was repeated three times with similar results.



Supplemental Figure 2.4 *Overview of the double barrel gene gun.* A. Picture of the double barrel gene gun with soybean leaf in position for bombardment. B. Cartoon depicting delivery of tungsten particles with the plasmids carrying Gus, Effector, and the elicitor Avr4/6 to soybean leaves. C. Reduced number of *Gus* expressing cells with the elicitor, as indicated in parentheses. D. Effector suppresses PCD induced by elicitor as indicated by the increase of Gus expressing cells over the control. Effector (Eff), Elicitor (Elic), Empty Vector (EV).



Supplemental Figure 2.5 *Quantification of transcript levels of transgene in multiple independently transformed lines containing 35S::HaRxL23 and 35S:PsAvh73, using quantitative PCR.* Transcript accumulation is expressed as a fold change relative to *AtActin*. Error bars represent Standard Error from technical replicates.

HaRxL23 NOSP	CACCATGGCAACGTCTACCGATCTGA
HaRxL23 NS	GGCGTCGACGTGCTTTAGGC
HaRxL23 S	CTAGGCGTCGACGTGCTTTA
Avh73 NOSP	GCTTCTGCTTCTTCAGAGCTCGTCGC
Avh73 NS	AGGCGGCTTTGCCTTCGAGG
Avh73 S	GTATTTGCCGTACTGGGTGA
35S Seq Fwd	CTAGTCGACCTGCAGGCGGCC
35S Seq Rev	GGACTCTAGCATGGCCGCGGG
pEDV6 Fwd	GGCACCCCAGGCTTTACACTTTATG
M13 Fwd	GTAAAACGACGGCCAGTG
M13 Rev	GGAAACAGCTATGACCATG
AtActin2 Fwd	AATCACAGCACTTGCACCA
AtActin2 Rev	GAGGGAAGCAAGAATGGAAC
Hpa Actin Fwd	GTGTCGCACACTGTACCCATTTAT
Hpa Actin Rev	ATCTTCATCATGTAGTCGGTCAAGT
PR-1 Fwd	GAACACGTGCAATGGAGTTT
PR-1 Rev	GGTTCCACCATTGTTACACCT
ACD6 Fwd	ATCCTTACATGTGGCCTTGC
ACD6 Rev	CGAAAAGGAAGAATCCACCA
MPK3 Fwd	ACGTTTGACCCCAACAGAAG
MPK3 Rev	ATTCGGGTCGTGCAATTTAG
WAK1 Fwd	GGCTAATGGGAGAGGAAAGG
WAK1 Rev	TTCGACCCTCAAGGCTTCTA

Supplemental table 2.1 Table of primers used in this study

		HR Triggered	
Ecotype	**Origin**	**PsAvh73**	**HaRxL23**
An-1	Antwerpen, Belgium	NO	NO
Be-0	Bensheim/Bergstr., Germany	NO	NO
Bs-5	Basel, Switzerland	NO	NO
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	WEAK	YES
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	NO	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	Weak
Oy-0	Oystese, Norway	NO	NO
Per-1	Perm, Russia	NO	NO
Petergof, 3L1	Petergof, Russia	NO	NO
Pla-1	Playa de Aro, Spain	NO	Weak
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	NO
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 2.2 Table of *Arabidopsis* ecotypes used for large scale HR screen by effectors HaRxL23 and PsAvh73 when delivered from *Pseudomonas syringae* EDV.

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Chapter 3

Functional similarity between the *Hyaloperonospora arabidopsidis* effector protein HaRxL23 and *Pseudomonas syringae* AvrE

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The data in this chapter will be combined with data from our collaborators' bioinformatic analyses, for a manuscript to be submitted to *Molecular Plant-Microbe Interactions*

Key Words: oomycete, *Hyaloperonospora arabidopsidis*, *Pseudomonas*, effector, hypersensitive response (HR), callose, lesion

Abbreviations: conserved effector locus (CEL), effector detector vector (EDV), endoplasmic reticulum membrane retention/retrieval signal (ER-MRS), effector to host analyzer (EtHAn), Effector-triggered immunity (ETI), empty vector (EV), *Hyaloperonospora arabidopsidis* (*Hpa*),hypersensitive response (HR), host-targeting (HT), pathogen-associated molecular patterns (PAMP), partial least square (PLS), pattern recognition receptor (PRR), *Pseudomonas phaseolicola* (*Pph*), *Pseudomonas syringae* pv. *tomato* (*Pst*), PAMP-triggered immunity (PTI), resistance protein (R), redundant effector group (REG), reactive oxygen species (ROS), salicylic acid (SA), signal peptide (SP), type three (T3), type III secretion system (TTSS), type III effectors (T3Es).

Abstract

Effector proteins are exported to the interior of host cells by diverse plant pathogens. Effector proteins have been well characterized in bacteria. Contrastingly, their functions and targets in oomycete pathogenicity are poorly understood. Bioinformatic analysis of genome sequences from oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) have led to the identification of candidate effector genes with a signal peptide, RxLR and dEER that target the effectors into plant cells. We have bioinformatics-driven evidence that suggests similarities between the oomycete effector HaRxL23 and the conserved bacterial effector protein AvrE1 from *Pseudomonas syringae.* Their predicted protein structures show common regions of structural similarity. Hence, this study aimed at establishing functional similarities between AvrE1 and HaRxL23, with the rationale that there is a limited set of common targets between effectors of plant pathogens of common ancestry like *P. syringae* and *Hpa*. Here, we show that HaRxL23 induces cell death in wild type *Arabidopsis* young plants like AvrE1, suppresses PAMP-triggered callose deposition through the same pathway as AvrE1, and can complement the reduced bacterial speck phenotype of the *avrE* mutant *in planta*. Together, these data suggest that HaRxL23 is functionally similar to AvrE1 and perhaps targets the same protein/pathway as does AvrE1.

Introduction

Plants have evolved a multilayered system of inducible defenses against pathogen attack. The first layer, PAMP-triggered immunity (PTI), is activated when conserved microbial molecules (pathogen-associated molecular patterns or PAMPs) are recognized by pattern recognition receptors (PRRs) in the host (Zipfel et al., 2006). This recognition event triggers an immune response highlighted by the production of reactive oxygen species (ROS), along with deposition of callose and phenolic compounds (Jones & Dangl, 2006; Zipfel & Robatzek, 2010). Plant pathogenic bacteria, fungi, oomycetes, nematodes and insects secrete effector proteins that suppress this immunity by directly targeting the PRRs or targeting important components in the PTI signaling pathway (Bos et al., 2006; Fabro et al., 2011; van der Hoorn & Kamoun, 2008). This pathogenic strategy is best understood in plant-pathogenic bacteria such as the tomato speck pathogen *Pseudomonas syringae* pv. *tomato* (Jin et al., 2001) that use a type III secretion system (TTSS) to inject effector proteins inside host cells. Fungi and oomycete pathogens secrete effectors from their feeding structures or "haustoria" (Whisson et al., 2007).

The second layer of plant immunity is based on direct or indirect recognition of effectors by corresponding host proteins known as resistance or "R" proteins, termed effector-triggered immunity (ETI) (Chisholm, Coaker, Day, & Staskawicz, 2006; Jones & Dangl, 2006; van der Hoorn & Kamoun, 2008). This recognition triggers a robust and effective suite of defense response that typically includes the hypersensitive response (HR), featuring programmed cell death at the site of infection that restricts the growth of the invading pathogen (Dodds & Rathjen, 2010). Pathogens, in turn, have evolved more effectors to counteract ETI by either avoiding R protein recognition or suppressing

downstream signaling events (Dodds & Rathjen, 2010; Jones & Dangl, 2006; Zipfel & Robatzek, 2010).

Oomycetes are filamentous eukaryotic pathogens that secrete effector proteins inside host cell to promote infection and colonization. Recently published genome sequences of species from some of the important oomycetes genera including *Phytophthora* (Haas et al., 2009; Raffaele et al., 2010; Tyler et al., 2006), *Pythium* (Levesque et al., 2010), *Albugo* (Kemen et al., 2011; Links et al., 2011) and *Hyaloperonospora* (Baxter et al., 2010) revealed large repertoires of candidate effectors in these pathogens. This indicates the evolution of elaborate and sophisticated pathogenicity machinery. A major class of oomycete effectors is named "RxLR", and is defined by an N-terminal signal peptide (SP) and a conserved host-targeting (HT) motif-, RXLR (where R is Arginine, X is any amino acid and L is leucine). These motifs are respectively thought to be required for effector secretion outside the pathogen and translocation to the interior of host cells (Bhattacharjee et al., 2006; Dou et al., 2010; Dou, Kale, Wang, Chen, et al., 2008; Dou, Kale, Wang, Jiang, et al., 2008; Grouffaud, van West, Avrova, Birch, & Whisson, 2008; Kamoun, 2006; Rehmany et al., 2005; Whisson et al., 2007). However, detailed functional analysis of only a handful of oomycete effectors has occurred to date. For example, the cytosolic effector of *Phytophthora infestans* Avr3a, suppress hypersensitive cell death induced by another *P. infestans* protein, INF1 (Bos et al., 2006) by stabilizing the plant E3 ligase CMPG1(Bos et al., 2010; Gonzalez-Lamothe et al., 2006), which regulates INF1-triggered cell death.

Bacterial pathogens such Pst DC3000, maintain 30-40 effector proteins (Buell et al., 2003; Cui, Xiang, & Zhou, 2009; Lindeberg, Cunnac, & Collmer, 2009, 2012). From

previous studies, it is known that bacterial effectors are functionally redundant (Lindgren, Peet, & Panopoulos, 1986), hence mutations in individual effector genes have subtle or no virulence phenotype (Cunnac, Lindeberg, & Collmer, 2009). Numerous bacterial effectors have been shown to suppress immunity in plants and promote bacterial virulence when overexpressed as single genes (Guo, Tian, Wamboldt, & Alfano, 2009; Jamir et al., 2004). Unlike oomycetes, significant progress has been made in elucidating the targets and establishing the enzymatic activities of bacterial effector proteins. For example, some effectors (eg:, *Pst* DC3000 AvrPto) directly target PRRs (Gohre & Robatzek, 2008; Rosebrock et al., 2007). Others such as AvrB, AvrPphB and HopAI1interfere with downstream PTI signaling components (Cui et al., 2010; Cui et al., 2009), while the HopI1 effector targets the heat-shock proteins in the plant chloroplast (Jelenska, van Hal, & Greenberg, 2010).

The bacterial effector protein AvrE1, is an atypical type three (T3) effector. It is one of the most conserved and widespread effector proteins found in *Pseudomonas*, *Pectobacterium*, *Erwinia*, *Pantoea* and *Dickeya*. *P. syringae* AvrE1 belongs to one of the redundant effector groups (REGs) comprised of AvrE1/HopM1/HopR1 and is known to block pathogen-induced callose deposition (DebRoy, Thilmony, Kwack, Nomura, & He, 2004) and elicit cell death in plants (Badel, Shimizu, Oh, & Collmer, 2006). AvrE1 resides within the conserved effector locus (CEL) region of *Pst* DC3000 that also harbors HopPtoM, HrpW and HopPtoA1. The CEL region is conserved amongst diverse *P. syringae* pathovars (Alfano et al., 2000), implying that the genes therein are functionally important. AvrE-family effectors are very large proteins with very low sequence identity, e.g., WtsE and AvrE have 27.1% amino acid identity (Ham et al., 2009). AvrE1-like

effectors share WxxxE motif and a C-terminal endoplasmic reticulum membrane retention/retrieval signal (ER-MRS). The WxxxE and ER-MRS motifs are both required for the virulence activities, elicitation of the hypersensitive response, and suppression of defense responses in plants (Ham et al., 2009). Despite the apparent importance of AvrE1-like effectors, the target(s) of this family is unknown.

This study was initiated in response to results of a collaboration that led to the identification of *Hpa* effector proteins with structural similarity to the AvrE-family of effector proteins. Candidate AvrE1 analogs were identified through a bioinformatics-driven approach based on partial least squares (PLS) regression alignment-free methods (**Supplemental Figure 1**, Opiyo and colleagues, unpublished data). Opiyo et al., predicted the structures of proteins identified from *Hpa* genome using I-TASSER server and compared them with AvrE1 predicted protein structure and found that *Hpa* effector, HaRxL23 was the best *Hpa* RxLR candidate in terms of having regions similar to AvrE1 protein structure (**Supplemental Figure 2**, Opiyo and colleagues, unpublished data). Hence, this study aimed at establishing functional similarities between AvrE1 and HaRxL23, with the rationale that there is a limited set of common targets between effectors of plant pathogens of common ancestry like *P. syringae* and *Hpa*. We also hypothesized that even though these two pathogens have evolved independent virulence mechanisms, they would have overlapping functions and have common set of targets *in planta*. Below, we present data from several experiments that suggest functional equivalence between HaRxL23 and AvrE1.

Results

HaRxL23 and AvrE1 induce cell death in young *Arabidopsis* young plants when delivered by *Pseudomonas phaseolicola* (*Pph*) 3121

Previous studies with AvrE1 of PtoDC3000 and its orthologue in *Pantoea stewartii*, WstE, revealed that it is capable of inducing a cell death response in *Nicotiana tabacum* and tomato plants at high inoculum (Badel et al., 2006; DebRoy et al., 2004; Ham et al., 2008).

As a first test of functional similarity between HaRxL23 and *Pst* AvrE1, we determined whether HaRxL23 was capable of inducing cell death in *Arabidopsis* when delivered from *Pph3121* at a high dose. We used the "effector detector vector (EDV)" system of *Pseudomonas* bacteria as a surrogate to deliver HaRxL23 via the type III secretion system (TTSS) to the interior of plant cells (Sohn, Lei, Nemri, & Jones, 2007). We used the bean pathogen, *P. syringae* pv. *phaseolicola* NPS3121 (*Pph*) for our experiments as it is non-pathogenic on *Arabidopsis*, has a functional TTSS and elicits robust defenses in *Arabidopsis* including deposition of callose and accumulation of pathogenesis related 1 (PR-1) protein without eliciting HR-like cell death. In accordance with previous results, a typical cell death symptom of leaf collapse was observed with *Pst* DC3000 carrying AvrRpt2 in the *Arabidopsis* ecotype Col-0 **(Figure 3.1A)** (Sohn et al., 2007). Both *Hpa* HaRxL23 and *Pph* AvrE1 triggered leaf collapse symptoms comparable to AvrRpt2 in young *Arabidopsis* plants **(Figure 3.1A)**. This response was not seen in older plants. This experiment indicates that both AvrE1 and HaRxL23 elicited cell death in young *Arabidopsis* plants.

Pph HaRxL23 and *Pph* AvrE1 individually suppress callose deposition in wild type *Arabidopsis* elicited by *Pph* 3121

Suppression of cell wall based defenses (eg: callose deposition) is considered to be one of the important functions of both bacterial and oomycete effectors. Callose is based on β-1, 3 glucans that get deposited between the cell wall and cell membrane near the invading pathogen, and hence are key indicators of PTI response. The EDV strategy was again used to determine whether the effectors can suppress callose deposition when delivered transiently (Sohn et al., 2007). The non-pathogenic *Pph* 3121 strain was used as the trigger for callose in these experiments. Wild type *Arabidopsis* Col-0 plants exhibit extensive callose deposition when syringe infiltrated with the *Pph* 3121 strain because this strain is significantly compromised in its ability to suppress PTI (**Figure 3.2A**). Contrastingly, a reduction close to 50% in callose deposits is observed in plants that were syringe infiltrated with individual strains of *Pph* expressing either HaRxL23 or AvrE1 (**Figure 3.2A-B**). It is interesting to note that this suppression is not enhanced when a double transformant, *Pph* HaRxL23-AvrE1, is used (**Figure 3.2A-B**). This result indicates that both the effectors are interfering with the same regulatory pathway in the host.

Neither Hpa HaRxL23 nor Pph AvrE1 enhance Pph3121 virulence

The above assay for macroscopic plant cell death was carried out with a high dose of bacterial inoculum (1 X 10^8 colony-forming units (CFUs) per milliliter). To test whether HaRxL23 enhances or suppresses bacterial growth *in planta*, we infiltrated leaves with a low dose of bacteria (1 X 10^5 CFUs/mm) and then measured bacterial

growth at three days after inoculation. We compared the growth, in Col-0, of virulent *Pph* expressing HaRxL23 and AvrE1 to *Pph* 3121 with an empty vector (EV) control. After three days post infiltration, there was no enhancement in bacterial growth in either *Pph* HaRxL23 or *Pph* AvrE1 compared to *Pph* 3121 (EV) **(Figure 3.3)** in young *Arabidopsis* Col-0 plants. Thus, despite the ability of HaRxL23 to suppress callose elicited by Pph3121, there is no net enhancement of *Pph* virulence in *Arabidopsis* by *HaRxL23*.

HaRxL23 can rescue the reduced virulence phenotype of the *ΔavrE1* strain in tomato Moneymaker

The most stringent genetic test for functional equivalence between *HaRxL23* and *AvrE1* is to assay whether *HaRxL23* can rescue an *AvrE1* loss-of-function mutant. However, this experiment is complicated by functional redundancy between *AvrE1* and other *Pst* effectors, such that a *ΔavrE1* mutant does not display a phenotype under most previously tested conditions. The only phenotype of an *AvrE1* mutant was reported by Badel et al., who observed that *P. syringae* pv. *tomato* DC3000 *ΔavrE1* deletion mutant was impaired in the formation of bacterial speck lesions in tomato (*Lycopersicon esculentum* cv. Moneymaker) plants (Badel et al., 2006). Thus, we hypothesized that if HaRxL23 is functionally similar to AvrE1, then HaRxL23 would be able to rescue the reduced bacterial speck lesion phenotype of the *avrE1* mutant in tomato. Four-week old tomato Moneymaker plants were dip-inoculated with bacterial suspensions containing wild-type DC3000, the *ΔavrE1* mutant, the *ΔavrE1* mutant carrying *HaRxL23* and the complemented *ΔavrE1*(*avrE1*) strain (**Figure 3.4**). Bacterial speck lesions ≥0.25 mm^2 were quantified from infected leaves. *HaRxL23* was able to rescue the reduced lesion

phenotype of the *ΔavrE1* mutant strain (**Figure 3.4)**. Interestingly, this rescue is at a higher level than the complemented strain of *ΔavrE1* (Badel et al., 2006) which is known to have a partial rescue phenotype, relative to the wild-type strain, due to the incomplete penetrance of the plasmid-encoded gene

Discussion

The study of effector proteins of plant pathogens is important as effectors can be used as molecular probes to decipher unknown aspects of plant biology and immunity (Bozkurt, Schornack, Banfield, & Kamoun, 2012; Feng & Zhou, 2012). So far, the studies on pathogen effectors have provided several significant information's in regard to pathogenicity that has led to the emergence of numerous concepts across a range of pathosystems. Unlike oomycetes, effector proteins have been elegantly studied in plant-pathogenic bacteria such as *P. syringae* pv. *tomato* DC3000, but this research presents an excellent opportunity to utilize information from bacterial effectors to accelerate understanding of oomycete effectors.

Since PAMPs such as flagellin, EF-Tu and chitin trigger similar signaling pathways in PTI, one important concept that has emerged is that effectors from unrelated pathogens such as bacteria, fungi, oomycetes, nematodes and insects can perturb similar processes and can have similar targets in the host. One example of this is the cysteine-rich protease RCR3 protein from tomato which is inhibited by effectors from three unrelated phytopathogens, namely Avr2 from *Cladosporium fulvum*, EPIC1 & EPIC2B from *Phytophthora infestans* and VAP1 from the root nematode *Globodera rostochiensis*

(Lozano-Torres et al., 2012; Song et al., 2009). This is an excellent example where RCR3 forms a "core" target or "hub" for effectors from unrelated plant pathogens such as fungi, oomycetes and nematodes. A recent protein interaction study involving large scale yeast-two-hybrid screen by Mukhtar et al., 2011 identified a set of 18 "core" proteins that were commonly targeted by effectors from the gram-negative *Pseudomonas syringae* bacterium and the obligate biotroph, *Hyaloperonospora arabidopsidis* (*Hpa*). Alternatively, it has been observed that in some cases pathogens have evolved effectors that influence or alter multiple steps within a single targeted pathway instead of focusing on a single "core" target. This has been exemplified in the case of plant viruses where instead of targeting individual proteins, they have evolved numerous mechanisms to target the entire RNAi machinery in the host (Burgyan & Havelda, 2011). More recently, it has been shown that effectors from both bacterial and oomycete pathogens target vesicle trafficking pathways to interfere with host immunity (Bozkurt et al., 2012; Lindeberg et al., 2012).

This study was initiated after the identification of effector proteins similar to the AvrE-family of effector proteins from *Hyaloperonospora arabidopsis* genome following a bioinformatics-driven approach (**Supplemental Figure 3.1;** Opiyo et al., unpublished data). Using partial least squares (PLS) alignment-free methods (Opiyo & Moriyama, 2007), they identified nine candidate RXLR genes from *H. arabidopsidis* genome that were similar to AvrE1 proteins.

Opiyo et al., (unpublished data) predicted the structures of proteins identified from *Hpa* genome using I-TASSER server and compared them with AvrE1 predicted protein structure and found that *Hpa* effector, HaRxL23 was the best candidate in having

regions similar to AvrE1 protein structure (**Supplemental Figure 3.2;** Opiyo et al., unpublished data). Hence, this study aimed at establishing functional similarities between AvrE1 and HaRxL23, with the rationale that there is a limited set of common targets between effectors of plant pathogens of common ancestry like *P. syringae* and *Hpa*.

Our first set of experiments was directed towards understanding whether HaRxL23 and AvrE1 could induce cell death when transiently delivered in plants. It has been previously shown that AvrE1 induces cell death when transiently expressed at high inoculum (OD_{600} = 0.2-0.5) in *N. tabacum* and tomato leaves (Badel et al., 2006). We delivered both effector proteins from *Pseudomonas phaseolicola* (*Pph*) 3121 and found that both the effectors could induce cell death in young *Arabidopsis* plants. Previous studies have hypothesized a strong correlation between suppression of basal defense and R protein-independent cell-death promotion by bacterial effectors AvrE1 and HopM1 (Badel et al., 2006), where both these effectors restored basal resistance suppression to the ΔCEL mutant and produced a delayed necrosis when transiently expressed in the host. It was hence proposed that the recognition event triggered by AvrE1 was due to an extension of the "guard hypothesis" whereby high levels of AvrE1 in the host lead to the strong basal defense suppression and triggered cell death. It is interesting to note that the cell death response by both AvrE1 and HaRxL23 was not seen in older plants. Perhaps these effectors are triggering an R protein that is active in young plants but not older plants. Another hypothesis can be that both these effectors are targeting similar regulatory proteins involved in developmental-based pathways, hence explaining this stage-specific cell death event. It is also interesting that AvrE1 does not trigger this response in older plants. The reason for this could be that the avrE1 allele of Pph 3121

does not trigger cell death but the Pto DC3000 allele does or that Pph 3121 is in fact missing avrE1. This, however, still remains to be tested.

We next found out that in *Arabidopsis*, both effectors were successful in suppressing *Pph* 3121-induced callose deposition when delivered at a low dose into older plants. It has been previously demonstrated that suppression of PTI readouts such as callose deposition by AvrE1 and HopM1 is SA-dependent (DebRoy et al., 2004), so it is possible that HaRxL23 also targets similar conserved SA-mediated immunity pathway in the host as a possible virulence mechanism.

Finally, we found that HaRxL23 was able to rescue the reduced bacterial speck lesion phenotype of the *avrE1* mutant at a level higher than the complemented strain of *ΔavrE* (Badel et al., 2006). This is interesting because the plasmid-encoded copy only partially complements the phenotype. It is also noteworthy to mention that HaRxL23 can partially rescue the Pst DC3000 (ΔCEL) mutant (Deb et al., unpublished) and this provides a degree of genetic specificity that makes this particular result more definitive.

In conclusion, these results along with the bio-informatic predictions suggest similarities, both structural and functional, between these two unrelated effectors. It is possible that these effectors from evolutionarily divergent and unrelated pathogens can perturb similar processes and are targeting similar proteins in their respective hosts. The targets of both these effectors are currently unknown; future studies will reveal insights into host proteins and processes that are manipulated by these putatively convergent effectors from bacteria and oomycete plant pathogens that have not shared a common ancestor for billions of years.

Figures

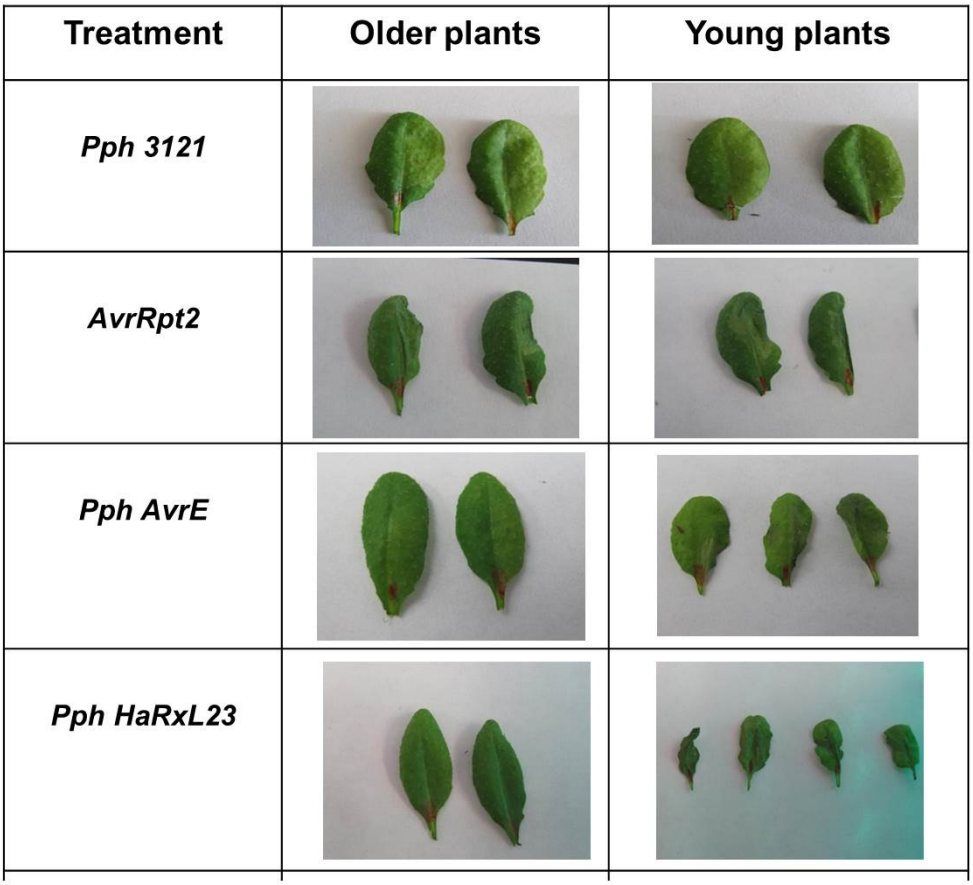


Figure 3.1 *Both HaRxL23 and AvrE1 induce cell death in young Arabidopsis plants when delivered by Pseudomonas phaseolicola.* Images from the Hypersensitive Response (HR) test from leaves of *Arabidopsis* wild type plants (Col-0). 5 week old plants were infiltrated with *P. phaseolicola* suspension expressing effectors (1 X 10^8 colony-forming units (CFUs) per milliliter). HR was visually monitored over a period of 20 hours after inoculation.

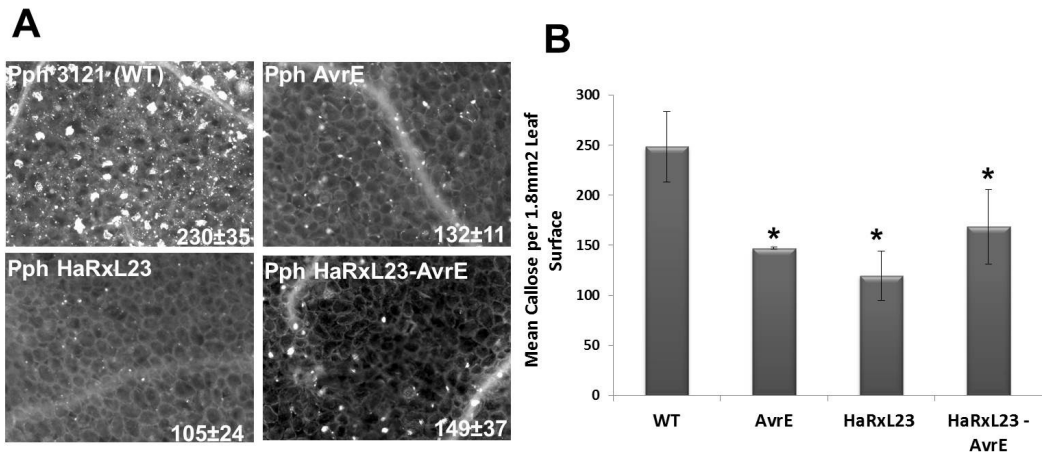


Figure 3.2 *Both effectors individually suppress callose deposition in Arabidopsis when delivered by P. phaseolicola via the effector detector vector system.* (A) Four-week old WT Col-0 plants were infiltrated with 5 X 10^7 cfu/ml *P. phaseolicola* (*Pph*) strains expressing effectors AvrE1, HaRxL23 and HaRxL23-AvrE1. Callose deposits were visualized by staining with aniline blue and (B) quantified using Autospots software program. Four pictures per leaf from six leaves were analyzed per treatment. P-value * < 0.01; t-test comparisons representing significant differences with Col-0. Error bars represent Standard Error of six independent leaf samples tested at the same time. This experiment was repeated three times with similar results.

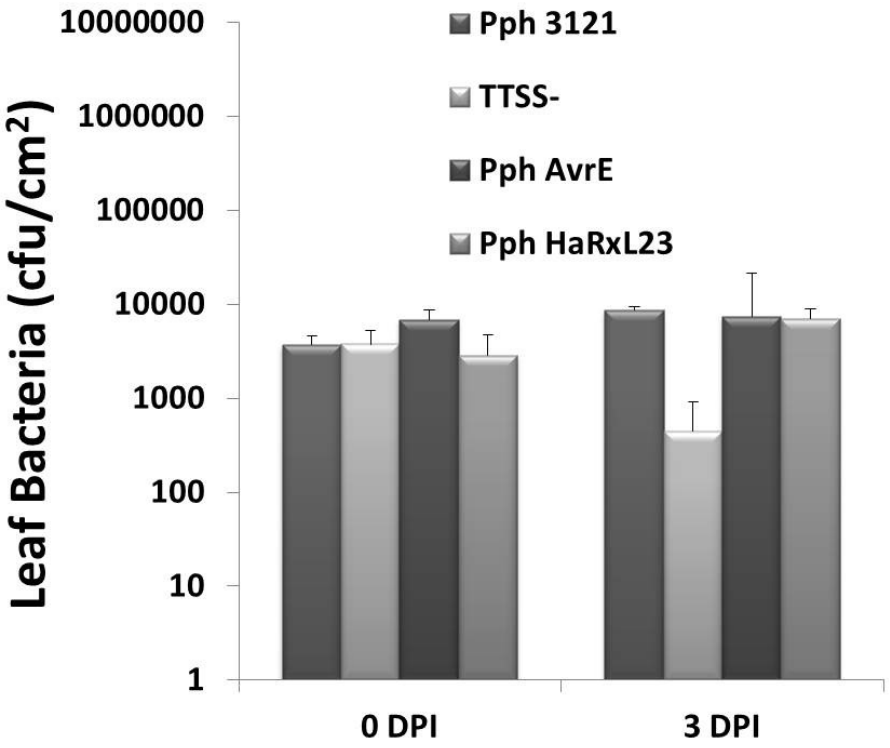


Figure 3.3 *Bacterial multiplication in leaves of wild type Arabidopsis plants (Col-0).* Plants were infiltrated with a bacterial suspension of 1 X 10^5 colony-forming units (CFUs) per milliliter. Bacterial populations were determined at day 0 and day 3 after inoculation. Error bars indicate Standard Error of six independent leaf samples tested at the same time. The experiment was repeated three times with similar results.

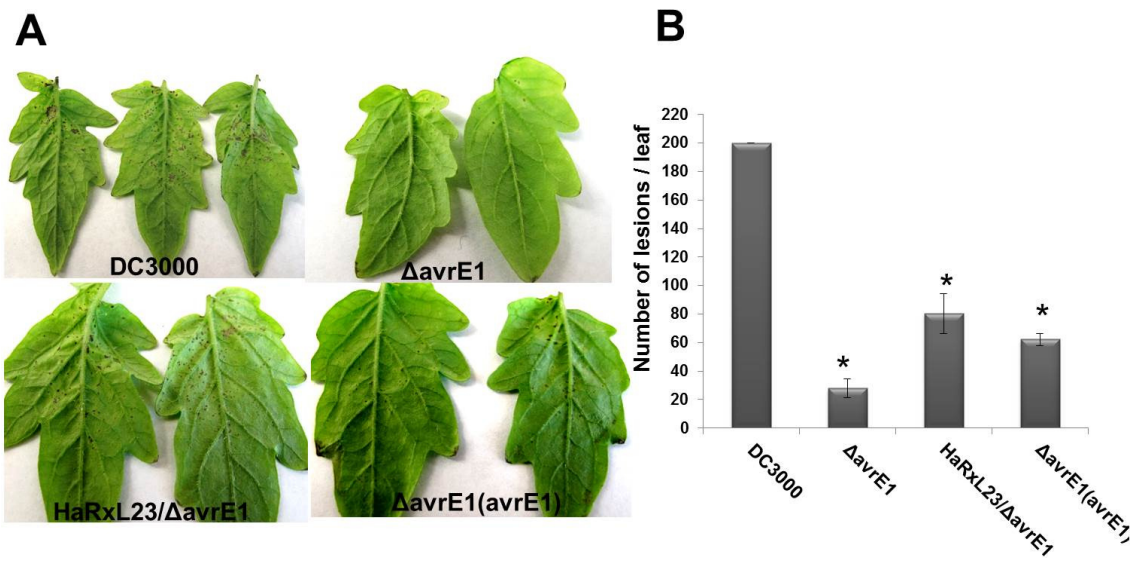


Figure 3.4 *HaRxL23 is able to rescue the reduced lesion phenotype of the ΔavrE1 strain in tomato Moneymaker plants.* (A) Disease symptoms (lesion production) on tomato cv. *Moneymaker* plants 8 days after dipping inoculation at $1x10^8$ cfu/ml bacterial culture. (B) Number of lesions ($\geq 0.25mm^2$) per whole leaf appearing on plants 8 days after dipping inoculation with the respective bacterial strains. Values indicate mean and error bars indicate standard error on at-least five whole leaves for each treatment. * = $p< 0.01$

Materials and methods

Construction of expression plasmids

HaRxL23 was amplified from genomic DNA extracted from *Arabidopsis* Oy-1 plants, infected with *Hpa* isolate *Emoy2,* using proofreading polymerase (Pfu, Invitrogen). Forward and reverse primers were designed to amplify from the signal peptide cleavage site (HaRxL23 NOSP, Supplemental Table 1) with (HaRxL23 S) or without the stop codon (HaRxL23 NS) depending on the type of fusion. For cloning into Gateway destination vectors, the sequence CACC was added at the 5' end of the forward primer and PCR was performed using the genomic DNA as template. PCR products were gel purified (Qiagen) and finally recombined into pENTR-D-TOPO Gateway entry vector following the manufacture's protocol (Invitrogen). This step was followed by transformation into *Escherichia coli* DH5α competent cells. Kanamycin resistant colonies were selected on agarose plates followed by colony PCR with plasmid specific M13 F and M13R primers. Colonies having the correct size were selected for plasmid purification and confirmed by sequencing. The pENTR clone generated was then used to create Gateway expression plasmids using LR recombination (Invitrogen).

For *Pseudomonas*-mediated transient studies, HaRxL23 gene was shuttled from pENTR into pEDV6 by LR recombination (Gateway, Invitrogen). pEDV6 contains the *AvrRPS4* promoter (Sohn et al., 2007). The EDV constructs with our effectors were transformed into *Pseudomonas phaseolicola* strains by standard tri-parental mating using *E. coli* pRK600 as a helper strain. *Pph* 3121*, Pph* AvrE1, *ΔavrE1* mutant (CUCPB5374)

and *ΔavrE1*(*avrE1*) (pCPP5246) strains were kindly provided by David Mackey at Ohio State University. All clones generated were confirmed by sequencing.

Plant materials and growth conditions

Arabidopsis and tomato (*Lycopersicon esculentum* cv. Moneymaker) plants were grown in Sunshine Pro-mix soil mixture number one. For experiments involving inoculation with *Pseudomonas spp.*, *Arabidopsis* was grown in controlled growth chambers under short day cycles (8h/16h light/dark and 150-200 $\mu E/m^2s$) at 22°C and 60% relative humidity. For all other experiments, *Arabidopsis* and tomato were grown under long day cycles (16h/8h light/dark at 90-100 $\mu E/m^2s$) at 22°C and 60% relative humidity.

Assays involving HR, bacterial virulence and callose suppression in *Arabidopsis*

For assays involving *Pseudomonas spp.*, *Arabidopsis* Col-0 plants were syringe infiltrated with $1x10^5$ cfu/ml (virulence assays) or $1x10^8$ cfu/ml (HR and callose suppression assays) bacterial solution in 10mM $MgSO_4$.

For HR assays, a total of 6 plants, 3 leaves each were infiltrated and visual scoring was performed 16-20 hours later.

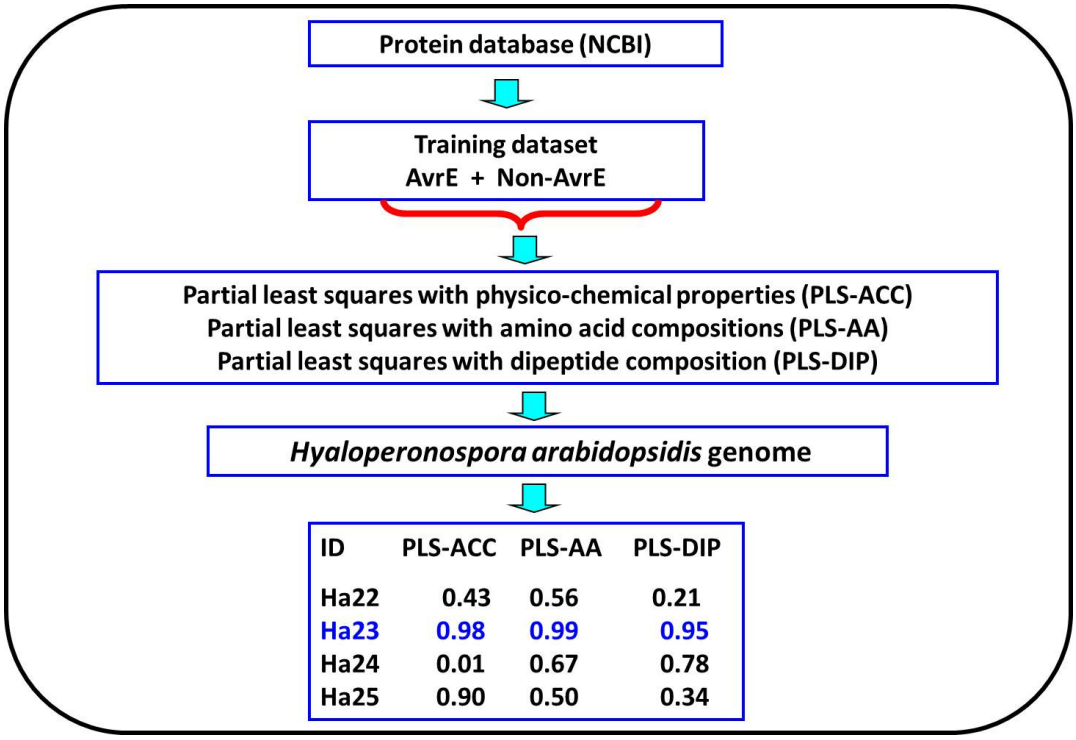
For bacterial growth assays, leaf discs were cored at zero and three dpi, surface sterilized with 70% ethanol and homogenized using a mini-bead beater (Biospec products). Serial dilutions were performed to count colony forming units. For each sample, three leaf discs were pooled three times per data point.

For callose suppression assays, whole leaves were harvested 16 hpi, treated with alcoholic lactophenol and stained with 0.01% (w/v) Aniline blue stain in K_2HPO_4 buffer as described previously (Sohn et al., 2007). Stained leaves were mounted on glass slides using 50% glycerol and imaged with a Zeiss Axio Imager.M1 using the filter settings for DAPI. Quantification of callose spots was performed using the Autospots software (Cumbie et al., 2010). Statistical analyses for growth curves were performed on means of log-transformed data using Student's t-test (*$p < 0.01$, **$p < 0.001$).

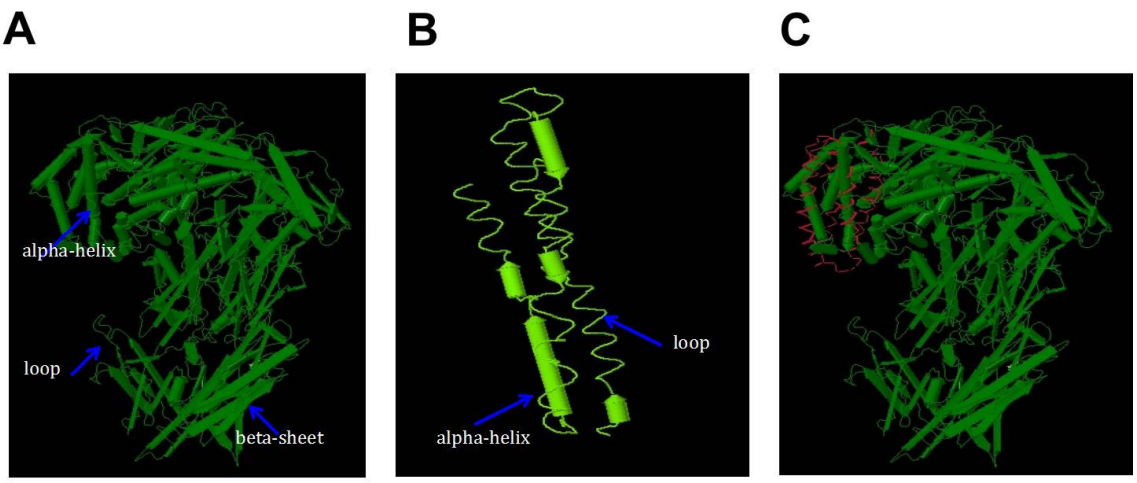
Bacterial lesion assay in tomato Moneymaker plants

For bacterial lesion assay, 4-5 week old *Lycopersicon esculentum* cv. Moneymaker (tomato) plants were dip inoculated for 30 seconds with $1x10^8$ cfu/ml bacterial solution in 10mM $MgSO_4$ containing 0.02% Silwet. A total of 3-4 plants were used for each treatment. Disease symptoms on leaves in the form of small, brown, necrotic lesions were monitored for a total of 6 days. 5 days after inoculation, the number of well-developed lesions (≥ 0.25 mm^2) per leaf was quantified.

Supporting information



Supplemental Figure 3.1 *Overview of mining AvrE1 from Hyaloperonospora arabidopsidis* (*Hpa*) *genome*. Using partial least squares (PLS) alignment-free methods nine candidates from *H. arabidopsidis* genome and 61 protein candidates from *Arabidopsis* proteome were found similar to AvrE1 proteins. Using information from gene expression data and metabolomics pathways 16 protein candidates were further investigated.



Supplemental Figure 3.2 *Structural predictions of HaRxL23 and AvrE1* (A) Predicted structure of AvrE1 by I-TASSER (B) Predicted structure of HaRxL23 by I-TASSER (C) Superimposed structure of HaRxL23 on AvrE1 by DaliLite. Structure of HaRxL23 is shown in red.

HaRxL23 NOSP	CACCATGGCAACGTCTACCGATCTGA
HaRxL23 NS	GGCGTCGACGTGCTTTAGGC
HaRxL23 S	CTAGGCGTCGACGTGCTTTA
Avh73 NOSP	GCTTCTGCTTCTTCAGAGCTCGTCGC
Avh73 NS	AGGCGGCTTTGCCTTCGAGG
Avh73 S	GTATTTGCCGTACTGGGTGA
pEDV6 Fwd	GGCACCCCAGGCTTTACACTTTATG
M13 Fwd	GTAAAACGACGGCCAGTG
M13 Rev	GGAAACAGCTATGACCATG

Supplemental table 3.1 Table of primers used in this study

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Chapter 4

An oomycete RXLR effector triggers antagonistic plant hormone crosstalk to suppress host immunity

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Contributions: J.W. is responsible for yeast-two-hybrid screens, R.G.A. is responsible for pathogen experiments with jaz3 knock out and JA signaling mutants, S.Y.H & J.M.M. initiated the project, are the principal investigators, J.M.M. contributed to manuscript preparation and editing.

This chapter comprises a manuscript in preparation for submission to *Science*

Key Words: oomycete, effector, phytohormone, jasmonic acid (JA), salicylic acid (SA), jasmonate-ZIM domain (JAZ), coronatine (COR), *Arabidopsis* plant-pathogen interactome (AtPPIN), Skp/Cullin/F box-coronatine 1 (SCF^{COI1}).

Abbreviations: *Arabidopsis* plant-pathogen interactome (AtPPIN), bimolecular fluorescence complementation (BiFC), SA methyltransferse (BSMT1), coding sequence (CDS), conserved effector locus (CEL), coronatine (COR), *Hyaloperonospora arabidopsidis* (*Hpa*), isochorismate synthase 1 (ICS1), jasmonic acid (JA), jasmonate-ZIM domain (JAZ), petunia NAM and *Arabidopsis* ATAF1, ATAF2, CUC2 (NAC), *Pseudomonas syringae* pv. *tomato* (*Pst*), salicylic acid (SA), SA glucosyl transferase gene 1 (SAGT1), Skp/Cullin/F box-coronatine 1 (SCF^{COI1}), tomato bushy stunt virus (TBSV), type III secretion system (TTSS), yellow fluorescent protein (YFP).

Abstract

Oomycete plant pathogens maintain large families of RXLR effector proteins that enter plant cells. The mechanisms through which these effectors promote virulence are largely unknown. Here, we show that the HaRxL10 effector protein from the *Arabidopsis* pathogen *Hyaloperonsopora arabidopsidis* (*Hpa*) targets Jasmonate-Zim Domain (JAZ) proteins that repress responses to the phytohormone jasmonic acid (JA). This manipulation activates a regulatory cascade that reduces accumulation of a second phytohormone, salicylic acid (SA), and thereby attenuates immunity. This virulence mechanism is functionally equivalent to but mechanistically distinct from activation of JA-SA crosstalk by the bacterial JA mimic coronatine. These results reveal a new mechanism underpinning oomycete virulence and demonstrate that the JA-SA crosstalk is an Achilles' heel that is manipulated by unrelated pathogens through distinct mechanisms.

All pathogens must evade or suppress their host's immune system. Understanding the mechanisms through which pathogens suppress host immunity is essential for complete understanding of host-pathogen interactions and will inform efforts to reduce the impact of diseases. Plants maintain a robust immune system that is activated when surveillance proteins perceive pathogen-derived signals (*1*). The phytohormones salicylic acid (SA) and jasmonic acid (JA) play central roles in immunity by regulating distinct signalling sectors that respectively provide resistance to biotrophic (SA) and necrotrophic (JA) pathogens (*2, 3*). The SA and JA sectors can be mutually antagonistic, such that activation of one sector can inhibit the other ((*4*), **fig. S4.1A**). This antagonism provides optimal defense through which immune responses can be tailored to specific types of pathogens, thereby reducing costs of resistance (*5*). However, JA-SA antagonism also provides a mechanism for pathogens to suppress one sector by inducing the other (*3*). This form of exploitation has been documented for the bacterial pathogen *Pseudomonas syringae*, which secretes a molecular mimic of JA (coronatine, COR) that promotes virulence in part by suppressing SA defenses ((*6, 7*), **fig S4.1C**).

The *Arabidopsis* downy mildew pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) is a reference organism for destructive oomycete pathogens and for the obligate biotrophic lifestyle, in which pathogens extract nutrients exclusively from living host cells and cannot survive apart from their hosts (*8*). In keeping with this lifestyle, the *Hpa* secreteome is configured for stealth in the host and includes a large family of RXLR effectors that enter plant cells (*9*). The molecular mechanisms through which *Hpa* and other oomycetes utilize RXLR proteins to subdue host immunity are only beginning to be explored (*10*).

Genetic experiments have demonstrated that SA-mediated responses are important in *Arabidopsis* for immunity against *Hpa*, while JA-mediated responses are ineffective against *Hpa* (*11*). Thus, we examined whether *Hpa* might activate JA

signalling to suppress SA-mediated responses, like *P. syringae*. Accordingly, the JA marker gene *Pdf1.2* is induced rapidly during infection by a virulent isolate of *Hpa* (**fig. S4.2A**). Moreover, mutants that compromise JA signalling (*jar1* and *jin1,* **fig. S4.1A**, (*12*)) display reduced susceptibility to virulent *Hpa* (**fig. S4.2B**), demonstrating that the JA signalling sector is genetically essential for full *Hpa* virulence. The reduced susceptibility phenotype in *jar1* and *jin1* is accompanied by enhanced plant cell death around *Hpa* infection structures (**fig. S4.2C-D**) and by elevated expression of the SA marker gene *PR-1* (**fig. S4.2E**), suggesting that the reduced susceptibility phenotype in these mutants is caused by de-repression of SA-mediated immunity due to removal of inhibition of the JA sector, consistent with previous reports.

To identify potential mechanisms through which *Hpa* could activate JA signalling, we examined the *Arabidopsis* Plant-Pathogen Interactome database (AtPPIN1) that documents putative targets of *Hpa* RXLR effectors (*13*). One *Hpa* effector, HaRxL10, interacts with the JA response respressor JAZ3 (**fig. S4.3A**). *Arabidopsis* encodes a family of 12 JAZ proteins, which act as transcriptional repressors of JA-responsive genes under conditions in which JA responses are not induced (*12*). This repression is relieved when pathogens, insects, or other signals induce biosynthesis of JA and its bioactive form, JA-isoleucine (**fig. S4.1B**, (*12*)). JA-Ile binds to and activates the Skp/Cullin/F box-Coronotine 1 (SCF^{COI1}) ubiquitin ligase complex. In turn, the activated SCF^{COI1} targets JAZ proteins for ubiquitination and subsequent destruction in the 26S proteasome, thereby de-repressing downstream responses (**fig. S4.1B**, (*12*)). We confirmed the previous report that a *jaz3* knockout mutant displays enhanced susceptibility to virulent *Hpa* (**fig. 4.1A**). This demonstrates that *JAZ3* is genetically necessary for basal resistance to virulent *Hpa*; thus, it is plausible that nullification of *JAZ3* function could promote *Hpa* virulence.

To obtain genetic evidence that HaRxL10 targets JA signalling, we transformed a *P. syringae* coronatine biosynthetic mutant (*Pst* DC3118) with a plasmid configured to express HaRxL10 in a form that can be delivered to plant cells through the Type 3 secretion system (T3SS, (*14*), **fig. S4.4A**). In this assay, secreted HaRxL10 partially rescued the virulence defects (*in planta* growth and disease symptoms) of the coronatine-deficient mutant when bacteria were sprayed onto the leaf surface (**fig. S4.4B-C**). The *in planta* growth defect of *Pst* DC3118 was also rescued when HaRxL10 was expressed from a plant transgene in stably transformed *Arabidopsis* Columbia (Col-0) plants (**fig. S4.4D**). HaRxL10 did not enhance the virulence of wild-type *Pst* DC3000 or rescue the virulence defect of the *Pst* DC3000(*ΔCEL*) mutant, suggesting that its mechanism of action is specifically equivalent to coronatine (**fig. S4.4B-D**). Additionally, Col:35S-HaRxL10 transgenic lines exhibited enhanced susceptibility to virulent *Hpa* (**fig. S4.4E**). Finally, the JA marker gene *Pdf1.2* is constitutively induced in uninfected Col:35S-HaRxL10 (**fig. S4.4F**), further demonstrating that transgenic expression of HaRxL10 is sufficient to activate JA responses, even in the absence of pathogen infection.

We confirmed that HaRxL10 interacts with JAZ3 in the yeast two-hybrid system and in an *in vitro* co-immunoprecipitation assay, indicating that the proteins bind directly to each other (**fig. 4.1B, S4.3B**). HaRxL10 also interacts with JAZ4 and JAZ9, but none of the other JAZ proteins, in yeast (**fig. S4.5A**). Deletion experiments with JAZ9 demonstrated that the conserved N-terminal domain (**fig. S4.5B**) is necessary for the interaction in yeast (**fig. S4.5C**). HaRxL10 does not interact with the SCF component COI1 (**fig. S4.5A**). We confirmed that HaRxL10 interacts with JAZ3 *in planta*, and bimolecular fluorescence complementation (BiFC, **fig. 4.1C**) assays. Fluorescently tagged HaRxL10 co-localizes with JAZ3 in subnuclear structures of unknown function (**fig. 4.1D**, (*15*)).

To test whether *JAZ4* and *JAZ9* are relevant for basal resistance to *Hpa*, we challenged *jaz4* and jaz9 mutants, along with a *jaz3*/*jaz4*/*jaz9* triple mutant, with virulent *Hpa*. We observed no enhanced virulence of *Hpa* in the single mutants. The triple mutant supported enhanced *Hpa* virulence relative to wild-type, but the degree of virulence was equivalent to the *jaz3* mutant (**fig. S4.6**). Thus, by genetic criteria, *JAZ3* plays a major, unique role in basal resistance to *Hpa.*

Because genetic loss of *JAZ3* is sufficient to enhance susceptibility to virulent *Hpa* ((*13*) and **fig. 4.1A**), we hypothesized that HaRxL10 degrades or otherwise nullifies JAZ3 to promote virulence. Accordingly, abundance of transgenically expressed YFP-JAZ3 was reduced during colonization of *Arabidopsis* by *Hpa*. (**fig. 4.2A**). Additionally, JAZ3-YFP abundance is reduced by co-expression of HaRxL10 in *N. benthamiana* (**fig. 4.2B-D**) or *Arabidopsis* (**fig. S4.7**). The HaRxL10-dependent destabilization of JAZ3 is reversed by the addition of proteasome inhibitor MG132 *in vivo* (**fig. 4.2E**). These data indicate that HaRxL10 targets JAZ3 for degradation by the 26S proteasome.

A recent study revealed the molecular cascade through which bacterial coronatine suppresses *Arabidopsis* SA responses ((*7*), **fig. S4.1C**): Coronatine mimics JA to induce COI1-SCF-dependent degradation of JAZ proteins, thereby derepressing the MYC2 transcription factor and activating three MYC2-regulated genes encoding homologous NAC (petunia NAM and *Arabidopsis* ATAF1, ATAF2, and CUC2) transcription factors: *ANAC019*, *ANAC055*, and *ANAC072*. In turn, the NAC proteins directly repress expression of a key SA biosynthetic gene (isochorismate synthase 1, *ICS1*) and activate genes encoding SA glucosyl transferase gene 1 (*SAGT1*) and SA methyltransferse (*BSMT1*). Together, this genetic reprogramming reduces the pool of bioactive SA and thereby compromises the SA immune sector. Because HaRxL10 genetically compensates for coronatine deficiency in *Pst* DC3118, we hypothesized that the HaRxL10-JAZ interaction suppresses SA through the same cascade. Accordingly, transcription of

ANAC019, *ANAC055*, *ANAC072*, and *SAGT1* is induced in Col:35S-HaRxL10, while *PR-1* and *ICS1* expression is reduced (**fig. 4.3A-B**). Similar effects were seen in infected plants (**fig. S4.8**). Moreover, the *jaz3* knockout mutant was similar to Col:35S-HaRxL10 in its effects on SA-associated gene expression (**fig. S4.8**). Conversely, *ANAC019*, *ANAC055*, *ANAC072*, and *SAGT1* transcripts are reduced in Col:35S-JAZ3, while *PR-1* and *ICS1* expression is induced (**fig. S4.8**). Together, these results demonstrate that JAZ3 is an important component of the SA suppression cascade with a non-redundant role, while *HaRxL10* overexpression phenocopies the downstream effects of coronatine, and of a jaz3 knockout, on the SA suppression cascade.

The robust configuration of plant immune networks imposes intense selective pressure on pathogens to exploit points of vulnerability in the network. It is now evident that every type of plant pathogen deploys effector proteins to disrupt the plant immune network. In oomycete phytopathogens, secreted RXLR effectors are thought to play a key role in manipulation of immune signalling hubs (*13*). The mechanisms of RXLR-mediated immune suppression are only beginning to be understood; Initial studies point to plant secretory pathways as a major target (*10*). Our experiments reveal a different oomycete virulence mechanism, based on exploitation of JA-SA antagonistic crosstalk (**fig. 4.4**): HaRxL10 is secreted into host cells, wherein it traffics to the nucleus and engages JAZ proteins to reduce their abundance via ubiquitin-mediated proteolysis. This results in derepression of JAZ targets, likely inducing MYC2, which in turn triggers a gene cascade that ultimately lowers the pool of active SA. Notably, JA-SA crosstalk is similarly manipulated by *P. syringae*, a bacterial phytopathogen that over two billion years diverged from *Hpa* (*7*). Thus, both pathogens have convergently evolved to exploit the same Achilles heel in the defense network of their host. However, the mechanisms used by bacteria and oomycetes are different: bacterial coronatine directly mimics JA and thereby activates the SCF^{COI1} complex that likely degrades all of the JAZ proteins, while

HaRxL10 acts further downstream and with more specificity, by binding to and destabilizing specific JAZ proteins. Our experiments implicate JAZ3 as a key player within the large JAZ family for regulation of cross-talk with the SA sector. Finally, this study validates the utility of the AtPPIN database for identifying effector targets (*13*) and opens the door for future studies to better understand how HaRxL10 alters JAZ stability and function for *Hpa*'s benefit, and to exploit HaRxL10 as a molecular probe to illuminate poorly understood aspects of JAZ function.

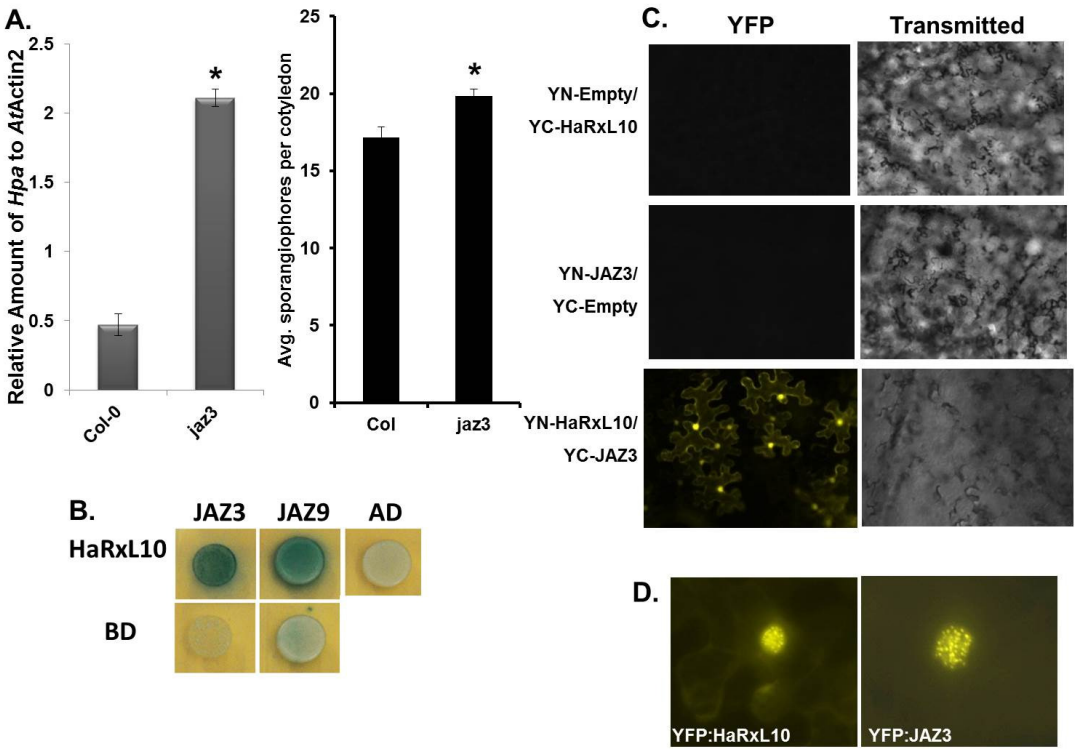

Fig. 4.1 *The Arabidopsis ZIM-domain protein JAZ3 is genetically necessary for basal resistance to virulent Hpa. The Hpa effector HaRxL10 interacts and co-localizes with JAZ3* (A) A *jaz3* knockout mutant displays enhanced susceptibility to virulent *Hpa* isolate *Emco5*. The graph on the left displays elevated *Hpa* hyphal biomass in *jaz3* relative to wild-type Col, based on a Q-PCR assay. The graph on the right displays enhanced *Hpa* reproduction in *jaz3* relative to Col-0. Disease progression was quantified 7 days post inoculation by visual sporangiophore counts. (B) HaRxL10 interacts with JAZ3 and JAZ9 in the yeast two-hybrid assay. AD and BD refers to control constructs containing activation and binding domains respectively. (C) BiFC assay in *Nicotiana benthamiana* of split YFP constructs of JAZ3-YC with an empty N-YFP vector, YN-RxL10 with an empty C-YFP vector and JAZ3-YC with YN-HaRxL10. (D) Confocal microscopic images of YFP-tagged HaRxL10 and JAZ3 in *N. benthamiana* epidermal cells, demonstrating subnuclear co-localization of RXL10 with JAZ3.

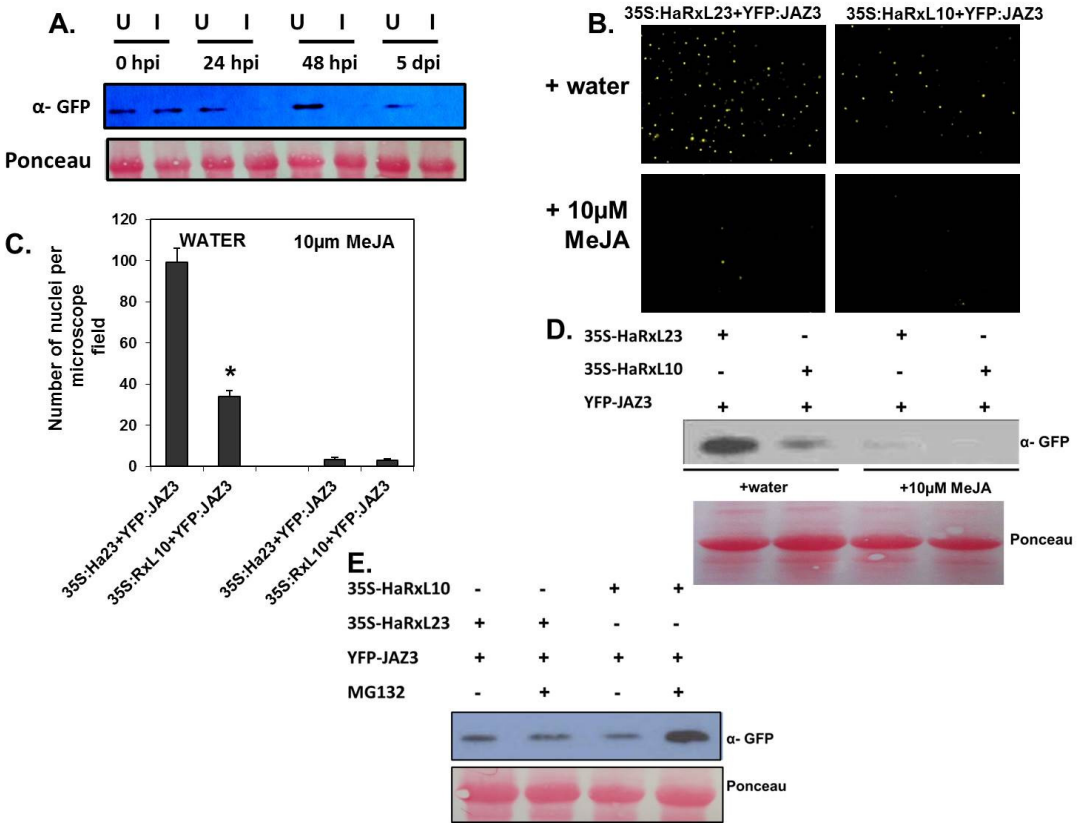

Fig. 4.2 *HaRxL10 de-stabilizes JAZ3 in a proteasome-dependent manner.* (**A**) Western blots showing reduced abundance of YFP-JAZ3 in transgenic *Arabidopsis* seedlings colonized by virulent *Hpa* Emco5. "U" refers to uninfected and "I" refers to infected seedlings (**B**) Confocal microscopic images depicted reduced signal from YFP-JAZ3 when co-expressed with HaRxL10 in *N. benthamiana* with water or methyl jasmonate (MeJA) treatment. HaRxL23 is a control RXLR effector that does not impact JA-SA crosstalk. (**C**) Quantification of YFP-JAZ3 signal from nuclei of *N. benthamiana* epidermal cells. (**D**) Western blot showing reduced abundance of JAZ3 when co-expressed with HaRxL10 in *N. benthamiana*. (**E**) Western blot showing that MG132 suppresses HaRxL10-dependent degradation of JAZ3 in *N. benthamiana*.

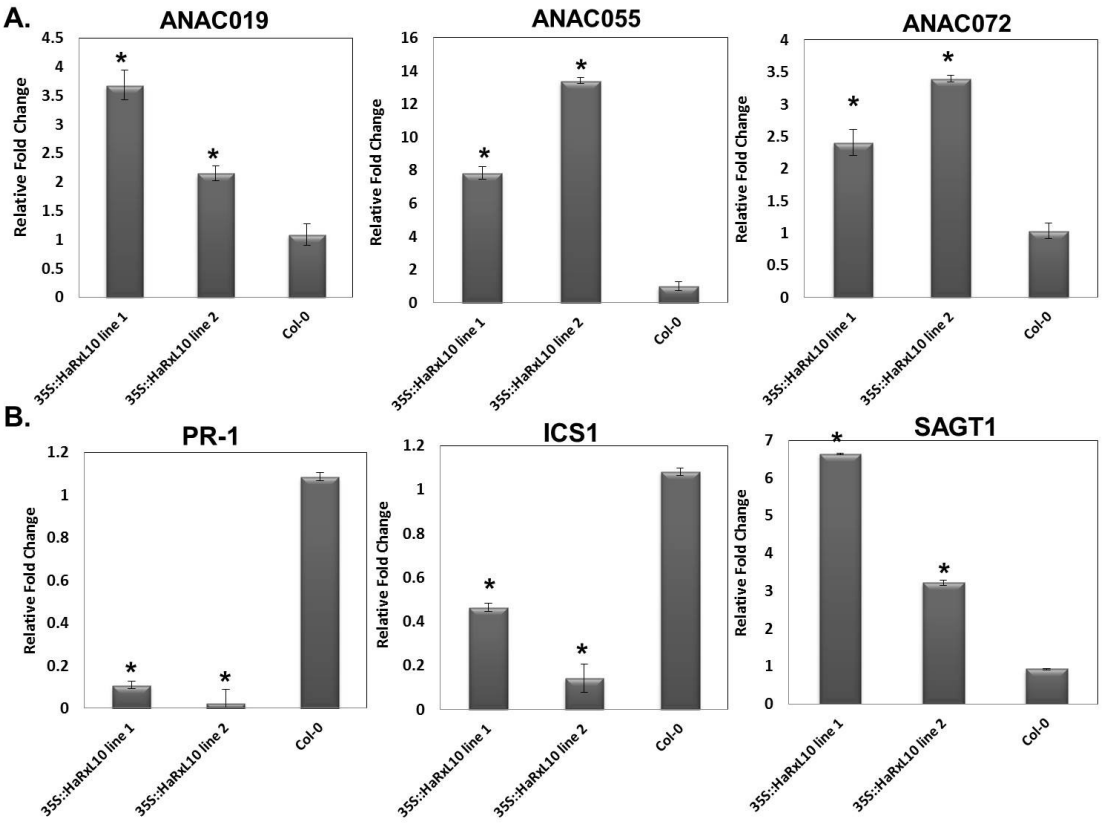

Fig. 4.3 *HaRxL10 activates a gene cascade that regulates bioavailable salicylic acid (SA).* qPCR data showing that Col:35S-RxL10 (**A**) de-represses or activates transcription of NAC TF genes; (**B**) represses transcription of PR-1 and SA biosynthesis gene ICS1; (**C**) activates transcription of SA metabolism gene SAGT1. Transcript abundance was measured using quantitative, real-time PCR using cDNA from uninfected Col:35S-RxL10 OX lines 1 and 2. Transcript abundance was normalized to *AtActin2*. * ddCt values representing statistically significant (*$P < 0.05$) differences with Col-0. Error bars depict variance among technical replicates. This experiment was repeated at least three times with similar results.

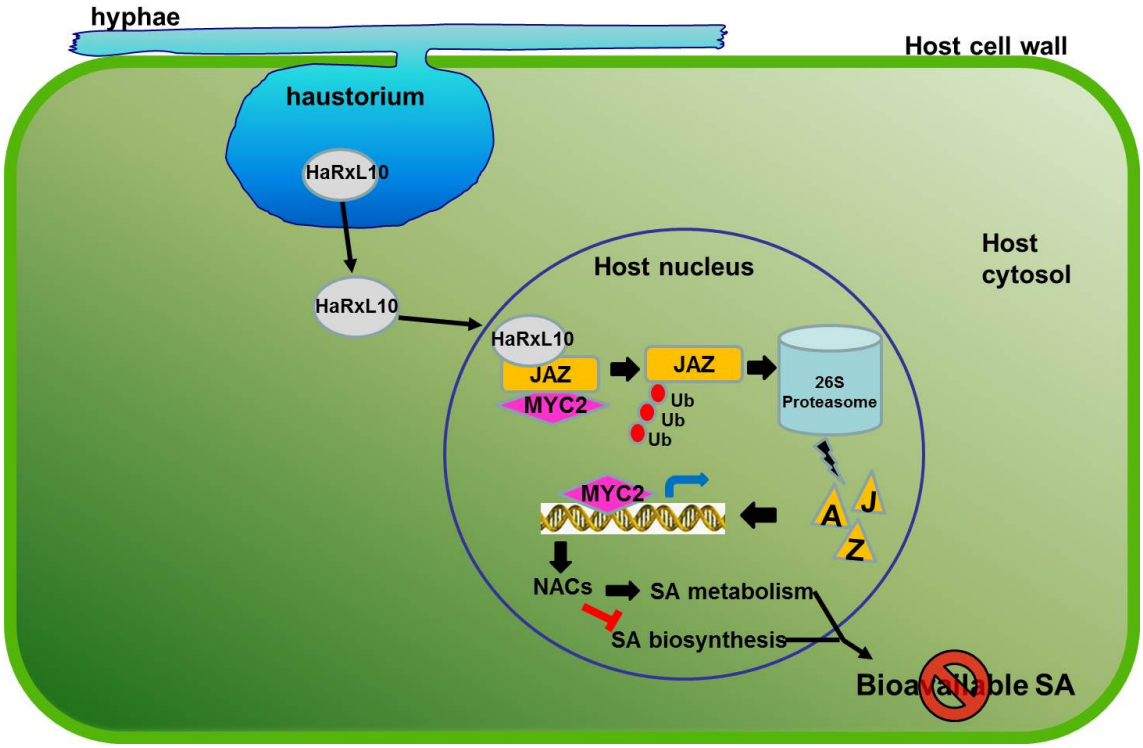

Fig. 4.4 *Hypothetical model showing the role of HaRxL10 in de-stabilizing JAZ3, thereby activating JA signalling and suppressing SA responses.*

Materials and methods

Construction of expression plasmids

The HaRxL10-pDONR207 clone was generated without the stop codon using standard Gateway cloning protocol (Invitrogen) and shuttled into expression plasmids using LR recombinase. HaRxL10:pDONR207 was kindly provided by B. M. Tyler. For *Agrobacterium*-mediated transient assays and subcellular localization studies, HaRxL10-pDONR207 was shuttled into pB2GW7 and pEarleyGate104 vectors respectively. For experiments involving *Pseudomonas syringae*, the entry construct of HaRxL10 were recombined into pEDV6 (Sohn et al., 2007). The expression plasmids obtained were mobilized from *E. coli* DH5α to PstDC3118 and PstDC3000(ΔCEL) by standard triparental mating using *E. coli* pRK600 as a helper strain. For BiFC experiments, the pDONR207 clone of HaRxL10 was recombined into the pE-SPYNE-GW binary vector, which fused the N-terminal half of YFP (nYFP) to the N terminus of HaRxL10. Similarly, the pENTR4 construct of JAZ3 was cloned into the pE-SPYCE-GW binary vector, which fused the C-terminal half of YFP (cYFP) to the N terminus of JAZ3. . The resulting binary expression plasmids were transformed into *Agrobacterium* GV3101. For *in-vitro* co-IP studies, the entry clones of HaRxL10 and JAZ3 were recombined into the pIX-HA and pIX-GST binary vectors. For yeast-two-hybrid experiments, the *JAZ* and *COI1* coding sequences (CDS) were originally amplified using RT-PCR from total RNA extracted from *Arabidopsis thaliana* (Col-0) and TA cloned into pCR2.1 (Life Technologies, Grand Island, NY). The coding sequences of the *JAZ* and *COI1* genes were released from plasmid pCR2.1 by digestion with *BamHI and EcoRI* restriction enzymes and the resulting fragments separated by agarose gel electrophoresis. The DNA fragments were purified using a Qiagen gel extraction kit (Qiagen, Valencia, CA). The *JAZ* coding sequences were ligated into the multi-cloning site of the Y2H vector pB42AD (Clontech, Mountain View, CA) to generate N-terminal fusions to the B42

transcriptional activation domain. The *RxL10* CDS was recombined from pDONR207 into a Gateway compatible version of the Y2H vector pGilda (Clontech, Mountainview, CA) to generate an N-terminal fusion to the LexA DNA binding domain. The Y2H constructs were transformed into *E. coli* DH5 alpha chemically competent cells and selected on LB plates with ampicillin. All clones were verified by sequencing.

Plant growth conditions and generation of transgenic *Arabidopsis* plants

Arabidopsis and *Nicotiana benthamiana* plants were grown in Sunshine Mix #1 for all experiments. For pathogen experiments, *Arabidopsis* was grown under short day conditions (8 hours (h) light 16 h dark) at 22˚C/20˚C. For all other experiments, *Arabidopsis* and *N. benthamiana* were grown at 16 h light, 8 h dark at 22˚C. *Arabidopsis* Col-0 were transformed following the floral dip method (Clough and Bent 1998). Primary transformed plants were selected on the basis of BASTA-resistance. The presence of transgene and transcript abundance was confirmed by PCR from genomic DNA and qPCR respectively. Segregation assays were performed in the T2 generation to identify lines with a single transgene locus. Homozygous T3 or T4 plants were used in all experiments.

***Hyaloperonospora arabidopsidis* maintenance, infection, and growth assays**

Weekly propagation and maintenance of *Hyaloperonospora arabidopsidis* isolates Emco5 and Emoy2 were performed on susceptible *Arabidopsis* ecotypes Ws-0 and Oy-1 respectively as described in (McDowell et al. 2011). For *Hpa* growth assays, 10-12 day old *Arabidopsis* seedlings were infected with conidial suspensions of $5x10^4$ spores/ml. Quantification of disease was performed as described previously (McDowell

et al. 2011). Trypan blue staining to visualize areas of cell death was performed as described previously (McDowell et al. 2011) .

RNA isolation, reverse-transcriptase PCR and qRT-PCR

Total RNA was isolated from uninfected and *Hpa* infected *Arabidopsis* seedlings using an RNeasy mini kit (Qiagen). To obtain cDNA for reverse-transcriptase and qRT-PCR, total RNA was first treated with DNase I (Ambion) and the first strand cDNA synthesis was performed using the OmniScript cDNA synthesis kit (Qiagen). Two micrograms of RNA were used aS starting template material for the cDNA synthesis. 1µl cDNA was used per well with SYBR Green PCR Master Mix (Applied biosystems) in 25µl reactions. Each PCR was performed in triplicate on the ABI7500 Real-time PCR system and transcript abundance was normalized to *AtActin2*.The primers used to detect specific transcripts are listed in Table S1. Statistical analyses were performed using Student's t-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Real Time PCR assay for growth of *H. arabidopsidis*

This assay followed the procedure described in (Anderson and McDowell 2012). Briefly, five individuals from each sample were pooled in extraction buffer (200mM Tris pH 7.5, 25mM EDTA, pH 7.5, 250mM NaCl, 0.5% SDS) and genomic DNA (gDNA) was extracted using a bead beater. gDNA samples were quantified and diluted to 10ng/uL final concentration. 25 µL samples were prepared by mixing 5 µL of gDNA sample with 12.5 µL of Sybr Green Mastermix (ABI, Carlsbad, California) along with primers and water. The primer sets *At*Actin Fwd/AtActin Rev were used for *At*Actin and *Ha*Act Fwd/*Ha*Act Rev were used for *Hpa*Actin (Brouwer et al. 2003). PCR reactions were

performed on an ABI 7500 device. Ct values were determined using ABI software. Relative abundance to AtActin was calculated as 2^-dCt.

Pseudomonas syringae infection

For spray inoculation assays, 4-5 week old *Arabidopsis* plants were sprayed with $1x10^8$ cfu/ml bacterial solution in 10mM $MgSO_4$ with 0.02% Silwet L-77. For syringe infiltration assays, 4-5 week old plants were infiltrated using a needleless syringe with $1x10^5$ cfu/ml bacterial solution in 10mM $MgSO_4$. Six plants were assayed for each data point. Leaf discs were cored at 0 days post infection (dpi) and 3dpi, surface sterilized with 70% ethanol and homogenized using a mini-bead beater (Biospec products). Serial dilutions were performed to count colony forming units. For each sample, three leaf discs were pooled three times per data point. Bacterial growth was measured as described previously. Statistical analyses were performed on means of log-transformed data using Student's t-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Transient assays using agro-infiltration in *N. benthamiana*

Recombinant *Agrobacterium tumefaciens* were grown as described previously (Van der Hoorn et al., 2000) with the appropriate antibiotics. *Agrobacterium* liquid cultures were grown overnight, centrifuged, and resuspended in MMA induction buffer (10mM $MgCl_2$, 10mM MES, 200mM Acetosyringone). The bacterial suspensions were incubated at room temperature for 1-3 hours. Infiltration using needleless syringe was performed on the abaxial side of 3-5 weeks old, *N. benthamiana* leaves. *Agrobacterium* strain containing pJL3-p19, a binary vector that expresses the suppressor of post-transcriptional gene silencing p19 of Tomato bushy stunt virus (TBSV; Voinnet et al.,

2003) was co-infiltrated with the transformed *Agrobacterium* strains for enhanced expression. *Agrobacterium* strains carrying the respective constructs were mixed in 1:1:1 ratio along with pJL3-p19 in MMA induction buffer to a final OD_{600} of 0.3 (for confocal microscopy) and 0.5 (for western blotting). For co-expression experiments, *Agrobacterium* carrying YFP tagged-JAZ3 and 35S-HaRxL10 or 35S-HaRxL23 were mixed in 1:1 ratio along with pJL3-p19 in MMA induction buffer and syringe-infiltrated in 4 week-old *N. benthamina* leaves. After 12 hours post infiltration, leaves were either syringe-infiltrated with water or 10µM MeJA solution. Imaging was performed at 15 minute intervals after water or MeJA treatment. Imaging for BiFC experiments were performed 4-5 days post infiltration. All other imaging was performed 1-2 days post infiltration. Images were taken using confocal microscopy using a Zeiss Z.1, 25x or 40x water immersion objective and 488 HeNe laser. Processing of fluorescent images was performed using the Zeiss Zen 2012 software.

Protein isolation and immunoblots

For western blotting, total proteins were extracted by grinding 3-4 leaf discs, 0.6cm in diameter in liquid nitrogen followed by boiling in SDS-loading buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% Triton X-100, 0.1% SDS, 1mM EDTA, and 1mM DTT) with 1% protease inhibitor. Equal amounts of protein were separated on an SDS-polyacrylamide gel followed by semi-dry transfers onto nitrocellulose membrane (Whatman) using Hoefer SemiPhor apparatus for 30 minutes at 35-40mA. Membranes were blocked for four hours in 4% non-fat dry milk in TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20). Overnight incubation at 4°C was performed with monoclonal anti-GFP antibodies (Covance Research) diluted with TBS-T (1:5000). After several washes with TBS-T the next day, the membrane was incubated with secondary

anti-mouse Ig antibody (GE Healthcare) diluted with TBS-T for 1 hour at room temperature. The antibody-antigen complex was detected using HRP conjugated Immobilin Western Chemiluminescent substrate (Millipore). For JAZ3 degradation experiments in *N. benthamiana*, 3-4 leaf discs, 0.6 cm in diameter were collected within 16 hours of agro-infiltration for protein isolation and consecutive western blotting. For YFP-JAZ3 assays in *Arabidopsis*, 10-11 day old seedlings overexpressing YFP::JAZ3 were challenged with 50,000 spores/ml of *Hpa* Emco5. Tissues from infected and uninfected seedlings were collected, flash frozen in liquid nitrogen at the indicated time points and harvested later for protein isolation and western blotting as described above.

In-vitro co-immunoprecipitation

In-vitro pull-down assays were performed according to the manufacture's protocol using the Pierce® HA Tag IP/Co-IP Kit (Thermo Scientific). Briefly, HA- and GST-tagged proteins were synthesized *in-vitro* using the TNT® Coupled Wheat Germ Extract Systems (Promega). For the pull-down assays, equal amounts of N-terminal GST-tagged JAZ3 or GST alone and N-terminal HA-tagged HaRxL10 were incubated with gentle end-over-end mixing at 4°C with 20 µL anti-HA agarose slurry (35 µg antibody) overnight. Next day, the samples were washed 2-3 times with TBS-T detergent (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20). After the final wash, the samples were subjected to SDS-PAGE followed by immunoblot analysis as described above using anti-GST antibody (Invitrogen).

Yeast-2-hybrid screens

pGilda:RxL10 was co-transformed along with pB42AD:JAZ, pB42AD:COI1, or empty pB42AD constructs into yeast strain EGY48 carrying the p8Op:LacZ reporter plasmid. Yeast transformation reactions were selected on plates containing SD minimal media (BD Biosciences, San Jose, CA) supplemented with -uracil (U)/-tryptophan (W)/-histidine (H) amino acid drop out solution. Following selection, colonies were cultured overnight in liquid SD-UWH drop out media. The overnight cultures were harvested, washed 2X in sterile water, adjusted to OD_{600} = 0.2 and 10 µl of each culture was spotted onto agar plates containing SD galactose/raffinose-UWH media supplemented with X-gal (80 µg/ml). Y2H plates were incubated at 30 °C for 5-7 days and positive interactions/colonies were identified by development of blue color.

Liquid Y2H assays were conducted using the □-Glo chemiluminescent system (Promega, Madison, WI) following the manufacturer's protocol. Yeast clones were cultured in minimal SD-UWH media overnight and cells were harvested by centrifugation at 3,500 rpm for 10 minutes and washed 2X in sterile water. The cells were then resuspended to OD_{600} = 0.2 in SD galactose/raffinose-UWH media and cultured for 18 hours before proceeding with the assays.

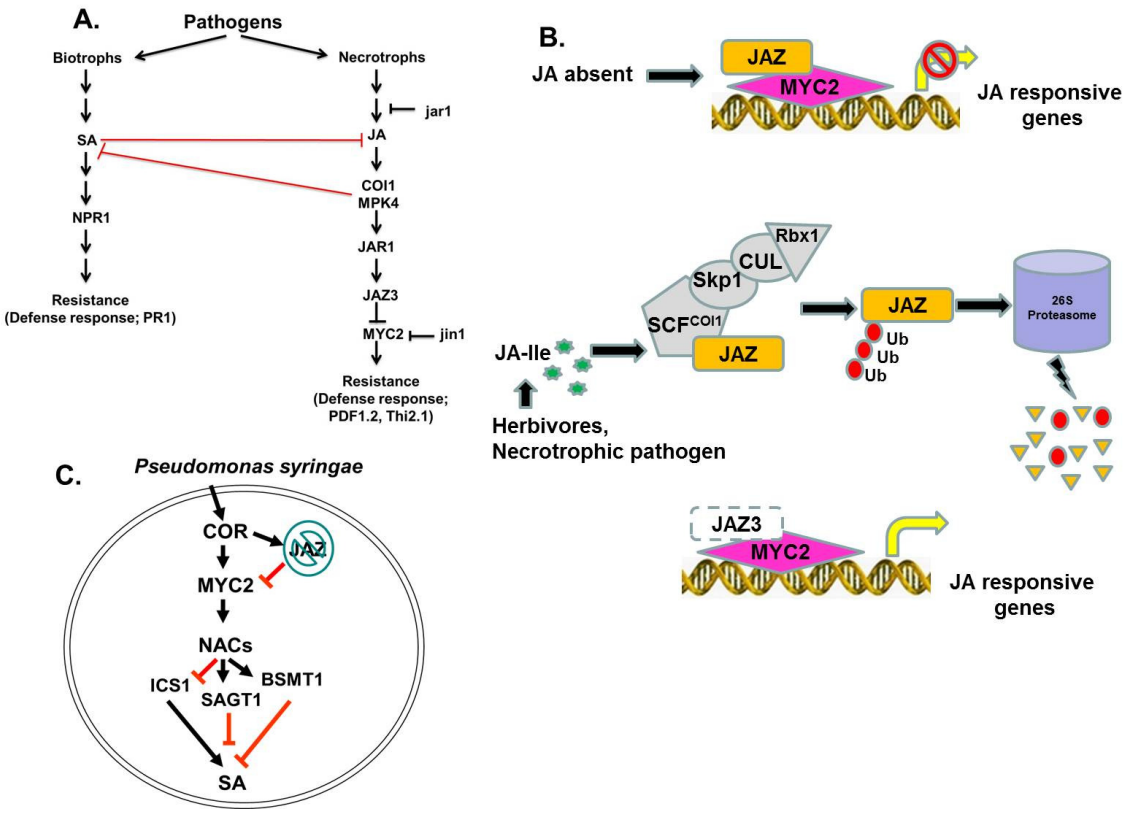

Fig. S4.1 *Schematic of JA biosynthesis, signalling, and physiological responses.* (**A**) Salicylic acid (SA) and jasmonic acid (JA) signaling sectors regulate resistance to biotrophic (SA) and necrotrophic (JA) pathogens respectively and are mutually antagonistic. (**B**) Model depicting major components of JA signalling. Low JA levels in plant cells enable repression of JA-responsive genes by JAZ proteins that counteract the activity of transcription factors (e.g., MYC2). Repression is relieved by the initiation of developmental or environmental cues that increase the accumulation of bioactive JAs (JA-Ile). JA-Ile binds to and activates the Skp/Cullin/F box-Coronotine 1 (SCF^{COI1}) ubiquitin ligase complex. In turn, the activated SCF^{COI1} targets JAZ proteins for ubiquitination and subsequent destruction in the 26S proteasome, thereby de-repressing downstream responses. (**C**) Mechanism for coronatine-induced suppression of SA accumulation in *Pseudomonas syringae*. COR acts as a molecular mimic of JA-Ile, thereby activating SCF^{COI1}which leads to degradation of JAZ proteins and activation of

the NAC transcription factors through MYC2. These TFs then repress SA biosynthesis gene ICS1 and de-repress SA metabolism genes BSMT1 and SAGT1 to inhibit SA accumulation and promote bacterial virulence.

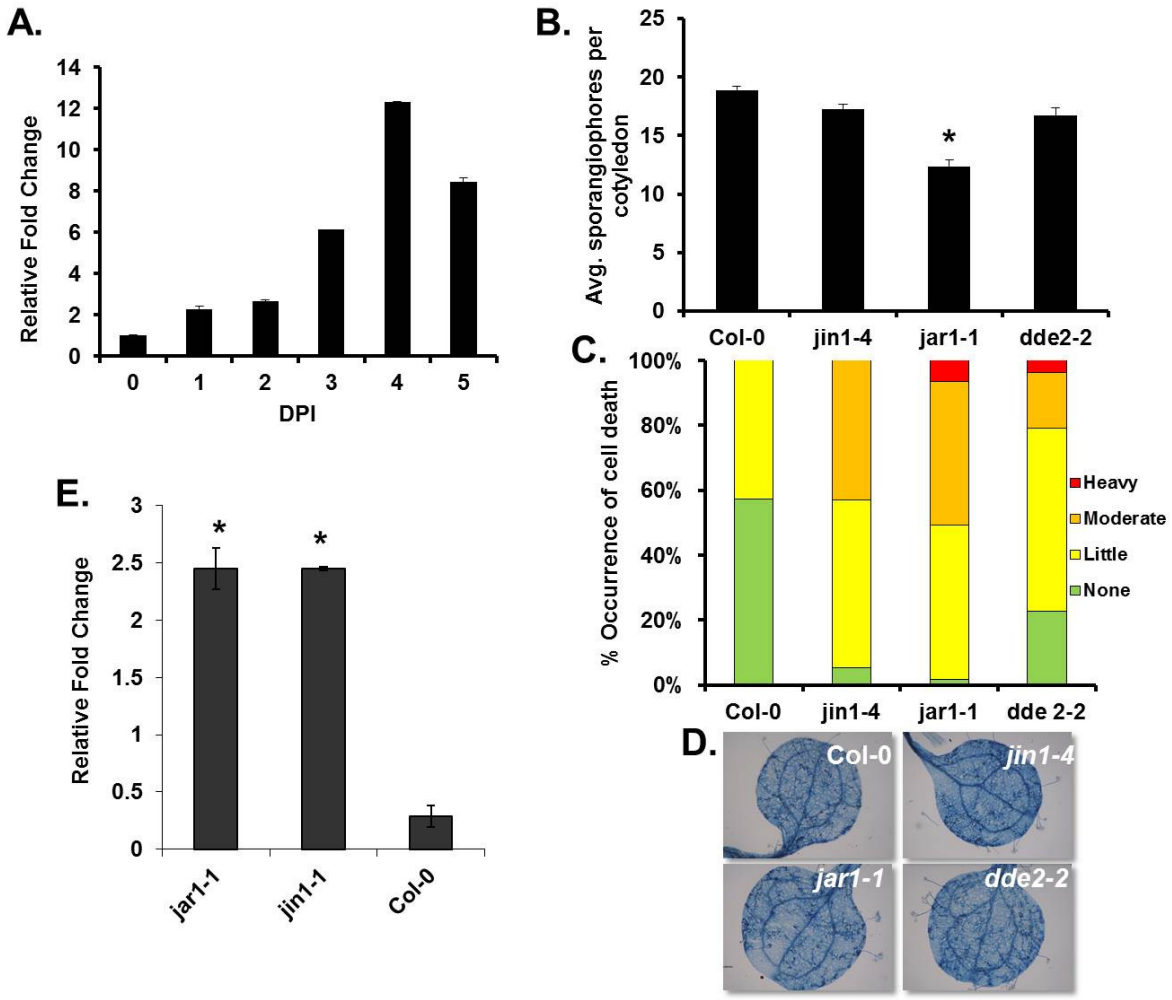

Fig. S4.2 *Evidence that Hpa engages the Arabidopsis jasmonic acid signalling sector to suppress SA-mediated immunity*. (**A**) Elevated transcription of JA marker gene *Pdf1.2* during virulent *Hpa* Emco5 infection. Q-PCR was used to measure transcript abundance of *PDF1.2* in response to virulent *Hpa* infection. Transcripts were normalized to *AtActin2*, and fold change was calculated relative to 0 DPI. Error bars represent standard deviation among technical replicates. Days post inoculation (DPI). (**B**) Reproduction of virulent *Hpa* Emco5 in JA biosynthesis (*dde1*) and signaling (*jar1*, *jin1*) mutants. Col-0

and JA mutant plants were challenged with virulent *Hpa* Emco5. Disease progression was quantified 7 days post inoculation by visual sporangiophore counts. (**C, D**) Enhanced host cell death in response to growth of virulent *Hpa* Emco5 in JA mutants.Visual quantification of trypan stained samples from the indicated *Hpa* Emco5 infected plants. (**E**) Elevated transcription of the SA marker gene *PR-1* in JA mutants. RNA was extracted from uninfected plants, and transcript abundance was measured using quantitative, real-time PCR. * ddCt values representing statistically significant (*$P <$ 0.05) differences with Col-0. Transcript abundance was normalized to *AtActin2*.

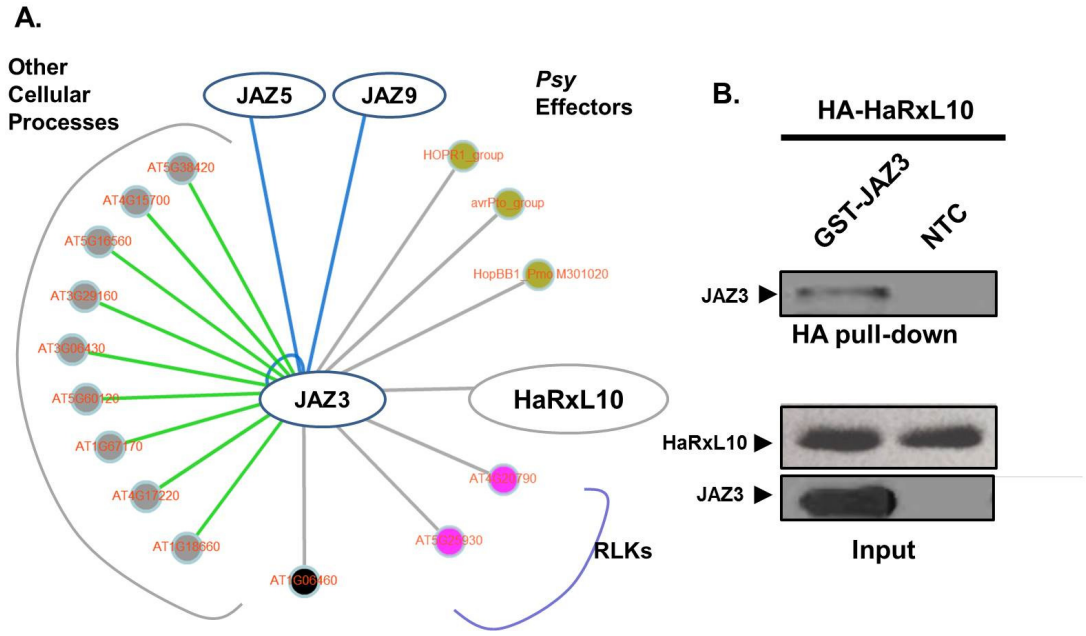

Fig. S4.3 *JAZ3 interacts with HaRxL10* (**A**) Cytoscape schematic of proteins that interact with *Arabidopsis* JAZ3 in AtPPIN version 1. JAZ3 interacts with a number of host proteins including, two other JAZ proteins, receptor like kinases (RLKs, pink), proteins involved in diverse cellular processes (grey), a defense related protein (black), and a number of pathogen effectors including 3 *P. syringae* effectors (gold) and *Hpa* HaRxL10. (**B**) HaRxL10 interacts with JAZ3 in an *in-vitro* co-immunoprecipitation assay (co-IP). HA-HaRxL10, GST-JAZ3 or NTC (no template control) were synthesized *in-vitro* using the TNT® Coupled Wheat Germ Extract Systems (Promega). Cell lysates were then

immunoprecipitated using anti-HA antibody. The immunoprecipitates were examined by Western blotting using anti-GST antibody. Input represented 10% of wheat germ lysates used in the Co-IP experiment.

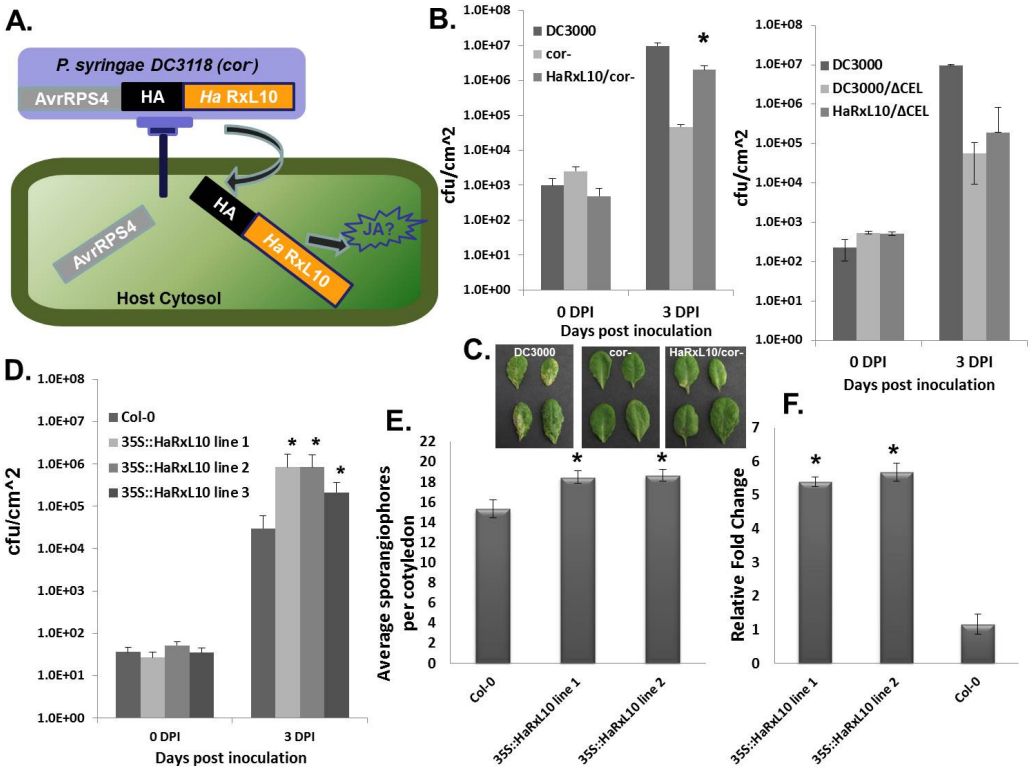

Fig. S4.4 *Genetic evidence that RXL10 targets JA signalling*. (**A**) Schematic of the effector detector system through which a fusion of RXL10 to the AvrRps4 leader is delivered from *P. syringae*, pathovar tomato (*Pst*) via Type III secretion (Sohn et al., 2007). (**B**) *In planta* growth of *Pst* strain DC3000 and mutants deficient in coronatine (*Pst* DC3118, designated as cor- in the legend) or lacking three important Type III effectors (*Pst* DC3000(*ΔCEL*)), with or without RxL10. (**C**) Plant disease symptoms triggered by *Pst* and mutants, with or without pEDV-RxL10. (**D**) *In planta* growth of *Pst* strains, without RxL10, on *Arabidopsis* Col:35S-RXL10. (**E**) Enhanced reproduction of virulent *Hpa* Emco5 on *Arabidopsis* Col:35S-RxL10. (**F**) Elevated transcription of the JA

marker gene *Pdf1.2* in uninfected Col:35S-RxL10 plants, assayed by quantitative RT-PCR as described above.

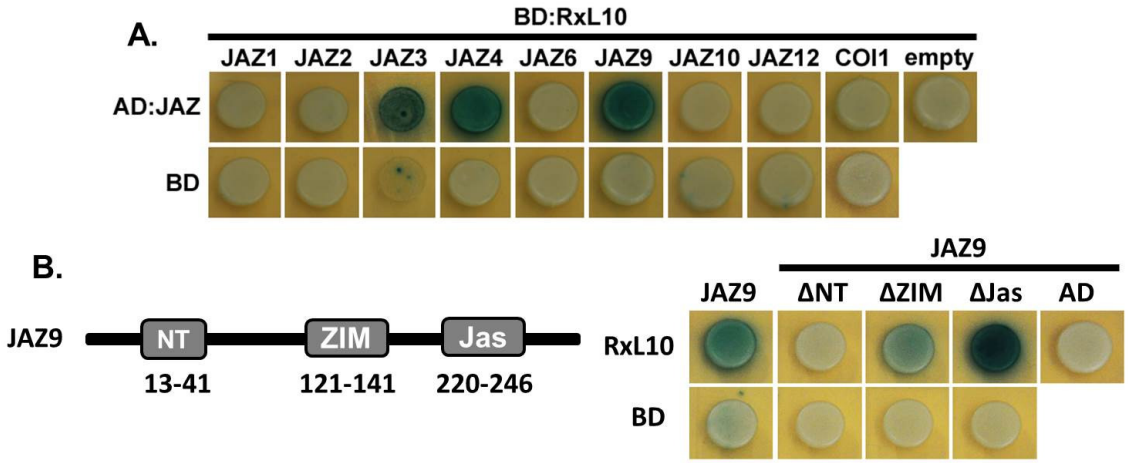

Fig. S4.5 *Yeast two-hybrid assays for interaction between HaRxL10 and JA signaling components.* (**A**) Assays for interaction between HaRxL10 and all 12 *Arabidopsis* JAZ proteins, demonstrating that HaRxL10 interacts with JAZ3, JAZ4, and JAZ9. Assays for interaction between HaRxL10 and the SCF component COI1 demonstrating no interaction (**B**) Schematics of *JAZ* gene and mutant derivatives used in these assays. (**C**) Assays for interaction between HaRxL10 and JAZ9 deletion derivatives, demonstrating necessity of the N- terminal (NT) domain for interaction.

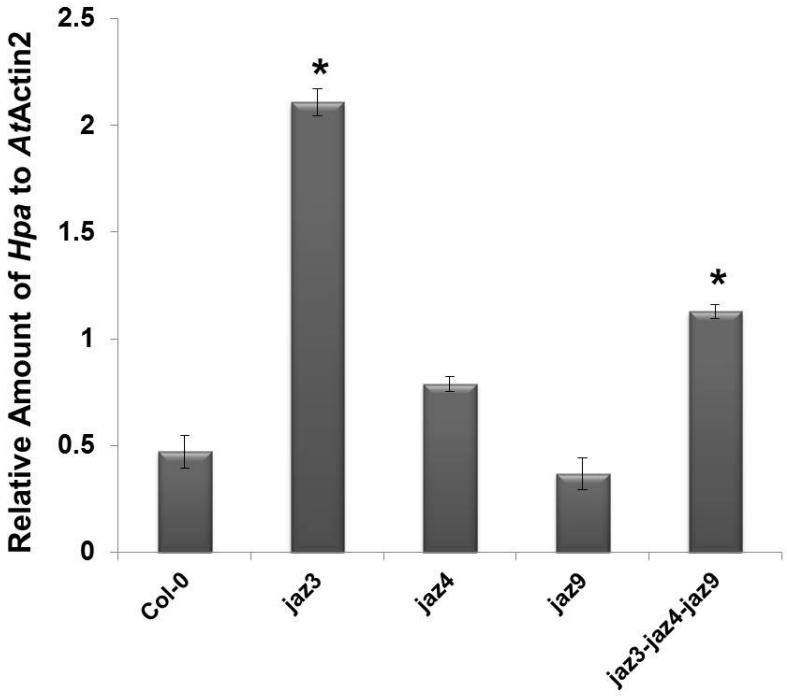

Fig. S4.6 *Hpa virulence is enhanced by mutations in JAZ3 but not in JAZ4 or JAZ9*. *Hp*a growth was quantified in *jaz3, jaz4*, *jaz9* and jaz3-jaz4-jaz9 seedlings infected with virulent *Hpa* Emco5. Genomic DNA was extracted from seedlings collected at six days post inoculation. qPCR was used to measure the relative abundance of *HpaActin* relative to *AtActin2*, as a proxy for pathogen biomass. Error bars represent SE of technical replicates. * $P < 0.05$; t-test comparisons with Col-0.

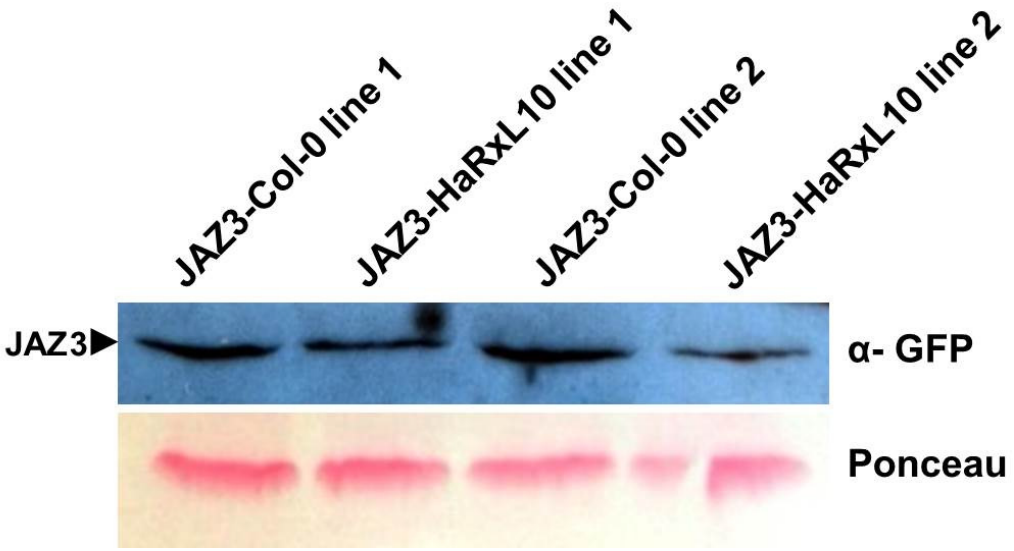

Fig. S4.7 *Abundance of YFP-JAZ3 is reduced by transgenically expressed 35S-HaRxL10.* Western blots showing reduced abundance of JAZ3-YFP in uninfected *Arabidopsis* F1 hybrids of a cross between Col:35S-JAZ3-YFP and Col:35S-RXL10.

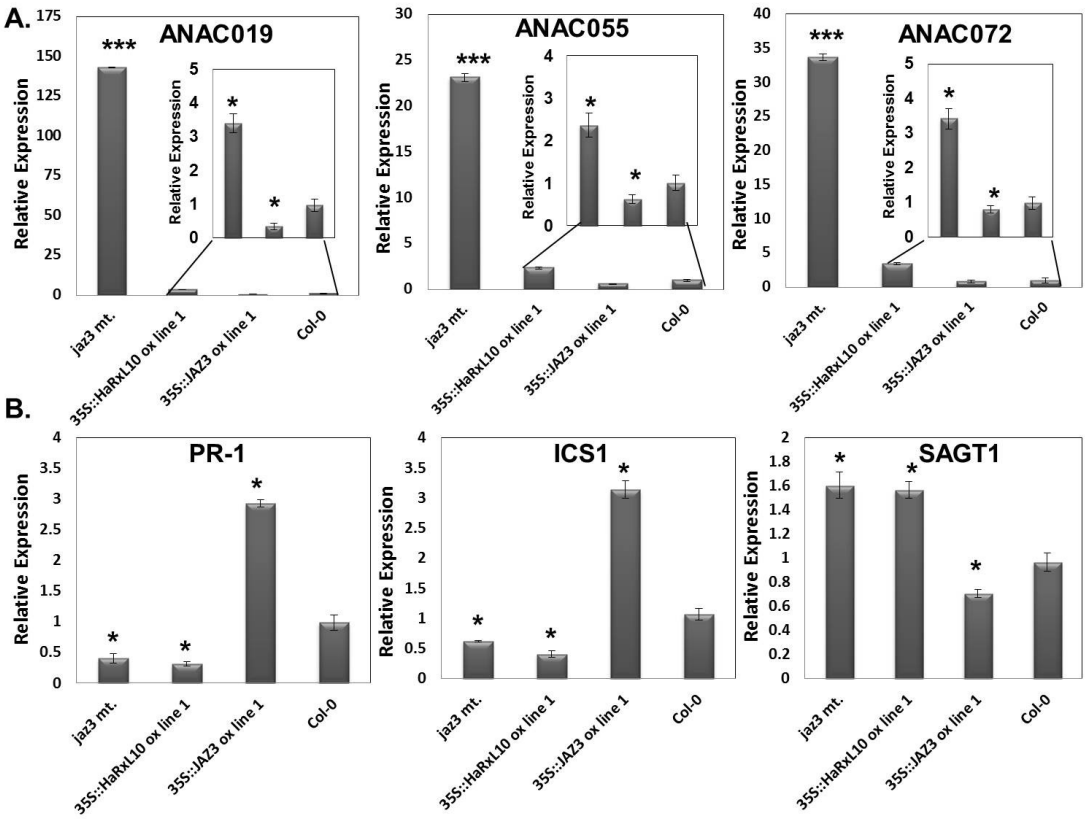

Fig. S4.8 *JAZ3 and HaRxL10 regulate expression of genes associated with SA biosynthesis and metabolism*. (**A**) (**B**) A *jaz3* knockout mutant (labeled as jaz3 mt) affects SA-associated gene expression similar to Col:35S-RxL10 (see **Fig. 3A-B**). A Col:35S-JAZ3 line affects SA-associated gene expression opposite to Col:35S-RxL10 and the *jaz3* knockout. Transcript abundance was measured using quantitative, real-time PCR using cDNA from *Hpa*-infected *jaz3* knockout, Col:35S-RxL10 OX line 1 and Col:35S-JAZ3 OX line 1 with primers specific for the indicated genes. Samples were collected 24hours post infection. * ddCt values representing statistically significant ($*P < 0.05$) differences with Col-0. Transcript abundance was normalized to *AtActin2*. This experiment was repeated at least three times with similar results.

Table S4.1: Oligonucleotide primers

HaRxL10 NOSP	GCCTCGGGACTCGCGAAACTAG
HaRxL10 NS	CGCTTTTTTACGCATTAGAGCGGAGG
JAZ3 Fwd	ATGGAGAGAGATTTTCTCGGG
JAZ3 Rev	TTAGGTTGCAGAGCTGAG
JAZ9 Fwd	ATGGAAAGAGATTTTCTGGGT
JAZ9 Rev	TGTAGGAGAAGTAGAAGAGTA
pG104 Fwd	ATGGGCAAGGGCGAGGAGCTGTTC
pG104 Rev	CGCATATCTCATTAAAGCAGGACTCTAGGGACTA
pEDV6 Fwd	GGCACCCCAGGCTTTACACTTTATG
pSPYNE Fwd	CTGGGGCACAAGCTGGAGTACA
pSPYCE Fwd	ATGGACGAGCTGTACAAGGTAAGC
pIX HA Fwd	CATTACATTTTACATTCT
pIX GST Fwd	CAACAACAACAACAAACA
pDONR207 Fwd	TCGCGTTAACGCTAGCATGGATCTC
pDONR207 Rev	GTAACATCAGAGATTTTGAGACAC
pGWB15 Fwd	GGGGACTCTAGAATGAGCGGGT
pGWB15 Rev	GTTTGAACGATCGGGGAAATTCG
M13 Fwd	GTAAAACGACGGCCAGTG
M13 Rev	GGAAACAGCTATGACCATG

HaRxL10 qPCR Fwd	GGAGATGGAATTGTCGCA
HaRxL10 qPCR Rev	CTTGAACAAAGTCGGGCA
JAZ3 qPCR Fwd	GAGTGAGGATGTTCCCTAATTCC
JAZ3 qPCR Rev	CTTCTTCCTCCTGGTGCATAAT
JAZ9 qPCR Fwd	CGGTTCGAGAAGCTGAAAGA
JAZ9 qPCR Rev	GTGTCCCTACACCTTGAGAAAT
ANAC019 Fwd	GCATCTCGTCGCTCAG
ANAC019 Rev	CTCGACTTCCTCCTCCG
ANAC055 Fwd	GCGCTGCCTCATAGTC
ANAC055 Rev	CGAGGAATCCCCTCAGT
ANAC072 Fwd	TGGGTGTTGTGTCGAAT
ANAC072 Rev	ATCGTAACCACCGTAACT
PDF1.2 Fwd	GGTGTCATGGTTGGTATGGGTC
PDF1.2 Rev	CCTCTGTGAGTAGAACTGGGTGC
PR-1 Fwd	GAACACGTGCAATGGAGTTT
PR-1 Rev	GGTTCCACCATTGTTACACCT
ICS1 Fwd	GGCAGGGAGACTTACG
ICS1 Rev	AGGTCCCGCATACATT
SAGT1 Fwd	TGGAGGAGCTTGCTTCAGCAGT
SAGT1 Rev	TGCCACCATGGGAACCCCGA
AtActin2 Fwd	AATCACAGCACTTGCACCA
AtActin2 Rev	GAGGGAAGCAAGAATGGAAC

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Chapter 5

Conclusions, future questions and directions and general outlook

Abbreviations: bimolecular fluorescence complementation (BiFC), coronatine (COR), crinkling and necrosis (CRN), signal peptide (SP), Effector-triggered immunity (ETI), *Hyaloperonospora arabidopsidis* (*Hpa*), jasmonic acid (JA), Jasmonate-Zim Domain protein (JAZ), pathogen-associated molecular patterns (PAMP), phosphatidylinositol-3-phosphate (PI3P), *Pseudomonas syringae* (*Psy*), PAMP-triggered immunity (PTI), resistance gene (R), salicylic acid (SA), systemic acquired resistance (SAR), type III secretion system (TTSS).

Conclusions

I believe the biggest challenges the field of molecular plant-microbial interaction research faces right now are the understanding of MAMPs, pathogen effectors and R protein mechanisms to develop and improve plant disease resistance. My PhD dissertation has primarily focused on functionally characterizing oomycete effectors and I feel that as more and more "essential" effectors are identified and characterized, new concepts of oomycete immunity, biology and evolution will be unraveled and will also contribute to the identification of the best resistance (R) genes to utilize for breeding programs (Bozkurt, Schornack, Banfield, & Kamoun, 2012; Feng & Zhou, 2012). In general, effectors from phytopathogens such as oomycetes are important molecular probes and can be used in a number of ways to understand unknown aspects of plant immunity and biology. The best examples for this are the type III effectors of plant-pathogenic *Pseudomona*s bacteria that have been elegantly studied to dissect several plant immune pathways (Feng & Zhou, 2012; Wei, Chakravarthy, Worley, & Collmer, 2012). Effectors can also be used as important molecular tools to understand plant cell biology and dynamic cellular processes such as vesicular trafficking (Bozkurt et al., 2011). Secondly, identifying host proteins that interact with oomycete effectors *in planta* will aid in understanding the virulence functions of these proteins (Mukhtar, Carvunis, Dreze, Epple, Steinbrenner, Moore, Tasan, Galli, Hao, Nishimura, Pevzner, Donovan, Ghamsari, Santhanam, Romero, Poulin, Gebreab, Gutierrez, Tam, Monachello, Boxem, Harbort, McDonald, Gai, Chen, He, European Union Effectoromics, et al., 2011). By identifying molecular targets of the effector proteins in the hosts, we can place those targets and their guardian R proteins into heterologous plant species and thereby change

the status of these pathogens from "host-adapted" pathogens to "non-adapted" pathogens. Other ways to utilize information from effector proteins include:- identification of host processes modified and altered to promote diseases leading to target modification, or using those genes for those host targets as important markers for efficient breeding. All these will further enhance our understanding of the molecular mechanisms of plant-microbe interactions which would, in turn, lead to its application in various applied-based research avenues.

Despite the substantial progress in oomycete effector research in recent years as a result of identification of hundreds of candidate effector genes by means of genome sequencing and bioinformatics screens, there is much that we still do not know about them. For instance, the molecular mechanism of infection, metabolism and defense suppression of the majority of these effectors are still unknown. Homology searches to known proteins offer insufficient information for effector targets or function which further delays our understanding of the complex interaction between oomycetes and plants. My dissertation research was designed to increase understanding of the molecular mechanisms that enable oomycete pathogens to cause diseases on plants. We focused on effectors that were conserved between the *Arabidopsis* downy mildew pathogen, *Hyaloperonospora arabidopsidis* (*Hpa*) and the soybean pathogen, *Phytophthora sojae*. We anticipated that as the majority of effector genes were divergent and rapidly evolving, analysis of conserved effectors will reveal virulence functions that were important for all oomycete plant pathogens. In short, Chapter 2 focused on the identification and detailed functional analysis of a pair of effectors from *Hpa*, HaRxL23 that had an identifiable homolog in *Phytophthora sojae*, PsAvh73. Chapter 3 focused on establishing functional

similarity between effector proteins AvrE and HaRxL23 from *Pseudomonas syringae* (*Psy*) and *Hpa* respectively and finally, chapter 4 focused on the interaction between an effector protein from *Hpa* and its molecular target in the host.

Recently, sequencing the genome of the *Hpa* isolate *Emoy2* revealed at least 134 candidate effectors (HaRxLs) (Baxter et al., 2010). Out of these, at least 42 have been found to be expressed during infection (Cabral et al., 2011). To date, only a few *Hpa* effector genes including *Arabidopsis thaliana* recognized 1 (ATR1) and ATR13 have been confirmed as *bona fide* effectors (Allen et al., 2004; Anderson et al., 2012; Badel et al., 2013; Caillaud et al., 2012; Rehmany et al., 2003). Hence it is necessary to validate the bioinformatic information experimentally and functionally characterize the candidate effectors. For my first project, the oomycete effector, HaRxL23 from *Hpa*, was identified on the basis of bioinformatics screens based on strong prediction for secretion (N-terminal signal peptide (SP) and host-targeting RXLR motif) (Baxter et al., 2010). HaRxL23 was also predicted to be a conserved and syntenic effector gene (Baxter et al., 2010). The first step in my project was to confirm the *bona fide* nature of HaRxL23 and its homolog PsAvh73 from *P. sojae* and we achieved this through expression of the effectors during compatible infection in the host plant. We further confirmed the *bona fide* nature of the effectors through a large-scale effector recognition screen in *Arabidopsis* and found that both these effectors were recognized by host surveillance proteins in an ecotype-specific manner. These results helped us outline my research proposal in which our primary objective centered on identifying effector functions and *in planta* targets using both transient assays and stably transformed plants. The specific aims of my proposal were i) to test functions of *HaRxL23* and *PsAvh73* by transiently

expressing in *Arabidopsis, Nicotiana benthamiana* and soybean, ii) perform a detailed analysis of stable *Arabidopsis* transformants expressing the effectors and finally iii) determine target(s) and specific function(s) of the effectors.

Due to the obligate biotrophic lifestyle of *Hpa*, we had to primarily rely on developing and performing transient assays to determine the molecular mechanism of how these effectors promoted diseases in plants. This was achieved through experiments conducted in the *Hpa* and *P. sojae* hosts *Arabidopsis* and soybean respectively and also in the unrelated *Nicotiana benthamiana* plants. We found that, in *N. benthamiana*, PsAvh73 was able to suppress immunity triggered by the pathogen associated molecular pattern (PAMP)-, INF1 and also by the *P. sojae* effector PsAvh163, whereas HaRxL23 was able to suppress PsAvh163 cell death and not INF1. In *Arabidopsis*, both the effectors, when delivered by the *P. syringae* type III secretion system (TTSS) (Sohn, Lei, Nemri, & Jones, 2007), were equally successful in suppressing the cell-wall based callose deposition, which is considered as one of the important readouts of PAMP-triggered immunity (PTI) (Jones & Dangl, 2006; Zipfel & Robatzek, 2010). We also found that both effectors partially enhanced bacterial virulence in *Arabidopsis* when delivered by the *P. syringae* TTSS. Finally, in soybean, both the effectors were successful in suppressing RPS4 or RPS6-mediated cell death elicited by the *P. sojae* effector, *Avr4/6* and in suppressing cell death or effector triggered immunity (ETI) by a crinkling and necrosis elicitor, CRN2 (Dou et al., 2010). Hence, using multiple assays, we were able to successfully show that these conserved oomycete RxLR effectors could suppress PAMP- and Effector-triggered immunity across diverse plants.

All these successful attempts motivated us to generate stably transformed *Arabidopsis* constitutively expressing the effectors and confirming the preliminary data that were generated through transient assays. To put our second aim into action, transgenic *Arabidopsis* plants expressing HaRxL23 and PsAvh73 were generated not only to confirm some of our preliminary data but also to study those aspects of immune suppression that was not possible with transient assays. Experiments with transgenic *Arabidopsis* suggested suppression of immunity triggered by pathogen associated molecular patterns (PTI), enhancement of bacterial and oomycete virulence and suppression of defense gene induction. Hence, it was established that homologous effectors HaRxL23 and PsAvh73 could suppress ETI in soybean and could suppress both PTI and ETI in *Arabidopsis* and *N. benthamiana*. We hypothesize that since PTI and ETI have overlapping regulatory pathways, the common target(s) of both these effectors act in both types of immunity.

Our final aim was to identify potential target(s) of our effectors in the host and determine the biological relevance of the targets. Till date, no host targets have been identified for HaRxL23 and PsAvh73 in the several protein interaction screens conducted by our many collaborators. However, we currently have bioinformatics-driven evidence that suggests similarities between the oomycete effector HaRxL23 and the conserved effector protein from the gram negative, plant-pathogenic *Pseudomonas syringae* bacteria, AvrE. We found evidence through a study that was initiated after the identification of effector proteins similar to the AvrE-family of effector proteins from *Hpa* genome following a bioinformatics-driven approach of partial least squares (PLS) regression alignment-free methods (Opiyo et al., unpublished data). Opiyo et al.,

predicted the structures of proteins identified from *Hpa* genome using I-TASSER server and compared them with AvrE predicted protein structure and found that *Hpa* effector, HaRxL23 was the best candidate in having regions similar to AvrE protein structure. Hence, we initiated a study aimed at establishing functional similarities between AvrE and HaRxL23, based on the rationale that there is a limited set of common targets between effectors of plant pathogens of common ancestry like *P. syringae* and *Hpa*. We also hypothesized that even though these two pathogens have evolved independent virulence mechanisms, they would have overlapping functions and have common set of targets *in planta*. Indeed, our results showed common functions between HaRxL23 and AvrE. Both induced cell death in wild type *Arabidopsis* young plants, suppressed PAMP-triggered callose deposition and finally HaRxL23 could complement the reduced bacterial speck phenotype of the avrE mutant *in planta*. All these results, along with the bio-informatic predictions suggest similarities, both structural and functional, between these two effectors.

The second project of my Ph.D. dissertation involved the identification and establishment of a different oomycete virulence mechanism, based on exploitation of jasmonic acid (JA) - salicylic acid (SA) antagonistic crosstalk. This is the first report of an obligate biotroph influencing JA signaling to suppress SA-mediated responses. This was achieved through confirming the interaction and identifying the functional role and biological relevance of the interaction between an *Hpa* effector, HaRxL10, and an *Arabidopsis thaliana* Jasmonate-Zim Domain (JAZ) protein that repressed responses to the phytohormone jasmonic acid (JA). The phytohormones SA and JA are not only important in immunity by regulating distinct signaling sectors that respectively provide

resistance to biotrophic (SA) and necrotrophic (JA) pathogens (Grant & Jones, 2009; Katagiri & Tsuda, 2010), but both the sectors can be mutually antagonistic. Hence, hemi-biotrophic pathogens such as *P. syringae* have exploited this antagonism to their benefit by suppressing the SA sector by inducing the JA sector through the use of the JA mimic coronatine (COR) (Brooks, Bender, & Kunkel, 2005) or by the use of effectors. The *P. syringae* effector, AvrB also targets a mediator of JA-SA crosstalk, MPK4 (Cui et al., 2010) and this action suppresses SA responses and enhances virulence.

Arabidopsis encodes a family of 12 JAZ proteins, which act as transcriptional repressors of JA-responsive genes (Browse, 2009). This repression is relieved when pathogens, insects, or other signals induce biosynthesis of JA and its bioactive form, JA-isoleucine (Browse, 2009). JA-Ile binds to and activates the Skp/Cullin/F box-Coronotine 1 (SCF^{COI1}) ubiquitin ligase complex. In turn, the activated SCF^{COI1} targets JAZ proteins for ubiquitination and subsequent destruction in the 26S proteasome, thereby de-repressing downstream responses (Browse, 2009). Previous genetic experiments have demonstrated that SA-mediated responses are important in *Arabidopsis* for immunity against *Hpa*, while JA-mediated responses are ineffective against *Hpa* (Glazebrook, 2005). Hence with quantitative Real-Time PCR (qRT-PCR) and *Hpa* infection experiments, we confirmed that *Hpa* activated JA signalling in order to suppress SA-mediated responses. We also established through *Hpa* infection experiments in JA signalling mutants that the JA signalling sector was genetically essential for full *Hpa* virulence. We identified the interaction between HaRxL10 and JAZ3 by examining the *Arabidopsis* Plant-Pathogen Interactome database (AtPPIN1) that documented putative targets of *Hpa* RXLR effectors (Mukhtar, Carvunis, Dreze, Epple, Steinbrenner, Moore,

Tasan, Galli, Hao, Nishimura, Pevzner, Donovan, Ghamsari, Santhanam, Romero, Poulin, Gebreab, Gutierrez, Tam, Monachello, Boxem, Harbort, McDonald, Gai, Chen, He, Vandenhaute, et al., 2011). We first confirmed the previously demonstrated result that *JAZ3* is genetically necessary for basal resistance to virulent *Hpa*. We also obtained evidence of HaRxL10 genetically targeting the JA signalling sector through several experiments in *Arabidopsis* involving delivering HaRxL10 by the *P. syringae* type III secretion system (TTSS) (Sohn et al., 2007) and also through experiments where HaRxL10 was expressed from a plant transgene in stably transformed *Arabidopsis* Columbia (Col-0) plants.

We next confirmed the interaction between HaRxL10 and JAZ3 in the yeast two-hybrid system and in an *in vitro* co-immunoprecipitation assay, indicating direct binding. HaRxL10 was also found to interact with two other JAZ proteins, JAZ4 and JAZ9, but none of the other JAZ proteins, in yeast. We also confirmed HaRxL10 interaction with JAZ3 and JAZ9 through bimolecular fluorescence complementation (BiFC,) assays. Fluorescently tagged HaRxL10 co-localized with JAZ3 and JAZ9 in sub-nuclear structures of unknown function (Withers et al., 2012). We next showed that colonization by *Hpa* resulted in the reduction in abundance of transgenically expressed JAZ3 and specifically, JAZ3 abundance was reduced significantly when co-expressed with HaRxL10 in *N. benthamiana* and *Arabidopsis*. Interestingly, the HaRxL10-dependent degradation of JAZ3 could be reversed by the addition of proteasome inhibitor, indicating proteasomal-based degradation of JAZ3 by the effector. Finally, several experiments confirmed JAZ3 to be a component of the SA suppression cascade, while HaRxL10 overexpression, mimicked the downstream effects of coronatine on the SA suppression

cascade further establishing that HaRxL10 nullified the ability of JAZ3 to promote SA accumulation. These results reveal a novel virulence mechanism by an oomycete effector protein through which manipulation of one hormonal pathway (JA) lead to the suppression of a second (SA) pathway in the host. This study also highlights the vulnerability of the existing JA-SA crosstalk in the host and how unrelated pathogens utilize it to its own benefit through distinct mechanisms.

Future questions and directions

Identifying target(s) and specific function(s) of HaRxL23 and PsAvh73

Plant cells require a remarkable level of structural organization to compartmentalize the diverse cellular processes and functions. Sub-cellular localizations determine the environments in which proteins operate. As such, sub-cellular localization influences protein function by controlling access to and availability of all types of molecular interaction partners. Thus, knowledge of protein localization often plays a significant role in characterizing the cellular function of newly discovered proteins. The current view is that dynamic changes in protein localization are critical for intra- and intercellular information exchange, which in turn enables proper cellular function and integration of extracellular signals. However, a key challenge in plant cell biology is to directly link protein localization to function. Subcellular localization studies of HaRxL23 and PsAvh73 using fluorescently-tagged proteins indicate that both of them localize to the nucleus and cytoplasm of *N. benthamiana* epidermal cells (Deb et al., unpublished). However, the functional relevance of nuclear localization is not known for these two

effectors which is one of the key future works for this project. One approach for reconciling protein localization with function is to alter protein distribution patterns and evaluate the impact on functionality. This can be achieved by removal or addition of known localization motifs such as secretion or translocation signals, nuclear localization signals (NLS) or nuclear export sequences (NES) followed by functional analysis of the mutated protein (Schornack, Minsavage, Stall, Jones, & Lahaye, 2008; Shen et al., 2007). Mis-localization can also be achieved using compartment-specific antibodies that generate artificial sinks (Conrad & Manteuffel, 2001). Mis-localization experiments can be very informative and complement loss-of-function experiments by establishing a direct link between biological function and cellular localization. However, the challenge with these approaches is to express, target and assemble the antibodies as well as to ensure sufficient specificity towards the protein targeted in vivo. We mis-localized both the effectors and performed some preliminary experiments and showed that nuclear localization is required for the proper virulence functions of HaRxL23 and PsAvh73 (data not shown). However, several questions still remain unanswered with regard to the target and function of these effectors in the nucleus. Finally, based on the functional and bioinformatically-predicted structural similarities between effectors from *Hpa* HaRxL23 and *Psy* AvrE, it is highly likely that these effectors from evolutionarily divergent and unrelated pathogens can perturb similar processes and are targeting similar proteins in their respective hosts. The targets of both these effectors are unknown till date and future studies will reveal insights into host proteins and processes that are manipulated by these conserved and similar effectors from bacteria and oomycete plant pathogens.

Multiple approaches can be taken as an attempt to identify targets of effectors. Large scale interactome screens based on yeast-two-hybrid (Y2H) or co-immuniprecipitation (co-IP) methods can be utilized to identify interacting proteins in the host. I recommend the co-IP method over the Y2H method, as it provides an unbiased approach and can also overcome the potential pitfalls of an Y2H assay. For confirmation of interaction experiments, transgenic *Arabidopsis* overexpression lines of the effectors can be used as the starting material. The assay can be further sensitized by having stringent conditions like *Hpa* infection to provide the ideal opportunity for target identification and confirmation.

Identifying the mechanism of JAZ3 degradation by HaRxL10, understanding the role of other interactors of HaRxL10 and elucidating unique function of JAZ3 in immunity

In the second project we demonstrate, for the first time, how an oomycete effector triggers antagonistic plant hormone crosstalk to suppress host immunity. This manipulation of one phytohormone, JA leads to the activation of a regulatory cascade that reduces accumulation of a second one, SA thereby weakening host immunity. This virulence mechanism is functionally equivalent to but mechanistically distinct from activation of JA-SA crosstalk by the bacterial JA mimic coronatine. Thus, both pathogens have convergently evolved to exploit the same Achilles heel in the defense network of their host. Future studies should be aimed at a better mechanistic understanding of how HaRxL10 destabilizes JAZ3. Secondly, HaRxL10 could be used as a molecular probe to illuminate poorly understood aspects of JAZ function. This can be achieved through

understanding the role(s) of other putative interactors of HaRxL10. Interestingly, the *Arabidopsis* PPIN revealed several transcription factors as putative HaRxL10 interactors. However, one of the only non-transcription factor targets of HaRxL10 is RING, an E3 ubiquitin ligase. We also confirmed the previous report of gene knockout of JAZ3 show enhanced susceptibility to *Hpa* and a variety of other pathogens (Gusmaroli, Feng, & Deng, 2004; Mukhtar, Carvunis, Dreze, Epple, Steinbrenner, Moore, Tasan, Galli, Hao, Nishimura, Pevzner, Donovan, Ghamsari, Santhanam, Romero, Poulin, Gebreab, Gutierrez, Tam, Monachello, Boxem, Harbort, McDonald, Gai, Chen, He, Vandenhaute, et al., 2011). Keeping both these in mind, along with the known function of RING, we hypothesize that HaRxL10 recruits RING to degrade JAZ3 and inappropriately activate JA signaling. Hence the next important question to be answered is whether HaRxL10, RING and JAZ3 interact in a complex that gets degraded by the 26S proteasome.

General outlook

In the past decade, significant discoveries and progress have been made in the field of molecular plant-microbial interactions not only in terms of basic science research but also in translating that knowledge and putting it to use for real-world agricultural practices. Most of the research so far has focused on identifying elicitors of plant immunity and their cognate resistance genes (R) to breed resistant plants. Stacking of multiple R genes that act against a single pathogen species is a commonly used agricultural practice. Some novel strategies, other than the conventional R-gene breeding are currently showing a lot of promise. Some of these include the development and

engineering of novel resistance determinants for certain pathogen associated molecular patterns (PAMP) receptors (Lacombe et al., 2010), some *Xanthomonas* effectors (Romer, Recht, & Lahaye, 2009; Romer et al., 2010) and systemic acquired resistance (SAR) activators (Jung, Tschaplinski, Wang, Glazebrook, & Greenberg, 2009). Plant pathogen effectors have always evolved to benefit the invading organism by mimicking plant processes. Two type III effector proteins from animal parasitic bacteria have been engineered to alter the kinase pathways in yeast and mammalian cells (Wei et al., 2012). Very recently, a novel approach was taken to engineer and custom design TAL effectors to bind to any target DNA sequence (Bogdanove & Voytas, 2011). In this approach, TAL effectors were fused to DNA nucleases in order to target a unique site in genomes of mammals, worms, flies and plants to produce precise genetic variations (Bogdanove & Voytas, 2011). In a first of its kind study in 2012, Li et al., used TAL-DNA nucleases and successfully engineered bacterial blight resistance in rice. Despite all these efforts, no one disease control strategy can ever be considered "the best one" due to the versatile nature of most plant pathogens in overcoming most resistance strategy put into practice by agriculturists in the field.

There is a lot that remains unanswered in the areas of biology, lifestyle, evolution and virulence mechanisms of oomycetes. For instance, it will be fascinating to compare effector functions between oomycetes of different lifestyles (hemi-biotroph vs obligate biotroph) and determine whether they adopt common infection strategies to evade recognition by the plant surveillance system. Secondly, identification of the transport mechanism deployed by oomycete effector proteins (RXLR, crinkler, and other cell-entering) and the role of PI3P binding needs to be confirmed. Once the cell entry

mechanism of RXLR effectors get solved, attempts can be then made to block the entry as an effective mechanism for oomycete disease prevention. Establishment of high-throughput transient and *in planta* assays have become a necessity as genome sequencing have revealed hundreds of "putative" effective candidates and it is important to validate and define their functions during infection. High-throughput cell biology techniques are also required for the assessment of virulence functions of oomycete effector candidates. There are several other questions related to effector activity, secretion and function that still need to be addressed. In terms of effector secretion, the mechanism of which is still unclear, it is unclear whether effectors get secreted at particular location(s) at the interface between the pathogen and its host plant and also whether effectors get secreted individually or in batches. Functionally, we are still unaware whether effectors have distinct functions at particular infection stage of the pathogen or whether effectors are capable of trafficking intracellularly after they have been secreted. Also, it has not yet been established how often evolutionarily similar and/or phylogenetically unrelated phytopathogens have common effector targets in their host. Finally, obtaining genetic manipulation capability in obligate biotrophs, which is currently the major limiting factor, will be an important step forward to study the dynamic nature and functions of effectors in these pathogens.

Despite all these above unanswered questions, we cannot overlook the remarkable progress the field of oomycete effector research has made in the past decade. The field has come a long way from the time the conserved RXLR motif of unknown function was first identified in 2005 (Rehmany et al., 2005). Also, following the traditional method of map-based cloning and avirulence functions, only a handful of oomycete effectors were

initially identified (Allen et al., 2004; Armstrong et al., 2005; Rehmany et al., 2005; Shen et al., 2007). But now, the situation is completely different due to the advancement made in the recent years in the areas of genome sequencing, bioinformatics screening and structural studies. Whole genome sequences of several oomycetes are now available which has led to the revelation of both common and unique features associated with oomycete biology and evolution (Baxter et al., 2010; Haas et al., 2009; Levesque et al., 2010; Links et al., 2011; Raffaele et al., 2010; Tyler et al., 2006). Answers to several questions regarding the diversity of oomycete lifestyles, gene composition, and horizontal gene transfer from bacteria and fungi have emerged from genome analysis and comparisons. We now know that genomes of most of the oomycete phytopathogens are made up of repetitive DNA and these regions of are often associated with rapidly evolving, plastic regions harboring genes including effector genes that are involved in virulence mechanisms. Secondly, reduction of pathogenicity genes seems to be one of the important adaptations to obligate parasitism by oomycetes such as *Hpa*. Some other important events that shaped oomycete genome evolution have been the loss of photosynthetic machinery and formation of novel domains and motifs (Tyler et al., 2006). We now know oomycete genomes maintain large number of RXLR effectors that are modular in nature and the conserved host-targeting RXLR motif is involved in cell entry in a pathogen-independent manner (Dou et al., 2008; Whisson et al., 2007). However an open debate that needs to be resolved is about the facilitation of host cell entry by oomycete effectors through binding of external phosphatidylinositol-3-phosphate by the RXLR motif (Kale et al., 2010; Kale & Tyler, 2011) or lysine residues. We now also have several evidences regarding the expression patterns, localization sites, structural

details and virulence functions of a few of the oomycete effectors namely Avr3a and AvrBlb1 from *P. infestans*, Avr1b, PsAvh73 from *P. sojae* and ATR1, ATR13, HaRxL96 from *Hpa*.

In sum, this research project ultimately represents a successful effort to broaden the understanding of poorly understood aspects of *Hpa* pathogenicity — PTI and ETI suppression by oomycete effectors in host and non-host (Chapter 2, 3) and uncovering a new mechanism of oomycete virulence — targeting hormonal pathways in the host plant (Chapter 4). I look forward to witnessing spectacular advances in this field in the years to come.

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