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Characterization of Genes Involved in Resistance and Susceptibility Against *Phytophthora infestans* and *Phytophthora cinnamomi*

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DEDICATION

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ABSTRACT OF THE DISSERTATION

Characterization of Genes Involved in Resistance and Susceptibility Against *Phytophthora infestans* and *Phytophthora cinnamomi*

by

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Challenges by pathogens and pests have presented major problems for crop production, posing major constraints to meeting the food requirements for the increasing global population. The most devastating pathogens for agriculture belong to the Phytophthora genus (The so-called "plant destroyers"). The goal of this dissertation research is to advance our understanding in ways to enhance resistance against two extremely destructive *Phytophthora* species, *P. infestans* and *P. cinnamomi.* Disease control/management strategies against these two oomycete pathogens remain unsustainable and argue for new control methods including the generation of crops with durable and effective resistance against them. This dissertation focuses on two main objectives: **1.** Identify and characterize specific plant proteins contributing to plant immunity to develop *P. infestans* resistant plants, **2.** Study the *in-planta* expression of the *P. cinnamomi* effector repertoire during its interaction with different host plants and conduct functional studies to identify effectors that contribute to *P. cinnamomi* virulence, and that can be eventually used to fetch key resistance and susceptibility plant targets to engineer *P. cinnamomi* resistant plants in the future.

In Chapter 2, we investigated the interacting proteins of microchidia 1 (MORC1) proteins from *Arabidopsis thaliana*, *Solanum tuberosum* (potato), and *Solanum lycopersicum* (tomato). We identified and characterized a MORC1 interacting protein, better known in Arabidopis as Drink Me-Like (DKML) basic leucine zipper (bZIP) transcription factor 29. Interestingly, DKML homologs in tomato, potato, and *Nicotiana benthamiana* interacted with the C-terminal region of MORC1 in tomato and potato. Additionally, we discovered that DKML regulates plant cell death and resistance for *P. infestans* using virus induced gene silencing (VIGS) and transient protein expression in *N. benthamiana* and tomato.

In Chapter 3, we identified and functionally validated the effector proteins that contribute to *P. cinnamomi* virulence. Through RNA-sequencing (RNA-Seq), five candidate effectors were highly expressed after *P. cinnamomi* infection in avocado, *N. benthamiana,* and *A. thaliana* when compared to the *in vitro* mycelia. Transcript expression was validated by quantitative real-time quantitative PCR (qRT-PCR) in avocado roots and *N. benthamiana* leaves inoculated with *P. cinnamomi.* Two of the five candidate effectors were functionally validated and shown to contribute to *P. cinnamomi* virulence.

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General Introduction

Plant Immunity

Plants have adapted clever ways to withstand the wide range of microorganisms encountered in its environment. Depending on the establishment of the interaction (whether it be mutualistic, commensal, or pathogenic) dictates the type of response that the plant may deploy (Nishad *et al.*, 2020). Additional to the physical barriers of defense, plants possess sophisticated immune response strategies that can induce defense mechanisms that are either nonspecific or specific (Reina-Pinto & Yephremov, 2009). This immune response strategy is composed of several layers, where the first line of defense occurs when the plants recognize conserved pathogen molecules known as pathogen associated molecular patterns (PAMPs). These PAMPs induce a PAMP-trigged immunity (PTI) response in the plant and restrict pathogen growth (Davis & Hahlbrock, 1987; Jones & Dangl, 2006). Pathogens have evolved ways to suppress PTI, thus plants have evolved mechanisms to counteract these pathogen effects by triggering a stronger resistance known as effector-triggered immunity (ETI) (Jones and Dangl, 2006). This occurs when the plant immune receptors (resistance proteins) recognize specific effector proteins named Avirulence (Avr) proteins secreted by the pathogen to interfere with PTI. These resistance proteins are mainly composed of polymorphic nucleotide binding-leucine rich repeat (NB-LRR) receptor proteins, encoded by resistance I genes that can directly or indirectly recognize the presence or activity of the pathogen effectors (Monteiro & Nishimura, 2018). During the evolutionary arms race between plants and pathogens, fast-evolving pathogen effectors can render the plant susceptible again if the plants R proteins are unable to recognize these newly evolved effectors. This intricate

play between plant and pathogen interaction is known as the zig-zag model (Jones and Dangl, 2006).

The genus *Phytophthora* "The Plant Destroyers"

The genus, *Phytophthora* has given rise to some of the most devastating crop diseases of oomycetes. Though morphologically *Phytophthora* resemble filamentous fungi, genetically *Phytophthora* have evolved independently from true fungi and in fact, belong to a class of eukaryotes within the kingdom Chromista (Hardham and Blackman, 2018). *Phytophthora* species can produce long-lived oospores (reproductive spores) and chlamydospores (long-term survival spores) that can lay dormant until favorable circumstances arise (Boevink *et al.*, 2020). When conditions permit, either the oospore or chlamydospore can produce multinuclear sporangia which can germinate directly or differentiate into swimming biflagellate zoospores (Boevink *et al.*, 2020). The specific conditions for zoospore formation and release varies among *Phytophthora* species (Boevink *et al.*, 2020). This dissertation explores the dynamic plant-pathogen interaction of two of the most devastating *Phytophthora* species, *Phytophthora infestans* and *Phytophthora cinnamomi.*

Potato, a food-security crop, is continuously threaten by *Phytophthora infestans*

Potato (*Solanum tuberosum*) cultivation was first established about 8000 years ago in the mountainous regions of Peru (Lutaladio & Castaldi, 2009). The high elevations and fluctuating temperatures of the region made it difficult to harvest wheat or corn, thus marking the beginning of potato cultivation (Lutaladio & Castaldi, 2009). It wasn't until the 16th century that potatoes were finally brought to Europe by the Spanish

conquistadors. At the time potatoes were not considered a suitable dish for the general public and were primarily used as food for the severely poor or as feed for livestock (Lutaladio & Castaldi, 2009). However, in the 1740s, potatoes were finally credited for their importance by the Europeans when famine struck and potato breeding and cultivation saved many from starvation (Lutaladio & Castaldi, 2009).

Due to potatoes hardy agricultural qualities and great nutrient-to-price ratio (Beals, 2019), now potato cultivation has spread to 160 countries around the world (Camire *et al.*, 2009). Today, the potato is the most important non-cereal crop consumed and the world's fourth most important food crop, with annual productions of over 350 million tons (FAO, 2014; Zhang *et al.*, 2017). As a vital food-security crop, protecting potato production is of upmost importance. The leading threat to potato production is caused by the oomycete pathogen, *P. infestans*, the causal agent of potato and tomato late blight disease. Although late blight is infamously known for the Irish potato famine and subsequent deaths and emigration of millions between 1845 and 1849 (Zadoks, 2008), late blight is still a major threat, causing million-dollar losses in potato and tomato cultivation worldwide. Potato late blight causes annual crop yield losses of about 16% globally which corresponds to a financial loss of over $6.5 billion annually (Haverkort *et al.*, 2008; Aguilera-Galvez *et al.*, 2018). Current methods in controlling *P. infestans* include the application of fungicides, generation of resistance crops, use of RNA interference against *P. infestans*, and cultural practices (Ivanov *et al.*, 2021).

Phytophthora infestans

Although two mating types persist in the environment (A1 and A2), allowing for the potential of sexual reproduction as an heterothallic oomycete, most *P. infestans* outbreaks are initiated by asexual sporangia (clonal populations) (Leesutthiphonchai *et al.*, 2018), enabling fast and extensive growth in susceptible host tissue (Fry, 2008). These sporangia can germinate directly or indirectly depending on environmental cues and conditions (Fry, 2008). At higher temperatures (20-25°C), sporangia directly germinate via germ tube, however, at lower temperatures (10-15°C), sporangia indirectly germinate through releasing single nucleated zoospores which are motile and biflagellate (Fry, 2008). These zoospores can swim for short periods of time before encysting and germinating via a germ tube and appressoria to penetrate the host tissue (Fry, 2008). As characteristic of most hemibiotrophic pathogens, during the biotrophic phase, the host shows little to no visible symptoms, typical for the first two days after the initial infection, until small areas of necrosis become apparent, indicative of the necrotrophic phase (Zuluaga *et al.*, 2016). During the biotrophic stage, after the appressoria has penetrated the host tissue and primary and secondary hyphae have formed, specialized structures called haustoria, which is the place for nutrient uptake and delivery of pathogen effectors to the apoplast or into the cytoplasm of plant cells (Zuluaga *et al.*, 2016).

Phytophthora infestans has a large repertoire of effectors due to its robust 240 Mb genome size and complex genome architecture consisting of gene dense regions, also known as the core genome usually containing housekeeping genes or genes that are

critical for their biological functions, and consisting of gene sparse regions, or the plastic genome which is low in the number of genes and is enriched with repetitive elements including transposons (Dong *et al.*, 2015; Ah-Fong *et al.*, 2017; Leesutthiphonchai *et al.*, 2018). Interestingly, within these gene sparse regions, many effectors of *P. infestans* including RxLRs (named for their arginine, any amino acid, leucine, and arginine motif) and Crinklers (CRNs, named for their "crinkle and necrosis" phenotype) are disproportionally present and are thought to undergo rapid selective pressures through nonallelic recombination and other forms of structural variation (Dong *et al.*, 2015; Leesutthiphonchai *et al.*, 2018). The fast-evolving nature of *P. infestans* proteins associated with pathogenicity, virulence, and host and environment adaptation provides sources of genetic diversity to this clonally propagated pathogen resulting in phenotypic variability needed to evade and suppress host defenses making the development of long lasting resistant crops difficult and unsustainable (Fry, 2008). Though the genetic diversity of *P. infestans* has proven to render *R* genes and PTI genes ineffective, efforts still continue in identifying durable *R* gene-based and quantitative trait loci (QTL)-based resistance to control *P. infestans* (Fry, 2008).

_R_ gene-based resistance against _Phytophthora infestans_

In the last century, many *R* genes cloned or introgressed into cultivated potatoes from wild Mexican species (particularly *S. demissum*) have provided initial resistance to *P. infestans.* Several Mexican *R* genes that have been investigated including: *R1-R11* from *S. demissum*, *Rpi-blb1-Rpi-blb3* from *S. bulbocastanum*, *Rpi-mch1* from *S. michoacanum*, *Rpi-sto1* and *Rpi-pta* from *S. stoloniferum*, *Rpi-amr3* from *S.*

Americanum, and *Rpi1* from *S. pinnatisectum* (Kuhl *et al.*, 2001; Hein *et al.*, 2009; Vleeshouwers *et al.*, 2011; Śliwka *et al.*, 2012; Jo *et al.*, 2015; Witek *et al.*, 2016; Aguilera-Galvez *et al.*, 2018). Additional to Mexican *Solanum* species, several *R* genes from wild species of the Andean region in South America have also been identified (Aguilera-Galvez *et al.*, 2018). In the wild relative of tomato *S. pimpinellifolium* (native to Ecuador and Peru), the dominant *P. infestans* resistance gene, *Ph-1,* was identified and mapped to chromosome 7 (Foolad *et al.*, 2008) and the incomplete late blight resistance gene, *Ph-2,* was identified and mapped to chromosome 10 (Moreau *et al.*, 1998). However, due to the fast-evolving nature of *P. infestans*, a new strain emerged, rendering these genes ineffective long term (Panthee & Chen, 2009). Another partially dominant gene, *Ph-3,* was mapped to chromosome 9, conferring race-specific resistance and was successfully introgressed into tomato breeding cultivars for commercial and farming use (Zhang *et al.*, 2014). Though single *R* genes are pathogen race specific and less durable, latest breeding approaches have turned to stacking individual *R* genes, as seen more recently with *Ph-2* and *Ph-3* crossed lines, to provide broad-spectrum and durable resistance against the fast-evolving late blight pathogen (Jo, 2013; Jo *et al.*, 2015).

QTL-based resistance against *Phytophthora infestans*

In addition to resistance genes, other factors such as QTLs are important for conferring resistance to late blight. QTL-based resistance mapping is an approach for studying sets of alleles affecting traits for plant disease resistance. With QTL-based resistance a more durable resistance can be established due to the additive effects of PTI and ETI-associated genes (Ivanov *et al.*, 2021). Although finding and mapping QTLs is

quite difficult when dealing with complex genomes, the need for a strong durable resistant variety against *P. infestans* is moving the application of QTLs in tomato and potato to promising feats (Ivanov *et al.*, 2021). At least six QTLs of resistance to *P. infestans* have been found in potato (Ivanov *et al.*, 2021) and two major QTLs have been associated with late blight resistance in tomato (Panthee *et al.*, 2017).

Microchidia proteins are key regulators of multiple layers of plant immunity

Microchidia (MORC) proteins are members of a GHKL (Gyrase, Hsp90, Histidine Kinase, MutL) superfamily, with homologs found in both prokaryotic and eukaryotic organisms (Iyer *et al.*, 2008; Dong *et al.*, 2018; Kim *et al.*, 2019). In general, MORC proteins contain a highly conserved GHKL and S5 fold domain at the N-terminal region, which together form an active ATPase module followed by a unstructured region and a coiled-coil (CC) domain which make up the C-terminal region (Iyer *et al.*, 2008; Dong *et al.*, 2018). MORC proteins play roles in silencing DNA methylated genes, chromatin compaction, gene silencing, and signal transduction (Iyer *et al.*, 2008; Bordiya *et al.*, 2016; Manohar *et al.*, 2017; Kim *et al.*, 2019; Bhadouriya *et al.*, 2021).

MORC proteins have been identified in both monocots and dicots with eight MORC members found in Arabidopsis to six members found in the solanaceous species tomato, potato, and *N. benthamiana* (Manosalva *et al.*, 2015; Koch *et al.*, 2017). Specifically, MORC1 has been described previously in Manosalva *et al.* (2015), indicating a single homolog of the MORC1 protein from tomato, potato, and *N. benthamiana* were grouped with three Arabidopsis MORC proteins (AtMORC1,

AtMORC2, and AtMORC3). Additionally, MORC1 and other MORC members has been to form homodimers and heterodimers (Manosalva et al. 2015, Moissiard *et al.*, 2014). MORC proteins also regulate several layers of plant immunity including non-host resistance, PTI, ETI, and systemic acquired resistance (SAR) against multiple pathogens, including the oomycete, *P. infestans* (Kang *et al.*, 2010, 2013; Langen *et al.*, 2014; Manosalva *et al.*, 2015). Interestingly, the C-terminal region of MORC1 containing the disordered structural region (L2) and CC-domain regulates PTI-induced cell death and is responsible for efficient DNA relaxation, catenation, and DNA binding upon ATPase activity stimulation (Manosalva *et al.*, 2015; Manohar *et al.*, 2017). Though MORC1 has demonstrated significant roles in plant immunity, further investigation in the mechanism(s) of how MORC1 regulates plant immunity is necessary.

Avocado, the major fruit tree crop worldwide, is continuously threaten by *Phytophthora cinnamomi*

Evidence of avocado (*Persea americana*) domestication occurred in Mesoamerica as early as 6,400 B.C., suggesting that the avocado tree may represent one of the first domesticated trees in the Neotropics (Smith, 1966; Galindo-Tovar *et al.*, 2008). The true origins of avocado domestication has been difficult to resolve since at least three separate events of domestication has occurred, highlighting the importance of avocado cultivation for nourishment and sustenance (Chen *et al.*, 2009). Additional to being great in flavor and texture, avocados are rich in nutrients and help increase the intake of important nutrients from other foods (Ford & Liu, 2020). Studies have shown that avocado consumption correlates to healthier diets, better weight management, and increased

cardiometabolic health through the reduction of total cholesterol, low-density lipoprotein cholesterol (LDL-C), and triglycerides (Peou *et al.*, 2016; Ford & Liu, 2020). Demands for avocados have skyrocketed over the past decade, nearly doubling in production since 2012 with approximately 8.06 million metric tons produced in 2020 (FAO, 2022).

The leading threat to the avocado industry is the soil-borne oomycete, *P. cinnamomi*, the causal agent of Phytophthora root rot (PRR) (Erwin, D. C., and Ribeiro, 1996). Reports of 50-90% of the orchards are affected by PRR in Australia, South Africa, California and in some regions of Mexico (Ploetz, 2013; Solís-García *et al.*, 2021). As there is no current method to eradicate *P. cinnamomi* once established in a given location, methods in controlling *P. cinnamomi* include the use of chemical treatments, such as potassium phosphite, good farming practices, and the planting of tolerant or resistant rootstocks (Hardham & Blackman, 2018; Joubert *et al.*, 2021). More recently, populations of *P. cinnamomi* have shown signs of resistance against the industry standard treatment of potassium phosphite (Belisle *et al.*, 2019), stressing the importance for a new *P. cinnamomi* treatment alternatives, such as resistance crops. To generate new *P. cinnamomi* resistant crops, it is useful to first identify and validate important effectors that help establish PRR disease.

***Phytophthora cinnamomi,* "The biological bulldozer"**

Phytophthora cinnamomi is a hemibiotroph, capable of infecting more than 5000 plant species, devastating forests, nurseries and other economically important horticultural crops like avocado, durian, chestnut, macadamia, peach, and pineapple (Hardham & Blackman, 2017; Engelbrecht *et al.*, 2021). *P. cinnamomi* is heterothallic,

with two mating types, A1 and A2, and has both sexual and asexual phases in its life cycle (Hardham, 2005). Surprisingly, even when both mating types are present in a given area, *P. cinnamomi* populations associated with avocado PRR are A2 type and clonal (Hardham, 2005; Hardham & Blackman, 2018).

Phytophthora cinnamomi infects fine, feeder roots, but can also take advantage of wounds or abrasions in the peridermal layer of woody stems producing cankers (O'Gara *et al.*, 2015; Hardham & Blackman, 2018). Typical disease symptoms include brittle, blackened feeder roots, with little symptoms in the larger roots, occasional stem cankers, yellowing of the leaves, and often times dieback of young shoots (Erwin, D. C., and Ribeiro, 1996; Hardham, 2005; Hardham & Blackman, 2018; van den Berg *et al.*, 2021). In optimal conditions (wet/moist soil), asexual sporulation occurs where multinucleated sporangia release mononucleate biflagellate zoospores, which in turn encyst, germinate and eventually penetrate the host tissue through appressorium-like swelling (Hardham, 2005). Upon entry into the host tissue and within the apoplast space, during the biotrophic stages of infection, haustoria develop and form a host-microbe interface (Huisman *et al.*, 2015; Redondo *et al.*, 2015). These haustoria function in a myriad of activities, including the intake of nutrients and secretion of effectors to aid in the infection process (Panstruga & Dodds, 2009; Martin & Kamoun, 2011; Hardham & Blackman, 2018; van den Berg *et al.*, 2018, 2021). Effectors are key elements in disease establishment, however, despite their importance, the identity and characterization of *P. cinnamomi* effectors contributing to pathogenicity, virulence, and host adaptation are unknown.

Oomycete effectors and effectoromics

Oomycete effectors are secreted proteins categorized as either apoplastic or cytoplasmic depending on the compartments where they function and target their corresponding plant targets (Kamoun, 2007; Martin & Kamoun, 2011). Among the apoplastic effectors, some major classes include protease inhibitors, Nep1-like proteins (NLPs), elicitins, proteases, cell wall degrading enzymes, lipases and phospholipases (Jiang *et al.*, 2008; Haas *et al.*, 2009; Baxter *et al.*, 2010; Lévesque *et al.*, 2010; Martin & Kamoun, 2011). In *P. infestans*, some of the characterized apoplastic effectors inhibit plant defense proteins like serine proteases, cysteine proteases and ß-1,3-glucanases which aid in resistance and battling pathogens in the apoplast region (Jashni *et al.*, 2015). NLP effectors contain a distinct necrosis-inducing *Phytophthora* protein (NPP) domain which form a class of cell death-inducing proteins found across multiple pathogens including bacteria, fungi, and oomycetes (Martin & Kamoun, 2011). Elicitins are structurally conserved, small proteins that can induce strong plant immune responses when recognized by a host plant protein (Kawamura *et al.*, 2009; Schornack *et al.*, 2009). Elicitins are especially important for *Phytophthora* species as they bind to sterols and other lipids essential for oomycete growth and sporulation (Schornack *et al.*, 2009). In 1989, the elicitin cinnamomin was purified from *P. cinnamomi* and upon application to *Nicotiana tabacum*, induced cell death as well as displayed some protective qualities against *P. nicotianae* (Huet & Pernollet, 1989; Li *et al.*, 2020). One of the most popular elicitins, infestin-1 (INF1) was first identified in *P. infestans* and upon inoculation induced cell death when recognized in *Nicotiana* genus and other *Solanaceae* species

(Kamoun *et al.*, 1998; Schornack *et al.*, 2009). With elicitins producing such striking phenotypes, it certainly begs the question of "how do oomycete effectors suppress elicitin triggered responses?" (Schornack *et al.*, 2009) and if cell death triggered by elicitins will accelerate the shifting between the biotrophic to necrotrophic stages enhancing pathogen virulence.

Two major classes of cytoplasmic effectors include RxLRs and CRNs, both with conserved motifs in their N-terminal regions, necessary for translocation into the host cells (Martin & Kamoun, 2011; Hardham & Blackman, 2018; Leesutthiphonchai *et al.*, 2018). The conserved amino acid motif for the RxLR family include an arginine, any amino acid, leucine, and arginine (RxLR), and for the CRN effector family it includes leucine, phenylalanine, alanine, and lysine (LFLAK) (Martin & Kamoun, 2011; Hardham & Blackman, 2018). During plant colonization, these effectors tend to show distinct expression patterns as the pathogen transitions from biotrophic to necrotrophic stages (Martin & Kamoun, 2011). RxLRs with avirulence activity like *Avr1*, *Avr2*, *Avr3a*, *Avr4*, *Avrblb1*, and *Avrblb2* peak in expression after 2-3 dp infection (during the biotrophic phase), but then reduce to basal levels of expression as the necrotrophic phase begins to dominate (Haas *et al.*, 2009; Martin & Kamoun, 2011). Recently, RxLRs from *Pythium oligandrum* (PyolRxLR32), *Phythium periplocum* (PypeRxLR22, PypeRxLR24), and *Pythium ultimum* (PyulRxLR22) were identified and functionally verified (Ai *et al.*, 2020). PyolRxLR32, PypeRxLR22, and PypeRxLR24 induced defense responses in *N. benthamiana* leaves and PyulRxLR22 showed necrosis-inducing activity (Ai *et al.*, 2020). In the latest *P. cinnamomi* genome annotation, 181 proteins were predicted as

putative RxLRs effectors (Engelbrecht *et al.*, 2021) and around 60 predicted *P. cinnamomi* RxLRs were expressed during avocado root *P. cinnamomi* infection (Joubert *et al.*, 2021). Dai *et al.* (2020) reported that the *P. cinnamomi* RxLR effector, PHYCI_587572, can be used a biomarker for *P. cinnamomi* detections assays. In this report, the authors also report that this RxLR effector acts as a suppressor of cell death induced by BAX (pro-apoptotic protein), and INF1 in *N. benthamiana* (Dai *et al.*, 2020). This is the only report conducting a functional study for *P. Cinnamomi* effectors.

Hypotheses and Objectives

To combat successful pathogens like *P. infestans* and *P. cinnamomi,* discovery of novel components of plant immunity is critical. With its role in broad-spectrum immunity, MORC1 is considered a critical regulator of plant immunity, however, due to its multifaceted roles in chromatin remodeling and epigenetics, targeting MORC1 for transgenic crops may subject the crop to deleterious secondary effects. Since the C-terminal region of MORC1 is so critical in regulating their resistance functions (Manosalva *et al.*, 2015; Manohar *et al.*, 2017), the first hypothesis of this thesis is that protein interactors of the C-terminal region of MORC1 may be involved in plant immunity and be better targets for developing transgenic *P. infestans* resistant plants. Through yeast-two hybrid (Y2H) screening, a basic leucine zipper (bZIP) transcription factor (TF) was identified to physically interact with the C-terminal region of MORC1. This interaction was further confirmed *in planta* using coimmunoprecipitation (Co-IP) assays. To determine its role in plant immunity, the bZIP TF was silenced in *N.*

benthamiana and tomato and tested for changes in PTI-, ETI- induced cell death and susceptibility to *P. infestans.*

Phytophthora cinnamomi is one of the most invasive oomycetes threatening natural ecosystems and agriculture. Despite the importance of this pathogen, the molecular basis

of plant-*P. cinnamomi* interactions are unknown due to the lack of high-throughput pathosystems to conduct functional studies. The second part of this thesis is the utilization of the *N. benthamiana-P. cinnamomi* pathosystem to conduct effector functional studies. Under the hypothesis that effectors of *P. cinnamomi* that are highly expressed only *in planta* across multiple hosts after pathogen inoculation are important for pathogenicity/virulence, we selected several candidate effectors based on the above criteria from RNA-Seq studies conducted in *N. benthamiana*, Arabidopsis, and avocado at different times post inoculation using a detached leaf assay. The five candidate effectors were validated by quantitative real-time polymerase chain reaction (qRT-PCR) in *N. benthamiana* leaves and avocado roots after *P. cinnamomi* infection. After validation, candidate effectors were used in functional studies to test their contributions in *P. cinnamomi* pathogenicity and/or plant immunity.

Aim 1: Identify MORC1 interactors and determine their roles if any during plant immunity against *P. infestans*

P. infestans is a fast-evolving pathogen, with a two-speed genome, capable of overcoming resistant crop varieties at alarming speeds. Known as the "*R* gene destroyer", currently *P. infestans* is out pacing the development of *P. infestans* resistant plants (Fry,

2008). In this study a new candidate for resistance against *P. infestans* has been identified and shows great potential in generating the next *P. infestans* resistant cultivars.

Aim 2: Identify and validate candidate *P. cinnamomi* effectors differentially expressed during infection for their contribution to pathogenicity or virulence

The identification of effectors and determining their direct contribution to pathogenicity/virulence in plants is critical to elucidate the mechanism(s) of how they regulate plant immunity so they can be used to fetch plant targets to enhance resistance or break susceptibility. Apart from one study, there have been no reports of functional validation and characterization of *P. cinnamomi* effectors. This will be the first study reporting the functional validation of *P. cinnamomi* RXLRs and elicitins for their roles in pathogenicity.

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CHAPTER 2

Arabidopsis bZIP transcription factor 29 (DKML) homolog interacts with MORC1 and positively regulates plant immunity to *Phytophthora infestans* in Solanaceae

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Natasha A. Jackson, Ji C. Nam, Hong-Gu Kang, and Patricia M. Manosalva. Arabidopsis bZIP transcription factor 29 (DKML) homolog interacts with MORC1 and positively regulates plant immunity to *Phytophthora infestans* in Solanaceae. New Phytologist. 2022 *In preparation.*

Abstract

Phytophthora infestans, the Irish potato famine pathogen from the 1840s, remains a global threat to food security, causing massive reduction in crop production and expenditures in disease control worldwide. We showed previously that microrchidia 1 (MORC1) regulates pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) against *P. infestans* in Solanaceae and that the coil-coiled (CC) domain at the C-terminal region was required for MORC1 phenotypes on resistance and PAMP-triggered cell death. CC-domains mediate protein-protein interactions. Therefore, we hypothesized that the MORC1 protein regulates plant immunity by interacting with positive and regulators at its CC-domain.

The *Arabidopsis thaliana* (At) Drink Me-Like (DKML) basic leucine zipper (bZIP) transcription factor (TF) 29 homolog in tomato (Sl) interacts with the tomato MORC1 CC-domain and regulates plant cell death and resistance to *P. infestans*, based on gene silencing and transient expression analyses in *Nicotiana benthamiana* (Nb) and tomato. DKML and MORC1 interactions occur at their C-terminal domains demonstrated by yeast two-hybrid (Y2H) and coimmunoprecipitation (Co-IP) experiments. Like MORC1, DKML lacks a canonical nuclear localization signal (NLS), but localizes in the nucleus and plasma membrane.

In *N. benthamiana*, silencing *NbDKML* increased *P. infestans* susceptibility while its transient overexpression enhanced resistance. Cell death triggered by the *P. infestans* PAMP, infestin 1 (INF1), was compromised when *NbDKML* was silenced by virus-induced gene silencing (VIGS). Moreover, in tomato, silencing *SlDKML* compromised

hypersensitive response (HR) triggered by several pathogen effectors and their corresponding resistance R-genes including *Pto-AvrPto* interaction, which was found to be modulated by MORC1 previously. Together, these results argue that DKML directly interacts with MORC1 and act as a positive regulator of *P. infestans* resistance as well as PTI- and ETI- triggered cell death.

Introduction

Plants are constantly overcoming the pressures of fast-evolving plant pathogens. Oomycetes are the most devastating eukaryotic parasites. *Phytophthora infestans* is the causal agent of potato and tomato late blight and was responsible for the 1840s Irish potato famine and is a recurring threat to food security causing multibillion-dollar losses (Haverkort *et al.*, 2008). Disease control/management strategies against these oomycete pathogens remain unsustainable and argue for new control methods including the generation of crops with durable and effective resistance against *P. infestans*.

In addition to physical and chemical barriers, plants have developed complex defenses to fight off pathogens at a molecular level. Analogous to the animal innate immunity, plants can recognize PAMPs by their extracellular surface receptors or pattern recognition receptors (PRRs), resulting in an active defense response known as PTI (Ingle *et al.*, 2006; Jones & Dangl, 2006). PAMPs are slowly evolving signature-pattern molecules that are widely distributed amongst microbial species. Examples of such PAMPs include flg22 from bacteria, chitin from fungi, and elicitins such as INF1 from *P. infestans* (Kamoun *et al.*, 1998; Heese *et al.*, 2007; Chaparro-Garcia *et al.*, 2011).

Although sometimes PTI is sufficient to prevent further colonization of harmful microbes, other times, pathogens have evolved effectors that can suppress PTI. If the plant has evolved R proteins that can either directly or indirectly target these effectors, then the plant can induce ETI, restricting the pathogens ability to spread throughout the plant by inducing programmed cell death or HR (Jones and Dangl, 2006; Muthamilarasan and Prasad, 2013; Katagiri and Tsuda, 2010). Several effectors have been isolated from bacteria, fungi, and oomycetes. In the gram-negative bacterial pathogen *Xanthomonas campestris pv. vesicatoria,* the avirulence gene, *avrBs3* is secreted by way of the type III secretion system (Marois *et al.*, 2002; Scheibner *et al.*, 2017). When AvrBs3 is inoculated in pepper plants containing the R protein Bs3, a clear ETI-related response is observed as evidenced by HR (Herbers *et al.*, 1992; Scheibner *et al.*, 2017). In the agent of bacterial speck disease, *Pseudomonas syringae*, strains that contain the effector AvrPto can be recognized by plants that express the R protein, Pto. When AvrPto is delivered into the plant cell, Pto directly interacts with AvrPto (Tang *et al.*, 1996; Bogdanove & Martin, 2000) and in conjunction with another R protein, Prf, confers resistance to *P. syringae* pv *tomato* by inducing HR (Martin *et al.*, 1993; Salmeron *et al.*, 1996).

MORC proteins belong to the GHKL (Gyrase, Hsp90, Histidine Kinase, and MutL) ATPases superfamily, containing a GHKL ATPase domain and an S5 fold domain. GHKL proteins play roles in chromatin remodeling, heat shock response, signal transduction, and DNA mismatch repair in animal and plants (Iyer *et al.*, 2008; Bordiya *et al.*, 2016; Manohar *et al.*, 2017; Kim *et al.*, 2019; Bhadouriya *et al.*, 2021). In addition to MORC's role in gene silencing, MORC family members are also critical regulators of

several layers of plant immunity, including non-host resistance, PTI, ETI, and systemic acquired resistance (SAR) against viral, bacterial, fungal, and oomycete pathogens (Kang *et al.*, 2010, 2013; Langen *et al.*, 2014; Manosalva *et al.*, 2015)**.** Previous studies have shown that MORC1 is required for INF1-induced cell death (Manosalva *et al.*, 2015), and HR induced by bacteria, viruses, and oomycete effectors and their corresponding R gene including HR mediated by AvrPto-Pto (Kang *et al.*, 2010).

The MORC1 C-terminal region, containing a disordered structural domain (2nd linker/L2) and a coiled-coil (CC) domain (Iyer *et al.*, 2008), is responsible for the regulation of PTI-induced cell death (Manosalva *et al.*, 2015), is phosphorylated, and is required for its DNA-modifying activities and DNA-mediated stimulation of SlMORC1 ATPase activity (Kang *et al.*, 2013; Manohar *et al.*, 2017). Moreover, we have previously shown that SlMORC1 ATPase activity it is required for INF1-triggered cell death in *N. benthamiana* (Manohar et al, MPMI, 2018). While an affinity purification with the SET domain-containing SU(VAR)3–9 homologs, SUVH2 and SUVH9, identified MORC1 and its homolog MORC6 (Liu *et al.*, 2016), no systematic screening for MORC1-interacting proteins has been carried out despite the importance of MORC1 in plant immunity. The dual roles of MORC1 in regulating several layers of broad-spectrum resistance and chromatin stability questions its utility as a desirable gene to generate resistant crops without affecting other important phenotypic epigenetic traits.

This study started with the identification of *A. thaliana* MORC1-interacting proteins (AtMIPs) via a yeast-two-hybrid screening and characterized one of the MIPs that regulate plant immunity to *P. infestans* in Solanaceae. We investigate the possibility

that these AtMIPs also interact with full-length and the C-terminal regions containing the CC-domain of tomato (Sl) and potato (St) MORC1 proteins using Y2H. We found that only a subset of these 14 AtMIPs interact with SlMORC1 and StMORC1 (Sl/StMORC1). This study focused on one AtMIP which corresponds to an Arabidopsis bZIP TF 29 (AtDKML) which physically interacts with the Sl/StMORC1 C-terminal region. Previous studies of AtDKML report DKMLs function in *A. thaliana* as a TF containing a bZIP domain with involvement in plant development (Lozano-Sotomayor *et al.*, 2016; Van Leene *et al.*, 2016). Further characterization of DKMLs involvement in plant immunity was explored. These analyses reveal that the MORC1 protein can directly interact with a bZIP TF protein. Specifically, this interaction was driven by the C-terminal region of MORC1 and the C-terminal region of the bZIP TF. Though the identification of bZIP TF family proteins has been established in several plant species, only a handful of these bZIP TFs have been extensively studied at the biochemical, molecular, and functional level. In this study, we identified a bZIP TF that plays a role in broad spectrum plant immunity. Using virus induced gene-silenced *N. benthamiana* and tomato plants we were able to test this bZIP TF's effects on PTI, ETI and resistance to *P. infestans.*

Our findings support our hypothesis that DKML and MORC1 regulate plant immunity and cell death via the same pathway. To our knowledge, this is the first study reporting the role of this specific member of the bZIP TF family on regulating plant immunity. Highlighting the potential of using DKML as target to develop P. infestans resistant solanaceous crops.

Material and Methods

Plant material and growth conditions.

N. benthamiana plants were grown under cycles of 16 h of light and 8 h of darkness at 22°C with 70% relative humidity. When preparing *Solanum lycopersicum* cv. M82 plants for VIGS, seeds were germinated and grown under cycles of 16 h of light and 8 h of darkness at 22°C with 70% for 12 days, then inoculated with VIGS constructs and subjected to cold temperatures at 20°C in the growth chamber under cycles of 16 h of light and 8 h of darkness with 70% relative humidity. After 4 weeks, plants were transferred to increased temperatures at 22°C under cycles of 16 h of light and 8 h of darkness with 70% for two weeks before inoculation experiments were administered. For maintenance of *P. infestans* and nucleic acid extractions, *S. lycopersicum* cv. Rutgers and *S. tuberosum* cv. Désirée seeds were germinated and grown under cycles of 16 h of light and 8 h of darkness at 22°C with 70% for 12 days, then transferred to glasshouse conditions at temperatures averaging 25 to 28°C at 40 to 50% relative humidity.

Yeast two-hybrid screen.

Systematic identification of MIPs was carried out in Y2H screening by Hybrigenics (France) using a cDNA library prepared from 1-week-old Arabidopsis seedlings. A FL coding sequence of *AtMORC1* was cloned into pB27 as a C-terminal fusion to the LexA DNA-binding domain, which was used as a bait for the screening. Fourteen cDNA clones in pP6, identified as MIPs, were provided at the conclusion of the screening by Hybrigenics.

pB27 bait and pP6 prey plasmid were transformed into Y187 and L40 yeast strains, respectively. The minimum SD media (-Trp and -Leu) containing histidine was used to show the growth, while those without were used to test the interaction between prey and bait. Sequences of cDNAs of Sl/StMORC1 were fused with GAL4-BD in the pB27 vector and were used as bait. The pB27 constructs were transformed into the yeast strain L40 and grown on the SD medium minus tryptophan (SD-Trp). Sequences of cDNAs of DKML were fused with GAL4-AD in the pP6 vector and were used as prey. The pP6 constructs were transformed into the yeast strain Y187 and grown on the synthetic dropout medium minus leucine (SD-Leu). All yeast transformations were performed using the Zymo Frozen-EZ Yeast Transformation Kit (T2001) following the manufacture's protocol. For yeast mating, positively interacting yeast strains were obtained by following Clontech Yeast Protocols (Clontech, 2009). Mated cells were plated on SD media lacking leucine, tryptophan, and histidine (SD-Leu/-Trp/-His).

Vector construction.

All oligonucleotides used for cloning and plasmid construction in this study are listed in Table S2.1. For Y2H cloning, the following PCR products were amplified: NbDKML1A$_{143\text{-}588}$ fragment using primer pairs 7/8, SlDKML$_{124\text{-}475}$ and SlDKML$_{124\text{-}372}$ fragments using primer pairs 9/10and 9/13, and StDKML$_{125\text{-}475}$ and StDKML$_{125\text{-}372}$ fragments using primer pairs 11/12 and 11/13. SlDKML$_{124\text{-}475}$ and StDKML$_{125\text{-}475}$ contained most of the gene, which included the leucine zipper region and one CC-domain. SlDKML$_{124\text{-}372}$ and StDKML$_{125\text{-}372}$ did not contain the C-terminal region, truncating the leucine zipper region and CC-domains. The resulting PCR products were

digested with *BamHI* and *XhoI* and cloned in the prey vector pP6 (Zhao *et al.*, 2020). Additionally, Arabidopsis, tomato, and potato MORC1 PCR products were amplified as follows: AtMORC1 using primer pairs 14/15 (Ji Chul Nam, 2020)SlMORC1 and StMORC1 using primer pairs 16/17, SlMORC1$_{475\text{-}644}$ and StMORC1$_{475\text{-}644}$ using primer pairs 17/18, and SlMORC1$_{485\text{-}644}$ and StMORC1$_{485\text{-}644}$ using primer pairs 17/19. SlMORC1$_{FL}$ and StMORC1 contained the full-length coding sequence of the MORC1 gene, which included the ATPase domain, the first linker (L1) domain, S5 fold, followed by the second linker (L2) and CC-domain. SlMORC1$_{475\text{-}644}$ and SlMORC1$_{475\text{-}644}$ did not contain the N-terminal region, truncating the ATPase and L1 domains. SlMORC1$_{475\text{-}644}$ and SlMORC1$_{475\text{-}644}$ did not contain the N-terminal region, truncating the ATPase and L1 domains. SlMORC1$_{485\text{-}644}$ and SlMORC1$_{485\text{-}end}$ constructs did not contain the S5 fold domain, in addition to the ATPase and L1 domains. The resulting PCR products were digested with *EcoRI* and *SpeI* to be cloned in the bait vector pB27 (Zhao *et al.*, 2020) to conduct the Y2H experiments.

To generate the β-estradiol-inducible over expressing (OE) constructs SlDKML$_{124\text{-}474}$ used for Co-IP experiments, cDNAs encoding SlDKML was amplified using primers 20/21 (Table S2.1). The resulting PCR products were digested using *AvrII* and *SpeI* and cloned in the pER8:myc vector (Manosalva, 2015).

To generate VIGS constructs, a multiple sequence alignment of *NbDKML1A, NbDKML1B, NbDKML2A*, *NbDKML2B* was generated using MultAlin (Corpet, 1988). The Tobacco rattle virus (TRV2) *NbDKML1&2* construct was generated using primers 22/23 and PCR products were digested with *XbaI* and *BamHI.* TRV2:*SlDKML* construct

to perform VIGS in tomato was generated using primer pairs 24/25 and PCR products were digested using *EcoRI* and *BamHI.* Finally, digested PCR products were cloned in the pTRV2/YL156 (Burch-Smith *et al.*, 2006) digested with *EcoRI* and *BamHI.*

To generate myc and YFP tagged constructs, gateway entry clones encoding SlDKML and StDKML FL were constructed by PCR amplification using primer pairs 26/27 that contained portions of the attB1 and attB2 sites. A second round of PCR amplification was conducted utilizing primer pairs 28/29, resulting in a final PCR product with the full integrity of the attB1 and attB2 sites. Gel-purified PCR products were introduced into pDONR221 (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) using BP Clonase II (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Recombinant entry clones were verified by restriction digest analysis and sequencing. LR recombination reactions were performed according to the manufacturer's instructions, mixing 1 µl of either pGW517-myc (150ng) or pGW541-YFP (150ng) 1 µl of entry clone (150ng), and 1 µl of LR-clonase II (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) to generate SlDKML C-terminally tagged with YFP. All oligonucleotides used for cloning are listed in Table S1.

DKML homologs phylogenetic analysis.

Full-length protein sequences corresponding to the 78 reported Arabidopsis bZIP TFs including AtDKML (Dröge-Laser *et al.*, 2018) were obtained from the Arabidopsis Information Resource (TAIR) on www.arabidopsis.org. The evolutionary history of these AtbZIP TFs were inferred using the neighbor-joining method (Saitou and Nei 1987) and was conducted in MEGAX (Tamura et al. 2011). A bootstrap consensus tree was inferred

from 1000 replicates. The evolutionary distances were computed using the Poisson correction method. The analysis involved 79 amino acid (aa) sequences corresponding to all the AtbZIP TF proteins. To determine the evolutionary history of the solanaceous DKML homologs, only the most closely related AtbZIP TF to AtDKML were used and phylogenic analyses were conducted as indicated above. The DKML homolog from moss (*Physcomitrium patens*) was used as an outgroup for the generation of the rooted phylogenetic using MEGAX.

Identification of Solanaceae DKML homologs.

To identify bZIP29 (DKML) homologs in tomato, DKML cDNA sequence from *A. thaliana* (At4g38900) was used as query for a tBLASTx search with the *S. lycopersicum* genome (ITAG4.0) available in the Sol Genomics Network (SGN) database. The resulting tomato *DKML* homolog (*SlDKML*) was then used for tBLASTx analysis against potato (version 3) and *N. benthamiana* (version 1.0.1) genome sequences. To confirm the *SlDKML* and *StDKML* homologs cDNA sequences obtained *in silico*, total RNA from *S. lycopersicum* cv. Rutgers and *S. tuberosum* cv. Désirée were extracted using TRIzol (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) and following the manufacturers guide. SuperScript III First-Strand Synthesis System (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) was used to synthesize cDNA following the manufacturer protocol. To clone FL sequences, primers were designed using Primer 3.0 (Koressaar & Remm, 2007) and used to amplify the DKML homologs in Solanaceae. RACE 5’ to 3’ and Sanger Sequencing analyses were conducted to confirm the 5’ and 3’ ends of DKML homologs using SMARTer RACE 5’/ 3’ kit

(TaKaRa, San Jose, CA, USA) following the manufacturer's protocol. Primer sequences used for RACE (Primer pairs 1-6) are shown in Supporting Information Table **S2.1**. Resulting PCR products were purified using QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) and sent for sequencing to Eurofins, Louisville, KY. DKML protein domains were identified with the Pfam and Interpro database (Mistry *et al.*, 2021). Phosphorylation sites DKML homologs were predicted using netphos-3.1b (Blom *et al.*, 1999). DKML homologs subcellular localization and identification of canonical and non-canonical NLS sites were predicted using cNLS mapper (Kosugi *et al.*, 2009), NucPred (Brameier *et al.*, 2007), and BaCelLo (Pierleoni *et al.*, 2006).

Agrobacterium-mediated transient expression in N. benthamiana and tomato.

Agrobacterium tumefaciens strain GV3101 was used for *N. benthamiana* and for tomato. Leaves of 4-week-old *N. benthamiana* plants were infiltrated with *A. tumefaciens* transformed with the different constructs in this study (Table 2.1) using 10 mM $MgCl_2$, 10 mM MES [pH5.5], and 200 µM acetosyringone. Optical density (OD) for each construct were adjusted as follows: MORC1 at 0.2; INF1, AvrBs3 and Bs3, and AvrPto and Pto at 0.15; $SlDKML_{124-475}$ and SlDKML at 0.7; and EV-GFP at 0.3. These Ods were selected based on immunoblot analysis at 2 and 3 dp estradiol induction to ensure similar levels of protein expression among all constructs. For the expression of proteins under β-estradiol inducible promoter, a solution of 30µM ß-estradiol and 0.01% tween-20 was sprayed on the leaves 1 day post agroinfiltration. Leaves expressing pGW541:SlDKML-YFP were infiltrated 1 day post agroinfiltration with proteosome inhibitor, MG132 (50 µM) (Supplier AdooQ).

Fluorescence spectrometry.

Confocal images were collected on a Leica TCS-SP5 confocal microscope as described (Kang *et al.*, 2010) in CFAMM at UC Riverside. GFP was excited with an argon laser (488nm), and emitted light was collected between 500 and 520nm. Images were processed using Leica LAS-AF software (version 3.1). Mean fluorescence intensity (MFI) was measured using ImageJ v1.53. Ten identical circles with an area of 0.078 marked ten different locations within each image and detected the MFI within each designated area.

Subcellular fractionation.

Enrichment of plant nuclei was performed as previously described (Chaturvedi et al., 2014) with the following modifications: ~4g of *N. benthamiana* leaves were homogenized in 10 ml of extraction buffer (50 mM Tris-HCl [pH 7.5], 5 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol [DTT], 0.3 M sucrose, 15 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 0.4% Triton-100, and 1 protease inhibitor mini tablet (Pierce) at 4°C with a mortar and pestle. Debris was removed by centrifugation at 300g. The supernatant was then filtered twice through a single layer of Miracloth (EMD Millipore) to obtain the total input. Nuclei were enriched by centrifugation at 5,000rpm for 10min at 4°C while the supernatant served as nucleus-depleted fraction.

Co-immunoprecipitation and immunoblot analyses.

Two to eight leaf discs were collected at 2dp or 1dp estradiol induction. Crude extracts were obtained by grinding the tissue in 50ul of 2X SDS buffer. Co-Ips and

immunoblots (Ibs) were performed as described (Kang *et al.*, 2008). Protein loading was checked by visualizing the large subunit of RuBPCase by staining the polyvinylidene fluoride membrane (Millipore) with Coomassie Brilliant Blue. Signals were detected by a chemiluminescence reaction using the ECL Prime Western Blotting Kit (Cytiva). Monoclonal anti-HA-Tag (Santa Cruz) antibody was used at a dilution of 1:5000. Monoclonal anti-c-Myc (Santa Cruz) antibody was used at a dilution of 1:500. Polyclonal anti-Histone H3 (Agrisera), polyclonal anti-Cytosolic fructose-1, 6-bisphosphatase (Agrisera), and secondary antibody conjugated to HRP, goat anti-Rabbit (Agrisera), were used at a dilution of 1:10,000.

VIGS in N. benthamiana and tomato.

For VIGS, the TRV2:EV constructs as described (Kang *et al.*, 2010), as well as a TRV2:*EC1* negative control construct described by Chakravarthy and associates (2010) were used. VIGS in *N. benthamiana* was performed as described (Velásquez *et al.*, 2009). Four weeks post VIGS, *N. benthamiana* and tomatoes were used for pathogen inoculations and *Agrobacterium*-mediated transient expression. The primers used for cloning *NbDKML1&2* and *SlDKML* were described above and listed in Table S1.

qRT-PCR analysis.

Silencing was tested by performing quantitative real-time PCR (qRT-PCR) using iQ SYBR Green mix (Bio-Rad) using gene-specific primer pairs for *NbDKML1A* (30/31), *NbDKML2A* (30/31), and *SlDKML* (33/35). Control reactions to normalize qRT-PCR amplifications were run with primer pairs for the constitutively expressed *translation elongation factor 1α* gene from *N. benthamiana* (36/37) and tomato (38/39). Total RNA

and cDNA synthesis were isolated as described above. *P. infestans* biomass was quantified using PiO8 primer pairs 40/41 as described in (Llorente *et al.*, 2010) to quantify *P. infestans* DNA.

Trypan blue staining.

Leaf tissue was processed 3dp INF1 inoculation as described in (Manosalva, 2015).

Pathogen inoculations.

P. infestans strain 1306 were maintained on tomato leaves under a cycle of 16h of light and 8 h of darkness at a temperature of 18°C. *P. infestans* strain 1306 was used as a suspension of 20,000 sporangia/ml for *N. benthamiana* inoculations. Detached-leaves were inoculated with 20µl droplets of the *P. infestans* suspension and incubated under a cycle of 16hrs of light and 8hrs of darkness at 18°C for 24hrs. Droplets were removed and pathogen infection was assessed and measured over a 2-week period.

Results

A Y2H screening identified 14 MORC1 interaction proteins

While MORC1 and its homologs have been shown to play important roles in plant immunity, a systematic screening for MIPs has not been performed. Thus, a conventional Y2H screen for MIPs were performed by using AtMORC1 as a bait, resulting in 14 candidates listed in Table 2.1. To confirm the physical interaction, AtMORC1 and candidate 14 MIPs were retested in a targeted Y2H assay and grown on SD -/Leu/-Trp/+His media or SD -/Leu/-Trp/-His selection media (Fig. 2.1a). AtMORC1, when used as a bait, interacted with all 14 AtMIP clones identified in the screen as indicated by

growth on SD -/Leu/-Trp/-His selection media. MORC1 showed a weak interaction with itself, indicating homodimerization. MIP6 showed an autoactivation with an empty bait vector, suggesting that MIP6 may carry a transactivation domain and bind to the reporter.

Interaction of MIPs with tomato and potato MORC1

Tomato (*S. lycopersicum*) and potato (*S. tuberosum*) MORC1 possesses about 70% aa homology to AtMORC1, and their C-terminal domains, which is required for homodimerization and phosphorylation, are considerably divergent from each other (Manosalva *et al.*, 2015). StMORC1 shares 96% aa identity and 98.5% similarity with SlMORC1. The C-terminal region of MORC1 proteins containing a second linker (L2) and a CC-domain is required for their enzymatic functions and cell death regulation in plants (Manosalva *et al.*, 2015; Manohar *et al.*, 2017). Considering that the L2 of MORC1 is a disordered region, we designed two constructs, one including the S5 fold domain (St/SlMORC1$_{475\text{-}644}$) to increase the protein stability and a second construct containing only the L2 and CC-domains (St/SlMORC1$_{485\text{-}644}$) (Fig. 2.1b). To gain insight into the role of MORC1 in solanaceous, StMORC1 and SlMORC1s were tested for interaction with the 14 AtMIPs. AtMIP3 (better known as MORC6) displayed a notable interaction with solanaceous MORC1, excluding autoactivation from MIP6 (Figure 2.1c). Poor interaction of SlMORC1 and StMORC1 with the 14 MIPs overall suggests that many MIP counterparts in solanaceous have diverged from those in Arabidopsis. In addition to MIP3 (AtMORC6) proteins (positive controls), the C-terminal regions of St/SlMORC1, but not with their corresponding FL proteins showed interaction with two

AtMIPs: AtMIP10 and AtMIP12 (Fig. 2.1c). AtMIP10 was identified as the bZIP TF 29 (DKML, At4g38900) and was further investigated in this study.

Identification of DKML homologs from solanaceous genomes

In *A. thaliana*, 78 bZIP TFs have been previously reported (Dröge-Laser *et al.*, 2018). A phylogenetic tree was generated, grouping all 78 AtbZIP TFs in eight different clades (Fig. S2.1). To identify the solanaceous AtDKML homologs, the tomato (*S. lycopersicum*) genome sequence was scanned with the AtDKML cDNA sequence. The resulting tomato homolog cDNA sequence was then used to capture the corresponding potato and *N. benthamiana* homologs. One AtDKML homolog was identified in the tomato (Solyc01g110480) and one in the potato (PGSC0003DMP400002986) genome sequence used in this study. *N. benthamiana*, which is an allotetraploid species resulting from the hybridization of two unknown progenitors (Bombarely et al. 2012), appears to have two DKML homologs (NbDKML 1 and 2) each of them having two different alleles (A and B), which presumably are derived from the two progenitor lines resulting in a total of 4 NbDKML homologs (Niben101Scf00466g04027 [NbDKML1A], Niben101Scf10919g01034 [NbDKML1B], Niben101Scf01852g0403 [NbDKML2A], and Niben101Scf18662Ctg00010 [NbDKML2B]) (Fig. S2.2**)**. To further validate the AtDKML solanaceous homologs, a phylogenetic tree was constructed using the 11 closest bZIP TFs to AtDKML (clade II) (Fig. S2.1) and the solanaceous bZIP AtDKML homologs from tomato, potato, and *N. benthamiana* (Fig. S2.2). As expected, AtDKML grouped together with all DKML homologs from Solanaceae, the DKML homolog from

moss (PpDKML), and the closest bZIP TF in Arabidopsis (At2g21230.1) and their corresponding homologs from Solanaceae (Fig. S2.2).

Sequence analysis indicated that AtDKML shares 57% and 58% aa identity and 68% and 69% amino acid similarity with SlDKML and StDKML, respectively. In addition, SlDKML and StDKML exhibited 98.1% and 98.3% aa identity and similarity, respectively. There are only 4 conservative and 2 nonconservative aa differences between these proteins at the N-terminal region and a 5-aa insertion at the C-terminal region of the SlDKML where a second CC-domain is not predicted when compared with the StDKML (Fig. S2.2 and Fig. 2.2). In addition, SlDKML shares 88% and 93% aa identity and similarity, respectively, with NbDKML1A; SlDKML and NbDKML1B shares 75% and 79% aa identity and similarity, respectively; SlDKML and NbDKML2A shares 79 and 84% aa identity and similarity, respectively; and SlDKML shares 78% and 85% aa identity and similarity, respectively (Fig. S2.3, and Fig. 2.2).

The protein structure for AtDKML bZIP TF includes a canonical NLS at the 298 aa position and the basic region/leucine zipper (bZIP) domain (392-463 aa) embedded with a single CC-domain at its C-terminal region (425-473 aa) (Fig. 2.2). Interestingly, the solanaceous homologs did not have canonical NLS sequences, however they do have a putative non canonical NLS predicted with low scores as described in the methods (Fig. 2.2). Similar to AtDKML, all homologs from Solanaceae possess one bZIP domain containing one CC-domain. Apart from NbDKML1B, all the others solanaceous homologs contain one or two (StDKML) extra CC-domains after the bZIP domain when compared with AtDKML protein (Fig. 2.2).

DKML is localized in plasma membrane and the nucleus

To gain a deeper understanding in DKML function, its subcellular location was investigated using transient expression of a SlDKML-YFP fusion gene in *N. benthamiana*. NLS sites were predicted, however, canonical sites were not found. cNLS mapper gave SlDKML a score of 3-4 for bipartite NLSs, suggesting that it is found in both the cytoplasm and the nucleus (Kosugi *et al.*, 2009). NucPred gave SlDKML a score of 0.78, meaning that over 81% of other proteins with similar scores were found in the nucleus (Brameier *et al.*, 2007). Another NLS predictor software, BaCelLo, simply suggested that SlDKML was most likely in the nucleus (Pierleoni *et al.*, 2006). Interestingly, similar to AtMORC1(Kang *et al.*, 2010, 2013), SlDKML-YFP, lacking of a canonical NLS, resides within the plasma membrane and nucleus (Fig. 2.3a) revealed by confocal microscopy and subcellular fractionation (Fig. 2.3b).

The C-terminal region of Solanaceae DKML homologs is required for MORC1 interaction

To assess whether Sl/StMORC1 interacts with DKML homologs from Solanaceae, a Y2H assay was performed using FL and C-terminal regions of Sl/StMORC1 as baits and DKML constructs as prey (Fig. 2.4, Fig. S2.4a). Yeast transformed with bait and prey vectors were grown on the SD -/Leu/-Trp/+His media to indicate the presence of both constructs (Fig. 2.4b, c, d). The prey vector, AtMORC6, was used as a positive control, interacting with all variations of the MORC1 bait constructs (Fig. 2.4b). Identical to the interaction observed with AtDKML and MORC1

from Solanaceae, yeast grew on SD -/Leu/-Trp/-His selection media that were coexpressing SlDKML$_{124-475}$ and the C-terminal regions of Sl/StMORC1 and the full-length of AtMORC1, indicating physical interaction between these proteins (Fig. 2.4b). No interaction was observed between SlDKML$_{124-475}$ and the Sl/StMORC1 FL proteins (Fig. 2.4b) as indicated by the absence of growth on the SD -/Leu/-Trp/-His selection media. Ten-fold serial dilutions revealed a stronger interaction between SlDKML$_{124-475}$ and C-terminal Sl/StMORC1 containing a portion S5 fold, L2 and CC-domain (St/SlMORC1$_{474-644}$) compared to the C-terminal Sl/StMORC1construct containing only the L2 and CC-domain (St/SlMORC1$_{485-644}$) (Fig. 2.4c).

Many members of the bZIP family of TFs interact with their corresponding targets via their CC-domains (Newman & Keating, 2003), thus, the CC-domain of DKML at its C-terminal region was hypothesized to be the driving force of the DKML interaction with MORC1. To test this hypothesis, a second SlDKML construct lacking the CC-domain at the C-terminal region (SlDKML$_{124-374}$) was generated (Fig. 2.4a). When truncated, an absence of growth on SD -/Leu/-Trp/-His selection media was observed in yeast coexpressing the truncated SlDKML$_{124-374}$ and the C-terminal regions of SlMORC1, indicating a loss of the interaction (Fig. 2.4d). To test the physical interaction between StDKML and MORC1, DKML constructs from potato (StDKML$_{139-475}$) were coexpressed in yeast with FL At/Sl/StMORC1 and C-terminal regions of Sl/StMORC1 bait vectors (Fig. S2.4a). Similar to SlDKML, StDKML constructs also interact with FL AtMORC1 and the C-terminal regions of Sl/StMORC1 (Fig. S2.4b). This interaction was lost when the CC-domain of the StDKML was truncated (Fig.

S2.4c). Together these results argue that DKML proteins directly interact with the C-terminal region of Sl/StMORC1 proteins via their CC-domains.

SlDKML and SlMORC1 are present in the same protein complex *in planta*

To determine if SlDKML and SlMORC1 interact *in planta*, these proteins were tagged with myc and HA epitopes and then co-expressed in *N. benthamiana via Agrobacterium*-mediated transient expression. The same fragments used in the Y2H assays (SlDKML$_{124-475}$, FL SlMORC1, and SlMORC1$_{485-644}$) were cloned into the inducible pER8-myc or pER8-HA vector, respectively, in which the ß-estradiol-responsive regulatory element of this plasmid controlled the expression of these proteins. The FL pER8:SlMORC1-HA and pER8:SlMORC1$_{474-644}$-HA were pulled down from the total leaf extracts using anti-HA-Agarose beads and the immunoprecipitants were subjected to immunoblot analysis with anti-HA and anti-myc antibodies. As expected, a band size of 68 kDa, corresponding to the pER8:SlDKML$_{124-475}$-myc protein was detected in leaves coexpressing pER8:SlMORC1$_{485-644}$-HA (28 kDa) (Manosalva *et al.*, 2015) and pulled down with HA-tagged agarose beads indicating positive physical interaction *in planta* (Fig. 2.5). Additionally, pER8:SlDKML$_{124-475}$-myc protein was detected in leaves coexpressing FL pER8:SlMORC1-HA (100 kDa) (Manosalva *et al.*, 2015) and pulled down with HA-tagged agarose beads also indicating positive physical interaction *in planta* (Fig. 2.5). Previously, we reported that MORC1 homodimerized, thus we used this interaction as a positive control (Manosalva *et al.*, 2015). As expected, pER8:SlMORC1-myc protein was pulled down in the positive control leaves coexpressing pER8:SlMORC1-HA and pER8:MORC1-myc (Fig. 2.4). Contrarily,

pER8:SlDKML$_{124-475}$-myc protein was not pulled down in the negative control leaves coexpressing pBIN:GFP-HA and pER8:SlDKML$_{124-475}$-myc, (Fig. 2.5). Together, these results indicate that DKML and MORC1 proteins are present in the same protein complex *in planta*.

Silencing *DKML* homologs in *N. benthamiana* compromised PAMP- triggered cell death

Before assessing the role of DKML in plant immunity through transient experiments in *N. benthamiana*, we first determined whether the *N. benthamiana* DKML homolog also interacted with Sl/St MORC1 proteins using a Y2H assay. Y2H assay was performed using FL and C-terminal regions of Sl/StMORC1 as baits and NbDKML1A$_{143-588}$ containing two CC-domains as prey vector (domains highlighted in Fig. S2.5). Yeast transformed with bait and prey vectors were grown on the SD -/Leu/-Trp/+His selective media to indicate the presence of both constructs (Fig. 2.6a). The prey vector, AtMORC6, was used as a positive control, interacting with FL At/Sl/StMORC1 and C-terminal regions of Sl/StMORC1 bait vectors (Fig. 2.6a). These results indicated that DKML homolog in *N. benthamiana* also physically interact with MORC1 C-terminal proteins.

To determine the role of DKML in basal resistance, *DKML* homologs in *N. benthamiana (NbDKML1A, NbDKML1B, NbDKML2A,* and *NbDKML2B)* were silenced by VIGS using a single silencing construct designed in a highly conserved region of the genes (Fig. S2.5). *NbDKML1A/B* and *NbDKML2A/B* gene expression was significantly reduced in the TRV2:*NbDKML* silencing plants when compared with the TRV2:*EC1* non silencing control plants (Fig. 2.6b). In *N. benthamiana,* upon the application of the

elicitin, INF1, a PAMP from *P. infestans*, defense responses are triggered, inducing accumulation of reactive oxygen species and cell death (Kamoun *et al.*, 1998; Bos *et al.*, 2006). TRV2:*NbDKML* and TRV2:*EC1* leaves were infiltrated with INF1, and compromised INF1-induced cell death was observed at day 3 in the TRV2:*NbDKML* plants when compared with the TRV2:*EC1* controls (Fig. 2.6c, d). These results indicate that similarly to MORC1, DKML was also required for the INF1-induced cell death in *N. benthamiana*.

Silencing DKML homologs in *N. benthamiana* compromised ETI- triggered cell death

Since DKML was important for INF1-induced cell death, we wanted to investigate whether DKML, like MORC1, played a role in regulating HR triggered by ETI. To do this, Pto and Bs3 resistant proteins were co-expressed with their corresponding AvrPto and AvrBs3 effector proteins in the TRV2:*EC1* control plants and in the TRV2:*NbDKML* silenced plants. Both Bs3-mediated and Pto-mediated HR were compromised on day 3 and day 4 in the TRV2:*NbDKML* silenced plants compared with the TRV2:*EC1* control plants (Fig. 2.7a,b). GFP and INF1 were used as negative and positive controls for cell death in this experiment.

Pto confers ETI to *Pst* in tomato and MORC1 was previously shown to regulate ETI against *Pst* in *N. benthamiana* and tomato (Kang *et al.*, 2010). To confirm the role of *SlDKML* during ETI against *Pst* in tomato, the *SlDKML* gene was silenced by VIGS using a 300 bp region at the 5’ end of the gene (Fig. S2.7). *SlDKML* expression was significantly reduced in the TRV2:*SlDKML* silenced tomato plants when compared with

the TRV2:*EC1* control tomato plants (Fig. 2.8a). Consistent with our results in *N. benthamiana*, tomato TRV2:*SlDKML* silenced plants showed a significant delay in HR mediated by Pto at 4 dpi compared to TRV2:*EC1* control tomato plants (Fig. 2.8b). Together our results in *N. benthamiana* and tomato argue that DKML plays an important role in cell death triggered also by ETI.

MORC1 interactor, DKML, is a positive regulator for disease resistance to *Phytophthora infestans*

Previously we have shown that MORC1 regulates plant immunity to *P. infestans* in solanaceous crops (Manosalva *et al.*, 2015). Thus, we determine if the MORC1 interactor, DKML, also plays a role during resistance to *P. infestans* in *N. benthamiana*. To do this, TRV2:*NbDKML* silenced *N. benthamiana* plants were used in a detached-leaf *P. infestans* inoculation assay. Six days after inoculation with the oomycete pathogen *P. infestans*, TRV2:*NbDKML* silenced plants developed significantly larger lesions when compared with the TRV2:*EC1* controls plants (Fig. 2.9a, b). To further confirm this result, *P. infestans* biomass was determined by quantifying the *P. infestans*-specific *PiO8* biomass marker gene at 4 and 6 dpi (Llorente *et al.*, 2010). Significantly higher levels of *PiO8* was detected in TRV2:*NbDKML* silenced plants than in TRV2:*EC1* control plants (Fig. 2.9c, d) indicating higher pathogen accumulation in plants with reduced levels of *DKML*.

In agreement with its role as a positive regulator of *P. infestans* resistance, transient expression of SlDKML-myc in *N. benthamiana* enhanced resistance to this oomycete pathogen (Fig. S2.7). *Nicotiana benthamiana* leaves were inoculated with *P.*

infestans using a detached leaf assay 24 hours after agroinfiltration with either SlDKML-myc or GFP expression constructs (Fig. S2.7a). At 10 dpi with *P. infestans*, leaves transiently expressing SlDKML had significantly smaller lesions compared to control GFP plants (Fig. S2.7b, c). This enhancement of resistance in the plants transiently expressing SlDKML correlates with the lower biomass of *P. infestans* observed in these plants when compared with GFP controls (Fig. S2.7d). These results indicate that DKML positively regulates resistance to *P. infestans* in *N. benthamiana.*

To investigate how DKML positively regulates plant immunity to *P. infestans* in Solanaceae, the gene expression levels of *pathogenesis-related class 1* (*PR1*, Niben101Scf04053g02006.1) and *phenylalanine ammonia-lyase* (*PAL2*, Niben101Scf03712g01008.1) defense response genes previously associated with *P. infestans* resistance (Eschen-Lippold *et al.*, 2012) was assessed in plants transiently expressing SlDKML after pathogen infection. Significant upregulation of these defense response genes was observed in leaves transiently expressing SlDKML at 9 dpi with *P. infestans* compared to control leaves transiently expressing GFP (Fig. 2.10). Together our results argue that DKML is a positive regulator of cell death triggered by PTI and ETI as well as resistance to the oomycete destructive pathogen *P. infestans* probably by interacting with MORC1 and up-regulating defense response genes previously associated with pathogen resistance highlighting the potential of using DKML to develop *P. infestans* resistant crops.

Discussion

MORC1 plays important roles in PTI and ETI against different pathogens including *P. infestans* and cell death regulation in plants. Thirteen new interactors (excluding MIP6) of AtMORC1, a key regulator of chromatin stability and broad-spectrum resistance, were identified in this study. Previously, other MIPs have been reported in other systems including proteins involved in DNA binding, chromatin remodeling, RNA-directed DNA methylation pathways, and several proteins associated with plant immunity including R proteins and PRR receptors (Kang *et al.*, 2008, 2010; Langen *et al.*, 2014; Jing *et al.*, 2016; Koch *et al.*, 2017). In this study we report thirteen new MIPs ranging from intracellular protein transporters, proton transport regulators, and several proteins with DNA-related and nuclear functions (Table 1).

MORC1 has been shown to form homodimers and heterodimers (Manosalva *et al.* 2015, Moissiard *et al.*, 2014). One of the interactors, MIP3, identified in this screening corresponded to AtMORC6 which has been shown to form stable heterodimers with AtMORC1 (Moissiard *et al.*, 2014). Interestingly, StMORC1 and SlMORC1 interact with only 3 of the 13 MIPs (excluding MIP6) including AtMORC6 (MIP3), AtDKML(MIP10) described previously as bZIP29 TF (Lozano-Sotomayor *et al.*, 2016; Van Leene *et al.*, 2016) and an aminotransferase-like, mobile domain protein (MIP12) (Ji Chul Nam, 2020). These results suggest that either MORC1 functions are regulated differently in different plant species or that the AtMIPs have some sequence differences with the Solanaceae homologs that are required for their interactions with St/SlMORC1.

In addition to our finding that DKML TF directly interacts with MORC1, we found that the DKML protein region containing the bZIP and a CC-domain are necessary for this interaction in our Y2H assays. These domains have been previously associated with binding activities of the bZIP TF to their plant targets (Reinke *et al.*, 2013; Llorca *et al.*, 2014). Surprisingly, in the Y2H assay, SlDKML only interacted with the C-terminal region of SlMORC1 whereas *in planta* Co-IP assays, SlDKML does interact with both the C-terminal and full-length of SlMORC1 protein. This suggested that modifications or domain exposure of SlMORC1 or SlDKML may be necessary for these proteins to interact, which could only be carried out when *in planta*.

The bZIP family of TFs have been reported in Arabidopsis describing 78 members (Jakoby *et al.*, 2002; Dröge-Laser *et al.*, 2018) and in tomato a total of 69 members (Li *et al.*, 2015), respectively. The AtDKML, described as bZIP 29 member, corresponds to bZIP 11 in tomoto (SlDKML). Similarly in tomato, potato has also one homolog (StDKML), however we found two homologs of DKML in *N. benthamiana* (NbDKML1 & NbDKML2), which each have two different alleles (A & B) due to their allotetraploid nature. Similar to MORC1 (Koch *et al.*, 2017), we found that the number of CC-domains varies among different DKML proteins from different plant species ranging from one in Arabidopsis, two in tomato and three in potato. Interestingly, only one CC-domain is sufficient for their interactions with MORC1. Future experiments are required to determine the importance of having different numbers of CC-domains in these proteins for their biological functions. Additionally, like SlMORC1, SlDKML does not have traditional canonical NLS sites, and yet, it is still localized to the nucleus (Fig. 2.2 & 2.3).

It is possible that DKML localizes in the nucleus through interactions with other proteins or that DKML is targeted to the nucleus using a non-canonical NLS signal. Using cNLS mapper, we predicted one bipartite non canonical NLS at the N-terminal regions of Solanaceae DKMLs possibly accounting for its nuclear localization, however more experiments need to be done to test this hypothesis.

Since DKML is involved in multiple biological processes, DKML must have a way to differentiate between its many roles. One way may be through post-translational modifications like phosphorylation. In Arabidopsis, quantitative phosphoproteomics data showed that AtDKML undergoes phosphorylation enrichment in four different serine amino acid sites, positions 196, 174, 321, and 342, (Van Leene *et al.*, 2019). In SlDKML, predicted phosphorylation sites were scattered throughout the entire protein, including seven within the bZIP domain and two within coiled-coil regions. Many of the kinases that were predicted to phosphorylate SlDKML were known activators of cell division control, but some, like proteinase kinase C (PKC), have a broad kinase activity, regulating transcription and mediating immune responses. Additionally, it is known that phosphorylation of adjacent NLS sites can dictate the import or export of TFs into the nucleus (Jans *et al.*, 2000). In SlDKML, the predicted NLS site in the C-terminal region was only 8 amino acids away from a predicted site phosphorylated by proteinase kinase C (PKC). It is possible that this phosphorylation site surrounding the NLS of SlDKML may be important in dictating SlDKMLs ability to enter the nucleus or become activated to help regulate host immune responses. Further experiments are required to determine if DKML can be phosphorylated, and if so, determine whether phosphorylation is necessary

for DKMLs localization and specific biological functions. Taken together, it would be interesting to determine the relevance of all these structural differences in DKML for their biochemical and biological functions in their corresponding plant species.

In *A. thaliana,* AtDKML was linked to leaf and root development and cell proliferation (Lozano-Sotomayor *et al.*, 2016; Van Leene *et al.*, 2016). The classification of the bZIP family in *A. thaliana* placed AtDKML in group I of the bZIP TFs, which was comprised of 13 members (Jakoby *et al.*, 2002). Many of the TFs in this group have been reported for their roles in development and osmosensory responses, including AtDKML (Tzfira *et al.*, 2001; Djamei *et al.*, 2007). Some members of this group of TFs have been associated with pathogen responses (Li *et al.*, 2015, Djamei *et al.*, 2007). In tomato, 69 non-redundant SlbZIP TFs were identified and were clustered into nine clades (Li *et al.*, 2015). Consistent with the grouping in our study, Li et al. 2015 showed that AtDKML clustered with one SlbZIP called SlbZIP11 (known as SlDKML in our study) when comparing the tomato bZIP TF family with other plant species. Among these SlbZIPs TFs, SlDKML was found to be differentially expressed after infection with *Botrytis canerea* or *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) (Li *et al.*, 2015). As far as our knowledge, no direct role of DKML on plant immunity was reported.

In this study, we determined that the new MORC1 interactor, DKML from Solanaceae, positively regulates PTI- and ETI- induced cell death and disease resistance to *P. infestans*. After transiently silencing *DKML* in *N. benthamiana* and inoculating with the INF1, AvrPto-Pto and AvrBs3-Bs3 combinations of effectors and R proteins, a delay in cell death was observed, respectively. In addition, transiently silencing and expressing

DKML proteins enhanced susceptibility or resistance to the oomycete pathogen *P. infestans*, respectively (Fig 2.9 & S2.7). Defense response genes including *PR1, PAL, MORC1* associated with pathogen resistance were significantly up-regulated after infection with *P. infestans* in leaves transiently expressing SlDKML and exhibiting enhancement of resistance when compared with control plants. This is interesting since *PR1* and *PAL* are markers of the salicylic acid (SA) pathway, which is required for cell death and PTI & ETI against *P. infestans* (Shibata *et al.*, 2010, Hõrak, 2020). Moreover, SlDKML transcript was shown to be up-regulated in tomato plants after SA treatment (Li *et al.*, 2015) suggesting that DKML regulation of plant immunity may require the SA signaling pathway. We previously have shown that MORC1 regulates INF1-induced cell death (Manosalva *et al.*, 2015; Manohar *et al.*, 2017), HR-induced cell death by different *R* genes and their corresponding effector combinations including AvrPto-Pto (Kang *et al.*, 2008, 2010), and plant immunity to *P. infestans* (Kang *et al.*, 2013; Manosalva *et al.*, 2015). It is consistent with our findings that DKML, as a MORC1 interactor, share similarity with MORC1 functions in plant immunity including the regulation of cell death, pathogen resistance (*P. infestans* and *Pst*), and its links with the SA signaling pathway (Li *et al.*, 2015; Bordiya *et al.*, 2016) suggesting that both proteins work together or in the same pathway to regulate these plant immune responses.

Together, our findings demonstrate for the first time, a direct role of DKML to plant immunity. SlDKML is the only bZIP TF in tomato that has been functionally characterized for its role in plant immunity. Future experiments will explore the benefits as well as any negative secondary effects that may come with the generation of a stable

transgenic plant. Some of the excitement observed with generating DKML transgenic plants stems from the fact that DKML is expressed across all types of tissues in *A. thaliana* and tomato, including roots, leaves, stems, cotyledons, and flowers (Li *et al.*, 2015; Van Leene *et al.*, 2016). Having expression throughout the plant like that makes DKML a great candidate to generate stable transgenic plants to fight off a multitude of pathogens with different modes of infection.

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Figure and Table legends

Table 2.1. MIPS identified in Y2H screening.

	Locus	Annotation
MIP1	At3g56190	Intracellular protein transport: αSNAP2
MIP2	At5g15450	Chloroplast-targeted HSP101 homologue
MIP3	At1g19100	MORC6
MIP4	At2g33540	CPL3 (C-terminal domain phosphatase-like 3)
MIP5	At2g35110	NAPP/WAVE
MIP6	At5g51600	Mitotic microtubule organizer
MIP7	At4g27500	Regulates proton transport
MIP8	At3g54670	SMC1- cohesion during DNA replication
MIP9	At3g10030	SANT- chromatin remodeling factor
MIP10	At4g38900	bZIP transcription factor
MIP11	At5g06140	SORTING NEXIN 1
MIP12	At1g50750	WRKY TFs. Mobile domain
MIP13	At1g55080	Mediator9 complex protein
MIP14	At1g31780	COG6 (Golgi retrograde transport)

Figure 2.1. Potato MORC1 (StMORC1) and tomato MORC1 (SlMORC1) proteins interact with fewer *A. thaliana* MORC1 interacting proteins (AtMIPs) compared to *A. thaliana* MORC1 (AtMORC1).

(a) Full-length AtMORC1 positively interacted with itself and 14 other AtMIPs. (b) Schematic presentation of StMORC1, SlMORC1 and AtMORC1 domains and the deletion constructs used as bait. (c) StMORC1 and SlMORC1 bait constructs interacting with fewer MIPs when compared with AtMORC1. Transformed yeast were mated and grown on synthetic dropout medium lacking leucin (Leu) and tryptophan (Trp) (-Leu/-Trp), and on SD medium lacking Leu, Trp, and histidine (His) (-Leu/-Trp/-His) and were cultivated for 3 days at 30°C. Experiments were repeated twice with similar results.

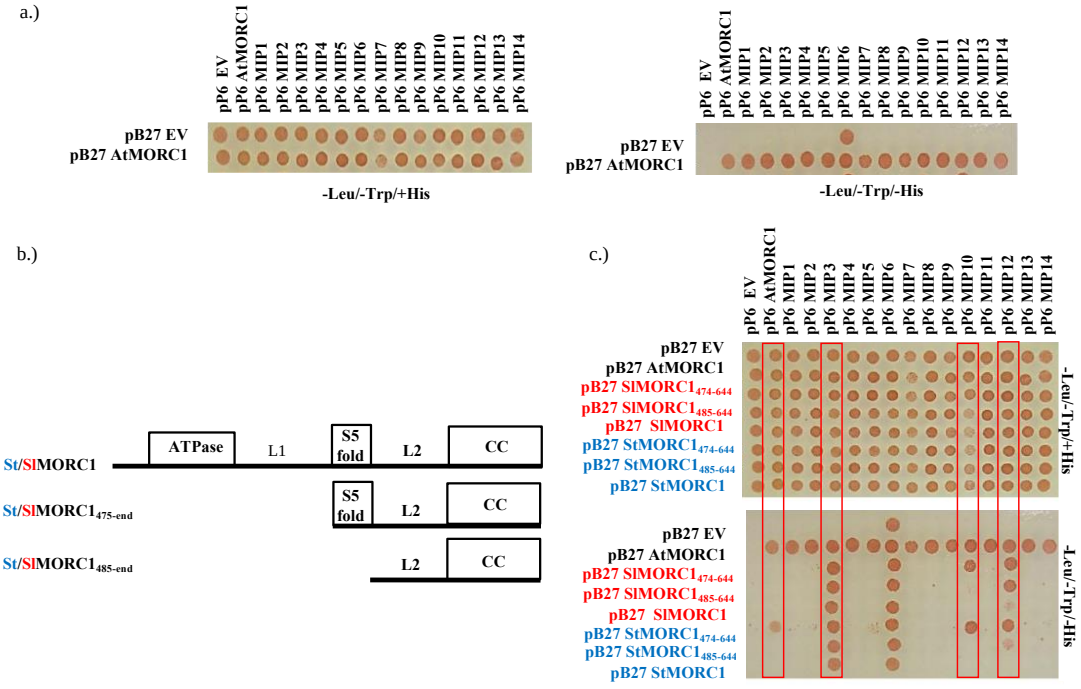

a.)
pP6 EV
pP6 AtMORC1
pP6 MIP1
pP6 MIP2
pP6 MIP3
pP6 MIP4
pP6 MIP5
pP6 MIP6
pP6 MIP7
pP6 MIP8
pP6 MIP9
pP6 MIP10
pP6 MIP11
pP6 MIP12
pP6 MIP13
pP6 MIP14
pB27 EV
pB27 AtMORC1
-Leu/-Trp/+His
pB27 EV
pB27 AtMORC1
-Leu/-Trp/-His
b.)
St/SlMORC1
ATPase
L1
S5 fold
L2
CC
St/SlMORC1475-end
S5 fold
L2
CC
St/SlMORC1485-end
L2
CC
c.)
pP6 EV
pP6 AtMORC1
pP6 MIP1
pP6 MIP2
pP6 MIP3
pP6 MIP4
pP6 MIP5
pP6 MIP6
pP6 MIP7
pP6 MIP8
pP6 MIP9
pP6 MIP10
pP6 MIP11
pP6 MIP12
pP6 MIP13
pP6 MIP14
pB27 EV
pB27 AtMORC1
pB27 SlMORC1474-644
pB27 SlMORC1485-644
pB27 SlMORC1
pB27 StMORC1474-644
pB27 StMORC1485-644
pB27 StMORC1
-Leu/-Trp/+His
pB27 EV
pB27 AtMORC1
pB27 SlMORC1474-644
pB27 SlMORC1485-644
pB27 SlMORC1
pB27 StMORC1474-644
pB27 StMORC1485-644
pB27 StMORC1
-Leu/-Trp/-His

Figure 2.2. Schematic diagram of the domain structure of DKML in *A. thaliana,* potato, tomato, and *N. benthamiana.*

The arrows indicate the primers used for the AtDKML, StDKML, SlDKML, and NbDKLM1A Y2H constructs. Total protein amino acid (AA) number for the full length proteins are indicated in the right.

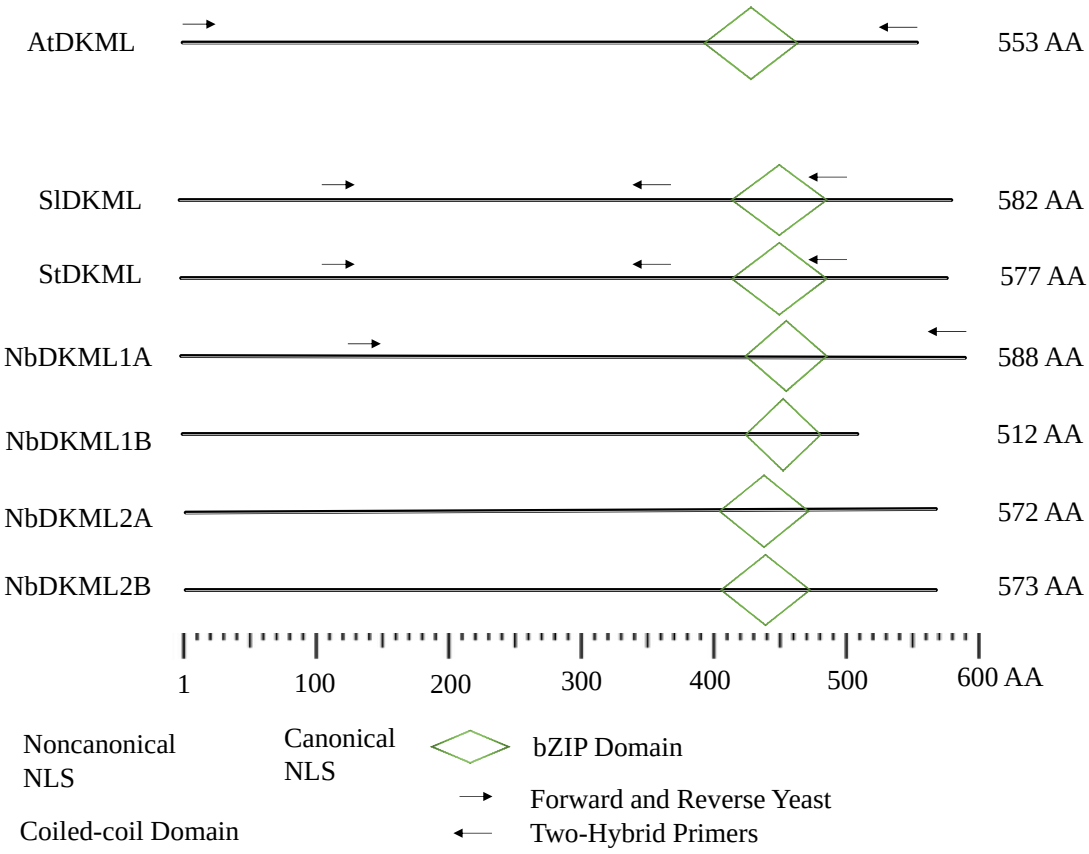
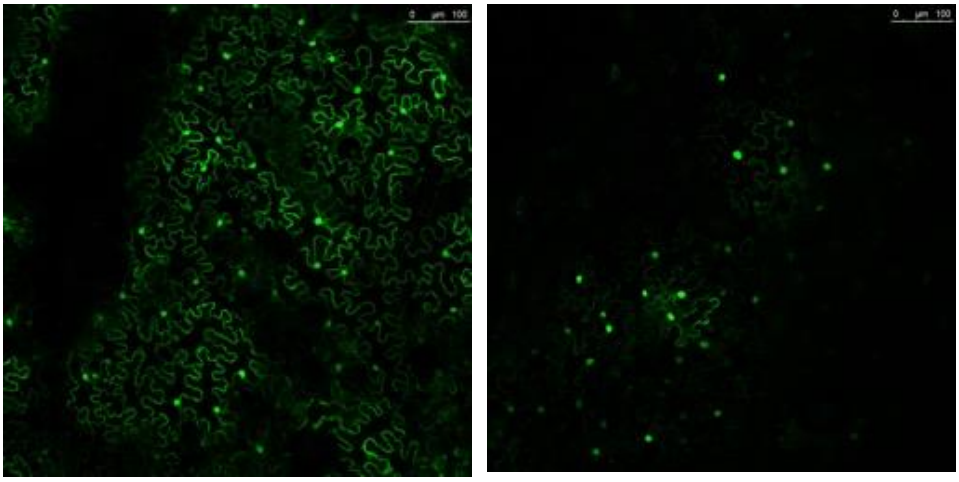

AtDKML
553 AA
SlDKML
582 AA
StDKML
577 AA
NbDKML1A
588 AA
NbDKML1B
512 AA
NbDKML2A
572 AA
NbDKML2B
573 AA
1
100
200
300
400
500
600 AA
Noncanonical NLS
Canonical NLS
bZIP Domain
Coiled-coil Domain
Forward and Reverse Yeast Two-Hybrid Primers

Figure 2.3. SlDKML localizes to the nucleus and plasma membrane.

(a) Confocal imaging was performed on wild-type *N. benthamiana* plants transiently expressing GFP or SlDKML-YFP. Scale bar, 100µm. (b) IB analysis with αMyc antibodies was carried out with total, nucleus-depleted and nucleus-enriched fractions. Subcellular fractionation was performed on wild-type plants transiently expressing SlMIP10-Myc. Plants were treated with water (mock) or 1µM flg22 treatment for 15 min. Antibodies against histone (Histone 3, a nuclear protein) and cytosolic fructose-1, 6-bisphosphatase (cFBPase, a cytosolic protein), were used to ensure appropriate enrichment or depletion of nuclei. Experiments were repeated twice with similar results.

a)



GFP SlDKML

b)

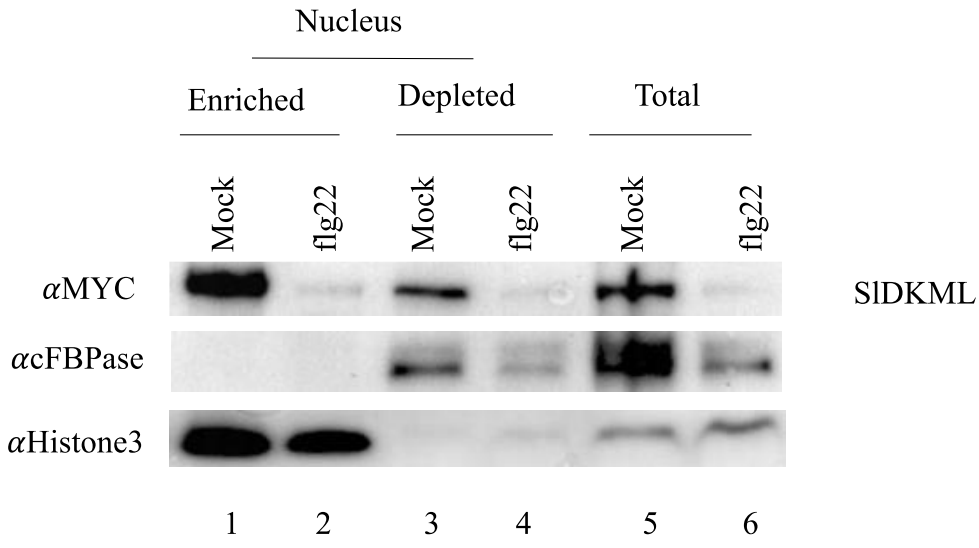
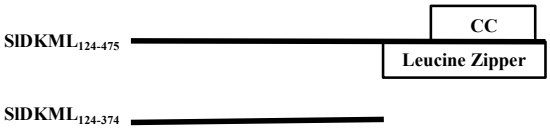


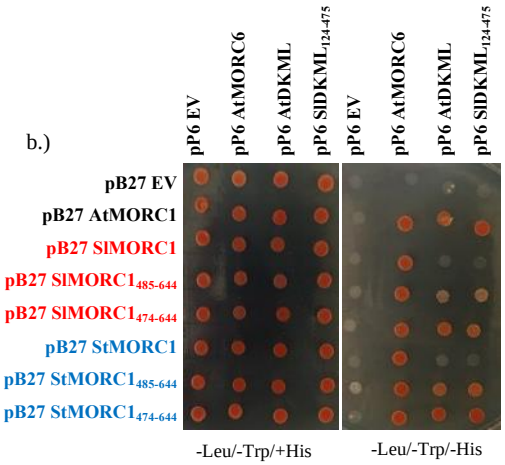
Figure 2.4. SlMORC1 and StMORC1 binds directly to SlDKML via their C-terminal regions.

(a) Schematic presentation of tomato SlDKML constructs cloned into the prey pP6 vector. (b) Yeast two-hybrid (Y2H) assay of full length AtMORC1, SlMORC1, and StMORC1 proteins as well as the C-terminal constructs SlMORC1$_{475-644}$, SlMORC1$_{485-644}$, StMORC1$_{475-644}$, StMORC1$_{485-644}$ as bait with the AtMORC6, AtDKML, and SlDKML$_{124-475}$ as prey. (c) Y2H assay between the Sl/St MORC1 constructs and ten-fold serial dilution of the SlDKML$_{124-475}$ construct. (d) The C-terminal region of SlDKML (SlDKML$_{124-475}$) containing the leucine zipper and CC-domain was necessary for Sl/StMORC1$_{475-644}$ and Sl/StMORC1$_{485-644}$ interaction. Experiments were repeated at least twice with similar results.

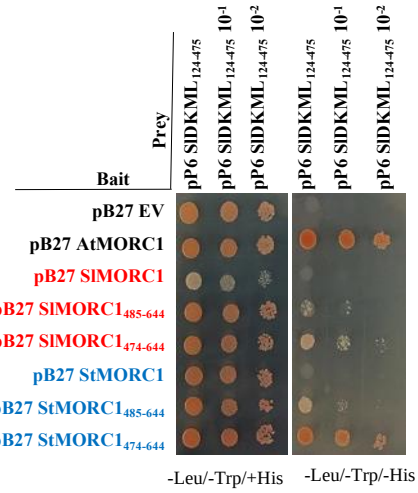
a.)


SlDKML124-475
CC
Leucine Zipper
SlDKML124-374

b.)


pP6 EV
pP6 AtMORC6
pP6 AtDKML
pP6 SlDKML124-475
pP6 EV
pP6 AtMORC6
pP6 AtDKML
pP6 SlDKML124-475
pB27 EV
pB27 AtMORC1
pB27 SlMORC1
pB27 SlMORC1485-644
pB27 SlMORC1474-644
pB27 StMORC1
pB27 StMORC1485-644
pB27 StMORC1474-644
-Leu/-Trp/+His
-Leu/-Trp/-His

c.)


Prey
Bait
pP6 SlDKML124-475
pP6 SlDKML124-475 10-1
pP6 SlDKML124-475 10-2
pP6 SlDKML124-475
pP6 SlDKML124-475 10-1
pP6 SlDKML124-475 10-2
pB27 EV
pB27 AtMORC1
pB27 SlMORC1
pB27 SlMORC1485-644
pB27 SlMORC1474-644
pB27 StMORC1
pB27 StMORC1485-644
pB27 StMORC1474-644
-Leu/-Trp/+His
-Leu/-Trp/-His

d.)

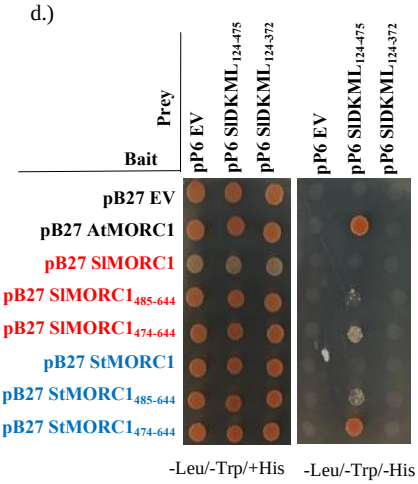

Prey
Bait
pP6 EV
pP6 SlDKML124-475
pP6 SlDKML124-372
pP6 EV
pP6 SlDKML124-475
pP6 SlDKML124-372
pB27 EV
pB27 AtMORC1
pB27 SlMORC1
pB27 SlMORC1485-644
pB27 SlMORC1474-644
pB27 StMORC1
pB27 StMORC1485-644
pB27 StMORC1474-644
-Leu/-Trp/+His
-Leu/-Trp/-His

Figure 2.5. SlDKML physically associates with SlMORC1 *in planta.*

SlMORC1 physically interacts with SlDKML *in planta* in immunoprecipitation assays. $SlDKML_{124\text{-}475}$ interacts with the full-length and C-terminal region of SlMORC1. Experiments were repeated at least twice with similar results. Equal loading was confirmed by Coomassie Brilliant Blue staining.

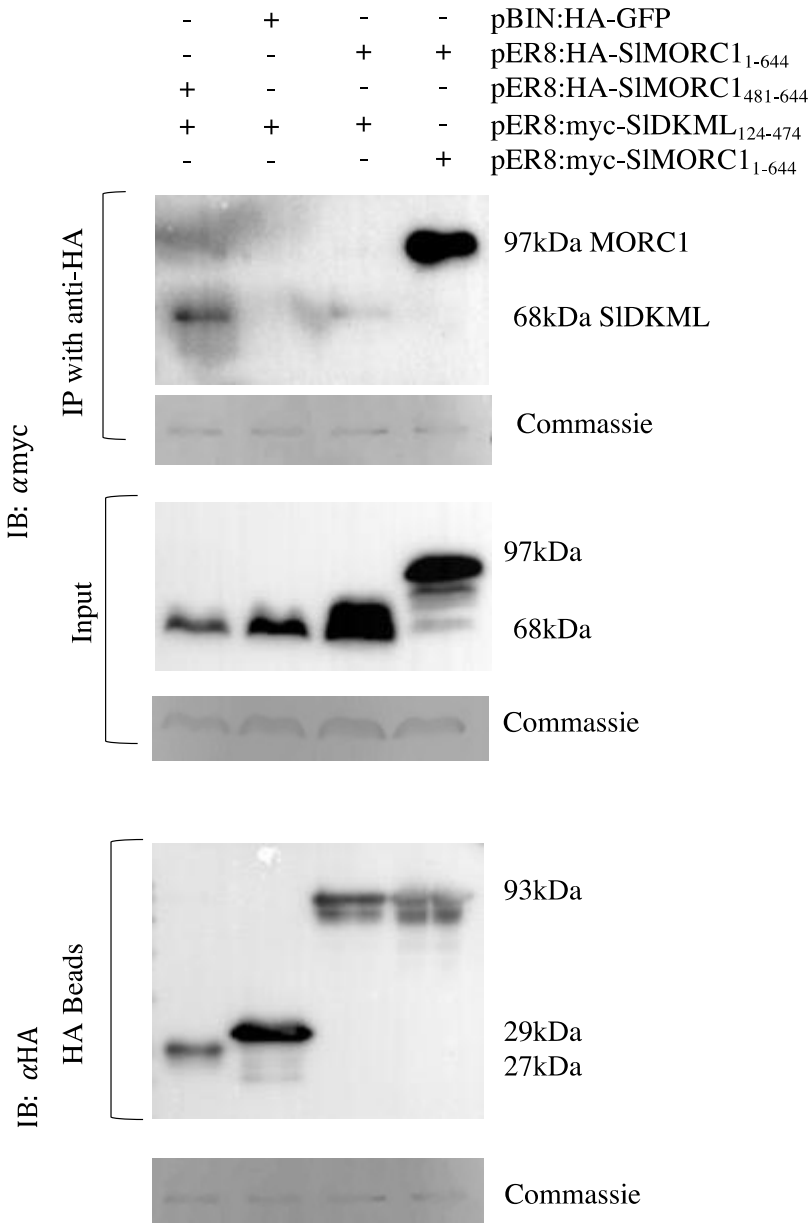

- + - - pBIN:HA-GFP
- - + + pER8:HA-SlMORC1$_{1-644}$
+ - - - pER8:HA-SlMORC1$_{481-644}$
+ + + - pER8:myc-SlDKML$_{124-474}$
- - - + pER8:myc-SlMORC1$_{1-644}$
IB: αmyc
IP with anti-HA
97kDa MORC1
68kDa SlDKML
Commassie
Input
97kDa
68kDa
Commassie
IB: αHA
HA Beads
93kDa
29kDa
27kDa
Commassie

Figure 2.6. Silencing DKML in *N. benthamiana* compromised INF1-induced cell death

Two DKML genes were identified in *N. benthamiana* and were silenced by *Tobacco rattle virus* (TRV2)-based VIGS (TRV2:*NbDKML*); TRV2:*EC1* was used as a negative control. (a) Yeast two-hybrid assay of AtMORC1$_{1\text{-}635}$, SlMORC1$_{1\text{-}644}$, SlMORC1$_{475\text{-}644}$, SlMORC1$_{485\text{-}644}$, StMORC1$_{1\text{-}644}$, StMORC1$_{475\text{-}644}$, StMORC1$_{485\text{-}644}$, with AtMORC6, SlDKML$_{124\text{-}475}$ NbDKML1A$_{143\text{-}588}$. (b) Level of expression for each DKML homolog, *NbDKML1A* and *NbDKML2A*, was assessed by quantitative polymerase chain reaction 4 weeks after infiltration. *NbEF1a* was used as an internal control. (c) Extent of INF1-induced cell death in TRV2:*NbDKML* plants was quantified as 0=none, 1=partial, and 2= complete cell death from leaves taken 3 days after infiltration (dai) with INF1. Data correspond to a total of 18 inoculation points from five control and five experimental plants. This experiment was repeated at least four times with similar results. **$P<0.01$ and *$P<0.05$. (d) Cell death was visualized by trypan blue staining of leaves rated in (c).

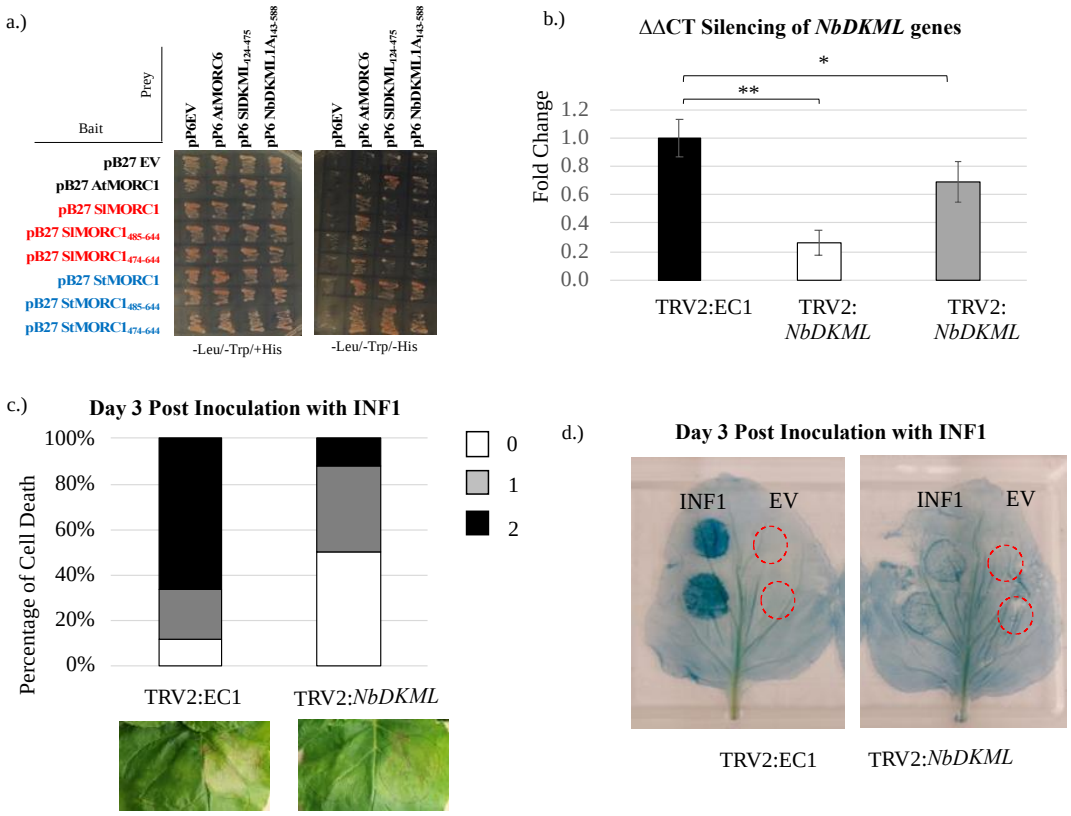

a.)
Prey
Bait
pP6EV
pP6 AtMORC6
pP6 SlDKML124-475
pP6 NbDKML1A143-588
pB27 EV
pB27 AtMORC1
pB27 SlMORC1
pB27 SlMORC1485-644
pB27 SlMORC1474-644
pB27 StMORC1
pB27 StMORC1485-644
pB27 StMORC1474-644
-Leu/-Trp/+His
-Leu/-Trp/-His
b.)
ΔΔCT Silencing of NbDKML genes
Fold Change
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
TRV2:EC1
TRV2: NbDKML
TRV2: NbDKML
c.)
Day 3 Post Inoculation with INF1
Percentage of Cell Death
100%
80%
60%
40%
20%
0%
0
1
2
TRV2:EC1
TRV2:NbDKML
d.)
Day 3 Post Inoculation with INF1
INF1
EV
INF1
EV
TRV2:EC1
TRV2:NbDKML

Figure 2.7. Effector Triggered Immunity was compromised in TRV2:*NbDKML* plants.

DKML-silenced plants were transiently overexpressed with cell death elicitors 4 weeks after infection with TRV2:*NbDKML,* TRV2:*EC1*, or TRV2:*SlDKML*. Cell death was scored at 3 dai and 4 dai using the following score system: 0=none, 1=partial, and 2= complete cell death. (a/b) TRV2:*NbDKML* plants showed significant delay in cell death at 3 dai and 4dai after transient expression of AvrBs3 and Bs3, AvrPto and Pto, and INF1. *P<0.05, **P<0.01 (*t* test). Experiments were repeated with similar results.

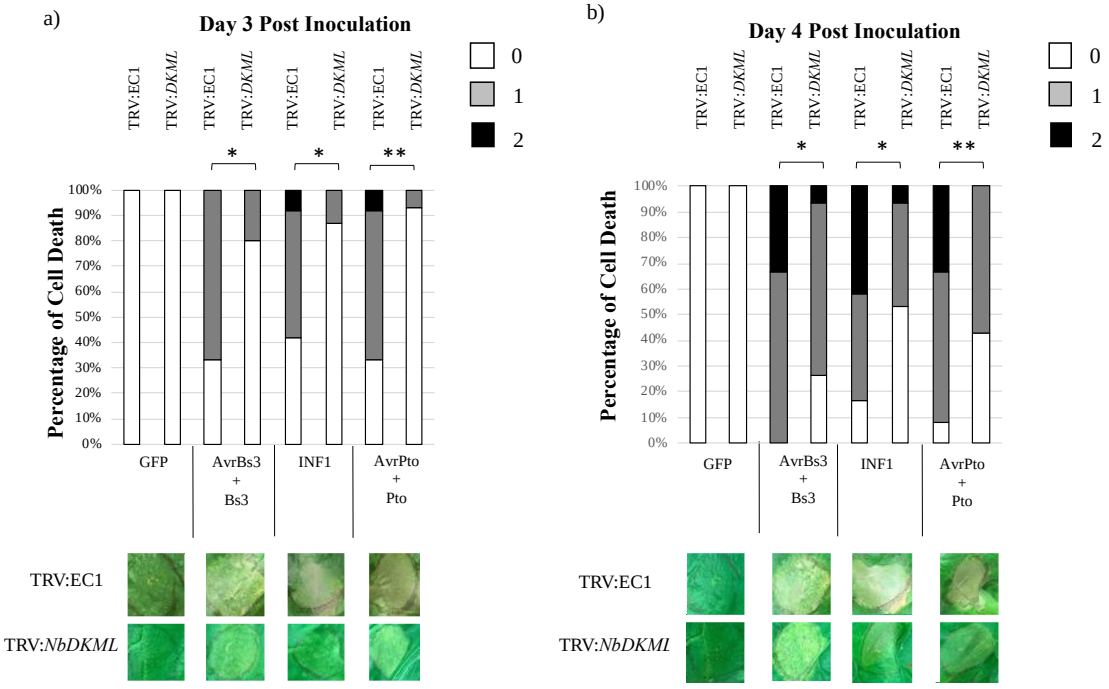
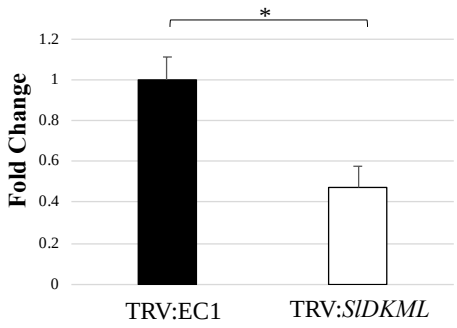

a)
Day 3 Post Inoculation
b)
Day 4 Post Inoculation
0
1
2
TRV:EC1
TRV:DKML
*
**
Percentage of Cell Death
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
GFP
AvrBs3 + Bs3
INF1
AvrPto + Pto
TRV:EC1
TRV:NbDKML

Figure 2.8. Effector Triggered Immunity was compromised in silenced DKML tomato plants (TRV2:*SlDKML)*.

(a) Level of expression for *SlDKML* in tomato, was assessed by quantitative polymerase chain reaction 4 weeks after infiltration. *SlEF1a* was used as an internal control. (b) TRV2:*SlDKML* plants showed significant delay in AvrPto-Pto cell death at 4 dpi. Cell death was scored at 3 dpi and 4 dpi using the following score system: 0=none, 1=partial, and 2=complete cell death. *P<0.05, **P<0.01 (*t* test). Experiments were repeated with similar results.

a.)


ΔΔCT Silencing of *SlDKML*
*
1.2
1
0.8
0.6
0.4
0.2
0
Fold Change
TRV:EC1
TRV:*SlDKML*

b.)

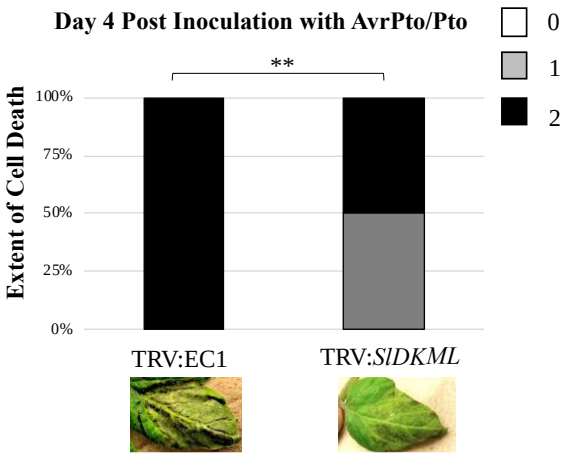
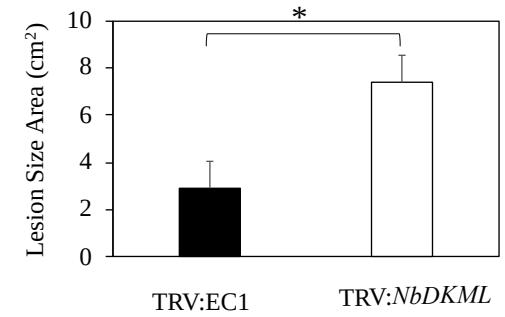

Day 4 Post Inoculation with AvrPto/Pto
**
100%
75%
50%
25%
0%
Extent of Cell Death
0
1
2
TRV:EC1
TRV:*SlDKML*

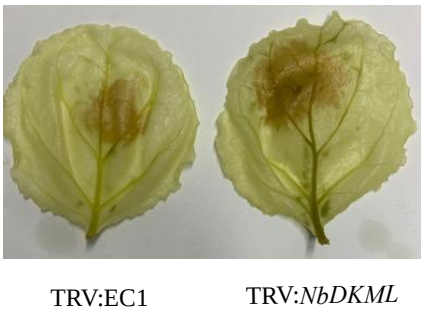
Figure 2.9. Silencing DKML in *N. benthamiana* significantly increases susceptibility to *Phytophthora infestans.*

Basal resistance was monitored in *DKML* silenced *N. benthamiana* plants (white bars) and in the corresponding *EC1* control plants (black bars) following inoculation with the 1306 isolate of *P. infestans (Pi).* (a) TRV2:*NbDKML* and TRV2:*EC1* plants were inoculated with the 1306 isolate of *Pi* (20,000 sporangia/ml) using a detached-leaflet assay. Disease symptoms were measured based on size of the blighted area (cm^2) at 6 dpi. (b) Photographs of inoculated areas undergoing cell death taken at 6 dpi. Carnoy's solution was used to visualize cell death. (c) Biomass of *Pi* in TRV2:*NbDKML* and TRV2:*EC1* leaves measured by qRT-PCR at 4 dpi. (d) Biomass of *Pi* in TRV2:*NbDKML* and TRV2:*EC1* leaves measured by qRT-PCR at 6 dpi. The levels of *PiO8* element and *NbEf1a* were used to quantify the levels of *Pi* and plant cells by qRT-PCR. Data represent the mean and standard error (n=4) from one experiment. Similar experiments were repeated at least twice with similar results. Statistical difference between TRV2:*EC1* and TRV2:*NbDKML* is indicated; *P<0.05 (*t* test).

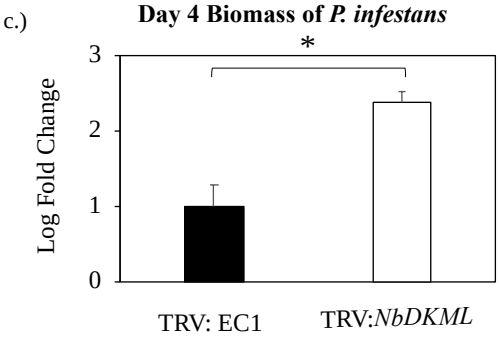
a.)


Day 6 Post Inoculation with P. infestans
Lesion Size Area (cm2)
10
8
6
4
2
0
*
TRV:EC1
TRV:NbDKML

b.)


Day 6 Post Inoculation with P. infestans
TRV:EC1
TRV:NbDKML

c.)


Day 4 Biomass of P. infestans
Log Fold Change
3
2
1
0
*
TRV: EC1
TRV:NbDKML

d.)

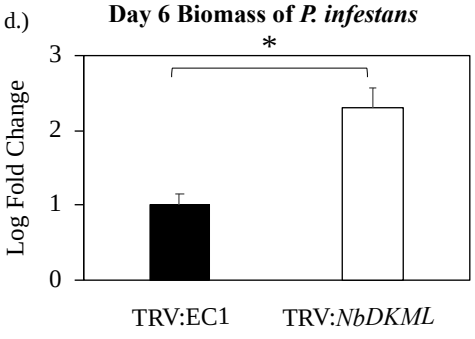
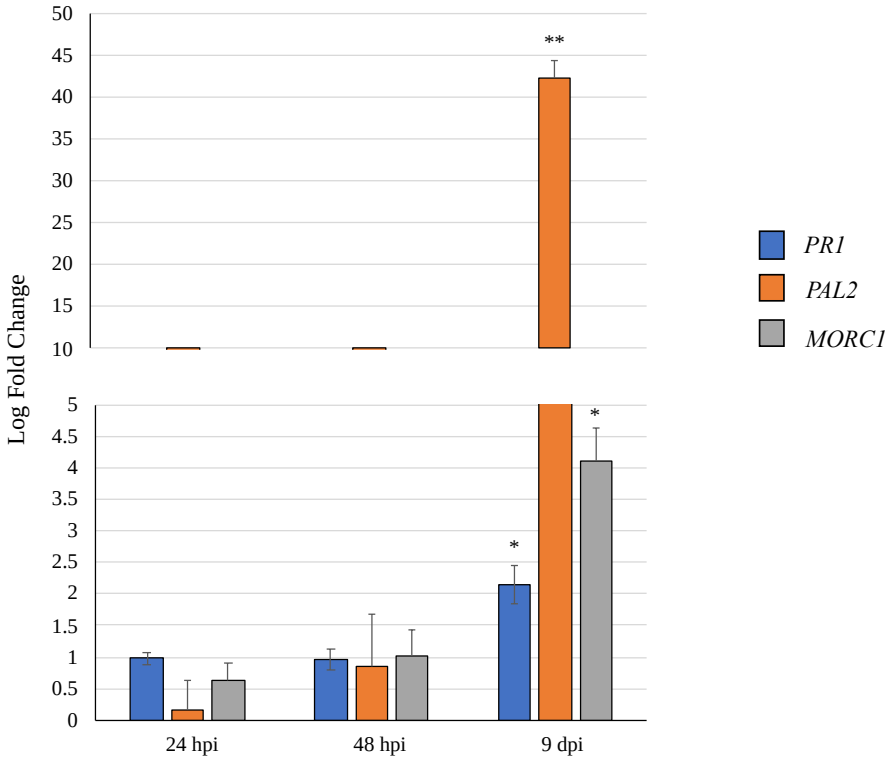

Day 6 Biomass of P. infestans
Log Fold Change
3
2
1
0
*
TRV:EC1
TRV:NbDKML

Figure 2.10. Transcript levels of defense related genes in *N. benthamiana* transiently expressing SlDKML after *P. infestans* infection.

qRT-PCR was used to validate expression patterns of defense-related genes, *PAL2, PR1,* and *MORC1*. *N. benthamiana* housekeeping gene *EF1-a1* was used as the reference gene for normalization. Experiments were repeated twice. *P-values ≤ 0.05 and **P-values ≤ 0.005 (*t* test).


Log Fold Change
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45
40
35
30
25
20
15
10
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
*
*
24 hpi
48 hpi
9 dpi
PR1
PAL2
MORC1

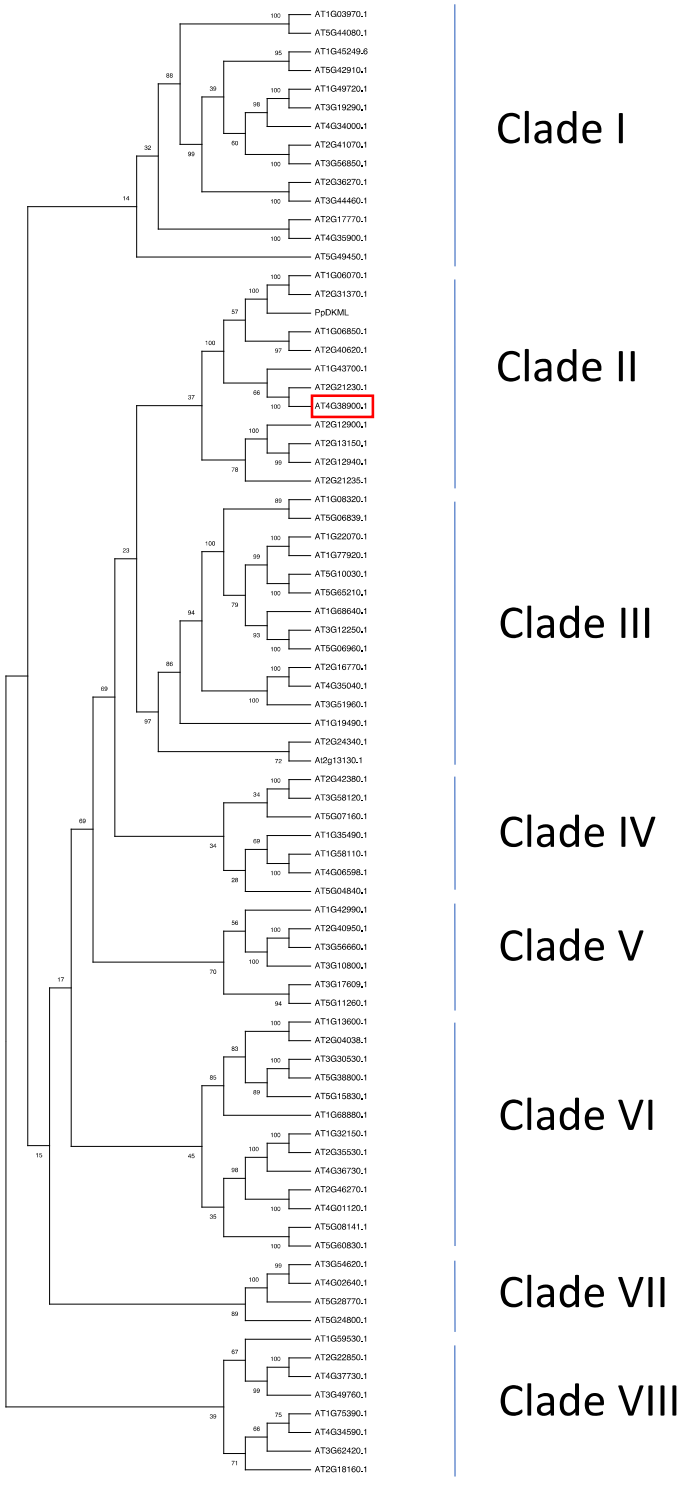
Supplemental Figures and Tables

Supplemental Table 2.1. List of oligonucleotides.

Primer Pair Number	Primer Name	Primer Sequence	Constuct
1	SlDKML5`Race_GSP1	GATTACGCCAAGCTTCTCTCTGCAAGAGGGTCAGTTGAGCAG	SlDKML gene specific primer for 5'Race. Includes cloning adapter for vector pPUC19
2	StDKML5`Race_GSP1	GATTACGCCAAGCTTctctctgcaagagggtcaattgagcag	StDKML gene specific primer for 5'Race. Includes cloning adapter for vector pPUC19
3	Sl/StDKML5`Race_GSP2	GATTACGCCAAGCTTGCAAGTTTTTCATTTGCCATAATTTTC	Sl/StDKML nested gene specific primer for 5'Race. Includes cloning adapter for vector pPUC19
4	Sl/StDKML3`Race_GSP1	GATTACGCCAAGCTTcagggaatcagcaaatgggtatgcagaac	Sl/StDKML gene specific primer for 3'Race. Includes cloning adapter for vector pPUC19
5	StDKML3`Race_GSP2	GATTACGCCAAGCTTgccgtatagggaatccccgtccacg	SlDKML nested gene specific primer for 3'Race. Includes cloning adapter for vector pPUC19
6	SlDKML3`Race_GSP2	GATTACGCCAAGCTTACCGTATAGGGAATCCCCGTCCACG	StDKML nested gene specific primer for 3'Race. Includes cloning adapter for vector pPUC19
7	Y2H_NbDKML1A_BAMHI_F	TAAGCAGGATCCATATGCCCCCTATTTCACCTTACTCTCAGA	Forward primer for cloning NbDKML1A$_{143\text{-}588}$ into Y2H prey vector pP6
8	Y2H_NbDKML1A_XhoI_R	TGCTTACTCGAGtcaCTACTTCGATTCATTCTTTGAAGGTGTAGAGCTGTT	Reverse primer for cloning NbDKML1A$_{143\text{-}588}$ into Y2H prey vector pP6
9	Y2H_SlDKML_124_BamHI_F	TAAGCAGGATCCATATGCCACCCTTGAGCCCTTCACC	Forward primer for cloning SlDKML region 124-475AA (SlDKML$_{124\text{-}475}$) into Y2H prey vector pP6
10	Y2H_SlDKML_475_XhoI_R	TGCTTACTCGAGtcaCAGCCCAGTCGCATCTCTC	Reverse primer for cloning SlDKML$_{124\text{-}475}$ into Y2H prey vector pP6
11	Y2H_StDKML_125_BamHI_F	TAAGCAGGATCCATATGCCCTTGAGCCCTTCGCCGTAT	Forward primer for cloning StDKML region 125-475AA (StDKML$_{125\text{-}475}$) into Y2H prey vector pP6
12	Y2H_StDKML_475_XhoI_R	TGCTTACTCGAGctaTAGCCCAGTCGCATCTCTC	Reverse primer for cloning StDKML$_{125\text{-}475}$ into Y2H prey vector pP6
13	Y2H_Sl/StDKML_372_XhoI_R	TGCTTACTCGAGTCAGCGTGGTCCAGGAGATGGAG	Reverse primer for cloning SlDKML region 124-372AA (SlDKML$_{124\text{-}372}$) or StDKMLregion 125-372AA (StDKML$_{125\text{-}372}$) into Y2H prey vector pP6
14	Y2H_AtMORC1_1_SfiI_F	CACCCCAATTTGTGGTTTCAATGTC	Forward primer for cloning AtMORC1 region 1-553AA into entry vector pDONR and recombined to pDEST-BD
15	Y2H_AtMORC1_644_SfiI_R	AACTTGTTGCATCTCCTTCTT	Reverse primer for cloning AtMORC1 region 1-553AA into entry vector pDONOR and recombined to pDEST-BD
16	Y2H_Sl/StMORC1_1_EcoRI_F	GGAAAC GAATTC ATGCCGCCTAAGGTAGAGAAGC	Forward primer for cloning Sl/StMORC1 region 1-644AA into Y2H bait vector pB27
17	Y2H_Sl/StMORC1_644_SpeI_R	GGAAACACTAGTCTATGCTTTCTGTCCAAGTTG 3	Reverse primer for cloning Sl/StMORC1 regions 1/474/484-644AA into Y2H bait vector pB27
18	Y2H_Sl/StMORC1_474_EcoRI_F	GGAAACGAATTCATG GATTACTGGAAAGGTCATTGTC	Forward primer for cloning Sl/StMORC1 region 474-644AA into Y2H bait vector pB27
19	Y2H_Sl/StMORC1_485_EcoRI_F	GGAAACGAATTCATGGGATTGAAGCCTCTTGAACCGC	Forward primer for cloning Sl/StMORC1 region 484-644AA into Y2H bait vector pB27
20	CoIP_pER_SlDKML_124_AvrII_F	GGAAACcctaggtATGCCACCCTTGAGCCCTTCACC	Forward primer for cloning SlDKML$_{124\text{-}475}$ into inducible vector pER8
21	CoIP_pER_St/SlDKML_475_SpeI_R	GGAAACactagtctaGCCCAGTCGCATCTCTC	Reverse primer for cloning SlDKML$_{124\text{-}475}$ into inducible vector pER8
22	VIGS_NbDKML_0466_8662_EcoRI_F	GGAAACGAATTCaGGAGAGAGAGTCTTCCTCCTCGTAAGG	Forward primer for cloning NbDKML1&2 into VIGS vector pTRV2/YL156
23	VIGS_NbDKML_0466_8662_BamHI_R	TAAGCAGGATCCATATGGAAAACAGGTCATCCACAACTTCTCCTTCAG	Reverse primer for cloning NbDKML1&2 into VIGS vector pTRV2/YL156
24	VIGS_SlDKML_EcoRI_F	GGAAAgaattcATGGCTGGTGACAATGACGAAG	Forward primer for cloning SlDKMLinto VIGS vector pTRV2/YL156
25	VIGS_SlDKML_BamHI_R	TAAGCAGGATCCGTTCTGCATACCCATTTGCTGATTCC	Reverse primer for cloning SlDKML into VIGS vector pTRV2/YL156
26	AttB1_Sl/StDKML_FL_F	AAAAAGCAGGCTTCATGGCTGGTGACAATG	Forward primer for cloning SlDKMLFL or StDKML.FL into vector pDONR221
27	AttB2_Sl/StDKML_FL_NoStop_R	AGAAAGCTGGGTGCTTTGATTCATGCTTTGAAG	Forward primer for cloning SlDKMLFL or StDKML.FL into vector pDONR221
28	Gateway_attB1F	GGGGACAAGTTTGTACAAAAAAGCAGGCT	Forward primer for two-step PCR of Gateway attB1F site
29	Gateway_attB2R	GGGGACCACTTTGTACAAGAAAGCTGGGT	Reverse primer for two-step PCR of Gateway attB1R site
30	qPCR_NbDKML1A_F	CCCAGGTTCTCTTGAAAGGTCAGT	Forward primer for qPCR to check NbDKML1A silencing effect
31	qPCR_NbDKML1AR	AGTCCCCGAAGAGTTTAATGCA	Reverse primer for qPCR to check NbDKML1A silencing effect
32	qPCR_NbDKML2AF	AAGTGATGAAGCTAATACTGAGATGATGCGG	Forward primer for qPCR to check NbDKML2A silencing effect
33	qPCR_NbDKML2AR	GTCGGCCTGCTAACTGGGATATGAGAA	Reverse primer for qPCR to check NbDKML2A silencing effect
34	qPCR_SlDKMLF2	TGCAGTCATCACCACCACTT	qPCR Forward primer to check SlDKML silencing effect
35	qPCR_SlDKMLR2	TCACCCATCCCTTCAACATT	qPCR Reverse primer to check SlDKML silencing effect
36	qPCR_NbEF1aF	AGCTTTACCTCCCAAGTCATC	qPCR Forward primer for NbEF1α to normalize RT-PCR amplifications
37	qPCR_NbEF1aR	AGAACGCCTGTCAATCTTGG	qPCR Reverse primer for NbEF1α to normalize RT-PCR amplifications
38	qPCR_SlEF1aF2	TCGTGGTCATTGGTCATGTT	qPCR Forward primer for SlEF1α to normalize RT-PCR amplifications
39	qPCR_SlEF1aR2	GCACCCAGGCATACTTGAAT	qPCR Reverse primer for SlEF1α to normalize RT-PCR amplifications
40	qPCR_PiO8F	CAATTCGCCACCTTCTTCGA	qPCR Forward primer for PiO8 to quantify P. infestans biomass
41	qPCR_PiO8R	GCCTTCCTGCCCTCAAGAAC	qPCR Reverse primer for PiO8 to quantify P. infestans biomass

Supplemental Figure 2.1. Phylogenetic analysis of the *A. thaliana* 78 bZIP transcription factors.

The evolutionary history was inferred using the Neighbor-Joining method using MEGAX. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.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AT1G45249.6
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AT2G41070.1
AT3G56850.1
AT2G36270.1
AT3G44460.1
AT2G17770.1
AT4G35900.1
AT5G49450.1
AT1G06070.1
AT2G31370.1
PpDKML
AT1G06850.1
AT2G40620.1
AT1G43700.1
AT2G21230.1
AT4G38900.1
AT2G12900.1
AT2G13150.1
AT2G12940.1
AT2G21235.1
AT1G08320.1
AT5G06839.1
AT1G22070.1
AT1G77920.1
AT5G10030.1
AT5G65210.1
AT1G68640.1
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AT4G35040.1
AT3G51960.1
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AT2G24340.1
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AT4G06598.1
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AT3G56660.1
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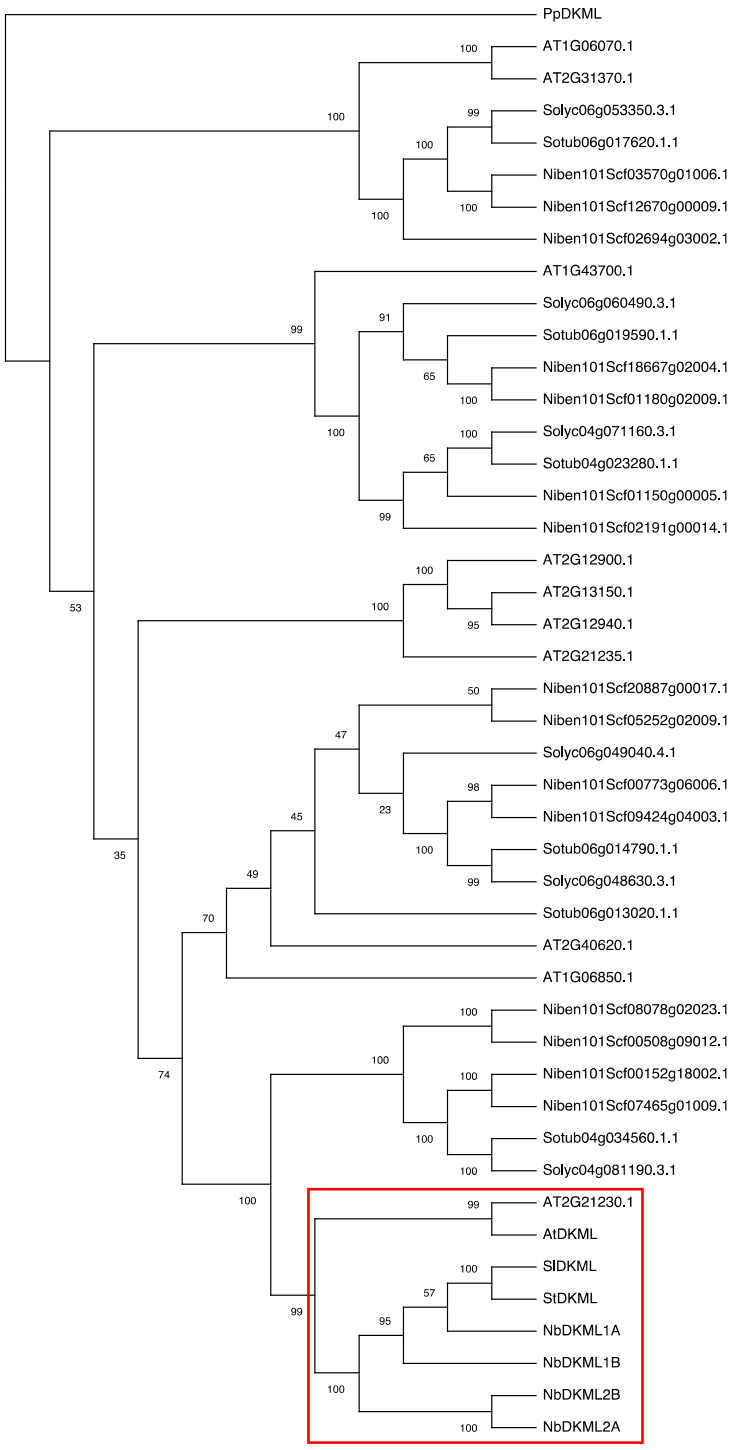
AT5G11260.1
AT1G13600.1
AT2G04038.1
AT3G30530.1
AT5G38800.1
AT5G15830.1
AT1G68880.1
AT1G32150.1
AT2G35530.1
AT4G36730.1
AT2G46270.1
AT4G01120.1
AT5G08141.1
AT5G60830.1
AT3G54620.1
AT4G02640.1
AT5G28770.1
AT5G24800.1
AT1G59530.1
AT2G22850.1
AT4G37730.1
AT3G49760.1
AT1G75390.1
AT4G34590.1
AT3G62420.1
AT2G18160.1
Clade I
Clade II
Clade III
Clade IV
Clade V
Clade VI
Clade VII
Clade VIII

Supplemental Figure 2.2. Phylogenetic analysis of the nine closest *A. thaliana* bZIP transcription factors to AtDKML and their corresponding homologs from Solanaceae family.

The analysis involved 50 amino acid sequences corresponding to bZIP TFs from the following species: *A. thaliana* (*At*), *S. tuberosum* (*St/Sotub*), *S. lycopersicum* (*Sl/Solyc*), and *N. benthamiana* (*Nb/Niben)*. The closest DKML homolog from *Physcomitrium patens* (*Pp*) was used as an outgroup. The evolutionary history was inferred using the Neighbor-Joining method using MEGAX. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.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Sotub06g017620.1.1
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Niben101Scf12670g00009.1
Niben101Scf02694g03002.1
AT1G43700.1
Solyc06g060490.3.1
Sotub06g019590.1.1
Niben101Scf18667g02004.1
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Solyc04g071160.3.1
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Niben101Scf02191g00014.1
AT2G12900.1
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AT2G21235.1
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Niben101Scf00773g06006.1
Niben101Scf09424g04003.1
Sotub06g014790.1.1
Solyc06g048630.3.1
Sotub06g013020.1.1
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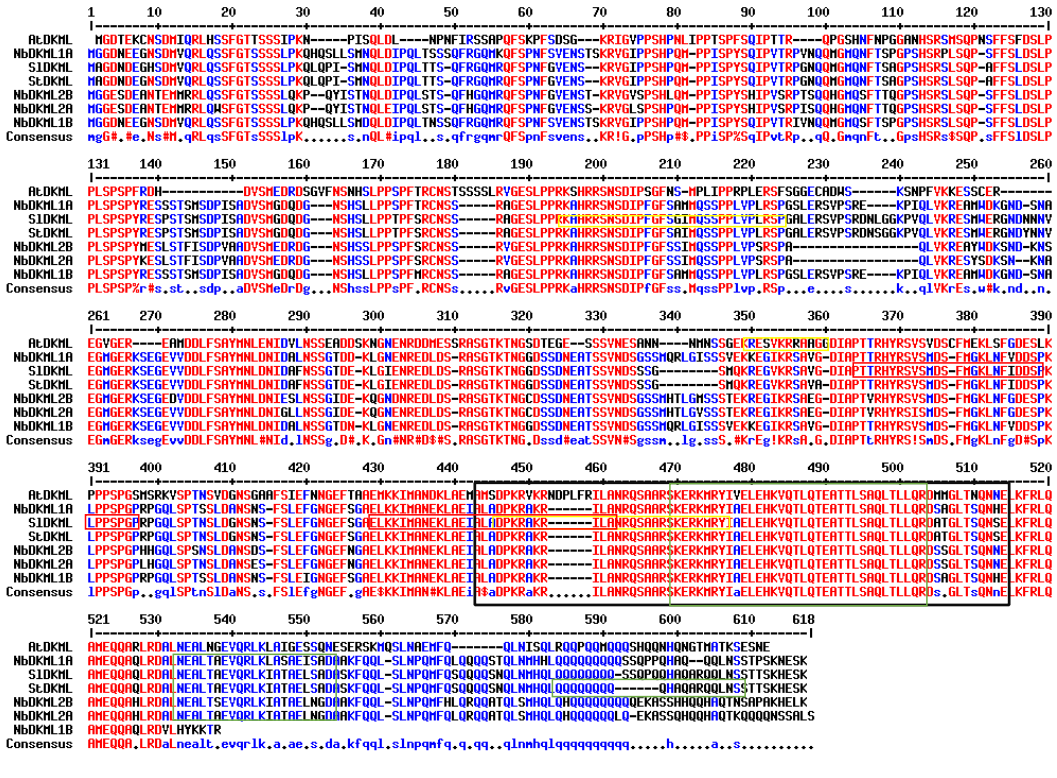
AT1G06850.1
Niben101Scf08078g02023.1
Niben101Scf00508g09012.1
Niben101Scf00152g18002.1
Niben101Scf07465g01009.1
Sotub04g034560.1.1
Solyc04g081190.3.1
AT2G21230.1
AtDKML
SlDKML
StDKML
NbDKML1A
NbDKML1B
NbDKML2B
NbDKML2A

Supplemental Figure 2.3. Multiple sequence alignment of DKML homologs from *A. thaliana* (*At*), *S. tuberosum* (*St*), *S. lycopersicum* (*Sl*), and *N. benthamiana* (*Nb*).

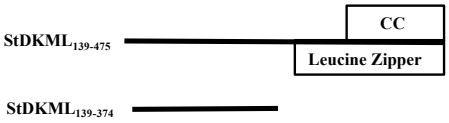
Canonical NLS are highlighted in orange and no canonical NLS are highlighted in yellow. The black outlined box indicates the bZIP domain and the green colored boxes represent the CC-domains.



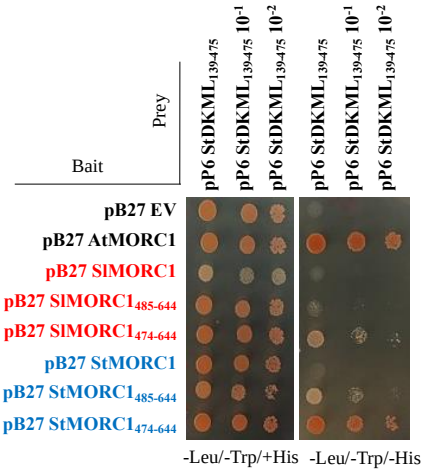
Supplemental Figure 2.4. SlMORC1 and StMORC1 binds directly to StDKML thought their C-terminal regions.

(a) Schematic presentation of the potato DKML constructs cloned into the prey vector pP6. (b) Yeast two-hybrid (Y2H) assay of full length AtMORC1, SlMORC1, and StMORC1 proteins as well as the C-terminal constructs SlMORC1$_{475-644}$, SlMORC1$_{485-644}$, StMORC1$_{475-644}$, StMORC1$_{485-644}$ as bait with the ten-fold serial dilution of the StDKML$_{139-475}$. (c) The bZIP and CC-domains of StDKML are required for the physical interaction with the MORC1 C-terminal regions.

a.)


StDKML139-475
CC
Leucine Zipper
StDKML139-374

b.)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Bait
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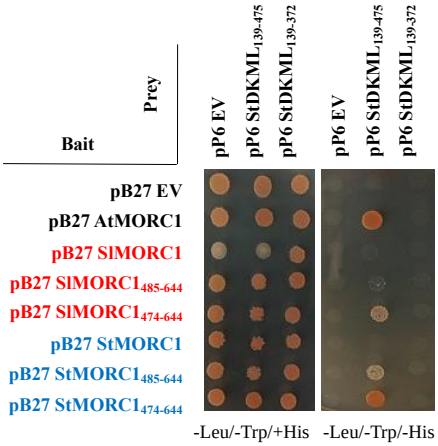
pP6 StDKML139-475 10-1
pP6 StDKML139-475 10-2
pP6 StDKML139-475
pP6 StDKML139-475 10-1
pP6 StDKML139-475 10-2
pB27 EV
pB27 AtMORC1
pB27 SlMORC1
pB27 SlMORC1485-644
pB27 SlMORC1474-644
pB27 StMORC1
pB27 StMORC1485-644
pB27 StMORC1474-644
-Leu/-Trp/+His
-Leu/-Trp/-His

c.)


Prey
Bait
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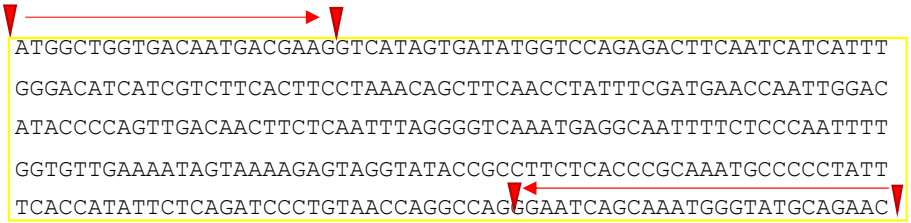
pP6 StDKML139-475
pP6 StDKML139-372
pP6 EV
pP6 StDKML139-475
pP6 StDKML139-372
pB27 EV
pB27 AtMORC1
pB27 SlMORC1
pB27 SlMORC1485-644
pB27 SlMORC1474-644
pB27 StMORC1
pB27 StMORC1485-644
pB27 StMORC1474-644
-Leu/-Trp/+His
-Leu/-Trp/-His

Supplemental Figure 2.5. Generation of Virus-Induced Gene Silencing NbDKML constructs.

The yellow box denotes the region cloned for VIGS silencing. The red arrows are the primers used to confirm DKML transcript silencing for NbDKML2A/B and the blue arrows are the primers used to confirm DKML transcript silencing for NbDKML1A/B. The green boxes indicate the CC-domains.

Supplemental Figure 2.6. Generation of Virus-Induced Gene Silencing DKML plants in tomato.

VIGS primers are denoted by the red arrow and the primers to confirm DKML transcript silencing are denoted by the blue arrows. The yellow box denotes the region cloned for VIGS silencing.



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ACGAATGGTGGTGACAGCAGTGATAATGAAGCTACAAGCAGTGTCAATGACAGTAGCAGC
GGCAGTATGCAGAAGAGAGAAGGGGTCAAAAGGAGTGCTGTGGGAGATATCGCTCCAACC
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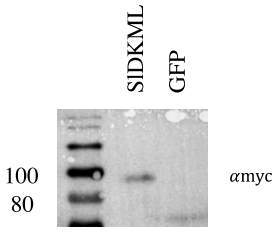
GCTTCCAAGTTTCAGCAGCTATCTCTAAATCCTCAGATGTTCCAATCGCAGCAGCAACAA
TCGAATCAGCTAAACATGCATCAGTTGCAACAGCAGCAGCAGCAGCAACAATCATCTCAG
CCACAACAACATGCTCAAGCACGACAACAACTTAACAGCTCGACGACTTCAAAGCATGAA
TCAAAGTAG

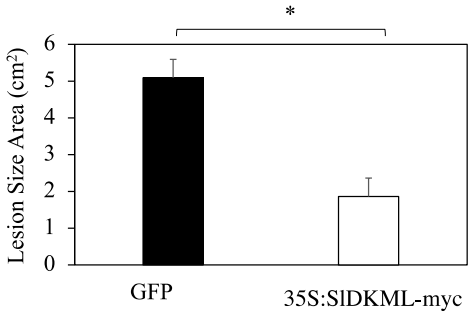
Supplemental Figure 2.7. Transiently expressing SlDKML in *N. benthamiana* significantly increases resistance to *Phytophthora infestans.*

Basal resistance was monitored in SlDKML overexpressed *N. benthamiana* plants (white bars) and in the corresponding EC1 control plants (black bars) following inoculation with the 1306 isolate of *Phytophthora infestans (Pi).* (a) Transient expression of $SlDKML_{FL}$ proteins for OE studies was confirmed and detected by immunoblot analysis using an anti-myc-HRP antibody. Lane 1 was loaded with $SlDKML_{FL}$ and lane 2 was loaded with GFP-HA. (b) TRV22:*NbDKML* and TRV2:*EC1* plants were inoculated with the 1306 isolate of *Pi* (20,000 sporangia/ml) using a detached-leaflet assay. Disease symptoms were measured based on size of the blighted area (cm^2) at 6 dpi. (c) Photographs of inoculated areas undergoing cell death taken at 6 dpi. (d) Biomass of *Pi* in TRV2:*NbDKML* and TRV2:*EC1* leaves measured by qPCR. The levels of *PiO8* element and *NbEf1a* were used to quantify the levels of *Pi* and plant cells by qPCR. Data represent the mean and standard error (n=4) from one experiment. Similar experiments were repeated at least twice with similar results. Statistical difference between TRV2:*EC1* and TRV2:*NbDKML* is indicated; *P<0.05 (*t* test).

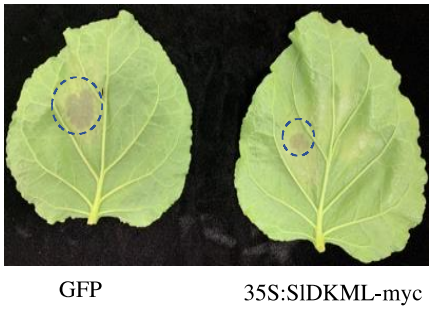
a.)



b.) **Day 10 Post Inoculation with *P. infestans***

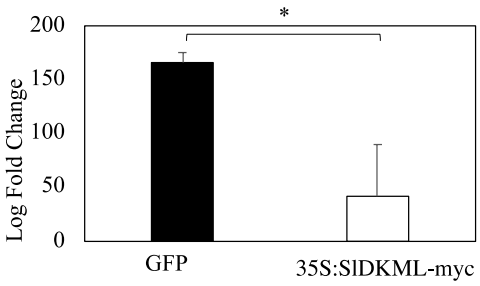


c.) **Day 10 Post Inoculation with *P. infestans***



d.)

Day 9 Biomass of *P. infestans*



CHAPTER 3

Functional studies of *Phytophthora cinnamomi* effectors in *Nicotiana benthamiana* reveal the contribution of Elicitin 2373 and RxLR 2279 effector to virulence

Abstract

Phytophthora cinnamomi is known as “the biological bulldozer” for its capacity to infect over 5000 plant species, threatening the sustainability of our crop industry, forestry, and natural ecosystems. To better understand the mechanisms that allow this oomycete to be so effective, identification and characterization of the “effector proteins” that contribute to its pathogenicity and virulence is necessary. To date, there have been no reports of functional validation of *P. cinnamomi* effectors that contribute directly to its virulence. In this study, identification of effectors contributing to *P. cinnamomi’s* virulence was carried out through transcriptomic approaches coupled with functional studies in the model plant, *Nicotiana benthamiana,* using a detached leaf assay. Five candidate effectors were investigated due to their significant expression levels upon *P. cinnamomi* infection in three different host plants, Avocado, *N. benthamiana*, and *Arabidopsis thaliana.* Synthesis of five candidate effectors were done and the functional validation of three effectors from *P. cinnamomi* were conducted, where the transient expression of the *P. cinnamomi* elicitin (Elicitin 2373) resulted in cell death and transient expression of the *P. cinnamomi* RxLR (RxLR 2279) resulted in faster and larger lesion size development after *P. cinnamomi* inoculation compared with the controls plants expressing GFP. Together, this study is the first report identifying and functionally validating two *P. cinnamomi* effectors with direct roles in the virulence of this destructive oomycete pathogen.

Introduction

The genus, *Phytophthora* is comprised of some of the most devastating oomycete pathogens threatening agriculture and food security. *P. cinnamomi* is considered one of the most destructive plant pathogens, with a broad host range of over nearly 5000 plant species (Cahill *et al.*, 2008; Hardham & Blackman, 2018). Many of these plant species include agricultural crops, forestry, and horticulture (Hardham and Blackman, 2018). Among the agricultural crops, *P. cinnamomi* has claimed fame to being the most destructive disease in the avocado industry (Wheller *et al.*, 1998). In California alone, it is estimated this pathogen has caused losses to avocado growers that exceed US$40 million annually (Ploetz, 2013).

P. cinnamomi is a soil-borne pathogen, which invades the small absorbing feeder roots of susceptible hosts, causing the roots to brown and rotten leading to plant wilting and dieback in tree crops (van den Berg *et al.*, 2021). Oomycetes like *P. cinnamomi* have distinct set of genes promoting pathogenicity and virulence through different mechanisms including the suppression of the plant immune response (Boutemy *et al.*, 2011). The first *P. cinnamomi* genome was generated through the joint genome institute (JGI) based on short read sequencing technology indicating a genome size of 78 Mb for *P. cinnamomi*, however recently, a new reference genome was published based on long and short reads indicating that the *P. cinnamomi* genome size was actually 109.7 Mb and exhibited a triploidy genome profile (Engelbrecht *et al.*, 2021). Van den berg *et al.* (2021), reported that the *P. cinnamomi* genome had 19,981 protein-coding genes, with 15,803 expressed *in vitro* and *in planta*. From these proteins, 1,347 were secreted proteins including 181

RXLRs effectors (Engelbrecht *et al.*, 2021). An earlier study in 2018 utilized the elicitin gene, PHYCI_98389, as query predicting 32 putative elicitin sequences (Hardham & Blackman, 2018). In the last decade, the sequencing of many fungal and oomycete pathogen genomes has allowed for the emergence of fundamental concepts such as the "two-speed genome" model. Under this model, some pathogen genomes exhibit a bipartite architecture with repeat-rich, transposon-rich, and gene-sparse regions (non-core genome), harboring genes involved in environmental and host adaptation as well as pathogenicity (Dong *et al.*, 2015). *P. cinnamomi* has also been characterized as having the genome architecture of a two-speed genome (Engelbrecht *et al.*, 2021). Recently, the Manosalva lab sequenced the genomes of two genetically and phenotypically distinct *P. cinnamomi* isolates from California (Pc2113 and Pc2109). Surprisingly, the genome size of these isolates ranged from 136-139 Mb based on flow cytometry (FC) and *de novo* assembly, which is larger than the currently available *P cinnamomi* genome sizes (Engelbrecht *et al.*, 2021). The Pc2113 genome is comprised of 21,403 genes, encoding 21,778 proteins in which 1,342 are secreted including 43 elicitins and 173 RxLR effectors (Shands *et al.*, *in preparation*).

Pathogen effectors can be secreted into their hosts and interfere with plant biological functions and host immunity in the extra-haustorial matrix (apoplastic effectors) or within the plant cells (cytoplasmic effectors) (Wang *et al.*, 2017; Engelbrecht *et al.*, 2021). In addition to its virulence functions, some effectors have avirulence functions through specific recognition by their corresponding resistance (R) protein, triggering ETI (Jones & Dangl, 2006; Naveed *et al.*, 2020). Based on motifs,

domains, and distinct amino acid content, apoplastic and cytoplasmic effectors are separated into different classes (Martin & Kamoun, 2011). Within apoplastic effectors there are seven classes which include small cysteine-rich (SCR) proteins, protease inhibitors, Nep1-like proteins (NLPs), elicitins, proteases (aspartyl cysteine and serine proteases), cell wall degrading enzymes, and lipases and phospholipases (Martin & Kamoun, 2011). Within cytoplasmic effectors, there are two major classes of effectors based on their N-terminal motifs, RxLRs and Crinkler (CRN) (Martin & Kamoun, 2011). In this study, a subset of effectors belonging to the classes of elicitins and RxLRs were characterized and functionally validated for their effects on *P. cinnamomi* virulence.

Elicitins are small proteins with six conserved cysteine residues that are secreted to the apoplasts or sometimes anchored to the oomycete plasma membrane and cell wall (RICCI *et al.*, 1989; Qutob *et al.*, 2003; Uhlíková *et al.*, 2017). Like most pathogen associated molecular patterns (PAMPs), if recognized by the hosts cell surface receptors, an induction of the host defense response is initiated, and a cell death response involving reactive oxygen species (ROS) burst occurs (Jones & Dangl, 2006). However, unlike typical PAMPs, studies have shown that elicitins induce a second sustained ROS burst that eventually leads to cell death (Mur *et al.*, 2008; Adachi *et al.*, 2015; Derevnina *et al.*, 2016). Additional to its involvement in triggering defense responses, a key feature of elicitins is its ability to bind to sterols and other lipids. This is a crucial element for *Phytophthora* species since they cannot synthesize sterols themselves so they rely on elicitins to bind and carry them from the plant membrane to the pathogen (Osman *et al.*, 2001). Infestin 1 (INF1) is a highly characterized elicitins from *P. infestans*, inducing cell

death upon recognition in certain host plants like *N. benthamiana* and tomato (Kamoun et al., 1997).

RxLRs are secreted proteins that include a signal peptide followed by a conserved motif in the N-terminal domain defined as RxLR (Arg-Xaa-Leu-Arg) and sometimes trailed by an EER (Glu-Glu-Arg) motif (Boutemy *et al.*, 2011; Bozkurt *et al.*, 2012). Though the RxLR domain is necessary for the translocation inside plant cells, the biochemical activity of the effector is not dependent on the RxLR motif, but rather the C-terminal region (Whisson *et al.*, 2007; Schornack *et al.*, 2009). Though RxLRs do have orthologs across many *Phytophthora* species, many RxLRs are highly diverse and can be exclusive to a given species (Engelbrecht *et al.*, 2021). This specificity is of great interest when developing precise diagnostic tools for detecting and differentiating pathogens infecting a given plant or crop. A recent study identified and briefly characterized a unique RxLR effector from *P. cinnamomi* for diagnostic tool improvement (Dai *et al.*, 2020). The study focused on the RxLR effector Avh87 of *P. cinnamomi,* which encompasses all the typical hallmarks of an RxLR including a signal peptide cleavage site and an RxLR motif followed by an EER motif. Additional to being a good candidate for diagnostic tool development, functional assays revealed that RxLR Avh87 behaves as a suppressor of programmed cell death when co-inoculated with the pro-apoptotic protein Bax (21kDa human protein of the *bcl-2* family) and INF1 (Kamoun *et al.*, 1997; Brady & Gil-Gómez, 1998; Dai *et al.*, 2020). However, no other studies have been reported on the characterization of more RxLR effectors or other types of *P. cinnamomi* effectors such as elicitins in depth. In this study, several datasets from RNA-sequencing (RNA-Seq)

experiments conducted by the Manosalva lab using several host plants infected by *P. cinnamomi* at different time points in order to select candidate effectors for further functional validation. Validation by qRT-PCR confirmed the transcript up-regulation of all the candidate effector genes after *P. cinnamomi* infection in *N. benthamiana* leaves and avocado roots. Lastly, functional studies were performed to gain more information on how some of these effectors contribute to *P. cinnamomi* virulence.

Materials and methods

Maintenance of *Phytophthora cinnamomi* isolates.

This study used the *P. cinnamomi* isolate Pc2113, obtained from the Manosalva Lab collection. Isolates were maintained as water agar plugs (Boesewinkel, 1976). To resume growth, agar plugs were removed and plated on 10% clarified V8 agar (10 g of $CaCO_3$ per 1 liter of V8 juice spun down at 4000 rpm for 20 min) and grown at 22°C in the dark.

Plant material.

Zutano avocado rootstock seeds were germinated in 7 cm deep pots with vermiculite. Seedlings were grown under glasshouse conditions at temperatures averaging 25 °C to 28°C at 40 % to 50% relative humidity. Seedlings were watered daily with industrial water and once a week with fertilized water. At 14 weeks (wks), the seed cotyledons were removed and at 16 wks the plants were inoculated using a millet inoculation method. *Nicotiana benthamiana* plants were grown in a growth room under cycles of 16 h of light and 8 h of darkness at 22°C with 70% relative humidity.

Nicotiana benthamiana detached leaf inoculations

Leaves from 4 to 4.5-wk old plants were used for all *P. cinnamomi* inoculation experiments in this study. For gene expression studies, leaves were inoculated in the abaxial side with four 20 µl drops of zoospores as described in Belisle *et al*. (2019). Samples for each biological replicate contained 3 inoculated leaf discs were pooled from 3 individual plants. At least three biological replications, both pathogen and mock inoculated, were produced for each timepoint using 6 plants for gene expression analyses of the candidate effectors. For experiments investigating the candidate effectors contribution in *P. cinnamomi* virulence, leaves were transiently expressed with the construct, detached at 12 hours post inoculation (hpi) and inoculated on the abaxial side of the leaves with six-day-old 7 mm plugs of *P. cinnamomi* isolate Pc2113. Leaves were then incubated in a plant growth chamber at 23°C on inverted 15-cm 1.5% water agar plates and evaluated over the course of 5 days. At least three biological replications, both pathogen and mock inoculated, were used in these experiments.

Avocado root millet inoculations

Millet inoculum was prepared as described in Belisle et. al (2018) with some modifications. *P. cinnamomi* isolate Pc2113 plugs and 50ml of 10% clarified V8 broth were added two twice autoclaved millet. After 21 days of incubation at 25°C in the dark, the millet was harvested, weighed, and mixed in a plastic bag. Two weeks prior to inoculation, fertilization was paused as it can disrupt the infection process. Each plant had a total of 3 g. of inoculum added to the surrounding of the root collar and covered

with vermiculite. A total of six biological replicates (6 plants) were used for each time point, pooled for gene expression analyses.

RNA extraction.

Different RNA extraction protocols were executed depending on the tissue sample. For *N. benthamiana* leaves, total RNA was extracted from both *P. cinnamomi* inoculated and mock leaves collected at 16 hpi and 24 hpi time points. Three leaf discs (9 mm in diameter) were cut using a # 4 cork borer from each leaf and frozen in liquid nitrogen immediately. RNA of each leaf disc was extracted using the RNeasy Plant Mini Kit (Qiagen, Germantown, MD) following the manufacturer's instructions and then treated with DNase using the Invitrogen TURBO DNA-free Kit (Thermo Fisher Scientific, Waltham, MA) to remove genomic DNA contaminations. RNA clean-up was performed using the Zymo RNA Clean and Concentrator Kit (Zymo Research Corp., Irvine, CA). For Avocado root tissue, total RNA was extracted from both inoculated and mock roots collected at 24 hpi, 48 hpi, 72 hpi, and 1wk post inoculation. Approximately 8 g. of root tissue were collected and frozen in liquid nitrogen immediately. RNA was extracted using a modified protocol of Chang *et al.* Modifications to the protocol included using 0.12 g. of tissue and a change in the extraction buffer to 2% CTAB (hexadecyltrimethylammonium bromide), 2% PVP K-30, 100mM Tris-HCl ph 8.0, 25mM EDTA, 2M NaCl and 2% ß-mercaptoethanol extraction buffer (Chang *et al.*, 1993). DNA removal and RNA clean-up was conducted as described above. RNA quantification was done using a nanodrop (DeNovix DS-11 Series).

DNA extraction.

For DNA extraction, 0.12 g. of avocado root tissue was incubated in 3% CTAB extraction buffer for one hour at 65°C and was processed as described in Barbier *et al*., (2019). RNase cocktail (Invitrogen) was used for RNA removal following the manufacturers protocol. DNA quantification was done using a nanodrop (DeNovix DS-11 Series) and was used to determine the relative biomass of *P. cinnamomi* within the collected root samples using quantitative real-time PCR (qRT-PCR) with the *LPV3* biomass marker (Kong *et al.*, 2003).

qRT-PCR validation.

qRT-PCR was used to validate RNA-Seq results with the primers in Supplemental Table 3.2. qRT-PCR analysis of five selected highly up-regulated *P. cinnamomi* effector genes were performed to validate RNA-Seq results and conduct functional validation. cDNA synthesis were done using the Invitrogen Superscript III Kit (Thermo Fisher Scientific) following the manufacture's protocol. Each 10-ul reaction included 4 µl of a cDNA (1:20 dilution) or 4 µl of DNA (12.5 ng/µl), 5 µl iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA), 0.5 µM of each forward and reverse qRT-PCR primers (Table S3.2). Reactions were amplified using a CFX384 Touch Bio-Rad Real Time PCR system (Bio-Rad Laboratories) at the following conditions: 95°C for 3 min (initial denaturation) followed by 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 55°C for 30 s. A dissociation curve was generated at the end of each qRT-PCR to verify single product amplification. Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ (Ct = cycle threshold) comparative method. The results were

reported as the mean ± standard error of three or five biological replications. Pearson's correlation test was used to evaluate significance. All primers in this study were designed using primer 3 (version 0.4.0) software. *Elongation factor 1* (*EF1-a1*) (1/2) was used as an endogenous reference gene for *N. benthamiana* (3/4) and *P. americana* (5/6). Primer efficiency for each pair was calculated via the slope of a standard curve constructed by the amplification of 10-fold dilution series of DNA (covering 5 dilution points, 1-10,000 pg). All primer sets were between 95-99% efficient.

Heatmap of candidate effector gene expression

The expression profiles of each candidate effector genes selected for this study was obtained from the different RNA-Seq datasets analyzed using DEseq2 by the Manosalva lab. Visualization of the expression pattern for each differentially expressed candidate effector at different time points after inoculation in different hosts were generated using Pheatmap v1.0.12 (Kolde, 2015).

Sequencing and synthesis of P. cinnamomi candidate effectors

Full-length CDS of the genes *RXLR 2407*, *RXLR 2279*, *RXLR 2266*, *elicitin 2373*, *elicitin 2418*, and *RXLR 2407-like* were amplified using Q5 High-Fidelity DNA Polymerase (New England BioLabs) with conditions as follows: 98°C for 3 min; followed by 35 cycles at 98°C for 10 s, 55°C for 10 s, 72°C for 50 s; and a final extension at 72°C for 5 min. Products were confirmed on a 1.5% agarose gel, excised, and purified with the Zymoclean Gel DNA Recovery Kit. Products were Sanger sequenced through Eurofins Genomics sequencing services. The sequencing results were used for the synthesis of these target genes through GenScript (New Jersey, USA). The five candidate

P. cinnamomi effectors selected from our RNA-Seq studies, a RXLR effector previously characterized by Dai *et al.* (2020), and the GFP negative control were synthesized by GenScript. Each effector was obtained in pUC57 vector and sub-cloned into a binary expression vector pJL-TRBO (Lindbo, 2007) using restriction enzyme sites *PacI* and *NotI* or *PacI* and *AvrII*. The final expression vectors were used to transform *Agrobacterium tumefaciens* strain GV2260 and used them for functional assays in *N. benthamiana.*

Agrobacterium tumefaciens*-mediated transient expression in *N. benthamiana.

Vector pJL48:TRBO containing effector candidates FLAG:RXLR2279 and FLAG:Elicitin2373, FLAG:Avh87 and negative control (FLAG:GFP) were electroporated into *A. tumefaciens* strain GV2260. *A. tumefaciens*-mediated transient expression of candidate effectors were performed similarly as described in Du and Vleeshouwers, (2014). Positive transformants were grown on LB Kan^+ for 48 h at 28°C. Individual bacterial colonies were grown in liquid LB Kan^+ for 18 h at 28°C and glycerol stocks were made. Transformed *A. tumefaciens* grown for 48 h LB Kan^+ agar plates were suspended in infiltration buffer (10mM $MgCl_2$, 10mM MES [pH5.5], and 200µM acetosyringone) and the abaxial side of leaves of four-week-old *N. benthamiana* plants were infiltrated using cultures at a final optical density (OD) of 0.4. For inducing cell death activities, FLAG:RXLR2279, FLAG:Elicitin2373, FLAG:Avh87 and negative control (FLAG:GFP) were inoculated in *N. benthamiana* leaves and cell death monitored for up to 7 days post (dp) *Agrobacterium* infiltration. For testing suppression of INF1-induced cell death by the effectors, a sequential *Agrobacterium* infiltration experiment

was designed. One infiltration zone had *A. tumefaciens* cells carrying RxLR 2279 alone, the second infiltration zone had co expression of both RxLR 2279 and INF1 at the same time (labeled as RxLR 2279 + INF1 0h), the third infiltration zone was first inoculated with RxLR 2279 then 12h later INF1 (labeled as RxLR 2279 + INF1 12h), and fourth zone was first inoculated with RxLR 2279 then 24h later INF1 (labeled as RxLR 2279 + INF1 24h) (Wang *et al.*, 2011). This design was also used for the negative control, GFP, and the RxLR Avh87 effector. Infiltration sites were observed over a period of 5 days.

Immunoblot analyses.

Two leaf discs were collected at 24, 48, and 72 hpi with *Agrobacterium* carrying effector constructs or GFP. Crude extracts were obtained by grinding the tissue in 50ul of 2X SDS buffer. Immunoblots were performed as described in Kang et al., (2008). Protein loading was checked by visualizing the large subunit of RuBPCase by staining the polyvinylidene fluoride membrane (Millipore) with Coomassie Brilliant Blue. Signals were detected by a chemiluminescence reaction using the ECL Prime Western Blotting Kit (Cytiva) and visualized in the ChemiDoc MP machine (BioRad). Monoclonal anti-FLAG-Tag (Invitrogen) antibody was used at a dilution of 1:5000.

Carnoy staining.

Cell death was visualized in leaves at 4 and 5 dpi INF1 infiltration and 5 dpi *P. cinnamomi* inoculation by removing the chlorophyll through 60% ethanol, 30% chloroform and 10% acetic acid treatment.

Results

Selection and transcriptional validation of P. cinnamomi effectors during infection

To identify *P. cinnamomi* effectors contributing to pathogenicity or virulence, five candidate effectors (*RxLR 2407*, *RxLR 2266*, *RxLR 2279*, *Elicitin 2418*, and *Elicitin 2373*) genes were selected based on their expression profiles after pathogen infections in more than one host plant (Fig. 3.1 & Table S3.1). Through the software EffectorP V3.0, each of the five selected candidates were classified as either a cytoplasmic or apoplastic effectors. Three of the candidates, (RxLR 2407, RxLR 2266, and RxLR 2279) were predicted to be cytoplasmic effectors (Table S3.1). Elicitin 2418 was classified as an apoplastic effector and Elicitin 2373 did not classify as either an apoplastic or cytoplasmic effector using this prediction program (Table S3.1). The selected candidate genes were among the most highly up-regulated effectors *in planta* across two or all hosts when compared with their expression in *in vitro* grow Pc2113 mycelium and sporangia (Fig. 3.1). Moreover, these effectors are not expressed in *P. cinnamomi* zoospores obtained from *in vitro* grow Pc2113 cultures (Fig. 3.1). Candidate effectors *RxLR 2266*, *RxLR 2407*, *Elicitin 2418*, and *Elicitin 2373* were up-regulated between 4.8-13.4 log fold change in all hosts after infection (Fig. 3.1 & Table S3.1). *RxLR 2279* was up-regulated between 10-12 log fold change in *N. benthamiana* and Avocado (Fig. 3.1 & Table S3.1).

During infection of *N. benthamiana* leaves, *RxLR 2407*, *RxLR 2266*, and *RxLR 2279* transcripts were significantly up-regulated at 16 hpi and 24 hpi (Fig. 3.2). *RxLR 2407* and *RxLR 2279* exhibited the highest up-regulation at 16 hpi while *RxLR 2266*

exhibited the highest expression at 24 hpi. *Elicitin 2418* and *Elicitin 2373* were nearly undetectable at 16 hpi then strongly up-regulated at 24 hpi (Fig. 3.2).

Taking in account that *P. cinnamomi* is mainly a root pathogen, we conducted similar experiments in avocado roots infected with Pc2113 and validated the expression profile of the candidate effectors in avocado roots after infection before conducting functional assays. We confirmed the successful Pc2113 root infection by root plating assays of the infected and mock inoculated avocado plants (*data not shown*) as well as conducting pathogen biomass quantification at different time points after root inoculations (Fig. 3.3). Successful disease progression correlated with an increase in pathogen biomass starting at 48 hpi, which is after haustoria formation (Belisle *et al.*, 2019) and significantly increased at 72 hpi with the highest biomass exhibited at 168 hpi indicating successful avocado root colonization (Fig. 3.3).

Consistent with the results in *N. benthamiana*, we observed that all the candidate effectors are differentially expressed in avocado roots after *P. cinnamomi* infection (Fig 3.4). Effectors *RxLR 2407* and *RxLR 2266* exhibited significant up-regulation at the latest time point after inoculation while *RxLR 2279* had shown significant up-regulation starting at 24 hpi which significantly increased over time (Figure 3.4). Interestingly, *Elicitin 2418* exhibited similar expression patterns as *RxLR 2279*, starting as early as 24 hpi with progressively higher levels of expression over time. High transcripts levels of *Elicitin 2373* were observed at 72 and 168 hpi (Fig 3.4). The significant up-regulation of all the effectors selected in this study after infection of *N. benthamiana* leaves and avocado roots suggest a role of these effector in *P. cinnamomi* virulence.

Synthetized P. cinnamomi effectors are successfully expressed in N. benthamiana after Agrobacterium infiltration assays

Given the strong expression patterns of *RxLR 2407*, *RxLR 2266*, *RxLR 2279*, *Elicitin 2418*, and *Elicitin 2373* after infection, we hypothesized that these effectors may contribute to *P. cinnamomi* infection. To test this hypothesis, these effectors were synthesized (GenScript) to perform *in planta* transient assays and investigate their roles in pathogen virulence (McLellan *et al.*, 2013; King *et al.*, 2014). Prior to gene synthesis, effector sequences were confirmed by PCR coupled with Sanger sequencing to provide accurate transcript sequences for each construct designed. Constructs for each RxLR effector proteins were designed after the RxLR domain or if present the EER motifs (Fig. 3.5). RxLR Avh87 was cloned as described in Dai *et al.* (2020), after the signal peptide site (Fig. 3.5).

For elicitin synthesis, constructs were designed after the signal peptide cleavage site (Fig 3.5). For negative control purposes, GFP full length was also synthetized by GeneScript. Each construct in this study included a FLAG tag fused at the N-terminal region of each protein to allow detection and confirmation of their expression *in planta.* High levels of protein expression *in planta* were observed at 72 hp *Agrobacterium* infiltration for FLAG:GFP (30 KDa), FLAG:RxLRAvh87 (17 kDa), and FLAG:Elicitin2373 (26 kDa) whereas FLAG:RxLR2279 (16 kDa) had high levels at 48 hp *Agrobacterium* infiltration (Fig. 3.6a, b).

Elicitin 2373 triggers cell death after transiently expressed in *N. benthamiana* leaves

To determine if the effector proteins selected trigger cell death *in planta,* signs of induced cell death was monitored over a period of 6 dp *Agrobacterium* infiltration in *N. benthamiana*. Transient expression of GFP and INF1 was used as negative and positive controls, respectively. As expected for a positive control, transient expression of INF-1 triggered cell death in every experiment at 3 dp *Agrobacterium* infiltration (Fig. 3.7, 3.8). Our GFP negative control did not triggered any cell death at any time post infiltration (Fig. 3.7, 3.8).

Transient expression of FLAG:RxLRAvh87 and FLAG:RxLR2279 did not show any signs of cell death after *Agrobacterium* infiltration (Fig. 3.7a, b, c). In contrary, similar to INF1, FLAG:Elicitin2373 triggered cell death at 4 dp *Agrobacterium* infiltration (Fig. 3.8a, b, c). Together, these results indicate that Elicitin 2373 can induce cell death in *N. benthamiana* whereas RxLR 2279 and RxLR Avh87 alone do not initiate a cell death response.

Effector activity on INF1-induced cell death

Oomycete effector proteins especially RxLRs, have been shown to suppress plant immunity to trigger pathogen susceptibility (Wang *et al.*, 2011). Several *Phytophthora* effectors including RxLR Avh87 from *P. cinnamomi* have been shown to suppress the cell death induced by the PAMP, INF1 (Bos *et al.*, 2006; Dai *et al.*, 2020). Thus, we assessed if some of the effectors in this study also have activity on suppressing INF-1 induced cell death. To test this, sequential *Agrobacterium* infiltrations were performed as

described in the methods. The same sequential *Agrobacterium* infiltrations were done with INF1 and GFP as a control. Though after 3 dp INF1 infiltration there was a slight trend of accelerated cell death in zones expressed with FLAG:RxLR2279 24 h prior to INF1 (Fig. 3.9a, b), this acceleration was not observed at 4dp INF1 infiltration (Fig S3.1a,b). Additionally, we tested the previously described suppressor of INF1-induced cell death, RxLR Avh87 effector (Dai *et al.*, 2020). Surprisingly, we did not observe a strong cell death suppressing phenotype as previously reported for RxLR Avh87 in our experiments (Fig. 3.9c, d & Fig. S3.1). However, we did observed a delay on cell death at 3 and 4 dp INF1 infiltration when FLAG:RxLRAvh87 effector was expressed 12 h and 24 h before INF1 when compared with GFP control infiltrated with INF1 at the same times (Fig. 3.9c, d & Fig. S3.1). Together these results suggest a weak effect of these *P. cinnamomi* RxLR effectors on INF1-Induced cell death.

Transiently expression of *Phytophthora cinnamomi* RxLR effectors enhance pathogen susceptibility in *N. benthamiana*

To test whether RxLR 2279 and RxLR Avh87 were contributors to *P. cinnamomi's* virulence, transient expression of FLAG:RxLR2279 and FLAG:RxLRAvh87 in *N. benthamiana* leaves were conducted 12 h prior to inoculation with Pc2113. Leaves transiently expressing FLAG:RxLR2279 had significantly larger lesion sizes after Pc2113 inoculation when compared to leaves transiently expressing FLAG:GFP controls at 4 and 5 dpi in two experiments conducted (Fig. 3.10, 3.11, & S3.2). This result argues that RxLR2279 effector positively contributes to *P. cinnamomi* virulence. Contradictory results were obtained for FLAG:RxLRAvh87 effectors in two

experiments conducted (Fig. 3.10, 3.11, & S3.2). In the first experiment, transiently expression of FLAG:RxLRAvh87 effector enhanced resistance to *P. cinnamomi* at 4 but not at 5 dpi (Fig. 3.10, 3.11). Contrarily, in a second experiment transiently expression of FLAG:RxLRAvh87 effector increased susceptibility to this pathogens was observed at 4 dpi, as indicated by the significantly larger lesion size compared to control plants expressing GFP (Fig. S3.2). Together these results indicate that RxLR 2279 significantly contributes to the virulence of *P. cinnamomi*, however, the role of RxLR Avh87 in *P. cinnamomi* infection is unconclusive. More experiments need to be conducted to confirm the roles of this effector during plant-*P. cinnamomi* interactions.

Discussion

In this study, we selected five *P. cinnamomi* candidate effectors, *RXLR 2407*, *RXLR 2279*, *RXLR 2266*, *Elicitin 2373*, and *Elicitin 2418* based on their high up-regulation at different time points after Pc2113 infection based on several RNA-Seq experiments in Avocado, *N. benthamiana*, and Arabidopsis conducted by several members from the Manosalva lab. Differential expression analysis of these multi-species RNA-Seq datasets revealed a total of 2378 pathogen genes up-regulated with a log fold change of ≥2 and adjusted P-values ≤0.05 in any of the three hosts (Avocado, *N. benthamiana*, and Arabidopsis) after infection. Of these 2378 up-regulated genes, 39 encoded for RxLRs effectors and 13 for elicitins (Shands et al., *in preparation*). As expected *RXLR 2407*, *RXLR 2279*, and *RXLR 2266* where classified as cytoplasmic and *Elicitin 2418* as apoplastic effector. With the exception of *RxLR 2279*, which showed up-

regulation in *N. benthamiana* and avocado, the other four selected effectors showed up-regulation in all the three hosts (Table S3.1).

With such abundance, it is expected that effectors can range in specificity to a given species. When comparing the five effectors to other *Phytophthora spp.* such as *P. sojae, P. capsici, P. fragariae, P. infestans, P. parasitica, and P. ramorum*, RxLR 2266 and RxLR 2279 were unique to *P. cinnamomi*, whereas the other three effectors had orthologs found in the other species (Table S3.1). Elicitin 2418, Elicitin 2373 and RxLR 2407 were found in all 6 species analyzed. Interestingly, the ortholog of RxLR 2407 in *P. sojae*, was identified as the well-studied RxLR, Avr1b (Shan *et al.*, 2004). *Avr1b*, is as a major virulence effector of *P. sojae* (Shan *et al.*, 2004). It is a small, secreted protein with a highly polymorphic C terminus that is necessary to overcome resistance in plants carrying the R-gene, *Rps*1b (Shan *et al.*, 2004). Depending on the expression of *Avr1b* in *P. sojae* transformants dictates the strains ability to be highly virulent or incapable of infection of soybean (Dou *et al.*, 2008).

In addition to standard predictive pipeline measures for identifying the effectors, Shands *et al.* (*in preparation*) separated the effectors into multiple categories. One category broadly assigned each effector gene a location within the genome through segregation between GDRs, GSRs, and GIB (Raffaele et al., 2010; Rojas-Estevez et al., 2020). This established a way of visualizing gene distribution of effector candidates within the genome. Not only did this highlight the complexity of *P. cinnamomi's* two-speed genome, but it provided us a clue in whether a particular gene was more or less likely to undergo evolutionary changes, critical for *P. cinnamomi's* survival and

pathogenesis (Engelbrecht *et al.*, 2021). Since genes within the GSR (and perhaps within the IBR) are more likely to be subjected to prompt evolutionary changes due to the richness in transposable elements in this region (Raffaele *et al.*, 2010; Rojas-Estevez *et al.*, 2020; Engelbrecht *et al.*, 2021), we were interested in how our five candidate effectors were categorized.

Elicitin 2418 was the only candidate effector gene found within the GDR, indicating that it was within a region of the genome consisting of high gene density, conserved gene order, and low transposable elements (Pais *et al.*, 2013). Consistent with its identification within the GDR, Elicitin 2418 orthologs were found in six other *Phytophthora spp*., indicating that it may in fact be a conserved gene ortholog. Considering Elicitin 2418 was also predicted to be an apoplastic effector and identified within the GDR, it is likely that Elicitin 2418 is a PAMP and alone can be recognized by plant receptors and induce a plant immune response (Derevnina *et al.*, 2016). The *RxLR 2407* gene was the only effector identified within the GSR. Genes within GSRs are typically important for virulence and pathogenicity as they are in highly plastic genomic regions, enriched with transposable elements and expanded repeats (Rojas-Estevez *et al.*, 2020). It would follow suit that this effector would be an important player in *P. cinnamomi* virulence and has probably experienced high evolutionary changes across the different strains of *P. cinnamomi*, as seen with its *P. sojae* ortholog, *Avr1b*, which has exhibited higher rates of sequence polymorphisms and positive selection among 26 different field isolates of *P. sojae* (Zhang *et al.*, 2019). RxLR 2279 was not designated within the GSR, GDR, GIB, but considered "not determined" (Raffaele *et al.*, 2010;

Rojas-Estevez *et al.*, 2020). Additionally, RxLR 2266 did not matched any orthologs in other *Phytophthora* species. labeling it a *P. cinnamomi* specific RxLR. Both candidate genes *RxLR 2266* and *Elicitin 2373* were designated within the GIB. Genes within the GIB have flanking intergenic regions larger than the distribution found in GDRs but smaller than that of GSRs (Raffaele *et al.*, 2010; Rojas-Estevez *et al.*, 2020). It is likely that these genes undergo a higher rate of evolutionary changes than genes found in the GDR, however, not as often as their GSR counterparts. The fact that all these candidate effectors were found within such different architectures of the genome and all candidate effectors were so highly expressed across multiple host species made our effector candidate list very appealing to further investigate, validating their expression profiles and exploring their involvement in pathogen virulence.

Previous studies have reported 61 RxLR candidate genes differentially expressed after *P. cinnamomi* root inoculation with zoospores in avocado (Joubert *et al.*, 2021). Interestingly, our three candidate RxLRs (RxLR 2279, RxLR 2266, and RxLR 2407) were not found in the Joubert et al. (2021) RxLR list. To our knowledge, there have been no reports showing the differential expression of *P. cinnamomi* elicitins during pathogen infection in any host plants. Thus, we were very excited to report two elicitins which were up-regulated in all three host plants analyzed reflecting their importance for plant-*P. cinnamomi* interactions.

In this study, we also validated the expression profile of our candidate effectors (*RxLR 2407, RxLR 2266, RxLR 2279, Elicitin 2418,* and *Elicitin 2373*) in *N. benthamiana* leaves and avocado roots infected with Pc2113 using qRT-PCR, further suggesting their

involvement in pathogen virulence. In *N. benthamiana* leaves infected with *P. cinnamomi* zoospores, biotrophic growth is dominant until 48 hpi where development of necrotic lesions can be observed (Belisle *et al.*, 2019). *Elicitin 2418* and *Elicitin 2373* showed significantly high expression after 24 hpi in *N. benthamiana* leaf inoculations, indicating important roles during the later stages of the biotrophic phase of *P. cinnamomi* infection. Additionally, *Elicitin 2418* and *Elicitin 2373* were highly expressed in later time points (72 hpi and 168 hpi) in avocado roots inoculated with *P. cinnamomi* millet further suggesting that *Elicitin 2418* and *Elicitin 2373* may be involved in critical pathways necessary for sustained pathogen growth. Both *RxLR 2407* and *RxLR 2266* in Pc2113 infected *N. benthamiana* leaves were highly expressed at early time points (16 and 24 hpi), however, high expression was only observed at later timepoints in avocado root inoculations (168 hpi) possibly reflecting differences in the kinetics of infection due to the different hosts analyzed. Interestingly, *RxLR 2279* was expressed earlier in both *N. benthamiana* leaf and avocado root infections, further stressing its importance in virulence likely through the suppression of host defense response genes, however, more experiments must be done to test this hypothesis. Together, the RNA-Seq transcriptome data in conjunction with the qRT-PCR validation in different hosts provided us with the confidence that these five candidate effectors are important for the interaction between the pathogen and their host. Functional validation of RXLR 2279 and Elicitin 2373 was performed by transiently expressing their corresponding proteins to assess any effect on cell death and immunity. Although synthesis of Elicitin 2418, RxLR 2266, and RxLR 2407 was completed, *Agrobacterium* transformations and functional validations have not

been executed. Future experiments will be performed to explore the context in which Elicitin 2418, RxLR 2266, and RxLR 2407 aid in *P. cinnamomi* virulence.

RXLR effectors constructs for this study were designed to express their corresponding proteins without the RXLR or DEER domains. Previously, *in planta* expression screens of RxLR effectors from *P. infestans* had shown that the RxLR effector AVRblb2 did not require the RxLR motif to cause cell death upon recognition by *R* gene *Rpi-blb2* (Oh *et al.*, 2009). Although the RxLR domain is required for translocation inside plant cells, when expressed directly inside host cells by *Agrobacterium* transformation, the RxLR motif is rendered unnecessary since the C-terminal region dictates the effector activity (Boutemy *et al.*, 2011). Interestingly, when expressed *in planta*, FLAG:RxLR2279 and FLAG:Elicitin2373 had slightly larger band sizes than expected suggesting post-translational modifications such as phosphorylation, ubiquitination, myristoylation, palmitoylation, oligoimerization or ADP-ribosylation (Tahir *et al.*, 2019). To test this possibility, we investigated if posttranslational modifications are predicted to occur in these two effector proteins by predicting putative phosphorylation sites at their C-terminal regions (*data not shown*) which are the protein region expressed *in planta* in this study and showing high molecular weight by immunoblot analyses (Fig. 3.6). Using NetPhos V3.1 four different amino acids were predicted to putative undergo phosphorylation by protein kinase C (PKC) and by protein kinase A (PKA) at the C-terminal region of RxLR 2279, possibly accounting for this change in band size from 12.5 kDa to 14 kDa. Additionally, several amino acids were predicted to undergo phosphorylation some of them by PKC and cyclin dependent kinase

(cdc) 2 at the C-terminal region of Elicitin 2373, possibly accounting for its change in band size from 18 kDa to 26 kDa. Together these results indicate that Elicitin 2373 and RxLR 2279 may undergo post-translational modifications when *in planta.*

To further elucidate the roles of RxLR 2279 and Elicitin 2373 during *P. cinnamomi* infection, functional studies were performed in *N. benthamiana* leaves. This was the first study looking into the effects of Elicitin 2373 and its potential role in *P. cinnamomi* virulence. *Elicitin 2373* was predicted as an apoplastic effector, with orthologs identified in other *Phytophthora* species. Predicted structural features of Elicitin 2373 included a N-terminal signal sequence, a core elicitin domain and a variable C-terminal region (InterPro). Similar elicitin domain structures were described in *P. sojae,* where five elicitins (SOJA-2, SOJB, SOJ2, SOJ3, SOJ5, SOJ6, and SOJ7) induced HR in *Nicotiana* species and were predominantly expressed in mycelia and during infection (Qutob *et al.*, 2003). Similar to the *P. sojae* elicitin proteins, upon transient expression of Elicitin 2373 in *N. benthamiana* leaves we also observed a cell death response. It would be interesting to identify the mechanisms in which Elicitin 2373 initiates this cell death response. Studies looking into INF1 from *P. infestans* have identified several plant proteins involved in recognizing and transducing INF1-induced cell death (Kanzaki *et al.*, 2008; Du *et al.*, 2015). Future studies should include whether these same plant proteins are also involved in the recognition of Elicitin 2373.

Upon transient expression of RxLR 2279, infiltration zones were sequentially infiltrated with INF1, a slight acceleration in cell death was observed. Interestingly, this suggested that RxLR 2279 did not suppress the plant response genes triggered by INF1.

However, *N. benthamiana* leaves transiently expressing RxLR 2279 did show increased lesion areas after infection with *P. cinnamomi* plugs compared to GFP control leaves, clearly suggesting that RxLR 2279 is an important player in *P. cinnamomi* virulence. To better understand the mechanisms in which RxLR 2279 behaves in *P. cinnamomi* biological function, future experiments should include identification of putative host targets as well as generation of *P. cinnamomi* knock-out mutants to determine its role in pathogenicity.

In this study we also included the previously reported *P. cinnamomi* specific RxLR, Avh87, to test its effects in suppressing INF1-induced cell death and contributions to *P. cinnamomi* virulence (Dai *et al.*, 2020). RxLR Avh87 was reported to suppress INF1-induced cell death upon transient expression in *N. benthamiana* leaves (Dai *et al.*, 2020). Surprisingly, our results did not show a strong suppression of INF1-induced cell death as described in the paper. When taking a closer look, we did realize our experimental design differed from Dai and colleagues (2020). In our study, the cell death comparisons were between zones inoculated with FLAG:GFP and INF1 compared to FLAG:RxLRAvh87 and INF1 whereas Dai and colleagues (2020) compared RxLR Avh87 and GFP compared to RxLR Avh87 and INF1. The comparison used in Dai et al. (2020) may have skewed their results. Additionally, when assessing RxLR Avh87's contribution to *P. cinnamomi* virulence, our results were inconclusive. In one experiment, transient expression of RxLR Avh87 did not influence *P. cinnamomi* virulence, however, upon repeating the experiment we did see a significant increase in lesion size areas at 4 dpi in leaves transiently expressing RxLR compared to GFP control leaves. Repeats of

this experiment with increased replicates are necessary to determine the true nature of RxLR Avh87 and its contributions to *P. cinnamomi* virulence.

Recently, an improved *P. cinnamomi* transformation protocol was established, bringing to light the potential for future gene knock-out mutant generation in *P. cinnamomi* (Dai *et al.*, 2021). It would be very exciting to test if these candidate effectors reported in this study were essential to *P. cinnamomi's* ability to infect. Although the effectors in this study were functionally validated in the model plant, *N. benthamiana*, future experiments should be validated in Avocado with said knock-out *P. cinnamomi* mutants. Additionally, this was the first study looking at effectors and *P. cinnamomi*-plant interactions in a multi-species fashion (more than one host), providing us with a glimpse into the workings of *P. cinnamomi's* broad spectrum pathogenicity. Together this study sheds light on some of the important players in *P. cinnamomi* virulence and potential directions to combat this devastating disease.

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Figure and Table legends

Figure 3.1. Heatmap of significantly up-regulated effectors after infection.

Five candidate effectors (RxLR 2407, RxLR 2266, RxLR 2279, elicitin 2418, and elicitin 2373) highly up-regulated effectors within multiple hosts. All genes had a log fold change of ≥ 2 and adjusted P-values ≤ 0.05 in plant hosts: Avocado, *N. benthamiana*, and Arabidopsis after infection.

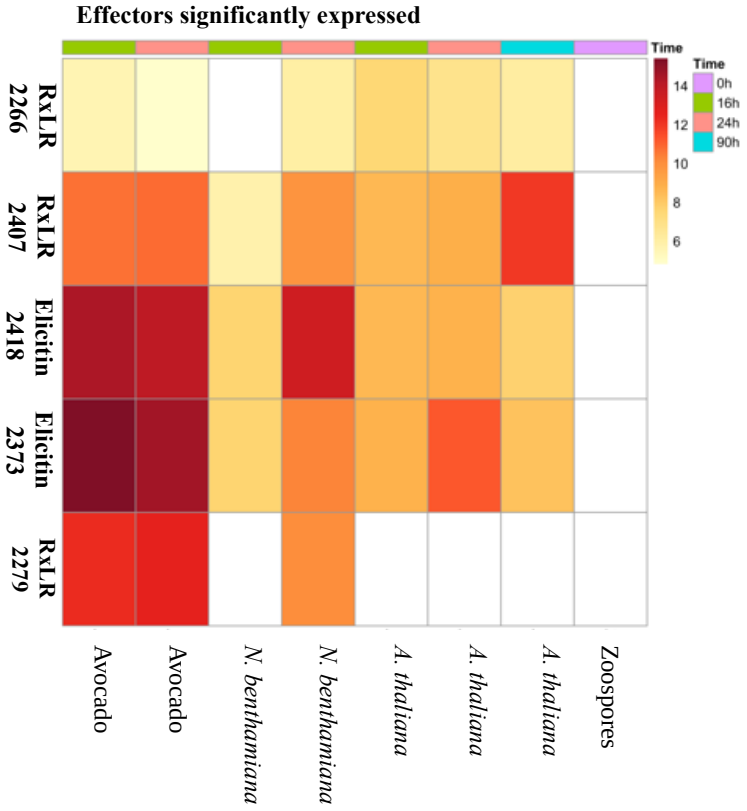

Effectors significantly expressed
Time
14
12
10
8
6
Time
0h
16h
24h
90h
RxLR 2266
RxLR 2407
Elicitin 2418
Elicitin 2373
RxLR 2279
Avocado
Avocado
N. benthamiana
N. benthamiana
A. thaliana
A. thaliana
A. thaliana
Zoospores

Figure 3.2. Effector transcript expression after *P. cinnamomi* infection in *N. benthamiana* leaves.

qRT-PCR was used to validate expression patterns identified by the RNA-Seq data. *N. benthamiana* housekeeping gene *EF1-a1* was used as the reference gene for normalization. Experiments were repeated twice. **P-values ≤ 0.005 and ***P-values ≤ 0.0005.

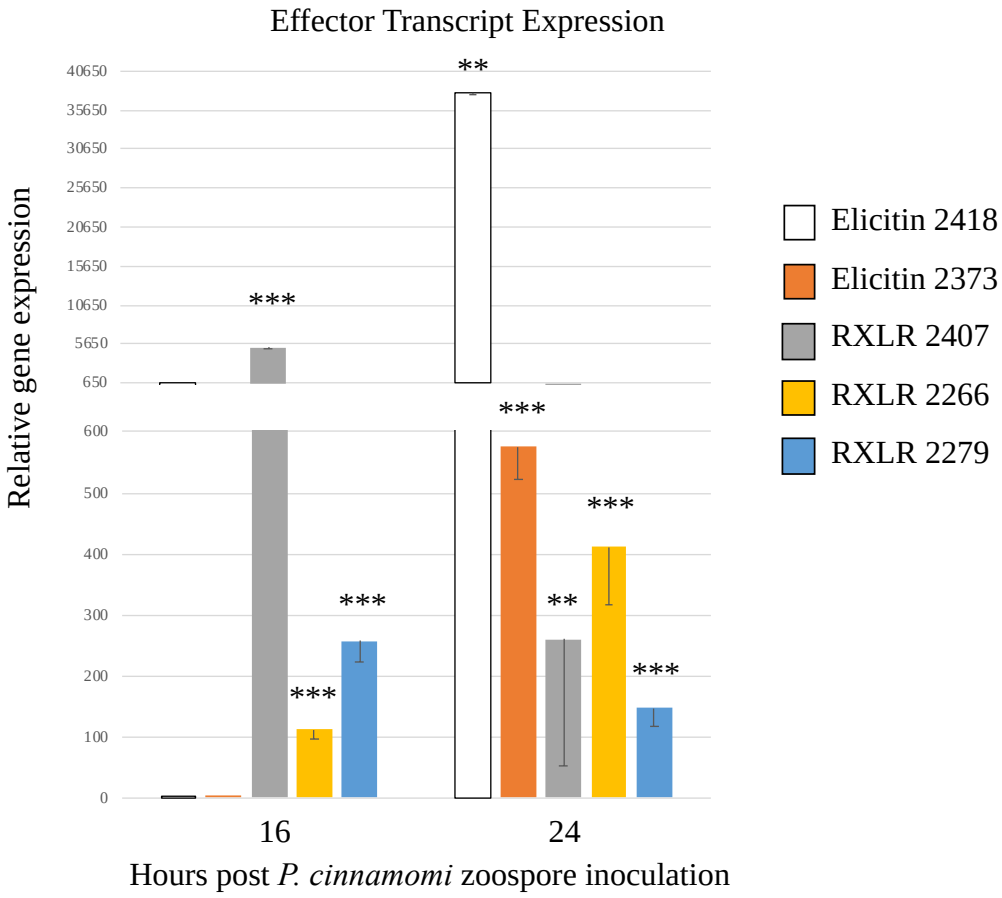

Effector Transcript Expression
Relative gene expression
40650
35650
30650
25650
20650
15650
10650
5650
650
600
500
400
300
200
100
0
**
***
***
***
***
**
***
16
24
Hours post P. cinnamomi zoospore inoculation
Elicitin 2418
Elicitin 2373
RXLR 2407
RXLR 2266
RXLR 2279

Figure 3.3. Relative expression of biomass marker LPV3 in avocado roots infected with *P. cinnamomi* over time.

Avocado housekeeping gene *EF1-a1* was used as the reference gene for normalization.

Experiments were repeated twice. **P-values ≤ 0.005 and ***P-values ≤ 0.0005.

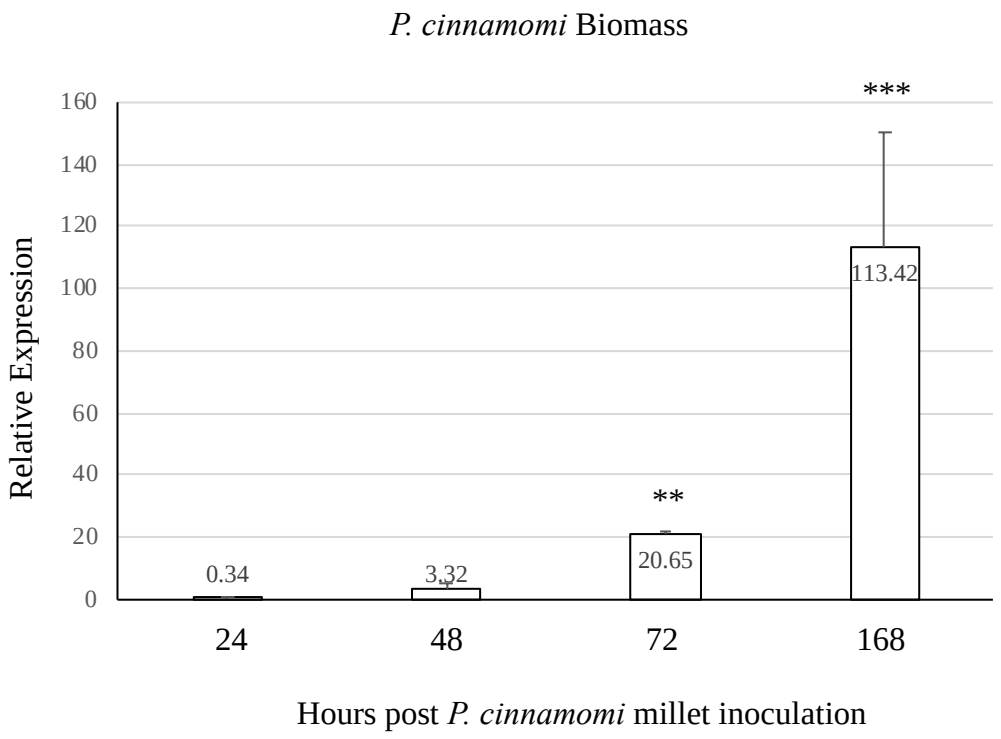

P. cinnamomi Biomass
Relative Expression
160
140
120
100
80
60
40
20
0
0.34
3.32
20.65
113.42
**
***
24
48
72
168
Hours post *P. cinnamomi* millet inoculation

Figure 3.4. Transcript levels of candidate effectors in avocado roots infected with *P. cinnamomi.*

Expression for candidate effectors was determined by qRT-PCR. Treatment of avocado roots with *P. cinnamomi* results in the accumulation of candidate effector transcripts relative to mock treatment. Avocado housekeeping gene *EF1-a1* was used as the reference gene for normalization. Experiments were repeated twice. *P- values ≤ 0.05 values **P-values ≤ 0.005 and ***P-values ≤ 0.0005.

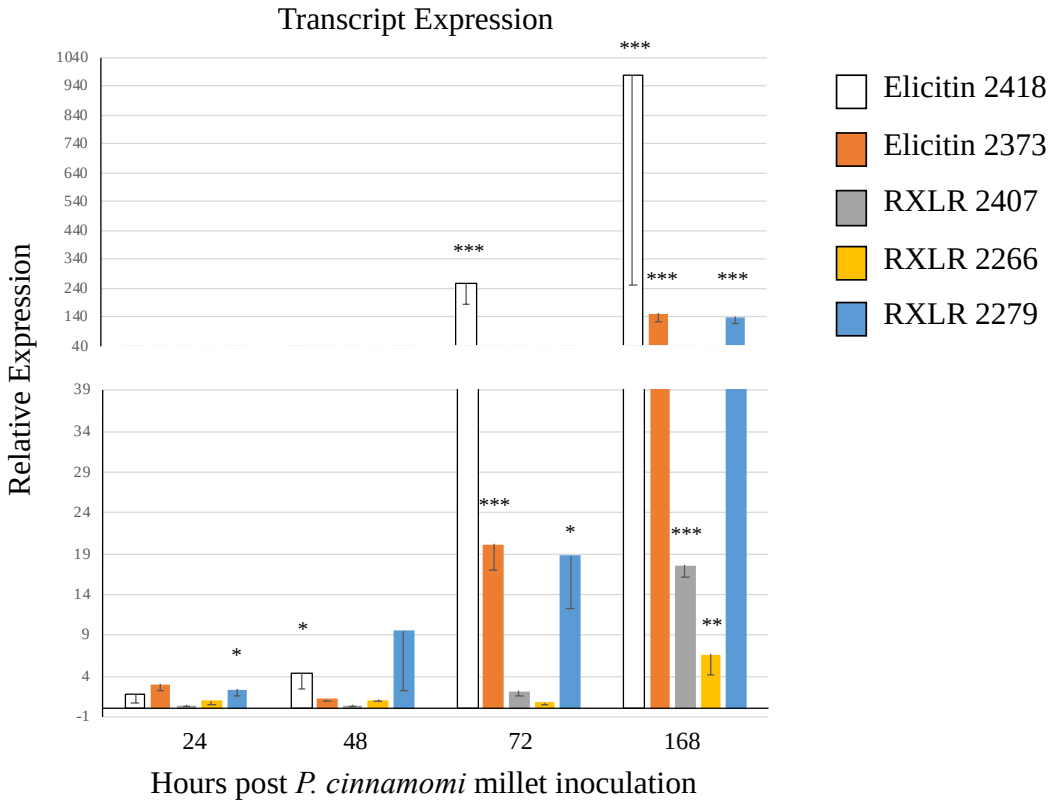

Transcript Expression
Relative Expression
1040
940
840
740
640
540
440
340
240
140
40
39
34
29
24
19
14
9
4
-1
***
***
***
***
***
*
*
*
***
*
**
24
48
72
168
Hours post P. cinnamomi millet inoculation
Elicitin 2418
Elicitin 2373
RXLR 2407
RXLR 2266
RXLR 2279

Figure 3. 5. Schematic diagram of the candidate effector predicted motifs.

Dotted boxes represent signal peptide sequence (SignalP V3.0), white boxes represent RxLR motifs, grey boxes represent the dEER motifs, grey triangles represent NLS site (cNLS mapper), arrows represent the synthesized region for each construct, and the numbers represent the amino acid (aa) location for each motif or protein length.

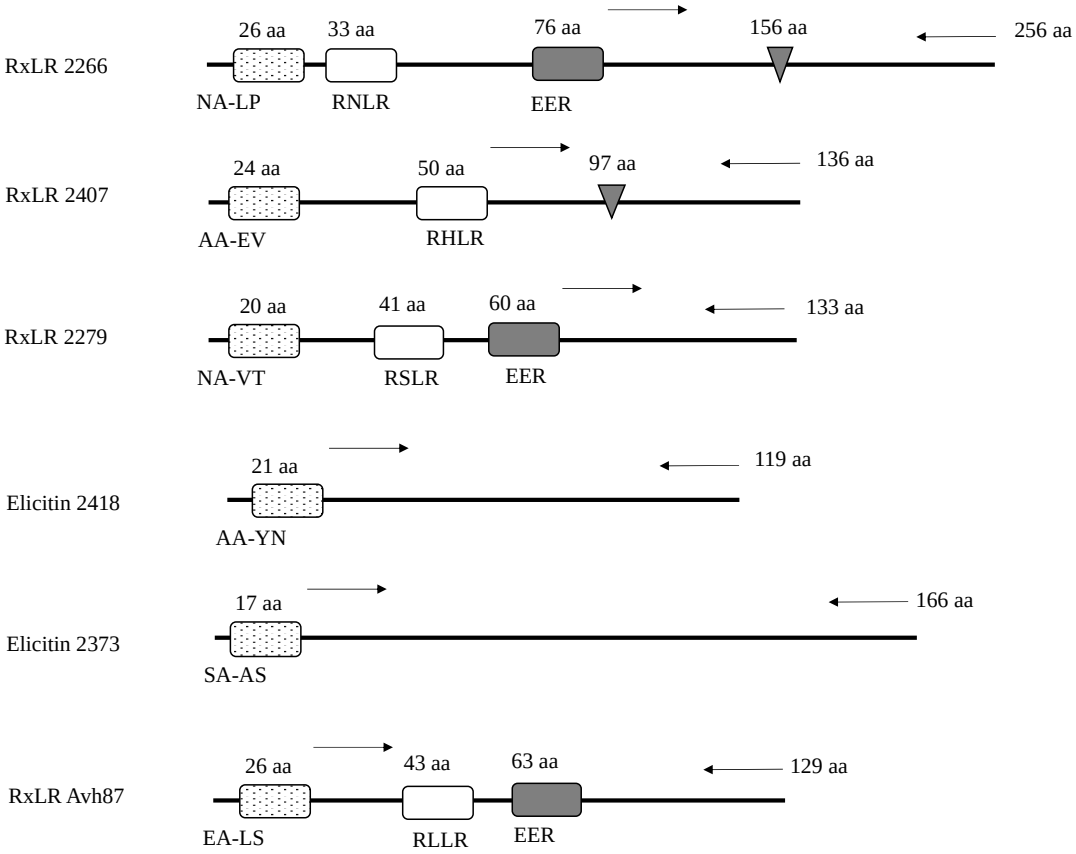

RxLR 2266
26 aa
33 aa
76 aa
156 aa
256 aa
NA-LP
RNLR
EER
RxLR 2407
24 aa
50 aa
97 aa
136 aa
AA-EV
RHLR
RxLR 2279
20 aa
41 aa
60 aa
133 aa
NA-VT
RSLR
EER
Elicitin 2418
21 aa
119 aa
AA-YN
Elicitin 2373
17 aa
166 aa
SA-AS
RxLR Avh87
26 aa
43 aa
63 aa
129 aa
EA-LS
RLLR
EER

Figure 3.6. Immunoblot detection of transiently expressed FLAG:Elicitin2373, FLAG:RxLR2279, and FLAG:RxLRAvh87 effectors in *N. benthamiana* leaves.

(a) FLAG:GFP (30 kDa) and FLAG:Elicitin2373 (26 kDa) were detected by immunoblot detection with α-FLAG conjugated with HRP antibody. (b) FLAG:RxLRAv87 (17 kDa) and FLAG:RxLR2279 (16 kDa) were detected by immunoblot detection with α-FLAG conjugated with HRP antibody. Experiments were repeated at least twice with similar results. Equal loading was confirmed by Coomassie blue staining.

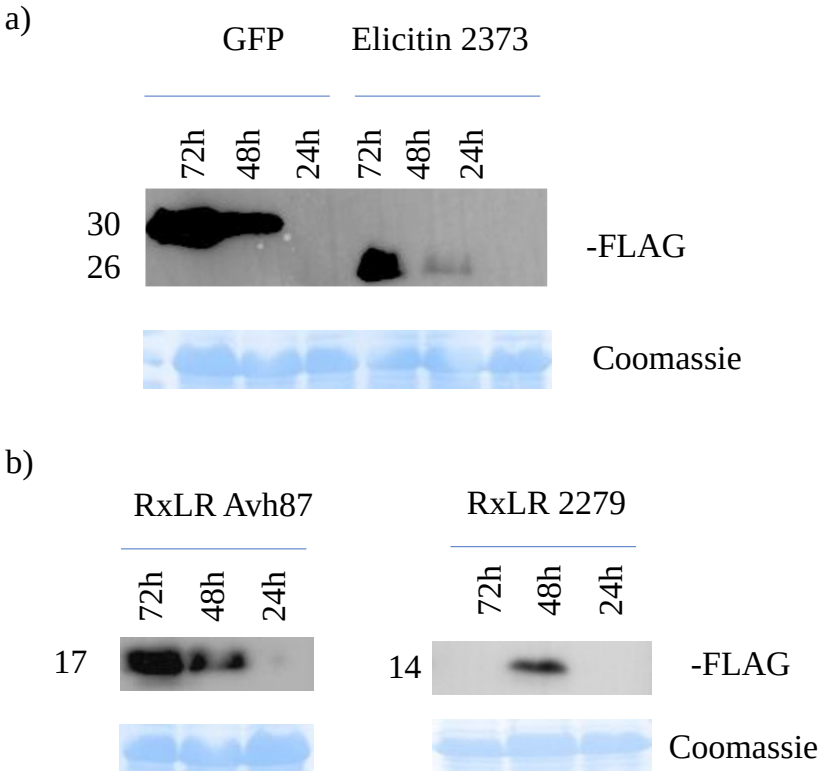

a)
GFP
Elicitin 2373
72h
48h
24h
72h
48h
24h
30
26
-FLAG
Coomassie
b)
RxLR Avh87
RxLR 2279
72h
48h
24h
72h
48h
24h
17
14
-FLAG
Coomassie

Figure 3.7. Transient expression of RxLR Avh87 and RxLR 2279 in *N. benthamiana* leaves.

(a) Graph depicting percentage of cell death after 4 dpi. Black represents complete cell death (71-100%), grey represents partial cell death (30-70%), and white represents no cell death (0-29%). (b) Photo of a *N. benthamiana* leaf with infiltration zones expressing FLAG:GFP, FLAG:INF1, FLAG:RxLR2279, FLAG:RxLRAvh87. (c) *N. benthamiana* leaf from (b) after carnoy staining. A total of 16 infiltration zones for each construct were evaluated from two leaves of 4 biological replicates. This experiment was repeated twice with similar results.

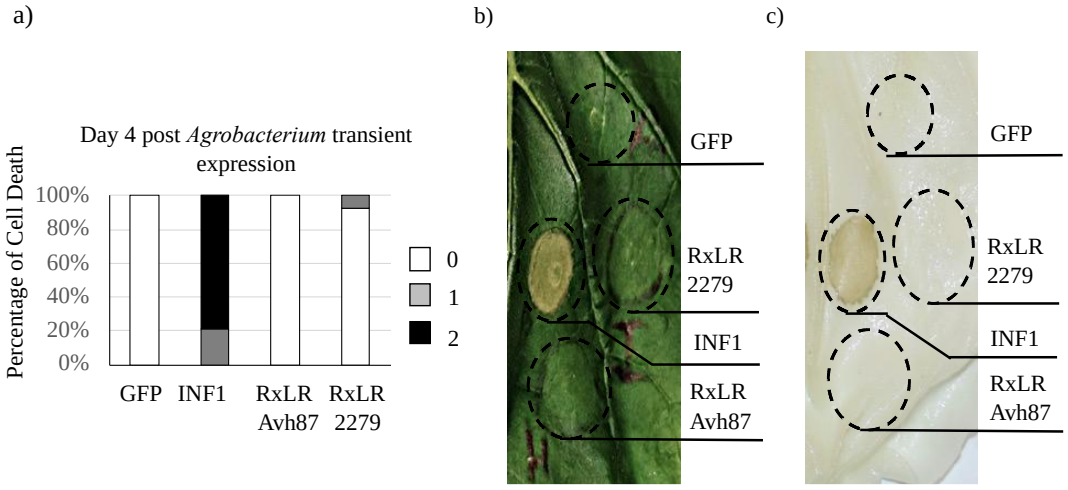

a)
b)
c)
Day 4 post *Agrobacterium* transient expression
Percentage of Cell Death
100%
80%
60%
40%
20%
0%
GFP
INF1
RxLR Avh87
RxLR 2279
0
1
2
GFP
RxLR 2279
INF1
RxLR Avh87
GFP
RxLR 2279
INF1
RxLR Avh87

Figure 3.8. Transient expression of Elicitin 2373 in *N. benthamiana* leaves induces cell death after 4 dpi.

(a) Graph depicting percentage of cell death after 4 dpi. Black represents complete cell death (71-100%), grey represents partial cell death (30-70%), and white represents no cell death (0-29%). (b) Photo of a *N. benthamiana* leaf with infiltration zones expressing FLAG:GFP, FLAG:INF1, and FLAG:Elicitin2373. (c) *N. benthamiana* leaf from (b) after carnoy staining. A total of 16 infiltration zones for each construct were evaluated from two leaves of 4 biological replicates. This experiment was repeated twice with similar results.

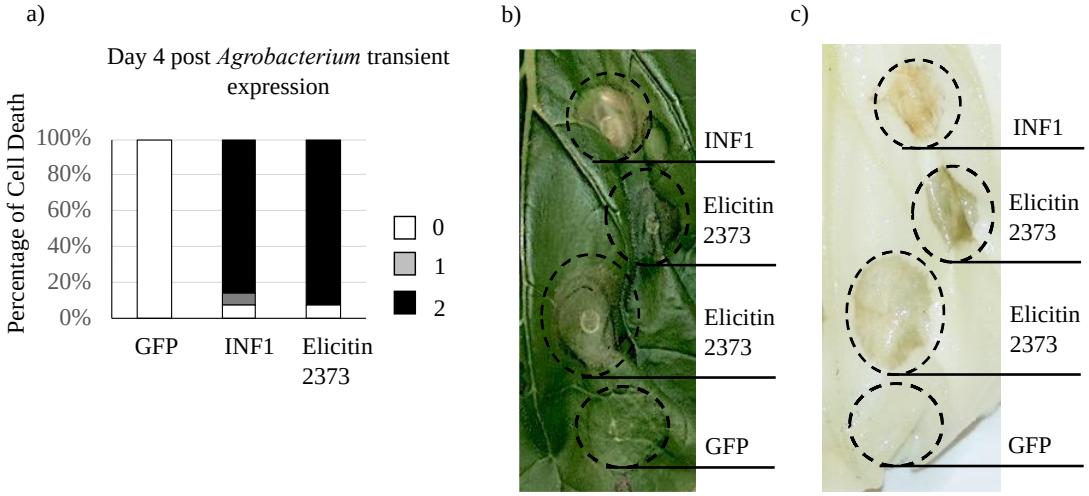

a)
Day 4 post *Agrobacterium* transient expression
Percentage of Cell Death
100%
80%
60%
40%
20%
0%
GFP
INF1
Elicitin 2373
0
1
2
b)
INF1
Elicitin 2373
Elicitin 2373
GFP
c)
INF1
Elicitin 2373
Elicitin 2373
GFP

Figure 3.9. RxLR2279 and RxLRAvh87 do not significantly alter INF1-induced cell death.

(a) Photos of infiltration zones at 3 dpi transiently expressing RxLR2279 or GFP in the presence of INF1. (b) A graph depicting the percentage of cell death resulting from zones inoculated with RxLR2279 then INF1 at 0 h, 12 h, or 24 h. (c) Photos of infiltration zones at 3 dpi transiently expressing RxLRAvh87 or GFP in the presence of INF1. (d) A graph depicting the percentage of cell death resulting from zones inoculated with RxLRAvh87 then INF1 at 0 h, 12 h, or 24 h. Black represents complete cell death (71-100%), grey represents partial cell death (30-70%), and white represents no cell death (0-29%). A total of 8 infiltration zones for each construct were evaluated from two leaves of 4 biological replicates. This experiment was repeated with similar results.

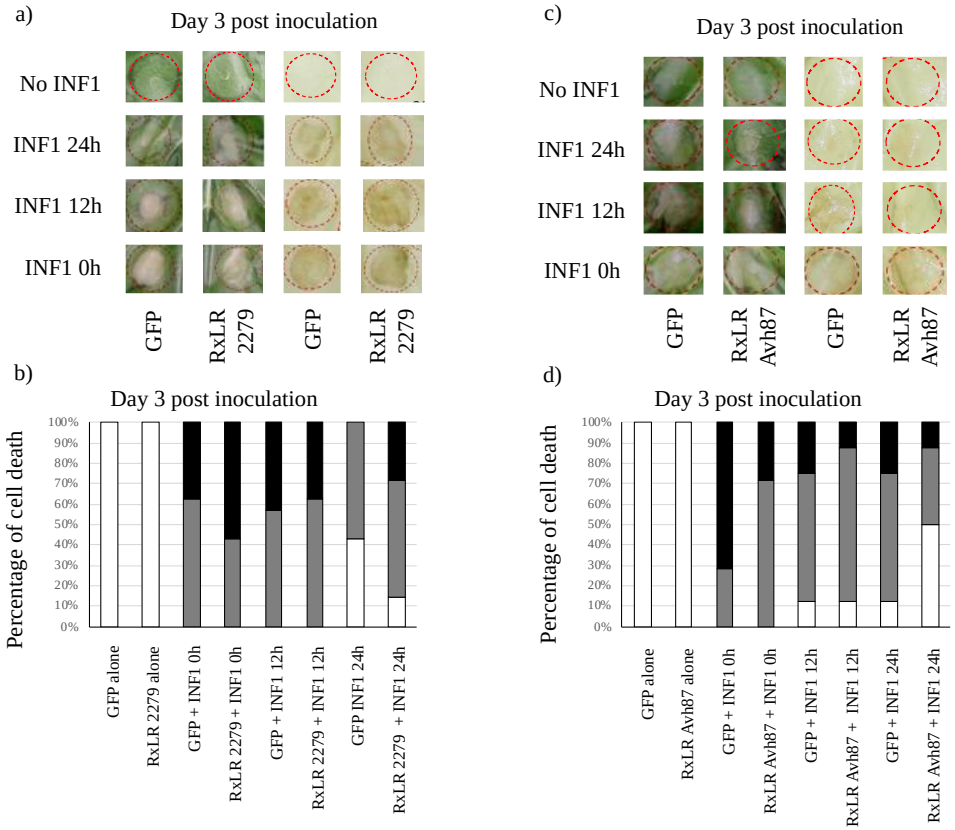
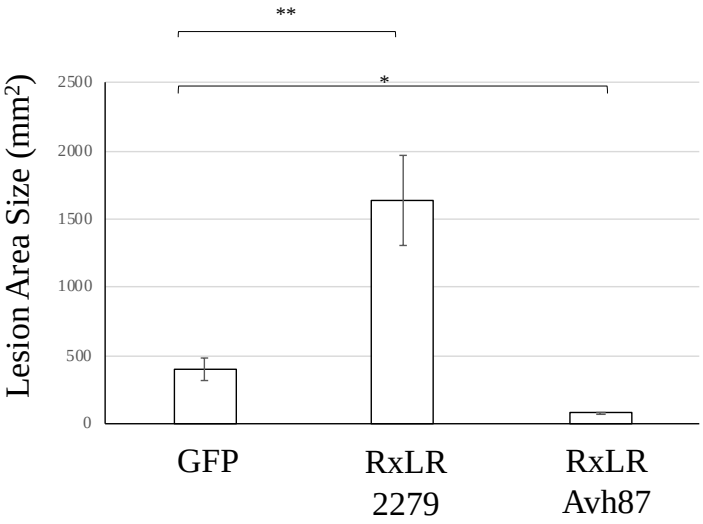

a)
Day 3 post inoculation
No INF1
INF1 24h
INF1 12h
INF1 0h
GFP
RxLR 2279
GFP
RxLR 2279
b)
Day 3 post inoculation
Percentage of cell death
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
GFP alone
RxLR 2279 alone
GFP + INF1 0h
RxLR 2279 + INF1 0h
GFP + INF1 12h
RxLR 2279 + INF1 12h
GFP INF1 24h
RxLR 2279 + INF1 24h
c)
Day 3 post inoculation
No INF1
INF1 24h
INF1 12h
INF1 0h
GFP
RxLR Avh87
GFP
RxLR Avh87
d)
Day 3 post inoculation
Percentage of cell death
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
GFP alone
RxLR Avh87 alone
GFP + INF1 0h
RxLR Avh87 + INF1 0h
GFP + INF1 12h
RxLR Avh87 + INF1 12h
GFP + INF1 24h
RxLR Avh87 + INF1 24h

Figure 3.10. RxLR 2279 contributes to *P. cinnamomi* virulence after 4 dpi.

(a) Graph depicting lesion area size in leaves transiently expressing FLAG:GFP, FLAG:RxLR2279, or FLAG:RxLRAvh87 and then inoculated with *P. cinnamomi* plugs after 4 dpi. (b) Representative leaf photos of leaves from (a). A total of 4 biological replicates were used. This experiment was repeated with FLAG:GFP, FLAG:RxLR2279 showing similar results.

Day 4 Post Inoculation with *P. cinnamomi*

a)



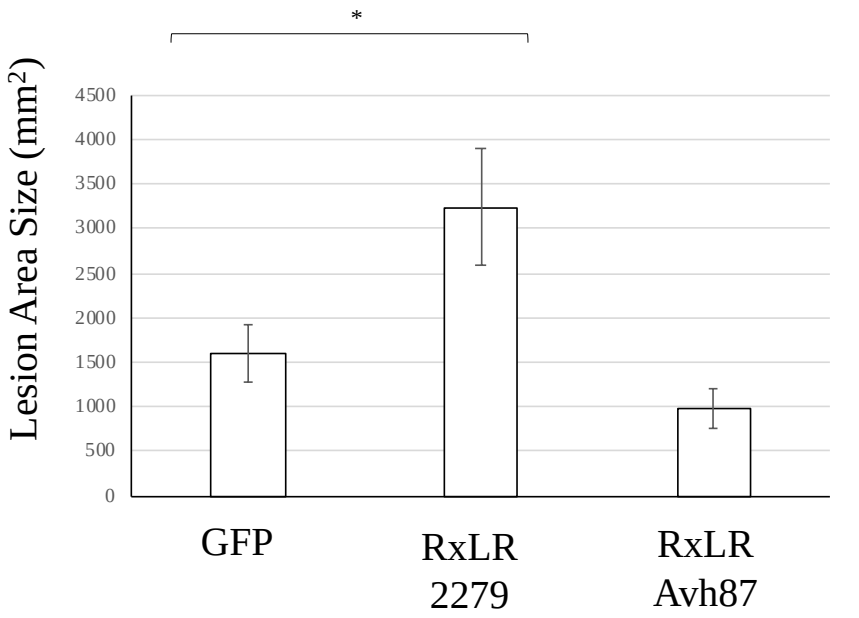
b)

GFP RxLR 2279 RxLR Avh87

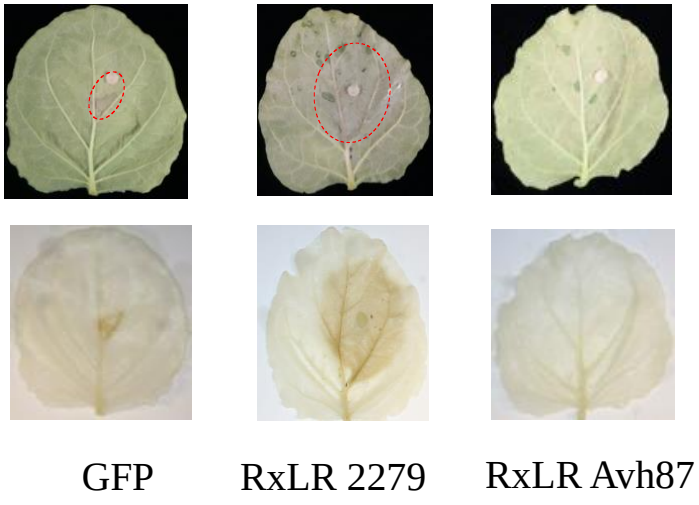
Figure 3.11. RxLR 2279 contributes to *P. cinnamomi* virulence after 5 dpi.

(a) Graph depicting lesion area size in leaves transiently expressing FLAG:GFP, FLAG:RxLR2279, or FLAG:RxLRAvh87 and then inoculated with *P. cinnamomi* plugs after 5 dpi. (b) Representative leaf photos of leaves from (a) along with photos of the leaves after carnoy staining. A total of 4 biological replicates were used. This experiment was repeated with FLAG:GFP, FLAG:RxLR2279 showing similar results.

a)


Day 5 Post Inoculation with P. cinnamomi
*
Lesion Area Size (mm²)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
GFP
RxLR 2279
RxLR Avh87

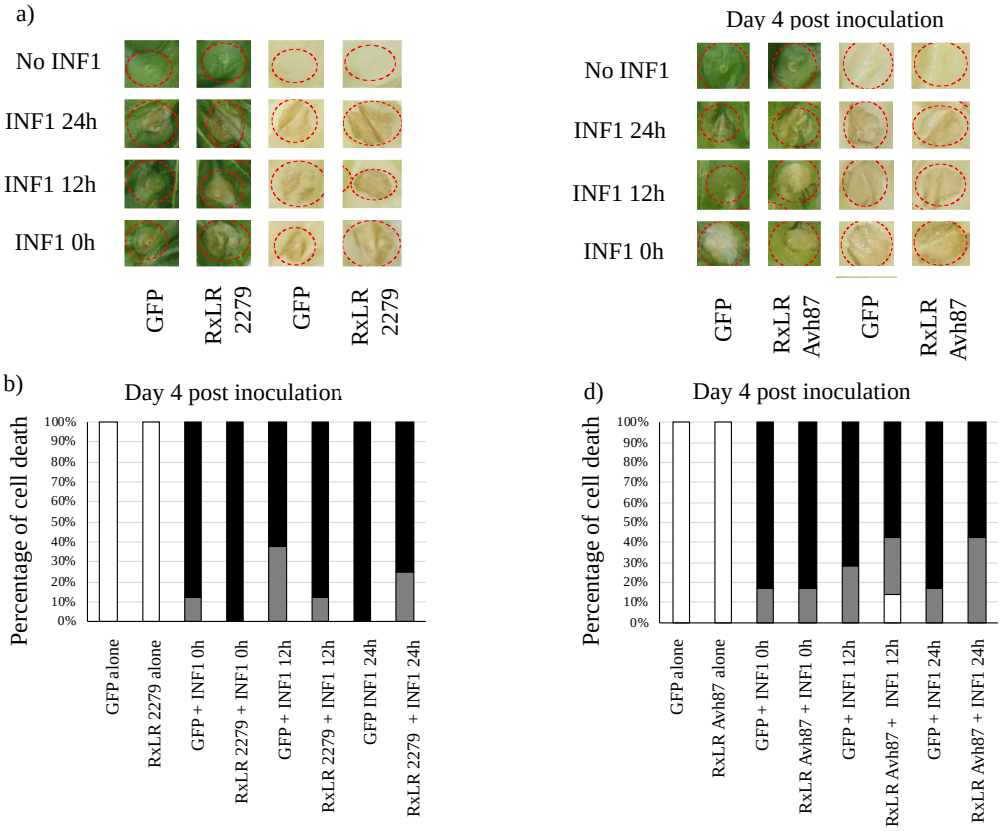
b)


GFP
RxLR 2279
RxLR Avh87

Supplemental Figures and Tables

Supplemental Figure 3.1. RxLR2279 and RxLRAvh87 do not significantly alter INF1-induced cell death.

(a) Photos of infiltration zones at 4 dpi transiently expressing RxLR2279 or GFP in the presence of INF1. (b) A graph depicting the percentage of cell death resulting from zones inoculated with RxLR2279 then INF1 at 0 h, 12 h, or 24 h. (c) Photos of infiltration zones at 4 dpi transiently expressing RxLRAvh87 or GFP in the presence of INF1. (d) A graph depicting the percentage of cell death resulting from zones inoculated with RxLRAvh87 then INF1 at 0 h, 12 h, or 24 h. Black represents complete cell death (71-100%), grey represents partial cell death (30-70%), and white represents no cell death (0-29%). A total of 8 infiltration zones for each construct were evaluated from two leaves of 4 biological replicates. This experiment was repeated with similar results.

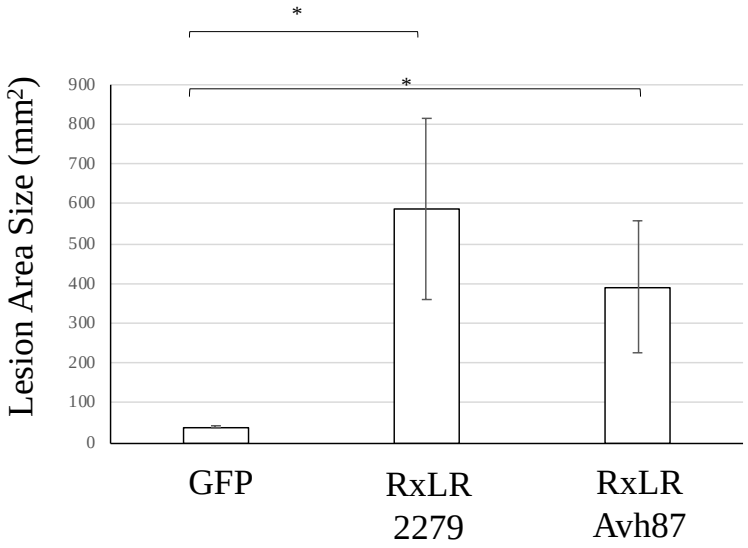

a)
No INF1
INF1 24h
INF1 12h
INF1 0h
GFP
RxLR 2279
GFP
RxLR 2279
Day 4 post inoculation
No INF1
INF1 24h
INF1 12h
INF1 0h
GFP
RxLR Avh87
GFP
RxLR Avh87
b)
Day 4 post inoculation
Percentage of cell death
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
GFP alone
RxLR 2279 alone
GFP + INF1 0h
RxLR 2279 + INF1 0h
GFP + INF1 12h
RxLR 2279 + INF1 12h
GFP INF1 24h
RxLR 2279 + INF1 24h
d)
Day 4 post inoculation
Percentage of cell death
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
GFP alone
RxLR Avh87 alone
GFP + INF1 0h
RxLR Avh87 + INF1 0h
GFP + INF1 12h
RxLR Avh87 + INF1 12h
GFP + INF1 24h
RxLR Avh87 + INF1 24h

Supplemental Figure 3.2. RxLR 2279 and RxLRAvh87 contributes to *P. cinnamomi* virulence after 4 dpi.

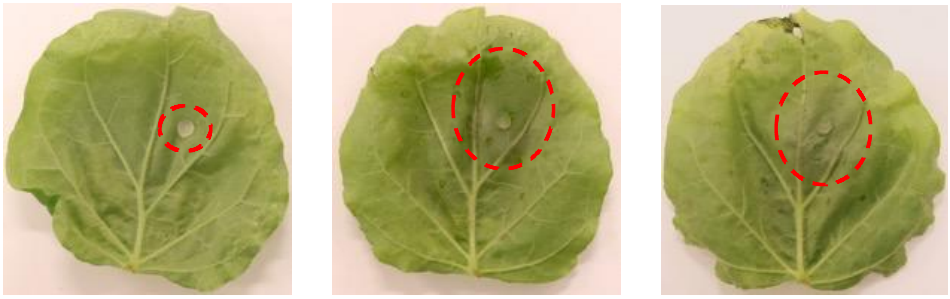
(a) Graph depicting lesion area size in leaves transiently expressing FLAG:GFP, FLAG:RxLR2279, or FLAG:RxLRAvh87 and then inoculated with *P. cinnamomi* plugs after 4 dpi. (b) Representative leaf photos of leaves from (a). A total of 4 biological replicates were used. This experiment was repeated with FLAG:GFP, FLAG:RxLR2279 showing similar results.

Day 4 Post Inoculation with *P. cinnamomi*

a)



b)



GFP RxLR 2279 RxLR Avh87

Supplemental Table 3.1. Summary of 5 candidate effectors.

Annotated name	Name	Gene family	EffectorP prediction	Orthologs with other *Phy.* Spp.	Significant DEGs Host
Pc2113T1C00002407g0001290.1	RxLR 2407	RxLR	Cytoplasmic Effector	Yes[#]	*Nb*, *At*, *Pa*
Pc2113T1C00002266g0000100.1	RxLR 2266	RxLR	Cytoplasmic Effector	No	*Nb*, *At*, *Pa*
Pc2113T1C00002279g0000010.1	RxLR 2279	RxLR	Cytoplasmic Effector	No	*Nb* & *Pa*
Pc2113T1C00002418g0002680.1	Elicitin 2418	Elicitin	Apoplastic Effector	Yes[#]	*Nb*, *At*, *Pa*
Pc2113T1C00002373g0000080.1	Elicitin 2373	Elicitin	N/A	Yes[#]	*Nb*, *At*, *Pa*

[#] Orthologs found in all six *Phytophthora* species analyzed

Supplemental Table 3.2. Oligonucleotides for qRT-PCR and sequencing.

Primer Pair Number	Primer Name	Primer Sequence	Constuct
1	Pc_Biomass_Marker_86101_F	CTGTTCTGCATCGCCTTCAC	qPCR Forward primer for biomass analyses
2	Pc_Biomass_Marker 86101_R	GCGGATGTACACGTTCTGGA	qPCR Reverse primer for biomass analyses
3	Nb_Ef1A_F	AGCTTTACCTCCCAAGTCATC	qPCR Forward primer for gene expression analyses
4	Nb_Ef1A_R	AGAACGCCTGTCAATCTTGG	qPCR Reverse primer for gene expression analyses
5	Av_Ef1A_F	CAACTGCAAGACTTAGCTGGGAAAA	qPCR Forward primer for gene expression analyses
6	Av_Ef1A_R	TGCTAAAGTGATGCCAAATGCTACA	qPCR Reverse primer for gene expression analyses