Functional Characterization of the HopF Family of Type III Effectors in *Arabidopsis thaliana*

by

Noushin Koulena

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Abstract

*Pseudomonas syringae* is a Gram-negative bacterial phytopathogen that causes disease in economically important crops. An essential virulence strategy of *P. syringae* involves injecting type III secretion effectors (T3SEs) into host cells to inhibit immune signaling. Advances in genomic sequencing have lead to an increase in the available number of sequences of plant bacterial pathogens, which has lead to identification of divergent homologs of known T3SEs. The HopF family of T3SE contains over 40 diverse family members. In order to explore the functional diversification of HopF family, this thesis undertakes the functional characterization of HopF T3SE in *Arabidopsis thaliana*. Functional analysis revealed multiple members with novel immune responses in *Arabidopsis*. A reverse genetics approach was used to identify the mechanism underlying the novel immune responses triggered by HopF family of T3SE. The *Arabidopsis* R protein X was required for recognition of a novel member of the HopF family.
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Abbreviations

ADP-RT – ADP-ribosylation factor

ARTIC – *Arabidopsis* R gene T-DNA Insertion Collection

Avr - avirulence

BAK1 – BRI1-ASSOCIATED RECEPTOR KINASE 1

BIK1 – BOTRYTIS-INDUCED KINASE1

CERK1 – CHITIN ELICITOR RECEPTOR KINASE 1

Cfu – colony-forming units

Dpi – days post-inoculation

EDS1 – ENHANCED DISEASE SUSCEPTIBILITY 1

EF – elongation factor-tu

ETI – effector-triggered immunity

FLS2 – FLAGELLIN-SENSITIVE 2

HA – haemagglutinin

Hop – *hrp* outer protein

Hpi – hours post-inoculation

HR – hypersensitive response
Hrp – hypersensitive response and pathogenicity

kDa – kilodalton

LRR – leucine-rich repeat

M/PAMP – microbe/pathogen associated molecular pattern

MAP Kinase – mitogen-activated protein kinase

PTI – PAMP-triggered immunity

NB – nucleotide-binding

NDR1 – NON-RACE-SPECIFIC DISEASE RESISTANCE 1

PAD4 - PHYTOALEXIN DEFICIENT 4

PR – pathogenesis-related

PRR – pattern-recognition receptors

Pto DC3000 – Pseudomonas syringae pv. tomato DC3000

Pph – Pseudomonas syringae pv. phaseolicola

RIN4 – RPM1-interacting protein

RLK – receptor-like kinase

RPM1 – resistance to Pseudomonas syringae pv. maculicola 1

RPS2 – resistance to Pseudomonas syringae pv. syringae 2
Shc – specific hop chaperone

TAO1 – TARGET of AVRB OPERATION 1

TTSS – type III secretion system

TTSE – type III secreted effector

TLR – Toll-like receptors

RAR1 – REQUIRED FOR MLA12 RESISTANCE 1

RIPK - RIN4-INTERACTING RECEPTOR-LIKE PROTEIN KINASE

Rpt2 – resistance to *Pseudomonas syringae* pv. *tomato* 2

ROS – reactive oxygen species
Chapter 1

1 Introduction

1.1 The plant immune system

The interaction between plants and disease-causing phytopathogens has been described as a co-evolutionary arms race. *Pseudomonas syringae* is a Gram-negative host specific bacterial phytopathogen that can cause disease on many economically important crops such as bean, and wheat (Hirano & Upper 1990; Jones & Dangl 2006). The *P. syringae* species complex is comprised of approximately fifty different pathovars each of which has the ability to infect a wide variety of plant hosts. Despite the ubiquity of disease responses caused by *P. syringae*, each individual pathovar has a limited host range delineated by plant immune systems (Hwang et al. 2005). For example, *P. syringae* pathovar tomato DC3000 (PtoDC3000) is pathogenic on both tomatoes and the model plant organism *Arabidopsis thaliana* whereas *P. syringae* pathovar phaseolicola is pathogenic on bean (Whalen et al. 1991).

Plants have evolved a sophisticated immune system to detect bacterial presence. The plant immune system is separated into two tiers of immunity. The first tier of immunity is called pattern recognition receptor (PRR)-triggered immunity (PTI), which consists of extracellular PRRs that can detect pathogen or microbe associated molecular patterns (PAMP/MAMP) (Jones & Dangl 2006; Zipfel & Felix 2005). The recognition of PAMP/MAMPs by surface-localized immune receptors leads to activation of downstream kinase pathways, production of reactive oxygen species (ROS), transient calcium fluxes and transcriptional reprogramming of defense genes and the formation of callose (Torres et al. 2002; Ranf et al. 2011; Ham et al. 2007; Schwessinger et al. 2011). To date, the best characterized PRRs that have been identified for MAMPs derive from bacterial flagellin, elongation factor-Tu, and fungal derived chitin (Gómez-
Gómez & Boller 2000; Zipfel et al. 2006; Miya et al. 2007). PTI is considered a broad-spectrum defense that is able to perceive patterns that are characteristic of entire groups or classes of microorganisms, which leads to restriction of growth of most pathogens and thus the restriction of their host range (Jones & Dangl 2006; Zipfel & Felix 2005).

Successful bacterial pathogens must overcome this tightly regulated first layer of immunity. As a result, to counteract a plant immune responses, *P. syringae* has evolved a needle-like secretion apparatus known as the type III secretion system (TTSS) to deliver virulent proteins known as type III secreted effectors (T3SEs) into host cytosol (Jones and Dangl, 2006; Lindeberg et al., 2009). The TTSS is used by many Gram-negative pathogens of plants and animals to inject effectors into host cells to suppress PTI and promote bacterial growth (Galán & Collmer 1999; Lindeberg et al. 2009). *P. syringae* strains carry variable numbers of T3SEs, with complements ranging from 3-38 T3SEs per strain (Guttman et al. 2002; O’Brien et al. 2011). T3SEs interact with host proteins to inhibit PTI. In many cases, T3SEs directly inhibit early signaling components of PTI to weaken immune signaling. Some T3SEs target PRR receptors directly. For example, AvrPtoB, AvrPto and HopAO1 all directly target FLS2 to either decrease its stability or specifically inhibit flg22 induced phosphorylation (Xiang et al. 2008; Göhre et al. 2008; Macho et al. 2014). Similarly, AvrPtoB and HopF2 can also alter the stability or inhibit flg22-induced phosphorylation of BAK1 (Brassinosteroid Insensitive 1-Associated Kinase 1) (Shan et al. 2008; Zhou et al. 2014). The RLCKs and MAPKs act downstream of PRR signaling are also common targets of T3SEs. The RLCK BIK1 is targeted by HopF2 and AvrPphB, while AvrPphB can also target PBS1 and multiple PBL family members (Zhou et al. 2014; Zhang et al. 2010).

Many successful pathogens translocate T3SEs into the plant cell in order to suppress PTI and thus provide the phytopathogen with a favorable environment in which to proliferate and cause
disease. Analysis of various *P. syringae* genomes has revealed that individual strains possess unique TTSS effector complements which define the host range of individual *P. syringae* pathovars (Alfano & Collmer 2004). In response, plants have evolved a second layer of immunity that monitors for the presence of T3SEs. Towards this end, plants deploy cytosolic immune receptors called resistance (R) proteins that recognize T3SE-induced modifications of specific host proteins (Jones & Dangl 2006; Lindeberg et al. 2009). Recognition of T3SEs by R proteins results in the activation of effector-triggered immunity (ETI). Both PTI and ETI result in similar immune responses, however the amplitude of ETI is much greater and often times results in a rapid and strong immune response that culminates in programmed cell death at the site of infection, termed the hypersensitive response (HR) (Dangl et al. 1996; Hammond-Kosack & Jones 1996; Spoel & Dong 2012). The recognition of T3SEs by R Proteins that do not induce HR can still induce defenses that effectively restrict pathogen growth (Gassmann 2005a; Ham et al. 2007). Overall, while bacteria use T3SEs to infiltrate host cells and interdict plant immunity at multiple stages, their presence can also betray the bacteria by being detected by plants intracellular immune receptors leading to activation of ETI. ETI is responsible for the race-specific resistance of plant cultivars that contain an R protein recognizing a T3SE utilized by the pathogen (Holub & Cooper 2004; Schulze-Lefert & Panstruga 2011).

1.2 NB-LRR proteins and the generation of ETI

Perception of T3SEs leading to ETI relies on R proteins, which characteristically contain either a coiled-coil (CC) or a *Drosophila* Toll-IL-1 (TIR) N-terminal domain linked to a nucleotide binding site, and a leucine-rich repeat at the C-terminus (NB-LRR) (Dangl & Jones 2001; Eitas & Dangl 2010). Currently, it is thought the N-terminal CC or TIR domain determines the downstream signaling pathway and the components involved in activating a resistance response.
R proteins that contain a CC domain such as RPM1, RPS2 and RPS5 have been shown to require NDR1/RAR1 for function, while R proteins such as RPS4 that contain a TIR domain require EDS1/PAD4 (Century et al. 1995; Aarts et al. 1998; Parker et al. 1996; Feys et al. 2001).

T3SEs can be recognized either via direct physical association with an R protein, similar to a ligand binding to a receptor, or via indirect recognition (Dangl & Jones 2001; Jia et al. 2000). Indirect R protein and T3SE interaction is often explained within the context of the guard hypothesis (Dangl & Jones 2001; Nimchuk et al. 2003). The guard hypothesis posits that R proteins act as ‘guards’, which indirectly perceive T3SEs by monitoring modifications to host proteins through protein-protein interactions. Plant host proteins monitored by R proteins are termed ‘guardees’. The guard hypothesis proposes that guardees are the targets of T3SEs, and that T3SE specific modification of guardees suppresses plant immunity to promote virulence (Dangl & Jones 2001).

An example of a ‘guardee’ is the *Arabidopsis thaliana* RPM1-interacting protein 4 (RIN4), which is a negative regulator of PTI and is guarded by two R proteins, RPM1 and RPS2 (Kim et al. 2005; Mackey et al. 2003a; Mackey et al. 2002). To date, four unrelated *P. syringae* T3SEs, AvrRpm1, AvrB, AvrR mass and HopF2_PtoDC3000 have been shown to target RIN4 (Wilton et al. 2010; Mackey et al. 2003a; Mackey et al. 2002). The R protein RPM1 recognizes the two sequence diverse T3SEs AvrRpm1 and AvrB, while AvrRpt2 is recognized by RPS2 (Belkhadir et al. 2004; Mackey et al. 2003a; Mackey et al. 2002). Both AvrRpm1 and AvrB are localized to the plasma membrane where they target RIN4 and modify its phosphorylation status, leading to the activation of RPM1-triggered ETI (Mackey et al. 2002). However, neither T3SEs possesses any kinase activity (Desveaux et al. 2007). Rather, RPM1-Induced Protein Kinase (RIPK), a RIN4-interacting cytoplasmic receptor-like protein kinase, phosphorylates RIN4 in response to
AvrRpm1 and AvrB (Liu et al. 2011). In the absence of RPM1, both T3SEs cause virulence in *Arabidopsis* (Mackey et al. 2002). Further, another R protein, TAO1, a TIR-NB-LRR resistance gene was identified to contribute to disease resistance response induced by AvrB (Eitas et al. 2008).

The T3SE protein AvrRpt2 is a cysteine protease that also targets RIN4 and causes its degradation while triggering RPS2-mediated ETI (Coaker et al. 2005; Coaker et al. 2006; Mackey et al. 2003b). AvrRpt2 is activated by the host cyclophilin ROC1 leading to the autoprocessing of 71 amino acids at the N terminal end of the effector (Coaker et al. 2005). Upon cleavage, AvrRpt2 acts as a mature protease that cleaves RIN4 at two sequences, resulting in RPS2 activation and ETI (Mackey et al. 2003b).

The HopF2 T3SE from *P. syringae* pv. *tomato* DC3000 (HopF2<sub>PtoDC3000</sub>) is yet another effector protein that has been shown to interact with RIN4 (Wilton et al. 2010). HopF2<sub>PtoDC3000</sub> has been shown to prevent the cleavage of RIN4 by AvrRpt2, and it can also promote bacterial virulence in *Arabidopsis* (Wilton et al. 2010). Targeting of RIN4 by these various T3SEs points to the fact that RIN4 is a point of convergence in the plant-pathogen evolutionary arms race (Mukhtar et al. 2011).

### 1.3 The HopF family of effector

The HopF family of effectors consists of over 40 different members (Figure 1). Literature of the HopF family so far has focused on HopF1<sub>Pph1449B</sub> (formely AvrPphF) from *P. syringae* pv. *phaseolicola* race 7 (1449B) and HopF2<sub>PtoDC3000</sub> from *P. syringae* pv. *tomato* DC3000 (Singer et al. 2004; Wang et al. 2010a; Wilton et al. 2010). The HopF T3SE family is broadly distributed among pathovars of *P. syringae*. HopF1<sub>Pph1449B</sub> is a bean pathogen that is recognized in bean
cultivars expressing the R1 resistance protein and causes disease in cultivars lacking the R1 resistance protein (Singer et al. 2004; Tsiamis et al. 2000). The crystal structure of the HopF1 locus from the bean pathogen *P. syringae* pv. *Phaseolicola* 1449B was one of the first structures of the type III chaperone and its corresponding T3SE to be solved. The structure of HopF has been revealed to resemble a mushroom-like structure with “head” and “stalk” subdomains. The head subdomain of the protein possesses slight structural similarities to the catalytic domain of ADP-ribosyltransferases (ADP-RT) such as diphtheria toxin (Singer et al. 2004). However, the stalk subdomain does not resemble any significant structural similarities to any known structures of known functions (Singer et al. 2004). Structural similarities observed between HopF and ADP-RT was used to predict two putative catalytic sites, Arg72 and Asp174. Mutations in either site abrogate the virulence and avirulence function of HopF1 (Singer et al. 2004; Wang et al. 2010a). In addition, two regions of conserved amino acids were identified with one in the “head” of the protein, while the other located at the head-stalk interface (Singer et al. 2004). The conserved residues were tested for function of HopF1<sub>Pph1449B</sub> and both regions demonstrated to be important for recognition and virulence of HopF1<sub>Pph1449B</sub>, though to a lesser extent than the ADP-RT catalytic residues (Singer et al. 2004).

The other well characterized HopF member, HopF2 from *PtoDC3000* utilizes a unique start codon, ATA, which limits protein production in *P. syringae* (Robert-Seilanianz et al. 2006). Mutation of the native ATA start codon to ATG results in increased virulence in tomato plants (Robert-Seilanianz et al. 2006). In *Arabidopsis*, HopF2<sub>Pto</sub> is capable of interacting with the plasma membrane localized plant protein RPM1-interacting protein 4 (RIN4) to suppress the HR associated with AvrRpt2, while promoting bacterial growth (Wilton et al. 2010). Similar to HopF1, the virulence function of HopF2<sub>Pto</sub> has been demonstrated to be dependent on the
predicted catalytic residues present in the head subdomain (Wang et al. 2010a; Wilton et al. 2010). HopF2\textsubscript{Pto} is also involved in induction of non-host HR on tobacco W38 plants. The recognition of HopF2\textsubscript{Pto} in tobacco W38 and virulence in tomato require an intact myristoylation sequence (Robert-Seilaniantz et al. 2006).

HopF2\textsubscript{Pto} also targets various PTI signaling components. It is been demonstrated that HopF2\textsubscript{Pto} can suppress PTI in \textit{Nicotiana benthamiana}, flagellin-induced NON-HOST1 (NHO1) induction in \textit{Arabidopsis}, AvrRpm1-dependent HR and callose deposition in transgenic \textit{Arabidopsis} plants overexpressing HopF2\textsubscript{Pto} (Li et al. 2005; Guo et al. 2009). Consistent with structural predictions, HopF2\textsubscript{Pto} has also been demonstrated to possess ADP-RT activity. HopF2\textsubscript{Pto} is capable of ADP-ribosylating and inactivating \textit{Arabidopsis} Map Kinase Kinase 5 (MKK5) leading to inhibition of PTI (Wang et al. 2010a). In addition, it has also been shown to target the \textit{Arabidopsis} co-receptor for flagellin perception, BAK1 to suppress PTI (Zhou et al. 2014). In both instances the putative ADP-RT catalytic resides are required for the inhibition of PTI (Wang et al. 2010a; Zhou et al. 2014).

1.4 Thesis objective

The rapid increase in the sequence of bacterial genomes of plant bacterial pathogens has lead to identification of a large number of divergent homologs of known T3SEs (Baltrus et al. 2011). To date, a majority of functional studies of T3SEs have focused on single representatives of a given T3SE family. However, in the few cases where multiple homologs of a single T3SE family have been characterized, it has been demonstrated that they exhibit variation in virulence and avirulence functions. A study of the HopZ family of T3SEs has revealed a coevolutionary arms race between HopZ effectors and their hosts (Ma et al. 2006). Ma et al. (2006) demonstrated that the genetic diversity within the HopZ family allowed \textit{P. syringae} to avoid host recognition while
still providing a virulence function. To further understand the mechanism of diversification that promotes the virulence of a pathogen, HopF alleles have been identified in numerous *P. syringae* strains. I propose that novel HopF alleles will contribute to both resistance and virulence within the model plant *Arabidopsis*. Specifically, my objectives were: (1) to identify a novel immune response for various hopF alleles in the non-host plant, *Arabidopsis*; and (2) to further identify the genetic requirements of the novel immune response using a reverse genetics approach. A functional screen of novel hopF alleles will help elucidate novel T3SE function and plant immune responses to T3SE recognition.
Chapter 2

2 Materials & methods

2.1 Growth of plants on soil and bacterial strains

*Arabidopsis thaliana* seeds for soil-grown plants were placed on moist soil (Sunshine Professional Growing MIX LC1, SunGro, Canada) amended with 20-20-20 fertilizer, stratified for two to seven days at 4 °C, then placed in a growth room with a nine-hour photoperiod and fifteen-hour darkness, along with a day/night temperature regime of 22°C/20°C at ~70% humidity. We would like to thank Prof. David Guttman for sending us HopF2\textsubscript{PacM302273PT} constructs, Prof. David Baltrus for sending us the HopF2\textsubscript{PamM302091} construct, and Prof. Pablo Rodríguez Palenzuela and Prof. Emilia López Solanilla for sending us the \textit{PsvNCPPB3335} strain. All \textit{P. syringae} strains were grown at 28°C in King’s B media (King et al. 1954) and \textit{hrp}-inducing minimal media. KB media was supplemented with the following antibiotics, where appropriate: 50 \(\mu\)g mL\(^{-1}\) rifampicin, 50 \(\mu\)g mL\(^{-1}\) kanamycin, and 50 \(\mu\)g mL\(^{-1}\) cycloheximide. \textit{Escherichia coli} or \textit{Agrobacterium tumefaciens} were grown in Luria-Bertani (LB) broth. Antibiotics were used at the following concentrations: for \textit{Escherichia coli} 50 \(\mu\)g mL\(^{-1}\) kanamycin; for \textit{A. tumefaciens}, 25 \(\mu\)g mL\(^{-1}\) gentamicin, 50\(\mu\)g mL\(^{-1}\) kanamycin and 50 \(\mu\)g mL\(^{-1}\) rifampicin.

2.2 Soil-grown plant inoculations and quantitation of bacterial growth

The leaves of five-week-old *Arabidopsis* plants were inoculated by pressure infiltration using a needleless syringe. Bacterial growth within inoculated tissues was quantified as described previously (Katagiri et al. 2002). \textit{P. syringae} strains were streaked from glycerol stocks onto KB
agar plates supplemented with kanamycin, rifampicin and cycloheximide. The cells were grown at 28°C overnight, and re-streaked onto fresh KB agar plates. For infiltration, *P. syringae* was re-suspended to an optical density at 600 nm of either 0.1 or 0.4 ($\sim 1 \times 10^8$ cfu/mL or $\sim 4 \times 10^8$ cfu/mL) for HR assays and trypan blue staining, or diluted to obtain a final concentration of $\sim 2 \times 10^7$ cfu/mL for ion leakage assays, or diluted to obtain a concentration of $1 \times 10^5$ cfu/mL for determination of growth curves.

### 2.3 Cloning and plasmids

All gene expression plasmids were created using Gateway cloning technology (Invitrogen). Unless otherwise indicated, *pfu* polymerase (Fermentas) was used for all cloning procedures, and the sequences of all constructs were confirmed via Sanger sequencing. Sequence analysis was performed using CLC Genomics Workbench and CLC Main Workbench. All constructs for *P. syringae* expression were expressed under their native promoters along with chaperones, and contained an in-frame HA tag at the C terminus, as described by (Lewis et al. 2008). All *hopF* alleles indicated by red arrow in Figure 1 were PCR amplified from their original strain genomic DNA using primers with attB1 and attB2 sites added on them to enter the Gateway system (Invitrogen). PCR products were recombined into the entry vector pDONR207 (Invitrogen, USA) using the BP clonase, followed by recombination using LR clonase (Invitrogen) into the multicopy broad-host range plasmid pBBR1 MCS-2 (Kovach et al. 1995). The resulting pBBR1 MCS-2 construct containing the genomic DNA of interest was then mated into *PtoDC3000ΔHopF2* for experiments (Wei et al. 2007). Site-directed mutagenesis was performed using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, USA) and cloned using Gateway cloning technology described above. Mutants were sequence-confirmed via Sanger sequencing.
2.4 Glycerol stocks

Glycerol stocks were prepared by adding 250μl of sterile 60% glycerol (v.v) to 750 μl of an overnight bacterial culture. The solution was mixed gently by pipetting up and down to ensure even dispersion of cells. The glycerol stocks were snap freeze and stored at -80°C.

2.5 Protein expression in *P. syringae*

*P. syringae* cultures were grown overnight in KB with kanamycin and rifampin, pelleted, and washed with *hrp*-inducing minimal medium. To induce the type III secretion system, cells were inoculated in minimal media at OD$_{600}$ of 0.3 and were grown overnight at 28°C. Cells from 1.5 mL of overnight cultures were pelleted and re-suspended in 40μl of 1X loading buffer. The samples were heated at 95°C for 5 min and vortexed for 1 min, followed by another 5 min incubation at 95°C. 30μl of each sample was loaded onto 12% SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes and probed with α-HA antibodies (Roche, 9102) at a dilution of 1:10,000. Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibodies at 1/25,000 and detected via chemiluminescence (Amersham ECL, GE).

2.6 Ion leakage assay

Leaves of five-week-old *Arabidopsis* Col-0 plants were syringe- infiltrated at OD$_{600}$ of 0.04 (4 × 10$^8$ cfu/mL). For each treatment, four leaves of six plants were inoculated. Following inoculation, four leaf discs (1.5 cm$^2$) were harvested from each plant, soaked in 4mL of double deionized water (ddH$_2$O) for 45 min on an agitator. Leaf discs from each plant were then transferred to 6 mL of fresh double deionized water (ddH$_2$O). Readings were obtained with an Orion 3 Star conductivity meter (Thermo Electron Corporation, Beverly, MA).
2.7 Bacterial growth assay

The leaves of five-week-old *Arabidopsis* plants were used for *in planta* bacterial growth assays. Plants were taken out of growth chambers 24hrs prior to infiltration and kept at <~45% humidity for the remainder of experiments. Humidity was monitored on day 0 through day 3. Prior to infiltration, *P. syringae* was re-suspended in 10mM MgCl\(_2\) and diluted to 1 x 10\(^5\) cfu/mL for growth curves. *Arabidopsis* plants of varying ecotypes in different genetic backgrounds were syringe-infiltrated with *PtoDC3000ΔHopF2* carrying MCS-2 vector, *AvrRpm2\(_{PsNCPPB3335}\)*, *HopF2\(_{PacM302273PT}\)*, *HopF2\(_{PtoT1}\)*, or *AvrRpm2\(_{PphY5-2}\)* at OD\(_{600}\) = 0.0002 (2 x 10\(^5\) cfu/mL). Four leaf-discs (1 cm\(^2\)) were harvested from each plant and homogenized in 10mM MgCl\(_2\) (BioSpec Mini Bead-beater). The leaf homogenates were serially diluted and plated on KB with rifampicin and cycloheximide (at the concentrations mentioned above) on days 0 and 3 for colony counting.

2.8 Dead cell assay

Leaves of five-week-old *Arabidopsis* Col-0 or Ws-2 plants were pressure infiltrated using a needles syringe. The HR was scored at 16-to-20 hours post infiltration. To visualize cell death, leaves were harvested at 18 hours and stained with lactophenol-trypan blue solution (Koch & Slusarenko 1990) by boiling them in a water bath for 5 minutes, and followed by one hour of incubation at room temperature. Leaf samples were cleared in chloral hydrate overnight, and leaves were stored in 80% glycerol. Experiments were repeated at least 3 times to verify results.

2.9 Spray inoculation assay

Spray inoculations were performed on four- to five- week-old plants as mentioned earlier. Prior to spray inoculation *P. syringae* was re-suspended in 10mM MgCl\(_2\) and diluted to 8 x 10\(^8\) cfu/mL (OD\(_{600}\) = 0.8), with 0.04% surfactant silwet L-77 added. 10-15 mL of bacterial inoculum was
used to spray on 4-5 plants. Plants were immediately domed after spray inoculation and up to 3 days. Photographs were taken on day 3. Then Plants were re-sprayed with the initial bacterial suspension of $8 \times 10^8$ cfu/mL on day 3, domed for one more day, and monitored for up to 7 days. The rosette of individual plants were detached on day 7 and weighted.

2.10 Statistical analysis

All statistical analysis was performed using Microsoft Excel 2011 (Microsoft Corporation). Significance groups were generated utilizing a Student’s t-test ($\alpha = 0.01$) to determine whether differences between data sets were statistically significant.

2.11 Sequence analysis

DNA sequences were edited using CLC Genomics Workbench 6. Amino acid sequence alignment were also performed using CLC Genomics Workbench 6.
3 Results

3.1 Phylogenetic tree and cloning

Due to advances in genomic sequencing, hopF alleles were made available from sequenced P. syringae genomes and the non-redundant protein sequence database from NCBI. A total of 50 sequenced genomes were examined and 41 different T3SEs that belong to the HopF family were identified and analyzed phylogenetically (T. Lo, Unpublished). Based on amino acid sequence similarity, the HopF family falls into 6 different groups: HopF1, HopF2, HopF3, HopF4, HopBB1, and AvrRpm2 (T. Lo, Unpublished). Amino acid alignments were used to build a phylogenetic tree of the 41 HopF family members (Figure 1). The HopF family contains two chimeric T3SE groups, HopBB1 and AvrRpm2. Chimeric HopF members are only homologous to the rest of the HopF family at the N-terminal end of the sequence, while their C-terminal end is not homologous to the HopF family. In the case of the HopBB1 effector group, the C-terminus resembles no known protein structures, domains, or motifs. However, members of the AvrRpm2 group show sequence similarity with AvrRpm1 T3SE family in the majority of its protein sequence (Figure 2). While The AvrRpm1 and HopF families are unrelated at the sequence level, both families are predicted to encode ADP-RT activity or at least function as a molecular mimic of an ADP-RT (Cherkis et al. 2012; Singer et al. 2004; Wang et al. 2010a). While HopBB1 maintains the first catalytic residue required for the proposed ADP-RT activity of HopF family members, AvrRpm2 doesn’t possess any catalytic residues. Predicted ADP-RT catalytic residues of AvrRpm1 (His63-Tyr122-Asp185) are required for its ability to either elicit an RPM1-dependent immune response or contribute to virulence on a susceptible host (Cherkis et al.,
2012). As evident in Figure 2, putative catalytic residues of AvrRpm1 are similarly conserved within the AvrRpm2 group of chimeric effectors, raising the question of whether they carry out a similar function (See section 3.4).

The phylogenetic tree was used as a reference to select hopF alleles to clone, with the objective of covering as much diversity within the family as possible. A total of 15 HopF family members were chosen for cloning: four members of HopF1, three members of HopF2, three members of HopF3, as well as two members each for HopF4, HopBB1, and AvrRpm2. I cloned a total of five hopF alleles, hopBB1_{PmaM6} from *Pseudomonas syringae* pathovar *maculicola* M6, hopF1_{Pci0788-9} and hopF4_{Pci0788-9} from *Pseudomonas syringae* pathovar *cilantro* 0788-9, avrRpm2_{PphY5-2} from *pseudomonas syringae* pathovar *phaseolicola* Y5-2, and hopF2_{PacM302273PT} from *Pseudomonas syringae* pathovar *aceris* M302273PT were cloned. Timothy Lo has previously cloned the remaining 10 hopF alleles that are indicated by green boxes in Figure 1. Each effector was cloned into the Gateway entry vector pDONR207 and recombined into the multicopy broad-host range cloning vector pBBR1MCS-2 with its native promoter along with its upstream type III chaperone SchF. A second construct containing only effector was cloned into pDONR207 as well for further biochemical work.
Figure 1. Phylogenetic analysis of the HopF family and schematic representations of chimeric effectors with HopF similarity. (a) Maximum-likelihood tree generated by RAxML using the JTT (Jones Taylor Thornton) substitution model determined by PROTTEST. Green boxes and read arrows represent the hopF alleles that have been cloned. Green arrows represent the hopF alleles cloned by Timothy Lo, and the red arrows represent the hopF alleles that were cloned by me. (b) Schematic representation of chimeric HopF effectors. The second glycine of the myristoylation signal is indicated by the purple arrow, ADP-ribosylation residue by red.
Figure 2. Protein sequence alignment of HopF family group AvrRpm2 with AvrRpm1. Putative catalytic residues of AvrRpm1 are indicated by purple arrowheads and corresponding residues of AvrRpm2 are indicated by red boxes.

3.2 Screening for phenotypes in Arabidopsis

To functionally characterize the HopF T3SE family, the hopF alleles of interest were expressed with an in-frame C-terminal HA epitope tag under their native promoters along with the type III chaperone SchF in PtoDC3000 lacking its native hopF2 allele (PtoDC3000ΔHopF2; Jamir et al.
bacterial growth assays were conducted to investigate the influence of each T3SE on the virulence of *P. syringae* in *Arabidopsis* ecotype Colombia-0 (Col-0). I tested two *hopF* alleles, HopF<sub>PacM302273PT</sub> and AvrRpm2<sub>PphY5-2</sub>, and also reconfirmed a decrease in virulence conferred by two previously screened *hopF* alleles, AvrRpm2<sub>PsvNCPPB3335</sub> and HopF<sub>PtOT1</sub>. The remaining *hopF* alleles were tested by PhD candidate Timothy Lo, and two undergraduate students, Derek Seto and Jennifer Duan (Table 1). While expression of HopF<sub>PtoDC3000</sub> promoted the virulence of *PtoDC3000ΔHopF2*, expression of AvrRpm2<sub>PsvNCPPB3335</sub>, AvrRpm2<sub>PphY5-2</sub>, HopF<sub>PacM302273PT</sub>, and HopF<sub>PtOT1</sub> reduced growth relative to empty vector controls (Figure 3; Wilton et al. 2010). This suggests that *Arabidopsis* Col-0 can recognize each one of these effectors to elicit an ETI response (Figure 3; Figure S 1).

In addition, *in planta* growth of *PtoDC3000ΔHopF2* expressing HopF<sub>PacM302273PT</sub> and AvrRpm2<sub>PsvNCPPB3335</sub> were also tested in *Arabidopsis* Ws-2 ecotype. Interestingly, similar to results observed in Col-0, both alleles grew significantly less than the strain with empty vector (Figure 4). Therefore, both Ws-2 and Col-0 express R proteins capable of recognizing HopF<sub>PacM302273PT</sub> and AvrRpm2<sub>PsvNCPPB3335</sub>.

During replication of the observed ETI response for all 4 *hopF* alleles, it was discovered that ETI was abolished when the relative humidity was greater than ~45% during the course of the experiment (Figure 5). Hence, remaining bacterial growth assays were done at a relative humidity of <45%. This was true of both ETI response observed in Col-0 and Ws-2 ecotypes. No macroscopic HR or HR-associated electrolyte leakage was observable with any of the tested *hopF* alleles (Figure 12; Figure 13).
The other hopF alleles tested showed no effect on bacterial growth (Table 1). This may be due to the fact that they do not have a function in Arabidopsis or perhaps the bacterial growth assay conducted is not sensitive enough to detect a significant difference. The remaining three alleles, which I have cloned, are yet to be tested.

Table 1. Summary of bacterial growth assays conducted on Arabidopsis using cloned hopF alleles. Red bar represents significant increase in growth (p<0.05), blue bar represents significant decrease in growth (p<0.01), and yellow bar represents no difference in growth. Asterisk represents previously published data (Wilton et al. 2010).

<table>
<thead>
<tr>
<th>Effector</th>
<th>Phenotype</th>
<th># of Trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>HopF1$_{Pta11528}$</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>HopF1$_{PmeN6801}$</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>HopF1$_{Pph1449B}$</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>*HopF2$_{ATG}^{PtdDC3000}$</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>HopF2$_{PvtHC-1}$</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>HopF2$_{PtoT1}$</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>HopF2$_{PacM302273PT}$</td>
<td></td>
<td>~10</td>
</tr>
<tr>
<td>HopF3$_{Pph1448A}$</td>
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</tr>
<tr>
<td>HopF3$_{Pph1302A}$</td>
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</tr>
<tr>
<td>HopF4$_{PaeNCPPB3681}$</td>
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<td>3</td>
</tr>
<tr>
<td>HopBB1$_{PavBPIC631}$</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>AvrRpm2$_{PsvNCPPB3335}$</td>
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</tr>
<tr>
<td>AvrRpm2$_{PphY5_2}$</td>
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</table>

Significant decrease | Significant increase | No difference
Figure 3. Four sequence diverse hopF alleles induce ETI in Arabidopsis when expressed heterologously in PtoDC3000ΔHopF2. Growth analysis of (a) HopF2_Pac, (b) AvrRpm2_Psv, (c) AvrRpm2_PphY5-2, (d) HopF2_PtoT1 infiltrated into Arabidopsis ecotype Col-0. Colony counts for all experiments were made at 0 and 3 days post-infiltration, and relative humidity (RH) was <~45%. EV represents PtoDC3000ΔHopF2 expressing the MCS-2 vector. H68A represents the putative catalytic mutation of AvrRpm2. The mutant protein is expressed at a similar level to AvrRpm2_Psv. AvrRpm1 represents positive control. Error bars represent standard deviation from the mean of 10 samples. Results have been replicated multiple times, indicated in Table.1. Two-tailed t-test was performed to test for significant differences, which is indicated by an asterisk (*p<0.01).
Figure 4. HopF2_{Pac} and AvrRpm2_{Psv} induce an ETI response in *Arabidopsis* ecotype Ws-2 when expressed heterologously in *PtoDC3000ΔHopF2*. (a) Growth analysis of HopF2_{Pac} infiltrated into *Arabidopsis* ecotype Ws-2. (b) Growth analysis of AvrRpm2_{Psv} infiltrated into *Arabidopsis* ecotype Ws-2. Colony counts for all experiments were made at 0 and 3 days post-infiltration, and relative humidity (RH) was ~<45%. EV represents *PtoDC3000ΔHopF2* expressing MCS-2 vector. Error bars represent standard deviation from the mean of 10 samples. Results have been replicated at least 3 times. Two-tailed t-test was performed to test for significant differences, which is indicated by an asterisk (*p<0.01).
Figure 5. ETI induced by four sequence diverse hopF alleles in *Arabidopsis* ecotype Col-0 is lost in relative high humidity. Growth analysis of (a) HopF2*Pac*, (b) AvrRpm2*Psv*, (c) AvrRpm2*Pph5-2, (d) HopF2*PtoT1* infiltrated into *Arabidopsis* ecotype Col-0. Colony counts for all experiments were made at 0 and 3 days post-infiltration and relative humidity (RH) was >45%. EV represents *PtoDC3000ΔHopF2* expressing the MCS-2 vector. Error bars represent standard deviation from the mean of 10 samples. Two-tailed t-test was performed to test for significant differences, which is indicated by an asterisk (*p<0.01).
3.3 Genetic requirements of ETI triggered by various hopF alleles in Arabidopsis

While four different hopF alleles, AvrRpm2\textsubscript{PsvNCPPB3335}, AvrRpm2\textsubscript{Pph\textsubscript{Y5-2}}, HopF2\textsubscript{PacM302273PT}, and HopF2\textsubscript{PtoT1} decrease the virulence of Pto\textsubscript{DC3000} in Arabidopsis; it is unclear what processes underlie these resistance responses. Therefore, I sought to uncover the required genetic elements contributing to decreased growth of \textit{P. syringae} expressing HopF2\textsubscript{PtoT1}, AvrRpm2\textsubscript{PsvNCPPB3335}, and HopF2\textsubscript{PacM302273PT} using a reverse genetic screen of known R proteins and accessory elements critical to ETI signaling.

Based on previous knowledge, HopF2\textsubscript{PtoDC3000} interacts with the Arabidopsis host protein RIN4 to hinder its cleavage by the AvrRpt2 effector, which subsequently prevents ETI and HR in response to AvrRpt2 (Wilton et al. 2010). As a result, to assess whether RIN4 and/or one or both of the known associated R proteins (RPM1 and RPS2) are responsible for the observed ETI, bacterial growth assays were conducted on rpm1rps2rin4 triple knockout plants (Belkhadir et al. 2004). The decreased bacterial growth observed in Col-0 plants infiltrated by HopF2\textsubscript{PtoT1} is lost in this genetic background suggesting that one or more of these genes are responsible for recognition of HopF2\textsubscript{PtoT1} and inducing an immune response (Figure 6c) (Lo et al. Submitted).

Next, to examine whether RPM1 and or RPS2 are required for HopF2\textsubscript{PtoT1} recognition, bacterial growth assays were done on both rpm1 and rps2 single knockout plants. The decreased bacterial growth observed in Col-0 plants infiltrated by HopF2\textsubscript{PtoT1} is observed in rps2 knockout plants, but lost in rpm1 plants (Figure 6a and b). This demonstrates that RPM1 is required for recognition of HopF2\textsubscript{PtoT1} in Arabidopsis (Lo et al. Submitted).

The genetic requirement of ETI triggered by HopF2\textsubscript{PacM302273PT} and AvrRpm2\textsubscript{PsvNCPPB3335} in Arabidopsis were also examined using a similar experimental design. Bacterial growth assays
were conducted on *rpm1rps2rin4* triple knockout plants (Figure 7; Figure S 2) with *PtoDC3000ΔHopF2* expressing HopF2\textsubscript{PacM302273PT} or AvrRpm2\textsubscript{PsvNCPPB3335}. Both HopF2\textsubscript{PacM302273PT} or AvrRpm2\textsubscript{PsvNCPPB3335} reduced bacterial growth on *rpm1rps2rin4* mutant plants, similar to what was observed in Col-0 plants. Therefore, unlike HopF2\textsubscript{PtoT1}, ETI induced by both AvrRpm2\textsubscript{PsvNCPPB3335} and HopF2\textsubscript{PacM302273PT} is independent of RIN4 or its known associated R genes, RPM1 and RPS2.

To further confirm this result and the role of two main R genes RPM1 and RPS2, growth assays were conducted with AvrRpm2\textsubscript{PsvNCPPB3335} on both *rpm1* and *rps2* single mutant plants. The ETI triggered by AvrRpm2\textsubscript{PsvNCPPB3335} is observed in both genetic backgrounds, which further confirms that RPM1, RPS2 and RIN4 are not responsible for the recognition of AvrRpm2\textsubscript{PsvNCPPB3335} in *Arabidopsis* (Figure S 3). Thus, the ETI induced by both HopF2\textsubscript{PacM302273PT} and AvrRpm2\textsubscript{PsvNCPPB3335} is independent of RIN4 complex and its associated R proteins.

Given that both HopF2\textsubscript{PacM302273PT} and AvrRpm2\textsubscript{PsvNCPPB3335} consistently show 5-7 fold decrease in bacterial growth in both wild type Col-0 and mutant plants lacking known R genes, *in vitro* growth of *PtoDC3000ΔHopF2* expressing both T3SEs were tested to rule out any possible general deleterious effects of their expression on bacterial fitness (Figure 8). The *in vitro* growth rate of both T3SEs in KB and *hrp*-inducing minimal media are the same as *PtoDC3000ΔHopF2* carrying an empty vector. This indicates that the weak reduction of bacterial growth observed with both effector proteins *in planta* is not due to deleterious effects of ectopic *hopF* allele expression (Figure 8). In addition, to further confirm that the observed results are a result of HopF expression, western blot analyses were conducted on *PtoDC3000ΔHopF2* expressing different *hopF* alleles. As shown in Figure 8e both HopF2\textsubscript{PacM302273PT} and AvrRpm2\textsubscript{PsvNCPPB3335}
are expressed equally in \( PtoDC3000\Delta HopF2 \) and comparable to the previously characterized \( HopF2_{PtoDC3000} \).

**Figure 6. Contribution of RPM1, RPS2 and or RIN4 to \( HopF2_{PtoT1} \) triggered ETI.** 
\( HopF2_{PtoT1} \) was expressed heterologously in \( PtoDC3000\Delta HopF2 \) and bacterial growth assays were conducted on (a) \( rpm1 \) and (b) \( rps2 \) single mutant plants and (c) \( rpm1rps2rin4 \) triple mutant plants. Colony counts were made at 0 and 3 days post-infiltration. EV represents \( PtoDC3000\Delta HopF2 \) expressing the empty MCS-2 vector. Error bars represent standard deviation from the mean of 10 samples. Results have been replicated at least three times. Two-tailed t-test was performed to test for significant differences, which is indicated by an asterisk (*p<0.01).
Figure 7. HopF2_{pac} and AvrRpm2_{psv} recognition in Arabidopsis is independent of RIN4, RPM1 and RPS2. (a) HopF2_{pac} was expressed heterologously in PtoDC3000ΔHopF2 and bacterial growth assays were conducted on the triple mutant plants, rpm1rps2rin4. (b) AvrRpm2_{psv} was expressed heterologously in PtoDC3000ΔHopF2 and bacterial growth assays were conducted on the triple mutant plants, rpm1rsp2rin4. Colony counts were made at 0 and 3 days post infiltration. EV represents PtoDC3000ΔHopF2 expressing the empty MCS-2 vector. Error bars represent standard deviation from the mean of 10 samples. Results have been replicated at least three times. Two-tailed t-test was performed to test for significant differences, which is indicated by an asterisk (*p<0.01).
Figure 8. Expression level of HopF2\textsubscript{Pac} and AvrRpm2\textsubscript{Psv}. Growth analysis of (a) HopF2\textsubscript{Pac} and (c) AvrRpm2\textsubscript{Psv} in hrp-inducing minimal medium, respectively. At cycle one the cultures were transferred from KB medium to hrp-inducing minimal medium and the OD600 of all cultures were set to ~0.1. Growth analysis of (b) HopF2\textsubscript{Pac} and (d) AvrRpm2\textsubscript{Psv} in KB medium, respectively. (e) Immunoblot analysis of HopF2\textsubscript{Pac}-HA and AvrRpm2\textsubscript{Psv}-HA protein expression in \textit{P. syringae} pv. tomato DC3000. \textit{PtoDC3000AHopF2} carrying an empty vector or one of the indicated HA-tagged proteins were grown in minimal medium to induce the type III secretion system. Equal amounts of protein were resolved on 12% SDS-PAGE gels, blotted onto nitrocellulose, and probed with HA antibodies. The Ponceau red-stained blot was used as a loading control.
3.4 AvrRpm2<sub>PsvNCPB3335</sub> recognition is dependent on predicted catalytic residues

As previously stated, AvrRpm2<sub>PsvNCPB3335</sub> is a chimeric T3SE, with N-terminal similarity to HopF family of effectors, and 60% amino acid similarity to AvrRpm1 family of effectors in the C-terminal end (Figure 2). AvrRpm1 has been shown to structurally resemble the catalytic domain of poly (ADP-ribosyl) polymerases (PARP) (Cherkis et al. 2012). It has been demonstrated that the predicted catalytic residues of AvrRpm1 (His63-Tyr122-Asp185) are required to elicit an RPM1-dependent immune response and contribute to virulence on a susceptible host (Cherkis et al. 2012).

Since the C-terminal end of AvrRpm2<sub>PsvNCPB3335</sub> shows close resemblance with the AvrRpm1 effector family, the amino acid sequences of the two were aligned (Figure 2). The catalytic triad of AvrRpm1, His63-Tyr122-Asp185, is conserved in AvrRpm2<sub>PsvNCPB3335</sub>. Hence, to investigate the functional significance of these sites in the function of T3SE AvrRpm2<sub>PsvNCPB3335</sub>, site-directed mutagenesis was used to substitute the three sites with an alanine. Two of the putative catalytic residues, His68 and Asp188 were successfully mutated to an alanine using the QuikChange II site-directed mutagenesis kit. Bacterial growth assays were performed using the His68A mutant to examine whether it is required for the ETI elicited by AvrRpm2<sub>PsvNCPB3335</sub>. As shown in Figure 3b the decreased bacterial growth observed in wild type Col-0 plants due to recognition of AvrRpm2<sub>PsvNCPB3335</sub> is abolished when the putative catalytic residue H68 is mutated to an alanine. This is not due to lack of expression since AvrRpm2<sub>PsvNCPB3335</sub>H68A was detected by western blot analysis (Figure 7). This suggests that the putative PARP enzymatic function of AvrRpm2 is required for elicitation of the ETI response. To further investigate the possible role of other two putative catalytic residues, site-directed mutagenesis and further
bacterial growth assays needs to be conducted to determine their contribution to ETI induced by AvrRpm2.

3.5 Assessing HopF2_{PacM302273PT} and AvrRpm2_{PsNCPPB3335} recognition via known signaling components of R gene-mediated immunity

To identify components of the signaling pathway with which HopF2_{PacM302273PT} and AvrRpm2_{PsNCPPB3335} induce immunity, a larger collection of characterized mutations in various defense signaling and response pathways were investigated. In plants, many of the disease resistance (R) proteins contain an NB-LRR structure. NB-LRR proteins are divided into two subclasses based on the presence of an N-terminal Coiled-coil (CC) or Toll interleukin receptor (TIR) domain. ETI induced by the CC-NB-LRR class of R proteins such as RPS2, RPM1, and RPS5 require NDR1, a membrane localized glycosylphosphatidylinositol (GPI)-anchored protein (Century 1997; Coppinger et al. 2004). Similarly, TIR-NB-LRR R proteins such as RPS4 require EDS1 and its interacting protein PAD4 for ETI signaling (Aarts et al. 1998; Feys et al. 2001). In addition, RAR1 has also been demonstrated to mediate R protein stability and accumulation (Tornero 2002). Further, NDR1 and RAR1 have been shown to operate in both linear and parallel signaling events, depending on the R protein involved (Tornero 2002).

As well, the plant hormone salicylic acid (SA) plays a number of important roles in the defense response, and is also implicated to be involved in the development of ETI (Delaney et al. 1994). Consequently, nahG transgenic plants, which express a bacterial salicylate hydroxylase that constantly degrades SA, are commonly used to investigate the involvement of SA in planta (Wees & Glazebrook 2003).
To examine the ETI triggered by AvrRpm2$_{PsvNCPPB3335}$ and HopF2$_{PaeM302273PT}$, bacterial growth assays were conducted on ndr1rar1, pad4, nahG and eds1 plants. The reduced bacterial growth elicited by both T3SEs on wild type plants was also observed in ndr1rar1, pad4, and eds1 mutant plants. This demonstrates that neither NDR1, RAR1, PAD4 nor EDS1 play a role in AvrRpm2$_{PsvNCPPB3335}$ and HopF2$_{PaeM302273PT}$ triggered immunity (Figure 9; Figure 10; Figure S 4; Figure S 5).

In addition, the AvrRpm2$_{PsvNCPPB3335}$- induced immunity was also observed in nahG plants, where SA is degraded (Figure 9). However, upon replication of this ETI response it was observed that at times the ETI response is abolished in nahG plants (Figure S 5). The nahG background has been reported to affect non-host resistance in Arabidopsis to P. syringae pv. phaseolicola, due to the accumulation of catechol (Wees & Glazebrook 2003). To further examine the loss of ETI response in nahG transgene, defense responses in eds16 plants (also called sid2 or ics1) impaired in isochorismate synthase responsible for the synthesis of SA during plant immunity was also tested. In contrast to nahG plants, AvrRpm2$_{PsvNCPPB3335}$ induced a resistance response in eds16 mutant plants, similar to the response observed in wild type Col-0 plants (Figure 11). This indicates that the loss of ETI observed in nahG transgene is possibly due to its effect on plant immunity and development (See discussion). Subsequent confirmation of results on eds16 result is required.

The ETI triggered by HopF2$_{PaeM302273PT}$ was also tested in nahG background (Figure 10). Contrary to AvrRpm2$_{PsvNCPPB3335}$, the ETI response observed with HopF2$_{PaeM302273PT}$ was lost in nahG background. This was further confirmed in subsequent trials (Figure S 4). To examine the loss of ETI response in nahG background, defense response in eds16 mutant plants were tested. Similar to AvrRpm2$_{PsvNCPPB3335}$, HopF2$_{PaeM302273PT}$ also triggered an ETI response in eds16
mutant plants (Figure 11). Again, this demonstrates that the loss of ETI observed in *nahG* transgene is possibly due to its effect on plant immunity in development.

![Bar graphs](image)

**Figure 9.** *AvrRpm2<sub>PsV</sub>* recognition is independent of known signaling components of *R* gene-mediated immunity. (a)–(d) *AvrRpm2<sub>PsV</sub>* was expressed heterologously in *PtoDC3000ΔHopF2* and bacterial growth assays were conducted on the indicated genotypes. Colony counts were made at 0 and 3 days post infiltration. EV represents *PtoDC3000ΔHopF2* expressing the empty MCS-2 vector. Error bars represent standard deviation from the mean of 10 samples. Results have been replicated at least three times. Two-tailed t-test was performed to test for significant differences, which is indicated by an asterisk (*p<0.01).
Figure 10. HopF2* recognition is independent of known signaling components of R gene-mediated immunity. HopF2* was expressed heterologously in PtoDC3000ΔHopF2 and bacterial growth assays were conducted on the indicated genotypes. Colony counts were made at 0 and 3 days post infiltration. EV represents PtoDC3000ΔHopF2 expressing the empty MCS-2 vector. Error bars represent standard deviation from the mean of 10 samples. Results have been replicated at least three times. Two-tailed t-test was performed to test for significant differences, which is indicated by an asterisk (*p<0.01).
Figure 11. Recognition of HopF2\textsubscript{Pac} and AvrRpm2\textsubscript{Psv} in Arabidopsis is independent of salicylic acid. (a) HopF2\textsubscript{Pac} and (b) AvrRpm2\textsubscript{Psv} were expressed heterologously in PtoDC3000ΔHopF2 and bacterial growth assays were conducted on eds16 mutant plants. Colony counts were made at 0 and 3 days post infiltration. EV represents PtoDC3000ΔHopF2 expressing the empty MCS-2 vector. Error bars represent standard deviation from the mean of 10 samples. Results have been replicated at least three times. Two-tailed t-test was performed to test for significant differences, which is indicated by an asterisk (*p<0.01).

3.6 Uncoupling HR

Plant cells undergoing ETI often show a hypersensitive response (HR), which is programmed cell death at the site of infection (Dangl et al. 1996; Hammond-Kosack & Jones 1996). HR is thought to deprive the pathogen of a supply of food and confine them to initial site of infection. However, HR is not always required for ETI and successful restriction of pathogen growth (Gassmann 2005a). HR results in the lysis of plant cells leading to ion leakage, which can be used as a proxy for ETI. In the case of HopF T3SEs, none of the alleles tested in Table 1 showed a macroscopic HR at 18 hours post-infection (Data not shown). AvrRpm2\textsubscript{PsvNCPPB335} and HopF2\textsubscript{PacM302273PT} similar to other hopF alleles did not show a macroscopic HR on Col-0 and or Ws-2 ecotypes at 18 hours when infiltrated at an OD\textsubscript{600} of 0.1 (~1 × 10\textsuperscript{8} cfu/mL) (Figure 12).
However, when tested at a higher OD₆₀₀ of 0.4 (4 x 10⁸ cfu/mL), AvrRpm2ₚₛₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₕ
Figure 12. Neither HopF2<sub>pac</sub> nor AvrRpm2<sub>psv</sub> induce a hypersensitive response in Arabidopsis ecotype Col-0 or Ws-2. (a) Half leaves of Arabidopsis ecotype Col-0 or Ws-2 plants were infiltrated with 10mM MgCl₂, or PtoDC3000ΔHopF2 expressing the empty vector (EV), or one of the hopF family members under the control of their endogenous promoter. The bacteria at an optical density of 0.1 (~1 × 10⁸ cfu/mL) were syringe infiltrated into the leaves. Photographs were taken at 18 h post-infiltration. The numbers of leaves showing an HR are indicated at the bottom of the leaves. HRs are indicated by an asterisk. (b) Same as (a) except the bacteria at an optical density of 0.4 (4 x 10⁸ cfu/mL) were syringe infiltrated into the leaves.
Figure 13. HopF2_{Pac} and AvrRpm2_{Psv} do not show microscopic HR. Trypan blue staining of Arabidopsis Col-0 leaves infiltrated with 10mM MgCl2, or PtoDC3000ΔHopF2 expressing the empty vector (EV), or one of the hopF family members under the control of their endogenous promoter. The bacteria were syringe infiltrated into the leaves at an optical density of 0.4 (4 x 10^8 cfu/mL). H68A represents the putative catalytic mutation of AvrRpm2. The mutant protein is expressed at a similar level to AvrRpm2_{Psv}.

Figure 14. Quantitative measures of ion leakage from leaf tissue on Col-0 following infection of PtoDC3000ΔHopF2 expressing HopF2_{Pac} and AvrRpm2_{Psv}. Electrolyte leakage of Arabidopsis Col-0 leaf discs after infiltration with PtoDC3000ΔHopF2 expressing either HopF2_{Pac} or AvrRpm2_{Psv}. AvrRpm1 represents positive control. The bacteria were syringe infiltrated into the leaves at 4 x 10^8 cfu/mL. Error bars indicate the standard deviation from the mean of 8 samples. Two-tailed t-test was performed to test for significant differences, which is indicated by an asterisk (*p<0.01).
3.7 Screening for the R gene – ARTIC collection

The inability of HopF effectors to elicit an HR response restricted the screening of functional phenotypes to one assay – the bacterial growth assay. Due to the time-consuming nature of syringe inoculation, it was not a feasible method for screening and identifying the R gene responsible for recognition. Thus, other high throughput inoculation methods were tested to identify conditions that reproduced resistance responses observed via growth assay in order to facilitate a genetic screening. Bacterial suspensions of either *P. syringae* carrying empty vector and or HopF2$_{Pac}$M302273PT or AvrRpm2$_{Psv}$NCPPB3335 were prepared with the 0.04% surfactant silwet L-77 (Kim et al. 2009). The inoculums were sprayed on Col-0 and Ws-2 ecotypes to inoculate bacteria onto the surface of leaves. Following spray inoculation the plants were monitored for the development of resistance and or susceptible phenotypes for seven days.

Because *Pto*DC3000 can successfully overcome stomatal immunity in *Arabidopsis*, susceptible plants should display disease symptoms in the form of leaf wilting and chlorosis (Melotto et al. 2008). In contrast, ETI should limit pathogen growth and prevent disease symptoms from developing. Therefore, I hypothesized that spray inoculation of *Pto*DC3000ΔHopF2 expressing HopF2$_{Pac}$M302273PT and AvrRpm2$_{Psv}$NCPPB3335 should result in less chlorosis relative to empty vector controls.

For both Col-0 and Ws-2 ecotypes sprayed with *Pto*DC3000ΔHopF2 expressing HopF2$_{Pac}$M302273PT or AvrRpm2$_{Psv}$NCPPB3335, development of resistance phenotypes were correlated with those observed with growth assays by syringe inoculation (Figure 15; Figure 16; Figure S 6). Plants sprayed with *P. syringae* carrying empty vector show light chlorosis with leaves yellowing on day 3, while plants sprayed with either HopF2$_{Pac}$M302273PT or AvrRpm2$_{Psv}$NCPPB3335 did not show any disease symptoms (Figure 15; Figure 16; Figure S 6). By
day 7, plants sprayed with empty vector show 100% mortality while plants sprayed with either hopF allele proteins appear healthy. Together, this suggests that induction of ETI by hopF alleles during syringe inoculation and bacterial growth assay can be recapitulated by spray inoculation and visual inspection of whole plant phenotypes.

In order to identify an R gene in Arabidopsis that determines resistance to HopF2_{PacM302273PT}, spray inoculation was used to screen through the Arabidopsis R gene T-DNA insertion collection (ARTIC) (Lewis et al. 2010). Approximately 61 R gene T-DNA knockout lines were tested by spray inoculation with PtoDC3000ΔHopF2 expressing empty vector (EV) and PtoDC3000ΔHopF2 expressing HopF2_{PacM302273PT} (Figure 17). Amongst the 61 R gene knockout lines tested, four of them, R-AX, R-AZ, R-X and R-BB lost their resistance phenotype and displayed disease symptoms similar to EV (Figure 17). This suggests that one or combination of all 4 R genes might be responsible for the recognition of HopF2_{PacM302273PT}. Upon repeating the spray inoculation the second time on the same set of plants, only R-X, which encodes the R protein X reconfirmed (Figure 18). This demonstrates that X is the potential R gene responsible for the recognition of the T3SE HopF2_{PacM302273PT} (Figure 18; Figure 19).
Figure 15. Phenotypes of Arabidopsis ecotype Col-0 plants sprayed with either HopF2\textsubscript{Pac} or AvrRpm2\textsubscript{PsV}. Five-week-old Arabidopsis Col-0 plants were sprayed with PtoDC3000ΔHopF2 expressing empty vector MCS-2 (EV) and or HopF2\textsubscript{Pac} or AvrRpm2\textsubscript{PsV} at OD\textsubscript{600} = 0.8 or OD\textsubscript{600} = 0.4. Plants were domed for 3 days and re-sprayed on day 3 with similar optical density as day 0. Domes were removed on day 4. Photographs were taken on day 3 before the re-spray and on day 7.
Figure 16. Phenotypes of *Arabidopsis* ecotype Ws-2 plants sprayed with either HopF2$_{pac}$ or AvrRpm2$_{psv}$. Five-week-old *Arabidopsis* Ws-2 plants were sprayed with *PtoDC3000ΔHopF2* expressing empty vector MCS-2 (EV) and or HopF2$_{pac}$ or AvrRpm2$_{psv}$ at OD$_{600}$ = 0.8 or OD$_{600}$ = 0.4. Plants were domed for 3 days and re-sprayed on day 3 with similar optical density as day 0. Domes were removed on day 4. Photographs were taken on day 3 before the re-spray and on day 7.
### Table 1: Plant Growth Analysis

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Figure 17. Phenotypes of various ARTIC collection lines sprayed with HopF2_{pac}.
(a) – (j) Five-week-old *Arabidopsis* Col-0 or R-gene T-DNA insertion collection plants were sprayed with *PtoDC3000ΔHopF2* expressing empty vector MCS-2 (EV) and or HopF2_{pac} at OD_{600} = 0.8. Plants were domed for 3 days and re-sprayed on day 3 with similar optical density as day 0. Domes were removed on day 4. Photographs were taken on day 3 before the re-spray and on day 7.
Figure 18. Phenotypes of various ARTIC collection lines sprayed with HopF2\textsubscript{Pac}. Five-week-old *Arabidopsis* Col-0 or R-gene T-DNA insertion collection plants were sprayed with *PtoDC3000\Delta HopF2* expressing empty vector MCS-2 (EV) and or HopF2\textsubscript{Pac} at OD\textsubscript{600} = 0.8. Plants were domed for 3 days and re-sprayed on day 3 with similar optical density as day 0. Domes were removed on day 4. Photographs were taken on day 3 before the re-spray and on day 7.
Figure 19. Phenotypes of *Arabidopsis* Col-0 or x mutant plants sprayed with HopF2<sub>Pac</sub>. Five-week-old *Arabidopsis* Col-0 or x mutant plants were sprayed with *PtoDC3000ΔHopF2* expressing empty vector MCS-2 (EV) and or HopF2<sub>Pac</sub> at OD<sub>600</sub> = 0.8. Plants were domed for 3 days and re-sprayed on day 3 with similar optical density as day 0. Domes were removed on day 4. Photographs were taken on day 3 before the re-spray and on day 7.
Chapter 4

4 Discussion

Type III effectors are the direct molecular interface between pathogen and plant host and are essential for the virulence of many pathogens. The recent advance in genomic sequencing has led to a rapid increase in the number of putative T3SEs available in databases. Subsequently, this has provided information regarding multiple homologs of T3SEs from distantly unrelated bacteria. While a majority of current research is focused on characterizing a single representative of a given T3SE family, we decided to characterize divergent homologs of a single T3SE family, HopF. We demonstrated that HopF family of T3SE contains various members that are capable of eliciting both virulence and avirulence response in *Arabidopsis* plants. We also revealed that the mechanism underlying the recognition of *P. syringae* T3SE, HopF2<sub>PacM302273PT</sub> and AvrRpm2<sub>PsvNCPPB3335</sub> is not associated with the RIN4 complex. Furthermore, we show that the putative catalytic residues of AvrRpm2<sub>PsvNCPPB3335</sub> are responsible for the recognition of the effector protein in *Arabidopsis*.

We show that four different T3SEs from HopF family, HopF2<sub>PtoT1</sub>, HopF2<sub>PacM302273PT</sub>, AvrRpm2<sub>PsvNCPPB3335</sub> and AvrRpm2<sub>PphY5-2</sub> are recognized by *Arabidopsis* to trigger ETI. The ETI triggered by these T3SE proteins are completely abolished under high humidity (over ~45% RH) (Figure 5). This response is similar to previous reports showing the suppression of ETI during high humidity (Freeman & Beattie 2009; Jambunathan et al. 2001). The *P. syringae* T3SE, AvrRpm1 is recognized by the *Arabidopsis* resistance protein, RPM1, and triggers an ETI response accompanied by reduction in the growth of bacteria. The ETI induced by AvrRpm1 is abolished in plants placed in high humidity (Freeman & Beattie 2009). This is similar to other
reports showing that maintaining leaves in high humidity environment can suppress macroscopic HR while also increasing the growth of bacteria (Young 1974; Cook 1977). Furthermore, high humidity has also been shown to influence the Arabidopsis lesion-mimic defense gene mutants, {\it cpr}22 and {\it cpn}1-1, which normally show elevated defense phenotypes (Jambunathan et al. 2001; Yoshioka et al. 2001). The suppression of ETI triggered by either HopF2{\it Pto}T1, HopF2{\it Pac}M302273PT, AvrRpm2{\it Ps}NCPPB3335 and or AvrRpm2{\it Pph}Y5-2 in high humidity conditions is consistent with the hypothesis that bacterial growth is limited by water availability during plant immune responses (Freeman & Beattie 2009).

ETI is often accompanied by programmed cell death or HR at the site of infection, while also limiting the growth of the bacteria. Interestingly, HR is not always required for the inhibition of bacterial growth. A number of T3SEs such as AvrRps4 (Hinsch 1996) and HopPsyA (Gassmann 2005b) are recognized by Arabidopsis ecotype Col-0 and inhibit the growth of bacteria in the absence of a visible HR. In addition, Arabidopsis dnd1 mutants also mediate resistance to some bacterial pathogens without inducing a visible HR response (Clough et al. 2000; Yu et al. 1998). Together, this supports the hypothesis that HR is not always associated with restricted pathogen growth or {\it vice versa}. My findings that hopF alleles inhibited the bacterial growth without inducing macroscopic or microscopic cell death are consistent with what previously has been observed with other T3SE. My data further adds to the hypothesis that HR is non-essential for the restriction of pathogen growth during ETI.

Previous work by Wilton et al. showed that a different hopF allele, HopF2 from {\it P. syringae} PtoDC3000 elicits a virulence response in Arabidopsis plants (Wilton et al. 2010). This is in contrast to what I observed with the four different hopF alleles that were tested in Arabidopsis
(Figure 3). This highlights the functional diversification of the HopF family and likely reflects the diversification of host proteins targeted by HopF family members.

HopF2<sub>PtoDC3000</sub> has been shown to interact with the <i>Arabidopsis</i> plasma-membrane protein RIN4 to cause virulence (Wilton et al. 2010). In addition, it has also been shown that HopF2<sub>PtoDC3000</sub> is capable of suppressing the resistance response induced by another effector protein AvrRpt2 through RIN4 (Wilton et al. 2010). Given that both HopF2<sub>PtoDC3000</sub> and HopF2<sub>PtoT1</sub> belong to the same subclade of the HopF family, I tested the role of two RIN4 associated R proteins, RPM1 and RPS2, in the recognition of HopF2<sub>PtoT1</sub> in <i>Arabidopsis</i>. We show that the ETI triggered in response to HopF2<sub>PtoT1</sub> is dependent on the RIN4 associated R protein, RPM1. Surprisingly, this suggests while one HopF family member can trigger ETI through targeting RIN4 a subclade member can promote virulence in <i>Arabidopsis</i>. While highlighting the functional diversification of this family, it is unclear how the molecular mechanisms underlying these responses differ.

With the addition of HopF2<sub>PtoT1</sub>, the R protein RPM1 is capable of recognizing three divergent T3SEs and inducing and ETI (Bisgrove et al. 1994; Grant et al. 1995).

In addition, to further examine whether the HopF2<sub>PtoT1</sub> ETI response is dependent on RIN4, bacterial growth assays were conducted on the <i>rps2/rin4</i> double knockout plants (Mackey et al. 2003b). The <i>rin4</i> mutant plants, where RIN4 is knocked out, RPS2 recognizes its absence and triggers a constitutive ETI response leading to seedling lethality (Mackey et al. 2003b). Therefore in order to assess the role of RIN4, the double knockout of <i>rin4rps2</i> must be used, since the loss of RIN4 in this background will no longer constitutively trigger RPS2-triggered ETI. In addition, since RPS2 is not required for HopF2<sub>PtoT1</sub> resistance this result can indicate whether RIN4 specifically is required for the HopF2<sub>PtoT1</sub> ETI response. Timothy Lo has done the aforementioned growth assays. The results demonstrated that the RIN4 and its associated R
protein RPM1 are responsible for the recognition of HopF2<sub>Pto</sub>T1 (T. Lo et al. submitted). Additional work on this T3SE protein has been done by Timothy Lo, which has been submitted for publication.

Based on the results observed for HopF2<sub>Pto</sub>T1, it was hypothesized that the other two members of the HopF family, HopF2<sub>Pac</sub>M302273PT and AvrRpm2<sub>Psv</sub>NCPPB3335 also confer resistance through the RIN4 complex and one or both of its associated R proteins. However, results from bacterial growth assays indicate that the ETI triggered by HopF2<sub>Pac</sub>M302273PT and AvrRpm2<sub>Psv</sub>NCPPB3335 does not require the <em>Arabidopsis</em> protein RIN4 (Figure 7). In addition, neither RPM1 nor RPS2 are required for recognition of either hopF alleles. Given that these two effector proteins are from a different subclade within the family, it suggests further functional diversification from the RIN4 targeting subclade containing HopF2<sub>Pto</sub>T1 and HopF2<sub>Pto</sub>DC3000. It is interesting to note that AvrRpm2<sub>Psv</sub>NCPPB3335 has ~60% amino acid similarity to AvrRpm1 effector protein which is recognized by RPM1. However, regardless of the sequence similarities AvrRpm1 and AvrRpm2 appear to be recognized by distinct R proteins.

What are the R proteins in <em>Arabidopsis</em> responsible for the recognition of the two T3SEs, HopF2<sub>Pac</sub>M302273PT and AvrRpm2<sub>Psv</sub>NCPPB3335? The NB-LRR class of R proteins is subdivided into two classes based on their N-terminal domain. Some proteins contain a coiled-coil (CC) domain, while others contain a Toll-interleukin 1 receptor homology region (TIR) domain (van der Biezen & Jones 1998; Meyers et al. 2003). Previously, mutant screens in <em>Arabidopsis</em> helped identify several loci that suppress the function of multiple R genes and are thought to be additional components of R gene-dependent resistance response involved in signal transduction (Belkhadir et al. 2004; Wiermer et al. 2005). The NDR1 and EDS1 loci were observed to be required for the function of different subclasses of NB-LRR genes (Century et al. 1995; Parker et
al. 1996; Aarts et al. 1998). The function of R genes suppressed by the NDR1 mutation are not affected by mutation of EDS1, and *vice versa* (Aarts et al. 1998). To date, the tested members of the TIR-NB-LRRs require the EDS1 protein for function (Parker et al. 1996). In contrast, NDR1 is required for the CC-NB-LRRs (Tornero 2002). It is important to note that other components have also been identified to be involved for resistance along with NDR1 and EDS1. The *Arabidopsis* TIR type R proteins RPS4, RPP2, RPP4, RPP5, and RPP21 recruit EDS1 and PAD4 to signal specific pathogen recognition and resistance (Falk et al. 1999; Aarts et al. 1998; Feys et al. 2001; Wiermer et al. 2005). On the other hand, NDR1 is required for resistance by CC-NB-LRRs (Century et al. 1995). RAR1 is another component within the signaling transduction that is been identified, however it is only required for distinct classes of R genes (Jørgensen 1996). The function of three different R genes, RPM1, RPS2 and RPS5 are dependent on RAR1 (Tornero 2002). In addition, RAR1 has been shown to act in combination with NDR1 and regulate the stability of NB-LRR proteins (Tornero 2002; Holt et al., 2006). The plant hormone salicylic acid (SA) also plays an important regulatory role in plant immune responses. SA is degraded in *nahG* transgenic lines via a bacterial salicylate hydroxylase (Delaney et al. 1994). To identify the signaling pathway involved in the recognition of HopF2$_{PacM302273PT}$ and AvrRpm2$_{PsNCPPB3335}$, well-known *Arabidopsis* defense-signaling lines such as *nahG*, *ndr1-rar1*, *pad4*, *eds1* and *eds16* were tested. The bacterial growth assays conducted demonstrate that *ndr1rar1*, *pad4*, and *eds1* do not contribute to HopF2$_{PacM302273PT}$ and or AvrRpm2$_{PsNCPPB3335}$ recognition, suggesting that recognition of these hopF alleles occurs by unknown mechanisms of R protein signaling (Figure 9; Figure 10).

However, the ETI triggered by both HopF2$_{PacM302273PT}$ and AvrRpm2$_{PsNCPPB3335}$ are completely or partially compromised in *nahG* plants, respectively (Figure S 4; Figure S 5). It is important to
note that the nahG transgene has pleiotropic effects on Arabidopsis plant development and the break down of SA products (Heck et al. 2003; Wees & Glazebrook 2003). As a result, to further understand the role of SA in the recognition of HopF2$_{Pac}$M302273PT and AvrRpm2$_{Psv}$NCPPB3335, bacterial growth was measured in plants lacking EDS16. In contrast to nahG plants, the growth assays conducted on eds16 mutant plants displayed the resistance response observed in wild-type plants (Figure 11). This result indicates that the ETI-triggered by both HopF2$_{Pac}$M302273PT and AvrRpm2$_{Psv}$NCPPB3335 are independent of SA and the loss of resistance that was observed in nahG mutant plants were due to the pleiotropic effects of nahG. However, the eds16 results observed for HopF2$_{Pac}$M302273PT need to be repeated to further confirm the findings.

There are several methods to inoculate Arabidopsis with P. syringae including pressure infiltration, vacuum infiltration and dip and spray inoculation (Katagiri et al. 2002). I used a spray inoculation approach to screen through the Arabidopsis R gene T-DNA Insertion Collection (ARTIC) to identify an R gene(s) responsible for the recognition of HopF2$_{Pac}$M302273PT (Lewis et al. 2010). This reverse genetic screen allowed for screening of the R gene collection, which includes the other well-known R genes, such as RPS4 and RPS5 that weren’t tested by bacterial growth assay using syringe inoculation. The first round of screening identified four different R genes that lost their resistance to HopF2$_{Pac}$M302273PT, which was evident by looking at the phenotype of plants that were sprayed by the T3SE HopF2$_{Pac}$M302273PT (Figure 17). A challenge of using spray inoculation technique aside from its advantage of being high throughput and requiring less time is its inability to obtain uniform disease symptoms. Plant-pathogen interactions are affected by environmental factors and the developmental stages of the plant (Ishiga et al. 2011). Therefore, considering these various aspects, the screen was repeated to further confirm the preliminary result (Figure 18). The observed preliminary data in more than
one trial demonstrated that one R-gene, the CC-NB-LRR resistance protein X is required for the resistance in *Arabidopsis* observed with HopF2*PacM302273PT* (Figure 18; Figure 19). Intriguingly, the recognition of the unrelated T3SE by X is independent of EDS1, NDR1, RAR1 and PAD4, and therefore consistent with our results for HopF2*PacM302273PT*. Given that previously an R gene, RPM1, was demonstrated to recognize two sequence diverse T3SEs, AvrB and AvrRpm1, our data further provides evidence that a single R gene is capable of recognizing sequence diverse T3SEs. It remains to be determined whether the loss of resistance observed via spray inoculation technique is also observed with bacterial growth assay technique using syringe infiltration. In a recent study by Cherkis et al. it was demonstrated that AvrRpm1 contains a fold homologous to the catalytic domain of poly (ADP-RT) polymerases (PARPs), suggesting that it may function as an ADP-RT, similar to the HopF family of effectors (Cherkis et al. 2012; Wang et al. 2010b). Putative catalytic residues of AvrRpm1 were tested, demonstrating that they are required for both elicitation of RPM1-dependent immunity or promotion of virulence on susceptible hosts (Cherkis et al. 2012). An alignment of the amino acid sequence of AvrRpm2*PsvNCPPB3335* with AvrRpm1 revealed that the putative catalytic residues of AvrRpm1 are conserved in AvrRpm2*PsvNCPPB3335* (Figure 2). We used a site-directed mutagenesis approach to identify whether any of the conserved residues are required for the recognition of AvrRpm2*PsvNCPPB3335* in *Arabidopsis*. Interestingly, similar to AvrRpm1, the mutational analysis of the conserved site of AvrRpm2*PsvNCPPB3335* revealed that it is important for its recognition in *Arabidopsis*. It is possible that AvrRpm2*PsvNCPPB3335* effector protein similar to HopF2*PtoDC3000* could possess ADP-RT activity (Wang et al. 2010b). Further experiments need to be carried out to test the enzymatic activity of the effector protein HopF2*PacM302273PT* and determine if predicted catalytic residues play a role in its recognition by the plant host protein.
Chapter 5

5 Conclusions & Future Directions

The Gram-negative bacterial phytopathogen *P. syringae* employs a large subset of T3SEs to cause disease to a wide range of plants, including *Arabidopsis*. However, T3SEs are a double-edged sword and can be recognized by NB-LRR proteins to elicit an immune response. To date, most attempts to understand pathogen virulence and avirulence determinants, the mechanisms underlying host recognition of pathogen avirulence factors, and host specificity have focused on single representative of T3SE families. The HopF family of effector is a highly polymorphic effector family distributed across different *P. syringae* pathovars, and so far literature has only focused on two members of this family, HopF1 and HopF2. This thesis explores the function of novel hopF alleles using *Arabidopsis* as a host. The diversification of HopF family provides a great model for understanding the mechanisms driving the evolution of effectors in response to plant resistance and host specificity.

In this thesis I explored the function of HopF2<sub>PtoT1</sub>, HopF2<sub>PacM302273PT</sub>, AvrRpm2<sub>PsvNCPPB3335</sub> and AvrRpm2<sub>PphY5-2</sub> in *Arabidopsis*. It was demonstrated that each T3SE protein elicited an immune response in *Arabidopsis*. The HopF2<sub>PtoT1</sub> ETI response was revealed to be dependent on the resistance protein RPM1 and the RPM1-associated protein RIN4. Further, the mechanism underlying the ETI response observed with HopF2<sub>PacM302273PT</sub> and AvrRpm2<sub>PsvNCPPB3335</sub> was evaluated. It was shown that unlike HopF2<sub>PtoT1</sub>, both HopF2<sub>PacM302273PT</sub> and AvrRpm2<sub>PsvNCPPB3335</sub> trigger an ETI response independent of RIN4 and its known interacting R proteins. In addition, it was also demonstrated that the ETI triggered by AvrRpm2<sub>PsvNCPPB3335</sub> is dependent on conserved putative catalytic residues. Together, this suggests HopF family members target diverse host
proteins, and can trigger immune responses through immune signaling pathways mediated by distinct R proteins.

Furthermore, the ETI triggered by HopF2<sub>PacM302273PT</sub> and AvrRpm2<sub>PsvNCPPB3335</sub> is unaffected in mutant Arabidopsis lacking known signaling components of R gene-mediated immunity. This suggests that an uncharacterized ETI signaling pathway is responsible for the recognition of both HopF2<sub>PacM302273PT</sub> and AvrRpm2<sub>PsvNCPPB3335</sub>.

A previously assembled Arabidopsis R gene T-DNA insertion collection was used to identify the R protein responsible for the recognition of HopF2<sub>PacM302273PT</sub> and AvrRpm2<sub>PsvNCPPB3335</sub>. Spray inoculation assay was adapted and optimized for the screen. A reverse genetic screen revealed that the Arabidopsis R protein X is genetically required and partially responsible for the recognition of HopF2<sub>PacM302273PT</sub> in Arabidopsis. Although, these results still need to be confirmed in replicate experiments, it is likely that X is partially responsible for the recognition of HopF2<sub>PacM302273PT</sub>. The confirmation of X as an R protein responsible for the recognition of HopF2<sub>PacM302273PT</sub> will undoubtedly provide an interesting outlook into the function of this effector protein. Furthermore, this thesis provides evidence that spray inoculation can be appropriate to screen through the mutant collections of Arabidopsis to identify the R protein responsible for the recognition of T3SEs that can induce ETI without macroscopic HR.

In the future it would be useful to test the role of X as a resistance protein to HopF2<sub>PacM302273PT</sub> with bacterial growth assay. It would also be interesting to look at the role of the predicted ADP-ribosyltransferase catalytic residues of HopF2<sub>PacM302273PT</sub> to see if they are required for the resistance observed. Additionally, it would be useful to further characterize the resistance response observed with the two chimeric effector proteins, AvrRpm2<sub>PsvNCPPB3335</sub> and
AvrRpm2_{rphY5-2}. Subsequent analysis will broaden our understanding of effector function and diversification and the underlying mechanisms driving their evolution.
References


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Figure S 1. HopF2<sub>Pac</sub> and AvrRpm2<sub>Psv</sub> induce an ETI response in Arabidopsis ecotype Col-0 when expressed heterologously in PtoDC3000ΔHopF2. (a) and (b) Growth analysis of HopF2<sub>Pac</sub> infiltrated into Arabidopsis ecotype Col-0. (c) and (d) Growth analysis of AvrRpm2<sub>Psv</sub> infiltrated into Arabidopsis ecotype Col-0. Colony counts for all experiments were made at 0 and 3 days post-infiltration and relative humidity (RH) was <~45%. EV represents PtoDC3000ΔHopF2 expressing MCS-2 vector. Error bars represent standard deviation from the mean of 10 samples. Two-tailed t-test was performed to test for significant differences, which is indicated by an asterisk (*p<0.01).
Figure S 2. HopF2<sub>Pac</sub> and AvrRpm2<sub>Psv</sub> recognition in <i>Arabidopsis</i> are independent of RIN4, RPM1 and RPS2. (a) and (b) HopF2<sub>Pac</sub> was expressed heterologously in <i>PtoDC3000ΔHopF2</i> and bacterial growth assays were conducted on the triple mutant <i>rpm1</i><i>rps2</i><i>rin4</i>. (c) and (d) AvrRpm2<sub>Psv</sub> was expressed heterologously in <i>PtoDC3000ΔHopF2</i> and bacterial growth assays were conducted on the triple mutant <i>rpm1</i><i>rps2</i><i>rin4</i>. Colony counts were made at 0 and 3 days post infiltration. EV represents <i>PtoDC3000ΔHopF2</i> expressing the empty MCS-2 vector. Error bars represent standard deviation from the mean of 10 samples. Two-tailed t-test was performed to test for significant differences, which is indicated by an asterisk (*p<0.01).
**Figure S 3. AvrRpm2<sub>Psv</sub> recognition in Arabidopsis is independent of RPM1 and RPS2.**

(a) AvrRpm2<sub>Psv</sub> was expressed heterologously in *PtoDC3000ΔHopF2* and bacterial growth assays were conducted on the *rps2* mutant plants. (b) Same as (a) except bacterial growth assays were conducted on the *rpm1* mutant plants. Colony counts were made at 0 and 3 days post infiltration. EV represents *PtoDC3000ΔHopF2* expressing the empty MCS-2 vector. Error bars represent standard deviation from the mean of 10 samples. Two-tailed t-test was performed to test for significant differences, which is indicated by an asterisk (*p<0.01).
Figure S 4. HopF2$_{Pac}$ recognition is independent of known components of R gene-mediated immunity. (a) – (d) HopF2$_{Pac}$ was expressed heterologously in $Pto$DC3000ΔHopF2 and bacterial growth assays were conducted on the indicated genotypes. Colony counts were made at 0 and 3 days post-infiltration. EV represents $Pto$DC3000ΔHopF2 expressing the empty MCS-2 vector. Error bars represent standard deviation from the mean of 10 samples. Two-tailed t-test was performed to test for significant differences, which is indicated by an asterisk (*p<0.01).
Figure S 5. AvrRpm2\textsubscript{Psv} recognition is independent of known components of R gene-mediated immunity. (a) – (d) AvrRpm2\textsubscript{Psv} was expressed heterologously in \textit{PtoDC3000ΔHopF2} and bacterial growth assays were conducted on the indicated genotypes. Colony counts were made at 0 and 3 days post-infiltration. EV represents \textit{PtoDC3000ΔHopF2} expressing the empty MCS-2 vector. Error bars represent standard deviation from the mean of 10 samples. Two-tailed t-test was performed to test for significant differences, which is indicated by an asterisk (*p<0.01).

![Figure S 5. AvrRpm2\textsubscript{Psv} recognition is independent of known components of R gene-mediated immunity.](image)

Figure S 6. HopF2\textsubscript{Pac} induces an ETI response in \textit{Arabidopsis} ecotype Col-0 when expressed heterologously in \textit{PtoDC3000ΔHopF2}. Four-week-old \textit{Arabidopsis} Col-0 plants were sprayed with \textit{PtoDC3000ΔHopF2} expressing the empty MCS-2 vector (EV) and HopF2\textsubscript{Pac} (OD\textsubscript{600} = 0.8). Plants were domed for 3 days and re-sprayed on day 3 (OD\textsubscript{600} = 0.8). Domes were removed on day 4. Photographs were taken at day 3 before the re-spray and on day 7.

![Figure S 6. HopF2\textsubscript{Pac} induces an ETI response in \textit{Arabidopsis} ecotype Col-0 when expressed heterologously in \textit{PtoDC3000ΔHopF2}.](image)