Environmental Requirements Underlying Effector-Triggered Immunity in *Arabidopsis thaliana*

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Cell and Systems Biology
University of Toronto

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2015

Abstract

*Pseudomonas syringae* is a Gram-negative bacterium that infects multiple plant species via injection of type three secreted effectors (T3SEs) into host cells. Nucleotide-binding leucine-rich repeat (NLR) resistance (R) proteins recognize specific T3SEs and trigger an immune response, called effector-triggered immunity (ETI). ETI is often associated with localized programmed cell death (PCD), known as the hypersensitive response (HR), which limits pathogen proliferation.

We examined two abiotic factors that differentially affect ETI outputs – humidity and temperature. We observed that high relative humidity or elevated temperature conditions capable of suppressing HR had minimal influence on ETI-associated virulence suppression, thereby uncoupling these two ETI responses. We have identified ecotypes of *Arabidopsis* that show suppression of ETI-associated virulence suppression at elevated temperature, highlighting the natural variation that exists in coping with elevated temperature. These results reinforce the influence of abiotic factors on plant immunity and emphasize the importance of carefully documented environmental conditions in studies of plant immunity.
Acknowledgments

I would like to thank my co-supervisors – Dr. Darrell Desveaux and Dr. David Guttman – for accepting me as a graduate student, and for providing guidance and support throughout these two years. I would also like to extend a gracious thank you to all members of the Desveaux/Guttman greater lab area for helpful advice, troubleshooting, and insightful discussion. Thank you to Dr. Daphne Goring and Dr. Keiko Yoshioka for helpful advice during committee meetings, and Dr. Nicholas Provar for acting as my external examiner.

I am indebted to my lab mates – Christine, Tim, and Nouisin – for providing constant entertainment during growth assay marathons, and always offering help, encouragement, and friendship without asking for anything in return. I will always fondly remember our album of lunch-time selfies. I would also like to extend a big thank you to Brenden, Jessica, and Nina for their support (and plant materials). I would like to gratefully acknowledge Pauline not only for being a constant source of motivation (lab/boot camp), but also for being a source of sage advice.

I need to thank my family for their unwavering support and encouragement at all times – knowing that I can go off into my life in any direction I choose with them behind me makes the scary world of “adult” life that much more bearable. Without their support and guidance, success would be unattainable.

And last, but certainly not least, I would like to thank Duncan who let me cry at him whenever I needed to. And I needed to a lot of times. Everything is better when you’re around.

Thank you, thank you, thank you!
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<th>Description</th>
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<tr>
<td><em>Arabidopsis</em></td>
<td><em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>CC-</td>
<td>Coiled-coil NB-LRR</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CIBC-5</td>
<td><em>Arabidopsis thaliana</em> ecotype CIBC-5</td>
</tr>
<tr>
<td>Col-0</td>
<td><em>Arabidopsis thaliana</em> ecotype Columbia-0</td>
</tr>
<tr>
<td>Cvi-0</td>
<td><em>Arabidopsis thaliana</em> ecotype Cape Verde Island-0</td>
</tr>
<tr>
<td>DEX</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>dpi</td>
<td>Days post-infiltration</td>
</tr>
<tr>
<td>dps</td>
<td>Days post-spraying</td>
</tr>
<tr>
<td>ET</td>
<td>Elevated temperature</td>
</tr>
<tr>
<td>ETI</td>
<td>Effector-triggered immunity</td>
</tr>
<tr>
<td>ETS</td>
<td>Effector-triggered susceptibility</td>
</tr>
<tr>
<td>EV</td>
<td>Empty vector</td>
</tr>
<tr>
<td>Fab-2</td>
<td><em>Arabidopsis thaliana</em> ecotype Faberget-2</td>
</tr>
<tr>
<td>hpi</td>
<td>Hours post-infiltration</td>
</tr>
<tr>
<td>HR</td>
<td>Hypersensitive response</td>
</tr>
<tr>
<td>HRH</td>
<td>High relative humidity</td>
</tr>
<tr>
<td>LMM</td>
<td>Lesion mimic mutant</td>
</tr>
<tr>
<td>LRH</td>
<td>Low relative humidity</td>
</tr>
<tr>
<td>MAMPs</td>
<td>Microbe-associated molecular patterns</td>
</tr>
<tr>
<td>NB-LRR</td>
<td>Nucleotide-binding domain leucine-rich repeat</td>
</tr>
<tr>
<td>NLR proteins</td>
<td>Nucleotide-binding domain leucine-rich repeat proteins</td>
</tr>
<tr>
<td>OX</td>
<td>Overexpression</td>
</tr>
<tr>
<td><em>P. syringae</em></td>
<td><em>Pseudomonas syringae</em></td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
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<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td><em>Pci</em>0788-9</td>
<td><em>Pseudomonas syringae</em> pv. <em>cilantro</em> 0788-9</td>
</tr>
</tbody>
</table>
PTI  PAMP-triggered immunity

*Pto*DC3000  *Pseudomonas syringae* pv. *tomato* DC3000

R genes  Resistance genes

RLCK  Receptor-like cytoplasmic kinase

RLK  Receptor-like kinase

RLK-PRRs  Receptor-like kinase pattern-recognition receptors

RT  Room temperature

SA  Salicylic acid

SAR  Systemic acquired resistance

T3SEs  Type III secreted effectors

T3SS  Type III secretion system

T-DNA KO  Transfer-DNA knockout

TIR-  Toll-interleukin-1 NB-LRR

Tsu-1  *Arabidopsis thaliana* ecotype Tsushima-1

Wei-0  *Arabidopsis thaliana* ecotype Weiningen-0

ZAR1  HopZ-ACTIVATED RESISTANCE1

ZED1  HopZ-ETI-DEFICIENT1

ZRK  ZED1-related kinase

**N.B.** Section 3.3 entitled “Investigating the Effects of Elevated Temperature on ETI” and corresponding Discussion Section 3.4.2 were submitted for publication as a peer-reviewed perspective:


It was prepared by AM, DSG, and DD under the guidance of DSG and DD.

This section includes expanded introduction, results, and discussion sections.
Chapter 1

1 Introduction: Plant Immunity

As vital members of various diverse ecosystems, plants interact with numerous biotic and abiotic factors daily (Fujita et al., 2006). Environmental conditions, highly dynamic microbial communities in the phyllosphere, and herbivory are a few examples of the stimuli to which plants must respond constantly (Fujita et al., 2006; Porras-Alfaro and Bayman, 2011). Some of these interactions may have mutually beneficial outcomes, while others require the plant to expend energy in order to protect its domain and ensure survival (Porras-Alfaro and Bayman, 2011). For example, environmental factors, such as wind, may favour the spread of seeds in order to ensure dispersal of progeny to introduce genetic variation into a species pool. Additionally, microbes may have negligible or mutually-beneficial relationships with plants (Bulgarelli et al., 2013; Porras-Alfaro and Bayman, 2011). In some cases, symbiotic or mutualistic microbe interactions are necessary for the plant’s survival (Bulgarelli et al., 2013; Porras-Alfaro and Bayman, 2011). However, instances exist where environmental factors and interacting microbes may have antagonistic effects on plant members of these ecosystems. Unfavourable environmental conditions reduce photosynthetic capability and seed dispersal; pathogenic microbes have evolved to prey on plants to ensure their own survival (Ingle et al., 2009). This complex network of environment-plant-microbe interactions is both present and vital in all ecosystems, and has resulted in the evolutionary development of a strictly regulated and highly evolved two-tiered plant immune system to respond to abiotic and biotic stresses accordingly.

*Pseudomonas syringae* (hereafter *P. syringae*) is a Gram-negative bacterial pathogen that causes disease on a wide variety of economically important crop species, resulting in millions of dollars
in global crop loss each year globally (Cunnac et al., 2009; Hwang et al., 2005). *P. syringae* is the causative agent of disease on crops such as kiwi, bean, tomatoes, and several others including the model plant *Arabidopsis thaliana* (hereafter *Arabidopsis*) (Cunnac et al., 2009; Hwang et al., 2005). *P. syringae* secretes proteinaceous virulence effectors, called type III secreted effectors (T3SEs), into the plant host cytosol via a needle-like structure, called the type III secretion system (T3SS), where they target cellular components to disrupt host immunity (Deslandes and Rivas, 2012; Dodds and Rathjen, 2010; Lewis et al., 2009).

Plants possess both pre- and post-invasive layers of defense to prevent pathogen invasion and disease. Structural barriers, such as waxy epidermal cuticles and cell walls, however, can be evaded by successful pathogens (Beattie, 2011; Jones and Dangl, 2006). Post-invasively, there are further lines of defense that detect the presence of these pathogens in order to mount an immune response to prevent bacterial proliferation (Dodds and Rathjen, 2010; Jones and Dangl, 2006). The first layer of plant immunity is triggered by the detection of pathogen-associated molecular patterns (PAMPs), also known as microbe-associated molecular patterns (MAMPs), such as flagellin or chitin (Jones and Dangl, 2006; Naito et al, 2008). These PAMPs/MAMPs are detected by receptor-like kinase pattern-recognition receptors (RLK-PRRs) to activate PRR-induced immunity (PTI) (Boller et al., 2009; Jones and Dangl, 2006). However, over evolutionary time, pathogens have evolved the ability to suppress this innate immunity by secreting T3SEs into the host cell (Jones and Dangl, 2006; Boller et al., 2009). T3SEs target vital cellular machinery and processes to disrupt general cellular function and suppress plant host immune responses during effector-triggered susceptibility (ETS) (Jones and Dangl, 2006; Dodds and Rathjen, 2010; Boller et al., 2009).
Plant hosts, such as *Arabidopsis*, have evolved specific resistance (R) genes to directly or indirectly recognize T3SEs and initiate the second layer of plant defense known as effector-triggered immunity (ETI) (Jones and Dangl, 2006; Dodds and Rathjen, 2010). Broadly, R genes are divided into two sub-classes based on N-terminal putative signaling domains possessing either a Toll-Interleukin-1 receptor (TIR) or a coiled-coil (CC) domain (Boller et al., 2009; Eitas et al., 2008; Jones and Dangl, 2006). These R genes are intracellular nucleotide-binding domain leucine-rich repeat (NLR) proteins that, upon effector recognition, will often induce localized programmed cell death (PCD), which manifests as a hypersensitive response (HR) in order to restrict bacterial proliferation (Coll et al., 2011; Jones and Dangl, 2006). HR is further associated with rapid ion fluxes, extracellular oxidative burst, and transcriptional reprogramming in order to localize the site of the infection and restrict biotrophic pathogenic spread. NLR-mediated ETI subsequently induces systemic acquired resistance (SAR) in distal regions from the site of infection to prepare plants for future pathogenic challenge (Dempsey et al., 1999; Dempsey and Klessig, 2012; Horváth et al., 2007; Vlot et al., 2009).

Many T3SEs elicit ETI through known mechanisms of plant immune signaling (Jones and Dangl, 2006). Upon secretion of these T3SEs, recognition via specific NLR-mediated signaling pathways results in a robust immune response, and as such they are known as “avirulent effectors” as they induce immunity (Boller et al., 2009; Dodds and Rathjen, 2010; Eitas et al., 2008; Jones and Dangl, 2006). Conversely, effectors that are able to evade host recognition and promote successful bacterial proliferation are known as “virulent effectors” (Boller et al., 2009; Dodds and Rathjen, 2010; Eitas et al., 2008; Jones and Dangl, 2006). The immune response elicited by an avirulent effector can be observed utilizing various assays. These assays serve as measures of ETI outputs, allowing the observation of timing, efficiency, and overall magnitude of the ETI response induced by a particular avirulent effector.
Firstly, upon avirulent effector recognition and subsequent elicitation of localized PCD, macroscopic HR is often visible on leaves infiltrated with high dosages of bacteria delivering the effector of interest (Coll et al., 2011; Heath, 2000; Morel and Dangl, 1997). In Arabidopsis, macroscopic HR resembles a leaf collapse characterized by dry, paper-like silvering at the area of infiltration (Coll et al., 2011; Heath, 2000; Morel and Dangl, 1997). Secondly, infiltrating with a lower dosage of bacteria more accurately mimics a natural infection, and allows quantification of ion flux associated with localized PCD using conductivity assays. In these assays, the leakage of intracellular ions due to the development of HR, which lyses plant cells, can be quantified as a quantitative proxy for HR, and ETI by extension (Lewis et al., 2010). Lastly, and perhaps most importantly, virulence suppression induced by avirulent effectors can be quantified using in planta bacterial growth assays, and macroscopically observed using disease resistance spray assays (Jones and Dangl, 2006). A major function of ETI is to reduce virulent bacterial growth, and thus the use of in planta bacterial growth assays allows for the quantification of bacterial growth when infiltrating with a low dosage of bacteria delivering the effector of interest. In quantifying the growth over the course of several days, one is able to quantify the virulence suppression by host machinery. In addition, by spraying plants with high dosages of bacteria delivering the effector of interest, one is able to observe the stark contrast between healthy plants that have induced ETI upon recognition of an effector, or diseased plants that are unable to recognize effectors and thus cannot launch an immune response. Infecting plants via spray assays mimics the manner in which plant hosts would encounter bacterial pathogens naturally. T3SEs that elicit robust ETI responses are of particular interest to this study, and the aforementioned assays will be used to qualify and quantify the environmental requirements for ETI elicited by the following T3SEs – HopZ1a, AvrRpt2, AvrRpm1, and AvrRps4.
T3SE HopZ1a is a member of the widely-distributed and evolutionarily conserved YopJ/HopZ/AvrRxv superfamily of bacterial effectors (Lewis et al., 2008, 2009, 2011; Ma et al., 2006; Zhou et al., 2009). The YopJ effector family is found in Yersinia pestis, the causal agent of Black Death, while the AvrRxv family is found in plant pathogen Xanthomonas campestris (Zhou et al., 2009). The HopZ family is widely present among P. syringae strains and exists as four homologs – HopZ1, HopZ2, HopZ3, and HopZ4 (Ma et al., 2006; Zhou et al., 2009; Üstün et al., 2014). It has been shown that HopZ1 homologs are ancestral to P. syringae (Ma et al., 2006). Additionally, HopZ2 and HopZ3 are believed to have been incorporated into P. syringae via horizontal transfer from other ecologically similar bacteria – Xanthomonas campestris and Erwinia spp. (pyrifoliae and amylovora), respectively (Ma et al., 2006). Under the pressure of diversifying selection, HopZ1 has radiated into three allelic forms in P. syringae via pathoadaptive mutational changes: HopZ1a (P. syringae pv. syringae A2), HopZ1b (P. syringae pv. glycinea BR1), and HopZ1c (P. syringae pv. maculicola ES4326) (Lewis et al., 2008, 2010, 2011; Ma et al., 2006; Zhou et al., 2009, 2011). While all three forms are functional, HopZ1a induces a consistently strong HR approximately 16 hours post infection in a salicylic acid (SA)-independent manner in wildtype Arabidopsis (Lewis et al., 2008, 2010, 2011; Zhou et al., 2009; Ma et al., 2006). Recognition of HopZ1a requires indirect interaction with an NLR protein called HopZ-ACTIVATED RESISTANCE1 (ZAR1) and a unique ETI-associated pseudokinase called HopZ-ETI-DEFICIENT1 (ZED1) (Lewis et al., 2010, 2013, 2014).

The T3SE AvrRpt2 – originally cloned from P. syringae pv. tomato JL1065 – is classified as a cysteine protease (Axtell et al., 2003; Dong et al., 1991; Mackey et al., 2003; Whalen et al., 1991). Upon translocation of this effector into the host cell, AvrRpt2 is cleaved in an autocatalytic event (Axtell et al., 2003; Dong et al., 1991; Mackey et al., 2003; Whalen et al., 1991). Following autocatalysis, membrane-associated AvrRpt2 cleaves Arabidopsis RPM1-
interacting protein 4 (RIN4) – an important hub of plant immunity (Axtell et al., 2003; Dong et al., 1991; Mackey et al., 2003; Whalen et al., 1991). The degradation of RIN4 leads to NLR-mediated defense signal transduction via the R protein RPS2 to initiate ETI in an SA-dependent manner (Axtell et al., 2003; Bent et al., 1994; Dong et al., 1991; Mackey et al., 2003; Mindrinos et al., 1994; Whalen et al., 1991). Importantly, AvrRpt2 proteolysis and RIN4 degradation occur independent of RPS2, indicating that RIN4 is most likely a direct substrate for AvrRpt2 (Axtell et al., 2003; Dong et al., 1991; Mackey et al., 2003; Whalen et al., 1991). However, to date, attempts to demonstrate direct interaction of AvrRpt2 with RIN4 or RPS2 have been unsuccessful (Hotson and Mudgett, 2004; Belkhadir et al., 2004; Kim et al., 2005, 2009). AvrRpt2 induces a strong HR phenotype approximately 12 hours post infiltration (Axtell et al., 2003; Dong et al., 1991; Mackey et al., 2003; Whalen et al., 1991).

AvrRpm1 is another T3SE that interacts with RIN4 to induce immunity in an SA-dependent manner (Boyes et al., 1998; Ritter and Dangl, 1995). Originally isolated from P. syringae pv. maculicola M2, this effector is directed to the plasma membrane via a myristoylation sequence and is recognized by RPM1 – a peripheral plasma membrane NLR protein (Boyes et al., 1998; Ritter and Dangl, 1995). Additionally, AvrRpm1 interacts with and induces phosphorylation of RIN4 to induce immune signaling (Boyes et al., 1998; Ritter and Dangl, 1995). Current working models suggest that RPM1 guards RIN4, which is a common target of multiple bacterial effectors (Mackey et al., 2003). AvrRpm1 induces a strong HR phenotype approximately 6-8 hours post infiltration (Boyes et al., 1998; Ritter and Dangl, 1995).

T3SE AvrRps4 was originally isolated from P. syringae pv. pisi 151, a causal agent of bacterial blight in pea (Hinsch and Staskawicz, 1996; Sohn et al., 2009, 2012). This effector is recognized by the TIR-NB-LRR protein RPS4 (Sohn et al., 2009, 2012). Upon delivery into plant cells,
AvrRps4 is cleaved and detected by RPS4 to induce transcriptional programming via transcription factor WRKY52 in order to induce immune signaling and initiate ETI in an SA-dependent manner (Sohn et al., 2012). AvrRps4 induces a strong HR phenotype approximately 8-10 hours post infiltration in the Arabidopsis Ws-0 ecotype (Hinsch and Staskawicz, 1996; Sohn et al., 2009, 2012).

In delivering the above described effectors from the virulent pathogen P. syringae pv. tomato DC3000 (hereafter, PtoDC3000), the avirulence function elicited by each effector can be closely examined. As previously mentioned, ETI-associated PCD and virulence suppression upon avirulent effector recognition is of interest to this project. The overall aims of this project were to address the influences of abiotic factors – specifically elevated temperature and elevated humidity – on the outputs of ETI associated with T3SEs HopZ1a, AvrRpt2, AvrRpm1, and AvrRps4.

Abiotic factors, such as temperature, humidity, and light, significantly impact reproductive fitness, as well as the host’s ability to robustly fend off pathogenic invasion during plant-pathogen interactions (Alcázar et al., 2011; Hua et al., 2013; Hua, 2014; Huang et al., 2014; Roden and Ingle, 2009; Wang et al., 2009; Wang et al., 2011). As plants are sessile organisms, they are subject to daily fluctuations in environmental conditions, and, therefore, must simultaneously balance environmental and pathogenic stress, alongside ensuring successful growth and development (Alcázar et al., 2011; Río-Álvarez et al., 2014). Many studies have shown that maternal environment is a key determinant in overall fitness and seed yield of subsequent generations (Hedhly et al., 2003). Temperature is a major factor that influences seed production, affecting seed size in some species or germination rates in others (Huang et al., 2014). Many studies have shown that environmental stresses, such as temperature, not only
affect the parent plant, but that signals with environmental information can be transmitted to seeds that would be maintained in future generations (Kendall and Penfield, 2012). In general, environmental stresses clearly have the ability to limit development and negatively impact fitness (Hedhly et al., 2003; Huang et al., 2014; Kendall and Penfield, 2012). Therefore, the ability to successfully integrate and respond accordingly to simultaneous biotic and abiotic environmental cues is likely to be a major driver towards fitness. As such, it is the goal of this thesis to characterize the environmental requirements underlying ETI, and demonstrate the ways in which changes in environmental conditions can impact how outputs of ETI are observed, measured, and interpreted.

1.1 Humidity and Temperature Modulate Plant Immunity

The physiological requirements underlying ETI remain unclear, and the extent to which specific environmental factors influence outputs of ETI is not well-documented. High humidity has been shown to suppress the macroscopic HR phenotype, as well as increasing pathogen growth to the point of abrogating ETI-associated virulence suppression (Cook and Stall, 1977; Young, 1974; Freeman and Beattie, 2009; Beattie, 2011). Temperature has been shown to modulate outputs of plant ETI in several systems. (Alcázar et al., 2011; Hua 2014; Huang et al., 2014; Wang et al., 2009; Wang et al., 2011). Specifically, many R-gene mediated cell death responses have been shown to be environmentally sensitive (Alcázar et al., 2011; Atkinson et al., 2012; Moeder and Yoshioka, 2008). It has also been proposed that PTI and ETI are preferentially activated at low and high temperatures, respectively (Cheng et al., 2013). Although examples exist to demonstrate the influence of abiotic factors on plant immunity, limited information is available about how pathogen invasion and ensuing ETI responses are specifically modulated by temperature or humidity.
*Cladosporium fulvum* is a fungal pathogen that causes leaf mold disease on tomato (Hammond-Kosack et al., 1996; May et al., 1996). Host recognition of cognate Avr genes carried by *C. fulvum* is associated with oxidative burst, lipid peroxidation, production of glutathione, alteration of the cellular redox reactions, as well as production of SA and ethylene followed by rapid stomatal opening leading to HR cell death (de Jong et al., 2002; Hammond-Kosack et al., 1996; Moeder and Yoshioka, 2008). The *Cf-9/Avr9*, *Cf-2/Avr2*, and *Cf-4/Avr4* interactions are either inhibited or delayed by high relatively humidity (≈95-100%) (de Jong et al., 2002; Hammond-Kosack et al., 1996; May et al., 1996; Moeder and Yoshioka, 2008; Wang et al., 2005). Additionally, temperature was found to have an additive suppressive effect on these interactions. (Moeder and Yoshioka, 2008; de Jong et al., 2002; Wang et al., 2005).

Various other systems have reported full or partial suppression of constitutive activation of defense responses or enhanced resistance in high relative humidity. In transgenic *Arabidopsis* lines overexpressing RPW8.1 and RPW8.2 genes, which confer resistance to a wide range of powdery mildews, the HR-like lesion formation, constitutive PR gene expression, SA accumulation, and enhanced resistance phenotypes were suppressed in high humidity (Xiao et al., 2003). In many of these systems, temperature and humidity have additive suppressive effects.

Lesion mimic mutants (LMMs) are widely used to study HR in order to further elucidate the contribution of HR to the bacterial resistance response triggered in the presence of a recognized pathogen (Clough et al., 2000; Moeder and Yoshioka, 2008; Bruggeman et al., 2015; Coll et al., 2011; Yu et al., 1998). Several of the initiation type LMMs have been reported to be environmentally-sensitive. The LMM *lesion simulating disease 6* (*lsd6*) was first reported to be sensitive to humidity by Weymann et al. (1995). This initiated the investigation of environmental sensitivity in LMMs, revealing both *constitutive expresser of PR genes 22* (*cpr22*) and
*copine1/bonzai1 (cpn1/bon1)* as sensitive to temperature and humidity (Jambunathan et al., 2001; Hua et al., 2001, Yang and Hua, 2004). That is, their LMM-associated phenotypes – such as morphology, enhanced accumulation of SA, SA-dependent defense responses, and homozygous lethality – are suppressed in high relative humidity (≈ 95%) and elevated temperature (28-30°C) (Moeder and Yoshioka, 2008; Yoshioka et al., 2001; Jambunathan et al., 2001; Hua et al., 2001). As another example, the phenotypes associated with the *suppressor of SA-sensitive 4 (ssi4)* gain-of-function mutant, including suppression of SA insensitivity, constitutive expression of PR genes, induced SA accumulation, lesion formation, and enhanced resistance to bacterial and oomycete pathogens, were found to be suppressed by high humidity (Shirano et al., 2002; Zhou et al., 2004).

Few studies exist reporting the suppressive effects of high relative humidity on the timing and magnitude of NLR-mediated ETI outputs induced by well-studied T3SEs. In 2009, Freeman and Beattie investigated the role of leaf water loss and localized cessation of vascular activity during host resistance to *P. syringae*. It was reported that reducing water availability is a major factor in restricting bacterial growth during R-gene mediated defense responses (Freeman et al., 2009). At only 3 hours post infiltration, leaves infected with *PtoDC3000 (AvrRpm1)* show restricted vascular activity in the infiltrated regions, indicating an important role for water flow through the vasculature during an ETI response (Freeman et al., 2009). By examining vacuum-infiltrated *Arabidopsis* Col-0 kept in a water-soaked (extreme high humidity) environment, it was reported that by 24 hours post infiltration *PtoDC3000* carrying AvrRpm1 and *PtoDC3000* carrying empty vector (hereafter EV) grew significantly more than in plants that were not maintained in a water-soaked environment (Freeman et al., 2009).
Several instances of specific NLR-mediated ETI responses have been demonstrated to be environmentally sensitive (Beattie, 2011; Hua, 2014). The tobacco N (necrosis) gene, from the TIR-NLR class of R genes, confers resistance to tobacco mosaic virus (TMV) and mounts a rapid HR after infection at a permissive temperature (21°) (Whitham et al., 1994). Shifting to a moderately elevated temperature (28°) prevents the development of this HR (Malamy et al., 1992; Samuel, 1931; Whitham et al., 1994). Similarly, the HR responses induced by the T3SEs AvrRpt2, AvrRpm1, and AvrRps4 in Arabidopsis are inhibited at elevated temperature (28°C) (Cheng et al., 2013; Freeman and Beattie, 2009; Goel et al., 2008; Wang et al., 2009).

Arabidopsis TIR-NLR gene pair RPS4 and RRS1 confer resistance to Pseudomonas syringae pv tomato (PtoDC3000) expressing AvrRps4, which is accompanied by PCD in Ws-0, but not in Col-0 (Heidrich et al., 2013). It has been shown that RPS4<sup>Col</sup>/RRS1<sup>Col</sup> contribute to temperature-conditioned autoimmunity (Heidrich et al., 2013). In elevated temperature, stunted growth and bacterial resistance response associated with autoimmunity in permissive temperature is inhibited (Heidrich et al., 2013). Shifting from elevated (28°C) to permissive (22°C) temperature induced activation of this RPS4 autoimmunity, revealing a transcriptional reprogramming in leaf tissues showing a mainly quantitative contribution of RRS1<sup>Col</sup> to differential regulation of immunity-related genes (Heidrich et al., 2013). Furthermore, constitutive expression of the NLR RPS4 results in temperature-conditioned autoimmunity that is suppressed at 28°C (Heidrich et al., 2013; Sohn et al., 2014). Furthermore, the abscisic acid (ABA)-deficient mutant aba2 shows enhanced resistance mediated by the NLR-like gene SUPPRESSOR OF npr1-1 CONSTITUTIVE1 (SNC1) and RPS4 at high temperatures, indicating temperature as an important modulator of the interplay between abiotic and biotic stress response pathways (Mang et al., 2012).
Other studies have attempted to show that effectors triggering immunity in an SA-dependent manner – AvrRpt2, AvrRpm1, and AvrRps4 – at permissive environmental conditions have been shown to be differentially inhibited at moderately elevated temperature (28°C), mainly based on suppression of HR phenotype (Cheng et al., 2013; Freeman et al., 2009; Goel et al., 2008; Wang et al., 2009). HR induced by overexpression of AvrRpt2 under a dexamethasone (DEX)-inducible promoter in Col-0 background has been shown to be partially inhibited at moderately elevated temperature (28°C), and entirely suppressed at elevated temperature (32°C) (Cheng et al., 2013). However, without biologically relevant in planta bacterial growth assays or expression analyses of immunity-related gene markers post-infection, this finding begs the question – is ETI entirely inhibited, or is it simply PCD that is suppressed by elevated temperature?

Several instances exist in which ETI occurs without apparent PCD (Lindeberg et al., 2009). For example, P. syringae pv. phaseolicola 1448A elicits a nonhost resistance response in planta, however this restriction of bacterial growth is not accompanied by an HR (Lindeberg et al., 2009). Further, studies show that some NLR-mediated ETI responses – such as those triggered by effectors AvrB and HopA1Psy61, recognized by NLRs TAO1 and RPS6, respectively – contribute to ETI without manifestation of an HR (Eitas et al., 2008; Kim et al., 2009; Naito et al., 2008; Shimizu et al., 2003). Therefore, despite the lack of localized PCD, ETI-associated virulence suppression – that is, reduced bacterial growth induced by recognition of a specific effector – is still observed in these cases.

In this study, we show that ETI-associated virulence suppression can be uncoupled from HR at elevated temperature or in high relative humidity conditions in Arabidopsis. Standard immunity assays were conducted to observe the development of macroscopic HR symptoms, quantify ion
leakage as a proxy for ETI, and monitor *in planta* bacterial growth in both permissive and elevated temperature conditions. In doing this, I have shown that while ETI outputs are differentially influenced by humidity or temperature shifting, the overall reduction of bacterial growth observed during ETI-associated virulence suppression remains intact. In summary, these findings indicate that in certain environmental contexts, ETI can still occur to restrict bacterial proliferation without the manifestation of PCD. As such, two facets of the ETI-response, HR and virulence suppression, can be differentially affected, and uncoupled, by high relative humidity or elevated temperature conditions.
Chapter 2

2 Materials and Methods

2.1 Plant materials and growth conditions

Arabidopsis plants were grown in 12 hours (h) of light (130-150 microeinsteins m$^{-2}$s$^{-1}$) and 12 h of darkness at 21-22°C and 50-65% humidity in Sunshine Mix 1 soil supplemented with 20:20:20 fertilizer at 1g/L. Except for assays conducted on various ecotypes or mutants when indicated, all assays were performed in the Col-0 background. Prior to assays, plants were subjected to high humidity or temperature priming for 24 h to avoid developmental differences.

2.2 Experimental conditions

2.2.1 High relative humidity and elevated temperature experiments

For high relative humidity (HRH) experiments, Arabidopsis plants were removed from original growth conditions and incubated for 24 h in a humidity-controlled chamber in 24 h light (130-150 microeinsteins m$^{-2}$s$^{-1}$) at 22°C and 95% humidity. Simultaneously, plants for ambient or low relative humidity (LRH) experimental comparisons were moved to ambient light, temperature, (22-26°C) and humidity (10-45%) conditions 24 h prior to the experiment. Specific environmental conditions indicated in each figure legend.

For elevated temperature (ET) experiments, Arabidopsis plants were removed from original growth conditions and incubated for 24 h in a temperature-controlled chamber in 24 h light (130-150 microeinsteins m$^{-2}$s$^{-1}$) at 30°C and 15-50% humidity. Simultaneously, plants for ambient or room temperature (RT) experimental comparisons were moved to ambient light, temperature (22-26°C), and humidity (10-45%) conditions 24 h prior to the experiment. Specific environmental conditions indicated in each figure legend.
2.3 Macroscopic HR, trypan blue staining, and ion leakage assays

2.3.1 Macroscopic HR

One day prior to experiments, plants were watered to ensure ease of infiltration. *PtoDC3000* strains carrying empty vector (pUCP20) (Ma et al., 2006), HopZ1a (Ma et al., 2006), AvrRpt2 (Mudgett and Staskawicz, 1999), or AvrRpm1 (Ritter and Dangl, 1995) were used for infiltrations. *PtoDC3000* carrying HopZ1a or AvrRpt2 strains were infiltrated in the evening (approximately 6-8pm), and HR symptoms monitored beginning the next morning. *PtoDC3000* carrying AvrRpm1 was infiltrated in the morning (approximately 8-9am) and monitored throughout the day. Four to five-week-old *Arabidopsis* Col-0 plants were pressure-infiltrated with *P. syringae* resuspended to an optical density at 600nm (OD$_{600}$) of 0.1 (5 x 10$^7$ CFU/mL). The right side of leaves were pressure-infiltrated, and immediately dried after infiltration. Macroscopic HR phenotypes were scored based on time points associated with ETI response onset associated with each effector (i.e., 12-24 h for AvrRpt2 and HopZ1a, 2-12h for AvrRpm1).

2.3.2 Trypan blue staining of PCD

*PtoDC3000* strains carrying empty vector (pUCP20) (Ma et al., 2006), HopZ1a (Ma et al., 2006), AvrRpt2 (Mudgett and Staskawicz, 1999), or AvrRpm1 (Ritter and Dangl, 1995) were used for infiltrations. Four to five-week-old plants were pressure-infiltrated at OD$_{600}=0.1$ (5 x 10$^7$ CFU/mL). *PtoDC3000* carrying HopZ1a or AvrRpt2 strains were infiltrated in the evening (approximately 6-8pm), and HR symptoms monitored beginning the next morning. Infiltrated *Arabidopsis* leaves were harvested at indicated time points and stained by submerging leaves in lactophenol-trypan blue solution and boiling for 5-10 minutes, followed by 1 h incubation on the bench at ambient temperature. Clearing was performed in chloral hydrate (100%, w/v) overnight, and leaves were mounted on glass slides in 60% glycerol.
2.3.3 ETI quantification via ion leakage assay

One day prior to experiments, plants are watered to ensure ease of infiltration. *PtoDC3000* strains carrying empty vector (pUCP20) (Ma et al., 2006), HopZ1a (Ma et al., 2006), AvrRpt2 (Mudgett and Staskawicz, 1999), or AvrRpm1 (Ritter and Dangl, 1995) were used for infiltrations. *PtoDC3000* carrying HopZ1a or AvrRpt2 strains were infiltrated in the evening (approximately 6-8pm), and ion leakage was quantified beginning the next morning. *PtoDC3000* carrying AvrRpm1 was infiltrated in the morning (approximately 8-9am) and ion leakage was quantified throughout the day. Leaves of four-week-old *Arabidopsis* Col-0 plants were syringe-infiltrated with *P. syringae* at OD$_{600}$=0.04 (2 x 10$^7$ CFU/mL). For each strain, eight plants were infiltrated – four leaves per each plant, one disk taken from each independent leaf. Immediately after infiltration, four leaf disks (1.5 cm$^2$) were harvested, washed in sterile distilled H$_2$O for 30 minutes on a bench-top shaker at 250 rpm, and transferred to 6 mL of sterile distilled H$_2$O. For temperature experiments, tubes containing leaf discs were returned to elevated temperature chamber to ensure that conductivity readings were taken in the same environmental conditions as other experiments. Readings were obtained with an Orion 3 Star benchtop conductivity meter (Thermo Fisher Scientific Inc., Fort Collins, CO, USA). This meter utilizes a 25°C reference temperature. Corrections of ion leakage readings to 30°C is negligible in ddH$_2$O (data not shown).

2.4 Disease resistance assay

2.4.1 In planta bacterial growth assay

One day prior to experiments, plants were watered to ensure ease of infiltration. *PtoDC3000* strains carrying empty vector (pUCP20) (Ma et al., 2006), HopZ1a (Ma et al., 2006), AvrRpt2 (Mudgett and Staskawicz, 1999), AvrRpm1 (Ritter and Dangl, 1995), or AvrRps4 (Hinsch and
Staskawicz, 1996) were used for infiltrations. PtoDC3000 carrying HopZ1a, AvrRpt2, AvrRpm1, or AvrRps4 strains were infiltrated in the morning or afternoon (10am-1pm). Leaves of three to four-week-old Arabidopsis Col-0 plants were syringe-infiltrated with P. syringae at OD$_{600}=0.0002$ (1 x 10$^5$ CFU/mL). On average, twelve plants are initially infiltrated on Day 0 – four leaves per plant, and, therefore, one leaf disk is taken from each independent leaf. For Day 0 (hereafter 0 days post infiltration, 0 dpi) quantification, four disks (1 cm$^2$) per plant were harvested approximately 1 hour after infiltration, ground in 1mL 10mM MgCl$_2$, and plated on KB with rifampicin (50µg/mL) and cycloheximide (50µg/mL) for colony quantification. On average, two to three plants were used for Day 0 quantification. 3 days post infiltration (3 dpi), four disks (1 cm$^2$) per plant were harvested, ground in 1mL 10mM MgCl$_2$, and plated on KB with rifampicin (50µg/mL) and cycloheximide (50µg/mL) for colony quantification. On average, eight to ten plants were used for 3 dpi quantification. Refer to figure legends for deviations from sample size numbers indicated here.

2.4.2 Disease resistance spray assay

One day prior to experiments, plants were watered to ensure ease of bacterial entry. PtoDC3000 strains carrying empty vector (pUCP20) (Ma et al., 2006), HopZ1a (Ma et al., 2006), AvrRpt2 (Mudgett and Staskawicz, 1999), or AvrRpm1 (Ritter and Dangl, 1995) were used for infiltrations. PtoDC3000 carrying HopZ1a, AvrRpt2, or AvrRpm1 strains were infiltrated in the morning or afternoon (10am-1pm) on the initial day of spraying and the subsequent challenge spray. For disease resistance assays testing effectors on Col-0 in elevated temperature, leaves of three-week old Arabidopsis wildtype were spray-infiltrated with P. syringae at OD$_{600}=0.4$ (2.0 x 10$^7$ CFU/mL). Leaves of three-week-old Arabidopsis ecotypes were spray-infiltrated with P. syringae at OD$_{600}=0.8$ (4 x 10$^8$ CFU/mL). Silwet L-77 was added to inocula at 0.04% (v/v). ) 0 days post spraying (0 dps) – that is, on the day of original spray – plants were sprayed using a
Prevall sprayer at an excess of 1.5mL of inoculum per plant, and domed immediately. On 3 dps plants were re-sprayed in the same manner as indicated on 0 dps, and domed once again immediately. On 4 dps, the dome was removed. Plants were monitored for disease symptoms up to 7-10 days. For indicated experiments, fresh weight of plants was determined by weighing individual plants using a Mettler Toledo AB54-S/FACT Analytical Balance at 10 dps.
Chapter 3

3 Environmental Factors Differentially Influence Effector-Triggered Immunity Outputs in *Arabidopsis*

3.1 Troubleshooting the Impact of High Relative Humidity on Effector-Triggered Immunity

During the initial screening of overexpression and knockout libraries for a separate project, ion leakage assays indicated severely decreased values as compared with published results, specifically for Col-0 plants infiltrated with *Pto*DC3000 (HopZ1a) (Lewis et al., 2010). In general, the HR elicited by *Pto*D3C3000 (HopZ1a) yields ion leakage values ranging from approximately 40-60μS/cm² at 12-15 hours post infiltration, and reaching a maximum of approximately 70-100μS/cm² at 18-21 hours post infiltration (Lewis et al., 2010). The ion leakage values associated with the failed experiments would reach a maximum average of 35-40μS/cm² at 20-22 hours post infiltration (Figure 1A, B). Due to this significant discrepancy, screening was initially put on hold in order to identify the underlying issues.
Figure 1. Ion leakage assay troubleshooting to match previously published data. Col-0 leaves were infiltrated with *PtoDC3000* expressing empty vector (EV) or HopZ1a at $2 \times 10^{7}$ CFU/mL, followed by harvesting four leaf disks per plant (one disk per leaf, total leaf tissue $1.5\text{cm}^2$) and washing in sterile distilled H$_2$O for 30 minutes on a bench-top shaker at 250 rpm. Disks were transferred to 6 mL of sterile distilled H$_2$O and readings were obtained until 20-22 hpi as indicated. (A) Previously published data demonstrating expected ion leakage values induced by T3SE HopZ1a delivered by *PtoDC3000* in Col-0 (Lewis et al., 2010). (B) An example of a failed experiment, in which ion leakage induced by *PtoDC3000* (HopZ1a) did not match previously published data. (C) Experiment demonstrating successful troubleshooting and recovered ion leakage induced by *PtoDC3000* (HopZ1a). Solid lines represent Condition #1, in which plants were removed from humid chamber 48 hours prior to infiltration ($\approx$LRH). Dashed lines represent Condition #2, in which plants were removed from humid chamber approximately 4 hours prior to infiltration ($\approx$LRH). Dotted lines represent Condition #3, in which plants were removed from humid chamber immediately before infiltration ($\approx$HRH).

Firstly, Col-0 seed stock from an external lab was tested in comparison to my seed stock to determine whether or not the issues with ion leakage was due to a contaminated seed stock.
(Figure 1C). This issue was ruled out, as there was no difference in ion leakage values across the tested seed stocks (Figure 1C). Secondly, in this experiment, environmental changes were also tested as flats were removed from the walk-in growth chamber, in which the relative humidity (RH) on average can range from 65-80%, at varying times prior to experimentation in order to determine whether or not timing of infiltration or environmental factors could play a role in development of *PtoDC3000* (HopZ1a)-induced HR (Figure 1C). In this experiment, Condition #1 was a set of Col-0 plants derived from seed stock taken from an external lab, grown in walk-in chamber Room 6, and left out in the lab over 48 hours. Condition #2 was a set of Col-0 plants derived from personal seed stock (Desveaux Lab), grown in walk-in chamber Room 6, and removed from the room approximately 4 hours prior to infiltration. Condition #3 was a set of Col-0 plants derived from seed stock taken from an external lab, grown in reach-in Chamber 31, taken out of chamber approximately 1 hour prior to infiltration and kept domed until the moment of infiltration. All three flats of plants were 5-6 weeks old. It was discovered that flats removed from the growth room four to five hours prior to infiltration displayed ion leakage values almost identical to those previously published (Figure 1A; Lewis et al., 2010). After subsequent experiments to confirm this, it was determined that removing the flats from a high humidity environment several hours prior to experimentation allowed acclimatization to the lower humidity environment of the lab (10-45%). Only plants that were treated in this manner and experimented on in a low relative humidity (LRH) environment exhibited a strong HR induced by *PtoDC3000* (HopZ1a), while plants kept covered with a dome after removal from the growth room, and thus were maintained in a high relative humidity (HRH) environment immediately until the start of experimentation, showed a dampened and almost entirely inhibited *PtoDC3000* (HopZ1a)-induced HR (Figure 1 B, C).
In these earlier experiments, high humidity appeared to also reduce ion leakage associated with \( PtoDC3000 \) (AvrRpt2)-induced HR, but in a less severe manner. This troubleshooting process revealed that the environmental context of experiments significantly impacts the results and, therefore by extension, the conclusions derived from each experiment. Troubleshooting in other experiments conducted by fellow lab mates revealed that weak bacterial resistance responses, elicited by effectors that do not cause an HR, demonstrated similar trends of HRH suppression of ETI outputs. For example, bacterial growth reduction in Col-0 induced by \( PtoDC3000\Delta HopF \) (HopF2\(_{PtoT1}\)) – which does not cause an HR in Col-0 – was entirely suppressed when \textit{in planta} bacterial growth quantification was conducted in an HRH environment (Lo et al., in review).

### 3.2 Investigating the Influence of High Relative Humidity on ETI

#### 3.2.1 Macroscopic HR delayed and ETI-associated ion leakage diminished in high relative humidity

We examined three effectors – HopZ1a, AvrRpt2, and AvrRpm1 – that have been previously shown to elicit strong ETI resistance responses and associated HR in \textit{Arabidopsis}. We tested the ability of all three effectors to induce ETI-associated HR in \textit{Arabidopsis Col-0} under either LRH (<13-50%) or HRH (≥95%)-primed conditions (Figure 2). Delivery of AvrRpm1, AvrRpt2, and HopZ1a from the virulent \( PtoDC3000 \) triggers a strong macroscopic HR in all leaves infiltrated by 4-6, 12, and 16 hours post infiltration (hpi), respectively, under normal ambient conditions.

At the crucial time point of 16 hpi, all leaves infiltrated with both \( PtoDC3000 \) (AvrRpt2) and \( PtoDC3000 \) (HopZ1a) should show a strong macroscopic HR. We observed that plants infiltrated with either effector incubated at ambient low relative humidity showed a strong HR as expected, \( PtoDC3000 \) (HopZ1a)-induced HR was significantly delayed in plants incubated in HRH (Figure 2). Additionally, \( PtoDC3000 \) (AvrRpt2)-induced HR appeared visible as expected in plants
incubated in HRH condition. Specifically, at 16 hpi, 0% of leaves infiltrated with \textit{PtoDC3000} (HopZ1a) showed HR, while 94% of leaves infiltrated with \textit{PtoDC3000} (AvrRpt2) showed HR in the HRH condition (Figure 2). At this same time point, over 50% of leaves infiltrated with \textit{PtoDC3000} (HopZ1a) showed HR and 94% of leaves infiltrated with \textit{PtoDC3000} (AvrRpt2) showed HR in the LRH condition. To confirm that PCD was suppressed by HRH, trypan blue staining of infiltrated leaves at 16 hpi was conducted (Figure 3). The staining reveals that \textit{PtoDC3000} (HopZ1a)-infiltrated leaves in the HRH condition exhibit negligible microscopic cell death at this time point.

At 19 hpi, 0% of leaves infiltrated with \textit{PtoDC3000} (HopZ1a) showed HR while 100% of leaves infiltrated with \textit{PtoDC3000} (AvrRpt2) showed HR in the HRH condition (not pictured, Tables 1a-c). Conversely, 94% of leaves infiltrated with \textit{PtoDC3000} (HopZ1a) and 100% of leaves infiltrated with \textit{PtoDC3000} (AvrRpt2) showed HR in the LRH condition. By 21 hpi, all leaves showed HR in both LRH and HRH conditions.

\textit{PtoDC3000} (AvrRpm1) elicits a strong HR at an earlier time point (approximately 6 hpi) in LRH, which was accurately observed in our experiments. At this time point, 80% of leaves showed HR in the LRH environment. However, a delayed and weakened HR elicited by \textit{PtoDC3000} (AvrRpm1) was observed in plants incubated in the HRH condition (Figure 2). At 6 hpi, the expected HR time point, only 12.5% of leaves showed HR in the HRH environment.

All replicates of macroscopic HR experiments conducted are summarized in heat maps with corresponding environmental conditions in Tables 1A-C. All replicates are visually presented as scatter plots with linear regression lines in Figure 4.
Figure 2. Macroscopic hypersensitive response (HR) is suppressed in high relative humidity (HRH) in wildtype Arabidopsis thaliana. Col-0 leaves were infiltrated with PtoDC3000 expressing empty vector (EV), HopZ1a, AvrRpt2, or AvrRpm1. For low relative humidity (LRH) or high relative humidity (HRH) conditions, plants were incubated in 15-25% and 95% humidity, respectively, for 24 hours prior to infiltration. The bacteria were syringe infiltrated into the leaves at $5 \times 10^7$ CFU/mL, and hypersensitive response (HR) phenotypes were scored until 10 (AvrRpm1) and 20 hours (HopZ1a, AvrRpt2) post infiltration (hpi). Asterisk (*) indicates leaves that show HR. Fractions indicate the number of infiltrated leaves showing HR phenotype out of total number of leaves infiltrated at the particular time point shown (16 hpi, and 6 hpi). Percentages shown below the figure are reflective of these fractions, indicating the percentage of leaves showing HR phenotype for each strain at the particular time points shown (16 hpi, and 6 hpi). Macroscopic HR assays were performed three times. Photos of each leaf taken independently, photos later combined into panel.
Figure 3. Trypan blue staining of programmed cell death (PCD) in wildtype Col-0 Arabidopsis thaliana leaves infiltrated with *PtoDC3000* (HopZ1a) or (AvrRpt2) in high relative humidity (HRH) at 16 hours post infiltration. Col-0 leaves were infiltrated with *PtoDC3000* expressing empty vector (EV), HopZ1a or AvrRpt2. For low relative humidity (LRH) or high relative humidity (HRH) conditions, plants were incubated in 15-25% and 95% humidity, respectively, for 24 hours prior to infiltration. The bacteria were syringe infiltrated into the leaves at $5 \times 10^7$ CFU/mL, and hypersensitive response (HR) phenotypes were scored and stained at 16 hours post infiltration (hpi). Trypan blue staining performed once. Darker blue indicates higher level of cell death. In HRH condition, cell death is either completely inhibited at this time point (*PtoDC3000* (HopZ1a)) or weakened (*PtoDC3000* (AvrRpt2)). Photos of each leaf taken independently, photos later combined into panel.
Figure 4. Scatter plot and linear regression summarizing all macroscopic HR experiments conducted with *PtoDC3000* (HopZ1a), (AvrRpt2), or (AvrRpm1) in low or high relative humidity (LRH, or HRH, respectively). Col-0 leaves were infiltrated with *PtoDC3000* expressing empty vector (EV, not shown), HopZ1a, AvrRpt2, or AvrRpm1. For low relative humidity (LRH) or high relative humidity (HRH) conditions, plants were incubated in 15-25% and 95% humidity, respectively, for 24 hours prior to infiltration. The bacteria were syringe infiltrated into the leaves at $5 \times 10^7$ CFU/mL. Data presented here represents the summary of (A) four or (B) two independent trials.
Table 1A. Heat map summarizing macroscopic HR experiments examining *PtoDC3000* expressing empty vector (EV), (HopZ1a), or (AvrRpt2) in low or high relative humidity (LRH or HRH, respectively). HR values calculated as a percentage of leaves showing HR out of total number of leaves infiltrated.

<table>
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<tr>
<th>Experiment #</th>
<th>Time (hpi)</th>
<th>EV-LRH</th>
<th>HopZ1a-LRH</th>
<th>AvrRpt2-LRH</th>
<th>EV-HRH</th>
<th>HopZ1a-HRH</th>
<th>AvrRpt2-HRH</th>
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Table 1B. Heat map summarizing macroscopic HR experiments examining *PtoDC3000* expressing empty vector (EV) or (AvrRpm1) in low or high relative humidity (LRH or HRH, respectively). HR values calculated as a percentage of leaves showing HR out of total number of leaves infiltrated.

<table>
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<th>Time (hpi)</th>
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<th>EV-HRH</th>
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Table 1C. Environmental conditions of experiments summarized in Tables 1A and 1B.

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To further observe the suppressive effects of HRH on ETI, we quantified ion leakage as a proxy for HR in plants primed at either LRH or HRH conditions (Figure 5). Similar time points to those observed for macroscopic HR were used as a guideline to quantify ion leakage. Previously published data shows *PtoDC3000* (AvrRpt2)-induced HR is associated with elevated ion leakage values compared to those associated with *PtoDC3000* (HopZ1a)-induced HR in ambient environmental conditions. Both effectors, however, result in HR-associated ion leakage values significantly different from that of the negative control, *PtoDC3000* (EV) (Figure 5A). In our LRH condition, these previously published trends were observed. In HRH-primed plants, however, ion leakage values of *PtoDC3000* (HopZ1a)-induced HR were severely decreased (Figure 5A). Specifically, ion leakage is severely decreased in such a way that these values are no longer significantly different from those ion leakage values of the *PtoDC3000* (EV) control at the crucial HR time points previously indicated (Figure 5A). In another trial, ion leakage values associated with *PtoDC3000* (AvrRpt2)-induced HR appeared no different across the LRH to HRH condition. In one trial, *PtoDC3000* (AvrRpt2)-induced HR ion leakage values were slightly decreased in a statistically significant manner, but the magnitude of the ion leakage in this experiment is still high enough that it is interpreted to indicate the occurrence of a strong HR – that is, ion leakage values exceeded ≥60µS/cm (data not shown).
Similarly, HR time points expected for *PtoDC3000* (AvrRpm1) were examined under both LRH and HRH conditions (Figure 5B). Ion leakage induced by *PtoDC3000* (AvrRpm1) in HRH was slightly decreased from that in LRH. This decrease was only statistically significant starting at 5 hpi and for all later time points (Figure 5B).

**Figure 5.** ETI-associated ion leakage indicates suppression of hypersensitive response (HR) in high relative humidity (HRH) in wildtype *Arabidopsis thaliana*. Col-0 leaves were infiltrated with *PtoDC3000* expressing empty vector (EV), (A) HopZ1a, AvrRpt2, or (B) AvrRpm1. For low relative humidity (LRH) or high relative humidity (HRH) conditions, plants were incubated in 15-25% and 95% humidity, respectively, for 24 hours prior to infiltration. The bacteria were syringe infiltrated into the leaves at 2 x 10^7 CFU/mL, followed by harvesting four leaf disks per plant (one disk per leaf, total leaf tissue 1.5cm^2^) and washing in sterile distilled H_2O for 30 minutes on a bench-top shaker at 250 rpm. Disks were transferred to 6 mL of sterile distilled H_2O and readings were obtained until 8 and 20.5 hpi, respectively. Ion leakage values induced by *PtoDC3000* (HopZ1a), (AvrRpt2), or (AvrRpm1) are significantly different from *PtoDC3000* (EV) in either condition according to a two-tailed homoschedastic t-tests (p-value < 0.01).

### 3.2.2 ETI-associated virulence suppression is not inhibited in high relative humidity

According to our macroscopic HR and ion leakage data, HR appears to be suppressed in HRH, corroborating previously published data examining HR induced by *PtoDC3000* (AvrRpm1) (Freeman et al., 2009). Similarly, this trend was also observed with HR induced by *PtoDC3000* (HopZ1a). *PtoDC3000* (AvrRpt2)-induced HR, however, appeared to be relatively intact and on
time. In order to examine whether ETI-associated virulence suppression was impaired by high relative humidity incubation, we conducted in planta bacterial growth assays (Figure 6). Plants incubated at LRH (13-21%) show a strong resistance response – that is, a reduction in bacterial growth – approximately ≥1-1.5 log CFU/cm² in magnitude induced by all three effectors tested when individually compared to the negative control, *PtoDC3000* (EV) (Figure 6). Plants incubated at HRH exhibit increased bacterial growth in all conditions tested. Despite this general increase in bacterial growth, the reduction in bacterial growth associated with virulence suppression during ETI observed in the LRH condition was still observed in the HRH condition. Increased bacterial growth for each effector was significantly different between Day 3 growth in plants in LRH or HRH conditions compared to *PtoDC3000* (EV) (Figure 6). Bacterial growth induced by *PtoDC3000* (EV) in HRH reaches approximately 7 log CFU/cm² of growth, while growth under the same experimental conditions induced by *PtoDC3000* (HopZ1a) reaches just under 5 log CFU/cm² of growth – indicating a very robust 2 log CFU/cm² reduction in bacterial growth, associated with a very strong resistance response. In LRH, this same reduction in *PtoDC3000* (HopZ1a)-induced growth was approximately 1.5 log CFU/cm² reduction in growth. Similar trends are observed for *PtoDC3000* carrying *PtoDC3000* (AvrRpt2) and *PtoDC3000* (AvrRpm1). Therefore, the ETI-associated virulence suppression, or bacterial resistance response, remains intact in HRH.
Figure 6. *In planta* bacterial growth assays demonstrate ETI-associated virulence suppression remains intact in high relative humidity (HRH). *Arabidopsis* Col-0 leaves were infiltrated with *PtoDC3000* expressing empty vector (EV), HopZ1a, AvrRpt2, or AvrRpm1. For low relative humidity (LRH) or high relative humidity (HRH) conditions, plants were incubated in 15-25% and 95% humidity, respectively, for 24 hours prior to infiltration. The bacteria were syringe infiltrated into the leaves at $1 \times 10^5$ CFU/mL and bacterial counts were determined one hour post-infection (0 dpi) and 3 days post-infection (3 dpi). Two-tailed homoscedastic t-tests were performed to test for significant differences between *PtoDC3000* (EV) and each single T3SE in both LRH and HRH conditions. *In planta* bacterial growth induced by T3SEs is significantly different when compared to *PtoDC3000* (EV) in either the ambient LRH or HRH conditions. Significant differences are indicated by an asterisk (*p*-value < 0.01). Error bars indicate the standard deviation from the mean of 2 (0 dpi) or 10 (3 dpi) samples. Growth assays were performed 4 times.

To better visualize this immune response in HRH, we performed disease resistance spray assays (Figure 7). Plants incubated in ambient room or HRH, in the manner previously described for all other assays, were sprayed at a high dosage bacterial density. Disease symptoms and chlorosis in leaves of plants sprayed with *PtoDC3000* (EV) become visible at approximately 2 to 3 days post spraying (dps). At 5 dps, disease symptoms become more severe, and susceptible plants are very obviously distinguishable from healthy resistant plants. In LRH, plants sprayed with *PtoDC3000*
carrying \textit{PtoDC3000} (HopZ1a) remain healthy, and developmental growth continues as expected (Figure 7). Plants sprayed with \textit{PtoDC3000} (EV) show signs of severe chlorosis by 5-6 dps, and plant growth slows or is completely halted. In HRH, sprayed plants show the same pattern of symptoms as those in ambient temperature for \textit{PtoDC3000} (EV) and \textit{PtoDC3000} (HopZ1a), pictured on 7 dps (Figure 7). This observation combined with the quantification of \textit{in planta} bacterial growth indicate that ETI-associated virulence suppression is still intact even in HRH conditions.

\textbf{Figure 7.} Disease resistance spray assays demonstrate macroscopic ETI-associated virulence suppression is intact in high relative humidity (HRH). \textit{Arabidopsis} Col-0 were sprayed with \textit{PtoDC3000} expressing EV, HopZ1a, AvrRpt2, or AvrRpm1. For low relative humidity (LRH) or high relative humidity (HRH) conditions, plants were incubated in 15-25\% and 95\% humidity, respectively, for 24 hours prior to infiltration. Plants were sprayed at $1 \times 10^8$ CFU/mL, and disease symptoms were monitored 3 to 7 days post infection. Photos shown were taken 7 days post infection. Spray assays were performed twice.
3.3 Investigating the Influence of Elevated Temperature on ETI

3.3.1 Macroscopic HR delayed and ion leakage suppressed in elevated temperature

We examined three T3SEs delivered by the virulent PtoDC3000 – HopZ1a, AvrRpt2, and AvrRpm1 – that have been previously shown to elicit strong resistance responses and associated hypersensitive responses (HR) in Arabidopsis characteristic of ETI. We tested the ability of all three T3SEs to induce ETI-associated HR in Arabidopsis Col-0 under either ambient room temperature (RT; 21-24°C) or ET (28-30°C)-primed conditions. Delivery of AvrRpm1, AvrRpt2, and HopZ1a from the virulent PtoDC3000 triggers a strong macroscopic HR in all leaves infiltrated by 6, 12, and 16 hours post infiltration (hpi), respectively, under normal ambient conditions.

We examined the ability of T3SEs HopZ1a, AvrRpt2, and AvrRpm1 to induce ETI-associated HR under RT and ET conditions (Figure 8). Delivery of PtoDC3000 (AvrRpt2) and PtoDC3000 (HopZ1a) triggers a strong macroscopic HR between 12 and 16 hours post infiltration (hpi) in ambient RT conditions, as expected (Figure 8). Specifically, at 16 hpi in RT, 85% (24/28) of leaves infiltrated with PtoDC3000 (HopZ1a) and 82% (23/28) of leaves infiltrated with PtoDC3000 (AvrRpt2) showing strong HR phenotypes (Figure 8). At ET, both the PtoDC3000 (AvrRpt2) and PtoDC3000 (HopZ1a)-induced HR was suppressed, with no leaves showing a strong HR, and only ~20% of leaves showing a weak HR-like collapse (Figure 8). Specifically, at 16 hpi in ET, 18% (5/28) of PtoDC3000 (HopZ1a)-infiltrated leaves and 25% (7/28) of PtoDC3000 (AvrRpt2)-infiltrated leaves showing HR phenotypes (Figure 8). At 18.5 hpi, 50% (14/28) of leaves infiltrated with PtoDC3000 (HopZ1a) showed a strong macroscopic HR, and 54% (15/28) of leaves infiltrated with PtoDC3000 (AvrRpt2) showed a macroscopic HR (photos not shown, Table 2A). By 20 hpi, only 85% (24/28) of leaves infiltrated with PtoDC3000
(HopZ1a) showed a strong macroscopic HR, while 93% (26/28) of leaves infiltrated with \textit{PtoDC3000 (AvrRpt2)} showed a macroscopic HR (photos not shown, Table 2A).

Similarly, \textit{PtoDC3000 (AvrRpm1)} elicits a strong HR at an earlier time point in ambient room temperature, which was accurately observed in our experiments. However, a slightly delayed and weakened HR elicited by \textit{PtoDC3000 (AvrRpm2)} was observed in plants incubated in the elevated temperature condition at 4.25 hpi (Figure 8). That is, of the leaves infiltrated with \textit{PtoDC3000 (AvrRpm1)} in both temperature conditions, 50% (12/28) showed strong HR in room temperature, and 35% (10/28) showed weak HR in elevated temperature. In earlier replicates of this experiment, a more significant delay was observed (Table 2B). Additionally, AvrRpm1-induced HR was observed in RT closer to 6 hpi, which matches expected results compared with the literature more accurately. However, the final two replicates of this experiment showed an earlier HR in both the RT and ET condition as indicated (approximately 4.25 hpi, Table 2B).

All replicates of macroscopic HR experiments are summarized in heat maps with corresponding environmental conditions in Tables 2A-C. All replicates are visually presented as scatter plots with linear regression lines in Figure 9.
Figure 8. Macroscopic hypersensitive response (HR) is inhibited or delayed by elevated temperature (ET) in wildtype Arabidopsis thaliana. Col-0 leaves were infiltrated with PtoDC3000 expressing empty vector (EV), HopZ1a, AvrRpt2, or AvrRpm1. For ambient room temperature (RT) or elevated temperature (ET) conditions, plants were incubated in 21-24°C and 30°C, respectively, for 24 hours prior to infiltration. The bacteria were syringe infiltrated into the leaves at $5 \times 10^7$ CFU/mL, and hypersensitive response (HR) phenotypes were scored until 10 and 20 hours post infiltration (hpi). Fractions indicate the number of infiltrated leaves showing HR phenotype out of total number of leaves infiltrated at the particular time point shown (16 hpi, and 6 hpi). Percentages shown below the figure are reflective of these fractions, indicating the percentage of leaves showing HR phenotype for each strain at the particular time points shown (16 hpi, and 6 hpi). Asterisk (*) indicates leaves that show HR. Numbers in brackets indicate the presence of a weak HR phenotype. Macroscopic HR assays were performed three times.
Figure 9. Scatter plot and linear regression summarizing all macroscopic HR experiments conducted with *PtoDC3000* (HopZ1a), (AvrRpt2), or (AvrRpm1) in ambient room or elevated temperature (RT or ET, respectively). Col-0 leaves were infiltrated with *PtoDC3000* expressing empty vector (EV, not shown), HopZ1a, AvrRpt2, or AvrRpm1. For ambient room temperature (RT) or elevated temperature (ET) conditions, plants were incubated in 21-24°C and 30°C, respectively, for 24 hours prior to infiltration. The bacteria were syringe infiltrated into the leaves at $5 \times 10^7$ CFU/mL. Data presented here represents the summary of (A) three or (B) two independent trials.
Table 2A. Heat map summarizing macroscopic HR experiments examining \textit{PtoDC3000} expressing empty vector (EV), (HopZ1a), or (AvrRpt2) in ambient room or elevated temperature (RT or ET, respectively). HR values calculated as a percentage of leaves showing HR out of total number of leaves infiltrated.

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Table 2B. Heat map summarizing macroscopic HR experiments examining \textit{PtoDC3000} expressing empty vector (EV) or (AvrRpm1) in ambient room or elevated temperature (RT or ET, respectively). HR values calculated as a percentage of leaves showing HR out of total number of leaves infiltrated.

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<td>0.00</td>
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<td>8</td>
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<td>1.00</td>
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<td>1.00</td>
</tr>
<tr>
<td>4</td>
<td>8.25</td>
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<td>1.00</td>
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</table>
Table 2C. Environmental conditions of experiments summarized in Tables 2A and 2B.

<table>
<thead>
<tr>
<th>Experiment #</th>
<th>RT Temperature (°C)</th>
<th>RT Humidity (%)</th>
<th>ET Temperature (°C)</th>
<th>ET Humidity (%)</th>
</tr>
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<td>27.7</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>21.9</td>
<td>50</td>
<td>30.0</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>23.7</td>
<td>13</td>
<td>30.3</td>
<td>23</td>
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<tr>
<td>6</td>
<td>21.9</td>
<td>50</td>
<td>30.0</td>
<td>50</td>
</tr>
</tbody>
</table>

To further observe the suppressive effects of elevated temperature on ETI, we quantified ion leakage as a proxy for HR in plants primed at either ambient or elevated temperature stress conditions (Figure 10). Similar time points to those observed for macroscopic HR were observed to quantify ion leakage. Previously published data shows HR elicited by *PtoDC3000 (AvrRpt2)* induces higher ion leakage values than those induced by *PtoDC3000 (HopZ1a)* in ambient environmental conditions. Both effectors, however, result in HR-associated ion leakage values significantly different from that of the negative *PtoDC3000 (EV)* control. In our ambient RT condition, these previously published trends were observed. In ET-primed plants, ion leakage values of *PtoDC3000 (HopZ1a)* and *PtoDC3000 (AvrRpt2)*-induced HR are significantly decreased. Specifically, ion leakage is severely decreased in such a way that these values are no longer significantly different from those ion leakage values of the *PtoDC3000 (EV)* control at the crucial HR time points previously indicated (Figure 10).
Figure 10. ETI-associated ion leakage indicates suppression of hypersensitive response (HR) in elevated temperature (ET) in wildtype Arabidopsis thaliana. Col-0 leaves were infiltrated with *Pto*DC3000 expressing empty vector (EV), (A) HopZ1a, AvrRpt2, or (B) AvrRpm1. For ambient room temperature (RT) or elevated temperature (ET) conditions, plants were incubated in 21-24°C and 28-30°C, respectively, for 24 hours prior to infiltration. The bacteria were syringe infiltrated into the leaves at 2 x 10⁷ CFU/mL, followed by harvesting four leaf disks per plant (one disk per leaf, total leaf tissue 1.5cm²) and washing in sterile distilled H₂O for 30 minutes on a bench-top shaker at 250 rpm. Disks were transferred to 6 mL of sterile distilled H₂O and readings were obtained until 8 and 20.5 hpi, respectively. Ion leakage values induced by *Pto*DC3000 (HopZ1a), (AvrRpt2), or (AvrRpm1) are significantly different from *Pto*DC3000 (EV) in either condition according to a two-tailed homoschedastic t-tests (p-value < 0.01).

3.3.2 ETI-associated virulence suppression is not inhibited in elevated temperature

According to our macroscopic HR and ion leakage data, HR appears to be inhibited or suppressed in ET, corroborating previously published data examining HR induced by both *Pto*DC3000 (AvrRpt2) and *Pto*DC3000 (AvrRpm1) (Cheng et al, 2013; Freeman and Beattie, 2009). Similarly, this trend was also observed with HR induced by HopZ1a (Figures 8-10). In order to examine whether the ETI response was impaired by ET incubation, we conducted *in planta* bacterial growth assays (Figure 11). Plants incubated at ambient room temperature show a strong resistance response indicated by a reduction in bacterial growth (~0.5-1 log CFU/cm²) induced by recognition of all three effectors tested when compared to the negative control (Figure 11). Plants incubated at elevated temperature (30°C) exhibit increased bacterial growth...
in all conditions tested. Nevertheless, the ETI-associated virulence suppression induced by all three T3SEs tested was still observed under ET conditions. (Figure 11) In fact, the magnitude of ETI-associated \textit{PtoDC3000} growth reduction was an order of magnitude greater for both T3SEs at ET (~2.5 log CFU/cm$^2$) than at ambient RT (~0.5-1 log CFU/cm$^2$). Statistical significance between day 3 growth in plants in RT or ET conditions varies between effectors (Figure 12). However, the strengths of the resistance response trends quantified remain consistent, as demonstrated by replicates of the \textit{in planta} bacterial growth assays shown (Figure 12).

**Figure 11.** \textit{In planta} bacterial growth assays demonstrate ETI-associated virulence suppression remains intact in elevated temperature (ET). \textit{Arabidopsis} Col-0 leaves were infiltrated with \textit{PtoDC3000} expressing empty vector (EV), HopZ1a, AvrRpt2, or AvrRpm1. For ambient room temperature (RT) or elevated temperature (ET) conditions, plants were incubated in 21-24°C and 30°C, respectively, for 24 hours prior to infiltration. The bacteria were syringe infiltrated into the leaves at 1 x 10$^5$ CFU/mL and bacterial counts were determined one hour post-infection (Day 0) and 3 days post-infection. Two-tailed homoschedastic t-tests were performed to test for significant differences between \textit{PtoDC3000} (EV) and each single T3SE in both RT and ET conditions. \textit{In planta} bacterial growth induced by T3SEs is significantly different when compared to PtoDC3000 (EV) in either the ambient RT or ET conditions. Significant differences are indicated by an asterisk (*p-value < 0.01). Error bars indicate the standard deviation from the mean of 2 (0 dpi) or 10 (3 dpi) samples. Growth assays were performed 4 times.
Figure 12. Replicates of *in planta* bacterial growth assays demonstrating ETI response induced by T3SEs in elevated temperature (ET). *Arabidopsis* Col-0 leaves were infiltrated with *PtoDC3000* expressing empty vector (EV), HopZ1a, AvrRpt2, or AvrRpm1. For ambient room temperature (RT) or elevated temperature (ET) conditions, plants were incubated in 21-24°C and 30°C, respectively, for 24 hours prior to infiltration. The bacteria were syringe infiltrated into the leaves at 1 x 10^5 CFU/mL and bacterial counts were determined one hour post-infection (Day 0) and 3 days post-infection. Two-tailed homoschedastic t-tests were performed to test for significant differences between *PtoDC3000* (EV) and each single T3SE in both RT and ET conditions. *In planta* bacterial growth induced by T3SEs is significantly different when compared to *PtoDC3000* (EV) in either the ambient RT or ET conditions. Significant differences are indicated by an asterisk (*p*-value < 0.01). Error bars indicate the standard deviation from the mean of 2 (0 dpi) or 10 (3 dpi) samples.

It is not yet fully understood how temperature modulates host disease resistance, or whether elevated temperatures modulate basal and R-gene mediated resistance via similar mechanisms. Furthermore, the molecular basis of the inhibitory or suppressive effects of temperature on immunity have not yet been elucidated. To understand whether or not the two major classes of
NLRs – CC-NB-LRRs and TIR-NB-LRRs – differentially modulate virulence suppression in elevated temperature, *in planta* disease resistance assays were also conducted using *PtoDC3000* carrying AvrRps4 (Figure 13). *PtoDC3000* (AvrRps4) induces a strong immune response *in planta* upon recognition by the TIR-NB-LRR protein, RPS4. A moderate increase in bacterial growth induced by *PtoDC3000* (AvrRps4) was observed in elevated temperature, however, as observed with the other effectors tested, there was still a strongly significant reduction in bacterial growth when compared to *PtoDC3000* (EV) (Figure 13).

![Graph showing bacterial growth](image)

**Figure 13.** ETI-associated virulence suppression mediated by both CC- and TIR-NB-LRRs remains intact in elevated temperature (ET). *Arabidopsis* Col-0 leaves were infiltrated with *PtoDC3000* expressing empty vector (EV), HopZ1a, AvrRpt2, or AvrRps4. For ambient room temperature (RT) or elevated temperature (ET) conditions, plants were incubated in 21-24°C and 30°C, respectively, for 24 hours prior to infiltration. The bacteria were syringe infiltrated into the leaves at 1 x 10⁵ CFU/mL and bacterial counts were determined one hour post-infection (Day 0) and 3 days post-infection. Two-tailed homoschedastic t-tests were performed to test for significant differences between *PtoDC3000* (EV) and each single T3SE in both RT and ET conditions. *In planta* bacterial growth induced by T3SEs is significantly different when compared to *PtoDC3000* (EV) in either the ambient RT or ET conditions. Significant differences are indicated by an asterisk (*p*-value < 0.01). Error bars indicate the standard deviation from the mean of 2 (0 dpi) or 10 (3 dpi) samples. Assay performed once.
In order to understand the timing of the immune response as demonstrated by *in planta* bacterial growth assays, bacterial growth induced by *PtoDC3000* carrying EV, HopZ1a, AvrRpt2, or AvrRpm1 was quantified on Day 0, Day 1, and Day 2 (Figure 14). Because the HR was shown to be delayed under elevated temperature conditions, we wondered whether this delay in HR manifested as a delay in bacterial growth reduction. It was predicted that if HR is delayed, perhaps ETI-associated virulence suppression is also delayed. *In planta* bacterial growth assays were conducted on plants in both ambient and elevated temperature conditions. On Day 0, bacterial growth quantification represents what was pressure infiltrated into the plant, and therefore all strains in all temperature conditions exhibited approximately the same amount of bacterial growth (Figure 14). On Day 1, plants in room temperature demonstrated almost equivalent bacterial growth across all effector treatments as well as that of empty vector. In elevated temperature, however, bacterial growth of *PtoDC3000* (EV) was almost at the equivalent level of 3 days’ worth of bacterial growth at ambient temperature – that is, just under 6 log CFU/cm² of growth (Figure 14). The T3SEs used in this experiment, however, did not induce bacterial growth of that level even in elevated temperature. By day 2, the effector treatments had reached essentially the maximum growth as compared to what is normally observed in both ambient room and elevated temperature on Day 3 – approximately 4-5 log CFU/cm² of bacterial growth. *PtoDC3000* (EV) induced growth, however, continued to increase slightly post-Day 1, and presumably continued to increase post-Day 2 based on other growth assays (Figure 14). This indicates that while HR is suppressed, the timing of the immune response appears to be relatively unaltered in elevated temperature conditions.
**Figure 14. In planta bacterial growth assays reveal the majority of increased growth in elevated temperature (ET) occurs two days post infection.** *Arabidopsis* Col-0 leaves were infiltrated with *PtoDC3000* expressing empty vector (EV), HopZ1a, AvrRpt2, or AvrRpm1. For ambient room temperature (RT) or elevated temperature (ET) conditions, plants were incubated in 21-24°C and 30°C, respectively, for 24 hours prior to infiltration. The bacteria were syringe infiltrated into the leaves at $1 \times 10^5$ CFU/mL and bacterial counts were determined one hour post-infection (Day 0) and 3 days post-infection. Two-tailed homoschedastic t-tests were performed to test for significant differences between *PtoDC3000* (EV) and each single T3SE in both RT and ET conditions. *In planta* bacterial growth induced by T3SEs is significantly different when compared to *PtoDC3000* (EV) in either the ambient RT or ET conditions. Significant differences are indicated by an asterisk (*p*-value < 0.01). Error bars indicate the standard deviation from the mean of 2 (0 dpi) or 10 (3 dpi) samples. Assay performed once.

To better visualize this immune response in elevated temperatures, we performed disease resistance spray assays (Figure 15). In ambient RT, plants sprayed with *PtoDC3000* carrying any of the T3SEs tested – HopZ1a, AvrRpt2, AvrRpm1 – remain healthy, and developmental growth continues as expected, pictured on 10 days post spraying (dps) (Figure 15). Plants sprayed with *PtoDC3000* (EV) show signs of severe chlorosis by 5 dps, and plant growth slows or is entirely halted by 10 dps (Figure 15). In elevated temperature, sprayed plants show the same pattern of
symptoms as those in ambient temperature for \( PtoDC3000 \) (EV) and \( PtoDC3000 \) carrying HopZ1a, AvrRpt2, or AvrRpm1 (Figure 15). This observation combined with the quantification of \textit{in planta} bacterial growth indicated that ETI-associated virulence suppression remained intact under ET conditions that suppressed ETI-associated HR.

![Image of disease resistance spray assays](image)

**Figure 15.** Disease resistance spray assays demonstrate macroscopic ETI-associated virulence suppression is intact in elevated temperature (ET). \textit{Arabidopsis} Col-0 were sprayed with \( PtoDC3000 \) expressing EV, HopZ1a, AvrRpt2, or AvrRpm1. For ambient room temperature (RT) or elevated temperature (ET) conditions, plants were incubated in 21-24°C and 30°C, respectively, for 24 hours prior to infiltration. Plants were sprayed on Day 0 and Day 3 at \( 2.0 \times 10^8 \) CFU/mL, and disease symptoms were monitored 3 to 10 days post-spraying (dps). Photos shown were taken 10 dps. Spray assays were performed three times. Scale bar indicates 1cm.

### 3.3.3 Arabidopsis ecotypes exhibit varying temperature sensitivity

We demonstrated that ETI-associated virulence suppression remains intact under elevated temperature conditions that otherwise suppress ETI-associated HR in the \textit{Arabidopsis} Col-0 ecotype. In order to examine whether local adaptation to varying climates can influence disease resistance under ET conditions, we tested \textit{Arabidopsis} ecotypes isolated from varying geographic
climate regions for loss of *PtoDC3000* (HopZ1a)-triggered immunity at elevated temperature (Bomblies and Weigel, 2007; Huang et al., 2010; Li et al., 2010) (Figures 16, 17). Although most ecotypes showed a Col-0 like retention of ETI at elevated temperature (Figure 16A, bottom left photo), we identified three ecotypes CIBC-5, Wei-0 and Tsu-1, that displayed a loss of ETI-associated virulence suppression at elevated temperature, despite showing a normal (Col-0 like) response at ambient temperature (Figure 17). As we were conducting our spray assays we noted a noticeable size difference in resistant (green) versus susceptible (chlorotic) plants. As disease progresses, plant growth essentially halts and plants eventually die, resulting in reduced fresh weight when compared to a resistant plant. It is important to note that in ET, *Arabidopsis* grows at a faster rate than in ambient RT. As a result, resistant plants in ET weigh more than those in ambient RT. Additionally, diseased plants sometimes may weight more in ET than those in ambient RT, due to increased growth rate of *Arabidopsis*. Importantly, plants that are not resistant to the delivered T3SE will weigh less than or the same as plants sprayed with *PtoDC3000* (EV) regardless of temperature condition.

*Arabidopsis* ecotypes listed in Table 3 were selected for further temperature sensitivity study based on the climate of the geographic region from which they were isolated. Disease resistance spray assays were conducted on all selected ecotypes (Figures 14, 15).
Table 3. Geographical locations and climate of ecotypes\(^a\)

<table>
<thead>
<tr>
<th>Ecotype</th>
<th>Location</th>
<th>Coordinates</th>
<th>Average Temperature(^b)</th>
<th>Altitude</th>
<th>Climate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0</td>
<td>Columbia, Missouri, USA</td>
<td>38.952°N, 92.334°W</td>
<td>20-36°C</td>
<td>100m</td>
<td>Humid continental-subtropical</td>
</tr>
<tr>
<td></td>
<td><strong>High temperature acclimated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cvi-0</td>
<td>Cape Verde Island, Republic of Cabo Verde</td>
<td>16.0°N, 24.0°E</td>
<td>23-29°C</td>
<td>1200m</td>
<td>Semi-desert,</td>
</tr>
<tr>
<td>Tsu-1</td>
<td>Tsushima, Japan</td>
<td>34.202°N, 129.29°E</td>
<td>20-26°C</td>
<td>100m</td>
<td>Humid subtropical</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIBC-5</td>
<td>Ascot, Berkshire, United Kingdom</td>
<td>51.4084°N, 0.6707°W</td>
<td>9-17°C</td>
<td>68m</td>
<td>Maritime temperate</td>
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<tr>
<td>Fab-2</td>
<td>Faberget, Sweden</td>
<td>63.016°N, 18.325°E</td>
<td>10-18°C</td>
<td>110m</td>
<td>Maritime temperate</td>
</tr>
<tr>
<td>Wei-0</td>
<td>Weiningen, Switzerland</td>
<td>47.419°N, 8.4326°E</td>
<td>9-20°C</td>
<td>413m</td>
<td>Maritime temperate</td>
</tr>
</tbody>
</table>

\(^a\)All data derived from [www.worldweatheronline.org](http://www.worldweatheronline.org), TAIR ([https://www.arabidopsis.org](https://www.arabidopsis.org)), [www.gps-coordinates.net](http://www.gps-coordinates.net), and the Köppen climate classification ([https://en.wikipedia.org/wiki/K%C3%B6ppen_climate_classification](https://en.wikipedia.org/wiki/K%C3%B6ppen_climate_classification)).

\(^b\)Temperature information describes warmest season of annual climate because this study is concerned with elevated temperature tolerance.

Several ecotypes were found to have increased temperature sensitivity, meaning that they showed impaired resistance induced by \textit{PtoDC3000} carrying HopZ1a in ET, despite showing a normal (Col-0)-like response at ambient RT. Compared to Col-0, ecotypes that showed impaired resistance are described as “elevated temperature-intolerant”, while those that exhibit the same resistance phenotypes as Col-0 are described as “elevated temperature-tolerant”.

Firstly, based on previous macroscopic HR screens conducted in the lab, the ecotype Cvi-0 was thought to be weakly resistant to \textit{PtoDC3000} (HopZ1a) (Figure 16A, top left). Spray assays revealed that neither in ambient room temperature nor elevated temperature is Cvi-0 resistant to \textit{PtoDC3000} (HopZ1a), either at a lower bacterial dosage of OD\textsubscript{600}=0.4 (photos not shown) or a
higher dosage at OD$_{600=0.8}$ (Figure 16A, top left). Therefore, Cvi-0 provided a consistent negative control in the experiments, while Col-0 represents our positive control of an ecotype that is strongly resistant to $P_{to}DC3000$ (HopZ1a) in both RT and ET conditions. Fab-2, isolated from Northern Sweden, robustly exhibited a resistant phenotype at both ambient RT and ET (Figure 16A, bottom left). Fresh weight values indicate trends of elevated temperature-intolerant and tolerant phenotypes quantitatively (Figure 16B).
Figure 16. See caption on following page.
Figure 16. Macroscopic disease resistance spray assays reveal warm-acclimated Cvi-0 is not resistant to *PtoDC3000* (HopZ1a) in ambient room temperature (RT) or elevated temperature (ET), while cold-acclimated Fab-2 demonstrates a robustly resistance phenotype in either condition. *Arabidopsis* Col-0, Cvi-0, and Fab-2 were sprayed with *PtoDC3000* expressing empty vector (EV) or HopZ1a. For ambient room temperature (RT) or elevated temperature (ET) conditions, plants were incubated in 21-24°C and 30°C, respectively, for 24 hours prior to infiltration. (A) Plants were sprayed on Day 0 and Day 3 at 4 x 10^8 CFU/mL. Photos shown were taken 10 days post-spraying (dps). Spray assays were performed three times. Scale bar indicates 1cm. (B) Fresh weight (mg) of sprayed plants measured on 10 dps.

Unlike Col-0, CIBC-5, Wei-0 and Tsu-1, developed a chlorotic, disease phenotype when sprayed with *PtoDC3000* (HopZ1a) in elevated temperature, similar to that of *PtoDC3000* (EV) (Figure 17A). That is, these ecotypes exhibited disease-like symptoms when sprayed with *PtoDC3000* carrying HopZ1a. All other ecotypes listed in Table 3 were found to be elevated temperature-tolerant when sprayed with high dosages of *PtoDC3000* (HopZ1a) (photos not shown). That is, they exhibited resistant phenotypes that resemble that of Col-0 when sprayed with high dosages of *PtoDC3000* (HopZ1a).
Figure 17. See Panel B and caption on following page.
Figure 17. Macroscopic disease resistance spray assays reveal ecotypes CIBC-5, Wei-0, and Tsu-1 lose PtoDC3000 (HopZ1a)-induced resistant phenotype in elevated temperature (ET), but not in ambient room temperature (RT). *Arabidopsis* Col-0, Cvi-0, and Fab-2 were sprayed with PtoDC3000 expressing empty vector (EV) or HopZ1a. For ambient room temperature (RT) or elevated temperature (ET) conditions, plants were incubated in 21-24°C and 30°C, respectively, for 24 hours prior to infiltration. (A) Plants were sprayed on Day 0 and Day 3 at 4 x 10^8 CFU/mL. Photos shown were taken 10 days post-spraying (dps). Spray assays were performed three times. Scale bar indicates 1cm. (B) Fresh weight (mg) of sprayed plants measured on 10 dps.
Therefore, in order to quantify this macroscopic loss of ETI-associated virulence suppression, fresh weight of plants was quantified at 10 days post-infection (Figure 17B). At both RT and ET, Col-0 plants sprayed with PtoDC3000 (HopZ1a) were of similar weight to untreated plants. CIBC-5, Wei-0 and Tsu-1 plants sprayed with PtoDC3000 (HopZ1a) also had fresh weights comparable to untreated plants at RT, but they had severely reduced fresh weight relative to untreated plants at ET and resembled those sprayed with PtoDC3000 (EV) (Figure 17B). Overall, these data demonstrate the ETI response of different Arabidopsis ecotypes is differentially affected by temperature.

3.4 Discussion

The data in this chapter corroborates previous findings that humidity and temperature are direct modulators of plant immunity. However, we find that different outputs of immunity are differentially sensitive to humidity and temperature. A previous study has proposed that lower temperatures favour the activation of ETI, whereas ETI is compromised at elevated temperatures which favour PTI (Cheng et al., 2013). In light of our results, we would add that different facets of ETI are differentially influenced by temperature or humidity, with HR being more sensitive than ETI-associated virulence suppression. In fact, ETI-associated virulence suppression is greater at elevated temperature and high relative humidity (~2.5 log CFU/cm²) relative to ambient conditions (~1.5 log/cm²; Figures 6, 11). This is likely a reflection of the increased growth of virulent PtoDC3000 observed at these conditions, but nevertheless emphasizes that ETI-associated virulence suppression is still robust in these conditions.

Temperature modulation of plant-pathogen interactions has been demonstrated in this chapter to impact disease resistance in varying ways. Importantly, the same increased temperature may
have differential impacts on various pathosystems. When evaluating the role of ET in modulation of ETI, all environmental factors that have inhibitory or suppressive effects must be considered simultaneously. For example, the LMM phenotypes that demonstrate sensitivity to temperature are also sensitive to high relative humidity (Lorrain et al., 2003; Moeder and Yoshioka, 2008). That is, the general enhanced disease resistance, elevated SA levels, and loss of PCD phenotype associated with LMMs is inhibited by both ET and HRH in an additive manner (Lorrain et al., 2003; Moeder and Yoshioka, 2008). A common conclusion exists that R gene-mediated resistance responses are inhibited by increased temperatures, however the particular environmental context in which we evaluate these disease resistance responses must be considered (Zhu et al., 2010).

In order to minimize plant developmental differences between experiments, plants were grown for 3-5 weeks under our standard plant growth conditions, and then acclimated to HRH or ET conditions for 24 hours prior to infection assays. In evaluating the impact of high relative humidity, temperature was controlled to be within a consistent range (20-25°C) in order to avoid the additive suppressive effects of elevated. Conversely, in evaluating the impact of elevated temperature, relative humidity was controlled to be within a moderate to low range (30-60%) in order to avoid the additive suppressive effects of HRH (this work; Beattie, 2011; Freeman and Beattie, 2009; Hangyang et al., 2015; Jambunathan et al., 2001; Lorrain et al., 2003; Moeder and Yoshioka, 2008; Mosher et al., 2010; Noutoshi et al., 2005; Samuel, 1931; Yoshioka et al., 2001; Zhou et al., 2004).

In evaluating the impact of high relative humidity, temperature was controlled to be within a consistent range (20-25°C) in order to avoid the additive suppressive effects of elevated temperature – which is demonstrated in the next chapter of this thesis. It is important to note that
in this chapter, elevated humidity does not negatively impact the growth of *Arabidopsis* or the bacterial pathogen *P. syringae*.

As demonstrated in this chapter, both macroscopic HR and ion leakage phenotypes associated with an ETI response are significantly delayed in plants subject to 24h of HRH or ET, corroborating previously published data (Freeman et al., 2009; Lorrain et al., 2003; Moeder and Yoshioka, 2008). However, *in planta* bacterial growth and overall macroscopic disease resistance appear to be unaffected by this incubation period. In this chapter, we showed that HR is delayed in ET according to macroscopic HR assay data, but appears to be inhibited when evaluating ion leakage caused by HR. While HR and ion leakage data indicate the suppression or inhibition of R-gene associated PCD in elevated temperature, the disease resistance response evaluated using *in planta* bacterial growth assays appears to be generally intact under our experimental conditions. Further corroborating this, our bacterial spray assays show that Col-0 still elicit a robust ETI response even in elevated temperature.

Two different assays to monitor the ETI-associated HR yielded slightly differing results. Macroscopic HR was significantly delayed in our experiments, but not entirely inhibited. While HR is delayed in our experiments, it is not entirely inhibited. In high relative humidity-incubated plants, ETI-associated ion leakage is diminished but not entirely suppressed. In elevated temperature-incubated plants, however, ion leakage assays would have us conclude that HR is entirely inhibited due to entirely suppressed ion leakage, similar to that of empty vector (*PtoDC3000*) (Figure 10). We believe this may be due to the nature of the assay. This is likely influenced by the fact that for ion leakage assay, infiltrated leaf disks are placed in water (i.e. 100% humidity) immediately post-infiltration in order to monitor changes in water conductance. As such, the ion leakage assays are conducted in both an ET and HRH environment. We believe
that the combined suppressive effects of elevated temperature and humidity result in a complete loss of HR-associated ion leakage, whereas macroscopic HR is delayed but still observed since these assays are conducted at ambient relative humidity (Beattie, 2011; Freeman and Beattie, 2009; Hangyang et al., 2015; Jambunathan et al., 2001; Lorrain et al., 2003; Moeder and Yoshioka, 2008; Mosher et al., 2010; Noutoshi et al., 2005; Samuel, 1931; Yoshioka et al., 2001; Zhou et al., 2004). Our results also indicate that high relative humidity or elevated temperatures can uncouple the HR response from ETI-associated virulence suppression. This is reminiscent of ETI examples that occur without apparent PCD. Therefore, despite the lack of localized PCD, virulence suppression is still observed in these cases, similar to our observations of ETI at these experimental conditions.

Additionally, it is important to note that in this chapter, and in many other studies that evaluate the temperature modulation of plant pathogen interactions, suppressed HR at ET is not due to inhibition of the type III secretion system since *P. syringae* type III effector expression assays are routinely conducted at 28-30°C. Additionally, the *in planta* growth rate of virulent *PtoDC3000* actually increased at ET, corroborating previously published data (Freeman and Beattie, 2009; Wang et al., 2009; Cheng et al., 2013). Other works provide evidence that lower temperatures favour the activation of ETI, as effectors are more likely secreted in temperatures closer to ambient (Cheng et al., 2013). Whereas basal immune responses – PTI – are favoured in elevated temperatures due to the increased bacterial population in a favourable growth temperature (Cheng et al., 2013). However, it has been shown that basal resistance to *P. syringae* is also compromised by even moderate increases in temperature (Wang et al., 2009). Therefore, in general there is a phenomenon that variances in temperature will directly impact outputs of basal or R gene-mediated immunity in varying ways. Most importantly, as demonstrated in this thesis, the environmental context in which the experiment is conducted is incredibly important,
and must be considered when drawing conclusions as far as the role of environmental factors and their modulation of plant-pathogen interactions.

Data presented in this chapter may differ from previously published data due to difference in pathosystems employed. In this study, the *P. syringae-Arabidopsis thaliana* pathosystem was used in order to evaluate the effects of humidity and temperature on immunity outputs. In other studies referenced in this thesis, model organisms used differ. For example, fungal pathogens such as *Cladosporium fulvum* or oomycetes such as *Hyaloperonospora arabidopsidis* have been used on various model plant organisms such as *Nicotiana benthamiana* to study effects of humidity and temperature on immunity. These pathogens have very different lifestyles from that of *P. syringae*. *P. syringae* is a hemibiotrophic pathogen, meaning that it causes disease on a healthy plant initially as a biotroph, and as its disease symptoms become more severe leaving the plant tissue chlorotic it can continue to proliferate in dead plant matter as a necrotroph. It is important to note that the findings detailed in this thesis are specific to this pathosystem, and the use of a different model host plant or pathogen may result in different sensitivities to various abiotic factors, specifically humidity and temperature.

Data presented in this chapter differs from previously published data in Freeman et al., 2009, which indicates that the ETI-associated reduction in bacterial growth induced by *PtoDC3000 (AvrRpm1)* is entirely abrogated in HRH. In Freeman and Beattie (2009), bacterial resistance associated with ETI was inhibited such that bacterial growth induced by *PtoDC3000 (AvrRpm1)* resembled that of *PtoDC3000 (EV)* in high humidity. This indicates that the ability of the host to recognize and respond to the presence of this effector – which, in ambient conditions, is accompanied by a strong HR, high ion leakage values, and 1.5-2 log CFU/cm² reduction in bacterial growth when compared to *PtoDC3000* – is entirely suppressed in high relative humidity.
(Freeman and Beattie, 2009). The data in this chapter does not corroborate this finding, however, it does point out that growth conditions and environmental conditions in which the experiment was conducted may have profound effects on the actual outcome. Further, the data in this chapter demonstrates that HRH, or other abiotic factors, may influence various outputs of ETI differentially. Freeman et al., 2009 also report a high level of PCD induced by \textit{PtoDC3000} (AvrRpm1) in HRH, accompanied by unrestricted bacterial growth levels. These two pieces of data conflict with each other and conflict with the data presented in this chapter. An explanation for these discrepancies most likely lies in the difference in assay set up. The assays used in this chapter and in Freeman et al., 2009 are quite different, as previously mentioned, which indicates that even seemingly negligible differences in assays that are attempting to quantify the same resistance response may provide conflicting evidence.

In order to test the influence of local adaptation on ETI-associated virulence suppression, various \textit{Arabidopsis} ecotypes were tested using spray assays and we identified three ecotypes that displayed compromised ETI at elevated temperature. All ecotypes tested in this study exhibit resistant phenotypes, and exhibit macroscopic HR when infected with \textit{PtoDC3000} (HopZ1a) in ambient conditions (unpublished data, Dr. Jennifer Lewis). Additionally, the ZAR1 protein for several ecotypes tested has been sequenced (unpublished data, Dr. Jennifer Lewis). Curiously, Cvi-0 was previously demonstrated in our lab to exhibit weak to moderate HR when infiltrated with \textit{PtoDC3000} (HopZ1a) (unpublished data, Dr. Jennifer Lewis). However, as previously mentioned, our spray assays indicated that this ecotype is not resistant to HopZ1a in either ambient or elevated temperature conditions. The ZAR1 protein sequence for Cvi-0 has not yet been obtained.
Ecotypes for screening were selected based on geographic location and climate from which they were isolated. CIBC-5 was isolated from Ascot, Berkshire, United Kingdom (51.4084°N, 0.6707°W), Tsu-1 from Tsushima, Japan (34.202°N, 129.29°E) and Wei-0 from Weiningen, Switzerland (47.419°N, 8.4326°E). The Col-0 ecotype which retained ETI-virulence suppression at elevated temperature was isolated from Columbia, Missouri, USA (38.952°N, 92.334°W). Another ecotype, Fab-2, also robustly retained ETI-virulence suppression at elevated temperature. Fab-2 was isolated from Faberget, Sweden (63.016°N, 18.325°E). The CIBC-5 and Wei-0 ecotypes were isolated from locations that would experience cooler average temperatures (highest monthly summer average temperatures are 23° and 24°, respectively [worldweatheronline.org]) than Col-0 (32°), which may explain the lower tolerance of their ETI responses. However, Tsu-1 (33°) was isolated from a location that experiences relatively similar temperatures to Col-0, so local adaptation may not provide the entire explanation for this elevated temperature intolerance.

ZAR1 protein sequence information is available for the ecotypes discussed here (unpublished data, Dr. Jennifer Lewis). The ZAR1 protein sequenced I Fab-2 has two amino acid substitutions compared to that of Col-0 (D557E and H739L). ZAR1 from Wei-0 has five amino acid substitutions compared to that of Col-0 (D557E, I582M, N611X, S618X, H739L), CIBC-5 has none, and Tsu-1 has two (D577E, H739L). It is curious that Tsu-1, an ecotype that loses resistance to HopZ1a in elevated temperature, has the same amino acid substitutions in ZAR1 as that of Fab-2, an ecotype that robustly retains resistance in elevated temperature. CIBC-5 has no amino acid substitutions, in its copy of ZAR1, which is unexpected if we believe different copies of ZAR1 could contribute to destabilization of ETI in elevated temperature. The ZAR1 sequence in Wei-0 shows the highest level of deviation from that of Col-0. Wei-0 and Tsu-1 have two overlapping amino acid substitutions, one of which is D577E – however, Fab-2 also has this
substitution. Aspartic acid (D) and glutamic acid (E) are both charged amino acids, so perhaps this substitution would have negligible functional effects. Wei-0 and Tsu-1 share another amino acid substitution, H739L – this switch from a histidine (H) to a leucine (L) could result in functional changes, as histidine is a polar amino acid that may participate in hydrogen bonding, whereas leucine is a hydrophobic amino acid normally buried inside the protein core. Wei-0 also has an isoleucine (I) substituted for a methionine (M) (I582M), which likely has no significant effects, and also has missing information for two amino acids compared to the Col-0 ZAR1 protein sequence (N611X, S618X). The X either means that a nucleotide base could not be read properly in that codon, and thus the amino acid could not be accurately predicted, or that amino acid is missing from the sequence. It seems that these amino acid substitutions are not particularly informative as far as demonstrating an alteration in the ability of ZAR1 to function in elevated temperature differentially in varying ecotypes. Therefore, the differences observed in the temperature tolerance of their respectively ETI responses is likely influenced by additional factors, and further investigation is necessary. Nevertheless, the ecotypic differences observed in ETI responses at elevated temperature indicate that temperature tolerance is an adapted trait that should be genetically mappable (Bomblies and Weigel, 2007; Huang et al., 2014; Li et al., 2010).

In this study, we have demonstrated that the virulence suppression and HR outputs of ETI are differentially sensitive to high relative humidity or elevated temperature. At either of these experimental conditions, HR is significantly delayed or inhibited depending on the assay method used, whereas immunity, measured as bacterial growth inhibition or disease symptom development, is relatively unaffected. These observations not only emphasize the importance of carefully documented plant growth conditions in studies of plant immunity, but also highlight the differential sensitivity of various immunity outputs to abiotic stresses.
Chapter 4

4 Conclusions and Future Directions

The environmental requirements underlying ETI in Arabidopsis thaliana were examined. Specifically, temperature and humidity were studied in order to understand how abiotic stresses impact the ability of a host to respond to pathogenic invaders. Standard ETI output assays were used to qualify and quantify the effects of elevated temperature and high relative humidity on wildtype Col-0 Arabidopsis. In this study, we have demonstrated that the virulence suppression and HR outputs of ETI are differentially sensitive to temperature and humidity. Macroscopic HR and ion leakage outputs induced by T3SEs that induced strong HR responses in ambient environmental conditions were shown to be either delayed or suppressed by elevated temperature or high relatively humidity. Our results also indicate that elevated temperature or high relative humidity can uncouple the HR response from ETI-associated virulence suppression.

The infiltration of these effectors – PtoDC3000 carrying HopZ1a, AvrRpm1, or AvrRpt2 – into the leaves of Arabidopsis plants induce macroscopic cell death responses indicative of ETI, which is also associated with a reduction in bacterial growth when compared to PtoDC3000 (EV). This in planta reduction in bacterial growth remained intact in both elevated temperature and high relative humidity conditions during our experiments. Further, when Col-0 is spray-infiltrated with high dosages of PtoDC3000 carrying an effector to which it is resistant, it displays a healthy phenotype and continued growth. When sprayed with the effectors used in this study, Col-0 demonstrates this resistant phenotype in ambient environmental conditions. Additionally, this resistant phenotype is also observed in plants spray-infiltrated in elevated temperature and high relative humidity conditions. This is a macroscopic indication that the
bacterial resistance response associated with an effector that induces a strong ETI response remains intact in these abiotic stress environments.

Based on these results, we determined that while the ETI induced by weak effectors may be more susceptible to inhibition by abiotic stressors, the effectors used in this study were able to elicit an ETI response strong enough to withstand that suppressive effects of elevated temperature and high relative humidity independent of one another. Future experiments should be conducted in order to specifically evaluate the additive suppressive effects of these abiotic stress factors on ETI outputs.

Many studies indicate that ETI is modulated by environmental factors via NLR proteins, indicating a molecular basis for temperature modulation. This thesis, alongside many other works, has attempted to characterize the differential effects of abiotic stressors on outputs of ETI, however, studies aimed towards understanding the genetic basis for this immunity modulation are currently lacking. The data presented in this thesis provide novel understanding of this modulation, however many questions still remain unanswered.

As observed in this thesis, T3SEs elicit robust ETI responses in both ambient and elevated temperature or high relative humidity conditions in Col-0 background. This Arabidopsis accession possess all the necessary NLR proteins required to mediate recognition and induce immune signaling pathways to elicit an ETI response in the presence of all T3SEs – HopZ1a, AvrRpt2, AvrRpm1 – tested in this study. In order to determine what genes are required for elevated temperature-tolerant ETI responses, ethyl methanesulfonate (EMS) mutagenesis of Col-0 background seeds may provide a useful tool for probing this question. Using high-throughput disease resistance spray assays described in this thesis, EMS Col-0 seeds could be sprayed with a mixture of all three T3SEs tested in this study in ambient, elevated temperature, and high relative
humidity conditions. In spraying with a mixture of these T3SEs at once, it would be ensured that any mutations that occur in the NLR genes necessary for that particular T3SE’s recognition would be recovered by the presence of the other T3SEs. If a mutant was identified that showed a diseased chlorotic phenotype in either abiotic stress condition – elevated temperature or high relative humidity – when sprayed with this T3SE mixture, then it is possible that a gene implicated in abiotic stress modulation could be uncovered. In a secondary screen, mutants of interest would retain ETI at ambient temperatures.

The ability of the wildtype Arabidopsis host Col-0 to accurately launch an ETI response under elevated temperature conditions lead to the examination of ETI under stress conditions in locally-adapted Arabidopsis ecotypes. In this study, we uncovered three Arabidopsis ecotypes that display compromised ETI in elevated temperatures. It was hypothesized that Col-0 was isolated from a geographical region characterized by humid and excessively warm summer temperatures, and this local adaptation may confer temperature tolerance advantages to this ecotype. If this proves true, then perhaps ecotypes locally-adapted to cold or arid geographical regions may not display this elevated temperature tolerance. It was found that ecotypes Tsu-1, Wei-0, and CIBC-5 demonstrated a susceptible phenotype in elevated temperature upon spray-infiltration with high dosages of PtoDC3000 (HopZ1a), but remained healthy and resistant in ambient temperature conditions. Interestingly, an ecotype from Northern Sweden, Fab-2, displayed a robustly healthy resistant phenotype in both elevated and ambient temperature conditions.

To probe the loss of T3SE-induced resistance in Arabidopsis ecotypes in elevated temperature, several experiments could be conducted. Firstly, ETI responses induced by the remaining T3SEs included in this study – AvrRpt2, AvrRPm1, and AvrRps4 – must be qualified and quantified
using aforementioned assays described in Chapters 2 and 3. In order to confirm whether the loss of resistance in elevated temperature demonstrated by ecotypes Tsu-1, Wei-0, and CIBC-5 corresponds with a quantitative loss in ETI-associated virulence suppression, *in planta* bacterial growth assays must be conducted on these ecotypes. Further, to complete the study, HR must be observed via macroscopic and ion leakage assays in order to compare the resistance responses demonstrated by these ecotypes in elevated temperature as compared to Col-0 and ambient temperature conditions.

It is possible that the loss of resistance demonstrated by these ecotypes in elevated temperature is specific to HopZ1a, or perhaps similar phenotypes in different ecotypes could be specific to other T3SEs. Sequencing of known genes necessary for recognition of the T3SE HopZ1a – such as NLR protein ZAR1, and ETI-associated pseudokinase ZED1 – is necessary in the ecotypes that demonstrate impaired HopZ1a-induced resistance in elevated temperature. If sequence data shows that an ecotype has amino acid substitutions in one or both of these genes that may affect structure and function of the predicted protein of interest, these substitutions could possibly lead to the destabilization and defunctionalisation of these proteins under elevated temperature stress. In this case, immunity mediated by NLRs and other ETI-associated proteins may be compromised in elevated temperature, but remain intact in ambient conditions. To address this question, EMS-mutagenized populations of these ecotypes could be screened using this established spray assay with *PtoDC3000* (HopZ1a) or other T3SEs that elicit strong HRs in both ambient and elevated temperature conditions in order to determine if there is a genetic basis for this elevated temperature tolerance that appears to be influenced by the natural variation of *Arabidopsis*. A mutant of a predicted elevated temperature-tolerant ecotype that shows a disease phenotype, or a mutant of a predicted elevated temperature-intolerant ecotype that shows a resistant phenotype, could potentially provide more information about the genetic requirements
for elevated temperature sensitivity or insensitivity. Further, this screen may shed light on genetic requirements in general for immunity functioning under conditions of various abiotic stressors, including temperature or humidity. However, while it is possible that mutations in NLRs may contribute to elevated temperature tolerance or susceptibility, it is unlikely since we see robust immunity phenotypes at room temperature in our experiments. Structural modelling of the NLR genes found in each of these ecotypes may further provide information about differing abilities to detect T3SEs under conditions of abiotic stress.

Lastly, using tools already available in the lab, the genetic segregation of this phenotype can be tested. All ecotypes used in this study have been crossed to Col-0, and have been carried through and harvested at the F2 generation (crosses done by André Santos-Severino). With these populations, we already have tools available to reveal if this elevated temperature-intolerance is a genetically mappable trait. If there is one genetic factor that contributes to this elevated temperature adaptation in Col-0 and ecotypes that behave like Col-0, then conducting these spray assays on crossed F2 populations will reveal genetic segregation of this gene. In this case, by screening crossed populations using disease resistance spray assays, we can easily screen for genetic segregation phenotypes and determine if temperature intolerance is conferred by a single genetic locus. However, if there are multiple genetic factors, or if perhaps a quantitative trait locus (QTL), is responsible for this adaptation, then the genetic segregation will not be visible at the expected ratios.

Overall, this thesis provides sufficient evidence that various outputs of ETI are differentially influenced by high relative humidity and elevated temperature, substantiating the idea that these abiotic factors uncouple HR from ETI-associated virulence suppression. Furthermore, it provides evidence that corroborates published data that temperature and humidity may have additive
suppressive effects on various pathosystems. The most important implication of this thesis is to further corroborate the findings that temperature and humidity can be viewed as important modulators of plant immunity. Our results reinforce the fact that the conditions in which experimental plants are grown, handled, and assayed profoundly affects the outcomes of plant immunity experiments. Therefore, we must be acutely aware of our growth conditions and experimental designs in order to consider all environmental factors at once when drawing conclusions about the interplay between temperature, humidity, and plant-pathogen interactions.
References


Appendices

A. Identifying the Role of ZED1-Related Kinases (ZRKs) in Plant Immunity

I. Introduction

*Pseudomonas syringae* is a Gram-negative bacterium that infects multiple plant species by manipulating cellular processes via injection of virulence factors into host cells (Cunnac et al., 2009; Hwang et al., 2005). These virulence factors are injected via a needle-like type three secretion system (T3SS), and are known as type three secreted effectors (T3SEs) (Boller et al., 2009; Deslandes and Rivas, 2012; Dodds and Rathjen, 2010; Jones and Dangl, 2006; Lewis et al., 2009). Plants in turn deploy nucleotide-binding leucine-rich repeat (NLR) resistance proteins that recognize specific T3SEs and trigger an immune response, called effector-triggered immunity (ETI) (Jones and Dangl, 2006). ETI is a rapid immune response capable of preventing pathogen spread that often manifests as localized programmed cell death, known as the hypersensitive response (HR) (Coll et al., 2011; Heath, 2000; Jones and Dangl, 2006; Morel and Dangl, 1997).

The YopJ superfamily of T3SEs is a widely-distributed and evolutionarily diverse superfamily of bacterial effectors present in both animal and plant pathogens (Lewis et al., 2008, 2009, 2011; Ma et al., 2006; Zhou et al., 2009). In *P. syringae*, the YopJ superfamily is represented by the HopZ family of T3SEs (Lewis et al., 2008, 2009, 2011; Ma et al., 2006; Zhou et al., 2009). The HopZ family is comprised of three homologs (HopZ1, HopZ2, and HopZ3), with the HopZ1 homolog diversifying into three allelic variants (HopZ1a, HopZ1b, and HopZ1c) (Lewis et al., 2008, 2010, 2011; Ma et al., 2006; Üstün et al., 2014; Zhou et al., 2009, 2011). Recognition of HopZ1a requires indirect interaction with an NLR protein called HopZ-ACTIVATED
RESISTANCE1 (ZAR1) to elicit ETI. In plants lacking ZAR1, HopZ1a reveals an ancestral virulence function to promote bacterial growth (Lewis et al., 2010). Additionally, a unique ETI associated gene called HopZ-ETI-DEFICIENT1 (ZED1) is essential for this ZAR1-mediated immunity (Lewis et al., 2010, 2013).

ZED1 is a non-functional pseudokinase that has been shown to directly interact with both HopZ1a and ZAR1, and is acetylated by HopZ1a. ZED1 belongs to the receptor-like kinase/Pelle (RLK/Pelle) kinase family in plants (Lehti-Shiu et al., 2012, 2012; Lewis et al., 2013). Because ZED1 lacks both a transmembrane 2 domain and an extracellular domain, it belongs to a subset of the RLK/Pelle family called the receptor-like cytoplasmic kinases (RLCKs) (Lehti-Shiu et al., 2012, 2012; Lewis et al., 2013). ZED1 forms a closely-related cluster with 26 additional homologs in Arabidopsis wildtype ecotype Columbia-0 (Col-0), known as the ZED1-related kinases (ZRKs) (Lewis et al., 2013). The ZRKs are part of two distinct RLCK subfamilies: the genomic cluster containing ZED1 belongs to the RLCK subfamily XII-2 and is composed of seven additional homologs, and the remaining ZRKs belong to the RLCK subfamily IXb (Lehti-Shiu et al., 2012, 2012; Lewis et al., 2013).

Current working models support two functions for ZED1 functions based on the observation that both zed1 and zar1 mutant plants are both specifically impaired in the recognition of HopZ1a (Lewis et al., 2010, 2013). Further, HopZ1a’s observed ancestral virulence function is retained in plants lacking ZED1, suggesting it is not a direct virulence target of HopZ1a (Lewis et al., 2013). Together, these imply ZED1 may function as a decoy for related kinases actively involved in immune signaling guarded by ZAR1, with recognition occurring after ZED1 acetylation by HopZ1a (Lewis et al., 2010, 2013, 2014; Roux et al., 2014). Based on sequence similarity, it has been proposed that ZED1 and other ZRKs may play a role in ZAR1-mediated immunity (Lewis
et al., 2010, 2013, 2014; Roux et al., 2014). In particular, it is possible that ZED1 may act as a decoy for other ZRKs, which are possible virulence targets of HopZ1a (Lewis et al., 2010, 2013, 2014; Roux et al., 2014).

Pseudokinases are important in plant immunity, and may possess weak catalytic activity *in planta* or perform a regulatory function despite lacking typical canonical residues necessary for phosphotransfer (Lewis et al., 2010, 2013, 2014; Roux et al., 2014). While ZED1 does not possess kinase activity, some of the ZRKs are predicted to be functional kinases. ZRK10 possesses kinase activity, while ZRK14 appears to be acetylated by HopZ1a *in vitro*, supporting this idea (unpublished data, Dr. B. Hurley, Dr. P. Bastedo). It is thought that ZED1 interacts with ZAR1 to initiate an ETI response, potentially by forming a resistance complex involving one or several ZRK(s) (Lewis et al., 2010, 2013, 2014; Roux et al., 2014).

Initially, the role of ZED1 and ZRK family members in plant immunity was investigated using a reverse genetics approach. Towards this end, both loss-of-function (T-DNA insertion knockout lines) and gain-of-function mutants (overexpression lines) were generated. The role of ZRKs in plant immunity was characterized using *in planta* bacterial growth assays in overexpression lines in Col-0 background. To test if any ZRK family members are targeted by HopZ1a in the absence of ZAR1, overexpression lines were crossed into *zar1-1* mutant background, and are currently available in the F1 generation, but have not yet been tested (crosses done by André Santos-Severino). This project will shed light on whether or not HopZ1a’s virulence response is modified by overexpression of ZRKs in the absence of ZAR1. Conductivity and *in planta* bacterial resistance response assays were used during initial screening to quantify the immunity in homozygous *zrk* T-DNA knockout and homozygous ZRK overexpression lines.
II Materials and Methods

i. zrk T-DNA knockout library

Knockout lines are obtained from the *Arabidopsis* Biological Resource Centre (ABRC), and genotyped to confirm homozygous T-DNA insertion in the gene of interest. Prior to this project, genotyping of part of the library was conducted by both Ph.D. candidate Timothy Lo and Dr. Jennifer Lewis. Lines with a confirmed T-DNA insertion within an exon were prioritized for functional experiments (Table 4). T-DNA knockout lines confirmed thus far as homozygous are summarized in Table 4.

ii. ZRK overexpression in Arabidopsis wildtype Col-0 background

Conditional overexpression lines were created by dipping Col-0 wildtype flowers into *Agrobacterium tumefaciens* carrying a dexamethasone (DEX)-inducible overexpression construct (pMAC14). Cloning of ZRK constructs was conducted by Dr. Jianfeng Zhang. Floral dipping conducted prior to the start of this project was done by both Ph.D. candidate Timothy Lo and Dr. Jianfeng Zhang. Status of overexpression lines for each ZRK is indicated and described in detail in Table 4. Experiments were conducted on homozygous confirmed overexpression lines (Figures 16-18, 20). Growth assays were conducted to expose any immunity-related phenotype in overexpression lines in Col-0 background. Growth assays on lines in *zar1-1* background were not conducted as crossing and dipping were completed, but transgene expression was not confirmed in time. Growth assays were conducted to quantify bacterial growth of two *P. syringae* pathovars – *PtoDC3000* and *Pci0788-9* – in order to observe virulence phenotypes associated with the overexpression of specific ZRKs in confirmed homozygous overexpression lines. Highly virulent *PtoDC3000* is useful to quantify larger scale differences in bacterial growth, while the slower-growing *Pci0788-9* will allow for easier detection of subtle
differences in pathogenesis (*Pci*0788-9 growth assays not shown for T3 growth assays, but discussed for T2 growth assays) (Hwang et al., 2005; Yucel et al., 1993).

iii. **Expression confirmation of overexpression transgenic lines via immunoblot analyses**

T1 seeds from dipped *Arabidopsis* Col-0 plants were harvested and screened on 1mL/L Basta-supplemented soil. Resistant plants were transplanted onto herbicide-free soil containing 1g/L 20-20-20 fertilizer. At approximately 3-4 weeks old, one leaf per resistant plant was clipped and submerged in a 5mL solution of 30µM dexamethasone for three days. Leaves were harvested in liquid nitrogen and stored at -80°C until use. Protein was extracted by grinding frozen leaf tissue in extraction buffer (20mM Tris-Cl pH 8.0, 100mM NaCl, 1mM DTT, and 1.25% Triton). Samples were centrifuged at 6000g for 10-15 minutes at 4°C. 1X SDS loading dye was added to extracts, and samples were boiled at 95-100°C for 5 minutes prior to loading. Samples were resolved on 10-12% SDS-PAGE gels for 1 hour at 150V. After run completion, gels were transferred onto nitrocellulose membranes for blotting. Following transfer, membranes were subject to 1 hour incubation in 1.5-5% either Amersham ECL blocking solution or skim milk protein powder solution at room temperature, followed by either overnight probing at 4°C or 1 hour incubation at room temperature in primary (1°) antibody – in this case, hemagglutinin (α-HA) – at a concentration of 1:10,000 on a bench-top rocker. Blots were washed for a half hour (one 10 minute wash, three 5 minute washes) in a 1X TTBS buffer (1X TBS [50mM Tris-Cl pH 8.0, 150mM NaCl], 0.5% Tween). Following the washes, blots were probed for one hour at room temperature in secondary (2°) antibody – in this case, goat anti-rat (α-GAR) – at a concentration of either 1/10,000 or 1/20,000. Deviations in concentration of 2° were sometimes used to optimize exposure of certain bands. Blots were washed in the same manner as previously described for half hour. After washing was complete, blots were detected using Amersham ECL
advance detection kit and exposed onto x-ray film for size analysis. Plants identified as those expressing the protein of interest were carried forward to the T2 generation.

T2 seeds were harvested from confirmed expressing plant lines, and sowed out onto bialaphos-supplemented 0.5x MS media for segregation counting. Lines determined to have one insert based on ratios were transplanted to 20-20-20 fertilizer (1g/L) supplemented soil and carried through to the T3 for further segregation counting in order to determine which T2 lines were derived from a homozygous population. Growth assays were conducted on both T2 and T3 plants of confirmed homozygosity, however, high rate of gene silencing and low protein expression consistently posed an obstacle.

iv. Ion leakage and *in planta* bacterial growth assays

One day prior to experiments, plants are watered to ensure ease of infiltration. *PtoDC3000* strains carrying empty vector (pUCP20) (Ma et al., 2006), HopZ1a (Ma et al., 2006), or AvrRpt2 (Mudgett and Staskawicz, 1999) were used for ion leakage assay infiltrations. *P. syringae* pv. *tomato* DC3000 (*PtoDC3000*) and *Pseudomonas syringae* pv. *cilantro* 0788-9 (hereafter *Pci0788-9*) were used for *in planta* bacterial growth assay infiltrations (Hwang et al., 2005; Yucel et al., 1993). Plants were sprayed with 30µM dexamethasone prior to infiltration (+DEX) were compared to plants lacking the dexamethasone treatment, which were sprayed with a mock solution (-DEX). Leaves of four-week-old *Arabidopsis* Col-0 plants were syringe-infiltrated with *P. syringae* at OD_{600}=0.04 (2 x 10^7 CFU/mL). For each strain, eight plants were infiltrated – four leaves per each plant, one disk taken from each independent leaf. Immediately after infiltration, four leaf disks (1.5 cm^2) were harvested, washed in sterile distilled H2O for 30 minutes on a bench-top shaker at 250 rpm, and transferred to 6 mL of sterile distilled H2O. Readings were
obtained with an Orion 3 Star benchtop conductivity meter (Thermo Fisher Scientific Inc., Fort Collins, CO, USA). This meter utilizes a 25°C reference temperature.

To determine the role of ZRK overexpression in basal plant immunity, *P. syringae* pathovars *Pto*DC30000 and *Pci*-07889 were used (Hwang et al., 2005; Yucel et al., 1993). Leaves of three to four-week-old *Arabidopsis* Col-0 plants were syringe-infiltrated with *P. syringae* at OD$_{600}$=0.0002 (1 x 10$^5$ CFU/mL). Plants were sprayed with 30µM dexamethasone prior to infiltration (+DEX) were compared to plants lacking the dexamethasone treatment, which were sprayed with a mock solution (-DEX). For T$_2$ growth assays, twelve plants were initially infiltrated on Day 0 – four leaves per plant, and, therefore, one leaf disk is taken from each independent leaf. For Day 0 quantification, four disks (1 cm$^2$) per plant were harvested approximately 1 hour after infiltration, ground in 1mL 10mM MgCl$_2$, and plated on KB with rifampicin (50µg/mL) and cycloheximide (50µg/mL) for colony quantification. On average, two to three plants were used for Day 0 quantification. On Day 3, four disks (1 cm$^2$) per plant were harvested, ground in 1mL 10mM MgCl$_2$, and plated on KB with rifampicin (50µg/mL) and cycloheximide (50µg/mL) for colony quantification. Nine plants were used for Day 3 quantification.

For growth assays conducted on T$_3$ plants, growth assays varied slightly from the method described above. Instead of Day 3 quantification, leaf tissue was harvested on Day 2, due to application of DEX, which caused leaves to become very wilted and difficult to work with by Day 3. Due to high rates of silencing and low levels of detectable protein expression, the sample size of individual +DEX plants used for Day 2 quantification was increased to 26, while on Day 0 only two plants were used. Two plants treated with the mock solution (-DEX) were used on Day 0, followed by another two plants for Day 2 quantification. This was done in order to
increase proportion of plants that were reliably expressing the protein of interest. Only data derived from plants that showed detectable protein expression determined via immunoblotting were used for bacterial growth quantification. Growth assays described were conducted using established methods (Lewis et al., 2008).

In order to test expression of ZRK of interest in plants used for growth assays, after leaves were harvested for Day 2 bacterial growth quantification, whole plants were weighed, harvested in liquid nitrogen, and stored at -80°C until use. Using a frozen mortar and pestle, whole plants were ground and extraction buffer (20mM Tris-Cl pH 8.0, 100mM NaCl, 1mM DTT, and 1.25% Triton) was added to a proportion of 1g fresh weight: 2mL extraction buffer. Samples were centrifuged at 6000g for 10-15 minutes at 4°C. 1X SDS loading dye was added to extracts, and samples were boiled at 95-100°C for 5 minutes prior to loading. Samples were resolved on 8-10% SDS-PAGE gels for 1 hour at 150V. Blotting and exposing conducted as previously described.

N.B. Summary of all zrk T-DNA KO and ZRK OX lines currently available and experiments conducted thus far summarized in Table 4.
Table 4. Summary of the status of zrk T-DNA knockout (KO) lines and ZRK overexpression (OX) lines in Col-0 background.

<table>
<thead>
<tr>
<th>ZRK #</th>
<th>in vitro or in planta Features</th>
<th>Current Status OX (Expression Confirmed)</th>
<th>Current Status KO (Genotype Confirmed)</th>
<th>T2 Growth Assay</th>
<th>T3 Growth Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZED1</td>
<td>Required for HopZ1a recognition in A. thaliana</td>
<td>Tested; none expressing</td>
<td>1 homozygous exon lines; several point mutation lines</td>
<td>Line #14-1 – PtoDC3000 showed reduced growth in +DEX treatment, significant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xc resistance (RKS1), strong interactor with HopZ1a via Y2H</td>
<td>1 confirmed homozygous OX line</td>
<td>2 homozygous lines (exon, intron)</td>
<td>Not tested</td>
<td>Not tested</td>
</tr>
<tr>
<td>ZRK1</td>
<td>Tested; none expressing</td>
<td>2 homozygous lines (exon, UTR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZRK2</td>
<td>Tested; none expressing</td>
<td>2 homozygous lines (exon)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZRK3</td>
<td>Tested; none expressing</td>
<td>2 homozygous lines (exon)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZRK4</td>
<td>Tested; none expressing</td>
<td></td>
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</tr>
<tr>
<td>ZRK6</td>
<td>Altered activation loop</td>
<td>Tested; none expressing</td>
<td>1 homozygous line (exon)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZRK7</td>
<td>Missing a C-terminal domain</td>
<td>Tested; none expressing</td>
<td>1 homozygous line (UTR)</td>
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<td></td>
</tr>
<tr>
<td>ZRK10</td>
<td>In vitro phosphorylation activity; putative in vitro acetylation sites detected</td>
<td>Tested; none expressing</td>
<td>1 homozygous line (UTR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZRK11</td>
<td>5 confirmed</td>
<td>1 homozygous line</td>
<td>Line #1 – PtoDC3000 no</td>
<td>Line #7-3 – PtoDC3000 reduced</td>
<td></td>
</tr>
<tr>
<td>ZRK12</td>
<td>Need to be dipped</td>
<td>2 homozygous lines (exon)</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>-------</td>
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<td>--------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZRK13</td>
<td>Shows differential expression patterns under biotic stress relative to other ZRK family members (BAR)</td>
<td>Need to be dipped</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZRK14</td>
<td>Putative <em>in vitro</em> acetylation sites detected</td>
<td>Need to be dipped</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZRK15</td>
<td>Need to be dipped</td>
<td>2 homozygous lines (exon)</td>
<td></td>
<td></td>
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<tr>
<td>ZRK16</td>
<td>2 confirmed homozygous OX lines</td>
<td>Need to be dipped</td>
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</table>

homozygous OX lines (exon) difference in growth; *Pci*0788-9 showed reduced growth in +DEX treatment, NOT significant Line #7 – DC3000 no significant differences in growth; *Pci*0788-9 showed significant increase in growth in +DEX treatment growth in + DEX treatment, significant Line #7-1 – *Pto*DC3000 reduced growth in +DEX treatment, not significant Line #15-1 – *Pto*DC3000 reduced growth in +DEX treatment, significant Line #7-4 – *Pto*DC3000 reduced growth in +DEX treatment, not significant Line #14-4 – *Pto*DC3000 showed reduced growth in +DEX treatment, significant

ZRK12
Need to be dipped
2 homozygous lines (exon)

ZRK13
Shows differential expression patterns under biotic stress relative to other ZRK family members (BAR)

ZRK14
Putative *in vitro* acetylation sites detected

ZRK15
Need to be dipped
2 homozygous lines (exon)

ZRK16
2 confirmed homozygous OX lines

Line #2 – No significant differences in growth in either *Pto*DC3000 or *Pci*0788 Line #9 – *Pto*DC3000 showed no significant differences in growth; *Pci*0788 showed significant
<table>
<thead>
<tr>
<th>ZRK17</th>
<th>Tested; none expressing</th>
<th>2 homozygous lines (exon)</th>
<th>increase in growth in +DEX treatment</th>
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<tr>
<td>ZRK18</td>
<td>5 confirmed homozygous OX lines</td>
<td>2 homozygous lines (exon, UTR)</td>
<td>Line #4 – <em>PtoDC3000</em> showed significant increase in growth in +DEX treatment; <em>Pci0788</em> showed no significant differences in growth Line #6 – <em>PtoDC3000</em> showed no significant differences in growth; <em>Pci0788</em> showed significant increase in growth in +DEX treatment</td>
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<td>ZRK19</td>
<td>2 confirmed homozygous OX lines</td>
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<td></td>
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<tr>
<td>ZRK20</td>
<td>Strongest interactor with HopZ1a via Y2H</td>
<td>Tested; none expressing</td>
<td></td>
</tr>
<tr>
<td>ZRK21</td>
<td>Tested; none expressing</td>
<td>2 homozygous lines (exon)</td>
<td></td>
</tr>
<tr>
<td>ZRK22</td>
<td>Tested; none expressing</td>
<td>1 homozygous line (intron)</td>
<td></td>
</tr>
<tr>
<td>ZRK23</td>
<td>Need to be dipped</td>
<td>2 homozygous lines (exon)</td>
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<tr>
<td>ZRK24</td>
<td>Need to be dipped</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZRK25</td>
<td>Tested; none expressing</td>
<td>1 homozygous line (UTR)</td>
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<td>ZRK26</td>
<td>Need to be dipped</td>
<td>4 homozygous lines (exon, intron, promoter, UTR)</td>
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<td>ZRK27</td>
<td>Additional domain at</td>
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<td>ZRK28</td>
<td>N-terminal end; potentially solubilizes protein</td>
<td>expressing</td>
<td>3 confirmed homozygous OX lines</td>
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</tr>
<tr>
<td>ZRK29</td>
<td>Need to be dipped</td>
<td>1 homozygous line (exon)</td>
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III Results and Discussion

v. zrk T-DNA knockout lines

Initial conductivity screening was conducted on confirmed homozygous knockout lines as they became available. Preliminary assays showed no significant differences between HopZ1a-induced ETI in knockout lines compared to Col-0. Several lines tested to date have T-DNA insertions located in a promoter or untranslated region (UTR) (data not shown). These lines show no significant difference in HopZ1a ETI phenotypes (data not shown). It is possible that T-DNA insertion was not sufficient to disrupt gene function or expression. Further, exon localized T-DNA lines tested thus far fail to show significant differences in HopZ1a-induced ETI relative to Col-0 (data not shown). This is most likely due to the fact that Col-0 possesses ZAR1 and ZED1, both required for recognition of HopZ1a. With these two proteins present, perhaps disrupting a possible virulence target is simply insufficient to affect the avirulence response induced by HopZ1a.

Because this family of pseudokinases is closely related, functional redundancy within the ZRKs could mask the phenotype exhibited by a single ZRK knockout. In this case, employing RNA interference (RNAi) or CRISPR/Cas9 technology to disrupt expression of function of several ZRKs at once could provide more information about these family members working in concert during immune response initiation and effector recognition. Furthermore, zrk T-DNA knockout lines or RNAi lines in zar1 or zed1 knockout backgrounds would be incredibly useful in understanding the role of one or multiple ZRKs in the specific recognition of HopZ1a.
vi. Overexpression of ZRK1 and ZRK11 reduce *P. syringae* pv. *tomato* DC3000 growth, but roles in plant immunity require further investigation

Initial growth assay screening was conducted on T₂ overexpression lines (data not shown). Several ZRK T₂ overexpression lines showed inconsistent data among individual lines of the same overexpressor. DEX-induced plants for both ZRK11 line #1 and ZRK16 line #2 both showed no significant difference in bacterial growth of either *Pto*DC3000 or *Pci*0788-9. One replicate of *in planta* bacterial growth assay has been conducted for ZRK11 line #1, with confounding results. In the repeated experiment, ZRK11 line #1 showed significantly decreased growth in *Pto*DC3000 in DEX-treated plants compared to untreated plants. Of the remaining lines, ZRK11 line #7, ZRK16 line #9, and ZRK19 line #6 showed a decrease in *Pci*0788-9 growth in uninduced plants relative to DEX-treated plants. These lines did not show significant differences in *Pto*DC3000 growth. Two replicate experiments of the ZRK11 line #7 growth assay were conducted, once showing a significant decrease in *Pto*DC3000 growth in untreated plants, while the other replicate showed a significant decrease in *Pci*0788-9 growth in DEX-treated plants. ZRK19 line #4 showed a significant decrease in *Pto*DC3000 growth in untreated plants compared to DEX-treated plants, and did not show differences in *Pci*0788-9 growth *in planta*.

A secondary set of growth assays was completed on T₃ generation plants (Figures 16-18, 20). Of the five confirmed ZRK overexpression lines – ZRK1, ZRK11, ZRK16, and ZRK19 – ZRK11 showed the lowest gene silencing, the highest rate of detectable protein expression, and the largest number of confirmed independent homozygous single-insertion lines. Because there were several lines with relatively reliable protein expression, screening began with independent lines for ZRK11. On average, for each growth assay, approximately 60-66% of ZRK11
overexpressing individuals exhibited detectable protein expression via immunoblot that were associated with a consistent growth assay phenotype when infiltrated with P. syringae DC3000 (blot not shown). ZRK11 line #7-3 showed a significant reduction in bacterial growth of PtoDC3000 without the removal of individuals that did not show detectable protein expression (Figure 18). Combined with expression analyses – that is, when considering individuals that are expressing rather than those that are not – the statistical significance of this reduction was strengthened. DEX-induced individuals that are not expression (+DEX, not expressing) were used as an internal positive control. If these individuals are not expressing the protein of interest, and if the protein of interest is biologically linked to the reduction in bacterial growth phenotype, then the bacterial growth for +DEX, not expressing individuals should resemble that of uninduced (-DEX) individuals.

Figure 18. In planta bacterial growth of PtoDC3000 is reduced in DEX-induced ZRK11 overexpression lines compared to uninduced lines in wildtype Arabidopsis Col-0 background. DEX-induced (+DEX) and uninduced (-DEX) ZRK11 Line #7-3 leaves were syringe-infiltrated with PtoDC3000 at 1 x 10^5 CFU/mL, and bacterial counts were determined one hour post-infection (0 dpi) and 3 days post-infection (3 dpi). Two-tailed homoschedastic t-tests were performed to test for significant differences, indicated by an asterisk (*p-value < 0.01). Error bars indicate the standard deviation from the mean of (A) 12 individuals for uninduced and induced individuals or (B) 12 individuals for uninduced and 8 individuals for
induced/expressing, and 4 individuals induced/not expressing. Assay presented here conducted once.

ZRK line 11 #7-1 showed a reduction in bacterial growth of *PtoDC3000* that was not significant with and without inclusion of individuals for which protein expression was detected (Figure 19). Without taking into account individuals that protein expression could be detected, ZRK11 lines #15-1 and #14-4 showed a significant reduction in bacterial growth of *PtoDC3000*, whereas ZRK11 line #7-4 showed a reduction in bacterial growth that was not significant (Figure 20).

![Figure 19](image.png)

**Figure 19.** *In planta* bacterial growth of *PtoDC3000* is reduced in DEX-induced ZRK11 overexpression lines compared to uninduced lines in wildtype *Arabidopsis* Col-0 background. DEX-induced (+DEX) and uninduced (-DEX) ZRK11 Line #7-1 leaves were syringe-infiltrated with *PtoDC3000* at 1 x 10^5 CFU/mL, and bacterial counts were determined one hour post-infection (0 dpi) and 3 days post-infection (3 dpi). Two-tailed homoscedastic t-tests were performed to test for significant differences, indicated by an asterisk (*p*-value < 0.01). Error bars indicate the standard deviation from the mean of (A) 12 individuals for uninduced and induced individuals or (B) 12 individuals for uninduced and 8 individuals for induced/expressing, and 4 individuals induced/not expressing. Assay presented here conducted once.
**Figure 20.** *In planta* bacterial growth of *PtoDC3000* is reduced in DEX-induced ZRK11 overexpression lines compared to uninduced lines in wildtype *Arabidopsis* Col-0 background. DEX-induced (+DEX) and uninduced (-DEX) ZRK11 Lines #15-1, #7-4, and #14-4 leaves were syringe-infiltrated with *PtoDC3000* at $1 \times 10^5$ CFU/mL, and bacterial counts were determined one hour post-infection (0 dpi) and 3 days post-infection (3 dpi). Two-tailed homoschedastic t-tests were performed to test for significant differences, indicated by an asterisk (*$p$-value < 0.01). Error bars indicate the standard deviation from the mean of either treatment on 0 dpi, and 12 individuals of either treatment on 3pi; (B, C) 2 individuals of either treatment on 0 dpi, and 2 –DEX individuals and 28 +DEX individuals on 3 dpi.

Due to the reduction in *PtoDC3000* growth observed when ZRK11 is overexpressed, the *zrk11* T-DNA knockout line, T910 #1, was re-evaluated (Figure 21). This assay showed that AvrRpt2-induced ion leakage is not significantly affected by loss of ZRK11. However, ion leakage induced by HopZ1a significantly increased in *zrk11* knockout plants after 16.5 h. This could potentially mean that the loss of ZRK11 increased the strength of the avirulence response. If ZRK11 is a potential virulence target of HopZ1a, the removal of the target could allow more HopZ1a to be recognized by the ZAR1/ZED1 immune complex, rather than ever making contact with a virulence target. This assay was only performed once and needs to be repeated under the current optimized environmental conditions.
Figure 21. Quantification of avirulent ion leakage in T-DNA exon knockout zrk11 line in comparison to wildtype Arabidopsis Col-0. Wildtype Col-0 and T-DNA knockout zrk11 in Col-0 background leaves were syringe-infiltrated at 2 x 10^7 CFU/mL with PtoDC3000 expressing empty vector (EV), HopZ1a, or AvrRpt2. Infiltration followed immediately by harvesting four leaf disks per plant (one disk per leaf, total leaf tissue 1.5cm^2) and washing in sterile distilled H2O for 30 minutes on a bench-top shaker at 250 rpm. Disks were transferred to 6 mL of sterile distilled H2O and readings were obtained until 21.25 hpi. Two-tailed homoschedastic t-tests were performed to test for significant differences compared to PtoDC3000 (EV) in either Col-0 or zrk11 background, indicated by an asterisk (*p-value < 0.01). Assay presented here performed once.

Only one independent T2 homozygous confirmed line overexpressing ZRK1 was available (ZRK #14), and various T3 propagated from this original line were tested using growth assays. ZRK1 #14-1 and ZRK1 #14-6 both showed significant reductions in bacterial growth of PtoDC3000 without taking into account which individuals were expressing the protein of interest or not (Figure 22). ZRK1 #14-2 showed a reduction in bacterial growth of PtoDC3000 that was not significant (Figure 22). No protein could be detected in samples tested for ZRK1 #14-1, however 83% of individuals tested showed detectable protein expression for ZRK1 #14-6 (blot not shown).
In planta bacterial growth of *PtoDC3000* is reduced in DEX-induced ZRK1 overexpression lines compared to uninduced lines in wildtype *Arabidopsis* Col-0 background. DEX-induced (+DEX) and uninduced (-DEX) ZRK1 Lines #14-1, #4-2, and #14-6 leaves were syringe-infiltrated with *PtoDC3000* at $1 \times 10^5$ CFU/mL, and bacterial counts were determined one hour post-infection (0 dpi) and 3 days post-infection (dpi). Two-tailed homoschedastic t-tests were performed to test for significant differences, indicated by an asterisk (*P<0.01). Error bars indicate the standard deviation from the mean of (A, B) 3 individuals for either treatment on 0 dpi, and 12 individuals for either -DEX or +DEX treatments on 3 dpi; (C) 4 individuals for either treatment on 0 dpi, and 37 +DEX individuals, and 4 -DEX individuals on 3 dpi.

Growth assay data showing insignificant or inconsistent phenotypes could be the result of several factors. Firstly, insignificant growth differences could indicate that these particular ZRKs do not play a role in plant immunity in general, or perhaps differences in bacterial growth *in planta* are simply too minute to detect or replicate. Conducting these experiments on ZRK overexpression lines in a *zar1-1* background provide information about the roles of ZRK family members in HopZ1a pathogenesis in the absence of the NLR gene necessary to launch an ETI. Secondly, inconsistent data could be the result of gene silencing at the T2 generation. If there is a high rate of gene silencing, it is possible that the immune-related phenotypes exhibited by plants expressing the protein could be masked if only a small number of individuals in the T2 or T3 populations are expressing the protein of interest.
Conductivity assays were used to quantify the ETI response in homozygous overexpression lines in plants infected with \textit{P. syringae PtoDC3000} expressing HopZ1a. It was initially hypothesized that one or multiple ZRKs may play the role of virulence target to HopZ1a, and if that target is overexpressed, then an enhanced avirulence response – observed as increased ion leakage – would be observed. However, this original hypothesis did not take into account the fact that these ZRK overexpression lines are in wildtype Col-0 background – meaning that ZAR1, responsible for HopZ1a’s recognition – is present. Perhaps this would mean that a slightly enhanced avirulence phenotype of an effector that already induces a very strong ETI response would be difficult to detect with the ion leakage assay being employed in this project. For these assays, plants used in conductivity assays were induced with 30\(\mu\)M DEX 24 hours prior to infection, and are compared to plants that have not been DEX-treated. All plants used in these experiments were infected with \textit{P. syringae PtoDC3000} expressing AvrRpt2 as a positive control, as well as empty vector as a negative control. Experiments conducted to date show no significant differences between DEX-treated and untreated plants.

**IV Conclusions and Future Directions**

In conclusion, the experiments conducted thus far have few biological implications but strengthen the argument of the ZRKs playing a role in plant immunity. The main objective in embarking on this project was not only to gather biological information, but to build a set of tools through which initiation of immune responses and effector recognition could be further studied. There are many examples in the literature recently that show plant proteins can act as virulence targets or immunity hubs, and this family of ZED1-related kinases is a potential family that could play an important role in HopZ1a recognition, and potentially plant immunity in
general. Furthermore, the “decoy model” is garnering increasing support, and studies such as this will impact our understanding of plant immune complexes that initiate ETI.

From these findings, we may postulate a potential role for ZRK1 and ZRK11 in plant immunity in general based on growth assay data. However, in the field of plant pathology in recent years, overexpression in an artificial system is becoming less and less favourable. In order to put weight behind such a conclusion, knockout lines of these ZRKs must be characterized in order to demonstrate a loss of this phenotype. Additionally, in order to ascribe any biological function to these ZRKs, we must show some sort of enzymatic or active function in planta. Specifically, it would be interesting to continue these studies of the immunity role of ZRKs 1 and 11 in a zar1 mutant background. As previously, mentioned whenever ZAR1 is present, HopZ1a will be readily recognized and an immune response will be launched. Therefore, in studying overexpression or knockout or knockdown of targeted ZRKs in the absence of ZAR1, we can acquire more detailed information about the role these family members play in the recognition of this effector. As indicated, homozygous confirmed overexpression lines of ZRK1, 11, 16, and 19 have been crossed into the zar1-1 background, and will require genotyping and expression testing to determine both the presence of the T-DNA transgene knocking out ZAR1 and the proper overexpression of the ZRK of interest. Building upon this toolbox of overexpression and knockout or knockdown lines available will allow us to characterize the true biological function of these family members in planta, and allow us to definitively ascribe any immunity-related role to these ZRKs.

Lastly, it is important to note that one of the most important aims of this project was tool building. Based on high rates of gene silencing and low detectable expression rates, the overexpression lines are cumbersome to work with and may not be the most suitable system to
study the role of the ZRKs. It has been suggested that the pMAC14 DEX-inducible vector may have unforeseen issues due to the fact that it has been made Gateway compatible. This may have interfered with the ability of this vector to express upon DEX induction, or, rather, express at a detectable level. Because of this, it would be difficult to conduct proteomic analysis with a system that is unreliable and difficult to work with. In addition, there are concerns with the knockout lines as well. As mentioned, perhaps creating RNAi lines will allow these ZRKs to be more informative and efficient to work with. Overall, there are certainly a set of tools available to study the ZRKs at this point, but further expanding would be both necessary and informative.

The implication of specific plant proteins as hubs of immunity unlocks information about the immune complexes necessary for effector recognition. In furthering our knowledge of such systems, we can better understand the genetic and proteomic requirements for effector recognition. Additionally, studies such as these allow us to broaden our models of understanding in order to fit the biological processes that are actually occurring in planta. For some time now, it has become relatively known that the “guard model” – which indicates that the R protein is directly contacted by the effector and thus switches into an “on” position initiates ETI – is probably not the most accurate, but something more like the “decoy model” or an “integrated decoy model” involving paired R proteins may be more accurately representative of the signaling events leading to effector recognition and thus ETI (Cesari et al., 2014). It is important to continue clarifying the events leading up to effector recognition and after it occurs in order to understand the mechanism of plant immunity from the broadest, most accurate, and most holistic point of view possible.