

DISSERTATION

LEVERAGING *PSEUDOMONAS SYRINGAE* TYPE III EFFECTORS TO UNDERSTAND
THE GENETIC REQUIREMENTS FOR DISEASE RESISTANCE IN ARABIDOPSIS

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ABSTRACT

LEVERAGING *PSEUDOMONAS SYRINGAE* TYPE III EFFECTORS TO UNDERSTAND THE GENETIC REQUIREMENTS FOR DISEASE RESISTANCE IN ARABIDOPSIS

Plants employ a sophisticated immune system to recognize and respond to infecting pathogens. One of the most well-studied aspects of this immune system is the intracellular nucleotide-binding site leucine-rich repeat (NLR) receptor family. These proteins mediate pathogen perception by recognizing pathogen type III effector proteins (effectors) in the plant cell. Hundreds of NLR-cognate effector pairs have been characterized, yet much less work has been devoted to understanding 1) what effectors do in plant cells to subvert immune responses and 2) how individual NLRs work to signal immune responses in the context of the broader plant immune system.

Here, I investigated the potential targeting of subfamily VII receptor-like cytoplasmic kinases (RLCK VIIs) in Arabidopsis and *Nicotiana benthamiana* by the *Pseudomonas syringae* effector, HopBA1. This work demonstrated that loss of function mutants in individual subgroups of RLCK VIIs in Arabidopsis polymutants were not sufficient to abolish HopBA1 recognition by the Arabidopsis immune system. I found that members of subgroup 6 of the *NbRLCK* VII family are apparently cleaved upon transient co-expression with HopBA1 in *N. benthamiana*. These data suggest that HopBA1 may act as a protease upon *NbRLCK* VII substrates, a previously undescribed enzymatic function for HopBA1.

Through study of HopBA1 recognition by two NLRs in Ag-0 Arabidopsis, Response to HopBA1 (RBA1) and HopZ-Activated Resistance 1 (ZAR1), I characterize an unequal partnership in initiating cell death and growth restriction responses by distinct classes of plant immune receptor. This work also revealed a partial dependence of ZAR1 upon the canonical Toll-interleukin receptor (TIR)-NLR immune response regulator, ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1). I followed this observation with a genetic dissection of ZAR1-mediated responses to other pathogen effectors in Col-0. I found that ZAR1 generally requires EDS1 and the PHYTOALEXIN DEFICIENT 4 (PAD4) -ACTIVATED DISEASE RESISTANCE 1 (ADR1) signaling node to varying degrees to initiate immune responses, depending on the effector used to activate ZAR1.

Taken together, the work presented here helps to fill some of the major gaps in understanding that exist within the field of molecular plant-pathology. I took steps to understand the particular enzymatic functions of HopBA1, which lays a foundation to better understand how effectors work to support pathogen colonization of their plant hosts. The finding that ZAR1 requires EDS1 to varying degrees to initiate complete immune responses in Arabidopsis represents a departure from the published work on ZAR1-mediated immune responses. These data support a more holistically integrated model for plant NLR-mediated immune responses and emphasize the importance of considering the whole immune system when describing signaling events initiated by a particular NLR.

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CHAPTER 1: INTRODUCTION¹

1.1 History of plant pathology

Plants are critical pieces of nearly every ecosystem on Earth, and the progression of human society is intimately linked with our collective cultivation of plants and their products. Since the development of agriculture by early humans ~12,000 years ago (Carey 2023), plant domestication and breeding has provided nutritional stability to the human race which spurred a string of advancements that can be directly traced back to those first innovations in plant husbandry. Some of humanity's most important steps towards grasping genetics and inheritance can be seen in Gregor Mendel's experiments on pea plants in the mid-19th century and Darwin's observations of both plant and animal trait inheritance around the same time. In more applied settings, the reliance of humans on plants for their agricultural and medicinal products has built an intuitive understanding of plant health amongst our civilizations that is evident in some of the earliest pieces of recorded human history (Yuen, Djurle, and Collinge 2020). Commensurate with humanity's reliance on plant agriculture, the impacts of plant pathogens on agricultural efforts have also shaped our history. Events like the Irish Potato Famine have shaped human history and will continue to do so as plant agricultural outputs remain essential (Goss et al. 2014; Mccook and Vandermeer 2015).

Despite having numerous recorded observations of plant disease throughout early human history, it was not until the scientific revolutions of the 18th and 19th centuries that the causative agents of these diseases were understood to be separate living entities that exploit plants for nutrients. In the early 20th century, H. H. Flor's study of the rust disease of flax led to the development of the gene-for-gene model for interactions between plants and disease-causing

pathogens (Flor 1971). The gene-for-gene model posits that plants which are resistant to a pathogen harbor a gene whose product allows for recognition of a cognate gene product encoded by the pathogen. The pathogen gene is referred to as an avirulence gene for its role in impairing pathogen growth on plants which harbor a cognate resistance or “*R*” gene. This seminal theory laid the foundation for the molecular understanding of plant-microbe interactions (Peter N. Dodds 2023). Elucidation of the molecular structure of DNA and progression into the molecular era of biology in the mid-20th century has largely supported the gene-for-gene hypothesis put forth by Flor but has also revealed a staggering complexity inherent to all biological systems. The first avirulence gene was cloned in 1984 from *Pseudomonas syringae* pv. *glycinea*, providing concrete support for one half of the gene-for-gene hypothesis (Staskawicz, Dahlbeck, and Keen 1984). The other half of the gene-for-gene hypothesis, the *R* gene, was first cloned 8 years later from maize in the form of the *HMI* disease resistance gene which confers resistance to the pathogenic fungus, *Cochliobolus carbonum* race 1 (Johal and Briggs 1992). Work primarily in the model plant *Arabidopsis thaliana* (hereafter Arabidopsis) involving continual cloning of disease resistance and pathogen recognition determinants from both plants and pathogens since the 1990s has built a more robust model of the plant immune system that can be broadly arranged into two tiers which serve to recognize and respond to pathogens.

1.2 The two-tiered model of the plant immune system

Plant cells first recognize conserved components of microbial pathogens (Pathogen/Microbe Associated Molecular Patterns (PAMPS/MAMPS)) via cell surface receptors that initiate a resistance response sufficient to restrict the growth of non-adapted pathogens. This first stage of the plant immune response is known as pattern triggered immunity (PTI). Adapted pathogens, however, will inject virulence proteins into the plant cell via a haustorium (in the case

of oomycetes and fungi) or via a type III secretion system (in bacteria). These virulence proteins, also called effectors, function to disrupt a wide range of signaling processes in the plant cell to alter its capacity to restrict pathogen growth and to adjust host physiology to make the environment more favorable for pathogen growth and proliferation. The increased pathogen growth afforded by effector activities is known generally as effector triggered susceptibility (ETS). In the case of incompatible gene-for-gene interactions as described by Flor, plants recognize effectors via intracellular immune receptors in the plant. Intracellular immune receptors, especially those of the nucleotide binding leucine rich repeat receptor (NLR) class, initiate a strong immune response typified by localized cell death, known as the hypersensitive response (HR). This second phase of the immune response is known as effector triggered immunity (ETI). Collectively, the progression of disease stages through these events is summarized in the “zig-zag model” for plant immunity, where plants first mount a PTI response, which is suppressed, then mount ETI responses, effectively leading to a “zig-zagging” of disease resistance up and down over the course of infection (Figure 1.1A) (Jones, J.D.G. 2006; Jones, Staskawicz, and Dangl 2024). With continued improvements of genetic tools and experimental techniques to study plant immunity, the field has come to appreciate a more holistic view of the immune responses initiated by components that are part of PTI and ETI, with immunity being understood as a network of interdependent signaling components that are activated simultaneously and continuously throughout plant infection by a pathogen. While the two-tiered view of the immune system is perhaps less representative of biological reality than previously thought, this distinction between PTI and ETI signaling components is still a useful way to divide the immune system for discussions.

1.3 Pattern triggered immunity

Plants first perceive contact with a pathogen via cell surface immune receptors, membrane-spanning modular proteins with extracellular sensor domains such as leucine-rich repeats (LRRs). These pattern recognition receptors (PRRs) recognize conserved PAMPs, for instance, fragments of bacterial flagella (Ngou, Ding, and Jones 2022). PRRs have diversified significantly as a result of the plant-pathogen molecular arms race; the Arabidopsis genome encodes hundreds of these receptors, demonstrating the amount of diversity generated as a result of plant-pathogen competition over evolutionary time (Fischer, Droc, and Chantret 2016; Shiu et al. 2004; Shiu and Bleecker 2003). PRRs typically also cooperate with a co-receptor carrying a truncated extracellular domain during PAMP binding. The most well-studied of these is BAK1, which cooperatively signals with many PRR receptors to initiate PTI responses in Arabidopsis (Heese et al. 2007; Jia Li et al. 2002; Mühlenbeck et al. 2024; Roux et al. 2011; Wu et al. 2020; Yuan et al. 2021). Interestingly, BAK1 has been found to form complexes with cell surface receptors involved in developmental signaling as well as with PTI-related PRRs. The dual roles of BAK1 are illustrative of the complex balancing act between maintenance of physiological signaling pathways and regulation of immunity that plants are required to reckon with as they contend with developmental and environmental stimuli (Wu et al. 2025).

Following PAMP binding by the extracellular domains of PRRs and their co-receptors, the intracellular domains of these proteins begin to signal with other intracellular kinases to initiate a signaling cascade that results in a variety of cellular responses. Work primarily conducted in Arabidopsis has shown that members of the receptor-like cytoplasmic kinase subfamily VII (RLCK VII) or PBS1-like kinases (PBLs) are some of the most relevant substrates of PRR kinases domains during PTI responses (Boutrot and Zipfel 2017; Defalco and Zipfel 2021; B. Song et al. 2026; Hongze Wang et al. 2026).

The RLCK VII family is quite large in many plant species, with 46 RLCK VIIs in *Arabidopsis*, and is further expanded in *Nicotiana benthamiana* and tomato (Hailemariam, Liao, and Mengiste 2024; W. R. H. Huang et al. 2023). Study of the PBL kinase, BIK1, and other RLCK VIIs has shown that these kinases orchestrate a series of phosphorylation events that fine-tune the PTI response against a given elicitor (Bai et al. 2023; Baudin et al. 2021; X. Ma et al. 2020; Rao et al. 2018; Swiderski and Innes 2001; W. Tian et al. 2019). Characteristic PTI responses include production of reactive oxygen species (ROS), callose synthesis and deposition at infection sites, production of antimicrobial compounds, nutrient scavenging from the plant apoplast, defense hormone production, and widespread transcriptional reprogramming. Many of these responses have been directly linked to RLCK VIIs, which have been shown to modulate the activities of the proteins which carry out these responses via phosphorylation of regulatory sites (Defalco and Zipfel 2021).

Together, these responses are sufficient to restrict the growth of non-adapted pathogens. In fact, recent advancements in techniques to understand these early responses in more detail have placed additional emphasis on the importance of PTI-induced transcriptional reprogramming for plant immune responses beyond early PTI signaling events (Albert et al. 2020; Pruitt et al. 2021; Yuan et al. 2021). This reframing of PTI responses has led to a conceptual framework for plant immunity where PTI responses represent the primary suite of

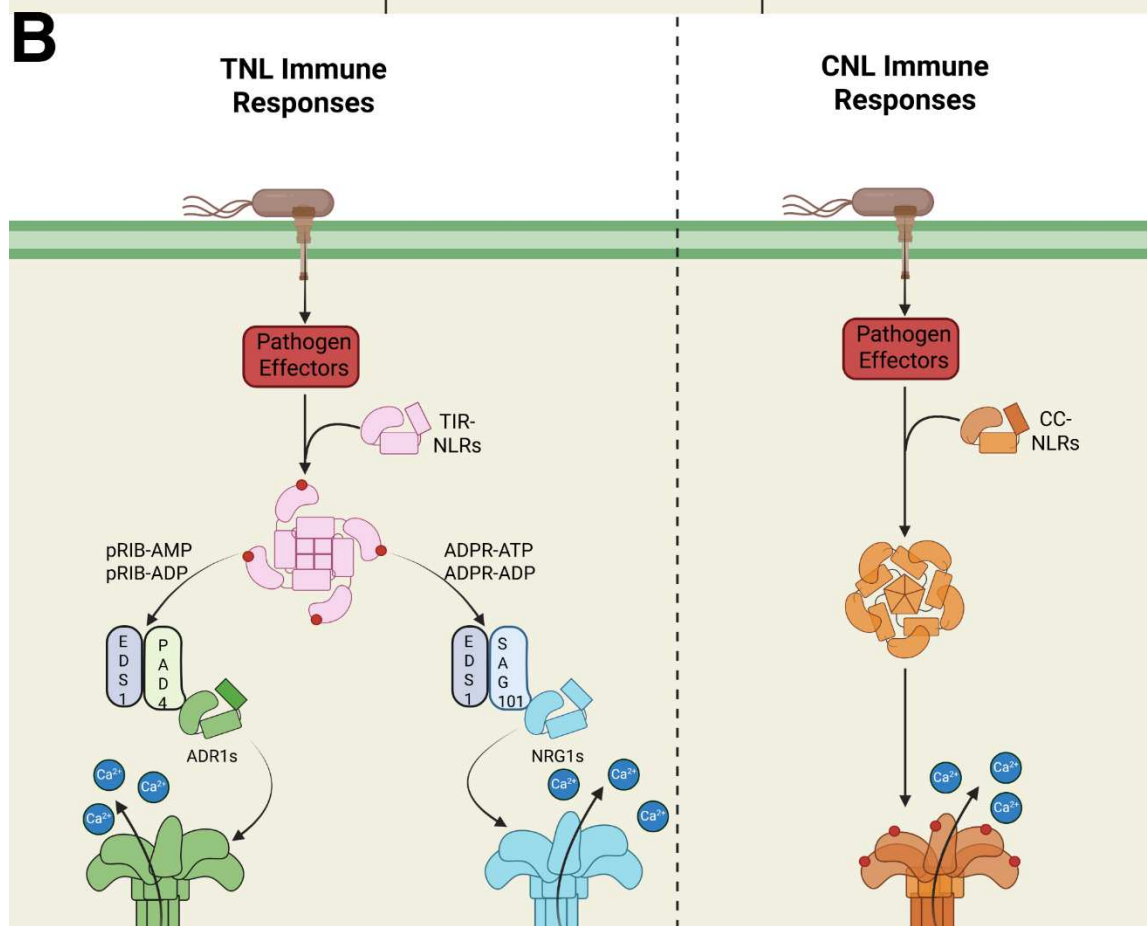
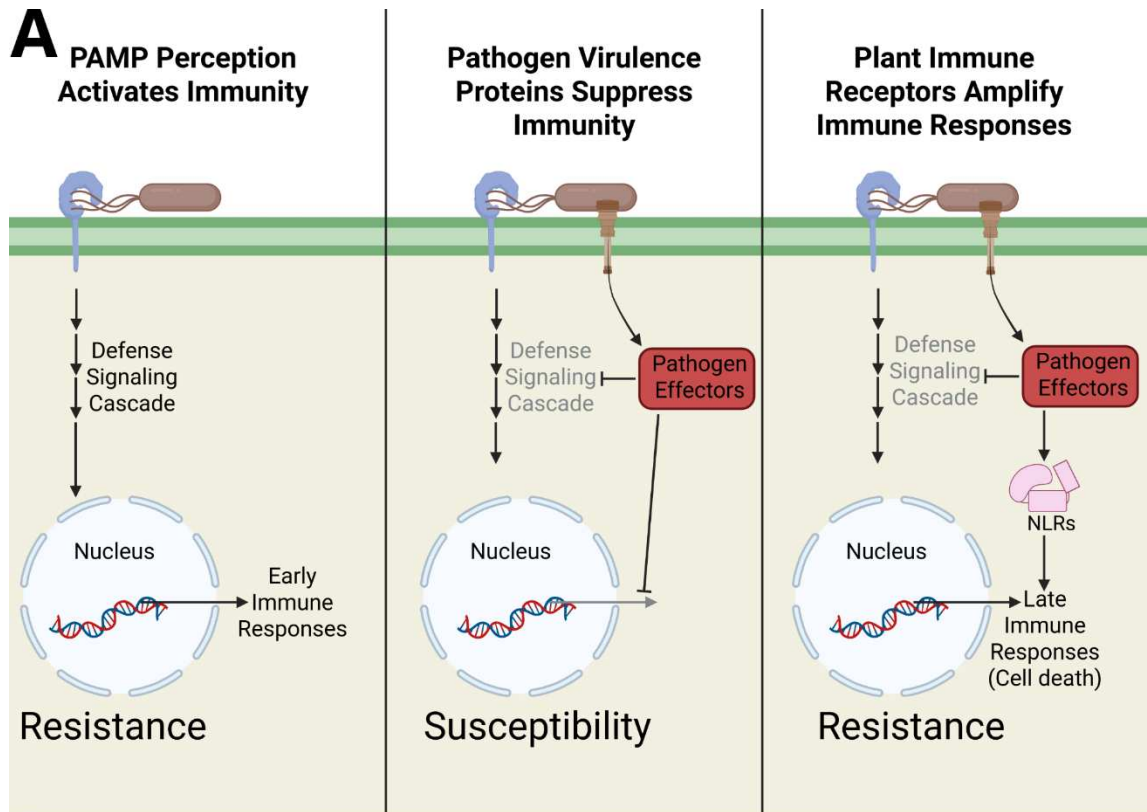


Figure 1.1. Generalized models of plant immune responses. **A)** The zig-zag model of plant immunity. Pathogens are initially perceived by cell surface receptors that trigger an intracellular signaling cascade that triggers early immune responses. Adapted pathogens secrete effectors into the plant cell that function to suppress the PTI signaling cascade initiated by cell surface PRRs. Plant NLRs directly or indirectly recognize pathogen effectors to initiate late immune responses, including cell death. **B)** More detailed models of NLR-mediated immune responses. TNL-mediated effector recognition induces TNL oligomerization and activation of TIR enzymatic activity. Enzymatically produced pRIB-AMP and pRIB-ADP are bound by the EDS1-PAD4-ADR1 heterotrimer, leading to activation and oligomerization of ADR1s and formation of a calcium-permeable channel. Enzymatically produced ADPR-ATP and ADPR-ADP are bound by the EDS1-PAD4-NRG1 heterotrimer, leading to activation and oligomerization of NRG1s and formation of a calcium permeable channel. CNL-mediated effector recognition induces CNL oligomerization. Oligomerized CNL complexes insert into plant membranes where they act as calcium-permeable channels.

implements that the plant cell is using to combat pathogen colonization. PTI transcriptional regulation then primes the proteomic landscape within the cell for immune responses that occur later in infection. These late immune responses can instead be thought as the results of feedback loops from this reordered transcriptional landscape, rather than as a distinct set of immune responses. The spectrum of observed immune responses can perhaps then be explained as variations in the magnitude of PTI activation and how this activation intersects with diverse pathogen virulence mechanisms and idiosyncratic NLR signaling, which will be expanded upon below.

1.4 Effector triggered immunity

The second tier of the plant immune system is frequently conceptualized as sitting downstream of early PTI responses, but as mentioned above, recent work has demonstrated that these two tiers are activated concurrently and similarly converge on a core set of downstream physiological responses in the plant. While these distinctions are indeed arbitrary, the two “tiers” of the plant immune system do diverge in their modes of activation. Where PTI responses are

initiated in response to pathogens and non-pathogens alike via recognition of conserved microbial epitopes by plant cell surface receptors (Colaïanni et al. 2021; Parys et al. 2021), adapted pathogens alone harbor the effector proteins required to elicit ETI responses. Given the efficacy of plant PTI responses in curbing the growth of infecting microbes, adapted pathogens have been forced over evolutionary time to accumulate genes which encode proteins that function to suppress PTI signaling and establish a favorable environment for their growth. Most commonly, pathogens secrete a group of structurally diverse proteins into the plant cytosol called effectors that achieve these goals (Jones, Staskawicz, and Dangl 2024). Since plants and their pathogens have been co-evolving and competing for hundreds of millions of years, plants in turn have evolved proteins of their own to detect the presence of effectors in their cells (Van de Weyer et al. 2019).

NLRs are the primary class of plant proteins that mediate effector recognition and initiate the second tier of the plant immune response, effector triggered immunity (ETI). The hallmark plant ETI response is localized cell death, termed the hypersensitive response (HR), surrounding the site of infection with a pathogen carrying an NLR-recognized effector (J. Morel and Dangl 1997; Pitsili, Phukan, and Coll 2020). HR is thought to restrict pathogen growth via nutrient deprivation that saves the main tissues of the plant from infection at the cost of a small area of locally infected cells. Despite HR being one of the earliest described plant responses to pathogens, the specific cell signaling events that result in cell death remain elusive (Aviv et al. 2002; Coll et al. 2010; Dietrich et al. 1997; Salguero-Linares et al. 2025). While HR is the prototypical response initiated by effector-activated NLRs, ETI does not definitionally include HR in the suite of responses (Kim et al. 2009). “Weak ETI”, as it has been termed, is characterized by NLR-activated immune responses that do not elicit cell death, but is sufficient

to restrict pathogen growth (Laflamme et al. 2020). The characterization of weak ETI has raised the question: what is the function of HR if it is not required for pathogen growth restriction? This question has yet to be fully answered, and the work presented in this dissertation serves to further texture the portrait of ETI responses in Arabidopsis.

1.5 NLRs

Canonical NLR protein structure is typified by the presence of a central nucleotide binding domain (NBS) and a C-terminal leucine rich repeat domain (LRR). The LRR domain typically functions to bind modified host proteins or effectors directly. Upon binding of modified host proteins or effectors, structural changes in the LRR are propagated to the NBS domain, which exchanges ADP for ATP, allowing the NLR to oligomerize and become active (Förderer et al. 2022; Martin et al. 2020; J. Wang et al. 2019) (Figure 1.1B). The immune-activating executor domains of NLRs are located at their N-termini, harboring either a coiled coil (CC) domain or Toll/interleukin-1 receptor (TIR) domain (Grant et al. 1995; Whitham et al. 1994). These executor domains become signaling competent via oligomerization-dependent induced proximity (Martin et al. 2020; J. Wang et al. 2019). Both CC-NLRs (CNLs) and TIR-NLRs (TNLs) work to directly or indirectly sense effector-mediated disruptions to cellular homeostasis and can broadly be classified as “sensor NLRs”.

Upon activation, CNLs oligomerize and the CC domains structurally rearrange to form a cation-permeable pore (Bi et al. 2021; J. Wang et al. 2019). CNLs have been reported at both the plasma membrane and endomembranes, but the functional relevance of NLR localizations pre- and post-activation is still unclear (Bi et al., 2021; Förderer et al., 2022; Jacob et al., 2021; Liu et al., 2024; Martin et al., 2020; Wang et al., 2019). Calcium influx through CNL pores leads to downstream disease resistance responses in the cell via undefined pathways (Cui et al., 2018;

Saile et al., 2020; Xu et al., 2014). TNL oligomerization causes production of a variety of nicotinamide adenine dinucleotide (NAD⁺)-derived small molecules via TIR domain enzymatic activities (Horsefield et al. 2019; S. Huang et al. 2022; Jia et al. 2022; L. Wan et al. 2019). TIR-produced small molecules are bound by a heterodimer consisting of members of the ENHANCED DISEASE RESISTANCE 1 (EDS1) protein family, which subsequently interacts with and promotes activation of another clade of CNLs with a RESISTANCE TO POWDERY MILDEW 8 (RPW8)-like CC-domain, known as RNLs or “helper NLRs”. Similar to CNLs, RNLs oligomerize to form cation-permeable pores that lead to downstream immune responses (Huang et al., 2022, 2025; Jia et al., 2022; Xiao et al., 2025; Yu et al., 2024). Thus, both CNL and TNL/RNL immunity ultimately function in parallel pathways to activate essentially identical immune outputs via Ca²⁺ signaling (Saile et al., 2020).

EDS1 has two closely related homologs in Arabidopsis: PHYTOALEXIN-DEFICIENT 4 (PAD4) and SENESCENCE-ASSOCIATED GENE 101 (SAG101). These three proteins mediate TIR signaling by forming two independent heterodimeric complexes (Wagner et al. 2013) (Figure 1.1B). In the presence of TIR-produced small molecules, the EDS1-PAD4 heterodimer interacts with and activates ACTIVATED DISEASE RESISTANCE 1 (ADR1) RNLs, while the EDS1-SAG101 heterodimer interacts with N REQUIREMENT GENE 1 (NRG1) family RNLs (Huang et al., 2022, 2025; Wang et al., 2023). In both cases, small molecule binding by the EDS1 complex leads to oligomerization and calcium channel activity by the corresponding helper NLR (S. Huang et al. 2025; W. Song et al. 2024a). Signaling through the EDS1-PAD4-ADR1 node is canonically associated with pathogen growth restriction outcomes and disease resistance without cell death (Lapin et al., 2020; Sun et al., 2021; Yu et al., 2024). Signaling through the EDS1-SAG101-NRG1 node is more so associated with cell death ETI responses.

These two branches of EDS1-dependent immunity are independently activated by related, but structurally distinct, TIR-produced small molecules (S. Huang et al. 2022; Jia et al. 2022). Despite observations that these two signaling complexes can lead to distinct immune outcomes, the structural and biochemical properties of both classes of RNLs (and most CNL proteins) have been shown to lead to broadly similar changes in plant cell physiology (Saile et al. 2020; Xiao et al. 2025). In some cases, the EDS1 family dichotomy between disease resistance and cell death is not strictly followed (Saile et al. 2020). ADR1s, NRG1s, and CNLs all form calcium-permeable channels, yet a wide spectrum of immune outcomes can be achieved through their activation. The extent to which these differences are qualitative, or quantitative, is not currently understood. Presumably, this complexity has evolved to allow the immune output level of each NLR to be appropriately tuned for the recognized pathogen.

In addition to individual NLRs recognizing multiple pathogen effectors, individual pathogen effectors can be convergently recognized by sequence-unrelated immune receptors (Contreras et al., 2023). The resulting network structure of NLR/effector interactions is poorly understood. In the Ag-0 accession of Arabidopsis, the *Pseudomonas syringae* effector HopBA1 was found to elicit an ETI cell death response via the noncanonical TIR-only NLR, RBA1 (Response to HopBA1). RBA1-mediated cell death responses require the canonical TIR signal receptor EDS1 (Nishimura et al., 2017). RBA1 is not expressed in Col-0 Arabidopsis and HopBA1 does not elicit a cell death response in this accession. Despite this, *Pseudomonas syringae* carrying HopBA1 is still growth-restricted in Col-0 Arabidopsis (Laflamme et al., 2020; Martel et al., 2020; Nishimura et al., 2017). HopBA1-dependent growth restriction in Col-0 requires the CNL immune receptor, ZAR1 (Laflamme et al., 2020). Dual recognition of HopBA1

by both RBA1 and ZAR1 in Ag-0 provides an opportunity to characterize points of overlap in TNL and CNL signaling pathways that are explored further in Chapter 3 of this dissertation.

1.6 ZAR1

Interestingly, the CC-NLR ZAR1 is also capable of tuned outputs in response to different pathogens. ZAR1 has been the focus of extensive research and was the first plant NLR to have its activated oligomeric structure determined by cryogenic electron microscopy (cryo-EM) (Bastedo et al., 2019; Bi et al., 2021; Laflamme et al., 2020; Lewis et al., 2008, 2013; Seto et al., 2021; Wang et al., 2019). Uniquely among characterized NLRs, ZAR1 recognizes bacterial effectors via cooperation with the ZED1-related (ZRK) family of receptor-like cytoplasmic kinases and pseudokinases (Martel et al., 2020). The ZRK pseudokinases ZED1 (HOPZ-ETI-DEFICIENT 1) and RKS1 (RESISTANCE-RELATED KINASE 1), have been found to conditionally interact with members of the RLCK-VII subfamily upon their modification by pathogen effectors (Bastedo et al., 2019; Liu et al., 2019; Wang et al., 2019). Thus, ZRKs function as co-receptor adapters that expand ZAR1's recognition specificity. So far, six structurally diverse pathogen effectors have been shown to be recognized by ZAR1, each activating ZAR1 through a different ZRK protein (Martel et al., 2020) (Figure 1.2). Interestingly, different effectors elicit different ETI outputs via ZAR1. For example, ZAR1-mediated responses to the effector HopZ1a result in cell death, while recognition of HopBA1 does not in Col-0 *Arabidopsis* (Laflamme et al., 2020). Given that ZAR1-mediated immune responses vary depending on the eliciting effector protein, ZAR1 and its cognate effectors present an ideal system to study the mechanisms that plants use to “make decisions” regarding cell death in immune responses.

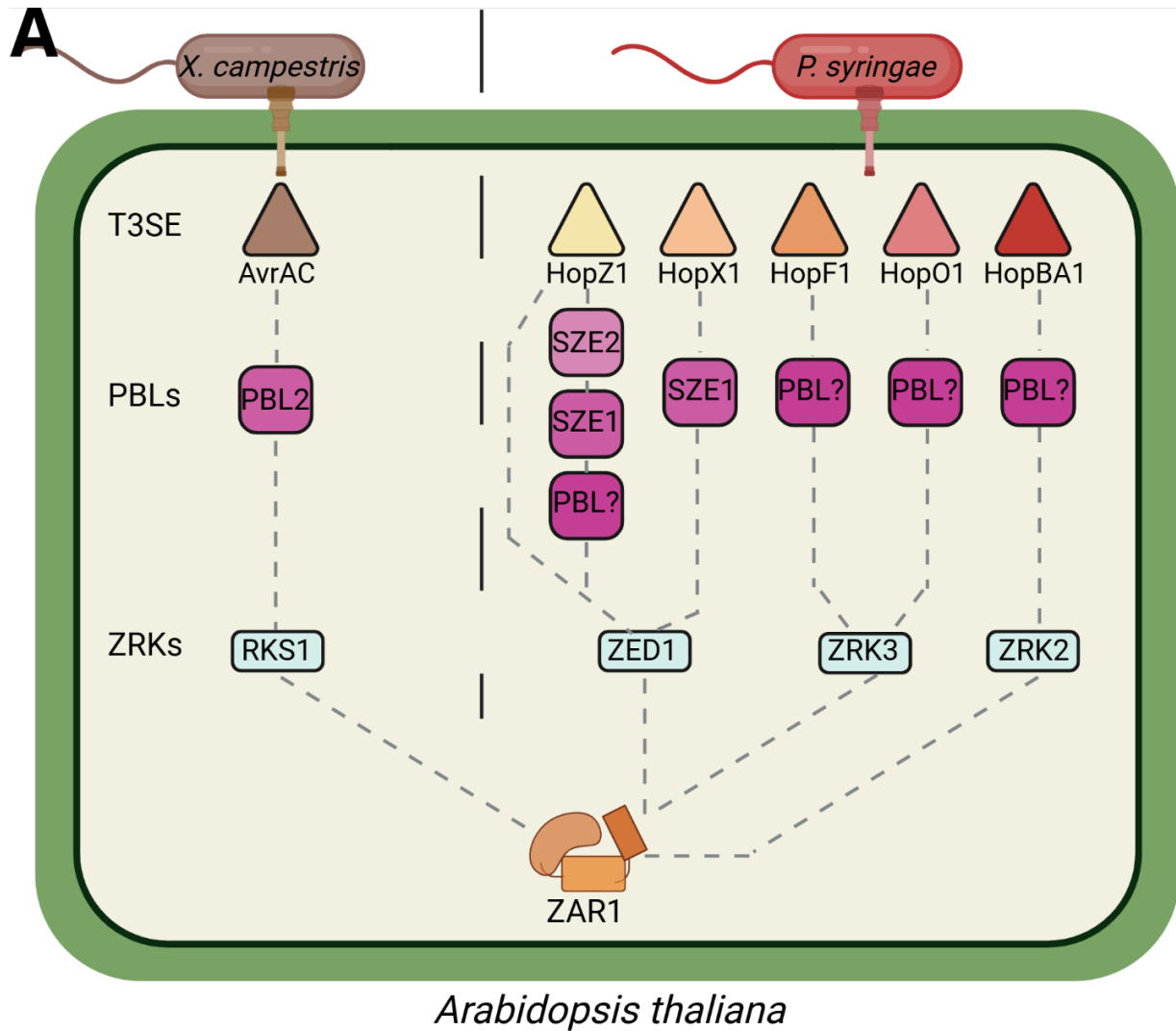


Figure 1.2. Model for ZAR1 perception of effectors in Arabidopsis. A) Effectors secreted by *Xanthomonas campestris* and *Pseudomonas syringae* have been shown to activate ZAR1-mediated immune responses. AvrAC, HopZ1 and HopX1 have been found to post-translationally modify members of the PBL kinase family in Arabidopsis. Modified virulence targets interact with RLCK XII pseudokinases, also called ZRKs. ZRKs form a complex with ZAR1 and are required for ZAR1-mediated immune responses. Figure adapted from Martel et al. 2020.

1.7 TIR domains in plant immunity

Extensive study of TIR-domain containing proteins across the tree of life has revealed consistent recruitment of the TIR domain into immunity-related signaling pathways (Essuman et al. 2022). More recently, the TIR domain has been appreciated as both a proximity-induced

oligomerization domain and as an enzymatic domain producing a range of small molecules. Discussion of these recent findings and how they fit into the larger picture of the plant immune system can be found below.

1.7.1 Plant TIRs exhibit intrinsic enzymatic activities

TIRs can function via at least three mechanisms. The first described mechanism was an inducible scaffold function in animal Toll-like receptors and their cytoplasmic TIR adapters (Nanson, Kobe, and Ve 2019). Physical interactions between these TIRs can result in high-order structures that concentrate signaling proteins such as kinases in a cell (Clabbers et al. 2021; Nanson, Kobe, and Ve 2019; Ve et al. 2017). This model might also apply to plants, as proximity labeling of a TNL, N, suggests physical interactions between its TIR domain and CNLs in *Nicotiana benthamiana*, which may contribute to TNL-dependent resistance (Y. Zhang et al. 2019). Additional work has found that TIR proteins in plants form phase-separated condensates that are required to induce HR in response to their cognate effector (W. Song et al. 2024b). Interpretation of these data should be done carefully, however, as the requirement for phase separation for cell death induction was only shown in the context of naturally occurring or artificially truncated TIR-only proteins. It is also not known what other proteins, if any, reside within these TIR condensates, so the inducible scaffold function of plant TIRs has yet to be fully demonstrated.

The second mechanism of TIR function is a recently discovered intrinsic TIR NAD⁺-degrading activity (Figure 1.3) (Essuman et al. 2022). Such activity was initially found in the animal TIR protein Sterile Alpha and TIR motif containing 1 (SARM1), which induces neuronal cell death via NAD⁺ depletion in response to injury (Essuman et al. 2017). Subsequently, TIR NADase activity was detected in plant and prokaryotic TIRs activating immunity (Essuman et al.

2018; Horsefield et al. 2019; L. Wan et al. 2019). In the case of prokaryotes, this TIR-produced signal can stimulate non-TIR NADases to substantially deplete NAD^+ (Ofir et al. 2021). By contrast, in plants, TIR NAD^+ derivatives activate EDS1-dependent immunity without a significant depletion of NAD^+ (Horsefield et al. 2019; S. Huang et al. 2022; Jia et al. 2022; L. Wan et al. 2019).

In the third mechanism, plant TIRs function as dsDNA/ RNA hydrolases to generate 2',3' cyclic adenosine/guanosine monophosphates (2',3'-cAMP/cGMP,). This mechanism is associated with oligomeric TIR filaments of indeterminate length rather than head-to-tail tetrameric complexes that hydrolyze NAD^+ (Figure 1b) (Maruta et al. 2023; Yu et al. 2022). This synthetase activity might not be directly relevant to TNL cell death activity (L. Tian, Lu, and Li 2022). Instead, 2',3'- cAMP/cGMP likely act as secondary messengers in plants to activate stress-related transcriptional reprogramming (Chodasiewicz et al. 2022). The role of these metabolites in immunity is supported by the observation that expression of *Arabidopsis* nudix hydroxylase homolog 7 (NUDT7) and other phosphodiesterases that degrade 2'3'-cAMP/ cGMP suppresses TIR cell death in *N. benthamiana* (Yu et al. 2022). Similarly, a *nudt7* loss-of-function *Arabidopsis* mutant displays autoimmunity (Bartsch et al. 2006), and at least one pathogen effector can degrade 2',3'-cAMP/cGMP (Yu et al. 2022). Although the 2',3'-cAMP/cGMP synthetase function has been demonstrated for TIRs of flowering plants including monocots, NUDT7 is *Brassicaceae*-specific (phylogen. org; PTHR13994). Thus, containment mechanisms of the TIR 2',3'-cAMP/cGMP synthetase activity likely vary across phylogeny.

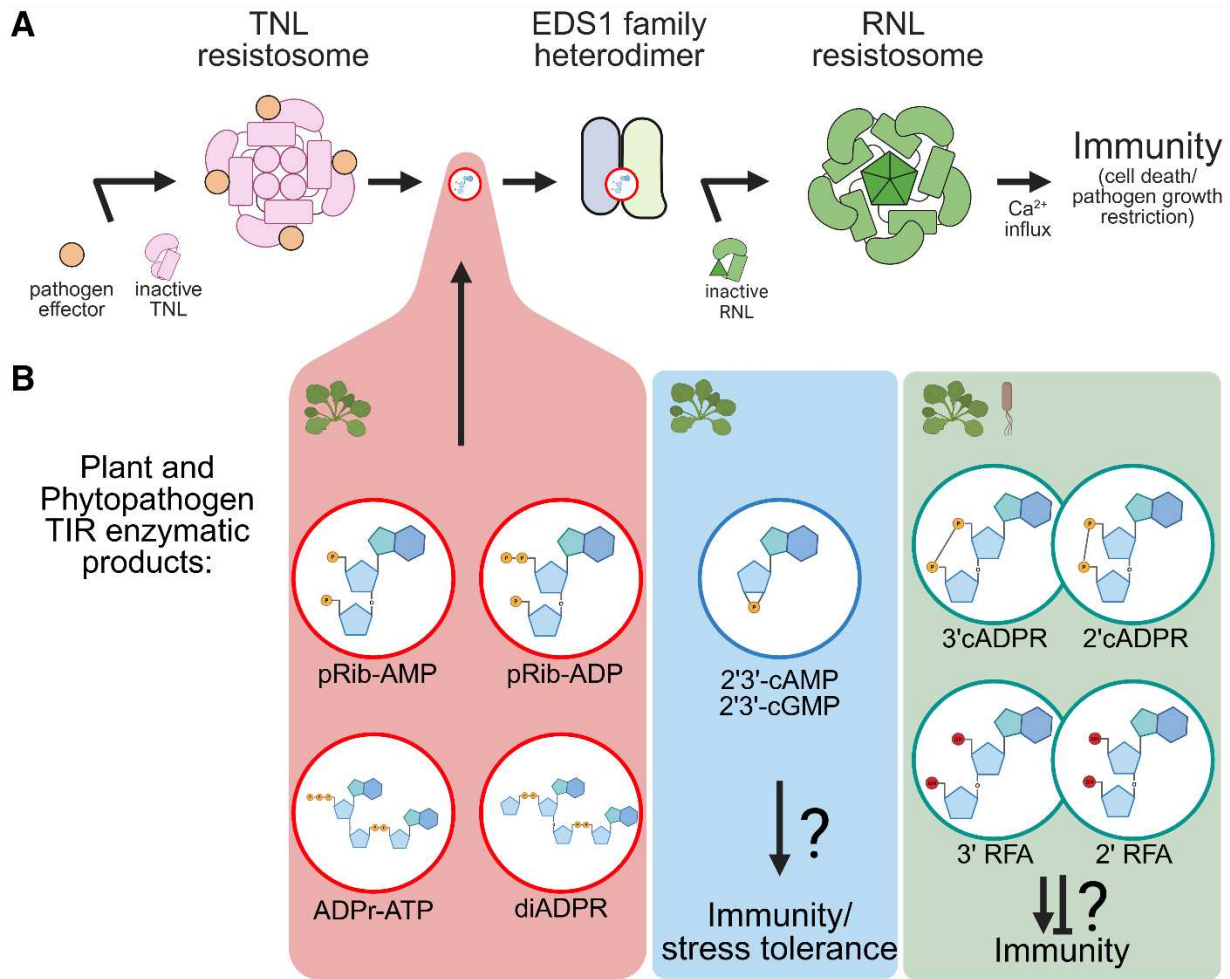


Figure 1.3. Plant and plant-pathogen TIR proteins generate diverse NADase products to regulate immunity. **A)** Intracellular TNL (TIR-NLR) immune receptors oligomerize into tetrameric resistosomes in response to pathogen effectors. Oligomerization of the TIR domains (green circles) activates their enzymatic activity to produce small molecules (red circle). TIR products signal downstream by activating the EDS1 heterodimer, which then promotes the oligomerization of an RNL resistosome. RNL resistosomes function as calcium channels to activate immunity via an unknown mechanism. **B)** Plant and plant pathogen TIRs produce a spectrum of nucleotide-related small molecules. Several TIR products (e.g. pRib-AMP, red) have been shown to function by activating the EDS1 complex. Plant TIRs can also degrade dsDNA/RNA to produce 2',3'-cAMP/cGMP previously linked to stress tolerance (blue). Plant and plant pathogen TIRs also produce isomers of cyclic ADPR and pRib-like RFA molecules (green). These molecules have been proposed to function as regulators of immunity, but any functions remain to be demonstrated. Small-molecule structures are graphically simplified to highlight their similarities and differences. Figure from Ogden et al. 2023.

1.7.2 Why do plant TIRs and pathogen TIR effectors produce an overlapping spectrum of NADase products?

As seen above, TIRs of flowering plants generate a diverse set of enzymatic products. Four of them (pRibAMP, pRib-ADP, ADPr-ATP, and diADPR) are immunogenic, bind EDS1 heterodimers both *in vitro* and *in planta*, and activate Ca^{2+} influx via RNLs (S. Huang et al. 2022; Jacob et al. 2021; Jia et al. 2022). Plant TIRs can also produce stable isomers of cyclic ADPR (2'-cADPR and 3'-cADPR) that are, surprisingly, identical to those produced by the bacterial TIR and TIR-like effectors HopAM1 and HopBY1 (Bayless et al. 2023; Eastman et al. 2022; Hulin et al. 2023; Manik et al. 2022). Plant TIRs (and likely HopAM1) also produce 3'-O-b-D-ribofuranosyladenosine (RFA), a molecule equivalent to a dephosphorylated version of the EDS1-activating TIR product pRib-AMP (Duxbury et al. 2020; Jacob, Hige, and Dangl 2023).

Why would a plant produce the same TIR products as the pathogen? Assuming that effector products are negative regulators of immunity, plant-produced cADPR isomers could be negative regulators of immunity (Duxbury et al. 2020; Eastman et al. 2022; Hulin et al. 2023; Manik et al. 2022). Perhaps plant TIRs are making both positive regulators of defense (i.e. EDS1- activating pRib, et al.) and negative regulators (cADPR isomers) to limit inappropriate immune activation or the cost of defense. This idea may be supported by single cell RNA-seq data showing that cells spatially organized at the nexus of an immune response are transcriptionally enriched in negative regulators of plant immune responses (Nobori et al. 2025). These data suggest that negative regulation of immunity is concomitant with intense immune activation – a result that is consistent with plant TIR proteins producing both positive and negative immunoregulatory small molecules. The production of the same negative regulatory molecules by pathogen and plant TIRs can be reconciled by considering the timing during

infection that the molecules are produced. Pathogen TIRs likely produce negative regulators of immunity early in the infection process to stop robust immune responses, while plant TIRs produce negative regulatory molecules later on. This late “backpedaling” in immunity may function to restrict plant HR from spreading beyond immediately infected tissues (Dietrich et al. 1997; Jacob et al. 2023; Jacob, Hige, and Dangl 2023). Alternatively, cADPR isomers and RFA produced by plant and pathogen TIRs may be enzymatic side-products or metabolites of EDS1 signals (Bayless et al. 2023; Drenichev et al. 2019; Jacob, Hige, and Dangl 2023). Finally, it seems possible that NADase effectors function by depleting NAD^+ to disrupt host metabolism rather than by generating signaling molecules (Bayless et al. 2023; Hulin et al. 2023). Differentiating between these hypotheses will require new bioassays to deliver small molecules into the plant cell or new TIR mutants that decouple the production of the various TIR enzymatic products from each other. Surveys of prokaryotic immune TIRs has led to the discovery of TIR proteins with a host of novel enzymatic outputs, so these tools might already exist in nature, waiting to be discovered (Rousset et al. 2025; Sabonis et al. 2024).

1.7.3 TIRs of flowering plants functioning as immunity boosters beyond effector recognition

Recent work using pharmacological inhibitors of TIR signaling in Arabidopsis has demonstrated that TIR enzymatic activity can be required for effector-triggered immunity even when CNLs initiate the effector-triggered immune responses (Figure 1.4) (Jacob et al. 2023). The data presented in Chapter 3 of this dissertation also supports this hypothesis, with EDS1-PAD4-ADR1 signaling being required for ZAR1-mediated immune responses. Many TIR loci are among the earliest transcriptionally activated defense genes in flowering plants (Bjornson et al. 2021; Johannndrees et al. 2023; Lapin et al. 2022; H. Tian et al. 2021). Together, these data

implicate TIRs in plant immunity beyond TIR or TNL-mediated effector recognition. One hypothesis that may explain these results is that certain TIR proteins act as “immune boosters” to enhance plant immune responses at the transcriptional level (Figure 1.4) (Yu et al. 2022). This model would also explain the importance of EDS1 and PAD4, receptors of some TIR enzymatic products, for signaling by the defense hormone salicylic acid (Cui et al. 2017; Fliegmann et al. 2025).

1.7.4 Emerging roles of TIRs in local acquired immunity in flowering plants

TIRs/TNLs have primarily been studied as initiators of a cell-autonomous EDS1-dependent response. However, recent studies of the TNL suppressor of ADR1-L2 1, (SADR1) also named suppressor of AITF1- induced autoimmunity 1 (SAA1), suggest that TIRs can have EDS1-independent functions essential for immune responses in areas next to the pathogen recognition site referred to as ‘local acquired resistance’ (LAR; (Jacob et al. 2023; Jacob, Hige, and Dangl 2023; Y. Wang et al. 2023)). SADR1 was identified as a suppressor of an autoactive D484V variant of the helper RNL, ADR1-L2. Surprisingly, *sadr1*’s suppression of ADR1-L2 D484V autoactivity was not fully phenocopied by an *eds1* mutant, indicating that at least some SADR1 function is EDS1- independent. Intriguingly, TIR functions supplied by two other autoactive TNLs were able to partially restore autoimmunity of the suppressed *ADR1-L2 D484V* *sadr1* double mutant, indicating that other TNLs share this SADR1 function (Jacob et al. 2023). SADR1 was also required to express the defense marker gene, *pathogenesis-related gene 1* (*PR1*) in a zone around the infected tissue. This non-cell autonomous expression was lost in an RNL polymutant that lacked ADR1 and NRG1 functions (Jacob et al. 2023).

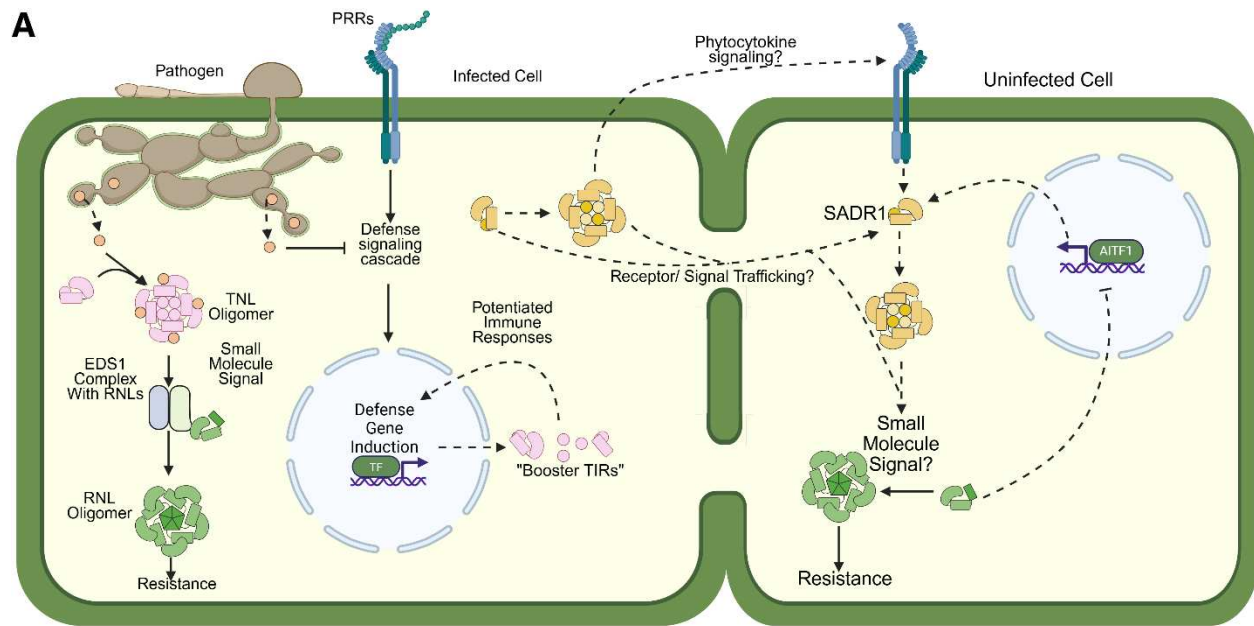


Figure 1.4. TIR proteins function in immune responses beyond effector recognition. A)

Upon activation of pattern-triggered immunity, “Booster TIRs” may be transcriptionally activated to further potentiate early immune responses. SADR1 would be activated in response to these same signals to regulate immune responses in neighboring uninfected cells, enhancing RNL-mediated immune responses to prevent pathogen spread. SADR1 could also be trafficked directly from pathogen-infected cells to neighboring cells, or small-molecule signals produced by SADR1 could move between cells through the apoplast or plasmodesmata to propagate a non-autonomous immune response. Figure from Ogden et al. 2023.

One could speculate that TIR-catalyzed signaling products or the downstream signals move from infected cells to induce immunity in surrounding uninfected cells. A biomarker for TIR signaling activity identified in this study, 3’-O β -D-ribofuranosyladenosine, could be a candidate for a mobile TIR-produced non-autonomous immune signal. These signals could plausibly travel intracellularly through plasmodesmata or function through an apoplastic route as phytochemicals perceived by PRRs (Figure 1.4).

1.7.5 TIRs in flowering plants differ in distribution across species and signaling requirements

Plant TIR differentiation in function and signaling was also elaborated using phylogenomics methods. They revealed two primary plant TIR classes (Johannndrees et al. 2023; Meyers, Morgante, and Michelmore 2002; Toshchakov and Neuwald 2020). The first class was introduced above as being associated with NBS and LRR domains. This class also includes “truncated” TIRs without LRR or NBS-LRR (i.e. TIR-NBS or TIR-only, respectively). We refer to this first class as “TNL” class TIRs (Johannndrees et al. 2023; Meyers, Morgante, and Michelmore 2002). This class is found in receptor TNLs and TIR-only proteins, putative booster TIR proteins, and TNL SADR1 acting in LAR. The second class includes TIR proteins with an NBS domain and a C-terminal tetratricopeptide repeat (TPR) rather than an LRR. This class has been named TIR-NBSTPR (TNP) TIRs (Johannndrees et al. 2023; Lapin et al. 2022; Y. M. Zhang et al. 2017). TNP-encoding genes are present in plant genomes in relatively low numbers, but they are the most widely distributed plant TIRs (Johannndrees et al. 2023; Y. M. Zhang et al. 2017). By contrast, the TNL-TIR class shows lineage-specific expansions and contractions. TNLs often have a C-terminal extension called C-JID (C-terminal jelly roll/ immunoglobulin-like domain), which binds effectors via a variable loop surface [(Lapin et al. 2022; S. Ma et al. 2020; P. N. Dodds, Lawrence, and Ellis 2001).

These observations support the notion that many TNLs recognize variable effectors. However, predicting more specific functions (e.g. as booster TIRs or LARs) based on the sequence seems difficult. For instance, SADR1 also has a C-JID domain and thus may bind an unknown ligand in addition to, or as part of, its EDS1-independent and LAR functions (Jacob et al. 2023). The TNL-TIR class shows clade-specific expansions and contractions. In contrast to dicots, monocots have lost nearly all TNL TIRs except for a low- or single-copy number TIR-only shared by most flowering plants (Johannndrees et al. 2023). The EDS1 paralog SAG101 and

SAG101-interacting RNL helper NRG1 were also lost in these genomes, indicating coevolution between TNLs, SAG101, and the helper NRG1 (Collier, Hamel, and Moffett 2011; Lapin et al. 2019). Still, many known TIR signaling components are retained in monocot genomes, including EDS1, PAD4, and ADR1 (Johannndrees et al. 2023). The rice EDS1, PAD4 and ADR1 protein complex has been shown to function in regulating immunity, suggesting that, despite contraction of the TNL family in monocots, some core functions of this signaling pathway have been retained (Yue et al. 2024). In contrast to TNL TIRs, TNP TIRs are present in plants without the EDS1 family and RNLs, suggesting that TNP activities are independent of them. Indeed, cell death by maize (*Zea mays*) TNP in *Nicotiana tabacum* requiring the TIR catalytic glutamate was not compromised in the *eds1* silencing line. The *tnp* mutants of *N. benthamiana* behaved like wild-type plants at the level of growth, early PRR receptor-like kinase signaling, and TNL cell death and resistance (Johannndrees et al. 2023), which leaves an open question about TNP physiological function(s). Given the extensive expansion of TIR-based immune signaling pathways in prokaryotes, it seems reasonable that plant genomes may house multiple classes of TIRs that signal via independent pathways (Doron et al. 2018; Yirmiya et al. 2025).

1.8 Perspectives on the future of plant immunity research

While the field of plant immunity research has made significant strides since the initial description of the gene-for-gene hypothesis in the early 1900s, many molecular mechanisms underpinning plant immune responses remain opaque. In particular, there is a major gap in understanding regarding the relevance and mechanisms of HR in defense. HR has long been described as the hallmark ETI response, but the specific mechanism of HR induction and its relationship to pathogen growth restriction are unclear. Work presented in Chapter 3 details an

example of TIR-induced HR being dispensable for pathogen growth restriction, a novel phenomenon that has not been previously described in the context of NLR-mediated cell death.

While many NLRs have been identified within plant genomes, the effectors that activate them remain largely unknown. Similarly, many effectors that have been identified have undescribed enzymatic functions. Both the sides of the effector-NLR relationship need to be more extensively characterized to complete our understanding of plant immunity. Given the inevitable threat that global climate change poses to human agricultural endeavors, it is as relevant as ever to leverage our understanding of plant-pathogen interactions to secure food production efforts. While supply chain logistics represent the main barrier to feeding the world's growing population, climate change induced variations in plant pathogen ranges and co-occurring stresses on crops mean that it is increasingly important to have a comprehensive understanding of the molecular basis of plant immunity. By building a complete picture of plant molecular processes through basic and applied research, we can improve our capacity as a society to adapt to future needs in agriculture.

1.9 Overview of dissertation aims

This dissertation addresses a few of the open questions discussed in the preceding sections. Chapter 2 of this work is focused on efforts to identify the virulence targets and biochemical function of a *Pseudomonas syringae* effector protein, HopBA1. By investigating the hypothesis that HopBA1 targets RLCK VII proteins in Arabidopsis and *Nb.*, we find that HopBA1 recognition is not lost in any single RLCK VII Arabidopsis polymutant, but that HopBA1 may act as a protease on specific members of the *Nb*RLCK VII family.

In Chapter 3, exploration of HopBA1's activation of both TIR- and CNL-mediated immune responses lead to the finding that ZAR1 broadly requires the EDS1-family mediated signaling pathway to initiate complete immune responses. We also characterize accession-specific differences in ZAR1-EDS1 dependence, highlighting the importance of considering the genetic context that receptor-effector interactions are studied in. This chapter also describes a scenario where RBA1-mediated cell death does not confer pathogen growth restriction – a unique case in the literature.

1.10 Author contributions

Section 1.7 and the associated subsections were originally published as a review titled “Functional diversity of Toll/interleukin-1 receptor domains in flowering plants and its translational potential” (Ogden, Nishimura, and Lapin 2023). The original manuscript was co-authored by Sam C. Ogden, Marc T. Nishimura, and Dmitry Lapin. All authors made roughly equal contributions to conceptualization, writing, figure generation, revising, and formatting for submission and revisions. Revisions between the published version and the version presented here were generated by Sam C. Ogden for the purpose of updating the manuscript to reflect progress in the field since publication and tailoring for this chapter. Pieces of sections 1.2-1.6 are part of the introduction section of a manuscript currently in preparation titled “ZAR1 requires canonical TIR-NLR signaling components to initiate complete immune responses against *Pseudomonas syringae* in Arabidopsis.” This manuscript is written by Sam C. Ogden with revision and editing by Marc T. Nishimura.

CHAPTER 2: ASSESSMENT OF HOPBA1 PHYSICAL AND GENETIC INTERACTIONS WITH RLCK VII PROTEINS

2.1 Summary

The work discussed below describes an investigation of the Arabidopsis receptor-like cytoplasmic kinase subfamily VII (RLCK VII) proteins, also known as AvrPphB SUSCEPTIBLE1-LIKE (PBL) kinases, as potential virulence targets of the *Pseudomonas syringae* effector, HopBA1. Given the recognition of HopBA1 by ZAR1 and ZAR1's recognition of multiple other pathogen effectors that have been found to post-translationally modify members of the RLCK VII family, HopBA1 was hypothesized to modulate the activities of one or more RLCK VIIs as its virulence function (Bastedo et al. 2019; Martel et al. 2020).

To test the hypothesis that recognition of HopBA1 by ZAR1 in Col-0 Arabidopsis requires modification of a RLCK VII virulence target, we conducted a yeast two-hybrid mating assay between HopBA1 and the Arabidopsis RLCK VII proteins and conducted pathogen growth curves in a panel of *RLCK VII* polymutants harboring loss of function mutations in multiple members of the RLCK VII family. Yeast two-hybrid assays did not consistently demonstrate HopBA1 interaction with any PBLs. Neither the work using Arabidopsis polymutants nor the work using transiently expressed PBL kinases supported the hypothesis that Arabidopsis PBL kinases are the virulence targets of HopBA1. Work completed in parallel silencing the *Nicotiana benthamiana* RLCK VII family, however, did reveal a requirement for some *NbRLCK VII* proteins in the *NbZAR1*-mediated response to HopBA1. Transient co-expression of two of these *NbPBL* kinases with HopBA1 led to a 4-9kDa migration shift on a western blot, consistent with

HopBA1-induced proteolytic cleavage. Furthermore, the presence of the putative cleavage product is abolished when co-expressed with an allele of HopBA1 that harbors point mutations in its hypothetical active site. Subsets of individual Arabidopsis RLCK VII proteins in RLCK VII subgroups closely related to NbRLCK VIIs required for induction of HR were also overexpressed in *N. benthamiana* with or without co-expression of HopBA1. Transiently expressed proteins were subjected to western blotting to determine if co-expression leads to any changes in PBL accumulation, however poor transient accumulation of Arabidopsis RLCK VIIs failed to yield any insight into potential HopBA1 substrates in Arabidopsis.

These data support the hypothesis that, at least in *N. benthamiana*, HopBA1 activity leads to proteolytic cleavage of these *NbPBL* proteins. Overall, the data are mixed regarding a HopBA1 virulence function via PBL kinases: in Arabidopsis we found no evidence for HopBA1-targeting of PBL kinases, however in *N. benthamiana* we found that PBL silencing impacted HopBA1 activation of ZAR1 and suggested that HopBA1 expression may directly or indirectly result in the cleavage of RLCK VIIs.

2.2 Results

2.2.1 HopBA1 does not consistently interact with Arabidopsis PBL kinases in a yeast two-hybrid assay

The Arabidopsis PBL kinase family consists of 48 members which can be organized into 9 sub-groups, named RLCK VII-1 to RLCK VII-9 (Figure 2.1) (Rao et al. 2018). Using a collection of cDNA clones generously provided by the Desveaux lab, the PBL kinases were assessed in a yeast two-hybrid mating assay for interaction with HopBA1, the predicted loss-of-function HopBA1 H56F mutant, or ZED1-RELATED KINASE 2 (ZRK2) as baits. ZRK2 is a

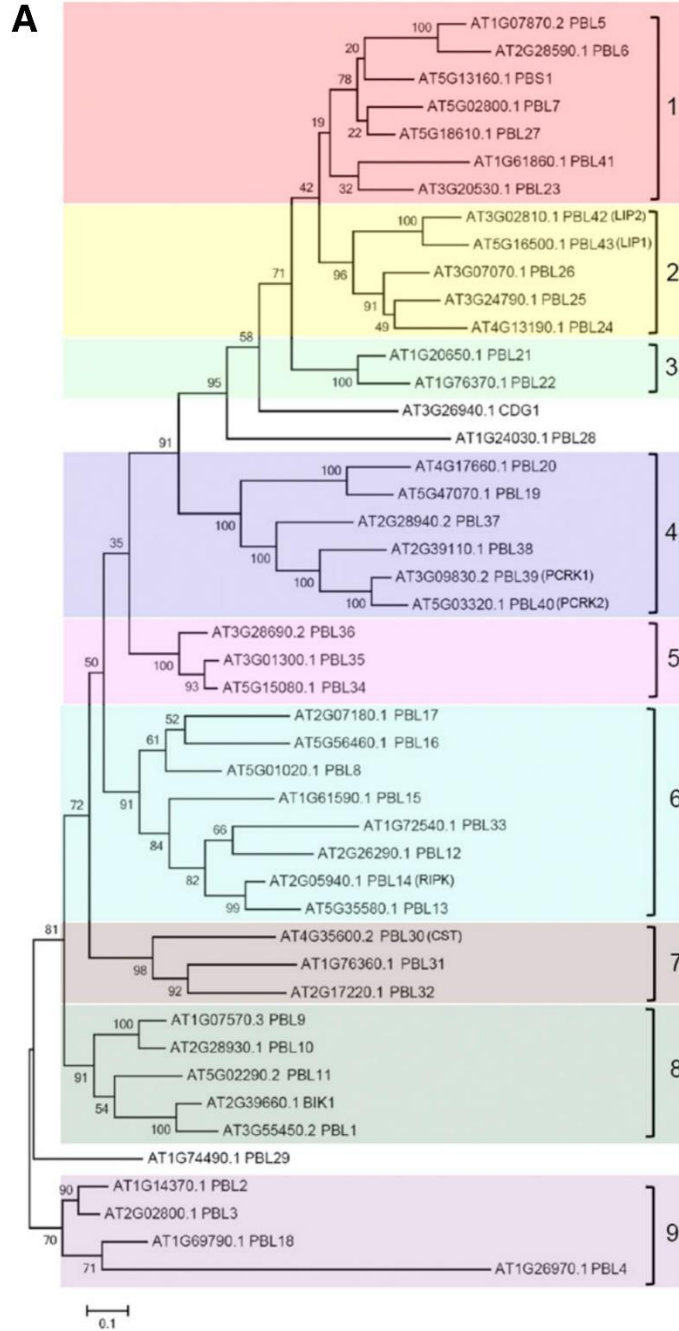


Figure 2.1. Phylogenetic clustering of the Arabidopsis RLCK subfamily VII. A) Phylogenetic tree of RLCK VII in Arabidopsis. The full-length sequences of RLCK VII members were used to generate the phylogenetic tree using MEGA. Subgroups are color-coded and indicated to the right of the phylogeny. Phylogeny from (Rao et al. 2018).

pseudokinase that is co-required with ZAR1 for HopBA1 recognition and is thought to bridge interactions between modified effector targets and ZAR1 (Diplock et al. 2023; Martel et al. 2020; J. Wang et al. 2019). We reasoned that some PBL kinases may interact with ZRK2 even in the absence of HopBA1-induced modification. The PBL kinase prey were expressed as a fusion protein with the B42 transcriptional activation domain (AD) in the prey vector pJG4-5, and the bait proteins were fused to the LexA transcription factor DNA binding domain (BD) in pEG202. Interaction between HopBA1 and a PBL kinase would lead to formation of a competent LexA transcription factor to drive expression of the Lac operon. When mated yeast colonies are grown on media containing galactose and raffinose to induce expression from pJG4-5, interacting protein pairs yield blue colonies from cleavage of the chromogenic substrate X-gal. To determine if any observed interactions were due to nonspecific association between the bait and prey domains, PBL prey constructs were also mated with an empty vector strain (EV) which simply expresses the AD as fusion protein as the bait. Autoactivity was assessed by growth on glucose plates lacking galactose/raffinose.

None of the PBLs consistently interacted with HopBA1 in this assay. While some PBL kinases did interact in technical replicates, these interactions were not maintained consistently across experiments (Figure 2.2 A). Like the matings with the WT HopBA1 allele, the predicted loss of function HopBA1 H56F allele did not consistently interact with any PBL kinase either (Figure 2.2 B). In mating assays with ZRK2 as bait, none of the PBL kinases interacted with ZRK2 (Figure 2.2 C). In addition to this, the EV matings consistently displayed a high degree of background signal, depending on the PBL mated to it (Figure 2.2 D). Collectively, these negative data do not support the hypothesis that HopBA1 forms a stable protein complex with the

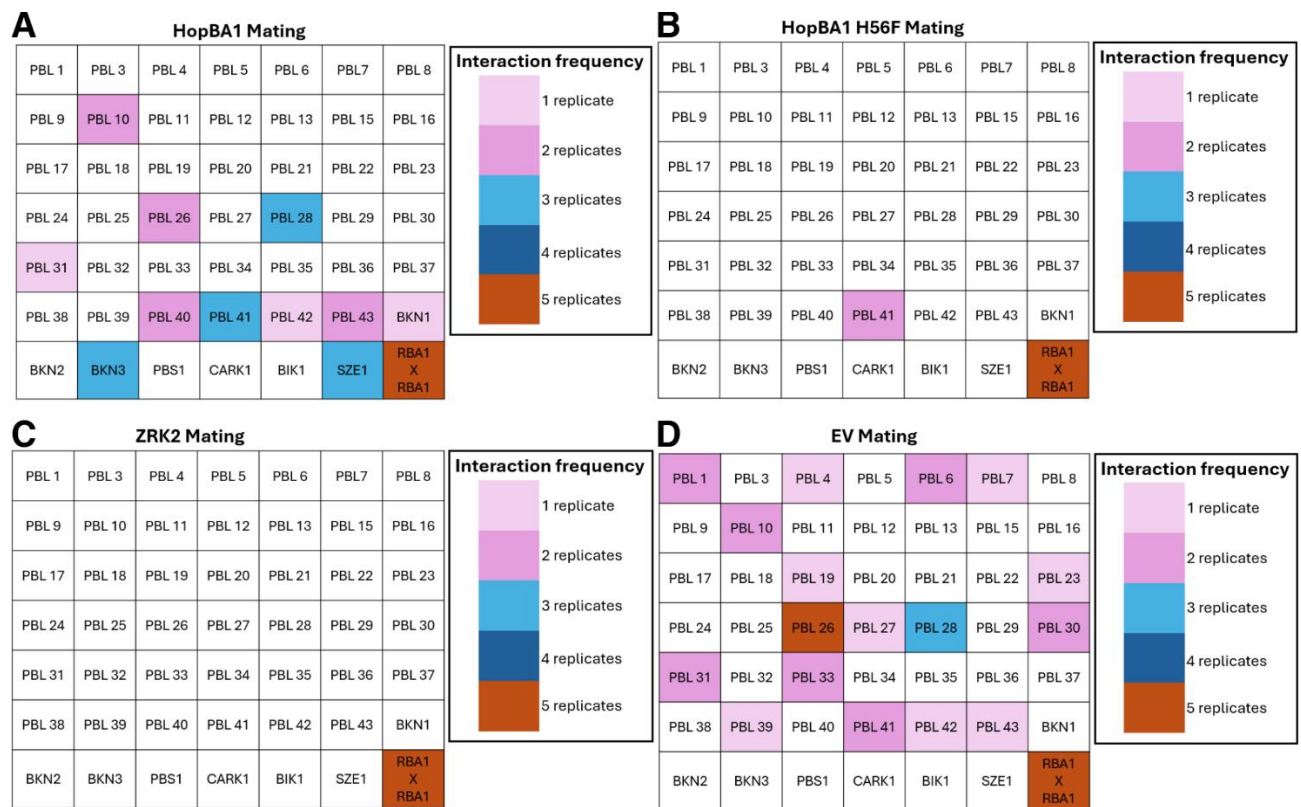


Figure 2.2. Summary of yeast two-hybrid mating results. **A)** Summary of yeast two-hybrid matings of Arabidopsis RLCK VII proteins with HopBA1. **B)** Summary of yeast two-hybrid matings of Arabidopsis RLCK VII proteins with HopBA1 H56F. **C)** Summary of yeast two-hybrid matings of Arabidopsis RLCK VII proteins with ZRK2. **D)** Summary of yeast two-hybrid matings of Arabidopsis RLCK VII proteins with an empty vector (EV) bait strain.

Arabidopsis PBL kinases. The hypothesis that the PBL kinases may form a stable resting state complex with ZRK2 was also not supported.

2.2.2 *Pseudomonas syringae* DC3000 carrying HopBA1 is growth restricted in Arabidopsis *rlck vii* polymutants

To test the hypothesis that HopBA1 targets one or more members of the Arabidopsis PBL kinase family, we obtained Arabidopsis mutants harboring knockout mutations in every member of a given PBL kinase family (Figure 2.1). If a particular clade of RLCK VII proteins is required for HopBA1 perception by ZAR1, Arabidopsis T-DNA lines with insertional mutations in those

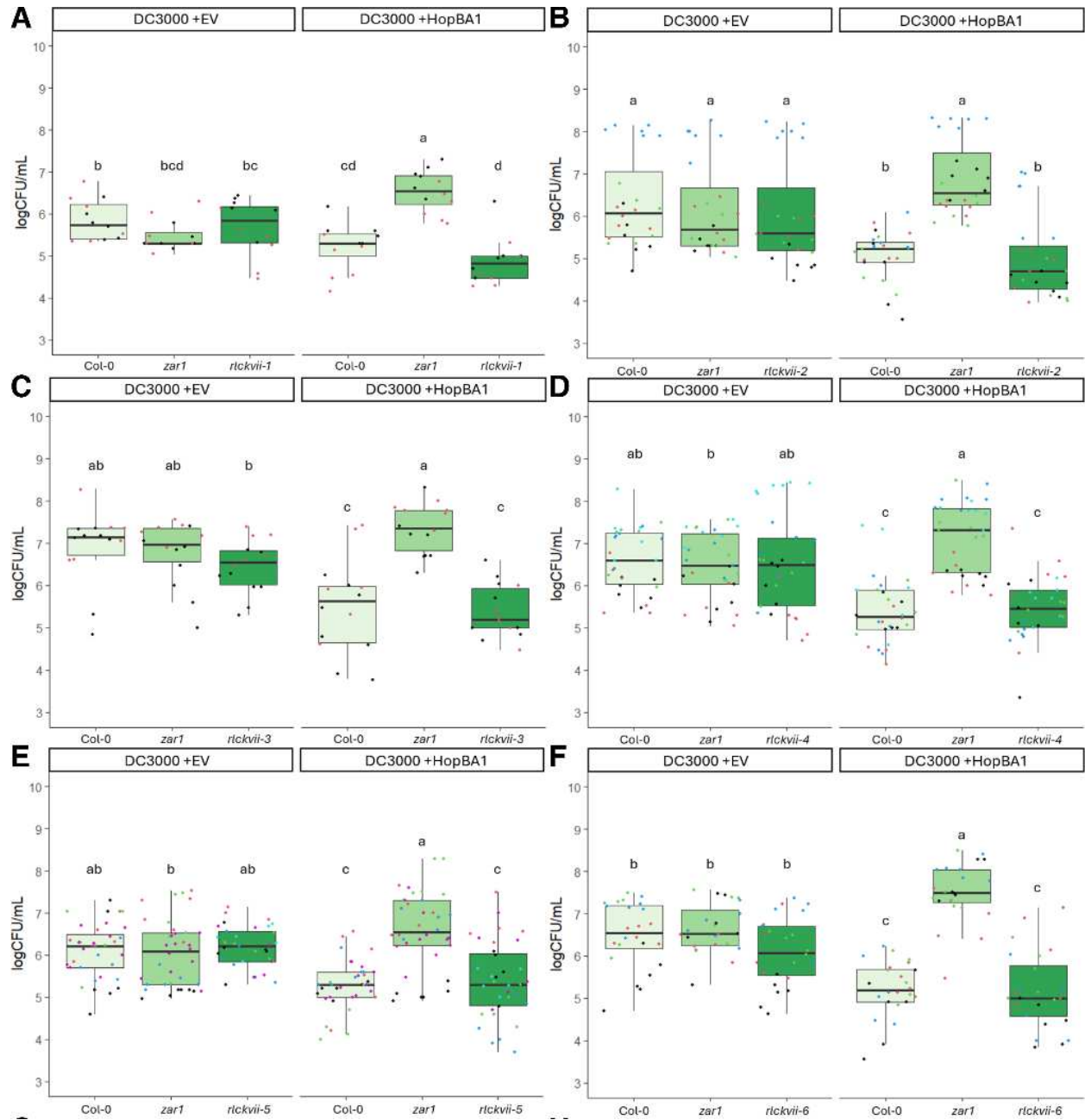
RLCK VII genes should at least partially lose the capacity to restrict the growth of *Pseudomonas syringae* DC3000 carrying HopBA1. We compared the growth of DC3000 EV and DC3000 HopBA1 in WT Col-0, *zar1* and the nine *rlckvii* polymutants four days post pathogen infiltration (Figure 2.3A-I). In these growth assays, each of the nine polymutants restricted the growth of DC3000 HopBA1 at least as much as WT Col-0. In all cases, the Col-0 *zar1* mutant line lost the capacity to restrict the growth of DC3000 carrying HopBA1 relative to WT Col-0, as an internal control for ZAR1-mediated immunity. While none of the *rlck* mutants lost HopBA1-dependent growth restriction, both *rlckvii-2* and *rlckvii-8* were less susceptible to DC3000 (both EV and HopBA1) than WT Col-0, consistent with these subgroups of PBL kinases having a general function as negative regulators of immunity.

2.2.3 Identification of *NbRLCK VII* genes required for the *NbZAR1* and *AtZRK2*-mediated hypersensitive response to HopBA1

To complement the work aimed at understanding HopBA1's biochemical function in Arabidopsis, Tyler Todd, a master's student in the Nishimura lab, tested the hypothesis that HopBA1 functions to manipulate RLCK VII proteins in the model plant, *Nicotiana benthamiana* (*Nb*) (Todd 2024). HopBA1 did not activate an NLR-mediated immune response in WT *Nb*, however transient co-expression of the Col-0 Arabidopsis allele of ZRK2 and HopBA1 induced a robust hypersensitive immune response. This HR was dependent upon *NbZAR1* and was abolished if the transient assay was conducted in a *Nb zar1* mutant (Figure 2.4).

The transient reconstruction of HopBA1-induced HR was leveraged as a platform to screen for other genes required for this response, with a focus on the *Nb RLCK VII* family. Nineteen RNAi silencing constructs that effectively silenced a 75-gene subset of the 91 kinases

with the highest degree of homology to Arabidopsis RLCK VIIs were constructed and employed to probe for a loss of HopBA1 HR in the reconstruction. Briefly, *NbRLCK VII* silencing hairpins



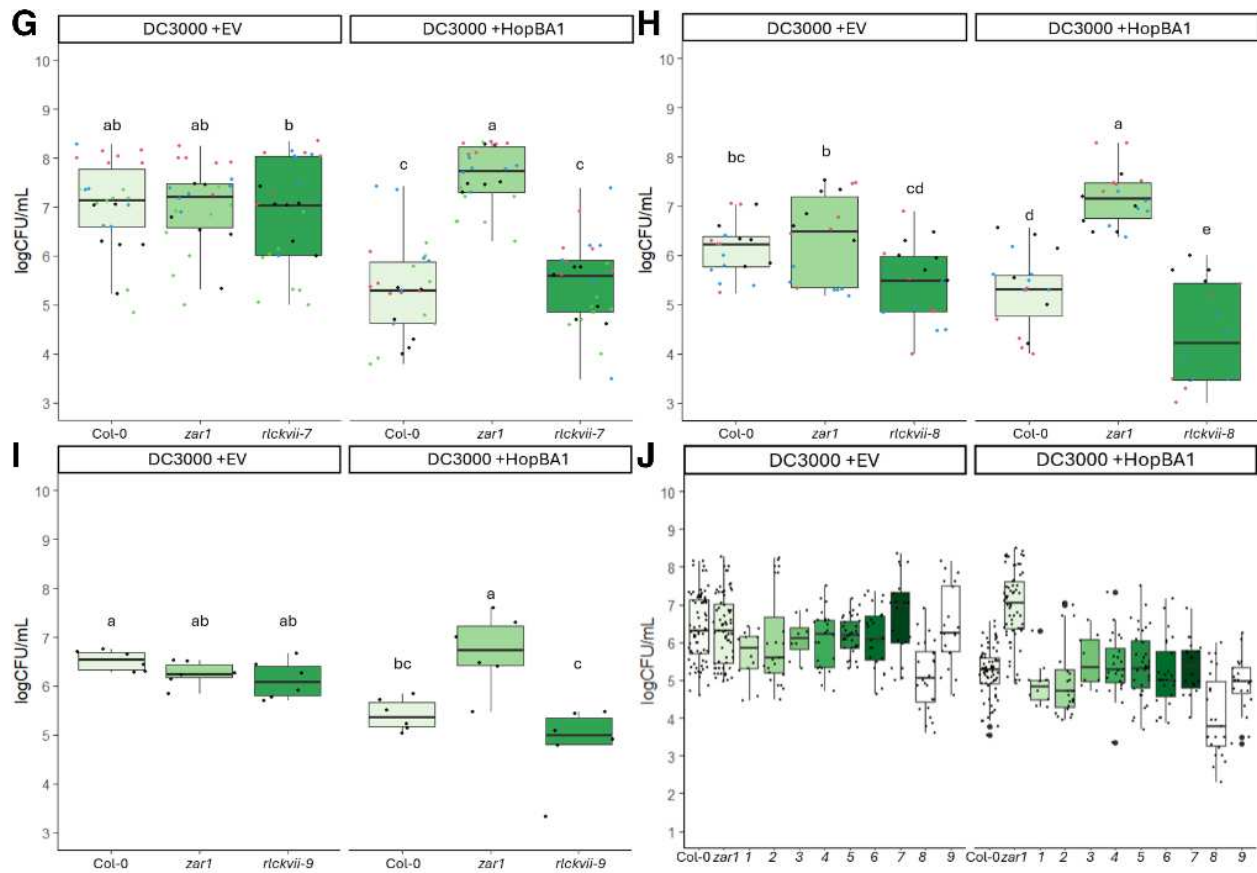


Figure 2.3. Assessment of DC3000 HopBA1 growth in RLCKVII subgroup polymutants. **A)** Growth restriction of DC3000 HopBA1 in the Col-0 WT, *zar1*, *rlckvii-1* mutants. *rlckvii-1* is a septamutant harboring knockout mutations in *PBL1*, *PBL 5*, *PBL6*, *PBL7*, *PBL 23*, *PBL 27* and *PBL 41*. **B)** Growth restriction of DC3000 HopBA1 in the Col-0 WT, *zar1*, *rlckvii-2* mutants. *rlckvii-2* is a pentamutant harboring knockout mutations in *PBL 24*, *PBL 25*, *PBL 26*, *PBL 42* and *PBL 43*. **C)** Growth restriction of DC3000 HopBA1 in the Col-0 WT, *zar1*, *rlckvii-3* mutants. *rlckvii-3* is a double mutant harboring knockout mutations in *PBL 21* and *PBL 22*. **D)** Growth restriction of DC3000 HopBA1 in the Col-0 WT, *zar1*, *rlckvii-4* mutants. *rlckvii-4* is a hexamutant harboring knockout mutations in *PBL 19*, *PBL 20*, *PBL 37*, *PBL 38*, *PBL 39* and *PBL 40*. **E)** Growth restriction of DC3000 HopBA1 in the Col-0 WT, *zar1*, *rlckvii-5* mutants. *rlckvii-5* is a triple mutant in *PBL 34*, *PBL 35* and *PBL 36*. **F)** Growth restriction of DC3000 HopBA1 in the Col-0 WT, *zar1*, *rlckvii-6* mutants. *rlckvii-6* is an octamutant harboring knockout mutations in *PBL 8*, *PBL 12*, *PBL 13*, *PBL 14*, *PBL 15*, *PBL 16* and *PBL 17*. **G)** Growth restriction of DC3000 HopBA1 in the Col-0 WT, *zar1*, *rlckvii-7* mutants. *rlckvii-7* is a triple mutant in *PBL 30*, *PBL 31* and *PBL 32*. **H)** Growth restriction of DC3000 HopBA1 in the Col-0 WT, *zar1*, *rlckvii-8* mutants. *rlckvii-8* is a pentamutant harboring knockout mutations in *PBL 1*, *PBL 9*, *PBL 10*, *PBL 11* and *BIK1*. **I)** Growth restriction of DC3000 HopBA1 in the Col-0 WT, *zar1*, *rlckvii-9* mutants. *rlckvii-9* is a tetramutant harboring knockout mutations in *PBL 2*, *PBL 3*, *PBL 4* and *PBL 18*. **J)** Growth restriction of DC3000 HopBA1 in the Col-0 WT, *zar1*, and *rlckvii 1 to 9* mutants. Data presented are collated from previous panels. Data are not representative of experiments where all *rlckvii* polymutants were assayed together. Numbers on the x-axis denote the specific *rlckvii*

subgroup plotted above. *P. syringae* DC3000 was infiltrated at OD 0.0002 ($\sim 10^5$ cfu) in 10mM MgCl₂.

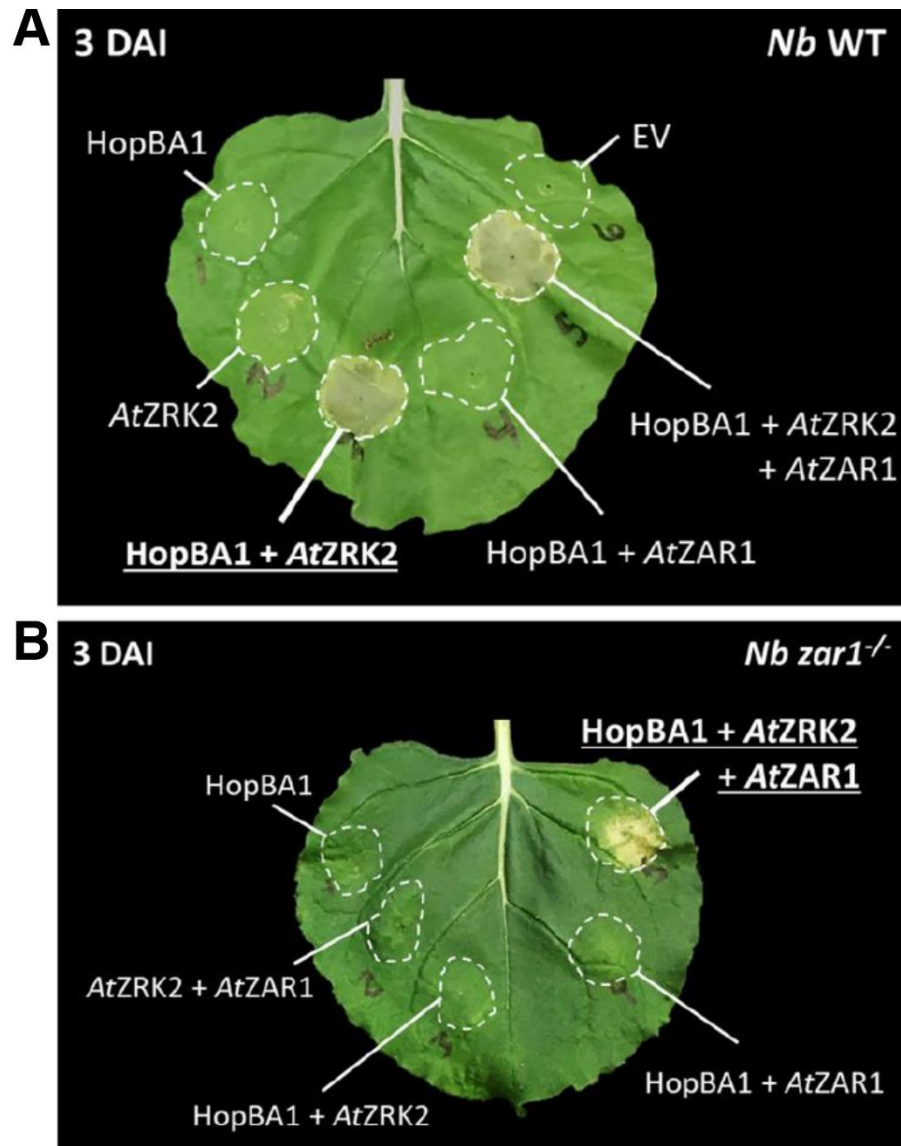


Figure 2.4. Transient expression of *AtZRK2* is sufficient to enable a *NbZAR1* mediated hypersensitive response to HopBA1. **A)** Transient expression of HopBA1 by Agrobacterium is insufficient to trigger immunity (i.e., cell death or hypersensitive response). Cell death occurs only when HopBA1 is co-expressed with the Arabidopsis pseudokinase adapter, ZRK2. **B)** Cell death is no longer triggered by transient co-expression of HopBA1 and At ZRK2 in the *Nb zar1* background, further indicating that recon-structed immunity requires *Nb ZAR1*. Host recognition of HopBA1 can be rescued by co-expression with At ZKR2 and At ZAR1. Leaves pictured 3 days after infiltration. Bacteria were delivered by needleless syringe at a total optical density (OD600) of 0.6. Experiments were replicated multiple times on 2 – 3 plants (3 leaves per plant) on each occasion. Figure from Todd et al. 2024.

were infiltrated into WT *Nb* and allowed to express for 2 days. Following this, HopBA1 and ZRK2 were co-infiltrated into the spots where hairpins were expressed, and leaf spots were monitored for 3 additional days to visualize HR development. *NbZARI* was silenced as a positive control for silencing capacity, and a non-targeting *HSP18* silencing construct was infiltrated as a negative control for silencing and a positive control for HR. From this screen, two silencing constructs, p259 and p273, were found to consistently suppress HR in the reconstruction (Figure 2.5). This group of kinases were found to have the highest degree of amino acid sequence homology to subgroups 5 (silenced by p259) and 6 (silenced by p273) Arabidopsis RLCK VIIs. Thus, we named these PBL kinases *NbPBL5-X*, and *NbPBL6-X* where *X* is the particular member of the NbRLCK VII subgroup silenced in the screen.

2.2.4 Transient co-expression of HopBA1 with *NbPBLs* reveals a HopBA1-dependent putative cleavage product

We successfully cloned the coding sequence of *NbPBL5-1* (*Niben101Scf03100g00002.1*), *NbPBL6-2* (*Niben101Scf03619g00005.1*), *NbPBL6-3* (*Niben101Scf08926g01017.1*), and *NbPBL6-4* (*Niben101Scf09211g00011.1*) from a genomic *Nb* DNA template with BP ends for Gateway cloning (Table A2.2). The *NbPBL6-2*, *NbPBL6-3* and *NbPBL6-4* sequences were then moved into binary vectors for *Agrobacterium*-mediated transient expression with a C-terminal FLAG epitope tag in *Nb*. We were unable to clone *NbPBL5-1* into an expression vector. The *NbPBL* subgroup 6 proteins readily accumulated in transient expression assays (Figure 2.6 A). In lanes where HopBA1 and *NbPBL6-2* and *NbPBL6-3* co-expression samples were run, a slightly smaller PBL band was observed, approximately 4-9kDa less than the expected 51.2 kDa (Figure 2.6 A). This HopBA1-dependent size shift was not observed for *NbPBL6-4* (Figure 2.6 A). These

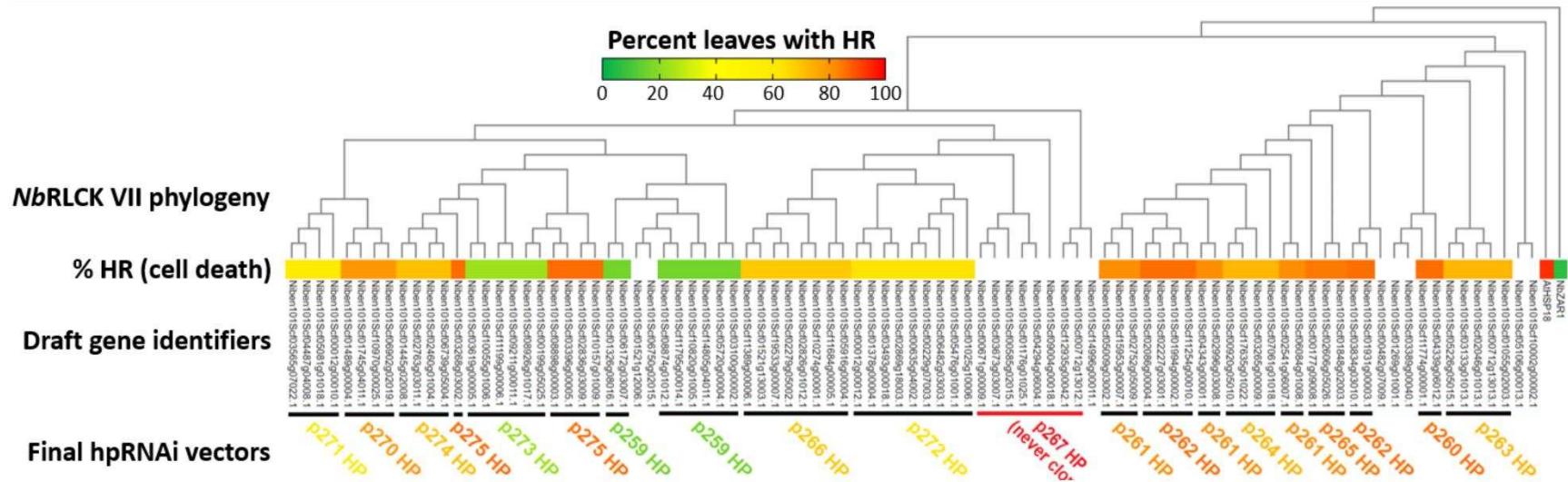


Figure 2.5. The draft *Nb* RLCK VII phylogeny based on homology to the published subfamily phylogeny in *Arabidopsis* with silencing constructs overlaid based on fragment coverage of the listed genes. The observed percent cell death is given by color, ranging from 0% cell death (green) to 100% cell death (red). The silencing construct “p267 HP” was never cloned and therefore was not tested in the RNAi screen. The positive control (ZAR1) and the negative control (Hsp18 3’ UTR) hairpin/silencing constructs are also provided for reference. “p259 HP” (clade 5) and “p273 HP” (clade 6) were identified as promising HopBA1 targets as their silencing resulted in the greatest reduction in % cell death. Figure from Todd et al. 2024.

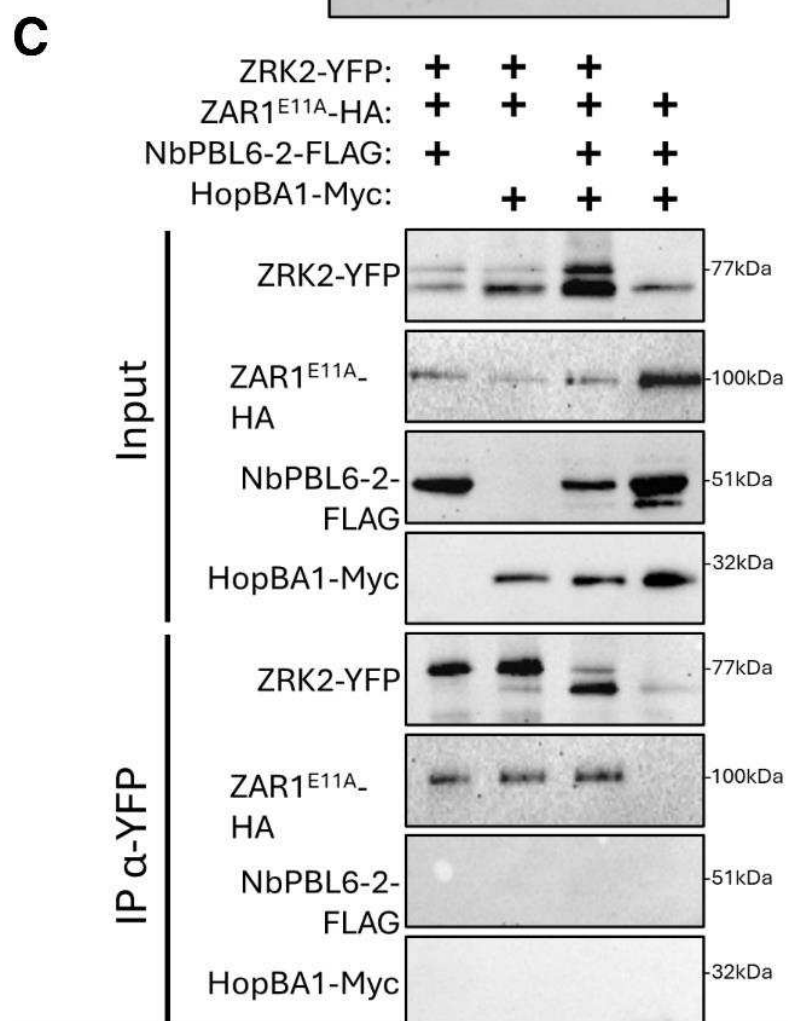
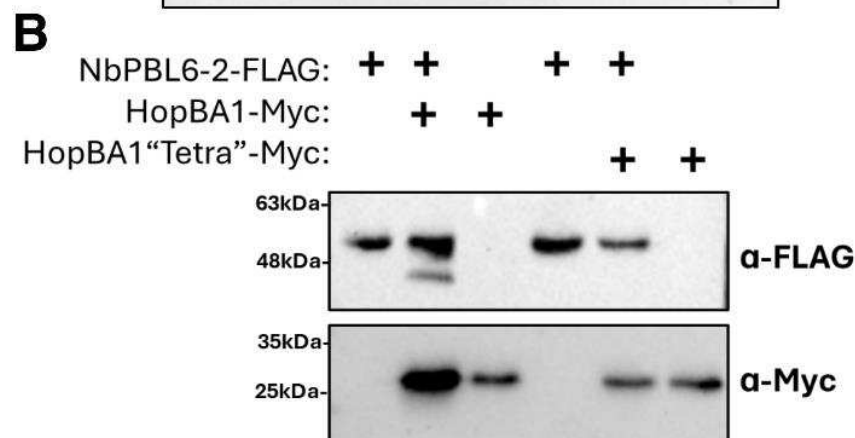
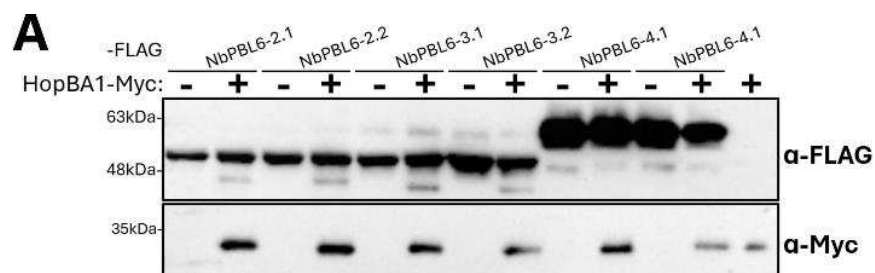


Figure 2.6. Transient expression of NbPBL kinases reveals potential proteolytic activity of HopBA1. A) Transient co-expression of NbPBL6-2 and NbPBL6-3 with HopBA1 led to a ~6kDa decrease in apparent molecular weight. Transient expression was conducted in WT *Nb*. Two independently transformed *Agrobacterium* colonies were used to seed NbPBL infiltration cultures, indicated by the number following the decimal in the name (i.e. NbPBL6-2.1, NbPBL6-2 etc.). **B)** Residues in the putative HopBA1 active site are required for production of the NbPBL6-2 size-shifted band. HopBA1 "Tetra" harbors mutations relative to the WT HopBA1 coding sequence that change the following amino acids: H56F, W112A, Y158A and R162A. **C)** The C-terminal end of NbPBL6-2 does not co-immunoprecipitate with the ZAR1-ZRK2 protein complex. The lower band in the anti-YFP blots is presumably a nonspecific band. Lysates were immunoprecipitated with anti-YFP beads and then were immunoblotted for anti-YFP, anti-HA, anti-FLAG and anti-Myc to assess input, immunoprecipitation, and

coimmunoprecipitation. Transient expression was conducted in *Nb zar1* to avoid cell death induction via endogenous *NbZAR1*.

data are consistent with the hypothesis that *NbPBL6-2* and *NbPBL6-3* are proteolytically cleaved (near their N-termini) upon co-expression with HopBA1.

To test the hypothesis that the putative *NbPBL* cleavage product requires HopBA1 enzymatic activity, we co-expressed *NbPBL6-2* with WT and the “Tetra” allele of HopBA1. The HopBA1 “Tetra” protein harbors four point mutations in the hypothetical active site of HopBA1: H56F, W112A, Y158A and R162A (Nishimura et al. 2017) (Further discussion of HopBA1 mutant alleles can be found in Chapter 3). Transient co-expression of *NbPBL6-2* with HopBA1 “Tetra” failed to produce the size shifted band observed upon co-expression with WT HopBA1 (Figure 2.6 B). We next sought to test the hypothesis that the putative *NbPBL6-2* cleavage product may physically interact with the ZAR1-ZRK2 protein complex. In order to avoid ectopic induction of cell death, we conducted co-immunoprecipitation experiments in *Nb zar1* with a ZAR1 E11A mutant that cannot induce cell death (Bi et al. 2021; Gong et al. 2022; J. Wang et al. 2019) (Figure 2.6 C). In these co-immunoprecipitation assays, ZAR1 and ZRK2 form a stable physical complex independent of HopBA1 co-expression. The C-terminal tagged portion of *NbPBL6-2*, however, was not found to interact with the ZAR1-ZRK2 complex in co-immunoprecipitation experiments (Figure 2.6 C).

We also used AlphaFold3 to generate structural models of HopBA1 and *NbPBL6-2* to gain insight into potential points of interaction between the two proteins (Abramson et al. 2024) (Figure 2.7). While AlphaFold3 generated a structure of HopBA1 that closely resembles its crystal structure (Nishimura et al. 2017), and the core of *NbPBL6-2* resembles a plant kinase protein, the structural model of the two proteins predicted interaction was largely uninformative.

The N-terminal region of *NbPBL6-2*, where cleavage by HopBA1 would likely occur, was not able to be confidently folded and is represented in the model as a disordered loop. The putative active site residues of HopBA1 (Figure 2.7, orange) are also not in contact with *NbPBL6-2*, suggesting that this model is likely not an accurate representation of biological reality, or that our hypotheses of HopBA1's active site are incorrect, or that *NbPBL6-2* cleavage is not directly mediated by HopBA1.

2.2.5 Transient co-expression of HopBA1 with Arabidopsis RLCK VII proteins does not induce accumulation changes

While Arabidopsis RLCK VII polymutants did not reveal any impacts on ZAR1-mediated growth restriction of DC3000 HopBA1, we were still interested in testing the hypothesis that some of the Arabidopsis RLCK VIIs might be targeted by HopBA1. If Arabidopsis RLCK VIIs function redundantly to activate ZAR1, we would still be able to observe HopBA1-induced cleavage via Western blot similar to what we saw in assays with transiently HopBA1 co-expressed with *NbPBL6-2*. The Arabidopsis RLCK VII kinase cDNA clones described above were cloned into binary expression vectors that allowed for constitutive

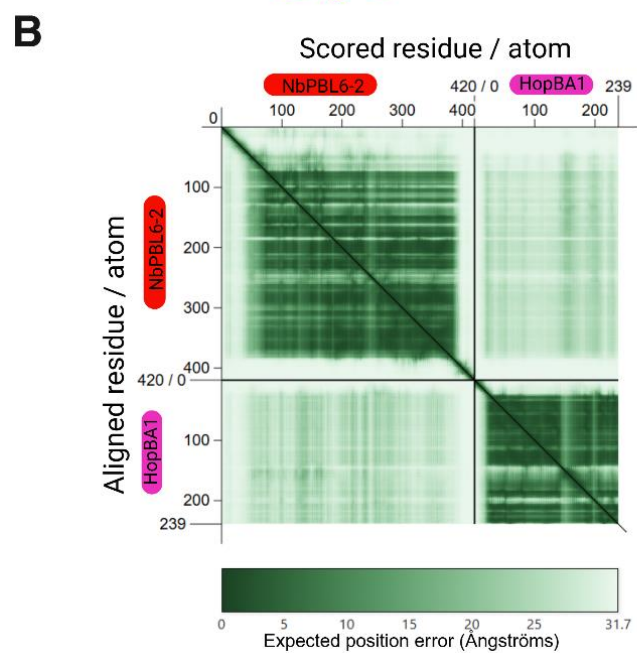
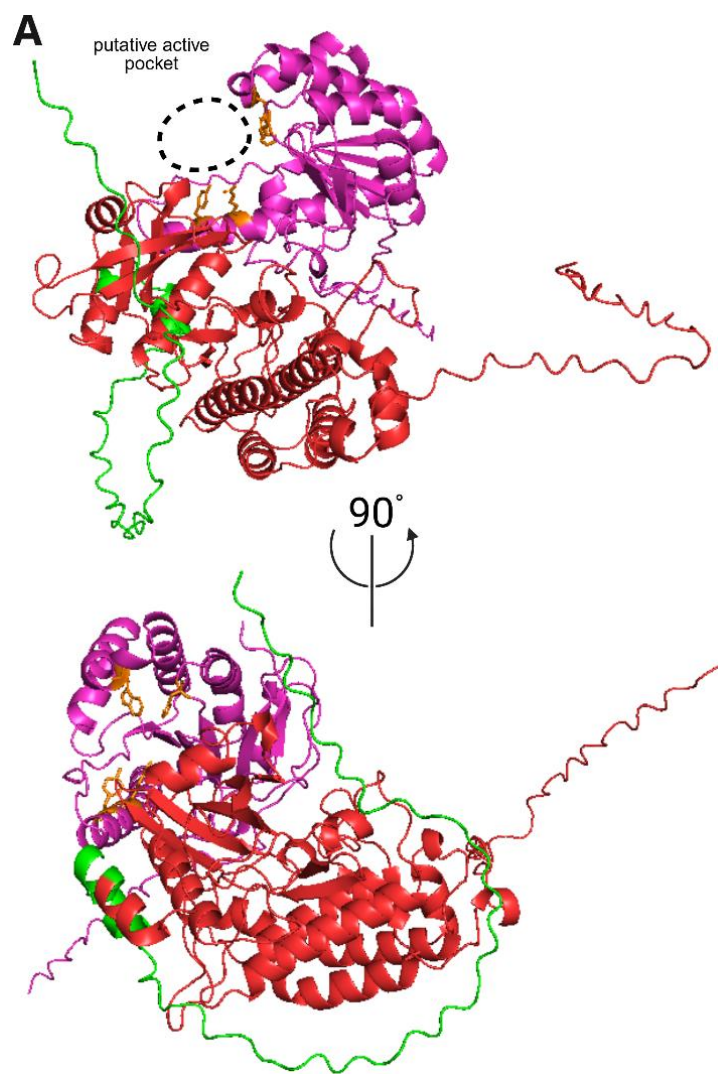


Figure 2.7. Structural modeling of a HopBA1-NbPBL6-2 complex does not align with experimental data. A) Alphafold3 predictions of HopBA1 and NbPBL6-2 structures. NbPBL6-2 is shown in red, with the first 60 amino acids, corresponding to a putatively cleaved ~6kDa peptide, colored green. HopBA1 is shown in magenta, with the four putative active site residues mutated in the “Tetra” allele shown in orange. The HopBA1 putative active site is outlined in a dashed circle in the top orientation. **B)** Predicted aligned error heatmap of the modeled complex.

overexpression of epitope-tagged RLCK VIIs in *N. benthamiana* transient expression assays.

Arabidopsis RLCK VIIs were transiently expressed with or without HopBA1 in WT *Nb* and their accumulation was assessed via western blot. In general, these RLCK VII proteins displayed accumulation defects, potentially because these proteins do not tolerate N-terminal tagging or perhaps due to intrinsic instability of some Arabidopsis RLCK VIIs when overexpressed in *Nb*.

2.3 Discussion

The role of RLCK VII proteins in immunity has long been appreciated, and many pathogen effectors have been found to post-translationally modify members of this family to facilitate host colonization (Bastedo et al. 2019; Lee et al. 2020; Z. D. Lin et al. 2015; X. Ma et al. 2020; Rao et al. 2018; B. Song et al. 2026; Swiderski and Innes 2001; Thor et al. 2020; W. Wan et al. 2018). Cleavage of PBS1 by AvrPphB and subsequent recognition of this cleavage by RESISTANT TO *P. SYRINGAE* 5 (RPS5) was an early example of the “guardee” hypothesis for NLR activation. Years after the AvrPphB-RPS5 relationship was first described, another CC-NLR, ZAR1 was found to “guard” members of the Arabidopsis RLCK VII family. ZAR1 in particular has been shown to indirectly monitor PBL kinase status through cooperation with ZRK family pseudokinases, both in Arabidopsis and *N. benthamiana* (Bastedo et al. 2019; Lewis et al. 2008; Martel et al. 2020; Schultink et al. 2017, 2019). ZAR1 is also somewhat unique among

NLRs in its capacity to induce immune responses to multiple pathogen effectors, including HopBA1 (Laflamme et al. 2020; Martel et al. 2020).

These previous works establishing the link between ZAR1 and RLCK VIIs led us to hypothesize that the unknown virulence targets of HopBA1 may be members of the RLCK VII family. Our yeast two-hybrid screen for physical interactions between HopBA1 and the Arabidopsis RLCK VIIs did not reveal any consistent interactions (Figure 2.2). These data may be explained by the subsequent finding that HopBA1 induces a 4-9kDa size shift in *NbPBL6-2* and *NbPBL6-3* (Figure 2.6). One hypothesis that could explain these data is that HopBA1 directly acts as a protease upon *NbPBL6-2* and *NbPBL6-3* substrates. Observation of the HopBA1 induced putative cleavage products in the context of C-terminally epitope tagged *NbPBL* substrates indicates that the cleavage of these *NbPBLs* is occurring at their N-termini. In our yeast two-hybrid assays, the Arabidopsis RLCK VII proteins are N-terminally tagged with the B42-AD. If HopBA1 is proteolytically processing *AtRLCK VII* proteins, proximity between LexA-DBD-tagged HopBA1 and the B42-AD of the bait proteins would likely be insufficient to yield consistently positive yeast two-hybrid results. Furthermore, assays using the HopBA1 H56F putative active site mutant may not be sufficient to fully abolish HopBA1 protease activity, as evidenced by *NbZAR1* activation by mutated HopBA1 alleles in reconstruction assays (Expanded upon in Chapter 3). Future work using the HopBA1 “Tetra” mutant may yield more informative yeast two-hybrid results as loss of function effector alleles have been found to more robustly interact with their targets in similar assays (Bastedo et al. 2019).

We leveraged existing Arabidopsis genetic resources to test the hypothesis that Arabidopsis RLCK VII mutants may lose the capacity to restrict the growth of *Pseudomonas syringae* DC3000 carrying HopBA1. Previously generated Arabidopsis RLCK VII polymutants

were subjected to pathogen growth assays with virulent DC3000 EV and ZAR1-recognized DC3000 HopBA1 (Figure 2.3). None of the *RLCK VII* polymutants exhibited a loss of DC3000 HopBA1-dependent growth restriction. These data imply that either the Arabidopsis RLCK VIIs are not “guarded” by ZAR1 and that the loss of them does not lead to a reduced capacity for ZAR1 activation, or that ZAR1-ZRK2 redundantly “guards” RLCK VIIs across subgroups, meaning that loss of any one subgroup may be insufficient to impact HopBA1 recognition by ZAR1. The potential for cross-subgroup RLCK VII targeting by HopBA1 is indeed supported by the results obtained in the *Nb* silencing assays (Figure 2.4). Both hairpin groups p259 and p273 were found to equivalently silence the *Nb*ZAR1-mediated cell death responses to HopBA1. These hairpins silence closely related groups of *Nb*RLCK VIIs, and it is possible that there is cross-subgroup silencing by both p259 and p273. Silencing was not assessed on a transcript level, so there are no data to indicate otherwise. Additionally, independent silencing of two clades of *Nb*RLCK VIIs being sufficient to abolish *Nb*ZAR1-mediated cell death is somewhat incongruent with existing models for NLR activation. Typically, if one effector target is present and modified, it is expected that NLR activation could occur even in the absence of a subset of these effector targets. There is a possibility that, for instance, a multi-membered RLCK VII complex is formed between individual RLCK VII proteins in the subgroups targeted by both p259 and p273 and the status of this complex is being guarded by the *Nb*ZAR1-*At*ZRK2 complex. In this model, loss members of each subgroup of *Nb*RLCK VII would be independently sufficient to silence the response to HopBA1, however there is also no evidence to support this model.

If the lower molecular weight band produced upon co-expression of *Nb*PBL6-2 and *Nb*PBL6-3 with HopBA1 is taken to be resultant of HopBA1’s activity as a protease, we can

hypothesize what HopBA1's function as a virulence factor may be. Given the well characterized functions of RLCK VII proteins in signal transduction during canonical PTI responses, it is likely that HopBA1 targets *NbPBLs* to disrupt these signaling events. It is less clear, however, what the consequences of *NbPBL6-2* cleavage may be. We have made preliminary attempts to purify *NbPBL6-2* to conduct *in vitro* cleavage and kinase activity assays, however these have yet to be completed. Arabidopsis BOTRYTIS INDUCED KINASE 1 (BIK1), a RLCK VII, has been found to be both monoubiquitinated and phosphorylated at residues in its N-terminus (W. Lin et al. 2014; X. Ma et al. 2020). Monoubiquitination of BIK1 regulates its turnover and ability to signal alongside PRR receptor complexes (X. Ma et al. 2020). While BIK1 was found to be N-terminally phosphorylated, the relevance of this phosphorylation in immunity has not been explored (W. Lin et al. 2014). Recent work has led to the creation of RLCK VII polymutants in *Nb*, analogous to those in Arabidopsis (W. R. H. Huang et al. 2024). This work assayed PTI responses in the RLCK VII subgroup 6 polymutant and found that these mutants are compromised in initiation of PTI reactive oxygen species (ROS) bursts in response to a range of elicitors (W. R. H. Huang et al. 2024). These data suggest that there may be a relevant "reason" for HopBA1 to target *NbPBL6-2* and -3 for cleavage, assuming that this cleavage negatively regulates *NbPBL* function in some way. Characterization of the immune signaling consequences of HopBA1-induced *NbPBL6-2* cleavage could also serve as a lens through which the functionality of the N-terminus of RLCK VII kinases may broadly be more well understood.

Taken together, these data from Arabidopsis polymutant growth assays and the *Nb* silencing screen support a view of ZAR1-RLCK VII "guarding" where members of both subgroups 5 and 6 are targeted by HopBA1 and recognition of HopBA1 by ZAR1-ZRK2 depends on the presence of members of either group of proteins.

2.4 Material and methods

2.4.1 Bacterial strains and growth conditions

Escherichia coli (*E. coli*) Top10 and *Agrobacterium tumefaciens* strain GV3101 were grown in LB medium at 37°C and 28°C, respectively. *Pseudomonas* strains were grown at 28°C in King's B medium. The *E. coli* antibiotic concentrations used (in micrograms per milliliter) were ampicillin 100, kanamycin 30, gentamycin 25, and spectinomycin 50. *Agrobacterium* antibiotic concentrations used (in micrograms per milliliter) were gentamycin 50, kanamycin 100, rifampicin 100, and spectinomycin 100. *Pseudomonas* antibiotic concentrations used (in micrograms per milliliter) were gentamycin 25, kanamycin 30, and rifampicin 50.

2.4.2 Plant materials and growth conditions

Arabidopsis plants were grown under 100μMol light intensity, 75% humidity at 22°C short day conditions (8h light 16h dark) in a Conviron growth chamber. Arabidopsis were seeded into pots where they germinated and were grown until they had four true leaves (approximately 9 days). Arabidopsis seedlings for use in growth curves were transplanted into flats in a grid. Arabidopsis were planted into 8 plant deep columns of a flat where each column is composed of a particular Arabidopsis genotype, with the genotype being different on each half of the flat. Between 3 and 5 Arabidopsis genotypes were included in a given flat, depending on the number of genotypes used in an assay. *N. benthamiana* plants were grown in Conviron growth chambers at 25 °C, 70% relative humidity, and 15 h photoperiod with a light intensity of ~80 μE m⁻² s⁻¹. *N. benthamiana* plants were sown at a density of ~100 seeds in a 4-inch pot and were grown until they had two true leaves (approximately 9 days). *N. benthamiana* seedlings were transplanted as individuals into 4-inch pots and were allowed to grow under a humid dome in the

growth chamber for ~2 weeks after transplanting, at which point the dome was slowly removed over the course of 3 days to acclimate plants to humidity conditions in the growth chamber.

2.4.3 Transient expression in *Nicotiana benthamiana* via *Agrobacterium* delivery

Agrobacterium tumefaciens strain GV3101 was infiltrated using needleless syringes into leaves of ~4-5 week-old *Nb*. GV3101 was infiltrated at OD₆₀₀ of 0.80. The viral suppressor of silencing, p19, was included in the inoculum at OD 0.1, as described previously (Nishimura et al. 2017; L. Wan et al. 2019). GV3101 was transformed via heat shock with ~500 ng of binary construct DNA or via triparental mating with *E. coli* harboring the binary plasmid of interest. GV3101 liquid cultures were grown overnight at 28°C under selection (50 µg/mL each of rifampicin, gentamicin, spectinomycin or kanamycin, respectively). Cultures were pelleted at 3000 x g for 3 min and then induced for ~3 h in 10 mM MES buffer (pH 5.6), 10 mM MgCl₂, and 100 µM acetosyringone prior to leaf infiltration.

2.4.4 Yeast two-hybrid mating assay

Protein interactions were assayed using Gateway-compatible versions of the pEG202 “bait” (N-terminal LexA DNA binding domain fusion, TRP⁺) and pJG4-5 “prey” (N-terminal B42 activating domain fusion, HIS⁺) vectors. pDONR207 or pENTR223 cDNA Gateway entry clones of the Arabidopsis PBL kinase family (Bastedo et al. 2019) were shuttled into pJG4-5 using LR Clonase II. HopBA1 and ZRK2 pDONR207 entry clones were similarly shuttled into pEG202. Resulting vectors were sequence verified and transformed into haploid EGY48 (‘α’ mating type) (pJG4-5) and haploid RFY206 (‘A’ mating type) containing the LacZ reporter pSH18-34, URA⁺ (pEG202). The resulting haploid strains were mated in YPD media shaking at 100 rpm at 30°C overnight, and diploids were selected on synthetic medium (SC-HUW).

Diploids were spotted in triplicate onto SC-HUW +X-gal (80 µg/mL) containing either galactose (inducing medium) or glucose (non-inducing medium). PBL kinase “prey” strains were also mated with RFY206 containing pSH18-34 and an insertion-free pEG202 “EV” plasmid. Positive control matings between EGY48 and RFY206 carrying RBA1 in pJG4-5 and pEG202 were also spotted onto each plate to confirm the integrity of the X-gal and galactose in the mating plates. All yeast plates contained 2% agar and were incubated at 30°C.

2.4.5 Immunoblots

Three 6 mm discs from three different *Nb* leaves were harvested ~36 h post infiltration into 2.0 mL tubes containing single glass beads and flash frozen in liquid nitrogen. Leaf tissues were homogenized in a TissueLyzer II (Qiagen) at 30 Hz for 30 s. Proteins were extracted in 200 µL of lysis buffer [50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.2% Triton X-100, 10% (vol/vol) glycerol, 1/100 Sigma protease inhibitor cocktail]. Lysates were centrifuged at 4°C for 5 min at 2300g (5K RPM, Beckman FA241.5P rotor) and then stored on ice. Equal lysate volumes were loaded for separation and analysis by SDS-PAGE. Samples were transferred overnight at 4°C in 1X Towbin buffer or using an Owl HEP-1 Semidry Electroblotting System for 90 minutes at room temperature. Following transfer, nitrocellulose blots were blocked for 1 h at RT (room temperature) in 3% (wt/vol) nonfat dry milk 1X TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween 20). Immunoblots for primary antibodies [HA (3F10, Roche) or GFP (Cat# 1181446001, Roche)] were incubated for 1 h at RT in 3% (wt/vol) nonfat dry milk TBS-T at 1:2,000, followed by 3X 5 min washes in 1X TBS-T. Secondary HRP-conjugated goat anti-rabbit or goat anti-mouse IgG (Millipore Sigma) was added at 1:10,000 in TBS-T (3% milk) and incubated for 1 h at room temperature on a platform shaker, followed by 3X 5 min washes in 1X

TBS-T. Chemiluminescence detection was performed using Clarity Western ECL Substrate (Bio-Rad) and developed with a ChemiDoc MP chemiluminescent imager (Bio-Rad).

2.4.6 Pathogen growth curves

Bacterial growth assays were performed as described in (J. B. Morel and Dangl 1999). Briefly, *Pseudomonas syringae* DC3000 was grown overnight on Kings B medium plates, washed off the plate for ~10 minutes in 10mM MgCl₂ and diluted to an OD₆₀₀ of 0.0002. Cultures were then injected as described previously for ion leakage assays. 4 days following infiltration, plants were cored with a number 2 cork borer. Each biological replicate contains 6 cores from 6 different plants in 200 μ L of 10mM MgCl₂; each growth curve experiment represents 6 biological replicates taken from 8 plants of a given Arabidopsis genotype. Samples were lysed for 30s at 30hz using a Qiagen TissueLyser II. Following processing, 800 μ L of 10mM MgCl₂ was added to each tube. 100 μ L of each sample was added to a 96 well plate for serial dilutions. 20 μ L of each sample was then transferred to 180 μ L of 10mM MgCl₂, with 20 μ L of each dilution being serially diluted a total of 6 times (range of 1-1000000-fold dilution). 10 μ L of each dilution was plated onto KB media agar carrying the appropriate antibiotics. Plates were allowed to grow for 48 h at 28°C before colonies were counted.

2.4.7 Protein structure modeling

Protein structure models for NbPBL6-2 and HopBA1 were generated using AlphaFold3 (Abramson et al. 2024) and visualized with PyMOL.

2.4.8 Statistical analyses

Multiple comparisons were analyzed via two-way ANOVA with post-hoc Tukey HSD (honestly significant differences) using R Studio (version 1.4.1717, RStudio Team (2021),

Boston, MA). Comparisons of significance are indicated with compact letter display (CLD). Over-lapping letters are non-significant ($p>0.05$), while separate letter classes indicate $p<0.05$ or better.

2.5 Author contributions

This chapter incorporates data from experiments conducted by Sam C. Ogden and Tyler S. Todd. Sam C. Ogden carried out yeast two-hybrid construct cloning and mating assays, *rlck vii* polymutant Arabidopsis growth curves, Western blot analysis, and Alphafold3 structural modeling. Tyler S. Todd conducted and photographed AtZRK2 transient HopBA1 recognition assays (Figure 2.4), generated NbRLCK VII RNAi silencing constructs, carried out silencing experiments, and produced the NbRLCK VII phylogeny with silencing results (Figure 2.5). All figures created using BioRender.com.

CHAPTER 3: ZAR1 REQUIRES CANONICAL TIR-NLR SIGNALING COMPONENTS TO INITIATE COMPLETE IMMUNE RESPONSES AGAINST *PSEUDOMONAS SYRINGAE* IN ARABIDOPSIS²

3.1 Summary

Decades of plant pathology research has identified hundreds of intracellular nucleotide-binding site leucine-rich repeat (NLR) receptor proteins that mediate pathogen perception. Historically, these receptors have been studied in the context of pathogen virulence proteins and a single cognate NLR receptor, and comparatively much less research has been devoted to understanding how these NLRs function in the broader context of a functioning immune system. Here, through study of the recognition of the *Pseudomonas syringae* type III effector protein, HopBA1, by two NLRs in Ag-0 Arabidopsis, Response to HopBA1 (RBA1) and HopZ-Activated Resistance 1 (ZAR1), we characterize an unequal partnership in initiating cell death and growth restriction responses by distinct classes of plant immune receptor. HopBA1 recognition was also found to be dependent on residues in its putative active site, indicating that HopBA1 enzymatic activity is required for recognition. This work also revealed a partial dependence of ZAR1 upon the canonical Toll-interleukin receptor (TIR)-NLR immune response regulator, ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1). We follow this observation with a genetic dissection of

²This chapter is a modified version of a manuscript in preparation for submission under the same title

Ogden S. C, Todd T. S., Gagnier T., Nishimura M. T. (2026) ZAR1 requires canonical TIR-NLR signaling components to initiate complete immune responses against *Pseudomonas syringae* in Arabidopsis. *In preparation*

ZAR1-mediated responses to other pathogen effectors in Col-0. We find that ZAR1 generally requires EDS1 and the PHYTOALEXIN DEFICIENT 4 (PAD4) -ACTIVATED DISEASE RESISTANCE 1 (ADR1) signaling node to varying degrees to initiate immune responses, depending on the effector used to activate ZAR1.

Taken together, these data support a more deeply interconnected model for plant immune responses, where even “singleton” NLR immune receptors have nuanced signaling dependencies, and caution against assumptions that immune receptors will behave equivalently across distinct genetic backgrounds.

3.2 Results

3.2.1 Immune responses to HopBA1 in Ag-0 Arabidopsis partially require RBA1 and ZAR1

Given the dual recognition of HopBA1 by both RBA1 and ZAR1, we sought to characterize the involvement of each receptor in pathogen growth restriction and cell death responses to HopBA1 as well as any potential interactions or synergy between the two receptors (Laflamme et al., 2020; Nishimura et al., 2017). To dissect the relative contributions of the Ag-0 alleles of RBA1 and ZAR1 to the response to HopBA1, we generated Ag-0 *rba1*, *zar1* and *rba1 zar1* mutants. Ag-0 is a late-flowering accession, so for practicality, we generated an early-flowering mutant of Ag-0 by Cas9 mutagenesis of the *Frigida* gene, which co-segregated with the Ag-0 delayed flowering phenotype (Figure 3.1A). The Ag-0 *fri* mutation results in an N-terminal deletion similar to what is found in early-bolting accessions such as Col-0, and it did not result in altered susceptibility to *Pseudomonas syringae* pv. *tomato* DC3000 (Hereafter DC3000) EV or DC3000 HopBA1 (Figure 3.1B). All Ag-0 lines used in this study are in the Ag-0 *fri*

background (hereafter “Ag-0”). We used Cas9 to generate a *zar1* knockout mutation by introducing a frameshift mutation in the 5’ end of the ZAR1 coding sequence to produce a stop codon at amino acid 55 in the ZAR1 coding sequence (Figure 3.1C). The previously published Ag-0 *rba1-1* line (Nishimura et al., 2017) was crossed with *zar1-6* to generate an Ag-0 *rba1 zar1* double mutant. The Ag-0 *rba1*, Ag-0 *zar1*, Ag-0 *rba1 zar1* and Ag-0 *eds1-2* lines were subsequently evaluated for their responses to HopBA1 delivery by DC3000 HopBA1 and the Pf0-1 EtHAN strain (Thomas et al. 2009).

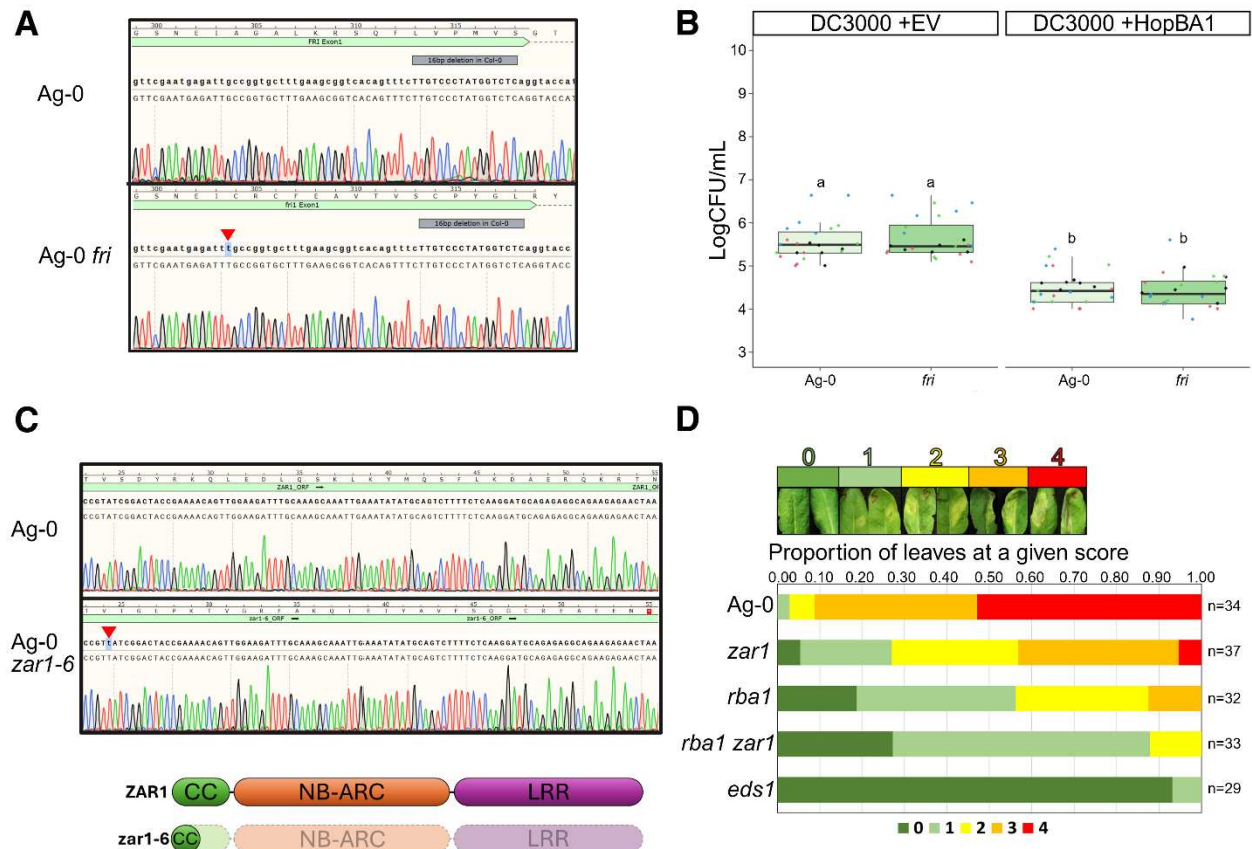


Figure 3.1. Comparison of Ag-0 WT and Ag-0 *fri*, construction of the Ag-0 *zar1-6* mutant and qualitative cell death responses in Ag-0 mutants. A) Ag-0 *fri-1* harbors a T base insertion following bp 909 in the *FRI* coding sequence (red arrowhead), leading to a frame shift after amino acid 303. This Ag-0 frameshift is upstream of the 16bp deletion responsible for early flowering in *Col-0* (gray box) **B)** WT Ag-0 and Ag-0 *fri* exhibit identical growth restriction responses to DC3000 EV and DC3000 HopBA1. Bacterial growth was assayed 4 days post infiltration into the indicated Ag-0 genotypes. *P. syringae* DC3000 was infiltrated at OD 0.0002

($\sim 10^5$ cfu) in 10mM MgCl₂. In boxplots, individual biological replicates are shown as dots, dots are colored according to independent experimental replicates. **C)** Ag-0 *zar1-6* harbors a T base insertion following bp 72 in the *ZAR1* coding sequence, leading to a frame shift at amino acid 24. **D)** Qualitative assessment of cell death in Ag-0 mutants. Cell death was qualitatively scored 3 days post infiltration into the indicated Ag-0 genotypes. Pf0-1 was infiltrated at OD 0.1 in 10mM MgCl₂.

When evaluating the growth of DC3000 HopBA1 in Ag-0, we saw that, compared to WT Ag-0, the *rbal* single mutant did not exhibit a significantly impaired capacity to restrict the growth of DC3000 HopBA1 (Figure 3.2B). Compared to the growth of virulent DC3000 EV, the Ag-0 *zar1* single mutant and the *rbal zar1* double mutant only partially lost HopBA1-dependent growth restriction (Figure 3.2B). To assess HopBA1-induced cell death quantitatively, we subjected our Ag-0 mutants to ion leakage assays. In these assays, we used a non-pathogen, *Pseudomonas fluorescens*, that has been engineered to express a type III secretion system along with our effector of interest (*Pf0-1*). Use of *Pf0-1* or the DC3000 D36E “effectorless” strain used later on, allows for evaluation of cell death elicited by a single effector without the contribution of other effectors to cell death responses confounding the results. These data aligned with the results of qualitative cell death assessment (Figure 3.1D), with the Ag-0 *rbal* and Ag-0 *zar1* mutants displaying partially reduced ion leakage in response to HopBA1 (Figure 3.2D). The Ag-0 *rbal zar1* and Ag-0 *eds1-2* mutants both fully lost the ion leakage response to HopBA1 (Figure 3.2D). While the ion leakage responses to HopBA1 in the *rbal zar1* mutant were statistically equivalent to cell death responses to *Pf0-1* EV, the overall ion leakage displays a distinct positive trend towards more significant ion leakage than the responses to *Pf0-1* EV. Qualitative cell death assays also show a slightly elevated cell death response to HopBA1 in the *rbal zar1* double mutant, supporting the hypothesis that the factors conferring residual resistance to DC3000 +HopBA1 in *rbal zar1* contribute to cell death responses as well. Cell death assays are

intrinsically more variable than pathogen growth assays, and this likely is why the *rbal zar1* ion leakage responses were statistically insignificant. Collectively, these data suggest that an additional receptor or receptors may be involved in mediating HopBA1-dependent growth restriction and possibly cell death in Ag-0 Arabidopsis. Additionally, the loss of immune responses to HopBA1 in the Ag-0 *eds1-2* mutant was unexpected as ZAR1 is expected to be EDS1-independent (Lewis et al. 2010; Macho et al. 2010).

The minimal contribution of RBA1 to DC3000 HopBA1 growth restriction in Ag-0 led us to consider that the traditional growth assay which measures pathogen growth 4 days following pathogen inoculation may miss the contribution of RBA1 to pathogen growth restriction. To observe any RBA1 or ZAR1-mediated pathogen growth restriction not captured in the typical single-timepoint growth curve assay, we measured DC3000 EV and DC3000 HopBA1 growth in the panel of Ag-0 mutants over the course of five days. Relative to DC3000 EV, we observed growth restriction of DC3000 HopBA1 starting one day post infiltration in all Ag-0 genotypes, with Ag-0 WT and Ag-0 *rbal* restricting pathogen growth more strongly than the other genotypes (Figure 3.3). This trend continued through day 5. Ag-0 *zar1*, *zar1 rbal* and *eds1* all displayed an equivalently reduced capacity to restrict DC3000 HopBA1 growth until 3 days post infiltration at which point *eds1* diverged at days 4 and 5, losing additional capacity to restrict DC3000 HopBA1 growth relative to *zar1* and *rbal zar1* mutants. We also observed a reduction in DC3000 HopBA1 burden between 4 and 5 days post infiltration in the *zar1* and *rbal zar1* mutants, suggesting that the uncharacterized factors conferring residual pathogen growth restriction in these backgrounds are particularly relevant at later timepoints (*zar1* HopBA1 D4 vs D5: $p=0.0100$, *rbal zar1* HopBA1 D4 vs D5: $p=0.0272$, two-way analysis of variance (ANOVA) with post hoc Tukey HSD (honestly significant difference)).

3.2.2 Mutation of putative active site residues in HopBA1 leads to altered disease resistance responses in Ag-0 Arabidopsis

To better understand the genetic requirements for HopBA1 recognition in Ag-0, we conducted growth assays in our mutant panel with HopBA1 alleles harboring mutations in its putative active site. Infection of WT, *rba1*, *zar1* and *rba1 zar1* Ag-0 Arabidopsis with DC3000 HopBA1 H56F led to a partial loss of growth restriction in WT and *rba1* Ag-0 compared with the complete growth restriction of DC3000 HopBA1 in these lines (Figure 3.4). In the Ag-0 *zar1* and *rba1 zar1* mutants, DC3000 HopBA1 H56F grew equivalently to DC3000 EV, suggesting that the observed HopBA1 H56F induced growth restriction in Ag-0 WT and *rba1* was mediated by ZAR1. In addition to this observation, it can also be inferred that the factors conferring

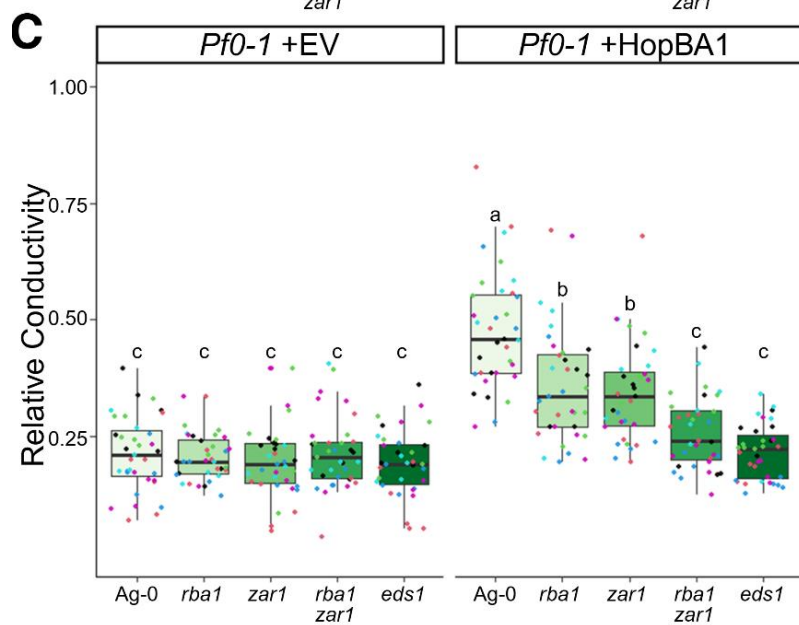
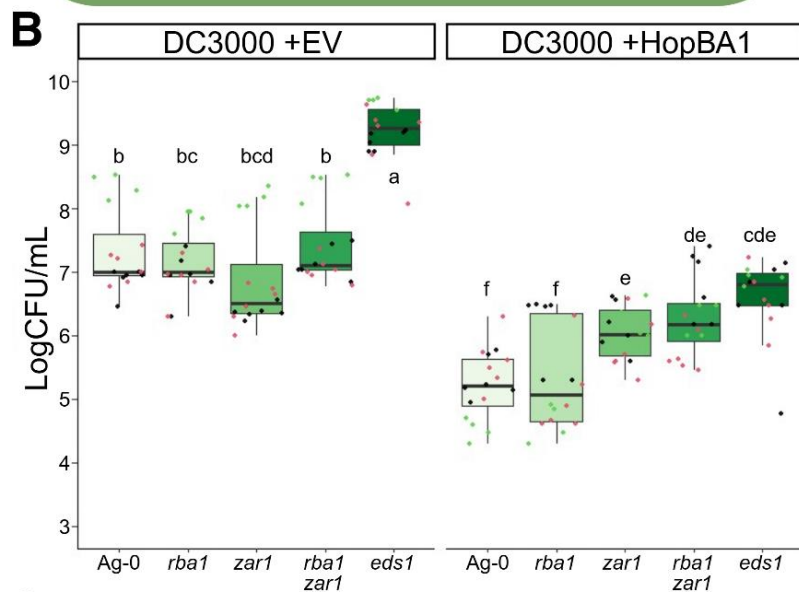
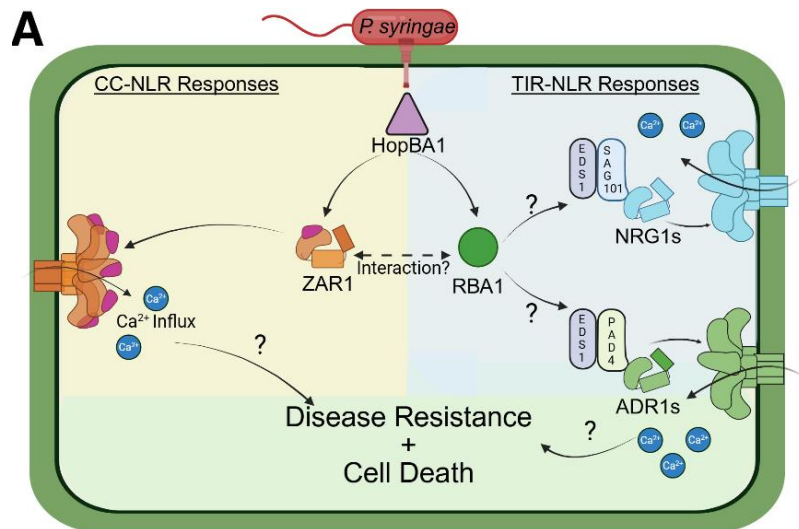


Figure 3.2. RBA1 and ZAR1 differentially contribute to responses to HopBA1. **A)** Model for dual recognition of HopBA1 by RBA1 and ZAR1 in Ag-0 Arabidopsis. **B)** Growth restriction of DC3000 HopBA1 in the Ag-0 WT, *rba1*, *zar1*, *rba1 zar1*, *eds1* mutants. Bacterial growth was assayed 4 days post infiltration into the indicated Ag-0 genotypes. *P. syringae* DC3000 was infiltrated at OD 0.0002 ($\sim 10^5$ cfu) in 10mM MgCl₂. **C)** Ion leakage responses to *Pf0-1* carrying HopBA1 in Ag-0 WT, *rba1*, *zar1*, *rba1 zar1*, *eds1-2* mutants. Cell death was quantitatively measured as a function of ion leakage from infiltrated leaves. Ion leakage was assayed 3 days post infiltration into the indicated Ag-0 genotypes. *Pf0-1* was infiltrated at OD 0.1 in 10mM MgCl₂. In boxplots, individual biological replicates are shown as dots, dots are colored according to independent experimental replicates.

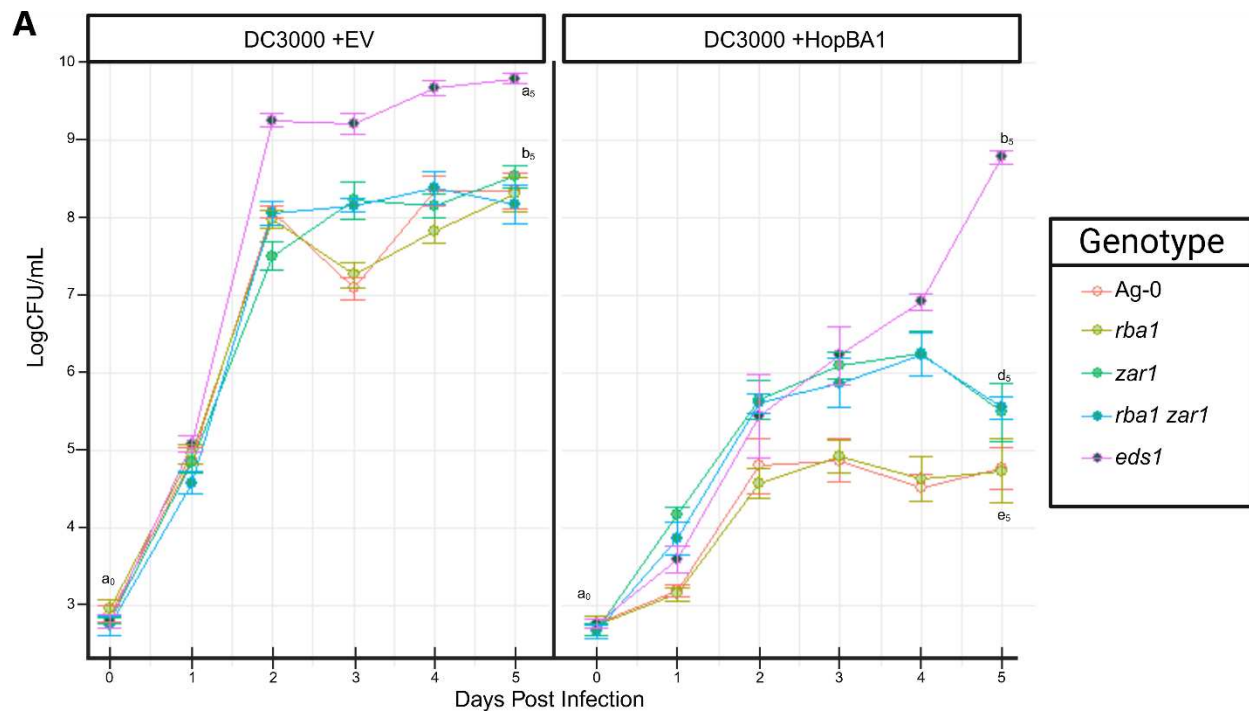


Figure 3.3 Growth dynamics of DC3000 EV and DC3000 HopBA1 over the course of a five-day time course in WT and mutant Ag-0 Arabidopsis. **A)** Growth restriction of DC3000 EV and HopBA1 in Ag-0, *rba1*, *zar1*, *rba1 zar1*, and *eds1* mutants. Bacterial growth was assayed every 24 hours for 5 days post infiltration into the indicated Ag-0 genotypes. *P. syringae* DC3000 was infiltrated at OD 0.0002 ($\sim 10^5$ cfu) in 10mM MgCl₂. Data are presented as the mean of 6 biological replicates $\pm 2 \times$ SE. Statistical analysis was conducted on day 0 and day 5 data. Data was analyzed via two-way analysis of variance (ANOVA) with post hoc Tukey HSD (honestly significant difference).

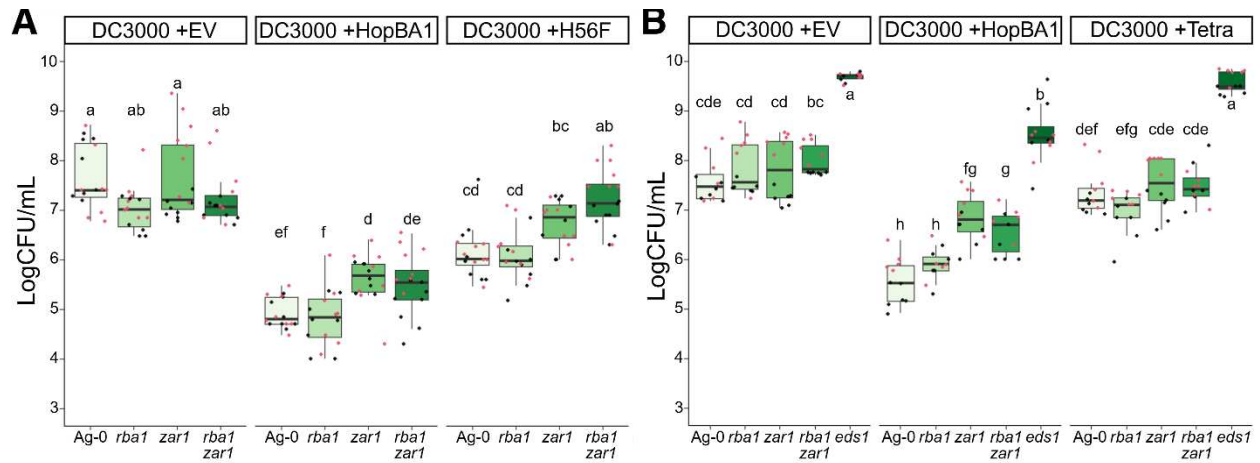


Figure 3.4. Predicted loss of function alleles of HopBA1 confer reduce growth restriction in WT and mutant Ag-0 compared to WT HopBA1. A) Growth restriction of DC3000 HopBA1 and H56F in Ag-0, *rba1*, *zar1*, and *rba1 zar1* mutants. DC3000 +H56F refers to DC3000 carrying the mutated H56F allele of HopBA1. **B)** Growth restriction of DC3000 HopBA1 and “Tetra” in Ag-0, *rba1*, *zar1*, *rba1 zar1*, and *eds1* mutants. DC3000 +Tetra refers to DC3000 carrying the quadruple mutant “Tetra” allele of HopBA1. Bacterial growth was assayed 4 days post infiltration into the indicated Ag-0 genotypes. *P. syringae* DC3000 was infiltrated at OD 0.0002 (~10⁵ cfu) in 10mM MgCl₂. In boxplots, individual biological replicates are shown as dots, dots are colored according to independent experimental replicates.

residual growth restriction in response to WT HopBA1 in Ag-0 *rba1 zar1* fail to recognize the H56F allele of HopBA1 (Figure 3.4A).

To further investigate the relevance of HopBA1’s active site we generated a mutant HopBA1 allele harboring all four previously described partial loss of function mutations in the HopBA1 putative active site (H56F, W112A, Y158A, R162A) (hereafter HopBA1 “Tetra”). We hypothesized that including these four mutations will cause this allele to be a stronger loss of function allele than the HopBA1 H56F mutant alone. Next, we assayed the growth of DC3000 carrying HopBA1 “Tetra” in our Ag-0 mutant lines. Consistent with the hypothesis that the “Tetra” allele of HopBA1 is a loss of function, DC3000 carrying the HopBA1 “Tetra” mutant allele grew equivalently to DC3000 EV in all Ag-0 lines (Figure 3.4B).

3.2.3 HopBA1 recognition in *Nicotiana benthamiana* requires *AtZRK2* to trigger robust and *NbEDS1*-independent HR

Upon finding that HopBA1 recognition by *AtZAR1* partially requires *AtEDS1*, we felt that it was relevant to test the requirement for *NbEDS1* in HopBA1 recognition in the *Nb* reconstruction assay (Figure 2.4). We transiently co-expressed *AtZRK2* with a range of doses of HopBA1 for two days in the *eds1* mutant of *Nb*. All tested HopBA1 doses triggered HR when co-expressed with *AtZRK2* (Figure 3.5). Given the ease of conducting cell death assays in *Nb*, we were interested in exploring whether previously characterized (Nishimura et al. 2017) putative active site HopBA1 mutants lost the capacity to trigger *NbZAR1*-mediated HR in the reconstruction assay. Prior to testing the HopBA1 mutant alleles for phenotypic

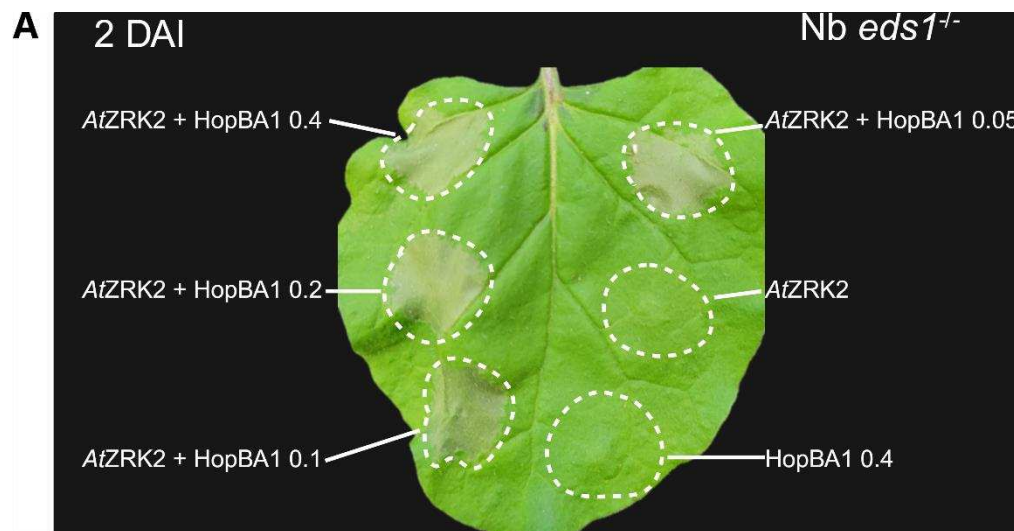


Figure 3.5 *NbZAR1*-mediated recognition of HopBA1 in *Nicotiana benthamiana* does not require *NbEDS1*. A) Cell death occurs when HopBA1 is co-expressed with the Arabidopsis pseudokinase adapter, ZRK2 in *Nb eds1* mutant plants. *AtZRK2* was infiltrated at OD 0.2, HopBA1 was infiltrated at the OD indicated on the label. Infiltrated leaf pictured 2 days after infiltration. Experiments were replicated multiple times on 2 – 3 plants (3 leaves per plant) on each occasion.

differences, we confirmed that they accumulate in transient assays (Figure 3.6A). We found that all HopBA1 mutants except for W112A, qualitatively accumulated to the same levels as WT HopBA1. The W112A mutant expression appeared to be slightly lower than that of WT HopBA1 and the other mutants, but the robust accumulation of HopBA1 “Tetra” suggests that this accumulation defect is not intrinsic to the W112A mutation. As before, we transiently co-expressed *AtZRK2* with WT HopBA1 and each of the previously characterized HopBA1 mutants (H56F, W112A, Y158A, R162A) across a range of inoculum doses to observe any dose-dependent changes in HopBA1 recognition. We started at a HopBA1 *Agrobacterium* OD of 0.4 and progressively halved the infiltrated OD until OD 0.0015 (Figure 3.6B). Surprisingly, we observed recognition of all alleles of HopBA1, even at very low infiltration ODs. Recognition of R162A was lost at the highest dose of all the mutants, with OD 0.0063 failing to trigger HR. The W112A mutant no longer triggered HR at OD 0.0031, and the other mutants lost HR-inducing capacity at OD 0.0015, while WT HopBA1 retained HR induction at this dose. These data show that *NbZAR1* is sensitive to even low doses of HopBA1 mutants, which presumably correlates with a high degree of sensitivity to HopBA1 enzymatic activity, or differences between native delivery from DC3000 and transient expression in host cells via *Agrobacteria*.

3.2.4 HopBA1 recognition by ZAR1 in Col-0 partially requires canonical TIR signaling components

The DC3000 HopBA1 growth results in Ag-0 *eds1-2* prompted us to further dissect a potential ZAR1-EDS1 relationship in disease resistance responses. To do this, we sought a less genetically complex background, given the apparent residual resistance to DC3000 HopBA1 in the Ag-0 *rba1 zar1* double mutant and the use of the extensively introgressed Ag-0 *eds1-2* in the initial observations of potential ZAR1 dependence on EDS1. To do this, we evaluated HopBA1-

elicited immune responses in Col-0, which lacks RBA1-dependent cell death and for which an extensive mutant collection already exists (Laflamme et al., 2020). In Col-0 *eds1-12*, we observed a partial loss of growth restriction (Figure 3.7A).

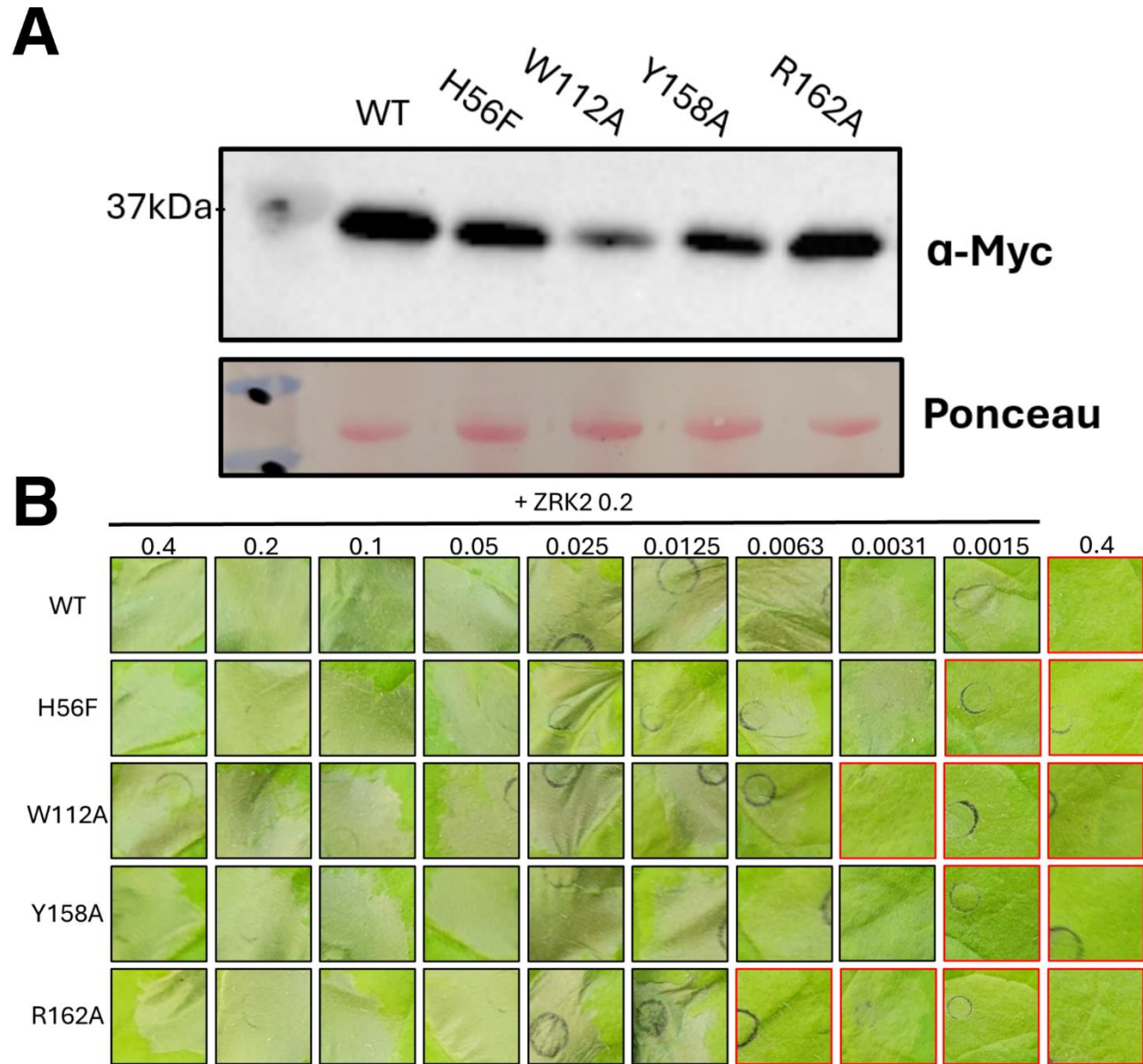


Figure 3.6. HopBA1 mutant alleles are robustly recognized in the *Nicotiana benthamiana* reconstruction assay. A) Transiently expressed mutant alleles of HopBA1 accumulate in Nb. The immunoblotted HopBA1 allele is indicated above the blot. Total protein loading was assessed by Ponceau S staining of the membrane, shown below the blot. **B)** Extensive dose titration of HopBA1 mutant alleles is required to abolish *NbZAR1*-mediated HR responses in *Nb eds1* transient expression assays. The infiltrated HopBA1 allele is indicated on the left side of the grid. The infiltrated OD of *Agrobacterium* carrying a particular HopBA1 construct is indicated

above the grid. Leaves that do not qualitatively display HR are boxed in red, ones with HR are boxed in black. Photos were taken 36 hours post infiltration. Experiments were replicated multiple times on 2 – 3 plants (3 leaves per plant) for each infiltration with similar results each time.

This loss of growth restriction was found to depend upon PAD4, with SAG101 being dispensable for DC3000 HopBA1 growth restriction (Figure 3.7A). EDS1 immunity typically requires the downstream function of RPW8-type “helper” NLRs (RNLs). Consistent with canonical EDS1 pathway function, a “*helperless*” polymutant (hereafter Col-0 *helperless*) lacking all ADR1 and NRG1 family genes displayed similar growth restriction to the *eds1* mutant (Figure 3.7B). Given the requirement of PAD4 for HopBA1 induced growth restriction, we observed an expected loss of DC3000 HopBA1 growth restriction in the *adr1 triple* mutant as well (Figure 3.7B). In a comparison of HopBA1-induced growth restriction responses between the *eds1* and *helperless* mutants, we observed a more significant loss of growth restriction in the *helperless* mutant (Figure 3.8A). These revealed that HopBA1-triggered EDS1-dependent growth restriction was specifically dependent on the PAD4-ADR1 branch of EDS1 immunity, while SAG101 and NRG1s were dispensable (Figure 3.7A-B). In growth assays in a Col-0 *zar1 eds1* double mutant, we observe a total loss of DC300 HopBA1 growth restriction, consistent with both ZAR1 and EDS1 being involved in mediating responses to HopBA1 (Figure 3.7C).

3.2.5 HopZ1a-induced growth restriction partially requires canonical TIR signaling components in Col-0, but not in Ag-0 or Ws Arabidopsis

To evaluate the hypothesis that ZAR1-mediated immune responses might generally require *EDS1*, we took the same approach as taken for evaluating DC3000 HopBA1 growth with other ZAR1-recognized *Pseudomonas syringae* effectors (HopZ1a, HopO1c, HopX1i and HopF1r) (Laflamme et al., 2020) by assessing their growth in the same panel of Col-0 mutants.

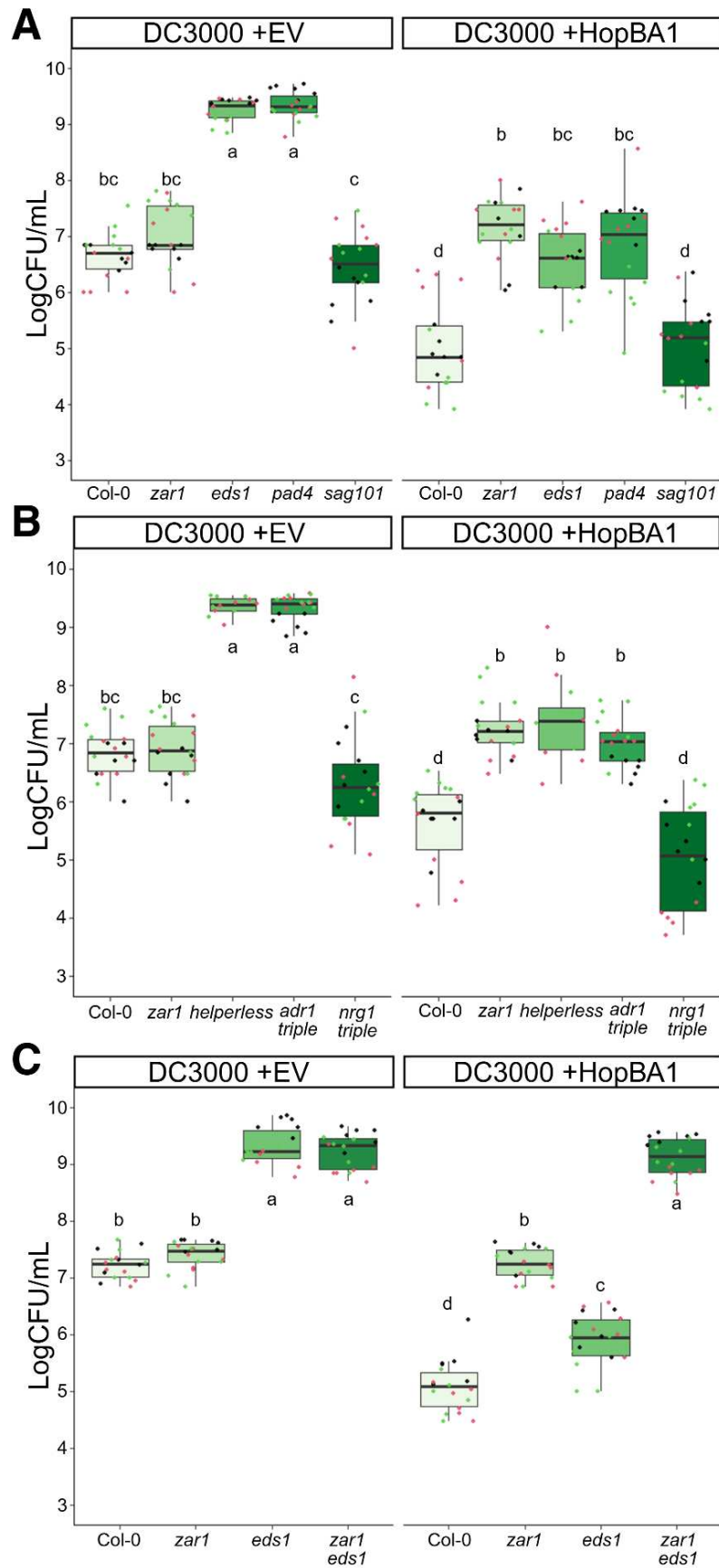


Figure 3.7 ZAR1-mediated recognition of HopBA1 in Col-0 partially requires the EDS1-PAD4-ADR1 signaling node of the immune system. **A)** Growth restriction of DC3000 HopBA1 in the Col-0 WT, *zar1*, *eds1*, *pad4*, *sag101* mutants. **B)** Growth restriction of DC3000 HopBA1 in the Col-0 WT, *zar1*, *helperless*, *adr1* triple, *nrg1* triple mutants. **C)** Growth restriction of DC3000 HopBA1 in the Col-0 WT, *zar1*, *eds1*, *zar1 eds1* mutants. Bacterial growth was assayed 4 days post infiltration into the indicated Col-0 genotypes. *P. syringae* DC3000 was infiltrated at OD 0.0002 ($\sim 10^5$ cfu) in 10mM MgCl₂. In boxplots, individual biological replicates are shown as dots, dots are colored according to independent experimental replicates.

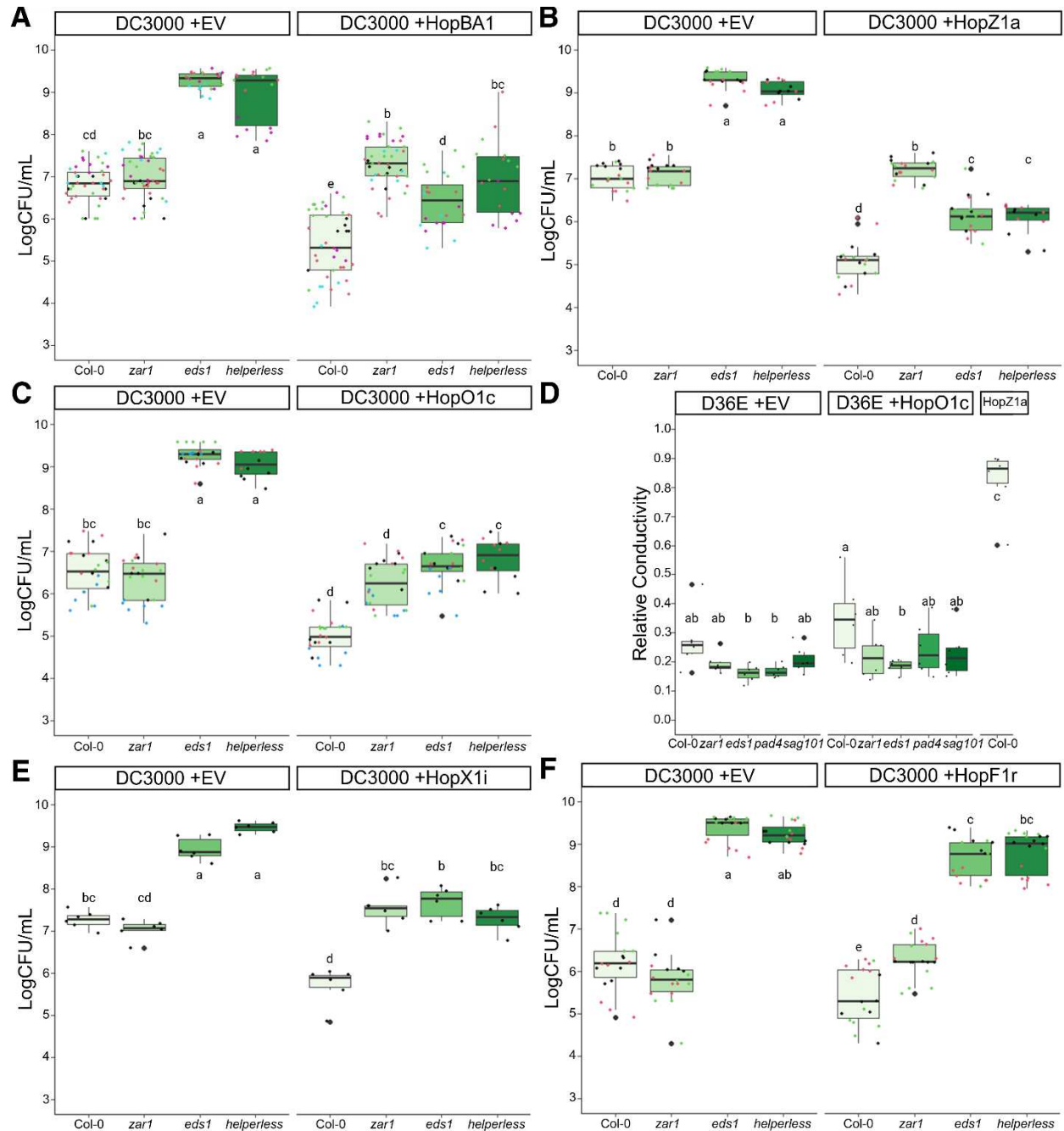


Figure 3.8 Loss of growth restriction of ZAR1-recognized effectors is similar in *eds1* and *helperless* mutants and HopO1c does not trigger significant ion leakage in Col-0. **A)** Growth restriction of DC3000 HopBA1 in the Col-0 WT, *zar1*, *eds1* and *helperless* mutants. **B)** Growth restriction of DC3000 HopZ1a in the Col-0 WT, *zar1*, *eds1* and *helperless* mutants. **C)** Growth restriction of DC3000 HopO1c in the Col-0 WT, *zar1*, *eds1* and *helperless* mutants. **D)** Ion leakage phenotypes of DC3000 D36E HopO1c in the Col-0 WT, *zar1*, *eds1*, *zar1 eds1* mutants. Ion leakage responses of Col-0 WT to DC3000 D36E HopZ1a were included as a positive control for the capacity of tested plants to induce cell death responses. Cell death was quantitatively measured as a function of ion leakage from infiltrated leaves. Ion leakage was assayed 3 days post infiltration into the indicated Col-0 genotypes. “Effectorless” *P. syringae* D36E was infiltrated at OD 0.5 in 10mM MgCl₂. **E)** Growth restriction of DC3000 HopX1i in the Col-0 WT, *zar1*, *eds1* and *helperless* mutants. **F)** Growth restriction of DC3000 HopF1r in the Col-0 WT, *zar1*, *eds1* and *helperless* mutants.

When evaluating the growth of DC3000 HopZ1a in WT Col-0, *zar1* and EDS1-family mutants, we observed a partial loss of pathogen growth restriction similar to that of DC3000 HopBA1 in the Col-0 *eds1-12* and *pad4* mutants (Figure 3.9A). Similarly, we found that only the Col-0 *helperless* and *adr1 triple* mutants displayed this partial loss of pathogen growth restriction when evaluating the “helper” family mutants (Figure 3.9B). In a comparison of HopZ1a-induced growth restriction responses between the *eds1* and *helperless* mutants, we observed an equivalent loss of growth restriction in the two mutant lines (Figure 3.8B). Like DC3000 HopBA1, we saw a total loss of DC3000 HopZ1a growth restriction in the *zar1 eds1* double mutant (Figure 3.9C).

In contrast to the loss of ion leakage responses to HopBA1 observed in the Ag-0 *eds1-2* mutant, HopZ1a-induced ion leakage did not require EDS1, PAD4 or SAG101 (Figure 3.9D). We hypothesized that ZAR1-EDS1 dependence is related to the strength of ZAR1 activation. Effectors that are more growth restricted in *eds1* mutants (Ex: HopZ1a) would be considered “strong” ZAR1 activators. Hypothetically, NLR activation strength is correlated with the number of oligomerized resistosomes in a pathogen-infected cell. We expected that lowering the pathogen infection dose would weaken an effector’s ZAR1 activation phenotype by reducing the number effector molecules available to induce resistosome formation in an infected cell. To test

this, we infected WT Col-0, *zar1*, *eds1* and *helperless* mutants with a 10-fold reduced dose of DC3000 EV and DC3000 HopZ1a (OD 0.00002).

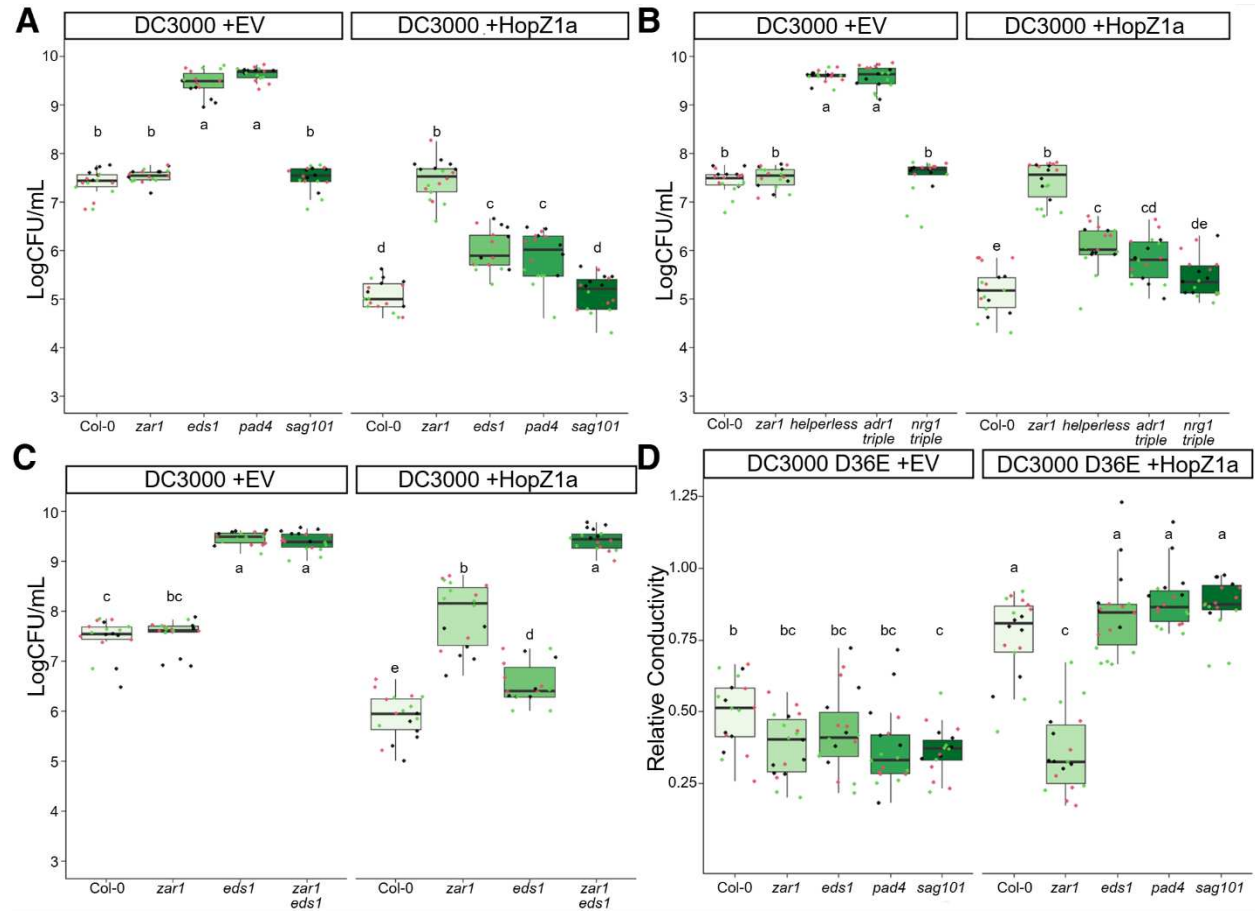


Figure 3.9. ZAR1-mediated recognition of HopZ1a in Col-0 partially requires the EDS1-PAD4-ADR1 signaling node of the immune system. **A)** Growth restriction of DC3000 HopZ1a in the Col-0 WT, *zar1*, *eds1*, *pad4*, *sag101* mutants. **B)** Growth restriction of DC3000 HopZ1a in the Col-0 WT, *zar1*, *helperless*, *adr1 triple*, *nrg1 triple* mutants. **C)** Growth restriction of DC3000 HopZ1a in the Col-0 WT, *zar1*, *eds1*, *zar1 eds1* mutants. Bacterial growth was assayed 4 days post infiltration into the indicated Col-0 genotypes. *P. syringae* DC3000 was infiltrated at OD 0.0002 ($\sim 10^5$ cfu) in 10mM MgCl₂. **D)** Ion leakage phenotypes of DC3000 D36E HopZ1a in the Col-0 WT, *zar1*, *eds1*, *pad4* and *sag101* mutants. Cell death was quantitatively measured as a function of ion leakage from infiltrated leaves. Ion leakage was assayed 3 days post infiltration into the indicated Col-0 genotypes. “Effectorless” *P. syringae* D36E was infiltrated at OD 0.5 in 10mM MgCl₂. In boxplots, individual biological replicates are shown as dots, dots are colored according to independent experimental replicates.

In these experiments, we observed a growth reduction of roughly 1 log for DC3000 EV, relative to the standard growth curve infection dose, in all tested genetic backgrounds (Figure 3.10). For

plants infected with DC3000 HopZ1a, we did not observe a reduced capacity for *eds1* or *helperless* mutants to restrict pathogen growth. In fact, we observed that the tested mutants restricted pathogen growth equivalently to WT Col-0. Additionally, DC3000 HopZ1a grew to a variable degree in *zar1* mutants across growth assays (Figure 3.10). These data suggest that adjusting pathogen inoculum OD may not be an effective method to understand receptor signaling dynamics during an immune response.

To flesh out the relationship between ZAR1 and EDS1 in Ag-0, we assessed the growth of DC3000 HopZ1a in our panel of Ag-0 mutants (Figure 3.11A). In accordance with our expectations, we did not see a loss of DC3000 HopZ1a growth restriction in Ag-0 *eds1-2*. We also did not see a change in HopZ1a-dependent pathogen growth restriction in *rba1* mutant genotypes. ZAR1's EDS1-independence was previously reported only in the context of activation by HopZ1a in the Ws *eds1-1* background and in the Col-0 *eds1-2* introgression (Lewis et al., 2010; Macho et al., 2010). We re-evaluated HopZ1a growth restriction in WT and the *eds1-1* mutant of Ws (Fig 3.11B). In Ws *eds1-1*, we observed a partial loss of HopZ1a-mediated growth restriction relative to WT Ws.

3.2.6 ZAR1-mediated recognition of HopO1c partially requires canonical TIR signaling components in Col-0 Arabidopsis

Growth restriction of DC3000 HopO1c was more strongly lost in the Col-0 *eds1* and *pad4* mutants than the observed partial loss of growth restriction of DC3000 HopBA1 and HopZ1a, with *eds1* and *pad4* mutants supporting pathogen growth equivalent to that of the *zar1* mutant (Figure 3.12A). In line with previous findings, we see again that this loss of growth restriction is present in the *helperless* and *adr1 triple* mutants *nrg1 triple* mutant (Figure 3.12B).

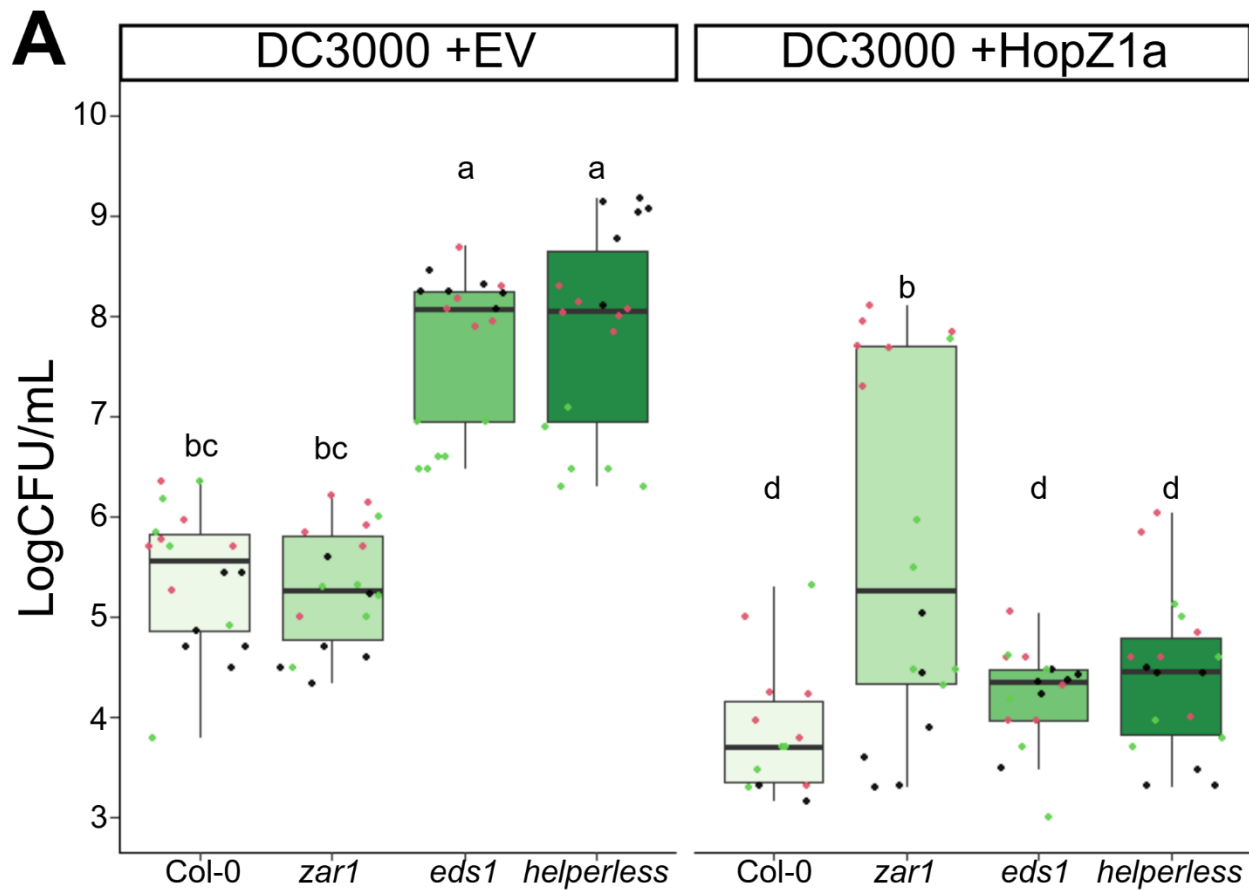


Figure 3.10. Pathogen dose titration does not increase ZAR1-EDS1 dependence phenotypes.

A) Growth restriction of DC3000 HopZ1a in the Col-0 WT, *zar1*, *eds1* and *helperless* mutants. Bacterial growth was assayed 4 days post infiltration into the indicated Col-0 genotypes. *P. syringae* DC3000 was infiltrated at OD 0.00002 in 10mM MgCl₂. In boxplots, individual biological replicates are shown as dots, dots are colored according to independent experimental replicates.

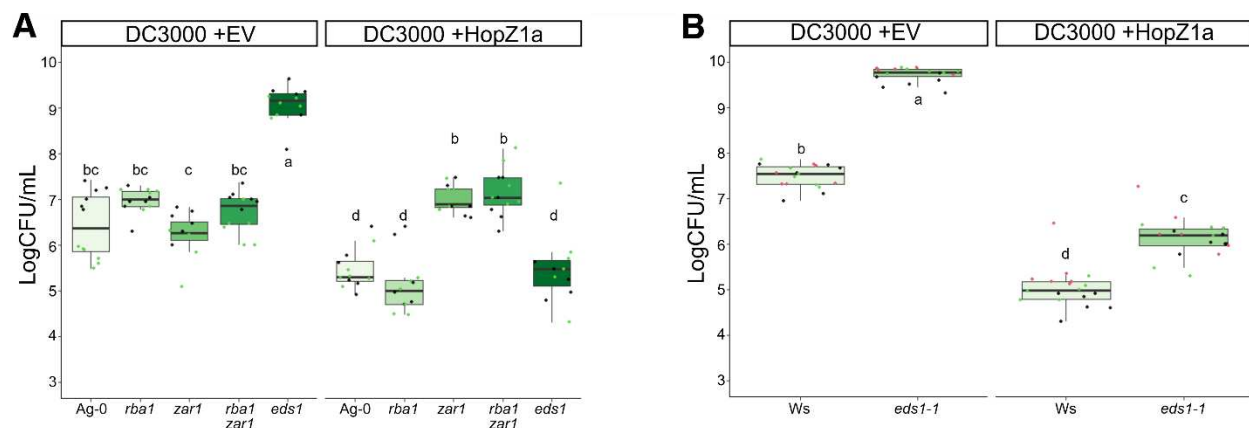


Figure 3.11. ZAR1-mediated recognition of HopZ1a in Ag-0 and Ws does not require EDS1.

A) Growth restriction of DC3000 HopZ1a in the Ag-0 WT, *rba1*, *zar1*, *rba1 zar1*, *eds1* mutants. Bacterial growth was assayed 4 days post infiltration into the indicated Ag-0 genotypes. *P.*

syringae DC3000 was infiltrated at OD 0.0002 ($\sim 10^5$ cfu) in 10mM MgCl₂. **B)** Growth restriction of DC3000 HopZ1a in Ws and the Ws *eds1-1* mutant. Bacterial growth was assayed 4 days post infiltration into the indicated Ws genotypes. *P. syringae* DC3000 was infiltrated at OD 0.0002($\sim 10^5$ cfu) in 10mM MgCl₂.

In a comparison of HopO1c-induced growth restriction responses between the *eds1* and *helperless* mutants, we observed an equivalent loss of growth restriction in the two mutant lines (Figure 3.8C). While HopO1c has previously been reported to trigger cell death responses in Col-0, we did not observe a significantly higher degree of ion leakage responses to HopO1c relative to EV (Figure 3.8D). The *zar1 eds1* double mutant lost all capacity to restrict DC3000 HopO1c growth, consistent with observations for HopBA1 and HopZ1a growth restriction in this mutant line (Figure 3.12C).

3.2.7 ZAR1-mediated recognition of HopX1i partially requires canonical TIR signaling components in Col-0 Arabidopsis

We observed a similar degree of growth restriction of DC3000 HopX1i in Col-0 *eds1* and *pad4* mutants as seen with DC3000 HopO1c (Figure 3.13A). Once again, we found that this growth restriction was tied to the PAD4-ADR1 branch of the EDS1 immune pathway, with no loss of growth restriction observed in *sag101* or *nrg1 triple* mutant plants (Figure 3.13A-B). In a comparison of HopX1i-induced growth restriction responses between the *eds1* and *helperless* mutants, we observed an equivalent loss of growth restriction in the two mutant lines (Figure 3.8E). As observed for previously tested effectors, we noted a total loss of growth restriction of DC3000 carrying HopX1i in the *zar1 eds1* double mutant (Figure 3.13C).

3.2.8 ZAR1-mediated recognition of HopF1r partially requires canonical TIR signaling components in Col-0 Arabidopsis

Surprisingly, we found that DC3000 HopF1r was significantly less growth restricted in the *eds1* or *pad4* mutants, growing ~1 log more than DC3000 HopO1c and HopX1i (Figure 3.14A). Like with the other ZAR1-recognized effectors, the loss of growth restriction was specific to the *pad4* and *adr1 triple* mutants in growth curves testing the relevance of EDS1-family and helper-family mutants, respectively (Figure 3.14B). In a comparison of HopF1r-induced growth restriction responses between the *eds1* and *helperless* mutants, we observed an equivalent loss of growth restriction in the two mutant lines (Figure 3.8F). The *zar1 eds1* double mutant fully lost the capacity to restrict DC3000 HopF1r growth, like observed losses in pathogen growth restriction observed for the other tested effectors. Taken together, these data suggest that ZAR1-mediated immune responses require the EDS1-PAD4-ADR1 node of the immune system to varied degrees depending on the activating effector protein (Figure 3.14C).

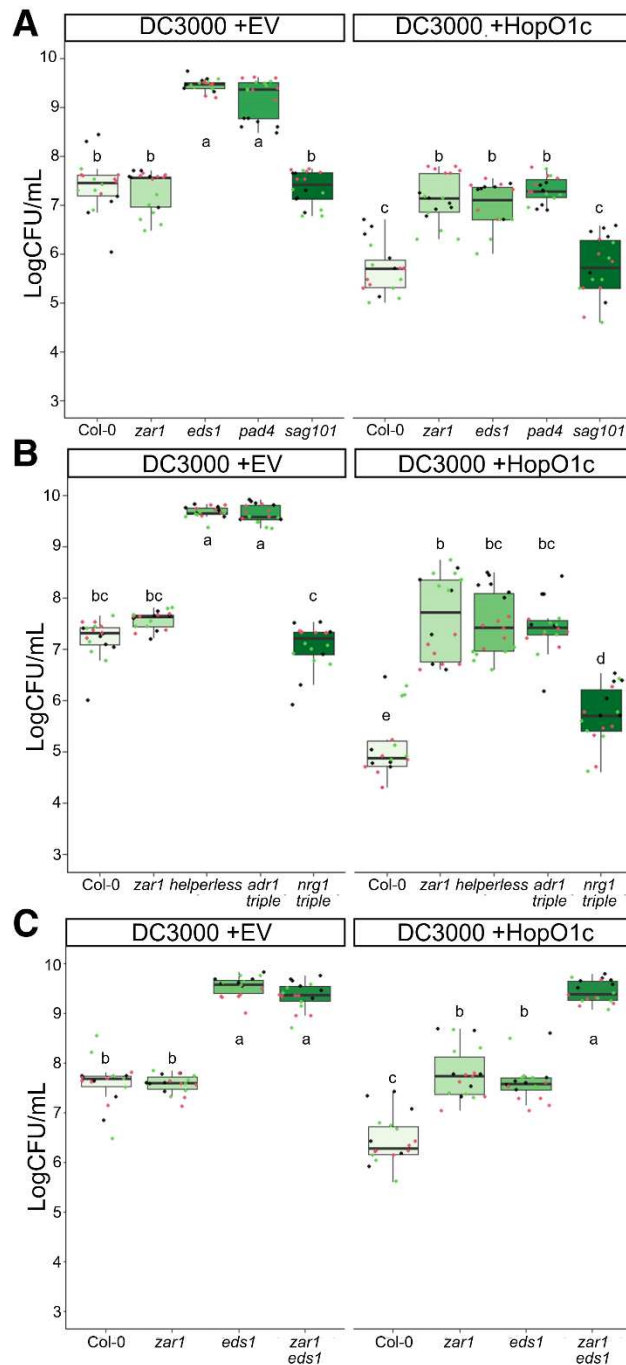


Figure 3.12. ZAR1-mediated recognition of HopO1c in Col-0 partially requires the EDS1-PAD4-ADR1 signaling node of the immune system. A) Growth restriction of DC3000 HopO1c in the Col-0 WT, *zar1*, *eds1*, *pad4*, *sag101* mutants. **B)** Growth restriction of DC3000 HopO1c in the Col-0 WT, *zar1*, *helperless*, *adr1 triple*, *nrg1 triple* mutants. **C)** Growth restriction of DC3000 HopO1c in the Col-0 WT, *zar1*, *eds1*, *zar1 eds1* mutants. Bacterial growth was assayed 4 days post infiltration into the indicated Col-0 genotypes. *P. syringae* DC3000 was infiltrated at OD 0.0002 (~10⁵ cfu) in 10mM MgCl₂. In boxplots, individual biological replicates are shown as dots, dots are colored according to independent experimental replicates.

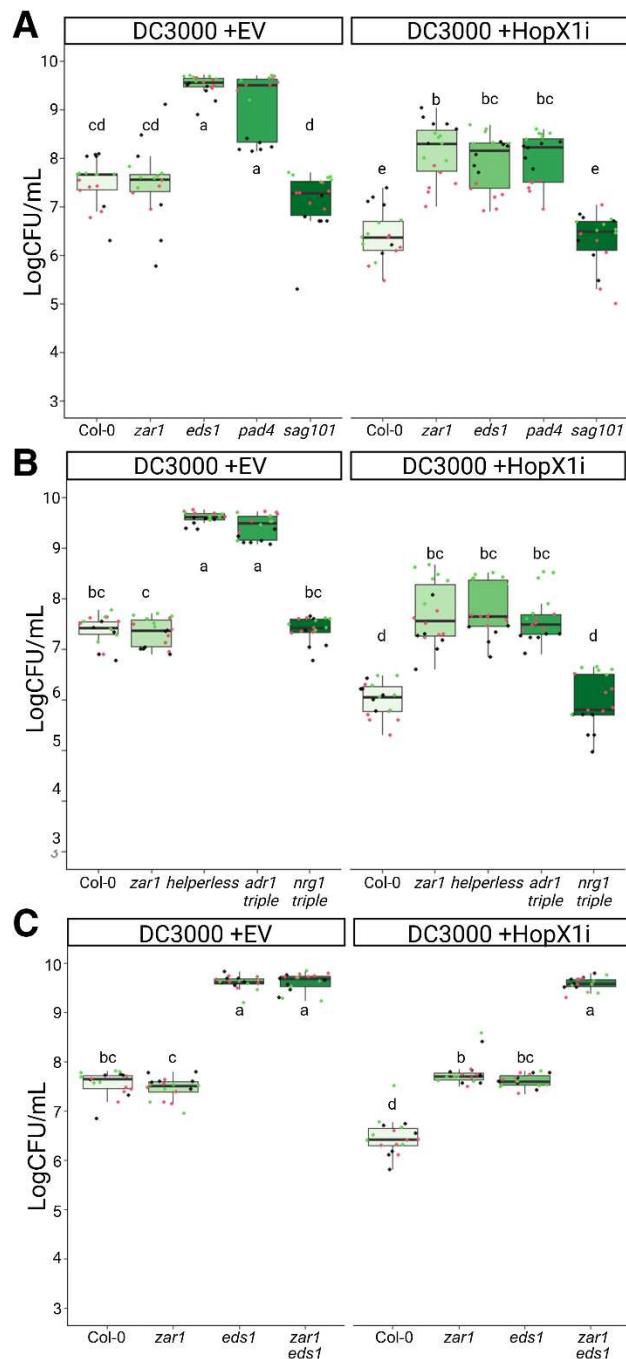


Figure 3.13. ZAR1-mediated recognition of HopX1i in Col-0 partially requires the EDS1-PAD4-ADR1 signaling node of the immune system. **A)** Growth restriction of DC3000 HopX1i in the Col-0 WT, *zar1*, *eds1*, *pad4*, *sag101* mutants. **B)** Growth restriction of DC3000 HopX1i in the Col-0 WT, *zar1*, *helperless*, *adr1 triple*, *nrg1 triple* mutants. **C)** Growth restriction of DC3000 HopX1i in the Col-0 WT, *zar1*, *eds1*, *zar1 eds1* mutants. Bacterial growth was assayed 4 days post infiltration into the indicated Col-0 genotypes. *P. syringae* DC3000 was infiltrated at OD 0.0002 (~10⁵ cfu) in 10mM MgCl₂. In boxplots, individual biological replicates are shown as dots, dots are colored according to independent experimental replicates.

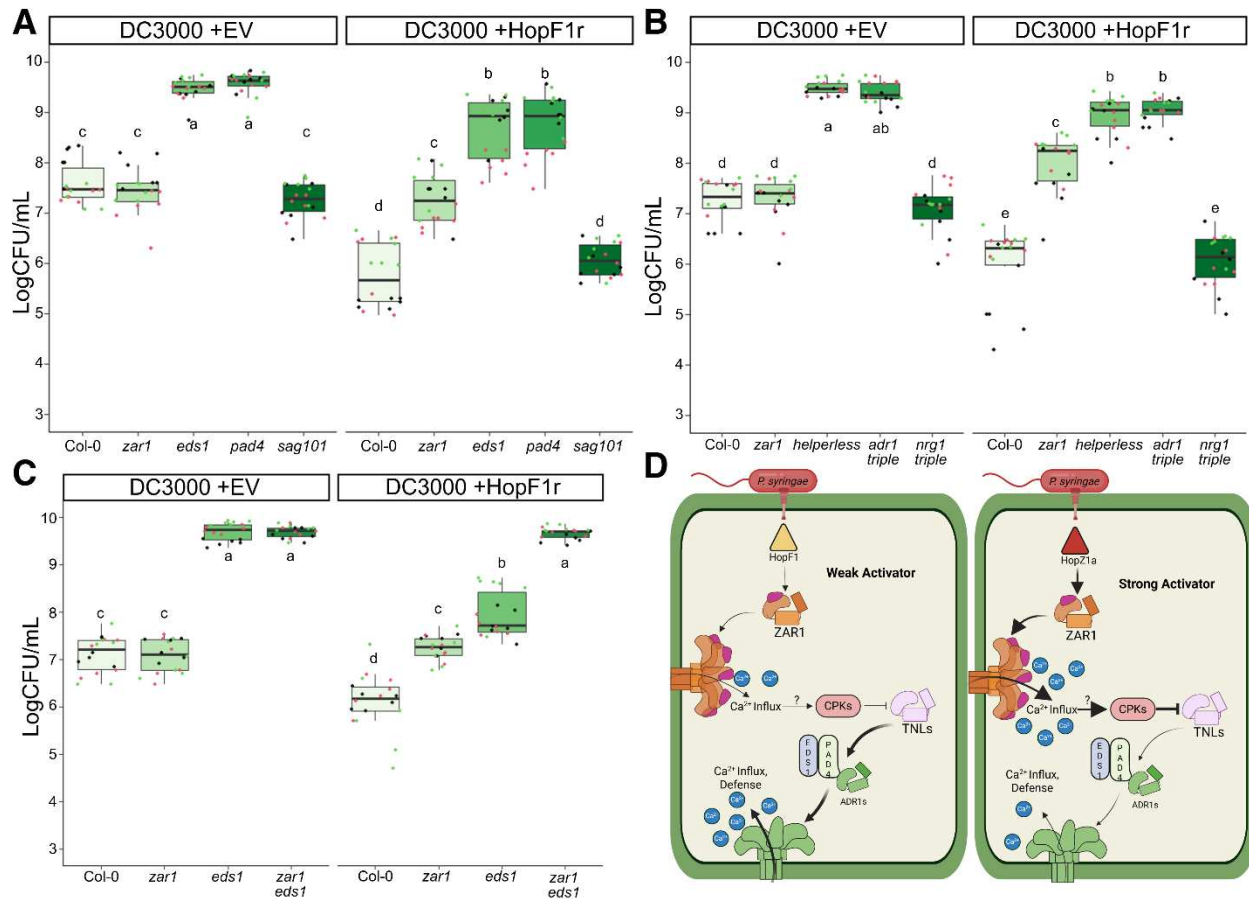


Figure 3.14. ZAR1-mediated recognition of HopF1r in Col-0 strongly requires the EDS1-PAD4-ADR1 signaling node of the immune system. **A)** Growth restriction of DC3000 HopF1r in the Col-0 WT, *zar1*, *eds1*, *pad4*, *sag101* mutants. **B)** Growth restriction of DC3000 HopF1r in the Col-0 WT, *zar1*, *helperless*, *adr1 triple*, *nrg1 triple* mutants. **C)** Growth restriction of DC3000 HopF1r in the Col-0 WT, *zar1*, *eds1*, *zar1 eds1* mutants. Bacterial growth was assayed 4 days post infiltration into the indicated Col-0 genotypes. *P. syringae* DC3000 was infiltrated at OD 0.0002 ($\sim 10^5$ cfu) in 10mM MgCl₂. In boxplots, individual biological replicates are shown as dots, dots are colored according to independent experimental replicates. **D)** Model for variable ZAR1 dependence on EDS1 in immune responses triggered by diverse *Pseudomonas syringae* effectors.

3.3 Discussion

Here we describe both the immune responses to the *Pseudomonas syringae* effector HopBA1 in the Ag-0 ecotype of Arabidopsis and requirement of the EDS1-PAD4-ADR1 node of the immune system for complete ZAR1-mediated immunity in Col-0. There are relatively few examples of multiple plant NLRs functioning to recognize a single effector, and our work in Ag-

0 serves as an example of a single effector recognized both by a CNL and a TIR immune receptor. The role of RBA1 in regulating the cell death, but not growth restriction, responses to HopBA1 demonstrates that hypersensitive cell death is not necessarily positively correlated with pathogen growth restriction (Pitsili, Phukan, and Coll 2021). Furthermore, these assays all use *Pseudomonas syringae* DC3000 as the pathogenic agent, where the hypersensitive response may not impact pathogen growth, but other pathogens, like oomycetes, may be susceptible to these responses.

These findings also highlight the need to further study RBA1-mediated immune responses at a mechanistic level, given its unique status as a TIR-only NLR with a known cognate effector protein. While previous work had established that Ag-0 *rba1* mutants can restrict DC3000 HopBA1 growth like WT Ag-0, these data did not address ZAR1's contribution to HopBA1-triggered immunity in Ag-0 (Nishimura et al. 2017). By assessing immune phenotypes in Ag-0 *zar1* and *rba1 zar1* mutants, we found that ZAR1 functions to induce immune responses to HopBA1, but RBA1 and ZAR1 are not the only immune receptors required for these immune outputs.

In addition to the dual recognition of HopBA1 by RBA1 and ZAR1, we also observe allelic differences in ZAR1-mediated responses to HopBA1. In Ag-0, ZAR1 contributes to both cell death and pathogen growth restriction, however in Col-0, ZAR1-mediated immunity to HopBA1 does not include hypersensitive cell death. Additionally, ZAR1 mediated growth restriction responses to HopZ1a are also EDS1-independent, unlike the partial EDS1-dependence observed in Col-0 (Figure 3.11A). This highlights that even among ecotypes, the same immune receptor, responding to the same elicitor, can initiate a range of immune responses with varied signaling dependencies. Furthermore, these data reiterate the importance of considering the

specific genetic background of the plants used to study immune responses and understanding that these data will not necessarily translate across even closely related plants. In addition to this, the data reveal that while our collective understanding of the plant immune system has made great strides through years of rigorous research, we are still far from being able to predict the behaviors of immune receptors when they are placed into different genetic contexts, likely due to an incomplete picture of the more minor genetic determinants driving a particular immune response.

Investigation of pathogen growth over a time course did not reveal any unobserved contribution of RBA1 to DC3000 HopBA1 growth restriction as we had hypothesized (Figure 3.3). These growth assays did reveal, however, that the Ag-0 *eds1* mutant loses additional capacity to restrict DC3000 HopBA1 growth at five days post infection, suggesting that Ag-0 ZAR1 may rely upon EDS1 to maintain pathogen growth restriction at later infection timepoints. The literature is almost entirely focused on assaying DC3000 growth at early infection timepoints (3-4 days post infection) while comparatively little work has been done to understand the later stages of *P. syringae* infection. *P. syringae* is considered to be a hemibiotrophic pathogen, meaning that only part of the bacteria's life cycle requires a living host. Despite this, the necrotrophic portion of the *P. syringae* life cycle remains virtually unstudied. Even by assaying growth one day beyond the typical growth assay timing, we found a greater loss of pathogen growth in the *eds1* mutant as well as contributions of residual resistance factors in the *zar1* and *rba1 zar1* mutants. These data are worth considering if efforts are made to map the residual *rba1* and *zar1* resistance responses to HopBA1, as assaying disease resistance at later timepoints could be the key to mapping these residual responses to specific genetic loci.

The finding that all the tested mutants of HopBA1 still elicit robust *Nb*ZAR1-mediated immune responses and the results of Ag-0 growth assays with HopBA1 H56F support the hypothesis that ZAR1 in *Nb* and *Arabidopsis* is sensitive to even low levels of HopBA1 enzymatic activity (Figure 3.4A, Figure 3.6B). In conjunction with the finding that the HopBA1 induced putative cleavage product of *Nb*PBL6-2 is abolished upon co-expression with HopBA1 Tetra, the idea that tested HopBA1 mutants are indeed loss of function alleles is additionally supported (Figure 2.6). These data are also consistent with the hypothesis that ZAR1-ZRK2 is guarding some HopBA1-modified virulence target to become active.

The lack of *Nb*ZAR1 dependence on *Nb*EDS1 for HopBA1-induced HR is consistent with the results in Col-0 where *At*EDS1 is dispensable for HopZ1a-triggered ion leakage (Figure 3.6, Figure 3.9D). Given the need to supplement *At*ZRK2 into *Nb* to confer responses to HopBA1, *At*ZRK2-transgenic *Nb* would be necessary to investigate the relevance of *Nb*EDS1 in *Nb*ZAR1-mediated growth restriction responses to DC3000 HopBA1. The *Nb*ZAR1-mediated responses to the *X. perforans* effector XopJ4, could also be assayed to gain insight into this question in a non-transgenic *Nb* genetic background (Schultink et al. 2019). Work investigating the role of *Nb*EDS1 and *Nb*RNLs found that they are not required for PRR signaling in *Nb*, which suggests that *Nb*EDS1 may not be as generally important for all facets of immunity as is observed for *At*EDS1 in *Arabidopsis* (Zönnchen et al. 2022).

Growth curves employing a reduced pathogen dose failed to support our hypothesis that ZAR1 dependence on EDS1 could be correlated with effective effector dose (Figure 3.10). These data displayed a high degree of variability in DC3000 HopBA1 growth in *zar1* plants, and overall very little growth in genetic backgrounds where HopBA1 was recognized, suggesting that this assay may not be well optimized for asking questions aimed at understanding disease

resistance in a reduced pathogen burden system. In fact, the relationship between pathogen infection OD and the quantity of effector molecules that are delivered to infected cells remains a major unresolved question in the field. Being able to quantify the number of effector molecules delivered by a particular dose of pathogen would prove to be extremely useful in clearly defining “strong” NLR activating versus “weak” NLR activating effectors. The idea of plant immune activation strength existing on an elicitor-dependent spectrum has long existed in the field. However, attempts to relate *in vitro* effector-receptor dynamics to actual plant immunobiology have scarcely been attempted and the tools to do this in the context of real infections are lacking.

By assaying the capacity for Col-0 *eds1* mutant plants to restrict the growth of *Pseudomonas syringae* carrying ZAR1-recognized effectors, we observed a surprising dependence of ZAR1 on the canonical TIR signaling pathways for complete immunity. While the Col-0 *eds1* mutant displayed significantly reduced capacity to restrict the growth of virulent DC3000 +EV, prior work in the field and the results of growth assays in Ag-0 *eds1* using DC3000 +HopZ1a (Figure 3.11A) demonstrate that EDS1-independent ETI leads to growth restriction that is equivalent to what is observed in WT Arabidopsis. With this background in mind, the increased growth of DC3000 strains carrying ZAR1-recognized effectors relative to WT Col-0 suggests that ZAR1 requires EDS1 to mediate its ETI responses. Interestingly, the degree of EDS1 dependence for ZAR1 immunity varied depending on the effector used to elicit immune responses, with HopBA1 and HopZ1a-elicited immunity only partially requiring EDS1, while HopF1r-induced immune responses strongly required the presence of the EDS1-PAD4-ADR1 signaling node. It is not clear based on our data how ZAR1 dependence on EDS1-PAD4-ADR1 functions at the mechanistic level, however the existing literature can inform a few plausible explanations for this dependence. One possible explanation is that each ZAR1-

recognized effector variably activates ZAR1 resistosome formation, with ostensibly stronger ZAR1-activating effectors leading to less dependence of ZAR1 on EDS1 (HopZ1a) and weaker ZAR1 activators leading to an increased dependence of ZAR1 on EDS1 (HopF1r). Mechanistic studies of the ZAR1 resistosome have demonstrated its action as a calcium channel in the plant cell (Bi & Zhou, 2021). Presumably, weak activators of ZAR1 lead to fewer resistosomes being formed and less calcium influx into the cytosol during ETI. This leads us to hypothesize that the strength of ZAR1 activation is inversely related to its dependence on EDS1 for complete immune responses.

Mechanistically, this could be reconciled by considering the role of calcium-dependent kinases (CPKs), which have been shown to phosphorylate plant TIR-NLR immune receptors in order to negatively regulate their activity (Li et al., 2025). This poses a plausible mechanism for which the degree of ZAR1-mediated calcium influx is inversely related to TIR-induced signaling through the EDS1-PAD4-ADR1 node of the immune system. Strongly activated ZAR1 and high calcium influx into the cell leads to quenching of TNL signaling mediated by their phosphorylation by CPKs and weak activation of EDS1-PAD4-ADR1 signaling. Weak activation of ZAR1 and low calcium influx leads to less CPK-restricted TNL signaling and a strong activation of EDS1-PAD4-ADR1 (Figure 6d). The EDS1-PAD4-ADR1 node of the immune system has been shown to be activated during some early immune PTI responses, and these functions are dependent on the residues in EDS1-PAD4 that mediate TIR-derived small molecule binding (Fliegmann et al., 2025; Pruitt et al., 2021). These data imply that, during immune responses to DC3000, there are active TIR-NLRs which could plausibly be the target of regulation by CPKs downstream of ZAR1 activation. These TIR-NLRs could be thought to serve as a key point of crosstalk between CC-NLR and TIR-NLR immune signaling that has been fine-

tuned through evolution to mediate consistent immune outputs in contexts where receptors may be activated to varying degrees. Identification of the substrates of the previously identified immune-relevant CPKs will be a critical step in understanding this proposed regulatory node of the plant immune system and the validity of this line of thinking, as this mechanism is currently purely hypothetical.

Likewise, much more work needs to be done to put a finer point on the definitions of “strong” versus “weak” immune responses and the particular biochemical underpinnings of those responses. We see a disconnect in the paradigm of cell death as a “strong” immune response in the observation that RBA1-mediated cell death in Ag-0 is totally dispensable for pathogen growth restriction. Similarly, we see that ZAR1-mediated responses to HopZ1a are associated with HR, but the cell death responses elicited by HopZ1a are EDS1-independent. These findings suggest either that cell death is not necessarily a consequence of strongly activated ZAR1, or that ZAR1-mediated cell death and disease resistance responses stem from the activation of parallel signaling pathways.

Clearly, a multitude of mechanistic questions regarding this relationship between ZAR1 and EDS1 signaling need to be addressed. Hopefully this work can serve as a starting point from which to ask more mechanistic questions about the generic requirement of EDS1 in ETI responses and disconnects between pathogen disease resistance and cell death responses.

3.4 Material and methods

3.4.1 Plant growth conditions

Arabidopsis plants were grown under 100 μ Mol light intensity, 75% humidity at 22°C short day conditions (8h light 16h dark) in a Conviron growth chamber. Arabidopsis were seeded

into pots where they germinated and were grown until they had four true leaves (approximately 9 days). Arabidopsis seedlings for use in growth curves and ion leakage assays were transplanted into flats in a grid. Arabidopsis were planted into 8 plant deep columns of a flat where each column is composed of a particular Arabidopsis genotype, with the genotype being different on each half of the flat. Between 3 and 5 Arabidopsis genotypes were included in a given flat, depending on the number of genotypes used in an assay. *N. benthamiana* plants were grown in Conviron growth chambers at 25 °C, 70% relative humidity, and 15 h photoperiod with a light intensity of $\sim 80 \mu\text{E m}^{-2} \text{s}^{-1}$. *N. benthamiana* plants were sown at a density of ~ 100 seeds in a 4-inch pot and were grown until they had two true leaves (approximately 9 days). *Nb* seedlings were transplanted as individuals into 4-inch pots and were allowed to grow under a humid dome in the growth chamber for ~ 2 weeks after transplanting, at which point the dome was slowly removed over the course of 3 days to acclimate plants to humidity conditions in the growth chamber.

3.4.2 Bacterial strains and growth conditions

Pseudomonas syringae DC3000 and *Pseudomonas fluorescens* strains were grown at 28 °C in King's B medium. Pseudomonas antibiotic concentrations used (in micrograms per milliliter) were gentamycin 25, kanamycin 30, tetracycline 10, and rifampicin 50.

3.4.3 Molecular cloning

Gateway-compatible Entry clones and Destination clones were generated by BP and LR cloning (Invitrogen) or by direct synthesis (TWIST). Site-directed mutants were generated polymerase incomplete primer extension (PIPE)(Klock, H.E., Lesley 2009). *Agrobacterium* 35S-promoter expression plasmids included pGWB615 (3×HA), pGWB617 (4×MYC), pGWB641

(eYFP). *Pseudomonas* expression plasmids used were pJC531 (native promoter:1×HA) and pUCP20 (native promoter:1×HA). pGWB vectors are from the Nakagawa laboratory (Nakagawa et al. 2007).

3.4.4 Pathogen growth curves

Pseudomonas syringae pv. *tomato* DC3000 was grown for 36h prior to pathogen infiltration on KB agar plates containing the appropriate antibiotics. Prior to infiltration, plates were soaked in 10mL 10mM MgCl₂ for 10 minutes and bacteria were scraped from the plate and OD₆₀₀ was measured on a spectrophotometer. Bacterial cultures were first diluted to OD 0.1 in 15mL of 10mM MgCl₂ and subsequently diluted to OD 0.0002. The abaxial side of whole leaves of 6-week-old *Arabidopsis* were infiltrated using a needleless syringe. Three leaves on each plant were infiltrated with a given bacterial strain, with all strains in an experiment infiltrated into each individual plant. Growth curve Infiltrations were completed by 11AM. Infiltrated plants were returned to the growth chamber for 4 days following infiltration. Infiltrated leaves were cut from the plant and 2mm diameter cores were taken from the leaves. Six leaf cores from six individual plants were combined to form a single sample and six samples were taken for each genotype. Cores were homogenized with a single glass bead in a Qiagen Tissuelyser II at 30hz for 30s. Pulverized samples were resuspended in 1mL of 10mM MgCl₂ and were serially diluted. 10μL of each dilution was plated on KB agar plates containing appropriate antibiotics and were allowed to grow for 48h at 28C before colonies were counted.

3.4.5 Ion leakage assays

Four *Arabidopsis* leaf discs (diameter 0.6 cm) were collected either 3 or 1 day(s) after infiltration with Pf0-1 EtHAN or DC3000 D36E, respectively, and floated on 10 ml of deionized

water in a clean scintillation vial for 3 h with continuous shaking (100 r.p.m.) at room temperature. The initial and final conductivity values after 15 min of boiling were measured using an OrionStar A112 conductivity meter (Thermo Fisher Scientific). Relative conductivity was calculated by comparing the initial and final conductivity values (protocol adapted from (Sun et al. 2022)).

3.4.6 Statistical analyses

Multiple comparisons were analyzed via two-way analysis of variance (ANOVA) with post hoc Tukey HSD (honestly significant difference) using R Studio [R version 4.4.1, RStudio Team (2021), Boston, MA]. Comparisons of significance indicated with compact letter display (CLD). Overlapping letters are nonsignificant ($P > 0.05$), while separate letter classes indicate $P < 0.05$ or better.

3.5 Author contributions

This chapter incorporates data from experiments conducted by Sam C. Ogden and Tyler S. Todd. Sam C. Ogden carried out Ag-0, Ws and Col-0 growth curves, ion leakage assays, western blot analysis, and *N. benthamiana* phenotyping assays. Tyler S. Todd conducted Ag-0 WT vs Ag-0 *fri1* growth curves, qualitative HR assays and generated the Ag-0 *zar1-6* mutant used throughout (Figure 3.1). All figures created using BioRender.com.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Conclusions

Since the proposal of the zig-zag model for the plant immune system in 2006, significant efforts have been made to describe the protein signaling components involved in regulating PTI and ETI responses (Jones, J.D.G. 2006). Identification of the proteins that carry out each of these two phases of the plant immune response has not only improved our understanding of the signaling events that take place in immune signaling, but increasingly, has supported an alternative conceptual view of the plant immune system's organization. Rather than a two-tiered immune system that functions via discrete groups of proteins activated at distinct times during an immune response, a more holistic view of the signaling events taking place during immunity is more supported by modern research in the field (Jones, Staskawicz, and Dangl 2024; Köster, DeFalco, and Zipfel 2022; Pruitt et al. 2021; H. Tian et al. 2021; Y. Wang et al. 2023; Yuan et al. 2021). Central to these points of crosstalk between traditional PTI and ETI-related signaling events is EDS1, which has emerged as somewhat of a “master regulator” of plant immunity – especially in *Arabidopsis*, where a majority of the field's work has been conducted.

4.1.1 EDS1 is a general regulator of plant immune responses

While EDS1 was first described as a signaling component required specifically for TNL-induced immunity, EDS1 has been found to be involved in a myriad of plant immunity signaling pathways over years of research (Bartsch et al. 2006; Cui et al. 2017; Falk et al. 1999; Pruitt et al. 2021; Saile et al. 2020). EDS1, and specifically the EDS1-PAD4-ADR1 signaling complex, has been found to act as a positive regulator of PRR signaling, although the mechanism for this

positive regulation is still relatively poorly understood (Feehan et al. 2022; Pruitt et al. 2021; Zönnchen et al. 2022). Despite these findings, some NLR immune receptors, like ZAR1 and RPM1, have continued to be thought of as standalone “singleton NLRs” that do not require EDS1 or other immune signaling components to induce defense responses (Lewis et al. 2010; Saile et al. 2020). In the case of ZAR1, the work presented in Chapter 3 presents a compelling argument that ZAR1 is in fact not a singleton NLR and requires the EDS1-PAD4-ADR1 node to initiate complete immunity in Arabidopsis. The requirement of EDS1 for ZAR1-mediated immune responses was only ever previously tested in the context of HopZ1a activated immunity (Lewis et al. 2010; Saile et al. 2020). If ZAR1-EDS1 dependence had been tested in the context of responses to HopF1r, for example, the requirement of EDS1 would probably have been caught sooner, highlighting the importance of evaluating signaling dependencies for a given immune receptor each time it is being assessed in a new context.

4.1.2 Genetic context as a major determinant of immune outcomes

While it is obvious that genetics underpin any biological phenomenon, it is a common temptation among researchers to assume that proteins studied in one context will behave equivalently in others. For example, work on EDS1 has established its role as an important positive regulator of PRR-mediated immune responses in Arabidopsis (Pruitt et al. 2021). Research focusing on the *Solanum lycopersium* and *Nicotiana benthamiana* alleles of EDS1, however, demonstrated directly conflicting results, finding that EDS1 is wholly dispensable for the tested PRR-mediated immune responses (Zönnchen et al. 2022). In Chapter 3, we see divergent requirements of EDS1 for immune responses between Ag-0 and Col-0 Arabidopsis (Figure 3.2C, 3.11A). These data demonstrate that homologous proteins cannot necessarily be expected to behave equivalently across even closely related genetic backgrounds. Furthermore,

the mechanisms underpinning observed differences in signaling between closely related proteins are poorly understood, representing another facet of plant immunity research that would benefit from more investigation.

4.1.3 Type III effectors: virulence factors or genetic burdens?

Pathogen-secreted type III effector proteins are typically thought of as virulence factors, meaning that by encoding type III effectors, a given pathogen will have increased virulence on at least some host plants. Indeed, work with DC3000 *hrcC* and effectorless mutants have demonstrated that type III effector secretion is required for virulence (Cunnac et al. 2011; Hauck, Thilmony, and He 2003). Surprisingly, work dissecting the requirement of specific groups of effectors in DC3000 found that a minimal effector repertoire of eight out of the 36 naturally occurring effectors was sufficient to reconstitute a majority of DC3000 virulence in *Nb*. (Cunnac et al. 2011). These results beg the question, why does DC3000 carry so many effectors if eight are sufficient for a majority of the virulence functions required for plant colonization – especially when a single recognized effector can completely abolish a pathogen’s ability to colonize a host?

One hypothesis is that lab assays are conducted using artificially high pathogen doses and that the relevance of most effectors is only apparent in low-dose infections where the pathogen’s colonization success is more precarious. In this case, the presence of many effectors may be necessary to more comprehensively suppress plant immune responses. Another hypothesis that explains effector repertoire size is that some effectors in a pathogen’s genome function to suppress immunity triggered by other effectors, allowing ETI-eliciting effectors to exist within a pathogen’s repertoire conditional upon the presence of ETI-suppressing effectors. This hypothesis is supported by work characterizing the effectors found across *Pseudomonas syringae* strains. This work found that a majority of strains harbor at least one ETI-eliciting effector

(Laflamme et al. 2020). Follow up work demonstrated that ETI can be suppressed by inclusion of certain effectors in *P. syringae*'s effector repertoire, but that this suppression is quantitative (i.e. ETI is still initiated but can be partially suppressed) (Martel et al. 2022). These data are further supported by findings that pathogen virulence can be created in aggregate by a population of *P. syringae* strains each harboring a single effector from the wild-type effector repertoire (Ruiz-bedoya et al. 2023). These data together support the hypothesis that in nature, where plant host immune genotypes may vary, pathogen populations may “cheat” ETI elicitation through this cooperative virulence. This would allow natural populations of a pathogen to tolerate some number of ETI-eliciting effectors to exist. The presence of “freeloader” strains in a pathogen’s population is beneficial, as the categorization of an effector as a virulence or avirulence factor is entirely dependent on the host plant’s genotype, which varies in nature.

Given both the conservation of ZAR1 throughout the plant phylogeny and its capacity to recognize a wide range of effectors, it is likely that effectors that function to directly suppress ZAR1 immune activation will be identified eventually (Gong et al. 2022; Laflamme et al. 2020). Indeed, direct inhibition of NLR function by pathogen effectors has been demonstrated already (Sugihara et al. 2025). Such an effector that inhibits ZAR1 activation could effectively suppress the ETI elicited by a significant subset of naturally occurring *P. syringae* strains.

4.1.4 Cell death in plant immunity

In Chapter 3, we describe incongruencies between the expected and observed relationships between plant HR and disease resistance. RBA1’s apparent lack of impact on disease resistance in Ag-0 is one example of a disconnect between the data in this dissertation and what is found in the literature (Figure 3.2). Another example of cell death and disease resistance having an unexpected relationship can be seen in ZAR1 requiring EDS1 for disease

resistance but not cell death in Col-0 (Figure 3.9). This example is further complicated by the observation that HopBA1-induced cell death responses are fully lost in Ag-0 *eds1* (Figure 3.2). Both of these pieces of data challenge the view that plant HR results from strong activation of immune receptors and that NLR activation is correlated with cell death. Presumably, there is reduced ZAR1 activity in Col-0 *eds1*, as evidenced by impaired DC3000 HopZ1a growth restriction, yet HopZ1a-induced cell death is equivalent in WT and *eds1* Col-0 (Figure 3.9). These data might point to the presence of a separate regulatory network governing Arabidopsis HR that can be activated along with or independently of signaling networks that confer pathogen growth restriction.

Activation of these networks is likely tied to subtle differences in cellular calcium signatures produced in response to a particular set of elicitors. Indeed, many calcium binding proteins with disease resistance and HR phenotypes have been identified (Prasad, Abdel-Hameed, and Anireddy 2024; L. Wang et al. 2011). The precise relationships between immune-induced calcium signatures and these calcium binding proteins and the downstream impacts on transcription and immune outputs have yet to be described. Description of these relationships is quite challenging for a number of reasons: the apparent sensitivity of these proteins to calcium signatures, the fact that these proteins likely function in feedback loops, where genetic ablation of calcium receptors would alter signaling dynamics beyond their specific signaling role in the cell, and genetic redundancy of many calcium binding proteins. Despite these difficulties, the tools available to dissect calcium signaling dynamics continue to improve, lending a positive outlook to those hoping to understand how calcium regulates cellular signaling in intimate detail.

In a broader sense, this work demonstrates the value of experimentally testing how receptors function in a specific context of interest and not necessarily relying on models for how

a system is thought to work. The differences in immune output of the Ag-0, Ws and Col-0 alleles of ZAR1 emphasize that even closely related alleles of the same gene may behave differently. In breeding efforts to improve crop yield and disease resistance traits, it is worth keeping this in mind, as leveraging genomics and screening a wide range of alleles for a gene of interest may be the key to identifying ones that give a desired phenotype. Improving our collective understanding of how the plant immune system works at a molecular level is critical to achieving the field's long-term goal of being able to rapidly engineer plant immunity to combat disease outbreaks in the field.

4.2 Future directions

The work outlined in the below sections would serve to fill in gaps in understanding raised by the work from Chapters 2 and 3. Some of this work has been started and could realistically be completed using existing tools created as part of the dissertation work, while other approaches would necessitate more significant time investment, including generation of new genetic material, and the proposed outcomes are more speculative.

4.2.1 Determination of HopBA1 virulence targets

From the outset of the work described in Chapter 2, description of the enzymatic activity of HopBA1 and the targets of its hypothetical function was a goal for this research. While significant strides were made in identifying a potential substrate of HopBA1 from *Nicotiana benthamiana*, virulence targets of HopBA1 in Arabidopsis remain enigmatic. One of the significant barriers to this is the apparent instability of many of these proteins in transient expression assays (chapter 2). Expression of *AtRLCK* VIIs with a range of epitope tags may alleviate the observed accumulation issues and allow for co-expression assays to take place,

similar to the approach used to identify HopBA1-dependent cleavage of *NbPBL6-2*. If *AtRLCK VII* transient expression remains intractable, confirmation of motifs required for HopBA1-dependent *NbPBL6-2* cleavage could be used to identify *AtRLCK VII*s (or other Arabidopsis genes) to knock out for genetic determination of HopBA1 virulence targets in Arabidopsis.

4.2.2 Confirmation of HopBA1 protease activity on NbPBL6-2

When transiently co-expressed with *NbPBL6-2* in *Nb*, HopBA1 appeared to act as a protease (Figure 2.6). While the low molecular weight band derived from *NbPBL6-2* is consistent with HopBA1 enzymatically cleaving the N-terminus of *NbPBL6-2*, the generation of these data in an *Nb. Agrobacterium* transient assay leaves open the possibility of HopBA1 indirectly leading to the cleavage of *NbPBL6-2*, rather than *NbPBL6-2* being a *bona fide* substrate of HopBA1. To rule this possibility out, an *in vitro* cleavage assay using purified HopBA1 and *NbPBL6-2* would definitively demonstrate that HopBA1 acts as a protease on this substrate. Expression constructs for purification of *NbPBL6-2* and HopBA1 from *E. coli* were created, and optimal expression conditions for HopBA1 were identified. Reasonable expression conditions for *NbPBL6-2* were identified, but expression was inconsistent and *NbPBL6-2* could not be purified in quantities sufficient for *in vitro* assays. The successful purification of HopBA1 for structural determination by Karen Cherkis in the Dangl lab suggests that biochemical assays with HopBA1 are feasible, and simply more troubleshooting of *NbPBL6-2* expression could yield the proteins required for the desired *in vitro* assays. Submission of cleaved purified *NbPBL6-2* for proteomic analysis would also allow for determination of the HopBA1 cleavage site, assuming that *in vitro* cleavage assays were successful.

Characterization of the HopBA1 cleavage site and substrate recognition motif would potentially allow for more straightforward identification of HopBA1 substrates in Arabidopsis

and other plant species via bioinformatic analysis of protein sequences from these species. Experimental validation of bioinformatically-derived HopBA1 substrates in agrotransient or *in vitro* assays would be required to confirm these results but could yield novel engineering targets for resistance to pathogens carrying HopBA1. Similar to approaches taken with AvrPphB and its protease substrate, PBS1, the HopBA1 substrate recognition and cleavage motif could be integrated into orthologous proteins, allowing for HopBA1 recognition to be expanded into new genetic contexts.

4.2.3 Functional characterization of *NbPBL6-2*

HopBA1-induced cleavage of *NbPBL6-2* could be leveraged to describe the functional role of this *NbRLCK* VII. Higher-order polymutants of subgroup 6 *NbRLCK* VIIs were compromised in PTI-induced ROS bursts, suggesting an important function of these *RLCK* VIIs in positive regulation of PTI (W. R. H. Huang et al. 2024). The *nbrlckvii-6* polymutant assayed here harbors a knockout mutation in the *NbPBL6-2* coding sequence, indicating that it likely functions to regulate PTI responses. More targeted biochemical assays using *NbPBL6-2* and HopBA1 could reveal a role of the N-terminus of *NbPBL6-2* in its function. The role of the N-terminus of *RLCK* VIIs has not been extensively characterized, meaning that these assays could yield novel insights into *RLCK* VII function.

4.2.4 Characterization of the ZAR1 active complex in response to HopBA1

The C-terminal fragment of *NbPBL6-2* was not found to co-immunoprecipitate with the ZAR1-ZRK2 protein complex in agrotransient assays. Expression of *NbPBL6-2* with a N-terminal epitope tag and subsequent co-IP assays could potentially show HopBA1-dependent interaction of the cleaved *NbPBL6-2* N-terminal fragment with the ZAR1-ZRK2 complex,

contributing to the field's understanding of ZAR1 activation by HopBA1. These assays could lead to the identification of the interface between HopBA1 virulence targets and ZRK2, which could be used as another platform for resistance engineering.

4.2.5 Untargeted searches for HopBA1 virulence targets in Arabidopsis

Targeted searches for HopBA1 substrates within the Arabidopsis RLCK VII family did not lead to identification of HopBA1's virulence target(s) in Arabidopsis (Chapter 2). Given these results, untargeted approaches should be used to identify potential non-RLCK VII virulence targets of HopBA1. Proximity-labeling based approaches could be taken to identify HopBA1's substrates in an unbiased way.

NbPBL6-2 was found to potentially be a substrate for HopBA1's protease activity, however other HopBA1 virulence targets may exist in *Nb*. These unknown virulence targets could be identified via proximity-labeling techniques in *Nb* like those described above or via immunoprecipitation of ZRK2 and HopBA1 and mass spectrometry of overlapping proteins in the pulldown assays. Subsequent confirmation of these putative virulence targets via biochemical and genetic assays would be required to demonstrate the validity of these as HopBA1 substrates.

4.2.6 Characterization of the RBA1 activation mechanism

While a variety of TNL activation mechanisms have been characterized, it remains unclear how TIR-only NLRs like RBA1 perceive effectors. Upon identification of the substrates of HopBA1, these substrates could be tested for their association with RBA1. Untargeted proteomic approaches in Ag-0 could also identify other unknown proteins required for RBA1 signaling.

4.2.7 Determination of mechanism for EDS1 dependence

The model proposed at the end of chapter 3 suggests that CPKs found in recent publications (Jun Li et al. 2025) may mediate ZAR1-dependence on EDS1 for complete immunity. This model is completely speculative and is not based on any data collected as part of this dissertation work. Examination of the disease resistance responses to pathogens carrying ZAR1 recognized effectors in the *cpk3/4/5/6/11* quintuple mutant and responses in a *zar1*, *cpk3/4/5/6/11* sextuple mutant would allow the hypothesis that CPKs modulate crosstalk between ZAR1 and EDS1-dependent signaling pathways to be tested. Additional experiments measuring calcium signaling via calcium reporters in response to ZAR1 activation by the five effectors assayed in chapter 3 could also help define the strength of ZAR1 activation and clarify differences proposed in the model figure.

4.2.8 Dissection of ZAR1 allelic differences

Studies of responses to HopBA1 in Ag-0 and Col-0 revealed surprising differences in the ZAR1-dependent responses to *P. syringae* carrying HopBA1. These differences are further emphasized when comparing the responses of *eds1* mutant Ag-0 and Col-0 Arabidopsis to DC3000 HopBA1 and HopZ1a, with growth restriction of DC3000 HopZ1a by ZAR1 in Ag-0 being fully EDS1-independent but partially requiring EDS1 in Col-0 (Chapter 3). Related, recognition of HopBA1 by Ag-0 ZAR1 induces an immune response that includes localized cell death, whereas HopBA1 recognition by Col-0 ZAR1 does not induce a hypersensitive response. Further complicating things, ZAR1-mediated cell death responses to HopBA1 in Ag-0 are fully lost in the *eds1-2* mutant, whereas ZAR1-mediated cell death responses to HopZ1a and HopO1c are fully EDS1-independent, suggesting further differences in ZAR1 signaling between the two ecotypes. Complementation of Col-0 *zar1* with the Ag-0 *ZAR1* allele and vice versa (complementation of Ag-0 *zar1* and *rbal zar1* mutants with Col-0 *ZAR1*) would allow for further

exploration of the sequence and genetic determinants of the responses to and other ZAR1-recognized effectors in these two ecotypes. Indeed, ion leakage and pathogen growth assays with the other ZAR1-recognized effectors in Ag-0 would potentially reveal additional ecotype-specific differences for ZAR1-mediated immune responses. The results of these assays would hopefully shed additional light on the sequence determinants of CNL-mediated immune responses in Arabidopsis. This is an underexplored area of plant immunity research and the study of ZAR1 allelic differences could explain how CNLs can yield different immune outcomes.

APPENDIX 1: INVESTIGATION OF *PSEUDOMONAS SYRINGAE* DC3000 Δ HOPQ VASCULAR COLONIZATION OF *NICOTIANA BENTHAMIANA*

A1.1 Results

A1.1.1 Evaluation of *Pseudomonas syringae* DC3000 Δ hopQ vascular colonization in *Nicotiana benthamiana*

Previous work in the lab revealed that DC3000 Δ hopQ can spread to and colonize the vasculature of *Nicotiana benthamiana* (Todd 2024). This work characterized colonization of *Nb* petioles and stems via pathogen growth assays and fluorescence microscopy, but these techniques lack the resolution to identify the specific anatomical structures that bacterial cells are inhabiting. We employed scanning electron microscopy (SEM) of infected tissues to identify the particular regions of these tissues that are colonized. At 9 days post bacterial infiltration (dpi), we observed robust colonization of vascular bundles in the distal petiole (DP, the end of the petiole near the leaf) and proximal petiole (PP, the end of the petiole near the stem) by DC3000 Δ hopQ +EV (Figure A1.1B-D). In contrast, we only observed DC3000 Δ hopQ +HopBA1 in DP vascular bundles, but none in PP tissue (Figure A1.1E-H).

A1.1.2 Generation of a type II secretion system mutant in *Pseudomonas syringae* DC3000 Δ hopQ

Given that a vascular habit of *Pseudomonas syringae* has not been previously described, we sought to characterize the genetic determinants required for this behavior. We hypothesized that the type II secretion system (T2SS) of DC3000 may function to facilitate vascular spread and colonization in a *Nb* disease system. The T2SS of other plant pathogenic bacterial species

has been shown to facilitate translocation of effector proteins from the bacterial cytosol into the extracellular milieu (Goll et al. 2025; Korotkov, Sandkvist, and Hol 2012). Frequently, these effectors are proteases or other enzymes that increase the virulence of the pathogen by degrading and interfering with the host immune response (Korotkov, Sandkvist, and Hol 2012). The T2SS and specific effector proteins of other bacterial plant pathogens like *Xanthomonas campestris* has been shown to be required for vascular colonization of their host plant species (Goll et al. 2025; Paauw et al. 2023), but the role of the T2SS in any aspect of *Pseudomonas syringae* disease has not been investigated.

In addition to the question of DC3000 vascular colonization, we were also interested in investigating a possible function of the T2SS in the unique upward petiole bending (hyponasty) phenotype described in (Todd 2024) induced by DC3000 carrying HopBA1. To test the hypothesis that the T2SS has a function in *Pseudomonas syringae* pathology, we used allelic exchange to generate a deletion mutant of the major subunit of the T2SS, *GspD*. In other species, $\Delta gspD$ mutants have been recognized to completely lose T2SS activity (Hohl, Tassinari, and Low 2021; Korotkov et al. 2009; Korotkov, Sandkvist, and Hol 2012; Lauber et al. 2024). Since we were interested specifically in assaying the spread of T2SS loss of function mutants in *Nb*, we generated our *GspD* KO in the DC3000 $\Delta hopQ$ 3xmCherry genetic background. Once we successfully generated the DC3000 $\Delta hopQ \Delta gspD$ 3xmCherry strain, we used triparental mating to add the type III effector delivery plasmid, pJC531 with HSP18UTR (empty vector), HopQ or HopBA1 for downstream assays.

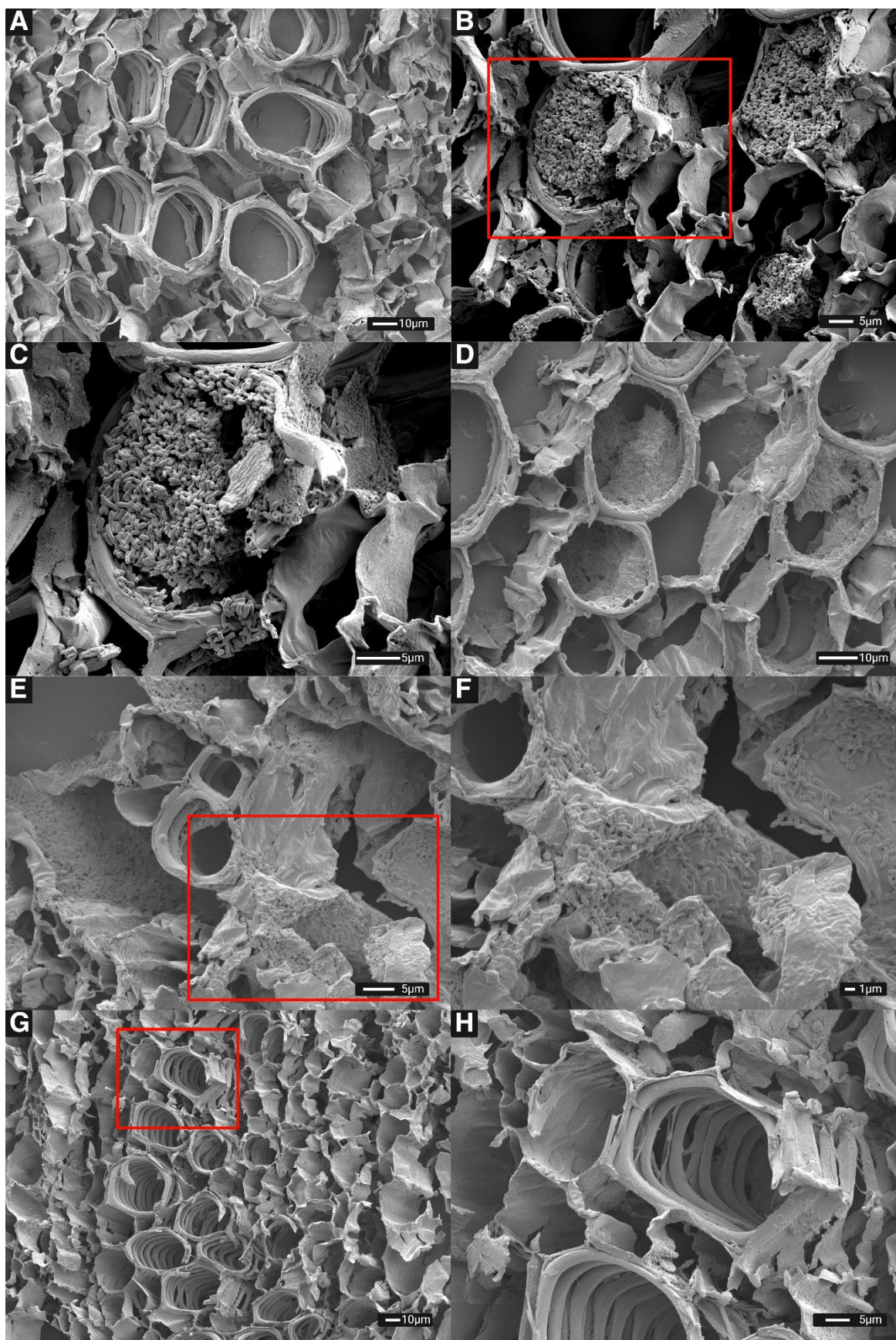


Figure A1.1. *Pseudomonas syringae* DC3000 $\Delta hopQ$ is capable of colonizing the vasculature of *Nicotiana benthamiana*. **A)** SEM imaging shows that distal petiole tissue associated with leaves infiltrated with a $MgCl_2$ control do not harbor any bacterial cells. Structures are pictured at 700x magnification 6 days post infiltration (dpi). **B)** SEM images of distal petiole tissues associated with leaves infiltrated with DC3000 $\Delta hopQ$ +EV show that they harbor bacterial cells in vascular bundles, up to the point of complete vascular occlusion, 1400x magnification 9 days post infiltration. **C)** SEM image of the box in (B) at 2,500x magnification showing a closer image of vascular colonization. **D)** SEM images of proximal petiole tissues associated with leaves infiltrated with DC3000 $\Delta hopQ$ +EV show that they harbor bacterial cells in vascular tissue, 1100x magnification 9 days post infiltration. **E)** SEM images of distal petiole tissues associated with leaves infiltrated with DC3000 $\Delta hopQ$ +HopBA1 harbor bacterial cells, 1800x magnification 9 days post infiltration. **F)** SEM image of the box in (E) at 3000x magnification focusing on bacterial cells in petiole tissue. **G)** SEM images of proximal petiole tissues associated with leaves infiltrated with DC3000 $\Delta hopQ$ +HopBA1 show a lack of bacterial colonization in these tissues, 430x magnification 9 days post infiltration. **H)** SEM image of the box in (F) at 1400x magnification focusing on bacteria-free vascular bundles.

To test the hypothesis that our $\Delta gspD$ mutant strain would be deficient in the capacity to systemically colonize *Nb*, we conducted bacterial growth assays in the petioles of *Nb* leaves infiltrated with our DC3000 strains of interest. To gain further insight into the specific colonization habits of these strains, we split petiole tissue into the DP and PP subgroups used to assess vascular colonization via SEM. We assessed growth of DC3000 $\Delta hopQ$ 3xmCherry EV, DC3000 $\Delta hopQ$ $\Delta gspD$ 3xmCherry EV, DC3000 $\Delta hopQ$ 3xmCherry HopQ and DC3000 $\Delta hopQ$ $\Delta gspD$ 3xmCherry HopQ in distal and proximal petiole tissues at 6 and 12 days post bacterial infiltration and in the stem above and below the petiole of the infiltrated leaf 12 dpi (Figure A1.2). We did not observe any significant impact of the $\Delta gspD$ mutant on bacterial petiole or stem colonization in the virulent EV or avirulent HopQ complementation strains of DC3000 $\Delta hopQ$ 3xmCherry relative to the strains with a functioning T2SS.

The other phenotype that we were interested in assessing was the potential requirement of the T2SS in HopBA1-dependent hyponasty. Preliminary qualitative evaluation of hyponasty

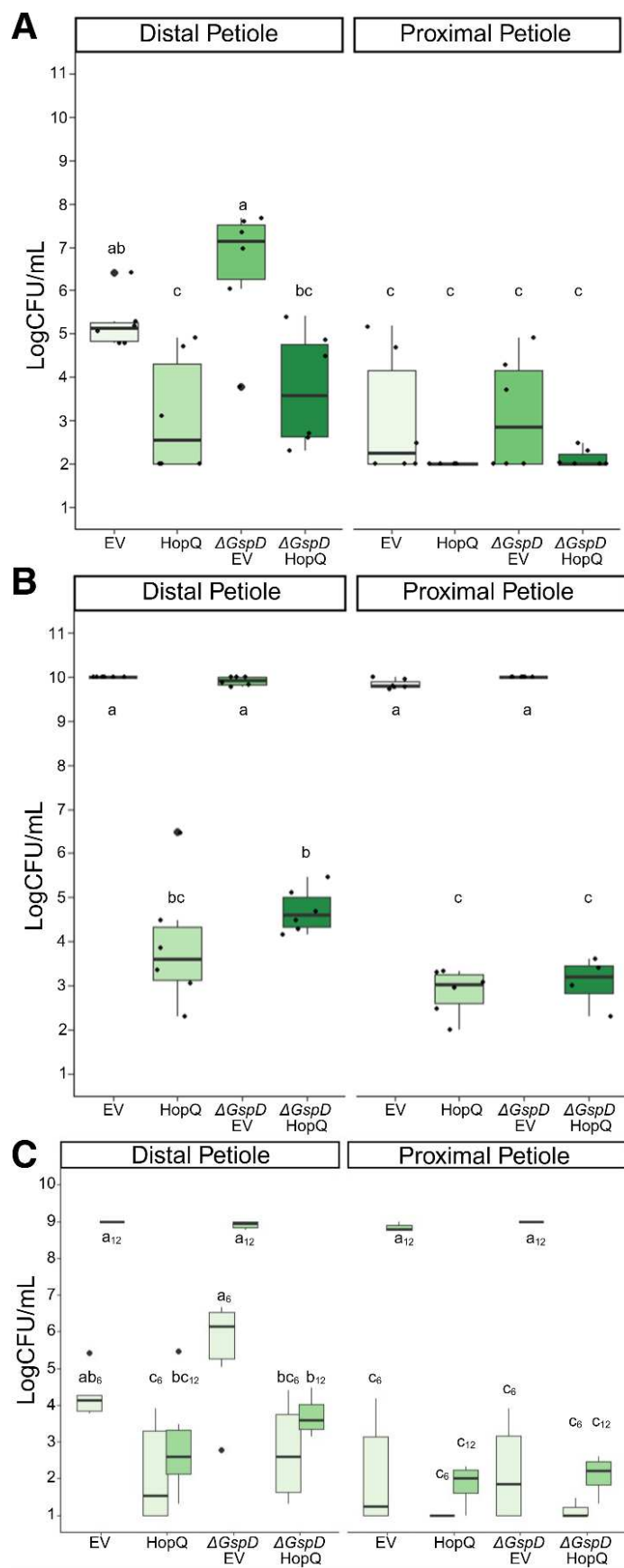


Figure A1.2 Deletion of the *Pseudomonas syringae* T2SS does not impact petiole colonization at 6 and 12 days post infiltration. A) Growth restriction of DC3000 $\Delta hopQ$ 3xmCherry with or without a functioning T2SS in *Nb* petioles 6 days post pathogen infiltration. **B)** Growth restriction of DC3000 $\Delta hopQ$ 3xmCherry with or without a functioning T2SS in *Nb* petioles 12 days post pathogen infiltration. **C)** Combined 6- and 12-day growth assays for comparison of pathogen growth over time. Pathogen strain is denoted on the x-axis. All strains used are *Pseudomonas syringae* DC3000 $\Delta hopQ$ 3xmCherry carrying pJC531 with EV or HopQ inserts. Strains with $\Delta GspD$ are knockout mutants for the GspD component of the T2SS.

showed that at six dpi, both DC3000 $\Delta hopQ$ 3xmCherry HopBA1 and DC3000 $\Delta hopQ$ $\Delta gspD$ 3xmCherry HopBA1 exhibited petiole bending characteristic of the hyponasty phenotype. More replication and quantification of hyponasty according to methods described in (Todd 2024) is required to fully characterize the impact of the T2SS deletion on hyponasty phenotypes.

A1.2 Discussion

Scanning electron microscopy of DC300 $\Delta hopQ$ -infected *N. benthamiana* petiole tissue revealed robust colonization of *Nb* vascular bundles (Figure A1). *Pseudomonas syringae* systemic spread throughout *Nb* was described in Todd 2024, but the precise location of bacteria in petiole tissues was not resolved. Here we confirm our expectation that DC300 $\Delta hopQ$ is capable of vascular colonization of petiole tissues. Further work needs to be done to establish the molecular determinants of vascular spread, and the factors that restrict DC300 $\Delta hopQ$ HopBA1 from reaching proximal petiole tissue.

Our assessment of systemic spread of DC3000 $\Delta hopQ$ $\Delta gspD$ does not establish a function for the T2SS in DC3000 virulence in *Nb*. Transcriptomic data suggests that the genes encoding the DC3000 T2SS are expressed and at least some of the genes are PTI-regulated (Chen et al. 2025; Haibi Wang et al. 2022). The method used in the lab for conducting bacterial growth assays involves whole-leaf infiltration of *Nb* leaves with the bacterial strain of interest. It

is possible that T2SS function is density-dependent and that a partial leaf infiltration or lower bacterial dose would reveal some activity that is currently obscured by the assay parameters. While we did not establish a role for the T2SS in *Pseudomonas syringae* disease in progression in *Nb*, evaluation of other pathosystems may be more well suited to dissect this underexplored secretion system. For example, the Arabidopsis accession Oy-0 has been found to be a suitable host for vascular colonization by *Xanthomonas campestris* and this was recently leveraged to describe multiple type-II secreted effector proteins required for this activity (Paauw et al. 2023, 2024). Similar experiments in this Arabidopsis ecotype may reveal a role for the *Pseudomonas syringae* T2SS not observed in our current body of work.

A1.3 Material and methods

A1.3.1 Bacterial strains and growth conditions

Escherichia coli (*E. coli*) Top10 was grown in LB medium at 37°C. *Pseudomonas* strains were grown at 28°C in King's B medium. The *E. coli* antibiotic concentrations used (in micrograms per milliliter) were ampicillin 100, kanamycin 30, gentamycin 25, and spectinomycin 50. *Pseudomonas* antibiotic concentrations used (in micrograms per milliliter) were tetracycline 5, gentamycin 25, kanamycin 30, and rifampicin 50. The in-frame deletion mutant of *Pseudomonas syringae* $\Delta hopQ$ 3xmCherry was generated via two-step allelic exchange. DNA fragments 500bp in length upstream and downstream of the *GspD* coding sequence, flanked by BP sites for Gateway cloning were synthesized by Twist Biosciences. This sequence was cloned into pDONR207 via a BP reaction, followed by a LR reaction to move the knockout sequence into the suicide plasmid pLVC-D7-trpSacB. This plasmid containing the *GspD* knockout sequence was moved into *Pseudomonas syringae* $\Delta hopQ$ 3xmCherry via triparental mating. Bacterial recombinants were selected on King's B agar containing 50 µg/mL

rifampicin and 5 µg/mL tetracycline. Integration of the suicide plasmid was verified via PCR (Table A2.2). Recombinant bacterial strains were counterselected on King's B agar containing 50 µg/mL rifampicin and 5% sucrose to induce excision of pLVC-D7 sequence from the *P. syringae* genome. Successful deletion of *GspD* was verified using PCR and bacterial whole-genome sequencing provided by Plasmidsaurus.

A1.3.2 Plant materials and growth conditions

N. benthamiana plants were grown in Conviron growth chambers at 25°C, 70% relative humidity, and 15 h photoperiod with a light intensity of $\sim 80 \mu\text{E m}^{-2} \text{s}^{-1}$. *N. benthamiana* plants were sown at a density of ~ 100 seeds in a 4-inch pot and were grown until they had two true leaves (approximately 9 days). *N. benthamiana* seedlings were transplanted as individuals into 4-inch pots and were allowed to grow under a humid dome in the growth chamber for ~ 2 weeks after transplanting, at which point the dome was slowly removed over the course of 3 days to acclimate plants to humidity conditions in the growth chamber.

A1.3.3 Pathogen growth curves

Petiole and stem growth curves were conducted according to the protocol published in (Todd 2024). Petioles were excised from infiltrated leaves and were divided into the sub-regions of distal petiole (DP) and proximal petiole (PP), which consisted of tissue that was closest to the infiltrated leaf and stem, respectively. Petiole tissue was allowed to dry after being cut from the plant using a sterile razor blade. Petiole tissue was then surface sterilized utilizing two washes of 1X TE (2 min. each), one wash of 70% EtOH (1 min.), one wash of 3% bleach (1 min.), and five washes of ddH₂O (~ 1 min. each).

Following surface sterilization, tissue from each sub-region was divided into three equal parts and a corresponding segment from each of three biological replicates was combined into a single sample. Three technical replicates were used for each strain and for each petiole sub-region. All samples were weight-adjusted to account for the variable dimensions of petioles and stems. Petiole tissue was placed in 2mL tubes containing a glass bead and 200 μ L 10mM MgCl₂. Samples were lysed for 30s at 30hz using a Qiagen TissueLyser II. Following processing, 800 μ L of 10mM MgCl₂ was added to each tube. 100 μ L of each sample was added to a 96 well plate for serial dilutions. 20 μ L of each sample was then transferred to 180 μ L of 10mM MgCl₂, with 20 μ L of each dilution being serially diluted a total of 6 times (range of 1-1000000-fold dilution). 10 μ L of each dilution was plated onto KB media agar carrying the appropriate antibiotics. Plates were allowed to grow for 48 h at 28°C before colonies were counted.

Stem growth curves were carried out as described above, with the exception of tissue divided into sub-regions from above and below the petiole/leaf that was infiltrated with bacteria.

A1.3.4 Scanning electron microscopy (SEM)

Samples for SEM were prepared according to (Caldwell and Iyer-Pascuzzi 2019). Briefly, infiltrated petiole tissue was placed into 2 to 3 mL of primary fixative solution (1.5% glutaraldehyde and 2% para- formaldehyde in 0.1M of cacodylate buffer) in a glass scintillation vial immediately following harvesting. Sample size was limited to 2 to 5 mm to ensure penetration of the fixative into the tissue. The scintillation vial (without the lid) was placed into a vacuum desiccator and low vacuum pressure was applied for 2 h. Samples were removed from the vacuum desiccator after 2 h by slowly releasing the vacuum pressure. The fixative was removed with a fine-tip disposable transfer pipette without touching the sample. Spent fixative was removed and replaced with 2 to 3 mL of wash buffer (0.1 M of cacodylate, pH 6.8). The

sample was not permitted to dry out between liquid changes. The scintillation vial without the lid was placed into a vacuum desiccator for 15 min and low vacuum pressure was applied. After 15 min, the samples were removed from the vacuum desiccator by releasing the vacuum pressure slowly. Spent wash buffer was removed and replaced with fresh wash buffer. The sample was rinsed with wash buffer a total of 3 times. Samples were kept in wash buffer in scintillation vials as they were transferred to the CSU experimental pathology facility for sample dehydration and paraffin embedding.

Paraffin embedded samples were placed in a -20°C freezer for 2 hours prior to sectioning on a Leica RM2125 RTS rotary microtome at room temperature. Samples were sectioned to a thickness of 20 µm. A square coverglass was taped to a glass slide with double-stick tape and a drop of water was added to the coverglass to allow the paraffin ribbon to be floated, expand and were subsequently dried for 24 h. Slides were deparaffinized in three changes of 100% xylene followed by 100% EtOH for 5 min each. The samples were removed from the EtOH, and the coverglass was removed from the slide and placed on a SEM stub with double-sided tape. To reduce charging of the sample, a line of silver conductive coating was applied from the pin of the specimen holder to the coverglass. Samples were sputter coated for 90s in a Quorum Q150T ES Plus Coater sputter coater with chromium to a thickness of 20 nm. Samples were imaged on a JEOL JSM-IT800(HL) Field Emission SEM.

A1.3.5 Statistical analyses

Multiple comparisons were analyzed via two-way analysis of variance (ANOVA) with post hoc Tukey HSD (honestly significant difference) using R Studio [R version 4.4.1, RStudio Team (2021), Boston, MA]. Comparisons of significance indicated with compact letter display

(CLD). Overlapping letters are nonsignificant ($P > 0.05$), while separate letter classes indicate $P < 0.05$ or better.

APPENDIX 2: SUPPLEMENTAL MATERIALS

Table A2.1 List of plant lines used in this dissertation.

Ecotype	Mutant	AGI Code(s)	Line	Published in
Col-0	<i>eds1-12</i>	AT3G48090	CAS9 deletion	Ordon et al. 2017
Col-0	<i>pad4-1</i>	AT3G52430	CS3806	Zhou et al. 1998
Col-0	<i>sag101-2</i>	AT5G14930		Tissier et al. 1999
Col-0	<i>zar1</i>	AT3G50950	SALK_031297C	Laflamme et al. 2020
Col-0	<i>adr1 triple</i>	AT1G33560, AT4G33300, AT5G04720	<i>adr1-1</i> (SAIL_842_B05), <i>adr1-L1-1</i> (SAIL_302_C06), <i>adr1-L2-4</i> (SALK_126422)	Bonardi et al. 2011
Col-0	<i>nrg1 triple</i>	AT5G66910, AT5G66900, AT5G66890	CAS9 deletion	Wu et al. 2018
Col-0	<i>helperle ss</i>	AT1G33560, AT4G33300, AT5G04720, AT5G66910,	<i>adr1-1</i> (SAIL_842_B05), <i>adr1-L1-1</i> (SAIL_302_C06), <i>adr1-L2-4</i> (SALK_126422); CAS9 <i>nrg1</i> deletion	Wu et al. 2018

		AT5G66900, AT5G66890		
Col-0	<i>zar1</i> <i>eds1</i>	AT3G50950, AT3G48090	Col-0 <i>zar1</i> (SALK_013297C) and <i>eds1-12</i>	This manuscript
Ag-0 (<i>fri1</i>)	<i>rba1-1</i>	AT1G47370	CAS9 deletion	This manuscript; <i>rba1-1</i> in WT Ag-0 from Nishimura et al. 2017
Ag-0 (<i>fri1</i>)	<i>zar1-6</i>	AT3G50950	CAS9 deletion	This manuscript
Ag-0 (<i>fri1</i>)	<i>rba1-1</i> <i>zar1-6</i>	AT1G47370, AT3G50950	Cross with Ag-0 <i>rba1</i> and <i>zar1-6</i>	This manuscript
Ag-0 (<i>fri1</i>)	<i>eds1-2</i>	AT3G48090	Cross with Col-0 <i>eds1-2</i>	<i>eds1-2</i> in WT Ag-0 from Nishimura et al. 2017
Ws	<i>eds1-1</i>	AT3G48090		Parker et al. 1996

Ag-0	<i>fril</i>	AT4G00650	CAS9 deletion	This manuscript
<i>Nicotiana benthamiana</i>	<i>eds1</i>	Nbv0.5scaffold193	CAS9 deletion	Schultink et al. 2017
Col-0	<i>rlckvii-1</i>	AT5G13160,AT1G07870, AT2G28590, AT5G02800, AT3G20530, AT5G18610, AT1G61860	<i>pbs1-1 pbl5</i> (SALK_045613) <i>pbl6</i> (CAS9) <i>pbl7</i> (SALK_114130) <i>pbl23</i> (SALK_112111) <i>pbl27</i> (GK_088H03) <i>pbl41</i> (SALK_150918)	Rao et al. 2018
Col-0	<i>rlckvii-2</i>	AT4G13190, AT3G24790, AT3G07070, AT3G02810, AT5G16500	<i>pbl24</i> (SALK_072589) <i>pbl25</i> (CAS9) <i>pbl 26</i> (SALK_023374) <i>pbl42</i> (SALK_009528) <i>pbl43</i> (SALK_055909)	Rao et al. 2018
Col-0	<i>rlckvii-3</i>	AT1G76370, AT1G20650	<i>pbl21</i> (SALK_025049) <i>pbl22</i> (SALK_045159)	Rao et al. 2018
Col-0	<i>rlckvii-4</i>	AT5G47070, AT4G17660, AT5G03320, AT3G09830,	<i>pbl 19</i> (SALK_021064) <i>pbl20</i> (SALK_049965) <i>pbl37</i> (GK_090A05) <i>pbl38</i> (SAIL_416_A03) <i>pbl39</i>	Rao et al. 2018

		AT2G39110, AT2G28940	(SALK_145629) <i>pbl40</i> (SAIL_129_D02)	
Col-0	<i>rlckvii-5</i>	AT5G15080, AT3G01300, AT3G28690	<i>pbl34</i> (SALK_067743) <i>pbl35</i> (SALK_039402) <i>pbl36</i> (SAIL_885_B03)	Rao et al. 2018
Col-0	<i>rlckvii-6</i>	AT5G01020, AT2G26290, AT5G35580, AT2G05940, AT1G61590, AT2G07180, AT5G56460	<i>pbl8</i> (GK_625H05) <i>pbl12</i> (SALK_017105) <i>pbl13</i> (GK_586B09) <i>pbl14</i> (CAS9 (<i>ripk</i>)) <i>pbl15</i> (SALK_055095) <i>pbl16</i> (SALK_201102) <i>pbl17</i> (CAS9)	Rao et al. 2018
Col-0	<i>rlckvii-7</i>	AT2G17220, AT1G76360, AT4G35600	<i>pbl30</i> (SAIL_296_A06) <i>pbl31</i> (SAIL_273_C01) <i>pbl32</i> (SALK_113804)	Rao et al. 2018
Col-0	<i>rlckvii-8</i>	AT2G28930, AT1G07570, AT5G02290, AT3G55450, AT2G39660	<i>pbl1</i> (SAIL_1236_D07) <i>pbl9</i> (GK_430G06) <i>pbl10</i> (SALK_001115) <i>pbl11</i> (SALK_046795) <i>bik1</i> (SALK_005291)	Rao et al. 2018
Col-0	<i>rlckvii-9</i>	AT1G69790, AT1G26970,	<i>pbl2</i> (SALK_149140) <i>pbl3</i> (SALK_039503)	Rao et al. 2018

		AT2G02800, AT1G14370	<i>pbl4</i> (SALK_097999) <i>pbl18</i> (SALK_097486)	
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Table A2.2 List of primers used in this dissertation.

Lab Stock Name	Sequence	Informative Name
MTN0193	TCGCGTTAACGCTAGCATGGATCTC	pDONR F
MTN0194	GTAACATCAGAGATTTTGAGACAC	pDONR R
SO021	AGGAGATGCCGATGGAAATG	pJG4-5 F backed out into AD F
SO022	AAGCCGACAACCTTGATTGG	pJG4-5 R backed out into AD R
MTN0173	GAAAAAACAGGGCAATAAAG	pEG202 MCS seq primers F
MTN0174	CGTTTTAAACCTAAGAGTCAC	pEG202 MCS seq primers R
MTN0408	GCATCGTGAAAAAGAAGACGTTC	pGWB6 genotyping F
MTN0409	TTCAGCTCGATGCGGTTCAC	pGWB6 genotyping R
SO015	ATTTTGCCGATTTCGGAAC	SALK T-DNA LBb1.3
SO016	CAGAGCATTTTCAGGATGAGC	SALK013297C Genotyping (zar1LP)

SO017	TACCTCAACATCGGAAGCTTG	SALK013297C Genotyping (zar1RP)
SO023	ttatgtagtagctagaacttggagcg	eds1-12 Genotyping F
SO024	tcttctatacagagaccaaacttgaaaacc	eds1-12 Genotyping R
SO025	ggggacaagttgtacaaaaagcaggctccaccATGGAAAAT AAGAAAAAGAATAAT	Niben101Scf03100g0000 2.1/NbPBL Clade 5-1 BP Cloning F
SO026	ggggaccactttgtacaagaaagctgggtgTTTCCTACTTGTT AAGTTCA	Niben101Scf03100g0000 2.1NbPBL Clade 5-1 BP Cloning R
SO027	ggggacaagttgtacaaaaagcaggctccaccATGGAAAAT AAGAAGGAAAAA	Niben101Scf05720g0000 4.1/NbPBL Clade 5-2 BP Cloning F
SO028	ggggaccactttgtacaagaaagctgggtgTTTACAATGTTG CCTG	Niben101Scf05720g0000 4.1/NbPBL Clade 5-2 BP Cloning R
SO029	ggggacaagttgtacaaaaagcaggctccaccATGGGGTTAT TAGCTTCTG	Niben101Scf06172g0300 7.1/NbPBL Clade 5-3 BP Cloning F
SO030	ggggaccactttgtacaagaaagctgggtgACTTTTCATGGT TGGTAAGT	Niben101Scf06172g0300 7.1/NbPBL Clade 5-3 BP Cloning R

SO031	ggggacaagtttgtaaaaaagcaggctccaccATGGGATTA GGTAGTGATG	Niben101Scf11795g00014 .1/NbPBL Clade 5-4 BP Cloning F
SO032	ggggaccactttgtacaagaaagctgggtgAGTTTTGCCATT TGGTT	Niben101Scf11795g00014 .1/NbPBL Clade 5-4 BP Cloning F
SO033	ggggacaagtttgtaaaaaagcaggctccaccATGGGCAAT TGTGGTACTAG	Niben101Scf00199g0502 5.1/NbPBL6-1 BP Cloning F
SO034	ggggaccactttgtacaagaaagctgggtgTCTAACGCGGCA TGC	Niben101Scf00199g0502 5.1/NbPBL6-1 BP Cloning R
SO035	ggggacaagtttgtaaaaaagcaggctccaccATGAAAGAC TCAAAATCTTGG	Niben101Scf03619g0000 5.1/NbPBL Clade 6-2 BP Cloning F
SO036	ggggaccactttgtacaagaaagctgggtgCTTGCTCTTACT ATGAACAG	Niben101Scf03619g0000 5.1/NbPBL Clade 6-2 BP Cloning R
SO037	ggggacaagtttgtaaaaaagcaggctccaccATGGGCAAT TGTGGTACTA	Niben101Scf08926g0101 7.1/NbPBL Clade 6-3 BP Cloning F

SO038	ggggaccactttgtacaagaaagctgggtgTCTAACACTGCA TGCTG	Niben101Scf08926g0101 7.1/NbPBL Clade 6-3 BP Cloning R
SO039	ggggacaagttgtacaaaaagcaggctccaccATGGTTTAA CTTGGAATCAG	Niben101Scf09211g00011 .1/NbPBL Clade 6-4 BP Cloning F
SO040	ggggaccactttgtacaagaaagctgggtgATGTCTTCTATAA AGAGCTGTATCC	Niben101Scf09211g00011 .1/NbPBL Clade 6-4 BP Cloning R
SO041	ggggacaagttgtacaaaaagcaggctccaccATGAAAGAC CCAAAACCTT	Niben101Scf10055g0100 6.1/NbPBL Clade 6-5 BP Cloning F
SO042	ggggaccactttgtacaagaaagctgggtgCTTGCCCTTACT ATGAACA	Niben101Scf10055g0100 6.1/NbPBL Clade 6-5 BP Cloning R
SO043	ggggacaagttgtacaaaaagcaggctccaccATGGTTTAA CTTGGAATCA	Niben101Scf11199g00006 .1/NbPBL Clade 6-6 BP Cloning F
SO044	ggggaccactttgtacaagaaagctgggtgATGTCTTCTATAA AGAGCTGT	Niben101Scf11199g00006 .1/NbPBL Clade 6-6 BP Cloning R
SO083	GGCTGTGACCAGAACGGC	GspD Downstream Genomic Primer

SO084	AGCCGTATCGTCAGGTCAAG	GspD Upstream Genomic Primer
SO085	ggggaccactttgtacaagaaagctgggtgTAGCTCATCATG GCGCCCT	3' Flank BP Primer
SO086	ggggacaagttgtacaaaaaagcaggctccacATTTTCAGCG GCGCTGC	5' Flank BP Primer
SO087	CATGACCTCTGTTGGACGCT	GspD KO R
SO088	CCTTAACCAACGATTCCACCCTG	GspD KO F

Table A2.3 *Pseudomonas* strains used in this dissertation.

Species	Plasmid	Insert
Pf-0_EtHAn	Tn7 insertion	Tn7 promoterless Citrean:HA
Pf-0_EtHAn	Tn7_HA	Atro_4494 (HopBA1)
DC3000	pJC531	EV (HSP18 UTR)
DC3000	pJC531	HopBA1
DC3000	pJC531	HopF1r Genomic
DC3000	pJC531	HopO1c Genomic
DC3000	pJC531	HopX1i Genomic
DC3000	pUCP20	HopZ1a
DC3000 D36E	pJC531	EV (HSP18 UTR)
DC3000 D36E	pJC531	HopBA1

DC3000 D36E	pJC531	HopF1r Genomic
DC3000 D36E	pJC531	HopO1c Genomic
DC3000 D36E	pJC531	HopX1i Genomic
DC3000 D36E	pUCP20	HopZ1a
DC3000 $\Delta hopQ \Delta gspD$ 3xmCherry	pJC531	EV (HSP18 UTR)
DC3000 $\Delta hopQ \Delta gspD$ 3xmCherry	pJC531	HopBA1
DC3000 $\Delta hopQ \Delta gspD$ 3xmCherry	pJC531	HopQ
DC3000 $\Delta hopQ$ 3xmCherry	pJC531	EV (HSP18 UTR)
DC3000 $\Delta hopQ$ 3xmCherry	pJC531	HopBA1
DC3000 $\Delta hopQ$ 3xmCherry	pJC531	HopQ

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