THE ROLE OF NDR1 IN PATHOGEN DEFENSE AND ARABIDOPSIS PHYSIOLOGY

By

Caleb Knepper

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
For the degree of

DOCTOR OF PHILOSOPHY

Genetics

2011

ABSTRACT

THE ROLE OF NDR1 IN PATHOGEN DEFENSE AND ARABIDOPSIS PHYSIOLOGY

By

Caleb Knepper

Arabidopsis thaliana NDR1 (NON-RACE SPECIFIC DISEASE RESISTANCE-1), a plasma membrane localized protein, plays a critical role in resistance mediated by the CC-NB-LRR class of resistance (R) proteins, which includes RPS2, RPM1 and RPS5. Infection with *Pseudomonas syringae* pv. *tomato* DC3000 expressing the bacterial effector proteins AvrRpt2, AvrB and AvrPphB activate resistance through the activation of the aforementioned R proteins. Whereas the genetic requirement for *NDR1* in plant disease defense signaling has been established, the global physiological role for NDR1 in Arabidopsis remained unknown. With the use of homology modeling, NDR1 was predicted to have a high degree of structural similarity to Arabidopsis LEA14, a protein implicated in abiotic stress responses, as well as to mammalian integrins, well characterized proteins involved in adhesion and signaling. This structural homology led to the examination of a physiological role for NDR1 in preventing fluid loss and maintaining the plasma membrane-cell wall continuum. Results demonstrated a substantial alteration in pathogen-induced electrolyte leakage in *ndr1-1* mutant plants. As an extension of these analyses, using a combination of genetic and cell biology-based approaches, a role was identified for NDR1 in mediating plasma membrane-cell wall (PM-CW) adhesions through a specific protein motif. With the establishment of a distinct physiological role in PM-CW adhesion for NDR1 and the identification of a compromised PAMP-triggered immune response in *ndr1-1*, the focus shifted to the integration of NDR1 in early resistance signaling including pathogen perception, PAMP-triggered immunity (PTI) and effector delivery, recognition and

signaling. To this end, the PAMP-specific flg22- and elf26-dependent signaling mechanisms were analyzed to elucidate the breadth of NDR1 function in PTI. Mechanisms identified included a role for NDR1 in the regulation of stomatal closure in response to the PAMP flg22 and the hormone abscisic acid as well as a reduction in MAPK3/6 expression after both flg22 and elf26 exposure. Furthermore, the loss of NDR1 alters the type-three secretion system mediated delivery of the *P. syringae* effector AvrRpt2 to the cell interior. The structural similarities of NDR1 to LEA14, an abiotic stress protein, coupled with the identification of roles for NDR1 in maintaining PM-CW adhesion and regulation of stomata led to the examination of potential abiotic stress related functions for NDR1. By monitoring leaf relative water content as well as electrolyte leakage under severe drought conditions, NDR1 was shown to be required for drought tolerance in Arabidopsis. Further analysis revealed that *NDR1* mediated drought tolerance is an ABA-dependent process and the over-expression of the NDR1 protein provides increased stress tolerance in Arabidopsis. Taken together, these data indicate not only a broad role for NDR1 in defense signaling through both PAMP and effector mediated pathways, but also in mediating basic physiological functions in Arabidopsis through the maintenance of the PM-CW continuum and a requirement in stomatal aperture dynamics.

I would like to dedicate this dissertation to my loving wife, Amy.
Without her I could not have completed my degree.

ACKNOWLEDGEMENTS

First I would like to thank Brad Day for taking me into his lab. As a new faculty Brad was definitely taking a chance on a 22 year old student fresh out of Kansas State University when he agreed to take me in. We both grew along with the lab. I would also like to thank the members of the Day lab, both past and present for all their help with experiments as well as manuscript writing, they put up with me for many years which was not always easy.

I would also like to thank my committee members Drs. George Sundin, Jonathan Walton, Beronda Montgomery, and formerly Markus Pauly for helping to keep me on track and supporting me when I needed it.

I also must thanks the National Science Foundation for their financial support of the Day lab during my time there.

I also want to thank my parents and in-laws for being supportive even though I relocated 900 miles away and my work often seemed totally foreign to them.

Finally I want to thank my wife for always being there for me on both the good days as well as the bad and staying encouraging through all our years in Michigan; also, our dog Missy for checking on me those late nights in the office and reminding me when it was time to call it a night.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

The *Arabidopsis thaliana-Pseudomonas syringae* Pathosystem: The Molecular-Genetic Landscape of Host-Pathogen Interactions.

This review was originally published in *The Arabidopsis Book*.
Knepper C and Day B (2010) From Perception to Activation: The Molecular-Genetic and Biochemical Landscape of Disease Resistance Signaling in Plants: May 14, 2010. In *The Arabidopsis Book*. Rockville, MD: American Society of Plant Biologists. doi: 10.1199/tab.0124, http://www.aspb.org/publications/arabidopsis/.

ABSTRACT

More than 60 years ago, H.H. Flor proposed the "Gene-For-Gene" hypothesis, which described the genetic relationship between host plants and pathogens. In the decades that followed Flor's seminal work, our understanding of the plant-pathogen interaction has evolved into a sophisticated model, detailing the molecular genetic and biochemical processes that control host-range, resistance signaling and susceptibility. The interaction between plants and microbes is an intimate exchange of signals that has evolved for millennia, resulting in the modification and adaptation of pathogen virulence strategies and host recognition elements. In total, plants have evolved mechanisms to combat the ever-changing landscape of biotic interactions bombarding their environment, while in parallel, plant pathogens have co-evolved mechanisms to sense and adapt to these changes. On average, the typical plant is susceptible to attack by dozens of microbial pathogens, yet in most cases, remains resistant to many of these challenges. The sum of research in our field has revealed that these interactions are regulated by multiple layers of intimately linked signaling networks. As an evolved model of Flor's initial observations, the current paradigm in host-pathogen interactions is that pathogen effector molecules, in large part, drive the recognition, activation and subsequent physiological responses in plants that give rise to resistance and susceptibility. In this Chapter, we will discuss our current understanding of the association between plants and pathogens, detailing the pressures placed on both host and microbe to either maintain resistance, or induce susceptibility and disease. From recognition to transcriptional reprogramming, we will review current data and literature that has advanced the classical model of the Gene-for-Gene hypothesis to our current understanding of basal and effector triggered immunity.

Introduction

Since the last *Arabidopsis Book* Chapter outlining the *Arabidopsis-Pseudomonas syringae* interaction (Katagiri et al., 2002), there have been a number of advances in our understanding of how plants perceive and respond to biotic stress. In this respect, *Arabidopsis* has continued the lead the way in these advances, both in regard to understanding host defenses, as well as uncovering pathogen virulence strategies. A plant's response to environmental pressures is guided by its ability to sense and process stimuli. So too is a plant's ability to detect and respond to pathogen infection. In total, these processes are regulated in large part by the genetic and biochemical exchange between host and pathogen. In this Chapter, we will outline our current understanding of how plants and pathogens communicate through the balance of resistance and susceptibility. A "dance", a "molecular arms race", or simply survival, the interaction between a plant and pathogen represents a sophisticated interplay of genetic and biochemical processes, ultimately leading to the demise of either the host or the invader. Here, we will focus on the architecture of the plant immune response, highlighting the key advances in our understanding of host cell physiology, the activation of specific defense responses, and too, the evolution of strategies by the invading pathogen to shut down defense signaling in plants.

In a recent review by Alan Jones and colleagues (Jones et al., 2008), a parallel is drawn between research advances in humans and those that can be directly attributed to studies first conducted in the model plant *Arabidopsis thaliana*. None too surprising, our understanding of human health and disease has been greatly advanced by our understanding of similar processes in plants. For example, approximately 70% of the genes associated with the development of cancer(s) in

humans have orthologues present in *Arabidopsis*. Furthermore, with respect to advances in research first undertaken in *Arabidopsis* and subsequently "translated" in human disease research, innate immune receptor identification in plants have made significant impacts in our understanding of disease signaling in humans; resistance proteins were first identified and characterized in *Arabidopsis* (ca. 1994) before their counterparts (e.g., NOD/CARD/CATERPILLAR) in humans (ca. 2000; Ting et al., 2006). Jones and colleagues cite additional examples where research findings in *Arabidopsis* have advanced the broader study of biology in humans, including research in the area of circadian rhythms (Ahmad and Cashmore, 1993), RNA silencing (Hamilton and Baulcombe, 1999) and G-protein signaling (e.g., Temple and Jones, 2007).

Disease and defense signaling in plants, as we will outline throughout this Chapter, is a complicated, highly regulated process, involving the coordinated signaling networks of both host and pathogen. In this regard, the development of model systems that are both tractable and translational have been critical to addressing the many facets of the host-pathogen interface. Below, we will give a broad overview of several of the processes that typify studies in the area of plant-pathogen interactions, and too, highlight their significance towards increasing our understanding of host defense signaling in response to pathogen infection.

a. *Pseudomonas syringae*

Pseudomonas syringae is a gram-negative plant pathogenic bacterium that causes bacterial speck disease on tomato (Pedley and Martin, 2003; http://pseudomonas-syringae.org/). Towards

developing the laboratory-based tools we now have at our disposal, several strains were identified in the 1980's that would infect *Arabidopsis* (Katagiri et al., 2002), giving birth to a new era in molecular plant pathology. Since the establishment of the *Arabidopsis-Pseudomonas* pathosystem, research has explored nearly all facets of the interaction, from epiphytic colonization of the leaf surface (Hirano and Upper, 2000), pathogen entry through, and manipulation of, stomata (Melotto et al., 2006), as well as the delivery of effectors (Lindeberg et al., 2009) and induction of cell death (Figure 1.1; reviewed in Kim et al., 2008). As a consequence of standard mechanisms of dispersal (i.e., rain splash, insects, animals, humans, etc.), *P. syringae* establishes itself on the surface of plants as an epiphyte, before gaining entry into the intercellular space (Hirano and Upper, 2000). Once inside the intercellular space, the pathogen employs a type III secretion system (T3SS) for the delivery of effectors proteins directly into the host cell. In total, it is the action of these effectors that promote pathogenicity, shutting down critical host processes required to fight pathogen infection. Thus, the T3SS is essential for the development of disease symptoms and bacterial multiplication (reviewed in Lindeberg et al., 2009).

In 2009, researchers in the field of plant-microbe interactions marked the 25th anniversary since the cloning of the first bacterial type III secreted effector protein. In the November 2009 issue of the journal *Molecular Plant Pathology*, Brian Staskawicz reflects on the advances in the field of molecular plant pathology since his lab's seminal discovery (Staskawicz et al., 1984; Staskawicz et al., 2001; Staskawicz, 2009). Since 1984, advances in the area of plant-pathogen interactions have shaped our understanding of microbial genetics and pathogenicity, as well as plant physiology and evolution (reviewed in Cui et al., 2009). Collectively, these bacterial proteins,

called “effectors”, function to manipulate host cell processes for the purpose of enhancing infection and pathogen proliferation. While the function of the full suite of effector proteins remains unknown, what is known is that the complex genetic and biochemical interactions between pathogen effectors and their cognate host proteins evokes specific responses, that when recognized, elicit resistance, or when evaded, promote susceptibility.

b. Fungal and Oomycete Pathogens

Much like the bacterial virulence strategies described above, fungi and oomycete pathogens have also evolved mechanisms to infect and colonize plants. Beyond the cell surface components, such as Pathogen Associated Molecular Patterns (PAMPs), fungal pathogens have the ability to stimulate the release of host cell wall molecules through the production of hydrolytic enzymes during host invasion. These molecules, termed DAMPs (Danger-Associated Molecular Patterns), can be recognized by the plant and subsequently activate the defense response (Matzinger, 2007; Denoux et al., 2008). In addition to secreted hydrolytic enzymes and toxins, fungal and oomycete pathogens also encode for a suite of effector proteins, putatively similar in function to their bacterial counterparts (reviewed in De Wit et al., 2009; Schornack et al., 2009). However, one of the major differences with regard to effector action between bacterial pathogens and fungal or oomycete pathogens lies in the delivery of the effectors themselves. While bacteria rely on the T3SS, fungal and oomycete pathogens do not utilize a T3SS, and must instead rely on other mechanisms for effector delivery. At present, the mechanism(s) required for fungal/oomycete effector delivery is unknown.

In general, fungal effectors fall into two groups: those that are secreted into the host apoplast, and those that are translocated into the host cells (De Wit et al., 2009). In fungal pathogens, the mechanism by which the effectors are translocated remains elusive, and in oomycetes, while the specific mechanism of translocation is unknown, a conserved motif has been identified as being sufficient for effector uptake by host cells (Whisson et al., 2007). In short, many of these cytoplasmic oomycete effectors consist of an N-terminal region involved in secretion and translocation, as well as a C-terminal domain possessing the biochemical activity of the effector itself (Morgan and Kamoun, 2007). In recent years, a signature motif at the N-terminus (i.e., Arg-X-Leu-Arg; RxLR) has been identified and characterized as a critical component that not only guides oomycete effector identification (i.e., bioinformatics), but is also a critical component in the function of these secreted proteins during host interactions (Whisson et al., 2007).

c. *Non-host Systems and Resistance*

As we will discuss in more detail below, a pathogen's ability to colonize any given host is regulated in large part by its ability to avoid structural and preformed defenses, as well as abrogate or circumvent induced host-specific defenses. This begs the question: What are the initial responses by both plant and pathogen that determine host-specificity? Moreover, what differentiates host-specific from non-host interactions, and how is defense signaling regulated in each? To answer this question, research in the area of non-host resistance has revealed at least two layers of signaling: pre- and post-invasion resistance (reviewed in Mysore and Ryu, 2004). While most plants are resistant to most pathogens, the cellular and genetic responses that tip this

balance in favor of the pathogen have been best characterized using non-adapted pathogens such as the cucurbit powdery mildew pathogen *Golovinomyces cichoracearum*, and *Blumeria graminis*, a powdery mildew of the grasses. As host-specific pathogens, pathogen entry is effective, with a penetration rate of approximately 70% on their respective hosts (reviewed in Lipka et al., 2008). However, when *Arabidopsis* plants are inoculated, this rate falls dramatically. Herein lies the premise for the further characterization and identification of components required for pathogen entry and host-mediated responses to infection.

To identify and define the host mechanisms associated with resistance to non-adapted pathogens, initial work began with an extensive mutagenesis screen to identify host factors responsible for abrogating pathogen entry (Collins et al., 2003). To this end, early work demonstrated that plants attempt to prevent penetration by fungal pathogens through the formation of cell wall appositions termed papillae (Aist and Bushnell, 1991). For example, ethyl methanesulfonate (EMS) mutagenized *Arabidopsis* populations were screened for increased penetration by the non-adapted powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006). Three penetration, or *PEN*, mutants have been characterized. *PEN1* encodes for an *Arabidopsis* syntaxin, which is predicted to function in the targeted trafficking of secretory vesicles to sites of papillae formation in response to attempted fungal pathogen penetration (Collins et al., 2003). *PEN2* and *PEN3* have been shown to function in the same pathway (Stein et al., 2006), also at sites of attempted fungal penetration. Subsequent work has gone on to show that PEN2 is a myrosinase functioning in the glucosinolate pathway (Clay et al., 2009), while PEN3 is an ABC (ATP-binding cassette) transporter (Stein et al., 2006) thought to be involved in the efflux of antimicrobial compounds to sites of attempted pathogen

penetration. In total, these observations demonstrate that preformed responses are critical to the ability of the plant to resist penetration. As such, the suite of pre-invasive defense responses present in plants is sufficient to limit non-host pathogen entry; these include generalized responses such the deposition of callose at the site of attempted pathogen entry (Aist and Bushnell, 1991; reviewed in Hématy et al., 2009), as well as a dynamic re-organization of the host actin cytoskeleton. These responses coincide with increased cellular trafficking of organelles and defense signaling molecules to the site of infection. As will be a common theme throughout this Chapter, considerable overlap in defense signaling exists both in the initial perception and activation of cell signaling to numerous pathogen species, as well as critical defense signaling nodes associated with signal transduction amplification and the onset of resistance. Interestingly, however, the *PEN* mutations have not been reported to compromise resistance to bacterial pathogens, such as *P. syringae* (Lipka et al., 2008). These results suggest that restriction of host range to phytopathogens is regulated by additional other mechanisms.

Host Architecture and Physiology

The plant cell is a remarkable evolutionary product of chemical, mechanical and electrical engineering. The structural capacity of the plant cell to resist mechanical forces from biotic and abiotic pressures is evidenced through the strength and elasticity of the cell wall (reviewed in Hématy et al., 2009). As discussed below, the cell wall can serve as a passive barrier to pathogen entry, as well as the site of first contact between host and pathogen. Serving in a more dynamic capacity, plants have the ability to actively reinforce their cells walls in response to a

pathogen, such as in attempted fungal penetration, by the deposition of callose at sites of infection (Aist and Bushnell, 1991).

Preformed Defenses

The leaf surface presents a formidable barrier to pathogen colonization and entry. Studded with trichomes, the leaf's waxy surface provides an unwelcoming environment from which pathogens must attempt to colonize and gain entry into the host. The outermost layers of the leaf epidermis consist of a modification to the cell wall known as a cuticle (Nawrath, 2006), which is comprised of cutin and waxes secreted onto the exterior surface of the cell (Jeffree, 2006). In addition to serving as a barrier to pathogen entry, the cutin is indispensible for the prevention of water loss from the leaf surface (Aharoni et al., 2004). There is growing evidence for the cuticle as a major player in *Arabidopsis* resistance to a wide variety of pathogen types from the bacterial pathogen *P. syringae* to the fungal pathogen *Botrytis cinerea* (reviewed in Reina-Pinto and Yephremov, 2009).

Once *P. syringae* gains entry to the leaf apoplast, it must still interact with the host cytoplasm in order to acquire nutrients; thus, the basic plant cell wall still proves a substantial barrier to pathogen entry. The rigid cell wall can therefore be viewed as a major constituent of resistance to non-adapted pathogens (discussed below; reviewed in Hématy et al., 2009). While the physical barriers to pathogen entry are substantial, additional preformed defenses, such as chemical defenses, play ubiquitous roles in basal defense responses against pathogen infection. Among the best-characterized modes of chemical defenses are the phytoanticipins, which

represent a diverse group of antimicrobial compounds present in the host before pathogen infection (VanEtten et al., 1994). This is in contrast to phytoalexins, which are by definition formed in response to pathogen infection, such as the well-characterized *Arabidopsis* phytoalexin, camalexin (reviewed in Glawischnig, 2007). Camalexin, 3-thiazol-2'yl-indole, was originally isolated from leaves of the crucifer *Camelina sativa* infected with *Alternaria brassicae* (Browne et al. 1991), and was subsequently identified in *Arabidopsis* challenged with *P. syringae* (Tsuji et al., 1992), and its production was found to be induced by a wide range of stress conditions (reviewed in Glawischnig, 2007). However, production levels (and concentration) vary greatly within and among associated stresses. As is the case with all pathogen-induced defense responses in plants, phytoalexins are not an impenetrable barrier to infection and subsequent proliferation. In support of this, multiple pathogens have been identified that are able to tolerate camalexin production in *Arabidopsis* through a variety of mechanisms. Isolates of the root rot fungus *Rhizoctonia solani* have the ability to degrade camalexin through the 5-hydroxlyation of its indole ring, or by the formation of an oxazoline derivative (Pedras and Khan, 1997, 2000). In the case of the fungal pathogen *B. cinerea*, both resistant and sensitive isolates have been identified (Kliebenstein et al., 2005). This mechanism of resistance is mediated in *B. cinerea* through the activity of an ABC transporter, BcatrB, which acts as an efflux pump for removing camalexin from the cell (Stefanato et al., 2009).

Hormones and Defense Signaling

Extensive research has unraveled the intimate link between plant development, responses to the environment and pathogen perception. Through all of this, the role of plant hormones has been

revealed as a central, key component in not only regulating defense signaling responses within infected cells, but also as a mediator of systemic signaling (reviewed in Spoel and Dong, 2008). At a primary level, plant hormones are responsible for the integration and processing of developmental and environmental cues. To this end, they are responsible not only for shaping the dynamic regulatory processes that control development, reproduction and death, but also priming the host cell for both biotic and abiotic stress responses. Of the major plant hormones, salicylic acid (SA), jasmonic acid (JA) and ethylene have been shown to play key roles in defense signaling in plants (reviewed in Bari and Jones, 2009).

It is widely known that pathogen infection affects plant development (Block et al., 2010; Chandra and Huff, 2010), and in large part, this effect is manifested through perturbations in hormone signaling within the host plant (Chen et al., 2007). As discussed above, pathogens have evolved elaborate mechanisms to colonize and infect their host; typically through the manipulation of host physiology by secreted pathogen effectors. During a typical infection, *P. syringae* delivers approximately 32 effector proteins inside its host (Lindeberg et al., 2009). Of these, one of the best characterized is AvrRpt2, a cysteine protease effector protein, whose catalytic activity sets into motion a series of defense signaling responses which have become hallmark tenets for the gene-for-gene and guard hypotheses. However, aside from AvrRpt2's well-established role in avirulence, studies investigating the manipulation of host physiology, and more specifically hormone signaling, have revealed an intimate link between pathogen effector action and hormone signaling. In 2007, Chen and colleagues (Chen et al., 2007) demonstrated a link between AvrRpt2-mediated defense signaling and the elicitation of host auxin biosynthesis. Phenotypically, plants expressing AvrRpt2 were found to be similar in

stature to plants over-expressing auxin; plants have longer primary roots, increased lateral root formation and enhanced sensitivity to exogenously applied auxin (Sato and Yamamoto, 2008). One interesting finding of this study was the link between AvrRpt2 action within the host cell and hormone biosynthesis. In short, AvrRpt2-expressing plants were found to have elevated levels of free indole-3-acetic acid (IAA). The link between host defense, pathogen virulence and hormone perception was further supported as a consequence of enhanced disease symptom development (Chen et al., 2007).

In an example analogous to manipulation of auxin biosynthesis by pathogens, described above, recent evidence also suggests that SA inhibits pathogen growth by suppressing auxin signaling (Wang et al., 2007). Through the use of expression profiling, Wang and colleagues (2007) found that SA inhibits auxin-mediated signaling, partially countering the pathogen's impact on hormone-associated defense signaling. This work showed that the SA analog BTH (benzothiadiazole-s-methyl ester) suppressed the expression of auxin responsive genes in an *NPR1*-dependent manner. In total, this work demonstrated the host plant's ability to antagonistically co-regulate multiple hormone signaling networks in response to pathogen infection, and with that, strengthens the hypothesis that plants may divert limited resources to defense-related processes at the expense of plant growth when attacked by a pathogen.

Endocytosis, Trafficking and Cellular Dynamics

In recent years, advances in imaging and cell biology technologies have made possible the observation of the dynamic responses to pathogen infection, such as increases in cellular

trafficking, (re)-localization of proteins following pathogen perception, as well as reorganization of the actin cytoskeleton. In total, these collective works have not only enabled researchers to glimpse the cellular processes that are impacted during pathogen infection, but to also identify additional signaling components required for defense and resistance activation in plants. As we discussed above, the primary defense response in plants following pathogen perception is collectively referred to as PAMP-triggered immunity (PTI; Chisholm et al., 2006; Jones and Dangl, 2006). In this regard, the flagellin recognition receptor, FLS2, is a key component in both the initiation and amplification of basal defense responses in plants following pathogen perception (Figure 1.2A). In an eloquent series of experiments by Robatzek and colleagues (Robatzek et al., 2006), FLS2 was found to enter the endocytic pathway upon flg22 perception, resulting in the rapid accumulation of FLS2 in intracellular vesicles. In total, this series of experiments has led to a more complete understanding of receptor endocytosis in plants, and too, the regulatory network that follows PAMP perception leading to activation of PTI (reviewed in Irani and Russinova, 2009).

Once receptor-mediated endocytosis occurs, as in the case of flg22 perception via FLS2 described above, a plant's response to pathogen perception is further amplified by the intercellular trafficking of defense-associated compounds. Thus, from the standpoint of resistance, the host plant must mobilize defense-associated components both to the site of infection, as well as within, and amongst, adjacent cells. From the standpoint of pathogen virulence, shutting down this response is key to continued infection and proliferation. Not surprisingly, changes in the host cell endomembrane system have also been observed during effector-triggered immunity (ETI; Chisholm et al., 2006; Jones and Dangl, 2006). For example,

work from the laboratory of Sheng Yang He demonstrated that the secreted effector protein HopM1 from *P. syringae* localizes to plant endomembrane fractions (Nomura et al., 2006). With this information, an investigation into possible host targets revealed an association between HopM1 and the *Arabidopsis* protein MIN7 (i.e., *At*MIN7). This work demonstrated that *AtMIN7* encodes for an adenosine diphosphate ribosylation factor (ARF) guanine nucleotide exchange factor (GEF), further solidifying the link between HopM1 function and the regulation of vesicle trafficking during plant-pathogen interactions. Confirmation of these observations, using a pharmacological approach, Nomura and colleagues found that application of the fungal-derived antibiotic Brefeldin-A phenocopied the activity of HopM1; Brefeldin A interferes with endomembrane protein transport from the Golgi apparatus to the endoplasmic reticulum. In short, HopM1 was found to trigger the degradation of AtMIN7 as part of its virulence function, leading to the hypothesis that *P. syringae* manipulates vesicle trafficking by targeted ARF-GEF (i.e., *At*MIN7) degradation.

Dynamic responses to bacterial phytopathogen perception have also recently been shown to engage components of the actin cytoskeleton (Tian et al., 2009). Using a reverse genetic and biochemical approach, Tian and colleagues identified a regulator of stochastic actin dynamics (i.e., ACTIN DEPOLYMERIZING FACTOR-4; ADF4) as being required for the perception of *P. syringae* expressing the cysteine protease effector protein AvrPphB. In mutant *Arabidopsis* plants lacking ADF4, pathogen growth was unchecked, resulting in an increase in bacterial multiplication, leading to increased disease symptoms. This work further characterized the biochemical activity of the protein, and has led to the hypothesis that subtleties in depolymerization activity (i.e., actin binding, F-actin severing and depolymerization) may in fact

account for some level of specificity regulating pathogen perception and the subsequent remodeling of the cortical actin cytoskeleton. Interestingly, this work also identified a link between actin depolymerization dynamics and the homeostatic control of hormone (i.e., SA and JA) physiology, further implicating the link between hormone signaling, host cell dynamics and the perception of pathogens by plants.

Transcriptional Regulation and Pathogenesis Related Genes

The common misconception is that plant defense responses are centrally regulated through protein-protein interactions. While protein dynamics certainly account for a large proportion of the overall defense response (e.g., Mackey et al., 2002, 2003; Axtell and Staskawicz, 2003; Day et al., 2005; Liu et al., 2009; Lu et al., 2009), transcriptional regulation is a critical component in controlling plant resistance responses to pathogen infection (reviewed in Eulgem, 2005). Microarray analyses investigating the transcriptional reprogramming of defense signaling in *Arabidopsis* following inoculation with a variety of pathogens has revealed that in addition to the well established pathogenesis related (*PR*) genes (Sels et al., 2008), several hundred, even thousands, of genes undergo differential expression both during and following pathogen perception (Glazebrook, 2001). In fact, up to 25% of all *Arabidopsis* genes display altered transcript levels in response to pathogen infection (Maleck et al., 2000; Tao et al., 2003). Among these altered transcripts, members of several transcription factor families have also been implicated in defense gene regulation (Eulgem, 2005).

An early observation in response to pathogen infection is the expression of *PR* genes. *PR* genes are defined as the genes encoding for host proteins that accumulate after pathological or related stimuli (Van Loon and Van Strien, 1999). Currently, PR genes are classified into seventeen distinct families (Van Loon et al., 2006), including some of the most commonly used resistance markers *PR1* and *PDF1.2* (Ryals et al., 1996; Lay and Anderson, 2005). The *PR1* family of genes are some of the most ubiquitous, showing a strong conservation across species, and as such, appear to be represented across all plant species, with homologues present in fungi, insects and vertebrates (Van Loon et al., 2006). Despite being such a widely conserved group, relatively little is known of PR-1 family protein function in *Arabidopsis* disease resistance. Part of the difficulty in studying these genes are the number in *Arabidopsis,* with 22 *PR-1*-type genes present, as well as a widely-varied expression pattern; only a single member of the *PR-1* gene family is activated by pathogen infection, insect feeding, or chemical treatment, while ten *PR-1*-type genes are constitutively expressed in roots and eight in pollen (Van Loon et al., 2006). In contrast to the *PR-1* family, several additional *PR* groups have also been widely studied, including members of the *PR-12* family, also known as defensins, which have members exhibiting antifungal activity. To this end, Terras et al. (1995) demonstrated *in vitro* antifungal activity to a wide range of fungi using purified PDF1.1. In a complementary series of experiments, Penninckx et al. (1996) showed *in vitro* antifungal activity to *Alternaria brassicicola* and *Fusarium culmorum*.

PR genes, in general, appear to be only a small portion of a larger defense-signaling network involving SA, JA and ethylene. Several compelling examples of this, discussed in Sels et al. (2008), include an analysis of resistance in *ein2* mutants, defective in JA/ET signaling, as well as

the SA signaling deficient *npr1* mutant. The *ein2* mutant was shown to have increased susceptibility to the necrotrophic fungal pathogen *B. cinerea* (Thomma et al., 1999), while showing decreased expression of several *PR* genes, including those from the *PR-12*, *PR-3* and *PR-4* gene families (Thomma et al., 2001). Likewise, the *npr1* mutant showed increased susceptibility to many biotrophic pathogens including the bacterium *P. syringae*, with decreases in *PR-1*, *PR-2* and *PR-5* (Thomma et al., 2001).

a. Transcription Factors

In addition to the large, ubiquitous family of *PR* genes described above, representatives of the *Arabidopsis* TGA-bZIP, ERF, Myb, Whirly and WRKY families have been shown to bind defense related gene promoter elements and regulate their expression (reviewed in Eulgem, 2005). Binding sites of WRKY factors (W boxes) are ubiquitously conserved in upstream regions of genes up regulated during a variety of defense responses including SAR, R-protein-mediated resistance and basal defense (Maleck et al., 2000; Eulgem et al., 2004; Zipfel et al., 2004). Dong et al. (2003) showed the promoters of pathogen-inducible *Arabidopsis* WRKY genes were strongly enriched for W boxes, suggesting a role for feedback regulation by WRKYs themselves. Interestingly, the conservation of binding sites in defense genes is not limited to the WRKY family of transcription factors. The consensus binding motif of Whirly factors and a motif with similarity to ERF binding sites are conserved in promoters of genes expressed during incompatible interactions with *Hyaloperonospora arabidopsidis* (Eulgem et al., 2004).

The transcriptional cascade leading to the SA-dependent expression of *PR1* is well established, and involves numerous transcription factors, such as WRKYs, NPR1 and TGAs (reviewed in Eulgem, 2005). For example, following activation of SA-responsive defense signaling, approximately 50 *WRKY* genes are activated, which in turn lead to the coordinate regulation of defense signaling; this represents both the accumulation and repression of differentially regulated transcripts (Eulgem and Somssich, 2007). Among the best-characterized responses linking perception of SA and the activation of defense signaling is the activation of *NPR1* transcription (Yu et al., 2001). Accumulation of SA also triggers a change in the redox status of NPR1, reducing it to a monomeric form that can then be translocated into the nucleus (Mou et al. 2003). Once inside the nucleus, NPR1 monomers are able to interact with members of the TGA-bZIP family of transcription factors (Fan and Dong, 2002), which in turn stimulates their binding to TGA boxes within the promoter of *PR1* (reviewed in Singh et al., 2002). In total, this multi-step process leads to the activation and regulation of SA-dependent gene expression.

b. MAPK Signaling

Once pathogen perception has occurred, amplification and precise regulation of the signaling cascade is required. In both PTI and ETI, this amplification step typically involves the function of a suite of Mitogen-Associated Protein Kinases (MAPK) for downstream resistance signaling. The utility of MAPK signaling in plants is not restricted to biotic interactions; indeed, current literature is rife with examples, including development, reproduction and response to environmental stress (reviewed in Andreasson and Ellis, 2010). *Arabidopsis* has 23 MAPKs, 10 MAPKKs and 60 MAPKKKs (hereafter collectively referred to as MAPKs; reviewed in

Cvetkovskai et al., 2005). In total, the primary function of MAPKs is the transduction of signals originating from perception (i.e., ligand binding; e.g., FLS2-flg22 interaction) to the activation and regulation of a downstream target. Whether through protein-protein interactions, regulation of cellular trafficking or transcriptional activation, MAPKs have ubiquitous roles in the amplification and processing of stimuli from biotic and abiotic responses. For example, responses regulated by MAPKs that are specifically required for defense signaling include the hypersensitive response (HR), systemic acquired resistance (SAR), generation of reactive oxygen species (ROS), and the induction of *PR* gene expression. As detailed above, one of the primary downstream responses regulated by MAPK signaling is the transcriptional regulation of numerous genes associated with defense activation (reviewed in Eulgem, 2005).

The Host-Pathogen Interface: Layered Defenses, Resistance and Susceptibility

Sequencing of the *Arabidopsis* genome opened the door to a plethora of resources enabling a detailed analysis of disease resistance signaling in plants. Homology-based analyses led to the identification of broadly conserved gene families, such as plant resistance genes (Aarts et al., 1998; Shen et al., 1998; Zhou et al., 2004). Coupled with forward and reverse genetic approaches, such as EMS mutagenesis and the subsequent functional characterization of candidate co-regulators (Century et al., 1995; Falk et al., 1999), signaling networks were soon assembled. Through all of this, what is now evident is that resistance signaling in plants is a multi-layered network of perception, signal amplification and regulation. Crosstalk between these layers of responses mediates perception and specificity, as well as regulates the strength and duration of the response.

Elicitors and PAMP-Triggered Immunity

Plants have evolved the ability to recognize the somewhat basic features of a pathogen for the purpose of eliciting defense responses (reviewed in Zipfel, 2009). Among the earliest elicitors of a plant defense response to be characterized were the oligosaccharide polymers that constitute the outer cell walls of pathogenic organisms (Hahn et al., 1981). From a historical standpoint, PAMPs were first observed and characterized in early experiments by Anderson-Prouty and Albersheim (1975), which described the ability of a fungal cell wall component, β-glucan, to induce a defense response in plants. These experiments were followed by in-depth studies to identify additional PAMPs and their associated responses, including oligogalacturonides (Davis and Hahlbrock, 1987), chitin (Baureithel et al., 1994; Shibuya et al., 1996; Day et al., 2001; Okada et al., 2002; Miya et al., 2007; Wan et al., 2008), and chitosan (Hadwiger et al., 1981).

In 1999, Thomas Boller's group identified a single genetic locus in *Arabidopsis* that mediates the perception of what has become the best-characterized PAMP recognition response in plants: the FLS2-bacterial flagellin interaction (Gómez-Gómez et al., 1999). Looking back, the discovery of the flagellin receptor (i.e., FLS2; FLAGELLIN SENSITIVE-2) in *Arabidopsis* represents one of the seminal discoveries in molecular plant pathology. While the study of PAMP recognition in plants has a long history, until the identification of a specific PAMP receptor, the classical R-protein-effector interaction(s) was seen as the penultimate mechanism of resistance signaling, controlling specificity, host-range, recognition and the activation of immunity in plants (reviewed in Staskawicz et al., 2001). With FLS2, researchers were now confronting the

possibility that plants coordinate parallel, and to a large extent, overlapping layers of defense signaling.

PTI occurs almost immediately following the physical interaction between host and pathogen (reviewed in Jones and Dangl, 2006). As noted above, the identification of FLS2 provided the first genetic evidence that PTI controls a broad range of both physiological and pathogen-specific resistance responses. In total, structure-function studies of the flg22-FLS2 interaction have contributed to the elucidation of signaling pathways and their associated mechanisms (Chinchilla et al., 2006; Robatzek et al., 2006; Göhre et al., 2008). However, what may be the greatest contribution of these studies is that they have provided an understanding of the spatial dynamics of signal perception and transduction (Robatzek et al., 2006; Heese et al., 2007).

FLS2 is a receptor protein kinase comprised of an extracellular leucine-rich repeat (LRR) domain and an intercellular cytoplasmic serine threonine kinase domain (Figure 1.2A; Gómez-Gómez and Boller, 2000). Following the perception of bacterial flagellin, FLS2 mediates the activation of broad-based plant defense responses, such as the activation of MAP kinase signaling (Asai et al., 2002), endosomal trafficking (Otegui and Spitzer, 2008) and regulation of stomatal closure (Melotto et al., 2006) (Figure 1.2B). In short, binding of flagellin to FLS2 promotes the association with the receptor-like kinase BAK1 (Chinchilla et al., 2007; Heese et al., 2007), which is believed to trigger the activation of at least two MAP kinase cascades. In terms of regulating basal defense, genetic evidence seems to suggest that the MAP kinase kinases MKK1 and MKK2 negatively regulate immune responses in response to FLS2 activation (Ichimura et al., 2006; Qiu et al., 2008), while MPK3 and MPK6 are thought to positively

regulate FLS2 immune responses (Bittel and Robatzek, 2007). To add to this complexity, evidence also points to the involvement of hormone signaling in regulating FLS2-mediated responses (Navarro et al., 2006; Tsuda et al., 2008).

As noted throughout this Chapter, the driving force behind the association of pathogens with plants is the acquisition of nutrients. In the first installment of the *Arabidopsis Book* Chapter, which focused on the *Arabidopsis-Pseudomonas* interaction. Fumi Katagiri and colleagues pointed to the role of nutrient restriction as a basal defense response that promotes host resistance (Katagiri et al., 2002). After all, pathogens are not teleological beings; they are not attacking the plant, they are simply in search of nutrients, which in turn, provide a means to an end. As such, disease may simply be a consequence of a pathogen's search for nutrients, and as such, barriers, obstacles or processes that prevent the acquisition of nutrients must be circumvented or disabled. Pathogen entry may therefore be viewed as a "filter" that determines the success or demise of a pathogen. In support of this hypothesis, Melotto et al. (2006) demonstrated that the recognition of flg22 by FLS2 induces the rapid closure of stomata, thus restricting pathogen entry and subsequent proliferation. Interestingly, this restriction can be lifted through the action of a *P. syringae*-specific toxin, coronatine, which interferes with abscisic acid (ABA) signaling and stimulates stomatal re-opening.

A common theme in the perception of pathogens by plants, as well as the subsequent signaling of defense-specific responses, is that there is a significant overlap in the regulation of general host physiology and the activation and regulation of defense responses. Whether through manipulating hormone balance, or through modulation of ubiquitous MAPK signaling pathways,

plants have evolved broad mechanisms to sense and abrogate pathogen infection and proliferation. As such, the basal defense response may represent the most basic and ancient form of plant immunity (reviewed in Chisholm et al., 2006). As discussed in the following section, the evolution and adaptation of highly specific defense responses has occurred through gene-for-gene interactions.

ETI: Gene-for-Gene Resistance and the Guard Hypothesis

The current paradigm in host-pathogen interactions is that the activation of primary defense responses is initiated by PAMP recognition, which in turn leads to the activation of PTI (reviewed in Jones and Dangl, 2006). With the discovery of the first bacterial avirulence protein (Staskawicz et al., 1984), a new discipline in plant biology was born: molecular plant pathology. Approaches such as EMS mutagenesis, transposon tagging, as well as advances in gene expression and DNA sequencing made possible our ability to identify genetic elements responsible for the recognition of plant pathogens. As our understanding of how plants recognize and respond to pathogen infection increases, models have evolved, paradigms have shifted, and too, our approaches have adapted to advances in technology. The sequencing of the *Arabidopsis* genome made possible many of these advances, and more importantly, provided a pool of candidate defense components for further characterization. In this regard, it soon became evident that the classical gene-for-gene hypothesis could not fully explain the complex interactions between *all* plants and *all* pathogens.

In 1998, Van Der Biezen and Jones proposed what is now known as the "Guard Hypothesis" to explain the role of Prf in the AvrPto-Pto interaction, a model that has evolved to explain the complex surveillance mechanism(s) that controls host-pathogen interactions (Van Der Biezen and Jones, 1998). While this model does not fully explain all aspects of the dominant (e.g., R-protein-mediated) resistance responses in plants, it does provide a benchmark for investigating the genetic interactions between host R-proteins and their cognate pathogen effectors. Just a few years later, in 2002, Van der Hoorn et al. (2002) made three observations that they believed would generally validate the Guard hypothesis. First, when an R-protein serves as a guardee, there would be no direct interaction with the cognate effector protein. This was a critical step in addressing the few identified instances of direct R-effector interactions. Secondly, that the indirect interaction requires an additional host protein that is specific for each effector-R-protein pair. And finally, that this additional host protein's structure, or general occurrence, would qualify it as a candidate virulence target of the pathogen. As is often the case, the lack of evidence, or in this case, the inability to demonstrate direct interaction between effector-R-protein pairs has limited our ability to further explain the processes required for pathogen recognition. To this end, the Guard Hypothesis finally offered an answer to explain the interaction between host resistance proteins and cognate pathogen effectors. Perhaps more interesting at the time was the possibility that there existed an additional host protein that was unique for each interaction. However, one surprising caveat to this paradigm is the finding that an absolute uniqueness does not exist in all guard-guardee interactions (Mackey et al., 2002, 2003; Axtell et al., 2003; Day et al., 2005; Chisholm et al., 2006). To this end, an additional level of co-regulation exists among shared signaling networks comprising ETI.

a. Structure

Despite the ability of plants to recognize a wide range of pathogens, the suite of R-proteins present in most plants is somewhat limited in both structural and operational diversity. As shown in Figure 1.3A, R-proteins share a number of basic, common features, and in *Arabidopsis*, approximately 150 proteins comprise this family of resistance signaling mediators (Baumgarten et al., 2003). The largest class of R-genes encode for a nucleotide binding site-leucine-rich repeat (NB-LRR) class of proteins (Chisholm et al., 2006; Jones and Dangl, 2006). At the amino-terminus, the conserved nucleotide-binding (NB) site has been shown to be critical for ATP or GTP binding (Saraste et al., 1990). At the C-terminus, the LRR domain, which exhibits variability both in spatial organization (Istomin and Godzik, 2009) and length (Matsushima et al., 2009), is likely a platform for protein-protein interactions and peptide/ligand binding (Jones and Jones, 1996; Kajava, 1998). Not surprising, LRR domains are found in a diverse suite of proteins, ranging in function as regulators of processes controlling both development and plant defense (reviewed in Padmanabhan et al., 2009). The NB-LRR class of R-proteins can be further sub-divided based on N-terminal structural features (Chisholm et al., 2006). Among these, one type contains an N-terminal domain with homology to the *Drosophila* Toll and mammalian interleukin 1 receptors (TIR-NB-LRRs) while the other class contains putative coiled-coil domains (CC-NB-LRRs) (reviewed in Dangl and Jones, 2001). In *Arabidopsis*, the best-characterized R-proteins are members of the CC-NB-LRR proteins, such as RPM1 (Resistance to *Pseudomonas syringae* pv. *maculicola*-1; Bisgrove et al., 1994), RPS5 (Resistance to *Pseudomonas syringae*-5; Simonich and Innes, 1995) and RPS2 (Resistance to *Pseudomonas syringae*-2; Kunkel et al., 1993). Functionally, R-protein recognition of pathogen effectors,

and/or the cellular perturbations elicited by the action of effectors, is critical to plant defense (Figure 1.3B). However, these actions alone (i.e., detection of perturbations) do not account for the full activation of resistance. To this end, additional plant proteins are required for proper R-protein function.

b. R-protein Stability and Activation

RAR1 (Required for MLA12 Resistance-1) was demonstrated to be required for resistance mediated by several CC-NB-LRR, as well as at least one TIR-NB-LRR class R-protein (Muskett et al., 2002; Tornero et al., 2002). Evidence suggests that RAR1 may function through its physical interaction with another protein (i.e., SGT1; Suppressor of G2 allele of suppressor of kinetochore protein 1; Azevedo et al., 2002) that is also required for resistance mediated by several CC-NB-LRR and TIR-NB-LRRs (Azevedo et al., 2002). In short, the simplest model for a role of RAR1 in R-protein function is that it directs either the removal of a negative regulator (Gray et al., 1999) or the activation of a positive regulator (Wang et al., 2001) by recruitment of that factor to the SCF complex via SGT1 and subsequent ubiquitination (Tornero et al., 2002).

SGT1 was originally identified as a regulatory component of the Skp1, Cullin, F-box (SCF) complex (Bachmair et al., 2001) that acts as an E3 ligase involved in the ubiquitination of target proteins (Tornero et al., 2002). Since its identification, numerous genetic studies have implicated SGT1 as a key component in pathogen resistance signaling, most likely through regulating the expression levels and activities of R proteins (Peart et al., 2002). With the characterization of SGT1 interactions with the chaperone HSP90, as well as with another protein,

RAR1 (Takahashi et al., 2003), the shape of the complex regulatory node involving R-protein stability is starting to emerge (Figure 1.4). RAR1 is a member of the conserved CHORD-containing family (CHP; Shirasu et al., 1999), and is distinguished by the presence of two cysteine- and histidine-rich zinc-binding domains (CHORD I and CHORD II). *In planta*, RAR1 associates with SGT1, and this interaction appears to be required for full functionality of associated R-proteins (Austin et al., 2002; Azevedo et al., 2002; Peart et al., 2002; Tör et al., 2002). In mammals, Nod1 was recently shown to associate with HSP90, further confirming that studies first conducted in plants are invaluable about pathways involving NLR proteins in non-plant systems (Hahn, 2005). More recently, da Silva Correia (2007) also demonstrated that SGT1 is a positive regulator of Nod1 activation, providing compelling evidence that SGT1 is required for signaling by Nod1 in human cells, just as it is required in innate immune signaling in plants.

c. Regulators and Amplifiers of R-protein Signaling

In addition to the requirement for stabilizing and directing R-protein function, additional R-protein accessory proteins have been identified as being required for the activation of disease resistance signaling in plants. Among these, the best-characterized examples include ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and NON-RACE SPECIFIC DISEASE RESISTANCE 1 (NDR1). Both have been shown to be indispensible for the activation of resistance mediated by nearly all TIR-NB-LRRs and CC-NB-LRRs, respectively (Aarts et al., 1998). EDS1 has homology to eukaryotic lipases (Falk et al., 1999), and serves as a central regulatory protein in biotic and oxidative stress signaling (reviewed in Wiermer et al., 2005).

Originally, *EDS1* was identified in a screen for loss of resistance in *Arabidopsis* to isolates of the oomycete pathogen *Hyaloperonospora arabidopsidis* (Parker et al., 1996), and was the first plant L-family lipase representative to be cloned and assigned a function (Falk et al., 1999). In subsequent studies, *eds1* mutants were also implicated in a loss of resistance to specific strains of *P. syringae* targeting TIR-NB-LRRs such as RPP2, RPP4, RPP5, RPP21 and RPS4 (Aarts et al., 1998; Feys et al., 2001). In addition to the identification of a required role for EDS1 as a signaling protein in the TIR-NB-LRR network, significant advances were also made as a result of the identification of the first of two important interacting partners of EDS1, PHYTOALEXIN DEFICIENT 4 (PAD4), followed by the identification of an interaction with SENESCENCE-ASSOCIATED GENE 101 (SAG101) (Feys et al., 2005). PAD4 and SAG101 functions appear partially redundant, yet functionally independent. Feys et al. (2005) also demonstrated nuclear localization for EDS1 as well as EDS1:PAD4 and EDS1:SAG101 complexes, suggesting a dynamic role for EDS1 in defense signaling. Evidence also supports a role for EDS1, along with PAD4, in the plant response to oxidative stress (Rusterucci et al., 2001; Mateo et al., 2004), as well as being required for the runaway cell death response observed in LESION SIMULATING DISEASE 1 (LSD1) mutant plants caused by photooxidative stress (Mateo et al., 2004).

In addition to EDS1, a key regulator of CC-NB-LRR R-protein activation was also identified. A mutation in *NDR1* was identified in a screen of fast-neutron mutagenized Col-0 *Arabidopsis* by screening for plants that became susceptible to *P. syringae* expressing the effector AvrB (discussed below; Century et al., 1995). The *ndr1-1* mutant plant contains an approximately 1 kilobase-pair deletion spanning the *NDR1* locus on *Arabidopsis* chromosome three (Century et al., 1997). Functional characterization of NDR1 has revealed it to be a plasma membrane-

localized protein of 219 amino acids (Century et al., 1997), which undergoes several post-translational modifications, including C-terminal processing and N-linked glycosylation (Coppinger et al., 2004). Interestingly, the proposed topology of NDR1 within the plasma membrane suggests that an approximate 18-amino acid portion lies within the cytoplasm, while the remainder of the NDR1 protein resides on the outside surface of the plasma membrane (Coppinger et al., 2004; Day et al., 2006). This hypothetical model raises the possibility that NDR1 positioning within the plasma membrane may serve to facilitate signaling from within the apoplast, across the plasma membrane, and into the cytoplasm, possibly through its interaction with RIN4 (RPM1 Interacting Protein-4; Day et al., 2006). In total, NDR1 is required for the activation of many CC-NB-LRRs including RPS2, RPM1 and RPS5 (Century et al., 1995), and in support of this, *ndr1-1* mutant plants are susceptible to *P. syringae* expressing the effector genes AvrB, AvrRpt2, AvrRpm1 or AvrPphB (Coppinger et al., 2004). As a required signaling component of multiple R-protein pathways in response to bacterial infection, NDR1 may also play a role in multiple resistance networks in plants. In support of this hypothesis, *ndr1-1* plants show higher growth of *P. syringae* DC3000 (Century et al., 1995) suggesting the role of NDR1 may not be limited to only R-gene-mediated resistance, but could also be a critical component of PTI.

d. R-protein-Effector Interactions

In mammalian innate immune signaling, TLRs are responsible for the recognition of PAMPs, while their plant counterparts (i.e., R-proteins) are responsible for the recognition of secreted pathogen effector proteins. As is the case in most receptor-ligand interactions, direct association

between receptor and elicitor results in the stimulation and activation of down-stream signaling events required for activation. In total, this interaction results in the activation of signaling required for the successful deployment of defense responses and resistance. To date, two mechanisms of pathogen effector perception have been described in plants: direct and indirect recognition. In 2000, Jia et al (2000) demonstrated a direct interaction between the rice CC-NB-LRR R-protein Pita and its cognate effector protein AvrPita. This interaction specifies resistance in rice to the blast fungal pathogen *Magnaporthe grisea*. In short, this work demonstrated for the first time a direct interaction between a R-protein (receptor) and its ligand, a secreted effector from an invading pathogen. Subsequent work, as conceptually described above, identified this interaction as being mediated by the LRR domain of Pita (Bryan et al., 2000). Additional direct interactions have also been demonstrated between other R-proteins and their cognate pathogen effectors, such is the case with RRS1-R and the bacterial wilt pathogen effector PopP2 (Deslandes et al., 2003), as well as becoming the best-characterized examples from the flax rust resistance loci, which recognize approximately 30 effector proteins from flax rust (reviewed in Ellis et al., 2007). However, direct recognition of pathogen effector proteins appears to be the exception, rather than the rule.

As described above, the "rules" governing the proposition of the guard hypothesis satisfied the lack of additional direct interactions between host R-proteins and pathogen effectors. As a first example of the guard hypothesis, work in Roger Innes's lab demonstrated a multi-protein interaction that seemed to satisfy the criteria of an indirect surveillance mechanism (Simonich and Innes, 1995; Swiderski and Innes, 2001; Shao et al., 2003; Ade et al., 2007). In this case, the association of the *Arabidopsis* R-protein RPS5 with a protein kinase, PBS1, fulfilled the all of

the requirements of the first experimentally validated example of the guard hypothesis. This mechanism requires that: RPS5 associates/interacts with PBS1 (Ade et al., 2007); the *P. syringae* effector AvrPphB, a cysteine protease, cleaves PBS1 (Shao et al., 2003); and, following cleavage of PBS1, the RPS5-PBS1 association is disrupted, leading to a (likely) conformational change in RPS5 and activation of ETI (Ade et al., 2007). Thus, the detection of the pathogen relies on the disruption, or perturbation, of a protein-protein surveillance mechanism by the action of the pathogen effector protein. This, in short, defines ETI.

Several years later, a series of studies presented a new twist in the guard hypothesis; one that presented a testable model to explain the co-regulation and interplay between potentially overlapping defense signaling pathways. In 2002, the laboratory of Jeff Dangl presented the identification of a protein isolated as interacting with the CC-NB-LRR R-protein RPM1 (Mackey et al., 2002). This protein, RIN4, was shown to not only associate with RPM1, yet was also demonstrated to satisfy the requirement(s) as a guard of RPM1 activation. In short, the *Pseudomonas* effector protein AvrRPM1, which activates resistance through RPM1 (Bisgrove et al., 1994), was also demonstrated to act upon RIN4, most likely as an intermediate signaling component in this pathway (Mackey et al., 2002). The AvrRpm1-RIN4 interaction leads to the hyper-phosphorylation of RIN4 (Mackey et al., 2003) that is in turn recognized by RPM1. This interaction alone would seem to perfectly represent one of the original tenets of the Guard Hypothesis (Figure 1.3B). In a parallel series of experiments, RIN4 was also identified as a negative regulator of the RPS2-AvrRpt2 signaling pathway (Axtell and Staskawicz, 2003; Mackey et al., 2003). In this example, RIN4 is cleaved by AvrRpt2 (a cysteine protease), which in turn leads to the activation of ETI (Axtell and Staskawicz, 2003; Mackey et al., 2003; Day et

al., 2005; Chisholm et al., 2006). While a host protein targeted by multiple effectors may not completely conform to the classical definition of the guard hypothesis, it provides an exceptional example of the overlapping regulation in parallel defense signaling networks. This demonstration of a shared intermediate (e.g., RIN4) has strengthened our understanding of the R-protein-effector interaction, and has paved the way for some of the newest concepts in molecular plant pathology. Additional recent studies have revealed a growing, almost ubiquitous, function for RIN4 in a variety of host-pathogen processes (Day et al., 2006; Liu et al., 2009; Luo et al., 2009; Wilton et al., 2010).

The case of RIN4 raises an interesting question: How does specificity, in terms of recognition and regulation, accommodate multiple processes (i.e., RPS2-specific vs. RPM1-specific) being guarded by a single protein? To this end, one new model that may help to explain these shared intermediates, or other putative virulence targets, is the decoy model proposed by Van der Hoorn and Kamoun (2008). In this model, an interesting hypothesis is proposed whereby plants have evolved the utility of proteins as decoys, whose function is, in essence, to dilute the activity of pathogen effector proteins, or, to serve as triggers for a central alarm system alerting the host to the presence of pathogens. Through the evolution, or adaptation of decoys, plants can, in essence, use a single protein to guard multiple targets. This would then explain, to some extent, the functional genetic overlap between parallel resistance signaling processes.

Final Thoughts

So, which is it: Gene-for-gene? The Guard Hypothesis? Are pathogen effector proteins the magic bullets that must be stopped? The more we learn, the more evident it becomes that we are only scratching the surface of what is to be understood in the field of plant-pathogen interactions. In the past few years, an explosion in the area of plant pathology has led to groundbreaking discoveries not only at the level of signaling and gene expression, but also in terms of the application of whole genome biology to non-model systems. In this regard, the early development and proposition that *Arabidopsis* is indeed a model system for translational agriculture is starting to become a reality. Moving forward in the next 10-20 years, a significant investment in the plant sciences will be to put into practice what has been learned from the studies highlighted above. Can we in fact tailor crops to recognize pathogens more efficiently, and too, can we engineer durable resistance to multiple pathogens across multiple crop species? Based on the overlapping specificity detailed above, one would imagine that through defining the shared mechanisms by which plants recognize diverse pathogens, the answer may be "YES!". However, the complexity of resistance signaling, as well as the intimate links shared between resistance signaling and standard processes such as development, reproduction and, tolerance to additional environmental (i.e., abiotic) pressures, a balance must be struck in engineering resistance versus sacrificing plant health and vigor. Thus, in total, the end goal of molecular plant pathology is to understand the processes that ultimately make breeding for durable resistance a possibility. Whether this means that through understanding the function of all pathogen effectors we will be able to breed plants that are more resistant, or that by defining and better understanding the processes in plants that are minimally required for resistance, is still up for debate. Fortunately, there is much we still do not understand and a wealth of knowledge waiting to be discovered.

Acknowledgements

We would like to thank all members of the Day laboratory for critical reading of the manuscript. Research in the Day lab is supported by a grant from the National Science Foundation (IOS-0641319).

Figure 1.1.

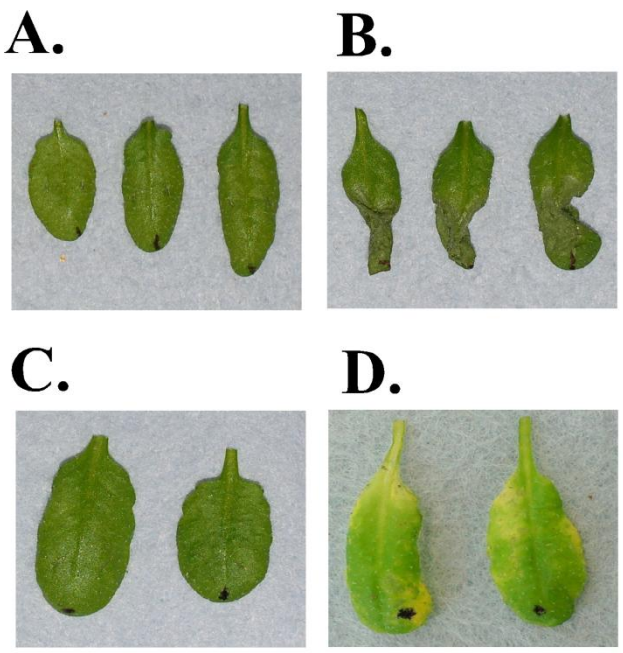


Figure 1.1. The *Arabidopsis thaliana-Pseudomonas syringae* pathosystem. A) Phenotype of the healthy *Arabidopsis* leaves, **B)** leaves undergoing the hypersensitive response (24 hpi), **C)** leaves inoculated with a non-disease eliciting *P. syringae* strain, and **D)** leaf symptoms of the bacterial speck disease. hpi, hours post-inoculation. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Figure 1.2.

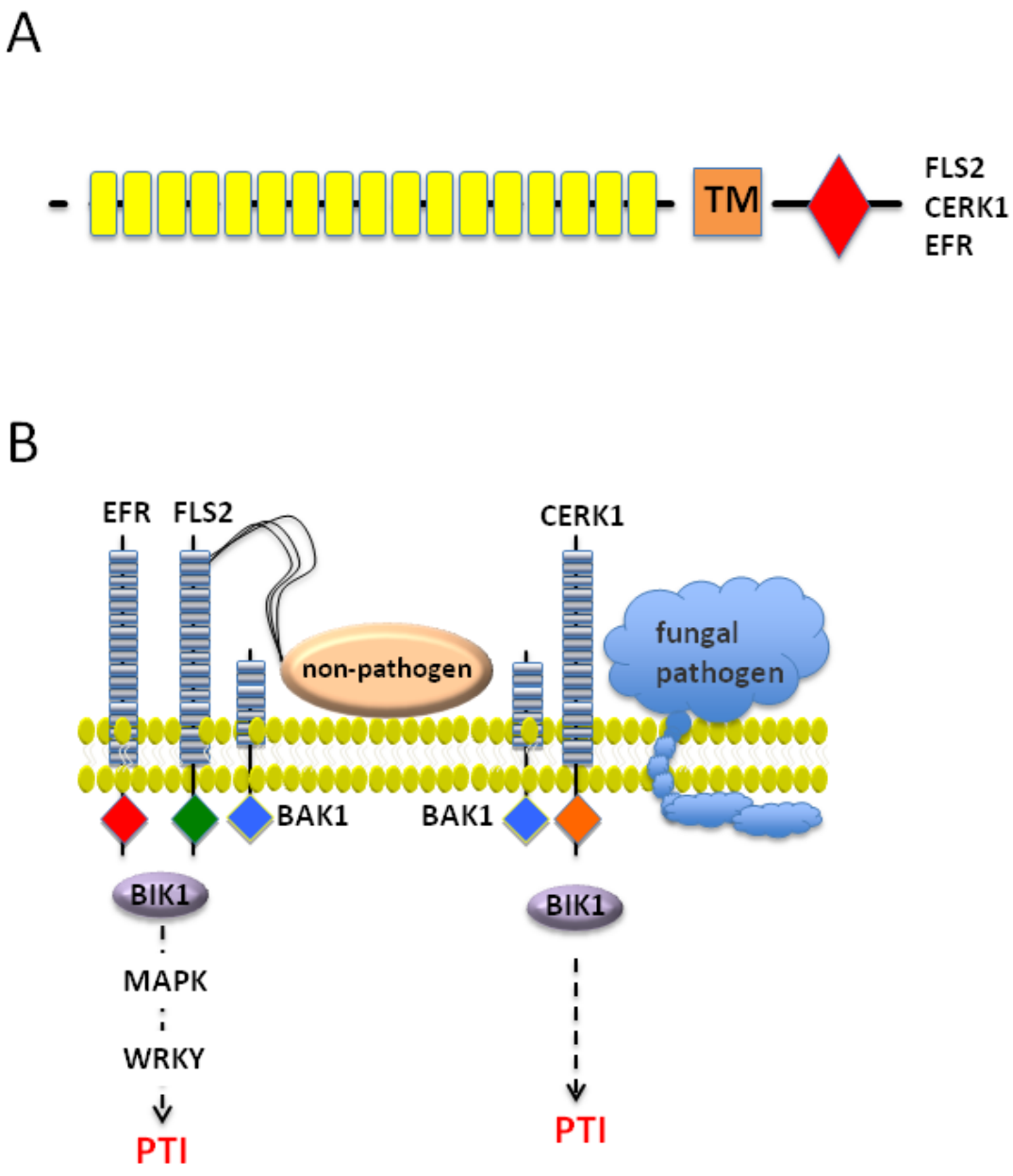

A
TM
FLS2
CERK1
EFR
B
EFR
FLS2
CERK1
non-pathogen
fungal
pathogen
BAK1
BAK1
BIK1
BIK1
MAPK
WRKY
PTI
PTI

Figure 1.2: Pathogen Associated Molecular Pattern Recognition and the Activation of PAMP-Triggered Immunity. A) The PAMP receptors FLS2, CERK1 and EFR. Yellow boxes denote leucine-rich repeats (LRRs); CC, coiled-coil; NB, nucleotide-binding site; TIR, Toll-Interleukin-1 Receptor. Red diamond denotes kinase domain. **B)** As the first layer of defense signaling in plants, PTI is activated via the recognition of conserved pathogen elicitors, generally referred to as PAMPs. Well-characterized PAMPs include flagellin, the bacterial elongation factor, EF-Tu, and the fungal cell wall component, chitin. Binding of PAMPs to their corresponding effectors (e.g., flagellin-FLS2; EF-Tu-EFR; chitin-CERK1) results in the activation of downstream defense signaling, via MAPK activation, resulting in the elicitation of immunity.

Figure 1.3.

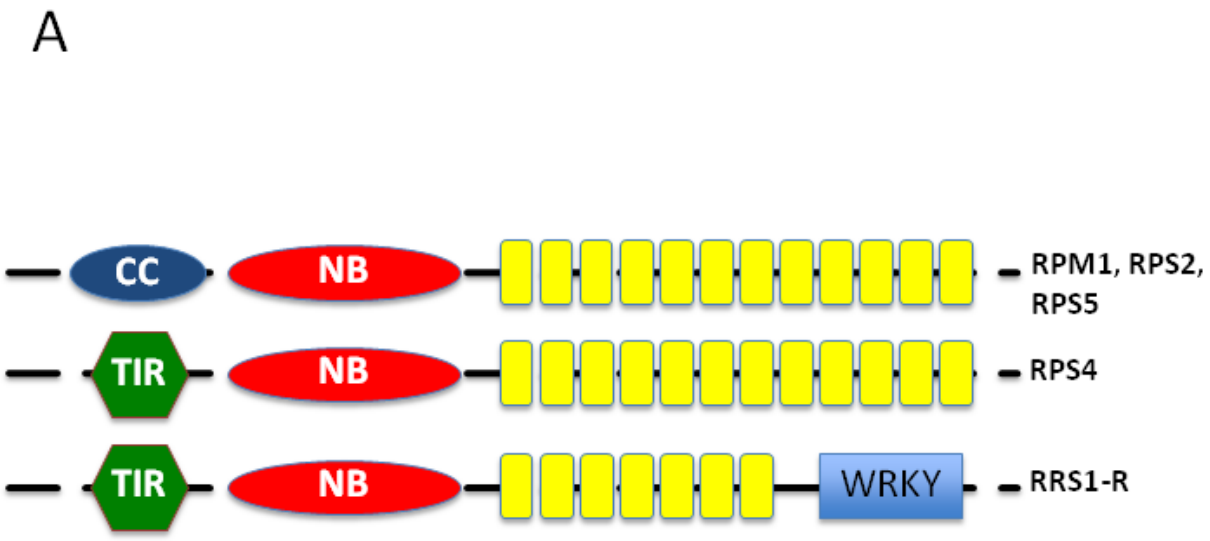


Figure 1.3. (cont'd)

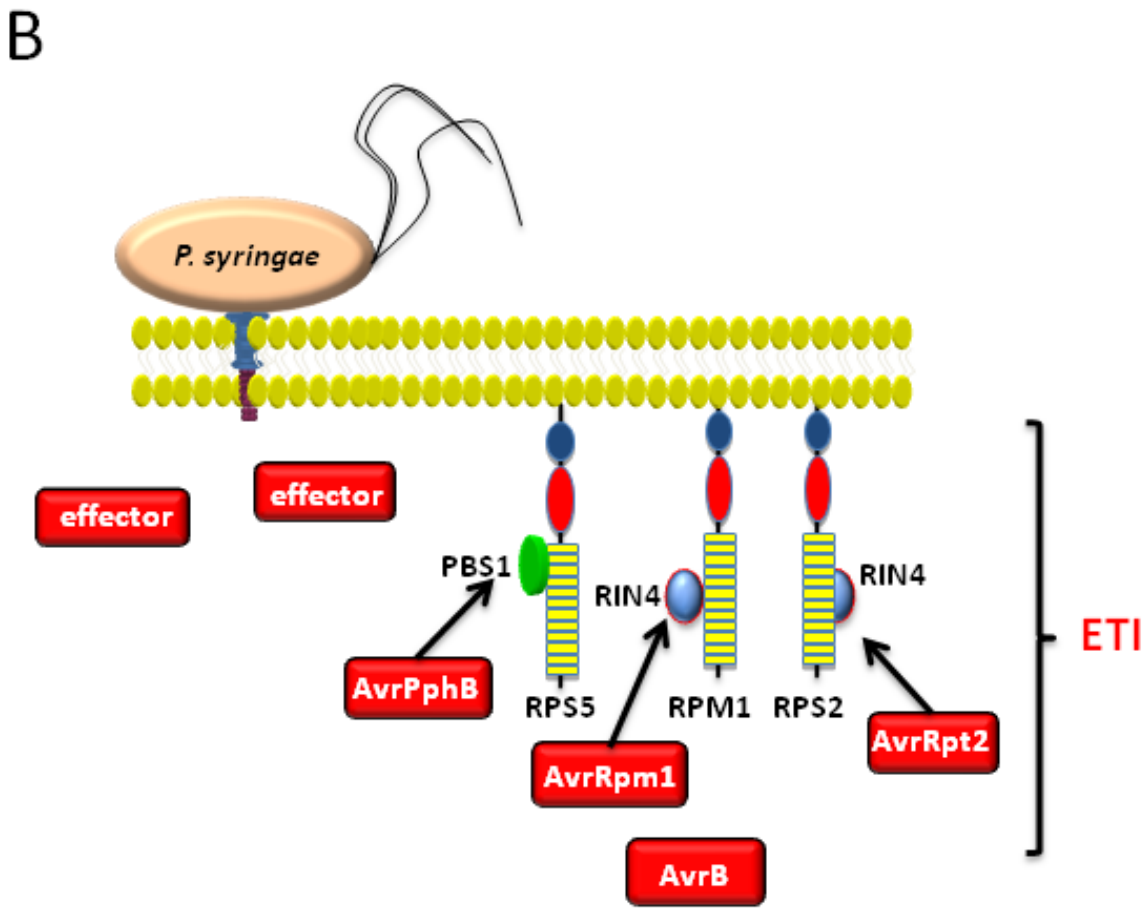


Figure 1.3. Pathogen Effector Recognition and the Activation of Effector-Triggered Immunity. A) The largest class of resistance proteins in *Arabidopsis* is the CC-NB-LRR class, whose members include the R-proteins RPM1, RPS2 and RPS5. The TIR-class of R-proteins are represented by the well-characterized R-protein RPS4. A recently identified variant of this class, RRS1-R, contains a WRKY domain believed to impart transcriptional regulation as part of its function following pathogen effector recognition. **B)** Similar to the activation of PTI, the elicitation of ETI results in the activation of defense signaling via the specific recognition of pathogen-derived elicitors. As a second layer of defense signaling, ETI is the culmination in the recognition of pathogen effector proteins. As shown, delivery of an effector protein via the type III secretion system (T3SS) and subsequent recognition by cognate host R-proteins, leads to the activation of an amplified defense response. The general role of pathogen effector proteins is thought to be the inactivation of PTI, while the role of ETI is to block all mechanisms of pathogen virulence.

Figure 1.4.

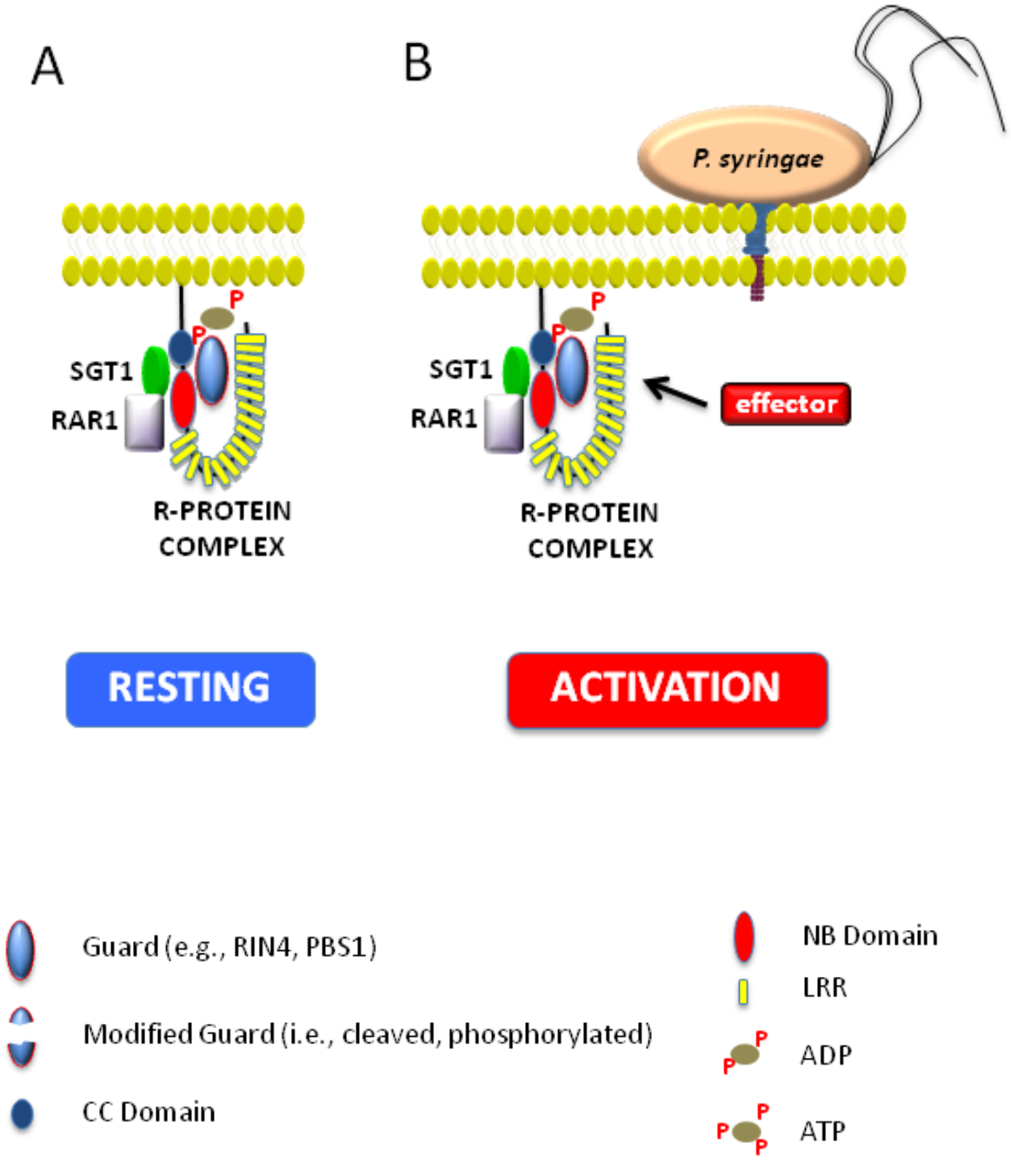

A
B
P. syringae
P
P
SGT1
RAR1
R-PROTEIN COMPLEX
effector
RESTING
ACTIVATION
Guard (e.g., RIN4, PBS1)
Modified Guard (i.e., cleaved, phosphorylated)
CC Domain
NB Domain
LRR
ADP
ATP

Figure 1.4. (cont'd)

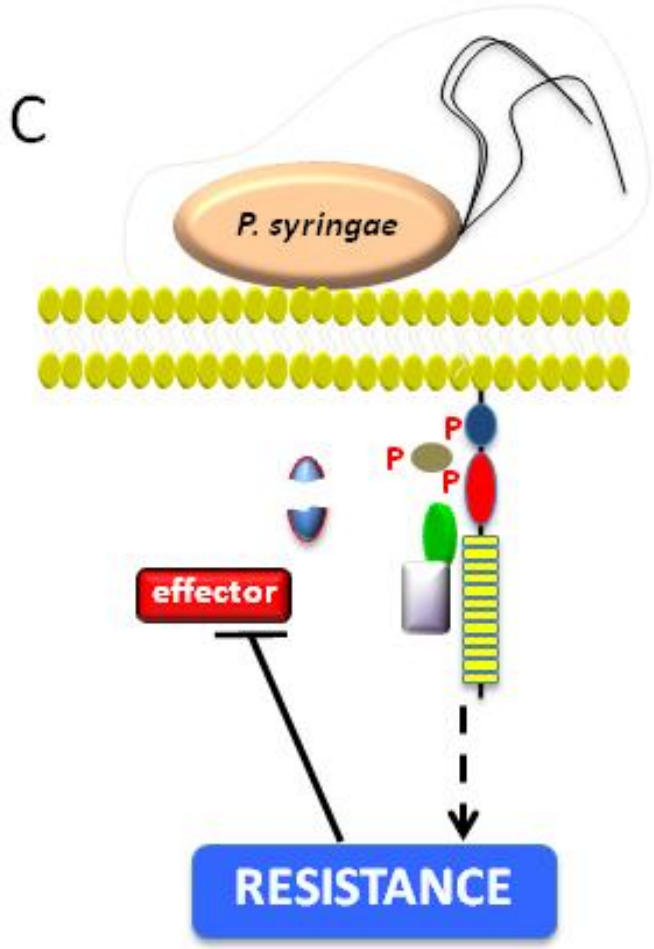


Figure 1.4. A Model for the Regulation and Activation of R-Protein Mediated Defense Signaling in Arabidopsis. A) In the absence of a pathogen, R-proteins are held in an inactive state. This conformation is the result of protein-proteins interaction(s), and as a consequence of these associations, binding of ATP/GTP to the NB domain is blocked. **B)** Following perception of the pathogen, via the activity of secreted effector molecules, an induced conformational change in the R-protein complex is induced. This change results in a possible shift in the stoichiometry of protein-protein interactions, leading to the binding of ATP/GTP to the R-protein NB domain. **C)** Once a pathogen effector is recognized, the activation of ETI results in the initiation of defense signaling, ultimately leading to the abrogation of pathogen growth. Once perturbations to the R-protein surveillance system are no longer perceived, the system resets back to the resting state depicted in "**A**".

LITERATURE CITED

LITERATURE CITED

Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J., and Parker, J.E. (1998). Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in *Arabidopsis*. Proc. Natl. Acad. Sci. U.S.A. **95**, 10306-10311.

Ade, J., DeYoung, B.J., Golstein, C., and Innes, R.W. (2007). Indirect activation of a plant nucleotide binding site-leucine-rich repeat protein by a bacterial protease. Proc. Natl. Acad. Sci. U.S.A. **104**, 2531-2536.

Aist, J.R.B., and Bushnell, W.R. (1991). In The Fungal Spore and Disease Initiation in Plants and Animals, G. T. Cole, H. C. Hoch, Eds. (Plenum, New York). 321-345.

Aharoni, A., Dixit, S., Jetter, R., Thoenes, E., van Arkel, G. and Pereira, A. (2004). The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in *Arabidopsis*. Plant Cell **16**, 2463-2480.

Ahmad, M., and Cashmore, A.R. (1993). HY4 gene of *A. thaliana* encodes a protein with characteristics of a blue-light photoreceptor. Nature **366**, 110-111.

Anderson-Prouty, A.J., and Albersheim, P. (1975). Host-Pathogen interactions: VIII. Isolation of a pathogen-synthesized fraction rich in glucan that elicits a defense response in the pathogen's host. Plant Physiol. **56**, 286-291.

Andreasson, E., and Ellis, B. (2010). Convergence and specificity in the *Arabidopsis* MAPK nexus. Trends Plant Sci. **15**,106-113.

Asai, T., Tena, G., Plotnikova., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., and Sheen, J. (2002). MAP kinase signaling cascade in *Arabidopsis* innate immunity. Nature **415**, 977-983.

Austin, M.J., Muskett, P., Kahn, K., Feys, B.J., Jones, J.D., and Parker, J.E. (2002). Regulatory role of *SGT1* in early *R* gene-mediated plant defenses. Science **295**, 2077–2080.

Axtell, M.J., and Staskawicz, B.J. (2003). Initiation of *RPS2*-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. Cell **112**, 369-377.

Azevedo, C., Sadanandom, A., Kitagawa, K., Freialdenhoven, A., Shirasu, K., and Schulze-Lefert, P. (2002). The RAR1 interactor SGT1, an essential component of *R* gene-triggered disease resistance. Science **295**, 2073–2076.

Bachmair, A., Novatchkova, M., Potuschak, T., and Eisenhaber, F. (2001). Ubiquitination in plants: A post-genomic look at post-translational modification. Trends Plant Sci. **6**, 463-470.

Bari, R., and Jones, J.D. (2009). Role of plant hormones in plant defence responses. Plant Mol. Biol. **69**, 473-488.

Baumgarten, A., Cannon, S., Spangler, R., and May, G. (2003). Genome-level evolution of resistance genes in Arabidopsis thaliana. Genetics **165**, 309-319.

Baureithel, K., Felix, G., and Boller, T. (1994). Specific, high affinity binding of chitin fragments to tomato cells and membranes. Competitive inhibition of binding by derivatives of chitooligosaccharides and Nod factor of Rhizobium. J. Biol. Chem. **269**, 17931-17938.

Bisgrove, S.R., Simonich, S.T., Smith, N.M., Sattler, A., and Innes, R.W. (1994). A disease resistance gene in *Arabidopsis* with specificity for two different pathogen avirulence genes. Plant Cell **6**, 927-933.

Bittel, P., and Robatzek, S. (2007). Microbe-associated molecular patterns (MAMPs) probe immunity. Curr. Opin. Plant Biol. **10**, 335-341.

Block, A., Guo, M., Elowsky, C., Clemente, T.E., and Alfano, J.R. (2010). The Pseudomonas syringae type III effector HopG1 targets mitochondria, alters plant development and suppresses plant innate immunity. Cell Microbiol. **12**, 318-330.

Browne, L.M., Conn, K.L., Ayer, W.A., and Tewari, J.P. (1991) The camalexins: new phytoalexins produced in the leaves of *Camelina sativa* (Cruciferae). Tetrahedron **47**, 3909-3914.

Bryan, G.T., Wu, K.S., Farrall, L., Jia, Y., Hershey, H.P., McAdams, S.A., Faulk, K.N., Donaldson, G.K., Tarchini, R., and Valent, B. (2000). A single amino acid difference distinguishes resistant and susceptible alleles of the rice blast resistance gene Pi-ta. Plant Cell. **12**, 2033-2046.

Century, K., Holub, E.B., and Staskawicz, B.J. (1995). NDR1, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and fungal pathogen. Proc. Natl. Acad. Sci. U.S.A. **92**, 6597-6601.

Century, K., Shapiro, A.D., Repetti, P.P., Dahlbeck, D., Holub, E., and Stascawicz, B.J. (1997). NDR1, a pathogen-induced component required for *Arabidopsis* disease resistance. Science **278**, 1963-1965.

Chandra, A., and Huff, D.R. (2010). A fungal parasite regulates a putative female-suppressor gene homologous to maize tasselseed2 and causes induced hermaphroditism in male buffalograss. Mol. Plant Microbe Interact. **23**, 239-250.

Chen, Y.F., Shakeel, S.N., Bowers, J., Zhao, X.C., Etheridge, N., and Schaller, G.E. (2007). Ligand-induced degradation of the ethylene receptor ETR2 through a proteasome-dependent pathway in *Arabidopsis*. **282**, 24752-24758.

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T., and Felix, G. (2006). The *Arabidopsis* receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. **18**, 465-476.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nurnberger, T., Jones, J.D., Felix, G., and Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature **448**, 497-500.

Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B.J. (2006). Host-Microbe Interactions: Shaping the Evolution of the Plant Immune Response. Cell **124**, 803-814.

Clay, N.K., Adio, A.M., Denoux, C., Jander, G., and Ausubel, F.M. (2009) Glucosinolate metabolites required for Arabidopsis innate immune response. Science **323**, 95-101.

Collins, N.C., Thordal-Christensen, H., Lipka, V., Bau, S., Kombrink, E., Qiu, J.L., Huckelhoven, R., Stein, M., Freialdenhoven, A., Somerville, S.C, and Schulze-Lefert, P. (2003). SNARE-protein-mediated disease resistance at the plant cell wall. Nature **425**, 973-977.

Coppinger, P., Repetti, P.P., Day, B., Dahlbeck, D., Mehlert, A., and Staskawicz, B.J. (2004). Overexpression of the plasma membrane-localized NDR1 protein results in enhanced bacterial disease resistance in *Arabidopsis thaliana*. Plant J. **40**, 225-237.

Cui, J., Bahrami, A.K., Pringle, E.G., Hernandez-Guzman, G., Bender, C.L., Pierce, N.E., and Ausubel, F.M. (2005). *Pseudomonas syringae* manipulates systemic plant defenses against pathogens and herbivores. Proc. Natl. Acad. Sci. U.S.A. **102**, 1791-1796.

da Silva Correia, J., Miranda, Y., Leonard, N., and Ulevitch, R. (2007). SGT1 is essential for Nod1 activation. Proc. Natl. Acad. Sci. U.S.A. **104**, 6764–6769.

Dangl, J.L., and Jones, J.D.G. (2001). Plant pathogens and integrated defence responses to infection. Nature **411**, 826-833.

Davis, K.R, and Hahlbrock, K. (1987). Induction of Defense Responses in Cultured Parsley Cells by Plant Cell Wall Fragments. Plant Physiol. **84**, 1286-1290.

Day, B., Okada, M., Ito, Y., Tsukada, K., Zaghouani, H., Shibuya, N., and Stacey, G. (2001). Binding site for chitin oligosaccharides in the soybean plasma membrane. Plant Physiol. **126**, 1162-1173.

Day, B., Dahlbeck, D., Huang, J., Chisholm, S.T., Li, D., and Staskawicz, B.J. (2005). Molecular basis for the RIN4 negative regulation of RPS2 disease resistance. Plant Cell **17**, 1292-1305.

Day, B., Dahlbeck, D., and Staskawicz, B.J. (2006). NDR1 interaction with RIN4 mediates the differential activation of multiple disease resistance pathways in *Arabidopsis*. Plant Cell **18**, 2782-2791.

De Wit, P.J., Mehrabi, R., Van den Burg, H.A., and Steriopoulos, I. (2009). Fungal effector proteins; past, present and future. Mol. Plant Pathol. **10**, 735-747.

Denoux, C., Galletti, R., Mammarella, N., Gopalana, S., Werck, D., DeLorenzo, G., Ferrari, S., Ausubel, F.M., and Dewdney, J. (2008). Activation of defense response pathways by OGs and Flg22 elicitors in *Arabidopsis* seedlings. Mol. Plant **1**, 423-445.

Deslandes, L., Olivier, J., Peeters, N., Feng, D.X., Khounlotham, M., Boucher, C., Somssich, I., Genin, S., and Marco, Y. (2003). Physical interaction between RRS-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. Proc. Natl. Acad. Sci. U.S.A. **100**, 8024-8029.

Dong, J, Chen, C., and Chen, Z. (2003) Expression profiles of the *Arabidopsis* WRKY gene superfamily during plant defense response. Plant Mol. Biol. **51**, 21-37.

Ellis, J.G., Dodds, P.N., and Lawrence, G.J. (2007). The role of secreted proteins in diseases of plants caused by rust, powdery mildew, and smut fungi. Curr. Opin. Microbiol. **10**, 326-331.

Eulgem, T. (2005). Regulation of the Arabidopsis defense transcriptome. Trends Plant Sci. **10**, 71-78.

Eulgem, T., Weigman, V.J., Chang, H.S., McDowell, J.M., Holub, E.B., Glazebrook, J., Zhu, T., and Dangl, J.L. (2004). Gene expression signatures from three genetically separable resistance gene signaling pathways for downy mildew resistance. Plant Physiol. **135**, 1129-1144.

Eulgem, T., and Somssich, I.E. (2007). Networks of WRKY transcription factors in defense signaling. Curr. Opin. Plant Biol. **10**, 366-371.

Falk, A., Feys, B.J., Frost, L.N., Jones, J.D.G., Daniels, M.J., and Parker, J.E. (1999). EDS1, an essential component of R gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. Proc. Natl. Acad. Sci. U.S.A. **96**, 3292-3297.

Fan, W., and Dong, X. (2002). *In vivo* interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in *Arabidopsis*. Plant Cell **14**, 1377-1389.

Feys, B.J., Moisan, L.J., Newman, M.A., and Parker, J.E. (2001). Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. EMBO J. **20**, 5400-5411.

Feys, B.J., Wiermer, M., Bhat, R.A., Moisan, L.J., Medina-Escobar, N., Neu, C., Cabral, A., and Parker, J.E. (2005). *Arabidopsis* SENESCENCE-ASSOCIATED GENE101 stabilizes

and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity Plant Cell **17**, 2601-2613.

Glawischnig, E. (2007) Camalexin. Phytochemistry **68**, 401-406.

Glazebrook, J. (2001). Genes controlling expression of defense responses in *Arabidopsis*-2001 status. Curr. Opin. Plant Biol. **4**, 301-308.

Göhre, V., Spallek, T., Haweker, H., Mersmann, S., Mentzel, T., Boller, T., de Torres, M., Mansfield, J.W., and Robatzek, S. (2008). Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. Curr. Biol. **18**, 1824-1832.

Gómez-Gómez, L, Felix, G, and Boller, T. (1999). A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. Plant J. **18**, 277-284.

Gómez-Gómez, L., and Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Mol. Cell. **5**, 1003-1011.
Gray, W.M., del Pozo, J.C. Walker, L., Hobbie, L., Risseeuw, E., Banks, T., Crosby, W.L., Yang, M., Ma, H., and Estelle, M. (1999). Identification of an SCF ubiquitin-ligase complex required for auxin response in *Arabidopsis thaliana*. Genes Dev. **13**, 1678-1691.

Hadwiger, L.A., Beckman, J.M., and Adams, M.J. (1981). Localization of fungal components in the pea-*Fusarium* interaction detected immunochemically with anti-chitosan and anti-fungal cell wall antisera. Plant. Physiol. **67**, 170-175.

Hahn, H., and Kaufmann, S.H.E. (1981). The role of cell-mediated immunity in bacterial infections. Rev. Infect. Dis. **3**, 1221-1250.

Hahn, J.S. (2005). Regulation of Nod1 by Hsp90 chaperone complex. FEBS Lett. **579**, 4513-45-19.

Hamilton, A.J., and Baulcombe, D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. Science **286**, 950-952.

Hematy, K., Cherk, C., and Somerville, S. (2009). Host-pathogen warfare at the plant cell wall. Curr. Opin. Plant Biol. **12**, 406-413.

Heese, A., Hann, D.R., Gimenez-Ibanez, S., Jones, A.M., He, K., Li, J., Schroeder, J.I., Peck, S.C., and Rathjen, J.P. (2007). The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. Proc. Natl. Acad. Sci. U.S.A. **104**, 12217-12222.

Hirano, S.S., and Upper, C.D. (2000). Bacteria in the leaf ecosystem with emphasis on Pseudomonas syringae-a pathogen, ice nucleus, and epiphyte. Microbiol. Mol. Biol. Rev. **64**, 624-653.

Ichimura, K., Casais, C., Peck, S.C., Shinozaki, K., and Shirasu, K. (2006). MEKK! Is required for MPK4 activation and regulates tissue-specific and temperature-dependent cell death in Arabidopsis. J. Biol. Chem. **281**, 36969-36976.

Irani, N.G., and Russinova, E. (2009). Receptor endocytosis and signaling in plants. Curr. Opin. Plant Biol. **12**, 653-659.

Istomin, A.Y., and Godzik, A. (2009). Understanding diversity of human innate immunity receptors: analysis of surface features of leucine-rich repeat domains in NLRs and TLRs. BMC Immunol. **3**, 1-15.

Jeffree, C.E. (2006). The fine structure of the plant cuticle. Biology of the plant cuticle; Annual Plant Reviews, Volume 23. Eds. Reiderer, M., and Muller, C. Blackwell publishing Ltd. p. 11-16.

Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P., and Valent, B. (2000). Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. EMBO J. **19**, 4004-4014.

Jones, A.M., Chory, J., Dangl, J.L., Estelle, M., Jacobsen, S.E., Meyerowitz, E.M., Nordborg, M., and Weigel, D. (2008). The Impact of *Arabidopsis* on Human Health: Diversifying Our Portfolio. Cell **133**, 939-943.

Jones, D.A. and Jones, J.D.G. (1996). The roles of leucine rich repeats in plant defences. Adv. Bot. Res. Adv. Plant Pathol. **24**, 90-167.

Jones, J.D., and Dangl, J.L. (2006). The plant immune system. Nature **444**, 323-329.

Kajava, A.V. (1998). Structural diversity of leucine-rich repeat proteins. J. Mol. Biol. **277**, 519-527.

Katagiri, F., Thilmony, R., and He, S.Y. (2002). The *Arabidopsis Thaliana-Pseudomonas Syringae* Interaction. In The Arabidopsis Book. Last, R., Eds. American Society of Plant Biologists.

Kim, C.Y., Bove, J., and Assmann, S.M. (2008). Overexpression of wound-responsive RNA-binding proteins induces leaf senescence and hypersensitive-like cell death. New Phytol. **180**, 57-70.

Kliebenstein, D.J., Rowe, H.C., and Denby, K.J. (2005). Secondary metabolites influence *Arabidopsis*/*Botrytis* interactions: variation in host production and pathogen sensitivity. Plant J. **44**, 25-36.

Lay, F.T., and Anderson, M.A. (2005). Defensins-components of the innate immune system in plants. Curr. Protein Pept. Sci. **6**, 85-101.

Lindeberg, M., Cunnac, S., and Collmer, A. (2009). The evolution of *Pseudomonas syringae* host specificity and type III effector repertoires. Mol. Plant. Pathol. **10**, 767-775.

Lipka, V., Dittgen, J., Bednarek, P., Bhat, R., Wiermer, M., Stein, M., Landtag, J., Brandt, W., Rosahl, S., Scheel, D., Llorente, F., Molina, A., Parker, J., Somerville, S., and Schulze-Lefert, P. (2005). Pre- and post-invasion defenses both contribute to nonhost resistance in *Arabidopsis*. Science **310**, 1180-1183.

Lipka, U., Fuchs, R., and Lipka, V. (2008). *Arabidopsis* non-host resistance to powdery mildews. Curr. Opin. Plant Biol. **11**, 404-411.

Liu, J., Elmore, J.M., Fuglsang, A.T., Palmgren, M.G., Staskawicz, B.J., and Coaker, G. (2009). RIN4 functions with plasma membrane H^+-ATPases to regulate stomatal aperatures during pathogen attack. PLoS Biol. **7**, e1000139.

Lu, X., Tintor, N., Mentzel., T., Kombrink, E., Boller, T., Robatzek, S., Schulze-Lefert, P., and Saijo, Y. (2009). Uncoupling of sustained MAMP receptor signaling from early outputs in an *Arabidopsis* endoplasmic reticulum glucosidase II allele. Proc. Natl. Acad. Sci. U.S.A. **106**, 22522-22527.

Luo, Y., Caldwell, K.S., Wroblewski, T., Wright, M.E., and Michelmore, R.W. (2009). Proteolysis of a negative regulator of innate immunity is dependent on resistance genes in tomato and Nicotiana benthamiana and induced by multiple bacterial effectors. Plant Cell. **21**, 2458-2472.

Mackey, D., Holt, B.F., Wiig, A., and Dangl, J.L. (2002). RIN4 interacts with Pseudomonas syringae type III effector molecules and is required for RPM1-mediated resistance in *Arabidopsis*. Cell. **108**, 743-754.

Mackey, D., Belkhadir, Y., Alonso, J.M., Ecker, J.R., and Dangl, J.L. (2003). *Arabidopsis* RIN4 is the target of the type III virulence effector AvrRpt2 and modulates RPS2 mediated resistance. Cell **112**, 379-389.

Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K.A., Dangl, J.L., and Dietrich, R.A. (2000). The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. Nat. Genet. **26**, 403-410.

Mateo, A., Muhlenbock, P., Rusterucci, C., Chang, C.C., Miszalski, Z., Karpinska, B., Parker, J.E., Mullineaux, P.M., and Karpinski, S. (2004). LESION SIMULATING DISEASE 1 is required for acclimation to conditions that promote excess excitation energy. Plant Physiol. **136**, 2818-2830.

Matsushima, N., Mikami, T., Tanaka, T., Miyashita, H., Yamada, K., and Kuroki, Y. (2009). Analyses of non-leucine-rich repeat (non-LRR) regions intervening between LRRs in proteins. Biochim Biophys Acta. **1790**, 1217-1237.

Matzinger, P. (2007). Friendly and dangerous signals: is the tissue in control? Nat. Immunol. **8**, 11-13.

Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S.Y. (2006). Plant stomata function innate immunity against bacterial invasion. Cell. **126**, 969-980.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu., K, Narusaka, Y., Kawakami, N., Kaku, H., and Shibuya, N. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. Proc. Natl. Acad. Sci. U.S.A. **104**, 19613-19618.

Morgan, W., and Kamoun, S. (2007). RXLR effectors of plant pathogenic oomycetes. Curr. Opin. Microbiol. **10**, 332-338.

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell **113**, 935-944.

Muskett, P.R., Kahn, K, Austin, M.J., Moisan, L.J., Sadanandom, A., Shirasu, K., Jones, J.D.G., and Parker, J.E. (2002). *Arabidopsis RAR1* exerts rate-limiting control of R gene mediated defenses against multiple pathogens. Plant Cell **14**, 979-992.

Mysore, K.S., and Ryu, C. (2004). Nonhost resistance: how much do we know? Trends Plant Sci. **9**, 97-104.

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J.D. (2006). A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science **312**, 436-439.

Nawrath, C. (2006). Unraveling the complex network of cuticular structure and function. Curr. Opin. Plant Biol. **9**, 281-287.

Nomura, K., Debroy, S., Lee, Y.H, Pumplin, N., Jones, J., and He, S.Y. (2006). A bacterial virulence protein suppresses host innate immunity to cause plant disease. Science **313**, 220-223.

Okada, M., Matsumura, M., Ito, Y., and Shibuya, N. (2002). High-affinity binding proteins for N-Acetylchitooligosaccharide elicitor in the plasma membranes from wheat, barley, and carrot cells: conserved presence and correlation with the responsiveness to the elicitor. Plant Cell Physiol. **43**, 505-512.

Otegui, M.S., and Spitzer, C. (2008). Endosomal functions in plants. Traffic **9**, 1589-1598.
Padmanabhan, M., Cournoyer, P., and Dinesh-Kumar, S.P. (2009). The leucine-rich repeat domain in plant innate immunity: a wealth of possibilities. Cell Microbiol. **11**, 191-198.

Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D., and Daniels, M.J. (1996). Characterization of eds1, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different RPP genes. Plant Cell **8**, 2033-2046.

Peart, J.R., Lu, R., Sadanandom, A., Malcuit, I., Moffett, P., Brice, D.C., Schauser, L., Jaggard. D.A.W., Xiao. S., Coleman. M.J., Dow. M., Jones. J.D.G., Shirasu., and Baulcombe, D.C. (2002). Ubiquitin ligase-associated protein SGT1 is required for host and nonhost disease resistance in plants. Proc. Natl. Acad. Sci. USA. **99**, 10865-10869.

Pedley, K.F., and Martin, G.B. (2003). Molecular basis of Pto-mediated resistance to bacterial speck disease in tomato. Annu. Rev. Phytopathol. **41**, 215-243.

Pedras, M.S., and Khan, A.Q. (1997). Unprecedented detoxification of the cruciferous phytoalexin camalexin by a root phytopathogen. Bioorg. Med. Chem. Lett. **7**, 2255-2260.

Pedras, M.S., and Khan, A.Q. (2000). Biotransformation of the phytoalexin camalexin by the phytopathogen *Rhizoctonia solani*. Phytochemistry **53**, 59-69

Penninckx, I.A., Eggermont, K., Terras, F.R., Thomma, B.P., De Samblanx, G.W., Buchala, A., Metraux, J.P., Manners, J.M., and Broekaert, W.F. (1996). Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. Plant Cell **8**, 2309-2323.

Qiu, J., Zhou, L., Yun, B., Nielsen, H.B., Fiil, B.K., Petersen, K., MacKinlay, J., Loake, G. J., Mundy, J., and Morris, P.C. (2008). *Arabidopsis* mitogen-activated protein kinases MKK1 and MKK2 have overlapping functions in defense signaling mediated by MEKK1, MPK4, and MKS1. Plant Physiol. **148**, 212-222.

Reina-Pinto, J.J., and Yephremov, A. (2009). Surface lipids and plant defenses. Plant Physiol. Biochem. **47**, 540-549.

Robatzek, S., Chinchilla, D., and Boller, T. (2006). Ligand-induced endocytosis of the pattern recognition receptor FLS2 in Arabidopsis. Genes Dev. **20**, 537-542.

Rusterucci, C., Aviv, D.H., Holt, B.F., Dangl, J.L., and Parker, J.E. (2001). The disease resistance signaling components *EDS1* and *PAD4* are essential regulators of the cell death pathway controlled by *LSD1* in Arabidopsis. Plant Cell **13**, 2211-2224.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H., and Hunt, M.D. (1996). Systemic acquired resistance. Plant Cell **8**, 1809-1819.

Saraste, M., Sibbald., and Wittinghofer, A. (1990). The P-loop-a common motif in ATP- and GTP-binding proteins. Trends Biochem. Sci. **15**, 430-434.

Sato, A., and Yamamoto, K.T. (2008). What's the physiological role of domain II-less Aux/IAA proteins? Plant Signal Behav. **3**, 496-497.

Schornack, S., Huitema, E., Cano, L.M., Bozkurt, T.O., Oliva, R., Van Damme, M., Schwizer, S., Raffaele, S., Chaparro-Garcia, A., Farrer, R., Segretin, M.E., Bos, J., Haas,

B.J., Zody, M.C., Nusbaum, C., Win, J., Thines, M., and Kamoun, S. (2009). Ten things to know about oomycete effectors. Mol. Plant Pathol. **10**, 795-803.

Sels, J., Mathys, K.J., De Coninck, B.M.A., Cammue, B.P.A., and De Bolle, M.F.C. (2008). Plant pathogenesis-related (PR) proteins: A focus on PR peptides. Plant Physiol. and Biochem. **46**, 941-950.

Shen, K.A., Meyers, B.C., Islam-Faridi, M.N., Chin, D.B., Stelly, D.M., and Michelmore, R.W. (1998). Resistance gene candidates identified by PCR with degenerate oligonucleotide primers map to clusters of resistance genes in lettuce. Mol. Plant Microbe Interact. **11**, 815-823.

Shibuya, N., Ebisu, N., Kamada, Y., Kaku, H., Cohn., and Ito, Y. (1996). Localization and binding characteristics of a high-affinity binding site for N-Acetlychitooligosaccharide elicitor in the plasma membrane from suspension-cultured rice cells suggest a role as a receptor for the elicitor signal at the cell surface. Plant Cell Physiol. **37**, 894-898.

Shirasu, K., Lahaye, T., Tan, M.W., Zhou, F.S., Azevedo, C., and Schulze-Lefert, P. (1999). A novel class of eukaryotic zinc-binding proteins is required for disease resistance signaling in barley and development in *C. elegans*. Cell **99**, 355-366.

Shoa, F., Golstein, C., Ade, J., Stoutemyer, M., Dixon, J.E., and Innes, R.W. (2003). Cleavage of *Arabidopsis* PBS1 by a bacterial type III effector. Science **301**, 1230-1233.

Simonich, M.T., and Innes, R.W. (1995). A disease resistance gene in *Arabidopsis* with specificity for the avrPph3 gene of *Pseudomonas syringae* pv. *phaseolicola*. Mol. Plant Microbe Interact. **8**, 637-640.

Singh, K., Foley, R.C., and Onate-Sanchez, L. (2002). Transcription factors in plant defense and stress responses. Curr. Opin. Plant Biol. **5**, 430-436.

Spoel, S.H., and Dong, X. (2008). Making sense of hormone crosstalk during plant immune responses. Cell Host Microbe **12**, 348-351.

Staskawicz, B.J., Dahlbeck, D., and Keen, N.T. (1984). Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinea* determines race-specific incompatibility on *Glycine max* (L.) Merr. Proc. Natl. Acad. Sci. U.S.A. **81**, 6024-6028.

Staskawicz, B.J., Mudgett, M.B., Dangl, J.L., and Galan, J.E. (2001). Common and contrasting themes of plant and animal diseases. Science **292**, 2285-2289.

Stasckawicz, B.J. (2009). First insights into the genes that control plant-bacterial interactions. Mol. Plant Pathol. **10**, 719-720.

Stefanato, F.L., Abou-Mansour, E., Buchala, A., Kretschmer, M., Mosbach, A., Hahn, M., Bochet, C.G., Metraux, J., and Schoonbeek, H. (2009). The ABC transporter BcatrB from

Botrytis cinerea exports camalexin and is a virulence factor on *Arabidopsis thaliana*. Plant J. **58**, 499-510

Stein, M., Dittgen, J., Sanchez-Rodriquez, C., Hou, B-H., Molina, A., Schulze-Lefert, P., Lipka, V., and Somerville, S. (2006). *Arabidopsis* PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant Cell **18**, 731-746.

Swiderski, M.R., and Innes, R.W. (2001). The *Arabidopsis* PBS1 resistance gene encodes a member of a novel protein kinase subfamily. Plant J. **26**, 101-112.

Takahashi, A., Casais, C., Ichimura, K., and Shirasu, K. (2003). HSP90 interacts with RAR1 and SGT1 and is essential for RPS2-mediated disease resistance in *Arabidopsis*. Proc. Natl. Acad. Sci. U.S.A. **100**, 11777-11782.

Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H.S., Han, B., Zhu, T., Zou, G., and Katagiri, F. (2003). Quantitative nature of *Arabidopsis* responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. Plant Cell **15**, 317-330.

Temple, B.R., and Jones, A.M. (2007). The plant heterotrimeric G-protein complex. Annu. Rev. Plant Biol. **58**, 249-266.

Terras, F.R., Torrekens, S., Van Leuven, F., Osborn, R.W., Vanderleyden, J., Cammue, B.P.A., and Broekaert, W.F. (1995). A new family of basic Cysteine-rich plant antifungal proteins from Brassicaceae species. FEBS Lett. **316**, 233-240.

Thomma, B.P., Eggermont, K., Tierens, K.F., and Broekaert, W.F. (1999). Requirement of functional ethylene-insensitive 2 gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. Plant Physiol. **121**, 1093-1102.

Thomma, B.P., Penninckx, I.A., Broekaert, W.F., and Cammue, B.P. (2001). The complexity of disease signaling in Arabidopsis. Curr. Opin. Immunol. **13**, 63-68.

Tian, M., Chaudhry, F., Ruzicka, D.R., Meagher, R.B., Staiger, C.J. and Day, B. (2009). *Arabidopsis* Actin-depolymerizing factor AtADF4 mediated defense signal transduction triggered by the *Pseudomonas syringae* effector AvrPphB. Plant Physiol. **150**, 815-824.

Ting, J.P., Kastner, D.L., and Hoffman, H.M. (2006). CATERPILLERs, pyrin and hereditary immunological disorders. Nat. Rev. Immunol. **6**, 183-195.

Tör, M., Gordon, P., Cuzick, A., Eulgem, T., Sinapidou, E., Mert-Turk, F., Can, C., Dangl, J.L., and Holub, E.B. (2002). *Arabidopsis SGT1b* is required for defense signaling conferred by several downy mildew resistance genes. Plant Cell **14**, 993-1003.

Tornero, P., Merritt, P., Sadanandom, A., Shirasu, K., Innes, R.W., and Dangl, J.L. (2002). *RAR1* and *NDR1* contribute quantitatively to disease resistance in *Arabidopsis*, and their relative contributions are dependent on the R gene assayed. Plant Cell **14**, 1005-1015.

Tsuda, K., Glazebrook, J., and Katagiri, F. (2008). The interplay between MAMP and SA signaling. Plant Signal. Behav. **3**, 359-361.

Tsuji, J., Jackson, E.P., Gage, D.A., Hammerschmidt, R., and Somerville, S.C. (1992). Phytoalexin accumulation in *Arabidopsis thaliana* during the hypersensitive reaction to *Pseudomonas syringae* pv *syringae*. Plant Physiol. **98**, 1304-1309

Van der Biezen, E.A., and Jones, J.D. (1998). Plant disease-resistance proteins and the gene-for-gene concept. Trends Biochem. Sci. **23**, 454-456.

Van der Hoorn, R.A., De Wit, P.J., and Joosten, M.H. (2002). Balancing selection favors guarding resistance proteins. Trends Plant Sci. **7**, 67-71.

Van der Hoorn, R.A., and Kamoun, S. (2008). From guard to decoy; a new model for perception of plant pathogen effectors. Plant Cell **20**, 2009-2017.

Van Loon LC, Rep M, Pieterse CMJ (2006). Significance of inducible defense-related proteins in infected plants. Annu. Rev. Phytopathol. **44**, 135-162.

Van Loon, L.C., and Van Strien, E.A. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol. and Mol. Plant Pathol. **55**, 85-97.

VanEtten, H.D., Mansfield, J.W., Bailey, J.A., and Farmer, E.E. (1994). Two classes of plant antibiotics: Phytoalexins versus "Phytoanticipins". Plant Cell **6**, 1191-1192.

Wan, J., Zhang., X.C., and Stacey, G. (2008). Chitin signaling and plant disease resistance. Plant Signal. Behav. **3**, 831-833.

Wang, D., Pajerowska-Mukhtar, K., Culler, A.H., and Dong, X. (2007). Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. Curr. Biol. **17**, 1784-1790.

Whisson, S.C., Boevink, P.C., Moleleki, L., Avrova, A.O., Morales, J.G., Gilroy, E.M., Armstrong, M.R., Grouffaud, S., van West, P., Chapman, S., Hein, I., Toth, I.K., Pritchard, L., and Birch, P.R. (2007). A translocation signal for delivery of oomycete effector proteins into host plant cells. Nature **450**, 115-118.

Wiermer, M., Feys, B.J., and Parker, J.E. (2005). Plant immunity: the EDS1 regulatory node. Curr. Opin. Plant Biol. **8**, 383-389.

Wilton, M., Subramaniam, R., Elmore, J., Felsensteiner, C., Coaker, G., and Desveaux, D. (2010). The type III effector HopF2Pto targets Arabidopsis RIN4 protein to promote *Pseudomonas syringae* virulence. Proc Natl Acad Sci U S A. **107**, 2349-2354.

Yu, D., Chen, C., and Chen, Z. (2001). Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. Plant Cell **13**, 1527-1540.

Zhou, T., Wang, Y., Chen, J.Q., Araki, H., Jing, Z., Jiang, K., Shen, J., and Tian, D. (2004). Genome-wide identification of NBS genes in japonica rice reveals significant expansion of divergent non-TIR NBS-LRR genes. Mol. Genet. Genomics **27**, 402-415.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D.G., Felix, G., and Boller, T. (2004). Bacterial disease resistance in *Arabidopsis* through flagellin perception. Nature **428**, 764-767.

Zipfel, C. (2009). Early molecular events in PAMP-triggered immunity. Curr. Opin. Plant Biol. **12**, 414-420.

CHAPTER 2

Arabidopsis NDR1 is an integrin-like protein with a role in fluid loss and plasma membrane-cell wall adhesion

This research was originally published in Plant Physiology.
Knepper, C., Savory, E.A. and Day, B. (2011) Arabidopsis NDR1 is an integrin-like protein with a role in fluid loss and plasma membrane-cell wall adhesion. Plant Physiol. **156**: 286-300. www.plantphysiol.org © American Society of Plant Biologists.

Abstract

Arabidopsis thaliana NDR1 (NON-RACE SPECIFIC DISEASE RESISTANCE-1), a plasma membrane localized protein, plays an essential role in resistance mediated by the CC-NB-LRR class of resistance (R) proteins, which includes RPS2, RPM1 and RPS5. Infection with *Pseudomonas syringae* pv. tomato DC3000 expressing the bacterial effector proteins AvrRpt2, AvrB and AvrPphB activate resistance by the aforementioned R proteins. Whereas the genetic requirement for NDR1 in plant disease resistance signaling has been detailed, our study focuses on determining a global, physiological role for NDR1. Through the use of homology modeling and structure threading, NDR1 was predicted to have a high degree of structural similarity to Arabidopsis LEA14, a protein implicated in abiotic stress responses. Specific protein motifs also point to a degree of homology with mammalian integrins, well characterized proteins involved in adhesion and signaling. This structural homology led us to examine a physiological role for NDR1 in preventing fluid loss and maintaining cell integrity through plasma membrane-cell wall adhesions. Our results show a substantial alteration in induced (i.e., pathogen inoculated) electrolyte leakage and a compromised PAMP triggered immune response in *ndr1-1* mutant plants. As an extension of these analyses, using a combination of genetic and cell biology-based approaches, we have identified a role for NDR1 in mediating plasma membrane-cell wall adhesions. Taken together, our data point to a broad role for NDR1 both in mediating primary cellular functions in Arabidopsis through maintaining the integrity of the cell wall-plasma membrane connection, as well as a key signaling component of these responses during pathogen infection.

Introduction

Defense signaling in plants following pathogen infection results in the activation of multiple, often parallel, signaling pathways. To accomplish this, the activation of plant resistance signaling requires the utilization of numerous preformed defense responses, systemic and cell-to-cell signaling, and in many cases, the initiation of Gene-for-Gene resistance (Knepper and Day, 2010). While examples of activation and regulation of parallel processes have been described (van Wees *et al.*, 2000), there are numerous instances in which bifurcation exists (Wiermer *et al.*, 2005). In plants, it is now evident that regulation and specificity often have overlapping nodes; the guard hypothesis is one such example (reviewed in Chisholm *et al.*, 2006; Jones and Dangl, 2006). In short, host resistance (R) proteins are responsible for the indirect recognition of pathogen effectors by means of "guarding" a virulence target and initiating an effective defense response.

The largest class of resistance (R) genes found in plants encode for nucleotide-binding site-leucine-rich repeat (NB-LRR) signaling molecules (Moffett, 2009). NB-LRR R-proteins have been historically divided into two subgroups, based on the amino-terminal presence of either a coiled-coil (CC) domain, or a domain with similarity to the Toll interleukin-1 receptor (TIR) family of proteins (reviewed in Takken and Tameling, 2009). While R-proteins play a central role in the activation of resistance following pathogen perception, R-proteins alone are not sufficient for the initiation of resistance. As such, numerous ancillary proteins and chaperones serving as co-activators of resistance have also been identified (Muskett *et al*., 2002; Tornero *et al*., 2002; Azevedo *et al.*, 2002; Hubert *et al*., 2003; Belkhadir *et al.*, 2004). For example, EDS1

(ENHANCED DISEASE SUSCEPTIBILITY-1; Parker *et al.*, 1996) has been shown to mediate defense signaling through the activation of the TIR domain-containing R-proteins, while NDR1 (NON-RACE SPECIFIC DISEASE RESISTANCE-1; Century *et al.*, 1995; Century *et al.*, 1997; Coppinger *et al.*, 2004) has been shown to be required for R-proteins that contain the CC domain (Aarts *et al.*, 1998). There are, however, exceptions to this rule; several CC-NB-LRR R-proteins that specify resistance to the oomycete pathogen *Hyaloperonospora arabidopsidis* function independently of NDR1 (McDowell *et al.*, 2000; Bittner-Eddy and Beynon, 2001). Thus, while there appears to be conservation in terms of specificity, function and activation of signaling through both EDS1 and NDR1 in terms of R-protein structure, the full mechanism(s) associated with these pathways are unknown.

Of the two primary signaling components required for the activation of R-protein-mediated resistance, the role of EDS1 in defense signaling is best understood. EDS1 serves as a central regulatory protein involved in both biotic and oxidative stress signaling (reviewed in Wiermer *et al.*, 2005). In this capacity, EDS1, through its interaction with PAD4 (PHYTOALEXIN DEFICIENT 4) and SAG (SENESCENCE ASSOCIATED GENE)-101, is required for elicitation of the hypersensitive response (HR) during bacterial infection (Feys *et al.*, 2001, 2005). EDS1 has also been shown to function in halting post-invasive growth of non-pathogenic fungi (Yun *et al.*, 2003), as well as functioning in oxidative stress signaling (Wiermer *et al.*, 2005). Thus, EDS1 can be classified as a broad-spectrum signaling mediator required for the activation of resistance to multiple pathogen types (Hu *et al.*, 2005).

In contrast, the function of NDR1 within the CC-NB-LRR signaling pathway remains unclear. NDR1 is a plasma membrane-localized protein (Century *et al.,* 1997) that undergoes multiple post-translational modifications, including C-terminal processing (i.e., Glycosylphosphatidylinositol (GPI) anchoring) and N-linked glycosylation (Coppinger *et al.*, 2004). Unlike most GPI-anchored proteins, NDR1 appears to be unique in that the anchor at the carboxyl-terminus is resistant to cleavage by phospholipase-C, and therefore possibly positions NDR1 within the plasma membrane as a "double anchored" protein (Coppinger *et al.*, 2004). This positioning further raises the possibility that NDR1 may play a role in signaling from within the apoplast, transducing the signal from the extracellular space to within the cell, possibly via its association with RIN4 (RPM1-INTERACTING PROTEIN-4; Day *et al.*, 2006), however, this hypothesis remains untested. NDR1 has been demonstrated to associate with RIN4 (Day *et al*., 2006) which has been shown to be required for both the regulation and activation of resistance mediated by at least two member of the CC-NB-LRR class of resistance proteins (Mackey *et al.*, 2002, 2003; Axtell and Staskawicz, 2003; Belkhadir *et al.*, 2004; Day *et al.*, 2005; Chisholm *et al.*, 2005). The association of RIN4 with the resistance proteins RPM1 (RESISTANCE TO *PSEUDOMONAS SYRINGAE* PV. *MACULICOLA*-1; Grant *et al.*, 1995) and RPS2 (RESISTANCE TO *PSEUDOMONAS SYRINGAE*-2; Kunkel *et al.*, 1993) is believed to maintain the proteins in a signaling-incompetent state (Ellis and Dodds, 2003; Chisholm *et al.*, 2006). The association of NDR1 with a key negative regulator of R-protein activation (RIN4) may in fact point to a role for NDR1 as a signal recognition element upstream of the activation of specific R-protein signaling (Belkhadir *et al.*, 2004), and moreover, may signify a role for NDR1 in broader defense and stress-associated response requiring both NDR1 and RIN4.

The activation of defense signaling through ligand-receptor interactions (e.g., pathogen recognition receptor (PRR)-pathogen associated molecular pattern (PAMP)) is likely preceded by the mechanical stimulation of responses at the onset of pathogen invasion. For example, in mammalian innate immune and stress signaling, integrins have been characterized as a central signaling component of the mechano-sensing apparatus of cells (Kumamoto, 2008). Integrins are ubiquitous plasma membrane receptors that recognize extracellular glycoproteins (e.g., collagen and fibronectins) via the conserved, solvent exposed *Arg-Gly-Asp* (RGD) motif (Gee *et al.*, 2008). On the cytoplasmic side, integrins connect the extracellular matrix to the cytoskeleton, and following signal recognition, can initiate signaling responses associated with cell differentiation (Streuli, 2009), growth and proliferation (Huveneers, *et al.*, 2007). In plants, peptides containing the RGD motif have been shown to block numerous processes, including fungal toxin penetration (Manning *et al.*, 2008), mechanoperception (reviewed in Telewski, 2006), cell wall adhesion to the plasma membrane (Canut, *et al.*, 1998) and abiotic stress responses (Zhu *et al.*, 1993; reviewed in Gao *et al.*, 2007). Similarly, RGD containing peptides have also been shown to interfere with cell signaling responses initiated during interactions with microorganisms, particularly those involving fungal and oomycete pathogens (Kiba *et al.*, 1998; Mellersh and Heath, 2001; Senchou *et al.*, 2004). While *bona fide* integrin proteins have not been identified in plants, high affinity RGD-binding sites have been identified (Canut *et al.*, 1998), and it is hypothesized that these sites mediate plasma membrane-cell wall adhesion.

In the current work, we describe a role for NDR1 in both defense and stress response signaling based on a predicted structure of NDR1 with homology to biotic and abiotic stress responsive proteins. Using this predicted structural homology, and the known positioning of NDR1 in

defense signaling, we demonstrate using molecular-genetic, physiological and cell biology approaches, that NDR1 plays a role in electrolyte release in response to bacterial pathogen infection, as well as in maintenance of the plasma membrane-cell wall junction. Mutational analysis of NDR1 supports a role for the RGD-like motif, NGD (*Asn-Gly-Asp*), as a key motif in NDR1 involved in defense signaling following *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) infection. Taken together, our data suggest a role for NDR1 in coordinating broader cellular processes in response to stress and bacterial pathogen infection.

RESULTS

NDR1 shares predicted structural homology with LEA14, an integrin-like protein

Two independent methods were used to generate a predicted structure for NDR1 (Figure 2.1A). First, the protein fold recognition server PHYRE (Kelley and Sternberg, 2009) was used to generate a predicted structural model. In the second approach, we utilized the automated homology modeling server I-TASSER to generate five predicted structures for NDR1 (Figure S2.1). Both PHYRE and I-TASSER generated similar homology model predictions for NDR1. The outputs of these predictions were then used as a template to guide our structure-function analyses described in the present study. Homology modeling and structure threading was used to predict a tertiary fold of NDR1. Once a secondary structure for NDR1 was predicted using PHYRE, homology modeling was performed using MODELLER (Marti-Renom *et al.*, 2000) and Chimera (Pettersen *et al.*, 2004). The output of this analysis was then threaded onto the solved structure of the Arabidopsis LEA14 protein (LATE EMBRYOGENESIS ABUNDANT-14;

Figure 2.1B; pdb_1yyc; Singh *et al.*, 2005), a member of the late embryogenesis family of proteins.

Further analysis of the predicted structure was performed, in a domain-by-domain manner, from which we identified several striking similarities with mammalian proteins involved in signal perception and innate immune responses (Figure S2.2). For example, the large β-sheet torus (Figure 2.1A; blue arrows) resembles the core structure of type III fibronectins (Potts and Campbell, 1994). Further modeling (Figure S2.2) suggests the primary core structure of NDR1 shares strong structural similarity with the membrane bound subunit (i.e., fibronectin FNIII domain) of integrin, with the putative transmembrane domains of both NDR1 and integrin connected to large single β-sheets (Hynes, 2009). Adjacent to the 3-amino acid α-helix (Figure 2.1B, small red/orange helix; compare homologous position in Figure 2.1C), we identified the presence of a solvent-exposed RGD-like motif (i.e., NGD) at amino acid position 178 to 180. In host-fungal interactions, the role of RGD motifs in defense signaling has been characterized as a potential ligand binding site involved in cell wall-plasma membrane adhesion (Manning *et al.*, 2008; reviewed in Dodds *et al.*, 2009). Moreover, RGD sites have also been postulated as both a target, and mode of action, for pathogen effector proteins and secretion systems, presumably to facilitate pathogen entry through disrupting cell wall-plasma membrane focal adhesions (Jimenez-Soto *et al*., 2009; Wang *et al*., 2009).

NDR1 plays a role in limiting electrolyte leakage in response to *Pst* DC3000 infection

Based on the proposed function of LEA proteins in Arabidopsis, we hypothesized that NDR1 may play a broad role in regulating fluid loss. While the precise function of the LEA family of proteins remains elusive, numerous studies have linked LEAs to a variety of biotic and abiotic stress responses (De Meutter *et al.*, 2005; Goyal *et al.*, 2005; Battaglia *et al.*, 2008). In support of this, Singh *et al*. (2005) proposed LEAs to have a role in stopping or slowing the process of fluid loss in response to wounding or dehydration. Based on the predicted structural homology of NDR1 to LEA14, we hypothesized that defense signaling may be regulated, in part through NDR1, as a function of controlling nutrient availability during pathogen infection (Katagiri *et al.*, 2002). When inoculated with the phytopathogenic bacterium *Pst* DC3000, *ndr1-1* plants show altered electrolyte leakage, compared with Col-0 plants. These data are in agreement with similar plant-pathogen studies that have utilized electrolyte leakage as a physiological marker for the HR as well as the activation of defense signaling in plants (Baker *et al.*, 1991; Mackey *et al*., 2002). While previous methods used to measure electrolyte leakage relied on simple conductance measurements over a relatively short time scale (i.e., 24 hours; Figure S2.3), we chose to examine the changes in leakage in response to disease progression, and not simply in correlation to the HR, by utilizing a method previously applied to abiotic stress (Gilmour *et al*., 1988). When *ndr1-1* plants were dip inoculated with *Pst* DC3000 the leakage measurements observed paralleled those of Col-0 (Figure 2.2A). These data are consistent with previously published bacterial growth data and the enhanced disease susceptibility phenotype(s) (Century *et al.* 1995), while also indicating a possible alteration in membrane permeability in the *ndr1-1* mutant. When plants were inoculated with *Pst* DC3000 expressing the cysteine protease effector protein AvrRpt2 (Axtell *et al.*, 2003), the difference in measured electrolytes between *ndr1-1* and Col-0 are in greater contrast, with the most striking difference observed at 2 days post-

inoculation (dpi; Figure 2.2B). This result provides further evidence for an alteration in membrane integrity in the *ndr1-1* mutant, as a simple increase in programmed cell death cannot explain the dramatic difference in leakage observed; *ndr1-1* does not undergo HR in response to AvrRpt2 (Century *et al*., 1995). The large observed difference in leakage also correlates with the onset of disease symptoms in *ndr1-1* (Figure 2.2E). To this end, while *ndr1-1* does not exhibit a significant increase in leakage, our findings would suggest that the *ndr1-1* mutant is unable to restrict leakage induced following *Pst* DC3000-AvrRpt2 inoculation. This apparent loss in restricting leakage may be a primary mechanism through which AvrRpt2 is able to enhance susceptibility in *ndr1-1*. Indeed, this hypothesis is further supported by previous results from Freeman and Beattie (2009), which shows that Arabidopsis responds to the effector AvrRpm1 by restricting vascular flow to the infected area. Related to the work presented herein, it is noteworthy that both AvrRpm1 and AvrRpt2 are known to target RIN4 (Mackey *et al.*, 2002; 2003), the only previously identified interacting partner of NDR1 (Day et al., 2006). *Pst* DC3000 expressing the effector proteins AvrPphB (Shao *et al.*, 2003) or AvrB (Mackey *et al.*, 2002) did not elicit a statistically significant difference in the electrolyte release response in *ndr1-1* plants compared with Col-0 (Figure 2.2C-D).

The *ndr1-1* mutant displays altered mRNA expression of several biotic and abiotic stress responsive genes

Given the differences between the disease phenotypes in the *ndr1-1* mutant versus Col-0 plants, as well as the observation of enhanced electrolyte leakage, we hypothesized that a relationship between these phenotypes and gene expression may exist. We reasoned that altered expression

of genes that are known to be associated with nutrient and water stress, as well as other general abiotic stresses associated with drought tolerance may be indicators of an altered stasis in *ndr1-1*. Based on the general role of LEA proteins in both biotic and abiotic stress responses, as well as the predicted similarity of NDR1 to LEA14 (Figure 2.1), we hypothesized that NDR1 may also play a role in abiotic stress tolerance, or may be functionally affected by these stresses. We compiled a candidate list of genes from publicly available microarray and expression data (Sato *et al.*, 2007) whose expression profiles may correlate with our observed electrolyte phenotypes. Two members of the *LEA* family met our search criteria and were investigated for altered mRNA expression in *ndr1-1* plants. *LEA* group 1 domain containing protein (At1g32560) showed a nearly 3-fold increase in expression in *ndr1-1* mutant plants as compared to Col-0 when inoculated with *Pst* DC3000 (Figure 2.3A). *LEA* group 1 domain containing protein also showed altered levels of expression in *ndr1-1* plants when inoculated with *Pst* DC3000 expressing AvrRpt2, but when inoculated with *Pst* DC3000 expressing AvrPphB, a rapid induction was observed in *ndr1-1* leading to a nearly 4 fold increase in mRNA expression at 48 hours post inoculation (hpi; Figure 2.3A). The expression patterns of *LEA14* were nearly identical between Col-0 and *ndr1-1* under all conditions with the exception of *Pst* DC3000 expressing AvrRpt2 which showed a nearly 3 fold increase in mRNA at 48 hpi in *ndr1-1* plants (Figure 2.3A). This differential expression in *LEA* family genes observed between Col-0 and *ndr1-1* suggests an increase in cellular stresses occurring within an *ndr1-1* cell following pathogen inoculation, and may also indicate a similar role for NDR1 in cellular stress responses as that of LEA14.

Genes associated with water regulation and ion release (e.g., aquaporin homologs; *AtPIP1;3*, *AtPIP1;4*, *AtPIP1;5*, *AtPIP2;5*, *AtPIP2;6*) were also examined. No significant differences in expression were observed between Col-0 and *ndr1-1* at 0 hpi (Figure 2.3B). However, two of the aquaporin homologs (*AtPIP1;4* and *AtPIP2;5*) showed a rapid induction in *ndr1-1* compared with Col-0 when infected with *Pst* DC3000 alone, or expressing AvrRpt2 (Figure 2.3B). This finding provides further evidence that NDR1 may play a role in mediating fluid loss as *AtPIP1;4* and *AtPIP2;5* have been previously demonstrated to be up-regulated in response to drought stress (Alexandersson *et al.*, 2005). Consistent with our observed electrolyte leakage data, we did not detect a significant differences in the aquaporin homologs tested between Col-0 and *ndr1-1* following inoculation with *Pst DC3000* expressing either AvrB or AvrPphB (Figure S4). These data further suggest increased water stress within the cells of *ndr1-1* plants following inoculation with *Pst* DC3000. Interestingly, we observed an increase in *PDF1.2* mRNA expression at 24 hpi with *Pst* DC3000 expressing AvrB in the *ndr1-1* mutant (Figure S5), which is consistent with previously published observations in RIN4-associated mutants (Cui et al., 2010),

NDR1 self-associates *in planta* via the formation of oligomers

One of the primary characteristics of integrins, and integrin-like proteins, is their association *in vivo* as dimers (Zhao and Newman, 2001). Based on the predicted structural similarity of NDR1 to integrin-like proteins, we hypothesized that NDR1 exists as multimers *in planta*. To test this, differentially epitope-tagged (e.g., T7 and HA epitope) NDR1 constructs were expressed in *Nicotiana benthamiana* using *Agrobacterium tumefaciens*-mediated transient expression. As shown in Figure 2.4A, using epitope-tagged NDR1 constructs, we detected a self-association in

reciprocal co-immunoprecipitation pull-downs, providing another example of integrin-like structure. The band intensity observed for the reciprocal self-associations correlated with differences in total tagged NDR1 protein detection rates (Figure S6).

The NGD motif is not required for the interaction of NDR1 with RIN4, nor self-association as a multimeric protein complex

RIN4 has been shown to interact with NDR1 (Day *et al.*, 2006). Based on the similarity of the NGD motif to known ligand binding sites (i.e., the RGD site found in integrins), the possibility exists that this site serves to stabilize the NDR1-RIN4 interaction, or as an additional point of interaction between the two associated proteins. Co-immunoprecipitation experiments were performed to isolate the NDR1-RIN4 protein complex (Figure 2.4D). T7-tagged NDR1, as well as variants with mutations within the NGD motif (i.e., 178AAA180 and 178RGD180), were tested for association with HA-tagged RIN4. In all cases, an interaction between NDR1 and RIN4 was detected, despite alterations to the NGD motif (Figure 2.4E-F). These data suggest that the NGD motif is not required for the NDR1 association with RIN4 *in planta*, in agreement with the previous finding that the association occurs within the N-terminal region of NDR1 (Day *et al.*, 2006). Based on the proposed topology of NDR1 (Coppinger *et al.*, 2004), our results are not surprising, and moreover, they are consistent with published data describing integrin dimerization and protein association (van der Flier and Sonnenberg, 2001; Zhao and Newman, 2001).

We also tested the impact of mutations in the NGD motif on the NDR1-NDR1 self-association identified in this study. Unlike the NDR1-RIN4 association, the specific residues or motifs required for the formation of multimeric complexes of NDR1 are unknown. HA-epitope-tagged NDR1 along with various T7-tagged NDR1 proteins (i.e., 178NGD180, 178AAA180 and 178RGD180) were transiently expressed in *N. benthamiana* using *Agrobacterium*-mediated transient expression. Complexes consisting of NDR1 along with NDR1 variants containing an altered NGD site could successfully be isolated using co-immunoprecipitation (Figure 2.4B-C). These data also demonstrate that the NGD site is not required for the formation of self-associated multimeric NDR1 protein complexes.

The *ndr1-1* mutation has a reduced response to the PAMP flg22

While it has long been known that *ndr1-1* mutant plants are compromised in resistance to *Pst* DC3000, as well as in addition to strains expressing several specific bacterial effector proteins (Century *et al*., 1995), the underlying mechanism(s) of this process have never been explored. In the current study, we explored this through the analysis of the expression of *FRK1* (FLG22-INDUCED RECEPTOR-LIKE KINASE 1), an early marker of MAPK (Mitogen-Activated Protein Kinase) pathway activation (Asai *et al*., 2002), in both Col-0 and *ndr1-1* plants treated with the PAMP flg22. As shown in Figure 2.5B, the ndr1-1 mutant showed a significantly weakened response to flg22 treatment, as measured by MAPK activation. Furthermore, total protein levels of MAPK3 and MAPK6, both of which are required for priming of the biotic stress response (Beckers *et al.*, 2009), were reduced in *ndr1-1* plants treated with flg22 as compared to Col-0 (Figure 2.5C).

Mutation of the NGD site in NDR1 may compromise resistance to *Pseudomonas syringae* DC3000

Our experimental approach assessing the role of the NGD site in mediating protein-protein interactions revealed no detectable impact on the NDR1-RIN4 or NDR1-NDR1 associations (Figure 2.4), the question remains whether mutations in this site alter the overall resistance response to *Pst* DC3000. To test this, we generated stable T7-tagged transgenic lines in the *ndr1-1* mutant background expressing the two mutant variants of NDR1 (i.e., RGD and AAA) at positions 178-180, as well as an NDR1-complemented line (i.e., NGD). As shown in Figure 2.6A, the NGD complemented line displayed resistance to *Pst* DC3000 and *Pst* DC3000 expressing each of the 3 effector proteins described above, at a level comparable to WT Col-0. Similarly, conversion of the NGD motif to RGD also resulted in a near WT level of resistance to all *Pst* DC3000 strains tested (Figure 2.6B). When the NGD motif was changed to AAA, a complementation of resistance to *Pst* DC3000 over-expressing any of the 3 effector genes was observed, however only an intermediate level of resistance to *Pst* DC3000 was detected (Figure 2.6C). The 1 log increase in bacterial growth observed in the AAA lines as compared to Col-0, while not statistically significant under our assigned p value ($p<0.05$; Table S2.3), is nonetheless an interesting finding and strengthens the case for the NGD site of NDR1 as playing a critical role in specific defense responses. *Pst* DC3000 demonstrates an increased growth on *ndr1-1* plants as compared to Col-0 (Century *et al.,* 1995). This, together with our observations of the AAA complemented line, suggests that NDR1 may be involved in mediating PAMP triggered, or basal, immunity. Indeed, this hypothesis is further supported by the alteration observed in the

MAPK pathway response to the PAMP flg22 in the *ndr1-1* mutant (Figure 2.5). The inability of the AAA complemented lines to fully complement resistance would suggest that this site may in fact play a role in resistance signaling to *Pst* DC3000, possibly through mediating association with an as yet-unidentified ligand, similar to the function of integrin RGD binding sites (Plow, *et al.*, 2000).

ndr1-1 mutant plants have altered cell wall adhesions

Several pieces of evidence point to a role for NDR1 in the adhesion of the plasma membrane to the cell wall; predicted structural similarity to integrins, as well as the putative orientation of NDR1 within the plasma membrane (Coppinger *et al.*, 2004). To test this hypothesis, 8-day-old *ndr1-1* and Col-0 hypocotyls were visualized pre- and post-$CaCl_2$-induced plasmolysis to assess the integrity of plasma membrane-cell wall adhesion. As shown in Figure 2.7A, a distinctive concave shape was observed in the membranes of Col-0 hypocotyls cells undergoing plasmolysis, with obvious attachments to the cell wall still visible (Figure 2.7A, arrows). These data are in agreement with previously observed results using hypocotyls or suspension-cultured cells (Canut *et al.*, 1998; Gouget *et al.*, 2006). Surprisingly, $CaCl_2$-induced plasmolysis of the *ndr1-1* mutant was significantly altered (Figure 2.7A, 2.7C), resulting in the complete detachment of the plasma membrane from the cell wall, yielding spherical protoplasts with no remaining attachments (convex plasmolysis). Complementation of the *ndr1-1* mutant with a constitutively expressed (i.e., 35S) GFP:NDR1 fusion protein restored the cell wall attachment phenotype to wild-type (Figure 2.7B).

Disruption of plasma membrane-cell wall adhesion has been previously correlated with the function of the RGD motif (Canut *et al.*, 1998). Moreover, it has been well-established that the conversion from concave to convex plasmolysis can be induced in cells with the addition of exogenous RGD-containing or RGD-binding peptides (Canut *et al.*, 1998; Mellersh and Heath, 2001; Senchou *et al.*, 2004; Gouget *et al.*, 2006). As shown in Figure 2.8, addition of exogenous peptides did not alter the plasmolysis phenotype in *ndr1-1* hypocotyls. Interestingly, the addition of 'VNGDG' peptide prior to the addition of $CaCl_2$ was able to alter the plasmolysis pattern of Col-0 hypocotyls from concave to convex in the same manner as the addition of 'VRGDG' peptide, suggesting that the NGD motif might function similarly to the RGD motif. In addition, complemented *ndr1-1* lines expressing either P_{NDR1}:NGD or P_{NDR1}:RGD behaved similarly to Col-0 without peptide treatment, as well as when treated with exogenous peptides. Conversely, P_{NDR1}:AAA complemented lines responded in the same manner as *ndr1-1* under both treatment conditions (Figure 2.8), strengthening the case for the NGD motif functioning in adhesion in NDR1.

DISCUSSION

NDR1 was identified more than 15 years ago in a screen for enhanced disease susceptibility mutants in Arabidopsis following *Pst* DC3000 inoculation (Century *et al.*, 1995). Numerous studies have speculated on a role for NDR1 in defense signaling (Belkhadir *et al.*, 2004; Coppinger *et al.*, 2004; Zhang *et al.*, 2004; Day *et al.*, 2006), however, a functional role has remained elusive. Our primary goal in this study was to look beyond the general defense

signaling processes with which *NDR1* is known to be associated, and focus on determining a broader physiological role for NDR1. This knowledge would both give insight into the general role of NDR1 and also enable us to hypothesize on its function in plant disease resistance signaling.

Studies have shown that *NDR1* is required for a specific subset of resistance signaling pathways in plants (Innes, 1998). A conceptual model has evolved that infers that a biochemical role for NDR1 must encompass a multitude of specific signaling pathways associated with the activation of resistance. For example, *NDR1* is required for the activation of a majority of CC-NB-LRR R-proteins in Arabidopsis (Aarts *et al.*, 1998). However, there are exceptions to this requirement (McDowell *et al.*, 2000; Bittner-Eddy and Beynon, 2001). With this in mind, how do we broadly assign a role for *NDR1* in defense signaling, while also explaining exceptions to the rule?

The association of NDR1 with RIN4 provides a possible mechanism through which NDR1 participates in defense signaling (Day *et al.*, 2006). As a required component of signaling mediated by three CC-NB-LRR resistance proteins (e.g., RPM1, RSP2 and RPS5), association with RIN4 physically links NDR1 to two of these R-proteins: RPM1 and RPS2 (Mackey *et al.*, 2003; Axtell and Staskawicz, 2003). Day *et al.* (2006) speculated that the NDR1-RIN4 association might serve as a protein scaffold, directly or indirectly linking multiple signaling components at the plasma membrane. The association of NDR1 with RIN4, as well as the association of RIN4 with both RPM1 and RPS2, provides the potential for the assembly of a multi-protein complex at the plasma membrane.

The control of electrolyte release during host-pathogen interactions (Baker et al., 1991; Mackey *et al.,* 2002) as well as the desiccation responses induced in plants as a result of pathogen infection (Wright and Beattie, 2004), led us to hypothesize that NDR1 may play a role in similar, associated responses. This proved successful, as our data suggest a role for NDR1 in electrolyte release following infection with *Pst* DC3000 (Figure 2.2A-D). However, our data do not fully define the precise role of NDR1 in the general process of nutrient release. The simplest explanation, given the proposed role of NDR1 in membrane adhesion, may be that the absence of NDR1 results in a weakened membrane system, one that is unable to prevent or restrict leakage in response to a virulent pathogen. Alternatively, NDR1 may be associated with additional proteins, such as membrane-associated transporters and/or regulatory processes, in the host plant which themselves are involved in nutrient release-associated mechanisms. In support of this, recent work by Liu *et al*., (2009) identified several interacting partners of RIN4, thereby potentially linking NDR1 to a multitude of host cell processes, including guard cell dynamics and membrane potential.

The predicted structural homology of NDR1 with LEA14 (Figure 2.1), and by inference, with integrins (Figure S2.2), prompted us to investigate a general role for NDR1 in stress responses that are regulated by the LEA family of proteins. In plants, LEA proteins have been described as playing a role in a broad range of biotic and abiotic responses, including salt tolerance, as well as cold, heat and drought stress (De Meutter *et al.*, 2005; Goyal *et al.*, 2005; Battaglia *et al.*, 2008). Our observation of an increase in *LEA14* mRNA expression in *ndr1-1* plants in response to *Pst* DC3000 infection may in fact suggest that *ndr1-1* plants undergo increased water stress during pathogen infection. We reason that this increase in *LEA14* expression may be an attempt by the

plant to compensate for the increased electrolyte leakage observed in *ndr1-1* plants. Therefore, we hypothesize that NDR1 plays a role in limiting fluid loss. A similar role was proposed for LEA14 in response to wounding and dehydration (Singh *et al.*, 2005). Changes in expression patterns of *LEA* group 1 domain containing transcripts further strengthens the argument for NDR1 in the regulation of abiotic stress tolerance, while increases in the expression of *PIP1;4* and *PIP2;5* (Figure 2.3) demonstrates an increase in drought stress within the cells of *ndr1-1* mutant plants inoculated with *Pst* DC3000 expressing AvrRpt2. Linking the physiological and phenotypic observations with the mRNA expression data provide a more complete picture of the role NDR1 plays in processes associated with electrolyte release, water movement, drought tolerance and pathogen response.

In mammals, integrins are viewed as the primary signal response elements having critical roles in adhesion signaling (Huveneers and Danen, 2009). Based on the predicted structural homology with the fibronectin protein fold, we hypothesized that NDR1 may be involved with the plasma membrane-cell wall network. Based on the "double anchor" model (Coppinger *et al*., 2004), we hypothesized that NDR1 may function by mediating signaling processes or play a role in plasma membrane-cell wall adhesion. During compatible or incompatible plant-pathogen interactions, adhesion between the plasma membrane and cell wall can be disturbed or strengthened, respectively, and elicitation of defense responses is dependent on adherence (Mellersh and Heath, 2001). Our work shows that *ndr1-1* mutant plants lack observable adhesion points between the plasma membrane and cell wall under $CaCl_2$-induced plasmolysis (Figure 2.7A). Interestingly, we observed that addition of peptides with the NDR1 motif NGD (i.e., 'VNGDG') to Col-0 hypocotyls prior to plasmolysis resulted in conversion from the concave to convex

plasmolysis phenotype. This is in agreement with previous work described by Canut *et al.* (1998), who characterized the role of RGD peptides in the disruption of plasma membrane-cell wall adhesion. Furthermore, complemented *ndr1-1* lines expressing *NDR1* with the wild type NGD, or mutant RGD, site behaves, as one would predict, with both NDR1 variants able to complement the mutation and thus restore normal plasmolysis. Conversely, by substituting AAA into the NGD site mirrored the lack of adhesion and convex plasmolysis observed in *ndr1-1* mutant plants.

In support of our proposed role for NDR1 in mediating these processes, there are numerous examples describing the role of plasma membrane-cell wall adhesion in basic plant physiology (Gouget *et al.,* 2006), mediating resistance during host-pathogen interactions (Mellersh and Heath, 2001), as well as a potential virulence target during pathogen invasion (Pieterse *et al.*, 1992; Senchou *et al.*, 2004). In each of these instances, exogenous application of RGD peptides altered host cell membrane dynamics. In the current study, the addition of exogenous peptides (e.g., VNDGG, VRGDG or VAAAG), while significantly altering plasma membrane-cell wall adhesion, did not alter the HR observed in either Col-0 or *ndr1-1* plants (Table S2.1). This finding indicates that the role of NDR1 in adhesion does not fully account for the *ndr1-1* mutant's loss of HR in response to the effector AvrRpt2. Based on the role(s) *NDR1* plays in defense signaling processes initiated by a diverse array of plant pathogens, we hypothesize that the role of *NDR1* in these processes are **1)** mediating plasma membrane-cell wall adhesion, **2)** regulation of fluid movement in response to pathogen infection, **3)** the perception of mechanical stimuli initiated at the plasma membrane-cell wall interface **4)** the transmission of this signal

from the apoplast to/through the plasma membrane either via its association with RIN4 or other associated proteins.

The proposed role of NDR1 in mediating leakage may partially explain the differential requirements for NDR1 in CC-NB-LRR mediated resistance to bacterial pathogens and to the oomycete pathogen *Hyaloperonospora arabidopsidis* (McDowell *et al.*, 2000; Bittner-Eddy and Beynon, 2001). Wright and Beattie (2004) have previously shown that limiting leaf water availability results in restricted *P. syringae* growth, thereby supporting our hypothesis that the inability of *ndr1-1* mutants to restrict leakage provides a more suitable environment to support bacterial growth. Given the different mechanisms of infection and disease progression between a bacterial and an obligate oomycete pathogen such as *H. arabidopsidis*, it is not surprising that NDR1 would have a much stronger effect on defense responses associated with a specific subset of CC-NB-LRR proteins.

While much work remains to fully define the role of NDR1 in plant defense signaling, the current work described herein provides a foundation for moving forward with additional biochemical analyses. To date, the genetic role of *NDR1* in defense signaling has been narrowly defined without having a biochemical function for NDR1. As such, the role of *NDR1* has been defined based almost exclusively on the absence (e.g., *ndr1-1* mutant) of the protein. In total, our work proposes a role for *NDR1* in mediating electrolyte leakage, and in part, regulating processes associated with desiccation and drought tolerance; processes that are primary tenants of plant disease resistance and abiotic stress response. As a role for LEA14 may be the preservation of nutrient and fluid loss during biotic and abiotic stresses (Singh *et al.*, 2005),

NDR1 may have a similar role, while also having evolved parallel functionality as an integral signaling component of the plant defense network, possibly linking both ETI and PTI induced signaling following *P. syringae* infection.

MATERIALS AND METHODS

Homology Modeling and Structure Threading

The primary amino acid sequence of NDR1 (AT3G20600) was submitted to the PHYRE (Protein Homology/analogY Recognition Engine) protein fold recognition server (http://www.sbg.bio.ic.ac.uk/phyre/). The predicted structure was analyzed using the MODELLER comparative homology modeling software (Marti-Renom *et al.*, 2000). The output of this analysis, a predicted model of all non-hydrogen atoms based on spatial restraints, was then viewed using Chimera (Pettersen *et al.*, 2004). Image overlays were performed using the solved NMR structure of the LEA (LATE EMBRYOGENESIS ABUNDANT protein)-14 (AT1G01470; 1yyca; Singh *et al.*, 2005). Further analysis (Figure S2.1) was performed by submitting the primary amino acid sequence of NDR1 to I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/), an internet-based structure prediction service.

Plant Growth Conditions

Arabidopsis thaliana plants were grown at 20°C under a 12 hour/12 hour light/dark cycle at 60% relative humidity in a Bio Chambers Incorporated model FLX-37 growth chamber. *Nicotiana benthamiana* plants were grown under the same conditions.

DNA Cloning and Mutagenesis

Cloning of DNA constructs was performed using standard protocols. Site-directed mutagenesis of NDR1 was performed according to previously published protocols (Day *et al.*, 2005), modified from the QuikChange PCR Mutagenesis Kit (Stratagene). Two site-directed mutations within the NGD site of NDR1, located at amino acids 178-180, were constructed. A template plasmid (i.e., pTOPO-NDR1) containing the open reading frame of *NDR1* flanked by *Sal*I (5') and *Sac*I (3') restriction enzyme sites was used in combination with the DNA oligonucleotide primer sets labeled 'RGD' and 'AAA' in Table S2.1. Following 18 cycles (95°C, 1 minute + 55°C, 3 minutes + 68°C, 5 minutes) on a BioRad MyCycler thermal cycler (BioRad Laboratories) using *Pfu* Turbo DNA polymerase (Clontech), the product was then treated with *Dpn*I for 1 hour at 37°C. Five µl of the digested DNA reaction was transformed into *Escherichia coli* DH5α cells and grown on Luria-Bertani media containing 100µg mL^{-1} kanamycin, overnight at 37°C. Mutant NDR1 constructs were cloned into either the native promoter vector pDD*NDR* (Coppinger *et al.*, 2004) or the 35S binary vector pMD-1 with an amino- terminal T7 epitope tag (Day *et al.*, 2005). To make pDD*NDR* native promoter constructs, primers were designed to add a 5' T7 epitope tag and *Sal*I site and a 3' *Spe*I site (Table S2.2). Amplicons from the site-directed mutant constructs were ligated into pGEM T-EASY vector (Promega) and subsequently digested with *Sal*I and *Spe*I and ligated into pDD*NDR* (Coppinger *et al.*, 2004).

DNA plasmids were transformed and maintained in *E. coli* DH5α cells. The fidelity of all DNA constructs was confirmed by DNA sequencing (ABI 3730 Genetic Analyzer, Applied Biosystems). *Agrobacterium tumefaciens* strain GV3101 (pMP90) (Lazo *et al.*, 1991) and strain C58C1 (Tai *et al.*, 1999; for *N. benthamiana* expression) were transformed with WT and *NDR1* mutant constructs by electroporation.

All native, WT NDR1 T7- and HA-fusion constructs were previously demonstrated to be functional and fully complement the *ndr1-1* mutation (Coppinger et al., 2004; Day et al., 2006).

Cloning of NDR1 mutant constructs.

Two site-directed mutations within the NGD site of NDR1, located at amino acids 178-180, were constructed using a quick change PCR approach. A template plasmid (i.e., pTOPO-NDR1) containing the open reading frame of *NDR1* flanked by *Sal*I (5') and *Sac*I (3') restriction enzyme sites was used in combination with the DNA oligonucleotide primer sets labeled 'RGD' and 'AAA' in Table S2.1. Following 18 cycles (95°C, 1 minute + 55°C, 3 minutes + 68°C, 5 minutes) on a BioRad MyCycler thermal cycler (BioRad Laboratories) using *Pfu* Turbo DNA polymerase (Clontech), the product was then treated with *Dpn*I for 1 hour at 37°C. Five µl of the digested DNA reaction was transformed into *Escherichia coli* DH5α cells and grown on Luria-Bertani media containing 100µg mL^{-1} kanamycin, overnight at 37°C. Resultant site-directed mutant constructs were digested with *Sal*I and *Sac*I restriction enzymes and ligated into the respective sites in the 35S binary vector pMD-1-T7, which incorporates a 5' T7 epitope tag (Day *et al.*, 2005). To make pDD*NDR* native promoter constructs, primers were designed to add a 5'

T7 epitope tag and *Sal*I site and a 3' *Spe*I site (Table S2.1). Amplicons from the site-directed mutant constructs were ligated into PGEM T-EASY vector (Invitrogen) and subsequently digested with *Sal*I and *Spe*I and ligated into pDD*NDR* (Coppinger *et al.*, 2004). DNA plasmids were transformed and maintained in *E. coli* DH5α cells. *Agrobacterium tumefaciens* strain GV3101 (pMP90) (Lazo *et al.*, 1991) and strain C58C1 (Tai *et al.*, 1999; for *N. benthamiana* expression) were transformed with WT and *NDR1* mutant constructs by electroporation.

All native, WT NDR1 T7- and HA-fusion constructs were previously demonstrated to be functional and fully complement the *ndr1-1* mutation (Coppinger et al., 2004; Day et al., 2006).

Arabidopsis thaliana Transformation

Flowering Arabidopsis plants were transformed and selected for homozygosity, as described by Clough and Bent (1998), on MS media containing 1% Bacto agar and 25µg mL^{-1} kanamycin.

Pathogen Inoculation and Growth Assays

Pseudomonas syringae pv. *tomato* DC3000 strains containing pVSP61 (empty vector), or pVSP61-containing AvrRpt2, AvrB or AvrPphB were described previously (Kunkel *et al.*, 1993; Simonich and Innes, 1995). To assay for bacterial growth, four-week-old plants were dip-inoculated in bacterial suspensions of 3×10^7 cfu mL^{-1}. Leaves were pre-selected and marked to ensure analyses were performed on developmentally similar leaves. At 0 and 4 days post-inoculation, 3 leaf discs of 0.7 cm diameter were collected from a single plant into a micro-centrifuge tube containing 1 mM $MgCl_2$ + 0.1% Triton X-100. Bacterial growth assays were

performed as described in Tornero and Dangl (2001) with the modification of plating 5 µl instead of 2 µl, as described in Tian *et al.* (2009). Results were analyzed for significance using SAS (Version 9.2; SAS Software) using an ANOVA model modified from Tsuda *et al.*, 2008 (Formula S2.1). Log_{10} transformed bacterial titer counts were compared using Tukey's test.

Electrolyte Leakage

Electrolyte leakage was measured in 4 to 5-week-old plants using a protocol modified from Gilmour *et al*., (1988). Leaves were pre-selected and marked based on similarity in size before plants were dip inoculated at 3×10^7 cfu mL^{-1}. After inoculation, plants were covered with a clear plastic dome for 30 minutes before the 0 hour time point leaves were removed. The remaining pots were left covered for another 2.5 hours. A single leaf was removed from a plant and a disc (0.7 cm diameter) was harvested using a number 3 size cork borer. Excised leaf discs were floated in a bath of sterile dH_2O and quickly swirled before being placed in a tube containing 3 mL of sterile dH_2O. Four plants were used for each replicate. Tubes containing leaf disks were shaken on an orbital rocker at 35 rpm for 3 hours. After 3 hours, leaf disks were removed and the solution was assayed for conductance using a conductance meter (Traceable 23226-505; VWR Scientific). Leaf disks were frozen at -80°C for 1 hour. After the freeze cycle, the leaf punch was returned to the original sample tube and rocked for an additional 3 hours at room temperature. After 3 hours, the leaf punch was removed and the conductance was measured, recorded as total leakage. Electrolyte leakage was recorded and calculated as percent leakage of total (i.e., first reading/second reading) adjusted to percent maximal.

RNA Isolation and qRT-PCR

Total RNA was extracted from leaves using the RNeasy Plant Mini Kit (Qiagen). First strand cDNA was synthesized from 1 µg total RNA using SuperScript II reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed on a Mastercycler ep Realplex real-time PCR (Eppendorf) using HotStart-IT SYBR Green qPCR Master Mix (2x) (USB). Cycle time for all replicates was 95°C 2 min followed by 40 cycles 95°C 15s, 60°C 15s, 72°C 45s. Data was analyzed by two-way ANOVA using Prism 4 (GraphPad Software) with outliers removed by Grubb's test (α= 0.05) utilizing the QuickCalcs online outlier calculator (GraphPad Software; http://www.graphpad.com/quickcalcs/Grubbs1.cfm). Aquaporin homolog primers used are described in Alexandersson *et al.* (2009). All primer sets utilized are listed in Table S2.2.

Hypersensitive Response Assay

The hypersensitive response was assayed as described in Century *et al.,* (1995) slightly modified, by hand infiltration of Arabidopsis leaves with *Pst* DC3000 using a needleless syringe at a concentration of 1x 10^7 cfu/cm^2, following a 6 or 12 hour pretreatment by hand infiltration of 5 mM 'VAAAG', 'VNGDG' or 'VRGDG' peptide solutions in 1 mM $MgCl_2$ buffer or a mock control of only buffer. The leaves were evaluated for tissue collapse 20 h after infiltration of the bacteria.

Plasmolysis

Plasmolysis experiments were performed based on the methods of Gouget *et al.* (2006) using 8-day-old etiolated hypocotyls with the cotyledons and roots removed. Hypocotyl sections were immersed in 50 mM Tris (pH 8.0) for 1 hour at room temperature, rinsed in sterile distilled water, and then stained with 0.05% neutral red for 5-30 minutes. Sections were rinsed in sterile distilled water and mounted on a coverslip in 15 µL sterile water. A second coverslip was placed on top of the section, offset to the first coverslip. To plasmolyze the cells, 15 µL of 1.0 M $CaCl_2$ was placed on the sample where the second coverslip overlapped the first, allowing the solution to cover the sample via capillary action. Stained, plasmolyzed hypocotyls were observed on an Olympus IX-71 inverted microscope, and images acquired with an Olympus DP70 camera. Images were processed and adjusted for contrast using Canvas X (ACD Systems). Approximately 100 hypocotyls of each genotype were observed in total from > 5 biological replicates. For peptide addition experiments, the same method as described above was followed, with the exception that the hypocotyls were mounted in 15 µL of 5 mM peptide in dH_2O. Peptides (VRGDG, VAAAG and VNGDG) were synthesized by EZ-Biolabs at a purity of >99%.

Tagged Protein Constructs and Co-Immunoprecipitation

Co-immunoprecipitation experiments were performed as described in Day et al (2006), with slight modifications. In brief, *Agrobacterium tumefaciens* strains expressing the epitope tagged (e.g., T7 or HA) NDR1, RIN4 and NDR1 mutant constructs fused to a 35S promoter were infiltrated into 5-week-old leaves on *N. benthamiana* at a final, individual, concentration of 4 x

10^8 cells/mL. Leaves were incubated at room temperature for 40 hours, after which time, 16-$1cm^2$ leaf disks were harvested into liquid nitrogen, and held at -80°C until processing. Samples were processed according to Day *et al.* (2005).

T7 epitope monoclonal and T7-HRP conjugated antibodies were purchased from Novagen. HA epitope monoclonal antibody was purchased from Covance. HA-HRP conjugated antibody and protease inhibitors were purchased from Roche.

MAPK Western blotting

To detect MAPK 3/6 activity, 40 µg total protein was loaded onto a 12% SDS-PAGE gel and transferred onto nitrocellulose membrane, followed by incubation for 1 hour with an antibody specific for anti-pTEpY (catalog number 9101S, Cell Signaling Technology). An anti-rabbit horseradish peroxidase secondary antibody was used for detection on film.

Acknowledgements

We would like to thank Katie Porter for technical support on qRT-PCR analyses as well as Wenzhao Yang for assistance with the statistical analysis of bacterial growth assays.

Figure 2.1.

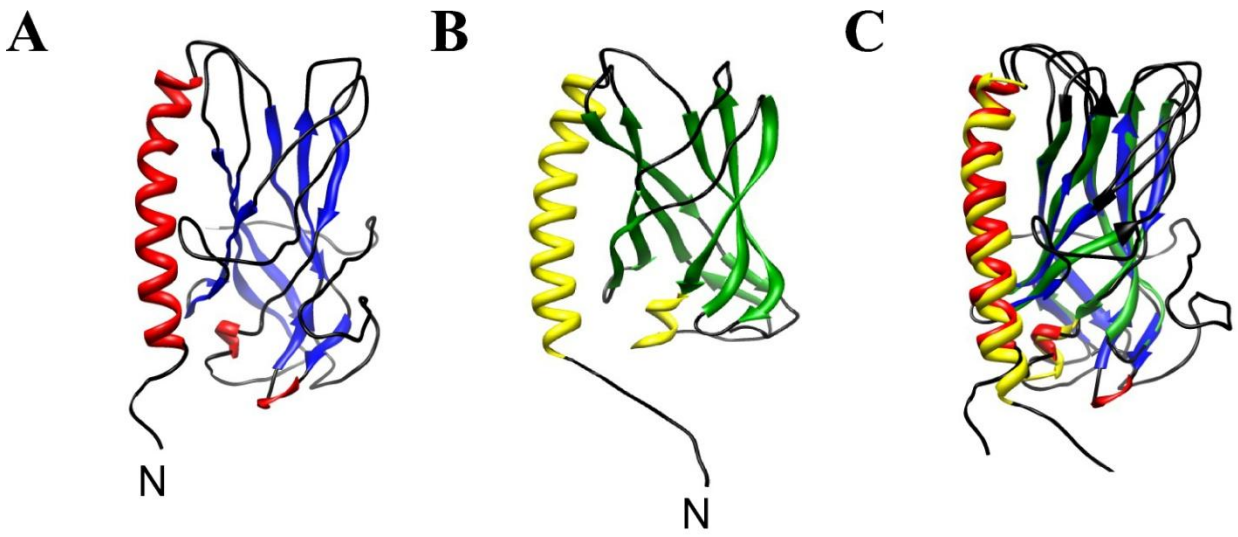


Figure 2.1. Homology modeling of NDR1 with the integrin-like late embryogenesis protein, LEA14.

(A) Predicted structure of NDR1. (B) The solved structure of LEA14 (pdb_1yyc; Singh *et al.*, 2005). (C) NDR1 predicted structure threaded onto the solved structure of LEA14 highlighting the predicted structural homology. 'N' denotes amino-terminal. PHYRE anaylsis returned an estimated structural homology precision of 95%, with an E-value of 0.008. Additional predicted models are shown in Figure S2.1.

Figure 2.2.

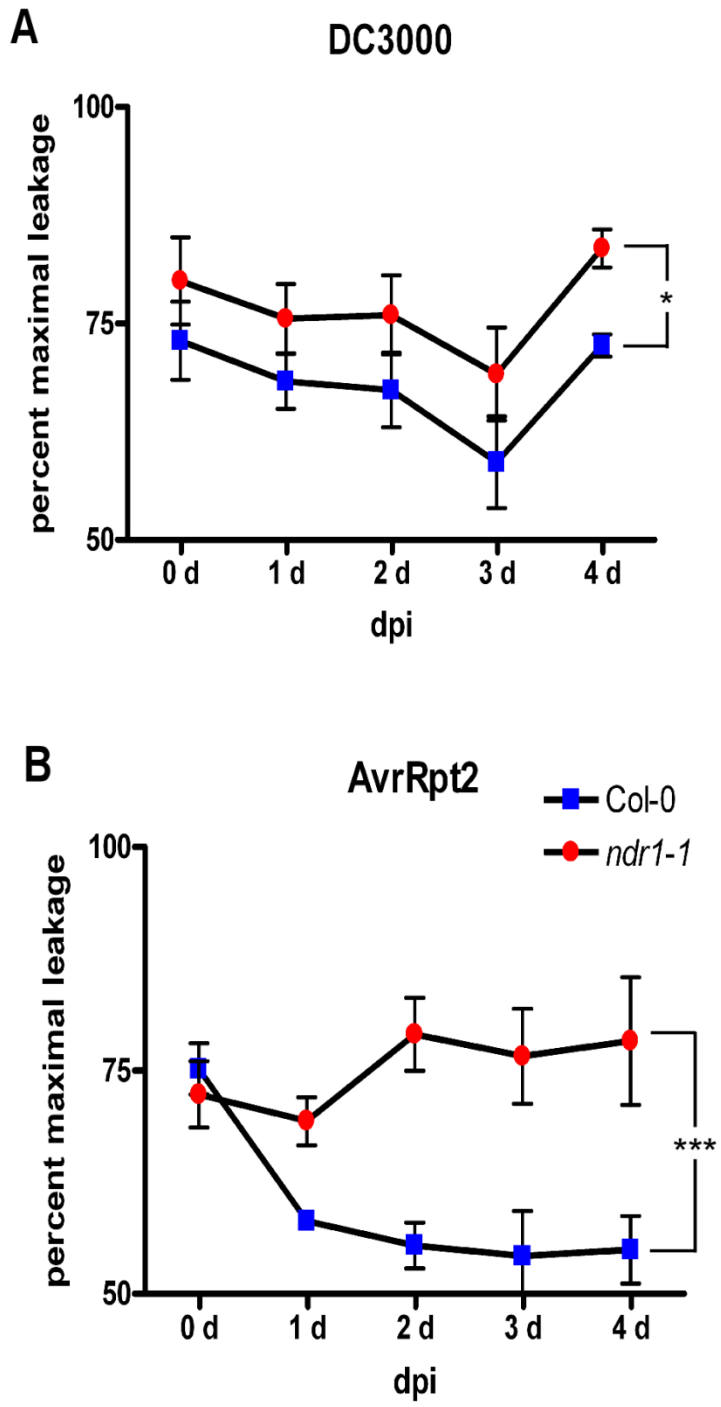

A
DC3000
percent maximal leakage
100
75
50
0 d
1 d
2 d
3 d
4 d
dpi
*
B
AvrRpt2
Col-0
ndr1-1
percent maximal leakage
100
75
50
0 d
1 d
2 d
3 d
4 d
dpi
***

Figure 2.2. (cont'd)

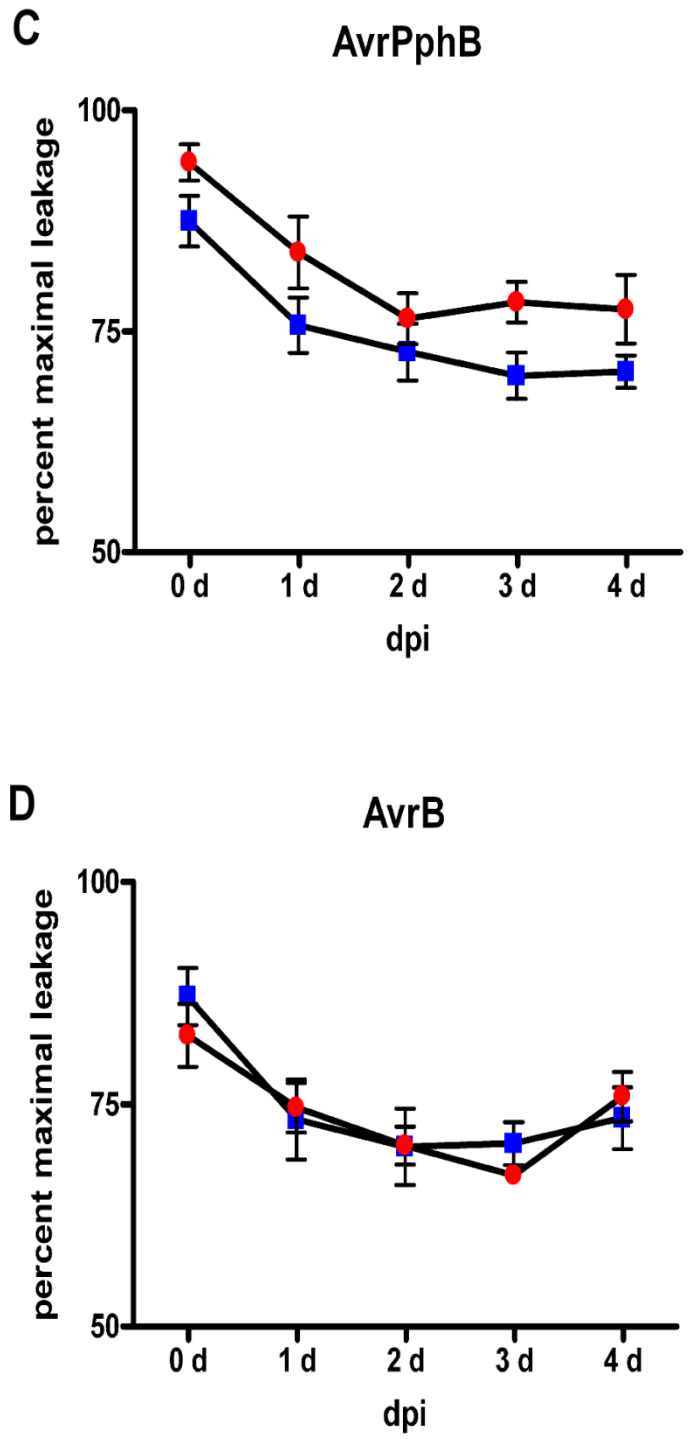


Figure 2.2 (cont'd)

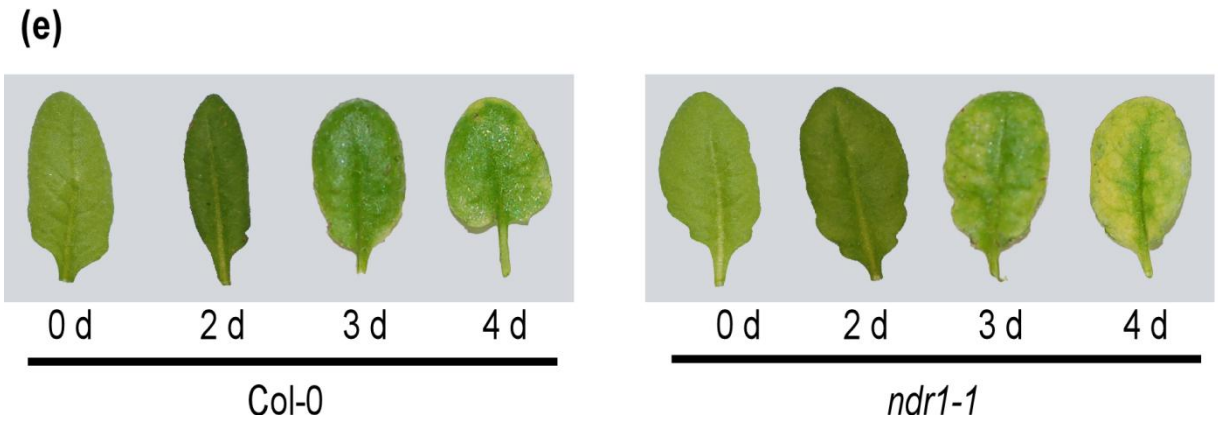


Figure 2.2. Enhanced nutrient leakage in the *ndr1-1* mutant following inoculation with *Pst* DC3000.

Levels of electrolyte leakage from Col-0 and *ndr1-1* plants in response to DC3000 inoculation are displayed as percent maximal leakage. Treatments include *Pst* DC3000 expressing (A) vector control, (B) AvrRpt2, (C) AvrB, (D) AvrPphB. The dramatic increase in leakage observed in *ndr1-1* as compared to Col-0 when inoculated with *Pst* DC3000 correlates with the onset of disease symptoms. (E) Col-0 and *ndr1-1* leaves at 0, 2, 3, and 4 days post inoculation (dpi) with *Pst* DC3000 expressing AvrRpt2. Error bars display standard deviation from 4 technical replicates from 2-3 biological replicates. Significance was determined using two-way ANOVA where * represents a statistically significant difference between Col-0 and *ndr1-1*. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure 2.3.

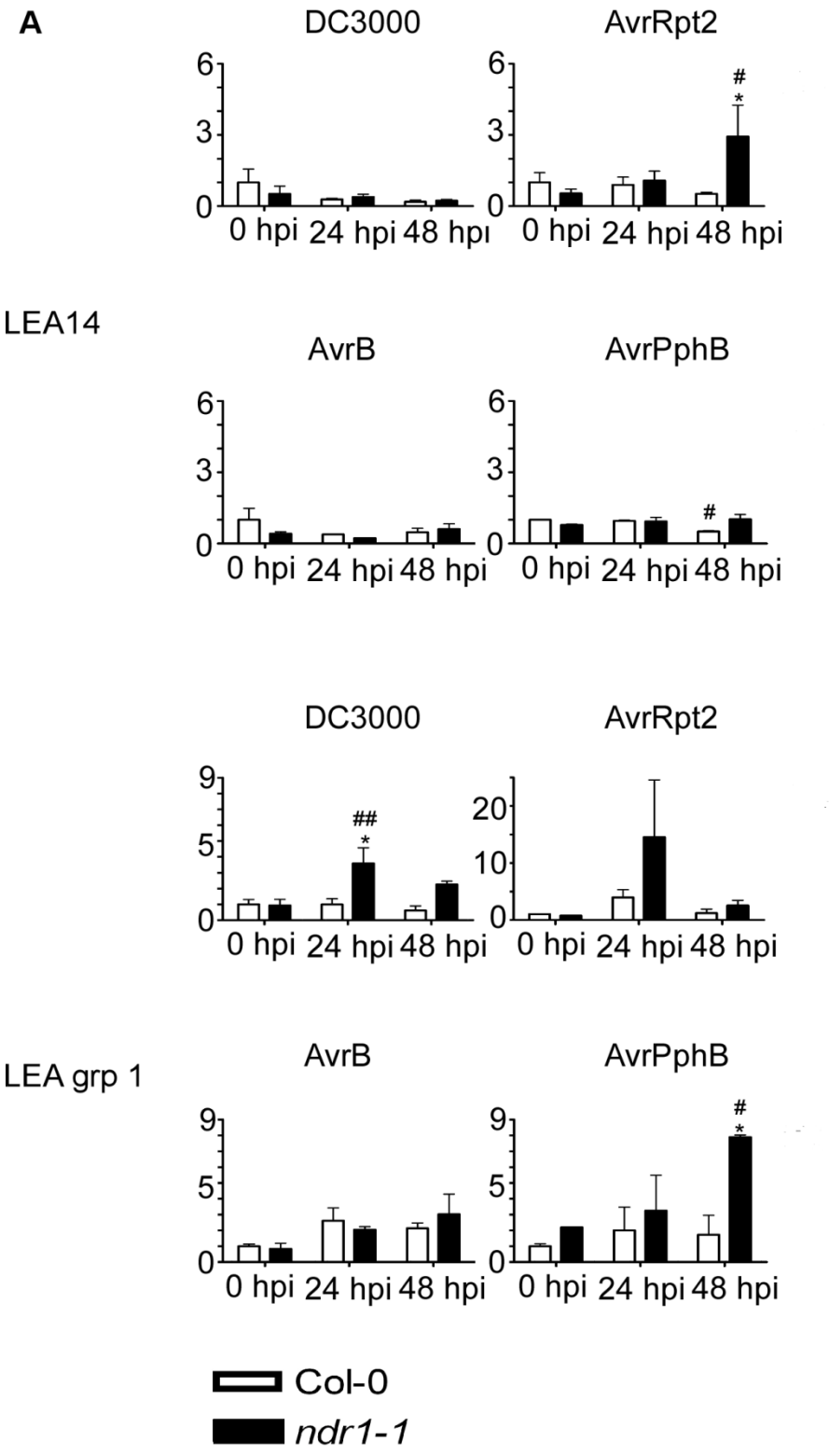

A
DC3000
AvrRpt2
LEA14
AvrB
AvrPphB
DC3000
AvrRpt2
LEA grp 1
AvrB
AvrPphB
0 hpi
24 hpi
48 hpi
Col-0
ndr1-1

Figure 2.3 (cont'd)

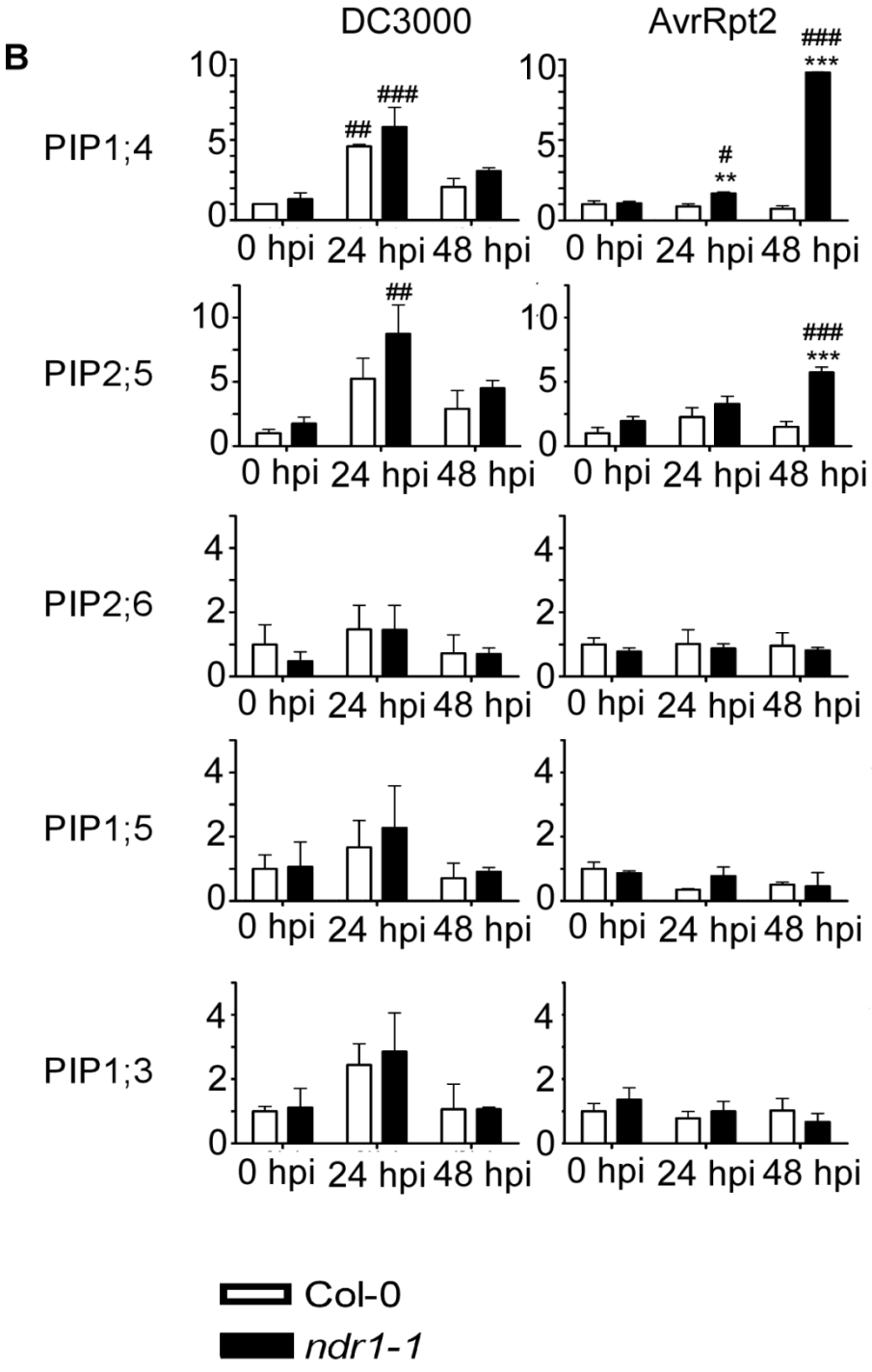


Figure 2.3. Relative mRNA expression of biotic and abiotic responsive genes in *ndr1-1* following *Pst* DC3000 inoculation.

(A) Altered levels of expression in WT Col-0 and *ndr1-1* mutant plants of *LEA* family genes in response to *Pst* DC3000 or *Pst* DC3000 expressing AvrRpt2, AvrB or AvrPphB. (B) Expression levels of aquaporin homologs known to be drought responsive in Arabidopsis when inoculated with *Pst* DC3000 or *Pst* expressing AvrRpt2. Error bars display standard deviation from 1-2 technical replicates from 2 biological replicates. Samples taken at 0, 24 and 48 hours post inoculation (hpi). Expression displayed as fold Col-0 0 h average. Significance was determined using two-way ANOVA where * represents a statistically significant difference between Col-0 and *ndr1-1* and # represents statistically significant change over time. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure 2.4.

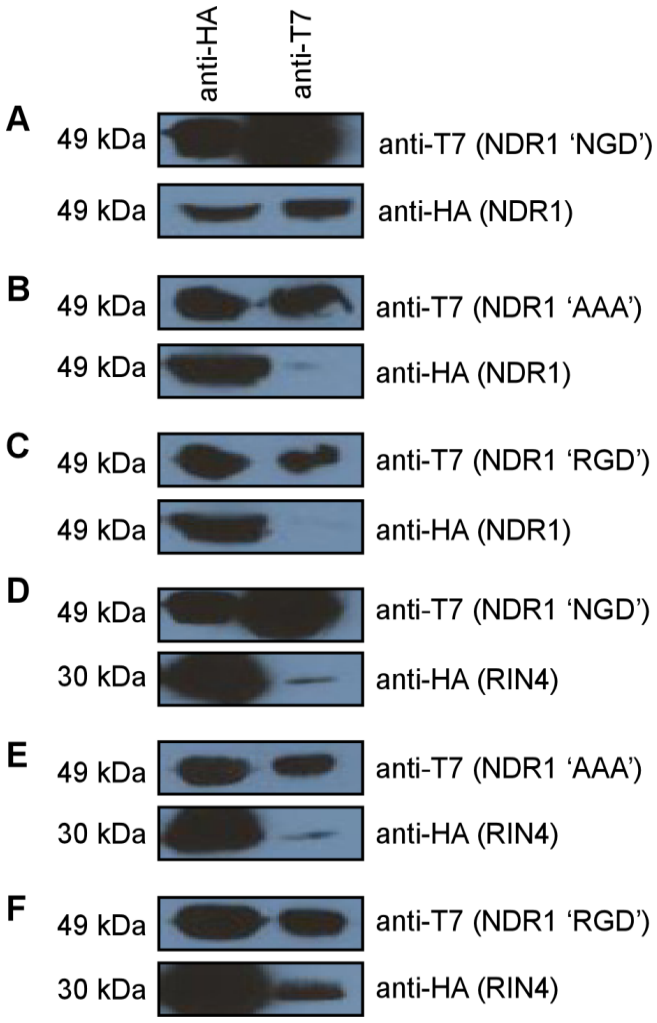

anti-HA
anti-T7
A
49 kDa
anti-T7 (NDR1 'NGD')
49 kDa
anti-HA (NDR1)
B
49 kDa
anti-T7 (NDR1 'AAA')
49 kDa
anti-HA (NDR1)
C
49 kDa
anti-T7 (NDR1 'RGD')
49 kDa
anti-HA (NDR1)
D
49 kDa
anti-T7 (NDR1 'NGD')
30 kDa
anti-HA (RIN4)
E
49 kDa
anti-T7 (NDR1 'AAA')
30 kDa
anti-HA (RIN4)
F
49 kDa
anti-T7 (NDR1 'RGD')
30 kDa
anti-HA (RIN4)

Figure 2.4. NDR1 associates with itself and RIN4 *in planta*, and this association is unaffected by mutations in the NGD site.

Agrobacterium expressing HA:NDR1 or HA:RIN4, along with *Agrobacterium* expressing T7:NDR1 with substitutions in the NGD motif (wild-type 'NGD', 'RGD', or 'AAA') were infiltrated into *N. benthamiana*. Tagged proteins were immunoprecipitated from samples taken 48 h after infiltration using anti-HA (NDR1 or RIN4; right lane) or anti-T7 (NDR1; left lane) antibodies. Proteins were detected by blotting with anti-T7 (upper blot) or anti-HA HRP (lower blot) conjugated antibody. (A) T7:NDR1 'NGD'-HA:NDR1, (B) T7:NDR1 'AAA' -HA:NDR1, (C) T7:NDR1 'RGD' -HA:NDR1, (D) T7:NDR1 'NGD'-HARIN4, (E) T7:NDR1 'AAA' -HA:RIN4, (F) T7:NDR1 'RGD' -HA:RIN4. Western blots in Figure S6 show relative detection limits.

Figure 2.5.

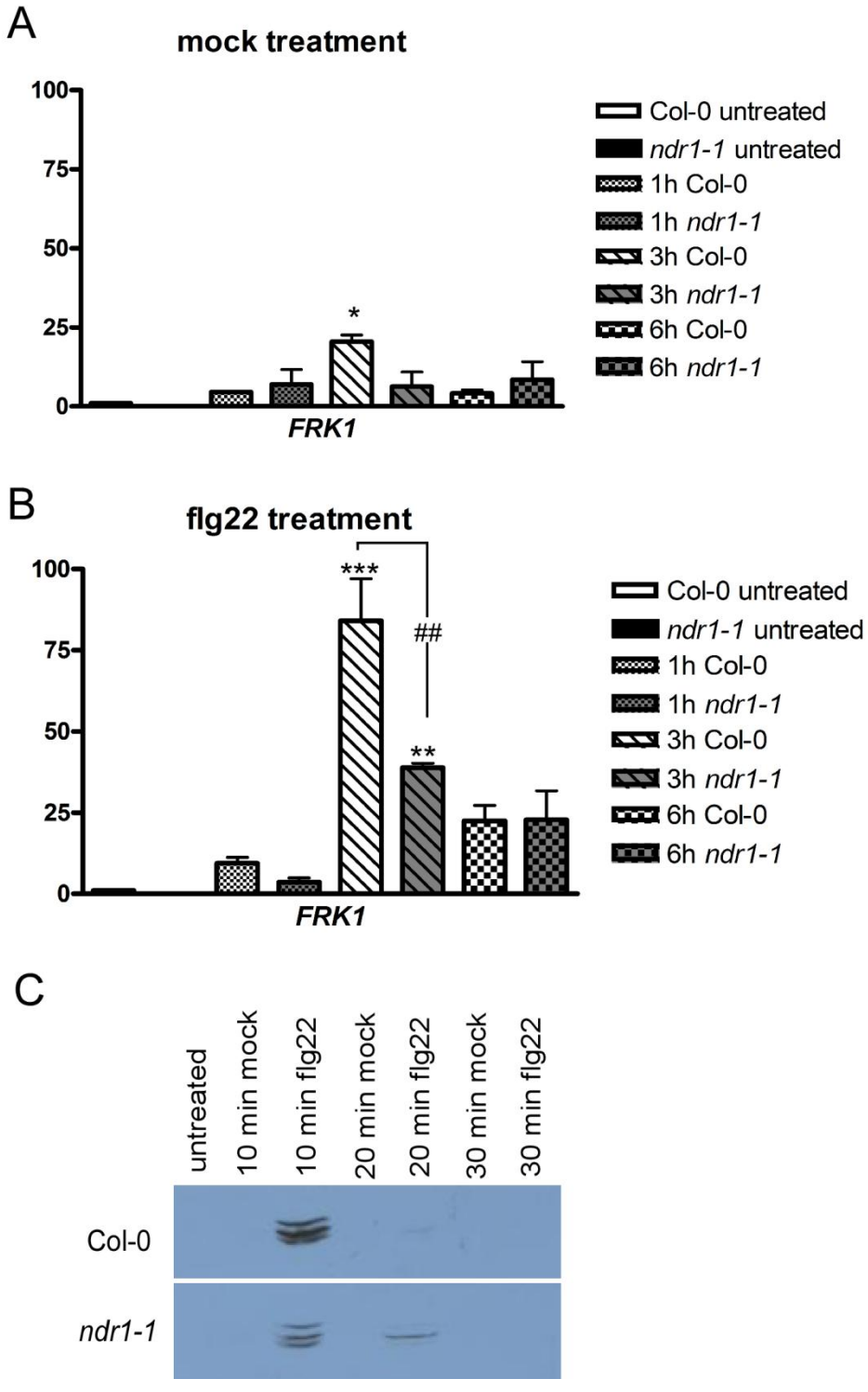

A
mock treatment
100
75
50
25
0
*
FRK1
Col-0 untreated
ndr1-1 untreated
1h Col-0
1h ndr1-1
3h Col-0
3h ndr1-1
6h Col-0
6h ndr1-1
B
flg22 treatment
100
75
50
25
0
***
##
**
FRK1
Col-0 untreated
ndr1-1 untreated
1h Col-0
1h ndr1-1
3h Col-0
3h ndr1-1
6h Col-0
6h ndr1-1
C
untreated
10 min mock
10 min flg22
20 min mock
20 min flg22
30 min mock
30 min flg22
Col-0
ndr1-1

Figure 2.5. *ndr1-1* exhibits altered PAMP responses.

(A-B) The expression levels of FRK1 mRNA was analyzed by qRTPCR in Col-0 and *ndr1-1* mutant plants in response to flg22 or mock treatment. Error bars display standard deviation from 2 technical replicates from 1 biological replicate. Expression displayed as fold Col-0 untreated average. Statistical significance was determined using one-way ANOVA followed by Tukey's test where * represents statistical difference as compared to Col-0 untreated and # represents a statistically significant difference between Col-0 and *ndr1-1*. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. (C) Western blot analysis of MAPK3/6 in Col-0 and *ndr1-1* mutant plants in response to flg22 or mock treatment.

Figure 2.6.

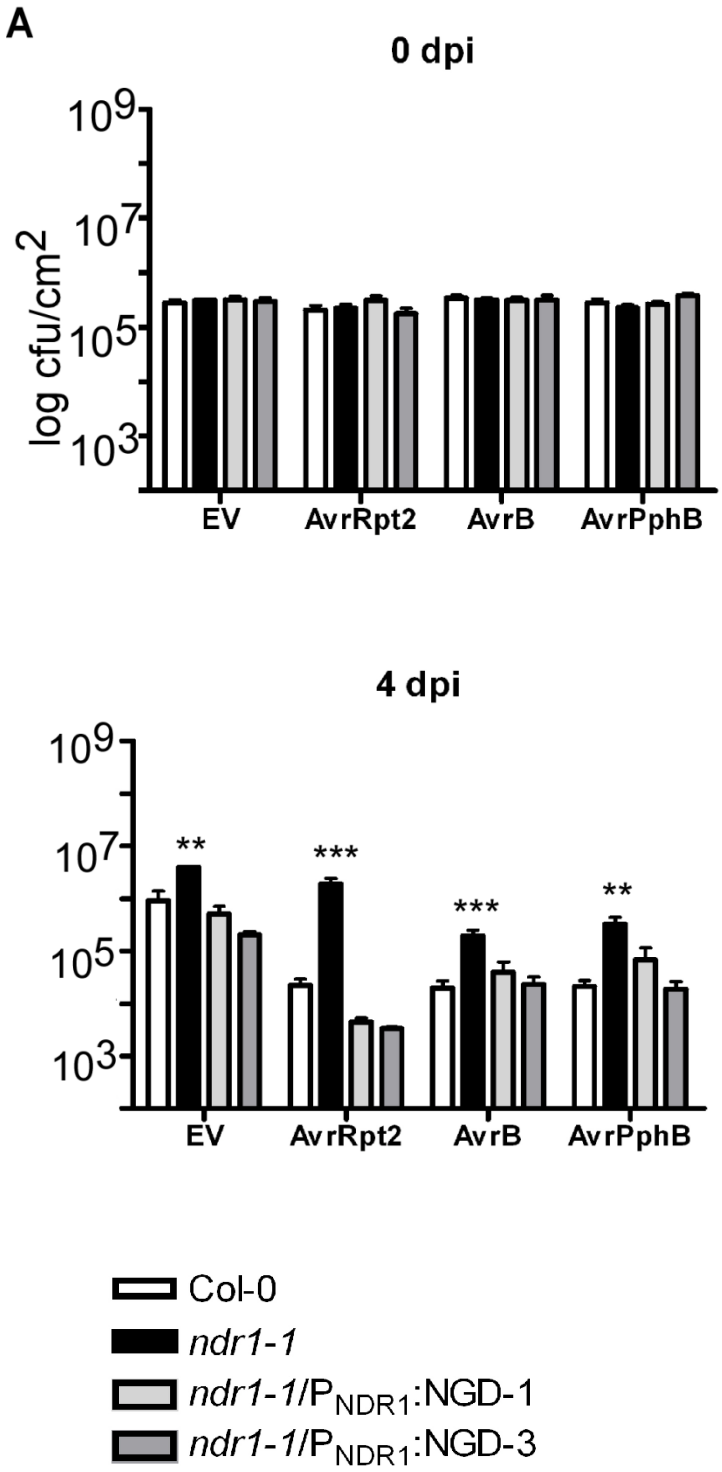

A
0 dpi
log cfu/cm2
10^9
10^7
10^5
10^3
EV
AvrRpt2
AvrB
AvrPphB
4 dpi
**
***
***
**
Col-0
ndr1-1
ndr1-1/P_NDR1:NGD-1
ndr1-1/P_NDR1:NGD-3

Figure 2.6. (cont'd)

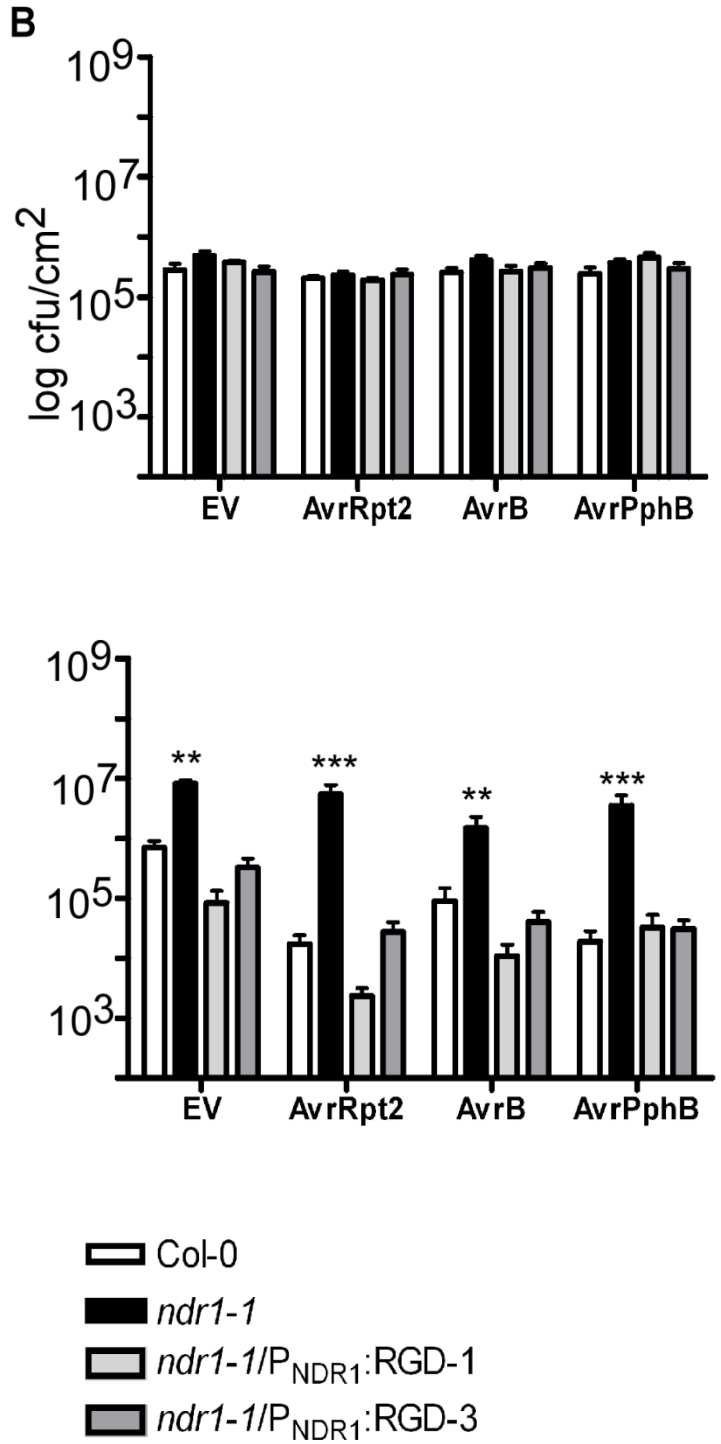


Figure 2.6. (cont'd)

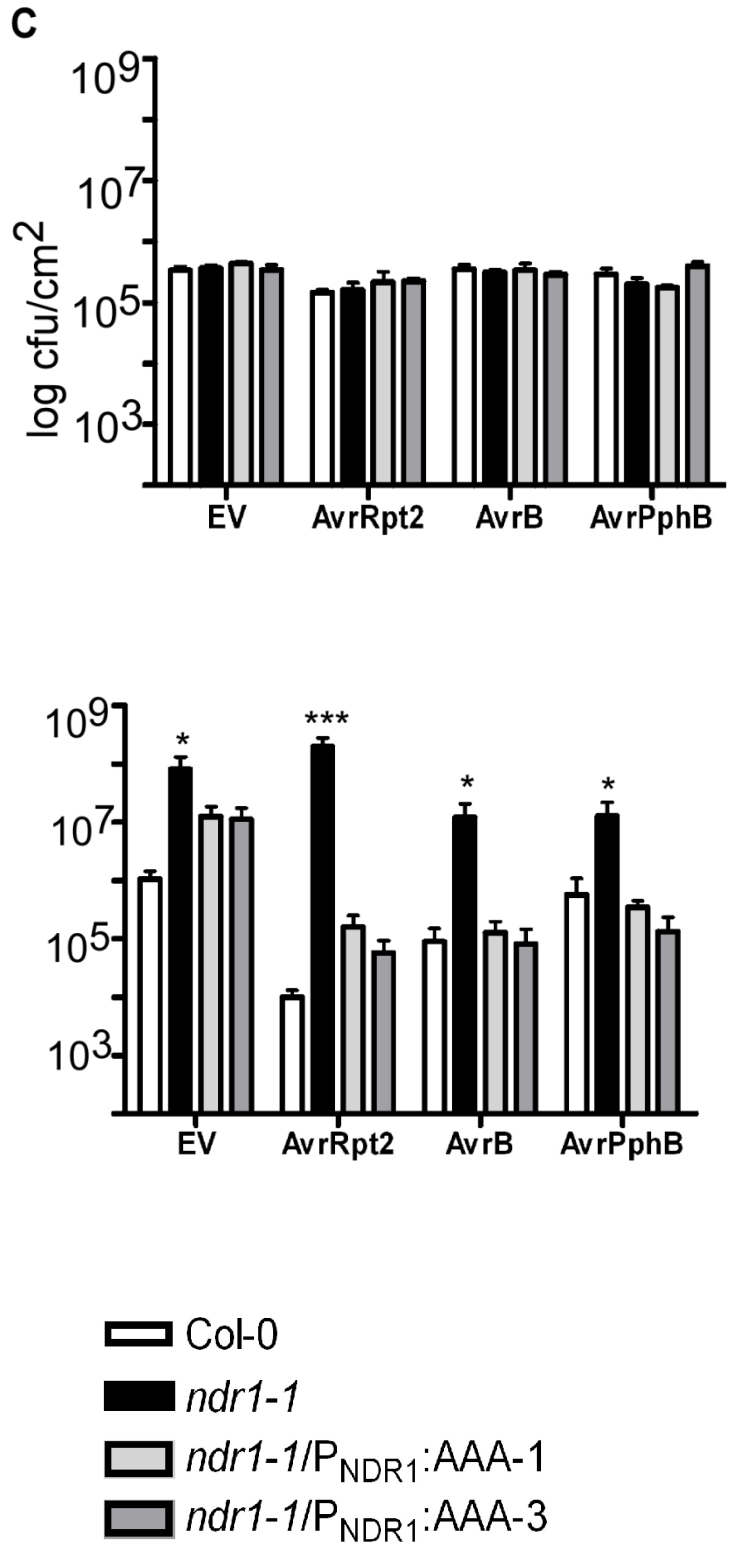


Figure 2.6. Growth of *Pst* DC3000 in Arabidopsis is altered by mutations to the NGD site of NDR1.

Bacterial growth assay of *Pst* DC3000 (EV) and *Pst* DC3000 expressing AvrRpt2, AvrB or AvrPphB dip inoculated on *ndr1-1* lines complemented with native *NDR1* promoter with *NDR1* NGD (*ndr1-1*/P_{NDR1}:NGD; A), *NDR1* RGD (*ndr1-1*/P_{NDR1}:RGD; B), or *NDR1* AAA (*ndr1-1*/P_{NDR1}:AAA; C). Growth was assayed at 0 days and 4 days post inoculation (dpi). Growth expressed as log cfu/cm^2. Error bars display standard deviation from 3 technical replicates from 2 biological replicates. Statistical significance was determined using unbalanced two-way ANOVA (model included Figure S7) where * represents a statistically significant difference between Col-0 and *ndr1-1*. * $p<0.1$; ** $p<0.01$; *** $p<0.0001$ (p values Table S2.3).

Figure 2.7.

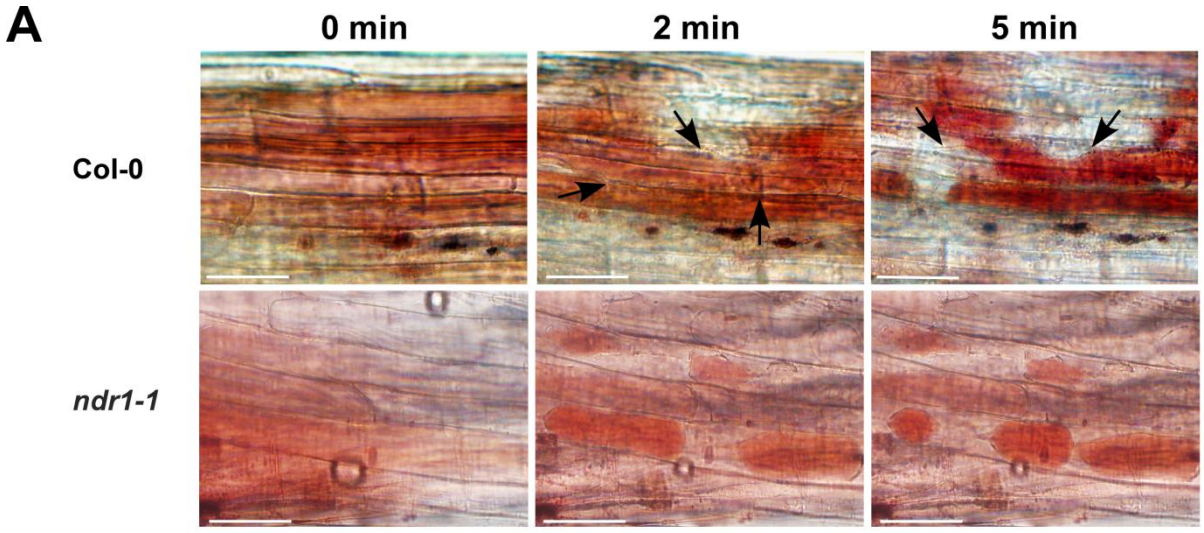


Figure 2.7. (cont'd)

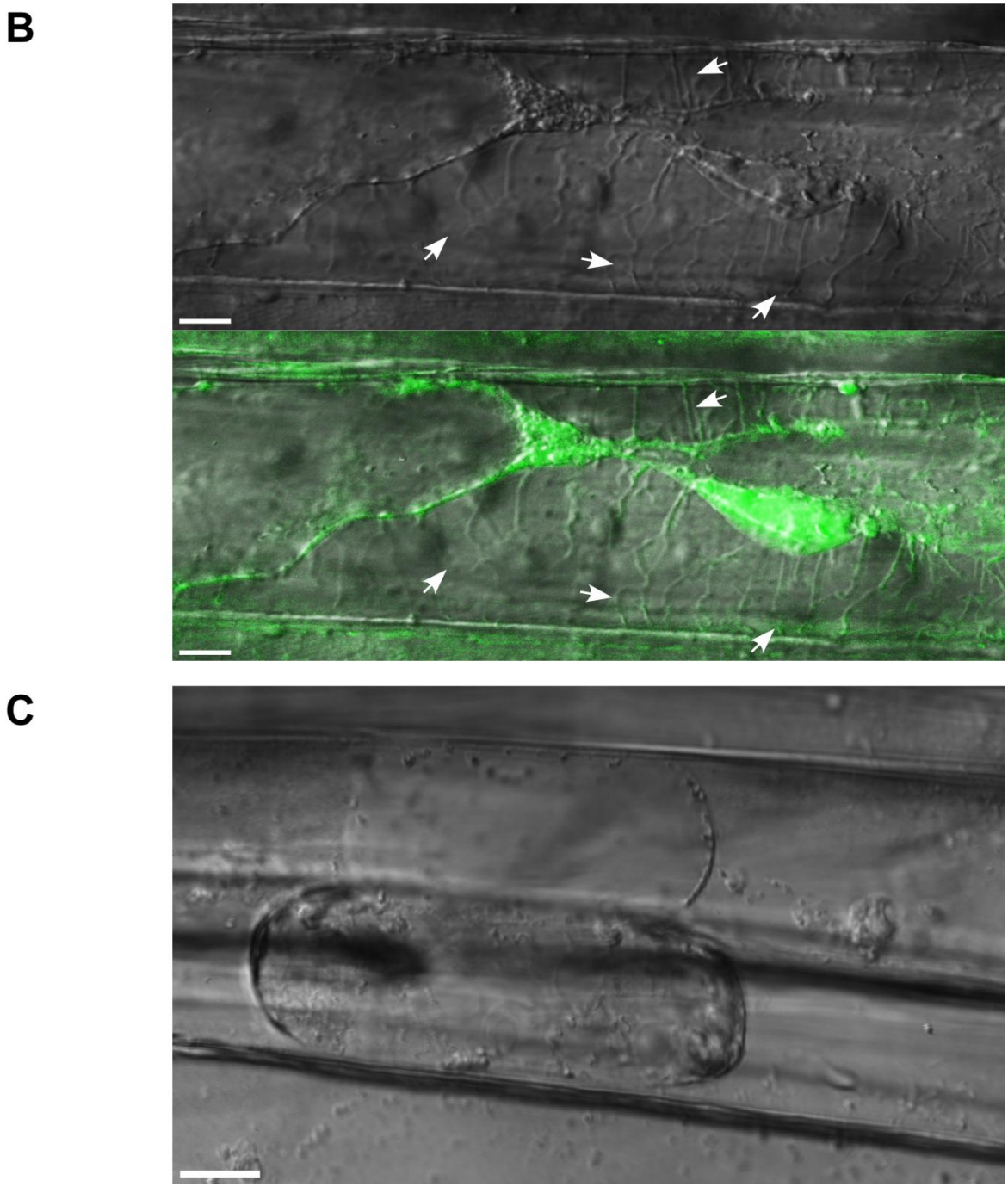


Figure 2.7. *ndr1-1* mutant plants exhibit altered plasmolysis and plasma membrane-cell wall focal adhesions.

(A) Time course (left to right: 0 (before), 2, and 5, min after treatment) of 8-day-old Col-0 or *ndr1-1* $CaCl_2$-plasmolyzed hypocotyls. In contrast to plasmolyzed WT Col-0 hypocotyl cells, which reveal plasma membrane-cell wall attachments (arrows), the plasma membrane of *ndr1-1* cells quickly loses adhesion following induction of plasmolysis, yielding spherical protoplasts within the cell wall. Scale bars = 25 µm. (B) Complemented *ndr1-1* mutant line constitutively expressing a GFP:NDR1 protein exhibits wild-type plasma membrane-cell wall adhesions (arrows). DIC (top panel) and confocal-DIC overlay (bottom panel) images of $CaCl_2$-induced plasmolysis. Arrows indicate Hechtian strand formation, illustrating significant physical linkages with the plasma membrane and cell wall. Scale bars = 30 µm. (C) Hechtian strands are absent, or significantly reduced, in the *ndr1-1* mutant plant. Scale bar = 10 µm.

Figure 2.8.

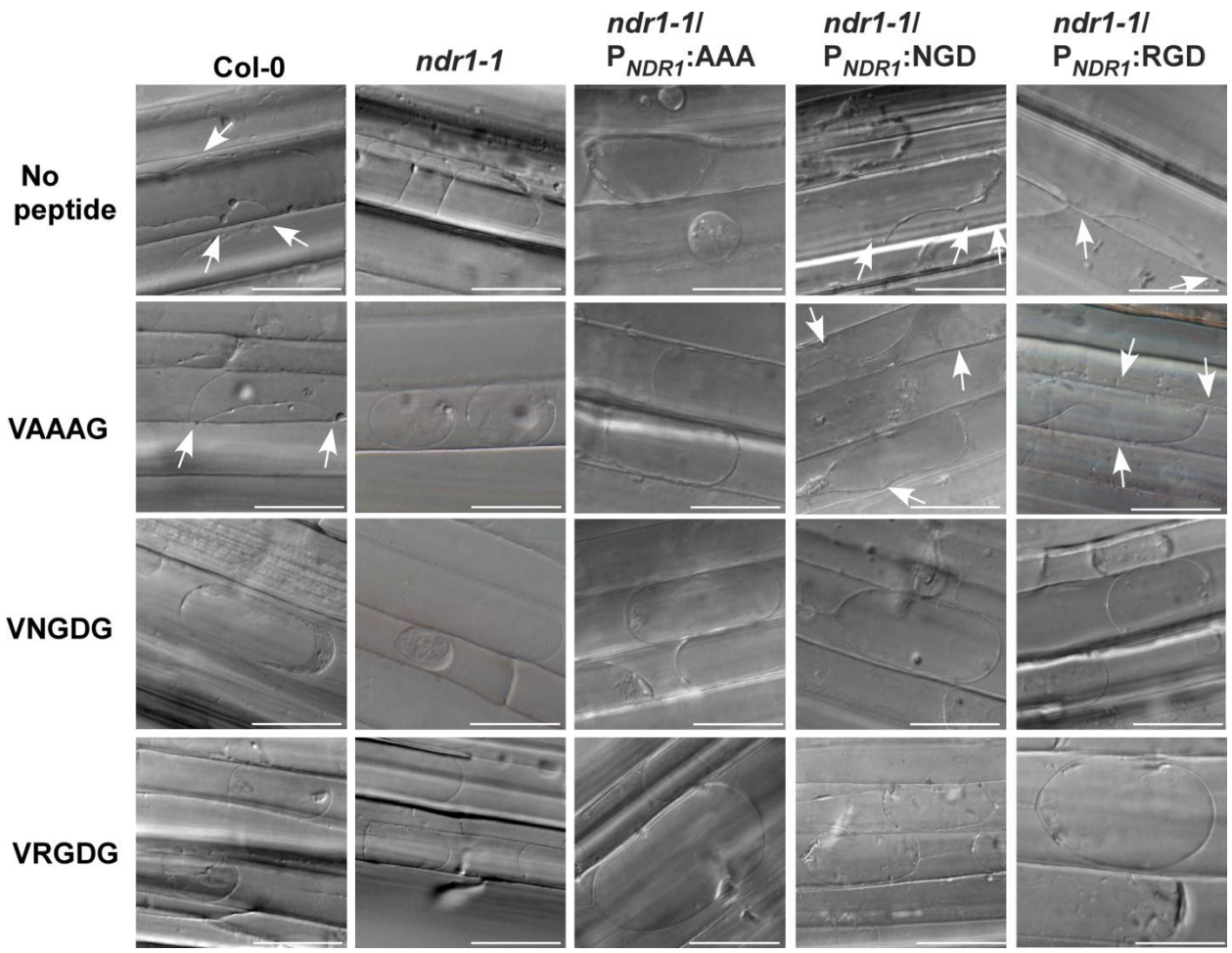

Col-0
ndr1-1
ndr1-1/ P_NDR1:AAA
ndr1-1/ P_NDR1:NGD
ndr1-1/ P_NDR1:RGD
No peptide
VAAAG
VNGDG
VRGDG

Figure 2.8. Application of exogenous peptides can alter the plasma membrane-cell wall adhesion in WT Col-0.

Application of exogenous 'VNGDG' or 'VRGDG' peptide results in reduced plasma membrane-cell wall adhesions in Col-0, *ndr1-1*/P_{NDR1}:NGD, and *ndr1-1*/P_{NDR1}:RGD hypocotyl cells. Both *ndr1-1* and *ndr1-1*/P_{NDR1}:AAA hypocotyls are unaltered in adhesion phenotypes upon addition of purified 'VRGDG', 'VNGDG', and 'VAAAG' peptides. Arrows indicate cell wall adhesion points. DIC microscopy images were collected 5 minutes after induction of plasmolysis. Scale bars = 25 μm.

LITERATURE CITED

Literature Cited

Aarts N, Metz M, Holub E, Staskawicz BJ, Daniels MJ and Parker JE (1998) Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in *Arabidopsis.* Proc Natl Acad Sci USA **95**: 10306-10311

Alexandersson E, Fraysse L, Sjovall-Larsen S, Gustavsson S, Fellert M, Karlsson M, Johanson U and Kjellbom P (2005) Whole gene family expression and drought stress regulation of aquaporins. Plant Mol Biol **59**: 469-484

Alexandersson E, Danielson JAH, Rade J, Moparthi VK, Fontes M, Kjellbom P and Johanson U (2010) Transcriptional regulation of aquaporins in accessions of Arabidopsis in response to drought stress. Plant J **61**: 650-660

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu W, Gomez-Gomez L, Boller T, Ausubel FM and Sheen J (2002) MAP kinase signaling cascade in Arabidopsis innate immunity. Nature **415**: 977-983

Axtell MJ and Staskawicz BJ (2003) Initiation of *RPS2*-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. Cell **112**: 369-377

Axtell MJ, Chisholm ST, Dahlbeck D and Staskawicz BJ (2003) Genetic and molecular evidence that the *Pseudomonas syringae* type III effector protein AvrRpt2 is a cysteine protease. Mol Microbiol **49**: 1537-1546

Azevedo C, Sadanandom A, Kitagawa K, Freialdenhoven A, Shirasu K and Schulze-Lefert P (2002) The RAR1 interactor SGT1, an essential component of *R* gene-triggered disease resistance. Science **295**: 2073–2076

Baker CJ, O'Neill NR, Keppler LD and Orlandi EW (1991) Early responses during plant-bacteria interactions in tobacco cell suspensions. Phytopathology **81**: 1504-1507

Battaglia M, Olvera-Carrillo Y, Garciarrubio A, Campos F and Covarrubias AA (2008) The enigmatic LEA proteins and other hydrophilins. Plant Physiol **148**: 6-24

Beckers GJM, Jaskiewicz M, Liu Y, Underwood WR, He SY, Zhang S and Conrath U (2009) Mitogen-Activated Protein Kinases 3 and 6 are required for Full Priming of Stress Responses in Arabidopsis thaliana. Plant Cell **21**: 944-953

Belkhadir Y, Subramaniam R and Dangl JL (2004) Plant disease resistance protein signaling: NBS-LRR proteins and their partners. Curr Opin Plant Biol **7**: 391-399

Bittner-Eddy PD and Beynon JL (2001) The Arabidopsis downy mildew resistance gene, RPP13-Nd, functions independently of NDR1 and EDS1 and does not require the accumulation of salicylic acid. Mol Plant Microbe Interact **14**: 416-421

Canut H, Carrasco A, Galaud J, Cassan C, Bouyssou H, Vita N, Ferrara P and Pont-Lezica R (1998) High affinity RGD-binding sites at the plasma membrane of *Arabidopsis thaliana* link the cell wall. Plant J **16**: 63-71

Century K, Holub EB and Staskawicz BJ (1995) NDR1, a locus of Arabidopsis thaliana that is required for disease resistance to both a bacterial and fungal pathogen. Proc Natl Acad Sci USA **92**: 6597-6601

Century K, Shapiro AD, Repetti PP, Dahlbeck D, Holub E and Stascawicz BJ (1997) NDR1, a pathogen-induced component required for Arabidopsis disease resistance. Science **278** 1963-1965

Chisholm S T, Coaker G, Day B and Staskawicz BJ (2006) Host-Microbe Interactions: Shaping the Evolution of the Plant Immune Response. Cell **124**: 803-814

Clough SJ and Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J **16**: 735–743

Coppinger P, Repetti PP, Day B, Dahlbeck D, Mehlert A and Staskawicz BJ (2004) Overexpression of the plasma membrane-localized NDR1 protein results in enhanced bacterial disease resistance in *Arabidopsis thaliana*. Plant J **40**: 225-237

Cui H, Wang Y, Xue L, Chu J, Yan C, Fu J, Chen M, Innes RW and Zhou JM (2010) *Pseudomonas syringae* effector protein AvrB perturbs Arabidopsis hormone signaling by activating MAP kinase 4. Cell Host Microbe **7**: 164-175

Day B, Dahlbeck D, Huang J, Chisholm ST, Li D and Staskawicz BJ (2005) Molecular basis for the RIN4 negative regulation of RPS2 disease resistance. Plant Cell **17**: 1292-1305

Day B, Dahlbeck D and Staskawicz BJ (2006) NDR1 interaction with RIN4 mediates the differential activation of multiple disease resistance pathways in Arabidopsis. Plant Cell **18**: 2782-2791

De Muetter J, Robertson L, Parcy F, Mena M, Fenoll C and Gheysen, G (2005) Differential activation of *ABI3* and *LEA* genes upon plant parasitic nematode infection. Mol Plant Pathol **6**: 321-325

Dodds PN, Rafiqi M, Gan PH, Hardham AR, Jones DA and Ellis JG (2009) Effectors of biotrophic fungi and oomycetes: pathogenicity factors and triggers of host resistance. New Phytol **183**: 993-1000

Ellis J and Dodds P (2003) Plant pathology: monitoring a pathogen-targeted host protein. Curr Biol 13: R400-402

Feys BJ, Moisan LJ, Newman MA and Parker JE (2001) Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. EMBO J 20: 5400-5411

Feys BJ, Wiermer M, Bhat RA, Moisan LJ, Medina-Escobar N, Neu C, Cabral A and Parker JE (2005) *Arabidopsis* SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. Plant Cell **17**: 2601-2613

Freeman BC and Beattie GA (2009) Bacterial Growth Restriction During Host Resistance to Pseudomonas syringae is associated with Leaf Water Loss and Localized Cessation of Vascular Acivity in Arabidopsis thaliana. Mol Plant Microbe Interact **22**: 857-867

Gao H, Gong Y and Yuan Y (2007) RGD-Dependent Mechanotransduction of Suspension Cultured *Taxus* Cell in Response to Shear Stress. Biotechnol Prog **23**: 673-679

Gee EP, Ingber DE and Stultz CM (2008) Fibronectin unfolding revisited: modeling cell traction-mediated unfolding of the tenth type-III repeat. PLoS One **3**: e2373. doi:10.1371/journal.pone.0002373

Gilmour SJ, Hajela RK and Thomashow MF (1988) Cold Acclimation in *Arabidopsis thaliana*. Plant Physiol **87**: 745-750

Gouget A, Senchou V, Govers F, Sanson A, Barre A, Rouge P, Pont-Lezica R and Canut H (2006) Lectin Receptor Kinases Participate in Protein-Protein Interactions to Mediate Plasma Membrane-Cell Wall Adhesions in Arabidopsis. Plant Physiol **140**: 81-90

Goyal K, Walton LJ and Tunnacliffe A (2005) LEA proteins prevent protein aggregation due to water stress. Biochem J **388**: 151-157

Grant MR, Godiard L, Straube E, Ashfield T, Lewald J, Sattler A, Innes RW and Dangl JL (1995) Structure of the Arabidopsis RPM1 gene enabling dual specificity disease resistance. Science **269**: 843-846

Hu G, deHart AK, Li Y, Ustach C, Handley V, Navarre R, Hwang CF, Aegerter BJ Williamson VM and Baker B (2005) EDS1 in tomato is required for resistance mediated by TIR-class R genes and the receptor-like R gene Ve. Plant J **42**: 376-391

Hubert DA, Tornero P, Belkhadir Y, Krishna P, Takahashi A, Shirasu K and Dangl JL (2003) Cytosolic HSP90 associates with and modulates the Arabidopsis RPM1 disease resistance protein. EMBO J **22**: 5679-5689

Huveneers S, Truong H and Danen EHJ (2007) Integrins: signaling, disease, and therapy. Int J Radiat Biol **83**: 743-751

Huveneers S and Danen EHJ (2009) Adhesion signaling-crosstalk between integrins, Src and Rho. J Cell Sci **122**: 1059-1069

Hynes RO (2009) The extracellular matrix: not just pretty fibrils. Science **326**: 1216-1219

Innes RW (1998) Genetic dissection of R gene signal transduction pathways. Curr Opin Plant Biol **1**: 299-304

Jiménez-Soto LF, Kutter S, Sewald X, Ertl C, Weiss E, Kapp U, Rohde M, Pirch T, Jung K, Retta SF, Terradot L, Fischer W and Haas R (2009) *Helicobacter pylori* type IV secretion apparatus exploits beta1 integrin in a novel RGD-independent manner. PLoS Pathog **5**(12): e1000684

Jones JD and Dangl JL (2006) The plant immune system. Nature **444**: 323-329

Kelley LA and Sternberg MJ (2009) Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc* **4**: 363-371

Kiba A, Sugimoto M, Toyoda K, Ichinose Y, Yamada T and Shiraishi T (1998) Interaction between cell wall and plasma membrane via RGD motif is implicated in plant defense responses. Plant Cell Physiol **39**: 1245-1249

Knepper C and Day B (2010) From Perception to Activation: The Molecular-Genetic and Biochemical Landscape of Disease Resistance Signaling in Plants: May 14, 2010. In *The Arabidopsis Book*. Rockville, MD: American Society of Plant Biologists. doi: 10.1199/tab.0124, http://www.aspb.org/publications/arabidopsis/.

Kumamoto CA (2008) Molecular mechanisms of mechanosensing and their roles in fungal contact sensing. Nat Rev Microbiol **6**: 667-673

Kunkel BN, Bent AF, Dahlbeck D, Innes RW and Staskawicz BJ (1993) RPS2, an Arabidopsis disease resistance locus specifying recognition of *Pseudomonas syringae* strains expressing the avirulence gene avrRpt2. Plant Cell **5**: 865–875

Lazo GR, Stein PA and Ludwig RA (1991) A DNA transformation-competent Arabidopsis genomic library in Agrobacterium. Bio/Technology **9**: 963-967

Liu J, Elmore JM, Fuglsang AT, Palmgren MG, Staskawicz BJ and Coaker G (2009) RIN4 functions with plasma membrane H+-ATPases to regulate stomatal apertures during pathogen attack. PLoS Biol **7**(6): e1000139. doi:10.1371/journal.pbio.1000139

Mackey D, Holt BF, Wiig A and Dangl JL (2002) RIN4 interacts with Pseudomonas syringae type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. Cell **108**: 743-754

Mackey D, Belkhadir Y, Alonso JM, Ecker JR and Dangl JL (2003) *Arabidopsis* RIN4 is the target of the type III virulence effector AvrRpt2 and modulates RPS2 mediated resistance. Cell **112**: 379-389

Manning VA, Hamilton SM, Karplus PA and Ciuffetti LM (2008) The Arg-Gly-Asp-containing, solvent-exposed loop of Ptr ToxA is required for internalization. Mol Plant Microbe Interact **21**: 315-325

Martí-Renom MA, Stuart AC, Fiser A, Sánchez R, Melo F and Sali A (2000) Comparative protein structure modeling of genes and genomes. Annu Rev Biomol Struct **29**: 291-325

McDowell JM, Cuzick A, Can C, Beynon J, Dangl JL and Holub EB (2000) Downy mildew (Peronospora parasitica) resistance genes in Arabidopsis vary in functional requirements for NDR1, EDS1, NPR1 and salicylic acid accumulation. Plant J **22**: 523-529

Mellersh DG and Heath MC (2001) Plasma Membrane-Cell Wall Adhesion is required for Expression of Plant Defense Responses during Fungal Penetration. Plant Cell **13**: 413-424

Moffett P (2009) Mechanisms of recognition in dominant R gene mediated resistance. Adv Virus Res **75**: 1-33, 228-229

Muskett PR, Kahn K, Austin MJ, Moisan LJ, Sadanandom A, Shirasu K, Jones JDG and Parker JE (2002) *Arabidopsis RAR1* exerts rate-limiting control of R gene mediated defenses against multiple pathogens. Plant Cell **14:** 979-992

Parker JE, Holub EB, Frost LN, Falk A, Gunn ND and Daniels MJ (1996) Characterization of eds1, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different RPP genes. Plant Cell **8**: 2033-2046

Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC and Ferrin TE (2004) UCSF Chimera--a visualization system for exploratory research and analysis. J Compt Chem **25**: 1605-1612

Pieterse CMJ, de Wit PJGM and Govers F (1992) Molecular aspects of the *potato-Phyrophthora infestans* interaction. Neth J Plant Pathol **98**: 85-92

Plow EF, Haas TA, Zhang L, Loftus J and Smith JW (2000) Ligand Binding to Integrins. J Biol Chem **275**: 21785-21788

Pont-Lezica RF, McNally JG and Pickard BG (1993) Wall-to-membrane linkers in onion epidermis: some hypotheses. Plant Cell and Environ **16**: 111-123

Potts JR and Campbell ID (1994) Fibronectin structure and assembly. Curr Opin Cell Biol **6**: 648-655

Sato M, Mitra RM, Coller J, Wang D, Spivey NW, Dewdney J, Denoux C, Glazebrook J and Katagiri F (2007) A high-performance, small-scale microarray for expression profiling of many samples in Arabidopsis-pathogen studies. Plant J **49**: 565-577

Senchou V, Weide R, Carrasco A, Bouyssou H, Pont-Lezica R, Govers F and Canut H (2004) High affinity recognition of a Phytophthora protein by Arabidopsis via an RGD motif. Cell Mol Life Sci **61**: 502-509

Shao F, Golstein C, Ade J, Stoutemyer M, Dixon JE and Innes RW (2003) Cleavage of *Arabidopsis* PBS1 by a bacterial type III effector. Science **301**: 1230-1233

Simonich MT and Innes RW (1995) A disease resistance gene in Arabidopsis with specificity for the avrPph3 gene of *Pseudomonas syringae* pv. *phaseolicola*. Mol Plant Microbe Interact **8**: 637–640

Singh S, Cornilescu CC, Tyler RC, Cornilescu G, Tonelli M, Lee MS and Markley JL (2005) Solution structure of a late embryogenesis abundant protein (LEA14) from *Arabidopsis thaliana*, a cellular stress-related protein. Prot Sci **14**: 2601-2609

Streuli CH (2009) Intergrins and cell fate determination. J. Cell Sci **122**: 171-177

Takken FL and Tameling WI (2009) To nibble at plant resistance proteins. Science **324**: 744-746

Telewski FW (2006) A unified hypothesis of mechanoperception in plants. Amer J Bot **93**: 1466-1476

Tian M, Chaudry F, Ruzicka DR, Meagher RB, Staiger CJ and Day B (2009) Arabidopsis Actin-Depolymerizing Factor AtADF4 Mediates Defense Signal Transduction Triggered by the *Pseudomonas syringae* Effector AvrPphB. Plant Physiol **150**: 815-824

Tornero P and Dangl JL (2001) A high-throughput method for quantifying growth of phytopathogenic bacteria in Arabidopsis thaliana. Plant J **28**: 475–481

Tornero P, Merritt P, Sadanandom A, Shirasu K, Innes RW and Dangl JL (2002) *RAR1* and *NDR1* contribute quantitatively to disease resistance in *Arabidopsis*, and their relative contributions are dependent on the R gene assayed. Plant Cell **14**: 1005-1015

Tsuda K, Sato M, Glazebrook J, Cohen JD and Katagiri F (2008) Interplay between MAMP-triggered and SA-mediated defense responses. Plant J **53**: 763-775

van der Flier A and Sonnenberg A (2001) Function and interactions of integrins. Cell Tissue Res **305**: 285-298

van Wees SC, de Swart EA, van Pelt JA, van Loon LC and Pieterse CM (2000) Enhancement of induced disease resistance by simultaneous activation of salicylate- and

jasmonate-dependent defense pathways in Arabidopsis thaliana. Proc Natl Acad Sci USA **97**: 8711-8716

Volgger M, Lang I, Ovecka M and Lichtscheidl I (2009) Plasmolysis and cell wall deposition in wheat root hairs under osmotic stress. Protoplasma **243**: 51-62

Wang J, Cao Y, and Zheng G (2009) Mutation in the RGD motif decreases the esterase activity of Xcc_est. Biotechnol Lett **31**: 1445-1449

Wiermer M, Feys BJ and Parker JE (2005) Plant immunity: the EDS1 regulatory node. Curr Opin Plant Biol **8**: 383-389.

Wright CA and Beattie GA (2004) *Pseudomonas syringae* pv. tomato cells encounter inhibitory levels of water stress during the hypersensitive response of *Arabidopsis thaliana*. Proc Natl Acad Sci USA **101**: 3269-3274

Yun BW, Atkinson HA, Gaborit C, Greenland A, Read ND, Pallas JA and Loake GJ (2003) Loss of actin cytoskeletal function and EDS1 activity, in combination, severely compromises non-host resistance in Arabidopsis against wheat powdery mildew. Plant J **34**: 768-777

Zhang Y, Dorey S, Swiderski M and Jones JDG (2004) Expression of RPS4 in tobacco induces an AvrRps4-independent HR that requires EDS1, SGT1 and HSP90. Plant J **40**: 213-224

Zhao T and Newman PJ (2001) Integrin activation by regulated dimerization and oligomerization of platelet endothelial cell adhesion molecule (PECAM)-1 from within the cell. J Cell Biol **152**: 65-73

Zhu JK, Shi J, Singh U, Wyatt SE, Bressan RA, Hasegawa PM and Carpita NC (1993) Enrichment of vitronectin- and fibronectin-like proteins in NaCl-adapted plant cells and evidence for their involvement in plasma membrane-cell wall adhesion. Plant J **3**: 637-646

CHAPTER 3

Arabidopsis NDR1 is required for robust activation of PAMP Triggered Immunity

Abstract

Much of the progress made towards the identification of a specific cellular role for Arabidopsis (*Arabidopsis thaliana*) NON-RACE-SPECIFIC DISEASE RESISTANCE1 (NDR1) has focused on effector-triggered immunity (ETI) signaling. Previous work in our laboratory provided the first mechanistic understanding of the global physiological role of NDR1 in plasma membrane-cell wall adhesion and its impact on disease resistance to *Pseudomonas syringae*. With distinct physiological and effector-dependent signaling roles for NDR1 now firmly established, our present study focuses on the integration of NDR1 in early pathogen perception and PAMP-triggered immunity (PTI), with effector delivery, recognition and signaling. To this end, we analyzed flg22- and elf26-specific signaling mechanisms to elucidate the breadth of NDR1 function in PTI. These mechanisms include a role for NDR1 in the regulation of stomatal closure in response to the PAMP flg22 as well as a reduction in MAPK3/6 expression after elf26 exposure. Furthermore, the loss of NDR1 alters the delivery of the *P. syringae* effector AvrRpt2 to the cell interior by the type-three secretion system. These findings, together with previous results, have allowed us to develop a basic model where NDR1 is involved at numerous stages of pathogen defense signaling from pathogen entry to ETI signaling.

Introduction

In 1995, Century *et al*. identified an Arabidopsis mutant that exhibited a loss in resistance to the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) expressing the bacterial effector proteins AvrB, AvrRpt2, AvrRpm1 or AvrPphB (Century et al. 1995). In the absence of host-effector recognition (i.e., compatible interaction), this mutant displayed an enhanced susceptibility phenotype, suggestive of a broad compromise in basal resistance activation. The gene, *NON-RACE-SPECIFIC DISEASE RESISTANCE1* (*NDR1*), has since been characterized as being required for signaling and activation of resistance through the coiled-coil nucleotide binding-site leucine-rich repeat (CC-NB-LRR) class of resistance (R) genes (Aarts et al. 1998). In terms of resistance and susceptibility, the difference(s) in pathogen growth between the *ndr1-1* mutant and WT Col-0 is most striking in an effector-dependent manner, particularly in the presence of *Pst* DC3000 expressing the effector AvrRpt2, where a significantly higher level of growth is observed in the *ndr1-1* mutant (Century et al. 1995). Much of this difference in resistance can be accounted for by *R*-gene mediated defenses (i.e., effector-triggered immunity; ETI; Chisholm et al. 2006). At present, the molecular-genetic basis for enhanced susceptibility in the *ndr1-1* mutant in the absence of effector recognition remains undefined.

A possible mechanism for NDR1 function in the regulation of disease resistance signaling was described when Day et al. (2006) identified an interaction between NDR1 and RPM1 INTERACTING PROTEIN4 (RIN4), an *R*-gene accessory protein that functions as a negative regulator of activation and signaling (Mackey et al. 2002, 2003; Axtell and Staskawicz, 2003). RIN4 is cleaved in the presence of AvrRpt2, a mechanism through which activation of the

AvrRpt2 cognate R-protein RESISTANCE TO PSEUDOMONAS SYRINGAE2 (RPS2) occurs (Axtell and Staskawicz, 2003; Chisholm et al. 2005). In the *ndr1-1* mutant, RIN4 is cleaved, yet effector-mediated resistance is not activated (Axtell and Staskawicz, 2003). This observation affirms that resistance signaling following the recognition of the effector AvrRpt2 by the host R-protein requires the presence of a functional NDR1. In total, this points to a broader role for NDR1, not in the direct physical recognition of the effector, but in the activation of the subsequent defense signaling cascades upon effector recognition. While ETI can account for a portion of the bacterial growth difference, Century et al. (1995) also observed that when plants are inoculated with *Pst* DC3000 higher growth is observed in the *ndr1-1* mutant, which is suggestive of a potential role for NDR1 in the activation of basal immunity. Subsequent work has corroborated this early observation (Coppinger et al. 2004; Knepper et al. 2011), and further suggests that ETI alone cannot account for all differences in resistance observed in the mutant line. These data, taken together, demonstrated that NDR1 is required for the full activation of not only a subset of *R*-genes, but also for a robust activation of basal immunity.

Among the initial defense responses activated during host-pathogen interactions are those mediated by the recognition of conserved pathogen motifs known as pathogen associated molecular patterns (PAMP; e.g., chitin, flagellin, LPS) by cell surface pattern recognition receptors (PRR) (reviewed in Zipfel, 2008). One of the best-characterized PAMP-PRR interactions is the recognition of the conserved 22 amino acid flagellin peptide flg22 by the receptor FLAGELLIN SENSITIVE2 (FLS2; Gomez-Gomez et al. 1999). Following receptor-ligand association (i.e., recognition of flg22), a defense signaling cascade is initiated, including the activation of MITOGEN ASSOCIATED PROTEIN KINASE (MAPK) signaling (Asai et al.

2002), stomatal closure (Melotto et al. 2006) and endosomal trafficking (Otegui and Spitzer, 2008). The activation of PAMP triggered immunity (PTI) is often classified as a separate, initial response within the broader, more robust activation of plant defense signaling. However, an increasing number of studies have begun to link PTI and ETI into a larger global defense signaling network (Xiang et al. 2008; Zhang et al. 2010; Knepper et al. 2011; Qi et al. 2011). For example, recent work identified a requirement for NDR1 in the signal cascade initiated following flg22 perception (Knepper et al. 2011). These data demonstrated that the *ndr1-1* mutant displays both a reduced and delayed MPK3/6 response to flg22, as well as significantly diminished expression of the MAPK cascade marker gene *FLG22-INDUCED RECEPTOR-LIKE KINASE1* (*FRK1*).

The identification of several roles for NDR1 in defense signaling, as well as in basic host physiological processes, first suggested that NDR1 may be involved in multiple steps throughout the infection process. While the molecular-genetic interactions described above are among the best-characterized mechanisms known to be required for the activation of resistance in plants, there are numerous basic physiological processes that are also required for pathogen perception and subsequent resistance signaling. In total, NDR1 appears to be involved in numerous defense mechanisms starting with the initial interaction between host and pathogen, at the level of stomatal aperture at the guard cells. Many plant pathogens, including *Pst* DC3000, gain access to the interior of host through stomata (Melotto et al. 2006). As an active defense response, the host has evolved mechanisms (e.g., PAMP detection) through which to recognize the presence of potential pathogens. Once recognition occurs, an abscisic acid (ABA)-dependent signaling cascade initiates the rapid closure of stomata, thus restricting pathogen entry. To counter this

response, *Pst* DC3000 has evolved a mechanism to drive stomata opening *via* the action of a jasmonic acid (JA) mimic, coronatine (Ma et al. 1991; Bender et al. 1999). As a function of PTI, the PAMP flg22 has been shown to induce stomata closure (Melotto et al. 2006). Previous studies have also shown that numerous PTI host signaling components can act in a PAMP-specific manner to regulate stomata closure (Desakin et al. 2008).

Plasma membrane (PM)-cell wall (CW) adhesion, a basic physiological process required for growth, development and response to the environment, is also required for host response to pathogen infection (Mellersh and Heath 2001). The maintenance of proper PM-CW adhesion is critical for the defense signaling cascade (Kiba et al. 1998; Mellersh and Heath 2001). When adhesion is defective, the host plant is reduced in the ability to detect and respond to pathogen infection. Recent work from our laboratory has shown that NDR1 functions in adhesion between the plasma membrane and cell wall (Knepper et al. 2011). A potential consequence of the loss of the PM-CW continuum in *ndr1-1* is a reduction in the ability of effectors to be delivered to the plant and concomitantly, a reduction in their detection and subsequent initiation of ETI. The reduction in adhesion observed in the *ndr1-1* mutant plants was shown to be coordinated with an increase in leakage across the membrane in response to pathogen infection, which potentially allows for a more favorable apoplastic environment for pathogen growth (Knepper et al. 2011).

The requirement for NDR1 in PM-CW adhesion provides much of the foundation for the analyses presented in the current study. The delivery of effectors by way of the type three-secretion system (T3SS) requires bacterial pathogens, such as *Pst* DC3000, to directly interact with both the CW and PM of the host. In the current study, we demonstrate that effector delivery

is reduced significantly in the *ndr1-1* mutant, as compared to WT Col-0. Following the initial perception of pathogens at the host cell surface, subsequent defense signaling is dependent upon membrane-bound PRRs (Gomez-Gomez et al. 1999; Zipfel et al. 2006). In the *ndr1-1* mutant, the activation of MAPK signaling following perception of flg22 by FLS2 is reduced (Knepper et al. 2011). Herein, we present a detailed analysis of the broad requirement for NDR1 across multiple steps of *Pst* DC3000 infection - from the first contact of host and pathogen to dissecting the specific requirement for NDR1 in PTI signaling. Our data demonstrate that NDR1, a critical signaling component of innate immune signaling in plants, engages *Pst* DC3000 at multiple steps of the infection process, linking primary physiological processes with the activation of defense-specific signaling.

Results

Flg22-induced stomata closure is reduced in the *ndr1-1* mutant.

Defense signaling downstream of flg22 perception has been demonstrated to be compromised in the *ndr1-1* mutant (Knepper et al. 2011). To examine the physiological implication of reduced flg22 signaling and/or perception, the stomatal aperture response following flg22 perception was analyzed in the *ndr1-1* mutant. As shown in Figure 3.1, when epidermal peels were exposed to flg22, WT Col-0 stomata exhibited a significant closure of stomatal aperture as compared to *ndr1-1*. One hour after peels were exposed to flg22 peptide, the stomatal apertures of *ndr1-1* were, on average, greater than 1 µm wider than those of Col-0. No significant differences in aperture width were identified in either the untreated controls or mock treated peels in either Col-

0 or *ndr1-1* (Figure 3.1). The stomata of the *ndr1-1* mutant appeared completely nonresponsive to flg22 peptide treatment one hour post treatment, indicating that the perception of flg22 in the *ndr1-1* mutant fails to initiate the wild-type response of stomatal closure usually observed in response to a PAMP trigger. With NDR1 apparently required for stomatal closure in response to a bacterial pathogen the mutant line is lacking the first active physical barrier to prevent pathogen entry.

NDR1 is required for a robust PTI response following elf26 perception.

Previous work (Knepper et al. 2011) demonstrated a reduced MAPK cascade in response to recognition of flg22 in the *ndr1-1* mutant as compared to Col-0. While the recognition of flg22 by the pattern recognition receptor FLS2 seems to be widely conserved across species (reviewed in Zipfel and Felix, 2005), the recognition of bacterial elongation factor-Tu (EF-Tu) is restricted to the *Brassicales* (Kunze et al. 2004). In Arabidopsis, the perception of EF-Tu by the EF-Tu RECEPTOR, EFR, activates a cascade similar to what is observed in the recognition of flg22 by FLS2 (Zipfel et al. 2006). In Col-0, PAMP perception elicits the robust activation of a MAPK cascade, resulting in the phosphorylation of MAPK3 and MAPK6 (Asai et al. 2002). To examine the specificity and role of NDR1 in PAMP-mediated signaling, leaves of Col-0 and *ndr1-1* were infiltrated with an active 26 amino acid subunit of EF-Tu (elf26; Kunze et al. 2004) in order to induce MAPK3 and MAPK6 phosphorylation. As shown in Figure 3.2, treatment of leaves with elf26 resulted in a reduced and delayed PTI response, similar to the previously observed flg22-induced response observed in the *ndr1-1* mutant (Knepper et al. 2011). The levels of phosphorylated MAPK3 and MAPK6, shown to be required for the priming of biotic stress

responses (Beckers et al. 2009), are reduced greater than 50 % in the *ndr1-1* mutant ten minutes after infiltration with elf26 (Figure 3.2). Twenty minutes after elf26 infiltration, *ndr1-1* showed slightly higher MAPK3 and MAPK6 phosphorylation, corresponding with the reduced and delayed response previously observed in *ndr1-1.* As determined by western blot analysis of MAPK phosphorylation, we observed both a delay in phosphorylation, as well as a slightly more diffuse response following PAMP elicitation in the *ndr1-1* mutant as compared to WT Col-0. The measured reduction in MAPK3 and MAPK6, while not clarifying a specific point of regulation in the PTI response or MAPK cascade in which NDR1 is involved, demonstrates that Arabidopsis plants lacking functional NDR1 cannot fully activate downstream resistance signaling following pathogen perception. Differences in the mRNA expression of markers for both MAPK signaling and CDPK signaling were also tested with corroborative results (Figure S3.1). These results, taken together with the established role for NDR1 in flg22 signaling (Knepper et al. 2011), confirm a broad requirement for NDR1 in PTI, in a manner that is not specific to a single PAMP, but indicative of a broader role in multiple PAMP recognition and signaling cascades.

Type III effector delivery is reduced in the *ndr1-1* mutant.

Knepper et al. (2011) identified a role for NDR1 in mediating plasma membrane (PM)-cell wall (CW) focal adhesion. From a structural standpoint, we hypothesized that a loss in adhesion could impact numerous host cell processes associated with defense signaling at the membrane, such as ROS production (Kiba et al. 1998) and penetration resistance (Mellersh and Heath, 2001). From the standpoint of pathogen infection, we further hypothesized that a loss in PM-CW adhesion

may also negatively impact the ability of *P. syringae* to efficiently delivery type III secretion system effectors (T3Es). As a result of a reduction in T3E delivery into the host cell, the pathogen would proliferate, unencumbered by the activation of ETI. To test this hypothesis, we investigated if type III secretion system (T3SS)-dependent delivery of effectors is altered as a result of the loss of PM-CW adhesion in the *ndr1-1* mutant. Utilizing a *P. syringae* DC3000 strain expressing an AvrRpt2-adenylate cyclase fusion (i.e., AvrRpt2:Cya; Casper-Lindley et al. 2002), the *in planta* translocation of the bacterial effector protein AvrRpt2 was monitored. As shown in Figure 3.3, by five hours post-inoculation, a substantial difference in cAMP levels was observed indicating a reduction in effector delivery to the *ndr1-1* mutant. The quantity of AvrRpt2 delivered to the *ndr1-1* mutant line remained lower when compared to Col-0 throughout the duration of the time course. Based on these data, we hypothesize that the loss of functional NDR1 reduces the ability of *P. syringae* to deliver T3SS effectors. Coupled with our previous analyses (Knepper et al. 2011), we posit that reduced adhesion between the PM and CW negatively impacts the pathogen's ability to interact with the host cell and therefore limits its ability to deliver effector proteins. The recognition of effectors by corresponding host R-proteins serves as a critical step in the initiation of ETI and the amplification of defense responses in Arabidopsis. This reduced delivery of effectors in addition to previous findings indicating a loss of defense signaling at the PM-CW interface when adhesion is reduced (Kiba et al. 1998; Mellersh and Heath, 2001; Knepper et al. 2011), indicate that the role of NDR1 in maintaining PM-CW adhesion may be critical for both PTI and ETI.

Effector delivery is not reduced by flg22 priming in the absence of NDR1.

The activation of basal immunity through the perception of PAMPs is the first line of defense for the host plant during pathogen infection; thus, a robust PTI response may serve to "prime" the host against escalating pathogen virulence. Recently, Crabill et al. (2011) showed that PTI can restrict T3SS delivery of effectors. This restriction can be overcome in a susceptible interaction, presumably *via* a mechanism whereby effectors are able to suppress the PTI response (Hauck et al. 2003; Kim et al. 2005). In resistant lines, the recognition of effectors and subsequent activation of ETI prevents PTI from being suppressed by the delivered effectors. Based on our previous findings describing a reduction in flg22-mediated signaling in the *ndr1-1* mutant (Knepper et al. 2011), as well as the original observation of a reduced response in *ndr1-1* to several *P. syringae* effectors (Century et al. 1995), we hypothesized that plants lacking functional NDR1 may be unable to actively suppress the delivery of effectors by the T3SS. To test this, both Col-0 and *ndr1-1* plants were inoculated with flg22 peptide concurrently with *P. syringae* AvrRpt2:Cya or plants were pre-treated with the flg22 peptide two hours before bacterial inoculation. In Col-0, the pre-treatment with flg22 led to a slight reduction in effector delivery, as measured by cAMP levels (Figure 3.4), in contrast to the *ndr1-1* mutant, in which no difference was observed between pre-treated and simultaneously inoculated samples. The fact that cAMP levels in *ndr1-1* were unaffected by flg22 pretreatment further supports a role for NDR1 in flg22 signaling. Taken together, these results suggest that the activation of layered defenses in Arabidopsis is critical for disease resistance and that NDR1 is an important component in the transition from basal immunity to the activation of a more robust ETI signaling response.

***Pst* DC3000 D28E shows increased growth in the *ndr1-1* mutant as compared to WT Col-0**.

Recently, Cunnac et al. (2011) developed a *Pst* DC3000 polymutant strain that permits a fine-tuned analysis of PTI without the need for cell-free systems (i.e., PAMP treatment) or T3SS-deficient bacteria. In short, the 28 expressed native effectors of *Pst* DC3000 were systematically deleted, leading to bacterium with an intact T3SS that is functionally effector-less, designated *Pst* DC3000 D28E (deficient in 28 effectors, CUCPB5585). To determine the impact of the *NDR1* mutation on PTI signaling in response to *Pst* DC3000 D28E inoculation, we analyzed the growth of the effector-less strain in the *ndr1-1* mutant. As shown in Figure 3.5, while a deletion of the native *Pst* DC3000 effectors yields a significantly weakened pathogen, we observed an increase in growth four days post inoculation (dpi) in the *ndr1-1* mutant, as compared to WT Col-0. This growth differential is mirrored in the *Pst* DC3000 D28E T3SS deficient line, CUCPB5589 (Figure 3.5). While not statistically significant using a threshold of $p<0.05$, a reproducible increase in bacterial growth was observed in the *ndr1-1* mutant in both CUCPB5585 and CUCPB5589 compared to Col-0. These data further support that the *ndr1-1* mutant is, as long-speculated, deficient in PTI.

The oxidative burst response is diminished in the *ndr1-1* mutant.

In Arabidopsis, the initiation of PTI leads to the activation of numerous defense signaling processes, including the generation of reactive oxygen species (ROS; reviewed in Bolwell and Wojtaszek 1997; Davies et al. 2006). The accumulation of ROS is a central component of plant defenses serving numerous functions including direct toxicity to pathogens (Keppler et al. 1989), cross linking of plant cell walls (Fry et al. 2002) and signaling (Grant and Loake, 2000).

Previously, *OXIDATIVE SIGNAL-INDUCIBLE1* (*OXI1*) was identified as being an integral signaling component of the MAPK pathway, which is induced in response to ROS stimuli (Rentel et al. 2004; Petersen et al. 2009). As a result, *OXI1* mRNA accumulation is a marker for the generation of ROS in response to pathogen infection. As shown in Figure 3.6B, qRT-PCR analysis revealed an increase in *OXI1* expression three hours after elf26 infiltration in WT Col-0, in agreement with ROS production in Col-0 following *Pst* DC3000 infection (Torres et al. 2002). Likewise, qRT-PCR analysis revealed a similar response in Col-0 three hours after infiltration with flg22 peptide (Figure 3.6D). In contrast, we did not observe a rapid increase in *OXI1* mRNA accumulation in the *ndr1-1* mutant, which is suggestive of a diminished ROS response following elf26 or flg22 recognition (Figure 3.6B, 3.6D). The failure of *OXI1* induction observed in the absence of NDR1 indicates that NDR1 is required for a robust PTI response, not only in the upstream activation of defense signaling, but also in the downstream activation of ROS production and subsequently in the activation of defense. This is further supported by 3,3'-diaminobenzidine (DAB) staining for ROS in leaves infiltrated with elf26 or flg22 peptide (Figure 3.6E), with more of the characteristic brown staining observed in Col-0 compared to *ndr1-1* mutant leaves.

BTH pre-treatment fails to restore ETI in the *ndr1-1* mutant background.

In plants, benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) is metabolized into a salicylic acid (SA) analog, and has been shown to elicit a response similar to exogenous SA application (Lawton et al. 1996). Previously, the downstream marker gene for SA signaling, *PR1*, was analyzed in the *ndr1-1* mutant background, showing that *PR1* mRNA expression was

reduced following inoculation with *Pst* DC3000 AvrRpt2 in the *ndr1-1* mutant as compared to WT Col-0 (Knepper et al. 2011). Shapiro and Zhang (2001) observed a potential link between the *ndr1-1* mutation and the activation of systemic acquired resistance (SAR). In short, this study found that pre-treatment of *ndr1-1* mutant plants with BTH restores resistance to *Pst* DC3000, which is likely the result of systemic basal defense responses (Shapiro and Zhang, 2001). In the present study, pre-treatment with BTH, while able to restore resistance to *Pst* DC3000, failed to fully restore resistance in the *ndr1-1* mutant following infection with *Pst* DC3000 expressing the cysteine protease effector AvrRpt2 (Figure 3.7). In addition, an increase in bacterial growth was observed in the *ndr1-1* line following BTH pre-treatment, indicating that while NDR1 appears to be important for the full activation of SA-dependent defense signaling, SA defense signaling alone cannot account for the resistance deficiencies observed in the *ndr1-1* mutant.

Discussion

NDR1 is a key component of the Arabidopsis defense-signaling network required for resistance to the phytopathogen *Pseudomonas syringae* pv. tomato DC3000 (Century et al. 1995; Century et al. 1997; Coppinger et al. 2004; Day et al. 2006; Knepper et al. 2011). While much progress has been made toward the identification of a specific cellular role for NDR1 in effector-triggered immunity (ETI) signaling, recent work represents the first mechanistic understanding of the role of NDR1 in defense signaling, linking basic host physiological processes to the activation of resistance following *Pst* DC3000 perception (Knepper et al. 2011). As a mediator of the host plasma membrane (PM)-cell wall (CW) continuum, NDR1 was shown to play a role in regulating fluid movement across the membrane in response to pathogen infection (Knepper et

al. 2011). This observation offers a possible mechanism to explain the enhanced bacterial growth phenotype in the *ndr1-1* mutant. As nutrient acquisition is the primary factor driving the virulence capacity of pathogens (Katagiri et al. 2002), restricting access by the host is a means to limit pathogen proliferation, and disease. Taken together, our previous work suggests that the specific physiological niche NDR1 occupies within Arabidopsis defense signaling provides a platform through which NDR1's role in maintenance of PM-CW adhesion ultimately impacts multiple steps of pathogen infection, perception and subsequent activation of defense signaling (Figure 3.8). In total, the loss of *NDR1* diminishes the temporal and spatial recognition of *Pst* DC3000 by Arabidopsis, resulting in both a delay in perception as well as a compromise in broad signaling of defense.

In the current study, we show that an interruption of the PM-CW continuum in the *ndr1-1* mutant not only affects fluid movement as previously demonstrated (Knepper et al. 2011), but also disrupts the host-pathogen interaction through an abrogation in the delivery of bacterial effectors by the type III secretion system (T3SS; Figure 3.3). In an analysis of defense signaling in plant-fungal interactions, Mellersh and Heath (2001) observed that signaling at the CW is reduced during the penetration of non-host plants by biotrophic fungal pathogens. Based on this observation, it was hypothesized that the expression of CW-associated defense responses is dependent upon proper PM-CW adhesion. In the case of host-phytopathogenic bacterial interactions, the role of PM-CW focal adhesion is largely unknown. As an intimate point of contact between the host and pathogen, it is reasonable to hypothesize that a disruption in the normal function of this interface would impact numerous processes, including host resistance signaling. Indeed, we demonstrate that a loss in adhesion between the PM and CW also affects

the ability of *Pst* DC3000 to efficiently deliver T3SS effectors (T3E) into the host, possibly *via* a disruption of the normal function of the T3SS. In support of this, we observed a reduction in the delivery of an AvrRpt2:Cya reporter construct, *in planta* (Figure 3.3). Despite the obvious differences in mechanisms of infection utilized by fungal and bacterial pathogens, the maintenance and function of PM-CW adhesion would appear to be a conserved point of function in defense signaling in plants. Our data support the hypothesis that in Arabidopsis, NDR1 is a mediator of this role, specifically in the case of phytopathogenic bacterial infection. To the best of our knowledge, an extensive analysis of the role of NDR1 in fungal penetration in plants has not been investigated. Based on our data presented herein, it is tempting to speculate that fungal penetration in the *ndr1-1* mutant would also be affected.

Crabill et al. (2010) found that the activation of PTI within the host leads to a reduction in the ability of *P. syringae* to deliver T3SS effectors. Our data support the hypothesis that PTI suppresses T3E delivery and demonstrate that NDR1 is required for this activity. Figure 3.4 shows that when *ndr1-1* mutant plants are pre-treated with flg22, we did not observe a reduction in effector delivery, which is in contrast to the reduction observed in WT Col-0. This observation supports the hypothesis that suppression of effector delivery in the *ndr1-1* mutant is likely due to the reduced flg22 response (Knepper et al. 2011). Crabill et al. (2010) argued that PTI in Arabidopsis is more effective at restricting effector injection by *P. syringae* strains in incompatible rather than compatible interactions because in compatible interactions, effectors are still delivered at a low level during PTI-induced injection restriction. Presumably, this would permit T3Es to suppress PTI in the compatible host, thus leading to a reduction of the PTI-

induced effector delivery restriction. Based on the results from this study it appears that NDR1 is required for Arabidopsis to actively restrict effector delivery after PTI activation.

When first identifying the *ndr1-1* mutation as being involved in resistance in an effector-specific fashion, Century et al. (1995) also made the observation that NDR1 contributed, to some degree, to the activation of basal immunity. In support of this early observation, Knepper et al. (2011) identified a distinct signaling function of NDR1 with respect to the perception and downstream signaling associated with flg22 perception, thereby establishing a mechanistic link between NDR1 and PTI signaling. In total, a reduction in *FRK1* mRNA expression, coupled with a delayed and weakened MPK3/6 protein expression profile provides the first definitive evidence of a requirement for NDR1 in PTI signaling (Knepper et al. 2011). Broadly considering the host-pathogen interface, downstream PTI signaling pathways appear to be shared following PAMP association with specific PRRs (reviewed in Schwessinger and Zipfel, 2008). In the *ndr1-1* mutant, it appears that the PTI signals measured in the *ndr1-1* mutant are reduced after elf26 treatment compared to those previously observed after flg22-induced defenses (Figure 3.2; Knepper et al. 2011). This seems to indicate that NDR1 plays a stronger role in PTI signaling downstream of flg22 perception as compared to elf26 perception. The connection between NDR1 and flg22 signaling is further strengthened by the results of the stomatal aperture assay (Figure 3.1) that indicates a disconnect between the perception of flg22 by FLS2 and the closure of stomata in the *ndr1-1* mutant plants. In the absence of NDR1 stomata fail to close in response to a potential pathogen signal which leaves the mutant plants vulnerable to pathogen entry.

Shapiro and Zhang (2001) observed that localized treatment of *ndr1-1* plants with BTH restores a moderate level of resistance, presumably as a function of the activation of SAR. Based on this observation and our data presented in Figure 3.7, we propose that the level of resistance observed in the *ndr1-1* mutant following BTH treatment (Shapiro and Zhang, 2001) is linked to basal immunity. In the present study, pre-treatment of *ndr1-1* with BTH fails to restore wild-type levels of resistance following inoculation with *P. syringae* expressing the effector AvrRpt2 (Figure 3.7). While the observation of moderate resistance in *ndr1-1* after BTH pre-treatment is intriguing, the specificity of this resistance to *P. syringae* DC3000 raises many new questions as to the nature of SAR and NDR1. These data indicate that while NDR1 is required for full pathogen resistance, alternative pathways may exist that allow for some level of activation, even in the absence of a functional *NDR1* gene.

A mechanistic analysis of plant defense activation and signaling following perception of the bacterial pathogen *P. syringae* has provided numerous insights into the broader role of host gene function and the regulation of resistance signaling (Chisholm et al. 2006). However, a full understanding of the shared and convergent regulation of ETI and PTI remains elusive. Recently, Cunnac et al. (2011) developed *P. syringae* strains that are essentially effector-less. While it is known that the *ndr1-1* mutant line is defective in the robust activation of basal resistance signaling, a precise understanding of the molecular-genetic mechanism(s) remains unknown. In the absence of the native 28 *P. syringae* effectors, the *ndr1-1* mutant supported an increase in bacterial growth compared with WT Col-0 (Figure 3.5). In total, this data supports a role for NDR1 in PTI activation, yet the precise signaling mechanism(s) underpinning this observation remain largely undefined.

The production of ROS is a well-established component of plant immunity (reviewed in Bolwell and Wojtaszek, 1997). While it has been suggested that *NDR1* functions downstream of ROS production (Shapiro and Zhang, 2001), our data presented herein indicates that in the absence of NDR1, ROS production is significantly reduced following perception of the PAMPs elf26 or flg22 (Figure 3.6B, 3.6D). This indicates that NDR1 is required for robust ROS production in response to PAMP signals. The reduction in ROS production may indicate that NDR1 functions upstream of ROS production, at least in a PTI-dependent manner.

Given the established physiological role for NDR1 in maintaining PM-CW adhesion and the mediation of fluid movement in response to pathogen infection, in addition to the mounting evidence linking NDR1 to an array of defense signaling pathways, it is becoming increasing likely that NDR1 may serve as a signaling hub within Arabidopsis. This hypothesis is further supported by recent findings physically linking the PRR FLS2 and the R-protein RPS2 (Qi et al. 2011); both of which require the presence of NDR1 for the full activation of downstream signaling. We set forth a model showing several critical steps in plant pathogen defense from pathogen entry to effector recognition and signaling in which NDR1 has been shown to impact the host response (Figure 3.8). NDR1 appears to be a critical component of defense signaling in both an effector- and PAMP-mediated fashion; it is due to this wide ranging requirement for NDR1 in defense signaling that the interconnected nature of basal and effector-triggered immunity becomes more clear. Given the success in experimentally associating NDR1 with a multitude of defense responses it may be possible that other plant defense components previously thought of as ancillary *R*-gene accessory proteins, may be involved in a multitude of

defensive functions. However, the extent to which NDR1 is so deeply integrated into nearly all aspects of defense, from stomatal aperture regulation to PTI activation and effector mediated defense, appears unique. The position and physiological function of NDR1 in Arabidopsis may help to explain the extensive nature of NDR1's involvement in plant pathogen perception and defense signaling.

Materials and Methods.

Plant Growth Conditions

Arabidopsis thaliana plants were grown in a BioChambers model FLX-37 growth chamber (Manitoba, Winnipeg, Canada) at 20°C under a 12 h/12 h light/dark cycle with 60% relative humidity and a light intensity of 120 μmol photons $m^{-2}s^{-1}$.

CyA Inoculation Assay and cAMP Extraction

CyA inoculation assays were performed as previously described (Casper-Lindley et al. 2002; Crabill et al. 2010), with slight modifications. Four- to five-week-old WT Col-0 and *ndr1-1* mutant leaves were hand-infiltrated with *Pst* DC3000 containing AvrRpt2:CyA at a concentration of 3 x 10^8 cells/ml in 5 mM MES pH 5.5. After inoculation, plants were kept under continuous light (100 $\mu E.m^{-2}.s^{-1}$) until 2 leaf discs (0.56 cm^2) per sample (3 samples per treatment) were harvested at 0, 3, 5, 8 and 24 hours post inoculation (hpi), frozen immediately in liquid nitrogen and stored at -80ºC until processed. Samples were ground in liquid nitrogen to a

fine powder and resuspended in 100 µl of 0.1 M HCl. Next, samples were mixed by vortexing and centrifuged at 1000 x g for 10 minutes. Supernatants were recovered and the total protein concentration was determined using a Bradford assay. Samples were adjusted to 10 ng/µl, and dilutions ranging from 1:2 to 1:7 were used. cAMP levels were measured using a direct cAMP immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. For flg22 priming studies leaves were hand-infiltrated with 1uM flg22 peptide with a needless syringe either immediately before *P. syringae* infiltration or 2 hours prior to bacteria infiltration. Samples were then collected and processed as described above. Results were analyzed using Prism 4 (GraphPad Software) with outliers removed by Grubb's test (α=0.05) using the QuickCalcs online outlier calculator (GraphPad Software; http://www.graphpad.com/quickcalcs/Grubbs1.cfm). Statistical significance was determined by either Student's t-test.

Pathogen Inoculation and Growth Assays

Pseudomonas syringae pv tomato DC3000 strains containing the pVSP61 vector (*Pst* DC3000) or pVSP61 containing AvrRpt2 were previously described in Kunkel et al. (1993). The effector-deficient *Pst* DC3000 strain CUCPB5585 (D28E) and the T3SS deficient strain CUCPB5589 were previously described in Cunnac et al. (2011). To assess bacterial growth, plants were handled using the method described in Knepper et al. (2011). Four- week-old plants were dip inoculated in bacterial suspensions of 3×10^7 colony forming units (cfu) mL^{-1}. Three leaf discs of 0.7 cm were harvested 0 and 4 days post-inoculation. Growth assays were performed as described in Tornero and Dangl (2001) and Tian et al. (2009). At day 0 leaves were surface

sterilized by submerging in 10% bleach for 30 seconds with gentle swirling followed by 30 seconds in sterile dH_20. Results were analyzed using Prism 4 (GraphPad Software) with outliers removed by Grubb's test (α=0.05) using the QuickCalcs online outlier calculator (GraphPad Software; http://www.graphpad.com/quickcalcs/Grubbs1.cfm). Statistical significance was determined by either one way ANOVA with Tukey's post test (Figure 3.7) or Student's t-test (Figure 3.5).

Benzo(1,2,3)-Thiadiazole-7-Carbothioic Acid S-Methyl Ester (BTH) pretreatment

Leaves were treated using a method modified from Shapiro and Zhang (2001). Leaves were hand-infiltrated with 0.12 mM benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester **(**BTH; Sigma, St. Louis, MO, USA) in 1 mM $MgCl_2$ buffer or 1 mM $MgCl_2$ buffer only (mock) using a needless syringe 48 h before being dip-inoculated for the bacterial growth assay.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from leaves using the PrepEase Plant RNA Spin kit (USB Affymetrix, Santa Clara, CA, USA). First-strand cDNA was synthesized from 1 μg Total RNA using the First-Strand cDNA Synthesis kit (USB Affymetrix). qRT-PCR was performed using the Mastercycler ep Realplex system (Eppendorf AG, Hamburg, Germany), as previously described (Knepper et al. 2011), using the Hot Start SYBR Master mix 2X (USB Affymetrix). Ubiquitin (*UBQ10*) was used as an endogenous control for amplification. All replicates were performed using a cycle, previously used in Knepper et al. (2011), of 95 °C for 2 min followed by 40 cycles

of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 45 s. Results were analyzed using Prism 4 (GraphPad Software) with outliers removed by Grubb's test (α=0.05) using the QuickCalcs online outlier calculator (GraphPad Software; http://www.graphpad.com/quickcalcs/Grubbs1.cfm). Statistical significance was determined by one-way ANOVA with Tukey's post test. All primer sets utilized are listed in Supplemental Table S3.1.

MAPK Phosphorylation and Western Blot Analysis

Arabidopsis leaves were hand-infiltrated with 1 µM elf26 peptide in 1 mM $MgCl_2$. Samples were collected 10, 20 and 30 min post inoculation. To detect the activity of MAPK3/6, 70 µg of total protein was loaded onto a 12% SDS-PAGE gel and transferred to nitrocellulose membrane. This was followed by 1 h incubation with an anti-pTEpY specific antibody (Cell Signaling Technology, Danvers, MA, USA). An anti-rabbit HRP conjugated secondary antibody was used for chemiluminescence detection. MAPK3/6 protein was quantified using Image J software (National Institutes of Health).

Stomatal Aperture Assay

Stomatal aperture was assayed using a method modified from Melotto et al. (2006). In brief, 4-5-week-old plants were placed under continuous light for 24 h, covered with a plastic dome to maintain humidity. Epidermal peels were taken from five separate leaves and placed on a glass

slide covered with either dH_2O or 1 µM flg22 peptide in dH_2O. Slides containing peels were placed in a petri plate covered to maintain humidity and placed under growth lights for 1 h (immediately for controls) before stomata were imaged using an Olympus IX-71 inverted microscope with an Olympus DP70 camera. Stomata apertures were measured from images using Image J software (National Institutes of Health). Results were analyzed using Prism 4 (GraphPad Software). Statistical significance was determined by Student's t-test.

3,3'-diaminobenzidine (DAB) staining

One half of Arabidopsis leaves were hand-infiltrated using a needless syringe with 1 µM flg22 or elf26 peptide in 1 mM $MgCl_2$ or 1 mM $MgCl_2$ alone (mock). Leaves were collected 24 hours post infiltration and placed submerged in 1mg/ml 3,3'-diaminobenzidine and rocked gently at room temperature overnight. The leaves were then cleared in 100% ethanol.

Acknowledgements

We thank Katie Porter for technical support on the analysis of MAPK3/6 expression, and Elizabeth Savory for technical support on the microscopy analysis. Alyssa Burkhardt is acknowledged for critical reading of the manuscript. This work was supported by the National Science Foundation (Early CAREER Award IOS-0641319 to B.D.). The authors acknowledge Fundação para a Ciência e Tecnologia, grant SFRH/BPD/43228/2008 to P.S. (co-financed by the Portuguese PIDDAC program and European Social Fund, under the third framework program).

Figure 3.1.

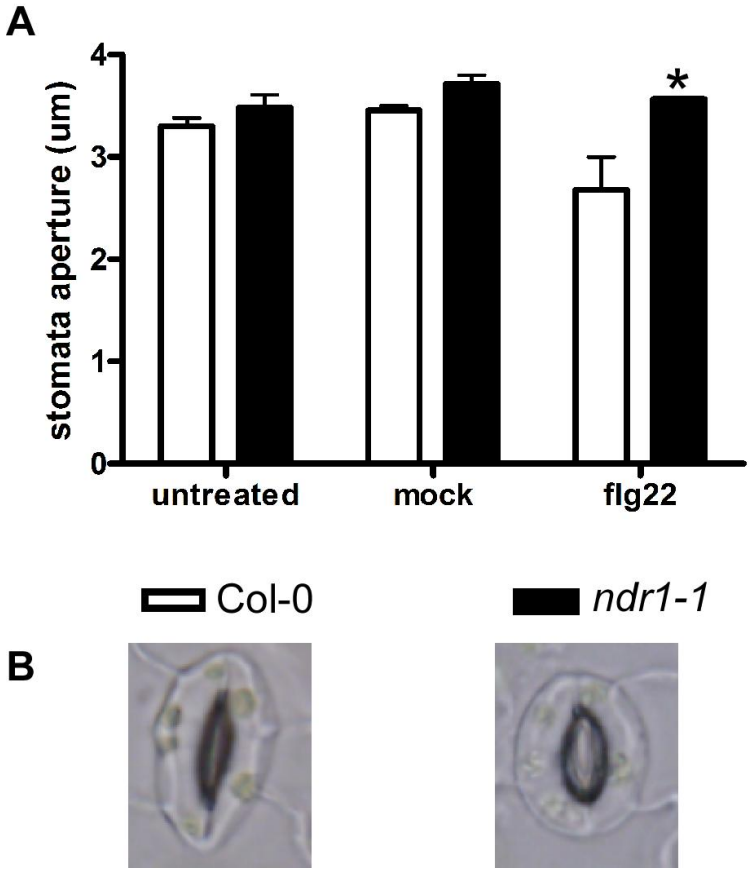


Figure 3.1. The stomata of *ndr-11* are non-responsive to flg22 peptide.

A, Stomatal aperture widths after Col-0 and *ndr1-1* epidermal peels were exposed to 1 µM flg22 peptide. Peels of untreated samples were scored for aperture width immediately following placement on slide. Mock (dH_2O alone) samples were included as controls. Error bars display standard deviation from two biological replicates with n=50-130 stomata. Significance was determined using Student's t-test, where * represents statistically significant differences between Col-0 and *ndr1-1*: * $P < 0.05$. B, Representative stomata of Col-0 (left) and *ndr1-1* 1 h after flg22 treatment.

Figure 3.2.

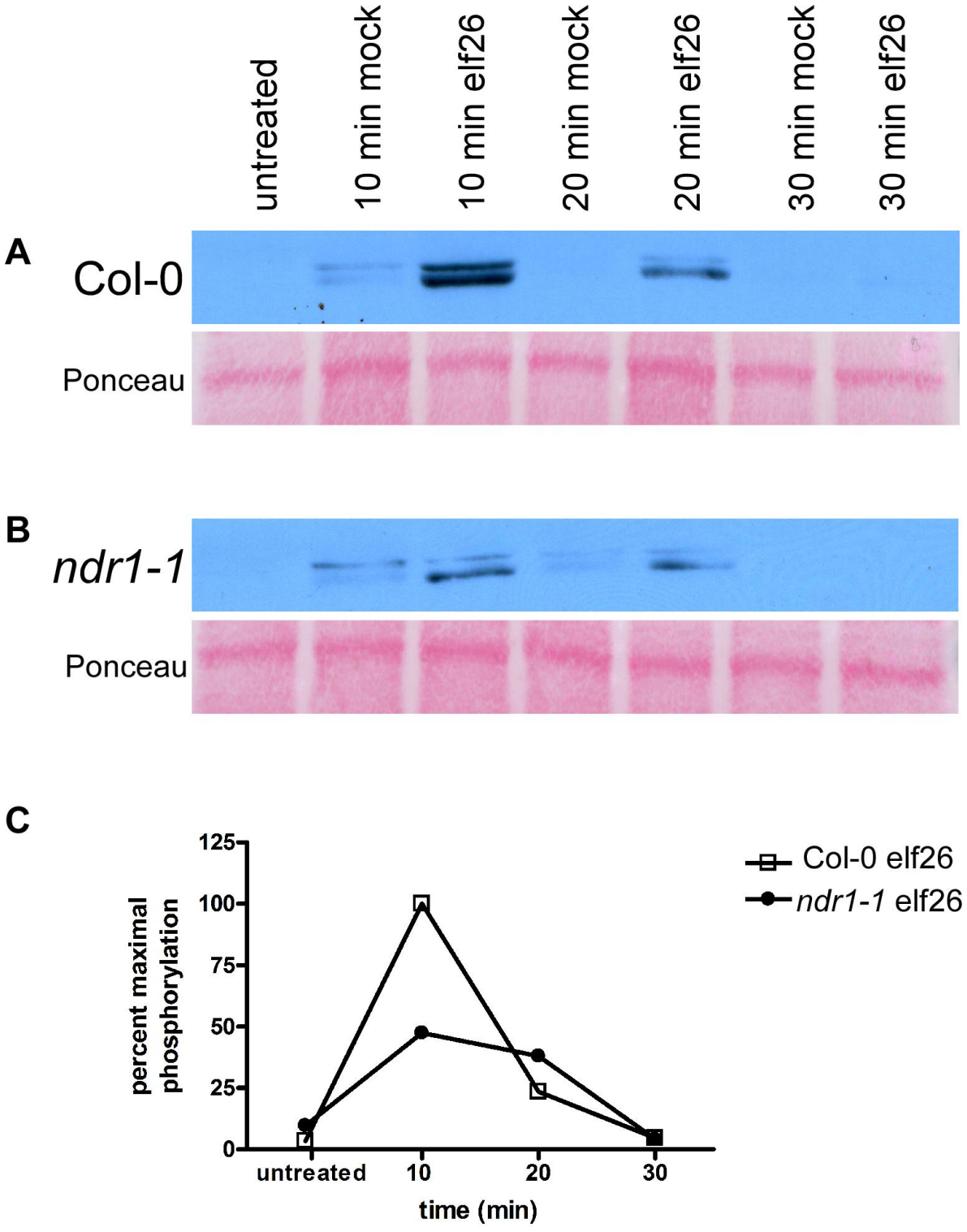

untreated
10 min mock
10 min elf26
20 min mock
20 min elf26
30 min mock
30 min elf26
A
Col-0
Ponceau
B
ndr1-1
Ponceau
C
percent maximal phosphorylation
125
100
75
50
25
0
untreated
10
20
30
time (min)
Col-0 elf26
ndr1-1 elf26

Figure 3.2. *ndr1-1* displays altered PAMP responses to elf26.

A and B, Western blot analysis of MAPK3 and MAPK6 phosphorylation in Col-0 and *ndr1-1* in response to 1 µM elf26 peptide. Mock (water only) inoculations were included as controls. Ponceau stained blots display equal loading of 70 µg total protein per lane. C, Percent maximal phosphorylation of MAPK3 and MAPK 6 protein expression after elf26 treatment. Blot and graph are representative of one of two replicates performed with similar results.

Figure 3.3.

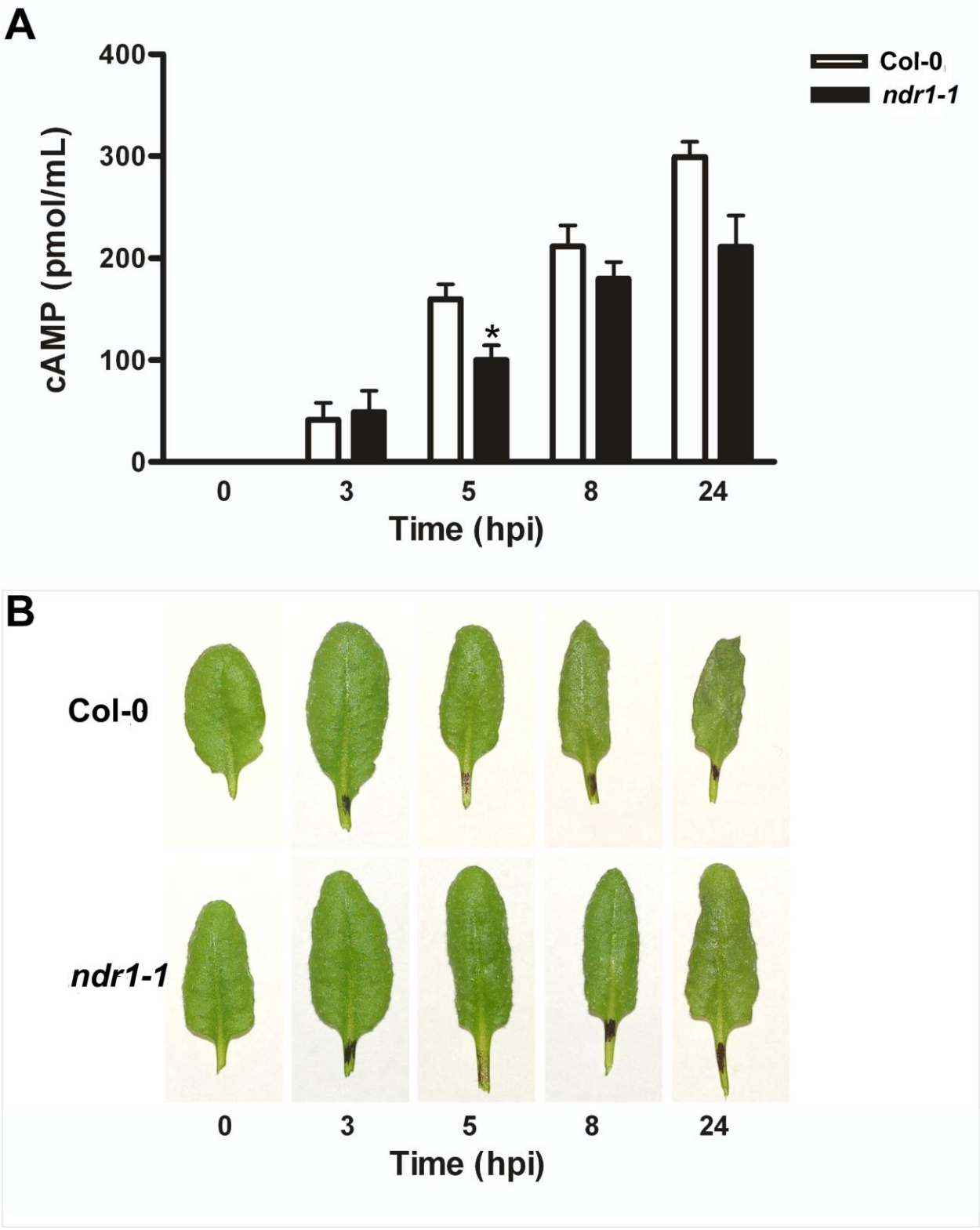

A
400
300
200
100
0
cAMP (pmol/mL)
Col-0
ndr1-1
*
0
3
5
8
24
Time (hpi)
B
Col-0
ndr1-1
0
3
5
8
24
Time (hpi)

Figure 3.3. AvrRpt2:Cya effector fusion delivery is diminished in the *ndr1-1* mutant.

A, Effector delivery expressed as cAMP activity was measured in wild-type Col-0 and *ndr1* at 0, 3, 5, 8 and 24 hours post inoculation (hpi) with *Pst* DC3000 expressing AvrRpt2:CyA. B, Leaves of Col-0 display the onset of the hypersensitive response (HR) by 24 hpi demonstrating recognition of the AvrRpt2:CyA. The HR response is absent in the *ndr1-1* mutant. Error bars display standard deviation from two biological replicates with n=3-6. Significance was determined using Student's t-test, where * represents statistically significant differences between Col-0 and *ndr1-1*: * $P < 0.05$.

Figure 3.4.

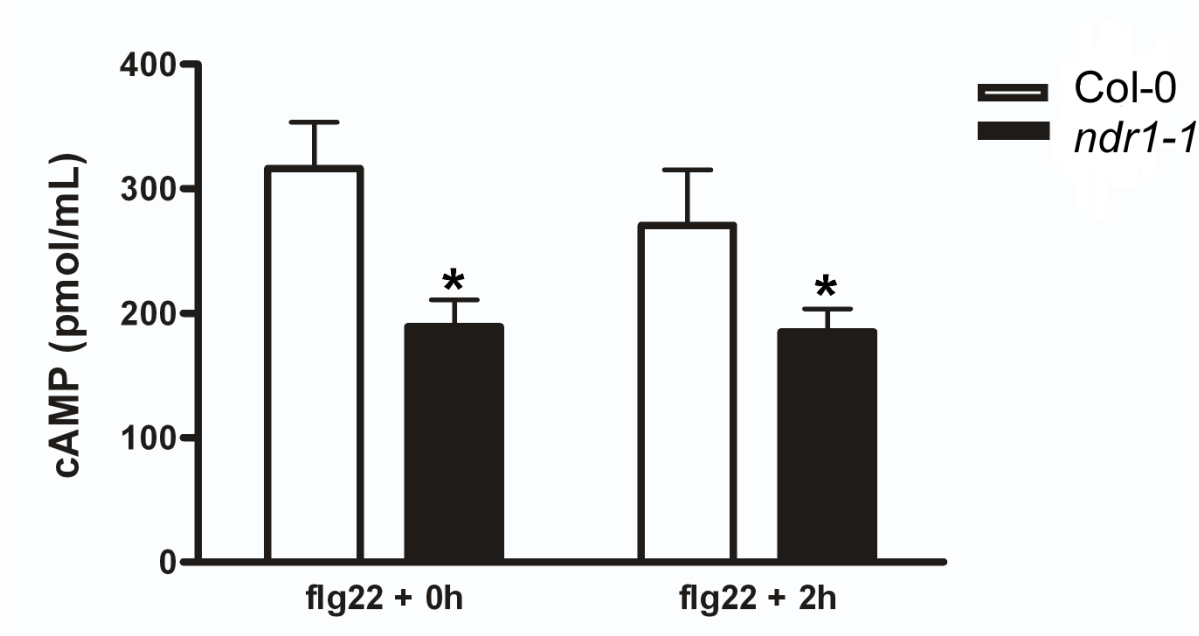


Figure 3.4. Delivery of AvrRpt2:Cya by *Pst* DC3000 is not reduced in the *ndr1-1* mutant by flg22 priming of PTI.

Leaves were infiltrated with 1 µM flg22 peptide either immediately prior (flg22 + 0 h) or 2 hours prior (flg22 + 2 h) to inoculation with *Pst* DC3000 expressing AvrRpt2:CyA. Effector delivery expressed as cAMP activity was measured in wild-type Col-0 and *ndr1-1* 16 hours post inoculation (hpi). Error bars display standard deviation from two biological replicates with n=7. Significance was determined using Student's t-test, where * represents statistically significant differences between non-pretreated Col-0 (flg22 + 0h) and *ndr1-1*: * $P < 0.05$.

Figure 3.5.

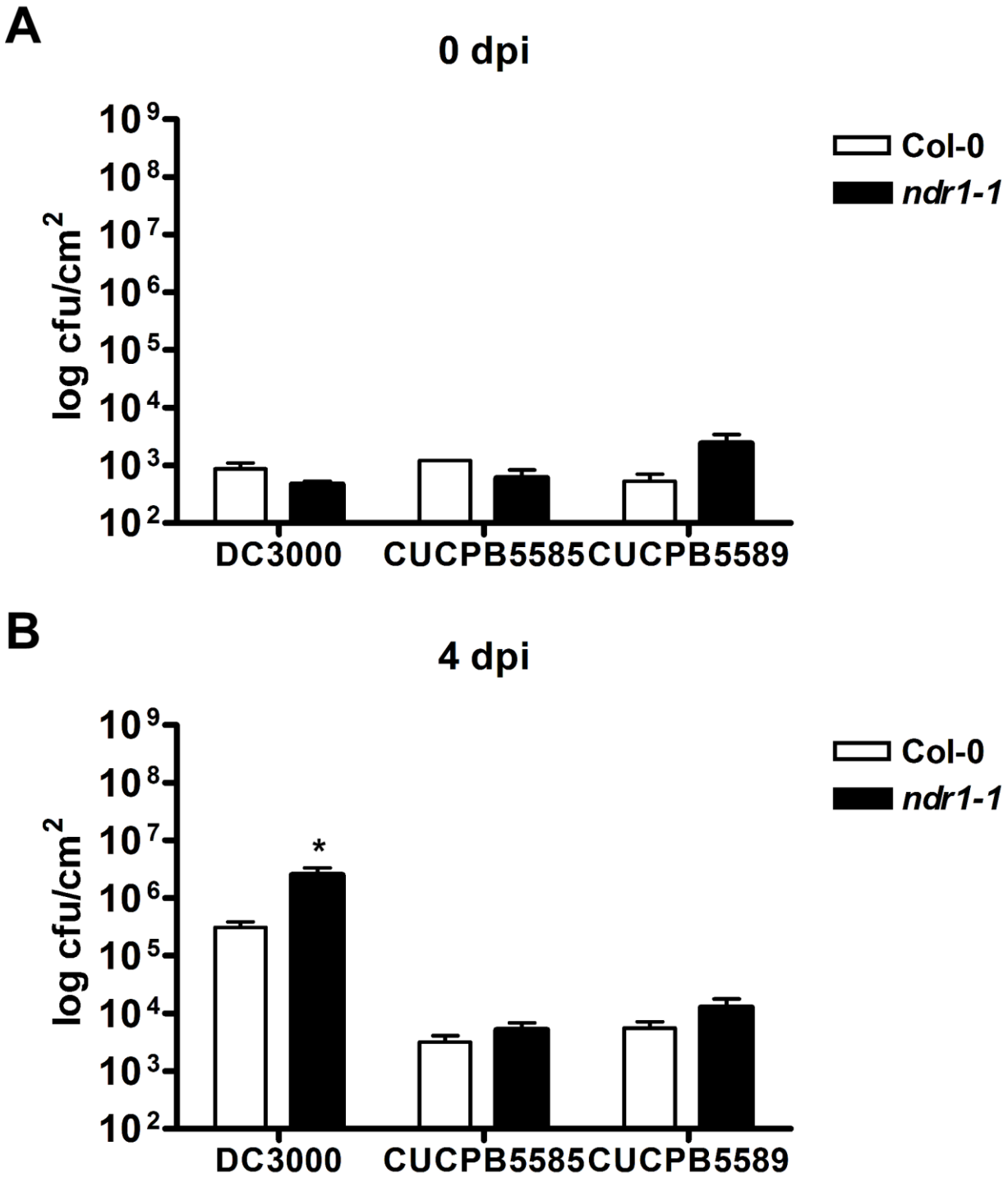

A
0 dpi
log cfu/cm2
10 9
10 8
10 7
10 6
10 5
10 4
10 3
10 2
Col-0
ndr1-1
DC3000
CUCPB5585
CUCPB5589
B
4 dpi
log cfu/cm2
*
Col-0
ndr1-1
DC3000
CUCPB5585
CUCPB5589

Figure 3.5. The growth of *P. syringae* DC3000 deficient in 28 effectors (D28E) is increased in the *ndr1-1* mutant.

A, Bacterial growth assay of *P. syringae* DC3000 D28E (CUCPB5585) or B, type III secretion system deficient D28E (CUCPB5589) at 0 and 4 days-post-inoculation (dpi). Error bars represent the standard deviation from n=3; day 0 and n=9; day 4. Significance was determined using Student's t-test, where * represents statistically significant differences between Col-0 and *ndr1-1*: * $P < 0.05$.

Figure 3.6.

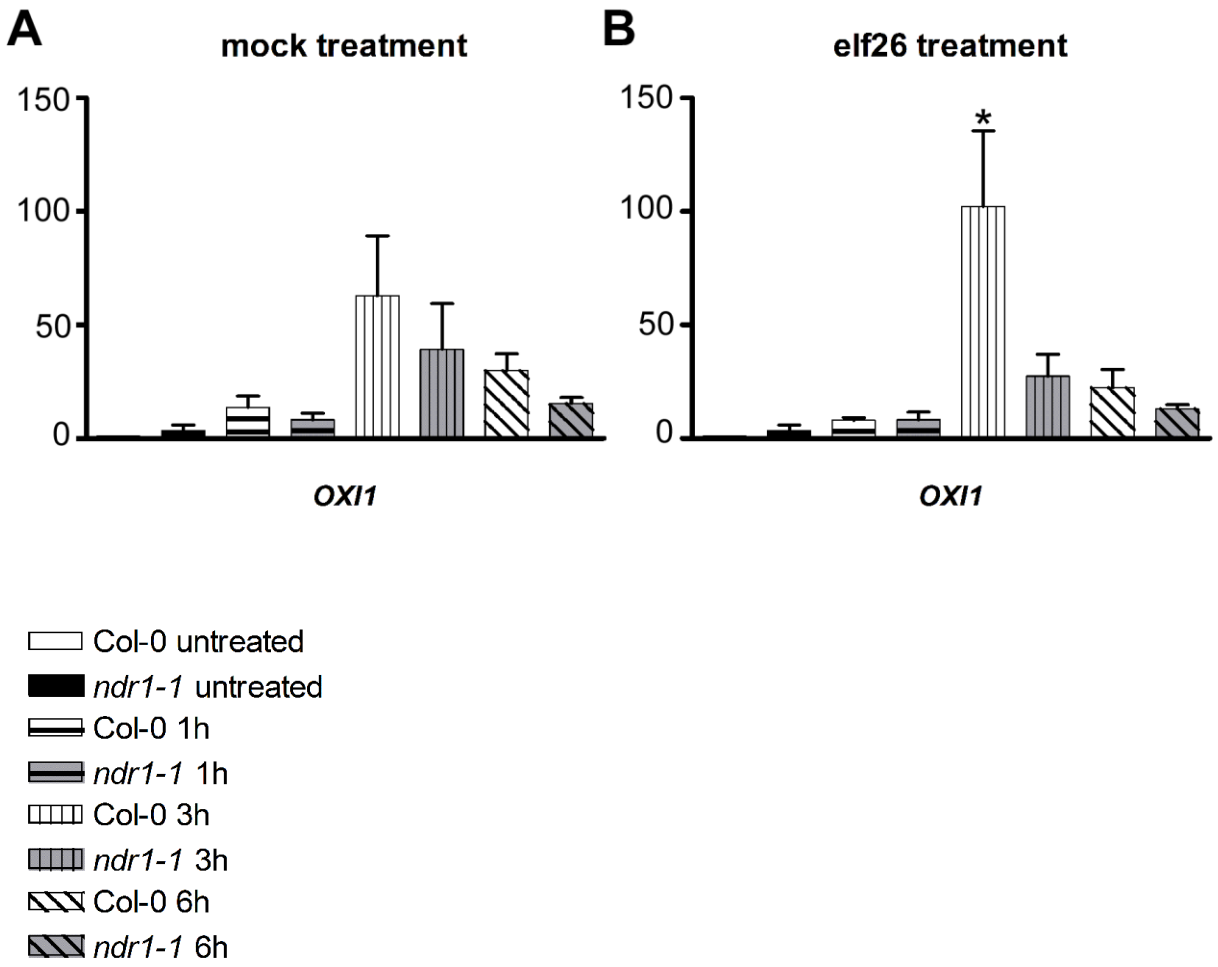

A
mock treatment
150
100
50
0
OXI1
B
elf26 treatment
150
100
50
0
*
OXI1
Col-0 untreated
ndr1-1 untreated
Col-0 1h
ndr1-1 1h
Col-0 3h
ndr1-1 3h
Col-0 6h
ndr1-1 6h

Figure 3.6. (cont'd)

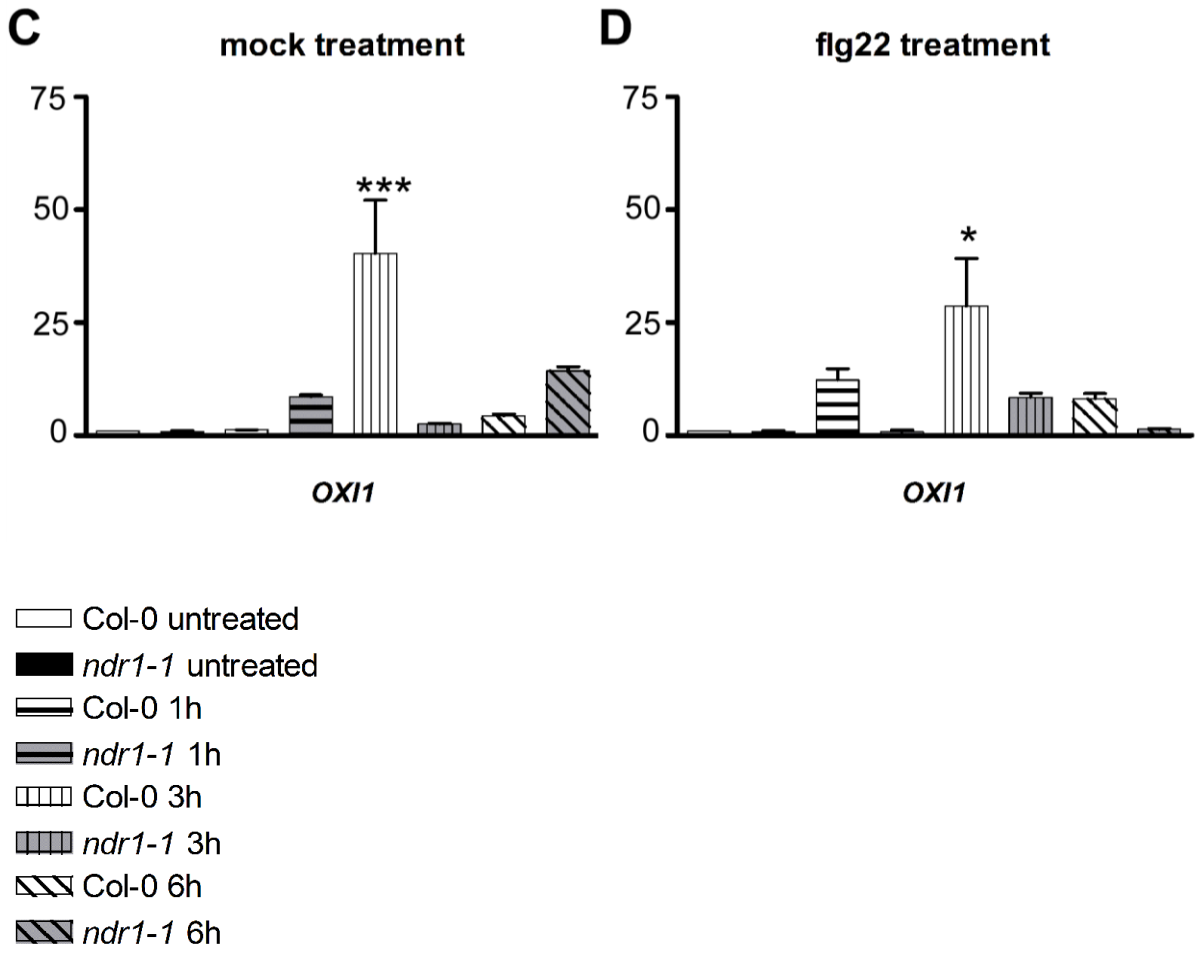


Figure 3.6. (cont'd)

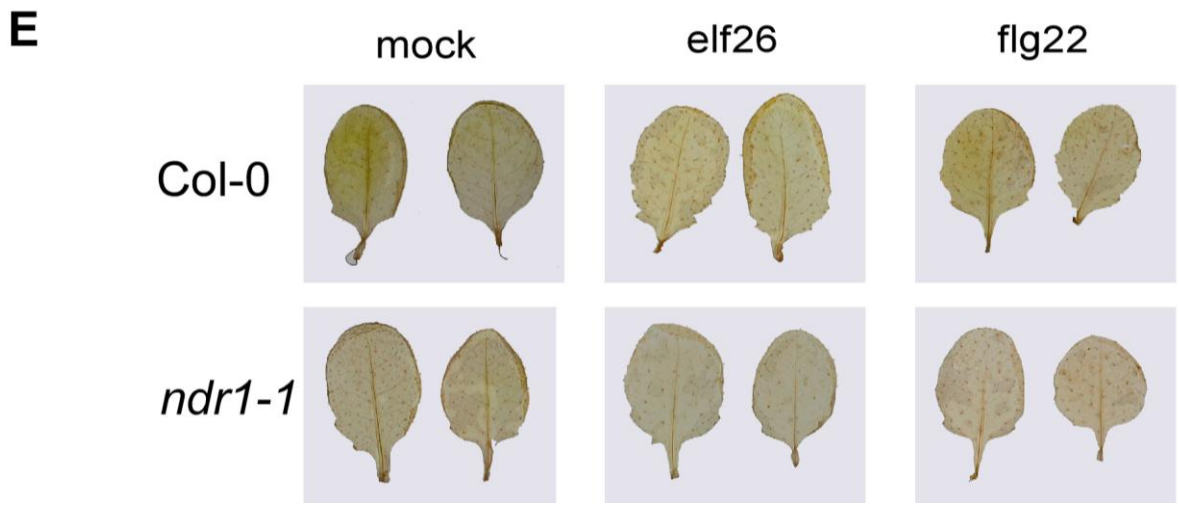


Figure 3.6. The production of reactive oxygen species is reduced in the *ndr1-1* mutant following PAMP perception.

Expression of *OXI1* mRNA, displayed as fold Col-0 untreated, following 1 µM elf26 (B) or 1 µM flg22 (D) treatment or mock treated corresponding with the PAMP treatments (A, elf26; C, flg22). E, 3,3'-diamainobenzidine (DAB) stained leaves showing altered reactive oxygen species generation in Col-0 (upper panel) and *ndr1-1* (lower panel) in response to mock, elf26 or flg22 treatment. Error bars display standard deviation from three biological replicates. Significance was determined using one-way ANOVA followed by Tukey's post test, where * represent statistically significant differences between Col-0 and *ndr1-1*: * $P < 0.05$ and *** $P < 0.001$.

Figure 3.7.

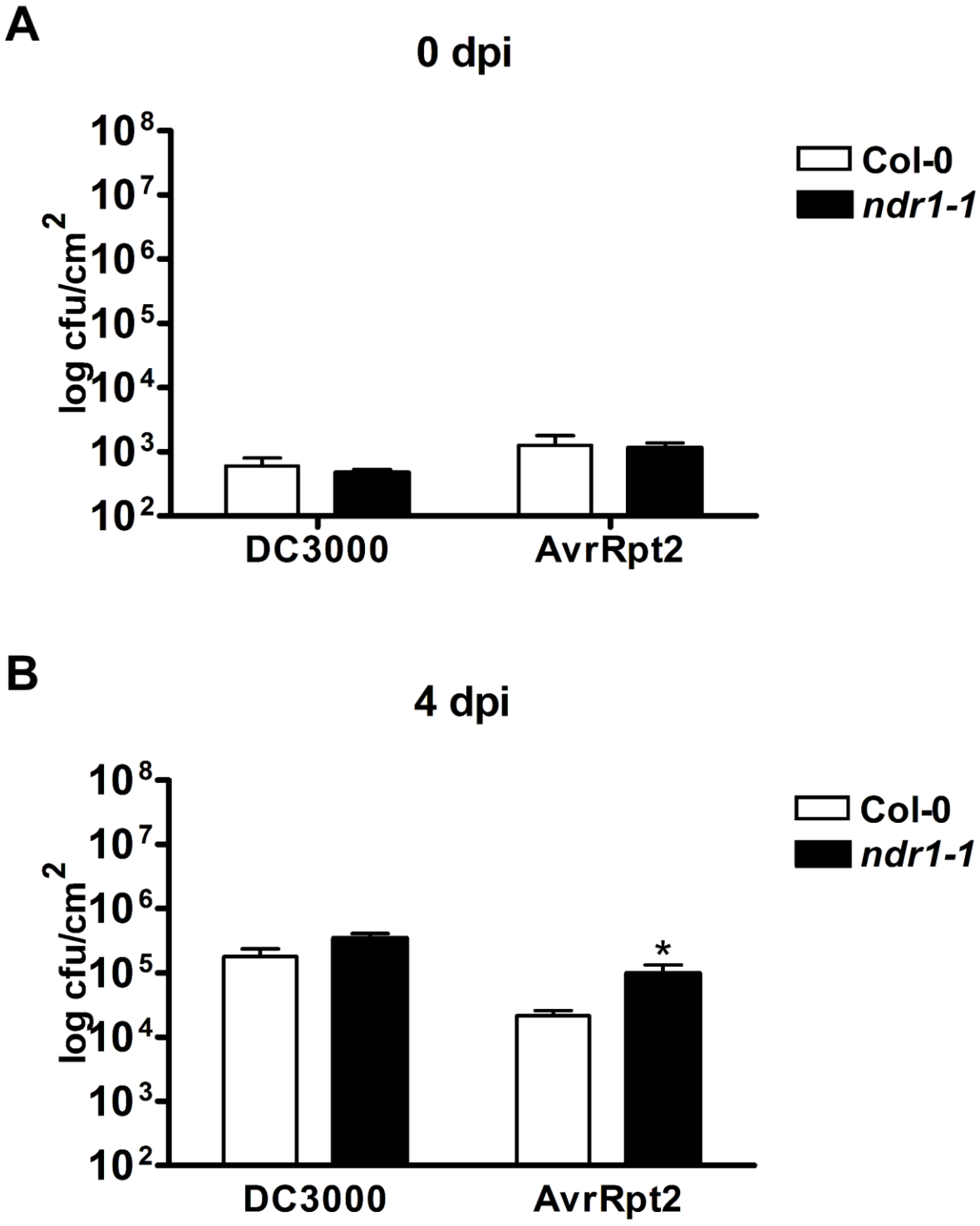

A
0 dpi
log cfu/cm2
10^8
10^7
10^6
10^5
10^4
10^3
10^2
DC3000
AvrRpt2
Col-0
ndr1-1
B
4 dpi
log cfu/cm2
10^8
10^7
10^6
10^5
10^4
10^3
10^2
*
DC3000
AvrRpt2
Col-0
ndr1-1

Figure 3.7. Benzo(1,2,3)-Thiadiazole-7-Carbothioic Acid S-Methyl Ester (BTH) restores resistance in *ndr1-1* in a PTI specific manner.

Leaves were hand infiltrated with 0.12 mM BTH 48 hours prior to bacterial dip inoculation. Assay for bacterial growth in Col-0 and *ndr1-1* of *P. syringae* DC3000 or DC3000 expressing the effector AvrRpt2 at 0 (A) and 4 (B) days post inoculation (dpi). Growth is expressed as log cfu cm^{-2}. Error bars display standard deviation from n=3; day 0 and n=9; day 4. Significance was determined using one-way ANOVA followed by Tukey's post test, where * represent statistically significant differences between Col-0 and *ndr1-1*: * $P < 0.05$.

Figure 3.8.

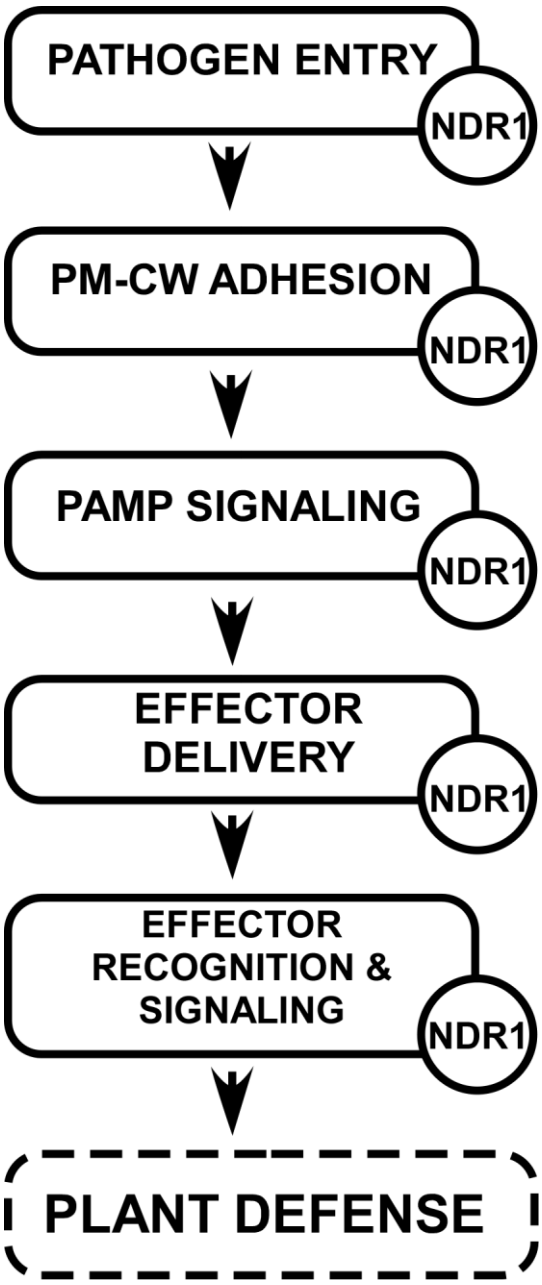

PATHOGEN ENTRY
NDR1
PM-CW ADHESION
NDR1
PAMP SIGNALING
NDR1
EFFECTOR DELIVERY
NDR1
EFFECTOR RECOGNITION & SIGNALING
NDR1
PLANT DEFENSE

Figure 3.8. Summary of NDR1's involvement in the perception of *Pst* DC3000 and the subsequent activation of defense signaling in Arabidopsis.

NDR1 has been shown to be involved in multiple steps of perception and resistance activation following *Pst* DC3000 inoculation. Steps in which NDR1 has been shown to be involved include pathogen entry (Figure 3.1), plasma membrane-cell wall (PM-CW) adhesion (Knepper et al., 2011), PAMP signaling (Figure 3.2; Knepper et al., 2011), effector delivery (Figure 3.3 and 3.4) and effector recognition and resistance signaling (Century et al., 1995, Coppinger et al., 2004; Day et al., 2006; Knepper et al., 2011).

LITERATURE CITED

LITERATURE CITED

Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J. and Parker, J.E. (1998) Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. **95**: 10306-10311.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W., Gomez-Gomez, L., Boller, T., Ausubel, F.M. and Sheen, J. (2002) MAP kinase signaling cascade in Arabidopsis innate immunity. Nature **415**: 977-983.

Axtell, M.J. and Staskawicz, B.J. (2003) Initiation of *RPS2*-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. Cell **112**: 369-377.

Bender, C.L., Alarcon-Chaidez, F. and Gross, D.C. (1999) *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol. Mol. Biol. Rev. **63**: 266-292.

Beckers, G.J.M., Jaskiewicz, M., Liu, Y., Underwood, W.R., He, S.Y., Zhang, S. and Conrath, U. (2009) Mitogen-activated protein kinases 3 and 6 are required for full priming of stress responses in *Arabidopsis thaliana*. Plant Cell **21**: 944-953.

Bolwell, G.P. and Wojtaszek, P. (1997) Mechanisms for the generation of reactive oxygen species in plant defence- a broad perspective. Physiol. Mol. Plant Pathol. **51**: 347-366.

Boudsocq, M., Willmann, M.R., McCormack, M., Lee, H., Shan, L., He, P., Bush, J., Cheng, S. and Sheen, J. (2010) Differential innate immune signaling via Ca^{2+} sensor protein kinases. Nature **464**: 418-423.

Casper-Lindley, C., Dahlbeck, D., Clark, E.T. and Staskawicz, B.J. (2002) Direct biochemical evidence for type III secretion-dependent translocation of the AvrBs2 effector protein into plant cells. Proc. Natl. Acad. Sci. U. S. A. **99**: 8336-8341.

Century, K., Holub, E.B. and Staskawicz, B.J. (1995) NDR1, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and fungal pathogen. Proc. Natl. Acad. Sci. U.S.A. **92**: 6597-6601.

Century, K.S., Shapiro, A.D., Repetti, P.P., Dahlbeck, D., Holub, E. and Staskawicz, B.J. (1997) NDR1, a pathogen-induced component required for Arabidopsis disease resistance. Science **278**: 1963-1965.

Chisholm, S.T., Coaker, G., Day, B. and Staskawicz, B.J. (2006) Host-microbe interactions: shaping the evolution of the plant immune response. Cell **124**: 803-814.

Coppinger, P., Repetti, P.P., Day, B., Dahlbeck, D., Mehlert, A. and Staskawicz, B.J. (2004) Overexpression of the plasma membrane-localized NDR1 protein results in enhanced bacterial disease resistance in *Arabidopsis thaliana*. Plant J. **40**: 225-237.

Crabill, E., Joe, A., Block, A., van Rooven, J.M. and Alfano, J.R. (2010) Plant immunity directly or indirectly restricts the injection of type III effectors by the *Pseudomonas syringae* type III secretion system. Plant Physiol. **154**: 233-244.

Cunnac, S., Chakravarthy, S., Kvitko, B.H., Russell, A.B., Martin, G.B. and Collmer, A. (2011) Genetic disassembly and combinatorial reassembly identify a minimal functional repertoire of type III effectors in *Pseudomonas syringae*. Proc. Natl. Acad. Sci. U.S.A. **15**: 2975-2980.

Davies, D.R., Bindschedler, L.V., Strickland, T.S. and Bolwell, G.P. (2006) Production of reactive oxygen species in *Arabidopsis thaliana* cell suspension cultures in response to an elicitor from *Fusarium oxysporum*: implications for basal resistance. J. Exp. Bot. **57**: 1817-1827.

Day, B., Dahlbeck, D. and Staskawicz, B.J. (2006) NDR1 interaction with RIN4 mediates the differential activation of multiple disease resistance pathways in Arabidopsis. Plant Cell **18**: 2782-2791.

Forzani, C., Carreri, A., de la Fuente van Bentem, S., Lecourieux, F. and Hirt, H. (2011) The Arabidopsis protein kinase Pto-interacting 1-4 is a common target of the oxidative signal-inducible 1 and mitogen-activated protein kinases. FEBS J. **278**: 1126-1138.

Fry, S.C., Willis, S.C. and Patterson, A.E.J. (2000) Intraprotoplasmic and wall localized formation of arabinoxylan-bound diferulates and larger ferulate coupling products in maize cell-suspension cultures. Planta **211**: 679-692.

Gómez-Gómez, L., Felix, G. and Boller, T. (1999) A single locus determines sensitivity to bacterial flagellin in Arabidopsis thaliana. Plant J. **18**: 277-284.

Grant, J.J. and Loake, G.J. (2000) Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. Plant Physiol. **124**: 21-29.

Hauck, P., Thilmony, R. and He, S.Y. (2003) A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible Arabidopsis plants. Proc. Natl. Acad. Sci. USA **100**: 8577-8582.

Katagiri, F., Thilmony, R. and He, S.Y. (2002) The *Arabidopsis thaliana-Pseudomonas syringae* interaction. The Arabidopsis Book 1: e0039, doi/10.1199/tab.0039.

Kiba, A., Sugimoto, M., Toyoda, K., Ichinose, Y., Yamada, T. and Shiraishi, T. (1998) Interaction between cell wall and plasma membrane via RGD motif is implicated in plant defense responses. Plant Cell Physiol. **39**: 1245-1249.

Kim, M.G., da Cunha, L., McFall, A.J., Belkhadir, Y., DebRoy, S., Dangl, J.L. and Mackey, D. (2005) Two *Pseudomonas syringae* type III effectors inhibit RIN4-regulated basal defense in Arabidopsis. Cell **121**: 749-759.

Keppler, L.D., Baker, C.J. and Atkinson, M.M. (1989) Activated oxygen production during a bacteria induced hypersensitive reaction in tobacco suspension cells. Phytopathology **79**: 974-978.

Knepper, C., Savory, E.A. and Day, B. (2011) Arabidopsis NDR1is an integrin-like protein with a role in fluid loss and plasma membrane-cell wall adhesion. Plant Physiol. **156**: 286-300.

Kunkel, B.N., Bent, A.F., Dahlbeck, D., Innes, R.W. and Staskawicz, B.J. (1993) RPS2, an Arabidopsis disease resistance locus specifying recognition of *Pseudomonas syringae* strains expressing the avirulence gene avrRpt2. Plant Cell **5**: 865–875.

Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T. and Felix, G. (2004) The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. Plant Cell **16**: 3496-3507.

Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T., Ryals, J. (1996) Benzothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. Plant J. **10**: 71-82.

Ma, S.W., Morris, V.L. and Cuppels, D.A. (1991) Characterization of a DNA region required for production of the phytotoxin coronatine by *Pseudomonas syringae* pv. *tomato*. Mol. Plant Microbe Interact. **4**, 69–77.

Mackey, D., Holt, B.F., Wiig, A. and Dangl, J.L. (2002) RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. Cell **108**: 743-754.

Mackey, D., Belkhadir, Y., Alonso, J.M., Ecker, J.R. and Dangl, J.L. (2003) *Arabidopsis* RIN4 is the target of the type III virulence effector AvrRpt2 and modulates RPS2 mediated resistance. Cell **112**: 379-389.

Mellersh, D.G. and Heath, M.C. (2001) Plasma membrane-cell wall adhesion is required for expression of plant defense responses during fungal penetration. Plant Cell **13**: 413-424.

Melotto, M., Underwood, W., Koczan, J., Nomura, K. and He, S.Y. (2006) Plant stomata function in innate immunity against bacterial invasion. Cell **126**: 969-980.

Otegui, M.S. and Spitzer, C. (2008) Endosomal functions in plants. Traffic **9**: 1589-1598.

Petersen, L.N., Ingle, R.A., Knight, M.R. and Denby, K.J. (2009) OXI1 protein kinase is required for plant immunity against *Pseudomonas syringae* in Arabidopsis. J. Exp. Bot. **60**: 3727-3735.

Qi, Y., Tsuda, K., Glazebrook, J. and Katagiri, F. (2011) Physical association of pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) immune receptors in Arabidopsis. Mol. Plant Pathol. **12**: 702-708.

Rentel, M.C., Lecourieux, D., Ouaked, F., Usher, S.L., Peterson, L., Okamoto, H., Knight, H., Peck, S.C., Grierson, C.S., Hirt, H. and Knight, M.R. (2004) OXI1 kinase is necessary for oxidative burst-mediated signaling in Arabidopsis. Nature **427**: 858-861.

Schwessinger, B. and Zipfel, C. (2008) News from the frontline: recent insights into PAMP-triggered immunity in plants. Curr. Opin. Plant Biol. **11**: 389-395.

Shapiro, A.D. and Zhang, C. (2001) The role of NDR1 in avirulence gene-directed signaling and control of programmed cell death in Arabidopsis. Plant Physiol. **127**: 1089-1101.

Tian, M., Chaudry, F., Ruzicka, D.R., Meagher, R.B., Staiger, C.J. and Day, B. (2009) Arabidopsis actin-depolymerizing factor *At*ADF4 mediates defense signal transduction triggered by the *Pseudomonas syringae* effector AvrPphB. Plant Physiol. **150**: 815-824.

Tornero, P. and Dangl, J.L. (2001) A high-throughput method for quantifying growth of phytopathogenic bacteria in Arabidopsis thaliana. Plant J. **28**: 475–481.

Torres, M.A., Dangl, J.L. and Jones, J.D.G. (2002) Arabidopsis gp91^{phox} homologues *AtrbohD* and *AtrbohF* are required for the accumulation of reactive oxygen intermediates in the plant defence response. Proc. Natl. Acad. Sci. U.S.A. **99**: 517-522.

Xiang, T., Zong, N., Zou, Y., Wu, Y., Zhang, J., Xing, W., Li, Y., Tang, X., Zhu, L., Chai, J. and Zhou, J. (2008) *Pseudomonas syringae* effector AvrPto blocks innate immunity by targeting receptor kinases. Curr. Biol. **18**: 74-80.

Zhang, J., Lu, H., Li, X., Li, Y., Cui, H., Wen, C., Tang, X., Su. Z. and Zhou, J. (2010) Effector-triggered and pathogen-associated molecular pattern-triggered immunity differentially contribute to basal resistance to *Pseudomonas syringae*. Mol. plant Microbe Interact. **23**: 940-948.

Zipfel, C. and Felix, G. (2005) Plants and animals: a different taste for microbe? Curr. Opin. Plant Biol. **8**: 353-360.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T. and Felix, G. (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. Cell **125**: 749-760.

Zipfel, C. (2008) Pattern-recognition receptors in plant innate immunity. Curr. Opin. Immunol, **20**: 10-16.

CHAPTER 4

NDR1 is involved in drought stress via an abscisic acid dependent pathway

INTRODUCTION

Numerous biotic and abiotic stresses impact plant health and crop production. Among these, disease pressure and water availability are two primary factors limiting agricultural productivity. To combat drought stress, plants have evolved a series of mechanisms to cope with low water availability. For example, one of the primary plant responses to drought stress is the reduction of transpiration, mediated by the rapid closure of stomata on the leaf surface. This process is regulated by the activity of the plant hormone abscisic acid (ABA; Shinozaki and Yamaguchi-Shinozaki, 2000; Finkelstein et al., 2002; Xiong et al., 2002), and is one of the primary mechanisms governing water loss in plants (Schroeder et al., 2001; Sirichandra et al., 2009). Membrane integrity is also a primary physiological process associated with dehydration, solute leakage and metabolic dysfunction (García-Gómez et al., 2000; Mahajan and Tuteja 2005). Solute leakage is a process by which plants attempt to overcome limited water availability by adjusting ionic as well as osmotic equilibrium of the cell in order to maintain cellular homeostasis under stress conditions (reviewed in Chaves et al., 2003). As plants attempt to respond to drought stress many changes occur in the cell including the activation of major stress responsive genes such as *RESPONSIVE TO DEHYDRATION* (*RD*) family genes (Yamaguchi-Shinozaki and Shinozaki, 1993), synthesis of LATE EMBRYOGENESIS ABUNDANT (LEA)/dehydrin-type proteins, synthesis of molecular chaperones which help in protecting the partner protein from degradation and proteinases that function to remove denatured and damaged proteins along with the synthesis of osmolytes (Mahajan and Tuteja 2005) and activation of enzymes involved in the production and removal of reactive oxygen species (ROS; Cushman and Bohnert 2000; Zhu 2002).

Many of these drought responsive genes are also activated by ABA (Ingram and Bartels, 1996; Seki et al., 2002). Recent evidence suggests the existence of a significant overlap between water stress and disease resistance pathways (Lee and McNellis 2009; Mosher et al. 2010). ABA is of particular importance in the overlap of these two seemingly different stress responsive pathways. ABA-dependent signaling appears to be able to modulate responses between drought and pathogen stresses leading to the suppression of defense responses during severe water stress (reviewed in Beattie 2011). Previously the exogenous application of ABA has been shown to enhance disease susceptibility (Yasuda et al. 2008; Fan et al. 2009) while an insensitivity to ABA enhances disease resistance (Cao et al. 2011).

Several of the resistance mechanisms by which plants respond to pathogen infection appear somewhat similar to those that occur early in drought stress. Arabidopsis has evolved multiple layers of both constitutive and induced defenses to respond to pathogen infection beginning on the cell surface where Pattern Recognition Receptors (PRRs) detect conserved Pathogen Associated Molecular Patterns (PAMPs) activating basal immunity including the closure of stomata to prevent pathogen entry (Melotto et al., 2006). Stomata also serve an important function in the regulation of water stress, by regulating the rate of transpiration through plant leaves (Chaves et al. 2003). The regulation of stomatal aperture is an ABA-dependent process in which ABA signaling causes a conformational change in the shape of the guard cells leading to a decrease in aperture size (review by Chaves et al. 2003). As such, modulation of stomatal aperture appears to be an important initial response for both disease resistance as well as the prevention of water loss, further supporting an overlap between biotic and abiotic stress responsive mechanisms

NON-RACE SPECIFIC DISEASE RESISTANCE1 (*NDR1*; Century *et al.*, 1995; Century *et al.*, 1997; Coppinger *et al.*, 2004) is one of many resistance (*R*)-gene accessory proteins that

serve as a co-activator of resistance. Recently, a physiological role for NDR1 in regulating fluid loss and plasma membrane-cell wall (PM-CW) adhesion has been proposed (Knepper et al. 2011). As a primary physiological process required for mediating cellular response to abiotic and biotic stress signaling, PM-CW adhesion has been well-characterized (Canut et al., 1998; Kohorn, 2000; Mellersh and Heath, 2001; Knepper et al., 2011). A loss of PM-CW adhesion in *ndr1-1* leads to a reduction in effector delivery by the type-three secretion system to the plant and a subsequent reduction in effector detection and initiation of robust defense responses including the initiation of Effector Triggered Immunity (ETI; Knepper et al., submitted).

Based on previous results, indicating a strong connection between NDR1 and PM-CW adhesion and electrolyte leakage (Knepper et al., 2011), along with the link between NDR1 and stomatal response (Knepper et al., submitted) we hypothesized that NDR1 may fulfill a physiological and/or signaling role in the response to drought stress. Furthermore, it has been shown that members of the *NDR1/HIN1-LIKE* (*NHL*) gene family, which includes *NDR1* and are involved in defense responses to pathogens, also have non-defensive roles (Zheng et al., 2004; Takahashi et al. 2004; Quirino et al. 2000; Pontier et al. 1999). Additionally, Zhang et al., (2011) reported that a related gene, *HRF1*, when over-expressed in rice, enhances resistance to both pathogens and abiotic stress. In this study we demonstrate NDR1 is in fact required for drought tolerance and serves as a mediator between disease resistance and abiotic stress signaling. Furthermore, the over-expression of NDR1 increases the resistance of Arabidopsis to drought.

RESULTS

NDR1 functions in drought stress tolerance in *Arabidopsis thaliana*.

Previous studies have demonstrated that an increase in electrolyte leakage following *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 infection in the *ndr1-1* mutant is correlated with a loss in focal adhesion between the plasma membrane (PM) and cell-wall (CW) in *ndr1-1* (Knepper et al. 2011). Based on these observations, we hypothesized that NDR1 would play a role in abiotic stress responses, specifically the response to drought stress. To assess the involvement of NDR1 in drought stress tolerance, two basic parameters for assaying drought stress in leaves were utilized; relative water content (RWC %) and electrolyte leakage. As shown in Figures 4.1A and 4.1C, Col-0, *ndr1-1* and the NDR1 over-expressing line (347; Coppinger et al., 2004) displayed similar RWC and electrolyte leakage measurements throughout the course of the experiment when soil moisture was maintained at non-stress conditions. However, under severe drought stress conditions, differences in RWC were observed (Figure 4.1B) in a genotype-dependent manner. In wild-type Col-0 no changes in RWC were observed until eight days after the initiation of drought stress after this period, the RWC in Col-0 fell to 34% and by day 12 Col-0 had lost all turgor pressure. The *ndr1-1* mutant showed a decline in RWC beginning four days after watering was ceased. By day eight, the RWC of *ndr1-1* was reduced to 45%, and by day 11 the *ndr1-1* plants were dead. In contrast to the loss in drought tolerance observed in the *ndr1-1* mutant, the NDR1 over-expressor, 347, maintained RWC through day eight. After eight days of drought stress the RWC of 347 began to decrease slowly reaching 50% by day 12. A striking difference in each genotype's response to drought stress can be observed in Figure 4.1F, where eight days after the initiation of drought stress both Col-0 and *ndr1-1* plants show substantial decreases in overall plant health as compared to the over-expression line. These results were further supported by the electrolyte leakage observed in the three lines (Figure 4.1D). An increase in leakage was observed just before plants lost turgor pressure and could not be recovered from the drought stress. In *ndr1-1*, this increase in electrolyte leakage was observed at day 10,

whereas a smaller increase was observed in Col-0 at day 10 aligning with plant death for both lines; Col-0 at day 11 and *ndr1-1* at day 10. In the NDR1 over-expression line,an increase in leakage was not observed until day 12 and plants survived until day 14. This finding further supports the hypothesis that NDR1 is involved in tolerance to drought stress. The loss of NDR1 reduced the ability of Arabidopsis to withstand drought while the over-expression of NDR1 yielded a plant more tolerant of drought conditions than the wild-type Col-0.

To understand the genetic basis of the role of NDR1 in drought tolerance, we examined the expression profiles of several well-characterized genes associated with the induction of drought stress in Arabidopsis. Analysis was performed using qRT-PCR utilizing two common drought stress markers genes including the abscisic acid (ABA)-dependent *RESPONSIVE TO DESICCATION* (*RD29B*; Yamaguchi-Shinozaki and Shinozaki 1993) and ABA-independent *EARLY-RESPONSIVE TO DEHYDRATION* (*ERD4*; Kiyosue et al. 1994) gene. As shown in Figure 4.1E, on day eight a significant increase ($P < 0.001$) in *RD29B* mRNA expression was observed in the *ndr1-1* mutant as compared to Col-0 and 347. No differences were observed in mRNA expression for the ABA-independent drought marker *ERD4*. These data indicate that the loss of *ndr1-1* increases drought stress within Arabidopsis in an ABA-dependent manner, suggesting that NDR1 functions in the ABA-dependent pathway linked to drought stress signaling.

NDR1 is required for ABA-dependent stomata closure

Based on two observations, the response of the ABA-dependent drought marker gene *RD29B* (Figure 4.1E) as well as the reduced stomatal response to a Pathogen Associated Molecular Pattern (PAMP) trigger previously observed in the *ndr1-1* mutant (Knepper et al., submitted), we hypothesized that NDR1 plays a role in the regulation of stomatal closure in an ABA-

dependent manner. To determine the role of NDR1 in stomatal closure, epidermal peels of Col-0 and *ndr1-1* were treated with 10 µM ABA, and after one hour, the guard cell aperture was measured. As shown in Figure 4.2, we observed a significant reduction in guard cell aperture in WT Col-0, as compared to *ndr1-1*, following ABA treatment. The stomata apertures of *ndr1-1* showed no change between mock and ABA treatments. These results indicate that guard cell dynamics and aperture are impacted in the *ndr1-1* mutant and that NDR1 is required for stomata closure in an ABA-dependent manner. It appears based on these data as well as previous results (Knepper et al., submitted) that in the absence of NDR1 there is a disconnect between the perception of a signal to close stomata (e.g. ABA or PAMP) and the physical change in guard cells leading to a reduction in aperture width.

A coronatine deficient *Pseudomonas syringae* strain can grow to higher levels in the *ndr1-1* mutant.

Many plant pathogens, including *P. syringae*, enter the host through stomata. In response, one of the first resistance mechanisms activated in Arabidopsis to potential pathogen invasion is the closure of stomata to prevent pathogen entry. Previous work has shown that stomata close within one hour after exposure to *Pst* DC3000 (Melotto et al., 2006). In *Pst* DC3000 the production of the jasmonic acid (JA) mimic coronatine can re-open stomata by three hours allowing pathogen entry (Melotto et al., 2006). Based on previous findings linking NDR1 to PAMP-induced as well as ABA-induced stomata closure (Knepper et al., submitted; Figure 4.2) we hypothesized that defects in stomatal closure in the *ndr1-1* mutant would allow for a coronatine deficient strain of *Pst* DC3000 (*Pst* DC3118; Ma et al., 1991) to gain access to the leaf interior and thus grow to higher levels in the mutant. The *ndr1-1* mutant showed an increase in growth of *Pst* DC3000 of greater than one log cfu/cm^2 (colony forming units/cm^2) as compared to wild-type Col-0 (Figure 4.3). When the growth of the coronatine mutant, *Pst*

DC3118, was assessed an increase in growth was observed of 0.75 log cfu/cm^2 as compared to Col-0. Brooks et al. (2004) demonstrated that coronatine-deficient mutant strains show a decrease in growth when surface inoculated; when syringe infiltrated, thus bypassing the stomata, the growth observed is similar to strains that produce coronatine. In the *ndr1-1* mutant, the increase in growth observed when surface inoculated with DC3118, as compared to Col-0, indicates that NDR1 is required for stomata aperture closure in response to *P. syringae*, which corroborates previous results with PAMP induced stomatal closure (Knepper et al., submitted). The ability of the coronatine-deficient mutant to enter the host, as measured by increased pathogen growth, indicates that the stomata in *ndr1-1* are open to bacterial entry. These data demonstrate a direct resistance consequence for NDR1 in stomata aperture regulation, as stomata that are unresponsive to potential pathogen invasion leave the host vulnerable to pathogen infection.

Disease resistance is not suppressed by ABA in the *ndr1-1* mutant.

The hormone ABA accumulates in response to drought stress, promoting physiological changes such as stomatal closure under conditions of low humidity. ABA also can interfere with plant-pathogen interactions leading to the suppression of defense responses (Mauch-Mani and Mauch, 2005; Asselbergh et al., 2008). Several studies have demonstrated the ability of ABA to suppress defense signaling, including the exogenous application of ABA being shown to enhance disease susceptibility (i.e., suppression of systemic acquired resistance; Yasuda et al., 2008; Fan et al., 2009) while ABA insensitive plants have demonstrated enhanced resistance (Cao et al., 2011). These varied responses to ABA appear to indicate a metabolic tradeoff between drought tolerance and disease resistance. As shown in Figure 4.2, stomata of the *ndr1-1* mutant are less responsive to the exogenous application of ABA. Therefore, we hypothesized that bacterial growth in *ndr1-1* plants will be unaltered in

response to the application of ABA, whereas growth of *Pst* DC3000 in Col-0 is likely to increase with ABA-induced defense suppression. To test this, four-week-old Col-0 and *ndr1-1* plants were pre-treated by soil drench with 400 μM ABA or water (control) five days prior to inoculation with *Pst* DC3000. Bacterial growth was assayed zero and four days post inoculation. As shown in Figure 4.4, wild type Col-0 plants pre-treated with ABA had an increase in growth of *Pst* DC3000 of greater than 0.5 log cfu/cm^2 as compared to control Col-0 plants. In *ndr1-1* only a negligible change in bacterial growth was observed with ABA pre-treatment. While the difference in bacterial growth in wild type Col-0 plants is not assigned statistically significance at $P<0.05$, it is a reproducible increase observed across three biological replicates (n = 9). The results of this analysis suggest that in the absence of NDR1, plants are less responsive to exogenous ABA treatment, and this can be observed in the lack of ABA-based defense suppression. In contrast, Col-0 shows a consistent increase in bacterial growth after ABA application showing the previously established suppression of defenses in response to exogenous ABA application (Yasuda et al., 2008; Fan et al., 2009). While the differences in bacterial growth changes were somewhat smaller than anticipated these data, taken with previous results (Figure 4.2), support a mechanistic link between ABA and NDR1 function.

DISCUSSION

For more than 15 years the identification of a functional role for NDR1 in plant disease resistance, to complement the strong genetic requirement first identified (Century et al., 1995), has been a slow but rewarding endeavor. Recent results from our laboratory (Knepper et al., 2011) were able to identify a specific requirement for NDR1 in the maintenance of PM-CW adhesion as well as a functional consequence in the mediation of fluid loss. With a basic physiological function for NDR1 beyond the scope of disease resistance established, our focus

shifted to the identification of specific consequences of NDR1 in Arabidopsis biology. Based on the functions assigned for NDR1 in adhesion and fluid movement, plus the link between NDR1 and stomata function (Knepper et al., submitted) we hypothesized that NDR1 would function in drought stress signaling. This was further strengthened by the structural homology between NDR1 and LATE EMBRYOGENESIS ABUNDANT-14 (LEA14; Knepper et al., 2011), a protein known to function in desiccation resistance (Singh et al., 2005) and a member of the LEA family of proteins closely associated with abiotic stress resistance (Goyal et al., 2005; Battaglia et al., 2008), as well as the recent report of the harpin-encoding *HRF1* increasing resistance to drought when over-expressed in rice (Zhang et al., 2011).

While the *ndr1-1* mutant line has been examined for years in the context of disease resistance, pursuing a function for NDR1 proved challenging. This study required the analysis of both a knock-out mutant (*ndr1-1*) and an over-expression line (347) to fully understand the functional significance of NDR1 in drought tolerance. By examining the RWC and electrolyte leakage within the leaves of Col-0, *ndr1-1* and 347 several trends were identified. In the *ndr1-1* mutant RWC began to decrease earlier and the plants lost turgor pressure and died earlier than either the Col-0 or 347 plants (Figure 4.1B, 4.1F). The electrolyte leakage data further supported this in that an increase in leakage corresponding with plant death is observed at day ten in *ndr1-1* which is prior to plant death in Col-0. In contrast, Arabidopsis over-expressing NDR1 demonstrated a reduction in water loss under drought stress conditions, observed as reduced loss of RWC through day 12 (Figure 4.1B). Plants over-expressing NDR1 also do not exhibit an increase in electrolyte leakage for nearly two days after the *ndr1-1* mutant and one day later than wild-type Col-0. Together with the increase in *RD29B* expression observed in drought stressed *ndr1-1* plants it is clear that NDR1 is a critical component of drought tolerance in Arabidopsis.

With a link now established between NDR1 and drought tolerance we began to examine the mechanism behind NDR1-dependent drought tolerance likely involving the regulation of stomata, which serve as both a critical regulator of pathogen entry (Melotto et al., 2006) and a regulator of water loss through the control of leaf transpiration (Chaves et al., 2003), based on the recent demonstration of a link between NDR1 and stomata aperture regulation (Knepper et al., submitted). Arabidopsis plants that are unable to regulate stomata aperture width, such as *ndr1-1*, could potentially lose leaf water content more rapidly than wild-type plants, leading to a reduction in tolerance to water stress. The regulation of stomatal closure is an ABA-dependent process leading to a conformational change in guard cells and a decrease in aperture size (reviewed in Chaves et al., 2003). Plants lacking a functional NDR1 were shown to be insensitive to ABA-induced stomatal closure (Figure 4.2). This may partially explain why the *ndr1-1* mutant shows increased rates of water loss, potentially due to improper regulation of leaf transpiration through stomata. The role of NDR1 in ABA signaling is further supported in the insensitivity to exogenous ABA-induced disease resistance suppression observed in *ndr1-1*.

With the defects observed in the *ndr1-1* mutant we hypothesized that a *Pst* DC3000 strain that is coronatine defective would be more virulent on *ndr1-1* plants as compared to Col-0, based on the inability of *ndr1-1* to close stomata in response to either PAMP signals (Knepper et al., submitted) or exogenous ABA application. *Pst* DC3118 which is defective in entry to the leaf, has been shown to grow to levels equivalent those of a coronatine producing strain if syringe-infiltrated and thus bypassing stomata entry (Brooks et al., 2004). The coronatine deficient strain is able to grow to higher levels in the *ndr1-1* mutant as compared to Col-0 indicating stomata do not serve as an effective barrier in *ndr1-1*. This result shows the interconnected nature of NDR1-dependent signaling in that both drought tolerance and disease resistance are strongly altered in plants lacking functional NDR1.

With the identification of a physiological role for NDR1 the way in which resistance associated proteins are viewed has begun to change. Based on the data presented herein, NDR1 can be considered a protein of physiological relevance first, and not simply an *R*-gene accessory protein as long thought. The examination of metabolic and physiological trade-offs within plants to biotic and abiotic stresses are and will continue to be a critical part of plant biology research. The identification of proteins such as NDR1 with functions in both disease resistance and drought stress tolerance will help to integrate our knowledge of biotic and abiotic stress signaling. By demonstrating a function for NDR1 in abiotic stress tolerance we have further cemented the importance of NDR1 not only in disease resistance signaling and PM-CW adhesion, but also in the mediation of tolerance to an abiotic stress of ever increasing importance: drought.

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis thaliana Col-0, *ndr1-1* (Century et al., 1995) and 347 (*NDR1* over-expressor; Coppinger et al. 2004) plants were grown in square potting trays (weighed and adjusted for initial moisture content) during four weeks under standard watering conditions (100% soil moisture), in a model GC8-2H growth chamber (Environmental Growth Chambers LTD., Winnipeg, Manitoba, Canada) at 20°C, with a 12 h/12 h light/dark cycle, 60% relative humidity and a light intensity of 120 μmol photons $m^{-2}s^{-1}$.

Drought stress treatment

After 4 weeks, the water content of the flats was adjusted to 100 % (day 0) and plants were then subjected to 2 watering regimes: 1) control (C), where plants were watered three times weekly, and 2) drought stress (DS) conditions, where plants received no additional water for the remainder of the experiment. Leaf samples were harvested at 0, 2, 6, 8, 10 and 12 days after drought stress and immediately frozen in liquid nitrogen and kept at -80ºC.

Relative Water Content Measurements

Relative water content (RWC %; Catsky, 1960) was measured in leaves from plants submitted to control and drought stress conditions as described: 4-8 leaves from 3-4 plants were harvest at 0, 2, 6, 8, 10 and 12 days after drought stress and immediately weighed to obtain the fresh weight (FW). Next, the leaves were left to float on a covered Petri dish containing deionized water, during 24 hours, in the dark. The turgid weight (TW) of these leaves was then measured. Finally, the leaves were dried in an oven at 80 ºC, for 24 hours, taken out 15 minutes before the dry weight (DW) was measured. RWC was calculated according to the formula: RWC (%) = (FW)-(DW)/(TW)-(DW) X 100.

Electrolyte leakage Assay

Electrolyte leakage was performed as previously described (Knepper et al., 2011), with samples harvested at 0, 2, 4, 6, 8, 10 and 12 days after drought stress. In brief, a single leaf disc (0.7 cm diameter) was harvested from each plant. Four plants were used for each replicate. Leaf discs were quickly washed by submerging and gently swirling in sterile dH_2O before being placed in a tube with 3 mL sterile dH_2O. Samples were rocked at 35 rpm for 3 h

on an orbital shaker. After 3 h, the leaf disc was removed and the solution was measured using a conductance meter (Traceable 23226-505; VWR Scientific). The leaf discs were frozen for 1 h at -80°C, and then returned to the original tubes and rocked for an additional 3 h before the disc is discarded and the final conductance measurement was taken. Readings were calculated as percent leakage of total (i.e. (first reading - background)/second reading) adjusted for background.

Stomatal Aperture Assay

Aperture width of stomata was assayed using a modified method from Melotto et al. (2006). Four-to-five-week old plants were covered with a clear plastic dome, to maintain high humidity, and kept under continuous light for 24 hours. Epidermal peels were collected from five leaves and placed on a glass slide covered with 10 μM abscisic acid (ABA) in dH_2O or dH_2O (mock). The prepared samples were then placed into a petri plate and covered to maintain humidity. These were placed back under growth lights for 1 h before peels were imaged using an Olympus IX-71 inverted microscope with an Olympus DP70 camera. The aperture widths of the stomata were measured using Image J software (National Institutes of Health). Results were analyzed using Prism 4 (GraphPad Software). Statistical significance was determined by one-way ANOVA followed by Tukey's post-test.

Pathogen Inoculation and Growth Assays

Pseudomonas syringae pv. tomato DC3000 containing the pVSP61 vector (*Pst* DC3000) was previously described in Kunkel et al. (1993). The *Pst* DC3118 coronatine deficient strain was previously described in Ma et al. (1991). Bacterial growth assays were performed as described

in Knepper et al. (2011). Four-week-old plants were dip inoculated in bacterial suspensions of 3×10^7 colony forming units (cfu) mL^{-1}. Three leaf discs of 0.7 cm were harvested 0 and 4 days post-inoculation. Results were analyzed using Prism 4 (GraphPad Software). Statistical significance was determined by ANOVA followed by Tukey's post-test.

Exogenous ABA treatment

Four-week-old plants were pretreated with water (Control) or ABA (400 µM in water; Yasuda et al. 2008)) by soil drenching five days prior to inoculation of *Pst* DC3000 as described before (see Pathogen Inoculation and Growth Assays**).**

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from leaves using the PrepEase Plant RNA Spin kit (USB Affymetrix, Santa Clara, CA, USA). First-strand cDNA was synthesized from 1 µg Total RNA using the First-Strand cDNA Synthesis kit (USB Affymetrix). qRT-PCR was performed using the Mastercycler ep Realplex system (Eppendorf AG, Hamburg, Germany), as previously described (Knepper et al., 2011), using the Hot Start SYBR Master mix 2X (USB Affymetrix). Ubiquitin (*UBQ10*) was used as an endogenous control for amplification. All replicates were performed using a cycle, previously used in Knepper et al. (2011), of 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 45 s. Results were analyzed using Prism 4 (GraphPad Software) with outliers removed by Grubb's test (α=0.05) using the QuickCalcs online outlier calculator (GraphPad Software; http://www.graphpad.com/quickcalcs/Grubbs1.cfm). Statistical significance was determined

by one-way ANOVA followed by Tukey's post test. All primer sets utilized are listed in Supplemental Table S4.1.

ACKNOWLEDGEMENTS

This work was supported by the National Science Foundation (Early CAREER Award IOS-0641319 to B.D.). The authors acknowledge Fundaçãopara a Ciência e Tecnologia, grant SFRH/BPD/43228/2008 to P.S. (co-financed by the Portuguese PIDDAC program and European Social Fund, under the third framework program).

Figure 4.1.

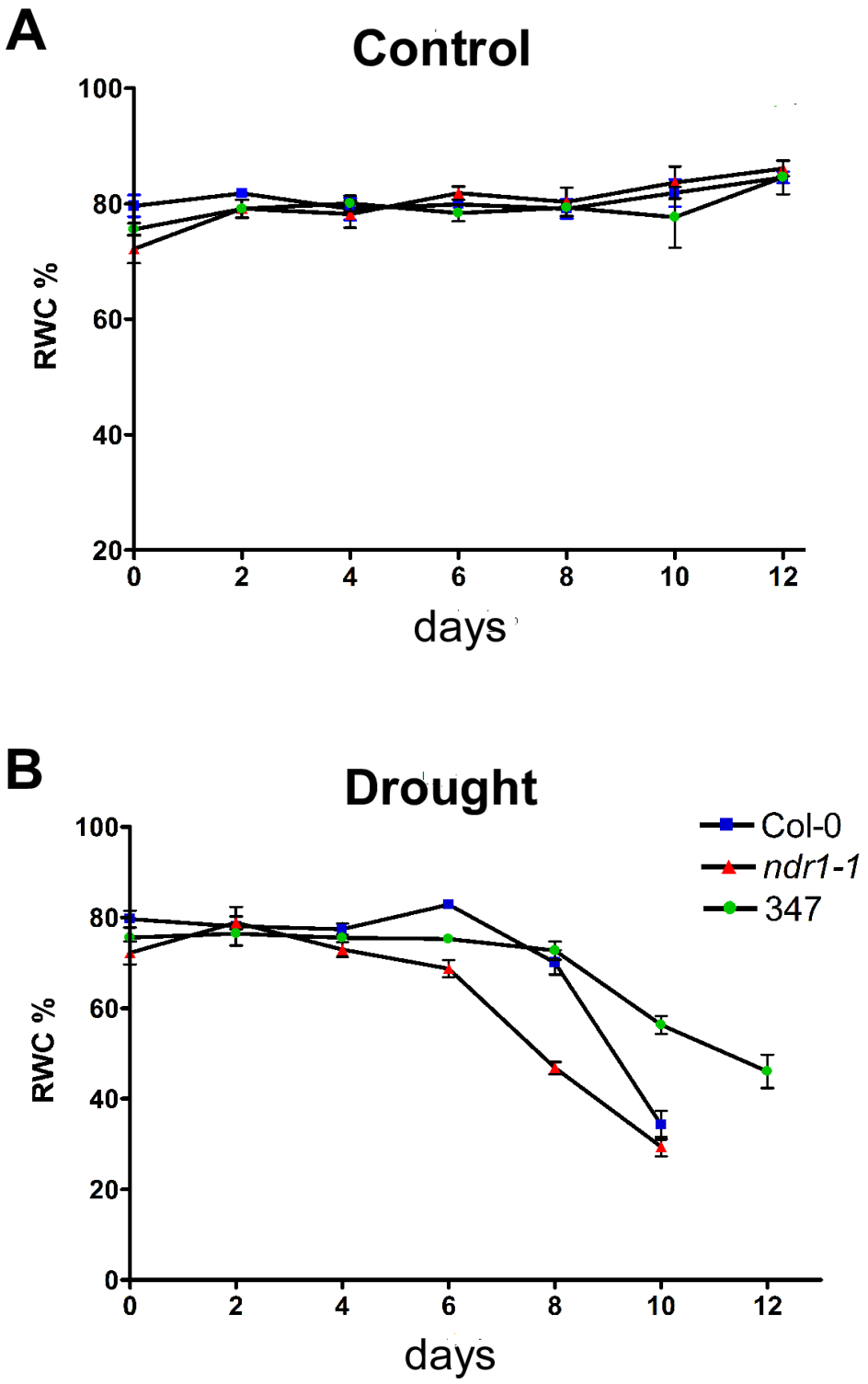

A
Control
100
80
60
40
20
RWC %
0
2
4
6
8
10
12
days
B
Drought
Col-0
ndr1-1
347
100
80
60
40
20
0
RWC %
0
2
4
6
8
10
12
days

Figure 4.1. (cont'd)

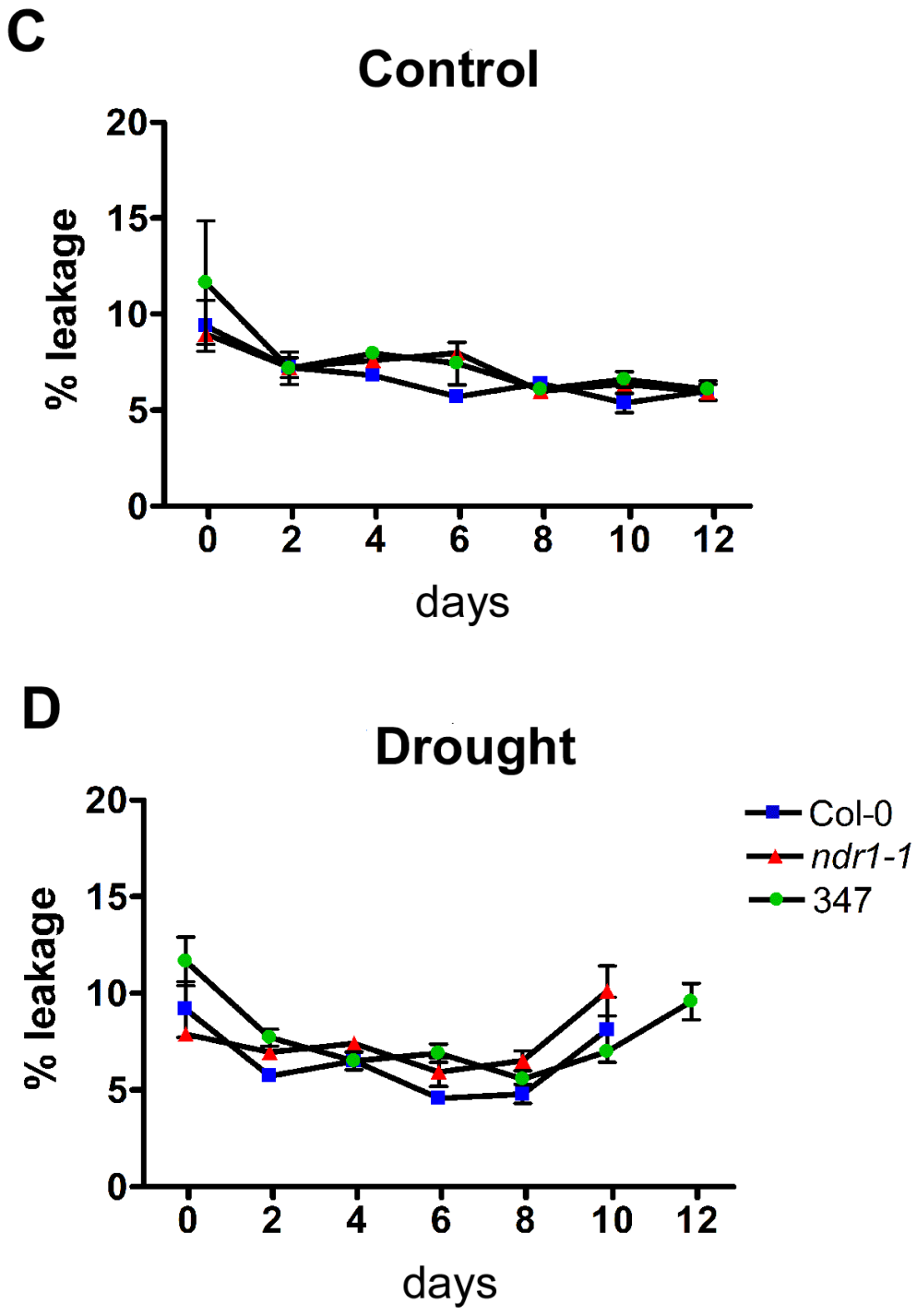
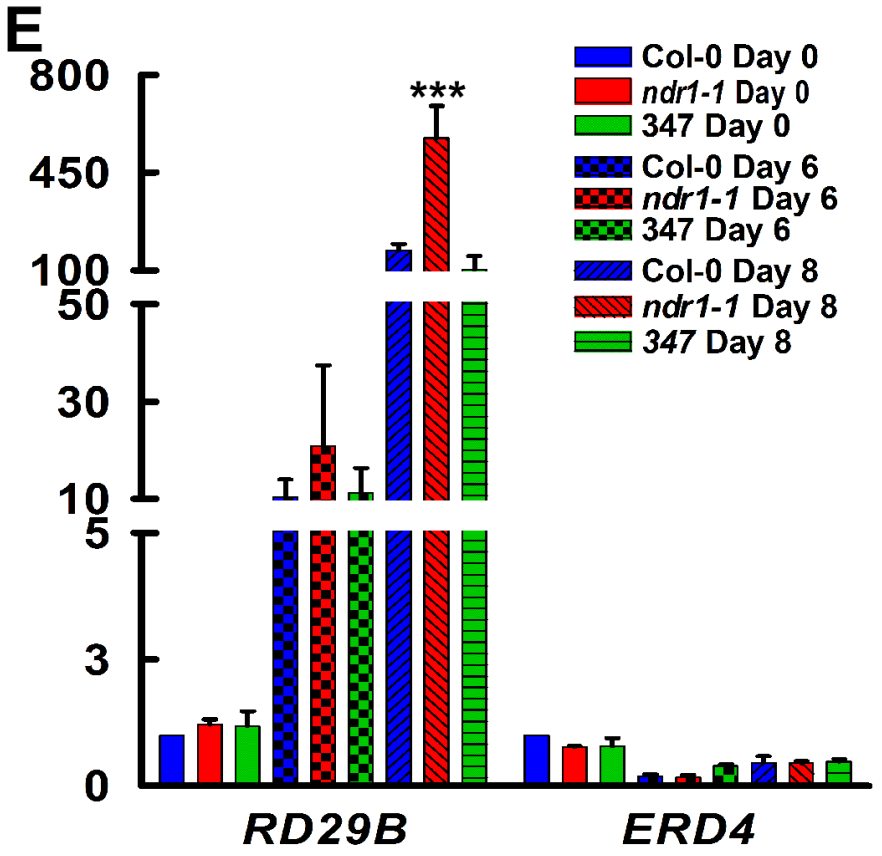


Figure 4.1. (cont'd)



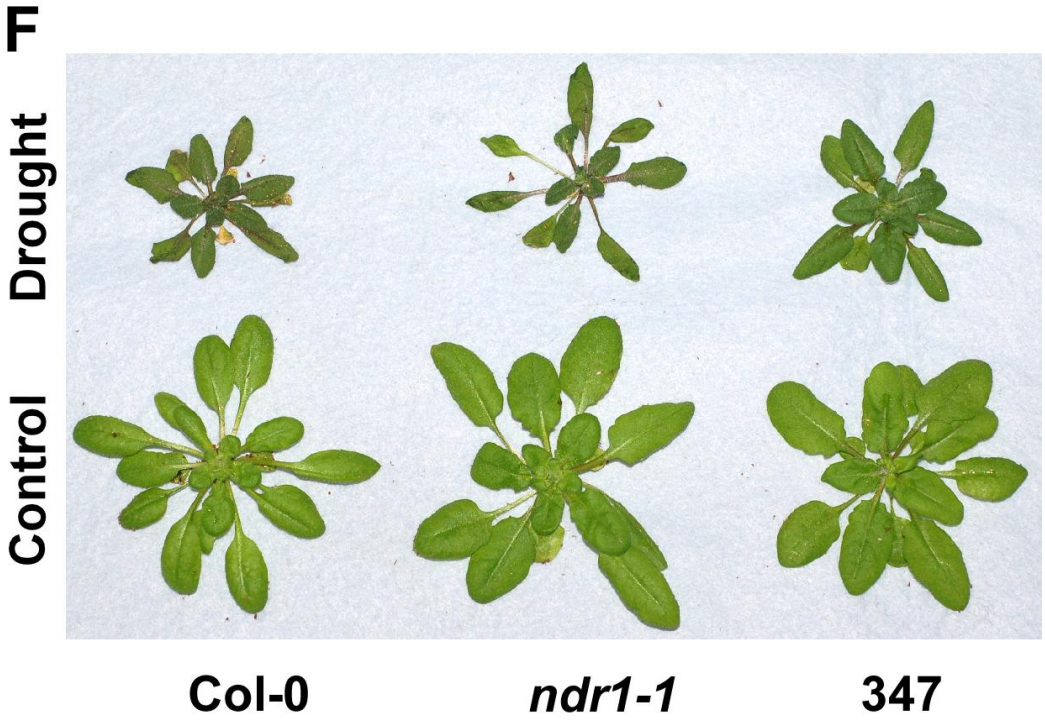


Figure 4.1. Relative water content, electrolyte leakage and *RD29B* and *ERD4* marker genes expression in response to drought stress.

A, Relative water content (RWC%) from leaves of wild type Col-0, *ndr1-1* and the NDR1 over-expressor (347) plants, 12 days after control (normal watering) or (B) drought stress (no water added after day 0; n = 3-4). (C) Electrolyte leakage (% leakage) from Col-0, *ndr1-1* and 347 under control or (D) drought stress conditions (n = 3). E, Levels of expression in Arabidopsis of the ABA-dependent drought stress marker *RD29B* and the ABA-independent drought marker *ERD4*. Expression is displayed as fold Col-0 0 day average (n = 7). F, Phenotypes of Arabidopsis plants at eight days after the iniation of drought stress or control. Samples were collected 0, 2, 4, 6, 8, 10 and 12 days after the initiation of drought stress. * represents a statistically significant difference between Col-0 and *ndr1-1*. *** $p<0.001$.

Figure 4.2.

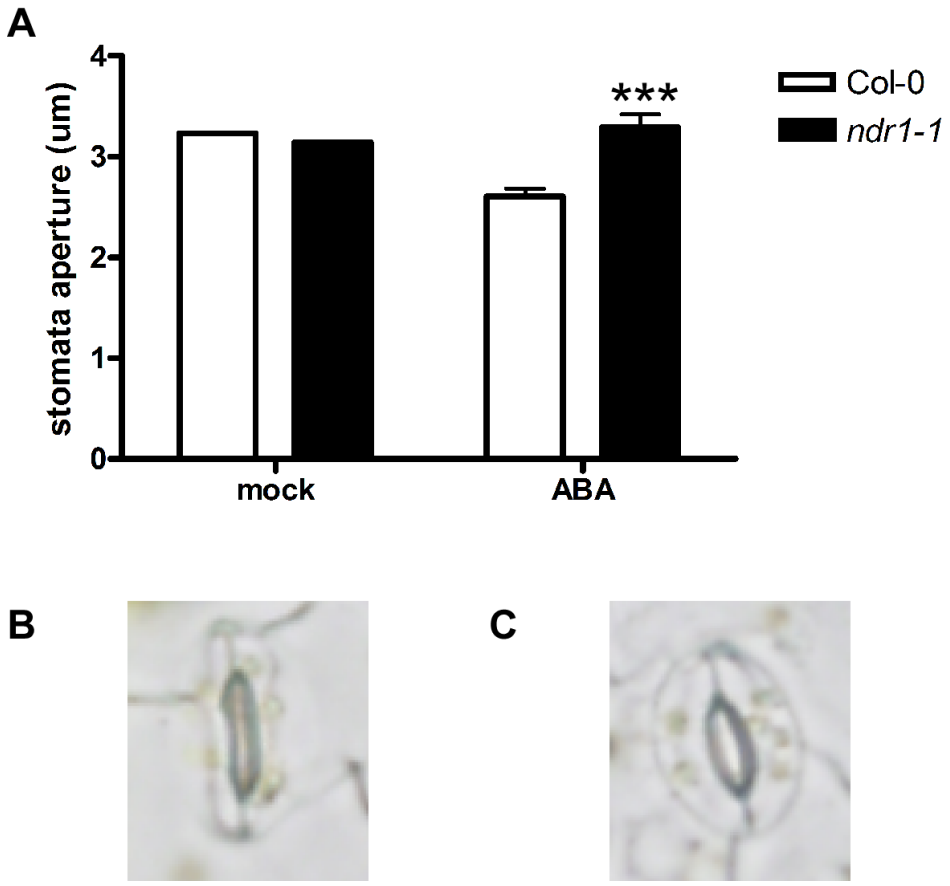


Figure 4.2. The stomata of *ndr1-1* are non-responsive to exogenous abscisic acid application. A, Stomatal aperture widths (µm) after Col-0 and *ndr1-1* epidermal peels were exposed to 10 µM abscisic acid (ABA) or dH_2O (mock) for 1 hour. Error bars display standard deviation from two biological replicates with n=50-100 stomata. Significance was determined using one-way ANOVA followed by Tukey's post test, where * represents statistically significant differences between Col-0 and *ndr1-1*: *** $P < 0.001$. Representative stomata of Col-0 (B) and *ndr1-1* (C) 1 h after ABA treatment.

Figure 4.3.

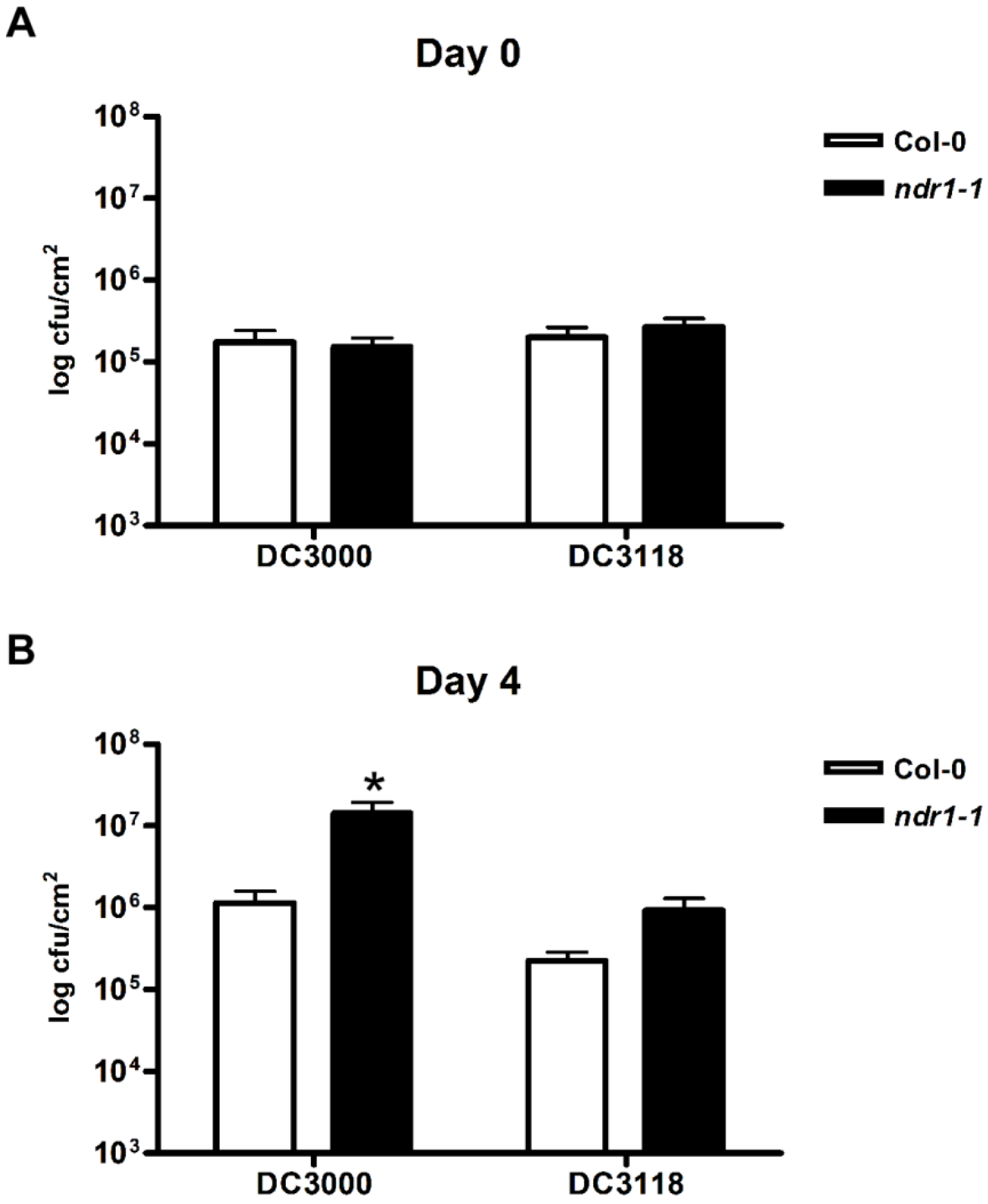

A
Day 0
log cfu/cm2
10^8
10^7
10^6
10^5
10^4
10^3
DC3000
DC3118
Col-0
ndr1-1
B
Day 4
log cfu/cm2
*
DC3000
DC3118
Col-0
ndr1-1

Figure 4.3. The coronatine-deficient mutant strain *Pseudomonas syringae* DC3118 reaches higher cell densities in the *ndr1-1* mutant.

Bacterial growth of *Pst* DC3000 and the coronatine deficient mutant *Pst* DC3118 at 0 (A) and 4 (B) days post inoculation in Col-0 and *ndr1-1* leaves. Bacterial growth is expressed as log cfu/cm^2. Error bars represent standard deviation from 3 biological replicates (n=9). Statistical significance was determined using one-way ANOVA followed by Tukey's post test, where * represents statistically significant differences between Col-0 and *ndr1-1*: * $P < 0.05$.

Figure 4.4.

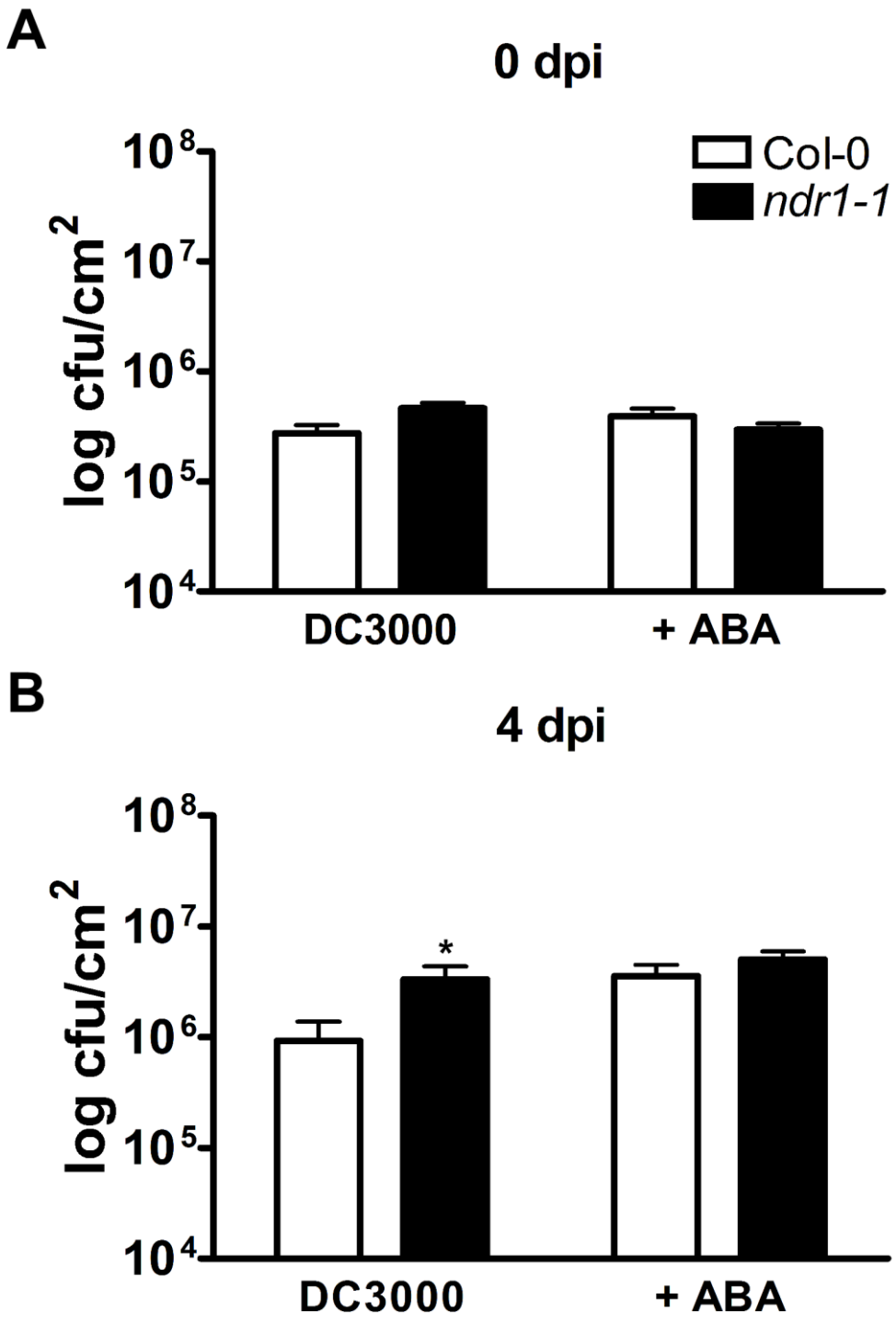

A
0 dpi
Col-0
ndr1-1
log cfu/cm2
10^8
10^7
10^6
10^5
10^4
DC3000
+ ABA
B
4 dpi
log cfu/cm2
10^8
10^7
10^6
10^5
10^4
*
DC3000
+ ABA

Figure 4.4. Abscisic acid pretreatment fails to suppress pathogen defense responses in *ndr1-1*. Col-0 and *ndr1-1* plants were pretreated with either water or 400 μM ABA in water for five days by soil saturation. Growth of *Pst* DC3000 was assayed from both the water control (DC3000) and the ABA treated plants (+ABA) at 0 (A) and 4 (B) days post inoculation (dpi). Bacterial growth is expressed as log cfu/cm^2. Error bars display standard deviation from 3 technical replicates from 3 biological replicates. Statistical significance was determined using two-way ANOVA where * represents a statistically significant difference between Col-0 and *ndr1-1*. * $P < 0.05$.

LITERATURE CITED

LITERATURE CITED

Aarts N, Metz M, Holub E, Staskawicz BJ, Daniels MJ, Parker JE (1998) Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene mediated signaling pathways in Arabidopsis. Proc Natl Acad Sci USA **95:** 10306-10311

Asselbergh B, De Vleesschauwer D, Hofte M (2008) Global switches and fine-tuning-ABA modulates plant pathogen defense. Mol Plant-Microbe Interact **21:** 709-719

Battaglia M, Olvera-Carrillo Y, Garciarrubio A, Campos F and Covarrubias AA (2008) The enigmatic LEA proteins and other hydrophilins. Plant Physiol **148:** 6-24

Beattie GA (2011) Water relations in the interaction of foliar bacterial pathogens with plants. Ann Rev Phytopathol **49**: 533-555

Boyer JS (1992) Plant productivity and environmental potential for increasing crop plant productivity, genotypic selection. Science **218:** 443-448

Brooks DM, Hernandez-Guzman G, Kloek AP, Alarcon-Chidez F, Sreedharan A, Rangaswamy V, Penaloza-Vazquez A, Bender CL, Kunkel BN (2004) Identification and characterization of a well-defined series of coronatine biosynthetic mutants of *Pseudomonas syringae* pv. *tomato* DC3000. Mol Plant Microbe Interact **17:** 162-174

Canut H, Carrasco A, Galaud J, Cassan C, Bouyssou H, Vita N, Ferrara P and Pont-Lezica R (1998) High affinity RGD-binding sites at the plasma membrane of *Arabidopsis thaliana* link the cell wall. Plant J **16**: 63-71

Cao FY, Yoshioka K, Desveaux D (2011) The roles of ABA in plant-pathogen interactions. J Plant Res **124:** 489–499

Catsky J (1960) Determination of water deficit in discs cut out from leaf blades. Biol Plant **2:** 76-77

Century K, Holub EB, Staskawicz BJ (1995) NDR1, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and fungal pathogen. Proc Natl Acad Sci USA **92:** 6597-6601

Century KS, Shapiro AD, Repetti PP, Dahlbeck D, Holub E, Staskawicz BJ (1997) NDR1, a pathogen-induced component required for Arabidopsis disease resistance. Science **278:** 1963-1965

Chaves MM, Maroco J, Pereira JS (2003) Understanding plant responses to drought- from genes to whole plant. Funct Plant Biol **30:**239-264

Chaves MM and Davies B (2010) Drought effects and water use efficiency: improving crop production in dry environments. Funct Plant Biol **37:** iii–vi

Coppinger P, Repetti PP, Day B, Dahlbeck D, Mehlert A and Staskawicz BJ (2004) Overexpression of the plasma membrane-localized NDR1 protein results in enhanced bacterial disease resistance in *Arabidopsis thaliana*. Plant J **40:** 225-237

Cushman JC and Bohnert HJ (2000) Genomic approaches to plant stress tolerance. Curr Opin Plant Biol **3:** 117–124

Fan J, Hill L, Crooks C, Doerner P, Lamb C (2009) Abscisic acid has a key role in modulating diverse plant-pathogen interactions. Plant Physiol **150:** 1750-1761.

Finkelstein RR, Gampala SSL, Rock CD (2002) Abscisic acid signaling in seeds and seedlings. Plant Cell **14:** S15–S45.

Goyal K, Walton LJ, Tunnacliffe A (2005) LEA proteins prevent protein aggregation due to water stress. Biochem J **388**: 151-157

Ingram J and Bartels D (1996) The molecular basis of dehydration tolerance in plants. Annu Rev Plant Physiol Plant Mol Biol **47:** 377–403

Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K (1994) Cloning of cDNAs for genes that are early-responsive to dehydration stress (ERDs) in *Arabidopsis thaliana* L.: identification of three ERDs as HSP cognate genes. Plant Mol Biol **25**: 791-798

Knepper C, Savory E, Day B (2011) Arabidopsis NDR1is an integrin-like protein with a role in fluid loss and plasma membrane-cell wall adhesion. Plant Physiol **156**: 286-300

Knepper C, Santos P and Day B (submitted) Arabidopsis NDR1 is required for robust activation of PAMP Triggered Immunity Mol. Plant Microbe Interact.

Kohorn BD (2000) Plasma membrane-cell wall contacts. Plant Physiol **124**: 31-38

Kunkel BN, Bent AF, Dahlbeck D, Innes RW, Staskawicz BJ (1993) RPS2, an Arabidopsis disease resistance locus specifying recognition of *Pseudomonas syringae* strains expressing the avirulence gene avrRpt2. Plant Cell **5:** 865–875

Lee T-F, McNellis TW (2009) Evidence that the BONZAI1/COPINE1 protein is a calcium- and pathogen-responsive defense suppressor. Plant Mol Biol **69:**155–166

Ma SW, Morris VL, Cuppels DA (1991) Characterization of a DNA region required for production of the phytotoxin coronatine by *Pseudomonas syringae* pv. *tomato*. Mol Plant Microbe Interact **4:** 69–77

Mahajan S, Tuteja N (2005) Cold, salinity and drought stresses: An overview. Arch Biochem Biophys **444:** 139–158

Mauch-Mani B, Mauch F (2005) The role of abscisic acid in plant-pathogen interactions. Curr Opin Plant Biol **8:** 409-414

Mellersh DG, Heath MC (2001) Plasma membrane-cell wall adhesion is required for expression of plant defense responses during fungal penetration. Plant Cell **13:** 413-424

Melotto M, Underwood W, Koczan J, Nomura K, He SY (2006) Plant stomata function in innate immunity against bacterial invasion. Cell **126:** 969-980

Mosher S, Moeder W, Nishimura N, Jikumaru Y, Joo S-H, Urquhart W, Klessig DF, Kim S-K, Nambara E, Yoshioka K (2010) The lesion-mimic mutant *cpr22* shows alterations in abscisic acid signaling and abscisic acid insensitivity in a salicylic acid-dependent manner. Plant Physiol **152:** 1901–1913

Pontier D, Gan S, Amasino RM, Roby D, Lam E (1999) Markers for hypersensitive response and senescence show distinct patterns of expression. Plant Mol Biol **39:** 1243–1255

Quirino BF, Noh Y-S, Himelblau E, Amasino RM (2000) Molecular aspects of leaf senescence. Trends Plant Sci **5:** 278–282

Schroeder JI, Allen GJ, Hugouvieux V, Kwak JM, Waner D (2001) Guard cell signal transduction. Ann Rev Plant Physiol Plant Mol Biol **52:** 627–658

Seki M, Ishida J, Narusaka M, Fujita M, Nanjo T, Umezawa T, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002) Monitoring the expression pattern of around 7,000 Arabidopsis genes under ABA treatments using a full-length cDNA microarray. Funct Integr Genomics **2:** 282-291

Shinozaki K, Yamaguchi- Shinozaki K (2000) Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. Curr Opin Plant Biol **3:** 217-223.

Singh S, Cornilescu CC, Tyler RC, Cornilescu G, Tonelli M, Lee MS, Markley JL (2005) Solution structure of a late embryogenesis abundant protein (LEA14) from *Arabidopsis thaliana*, a cellular stress-related protein. Prot Sci **14:** 2601-2609

Sirichandra C, Wasilewska A, Vlad F, Valon C, Leung J (2009) The guard cell as a single-cell model towards understanding drought tolerance and abscisic acid action. Journal of Experimental Botany **60:** 1439–1463

Takahashi Y, Berberich T, Yamashita K, Uehara Y, Miyazaki A, Kusano T (2004) Identification of tobacco HIN1 and two closely related genes as spermine-responsive genes and their differential expression during the Tobacco mosaic virus-induced hypersensitive response and during leaf- and flower-senescence. Plant Molecular Biology **54:** 613–622

Tian M, Chaudry F, Ruzicka DR, Meagher RB, Staiger CJ and Day B (2009) Arabidopsis Actin-Depolymerizing Factor AtADF4 Mediates Defense Signal Transduction Triggered by the*Pseudomonas syringae* Effector AvrPphB. Plant Physiol **150**: 815-824

Tornero P, Dangl JL (2001) A high-throughput method for quantifying growth of phytopathogenic bacteria in Arabidopsis thaliana. Plant J **28:** 475–481

Xiong L, Schumaker K, Zhu J-K (2002) Cell signaling during cold, drought and salt stress. Plant Cell **14 (suppl.):** S165–S183

Yamaguchi-Shinozaki K, Shinozaki K (1993) Arabidopsis DNA encoding two desiccation-responsive *rd29* genes. Plant Physiol **101:** 1119-1120

Yasuda M, Ishikawa A, Jikumaru Y, Seki M, Umezawa T, et al. (2008) Antagonistic interaction between systemic acquired resistance and the abscisic acid-mediated abiotic stress response in Arabidopsis. Plant Cell **20:** 1678-1692

Zhang L, Xiao S, Li W, Feng W, Li J, Wu Z, Gao X, Liu F Shao M (2011) Overexpression of a harpin-encoding gene hrf1 in rice enhances drought tolerance. Journal Exp Biol **62:** 4229- 4238

Zheng MS, Takahashi H, Miyazaki A, Hamamoto H, Shah J, Yamaguchi I and Kusano T (2004) Up-regulation of *Arabidopsis thaliana NHL10* in the hypersensitive response to *Cucumber mosaic virus* infection and in senescing leaves is controlled by signaling pathways that differ in salicylate involvement. *Planta* **218**:740-750

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol **53:** 247–273

CHAPTER 5

Conclusions and Future Directions

Conclusions

Since the identification of NDR1 in 1995 (Century et al., 1995), followed by the publication of Knepper et al., (2011a), NDR1 had been primarily associated with plant-pathogen defensive functions. This narrow focus had made the study of NDR1 difficult, and as a result, little progress had been made toward the elucidation of NDR1's biochemical and genetic function. The focus of this dissertation project was to broaden the scope of our understanding of NDR1 function in order to identify a global physiological role for NDR1 in Arabidopsis. Our hypothesis was that NDR1 had a primary function outside of its role in defense and was co-opted into the defense signaling pathway. This proved at least partially correct as NDR1 was shown to be critical for proper plasma membrane-cell wall (PM-CW) adhesion. That, combined with its ability to mediate fluid loss in response to pathogen infection marked a true paradigm shift in how pathogen defensive proteins are viewed.

Prior to the work described herein, the genetic requirement for NDR1 in pathogen defense had been established, but the mechanisms underlying the requirement were not understood. To attempt to understand these mechanisms we began with a detailed analysis of the NDR1 protein itself, ignoring its pre-established role in defense. The use of homology modeling led to the identification of a strong structural homology between NDR1 and the abiotic stress related protein LEA14 (Figure 2.1). Perhaps more interestingly, NDR1 also showed a high degree of similarity to mammalian integrins, a class of proteins not known to exist in plants. Based on these structural models, multiple protein motifs were identified that appeared to be functionally relevant in NDR1. One such motif was the three amino acid NGD peptide sequence. The NGD

motif, similar in both peptide sequence and location within the protein to the known mammalian ligand binding RGD motif, indicated that NDR1 could be involved in ligand binding.. This motif proved to be of critical importance in the physiological role identified for NDR1, that of mediating PM-CW adhesion. The identification of a role for NDR1 in PM-CW adhesion presented an entirely new perspective on defense associated proteins. To this end, we have now described a physiological role for NDR1, revealed through analyzing electrolyte leakage in response to pathogen infection (FIGURE 2.2). Cells of the *ndr1-1* mutant, when inoculated with *P. syringae* DC3000 expressing the effector AvrRpt2, are unable to restrict fluid leakage across the membrane. This evidence, showing a strong physiological requirement for NDR1 outside of defense, supports the hypothesis that NDR1 may have been co-opted into the defense network, potentially through its interaction with RIN4. This would support the hypothesis that NDR1 serves a non-pathogen-defensive role, in addition to its role in innate immune signaling.

Interestingly, the role of NDR1 in maintaining the PM-CW continuum proved to have function beyond that of the regulation of fluid loss. Previous studies have shown that PM-CW adhesion can be important for interaction of plant and pathogen and the subsequent defense signaling (Mellersh and Heath 2001). In the absence of NDR1 mediated PM-CW adhesion it was hypothesized that the interaction between the pathogen type III secretion system may be altered. By monitoring effector delivery in the *ndr1-1* mutant it was shown that the loss of PM-CW adhesion reduces the ability of effectors to be delivered into the cell interior. The recognition of pathogen effectors by R-proteins is an important step in the activation of plant defense, and with a reduction in the volume of effectors delivered the ability of the host to recognize and respond appropriately may also be reduced.

Beyond the identification of a role in adhesion, work to examine the function of NDR1 yielded a unique result that helped to bridge the connection between ETI and PTI. When challenged with the PAMP flg22, *ndr1-1* mutant plants showed significant reductions in expression of the MAPK pathway marker *FRK1*, along with a reduction in phosphorylated MAPK3/6 protein as compared to Col-0. This novel discovery served as a basis on which much of my future research was based.

While NDR1 has long been associated with defense signaling linked to the recognition of specific effectors and the subsequent activation of defense, the level at which NDR1 was integrated into both PTI and ETI was not well established. To begin to analyze the specifics of NDR1's role in PAMP-mediated defense signaling, the requirement for NDR1 was examined at every stage of the infection process. At the first stage, that of pathogen entry, the *ndr1-1* mutant plant was shown to be insensitive to the PAMP flg22 (Figure 3.1). The failure of the mutant to respond to a potential pathogen stimuli results in stomatal aperture that do not close appropriately leaving the plant "open" to pathogen infection.

After *P. syringae* successfully enters the interior space of the leaf, the next stage of defenses involves the recognition of conserved protein motifs of the pathogen by plant cell surface receptors. NDR1 was shown to be required for the robust activation of the MAPK signaling cascade after the perception of the PAMPs flg22 and elf26. The demonstration of the requirement for NDR1 in PAMP signaling was able to answer a curious previous observation in which *ndr1-1* mutant plants when inoculated with *Pst* DC3000 showed an increase in bacterial growth as compared to wild-type Col-0. Additionally, the production of Reactive Oxygen

Species (ROS) is often associated with the activation of PTI. In the *ndr1-1* mutant ROS generation was reduced in response to multiple PAMP elicitors, providing more evidence that the signaling cascade initiated in response to PAMP detection is abrogated in *ndr1-1*. Furthermore, by positively linking NDR1 to PAMP triggered immunity we are able to link basal immunity directly to effector triggered immunity, with NDR1 serving as an important point of connectivity between the two (Figure 5.1)

Beyond the identification of a role for NDR1 in mediating both basal immunity and *R*-gene mediated defenses the role of NDR1 in stomata aperture signaling and PM-CW adhesion led to perhaps the most significant and unexpected finding of this dissertation; the association of NDR1 to drought stress tolerance. After initial homology modeling revealed a high degree of structural homology to LEA14 a protein involved in desiccation resistance, and a member of an abiotic stress responsive protein family we began to speculate on a role for NDR1 in abiotic stress responses. With the demonstration of NDR1 as a critical component of PM-CW adhesion followed by the definitive connection between NDR1 and stomata aperture (Figure 3.1) the potential for an association between NDR1 and drought stress became increasingly likely. By analyzing several metrics associated with free water potential including relative water content, electrolyte leakage and drought responsive gene expression a link between NDR1 and drought stress was established (Figure 4.1). Furthermore, the over-expression of NDR1 was shown to increase the tolerance of Arabidopsis to severe drought stress. The connection of a disease resistance associated gene, *NDR1*, with abiotic stresses is a major step forward in integrated research, which in the future will allow for the examination of metabolic and physiological trade-

offs associated with resistance to multiple varied stresses; more closely mirroring natural conditions.

Future directions

While much progress has been made during the course of my dissertation work in the area of NDR1 function, several important questions remain unanswered. Several of these remaining questions are specific to the NDR1 protein itself. The localization of the NDR1 protein in the plasma membrane has been previously established (Coppinger et al., 2004), but the specific orientation of the protein within the membrane remains unknown. Two models currently exist, one in which a single transmembrane domain and a C-terminal GPI anchor link NDR1to the membrane with nearly the entirety of the protein on the cell exterior. The other model utilizes a predicted second transmembrane domain, which places the majority of the protein within the cytoplasm and only a small loop on the exterior. Current evidence from Knepper et al. (2011) supports the former model. The role of NDR1 in PM-CW adhesion was shown to require the NGD protein motif, which would necessitate this motif's location in the apoplast, thus supporting the former model with the majority of the protein outside the cytoplasm. Establishing the specific orientation of NDR1 will be necessary in the future in order to identify both the mechanism by which NDR1 functions in PM-CW adhesion as well as its interacting partner(s), whether they are cell wall components or membrane proteins.

Another important question that remains to be answered in regards to the NDR1 protein is the number and nature of protein modifications present in NDR1. NDR1 was identified as containing

seven putative glycosylation sites (Coppinger et al., 2004) and treatment of NDR1 to remove N-linked glycosylations resulted in a downshift in protein size, thus showing at least some of these putative sites are, in fact, glycosylated. An in-depth analysis is needed to identify the number and nature of N-linked glycosylations along with other potential protein modifications to NDR1 that may impact its function, including PM-CW adhesion and defense activation and signaling.

A final NDR1 protein-related question that must be examined is the identification of the motif involved in the self-association of NDR1. A strong similarity, previously described in this dissertation (Chapter 2), is that between NDR1 and mammalian integrins, including the formation of multimers. While the self-association of NDR1 proteins has been established (Knepper et al., 2011) the precise number involved in the multi-protein complex, as well as the specific protein motif(s) involved in this interaction has not been established. The identification of the self-associating motif in NDR1 could allow for a better understanding of complex formation and potential signaling functions that can be linked to the formation of or disillusion of protein multimers. This is possibly one more link to the integrin-like function of NDR1 in Arabidopsis. Furthermore, the identification of a motif required for NDR1 self-association could allow for data mining in Arabidopsis as well as in related species, looking for other multimer forming proteins that could be involved in signaling mechanisms similar to those of NDR1.

Beyond the scope of NDR1 protein analysis, several physiological functions of NDR1 warrant exploration. While the link between NDR1 and drought stress (Chapter 4) has proved to be a significant shift in how *R*-gene accessory proteins are viewed, there may remain links to other abiotic stress signaling pathways in which NDR1 could function. Given the strong connection between NDR1 and drought stress, it is likely that NDR1 may also function to mediate other

abiotic stresses. An interesting long term project would be to thoroughly assay for abiotic stress phenotypes other than drought in the *ndr1-1* mutant. NDR1 could potentially serve as a regulatory hub in both biotic and abiotic stress signaling.

When this dissertation project began, NDR1 was merely an *R*-gene accessory protein with an established genetic requirement in pathogen defense. It was known that many CC-NB-LRR R-genes required the presence of a functional NDR1 protein to properly initiate defensive cascades upon pathogen effector detection. Working from this important, but narrow knowledge base, I was able to expand the very idea of what a defense-related protein could be, starting with the identification of a specific physiological function for NDR1 in PM-CW adhesion. Beyond a mechanical role in adhesion, NDR1 was also identified as a required component to mediate fluid loss in response to a pathogen. NDR1 proved to be a required component in PAMP signaling, necessary for robust PTI activation, as well as playing a role in the stomatal aperture response. Currently, many projects focusing on plant-pathogen interactions are beginning to examine cross-talk between the PTI and ETI signal cascade, which were once thought of as separate but complementary systems. The findings herein, which connect NDR1 to both basal and effector-mediated resistance, will likely lend support to the hypothesis of a strongly interconnected defense network that is reliant on both PAMP and effector signals for robust activation. Furthermore, showing a definitive connection between NDR1 and drought stress suggests that the primary function of NDR1 may, in fact, be of a physiological capacity where it serves to mediate PM-CW adhesion and regulate fluid movement, and that through interactions with defensive regulatory proteins, such as RIN4, NDR1 was co-opted into the defense network where it serves as a signaling hub. In conclusion, with the knowledge generated through this

dissertation project the NDR1 paradigm has truly shifted. Where NDR1 was formerly recognized as simply an *R*-gene accessory protein, it can now stand on its own as a critical component of Arabidopsis biology.

Figure 5.1.

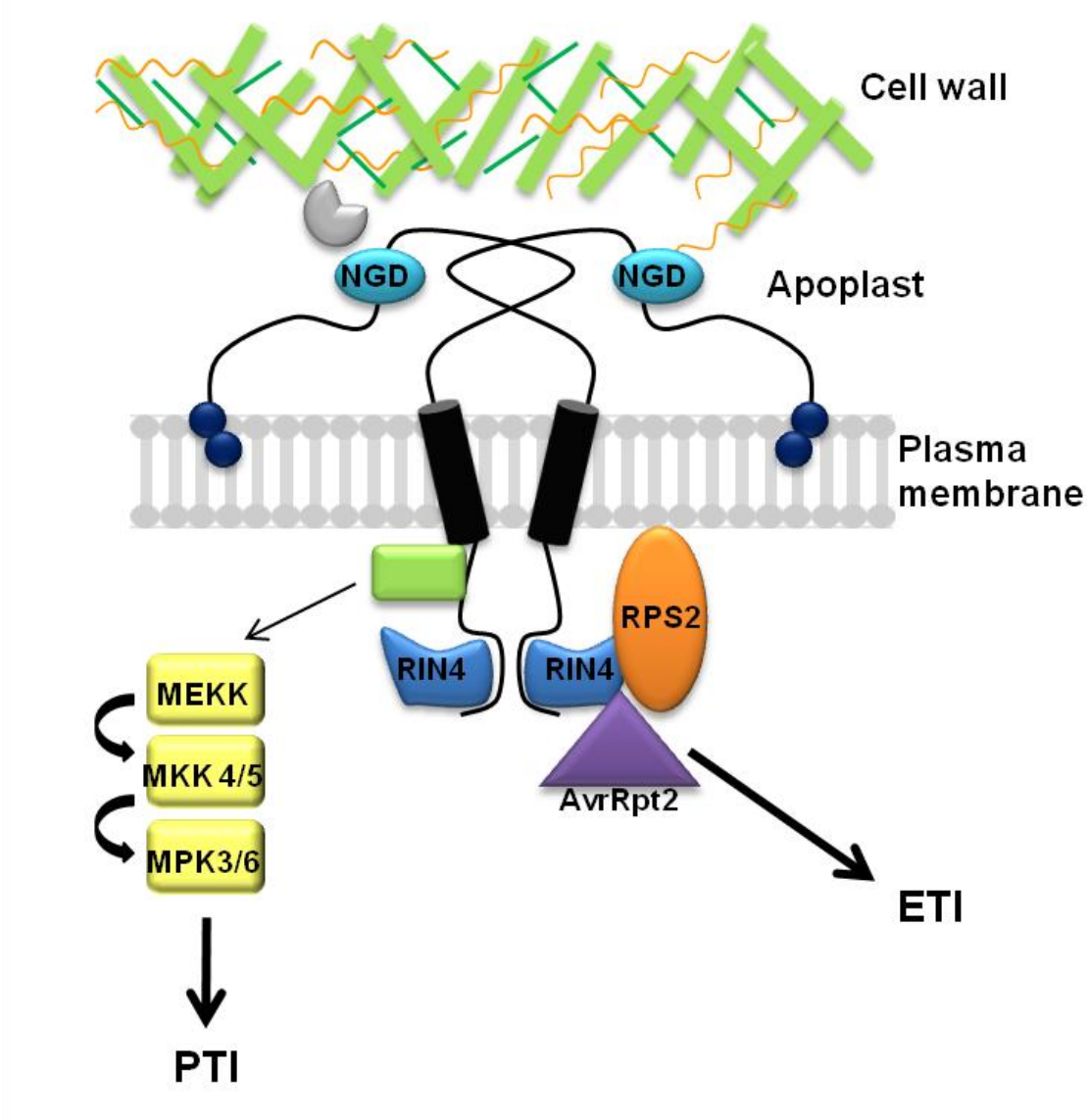

Cell wall
NGD
NGD
Apoplast
Plasma membrane
RIN4
RIN4
RPS2
AvrRpt2
MEKK
MKK 4/5
MPK3/6
PTI
ETI

Figure 5.1. Model of NDR1 location and interconnectivity.

NDR1 (NON-RACE SPECIFIC DISEASE RESISTANCE-1) is a plasma membrane localized, GPI-anchored protein that is capable of forming multimers. NDR1 contains an NGD (*Asn-Gly-Asp*) motif located in the putative apoplastic-localized region of the protein, shown to be important for maintaining cell wall-plasma membrane adhesion points through either direct or indirect interaction with components of the cell wall that are as yet unknown. The role of NDR1 in ETI has been further strengthened through the identification of an interaction between NDR1 and RIN4 (RPM1 INTERACTING PROTEIN 4; Day et al., 2006). RIN4 has been shown to associate with the R-protein, RPS2 (RESISTANCE TO *PSEUDOMONAS SYRINGAE* 2), and it is through RIN4's cleavage by the *P. syringae* DC3000 effector protein AvrRpt2 and the subsequent recognition of this event by RPS2 (Kunkel et al., 1993), that ETI is activated. NDR1 has also been linked directly to the activation of the MAPK (MITOGEN ACTIVATED PROTEIN KINASE) pathway and PTI (PAMP Triggered Immunity). Although the specific interaction of NDR1 in the upstream components of PTI signaling remain unknown. Figure originally printed in Knepper et al., (2011b).

LITERATURE CITED

LITERATURE CITED

Century, K., Holub, E.B. and Staskawicz, B.J. (1995) NDR1, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and fungal pathogen. Proc. Natl. Acad. Sci. U.S.A. **92**: 6597-6601.

Coppinger, P., Repetti, P.P., Day, B., Dahlbeck, D., Mehlert, A. and Staskawicz, B.J. (2004) Overexpression of the plasma membrane-localized NDR1 protein results in enhanced bacterial disease resistance in *Arabidopsis thaliana*. Plant J. **40**: 225-237.

Day, B., Dahlbeck, D. and Staskawicz, B.J. (2006) NDR1 interaction with RIN4 mediates the differential activation of multiple disease resistance pathways in Arabidopsis. Plant Cell **18**: 2782-2791.

Knepper, C., Savory, E.A. and Day, B. (2011a) Arabidopsis NDR1is an integrin-like protein with a role in fluid loss and plasma membrane-cell wall adhesion. Plant Physiol. **156**: 286-300.

Knepper, C., Savory, E.A. and Day, B. (2011b) The role of NDR1 in pathogen perception and plant defense signaling. Plant signal. behav. 6: 1114-1116.

Kunkel, B.N., Bent, A.F., Dahlbeck, D., Innes, R.W. and Staskawicz, B.J. (1993) RPS2, an Arabidopsis disease resistance locus specifying recognition of *Pseudomonas syringae* strains expressing the avirulence gene avrRpt2. Plant Cell **5**: 865–875.

Mellersh, D.G. and Heath, M.C. (2001) Plasma membrane-cell wall adhesion is required for expression of plant defense responses during fungal penetration. Plant Cell **13**: 413-424.

APPENDIX

Supplemental Methods

Electrolyte Leakage: supplemental conductance method

Electrolyte leakage was measured for Figure S3 in 4-week-old plants using a protocol modified from Mackey *et al.* (2002). Plants were dip inoculated at 3×10^7 cfu mL^{-1}. After inoculation, plants were covered with a clear plastic dome for 1 hour before the 0 hour time point leaves were removed. A leaf disc (0.7 cm diameter) was harvested using a number 3 size cork borer from 2 leaves each from 4 plants. The punches were added to a glass container containing 50 mL of sterile dH_2O and allowed to rotate on orbital shaker for 30 minutes at 20 rpm. After this wash step the dH_2O was removed and replaced by 10 mL sterile dH_2O that had been previously measured for conductance to allow for background to be removed. The samples were allowed to remain on shaker with measurements conducted at 3 hour intervals for 24 hours. Electrolyte leakage was recorded as conductance (µS). To allow for the calculation of percent maximal leakage, after the final measurement was taken the leaf punches were frozen at -80°C for 24 hours before being returned to their respective samples, followed by 3 hours on the shaker and a final measurement recorded as total leakage. Total percent leakage can be calculated as first reading/second reading adjusted to percent maximal leakage.

Supplemental Formulas

Formula S2.1

$$Log_{10}(n)_{ijk}=T_i+ G_j + T_iG_j + e_k$$

Formula S2.1. Statistical model for analysis of bacterial growth assays.

Where T, treatment; G, genotype; TG, treatment:genotype; e, residual; i, treatment index (1 to 4); j, genotype index (1 to 4), k, replicate index (4 to 6).

Supplemental Tables

Table S2.1.

		6 h pretreatment			12 h pretreatment	
Line	peptide pretreatment	HR +	HR -		HR +	HR -
Col-0	mock	31	1		30	2
	VNGDG	32	0		29	1
	VRGDG	32	0		28	2
	VAAAG	33	0		28	3
ndr1-1	mock	1	31		2	27
	VNGDG	2	28		1	30
	VRGDG	7	24		2	25
	VAAAG	4	29		0	34

Table S2.1. The effect of exogenous peptide application on the hypersensitive response (HR) in WT Col-0 and *ndr1-1* mutant plants.

Pre-treatment of Col-0 or *ndr1-1* mutant plants, at either 6 or 12 h with 5 mM 'VAAAG', 'VNGDG' or 'VRGDG' peptide solutions in 1 mM $MgCl_2$ or 1 mM $MgCl_2$ alone did not significantly alter the number of leaves undergoing HR after inoculation with *Pst* DC3000 expressing AvrRpt2.

Table S2.2.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
NDR1 (AT3g20600)	CGGTTTTACGAGCGGTTTTG	CCAACTTCAACCCCATACCTC
LEA14 (At1g01470)	ACCTCAAAGACGTGAACCGTGACT	GGCGCTGTGGAAAGTGAAACTGAT
LEA group 1 domain containing protein (At1g32560)	AAGAGATAGCGCACCAACGGAGAA	ATTATGCCCGTAACCGTGTCCCAT
PIP2;6 (At2g39010)	GGCATCTCTGGTGGACACATC	CAACTCCACAAGTGGCTCCG
PIP2;5 (At3g54820)	TGGTGGGCATATTAATCCGGCAGT	TGACCAAAGCCACACCACAAATGG
PIP1;5 (At4g23400)	GCTGGAATCTCAGGAGGACATATT	AGCTCCAAGGCACTGCATTACT
PIP1;4 (At4g00430)	TGGGATGACCATTGGATTTT	TCTGGACCGTGGAATCTTTC
PIP1;3 (At1g01620)	GGAATCTCTGGTGGGCACAT	CTCCGAGACATTGCATCACG
PR1 (At2g14610)	CTGCGAACACGTGCAATGGAGTTT	TACACCTCACTTTGGCACATCCGA
PDF1.2 (At5g44420)	GCTGCTCTTGTTCTCTTTGCTGCT	AACTTCTGTGCTTCCACCATTGCC
RPS2 (At3g03600)	TCTTATCGTTGGCTGTGCTCAGGT	ACGTATGGCCTTCAAGTCACCGAT
FRK1 (At2g19190)	CGGTCAGATTTCAACAGTTTGTC	AATAGCAGGTTGGCCTGTAATC
'RGD'- site directed mutagenesis	GTTGAAGTCAGGGGTGATGGAG	CTCCATCACCCCTGACTTCAAC
'AAA'- site directed mutagenesis	GTTGAAGTCGCCGCTGCTGGAG	CTCCAGCAGCGGCGACTTCAAC
NDR1 T7 Sal1 For	GTCGACATGGCTTCAATGACAAGGTGGTCAACAAATGGGTATGAATAATCAAAATGAAGACACAG	
NDR1 SpeI Rev		ACTAGTTTAACGAATAGCAAAGAATAC

Table S2.2. Primers used in Chapter 2 for qRTPCR and for the construction of the RGD and AAA site-directed mutant constructs in *NDR1*.

All primer sets utilized including primers for qRTPCR and primers for site directed mutagenesis for development of the *NDR1* NGD (RGD and AAA) site mutants. Aquaporin homolog primers, *PIP*s, are from Alexandersson *et al.*, 2010.

Table S2.3.

Arabidopsis lines compared 'NGD'		***Pst* DC3000**	**Adjusted p values**
Col-0	*ndr1-1*	EV	0.002
Col-0	*ndr1-1*/P_{NDR1}:NGD-1	EV	0.9128
Col-0	*ndr1-1*/P_{NDR1}:NGD-3	EV	0.3045
ndr1-1	*ndr1-1*/P_{NDR1}:NGD-1	EV	0.0002
ndr1-1	*ndr1-1*/P_{NDR1}:NGD-3	EV	<.0001
ndr1-1/P_{NDR1}:NGD-1	*ndr1-1*/P_{NDR1}:NGD-3	EV	0.6931
Col-0	*ndr1-1*	AvrRpt2	<.0001
Col-0	*ndr1-1*/P_{NDR1}:NGD-1	AvrRpt2	0.064
Col-0	*ndr1-1*/P_{NDR1}:NGD-3	AvrRpt2	0.0341
ndr1-1	*ndr1-1*/P_{NDR1}:NGD-1	AvrRpt2	<.0001
ndr1-1	*ndr1-1*/P_{NDR1}:NGD-3	AvrRpt2	<.0001
ndr1-1/P_{NDR1}:NGD-1	*ndr1-1*/P_{NDR1}:NGD-3	AvrRpt2	0.9825
Col-0	*ndr1-1*	AvrB	0.0001
Col-0	*ndr1-1*/P_{NDR1}:NGD-1	AvrB	0.9912
Col-0	*ndr1-1*/P_{NDR1}:NGD-3	AvrB	0.9799
ndr1-1	*ndr1-1*/P_{NDR1}:NGD-1	AvrB	0.0007
ndr1-1	*ndr1-1*/P_{NDR1}:NGD-3	AvrB	0.0009
ndr1-1/P_{NDR1}:NGD-1	*ndr1-1*/P_{NDR1}:NGD-3	AvrB	0.9997
Col-0	*ndr1-1*	AvrPphB	0.0003
Col-0	*ndr1-1*/P_{NDR1}:NGD-1	AvrPphB	0.9428
Col-0	*ndr1-1*/P_{NDR1}:NGD-3	AvrPphB	0.883
ndr1-1	*ndr1-1*/P_{NDR1}:NGD-1	AvrPphB	0.0011
ndr1-1	*ndr1-1*/P_{NDR1}:NGD-3	AvrPphB	<.0001
ndr1-1/P_{NDR1}:NGD-1	*ndr1-1*/P_{NDR1}:NGD-3	AvrPphB	0.5282

Table S2.3. (cont'd)

Arabidopsis lines compared 'RGD'		***Pst* DC3000**	**Adjusted p values**
Col-0	*ndr1-1*	EV	0.0067
Col-0	*ndr1-1*/P_{NDR1}:RGD-1	EV	0.0047
Col-0	*ndr1-1*/P_{NDR1}:RGD-3	EV	0.5256
ndr1-1	*ndr1-1*/P_{NDR1}:RGD-1	EV	<.0001
ndr1-1	*ndr1-1*/P_{NDR1}:RGD-3	EV	<.0001
ndr1-1/P_{NDR1}:RGD-1	*ndr1-1*/P_{NDR1}:RGD-3	EV	0.1422
Col-0	*ndr1-1*	AvrRpt2	<.0001
Col-0	*ndr1-1*/P_{NDR1}:RGD-1	AvrRpt2	0.0835
Col-0	*ndr1-1*/P_{NDR1}:RGD-3	AvrRpt2	0.9843
ndr1-1	*ndr1-1*/P_{NDR1}:RGD-1	AvrRpt2	<.0001
ndr1-1	*ndr1-1*/P_{NDR1}:RGD-3	AvrRpt2	<.0001
ndr1-1/P_{NDR1}:RGD-1	*ndr1-1*/P_{NDR1}:RGD-3	AvrRpt2	0.0348
Col-0	*ndr1-1*	AvrB	0.0004
Col-0	*ndr1-1*/P_{NDR1}:RGD-1	AvrB	0.1828
Col-0	*ndr1-1*/P_{NDR1}:RGD-3	AvrB	0.9999
ndr1-1	*ndr1-1*/P_{NDR1}:RGD-1	AvrB	<.0001
ndr1-1	*ndr1-1*/P_{NDR1}:RGD-3	AvrB	0.0002
ndr1-1/P_{NDR1}:RGD-1	*ndr1-1*/P_{NDR1}:RGD-3	AvrB	0.1309
Col-0	*ndr1-1*	AvrPphB	<.0001
Col-0	*ndr1-1*/P_{NDR1}:RGD-1	AvrPphB	0.9999
Col-0	*ndr1-1*/P_{NDR1}:RGD-3	AvrPphB	0.9063
ndr1-1	*ndr1-1*/P_{NDR1}:RGD-1	AvrPphB	<.0001
ndr1-1	*ndr1-1*/P_{NDR1}:RGD-3	AvrPphB	<.0001
ndr1-1/P_{NDR1}:RGD-1	*ndr1-1*/P_{NDR1}:RGD-3	AvrPphB	0.8816

Table S2.3. (cont'd)

Arabidopsis lines compared 'AAA'		***Pst* DC3000**	**Adjusted p values**
Col-0	*ndr1-1*	EV	0.0713
Col-0	*ndr1-1*/P_{NDR1}:AAA-1	EV	0.3311
Col-0	*ndr1-1*/P_{NDR1}:AAA-3	EV	0.4212
ndr1-1	*ndr1-1*/P_{NDR1}:AAA-1	EV	0.8447
ndr1-1	*ndr1-1*/P_{NDR1}:AAA-3	EV	0.7546
ndr1-1/P_{NDR1}:AAA-1	*ndr1-1*/P_{NDR1}:AAA-3	EV	0.9982
Col-0	*ndr1-1*	AvrRpt2	<.0001
Col-0	*ndr1-1*/P_{NDR1}:AAA-1	AvrRpt2	0.3475
Col-0	*ndr1-1*/P_{NDR1}:AAA-3	AvrRpt2	0.8406
ndr1-1	*ndr1-1*/P_{NDR1}:AAA-1	AvrRpt2	<.0001
ndr1-1	*ndr1-1*/P_{NDR1}:AAA-3	AvrRpt2	<.0001
ndr1-1/P_{NDR1}:AAA-1	*ndr1-1*/P_{NDR1}:AAA-3	AvrRpt2	0.7901
Col-0	*ndr1-1*	AvrB	0.0102
Col-0	*ndr1-1*/P_{NDR1}:AAA-1	AvrB	0.9966
Col-0	*ndr1-1*/P_{NDR1}:AAA-3	AvrB	0.921
ndr1-1	*ndr1-1*/P_{NDR1}:AAA-1	AvrB	0.0192
ndr1-1	*ndr1-1*/P_{NDR1}:AAA-3	AvrB	0.0007
ndr1-1/P_{NDR1}:AAA-1	*ndr1-1*/P_{NDR1}:AAA-3	AvrB	0.8277
Col-0	*ndr1-1*	AvrPphB	0.0231
Col-0	*ndr1-1*/P_{NDR1}:AAA-1	AvrPphB	0.5109
Col-0	*ndr1-1*/P_{NDR1}:AAA-3	AvrPphB	0.9332
ndr1-1	*ndr1-1*/P_{NDR1}:AAA-1	AvrPphB	0.4553
ndr1-1	*ndr1-1*/P_{NDR1}:AAA-3	AvrPphB	0.0023
ndr1-1/P_{NDR1}:AAA-1	*ndr1-1*/P_{NDR1}:AAA-3	AvrPphB	0.1814

Table S2.3. p values of *Pst* DC3000 growth assays in WT Arabidopsis and NGD/RGD/AAA-complemented lines.

Adjusted p values were determined in SAS version 9.2 by the use of an unbalanced two-way ANOVA (model Formula S2.1) and Tukey's test.

Table S3.1.

Gene	Locus tag	Forward primer (5'-3')	Reverse primer (5'-3')
FRK1	At2g19190	CGGTCAGATTTCAACAGTTTGTC	AATAGCAGGTTGGCCTGTAATC
PHI1	At1g35140	TTGGTTTAGACGGGATGGTG	ACTCCAGTACAAGCCGATCC
OXI1	At3g25250	GACGAGATTATCAGATTTTACGC	AACTGGTGAAGCGGAAGAGAC

Table S3.1. DNA primers used in Chapter 3 for quantitative real-time PCR.

FRK1 and *PHI1* primer sequences are from Boudsocq et al. (2010). *OXI1* primer sequences are from Forzani et al. (2011).

Table S4.1.

Gene	Locus tag	Forward primer (5'-3')	Reverse primer (5'-3')
RD29B	At5g52300	GATACCTTCCGACCAGATAGC	TTCCTTCTCCACCTTTTCCTTC
ERD4	AT1G30360	CGCAACAGAAAACAGCAAGG	TTGTTTACCGACTAGCCCAC

Table S4.1. DNA primers used in Chapter 4 for quantitative real-time PCR.

RD29B and *ERD4* primer sequences for qRT-PCR.

Supplemental Figures

Figure S2.1.

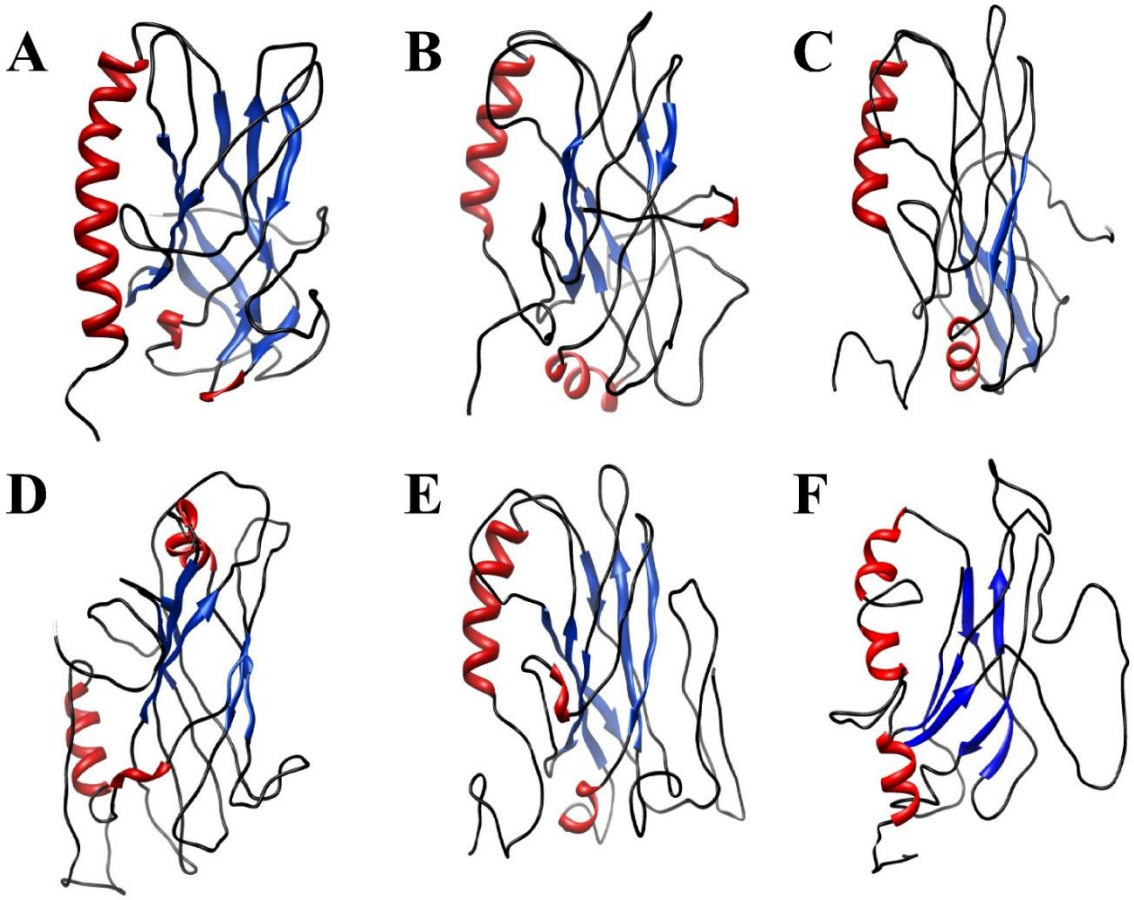


Figure S2.1. Predicted structural models of NDR1.

(A)-(E), I-TASSER-generated homology models of NDR1. I-TASSER analysis returned results of comparison Model A and closest Protein Database result 1yycA (LEA14; Singh *et al.*, 2005) with a TM-score of 0.6393 and RMSD 2.96. (F) PHYRE-based prediction of NDR1 structure.

Figure S2.2.

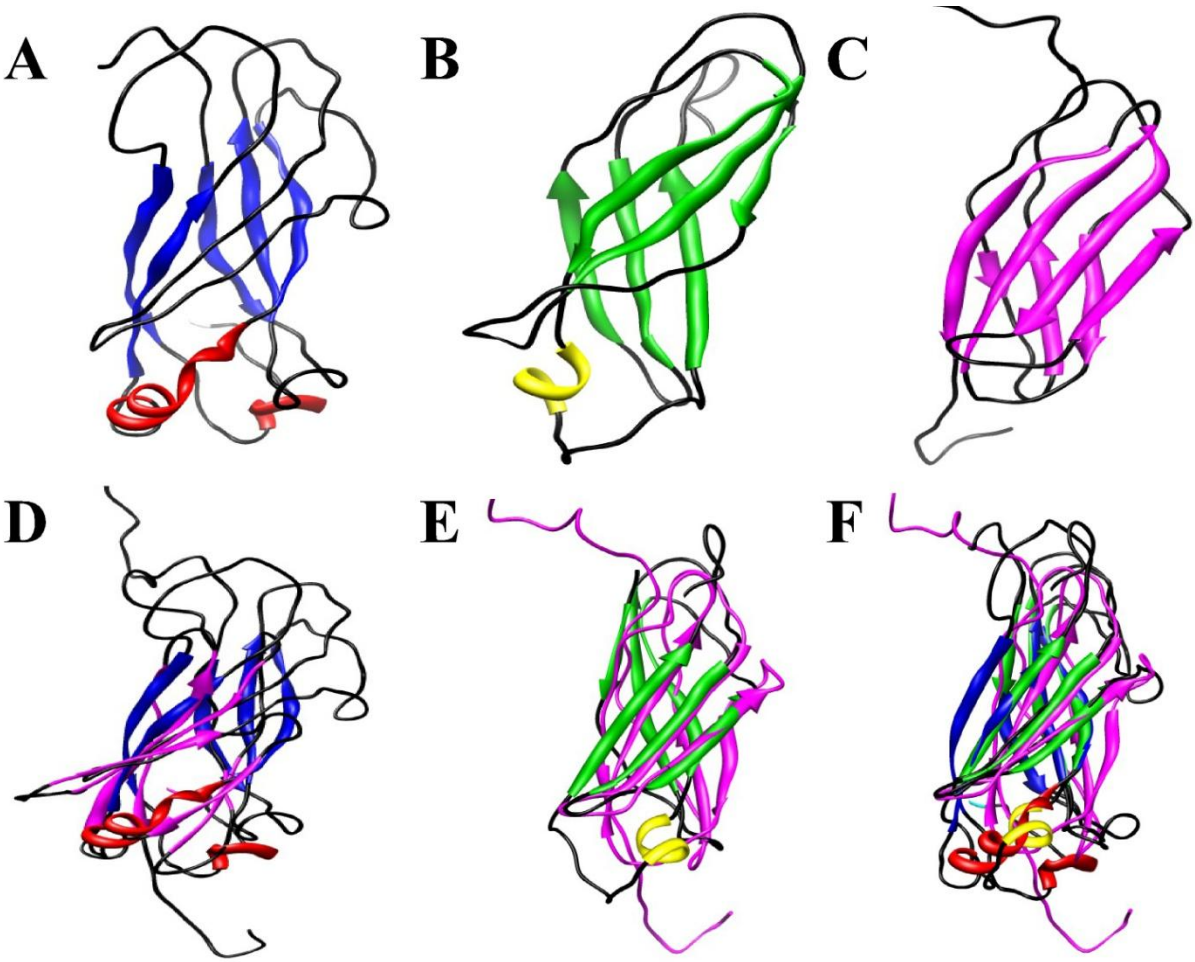


Figure S2.2. Predicted (NDR1; I-TASSER) and solved (LEA14 and fibronectin type III domain of human Integrin beta-4.) structures of the β-sheet torus.

(A) NDR1. (B) LEA14. (C) Fibronectin type III (FNIII) domain of human Integrin beta-4 (10.2210/pdb2yrz/pdb). (D) NDR1- fibronectin type III domain of human Integrin beta-4 (10.2210/pdb2yrz/pdb) overlay. (E) LEA14- fibronectin type III domain of human Integrin beta-4 (10.2210/pdb2yrz/pdb) overlay. (F) Overlay of NDR1, LEA14 and fibronectin type III domain of human Integrin beta-4 (10.2210/pdb2yrz/pdb). Overlays of predicted and solved structures were generated using CHIMERA. For generation of the NDR1 β-sheet model, amino acids 34-195 were used. For LEA14, amino acids 40-162 were used, based on the solved structure pdb_1yyca (Singh *et al.*, 2005).

Figure S2.3.

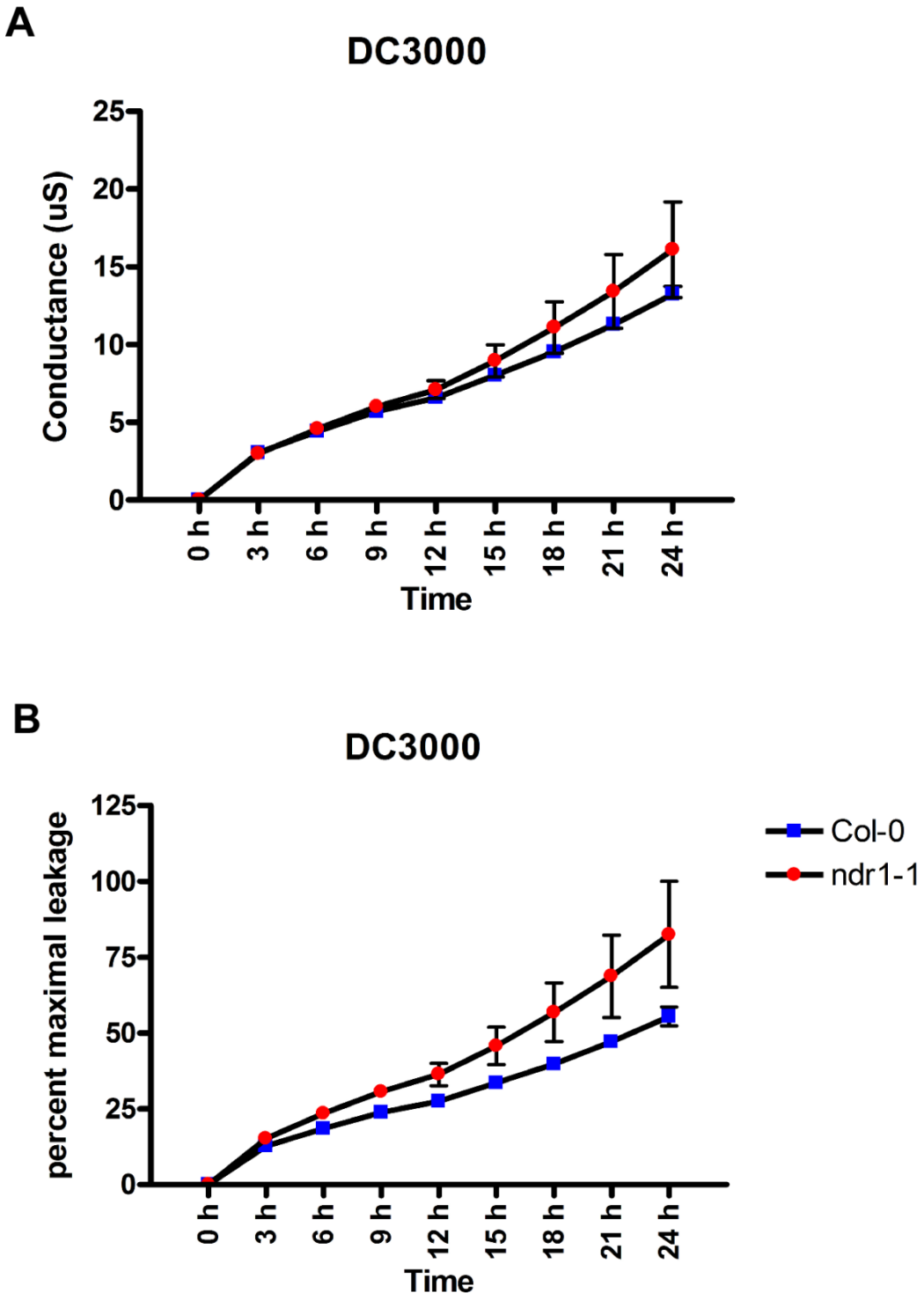

A
DC3000
Conductance (uS)
25
20
15
10
5
0
0 h
3 h
6 h
9 h
12 h
15 h
18 h
21 h
24 h
Time
B
DC3000
percent maximal leakage
125
100
75
50
25
0
0 h
3 h
6 h
9 h
12 h
15 h
18 h
21 h
24 h
Time
Col-0
ndr1-1

Figure S2.3. (cont'd)

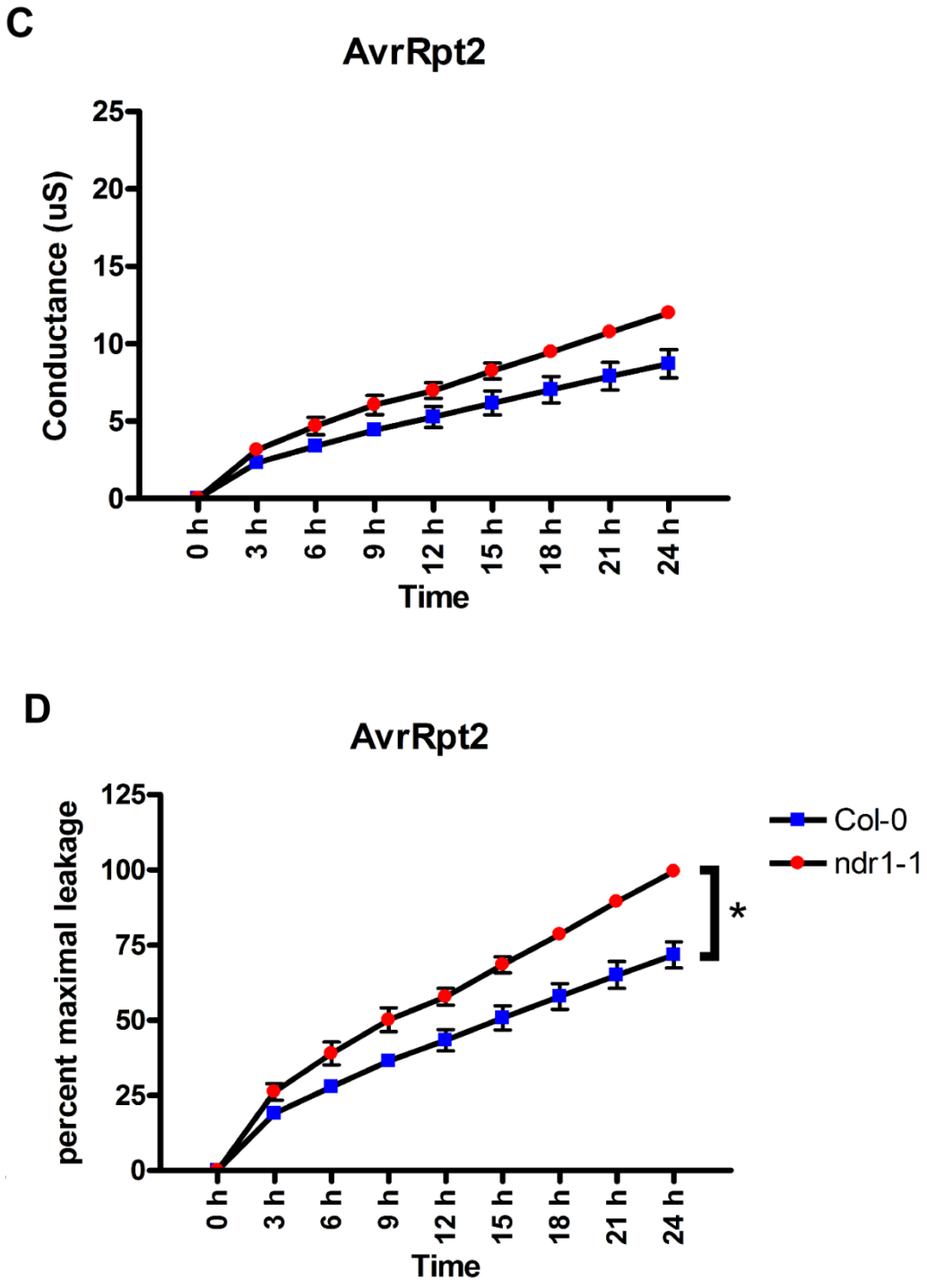


Figure S2.3. Electrolyte leakage Conductance measurements.

As a control for the method of electrolyte leakage utilized in this study, an experiment was performed using a technique adapted from Mackey *et al.* (2002; Appendix). Levels of electrolyte leakage from Col-0 and *ndr1-1* plants in response to DC3000 inoculation are displayed as conductance adjusted for background. Treatments include *Pst* DC3000 expressing (A) vector control and (C) AvrRpt2. (B,D) data from the same experiments displayed in percent maximal leakage the method of calculation used in this study. Error bars display standard deviation from 2 technical replicates from 1 biological replicate. Significance was determined using two-way ANOVA where * represents a statistically significant difference between Col-0 and *ndr1-1*. * $p<0.05$.

Figure S2.4.

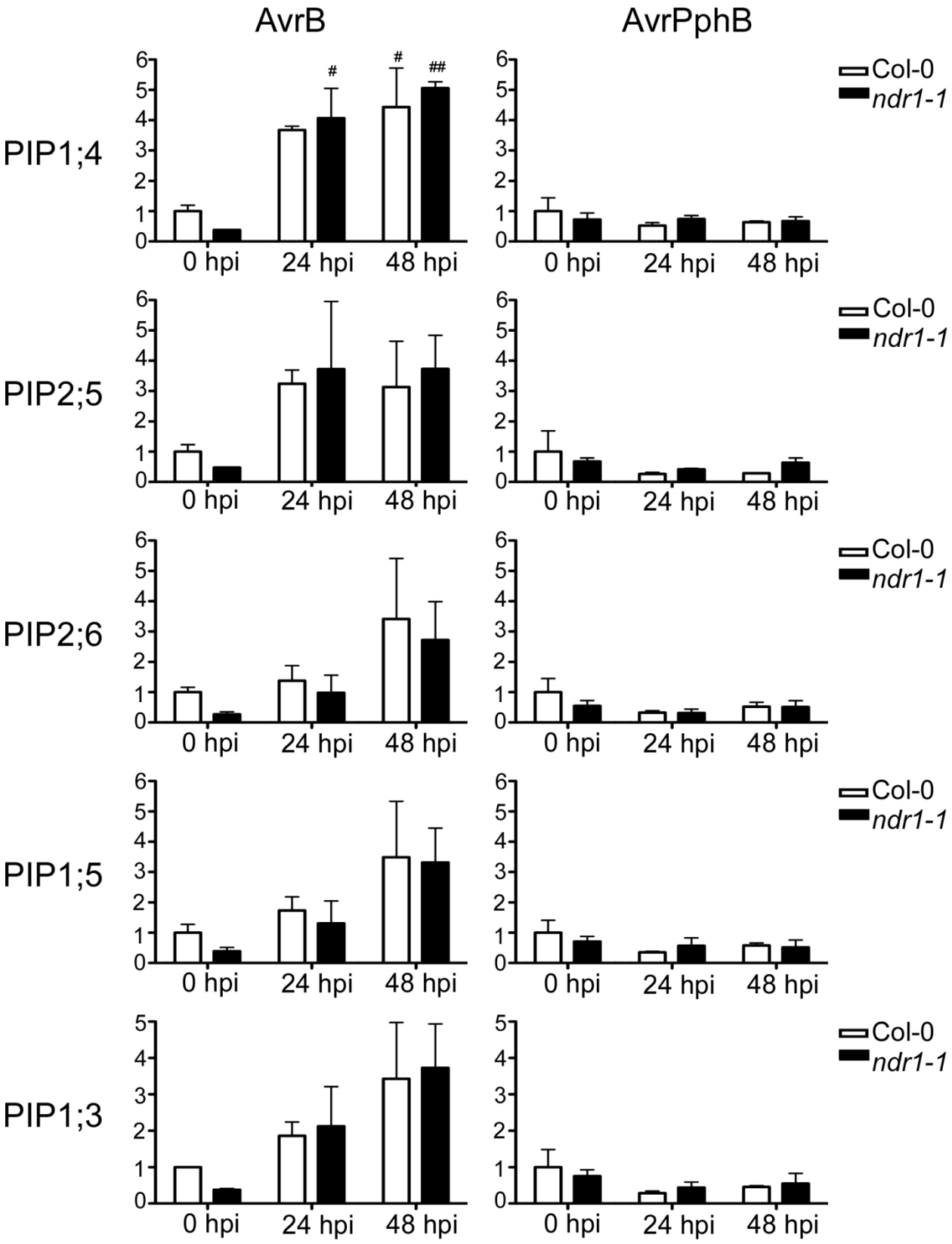

AvrB
AvrPphB
PIP1;4
PIP2;5
PIP2;6
PIP1;5
PIP1;3
Col-0
ndr1-1
0 hpi
24 hpi
48 hpi
#
#
##

Figure S2.4. Expression of aquaporin homolog family genes in response to *Pst* DC3000 expressing AvrB or AvrPphB.

mRNA expression levels of aquaporin homolog genes observed in WT Col-0 and *ndr1-1* plants in response to inoculation with *Pst* DC3000 expressing either AvrB or AvrPphB. Error bars display standard deviation from 1 technical replicate from 2 biological replicates. Samples taken at 0, 24, and 48 hours post inoculation (hpi). Expression values displayed as fold Col-0 0 h average. Statistical significance was determined using two-way ANOVA where * represents statistical difference between Col-0 and *ndr1-1* and # represents statistical change over time. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure S2.5.

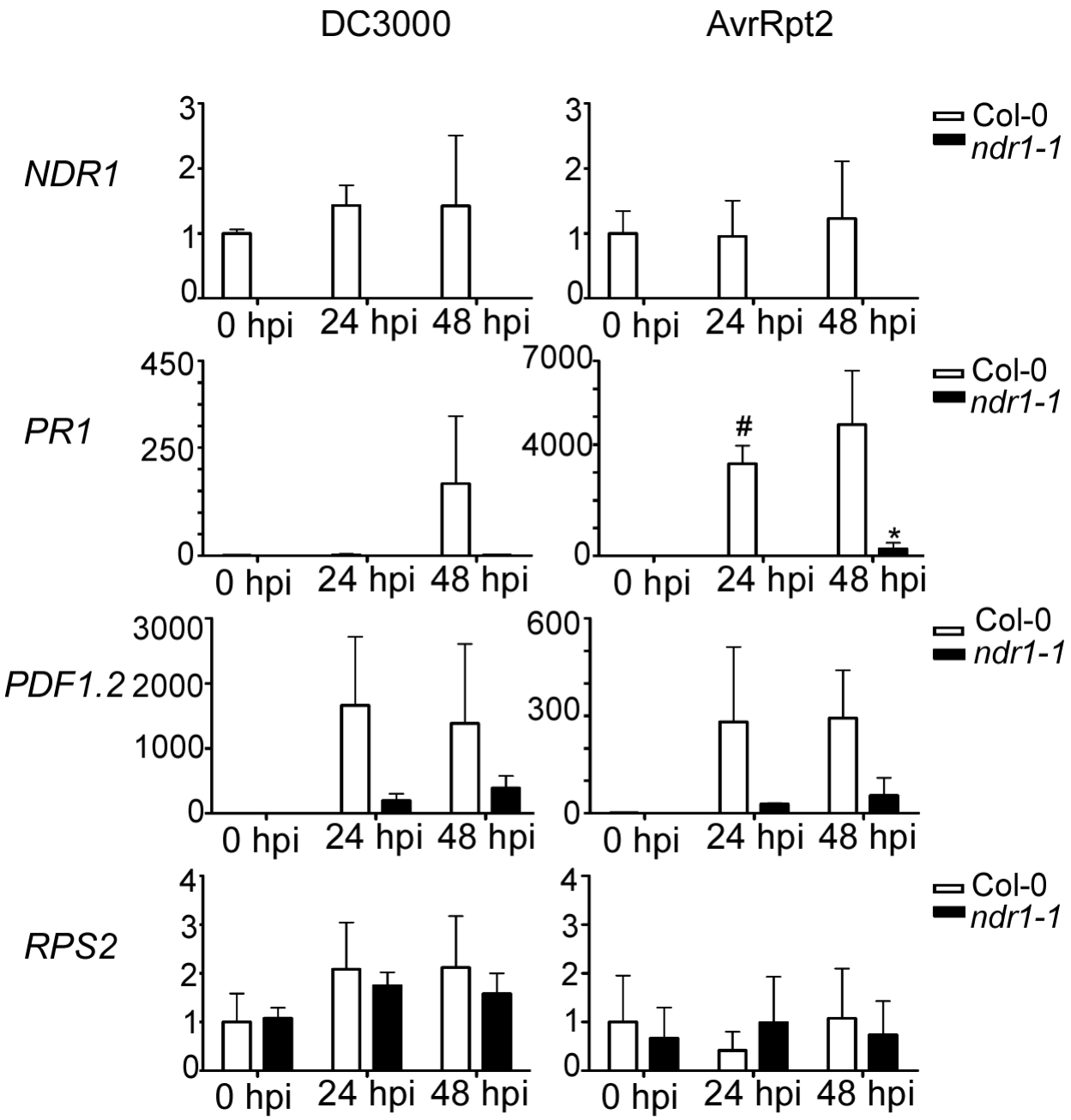

DC3000
AvrRpt2
NDR1
PR1
PDF1.2
RPS2
Col-0
ndr1-1
0 hpi
24 hpi
48 hpi
#
*

Figure S2.5. (cont'd)

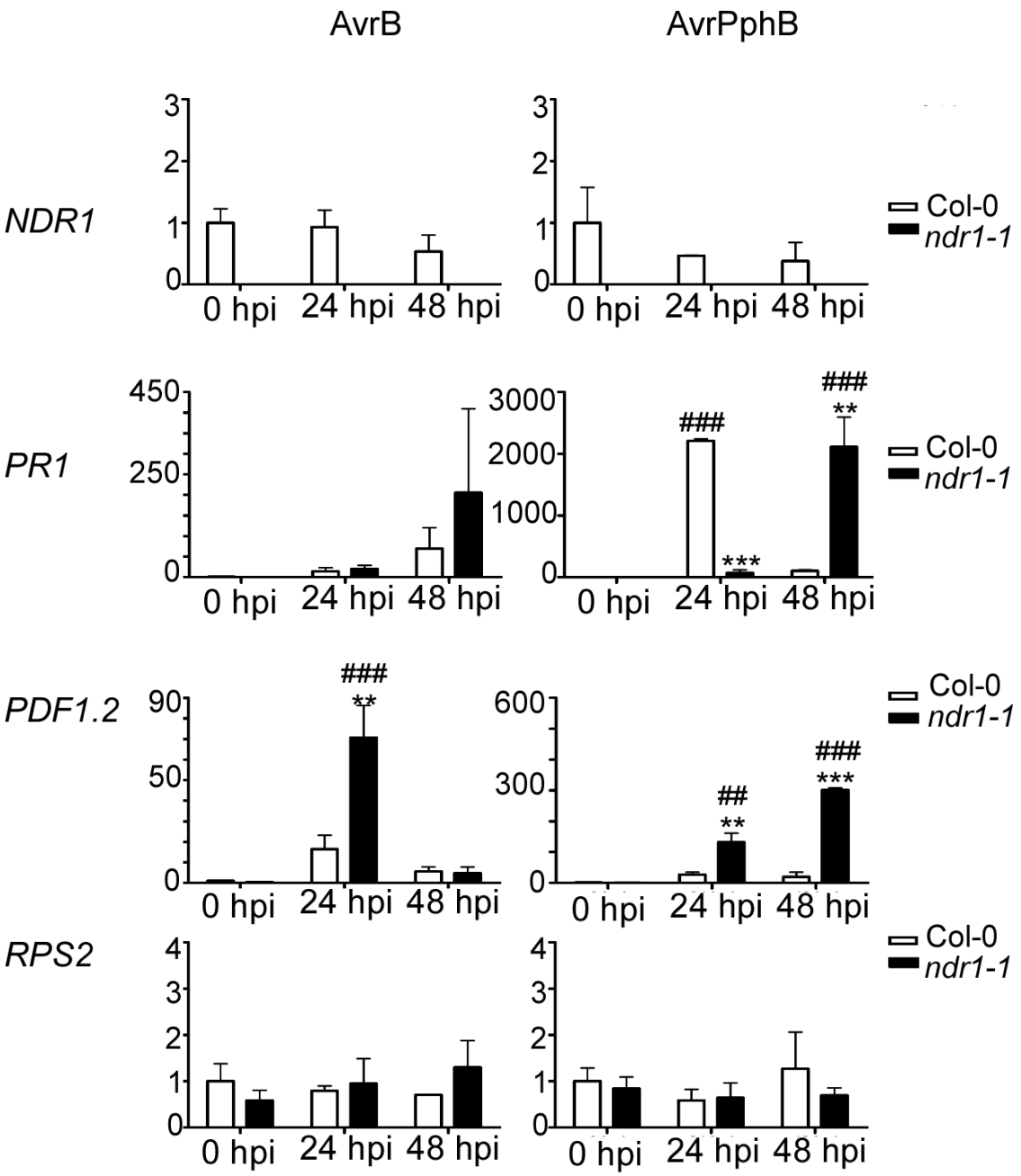


Figure S2.5. Expression of defense marker genes.

mRNA expression levels of *NDR1*, the defense marker genes *PR1* and *PDF1.2*, and the R-gene *RPS2* in WT Col-0 and the *ndr1-1* mutant plants in response to inoculation with *Pst* DC3000 and *Pst* DC3000 expressing the effectors AvrRpt2, AvrB or AvrPphB. Error bars display standard deviation from 1 technical replicate from 2 biological replicates. Samples taken at 0, 24 and 48 hours post inoculation (hpi). Expression values are displayed as fold Col-0 0 h average. Statistical significance was determined using two-way ANOVA where * represents statistical difference between Col-0 and *ndr1-1* and # represents statistical change over time. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure S2.6.

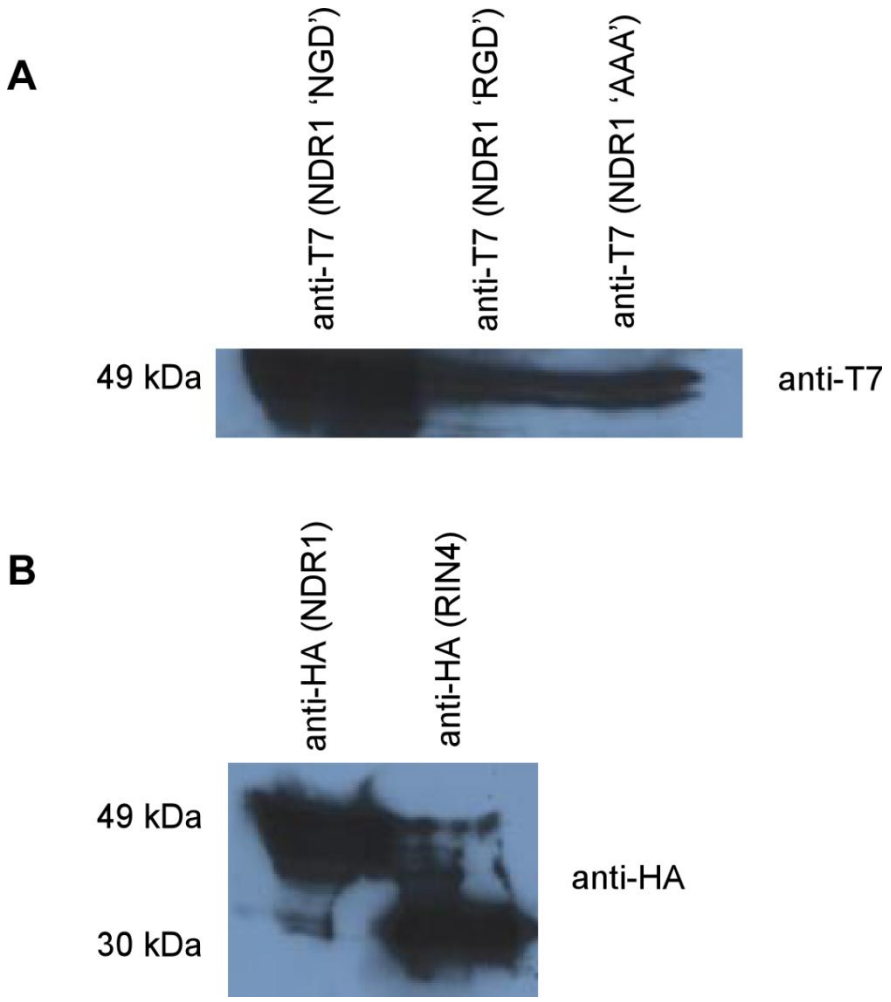


Figure S2.6. Expression levels of HA- and T7-tagged NDR1 constructs utilized.

Expression of all constructs showed modest variability, which accounts for, in part, the variation of band intensity observed in Figure 2.4. Differences in antibody affinities (i.e., T7-HRP versus HA-HRP) are also in part responsible for the observed differences. (A) T7-tagged NDR1 constructs (wild type NDR1 with the NGD site along with NDR1 altered in the NGD site to either AAA or RGD) along with (B) HA-tagged NDR1 and HA-tagged RIN4 utilized in this project.

Figure S3.1.

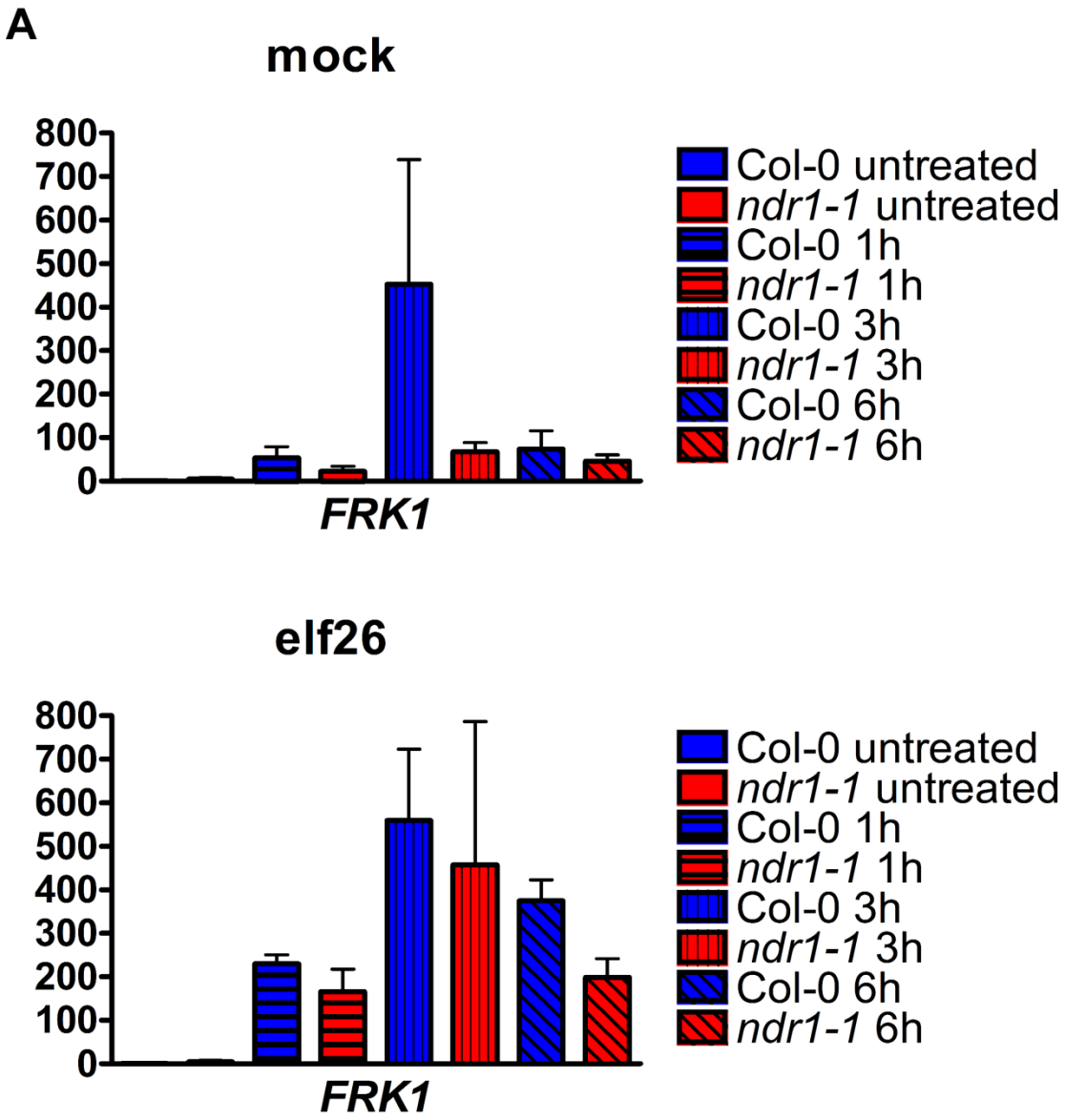

A
mock
800
700
600
500
400
300
200
100
0
FRK1
Col-0 untreated
ndr1-1 untreated
Col-0 1h
ndr1-1 1h
Col-0 3h
ndr1-1 3h
Col-0 6h
ndr1-1 6h
elf26
800
700
600
500
400
300
200
100
0
FRK1
Col-0 untreated
ndr1-1 untreated
Col-0 1h
ndr1-1 1h
Col-0 3h
ndr1-1 3h
Col-0 6h
ndr1-1 6h

Figure S3.1.

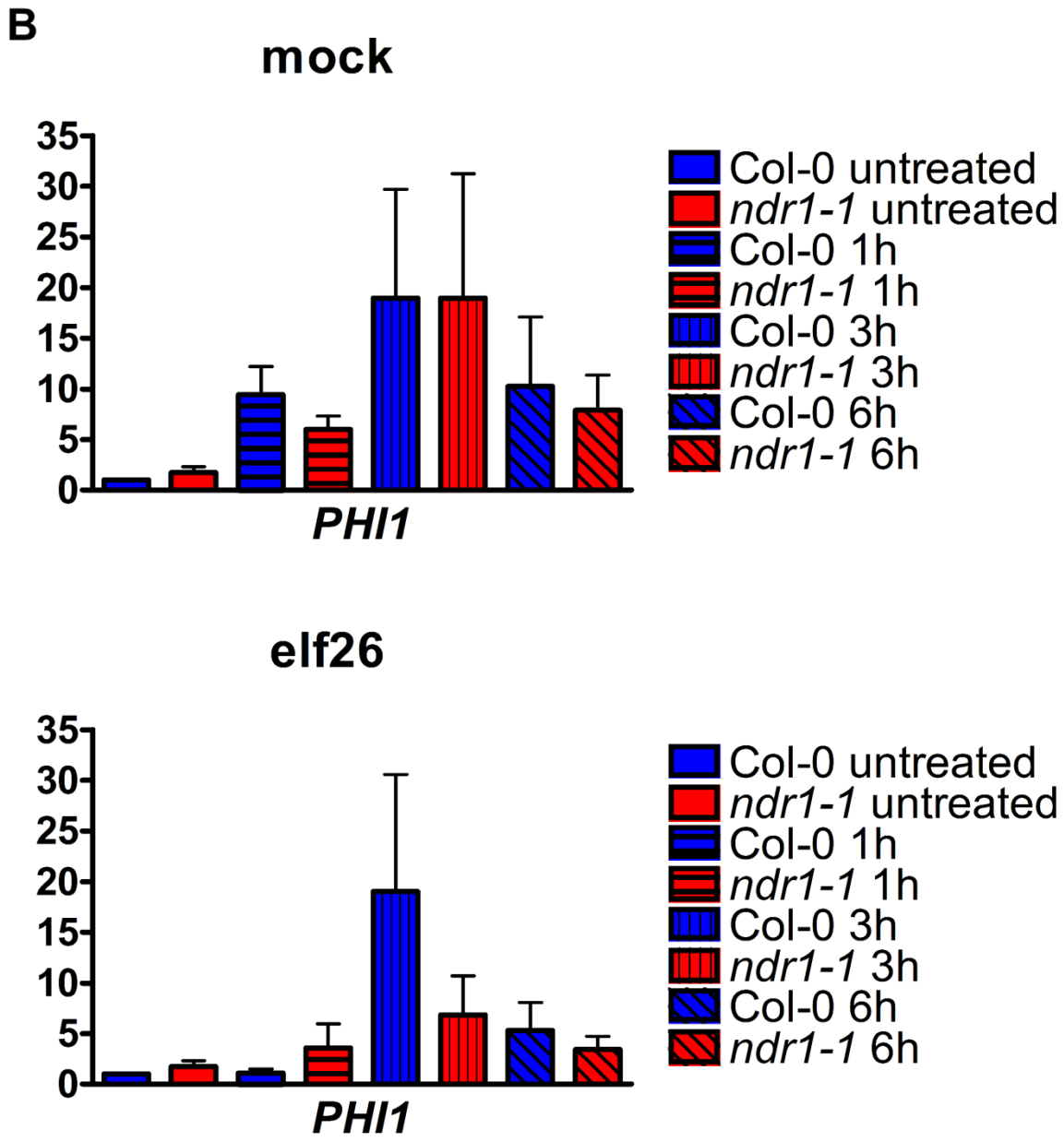

B
mock
35
30
25
20
15
10
5
0
PHI1
Col-0 untreated
ndr1-1 untreated
Col-0 1h
ndr1-1 1h
Col-0 3h
ndr1-1 3h
Col-0 6h
ndr1-1 6h
elf26
35
30
25
20
15
10
5
0
PHI1
Col-0 untreated
ndr1-1 untreated
Col-0 1h
ndr1-1 1h
Col-0 3h
ndr1-1 3h
Col-0 6h
ndr1-1 6h

Figure S3.1. mRNA expression of *FRK1* and *PHI1*.

Expression of the MAPK marker *FRK1* (A) and the CDPK marker *PHI1* (B) after 1 µM elf26 treatment. Samples were collected 1, 3 and 6 hours post infiltration. Expression is displayed as fold Col-0 untreated. Error bars display SD from three biological replicates. No statistical significance was observed using one-way ANOVA followed by Tukey's post-test.

LITERATURE CITED

LITERATURE CITED

Alexandersson, E., Fraysse, L., Sjovall-Larsen, S., Gustavsson, S., Fellert, M., Karlsson, M., Johanson, U. and Kjellbom, P. (2005) Whole gene family expression and drought stress regulation of aquaporins. Plant Mol Biol **59**: 469-484.

Boudsocq, M., Willmann, M.R., McCormack, M., Lee, H., Shan, L., He, P., Bush, J., Cheng, S. and Sheen, J. (2010) Differential innate immune signaling via Ca^{2+} sensor protein kinases. Nature **464**: 418-423.

Forzani, C., Carreri, A., de la Fuente van Bentem, S., Lecourieux, F. and Hirt, H. (2011) The Arabidopsis protein kinase Pto-interacting 1-4 is a common target of the oxidative signal-inducible 1 and mitogen-activated protein kinases. FEBS J. **278**: 1126-1138.

Mackey, D., Holt, B.F., Wiig, A. and Dangl, J.L. (2002) RIN4 interacts with Pseudomonas syringae type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. Cell **108**: 743-754.