NB-LRR Regulation and Function in Arabidopsis

Timothy Kosarick Eitas

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Approved by:

Jeff Dangl

Joe Kieber

Jeff Frelinger

Robert Dietrich

Jason Reed

Abstract

Tim Eitas: NB-LRR Regulation and Function in Arabidopsis
(Under the direction of Jeff Dangl)

Across multi-cellular eukaryotes, Nucleotide-binding, Leucine Rich Repeat (NB-LRR) proteins mediate cell death and responses to pathogens. NB-LRR protein function influences many topics in human health, including vaccine development and autoimmune disorders. For plants, NB-LRR proteins mediate defense responses to a variety of pathogens, such as viruses, bacteria, and fungi. Recent reports estimate that approximately 20-40% of worldwide agricultural production is reduced by plant pathogens and pests. Therefore, basic findings in NB-LRR biology impact topics ranging from biomedical research to crop protection. In the first chapter of my dissertation, I will provide an overview of the genetic and biochemical regulation of NB-LRR proteins and describe how NB-LRR proteins perform signal transduction. My dissertation research characterizes the NB-LRR protein RPM1 in the model plant Arabidopsis. Arabidopsis can be infected by the bacterial pathogen *Pseudomonas syringae*. *P. syringae* pathogenesis is largely caused by secretion of proteins called effectors into host cells. Two *P. syringae* effectors, AvrB and AvrRpm1, induce RPM1-mediated defense responses. In chapter 2, I will describe Arabidopsis mutants that lose recognition of AvrRpm1 (*lra*) because of epigenetic silencing of *RPM1*. In chapter 3, I will evaluate RPM1 regulation by nucleotide exchange and through interaction with the host factor RIN4. In chapter 4, I

characterize the NB-LRR protein TAO1, which perceives AvrB and additively functions with RPM1. In conclusion, my dissertation describes the regulation and function of the NB-LRR protein RPM1 in order to learn new aspects of NB-LRR biology.

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ADP	Adenosine Di-phosphate
ATP	Adenosine Tri-phosphate
Apaf-1	Apoptotic Protease-activating Factor 1
AVR	Avirulence
CC	Coiled Coil
CED4	Cell-death Abnormal-4
DEX	Dexamethasone
EDS1	Enhanced Disease Susceptibility 1
ETI	Effector Triggered Immunity
HSP90	Heat Shock Protein 90
IB	Immunoblot
Lra	Lose Recognition of AvrRpm1
MalT	Maltose Transcription
NALP	NACHT-LRR-PYRIN-containing
NB-LRR	Nucleotide-binding, Leucine Rich Repeat
NDR1	Non-race-specific Disease Resistance 1
NLR	NACHT-LRR
ORF	Open Reading Frame
PAD4	Phytoalexin Deficient 4
PAGE	Polyacrylamide Gel Electrophoresis
PAMP	Pathogen Associated Molecular Pattern
PCR	Polymerase Chain Reaction

PTI	PAMP Triggered Immunity
Pto	*Pseudomonas syringae* pv. tomato
PR-1	Pathogenesis Related 1
R	Resistance
RAR1	Required for *Mla-12* resistance
RIN	RPM1 Interactor
RPM	Resistance to *Pseudomonas syringae* pv *maculicola*
RPP	Resistance to *Peronospora parasitica*
RPS	Resistance to *Pseudomonas syringae* pv *syringae*
SA	Salicylic Acid
SAG101	Senescence Associated Gene 101
SGT1	Yeast dosage suppressor of *skp1–4*
STAND	Signal Transduction ATPase with Numerous Domains
SNC1	Suppressor of npr1-1, constitutive
TAO1	Target of AvrB Operation
TIR	Toll and interleukin Receptor-like
TLR	Toll-like Receptor
WB	Western Blot

CHAPTER 1: Regulation and Signaling of NB-LRR Proteins

Preface

Much of the following chapter will be published in the biotic interactions section of the August 2010 issue of Current Opinion in Plant Biology. I am the sole first author of this publication and wrote the majority of the article. Topics describing the plant immune system that were not covered in the review article but will be included in this chapter are 1) further explanation of PAMP-Triggered Immunity (PTI), 2) mechanisms of pathogen effector perception by plant NB-LRR proteins, 3) regulation of the NB-LRR proteins in the absence of pathogens, and 4) activation-induced NB-LRR localization. These topics have been included in order to provide context for how the additional chapters link to the broad study of the plant immune system.

Abstract

Plants have evolved mechanisms to resist attack by pathogens. The first level of defense consists of Pattern Recognition Receptors (PRRs) that perceive Pathogen Associated Molecular Patterns (PAMPs) and initiate PAMP Triggered Immunity or PTI. Pathogens have evolved the ability to suppress PTI, in many cases through the deployment of proteins generically termed effectors. In response to effectors, plants have evolved NB-LRR (Nucleotide-binding site, Leucine-rich repeat) proteins which are encoded by disease resistance (*R*) genes. NB-LRR

proteins recognize effectors and initiate Effector Triggered Immunity (ETI). The regulation and signaling of NB-LRR proteins are summarized in this chapter.

The Plant Immune System

To defy infection by pathogens, plants have evolved a multi-layered defense system. The first layer of resistance comprises of plant receptors that recognize PAMPs (Jones and Dangl, 2006). Examples of PAMPs include bacterial flagellin, bacterial EF-Tu, and fungal chitin (Zipfel et al., 2004; Ramonell et al., 2005; Zipfel et al., 2006). PAMP-activated defense or PTI results in the production of an oxidative burst, Ca^{2+} influx, Mitogen-activated Protein Kinase (MAPK) cascade, transcriptional re-programming and callose deposition (Gomez-Gomez and Boller, 2002; Navarro et al., 2004; Boudsocq M, 2010). PTI is likely responsible for the growth restriction of non-pathogenic organisms.

Effectors Suppress PTI

Plant pathogens have evolved mechanisms to suppress host PTI through the expression of proteins called effectors (Jones and Dangl, 2006). An example of this is the bacterial effector AvrPtoB which mediates degradation of the Flagellin receptor FLS2 (Gohre et al., 2008). Additionally, the effector HopM1 was shown to cleave the PTI-associated host protein AtMIN7 (Nomura et al., 2006). Over-expression of some effectors *in planta* can enhance the growth of non-pathogenic bacteria approximately 1000 fold (Kim et al., 2005b). Although many effectors have been shown inhibit PTI, a host target that enhances the fitness of a pathogen has not been discovered. Therefore, a possibility is that plant pathogens have evolved effectors for the sole purpose of host defense suppression.

Effectors Elicit ETI

Effectors that are perceived by a plant host are called Avirulence (AVR) proteins. AVR proteins activate Disease Resistance (R) proteins resulting in Effector Triggered Immunity (ETI). In many cases, ETI is produced from the elicitation of disease resistance proteins (R) of the Nucleotide binding site-Leucine Rich Repeat (NB-LRR) protein class. This AVR-R protein interaction can occur directly (Deslandes et al., 2003; Dodds et al., 2006) or though an intermediate protein called a "Guardee" (Dangl and Jones, 2001). Effector-mediated modifications on guardee proteins include proteolytic cleavage, phosphorylation, and ubiquitination (Mackey et al., 2002; Mackey et al., 2003; Shao et al., 2003; Rosebrock et al., 2007). Interestingly, it still remains an open question whether guardees have functional roles in PTI or act as decoys (Kim et al., 2005b; Moffett, 2009). It is also possible that there is variation among the suite of plant guardees for function in either PTI or as decoys. Nevertheless, guardees allow for the detection of effectors by NB-LRR proteins. After effector-induced activation, NB-LRR-mediated ETI results in pathogen growth restriction, Systemic Acquired Resistance (SAR), and typically cell death referred to as a Hypersensitive Response (HR) (Jones and Dangl, 2006).

NB-LRR Protein Family

Plant NB-LRR proteins are a sub-group of the STAND (Signal Transduction ATPase with Numerous Domains) family of proteins (Danot O, 2009). STAND proteins are conserved across both prokaryotes and eukaryotes and mediate biological processes such as transcriptional regulation and apoptosis (Danot O, 2009). In mammalian cells, NB-LRR (NLR) proteins initiate inflammation, responses to pathogens, and perception of danger associated molecular patterns (DAMPs) (Ting JP, 2008). For plants, NB-LRR proteins mediate responses to pathogens in both model systems (Arabidopsis) as well as agriculturally important crops (Tobacco, Wheat, Rice, Tomato, etc) (Sinapidou et al., 2004; Peart et al., 2005; Gutierrez JR, 2009; Loutre et al., 2009; Sang-Kyu Lee, 2009). Therefore, basic knowledge regarding NB-LRR protein regulation and function has important implications for topics ranging from crop protection to human health.

NB-LRR Regulation

Since NB-LRR protein activation typically leads to cell death and severe morphological defects (Zhang et al., 2003; Igari et al., 2008), plants must keep their suite of NB-LRR proteins under tight regulation. Important areas of NB-LRR regulation include mRNA transcript accumulation and protein stabilization. Additionally, NB-LRR proteins are regulated by nucleotide binding, nucleotide hydrolysis, and intramolecular interactions. Finally, some NB-LRR proteins undergo homotypic interactions in either the absence or presence of pathogens. These aspects of NB-LRR regulation allow for the fine balance between the maintenance of signal competency and the prevention of ectopic activation.

Transcript Accumulation

In Arabidopsis, there have been numerous mutants isolated that have altered levels of *NB-LRR* mRNA transcript accumulation. A mutation in the host gene *SSI2*, a stearoyl-acyl carrier protein-desaturase, was demonstrated to induce higher transcript accumulation for several *NB-LRR* genes though alteration of fatty acid levels (Chandra-Shekara AC, 2007). In addition, mutations in the *TIR-NB-LRR* genes, *ssi4* (Suppressor of salicylic acid insensitivity of *npr1-5*) and *snc1* (Suppressor of *npr1-1*, Constitutive 1), cause higher transcript accumulation for multiple *NB-LRR* genes (Shirano et al., 2002; Yi and Richards, 2007). Importantly, all of these mutants (*ssi2*, *ssi4*, *snc1*) have dwarfed morphology, constitutive defense gene expression, and enhanced resistance to virulent pathogens [25, 26, 27]. Therefore, higher mRNA transcript accumulation of some *NB-LRR* genes correlate with enhanced plant defense. *edm2* (Enhanced downy mildew 2) mutants regulate *NB-LRR* transcript accumulation independent of defense activation (Eulgem et al., 2007). *EDM2* encodes a protein that resembles a transcription factor and is required for mRNA transcript accumulation of the *NB-LRR* gene *RPP7* (Eulgem et al.). Collectively, these results show that *NB-LRR* mRNA transcript accumulation is regulated in both the absence and presence of defense activation. Mutants associated with *NB-LRR* transcript accumulation will be described in Chapter 2.

Post-transcriptional Regulation

NB-LRR proteins are subject to post-transcriptional regulation by the host proteins HSP90, RAR1, and SGT1 (Azevedo et al., 2002; Tornero et al., 2002b; Hubert et al., 2003). HSP90 and RAR1 are required for protein accumulation and

function of numerous NB-LRR proteins (Hubert et al., 2003; Bieri et al., 2004; Liu et al., 2004; Holt III et al., 2005). Interestingly, there are NB-LRR proteins that can still function despite having reduced protein accumulation in a *rar1* background (Bieri et al., 2004; Holt III et al., 2005). These results led to the 'threshold model' of NB-LRR protein accumulation which proposes that different NB-LRR proteins are maintained at different accumulation levels (Bieri et al., 2004; Holt III et al., 2005). Additionally, differences in NB-LRR protein accumulation correlate with the amount of disease resistance and also whether an NB-LRR protein requires RAR1 for function (Bieri et al., 2004; Holt III et al., 2005). SGT1 has a more complicated role in NB-LRR protein regulation in that transient silencing of SGT1 reduces protein accumulation for some NB-LRRs (Peart et al., 2002), but SGT1 also negatively regulates NB-LRR protein accumulation in a *rar1* background (Holt III et al., 2005). Additionally, SGT1 has a positive role in NB-LRR-mediated cell death (Holt III et al., 2005). HSP90, RAR1, and SGT1 associate in a protein complex and biochemically regulate each other's function (Boter et al., 2007; Kadota et al., 2008; Hubert et al., 2009).

Nucleotide Binding and Hydrolysis

NB-LRR proteins can bind and hydrolysis nucleotides (Tameling et al., 2002; Ueda H, 2006). Furthermore, a large subset of NB-LRR proteins has been shown to require the nucleotide binding amino acids of the Walker A motif for function (Tornero et al., 2002a; Mestre and Baulcombe, 2006; Tameling et al., 2006; Ade et al., 2007; Wirthmueller et al., 2007; Rairdan et al., 2008). Mechanistically, it has been proposed that NB-LRR proteins undergo cycles of ADP-ATP transitions

(Takken et al., 2006; Tameling et al., 2006). In this model, the stable, resting state NB-LRR is bound to ADP (Tameling et al., 2006). Exposure to a pathogen elicitor causes ADP to be expelled from the NB domain and replaced by ATP. The activated, ATP bound, NB-LRR then signals (Tameling et al., 2006). ATP bound NB-LRR proteins can revert back to the resting ADP-bound state by hydrolyzing ATP (Tameling et al., 2006). This model is supported by data showing that mutations predicted to stabilize ADP binding to the NB, or mutations that block ATP hydrolysis, cause ectopic NB-LRR activation (Tameling et al., 2006). These topics will be addressed for the NB-LRR protein RPM1 in Chapter 3.

Intra-molecular Interactions

NB-LRR proteins are negatively regulated by intramolecular interactions. Examples of this include RPS2, RPS5, RPP1A, RPS4, Rx, and N in which expression of protein fragments that lack the LRR domain result in ectopic signaling (Tao et al., 2000; Michael Weaver L, 2006; Ade et al., 2007; Swiderski MR, 2009). NB-LRR regulation by intra-molecular interactions has been thoroughly characterized for the CC-NB-LRR protein Rx (Moffett et al., 2002; Rairdan and Moffett, 2006). Co-expression of the Rx CC-NB and LRR fragments, or the CC and NB-LRR fragments, results in fragment association and functional complementation (Moffett et al., 2002). Additionally, co-expression of the Rx elicitor abrogates the intramolecular association between the Rx fragments (Moffett et al., 2002). These data indicate that an elicitor can break the intramolecular interactions, releasing negative regulation and opening the protein for signaling. Conversely, activation of the NB-LRR proteins Bs2 or Mi1-2, respectively, does not correlate with a loss of

protein fragment association (Leister et al., 2005; van Ooijen G, 2008). These data collectively indicate that there seems to be variation for the role of intramolecular interactions in the regulation of NB-LRR proteins.

Homotypic Association

NB-LRR proteins can undergo homotypic association in both the presence and absence of pathogens. In the absence of elicitation, the CC-NB-LRR protein RPS5 and the N-terminal domain(N-term)-Solanaceous Domain(SD)-CC-NB-LRR protein Prf form homotypic association (Ade et al., 2007; Gutierrez JR, 2009). For RPS5, the NB domain is sufficient to form homotypic association whereas multi-merization of Prf is mediated by the N-term domain (Ade et al., 2007; Gutierrez JR, 2009). Conversely, the TIR-NB-LRR protein N forms homotypic interactions only after activation by the viral protein p50 (Mestre and Baulcombe, 2006). Mutation of the nucleotide binding residues of N abolished homotypic associaiton, indicating that nucleotide binding can be required for this event (Mestre and Baulcombe, 2006). Collectively, these data indicate that homotypic associaiton can influence plant NB-LRR resting state regulation and signaling. Homotypic interactions of the CC-NB-LRR protein RPM1 will be demonstrated and discussed in Chapter 3.

NB-LRR Signaling

After the negative regulation on a NB-LRR protein is released, these proteins must signal in order to produce ETI. Recent results have demonstrated which NB-LRR protein fragments are sufficient to initiate defense signaling. Importantly, distinct fragments of different NB-LRRs are sufficient for function. Additional factors that are required for NB-LRR signaling include guardees and the

host proteins EDS1, NDR1, MOS7, and SID2. Translocation to the nucleus after activation is required for the function of some NB-LRRs. Finally, disease resistance to a pathogen isolate sometimes requires a pair of NB-LRR proteins.

Pieces: Modularity in NB-LRR Signaling

Given that NB-LRR proteins are modular (Lukasik E, 2009), two reasonable questions are which portion(s) of the protein mediates downstream signaling, and whether these requirements are generalizable across the NB-LRR superfamily. Swiderski et al., (2009) demonstrated that two N-terminal protein fragments of the TIR-NB-LRR protein RPS4, TIR+45 (AA1-205) and TIR+80 (AA1-240), were sufficient to induce cell death. The TIR+80-induced cell death required EDS1, SGT1, and HSP90, indicating that cell death mediated by this fragment had the same genetic requirements as cell death induced by the full-length protein (Zhang Y, 2004; Wirthmueller et al., 2007). Interestingly, cell death was also induced by TIR+80 fragments of RPP1A and N but not RPP2A or RPP2B. Collectively, these data showed that the TIR+80 fragment was sufficient to initiate cell death induced by some but not all TIR-NB-LRR proteins.

Characterization of the CC-NB-LRR protein Rx revealed that a sub-domain (AA139-293) of the NB domain was sufficient to produce cell death (Rairdan et al., 2008). Strikingly, the NB-mediated cell death occurred with a variant that contained multiple mutations in the nucleotide binding residues of the Walker A motif (Tameling et al., 2006; Rairdan et al., 2008). Therefore, ectopic cell death activity of this fragment was likely independent of nucleotide binding. NB-induced cell death

was dependent on SGT1, consistent with previous data for cell death induced by the full-length Rx protein (Boter et al., 2007; Rairdan et al., 2008).

These studies collectively demonstrate that over-expression of fragments from both TIR and CC-containing NB-LRR proteins can initiate cell death. Notably, cell death does not always correlate with disease resistance (Century et al., 1995; Holt III et al., 2005; Michael Weaver L, 2006). Therefore, it will be important to evaluate if expression of the TIR+80 fragments or the NB domain fragment of Rx is also sufficient for ectopic disease resistance. Interestingly, signaling for these fragments (TIR+80 (RPS4, N, RPP1A), NB fragment (Rx)) is likely independent of nucleotide binding. Nucleotide binding and hydrolysis regulate the on-off states and stabilization for some NB-LRR proteins (Tameling et al., 2006; Wirthmueller et al., 2007). Therefore, the recent results for the TIR+80 (RPS4, N, RPP1A) and NB (Rx) fragments may indicate that these fragments bypass regulation at the resting state, and thus represent the exposed signaling platform normally unleashed by activation. Importantly, NB-containing protein fragments of RPS2 and RPS5 require the CC domain in order to initiate ectopic cell death (Tao et al., 2000; Ade et al., 2007). Additionally, the CC domain is required for ectopic cell death and disease resistance mediated by the CC-NB-LRR protein NRG1 (Peart et al., 2005). Collectively, these results indicate that there is a lack of uniformity for NB-LRR fragment-mediated cell death. This suggests that the mechanism for NB-LRR signaling might be particular for each NB-LRR protein.

Perception and Partners: Roles of Accessory Proteins in NB-LRR Signaling

NRIP (N-Receptor-Interacting Protein) was recently demonstrated to be required for disease resistance mediated by the TIR-NB-LRR protein N (Caplan et al., 2008). NRIP interacted with both N and the corresponding viral AVR, p50 (Caplan et al., 2008). Expression of p50 *in planta* caused NRIP to move from chloroplasts to the cytoplasm and nucleus, resulting in association with N (Caplan et al., 2008). The NRIP-N interaction differs from the typical guardee-NB-LRR relationship in that the association of the full length proteins only occurred in the presence of p50 (Caplan et al., 2008). Further studies may reveal if NRIP induces N activation or if NRIP has a downstream role in N signaling. Additionally, NRIP may be important for both aspects of N-mediated defense.

The accessory protein Pto and highly related Pto-like kinases regulate the function of the N-term-SD-CC-NB-LRR protein Prf (Gutierrez JR, 2009). In the absence of pathogen, Pto is required for Prf to form homotypic association that mediate the formation of a signal competent protein complex of ~600 kDA (Gutierrez JR, 2009). The Pto-Prf complex is targeted by the bacterial effectors AvrPto and AvrPtoB, leading to cell death and disease resistance (Gutierrez JR, 2009). In the absence of AvrPto and AvrPtoB, co-expression of an N-terminal domain fragment (AA1-537) and a SD-CC-NB-LRR fragment (AA537-1824) of Prf caused weak cell death, suggesting that the split domains could interact but in a manner that did not fully recapitulate the “off” state (Gutierrez JR, 2009). Interestingly, this Prf-mediated cell death was dependent on Pto, revealing that Pto had a positive role in ectopic Prf signaling (Gutierrez JR, 2009). Co-expression of AvrPtoB, Pto, the N-term domain Prf fragment, and the SD-CC-NB-LRR Prf

fragment also caused cell death, indicating that elicitor-mediated Prf signaling was also dependent on Pto (Gutierrez JR, 2009). These data indicate that Pto has a critical role in ectopic and elicitor-mediated Prf signaling.

Activation Causes NB-LRR Proteins to Change Sub-cellular Localization

Signaling of some NB-LRR proteins has been linked to nuclear accumulation. The barley CC-NB-LRR protein MLA10 was shown to relocate to the nucleus after elicitation and can associate with WRKY transcription factors (Shen et al., 2007). Interestingly, some of the MLA10-interacting WRKY transcription factors negatively regulate PTI (Shen et al., 2007). These results led to the model in which MLA10-mediated ETI was mechanistically due to a de-repression of PTI (Shen et al., 2007). The TIR-NB-LRR proteins N, SNC1, and RPS4 have also been shown to have nuclear accumulation after activation (Burch-Smith et al., 2007; Wirthmueller et al., 2007; Yu Ti Cheng, 2009). For RPS4, the residues in the C-terminal extension domain are required for both nuclear accumulation and cell death (Wirthmueller et al., 2007). However, the RPS4 TIR+80 fragment lacks this C-terminal extension domain and was sufficient to initiate cell death (Swiderski MR, 2009). Therefore, two possibilities from these data are that the small size of the RPS4 TIR+80 fragment may allow for passive diffusion to the nucleus or that the TIR+80 fragment may signal differently that the full length protein. Finally, a striking connection between NB-LRR proteins and nuclear accumulation is RRS1 (Deslandes et al., 2002). RRS1 is localized to the nucleus and consists of a TIR-NB-LRR structure with a C-terminal WRKY transcription factor domain (Deslandes et al., 2002).

Pathways: Downstream Requirements for NB-LRR Function

NB-LRR proteins in plants are divided into two sub-classes based on the presence of an N-terminal Coiled-coil (CC) or Toll and human interleukin receptor (TIR) domain (Meyers et al., 2003). The presence of a CC or TIR domain typically determines whether an NB-LRR-mediated defense response requires either NDR1 (Non-race-specific Disease Resistance) or the EDS1 (Enhanced Disease Susceptibilty 1) /PAD4 (Phytoalexin Deficient 4) /SAG101 (Senescence Associated Gene 101) complex, respectively (Aarts et al., 1998; Feys et al., 2005). NDR1 is a GPI (glycophosphatidyl-inositol) anchored protein that is localized to the plasma membrane (Century et al., 1997; Coppinger et al., 2004; Day et al., 2006). Interestingly, in some cases (RPM1, RPS5), NDR1 is required for NB-LRR-mediated disease resistance but not cell death whereas in other cases (RPS2), NDR1 is required for both disease resistance and cell death (Century et al., 1997). This indicates that NB-LRR-mediated cell death and disease resistance can be genetically separated and that there is variation in how NB-LRR protein function requires NDR1. TIR-NB-LRR proteins require the EDS1/PAD4/SAG101 node for function (Aarts et al., 1998; Feys et al., 2005; Venugopal et al., 2009). Interestingly, EDS1, PAD4, and SAG101 stabilize each other in a heterotypic protein complex that is localized to both the cytoplasm and the nucleus (Feys et al., 2005). Although there has been extensive genetic characterization of NDR1 and the EDS1/PAD4/SAG101 node in NB-LRR signaling, a direct molecular link remains elusive.

The Arabidopsis *snc1-1* (*suppressor of npr1-1, constitutive*) mutation lies in the linker region between the NB and LRR domains of a TIR-NB-LRR protein, resulting in constitutive defense activation (Zhang et al., 2003). A forward genetic screen for suppressors of *snc1-1* has resulted in the isolation of a series of *mos* (*modifier of snc1*) mutants (Palma et al., 2005; Zhang and Li, 2005; Zhang Y, 2005; Yu Ti Cheng, 2009). The *mos* mutants suppress the *snc1-1*-conferred phenotypes of dwarf morphology, constitutive PR-1 expression, and enhanced resistance (Palma et al., 2005; Zhang and Li, 2005; Zhang Y, 2005; Yu Ti Cheng, 2009). Interestingly, the MOS proteins are associated with the plant nucleus although a direct MOS-SNC1 association has not been demonstrated. Nuclear function was shown for *mos7-1*, in that this mutant has lower nuclear protein accumulation of SNC1 (Yu Ti Cheng, 2009). In the absence of the *snc1-1* mutation, *mos7-1* plants were also partially compromised for disease resistance mediated by multiple CC-NB-LRR and TIR-NB-LRR proteins (Yu Ti Cheng, 2009). These data led to the proposal that MOS7-mediated nuclear export pathway has a critical role in NB-LRR signaling (Yu Ti Cheng, 2009).

An important genetic redundancy involving SA (Salicylic Acid) and EDS1 for NB-LRR function was recently uncovered (Venugopal et al., 2009). This study demonstrated that *sid2* (SA induction deficient) or *eds1* mutants did not alter NB-LRR-mediated disease resistance (HRT, RPS2, or RPP8) whereas disease resistance was compromised in the double *sid2 eds1* mutant (Venugopal et al., 2009). For the CC-NB-LRR protein RPS2, loss of disease resistance in the *sid2 eds1* line was not attributed to the loss of RPS2 protein accumulation (Venugopal

et al., 2009). These data led to the model that SA and EDS1 have redundant but critical roles in the signaling of some NB-LRR proteins. Data showing a loss of NB-LRR-mediated cell death (RPM1, RPS2, RPS5) in the *mos7-1* or *eds1 sid2* double mutants would further indicate a direct involvement of MOS7, EDS1, and SID2 in NB-LRR signaling.

It Takes Two to Tango: Disease Resistance Mediated by NB-LRR Pairs

Early research in plant pathology characterizing the interaction between the fungal pathogen flax rust (*Melampsora lini*) and flax (*Linum usitatissimum*) revealed the gene-for-gene relationship, in which the outcome of a pathogen-plant interaction is determined by whether a pathogen avirulence gene (*avr*) coincides with a corresponding plant resistance gene (*R*) (Flor, 1971). However, an emerging theme from both model and agriculturally important plants is that disease resistance against a pathogen isolate, or response to a single AVR, can require pairs of *NB-LRR* genes. Interestingly, these *NB-LRR* pairs differ for their 1) encoded protein domain structures, 2) pathogen isolate and 3) genomic locations.

The first demonstration that a pair of *NB-LRR* genes are required for disease resistance against a pathogen isolate was the finding that both *RPP2A* and *RPP2B* are necessary for disease resistance to an oomycete pathogen isolate (Sinapidou et al., 2004). Since there was no evidence that RPP2A and RPP2B perceived a single AVR, RPP2A and RPP2B may become activated by multiple AVRs. Characterization of *N-NRG1* and *RPM1-TAO1* revealed that disease resistance to viral and bacterial pathogens expressing a single AVR (p50-Tobacco Mosaic Virus, AvrB-*Pseudomonas syringae,* respectively) can be mediated by an *NB-LRR* pair

encoding proteins of the TIR and CC sub-classes (Peart et al., 2005; Eitas et al., 2008). Recent investigation of *RRS1* and *RPS4* demonstrated that this *TIR-NB-LRR* pair is required for disease resistance against multiple pathogen isolates (Gassmann et al., 1999; Deslandes et al., 2002; Narusaka M, 2009) Examples of CC-NB-LRR-encoding gene pairs mediating disease resistance to fungal pathogen isolates came from the identification of *Lr10-RGA2* and *Pi5-1-Pi5-2* (Lee SK, 2009; Loutre et al., 2009). Finally, characterization of *Pikm1-TS* and *Pikm2-TS* demonstrated that two *NB-LRR* genes encoding N-terminal non-TIR domains are required for disease resistance against a fungal pathogen isolate (Ashikawa I, 2008). Similar to RPP2A-RPP2B, it has not been shown whether the Lr10-RGA2, Pi5-1-Pi5-2, and Pikm-1-TS-Pikm2-TS pairs are activated by a single or multiple AVRs. The domain structure, pathogen isolate, and AVR elicitor for these NB-LRR pairs are summarized in Figure 4.6.

Since *NB-LRR* gene families can exist in genomic clusters, a possibility is that a *NB-LRR* pair resides within a single locus. In fact, many of the *NB-LRR* pairs are tightly linked (*RPP2A-RPP2B*, *RRS1-RPS4*, *LR10-RGA2*, *Pikm-1-TS-Pikm-2-TS, Pi5-1-Pi5-2*) (Sinapidou et al., 2004; Ashikawa I, 2008; Lee SK, 2009; Loutre et al., 2009; Narusaka M, 2009). Over-expression of NRG1 or an RPS4 truncation can initiate ectopic cell death in the absence of N or RRS1 activation, respectively (Peart et al., 2005; Swiderski MR, 2009). These data demonstrate that NRG1 or RPS4 either signal downstream of their respective partner NB-LRR, or that over-expression of these NB-LRRs can overcome the requirement for the partner NB-LRR. Interestingly, the *TAO1-RPM1* pair is not linked and these NB-LRR proteins

can independently produce disease resistance following recognition of AvrB (Eitas et al., 2008) (Chapter 4, Figures 2 and 4). These results indicate that the function of one NB-LRR does not always require the partner NB-LRR.

Parting shots: Perspectives

A number of recent reports have demonstrated that pairs of *NB-LRR* genes are required for disease resistance to a pathogen isolate or a single AVR. These NB-LRR pairs function in disease resistance against multiple pathogens, include homo- and hetero-typic N-terminal domain pairs, and can be genetically linked or unlinked. When both *NB-LRR* genes of are required for disease resistance, a possible model is that the NB-LRR pair form hetero-multimers that allow for pathogen detection. Heterotypic interactions of both Toll-like receptors (TLRs) and NLRs have been demonstrated in mammals (Takeuchi O, 2002; Hsu LC, 2008; Ting JP, 2010). Downstream of AVR perception, activation of multiple NB-LRR proteins may lead to an increase or diversity of signal(s) that is required for an effective defense response.

The recent work characterizing NB-LRR signaling provokes some compelling questions. First, why is there a lack of uniformity for signaling among fragments of TIR-NB-LRR and CC-NB-LRR proteins? Second, how do these NB-LRR fragments biochemically initiate cell death? Third, are the same NB-LRR fragments required for both cell death and disease resistance? Fourth, does signaling leading to cell death and pathogen growth restriction occur in the same sub-cellular compartment? Fifth, how do accessory proteins specifically influence effector-mediated NB-LRR signaling? Finally, what is the molecular mechanism

underlying the loss of NB-LRR-mediated disease resistance in the *ndr1*, *eds1*, *mos7*, and *eds1 sid2* mutants? As is typically the case in science, these initial findings have provided fodder for further investigation.

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CHAPTER 2: Epigenetic Regulation of *RPM1*

Preface

The following chapter will be submitted for publication in next 2-3 months. David Hubert performed the forward genetic screen and isolated the *lra6* mutants. Additionally, David Hubert positionally cloned *lra6-1*. All figures and tables presented in this chapter represent my work. Kyle Gaulton performed the bioinformatical analysis that will be described in the Discussion section.

Abstract

Many phytopathogenic bacteria inject type III effectors into host plant cells for the purpose of enhancing pathogen virulence. In response, plants have evolved disease resistance (*R*) genes which confer defense to bacterial pathogens expressing specific effectors. Many disease resistance genes encode proteins of the Nucleotide-binding site, Leucine Rich Repeat (NB-LRR) class. AvrRpm1 is an extensively studied type III effector that is perceived by the Arabidopsis NB-LRR protein RPM1. A forward screen for host gene products that Lose Recognition of AvrRpm1 (*lra*) was initiated using a two component β-Estradiol-inducible *avrRpm1* transgenic system and two gene copies of *RPM1*. This screen yielded two allelic mutants, *lra6-1* and *lra6-2*. In both *lra6* alleles, the loss of AvrRpm1 perception is due to loss of *RPM1* transcript accumulation. *lra6-1,* but not *lra6-2,* is also partially compromised for the function of the unlinked, *NB-LRR RPS2*. The loss of *RPM1* transcript accumulation in the *lra6* mutants is accompanied by methylation of the

RPM1 locus. Therefore, our findings indicate that *NB-LRR* genes can be regulated by epigenetic silencing in Arabidopsis.

Introduction

DNA methylation influences on gene regulation across eukaryotic cells. For the model plant Arabidopsis, DNA methylation is exclusively targeted to cysteine residues of 'CG', 'CNG', and 'CNN' motifs (Zhang X, 2006). Methylation patterns differ between Arabidopsis and vertebrates in that the Arabidopsis genome has large non-methylated regions of 'CG' motifs (Zhang X, 2006; Suzuki MM, 2008). DNA methylation in coding sequences positively correlates with mRNA transcript accumulation whereas promoter methylation negatively correlates with mRNA transcript accumulation (Zhang X, 2006). Therefore, DNA methylation in Arabidopsis is specifically regulated and can influence gene expression.

Approximately 40% of methylated regions in Arabidopsis have corresponding siRNA species (Aufsatz W, 2002; Matzke M, 2007). The Arabidopsis siRNA transcriptome is large and diverse relative to other organisms (Kasschau KD, 2007). Functions of siRNA species include silencing of transposons and repetitive elements (Vaucheret H, 2004; Suzuki MM, 2008). Mechanistically, RNA-directed DNA Methylation (RdDM) occurs through short stretches (19-25 basepairs) of corresponding sequence between small RNA species and target sequences (Vanitharani R, 2003; D., 2004). RdDM is sub-divided into Transcriptional Gene Silencing (TGS) or Post-Transcriptional Gene Silencing (PTGS) based upon whether the silencing siRNA species targets a promoter or coding sequence, respectively (D., 2004; Huettel B, 2007).

Few studies have evaluated the relationship between host gene silencing and regulation of *NB-LRR* genes. One study characterized the relationship between the Arabidopsis *RPP5* gene cluster and the *NB-LRR SNC1* (Yi H, 2007). Transgenic over-expression of SNC1 caused inhibition of mRNA transcript accumulation for both *SNC1* and two additional *NB-LRR* members of the *RPP5* gene cluster (Yi H, 2007). The *RPP5* gene cluster was shown to produce siRNA species, and host genes associated with PTGS (*DCL4* and *AGO1*) were required for inhibiting mRNA transcript accumulation of *SNC1* (Yi H, 2007). Importantly, over-expression of SNC1 caused ectopic defense activation (Yi H, 2007). This result led to the proposal that silencing of both *SNC1* and two additional members of the *RPP5* gene cluster was induced by stress associated with ectopic defense activation (Yi H, 2007). Another example of transgene-induced silencing of *NB-LRR* genes came from the characterization of the lettuce *RGC2* gene cluster (Wroblewski T, 2007). This study demonstrated that transgenic over-expression of an interfering hairpin RNA (ihpRNA) of *RGC2B* reduced transcript accumulation for eight *NB-LRR* genes of the *RGC2* cluster (Wroblewski T, 2007). In both of these studies, there was no demonstration that the loss of transcript accumulation was due to methylation of the *NB-LRR* loci analyzed. There has also been a demonstration that production of a siRNA species positively contributes to RPS2-mediated disease resistance (Katiyar-Agarwal S, 2006). Therefore, small RNA species can both positively and negatively regulate *NB-LRR* function.

Our study is focused on the regulation of the *NB-LRR* gene *RPM1* in Arabidopsis. The bacterial effector AvrRpm1 activates RPM1, resulting in host

defense responses. A forward genetic screen was initiated for the purpose of isolating mutants that lose perception of AvrRpm1 (Hubert et al., 2009). The Col-0 accession of Arabidopsis, containing both a two component β-Estradiol-inducible a*vrRpm1* transgenic system and a *RPM-MYC* transgene, was selected for the screen (Boyes et al., 1998; Hubert et al., 2009). Since the Col accession has a native *RPM1* gene, the mutagenized line had two gene copies (four alleles) of *RPM1* (Hubert et al., 2009). After EMS-mutagenesis, selection of mutants that lose perception of AvrRpm1 resulted in isolation of the *lra6* mutants.

In this work, we characterize two alleles of *lra6*, *lra6-1* and *lra6-2*. Both *lra6-1* and *lra6-2* lose *RPM1* transcript accumulation. The loss of *RPM1* transcript accumulation is accompanied by methylation of the *RPM1* promoter and coding region. Interestingly, *lra6-1* is also compromised for RPS2 function but does not affect RPS5 function or basal disease resistance to *Pto* DC3000. We propose that silencing of *RPM1* in the *lra6* mutants was caused by the summation of ectopic activation of RPM1, EMS mutagenesis, and the presence of two gene copies of *RPM1*.

Results

Genetic Analysis of the *lra6* Mutants

lra6-1 and *lra6-2* were crossed to the La-*er* (Landsberg *erecta*) accession for map-based positional cloning (Table 2.1). Analysis of the F1 and F2 progeny indicated that both *lra6* mutants are recessive and segregate as a mutation in a single locus (Table 2.1). Immunoblot analysis revealed that the *lra6* mutants lost RPM1-MYC protein accumulation (D. Hubert, unpublished), leading to speculation

that the *lra6* mutants were allelic. Analysis of the F1 and F2 progeny from a cross between *lra6-1* and *lra6-2* demonstrated that these two mutants are allelic (Table 2.1).

Cross	Progeny	No. of Plants Analyzed	Resistant	Susceptible	X^2	*P* Value
lra6-1 **x La-*er***	**F1**	**6**	**6**	**0**	**ND**	**ND**
lra6-1 **x La-*er***	**F2**	**130**	**100**	**30**	**1.6[a]**	***P*** **> .05**
lra6-2 **x La-er**	**F1**	**6**	**6**	**0**	**ND**	**ND**
lra6-2 **x La-er**	**F2**	**72**	**52**	**20**	**1.18[a]**	***P*** **> .05**
lra6-1 **x *lra6-2***	**F1**	**6**	**0**	**6**	**ND**	**ND**
lra6-1 **x *lra6-2***	**F2**	**54**	**0**	**54**	**ND**	**ND**

a. X^2 value given for expected ratio of 3:1

Table 2.1. *lra6* Genetic Analysis. 3-week-old Arabidopsis plants were spray inoculated with *Pto* DC3000(*avrRpm1*). "Resistant" or "Susceptible" was based on the absence or presence of *Pto* DC3000(*avrRpm1*)-induced disease symptoms. ND represents "Not" "Done".

lra6 Mutants Alter *RPM1* and *RPS2*-mediated Function

Instead of performing a traditional backcross to the parental mutagenized line, we crossed the *lra6* mutants to the Col-0 accession and selected F2 lines that lost RPM1 function. The β-Estradiol-inducible *avrRpm1* transgenic system consists of an inducible promoter driving expression of an *avrRpm1* transgene and a 'Driver'

transgene. The 'Driver' transgene expresses a transcription factor which, in the presence of β-Estradiol, drives expression of *avrRpm1* from the inducible promoter (Tornero et al., 2002a). After the isolation of *lra6* lines, we used PCR to select lines that lacked the *avrRpm1* and *RPM1-MYC* transgenes. The selected *lra6* lines still contained the 'Driver' transgene and were used for all figures presented in this work except Figure 2.3B. Inoculation of the *lra6* lines with either a high or low titer of *Pto* DC3000(*avrRpm1*) showed that both *lra6-1* and *lra6-2* are fully compromised for RPM1 function (Fig. 2.1A,B). Additionally, inoculation with either a high or low titer of *Pto* DC3000(*avrRpt2*) revealed that *lra6-1* partially compromised RPS2 function (Fig. 2.1C,D). *lra6-2* did not affect RPS2 function (Fig. 2.1C,D). Collectively, these data indicate that RPM1 function is lost in both *lra6* mutants and RPS2 function is partially compromised in *lra6-1.*

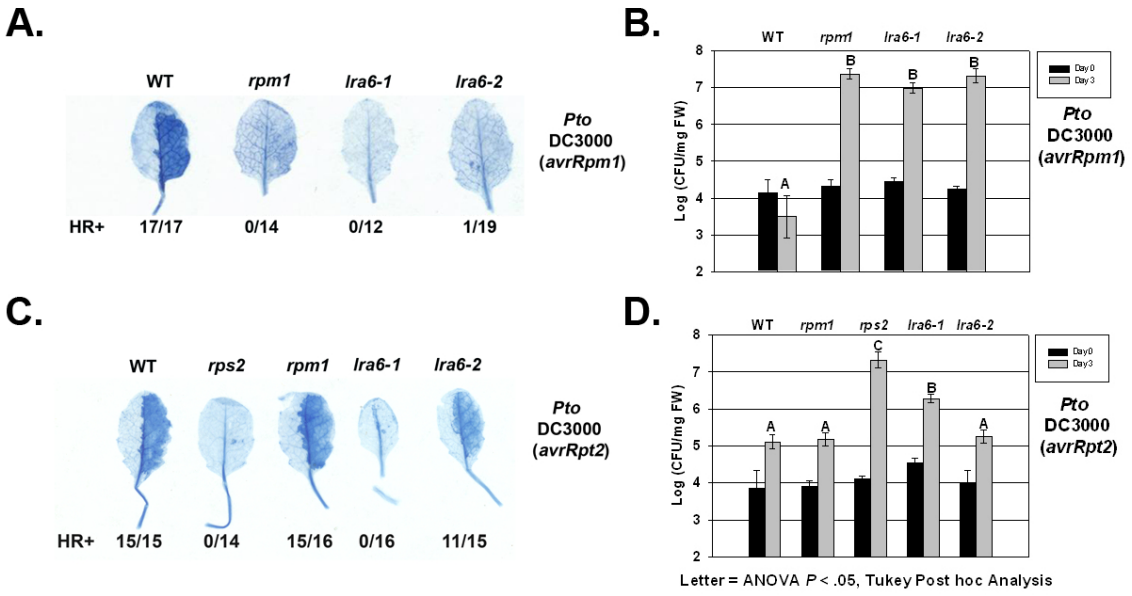

Figure 2.1 *lra6* Mutants Lose RPM1 and RPS2 Function. A. ~4-week-old plants were hand inoculated with *Pto* DC3000(*avrRpm1*) and leaves were stained with trypan blue ~6 hours post-inoculation. The number of leaves displaying a RPM1-mediated hypersensitive response (HR) are below each leaf picture. B. ~2-3 week-old-seedlings

were dip inoculated with *Pto* DC3000(*avrRpm1*) according to previously published conditions (Tornero and Dangl, 2001). Error bars represent 2X Standard Error. A one-way analysis of variance (ANOVA) was applied to the values. Letters represent different classes based on Tukey Post-hoc analysis, $P < .05$. C. ~4-week-old plants were hand inoculated with *Pto* DC3000(*avrRpt2*) and leaves were stained with trypan blue ~12 hours post-inoculation. The number of leaves displaying a RPS2-mediated hypersensitive response (HR) are below each leaf picture. D. ~2-3 week-old-seedlings were dip inoculated with *Pto* DC3000(*avrRpt2*) according to previously published conditions (Tornero and Dangl, 2001). Error bars represent 2X Standard Error. A one-way analysis of variance (ANOVA) was applied to the values. Letters represent different classes based on Tukey Post-hoc analysis, $P < .05$.

lra6 Mutants Do Not Affect RPS5 Function or Basal Resistance.

We next tested if RPS5 function was compromised in the *lra6* lines. Measurement of bacterial growth after inoculation with *Pto* DC3000(*avrPphB*) showed that RPS5-mediated disease resistance was not altered in either *lra6-1* or *lra6-2* mutant lines (Fig.2.2A). Since *NB-LRR* genes likely have roles in basal defense to *Pto* DC3000 (Holt III et al., 2005), we also tested if the *lra6* mutants were altered for basal resistance to *Pto* DC3000. Results displayed in Figure 2.2B demonstrate that *lra6-1* and *lra6-2* are not altered for basal resistance to *Pto* DC3000. Therefore, the *lra6* mutants may only affect defense responses mediated by RPM1 and RPS2.

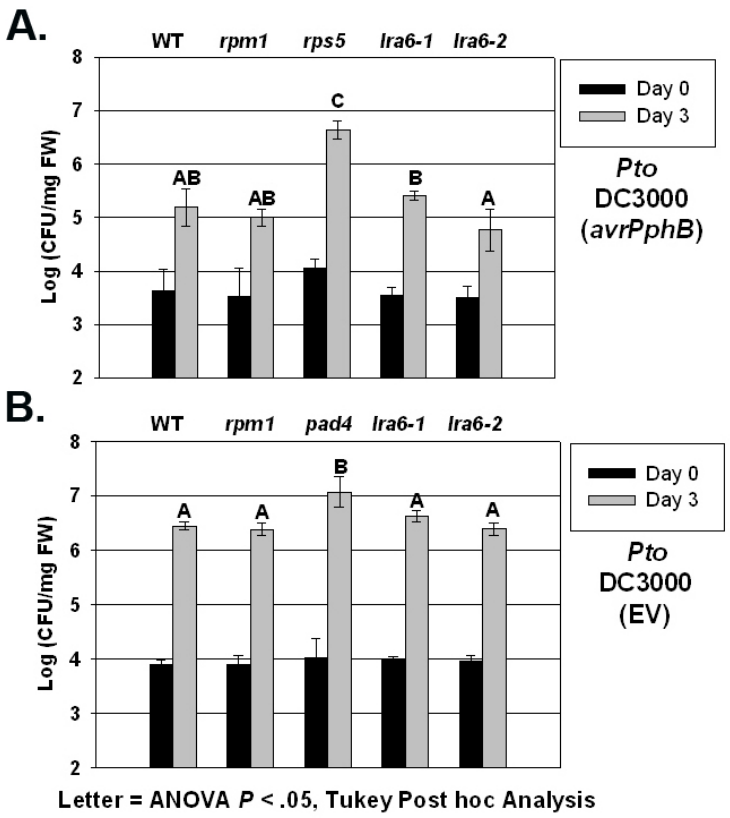

Figure 2.2. *lra6* Mutants Do Not Affect RPS5 Function or Basal Resistance. A. ~2-3 week-old-seedlings were dip inoculated with *Pto* DC3000 (*avrPphB*) according to previously published conditions (Tornero and Dangl, 2001). Error bars represent 2X Standard Error. A one-way analysis of variance (ANOVA) was applied to the values. Letters represent different classes based on Tukey Post-hoc analysis, $P < .05$. B. Same as A, but with *Pto* DC3000 (EV) (empty vector).

lra6 Mutants Alter *RPM1* and *RPS2* Transcript Accumulation.

A potential reason why RPM1 function is lost in the *lra6* mutants is due to a lack of *RPM1* mRNA accumulation. Real-time PCR analysis revealed that *RPM1* mRNA accumulation is abolished in both *lra6-1* and *lra6-2* (Fig. 2.3A).

In presence of both the β-Estradiol-inducible a*vrRpm1* transgenic system and the *RPM1-MYC* transgene, *RPS2* transcript accumulation was reduced in *lra6-1* (Fig. 2.3B). Surprisingly, when the same analysis was performed with *lra6-1* lines that lacked both the *avrRpm1* and *RPM1-MYC* transgenes, there was no difference in

RPS2 transcript accumulation (Fig. 2.3C). These data indicate that the reduced accumulation of the *RPS2* transcript in *lra6-1* correlated with the presence of the a*vrRpm1* and *RPM1-MYC* transgenes.

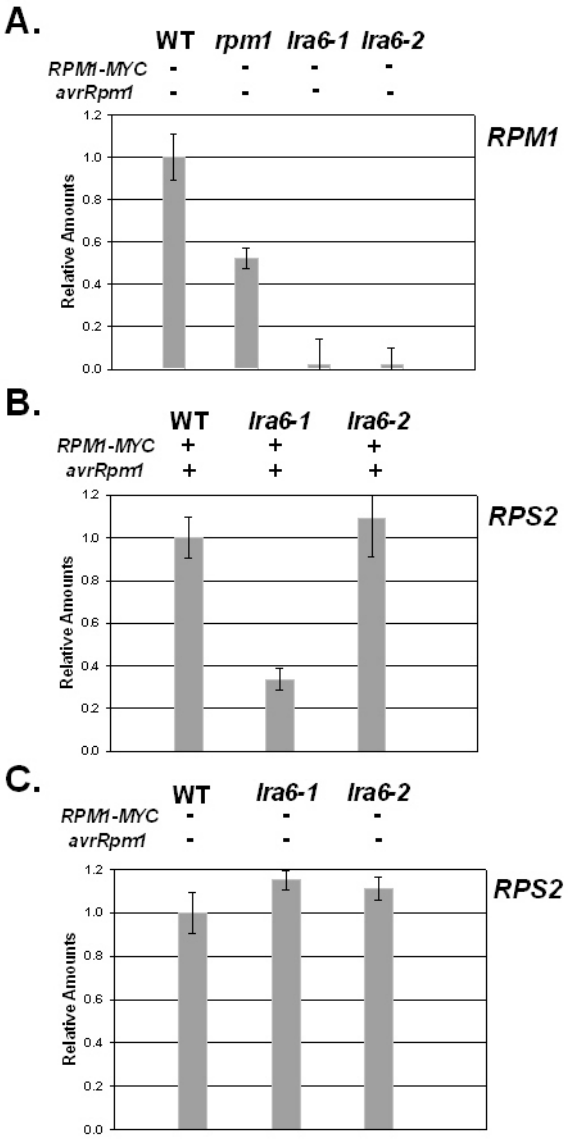

Figure 2.3. *lra6* Mutants Alter *RPM1* and *RPS2* Transcript Accumulation. A. Various plant lines that lack the *avrRpm1* and *RPM-MYC* transgenes were subject to Real-time PCR analysis for the *RPM1* transcript. Ct values were normalized to EF-1 (Baud S, 2003). Bar graphs depict the amount of *RPM1* transcript relative to WT(Col). Error bars represent Std. Error. B. Real-time PCR analysis for the *RPS2* transcript in various lines containing both the *RPM1-MYC* and *avrRpm1* transgenes. Bar graphs depict the amount of *RPS2* transcript accumulation in relation to WT(Col). Error bars represent Std. Error. C. Same as B., but lines do not contain *RPM1-MYC* or *avrRpm1* transgene.

The *lra6* Mutants are Epialleles of *RPM1*

Mapping revealed that the *lra6-1* mutation was in a ~800 kilobase interval that contained the native *RPM1* gene. Therefore, we hypothesized that the loss of *RPM1* mRNA transcript accumulation in the *lra6* mutants could be due to epigenetic modification of the *RPM1* locus. We tested this possibility genetically, by crossing *lra6* to the recessive, loss-of-function *rpm1-3* allele (Grant et al., 1995). The F1 progeny lost RPM1 function, indicating a lack of complementation between the *lra6* mutants and *rpm1-3* (Fig. 2.4A). The same experimental approach was used to demonstrate epigenetic silencing of *SUPERMAN* in Arabidopsis (Jacobsen SE, 1997). Since sequencing of the 1 kb promoter, coding sequence, and 3' UTR of the native *RPM1* locus revealed no nucleotide mutation, we speculated that the loss of *RPM1* mRNA transcript accumulation was due to methylation of the *RPM1* locus.

We digested genomic DNA with methylation-sensitive restriction enzymes and subjected the digestion products to PCR analysis. In this experiment, the presence of a PCR product indicates that the template DNA was methylated at the restriction enzyme cut-site (Saze H, 2008; Martin A, 2009). After digestion with AclI, the wild-type(*RPM1*) line lacked a PCR product for either the *RPM1* promoter or *RPM1* ORF(1-1504) whereas the *lra6* mutants had PCR products for both regions (Fig. 2.4B). These results indicate that the *lra6* mutants were methylated at the cut sites for AclI in both the *RPM1* promoter and *RPM1* ORF(1-1504) (Fig. 2.4B). As a

control for the presence of DNA in the wild-type(*RPM1*) sample, PCR was performed on a *RPM1* region that did not have an AclI cut site (Fig. 2.4B, right). Similar results were demonstrated using the methylation-sensitive enzyme HpaII (Fig. 2.4B). Therefore, our data show that *lra6-1* and *lra6-2* are epigenetically silenced due to methylation at the *RPM1* locus.

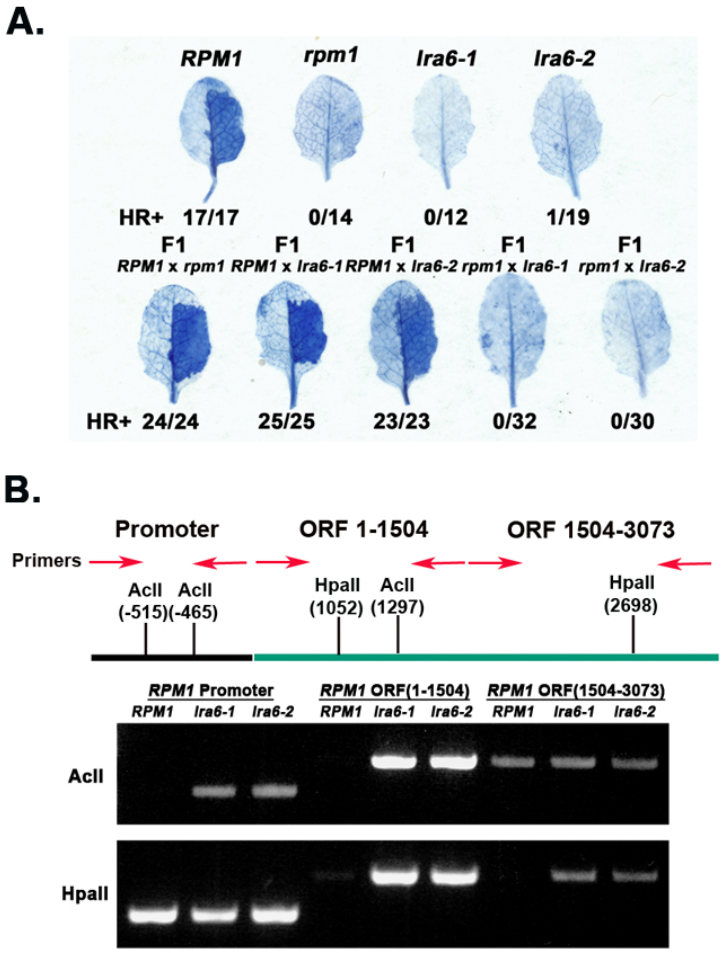

Figure 2.4. The *lra6* Mutants are Epialleles of *RPM1*. A. Lack of complementation between *lra6* mutants and *rpm1*. ~4-week-old plants were hand inoculated with *Pto* DC3000(*avrRpm1*) and leaves were stained with trypan blue ~6 hours post-inoculation. The number of leaves displaying a RPM1-mediated hypersensitive response (HR) are below each leaf picture. B. (top) Schematic of *RPM1* promoter(black) and coding region(green). Primers are shown in red and AclI and HpaII cut sites are shown. (bottom) DNA from indicated lines was digested with methyl-sensitive restriction enzymes (left) and then subjected to PCR analysis. For AclI, the control for the presence of DNA in samples was shown by PCR amplification of the *RPM1* ORF(1504-3073) which did not have AclI cut

site. For HpaII, the control for the presence of DNA in samples was shown by PCR amplification of *RPM1* Promoter region which did not have HpaII cut site.

Discussion

In this work we characterize two epialleles of *RPM1*, *lra6-1* and *lra6-2*. The presence of both the β-Estradiol-inducible *avrRpm1* transgenic system and one gene copy of *RPM1* has been shown induce low levels of ectopic defense activation in Arabidopsis (Tornero et al., 2002a). Ectopic defenses caused by over-expression of the NB-LRR SNC1 correlates with silencing of *SNC1* and additional *NB-LRR* genes (Yi H, 2007). Notably, no epigenetic alleles of *RPM1* were isolated from the forward screen using the β-Estradiol-inducible *avrRpm1* transgenic system and one gene copy of *RPM1* (Tornero et al., 2002a). The key difference to the forward screen that produced the *lra6* mutants was the presence of the *RPM1-MYC* transgene (Hubert et al., 2009). Therefore, the RPM1-mediated ectopic defense activation, coupled with an additional *RPM1* gene copy, may have induced silencing of *RPM1* in the *lra6* mutants.

The *RPM1-MYC* transgene likely served as the 'trigger' to induce silencing in the *lra6* mutants. Transgenes with inverted repeats can cause silencing of multiple *NB-LRR* genes (Wroblewski T, 2007; Brodersen P, 2008), and our preliminary data indicate that the *RPM1-MYC* transgene is inserted as an inverted repeat. Regardless of the silencing 'trigger', the *RPM1* promoter and coding regions are methylated in the *lra6* mutants (Fig. 2.4). Transcript repression of both *RPM1* and *RPS2* in *lra6-1* could be due to either transcriptional gene silencing (TGS) or post-transcriptional gene silencing (PTGS) (Fig. 2.3A,B). This question could be

addressed genetically since there are host genes specifically associated with either TGS or PTGS (TGS- AGO4, DCL3; PTGS- AGO1, DCL4) (Zilberman D, 2003; Chan SW, 2004; Vaucheret H, 2004; Gasciolli V, 2005). Additionally, demonstration that an *lra6*-specific siRNA species that corresponds to either the promoter or coding region of *RPM1* would indicate TGS or PTGS, respectively (Mourrain P, 2007b). Another possibility is that the silencing 'trigger' in the *lra6* mutants could initiate both TGS and PTGS of target loci (Mourrain P, 2007b).

Studies have shown that silencing 'triggers' require a minimum of 19 corresponding basepairs (Vanitharani R, 2003). Additionally, it has been shown that sequence homology to the silencing 'trigger' for the *RGC2 NB-LRR* gene cluster positively correlated with reduction of transcript accumulation (Wroblewski T, 2007). Therefore, we used Nucleic Acid Dot Plot analysis to determine whether a 19 basepair stretch of homologous sequence exists between *RPM1* and *RPS2*. For this analysis, we chose *RPM1* and *RPS2* sequences that included 3 kilobases upstream of the coding region, the coding region, and 3 kilobases downstream of the coding region. In addition, both sense and anti-sense configurations were analyzed for the *RPM1* and *RPS2* sequences. The analysis revealed that there was no homologous sequence of 19 basepairs or greater between the *RPM1* and *RPS2* regions.

We speculate that regulation between *RPM1* and *RPS2* occurs through an indirect or 'transitive' RNA silencing mechanism. Studies have shown that a silencing 'trigger' can initiate repression of homologous primary target sequences, resulting the production of siRNA species (Van Houdt H, 2003). New siRNA

species then arise from both the primary homologous sequences and adjacent non-homologous sequences, causing silencing of secondary targets (Van Houdt H, 2003). Importantly, due to the silencing of non-homologous adjacent sequences in the primary target, the silenced secondary targets may not contain homologous sequences to the original 'trigger' (Van Houdt H, 2003). Since there is not a 19 basepair stretch of homologous sequence between *RPM1* and *RPS2*, silencing of these loci may occur through an intermediate sequence(s) that has sequence homology to both *RPM1* and *RPS2*. We tested this possibility *in silico* by creating a sliding 19 basepair window for the 1 kilobase promoter and coding regions of *RPM1* and *RPS2*, queried each 19 basepair sequence against the Arabidopsis genome, and compared the results for both *RPM1* and *RPS2*. Unfortunately, we did not find a gene or locus that had sequence homology to both the *RPM1* and *RPS2* regions.

Although both *lra6-1* and *lra6-2* lack *RPM1* transcript accumulation in the absence of the *RPM1-MYC* and *avrRpm1* transgenes (Fig. 2.3A), the repression of *RPS2* transcript accumulation in *lra6-1* coincides with the presence of the *avrRpm1* and *RPM1-MYC* transgenes (Fig. 2.3B,C). These results agree with the proposal that the *RPM1-MYC* transgene acts as a silencing 'trigger' that induces repression of *RPS2*. Silencing 'triggers' can mediate both TGS and PTGS (Mourrain P, 2007a), with TGS being meiotically heritable while PTGS is reset after meiosis (Vaucheret H, 1998).Therefore, our findings could be due to TGS of *RPM1* in both *lra6* mutants and PTGS of *RPS2* in the *lra6-1* background. Although *lra6-1* does not alter *RPS2* transcript accumulation in the absence of the *RPM1-MYC* and *avrRpm1*

transgenes (Fig. 2.3C), RPS2 function is compromised (Fig. 2.1C,D). Two explanations for these results are 1) repression of RPS2 translation or 2) repression of another gene required for RPS2 function. Although typically associated with micro(mi)RNA-mediated silencing, siRNAs can cause translational inhibition of targets (Brodersen P, 2008). Transgenic lines expressing RPS2 with an epitope tag could be used to address if RPS2 translation is repressed in *lra6-1*. Interestingly, an siRNA species (nat-siRNAATGB2) is expressed after RPS2-mediated defenses and is required for RPS2-mediated disease resistance (Katiyar-Agarwal S, 2006). This siRNA species represses the expression of *PPRL*, a gene that represses RPS2 disease resistance when over-expressed (Katiyar-Agarwal S, 2006). Therefore, further experiments will address transcript accumulation of *PPRL* in *lra6-1*.

A complete characterization of the *lra6* mutants would require knowledge of both global methylation patterns and the siRNA and mRNA transcriptomes (Meyers et al., 2003; Zhang X, 2006). These large-scale analyses could reveal alterations of siRNA populations, differences in expression of mRNA species, and global methylation pattern shifts in the *lra6* mutants. These analyses could also potentially identify the silencing intermediate(s) between *RPM1* and *RPS2* in *lra6-1*.

Methods

Bacterial Infections. For Table 2.1, 3-week-old plants were spray inoculated with *Pto* DC3000(*avrRpm1*) at a concentration of 2.5 x 10^7 cfu/ml. Plant were scored as "Resistant" or "Susceptible" based on the presence or absence of disease symptoms ~5 days-post-inoculation, respectively. For Figure 2.1 and Figure 2.2, dip

inoculations were performed as previously described (Tornero and Dangl, 2001). Briefly, 2-to 3-week-old seedlings were inoculated by dipping in solutions of bacteria at a concentration of 2.5 X 10^7 cfu/ml. 4 replicates of three seedlings were placed in 1 ml of 10 mM MgCl2 with 0.2% Silwet L-77. The weights of the tubes with seedlings were measured, and the tubes were then shaken for 1 h at 28°C. Serial dilutions in 10 mM MgCl2 were made and plated on KB plates with rifampicin selection. For Figure 2.1A, C, HR assays were performed as previously described (Grant et al., 1995).

RNA Analysis. RNA was isolated from various plants lines by using TRIZOL Reagent (GIBCO_BRL) according to the manufacturer's instructions. Real-time PCR (SYBR Green) reactions in Figure 2.3 were set-up according to the manufacturer's instructions (Applied Biosystems). For measurement of *RPM1* transcript accumulation the PCR conditions used were 94°C 10 s, 58°C 15 s, 72°C 15 s, 50 cycles. Ct values were normalized to Elongation Factor 1 (Baud S, 2003).

Methylation Analysis. DNA was prepared using DNase Easy Kit(Quigen) according to manufacture's instructions, and then subjected to a Phenol-Chloroform extraction. 2µg of DNA for various lines were digested with either HpaII or AclI in a 40µL reaction for ~6 hours. Samples were then precipitated with 2X 100% EtOH and suspended in 20µL water. PCR analysis was then performed for different regions of either *RPM1*.

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CHAPTER 3: RPM1 is Regulated by Interaction with Nucleotides and Undergoes Homotypic Association

Preface

Much of the following chapter will be submitted for publication in the next 2-3 months. Zhiyong Gao initiated this project and will be the first author on the publication. Zhiyong's work is presented in Figures 3.1, 3.4, 3.7A, and 3.8A. The protein homology models in Figure 3.3 were built by Karen Cherkis. This work characterizes RPM1 in the context of nucleotide regulation and homotypic association. We show the first example that an ectopically active plant NB-LRR protein can be suppressed by a host guardee protein. Our results lead us to speculate that the guardee protein RIN4 can regulate the interaction between RPM1 and nucleotides.

Abstract

The plant NB-LRR protein RPM1 is activated by either of the type III bacterial effectors AvrRpm1 and AvrB. The host protein that links RPM1 and these bacterial effectors is RIN4. In addition to being required for RPM1 perception of AvrRpm1 and AvrB, RIN4 also negatively regulates RPM1 in the absence of pathogens. In this work, we generate RPM1 variants that produce defense responses in the absence of both RIN4 and bacterial effectors. The ectopic activation of RPM1 is likely due to alteration of the RPM1's interactions with nucleotides. Interestingly, RIN4 can suppress the signaling of an ectopically active

RPM1 variant. We also show that RPM1 undergoes homotypic association before activation, and that the RPM1 NB domain is sufficient for homotypic association. Finally, we provide evidence that RPM1 fragments are soluble in bacteria and therefore our work could be a first-step to an *in vitro* biochemical analysis of RPM1.

Introduction

A component of the plant immune system is the suite of Nucleotide-binding site and Leucine Rich Repeat (NB-LRR) proteins. NB-LRR proteins are a sub-class of the Signal Transduction ATPases with Numerous Domain (STAND) family of proteins (Danot O, 2009). STAND proteins function in basic processes such as transcriptional regulation and programmed cell death in both prokaryotes and eukaryotics (Danot O, 2009). Some of the biochemical evaluation of STAND proteins has come from the characterization of the *C. elegans* Caspase Recruitment Domain (CARD)-NB protein Cell-death Abnormal- 4 (CED4) and the mouse/human CARD-NB-WD40 protein Apoptotic Protease-activating Factor (Apaf-1) (Riedl SJ, 2005; Yan N, 2005). Crystal structures have been resolved for CED4 and Apaf-1 and therefore these structures are important tools for understanding STAND protein regulation (Riedl SJ, 2005; Yan N, 2005). A subset of STAND proteins that are homologous to the plant disease resistance (R) proteins are the mammalian NB-LRR (NLR) proteins (Ting JP, 2008). Functions of the NLR proteins include responses to pathogens, perception of Danger Associated Molecular Patterns (DAMPs), and initiation of adaptive immune responses (Eisenbarth SC, 2008; Halle A, 2008; Ting JP, 2008). Therefore, STAND protein

regulation has important roles in topics ranging from crop protection to human health.

Nucleotide binding and hydrolysis regulates the function of many STAND proteins (Yan N, 2005; Tameling et al., 2006; Duncan et al., 2007; Marquenet E, 2007). To explain this phenomenon, a model has been proposed in which STAND proteins are regulated by association with different nucleotides. Specifically, STAND proteins are regulated as molecular switches, with the “off” mode coinciding with the binding of ADP whereas the “on” mode coincides with association of ATP (Takken et al., 2006; Danot O, 2009). For plant NB-LRR proteins, the “on” mode initiates both host cell death and disease resistance.

Evidence for this ‘molecular switch’ model in the regulation of plant NB-LRR proteins has largely come from the characterization of the Tomato I-2 protein (Tameling et al., 2006). A amino acid change in the MHD motif, D495V, of the I-2 NB domain causes ectopic defense signaling (Tameling et al., 2006). Homology modeling of I-2 revealed that the MHD motif is potentially associated with the coordination of ADP to the nucleotide binding pocket of I-2 (Tameling et al., 2006).Therefore, the D495V mutation was proposed to inappropriately expel ADP from the I-2 NB domain, allowing for the binding of ATP (Tameling et al., 2006). Amino acid changes in the MHD motif also cause ectopic activation of the Potato CC-NB-LRR protein Rx and the Flax TIR-NB-LRR protein L6 (Bendahmane et al., 2002; Howles et al., 2005). Therefore, potential nucleotide regulation by the MHD motif seems to be conserved across plant NB-LRR proteins.

Mutations in the Walker B motif also cause constitutive activation of some STAND proteins. In *E. coli*, mutation of the catalytic base residue of the Walker B motif, aspartic acid residue 129, to an alanine renders MalT ectopically active and resistant to negative regulation by host factors (Marquenet E, 2007). Mutation of the analogous residue of the Walker B motif for the plant NB-LRR proteins RPS5 and I-2 also causes constitutive activation (Tameling et al., 2006; Ade et al., 2007). Biochemical analysis of the autoactive MalT and I-2 variants revealed that these proteins can bind, but not hydrolyze, ATP (Tameling et al., 2006; Marquenet E, 2007). Therefore, the autoactive MalT, I-2, and RPS5 variants are likely locked in the "on" mode due to constitutive association with ATP. These studies demonstrate that ATP hydrolysis has an important role in negative regulation or turning "off" of STAND proteins.

Studies that characterized the ectopically active variants of Rx, I-2, L6, I-2, RPS5, and MalT also evaluated amino acid residues *in cis* that are required for function (Bendahmane et al., 2002; Howles et al., 2005; Tameling et al., 2006; Ade et al., 2007; Marquenet E, 2007). With the exception of MalT, activity for all of these autoactive variants is suppressed by mutations in the Walker A motif of the NB domain (Bendahmane et al., 2002; Howles et al., 2005; Tameling et al., 2006; Ade et al., 2007). The residues of the Walker A motif have been demonstrated to be required for nucleotide binding in many STAND proteins, including I-2 (Tameling et al., 2006; Duncan et al., 2007; Ye Z, 2008). The autoactive MalT variant was suppressed *in cis* by the addition of a mutation in the Walker B motif that was required for nucleotide binding (Marquenet E, 2007). Collectively, these studies

demonstrate that STAND proteins require nucleotide binding for signaling and that cycling between ATP-bound and ADP-bound states regulates the “on” and “off” modes, respectively.

Many STAND proteins undergo homotypic association. In the resting state, CED4, NALP3, Monarch-1, and CIITA form homotypic association *in vivo* (Yang X, 1998; Linhoff MW, 2001; Duncan et al., 2007; Ye Z, 2008). Additionally, activation induces multi-merization of CED4, Apaf-1, and NALP1 *in vitro* (Kim HE, 2005; Yan N, 2005; Faustin B, 2007). For plant NB-LRR proteins, RPS5 and Prf undergo homotypic association in the resting state whereas the N protein multi-merizes after pathogen elicitation (Mestre and Baulcombe, 2006; Ade et al., 2007; Gutierrez JR, 2009). These studies demonstrate that many STAND proteins undergo homotypic association in either the resting or active state.

The influence of nucleotide binding on homotypic association has also been tested for many STAND proteins. The *in vivo* homotypic association between NALP3, N, and Monarch-1 are abolished by mutations of the Walker A motif whereas similar variants of CIITA and CED4 retain *in vivo* multi-merization (Yang X, 1998; Linhoff MW, 2001; Mestre and Baulcombe, 2006; Duncan et al., 2007; Ye Z, 2008). The *in vitro* assembly of homotypic complexes requires nucleotide binding for NALP1 and Apaf-1 (Kim HE, 2005; Faustin B, 2007). Since these studies show that multi-merization can be either dependent or independent of nucleotide binding, the influence of nucleotide binding on homotypic association is particular for each STAND protein.

RPM1 is an Arabidopsis CC-NB-LRR protein that mediates defense responses to bacterial pathogens expressing either of the type III effectors AvrRpm1 or AvrB (Grant et al., 1995). After delivery into the plant cell, AvrRpm1 or AvrB associate with and cause phosphorylation of the host protein RIN4 (Mackey et al., 2002). Alteration of RIN4 likely causes activation of RPM1, resulting in the hypersensitive response (HR) and disease resistance (Mackey et al., 2002). In yeast, RIN4 interacts with a CC-containing fragment (AA 1-176) of RPM1 (Mackey et al., 2002). The biological relevance of the RIN4-RPM1 association has been demonstrated from studies showing that RIN4 positively regulates protein accumulation and prevents ectopic activation of RPM1 (Mackey et al., 2002; Belkhadir et al., 2004). Independent of RPM1, RIN4 associates with and negatively regulates the CC-NB-LRR protein RPS2 (Mackey et al., 2003; Belkhadir et al., 2004). It is unknown whether RIN4 can associate with both RPM1 and RPS2 in the same protein complex or if different pools of RIN4 are associated with either RPM1 or RPS2. In either case, RIN4 has an important role in the regulation and activation of both RPM1 and RPS2 (Mackey et al., 2002; Mackey et al., 2003).

In this work, we characterize RPM1 in the context of regulation by nucleotides and homotypic association. Similar to other STAND proteins, we show that RPM1 is likely to be regulated by nucleotide binding and hydrolysis. Additionally, RPM1 undergoes homotypic association in the resting state. We also demonstrate new findings regarding the regulation of RPM1 by RIN4. Importantly, we show that all of these findings occur under native expression conditions in

Arabidopsis. Finally, we demonstrate that RPM1 protein fragments are soluble in *E. coli* and thus a biochemical, *in vitro* analysis of RPM1 is likely possible.

Results

The RPM1 D505V Variant is Ectopically Active in *Nicotiana benthamiana*

Since mutation of the conserved Aspartic acid residue in the MHD motif causes some plant NB-LRR proteins to be ectopically active (Bendahmane et al., 2002; Tameling et al., 2006), we investigated if mutation of this same Aspartic acid residue, D505V, would cause autoactivation of RPM1 (Fig. 3.1A). Transient expression of RPM1 D505V in *Nicotiana benthamiana* resulted in visual cell death after ~12 hours post-induction (Fig. 3.1B). The RPM1 D505V-mediated cell death was quantified by ion leakage measurements from the dying cells (Fig. 3.1C). These data indicate that RPM1 D505V is ectopically active and that the host components required for RPM1-mediated cell death are conserved between Arabidopsis and *Nicotiana benthamiana*. Western blot analysis revealed that RPM1 D505V accumulated to lower levels relative to wild-type RPM1 (Fig. 3.1D). This data is consistent with previous studies showing that RPM1 activation leads to the loss of RPM1 protein accumulation (Boyes et al., 1998). Collectively, these data demonstrate that RPM1 D505V produces pathogen-independent cell death.

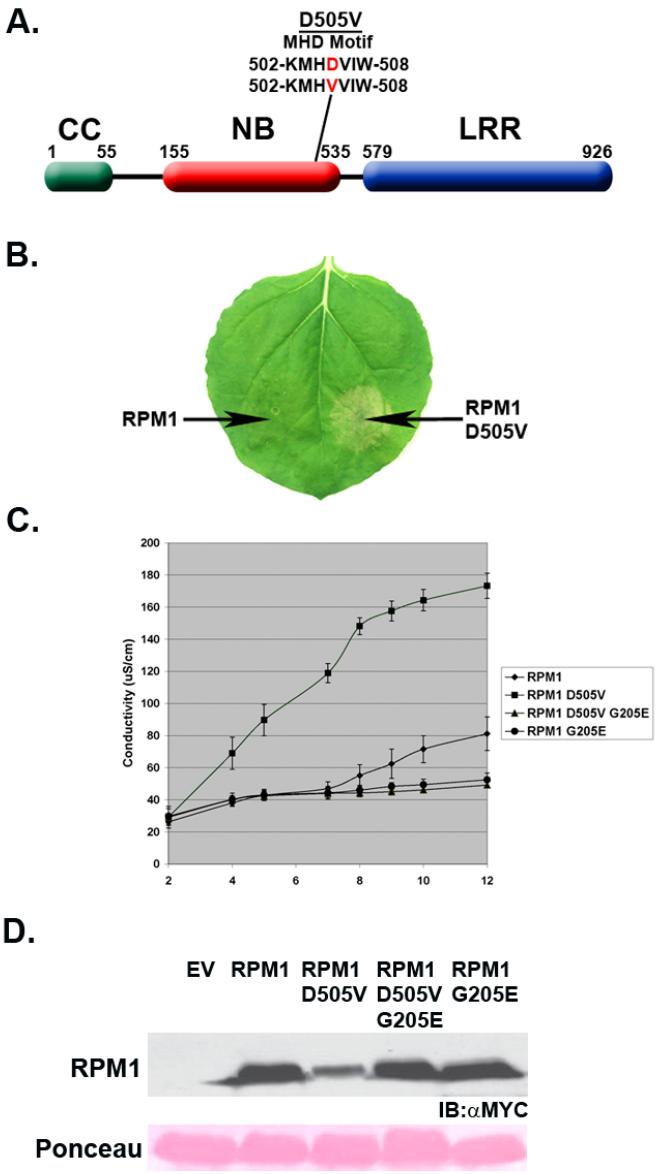


Figure 3.1. RPM1 D505V is Ectopically Active in *Nicotiana benthamiana.* A. Schematic of the RPM1 D505V mutation. B. Visual RPM1 D505V-mediated cell death. *N. benthamiana* leaves were transiently transformed with either RPM1-MYC or RPM1 D505V-MYC, driven by an Estradiol-inducible promoter. Photo was taken ~12 hours post-induction. C. Ion leakage measurement of cell death induced by RPM1-MYC, RPM1 D505V-MYC, RPM1 D505V G205E-MYC, and RPM1 G205E. All constructs were driven by an Estradiol-inducible conditional promoter. Numbers on X axis represent hours post Estradiol induction. Error bars represent 2X Standard Error. D. Immunoblot analysis showing accumulation of transiently expressed proteins ~6 hours post estradiol induction.

A Walker B Motif Mutation Renders RPM1 Ectopically Active in *Nicotiana benthamiana*

Mutation of the second aspartic acid residue in the Walker B motif induces pathogen-independent activation of some plant NB-LRR proteins (Tameling et al., 2006; Ade et al., 2007). We mutated the analogous residue in RPM1, D287, into an alanine and evaluated whether this mutation rendered RPM1 autoactive (Fig. 3.2A). Transient expression of RPM1 D287A in *N. benthamiana* resulted in leaf chlorosis and cell death (Fig. 3.2B). The RPM1 D287A-mediated cell death occurred ~72 hours after induction, indicating that the RPM1 D287A-mediated cell death was slower than the RPM1 D505V-mediated cell death (Fig 3.1,2). Perhaps because of a slower onset of cell death, we did not observe a reduction in the protein accumulation of RPM1 D287A relative to wild-type RPM1 (Fig. 3.2C). Nevertheless, our results collectively indicate that we have generated two ectopically active RPM1 variants (Fig. 3.1,2).

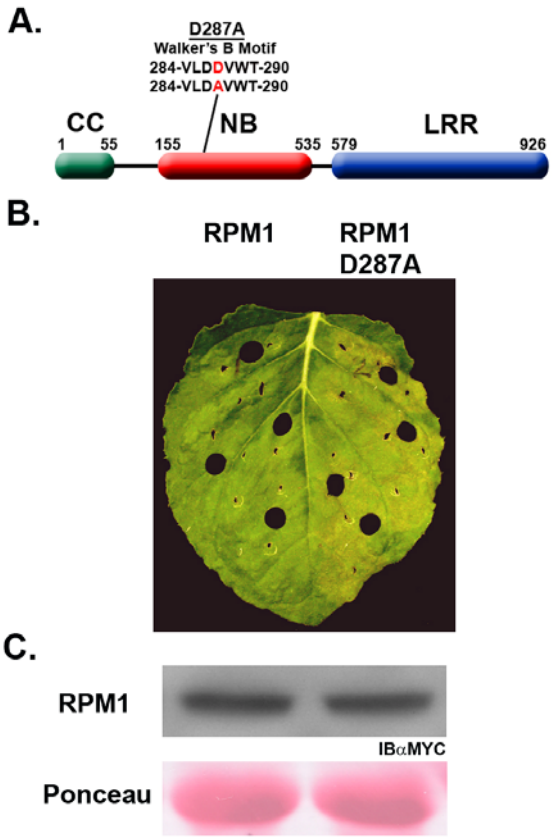


Figure 3.2. A Walker B Box Mutation Renders RPM1 Ectopically Active in *Nicotiana benthamiana.* A. Schematic of the RPM1 D287A mutation. B. Visual RPM1 D287A-mediated cell death. *N. benthamiana* leaf was transiently transformed with either RPM1-MYC or RPM1 D287A-MYC, driven by an Estradiol-inducible promoter. Picture was taken ~72 hours post-induction. C. Immunoblot analysis showing accumulation of transiently expressed protein 6 hours post induction shown in B.

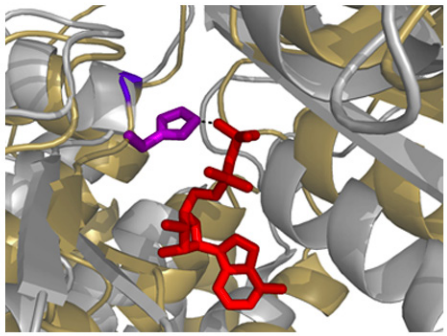
The D505V and D287A RPM1 Variants are Mechanistically Distinct

Since the D505V mutation renders RPM1 ectopically active, potentially through altering the stability of ADP association with RPM1, we analyzed these residues *in silico* by building homology models of the RPM1 NB domain based on the crystal structure of Apaf-1 (Riedl SJ, 2005). We chose to model RPM1 on Apaf-1 because the Apaf-1 crystal structure was made in the presence of ADP (Riedl SJ, 2005). Homology modeling revealed that RPM1 D505 corresponds structurally to D439 in Apaf-1 (Fig. 3.3A). Importantly, the adjacent histidine residue that is

present in both RPM1 and Apaf-1 forms a hydrogen bond with the β-phosphate of ADP for Apaf-1 (Fig. 3.3A)(Riedl SJ, 2005). This hydrogen bond helps to coordinate and bury ADP in the NB domain of Apaf-1 (Riedl SJ, 2005). Therefore, we speculate that RPM1 D505V de-stabilizes the adjacent histidine residue and therefore inhibits the association between ADP and RPM1. The de-stabilization of ADP could then cause RPM1 to transition to the active, ATP-bound state.

Since the Apaf-1 crystal structure was made with ADP and not ATP, no germane information regarding ATP hydrolysis could be derived from modeling RPM1 over Apaf-1 (Riedl SJ, 2005). Nevertheless, mutation of the analogous aspartic acid residue to a glutamic acid residue for the plant NB-LRR proteins I-2 and RPS5 causes ectopic activation of these proteins (Tameling et al., 2006; Ade et al., 2007). The I-2 D283E variant was biochemically demonstrated to bind but not hydrolyze ATP (Tameling et al., 2006). Importantly, previous Walker B motif mutations in RPS5 and I-2 differ from the Walker B motif mutation in RPM1 (aspartic acid to glutamic acid, aspartic acid to alanine, respectively) (Tameling et al., 2006; Ade et al., 2007). The D287A variant of RPM1 is analogous to a mutation (D129A) that was generated in the *E. coli* transcription factor MalT (Marquenet E, 2007). MalT D129A is constitutively active, and binds but does not hydrolyze ATP (Marquenet E, 2007). These findings lead us to speculate that the RPM1 D287A variant can bind, but not hydrolyze ATP, while RPM1 D505V likely favors exchange of ADP for ATP. Therefore, while both RPM1 D287A and RPM1 D505V are ectopically active, the mechanisms determining the autoactivation are likely to be different.

A.



B.

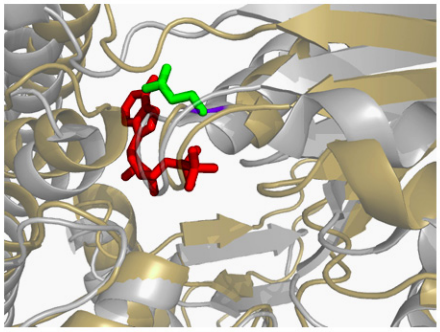


Figure 3.3. Homology Model of RPM1 Based on Apaf-1. A. Protein modeling of the RPM1 MHD motif over the crystal structure of Apaf-1 (Riedl SJ, 2005). The aspartic acid residue of the MHD motif is shown in blue. The imidazole side chain of Histidine 439 is shown in purple. A black dashed line represents the hydrogen between the Histidine 439 and the β-phosphate of ADP. B. Protein modeling of the RPM1 G205E mutation in the Walker A motif over the crystal structure of Apaf-1 (Riedl SJ, 2005). The Glycine residue is shown in blue, whereas the mutation to Glutamic acid is shown in green. The amide side chain of the glycine residue forms a hydrogen bond with the β-phosphate of ADP.

Ectopic Activity of RPM1 D505V is Suppressed by the G205E Mutation *in cis*

Nucleotide binding by NB-LRR proteins is mediated by the amino acid residues of the Walker A motif (Tameling et al., 2006; Duncan et al., 2007; Ye Z, 2008). A mutation in the Walker A motif of RPM1, G205E, was isolated in a forward screen for mutations that lose RPM1 function (Tornero et al., 2002a). The G205E mutation increases the length of the side chain and adds a negative charge to the

Walker A motif (Fig. 3.2). Therefore, it is likely that the G205E mutation disrupts the ability of RPM1 to bind nucleotide. We evaluated whether the activity of RPM1 D505V could be suppressed by addition of the G205E mutation *in cis*. Interestingly, RPM1 D505V G205E was suppressed for the ability to induce ectopic cell death (Fig. 3.1C, D). This indicates that ectopic activity of the RPM1 D505V variant likely requires nucleotide binding.

RIN4 Suppresses RPM1 D505V Activity in *Nicotiana benthamiana*

RIN4 is required for RPM1 to perceive the bacterial effectors AvrB and AvrRpm1 (Mackey et al., 2002). RIN4 also stabilizes and prevents ectopic activation of RPM1 (Belkhadir et al., 2004). Therefore, we assessed whether RIN4 could affect the activity of RPM1 D505V. Interestingly, transient co-expression of RIN4 with RPM1 D505V resulted in the inhibition of cell death (Fig. 3.4). Therefore, in addition to being required for effector perception by RPM1 (Mackey et al., 2002), RIN4 can also negatively regulate the function of an autoactive RPM1 variant.

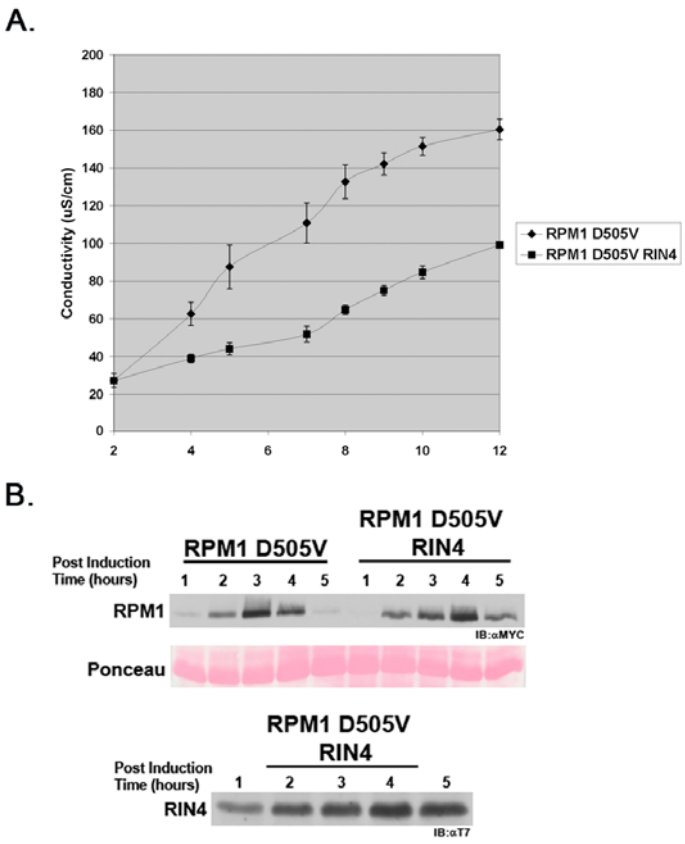


Figure 3.4. RIN4 Suppresses RPM1 D505V Activity in *Nicotiana benthamiana.* A. Ion leakage measurement of cell death induced by RPM1 D505V-MYC and RPM1 D505V-MYC co-expressed with T7-RIN4. RPM1 D505V-MYC was driven by an Estradiol-inducible promoter whereas T7-RIN4 was expressed by 1.2 Kb of the native RIN4 promoter. Numbers on the X axis represent hours post Estradiol treatment. Error bars represent 2X Standard Error. B. Immunoblot analysis showing expression of RPM1 D505V-MYC and T7-RIN4.

RPM1 D505V is Ectopically Active in Arabidopsis

We next evaluated whether RPM1 D505V could initiate ecoptic defense responses at native expression levels in Arabidopsis. A *RPM1 D505V-MYC* gene, driven by the *RPM1* promoter, was transformed into Arabidopsis *rin4 rpm1 rps2* mutants. The *rin4 rpm1 rps2 RPM1 D505V* lines had leaf lesions and were dwarfed relative the control *rin4 rpm1 rps2* lines (Fig. 3.5A). These *RPM1 D505V*-mediated phenotypes are indicative of constitutive defense activation (Zhang et al., 2003;

Howles et al., 2005). We then selected two lines, #63 and #49, which segregated the *RPM1 D505V* transgene as an insertion into a single locus (Table 3.1). Analysis of the T2 generation indicated that the *RPM1 D505V-MYC* trangene altered Arabidopsis morphology in a dose-dependent manner (Fig. 3.5A). Importantly, in addition to morphological alterations, the *rin4 rps2 rpm1 RPM1 D505V-MYC* lines had enhanced disease resistance as demonstrated by inoculation with virulent *P.syringae* (Fig. 3.5B). These data show that cell death induced by RPM1-D505V in *N. benthamiana* correlates with enhanced disease resistance in Arabidopsis.

Line #	Generation	No. of Plants Analyzed	Transgene +	Transgene -	X^2	*P* value
49	**T2**	**42**	**31**	**11**	**.0079[a]**	***P* > .05**
63	**T2**	**42**	**31**	**11**	**.0079[a]**	***P* > .05**

a. X^2 value given for expected ratio of 3:1

Table 3.1. Genetic Analysis of *RPM1*p:*RPM1 D505V-MYC* Arabidopsis Lines. T2 Arabidopsis lines were subjected to PCR analysis for *RPM1*p:*RPM1 D505V-MYC* transgene.

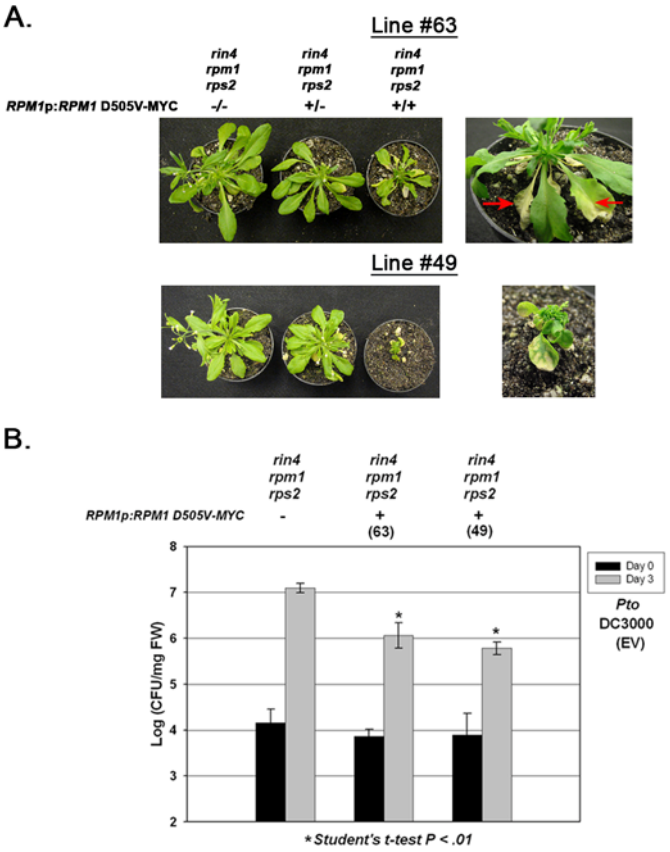


Figure 3.5. The RPM1 D505V Variant is Ectopically Active in Arabidopsis. A. Plants morphology of ~ 4 week-old T2 plants that either lack or contain the *RPM1 D505V-MYC* transgene. Plants were grown in long-day conditions in greenhouse. Plant genotypes are listed above plant pictures. Line #63 and line #49 were independently selected. B. ~2-3 week-old-seedlings were dip inoculated with *Pto* DC3000 (EV) according to previously published conditions (Tornero and Dangl, 2001). Error bars represent 2X Standard Error. Student's t-tests were used to verify differences between the lines lacking (Left) and possessing (Right) the *RPM1 D505V-MYC* transgene.

RIN4 Suppresses RPM1 D505V in Arabidopsis

Since co-expression of RIN4 suppresses RPM1 D505V activity in *N. benthamiana*, we wanted to evaluate whether RIN4 could suppress RPM1 D505V in Arabidopsis. The *rin4 rps2 rpm1 RPM1 D505V* Line #49 was crossed to both *rin4 rps2 rpm1* and *RIN4 rps2 rpm1*. Evaluation of the F1 progeny revealed that RIN4 suppressed the RPM1 D505V-mediated morphological defects and expression of

the defense-associated protein marker, Pathogenesis-Related 1 (PR-1) (Fig. 3.6A,B). These data indicate that RIN4 can suppress RPM1 D505V under native expression conditions in Arabidopsis.

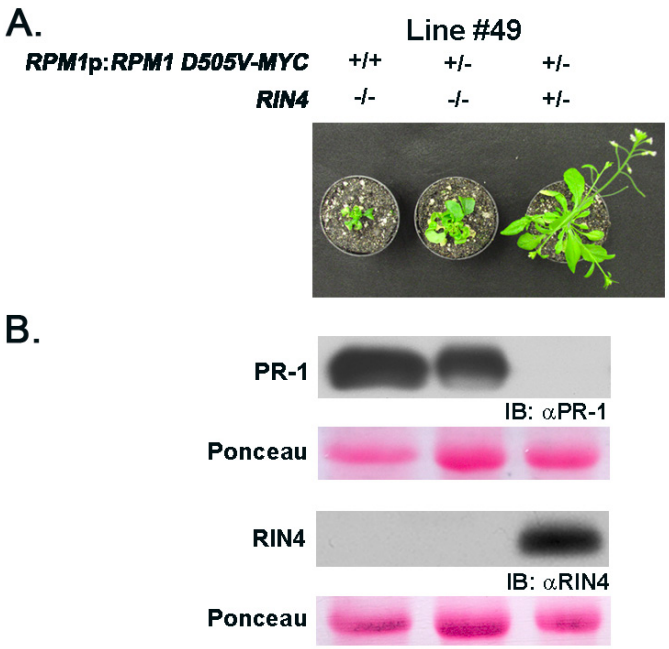


Figure 3.6. RIN4 Suppresses RPM1 D505V Activity in Arabidopsis. A. Plant morphology of ~4 week-old F1 plants that either lack or contain the *RPM1 D505V-MYC* transgene. Plants were grown in long-day conditions in the greenhouse. Genotypes are listed above plant pictures. B. Immunoblot analysis for either PR-1 (top) or RIN4 (bottom) in various Arabidopsis lines.

RPM1 Undergoes Homotypic Association in *Nicotiana benthamiana*

Some NB-LRR proteins undergo homotypic association and therefore we wanted to evaluate if RPM1 can multi-merize. Transient assays were set-up in which the combination of RPM1-MYC, RPM1-GFP, and RIN4 or the combination of RPM1-MYC and RIN4 were co-expressed in *N. benthamiana*. Protein extracts from the transformed tissue were then subjected to immunoprecipitation with anti-GFP beads. The presence of RPM1-MYC in the elution from the anti-GFP beads indicated that RPM1-GFP and RPM1-MYC associate (Fig. 3.7A). We next

assessed which fragment of RPM1 is sufficient to form homotypic interactions. Immunoprecipitation with anti-GFP beads after co-expression of the CC-NB-MYC, CC-NB-GFP, and RIN4 or CC-NB-MYC and RIN4 revealed that both the CC-NB-RPM1-MYC and CC-NB-GFP fragments can associate (Fig. 3.7B). Furthermore, subjecting protein extracts from plant tissue transiently expressing NB-MYC, NB-GFP, and RIN4 to immunoprecipitation with anti-GFP beads demonstrated that the NB-MYC and NB-GFP domains associate (Fig. 3.7C). Collectively, these data indicate that RPM1 multi-merizes in the presence of RIN4 and that the RPM1 NB domain is sufficient to form homotypic interactions.

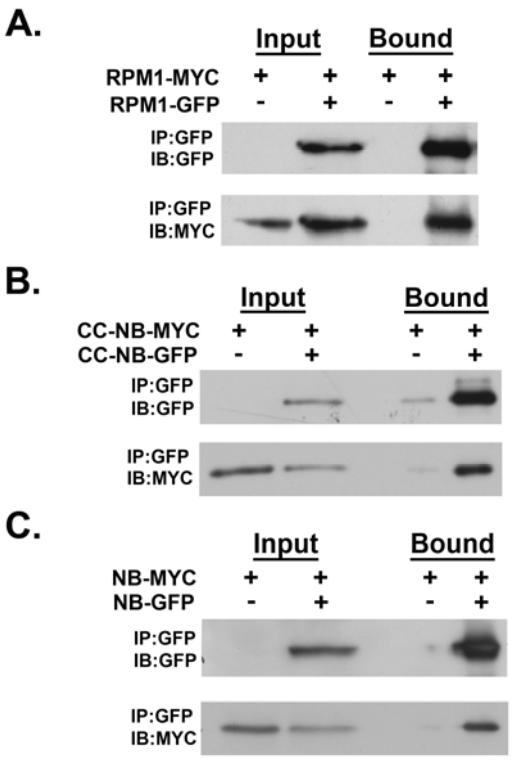


Figure 3.7. RPM1 Undergoes Homotypic Association in *Nicotiana benthamiana.* A. *N. benthamiana* leaves were transiently transformed with RIN4, RPM1-MYC, or RIN4, RPM1-MYC and RPM1-GFP (right). , driven by an Estradiol-inducible promoter. Protein samples were taken ~6 hours post-estradiol induction and subjected to microsomal purification and solubilization (See methods). anti-GFP beads were used to

isolate proteins associated with RPM1-GFP. Eluted samples were then subjected to immunoblot analysis for either GFP (top) or MYC (bottom). B. *N. benthamiana* leaf was transiently transformed with either RPM1-MYC or RPM1-GFP, driven by the constitutive 35S promoter. Protein samples were taken ~48 hours post inoculation with *A. tumefaciens* (See Methods), subjected to co-immunoprecipitation and immunoblot analysis as described in A. Since *A. tumefaciens* cultures were used to inoculate the same *N. benthamiana* leaf, the contaminating GFP band in the GFP pulldown for in the RPM1-MYC samples is likely due to contamination of the RPM1-GFP, RPM1-MYC *A. tumefaciens* culture from the other side of the *N. benthamiana* leaf. C. *N. benthamiana* leaf was transiently transformed with either RPM1-MYC or RPM1-GFP, driven by the constitutive 35S promoter. Protein samples were taken ~48 hours post inoculation with *A. tumefaciens* (See Methods), subjected to co-immunoprecipitation and immunoblot analysis as described in A. The Bound fraction was loaded at ~10X relative to the Input.

RPM1 Forms Homotypic Association in Arabidopsis

We next assessed whether RPM1 could self-associate under native expression conditions in Arabidopsis. A *RPM1*p:*RPM1-MYC* line that functionally complements an *rpm1* mutant and segregates as a single locus insertion has been previous characterized (Boyes et al., 1998). Since evaluation of homotypic interactions requires two differentially-tagged RPM1 proteins, we transformed an *RPM1*p:*RPM1-GFP* transgene into an *rpm1* line. A *RPM1*p:*RPM1-GFP rpm1* line that segregated as a single locus insertion and complemented the RPM1-mediated hypersensitive response (HR) was selected (Table 3.2). This *RPM1*p:*RPM1-GFP rpm1* line self-pollinated and a homozygous T3 line was isolated. Inoculation of these T3 progeny with *Pto* DC3000(*avrRpm1*) revealed that the *RPM1*p:*RPM1-GFP* transgene complements *rpm1* (Table 3.1)(Fig. 3.8A). This *RPM1*p:*RPM1-GFP rpm1* line was then crossed to the previously generated *RPM1*p:*RPM1-MYC rpm1* line and F3 lines homozygous for both transgenes were selected. Protein extracts from the *RPM1*p:*RPM1-MYC*, *RPM1*p:*RPM1-GFP*, *rpm1* line were then subjected to immunoprecipitation with anti-GFP beads. Elution from the anti-GFP beads

revealed the presence of RPM1-MYC (Fig 3.8B). Therefore, RPM1 undergoes homotypic association when expressed at native levels in Arabidopsis.

Line #	Generation	No. of Plants Analyzed	HR +	HR -	X^2	*P* value
4	**T2**	**44**	**29**	**15**	**1.55[a]**	***P* > .05**

a. X^2 value given for expected ratio of 3:1

Table 3.2. Genetic Analysis of the *RPM1*p:*RPM1-GFP* Arabidopsis Line. T2 Arabidopsis lines were inoculated with *Pto* DC3000(*avrRpm1*) at 5 x 10^7 CFU/mL. RPM1 HR was scored 6 hours-post inoculation. PCR analysis for *RPM1*p:*RPM1 D505V-MYC* transgene revealed that all lines that were HR + contained the *RPM1*p:*RPM1-GFP* transgene whereas all lines that were scored as HR – lacked the *RPM1*p:*RPM1-GFP* transgene.

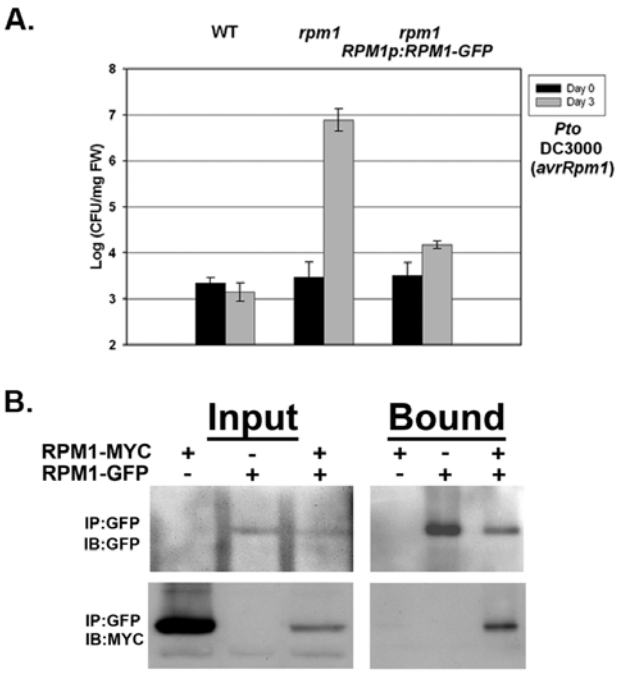


Figure 3.8. RPM1 Undergoes Homotypic Association in Arabidopsis. A. ~2-3 week-old-seedlings were dip inoculated with *Pto* DC3000(*avrRpm1*) according to previously published conditions (Tornero and Dangl, 2001). Error bars represent 2X Standard Error. B. Protein samples from indicated genotypes were subjected to microsomal purification and solubilization (See methods). anti-GFP beads were used to isolate proteins associated with RPM1-GFP. Eluted samples were then subjected to

immunoblot analysis for either GFP (top) or MYC (bottom). The Bound fraction represents ~10X enrichment of the Input.

RPM1 Truncations Are Soluble in *E. coli*

For the purpose of biochemical characterization of RPM1, we expressed the CC-NB and NB fragments with glutathione *S*-transferase(GST) and HIS epitope tags in *E. coli* (Fig. 3.9A). After optimization of protein expression conditions, we were able to produce soluble protein (Fig. 3.B,C). Bands representing both the soluble GST-6XHIS-CC-NB and GST-NBS fragments were visible after coomassie staining of protein gels loaded with *E. coli* extracts (Fig. 3.9B). Western blot analysis showed that the expected-sized protein bands, visible after coomassie staining, corresponded to GST and HIS tagged proteins (Fig. 3.B,C). These data demonstrate that the RPM1 NB and CC-NB fragments are soluble and can likely be purified in *E. coli*.

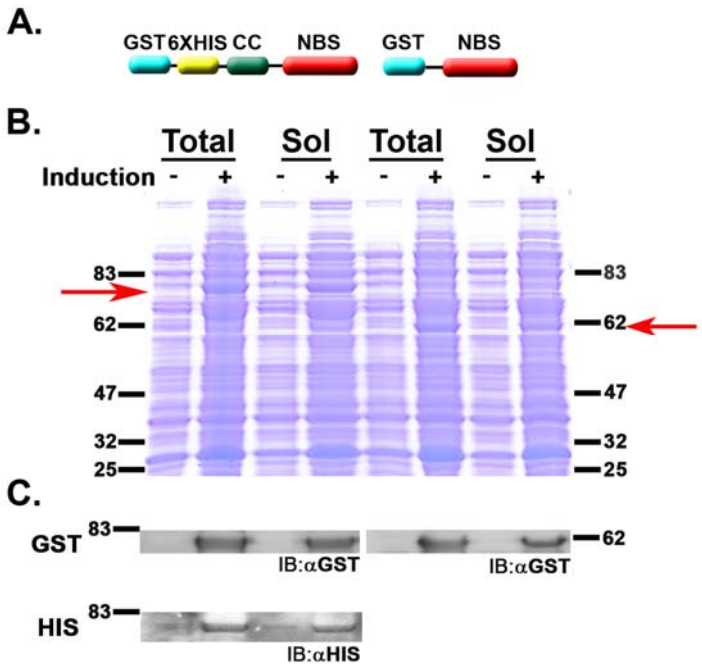


Figure 3.9. RPM1 Truncations are Soluble in *E. coli*. A. Schematic of expressed RPM1 fragments. B. Coomassie blue staining of a polyacrylamide gel loaded with total (left) or soluble (right) protein extracts. Protein samples were taken at 7 hours post-induction and equal volumes of total and soluble protein were loaded into the gel. Induction indicates the presence (+) or absence (-) of IPTG. Red arrows point to protein bands that correspond to the appropriately sized RPM1 fragment. C. Immunoblot analysis for either GST or HIS in the *E. coli* protein extracts.

Discussion

In this work we describe the characterization of the NB-LRR protein RPM1. We have generated two RPM1 variants, D505V and D287A, that have bypassed the requirements for both bacterial effectors and RIN4 in order to initiate defense responses. The D505V and D287A variants likely differ mechanistically in that D505V probably de-stabilizes ADP association whereas D287A likely prevents ATP hydrolysis (Tameling et al., 2006; Marquenet E, 2007). An additional important experiment would be to evaluate whether the G205E mutation could block RPM1 D287A *in cis*. ATP binding is required for the constitutive activity of the Walker B motif mutations in both I-2 and Mal-T, and therefore it would be expected that the G205E mutation would suppress signaling induced by D287A (Tameling et al., 2006; Marquenet E, 2007). Additionally, it would be interestingly to evaluate if RIN4 could suppress the activity of the RPM1 D287A.

Given that RIN4 is not required for signaling of either RPM1 D505V or wild-type RPM1 (Fig. 3.1)(Belkhadir et al., 2004), it is interesting that RIN4 can suppress RPM1 D505V (Fig. 3.4,6). These results indicate that RIN4 is likely to still associate with the activated RPM1 D505V variant. In order to further characterize the RPM1 D505V-RIN4 relationship, we are currently selecting two F2 lines from the progeny of the *rin4/RIN4 rpm1/rpm1 rps2/rps2 RPM1 D505V/* - line shown in Figure 3.6. First, isolation of lines that are homozygous for *RIN4* and *RPM1 D505V* will allow

testing of whether RIN4 can suppress the RPM1 D505V-mediated enhanced disease resistance to virulent *P. syringae* (Fig. 3.5). Secondly, selection of lines that are homozygous for the *RPM1 D505V* transgene but are heterozygous for *RIN4* in the F2 progeny, will allow us to evaluate of whether RIN4 suppresses RPM1 D505V in a dose-dependent manner. We also crossed the *rin4 rpm1 rps2 RPM1 D505V* line to the *RIN4 rpm1 RPS2 RPM1*p:*RPM1-GFP* line described in Figure 3.8. Selection of F2 lines that are *RIN4, rpm1, rps2, RPM1 D505V*, *RPM1-GFP* will allow us to determine whether RPM1 D505V can influence the functional relationship between RIN4 and RPM1-GFP. Additionally, isolation of *RIN4, rpm1, RPS2, RPM1 D505V* lines will allow us to test if RPM1 D505V can influence RIN4's regulation of RPS2. Interestingly, it has been demonstrated that RPM1 negatively influences RPS2 function (Ritter and Dangl, 1996).

Given our results indicating the potential underlying mechanism and the suppression of RPM1 D505V, we propose the following model regarding the biochemical regulation of RPM1. First, through destabilization of the binding of ADP to RPM1, the D505V mutation likely causes RPM1 to transition to the active, ATP-bound state (Fig. 3.1, 3-6). RPM1 D505V likely requires nucleotide binding for signaling since the G205E mutant suppresses D505V *in cis* (Fig. 3.1D). Additionally, RIN4 can suppress RPM1 D505V activity (Fig. 3.4, Fig. 3.6). Two explanations for these results are 1) RIN4 is stabilizing the binding of ADP to RPM or 2) RIN4 is inhibiting the binding of ATP to RPM1. If RIN4 functions to stabilize the binding of ADP to RPM1, it would be expected that RIN4 could not suppress signaling by the RPM1 D287A variant since ectopic activation of the RPM1 D287A

variant is likely due to a lack of ATP hydrolysis and not ADP de-stabilization (Tameling et al., 2006; Marquenet E, 2007). Alternatively, if RIN4 functions to prevent ATP binding to RPM1, it would be expected that RIN4 could also suppress the ectopic activity of the RPM1 D287A variant.

There are some striking similarities between regulation of RPM1 by RIN4 and the regulation of the mammalian NB-LRR protein NALP1 by Bcl-2 and Bcl-X_L. NALP1 can initiate inflammatory responses and is biochemically regulated by the anti-apoptotic proteins Bcl-2 and Bcl-X_L (Bruey JM, 2007; Faustin B, 2009). Negative regulation of NALP1 by Bcl-2 and Bcl-X_L is mediated by unstructured loop regions that suppress ATP from binding to NALP1 (Faustin B, 2009). Interestingly, the unstructured loop regions of Bcl-2 and Bcl-X_L are subject to numerous post-translational modifications, including phosphorylation (Cheng EH, 1997; Ojala PM, 2000). Similarly, RIN4 is an unstructured protein, is subjected to phosphorylation in the presence of the bacterial effectors AvrRpm1 and AvrB, and negatively regulates the NB-LRR protein RPM1 (Fig. 3.4,6)(Mackey et al., 2002; Belkhadir et al., 2004; Kim et al., 2005a). Therefore, a potential set of events could be; 1) RIN4 negatively regulates RPM1 through suppression of ATP binding; 2) RIN4 modification by either AvrRpm1 or AvrB causes a conformation change that represses (1); and 3) RPM1 binding to ATP induces defense signaling. This model indicates that a guardee protein can act as a regulator of the “on” and “off” nucleotide switch of a plant NB-LRR protein.

In the absence of activation, RPM1 undergoes homotypic association in both transient assays in *N. benthamiana* and also at native expression levels in

Arabidopsis. Examples of plant NB-LRR proteins that multi-merize under conditions of transient expression in *N. benthamiana* are the CC-NB-LRR protein RPS5 and the N-Term-SD-CC-NB-LRR protein Prf (Ade et al., 2007; Gutierrez JR, 2009). Similar to RPS5, the NB domain of RPM1 is sufficient for homotypic association (Ade et al., 2007) (Fig. 3.7C). Interestingly, the Prf N-terminal sub-domain is necessary and sufficient for homotypic association (Gutierrez JR, 2009). For full-length Prf, multi-merization requires the host guardee protein Pto (Gutierrez JR, 2009). Since RPM1 homotypic association occurs in the presence of RIN4, it will be interesting to evaluate if homotypic association of RPM1 require RIN4. Multi-merization of the mammalian NB-LRR proteins NALP3 and Monarch-1 requires nucleotide binding (Duncan et al., 2007; Ye Z, 2008). Therefore, future experiments will evaluate if the RPM1 G205E variant can multi-merize.

Many STAND proteins undergo homotypic association after activation including Apaf-1, CED4, and NALP1 (Riedl SJ, 2005; Yan N, 2005; Faustin B, 2007). For plants, activation-induced multi-merization has been shown for the TIR-NB-LRR protein N (Mestre and Baulcombe, 2006). Therefore, future experiments will address whether bacterial effector-induced or autoactivation influences RPM1 multi-merization. Additionally, it would be interesting to evaluate the size of the RPM1-containing protein complex in both the resting and active states. Experiments using size exclusion chromatography and native gel analysis have been shown to effectively measure size changes in NB-LRR protein complexes (Duncan et al., 2007; Hsu LC, 2008; Gutierrez JR, 2009).

A weakness of this work is the lack of biochemical data demonstrating RPM1 interactions with nucleotides. As both full-length or fragments of NB-LRR proteins are notorious difficult to purify, there have been limited studies characterizing the nucleotide-NB-LRR relationship (Lukasik E, 2009). Therefore, the finding that the RPM1 CC-NB and NB fragments are soluble in *E. coli* is a significant initial step to the biochemical characterization of RPM1 (Fig. 3.9). Large-scale purification of the CC-NB and NB fragments, along with the D505V, D287A, and G205E mutations, would allow for the predictions concerning how these RPM1 variants interact with nucleotides to be experimentally tested. Furthermore, biochemically showing that RIN4 can modulate the interaction between RPM1 and nucleotide binding or hydrolysis would an important step in the characterization of guardee-NB-LRR relationship. Finally, previous studies with fragments of STAND proteins have demonstrated roles of nucleotide binding and hydrolysis in the formation of higher-order structures *in vitro* (Kim HE, 2005; Faustin B, 2007). Therefore, it would be interesting to observe the roles of nucleotide binding and hydrolysis in the potential formation of higher-order homotypic RPM1 complexes *in vitro*.

Methods

***A. tumefaciens*-Mediated Transient Expression Assays in *N. benthamiana*.** *A. tumefaciens* containing various constructs were grown for ~24 hours on KB plates with the appropriate antibiotic selection. Cultures were then grown for ~24 hours in KB liquid media with the appropriate antibiotic selection. *A. tumefaciens* cells were then pelleted by centrifugation(10,000 x g, 1 min.), and resuspended in a solution of

10mM MgCl, 10mM MES. Cells were then pelleted again by centrifugation (10,000 x g, 1 min), resuspended in 10mM MgCl, 10mM MES with 1% acetosyringone. Cultures were then incubated for ~2 hours at room temperature while rotating on a shaker. *A. tumefaciens* cultures were adjusted to various OD_{600} and inoculated into *N. benthamiana* leaves.

Generation of Transgenic Arabidopsis Lines. Stable transgenic plant lines were generated by the floral dip method (Clough and Bent, 1998). Lines containing single transgene insertions were selected in the T2 generation by PCR analysis.

Ion leakage Analysis. Ion leakage analysis was performed as previously described (Mackey et al., 2002) except that 3 experimental replicates containing 6 leafs disks were floated in 6 mL of fresh water.

Bacterial Infections. For Fig. 3.5B and Fig. 3.8A, dip inoculations were performed as previously described (Tornero and Dangl, 2001). Briefly, 2-to 3-week-old seedlings were inoculated by dipping in solutions of bacteria at a concentration of 2.5 X 10^7 cfu/ml. Three seedlings were placed in 1 ml of 10 mM MgCl2 with 0.2% Silwet L-77. The weights of the tubes with seedlings were measured, and the tubes were then shaken for 1 h at 28°C. Serial dilutions in 10 mM MgCl2 were made and plated on KB plates with rifampicin selection. For Table 3, HR assays were performed as described (Grant et al., 1995).

Protein Analysis. For detection of PR-1, RIN4, and RPM1-myc, total protein extracts were prepared by grinding ~200 mg of leaf tissue in buffer (50 mM Tris/HCl

(pH 8.0), 1% SDS, 1 mM EDTA, 1 mM DTT, and plant protease inhibitor mixture (Sigma)). Protein concentrations were determined by using the Bradford-Lowry method and quantification buffer from BioRad. Ten microliters of 6X Laemmli buffer (final concentration 1X) was added to all measured samples after quantification. For immunodetection of RIN4 and PR-1, protein samples were electrophoresed on 14% SDS polyacrylamide gels. For immunodetection of RPM1-myc, protein samples were electrophoresed on 8% SDS polyacrylamide gels.

Co-immunoprecipitation Analysis. ~1 gram of leaf tissue from either *N. benthamiana* or Arabidopsis was ground in a solution of 20mM HEPES pH7.5, 0.33M sucrose, 10mM EDTA, 5mM DTT, and protease inhibitors(Sigma). Solutions were filtered through one layer of miracloth and centrifuged at 2000 x g for 5 min. Supernatants were then centrifuged at 6000 x g for 10 min. In order to purify microsomes, supernatants were centrifuged at 20,000 x g for 70 min. Pellets were then solubilized in 1mL of 50mM HEPES pH7.5, 50mM NaCl, 5mM EDTA, 1mM DTT, protease inhibitors (Sigma), and 1% Triton. Samples were then incubated on swinging rotator for ~2 hours at 4°C. After incubation, samples were centrifuged at 20,000 x g for 20 min, and the insoluble pellets were discarded. The supernatants were diluted to 50mM HEPES pH7.5, 50mM NaCl, 5mM EDTA, .05mM DTT, protease inhibitors (Sigma), and .05% Triton. 1mL of supernatents were then incubated with 50 µL of anti-GFP magnetic beads (MACS) overnight at 4°C while rotating. Samples were then run over magnetic columns (MACS), washed, and eluted according to the manufactures' protocol.

RPM1 CC-NB and NB Fragment Expression in *E. coli*. HIS-CC-NB and NB RPM1 fragments were cloned into pDEST15(N-Term GST fusion)(Invitrogen). Vectors were then transformed into Rosetta cells(Novagen). Transformed clones containing either the HIS-CC-NB or NB RPM1 fragment were grown ~12 hrs. in 6 ml cultures of 2XYT media with ampicillin selection. The cultures were then diluted 1:200 and grown in 10mL cultures with 2XYT media with ampicillin until OD_{600} was .51 for the HIS-CC-NB fragment and OD_{600} was .47 for the NBS fragment. The cultures were then grown at 21°C for ~45 minutes and then protein expression was induced with 1mM IPTG. In order to evaluate whether the RPM1 fragments were soluble, 2 mL of cells were centrifuged at 5,000 x g for 5 min. Pellets were then re-suspended in 500 µL of Wash Buffer (25mM Tris pH 8.0, 2mM EDTA, 100mM NaCl), centrifuged at 20,000 x g for 5 min, and frozen in liquid nitrogen. Cell pellets were then stored at -80°C. Cell pellets were then placed on ice and were suspended in 500 µL of Lysis Buffer (50mM Tris pH 8.0, 2mM EDTA, 25% Sucrose). Cell lysis was performed by 2X sonication for ten seconds. Soluble protein was isolated by centrifugation at 20,000 x g for 10 min and an equal volume of soluble protein was boiled in 1X Loading Buffer (60mM TRIS:HCL pH 6.8, 25% Glycerol, 2% SDS, 0.1% Bromophenol Blue). Samples were loaded into polyacrylamide gels and subjected to both coomassie blue straining (Fig. 3.9A) and immunodetection analysis (Fig. 3.9B) with either anti-GST or anti-HIS antibodies (Santa Cruz).

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CHAPTER IV: A Pair of NB-LRR Proteins Mediates Defense Responses to the Bacterial Effector AvrB

Preface

Much of the following chapter was published in the Proceedings of the National Academy of Sciences volume 105, issue 17 for which I shared co-authorship with Zack Nimchuk. My contribution to the publication, as with this chapter, was that I wrote the manuscript and generated the data for all figures except Figures 1A and 1B. This work was the first example that multiple NB-LRR proteins can function in response to a single bacterial effector.

Abstract

The type III effector protein encoded by avirulence gene B (AvrB) is delivered into plant cells by pathogenic strains of *Pseudomonas syringae*. There, it localizes to the plasma membrane and triggers immunity mediated by the Arabidopsis Coiled-coil (CC)-nucleotide binding (NB)-leucine-rich repeat (LRR) disease resistance protein RPM1. The sequence unrelated type III effector avirulence protein encoded by avirulence gene Rpm1 (AvrRpm1) also activates RPM1. AvrB contributes to virulence after delivery from *P. syringae* in leaves of susceptible soybean plants, and AvrRpm1 does the same in Arabidopsis *rpm1* plants. Conditional over-expression of AvrB in *rpm1* plants results in leaf chlorosis. In a genetic screen for mutants that lack AvrB-dependent chlorosis in an *rpm1* background, we isolated *TAO1* (Target of AvrB Operation), which encodes a Toll

and human interleukin receptor (TIR)-NB-LRR disease resistance protein. In *rpm1* plants, TAO1 function results in the expression of the pathogenesis-related protein 1 (PR-1) gene and growth restriction of virulent *P.syringae*. In *RPM1* plants, TAO1 contributes to disease resistance in response to *Pto* (*P*. *syringa*e pathovars tomato) DC3000(*avrB*), but not against *Pto* DC3000(*avrRpm1*). Therefore, our data provide evidence of genetically separable disease resistance responses to AvrB and AvrRpm1 in Arabidopsis. The *tao1–5* mutant allele, a stop mutation in the LRR domain of TAO1, post-transcriptionally suppresses RPM1 accumulation. We propose a model in which AvrB activates both RPM1 (CC-NB-LRR) and TAO1 (TIR-NB-LRR), and that these NB-LRR proteins act additively to produce a full disease resistance response to *P*. *syringae*.

Introduction

Exposure to pathogens has driven the evolution of a sophisticated plant immune system that controls complex defense mechanisms for limiting infection (Jones and Dangl, 2006). Plants express disease resistance proteins whose activation results in the restriction of pathogen growth and, typically, a localized cell death termed the hypersensitive response (HR). The most common class of disease resistance proteins consists of a central nucleotide binding (NB) site and a C-terminal leucine-rich repeat (LRR). NB-LRR proteins can be further subdivided based on the presence of a Coiled-coil (CC) or a Toll and human interleukin receptor (TIR) domain at the N terminus (Meyers et al., 2003). In response to infection by Gram-negative bacterial phytopathogens, specific NB-LRR proteins are activated via recognition of a pathogen-encoded type III effector protein. This

recognition can occur in an indirect manner, whereby type III effectors are perceived via their modification of host target proteins. Hence, NB-LRR proteins can survey the integrity of intracellular host proteins targeted by type III effectors acting as virulence factors. When a particular type III effector activates an NB-LRR protein, that type III effector is defined as an ‘‘avirulence protein.’’ This interaction is specific, in that a single allele of a NB-LRR gene is typically responsible for mediating resistance to a pathogen isolate expressing a particular type III effector (Jones and Dangl, 2006).

One exception is provided by the *Pseudomonas syringae* type III effector proteins avirulence gene B (AvrB) and avirulence gene RPM1 (AvrRpm1); each can elicit disease resistance mediated by the Arabidopsis CC-NB-LRR protein RPM1 (Bisgrove et al., 1994; Grant et al., 1995). After their delivery into host cells by the type III secretion pilus, AvrB and AvrRpm1 undergo N-terminal myristoylation that facilitates association with the plant plasma membrane (Nimchuk et al., 2000). Here, these type III effectors associate with the host target protein, RIN4 (RPM1-interacting protein 4) (Nimchuk et al., 2000). AvrB-RIN4 or AvrRpm1-RIN4 interactions are correlated with the phosphorylation of RIN4 and activation of RPM1 (Mackey et al., 2002). Interestingly, *Glycine max* (soybean) resistance to pathogen strains expressing AvrB or AvrRpm1 can be encoded by two different CC-NB-LRR genes, *Rpg1-b an*d *Rpg1-r*, respectively (Ashfield et al., 1995; Ashfield et al., 2004). Genetic data confirms that AvrB and AvrRpm1 have multiple host targets in Arabidopsis *rpm1* plants (Belkhadir et al., 2004), suggesting that different NB-LRR receptors may have evolved to monitor

the integrity of independent targets of these two type III effectors. Yet, the ability of AvrRpm1 and AvrB to trigger disease resistance in Arabidopsis has not been separated.

Transgenic *in planta* conditional expression of AvrB in *rpm1* plants leads to leaf chlorosis (Nimchuk et al., 2000). AvrB-induced chlorosis is RIN4-independent (Belkhadir et al., 2004), but requires the host protein RAR1 (Required for Mla-resistance 1) (Shang et al., 2006). RAR1 is part of a chaperone complex that functions to positively regulate steady-state accumulation of numerous NB-LRR proteins (Bieri et al., 2004; Holt III et al., 2005). Additionally, RAR1 is a positive regulator of basal disease resistance against virulent pathogens (Holt III et al., 2005). Because AvrB can coimmunoprecipitate RAR1, the suggestion has recently arisen that RAR1 might be a virulence target of AvrB (Shang et al., 2006).

We performed a genetic screen to isolate mutants compromised for AvrB-induced chlorosis. This screen was performed in Mt-0, an ecotype (inbred genetic line) of Arabidopsis from which *RPM1* is naturally deleted (Grant et al., 1998), but which responds to AvrB with leaf chlorosis (Nimchuk et al., 2000). We defined and isolated *TAO1* (Target of AvrB Operation), a gene encoding a disease resistance protein of the TIR-NB-LRR class. TAO1 activation correlates with defense gene expression and disease resistance. A specific mutant allele of *TAO1* post-transcriptionally suppresses the steady-state accumulation of RPM1. In the Col-0 (*RPM1*) accession, TAO1 is required for a full resistance response to *Pto* DC3000(*avrB*) but not to *Pto* DC3000(*avrRpm1*), showing that recognition of these two type III effectors is genetically separable in Arabidopsis. TAO1-dependent

phenotypes, like disease resistance mediated by all TIR-NB-LRR proteins, requires phytoalexin deficient 4 (PAD4). Our data provide further evidence that CC and TIR subclasses of NB-LRR proteins can function additively in disease resistance responses against biotrophic pathogens.

Results

TAO1 Encodes a TIR-NB-LRR Protein Required For AvrB-Induced Chlorosis in *rpm1* Host Plants.

We designed a conditional genetic screen to isolate loci required for AvrB-induced chlorosis in Mt-0 (*rpm1*). A stable single insertion transgenic line carrying a dexamethasone (DEX)-inducible *avrB* expression system (*DEX*:*avrB-HA* (Aoyama and Chua, 1997)) was established in Mt-0 (see Methods). Seed from this line was mutagenized, and ~400,000 M2 plants were screened for loss of AvrB-induced chlorosis after DEX treatment (Fig. 4.1A). DEX-inducible AvrB-HA expression was confirmed in mutant (fully green) M3 progeny by Western blot analysis (Fig. 4.1B). We used transient *Agrobacterium* transformation of *DEX*:*avrB-HA* on several M3 progeny from each putative M2 mutant plant to eliminate mutations in the *DEX*:*avrB-HA* expression system. In this assay, plants mutated in the *DEX*:*avrB-HA* transgene were complemented by the transient expression of *DEX*:*avrB-HA* and display chlorosis after DEX treatment. These lines were not studied further.

Genetic complementation crosses indicated eight allelic, recessive *tao1* mutants that were also allelic to the naturally occurring *tao1* phenotype of Cvi-0 (Nimchuk et al., 2000). Transgenic *DEX*:*avrB-HA rpm1 tao1–2* (Mt-0) and *DEX*:*avrB-HA rpm1 tao1–5* (Mt-0) lines were crossed independently to *rpm1–3*

TAO1 (Col-0) for map-based cloning of *TAO1*. After DEX induction, F1 plants exhibited host cell chlorosis, confirming that both *tao1–2* and *tao1–5* were recessive. We isolated non-chlorotic plants in the F2 generation. Two genotypes were present among these non-chlorotic F2 plants: (*i*) those that lacked the *DEX*:*avrB-HA* transgene and (*ii*) those that contained the *DEX*:*avrB-HA* transgene and were homozygous *tao1*. PCR with *avrB*-specific primers distinguished between these classes; plants lacking the *DEX*:*avrB-HA* transgene were discarded. From the *tao1–5* cross, ~1,000 informative F2 plants showed that *TAO1* was tightly linked to the bottom of chromosome 5. In a smaller F2 population, the *tao1–2* mutant allele also mapped to the same location, which was in agreement with data previously presented for the Cvi-0 loss of function allele of *TAO1* (Nimchuk et al., 2000). To avoid mis-scoring caused by transgene silencing, we used transient assays with *Agrobacterium tumefaciens* (*DEX*:*avrB-HA*) of F3 progeny to confirm the *tao1* genotypes of F2 individuals. This approach, combined with the sequencing of mutations in eight *tao1* alleles (designated *tao1–1* to *tao1–8*; Fig. 4.1C), identified *TAO1* as the Mt-0 allele of the Col-0 gene At5g44510.

TAO1 encodes a TIR-NB-LRR protein of the TNL-G clade (16). There are no other *NB-LRR* genes at the *TAO1* locus in the reference Col-0 genome sequence. The deduced TAO1 protein is only 26% similar to RPM1 and 25% similar to RPG1-B (soybean). For further analyses, we chose three alleles: *tao1–2*, an early stop mutation after Q36 and a likely null; *tao1–1*, a missense mutation at G238E in the NB domain; and *tao1–5*, a nonsense mutation at W892 that results in the deletion of the last five LRRs (13 through 17) (Fig. 4.1C). Sequencing of the naturally

occurring *tao1* allele in Cvi-0 revealed three amino acid changes compared with the Mt-0 TAO1 sequence at 703, 781, and 957 (Fig. 4.1C). Col-0 TAO1 shares these amino acids with Mt-0, while also having a polymorphism at amino acid 489 (Fig. 4.1C).

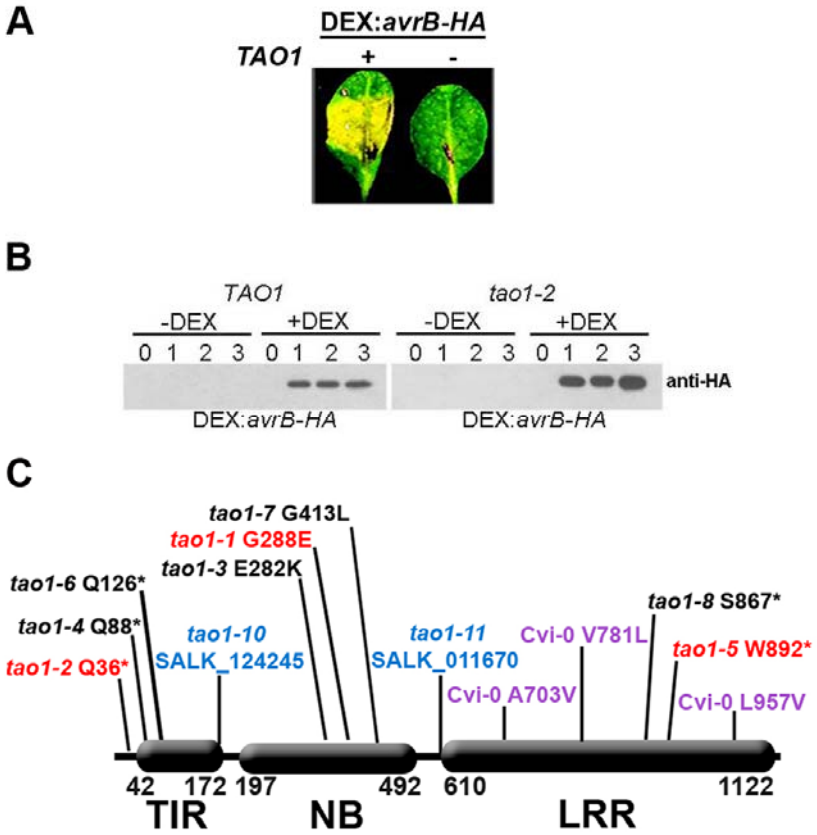

Figure 4.1. TAO1 is a TIR-NB-LRR Required for AvrB-induced Chlorosis in *rpm1* Host Plants. (A) Mt-0 leaves were inoculated with *Agrobacterium* containing T-DNA with a *DEX:avrB-HA* transgene (Nimchuk et al., 2000). Leaves were treated with dexamethosone 48 hours post inoculation. Picture was taken 72 hours post inoculation. (B) Western blot showing accumulation of AvrB-HA post-DEX treatment. (C) Deduced structure of *TAO1* alleles recovered in Mt-0 (red and black), Col-0 Salk T-DNA insertion lines (blue), and as polymorphisms in Cvi-0 (purple). For all missense mutations, the wild type Mt-0 residue is listed first. TIR, Toll Human Interlukin-1 like receptor domain, amino acids 42 to 172. NB, nucleotide binding domain, amino acids 197 to 492. LRR, leucine rich repeats, amino acids 610-1122. All *TAO1* alleles in Mt-0 were generated by EMS mutagenesis except *tao1-8*, which is a fast neutron deletion of 1 bp in codon S667. Red *tao1* alleles represent alleles which were out-crossed away from the

DEX:avrB-HA transgene and used for further analyses. The *tao1-10* (Salk_124245) insertion begins at amino acid 168. The *tao1-11* (Salk_011670) insertion begins at amino acid 597. The only amino acid difference between Mt-0 and Col-0 is V489M, respectively. Genomic *TAO1* sequences for Mt-0 (bankit100032-EU031442) and Cvi-0 (bankit1000326-EU031443) were deposited in GenBank.

TAO1 Activation Triggers Plant Defenses

Since *TAO1* encodes a TIR-NB-LRR protein, we evaluated whether TAO1 activation produced a defense response. To this end, we measured PR-1 expression and the growth of virulent *P.syringae* after *in planta* expression of AvrB. Five days after induction with DEX, *DEX*:*avrB-HA rpm1 tao1–2* plants were non-chlorotic and had low levels of PR-1 expression whereas the *DEX*:*avrB-HA rpm1 TAO1* plants displayed strong leaf chlorosis and had high levels of PR-1 expression (Fig. 4.2A). Furthermore, TAO1 activation caused growth restriction of virulent *P.syringae* (Fig. 4.2A).

To analyze TAO1 function in Col-0, a better-studied genetic background for disease resistance studies than Mt-0, we used two mutant alleles designated *tao1–10* (SALK_124245), and *tao1–11* (SALK_011670) that carry exon insertions in At5g44510 (Fig. 4.1C). Both mutants lose both *TAO1* transcript accumulation and TAO1 function (Fig. 4.2, Fig. 4.4.). The *tao1-10* and *tao1-11* lines were crossed to an isogenic *DEX:avrB-HA rpm1–3 TAO1* line, and *DEX:avrB-HA rpm1–3 tao1–10* and *DEX:avrB-HA rpm1–3 tao1–11* lines were selected from the resulting progeny. Five days after induction with DEX, the *DEX:avrB-HA rpm1–3 TAO1* plants were chlorotic, whereas the *DEX*:*avrB-HA rpm1–3 tao1–10* lines were nonresponsive (Fig. 4.2B). Furthermore, TAO1 activation correlated with an increase in PR-1 expression and growth restriction of virulent *P.syringae* (Fig. 4.2B).

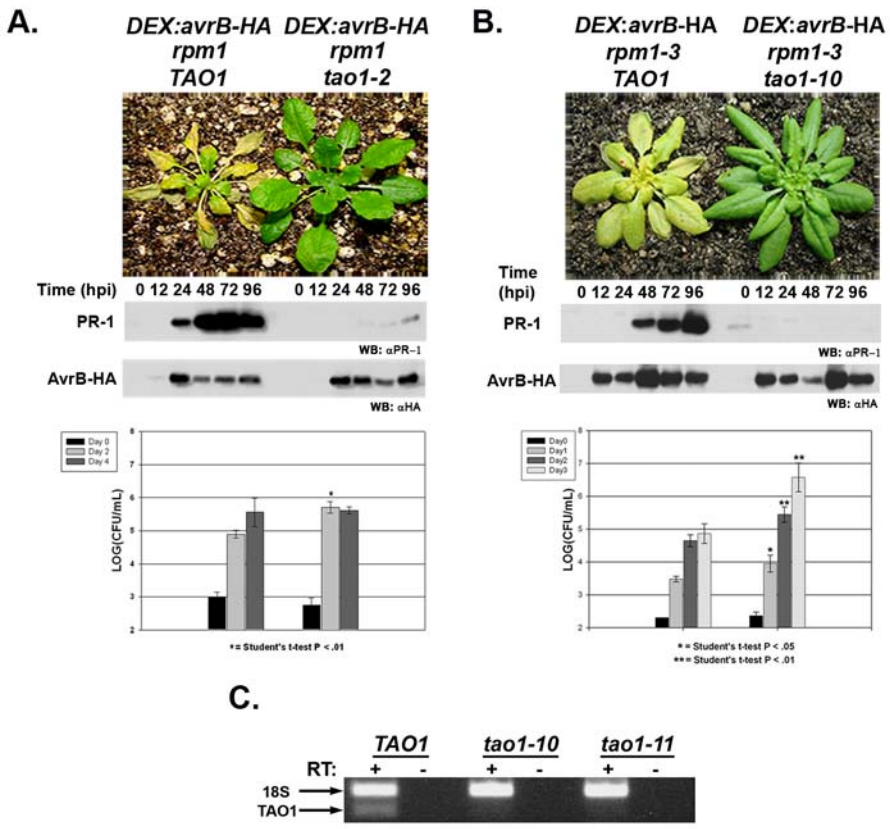

Figure 4.2. TAO1 Activation Causes Plant Defenses. (A-B) 5-week old plants were sprayed with 20 µM dexamethosone to induce expression of AvrB-HA. (A) Mt-0 and *tao1-2*. (B) Col-0(*rpm1-3*) and *rpm1-3 tao1-10*. Top panel, photograph was taken 5 days post-dexamethosone treatment. Middle panel, western blot showing PR-1 and AvrB protein levels. Lower panel, 4-week old plants were hand infiltrated with *Pto* DC3000 24 hours post-dex treatment at 10^5 cfu/mL. Error bars represent the standard deviation among four samples. Student's t-tests were used to verify differences between the *TAO1* and *tao1* lines. The experiment is representative of three independent replicates. (C) Leaf RNA from various plants lines was subject to RT-PCR analysis for the *TAO1* transcript (Bottom). Equivalent amounts of template cDNA loading shown by 18S control primer band (Top). RT + or – indicates presence or absence of reverse transcriptase in the cDNA synthesis reaction step. This experiment is representative of two independent replicates.

RPM1 Function is Lost in *tao1–5*.

We next addressed whether TAO1 contributed to RPM1-mediated disease resistance. For this, we introduced an *RPM1* transgene containing a C-terminal myc epitope tag, expressed from the native *RPM1* promoter (17), into Mt-0 (*rpm*1 *TAO1*). A line carrying a single copy of the *RPM1-myc* transgene was selected and

then crossed into the *tao1–1*, *tao1–2*, and *tao1–5* mutant backgrounds (previously back-crossed and selected for loss of the *DEX:avrB-HA* transgene) (see Methods). Stable *RPM1-myc TAO1*, *RPM1-myc tao1–1*, *RPM1-myc tao1–2*, and *RPM1-myc tao1–5* lines were selected, and RPM1 function was assessed. After hand infiltration with *Pto* DC3000(*avrB*) or *Pto* DC3000(*avrRpm1*), RPM1 function resulted in only a ~5-and 10-fold growth restriction of bacteria, respectively (Fig. 4.3A), which is much less bacterial growth restriction than observed in Col-0 (*RPM1*) (Fig. 4.4A). This weak RPM1-mediated growth restriction is likely caused by lower accumulation of RPM1-myc in Mt-0 relative to Col-0 background or transgene positional effects (Fig. 4.3D). In *RPM1-myc tao1–1* and *RPM1-myc tao1–2*, there was little to no effect on the weak *RPM1*-mediated growth restriction of *Pto* DC3000(*avrRpm1*) or *Pto* DC3000(*avrB*) (Fig. 4.3A). Interestingly, RPM1 function was fully lost in the *RPM1-myc tao1–5* background in response to either *Pto* DC3000(*avrB*) or *Pto* DC3000(*avrRpm1*) (Fig. 4.3A)

tao1-5 Loses RPM1 Protein Accumulation

We postulated two reasons why RPM1 function is lost in the *tao1–5* background. The *tao1–5* mutant protein could either prevent RPM1 signaling, or the *tao1–5* mutant protein might negatively regulate RPM1 steady-state accumulation. To distinguish between these possibilities, the *RPM1-myc TAO1*, *RPM1-myc tao1–1*, *RPM1-myc tao1–2*, and *RPM1-myc tao1–5* lines were subjected to Western blot analysis for RPM1-myc. Despite some experimental variation, RPM1 accumulated normally in *RPM1-myc tao1–1* and *RPM1-myc tao1–2* (Fig. 4.3B). By contrast, RPM1 accumulation was abolished in *RPM1-myc tao1–5* (Fig. 4.3B). RT-PCR

analysis revealed that this effect on RPM1 is post-transcriptional (Fig. 4.3E). Interestingly, the effect of *tao1–5* on RPM1 accumulation was recessive (Fig. 4.3C).

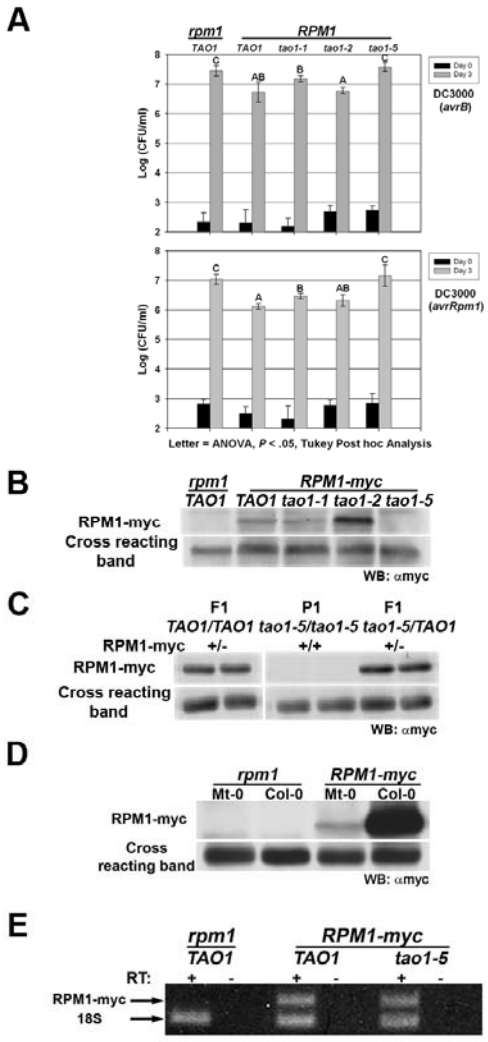

Figure 4.3. *tao1-5* Loses RPM1 Protein Accumulation (A) 4-week old plants were hand infiltrated with *Pto* DC3000(*avrB*) (Top) or *Pto* DC3000(*avrRpm1*) (Bottom) at 10^5 cfu/mL. Error bars represent the standard deviation among four samples. A one-way analysis of variance (ANOVA) was applied to the values. Letters represent different classes based on Tukey Post-hoc analysis, $P < .05$. This experiment is indicative of three independent replicates. (B-C) Western blot analysis showing RPM1-myc accumulation in plant lines with the noted genotypes. Cross reacting band indicates equivalent amounts of total protein loading. Samples from hybrid (F1) and parental (P1) lines are noted above their respective genotypes. These experiments are indicative of four (B) and two (C) independent replicates. The higher than wild type level of RPM-myc accumulation in the *tao1-2* background shown in (B) was not reproducible. (D) Western blot analysis showing RPM1-myc accumulation in various plant lines. The cross reacting band serves as an indicator of equivalent amounts of total protein loaded. This experiment is indicative of two independent replicates. Note that we screened several independent transgenic Mt-0 lines

for RPM1-myc accumulation and this was the highest level observed. (E) Leaf RNA from various plants lines, genotypes above, was subjected to RT-PCR analysis for the *RPM1* transcript (Top). Equivalent amounts of template cDNA loading shown by 18S control primer band (Top). RT + or – indicates presence or absence of reverse transcriptase in the cDNA synthesis reaction step. This experiment is representative of three independent replicates.

TAO1 Is Required for Full Disease Resistance Against *Pto* DC3000(*avrB*) in Col-0.

We crossed the *tao1–10* and *tao1–11* alleles into *rpm1–3*. Homozygous *rpm1–3 tao1–10* and *rpm1–3 tao1–11* lines were selected to evaluate TAO1 function in both the presence and absence of RPM1. After dip inoculation in a bacterial suspension of *Pto* DC3000(*avrB*), both the *RPM1 tao1–10* and *RPM1 tao1–11* lines displayed ~10-fold less disease resistance than *RPM1 TAO1* (Fig. 4.4A). Therefore, TAO1 contributes ~1–5% of the full defense response to *Pto* DC3000(*avrB*) in the presence of RPM1. There was no enhanced susceptibility to *Pto* DC3000(*avrB*) in the *rpm1–3 tao1–10* and *rpm1–3 tao1–11* compared to the *rpm1-3 TAO1* line (Fig. 4.4A). This indicates that when AvrB is delivered from *P.syringae*, the TAO1 contribution to disease resistance is too low to measure in the absence of RPM1. Conversely, *in planta* over-expression of AvrB resulted in TAO1-mediated growth restriction of virulent *P.syringae* in the absence of RPM1 (Fig 4.2). Collectively, these data indicate that TAO1 produces a defense response in both the absence and presence of RPM1, but that the TAO1-mediated defense response alone is too weak to observe when AvrB is delivered from *P.syringae*. We observed full growth restriction in the *RPM1 tao1–10* and *RPM1 tao1–11* lines after dip inoculation of *Pto* DC3000(*avrRpm1*) (Fig. 4.4B). Therefore, TAO1 contributes specifically to disease resistance triggered by *Pto* DC3000(*avrB*) and does not

contribute to RPM1-mediated disease resistance in response to *Pto* DC3000(*avrRpm1*). As with *Pto* DC3000(*avrB*), we observed no enhanced susceptibility of the *rpm1–3 tao1–10* and *rpm1–3 tao1–11* lines to *Pto* DC3000(*avrRpm1*) (Fig. 4.4A).

Although RPM1-mediated resistance to *Pto* DC3000(*avrRpm1*) was unaffected in the *tao1–10* and *tao1–11* lines, the possibility existed that TAO1 alters RPM1 steady-state accumulation. This scenario would indicate that different accumulation levels of RPM1 are required for resistance to *Pto* DC3000(*avrB*) versus *Pto* DC3000(*avrRpm1*). To address this possibility, the *rpm1–3 tao1–10* and *rpm1–3 tao1–11* lines were crossed to a *rpm1–3 RPM1-myc TAO1* line (Boyes et al., 1998). Stable *rpm1–3 RPM1-myc tao1–10* and *rpm1–3 RPM1-myc tao1–11* lines were selected. Western blot analysis revealed that RPM1 accumulation was approximately equivalent in the *tao1–10* and *tao1–11* lines relative to the *TAO1* parental line (Fig. 4.4B). These data demonstrate that TAO1 does not alter RPM1 steady-state accumulation. Therefore, the contribution of TAO1 to disease resistance against *Pto* DC3000(*avrB*) in Col-0 is likely to be at the signaling level.

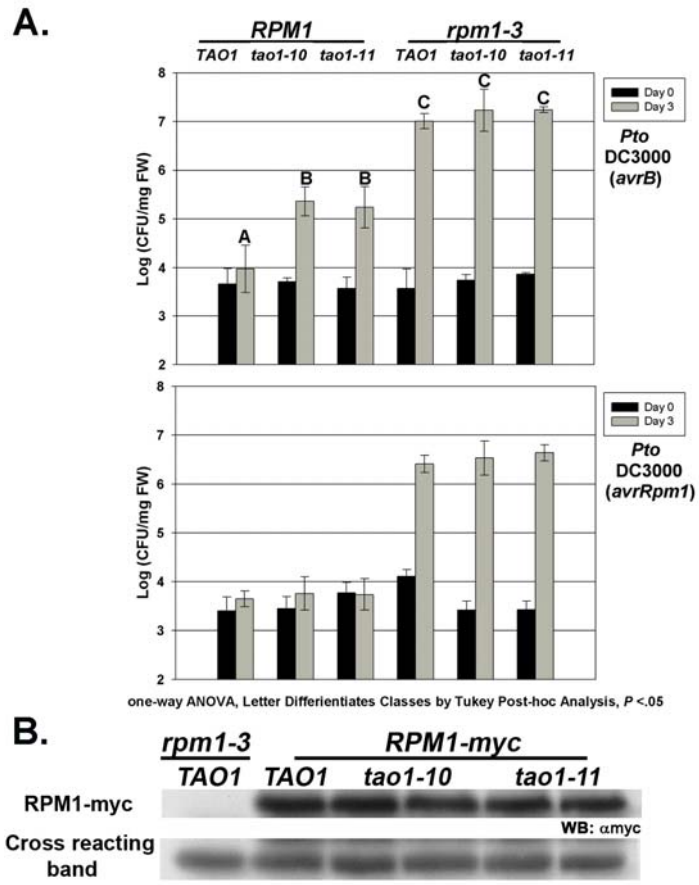

Figure 4.4. TAO1 Contributes to Disease Resistance Against *Pto* DC3000(*avrB*) (A) 2-3 week old plants were dip infiltrated with *Pto* DC3000(*avrB*) (Top) or *Pto* DC3000(*avrRpm1*) (Bottom) at a bacterial concentration of 2.5 x 10^7 cfu/mL. Error bars represent the standard deviation among four samples. Error bars represent the standard deviation among four samples. A one-way analysis of variance (ANOVA) was applied to the values. Letters represent different classes based on Tukey Post-hoc analysis, $P < .05$. This experiment is representative of three independent replicates. (B) Western blot analysis showing RPM1-myc accumulation in plant lines with the noted genotypes. The cross reacting band serves as an indicator of equivalent amounts of total protein loaded. This experiment is representative of two independent replicates.

TAO1 Requires PAD4 For Function.

The *Arabidopsi*s PAD4 protein functions in both basal defense and TIR-NB-LRR-mediated disease resistance (Glazebrook et al., 1997; Feys et al., 2005). To assess whether PAD4 is required for TAO1 function, we crossed the *DEX*:*avrB-HA*

rpm1–3 line to the *pad4–1* mutant line (Glazebrook et al., 1996) and selected homozygous F2 *rpm1–3 pad4–1* lines that contained the *DEX*:*avrB-HA* transgene. After inducing AvrB expression *in planta*, both TAO1-mediated chlorosis and PR-1 expression were lost in the *rpm1–3 pad4–1* lines (Fig. 4.5A). Furthermore, inoculation with *Pto* DC3000(*avrB*) and *Pto* DC3000(*avrRpm1*) on *pad4–1 TAO1 RPM1* lines revealed that the TAO1 defense function against *Pto* DC3000(*avrB*) is also lost in *pad4–1* (Fig. 4.5B). These results demonstrate that TAO1 requires PAD4 for function.

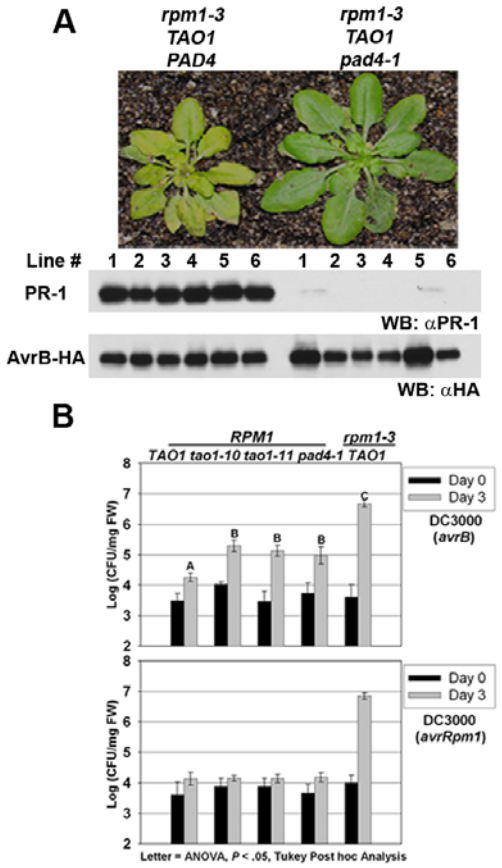

Figure 4.5. TAO1 Requires PAD4 for Function. (A) Six F2 *rpm1-3 pad4-1* lines containing the *DEX:avrB-HA* transgene were selected. The picture was taken 3 days post dexamethosone treatment. Protein extracts from six individual plants were subjected to western blot analysis for both PR-1 and AvrB-HA. (B) 2-3 week old plants were dip infiltrated with *Pto* DC3000(*avrB*) (Top) or *Pto* DC3000(*avrRpm1*) (Bottom) at a bacterial

concentration of 2.5 x 10^7 cfu/mL. Error bars represent the standard deviation among four samples. Error bars represent the standard deviation among four samples. A one-way analysis of variance (ANOVA) was applied to the values. Letters represent different classes based on Tukey Post-hoc analysis, $P < .05$. This experiment is representative of three independent replicates.

Discussion

AvrB is a bacterial type III effector protein that causes modification of the Arabidopsis host protein RIN4, subsequently activating the NB-LRR protein RPM1 (Mackey et al., 2002; Desveaux et al., 2007) or contributing to pathogen virulence in susceptible plants (Ashfield et al., 1995). The sequence-independent type III effector AvrRpm1 acts similarly (Ritter and Dangl, 1995). In the absence of RPM1, *in planta* expression of AvrB produces a host cell chlorosis originally speculated to be indicative of an AvrB virulence function (Nimchuk et al., 2000). Because AvrB-mediated chlorosis is RIN4 independent, AvrB must have additional targets within the Arabidopsis cell (Belkhadir et al., 2004). Our aims were to (*i*) identify host proteins required for AvrB-induced chlorosis, (*ii*) assess whether and how these host components affect RPM1 function, and (*iii*) clarify whether these host factors are specific for the responses to AvrB. We identified only one gene in our mutant screen, *TAO1*, which encodes a TIR-NB-LRR disease resistance protein.

Our data demonstrate that TAO1 activation contributes to disease resistance in response to *Pto* DC3000(*avrB*), but not in response to *Pto* DC3000(*avrRpm1*) (Fig. 4.4). To explain the functional relationship between AvrB, TAO1, and RPM1, we propose the following model. In *rpm1* plants, TAO1 activation by over-expression of AvrB *in planta* causes chlorosis, PR-1 expression, and growth restriction of virulent *Pto* DC3000 (Fig. 4.2). However, TAO1 activation when AvrB

is delivered from *Pto* DC3000 is insufficient to restrict *Pto* DC3000(*avrB*) growth in the absence of RPM1 (Fig. 4.4). These data indicate that TAO1 is a weak disease resistance protein. In *tao1* plants, RPM1 activation in response to AvrB results in some restriction of *Pto* DC3000(*avrB*) growth (Fig. 4.4). Therefore, the additive functions of RPM1 and TAO1 facilitate full disease resistance to *Pto* DC3000(*avrB*).

Since TAO1 function is RIN4 independent (Belkhadir et al., 2004), and RIN4 is required for RPM1 function (Mackey et al., 2002), TAO1 perception of AvrB might occur at a cellular site that lacks RIN4 and RPM1. Recently, two studies (Ong and Innes, 2006; Desveaux et al., 2007) identified AvrB mutants that are unable to activate RPM1, but still activate TAO1. Interestingly, one of the AvrB mutant variants, T125A, lost the ability to interact with RIN4 whereas the other AvrB mutant variant, D297A, retained the ability to interact with RIN4 (Ong and Innes, 2006; Desveaux et al., 2007). These data suggest that there are likely to be structural differences in how AvrB targets host proteins and consequently triggers either RPM1 or TAO1. Despite potentially separate mechanisms of AvrB perception by TAO1 and RPM1, both TAO1 and RPM1 activation requires AvrB localization to the plasma membrane (Nimchuk et al., 2000). These data may indicate that like RPM1 and RIN4, a second putative protein(s), targeted by AvrB and associated with TAO1, is present at the plasma membrane. Alternatively, it is possible that TAO1 directly interacts with AvrB at the plasma membrane.

Interestingly, the resting protein accumulation of RPM1 is lost in the *tao1-5* background (Fig. 4.3B). Because the other loss-of-function mutant alleles tested, *tao1–1* (presumptive NB catalytic dead) and *tao1–2* (presumptive null), do not

negatively alter RPM1 accumulation and *tao1–5* homozygosity is required for its effects on RPM1, *tao1–5* acts as a recessive gain-of-function allele (Fig. 4.3B,C). This phenotype is reminiscent of the effects of specific alleles of *hsp90.*2 on RPM1 accumulation (Holt III et al., 2003). The *tao1–5* allele exhibits no other observable phenotypic defects. A *tao1–5*-encoded protein could sequester RPM1 directly or negatively affect RPM1 stability through its effects on an intermediate host protein. Host proteins shown to be required for RPM1 steady-state accumulation are RIN4 and the general NB-LRR chaperone complex members RAR1 and HSP90.2 (Mackey et al., 2002; Tornero et al., 2002b). Because neither RPS2 function nor RPS5 steady-state accumulation are compromised in the *tao1–5* background, it is unlikely that the *tao1–5* mutant protein sequesters RIN4 or RAR1. Hence, we speculate that HSP90.2 or an as-yet-undiscovered protein that contributes to RPM1 steady-state accumulation is altered by the mutant *tao1–5*-encoded product.

Recently, Shang *et al*. (Shang et al., 2006) demonstrated that RAR1 is required for AvrB-dependent chlorosis in *rpm1* plants (Shang et al., 2006). The authors also showed that AvrB-induced chlorosis correlated with increased growth of a *Pto* DC3000(*hrpL*) mutant that lacks the type III pilus and therefore cannot deliver type III effectors to the plant cell. This relaxation of basal defense responses was RAR1 dependent. Also, when over-expressed, AvrB and RAR1 were coimmunoprecipitated (Shang et al., 2006) and also allow reactivation of a split-luciferase reporter (albeit under conditions where the AvrB myristoylation site is buried in the reporter fusion and mislocalization of AvrB is likely) (Chen et al., 2008). These results led the authors to conclude that RAR1 is a ‘‘virulence target’’

of AvrB, although no direct interaction of AvrB and RAR1 was demonstrated (Shang et al., 2006). Our data demonstrate that AvrB-induced chlorosis is mediated by the TIR-NB-LRR protein TAO1 and that TAO1 functions as a weak R protein (Figs. 4.2 and 4.4). Thus, there is a contradiction: is the observed host cell chlorosis indicative of AvrB virulence targeting of RAR1, or does it represent a TAO1-mediated disease resistance response, which in turn requires RAR1?

There are several possible explanations for the differing interpretations presented in Shang *et al*. and here regarding what AvrB-mediated chlorosis represents. First, the increase in *Pto* DC3000(*hrpL*) growth observed after induced AvrB over-expression *in planta* was COI1 dependent, whereas AvrB-induced chlorosis was COI1 independent (Shang et al., 2006). Therefore, our observation of AvrB-mediated, TAO1-dependent chlorosis can be genetically separated from AvrB-mediated, COI1-dependent suppression of basal defense (Shang et al., 2006). Second, our data show that AvrB-mediated chlorosis requires the host protein PAD4 (Fig 4.5), a positive regulator of both basal defense and TIR-NB-LRR-mediated disease resistance (Glazebrook et al., 1997; Feys et al., 2005). Third, a very strong regimen of conditional over-expression of AvrB, 30 µM DEX spray 2 days before the infection, and also continually throughout the Pto DC3000(*hrpL*) infection, was used in Shang *et al*. Using a regimen of two times 20 µM DEX treatment, but only 1 day before infection, we were able to fully induce AvrB-dependent chlorosis in *TAO1* plants and observed growth restriction of virulent *Pto* DC3000 (Fig. 4.2). These data suggest that the increased growth of *Pto* DC3000(*hrpL*) shown in Shang et al. is caused by extraordinary levels of AvrB

expression *in planta*, resulting in extensive tissue collapse. Our data also demonstrate that TAO1 contributes to the restriction of *P.syringae* growth in both the presence and absence of RPM1 (Fig. 4.2, Fig. 4.4). RAR1 regulates the function of many NB-LRR proteins by acting as a positive regulator of NB-LRR accumulation (Tornero et al., 2002b; Belkhadir et al., 2004; Bieri et al., 2004; Holt III et al., 2005). Hence, the simplest scenario to explain the sum of the data presented here and in Shang *et al*. is that the loss of AvrB-dependent chlorosis in a *rar1* background indicates that TAO1 requires RAR1 for function.

The bacterial type III effector proteins AvrB and AvrRpm1 each trigger RPM1-mediated disease resistance. Yet the activation of TAO1 by AvrB suggests that additional layers of recognition in response to infection can effectively add to the plant's defense response. In a mechanistic sense, if a given type III effector has multiple cellular targets, then direct or indirect recognition by more than one NB-LRR may add to both the flexibility and the amplitude of the host's overall response. Interestingly, there have been many recent examples of the combined action of two NB-LRR proteins in response to a single pathogen isolate (Fig. 4.6, Table 4.1) (Sinapidou et al., 2004; Peart et al., 2005; Ashikawa I, 2008; Lee SK, 2009; Loutre et al., 2009; Narusaka M, 2009). These *NB-LRR* pairs differ for their 1) encoded N-terminal domains, 2) pathogen isolate, and 3) genomic location (Fig. 4.6, Table 4.1.). Some remaining open questions include 1) are any of these NB-LRR pairs present in the same protein complex(es)? and 2) what is molecular mechanism underlying the benefit of two NB-LRR proteins? Our findings further the concept that a CC-NB-LRR and a TIR-NB-LRR, which typically trigger different signaling

pathways in plants (Aarts et al., 1998), can additively contribute to disease resistance in response to a single bacterial effector.

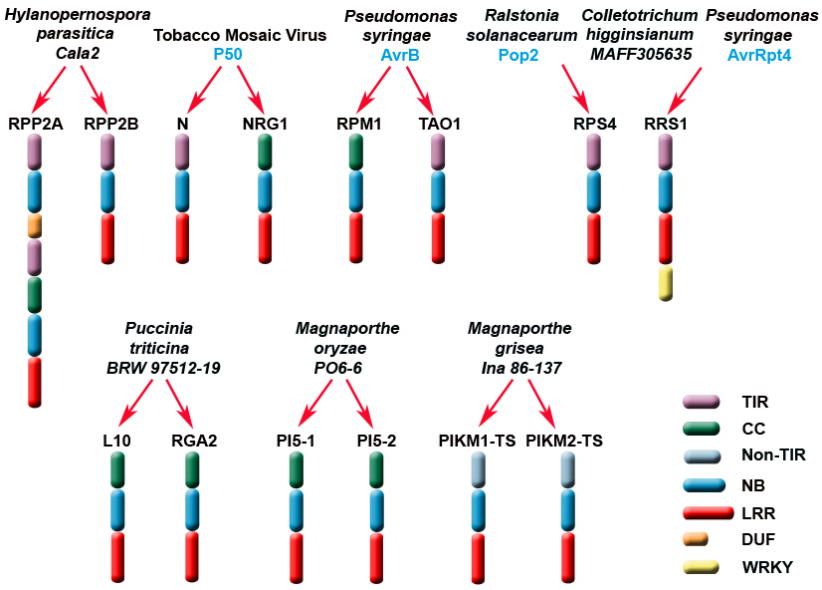

Figure 4.6. Domain Structure and Pathogen Isolates of NB-LRR Pairs. *Top row*: NB-LRR pairs in Arabidopsis and Tobacco. Black lettering represents pathogen isolate. Blue lettering represents *avr* gene product. *Bottom row*: NB-LRR pairs in Wheat and Rice. Black lettering represents pathogen isolate.

***NB-LRR* Pair**	**Plant**	**Pathogen Isolate**	**Linkage**
RPP2A/ *RPP2B*	Arabidopsis	Oomycete	Yes
TAO1/ *RPM1*	Arabidopsis	Bacterial	No
N/ *NRG1*	Tobacco	Viral	NA
RPS4/ *RRS1*	Arabidopsis	Bacterial Fungal	Yes
L10/ *RGA2*	Wheat	Fungal	Yes
Pi5-1/ *Pi5-2*	Rice	Fungal	Yes
Pikm1-TS/ *Pikm2-TS*	Rice	Fungal	Yes

Table 4.1. Characteristics of *NB-LRR* Pairs

Methods

***A. tumefaciens*-Mediated, DEX-Inducible Transient Expression Assays.** Transient transformation assays were performed as described (Nimchuk et al., 2000). Arabidopsis chlorosis response in this assay was observed at 72 h after induction. Protein was extracted 8 h after induction from four transformed leaf discs.

Generation of Stable Transgenic Lines, Mutagenesis, and Screening. Stable transgenic plant lines were generated by the floral dip method (Clough and Bent, 1998). Segregation of hygromycin resistance was used to isolate lines containing single-copy insertions of both the *DEX*:*avrB-H*A and *RPM1-my*c transgenes in Mt-0. For ethane methyl sulfonate (EMS) mutagenesis, ~30,000 seeds were incubated in a 0.25% EMS solution for 8 h, washed in distilled water, and dried on filter paper. Fast neutron mutagenesis was performed by the International Atomic Energy Agency research laboratory in Vienna, Austria. Approximately 700,000 M2 seeds in 100 lots (80 EMS, 20 fast neutron) were sprayed with 20µM DEX as described (McNellis et al., 1998). Putative mutants were isolated and allowed to self-pollinate. M3 progeny were retested by *Agrobacteriu*m transient delivery of *DEX*:*avrB-H*A as described above.

Positional Cloning of *TAO1* and Out-Crossing of the *DEX:avrB-HA* Transgene. Mapping of *TAO*1 was performed as described (Lukowitz et al., 2000). Candidate genes within the *TAO1* interval were PCR-amplified from the *tao1* mutants, and

PCR products were sequenced directly. To obtain *tao1* lines lacking the *DEX*:*avrB-H*A transgene, M3 *tao1* lines were crossed to nontransgenic *TAO1* Mt-0 plants. F2 plants containing the *DEX*:*avrB-H*A transgene were selected and sprayed with 20 µM DEX. Nonchlorotic (*tao1*) lines were selected and allowed to self-pollinate. F3 individuals that lacked the *DEX*:*avrB-H*A transgene were selected and allowed to self-pollinate. F4 seed was confirmed for *tao1* genotype by *Agrobacteriu*m transient transformation of *DEX*:*avrB-HA*. Subsequent confirmation of *tao*1 mutant alleles was performed by using dominant cleaved amplified polymorphic sequences specific to the *tao1* allele in question.

Bacterial Infections. For Fig. 4.2 and 4.3, 4-week-old plants were hand-infiltrated with bacteria at a concentration of 10^5 cfu/ml. For each sample, four leaf disks were pooled per time point (16 total). Leaf disks were ground in 10 mM MgCl2, subject to serial dilutions, and plated on King's Broth (KB) plates with rifampicin selection. For Fig 4.2, 20µM DEX was sprayed twice, and the lines were inoculated with *Pto* DC3000 24 hours after DEX spraying. For Fig. 4.4, 4.5, 4.6, dip inoculations were performed as previously described (Tornero and Dangl, 2001). Briefly, 2-to 3-week-old seedlings were inoculated by dipping in solutions of bacteria at a concentration of 2.5 X 10^7 cfu/ml. Three seedlings were placed in 1 ml of 10 mM MgCl2 with 0.2% Silwet L-77. The weights of the tubes with seedlings were measured, and the tubes were then shaken for 1 h at 28°C. Serial dilutions in 10 mM MgCl2 were made and plated on KB plates with rifampicin selection.

Protein Analysis. For detection of AvrB-HA, PR-1, and RPM1-myc, total protein extracts were prepared by grinding ~200 mg of leaf tissue in buffer [50 mM Tris/HCl

(pH 8.0), 1% SDS, 1 mM EDTA, 1 mM 2-mercaptoethanol, and plant protease inhibitor mixture (Sigma)]. Protein concentrations were determined by using the Bradford-Lowry method and quantification buffer from BioRad. Ten microliters of 6X Laemmli buffer (final concentration 1X) was added to all measured samples after quantification. For immunodetection of AvrB-HA and PR-1, 10-µg protein samples were electrophoresed on 14% SDS polyacrylamide gels. For immunodetection of RPM1-myc, 40-µg protein samples were electrophoresed on 8% SDS polyacrylamide gels.

RT-PCR. RNA was isolated from various plants lines by using TRIZOL Reagent (GIBCO_BRL) according to the manufacturer's instructions. RT-PCR (RETROscript, Ambion) analysis in Figures 4.2 and 4.3 was performed according to the manufacturer's instructions. Plant 18S Competimer Primers (Ambion) were used to co-amplify the 18S internal loading control. For Figure 4.2, primers flanking the first intron of *TAO1* 5' tatgaaatgcagagaagagttgg 3' and 3' ataaccgttctgctggtagag 5' were used with the PCR conditions 94°C 10 s, 55°C 60 s, 72°C 60 s, 31 cycles. A ratio of 9:1 Competimer:18S was used. For Figure 4.3, the RPM1 transcript primers 5' caccatggcttcggctactgttgattttg3' and 3' cactttgcatcgccatcatcaatagg 5' RPM1 transcript primers were used with the PCR conditions 94°C 10 s, 55°C 60 s, 72°C 60 s, 31 cycles. A ratio of 9.5:1 Competimer:18S was used. Products were separated on a 1% agarose gel.

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CHAPTER 5: Conclusions and Further Directions

Abstract

There has been a huge expansion in basic knowledge regarding NB-LRR biology during my graduate matriculation. I feel fortunate to have been able to participate in three diverse areas of NB-LRR regulation and function. Central to this work is the characterization of the CC-NB-LRR protein RPM1. We demonstrated that *RPM1* can be silenced by an epigenetic mechanism and that RPM1 is regulated through nucleotide interactions, homotypic association, and by the guardee protein RIN4. Furthermore, we showed that RPM1 can function additively with another NB-LRR protein in response to a single bacterial effector. My hope is that our work with RPM1 can be used to explore further aspects of NB-LRR biology.

Epigenetic Regulation of *RPM1*

The most disappointing component of my dissertation was the *lra6* project. Initially analysis of the *lra6* mutants was quite promising because the mutants had reduced transcript accumulation for multiple, unlinked *NB-LRR* genes. Although it was unfortunate that the loss of multiple NB-LRR function in the *lra6* mutant was due to silencing, this work should still have value in the context of plant biology and potentially biotechnology. Two gene copies of *RPM1* were chosen for the forward screen used to isolate the *lra6* mutants in order to circumvent the high number of

loss-of-function *RPM1* alleles isolated in the initial loss of AvrRpm1 recognition screen (Tornero et al., 2002a; Hubert et al., 2009). Therefore, characterization of the *lra6* mutants serves as a cautionary tale for future forward screens performed with multiple copies of a target gene. In a broader sense, characterization of the *lra6* mutants may also serve as a cautionary flag concerning off-target effects of transgenes and potentially siRNA-mediated silencing (Auer C, 2009). In combination with other studies (Van Houdt H, 2003; Mourrain P, 2007b), our work further demonstrates that off-target and sometimes even non-corresponding gene sequences to silencing 'triggers' can be repressed.

Further directions of the *lra6* project would be to globally evaluate levels of transcript accumulation and methylation (Zhang X, 2006). These analyses would not only provide a more thorough understanding of the *lra6* alleles, but could also yield information to the possible intermediate silencing targets between *RPM1* and *RPS2* in *lra6-1*. Additionally, the local genomic effects on gene expression and methylation adjacent to the *RPM1* and *RPS2* loci could be determined. Finally, these analyses would yield important information regarding the off-target effects of transgene induced silencing on an entire genome.

RPM1 is Regulated by Nucleotide Binding and Undergoes Homotypic Association

The chapter characterizing RPM1 in the context of nucleotide interactions and homotypic association is probably the most important work in my dissertation. The main reason for this is that the chapter extends characterization of RPM1 to broader STAND protein biology. Our results demonstrate that we can engineer

RPM1 to ectopically signal, and that ectopic signaling of RPM1 can be suppressed by either the loss of nucleotide binding, or by RIN4. In mammals, ectopically active NB-LRR variants are associated with autoimmune disorders including Crohn's Disease and Familial Cold Autoinflammatory Syndrome 1 (Fukata M, 2009). Similar to the RPM1 D505V variant, autoactive variants of the mammalian NB-LRR NALP3 are suppressed *in cis* by mutations in the nucleotide binding residues of the Walker A motif (Duncan et al., 2007). This finding led to speculation that phamacological targeting ATP binding to NALP3 could be an effective strategy to combat NALP3-mediated autoimmune disease (Duncan et al., 2007). Our results demonstrate that an ectopically active variant of RPM1 can be suppressed by the guardee protein RIN4 and hence a mechanistic understanding of this result could be important for the biomedical community. Additionally, these results are significant for the plant defense field because they represent a novel finding regarding the guardee-R protein relationship. Finally, we have taken the initial steps and laid out the path to characterize RPM1 *in vitro* for both nucleotide interactions and regulation by RIN4.

Further directions include experiments both *in planta* and *in vitro*. For the RPM1 homotypic association, future work will address if the loss-of-function mutations, gain-of-function mutations, RIN4, or effector activation alters RPM1 multi-merization. It would be also be interestingly to evaluate if either the RPM1 D287A or RPM1 D505V variants could be suppressed by co-expression with the RPM1 G205E variant. These data could connect the RPM1 nucleotide relationship with RPM1 homotypic association. Additionally, given the likely mechanistic difference between RPM1 D505V and RPM1 D287A, it will be important to address

if RIN4 can also suppress the RPM1 D287A variant. Since the STAND proteins Apaf-1 and CED4 interact differently with nucleotides (Kim HE, 2005; Yan N, 2005), correlating our *in planta* findings with RPM1 biochemical activity *in vitro* is crucial. Additionally, demonstration that RIN4 can biochemically influence the RPM1 nucleotide relationship would be a major step forward in the characterization of the guardee-R protein relationship.

A Pair of NB-LRR Proteins Mediates Defense Responses to the Bacterial Effector AvrB

Our work with TAO1 was the first example that a pair of NB-LRR proteins can perceive a single bacterial effector and additively contribute to defense. Since our publication, there have been four additional examples in which a pair of NB-LRR proteins is required for disease resistance against a pathogen isolate (Ashikawa I, 2008; Lee SK, 2009; Loutre et al., 2009; Narusaka M, 2009). An important characteristic to the TAO1 and RPM1 pair is that TAO1 is a TIR-NB-LRR whereas RPM1 is a CC-NB-LRR. NB-LRR proteins containing either a TIR or CC domain typically have different genetic requirements for function (Aarts et al., 1998), and therefore these sub-classes of NB-LRR proteins may have distinct signaling pathways. In mammalian innate immunity, Toll-like receptors (TLRs) and Nod-like receptors (NLRs) have been shown to respond to the same pathogen elicitor (Akira S, 2006; Miao EA, 2006). TLR signaling and NLR signaling complement each other in that TLR activation induces transcription of defense-associated protein called cytokines, while NLR activation results in the processing and secretion of cytokines (Mariathasan S, 2006; Miao EA, 2006; R., 2010). This is

the basis for the ‘two step’ model of cytokine release (Mariathasan S, 2006; R., 2010). Although there is no established downstream mechanism for the coupling of TIR-NB-LRR and CC-NB-LRR signaling in plants, it is tempting to speculate that such signaling interactions occur.

Many open questions remain for the *TAO1* project. First and perhaps most important, how does TAO1 perceive AvrB? TAO1’s perception of AvrB could be direct or could occur through association with a guardee protein. The guardee RIN4 was found in a yeast-two-hybrid screen for host proteins that interact with both AvrB and a fragment of RPM1 (Mackey et al., 2002). Therefore, a similar approach could find a potential guardee protein(s) between AvrB and TAO1. Another future step will be the generation of a transgenic line that has a natively expressed, epitope-tagged, TAO1 protein. This line could be used to isolate host proteins associated with TAO1 through mass spectrometry analysis. Comparison between the AvrB-RIN4-RPM1 complex and the AvrB-TAO1 or AvrB-guardee-TAO1 complex would be interesting since TAO1 function is so weak in response to AvrB relative to RPM1. Since the amplitude of NB-LRR-mediated defense response correlates with NB-LRR resting state protein accumulation (Holt III et al., 2005), a possible reason why TAO1 function is so weak relative to RPM1 is that TAO1 has a lower level of resting protein accumulation. Transgenic lines natively expressing an epitope-tagged TAO1 protein could be used to address this possibility.

RPM1 as a Model System for Understanding NB-LRR Biology

The primary goal of my dissertation was to provide new basic knowledge in the regulation and function of STAND proteins. Our attempt to characterize RPM1 in

the broadest context possible is to impact studies as diverse as transcription regulation by MalT in *E. coli* to research evaluating how anthrax toxin activates NALP1 in mammalian cells. The genetic tool kit that is Arabidopsis has not only been crucial to my dissertation research but has been significant in finding new general aspects of NB-LRR biology. Examples of this include the isolation of *SGT1* and *HSP90*, which were initially found in forward genetic screens in Arabidopisis and *N. Benthamiana*. Although SGT1 and HSP90 were found to be required for NB-LRR function in plants, these proteins were subsequently shown to regulate numerous mammalian NB-LRR proteins (Tör et al., 2002; Hubert et al., 2003; Liu et al., 2004; da Silva Correia et al., 2007; Mayor et al., 2007). These studies demonstrate the power of plant genetics in finding new players associated with general NB-LRR biology. Despite the strength of genetics, the plant NB-LRR field significantly trails the mammalian field regarding biochemical characterization of NB-LRR proteins. Chapter 3 is a microcosm of this in that the *in planta* findings and speculation regarding RPM1 regulation would be greatly enhanced by biochemical demonstration *in vitro*. Although time-consuming, elucidating the regulation of RPM1 by RIN4 to an atomic resolution would not only impact the plant defense field but would be valuable to the general study of STAND biology.

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