REGULATION OF THE STABILITY OF PLANT DISEASE RESISTANCE PROTEINS

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biology

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ABSTRACT

David Anthony Hubert: Regulation of the Stability of Plant Disease Resistance Proteins
(Under the direction of Jeffrey L. Dangl)

Plants, like animals, can recognize specific pathogens by virtue of a highly-evolved immune system. Recognition of pathogens by plants leads to a well-defined series of outputs, including production of reactive oxygen species, cell wall thickening, and a form of programmed cell death called the Hypersensitive Response (HR). At the forefront of this recognition process are the disease Resistance (R) genes. The proteins encoded by R genes are positive regulators of cell death and thus their accumulation is tightly regulated. Here I present evidence implicating cytosolic HSP90 as a central figure in the maintenance of appropriate R protein levels. Cytosolic HSP90 is encoded by four genes in our model plant system, Arabidopsis thaliana. I present data for genetic interactions between two of these HSP90 paralogs. I also demonstrate a physical interaction between HSP90 and RAR1 and SGT1, two proteins previously identified as playing a role in R protein accumulation. Consistent with the physical interaction, double mutant analyses uncovered a genetic interaction between HSP90 and SGT1. Furthermore mutant forms of HSP90 and RAR1 reveal that changes in their ability to physically interact with each other and with SGT1 can explain the phenotypes of the mutants in planta. I also describe a genetic screen to identify new additional loci required for the
function of the Arabidopsis R gene, RPM1. The phenotype of one mutant identified in this screen suggests that transcriptional regulation of R genes may play a more significant role in maintaining R protein levels than previously expected.
ACKNOWLEDGEMENTS

Many people have contributed their time and energies to help me in the completion of the work presented here. I could not even have begun had it not been for my family. My parents, Tony and Mary Jo, made many sacrifices for my brother, my sisters and I, and for that I will always be deeply grateful. My sister, brother, sister-in-law, and brothers-in-law have always been there anytime I needed advice or just to be cheered up.

My advisor, Jeff Dangl, has provided me with all the resources necessary for successful graduate research. He has created a supportive and generally wonderful lab environment. He has also showed extreme patience with me when my mind has skipped important steps in a thought process.

I’d like to thank Sarah Grant for being an inspiration to me on a daily basis. Your time management abilities are a minor miracle, but your ability to always keep a smile is a major one!

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<td>aa</td>
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<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>APAF1</td>
<td>apoptotic protease activating factor 1</td>
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<td>LRR</td>
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<td>Mbp</td>
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<td>Mi</td>
<td>Meloidogyne incognita tomato resistance</td>
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<td>massively parallel signature sequencing</td>
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<td>non-race specific disease resistance</td>
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<td>suppressor of kinetochore protein mutant</td>
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<td>Toll and Interleukin 1 receptors</td>
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CHAPTER 1

Introduction

Preface

The following chapter was published in Current Opinion in Immunology in February 2003 under the title “Resistance gene signaling in plants- complex similarities to animal innate immunity.” It is included here with several minor changes. I was second author on the paper, contributing ideas to the whole manuscript and much of the text to the section on RAR1 and SGT1. This review was published at the same time as I was working on the data in Chapter 2 and prior to work on the other chapters in this dissertation. As such it provides a good introduction and serves as a good reference point for the contribution of the subsequent chapters to the field of plant disease resistance.

Summary

During the past year, several important publications have significantly enhanced our current understanding of plant disease resistance. Among the most important discoveries are the role of SGT1 in resistance (R) gene mediated defenses, mounting support for the so-called “guard hypothesis” of R gene function, and evidence for intramolecular interactions within R proteins as a mode of signaling control. There are many emerging parallels between the plant R gene and animal
innate immunity receptor complexes. Plant SGT1 shows similarity to co-chaperones of the animal HSP90 complex and many receptor-like \textit{R} gene products appear to indirectly interact with their pathogen-derived signal. Considering these and other similarities, researchers from both fields should be looking carefully over each other’s shoulders.

\textbf{Introduction}

Like animals, plants offer up a bountiful and concentrated supply of resources for would be pathogens. Until recently, research on animal immunity has focused on so-called “acquired immunity.” Acquired immunity is the process by which the circulating immune system of animals somatically generates new resistance specificities against non-self invaders. Underlying this level of resistance in animals is the more evolutionarily ancient innate immune system, which relies on preformed receptors to detect conserved microbial specific patterns and trigger downstream defense responses (Kimbrell and Beutler, 2001). Plants, on the other hand, lack the ability to somatically generate new resistance specificities and rely on meiotically formed pathogen recognition mechanisms. This does not mean that plants have a less active or effective response to pathogens. Triggering of \textit{R} gene pathways gives rise to massive cellular ion influxes, an oxidative burst leading to the accumulation of superoxide and hydrogen peroxide, nitric oxide production, the hypersensitive response (HR; a form of programmed cell death thought to limit pathogen ingress), and the production of toxic antimicrobial metabolites (Dangl and Jones, 2001). We are just beginning to understand the elegant and varied defensive mechanisms
plants utilize to get around their biggest limitations—lack of a circulating immune system and immobility.

In this review, we hope to give the reader a brief look at some of the most interesting recent discoveries concerning R gene mediated defense signaling. Additionally, we provide historical context for these discoveries and comparisons to the animal innate immune response. For those interested in further reading on broader topics in plant disease resistance, we recommend the yearly “Biotic Interactions” special issue in the journal Current Opinions in Plant Biology.

**Resistance Genes - Sentries at the Gate**

For plant pathogens from every kingdom, from viruses to bacteria, fungi and aphids, to utilize host plant resources they must first circumvent preformed defense mechanisms such as the dense epidermal layer and waxy coverings on most leaves. Successful pathogens avoid these early obstacles by stealth (e.g., slipping in through stomatal openings on leaves and stems) and brute force (e.g., fungal and oomycete “penetration pegs” which literally drive a growing spike through the epidermis). Having found their way into the plant apoplast (the space between cells), pathogens must interact directly with individual plant cells to gain access to nutrients necessary for growth and reproduction. This is achieved by the production of virulence factors (also called effector proteins) that are released into the apoplast or injected directly into plant cells. Bacterial plant pathogens use a type III secretion system (like those of Salmonella, Shigella, Yersinia and pathogenic E. coli) to deliver type III effectors directly into the host cell (Nimchuk et al., 2001). Due to the lack of a
circulating immune system, essentially all plant cells must be individually capable of recognizing pathogens and activating an effective defense system.

The sentries at the gate are plant resistance (R) proteins, which either directly or indirectly (see guard hypothesis below) recognize pathogen effectors. In this role, pathogen effectors are called avirulence (avr) proteins. Avr genes are structurally diverse and are theoretically maintained in their respective genomes by virtue of virulence roles advantageous to the pathogen. Evidence for virulence function has been demonstrated for several, but not all, Avr proteins (Nimchuk et al., 2001). Recognition is typically “race specific,” meaning that a given R protein recognizes the Avr proteins from one or very few pathogen isolates. This R–avr genetic interaction initiates what is referred to as gene-for-gene resistance (Flor, 1971).

Five classes of highly polymorphic, but mostly structurally conserved R proteins have been identified and these can be broadly grouped into those with extracellular or intracellular leucine rich-repeat (LRR) regions (Dangl and Jones, 2001). Thus far, the putative intracellular class is the largest. The model plant species *Arabidopsis thaliana* is predicted to encode approximately 150 related R-like genes with central nucleotide binding (NB) domains and C-terminal LRRs. The putative encoded proteins differ structurally in their N-termini where they have either a region of Toll and Interleukin 1 Receptor homology (TIR) or a predicted coiled-coil motif (CC). Loss of function mutations in genes of this NB-LRR class only give loss of disease resistance phenotypes, suggesting that it is a gene family dedicated to R function. In contrast, there is another class of extracellular LRR proteins that are multifunctional (Dangl and Jones, 2001).
Although no plant resistance gene has been crystallized, the three dimensional structure has been solved for LRR containing proteins from numerous other species (Kobe and Kajava, 2001). LRRs are fairly uniform in structure across kingdoms and typically consist of a ligand binding face composed of repeating β strands backed by α helices. The LRR is under diversifying selection in plant R genes (Michelmore and Meyers, 1998) and potentially provides an evolutionarily flexible interface for ligand binding. In mammalian ribonuclease inhibitor, which is essentially one large LRR, the β strands flexibly combine to efficiently bind and inhibit a wide variety of ribonucleases with sequence identities as low as 24% (Kobe and Deisenhofer, 1996). In vitro generated (Thomas et al., 1997) and naturally occurring recombination events (Dodds et al., 2001) between the LRRs of highly related R genes can result in reversals of Avr recognition specificities. These data strongly implicate the LRR as the pathogen recognition specificity determinant, but what do R proteins recognize?

Guard Hypothesis – Gone Fishing

Almost every lab studying R-avr genetic interactions has tried their hand at proving a physical interaction with their favorite R-Avr combination. With the exception of the in vitro interaction between Pi-ta (a CC-NB-LRR R protein) from rice and its corresponding avirulence protein AvrPita (Bryan et al., 2000) from the fungal pathogen Magnaporthe grisea, no direct interaction between a pathogen avirulence product and an NB-LRR type R protein has been demonstrated. Pto, from tomato, encodes a serine/threonine kinase that can phosphorylate a variety of targets, some
with defense functions. It is probably an atypical R gene. Pto requires the NB-LRR type protein Prf to trigger defense responses against *Pseudomonas syringae* bacterial strains expressing *avrPto* (Salmeron et al., 1996). Yeast two-hybrid and genetic data strongly suggest a physical interaction between Pto and AvrPto, but AvrPto does not appear to interact with Prf. The *Prf/Pto-avrPto* genetic and Pto-AvrPto physical interactions may have been the Rosetta Stone for *R-avr* interactions all along.

The “guard hypothesis” suggests that the NB-LRR protein Prf detects and potentially “guards” or monitors, the Pto-AvrPto physical interaction (Dangl and Jones, 2001; van der Biezen and Jones, 1998). If AvrPto functions as a virulence factor targeting the Pto kinase and altering its ability to activate defenses, then Prf might be activated as a consequence of this interaction. So, Prf may act like a fishing pole with Pto as the bait and AvrPto as the trophy catch. Additionally, evidence is mounting that this model could be generalized to other *R-avr* interactions, suggesting that many of us have been fishing without bait for years. The explicit assumptions of the guard hypothesis are: 1) there is specificity in the interaction of R protein/host target (or “guardee”) pairings, and 2) the Avr/guardee interaction in the absence of an R protein is a positive virulence mechanism for the pathogen.

*Arabidopsis* plants with functional RPM1 are resistant to *Pseudomonas syringae* pathogens expressing *avrRpm1*. Despite considerable investigator effort, there is no evidence for a direct RPM1-AvrRpm1 physical interaction. The recently described RIN4 protein, which has no known motifs, interacts in yeast two-hybrid
assays and in vivo with both proteins and is necessary for the RPM1-avrRpm1-mediated HR (Mackey et al., 2002). All three of these proteins localize to the membrane fraction and mutations in AvrRpm1 that inhibit proper localization also strongly reduce the associated HR. RIN4 is not necessary for the function of the closely related RPS2 gene, demonstrating the expected specificity for a putatively guarded host target of AvrRpm1 virulence function. Nevertheless, RIN4 and RPS2 do interact in vivo although they do not interact by yeast two-hybrid analysis. This suggests that RPM1 and RPS2 may be found within conserved protein complexes, although their specific binding to individual components differ (Mackey, Belkhadir, Dangl unpublished). Parallel observations have been made for the RAR1 and SGT1b proteins (see below).

The tomato Cf2 gene conditions resistance to the fungal pathogen Cladosporium fulvum carrying Avr2 and also requires the recently cloned RCR3 gene for its resistance function (Kruger et al., 2002). RCR3 encodes a functional cysteine protease suggesting interesting parallels to the Drosophila Toll receptor. Toll requires a ligand processed by a cysteine protease for downstream signaling (Levashina et al., 1999) and, like Cf2, is required for innate immunity to fungal pathogens. Because RCR3 is not required for other highly related Cf resistance genes, it is also a good candidate for a guarded protein. Nevertheless, no direct physical contacts have been demonstrated for this three-way genetic interaction. Several other candidate host genes for the role of “guardee” exist for various R-avr pairs and have been summarized in a review by Van der Hoorn et al., 2002. To date, none of the guardee candidate proteins has a clear function as the target of a
pathogen virulence factor, though RIN4 does have a deduced function in plant defense (Mackey et al., 2002). Therefore, while evidence mounting in its favor, the jury is still out on the guard hypothesis.

**Resistance Gene Signaling – A Little Self Control**

Once the *R-avr* interaction is triggered, how does signal transduction proceed? Circumstantial evidence has accumulated that R proteins have intramolecular interactions that might affect their ability to signal downstream and bind other molecules. The tomato *Mi* gene, a CC-NB-LRR, is required for resistance to potato aphids and root-knot nematodes (Rossi et al., 1998). N-terminal domain swapping experiments between the functional *Mi-1.1* allele and the non-functional *Mi-1.2* allele generated lethal phenotypes (Hwang et al., 2000). When these same constructs were transiently expressed, pathogen-free cell death phenotypes similar to the HR were observed and the cell death phenotype could be suppressed by co-expression of the N-terminal domain from the parent allele. These data suggest that the N-terminal domain of *Mi* regulates signaling from the LRR leading to cell death. RIN4 and AtTIP49a, a recently described negative regulator of some *R* genes (Holt III et al., 2002), each interact by yeast two-hybrid assay with the N-terminal portion of RPM1. Both of these proteins demonstrate a marked reduction in their interaction with RPM1 when the two hybrid bait includes the NB domain, suggesting that this domain may inhibit some RPM1 interactions. The best, but still incomplete, evidence for the importance of intramolecular interactions as a regulator of R protein function has only just emerged.
Moffett et al., 2002 worked with potato Rx, a CC-NB-LRR protein necessary for recognition of potato virus X (PVX) via its coat protein (CP). They demonstrated that functional Rx could be reconstituted by transiently co-expressing separate portions of the protein. Functional Rx was generated in trans using combinations of both CC-NB and LRR or CC and NB-LRR. Additionally, they utilized immunoprecipitation experiments to show that these molecules physically interact in vivo when transiently co-expressed. Whether they directly interact or are brought together by shared interactions with other proteins is unknown because in vitro interaction experiments were not reported. Importantly, expression of biologically active CP triggered a normal HR response and eliminated the interaction between the separately expressed Rx peptides. Taken together, these data suggests that Rx intramolecular interactions are modified as a result of Avr protein perception, and that this renders Rx competent for further downstream signaling. CP did not immunoprecipitate with Rx, suggesting that 1) Rx is in complex with or, rapidly recruits other proteins following PVX infection (guard hypothesis) or 2) Rx only transiently interacts with CP, which is not detectable by immunoprecipitation.

Interestingly, two recently identified R genes, RPW8.1 and RPW8.2, which confer resistance to numerous powdery mildew isolates, consist of only a CC motif and a single transmembrane domain (Xiao et al., 2001). Because these proteins require EDS1 for their function, it will be interesting to see if they additionally require and interact with an LRR type protein, thus naturally recapitulating the Rx findings.
RAR1 and SGT1b – Complex Arrangements

Most NB-LRR resistance genes have been demonstrated to signal predominately through one of two pathways (Aarts et al., 1998). These two pathways were initially defined by mutations in the *EDS1* (enhanced disease susceptibility) (Parker et al., 1996) and *NDR1* (non-race specific disease resistance) (Century et al., 1997) genes. CC-NB-LRRs signal through NDR1 while TIR-NB-LRRs signal through *EDS1* (Aarts et al., 1998), although there are exceptions (McDowell et al., 2000). When either *NDR1* or *EDS1* are non-functional, *R* gene signaling through these pathways is abolished and the result is complete susceptibility. PAD4, which interacts *in vivo* with EDS1 (Feys et al., 2001), is also required for the function of TIR-NB-LRRs. *EDS1* and *PAD4* encode proteins with homology to catalytic lipases, and may be involved in lipid signaling (Falk et al., 1999; Zhou et al., 1998). The involvement of lipid signals in both animal (Wang et al., 1996) and plant (Rustérucci et al., 1996) cell death has been previously documented. Nevertheless, no catalytic function has been demonstrated for either EDS1 or PAD4. *NDR1* encodes a putative glycosylphosphatidylinositol (GPI)-anchored protein (B. Staskawicz, personal comm.), although nothing is known about its biochemical function.

Adding significantly to our knowledge of *R-avr* signal transduction in R-Avr signaling are numerous recent publications concerning the *RAR1* and *SGT1* genes. *RAR1* was initially identified in barley and acts as a non-redundant convergence point for race specific disease resistance to numerous powdery mildew isolates (Shirasu et al., 1999). The predicted RAR1 protein has two novel 60 amino acid zinc
binding domains (designated CHORD for Cys- and His-rich domain) and a plant-specific C-terminal extension. Animal proteins sharing this CHORD domain all have a C-terminal domain not found in plant RAR1 proteins, a region of so-called SGT1 homology. The SGT1 protein in yeast is a component of the SCF complex, which is an integral component in protein ubiquitylation (Kitigawa et al., 1999).

There are two SGT1 genes in Arabidopsis, designated SGT1a and SGT1b. Two papers have now revealed the importance of the Arabidopsis SGT1b gene in the resistance response mediated by numerous R genes, including both CC-NB-LRR and TIR-NB-LRR pathways (Austin et al., 2002; Tör et al., 2002). Additionally, SGT1 also has a role in non-host resistance, which refers to a presumably non-specific class of resistance where a plant species is resistant to all known isolates of a given pathogen. For example, *Nicotiana benthamiana* plants silenced for SGT1 become susceptible to bacterial pathogens normally pathogenic to members of the Brassicaceae (Peart et al., 2002). These data strongly suggest that SGT1, like RAR1, serves as a convergence point for numerous defense related pathways. Not all non-host resistance is compromised in SGT1 silenced *N. benthamiana* plants and there are Arabidopsis R genes that do not require SGT1b (Austin et al., 2002). In some cases these R genes do require RAR1. For other R genes, both (RPP5) or neither (RPP1A) gene are required. These results are presently puzzling, especially considering that RAR1 and SGT1b interact *in vivo* in Arabidopsis. One possible explanation for these observations is that SGT1a and b act *together* as a convergence point for these pathways. This is unlikely as sgt1a loss-of-function mutants do not alter the response of the SGT1b-independent R genes (K. Shirasu,
personal comm.). Unfortunately, an sgt1a/sgt1b double mutant is lethal (K. Shirasu, personal comm.).

SGT1b also interacts \textit{in vivo} with two E3 ubiquitin ligase subunits, SKP1 and CUL1 (Azevedo et al., 2002; Liu et al., 2002). SCF complexes have E3 ligase activity and define substrate specificity for ubiquitylation (Gray and Estelle, 2000). These interactions prompted Azevedo et al. (Azevedo et al., 2002) to test SGT1b for interaction with CSN4 and CSN5, two components of the COP9 signalosome. The COP9 signalosome resembles the lid portion of the 19S regulatory subunit of the 26S proteasome and was originally identified for its role in photomorphogenesis (Schwechheimer and Deng, 2001). CSN4 and CSN5 interacted with SGT1b and, although RAR1 additionally interacted with these components, RAR1 was not required for the SGT1-CSN4/5 interactions. Furthermore, silencing of CSN3 and CSN8, two additional components of the COP9 signalosome, inhibits \textit{N} gene-mediated resistance to tobacco mosaic virus in \textit{N. benthamiana} (Liu et al., 2002).

By way of analogy, auxin, a phytohormone involved in multiple developmental processes, induces the expression of the \textit{AUX/IAA} genes (Abel et al., 1994). AUX proteins can negatively regulate their own expression and auxin relieves this negative regulation by inducing the degradation of AUX/IAA repressor proteins at the COP9 signalosome (Schwechheimer and Deng, 2001). It is not yet clear if the COP9 signalosome might mechanistically regulate defense responses in a similar fashion. There is evidence that at least one R protein, RPM1, is degraded following elicitor perception just prior to onset of the HR (Boyes et al., 1998). Intriguingly, RPM1 does not accumulate in an Arabidopsis \textit{rar1} mutant (Tornero et al., 2002), suggesting that
RAR1 is required for either RPM1 stability or accumulation. Presently, no data concerning the degradation of other resistance proteins is available and potential targets of regulation by the COP9 signalosome are unknown. There is also rapidly growing evidence that ubiquitylation controls much more than protein turnover. For example, ubiquitylation of VP16 in yeast simultaneously activates its transcriptional activities and drives its future degradation (Salghetti et al., 2001).

Are there functional similarities between these R gene signaling molecules and the animal innate immune response? Actually, there are quite a few. For example, bacterial lipopolisaccharide (LPS) is a potent activator of animal innate immune responses. LPS associates with the LPS binding protein and CD14 in the plasma membrane (Wright et al., 1990). CD14 is, perhaps like NDR1, a GPI-linked protein. CD14 is associated with triton insoluble, heterogeneous regions of the plasma membrane called lipid rafts and is not competent to transduce the LPS signal on its own (Triantafilou et al., 2002; Triantafilou and Triantafilou, 2002). Numerous other signaling proteins are also constitutively localized with CD14 or rapidly recruited into the CD14 lipid raft, including Hsp70, Hsp90, and the Toll-like receptor TLR4 (Byrd et al., 1999; Jiang et al., 2000). Co-localization of these proteins in lipid rafts appears necessary for full cellular LPS stimulation (Triantafilou et al., 2002). TLR4 is a Toll-like receptor with homology to the N-terminal regions of plant TIR-NB-LRRs and it will be interesting to see whether other partners in the CD14 signalosome function in R-dependent responses in plants.

As with TLR4, steroid hormone receptors, such as the progesterone receptor, form mature complexes with HSP70 and HSP90 and these complexes require the
proteins HOP and p23 (Hernandez et al., 2002; Lange et al., 2000). Human and Arabidopsis SGT1, like HOP and p23, have tetratricopeptide repeats (TPR), a degenerate 34 amino acid sequence involved in protein-protein interactions (Richter and Buchner, 2001). Strengthening the links tying these numerous proteins together, Arabidopsis HSP90 physically interacts with human p23 via the TPR (Owens-Grillo et al., 1996). Excitingly, HSP90 and SGT1-like proteins were recently isolated in a screen for RAR1 interactors from N. benthamiana (Liu et al., 2002). Potentially coming full circle to the SGT1/COP9 interaction, the mature progesterone receptor in complex with HSP90 and p23 binds ligand and is subsequently phosphorylated, ubiquitinated and degraded by the 26S proteasome (Lange et al., 2000).

**Conclusion**

Although some of these comparisons to the mammalian innate immune response are presently circumstantial, the inclusion of SGT1 and RAR1 in the stable of R gene-mediated signaling components is exciting and full of potential. The breadth and quality of biochemical data in the animal literature should supply the plant disease resistance field with an abundance of launching points for future experiments. Due to the many unique advantages of Arabidopsis as a genetic model, such as rapidly progressing efforts to identify loss of function (T-DNA) alleles for every gene and the relative ease of working with whole organisms, the animal innate and acquired immune response community should also keep a watchful eye on molecular plant pathologists.
Bibliography


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CHAPTER 2

Cytosolic HSP90 associates with and modulates the Arabidopsis RPM1 disease resistance protein

Preface

The following chapter was published, as titled with several minor changes, in The EMBO Journal (2003, 21, 5679-5689). I share co-first authorship with Pablo Tornero and Youssef Belkhadir. For this paper I contributed the data presented in Figures 2.2, 2.4-2.8A, and 2.10, and corroborated independently the results presented in Figures 2.1 and 2.3. Additionally, I created all the figures and wrote the manuscript with input from all authors.

Abstract

The Arabidopsis protein RPM1 activates disease resistance in response to Pseudomonas syringae proteins targeted to the inside of the host cell via the bacterial type III delivery system. We demonstrate that specific mutations in the ATP-binding domain of a single Arabidopsis cytosolic HSP90 isoform compromise RPM1 function. These mutations do not affect the function of related disease resistance proteins. RPM1 associates with HSP90 in plant cells The Arabidopsis proteins RAR1 and SGT1 are required for the action of many R proteins, and display some structural similarity to HSP90 co-chaperones. Each
associates with HSP90 in plant cells. Our data suggest that 1) RPM1 is an HSP90 client protein and that 2) RAR1 and SGT1 may function independently as HSP90 co-factors. Dynamic interactions among these proteins can regulate RPM1 stability and function, perhaps similar to the formation and regulation of animal steroid receptor complexes.

**Introduction**

Our understanding of disease resistance specificity in plants centers on the structure and function of pathogen-specific Resistance (R) gene products. R proteins confer resistance to pathogen strains expressing a molecule that specifically triggers its action (Dangl and Jones, 2001). The largest class of R protein contains a nucleotide binding site (NB) and leucine-rich repeats (LRR), and are termed NB-LRR (Dangl and Jones, 2001). The repertoire of R proteins, though deployed with broad population polymorphism, may still not be sufficiently diverse to mediate direct recognition of all relevant pathogens. The question of repertoire size, among others, drove the formulation of the “guard hypothesis.” Here, pathogen molecules that trigger R action are most easily thought of as virulence factors whose presence is sensed by the host cell. Experimental evidence supports this model, though it is not yet fully generalizable (Dangl and Jones, 2001; Holt et al., 2003; Shirasu and Schulze-Lefert, 2003; Van der Hoorn et al., 2002). NB-LRR activation may include a large conformational change, perhaps akin to the "jackknife model" leading to proximity induced activation as proposed for Apaf-1 (Hwang and Williamson, 2003; Moffett et al., 2002). This, in turn, leads to a series of cellular events that collectively
form the defense response (Hammond-Kosack and Parker, 2003). It is unclear what portion of this diverse defense response is actually required to halt pathogen growth.

Arabidopsis RPM1 is an NB-LRR protein that confers recognition to bacterial strains expressing either of two divergent type III effector genes, *avrRpm1* or *avrB* (Grant et al., 1995). These two type III effector proteins have virulence function on hosts lacking *RPM1* (*rpm1*; disease susceptible; Ashfield et al., 1995; Ritter and Dangl, 1995). Recognition of AvrRpm1 or AvrB by RPM1 may be the consequence of their action on the Arabidopsis RIN4 protein. RIN4 is a protein of unknown function and is phosphorylated in response to the presence of either AvrRpm1 or AvrB (Mackey et al., 2002). RIN4 can interact with RPM1, AvrRpm1, and AvrB in vivo. All four of these proteins localize to the plasma membrane (Boyes et al., 1998; Mackey et al., 2002; Nimchuk et al., 2000).

A limited set of genetically defined proteins are broadly required for the action of Arabidopsis *R* genes subsets (Hammond-Kosack and Parker, 2003). The *ndr1* mutation compromises the function of a subset of NB-LRR R proteins (Century et al., 1997). NDR1 is a putative GPI-anchored protein (B. Staskawicz, pers. comm.). Mutations in *RAR1* and *SGT1b*, one of two *SGT1* orthologues in Arabidopsis, compromise the function of many R proteins (Azevedo et al., 2002; Muskett et al., 2002; Tör et al., 2002; Tornero et al., 2002b). Plant SGT1 proteins share similarity with the yeast SGT1 protein, a regulator of the SCF ubiquitin ligase complexes in a variety of cellular processes (Kitigawa et al., 1999). RAR1 and SGT1 can interact in vivo, and presumably function together (Azevedo et al., 2002).

*RPM1* function is compromised by *ndr1* and by *rar1* but, surprisingly, is not
compromised by either sgt1a or sgt1b. Because an sgt1a / sgt1b double mutant is lethal (Takahashi et al., 2003), overlapping contributions of these genes to RPM1 function cannot be determined. We performed a large-scale screen for loss of RPM1-mediated hypersensitive cell death (HR) in response to conditional expression of an avrRpm1 transgene (Tornero et al., 2002a). We describe here four mutant alleles of the Arabidopsis HSP90.2 gene that caused loss of RPM1-specified HR and disease resistance in that screen. This represents the first phenotype attributed to mutation of a plant cytosolic HSP90.

We demonstrate that RPM1 is the first client protein described for plant cytosolic HSP90, using as criteria 1) in vivo HSP90-RPM1 interaction, 2) modulation of RPM1 function by particular mutant alleles of the HSP90.2 isoform, and 3) greatly decreased steady state RPM1 levels in these hsp90.2 mutant backgrounds. We describe genetic interactions between HSP90.2 and both RPM1 and NDR1. We provide evidence for in vivo interactions between HSP90s and both RAR1 and SGT1. Thus, we describe a possible mechanism by which RAR1 and SGT1 affect disease resistance protein signaling through probable co-factor interactions with cytosolic HSP90s.

Results

Ira2 mutations specifically affect RPM1-mediated pathogen recognition

We screened ~500,000 EMS mutagenized Arabidopsis M2 individuals for mutants affecting recognition of avrRpm1. Among others, we identified four allelic mutations that we called Ira2-1, Ira2-2, Ira2-3, and Ira2-4 (Ira2; loss of recognition of
avrRpm1; Tornero et al., 2002a). Two of these, independently isolated, were later found to carry the same mutation.

Both the Ira2-2 and Ira2-3 alleles have an intermediate effect on RPM1 function (as did the other two alleles, data not shown) measured by pathogen growth and disease symptoms following challenge with Pseudomonas syringae pv. tomato (Pto) strain DC3000 expressing either avrRpm1 or avrB (Figure 2.1, and data not shown). We did not see a significant effect on other R genes active against different type III effectors from P. syringae (Figure 2.1). RPM1-mediated HR was also altered, but not completely abolished, in the Ira2 mutants in response to infiltration with Pto DC3000 (avrRpm1). This is similar to the effect of rar1 on RPM1-mediated HR (Tornero et al., 2002b). While normal RPM1-mediated HR occurs 5 to 8 hr after inoculation, we observed a low frequency of HR on leaves from all Ira2 alleles by 20 hours (data not shown). The effect of Ira2 mutations on the HR was also specific to RPM1.

Basal resistance against virulent pathogens (Pto DC3000) was not significantly affected (Figure 2.1). We also observed no alteration in the responses of Ira2 alleles to infection with a series of Peronospora parasitica isolates (Holub et al., 1994). This included isolates that were either specifically recognized by various R genes in the Ira2 parental background (Calal2 (recognized by RPP1a), Emoy2 (RPP4), Emwa1 (RPP4), and Hiks1 (RPP7)) or caused downy mildew disease (Emco5 and Noco2) (data not shown).

We observed that Ira2-2 and Ira2-4 displayed a partial penetrance phenotype
Figure 2.1: Mutations in *lra2* specifically affect *RPM1* signaling. Growth of *Pto* DC3000 containing the indicated avirulence genes in *lra2* mutants and corresponding controls (used throughout). The a11 line is the Col-0 parent containing the estradiol-inducible *avrRpm1* transgene (Tornero et al., 2002a). The a11;*rpm1-1* line is the a11 transgene crossed into an isogenic *rpm1-1* background. Bacterial numbers here and in Figures 2.2 and 2.3 are expressed as the log10 of colony forming units (cfu) per milligram fresh weight (f.w.) (Tornero and Dangl, 2001). Error bars indicate ±SE. Growth of *Pto* DC3000(vector) did not show a significant difference in growth in *lra2* mutants. *Pto* DC3000(*avrRpm1*) and *Pto* DC3000(*avrB*) exhibited consistent increased growth in *lra2* mutants intermediate to growth observed in *rpm1* mutants in four independent experiments.
for both HR and onset of disease symptoms. After inoculation with low doses of *Pto* DC3000(*avrRpm1*), *lra2*-2 and *lra2*-4 plants either developed symptoms characteristic of disease (in 0-80% of plants), or were completely asymptomatic (data not shown). Progeny from self-fertilization of either symptomatic or asymptomatic individuals displayed similar variable penetrance in the next generation (data not shown). The partially penetrant disease phenotype did influence the standard deviation in our bacterial growth assays (Figure 2.1).

**LRA2 and RPM1 interact genetically**

We made test-cross F1s between *rpm1* and all *lra2* alleles and assayed two phenotypes for allelism: the HR resulting from estradiol-induction of the *avrRpm1* transgene contained in these lines (Tornero et al., 2002a) and bacterial symptoms resulting from *Pto* DC3000(*avrRpm1*) infection. Both of these assays initially suggested that *lra2* was allelic to *rpm1* in the F1 generation. However, F2 progenies from these F1s contained a large percentage of phenotypically wild type individuals. This is inconsistent with allelism, and is consistent with non-allelic non-complementation. In this condition, F1 individuals of a cross between two unlinked recessive mutants (each mutation thus heterozygous) display a phenotype similar to either homozygous single mutant. This often indicates that the two genes act together, that the protein products often physically interact or are part of the same protein complex, or that half the wild type dose of the two, in combination, is insufficient for wild type function (e.g. Belanger et al., 1994; Larkin et al., 1999).

We assayed HR response and bacterial growth in the *lra2* alleles and in
various F1 progeny (Figure 2.2) to address the apparent genetic interaction between LRA2 and RPM1. The wild type parental plant line, containing the estradiol-inducible avrRpm1 transgene (called a11; Tornero et al., 2002a) responded to estradiol with a strong HR (Figure 2.2A). The isogenic a11;rpm1-1 control (Tornero et al., 2002a), and the tested lra2 alleles exhibited no HR. The obvious induction of HR in F1 progeny of backcrosses to a11 demonstrated that both rpm1 and the tested lra2 alleles were recessive in this assay. Strikingly, F1 progeny of (a11;rpm1-1 x lra2-2, or x lra2-3) did not respond to estradiol (Figure 2.2A). Furthermore, lra2-2 and lra2-3 partially compromised RPM1 inhibition of bacterial growth, and were fully recessive for this phenotype when assayed as F1s backcrossed to the a11 parental line (Figure 2.2B). The F1 progeny of (a11;rpm1-1 x lra2-x) exhibited modest, but reproducible, reduction of RPM1 function. These plants allowed bacterial growth between that of the lra2 parent and the (a11 x a11;rpm1-1) F1 control. This genetic interaction is specific to lra2 and rpm1, as we did not observe non-allelic non-complementation in other trans-heterozygous combinations of mutants affecting the RPM1 pathway tested (tested were rar1 x rpm1, ndr1 x rpm1, rar1 x ndr1, rar1 x lra2 and ndr1 x lra2 (data not shown)). The data in Figure 2.2 strongly support the conclusion that rpm1 and lra2 exhibit non-allelic non-complementation. Our data thus suggest that the respective wild type RPM1 and LRA2 proteins work in the same pathway, and potentially interact physically.

**ndr1 and lra2 display both synergistic and epistatic interactions**

Like lra2, ndr1 supported an intermediate level of bacterial growth when
Figure 2.2: LRA2 and RPM1 interact genetically. (A) Trypan blue staining of an HR assay following estradiol-induction of avrRpm1 expression. Two week old plants were treated with 10µM ED with 0.02% Silwet, and stained with trypan blue 2 days later. Row one displays parental responses to conditional expression of avrRpm1. Row two displays that the lra2 and rpm1 mutants are recessive. Row 3 shows that rpm1;lra2 trans-heterozygotes do not express HR. Three independent repetitions were performed. (B) Growth of Pto DC3000(avrRpm1) in same genotypes as (A). Error bars indicate ±SE of triplicates from this experiment. The experiment was performed six times with similar results.
challenged with *Pto* DC3000(*avrRpm1*) (Figure 2.3). Additionally, the *RPM1*-mediated HR is severely attenuated in *lra2* (see above), but not compromised in *ndr1* (Century et al., 1995; Tornero et al., 2002b). These incomplete phenotypic effects allowed us to assay for genetic interaction between *lra2* and *ndr1*. We constructed *lra2*;*ndr1* double mutants and tested them for bacterial growth and HR. The *lra2*;*ndr1* double mutants were completely compromised for *RPM1* function, allowing as much pathogen growth as an *rpm1* mutant following application of *Pto* DC3000(*avrRpm1*) (Figure 2.3; note log scale). The *lra2*;*ndr1* double mutant also displayed full loss of *Pto* DC3000(*avrRpm1*) triggered HR, suggesting that *LRA2* is required for the *RPM1*-dependent HR remaining in *ndr1* mutants. Note that while the *ndr1* allele is a null (Century et al., 1997), the *lra2* mutants presented here are not (see below).

**LRA2 is HSP90.2**

We cloned *LRA2* based on its map position. Our mapping population of disease susceptible (*lra2*;*lra2*) individuals narrowed the *LRA2* interval to a ~52 kb region on the bottom arm of chromosome V (see Materials and Methods and Figure 2.4). We sequenced candidate genes from this interval in the *lra2*-1 mutant and found a G/A transition at position 21937 (nucleotide positions relative to the published sequence of P1 clone MDA7, Genbank Accession: AB011476). This created a G95E mutation in the cytosolic *HSP90.2* (At5g56030; Figure 2.5A). We sequenced this gene from the other three independently isolated *lra2* alleles and found mutations in *lra2*-2 (C21952T; S100F, independently isolated in
Figure 2.3: *Ira2* and *ndr1* affect *RPM1* function synergistically. Growth of *Pto DC3000 (avrRpm1)* in *Ira2;ndr1* double mutants with corresponding controls, as in Figures 2.1 and 2.2. Both *Ira2* and *ndr1* single mutants exhibit intermediate growth, while the double mutant exhibits complete susceptibility.
Figure 2.4: **LRA2 is HSP90.2.** Map based cloning of *Ira2* mutants began with genetic definition of a 52 kB interval on the lower arm of Chromosome V using the markers shown on the left and right. This region was contained on the P1 clone MDA7 (Kaneko et al., 1998). Candidate clones, including all three of the constitutively expressed cytosolic HSP90s in Arabidopsis, are given in green with the arrow pointing in the direction of transcription.
*lra2-4*); and in *lra2-3* (G21785A; D80N) (Figure 2.5A). To avoid confusion and to follow accepted nomenclature conventions, we re-designate the *lra2* alleles *hsp90.2-1*, *hsp90.2-2*, and *hsp90.2-3* for *lra2-1*, *lra2-2*, and *lra2-3*, respectively.

All mutations were within the conserved ATPase domain of this HSP90 (Figure 2.5A). The *hsp90.2-3* change (D80N; yellow in Figure 2.5B) alters a residue previously shown to make multiple ATP contacts in the crystal structure of yeast HSP90 (Prodromou et al., 1997). The *hsp90.2-1* (G95E) and *hsp90.2-2* (S100F) changes are both adjacent to residues that make direct contact with ATP (N93 and R99). The G95E change should alter the local charge density while the S100F change results in addition of a large hydrophobic side chain. We used molecular markers based on the mutation to outcross *hsp90.2-3* from the conditional *avrRpm1* expression transgenes (Materials and Methods). This line expressed the same significant reduction of RPM1 function as its parent line, measured by both disease symptoms (Figure 2.6A, compare to Figure 2.1) and pathogen growth (Figure 2.6B). Thus, the transgenes carrying the estradiol inducible *avrRpm1* system have no effect on the mutant phenotype. Finally, an insertion allele, *hsp90.2-5* (a T-DNA insertion at approximately nt23453, aa position 601 of 699, SIGNAL line SALK_058553; Figure 2.4) was viable and exhibited no alteration of *RPM1*-mediated resistance (Figure 2.6B). We did not detect a truncated form by Western blot (data not shown), and assume that this allele is a null. We infer that one of the other three highly homologous cytosolic HSP90s compensates for the loss of HSP90.2 (Borkovich et al., 1989). *HSP90.2* is constitutively expressed, especially in flower structures and roots (Yabe et al., 1994). We observed only very modest pleiotropic
Figure 2.5: Clustering of *hsp90.2* mutations in the highly conserved N-terminal ATPase domain. (A) Alignment of the N-terminal ATPase domains of HSP90 orthologues across diverse kingdoms, highlighting the residues mutated in our *hsp90.2* alleles in red. Proteins compared: *At*HSP90.2 (swissprot id: HS82_ARATH), *Sc*HSP82 (swissprot id: HS82_YEAST), *Dm*HSP83 (swissprot id: HS83_DROME), *Hs*HSP90a (swissprot id: HS9A_HUMAN), *Ec*HptG (swissprot id: HTPG_ECOLI). (B) Hsp90.2 threaded over the structure of yeast HSP90 bound to ADP (center space filling structure) viewed from two different angles. Residues that make direct interactions with ADP are in violet. The three residues mutated in our *hsp90.2* alleles are in yellow. Note that D80 makes direct contacts with ADP, and is mutated in *hsp90.2*-3.
Figure 2.6: RPM1 function is not compromised by an hsp90.2 insertion allele and the hsp90.2-3 phenotype is independent of the conditional avrRpm1 expression system. (A) Bacterial growth assay with Pto DC3000(avnRpm1). The hsp90.2-5 (Salk 058553) T-DNA insertion allele displays a wild type response to Pto DC3000(avnRpm1). By contrast, the hsp90.2-3 allele outcrossed from the transgenic, conditional avrRpm1 expression system is still compromised for RPM1 function. (B) This line, and controls, were spray infected with Pto DC3000(avnRpm1), and still exhibits symptoms intermediate to a full loss of function rpm1 mutant.
developmental changes in the hsp90.2 mutants (Figure 2.7; Queitsch et al., 2002). Thus, the hsp90.2 alleles identified in our screen are rare, specific, and not compensated for by other HSP90 isoforms.

**HSP90 associates with RPM1 in vivo**

An RPM1-myc epitope-tagged protein is a peripheral plasma membrane protein (Boyes et al., 1998), and fails to accumulate in rar1 plants (Tornero et al., 2002b). We crossed an RPM1-myc transgene (Boyes et al., 1998) into both the fully penetrant hsp90.2-3 allele and the partially penetrant hsp90.2-2 allele. Both alleles exhibited greatly decreased RPM1-myc levels compared to wild type plants (Figure 2.8A). Curiously, hsp90.2-2 consistently accumulated less RPM1-myc than hsp90.2-3. This suggests that the partial penetrance phenotype of hsp90.2-2 is not simply correlated to RPM1 levels.

Given the genetic interaction between rpm1 and hsp90.2, we asked whether HSP90s could co-immunoprecipitate (co-IP) with RPM1-myc. We probed protein blots of anti-myc IPs with an antibody raised against cytosolic HSP90 from Pharbitis nil, which should detect all four isoforms of cytosolic HSP90 in Arabidopsis (Krishna et al., 1997). We successfully detected HSP90 in IPs from RPM1-myc plant extracts, but not in IPs from rpm1 mutant extracts (Figure 2.8B). The HSP90 antibody failed to detect a clear difference in protein levels between our point mutants or insertion allele and wild type (data not shown). We therefore cannot ascertain whether the HSP90 detected in our co-IPs is, or contains, HSP90.2. We were unable to detect HSP90 in anti-RIN4 co-IPs, but could co-IP RPM1-myc with anti-RIN4 from the
Figure 2.7: Phenotypic pleiotropy in hsp90.2 mutants suggests functions in additional Arabidopsis processes. While overall morphology is unaffected (row 1), hsp90.2 mutants show slight flattening of the leaves (adaxial view row 2 and abaxial view row 3), and young flower buds are not completely closed (row 4). Leaves often appeared somewhat flattened in hsp90.2. We often found that all the buds in a flower cluster were slightly opened prematurely. We did not observe a difference in gross morphological architecture of the roots. These phenotypes were weak and the bud phenotype was variably penetrant in all hsp90.2 alleles, in contrast to the disease resistance phenotype. We recapitulated heat shock conditions known to cause loss of R function in other systems, notably the tobacco N gene, but failed to see an effect on RPM1 function in wild type plants at 30°C. These same conditions also revealed no pronounced phenotypic effects in hsp90.2 plants.
Figure 2.8: HSP90 Interacts with RPM1. (A) *hsp90.2* mutations severely affect RPM1-myc accumulation. Forty (40) μg total protein were loaded and western blots were probed with anti-myc monoclonal antibody. (B) Top: anti-myc and co-IPs demonstrate that Hsp90 associates with RPM1-myc in planta. The relative amounts of protein from the immune pellet and the total extracts are not equivalent. The pellet is over represented by 20-fold. This experiment is representative of six independent replicates for RPM1-myc. Bottom: Control showing specificity of IP reagents, and that RPM1-myc is extracted from transgenic lines in appropriate mutant backgrounds under the conditions used for the co-IP.
same extracts (data not shown). These data suggest that RIN4 and HSP90 interactions with RPM1 might be mutually exclusive.

**Disease signaling components RAR1 and SGT1 also associate with HSP90 in planta**

RAR1 and SGT1 can be co-immunoprecipitated from plant cell extracts (Azevedo et al., 2002). As well, there are structural similarities between plant SGT1 and animal proteins required for HSP90 assembly and function (Dubacq et al., 2002; Garcia-Ranea et al., 2002; see Discussion). These observations prompted us to explore the physical relationships between RAR1, SGT1, and HSP90.

We used antibodies raised against either RAR1 or SGT1 (Azevedo et al., 2002) to IP HSP90 from total plant extracts. We found that anti-RAR1 is able to co-IP HSP90 from wild type extracts, but not from *rar1-20* extracts (Figure 2.9A). Arabidopsis contains two *SGT1* genes encoding proteins of slightly different mobility (Austin et al., 2002; Tör et al., 2002); both are detected by our antisera under the IP conditions used. We found that anti-SGT1 antibody is consistently able to co-IP HSP90 from wild type Col-0 and Ws-0 extracts (6/6 experiments; Figure 2.9B). However, we detected no, or only very low amounts, of HSP90 in anti-SGT1 IPs from *sgt1b* extracts in multiple experiments (Figure 2.9B). This suggests that the majority of HSP90 we detected is associated with SGT1b, and that SGT1a may weakly associate with HSP90. We invariably detected HSP90 in IPs from *sgt1a* extracts (Figure 2.9B), strengthening this conclusion. Thus, in our experimental conditions, there is a preference for HSP90 to associate with SGT1b compared to SGT1a,
Figure 2.9: HSP90 associates with RAR1 and SGT1. (A) Hsp90 associates with RAR1 in planta (top). The relative amounts of protein from the co-IP pellet and the total extracts are not equivalent. The pellet is over represented by 10-fold. This experiment is representative of two independent replicates. An extraction control is displayed below the co-IP. (B) HSP90 displays an apparent preference for SGT1b over SGT1a. The relative amounts of protein from the co-IP pellet and the total extracts are not equivalent. The pellet is over represented by 10-fold. This experiment is representative of 3/7 independent replicates for sgt1b and 4/4 independent replicates for sgt1a. Extraction controls beneath the co-IPs demonstrate the differential mobilities of SGT1a and SGT1b and the effect of the respective mutations.
consistent with yeast two hybrid data (Takahashi et al., 2003).

Additionally, we detected HSP90 in anti-SGT1 IPs from \textit{rar1} mutant extracts and in anti-RAR1 co-IPs from \textit{sgt1b} mutant extracts (Figure 2.9C). This suggests that RAR1 and SGT1b can independently associate with HSP90. We corroborated this finding in the converse experiment (IP with anti-RAR1 and blot with anti-HSP90) using the null \textit{rar1-20} allele (data not shown). We were, surprisingly, able to detect consistently HSP90 from anti-RAR1 IPs in \textit{rar1-21} mutant extracts (Figure 2.9C). The \textit{rar1-21} mutation introduces a stop codon near the end of the CHORD I domain (Tornero et al., 2002b), and consequently lacks the CHORD II domain, known to be the RAR1-SGT1 interaction platform (Azevedo et al., 2002). While we were unable to detect this small RAR1 fragment by direct immunoblot, it appears sufficient to IP HSP90, consistent with two hybrid interaction data presented elsewhere (Takahashi et al., 2003).

Artifactual detection of HSP90 in immunoblots is a concern, because it can represent up to 1-2% of total cellular protein (Lai et al., 1984). We therefore used several different antisera to test this and found that none of them could co-IP HSP90 (see Materials and Methods), thus suggesting strongly that our data represent specific interactions.

\textbf{Discussion}

We provide genetic and biochemical data demonstrating that RPM1 is a cytosolic HSP90 client. We demonstrate that mutations in the \textit{Arabidopsis HSP90.2} can specifically modulate RPM1 function. RPM1 accumulation is greatly diminished
in specific *hsp90.2* missense mutants. We provide pairwise co-IP data demonstrating interactions between HSP90s and RPM1, RAR1 and SGT1. We show that the HSP90 interaction with RAR1 does not require SGT1, nor does the HSP90 interaction with SGT1 require RAR1. Surprisingly, we did not find an association of HSP90s with RIN4 (*RPM1 Interacting Protein 4*), a protein clearly implicated in RPM1 and RPS2 function (Axtell and Staskawicz, 2003; Mackey et al., 2003), suggesting that RIN4 and HSP90 association with RPM1-myc might be mutually exclusive.

Our data suggest that RAR1, SGT1 and HSP90 may work together to coordinate RPM1 function. The key questions emerging from our work are: why do specific *hsp90.2* missense mutations result in diminution of RPM1 levels, and hence RPM1 function? Is the requirement for HSP90 in RPM1 signaling indicative of a common regulatory mechanism among the NB-LRR class of R proteins? And, do RAR1 and SGT1 act as co-factors for HSP90 in R protein signaling?

**Genetic interactions support a quantitative function for *HSP90.2* in R-mediated disease resistance**

Our four *hsp90.2* missense alleles are recessive and significantly reduce, but do not eliminate, *RPM1* function. They do not reproducibly affect any of the seven other *R* functions tested. The *hsp90.2* alleles exhibit non-allelic non-complementation with *rpm1*, suggesting that the two wild type proteins act together. The fact that far less steady state RPM1-myc protein accumulates in two *hsp90.2* alleles provides a simple, dosage-based mechanistic explanation for this genetic
Our results suggest that specific HSP90.2 missense mutations alter RPM1 function. The four hsp90.2 alleles reported here compare to 95 rpm1 alleles also identified in this screen (Tornero et al., 2002a). RPM1 is 926 amino acids long, HSP90.2 is 699; thus target size cannot explain this mutant ratio. Furthermore, the insertional hsp90.2-5 allele is viable, apparently null, and exhibited full RPM1 function. We assume that one of the other three cytosolic HSP90s can compensate for the full loss of HSP90.2, as observed in other systems (Borkovich et al., 1989). Thus, our hsp90.2 alleles also prohibit functional compensation. Significantly, we did not recover alleles in any of the other HSP90 genes as loss of RPM1 function mutants. Thus, our hsp90.2 mutants are rare, and suggest a preferential utilization of HSP90.2 in RPM1 accumulation and, hence, in RPM1 function.

Importantly, a mutation exactly orthologous to hsp90-2.3 (D80N) has been studied in yeast HSP90 (D79N). This mutant yeast protein homo-dimerizes properly, and a wild type / mutant mixed dimer exhibits wild type levels of ATP hydrolysis (Richter et al., 2001). But mutant dimers are unable to bind or hydrolyze ATP (Obermann et al., 1998; Panaretou et al., 1998), and in competition assays are unable to interfere with ATP hydrolysis, even in 8-fold excess (Richter et al., 2001). Interestingly, a transcriptionally inducible yeast hsp90(D79N) also prevented phenotypic compensation by a wild type, constitutively expressed isoform (Panaretou et al., 1998). This may explain how we identified our mutants in plants that normally contain four copies of cytosolic HSP90, as well as suggesting that HSP90 isoforms might have non-overlapping functions in Arabidopsis.
Our other two mutations, *hsp90.2-1* (G95E) and *hsp90.2-2* (S100F) have not been identified in screens in other systems. Both are solvent exposed and adjacent to ATP interacting residues. It is possible that these mutations also interfere with ATP binding. Altered ATP binding/hydrolysis can also directly affect client protein binding and release by HSP90, as well as the interaction of HSP90 with its co-chaperones, thus de-stabilizing the complex (Obermann et al., 1998; Panaretou et al., 1998).

Our evidence of both synergism (in pathogen growth assays) and epistasis (in an HR assay) between *hsp90.2* and *ndr1* suggests that HSP90 and NDR1 act together in the RPM1-dependent disease resistance response. A requirement for HSP90.2 early in RPM1 signaling is consistent with our proposal that it is part of, or required for the assembly of, a poised RPM1 receptor complex. These results suggest an interesting parallel with animal innate immunity. NDR1 is a putative GPI-anchored protein. In animal systems, HSP90 co-localizes with the GPI-anchored protein CD14, and both act in the TLR4-dependent innate immune response to lipopolysaccharide (Triantafilou et al., 2002). As well, HSP90 inhibitors prevent the innate immune response activation by bacterial DNA (Zhu and Pisetsky, 2001). We propose that NDR1 and HSP90 function together, perhaps transiently, during RPM1 signaling.

**Could RAR1 and SGT1 function as HSP90 co-factors in disease resistance pathways?**

Several observations indicate that RAR1 and HSP90 function together in
RPM1-dependent HR. We demonstrated that RAR1 and SGT1 can associate with HSP90 in vivo. We noted a severe attenuation of RPM1-dependent HR in *hsp90.2* mutants and we found decreased RPM1-myc stability seen in *hsp90.2* mutants, two phenotypes observed in *rar1* plants (Tornero et al., 2002b). The epistasis of *hsp90.2* over *ndr1* with respect to HR was also observed with *rar1;ndr1* double mutants assayed for *RPM1* function, and for the synergistic interaction of *rar1* and *ndr1* in *RPP7* mediated resistance (Tornero et al., 2002b). These data are, in sum, consistent with RAR1 and HSP90 acting together in both RPM1-dependent HR, and possibly more broadly, in NB-LRR function. Our data are further consistent with recent findings demonstrating that RAR1 and NDR1 contribute quantitatively to the function of various NB-LRR R proteins (Muskett et al., 2002; Tornero et al., 2002b).

Because an *sgt1a;sgt1b* double mutant is lethal in *Arabidopsis* (Takahashi et al., 2003), it is impossible to determine if SGT1 isoforms have overlapping function in RPM1 signaling. Recent gene silencing experiments corroborate a role for HSP90 in the function of several *R* genes that also require SGT1 and RAR1 for their function (Liu et al., 2004). Structural modeling also supports the contention that RAR1 and SGT1 might act as co-factors of HSP90 (Shirasu and Schulze-Lefert, 2003). SGT1 and RAR1 homologs in animals have predicted structural homology to the HSP90 partner protein, p23 (Dubacq et al., 2002; Garcia-Ranea et al., 2002; Figure 2.10). Additionally, the TPR domain of SGT1 shares structural homology with other HSP90 partner proteins including HOP/STI1 (Garcia-Ranea et al., 2002).

We propose that RAR1 and HSP90 normally act to not only maintain RPM1 in a signal competent conformation, but also stabilize RPM1 against degradation. This
is reminiscent of the assembly of activation competent steroid receptors with an HSP90 isoform-homodimer and various co-chaperones (Picard, 2002; Pratt and Toft, 2003). HSP90 binding to the steroid receptor is not sufficient to render the receptor competent; co-factor binding and continual ATP turnover are required to maintain the steroid binding cleft in a receptive conformation (Pratt and Toft, 2003). Further conformational change accompanies ligand binding.

Inhibition of ATP binding and/or turnover in our hsp90.2 mutants should result in a locked HSP90 conformation, bound to RPM1 but unable to hold it appropriately, thus leading to RPM1 disappearance. This would mimic the effect on client proteins observed after treatment with the ATP binding inhibitor geldanamycin in other systems. RPM1 instability is consistent with results showing that HSP90 can rapidly shut off transcriptional responses by binding transcription factors and causing their degradation (Freeman and Yamamoto, 2002). Steroid receptor levels, like RPM1 (Boyes et al., 1998), drop after signaling (Lange et al., 2000; Wallace and Cidlowski, 2001).

Evidence for a second HSP90 function in protein stability is also emerging. First, human SKP2, a member of the SCF complex, is able to co-immunoprecipitate HSP90b in mouse NIH 3T3 cells (Lyapina et al., 1998). Degradation of HSP90 client proteins, triggered by either geldanamycin treatment or over-expression of the E3 ligase CHIP, can be inhibited by the addition of proteasome inhibitors (Connell et al., 2001; Schneider et al., 1996; Segnitz and Gehring, 1997; Whitesell and Cook, 1996). However, full steroid binding is not recovered in these experiments, also suggesting two functions for HSP90. One, reversible by lactacystin, is required for
Figure 2.10: SGT1a and SGT1b contain strong structural similarity to two different HSP90 co-chaperones, HOP and p23. (A) The TPR motifs of AtSGT1s are predicted to fold like the TPR motif of the HSP90 co-chaperone, HOP (seen here). (B) The CS domain found in AtSGT1s is predicted to fold in a similar conformation as p23 (seen here).
degradation, and another, not completely reversed by lactacystin, is required to mold a steroid-binding complex. Initiation of RPM1 function leads to RPM1 degradation (Boyes et al., 1998), perhaps involving SGT1 that is first recruited to an HSP90-RPM1 complex, and then guides RPM1 to the proteasome. RAR1 may normally block this conversion, perhaps in conjunction with (or antagonistically to) SGT1. This notion is consistent with our findings that RAR and SGT1 do not require each other to associate with HSP90.

A model where HSP90, in association with RAR1 and SGT1, controls levels of properly poised R protein complexes is consistent with the two functions of HSP90 discussed above—conformational molding and trafficking to the proteasome. Because of both R protein sequence polymorphism, and the possibility that R proteins might associate with additional cellular proteins, one could expect differential functional requirements for maintenance of this poised complex. For example, we also could co-IP HSP90 with anti-HA monoclonal antibody detecting an RPS2-HA fusion (data not shown) suggesting that at least one other NB-LRR protein can interact with a cytosolic HSP90. But we observed no change in RPS2 function in our mutants. This model is consistent with the fact that RPM1-mediated HR is very fast compared to others, including RPS2, and that RPM1 is degraded following triggering, unlike RPS2 (Axtell and Staskawicz, 2003). A requirement for a finely tuned conformational poise in NB-LRR R protein function was recently suggested using split Rx molecules (Moffett et al., 2002).

We thus favor an overall model whereby different NB-LRR R proteins, perhaps in association with the cellular proteins they guard, are kept in active
sentinel mode by varying degrees of dynamic re-shaping and maintenance of appropriate steady state levels driven by HSP90, RAR1 and SGT1. This model can encompass a continuous quantitative function for HSP90 in both the assembly of conformationally charged R protein complexes and the regulation of signal flux through those complexes.

**Materials and Methods**

**Plant lines**

Transgenic Arabidopsis ecotype Columbia (Col-0) (line a11) and rpm1-1 (line a11r) containing estradiol inducible avrRpm1 have been described in (Tornero et al., 2002a). Mutant lines used (all in Col-0 unless noted) were ndr1-1 (Century et al., 1997), rar1-20; a null allele, originally pbs2 (Warren et al., 1999), rar1-21 (Tornero et al., 2002b), rps2-101C (Mindrinos et al., 1994), rps5-2 (Warren et al., 1998), ecotype RLD (Hinsch and Staskawicz, 1996) as a rps4 mutant control, sgt1a (T-DNA insertion in Ws-0 ecotype), and sgt1b (edm1-1; Tör et al., 2002). We constructed double mutants of hsp90.2 and rpm1-1 by identifying F2 individuals susceptible to Pto DC3000(avrRpm1) which were molecularly heterozygous for rpm1-1, yet did not give rise to resistant offspring in the next generation. The F3s from such a line were then selected for a homozygous rpm1-1 mutant. These lines were confirmed after identification of the hsp90.2 mutations using PCR based markers. A similar procedure was used for creation of hsp90.2 and ndr1 double mutants. A homozygous insertion in the SALK T-DNA insertion line 058553 was identified by
molecular analysis of a segregating pool. The insertion site was confirmed by sequencing of T-DNA specific product. Primer sequences for selection of mutations available on request.

**Bacterial strains, inoculation and growth quantification**

*Pto* DC3000 derivatives containing pVSP61 (empty vector), *avrRpm1, avrB, avrRpt2, avrPphB,* or *avrRps4* were maintained as described (Ritter and Dangl, 1996). Plant inoculations and counting of the bacteria were performed as described (Tornero and Dangl, 2001). Where indicated, high concentrations of bacteria (OD$_{600}$=0.075, $3.75 \times 10^7$ colony-forming units/mL) were infiltrated into the bottom part of the leaf with a blunt syringe to test for the induction of HR.

**Estradiol induction**

Two-week-old plants grown under short day (8 hr.) conditions were sprayed with 0.02% Silwet L-77 (CKWitco Corporation) and 10μM β-Estradiol (Sigma E 8875) in distilled water from a 10 mM β-Estradiol stock dissolved in 100% ethanol (Tornero et al., 2002a).

**Mapping and tests for disease symptoms**

Rough mapping was performed by crossing *hsp90.2* mutants and Landsberg erecta (La-er). F2s were tested for *lra2* like disease symptoms. One to three week old F2 plants were sprayed with a 10mM MgCl$_2$ suspension containing *Pto* DC3000(*avrRpm1*) at concentration OD$_{600}$=0.1 ($5 \times 10^7$ colony-forming units/mL)
with 0.02% Silwet L-77, covered with a clear lid for 4 hrs, and assessed for chlorosis and other symptoms of \textit{P. syringae} infection 4-6 days later under short day conditions. Chi-square analyses showed that all mutations were recessive; \textit{lra2-1}: 480 wt, 612 mutant $X^2=3.843$, $p=0.05$; \textit{lra2-2}: 217 wt, 89 mutant $X^2=2.723$, $p=0.099$; \textit{lra2-3}: 457 wt, 155 mutant $X^2=0.035$, $p=0.85$. Susceptible F2 individuals were allowed to self and were confirmed in the F3 generation. DNA (Ausubel et al., 1987) from 41 of these individuals was used in PCR amplification of known PCR-based molecular markers (www.arabidopsis.org) to obtain approximate mapping positions. This interval was refined using molecular markers we developed (available upon request). We used DNA from 939 susceptible F2 individuals to define a 52 kB interval on P1 clone MDA7. The ~52kb \textit{LRA2} interval lies between a T/A polymorphism at position 551 (Jander et al., 2002) and the published CAPS marker MDA7 (www.arabidopsis.org) at position 52632 (C/A) relative to the published sequence for P1 clone MDA7 (Kaneko et al., 1998). Independent mapping of the \textit{lra2-2} (195 susceptible individuals) and \textit{lra2-3} (312 susceptible individuals) alleles showed similar linkage. All mutations where confirmed by sequencing of both DNA strands.

\textbf{Alignments and threading analysis}

Protein alignments of HSP90 N-termini were made using Align X (a component of Vector NTI Suite 7.1; Informax, Inc. (Frederick, MD)). This program uses CLUSTALW to make alignments. Parameters used were: Gap Opening
Penalty=10, Gap Extension Penalty=0.05, Gap Separation Penalty Range=8, % Identity for Alignment Delay=40, and Hydrophobic Residue Gap=GPSNQEKR.

The sequences of AtSGT1a and AtSGT1b were submitted to the threading Meta server [META] (http://bioinfo.pl/meta/) to identify structural templates for homology modeling. The meta server accessed the following fold-recognition servers and reported the consensus: bioinbgu [BIOINBGU], 3D-PSSM [3D-PSSM], GenTHREADER [GENTHREADER], FUGUE [FUGUE], and Sam-T99 [SAMT99]. The meta server identified the crystal structure of the TPR1-domain of Hop (PDB ID 1elw) as a structural template for residues 1-115 of AtSGT1a; and the crystal structure of the human co-chaperone P23 (PDB ID 1ejf) as a structural template for residues 156-237 of AtSGT1a. Models of the P23 and TPR domains were built using the Modeler module of the InsightII molecular modeling system from Accelrys Inc. (www.accelrys.com). Figures were created with SPOCK.

**Protein Blots and co-immunoprecipitations**

For detection of RPM1-myc in *hsp90.2-2* and *hsp90.2-3*, we introgressed these mutants into plants expressing *RPM1-myc* from the native *RPM1* promoter (Boyes et al., 1998) as described in (Tornero et al., 2002b). Total protein was extracted in 50 mM Tris-HCl pH 8.0, 1% SDS, 1mM EDTA, 1 mM 2-mercaptoethanol, and 1 X plant protease inhibitor cocktail (Sigma). For immunodetection, 40 μg protein samples were electrophoresed on 8% SDS-PAGE gels. Western blots were performed using standard methods and detected with ECL+ (Amersham).
For co-immunoprecipitations, tissue was first ground in liquid nitrogen with a mortar and pestle. This material was then homogenized by alternate rounds of Polytron (Kinematica) and glass douncer (Kontes Glass Company) in 2 ml of sterile buffer 20mM Tris-HCL pH 8.0, 0.33M Sucrose,10 mM EDTA, 5mM DTT and 1 X plant protease inhibitor cocktail (Sigma) per 1 g of tissue. Debris was removed by centrifugation at 5,000XG for 40 minutes at 4°C. 1.5 ml of this supernatant was first pre-cleared by adding 50 μl of protein G-agarose (Boehringer Mannheim) and incubated at 4°C for 60 minutes on an orbital shaker. The cleared supernatant was then removed and combined with one of the following: 5 μl of the anti-RAR1 (Muskett et al., 2002) antibody, 5 μl anti-SGT1 (Azevedo et al., 2002) antibody, 30 μl of a re-suspended anti-HA Affinity Matrix (3F10, Roche), or 30 μl of a re-suspended anti-c-Myc Agarose (9E10, Santa Cruz Biotechnology). This was followed by incubation at 4°C for 2 hour. 50 μl of protein G-agarose was then added to the reactions containing the anti-RAR1 and anti-SGT1 antibodies. All reactions were then rolled at 4°C overnight. Beads were pelleted at 1000XG for five minutes. This was followed by four washes in 1.5 ml of 50mM HEPES (pH 7.4), 100 mM NaCl, and 10 mM EDTA pH 8.0. Bound proteins were eluted with 50 μl of sample buffer and run on an 8% polyacrylamide gel, and probed with a polyclonal antibody raised against the C-terminal portion of *Pharbitis nil* HSP90 (Krishna et al., 1997).

HSP90 was not detected in control immunoprecipitations with four different antibodies, three of which do immunoprecipitate HSP90 via RPM1-myc, RAR1, or SGT1b. Thus, HSP90 is not non-specifically sticking to Agarose-Protein A/G beads or other matrix reagents. Furthermore, we used antibodies to actin (soluble)
ascorbate peroxidase (soluble), BiP (soluble and ER), topoisomerase II (nuclear), RD28 (intergral plasma membrane; Daniels et al., 1994), and Tip (tonoplast intrinsic protein). None of these co-IP’ed HSP90. The absence of HSP90 in these IPs (data not shown) argues for the specificity of our co-IP data.
Bibliography

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CHAPTER 3

RIN4 negatively regulates RPS2 and RPM1 downstream or independent of NDR1 and is not required for AvrRpt2 or AvrRpm1 virulence functions

Preface

The following chapter was previously published as “RIN4 negatively regulates RPS2 and RPM1 downstream or independent of NDR1 and is not required for AvrRpt2 or AvrRpm1 virulence functions” in Plant Cell (2004, 10, 2822-35). I made intellectual contributions to the whole paper and produced the genetic material for Figure 3.3C, 3.4, 3.5, and 3.6B. Additionally, I performed independent repetitions of Figure 3.3C and 3.5, and designed the experiments for Figure 3.4B and 3.6B. At first glance, this chapter may not appear to build on the larger story presented in this dissertation. However, the data presented here generalized rar1’s affect on NB-LRR accumulation by showing a second Arabidopsis NB-LRR accumulated less protein in a rar1 mutant. We show here for the first time in any system that increasing NB-LRR protein levels/signaling can partially overcome the genetic effect of rar1. This finding suggested for the first time that rar1 may not function in a direct linear signal transduction pathway and thus built the groundwork of the threshold model discussed in Chapters 4 and 5. Additionally, this paper showed that ndr1, another of the few genes known to play a direct role in disease Resistance gene function, did not play a role in accumulation of NB-LRRs.
Abstract

Bacterial pathogens deliver type III effector proteins into the plant cell during infection. On susceptible (r) hosts, type III effectors can contribute to virulence. Some trigger the action of specific disease resistance (R) gene products. The activation of R proteins can occur indirectly via modification of a host target. Thus, at least some type III effectors are recognized at site(s) where they may act as virulence factors. These data indicate that a type III effector’s host target might be required for both initiation of R function in resistant plants and pathogen virulence in susceptible plants. In Arabidopsis, RIN4 associates with both the RPM1 and RPS2 disease resistance proteins. RIN4 is post-translationally modified following delivery of the *Pseudomonas syringae* type III effectors AvrRpm1, AvrB, or AvrRpt2 to plant cells. Thus, RIN4 may be a target for virulence functions of these type III effectors. We demonstrate that RIN4 is not the only host target for AvrRpm1 and AvrRpt2 in susceptible plants, as its elimination does not diminish their virulence functions. In fact, RIN4 negatively regulates AvrRpt2 virulence function. RIN4 also negatively regulates inappropriate activation of both RPM1 and RPS2. Inappropriate activation of RPS2 is NDR1-independent, in contrast to the established requirement for NDR1 during AvrRpt2-dependent RPS2 activation. Thus, RIN4 acts either at, downstream or independently of NDR1 to negatively regulate RPS2 in the absence of pathogen. We propose that many *Pseudomonas syringae* type III effectors have more than one target in the host cell. We suggest that a limited set of these targets, perhaps only one, are associated with R proteins. Thus, while any pathogen virulence factor may
have multiple targets, the perturbation of only one is necessary and sufficient R activation.

**Introduction**

In response to the pressures of infection, plants evolved an immune system to specifically detect pathogens and induce defenses against them. The most efficient sentinels of the plant immune response are proteins encoded by the disease resistance (R) genes (Flor, 1971). The most common and widely distributed class of R-proteins has a central nucleotide binding site (NB) domain and carboxy-terminal leucine rich repeats (LRR). Some of these so-called NB-LRR R-proteins have amino-termini with homology to the intracellular portion of the Drosophila Toll and mammalian interleukin (IL-1) receptors (TIR-NB-LRR). Other R-proteins have a coiled-coil motif at their amino-termini (CC-NB-LRR) (Dangl and Jones, 2001). Activation of NB-LRR proteins induces a defense response consisting of a series of biochemical and cellular events, and massive transcriptional re-programming within and surrounding the infection site (Dangl and Jones, 2001; Hammond-Kosack and Parker, 2003; McDowell and Dangl, 2000; Nimchuk et al., 2003). These often, but not always, culminate in a localized programmed cell death called the hypersensitive response (HR).

Plant pathogenic bacteria express genes whose products trigger activation of specific NB-LRR R proteins. These were historically termed *avr* genes because their presence rendered strains expressing them avirulent on plants expressing the corresponding R gene (Staskawicz et al., 1984). These Avr proteins are substrates
of the evolutionarily conserved type III secretion system used by a variety of Gram-negative animal and plant pathogens to deliver type III effector proteins to the eukaryotic host cell (Collmer et al., 2002; Greenberg and Vinatzer, 2003; Staskawicz et al., 2001). Thus, type III effector proteins in general, including the operationally defined Avr proteins, are likely to function primarily as virulence factors contributing to pathogen fitness on susceptible hosts. A growing base of experimental evidence supports this notion (Chang et al., 2000; Kearney and Staskawicz, 1990; Lorang et al., 1994; Ritter and Dangl, 1995; reviewed in Nimchuk et al., 2001).

The simplest molecular explanation for the genetics of \( \text{avr-R} \) disease resistance systems postulated a direct ligand-receptor interaction, but there is little experimental evidence to support this model generally with respect to NB-LRR proteins. This paucity of data led to the articulation of an alternative hypothesis in which R-proteins monitor the integrity of host targets of pathogen virulence factors (Dangl and Jones, 2001; van der Biezen and Jones, 1998; Van der Hoorn et al., 2002). Experimental support for this “Guard Hypothesis” is mounting (Axtell and Staskawicz, 2003; Chen et al., 2000; Kruger et al., 2002; Mackey et al., 2003; Mackey et al., 2002; Shao et al., 2003).

\( RPM1 \) encodes a CC-NB-LRR R-protein that confers resistance against \( P. \) syringae expressing either of two sequence unrelated type III effectors, AvrB and AvrRpm1 (Bisgrove et al., 1994; Grant et al., 1995). \( \text{RIN4} \) is a plasma membrane localized, evolutionarily conserved protein of 211 amino acids. Its sequence provides no clues to its function. \( \text{RIN4} \) is required for \( RPM1 \)-mediated disease resistance because it is required for \( RPM1 \) accumulation before infection. \( \text{RIN4} \) is
phosphorylated upon infection with *P. syringae* expressing either AvrB or AvrRpm1, though neither of these type III effectors has homology to known kinases (Lee et al., 2004). AvrB and AvrRpm1-dependent phosphorylation of RIN4 occurs in both *RPM1* and *rpm1* plants. These results suggested that RIN4 phosphorylation may result from the virulence activity of AvrB and AvrRpm1, and that this event leads to RPM1 activation when it is present (Mackey et al., 2002).

RIN4 is also involved in the activation of RPS2 (another CC-NB-LRR protein), with which it associates in vivo (Axtell and Staskawicz, 2003; Mackey et al., 2003). RPS2 confers resistance against *P. syringae* expressing the type III effector AvrRpt2 (Axtell and Staskawicz, 2003; Bent et al., 1994; Mackey et al., 2003; Mindrinos et al., 1994). AvrRpt2 is a putative cysteine protease (Axtell et al., 2003) that causes post-transcriptional disappearance of RIN4 (Axtell and Staskawicz, 2003; Mackey et al., 2003). Over-expression of RIN4 delays its disappearance in the presence of AvrRpt2 and, consequently, inhibits RPS2 activation. Thus, RIN4 disappearance is required for full RPS2 activation. A *rin4* null mutation is lethal, and this lethality is rescued in a *rin4 rps2* double mutant, indicating that RIN4 negatively regulates inappropriate activation of RPS2 (Mackey et al., 2003). We term this “inappropriate activation” to distinguish it from normal, AvrRpt2-dependent RPS2 activation (Belkhadair, et al.2004). Collectively, these data indicate that RIN4 is a target of multiple, unrelated bacterial type III effector proteins, and that RIN4 associates with two different NB-LRR proteins. Both findings are consistent with the “Guard Hypothesis” for NB-LRR activation (Dangl and Jones, 2001).
Plant genes required for disease resistance were defined via genetic screens for loss of specific \textit{R} functions (Glazebrook et al., 1997; Hammond-Kosack and Parker, 2003). Relevant to this work are \textit{NDR1} and \textit{RAR1}, genes required for the function of various NB-LRR proteins. \textit{RAR1} is the founding member of the CHORD protein family, containing two novel zinc-coordinating domains (Muskett et al., 2002; Shirasu et al., 1999; Tornero et al., 2002). \textit{RAR1} may modulate NB-LRR protein levels (Tornero et al., 2002) through its association with HSP90 and other components of a signal-competent NB-LRR protein complex (Hubert et al., 2003; Liu et al., 2003; Lu et al., 2003; reviewed in Holt et al., 2003; Schulze-Lefert, 2004; Shirasu and Schulze-Lefert, 2003). \textit{RAR1} can associate with SGT1, a possible proteasome regulator required for the action of some, but not all, NB-LRR proteins (Austin et al., 2002; Azevedo et al., 2002; Tör et al., 2002). \textit{NDR1} modulates the intensity of signaling through specific NB-LRR proteins (Tornero et al., 2002). \textit{NDR1} may be a glycosylphatidylinositol (GPI) membrane anchored protein (Century et al., 1995; Century et al., 1997). At least three CC-NB-LRR proteins, RPM1 (Boyes et al., 1998), RPS2 (Axtell and Staskawicz, 2003) and RPS5 (B. Holt unpublished), and their corresponding Avr proteins have been localized to the plasma membrane, or to a membrane fraction (Axtell and Staskawicz, 2003; Nimchuk et al., 2000). Thus, \textit{NDR1} localization at the same sub-cellular address via a GPI anchor would place it in an excellent position to participate in the integration and transduction of NB-LRR signaling during infection.

Here, we assess whether \textit{RIN4} has any negative regulatory effect on inappropriate activation of RPM1, in addition to its requirement for RPM1
accumulation and its established negative regulatory effect on RPS2. We address the requirements for RAR1 and NDR1 for the inappropriate activation of RPS2 observed in the absence of RIN4. And finally, we address whether the virulence activities of AvrRpm1 and AvrRpt2 in susceptible plants lacking RIN4 are altered. Our results establish novel functions for RIN4 in the regulation of RPM1 and RPS2 activity and prompt a modification of the tenets of the Guard Hypothesis for disease resistance protein activation.

Results

Normal RPM1 function is abrogated in rin4 null plants

We previously reported that a homozygous T-DNA insertion into the RIN4 ORF was embryo lethal. We demonstrated that the lethality of this rin4 null allele (hereafter rin4; see Methods for allele designations of all mutants and transgenic lines used in this study) is largely suppressed in rin4 rps2 plants. This indicated that elimination of RIN4 results in inappropriate RPS2 activation (Mackey et al., 2003). We tested whether RPM1 is required for inappropriate RPS2 activation and the consequent lethal phenotype in selfed progeny from RIN4/rin4 RPS2/RPS2 rpm1/rpm1 plants. One quarter of these plants died as embryos or early seedlings. Thus, the lethality in rin4 plants does not require RPM1 (not shown).

We tested whether or not rpm1, like rps2, could suppress part or all of the rin4 lethal phenotype. Plants with reduced levels of RIN4 (rin4K-D; RIN4 knock down plants due to an insertion in the RIN4 promoter; Ws-0 background (Mackey et al., 2002)) are partially compromised for RPM1-mediated inhibition of bacterial growth
because they accumulate lowered levels of RPM1. We extended these analyses to RPM1 function in rin4 rps2 plants (Figure 3.1). P. syringae pv. tomato (Pto) DC3000(vector) grew to high levels by three days after infection on wild type Col-0 plants. Importantly, this growth was reduced reproducibly by 10-fold in rin4 rps2, indicating that these plants expressed enhanced basal disease resistance against Pto DC3000 (see below). Growth of Pto DC3000 expressing AvrRpm1, AvrB, or AvrRpt2 was inhibited on wild type Col-0 plants, due to RPM1 or RPS2 action, respectively. The growth of each strain was enhanced in rpm1 rps2 (Figure 3.1), as expected in the absence of the respective R proteins.

Importantly, the growth of Pto DC3000(\textit{avrRpm1}) (Figure 3.1) or Pto DC3000(\textit{avrB}) (not shown) was the same in rin4 rps2 plants as in rin4 rpm1 rps2 plants, indicating a full loss of RPM1 function in the former plants, even though they are genotypically RPM1. Finally, the enhanced resistance against Pto DC3000 that we noted above in rin4 rps2 plants was not apparent against Pto DC3000 expressing \textit{avrRpm1} or \textit{avrRpt2} (Figure 3.1). Thus, these type III effectors (and \textit{avrB}; not shown) allow Pto DC3000 to overcome the enhanced basal disease resistance we observed in rin4 rps2 plants, presumably by suppressing an ectopic defense response (Figure 3.1).

**Enhanced resistance against Pto DC3000 in rin4 is due to ectopic activation of residual RPM1**

Numerous mutants exhibiting enhanced heightened resistance to pathogens also constitutively express pathogenesis-related (PR) genes, due to activation of basal defense responses (Glazebrook et al., 1997; Lorrain et al., 2003). The
Figure 3.1: RPM1 function is abrogated in rin4 null plants. Growth of the Pto DC3000 strains expressing the indicated type III effector genes, displayed on the right, was measured on wild-type and mutant Arabidopsis lines, indicated at bottom. Four-week-old plants were infiltrated with $10^5$ cfu/ml and the number of bacteria per area of leaf plotted on a log$_{10}$ scale for day 0 (white columns) and day 3 (black columns) (see Methods). Error bars represent the standard deviation among four samples. This experiment is representative of four independent replicates. The absence of error bars indicates low errors. A one-way ANOVA test was applied to each pair of values, and $p < 0.01$ for rin4 rps2 inoculated with Pto DC3000(vector) compared to all the others (*).
enhanced resistance we observed in rin4 rps2 plants against Pto DC3000(vector) indicated a possible cpr (constitutive expression of PR) phenotype (Bowling et al., 1994). Therefore, we analyzed PR1 protein expression as a convenient marker typical of cpr phenotypes (Figure 3.2A). We observed some residual constitutive PR1 protein accumulation in rin4 rps2 plants (Figure 3.2A). No PR1 expression was observed in Col-0, rpm1 rps2 or, most importantly, rin4 rps2 rpm1 plants (Figure 3.2A). For comparison, and as demonstrated previously (Mackey et al., 2002), rin4K-D plants express constitutively high levels of PR1. Note however, that the rin4K-D plants are in Ws-0, precluding direct comparison of PR-1 levels in Col-0 and Ws-0. Nevertheless, our results in the isogenic Col-0 lines in Figure 3.2A demonstrate a low level of residual RPM1-dependent PR1 expression in rin4 rps2 plants. Ectopic RPM1 activation thus explains both the enhanced resistance to Pto DC3000 in rin4 rps2 and the loss of that enhanced resistance in rin4 rps2 rpm1 plants (Figure 3.1).

We also tested whether or not ectopic RPM1 activation could be enhanced by increasing the RPM1 dose in the context of lowered RIN4 levels represented in the rin4K-D plants. We doubled the RPM1 dose by crossing an isogenic RPM1-myc transgene (driven by the native RPM1 promoter) into rin4K-D plants. We probed protein blots with anti-RIN4, anti-myc, and anti-PR1 antibodies (Figure 3.2B). As previously noted, rin4K-D plants accumulated reduced levels of RIN4 compared to wild type isogenic RPM1-myc plants (Figure 3.2B). Figure 3.2B also demonstrates, however, that rin4K-D (RPM1-myc) plants expressed significantly more PR1 than rin4K-D plants. The rin4K-D (RPM1-myc) plants also exhibited accentuated phenotypes relative to rin4K-D (data not shown). These included smaller stature,
A

Col-0 rpm1 rin4 rin4 Ws-0 rin4K-D
rpm2 rpm2 rpm2 rpm1

RIN4
WB.RIN4

PR1
WB.PR1

Rubisco
Ponceau

rin4

RPM1

RPS2

Embryonic lethality due to full RPM1 and RPS2 activation.

rin4 rps2

RPM1

Suppressed embryonic lethality; Residual RPM1 is still activated and sufficient for minor CPR phenotype.

B

Ws-0 rin4K-D

- RPM1 - RPM1
myc myc

RIN4
WB.RIN4

PR1
WB.PR1

Rubisco
Ponceau

RPM1-myc
WB:myc

rin4K D

RPM1

RPS2

Reduced viability, increased CPR phenotype, compared to RIN4 plants, due to moderate RPM1 and RPS2 activation.

rin4K-D RPM1myc

RPM1

RPS2

Enhanced CPR phenotype due to RPS2 activation and increased RPM1 activation.
Figure 3.2: Residual RPM1 is sufficient for constitutive defense response in *rin4* null plants. (A) Total protein extracts were prepared from wild type Col-0, rpm1 rps2, rin4 rps2, rin4 rps2 rpm1, Ws-0 and rin4 Knock-Down (*rin4K-D*) plants. These extracts were subjected to anti-RIN4 (top, WB:RIN4) or anti-PR1 (middle, WB:PR1) Western blot. Ponceau staining of ribulose-1,5-bisphosphate carboxylase_oxygenase (Rubisco) (Bottom) was for confirmation of equal loading in each lane. This experiment is representative of three independent replicates.

The models summarize the protein blot data. Grey balls represent the plasma membrane. Red shapes represent RPM1 and RPS2 potentially in complex with other cellular proteins, light and dark blue. In *rin4* null plants (left), RPM1 and RPS2 are inappropriately activated in the absence of pathogens. In *rin4 rps2* plants (right) the residual RPM1 present is activated by the lack of RIN4. The pale blue and red arrows represent respectively RPM1 and RPS2 activation. The levels of activation are proportional to the thickness of the arrows. (B) Total protein extracts were prepared from wild type Ws-0, and isogenic RPM1-myc, *rin4K-D* and *rin4K-D* RPM1-myc plants. These extracts were subjected to anti-RIN4 (top, WB: RIN4), anti-PR1 (middle, WB: PR1) and anti-myc (bottom, WB:myc) Western blots. Ponceau staining of ribulose-1,5-bisphosphate carboxylase_oxygenase (Rubisco) (middle 2 panel) demonstrates equal loading in each lane for the anti-RIN4 and anti-PR1 antibodies. For the myc western blot the nonspecific band detected below RPM1-myc was used as an equal loading control. Note that the PR1 immunoblot in Figure 3.2A is slightly over-exposed relative to that in Figure 3.2B. This experiment is indicative of three independent replicates. The models (symbols as in Figure 3.2A) show that RPM1 and RPS2 are inappropriateal activate when levels of RIN4 are lowered in *rin4K-D*. When more RPM1 is expressed (right, note bigger red RPM1 in model), it expresses a higher amplitude of inappropriate activation.
lower fertility, loss of apical dominance, and sporadic lesions (Mackey et al., 2002). By contrast, doubling the \textit{RPM1} dose in the \textit{RIN4} (\textit{RPM1}-myc) control plants did not result in detectable PR1 expression (Figure 3.2B), or in any other macroscopic phenotype observed in \textit{rin4K-D}. Thus, the additional copy of \textit{RPM1} enhances all aspects of the \textit{rin4K-D} phenotype.

The level of PR1 expression in both \textit{rin4 rps2} and \textit{rin4K-D} (\textit{RPM1}-myc) plants was influenced by environment. Growth in 16 hr days resulted in more PR1 expression compared to 8 hr day conditions. This is consistent with our previous observation that \textit{rin4K-D} plants show an exacerbated morphology when grown in long day conditions compared to short day conditions (Mackey et al., 2002). We also consistently observed a lower mobility of RIN4 in Ws-0 compared to in Col-0 (Figure 3.2A). This lower mobility is due to constitutive phosphorylation of RIN4 as phosphatase treatment restored the mobility of modified RIN4 to that of not modified (data not shown).

Collectively, the results in Figure 3.2 indicate that 1) when levels of RIN4 are reduced, residual RPM1 is activated inappropriately, and PR1 expression and enhanced resistance are consequently induced. 2) Wild type RIN4 levels are necessary and sufficient for both the proper accumulation of RPM1 and for prevention of its inappropriate activation; hence RIN4 negatively regulates RPM1. 3) The constitutive expression of PR1 in \textit{rin4K-D} plants is due to the sum of inappropriate activation of both RPS2 and RPM1.
RAR1 and NDR1 are differentially required for ectopic RPS2 activation in rin4

*rps2* suppresses lethality in *rin4* (Mackey et al., 2003). We addressed whether mutation in signaling components required for AvrRpt2-dependent activation of *RPS2* could suppress the ectopic RPS2 activation in *rin4*. *RAR1* and *NDR1* are both required for RPS2 signaling, and presumably act in the same pathway (see Introduction). We therefore followed lethality in selfed progeny from *RIN4/rin4 rar1/rar1* and *RIN4/rin4 ndr1/ndr1* plants (Figure 3.3A).

The *rar1* mutation delayed *rin4* lethality and we were able to isolate *rin4 rar1* plants. These plants had limited viability, were dwarfed relative to their *RIN4 rar1* siblings by ~2 weeks of age, formed numerous dead cell lesions spontaneously, and died before 3 weeks of age (Figure 3.3B). We previously demonstrated that RPM1 accumulation is severely reduced in *rar1* plants (Tornero et al., 2002). To address whether RPS2 levels were similarly affected, we crossed *rar1* to a transgenic line carrying an HA-epitope tagged version of RPS2 (driven by the native promoter in *rps2*; Axtell and Staskawicz, 2003). This line expresses an accelerated HR and enhanced inhibition of bacterial growth compared to wild type Col-0 following inoculation with *Pto DC3000(avrRpt2)*, presumably due to slight RPS2 protein over-expression (Axtell and Staskawicz, 2003). We PCR-selected a *rar1 rps2* (*RPS2-HA*) triple homozygous line (see Methods). As with RPM1-myc, we detected severely reduced levels of RPS2-HA protein in *rar1 rps2* (*RPS2-HA*) plants (Figure 3.3C). These results indicate that 1) RAR1 is required for accumulation of at least two CC-NB-LRR proteins and 2) *rar1* does not fully suppress the *rin4* lethality because the
Figure 3.3: RAR1, but not NDR1, delays the lethality in rin4 null plants. (A) F2 plants of the genotypes shown at left were allowed to self pollinate. The segregation of RIN4 in these progenies was scored on 100 F3 plants by RIN4 Western blot. Segregation data was evaluated with chi-square analysis. (B) Pictures of representative progenies from selfed RIN4/rin4 rar1/rar1 F2 plants. Note that rar1 rin4 are smaller and develop spontaneous lesions compared to rar1 RIN4 plants. (C) Total protein extracts were prepared from the genotypes listed at the top. These extracts were subjected to an anti-HA Western blot (top). The Ponceau stain of the ribulose-1,5-bisphosphate carboxylase_oxygenase (bottom) shows that the differences observed in rar1 rps2 (RPS2-HA) plants are not due to loading errors.
residual RPS2 in *rin4 rar1* plants remains ectopically activated. These results are consistent with a quantitative role for RAR1 in NB-LRR accumulation.

We did not recover any *rin4 ndr1* plants in the analyzed progenies (Figure 3.3A). Thus, *ndr1* cannot suppress inappropriate RPS2 activation in *rin4*, although it is clearly required for AvrRpt2-dependent RPS2 activation (Century et al., 1995). Additionally, there is no diminution of RPS2-HA levels in *ndr1 rps2* (*RPS2-HA*) plants (Figure 3.3C).

RPS2-HA is a plasma membrane protein, and this localization is retained in the absence of RIN4 following infection with *Pto DC3000*(*avrRpt2*) (Axtell and Staskawicz, 2003). NDR1 is a predicted GPI anchored protein (B. Staskawicz, pers. comm.). We tested whether NDR1 is responsible for RPS2 localization, since RPS2 mis-localisation could account for the differential NDR1 requirement during AvrRpt2-dependent RPS2 activation compared to its inappropriate activation in *rin4*. We fractionated crude lysates from *rps2* (*RPS2-HA*), *ndr1 rps2* (*RPS2-HA*) and *rar1 rps2* (*RPS2-HA*) transgenic plants into total, soluble, and microsomal fractions and analyzed protein blots (Figure 3.4A). RPS2-HA remained localized in the microsomal fraction in *ndr1* and *rar1* plants. Thus, gross mis-localization of RPS2 cannot explain either the loss of AvrRpt2-dependent RPS2 activation in *ndr1*, or the differential requirement for NDR1 in the two modes of RPS2 activation. Collectively, the results in Figures 3.3 and 3.4 indicate that 1) NDR1 is either upstream or independent of the inappropriate RPS2 activation in *rin4* and 2) NDR1 does not regulate RPS2 function by controlling its accumulation, as does RAR1, or its localization.
Figure 3.4: Microsomal RPS2 localization and interaction with RIN4 do not require NDR1 or RAR1. (A) Total protein extracts (T) from genotypes shown at the top were fractionated into soluble (S) and microsomal (M) extracts (see Methods). The fractionated samples were analyzed by Western blot with anti-HA, anti-RIN4, anti-APX (Ascorbate Peroxidase; control soluble protein) and anti-RD28 (control integral membrane protein) anti-sera (Boyes et al., 1998). Microsomal fractions are approximately 5 times concentrated relative to total (T) and soluble (S) fractions. (B) Protein from genotypes shown at top were immunoprecipitated (IP: RIN4) with anti-RIN4 sera (I) or with pre-immune sera (PI). Total extracts (T) from rps2 and rps2 (RPS2-HA) as well as immunoprecipitated samples were analyzed by Western blot with an anti-HA antibody (WB: HA). The relative amounts of protein from the immune pellet and the total extracts are not equivalent. The pellet is over-represented by 30-fold. This experiment is representative of two independent replicates.
We conducted co-immunoprecipitation experiments to test whether RIN4 also interacts with RPS2 in rar1 and ndr1 mutants (Figure 3.4B). Extracts from rps2 (RPS2-HA), ndr1 rps2 (RPS2-HA) and rar1 rps2 (RPS2-HA) transgenic plants immunoprecipitated with anti-RIN4 antisera were analyzed for RPS2-HA in protein blots. Neither ndr1 nor rar1 affected the ability of RIN4 to co-immunoprecipitate RPS2-HA, despite the overall lower levels of RPS2-HA accumulating in rar1 (Figure 3.4B). The data presented in Figures 3.3 and 3.4 indicate that neither RAR1 nor NDR1 affects the mechanism of inappropriate RPS2 activation in rin4 plants, though RAR1 apparently dampens it by modulating RPS2 accumulation.

Wild type levels of NDR1 are sufficient to transduce enhanced RPS2 function

Our data indicate that NDR1 acts upstream or independently of inappropriate RPS2 activation in rin4. There is, however, a possible alternative explanation for the inability of ndr1 to suppress rin4 lethality, where NDR1 would act downstream of RPS2 activation. Recall that NDR1 acts quantitatively during NB-LRR activation (see Introduction). There is obviously sufficient NDR1 in a wild type plant to transduce a normal, AvrRpt2-driven RPS2 response. It might be that the quantity of signal flux during inappropriate RPS2 activation in rin4 is greater, or more sustained, than during infection. Thus, the signal flux during inappropriate RPS2 activation may overcome the normal requirement for NDR1 such that the lethal rin4 phenotype is generated via bypass in an ndr1 mutant.

To address this possibility, we took advantage of the accentuated RPS2 function in our rps2 (RPS2-HA) transgenic line (introduced above; Axtell and
Staskawicz, 2003). This line should produce more flux through RPS2 during an AvrRpt2-driven response than wild type. We established this point by comparing RPS2 function in rar1 rps2 (RPS2-HA) and ndr1 rps2 (RPS2-HA) to rar1 and ndr1 (Figure 3.5). Pto DC3000(avrRpt2) growth was restricted in wild type Col-0, and even more restricted in rps2 (RPS2-HA), reflecting enhanced RPS2 action as previously noted (Axtell and Staskawicz, 2003). Pto DC3000(avrRpt2) grew to high levels on rps2. This growth was 90% reduced in rar1, indicating that the residual RPS2 in rar1 plants still functions. Importantly, Pto DC3000(avrRpt2) growth was reduced by more than 99.5% in rar1 rps2 (RPS2-HA), indicating that the enhanced AvrRpt2-dependent RPS2 activation in this line is sufficient to partially overcome the lack of RAR1 in rar1 rps2 (RPS2-HA). By contrast, the growth of Pto DC3000(avrRpt2) was identical on ndr1 and ndr1 rps2 (RPS2-HA), demonstrating that the enhanced RPS2 signal was still fully NDR1-dependent. These results are also consistent with a role for RAR1 in modulating RPS2 stability or accumulation. Further, they indicate that wild type levels of NDR1 are necessary and sufficient to mediate even the enhanced signaling observed in rar1 rps2 (RPS2-HA). The latter result argues against a bypass of NDR1 function during inappropriate RPS2 activation in rin4.
Figure 3.5: Enhanced RPS2 function modulates its requirement for RAR1, but does not overcome its requirement for NDR1. Growth of Pto DC3000(avrRpt2) was measured on wild-type and mutant Arabidopsis lines, indicated at bottom. Bacterial growth was measured as described in Figure 3.1, legend. Error bars represent the standard deviation among four samples, and this experiment is representative of two independent replicates. The absence of error bars indicates low errors.
RIN4 levels modulate AvrRpt2 virulence function, but RIN4 is not the only target of AvrRpt2

If RIN4 is the only target for AvrRpt2 when this type III effector acts as a virulence factor in rps2, then it could be the case that elimination of RIN4 would result in loss of that virulence activity. We used a weak pathogen strain, Pma M6CΔE (Rohmer et al., 2003), to examine the contribution of AvrRpt2 to bacterial virulence on plants with altered levels of RIN4. Note that we observed only a weak RPS2-dependent inhibition of bacterial growth with Pma M6CΔE(avrRpt2) at low bacterial doses (Figure 3.6A). However, using a higher titer of bacteria we observed consistently RPS2-mediated HR (data not shown). The weak RPS2-mediated inhibition of bacterial growth is likely due to the weak intrinsic virulence of Pma M6CΔE.

We reproducibly observed a very slight increase in the virulence of Pma M6CΔE(avrRpt2) on rps2 compared to Col-0 (Figure 3.6). AvrRpt2 delivered from Pma M6CΔE promotes increased bacterial growth in rin4 rps2 plants compared to rps2 plants (Figure 3.6A). This enhanced virulence function of AvrRpt2 is reversed in rps2 plants that over-express RIN4 (OxRIN4 rps2 plants; Mackey et al., 2003) (Figure 3.6A). These data indicate that 1) RIN4, in a formal sense, negatively regulates one or more AvrRpt2 virulence activities; 2) wild-type levels of RIN4 are apparently saturating for this negative regulation; and 3) RIN4 is not required for this AvrRpt2 virulence activity.
Figure 3.6: RIN4, RAR1 and NDR1 modulate AvrRpt2 virulence function(s). (A) RIN4 is not required for AvrRpt2 virulence function. Growth of *Pma* M6CΔE carrying either empty vector or *avrRpt2* (indicated at bottom) was measured on the genotypes indicated at top. Bacterial growth was measured as described in Figure 3.1, legend. A one-way ANOVA test was applied to each pair of values, and p < 0.01 for *rin4 rps2* inoculated with *Pma* M6CΔE(*avrRpt2*) compared to all others (*). Error bars represent the standard deviation among four samples and this experiment is representative of six independent replicates. (B) RAR1 and NDR1 negatively regulate AvrRpt2 virulence function. Inoculations and labels are as in (A). A one-way ANOVA test was applied to each pair of values, and p < 0.01 for *rin4 rps2, ndr1 rps2 and rar1 rps2* inoculated with *Pma* M6CΔE(*avrRpt2*) compared to all the others (*). Error bars represent the standard deviation among four samples and this experiment is representative of two independent replicates.
**The absence of RAR1 and NDR1 enhance AvrRpt2 virulence function(s)**

AvrRpt2 is able to promote the virulence of *Pto* DC3000 by suppressing plant defenses downstream or independently of SA-dependent basal defenses (Chen et al., 2004). RAR1 and NDR1 can regulate basal plant defense (see Introduction). We therefore addressed the contribution of RAR1 and NDR1 to AvrRpt2 virulence activities by inoculating *Pma M6CΔE(avrRpt2)* onto *rar1 rps2* and *ndr1 rps2* (Figure 3.6B). Again, *Pma M6CΔE(avrRpt2)* grew reproducibly to higher titers on *rps2* than did *Pma M6CΔE(vector)*, indicative of an AvrRpt2 virulence function. This was enhanced in *rin4 rps2*, as in Figure 3.6A. Importantly, AvrRpt2 promoted more bacterial growth in *rar1 rps2* and *ndr1 rps2* compared to *rps2* (Figure 3.6B). These results indicate that RAR1 and NDR1 negatively regulate one or more AvrRpt2 virulence activities, presumably via their functions in the induction of basal defense.

**RIN4 is not the only target of AvrRpm1 and AvrB in Arabidopsis**

The ability of AvrRpm1 and AvrB to interact with RIN4 and to induce its phosphorylation may contribute to their ability to enhance bacterial virulence in *rpm1* plants (Mackey et al., 2002). Thus, RIN4 might be the target, or be a partner in a complex with the target(s), of the AvrRpm1 and AvrB virulence function(s). To study the relationship between the virulence activities of these type III effectors and RIN4, we tested whether the absence or over-expression of RIN4 alters the phenotypes associated with AvrRpm1 and AvrB in *rpm1 rps2*, *rin4 rpm1 rps2* or *OxRIN4 rpm1* plants (Mackey et al., 2003). *Pma M6CΔE(vector)* grew to intermediate levels (Figure
Figure 3.7: RIN4 is not the only virulence target for AvrRpm1 and AvrB in Arabidopsis. (A) Growth of *Pma* M6CΔE carrying empty vector or *avrRpm1* indicated at bottom was measured on the genotypes indicated at top. Four-week-old plants were infiltrated with $10^4$ cfu/ml and the number of bacteria per area of leaf plotted on a log$_{10}$ scale for day 0 (white columns) and day 3 (black columns) (see Methods). Error bars represent the standard deviation among four samples and this experiment is representative of three independent replicates. The absence of error bars indicates insignificant differences. (B) *Agrobacterium* carrying empty vector or dexamethasone (DEX) inducible *avrB-HA* as indicated at bottom were inoculated onto leaves of various genotypes indicated at top, at $10^{10}$ cfu/ml. Leaves were sprayed 24 hours post-inoculation (hpi) with DEX (20μM), and photographed 96 hours after that. Total protein extracts were prepared 96 hours after DEX and subjected to an anti-HA Western blot.
This growth was unaffected by the expression level of RIN4, and was RPM1 and RPS2 independent (data not shown). *Pma M6CΔE(avrRpm1)* growth in wild type Col-0 was significantly reduced, due to RPM1 action, compared to growth in *rpm1 rps2, rin4 rps2 rpm1* or *OxRIN4 rpm1* plants. The virulence activity of AvrRpm1 (Ritter and Dangl, 1995; Rohmer et al., 2003) causes *Pma M6CΔE(avrRpm1)* to grow reproducibly 10-fold more than *Pma M6CΔE(vector)* in *rpm1*. This was observed on each *rpm1* genotype tested, including *rin4 rpm1 rps2* (Figure 3.7A). We conclude that the lack, or over-expression, of RIN4 does not affect this virulence activity of AvrRpm1.

We carried out a similar set of experiments with *Pma M6CΔE(avrB)* (not shown). Unlike AvrRpm1, AvrB is not able to promote pathogen growth on *rpm1*, though it can add to *P. syringae* virulence on susceptible soybean genotypes (Ashfield et al., 1995). Altered levels of RIN4 did not alter the growth of this strain compared to *Pma M6CΔE (vector)* on any tested plant line (data not shown).

AvrB can cause a chlorotic response when expressed in *rpm1*, potentially indicative of its virulence activity (Nimchuk et al., 2000). We addressed whether modifications of RIN4 levels alter this phenotype. Figure 3.7B demonstrates that AvrB-dependent chlorosis in *rpm1* is RIN4 independent. Further, AvrB accumulates in a RIN4-independent manner (the modest difference in the levels of AvrB in this experiment is sporadic and does not correlate with expression of RIN4, not shown). The results presented in Figure 3.7 indicate that while RIN4 is certainly an avirulence target for both AvrRpm1 and AvrB, it is not their only virulence target.
Alternatively, a direct requirement of RIN4 for the virulence activities of AvrRpm1 and AvrB cannot be measured in our assays.

**DISCUSSION**

This work was aimed at clarifying the role of the Arabidopsis RIN4 protein in the control of RPM1 and RPS2 activation. We further tested whether RIN4 is the unique target of AvrRpm1, AvrB and AvrRpt2 when these type III effectors function as virulence factors. We show that RIN4 has a negative regulatory function that blocks the inappropriate activation of RPM1, in addition to a similar regulatory function previously established for RIN4 in RPS2 activation (Axtell and Staskawicz, 2003; Mackey et al., 2003). We propose that wild type levels of RIN4 are required to maintain RPM1 and RPS2 in a non-signaling configuration. We demonstrate that inappropriate RPS2 activation, leading to lethality in rin4 plants, is quantitatively dependent on RAR1, but independent of NDR1. The latter observation differentiates this mode of RPS2 activation from its normal, AvrRpt2-driven activation and strongly indicates that RIN4 functions at, downstream, or independently of NDR1 to control RPS2 activity. We also demonstrate that RIN4 is not the only target of AvrRpm1, AvrB and AvrRpt2 with respect to the virulence activities of these three type III effectors. Surprisingly, RIN4 negatively regulates at least one virulence activity of AvrRpt2. We propose that *P. syringae* type III effector proteins may frequently have multiple targets in susceptible plants. Their manipulation of a subset of these targets (one, in fact), is demonstrably sufficient for activation of at least RPM1 and RPS2.
Our data extend the notion that NB-LRR proteins monitor the activities of type III effector proteins expressed by pathogenic bacteria, and have implications for the evolution of the plant immune system.

**RIN4 negatively regulates inappropriate RPM1 activation**

The *rin4* lethality was largely suppressed in a *rin4 rps2* double mutant, proving that inappropriate RPS2 activation is negatively regulated by RIN4 (Mackey et al., 2003). Yet residual signaling in *rin4 rps2* is sufficient to drive enhanced basal defense against *Pto* DC3000 (Figure 3.1) and PR1 expression (Figure 3.2A). The residual RPM1 present in *rin4 rps2* is responsible for these phenotypes, as they are eliminated in *rin4 rps2 rpm1* triple mutants. Note that this residual RPM1 is not competent to transduce AvrRpm1- or AvrB-dependent signals (Figure 3.1; and not shown). Thus, RIN4 also negatively regulates inappropriate RPM1 activity. Wild type RIN4 levels are apparently saturating for maintaining RPM1 in an inactive state, as neither a doubling of the RPM1 dose (Figure 3.2B) nor RIN4 over-expression (Mackey et al., 2002) affects RPM1 function. RPM1 was inappropriately active in wild-type plants when over-expressed (Leister and Katagiri, 2000), possibly due to an elevated RPM1/RIN4 ratio.

Four related models can explain these data. 1) RPM1 is activated in *rin4* plants because RIN4 is a negative regulator of RPM1 activation and that regulation is lacking. The lowered RPM1 levels we observed in *rin4K-D* (Figure 3.2B) would then be a consequence of RPM1 disappearance following its activation (Boyes et al., 1998). 2) Specific RPM1 activation might require the physical interaction of AvrRpm1
or AvrB with RIN4 (Mackey et al., 2002) or a RIN4-containing complex, and that interaction could be disrupted when residual RPM1 mis-accumulates in the absence of RIN4. 3) Residual, activated RPM1 might lose its responsiveness to AvrRpm1 and AvrB. This would be analogous to CARD15/NOD-2 variants that ectopically activate the NF-κB pathway, but lose responsiveness to LPS and subsequent, appropriate NF-κB activation (Tanabe et al., 2004). 4) RPM1 simply might not accumulate enough in the absence of RIN4 to allow a robust AvrRpm1- or AvrB-specific response in rin4 plants. This possibility, though, is inconsistent with the established notion that NB-LRR protein activation requires a lower threshold of signal than does activation of basal defense (Tao et al., 2003).

Lowering of RPM1 levels, however, is not necessarily accompanied by activation of basal defense. Arabidopsis rar1 mutants accumulate very low levels of RPM1, but display normal susceptibility to Pto DC3000 (Tornero et al., 2002), rather than the enhanced resistance that we observed in rin4 rps2. Arabidopsis athsp90.2 mutants also express severe RPM1 reduction that is correlated with a diminution of RPM1 function (Hubert et al., 2003). Thus, RPM1 is destabilized in atrar1 or athsp90.2 without concomitant activation of basal defense. This is consistent with a proposed function of RAR1/SGT1/HSP90 for assembly of signal-competent RPM1 "upstream" of any activation (Hubert et al., 2003; Schulze-Lefert, 2004).

Activation of the Rx NB-LRR protein is dependent on finely tuned intramolecular interactions (Moffett et al., 2002; Rathjen and Moffett, 2003). Intramolecular interactions are often conditioned and modulated by intermolecular interactions (Autiero et al., 2003; Djordjevic et al., 1998). The inappropriate RPM1
activation in *rin4 rps2* might also be due to the consequences of intra-molecular changes induced by the absence of normal interactions between RPM1, RIN4 and other putative components. This model is consistent with a possible requirement for RIN4 phosphorylation during AvrRpm1- or AvrB-induced activation of RPM1, as phosphorylation events are known to induce changes in protein-protein interactions (Djordjevic et al., 1998).

**Inappropriate RPS2 activation is independent of NDR1 and modulated by RAR1**

NDR1 is required for AvrRpt2-driven activation of RPS2. It was previously shown that NDR1 is not required for the AvrRpt2-induced disappearance of RIN4 (Axtell and Staskawicz, 2003). Here, we show that NDR1 is not required for RPS2 accumulation, gross localization, or association with RIN4. Thus, three important requirements for the RIN4-dependent activation of RPS2 by AvrRpt2 are NDR1-independent. These results corroborate our genetic demonstration that *ndr1* is not able to suppress inappropriate RPS2 activation in *rin4*. Thus, the events leading to either AvrRpt2-driven RPS2 activation or its inappropriate activation in *rin4* are separable. Very little is known about how NDR1 functions in NB-LRR activation. Based on our data, we propose that NDR1 does not affect 1) NB-LRR stability, or 2) NB-LRR localization, and 3) that NDR1 is not required for signaling downstream of NB-LRR protein activation. Instead we envision that NDR1 functions upstream of NB-LRR activation by various pathogens.

RAR1 is required for RPS2 and RPM1 signaling in Arabidopsis (see Introduction). The accumulated data indicates that RAR1 limits defense signal flux,
perhaps by modulating NB-LRR stability or accumulation (Tornero et al., 2002). Our results indicate that RAR1 also modulates RPS2-HA accumulation (Figure 3.3). Heightened RPS2 signaling capacity, presumably achieved by slight over-expression, can partially overcome the lack of RAR1 in rar1 rps2 (RPS2-HA) plants (Figure 3.6). We propose that RAR1 acts generally on NB-LRR proteins by controlling their accumulation and/or stability, and not by modulating a common downstream signal.

**AvrRpt2, AvrRpm1 and AvrB manipulate basal defense**

The enhanced resistance against Pto DC3000 in rin4 rps2 plants is abrogated when the bacteria express AvrRpm1 or AvrRpt2 (Figure 3.1), or AvrB (not shown). Thus, these proteins can presumably suppress the basal defense activated in rin4 rps2. Our findings are also consistent with recent data indicating that AvrRpt2 acts as a virulence factor downstream, or independent, of SA accumulation (Chen et al., 2004) and with recent data suggesting that a variety of P. syringae type III effectors manipulate plant basal defense responses (Abramovitch and Martin., 2004).

*Pto* DC3000(avrRpm1) and *Pto* DC3000(avrRpt2) suppress the enhanced basal resistance against *Pto* DC3000 observed in *rin4 rps2* (Figure 3.6). These data clearly indicate that RIN4 is either not a virulence target or not the only target for AvrRpm1 and AvrRpt2 in *rin4 rps2*. In fact, AvrRpt2-dependent virulence is enhanced in *rin4 rps2* (Figure 3.6; see below). The enhancement of AvrRpt2-dependent virulence on *rin4 rps2* was also observed when it was delivered from Pma M6CΔE (Figure 3.6). Because we did not observe enhanced resistance against
*Pma* M6CΔE on *rin4 rps2*, AvrRpt2 may enhance the growth of this strain in a manner distinct from its function in *Pto* DC3000.

**rar1 and ndr1 mutations enhance AvrRpt2 virulence function(s)**

*ndr1* plants are impaired in basal defense responses (our unpublished data). AvrRpt2 was recently shown to promote virulence in *rps2* by suppressing defense gene expression downstream or independent of SA (Chen et al., 2004). We extend these results by demonstrating that *ndr1 rps2* and *rar1 rps2* support significantly more AvrRpt2-dependent *Pma* M6CΔE growth than *rps2* (Figure 3.6). Hence, the loss of basal defense signaling normally induced via *NDR1* and *RAR1* enhances the observed effect of AvrRpt2. We therefore propose that there are multiple basal defense pathways that are downstream or independent of SA. Some of these are targeted by AvrRpt2, while others are *NDR1* and/or *RAR1* dependent.

**RIN4 is not the only target of AvrRpm1, AvrRpt2, or AvrB**

If each type III effector has a specific, single host target, then it follows that elimination of that target would diminish pathogen virulence. We hypothesized that elimination of RIN4 in the *rin4 rps2 rpm1* triple mutant would allow us to determine whether the known virulence function of AvrRpm1 requires RIN4. Our data clearly indicate that AvrRpm1 virulence function and AvrB-dependent chlorosis are maintained (Figure 3.7), and that AvrRpt2 function is unexpectedly enhanced (Figure 3.6) in *rin4 rps2*. Thus, while RIN4 is assuredly a target of AvrRpm1, AvrB and AvrRpt2 (Axtell and Staskawicz, 2003; Mackey et al., 2003; Mackey et al., 2002), it
is not the only target for any of them. We propose that type III effectors from *P. syringae*, like those from *Shigella flexneri*, have multiple host cellular targets (Hilbi et al., 1998; Lafont et al., 2002).

We established that, surprisingly, RIN4 negatively regulates virulence mediated by AvrRpt2 (Figure 3.6). AvrRpt2 encodes a probable cysteine protease, and it was proposed that this activity destabilizes RIN4 or a RIN4 containing complex (Axtell et al., 2003). Our observations of 1) increased bacterial growth mediated by AvrRpt2 on rin4 rps2 plants, and 2) reversal of that effect by RIN4 overexpression fit a model where a limited number of translocated AvrRpt2 molecules could operate on several cellular substrates. We envision that the specific activity of the AvrRpt2 protease for other substrates is increased in rin4 plants. As a result, the other targets are neutralized more quickly or more efficiently and the fitness of the bacteria on rin4 plants is increased. Alternatively, RIN4, like NDR1, regulates a basal defense pathway that is possibly targeted by AvrRpt2.

**Is RIN4 the only bacterial type III effector target “guarded” by RPM1 and RPS2?**

RIN4 is evolutionarily conserved in at least rice, maize, tobacco, tomato and potato (unpublished). The functional association of two NB-LRR proteins (RPM1 and RPS2) with RIN4 in Arabidopsis, combined with RIN4’s conservation, raises the possibility that RIN4 regulates defense responses in those plant species as well. Our work indicates, though, that RIN4 is not the only virulence target of AvrRpm1, AvrB and AvrRpt2. It is thus legitimate to question whether RPS2 and RPM1 monitor the
homeostasis of RIN4 alone or, alternatively, of RIN4 and a subset of other AvrRpm1, AvrB, and AvrRpt2 targets.

Ashfield et al., 2004 very recently demonstrated that the NB-LRR protein that recognizes AvrB (but not AvrRpm1) in soybean, \textit{Rpg1-b}, is not the closest ortholog of RPM1. They further showed that AvrRpt2 could interfere with AvrB dependent activation of Rpg1-b, consistent with results in Arabidopsis (Ritter and Dangl, 1996), but that this interference may not be due to the AvrRpt2-dependent elimination of RIN4, as observed in Arabidopsis (Mackey et al., 2003). Further, they saw no clear AvrB-dependent mobility changes in anti-RIN4 cross-reacting bands in soybean protein extracts. Thus, although more work remains to be done, it may be that Rpg1-b is not associated with RIN4, but rather with another host target of both AvrB and AvrRpt2.

The evolution of a single NB-LRR protein guarding any of the several potential targets of a given virulence factor is demonstrably sufficient to initiate successful disease resistance against pathogen strains expressing that virulence factor. Particularly effective virulence factors would presumably spread through the pathogen population, at frequencies balanced by the rate of evolution of NB-LRR proteins that detect their action. This might drive evolution of multiple NB-LRR genes, whose products recognize the action of a single virulence factor at different targets.

There may be, however, fundamental evolutionary pressures limiting the number of targets that a particular NB-LRR protein can simultaneously guard. The first is structural. If the various virulence factor targets are divergent, a single NB-
LRR protein might not be able to productively interact with all of them. The second may be reflected by the fact that maintenance of RPM1 expression in Arabidopsis results in a substantial fitness cost for the plant (Bergelson et al., 2001; Grant et al., 1998; Stahl et al., 1999). This might be generally true, since constitutive NB-LRR activation results in cell death (Collins et al., 1999; Hu et al., 1996; Shirano et al., 2002; Zhang et al., 2003). Thus, a potential explanation for the apparently limited number of AvrRpm1, AvrB and AvrRpt2 targets that are effectively guarded by RPM1 and RPS2 could be an inherent fitness cost associated with increasing NB-LRR expression levels. An increase in the number of host targets guarded by a particular NB-LRR protein might result in an increase in overall levels of that protein and an attendant fitness costs.

Materials and Methods

*Pseudomonas syringae*

*Pto* DC3000 carrying either pVSP61 or derivatives of this plasmid containing *avr* genes have been described (Mackey et al., 2003; Mackey et al., 2002) and *Pma* M6CΔE is a derivative of a weakly virulent isolate of *P. syringae* pv. *maculicola* (Rohmer et al., 2003). Bacterial growth in plant leaves was measured by two methods. Figure 3.1 was done by inoculating four weeks old plants with $10^5$ cfu/ml. In Figures 3.5 and 3.6, four weeks old plants were inoculated with $10^4$ cfu/ml. For each sample, four leaf discs were pooled, four times per data point (16 leaf discs
total). Leaf discs were bored from the infiltrated area, ground in 10 mM mgCl₂, and serially diluted to measure bacterial numbers.

Protein

Total protein extracts were prepared, and cell fractionation and co-immunoprecipitation assays performed, as described in (Mackey et al., 2003; Mackey et al., 2002). Anti-RIN4 serum was used at a dilution of 1:5000. The Anti-PR-1 serum (gift of Dr. Robert A. Dietrich, Syngenta) was used at a dilution of 1:10000. The anti-RD28 and anti-APX (respectively gifts of Dr Maarteen Chrispeels and Dr Daniel Kliebenstein) antibodies were used at a dilution of 1:5000. Detection of HA and myc epitope tags was with supernatants from cultures of hybridoma 3F10, monoclonal anti-HA antibody (Roche), at a dilution of 1:1000 and the hybridoma 9E10, monoclonal anti-myc antibody, at a dilution of 1/10 (Boyes et al., 1998).

Plants and mutant construction

The following plant genotypes were used in this work: rps2-101C in an allele of RPS2 in Col-0 with a stop codon following amino acid 235 (Bent et al., 1994); rpm1-3 is an allele of RPM1 with a stop codon following amino acid 87 (Grant et al., 1995). The rin4 null is a T-DNA insertion in the RIN4 ORF in Col-0 (Mackey et al., 2003). The rin4K-D is a T-DNA insertion in the promoter of RIN4 in Ws-0 (Mackey et al., 2002). The triple mutant rin4 rpm1 rps2 was constructed like the rin4 rps2 double
mutant described in (Mackey et al., 2003) using the Col-0 *rin4* null allele. The RPM1 PCR product was digested with EcoRV which cut the wild type, but not *rpm1-3*, into a doublet. The *rin4K-D RPM1-myc* line was made by crossing a Ws-0 based *RPM1-myc* transgenic line to the Ws-0 *rin4 K-D* plants. The *rin4 K-D* plants were used as a pollen source. *RPM1-myc* was followed by hygromycin resistance and *rin4K-D* was followed phenotypically. The *RPM1-myc* and *rin4 K-D RPM1-myc* plants in the Ws-0 background have both an endogenous and the transgenic copy of *RPM1*. Mutant alleles of the *ndr1-1* null (Century et al., 1997) and the premature stop in *rar1-21* (Tornero et al., 2002) were PCR selected using primers and conditions are available on request.

**Agrobacterium Transient Expression Assays**

2 ml overnight *Agrobacterium* cultures were grown at 30°C in YEB (5 g bacto beef extract, 1 g bacto yeast extract, 5 g bacto peptone, 5 g sucrose, 2 mM MgSO₄, pH 7.2, per liter) containing 100 mg/ml each of rifampicin, kanamycin, and gentamycin for strain GV3101. The following day, 150 ml of saturated culture was inoculated into 3 ml of YEB plus antibiotics, and grown for 13 hr. Two milliliters were collected and re-suspended in 3 ml *Agrobacterium* induction medium (10.5 g K₂HPO₄, 4.5 g KH₂PO₄, 1 g (NH₄)₂SO₄, 0.5 g (NaCitrate), 1 mM MgSO₄, 1 g glucose, 1 g fructose, 4 ml glycerol, 10 mM MES, pH 5.6, per liter, 50 mg/ml acetylsyringone), grown at 28°C for 5–7 hr., collected and resuspended in infiltration medium (1/2 MS-MES) to an OD600 of 0.4. The underside of 3-week-old leaves was inoculated using a needle less syringe. Plants were grown in 120 µE of light and
sprayed with 20 μM DEX (Sigma, St. Louis, MO). To inducibly express AvrB in planta, the gene with a carboxy-terminal HA-tag was cloned into pTA7002 (Aoyama and Chua, 1997).
Bibliography


CHAPTER 4

A second generation forward screen for Arabidopsis mutants unable to recognize avrRpm1

Preface

This chapter represents all unpublished work. Over the years several people have contributed towards it progress. Undergraduate Jonny Chen helped with the initial screening. Allison Osborne helped with finishing the confirmatory Estradiol retest and the initial bacterial tests. Anna Newton also helped with the bacterial tests. David Rybnicek and Tim Eitas have worked on the characterization of lra6.

Abstract

Plants recognize pathogens by an evolutionarily conserved innate immune system. Central to the immune response are disease Resistance (R) genes required by the plant to recognize specific avirulence (avr) proteins encoded by the pathogen. In this paper, we present the results of a second generation genetic screen to identify additional loci required for the function of the Arabidopsis R gene RPM1, which confers resistance to Pto DC3000 (avrRpm1). The screen builds on a previous screen for mutants unable to respond to inducible expression of AvrRpm1. This screen improves on this previous screen by utilizing an additional, transgenic copy of RPM1 to prevent the repeated identification of multiple rpm1 alleles.
Additional changes in screening methodology are described which aided in identifying several novel loci, one of which, \textit{lra\textit{6}}, compromises the function of several \textit{R} genes. As opposed to previously identified mutants that block accumulation of \textit{R} proteins, \textit{lra\textit{6}} mutants appear to block accumulation of \textit{R} gene transcript.

\textbf{Introduction}

Plants must fight pathogens or die. While the plant arsenal for use against pathogens ranges from a build-up of cell wall fortifications to the release of toxins, nearly all resistance responses require pathogen recognition to function maximally. The basis of that recognition can be found in plant disease \textit{Resistance (R)} genes (Flor, 1971). \textit{R} proteins are highly specific and recognize at most one or two pathogen gene products. Given that \textit{R} protein recognition of pathogen gene products enables the plant to mount an effective defense response, these pathogen genes have been dubbed \textit{avirulence (avr)} genes.

Immediately, one questions why pathogens maintain genes that trigger a plant resistance response. Obviously, they must confer to the pathogen some evolutionary advantage. After the first \textit{avr} genes were cloned, it became apparent that, in the absence of pathogen recognition by the plant, these \textit{avr} genes allow for better pathogen growth in plants (Ritter and Dangl, 1995). Thus, in the absence of their corresponding \textit{R} gene, \textit{avr} genes act as virulence factors.

While \textit{avr} gene products are structurally and sequence diverse, \textit{R} proteins have a much more conserved structure. The majority of \textit{R} proteins contain a nucleotide binding site (NB) domain and a C-terminal leucine-rich repeat (LRR)
domain. The NB domain has been shown to bind and hydrolyze ATP, while LRRs are often protein-protein interaction domains (Kobe and Kajava, 2001; Tameling et al., 2002). The N-terminal domain differentiates the two major classes of R proteins. One class contains a domain with homology to Toll and the Interleukin-1 receptor and is referred to as TIR domain (Dangl and Jones, 2001). The other class has an N-terminal coiled-coil domain. R genes in this class are referred to as CC-NB-LRR. These two classes of R genes differ in their functional requirement for additional loci.

Three loci, EDS1, PAD4, and SAG101, are specifically required for TIR-NB-LRR signaling, and are not required for the function of CC-NB-LRR type R proteins (Feys et al., 2005). While no locus has been shown to be specifically required for CC-NB-LRR function, several loci have been shown to be required for the function of both classes of NB-LRR protein. These include the genes NDR1, RAR1, SGT1, and HSP90 (Azevedo et al., 2002; Hubert et al., 2003; Liu et al., 2004; Schornack et al., 2004; Tornero et al., 2002b; van der Biezen et al., 2002). Interestingly, the majority of these loci play a role in NB-LRR protein stability (Holt III et al., 2005; Hubert et al., 2003; Tornero et al., 2002b).

The Arabidopsis R gene RPM1 encodes a CC-NB-LRR protein that recognizes the Pseudomonas gene products AvrB and AvrRpm1 (Grant et al., 1995). No direct interaction has been reported between RPM1 and either of these two avirulence proteins. However, another plant protein, RIN4, can interact with both RPM1 and AvrB (Mackey et al., 2002). RIN4 becomes phosphorylated in the presence of either AvrB or AvrRpm1. Currently, it is understood that this post-translational modification of RIN4 serves as the trigger of RPM1 action.
Consistent with the genetic requirements of other CC-NB-LRR type \textit{R} genes, \textit{RPM1} does not require \textit{EDS1}, \textit{PAD4}, or \textit{SAG101} (Feys et al., 2005), nor has any requirement for either of the two paralogs of Arabidopsis \textit{SGT1} been observed (Holt III et al., 2005). On the other hand, there are clear requirements for \textit{NDR1}, \textit{RAR1}, and \textit{HSP90} for \textit{RPM1} function (Hubert et al., 2003; Tornero et al., 2002b). Additionally, null mutations in \textit{rar1} and specific mutations within the ATPase domain of one of the four paralogs of Arabidopsis cytosolic \textit{HSP90}, \textit{hsp90.2}, lead to loss of accumulation of \textit{RPM1} protein (Hubert et al., 2003; Tornero et al., 2002b). \textit{SGT1}, \textit{RAR1}, and \textit{HSP90} can physically interact, and \textit{HSP90} can physically interact with \textit{RPM1} (Hubert et al., 2003). Thus, loss of \textit{RPM1} accumulation in \textit{rar1} and \textit{hsp90.2} likely occurs through a similar, though currently unknown, mechanism.

We were interested in finding additional loci required for \textit{RPM1} function. In order to uncover these genetically-required loci, we performed a large-scale mutant screen, based on a previously described transgenic system (see below; Tornero et al., 2002a). Our new screen was modified to solve several problems present in the original screen. These problems included a high number of mutations in \textit{RPM1} (over ninety), identification of few other loci (only four), loss of several potentially interesting mutants due to plant stress, and loss of time due to a need to introgress in necessary genetic material. This new screen attempted to rectify these problems with the addition of a second, transgenic copy of \textit{RPM1} and major changes in methodology.

We identified several, apparently novel loci based on the accumulation of \textit{RPM1} protein and sequencing of candidate genes. These mutants fell into three
classes: low accumulators of RPM1 reminiscent of rar1 and hsp90.2, mutants that showed no accumulation of RPM1, and, finally, mutants with normal accumulation of RPM1. This last class is perhaps the most exciting, since they are likely to define genes required for RPM1 signal transduction. We also describe new alleles of rar1 and hsp90.2. Finally, we provide initial genetic and pathology characterization data for lra6, a recessive mutant falling into the “no RPM1 accumulation” class. We present evidence that lra6 affects RPM1 transcript levels and is required for maximal function of several other CC-NB-LRR type R genes.

Results

A novel genetic screen to identify genes required for RPM1 function

In order to identify new genes required for RPM1-mediated recognition of avrRpm1, we undertook a second generation forward genetic screen. This screen relied on a previously described β-Estradiol-inducible avrRpm1 transgenic system (Tornero et al., 2002a). In the initial screen utilizing this system, several problems were encountered, as explained above, which we attempted to ameliorate in this new screen.

In the previous screen, over ninety loss-of-function alleles of rpm1 were identified. While this over-abundance of rpm1 alleles enabled an elegant structure-function analysis of RPM1, the excess undoubtedly prevented the identification of rare mutations in additional loci required for RPM1 function (Tornero et al., 2002a). To prevent the identification of more loss-of-function mutations in rpm1 in our new
screen, we used plants containing an additional, transgenic copy of RPM1 (Boyes et al., 1998). Since these plants effectively carry four copies of RPM1 at two loci, we hoped to greatly reduce the possibility of identifying loss-of-function mutations in rpm1.

Mutations in previously identified loci affecting RPM1 function have often affected the accumulation of RPM1 protein (Hubert et al., 2003; Tornero et al., 2002b). For instance, mutations in both rar1 and hsp90.2 resulted in significantly lower accumulation of RPM1 protein (Figure 4.3A). Unfortunately, a native RPM1 antibody which would easily allow us to monitor RPM1 accumulation in any newly identified mutant is unavailable. Thus, the additional, transgenic copy of RPM1 used in this new genetic screen was expressed from one kb of the native RPM1 promoter and translationally fused to a C-terminal c-myc-epitope (Boyes et al., 1998). RPM1 protein levels in the newly identified mutants can thus be monitored with anti-myc antibodies. Hence, the genotype of the plants used in this new screen was Arabidopsis ecotype Col-0 containing an Estradiol-inducible copy of avrRpm1 and carrying both the wild-type endogenous copy of RPM1 as well as an additional transgenic copy of RPM1-myc.

A corollary of the large number of rpm1 alleles obtained in the previous screen was the comparatively low number of alleles of other genes (Tornero et al., 2002a). The previous screen yielded 8 alleles of rar1 and 4 alleles of hsp90.2 (Hubert et al., 2003; Tornero et al., 2002a). One possible explanation for the observed low frequency of alleles in other loci required for RPM1 function was that the high mutation rate of rpm1 made finding an allele of rpm1 far more likely than
finding a rarer allele of another locus. Other possibilities include redundancy and/or lethality of additional components required for RPM1 function. Additionally, the Estradiol-driven *avrRpm1* transgene is itself a target for mutagenesis, and hits therein would lead to a false positive “loss of RPM1 function” phenotype. Our previous screen uncovered a great number of these (Tornero et al., 2002a).

We choose to attack these limitations through both the addition of the second copy of *RPM1* detailed above and through changes in the methodology of the second generation screen. Normally, in Arabidopsis screens, seeds are treated with mutagen and plants grown from them that contain mutated gametes. Small numbers of resulting M1 generation plants are allowed to self-fertilize, generating pools of M2 seed that may contain either homo- or heterozygous mutant alleles. The dose of Ethyl methanesulfonate (EMS) we used is thought to give rise to roughly 30 hits per haploid genome (Meyerowitz and Somerville, 1994). Each such M2 seed collection is termed an independent seed “lot”. Hence, one could isolate from any given lot sibling mutations, or independent mutations. Because sibling mutations are more likely in a given lot than independent mutations, Arabidopsis screens typically collect only 2-3 putative mutants per seed lot (Page and Grossniklaus, 2002). We arbitrarily choose to select eight putative mutants from each lot.

Sibling mutants likely display similar phenotypes. Thus, in order to increase the likelihood of isolating multiple mutants per mutagenized seed lot, we selected a range of phenotypes by arbitrarily collecting eight putative mutants per M2 seed lot screened. By choosing individuals of varying phenotypic severity, we sought to
decrease our likelihood of simply collecting eight individuals containing the same mutation. As an added benefit, choosing mutants of varying phenotypic severity allowed us to recover loci not recovered in the previous screen, which relied on strong loss-of-function phenotypes. We expected to consequently recover mutations in genes which contribute quantitatively to \( \text{RPM1} \) function.

We also anticipated that recovery of more rare alleles would be increased if we increased the number of total plants and lots screened. The previous screen was performed on roughly \( 5 \times 10^5 \) plants in 172 independent M2 seed lots (Tornero et al., 2002a). In this new screen we looked at approximately \( 1 \times 10^6 \) plants from 200 independent M2 seed lots.

Putative M2 mutant plants were recovered in the previous screen as loss of response mutants following Estradiol application (and transcriptional activation of \( \text{avrRpm1} \)) (Figure 4.1, Step 1). These were subjected to a secondary screen three weeks later (in the same generation) that entailed infection of leaves with \( \text{Pto DC3000 (avrRpm1)} \) (Figure 4.1, Step 3) (Tornero et al., 2002a). This secondary screen was performed to remove mutations in the Estradiol-inducible system since such plants would still respond to bacterially-delivered AvrRpm1. This method, though effective in removing false loss of response mutants, also caused a great deal of physiological stress, resulting in a high mortality rate. Therefore, in the current screen we decided to perform two secondary screens in the M3 generation (following self-fertilization of the eight single M2 putative mutants collected per seed lot). In this manner, we hoped to increase generally our recovery of mutants, but more importantly to increase the recovery of mutants with intermediate phenotypes.
This change is important because mutants with intermediate phenotypes are precisely those that would have experienced the most physiological stress from the original screen’s selection regime.

**Performance of screen and identification of putative mutants**

Our primary screen recovered 1187 putative mutants from 192 independent M2 seed lots (Table 4.1, “Primery Screen”). To confirm their lack of response to Estradiol-driven expression of *avrRpm1*, we first re-applied Estradiol to the M3 families obtained by selfing of the M2 putative mutants from the primary screen (Figure 4.1, Step 2). This confirmatory step was necessary since choosing mutants with subtle phenotypes increased our likelihood of recovering non-mutant individuals. In fact, this step removed 164 M3 mutant families (Table 4.1, “Confirmation of Phenotype”). While roughly 16% of the putative mutants were thus removed in this retest, 189 independent M2 seed lots were still represented. However, due to low fertility in some M3 families, we had to obtain more seeds, and consequently perform our secondary screens on these families in the M4 generation (Table 4.1, “Bulked”). Given the added time required to obtain testing material for these lines, we did not perform the Estradiol re-test on these lines and carried them directly forward to the second, secondary screen.

The purpose of our second, secondary screen was the removal of mutations in the *avrRpm1* inducible system. Previously, Tornero et al., 2002a removed Estradiol-inducible *avrRpm1* system mutations by hand inoculation of high titres of
Step 1 - M2
Primary Screen

Step 2 - M3
Confirmation of Phenotype

Step 3 - M3
Removal of Estradiol System mutants

Step 4 - M3
Removal of Contaminants

Step 5 - M3
Characterization of RPM1-myc Levels
Figure 4.1: Flowchart depicting the screening process used to identify new loci required for *RPM1* function. The action undertaken in each major step of the screening process, the generation of plants used for each step, and the purpose of each step are indicated to the right. Those plants which do not respond to Estradiol, or exhibit disease resistance to *Pto* DC3000 (*avrRpm1*) are indicated as dark green. Plants responding to Estradiol, or are disease susceptible in bacterial dip assays are indicated by their pale green color. Analysis of PCR marker amplification is indicated by white bands on a dark grey field, while western blot analysis of RPM1-myc levels is indicated as dark bands on a pale grey field.
avrRpm1 expressing Pto DC3000, followed by observation of the hypersensitive response (HR), a form of programmed cell death thought to limit the spread of some pathogens. Since we decided to perform all of our secondary screens in the M3 generation, hand inoculation of more than 6000 plants in 90 flats was not an appealing option. Instead, we analyzed disease symptoms in plants dipped into a suspension of Pto DC3000 expressing avrRpm1 (Figure 4.1, Step 3). We expected that true mutants that lost RPM1 function would be disease susceptible, while “system hits” in the Estradiol-inducible avrRpm1 expression transgenes would retain RPM1 function and remain resistant to Pto DC3000 (avrRpm1). Of the 1023 putative mutant progenies tested in this manner, 892 families, or 87%, were found to be resistant, indicative of system hits. These families were not studied further.

33 independent mutants were found to have intermediate phenotypes after bacterial inoculation, in that their level of disease symptoms lay between full susceptibility and resistance when compared to appropriate genetic controls (Figure 4.2, Table 4.1, Step 3). A further group of 28 independent mutants were fully disease susceptible. Finally, 30 independent mutants showed clear segregation in their response to bacteria. Only 14 independent seed lots contained individuals which fell into more than one loss of resistance phenotypic category (Figure 4.2), suggesting a total of 76 independent mutants. Given the large number of mutants obtained, we decided to focus our energies on the 28 mutants displaying a fully disease susceptible phenotype (Figure 4.2, Table 4.1, Step 3).

Removal of contaminants and initial characterization of mutants
<table>
<thead>
<tr>
<th>Step 1 Primary Screen</th>
<th>Step 2 of Phenotype- Estradiol Retest</th>
<th>Confirmation</th>
<th>Step 3 DC3000 (AvrRpm1) Dip Assay</th>
<th>Plo</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1187 (192) Responder</td>
<td>164 Non-Responder 753</td>
<td>665 Resistant</td>
<td>33 (22) Intermediate 33 (24) Segregating 22 (22)</td>
<td>88 (55)</td>
<td></td>
</tr>
<tr>
<td>24 Intermediate</td>
<td></td>
<td>16 Resistant</td>
<td>5 (5) Intermediate 0 (0) Segregating 4 (3)</td>
<td>9 (6)</td>
<td></td>
</tr>
<tr>
<td>62 Segregating Response</td>
<td></td>
<td>51 Resistant</td>
<td>2 (2) Intermediate 6 (5) Segregating 3 (3)</td>
<td>11 (10)</td>
<td></td>
</tr>
<tr>
<td>184 Bulked- Not Retested with Estradiol</td>
<td></td>
<td>160 Resistant</td>
<td>10 (9) Intermediate 2 (2) Segregating 12 (11)</td>
<td>24 (18)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total Resistant 892</td>
<td>Total Intermediate 50 (33)</td>
<td>Total Segregating 41 (30)</td>
</tr>
</tbody>
</table>
Table 4.1: Identification of 28 independent putative disease susceptible mutants, grouped by screening step and phenotypic class. Indicated steps correspond to those shown in Figure 4.1. M3 families, defined as progenies from self-fertilized individual M2 plants, which showed full response to \textit{avrRpm1} induction are indicated as Responder under the column corresponding to Step 2. M3 families in which all individuals showed no response to Estradiol induction of \textit{avrRpm1} are classed as Non-responder. M3 families which only weakly responded or families displaying a mixture of responding and non-responding plants are classed as Intermediate or Segregating Response, respectively. In Step 3, M3 families in which all individuals were resistant to \textit{Pto DC3000 (avrRpm1)} in a dip assay are placed in the Resistant class and are discarded as mutations in the inducible system. Individuals in Intermediate families showed slight loss of disease resistance to \textit{Pto DC3000 (avrRpm1)}. Segregating families contained both individuals resistant to \textit{Pto DC3000 (avrRpm1)} and individuals which were susceptible. In Susceptible families, all infected individuals showed complete loss of resistance when compared to a negative genotypic control. The number of M3 families, and hence M2 mutants, falling into each phenotypic class is indicated, and the number of M2 seed lots represented by these phenotypic classes is shown in parentheses.
Any genetic screen of the magnitude presented here is prone to contamination from outside genetic material. Fortunately, given the several transgenes present in the parental line used for this screen, we were easily able to distinguish contaminating genotypes from true mutants. To determine if the M3 family (progeny from a self fertilized M2 mutant) represented a contamination event, we used dominant PCR-based molecular markers which amplify transgenes present in the screen’s parental line (Figure 4.1, Step 4). The first was a primer pair which amplifies the conditional expression cassette that contains avrRpm1, while the second was a primer pair which specifically amplifies the 3’ end of the RPM1-myc transgene. At the time of our screen, only three avrRpm1-susceptible plant lines containing both of these transgenes were present in the lab, one allele of rar1 and two alleles of hsp90.2, which could be checked for by using specific molecular markers. One mutant lacked RPM1-myc and avrRpm1 transgenes, and was hence a contaminant. Another represented contamination from a known allele of hsp90.2, based on a molecular marker specific for this allele. Seven mutants were removed from further study due to heavy pleiotropism. This pleiotropism included severe dwarfism and high basal anthocyanin content which can be misconstrued as evidence of disease in bacterial dip assays. This left nineteen independent mutants derived from the 28 at the bottom of Step 3, Table 4.1.

Since mutants affecting RPM1 function can also affect RPM1 protein accumulation (Figure 4.3A), we took advantage of the fact that the parental line used in this screen contained the RPM1-myc transgene. We began characterizing our mutants by observing their RPM1-myc levels (Figure 4.1, Step 5). Western blot
Figure 4.2: Venn diagram presenting the number of lots represented by each putative mutant class based on susceptibility to Pto DC3000 (avrRpm1). Mutants were classed as either segregating susceptible (Segregating), intermediate susceptible (Intermediate), or fully susceptible (Susceptible) to Pto DC3000 (avrRpm1) in a bacterial dip assay. Total number of M2 seed lots represented by each class is shown next to the circle representing the respective class. Mutants falling into the same mutant class, recovered from the same independent M2 seed lot, are presumed to carry the same mutation and were excluded from the calculations used in the creation of this diagram. The number of total independent mutants was calculated as the number of independent lots which contained a mutant falling into any of the three classes.
analysis using α-myc antibody showed that the putative mutants differed in their accumulation of RPM1. *rar1* and *hsp90.2* mutations recovered from previous screens fall into the class which accumulate lower levels of RPM1-myc (compare Figure 4.3A and B). Among the new mutants, three classes could be distinguished based on this assay: a class which accumulated wild type levels of RPM1-myc (8 independent mutants), a class which accumulated lower levels of RPM1-myc (9 independent mutants), and a class without discernible RPM1-myc accumulation (3 independent mutants, Figure 4.3B). This totals 20 mutants, not 19. The reason for this is as follows. We noted that one seed lot contained mutants from two different RPM1-myc accumulation classes: one exhibiting normal RPM1-myc levels, the other displaying low levels of RPM1-myc. Neither of these mutants is segregating and we therefore consider them independent mutants from the same seed lot. Hence, there are 20 interesting independent new mutants derived from this screen (Table 4.1, at bottom of Step 3).

**Identification of new alleles of hsp90.2 and rar1**

We next decided to sequence possible candidate genes. We began by sequencing *RAR1* and *HSP90.2* in all 20 putative mutants. Given the depth of this screen, we considered the possibility of uncovering alleles of both genes that might separate their function in RPM1 accumulation from any requirement for RPM1 signaling. Consequently, we decided the safest course would be to not only sequence these two genes in the mutants which showed less than normal RPM1-
Figure 4.3: Mutants affecting *RPM1* function show differential RPM1-myc accumulation. (A) *hsp90.2* and *rar1* mutations severely affect RPM1-myc accumulation. Western blot probed with α-myc monoclonal antibody of total protein extracts from the indicated genotypes. (B) Three classes of mutants are observable based on RPM1-myc accumulation. Representative α-myc-probed western blot showing the three observed RPM1-myc accumulation classes. The class marked as Normal had levels of RPM1-myc accumulation indistinguishable from wild type. The Lower class shows similar accumulation as seen in *rar1* and *hsp90.2* mutants seen in (A). Finally in the class marked None, no RPM1-myc protein could be detected.
myc, but also in those mutants which showed normal RPM1-myc accumulation. Of the 20 mutant families remaining, four contained typical EMS mutations in \textit{rar1} (Figure 4.4A), and one contained a mutation in \textit{hsp90.2} (Figure 4.4B). These five mutant families all showed lower accumulation of RPM1-myc.

The previous screen failed to identify mutations in the other three paralogs of cytosolic HSP90 (Hubert et al., 2003). The reason remains unclear. We decided that such a mutation remained a possibility, and therefore sequenced the genes encoding the other three isoforms of HSP90 in all mutants showing lower than normal levels of RPM1-myc. We failed to identify any mutations in these other HSP90 paralogs in this new screen.

\textit{RIN4} is required for \textit{RPM1} function and protein accumulation and currently thought to serve as an intermediary between AvrRpm1 and RPM1. In essence RPM1 detects post-translational changes in RIN4 caused by RIN4’s affiliation with AvrRpm1 (Mackey et al., 2002). RPS2, another CC-NB-LRR disease resistance protein which recognizes the bacterial cysteine protease AvrRpt2, recognizes the disappearance of RIN4 due to cleavage by AvrRpt2 (Axtell et al., 2003). Loss of \textit{rin4} leads to constitutive \textit{RPS2} activation and resultant lethality (Mackey et al., 2003).

Recent experiments in our lab have shown that \textit{RPM1} also requires for its function a protein with homology to RIN4, NOI5 (A.J. Wu, unpublished data). However, unlike \textit{rin4}, loss of \textit{noi5} is not lethal, presumably because NOI5 levels are not monitored by RPS2. For these reasons, \textit{NOI5} was a candidate gene. Sequencing of \textit{NOI5} in the fifteen remaining mutants failed to uncover a mutation.
Figure 4.4: Newly identified *rar1* and *hsp90.2* mutations. (A) Schematic diagram of RAR1 protein showing all known *rar1* mutations. The CHORDI domain is shown in red, the CCH domain is shown in yellow, and the CHORDII domain is shown in blue. Mutations found in this study are highlighted in red type. Two cysteine mutations and one premature stop were uncovered. The fourth new mutation is in Intron 4. (B) The crystal structure of the ATPase domain of yeast HSP90 bound to ADP. Residues shown as green ball and stick models were found to be altered in previously identified *hsp90.2* mutants (Hubert et al., 2003). Alanine 42, shown in yellow, was changed to threonine in the newly identified *hsp90.2* mutant. ADP is shown in the middle of the structure as the space filling molecule.
Dominant negative mutations in $R$ genes have been uncovered in reverse genetic screens (Dinesh-Kumar et al., 2000; Tao et al., 2000). This kind of mutation in $RPM1$ was also a possibility in our screen. In order to address this possibility, we next sequenced both the endogenous copy of $RPM1$ and the transgenic copy of $RPM1$-myc. No mutations were recovered in the $RPM1$-myc transgene, however two mutations were uncovered in the endogenous copy of $RPM1$. Both mutants display normal levels of RPM1-myc. Both mutations are nonsense mutations which lead to an immediate stop (W109STOP and W681STOP) in either the coiled-coil domain or the leucine rich repeat domain. However, very recent segregation analysis suggests that these mutants may not be responsible for the mutant phenotype (see Discussion, T. Eitas, unpublished data).

Preliminary characterization of a mutant that accumulates no detectable RPM1-myc

Test-crosses to determine the number of complementation groups represented by the identified mutants are ongoing. However, initial results indicate that mutants that accumulate different levels of RPM1-myc fall into different complementation groups. Specifically, and interestingly, mutants that accumulate low levels of RPM1-myc and mutants that accumulate no detectable RPM1-myc fall into different complementation groups. However, based on F1 allelism tests, two independent mutants that do not accumulate any RPM1-myc, lra6-1 and lra6-2, fall into the same complementation group. Since this mutant phenotype has not been previously observed we decided to investigate these mutants further.
We began our analysis by determining the specificity of these alleles' loss of disease resistance. Plants were inoculated with bacteria expressing either \textit{avrRpm1} recognized by \textit{RPM1}, \textit{avrRpt2} (RPS2), or \textit{avrPphB} (RPS5). After three days, the resultant bacterial growth was quantified. Both alleles displayed a complete loss of \textit{RPM1} protein and loss of resistance to \textit{Pto DC3000 (avrRpm1)} when compared to an \textit{rpm1} null mutant (Figure 4.5A). However, the alleles behaved differently in response to the other two bacterial genotypes. \textit{lra6-1} was partially impaired in its response to both \textit{avrRpt2} and \textit{avrPphB} (Figure 4.5B and C). This suggests that \textit{lra6-1} plays a broad role in disease resistance to bacteria. However, \textit{lra6-2} displayed little, if any, loss of resistance to these two pathogens. Together these data suggest that \textit{RPM1} has a greater requirement for \textit{LRA6} than other \textit{R} genes. Also, \textit{lra6-2} may represent an allele with less severe effects on the function of the encoded protein. The increased requirement of \textit{RPM1} for \textit{LRA6} may be so high that the weak activity maintained in \textit{lra6-2} is insufficient to have an observable effect on \textit{RPM1} function.

Given the severe effect on \textit{RPM1}-myc accumulation, the locus affected in these mutants might either represent a novel mode of \textit{RPM1} regulation or be involved in the same stability regulating mechanism as \textit{RAR1} and \textit{HSP90.2} (see Chapter 2) but with a closer physical association with \textit{RPM1}. It has been previously shown that \textit{rar1} does not affect transcript levels of a barley \textit{rar1}-dependent \textit{NB-LRR} gene, \textit{Mla6} (Bieri et al., 2004). The same is true of \textit{RPM1} transcription in \textit{rar1} (T. Eitas, unpublished data). Thus, we predicted that \textit{RPM1} transcript levels may allow insight into the phenotypes of \textit{lra6-1} and \textit{lra6-2}. Semi-quantitative RT-PCRs were
Figure 4.5: A newly identified mutant, *lra6*, affects the function and transcript levels of several *R* genes. Bacterial growth assays measuring resistance to *Pto DC3000* expressing *avrRpm1* (A), *avrRpt2* (B), or *avrPphB* (C). The two allelic mutants *lra6-1* and *lra6-2* are impaired in *RPM1*-mediated resistance (A). *RPS2* (B) and *RPS5* (C) are affected to a lesser extent. (D) Semi-quantitative RT-PCR reaction of the CC-NB-LRR type Resistance genes *RPM1*, *RPS2*, and *RPS5* and the TIR-NB-LRR type *R* gene, *TAO1*. Changes in transcript levels correlate with changes in bacterial resistance. (E) Schematic representation of the third chromosome of Arabidopsis. The area showing strong genetic linkage disequilibrium between the mutant phenotype and molecular markers is indicated by a green bar, and is demarcated by the telomere and an INDEL molecular marker contained within the P1 clone MQD17. The location of *RPM1* is also indicated. The location of the centromere is indicated by a red circle.
performed using primers specific to \textit{RPM1}. Both mutants appear to lack \textit{RPM1} transcripts (Figure 4.5D, top panel). We next determined whether effects on transcript levels of other \textit{R} genes could explain the observed changes in resistance. \textit{lra6-1} appears to strongly affect \textit{RPS2} transcript level, and has a more moderate affect on \textit{RPS5} transcript levels (Figure 4.5D, two middle panels). \textit{lra6-2} has little or no observable effect on these two genes’ transcript levels, consistent with its phenotype. \textit{RPM1}, \textit{RPS2}, and \textit{RPS5} encode \textit{R} proteins belonging to the CC-NB-LRR type. Another class of \textit{R} proteins, TIR-NB-LRRs, represent an ancient divergence, as evidenced by their absence in monocots (Myers et al., 1999; Zhou et al., 2004). This ancient divergence may have led to differences in gene regulation. Consequently, we next looked at transcript levels of an \textit{R} gene belonging to the TIR-NB-LRR class, \textit{TAO1}. We could not discern any affect on \textit{TAO1} transcript levels in either mutant (Figure 4.5D, bottom panel). Phenotypic analysis of \textit{TAO1} effects must await creation of appropriate genetic material.

In order to isolate the lesion responsible for these phenotypes in \textit{lra6-1}, we created a mapping population by crossing this mutant to the ecotype Landsberg-\textit{erecta} which also carries a functional \textit{RPM1} gene (Grant et al., 1995). F2 individuals were then screened for loss of \textit{RPM1}-mediated resistance. Based on phenotypic analysis of the segregating F2 population, \textit{lra6-1} appears to be recessive. Two pools of DNA representing 12 susceptible individuals each were created. Bulk segregant analysis was performed on these two pools using molecular markers arrayed across all five chromosomes of the Arabidopsis genome (Michelmore et al., 1991). Linkage was only observed between the mutant phenotype and molecular
markers corresponding to the Col-0 ecotype on the north arm of Chromosome III. The strongest linkage was found between the mutant phenotype and a marker on the BAC clone F8A24 (4 recombination events in 29 plants or 58 meioses). Additional minor linkage to the Landsberg ecotype was observed on the bottom of Chromosome III, and may represent a genetic modifier.

**Discussion**

We describe here a novel genetic screen to discover new loci required for *RPM1*-mediated disease resistance. This screen takes advantage of tailored genetic materials and unconventional methodology to achieve this goal. There are many screens which have suffered from the problem of an overabundance of mutations in a single locus (Gil et al., 2001; Lease et al., 2001). In our case we had to contend with three very large mutagenesis targets, RPM1 and the two T-DNAs comprising our inducible *avrRpm1* gene expression system. We overcame these issues using a combination of engineered genetic redundancy for *RPM1* and by increasing per lot sampling. Based on the total number of mutants and the phenotypes obtained, this strategy was successful. We additionally describe initial characterization of one mutant class with a previously unobserved phenotype. The implications of this mutant class are unexpected, and could profoundly impact the field of plant disease resistance.

While previous analyses have shown little in the way of regulation of *R* gene transcript levels, our data suggest that CC-NB-LRR type *R* genes may be under a
common pre-translational regulatory mechanism. The lack of an observable effect on a TIR-NB-LRR R gene could be indicative of another force driving diversification of different R gene types in dicotyledonous plants. Since structural variations of plant R proteins are part of animal innate immune systems (Ting and Davis, 2005), it is also possible that these results could have implications outside of the plant research community.

**An effective approach to identifying new loci in previously screened pathways**

One of the main differences between this genetic screen and many others performed in Arabidopsis is the high level to which the parental material was genetically modified. The plants used in this screen contain three separate T-DNAs. While these refinements solved several problems, the main genetic advantage inherent in this screen is a reiteration of a previous screen (Tornero et al., 2002a).

Given the near lethal phenotype of *avrRpm1* expression in Arabidopsis, its use can be described as being more akin to a genetic selection process than a genetic screen. We found one must select for mutants with extremely weak phenotypes to recover false positives. We selected eight plants per independent seed lot in this screen, representing a range of phenotypes from a near full loss of function in RPM1 mediated cell death response (e.g. fully green) to a near full response (e.g. only slightly more green than background). If the eighth plant selected in each lot was a false positive, we would expect a false positive rate of 12.5%. The observed rate was 13.8%, only slightly higher than expected. This suggests that we were capturing the full range of phenotypes within each lot.
The selection of multiple individuals from each seed lot proved an effective filter for the expected high rate of mutations in the Estradiol-inducible system gene expression system. In nearly every lot containing a true susceptible mutant, false susceptible system hits were also recovered. Had we only chosen one or two individuals per lot, our mutant yield would have assuredly been diminished. This bias towards system mutations is even more evident when one considers that 87% of all \textit{avrRpm1} non-responsive mutants obtained from the primary screen were found to be completely disease resistant to \textit{Pto DC3000 (avrRpm1)} (Table 4.1).

Unfortunately, the necessity of using disease symptoms rather than the HR that follows hand inoculation of bacteria to screen many progeny families probably led to the loss of an important phenotypic class. Our secondary screening strategy prevented us from identifying mutants which had normal disease resistance, but lacked the ability to perform the hypersensitive response. One mutant of this class was identified in the previous screen utilizing inducible \textit{avrRpm1}, from the over 100 mutants identified. While the hypersensitive response is not required for resistance to \textit{P. syringae}, it is necessary for full resistance to \textit{Hyaloperonospora parasitica} (Holt III et al., 2005). This lack of effect on \textit{P. syringae} resistance increases the likelihood that such mutants were lost. In hindsight, the loss of these mutants is perhaps distressing given our current knowledge that a gene that negatively regulates R protein accumulation, \textit{SGT1B}, has such a phenotypic profile (Holt III et al., 2005).

**Mutations in known genes**
On the other hand, we did uncover several loci that act to positively regulate NB-LRR protein levels, including \textit{rar1} and \textit{hsp90.2}. Our recovery of new mutations in these two genes, however, does not shed new light on their function in disease resistance. Our recovered \textit{hsp90.2} allele, G95E, is a new mutation in the ATPase domain, as were all previously identified mutations (Figure 4.4B and Hubert et al., 2003). Recovered \textit{rar1} alleles either represent non-functional proteins encoding protein truncations or alter conserved cysteines required for zinc coordination (Figure 4.4A). The nature of our newly recovered \textit{rar1} alleles holds some interest. The C66S mutation is caused by an A to T transversion mutation. This type of mutation is quite rare for EMS mutants in Arabidopsis, occurring at a rate of much less than 1\% (Greene et al., 2003). This mutation is probably more likely the result of a spontaneous mutation event, which occur in Arabidopsis at a rate of between $10^{-7}$ and $10^{-8}$ base pairs per generation (Kovalchuk et al., 2000). Another interesting mutation found in \textit{rar1} occurred within the fourth intron in neither a splice donor nor an acceptor site. One would presume that such a mutation would have little or no effect on splicing. However, a small deletion in the middle of an intron in an Arabidopsis syntaxin gene led to a larger transcript, showing that such mutations can impact splicing (Ohtomo et al., 2005). To see if the case is the same here requires further experimentation. All of the mutations we recovered in \textit{rar1} and \textit{hsp90.2} led to decreased RPM1-myc levels as was observed in previously identified mutations in these genes (Hubert et al., 2003; Tornero et al., 2002b).

At first glance, it appears surprising that we only recovered four alleles of \textit{rar1} and one allele of \textit{hsp90.2}. Extrapolating from the previous screen using the
inducible avrRpm1 system, we expected to recover approximately ten alleles of rar1 and five alleles of hsp90.2. However, this result may be easily explained by the additional copy of RPM1. It has been shown that both rar1 dependent and rar1 independent NB-LRR proteins show reduced accumulation in a rar1 mutant background (Bieri et al., 2004). It has also been shown that increases in NB-LRR protein amount can partially overcome rar1 dependency (Belkhadir et al., 2004). This has led to the development of a “threshold” model for NB-LRR protein function. In this model, NB-LRR protein levels may be modulated by RAR1, but these levels must drop below a certain threshold before phenotypic effects can be seen in rar1 mutant plants (Holt III et al., 2005). By adding an additional copy of RPM1, we may have created a situation in which only very strong mutations in either rar1 or hsp90.2 have an observable phenotype. This decreases our likelihood of identifying mutations in these two genes.

Two mutations were identified in the endogenous copy of RPM1. It is unclear whether they cause the loss of RPM1 function we selected (though see below). If so, they must be dominant in order for them to have been recovered. Previous dominant negative mutations in two R genes, N and RPS2, have been uncovered through reverse genetic strategies (Dinesh-Kumar et al., 2000; Tao et al., 2000). However, these mutations have all been point mutations in the P-loop of the NB domain, in the case of N gene, or the coiled-coil domain, in the case of RPS2. One truncation causing dominant negative effects was uncovered in RPS2. Unlike the new mutants of RPM1 presented here, that truncation was an N-terminal truncation. Furthermore, it was found that C-terminal truncation products of RPS2 showed
simple loss-of-function phenotypes (Tao et al., 2000). This may also be the case here. Very recent segregation analysis suggests that these mutations are not responsible for the mutants’ phenotype (T. Eitas, unpublished results).

In a genetic screen as large as the one presented here, one can isolate mutations in two genes, both of which have roles in plant disease resistance. In the previous screen utilizing the Estradiol-inducible \textit{avrRpm1} expression system, a mutant was isolated that compromised both \textit{RPM1}- and \textit{RPS2}-mediated resistance. After many testcrosses and sequencing, it was discovered that this phenotype was not caused by mutation of a single gene, but rather two point mutations, one in \textit{RPM1} and the other in \textit{RPS2} (P. Tornero, unpublished data). Unfortunately, it will be necessary to remove these two \textit{RPM1} mutations by backcrossing before a mapping of the pertinent new loci can commence.

**A new mutant affecting \textit{NB-LRR} gene transcript levels**

Proper control of \textit{NB-LRR} protein levels has been shown to be crucial for proper disease resistance signaling (Belkhadir et al., 2004; Bieri et al., 2004; Holt III et al., 2005; Hubert et al., 2003; Tornero et al., 2002b). However, in all of these cases the control was at the post-transcriptional level. Our identification of a new phenotypic class of mutant, represented by \textit{lra6}, shows that \textit{R} protein accumulation can also be regulated at the transcriptional level.

Instances of transcriptional control of \textit{NB-LRR} genes have been rare, and rarely appear to have functional consequences (Levy et al., 2004; Yoshimura et al., 1998). One case of functional transcriptional control can be found in the \textit{Xa27 R}
gene in rice (Gu et al., 2005). There are two characterized alleles of Xa27, which does not encode an NB-LRR protein but rather a protein of unknown function. One allele confers resistance, while rice plants containing the other are susceptible to bacteria expressing avrXa27. The resistant allele has the same predicted protein sequence as the susceptible allele. However, while both have undetectable constitutive expression, presence of avrXa27 carrying bacteria is sufficient to induce expression of the resistant allele but not of the susceptible. Constitutive expression of Xa27 leads to resistance both to avrXa27 containing bacteria, but also to bacteria which are normally able to colonize rice. It is possible that avrXa27 itself is directly responsible for the induction of Xa27 expression, as this effector contains nuclear localization signals and a transcription activation domain (Gu et al., 2005). However, tests to determine if avrXa27 can bind to the promoter sequence of Xa27 have, to our knowledge, not been performed.

A more pertinent example of a second site locus controlling NB-LRR gene transcription was recently found in our lab. edm2 was isolated in a screen for loss of disease resistance to a Hyaloperonospora parasitica isolate, Hiks1, that is recognized by RPP7 (Eulgem et al., 2007). Interestingly, RPP7 transcript levels are reduced in the edm2 mutant background. EDM2 was isolated and found to encode a protein with plant homeodomain (PHD)-finger-like domains, which are zinc finger-like motifs that could act in regulation of transcription (Aasland et al., 1995; Kalkhoven et al., 2002). However, there are some important distinctions between the effects of the edm2 mutation and our lra6 mutant. The first is that edm2 suppresses only RPP7. Microarray data comparing wild type, edm2, and rpp7
shows that any effect by edm2 on the transcript levels of other NB-LRR genes can be explained by edm2’s effect on RPP7. This is in contrast to the effect of lra6-1 on the transcript level of several other tested NB-LRR genes. The second distinction is that rpp7 transcript levels are only slightly down-regulated in edm2. The wholesale loss of RPM1 transcript in lra6 is quite striking in comparison. However, these disparities could be explained by differences in functional overlap between family members. It is interesting to note that while the interval containing lra6-1 is currently rather large (~5 Mbp), it does contain a gene with homology to edm2 (At3g14980, 3e-04) that is also annotated as a PHD finger transcription factor.

The identification of new loci required for RPM1 function is central to continued progress in this field. We have shown that through unique genetic tailoring and screen methodology, one can successfully achieve this goal. Different phenotypic requirements for various NB-LRR genes for different loci demonstrate that pursuing a similar screen for other NB-LRR gene pathways may be equally successful. Additionally, the principles underlying this screen can be carried forward to the study of other processes in plants.

**Materials and Methods**

**Plant lines**

The parental plant line used for this screen, Col-0 containing β-Estradiol inducible avrRpm1 and transgenic RPM1-myc, has been previously described (Tornero et al., 2002b). rar1-21 and hsp90.2-3 containing RPM1-myc shown in
Figure 4.3 have been described in (Huber et al., 2003; Tornero et al., 2002b). Additional mutant alleles used in Figure 4.5 were *rpm1-1* containing inducible *avrRpm1* (Tornero et al., 2002a), *rps2-101c* (Mindrinos et al., 1994), and *rps5-2* (Warren et al., 1998).

**Estradiol induction and screening**

M2 plants grown under long day conditions (greenhouse) were sprayed twice at one and two weeks of age with 10μM β-Estradiol (Sigma E 8875) in distilled water from a 10 mM β-Estradiol stock dissolved in 100% ethanol. Since Estradiol does not move systemically through plants, the absence of a surfactant allowed for recovery of weak HR response mutants (Tornero et al., 2002a). Eight individual M2 plants showing a range of phenotypic severity (less HR than parental) were selected from each of 200 lots and M3 seeds were collected from each M2 individual. Secondary screening was similarly performed, except that plants were grown under short day (8 hr.) conditions.

**Bacterial strains, inoculation, and growth quantification**

Maintenance of *Pto* DC3000 containing the kanamycin selectable plasmid pVSP61 with an *avrRpm1*, *avrRpt2*, or *avrPphB* insert was as described (Ritter and Dangl, 1996). Secondary screening of M3 families was performed by dipping two-week-old plants grown in short day conditions in a suspension of *Pto* DC3000 (*avrRpm1*) at an inoculum of OD$_{600}$=0.1 (5 X 10$^7$ colony-forming units/mL) in 10 mM MgCl$_2$ and 0.02% Silwet L-77 (CKWitco Corporation). Plants were assayed for
disease symptoms between four and seven days post inoculation compared to wild type and *rpm1-3* controls. Plants used for mapping were assayed for disease symptoms by spraying four- to five-week-old, short-day-grown plants with the same solution of bacteria described above for dip assays. These plants were then covered for four hours with a clear plastic lid and assayed for symptoms between four and seven days. Bacterial quantifications were performed as described (Tornero and Dangl, 2001).

**Sequencing of candidate genes**

Genomic fragments corresponding to candidate genes were amplified with specific oligonucleotides (sequence available upon request) using LA Taq (TaKaRa). Sequencing was performed at the University of North Carolina at Chapel Hill Genome Analysis Facility using primers spaced approximately 300 base pairs apart allowing for a minimum of two-fold coverage. Known mutations in *rar1* were compiled from various sources including this study (Muskett et al., 2002; Shang et al., 2006; Tornero et al., 2002b), and mapped onto a schematic of the RAR1 protein using Vector NTI 10.3.0 (Invitrogen Corporation) and Photoshop 7.0 (Adobe Systems Incorporated). Residues mutated in *hsp90.2* were displayed on the crystal structure of the ATPase domain of yeast HSP90 (PDB ID 1A4H) using Viewerlite 5.0 (accelrys) and Photoshop 7.0.

**Protein blots**
A buffer containing 50 mM Tris-HCl pH 8.0, 1% SDS, 1mM EDTA, 1 mM 2-mercaptoethanol, and 1 X plant protease inhibitor cocktail (Sigma) was used to extract total protein. 40 μg protein samples were separated on 8% SDS-PAGE gels. Western blots were performed with anti-myc monoclonal antibody (9E10) utilizing standard methods and detected with ECL+ (Amersham).

**Semi-quantitative reverse transcription analysis**

*NB-LRR* gene transcript levels were analyzed by grinding two-week-old seedlings in liquid nitrogen and extracting their RNA with Trizol according to the manufacturer’s instructions (GibcoBRL). Reverse transcription was then performed on RNA samples (RETROscript, Ambion). RT-PCRs were performed according to the manufacturer's instructions. Plant 18S Competimer Primers (Ambion) were used at ratios appropriate for each specific RT product to co-amplify the 18S internal loading control.
Bibliography


CHAPTER 5

Analysis of the genetic and physical interactions underlying the RAR1/SGT1/HSP90 complex

Abstract

Plants recognize specific pathogens through the action of specific disease Resistance (R) proteins, which positively regulate cell death. Maintaining appropriate steady state levels of these R proteins is of tremendous importance to this recognition. Three genes, HSP90, RAR1, and SGT1, involved in maintenance of NB-LRR type R protein levels have been previously identified. Cursory genetic and physical interaction studies have been performed, but little data is available that links these three NB-LRR accumulation regulators to a single biological function. We present here a detailed analysis of the genetic and physical interactions underlying the HSP90/RAR1/SGT1 machinery responsible for maintaining steady state levels of NB-LRR protein accumulation.

We present data demonstrating a functional relationship between HSP90 isoforms in Arabidopsis. We demonstrate genetic interactions between hsp90.2 and sgt1b. We explore the functional role of HSP90 in general, and provide evidence that contradicts the widely-held view that loss of ATP binding and subsequent hydrolysis abolishes HSP90 function. In addition, the physical interactions between
HSP90, RAR1, and SGT1 are explored using point mutations in *HSP90.2* and *RAR1* and truncations of RAR1 in an attempt to biochemically explain phenotypic results.

**Introduction**

The watchmen standing ready to trigger a plant defense response are known as disease *Resistance (R)* genes. These *R* genes are responsible for the detection of specific pathogen gene products which betray the pathogen’s presence. Over-expression and inappropriate activation of *R* genes has been repeatedly shown to lead to an ectopic defense response, evidenced by spontaneous cell death and generally lower fecundity (Bendahmane et al., 2002; Mackey et al., 2003; Shirano et al., 2002; Tao et al., 2000; Zhang et al., 2003). Consequently, plants must maintain appropriate *R* protein levels to remain healthy. Forward genetic screens have yielded several genes shown to play a role in maintaining the accumulation of proteins of the NB-LRR class of *R* proteins. One of these genes encodes the RAR1 protein. Originally identified in barley, RAR1 has been shown to be required for disease resistance in diverse plant species (Liu et al., 2002a; Muskett et al., 2002; Shirasu et al., 1999; Tornero et al., 2002b). It is composed of two tandem zinc-binding CHORD domains separated by a plant conserved CCCH domain allowing for the binding of five zinc ions (Heise et al., 2007). *rar1* mutants show loss of accumulation of all NB-LRR proteins tested thus far (Belkhadir et al., 2004; Holt III et al., 2005; Tornero et al., 2002b). This is also the case for NB-LRR proteins which do not genetically require *RAR1* for their function (Bieri et al., 2004). This suggests that
rar1 reduces the levels of most, if not all, NB-LRR proteins, but only affects their function if NB-LRR levels drop below a threshold level.

Another protein shown to be involved in NB-LRR protein stability is SGT1. SGT1 was originally identified in yeast as a suppressor of the G2 allele of skp1, implicating SGT1 as part of the SCF-ubiquitin ligase complex which targets proteins to the proteasome (Kitigawa et al., 1999). SGT1 is composed of three domains, an N-terminal TPR domain, an SGT1 specific SGS domain, and a C-terminal CS domain. Each domain is separated by a region highly variable between all species of plants and animals. The CS domain led to SGT1’s functional characterization in Arabidopsis by reverse genetics because this domain is homologous to a domain in mammalian RAR1 homologs that is absent in plant RAR1 (Azevedo et al., 2002). The lack of a CS domain in plant RAR1 proteins suggested that plant RAR1 may require a CS-containing accessory protein in order to function properly. Simultaneously, SGT1 was also identified in forward genetic screens as a positive regulator of resistance to Hyaloperonospora parasitica (Austin et al., 2002; Tör et al., 2002).

SGT1 is encoded by two isoforms in Arabidopsis, SGT1a and SGT1b, but only SGT1b has been shown to play a role in disease resistance (Azevedo et al., 2002). Mutations in sgt1b cause loss of the hypersensitive response (HR), a pathogen triggered form of programmed cell death, which is mediated by several NB-LRR proteins. On its own, loss of SGT1b appears to not affect NB-LRR protein levels (Holt III et al., 2005). However, sgt1b mutation was found to suppress rar1 leading to recovery of nearly wild-type levels of some NB-LRR proteins, suggesting
that the wild-type function of \textit{SGT1b} is to stabilize at least some R protein levels (Holt III et al., 2005). This genetic interaction between \textit{RAR1} and \textit{SGT1b} is corroborated by \textit{in planta} evidence showing that SGT1 and RAR1 can physically interact (Azevedo et al., 2002; Liu et al., 2002b).

The third gene found to affect NB-LRR protein levels in plants is \textit{HSP90}. In yeast and animals, cytosolic HSP90 is a well-characterized protein which plays a role in the maturation of steroid receptors (Pratt and Toft, 1997). Its role in plant disease resistance was demonstrated in three ways:

1. HSP90 was shown to interact with RAR1 in a yeast two hybrid screen (Liu et al., 2004; Takahashi et al., 2003).
2. Silencing of \textit{hsp90} in tobacco caused loss of \textit{Pto}-mediated disease resistance (Lu et al., 2003). Both Liu et al (2004) and Lu et al (2003) further demonstrated that silencing of HSP90 caused loss of \textit{N}- and \textit{Rx}-mediated resistance, respectively. However, results supporting an effect of HSP90 on \textit{RPS2}-mediated resistance (Takahashi et al., 2003), could not be reproduced by outside labs (this manuscript, B. Staskawicz, personal communication and Holt III et al., 2005).
3. HSP90 was identified in a genetic screen for mutants that lose \textit{RPM1}-mediated disease resistance. This screen provided three different mutations, all within the ATPase domain of \textit{hsp90.2}, one of four Arabidopsis isoforms of \textit{HSP90} (see Chapter 2 and Hubert et al., 2003). These mutations seemed to have specific effects on \textit{RPM1}-mediated resistance, and did not affect any other pathway tested.

Hence, four independent labs reported physical interaction between HSP90 and RAR1, SGT1, and the respective NB-LRR protein, in either yeast two hybrid or
co-immunoprecipitation experiments. Thus, HSP90 provided a physical link between RAR1 and SGT1 action and NB-LRR protein stability. In addition, NB-LRR protein levels were found to decrease in both HSP90-silenced tobacco plants and in the specific hsp90.2 ATPase mutants in Arabidopsis (Hubert et al., 2003; Lu et al., 2003).

Previous work in yeast indicated that ATPase activity was essential for HSP90 function. In yeast, an ATPase-mutant allele of HSP90 was unable to complement an hsp90 null mutant (Borkovich et al., 1989; Obermann et al., 1998; Panaretou et al., 1998). Further work showed that yeast carrying the ATPase-mutant allele resembled those with the null mutant even in the presence of wild-type HSP90, indicating the presence of ATPase-mutant HSP90 protein negatively regulates the function of wild-type HSP90 protein. Therefore, it was reasoned that chemicals that blocked ATP binding and hydrolysis were inhibitors of HSP90 (Roe et al., 1999). However, we present evidence below that ATPase domain mutations in hsp90 act differently than null alleles. Our data resurrect a previously dismissed theory that HSP90 is an ATP-independent molecular chaperone (Wiech et al., 1992).

Additionally, we show genetic interaction between HSP90.2 and SGT1b. This demonstrates for the first time a biological relevance for the SGT1/HSP90 physical interaction with respect to disease resistance phenotypes. We identify a previously unobserved role for HSP90.2 and SGT1b in RPS2 function, partially resolving the paradox presented by the previously observed specificity of hsp90.2 mutation on RPM1 function. We also explore diversification of HSP90 isoform function, and a requirement for a minimum level of HSP90 for Arabidopsis survival. Finally, we
present an analysis of RAR1/HSP90/SGT1 interactions using yeast two hybrid methods which potentially explains the phenotypes of previously recovered mutants of rar1 and hsp90.2.

**Results**

**HSP90 expression and analysis of loss-of-function mutations in Arabidopsis HSP90 paralogs**

We previously demonstrated that specific point mutations within the ATPase domain of Arabidopsis *hsp90.2* lead to specific decline of *RPM1* function (see Chapter 2 and Hubert et al., 2003). No such mutations were found in the other three paralogs of cytosolic *HSP90*. Differences in the expression level of the different isoforms might explain this result. Due to the extremely high degree of homology between the four *HSP90* genes (79-96% nucleotide identity), we were unable to generate unique primer pairs to differentiate 3 different isoforms of HSP90 (*HSP90.1, HSP90.2, and HSP90.3*), therefore semi-quantitative RT-PCRs were unsuccessful.

Rather than directly measure transcript abundance, we used the publicly-available Arabidopsis Massively Parallel Signature Sequencing (MPSS) database (Meyers et al., 2004). Since this dataset is created through the direct sequencing of unique signature sequences rather than through hybridization techniques, the MPSS database is one of the few datasets able to address the differential abundance of these highly-homologous transcripts. We found that while all isoforms are
Table 5.1: *HSP90.2* shows higher levels of expression than any other *HSP90* isoform. Total number of unique sequence tags identified for each *HSP90* isoform in untreated 3-week-old leaves or 52 hours after treatment with 0.3 mM salicylic acid. Data obtained from the Arabidopsis MPSS database. 20 bp signature sequence data is shown.

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expressed in leaves, \textit{HSP90.2} showed the highest expression levels (Table 5.1). Interestingly, after treatment with salicylic acid, a plant hormone involved in disease resistance, both \textit{HSP90.1} and \textit{HSP90.2} showed a five- to ten-fold increase in levels, while \textit{HSP90.3} and \textit{HSP90.4} actually showed a decrease in expression.

We previously observed that a T-DNA insertion within \textit{hsp90.2} had no obvious phenotype. We assumed that this lack of phenotype was due to compensation by other isoforms of HSP90. We next looked to see if this was the case for all isoforms of cytosolic \textit{HSP90}. In order to address this question, we obtained T-DNA insertions within all isoforms of cytosolic \textit{HSP90} and looked for an effect on plant disease resistance.

We initially investigated the effect of these mutations on the function of \textit{RPM1}. The T-DNA insertion allele of \textit{hsp90.2}, henceforth \textit{hsp90.2} KO, showed complete resistance to \textit{Pto DC3000 (avrRpm1)}, similar to wild-type plants. Insertions in the other three isoforms of \textit{HSP90} also showed full resistance to this pathogen. This suggests redundancy—that other isoforms can compensate for the loss of any particular \textit{HSP90} isoform (Figure 5.1A). This result is consistent with the yeast data that suggested that expression of ATPase-impaired \textit{hsp90} can attenuate the function of wild-type HSP90.

We next determined whether this pattern held true for the function of another \textit{R} gene. The \textit{RPS2} protein recognizes the avirulence protein AvrRpt2. It has been previously reported that a T-DNA insertion in \textit{hsp90.1} partially compromises the \textit{RPS2} pathway (Takahashi et al., 2003). We independently obtained homozygous mutant plants carrying the same T-DNA insertion previously reported to have the
Figure 5.1: Null alleles of hsp90 isoforms do not affect disease resistance. Bacterial growth assays assaying (A) RPM1- and (B) RPS2-mediated resistance for T-DNA insertion lines corresponding to each of the four isoforms of HSP90. Note that the slight increase in growth seen in the hsp90.4 insertion is not reproducible. None of the insertions show an increase in bacterial growth compared to wild-type.
strongest effect on RPS2 function. In contrast to the results reported by Takahashi et al., we did not detect any difference in growth of bacteria expressing avrRpt2. Additionally, we were unable to observe an effect on the RPS2 pathway by knock-outs in any of the other three isoforms of HSP90, identical to the results observed for the RPM1 pathway (Figure 5.1B).

**Genetic interactions between hsp90.1 and hsp90.2**

HSP90.1 is the most highly divergent HSP90 isoform and shows the second highest level of expression of all HSP90 paralogs (Table 5.1 and Krishna and Gloor, 2001). Although HSP90.1 is located on Chromosome V like the other three isoforms of HSP90, it does not belong to the same syntenic group. The combination of these three attributes led us to explore HSP90.1’s relationship to the other three isoforms.

As stated previously, we did not observe a phenotype directly attributable to loss of hsp90.1, possibly because the other isoforms are able to compensate for hsp90.1’s absence. We crossed hsp90.2KO, hsp90.3KO, and hsp90.4KO to hsp90.1KO. In the F2 generation, we were able to identify individuals of genotypes: hsp90.1KO/hsp90.1KO, hsp90.2KO/HSP90.2, hsp90.1KO/hsp90.1KO, hsp90.3KO/HSP90.3, and hsp90.1KO/hsp90.1KO, hsp90.4KO/HSP90.4. In the context of a homozygous hsp90.1KO mutant, hsp90.3KO heterozygous and hsp90.4KO heterozygous genotypes resulted in wild-type appearance. However, hsp90.1KO homozygous hsp90.2KO heterozygous plants exhibited dwarfed stature and low fecundity, producing less than 50 µL of seeds per plant compared to the
greater than 500 µL of seeds per plant produced by its siblings grown in the same flat.

Furthermore, we were able to identify homozygous \( hsp90.1^{\text{KO}} \) \( hsp90.3^{\text{KO}} \) and \( hsp90.1^{\text{KO}} \) \( hsp90.4^{\text{KO}} \) double mutant plants which were wild-type in appearance. However, we were unable to isolate \( hsp90.1^{\text{KO}} \) \( hsp90.2^{\text{KO}} \) double mutant plants. This synthetic lethality suggests that for at least one normal physiological function, either there is no longer enough total HSP90 left in these plants to maintain HSP90 function, or that HSP90.2 and HSP90.1 play redundant roles for which the remaining two isoforms are unable to compensate.

Drugs that inhibit HSP90 ATP hydrolysis are referred to as HSP90 inhibitors (Jez et al., 2003; Roe et al., 1999; Stebbins et al., 1997). Contrary to this nomenclature, our results indicate that loss of ATPase activity leads to a gain-of-function phenotype inconsistent with a simple loss-of-function mutation (Hubert et al., 2003). However, we could not exclude the possibility that the gain-of-function nature of our mutation was due to the mutation acting in a dominant negative fashion over the other HSP90 isoforms. The lethality of \( hsp90.1^{\text{KO}} \) \( hsp90.2^{\text{KO}} \) gave us a clear phenotype for \( hsp90.2 \) loss of function (albeit in combination with \( hsp90.1 \)), and afforded us the possibility of directly testing the functional nature of our \( hsp90.2^{\text{ATPase}} \) mutant.

We immediately saw a phenotypic difference between the knock-out and ATPase alleles in the F2 generation of a cross between \( hsp90.1^{\text{KO}} \) and \( hsp90.2^{\text{ATPase}} \). Using allele-specific markers, we found that whereas \( hsp90.1^{\text{KO}}/\text{hsp90.1}^{\text{KO}} \) \( hsp90.2^{\text{KO}}/\text{HSP90.2} \) plants exhibited the dwarf phenotype
mentioned above, plants of genotype $hsp90.1^{KO}/hsp90.1^{KO}$ $hsp90.2^{ATPase}/HSP90.2$ were completely wild-type in appearance. If the ATPase mutation was a dominant negative, we would expect the dwarf phenotype of the knock-out or a stronger phenotype if this allele interfered with the function of $HSP90.3$ and $HSP90.4$ as has been previously asserted (Panaretou et al., 1998).

We continued to the next generation, and were easily able to identify the $hsp90.1^{KO}/hsp90.1^{KO}$ $hsp90.2^{ATPase}/hsp90.2^{ATPase}$ double mutant, again in contrast to the lethality of $hsp90.1^{KO}$ $hsp90.2^{KO}$. This implies that loss of ATPase domain activity is not equivalent to full loss of $HSP90$ activity, and as a corollary, that ATPase inhibitors are not necessarily inhibiting all HSP90 activities.

ATPase mutants of $hsp90.2$ cause intermediate loss of $RPM1$ function when compared to an rpm1 mutant and are wild-type for $RPS2$ function. Given our demonstration of a strict requirement for $HSP90.1$ and $HSP90.2$ in viability, the possibility existed that this partial loss of RPM1 function and absence of effect on the $RPS2$ pathway was due to interference or compensation by $HSP90.1$ with the activity of $hsp90.2^{ATPase}$. Consequently, we assayed our $hsp90.1^{KO}$ $hsp90.2^{ATPase}$ double mutant for effects on disease resistance. This double mutant displayed the same phenotypes as the $hsp90.2^{ATPase}$ single mutant: an intermediate phenotype for the $RPM1$ pathway (Figure 5.2A) and no effect on $RPS2$ function (Figure 5.2B). These data strongly suggest that the intermediate affect on $RPM1$-mediated resistance and the lack of effect on $RPS2$-mediated resistance is not due to interference or compensation by $HSP90.1$. 

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Figure 5.2: \textit{hsp90.2}^{ATPase} mutations do not show additivity with \textit{hsp90.1}^{KO} mutants and do not act in a dominant fashion over other \textit{HSP90} isoforms. Bacterial growth assays assaying (A) \textit{RPM1}- and (B) \textit{RPS2}-mediated disease resistance. While \textit{hsp90.1} \textit{hsp90.2} double mutants are nonviable, \textit{hsp90.1} \textit{hsp90.2}^{ATPase} double mutants display the \textit{hsp90.2}^{ATPase} mutant phenotype.
Genetic interaction of hsp90.2\textsuperscript{ATPase} with sgt1b

rar1 and hsp90.2\textsuperscript{ATPase} mutants share a similar phenotype: loss of RPM1 accumulation (Figure 4.3; Hubert et al., 2003; Tornero et al., 2002b). These two proteins interact in yeast two hybrid and co-IP experiments, suggesting that the loss of RPM1 in these mutant backgrounds is likely due to a common mechanism.

SGT1b, another protein shown to have a role in plant disease resistance, also interacts with both RAR1 and HSP90 in yeast two-hybrid and co-IP experiments (Hubert et al., 2003; Liu et al., 2004; Takahas hi et al., 2003). In addition, SGT1b interacts with several proteins shown to play a role in proteasome-dependent protein degradation (Azevedo et al., 2002), suggesting that it might direct substrates to the proteasome. Consistent with this observation, sgt1b can suppress rar1 deficiency for several NB-LRR functions, including resistance mediated by \textit{RPP4, RPP8}, and \textit{RPS5} (Holt III et al., 2005). Additionally, these authors demonstrated that RPS5 re-accumulated in the \textit{rar1 sgt1b} double mutant, showing that \textit{RAR1} and \textit{SGT1b} act antagonistically.

We were therefore interested in the genetic interactions between \textit{rar1}, \textit{sgt1b}, and hsp90.2\textsuperscript{ATPase} mutants. We generated the \textit{rar1 hsp90.2\textsuperscript{ATPase}} double mutant. This double mutant exhibited the \textit{rar1} phenotype: full loss of \textit{RPM1} function (data not shown). No additivity was discernible, consistent with these two genes operating in the same pathway. Quantification of bacterial growth confirmed the visual assay (Y. He, unpublished data).
Figure 5.3: \textit{hsp90.2}^{\text{ATPase}} and \textit{sgt1b} display a synthetic genetic interaction and both play a role in \textit{RPS2}-mediated resistance. Bacterial growth assays assaying (A) \textit{RPM1}- and (B) \textit{RPS2}-mediated resistance for the \textit{hsp90.2}^{\text{ATPase}} \textit{sgt1b} double mutant. While there is no consistent difference for \textit{RPM1}-mediated resistance, the \textit{hsp90.2}^{\text{ATPase}} \textit{sgt1b} double mutant displays decreased \textit{RPS2}-mediated resistance.
When \( hsp90.2^{\text{ATPase}} \) \( sgt1b \) plants were assayed for visible symptoms indicative of disease susceptibility following inoculation with \( Pto \) DC3000 (\( \text{avrRpm1} \)), this double mutant appeared to be more susceptible than the \( hsp90.2^{\text{ATPase}} \) single mutant and certainly more susceptible than the fully resistant \( sgt1b \) single mutant. Quantification of bacterial growth was less consistent. While we were able to see slightly more growth (5X greater than \( hsp90.2^{\text{ATPase}} \)) in one experiment, two subsequent experiments showed no difference (Figure 5.3A). On the other hand, a clear and reproducible difference was seen when this double mutant was assayed for \( RPS2 \) function by infection with DC3000 (\( \text{avrRpt2} \)). While neither single mutant affects \( RPS2 \) function, we were consistently able to see 100X more growth of bacteria in the double mutant (Figure 5.3B). Such a synthetic interaction is not unexpected for two genes whose products physically interact (Han et al., 2004). However, this result was surprising, since we expected a \( sgt1b \) mutant to have either no effect on the \( hsp90.2^{\text{ATPase}} \) phenotype or for \( sgt1b \) to suppress \( hsp90.2^{\text{ATPase}} \) as it does for \( rar1 \) (Holt III et al., 2005). When we probed \( RPS5 \) function using DC3000 (\( \text{avrPphB} \)), we found between 5X-10X more growth (data not shown), a similar though less dramatic result as found with DC3000 (\( \text{avrRpt2} \)). These results validate the previously observed physical interaction between HSP90 and SGT1b, and also show that this physical interaction has previously undemonstrated functional consequences for NB-LRR function. Furthermore, these results show that both \( HSP90.2 \) and \( SGT1b \) play a previously unobserved role in \( RPS2 \) and \( RPS5 \) mediated resistance.
We were interested in addressing how the different ATPase domain mutants affected the physical interaction between HSP90.2 and RAR1 and SGT1 for two reasons. First, we wanted to further investigate the synthetic genetic interaction observed between sgt1b and hsp90.2 ATPase mutations described in the previous section. This observation raises the possibility that the ATPase point mutations function by altering the interaction between HSP90.2 and SGT1b. Secondly, one of the hsp90.2 ATPase point mutations, S100F, was previously found to have a partially penetrant phenotype as opposed to the fully penetrant phenotype of the other hsp90.2 ATPase alleles (Hubert et al., 2003). I proposed that this phenotypic difference may be attributable to differences in how the partially penetrant allele interacts with RAR1 and/or SGT1.

Full length cDNAs corresponding to RAR1, SGT1a, SGT1b and HSP90.2 were cloned into LexA yeast two hybrid system vectors, and all four isolated ATPase mutations were introduced into HSP90.2 by site-directed mutagenesis (see Chapter 4 and Hubert et al., 2003). As previously reported for the in vivo interactions between these proteins (Hubert et al., 2003), HSP90.2 interacted with RAR1 and SGT1b but not SGT1a (Figure 5.4A, B and C, first column). HSP90 shows a preference to interact with SGT1b even though the two SGT1 isoforms are 87% similar at the amino acid level (Azevedo et al., 2002). Because these controls worked as expected, the yeast interaction system can serve as a suitable proxy for
Figure 5.4: hsp90.2\textsuperscript{ATPase} domain mutants lose the ability to interact with RAR1 and SGT1b. The interaction of HSP90.2 and hsp90.2 ATPase domain mutants with (A) RAR1, (B) SGT1b, or (C) SGT1a as quantified by β-galactosidase activity in yeast-two-hybrid assays. Miller units were calculated as 1000 X OD\textsubscript{420}/(OD\textsubscript{600} X time in minutes). Fully penetrant point mutants completely lose interaction with RAR1 and SGT1b, while the partially penetrant S100F mutant maintains strong interaction with RAR1. (D) Western blot analysis of yeast protein extracts probed with anti-HA antibody to show HSP90 levels. This panel shows that the differences observed in A and B are not due to differences in expression level.
in planta interactions. We found that the three fully-penetrant alleles of hsp90.2\(^{\text{ATPase}}\) were unable to interact with either RAR1 or SGT1b. This includes the D80N mutant which has been shown to be unable to bind ATP in vitro (Panaretou et al., 1998). Western blot analysis of accumulation of HSP90 showed that these results were not due to changes in accumulation level. Since the three fully penetrant hsp90.2\(^{\text{ATPase}}\) mutant proteins are completely unable to interact with SGT1b, the hsp90.2\(^{\text{ATPase}}\) mutant phenotype is unlikely be due to titration of SGT1b from a RAR1/SGT1b complex. Similarly, the synthetic phenotype observed in the sgt1b hsp90.2\(^{\text{ATPase}}\) double mutants with respect to RPS2 function is likely not due to a loss of residual SGT1b binding to hsp90.2\(^{\text{ATPase}}\) mutant proteins.

Interestingly, while the partially-penetrant allele S100F also lost the ability to interact with SGT1b, it maintained a strong interaction with RAR1 (Figure 5.4A, last column). This suggests that loss of interaction with RAR1 is a likely cause of the fully penetrant hsp90.2\(^{\text{ATPase}}\) phenotype.

**Detailed analysis of RAR1 and HSP90.2 physical interactions**

The apparent importance of RAR1 for HSP90 function led us to investigate the interaction between these two proteins in more detail. As has been previously shown (Azevedo et al., 2002), we observed RAR1 interaction with HSP90, and also with both SGT1a and SGT1b. Unlike HSP90, RAR1 does not show a preference for either isoform of SGT1. RAR1 is composed of three domains: two CHORD domains separated by the CCCH domain. By creating truncations of RAR1, we were able to isolate the interaction between RAR1 and SGT1a or SGT1b to the
Figure 5.5: Structure/function analysis of RAR1. (A) Results of yeast-two-hybrid assays between HSP90, SGT1a, or SGT1b and various RAR1 truncations and point mutations. Strong interaction is shown as dark blue rectangles marked with a “+”, weak interaction is shown as light blue rectangles marked with “+/-“, and loss of interaction is signified as a light yellow rectangle marked with “-“. Interaction with HSP90 maps to the CHORDI domain, while interaction with SGT1 maps to the CHORDII domain. C49, within CHORDI, is necessary for the HSP90 interaction, and H217, within CHORDII, is fully required for the interaction with SGT1b, but only partially required for the interaction with SGT1a. (B) Alignment of the CHORDI and CHORDII domains of RAR1 displaying position of point mutants shown in panel A. Positions of nonsense and missense mutations described in text are also shown.
CHORDII domain, consistent with previous analysis (Azevedo et al., 2002). Additionally, we found that the CHORDI domain was necessary and sufficient for the HSP90/RAR1 interaction as was inferred previously in planta (Hubert et al., 2003).

We previously reported that a nonsense mutant of RAR1 leading to a premature stop at amino acid position 52, *rar1-21*, was able to co-immunoprecipitate HSP90 even though no RAR1 protein was detectable by western blot (Figure 2.9; Figure 5.5B; Hubert et al., 2003). However, it was also discovered at the same time that another nonsense mutant only five amino acids shorter, *rar1-28*, was unable to co-immunoprecipitate HSP90 (Y. Belkhadir, unpublished data). Two possible explanations existed for this result. Either this shorter fragment was less stable than the *rar1-21* fragment, or those five amino acids were required for the interaction. We noticed that the previously isolated point mutant *rar1-26* (C49Y), was able to accumulate a significant amount of RAR1 protein, but still had a complete loss-of-function phenotype (Tornero et al., 2002b). This suggested that this residue could account for the differential interaction between *rar1-21* and *rar1-28* and HSP90.

We introduced the C49Y mutation into our yeast-two-hybrid clones and found that this Cysteine residue alone was necessary for the interaction with HSP90, but not for interaction with SGT1a and SGT1b (Figure 5.5A). A mutation with a presumably less-severe effect on protein structure, C49A, was also introduced with the same result. Additionally, by introducing this mutation into our truncated RAR1 variants, we were able to engineer a peptide which completely lost interaction with both HSP90 and SGT1 (Figure 5.5A).
A mutation in the second cysteine of this pair (the analogous CHORDII residue of C50), C197, in the CHORDII domain has also been isolated, rar1-15 (Figure 5.5B). We recreated this C197Y mutation to test if a mutation in this cysteine pair in CHORDII would block interaction with SGT1. Surprisingly, this mutation did not affect the RAR1 interaction with SGT1a, SGT1b or with HSP90. We then created a CHORDII mutation analogous to the CHORDI C49Y mutation, C196Y, and the double cysteine mutation C196Y C197Y to determine if either of these cysteines in CHORDII is necessary for the interaction with SGT1 like the first cysteine in this pair in CHORDI is necessary for the interaction with HSP90. Neither of these affected the RAR1 interaction profile (Figure 5.5A). To rule out the possibility of CHORDI compensating for the loss of CHORDII interaction with SGT1, we created a triple mutant, C49Y C196Y C197Y. While this mutant lost interaction with HSP90 as expected, the interactions with SGT1 and SGT1b were unaffected. Taken together, this data suggests that RAR1 interacts with HSP90 in a different way than with SGT1, since a residue (C49) in CHORDI necessary for interaction with HSP90 is not necessary in CHORDII (C196) for the interaction with SGT1.

Recently, a new mutation of RAR1 has been reported, H217Y (Shang et al., 2006). The authors show that, while RPM1 function is abolished, this allele is still able to produce a significant amount of RAR1 protein. Furthermore, they demonstrate in yeast two hybrid assays that while interaction with HSP90 was maintained, the H217Y mutant allele lost the ability to interact with SGT1b. We recreated these constructs and independently confirmed these results. However, in contrast to the loss of interaction with SGT1b, RAR1 interaction with SGT1a is not
completely abolished. This suggests that like HSP90, RAR1 interacts differently with SGT1a and SGT1b. This mutation was also introduced into our RAR1 truncation variants with expected results, namely loss of SGT1b interaction and a weak SGT1a interaction. Finally, we introduced both the C49Y and H217Y mutations in a single RAR1 construct. As expected, this construct was unable to interact with either HSP90 or SGT1b.

Discussion

We present here a detailed analysis of the genetic and physical interactions underlying the HSP90/RAR1/SGT1 machinery responsible for maintaining steady state levels of NB-LRR protein accumulation. Our data suggest that different HSP90 “client” proteins may have distinct requirements for HSP90 isoform homo- and heterodimers in Arabidopsis. Our data reveal genetic interactions between two isoforms of HSP90 and between hsp90.2 and sgt1b. We prove that, contrary to accepted dogma, loss of ATP binding and subsequent hydrolysis does not equate to full loss of HSP90 function. Furthermore, we demonstrate a novel synthetic role for HSP90.2 and SGT1b in RPS2-mediated disease resistance in Arabidopsis. In addition, we explored the physical interactions between HSP90, RAR1, and SGT1 using point mutations in HSP90.2 and RAR1 and truncations of RAR1. These results may explain the phenotypes of hsp90.2\textsuperscript{ATPase} mutants and of rar1 missense mutations.

The role of individual HSP90 isoforms in disease resistance and plant growth
Based on expression data, one likely reason that only mutations in \textit{hsp90.2} are recovered in forward genetic screens for loss of RPM1 function becomes apparent. \textit{HSP90.2} is expressed at higher levels (nearly 40-fold greater) than all other isoforms combined. It may be counterintuitive, then, that the seemingly insignificant additional decrease in total HSP90 levels caused by combining T-DNA insertions in \textit{hsp90.1} and \textit{hsp90.2} leads to lethality. However, it proves that this system is finely balanced, and while redundancy allows for major perturbation of HSP90 levels without obvious effect as seen in Figure 5.1, some HSP90 threshold level needs to be maintained. This is perhaps most saliently seen in HSP90 silencing experiments in tobacco (Liu et al., 2004; Lu et al., 2003). Adult plants silenced for HSP90 show heavy pleiotropism including stunting and leaf deformation. Effects were so severe that at times they proved lethal to the plants.

\textbf{Loss of HSP90 ATPase activity does not equal loss of HSP90 function}

While gene duplications in plants are often a hindrance to studies of gene function, the duplication of \textit{HSP90} in Arabidopsis has enabled us to obtain data that are impossible to acquire in systems with only one or two copies of \textit{HSP90}. The hypothesis that loss of ATP hydrolysis is synonymous with loss of HSP90 function arose when point mutations in the ATPase domain of HSP90 were first assayed for their ability to rescue yeast lacking any wild-type HSP90. Yeast lacking HSP90, as well as yeast expressing only the ATPase domain mutant, synonymous with the ATPase domain mutant used here, displayed the same lethality (Obermann et al., 1998; Panaretou et al., 1998). Dead men tell no tales, and it is not possible to assay
dead $hsp90^{ATPase}$ mutant yeast to discern if they die in the same way as HSP90 nulls. If the $hsp90.1^{KO} hsp90.2^{ATPase}$ double mutant used here had been lethal, we would have reached the same conclusion. Even in yeast, however, there were clues that ATPase mutations are not null mutations, specifically when it was found that overexpression of these ATPase mutants showed a semi-dominant negative effect on yeast growth in the presence of wild-type HSP90 (Panaretou et al., 1998). At the time, this was attributed to sequestering of client proteins or co-chaperones, i.e. that an ATPase mutant is null for function and also blocks wild-type HSP90 by binding/titrating other proteins required for function. However, this would imply that these mutants are “more null than a null.”

Our results suggest another hypothesis. When HSP90 chaperone activity was first described in vitro, it was shown to work in an ATP-independent manner (Jakob et al., 1995; Wiech et al., 1992; Yonehara et al., 1996). While previous reports have tried to reconcile the in vitro results with the in vivo results by suggesting ATP-dependent and ATP-independent steps in chaperone activity, that hypothesis is inconsistent with our results (Panaretou et al., 1998). If such were the case, our ATPase mutants would nevertheless be impaired in chaperone activity, and would have a phenotype at least as severe as the null.

Rather, our results suggest that HSP90 actually has two separate activities: one ATP-independent activity and one ATP-dependent or, to be more precise, ATP regulated activity (see below). Since in vitro “chaperoning” has been shown to occur in an ATP-independent fashion, it is possible that this represents the apparent ATP independent activity. This ATP-independent activity is absent in HSP90-silenced
plants and in \( hsp90.1^{KO} \) \( hsp90.2^{KO} \) double mutants, and is thus necessary for plant survival as has been previously shown for yeast (Borkovich et al., 1989). R proteins are unstable in both \( HSP90 \)-silenced lines and in our ATPase domain mutant suggesting that both activities are required for R protein accumulation.

**HSP90 maintains client protein steady state levels by an active mechanism**

We will now consider the ATP-dependent activity of HSP90. Loss of ATP binding leads to a gain-of-function phenotype based on the comparison between the \( hsp90.2^{ATPase} \) and the \( hsp90.2^{KO} \) single mutants (Hubert et al., 2003). This excludes the possibility of ATPase mutations being hypomorphic. Thus, the loss of ATP binding and subsequent hydrolysis is either neomorphic or hypermorphic. It is highly unlikely, given the conservation of the ATPase domain, that changes in ATP hydrolysis rates can be truly neomorphic. In fact several known co-chaperones are known to modulate ATPase rates either positively or negatively (Panaretou et al., 2002; Richter et al., 2004). This suggests that, in contrast to the accepted belief that loss of ATPase activity is equivalent to loss of HSP90 activity, loss of ATPase activity actually leads to higher HSP90 activity, i.e. that the ATPase domain is a negative regulatory domain. Given that the phenotypic output of a gain-of-function ATPase mutant is loss of client protein accumulation, the wild type ATP-regulated activity must negatively regulate client protein levels.

It has previously been shown that loss of ATPase activity causes HSP90 to have higher affinity for its client proteins, which could provide a mechanism for how the ATP-independent and ATP-regulated activities are linked in one protein (Young...
and Hartl, 2000). If a protein is grossly misfolded or at too high a concentration, one would expect that the time spent in interaction with HSP90 would increase, either due to increased chaperoning or due to simple concentration-dependent on/off rates. If increased interaction time also leads to higher ATP-regulated activity, then HSP90 would cause removal of the client protein from the cellular pool in both cases. In fact, we are unable to exclude the possibility that HSP90 is acting as an additional subunit of the SCF or proteasome complex (Eleuteri et al., 2002), as HSP90 can interact with SKP1 via SGT1 (Catlett and Kaplan, 2006; Lingelbach and Kaplan, 2004) and is required for the assembly of the 26S proteasome (Imai et al., 2003).

**HSP90 co-factors also separate HSP90 nurturing and destroying activities**

If the ATPase domain acts to balance HSP90 between its ATP-independent function and ATP-regulated functions, then one would expect HSP90 co-factors to also be able to differentiate between these two activities. Since the *in vitro* chaperoning function can occur in the absence of other co-factors, one would expect that many of these ATPase modulating co-factors would be non-essential to living cells. In fact, while it was found very early that HSP90 is an essential protein, phenotypes for loss of HSP90 co-factors have been much harder to identify (Borkovich et al., 1989). The yeast homolog of the HSP90 co-factor P23, SBA1, has been shown to decrease HSP90 ATPase activity. However, deletion of SBA1 has no immediate effect on client protein function and the only phenotype found for SBA1 deletion mutants is an increased sensitivity to inhibitors of HSP90 ATPase activity (Bohen, 1998).
Figure 5.6: Schematic genetic models of possible RAR1, SGT1, and HSP90 interactions and their relative effects on R protein stability. All three models show the HSP90 ATPase domain acting as a negative regulator of HSP90 activity consistent with the data presented in this manuscript. This also implies that HSP90 normally functions to decrease R protein levels. (A) shows the possibility of a direct linear pathway controlling HSP90 function. (B) shows a pathway in which RAR1 and SGT1 antagonize each other via HSP90. (C) shows a hybrid model of (A) and (B) in which RAR1 and SGT1 antagonize each other not only via HSP90 but also directly.
RAR1 has been shown to be required for maximal accumulation of R proteins. It has also been shown that the decrease in R protein accumulation in a rar1 mutant is dependent on SGT1b (Holt III et al., 2005), and that functional R proteins are produced in the absence of both RAR1 and SGT1b. Consequently, these proteins may not be necessary for HSP90’s role in chaperoning, and must be regulating some other aspect of HSP90 function. Therefore, these proteins might act to control HSP90 ATPase rates. Effects of RAR1 on HSP90 ATPase rates have not been reported so far. While one report shows no SGT1 effect on HSP90 ATPase rates, it did not take demonstrated concentration ratios necessary for effects on HSP90 ATPase rates into account (Catlett and Kaplan, 2006; Lee et al., 2004; Richter et al., 2004).

Possible models explaining the functional interactions between RAR1/SGT1/HSP90

In this manuscript we show a clear genetic interaction between HSP90 and SGT1b. This proves that our previously described physical interaction between these two proteins is biologically relevant (Hubert et al., 2003). sgt1b is able to suppress a rar1 mutant which supports the functional validity of their physical interaction (Azevedo et al., 2002; Holt III et al., 2005; Liu et al., 2002b). Our result that a partially-penetrant allele of hsp90.2 maintains physical interaction with RAR1 supports the requirement of this physical interaction for HSP90 function. Recently, mutations outside the ATPase domain of hsp90.2 were found to suppress a rar1 mutant phenotype (Y. He, unpublished data). While the consequences of these
missense mutations on \textit{hsp90.2} function are currently unclear, it supports evidence presented here in two ways. First, \textit{HSP90.2} represents the predominant isoform involved in plant disease resistance with six total mutations in \textit{hsp90.2} versus none in another isoform. Second, the previously observed physical interaction between RAR1 and HSP90.2 also has biological relevance (Hubert et al., 2003). Consequently these three proteins all interact with each other both physically and genetically.

Based on our genetic data we generated models explaining the relationships between HSP90, RAR1 and SGT1 (Figure 5.6). \textit{hsp90.2} and \textit{sgt1b} likely act downstream of RAR1 as they both can act to suppress \textit{rar1}. While \textit{sgt1b} is unable to suppress an \textit{hsp90.2}^{\text{ATPase}} mutant, the structural homology of the CS domain of SGT1 to the HSP90 co-chaperone P23 suggests that \textit{SGT1b} also modulates HSP90 ATPase activity.

As stated previously, it appears that the ATPase domain of HSP90 acts as a negative regulatory domain of HSP90 function. The loss of client protein accumulation found after treatment with HSP90 ATPase inhibitor has been shown to be reversible by proteasome inhibitors (Segnitz and Gehring, 1997; Whitesell and Cook, 1996). To our knowledge, this has not been demonstrated for ATPase domain mutants, but we expect that it will be the case since both mutation and inhibitor block ATP hydrolysis. Thus it is reasonable to predict that the ATP-regulated activity feeds client proteins into the proteasome. This output and its inhibition by HSP90 ATPase activity is the basis of all of our models (Figure 5.6).
The relationship of HSP90 ATPase activity with RAR1 and SGT1 is less clear. Since the CS domain of SGT1 folds similarly to P23, a protein with known effects on HSP90 ATPase rates, it is plausible that SGT1 directly modulates HSP90 ATPase activity. Thus all of our models show a direct effect of SGT1 on HSP90 ATPase rates. We can also predict that SGT1 negatively regulates ATPase rates for two reasons. The first is that P23 decreases ATP hydrolysis rates, and the other is that sgt1b mutants cause R protein levels to increase in a rar1 mutant background which can be explained by higher rates of HSP90 ATP hydrolysis in a sgt1b mutant (Holt III et al., 2005).

The placement of RAR1 in this pathway is problematic. We know that functionally it acts upstream of HSP90, since hsp90.2 mutants can act as suppressors of rar1. However, the ability of sgt1b to suppress rar1 places SGT1b downstream of RAR1 function. A direct linear model where SGT1 acts as an intermediary between RAR1 and HSP90 explains the genetic data, but discounts the physical association between RAR1 and HSP90 (Figure 5.6A). To explain the lower R protein levels observed in a rar1 mutant, RAR1 may either directly increase ATPase rates (Figure 5.6B) or indirectly increase ATPase rates by negatively regulating SGT1 (Figure 5.6A). These two models are not mutually exclusive, as RAR1 and SGT1 may be able to antagonize each other directly through their interaction, while at the same time antagonizing each other indirectly through their interaction with the ATPase domain of HSP90 (Figure 5.6C). This last model also explains the correlation between our partially-penetrant mutant phenotype and its loss of interaction with SGT1b but not RAR1.
These models are unable to explain two observations. The first is the synthetic genetic interaction between \( hsp90.2^{ATPase} \) and \( sgt1b \). They also cannot explain the fact the \( sgt1b \) alone cannot suppress \( rar1 \) for all R gene pathways tested, or that the two mutants show additivity for some pathways. However, the action of other isoforms of HSP90 and of the other isoform of SGT1 may explain these inconsistencies.

While the fact that these proteins work together was found using biochemical techniques, the phenotypic relevance of these interactions has been shown utilizing epistasis analysis. To test these models directly requires a return to biochemical techniques, specifically measurement of the effects of RAR1 and SGT1 on HSP90 ATPase hydrolysis rates. To that end we have set up in our lab a previously described method for determining HSP90 ATPase hydrolysis rates (Panaretou et al., 1998). We have purified wild-type HSP90.2 and found that its ATPase hydrolysis rates fall within previously described parameters (B. McNulty and DH, unpublished results). The identification of residues which block RAR1’s ability to interact with HSP90 or SGT1 described here will enable us to directly test each of these models \textit{in vitro} for RAR1’s role. Mutations of SGT1b which block HSP90 and RAR1 interaction separately are also available (Jack Peart, personal communication). These mutations have been introduced into protein expression vectors containing the cDNAs corresponding to each of these proteins, and protein expression and purification is underway.

This manuscript offers a revealing look inside the genetic machinery regulating R protein accumulation. There are much broader implications for these
results within plant science and medical science. \textit{sgt1b} was found to be an enhancer of a \textit{tir1} F-box mutant, and \textit{SGT1b} overexpression can partially suppress \textit{tir1} (Gray et al., 2003). HSP90 and the mammalian homolog of RAR1 have also recently been shown to play a role in regulation of NOD proteins, animal proteins involved in animal innate immunity similar to R proteins (Hahn, 2005). Findings presented here may find applications in the treatment of human disease, as they are analogous to the innate immunities of animals. Additionally, our findings of the difference between HSP90 ATPase inhibition and general HSP90 inhibition implies that suggested gene therapy approaches may have different and unexpected effects compared to pharmacological approaches currently being tested (Latchman, 2001; Sharp and Workman, 2006).

\textbf{Materials and Methods}

\textbf{Plant lines}

All plant lines used in this manuscript are in the genetic background Col-0. The a11 line (Tornero et al., 2002b) contains Estradiol inducible \textit{avrRpm1}. \textit{hsp90.2\textsuperscript{ATPase}} refers to \textit{hsp90.2-3} unless otherwise noted (Hubert et al., 2003). Additional mutant alleles used were \textit{rpm1-3} (Grant et al., 1995), \textit{rps2-101c} (Mindrinos et al., 1994), and the \textit{edm1} allele of \textit{sgt1b} (Tör et al., 2002). \textit{hsp90\textsuperscript{KO}} lines (HSP90.1 At5g52640 SALK_075596, HSP90.2 At5g56030 SALK_058553, HSP90.3 At5g56010 SALK_013240 and SALK_040191, HSP90.4 At5g56000 SALK_036835) were obtained as segregating populations from the Arabidopsis
Biological Resource Center (ABRC) and were originally created by the J. Ecker lab at the Salk Institute for Biological Studies. Homozygous insertions were determined by presence of a PCR product for a primer pair corresponding to the left border of the T-DNA insert and a gene specific primer and absence of a PCR product for a primer pair found to give specific isoform amplification. Homozygosity was confirmed in the next generation for all lines. Primers are available upon request. Selection of homozygous \( sgt1b^{edm1} \) plants was made by western blot analysis utilizing a native antibody to SGT1 (D. Hubert, unpublished data), and confirmed in the next generation. \( hsp90.2-3 \) homozygous plants were selected by a specific dCAPS marker. (Primers and conditions available upon request.)

**Bacterial strains, inoculation, and growth quantification**

Maintenance of \( Pto \) DC3000 containing the kanamycin selectable plasmid pVSP61 with an \( avrRpm1 \) or \( avrRpt2 \) insert was as described (Ritter and Dangl, 1996). Four- to five-week-old plants were spray-inoculated with \( 5 \times 10^7 \) colony-forming units/mL of \( Pto \) DC3000 \( (avrRpm1) \) in 10 mM MgCl\(_2\) and 0.02% silwet L-77 (CKWitco Corporation). These plants were then covered for four hours with a clear plastic lid and assayed for symptoms between four and seven days after treatment. Bacterial quantifications were performed as described (Tornero and Dangl, 2001).

**Estradiol induction and screening**

Two-week-old short-day-grown plants were sprayed with 10\( \mu \)M \( \beta \)-Estradiol (Sigma E 8875)/ 0.02% Silwet L-77 in distilled water, diluted from a 10 mM \( \beta \)-
Estradiol stock dissolved in 100% ethanol (Tornero et al., 2002a). Trypan Blue staining was performed 5 days later (Koch and Slusarenko, 1990). A light microscope with attached camera was used to obtain pictures of stained leaves (Nikon Eclipse).

**Semi-quantitative reverse transcription analysis**

To analyze HSP90 transcript levels, two-week-old seedlings were ground in liquid nitrogen and RNA was extracted with Trizol according to the manufacturer’s instructions (GibcoBRL). RNA reverse transcription and RT-PCRs were performed according to the manufacturer's instructions (RETOscript, Ambion).

**Yeast two-hybrid**

cDNAs for *RAR1* from an RT-PCR reaction are described above. cDNAs corresponding to *HSP90.2, SGT1a* and *SGT1b* were obtained from ABRC. These cDNAs were placed in pENTR D-TOPO cloning vectors (Invitrogen). From the entry clone, cDNAs were recombined into pEG202gw and pJG4-5gw (kind gift of Hironori Kaminaka; described in Holt III et al., 2005). Mutations were engineered using site-directed mutagenesis with overlapping primer pairs containing a mismatch corresponding to the mutation (Ausubel et al., 1987). These PCR products were then transferred into pENTR D-TOPO. Directed interactions were performed using yeast strain EGY48 (Bieri et al., 2004). β-galactosidase activity was performed as previously described (Kaminaka et al., 2006).
Protein blots

3 mL overnight culture of HSP90 wild-type and mutant forms cloned into pJG4-5gw and transformed into EGY48 containing pEG202 were grown in Ura⁻His⁻ Trp⁻ selective media. Cultures were centrifuged and washed in 1 mL of cold distilled water containing 1 mM PMSF and 1 mM EDTA. Samples were resuspended in 250 μL 1X SDS-PAGE loading buffer, boiled for 5 minutes and vortexed for 1 minute with 0.3 mL glass beads to lyse cells. Samples were then centrifuged again to precipitate cellular debris. 30 μL of each sample was run on 8% SDS-PAGE gels. Western blots were performed with anti-HA (Roche) monoclonal antibody utilizing standard methods and detected with ECL (Amersham).
Bibliography


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Chapter 6

Conclusions and Future Directions

Background and Significance

The pace of discovery in the field of plant disease resistance has continued to accelerate over the last decade. During the same period our view of the molecular mechanisms underlying the activity of disease Resistance (R) proteins has radically changed. We no longer believe that a simple receptor-ligand model holds true for recognition of all pathogen *avr* genes by *R* genes (Baker et al., 1997). It is now apparent that many *avr* genes are recognized indirectly by their virulence effect on plant cellular targets (Dangl and Jones, 2001).

Upon their identification, it was widely believed that most if not all identified mutants affecting *R* gene function did so by directly affecting the resistance signal transduction cascade (Century et al., 1995; Tornero et al., 2002; Warren et al., 1999). Since then it has become evident that several of these loci act indirectly in the pathway by modulating the levels of NB-LRR type R proteins (Holt III et al., 2005).

The work presented in this dissertation focuses on studying whether these mutants affecting *R* gene function alter R-protein levels. Specifically, I established that one gene, *LRA2*, affects accumulation of the Arabidopsis R protein RPM1 and encodes cytosolic *HSP90* (Chapter 2). In contrast, I ascertained that the previously described mutant, *ndr1*, does not affect the levels of an R-protein known to fully
require \textit{NDR1} for function (Chapter 3). This suggests that NDR1 may in fact act in the \textit{R} gene signal transduction pathway. I helped extend the previously observed effect of \textit{rar1} on NB-LRR accumulation by showing that this finding was not specific for RPM1 accumulation (Chapter 3). I additionally helped demonstrate that the \textit{rar1} mutant phenotype can be suppressed by increasing levels and/or signaling of NB-LRR proteins (Chapter 3). I also identified new mutants affecting NB-LRR accumulation, including one that reveals the importance of transcriptional control (Chapter 4).

As well as identifying an effect on NB-LRR levels by different mutants, I also studied the mechanism involved in this process. Data presented in Chapter 2 demonstrates that RAR1/SGT1/HSP90 physically interact. Genetic interactions between HSP90 and SGT1 presented subsequently in Chapter 5 validate the physical interaction’s biological relevance. Additionally, I show that several phenotypic mutants of \textit{rar1} and \textit{hsp90.2} have defects in their ability to interact with other members of the RAR1/SGT1/HSP90 trinity (Chapter 5). I also importantly demonstrate that the belief that \textit{HSP90} ATPase mutants are null or dominant negative alleles is ill-founded (Chapter 5). This has important implications for our continued study of the role of \textit{HSP90} in plant disease resistance. We are currently studying an ATPase allele of \textit{hsp90.2}, whereas many groups are studying \textit{HSP90}'s function using transient gene silencing. It is important to realize that the conclusions drawn from these experiments are not necessarily comparable. I have shown that \textit{SGT1b} plays a broader role in disease resistance than previously thought, and I also extended the known role of \textit{HSP90.2} (Chapter 5).
Future Directions

Much work remains to be done, especially for the work presented in Chapters 4 and 5. The new mutants described in Chapter 4 will allow us to identify new genes involved in \( R \)-gene mediated disease resistance, as they have already revealed new processes at work. To this end, complementation analysis is ongoing. Mapping has already commenced on another mutant with normal levels of RPM1 protein. Additionally, the 30 segregating mutant lines shown in Table 4.1 will be homozygotized, and included in our ongoing mutant analyses. The transition of this project to other hands is well underway.

The models I describe in Chapter 5 are clearly and easily testable. I have set up a system for testing these models and will soon know how the different HSP90 mutations affect ATPase activity. In order to determine the effects of RAR1 and SGT1 on ATP hydrolysis rates, I have already established conditions to express and solubilize RAR1 and SGT1. This work is in conjunction with research being performed by Y. He in our lab on alleles of \( hsp90.2 \) that suppress a \( rar1 \) mutant phenotype. Together we are currently extending the analysis presented in Chapter 5 to these new alleles.
Publications in chronological order


Bibliography


