Chapter 1:

Introduction
Overview of defense response regulation

Plants are attractive targets for a variety of pathogens, which seek to exploit them as a source of nutrients. As a consequence, plants have evolved complex defense mechanisms. The first lines of plant defense are “preformed” physical and chemical barriers, such as waxy leaf cuticles and cell walls. If a pathogen bypasses these preformed defenses, plants can then activate a second line of inducible defensive responses. These include a rapid burst of reactive oxygen intermediates (ROIs) and antibiotic release at the infection site, along with localized cell wall strengthening to impede pathogen growth (Hammond-Kosack and Jones, 1996). Genetically programmed cell death is also triggered at the site of infection; this process is known as the hypersensitive response (HR). These local responses can induce systemic acquired resistance (SAR), which provides long-lasting, broad-spectrum resistance.

These inducible defenses can be activated by plant R proteins, which are thought to recognize pathogen factors known as avirulence (Avr) proteins. After recognition of an Avr protein, R proteins activate a signal transduction cascade that regulates the inducible defenses described above. R proteins respond specifically to Avr proteins from a particular pathogen or pathogen isolate, providing so-called gene-for-gene resistance (Flor, 1971, Holt et al., 2000). This recognition specificity is often very narrow. Individual R gene alleles typically recognize individual Avr alleles, as pathogens maintain extensive allelic diversity at Avr loci (Rehmany et al., 2005). Plants must therefore maintain large arrays of R genes to recognize a broad range of pathogens.
In the absence of \( R \) gene-mediated recognition, plants can also activate so-called "basal defenses". These defenses are triggered by the recognition of general elicitor molecules produced by pathogens, such as flagellin or chitin (Gomez-Gomez and Boller, 2002). Although basal defenses activate the same defense pathways as many \( R \) genes, the response is slower and weaker. As a result, basal defenses are often unable to halt pathogenesis (Hammond-Kosack and Parker, 2003, Belkhadir et al., 2004). Effective plant resistance is therefore dependant upon proper \( R \) gene expression.

\( R \) proteins were originally thought to bind specific Avr proteins. However, few studies have found evidence for direct \( R/Avr \) protein interaction. An alternate model proposed by Van der Biezen and Jones (1998), called the “guard theory”, predicts indirect interaction between \( R \) and Avr proteins. In this model, pathogens secrete Avr proteins during infection. Some Avr proteins target and modify plant proteins, effectively reprogramming the plant to favor pathogen growth. These modifications are detected by \( R \) proteins, which interact with (“guard”) plant virulence targets. \( R \) proteins subsequently activate defense cascades. There are several variations of this hypothesis (protein phosphorylation, degradation, dual recognition between Avr protein and \( R \) protein), and different classes of \( R \) proteins may use distinct mechanisms (Martin et al., 2003).

Surprisingly few different structural classes of \( R \) genes have been discovered thus far. The majority encode cytoplasmic nucleotide-binding site – leucine-rich repeat (NBS-LRR) proteins. NBS domains are able to bind and hydrolyze ATP (Tameling et al.,
2002), and are potentially involved in signaling interactions (Shirano et al., 2002). LRR domains appear to be under diversifying selection (Michelmore and Meyers, 1998), and may have multiple functions, including ligand recognition, and signaling control via intramolecular interactions (Belkhadir et al., 2004, Martin et al., 2003) The NBS-LRR superfamily can be divided into two subclasses, based on N-terminal motifs: TIR-NBS-LRR proteins, so named for their similarity to Toll and Interleukin-1 receptors in mammalian innate immunity; and CC-NBS-LRR proteins, which form putative coiled-coil structures such as leucine zippers. These two subclasses will hereafter be referred to as “TIR” and “CC” proteins.

R proteins activate defense responses through a complex network of signaling pathways (summarized in Figure 1.1), which have been extensively characterized in Arabidopsis. Most R protein signaling pathways involve RAR1 and SGT1; these interacting proteins appear to control the ubiquination and destruction of regulatory proteins (Muskett and Parker, 2003). RAR1 may also play a crucial role in mediating R protein levels, possibly through protein stabilization (Bieri et al., 2004). SGT1 is closely related to HSP90 proteins, and may have a co-chaperone function (Muskett and Parker, 2003). The TIR and CC subclasses have distinct downstream signaling mechanisms, although there is extensive cross-talk between pathways (Aarts et al., 1998; McDowell et al., 2000). TIR proteins signal primarily through the putative lipase EDS1 (enhanced disease susceptibility 1) (Falk et al., 1999). EDS1 physically interacts in planta with another putative lipase, PAD4 (phytoalexin deficient 4), which reinforces the early defense response of EDS1 (Feys et al., 2001). CC protein signaling usually employs NDR1
(Non-race-specific disease resistance 1) (Century et al., 1997), although this protein also weakly contributes to TIR protein signaling (Aarts et al., 1998). NDR1 is a membrane-associated protein, and could potentially interact with pathogens directly (Coppinger et al., 2004). NDR1 expression is increased during responses to both avirulent and virulent bacterial strains (Century et al., 1997).

Other proteins and small molecules activate further defense functions downstream of primary signaling. One key player is the phenolic compound salicylic acid (SA), which is essential for many defense responses. Its importance was underscored by analysis of NahG plants, which express a bacterial transgene that breaks down SA; these plants are highly susceptible to numerous pathogens, and are unable to activate SAR (Lawton et al., 1995). SA promotes the production of ROIs and activates PR (pathogenesis related) mRNA accumulation (Ryals et al., 1996). SA accumulation after pathogen recognition plays a major role in several (but not all) Arabidopsis gene-for-gene resistance pathways (McDowell et al., 2000). Exogenous application of SA or its active homologs is sufficient to activate SAR in the absence of pathogen attack (Lawton et al., 1996). SA synthesis is therefore tightly controlled to prevent unnecessary defense activation, which would have a deleterious effect on plant growth (Bowling et al., 1994). Recently, SA has also been implicated as a component of positive feedback loops that enhance defense responses (Ryals et al., 1996, Shirano et al., 2002, Xiao et al., 2003; also see below).

Further downstream in the defense cascade is the transcriptional regulator NPR1 (non-expressor of PR genes 1) (Cao et al., 1994, Delaney et al., 1995). This protein enters the
nucleus after defense activation and induces pathogen resistance genes through interactions with transcription factors (Kinkema et al., 2000). In Arabidopsis, SA signals are transmitted by \textit{NPR1} (Ryals et al., 1996), but may also act through other pathways, as evidenced by SA accumulation and SAR in \textit{npr1} mutants (Shirano et al., 2002). A recent study by Wang et al. (2005) used microarray analysis to characterize genes directly influenced by NPR1; transcriptional targets included \textit{PR} genes and numerous protein folding and secretion factors. NPR1 thus appears to regulate appropriate secretion of PR proteins in systemic response. Surprisingly, NPR1 may also negatively regulate some defense genes under normal growing conditions (Eulgem et al., 2004). NPR1 therefore has a complex and important role in defense regulation.

In addition to SA-mediated defenses, some plant defense pathways rely upon the small molecules jasmonic acid (JA) and ethylene. These compounds have roles in basal and \textit{R}-gene-mediated resistance against some fungal, bacterial, and viral pathogens (Ellis et al., 2002, Takahashi et al., 2002, Ton et al., 2002). Another form of pathogen defense, termed ISR (induced systemic resistance), is activated by infection of plant roots with nonpathogenic bacteria, and requires JA and ethylene pathways (Pieterse et al., 1998). ISR, which can also be activated by treating plants with methyl jasmonate, enhances basal resistance to fungi and bacteria (Ton et al., 2002). Interestingly, both ISR and SAR require NPR1 (Pieterse et al., 1998), suggesting some overlap between these two defense pathways. Efficient plant defenses against many pathogens may require a balance between SA, JA, and ethylene defense signaling pathways (Devadas et al., 2002, Ton et al., 2002, Navarro et al., 2004). The plant hormone auxin may also be involved in some
JA resistance signaling pathways (Tiryaki et al., 2002, Navarro et al., 2004). The interaction between these different defense pathways is not yet understood.

Reactive oxygen intermediates (ROIs) may also regulate aspects of disease resistance, as oxidative stresses are able to induce some defense signaling molecules (Nawrath et al., 2002, Laloi et al., 2004). ROIs may be involved in SA-regulated feedback loops to control the HR (Delledonne et al., 2001), but their exact roles in gene regulation remain to be clarified.

*Regulation of R gene expression*

Plants must express R genes at an optimal level for proper defense activation. Constitutive overexpression of R genes can cause spontaneous cell death lesions and severely stunted growth (Tang et al., 1999, Xiao et al., 2003). On the other hand, underexpression of R genes can inhibit effective resistance responses (Parker et al., 1993, McDowell et al., 1998, Bieri et al., 2004, B. Holt and J. Dangl, unpublished; also see discussion). Most R genes are constitutively expressed at low levels in unchallenged plants (Van der Biezen and Jones, 1998, Tang et al., 1999, Halterman et al., 2003). However, several recent studies have found that R genes can be upregulated during defense responses. For example, Halterman et al. (2003) used real-time PCR to show that barley MLA genes are induced during pathogen attack. They examined the expression of the closely related genes MLA6 and MLA13, which belong to the CC class and provide resistance against two different isolates of barley powdery mildew (Bgh).
These $R$ genes are induced only after $R$ gene-dependant recognition, not as a result of basal defensive responses. A second example of $R$ gene upregulation is provided by the tobacco resistance protein N, which belongs to the TIR class and provides resistance against tobacco mosaic virus (TMV). Levy et al. (2004) found that $N$ mRNA levels are strongly increased by TMV; 72 hours after TMV infection, transcript levels increased 38-fold in infected leaves. Interestingly, TMV also provoked a strong, systemic upregulation of $N$, as it was induced 165-fold in uninfected leaves. This induction was specifically caused by TMV, as infection with potato virus X did not affect $N$ transcript accumulation. $N$ thus appears to be strongly induced upon activation. The activation of specific $R$ genes during infection presumably leads to a stronger defense response, but the mechanisms behind this upregulation are currently unknown.

*Feedback regulation of defense responses*

Positive feedback appears to be a common regulatory mechanism in defense responses. Numerous signaling genes are induced by pathogens, such as $NDR1$ (Century et al., 1997), $NPRI$ (Ryals et al., 1997, Cao et al., 1998), $RAR1$ and $SGT1$ (Halterman et al., 2003). SA alone is sufficient to induce many defense signaling genes, such as $EDSI$, $PAD4$, and $EDS5$, although these genes are genetically defined as acting upstream of SA (Feys et al., 2001, Shirano et al., 2002). These regulatory mechanisms may amplify weak recognition signals to insure an effective defense response.
R proteins may also be subject to positive feedback regulation. Xiao et al. (2003) examined the Arabidopsis R genes RPW8.1 and RPW8.2 (hereafter called RPW8), which confer resistance to powdery mildew. Col::RPW8 plants have SHL (spontaneous HR-like lesions) and accumulated very high levels of SA under normal environmental conditions, which corresponded with strong RPW8 expression. SA treatment amplified RPW8 transcript approximately 8-fold, indicating that RPW8 expression can be regulated by a SA-dependant feedback loop. Interestingly, SHL and resistance vary with environment; conditions that minimize or eliminate SHL also block powdery mildew resistance and lower RPW8 mRNA levels. However, SA treatment was sufficient to induce SHL and RPW8 expression and resistance regardless of environmental conditions. SA is thus essential for RPW8 amplification and its SHL phenotype in Columbia plants.

Studies of ssi4, a constitutively active TIR mutant, suggest that R gene feedback can amplify other defense responses. Shirano et al. (2002) isolated ssi4, during a search for suppressors of the npr1-5 mutant. ssi4 is a semidominant mutation caused by a single amino acid change in a non-conserved region of the NBS domain, and is transcribed at high levels in both heterozygotes and homozygotes. This mutant R protein exhibits constitutively active PR gene expression, spontaneous lesions, and stunted growth. ssi4 mutants accumulate high levels of SA in both npr1 and wild-type backgrounds, and exhibit strong resistance to both P. syringae and P. parasitica. SA is essential for ssi4 upregulation and the SHL phenotype. The TIR gene RPP1 is also induced by SA treatment, suggesting that SA may regulate a feedback loop controlling the expression of multiple R genes.
Additionally, recent microarray studies have revealed that dozens of $R$ genes and $R$ gene analog transcripts are upregulated by the bacterial PAMP (pathogen-associated molecular pattern) flagellin protein flg22 (Zipfel et al., 2004, Navarro et al., 2004). Many defense signaling components are also rapidly induced upon flg22 treatment. Pretreatment of plants with flg22 inhibited growth of the virulent bacterial pathogen *Psuedomonas syringae* pv. *tomato* DC3000, indicating that upregulation of these proteins is sufficient to activate defense responses (Navarro et al., 2004). Several $R$ gene homologs are also upregulated in response to CMV-Y (cucumber mosaic virus strain $Y$) infection (Marathe et al., 2004). These data suggest that positive feedback is a major regulator of plant defense responses, including $R$ genes; however, no positive feedback mechanism has been well characterized in the plant defense system.

**W-box elements**

Several cis regulatory elements have been associated with the transcriptional regulation of defense-related genes. W-box elements, which have the conserved sequence [T/C]TGAC[T/C], are found at high statistical frequency in SAR- and flg22-induced gene promoters (Maleck et al., 2000, Zipfel et al., 2004). These regulatory elements also appear to upregulate transcription after wounding (Rushton et al., 2002). The arrangement of W-boxes can influence the strength and inducibility of a promoter (Rushton et al., 2002, Cormack et al., 2002). W-boxes often occur in clusters and work
synergistically, as in the *NPR1* promoter (Yu et al., 2001). These *cis* elements are thus a major regulatory component of many plant defense genes.

*W*-boxes are recognized by the WRKY family of plant-specific transcription factors. WRKY proteins are defined by a conserved domain beginning with WRKYGQK at the N-terminus, and they contain zinc finger motifs (Eulgem et al., 2000). These proteins can mediate both upregulation and downregulation of gene expression (Turck et al., 2004, Ülker and Sommssich, 2004). Many WRKY proteins have important roles in defense responses, and have been associated with wound and pathogen responses (Yang et al., 1999, Rushton et al., 2002, Nishiuchi et al., 2004). In addition, many WRKY genes are rapidly induced by bacterial PAMPs (Cormack et al., 2002, Zipfel et al., 2004, Navarro et al., 2004). Overexpression of WRKY proteins is sufficient to upregulate multiple defense response proteins, including thioredoxins and PR proteins (Laloi et al., 2004). A recent study of the *NPR1* promoter revealed that WRKY proteins are essential for basal and inducible expression of the *NPR1* gene (Yu et al., 2001). In sum, WRKY proteins are part of a complex regulatory network that has yet to be fully elucidated.

**Hyaloperonospora parasitica and RPP8**

My experimental system is based on the interaction between *Hyaloperonospora parasitica* and *Arabidopsis thaliana*. *H. parasitica* (recently reclassified from *Peronospora parasitica*) is an oomycete pathogen that causes downy mildew disease on Arabidopsis and cultivated Brassicas, and is closely related to mildew pathogens that
cause major damage to a variety of commercial crops. Multiple isolates of *H. parasitica* have been discovered and characterized, and are used to identify and characterize race-specific resistance genes in multiple Arabidopsis ecotypes. This plant/pathogen interaction has also been employed to define the molecular basis of many *R* gene signaling pathways.

*H. parasitica* propagates by producing asexual conidiospores, which germinate on leaf surfaces, form infection pegs, and penetrate the host leaf. The peg differentiates into branched, filamentous hyphae that grow in the intercellular spaces. The hyphae produce structures called haustoria, which invaginate mesophyll cells and are thought to draw nutrients from the plant. Five to six days after the initial infection, hyphae emerge from open stomata and differentiate into tree-like sporangiophores. These structures, which are visible to the naked eye, produce conidiospores that are carried by wind and water to new hosts, thereby renewing the cycle of infection. The molecular mechanisms of *H. parasitica* pathogenicity remain to be explained.

*R* genes that mediate race-specific resistance to isolates of *H. parasitica* are called *RPP* genes (*Resistance to Hyaloperonospora parasitica*). *RPP* genes likely identify Avr proteins secreted by *H. parasitica* into the plant cell cytoplasm (Rehmany et al., 2005). Resistant plants activate a rapid HR at sites of infection, quickly killing the pathogen. Susceptible plants, on the other hand, are unable to mount effective defenses to this pathogen, and support vigorous pathogen growth and reproduction. Five to six days after infection, dozens of sporangiophore structures are visible on susceptible plant leaves.
Plants with incomplete resistance have an intermediate phenotype, and support relatively few sporangiophores.

*RPP8-Ler* is a CC gene from the Ler ecotype, and it provides resistance against the *H. parasitica* isolate Emco5. McDowell et al. (1998) cloned *RPP8* in Ler and found two closely related genes, *RPP8* and *RPH8A* (*RPP8* homolog A), at the locus (Figure 3.1A). The *RPP8* cluster in Columbia has only one gene, *RPP8-Col*, which appears to be the result of a recombination between ancestral *RPP8* and *RPH8A* genes. Two additional *RPP8* homologs of unknown function are present in the Columbia genome (McDowell et al., unpublished). The recognition specificities of *RPP8-Col* and *RPH8A* have not been defined. However, two other alleles of known function have been identified at the *RPP8* locus: *HRT*, which provides resistance to turnip crinkle virus (Cooley et al., 2000), and *RCY1*, which provides resistance to cucumber mosaic virus Y (Takahashi et al., 2002). Although highly similar, these alleles appear to require different downstream signaling pathways (Takahashi et al., 2002).

*RPP8-Ler* signaling is not dependant on any of the signaling molecules that are employed by other R genes. *RPP8-Ler*-mediated resistance to Emco5 is unaffected by mutations in *EDS1* and *NDR1*, and only weakly impaired by an *eds1/ndr1* double mutant (McDowell et al., 2000). *RPP8-Ler* is also not strongly dependant on SA or SAR for its upregulation, as its function is only slightly impaired by the *NahG* transgene and the *npr1* mutation. In addition, *rar1* and *sgt1b* mutants minimally impact *RPP8* function (B. Holt, pers. comm.). These results imply that *RPP8-Ler* uses a novel signaling mechanism in
Arabidopsis.

Previous studies of RPP8 alleles

The *RPP8* allele *HRT*, which mediates resistance against TCV (*turnip crinkle virus*), is upregulated by SA (Chandra-Shekara et al., 2004). SA treatment of Di-17 plants upregulates *HRT* mRNA levels at 24 hours and improves resistance to TCV. However, TCV infection alone does not appear to upregulate HRT. In addition, genes similar to *RPP8* are upregulated fourfold in response to exogenously applied flagellin (Zipfel et al., 2004, Navarro et al., 2004), and *RPP8-Col* is upregulated 2.5-fold in response to NPR1 overexpression (Wang et al., 2005). However, the mechanism regulating the increase in *RPP8* mRNA levels has not been examined.

Previous studies of RPP8 expression

Nicole Mammarella, an undergraduate in the McDowell lab, initially characterized *RPP8* expression in response to defense-inducing stimuli. To easily visualize gene expression, she employed plants transformed with *RPP8* reporter gene constructs. These constructs contain the coding region of the firefly luciferase gene, which were fused in frame with the 3’ end of the *RPP8-Ler* or *RP8A* coding region. The *RPP8-Ler* constructs have 679 bp of 5’ flanking sequence, which is sufficient for full gene function (McDowell et al., 1998). The luciferase gene fusion constructs were comprised of *RPP8* or *RP8HA* genomic DNA (including introns), to retain as much potential regulatory information as
possible (Figure 3.1 C). The translated RPP8-LUC and RPH8A-LUC products have luciferase activity. These constructs were created by Dr. Bonnie Woffenden, and were transformed into the Columbia ecotype.

Ms. Mammarella began her analysis of RPP8 expression by monitoring basal luciferase activity in three independently transformed lines of Col::RPP8-LUC and Col::RPH8A-LUC for thirty days post-germination (Figure 1.2). RPP8-LUC expression was low, but above background, for all three independent lines; RPH8A-LUC expression was lower, as only one line had luciferase activity significantly above background. RPP8-LUC was observed in inflorescence stems and leaves, but not in roots. Interestingly, Col::RPH8A-LUC plants exhibited strong luciferase activity in roots, and low levels in inflorescence stems and leaves. The divergence between RPH8A and RPP8-Ler thus includes organ-specific expression. Based on its expression pattern, RPH8A may provide resistance against a soil pathogen.

Ms. Mammarella then examined changes in luciferase activity after treatment with defense-inducing stimuli (Figure 1.2). Thirty-day-old plants were challenged with water, H. parasitica Emco5, or the synthetic SA homolog BTH. RPP8-LUC and RPH8A-LUC reporter gene expression increased when challenged with Emco5; luciferase activity increased rapidly at 24 and 48 hours to nearly fourfold background levels, and gradually decreased after 96 hours. Luciferase activity remained above background levels eleven days after pathogen challenge. Thus, RPP8-LUC and RPH8A-LUC expression levels
rapidly increased upon during pathogen attack and were maintained above basal levels long after the initial treatment.

Treatment with BTH led to a faster, but weaker, amplification of \textit{RPP8-LUC} activity. Luciferase activity peaked at 12 hours, diminished rapidly after 96 hours, and returned to baseline levels after 11 days. Thus, SA may induce a feedback loop to amplify \textit{RPP8} and other \textit{R} genes as part of a systemic defense, such as that seen for \textit{RPW8.1} and \textit{RPW8.2} (Xiao et al., 2003). \textit{RPH8A-LUC} did not strongly respond to BTH treatment, indicating that it may use different signaling feedback mechanisms than \textit{RPP8-Ler}.

\textit{Definition of the RPP8 promoter}

Dr. Ben Holt (former Ph.D. student, Dangl lab, University of North Carolina) initiated an EMS mutagenesis screen of Col::\textit{RPP8-Ler} to identify mutations that reduce or eliminate \textit{RPP8-Ler} function. He isolated three independent lines with a cytosine to thymine transition at -189 from the \textit{RPP8-Ler} start codon (Holt Ph. D. dissertation, 2002, University of North Carolina). The C to T mutation greatly reduces \textit{RPP8-Ler} mRNA levels, and mutant plants were completely susceptible to Emco5. This essential C base lies within a 9-10 bp inverted repeat (Figure 3.1 B), which is highly conserved between \textit{RPP8} alleles (Figure 3.7). This region represents a unique \textit{cis} element found only in select CC-NBS-LRR genes, hereafter referred to as the “X-box”. The promoter of wild-type \textit{RPP8-LUC} was mutated, using site-directed mutagenesis, to recreate this mutation. The resulting construct, \textit{xbox-LUC}, was transformed into the CW84 ecotype (S. Simon,
unpublished). She observed that this mutation greatly reduces RPP8-LUC expression, but did not test whether it also affects RPP8 upregulation.

Overview of my research

Ms. Mammarella observed RPP8 induction in response to pathogen challenge and BTH treatment. RPP8 expression was amplified under conditions of defense response activation, which suggests that its regulation involves a positive feedback loop. My research builds upon Ms. Mammarella’s work and focuses on RPP8 regulation. In the sections that follow, I report that RPP8-LUC is induced by a wide range of stimuli, including the Emco5 and Hiks1 isolates of H. parasitica, the virulent pathogen Pseudomonas syringae pv. tomato DC3000, wounding, and heat shock. Endogenous RPP8 genes in Ler are upregulated by Emco5 treatment. I also analyzed RPP8-Ler transcript abundance in Col::RPP8-Ler transgenic lines, and found that it is consistently elevated in response to Emco5 and SA treatment. Preliminary data indicates that RPP8-Ler expression is both systemically and locally induced by DC3000 infection. I also analyzed the expression of endogenous RPP8 in Columbia (RPP8-Col), which appears to be expressed and regulated similarly to RPP8-Ler.

To assay the role of W-box cis elements in RPP8 regulation, I created an RPP8 construct with mutated W-boxes in the promoter (wbox). Basal and induced expression is greatly reduced in Col::wbox lines, and plants are fully susceptible to Emco5. W-boxes are thus essential for RPP8-Ler function.
Finally, I examined the expression of \textit{RPP8-LUC} in plants carrying the X-box mutation, (CW84::\textit{xbox-LUC}). Plants carrying this mutation are fully susceptible to Emco5. These plants express very low levels of luciferase activity, and display altered organ-specific expression. Interestingly, CW84::\textit{xbox-LUC} still induces luciferase activity in response to Emco5 and DC3000 challenge, SA treatment, and heat shock. However, luciferase activity is not induced by wounding in the \textit{xbox-LUC} mutant.

In sum, I have shown that both \textit{RPP8-Ler} and \textit{RPP8-Col} genes are induced during defense activation. I have also defined the first known \textit{cis} regulatory elements regulating \textit{R} gene expression, and laid the groundwork for examining signaling mutants that potentially impair \textit{RPP8} regulation.

\textit{Overview of transgenes and plant genotypes used in this study:}

Our analysis of \textit{RPP8} expression employed a variety of genetic backgrounds. As previously described, the Col ecotype carries an \textit{RPP8} allele of unknown function, while the Ler ecotype has an \textit{RPP8} allele with functional resistance to Emco5. \textit{RPP8} expression was initially examined in Columbia transgenic plants, which expressed a reporter construct of \textit{RPP8-Ler} fused to luciferase (Col::\textit{RPP8-LUC}). To validate the findings of the reporter gene assay, I used a Taqman assay to examine the steady-state mRNA levels of the endogenous \textit{RPP8} allele in Ler. However, it is impossible to design a Taqman assay specific for \textit{RPP8} in Ler, due to its high sequence similarity with its
homolog, RPH8A. Therefore, I also analyzed RPP8-Ler upregulation in Col::RPP8-Ler transgenic lines, which do not contain RPH8A, and thus enable gene-specific assays. I also analyzed multiple lines of Col::wbox, which contains mutations in three W-boxes found in the RPP8-Ler promoter (transgenic line designations are explained in table 2.1). To compare the expression of multiple RPP8 alleles, I also analyzed the expression of endogenous RPP8 in Columbia (RPP8-Col). Finally, I examined the expression of CW84::xbox-LUC; this construct is RPP8-LUC with a mutation in the X-box promoter element at base pair –189. These transgenic lines were used to examine the role of the X-box element in RPP8-Ler expression.
Figure 1.1. Resistance gene signaling pathways.

General signaling mechanisms used by the TIR and CC subclasses of NBS-LRR R proteins. TIR proteins activate EDS1 and PAD4 signaling pathways, while CC protein defense responses are mediated by NDR1. R proteins from both pathways exhibit a requirement for RAR1 and SGT1. These signaling pathways converge in a mutual requirement for SA and NPR1. The curved red arrow denotes the positive feedback loop mediated by NPR1, which amplifies the TIR signaling pathway.
Figure 1.2. RPP8-LUC and RP8HA-Luc luciferase activity in response to *H. parasitica* Emco5 and BTH.

Luciferase assays performed by Ms. Nicole Mammarella. Basal luciferase activity was monitored for 29 days. Treatments were applied on day 30. Plants were sprayed with *H. parasitica* Emco5 (1\times10^5 conidiosporangia mL^{-1}), 250 uM BTH, water alone, or
wounded by crushing leaves a single time on both sides of the leaf midrib with forceps. Bars represent mean and standard error from five individual plants.

A. Luciferase expression in a single transgenic Col::RPP8-LUC line.

B. Luciferase expression in a single transgenic Col::RPH8A-LUC line.
Chapter 2:

Materials and Methods
Pathogen Challenge Assays

*H. parasitica* pathogen strains were propagated weekly on genetically susceptible Arabidopsis. Emco5 was grown on the Wassilewskija ecotype (Ws-0). Hiks1 was grown on either *eds1* or *Col::rpp7-7*, which contains a loss-of-function mutation in the *RPP7* resistance gene that provides resistance to Hiks1. For propagation, a suspension of conidiospores (5 X 10^4 mL\(^{-1}\)) was prepared by vortexing heavily sporulating leaves in distilled water. Conidiospore suspensions were sprayed onto leaves to the point of imminent runoff, using a Preval power sprayer (Precision Value Corporation, Yonkers, NY). Inoculated plants were kept under a sealed clear plastic lid to maintain high humidity, and grown in a Percival Scientific CU-36L4 growth chamber, with light intensities of approximately 85 microEinstiens, under 8 hour days at 20°C, 16 hour nights at 18°C.

*RPP8-Ler* and *wbox* transgene function was assayed by inoculating 7-day-old T2 or T3 transgenic seedlings with 5 X 10^4 conidiosporangia mL\(^{-1}\) of Emco5 in distilled water. *H. parasitica* growth was determined by visual assessment of sporangiophore production at 7 days post-infection. T2 plants were scored qualitatively; T3 plants were scored quantitatively. T2 plants exhibiting heavy sporulation were scored as susceptible; plants exhibiting 0-2 sporangiophores per cotyledon were scored as resistant. Mean sporangiophores per cotyledon of T3 seedlings were determined in a manner similar to McDowell et al. (1998): real numbers of sporangiophores per cotyledon were counted on cotyledons that supported 0-10 sporangiophores. Cotyledons with 11 or greater
sporangiophores were classified as “heavy” sporulation and assigned values of 15. Means and standard deviations were then calculated.

RPP8-LUC induction by *H. parasitica* was initially measured by challenging Col::RPP8-LUC plants with Emco5 (5 X 10⁴ conidiosporangia mL⁻¹ in water). All subsequent gene expression assays employed higher concentrations of Emco5 or Hiks1 (5 X 10⁵ conidiosporangia mL⁻¹) to insure a strong response.

*Pseudomonas syringae* pv. *tomato* DC3000 was a gift from Dr. Boris Vinatzer (Virginia Tech, Blacksburg, VA). Bacteria were grown for two days at 30°C on KB plates without antibiotic. Colonies from freshly grown plates were used to inoculate 5 ml of KB broth; liquid cultures were grown overnight at 30°C with shaking at 200 RPM. This overnight culture was diluted 1:10 in 5 ml fresh KB broth, and grown for an additional four hours at 30°C with shaking at 200 RPM until cultures reached midlog phase growth (OD₆₀₀ from 0.7 to 1.2). One ml of culture was spun down for five minutes at room temperature at 5000 RPM, and resuspended in one ml sterile 10 mM MgSO₄. Bacteria were diluted to the appropriate concentration (OD₆₀₀=0.01, 0.001, or 0.0001) in 10 mM MgSO₄, and then pressure infiltrated into leaves using a 1 ml syringe. Control plants were infiltrated with 10 mM MgSO₄ alone. Infiltrated leaves were marked with a permanent marker to distinguish them from uninfected leaves. For quantitative RT-PCR experiments, one leaf per plant was infiltrated with MgSO₄ or DC3000 (OD₆₀₀=0.001). Above ground plant materials were collected after 72 hours. Infiltrated leaves were removed and collected.
separately from untreated material. Samples were frozen in liquid N\textsubscript{2}, and stored at -80°C until processed.

**Plant Cultivation and Treatment**

Plants were grown in Sunshine Mix #1 (Sungro Horticulture, Seba Beach, Canada). Seeds were stratified at 4°C for at least two days, to synchronize seed germination before transfer to growing conditions. Plants for luciferase assays were grown in a Percival Scientific CU-36L4 growth chamber (Boone, IA), with light intensities of approximately 50 microEinstein\textperthousand s, under 14 hour days at 23°C, 10 hour nights at 21°C, and ambient humidity. Ler plants for quantitative RT-PCR were grown as described above for three weeks. Plants were then transferred to racks at ambient room temperature (approximately 19°C) and grown under light intensities of approximately 45 microEinstein\textperthousand s under a 20 hour day, 4 hour night cycle. All other plants were grown entirely on racks under long-day conditions as described above. After treatment, all plants were maintained in a Percival Scientific CU-36L4 growth chamber at ambient humidity, with light intensities of approximately 85 microEinstein\textperthousand s, under 8 hour days at 20°C, 16 hour nights at 18°C.

Ler plants were treated and collected at approximately 2 PM. Above ground material from four to six plants were taken at 0 hr (before treatment), 24 hr, and 48 hr timepoints, frozen in liquid N\textsubscript{2}, and stored at -80°C. Samples were collected from three independent biological replicates.
Col::RPP8-Ler and Col::wbox plants were treated and collected at approximately 9:30 PM. Three week old plants were treated with water, 1 mM SA, Emco5 (5 X 10^5 conidiosporangia mL^{-1}), or left untreated. After treatment, plants were covered with a clear plastic dome to maintain high humidity and transferred to the CU-36L4 growth chamber. Above ground materials from at least two plants were taken at 0 hr (before treatment), 24 hr, 48 hr, and 72 hr. timepoints, frozen in liquid N\textsubscript{2}, and stored at -80°C. Wounded leaves were collected at 0, 2, and 24 hr. Heat-treated plants were collected at 0 hr and 24 hr. Plants treated with Hiks1 (5 X 10^5 conidiosporangia mL^{-1}) were collected at 0 and 48 hrs.

WTA plants for the organ-specific expression assay (Figure 3.12) were grown on germination medium (GM) plates prepared with 1.95 g/L Phytagel (Sigma) and Murashige and Skoog vitamin solution to 1X final concentration (Sigma). Sterilized seeds were sown horizontally across the top of rectangular plates. The plates were vernalized at 4°C for two days, then grown in a Percival Scientific CU-36L4 growth chamber at ambient humidity, with light intensities of approximately 110 microEinsteins, under 16 hour days at 24°C, 8 hour nights at 21°C, for three weeks. Plates were inclined so that roots grew downward along the media surface. Aboveground materials were removed and collected first to avoid contamination of the roots. Samples were frozen in liquid N\textsubscript{2} and stored at -80°C until processed.

**Chemical Treatments and Abiotic Stress Assays**
Nicole Mammarella's initial experiments employed the synthetic SA analog benzothiadiazole (BTH). To conserve limited BTH stock, I compared luciferase activity after treatment with 1 mM SA (Sigma) or 250 uM BTH in two independent Col::RPP8\textit{-LUC} lines. These treatments induced luciferase activity to similar levels (data not shown) and did not harm plants. I therefore used 1 mM SA for subsequent experiments.

To assay potential chemical inducers, Col::\textit{RPP8-}LUC plants were sprayed to the point of imminent runoff with 50 mM methyl jasmonate (Sigma), 20 mM 2,4 D (Sigma), 150 uM of the ROI generator rose bengal (Sigma), or 1% hydrogen peroxide. All solutions were dissolved in water.

Wound assays were performed on rosette leaves of six week old Col::\textit{RPP8-}LUC plants or three week old Col::\textit{RPP8-Ler} plants. Leaves were damaged either by cutting incisions with scissors or by crushing leaf margins with flat forceps once on either side of the leaf midrib.

Drought-stressed plants were allowed to dehydrate gradually in the short-day CU-36L4 growth chamber. Plants were assayed at 3 days, 7 days, and 15 days after cessation of watering. Each plant was only assayed once over the timecourse of dehydration, to insure that luciferin treatment did not alter drought conditions.

Heat-treated and cold-treated plants were kept in plastic containers to maintain relative humidity. Plants were moved into the short-day CU-36L4 growth chamber during the
day; once the dark phase began at 5 PM, plants were moved to 37°C or 4°C environments for 16 hours. Plants were removed at 9 AM. RPP8-LUC plants were assayed immediately after they were removed from 37°C or 4°C environments. For RT-PCR, heat-stressed plants were collected immediately after they were removed from the 37°C environment, frozen in liquid N₂, and stored at -80°C until processed.

**Luciferase Assays**

Luciferase expression was assayed in six week old plants individually grown in 2” pots. Two independently transformed lines were used for each experiment, and three to five plants from each line were assayed per treatment. Plants were sprayed with a solution of 1 mM luciferin (Biosynth AG, Staad, Switzerland)/0.01% Triton-X (Fisher Scientific, Fair Lawn, NJ) and were incubated at room temperature for twenty minutes, to allow the substrate to enter plant organs. Photon emission was then measured for twenty minutes in a lightproof chamber, using a VIM-50 imaging system (Hamamatsu, Japan). Because the camera is able to detect a low level of infrared emission, a pot of soil was assayed in each experiment to estimate background. Background levels of emission were subtracted from the values depicted in Figure 3.2 B. Luciferase activity was quantitatively measured by using the gravity function of the Argus 50 software, which counted all light emitted in a 100 by 100 pixel square containing each plant. Qualitative images of luciferase activity were superimposed over background black-and-white images of plants. The luciferase overlays were adjusted to a bit range of 3-5 with the camera Argus-50 software; this setting eliminated background photon activity from soil in qualitative images. Initial luciferase activity (0 hours) was measured immediately before treatment.
All plants were initially assayed and treated in the morning (7:30 – 10:00 AM).
Wounded plants were sprayed with luciferin and imaged before wounding (0 hours), and
were imaged for three consecutive hours afterward without extra luciferin to monitor
rapid changes in luciferase activity. All other timepoints were imaged after plants were
sprayed with luciferin.

**Luciferase Constructs**

*RPP8-LUC* constructs (Figure 3.1 C) were created by Dr. Bonnie Woffenden, using the
pCambia 3300 binary vector.

CW84::*xbox-LUC*, featuring a promoter mutation described by Dr. Ben Holt (Holt et al.,
2002), was created by Stacey Simon. The C residue at -189 in the *RPP8-LUC* gene
(Figure 3.1 B) was changed to a T using site-directed mutagenesis. The mutagenized
promoter was subcloned into the pCambia-derived *RPP8-LUC* vector described above as
an XmaI/BsmBI fragment.

**Construction of W-box Mutations**

The *wbox* triple mutant was created by site-directed mutagenesis with the Quickchange II
XL Site-Directed Mutagenesis kit (Stratagene). Primers for mutagenesis were designed
with the Stratagene online tool ([http://labtools.stratagene.com/QC](http://labtools.stratagene.com/QC)) (shown in Table 2.1).
PAGE-purified primers were ordered from IDT (Coralville, IA) and were used with the
Quickchange kit as per manufacturer’s instructions. The template plasmid was pJGJ329,
which contains *RPP8-Ler* (cloned as an EcoR1 fragment from *RPP8*-pBAR1) in pBSK+.
W-boxes were individually mutated in the following order: W2, W3, W1 (as labeled in Figure 3.1 B). All mutations were confirmed by sequencing. The final wbox promoter was excised as a BsmBI/XmaI fragment, and was used to replace the corresponding wild-type BsmBI/XmaI fragment in the original pJGJ329 construct. This strategy avoided introduction of unwanted mutations into the RPP8-Ler coding region over the course of mutagenesis. This construct was named pTJB004. The ends of the Xma1/BsmB1 fragment were sequenced to confirm correct insertion. A 5.5 kb EcoRI/XmaI fragment containing the mutated RPP8-Ler was excised from TJB004, and was subsequently cloned into the pBAR1 binary plasmid (McDowell et al., 1998).

**Agrobacterium Transformation, and Herbicide Resistance**

Wild-type RPP8-Ler (described in McDowell et al., 1998) and wbox mutant constructs in the pBAR1 plasmid were transformed into the Agrobacterium strain GV3101 by electroporation. Agrobacterium containing these constructs were used to transform plants using the floral dip protocol, as previously described (Clough and Dent, 1998; Bechtold et al., 1993). BASTA (glufosinate-ammonium) selection was performed as previously described (McDowell et al., 1998) to identify transgenic plants and homozygous transgenic populations.

**RNA and cDNA Preparation**

Total RNA was isolated using the Aurum RNA kit (Bio-Rad) or Tri Reagent (Sigma, St. Louis, MO). RNA quality was analyzed by spectrophotometry and by electrophoresis on a denaturing MOPS gel. RNA was treated with the DNA-free DNase kit or the TURBO
DNA-free DNase kit (Ambion, Austin, TX), as per manufacturer’s recommendation for "rigorous treatment". First strand cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) or the Omniscript Reverse Transcription kit (Qiagen), using RNase Out recombinant ribonuclease inhibitor (Invitrogen) and oligo(dT)\textsubscript{12-18} (Invitrogen) as per manufacturer’s recommendation. Samples were tested for genomic DNA contamination by PCR with the \textit{ACT2} primers, which span an 86 base pair intron.

**PCR**

Quantitative PCR was performed using Taqman probes from Applied Biosystems (Foster City, CA). Primers and probes for \textit{ACT2}, \textit{RPP8-Ler}, \textit{PR-1}, and \textit{RPP8-Col} were designed using Primer Express software version 1.5 (Applied Biosystems) (listed in Table 2.2). All primers in this study were ordered from IDT (Coralville, IA). Applied Biosystems Taqman Universal PCR Master Mix and MicroAmp Optical 96-Well Reaction Plates with Optical Adhesive Covers were used for real-time PCR reactions. Measurements were made in an ABI PRISM 7700 Detection System. The threshold of each probe was kept constant to insure consistent results between plates. \textit{RPP8-Ler} was normalized to \textit{ACT2} for each sample, as described in Halterman et al. (2003). Data was analyzed in Microsoft Excel.

Semiquantitative PCR was performed using the same primers used for real-time PCR. \textit{ACT2} was used as a control for equal cDNA loading. PCR conditions for \textit{ACT2} and \textit{RPP8-Col} are as follows: 94°C/3 min, followed by 28 cycles of 94°C/15 sec, 60°C/15 sec,
72°C/30sec, and a 10 minute final extension time at 72°C. The same program was used for *RPP8-Ler*, with 35 cycles of amplification.
Table 2.1: Origin and designation of plant lines used for gene expression analysis

<table>
<thead>
<tr>
<th>Original designation</th>
<th>New name</th>
<th>Transgene</th>
<th>Source</th>
<th>Functional phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT RPP8 25.5.T3</td>
<td>WT1</td>
<td>WT \textit{RPP8-Ler}</td>
<td>This study</td>
<td>R</td>
</tr>
<tr>
<td>WT RPP8 46.2.T3</td>
<td>WT2</td>
<td>WT \textit{RPP8-Ler}</td>
<td>This study</td>
<td>R</td>
</tr>
<tr>
<td>WT RPP8 23.1.T3</td>
<td>WT3</td>
<td>WT \textit{RPP8-Ler}</td>
<td>This study</td>
<td>R</td>
</tr>
<tr>
<td>WT RPP8 11.4.T3</td>
<td>WT4</td>
<td>WT \textit{RPP8-Ler}</td>
<td>This study</td>
<td>R</td>
</tr>
<tr>
<td>WT RPP8 35.9.T3</td>
<td>WT5</td>
<td>WT \textit{RPP8-Ler} (silenced)</td>
<td>This study</td>
<td>S</td>
</tr>
<tr>
<td>WT RPP8 16.1.T3</td>
<td>WT6</td>
<td>WT \textit{RPP8-Ler}</td>
<td>This study</td>
<td>R</td>
</tr>
<tr>
<td>WT RPP8 45.2.T3</td>
<td>WT7</td>
<td>WT \textit{RPP8-Ler}</td>
<td>This study</td>
<td>R</td>
</tr>
<tr>
<td>WT RPP856.2.T3</td>
<td>WT8</td>
<td>WT \textit{RPP8-Ler}</td>
<td>This study</td>
<td>R</td>
</tr>
<tr>
<td>WT RPP860.6.T3</td>
<td>WT9</td>
<td>WT \textit{RPP8-Ler}</td>
<td>This study</td>
<td>R</td>
</tr>
<tr>
<td>TJB005 2.3.T3</td>
<td>WB1</td>
<td>\textit{WBOX RPP8-Ler}</td>
<td>This study</td>
<td>S</td>
</tr>
<tr>
<td>TJB005 31.1.T3</td>
<td>WB2</td>
<td>\textit{WBOX RPP8-Ler}</td>
<td>This study</td>
<td>S</td>
</tr>
<tr>
<td>TJB005 51.10.T3</td>
<td>WB3</td>
<td>\textit{WBOX RPP8-Ler}</td>
<td>This study</td>
<td>S</td>
</tr>
<tr>
<td>TJB005 42.3.T3</td>
<td>WB4</td>
<td>\textit{WBOX RPP8-Ler}</td>
<td>This study</td>
<td>S</td>
</tr>
<tr>
<td>TJB005 37.4.T3</td>
<td>WB5</td>
<td>\textit{WBOX RPP8-Ler}</td>
<td>This study</td>
<td>S</td>
</tr>
<tr>
<td>TJB005 15.1.T3</td>
<td>WB6</td>
<td>\textit{WBOX RPP8-Ler}</td>
<td>This study</td>
<td>S</td>
</tr>
<tr>
<td>TJB005 28.4.T3</td>
<td>WB7</td>
<td>\textit{WBOX RPP8-Ler}</td>
<td>This study</td>
<td>S</td>
</tr>
<tr>
<td>TJB005 43.10.T3</td>
<td>WB8</td>
<td>\textit{WBOX RPP8-Ler}</td>
<td>This study</td>
<td>S</td>
</tr>
<tr>
<td>TJB005 46.6.T3</td>
<td>WB9</td>
<td>\textit{WBOX RPP8-Ler}</td>
<td>This study</td>
<td>S</td>
</tr>
<tr>
<td>TJB005 50.5.T3</td>
<td>WB10</td>
<td>\textit{WBOX RPP8-Ler}</td>
<td>This study</td>
<td>S</td>
</tr>
</tbody>
</table>
Table 2.2: Primers used for site-directed mutagenesis

<table>
<thead>
<tr>
<th>Target W-box</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>#W1 forward</td>
<td>AATATAAGCAATGATAGGACTGATTCAATAGTTCAAGAAGGTAAG</td>
</tr>
<tr>
<td>#W1 reverse</td>
<td>CTTCACGTTCTTGAATCTATTGAACGTCCTATCATTGCTTATATT</td>
</tr>
<tr>
<td>#W2 forward</td>
<td>TAGCTAGATAGATAAGGTTGAATTGGAGTCAACTTCTTGCTTCTT</td>
</tr>
<tr>
<td>#W2 reverse</td>
<td>CTAAGCAAGAAAGTGACTCCAATTCACCTATCCTATCTCTAGCTA</td>
</tr>
<tr>
<td>#W3 forward</td>
<td>AGATAGATAAGGTTGAATTGGATTCAACTTCTTGCTTAGGATTT</td>
</tr>
<tr>
<td>#W3 reverse</td>
<td>CAAAACCTCTAAGCAAGAAGTTGAATTTCTTACCTATCTATCTT</td>
</tr>
</tbody>
</table>

35
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward strand primer</th>
<th>Reverse strand primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT2</td>
<td>TCGGTGTTCCATTCTTGCT</td>
<td>GCTTTTTAAGCCTTTGATCTTGAGAG</td>
<td>AGCACATTCAGAGATGTGGATCTCCAA</td>
</tr>
<tr>
<td>PR-1</td>
<td>AGAGGCAACTGCAGACTCATAACAC</td>
<td>AGCCTTCTCGCTAACCCACAT</td>
<td>TTCAGGGCGGAGACGCAGACAGACAGTC</td>
</tr>
<tr>
<td>RPP8-Ler</td>
<td>CGAAGAATCTCTCTATGATTGCTATGATTTA</td>
<td>TCTGAGCCACACAACATACGAT</td>
<td>CCAGTCTTGGAGCTCTCGCTTTCAGTG</td>
</tr>
<tr>
<td>RPP8-Col</td>
<td>CATGGCATCGACCCTTCTTC</td>
<td>CGTTTAGACGTAAGGCATTTC</td>
<td>N/A</td>
</tr>
<tr>
<td>RPP1</td>
<td>GTGGAGCTCCCGCTATCGAGATGCGAC</td>
<td>GCAAGGGAATCTGGAAGTTGGGAGTGATACCG</td>
<td>N/A</td>
</tr>
<tr>
<td>(from Shirano et al., 2002)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3: Primers and probes used for Q-PCR and PCR
Chapter 3:
Results
Expression of the RPP8-LUC reporter gene is induced by H. parasitica isolates and SA

My first experiment was to verify Ms. Mammarella’s preliminary observations that luciferase activity increases in response to Emco5 treatment. I also examined RPP8-LUC expression after treatment with the defense compound SA. Using two independent Col::RPP8-LUC transgenic lines, I observed that 1 mM SA causes a rapid upregulation of RPP8-LUC luciferase activity at 12 to 24 hours, which declines at 48 and 72 hours (Figure 3.2 A, B). Plants treated with Emco5 exhibit a slower upregulation of RPP8-LUC that peaks at 48 hours\(^1\). RPP8-LUC expression did not change in water-treated and untreated controls, except for a small upregulation at 12 hours that could be due to circadian regulation. These experiments verify Ms. Mammarella's previous observations that Emco5 induces Col::RPP8-LUC expression. Additionally, SA is sufficient to induce luciferase activity in Col::RPP8-LUC lines.

The RPP8-LUC construct is unable to provide resistance to Emco5 directly (B. Woffenden, pers. comm.), likely because the fusion with luciferase disrupts the structure of the LRR domain. Instead, Emco5 resistance in adult Col::RPP8-LUC plants is mediated by the developmentally regulated RPP31 gene (McDowell et al., 2005, in press). Thus RPP8-Ler may be upregulated as part of a general defense response. To confirm this idea, I challenged two independent transgenic Col::RPP8-LUC lines with the H. parasitica isolate Hiks1. Hiks1 is recognized by the R gene RPP7 in the Columbia

\(^{1}\) Note: Plants in Figure 3B were treated with 5x10\(^4\) conidiosporangia mL\(^{-1}\) Emco5; all subsequent experiments used 5x10\(^5\) conidiosporangia mL\(^{-1}\) to insure strong pathogen responses between transgenic lines. This higher pathogen concentration caused moderately stronger luciferase emission than that shown in Figure 3.2 B.
background, and is incompatible with adult Columbia plants. \textit{RPP8-LUC} is reproducibly induced by Hiks1 (Figure 3.2 A). The luciferase response is similar to that of Emco5, although it is of slightly weaker magnitude. Thus, \textit{RPP8} can be induced by defense responses activated by two different \textit{R} genes: \textit{RPP31} and \textit{RPP7}.

\textbf{RPP8-LUC is not induced by several defense signaling molecules}

Numerous small molecules have been associated with \textit{R} protein signaling pathways, including jasmonic acid, auxin, and reactive oxygen intermediates. Therefore, I tested whether these signaling molecules could upregulate \textit{RPP8-LUC} expression. I treated two independent Col::\textit{RPP8-LUC} transgenic lines with 50 mM methyl jasmonate (a synthetic version of jasmonic acid), 20 mM 2,4 D (a synthetic auxin), 150 uM of the ROI generator rose bengal, and 1% hydrogen peroxide. None of these compounds significantly induced \textit{RPP8-LUC} expression (data not shown). These molecules are therefore insufficient for \textit{RPP8-Ler} upregulation. However, these experiments do not rule out the possibility that these molecules are necessary co-activators of \textit{RPP8} expression in infected cells.

\textbf{RPP8/RPH8A mRNA levels in Ler-0 are increased by Emco5 challenge}

Having established that the \textit{RPP8-LUC} transgene fusion is upregulated in response to Emco5 and SA, I next examined transcript abundance from the endogenous \textit{RPP8} gene in the Ler-0 ecotype. I analyzed transcript levels with quantitative RT-PCR, using a Taqman probe. As described in the introduction, we were only able to design
appropriate Taqman probes and PCR primers in the 3' untranslated region (UTR) of RPP8. RPP8 and RPH8A in Ler-0 have nearly identical 3' UTRs; therefore, the RPP8-Ler primers amplify both transcripts, and the assay reflects the abundance of transcript from both genes. RPP8/RPH8A mRNA levels in Ler-0 are increased approximately twofold at 24 and 48 hours after pathogen challenge (Figure 3.3 A). This response was consistent across three biological replicates, each with two technical replicates. Water-treated and untreated control plants do not appear to have upregulated RPP8/RPH8A mRNA levels over this period. RPP8-Col is also consistently induced by Emco5 and SA, and is regulated very similarly to RPP8-Ler (Figure 3.9, and see page 49). Steady state mRNA levels of endogenous RPP8 alleles are thus modestly upregulated by Emco5 challenge.

I also assayed expression of the defense marker gene PR-1 in these samples, to determine the time course of general defense responses during Emco5 challenge (Figure 3.3 B). Untreated and water-treated plants have relatively stable expression of PR-1. Plants challenged with Emco5, on the other hand, strongly induce PR-1 at 24 and 48 hours after treatments. Expression levels of PR-1 are strongest at 48 hours (approximately 32-fold). Although highly variable between biological replicates, PR-1 expression was significantly upregulated in Emco5-treated samples (p<0.05). General defense responses thus appear to be strongly activated after RPP8/RP8HA expression is induced.

*Transgenic RPP8-Ler is induced by Emco5 challenge and SA*
As mentioned above, the identity between \textit{RPP8-Ler} and \textit{RPH8A} in the Ler-0 ecotype does not permit gene-specific quantitative RT-PCR analysis. Thus, the assay in Figure 3.3 A does not distinguish between transcripts from \textit{RPP8-Ler} and \textit{RPH8A}. Further analysis of \textit{RPP8-Ler} expression was therefore carried out in Col::\textit{RPP8-Ler} transgenic lines, to specifically assay \textit{RPP8-Ler} transcript levels. I examined \textit{RPP8-Ler} expression in homozygous T3 populations from five independent transformants: a single copy Col::\textit{RPP8-Ler} line described by McDowell et al. (1998), designated "WTA" (for wild type \textit{RPP8 A}), and four additional Col::\textit{RPP8-Ler} lines that I created, designated WT1-WT4 (for wild type \textit{RPP8} line 1, etc.). Col::\textit{RPP8-Ler} lines were treated with Emco5, SA, water alone, or left untreated. \textit{RPP8-Ler} expression was analyzed with quantitative RT-PCR, using the same Taqman assay described in the previous section. \textit{RPP8-Ler} expression levels were normalized to \textit{ACT2}.

Each of the five lines had modestly elevated \textit{RPP8-Ler} transcript levels in response to Emco5 and SA treatment (Figure 3.4). Two lines (WTA and WT3) had consistently elevated expression after Emco5 and SA treatment in two independent biological replicates (Figure 3.4 A, B). The other three lines (WT1, WT2, WT4) had similar upregulation, although they were only assayed once due to time constraints. Both Emco5 and SA treatment led to increased \textit{RPP8-Ler} mRNA levels at 24 hours, which appeared to be downregulated again at 48 hours. I also examined the expression of \textit{RPP8-Ler} in water-treated or untreated control plants in two independent lines (Figure 3.4 B, C). One line exhibited weak upregulation in untreated plants (Figure 3.4 C), but
expression appeared to be downregulated in other control plants during this period. Emco5 and SA treatment appear to induce a modest increase in transgenic \textit{RPP8-Ler} mRNA levels. These increases are consistent in timing and magnitude with those seen in endogenous \textit{RPP8-Ler} (Figure 3.3 A). \textit{RPP8-Ler} expression thus appears to be consistently induced during pathogen challenge and defense induction.

I also attempted to analyze \textit{RPP8-Ler} expression after challenge with Hiks1, using quantitative RT-PCR. However, I only analyzed plants treated at 0 and 48 hours in two independent Col::\textit{RPP8-Ler} lines, and \textit{RPP8-Ler} was not consistently induced at 48 hours in these samples (data not shown). I expect Hiks1 and Emco5 to activate \textit{RPP8-Ler} expression with similar kinetics, as observed in luciferase assays. Hiks1 treatment would thus induce \textit{RPP8-Ler} at 24 hours rather than 48 hours. Therefore, it would be necessary to assay the 24 hour timepoint to confirm that Hiks1 treatment increases \textit{RPP8-Ler} mRNA levels.

\textit{RPP8-LUC} is induced by infiltration and virulent \textit{Pseudomonas syringae pv tomato} \textit{DC3000}

Some \textit{R} genes are upregulated in response to both virulent and avirulent pathogens, suggesting that induction could be controlled by basal defense responses, rather than \textit{R} gene-dependant recognition (Wang et al. 2001). To evaluate whether \textit{RPP8} expression is also upregulated by virulent pathogens, I challenged two independent Col::\textit{RPP8-LUC} lines with \textit{Pseudomonas syringae pv. tomato} DC3000. This strain is virulent on Col-0.
Plants were infected by infiltration of DC3000 in 10 mM MgSO₄, or MgSO₄ alone, into 7-10 rosette leaves per plant. Interestingly, luciferase activity was provoked in both DC3000 and MgSO₄ infiltrated leaves (data not shown). Infiltration of leaves may therefore cause a wound response. DC3000 infiltration also activated RPP8-LUC expression in uninfiltreated leaves (data not shown), suggestive of a systemic defensive response. The systemic response began at 24 hours, and was still strong at 72 hours. This systemic induction appeared to be dose-dependant, as luciferase activity increased with increasing concentrations of DC3000 (data not shown).

To further characterize this apparent systemic upregulation, I examined luciferase activity in plants in which a single leaf was infiltrated with 10 mM MgSO₄ or DC3000 (OD₆₀₀=0.001). Infiltration of MgSO₄ induces a weak systemic response, perhaps as a result of wounding (Figure 3.5 A). However, DC3000 clearly induces a strong systemic response throughout the plant when infiltrated into a single rosette leaf (Figure 3.5 A). Luciferase expression appears to be strongest in the younger leaves. RPP8-LUC is therefore systemically induced by DC3000. Neither Emco5 nor SA was sufficient to consistently induce systemic upregulation of RPP8-LUC (data not shown).

I confirmed the systemic RPP8 upregulation observed in DC3000-treated luciferase plants by assaying RPP8-Ler transcript levels during DC3000 infection. Due to time constraints, this experiment was only performed once. I infiltrated the single-copy Col::RPP8-Ler line WTA with either DC3000 (OD₆₀₀=0.001) or MgSO₄, into a single leaf on each plant. Aboveground plant material was collected at 72 hours after treatment,
because luciferase assays indicated strong upregulation at this timepoint. Leaves infiltrated with MgSO$_4$ were healthy at the time of collection except for small wounds caused by infiltration. Leaves infiltrated with DC3000 exhibited chlorosis typical of *P. syringae* pathogens. Infiltrated leaves were removed from the plant and collected, and the untreated above ground plant materials were collected separately. This allowed me to compare *RPP8-Ler* expression in infected leaves with expression in the rest of the aboveground plant material.

*RPP8-Ler* transcript levels were assayed with quantitative RT-PCR (Figure 3.5 B), and normalized to *ACT2*. MgSO$_4$-infiltrated leaves exhibited tenfold higher *RPP8-Ler* transcript levels, compared to uninfiltrated leaves. *RPP8-Ler* may therefore be induced by a wound responsive pathway. Unexpectedly, *RPP8-Ler* mRNA levels appear to be more strongly upregulated in MgSO$_4$-treated leaves than indicated by luciferase assays (Figure 3.10). Thus, it is possible that *RPP8-Luc* expression may be modified at the post-translational level after wounding. Interestingly, challenge with DC3000 induced stronger *RPP8-Ler* expression than wounding alone in treated leaves, approximately 17-fold over untreated control material. *RPP8-Ler* expression was induced even more strongly in untreated leaves, over 25-fold greater than untreated control material. *RPP8-Ler* can be locally and systemically induced by DC3000 and by wounding.

*RPP8-LUC responds to wounding*
The *RPP8-LUC* experiments described in the previous section indicated that the *RPP8* promoter is wound-responsive, as it is induced by MgSO$_4$ infiltration. I explored this possibility further by analyzing other wound responses in *RPP8-LUC* plants. I wounded Col::*RPP8-LUC* transgenic lines by cutting or crushing leaves on either side of the leaf midrib. Three leaves were treated per plant; this experiment was repeated in triplicate in two transgenic lines. Previous studies in other labs showed that W-box elements mediate a rapid response within one hour of wounding (Rushton et al., 2002). I therefore observed plants for three continuous hours after injury. I also measured luciferase activity at 6, 12, 24, and 48 hours. It is possible that luciferin could more easily enter wounded tissues, so that luciferase activity would increase simply as a result of higher substrate availability. However, this would have immediately enhanced luciferase activity after wounding. Instead, cutting and crushing induced expression at the wound site approximately thirty to forty minutes after treatment (Figure 3.6 A). This response was strongest two to three hours after injury, and diminished to background levels after 12 hours. Luciferase activity was not systemically induced with these treatments. This result, combined with infiltration wound responses, strongly implies that *RPP8* is induced after injury. *R* genes may be activated after wounding to safeguard against opportunistic pathogen invasion.

Wound responses were analyzed at the transcript level in two Col::*RPP8-Ler* lines, WTA and WT3, with two biological repeats. *RPP8-Ler* transcript levels were characterized using quantitative RT-PCR. Wound responses were examined at 0 and 2 hours, when luciferase activity is strongest. However, *RPP8-Ler* expression was inconsistently
upregulated (data not shown), perhaps because induction was confined to a small number of responding cells.

*RPP8-LUC responds to heat stress, but not cold or drought*

Some *R* genes are regulated in response to environmental cues (Wang et al., 2001, Yang et al., 2004). To examine environmental effects on *RPP8* expression, I subjected two independent Col::*RPP8-LUC* transgenic lines to drought, cold (4°C), and heat (37°C) stresses. Heat- and cold-stressed plants were placed in plastic trays to avoid dehydration. Plants appeared healthy during the experiment, except for the expected wilting seen in late-stage drought plants. Drought-stressed plants were examined at several stages of dehydration, but did not exhibit significant changes in luciferase expression (data not shown). Some cold-stressed plants exhibited weak upregulation of luciferase activity, but this response was not consistent between lines (data not shown). Interestingly, heat stress induced a dramatic upregulation of RPP8-LUC luciferase activity in both transgenic lines (Figure 3.6 B). When plants were returned to normal temperatures, the luciferase activity diminished to baseline levels within 24 hours. This response was consistent between two biological replicates.

Surprisingly, *RPP8-Ler* transcript does not appear to accumulate after heat stress. Two independent Col::*RPP8-Ler* transgenic lines, WTA and WT3, were used to evaluate heat stress upregulation of *RPP8-Ler* mRNA levels using quantitative RT-PCR. Plants were collected at 0 hours, and immediately after 16 hours of overnight treatment at 37°C.
RPP8-Ler transcript levels were strongly downregulated at this timepoint after heat stress in the WTA line, while WT3 had inconsistent regulation between replicates (data not shown). As I only assayed one timepoint, it is possible that RPP8-Ler is transiently upregulated early in the heat stress response, and is then rapidly downregulated. Another possibility is that RPP8-LUC upregulation occurs at a post-translational level.

W-boxes in the RPP8 promoter are essential for RPP8 expression and function

To identify candidate cis elements controlling the transcriptional regulation of RPP8-Ler, the RPP8-Ler promoter, beginning 679 base pairs upstream of the start site, was searched using the PLACE database of plant-specific cis elements (Higo et al., 1999). This search revealed three W-boxes. W-box cis elements and their associated trans elements, WRKY proteins, play a major regulatory role for many plant defense genes. We therefore focused on W-boxes as a likely candidate for regulation of RPP8-Ler. Two of the W-box elements, W2 and W3, are clustered together. The arrangement and spacing of W-boxes is important for inducibility (Rushton et al. 2002); thus, these W-boxes could be important in regulating RPP8. These elements are retained in multiple RPP8 alleles, and the clustered W2 and W3 boxes are also conserved in the sister species Arabidopsis lyrata (Figure 3.7 and data not shown). This evolutionary conservation further suggests that these W-boxes are functionally important.

We hypothesized that mutagenizing W-boxes would impair RPP8 upregulation and/or function. Our mutational approach was based on a previous study, which demonstrated
that mutation of the core W-box sequence from TTGACT to TTGAAT abolished expression of the *NPR1* gene (Yu et al., 2001). The investigators used gel-shift assays to confirm that WRKY proteins are unable to bind this mutant W-box. I therefore used site-directed mutagenesis to inactivate all three W-boxes in the *RPP8*-Ler promoter with this mutation. The resulting construct, containing mutations in all three W-boxes, was named “*wbox*”, and was transformed into Col-0 plants. I also transformed other Col-0 plants with a wild-type *RPP8*-Ler transgene, to enable comparison of mutant and wild-type *RPP8*-Ler expression and function in a large number of independently transformed lines.

T2 seed was collected from 56 *wbox* construct transformants and 19 wild-type *RPP8*-Ler transformants. 7-day-old seedlings were sprayed with Emco5, and resistance was quantified one week later by counting sporangiophores on cotyledons. Wild-type *RPP8*-Ler function should segregate 3R:1S, assuming a single transgene locus, in the T2 generation. This segregation ratio was observed in 14 of 19 *RPP8*-Ler lines (Table 3.1). Four lines exhibited higher proportions of resistant plants, indicating multiple, unlinked transgene insertion sites. These lines were not examined further. One wild-type line was fully susceptible, suggesting that the *RPP8*-Ler transgene in this line was silenced; this was later confirmed by RT-PCR (see below). Significantly, all 56 *wbox* lines were as susceptible to Emco5 as wild-type Columbia. As silencing appears to be a relatively uncommon event, the severe and consistent impairment of resistance in the *wbox* lines strongly suggests that W-boxes are essential for proper *RPP8* function. I identified homozygous T3 populations, based on BASTA resistance, from ten independently transformed lines of each construct. I then confirmed the phenotype of each line by
challenging with Emco5 and counting sporangiophores one week later (Figure 3.8 A). As in the T2 generation, all ten Col::wbox T3 populations were completely susceptible to Emco5.

I next examined RPP8 expression in three week old T3 plants in the homozygous populations of Col::RPP8-Ler and Col::wbox. Untreated and Emco5-challenged plants were collected 48 hours after treatment, and RPP8-Ler mRNA was analyzed with semiquantitative RT-PCR. Almost all wild-type lines express RPP8-Ler, at levels comparable to the endogenous RPP8 in the Ler background, after challenge with Emco5 (Figure 3.8 B and data not shown). The exception was the fully susceptible line, WT5, which expressed no detectable transcript (data not shown). The transgene was presumably silenced in this line. Strikingly, wbox plants exhibited no detectable expression at 35 cycles of amplification. However, a faint band could be amplified from several lines at 40 cycles (data not shown). This experiment suggests that mutation of the three W-boxes dramatically reduced RPP8-Ler expression, relative to wild-type Col::RPP8-Ler.

Four T3 wbox lines were further analyzed by treatment with Emco5 or SA. Expression of both RPP8-Ler and the endogenous RPP8-Col allele were evaluated with gene-specific, semiquantitative RT-PCR. RPP8-Ler transcript was not detected during treatment with Emco5 or SA at 35 cycles in three independent lines (Figure 3.9 A). As expected, endogenous RPP8-Col transcripts follow the same pattern of upregulation as wild-type RPP8-Ler. Surprisingly, a fourth line (WB1) has detectable but very low
RPP8-Ler expression (Figure 3.9 B). I attempted to assay this expression with quantitative PCR; however, RPP8-Ler expression was too low to be reliably detected in any wbox line. The wbox mutations thus greatly inhibit basal expression, but may not preclude upregulation of the RPP8-Ler gene.

xbox-LUC is compromised in basal and organ-specific expression, but not in its capacity for pathogen, SA, or heat stress induction

As described in the introduction, the X-box promoter element is essential for RPP8-Ler function and for basal expression (B. Holt, Ph. D dissertation, 2002, University of North Carolina). However, Holt did not test the effect of this mutation on inducible expression. To determine the role of this cis element in RPP8-Ler regulation, I challenged homozygous xbox-LUC T3 plants with stimuli that upregulate RPP8-LUC. Plants were challenged with Emco5, SA, MgSO₄ and P. syringae DC3000 infiltration, wounding, and heat shock.

xbox-LUC mutants have very low basal expression at 6 weeks of age, but weak luciferase activity is detectable in younger leaves (Figure 3.10, 0 hr images). Unexpectedly, these plants are still able to upregulate RPP8-LUC in response to biotic stress. Although expression is barely detectable in untreated or water-treated controls, plants challenged with Emco5 or sprayed with SA have higher levels of luciferase activity (Figure 3.10 A). Plants also respond to high levels of P. syringae DC3000 (OD₆₀₀=0.01) infiltrated into multiple leaves (Figure 3.10 B), and heat shock upregulation is still very strong (Figure
Interestingly, these mutants do not significantly respond to MgSO$_4$ infiltration (Figure 3.10 B) or localized leaf wounds (Figure 3.11 A). Although *xbox-LUC* plants respond to some stimuli, luciferase activity is still much lower in mutant plants than in wild-type plants after induction.

Organ-specific expression is also disrupted in *xbox* mutant plants; luciferase activity is strongest in leaf petioles (Figures 3.10, 3.11). In contrast, wild-type *RPP8-LUC* expression occurs throughout the aboveground organs (Figure 3.2 A).

**RPP8-Ler and RPP8-Col are expressed at higher levels in leaves than in roots**

Little is known about the organ-specific expression of *RPP* genes. As described in the introduction, Nicole Mammarella examined the expression of *Col::RPP8-LUC* transgenic plants, which exhibited strong luciferase activity in rosettes and inflorescence stems. However, no luciferase expression was seen in roots. Consistent with this result, MPSS (massively parallel signature sequencing, Meyers et al., 2004) did not detect *RPP8-Col* transcript in roots. However, these analyses may not detect low levels of expression. To confirm the organ-specific expression of *RPP8-Ler* and *RPP8-Col*, I examined transcript levels in the single copy *Col::RPP8-Ler* line WTA. Allele-specific *RPP8* expression was analyzed by semiquantitative RT-PCR on roots and aboveground material from three week old plants. As shown in Figure 3.12, *RPP8-Ler* and *RPP8-Col* are strongly expressed in leaves. However, both alleles were also detected at low levels in the roots.
This data indicates that *RPP8-Col* and *RPP8-Ler* have similar expression patterns, and are not equivalently expressed in all plant organs.
Figure 3.1. Structures of endogenous RPP8 alleles and of RPP8-Ler transgenes used in this study.

A. Structure of the RPP8 locus in the Landsberg erecta (Ler-0) and Columbia (Col-0) ecotypes of Arabidopsis thaliana. 5’ and 3’ untranslated regions are shown as open boxes; protein coding regions are shown as filled boxes. Introns are depicted by diagonal lines. CycCH indicates a pseudogene resembling a rice cyclin C gene, while NF22H is homologous to a hypersensitive response-inducing gene in tobacco. Dashed lines indicate the region deleted in the Col-0 ecotype, relative to Ler-0.

B. Structure of the wild-type RPP8-Ler transgene used in this study. Important
regulatory elements and their locations are shown in the expanded section of the promoter. The *RPP8-Ler* promoter contains three W-boxes (orientation indicated by arrows) and the X-box (inverted repeats indicated by italics; orientation indicated by arrows; oversize, bold italic C indicates the essential residue at -189).

C. Structure of the *RPP8-LUC* construct. The firefly luciferase gene (LUC) is translationally fused to the 3’ end of the *RPP8-Ler* coding region, and is followed by the nopaline synthase (NOS) transcription terminator.
Figure 3.2. RPP8-LUC luciferase activity in response to *H. parasitica* and salicylic acid.

A. Images of light emitted from a single Col::RPP8-LUC line, imaged at 0, 24, and 48 hours after treatment. Plants were sprayed with *H. parasitica* Emco5 or Hiks1 (5 X 10^5 conidiosporangia mL^-1), 1 mM SA, water alone, or were untreated. Images are representative of at least three experiments, performed on two independent transgenic
B. Time course of luciferase activity in a single Col::RPP8-LUC line. Bars depict standard deviation between individual plants; at least three plants were assayed for each treatment. Plants were sprayed with *H. parasitica* Emco5 (5 X 10^4 conidiosporangia mL^-1), 1 mM SA, water alone, or were untreated. This graph is representative of at least three experiments, performed on two independent transgenic lines.
Figure 3.3. Quantitative RT-PCR of endogenous *RPP8* (A) and *PR-1* (B) in Ler-0.

Taqman assays were used to quantify abundance of transcript from *RPP8-Ler* or *PR-1* in the Landsberg *erecta* ecotype. Plants were sprayed with *H. parasitica* Emco5 (5 X 10^5 conidiosporangia mL^{-1}), water alone, or were untreated. *ACT2* transcript was used for normalization. Bars depict mean and standard deviation from three independent
biological replicates, each with two technical replicates from independent cDNA preparations.
Figure 3.4. Quantitative RT-PCR of RPP8-Ler in Col::RPP8-Ler transgenic lines.

Transcript abundance was measured by gene specific Taqman assay. ACT2 transcript was used for normalization. Plants were sprayed with H. parasitica Emco5 (5 × 10⁴ conidiosporangia mL⁻¹), 1 mM SA, water alone, or were untreated.

A. RPP8-Ler expression in WTA. Bars depict standard error between two biological replicates.
B. *RPP8-Ler* expression in WT3. Bars depict standard error between two biological replicates. Note that expression in water-treated and untreated plants was only assayed once.

C. *RPP8-Ler* expression in WT4. Expression was only assayed once.

D. *RPP8-Ler* expression in WT1. Expression was only assayed once.

E. *RPP8-Ler* expression in WT2. Expression was only assayed once.
A. Images of luciferase activity in a single Col::RPP8-LUC line, imaged at 0, 24, 48, and 72 hours. Single leaves (indicated by white arrows) were infiltrated with 10 mM MgSO$_4$ or DC3000 (OD$_{600}$=0.001). Images are representative of duplicate experiments, on two independent transgenic lines.

B. Quantitative PCR of RPP8-Ler expression in the WTA Col::RPP8-Ler line. A
single leaf on each plant was infiltrated with 10 mM MgSO$_4$ or DC3000 (OD$_{600}$=0.001). Plant materials were collected 72 hours after infiltration. ACT2 transcript was used for normalization. The graph shows $RPP8$-$Ler$ expression relative to untreated leaves from MgSO$_4$-infiltrated control plants. This experiment was performed once.
Figure 3.6. RPP8-LUC luciferase activity in response to mechanical wounding and heat stress.

A. Images of luciferase activity from a single Col::RPP8-LUC line in response to mechanical wounding. Leaves were wounded a single time on both sides of the leaf midrib with scissors (“Cut”) or with flat forceps (“Crush”). Plants were imaged at 0 hours (before wounding), 3, 6, 24, and 48 hours. Images are representative of
experiments performed in triplicate, on two independent transgenic lines.

B. Images of luciferase activity from a single Col::RPP8-LUC line in response to
overnight (16 hours) heat stress at 37°C. Plants were imaged eight hours before
treatment (0 hours), immediately after the treatment ended (24 hours), and 24 hours
after the treatment ended (48 hours). Images of luciferase activity in untreated controls
are also shown. Images are representative of experiments performed in duplicate, on
two independent transgenic lines.
Figure 3.7. Multiple sequence alignment of RPP8 allele promoters.

The functional RPP8-Ler promoter, beginning 679 nucleotides upstream of the translation start site, was aligned to sequences from RPP8-Col and to the RPP8 homolog from Arabidopsis lyrata (sequence kindly provided by D. Tian and J. Bergelson, Univ. of Chicaco). Dots represent identical bases, and dashes represent gaps in the alignment. W-box elements are designated by boxed regions and red text; mutated regions in the wbox construct are shown above the alignment in bold. The X-box element is denoted...
by blue text; inverted repeats are italicized, and the essential C residue at –189 is underlined. The C to T mutation in *xbox-LUC* is shown above the alignment, in bold and underlined. The translation initiation codon is underlined.
Table 3.1: T2 Segregation of wbox and WT RPP8-Ler resistance to Emco5

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Multiple insertion</th>
<th>3R:1S</th>
<th>Fully susceptible</th>
<th>Total lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBOX RPP8-Ler</td>
<td>4</td>
<td>14</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>RPP8-Ler</td>
<td>0</td>
<td>0</td>
<td>56</td>
<td>56</td>
</tr>
</tbody>
</table>
Figure 3.8. Function and expression of the wbox mutant, compared to wild-type RPP8-Ler.

A. Asexual sporulation in homozygous, independent T3 populations, scored 7 days after inoculation with Emco5. Lines designated WT1-9 are Columbia lines that contain the wild-type RPP8-Ler transgene (note that the transgene in line WT5 was silenced). WTA designates the single copy wild-type RPP8-Ler line. Lines designated WB1-10 contain the wbox mutant transgene. Bars depict mean and standard deviation of sporangiophores per cotyledon, from 40 cotyledons scored per line. C designates Columbia control (susceptible), L designates Ler control (resistant). The assay was performed in duplicate, with similar results.
B. Gene-specific, semiquantitative RT-PCR of transcript levels in wild type

*Col::RPP8-Ler* or *Col::wbox* transgenic lines. mRNA was isolated at 48 hours from Emco5-infected (+) or untreated plants (-). L designates Ler positive control cDNA, isolated from untreated plants. *ACT2* was used as a control for equal cDNA input. *ACT2* and *RPP8-Ler* were amplified for 35 cycles.
Figure 3.9. *RPP8-Ler* and *RPP8-Col* expression in Col::wbox lines.

*RPP8* expression in homozygous, T3 lines was analyzed with gene-specific, semiquantitative RT-PCR. Plants were treated with *H. parasitica* Emco5 (5 X 10^5 conidiosporangia mL^{-1}), or 1 mM SA. *ACT2* was used as a control for equal cDNA input. NTC denotes no template control. *ACT2* was amplified for 28 cycles, *RPP8-Ler* was amplified for 35 cycles, and *RPP8-Col* was amplified for 28 cycles.

A. *RPP8* expression in line WB5, representative of two additional WB lines.

B. *RPP8* expression in the atypical line WB1.
Figure 3.10. Luciferase activity in CW84::xbox-LUC in response to *H. parasitica*

Emco5, SA, and infiltration of MgSO₄, and *P. syringae* pv. *tomato* DC3000.

Plants were imaged at 0, 24, and 48 hours. This experiment was performed only once, on one transgenic line.
A. Luciferase activity in response to *H. parasitica* Emco5 (5 X 10^5 conidiosporangia mL^-1), 1 mM SA, or water alone.

B. Luciferase activity in response to infiltration of multiple leaves with 10 mM MgSO_4 or DC3000 (OD_600=0.01).
Figure 3.11. Luciferase activity in CW84::xbox-LUC in response to mechanical wounding and heat stress.

This experiment was only performed once, on one transgenic line.

A. Luciferase activity in response to wound treatment, imaged at 0 hours (before wounding), 1, 2, and 3 hours. Leaves were wounded a single time on both sides of the leaf midrib with scissors (“Cut”) or with flat forceps (“Crush”).
B. Luciferase activity in response to overnight (16 hours) heat stress at 37°C.

Luciferase activity was imaged eight hours before treatment (0 hours), immediately after treatment (24 hours), and 24 hours after the treatment ended (48 hours). Images of luciferase activity in untreated controls are also shown.
Figure 3.12. Expression of *RPP8-Ler* and *RPP8-Col* in leaves and roots.

Roots and aboveground plant materials ("leaves") were collected from three week old plants of the WTA Col::*RPP8-Ler* line. *RPP8* allele expression was examined using gene-specific, semiquantitative RT-PCR. Two independently collected root samples are shown. NTC denotes no template control. *ACT2* was amplified for 28 cycles, *RPP8-Ler* was amplified for 35 cycles, and *RPP8-Col* was amplified for 40 cycles.
Chapter 4:

Discussion
Plants constitutively express hundreds of $R$ genes, to recognize a broad range of prospective pathogens. These genes must be carefully regulated for the defense system to function properly. Overexpressed $R$ genes can activate ectopic defenses under inappropriate conditions, and cause damaging responses such as spontaneous HR lesions (Shirano et al., 2002, Xiao et al., 2003). This suggests that $R$ genes have an upper threshold of expression: if this threshold is exceeded, then deleterious effects on plant growth may occur. It therefore seems necessary for the plant to tightly control $R$ gene expression, so that these metabolically expensive, potentially destructive defense pathways are only activated under the proper circumstances.

On the other hand, underexpression of $R$ genes can compromise rapid defense responses and permit pathogen growth. For example, some $R$ genes, such as $RPP8$ (Parker et al., 1993, McDowell et al., 1998, B. Holt and J. Dangl, unpublished), exhibit incomplete dominance. Plants carrying a single copy of $RPP8$ have a slightly delayed defense response compared to homozygous plants. Thus, Emco5 conidiospores occasionally establish a successful infection of single copy $RPP8$ plants, as evidenced by light sporangiophore growth. These genetic data suggest that effective $R$ gene resistance requires a minimal threshold of expression.

Molecular evidence for a minimal threshold of $R$ gene expression comes from a recent study of the barley $MLA$ genes, which confer resistance to barley powdery mildew ($Bgh$). In a $rar1$ background, two copies of $MLA1$ provide effective resistance against $BghMla1$. However, a single copy of $MLA1$ is insufficient for full resistance. This loss of
resistance correlates with protein levels; single copy lines express half as much protein as homozygous lines (Bieri et al., 2004). Thus, a minimal threshold of R protein abundance is required to activate an effective defense response. This study further demonstrates that posttranslational control is an essential mechanism for proper regulation of R genes (Boyes et al., 1998, Bieri et al., 2004).

Growing evidence indicates that R gene regulation may also occur at the mRNA level. R genes were originally thought to be constitutively expressed at low levels (Ayliffe et al., 1999). In contrast, recent experiments demonstrate that some R genes are differentially regulated by external cues. As described in the introduction, some R genes, such as the tobacco N gene and the barley MLA genes, are regulated by an R gene-dependant feedback loop (Halterman et al., 2003, Levy et al., 2004). Additionally, a significant fraction of R genes are induced by a variety of stimuli (Zipfel et al., 2004, Navarro et al., 2004). RPP8 falls into this category, as I observed that RPP8-Ler expression is transiently upregulated by multiple isolates of H. parasitica, P. syringae pv. tomato DC3000, wounding, and heat stress.

It is intriguing that RPP8-Ler also appears to be induced by the defense compound SA, because SA accumulation occurs downstream of pathogen recognition. Several other R genes have been recently documented as SA-responsive, including the RPP8 allele HRT (Chandra-Shekara et al., 2004), RPW8, Pib, and SNC1 (Wang et al. 2001, Xiao et al., 2003, Yang et al. 2004). SA-dependant feedback thus appears to be a common method for upregulating R gene expression. Interestingly, a recent study by Wang et al. (2005)
reported a 2.5 fold induction of $RPP8$-Col transcript in $npr1$-3/35S::NPR1 overexpressing mutants. NPR1 acts downstream of SA in resistance signaling (Cao et al., 1998) and may be a component of the SA-dependant feedback loop that upregulates $RPP8$.

SA feedback regulation also impacts the expression of genes that act between R proteins and SA in the signaling hierarchy. For example, the defense signaling proteins PAD4, EDS1, and EDS5 are each necessary for pathogen-induced SA accumulation. However, these genes are induced after pathogen challenge and SA treatment (Feys et al., 2001, Nawrath et al. 2002), and SA accumulation is required for the inducibility of $EDS1$ and $EDS5$. PAD4 may have both SA-dependant and SA-independent feedback loops through EDS1 (Jirage et al., 1999, Feys et al., 2001). Although these defense proteins are important for basal and R-gene-mediated resistance, the significance of pathogen and SA inducibility in the function of these genes has not yet been examined. Moreover, it is not known whether the feedback module(s) regulating these genes is the same module that regulates $R$ genes like $RPP8$.

Although SA and NPR1 appear to be sufficient to upregulate $RPP8$ expression, it is not known whether they are necessary for $RPP8$ upregulation. To establish the mechanism of $RPP8$ upregulation, $RPP8$ expression could be examined in mutants impaired in SA signaling, such as pad4, npr1 and NahG. In this regard, it is significant that my work has shown the Ler and Col alleles of $RPP8$ to be similarity regulated. R gene signaling mutants are typically assayed by pathogen resistance. The majority of mutants that
disable resistance signals are in the Col-0 ecotype. Therefore, RPP8-Ler function cannot be directly tested in most mutants unless RPP8-Ler transgenes are introduced into each mutant. My work suggests that RPP8-Col may therefore be used to identify mutations essential for RPP8 transcriptional regulation. Semiquantitative PCR of RPP8-Col in Columbia plants challenged with Hiks1 has revealed several potential mutants with impaired RPP8 transcription and upregulation (T. Hoff, pers. comm.). These assays will identify mutants that may be important for RPP8-Ler upregulation, which can be further tested with RPP8-Ler transgenes.

Functional significance of RPP8 induction

The significance of RPP8 upregulation for its function as a resistance gene remains to be established. The simplest hypothesis is that the modest increase of RPP8-Ler expression contributes to a faster, more effective defense response against Emco5. The dose dependency of RPP8 suggests that expression of this gene is close to the minimum threshold required for full resistance. Upregulation may incrementally increase the timing and efficiency of RPP8-dependant resistance, and may be essential for completely blocking Emco5 infection. A recent study of the RPP8 allele in Di-17, HRT, suggests reported indirect evidence that SA-dependant regulation is important for HRT function. HRT provides resistance against TCV, but only prevents viral spread in ~85% of Di-17 plants. However, SA treatment enhances the HRT-activated resistance against TCV. Moreover, transgenic lines that strongly overexpress HRT have fully efficient resistance to TCV. SA might thus enhance resistance by upregulating HRT. However, the authors
do not provide direct proof that SA upregulation is part of normal TCV resistance. Interestingly, TCV infection does not appear to upregulate HRT expression (Chandra-Shekara et al., 2004). The authors also conclude that RPP8-Col is not upregulated by SA, but they only examined expression at the 48 hour time point. My research shows that RPP8-Col appears to be transiently induced by SA at 24 hours, and is downregulated at 48 hours. Further study could assess the functional significance of RPP8 upregulation, and whether this significance differs between alleles.

Promoter swapping experiments represent one approach to directly assess the functional impact of transcript regulation. The MPSS database and microarray data could be searched for a gene with similar basal transcription as RPP8-Ler, but which is not induced by pathogen or SA. The promoter from this gene could be used to drive the expression of RPP8-Ler in transgenic lines, and RPP8 function could be assayed by Emco5 resistance. We expect that such an experiment would impair, although perhaps not fully abolish, RPP8-Ler-mediated resistance.

Role in global pathogen response

RPP8 may be induced as part of basal defense activation, which is supported by its systemic upregulation by the virulent pathogen P. syringae pv. tomato DC3000. This global upregulation may activate a more effective resistance against pathogens by two different mechanisms. One possibility is that increased expression could effectively activate weak R protein-dependant recognition, allowing the plant to more effectively
recognize and defend against the pathogen. Alternately, elevated levels of R proteins could activate a resistance response through nonspecific signaling activity, as seen in transgenic plants overexpressing R proteins. This nonspecific defense signaling could prime the plant to respond to pathogen attack.

Further research will examine the role of SAR mutants, such as npr1, in the systemic response observed during DC3000 infection. Col::RPP8-LUC expression can be used as a screen to determine pathogens that are capable of upregulating RPP8, including viral pathogens such as TCV and CMV-Y, avirulent bacterial pathogens, parasitic plants, and nematodes.

Wound-inducible anticipatory control

RPP8 is also induced by wounding. The extent of RPP8 upregulation varies with treatments; light damage caused by cut or crush wounds cause a rapid, wound-specific response, while infiltration causes a stronger, long-term upregulation of RPP8 in the injured leaf. Wounding by infiltration also causes slight upregulation of XA1 in rice leaves (Yoshimura et al., 1998). Interestingly, the flagellin receptor FLS2 is also induced after wounding (Gomez-Gomez and Boller, 2002). Plants may therefore upregulate defense genes in anticipation of opportunistic pathogen invasion (Wang et al., 2001, Gomez-Gomez and Boller, 2002). The pathway that induces this wound response is not well understood. It is possible that SA mediates this wound response, as seen in the Brassica oleracea receptor kinase gene SFR2 (Rocher et al., 2005). Basal, local, and
systemic expression of $SFR2$ in response to wounding were all severely compromised in a $NahG$ background. The role of SA in wounding pathways can be addressed by the experiments described above.

*Role of W boxes in the RPP8 promoter*

Surprisingly little is known about the *cis* or *trans* factors that regulate transcription of *R* genes. Examination of the *RPP8* promoter led us to hypothesize that W-box *cis* elements could have important roles in its regulation. Most of the external cues that increase *RPP8* transcript have been previously shown to induce synthetic promoters containing concatenated W-boxes (Rushton et al., 2002). My data clearly demonstrate that W-boxes are important for *RPP8* function, as the *wbox* promoter mutant provides no resistance to Emco5. Given the previously documented role of W-boxes in pathogen-induced upregulation, we had expected that the *wbox* construct would only be impaired in pathogen- and/or SA-mediated inducibility. Surprisingly, the promoter mutation drastically reduces both basal and inducible expression of *RPP8*. This effect is consistent with results from a mutational analysis of the *NPR1* promoter, in which W-box mutations abolished both basal and SA-inducible transcription (Yu et al., 2001). Similarly, a W-box in the *SFR2* promoter was shown to be important for basal gene expression, but was not required for inducibility (Rocher et al., 2005). My results are also consistent with ChIP analysis of W-boxes in the parsley WRKY1 promoter (Turck et al., 2004); WRKY proteins constitutively occupy W-boxes in the WRKY1 promoter, and appear to compete for binding sites. Some of the constitutively bound WRKY proteins may be responsible
for basal expression. Over the course of defense responses, changes in the WRKY population could lead to induction or repression of the promoter relative to basal expression (Turck et al., 2004). Thus, W-boxes have complex roles in gene regulation.

At present, we do not know how individual W-boxes contribute to RPP8 expression. Previous studies have shown that W-boxes can have positive or negative roles in a promoter, while some W-boxes do not affect transcription at all (Yu et al., 2001, Chen and Chen, 2002, Turck et al., 2004). WRKY binding appears to be sensitive to the context of the W-box (Yu et al., 2001, Rushton et al., 2002, Cormack et al., 2002, Dong et al., 2003, Turck et al., 2004), but the determinant of this specificity has not been described. Thus, we are unable to predict the transcriptional role of individual W-boxes in the RPP8 promoter. Future research will examine the effect of individual W-box mutations on RPP8 expression and function. W-box mutations will be assayed, both singly and in combination with each other, for effects on RPP8 regulation, as was done with the wbox mutant. This experiment will broaden our understanding of W-boxes, and their specific contributions to RPP8 regulation.

The involvement of WRKY proteins in RPP8 regulation should also be directly demonstrated, using EMSAs (electrophoretic mobility-shift assays) to analyze WRKY binding in nuclear extracts from different treatments. An all-WRKY antibody against the conserved WRKYGQK domain (developed by Turck et al., 2004) could be utilized to confirm that WRKY proteins interact with the RPP8 W-boxes. Although we have established the importance of W-boxes in the RPP8 promoter, we have not yet shown
that this mutation alters the binding of WRKY proteins. EMSAs could also be used to
demonstrate changes in WRKY binding caused by W-box mutations. Additionally, the
occupancy of W-boxes on the RPP8 promoter could be analyzed in detail over the time
course of resistance using ChIP assays, as in Turck et al. (2004).

Additional approaches could be taken to identify the specific trans factors involved in
RPP8 regulation. A genetic approach is to screen individual WRKY loss-of-function
mutants with RPP8-Col PCR. This approach may not be effective, because of the
complexity and probable redundancy of the WRKY family. Several WRKY-
overexpressing lines have been generated, such as AtWRKY18 (Chen and Chen, 2002),
and these lines could be screened for possible effects on RPP8 expression. However, this
approach does not distinguish between direct and indirect regulation. Yeast-one-hybrid
screens would likely be the best approach to identify transcription factors that interact
directly with the RPP8 W-boxes. Genes identified by this method could be analyzed by
examining RPP8 transcript levels in T-DNA knockout or RNA-silenced lines.

RPP8 transcription is also dependant upon the X-box promoter element. As xbox
mutants primarily express RPP8-LUC in the petiole, we hypothesize that the X-box is
necessary for organ-specific expression in the leaf blade. Interestingly, the X-box mutant
does not to appear to alter RPP8 inducibility, in the petiole, to heat or biotic responses.
This result suggests that W-boxes confer inducibility on RPP8, but interactions between
W-box and X-box trans factors may be required to maintain strong basal resistance.
However, the X-box mutation appears to abolish wound responsiveness. As W-boxes
have been characterized in multiple wound responses, regulation of RPP8 wound responsiveness might depend on coordination between the X-box and W-boxes. Given its essential role in RPP8 transcription, it is important to characterize this unique promoter element. First, the inducibility of the X-box promoter mutant must be repeated and confirmed at the mRNA level, using RT-PCR. It is presently unknown what type of protein interacts with the X-box. Yeast-one-hybrid studies could identify a specific transcription factor that binds to this element, and EMSAs could provide information on cis element occupancy during different treatments. ChIP assays of the RPP8 promoter may also provide insight into dynamic X-box occupancy. The role of this promoter element in organ-specific expression should also be examined.

Temperature-dependant upregulation of RPP8-LUC

Some defense and R genes can be regulated by temperature, although the significance of this upregulation is unknown. Transcripts of the NBS-LRR Pib gene family in rice are barely detectable by Northern blot at 5°C. However, transcript levels increase with temperature, and they are strongly expressed at 35°C (Wang et al., 2001). In contrast, other R genes are negatively regulated by high temperatures. Copines, a class of calcium-dependant phospholipid-binding proteins highly conserved in eukaryotes, appear to repress defense signaling in a temperature-dependant fashion. Interestingly, the copine mutant bon1 has a stunted phenotype, which is caused by a constitutively active defense response at 22°C. This defense response was correlated with increased expression of the RPP5 homolog SNC1 (Yang et al., 2004), which is upregulated at 22°C compared to
28°C. In the absence of BON1-mediated repression, SNC1 upregulates its own expression by an SA-mediated positive feedback loop. Overexpression of SNC1 then leads to a constitutive defense response. Thus, R genes can be regulated by abiotic responses. Interestingly, BON1 is itself regulated by both temperature and basal defense responses; these proteins therefore represent an overlap between biotic and abiotic stresses (Jambunathan and McNellis, 2003). A heat shock transcription factor was upregulated by RPP7- and RPP8-activated defense (Eulgem et al., 2004), which may indicate overlap between heat stress and defense responses. The defense signaling protein EDS1 appears to have temperature-regulated mRNA accumulation as well (Yang et al., 2004), raising the possibility that defense signaling pathways are also temperature-dependant. The cross-talk between environmental and biotic stress signaling may indicate that plants prepare themselves for the possibility of pathogen attack during other stresses (Wang et al., 2001, Jambunathan and McNellis, 2003).

Although I did not see upregulation of RPP8 transcript after 16 hours of heat shock, early transcriptional responses may already be downregulated at this timepoint. Thus, future experiments will examine RPP8 expression over a timecourse of heat response, to determine if and when RPP8 transcription is activated during heat stress. RPP8 expression should also be examined at the protein and transcript level over a range of different temperatures. Signaling mutant plants could be screened as described above to examine the mechanism controlling the heat shock response, if it is regulated at the transcript level.
The upregulation of \textit{RPP8-LUC} activity during heat shock may also be post-translationally regulated. Protein turnover rates of RPP8-LUC may be slowed during heat stress, as chaperonin binding could stabilize \( R \) proteins. Interestingly, the heat shock protein HSP90 interacts with some \( R \) proteins (Muskett and Parker, 2003). A recent study on the MLA1 and MLA6 resistance proteins show that these proteins rapidly decreased and remained low upon exposure to 37°C (Bieri et al., 2004), which is not a strong heat shock for barley. These \( R \) proteins therefore appear to undergo rapid turnover. Interestingly, the C termini of these proteins interact with the heat shock protein HSP90. The normal rapid turnover of these proteins may be prevented by their interactions with heat shock proteins, indicating that \( R \) proteins can be regulated at the protein level. Interestingly, \textit{RPP1-LUC} transgenic plants also exhibit increased luciferase activity after heat shock (J. Sun and J. Jelesko, pers. comm.). The heat induction of \textit{RPP1-LUC} is not directly explained by similarities between the \textit{RPP1} and \textit{RPP8} promoters, as they are greatly diverged from each other. If \textit{RPP8} mRNA is not upregulated by heat shock, protein assays such as yeast-two-hybrid screens may reveal potential interactions between RPP8 and chaperonins that may play a key role in stabilizing \( R \) proteins at high temperatures.

\textit{Conclusions}

In sum, I have shown that \textit{RPP8} alleles from the Columbia and Landsberg \textit{erecta} ecotypes are consistently upregulated by treatment with \textit{H. parasitica} and the signaling molecule SA. \textit{RPP8} expression also appears to be upregulated in response to infiltration,
and systemically and locally upregulated in response to the virulent bacterial pathogen \( P. syringae \) pv. \( tomato \) DC3000. \( RPP8-LUC \) expression is also upregulated by leaf wounding and heat shock treatment. In addition, I have shown that W-box \( cis \) elements are essential for \( RPP8 \) function and expression at the transcript abundance level. Further research will uncover the significance of these defense responses, and will pave the way for future studies connecting \( R \) gene regulation with \( cis \) and \( trans \) elements, providing a more comprehensive view of plant defense mechanisms.
Literature Cited


Boyes DC, Nam J, Dangl JL. The Arabidopsis thaliana RPM1 disease resistance gene product is a peripheral plasma membrane protein that is degraded coincident with the hypersensitive response. Proc Natl Acad Sci U S A. 1998 Dec 22;95(26):15849-54.


Ryals J, Weymann K, Lawton K, Friedrich L, Ellis D, Steiner HY, Johnson J,


Wang ZX, Yamanouchi U, Katayose Y, Sasaki T, Yano M. Expression of the Pib rice-blast-resistance gene family is up-regulated by environmental conditions favouring


Vita

Toni J. Mohr (formerly Toni J. Burbidge) was born in Breckenridge, Minnesota, on April 21, 1978. She was raised in Colorado Springs, Colorado, and graduated from Palmer High School in 1996 with honors from the International Baccalaureate (IB) program. She received a B.A. from the University of Colorado at Boulder in 2000, majoring in Molecular, Cellular, and Developmental Biology, and Biochemistry, with a minor in Chemistry. After graduation, she worked as a technician in several labs on diverse projects: magnetic labeling of \textit{E. coli} with Dr. George Bajszar (University of Colorado at Colorado Springs), heterologous expression of the spinach protein PsRP1 with Dr. Peter Gegenheimer (University of Kansas at Lawrence), and neural crest cell migration in chicken embryos with Dr. Elizabeth Hay (Harvard Medical School, Boston, MA). Toni completed her Master of Science degree in the department of Plant Pathology, Physiology, and Weed Science, with a major emphasis in plant physiology, in July 2005, under the direction of Dr. John McDowell.