UNIVERSITY OF CALIFORNIA, SAN DIEGO

Exploring plant immune signaling through the Arabidopsis and *Pseudomonas syringae*

interaction

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by

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The Thesis of Amanda Gayle Mason is approved and it is acceptable in quality and form

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DEDICATION

In recognition of Steven P. Briggs for giving me the opportunity to carry out research in his lab and constantly coming up with ideas for me to ponder and consider. An additional thanks to all the Briggs lab members whom I enjoy spending my days with and who have given me the nick-name Crash, not because of anything I do in lab, but because of my constant clumsiness and ability to trip, fall, run into things, and spill stuff on myself during our short excursions from the lab (and sometimes, very rarely, in lab). With special thanks to Zouxin for all his advice and help with my experiments and always giving me half of his palmier at coffee.

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ABSTRACT OF THE THESIS

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Professor Steven Briggs, Chair

In this thesis I describe the results of experiments aimed at characterizing different aspects of the immune system in plants, through the investigation of the Arabidopsis *thaliana* and *Pseudomonas syringae* interaction. First, we explored the ability of different microbe associated molecular patterns, MAMPS to induce certain defense marker genes. Additionally we explored specific single bacterial avirulence effectors for their ability to suppress MAMP induced defense signaling in R-gene-deficient plants (Chapter 1). Flagellin (flg22), cellulase, and salicylic acid induced the

chosen marker genes in different ways, but plants seemed to respond to a lesser extent to the E.coli compound Kdo_2-lipid A, which induces defense in mammalian cells. The Pseudomonas effectors avrRpm1, avrRpt2, avrRps4, avrB, and avrPphB did not clearly suppress any of the marker-gene inductions. Moreover, some induction of markers was observed, suggesting recognition of the effectors by unknown (R-) proteins. We also explored the effects that light and temperature have on the hypersensitive response (HR) during the resistance (R)-gene defense response induced by expression of a single bacterial effector (Chapter 2). As expected deprivation of light delayed the HR. Surprisingly, and in contrast to other studies, HR was functional at high temperatures, and instead HR was delayed at low temperature (17°C). Since the function of many proteins in immune signaling is unknown, as well as potential protein-protein interactions, we explored the interactome of the protein NDR1. We identified 286 potential NDR1 interacting proteins with potential roles in plant immunity. Currently, candidate proteins are being tested for their role in defense.

General Introduction

Plants and pathogens have co-existed and interacted since they first appeared on land, co-evolving their means of defense and defense sabotage. Plants have evolved two main lines of defense against pathogen infection including a basal defense response and an effector triggered defense response [1,2]. A plant's first line of defense is its basal defense system wherein plant surface receptors recognize conserved microbe-associated molecular patterns (MAMPs) causing immunity in the plant [1] (figure 1a). One conserved MAMP is flagellin, which is recognized by the cell surface receptor, FLS2, causing a cascade of cellular signaling rapidly inducing an immune response [3]. Pathogen MAMPS are constantly changing as are plant receptors in a constant arms race for virulence and resistance [1].

Pathogens have evolved an effector protein secretion system, as a means of inhibiting basal defense to increase their virulence (figure 1b) [4]. Effectors are either secreted and then taken up by the host, or they are directly secreted into the host via a type III secretion system (TTSS) [4,5]. Effector proteins work within the plant to sabotage MAMP signaling in order to suppress the plants defense response, causing effector-triggered susceptibility (ETS) [1,6]. In response, plants evolved a resistance (R)-protein system wherein R-proteins recognize bacterial effectors (or their actions within the host) either directly or indirectly by "guarding" host effector targets [7,8]. R-proteins in Arabidopsis generally contain a series of leucine-rich repeats (LRR), a nucleotide binding site (NB), and a putative amino-terminal signaling domain either Toll-IL-1 receptor (TIR) or coiled-coiled (CC) thus terming them CC or TIR-NB-LRR proteins [9]. To date most R-proteins only guard one or two pathogen effectors in a gene-for-gene

resistance model that confers specificity in R-protein recognition and initiates a specific defense response based on the R-protein and effector involved [4]. R-gene mediated defense leads to a rapid hypersensitive response (HR) and the restriction of pathogen growth [10]. The hypersensitive response causes local cell death in the area of infection, appearing as dehydrated regions as well as necrotic lesions on plant leaves [11] and it also causes systemic defense signaling to the rest of the plant making them more resistant to future infections. Reactive oxygen species (ROS) are important for the Arabidopsis immune response. ROS have been shown to act in disease resistance signaling, and were found to be required for HR, with ROS absence causing a delay in the HR [12]. A large generation of ROS in defense comes from an enzyme, NADPH oxidase, that uses NADPH produced by photosystem I [13,14].

Signaling mechanisms downstream of R-proteins are largely unknown. R-proteins might regulate nucleo-cytoplasmic trafficking or translocate to the nucleus themselves where they might regulate a transcriptional response [15,16]. Understanding how R-proteins signal to cause resistance has become one of the greatest challenges faced in plant disease research.

The protein NDR1 (non-race-specific disease resistance 1) is an essential part of the R-gene mediated defense response involving the R-proteins, RPM1 and RPS2, which monitor the actions of the bacterial effectors, avrRpm1/avrB and avrRpt2, respectively [17,18] (figure 1 c). Using an *ndr1-1* mutant, it was observed that in an avrRpm1-RPM1 or avrB-RPM1 mediated defense response, NDR1 is essential for pathogen growth restriction but not HR whereas in an avrRpt2-RPS2 defense response, NDR1 is required for both pathogen restriction as well as HR. [17,19]. This is intriguing since it means that

pathogen growth restriction and HR are independent of each other and the role of NDR1 in RPM1 and RPS2 mediated defense responses is different.

The link between the two R-genes, RPM1 and RPS2, in defense is the protein RIN4 (RPM1-interacting protein 4), which also directly interacts with NDR1[20] (figure 1c). RIN4 is a negative regulator of defense, and it plays roles in both MAMP and R-protein signaling by being phosphorylated upon treatment with flagellin or with its conserved peptide, flg22 [21-23]. The effectors avrRpm1 and avrB both trigger the phosphorylation of RIN4, putatively resulting in hyperphosphorylated RIN4 in order to inhibit defense [24]. The MAMP and effector-mediated phosphorylation of RIN4 may act on different residues as they have different outcomes, with avrRpm1 causing the activation of RPM1. AvrRpm1 was also shown to activate the R-protein, RPS2, but to a lesser extent than RPM1 [25]. RPS2 is mainly activated by the effector avrRpt2 which causes the cleavage of RIN4 [23]. Down-stream signals form the proteins, RPM1 and RPS2, are unknown but we can learn about R-protein signaling by examining one of the common factors, NDR1, and describing its interactome.

There are many unanswered questions in plant immunity research. It is unclear to what extent different MAMPs are activating the same conserved defense pathways or if certain MAMPS are activating different pathways that are not highly conserved. The means by which specific effectors inhibit these MAMP signaling pathways is largely unknown as most studies have been done using a collection of effectors. Furthermore, it is unknown if all secreted effectors individually target different proteins to a level sufficient enough to cause suppression or if many effectors act cooperatively to sufficiently cause the suppression of MAMP signaling. Additionally, the mechanisms of

R-protein signaling are unknown. The protein, NDR1, is known to be essential for R-protein mediated immunity but it is unclear what NDR1's exact role and contribution to R-protein mediated immunity is. The mechanisms by which HR occurs and the external conditions required to cause an HR are largely unknown.

This thesis describes cross-talk between several MAMP-signaling pathways and the interference with MAMP-signaling caused by bacterial effector proteins. The environmental conditions necessary for HR are explored by changing both temperature and light conditions during an R-protein mediated defense response. Lastly, I describe the NDR1 interactome to explore the role of NDR1 in RPS2 mediated immunity.
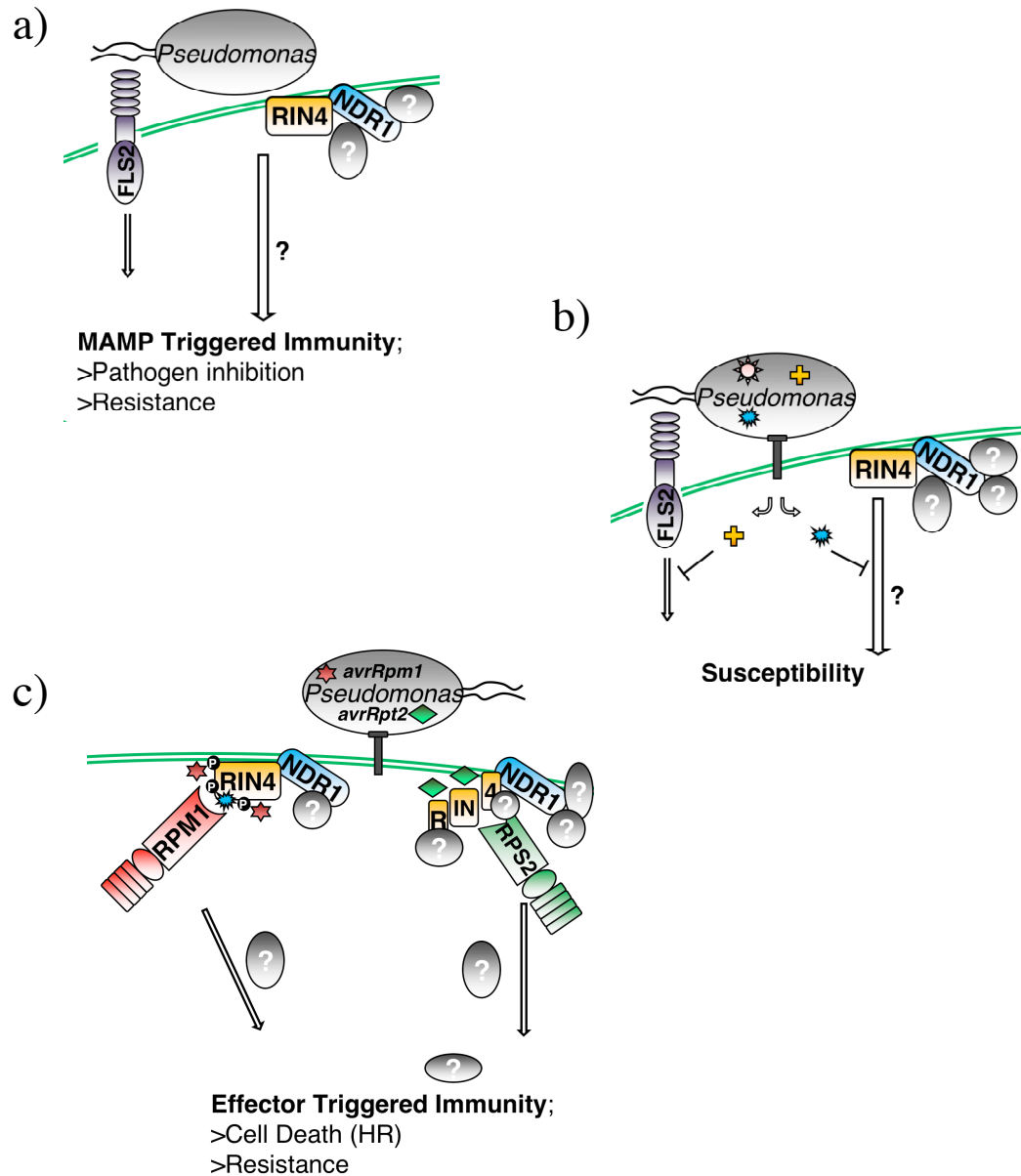
Figure 1: Plant Defense Response
a) Plants have a basal defense response which consists of surface receptors, for instance FLS2, recognizing conserved microbe-associated molecular patterns (MAMPs) causing MAMP-triggered immunity in the plant. RIN4 is also involved in basal defense and associates with NDR1. b) Pathogens can secrete effectors to block MAMP signaling making the host susceptible. c) Plants have evolved a R-gene recognition system wherein host R-proteins recognize the actions of pathogen effectors causing effector triggered immunity. The R-proteins RPM1 and RPS2 recognize effector modification of RIN4, which requires the protein NDR1 for successful effector triggered resistance.

Chapter 1 MAMP Response

1.1: Defense gene regulation by MAMPs

Introduction:

Plants have evolved many different ways to sense pathogen presence including a basal and an R-gene mediated defense system. Both defense systems respond to pathogen infections enabling plant resistance. Basal plant defense is triggered by microbe-associated molecular patterns (MAMPs) such as flagellin, chitin, lipopolysaccharides (LPS), bacterial elongation factor TU (EF-Tu), as well as other microbial surface proteins [26]. These surface proteins are recognized by pattern-recognition receptors (PRR) on the plants surface causing MAMP-triggered immunity [1,4]. After recognition a unique signaling pathway is triggered based on the MAMP perceived, however there is much cross-talk between pathways and the mechanisms that arrest infection may be the same. MAMP-triggered immunity causes cell wall thickening, callose deposition, production of toxic metabolites including reactive oxygen species (ROS), and the induction of pathogenesis-related (PR) proteins all of which work to inhibit pathogen growth [27]. MAMP responses have been studied thoroughly and many genes have been proven to be regulated by MAMP-triggered immunity and have been used as markers. A few of these genes are: pathogenesis related gene-1 (PR1) [28], non host resistance to P.S. Phaseolicola 1 (NHO1) [27], and WRKY DNA binding protein 29 (WRKY29) [29]. By monitoring the induction level of these MAMP induced PR genes it is possible to test if other compounds are recognized as MAMPs by plant cell surface PRRs. This chapter describes the induction pattern of defense genes in Arabidopsis in response to four different MAMP treatments. The pattern of defense gene induction is explored to see if

the same pattern of defense gene induction is seen regardless of the MAMP used to trigger immunity.

Results:

Experiments were performed to test whether previously reported defense gene patterns observed using cell suspensions or whole seedlings could be replicated in attached leaves. Also it is important to be able to replicate experiments in our lab's different growth conditions. The elicitors chosen for the experiments were compounds previously reported to be recognized as MAMPs in Arabidopsis, or compounds that initiated defense in a different system. Four different MAMP treatments were chosen to. (1) Cellulase is an enzyme that causes the hydrolysis of cellulose, a cell wall compound, and when plants recognize fractions of cell wall they initiate a defense response [30]. (2) Kdo_2-Lipid A (3-deoxy-D-*manno*-octulosonic acid) is a lipo-oligo saccharide endotoxin from E.coli, known to activate macrophages [31]. Since it is known that lipopolysaccharides (LPS) are involved in plant defense [32] we thought it would be interesting to test if this LPS that activates defense in mammalian cells also causes a defense response in plants. (3) Flg22 is a flagellin peptide that is recognized by the Arabidopsis FLS2 receptor kinase [3] causing a cascade of events that leads to the regulation of defense responsive genes [33]. (4) Salicylic acid is a hormone known to activate defense pathways causing the induction of many defense genes and defense compounds [34,35].

The expression level of genes known to be regulated in defense, were compared at three time points: 2, 6, and 24 hours following MAMP treatment. Gene expression levels in 3 control treatments (vacuum infiltration with a control solution, no vacuum

infiltration but submersion in the control solution, and no treatment) were compared to the vacuum infiltration with cellulase (0.1mg/ml), Kdo$_2$-lipid A (0.5μg/ml), flg22 (1μM), and SA (5mM), every solution contained 0.01% Silwet-L77. The defense genes chosen for the experiments were: PR1 (pathogenesis related gene-1) a gene previously shown to be up-regulated during defense [28], NHO1 (non host resistance to Pseudomonas phaseolicola 1) previously shown to have higher expression levels in plants treated with flagellin [27], and WRKY29 (WRKY DNA binding protein 29) a transcription factor known to act downstream of flg22 perception in basal defense [29]. Gene expression levels in treated plants were determined using RT-PCR. To ensure that an equal amount of template was used for gene expression level comparison, Actin2 RT-PCR of 26 cycles was used as a constitutively expressed control gene.

PCR results indicate that there is always a low basal level of PR1 present in the Arabidopsis *Col-0* plants (Figure 2). There was up-regulation of PR1 in all treated plants with noticeably higher levels in SA treated samples. An up-regulation of PR1 in cellulase treated plants is seen at 6 hours post-infiltration (6hpi) that stays constant through 24hpi. Kdo$_2$-Lipid A treatment shows a slight level of increased PR1 expression at 6 and 24hpi but flg22 treated plants only show a slight elevation in PR1 above basal level that stays constant throughout the sample time-points. It should be noted that the "nothing" control at 6hrs does seem to have a PR1 level a bit higher than expected. This can partially be explained by the higher amount of total cDNA, as seen by the Actin control primers, and in addition these plants might have coincidentally been stressed by an unknown source. If we look at the 6hr submerged with no vacuum sample we do not see this elevated level of PR1.

There is a low level of NH01 expression seen in all control plants indicating that NHO1 is either always present at a low level or it is slightly up-regulated due to the stress/damage of the vacuum infiltration process. The expression level of NHO1 seems to be transiently regulated in plants treated with cellulase and flg22. In these samples there is a very high level of expression seen 2hpi that then drops down to a low level by 6hpi. By comparing to the 2hpi vacuum control we see that although there is a low level on induction of NHO1 in the control it is clearly increased in the cellulase and flg22 treatments. The same pattern of regulation is not seen in Kdo_2-Lipid A and SA treated plants that express a steady low level of NHO1.

WRKY29 expression showed a rapid appearance and disappearance in cellulase, and flg22 treated plants and a possible elevation in Kdo_2-Lipid A treated plants. There was a low level of WRKY29 seen in the 2hpi vacuum inoculated control plants indicating that WRKY29 was slightly up-regulated due to the stress/damage of the vacuum inoculation process. Despite this there is a higher level of expression in the 2hpi treatment samples except for SA. WRKY29 must be a fast response gene which then gets quickly down regulated since we see a very high level of expression at 2hpi and a total disappearance by 6hpi. Thus we see transient expression of WRKY 29 by cellulase and flg22 with a possible transient expression of WRKY29 by Kdo_2-Lipid A, but no expression in SA treated plants. From these results we can conclude that the MAMP induced expression of NHO1 and WRKY29 seem to be similar.
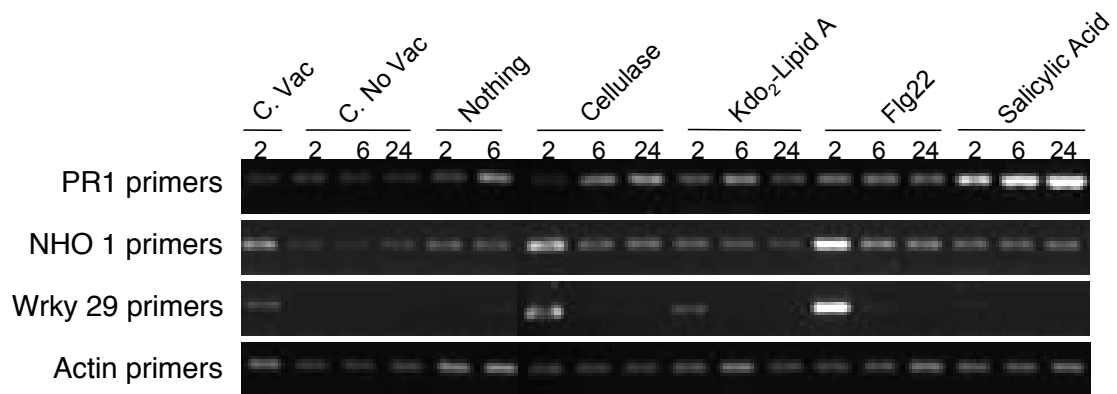
Figure 2: **MAMP induced expression of Defense Genes**
Levels of defense marker-genes in widtype plants post MAMP treatment, determined by
RT-PCR at varying hours post infiltration. The Actin2, NHO1, and WRKY29 primers
were run for 26 cycles of PCR with an additional cycle for PR1 primers (27 cycles).

Discussion:

In the RT-PCR experiments comparing the effects of different MAMP treatments on Arabidobsis *Col-0* (wild type) plants, changes in the expression of defense marker genes PR1, NHO1, and WRKY29 during MAMP induction were observed. It was found that PR1 is sensitive and moderately induced by most treatments with a strong induction by SA that increased through 24hpi. NHO1 and WRKY29 are rapidly and transiently induced by cellulase and Flg22, and also seem to respond slightly to the control vacuum infiltration. Kdo_2-Lipid A may activate WRKY29 expression but from our experiments it is inconclusive. If you compare the levels of Actin in the control verses Kdo_2-Lipid A 2hpi sample there are lower levels of Actin in the Kdo_2-Lipid A sample so you can associate that with less template in that sample. Thus when comparing WRKY29 levels of these two samples you must correct for this factor in your induction estimation, so there possibly is an induction of WRKY29 by Kdo_2-Lipid A. The RT-PCR experiments are semi-quantitative as induction level is based on band intensity read from a gel. This method of analysis results in the reliable detection of large differences in induction but not small differences. In the case of a slight WRKY29 induction by Kdo_2-Lipid A, a result would be more reliable and conclusive using Q (quantitative)-PCR.

The goals of these experiments were to see if previously reported defense gene patterns could be replicated in our lab, and also to test if Kdo_2-Lipid A would induce defense genes in plants. PR1 was first associated with Arabidopsis resistance in experiments using INA (2,6-dichloroisonicotinic acid), SA, and Pseudomonas syringae infection all of which caused a 20-fold or larger up-regulation of PR1 mRNA [28]. PR1

has been used as a defense marker gene in many Arabidopsis studies with documented induction by flg22 [36]. In our study the most potent inducer of PR1 was SA (with expression increasing to 24hpi). PR1 appears to be an insensitive gene with the MAMP treatments of cellulase, Kdo_2-Lipid A, and flg22 only slightly elevating the induction of PR1 above control levels. This is an unexpected result as other papers have shown a large induction by flg22 but this may be due to the fact that we used a lower concentration of flg22 (1µM vs.2.5µM) [36]. Another possibility for this discrepancy could be that our vacuum infiltration was not as efficient. Cellulase has been shown to cause an immune response and since PR1 is a common marker for defense we expected to see a high level of induction in cellulase treated plants. Instead we saw a low level induction that increase over time from 2 to 24hpi. Kdo_2-Lipid A causes an induction similar to the levels of flg22 and cellulase indicating that it is recognized as a MAMP in plants. Our finding that the E.coli defense inducer, Kdo_2-Lipid A, which activates a defense response in mammalians, also works as a defense inducer in Arabidopsis has not previously been reported. Plant pathogenic bacteria have surface LPSs which induce plant defense [37], but Kdo_2-Lipid A is an E-coli LPS and e-coli do not infect plants. Since LPS have conserved structure Kdo_2-Lipid A is likely recognized by receptors that normally recognize LPS's from plant pathogenic bacteria.

NHO1, a glycerol kinase, has been shown to be transcriptionally activated by flagellin, and flg22 [38]. Flg22, the 22AA conserved residue of flagellin is recognized by the receptor FLS2, implying that NHO1 activation must be a downstream target of the FLS2 receptor. Since NHO1 is regulated by one MAMP it is interesting to compare its regulation in regards to other MAMPS. We see a strong induction of NHO1 by flg22 as

well as by cellulase at 2hpi implying that the sensor for cell wall fractions [39] may act in a similar way as FLS2 since it appears they share the same downstream target. For the MAMP treatments of SA and Kdo_2-Lipid A we do not see an induction of NHO1 but NHO1 may be induced by the stress of the vacuum infiltration.

The transcription factor WRKY29 binds the promoter elements of defense genes, and is activated by many MAMPS including flg22 [29,40]. From my results the induction pattern of defense-related genes are overlapping in response to different MAMPs. This indicates a point of convergence between MAMP signaling pathways. It appears that many receptors feed into the same pathway initiating the same downstream targets. However, there is must be more than one pathway since SA does not induce NHO1 or WRKY29 but very strongly up-regulates the expression of PR1. The defense pathways show overlap (NHO1, WRKY29), yet they also show specificity (PR1) indicating that there is much yet to be discovered about MAMP perception and signaling pathways.

In these experiments we only examined the expression of three genes known to be involved in Arabidopsis defense to MAMP treatment at a semi quantitative level. To improve the study on defense gene regulation with different MAMPs the gene regulation could be studied at the quantitative level by doing Q-PCR. The comparison of induction values could then be made to fully understand the level to which the defense genes are being activated in response to MAMPs. Also it might be interesting to compare other know defense elicitors from Arabidopsis such as EF-Tu, xylanse, and chitin [27], to our studied MAMPs cellulase, flg22, and SA to see if they also cause a similar defense-gene regulation pattern. Also expanding the number of regulated defense genes compared would create a broader signaling network to examine and find possible signaling

convergence and overlap such as the cellulase and flg22 overlap we found with the regulation of NHO1 and WRKY29.

In the experiments done with wildtype plants we were only looking at the defense response initiated by plant MAMP recognition. Plants developed their MAMP recognition system to defend against bacteria, but as this system evolved bacteria developed a type III secretion system (TTSS) to deliver effector (avirulence or avr) proteins, that could defeat MAMP-triggered immunity. In response to the avr proteins plants evolved resistance (R)-proteins that recognize bacterial avr's and trigger immunity [6]. In the next Chapter (Chapter 1.2), I want to explore the effects that specific avr proteins have on the regulation of defense genes in the absence of the corresponding R-protein.

1.2: Bacterial Avrirulence Protein Interference in MAMP Signaling

Introduction:

Basal defense is triggered by MAMPs (see chapter 1.1) and can be inhibited by effector proteins produced by pathogens to increase their virulence. Effectors are either secreted from the pathogen and are dependent on uptake by the host or they can be actively injected into the plant cells by the bacterial type-III secretion system (TTSS) [4,5]. Effectors have many different modes of action incluting: altering plant hormone levels, altering RNA metabolism, acting as transcription factors, and blocking defense signaling, illustrating the diversity of effector action [41-47]. Bacteria that have a TTSS can secrete from 20-100 different effectors into a plant [42]. Resistance (R)-proteins can defeat effectors by guarding host targets of effectors or by directly recognizing effectors [7,8]. R-protein activation leads to a rapid hypersensitive response (HR) and restriction of pathogen growth. The HrpA mutant of Pto syringae, lacks an essential component of the TTSS [48]. This bacterial mutant can be used to study MAMP responses, and act as a negative control for effector-producing bacteria. HrpA mutant bacteria still contain surface MAMPs, like flagellin, that are recognized by PRRs but they cannot activate effector triggered immunity, only MAMP-triggered.

We make use of Arabidopsis plant lines that contain a dex-inducible effector in a genetic background with or without the cognate R-protein (i.e., dex::avrRpm1 with RPM1 or rpm1). When inoculated with the HrpA mutant of Ps. Syringae pv. Tomato (Pto) strain DC300, we can observe the effects of the avrRpm1 protein on both virulence (with rpm1, avrRpm1 acts as an effector) and on effector triggered immunity (with RPM1, avrRpm1 triggers immunity). Thus far people have done experiments using

DC3000 with or without an effector of interest (for instance avrRpm1) to study the role of effectors on MAMP-induced defense signaling. A disadvantage of this approach is that many other effectors are injected into the plant and may be acting together with the effector of interest. Also the other effectors may be triggering some R-gene activation, which would be indistinguishable from the actions of the effector of interest. Other studies have compared HrpA infection to infection with wildtype Pto, but again this is comparing a basal defense response to a basal defense response in the presence of every effector. With our engineered plant lines and the use of the HrpA mutant bacteria it was possible to study the effects of a single effector on MAMP induced signaling, without the presence of the R-gene normally recognizing the effector. Here we describe the effects of individual effectors on the expression of defense genes induced by MAMP-triggered immunity.

Results:

We have seen that there is regulation of defense-associated genes by MAMP treatments, in particularly by flg22. In the previous experiments we worked solely with defense elicitors, causing only a MAMP-triggered response. In these next experiments we focused on work with bacterial effectors in conjunction with MAMP-triggered defense responses to examine the effects of specific bacterial effectors on MAMP-regulated genes. When plant pathogenic bacteria secrete effector proteins via their TTSS, known as type-III effectors, they secrete anywhere from 20-100 effectors [42], making it difficult to decipher the action of an individual effector. It has been common practice to use bacteria containing a specific effector as a trans-gene to explore the actions of a specific effector. A disadvantage is that many other effectors are also being secreted into the plant and may

contribute to the gene regulation events seen. To specifically study the effect of one effector on bacterial induced MAMP signaling we used the Pseudomonas syringae pv tomato HrpA mutant, which lacks a TTSS. In HrpA mutant bacterial infections plants still recognize surface MAMPS of the bacteria including their flagellum and initiate a MAMP-triggered defense response, but no effectors are secreted into the plant. Different engineered plant lines were used in our experiments each lacking a resistance (R)-gene while containing the effector gene normally recognized by the R-gene under the control of an inducible dexamethasone (dex) promoter. This plant system was used in conjunction with the HrpA mutant bacteria to explore the actions of the individual effector proteins on the MAMP signaling cascade. Five plant lines were chosen expressing the effectors avrRpm1, avrB, avrRpt2, avrRps4, and avrPphB under the control of a dex promoter in their respective mutant R-gene background. Plants were infected with the HrpA mutant bacteria to study patterns of MAMP related defense genes in the engineered plant lines. Additionally plants from the same line were sprayed with dex 2 hours before the HrpA infection to study defense gene regulation differences caused by a specific effector on MAMP signaling.

To analyze the role of each effector on the MAMP signaling pathway we chose six defense genes and determined their expression via RT-PCR. The defense genes were chosen based on literature that had used bacteria containing an effector trans-gene and showed differentiated regulation of the gene or on the previously cited literature that showed gene induction by MAMPs (Ch.1.1). Published experiments have mainly been done using the effectors avrPto, avrRpm1, avrB, and HopAL expressed in bacteria that secreted other effectors, confounding the role of any given effector. We were interested

in seeing if the effectors avrRpm1, avrB, avrRpt2, avrRps4, and avrPphB could individually disrupt MAMP-triggered gene regulation. In particular we were interested in defense genes whose level was induced by bacterial infections and then subsequently decreased in the presence of an effector. The gene *WRKY47* was chosen since it was shown to be up-regulated by DC3000::hrp infection and then subsequently down regulated by infection with DC3000 containing avrRpm1 [49]. In another case it was found that flg22-induced expression of *NHO1* was inhibited by nine different effectors each as a transgene within DC3000 [6]. Interestingly it was previously shown that infection with DC3000 alone suppressed the expression of *NHO1* this was restored by avrB in DC3000 [50], associating *NHO1* with both MAMP induced and R-gene defense, making it a good candidate for our studies on effector specific action. *PR1*, a protein closely correlated with resistance, especially in SA induced defense, has expression levels that have been shown to be decreased during pathogen infection in plants lacking *npr1*, a protein involved in R-gene defense that blocks SA accumulation [51]. These findings make PR1 a good candidate for finding effector specific action that may block this same pathway that NPR1 is involved in. *WRKY29* and *FRK1* were found to be flg-22-induction regulated genes whose expression was suppressed in the presence of the effector HopAL1[46]. *FRK1* expression was also shown to be inhibited by the effector avrPto [52], and by infection with DC3000 containing many effectors [53]. Since *FRK1* and *WRKY29* were shown to be suppressed by specific effectors it is interesting to see if the effectors we are working with also regulate these genes. *MLP* was shown to be regulated by DC3000 infection, with the presence of the effector trans-genes avrRpm1, and avrB causing the up-regulation of *MLP,* while in the absence of the R-gene RPM1

(recognizes avrRpm1 and avrB) there was no expression of *MLP* [54]. Thus *MLP* is a part of R-gene defense regulation and is an interesting candidate for studies involving other effector R-gene couples in order to see if *MLP* is also regulated by individually expressed avrRpm1 and avrB, a different system than previously investigated. Also we wanted to know if other individually expressed effectors induce *MLP* or downregulate its expression.

Three control treatments were used in the experiments to compare the level of defense-related gene expression. The control treatments were: undisturbed plants (nothing), mock injected plants (10mM MgSO$_4$), and plants that were just sprayed with dex causing avr accumulation in the absence of infection. Defense-related gene expression of plants injected with HrpA mutant bacteria (OD= 0.1), triggering a MAMP defense response, were compared to the levels of expression in plants that had been sprayed with dex (50μM, 0.05% silwet) two hours prior to HrpA infection, as well as to the controls. Therefore we examined MAMP signaling with HrpA bacteria and determined if there was an effect by a specific avr on the MAMP signaling pathways, as well as if there was an effect on defense by a specific avr.

AvrRpm1:

The first experiments done were using the line containing dex::avrRpm1 in *Col-0, rpm1-3* background (lacks the R-gene RPM1). For each sample two internal replicates were taken for the time points 2, and 10 hpi for controls and 2, 4, and 10 hpi for HrpA infection with and without avrRpm1. The MAMP response from the HrpA mutant bacterial infection caused a strong induction of the defense-related genes *NHO1*, and *PR1* and a slight induction of *WRKY29, MLP,* and *FRK1* (figure 3), compared to mock

injection. The effect of the effector avrRpm1's presence in the HrpA defense response caused the slight decrease in the expression of *WRKY47* by 10hpi, and an increase of *PR1* and *MLP* expression levels by 10hpi. Also there seems to be a slight increase in *NHO1*, and *WRKY29* expression levels with avrRpm1 presence during HrpA infection. This is surprising as we would expect to see the suppression of more MAMP defense related genes by effectors rather than their increase.

There was also an effect seen on defense gene expression by the presence of avrRpm1 alone, with an increased expression level of *PR1* and *MLP* seen in the dex sprayed controls. This might partly explain the effect of avrRpm1 on the observed MAMP response gene expression increases discussed above. This result could also indicate that dexamethasone or silwet is causing some stress or slight defense response in the plants. In addition it should be noted that *FRK1* expression seems to be at an elevated level in all samples except the nothing control plants insinuating that it is a very sensitive gene and responds to the stress of injection as well as effector avrRpm1's presence. Also note that avrRpm1 is accumulated in plants that are sprayed with dex proving that our inducible system works. The four other studied effectors are under the same dex promoter as avrRpm1, so we assume their accumulation upon dex spray to a similar level as avrRpm1.

After the dex::avrRpm1 plant line experiment was done we chose to continue the same experiments using the inducible effectors avrB, avrRpt2, avrRps4, and avrPphB but on a smaller scale. From the first avrRpm1 experiments it was observed that the most highly regulated time point, where the clearest expression differences were seen, was at 10hpi. Since we are interested in significantly large differences in transcriptional

regulation we chose this time point to compare for treatments between the subsequent plant lines.

AvrB:

In the plant line containing the dex::avrB inducible construct in *rpm1-3* background, MAMP responses to HrpA mutant bacteria caused the induction of *FRK1*, and possibly a slight induction of *MLP* (figure 4). Surprisingly for many of the genes we see a lowered level of expression in the HrpA response, compared to the control mock injection. The effector avrB's presence on the HrpA MAMP defense response did not clearly cause down regulation of any of our chosen defense-related genes. An increased expression level of *WRKY47*, and *WRKY29* was seen with avrB's presence during HrpA infection. *FRK1* and *MLP* were induced to high levels in both cases of infection regardless of effector presence, meaning the MAMP response to HrpA caused an induction of *FRK1* and *MLP* that avrB does not block.

There was also an effect seen on defense gene expression by the presence of avrB alone with an increased expression level of *FRK1, WRKY47, NHO1,* and *PR1* in the dex sprayed controls compared to the nothing control. This level of increased induction may partially explain the increase of expression upon avrB's presence during MAMP response described above. Another interesting observation from the data is that *PR1* expression is up-regulated by the presence of avrB yet there is no significant level of *PR1* seen when HrpA infection accompanies avrB presence.

AvrRpt2:

In the Arabidopsis Colombia plant line lacking the R-gene RPS2 and containing the dex::avrRpt2 inducible construct, MAMP responses with HrpA mutant bacterial

infection caused an induction of the defense-related genes *FRK1*, *WRKY29*, and *PR1* (figure 5). There is also a possible slight induction of *MLP* and *WRKY47* when taking into account differences in the actin control levels. The effector avrRpt2 had an effect on the HrpA MAMP defense response, causing a slight down-regulation of the defense-related genes *FRK1*, *WRKY47*, *WRKY29*, and *PR1* compared to HrpA infection alone. This indicates that there is some block by avrRpt2 of MAMP induced defense signaling pathways, which effects the expression levels of our defense marker genes. Additionally *NHO1* and *MLP* levels remain induced to the same degree regardless of avrRpt2 presence during HrpA infection.

There was also an effect seen on defense gene expression by the presence of avrRpt2 alone, with an observed increased expression levels of *FRK1, WRKY47, MLP,* and *PR1*. There was also a possible slight induction of *NHO1,* as seen in the dex spray control compared to the nothing sample. Interestingly with avrRpt2 presence, as in avrB presence, we see the phenomena of high *PR1* induction by the effector avrRpt2 that is subsequently lowered in the HrpA infection with avrRpt2 induction.

AvrRps4:

In the Arabidopsis RLD ecotype plant line which lacks the R-gene RPS4 and contains the dex::avrRps4 inducible construct, MAMP responses to the bacterial infection using HrpA mutant bacteria caused the induction of *MLP* and a possible slight induction of *WRKY47* (figure 6). In this plant line there seems to be a elevated basal expression level of our chosen defense marker genes *FRK1, WRKY47, NHO1,* and *MLP,* which makes deciphering MAMP induction levels difficult. There was no clear effect of the effector avrRps4 when present during the HrpA MAMP induced defense response. This

indicates that avrRps4 does not affect any HrpA MAMP signaling that involves our 6 marker defense genes.

There was an effect seen on defense gene expression levels by the presence of avrRps4 alone, with an increased expression level of *FRK1*, *WRKY47*, *NHO1*, *MLP*, and *PR1* seen in dex prayed plants. However there was also an elevated level of all the aforementioned genes and *WRKY49* in mock injected plants suggesting that the RLD ecotype is very sensitive to and type of stress, including effector presence and injection.

AvrPphB:

In the Arabidopsis Colombia plant line lacking the R-gene RPS5 and containing the dex::avrPphB inducible construct, the MAMP response to HrpA mutant bacterial infection caused the induction of *FRK1, WRKY47, WRKY29*, and a possible induction of *MLP* (figure 7). The effect of the effector avrPphB's presence on the HrpA MAMP defense response caused the slight down regulation of *WRKY47* and *WRKY29,* and an up-regulation of *PR1*. The level of induction of *FRK1*, *NHO1*, and *MLP* stayed constant during HrpA infection regardless of avrPphB's presence during infection.

An effect was seen on defense gene expression by the presence of avrPphB alone with an increase of expression levels of *FRK1, MLP*, and *PR1,* and a possible induction of *NHO1*. As with the effectors avrB and avrRpt2 a high expression level of *PR1* was seen during avrPphB presence that is subsequently expressed to a lower level during HrpA infection with avrPphB induction. Since we see this pattern of elevation and then repression of *PR1* expression, there maybe some sort of competition of signaling happening in the plant where MAMP signaling interferes and reduces effector triggered signaling.

Figure 3: Effects of avrRpm1on MAMP induced signaling
RT-PCR of different treatments of plants containing dex::avrRpm1 in *rpm1-3* background. Plants were infected with HrpA mutant bacteria with and without the presence of the effector avrRpm1. Dex spray causes the production and accumulation of the effector protein meaning dex spray control is essentially the avrRpm1 presence without infection.

Figure 4: Effects of avrB on MAMP signaling
RT-PCR of different treatments of plants containing dex::avrB in *rpm1-3* background. Plants were infected with HrpA mutant bacteria with and without the presence of the effector avrB

Figure 5: Effects of avrRpt2 on MAMP signaling
RT-PCR of different treatments of plants containing dex::avrRpt2 in *rps2-101c* background. Plants were infected with HrpA mutant bacteria with and without the presence of the effector avrRpt2
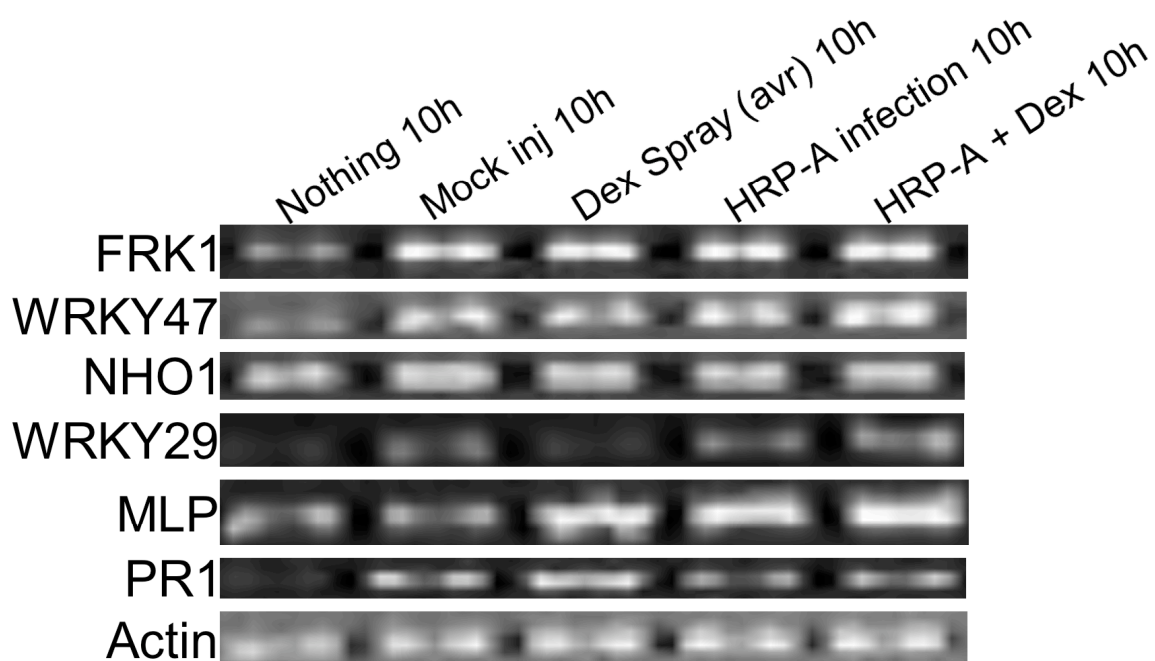
Figure 6: Effects of avrRps4 on MAMP signaling
RT-PCR of different treatments of plants containing dex::avrRps4 in RLD background, an ecotype that does not contain RPS4. Plants were infected with HrpA mutant bacteria with and without the presence of the effector avrRps4
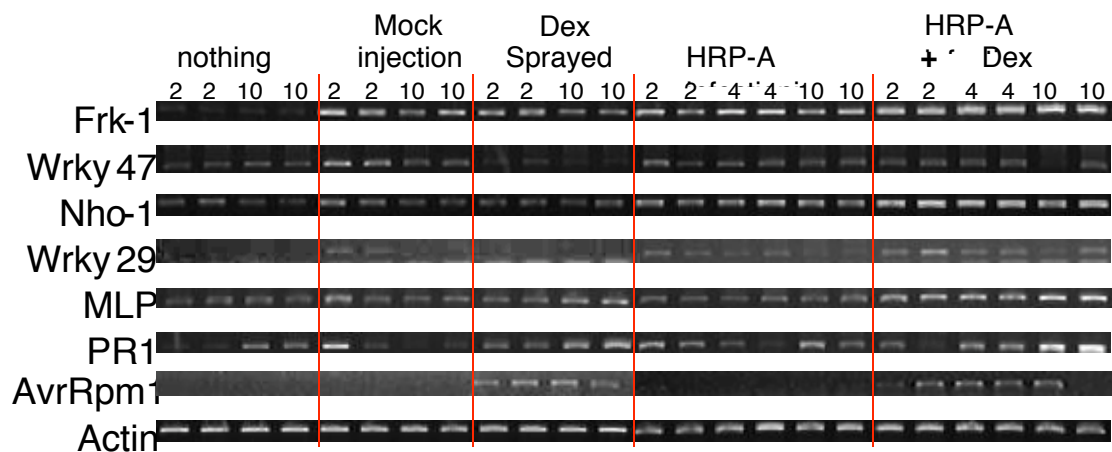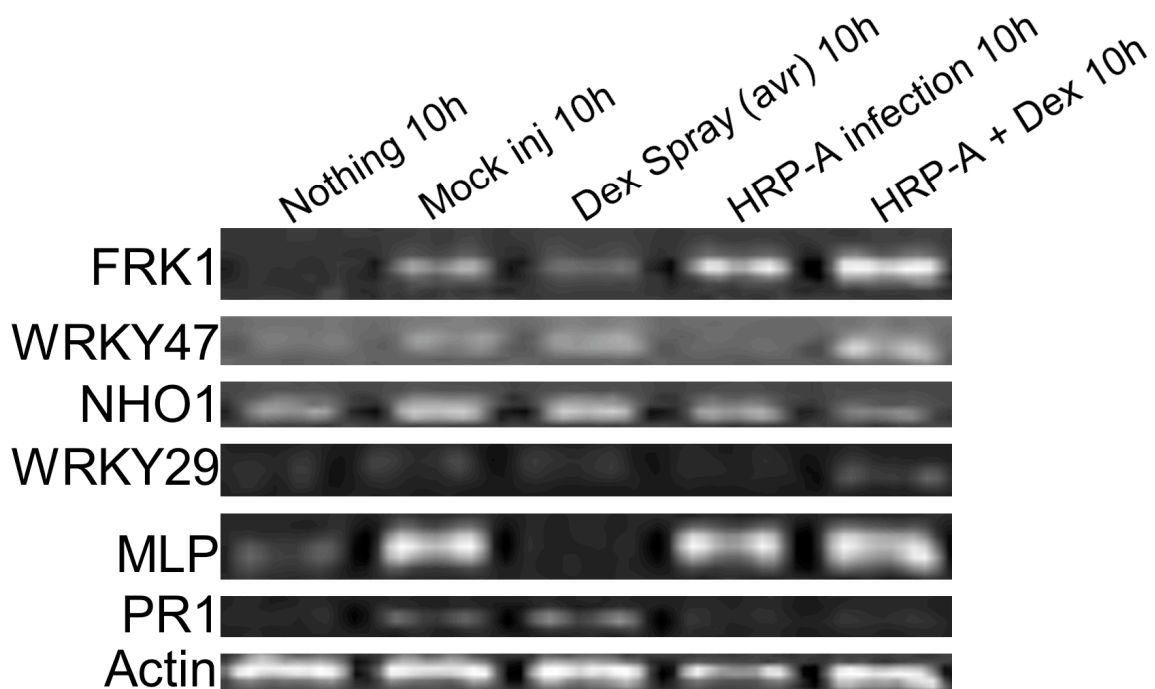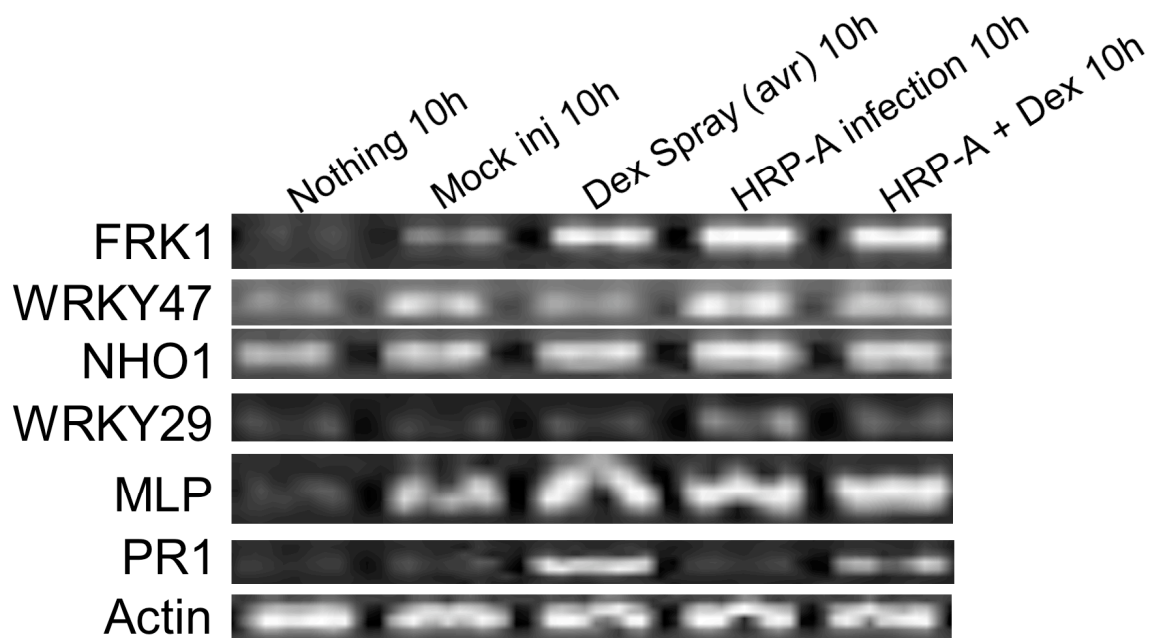
Figure 7: Effects of avrPphB on MAMP signaling
RT-PCR of different treatments of plants containing dex::avrPphB in *rps5* background.
Plants were infected with HrpA mutant bacteria with and without the presence of the
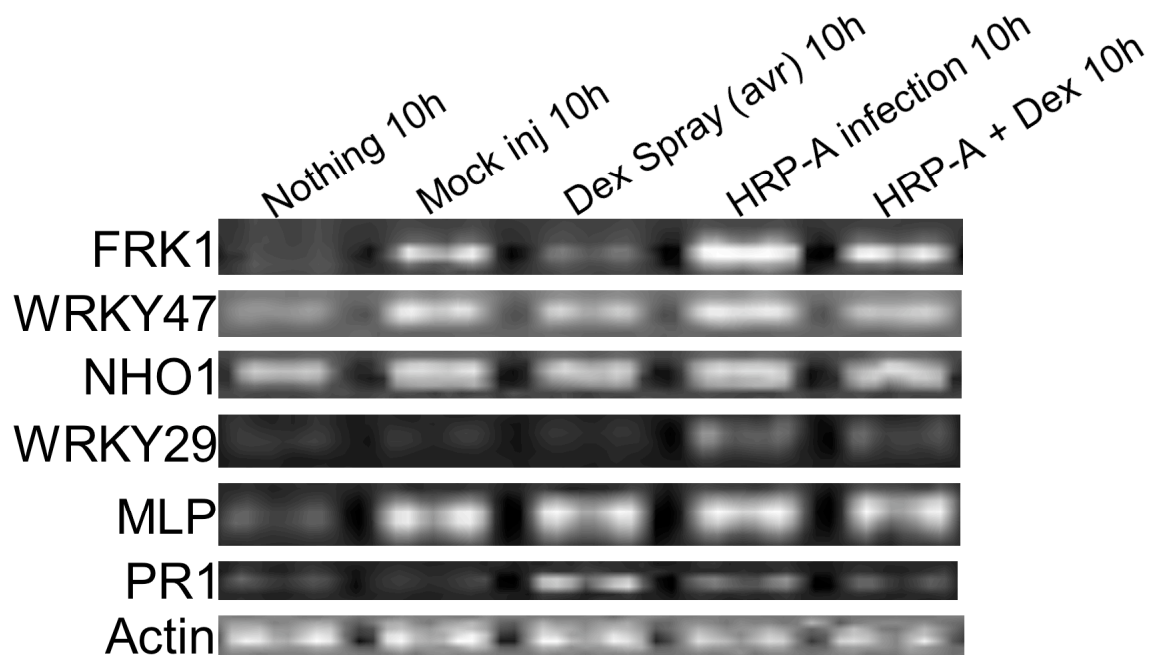effector avrPphB.

Discussion:

In the RT-PCR experiments examining the effects of different effectors on HrpA MAMP induced defense signaling, conclusions were made on the action of the effectors avrRpm1, avrB, avrRpt2, avrRps4, and avrPphB. Experiments were done in plant lines with an inducible effector in the absence of its' respective R-gene, in order to be able to decipher the actions that each individual effector acting alone had on MAMP signaling. In all of our five different inducible effector plant lines at least one if not more of our chosen defense marker genes was induced by HrpA infection. Suppression of HrpA MAMP induced defense signaling was seen in three out of our five tested effectors, but each effector only suppressed a few of our marker genes. The effector avrRpt2 had the widest range of suppression causing the down regulation of *FRK1*, *WRKY47*, *WRKY29*, and *PR1*. We see a conserved suppression of W*RKY47* by the effectors avrRpm1, avrRpt2, and avrPphB. In addition it was observed that generally all the effectors themselves caused an induction of some of our chosen defense marker genes. All conclusions were based on observations made from ethidium bromide gels where RT-PCR products were run, leading to a semi-quantitative evaluation of gene induction level. This type of evaluation allows for the observation of significantly large induction differences and is less reliable for small induction differences. For a more accurate understanding of the actions of effectors, based on subtle changes, quantitative PCR would need to be preformed on the samples.

A very interesting finding from the experiments was that the effectors avrRpm1, avrB, avrRpt2, avrRps4, and avrPphB all caused an induction in some of our chosen

marker defense genes. The accepted mechanism by which effectors work is that they function to block MAMP induced defense signaling. If there is an R-protein present within the plant that can recognizes the effector, either directly or indirectly, then a different R-gene mediated effector triggered defense response is initiated causing the regulation of defense-related genes [4]. In our system the experimental plants lacked the R-gene that recognized the respective inducible effector, meaning no R-gene defense signaling should have been activated. Therefore there should not have been a response to the effectors presence in the plant (control dex spray), or an increase in defense gene response levels during HrpA infection due to effector presence, if the defense system was working directly as modeled. An explanation for these events of defense gene up-regulation may be that an effector (or its actions) is actually recognized by multiple R-genes, so there is an R-gene response despite the absence of the supposedly only responsive R-gene. The effectors avrRpm1 and avrB (both monitored by RPM1), and avrRpt2 (monitored by RPS2) are known to target the protein RIN4. AvrRpm1 and avrB cause phosphorylation of RIN4 and avrRpt2 causes the cleavage of RIN4 with their respective R-proteins guarding RIN4 and recognizing changes in RIN4 [4]. It was shown that avrRpm1 is recognized by RPS2 (in addition to RPM1) causing a defense response [25], so it is quite possible that this duel R-gene recognition could extend to avrRps2 and avrB recognition. Since we do see defense activation by all three of these effectors, regardless of their respective R-gene absence, we hypothesize that RPM1 and RPS2 recognize any change in RIN4, thus either R-gene is able to recognize the action of the effectors avrRpm1, avrB, and avrRpt2. This could be true for other effector R-gene pairs and would explain why the presence of all of our effectors triggered some level of

defense response, even though the plant is lacking the recognized interacting R-protein. Another possibility is that many effectors target the same proteins that are monitored by R-genes, thus resulting in R-protein monitoring overlap. This means that R-gene mediated defense response may have less specificity than previously thought. Furthermore since we are using an inducible system we do not know if the level of effector presence caused by dex induction in the cells is similar to what would normally be injected naturally. If the effector is accumulated at an abnormally high level it might cause defense signaling to be activated by a different means than gene-for-gene recognition. Also when inducing the effector a 50μM dexamethasone (dex) with 0.05% silwet solution is used, so it is possible that the plant is actually responding to the dex or to the silwet. In order to test this possibility plants without a dex inducible construct, for instance wild type plants, should be sprayed with a 50μM dex, 0.05% silwet solution as well as with unmixed individual solutions of dex and silwet. The expression level of the same defense marker genes used in these effector studies should then be examined in all three of the samples mentioned above.

From these experiments we also concluded that there is not a complete block of any of our chosen marker defense-related genes by any of our individually induced effectors. There are many observed gene down regulation events, as described above, but no complete blockage of a gene induced by MAMP defense signaling. Other studies using the effectors avrPtoB, and HopAL1 have shown the complete blockage of defense genes (*FRK1*-avrPtoB, *FRK1* and *WRKY29*-HapAL) during bacterial infection [46,52]. One of our aims was to see if any of our effectors blocked a similar signaling response during HrpA infection. The conclusion from our data is that none of our chosen effectors

are capable of completely blocking signaling caused by MAMP infection on any of our chosen marker genes. A reason that we may not see the same defense gene signaling blockages as other studies have is because in all other studies the effector studied was injected into the plant by bacteria containing an effector transgene, through the bacteria's TTSS. In this experimental system many other effectors are also injected into the plant along with the effector of interest, so although there are controls of infection without the transgene just having a variety of effectors in combination with the effector of interest will give different results than just the effector alone. If we expanded the number of marker genes involved in defense used it is possible that we would find that a single effector acts to totally suppress the expression of certain defense-related genes, but at this point we have no evidence for it. Also it would be interesting to look at a combination of different effectors in multiple R-gene mutant plants to examine if certain effectors work together to cause the suppression of a specific defense related gene. Overall it appears that none of the single effector proteins we worked with are sufficient enough to completely suppress the expression of our chosen defense genes *FRK1*, *WRKY47*, *NHO1*, *WRKY29*, *MLP*, or *PR1* although in some cases they did cause expression down regulation.

There is a down regulation of *WRKY47* by the effectors avrRpm1, avrRpt2, and avrPphB. *WRKY47* was chosen as a marker because it had been shown to have elevated expression during DC3000:hrp infection that was then down regulated by induction with DC3000 containing the avrRpm1 transgene [49]. From our results we conclude that the effector avrRpm1 alone causes the down regulation of *WRKY47* and that the down regulation is not contingent on the presence of the other injected effectors from DC3000.

In addition this proves that the effectors avrRpt2 and avrPphB act in a similar way inhibiting MAMP signaling of *WRKY47*. It should be noted that the suppression of *WRKY47* is only a partial suppression of gene expression that may have a greater block of expression with multiple effectors. Meaning that the actions of a single effector are magnified by a collection of effectors and in reality there is no true complete suppression of defense signaling by a single effector. In addition the same study by Truman *et al.* found that DC3000:avrRpm1 infection caused the down regulation of *WRKY29* compared to DC3000:hrp injection. Interestingly we do not see this same down regulation by the effector avrRpm1 alone and additionally the effector avrB, which is recognized by the same R-gene as avrRpm1 (RPM1), actually causes an up-regulation of *WRKY29*. We do however see the described *WRKY29* down regulation by the effectors avrRpt2 and avrPphB both of which are induced in the presence of RPM1. In summary we see the absence of WRKY29 down regulation by effectors when RPM1 is missing, but we see the effector down regulation rescue when RPM1 is present in HrpA infection with effectors that RPM1 is not known to recognize. Perhaps the presence of the R-gene RPM1 is essential in causing the down regulation of *WRKY29* by effectors, regardless of if it is the recognized effector known for its interacting gene-for gene recognition with RPM1. So we conclude that *WRKY29* down regulation is an effect of R-gene signaling. This again supports our hypothesis that certain R-genes monitor cell changes caused by all effectors. *NHO1* was shown to be down regulated by nine effectors (present as transgenes within DC3000) [6], but in another study was shown to be up-regulated by the effector avrB within DC3000 [50]. Interestingly in our study *NHO1* was not regulated at all by any of our effectors alone. The induction of *NHO1* is already known to be a

confusing induction system, with both MAMP and R-gene defense regulating *NHO1,* but we show that none of our five chosen effectors are sufficient to cause regulation.

In the absence of dex spray all our different plant lines can essentially be considered as biological replicates, with mock injection (vs. nothing control) acting as an indication of stress induction, and HrpA infection verses mock injection as an indication of MAMP defense gene induction. There is a consistent induction of the four genes *MLP* (5/5)*, FRK1* (4/5)*, WRKY29* (3/5)*,* and *WRKY47* (3/5), indicating that they are good marker genes for MAMP induced defense, and it seems that our treatment was pretty reproducible. There is a consistent low level of induction of five of our chosen defense response genes to mock injection *NHO1* (5/5)*, FRK1* (5/5)*, WRKY47* (5/5), *MLP* (4/5)*,* and *PR1* (3/5), indicating that most of our marker defense genes also respond slightly to stress. Since we saw gene expression with our system, as well as suppression, we can confidently say that we choose good defense markers genes, but more markers should be used for a more thorough study. To choose more marker genes we would want to look at more effector studies involving multiple effectors and choose genes based on expression increase during R-gene mediated response, as well as MAMP induced response. In general doing RT-PCR with our marker genes was the correct approach to study our question of how individual effectors influence MAMP signaling. By looking at gene expression we were able to decipher the individual actions of effectors on specific defense related genes, but there are other ways to study the entire effect of a single effector of MAMP response. It is possible to monitor MAMP responses by monitoring other characteristics of a MAMP-triggered defense response such as ROS production, callose formation, and measuring bacterial titer [55]. Assays are performed for each of

the aforementioned characteristics of MAMP response and levels of each can be compared between treatments (MAMP response with or without effector), allowing the evaluation of the total effect of single effector on a MAMP-triggered immune response.

In our experiments we chose to use HrpA mutant bacteria to elicit a MAMP defense response (mix of multiple MAMPs) with our dex inducible effector system as opposed to using flg-22 to elicit a MAMP response (only one specific MAMP). This was done because effector avirulence proteins may inhibit MAMP signaling that is not elicited by flg-22. Also we assume that MAMPs trigger responses that overlap and therefore to get a complete idea of an effectors actions on MAMP signaling we would wants to see its effect on multiple overlapping pathways, as they may induce genes to a greater extent than just one MAMP-triggered pathway.

Materials and Methods Chapter 1:

Plant Material and growth conditions:

Arabidopsis thaliana ecotype Col-0 was used while testing for the defense gene induction with different elicitors. Plants were grown in soil placed in 2x2in small pots covered with mesh for four weeks. The pots were placed in a conviron growth chamber with a light cycle of 16h light 8h dark and held at the constant conditions of 25°C and 60% humidity.

Arabidopsis thaliana resistance (R) gene mutants that express corresponding Avr genes under a dexamethasone inducible promoter were used for observing the regulation of defense genes in MAMP (microbe associated molecular patterns) response as well as in MAMP response with avirulence protein (avr) presence when there is no R-gene recognition.

Five different mutant plant lines were used all obtained courtesy of Jeff Dangl:

Line 8: *rpm1-3*, dex::avrRpm1

Line 9: *rpm1-3*, dex::avrB

Line 11: *rps2-101c*, dex::avrRpt2

Line 13: *RLD* (no RPS4), dex::avrRps4

Line 14: *rps5-2*, dex::avrPphB.

Plants were grown in soil for four weeks within a Percival growth chamber having a light cycle of 11h light at 22°C and 13h dark at 20°C, the humidity was uncontrolled but stayed around 75%.

Bacterial Growth and preparation :

Pseudomonas syringae pv tomato hrpA mutant bacteria obtained courtesy of Sheng Yang He [48] (they lack a type three secretion system TTSS) were kept in a glycerol stock at –80°C, two days before use some of the glycerol stock was removed and plated on King's B plates (proteose peptone 15g/L, 10mM K2PO4, 1% glycerol, 12g/L agarose, PH ~7.2 :after autoclaving 10mM MgSO4 was added) containing rifampicin (25mg/L) and kanamycin (50mg/L) and allowed to grow for 2 days at 28°C. When ready to use 10ml of 10mM MgSO4 was poured onto the plate and agitated on a shaker for 5 minutes, the liquid was then removed to a tube and the OD of a 10x dilution taken. For these experiments the bacteria were then diluted with 10mM MgSO4 to an OD of 0.1. Plant treatments: Gene induction by defense inducing compounds:

To determine defense related gene expression by different treatments four-week-old WT plants were vacuum inoculated in solutions containing different defense inducing compounds. The plant pots were inverted over small round dishes containing 40ml of solution so that leafs were submerged but soil and roots did not have contact with the solution. Dishes were placed in a vacuum chamber and vacuum was applied for 5 minutes, plants were then returned to their controlled growth conditions. Plants were sampled at 2, 6, and 24 hours post inoculation by removing plant leaves to tubes and freezing them in liquid nitrogen. Five solutions were used, a control of just water, salicylic acid (SA) at a concentration of 5mM (note it must first be dissolved in ethanol and then diluted to volume with water), flg 22 (ABGENT flagellin peptide) at a concentration of $1\mu M$, cellulase (SIGMA) at a concentration of 0.1mg/ml, and Kdo_2-lipid A (an E.coli lipo-oligo saccharide, obtained from Avanti polar lipids inc) at a concentration of $0.5\mu g/ml$. All solutions also contained 0.01% silwet.

Plant treatments: Gene induction my MAMP signaling and effect of Avr Proteins:

To analyze the levels of defense genes induced by MAMPs and possible suppression by Avr proteins four-week-old R-gene mutant/inducible Avr plants (described above) were used. To get a MAMP response plants were injected with hrpA mutant bacteria (lack a TTSS) suspended in 10mM MgSO$_4$ to an OD of 0.1, control plants were injected with just MgSO$_4$. In plants where Avr effect was being studied plants were sprayed with a 50μM dexamethasone solution containing 0.05% silwet two hours before hrpA bacterial infection. Injection was done to the bottom sides of leaves with a 1ml syringe applying minimal pressure in order to avoid leaf damage. After treatment plants were returned to their controlled growing environment and sampled at 2, 4, and 10 hours after treatment by harvesting leaves into tubes and freezing in liquid nitrogen.

RNA Isolation:

Acid RNA isolation was used to extract the total RNA from ground nitrogen frozen leave tissue. Three milliliters of cell lysis buffer (PH<3.5: 2% Sodium dodecyl sulfate (SDS), 10mM EDTA, 50mM Sodium Citrate, and 150 mM Citric Acid) was added per 100mg of N$_2$ frozen ground leaf tissue (tissue was ground by placing a metal rod in the tube and vortexing). Tissue and buffer were then homogenized, allowed to warm up to room temperature, and left to sit for 5 minutes or until the mixture was clear. One milliliter of Protein-DNA Precipitation buffer (PH<2.5: 4M sodium chloride, 10mM sodium citrate, 40mM citric acid) was then added per 3ml of cell lysis buffer used, the solution was then mixed by inverting gently and left on ice for 30 minutes to precipitate protein and DNA. After the 30 minute incubation the sample was then centrifuged at

maximum speed for 5 minutes. The supernatant was removed to a new tube and one volume of isopropanol added and mixed in order to precipitate RNA. Precipitation was done at −20°C for 20 minutes. The sample was then centrifuged at maximum speed for 15 minutes and an RNA pellet seen. To obtain cleaner RNA with less genomic DNA contamination the RNA pellet was redissolved in 1ml of water (RNAse free) and re-precipitated with LiCL (400μl of 8M LiCL, end concentration needs to be 2M) for 3 hours at −20°C. The sample was then spun for 30 minutes at max speed and the pellet was washed twice with 70% ethanol, dried, and then redissolved in 25μl of water. RNA concentration was obtained via spectrophotometer (d=1cm; 1/100dil) and quality checked by loading 0.5μg of RNA on a 1.5% RNAse free 0.5x TBE agarose gel.

CDNA Synthesis

A DNAse treatment was done on 8μg of isolated RNA with Ambion Turbo DNA-free. CDNA synthesis was then done by using 10μl (4μg) of the DNA-free RNA. 1.5μl of 10mM dNTP's and 0.5μl of 100μM oligo dT primer were added to the RNA and the mixture incubated for 3 minutes at 94°C to denature the RNA. Samples are immediately cooled on ice and droplets spun down from the lid. A mix of 5μl reverse transcription (RT) buffer 5X, 2.5μl of DTT (0.1M), 2.3μl of water, and 0.2μl of Invitrogen superscript II RTase (added just before use) is added to the cooled samples. The solution is then mixed and left to incubate at 42°C for 2 hours. The cDNA is then diluted to 50μl and stored at -20°C until needed.

RT-PCR

Gene expression was analyzed via RT-PCR done on cDNA created from total extracted RNA, process described above. A 26 cycle (non saturating) PCR with Actin (a constitutively expressed gene) primers was done and band intensities were compaired on an ethidium-bromide-stained gel. Samples with higher cDNA levels were then diluted to the level of the lowest cDNA concentration to ensure an equal amount of template is used in each reaction (level of actin should be checked again after dilutions are done to ensure equality). PCRs were then performed using primers for genes related to defense to a non-saturated level of amplification. Primers spanning intron/exon borders were made so genomic DNA would not be amplified but only cDNA, refer to table 1. PCR reactions were a total of 25μl containing 1μl of cDNA, 2.5μl of 10x taq buffer (200mM Tris pH8.4, 500mM KCL, 20mM MgCL2, 1% TritonX100), 2.5μl of 2mM DNTP's, 1μl of each forward and reverse primer (10μM), 0.5μl of Taq polymerase (home made, approximately 1-2U/μl), and 17.5μl of water. Cycle schematic: Step 1: 95°-3min, Step 2: 94°-30sec, Step 3: 55° -40 sec, Step 4: 72° for 1 min, Step 5: 72° for 10 minutes. Steps 2-4 were cycled and the cycle number varied depending on the gene being amplified.

Primer Design

Primers were designed to prevent genomic DNA PCR products of the same size or larger than the desired cDNA products. This was done by looking at intron position relative to two exons and designing primers for a gene that span an intron-exon (figure 8). This allows for the specific amplification of cDNA. Also primers were designed to amplify a product of 300-800bp with a melting temperature of 60°C and G/C content of 40-60%.

Table 1: **Gene Primers used in RT-PCR**

Primer	Sequence	ATG numbers
NHO1_Fa.	CGGAGAAGTGCCAATCAAATC	
NHO1_Ra.	CCATCAGATCAGCCTGAATC	
FRK1_F	GTACACTTGGTGAGAGGAATG	
FRK1_R	AGATACTCTAGTCCTTGCGCTG	
WRKY47_F	CACAAGTGGAAGGTCTTCATC	
WRKY47_R	GCATCGTTGGACCTGTTTAC	
MLP_F	CTACGTCCACTGAGGGAACAAC	AT1G13340
MLP_R	CACAGGGTCTTCTCTGAATGAC	AT1G13340
WRKY29_F	GATCTCCATACCCAAGGAGTTA	
WRKY29_R	CCCACTGAAGAACTCTTGGCTA	
NHO1_Fb.	CTGATGCAGATTCAGGCTGATC	
NHO1_Rb.	TATGTGACTTGGAAGACAGAGC	
avrRpm1_R2	TACTTGCCACCCTTGGCTC	
avrRpm1_F2	TCCAATGCGGGCTTTGAAGT	
PR1-f	CTCGGAGCTACGCAGAACAACT	
PR1-r	ATACATCCTGCATATGATGCTC	
Act2-F	GTCGTACAACCGGTATTGTGC	
Act2-R	CACATCACACTTCATGATTGAG	
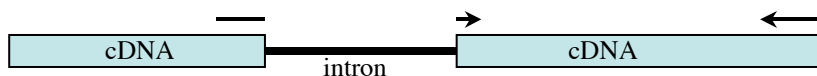
Figure 8: **intron-exon primer design**

Chapter 2: Effects of Light and Temperature on HR

Introduction:

Photosynthesis is an important process in plants allowing them to harvest the energy of sunlight and use it as energy within the plant cell to synthesize organic compounds. Photosynthesis is also important in the production of reactive oxygen species (ROS) during a plants defense response. Plants produce ATP and NADPH from photosynthesis that can be used for carbohydrate biosynthesis. NADPH is normally used in the light-dependent photosynthesis, the Calcin-cycle, where NADPH gets used for the production of glyceraldrhydes-3- phosphate. During pathogen infection the enzyme NADPH oxidase can use NADPH for the production of the ROSs O_2- and H_2O_2 resulting in the oxidative burst thought to play many roles in the defense response, including involvement in the hypersensitive response (HR) [12,56]. Therefore ROS production from NADPH oxidase is dependent on a product of photosynthesis, NADPH, and therefore photosynthesis. It has been shown that during a viral infection of tobacco in the absence of light plants did not accumulate the ROS H_2O_2, and had a significantly delayed HR [57]. It was also shown that the HR resulting from a viral infection in Arabidopsis ecotype Dijon was suppressed in the absence of light [58]. HR is partially light dependent, with delayed HR resulting if plants are infected in the absence of light. In another experiment it was shown that in Arabidopsis infection using Pseudomonas containing avrRpm1 it is crucial to start defense experiments in the early morning because defense is linked to a plants light/dark cycle, with limited HR occurring if defense is induced later in the day [59]. Additionally recent studies have shown a connection between chloroplastic peroxidases in photosynthesis and a negative regulation

of basal defense further solidifying a connection between photosynthesis and the plant immune response [60]. These experiments also suggest that light is crucial for the HR to occur, linking photosynthesis and HR.

Another external factor that has been shown to be involved in a successful HR upon R-protein mediated defense signaling is temperature. It was found that in tobacco plants, where both effector and effector recognition protein were expressed, plants did not die at higher temperatures due to a lack of effector receptors (HABS) [61]. Another study found that the protein HSP90, which is involved in the disposal of mis-folded R-genes, was non functional at higher temperatures causing a suppression of R-gene defense [62,63]. Also it was found in Arabidopsis that in an infection with DC3000 containing the effectors, avrRpm1, avrRps2, and avrRps4, HR was delayed at 28°C compared to infection at 22°C [64]. These studies all suggest that R-protein mediated defense is dependent upon the temperature at which the infection is preformed. Another reason temperature is an external factor of interest in HR is that at high temperatures it has been shown that net photosynthesis is inhibited [65], which as previously described, has been associated with a successful HR in R-protein mediated defense. The disadvantages of these studies are the same as previously described for the HR studies, as the same infection systems were used. Additionally many of the temperature experiments were done in another organism than Arabidopsis.

In this chapter we describe the relation between light and the HR induced by avrRpm1 and RPM1, using Arabidopsis ecotype Colombia (*rpm1-3*) containing a dexamethasone (dex) inducible avirulence transgene avrRpm1, as well as a RPM1 transgene (native promoter, myc tagged), which recognizes the actions of avrRpm1. Also

in this chapter we describe the relation between temperature and the HR induced by three effector-R-protein couples: avrRpm1 in the presence of the transgene RPM1 (native promoter, myc tagged), avrRpt2 in the presence of the endogenous RPS2, and avrRps4 in the presence of the endogenous RPS4.

Results:

Since light has been shown to be important in other plant systems for a successful HR during defense responses, we wanted to see if HR in our plant system was affected by the absence of light when R-gene mediated defense was initiated by the presence of the effector avrRpm1 alone. Plants were sprayed in the morning with a 50μM Dex and 0.05% silwet solution to induce the transcription of avrRpm1. Pictures were then taken at 4, 6, 10, and 24 hours post induction (hpi). Plants were assigned to one of three different treatments during avrRpm1 induced defense: placement in the light, placement in the dark just after dex spray, or placement in the dark 32 hours prior to dex spray with continued placement in the dark after avrRpm1 induction. HR symptom levels were compared between the different treatments to decipher the effects of light/photosynthesis on a R-gene mediated defense response initiated by the effector avrRpm1.

It was found that the hypersensitive response (HR) was significantly delayed in plants that had been in the dark for 32hours prior to effector induction and remained in the dark after effector induction (dark 32), compared to plants that remained in the light before and after effector induction (light) (figure 9). HR was also delayed in the 32hour dark pre-treatment plants compared to plants that were placed in the dark following the dex effector induction (dark). Dark treated plants however did display delayed HR compared to the light treated plants, but to a lesser extent than dark32 treated plants. At 4

hours post induction slight HR (wilting of the leaves) was seen in the light treated plants but no HR was see in the dark and dark 32 treated plants. Therefore a delay in HR was already noticeable at 4 hpi, with a difference seen between light plants and both the dark and dark 32 treated plants. By 6 hours post induction there was a fairly high level of HR (severe dehydration, browning, and wilting) on approximately 40% or more of each leaf's surface area in light treated plants. Dark treated plants had approximately 5% of each leaf's surface area showing signs of HR, a significantly lower amount than the light plants. The dark32 treated plants still did not show any visible signs of HR compared to the other treated plants. At the time point 10 hours post induction HR symptoms affected 90-100% of each leaf's surface area in the light treated plants. In dark treated plants 10hpi some leaves showed as severe HR as the light plants at this time point, but some leaves still had less surface area HR symptoms per leaf and leaves were not dehydrated to the extent of the light treated plants. Dark32 treated plants had on average 20% of a leaf's surface area effected by HR, meaning HR symptoms are beginning to appear at 10 hpi where as HR symptoms appeared at 6 hpi in the light and dark treated plants. In summary, by 10 hpi dark treated plants show almost the same extent of HR as light plants, but dark32 treated plants still show a significantly reduced level of HR symptoms. At 24 hours post induction light, dark, and dark32 treated plants show on average equal amounts of HR symptoms per leaf. This indicates that although HR was delayed in the dark treated plants by around 4 hours, and in the dark32 treated plants by around 14 hours, HR still occurs in our avrRpm1 effector inducible plant system regardless of light and thus functioning photosynthesis.

It has been shown that high temperatures can inhibit net photosynthesis [65], and since we see a delay in HR due to shutting down photosynthesis (light deprivation), we hypothesize that we might see this same delay at some temperatures. Also it has been shown that in tobacco the R-protein N does not accumulate at 30°C, but it does accumulate at 20°C [66]. If there is no accumulation of an R-gene in Arabidopsis at certain temperatures, then we would not expect to see HR with the respective effector accumulation. Since we looked at the effects of light and thus photosynthesis on our inducible effector plant system we decided to investigate the effects that different temperatures had during an R-gene mediated defense response on our inducible plant system. Again we are working with Arabidopsis Colombia with dex inducible avirulence effectors in the presence of the respective R-gene. We decided to test the effects of temperature on three different effector induced R-gene mediated defense responses: avrRpm1 in the presence of the transgene RPM1 (native promoter, myc tagged), avrRpt2 in the presence of the endogenous RPS2, and avrRps4 in the presence of the endogenous RPS4.

Experiments were done with our three chosen plant lines, containing the dex inducible effectors avrRpm1, avrRpt2, and avrRps4, wherein plants were transferred to one of six different growth chambers, each at a different temperature. The temperatures and relative humidity (RH) conditions for the experiment were chosen based on growth chamber availability. The chambers had the following varying levels of temperatures and RH: 17°C with 60%RH, 22°C with 75%RH, 24°C with 25%RH, 25°C with 60%RH, 30°C with 15%RH, and 33°C with 30%RH. Plants were placed in the chosen growth conditions three days prior to dex spray effector induction in order to allow the plants to

adjust to the growth conditions before infection. Pictures were taken at 10 and 24 hours post effector induction in order to compare the levels of HR at different temperatures, and to look for a temperature dependent phenotype in our effector R-gene signaling system.

In the plant line containing dex::avrRpm1 in the presence of the R-gene RPM1 HR was seen at the temperatures of 22°C, 24°C, 25°C, 30°C, and 33°C by 10 hours post effector induction, with the most severe levels of HR symptoms seen at 33°C (figure 10). In these plants, at the previously listed temperatures, HR was more severe with HR symptoms covering most of the leaves surface area by 24hours post effector induction. At the temperature of 17°C at 10 hours post effector induction there were no significant HR symptoms present on the leaves of dex:avrRpm1 plants. By 24 hours post effector induction at 17°C a small degree of HR (slight dehydration on some leaves) was seen on the dex::avrRpm1 plants, suggesting that at 17°C HR is severely delayed and primarily inhibited at this temperature.

In the plant line containing dex::avrRps2 in the presence of the R-gene RPS2 at 10 hours post effector induction no HR was seen in plants at 17°C, and limited HR at 25°C. Slight to moderate HR symptoms were seen at the temperatures of 22°C, 24°C, and 30°C, with severe HR present in plants at 33°C (figure 10). By 24 hours post effector induction some HR symptoms were visibly in all plants with slight HR at 17°C, moderate HR at 22°C, 24°C, 25°C, and 30°C, and severe HR seen only at 33°C.

Results from the temperature test revealed that regardless of the temperature in which dex induction was done the plant line containing dex::avrRps4 in the presence of the R-gene RPS4 showed no HR (figure 10). This is likely not due to temperature, but rather there is probably a problem with the plant line having lost the effector transgene

construct. We do see that the dex::avrRps4 plants seem to be unhappy at 33°C showing some wilting of leaves, but this is also the case for the other plant lines in addition to HR symptoms. This suggests that Arabidopsis are highly stressed at 33°C and get damaged aspecifically. Relative humidity (RH) varied between the different growth conditions but there did not seem to be any major effect on HR. The delayed HR at 17°C is probably not caused by RH since in the 17°C growth chamber has a 60% RH, a RH within the "normal" range. Additionally plants that were grown at higher temperatures than 17°C (22°C and 25°C), with similar levels of RH as the 17°C growth chamber (75% and 60% RH respectively) displayed normal HR symptoms.
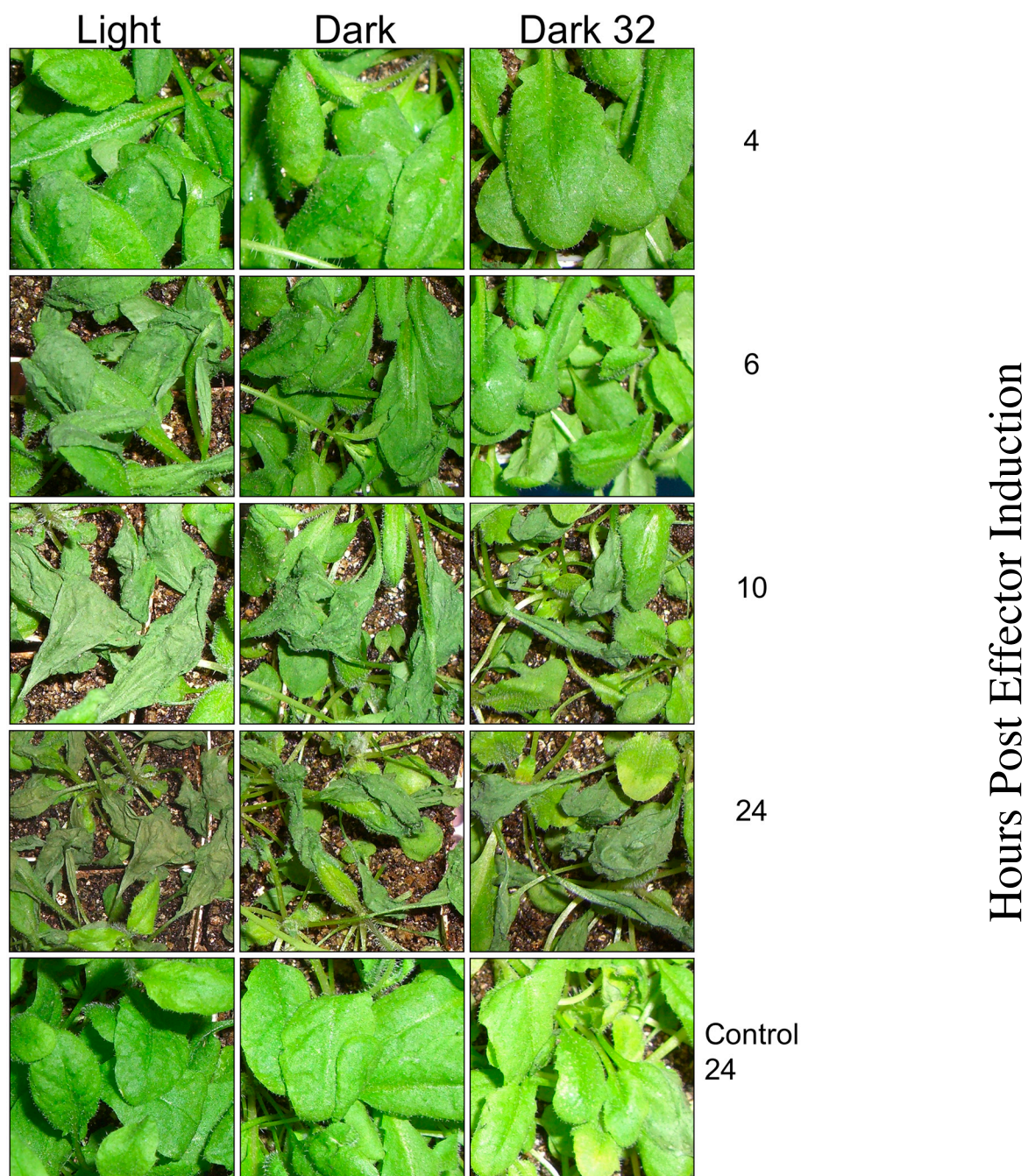
Figure 9: Effects of light deprivation on HR in avrRpm1 triggered defense
Arabidopsis plants with dex::avrRpm1 (*rpm1-3*, RPM1-myc) were sprayed with dex causing the transcription of the avirulence gene avrRpm1 and thus initiating an effector induced R-gene defense response. Plants were placed either in the light or dark (some 32hours prior to induction) during the R-gene defense response. A series of pictures were taken over the course of the defense response at 4, 6, 10, and 24 hours post induction and HR between the conditions was compared. Representative leaves/plants from 5 replicates are shown.
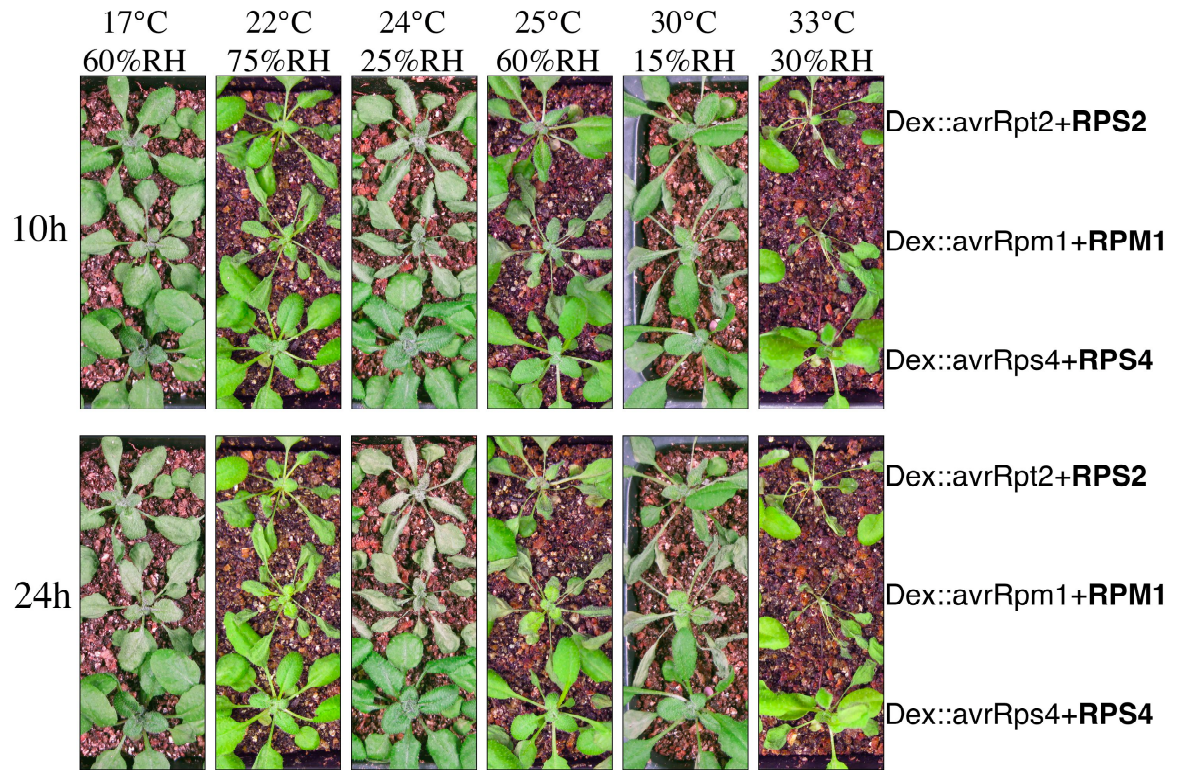
Figure 10: **Temperature effects on HR in effector triggered defense**
Arabidopsis plants with either dex::avrRpm1, dex::avrRpt2, or dex::avrRps4 were placed into growth chambers of different temperatures and then observed 10 and 24hours post effector induction by dex spray. Representative plants from 5-6 replicates are shown.

Discussion:

In the experiments exploring the effects of photosynthesis on our Arabidopsis plant line containing the inducible effector avrRpm1, it is possible to make conclusions on the RPM1 mediated defense response. HR was delayed by 14 hours if the plants were placed in the dark 32 hours prior to avrRpm1 induction and remained in the dark after induction. HR was delayed by only 4 hours in plants that were switched into the dark after avrRpm1 induction. From these observations we can conclude that our Arabidopsis Colombia system works in a similar way as previously reported in tobacco and the Arabidopsis ecotype Dijon system, with a partial light dependence on the HR resulting in a delayed defense response in the absence of light. A possible reason that the HR is delayed but not absent is because although the chloroplast, involved in photosynthesis, are responsible for the production of some ROS like H_2O_2, many other ROS are generated from different sources, such as mitochondria, which also play a role in HR [57]. The ROS from other sources than chloroplast appear to be sufficient enough to supplement the role of H_2O_2 when they accumulate to high enough levels. It has been suggested that ROS only act to facilitate and speed up the HR [57], with defense related genes being the true cause of the HR. Therefore defense induced genes may be sufficient over longer periods of time in the absence of ROS to cause full HR symptoms. The production of ROS by photosynthesis is only one hypothesis as many other photosynthetic products, such as ATP, NADPH, and the resulting electron gradient may also be important to the plants defense response [67]. Also it should be mentioned that the photosynthesis experiments were also preformed with an additional test of placing plants in dim/partial light to see if there was any delay in HR. There was no significant difference in the HR levels between

plants in partial light compared to plants in full light meaning that as long as the photosystems are working, regardless of full light damage, HR is not delayed.

For further experiments regarding photosynthesis it would be interesting to see if a dark pretreatment of 32 hours or greater to the plants with a subsequent switch to the light following effector induction would cause a delay in HR. This would help in understanding whether there are products from photosynthesis that require a certain level of accumulation overtime for a successful rapid HR, or if the plants are able to adjust quickly to light presence, giving the same level of HR on the same time scale as plants that were not dark pretreated. Additionally Photosynthesis-generated products required for HR, such as ROS and ATP, may stay present for a long period of time in the plant cells only slowly declining after hours or possibly even days without light exposure. It would be interesting to test if it is possible to inhibit HR completely by having a longer dark treatment (greater than 32hours), or by using a chemical photosynthesis inhibitor, thus eradicating all photosynthesis-generated products before effector induction.

In the experiments regarding the effects of different temperatures on the R-gene mediated hypersensitive response, with regards to dex inducible effectors, we find that at 17°C HR is delayed in avrRpm1 and avrRpt2 R-gene mediated responses. Delayed HR is also seems to be present in avrRpt2 plants at 25°C, but this is not as evident as the 17°C delay. We do not understand what is going on with the dex::avrRpt2 plants at 25°C and 60%RH as we would not expect two peaks of disfunctionality, it could merely be a coincidence or the plants in that line of the pot may not have received a sufficient amount of dex spray. Additionally we conclude that there must be a problem with the plant line containing dex::avrRps4 since no HR was seen at any temperature, not even at normal

growth conditions of 22°C with 75%RH. It is reasonable to hypothesis that since the effector avrRps4 is a transgene, it is possible that during segregation the construct became nonfunctional either by truncation, silencing, or total loss of the transgene. For this reason we ignore the results from the plant line dex::avrRps4 in the presence of the endogenous RPS4.

A draw back to conclusions in the temperature experiments is that the humidity within the growth chambers of different temperatures was not held constant. It has been shown that in the tomato-fungal interaction system humidity does effect HR in R-gene recognition defense with high levels of humidity (around 95%RH) causing delay and in some cases repression of HR in addition to defense gene repression [68]. Since we have varying temperatures and humidity simultaneously differences in HR seen it is difficult to attribute changes just to temperature, while ignoring humidity. In our experiments the most significant delay in HR was seen at 17°C in both avrRpm1 and avrRpt2 plants, but the humidity at this temperature was normal for Arabidopsis growth, 60%RH. From this we conclude that the delayed HR we saw was not due to the 60% RH since that is normal conditions and it is unlikely to affect HR. Also we did not use any higher humidity chambers but we did have chambers of lower humidity that could have accelerated and worsened HR. If the chambers were at a higher humidity then we could speculate that it could be causing the delayed HR seen, as it could by hypothesized that there is slower evaporation at higher humidity levels. Since the humidity is at a normal level we can confidently attribute the delayed HR at 17°C to the temperature at which the plants were treated. In conclusion at the temperature of 17°C we can hypothesize that there is a gene

that is not transcribed, or possibly a protein that is mis-folded and is important in the R-gene defense signaling.

It is an interesting finding that we see delayed HR at 17°C as most cases of HR delays and suppression findings caused by temperature have been associated with higher temperatures (29-30°C) rather than lower (17°C). It was found that in tobacco plants, where both effector and effector recognition protein were expressed, plants did not die at higher temperatures due to a lack of the effector receptors (HABS) [61]. Another study found that the protein HSP90, which is involved in the disposal of mis-folded R-genes, was non functional at higher temperatures causing a suppression of R-gene defense [62,63]. Since protein folding is related to temperature and more mis-folding occurs at higher temperatures the action of genes regulating and disposing of incorrect folding becomes more essential as temperature increases. When proteins that remove mis-folded R-genes become nonfunctional literature suggests that a successful defense response is inhibited. Knowing that this protein monitoring and detecting mechanism exists we can speculate that there are other chaperone proteins or receptors like HSP90 and HABS that are involved in defense that possibly become nonfunctional at different temperatures. One hypothesis is that a low temperature of 17°C could render such a protein nonfunctional rather than at high temperature causing a backup of incorrectly folded or damaged proteins involved in defense, thus inhibiting a successful defense response. In another study it was shown that a wheat resistance gene Yr36 in non functional at 15°C but gains functionality at 25°C-33°C causing resistance to a fungal rust [69]. This grain resistance protein is not structurally the same as Arabidopsis R-proteins (CC/TIR-NB-LRR) but demonstrates that lower temperatures could possibly affect resistance proteins.

At 17°C the R-proteins RPM1 and RPS2 may not function correctly or may have limited effector recognition thus causing delayed HR. Another possibility is that at lower temperatures the dex effector induction is not as robust as it is at higher temperatures, so at 17°C there is a lower and slower accumulation of avrRpm1 or avrRps2 thus resulting in delayed HR. To test this hypothesis westerns would have to be run to check avirulence gene accumulation levels at the different temperatures over time. Another Hypothesis is that at low temperatures, such as 17°C, water evaporation is slower so leaves dehydrate slower resulting in a delay in HR. Another test to give the results of 17°C more significance would be to relate the finding to a more relevant system, like wildtype plants. Wildtype plants would be grown at 17°C and then a bacterial infection done to see if at 17°C all HR is blocked. Another thing we propose to do is grow constitutively active RPM1 plants at 17°C to see if there is a problem with the RIN4 pre-activation complex possibly being frozen in place and not triggering HR. Another hypothesis is that there is a down stream block of the RPM1 defense signaling pathway at 17°C.

A finding contrary to our results is that it was found that in an infection of Arabidopsis with DC3000 containing the effectors, avrRpm1, avrRps2, and avrRps4, HR was delayed at 28°C compared to infection at 22°C [64]. We do not clearly see this same HR delay at higher temperatures that this study claims. This may be because we used a different measure of HR and did not score the % of leaf covered by HR symptoms. Also HR was not compared between temperatures but merely observed at each temperature to see that HR had occurred at the temperature the plants were treated at. Also we are using a dex induction of a single effector to trigger R-gene mediated defense instead of a collection of effectors. This is a different system than was used in the aforementioned

study and can possibly account for the descrepanct. It is quite possible that there was some sort of slight HR delay at higher temperatures that we did not catch due to the fact that we did not score HR for each individual leaf and look for the extent of HR between the different temperatures.

In our experiments we found that the Arabidopsis R-genes RPM1 And RPS2 are functional at 30°C -33°C in contrast to the tobacco R-genes N which does not function at these temperatures [66]. Both Arabidopsis R-genes and the tobacco R-gene N have the conserved NB-LRR motif yet we show effector recognition functionality by RPM1 and RPS2 at high temperatures, where as it is lost with the R-gene N. Additionally seeing HR at high temperatures in RPM1 and RPS2 plants means that there is no significant decrease in elicitor binding sites as is seen at high temperatures in the Cf-mediated defense of tomatoes [61]. Therefore we conclude that Arabidopsis R-genes RPM1 and RPS2 function differently than other R-genes studied in tobacco and tomato in relation to temperature dependence. Temperature dependence in Arabidopsis R-genes has not been previously studied.

To have a better understanding of the roles of temperature on R-gene mediated defense in our inducible effector plant systems we would want to perform experiments at a consistent humidity and only vary the temperature. Also we would want to use more controls to check if temperatures above or below the normal range are permissible for extended growth of untreated Arabidopsis plants.

Materials and Methods Chapter 2:

Plant Material and growth conditions:

Arabidopsis thaliana ecotype Col-0 with mutant rpm1-3, RPM1-myc (native promoter), and dexamethasone (dex) inducible avrRpm1 were used in the HR photosynthesis experiments. Plants were grown in soil with 5 plants per 2x2in small pot for four weeks. The pots were placed in a conviron growth chamber with a light cycle of 16h light 8h dark and held at the constant conditions of 25°C and 60% humidity.

In the HR temperature experiments, three plant lines were used: the above mentioned RPM1 dex::avrRpm1, a line containing RPS4 dex::avrRps4 and a line containing RPS2 dex::avrRpt2. Plants were grown in soil within 3x5in pots containing 5 plants from each line (15 plants total) for three and a half weeks. Plants were grown in the Percival growth chamber having a light cycle of 11h light at 22°C and 13h dark at 20°C, the humidity was uncontrolled but stayed around 75%.

Plant Treatments: Photosynthesis effects on HR:

Plants that were grown in the described growth conditions were divided into three treatment groups: no light, dim light, and full light. Plants were then either placed under a non-transparent box (cardboard=dark), a semi transparent box (cardboard sides/paper top = dim), or left uncovered (light). For the dark treatments plants were covered 16 or 32 hours before the planned avirulence gene induction or switched into the dark right after induction. Dim treated plants were covered 16 hours before induction. The avirulence gene avrRpm1 was induced by spraying plants with a 50μM dexamethasone, and 0.05% silwet solution. Control plants were also maintained in each condition and sprayed with a 0.05% silwet solution.

Plant Treatments: Temperature effects on HR:

Plants were grown in the described growth conditions and then divided into six temperature treatment groups: 17°C, 22°C, 24°C, 25°C, 30°C, and 33°C. Plants were conditioned before their dex treatment by transfer to desired temperature environment three days prior to treatment. The six different environments chosen for the temperature tests: 17°C with 60% relative humidity (RH), 22°C with 75% RH, 24°C with 25% RH, 25°C with 60% RH, 30°C with 15% RH, and 33°C with 30% RH. After spending three days in the appropriate growth conditions plants were sprayed with a 50μM dexamethasone, and 0.05% silwet solution, or with a 0.05% silwet solution as a control.

Chapter 3: The NDR1 Interactome

Introduction:

The protein NDR1 (non-race-specific disease resistance 1), a plasma membrane glycophosphatidyl-inositol (GPI)-anchored protein [70], is essential for plant resistance to Pseudomonas syringea (Pto). NDR1 is essential for R-protein mediated resistance but the role of NDR1 in basal defense is unclear. [17-20]. Arabidopsis *ndr-1* plants are impaired in resistance against Pseudomonas syringea (Pto) containing avrRps2 and avrRpm1/avrB. However upon infection of *ndr1-1* with Pto avrRpm1/avrB, HR still occurs while no HR occurs with Pto avrRps2 [17]. It is interesting that the role of NDR1 is different in RPS2 and RPM1 mediated response with NDR1 playing a broader role in the RPS2 mediated defense response. Additionally NDR1 is known to have an association with the membrane-localized protein RIN4 (RPM1-interacting protein 4). RIN4 also interacts with the R-proteins RPM1 (resistance to P. Syringae PV Maculicola1) and RPS2 (resistance to P. Syringae2). RPM1 and RPS2 guard RIN4 and respond to the actions of the effectors avrRpm1/avrB and avrRps2 respectively (phosphorylation and cleavage of RIN4) [20,71,72]. Aside from the RIN4-NDR1 interaction, to date no other direct protein-protein interactions with NDR1 have been described. Proteins rarely work alone within a cell but potentially form many interactions with other proteins to carry out cellular functions [73]. NDR1 interactions with other proteins might also be essential for NDR1 function and successful resistance. To decipher protein-protein interactions many techniques have been used but the current most sensitive and accurate way to discover a protein interactome is to perform co-immunoprecipitations followed by mass-spectrometry identification of the interacting proteins [74].

In this chapter co-immunoprecipitation (co-IP or pulldown) experiments with NDR1, before and during infection with Pto containing avrRps2, causing R-gene (RPS2) mediated defense response were performed to describe the NDR1 interactome. The role of NDR1 in defense is not clear and little is known about the proteins that NDR1 interacts with during R-gene mediated defense. Our experiments and can help in understanding the function of NDR1.

Results:

To study the interactions of NDR1 during R-gene mediated defense we chose to do pulldowns of the NDR1 protein. In order to do this we created a plant line that contained a tagged NDR1 protein. NDR1 was introduced into the pCHF3 vector which contains a 35S constitutively expressed promoter and that already contained two Strep and two HA tags. The HA-HA-Strep-Strep tagged NDR1 protein under the control of a 35S constitutively active promoter was then introduced into Arabidopsis Colombia *ndr1-1* plants via agrobacteria containing the constructed pCHF3 vector infection via the floral dip method [75]. Many of the plants were successfully transformed and NDR1 expression levels were checked using western blot analysis (figure 11). Three different plant lines with different levels of NDR1 expression were then chosen to complete experiments on. Multiple lines were chosen since we were unsure if using a 35S promoter that does not mimic natural transcription levels and causes elevated levels of NDR1 in a cell might cause a growth defect, or other problem within the plants. Additionally it is necessary to have independent plant lines to insure that phenotype effects are not due to the T-DNA integration in the plants genome, which might cause gene disruptions. We chose to work with the plant lines 10 (a high NDR1 expresser), line 12 (a low NDR1 expresser) and line

18 (a medium NDR1 expresser). A pool of T3 generation plants were germinated on kanamycin plates, and surviving homozygous and heterozygous NDR1 expressers were transferred to soil and grown for use in our experiments. Each line grew to normal size and appeared healthy so the different lines were kept separate during the experiments to serve as biological replicates. Arabidopsis Colombia *ndr1-1* plants are known to have a reduced defense phenotype during infection with bacteria containing avrRpt2 resulting in no HR symptoms, limited ROS production, and decreased resistance [17,19]. If the transgene 35S:NDR1 is functioning normally in our transformed *ndr1-1* lines then it should compensate for the lack of defense in *ndr1-1* plants. It is essential that our 35S:NDR1 plant lines display a normal defense response (similar to wildtype levels), as we want to discover NDR1 interacting proteins during a normal R-gene defense response.

In order to test that our transformed 35S:NDR1 plants were able to rescue the defense phenotype back to wildtype levels we preformed HR and bacterial titer assays on each of the three chosen over expression lines. In the HR assay 35S:NDR1 insertion lines, wildtype, and *ndr1-1* plants were injected with Pto. containing avrRpt2 at an OD of 0.02. Wildtype and *ndr1-1* plants were the controls used to compare our insertion lines to. HR was then scored for each leaf injected as a measure of the surface area of the leaf showing symptoms of HR at 24 hours post inoculation (hpi) (figure 12). After 24 hours no HR was seen on any of the *ndr1-1* plant leaves where as all wildtype, and 35S:NDR1 plant leaves showed some level of HR. It was found that all three of our 35S:NDR1 insertion lines rescued *ndr1-1* plants HR defense phenotype back to wildtype HR defense levels, and in fact all three NDR1 over expression lines increased the amount of HR symptoms seen per leaf compared to wildtype (figure 12a). A higher percentage of

leaves showed 70-100% HR in the 35S:NDR1 lines than wildtype and no leaves were in the lowest category of 0-20%HR for the 35S:NDR1 plant lines.

Bacterial growth assays were done on the three chosen 35S:NDR1 plant lines, wildtype, and *ndr1-1* plants by inoculating the plants with Pto. containing avrRpt2 bacteria at an OD of 0.00005. Again the wildtype and *ndr1-1* plants act as our controls to ensure the NDR1 insertion lines bacterial growth is restricted to a normal level. Bacterial growth was measured at 0, 2, and 4 days post inoculation (dpi) by calculating the number of bacterial colony forming units (cfu) per cm^2 of leaf for each plant line. Bacterial titer of *ndr1-1* control plants was 20 fold higher than in wildtype plants (figure 13). The 35S:NDR1 transgenic lines (in *ndr1-1* background) were rescued back to normal bacterial growth levels and had on average a 2-fold lower bacteria titer than wildtype plants. This shows that resistance is restored by our NDR1 transgene in *ndr1-1* background plant lines back to wildtype levels. Therefore, as with our HR assays, over-expressed NDR1-Strep-HA restores the wildtype defense phenotype in *ndr1-1* plants and even causes a slightly heightened defense response (more HR, less bacterial growth than wildtype). We concluded that the introduced NDR1-Strep-HA protein is functional in defense and thus we proceeded with the planned NDR1 pulldown experiments.

Pulldowns were performed on samples from 0 hpi and a pool of 2/3 hpi for wildtype control plants, line 10 plants, line 12 plants, and line 18 plants. Pulldowns were attempted with both anti-HA beads and strep-tactin beads, but NDR1 was only successfully pulled down using anti-HA beads as seen by our western blot analysis (figure 14a.). In our first trial pulldowns we sampled the pulldowns at many steps during the process including: the flow through of the pulldowns after overnight incubation with

the antibody beads (flowthrough), a sample of the antibody beads after overnight binding (Bound fraction), the 50mM NaCl wash solution post antibody-bead wash (wash 50), a sample of the beads after being washed in the 50mM NaCl solution (Bound Wash 50), the 150mM NaCl wash solution post antibody bead wash (wash 150), and a sample of the beads after being washed in the 150mM NaCl solution (Bound wash 150). Additionally controls sample were loaded that contained a sample of the total powder before protein extraction from the pooled NDR1 lines (total powder) to test for NDR1 presence, and also a sample of unused HA antibody beads to see if when denatured the detached bead antibody would give a band (HA beads). In the three lanes of bound fractions using anti-HA beads (bound fraction, bound wash 50, and bound wash 150) we see a very intense band around ~ 48 kDa, which is our tagged NDR1 protein (figure 14a.). This band is absent in the same three bound fractions using strep-tactin beads, meaning NDR1 was not pulled down using strep-tactin beads. Before running our final pulldown samples through the mass spectrometer (mass spec/ MS) to identify NDR1 interacting proteins a small fraction of each anti-HA pulled down sample was first checked for NDR1 presence via western blot analysis using an anti-HA antibody. The samples taken to check for NDR1 via western blot were: the total protein extract solution before antibody bead addition (total), the flow through of the pulldowns after overnight incubation with the antibody beads (flowthrough), and a sample of the antibody beads after a 50mM NaCl wash step (Resin). In all three 35S:NDR1 lines the NDR1 protein was detected in all final bound resin samples, in most of the total extraction samples, and in none of the flow through samples (figure 14 b.c.). This indicates that NDR1 was successfully pulled down by the anti-HA beads, and that we were able to bind the majority of the present NDR1 protein in

the sample. Also note that we did not get a band of this size in the wildtype samples which did not contain an HA tagged NDR1 protein, but we do see a faint band above the NDR1 band at approximately 55kDa that is most likely the large subunit of the HA antibody that gets dissociated from the anti-HA beads during protein denaturing, also seen as an extra band in the NDR1 pulldown samples.

After confirming NDR1 protein presence in our pulldown samples they were run through LC (liquid chromatography)-MS in order to detect NDR1 interacting proteins. A total of six wildtype control runs were done, three replicates of time point 0 and three replicates of time point 2/3. A total of twelve 35S:NDR1 runs were done with two replicates per plant line at each time point 0 and 2/3, meaning there were six replicates of each time point including two replicates from each of the three lines. We compared all tagged NDR1 sample runs to wildtype runs since many proteins probably aspecifically interact with the anti-HA beads and we are looking for proteins that are specifically interacting with NDR1. We were able to detect 286 interacting proteins unique to NDR1.

These proteins were analyzed by AmiGO, an online tool for sorting proteins into different ontology's that describe the proteins fundamental characteristics giving each protein a GO annotation [76]. Using AmiGO we were able to sort and analyze the NDR1 pulled down proteins to see if proteins of a certain class were over represented (2.5 fold or greater), or under represented (2 fold lower a value of 0.5) in our data (Table 2). There are three main AmiGO ontology organizing categories (amigo Goclass); cellular component (C), molecular function (F), and biological process (P) that are then further divided into subclasses (amigo description). We chose to only display a sub selection of all the categories generated from the NDR1 pulled down proteins. The classes displayed

are those that are over-or-under-represented, or otherwise of interest. From the GO annotations in the cellular component GO class we see that proteins associated with the membrane and plasma membrane are not over represented. This is important since NDR1 is a membrane associated protein and these results show we are not pulling down membrane pieces containing contaminating membrane proteins with our protocol. Also it shows that we are able to successfully extract NDR1 from the membrane. An over represented protein subclass of interest within the molecular function Go class is the lipid binding class. Other overrepresented protein subclasses of interest within the biological process Go class are lipid metabolic processes, photosynthesis, metabolite/energy precursors, cell death, and response to biotic stress proteins. Calcium dependent lipid binding/signaling domains have been associated with defense [77] insinuating that the lipid binding, and lipid metabolic processes proteins we find in our data may be important to defense. In Arabidopsis when a defense response is mounted it requires a shift in metabolism to supply more energy to the plant to fight off infection [78]. In seeing an overrepresentation of photosynthesis and metabolite/energy precursors, both of which are supplying energy to the plant, it suggests the plants were in fact mounting a defense response when protein was sampled. Additionally seeing an overrepresentation of genes related to biotic stimulus and cell death confirms that a defense response was in fact caused by our bacterial infection and the genes pulled down with NDR1 are likely related to an active defense response.

The subclass of proteins related to nucleotide binding was slightly overrepresented (1.8fold) but transcriptional regulation and transcription related proteins were under-represented. This suggests that many of our regulatory proteins within our

NDR1 data bind single nucleotides (like GTP and ATP) but are not transcription factors. Due to these findings it makes the transcription factors that were present within our data less likely to be artifacts since they are from an underrepresented protein class and generally very low abundant/expressed.

From the 286 NDR1 specific proteins we made a sub classification of the proteins by distinguishing proteins that were the most reproducibly pulled down (abundant), proteins that increased over the time course of infection (UP), and proteins that decreased over the infection time course (DOWN). There were 34 observed proteins that were classified as abundant (table 3), with NDR1 being the most abundant protein observed. A protein was considered abundant if it was present in at least three of the twelve NDR1 pulldown samples, making them the most reproducible and reliable interactors of NDR1. A classification of increase was then done with the 286 proteins by calculating the fold increase of the protein's intensity (number of spectra) in relation to NDR1 over the time course of infection. NDR1's intensity increased by 1.9 fold over the time course of infection (from time point 0 to time point 2/3), so all proteins were then corrected by this factor. If a protein's intensity increased by a factor greater than one and intensity at time point 0 was greater than zero, or if a proteins initial intensity was zero and increased by at least three spectra then the protein was considered to increase over the course of infection. By our classification 24 proteins increased in intensity (table 4) over the course of infection, of which 12 were also considered abundant. A classification of decrease was performed on the 286 proteins being defined by an intensity decrease of at least 2 spectra over the course of infection. By our classification 9 proteins decreased (table 5) over the

course of infection, of which 2 were also considered abundant. Proteins in each class are depicted in figure 15.

To compare our NDR1 interactome data to known and predicted or functional protein-protein interactions we used the on-line tool STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) [79]. The protein NDR1 was inputted into the database and the second level of interaction was chosen. The second level of interaction gives an interaction diagram of both NDR1 direct interactors and interactors of those direct NDR1 interactors (Figure 16). Interactors are proteins that are predicted or experimentally verified to have either a physical or functional interaction with the proteins of interest. CRK11 is a predicted NDR1intereactor and we see the protein CRK35 in our list of NDR1 specific proteins, which shares the most homology (~60%) (along with CRK22) to CRK11 out of more than 40 CRK family members (www.uniprot.org). Additionally CRK13, which also has ~50 % homology to CRK35 causes an enhanced resistance phenotype to Pseudomonas when over-expressed [80]. CRK11, CRK13 and CRK34 are part of a family of cysteine rich receptor like kinases known to be targeted by WRKY DNA-binding transcription factors (~100 family members [40]), known to be induced in the defense response [81]. Due to CRK11 and CRK13's known interactions in defense CRK35 is an interesting defense related protein in the NDR1 interactome. Another three predicted NDR1 interacting proteins were found within the total pulldown generated data from both wildtype and NDR1 runs: rps3 (ATCG00800), rps4 (ATCG00380), and EIN2 (AT5G03280), but they were present in both the wildtype control pulldowns as well as the NDR1 pulldowns. The proteins rps3 and rps4 are both chloroplast 30S ribosomal proteins S3 and S4 respectively while EIN2

(ethylene insensitive) is a metal ion trans-membrane transporter protein involved in ethylene signal transduction. These proteins were aspecifically pulled down and are not likely interacting with NDR1 as STRING suggests. In summary, the overlap of predicted interactions in STRING and our NDR1-pulldown data is very limited.

Next all 286 proteins were inputted into STRING level 0 (level 1 in multiple input setting) to see if among the NDR1 specific proteins clusters of physically or functionally interacting proteins existed (figure 17). From the search five clusters were identified but most of our 286 NDR specific proteins were not shown to interact with each other or NDR1. The clusters consisted of a group of proteasome proteins (cluster I.), a group of ribosomal proteins (cluster II.), a group of RNA binding and processing proteins (cluster III.), a group of photosynthesis related proteins (cluster IV.), and a group of proteins involved in sugar metabolism (cluster V). These five clusters do not have functions that have been associated with defense. In our NDR1 pulldown data we did not observe evident clusters of proteins or protein complexes that have been implicated in defense.
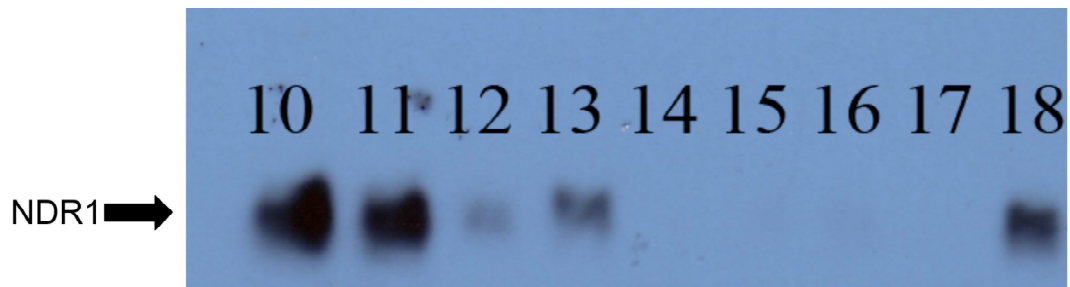
Figure 11: Expression level of NDR1
Level of NDR1 expression shown via anti HA western of total extracted protein from HA-HA-Step-Strep-NDR1 transformed Arabidopsis *ndr1-1* plants. High expressers and lines 10 and 11, medium expressers are lines 13 and 18, low expression line is line 12.

A)
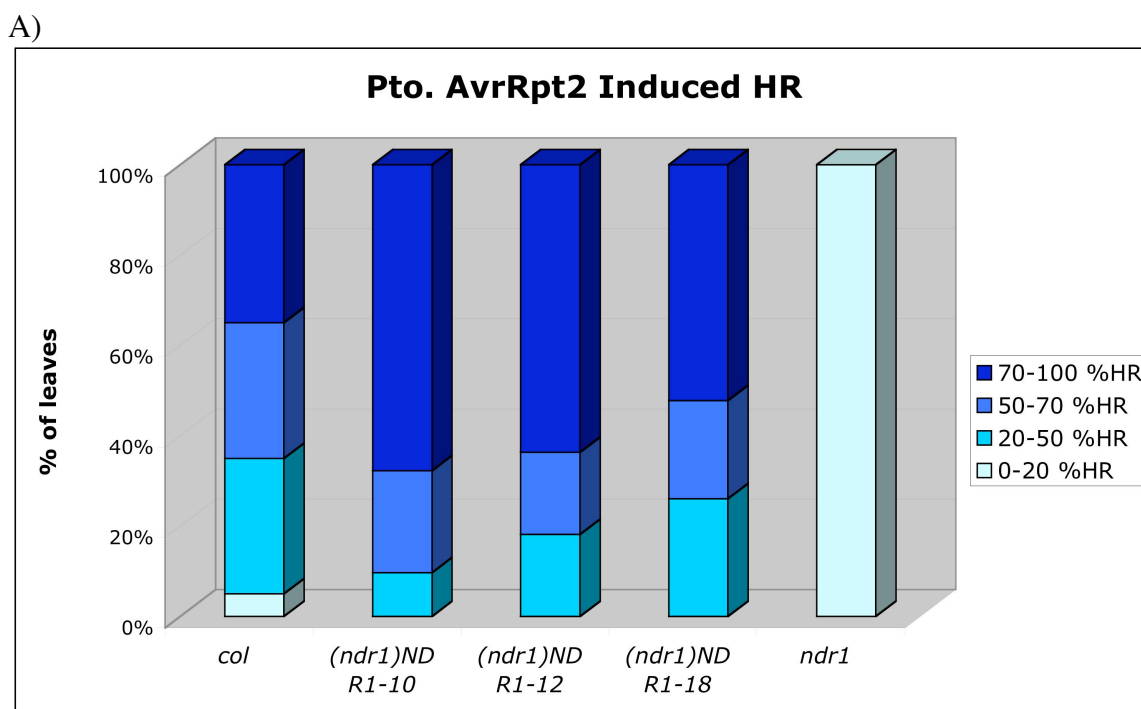
B)
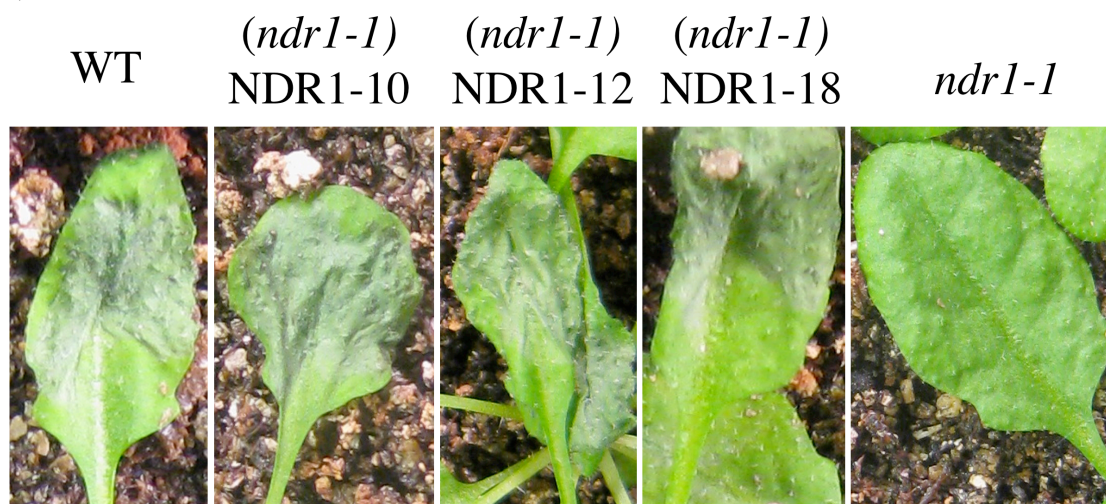
Figure 12: **35S:NDR1 plant lines rescue *ndr1-1* phenotype**
A) Plants were inoculated with Pto. avrRpt2 bacteria and then HR was scored as a percentage of the leaf's surface that showed HR. The graph represents the cumulative percentage of leaves in each HR category. B) A picture of a representative leaf from each plant line injected with Pto.DC3000:avrRpt2 bacteria. HR symptoms cover a percentage of each 35S:NDR1 over-expresser line to a similar degree as wildtype inoculated plants and *ndr1* plants do not show any HR symptoms.
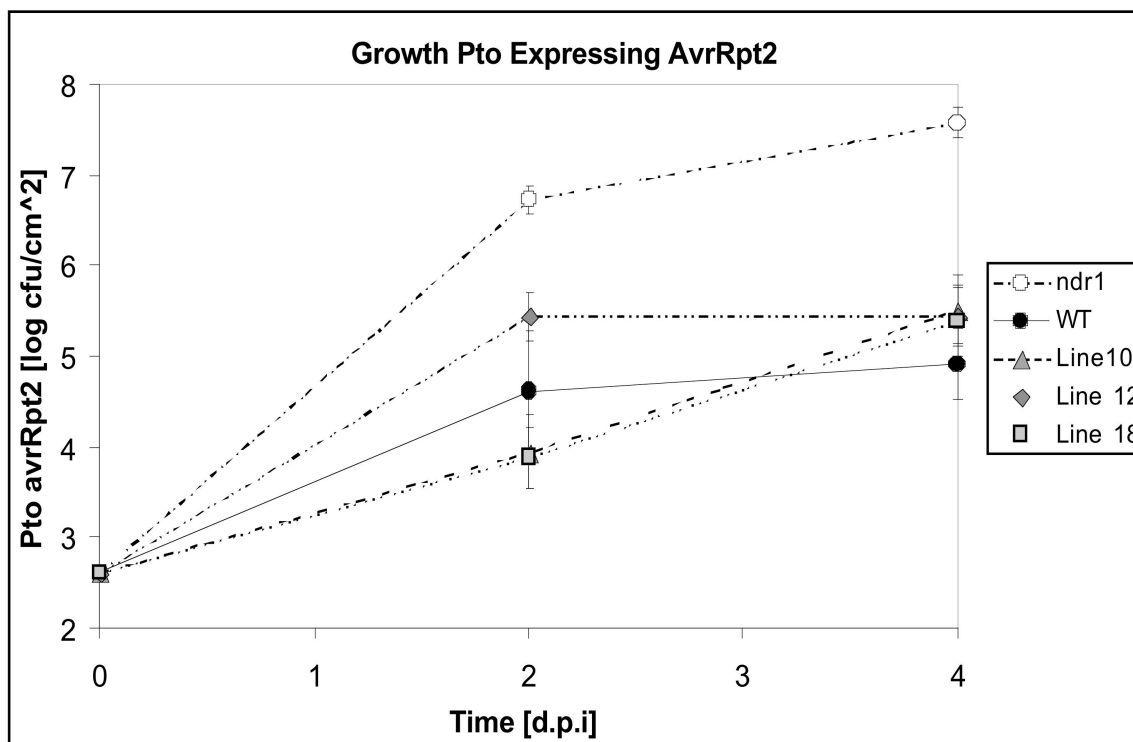
Figure 13: Growth of Pto expressing avrRpt2 in 35S:NDR1 plants
A bacterial growth assay was done with the three 35S:NDR1 plant lines as well as wildtype and *ndr1* plants. Pto. AvrRpt2 bacterial growth was then measured over a period of 4 days and the number of colony forming units per centimeter^2 (cfu/cm^2) of leaf was calculated. Numbers are an average of 8 replicates per sample except for day 0 where 3 replicates were used. Error bars represent standard deviations.
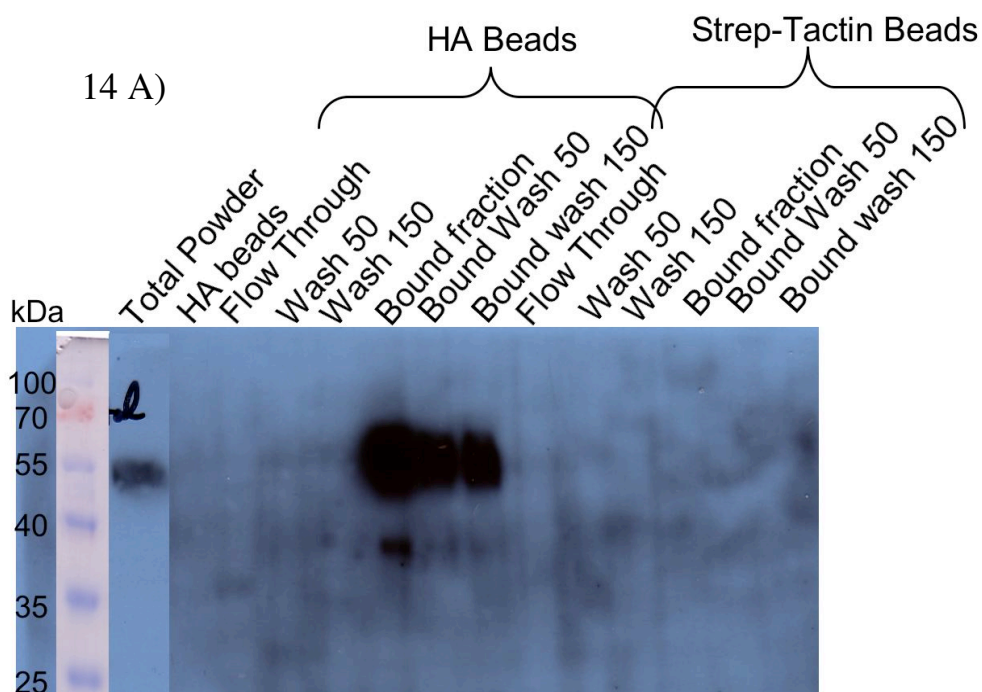
Figure 14: Detection of NDR1 in pulldown experiments
Anti-HA westerns of pulled down Strep-Strep-HA-HA tagged NDR1 A)Anti-HA and
strep-tactin beads (binds to the strep tag) were used to pulldown the tagged NDR1 protein
in a pooled sample of all three 35S:NDR1 lines. B)C) Pulldowns of tagged NDR1 using
anti HA beads in wildtype control, line 10, line12, and line 13 samples. Pulldowns were
done on the samples from the timepoint 0 hpi and a pool of timepoints 2, and 3hpi. Blots
show NDR1 protein from the total extract that beads were added to (total), the flow
through after bead binding (flowthrough), and the resin after binding and washing for all
samples run through mass spectrometer.

14 B)

14 C)
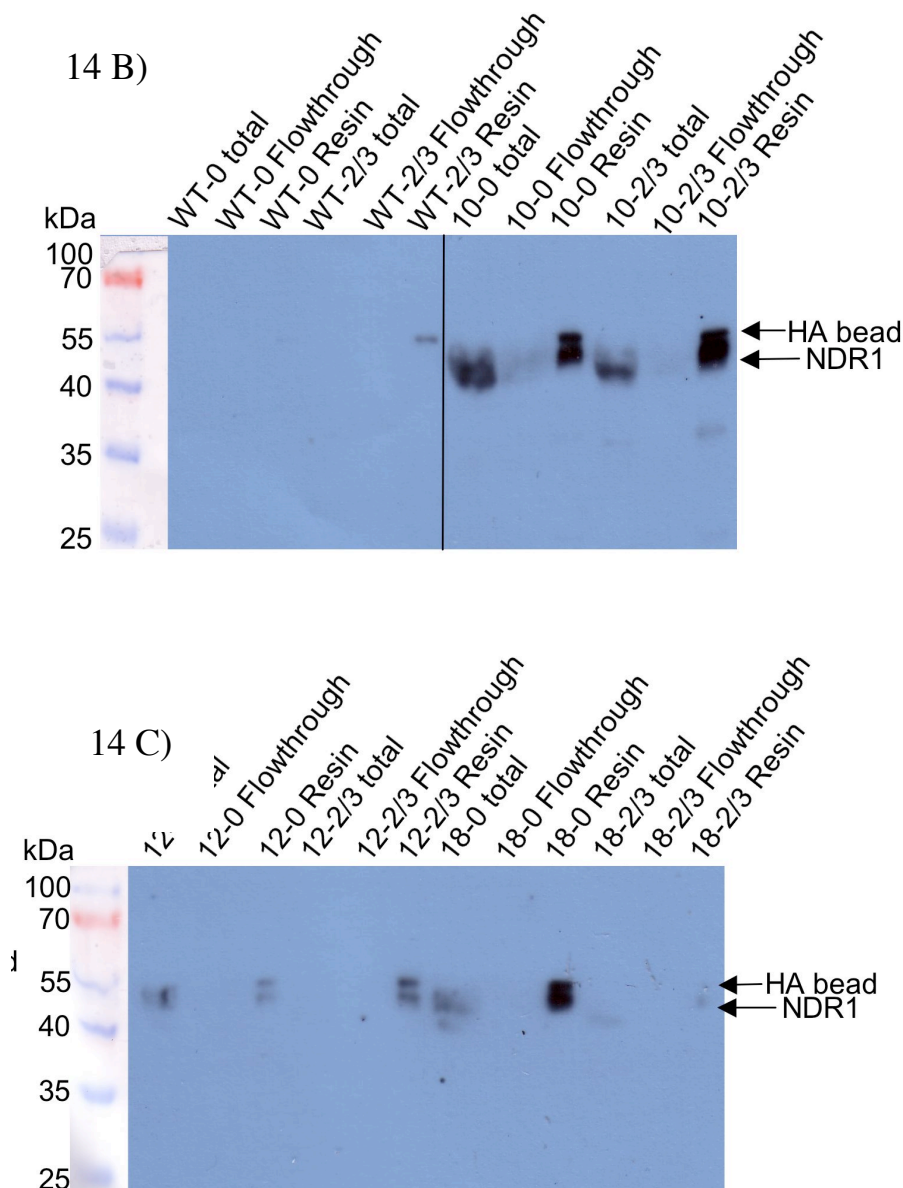
Figure 14 continued: Detection of NDR1 in pulldown experiments

Anti-HA westerns of pulled down Strep-Strep-HA-HA tagged NDR1 A)Anti-HA and strep-tactin beads (binds to the strep tag) were used to pulldown the tagged NDR1 protein in a pooled sample of all three 35S:NDR1 lines. B)C) Pulldowns of tagged NDR1 using anti HA beads in wildtype control, line 10, line12, and line 13 samples. Pulldowns were done on the samples from the timepoint 0 hpi and a pool of timepoints 2, and 3hpi. Blots show NDR1 protein from the total extract that beads were added to (total), the flow through after bead binding (flowthrough), and the resin after binding and washing for all samples run through mass spectrometer.

Table 2: NDR1 Specific Protein GO annotations
Proteins were sorted and analyzed using the web based tool AmiGO: online access to ontology and annotation Data. The proteins were first categorized by ontology organizing categories of cellular component (C), molecular function (F), and biological process (P) that were then further divided into subclasses called GO classes. A sub selection of the GO classes are shown in the table. The first column displays the ontology organizing categories (amigo GoClass), the second column is a description of the subclass within the larger amigo GO class (amigo description), the third column tell the number of proteins within that GO class out of total 32,348 Arabidopsis proteins (amigo all Goslim (32348)), the fourth column tells the number of proteins out of the 286 proteins in the NDR1 pulldowns that were present in that GO class (NDR1 pulldown (286)). NDR1 specific proteins were then analyzed based on if they were over represented or under represented based on each subclass in our data as compared to the entire genome as a control. The last two columns of the table represent the fold difference between the number of proteins expected and the number of proteins found in a certain GO class (fold) and a P-value for this fold difference is listed in the last column. P-vales are cut-off at 2 decimals, dark green highlighted p-values mean p<0.01, light green means 0.01< p<0.1. If values of a GO class are greater than 2.5 fold up-regulated they are highlighted in red and if a GO class is greater than 2 fold down regulated, a value of 0.5 they are highlighted in blue.

amigo Goclass	amigo description	amigo all Goslim (32348)	NDR1 pulldown (286)	fold	p
C	nuclear envelope	41	2	6.1	0.00
C	mitochondrion	998	22	2.8	0.00
C	thylakoid	779	14	2.2	0.00
C	plastid	3137	42	1.7	0.00
C	membrane	5178	45	1.1	0.55
C	plasma membrane	2713	14	0.6	0.08
F	motor activity	85	3	4.4	0.00
F	lipid binding	149	3	2.5	0.10
F	transporter activity	1133	19	2.1	0.00
F	nucleotide binding	846	12	1.8	0.04
F	transcription regulator activity	1815	3	0.2	0.00
P	pollination	64	3	5.9	0.00
P	photosynthesis	122	5	5.1	0.00
P	cell growth	220	8	4.5	0.00
P	cellular amino acid and derivative metabolic process	417	15	4.5	0.00
P	anatomical structure morphogenesis	415	14	4.2	0.00
P	lipid metabolic process	497	15	3.8	0.00
P	growth	269	8	3.7	0.00
P	post-embryonic development	519	15	3.6	0.00
P	cell death	109	3	3.4	0.02
P	generation of precursor metabolites and energy	183	5	3.4	0.00
P	cellular component organization	946	22	2.9	0.00
P	secondary metabolic process	351	8	2.8	0.00
P	carbohydrate metabolic process	450	10	2.8	0.00
P	response to external stimulus	283	6	2.6	0.01
P	response to biotic stimulus	597	12	2.5	0.00
P	transcription	1295	4	0.4	0.04
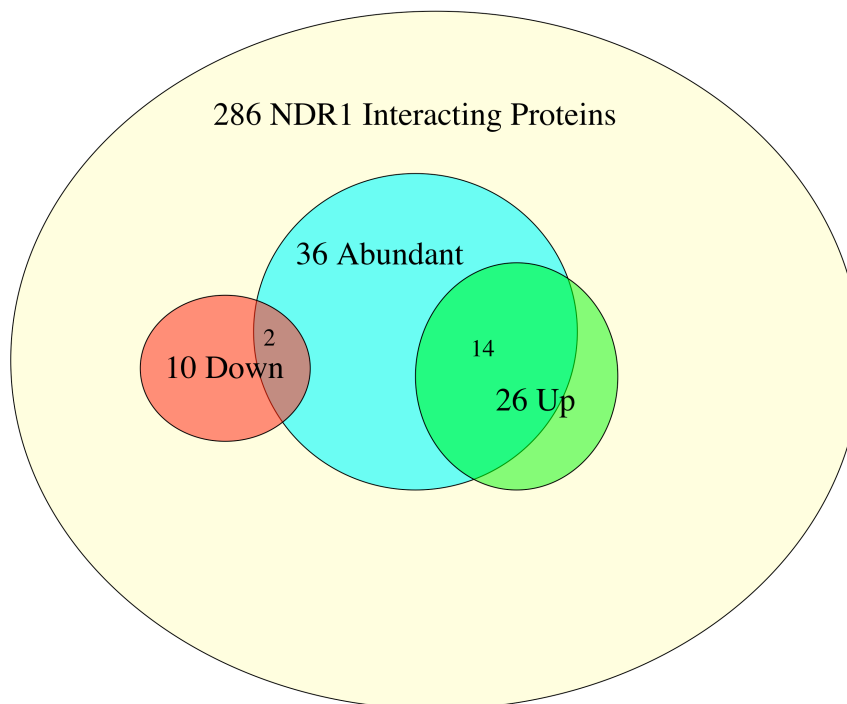
Figure 15: NDR1 interacting proteins schematic
286 unique NDR1 interacting proteins were identified via LC-MS and a sub-selection was made into three categories: Abundant (3 or more observed spectra), UP (increased by two or more spectra over infection course), and down (decreased by two or more spectra over infection course). Categories are depicted in a Venn diagram based on number and overlap between the categories.

Table 3: **Abundant NDR1 pulled down proteins**
Of the 286 NDR1 specific proteins we classified 34 of the observed proteins as abundant, with NDR1 being the most abundant protein observed. A protein was considered abundant if it was present in at least three of the twelve NDR1 pulldown samples (total # column is 3 or greater). The first column of the table gives the Arabidopsis IPI (international protein index) accession number for the identified protein (IPIaccession_number), while the second column gives the TAIR gene model name for the proteins (TAIR Model name). The third column gives the proteins name if it has one or a short description if the protein does not have an official name (entry-name). Column "# of T=0's found in" tells the number of time = zero hpi sample runs out of six total runs that the protein was present in, while column "intensity NDR1 0" gives the total number of spectra observed for a protein at time = zero hpi. Column "# of T=2/3's found in" tells the number of time = two/three hpi sample runs out of six total runs that the protein was present in, while column "intensity NDR1 t2t3" gives the total number of spectra observed for a protein at time = two/three hpi. The last two columns are sums of both the total number of samples in both sampled time points that the protein appears (total #), as well as the total number of spectra that were observed in both samples time points (total intensity).

IPI accession_number	TAIR Model name	entry-Name	#of T=0's found in	intensity NDR1 0	#of T=2/3's found in	intensity NDR1 t2t3	total #	total intensity
IPI00545993	AT3G20600	NDR1 NDR1 (NON RACE-SPECIFIC DISEASE RESISTANCE 1)	4	13	6	25	10	38
IPI00516684	AT4G15560	CLA1 Probable phosphate synthase, chloroplast precursor	3	6	3	4	6	10
IPI00520787	AT5G46580	Pentatricopeptide (PPR) repeat-containing protein	3	4	3	6	6	10
IPI00523612	AT4G34830	Binding;similar to EMB2745 (EMBRYO DEFECTIVE 2745), binding	3	3	3	4	6	7
IPI00518075	AT5G40490	RNA recognition motif (RRM)-containing protein	2	3	3	3	5	6
IPI00523546	AT5G11170	DEAD-box ATP-dependent RNA helicase 56	2	3	3	10	5	13
IPI00539947	AT3G61260	DNA-binding family protein/remorin family protein	2	3	3	9	5	12
IPI00544214	AT2G01250	60S ribosomal protein L7-2	2	3	3	5	5	8
IPI00516965	AT1G02740	Chromatin binding	2	3	2	2	4	5
IPI00518377	AT5G15090	Outer mitochondrial membrane protein porin 2	2	4	2	4	4	8
IPI00525150	AT3G48350	Cysteine proteinase	2	2	2	2	4	4
IPI00528496	AT3G02260	BIG BIG (DARK OVER-EXPRESSION OF CAB 1)	2	3	2	4	4	7
IPI00534266	AT4G16830	Nuclear RNA-binding protein (RGGA)	1	1	3	3	4	4
IPI00536785	AT4G32410	CESA1 Cellulose synthase A catalytic subunit 1	2	2	2	5	4	7
IPI00542810	AT1G77490	TAPX L-ascorbate peroxidase T, chloroplast precursor	1	1	3	3	4	4
IPI00786006	AT1G33680	Nucleic acid binding	2	2	2	7	4	9
IPI00520971	AT5G43060	Cysteine proteinase	1	1	2	2	3	3
IPI00521653	AT3G08010	ATAB2 ATAB2	1	1	2	3	3	4
IPI00522988	AT4G00630	KEA2 KEA2 (K+ efflux antiporter)	1	2	2	2	3	4
IPI00525537	AT5G53000	TAP46 PP2A regulatory subunit TAP46	1	1	2	2	3	3
IPI00526241	AT3G13060	ECT5 ECT5	0	0	3	3	3	3
IPI00528047	AT1G51690	ATB ALPHA Isoform 1 of Serine/threonine protein phosphatase	0	0	3	3	3	3
IPI00528780	AT5G67220	Nitrogen regulation family protein	1	1	2	2	3	3
IPI00534960	AT2G20020	CRS2-associated factor 1, chloroplast precursor	1	1	2	2	3	3
IPI00536168	AT3G08510	ATPLC2 ATPLC2 (PHOSPHOLIPASE C 2)	1	2	2	3	3	5
IPI00537308	AT2G24050	MIF4G domain-containing protein	2	2	1	2	3	4
IPI00537548		- Genomic DNA, chromosome 3, P1 clone:MFD22	1	1	2	2	3	3
IPI00538047	AT5G05780	ATHMOV34 26S proteasome non-ATPase regulatory subunit 7	1	1	2	4	3	5
IPI00541351	AT3G44620	Protein tyrosine phosphatase	1	1	2	2	3	3
IPI00544519	AT3G06400	CHR11 Putative chromatin remodelling complex ATPase chain	1	1	2	3	3	4
IPI00546719	AT2G41560	ACA4 Calcium-transporting ATPase 4, plasma membrane-type	0	0	3	4	3	4
IPI00546886	AT1G55670	PSAG Photosystem I reaction center subunit V, chloroplast precursor	1	2	2	3	3	5
IPI00547812	AT4G37800	Xyloglucan endotransglucosylase/ hydrolase protein 7 precursor	1	1	2	2	3	3
IPI00548857	AT1G08880	Probable histone H2AXa	2	4	1	2	3	6

Table 4: Proteins that increased over the time course pathogen infection

A classification of increase was then done with the 286 proteins by calculating the fold increase of the protein's intensity (number of spectra) in relation to NDR1 over the time course of infection. NDR1's intensity increased by 1.9 fold over the time course of infection (from time point 0 to time point 2/3), so all proteins were then corrected by this factor. If a protein's intensity increased by a factor greater than one and their intensity at time point 0 was greater than zero, or if a proteins initial intensity was zero and increased by three spectra then the protein was considered to increase over the course of infection. By our classification 24 proteins increased in intensity over the course of infection, of which 12 were also considered abundant. The first seven columns are the same as previously described in table 3. The eighth column gives the total intensity observed in both time =zero and time =two/three. The next column gives the changed intensity calculated be subtracting the intensity at time=zero from the intensity at time=two/three, followed by a column indicating if the protein was also previously classified as abundant. A fold change was calculated by dividing the intensity at time=two/three by the intensity at time=zero (fold change intensity) and since NDR1 increased by a fold of 1.92, all calculated fold increases were divided by 1.92 to give an NDR1 corrected fold increase. The last column gives the number of unique peptides detected in the MS runs that identified the protein.

IPIaccession_number	TAIR Model name	entry_name	#of T=0's found in	intensity NDR1 0	#of T=2/3's found in	intensity NDR1 t2t3	total intensity	changed intensity	abundant? (3# or more)	fold change intensity	NDR corrected	numPepsUnique
IPI00545993	AT3G20600	NDR1 NDR1 (NON RACE-SPECIFIC DISEASE RESISTANCE	4	13	6	25	38	12	YES	1.92	1	4
IPI00546705	AT1G51980	Probable mitochondrial-processing peptidase	0	0	2	8	8	8				3
IPI00545262	AT3G29360	UDP-glucose 6-dehydrogenase	0	0	2	6	6	6				4
IPI00521518	AT1G48620	HON5 (HIGH MOBILITY GROUP FAMILY A 5); DNA binding	0	0	2	5	5	5				3
IPI00532313	AT1G19870	IQD32 F6F9.8 protein, calmodulin binding	0	0	2	5	5	5				3
IPI00546719	AT2G41560	ACA4 Calcium-transporting ATPase 4, plasma membrane-type	0	0	3	4	4	4	YES			2
IPI00545671	AT2G02740	PTAC11 ATWHY3/PTAC11, DNA binding	0	0	2	4	4	4				3
IPI00531093	AT1G14380	IQD28 F14L17.15 protein, calmodulin binding	0	0	1	4	4	4				2
IPI00526241	AT3G13060	ECT5, protein binding	0	0	3	3	3	3	YES			1
IPI00528047	AT1G51690	Serine/threonine protein phosphatase regulatory subunit	0	0	3	3	3	3	YES			1
IPI00526568	AT1G80000	Hypothetical protein F19K16.3, similar to glycine-rich protein	0	0	2	3	3	3				1
IPI00534051	AT3G61050	NTMC2TYPE4; Lipid binding	0	0	2	3	3	3				2
IPI00546532	AT5G09510	40S ribosomal protein S15-4	0	0	2	3	3	3				2
IPI00547045	AT3G14210	ESM1 (EPITHIOSPECIFIER MODIFIER 1),	0	0	2	3	3	3				2
IPI00538047	AT5G05780	ATHMOV34 Probable 26S proteasome non-ATPase regulatory	1	1	2	4	5	3	YES	4	2.08	4
IPI00786006	AT1G33680	Nucleic acid binding	2	2	2	7	9	5	YES	3.5	1.82	3
IPI00523546	AT5G11170	AT5G11200 DEAD-box ATP-dependent RNA helicase 56	2	3	3	10	13	7	YES	3.33	1.73	5
IPI00539947	AT3G61260	DNA-binding family protein	2	3	3	9	12	6	YES	3	1.56	3
IPI00520158	AT4G30220	RUXF Probable small nuclear ribonucleoprotein F	1	2	1	6	8	4		3	1.56	1
IPI00534266	AT4G16830	Nuclear RNA-binding protein (RGGA)	1	1	3	3	4	2	YES	3	1.56	1
IPI00542810	AT1G77490	TAPX L-ascorbate peroxidase T, chloroplast precursor	1	1	3	3	4	2	YES	3	1.56	1
IPI00521653	AT3G08010	ATAB2; RNA binding	1	1	2	3	4	2	YES	3	1.56	3
IPI00544519	AT3G06400	CHR11 Putative chromatin remodelling complex ATPase chain	1	1	2	3	4	2	YES	3	1.56	1
IPI00543013	AT1G14410	ATWHY1/PTAC1 DNA binding / telomeric DNA binding	1	1	1	3	4	2		3	1.56	3
IPI00536785	AT4G32410	CESA1 Cellulose synthase A catalytic subunit 1	2	2	2	5	7	3	YES	2.5	1.3	2

Table 5: Proteins that decreased over the time course of pathogen infection
A classification of decrease was performed on the 286 proteins. A protein decrease was defined by an intensity decrease of at least 2 spectra over the course of infection. By our classification 9 proteins decreased over the course of infection, of which 2 were also considered abundant. The first seven columns are the same as described in table 3. The eighth column gives the change in number of samples that the protein was present in, the number of time=2/3 samples present in minus the number of time=zero samples the protein was present in (changed sample #). The next column gives the changed intensity of a protein over the time course of infection calculated by subtracting the intensity at time=zero from the intensity at time=two/three (changed intensity). The last two columns are classifying if the protein was characterized as abundant and the final column gives the number of unique peptides detected in the MS runs that identified the protein.

IPIaccession_number	TAIR Model name	entry_name	#of T=0's found in	intensity NDR1 0	#of T=2/3's found in	intensity NDR1 t2t3	changed sample #	changed intensity	Abundant	numPepsUnique
IPI00679306	AT3G59020	AT3G59020 Hypothetical protein F17J16_70	2	4	0	0	-2	-4		2
IPI00527183	AT1G22930	AT1G22930 T-complex protein 11	1	3	0	0	-1	-3		2
IPI00534716	AT3G19020	AT3G19020 Leucine-rich repeat family protein	1	3	0	0	-1	-3		2
IPI00516684	AT4G15560	CLA1;, chloroplast precursor	3	6	3	4	0	-2	YES	4
IPI00548857	AT1G08880	AT1G08880 Probable histone H2AXa	2	4	1	2	-1	-2	YES	2
IPI00521561	ATCG00350	PSAA Photosystem I P700 chlorophyll a apoprotein A1	1	4	1	2	0	-2		4
IPI00520428	AT1G50140	AT1G50140 AAA-type ATPase family protein	1	2	0	0	-1	-2		2
IPI00520520	AT2G38700	MVD1 MVD1 (mevalonate diphosphate decarboxylase 1)	1	2	0	0	-1	-2		1
IPI00524453	AT4G24580	Pleckstrin homology (PH) domain-containing protein-related	1	2	0	0	-1	-2		2

Figure 16: NDR1 interactors from the STRING database
Depiction of known and predicted protein-protein interactions with the protein NDR1
with STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) at level 3
which shows interactors of NDR1 and interactors of NDR1 interactors (STRING calls it
level 2 in single name input settings). Colored spheres represent proteins that directly
interact with NDR1 and grey spheres represent indirect protein interactors of NDR1.
Connection line color refers to what information the protein interaction connection is
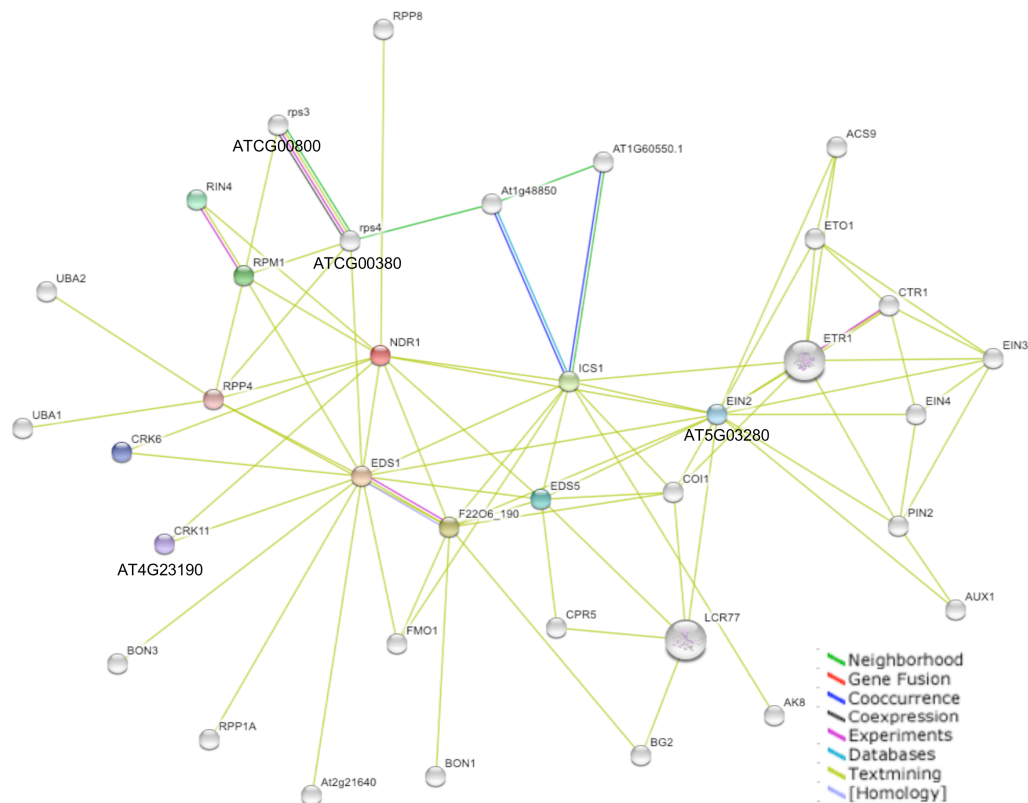based on, see ledgend.
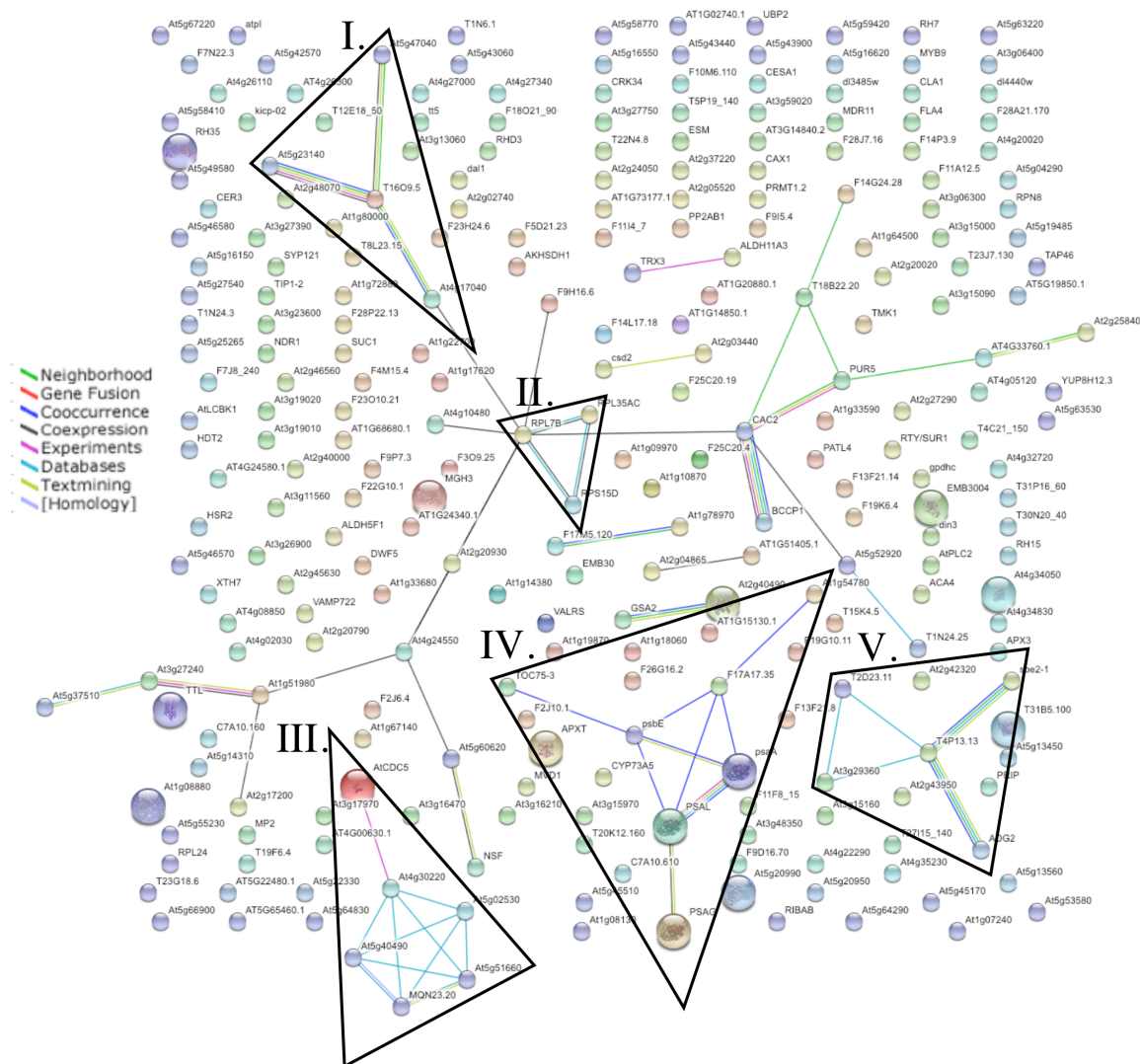
Figure 17: NDR1 specific protein interactions from the STRING database
Depiction of known and predicted protein-protein interactions with the NDR1 specific 286 proteins in STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) to level 0 interactions between inputted proteins only (STRING calls it Level 1 multiple names input). Each protein is depicted as a colored sphere and the type of interaction documented is depicted by different colored lines (see legend). Cluster I. represents a group of interacting proteasome proteins found in our data. Cluster II. represents a group of interacting ribosomal proteins within the data. Cluster III. shows a group of RNA binding and processing proteins. Cluster IV. shows a group of proteins related to photosynthesis and the chloroplast associating with each other. Cluster V. is a group of proteins from our data involved in sugar metabolism.

Discussion:

In our experiments validating the functionality of our created NDR1 over expression lines 10, 12, and 18 we see the *ndr1-1* (no HR or bacterial resistance to Pto. avrRpt2 [17]) defense phenotype rescued past that of wildtype levels. It has previously been reported that over expression of the NDR1 protein in Arabidopsis causes an enhanced bacterial disease resistance to bacteria carrying the effectors avrRpm1, avrRpt2, and avrpPhB [70]. Our over expression lines act like those previously reported proving defense response functionality of our engineered Arabidopsis NDR1 lines. It should be noted that since we are using NDR1 over expression lines there is more NDR1 protein present in the cells compared to wildtype levels. Due to a high level of NDR1 we may get proteins binding and interacting with NDR1 that are normally not interacting in defense. This may be because there are more available NDR1 binding sites and perhaps not enough endogenous normally interacting proteins. Therefore we have to be cautious with our results and we suggest further verification of NDR1 interacting proteins. In order to verify protein interactions with NDR1 reciprocal pulldowns could be done in either tobacco or E.coli. It is possible to do the reciprocal pulldowns in Arabidopsis as well, but it would take much longer to do than in the other two suggested systems. In reciprocal pulldowns tagged NDR1 and a differently tagged suspected protein interactor are both transiently expressed in tobacco using Agrobacterium and before or after Pseudomonas infection you perform a pulldown using one of the tags and then probe the western blot with an antibody for the other tag. In E.coli both the NDR1 tagged and differently tagged protein of interest are expressed in the bacteria followed by protein extraction and then interaction between the proteins is determined as described above. If the protein of

interest is in fact interacting with NDR1 then both NDR1 and the suspected interacting protein should appear on a western blot. Additionally we are currently testing if some of the suspected NDR1 interacting proteins are important to defense using T-DNA insertion mutants and performing bacterial titer and HR bioassays on the mutant plants.

In our experiments with the NDR1 over expression lines we choose to use Pto. containing avrRpt2 because in *ndr1-1* lines infection with Pto. avrRpt2 causes a total absence of HR as well as bacterial resistance [17], where as *ndr1-1* infected with bacteria containing avrRpm1, and avrB lose bacterial resistance but still induces/shows HR. Therefore with an infection using bacteria containing avrRpt2 NDR1 is functioning both in bacterial resistance as well as HR and we would expect more defense related interactors than with bacteria containing avrRpm1 or avrB. An additional note about our system is that in our pulldowns our HA:HA:Step:Strep:NDR1 protein has a predicted mass of ~32kDa but we see NDR1 migrating at around 45-50kDa when run on a denaturing SDS-PAGE. It has previously been reported that untagged NDR1 migrates at ~48kDa and is suggested that this may be due to post-translational modification of NDR1, specifically N-linked glycosylation [70]. This is consistent with the up shifted NDR1 we see on our anti-HA westerns and we also expect it is due to post-translational modification of NDR1ncluding glycosylation.

From the 286 proteins we selected a list of 11 interesting candidate proteins (table 6) including proteins that are abundant, up regulated, or previously shown to be related to defense, making their relation to NDR1 interesting. AtREM1.2 (AT3G61260), a member of the remorin family involved in DNA binding, was found to be both abundant and up regulated in our data. It has previously been shown that AtREM1.2 is upregulated during

the avrRpm1-RPM1 defense response as well as in MAMP treatment and it is thought to be important in early defense signaling [82,83]. Since AtREM1.2 has been associated with R-gene defense response that involves the protein NDR1 (RPM1 mediated defense), we predict that AtREM1.2 is actually interacting with NDR1 during the R-gene mediated defense response. Two other transcription factors WHY1/WHIRLY1 (AT1G14410) and WHY3/WHIRLY3 (AT2G02740) were found to be upregulated over the course of infection with avrRpt2 in our NDR1 specific data. WHY1 (77% identical to WHY3) has been associated with defense in Arabidopsis with *why1* mutants having an increased susceptibility to pathogens due to the mutant proteins lowered DNA binding activity [84]. It is not known what proteins WHY1 and WHY3 are associating with in the defense response but since they are specifically NDR1 pulled down we hypothesize that the WHIRLY transcription factors are associated with NDR1 in R-gene mediated defense. NTMC2T4 (AT3G61050) is a lipid binding protein with a C2 domain thought to be involved in calcium dependent phospholipid binding. The protein BAP1 has a C2 domain like NTMC2T4 and has been associated with negative regulation the R-gene defense response with *bap1* plants having an enhanced disease resistance [85]. Since NTMC2T4 has structural homology to the protein BAP1 it would be interesting to test if NTMC2T4 is negatively regulating defense though an association with NDR1. T18B22.20 (AT3G44620) is a protein tyrosine phosphatase that modifies proteins by removing phosphate groups attached to tyrosine residues. Protein phosphatases are involved in many plant-signaling pathways including defense by causing the regulation of different genes through de-phosphorylation [86], but the role of tyrosine phosphatases in defense has not been studied. Since phosphatases can regulate protein activity and signaling and

we specifically pulled down T18B22.20 with NDR1 this suggests that T18B22.20 may be involved in defense signaling. MUD21.16 (AT5G66900) is a probable disease resistance (R)-protein of the CC-NBS-LRR class. This R-protein might be involved in the defense response against Pto. or in general defense in relation to NDR1. AT1G33680 is a nucleic acid binding protein containing two KH domains, which can bind to RNA and function in RNA recognition. Based on our data AT1G33680 might be involved in defense through its RNA bindings functions. ATTIP49A/RIN1 (RPM1 interactor 1) (AT5G22330) has a protein binding domain, is similar to a DNA helicase, and has been associated with negative regulation of the R-protein RPM1 as well as normal Arabidopsis development [87]. Since plant development is inhibited in the absence of RIN1 it is difficult to study its effects on defense further, but it is likely associated with NDR1. CRK35 (AT4G11530) is a member of the CRK cysteine rich protein kinase family which, as previously discussed, are involved in defense and a family member (CRK11) has been directly associated with NDR1 thus making it likely that CRK35 is also involved with NDR1 in defense. SYR1 (syntaxin related protein 1)/PEN1 (AT3G11820) is a SNARE family protein known to be involved in vesicle fusion. PEN1 was shown to be involved in penetration resistance against pathogenic fungi [88]. In addition, our proteome profiling experiments of the RPM1 response showed upregulation of PEN1 protein levels (data now shown), indicating that PEN1 might be involved in defense against Pto. infection and the signaling pathway involving NDR1 and RPM1/RPS2. TRX3 (AT5G42980), thioredoxin H-type 3 was shown to be essential in the function of NPR1 (along with TRX5), a protein that has been described as essential for SA mediated pathogen defense [89,90]. Due to TRX3's relation to NPR1 defense it may also be essential to the function

of NDR1 in defense. In general NDR1 interactors might be involved in basal defense, where NDR1 and RIN4 have unclear roles, or the NDR1 interactors may be involved in R-protein defense signaling. We observe DNA and RNA binding proteins as NDR1 interactors which are proteins normally found in the nucleus or cytoplasm raising the question as to whether NDR1, a plasma membrane localized protein, translocates during defense signaling. Alternatively NDR1 interactors might dissociate from NDR1 during a defense response and translocate to the nucleus, or conversely they are recruited to the membrane during defense. Ultimately the actions of NDR1 and its interactors during defense are interesting but at this point unknown.

The 11 proteins of interest were then analyzed using STRING to the second level, which depicts interactions between imputed proteins as well as other direct interactors of the imputed proteins (STRING calls it level 2 in multiple names input setting) (Figure 18). None of our 11 proteins of interest were interacting directly but 3 proteins (TRX3, T18B22.20, and RIN1) were connected indirectly through secondary interactors. Additionally three clusters of genes were present in the STRING protein-protein interaction output but they do not seem to be directly related. Cluster I. represents a cluster of redox signaling proteins associated with TRX3 (Thioredoxin H-type 3), one of our proteins of interest. Cluster II. is a group of sugar metabolism proteins that indirectly links three of our proteins of interest, TRX3, T18B22.20, and RIN1. We believe that it is unrelated to defense that the three proteins of interest are connected via sugar metabolism proteins. Cluster III. is a group of DNA modification and transcription proteins including our protein of interest RIN1.

STRING is a useful online tool to give a visual representation of suggested and proven protein-protein interactions but its database is incomplete. It is known that the protein RPS2 interacts with NDR1 as well as RIN4 [20] but the STRING database does not show any RPS2 protein-protein interactions. There is another online protein-protein interaction database called AtPID [91] that does show the RPS2-NDR1 interaction but lacks other NDR1 interactions that STRING does show. The AtPID site also lacks the function of extending interaction parameters that STRING offers thus making it less user friendly. Additionally NDR1 and RIN4 have been shown to functionally as well as physically interact [20], yet in STRING the interaction is only shown as a result of text-mining meaning that means of interaction are not up-to-date and complete in the STRING database. Overall the online protein-protein interaction sites give a good indication of protein interactors but their databases are incomplete and many of the interactions are predicted and thus may be inaccurate.

It is known that NDR1 is involved in the R-gene mediated defense response and has a close association with the protein RIN4 [23]. RIN4 is monitored by the R-genes RPS2 and RPM1 and is also modified by the avirulence proteins avrRpm1, avrB, and avrRpt2 [4]. Additionally the direct interaction of RIN4 and NDR1 and the necessity of the RIN4-NDR1 interaction for the normal resistance activation against the effector avrRpt2 has been shown [20]. Since RIN4 is associated with NDR1 in defense signaling we would expect to pull it down with NDR1 after infection with bacteria containing avrRpt2, but this was not the case. We decided to check for RIN4 presence in our samples via western using an anti-RIN4 antibody but we did not find RIN4 in our pulled down samples (figure 19). We do see a band in our westerns that runs at ~25-30kDa

where we would expect to see RIN4. We also see additional bands that we cannot explain, which are probably just other proteins cross-reacting with the RIN4 antibody. The possible RIN4 band is present in only the total extract samples before antibody bead addition and is not present in the subsequent flow through and total resin samples. This would suggest that we are in fact pulling down RIN4 with NDR1 and it is subsequently washed off in the 50mM NaCl wash. What makes us cautious to jump to this conclusion is that we see the same band in one of our wildtype control total samples (no NDR1 pulled down) but again not in the flow through leading us to believe that this band that appears to be RIN4 is actually a protein that is aspecifically binding the anti-HA beads and is subsequently getting washed off. Therefore, it remains unclear whether RIN4 can be pulled down with NDR1. RIN4 Western blot conditions should be optimized and the experiment repeated, including all pulldown fractions and washes, and a RIN4 control sample, for instance a knockout mutant or a recombinant RIN4. A possible explanation of why we don't find RIN4 after treatment is that when avrRpt2 cleaves RIN4 it loses its association with NDR1 and that is why we do not pull the protein down. However this does not explain why we do not pull down RIN4 in our time zero pulldowns since RIN4 should be intact. RIN4 is a plasma membrane localized while NDR1 is predicted to be plasma membrane anchored and if their association is not strong it is possible that RIN4 was dissociated from NDR1 during the pulldown extraction or washing processes. We would like to re-try Western blots of pulldowns to see if RIN4 can be pulled down in our experiments with NDR1-Strep-HA. Western-blot conditions for the use of the RIN4 antibody have not yet been optimized. The fact that we did not observe RIN4 in the pulldown MS data might also be explained by the difference in sensitivity of Western

Blot [20] vs. MS. RIN4 might be a minor interactor, but Western is sensitive enough to detect a signal. Where as with MS, the most abundant interactors give the strongest MS signals (most spectra) and RIN4 might have been missed by the MS detector (below detection limit).

NDR1 was successfully expressed and pulldowns identified several interesting candidate proteins, which will be verified and functionally tested in follow up experiments, hopefully resulting in the identification of novel proteins important in the R-protein response or basal defense. Additionally pulldowns are being done in our lab with the proteins RPM1 and RIN4 in order to complete our knowledge of the defense interactome. In the future we hope to have described the protein interactome surrounding the RIN4 complex involving NDR1 in RPM1 and RPS2 R-protein mediated defense

Table 6: NDR1 interacting proteins of interest

From the 286 proteins 11 interesting candidate proteins were selected. The table lists these 11 proteins and includes proteins that are abundant, up regulated, or proteins that have previously been shown to be related to defense thus making their relation to NDR1 interesting. The columns in the table have all been previously described in table 3 and additional columns indicate whether the proteins were also classified in the other catagories as increased (UP), or decreased (down) over the time course of infection, and also if the protein was classified as abundant.

IPIaccession_number	TAIR Model name	entry_name	#of T=0's found in	intensity NDR1 0	#of T=2/3's found in	intensity NDR1 t2t3	total #	total intensity	changed #	changed intensity	abundant? (3# or more)	UP/DOWN?	numPepsUnique
IPI00539947	AT3G61260	AtREM1.2 DNA-binding family protein/ remorin family protein	2	3	3	9	5	12	1	6	YES	UP	3
IPI00545671	AT2G02740	ATWHY3/PTAC11 (A. THALIANA WHIRLY 3)	0	0	2	4	2	4	2	4		UP	3
IPI00534051	AT3G61050	NTMC2T4; lipid binding	0	0	2	3	2	3	2	3		UP	2
IPI00543013	AT1G14410	ATWHY1/PTAC1 (WHIRLY 1), DNA binding	1	1	1	3	2	4	0	2		UP	3
IPI00541351	AT3G44620	T18B22.20 Protein tyrosine phosphatase	1	1	2	2	3	3	1	1	YES		1
IPI00526823	AT5G66900	MUD21.16 Probable disease resistance protein (CC-NBS-LRR class)	1	1	0	0	1	1	-1	-1			1
IPI00786006	AT1G33680	AT1G33680 Nucleic acid binding	2	2	2	7	4	9	0	5	YES	UP	3
IPI00528249	AT5G22330	RIN1/ATTIP49A (RESISTANCE TO PSEUDOMONAS SYRINGAE1)	1	1	1	1	2	2	0	0			1
IPI00544267	AT4G11530	CRK35 cysteine-rich Protein kinase family protein	0	0	1	1	1	1	1	1			1
IPI00517442	AT3G11820	SYR ;Syntaxin-121	0	0	1	2	1	2	1	2			2
IPI00523104	AT5G42980	TRX3 Thioredoxin H-type 3	0	0	2	2	2	2	2	2		UP	1

Figure 18: Depiction of NDR1 interesting candidate interactors
Depiction of known and predicted protein-protein interactions of 11 interesting candidate proteins chosen from the NDR1 specific 286 proteins in STRING (Search Tool for the Retrieval of Interacting Geans/Proteins) to the second level which depicts interactions between imputed proteins as well as other direct interactors of the proteins used as input (STRING calls it level 2 in multiple names input setting). Proteins are depicted as spheres, with colored spheres representing inputted search proteins and grey spheres representing non-inputted interacting proteins. Cluster I. represents a cluster of redox signaling proteins associated with TRX3 (Thioredoxin H-type 3) one of our proteins of interest. Cluster II. is a group of sugar metabolism proteins that indirectly links three of our proteins of interest, TRX3, a protein tyrosine phosphatase, and RIN1. Cluster III. is a group of DNA modification and transcription proteins including our protein of interest RIN1.
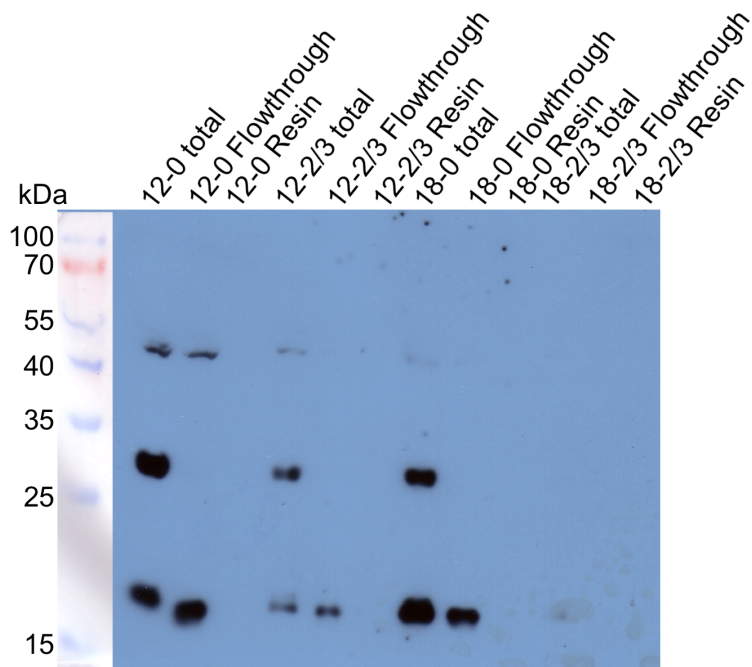
Figure 19: RIN4 Westerns
Anti-RIN4 westerns of pulled down NDR1-Strep-HA a)b) The blots previously used for NDR1 detection with anti-HA antibody were stripped and then re-hybridized with an anti-RIN4 antibody.

Materials and Methods:

Plant material and growth conditions:

Arabidopsis thaliana ecotype Col-0, and *Arabidopsis thaliana* ecotype Col-0 *ndr1* mutants with an over expression of NDR1 with a 2xHA-2xStrep tag were used for the NDR1 experiments. Plants for pulldown experiments were grown in 10 x 21in trays with 112 plants per line (four sections of 28), 168 plants per tray (six sections). The trays were placed in the Percival growth chamber having a light cycle of 11h light at 22°C and 13h dark at 20°C, the humidity was uncontrolled but stayed around 75%. For measuring bacterial growth and scoring HR differences all the previously mentioned plants as well as *Arabidopsis thaliana* ecotype Col-0 *ndr1* plants were grown. For bacterial growth assays 20 plants per line were grown in 10x21in trays and for HR scoring 4 plants per line were grown in 4x4in pots. Both were grown for four weeks in the aforementioned Percival growth chamber.

Bacterial Growth conditions:

Pseudomonas syr. pv tomato bacteria containing avrRpt2 were grown on King's B plates containing antibiotic selections of rifampicin (25mg/L) and tetracycline (10mg/L) for two days at 28°C. The bacteria were then grown in 5ml of King's B liquid overnight. In the morning the culture was spun down, supernatant removed, and then the bacteria were resuspended in 10ml of 10mM $MgSO_4$. An OD is then taken and bacteria can be diluted to desired OD with 10mM $MgSO_4$.

Plant treatments: Bacterial inoculation:

Bacterial inoculations were done in the morning on four-week-old plants. Bacteria were diluted to on OD of 0.05 for inoculating plants that were used in pulldowns, an OD

of 0.00005 for plants used in measuring bacterial growth, and an OD of 0.02 for plants used in scoring HR. Bacteria were injected with a 1ml syringe on the underside of the leaves with minimal pressure so that the liquid filled the leaf spaces.

Bio Assays: Measuring Bacterial growth:

Four weeks after planting four to five leaves (the fourth true leaf and younger) of the plant lines mentioned above were inoculated with bacteria containing avrRpt2. Bacterial growth was measured at 0, 2, and 4 days after infection by grinding two leaf disks (surface area per disk = 0.22cm^2) per replicate (8 replicates done) in 500µl of 10mM MgSO$_4$ and then plating 10 fold dilutions in duplicate on King's B plates containing rifampicin (25µg/ml). NOTE: day 0 leaf disks were first quickly rinsed in 70%ETOH and immediately dried using a tissue before grinding. Plates were then left at room temperature for three days before counting the bacterial colonies present in each replicate for each dilution.

Scoring HR:

Four weeks after planting, five to nine leaves per plant of the plant lines mentioned above were inoculated with bacteria containing avrRpt2. HR was then scored on these leaves 24 hours after infection as a percentage of the leaf covered by HR.

Plant Protein extraction and immuno precipitation (IP):

Flash frozen treated leaf tissue was ground with a mortar in liquid nitrogen and then transferred to a 50 ml Greiner tube. From this ground tissue an aliquot of 2 grams was transferred to a 15ml Greiner tube where 4ml of extraction buffer (50mM NaPO4 pH~7, 50mM NaCl, 5mM EDTA, 10% glycerol, 0.2% Triton X100, and Roche plant protease inhibitors complete) was added. Tissue/buffer were homogenized, 5% PVPP

(poly(vinyl(poly)pyrrolidone)) was added to the tube, and then the tubes were rotated for 10 minutes at room temperature. After rotation the tubes were spun at 3,220g for 20 minutes at 4°C and supernatant containing the total extracted protein was passed through a 5.0 micron nylon filter into a new 15ml tube (a 50μl sample of this total extracted protein was taken). 40μl of 50% antibody bead slurry, either Anti-HA (Sigma EZview) or strep-tactin (IBA), was then added to the extracted protein and incubated at 4°C on a rotating rack overnight. Beads were then spun down by centrifugation at 1000g for 5 minutes and a sample of the flow through was taken. Flow through was removed and beads were transferred to filter spin columns (Pierce screw cap) where the rest of the flow through was spun out.

Beads were washed twice for 10 minutes with 700μl of wash buffer #1 (50mM NaPO4 pH~7, 50mM NaCl, 5mM EDTA, 10% glycerol, and 0.2% tritonX100 for the first wash only). All liquid was spun out of the column after each wash. After the second wash the beads were re-suspended in 200μl of 50mM HEPES pH7.5 (A 20μl sample was taken for Western blot) . To the resuspention 10μl of 100mM TCEP (tris(2-carboxyethyl) phosphine hydrochloride) was added and then incubated at 95°C for 5 minutes to reduce cysteines. Samples were then cooled to room temperature before adding 5μl of 0.5M IAA (Indoacetamide), mixed, and then 2μl of Trypsin 1μg/μl (Roche) was added. Beads were then resuspended within the solution and left to shake (1000rpm) at 37°C overnight. Trypsinized peptides are then harvested (beads removed) using a spin column where they are subsequently acidified with 0.1% Formic Acid and stored at -20°C before being run on an LC column and through the mass spectrometer.

For pilot experiments beads were washed twice for 10 minutes with 700µl of wash buffer # 1 (50mM NaPO4 pH~7, 50mM NaCl, 5mM EDTA, 10% glycerol, and 0.2% tritonX100) and twice for 10 minutes with 700µl of wash buffer # 2 (50mM NaPO4 pH~7, 150mM NaCl, 5mM EDTA, and 10% glycerol). After each wash the liquid was spun out and sampled, washed resin was also sampled after the second wash of each wash set. After the final wash beads were re-suspended in 50µl of wash buffer#2. All samples/fractions were analyzed by Western blot.

Western Blots:

Western blots were done on the samples described above to check if the pull down procedure worked. 5x SDS protein loading buffer was added to the samples and they were boiled at 94°C for 5 minutes. 15µl of each sample was then run on a 12% SDS PAGE gel and transferred onto a PVDF membrane using a BioRad Trans-Blot semi-dry transfer cell. The membrane was then blotted in 5% milk dissolved in PBS for 1 hour before the primary antibody, 1/1000 αHA mouse (SIGMA), was added in 2% milk in PBS and incubated overnight at 4°C. The blot was then rinsed with PBS and washed three times for 10 minutes in PBS before the secondary, 1/10,000 α-Mouse IgG (GE healthcare), was added in 2% milk in PBS for 2 hours. The membrane was washed in the same fashion as before and then exposed to film using 2ml of ECL for 1 hour before developing.

Mass Spectrometer

Peptides were separated using 2D-LC and charged with electrospray ionization. Spectra were acquired using a LTQ linear ion trap tandem mass spectrometer.

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