Genetic Regulation of Cell Death and Disease Resistance in Arabidopsis

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

Chapel Hill
2012

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ABSTRACT

MELINDA MARGARET ROBERTS: Genetic Regulation of Cell Death and Disease Resistance in Arabidopsis
(Under the direction of Jeff Dangl)

Plants are constantly identifying and responding to cues and threats from their surroundings, such as changes in light, temperature, and humidity, mechanical damage from herbivores and insect, and pathogen attack. Resistance to plant pathogens involves both passive barriers and active, inducible disease resistance responses. Induction of immune responses in plants leads to, for example, cellular redox changes, activation of MAP kinase cascades, massive transcriptional reprogramming, and frequently culminates in a form of programmed cell death known as the hypersensitive response. In my dissertation work, I characterized proteins involved in the regulation of cell death and disease resistance in the model plant *Arabidopsis thaliana*.

My first project involved the zinc finger protein LSD1, a cytosolic scaffolding protein which is a negative regulator of cell death and disease resistance. *lsd1* mutant plants exhibit inappropriately triggered cell death and increased resistance to multiple pathogens. LSD1 was used in a Y2H screen which identified the LSD1 interactor NF-YC3, a CAAT-binding transcription factor. *nf-yc3* mutants have moderately increased susceptibility to the oomycete pathogen *Hyaloperonospora arabidopsidis*, and overexpression of NF-YC3 increases resistance to this pathogen, demonstrating that NF-YC3 is a positive regulator of disease resistance, likely via transcriptional regulation.

This activity could be partially controlled by LSD1 sequestering NF-YC3 in the cytosol, thereby preventing its nuclear relocalization and subsequent disease resistance function.

The latter half of my work involved the characterization of a positive regulator of *lsd1* rcd, ADR1-L2. ADR1-L2 belongs to a small family of NB-LRRs, the main class of resistance proteins that are required to recognize specific pathogen effector proteins, leading to pathogen recognition and defense responses. I created an autoactive mutant of ADR1-L2, which required P-loop dependent ATPase activity for function and exhibited increased resistance to infection with virulent pathogens. I then used this autoactive mutant to try to understand the genetic requirements of the signaling pathway involved in this resistance response, finding that ADR1-L2 functions in a feedback loop involving the defense-related hormone salicylic acid, LSD1, and the *lsd1* regulator EDS1. Together, my results refined the model of pathogen-triggered immunity in Arabidopsis.

To Stevie, who believed in me from day one. I know you are proud of me, and that means the most of all.

ACKNOWLEDGEMENTS

My first thanks must go, of course, to Jeff. Throughout my years in his lab he has challenged me, taught me, supported me, and overall made me a much better scientist, and much, much more of a critical thinker, than I ever thought I could be. He also gave me the best gift of all: a great scientific home in which I was able to learn from a huge variety of individuals, about science, about life, and about the type of person I want to be. The Dangl lab is truly a unique place, and a fitting tribute to its leader.

My gratitude also goes to the other members of my committee: Joe, Jason, Greg, and Mark. Each of you has supported me in many ways during my time here, both through great scientific ideas and insights, and by being excellent members of a caring department. When, as a naive first year, I chose my committee, I had no idea how interesting it would be to have Joe, Mark, and Jeff all together at the same meetings, frequently talking over each other to make their points. This combination of friendship and scientific curiosity is really one of the things that makes the Biology department at UNC a wonderful place to study. Plus, Joe always let me crash his lab's happy hours, which was a welcome release on several Fridays after some very long weeks.

From day one, the Dangl lab has been an awesome place to work. The friendships I have made helped me to get through grad school, both in an academic and a personal sense, and I am happy that they have continued with those who have left the lab before me, and confident that the same will happen once I am in Texas. Emily and Ben, and then

Emily, Marc, and Baltrus, made for excellent pit-going friends and I still miss sitting around there on warm days discussing anything and everything. Emily, you are one of my most favorite people in the world, and to quote another one of your fans "when I grow up, I want to be like Emily Fisher". Vera and Anna made my last project, and last two years, so much better than they otherwise would have been. And to Terry and Erica, for helping me all along but being especially supportive at the end: I don't think I would've made it through the last 18 months in lab without you guys. Thank you!

Outside of our lab, there were so many people who made my time here great. The upper-level grad students who were in the department when I arrived made my move to the East Coast so easy, and my fellow cohorts ("the first years", as some people still insist on calling us) gave me one of the most fun years of my life when we first arrived here, and then have remained good friends all along. And special thanks go to the Kieber lab girls (Cris, Blaire, and Tracy) and to Erin Friedman for helping to make Coker Hall more tolerable, and to the Chemistry Boys, for letting me crash your parties and being great friends (who also happened to introduce me to Michael). I also have to thank all of the people involved with Central Carolina Synchro-getting away from lab and to the rink really helped me stay sane! Kim and Dan of course get a very special thank you. Our friendship means so much to me, and I will miss you most of all. Good thing we have Yosemite, Harry Potter World, and an epic road trip in the future so that there will be lots more time to hang out (and yes Kim, Bridal Mart too-we'll figure out a way to make it all work).

To my friends from before grad school: thank you for your support as I went through this process. I know it's sometimes been hard to understand why I've kept this up

though the hard times, or what exactly I spend all of my time doing, or if I was ever going to finish, but you all have been wonderful and understanding, and I can't wait to talk to you and see you more now that it's over. Special thanks go to Erin, who knew what I was getting myself into and let me do it anyway. Thank you for leading me down this path and doing everything first so I could see what it was all like. And to Cristina, whose life has changed more during the time I've been here than ever before, thank you for all of your help and support and listening and understanding. I'm so proud of you in everything that you do, and so thankful to have you in my life. Now it's your turn to go back to school!

This process, especially moving across the country and starting a new life, has made me so very thankful for the support of my family. Suffice it say that I never would have gotten through this without you. My parents have been so supportive, and I really can't thank them enough. For my whole life my mom has been a shining example of what it means to be an excellent educator, and I hope that in my next endeavors I can follow that example and make people as excited to learn as she does. My stepdad, Robin, has always been there to listen when things were tough, encourage me when I needed it, and celebrate my successes. And he always offered me a plane ticket home any time I needed one; frequently the offer alone was enough to solve my problems. My dad has been a great encourager of this endeavor as well, and always made me feel like what I was doing was worth it. He never failed to tell me that he was "lucky to understand half of what [I] was saying" when I tried to explain my project to him, but always made me feel like that was because it was so advanced, and not because I was doing a terrible job with my explanation. Johannie has helped me, prayed for me, and understood what I have gone

through in a way that nobody else could. Bryan gives me the best advice, no matter the topic, and is the older brother I never had. He always had a fantastic outsider's prospective, and there were many, many times where talking to him allowed me to see things for what they were, rather than what I was making them out to be. And to Michelle, who is the best of sisters and the best of friends, I am thankful for all of your support and love, and so so so happy to have the two (almost three!) new people you have blessed us with in my life. There's not much better when I am tired or frustrated than to video chat with Owen and Emma. They make everything better.

Last but not least, I of course have to thank Michael, for being my best friend, and for making me laugh when I wanted to cry, and for letting me be exactly who I am. I think that grad school is hard for everyone, and at the end you have to weigh the good against the bad to see if it was worth it. You made every second worth it. I am so excited for all of our new adventures!

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List of Terms

ADR1	Activated Disease Resistance 1
BiFC	Bimolecular fluorescence complementation
BTH	Benzothiadiazole
CC	Coiled-coil
Dex	Dexamethasone
EDS1	Enhanced Disease Susceptibility 1
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
Hpa	*Hyaloperonospora arabidopsidis*
HR	Hypersensitive response
ICS1	Isochorismate Synthase 1
LSD1	Lesion Simulating Disease 1
MAMP	Microbe-associated molecular patterns
MTI	MAMP-triggered immunity
NB-LRR	Nucleotide-binding leucine-rich repeat
NPR1	Nonexpressor of PR Genes 1
NLR	Nucleotide-binding domain and leucine-rich repeat-containing
NF-Y	Nuclear factor Y
PCD	Programmed cell death
PR	Pathogenesis related
PRR	Pattern recognition receptors
Pto	*Pseudomonas syringae* pathovar tomato
R	Resistance gene

RCD	Runaway cell death
RLK	Receptor-like kinase
RLP	Receptor-like protein
ROI	Reactive oxygen intermediates
SA	Salicylic acid
SAR	Systemic acquired resistance
STAND	Signal transduction ATPases with numerous domains
TF	Transcription factor
TIR	Toll/interleukin-1
TTSS	Type three secretion system

Chapter 1

Introduction

Plants, like all other organisms, must properly respond to changes in their surroundings. Examples of these responses include finely-tuned tropism reactions to water gradients, light, and gravity (Eapen et al., 2005; Holland et al., 2009; Moulia and Fournier, 2009); proper timing of seed germination (Penfield and King, 2009); and correct responses to attacks from herbivores, phytophagous insects, and pathogens. As sessile organisms without adaptive or circulatory immune systems, plants have had to evolve a set of cell-autonomous defense responses. Many times, these endogenous disease resistance mechanisms are not successful: plant pathogens alone contribute up to $30 billion in annual losses to the US agriculture industry (Pimentel et al., 2000). However, despite the inherent limitation of not having an adaptive or circulatory immune system, most plants are resistant to most pathogens (McDowell and Simon, 2008).

Plant disease resistance arises from both pre-formed mechanical barriers and pathogen-induced responses. The former includes basic pathogen defense mechanisms such as the waxy cuticle on the outside of the plant leaves which blocks the entry of pathogens and a suite of secondary metabolites with strong anti-microbial activity (Taiz and Zeiger, 2002). In addition to these intrinsic barriers, plants are also able to organize and produce a series of inducible defense responses. These reactions may occur both

locally and systemically, and can be divided into two parts. The first of these branches includes recognition of microbe-associated molecular patterns (MAMPs) by transmembrane pattern recognition receptors (PRRs) in the host plant. PRRs frequently bind proteins and other molecules that are particularly important to the pathogen's function (Zipfel, 2009). While MAMP-triggered immunity, or MTI, is an effective and robust defense strategy, pathogens have, by definition, evolved methods of evading it and are thus able to colonize their hosts. Pathogens, such as the model bacteria *Pseudomonas syringae* pathovar tomato (*Pto*), secrete effector proteins into the host plant. These specialized proteins antagonize MTI responses by, for example, blocking cell wall callose deposition, interrupting plant hormone signaling important for a proper defense response, and interfering with cell death responses triggered by other effectors (Grant et al., 2006). Effector proteins that are able to suppress MTI help in a successful colonization of the host plant; this process is known as effector-triggered susceptibility (ETS). Plants have, in turn, developed a system for responding to ETS. This response depends on disease resistance, or *R*, genes.

Plant *R* gene products, frequently referred to as NB-LRR proteins, contain a nucleotide-binding (NB) domain followed by a leucine-rich repeat domain (LRR) at their C-terminus. They share homology to animal NLRs (nucleotide-binding domain leucine-rich repeat proteins) and recognize, either directly or indirectly, the effector proteins injected into the plant cell by the pathogen. In a direct interaction, the effector and NB-LRR interact with each other. Conversely, indirect interactions involve a host target protein which is modified by the effector, and it is this change that is perceived by the NB-LRR. Either type of NB-LRR-mediated effector recognition leads to effector-

triggered immunity (ETI), resulting in a disease resistance response that is both faster and stronger than MTI (Jones and Dangl, 2006). ETI responses include calcium influx, protein kinase activation, production of reactive oxygen intermediates, transcriptional reprogramming, and, frequently, the hypersensitive response (HR), a type of programmed cell death (Dangl and Jones, 2001).

Basal defense, or the responses triggered by virulent pathogens on susceptible hosts, and ETI are easily thought of as different magnitudes of the same defense responses. This is best visualized by the zigzag model put forth by Jones and Dangl in 2006 (Figure 1). Their model presents MTI as the primary, low-level amplitude reaction to pathogens. Successful pathogens utilize effector proteins to overcome this first response, and resistant plants employ NB-LRRs to recognize these intruder proteins and mount a stronger defense, including localized cell death (HR). Inherent in this model is the resulting evolutionary "arms race" between pathogens and their potential hosts. When plants begin to recognize existing effector proteins, the pathogen will evolve a new array of effectors that cannot be recognized or which can counteract the plant's original ETI. Plants, in turn, evolve new NB-LRRs, capable of recognizing the new effectors, and the cycle will begin again.

The remainder of this introductory chapter will focus on the specifics of MTI and ETI, including key signaling molecules and responses involved in both, and will discuss the overlap between the two, which leads to the conclusion that basal and effector-triggered defenses are not separate pathways, but rather represent different levels of activation of the same responses.

PRRs and MAMP Triggered Immunity

The first layer of inducible defense responses involves direct perception of non-host elicitors, or MAMPs, by PRRs. To avoid confusion between MAMPs and effectors, MAMPs are defined as being "conserved among a large group or class of microbes", whereas effectors evolve within a single or small group of microbial species (Zipfel, 2009). Continuous addition to the body of knowledge about elicitors and effectors makes categorizing these molecules an ongoing effort. Interactions between MAMPs and their receptors occur at the plant cell's plasma membrane, and all currently identified PRRs are transmembrane receptor-like proteins (RLPs) or kinases (RLKs). RLKs and RLPs have similar extracellular structures with multiple LRR domains and similar transmembrane helices, but RLKs posses a cytoplasmic kinase domain (Tor et al., 2009). The two best-studied examples of MAMP receptors are FLS2 and EFR, RLKs which bind flagellin and elongation factor-Tu (EF-Tu), respectively. Additional PRRs include XA21, a rice protein whose ligand Ax21 was recently discovered (Lee et al., 2009) and CERK1, a LysM-RLK which recognizes chitin (Petutschnig et al., 2010). Activation of any of these receptors leads to a common set of downstream defense responses, and *efr* and *fls2* mutants are more susceptible to a range of pathogens (Zipfel, 2009).

Individual pathogens each have multiple MAMPs which may be perceived by a potential host. Current knowledge of MTI is based on experiments which used single elicitors and/or single PRR knockout lines. These experiments do not provide information on the specific defense effects of each PRR-mediated MAMP recognition event in a

natural plant-pathogen interaction, but do offer an overall picture of MAMP-initiated defense effects (Segonzac and Zipfel, 2011). The most frequently studied elicitor-receptor interactions are flagellin-FLS2 and EF-Tu-EFR; the former will be used as an example here. Flagellin, or the minimal signaling epitope known as flg22 which is derived from the N-terminus of flagellin, is recognized at the cell surface by FLS2, a glycosylated, transmembrane RLK (Gomez-Gomez and Boller, 2000; Chinchilla et al., 2006). This extracellular detection leads to a heteromerization between FLS2 and BAK1, a short LRR RLK which is a member of the Somatic Embryogenesis Receptor Kinase (SERK) family (Chinchilla et al., 2007). This PRR/RLK complex also binds other SERKs (Roux et al., 2011). In addition to the formation of the receptor/kinase complex, phosphorylation of both FLS2 and BAK1 quickly follows elicitor recognition (Schulze et al., 2010), though the relevant residues are currently unknown.

Proper elicitor-triggered hetero-complex formation and phosphorylation of unidentified key residues leads to a network of downstream defense responses. These include callose deposition to strengthen cell walls, accumulation of defense-related hormones such as salicylic acid (SA) and jasmonic acid (JA), massive transcriptional reprogramming, activation of MAP kinase cascades, and a biphasic oxidative burst (Segonzac and Zipfel, 2011). FLS2/flg22-induced protein kinases include MEKK1, MKK4/MKK5 and MPK3/MPK6 (also involved in SA signaling, below), and these cascades trigger changes in many defense-related transcription factors, including WRKY22 and 29. The oxidative burst is a strong and rapid cell-to-cell increase in the amount of reactive oxygen species (ROS) in the plant cells. This induction requires the

NADPH oxidase *RbohD* in a cell autonomous manner, and is a way for the initial defense signal to be propagated across the leaf (Torres et al., 2002; Miller et al., 2009).

Classification of pathogen molecules as MAMPs versus effectors, and, in a related manner, PRRs versus R proteins, is an important and constantly evolving process. Initial categorization can be used as preliminary insight into the role of a new pathogenesis-related protein, but if incorrect can lead to faulty assumptions about that protein. For instance, classifying a protein as an effector leads to the conclusion that it will act within the plant cell, whereas a MAMP functions at the extracellular membrane. The experimental approaches used to test the functions of these two proteins are inherently different. Therefore, incorrectly identifying a protein makes it difficult to properly dissect the genetics and biochemistry of the defense processes in which it is involved. As more MAMP/PRR pairs are identified, there will be a better understanding of the common signaling components involved in MTI. Proper classification of MAMPs and effectors also allows for robust evolutionary studies, which will further inform the overall picture of plant-pathogen interactions.

The specific immunity contribution of each signaling event may still be unclear, but future research should be able to unravel these interactions and their respective significance to disease resistance. Importantly, even without proper classification of each defense-related protein, the common set of downstream host responses exhibited by all studied PRRs and shown to be functionally relevant to disease resistance are potential sources of real-world agricultural application. Genes from non-susceptible species can be transferred to previously susceptible plants as a possible means of boosting disease resistance. One particularly exciting study showed that transgenic expression of *EFR* in

tomato, a species that does not normally carry this PRR, leads to broad-spectrum bacterial resistance in these plants (Lacombe et al., 2010). Examples such as this prove that MTI-related research has developed rapidly over the course of the last 10 years. Continuing efforts should uncover a much more complete view of the path from ligand perception to disease resistance.

NB-LRRs and Effector Triggered Immunity

Pathogens, in an evolutionary response to MTI, evolved effector proteins to combat the defense responses triggered by their MAMPs. Bacteria utilize the type three secretion system (TTSS), a syringe-like apparatus that sends 15-30 such effector proteins into the host plant (Cornelis and Van Gijsegem, 2000; Alfano and Collmer, 2004). Effectors target the function of proteins important for MTI, thereby increasing pathogen virulence and causing ETS. For instance, the *Pto* effector *AvrPto* suppresses basal defenses in tomato, Arabidopsis, and the model plant *Nicotiana benthamiana*. AvrPto binds EFR and FLS2 (Xiang et al., 2008), and targets BAK1 (Shan et al., 2008), disrupting FLS2-BAK1 interactions and suppressing flg22-induced MPK3 and MPK6 activation, cell death, and callose deposition (Hann and Rathjen, 2007).

Another example of effectors targeting MTI involves the *Pto* effector *hopM1*. HopM1 is a highly conserved, TTSS effector which is required for full *Pto* virulence (DebRoy et al., 2004). HopM1 was found to interact with MIN7, an Arabidopsis adenosine diphosphate ribosylation factor guanine nucleotide exchange factor (ARF-GEF) protein (Nomura et al., 2006). ARF-GEF proteins are involved in vesicle

trafficking, and MIN7 is required for full bacterial resistance in Arabidopsis. HopM1 uses the proteaosome of the host plant to degrade MIN7, thereby increasing bacterial virulence (Nomura et al., 2006). Left unchecked, effectors can overcome MTI and lead to host plant susceptibility. Thus, plants have evolved a way to recognize and respond to pathogen effector proteins.

Recognition of pathogen effectors by the host requires the proper detection by and function of NB-LRRs, and proper recognition leads to ETI. Interaction between NB-LRR and effector proteins is hypothesized to occur in one of two ways: directly or indirectly. Direct interactions occur when an effector and an NB-LRR bind to each other. Examples of this include the Arabidopsis protein RRS1-R directly interacting with the *Ralstonia* Avr protein PopP2 (Deslandes, PNAS 2003), the rice Pi-ta NBS-LRR directly associating with Avr-Pita from rice blast (Jia et al., 2000), and L5, L6, and L7 proteins from flax, which directly recognize the products of the rust flax *AvrL567* genes (Dodds et al., 2006).

Alternatively, as described in the guard hypothesis, there is no direct interaction between effectors and NB-LRR proteins (Van der Biezen and Jones, 1998). Rather, the effector protein modifies its host target protein, and it is this change that activates the NB-LRR protein. The host target protein is thus “guarded” by the NB-LRR protein, and NB-LRR recognition of host target modification is what leads to the downstream defense responses (Dangl and Jones, 2001; Holt et al., 2003). Utilizing a common target for multiple effectors could allow the host plant to best exploit its recognition potential with a limited set of NB-LRRs. Maximizing the utility of each NB-LRR is very important for an organism that does not possess an adaptive immune system.

One well-studied example of the guard hypothesis involves the *P. syringae* effectors AvrB and AvrRpm1, along with the Arabidopsis proteins RIN4 and RPM1. *RPM1* encodes a CC-NB-LRR and guards RIN4, a small, membrane-bound protein that is a negative regulator of basal defense (Mackey et al., 2002). RIN4 is modified when either of the two sequence-unrelated effector proteins, AvrB or AvrRpm1, is introduced to the system via delivery by the TTSS of *Pto* DC3000. Neither effector is a kinase, but their interaction with RIN4 leads to phosphorylation of RIN4 at threonine 166 (Chung et al., 2011). This phosphorylation of RIN4 is perceived by RPM1, which then triggers a series of pathogen defense responses, including HR (Boyes et al., 1998; Chung et al., 2011). In an *rpm1* mutant, the lack of RPM1 protein allows AvrRpm1 or AvrB to enter the cell undetected. From there at least AvrRpm1 acts as a virulence factor, promoting bacterial growth and disease (Ritter and Dangl, 1995). RIN4 is also guarded by a second, independent NB-LRR, RPS2. RPS2 is triggered when AvrRpt2, a third *P.syringae* effector, cleaves RIN4 at two sites. This cleavage is detected by RPS2, triggering a similar series of defense responses to those activated by RPM1. A fourth effector, HopF2, also targets RIN4 (Wilton et al., 2010). These interactions involve four different effector proteins that are all found to trigger defense responses through the same protein, in fact, by their action on the same ~30 amino acid domain of RIN4. RIN4 is guarded by at least two different NB-LRRs. The RIN4 example provides proof that Arabidopsis is able to maximize its pathogen recognition specificity utilizing a small, non-adaptive set of NB-LRRs.

Constitutive disease resistance responses can be of high fitness cost to the host plant (Tian et al., 2003). It is therefore very important that these responses are only

triggered when necessary, and thus they must be under finely-tuned control. One model of disease resistance shows NB-LRRs functioning as molecular switches, with multiple subdomains responsible for keeping the protein in the resting, or "off", state, thereby preventing spurious NB-LRR activation (Takken et al., 2006). NB-LRR proteins, and NLR homologs in animals, are members of the NTPase superfamily and belong to the signal transduction ATPases with numerous domains (STAND) subclade (Leipe et al., 2004). In the "off" conformation, STAND proteins bind ADP, which must be exchanged for ATP in order to trigger defense responses (Takken et al., 2006).

Plant NB-LRR proteins consist of three distinct domains: either a CC or TIR N-terminal domain, which is involved in downstream signaling events; followed by a central nucleotide-binding domain (NB), where ADP or ATP binding occurs; which is fused to a leucine-rich repeat (LRR) domain at the C-terminus that provides recognition specificity (Takken et al., 2006). The P-loop and MHD, two subdomains within the central NBS domain, are particularly important for proper function. The P-loop motif is critical for nucleotide binding, and in most cases, mutations in this domain result in an inactive NB-LRR (Tameling et al., 2002; Hanson and Whiteheart, 2005; Rairdan and Moffett, 2006). Mutations in the MHD domain, on the other hand, typically result in autoactivity (Bendahmane et al., 2002; Howles et al., 2005; Tameling et al., 2006; Gao et al., 2011; Williams et al., 2011; Zhang et al., 2012). This is due to either a preference towards ATP binding or a lack of ATPase activity which keeps the protein in the "on" state (Tameling et al., 2006). The inactive conformation is further maintained by proper physical interactions between the NB and LRR domains (Bendahmane et al., 2002; Ade et al., 2007). In this "off" conformation, the LRR inhibits the NB from undergoing

nucleotide exchange. After pathogen recognition, where specificity is typically conferred by the LRR, this autoinhibition is released, allowing ADP to be exchanged for ATP and initiation of defense signaling events.

Attempts to study autoactive mutants have been made in Arabidopsis, flax, and tobacco (Table 1). While the majority of NB-LRR autoactive mutations recovered have been in the MHD domain (Bendahmane et al., 2002; Howles et al., 2005; Tameling et al., 2006; Gao et al., 2011; Williams et al., 2011; Zhang et al., 2012), there are also mutations that lead to autoactivity which occur outside of this domain (Zhang et al., 2003; Igari et al., 2008; Huang et al., 2010). Much of the work done with these autoactive alleles has been carried out in transient over-expression assays in flax or tobacco systems, making it difficult to test their biological relevance. However, some key work in Arabidopsis and flax has shown that these autoactive mutations lead to lethality or dwarfed morphology (Howles et al., 2005; Gao et al., 2011; Zhang et al., 2012). Additionally, there is evidence that NB-LRR autoactivity directly affects the immune system signaling pathway, as some of these mutants exhibit hallmarks of defense activation, including high steady-state SA levels (Zhang et al., 2003; Huang et al., 2010) and increased resistance to infection with virulent pathogens (enhanced basal defense) (Gao et al., 2011). Overall, these autoactive mutants clearly show that correctly controlled function of NB-LRRs is necessary for both plant fitness and defense activation.

While canonical ATP-driven activity of NB-LRRs is clearly essential for a complete defense response, examples in plants (Bonardi et al., 2011) and animals (Kofoed and Vance, 2011; Zhao et al., 2011) of NB-LRRs that do not require the canonical P-loop for function indicate that there are functions for these proteins beyond

typical ATPase activities. In these cases, NB-LRRs may not work as canonical 'sensors', but might instead act as 'helper' proteins. These 'helpers' potentially function as scaffolding proteins, perhaps working with other immune-related proteins, including canonical NB-LRRs, to trigger defense responses (Bonardi et al., 2012). These examples show us that there is still much to be learned about the overall role that NB-LRR proteins play in defense. Chapter 3 addresses the characterization of a unique NB-LRR with both 'helper' and P-loop dependent functions in disease resistance.

Proper accumulation and stabilization of NB-LRR proteins is also important for their activity, and control of NB-LRR protein levels requires additional plant proteins. Three proteins required for NB-LRR stability are HSP90, SGT1, and RAR1. *RAR1* encodes a zinc binding protein consisting of two CHORD domains, and is highly conserved among all eukaryotes except yeast (Shirasu et al., 1999). RAR1 protein is required for full accumulation of almost all tested NB-LRRs. However, only a subset of NB-LRRs are functionally suppressed in a *rar1* background. This dichotomy is explained by the "threshold model": proper defense response requires a certain, set level of NB-LRR protein, and the expression level of some NB-LRR proteins is very high in an unchallenged plant. In a *rar1* mutant, the expression level of this class of 'high-accumulating NB-LRR proteins' is reduced, but remains above the required threshold to trigger a defense response. Therefore, defense responses are not compromised (Bieri et al., 2004; Holt et al., 2005). Conversely, the steady state protein expression level of NB-LRR proteins that require RAR1 for their function falls below the threshold point in a *rar1* mutant, leading to significantly reduced defense responses.

SGT1 is also required for proper NB-LRR stability. RAR1 and SGT1 interact via the C-terminal CHORDII domain of RAR1 and the CS domain of SGT1. Arabidopsis contains two orthologues of this gene; mutations in *SGT1b*, but not *STG1a*, can alter the functions of some NB-LRR proteins. *SGT1* double mutants are lethal (Azevedo et al., 2006).

In planta, both RAR1 and SGT1 associate independently with the cytosolic protein HSP90. HSP90 is a chaperone protein that is responsible for the proper folding of its "client" proteins, and it is known to regulate accumulation of wild-type amounts of protein for all tested NB-LRRs. The ATPase domain of cytosolic HSP90 associates with both CHORDI of RAR1 and the CS domain of SGT1b. This association is clearly important to NB-LRR function, as point mutations in the ATPase domain of one isoform of HSP90, *hsp90.2*, cause a large reduction in the accumulation of the NB-LRR RPM1 (Hubert et al., 2003). This data lead to a model where NB-LRR proteins are clients of HSP90, and are held in proper conformation and therefore maintain proper protein levels with the co-chaperones RAR1 and SGT1b. Additional *hsp90.*2 alleles were identified that suppressed *rar1* phenotypes, allowing accumulation of functional levels of NB-LRRs in this background (Hubert et al., 2009). These alleles furthered the model of HSP90-regulated protein accumulation, showing that RAR1 normally functions to physically regulate HSP90-dependent dynamic protein turnover. Overall, disruption to RAR1, HSP90, or SGT1 can lead to an alteration in NB-LRR accumulation, and potentially affects disease resistance.

In addition to these proteins which are required for NB-LRR stabilization, the signaling pathways activated by NB-LRRs require further factors for proper activation.

Proper signal transduction from TIR-NB-LRRs is dependent on EDS1 (Enhanced Disease Susceptibility 1), PAD4 (Phytoalexin Deficient 4) and SAG101 (Senescence-Associated Gene 101), while CC-NB-LRRs require NDR1 (Non-race-specific Disease Resistance 1) for proper function (Glazebrook, 2001). Together, these NB-LRR regulatory proteins and domains attempt to balance plant cell damage caused by virulent pathogens with fitness costs stemming from disease responses whose amplitude is too high.

Salicylic Acid, a Central Molecule in Plant Defense Responses

Induction of SA, a phenolic plant hormone, has a very wide range of effects in plants. It directly and/or indirectly influences seed germination, cell growth, stomatal aperture, and fruit yield (Vlot et al., 2009). It is also important for proper defense responses, including basal defense and some effector-triggered disease resistance responses. SA levels increase after pathogen attack, and exogenous application of SA or BTH leads to increased disease resistance (Lu, 2009). Activation of either NB-LRR-mediated or MAMP-triggered disease resistance pathways leads to an increase in SA (Glazebrook, 2005; Tsuda et al., 2008). The majority of SA is generated by conversion of chorismate to isochorismate via the isochorismate synthase (ICS) pathway (Lu, 2009). Mutations in the Arabidopsis gene *SID2*, which encodes isochorismate synthase 1 (ICS1), block SA production and lead to pathogen-induced SA induction levels that are ~10% of wildtype (Wildermuth et al., 2001). *sid2* plants are more vulnerable to a variety of pathogens, and this increased susceptibility can be rescued by exogenous application of SA or its synthetic homolog benzothiadiazole (BTH) (Nawrath and Metraux, 1999;

Dewdney et al., 2000). An additional isochorismate synthase, ICS2, also exists in Arabidopsis, and it is responsible for generating the SA measured in *sid2* mutants (Garcion et al., 2008). There is also an ICS-independent pathway for SA synthesis, as *ics1 ics2* double mutants still display very low levels of SA (Garcion et al., 2008).

In addition to those genes encoding the proteins required for the biosynthesis of SA, several other genes are positive regulators of SA. The best characterized of these include EDS1, PAD4, and NDR1, though a handful of additional positive regulators have been recently characterized (Lu, 2009). Both EDS1 and PAD4, as well as several other positive regulators of SA, are also SA-inducible, and the loss of resistance phenotypes seen in *eds1*, *pad4*, and *ndr1* plants can be reversed by exogenous application of BTH (Zhou et al., 1998; Falk et al., 1999; Shapiro and Zhang, 2001). This suggests that SA regulation occurs in a feedback loop: many positive regulators of SA are induced by SA, leading to dramatic increases in this molecule after disease resistance pathways are triggered.

Much of the signaling downstream of SA requires *NONEXPRESSOR OF PR GENES 1* (*NPR1*) (Cao et al., 1997). NPR1 is found in both the cytosol and the nucleus, and in the latter location it functions as a transcriptional regulator of pathogen-related (PR) genes (Dong, 2004). In steady-state conditions, the majority of NPR1 is present in the cytosol as oligomers. Pathogen challenge promotes a conformational change of NPR1 from oligomers to monomers, allowing this molecule to enter the nucleus (Mou et al., 2003). Once in the nucleus, NPR1 indirectly activates transcription of defense-related genes via interaction with transcription factors (TFs), including the TGA family of bZIP TFs (Despres et al., 2000). As previously discussed, inappropriately triggered defense

responses can be of high fitness cost to a plant, and thus, inducers of defense must be tightly regulated. In the case of NPR1, this regulation comes in the form of proteasome-mediated degradation, which uniquely both prevents spurious gene activation in plants not undergoing pathogen attack and stimulates defense-related gene expression when plant defense responses are turned on (Spoel et al., 2009). Very recently, a potential mechanism for SA perception and monitoring was proposed (Fu et al., 2012). The data in this paper demonstrates that NPR3 and NPR4, paralogues of NPR1, are SA receptors with different binding affinities for the molecule. These two proteins function in the SA-mediated degradation of NPR1, and the authors propose that their different affinities for NPR1 sets up the proper regulation of NPR1 protein levels mentioned above.

NPR1 and SA also are essential for long-term, systemic activation of disease resistance. Endogenous increases in or exogenous application of SA lead to the transcriptionally-based defense responses that constitute systemic acquired resistance (SAR), and SAR requires NPR1. SAR confers broad-spectrum pathogen resistance, and is activated systemically after local pathogen infection or SA application (Shah, 2009). One key hallmark of SAR, and defense responses in general, is increases in PR protein levels (Sels et al., 2008). PR proteins can be induced by SA, jasmonic acid (JA), and ethylene (ET) (Dong, 2004). There are fourteen different classes of PR genes in Arabidopsis (van Loon and van Strien, 1991), and potentially hundreds of different members of some classes (Silverstein et al., 2005); these large numbers complicate the understanding of PR activation and activity. Some *PR* genes have been found to have specific antimicrobial activity (van Loon and van Strien, 1991; van Loon et al., 2006), though the precise role that the majority of these proteins play is still under investigation.

Much work has gone into trying to identify the molecule responsible for the spread of SAR. Early studies of SA showed that large amounts of the compound accumulate in and around the lesions that form at the site of pathogen infection (Enyedi et al., 1992). SA levels are also known to increase throughout the plant after pathogen recognition, including in the phloem (Yalpani et al., 1991). Given this data, it was originally thought that SA might be the SAR potentiation signal. However, grafting studies showed that SA is not necessary for development of the signal at the site of infection, though it is required for SAR at distal sites in the plant (Vernooij et al., 1994). SA can be reversibly turned into methylsalicylic acid (MeSA), and studies in tobacco found that this compound fit all the requirements to be the SAR signaling compound (Park et al., 2007). However, in Arabidopsis, studies showed that MeSA accumulation mutants still could induce SAR (Attaran et al., 2009). Thus, the search for the SAR systemic signal continues.

Programmed Cell Death in Plant Pathogenesis

Programmed cell death (pcd) in plants can be induced by a variety of abiotic and biotic stressors, including high light, heat shock or chilling, and the chemical inducers H_2O_2 and paraquat. One of the hallmarks of pathogen recognition is the HR, a type of pcd that includes rapid, localized cell death at and around the site of infection. HR is mainly associated with ETI, although cell death also can be induced by other high levels of flg22 (Naito et al., 2008). Early events after pathogen invasion include production of the ROS superoxide (O_2^-) and hydrogen peroxide (H_2O_2), as well as synthesis of nitric oxide (NO)

(Levine et al., 1994; Delledonne et al., 1998). Increases in ROS occur in and around the infected cell, and they are important signaling molecules for HR propagation across the leaf (Nanda et al., 2010). *AtRbohD* is required for the oxidative burst, and in wild-type plants this burst signals the cells proximal to sites of infection to induce transcription of defense genes and suppress cell death (Jabs et al., 1996; Torres et al., 2005). In distal cells, ROS and SA function as signal transduction molecules, potentiating cell death throughout the leaf. This cell death must be kept in check to prevent unnecessary death of parts of or the whole plant.

The Arabidopsis *lesions simulating disease1* (*lsd1*) mutant provides an excellent background to study the roll of cell death in disease resistance. LSD1 is a cytosolic zinc finger protein, and in wild-type plants it functions as a negative regulator of cell death. *lsd1* mutant plants exhibit inappropriately regulated cell death, also known as runaway cell death (rcd), and increased resistance to multiple pathogens (Dietrich et al., 1997). Triggers of rcd in an *lsd1* mutant include pathogen infection, changes in day length, and exogenous application of SA or BTH; *lsd1* plants are unable to stop the propagation of cell death from any trigger. As a cytosolic zinc finger protein, LSD1 functions as a potential interacting platform for other proteins involved in pcd (Kaminaka et al., 2006). Yeast two-hybrid and phage display screens identified several other LSD1 interactors, including NF-YC3 and NF-YC2, both encoding CAAT Box-binding Factor CBF-C subunits of heterotrimeric CAAT-binding TFs. These proteins and their roles in plant defense are further explored in chapter 2.

Several different proteins are required for *lsd1* rcd. These include EDS1 (Rusterucci et al., 2001), PAD4 (Rusterucci et al., 2001), and AtMC1 (Coll et al., 2010).

Runaway cell death in *lsd1* also requires both SA and NPR1 (Aviv et al., 2002). In *lsd1*, but not wild-type plants, SA is able to trigger rcd, indicating that LSD1 is normally working as a negative regulator of SA-dependent cell death (Dietrich et al., 1994). These results position LSD1 and SA in a feedback loop, where the presence of LSD1 is necessary and sufficient to stop SA-potentiated rcd. NPR1 is also necessary for *lsd1*-mediated rcd, making it clear that SA is at least partially required as a signal initiator in rcd. It is also important to note that these SA requirements are not the same for the *lsd1*-related basal defense phenotypes: SA-depleted *lsd1* plants still show increased resistance (Aviv et al., 2002).

Experiments looking for positive regulators of *lsd1* rcd also uncovered the *ADR1* family of NB-LRRs, members of which function as 'helper' NB-LRRs in basal defense (Bonardi et al., 2011). As previously stated, at least one member of this family also has canonical, P-loop dependent immune functions which are discussed chapter 3.

Conclusions

Initial studies of plant disease resistance responses led researches to believe that, for instance, effector-NB-LRR protein interactions were direct, and MAMP and effector triggered immunity were totally separate events. As genetic and molecular mechanisms have been uncovered, a more robust model of the system has been created. Once separated pathways are now seen as part of a larger network. For example, wound response, SA, ROS, MTI, and ETI all result in transcriptional reprogramming, and though the levels of these reactions may differ they frequently involve the same genes or

gene families (Tsuda et al., 2008; Miller et al., 2009). Another example of overlap, published recently, shows that there is an *in planta* association between the PRR FLS2 and the R proteins RPM1, RPS2, and RPS5 (Qi et al., 2011), though the functional consequences of this, if any, remain to be defined.

Defense-related hormone crosstalk, NB-LRRs with multiple independent functions, and positive and negative feedback loop pathways are all further evidence that the picture of disease resistance signaling outputs is more of a web than a linear pathway. As more data is collected, more of these overlaps will be uncovered, further complicating the disease resistance network.

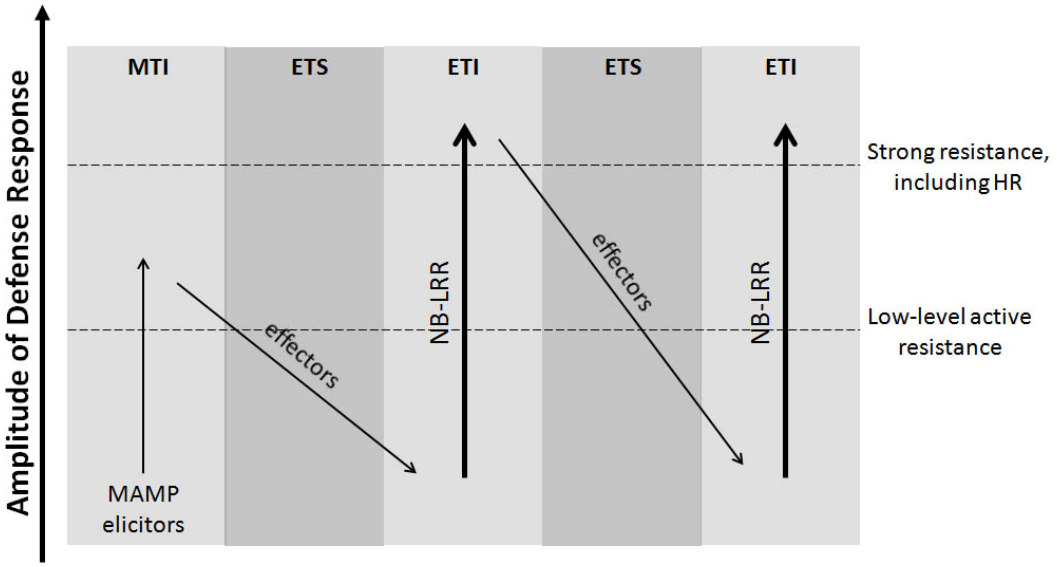

Figure 1.1. The zig-zag model of plant defense. Adapted from Jones and Dangl, 2006. Pathogens are initially recognized by potential hosts via MAMP recognition, leading to induction of low-level disease resistance responses, or MTI (MAMP-triggered immunity). These pathogens have evolved effector proteins, which are delivered using various mechanisms in various pathogens, and block MTI. Recognition of a single effector from the delivered suite by an NB-LRR (middle) leads to stronger resistance responses, including HR, known as ETI (effector-triggered immunity). The recognized effector is deleted by selection from the pathogen's genome, and remaining effectors cause ETS (effector-triggered susceptibility), and one of these effectors is recognized by a second, newly evolved NB-LRR (far right). This cycle of ETI and ETS is repeated both in a single plant-pathogen interaction, and is also the basis for the evolutionary 'arms race' that drives the evolution of new effectors, by the pathogen, and new effector-recognition proteins, from the plant.

Gene	Mutation	Description	Dominant/Recessive	Resistnace Phenotype	Epistasis Analysis
Rx (CC)	MHDV--->MHVV (D460V)	N/A, transient system used (In potato, Rx provides resistnace to Potato Virus X via Coat Protein)	N/A, done in transient tobacco system	Done in benth; get HR; no resistance data	P-loop and SGT1 both required for gain-of function phenotype
snc1 (TIR)	Mutation in NB/LRR linker of At4g16890, which looks like RPP4/5	Dwarfed plants with curly leaves, high SA levels, constituative PR1	snc1/SNC1 plants have constituative PR1 but look normal (Homozygote required for morphological phenotypes)	Increased resistance to *Hpa* Noco2 and *Psm*	PAD4 dependent; EDS1 dependent; NDR1 independent; partially SA dependent (snc1 eds5-3 (SA mutant) had low levels of SA, smaller than wt, and curly leaves, and wasn't resistant to *Psm)*
L6 (TIR)	Multiple different mutations to "D"; Strongest was MHV (MHDQ--->MHVQ)	MAD mutant had dwarf phenoypte, constituative expression of PR1, and resistance to normally virulent flax rust with HR at infection site	Dosage dependent (semi-dominant)	Could not test because could not recover MHV flax plants, death due to "severe leaf necrosis"	EDS1 dependent; P-loop dependent; possibly LRR dependent
I-2 (CC)	MHD--->MHV (D495V)	N/A, transient system used (I-2 provides resistance to Fusarium)	N/A, done in transient tobacco system	Done in benth; get HR; no resistance data	SGT1, RAR1, Hsp90, P-loop dependent
UNI (CC)	Mutation in Ws CC-NB-LRR (in LRR)	Dwarfed, bushy plants with late senescense	Semi-dominant	PR1/5 on	RAR1 dependent; partially SA dependent; independent of EDS1; NDR1 is a suppressor of UNI (*uni ndr1* double mutants are smaller than *uni* plants)
RPP4 (TIR)	Mutation near GLPL domain	Wild-type at 22 degrees; yellowing leading to lethality at 4 degrees; dwarfed and bushy at 16 degrees; high PR1 and SA in induced conditions	Dominant gain of function	Cold-induced responses that resemble HR, including ROS (DAB staining) and cell death	Mutation near RPP4 Walker B mutation suppresses; EDS1 and RAR1 dependent; partially dependent on SA, NPR1, and SGT1b; PAD4 independent
M	MHD--->MHV(D555V)	N/A, transient system used (M confers resistance to flax rust)	N/A, done in transient flax system	Necrosis in flax	P-loop dependent, RIN4 dependent
RPM1 (CC)	MHDV--->MHVV (D505V)	Dwarfed, bushy plants with curly leaves	Dosage-dependent (semi-dominant)	Increased resistance to *Pto* DC3000	P-loop dependent, RIN4 dependent
ADR1-L2 (CC)	MHDV--->MHVV (D484V)	Dwarfed, bushy plants with curly leaves, high SA levels	Recessive (dosage-dependent)	Increased resistance to *Hpa* Noco2 and Emco5, and *Pto* DC3000	RAR1 independent; EDS1 and AtMC1 suppress; partially SA independent

Table 1.1 Autoactive NB-LRR mutants in plants

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Chapter 2

NF-YC3 is a positive regulator of plant disease resistance to *Hyaloperonospora arabidopsidis* that is negatively regulated by LSD1

Preface

For the second chapter, I have included work that will be submitted to PLoS One at the completion of some additional experiments currently being performed by a collaborator (see Discussion for further details). The authors on this paper will be myself, Hiro Kaminaka, Kengo Takabayashi, Fumi Arase, Nicholas Siefers, Ben Holt, and Jeff Dangl. This work was started by Ben Holt at the beginning of his PhD work, when he helped with the initial screens for LSD1 interactors. Ben then performed the pull-down in Supplemental Fig. 2, and then performed the initial characterization of *nf-yc3*. Hiro Kaminaka and his students performed the yeast-2 hybrid experiment and the protoplast localization experiments. I helped with additional characterization of the *nf*-yc3 mutant, performed the NF-YC3 over-expression experiments and the *lsd1* Western blot, created the alignment for Fig. 1, and wrote the manuscript with the help of Ben Holt and Jeff Dangl. This work was performed under the direction of Jeff Dangl.

Abstract

Plants induce a variety of defense responses upon pathogen recognition. A hallmark of disease resistance in plants is the hypersensitive response (HR), a type of programmed cell death. Genetic regulators of cell death have been identified and include the cytosolic zinc finger protein LESION SIMULATING DISEASE 1 (LSD1), a negative regulator of cell death and disease resistance. Here we demonstrate that LSD1 can interact with NF-YC3, a NUCLEAR FACTOR Y, subunit C protein. NF-YC proteins are components of NF-Y transcription factor complexes that regulate many genes in diverse eukaryotic lineages. The LSD1 interaction could sequester NF-YC3 in the cytosol, which would prevent the formation of active NF-Y complexes. Using the combined techniques of yeast two-hybrid, phage display, and site directed mutagenesis, we define a single GxP motif in NF-YC3 as necessary for the LSD1 interaction. *nf-yc3* mutants display moderately increased susceptibility to the oomycete pathogen *Hyaloperonospora arabidopsidis (Hpa)*. Alternatively, plants conditionally over-expressing *NF-YC3* exhibit increased nuclear accumulation of NF-YC3 and corresponding enhancement of resistance to *Hpa*. Therefore, *NF-YC3* is a positive regulator of disease resistance.

Introduction

Plants possess a quick-acting, well-regulated immune system with which they respond to pathogen attacks (Jones and Dangl, 2006). Pathogen recognition is often mediated by plant resistance gene (*R* gene) products. Most R proteins belong to the nucleotide-binding leucine-rich repeat (NB-LRR) superfamily (Ellis and Jones, 1998); these directly or indirectly recognize specific pathogen effector proteins. This recognition

initiates a defense signaling cascade that leads to disease resistance (Pitzschke et al., 2009). Successful disease resistance requires transcriptional re-programming and consequently the production of myriad proteins and cell wall re-enforcements to stop pathogen growth and colonization (Dangl and Jones, 2001). Signal transduction subsequent to recognition thus is likely to culminate in activation of latent transcription factors to up- or down-regulate the transcription of disease resistance-related genes.

The *Arabidopsis thaliana* (Arabidopsis) genome encodes more than 1500 transcription factors (Riechmann et al., 2000). Of these, five families of transcription factors are known to play roles in defense responses: AP2/ERF (APETALA2 /Ethylene-response factors), bHLH (basic helix-loop-helix), bZIP (basic leucine zipper), MYB (myeloblast), and WRKY (characterized by the amino acids tryptophan (W), arginine (R), lysine (K), and tyrosine (Y)) (van Verk et al., 2009). Another transcription factor found in Arabidopsis is the heterotrimeric Nuclear Factor Y (NF-Y), also referred to as the heme-activated protein (HAP) or CCAAT binding factor (CBF). This transcription factor is found in all eukaryotes and regulates a diverse set of genes. In most organisms, each of the three unique NF-Y subunits (NF-YA, NF-YB, and NF-YC) is encoded by one or two genes (Riechmann et al., 2000). However, in Arabidopsis there are 10 NF-YA, 13 NF-YB, and 13 NF-YC subunits (Siefers et al., 2009b). *Brachypodium distachyon* and *Triticum aestivum* also have 35 or more NF-Ys in each of their genomes (Cao et al., 2011; Stephenson et al., 2007), indicating that there has been a generalized NF-Y expansion in the plant lineage.

The NF-Y heterotrimer assembles in a specific, stepwise manner (Maity et al., 1992; Sinha et al., 1996). NF-YB and NF-YC subunits are typically both found in the

cytosol, where they initially form a dimer (Frontini et al., 2004; Goda et al., 2005; Tuncher et al., 2005). NF-YB/C heterodimerization is required for translocation into the nucleus; once there the heterodimer binds the third subunit of the NF-Y family (NF-YA). The mature NF-Y complex binds DNA at the nucleotide sequence *CCAAT* (the "*CCAAT* box") (Ceribelli et al., 2008). The *CCAAT* box is a frequent and widespread promoter element, with functional sites minimally occurring in ~7-8% of mammalian promoters (FitzGerald et al., 2004; Testa et al., 2005). There is no accurate estimate for the number of functional *CCAAT* sites in plants, but Arabidopsis promoters have a higher frequency of this simple pentamer sequence than what is found in humans (Siefers et al., 2009a). NF-Y transcription factors are able to both up- and down-regulate the transcription of *CCAAT* box containing genes (Mantovani, 1999).

Compared to analyses in yeast and mammals, an understanding of whether or how the plant-specific NF-Y expansion leads to mechanistically diverse outputs is lacking. However, mutations in several single-subunit genes display phenotypes. For example, in Arabidopsis and maize, genes encoding NF-YA and NF-YB subunits have been found to promote drought resistance (Li et al., 2008; Nelson et al., 2007). NF-YB and NF-YC subunits both play roles in Arabidopsis flowering time regulation (Kumimoto et al., 2008; Kumimoto et al., 2010a). NF-Y subunits also regulate embryo development (Kwong et al., 2003), as well as blue light and abscisic acid responses (Warpeha et al., 2007). Furthermore, NF-YA and NF-YC subunits are required for proper rhizobial infection and formation of nitrogen fixing nodules in *Medicago truncatula* and *Phaseolus vulgaris* (Combier et al., 2008; Combier et al., 2006; Zanetti et al., 2010). In light of these important and numerous functions, and due to the potential cost of unnecessary

transcriptional activation, it follows that transcription factors such as NF-Ys must themselves be under some form of control.

Localization can regulate transcription factor activity (Whiteside and Goodbourn, 1993); cytoplasmic retention prevents transcription factors from entering the nucleus, thereby thwarting transcriptional activation. In some cases, transcription factors are retained in the cytosol until an appropriate signal causes them to move into the nucleus (Whiteside and Goodbourn, 1993). Such retention can result from the binding of transcription factors to cytosolic proteins that function as interaction modules. One known group of cytosolic interaction modules are zinc finger proteins (Krishna et al., 2003). These molecules use zinc ions to stabilize their protein folds and can bind DNA, RNA and small proteins (Krishna et al., 2003). In Arabidopsis, one such cytosolic zinc finger protein is LSD1, a proposed interaction module and a negative regulator of cell death (Dietrich et al., 1997).

lsd1 mutant plants exhibit inappropriately activated and uncontrolled cell death (Dietrich et al., 1994), leading to runaway cell death (rcd). These mutants express additional defense response phenotypes, including the production of pathogenesis-related (PR) proteins and increased resistance to multiple pathogens (Greenberg, 1997). *lsd1* rcd can be initiated by exposure to pathogens, exogenous application of the plant defense hormone salicylic acid (SA), and changes in growth conditions (Dietrich et al., 1994). The rcd phenotype requires SA, superoxide, and other key genetic components of disease resistance (Aviv et al., 2002). SA-dependent signaling during defense responses leads to increased local and systemic cell death. LSD1, in concert with reactive oxygen intermediates, prevents rcd during this process (Torres et al., 2005). As an interaction

module, LSD1 is known to interact with both transcription factors (Kaminaka et al., 2006) and positive regulators of cell death (Coll et al., 2010; Epple et al., 2003). These interactions take place in the cytosol. The transcription factor bZIP10 is a positive mediator of rcd. LSD1 functions to sequester bZIP10 in the cytosol, thereby preventing its function in transcription of a pro-cell death regulon (Kaminaka et al., 2006). LOL1 and AtMC1, two proteins with LSD1-like zinc-finger motifs, also interact with LSD1 (Coll et al., 2010; Epple et al., 2003). These proteins are also positive regulators of cell death, and in the absence of LSD1 each protein is required for rcd. Taken together, these data indicate that LSD1 may act as a cytoplasmic scaffolding protein, sequestering proteins necessary to appropriately balance cell death and defense responses. As such, other proteins which interact with LSD1 could be important for rcd and/or disease resistance.

We found that LSD1 interacted with the Arabidopsis NF-YC3 subunit in a yeast two-hybrid (Y2H) library screen. Using phage display techniques and directed Y2H assays, we noted that this interaction depended on a plant-specific NF-YC interaction motif. Additionally, *nf-yc3* mutants were more susceptible to infection with the oomycete parasite *Hpa* isolate Cala2. Parallel to loss-of-function analyses, we created a line conditionally over-expressing wild-type (wt) *NF-YC3*. This line exhibited enhanced disease resistance to *Hpa* isolate Emco5. Conditional over-expression of two *NF-YC3* mutants, one unable to form the B/C dimer and another that cannot bind DNA, did not result in enhanced *Hpa* resistance. Thus, using both mutant and over-expression lines, we demonstrate that *NF-YC3* is a positive regulator of disease resistance, likely via transcriptional regulation of defense-related genes. This transcriptional activation of NF-

YC3 could be partially controlled by LSD1 sequestering it in the cytosol, thereby preventing NF-YC3 movement into the nucleus and its subsequent disease resistance function.

Results

LSD1 interacts with the transcription factor NF-YC3. LSD1 is necessary for proper regulation of defense responses and interacts with proteins important in disease resistance (Coll et al., 2010; Dietrich et al., 1994; Kaminaka et al., 2006). To identify additional LSD1-interacting peptides, we performed a phage display using a library of random 12aa epitopes (Kay et al., 1996). GST:LSD1 fusion proteins were purified on glutathione sepharose beads and incubated with the phage library. Phage that bound to LSD1 were isolated and independent phage plaques were sequenced, yielding fifteen unique LSD1-interacting peptides (Figure 2.1A). The consensus sequence **WVWGxP** was found in 11 of the sequenced epitopes, and the G and P positions were invariant in all 15 LSD1 interacting peptides (Figure 2.1B). One of the sequenced variants was a near exact match to a peptide in NF-YC3, which had previously been isolated as an LSD1 interacting protein in Y2H assays. Arabidopsis NF-YC3 has homology to mammalian NF-YC, including the residues required for proper NF-Y formation (Figure 2.2).

To confirm the interaction between LSD1 and NF-YC3, we used a combination of *in vitro* and semi-*in vivo* methods. We first confirmed the Y2H interaction between LSD1 and full-length NF-YC3 (Figure 2.3A, top line). Additionally, we performed a protein immunoprecipitation experiment using *E. coli*-purified GST-NF-YC3 fusion proteins

(Figure 2.4). Purified GST-NF-YC3 was incubated with total protein extracts from Arabidopsis expressing LSD1-Myc under control of the 35S promoter. Excess protein was washed off and proteins bound to GST-NF-YC3 were eluted and separated on an SDS-PAGE gel. GST-NF-YC3 pulled down myc-tagged LSD1 protein, whereas a GST control did not (Figure 2.4A). Protein blots of input proteins showed that these two bands were specific to LSD1-Myc (Figure 2.4B).

NF-YC3 localization is dependent on GxP-mediated LSD1 interaction. As an additional test of whether LSD1 interacts with NF-YC3, we utilized the plant-specific GxP motif found in the phage display. This sequence was found in all phage display clones that bound LSD1 (Figure 2.1B), leading us to hypothesize that it would be necessary for the interaction between LSD1 and NF-YC3. There are 4 sequential GxP motifs in a Q-rich region at the C-terminus of NF-YC3. A truncation containing only this region retained interaction with LSD1 in Y2H assays (Figure 2.3A). Further, using a series of truncation mutations and a point mutation in the second GxP motif (GP2, labeled in the Figure 2.2 alignment), we found that this motif is necessary and likely sufficient for the interaction with LSD1. We note that this particular GxP motif is in a region divergent from human NF-YC.

To test the proposed functionality of the GxP interacting domain, we used different versions of GFP-tagged NF-YC3 transiently expressed in protoplast cells. NF-YC3-GFP was observed in both the nucleus and the cytoplasm (Figure 2.3B). A mutant of NF-YC3 lacking the second GxP motif was also expressed in protoplasts (*p35S*:*NF-*

YC3ΔGP2-GFP, expressing a G182A/P184A mutation). Interestingly, NF-YC3ΔGP2-GFP was only present in the nucleus (Figure 2.3B, right panel), indicating that the GxP motif was required for accumulation in the cytosol. As LSD1 is a known cytosolic protein and previous studies have shown that it works to sequester other transcription factors out of the nucleus (Kaminaka et al., 2006), these results are consistent with the suggestion that LSD1 could retain NF-YC3 in the cytosol.

If LSD1 interacts with NF-YC3, there must be direct interaction between these two proteins in plant cells. To test this hypothesis, we used a bimolecular fluorescence complementation (BiFC) assay to check for direct interaction between the two proteins, albeit under conditions of transient over-expression. LSD1 fused to N-terminal YFP (YFP^N-LSD1) and empty vector C-terminal fragments of YFP (YFP^C) did not produce YFP fluorescence (Figure 2.3C, top). However, strong YFP fluorescence was observed in protoplasts expressing both YFP^N-LSD1 and YFP^C-NF-YC3, indicating that these two proteins are interacting (Figure 2.3C, middle). When the GP2 mutant construct YFP^C-NF-YC3ΔGP2 was expressed in the same cells as YFP^N-LSD1, there was no fluorescent signal (Figure 2.3C, bottom), further indicating that the GxP motif is necessary for interaction between LSD1 and NF-YC3.

To further test the interaction between LSD1 and NF-YC3, we analyzed the nuclear accumulation of NF-YC3 in defense-induced wild-type and *lsd1* mutant plants. Given that NF-YC3 should enter the nucleus in order to affect transcriptional regulation after pathogen recognition in our model, and that LSD1 could sequester NF-YC3 in the cytoplasm, we hypothesized that i) the amount of nuclear NF-YC3 would increase after rcd was triggered, and ii) this increase would be stronger in *lsd1*, where NF-YC3 could

not be as effectively retained in the cytoplasm. Five week old Col-0 (wild-type) and *lsd1-2* plants were sprayed with benzothiadiazole (BTH), a synthetic SA functional analog that induces rcd in *lsd1* (Lawton et al., 1996), and leaf tissue was collected at regular intervals. Protein blots with an NF-YC3-specific antibody (Kumimoto et al., 2010b) demonstrated that NF-YC3 was detectable in the nuclear-enriched fraction of both wild-type and *lsd1-2* plants, and that the amount of protein increased after BTH activation (Figure 2.3D). However, the *lsd1-2* plants showed an overall increased level of nuclear-localized NF-YC3 compared to Col-0, indicating that LSD1 can function to keep NF-YC3 in the cytosol. The lack of hyper-accumulation of NF-YC3 in the nucleus of non-induced (0 time) *lsd1-2* plants suggests that there are likely additional factors besides LSD1 involved in the cytoplasmic retention of NF-YC3.

NF-YC3 is a positive regulator of disease resistance. LSD1 interactors can regulate pathogen responses (Coll et al., 2010; Kaminaka et al., 2006); therefore, NF-YC3 may also play a role in disease resistance. To test this hypothesis, we looked at the effects of NF-YC3 on disease resistance using the obligate biotrophic oomycete *Hpa.* We used the *Hpa* isolate Cala2, which is virulent on the Arabidopsis La-*er* ecotype (Holub et al., 1995). On the Ws ecotype, relatively weak resistance to *Hpa* Cala2 is conferred by *RPP1A* (Botella et al., 1998). We isolated *nf-yc3* homozygous mutant plants in the Ws background from publicly available stocks (Krysan et al., 1999) and demonstrated that they are protein nulls (Figure 2.5A). We inoculated Ws, La-*er*, and *nf-yc3* plants with 5 x 10^4 spores/ml of *Hpa* Cala2. After seven days, the number of sporangiophores per cotyledon was counted. Weak resistance phenotypes, like those seen in Ws (*RPP1A*), are

characterized by little or no sporulation. By contrast La-*er* (*rpp1a*) plants were highly susceptible to pathogen growth, as measured by profuse sporulation (Figure 2.5B). The *nf-yc3* plants exhibited an intermediate level of *Hpa* sporulation.

Infected cotyledons were also stained with trypan blue to study plant cell death and hyphal growth. Strong disease resistance responses exhibited no hyphal growth. However, intermediate resistance was characterized by trailing necrosis, where *Hpa* hyphal growth is accompanied by cell death that "trails" behind the growing hyphae (Davis and Hammerschmidt, 1993). Ws displayed resistance with a minimal amount of hyphal growth and trailing necroses, while La-*er* exhibited significant free hyphal growth (Figure 2.5C). As indicated by the sporangiophore counts, *nf-yc3* mutants displayed an intermediate level of disease resistance with more extensive trailing necroses than Ws, but less total hyphae growth than La-*er*.

To prove that the suppression of *RPP1A*-mediated resistance was due to the loss of NF-YC3, we transformed *nf-yc3-1* plants with a construct containing full length genomic *NF-YC3* driven by its own promoter (*pNF-YC3:NF-YC3*). A protein blot was performed to confirm that NF-YC3 protein accumulation was rescued by the transformation (Figure 2.5A). This complementation line was infected with *Hpa* Cala2, and displayed a low level of sporulation, similar to Ws (Figure 2.5B, far right). Together, this data indicates that NF-YC3 is necessary for full *RPP1A*-mediated resistance to *Hpa* Cala2.

If NF-YC3 is a positive regulator of disease resistance, then its over-expression should lead to increased disease resistance. To test this, we generated a dexamethasone

(Dex)-inducible, HA-tagged version of NF-YC3 and transformed it into wild-type Col-0 plants. When sprayed with 20uM of Dex, pDex:NF-YC3-HA plants expressed significantly more total NF-YC3 protein than non-transgenic parental Col-0 (Figure 2.6A). To assay for an enhancement of disease resistance in these transgenic lines, we used *Hpa* isolate Emco5, which is highly virulent on parental Col-0. For an Emco5 resistant control we used La-*er* plants, which exhibit strong *RPP8*-mediated resistance (McDowell et al., 1998). As expected, Col-0 plants exhibited high levels of sporulation when inoculated with *Hpa* Emco5, and La-*er* plants were resistant to this isolate (Figure 2.6B). Dex application 24 hours pre-inoculation did not affect the results for either control. Sporulation levels on *pDex:NF-YC3-HA* cotyledons that had not been sprayed with Dex were essentially identical to Col-0. However, when *Dex:NF-YC3* plants were sprayed with Dex, the number of *Hpa* Emco5 sporangiophores per cotyledon was reduced to La-*er* levels (Figure 2.6B). Additionally, trypan blue staining of these lines showed that in Col-0 plants with or without Dex, and *pDex:NF-YC3-HA* plants without Dex, there were high levels of free hyphae (Figure 2.6C). Conversely, Dex-induced *pDex:NF-YC3-HA* cotyledons exhibited no free hyphal growth, and were therefore disease resistant. While these plants exhibit some increases in cell death post Dex induction, disease resistance occurs prior to the appearance of cell death symptoms. This was demonstrated by the presence of numerous *Hpa* spores arrested at the penetration peg stage (prior to production of hyphae or death of surrounding cells; see magnified view in Figure 2.6C). Therefore, increased *Hpa* resistance is due to over-expression of NF-YC3. This finding, along with the opposing phenotype expressed by the *nf-yc3*

mutant data, supports our conclusion that *NF-YC3* is a positive regulator of disease resistance.

NF-YC3 function requires proper heterotrimeric NF-Y formation. As noted in the Introduction, NF-Y-containing transcription factors assemble in a specific manner and this formation is required for proper NF-Y-related transcriptional regulation. Specific conserved residues in the NF-YC subunits are required for both dimerization and DNA binding (Sinha et al., 1996). Dimerization is coordinated by conserved isoleucine (I) and leucine (L) residues that are highly conserved between plant and animal NF-YC proteins (Figure 2.2, arrows; (Cao et al., 2011; Siefers et al., 2009a)). Mutations in these residues disrupt dimerization, subsequent NF-Y formation, and transcriptional regulation in mammals (Sinha et al., 1996). A second conserved site in NF-YC is composed of alanine (A) and arginine (R) residues. Disruption of these residues prevents mature heterotrimeric NF-Y complexes from binding DNA. We predicted that disruptions in these residues in NF-YC3 would interfere with NF-Y complex formation, but not the LSD1 interaction, therefore eliminating the increased resistance to *Hpa* Emco5 observed when wild-type NF-YC3 is overexpressed.

To test this hypothesis, we created transgenic Col-0 expressing Dex-inducible NF-YC3 with either the I105D/L108E (predicted to interrupt NF-YB/C dimerization) or A74D/R75P (predicted to interrupt DNA binding) mutations (*pDex:NF-YC3ΔIL-HA* and *pDex:NF-YC3ΔAR-HA*, respectively). As expected, these proteins still associated with LSD1 at or near wild-type levels, as shown via Y2H assays (Figure 2.3A). Although NF-

YC3ΔAR-HA accumulated in the nucleus, we did not measure significant nuclear accumulation of NF-YC3ΔIL-HA (Figure 2.6A). Next, we challanged *pDex:NF-YC3ΔIL-HA* and *pDex:NF-YC3ΔAR-HA* plants with the *Hpa* isolate Emco5, with and without Dex induction. Dex-induced accumulation of these mutant proteins did not lead to substantially increased resistance to *Hpa* Emco5 (Figure 2.6D). These data suggest that NF-YC3 functions in a heterotrimeric complex and binds DNA to confer disease resistance to *Hpa*.

Discussion

Our key finding is that Arabidopsis NF-YC3 functions as a positive regulator of *Hpa* disease resistance, presumably by contributing to the overall up-regulation of disease resistance-related genes and/or cell death genes. Additionally, we discovered that LSD1 potentially participates in NF-YC3 cytosolic retention. As there is no hyper-accumulation of NF-YC3 in the nucleus of *lsd1* plants, we hypothesize that LSD1 may be working with other factors to regulate NF-YC3 nuclear accumulation. These retention factors may prevent interactions between NF-YB and NF-YC subunits, and thus their subsequent movement into the nucleus to form a functionally active NF-Y heterotrimer, as previously described in mammals and yeast (Ceribelli et al., 2008). LSD1 was previously shown to antagonize the nuclear shuttling of a defense-related transcription factor, AtbZIP10, resulting in increased disease resistance (Kaminaka et al., 2006), and also associates with other positive mediators of disease resistance and cell death (Coll et al., 2010). In this work, we provide additional evidence that LSD1 may function as a

transcriptional regulatory scaffold, sequestering defense-related proteins in the cytosol, and dampening their functions.

There is, however, one main caveat with the current data, specifically with the data indicating that LSD1 is functioning as a retention factor of NF-YC3. The key problem is that the current BiFC data shows that all the fluorescence is in the nucleus when both LSD1 and NF-YC3 are co-expressed (Figure 2.3C). This is in contrast to previously published data, which has shown LSD1 to be a cytosolic protein, and is also in opposition to the data from Figure 2.3B, which indicates that an intact LSD1 interaction motif is necessary for NF-YC3 to accumulate in the cytosol. The nuclear localization currently seen in Fiure 2.3C could be due to false nuclear localization of the xFP fusions, or could be a localization artifact of BiFC, either of which could be due to the over-expression of both fusions in the protoplast assay.

To address these problems, additional experiments are being performed by our collaborator Hiro Kaminaka. First, we will add a panel of LSD1-GFP to be the third row in Figure 2.3B to demonstrate LSD1 localization on its own. We will then set up a new Figure 2.3C, which will use co-localization of two xFP colors to address the specificity of the interaction between LSD1 and NF-YC3. This new figure would be co-over-expression of LSD1-xFP with, first NF-YC3-xFP, and second with NF-YC3ΔGP2-xFP. These constructs must express different FPs for LSD1 and the NF-YC3 constructs so that they can each be imaged at the same time, and a merged image can then be made for the figure. The anticipated result is that the LSD1 / NF-YC3 will co-localize in the cytosol (with perhaps some NF-YC3 signal in the nucleus) and that LSD1/ NF-YC3ΔGP2 will

show that LSD1 is in the cytosol and NF-YC3ΔGP2 is in the nucleus, as predicted by the current Figure 2.3B.

Even with these caveats, our data suggests that NF-YC3 positively regulates plant disease resistance. Over-expression of NF-YC3 clearly led to strong resistance to a normally highly virulent *Hpa* strain, although the loss of function phenotypes were relatively mild, possibly due to overlapping functionality with other NF-YC family member. Indeed, NF-YC4 was also identified as a potential LSD1 interactor via our Y2H screen, and this protein has the requisite LSD1-binding GxP sequence (unpublished data). Six additional Arabidopsis NF-YCs in Arabidopsis also contain the conserved GxP interaction motif, though their association with LSD1 has not yet been demonstrated. Functional overlap between these, or other NF-YA and NF-YB subunits, and NF-YC3 and LSD1 are the target of future work. However, this may be difficult to parse, due to the inherit redundancy of the large NF-Y gene families. Therefore, serial deletion or mutation of the *NF-Y* subunits may help to broaden the understanding of the function of these genes in plant defense.

Our studies also suggest that the specific amino acid residues identified in other systems for NF-Y assembly are conserved in Arabidopsis. The step-by-step assembly of the mature NF-Y has been extensively studied (Maity et al., 1992; Sinha et al., 1996). Here we provide genetic evidence demonstrating that NF-Y transcription factor formation is likely to proceed similarly in plants. The conserved IL and AR residues, previously shown to be necessary for NF-YB/C interaction and NF-Y DNA binding, respectively (Sinha et al., 1996), are required for at least the disease resistance phenotype we measure,

suggesting that the Arabidopsis proteins act analogously to their yeast and animal counterparts.

In the simplest model consistent with our data, LSD1, likely working with other immune-related proteins, retains NF-YC3 in the cytoplasm, preventing it from forming a functional NF-Y complex capable of driving defense-related gene transcription in the nucleus. A pathogen-induced signal, provided here by inoculation with *Hpa*, causes dissociation of NF-YC3 from LSD1. NF-YC3 is then able to bind an NF-YB, enter the nucleus, and form the active NF-Y complex which regulates the transcription of pro-defense genes. Further work to explore both the interactions between proteins regulated by LSD1 retention, as well as studies designed to define the relevant NF-YA and NF-YB subunits, and to indentify the set of defense genes induced by the NF-YC3-containing NF-Y, will allow a better understanding of the role of NF-Y transcriptional regulation in the plant defense response.

Materials and Methods

Plant Materials and Growth Conditions

We used *Arabidopsis thaliana* Columbia (Col-0), Landsberg erecta (La-*er*), and Wassilewskija (Ws) ecotypes. Mutant *nf-yc3* in the Ws background is a protein null T-DNA insertion line (-492bp from ATG). *nf-yc3 pNF-YC3:NF-YC3* (Ws), *Dex:NF-YC3-HA* (Col-0), *Dex:NF-YC3ΔIL-HA* (Col-0), and *Dex:NF-YC3ΔAR-HA* (Col-0) were cloned in the pGWB1 Gateway vector, and Arabidopsis transgenics were generated using Agrobacterium (GV3101)-mediated floral dip transformation (Clough and Bent, 1998). *lsd1-2* is in the Col-0 background. Plants were grown under short day conditions (9 hrs light, 21°C; 15 hrs dark, 18°C).

Phage Display

All phage display techniques used were performed as previously described (Kay et al., 1996). GST-LSD1 fusion proteins were purified with glutathione sepharose beads as per the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ). GST-LSD1 was eluted from the sepharose beads with glutathione prior to binding to the wells of high protein binding ELISA plates (Fisher Scientific, Atlanta, GA) and screening of the phage library. The phage library consisted of random 12 amino acid insertions into the pIII gene of M13 phage and was supplied as a generous gift from Brian Kay (University of Wisconsin, Madison). The vector pMYAP was used for expression of the phage epitopes fused to alkaline phosphatase as previously described (Yamabhai and

Kay, 2001). Epitopes from randomly selected independent phage plaques were sequenced, and 15 unique sequences were confirmed.

Yeast Two-Hybrid Assay

The fragments of mutated or deleted NF-YC3 cDNA were created by PCR-based mutagenesis. All NF-YC3 fragments including full-length, mutated and deleted cDNAs were cloned into pENTR-D-TOPO (Invitrogen). After verifying the nucleotide sequence of PCR fragments by sequencing, all NF-YC3 fragments were then transferred into pJG4-5gw (Holt et al., 2005) using LR clonase II (Invitrogen). Yeast two-hybrid assay using LexA-based two hybrid system was basically carried out as described previously (Kaminaka et al., 2006). Briefly, the transformation of yeast cells EGY48 (*MATα ura3 trp1 his3 3LexAop-leu2*) harboring pJK103 [*2lexAop-lacZ*]) reporter plasmid was carried out with the Frozen-EZ Yeast Transformation II Kit (Zymo Research). Transformants with both LSD1 in pEG202 (Kaminaka et al., 2006) and NY-FC3 fragments in pJG4-5 were grown on glucose base selection medium [SD(Glu)/-Ura/-His/-Trp], and then independent clones of each transformant were plated on galactose and raffinose base selection medium [SD(Gal)/-Ura/-His/-Trp] containing X-gal to perform semi-quantitative β-galactosidase activity assay on gel. Level of each interaction was also evaluated by measurement of β-gal activity using o-Nitrophenyl-β-D-galactopyranoside (ONPG) method according to Yeast Handbook (Clonetech).

Subcellular localization analysis using GFP and BiFC analysis in *Arabidopsis* mesophyll protoplasts

For the construction of NF-YC3-GFP fusion genes driven by the CaMV 35S promoter, NF-YC3 wild-type and NF-YC3ΔGP2 cDNA fragments made as described above were transferred into p2GWF7 (Karimi et al., 2002), using LR clonase II (Invitrogen). Similarly, for BiFC experiments, YFP^N-LSD1 and YFP^C-NF-YC3 genes driven by the CaMV 35S promoter were made by transferring LSD1 full-length cDNA and NF-YC3 wild-type or NF-YC3ΔGP2 cDNA fragments into nYFP/pUGW0 and cYFP/pUGW0 (Singh et al., 2009), respectively, using the Gateway LR recombination reaction. As a negative control, YFP^C alone driven by the CaMV 35S promoter was also created by PCR-based mutagenesis using YFP^C/pUGW2. NLS-tdTomato driven by the CaMV 35S promoter was used as a nuclear organelle marker and a control for transformation (Arase et al., 2012). Transient expression in *Arabidopsis* mesophyll protoplasts and assay for fluorescence using a confocal laser scanning microscopy was carried out as described (Arase et al., 2012).

Immunoblot Analysis.

Leaves from 2-wk-old plants were harvested, and total proteins were extracted by grinding frozen tissue in a buffer containing 20mM Tris (pH8), 0.33M Sucrose, 1mM EDTA (pH8), 5 mM DTT, and plant protein protease inhibitor mixture (Sigma-Aldrich). Samples were centrifuged at 2,000 × g for 5 min at 4°C to pellet debris, and a portion of the supernatant was set aside (total protein). The remaining supernatant was centrifuged

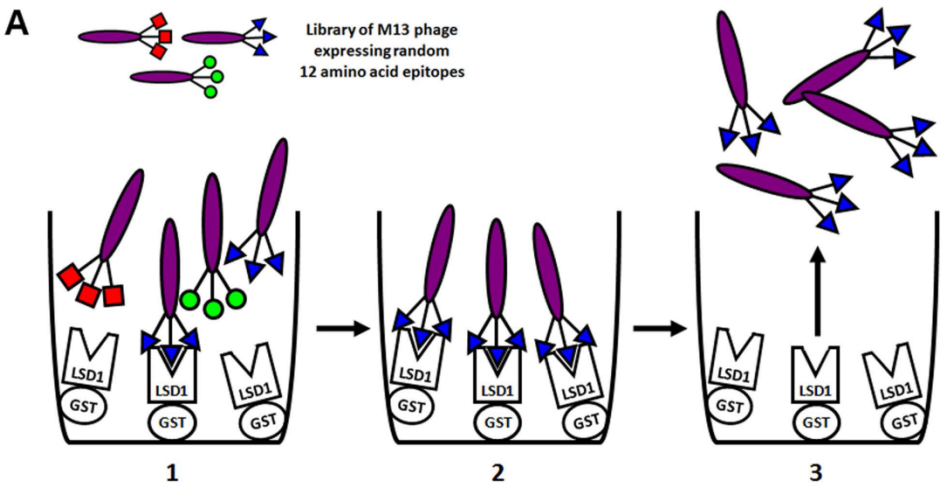
at full speed (~20G) for 30 min at 4°C. The resulting supernatant was transferred to a new tube (soluble fraction), and the pellet was resuspended (nuclear-enriched fraction). Proteins were separated on 12% SDS/PAGE gels and were transferred to polyvinylidene difluoride membrane. Protein blots were performed using standard methods. Anti-NF-YC3 antibody was used at a 1:3,000 dilution. Signals were detected by enhanced chemiluminescence using ECL Plus (Amersham Biosciences).

Hpa infection assays

Twelve- to fourteen-day-old seedlings were inoculated with 50,000 spores/ml of *Hyaloperonospora arabidopsidis* isolate Emco5 or Cala2. These plants were covered with a lid to increase humidity during inoculation and pathogen growth. Sporangiophores counted at 7 dpi as described (Holt et al., 2002). Trypan blue staining for cell death and hyphal growth as previously described.

Semi-*in vivo* Pulldown

Total protein was extracted from Arabidopsis expressing a 35S:LSD1-myc transgene. GST:NF-YC3 was purified from *E.coli* using glutathione sepharose beads. This gel matrix was incubated with the protein extract, and then the beads were precipitated from the solution. Bound proteins were eluted and run on an SDS-PAGE gel.



B

LSD1 Interacting Phage Clones

S	K	Q	D	**W**	**V**	**W**	**G**	A	**P**	V	R	
Y	D	Q	S	**W**	**V**	**W**	**G**	M	**P**	G	T	
			D	**W**	**V**	**W**	**G**	F	**P**	R	S	D	P	V
G	E	D	T	**W**	**V**	**W**	**G**	V	**P**	M	G	
		D	G	T	**V**	**W**	**G**	W	**P**	V	G	N	V	
			D	**W**	**V**	**W**	**G**	D	**P**	E	T	D	P	P
			G	**W**	E	**W**	**G**	G	**P**	I	E	S	N	S
		E	S	**W**	**V**	**W**	**G**	I	**P**	T	A	S	G	
	S	Q	K	T	**V**	**W**	**G**	F	**P**	L	W	E		
Y	D	Q	S	**W**	**V**	**W**	**G**	M	**P**	A	T			
L	D	G	G	**M**	**V**	**M**	**G**	Y	**P**	V	T			
		G	D	**W**	**V**	**W**	**G**	T	**P**	V	S	G	S	
A	S	F	D	K	**W**	**V**	**W**	**G**	T	**P**	V			
	G	G	Q	D	**W**	**V**	**W**	**G**	M	**P**	F	G		
G	H	A	G	E	T	**V**	**W**	**G**	W	**P**	V			

Consensus: WVWGxP

Figure 2.1: LSD1, a negative regulator of cell death, interacts with members of the NF-Y transcription factor family. A) Phage display: 1. GST-tagged LSD1 is bound to wells and a phage library of randomly-generated, 12 amino acid-long epitopes is added to the wells; 2. Interacting proteins bind to LSD1 and other phage are washed off; 3. Proteins attached to LSD1 are eluted and sequenced. B) A consensus sequence (WVWGxP) was found in a majority of sequenced epitopes.

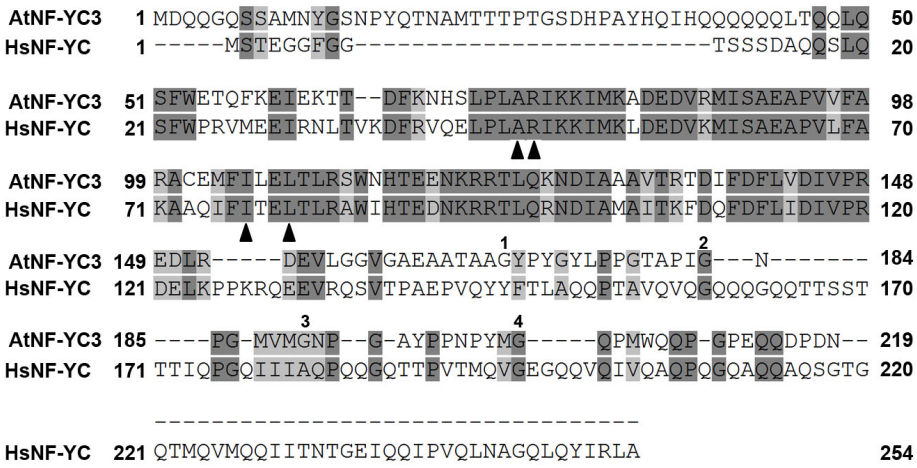
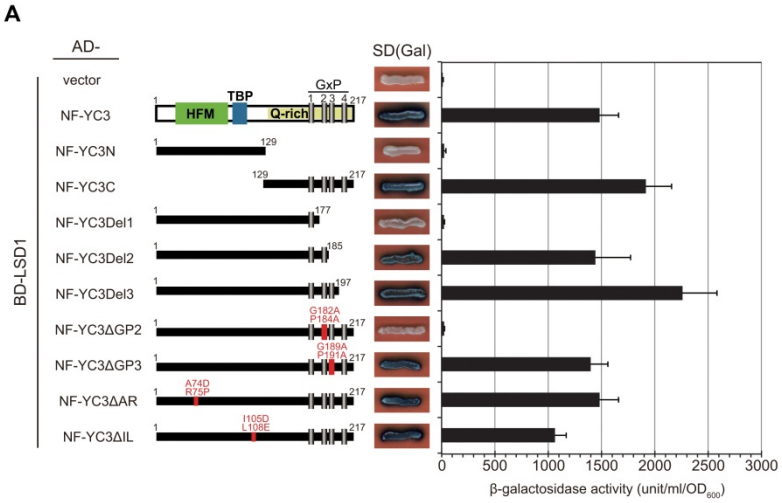
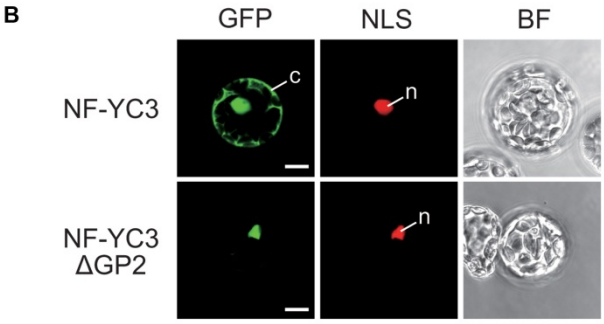
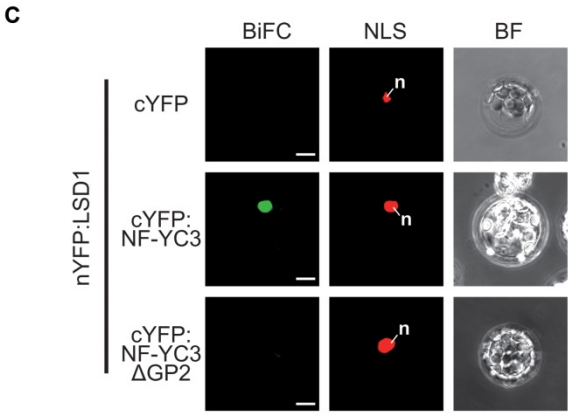


Figure 2.2: NF-YC Transcription factor subunits are conserved across eukaryotes. A) Alignment of *Homo sapiens* NF-YC and Arabidopsis NF-YC3 deduced protein sequences. Alignment was created using VectorNTI AlignX (Invitrogen). Residues shaded in dark grey are identical between species, those in light grey are similar. The NF-YC3 and mammalian NF-YC histone fold motifs are highly conserved; A74 and R75 are required for the complex to bind DNA and I105 and L108 are required for NF-YC to bind NF-YA. Arrowheads mark A74, R75, I105, and L108; numbers 1-4 indicate GxP motifs (potential LSD1 interacting motifs).


A
AD-
SD(Gal)
vector
NF-YC3
NF-YC3N
NF-YC3C
NF-YC3Del1
NF-YC3Del2
NF-YC3Del3
NF-YC3ΔGP2
NF-YC3ΔGP3
NF-YC3ΔAR
NF-YC3ΔIL
BD-LSD1
TBP
HFM
Q-rich
GxP
1 2 3 4
G182A
P184A
G189A
P191A
A74D
R75P
I105D
L108E
0 500 1000 1500 2000 2500 3000
β-galactosidase activity (unit/ml/OD600)


B
GFP
NLS
BF
NF-YC3
NF-YC3 ΔGP2
c
n


C
BiFC
NLS
BF
nYFP:LSD1
cYFP
cYFP: NF-YC3
cYFP: NF-YC3 ΔGP2
n

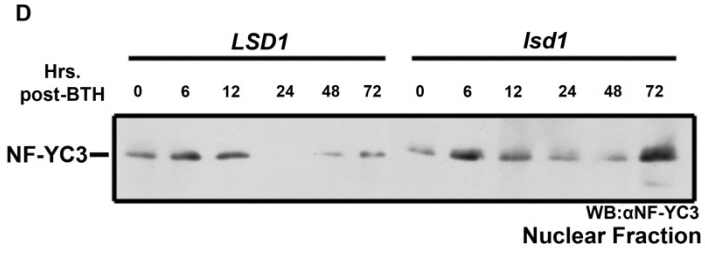

D
LSD1
lsd1
Hrs. post-BTH
0 6 12 24 48 72 0 6 12 24 48 72
NF-YC3
WB:αNF-YC3
Nuclear Fraction

Figure 2.3: LSD1 interacts with the NF-Y subunit NF-YC3. A) β-gal activity assay based on two-hybrid system in yeast showing specific interaction between LSD1 and NF-YC3 through GxP motif. Yeast (EGY48:pJK103) cells were co-transformed with BD-LSD1 bait plasmid and AD-NF-YC3 prey plasmid d with bait plasmid including LexA DNA-binding domain (BD)-LSD1 fusion (BD-LSD1) in pEG202 and prey plasmid including activation domain (AD)-NF-YC3 fusions (AD-NY-FC3s) in pJG4-5. To observe the interaction, semi-quantitative β-gal activity assay was carried out by plating transformants on SD(Gal)/-Ura/-His/-Trp medium containing X-gal. The level of each interaction was also evaluated by measurement of β-gal activity using the ONPG method. Vector indicates empty vector (negative control experiment). Strong, approximately equivalent expression of the NF-YC3 truncation and point mutant proteins in yeast was verified by protein blot analysis (data not shown). B) Subcellular localization of NF-YC3-GFP and NF-YC3ΔGP2-GFP. GFP fusions of NF-YC3 or NF-YC3ΔGP2 and NLS-tdTomato, as a nucleus marker and as a control for transformation, were co-introduced in *Arabidopsis* mesophyll protoplasts. GFP, NLS, and BF (top) represent GFP and tdTomato fluorescence and bright field images, respectively. n: nucleus, c: cytosol. Bars = 10 μm. C) BiFC assay was used for the detection of *in vivo* protein-protein interaction between LSD1 and NF-YC3. YFP^N-LSD1 and YFP^C:NY-FC3 or YFP^C fusions were transiently co-expressed in *Arabidopsis* mesophyll protoplasts with a nuclear marker NLS-tdTomato. BiFC, NLS, and BF (top) represent YFP and tdTomato fluorescence and bright field images, respectively. c: cytosol. Bars = 10 μm. D) *lsd1* plants have stronger NF-YC3 induction than wild-type. 5 week old Col-0 and *lsd1-2* plants were sprayed with 300μM BTH and collected at time points indicated. Protein was extracted and this extract was spun to separate the nuclear fraction, which was run on SDS-PAGE gels and immunoblotted with anti-NF-YC3 antibody.

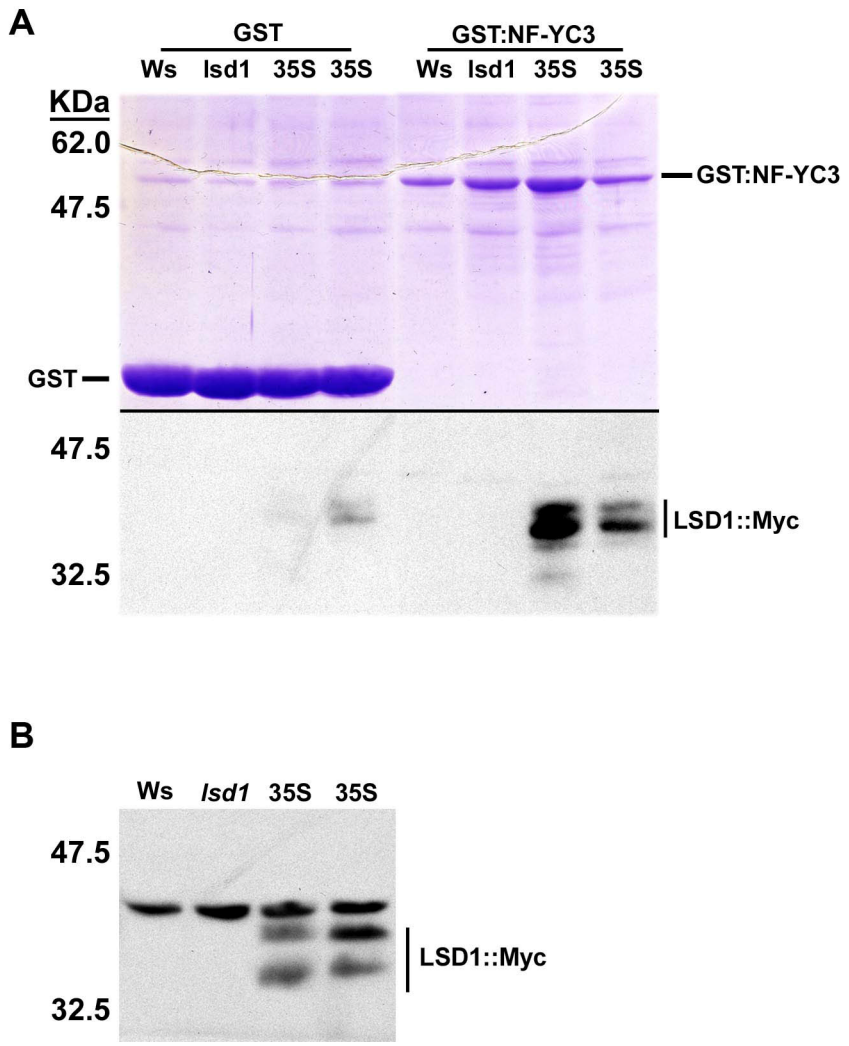


Figure 2.4: Semi-in vivo pulldown. A) Semi-*in vivo* pulldown using GST-bound NF-YC3, and adding plant extract containing 35S-overexpressed LSD1-myc. GST:NF-YC3 is able to pull down myc-tagged LSD1. B) Protein blot showing specificity of myc-tagged proteins used in (A).

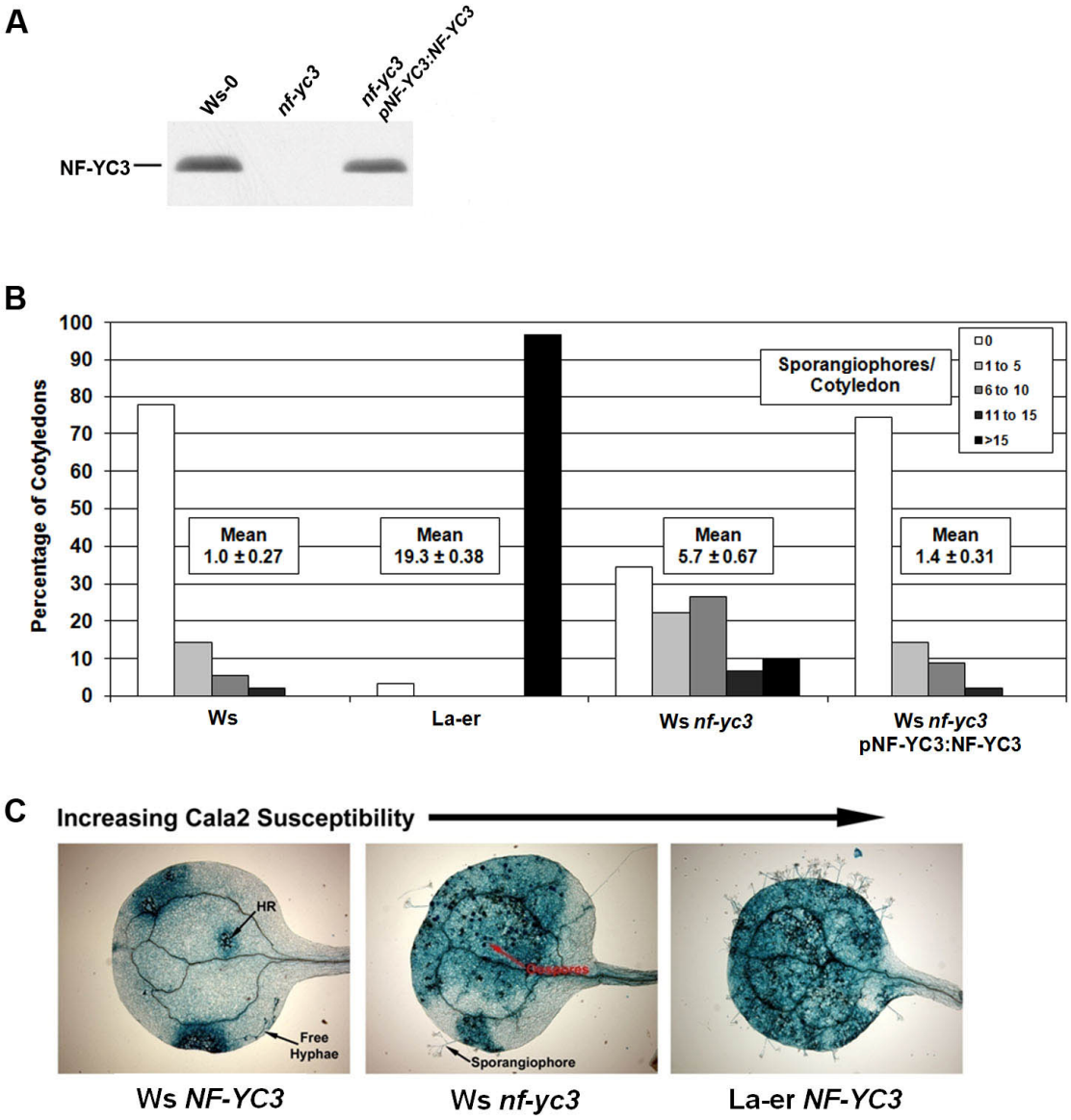
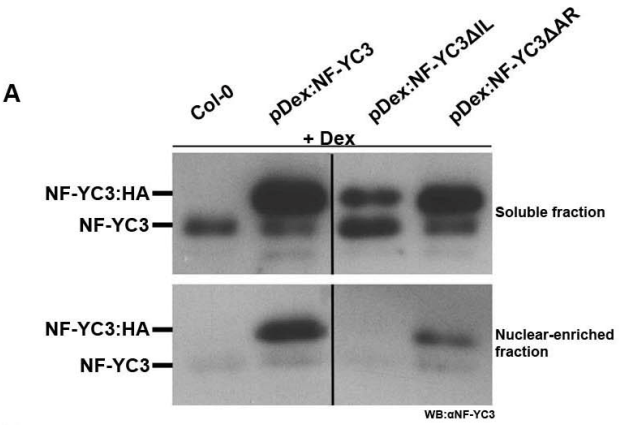
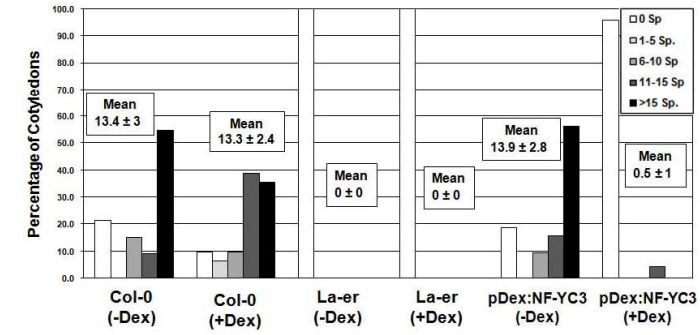
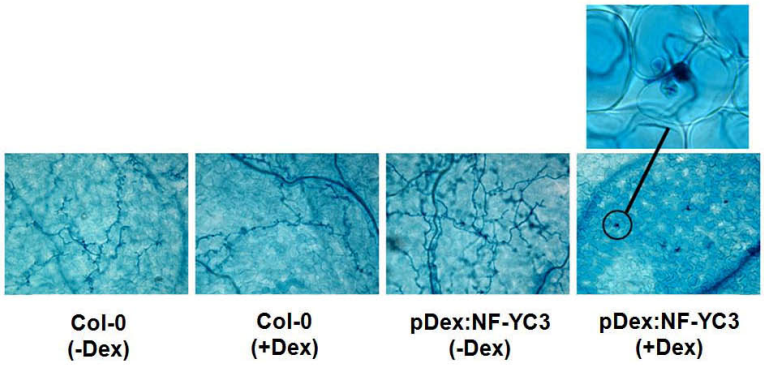


Figure 2.5: NF-YC3 is required for full pathogen resistance. A) Protein extracted from plants in (B) was run on an SDS-PAGE gel and immunoblotted with anti-NF-YC3. B) Two week old plants were sprayed with *Hpa* isolate Cala and sporangiophores counted 6dpi. *NF-YC3* is in Ws background; Ws *pNF-YC3:NF-YC3* is the *nf-yc3* mutant complemented with native-promoter-driven *NF-YC3*. C) Plants from (B) were stained with trypan blue to assay *Hpa* HR, free hyphae, and sporangiophore growth.


A
Col-0
pDex:NF-YC3
pDex:NF-YC3ΔIL
pDex:NF-YC3ΔAR
+ Dex
NF-YC3:HA
NF-YC3
Soluble fraction
NF-YC3:HA
NF-YC3
Nuclear-enriched fraction
WB:αNF-YC3


B
Percentage of Cotyledons
0 Sp
1-5 Sp.
6-10 Sp
11-15 Sp
>15 Sp.
Mean 13.4 ± 3
Mean 13.3 ± 2.4
Mean 0 ± 0
Mean 0 ± 0
Mean 13.9 ± 2.8
Mean 0.5 ± 1
Col-0 (-Dex)
Col-0 (+Dex)
La-er (-Dex)
La-er (+Dex)
pDex:NF-YC3 (-Dex)
pDex:NF-YC3 (+Dex)


C
Col-0 (-Dex)
Col-0 (+Dex)
pDex:NF-YC3 (-Dex)
pDex:NF-YC3 (+Dex)

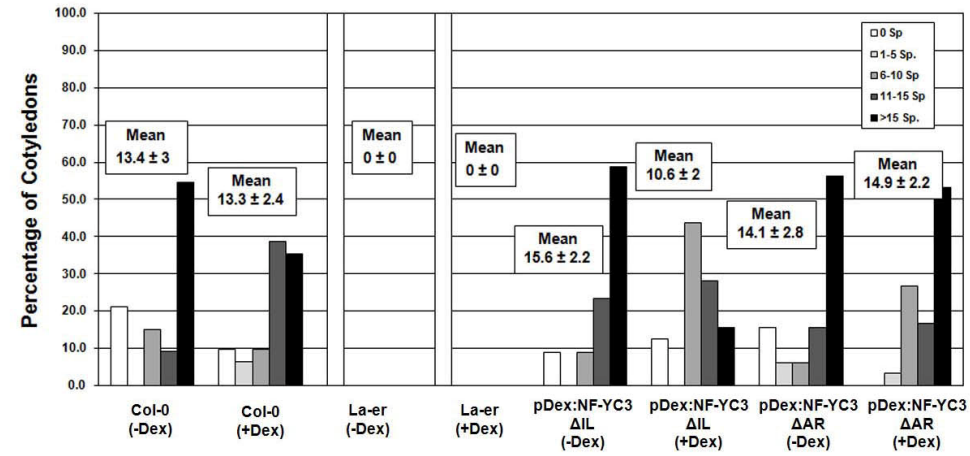

D
Percentage of Cotyledons
0 Sp
1-5 Sp.
6-10 Sp
11-15 Sp
>15 Sp.
Mean 13.4 ± 3
Mean 13.3 ± 2.4
Mean 0 ± 0
Mean 0 ± 0
Mean 15.6 ± 2.2
Mean 10.6 ± 2
Mean 14.1 ± 2.8
Mean 14.9 ± 2.2
Col-0 (-Dex)
Col-0 (+Dex)
La-er (-Dex)
La-er (+Dex)
pDex:NF-YC3 ΔIL (-Dex)
pDex:NF-YC3 ΔIL (+Dex)
pDex:NF-YC3 ΔAR (-Dex)
pDex:NF-YC3 ΔAR (+Dex)

Figure 2.6: NF-Y assembly and DNA interactions are required for induced pathogen resistance. A) Two week old Col-0, *pDex:NF-YC3-HA*, *pDex:NF-YC3ΔIL-HA* and *pDex:NF-YC3ΔAR-HA* were sprayed with silwet or silwet and 20uM dexamethasone. 24 hours later, protein was extracted from these plants and centrifuged to separate the soluble and nuclear fractions, which were run on SDS-PAGE gels and immunoblotted with anti-NF-YC3 antibody. Labels indicate endogenous NF-YC3 protein and HA-tagged, Dex induced NF-YC protein. Nuclear fraction is 4 times overloaded as compared to soluble fraction. B) Col-0, La-*er*, and *pDex:NF-YC3-HA* were sprayed with silwet or silwet and 20uM dexamethasone, and 24 hours later inoculated with *Hpa* isolate Emco5. Sporangiophores were counted 6dpi. C) Plants from (B) were stained with trypan blue; close-up is of arrested growth of Emco5 in dexamethasone-induced *pDex*:*NF-YC3-HA* plant. D) Two week old Col-0, La-*er*, *pDex:NF-YC3ΔIL-HA* and *pDex:NF-YC3ΔAR-HA* plants were sprayed with silwet or silwet and 20uM dexamethasone, and 24 hours later inoculated with *Hpa* isolate Emco5. Sporangiophores were counted 6dpi.

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Chapter 3

Genetic requirements for signaling from an autoactive plant NB-LRR intracellular innate immune receptor

Preface

Prior to the work reported in this chapter we published a paper on the mutant phenotypes of the ADR1 family (Bonardi et al., 2011). This preface quickly summarizes that paper.

Abstract Plants and animals deploy intracellular immune receptors that perceive specific pathogen effector proteins and microbial products delivered into the host cell. We demonstrate that the ADR1 (Activated Disease Resistance 1) family of Arabidopsis NB-LRR receptors regulates accumulation of the defense hormone Salicylic Acid (SA) during three different types of immune response: (i) they are required as 'helper NB-LRRs' to transduce signals downstream of specific NB-LRR receptor activation during effector-triggered immunity (ETI), (ii) they are required for basal defense against virulent pathogens, and (iii) they regulate microbial associated molecular pattern (MAMP)-dependent SA accumulation induced by infection with a disarmed pathogen. Remarkably, these functions do not require an intact P-loop motif for at least one ADR1 family member. Our results suggest that some NB-LRR proteins can serve additional functions

beyond canonical, P-loop-dependent activation by specific virulence effectors, extending analogies between intracellular innate immune receptor function from plants and animals.

Conclusions ADR1-L2, a positive regulator of *lsd1* rcd, is a part of a small family of NB-LRRs. This protein functions downstream of ROI production, and upstream of SA accumulation in basal defense and MAMP-triggered SA accumulation. ADR1-L2 also functions as a 'helper' protein during some, but not all ETI responses driven by effector-mediated activation of other NB-LRR.proteins. Surprisingly, none of these defense functions require an intact P-loop. We speculate that in these contexts, ADR1-L2 may be working in association with an additional, P-loop dependent NB-LRR, perhaps as a scaffold protein in a signal transduction pathway.

My Contributions For this paper, I characterized the *adr1* family mutant lines, represented in Supplemental Fig 1. I also helped with design and set-up of the ROS burst experiments, edited the paper, and contributed to the writing of the Material and Methods section.

Abstract

Plants react to pathogen attack via recognition of and response to pathogen-specific molecules at the cell surface and inside the cell. Pathogen effectors (virulence factors) are monitored by intracellular nucleotide-binding leucine-rich repeat (NB-LRR) sensor proteins in plants and mammals. Here, we study the genetic requirements for defense responses of an autoactive mutant of ADR1-L2, an Arabidopsis coiled-coil (CC)-NB-LRR protein. ADR1-L2 functions upstream of salicylic acid (SA) accumulation in several defense contexts, and can act as a 'helper' to transduce specific microbial activation signals from 'sensor' NB-LRRs. ADR1-L2 and another of two closely related members of this small NB-LRR family are required for propagation of unregulated runaway cell death (rcd) in an *lsd1* mutant. We demonstrate that, in this context, ADR1-L2 function is P-loop dependent. We generated an autoactive missense mutation, ADR1-$L2_{D484V}$, in a small homology region termed MHD. Expression of ADR1-$L2_{D848V}$ leads to dwarfed plants that exhibit increased disease resistance and constitutively high SA levels. The morphological phenotype also requires an intact P-loop, suggesting that these ADR1-$L2_{D484V}$ phenotypes reflect canonical activation of this NB-LRR protein. We used ADR1-$L2_{D484V}$ to define requirements for signaling. Signaling from ADR1-$L2_{D484V}$ does not require NADPH oxidase, and is negatively regulated by EDS1 and AtMC1. Transcriptional regulation of *ADR1-L2*$_{D484V}$ is correlated to its phenotypic outputs; these outputs are both SA-dependent and -independent. The genetic requirements for ADR1-$L2_{D484V}$ activity resemble those that regulate the SA-gradient-dependent signal amplification of defense and cell death signaling observed in the absence of LSD1. Together, these data allows us to propose a genetic model which provides further insight

about the proteins that function in an SA-dependent feedback regulation loop, which surprisingly includes ADR1-L2.

Introduction

Plants encounter a wide variety of pathogens. To defend against infection, plants evolved an active, two-layered immune system (Jones and Dangl, 2006). The first branch utilizes transmembrane receptors (PRRs, or pattern recognition receptors) which detect microbe-associated molecular patterns (MAMPs) of various pathogens (Segonzac and Zipfel, 2011). MAMP detection elicits a rapid, relatively low-amplitude host transcriptional response resulting in MAMP-triggered immunity (MTI) which is sufficient to halt growth of many microbes (Jones and Dangl, 2006; Boller and Felix, 2009). Successful pathogens can suppress or delay MTI via delivery of effector molecules into host cells. Effectors are virulence proteins (Dodds and Rathjen, 2010). Gram-negative bacterial pathogens deliver effectors via injection into the plant cell by the Type III Secretion System (TTSS). Plants respond to effectors with the second tier of recognition, which is dependent on highly polymorphic intracellular disease resistance (R) proteins of the NB-LRR family. NB-LRRs are specifically activated by the presence and/or action of effectors to trigger robust defense responses termed Effector-Triggered Immunity (ETI), which can include localized hypersensitive cell death (Jones and Dangl, 2006).

NB-LRR proteins are members of the signal transduction ATPases with numerous domains (STAND) superfamily, which also includes animal innate immune sensors of the

nucleotide-binding domain and leucine-rich repeat-containing (NLR) class (Leipe et al., 2004; Lukasik and Takken, 2009). STAND proteins are ATPases that function as molecular switches: in the "off" position they bind ADP, and in the "on" position they bind ATP, activating nucleotide hydrolysis and triggering downstream defense responses (Takken et al., 2006). Two essential, conserved homology regions necessary for proper plant NB-LRR activity are the P-loop (Walker-A) and the thus far plant-specific MHD domain located between the NB domain and the start of the LRRs. Mutations in the P-loop typically lead to loss of function (Tameling et al., 2002; Hanson and Whiteheart, 2005). Conversely, mutation of the Asp (D) in the MHD domain often leads to autoactivity of the NB-LRR (Bendahmane et al., 2002; Howles et al., 2005; Tameling et al., 2006; Gao et al., 2011; Williams et al., 2011; Zhang et al., 2012) resulting in either lethality or a severely dwarfed morphology thought to be the consequence of ectopic accumulation of SA, a key defense hormone whose synthesis from chorismate is controlled by the isochorismate synthase gene (ICS1) (Wildermuth et al., 2001), and consequent defense activation (Howles et al., 2005; Gao et al., 2011; Zhang et al., 2012). Several NB-LRRs, in both plants and animals, work in pairs: one functions as an effector-specific 'sensor', and the other as a 'helper' protein. This may allow or drive the formation of higher-order protein complexes necessary for defense activation (Eitas and Dangl, 2010; Kofoed and Vance, 2011; Zhao et al., 2011; Bonardi et al., 2012).

ADR1-L2 (Activated Disease Resistance 1-Like 2) is part of a small family of NB-LRR proteins that includes ADR1 and ADR1-L1 (Chini and Loake, 2005). We recently noted that ADR1-L2 functions downstream of reactive oxygen intermediates (ROI) production and upstream of SA accumulation in basal defense (defined as the

response that limits growth of genetically virulent pathogens), in MAMP-triggered SA accumulation, and as a 'helper' protein during some, but not all ETI responses driven by effector-mediated activation of specific sensor NB-LRR proteins (Bonardi et al., 2011).

Surprisingly, none of the functions of ADR1-L2 detailed above required an intact P-loop (Bonardi et al., 2011). In addition to these 'non-canonical' defense activities, we suggested that ADR1-L2 could have P-loop dependent, 'canonical' functions that are as yet undefined in the absence of the specific effector required for activation. ADR1-L2 would not be the first NLR protein to have multiple, independent functions. The mouse NLR protein NLRC4 has two separate functions: it functions as a 'helper' protein in the recognition of both the MAMP flagellin and PrgJ, a component of the Salmonella TTSS. These separate activities require two different sensor NLRs: NAIP5 is necessary for flagellin perception, and NAIP2 is required for PrgJ recognition (Kofoed and Vance, 2011; Zhao et al., 2011). Importantly, NLRC4 'helper' activity is also P-loop independent (Kofoed and Vance, 2011; Zhao et al., 2011).

Canonical, effector-driven NB-LRR activation drives an NADPH oxidase-dependent ROI burst (Torres et al., 2005). The ADR1-L2 helper function noted above is downstream or independent of this oxidative burst. Thus, the *adr1* triple mutant (*adr1 adr1-L1 adr1-L2)* exhibits normal ROI production after successful pathogen recognition (Bonardi et al., 2011). However, *adr1* triple mutants fail to accumulate the wild-type levels of SA required for ETI in this context (Bonardi et al., 2011). Another protein that functions downstream of effector-driven oxidative bursts and both regulates and responds to upstream of SA accumulation is *L*esion *S*imulating *D*isease resistance 1 (LSD1) (Dietrich et al., 1994; Torres et al., 2005). Loss of LSD1 leads to improper triggering and

regulation of runaway cell death, or rcd (Dietrich et al., 1994). The Arabidopsis NADPH oxidase AtRbohD, which is required for the effector-driven oxidative burst, is not required for *lsd1*-mediated cell death (Torres et al., 2005). On the other hand, *lsd1* rcd is both induced by and requires SA (Dietrich et al., 1994; Aviv et al., 2002). *lsd1* rcd is also regulated by Enhanced Disease Susceptibility 1 (EDS1) and a type I metacaspase, AtMC1; *eds1 lsd1* and *atmc1 lsd1* plants do not exhibit rcd (Rusterucci et al., 2001; Coll et al., 2010). EDS1 is a defense response regulator, required for both basal defense and Toll/interleukin-1 (TIR)-NB-LRR mediated ETI (Wiermer et al., 2005). EDS1 and SA act in a regulatory feedback loop, with SA up-regulating EDS1 and EDS1 functioning as a potentiator of SA-mediated signaling (Falk et al., 1999; Venugopal et al., 2009). AtMC1 is a positive regulator of ETI-mediated cell death (Coll et al., 2010).

To define the genetic requirements of the putative canonical functions of ADR1-L2, we created an autoactive MHD mutant, ADR1-L2$_{D484V}$. This allele displayed the dwarfed morphology that is the hallmark of such mutants (Howles et al., 2005; Gao et al., 2011; Zhang et al., 2012). We demonstrate that this autoactivity is P-loop dependent, downstream of AtRbohD-mediated ROI production, partially dependent on SA synthesis, and negatively regulated by EDS1 and AtMC1. We then present a model for the interaction of EDS1, LSD1, and ADR1-L2, showing that these proteins interact in both SA-dependent and SA-independent feedback loops.

Results

Members of the ADR1 family of NB-LRRs are required for runaway cell death in lsd1. ADR1-L2 is a CC-NB-LRR that suppresses *lsd1* rcd (Bonardi et al., 2011). It is part of a small family of NB-LRRs that includes ADR1 and ADR1-L1 (Chini and Loake, 2005; Bonardi et al., 2011). To test whether ADR1 and ADR1-L1 also suppress the initiation and propagation of *lsd1* rcd, we generated *adr1 lsd1-2* and *adr1-L1 lsd1*-2 double mutants and sprayed them with the SA analog benzothiadiazole (BTH) (Gorlach et al., 1996). Col-0 wild-type plants were unaffected by BTH treatment, whereas *lsd1-2* plants sprayed with BTH showed typical rcd (Dietrich et al., 1994). As previously reported, the *adr1-L2 lsd1-2* double mutants fully suppressed *lsd1* rcd (Bonardi et al., 2011). *adr1-L1* also fully suppressed *lsd1-2* rcd, while *adr1* only had a slight effect (Figure 3.1A,B). We quantified this phenotype by monitoring cellular ion leakage via changes in media conductivity, an established proxy for membrane damage associated with cell death (Dellagi et al., 1998). Col-0 plants did not exhibit significant changes in media conductivity, but *lsd1-2* plants showed increasing conductivity, with the highest reading at 92 hours post-BTH treatment. *adr1-L1 lsd1-2* and *adr1-L2 lsd1-2* both exhibited complete ion leakage suppression, while *adr1 lsd1-2* exhibited a marginal effect (Figure 3.1C). Thus, ADR1-L1 and ADR1-L2 are each required for *lsd1* rcd.

We noted that *adr1-L1* and *adr1-L2* exhibited non-allelic non-complementation (NANC), a rare genetic condition where plants which are heterozygous at both loci phenotypically resemble either homozygous single mutant. Thus, plants homozygous for *lsd1-2* and heterozygous for both *ADR1-L1* and *ADR1-L2* were found to exhibit full suppression of *lsd1* rcd (Figure 3.1D). We also found that *adr1-L2* was fully recessive,

whereas *adr1-L1* appeared to be semi-dominant (Figure 3.1D). NANC frequently indicates that the two genes act closely together or that the two proteins physically interact or are a part of the same protein complex (Stearns and Botstein, 1988). Because all three ADR1 proteins share significant amino acid identity, we speculated that lowering of the overall ADR1 dose might be sufficient to suppress *lsd1* rcd. Thus, the weak *adr1* rcd suppression phenotype might simply reflect low expression of *ADR1* relative to *ADR1-L1* and *ADR1-L2*. Quantitative RT-PCR analysis of gene specific mRNA levels confirmed that *ADR1* is expressed at lower levels than *ADR1-L1* and *ADR1-L2* under our growth conditions, consistent with this model (Figure 3.1E).

ADR1-L2 is required at the specific site undergoing cell death. ADR1-L2 is a positive regulator of *lsd1*-mediated cell death. This could be due either to (a) a requirement for ADR1-L2 activation in cells destined to die, followed by its continued activation in neighboring cells, as the SA-dependent signal for rcd spreads in the absence of LSD1 (Jabs et al., 1996; Torres et al., 2005); or (b) ADR1-L2 being required and activated in cells initially triggered to die, with this activation contributing to the spread of an ADR1-L2-independent cell death signal beyond the primary cell death site. To distinguish between these two hypotheses, we generated an estradiol-driven (Est) conditional expression system, which induces local target gene expression (Brand et al., 2006). *adr1-L2 lsd1-2* plants expressing an estradiol-induced, HA epitope-tagged *ADR1-L2* transgene were constructed (Methods). Expression of ADR1-L2 was activated by local application of estradiol on only part of a leaf, thus creating an artificial chimera containing both *adr1-L2 lsd1-2* and *ADR1-L2 lsd1-2* sectors (Figure 3.2A). ADR1-L2

expression was limited to the area of estradiol application as measured via Western blot (Figure 3.2B). BTH treatment was then used to induce *lsd1*-mediated rcd; we observed that cell death was limited to the zone of estradiol treatment and did not expand into the *adr1-L2 lsd1-2* sector (Figure 3.2C). This result supports our first hypothesis: ADR1-L2 expression is required in cells undergoing *lsd1*-mediated runaway cell death.

The function of ADR1-L2 in lsd1 rcd is P-loop dependent. We previously noted that ADR1-L2 is required for SA accumulation following effector and MAMP recognition, and that this does not require an intact P-loop motif (Bonardi et al., 2011). However, these results do not preclude additional, canonical P-loop-dependent functions for ADR1-L2. Thus, we tested whether or not the positive regulatory function of ADR1-L2 in *lsd1* rcd is P-loop dependent. We generated *adr1-L2 lsd1-2* plants expressing ADR1-L2$_{AAA}$, a mutated allele of ADR1-L2 which carries alanine (A) substitutions in the three consecutive conserved residues within the P-loop motif which are essential for nucleotide binding (Bonardi et al., 2011). Interestingly, ADR1-L2$_{AAA}$ is not sufficient to trigger *lsd1* rcd following BTH treatment (Figure 3.3A), suggesting that the ADR1-L2 function in *lsd1* rcd proceeds in a canonical, P-loop dependent manner.

An autoactive version of ADR1-L2 displays P-loop dependent, ectopically activated immune responses. Mutations of the aspartic acid (D) in the conserved MHD domain in plant NB-LRRs typically lead to autoactivity (Bendahmane et al., 2002; Howles et al., 2005; Tameling et al., 2006; Gao et al., 2011; Williams et al., 2011).

Mechanistically, this is thought to reflect either a preference for ATP binding or a lack of ATPase activity, either of which would favor the "on" state, according to current models of NB-LRR activation (Takken et al., 2006; Bonardi et al., 2012). Thus, a similar mutation in the MHD motif of ADR1-L2 should result in a permanent 'on' state, resulting in ectopic autoactivity. In the few cases where it has been examined, NB-LRR autoactivity via MHD mutation has been shown to require an intact P-loop (Bendahmane et al., 2002; Howles et al., 2005; Tameling et al., 2006; Gao et al., 2011; Williams et al., 2011). Thus, given the P-loop dependent function of ADR1-L2 in *lsd1* rcd, we speculated that ADR1-L2 activity in additional defense contexts might also require an intact P-loop.

We generated *adr1-L2* plants expressing *ADR1-L2* with a Val (V) for Asp (D) substitution at amino acid 484 (Figure 3.4A; hereafter *ADR1-L2*$_{D484V}$). As expected, *ADR1-L2*$_{D484V}$ transgenics exhibited a dwarfed, *cpr* (Constitutive PR1 expression)-like phenotype (Bowling et al., 1994) with short hypocotyls, pointed leaves (Figure 3.4B), and a very bushy appearance after bolting. In contrast, *adr1-L2* plants expressing wild-type *ADR1-L2* appeared morphologically similar to wild-type Col-0 plants (Figure 3.4B). Both transgenes were expressed from the native *ADR1-L2* promoter, with C-terminal HA epitope tags (Figure 3.4C). We note that the majority of ADR1-L2$_{D484V}$ transgenic lines accumulated higher protein levels than those expressing the wild-type ADR1-L2 allele. However, to show that the *cpr*-like phenotype is not simply a result of higher protein levels in the autoactive mutant, we specifically selected *ADR1-L2* and *ADR1-L2*$_{D484V}$ lines expressing similar levels of protein (Figure 3.4C); the differences in morphology persist. Additional *ADR1-L2*$_{D484V}$ lines expressing even less ADR1-L2$_{D484V}$ protein were also recovered; these did not exhibit strong *cpr*-like phenotypes, suggesting that there is a

threshold amount of ADR1-L2$_{D484V}$ required for the associated phenotypes (data not shown).

The *ADR1* family members work additively to limit pathogen growth, with *adr1* triple mutant plants exhibiting increased susceptibility to virulent pathogens (Bonardi et al., 2011). We therefore tested the ability of autoactive ADR1-L2$_{D484V}$ to confer enhanced basal defense against otherwise virulent pathogens. *ADR1-L2$_{D484V}$* plants displayed increased resistance to both *Hyaloperonospora arabidopsidis* (*Hpa*) Emco5 and *Pseudomonas syringae* pv tomato (*Pto*) DC3000 (Figure 3.4D,E). Trypan blue staining of cotyledons after inoculation with *Hpa* Emco5 revealed predominantly free hyphal growth in the wild-type Col-0 control and *adr1-L2* which was enhanced in the fully susceptible control, *eds1* (Figure 3.4F). ADR1-L2$_{D484V}$ plants, on the other hand, exhibited only localized hypersensitive cell death (HR). ADR1-L2$_{D484V}$ plants also exhibited a basal level of cell death (Figure 3.4F, top row) not seen in the other genotypes. Thus, ADR1-L2$_{D484V}$ constitutively triggers downstream signaling and increased immune function.

We next examined the dependence of the *ADR1-L2$_{D484V}$ cpr*-like phenotype on the P-loop. The triple missense P-loop dead mutation, *ADR1-L2$_{AAA}$* (Bonardi et al., 2011), and the autoactive *ADR1-L2$_{D484V}$* mutation were combined in *cis* and transformed into *adr1-L2* plants. *ADR1-L2$_{AAA\ D484V}$* plants did not exhibit the *cpr*-like phenotype (Figure 3.5A), despite the fact that they expressed levels of ADR1-L2$_{AAA\ D484V}$ protein that are similar to ADR1-L2$_{D484V}$ levels sufficient to cause the dwarfed phenotype (Figure 3.5B). Thus, an intact P-loop domain is required for ADR1-L2$_{D484V}$ autoactivity. We infer that ADR1-L2$_{D484V}$ is an activated version of this NB-LRR which can be used to study the canonical (P-loop dependent) functions of ADR1-L2.

ADR1-L2$_{D484V}$ autoactivity is regulated by lsd1 suppressors. ADR1-L2 was identified as an *lsd1* suppressor ((Jabs et al., 1996), above). LSD1 and ADR1-L2 both function downstream of the NADPH oxidase-dependent ROI burst driven by NB-LRR sensor activation, but upstream of SA accumulation (Rusterucci et al., 2001; Aviv et al., 2002; Bonardi et al., 2011). Additionally, ADR1-L2 is locally required for *lsd1*-mediated rcd (above) and its function in this context is P-loop dependent. Thus, we hypothesized that additional genetic components known to regulate *lsd1* rcd might also be required for activity of ADR1-L2$_{D484V}$. We generated double mutants between *ADR1-L2$_{D484V}$* and the *lsd1* suppressors *sid2*, *eds1*, and *atmc1* to try to define a genetic network required for the ADR1-L2$_{D484V}$ phenotypes. We also generated *ADR1-L2$_{D484V}$ atrbohD* double mutants to define whether an oxidative burst is required for the ADR1-L2$_{D484V}$ phenotypes. We examined these double mutants for ADR1-L2$_{D484V}$ protein accumulation, alterations in the *ADR1-L2$_{D484V}$ cpr*-like morphology, enhanced resistance to the virulent *Hpa* isolate Emco5, and steady-state SA levels.

AtRbohD is generally required for effector-driven, NB-LRR-dependent superoxide production, but not for *lsd1* rcd (Torres et al., 2005). In fact, *lsd1-2 atrbohD* plants exhibit increased rcd compared to *lsd1-2* single mutants, a phenotype that depends on SA accumulation (Aviv et al., 2002). This result suggests that the NADPH oxidase can down-regulate the spread of cell death as SA-dependent signals emanate from an infection site (Torres et al., 2005). *atrbohD ADR1-L2$_{D484V}$* plants morphologically resembled the *ADR1-L2$_{D484V}$* parent and expressed a similar level of ADR1-L2$_{D484V}$ protein (Figure 3.6A,B). Like the *ADR1-L2$_{D484V}$* parent, *atrbohD ADR1-L2$_{D484V}$* plants were significantly more resistant to *Hpa* Emco5 (Figure 3.6C), and had extremely high

steady-state levels of SA (Figure 3.6D). We conclude that ADR1-L2$_{D484V}$ autoactivity, unlike effector-driven NB-LRR activation, is downstream, or independent, of AtRbohD.

SA is required for *lsd1* rcd (Aviv et al., 2002) and mediates basal defense in plants (Loake and Grant, 2007). Additionally, SA levels are reduced in *adr1*-family triple mutant plants, corresponding to diminished basal defense and an increase in disease susceptibility (Bonardi et al., 2011). Thus, it seemed likely that the increased basal defense in *ADR1-L2$_{D484V}$* plants could be due to the massive increase in SA observed in this line (Figure 3.6D). We tested this hypothesis using the *sid2* mutant, which is unable to synthesize SA due to a mutation in the biosynthetic isochorismate synthase gene, *ICS1* (Wildermuth et al., 2001). *sid2 ADR1-L2$_{D484V}$* plants were smaller than wild-type plants, yet larger than *ADR1-L2$_{D484V}$* parents, despite accumulating similar amounts of ADR1-L2$_{D484V}$ protein (Figure 3.6A,B). *sid2 ADR1-L2$_{D484V}$* plants exhibited enhanced basal defense to *Hpa* Emco5, though not to the same extent as *ADR1-L2$_{D484V}$* (Figure 3.6C). As expected, *sid2 ADR1-L2$_{D484V}$* plants did not accumulate SA (Figure 3.6D). These observations indicate that the *cpr*-like phenotypes of *ADR1-L2$_{D484V}$* consist of both SA-dependent and SA-independent components.

EDS1 is required for *lsd1*-mediated rcd (Rusterucci et al., 2001) and is an essential regulator of both basal defense against virulent pathogens (Aarts et al., 1998; Feys et al., 2005) and TIR-NB-LRR dependent ETI (Feys et al., 2001; Zhang et al., 2003; Wirthmueller et al., 2007). Provision of an exogenous SA analog rescues *eds1* basal defense phenotypes, suggesting that EDS1 acts upstream of ICS1, at least for the phenotypes assayed (Parker et al., 1996; Feys et al., 2001). *eds1 ADR1-L2$_{D484V}$* plants were significantly more dwarfed than *ADR1-L2$_{D484V}$* (Figure 3.6A), though these two

lines expressed similar levels of ADR1-L2$_{D484V}$ protein (Figure 3.6B). *eds1 ADR1-L2$_{D484V}$* double mutants were completely resistant to *Hpa* Emco5 (Figure 3.6C), and had steady-state SA levels that were higher than the *ADR1-L2$_{D484V}$* single mutant (Figure 3.6D). These surprising results demonstrate that EDS1 is a negative regulator of the SA-accumulation observed in ADR1-L2$_{D484V}$.

AtMC1 is a metacaspase required for *lsd1* rcd; AtMC1 also contributes significantly to ETI-dependent HR (Coll et al., 2010). *atmc1 ADR1-L2$_{D484V}$* plants were extremely dwarfed (Figure 3.6A). However, these plants were not sterile; they produced small amounts of seed and had a very long life cycle compared to wild-type Col-0 or *ADR1-L2$_{D484V}$* plants (data not shown). They also accumulated more ADR1-L2$_{D484V}$ protein than the *ADR1-L2$_{D484V}$* parent (Figure 3.6B). Cotyledons of the *atmc1 ADR1-L2$_{D484V}$* plants were similar in size to those of *ADR1-L2$_{D484V}$* plants, and we were thus able to perform *Hpa* infection assays; we determined that *atmc1 ADR1-L2$_{D484V}$* cotyledons are completely resistant to *Hpa* Emco5 (Figure 3.6C). Due to the extremely small size of the *atmc1 ADR1-L2$_{D484V}$* double mutant, we were unable to perform SA analysis on this line. However, we measured SA levels from *atmc1* plants that were heterozygous for *ADR1-L2$_{D484V}$* and resembled the *ADR1-L2$_{D484V}$* parent in size. We noted significantly less SA in the *atmc1 ADR1-L2$_{D484V}$* +/- than in the *ADR1-L2$_{D484V}$* parent (Figure 3.6D). We noted significantly higher SA in the *atmc1 ADR1-L2$_{D484V}$* +/- than in the wild-type Col-0 plants (Figure 3.6D). Collectively, these data indicate that AtMC1 negatively regulates ADR1-L2$_{D484V}$ protein accumulation, thereby inhibiting ADR1-L2$_{D484V}$ accumulation, activity and likely subsequent SA accumulation.

lsd1 ADR1-L2$_{D484V}$ lethality requires EDS1. ADR1-L2 is required for *lsd1*-mediated rcd (Bonardi et al., 2011). We therefore examined whether ADR1-L2$_{D484V}$ affects the *lsd1* phenotpye. We crossed *lsd1-2* and *ADR1-L2$_{D484V}$* plants, and in the F3 generation homozygous *ADR1-L2$_{D484V}$* plants were selected via Basta resistance markers on the transgene (see Methods). *ADR1-L2$_{D484V}$* homozygotes were genotyped for *lsd1-2*; none were *lsd1-2* homozygous (Supplementary Table 1). Additionally, we carried *lsd1-2* homozygous, *ADR1-L2$_{D484V}$* heterozygous plants forward an additional generation, and again used the Basta resistance marker to find homozygous *ADR1-L2$_{D484V}$* plants. None were recovered. Next, we attempted to transform *lsd1-2* mutant plants with the same *ADR1-L2$_{D484V}$* construct used in the *adr1-L2* transformation. No lines were recovered that expressed detectable levels of ADR1-L2$_{D484V}$ protein, and no plants that were recovered displayed the dwarfed phenotype (data not shown). We conclude that *lsd1-2 ADR1-L2$_{D484V}$* is lethal, probably due to an overwhelming amount of constitutively active SA accumulation, and consequent cell death signaling.

We therefore looked for genetic determinants required for *lsd1 ADR1-L2$_{D484V}$* lethality. As stated above, *eds1* and *atmc1* are both suppressors of *lsd1* rcd. To determine if these two genes were necessary for *lsd1-2 ADR1-L2$_{D484V}$* lethality, we crossed *atmc1 lsd1-2* or *eds1 lsd1-2* plants to *ADR1-L2$_{D484V}$*. *atmc1 lsd1-2 ADR1-L2$_{D484V}$* plants could not be recovered (data not shown), indicating that *AtMC1* is not required for *lsd1-2 ADR1-L2$_{D484V}$* lethality. However, we did recover *eds1 lsd1-2 ADR1-L2$_{D484V}$* plants. These plants surprisingly exhibited wild-type morphology, effectively resembling *eds1 lsd1* (Rusterucci et al., 2001) (Figure 3.7A). The suppression of the *ADR1-L2$_{D484V}$ cpr*-like phenotype is likely due to a much lower level of steady state ADR1-L2$_{D484V}$

accumulation in the *eds1 lsd1-2 ADR1-L2$_{D484V}$* plants compared to parental plants (Figure 3.7B). Despite examining many *eds1 lsd1-2 ADR1-L2$_{D484V}$* plants from 4 independent progenies, no plant with *ADR1-L2$_{D484V}$* parental expression levels was recovered. Additionally, *eds1 lsd1-2 ADR1-L2$_{D484V}$* plants did not accumulate the high levels of SA observed in *ADR1-L2$_{D484V}$* (Figure 3.7C).

In light of the surprising result that *eds1 lsd1-2 ADR1-L2$_{D484V}$* plants are essentially wild-type, we re-confirmed the genotypes and phenotypes of *eds1* ADR1-L2$_{D484V}$ and *eds1 lsd1-2* ADR1-L2$_{D484V}$. We used a line that was homozygous for *eds1* and ADR1-L2$_{D484V}$ but heterozygous for *LSD1*. In the next generation, both dwarfed and wild-type size plants were identified (Figure 3.8A). These plants were genotyped for *LSD1*, and all dwarfed plants were found to be *LSD1* homozygotes (Figure 3.8B, 20 of 70 plants were *LSD1* homozygotes). Wild-type size plants were either *LSD1* heterozygotes (34 of 70 plants) or *lsd1* mutants (16 of 70 plants), suggesting that the dominant loss of function mutation in this context is the result of *LSD1* haploinsufficiency. We therefore conclude that the difference in the growth phenotype between *eds1 lsd1-2 ADR1-L2$_{D484V}$* (wild-type) and both *eds1 ADR1-L2$_{D484V}$* (nearly lethal) and *lsd1 ADR1-L2$_{D484V}$* -(lethal) is genuine, and that in the autoactive *ADR1-L2$_{D484V}$* mutant, the combined absence of EDS1 and the loss of, or reduction in, LSD1 leads to down-regulation of ADR1-L2$_{D484V}$ protein accumulation and restoration of wild-type morphology.

We addressed whether the lowered accumulation of ADR1-L2$_{D484V}$ protein in *eds1 lsd1-2 ADR1-L2$_{D484V}$* was due to transcriptional regulation. We performed quantitative RT-PCR, and discovered that the *ADR1-L2$_{D484V}$* transcript levels in *lsd1 eds1 ADR1-L2$_{D484V}$* plants were slightly lower than in *ADR1-L2$_{D484V}$* (Figure 3.7D), generally

consistent with the diminution of ADR1-L2$_{D484V}$ protein in *eds1 lsd1-2 ADR1-L2$_{D484V}$* (Figure 3.7B). We also noted that although the ADR1-L2$_{D484V}$ protein level in *eds1* is indistinguishable from the parental ADR1-L2$_{D484V}$ by western blot (Figure 3.7B), the *ADR1-L2$_{D484V}$* transcript accumulated to higher levels (Figure 3.7D). This apparently contradictory result suggests that ADR1-L2$_{D484V}$ protein stability requires EDS1, or an EDS1-dependent process. LSD1 and EDS1 are known to work together in an SA regulatory feedback loop (Rusterucci et al., 2001). Given that *lsd1 eds1 ADR1-L2$_{D484V}$* plants are morphologically normal, express lower levels of SA than *ADR1-L2$_{D484V}$*, and accumulate lower levels of both ADR1-L2 transcript and protein than *ADR1-L2$_{D484V}$* (Figure 3.7), and that ADR1-L2 accumulation is up-regulated by BTH application (Figure 3.4C), we speculate that this loop also regulates ADR1-L2 expression. However, we also observed that *sid2* had no effect on either ADR1-L2$_{D484V}$ mRNA or protein levels (Figures 3.6 and 3.7), suggesting that there are also SA-independent regulators of ADR1-L2. We also noted that *ADR1-L2$_{D484V}$* transcript accumulated to significantly higher levels than the endogenous ADR1-L2 transcript in wild-type Col-0 plants, indicating that plants expressing the activated ADR1-L2 allele constitutively up-regulate *ADR1-L2* transcription.

RAR1 is dispensable for accumulation of ADR1-L2. The autoactive phenotypes of *ADR1-L2$_{D484V}$* plants require ADR1-L2$_{D484V}$ protein accumulation above a threshold. This indicates that the expression level of wild-type ADR1-L2 may also be under exquisite control. The co-chaperone RAR1, while not necessary for the function of all NB-LRRs, is required for the steady state accumulation of all NB-LRRs tested to date

(Tornero et al., 2002; Belkhadir et al., 2004; Bieri et al., 2004; Holt et al., 2005). We thus crossed *adr1-L2 pADR1-L2:ADR1-L2-HA* to *rar1-21* (Tornero et al., 2002). Plants genotyped as homozygous *rar1-21* and homozygous *RAR1* exhibited similar levels of ADR1-L2-HA protein (Figure 3.9A), indicating that RAR1 is not required for ADR1-L2 accumulation. The *rar1* genotype was confirmed by Western blot for RAR1 protein (Figure 3.9B). ADR1-L2 expression can be up-regulated with BTH (Bonardi et al., 2011). We therefore also tested whether RAR1 is required for the high levels of ADR1-L2 accumulating after BTH treatment. BTH induced ADR1-L2 protein in *rar-21 ADR1-L2-HA* plants accumulated to levels at least as high as those in *RAR1 ADR1-L2-HA* plants (Figure 3.9A). Therefore, RAR1 is dispensable for both steady-state ADR1-L2 accumulation, in contrast to other assayed NB-LRR proteins (Tornero et al., 2002; Belkhadir et al., 2004; Bieri et al., 2004; Holt et al., 2005), and for its BTH-induced up-regulation.

Discussion

We previously demonstrated that the plant NB-LRR immune receptor ADR1-L2 can have a non-canonical 'helper' role in plant defense (Bonardi et al., 2011). Here, we sought first to define canonical, P-loop dependent function(s) for ADR1-L2, and then to understand the genetic requirements for these functions. We demonstrated that wild-type ADR1-L2 is required locally at the site of BTH-driven cell death activation in the *lsd1* cell death control mutant; this activity requires an intact P-loop. In this context, *ADR1-L2* genetically interacts with *ADR1-L1* to control runaway cell death, as shown by NANC, further suggesting that members of the ADR1 family might function together in cell

death signaling. Interestingly, ADR1-L2 does not require RAR1 for either its steady state accumulation, nor for its induced accumulation following BTH treatment. This is the first report of either steady state or inducible NB-LRR accumulation that is not RAR1-dependent. This result may differentiate 'helper' NB-LRRs from 'sensor' NB-LRRs, in that levels of the former might be dictated by the signaling partners with which they function, while the latter, acting as effector-sensors, are threshold-regulated by the co-chaperone complex (Shirasu, 2009).

Given the canonical P-loop-dependent function of ADR1-L2 as a positive regulator of *lsd1* cell death, we inferred that ADR1-L2, like other NB-LRRs studied to date, retains the ability to undergo a nucleotide-dependent conformational switch to regulate its activation. Thus, we sought a context in which we could analyze canonical ADR1-L2 P-loop dependent functions, despite the absence of an effector to trigger it. We created an autoactive allele, ADR1-L2$_{D484V}$. *ADR1-L2$_{D484V}$* plants exhibit the dwarfed morphology seen in other autoactive NB-LRR mutants. We showed that this autoactivity requires an intact P-loop. We then used this allele as a proxy for canonical activation of ADR1-L2 in a series of epistasis experiments.

Canonical, P-loop dependent, 'sensor' NB-LRR functions typically drive both the AtrbohD-dependent NADPH-dependent oxidative burst following effector perception and SID2-dependent SA accumulation (Torres et al., 2005). By contrast, ADR1-L2$_{D484V}$ autoactivity is downstream, or independent, of AtrbohD, yet still drives SID2-dependent SA accumulation. This is consistent with the previously defined, P-loop independent 'helper' activity of ADR1-L2 (Bonardi et al., 2011). Resting state NB-LRRs are localized to diverse sub-cellular compartments, and dynamic re-localization may accompany

effector-driven activation of some (Bonardi et al., 2012). We note that ADR1-L2 is soluble, and we have no evidence of activation-dependent re-localization (data not shown). Thus, our data support a scenario in which the P-loop-independent ADR1-L2 'helper' functions (Bonardi et al., 2011), and the P-loop-dependent functions we define here can be differentiated from the typical effector-driven activation of NB-LRR 'sensors' described to date (see also below).

Plants expressing *ADR1-L2*$_{D484V}$ exhibit increased disease resistance and very high steady-state levels of SA. *sid2 ADR1-L2*$_{D484V}$ plants expressed, as expected, very low levels of SA, but these plants did not completely revert to wild-type morphology, and they maintained an increased level of enhanced disease resistance. Thus, there must be SA-independent regulation of activated ADR1-L2. Redundant functions of EDS1 and SA in plant defense mediated by 'sensor' NB-LRR functions have been reported (Venugopal et al., 2009). In that work, *sid2* or *eds1* mutants were insufficient to disrupt CC-NB-LRR-mediated disease resistance, while combined loss of both gene products led to loss of resistance (Venugopal et al., 2009). Our results support this model, since the constitutive activation of ADR1-L2$_{D484V}$ results in both SA-dependent and SA-independent phenotypes. Given this data, as well as the fact that *eds1 lsd1 ADR1-L2*$_{D484V}$ phenocopies *sid2 ADR1-L2*$_{D484V}$ with respect to SA levels, but not the morphological phenotype, we conclude that the SA-independent pathway we describe here may require EDS1.

Our most surprising observation is the phenotypic rescue of both the lethal *lsd1 ADR1-L2*$_{D484V}$ phenotype and the nearly lethal *eds1 ADR1-L2*$_{D484V}$ phenotype in *eds1 lsd1 ADR1-L2*$_{D484V}$ plants. It is important to recall that loss of either *adr1-l2* or *eds1* function suppresses *lsd1* rcd (Rusterucci et al., 2001; Bonardi et al., 2011). Recall also

that the P-loop independent function of ADR1-L2 as a 'helper' is downstream of AtRbohD, but upstream of SA accumulation (Bonardi et al., 2011). This is in agreement with the autoactive *ADR1-L2*$_{D484V}$ phenotype, which bypasses AtRbohD but still drives enhanced SA levels, as expected.

We present a model consistent with our new findings and previous genetic analyses (Rusterucci et al., 2001; Aviv et al., 2002; Torres et al., 2005; Venugopal et al., 2009; Bonardi et al., 2011) (Figure 3.10). P-loop-dependent activation of ADR1-L2 results in ICS1/SID2-mediated SA accumulation via two separate pathways. We speculate that in the first pathway ADR1-L2$_{D484V}$ constitutively signals to EDS1, which in turn positively regulates ICS1/SID2, increasing SA levels. ADR1-L2$_{D484V}$ also triggers additional SA production in a parallel pathway that is both antagonized by EDS1 and is under the control of LSD1. In support of our model, SA regulates EDS1 transcription (Falk et al., 1999), which in turn regulates ICS1/SID2 (Bartsch et al., 2006). Once activated, ADR1-L2 causes cell death, which drives more AtRbohD-dependent ROI (Jabs et al., 1996) and SA accumulation in surrounding cells (Enyedi et al., 1992; Jabs et al., 1996). In both pathways, SA is part of a feedback loop that further potentiates the P-loop dependent activity of ADR1-L2, as indicated by the fact that ADR1-L2 is BTH inducible. Thus, ADR1-L2 is also both upstream and downstream of SA accumulation.

In an otherwise wild-type plant expressing activated ADR1-L2, the antagonism between EDS1 and LSD1 maintains SA production below toxic levels. In an *lsd1* plant, the level of SA surpasses this level due to the fact that LSD1 is not there to down-regulate ADR1-L2-driven SA production. This increased SA in turn drives ADR1-L2 expression, and the cycle repeats, leading to the lethality seen in *lsd1 ADR1-L2*$_{D484V}$. *eds1*

and *sid2* normally suppress *lsd1* because the feed forward regulation of the SA accumulation cycle is blocked. Thus, the surprising *eds1 lsd1 ADR1-L2*$_{D484V}$ phenotype is consistent with the low level of SA in this line being insufficient to up-regulate *ADR1-L2* expression: even though there is chronic signaling feeding the cycle, the cycle is interrupted. How LSD1 and EDS1 negatively regulate each other has yet to be determined, although our data suggest that LSD1 might regulate EDS1 function through transcriptional control, as EDS1 transcription levels are increased in an *lsd1* mutant (Figure 3.11). Together, our data support and refine the currently proposed roles of EDS1 and LSD1 as regulators of an SA feedback loop (Rusterucci et al., 2001; Aviv et al., 2002). In an *eds1 ADR1-L2*$_{D484V}$ plant, the ADR1-L2$_{D484V}$ phenotype is enhanced because of slightly higher SA levels due to the lack of EDS1 inhibitory function on the LSD1-regulated pathway. Our data also suggest that AtMC1 functions as a negative regulator of ADR1-L2 accumulation and activity. Unfortunately, due to the extremely dwarfed morphology of the *atmc1 ADR1-L2*$_{D484V}$ plants, we were unable to carry out the phenotypic assays performed on the other lines, and therefore are unable to place AtMC1 in our model.

Our model supports a scenario in which in wild-type, P-loop dependent NB-LRR activation leads to local increased levels of SA via an AtRbohD-dependent ROI burst and SID2-dependent SA accumulation. The spread of this SA accumulation is spatially down-regulated through a combined action of EDS1 and LSD1 at increasing distance from the infection site. As stated above, our model also implies that SA functions both up- and down-stream of ADR1-L2. This may seem difficult to reconcile with our previous finding that ADR1-L2 is required for SA accumulation and cell death (Bonardi et al.,

2011) following ‘sensor’ activation, but we point out that the phenotypes uncovered in our initial findings are P-loop independent, and thus potentially mechanistically different than the P-loop dependent ADR1-L2 phenotypes described here.

Overall, we present a general approach to characterize canonical, P-loop dependent functions of NB-LRR proteins in the absence of a specific effector. We applied this to a recently characterized ‘helper’ NB-LRR protein, ADR1-L2. We identified genetic components that regulate its P-loop-dependent, canonical functions, and found that they, in turn, are regulated by suppressors of the *lsd1* rcd phenotype. Our work suggests that the genetic requirements for ‘helper’ NB-LRR function may differ from the effector-driven activation of canonical ‘sensor’ NB-LRRs. Given that ADR1-L2, unlike other NB-LRRs, is required for *lsd1* rcd, we note that our results may be mainly relevant to the dissection of the functions of ADR1-L2 and its paralogues, rather than being broadly applicable to understanding of ‘sensor’ NB-LRRs. Nevertheless, in agreement with previous reports on ‘sensor’ NB-LRR function (Venugopal et al., 2009), we conclude that the P-loop-dependent autoactivity of ADR1-L2 relies on signaling pathways that differ in their requirement for SA accumulation, but which are both regulated by EDS1. Thus, though the requirements for ‘sensor’ and ‘helper’ NB-LRR functions may be separable, they could still share some overlapping features.

Materials and Methods

Plant lines and pathogen strains.

All *Arabidopsis* lines are in the Columbia (Col-0) ecotype. *adr1-1* (Bonardi et al., 2011), *adr1-L1-1* (Bonardi et al., 2011), *adr1-L2-4* (Bonardi et al., 2011), *eds1-2* (Parker et al., 1996), *sid2-1, atrbohD* (Torres et al., 2005), *lsd1-2* (Dietrich et al., 1994), *atmc1* (Coll et al., 2010), and *rar1-21* (Tornero et al., 2002) are described elsewhere; primers used to genotype these lines are in Supplemental Table 2. For generation of *adr1-L2* plants expressing *ADR1-L2-HA*, ADR1-$L2_{D484V}$-*HA*, and *ADR1-L2*$_{D484V}$ *ADR1-L2*$_{AAA}$ lines, the C-terminal HA-tagged coding sequence of wild-type *ADR1-L2* or the mutated alleles were fused to its native promoter (500 bp) and cloned in the pBAR (Basta resistant) Gateway vector (Nakagawa et al., 2007). For generation of *adr1-L2 lsd1-2* plants expressing an estradiol inducible ADR1-L2-HA, the coding sequence of ADR1-L2 was cloned into a modified pMDC7 (hygromicin resistant) Gateway vector carrying a C-terminal HA tag. Arabidopsis transgenics were generated using Agrobacterium (GV3101)-mediated floral dip transformation (Clough and Bent, 1998). Basta selection of transgenic plants was performed by spraying 10-day-old seedlings. Plants were grown under short day conditions (9 hrs light, 21°C; 15 hrs dark, 18°C).

Immunoblot Analysis.

Leaves from 4-week-old plants were harvested and total proteins were extracted by grinding frozen tissue in a buffer containing 20 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1mM EDTA (pH 8.0), 1% Triton X-100, 0.1% SDS, 10mM DTT, and plant

protein protease inhibitor cocktail (Sigma-Aldrich). Samples were centrifuged at 14,000 rpm for 15 min at 4°C to pellet debris. Proteins were separated on 7.5% (ADR1-HA) or 12% (RAR1) SDS-PAGE gels and were transferred to polyvinylidene difluoride membrane. Western blots were performed using standard methods. Anti-HA (Santa Cruz Biotechnology) antibody was used at a 1:3,000 dilution; anti-RAR1 (custom anti-RAR1 polyclonal antibody was made against the full length RAR1 with C-terminus GST tag by Cocalico Biologicals, Inc.) was used at a 1:2,000 dilution. Signals were detected by enhanced chemiluminescence using ECL Plus (Amersham Biosciences). For BTH induction experiments (300 µM), plants were collected 24 hpi.

SA measurement.

SA and SAG measurements were performed as described (Defraia et al., 2008). Briefly, 100 mg of leaves were collected from 4-week-old plants and frozen in liquid nitrogen. Samples were ground and tissue was homogenized in 200 µl 0.1M acetate buffer pH 5.6. Samples were centrifuged for 15 min at 16,000 g at 4°C. 100 µl of supernatant was transferred to a new tube for free SA measurement, and 10 µl was incubated with 1 µl 0.5 U/µl β-glucosidase for 90 min at 37°C for total SA measurement. After incubation, plant extracts were diluted 5-fold with 44 µl acetate buffer for free SA measurement. 60 µl of LB, 5 µl of plant extract (treated or not with β-glucosidase), and 50 µl of *Acinetobacter sp.* ADPWH-lux (OD = 0.4) were added to each well of a black 96-well plate (BD Falcon). The plate was incubated at 37°C for 60 min and luminescence was read with Spectra Max L (Molecular Devices) microplate reader. For the standard

curve, 1 µl of a known amount of SA (Sigma; from 0 to 1000 µg/ml) was diluted 10-fold in *sid2-1* plant extract, and 5 µl of each standard (undiluted for free SA measurement, or 5-fold diluted for total SA) was added to the wells of the plate containing 60 µl of LB and 50 µl of *Acinetobacter*. SA standards were read in parallel with the experimental samples. For BTH induction experiments (300 µM), plants were collected 24 hpi.

Pathogen strains and growth quantification.

Ten-day-old seedlings were spray-inoculated with 50,000 spores/ml of *Hyaloperonospora arabidopsidis* isolate Emco5 or 20,000 spores/ml of isolate Noco2. Pots were covered with a lid to increase humidity during inoculation and pathogen growth. Sporangiophores were counted at 4 dpi as described (Holt et al., 2002). *Pto* DC3000(EV) was resuspended in 10 mM $MgCl_2$ to a final concentration of 2.5 x 10^5 cfu/ml (OD_{600}=0.0005). Twenty-day-old seedlings were dipped in the bacterial solution and growth was assessed as described (Tornero and Dangl, 2001).

Cell death Assays.

4-week-old plants were sprayed with 300 µM BTH, or 10-day-old plants were inoculated with *Hpa* Emco5 as described above. Leaves were harvested and stained with lactophenol Trypan Blue (TB) to visualize dead cells as described (Koch and Slusarenko, 1990). For the conductivity measurements, 4-week-old plants were sprayed with 300 µM BTH. Plants were harvested and 4 leaf discs (7 mm) were cored and then floated in water

for 30min. These leaf discs were transferred to tubes containing 6 ml distilled water. Conductivity of the solution (μSiemens/cm) was determined with an Orion Conductivity Meter at the indicated time points (Epple et al., 2003).

Creation of an artificial chimera.

The central portion of the right halves of leaves from 4-week-old transgenic *adr1-L2 lsd1-2* plants expressing an estradiol inducible allele of ADR1-L2 were hand-infiltrated with Est (20 μM) using a needleless syringe. 300 μM BTH was sprayed on the whole plant 24h post-Est application. 20 μM Est was then hand-infiltrated on the same portion of the leaves 2 dpi to ensure expression of ADR1-L2. Leaves were collected 5 dpi from the first Est infiltration.

Quantitative RT-PCR.

Leaves from 4-week-old plants were collected, frozen into liquid nitrogen and ground into powder with a mortar and pestle. RNA was extracted using TRIzol (Invitrogen), DNased (Ambion Turbo DNase), and cleaned up with Qiagen RNeasy Mini kit. Reverse transcription was performed (Ambion RETROscript) using 1 μg/μl total RNA, and cDNA was analyzed with SYBR green (Applied Biosystem) using an Applied Biosystems ViiA7. Primers used are listed in Table 3.2.

Selection of segregating plants.

Pots of sibling plants fixed for *eds1* and segregating *lsd1-2* (*LSD1* heterzygotes) were Basta sprayed to check for segregation of *ADR1-L2*$_{D484V}$. Those found to be *eds1* *ADR1-L2*$_{D484V}$ were transplanted individually into pots, monitored for size, and genotyped for the T-DNA insertion of the *lsd1-2* mutation.

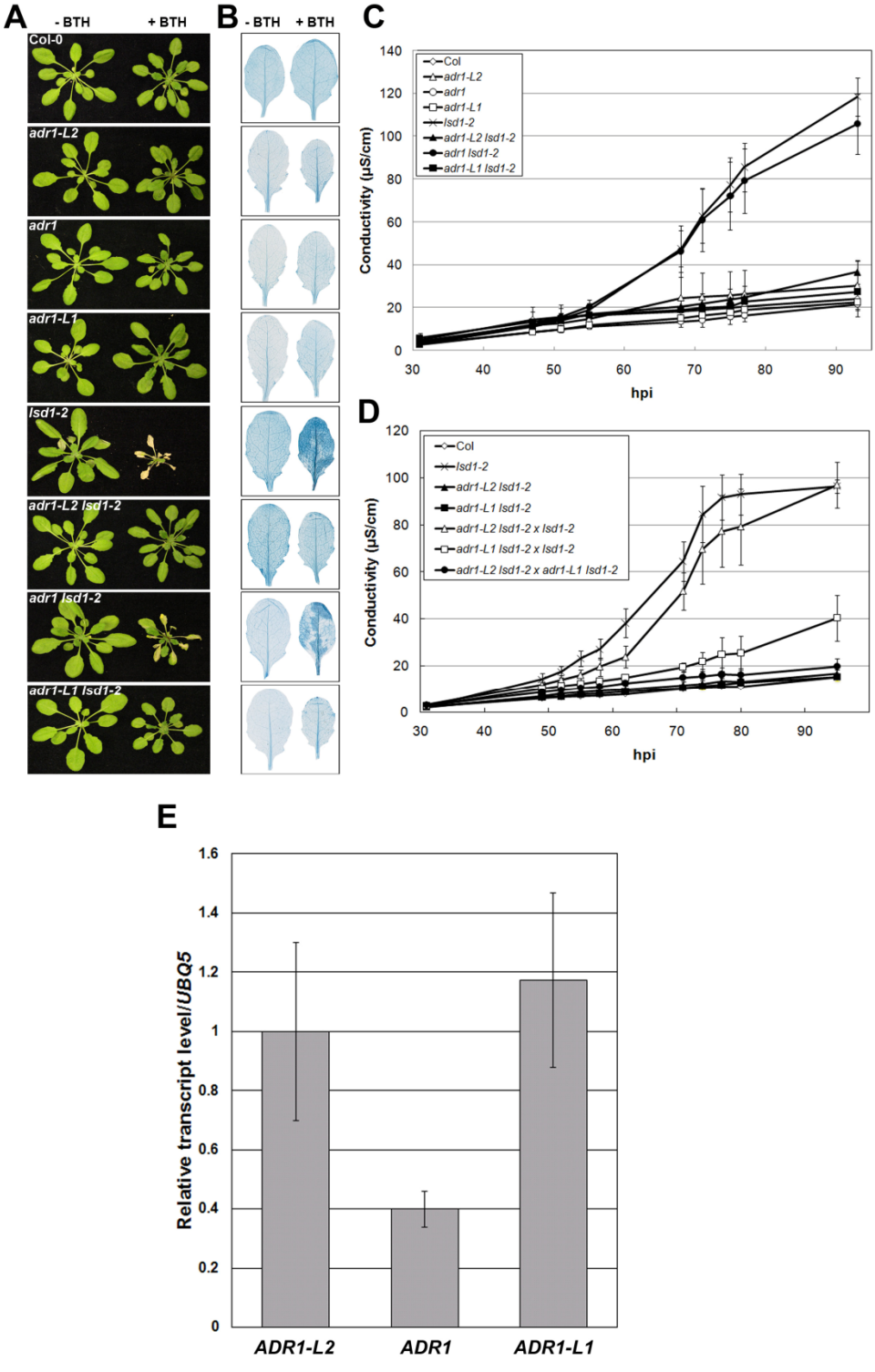
A
- BTH
+ BTH
Col-0
adr1-L2
adr1
adr1-L1
lsd1-2
adr1-L2 lsd1-2
adr1 lsd1-2
adr1-L1 lsd1-2
B
- BTH
+ BTH
C
Conductivity (µS/cm)
hpi
Col
adr1-L2
adr1
adr1-L1
lsd1-2
adr1-L2 lsd1-2
adr1 lsd1-2
adr1-L1 lsd1-2
D
Conductivity (µS/cm)
hpi
Col
lsd1-2
adr1-L2 lsd1-2
adr1-L1 lsd1-2
adr1-L2 lsd1-2 x lsd1-2
adr1-L1 lsd1-2 x lsd1-2
adr1-L2 lsd1-2 x adr1-L1 lsd1-2
E
Relative transcript level/UBQ5
ADR1-L2
ADR1
ADR1-L1

Figure 3.1. A family of CC-NB-LRR proteins is required for lsd1 runaway cell death. (A) Four-week-old plants were sprayed with BTH or water. Pictures of plants were taken 5 days post-inoculation (dpi). (B) Leaves from plants in (A) were stained with trypan blue to visualize cell death. Leaves on the left are untreated controls, leaves on the right are sprayed with BTH. (C) Ion leakage measurements from (A), 5 days post-BTH treatment. Values are means ± 2 × SE (n = 5). (D) Ion leakage measurements for NANC. *adr1-L1 lsd1-2* x *lsd1-2*, *adr1-L2 lsd1-2* x *lsd1-2*, *adr1-L1 lsd1-2* x *adr1-L2 lsd1-2* represent F1 plants of the indicated crosses, and are thus *lsd1* homozygous and heterozygous for the indicated *adr* mutations. (E) Quantitative real time PCR for the transcript amounts of the three members of the *ADR* family in wild-type Col-0 plants, normalized to *UBQ5*.

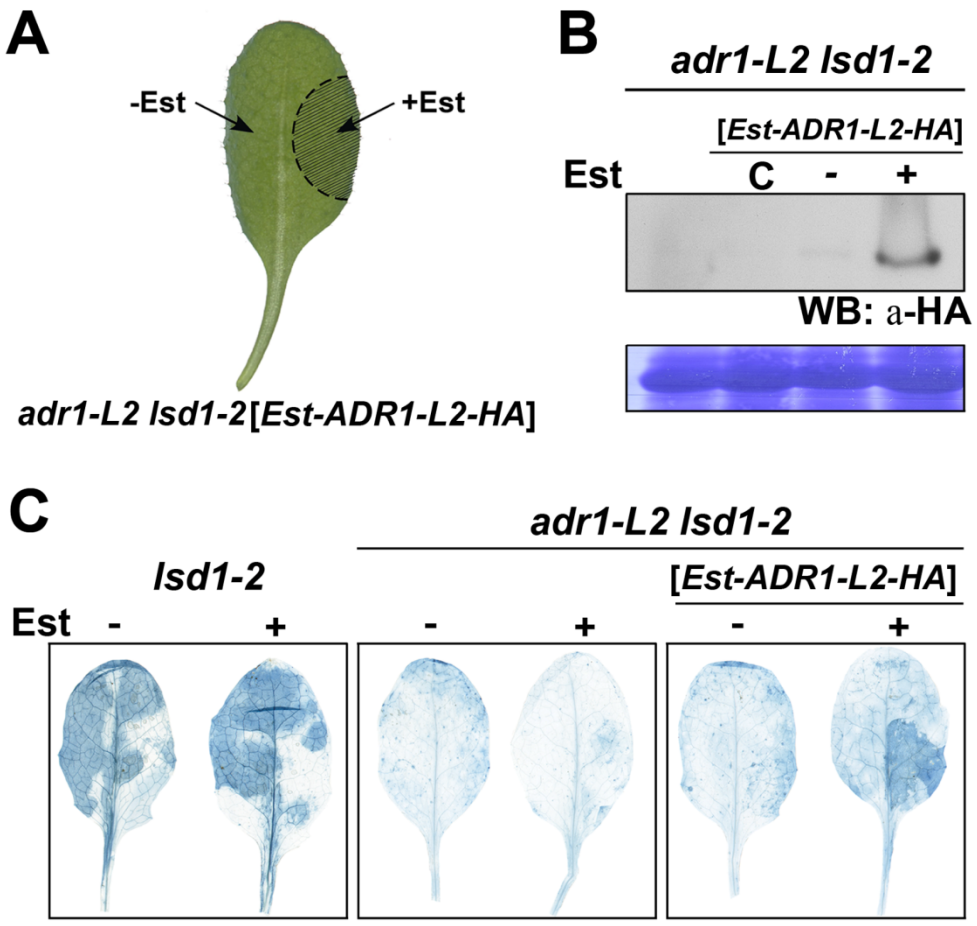

Figure 3.2. ADR1-L2 is required at the site undergoing cell death. (A) Schematic of the chimera. *adr1-L2 lsd1-2* expressing an estradiol inducible C-terminal HA-tagged ADR1-L2 were infiltrated in the indicated area with 20 µM estradiol, making that portion of the leaf *ADR1-L2 lsd1-2*. (B) Western blot to confirm expression of ADR1-L2 was limited to the estradiol-induced area. Estradiol + and – leaf areas were cored and protein was extracted from these cores. Proteins were run on SDS-Page gels and immunoblotted with anti-HA antibody; C, samples from un-infiltrated leaves; +,estradiol-infiltrated plant tissue; -, un-infiltrated tissue from the same leaf. In all samples, the entire leaf was treated with 300 µM BTH. (C) Trypan blue staining to show cell death in *lsd1* control and tissue chimera plants. Leaves from four-week-old plants were treated as indicated in (A). Plants were sprayed with BTH 16 hours after estradiol treatment, and leaves were stained with trypan blue 5 days after BTH treatment.

Figure 3.3. ADR1-L2AAA is not sufficient to trigger lsd1 rcd following BTH treatment. (A) Four-week-old plants were sprayed with BTH or water. Pictures of plants were taken 5 dpi. (B) Proteins from plants in (A) were extracted, run on SDS=Page gel, and probed with anti-HA antibody. Ponceau-stained blot shows relative loading.

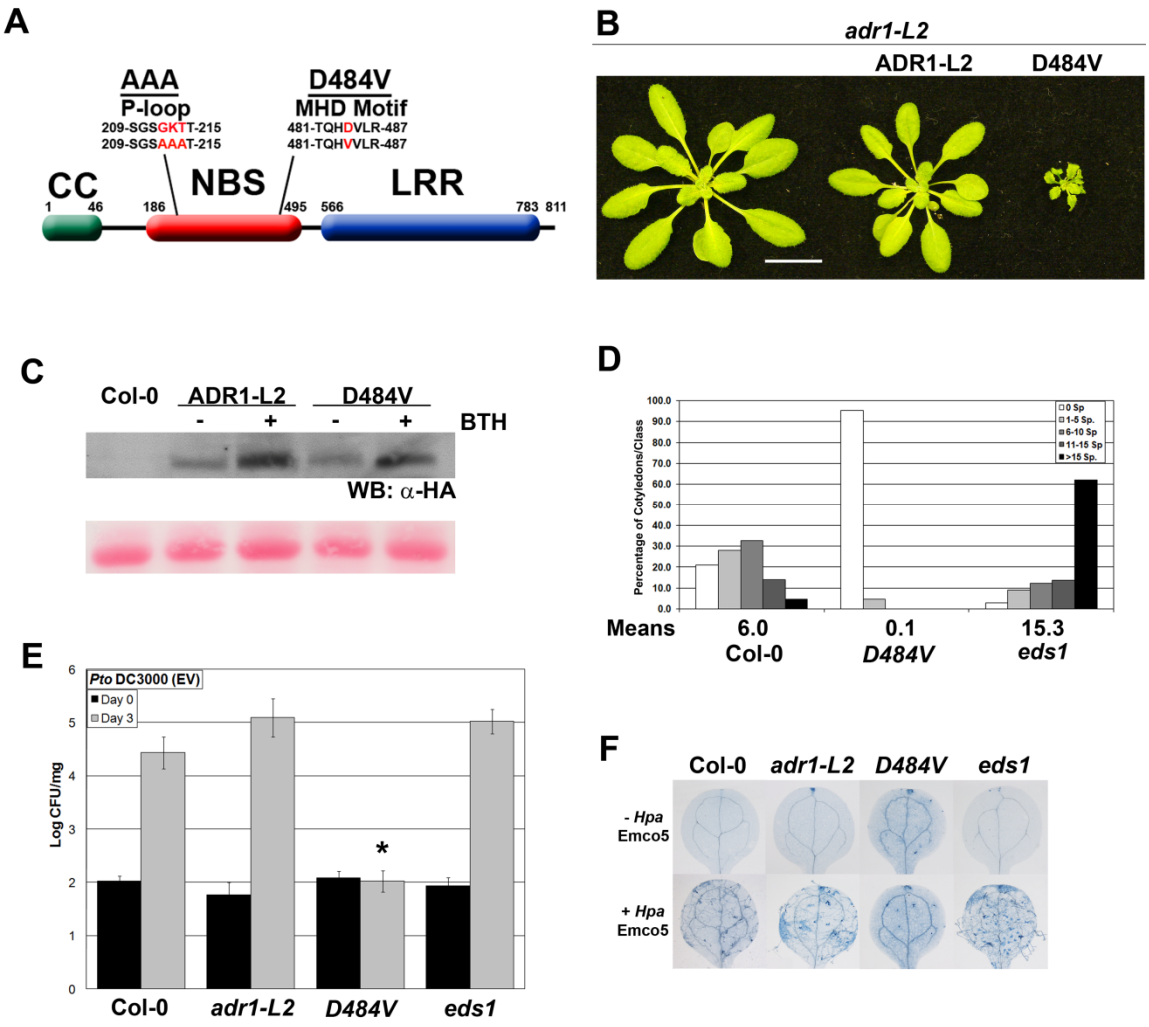

Figure 3.4. ADR1-L2D484V ectopically activates basal defense. (A) Schematic representation of ADR1-L2 showing the P-loop and MHD mutations used in this study. (B) Morphology of five-week-old *adr1-L2*, and *adr1-L2* complemented with *ADR1-L2* or *ADR1-L2*$_{D484V}$, showing relative size. White bar is 2 cm. (C) Western blot of HA-tagged proteins from plants in (B) + and - BTH. Proteins were extracted from plants and run on SDS-Page gel and probed with anti-HA antibody. Ponceau-stained blot shows relative loading. (D) Ten-day-old seedlings were inoculated with 5 x 10^4 spores/mL *Hpa* Emco5 via spray inoculation. Sporangiophores per cotyledon were counted 4 dpi, with an average of 80 cotyledons per genotype counted. Sporangiophore counts were classified into: no sporulation (0 sporangiophores/cotyledon), light sporulation (1-5), medium sporulation (6-10), heavy sporulation (11-15), or very heavy sporulation (>15). Means of sporangiophore per cotyledon are listed below the graph. (E) Twenty-day-old seedlings were dip-inoculated with *Pto* DC3000(EV). Bacterial growth was assayed at 0 and 3 dpi. Values are mean cfu/mg ± 2 x SE, n=4. (F) Trypan blue stained leaves from (D). Leaves were collected and stained 4 dpi.

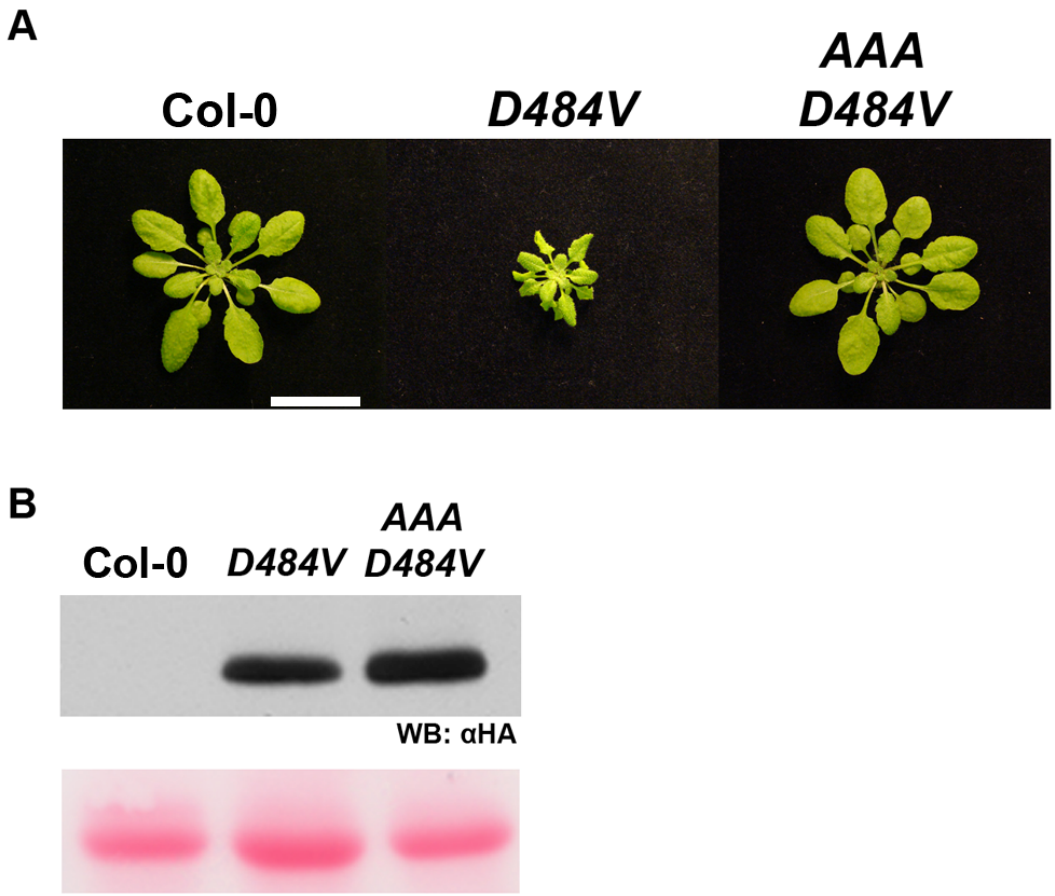

Figure 3.5. An intact P-loop catalytic domain is required for the ADR1-L2D484V morphological phenotype. (A) Pictures of 5-week-old Col-0, *ADR1-L2*$_{D484V}$, and *ADR1-L2*$_{AAA\ D484V}$ plants show relative morphology. White bar is 2 cm. (B) Western blot of Col-0 and HA-tagged ADR1-L2$_{D484V}$ and ADR1-L2$_{AAA\ D484V}$ proteins from plants in (A). Relative loading indicated by Ponceau stained blot.

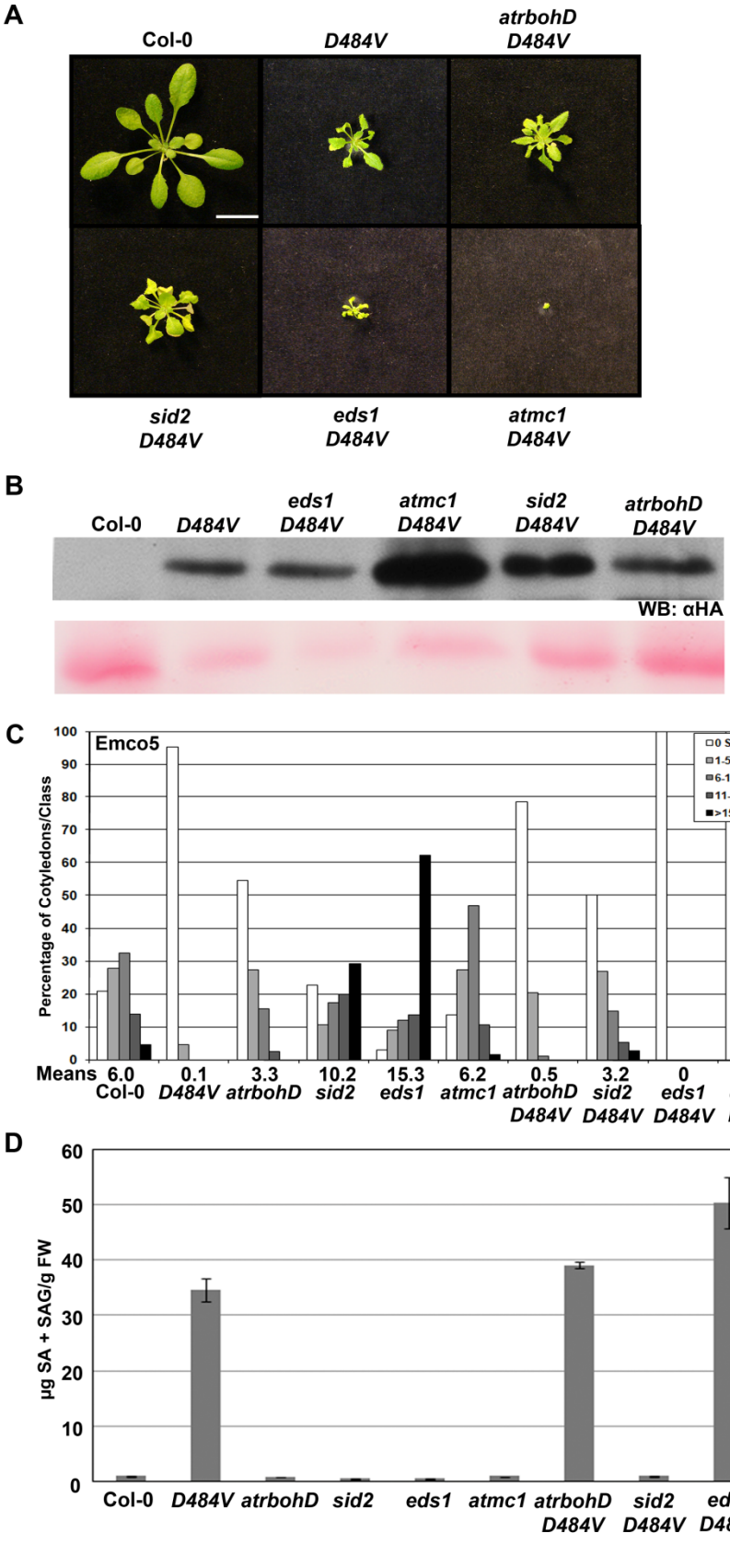
A
Col-0
D484V
atrbohD D484V
sid2 D484V
eds1 D484V
atmc1 D484V
B
Col-0
D484V
eds1 D484V
atmc1 D484V
sid2 D484V
atrbohD D484V
WB: αHA
C
Emco5
0 Sp
1-5 Sp.
6-10 Sp
11-15 Sp
>15 Sp.
Percentage of Cotyledons/Class
Means 6.0 0.1 3.3 10.2 15.3 6.2 0.5 3.2 0 0
Col-0 D484V atrbohD sid2 eds1 atmc1 atrbohD D484V sid2 D484V eds1 D484V atmc1 D484V
D
µg SA + SAG/g FW
Col-0 D484V atrbohD sid2 eds1 atmc1 atrbohD D484V sid2 D484V eds1 D484V

Figure 3.6. lsd1 suppressors are regulators of ADR1-L2D484V autoactivity. (A) Pictures of five-week-old Col-0, *ADR1-L2*$_{D484V}$, *atrbohD ADR1-L2*$_{D484V}$, *sid2-1 ADR1-L2*$_{D484V}$, *eds1-2 ADR1-L2*$_{D484V}$, or *atmc1-1 ADR1-L2*$_{D484V}$ plants, showing morphological differences between the genotypes. White bar is 2 cm. (B) Western blots of HA-tagged ADR1-L2$_{D484V}$ proteins from plants in (A). Ponceau staining shows relative loading. (C) Ten-day-old seedlings from plant lines as in (A) were inoculated with 5 x 10^4 spores/mL *Hpa* Emco5. At 4 dpi, sporangiophores were counted and classified as in Fig. 4. Means per cotyledon are listed below the graph. (D) Steady-state total SA levels were measured for leaves from plants as in (A). Values are average □g of total SA from 4 replicates, ± 2 x SE.

Self cross of ADR1-*L2*$_{D484V}$ *lsd1* +/-		
Genotype	**Actual**	**Expected**
LSD1/LSD1	**50**	**31**
LSD1/lsd1	**74**	**62**
lsd1/lsd1	**0**	**31**
Total	**124**	**124**

Table 3.1. *ADR1-L2*$_{D484V}$ is lethal in an *lsd1-2* background. Table of actual and expected genotypes of F3 progeny from a cross between *lsd1-2* and *ADR1-L2*$_{D484V}$ shows that no *lsd1-2* homozygous plants were recovered from plants that were homozygous for *ADR1-L2*$_{D484V}$. *ADR1-L2*$_{D484V}$ was also transformed into *lsd1-2*, but no plants with a detectable amount of ADR1-L2$_{D484V}$ protein were recovered.

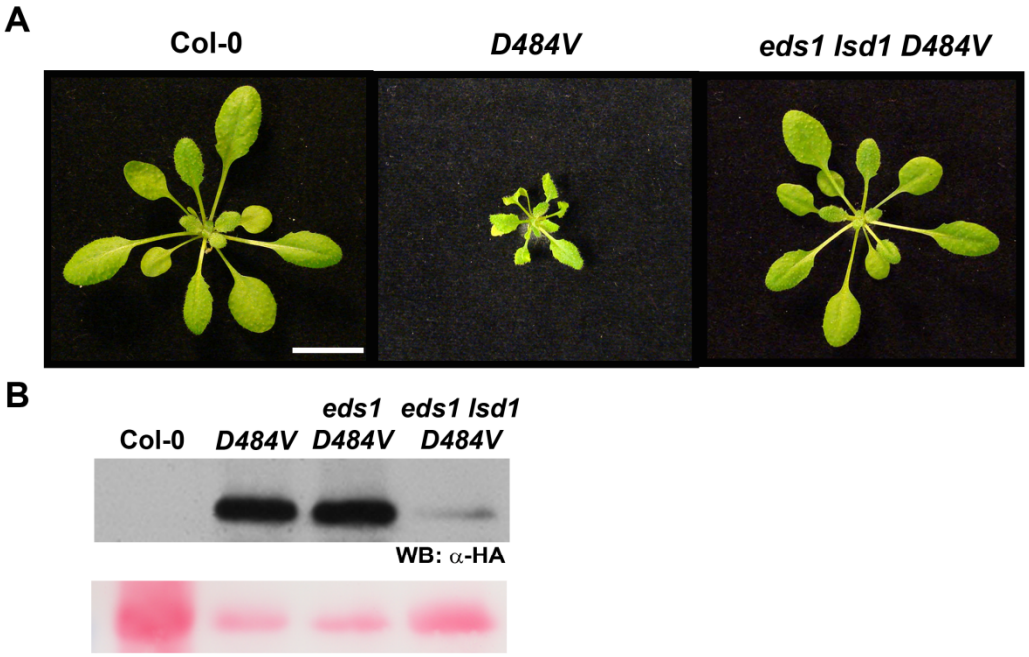
A
Col-0
D484V
eds1 lsd1 D484V
B
Col-0
D484V
eds1 D484V
eds1 lsd1 D484V
WB: α-HA
C
µg SA + SAG/g FW
0
10
20
30
40
50
Col-0
D484V
lsd1
eds1
eds1 D484V
eds1 lsd1 D484V
D
ADR1-L2/Ubq5 transcript level
0
2
4
6
8
10
12
14
Col-0
D484V
eds1 D484V
eds1 lsd1 D484V
sid2 D484V

Figure 3.7. eds1 lsd1 ADR1-L2D484V plants lose ectopic activation phenotypes. (A) Pictures of five-week-old Col-0, *ADR1-L2*$_{D484V}$, and *eds1-2 lsd1-2 ADR1-L2*$_{D484V}$ plants showing reversion of *eds1-2 lsd1-2 ADR1-L2*$_{D484V}$ to wild-type morphology. (B) Western blot of HA-tagged ADR1-L2$_{D484V}$ protein from plants in (A). Ponceau stain shows relative loading. (C) Total SA amounts (mean ± 2 x SE) were measured from plants of the indicated genotypes. Values are average µg of total SA from 4 replicates. Error bar represents ± 2 x SE. Controls here are from same experiment as data shown in Fig. 6C. (D) Quantitative real time PCR for the transcript amounts of *ADR1-L2* in Col-0, *adr1-L2 ADR1-L2*$_{D484V}$, *eds1 adr1-L2 ADR1-L2*$_{D484V}$, *eds1 lsd1 adr1-L2 ADR1-L2*$_{D484V}$, and *sid2 ADR1-L2*$_{D484V}$.

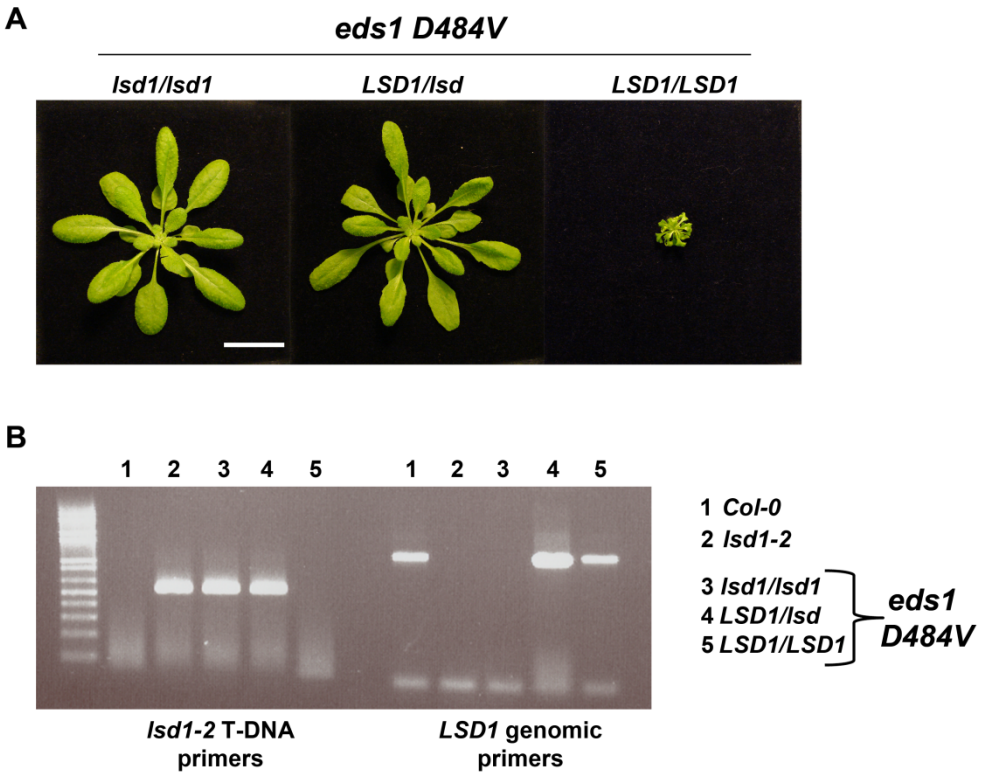

Figure 3.8. eds1 D484V plants segregating LSD1 show both wild-type and extreme cpr phenotypes. (A) Pictures of plants homozygous for *eds1* and *ADR1-L2*$_{D484V}$ and segregating *lsd1*. From the left: homozygote lsd1, heterozygote lsd1, homozygote LSD1. (B) PCR genotyping of plants in (A) shows that only *LSD1* homozygous *eds1 ADR1-L2*$_{D484V}$ plants have the severely stunted growth phenotype.

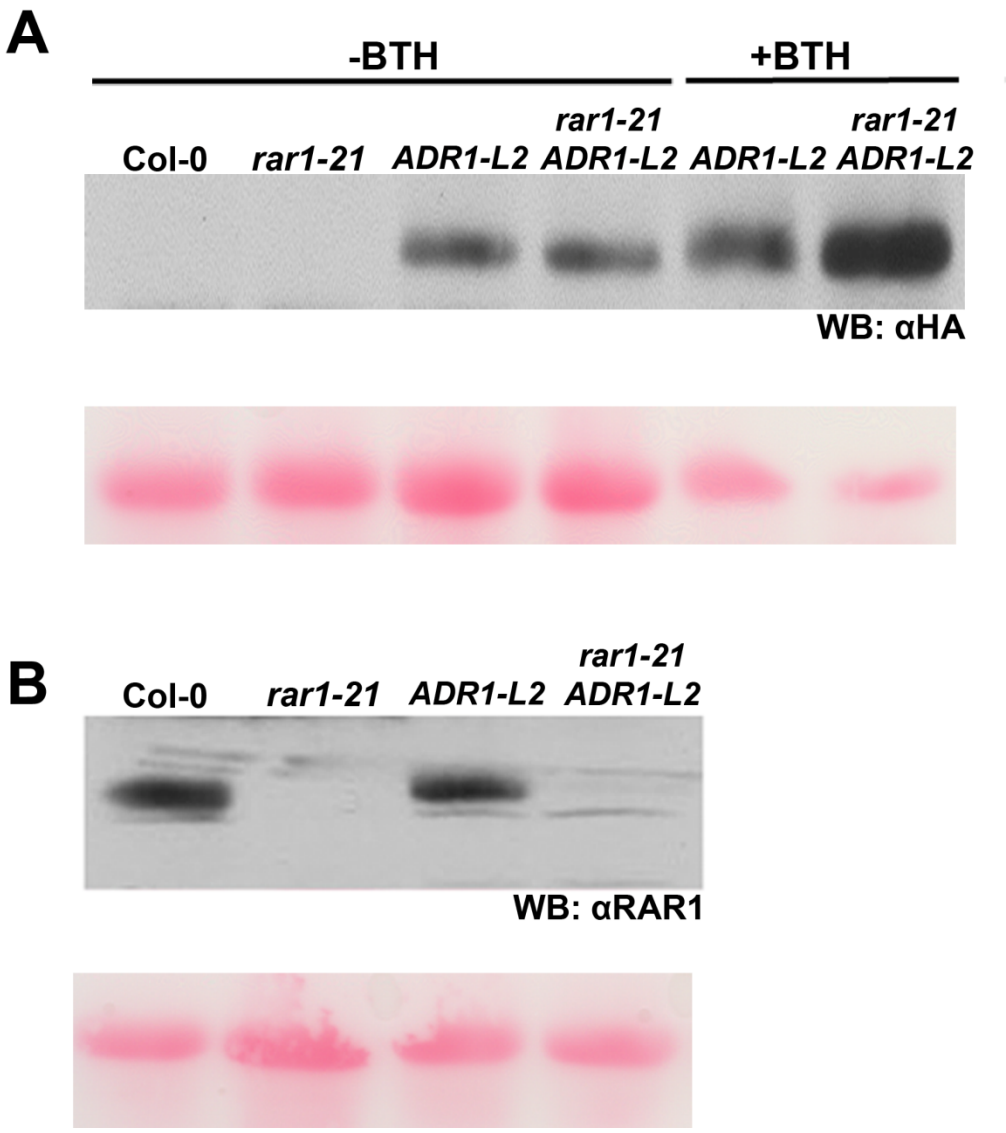

Figure 3.9. RAR1 is not required for either steady state ADR1-L2 accumulation or BTH-mediated induction. (A) *ADR1-L2-HA* and *rar1-21 ADR1-L2-HA* plants were sprayed with 300 µM BTH. Plants were collected for protein extraction 24 hpi. Proteins from Col-0, *rar1-21*, and *ADR1-L2-HA* and *rar1-21 ADR1-L2-HA* plants + and –BTH were run on SDS-Page gels and probed with anti-HA antibody. (B) Protein from plants in (A) were also used in an anti-RAR1 Western blot to ensure that *rar1-21* plants were not expressing RAR1. Ponceau stained blots in (A) and (B) show relative loading.

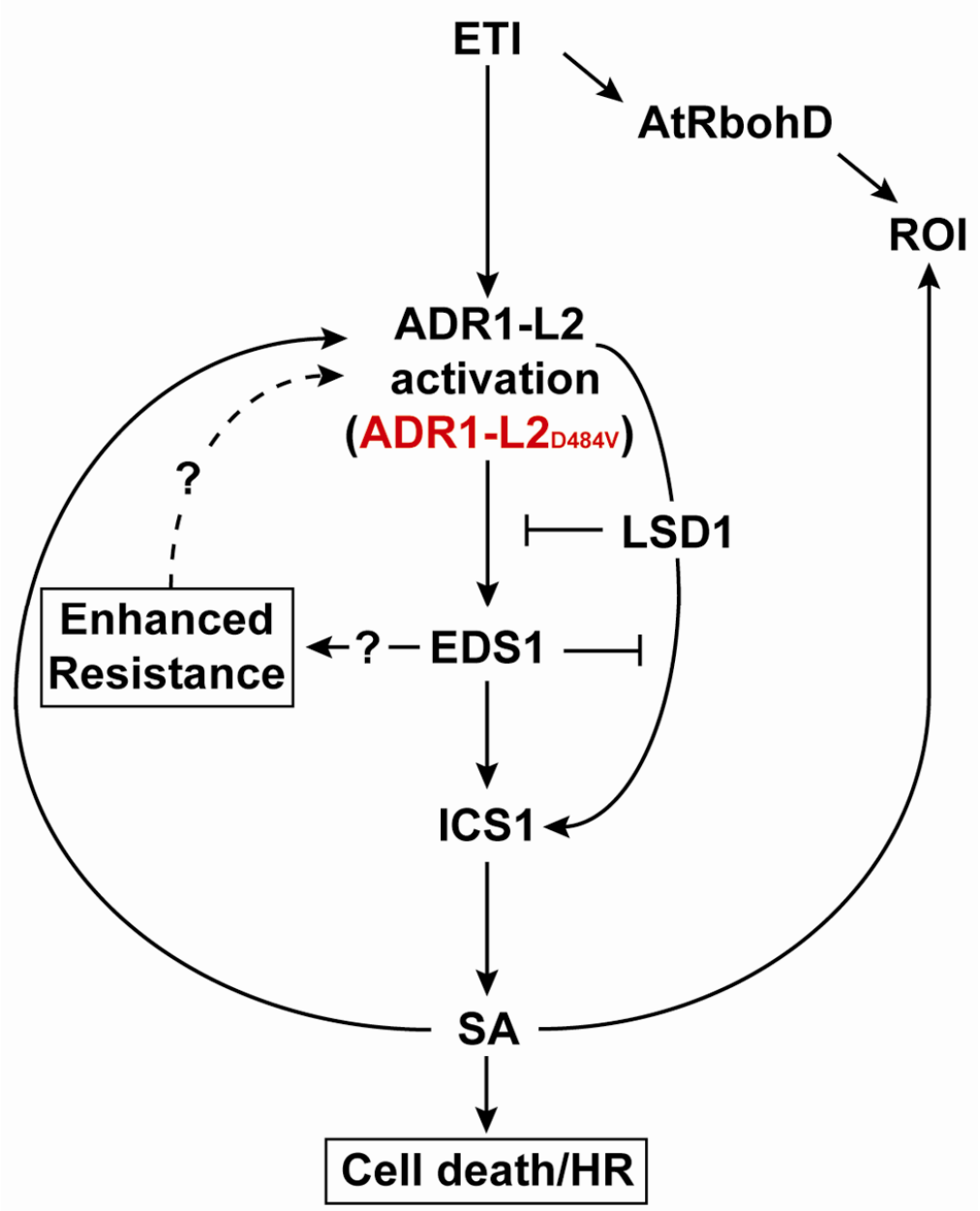

Figure 3.10. A model for the regulation of ADR1-L2D484V activity. ETI activates both an AtRbohD-dependent ROI burst and SID2-dependent SA accumulation via ADR1-L2. Activated ADR1-L2 initiates cell death and disease resistance via SA-dependent and -independent pathways. EDS1 functions downstream of activated ADR1-L2 as a positive regulator of both SA accumulation and the SA-independent pathway. ADR1-L2 also triggers SA via a pathway that is controlled by LSD1 and antagonized by EDS1. Therefore, the spread of this SA accumulation is spatially down-regulated through a combined action of EDS1 and LSD1. Due to its position in these feedback loops, SA functions both up- and down-stream of ADR1-L2.

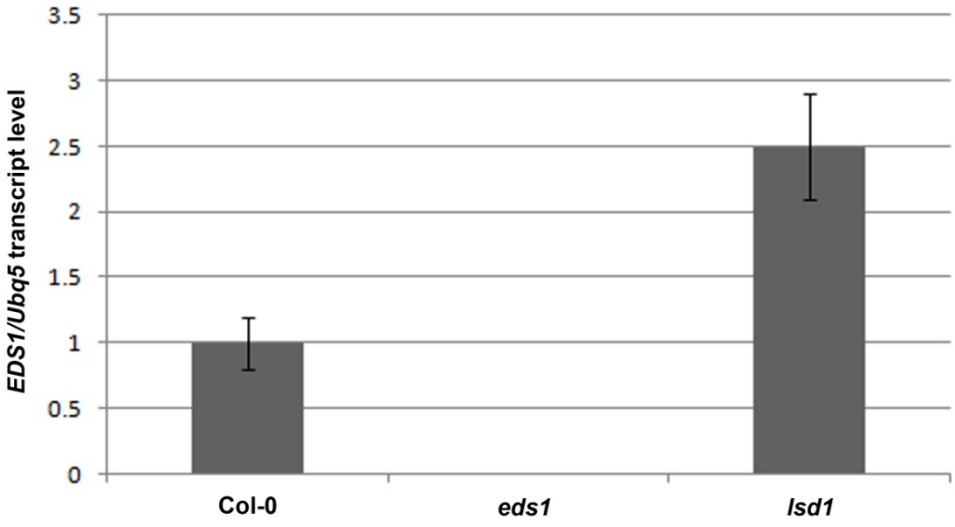

Figure 3.11. LSD1 negatively regulates EDS1 transcript. Quantitative real time PCR for the transcript amounts of *EDS1* in Col-0, *eds1-2*, and *lsd1-2*.

Primer Name	Primer Sequence
For genotyping	
eds1-2F	AAGGCGTCTGTAGAGGAAAC
eds1-2R	CATATAGTCTCGCAGAGGAG
rar1-21F	TCACGACGGAATGAAAGAGTGGAGCTGCTACTAG
rar1-21R	TTTTGGAACCGATTTGGCCAGAACTGGTTTCTCAG
sid2-1F	AAGCTTGCAAGAGTGCAACA
sid2-1R	AAACAGCTGGAGTTGGATGC
AtMC1F	GCGTCACCTTCTCATCAACA
AtMC1R	ACGGTACCACTATGGCAAGC
LSD1F	CTGGGATTTGTAAAGCAGCTG
LSD1R	TCAAGTTCCATGGAGCAAAAG
ADR1-L2F	TTCTTACTGTGTGTCCCCAG
ADR1-L2R	CCTTCCTATCAATCCGATCG
For quantitative PCR analysis	
EDS1F	GACGGGGAAGTAGATGAGAAG
EDS1R	TCATCCATCATACGCTCACG
ADR1F	ATGGCTTCGTTCATAGATCTTTTC
ADR1R	CACATTGTAGGTGGTTCTAGG
ADR1-L1F	AAACCACTCTTGCCAAAGAAC
ADR1-L1R	GGATTTCCAGCTTCACAACC
ADR1-L2F	CCTCTTGATGTTCTCATCAAC
ADR1-L2R	GTAGCTAGTGTACATCTGTCC

Table S2. Primer sequences used in this work.

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Chapter 4

Conclusions and Future Directions

Plants are the backbone of our environment, providing oxygen, preventing erosion, and functioning as the base of nutrition for all animals. As such, it is vitally important that these species are able to survive environmental threats that they encounter, such as pathogen attack. Studying the process of disease resistance in plants is of great importance, as it allows insight into the biochemical and mechanical approaches used by plants to combat potential pathogens. With such knowledge we are able to develop specific and direct approaches for improving disease resistance in plants. In addition, plants and animals share common disease resistance mechanisms, and therefore studying these processes in plants can inform our understanding of animal, and human, immune responses.

During my thesis work, I participated in two main projects, both of which stem from studies on the plant cell death regulator LSD1. In the first part of my work, several assays were used to identify potential LSD1 interactors. One of these, the NF-Y transcription factor subunit NF-YC3, was used in further studies and found to be a positive regulator of disease resistance. *nf-yc3* plants exhibit increased susceptibility to *Hpa*, whereas over-expression of functional NF-YC3 leads to increased resistance, presumably by contributing to the overall up-regulation of disease resistance-related

genes and/or cell death genes. Proper function of NF-YC requires its relocalization from the cytosol to the nucleus, and we discovered that LSD1, probably working with other factors, potentially participates in NF-YC3 cytosolic retention. Therefore, this work provides additional evidence that LSD1 may function as a transcriptional regulatory scaffold, sequestering defense-related proteins in the cytosol, and dampening their functions.

Immediate future work on this project focuses on the data indicating that LSD1 is functioning as a retention factor of NF-YC3. The key problem is that the current BiFC data shows that all the fluorescence is in the nucleus when both LSD1 and NF-YC3 are co-expressed, which is in contrast to previously published data, and in opposition to our data which indicates that an intact LSD1 interaction motif is necessary for NF-YC3 to accumulate in the cytosol. To address these problems, additional experiments are being performed which will use co-localization of two xFP colors to address the specificity of the interaction between LSD1 and NF-YC3. This new experiment will show co-over-expression of LSD1-xFP with, first NF-YC3-xFP, and second with NF-YC3ΔGP2-xFP. These constructs will express different FPs for LSD1 and the NF-YC3 constructs so that they can each be imaged at the same time, and a merged image can then be made for the figure. The anticipated result is that the LSD1 / NF-YC3 will co-localize in the cytosol (with perhaps some NF-YC3 signal in the nucleus) and that LSD1/ NF-YC3ΔGP2 will show that LSD1 is in the cytosol and NF-YC3ΔGP2 is in the nucleus, as predicted by our current FP localization data.

Beyond this immediate work, future studies for this project should focus on the redundancy in the NF-Y transcription factor family, looking to see whether other

members of this family, alone or in combination with one another, also play a role in disease resistance. Another NF-YC, NF-YC 4, was identified in the LDS1 interactor screen, and six additional Arabidopsis NF-YCs contain the GxP LSD1 interaction motif. Single and combinatorial mutants of these genes could be made to look at the contribution of these other NF-YC subunits to disease resistance.

In the second part of my work, I focused on ADR1-L2, a positive regulator of *lsd1* rcd. ADR1-L2 is an NB-LRR, one of the main class of disease resistance proteins that are about to recognize specific proteins injected into the cell by pathogens. We first showed that, in addition to the non-canonical, P-loop independent functions previously reported, ADR1-L2 had P-loop dependent functions in *lsd1* rcd. By creating an autoactive version of this protein, ADR1-L2$_{D484V}$, we were able to characterize the canonical, P-loop dependent functions of this protein in the absence of a specific effector that would normally be required to activate it. *ADR1-L2$_{D484V}$* plants are dwarfed, bushy plants with short hypocotyls and pointed leaves, and they exhibit high steady-state levels of SA and increased resistance to virulent pathogens. We then used this autoactive mutant to help define the genetic requirements of the signaling pathway that contains ADR1-L2. Our data led us to position ADR1-L2 in a feedback loop involving SA, LSD1, and EDS1. Our results also indicate that this protein is additionally regulated by SA-independent factors, as well as by the cell death executioner AtMC1.

The next experiments using the autoactive ADR1-L2$_{D484V}$ mutant should further examine the placement of ICS1/SA in our pathway. To do this, we will make *sid2 lsd1 ADR1-L2$_{D484V}$* and *sid2 eds1 ADR1-L2$_{D484V}$* plants. If our model is correct, loss of SA in both of these contexts should lead to a reduction in the rcd phenotype. Thus, we should

be able to recover *sid2 lsd1 ADR1-L2$_{D484V}$* plants, and *sid2 eds1 ADR1-L2$_{D484V}$* plants should not be severely dwarfed like the *eds1 ADR1-L2$_{D484V}$* plants presented here. *sid2 atmc1 ADR1-L2$_{D484V}$* plants should also be created, as the phenotypes of this plant could help to position AtMC1 in our current model. In parallel with this, forward genetic screens using EMS mutagenized seed could help us to identify other genes that are necessary for the autoactive phenotype. Using both the *ADR1-L2$_{D484V}$* parental line and the SA-deficient *sid2 ADR1-L2$_{D484V}$* line would allow us to discover genes important in both the SA-dependent and -independent pathways.

Uniquely, ADR1-L2 is the first NB-LRR to exhibit RAR1-independent accumulation. In light of this result, future experiments could also test the requirements of other proteins, such as SGT1b, that are typically required for NB-LRR protein stability. Additional work is also being carried out by Dr. Vera Bonardi to try and understand the mechanism behind ADR1-L2 function. She is currently analyzing proteomics data that examines both the phosphorylation state of inactive and activated ADR1-L2, as well as potential protein interactors.

Overall, my work has helped to refine the model of pathogen-triggered plant resistance, especially in terms of LSD1. I provided additional data that supports the idea of LSD1 as a cytosolic retention factor, and uncovered data that supports the role of the NF-Y transcription factor family in disease resistance. Importantly, I contributed a model that tries to add to the understanding of how a single protein could be involved as both a positive regulator of *lsd1* rcd and retain its function as a canonical NB-LRR. Additionally, my work presents a way around the problem of characterizing a NB-LRR without the benefit of knowing the effector that triggers it, as it provides a general

approach to characterize canonical, P-loop dependent functions of NB-LRR proteins in the absence of a specific effector.