Dissecting Plant Immune Signaling

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Abstract

Pathogen infection in plants can result in plant disease. In crop plants, this can cause large losses in yield and profit, as well as endanger food security. The plant immune system that has evolved to fight off these infections differs from that of mammals greatly, but also involves tight regulation and complex signaling networks, to prevent under- or over-active immune expression. The many layers of plant immune signaling are still not completely understood. Here, two aspects of immune signaling are considered. Firstly, the theoretical explanations and practical considerations for the clustering of NLRs in genomes, both in pairs and in larger clusters were examined. I put forward that the frequency with which functionally linked NLRs are found to also have close genomic proximity is not coincident, and thus should be taken into consideration when examining the functions of NLRs located adjacent to other NLRs, and broader recognition capabilities considered in these cases.

Secondly, mutants found through a suppressor screen of the autoimmune triple mutant *camta1/2/3* were studied in order to better understand downstream signaling and immune crosstalk in *Arabidopsis thaliana*. Complementation tests revealed that most of the mutants identified carry mutations in known genes. However, a handful of mutants were identified that will pave the way to future discoveries of novel mechanisms of plant immune regulation. This suggests that new mutants could be found through continued screening, as we are moving closer to screen saturation.

Lay Summary

Understanding plant immunity is an important part of our ability to combat plant diseases affecting crops and other plants. The process through which plants turn on and off immunity is incredibly complex. Here, I examine how plants may have evolved to have pathogen recognition proteins which interact together reside next to one another in the genome, and how that should influence our study of these proteins. I also examine mutants able to suppress the hyperactive immunity of another mutant in an attempt to learn more about how immune signaling is regulated in the model organism *Arabidopsis thaliana*.

Preface

The project in chapter 3 was originally conceptualized by Xin Li. The original mutagenesis and screening steps were organized and carried out by Yan Xu. I was involved in the transplanting of the M1 and the growing of the M2. The M2 were then split into two groups, one of which I was solely responsible for and is the group I discuss in this thesis. The work detailed was done by me, although the HPLC machine for the first SA measurements was operated by Yulin Sun.

A version of chapter 1 has been published. van Wersch, S., Tian, L., Hoy, R. and Li, X., 2020. Plant NLRs: the whistleblowers of plant immunity. *Plant Communications*, *1*(1), p.100016. I wrote most of the manuscript. Sections written by fellow students were either removed or re-worked.

A version of chapter 2 has been published. van Wersch, S. and Li, X., 2019. Stronger when together: clustering of plant NLR disease resistance genes. *Trends in plant science*, *24*(8), pp.688-699. It was originally conceived by Xin Li, and we planned the Opinion piece together. I wrote most of the manuscript and compiled the information contained in the figures.

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List of Abbreviations

ADP/ATP	adenosine diphosphate/adenosine triphosphate
ADR1	ACTIVATED DISEASE RESISTANCE 1
ALD1	AG2D-like defense response protein 1
ATR1	*Arabidopsis thaliana* cytochrome reductase 1
Avr	*Avirulence* gene
bal1	*SNC1* mutant line
CAMTA	Calcium/calmodulin binding transcriptional activator
CBP60G	Calmodulin binding protein 60-like g
CC	Coiled-coil domain
CDC5	Cell division cycle 5
CNL	CC-NB-LRR
Cf-9	*Cladosporum fulvum resistance* gene *9*
CHS	chilling sensitive
CPR1	CONSTITUTIVE EXPRESSER OF *PR GENES* 1
DDM1	Decreased DNA methylation 1
EDS1	ENHANCED DISEASE SUSCEPTIBILITY 1
EPS1	Enhanced *Pseudomonas* susceptibility 1
ETI	Effector-triggered immunity
FLS2	FLAGELLIN-SENSITIVE 2
FMO1	Flavin-dependent monooxygenase 1
HSP90s	HEAT SHOCK PROTEIN 90 FAMILY
ICS	Isochorismate synthase
LRR	leucine-rich repeat
MAC	MOS4-associated complex
MAMP	Microbe-associated molecular pattern
MIR1	microRNA 1
MLA	Mildew Resistance locus a
MOS	MODIFIER OF SNC1
MPK4	MAP KINASE 4
MUSE	Mutant *snc1*-enhancing
NAIP	NLR-family apoptosis inhibitory protein
NDR1	NON-RACE SPECIFIC DISEASE RESISTANCE 1
NHP	N-hydroxy pipecolic acid
NB	nucleotide-binding
NLRC4	helper NLR, CARD domain containing 4
NLRs	nucleotide-binding leucine-rich repeat immune receptors
NRG1	N REQUIREMENT GENE 1

PAD4	PHYTOALEXIN DEFICIENT 4
PAMP	pathogen-associated molecular pattern
PBL2	PBS1-LIKE PROTEIN 2
PBS3	Arabidopsis AVRPPHB SUSCEPTIBLE3
PR	*Pathogen response* gene
Prf	Pesudomonas resistance and fenthion sensitivity
PRRs	pattern-recognition receptors
R	*resistance* gene
RAR1	REQUIRED FOR MLA12 RESISTANCE 1
RGA	R-GENE ANALOG
RIN	RPM1-INTERACTING PROTEIN
RING	Really Interesting New Gene-like domain
RKS1	Resistance related kinase
RPM1	RESISTANCE TO *P. SYRINGAE* PV. *MACULICOLA* 1
RPP	RECOGNITION OF *Peronospora parasitica*
RPS	RESISTANCE TO *P. SYRINGAE*
RPW8	RESISTANCE TO *POWDERY MILDEW* 8
RRS1	RESISTANT TO *RALSTONIA SOLANACEARUM* 1
SA	salicylic acid
SAG101	SENESCENECE ASSOCIATED GENE 101
SAUL1	Senescence-associated E3 ubiquitin ligase 1
SAR	Systemic acquired resistance
SARD	Systemic acquired resistance deficient
SGT1	SUPPRESSOR OF G2 ALLELE OF SKP1
SIKIC	Sidekick SNC1
SIZ1	SAP and MIZ1 domain containing ligase 1
SNC1	Suppressor of *npr1*, Constitutive 1
sNLRs	sensor NLRs
SOC3	Suppressors of *chs1-2*, 3
SPLAYED	Chromatin remodeling complex subunit R3
SR	Stem rust resistance gene
SSI4	Suppressor of *npr1-5*-based salicylic acid insensitivity
SUMM2	Suppressor of *mkk1 mkk2*, 2
SUMO	Small ubiquitin-like modifier
TIR	Toll/interleukin-1 receptor
TN	TIR-NB
TNL	TIR-NB-LRR
ZAR1	HOPZ-ACTIVATED RESISTANCE 1

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In addition, I want to extend my appreciation to my fellow Li lab members, present and former, who helped to guide and teach me, especially Karen, for his endless patience with my endless questions, and Yan and Jianhua who laid much of the groundwork for my part in the *camta1/2/3* screen.

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I also want to extend love and thanks to my friends and family, who helped keep me sane.

To anyone who helped guide me along the way, thank you.

And to anyone else who has achieved something during this pandemic, well done.

Chapter 1: Introduction

1.1 Plant Pathology Background

Humans have relied on agriculture as our primary food source for over 10 000 years. For just as long, plant pathogens have posed a threat to livelihoods and societal growth. As such, the primitive study of phytopathological phenomena has also been around for thousands of years. Theophrastus, a pupil of Aristotle, wrote extensively on the subject of botany, noting that wild trees were much more "vigorous" than their cultivated counterparts[1]. Most of this early research into plant diseases, however, centered on descriptivism, with little understanding about the actual relationship between plants and their pathogens. Despite the interest of some early scholars such as Theophrastus, the impact of pathogens on crops was often overlooked, both scientifically and socially. Agricultural records throughout the Middle Ages rarely contained references to specific plant diseases[2]. In addition, much like their contemporaries who studied humans, most botanists up until the 19th century believed that plant diseases were caused by internal factors within the plant[3]. It is perhaps unsurprising then, that very little progress was made in preventing plant disease.

The discovery that human and animal diseases are caused by external factors, also known as the germ theory, occurred in the mid to late 1800s. The first strong evidence and application of this came from a decrease in mortality rates after Ignaz Semmelweis instructed doctors at the hospital where he worked to wash their hands before treating patients. It would take the work of several other scientists, including Lister, Koch, and Pasteur, before the theory became widely accepted[4]. Fascinatingly, the scientific debate and change in paradigm that occurred after Koch's

and Pasteur's research happened several decades earlier with botanists[5]; work by Berkeley and others showed that potato late blight was caused by a parasitic organism rather than solely environmental conditions[6]. This notorious pathogen was later named *Phytophthora infestans* by Anton de Bary[7]. Once the causal relationship of fungi and bacteria in plant diseases was established, breeding for plants that were capable of resisting relevant pathogens became possible. As the scientific understanding of genetics improved, such plants were suggested to possess *resistance* (*R*) genes; resistance could often be conferred following a simple dominant Mendelian inheritance pattern[8].

In the 1940s, after observing differences in susceptibility between isolates of *Melampsora lini* (flax rust) on cultivars of flax, Flor came up with the gene-for-gene hypothesis[9]. Oort simultaneously came to a similar conclusion by studying the interaction between wheat and *Ustilago tritici* (loose smut of wheat)[10]. More fully defined by Person, Samborski, and Rohringer in the 1960s, the gene-for-gene hypothesis suggested that individual gene products from pathogens (known as *avirulence* [*avr*] genes, now referred to as effector-encoding genes) interacted with R proteins in plants, and that the presence or absence of one or the other could predict whether successful biotrophic infection would occur[11]. The evolutionary arms race relationship between *avr* and *R* genes has always been part of the gene-for-gene hypothesis, but it is perhaps best illustrated by the zig-zag model drawn by Jones and Dangl in 2006[12]. In this model, *avr* genes encode effectors, molecules that often target elements of plant immune response and allow for pathogen infection, and R proteins are molecules that were evolved to recognize these effectors from biotrophic pathogens and trigger a stronger defense response. In other words, pathogens gained effectors to combat the plant immune system and successful

plants fought back by developing new *R* genes, the product of which could detect those effectors and re-establish a successful immune response, zig-zagging with each gain of a new *avr* or *R* gene. Though the evidence that all effectors target elements of basal immunity is not conclusive, this explanation provides an evolutionary link between the two layers of plant immunity: PAMP-triggered immunity (PTI) and effector triggered immunity (ETI), where PTI is triggered by pathogen/microbe-associated molecular patterns (PAMPs/MAMPs; examples include fungal chitin and bacterial flagellin, and elongation factor proteins) receptors at the plasma membrane while ETI is mediated by intracellular R genes, typically nucleotide-binding leucine-rich repeat (NLR) receptors[12–14].

1.2 NLRs in Plant Immunity

1.2.1 *Avr* and *R* Gene Identities

With the dawn of molecular biology, scientists were able to clone first the *Avr* genes (likely due to the small size of pathogen genomes) and soon after the *R* genes. In 1984, cosmids from wild-type *Pseudomonas syringae* pv. *glycinea* were used in complementation experiments to find a causal gene determining race 6 race-specificity on *Glycine max*, *AvrA*[15]. Though only showing one side of the interaction, this clear evidence for a single gene conferring race-specific host–pathogen interaction was the first strong molecular evidence in support of the gene-for-gene hypothesis. Over the next few years many more *avr* genes were cloned, including those from fungi and oomycetes such as *Avr9*[16] and *Avr1b*[17]. The *avr* identities vary greatly and are unpredictable, coming from many different protein families. For example, the *Pseudomonas* effector AvrF is from the protein chaperone family[18], while AvrPto interacts with kinases[19] and

the *Xanthomonas* effector AvrXa7 contains DNA-binding domains[20]. Even though effectors may have similar or related targets in plants, they often evolve independently[21]. Perhaps because of this, predicting the function and targets of effectors can be a challenge[22].

The first cloned *R* gene is generally considered to be *HM1*, which was reported in 1992[23]. The gene product of its *avirulence* counterpart, HC toxin, had been characterized previously in *Helminthosporium carbonum*, a pathogen that infects corn[24]. Presence of the carbonyl reductase *HM1* gene resulted in resistance against the HC toxin due to its detoxification effects. By today's standard, *HM1* is not a true *R* gene, as it does not trigger defense through detection of effector activity, but its cloning is a landmark moment in plant pathology. In 1993 a second *R* gene was cloned; researchers discovered that the kinase Pto in tomato conferred defense responses against pathogens carrying the effecter AvrPto[25]. These early well-studied cases seemed to confirm Flor's gene-for-gene hypothesis, and as such the theory continued as the primary model for understanding *R* gene activity for the remainder of the century. In 1994, several *R* genes that featured leucine-rich repeats (LRRs) were cloned, including *Cf-9*, a predicted membrane protein with an extracellular LRR domain from tomato[26], the *N* gene in tobacco[27], and *RPS2* from *Arabidopsis thaliana*[28,29]. In 1995, another *Arabidopsis* gene, *RPM1*, was also cloned[30], as well as the *L6* gene in flax[31] and *Xa21* from rice[32]. N, RPS2, RPM1, and L6 are all nucleotide-binding LRR (NLR) proteins. As more *R* genes were identified, it became clear that NLRs represented a majority of plant *R* genes, and that proteins such as HM1 and Pto were exceptions (see later discussion of Pto in section 1.2.4.1).

1.2.2 Discovering Plant NLRs

The completion of the full genome sequence of *A. thaliana* in the year 2000 revealed its possession of more than 150 NLR-encoding genes[33]. Since then, whole-genome sequencing has revealed that higher plant species contain anywhere from 50 (papaya) to over 1500 (wheat) *NLR* genes, with many non-vascular plants having fewer[34–36]. The number, arrangement, and domain combinations of these genes can vary drastically even among ecotypes, indicating that *NLR*s can be rapidly gained or lost[37]. NLR proteins were discovered to be present in humans and other animals about 5 years after they were found in plants[38]. In animals, NLRs play a role much like plant membrane-localized pattern recognition receptors (PRRs), detecting PAMPs or DAMPS (damage associated molecular pattern) and triggering innate immunity responses such as inflammation[39,40]. Though there may be plant NLRs not involved in immunity, for the remainder of this defence NLR will refer solely to those involved in plant defence.

Plant NLRs are also structurally distinct from animal NLRs. Both feature a nucleotide-binding domain believed to be involved in oligomerization, and an LRR domain that is generally thought to be involved in effector recognition and autoinhibition[38]. Typical plant NLRs almost universally feature the additional coiled-coil (CC) or Toll/interleukin-1 receptor (TIR) N-terminal domain, while many mammalian NLRs carry a caspase activation and recruitment domain at their N termini, enabling caspase activity as a major way of NLR activation. These N-terminal domains are used to sort plant NLRs into two main groups termed CNLs (CC-NLRs) and TNLs (TIR-NLRs). Both CC and TIR domains have been demonstrated to play key roles in the formation of dimers and oligomers. The structure–function relationship of NLRs is discussed in detail in section 1.2.6, but it is important to recognize that conformational changes (caused by

effector interactions) that result in different levels of nucleotide-binding domain affinity for ATP/ADP are considered the most likely mode of NLR activation in both plants and animals.

1.2.3 NLR Signalling Coworkers

Although NLRs themselves are responsible for allowing plants to detect specific pathogen threats, the process of actual triggering ETI involves many other components. Indeed, recognition of effectors is only the first step in activating the immune response in plants. Without caspase orthologs encoded in higher plant genomes, much of the downstream signaling remains a mystery, although several key players have been revealed from various genetic screens. The first major non-*R* gene discovered was *NON-RACE SPECIFIC DISEASE RESISTANCE 1* (*NDR1*) in 1995. Loss of function in *NDR1* leads to susceptibility against pathogens carrying a variety of effectors, both bacterial and fungal in origin[41]. The following year, another gene was discovered named *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*), which was found to be necessary for the immunity conferred by several *NLR*s in the *Resistance to Peronospora parasitica* (*RPP*) family[42]. CNLs seem to signal through NDR1, and TNLs signal through EDS1, suggesting at least two distinct downstream signaling branches for NLRs[43]. Further research into EDS1 revealed that it partners with either PAD4 or SAG101 downstream of known TNLs in order to trigger immunity[44]. The exact mechanisms of how these proteins aid in signaling is still unclear. EDS1, PAD4, and SAG101 are homologous lipase-like proteins; however, the lipase activity does not seem to be required for their immune-signaling activity. NDR1 has been shown to be required for the immunity of some, but not all, CNLs. Interestingly, among those CNLs that do not require NDR1 is RPP8, which shows decreased immunity only when both EDS1 and NDR1 are knocked out[43]. This suggests that the pathways are not entirely distinct for all NLRs.

Plant immune activation also relies on the presence of chaperone proteins. HSP90, SGT1b, and RAR1 are all known to contribute to NLR-triggered immunity, playing key roles in ETI triggered by both CNLs[45,46] and TNLs[47]. HSP90 is capable of interacting with both RAR1 and SGT1b in a non-exclusive manner, suggesting a potential for parallel pathways[45]. Although it is believed that these chaperones contribute to assembly of NLR activation complexes and therefore affect NLR homeostasis, concrete biochemical evidence is lacking.

1.2.4 Beyond the Gene-for-Gene Hypothesis for ETI

Although the gene-for-gene hypothesis has heavily influenced the way in which NLRs were studied, evidence from the last two decades has led many investigators to re-examine it. As its name suggests, the gene-for-gene hypothesis implies a direct connection between a pathogen effector and plant R protein, but it has become increasingly clear that NLR detection of effectors is much more complicated. Direct interaction between the R protein and effector has been observed in a number of cases, such as classic examples of rice Pi-Ta and its corresponding effector AvrPi-Ta in *Magnaporthe oryzae*[48], *Arabidopsis* RPP1, *Hyaloperonospora arabidosidis* ATR1[49,50], tobacco N, and tobacco mosaic virus (TMV) P50[51]. Several other NLRs that were more recently shown to directly interact with their cognate effectors include Roq1[52], L6[48], M[53], Sr35[54], Sr50[55], and the powdery mildew-resisting MLAs[56]. In general, however, it seems to be just as common to detect indirect protein–protein interactions between the NLR and its cognate effector.

1.2.4.1 The Guard Hypothesis

The first suggestion that R proteins can work through indirect detection came in 1998, when Van der Biezen and Jones examined the relationship between *Pseudomonas syringae* AvrPto, the kinase Pto, and the NLR Prf. Pto, as previously mentioned, had originally been considered to be an R protein because it was found to be required for AvrPto-triggered immunity. However, although Pto did appear to act in defense, its kinase activity was not what prevented infection; instead it was the activity of the NLR Prf which mediated the strong defense response that conferred immunity in tomato. Van de Biezen and Jones, in 1998[57], hypothesized that AvrPto targets Pto due to its role in non-effector-triggered immunity, and that Prf can sense this interference and turn on a stronger immune response. By 2001, this model had been christened the "Guard Model" and was regarded as another mechanism for effector detection: Avr proteins might target general plant immune proteins in order to increase virulence, and plant R proteins might function by detecting this interaction[13] (Figure 1 A). This model suggests a very clear and plausible evolutionary trajectory: pathogens evolve effectors to target plant defense proteins (or to aid virulence and growth in some other scenario), while host plants evolve R proteins to detect this threat and trigger a more powerful defense.

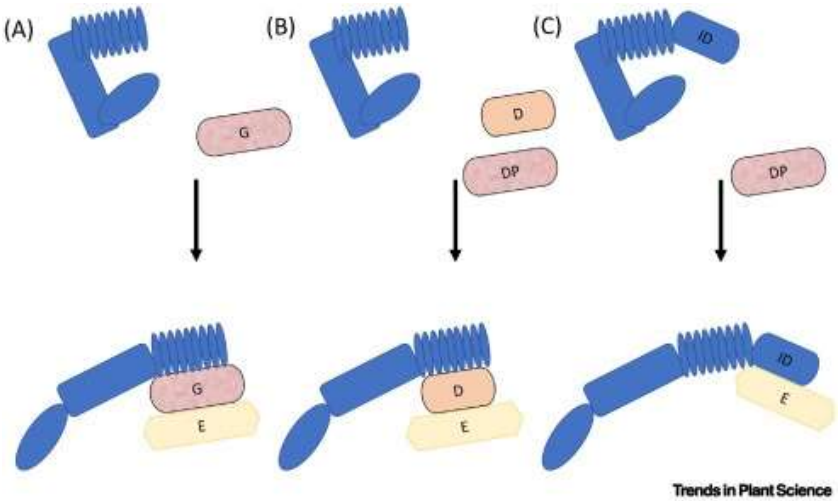

Figure 1. Three methods of indirect effector recognition by NLRs.

Figure from van Wersch and Li, 2019[58]. Note that this is only a representative, simplified model; effectors (E) do not necessarily stay bound to their targets and NLRs may not interact directly with their modified guardee (G). Activation of NLRs is likely to require the conversion of bound ADP to ATP and oligomerization. (A) Guard model: The NLR guards a defense-related protein and is activated when this guardee (G) is modified or influenced by a pathogen effector (E). (B) Decoy model: The NLR guards a decoy (D) protein, which mimics a defense-related protein (DP), and is activated when that decoy is modified or influenced by a pathogen effector. The decoy serves to protect the host immune protein DP from being targeted by effectors. (C) Integrated decoy model: The NLR is activated when an integrated decoy domain (ID) in the NLR is modified or influenced by a pathogen effector. The D or ID in (B) or (C) share sequence homology with DP.

The biochemical details of the guard model are versatile and are explored further in chapter 2. For some examples here, *Arabidopsis* protein RIN4 is targeted by a range of different pathogen effectors and is guarded by several R proteins[59]. In comparison, both R proteins TAO1 and RPM1 respond to the same effector, but their actual guardees appear to be different[60]. The R protein SUMM2 guards the phosphorylation product of a mitogen-activated protein kinase cascade, and thus detects interference of any of the proteins that make up the cascade[61]. Another interesting example of how the guard model differs from the basic form of the gene-for-gene

hypothesis is the case of the *Arabidopsis* guardee SAUL1, which is guarded by the SOC3–CHS1 and the SOC3–TN2 pairs depending on whether SAUL1 levels decrease or increase, respectively[62]. A major addition of the guard model is that NLRs detect effector activity, not effectors themselves, which more directly ties these interactions to the overall immune response; ETI will likely not be triggered unless a pathogen appears to be overcoming PTI.

1.2.4.2 Decoy and Integrated-Decoy Models

In 2008, a modification of the guard model, known as the decoy model, was proposed. Once again, a close examination of AvrPto played a key role in the development of this model[63–65]. In short, the researchers noticed that the effector triggered ETI by interacting with the kinase Pto, but that Pto did not seem to play a substantial role in PTI. AvrPto also appeared to be capable of interacting with PRRs on the plasma membrane that did play key roles in PTI, such as FLS2, which perceives bacterial flagellin. This observation led to the concept that perhaps guardees of some R proteins could act as decoys which are able to interact with pathogen effectors and thus trigger ETI through their guard (Figure 1 B). Kay *et al*.[66,67] also noted that the promoter sequence of the *R* gene *Bs3* mimics the promoter region of another gene, *upa20*, whose promoter sequence is bound by AvrBs3 in susceptible pepper lines. When *Bs3* is present, AvrBs3 binds to its promoter sequence instead, leading to resistance. One of the suggested benefits of decoys is that they are subject to fewer evolutionary pressures than traditional guardees[65]. Traditional guardees must maintain their immune function while remaining recognizable by their cognate NLRs and remaining a target of their effector. A decoy, on the other hand, only needs to

have its effector-induced changes recognized by its NLR guard, thus protecting its paralogs with immune functions.

While the decoy model is widely accepted by plant pathologists as a mode of effector detection by NLRs, proving the existence of a specific decoy is rather challenging. To do so, it must be proven that the protein targeted by the effector plays no functional role besides that of being a decoy. However, the decoy model is a simple explanation that can be applied when a guardee appears to have no role in PTI or ETI, or no influence on virulence[65].

The decoy model became more evident with the discovery of integrated domains within NLRs and the way in which they influence effector sensing and immune activation. A small percentage of plant NLRs feature atypical domains that resemble domains of proteins targeted by pathogen effectors[68]. The integrated-decoy model, put forward by Cesari[69], suggests that these domains may often act as decoys themselves, interacting with effectors and thus causing activation of their attached NLR (Figure 1 C). Such a model was mainly derived from the mechanistic studies of the RPS4–RRS1 NLR pair found in *Arabidopsis* and the RGA4–RGA5 pair from rice. As is discussed in more detail in chapter 2, these and other well studied NLRs with integrated domains arranged tandemly in the genome pair up to act together in plant immunity.

1.2.5 Regulation of NLRs

In healthy plants not being attacked by pathogens, NLRs are in low abundance and/or inactive, serving a basal surveillance role. While lack of the appropriate *R* genes can lead to susceptibility against certain pathogens, improper regulation of NLRs can result in

autoimmunity; these plants, if they survive, tend to be dwarfed in size, often with additional morphological phenotypes such as twisted leaves and macroscopic lesions[70]. One well-studied autoimmune mutant is *snc1*, which carries a point mutation resulting in a more stabilized TNL and displays autoimmunity. The *bal1* variant has an extra copy of *SNC1* due to genomic duplication, leading to increased transcription and enhanced immunity[71]. Other gain-of-function mutations in NLRs have been shown to result in autoimmunity, exemplified in *ssi4*[72], *uni*[73], *chs1*[74,75], *chs2*[76,77], and *chs3*[78]. The use of suppressor and enhancer screens with these mutants has proven invaluable in establishing many of the NLR homeostasis control mechanisms and downstream elements in ETI signaling. The morphological side effects of autoimmunity show why it is so necessary for plants to tightly regulate their NLRs, finding the balance between quick pathogen detection and normal growth[70].

1.2.5.1 Transcriptional Regulation

NLRs are heavily regulated at the transcriptional level[79]. Plants challenged by pathogens show large-scale changes in the expression levels of many NLRs, often in an organ- and tissue-specific manner. The binding sites of certain transcription factors, such as WRKYs, are enriched among NLR promoters, which is perhaps unsurprising considering WRKY transcription factors are associated with many defense processes[80]. However, there is variety between regulatory triggers. Some NLRs, such as *Mla6* and *Mla13*[81], seem to form effector-specific feedback loops regulating their own expression, while others respond to changes through the feedback regulation from the defense hormone salicylic acid (SA) alone[72,82].

Epigenetic modifications such as DNA methylation also have strong effects on immunity. Often, less methylation leads to more defense while increased methylation leads to plant

susceptibility. Mutations in the *Arabidopsis* proteins DDM1 and MOS1 both result in decreased cysteine methylation, which leads to changes in *SNC1* transcription[83,84]. DDM1, however, seems to play a more direct role in chromatin remodeling[85]. The MUSE (mutant *snc1*-enhancing) screen, which searched for enhancers of the *snc1* autoimmune phenotype, also discovered a chromatin-remodeling protein affecting *SNC1* transcription. This protein, SPLAYED, appears to negatively regulate the transcription of *SNC1*[86]. The case of CNL RPW8 offers a more direct link between pathogen infection and regulation, whereby treatment of plants with a pathogen results in an altered methylation state of *RPW8* DNA[87]. Interestingly, areas of the genome featuring NLRs also frequently contain high densities of transposons, which may attract epigenetic modifications to reduce transcription in the area[88], keeping some NLRs from being overexpressed and causing autoimmunity[89,90]. A good example of complex regulation of NLR genes in a cluster is that of *PigmR* and *PigmS*, where both reside in the same epigenetically regulated gene cluster, but PigmR activity is antagonistically regulated by PigmS in a tissue-specific manner[91,92].

1.2.5.2 Post-Transcriptional Regulation

Transcribed mRNAs of *NLRs* are also regulated post-transcriptionally. Mutations in proteins necessary for nuclear export of transcripts can result in changes in immune status. mRNA homeostasis can also be regulated through alternative splicing. Mutants with defects in splicing often exhibit altered NLR gene-splicing patterns and enhanced susceptibility phenotypes such as those reported in *mos4*[93], *cdc5*[94], *prl1*[93,95], *mac3a mac3b*[96], *mos14*[97], and *mos12*[98]. Interestingly, the alternative isoforms of some *NLRs* show strong variation in response to

pathogen infection. These alternative transcripts are typically aberrant, triggering their own degradation and preventing an overaccumulation of NLR protein in the plant cell. When nonsense-mediated decay is disrupted, plants may display autoimmunity. The activity of small RNAs has also been extensively linked to NLR transcript levels[99]. In fact, in spruce, small RNAs lead to some level of degradation in over 90% of TNLs[99]. MicroRNAs, in particular, have been associated with the regulation of many specific NLRs in a broad range of species[100–102].

1.2.5.3 Post-Translational Regulation

As conformational shifts are important for the process of effector activity detection and NLR activation, it is perhaps unsurprising that several chaperone proteins are needed for NLR-triggered immunity. ETI often relies heavily on the RAR1–SGT1–HSP90 chaperone complex[45–47,103–105]. Also unsurprising is that these might become targets for effectors. Recently, the HopBF1 family of effectors in bacteria have been shown to phosphorylate HSP90, preventing proper NLR activation and resulting in disease symptoms in the plant[106]. This targeting is both specific to HSP90 and observable using HSP90s from other eukaryotes.

Another strong post-translational effect on NLR protein levels comes in the form of the ubiquitin-proteasome-mediated degradation pathway, and other similar pathways such as the SUMO (small ubiquitin-like modification) pathway[107–109]. While three of the four pathway components (E1s, E2s, and E4s) are largely non-specific, some E3 ligases, which are responsible for bridging the gap between the E2 and substrate and transferring the ubiquitin, appear to target specific NLRs[110,111]. SNC1, for example, is either SUMOylated directly by SIZ1 or affected by the SUMOylation of an upstream positive regulator[112]. SNC1 is targeted by SCF^{CPR1} E3 complex[113,114] (Figure 2), while its partners SIKIC1/2/3 are targeted by simple RING-type E3

MUSE1/2 for ubiquitination and degradation[115]. Using the new Turbo-ID technology, which can detect more transient interactions, it was recently revealed that the E3 ligase UBR7 negatively regulates the levels of TNL N[116]. In contrast, the duplicated E3s RIN2 and RIN3 are necessary for wild-type levels of defense response triggered by RPM1 and RPS2, and thus serve as positive regulators of immunity[117]. Immune activation has been observed to lead to an upregulation in the components of the ubiquitin-proteasome system, which in turn explains the decrease in many defense-related gene products after infection. Both E3s, such as MIR1[118], and other members of the E3 complex, such as MUSE13/14[119], have been identified as specific regulators of immunity. Like with chaperone proteins, the ubiquitination pathway can be targeted by pathogen effectors. The previously mentioned SOC3 guardee SAUL1 is an E3 ligase and is far from the only example[120].

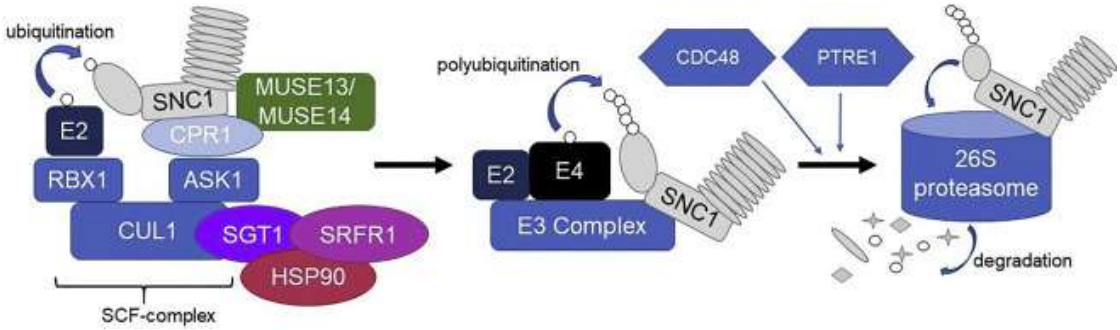

Figure 2. A pathway model for degradation of SNC1 through the ubiquitin-proteosome system.

Figure from van Wersch and Li, 2019[121]. The E3 ligase complex recognizes and binds to the substrate molecule (SNC1) (Copeland and Li, 2019). An E2 ligase then begins to ubiquitinate the substrate. Here, the E3 is a complex E3 of the SCF type (Cheng et al., 2011), with chaperones SGT1 and HSP90 (Copeland et al., 2016a), along with the adaptor proteins MUSE13/14 (Huang et al., 2016) and SRFR1 (Li et al., 2010a). The ubiquitination chain is elongated by an E4 ligase (MUSE3), which associates with the complex. The substrate is then released, recognized by the 26S proteasome due to its ubiquitination status, and degraded. Both the unfoldase CDC48 and PTRE1 positively regulate this process (Copeland et al., 2016b, Thulasi Devendrakumar et al., 2019). CDC48 likely assists in extracting the polyubiquitinated substrate from the E3/E4 complex.

1.2.6 New Structural Insights

The three domains of a typical plant NLR protein all play important roles in detection and signaling. The NB-ARC domain has been thought to be important for oligomerization and ATP binding for some time. The LRR domain, on the other hand, has long been considered the NLR domain that likely undergoes divergent evolution and interacts with, or recognizes, the effector/guardee/decoy. Although NLRs have been studied for over 25 years, there is still much we do not know about the way in which they function mechanistically in effector recognition and defense activation. The cooperative behavior of many NLRs, their ability to recognize multiple effectors/guardees/decoys, and the observed specificity changes not due to different LRR domains make this question especially intriguing to pursue. To this end, efforts have been made to further understand the structural biology of plant NLRs.

1.2.6.1 N-Terminal Domain Ologomerization

In 2011, crystal structures of both TIR and CC domains were revealed. Both the CC domain of MLA[122] and TIR domain of L6[123] were found to self-associate. Mutations to these domains that resulted in loss of homo-oligomerization led to loss of immune signaling by the full-length protein *in planta*. In fact, abolishing self-association prevented a defense response even in autoactive mutants of MLA. These findings, coupled with other known examples of hetero- and homo-oligomerization of full-length NLRs, showed that oligomerization not only played a key role in activation of NLRs but was also necessary for their ability to signal downstream responses. Whether the self-association helps NLRs to interact with downstream signaling components or simply allows the NLRs to perform some other defense-activating action remains to be fully clarified. Two recent papers shed some further light on this topic[124,125].

TIR domains of both animal and plant NLRs were shown to have NAD^+-cleaving capabilities that were required for cell death activity[124]. In addition, self-association between TIR domains was required for NAD^+ cleavage to occur[125]. Whether such enzymatic activity is fully responsible for TNL activation and the relationship with downstream EDS1/PAD4/SAG101 and other NLRs awaits further investigation.

1.2.6.2 "Resistosome" Formation

The full-length structure of animal NLRs was revealed before that of plants. In 2015, cryoelectron microscopy imaging of the NAIP-NLRC4 inflammasome revealed that it consisted of more than 10 activated NLRs[126]. The investigators observed that ligand binding activated members of the NAIP NLR family, which in turn activated and oligomerized to NLRC4. Additional NLRC4s were activated and oligomerized in order to form a doughnut-shaped structure containing a single NAIP (sensor) NLR and 10 NLRC4 (adaptor) NLRs. Due to the role of oligomerization in animal NLR signaling, it seems quite possible that plant NLRs act similarly.

Indeed, when two 2019 papers detailed the first full-length NLR structure, that of CNL ZAR1, the end finding was somewhat similar[127,128]. The biology of *ZAR1* had previously been well studied; this CNL is known to guard the pseudokinase PBL2 and its homologs, all of which appear to be decoys. The pseudokinases are targeted by the effector AvrC, which uridylylates them, and the modified decoy then interacts with the ZAR1 complex. The first paper by Wang et al. details the conformational change that occurs due to this interaction. When PBL2 interacts with the pseudokinase RKS1 in the ZAR1–RKS1 complex, the ZAR1 nucleotide-binding domain rotates slightly outward and releases ADP[127]. This conformational change and ADP release is

caused by changes in the interaction between the LRR domain of ZAR1 and RKS1, and transforms the NLR into its active state. The second paper shows that this active ZAR1 is able to pentamerize to form a ring-like structure resembling the NLRC–NAIP inflammasome[128] (Figure 3).

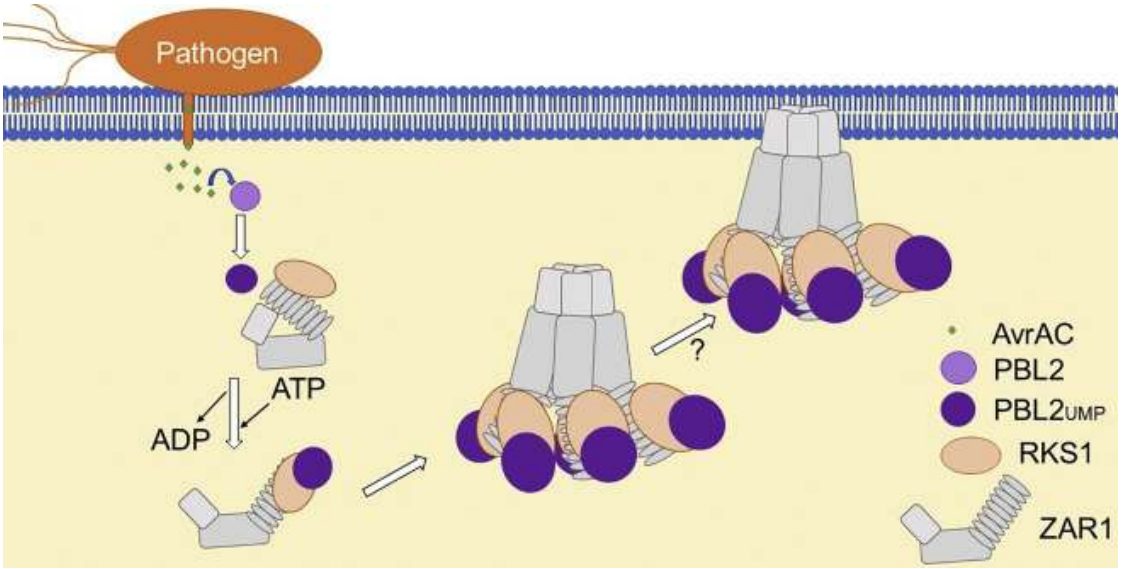

Figure 3. ZAR1 Resistisome formation in response to pathogen invasion.

Uridylylation of PBL2 by the effector AvrAC leads to changes in the interactions between PBL2 and ZAR1 bound RKS1. This in turn alters the exposure level of the nucleotide-binding domain of ZAR1, allowing the CC domains of ZAR1 to oligomerize. The resulting pentamer has been referred to as the plant "resistosome"[127,128].

The researchers named this structure the plant "resistosome." They hypothesized that the resistosome may form a pore through the membrane, allowing influx of calcium ions and triggering a defense response. This particular hypothesis would not explain why many NLRs require the presence of downstream elements such as EDS1 or NDR1 in order to function, but it could be the explanation for why some CNLs do not seem to require these downstream elements to trigger immunity. The membrane-associated portion of the resistosome also seems too short to form a channel crossing the membrane. NLR pentamerization, on the other hand, seems to be a more common event. For example, the NLR RPP7 was recently found to self-associate in

clusters of five[129]. It may be interesting to discover whether other NLRs can be found to also oligomerize into these larger structures. Overall, the findings of this first full-length structure have reaffirmed many thoughts about NLR biology, such as the importance of the LRR domain in sensing effector activity, and have also provided the community with some fascinating new ideas about downstream activation.

1.3 Other Elements of Immunity

Both ETI and PTI involve signalling and immune response networks that extend far beyond recognition of pathogens by PRRs and NLRs[130–132]. After a threat is detected, suites of transcriptional regulators[133–137], ion flux[130,138,139], reactive oxygen species[140] production and plant hormones[130,141] all work together to ensure that sufficient immune response occurs without overwhelming the plant.

1.3.1 Salicylic Acid

One of the key hormones that helps to regulate the plant defense response is salicylic acid (SA), which contributes heavily to basal immunity, pathogen-host specific resistance and systemic acquired resistance (SAR) against biotrophic pathogens[142]. During infection, SA accumulates locally and distally. Around 95% of this SA is created through the isochorismate synthase (ICS) pathway, with ICS1 (also known as SID2) responsible for at least 90% of the conversion of chorismite to isochorismate[143]. Plants not expressing functional ISC1 have increased susceptibility to a variety of pathogens as well as reduced expression of *pathogen response gene 1* (*PR1*). EDS5 (enhanced disease susceptibility 5), a membrane embedded protein required for the export of several hormonal precursors from the chloroplast[144,145], displays a similar phenotype when knocked out. The final stages of SA biosynthesis were recently

discovered to occur after this export, with PBS3 and EPS1 performing the final steps in the cytosol[146].

1.3.2 N-Hydroxy Pipecolic Acid

SA-independent pathways also trigger plant responses; for example, NHP (N-hydroxy pipecolic acid) accumulates in both local and distal tissues during infection, and has been shown to be required for full SAR[147]. Some research suggests that NHP may act as the mobile signal which triggers SAR in those distal tissues. The enzymes responsible for the biosynthesis of NHP are, in order, ALD1, SARD4 and FMO1[148]. Knocking out any of the genes encoding these enzymes results in SAR deficiencies. Interestingly, it was first hypothesized that pipecolic acid, the immediate precursor to NHP, might cause positive feedback for the NHP biosynthesis pathway whilst SA, due to some structural similarities to pipecolic acid, might negatively inhibit conversion of pipecolic acid into NHP by FMO1[149].

SA has for some time been known to be positively regulated by the master transcription factors SARD1 and CBP60g[150]. Recent data by Sun et al. (2020) shows that the NHP biosynthesis genes are also regulated by these transcription factors, suggesting that downstream regulation is likely more complicated than simple repression of NHP synthesis by SA.

Chapter 2: Clustering of NLR Genes and Functional Implications

2.1 Gene Clustering

Gene clusters appear in genomes of both prokaryotes and eukaryotes. In prokaryotes, they often take the form of operons. These tightly packed functionally related genes share a single regulatory sequence[151], saving both genomic space and regulatory machinery while ensuring that proteins necessary for a specific process are transcribed together. In eukaryotes, with both larger cells and genomes, such arrangements are less common[152] and very few genes exist as traditional operons[153,154]. Single-gene transcription and translation is more prevalent. One hypothesis for the high frequency of operons in prokaryotes is explained by the 'selfish' gene model, where all genes involved in a single process need to be transferred together to be adaptively beneficial[155]. As horizontal gene transfer is, debatably[156], a less-prevalent contributor to eukaryotic genomes, operons may lose some of their benefits. Because eukaryotes have the cellular space for the additional genomic material and regulatory machinery, controlling genes individually rather than in large groups may become a benefit rather than a drawback[152]. It could, for example, allow tissue-specific regulation of certain pathway elements or allow switching between pathways more easily.

Although operons are rarely found, general gene clustering can still be observed in most eukaryotes. This tends to fall into two categories: homologous gene duplications and functionally linked genes with little sequence similarity[157]. Tandem repeats of gene duplicates are often seen for essential and conserved genes, such as those encoding histone proteins, perhaps due to the large amounts of these gene products required[158]. By contrast, the functional gene clusters, which

in some sense resemble operons, tend to contain the genes required for metabolic or signaling pathways[159,160]. In plants, clustering of metabolic or hormone biosynthesis genes has been frequently observed[161]. Intriguingly, plant defense-related genes, especially those encoding NLR immune receptor proteins, are frequently found in clusters[33,36,162].

2.2 Clustering of *NLR*s

*NLR*s are found from charyophytes to flowering plants[35], but their numbers vary greatly among the genomes of even closely related species[162] in a manner that does not correlate with genome size or ploidy level[163]. Within the same species, some *NLRs* may be highly conserved while others contain high levels of polymorphism[164]. In early plant lineages, there is less *NLR* clustering, but it is extremely prevalent in the genomes of well-studied higher plants (Figure 4 and Figure 5), indicating an evolutionary benefit for such gene arrangements. Many clusters, including some of the pairs discussed below, contain more diverged *NLR*s. Contrary to more operon-like clusters, *NLR* gene clusters do not seem to contain downstream components necessary for signaling pathway completion. Interestingly, *NLR*s can be found in both pairs, often in a head-to-head arrangement, and larger clusters, some reaching several megabases in size[165] (Figure 5).

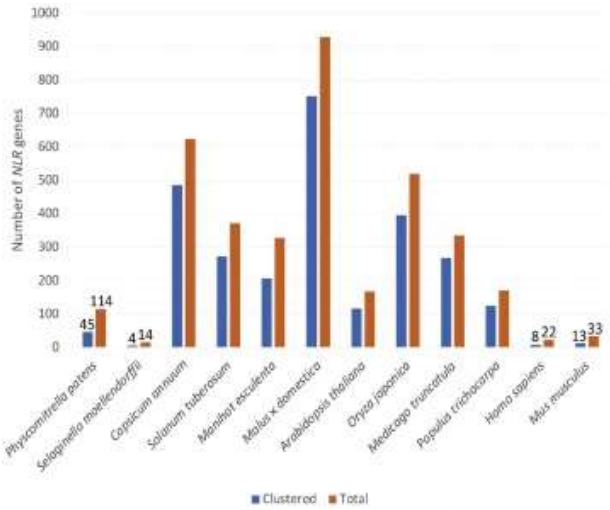

Figure 4. Number of NLRs observed to cluster in plant species.

Figure from van Wersch and Li, 2019[58]. Blue bars are the numbers of clustered NLRs, while the orange bars are total numbers of NLRs encoded in the indicated genome. Numbers of clustered NLRs taken from the literature for Capsicum annuum[162], Solanum tuberosum[166], Manihot esculenta[167], Malus × domestica[168], Arabidopsis thaliana[169], Oryza sativa[170], Medicago truncatula[171], and Populus trichocarpa[172]. These papers followed similar definitions of clustering, mostly involving both a distance of less than 200 kb and fewer than eight non-NLR genes. The same method was applied to the Physcomitrella patens, Selaginella moellendorffii* (https://phytozome.jgi.doe.gov/jbrowse/index.html), Homo sapiens and Mus musculus (https://www.ncbi.nlm.nih.gov/genbank/) genomes available. Although the total numbers of NLR genes differ, there is consistently over 60% clustering for seed plant NLRs. *The S. meollindorffii genome is still in scaffolds.

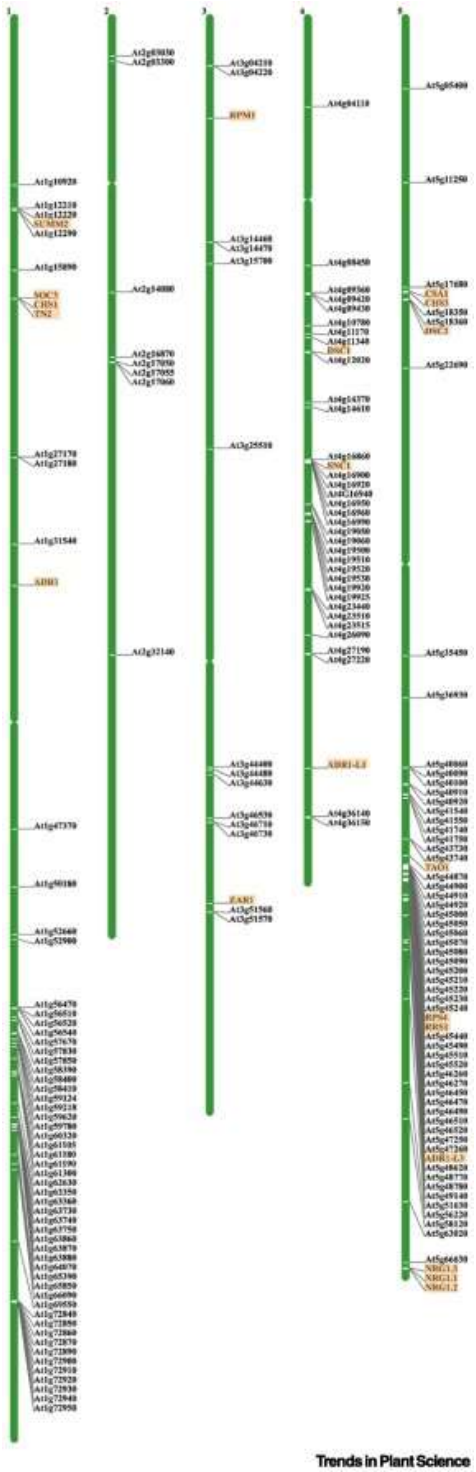

Figure 5. Genomic distribution of NLRs in Arabidopsis thaliana Col-0.

Figure taken from van Wersch and Li, 2019[58]. Image generated using the TAIR Chromosome Map Tool (https://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp). Less than a quarter of Col-0 *NLR* genes exist as singletons.

NLR proteins are also found in metazoans, where they appear to play a role in PAMP recognition and innate immunity activation. However, animal NLRs seem to have evolved independently[173] and higher plant species have far more NLR-encoding genes than mammals. Gao et al. traced plant NLRs back to charyophytes[35]. Despite all plants facing threats from pathogens, the number and type of NLRs found in a genome can vary greatly. For example, papaya has 54 NLRs[34] whereas wheat has at least 1540[36]. As shown in Figure 4, pepper (*Capsicum annum*) and potato (*Solanum tuberosum*), both members of the Solanaceae family, differ substantially in their total NLR gene counts. In addition, TNLs are completely absent from monocots, although they seem to have evolved alongside the first NLRs in charyophytes[35]. This high variation could be explained by the same 'birth–death' model[174] that was first proposed to explain the variability in NLR clustering among plants. It is interesting to note that although the total number of NLRs and NLR clusters varies among higher plant species, the proportion of NLRs that cluster together is relatively constant (Figure 4). The clustering observed in these genomes includes several tightly linked genes as well as large genomic regions with high NLR densities (Figure 4).

As previously mentioned, inappropriate activation of NLRs can result in reduced growth and cell death. Such autoimmunity caused by NLR activation can come from loss of regulation or can be triggered by interactions with foreign NLRs[175–177]. There are many examples of hybrid necrosis or lethality caused by interactions between two NLRs or an NLR and a guardee from their completely healthy parental lines[178–181]. This further reinforces that *NLR* genes are likely to be impacted by strong fitness pressures, and it has been proposed that these epistatic forces limit which NLRs (and other defense proteins) can be combined in a healthy individual organism[182].

Considering this variability and evolutionary pressure, it seems unlikely that *NLR* genomic arrangements are maintained primarily by chance. The goal of this chapter is to explore what is known about *NLR* gene clustering in higher plants in such a way as to highlight the functional mechanisms that are often associated with these genomic arrangements. My examination of the evidence has led me to the opinion that genomic proximity can be used as an initial guide for predicting the biological function and interaction patterns of plant NLRs.

2.2.1 Head-to-Head Gene Pairs

Head-to-head gene pairs have been found in many eukaryotes, often for genes with gene products that should be transcribed at equal rates, suggesting a pattern of shared regulatory regions. In humans, there have even been bidirectional promoters found for these pairs[183,184]. Although, so far, no *NLR*s have been shown to possess bidirectional promoters, the adaptive benefit of sharing regulatory regions does provide the most logical explanation for the high prevalence of such a configuration. Coexpression from a head-to-head cluster has been observed for the *SOC3–CHS1–TN2 NLR* cluster in arabidopsis[62], in which the gene products do interact. A brief examination of microarray data (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) for other arabidopsis head-to-head *NLR* gene pairs suggests that this proposal may be plausible under certain conditions, but likely does not apply in all cases. Although further experimental evidence would be required to confirm this as a general trend for plant head-to-head-arranged *NLR*s, it seems to be a reasonable explanation for such a genomic pattern. As these paired *NLR*s tend to be highly diverged, or not from the same phylogenetic cluster, their origin could be explained by a 'true love' model (i.e., meet randomly and live happily ever after) where initially they came

together by random evolutionary accidents that enabled them to withstand certain pathogen stresses. Selection for coregulation might then explain why these evolutionarily diverged pairs persist in plant genomes. This, at least, seems more plausible than two evolutionarily unrelated genes, that happen to function together, consistently ending up as next door neighbours to one another in the genome.

2.2.2 Large NLR Gene Clusters

Although there may also be coexpression benefits to larger clusters of *NLR* genes, there is little evidence to support such a hypothesis for plant *NLR*s. Regulation of NLR protein levels is clearly important for plant survival, as can be observed through the various pre- and post-transcriptional and post-translational mechanisms that plants use to control them[163]. As mentioned above, the sizes of both medium and large clusters can differ drastically among ecotypes[185,186] and cultivars[187], suggesting that there may be some local adaptation at play. For clusters comprised of highly homologous *NLR*s of a single gene family without too many inversions, the initial clustering is likely to have come from direct gene duplication, and later, a higher frequency of unequal recombination during meiosis could have supplied the raw genetic material for faster evolution of more diversity in immune sensors and thus larger clustering. These gene clusters could shrink or expand quickly under different pathogen stresses, explaining the huge differences in clustering patterns among ecotypes.

By contrast, for clusters that are more diverged, close genomic proximity could prevent recombination between compatible alleles of co-functioning NLRs, such as in the cases of head-to-head arrangements. To better understand these differences, it may be helpful to study clusters in more mechanistic detail and compare their differences in and sensitivity to defense activation.

2.3 Integrated Domains and NLR Pairing

As previously mentioned, a small portion (3.5%) of studied *NLR*s feature an additional non-NLR domain[68]. The identity of such regions varies greatly, and it is hypothesized that most serve as integrated decoys, mimicking the functional targets of pathogen effectors[188] (see Figure

1 C). Some atypical NLR domains are even specific to a certain plant lineage, such as the Solanaceae domain (SD) for Solanaceae plants. Intriguingly, for all studied cases of NLRs encoded by head-to-head gene pairs, one member carries an integrated domain while the other member is a typical NLR, suggesting a collaborative sensing and signaling function (Figure 6 B). This functional significance is even more evident considering that many of these domains were integrated completely independently[68]. Interestingly, head-to-head pairs featuring one member with an integrated decoy domain often have similar phylogenetic histories; more specifically, members of the same two phylogenetic clusters of NLRs consistently pair together in many lineages[33,36].

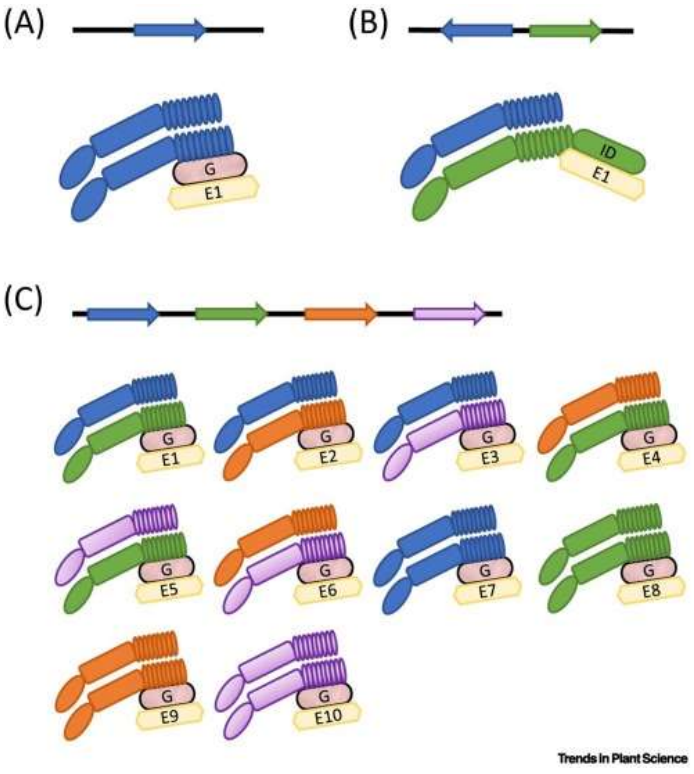

Figure 6. Models for NLR recognition possibilities assuming dimerization.

Figure from van Wersch and Li, 2019[58]. (A) NLRs encoded by singleton genes can homo-dimerize to sense a pathogen effector (E) on disturbance of their guardee (G) and trigger a defense response. (B) Paired NLRs encoded by head-to-head-arranged genes, where one features an integrated domain (ID), act together. The ID serves as a

decoy and effector–ID interactions result in altered heterodimerization interactions between the two NLRs, resulting in defense signaling. (C) NLRs encoded by large gene clusters allow increased heterodimerization options. The example here is a four-gene cluster. Each combination could possess different recognition capabilities, resulting in a greater capacity to recognize a large variety of different pathogen effectors, triggering immune responses.

The best-studied integrated decoy pair is RPS4 and RRS1 from *Arabidopsis thaliana*. RRS1 is a TNL with an integrated WRKY domain. WRKY transcription factors have been frequently implicated in transcriptional defense responses[189] and WRKY domains appear to be one of the more common integrated NLR domains[68,190]. RPS4 and RRS1 interact through their TIR domains[191] and the WRKY domain of RRS1 was shown to be able to interact with several effectors from three pathogens (*Pseudomonas syringae* AvrRps4, *Ralstonia solanacearum* PopP2, and a *Colletotrichum higginsianum* unknown effector)[192], serving as bait for these effectors to activate ETI. The ability of RPS4 and RRS1 to activate a normal defense response depends on both proteins being present and functional[193]. Interestingly, the head-to-head *RRS1–RPS4* pair mentioned above is part of a larger gene cluster of seven *NLR*s, the remainder of which have not been well studied. It would be interesting to test whether any of them contribute to immunity through interactions with RRS1 or RPS4. Intriguingly, there is a similar phylogenetic history between the *RRS1–RPS4* pair and another head-to-head pair, *CHS3* and *CSA1*[33], where the cluster contains only these two *NLR* genes. Although direct effector interaction with the integrated LIM domain of CHS3 has not been shown, and the domain's functions remain unknown, autoimmunity in the *chs3-2D* mutant line depends on the presence of a functional CSA1 protein[194], suggesting a cooperative activation mechanism requiring both CSA1 and CHS3.

Outside Arabidopsis, examples of such head-to-head gene pairs include rice NLRs encoding RGA4 and RGA5[69] and Pik-1 and Pik-2[195], which have been similarly demonstrated to function together. RGA5 and Pik-1 both possess Related to Yeast ATX1 (RATX1) [also known as heavy metal-associated (HMA)] domains whose physical interaction with effectors has been characterized[196,197]. In these cases, the NLR with the integrated decoy appears to serve as the effector sensor while its partner is responsible for activating the immune response. RGA5 inhibits RGA4 activation until effector recognition occurs[69]. The sensing and signaling function of NLR pairs being divided between the two proteins might further suggest a coevolutionary benefit for the pairs, with their close genomic proximity helping to prevent them from separation during replication errors.

2.4 Recognition of Guardees by NLRs from Large Clusters

For clusters lacking integrated decoy domains, the most common and best understood mode of effector recognition is through the guard model, where an NLR monitors the status of another host protein that may contribute to PTI[13] (Figure 6 A). In these cases, heterodimerization of NLRs encoded by the closely related gene cluster members might allow differential pairings, resulting in a broader detection spectrum (Figure 6 C). Besides homodimerization or oligomerization, SNC1 proteins are likely able to pair with three different proteins encoded by the *SIKIC1*/*2*/*3* genes from its cluster, and all three genes must be knocked out to suppress the autoimmunity of the *snc1* mutant[198]. Interestingly, SNC1 and its cluster partners (SIKIC1/2/3) are all typical TNLs, indicating that they could theoretically function independently. It has not been investigated whether the SIKICs interact with one another, and the guardee for the SNC1–

SIKIC2 pair is also unknown, but it is possible that there are alternative pairings among cluster members that serve additional recognition roles in immunity. Such diverse NLR pairing would allow for exponential growth of recognition possibilities using a limited number of genes residing in the cluster (Figure 7), especially if interactions between more than two NLRs at a time are allowed. The evidence from ZAR1 and RPP7 that oligomerization could be a required part of some NLR functions adds another layer to this. Whether all NLRs can pentamerize homogeneously or heterogeneously upon activation awaits further testing, but combined with possible protein variants from a single gene that can be generated through alternative splicing, alternative translation initiation, and protein modifications, these mechanisms provide an explanation for how plants can use a limited number of *NLR* genes to recognize an almost unlimited number of pathogen effectors.

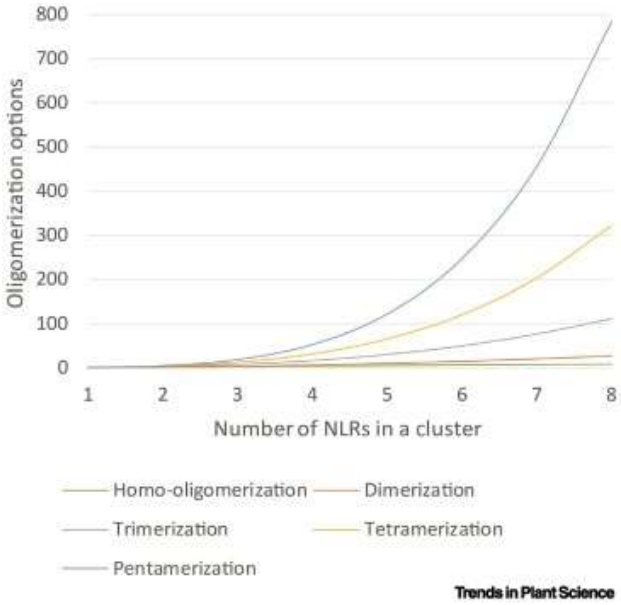

Figure 7. Oligomerization possibilities for clustered NLRs.

Figure from van Wersch and Li, 2019[58] For clusters of *NLR*s, the option of hetero-oligomerization results in far more potential pathogen recognition complexes than singletons.

There are also examples of NLRs encoded by head-to-head genes that function in a guard model. For example, the truncated Arabidopsis TIR-NB (TN) NLR CHS1 relies on the typical TNL SOC3 to confer immunity[199]. Interestingly, *SOC3* and *CHS1* are in a head-to-head orientation, with an additional truncated *TN* gene, *TN2*, closely following *CHS1* (Figure 5). Further studies into the interactions of the three genes in this cluster found that while SOC3 can associate with CHS1 to monitor their guardee SAUL1, it can also work with TN2 to sense overaccumulation of the E3 ligase SAUL1[62]. As the LRR domain of NLRs has generally been implicated in effector sensing, and both CHS1 and TN2 lack such a domain, SOC3 is likely the sensor NLR. The SOC3–CHS1 pair guards SAUL1 while the SOC3–TN2 pair is likely to guard the yet-to-be-identified ubiquitination target protein of SAUL1. A mechanism like this might allow plants more precise control over their defense response as well as refined sensitivity to all possible effector actions against their guardees.

2.5 Effector Sensing by NLRs encoded by Singleton Genes

Although the majority of plant *NLR*s cluster, many still exist as singletons. Among these, there are some that display functional ties to other NLRs. CNL RPM1 and TNL TAO1, for example, are two singletons that both trigger immunity in response to the *Pseudomonas syringae* effector AvrB. Unlike many of the functionally related genes discussed above, however, these proteins do not co-operate together to guard a single target but instead monitor two different guardees affected by *AvrB*. This independence implies far less evolutionary advantage in keeping these genes clustered.

In theory, although NLRs encoded by singleton genes may be able to pair with another NLR encoded distantly in the genome for effector sensing, we are not aware of such examples

except in cases of hybrid necrosis. Rather, the existing examples of NLRs encoded by singleton genes seem to operate by forming homo-oligomers, such as in the case of RPM1[200] and ZAR1[128]. Such a homo-oligomerization mechanism may explain their persistence as singletons in plant genomes, as they do not normally need other partners for activation.

Research into the NRG1s, ADR1s, and NRCs in various plant species has led to the identification of 'helper' NLRs. They may act as scaffolds for 'sensor' NLRs in a similar manner as with NLRC4 in the NLRC4–NAIP inflammasome in animals; In fact, it was recently shown that when activated, NRG1 and ADR1 did oligomerize to form calcium channels in the way that ZAR1 is hypothesized to[138]. Helper NLRs may not have recognition capacity themselves, but are required for the immune signaling capabilities of other NLRs[115,201–204]. Intriguingly, although many *NRC*s in tomato[205] and the *NRG1*s in *Arabidopsis* and related species cluster, the *ADR1*s do not[203]. The ADR1s and NRG1s show specificity in differential TNL signalling but also have redundancy within family members[115,202], so there is likely far less evolutionary pressure for them to remain clustered.

2.6 Transposable Elements in Gene Clusters

It is well known that the larger *NLR* clusters usually carry TEs interspersed throughout[187]; even some of the two-gene clusters in *A. thaliana* contain neighboring TEs[33]. This mysterious connection between *NLR*s and TEs has not yet been closely examined. As TEs can result in segmental duplications, deletions, and genomic rearrangements[199,200], they may be responsible for the duplications and positioning of the *NLR* clusters as well as some of the differences in the copy numbers of plant *NLR* genes. There is also evidence that TEs have contributed regulatory

elements to *NLR*s or *NLR* clusters[206]. For example, the promoter region of *PigmS* contains two tandem miniature transposons (*MITE1/2*), which are likely responsible for the low expression levels of *PigmS*, preventing it from inhibiting the defense activity of its partner *NLR PigmR*[91]. However, such a mechanism does not appear to be a common phenomenon, nor are there known examples of TE insertions directly influencing effector recognition. It seems more likely that the extra genomic diversity provided by TEs has contributed to the great variation among the *NLR*s of different plants, potentially resulting in more rapid evolution of functionally important clusters.

2.7 Challenges in Analyzing Gene Clusters

One of the biggest challenges in studying larger *NLR* clusters is the acquisition of accurately aligned and annotated genomic sequences. Because *NLR* gene quantity and clustering patterns vary so much among species, and even ecotypes, accurate genomic data are necessary to dissect the different roles of the encoded proteins, especially where functions are more subtle or redundant. Tools like RENseq, which are targeted towards accurate sequencing of NB-LRR genes, have been developed to improve *NLR* gene identification[207]. While RENseq can work well for more diverged *NLR*s, it may still fail to differentiate between those more recently duplicated. When trying to assemble longer clusters with similar *NLR*s, this could result in inaccurate predictions and annotations. Therefore, longer and more accurate sequence reads are of utmost demand. Improved technologies, such as Oxford Nanopore[208] or PacBio sequencing[209], may help to more accurately map the *NLR* clusters. In addition, it would be useful to gain a better understanding of which sequences are likely to represent functional *NLR*s and which represent

pseudogenes, as gene numbers can vary greatly, even reaching into the thousands, depending on annotation strategies [13].

2.8 Concluding Remarks

Taken together, the current knowledge of NLR genomic arrangements and molecular activities suggest an evolutionary connection between the two. Though further research is necessary to properly explore the importance of this relationship, the correlations seen can be used as a guide when predicting NLR interaction partners.

Chapter 3: *camta1/2/3* Suppression Screen

3.1 Background on *CAMTA1/2/3*

Upon detection of pathogens during both PTI and ETI, rapid changes in calcium ion (Ca^{2+}) concentration are triggered[139,210]. The influx of Ca^{2+} into the cell in turn results in the activation of various proteins. Calmodulin, a small protein with a high affinity for Ca^{2+} binding, appears to act as an intermediary between changes in Ca^{2+} concentration and other proteins in the cell. Many transcription factors, for example, contain calmodulin binding domains which interact with Ca^{2+} bound calmodulin[211]. The Ca^{2+}/calmodulin-binding transcription factors (CAMTAs) are one such family. CAMTAs are found across eukaryotes and the model organism *Arabidopsis thaliana* contains 6 CAMTA encoding genes[212]. *AtCAMTA1, 2* and *3* (hereafter listed only as *CAMTA1, 2* and *3*) display a high level of homology.

CAMTA1, 2 and 3 appear to play redundant negative roles in plant immunity, with *camta1/2/3* triple mutant plants severely dwarfed with elevated SA levels[135,213]. Conversely, a gain-of-function *camta3* mutant had compromised immunity to several known bacterial PAMPs and effectors and defects in SAR[214]. Expression of the SA degrading enzyme NahG in *camta1/2/3* plants results in suppression of the dwarfism and defence phenotypes[135], indicating that the immunity caused by *camta1/2/3* is reliant upon SA. *SARD1*, *CBP60g* and *ICS1* transcripts were also found to be upregulated in the triple knockout[135]. This suggests that *camta1/2/3* dwarfism is reliant upon SA and that these transcriptional activators might be negatively regulating SA production.

The strong phenotype of the triple mutant allowed the use of a suppressor screen to be carried out in the Li lab in order to further study the elements downstream of these regulators[215]. EMS mutagenesis of *camta1/2/3* seeds was carried out and suppressors were examined beginning in the M2 generation. Several of the *camta1/2/3* suppressors found in the screen are what would be expected based on previous data: both *ics1* and *eds5* show suppression of the dwarf phenotype, further confirming the SA dependence of *camta1/2/3* autoimmunity[216]. Mutations in several SA responsive genes also show suppression: *npr1* and *med15* are both required for the increased expression of *PR* genes which occurs in response to higher SA levels[217,218]. These mutants show suppression of the increased immunity and are slightly larger than the triple mutants but also display a bleaching phenotype in their stems due to SA levels remaining higher than normal. The screen revealed that *cdk8*, a member of the mediator complex, acts as a positive regulator of SA biosynthesis[215]. The TNL signalling node EDS1[44] plays a role in SA positive feedback, and *eds1* was found to suppress *camta1/2/3* dwarfism as well. One of EDS1s partnering proteins, PAD4, shows similar suppression when knocked out.

Interestingly, knocking out *sard1cbp60g* also suppressed immunity, and CAMTA3 could bind to the *cbp60g* promoter sequence, indicating that CAMTA1/2/3 operate upstream of these master transcription factors. In addition, knockouts of NHP biosynthesis genes *FMO1* (found in screen) and *ALD1* (not found in screen) also result in almost complete morphological suppression, comparable to that seen in *ics1 camta1/2/3*. This finding, that both loss of SA and loss of NHP are independently capable of mostly suppressing the *camta1/2/3* autoimmune phenotype, suggests that these hormonal pathways are more co-reliant than was previously thought[216]. These results tease at the potential discoveries about immune regulators which could

be found through this screen and raises the question of what additional hormone pathway interactions might be important for *camta1/2/3* autoimmunity.

I continued work on a second *camta1/2/3* triple mutant suppressor screen in the hopes of discovering new potential regulators of immunity downstream of CAMTA1/2/3. The previous *camta1/2/3* screen from our lab did not yield the SA biosynthesis enzyme PBS3 or the NHP biosynthesis enzymes SARD4 or ALD1 as suppressors, suggesting the screen had yet to reach saturation.

3.2 Results

3.2.1 Morphology and Salicylic Acid Levels in the new suppressors

A population of camta1/2/3 seeds was treated with EMS to induce single nucleotide mutations. In the new EMS M2 population, I found 49 complete and partial suppressors of the *camta1/2/3* dwarf phenotype (Figure 8). To help organize these mutants, and make it easier to rule out known suppressors, I measured total SA levels in many of these mutants (induced by *Psm*. ES4326 if plants were large enough, uninduced if plants were smaller), and found that most of the suppressors fell into similar categories as the known suppressors: they either had less SA than wildtype plants, like *ics1* and *pad4*, or they had SA levels higher than wildtype but still significantly reduced in comparison to *camta1/2/3* plants, like *fmo1* or *ald1* (Figure 9). The lone exception to this was suppressor *8-4#1*, which displayed higher SA levels than the *camta1/2/3* triple mutant did, as well as a mild bleaching phenotype. Unfortunately, this mutant also displayed poor fertility and seed germination, and I was unable to perform crosses between it and other lines. Given the phenotype, it is likely that it was a partial knockout of *NPR1* or *MED15*.

Figure 8. Morphology of camta1/2/3 suppressor lines.

Known and unknown suppressor lines used in this study, with Col-0 and *camta1/2/3* as size controls. All, except for Col-0 are in the *camta1/2/3* background (this is denoted by *x3* in the known suppressors).

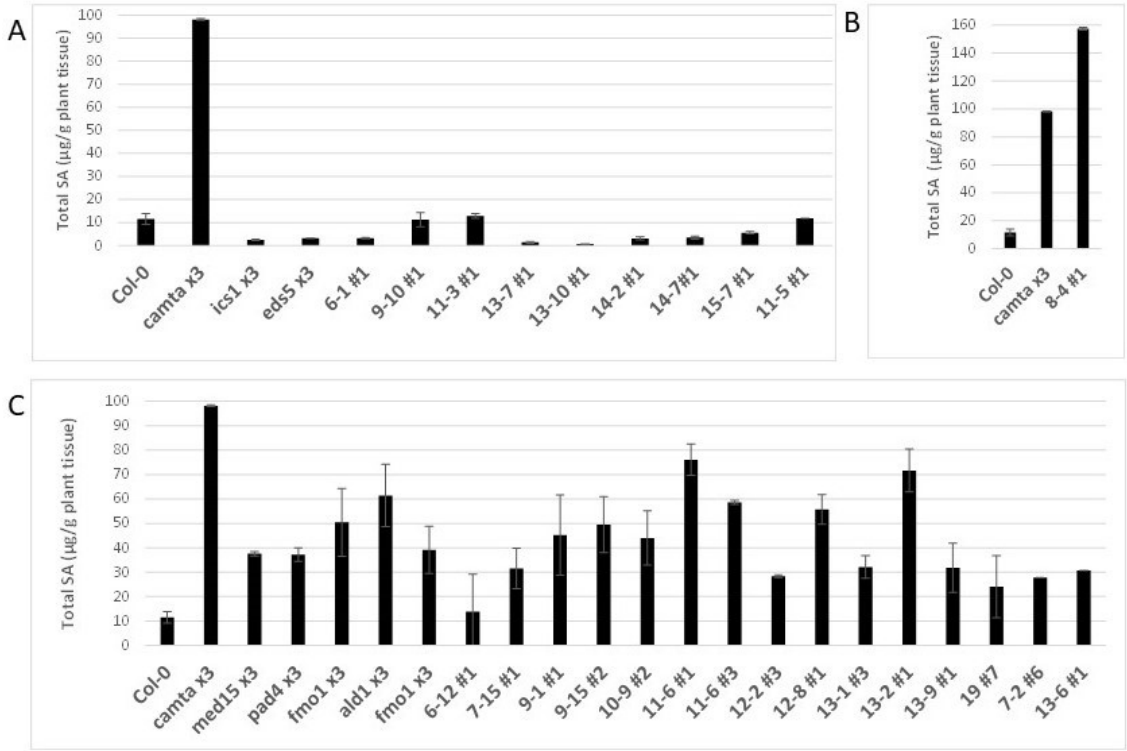

Figure 9. Salicylic acid levels in suppressor lines.

The total SA levels of the suppressor mutants can be seen, with the low SA suppressors in (A), the high SA suppressor in (B) and the mid-range suppressors in (C). All, except for Col-0 are in the *camta1/2/3* background (this is denoted by *x3* in the known suppressors).

3.2.2 Elimination of Known Suppressors

I next began to perform complementation crosses between the suppressor lines in order to rule out as many redundant known mutants as possible, in order to prevent sequencing mutants unnecessarily. I used both crosses between known and unknown mutants, as well as between two unknown mutants, to determine which of my unknown suppressors were allelic to the known mutants (Table 1). All of the low SA mutants failed to complement either the *ics1* or *eds5 camta1/2/3* quadruple mutant. Many of the mid SA mutants failed to complement either the *fmo1* or *pad4 camta1/2/3* quadruple mutant. Notably, *13-6#1* failed to complement *ald1 camta1/2/3*. The mutants which failed to complement can be seen in Figure 10 and Table 2.

Table 1. Table of complementation crosses. Crosses with suppressors dropped for reasons other than allelism are not shown. Crosses which failed to complement are labelled as big, and crosses which complemented are labelled as small, indicating a *camta1/2/3*-like appearance.

	6-1#1	*13-6#1*	*9-10#1*	*14-2#1*	*14-7#1*	*11-5#1*	*11-3#1*	*13-9#1*	*7-2#6*	*7-15#2*	*15-7#1*	*13-7#1*	*11-6#1*	*9-1#1*	*13-10#1*	*9-15#2*	*19#7*	*10-9#2*	*13-2#1*	*8-4#1*
ics1 x3	big		small	small		small	small	small			big				big					
eds5 x3	small			big	big	big	big				small	small								
pad4 x3								big	small				big			big				
fmo1 x3								small	small	big			small			small	big	big		
ald1 x3		big							small				small	small						
med15 x3																			big	small
6-1#1		small		small																
7-2#6			small																	
13-1#3		small																		
14-7#1			big	big		big														
11-3#1				big		big														
13-7#1								small			big									
9-15#2										small										
13-6#1	small			small																

Figure 10. Suppressor lines which failed to complement.

Suppressor lines which failed to complement *ics1* (A), *eds5* (B), *fmo1* (C), *ald1* (D), *pad4* (E) or *med15* (F) in the *camta1/2/3* background.

Table 2. Suppressor lines which failed to complement.
Suppressors are listed under the known mutant they appear to be allelic with.

ics1camta1/2/3	***eds5camta1/2/3***	***fmo1camta1/2/3***	***ald1camta1/2/3***	***pad4camta1/2/3***	***med15camta1/2/3***
13-10#1	*11-3#1*	*7-15#1*	*13-6#1*	*11-6#1*	*13-2#1*
13-7#1	*11-5#1*	*10-9#2*		*13-9#1*	
15-7#1	*14-2#1*	*19#7*		*9-15#2*	
6-1#1	*14-7#1*				
	9-10#1				

Some mutants were also eliminated because they were too small to differentiate from the *camta1/2/3* background (such as *7-1#4*), because their phenotype was too variable (*19#4*) or because of too low germination rates (*12-2#3*). A few mutants that I have been following have complemented all the known mutants they have been crossed with to date, but due to time constraints, these mutants were passed off to a fellow lab member to continue following. These mutants could represent novel genes involved in regulating immunity (Table 3), as will be discussed in chapter 4. Altogether, though novel suppressors may still be found, my research has only, thus far, conclusively found alleles of know suppressors.

Table 3. Potential novel suppressors of *camta1/2/3*. SA and other phenotypes of mutants which have not yet been shown to be allelic to any known mutants.

Mutant	**SA Grouping**	**Notes**
8-4#1	High	Only slightly larger than *camta1/2/3*. Slight bleaching phenotype. Very difficult to cross. Not *med15*
6-12#1	Mid	A little smaller than WT size usually, but variable.
7-2#6	Mid	Slightly larger than *camta1/2/3*. Doesn't appear to be *ald1, fmo1* or *pad4.*
9-1#1	Mid	Slightly larger than *camta1/2/3*.
11-6#3	Mid	Short and bushy. Could be *pad4* (similar phenotype to 11-6#1 which is *pad4*).
13-1#3	Mid	Later flowering.

3.3 Methods

3.3.1 Plants and Growth Conditions

Unless otherwise specified, Arabidopsis plants were grown in growth rooms at 22°C under a 16-h day/8-h night cycle. In complementation crosses, *camta1/2/3* known mutants were used as pollen donors and unknown suppressor plants as females. F1 seeds were sterilized with 15% (vol/vol) bleach, rinsed with water and plated on ½ MS media for two weeks before being transferred to soil. Other seeds were similarly sterilized but planted on soil.

3.3.2 EMS Mutagenesis and Mutant Screen

EMS mutagenesis of the *camta1/2/3* seeds was carried out following a previously described protocol[219]. In short, *camta1/2/3* seeds were suspended and shaken in 20 mM EMS for 16 h. The mutagenized seeds were sterilized and plated on 1/2 MS media. M1 seedlings were transplanted to soil at about 10 days old and were allowed to self. The resulting seeds were pool-harvested, and M2 plants were screened for those with increased rosette size compared to the dwarf *camta1/2/3* mutants.

3.3.3 SA Extraction Protocol

Total SA was extracted using a modified protocol described previously[220]. Roughly 0.05 grams of tissue were collected per sample, with two replicates for each genotype. Tissue was ground and extraction was performed first with 0.6 mL 90% ethanol and then 0.4 mL 100% ethanol, with samples being mixed and placed in a sonicator for 20 min. prior to being spun at 13000 rpm for 20 min. The supernatants from both extractions were combined and dried in fume hood overnight. 0.1 mL betaglucosidase solution (80 units/mL) was then added in order to cleave

salicylic acid glucoside into free SA, and samples were mixed, sonicated for 5 min and incubated at 37°C for 90 min. After incubation, 0.5 mL of 5% trichloroacetic acid was added to each sample, which were then spun down at 13000 rpm for 15 min. The supernatant was transferred to a new tube and extracted: 0.3 mL of a 100:99:1 ethylacetate:cyclopentant:isopropanol mixture was added each time, followed by vortexing and centrifuging for 1 min to encourage separation. The upper phase was transferred to separate tubes and the process was repeated twice more on the remaining sample. The cumulative upper phase was then dried overnight and resuspended in HPLC mobile phase (0.2 M potassium acetate, 0.5 mM EDTA, pH 5). Samples were mixed, sonicated for 5 min and then spun at 13000 rpm for 5 min. The clear supernatant was then transferred to new tubes and HPLC analysis was performed.

Chapter 4: Conclusion

3.1 Chapter 2 Conclusions

In this chapter, I argued that the genomic proximity of clustered NLRs is likely to have functional significance. Although the concept of clustered genes functioning together is not new[205], its influence on the study of *R* genes has been limited. Many paired or clustered genes that have been shown to function together were discovered through forward genetic screens or crosses rather than through deliberate testing of adjacent genes. Different types of clusters seem to have specific modes of action that can help to guide research. While NLRs encoded by singleton genes detect pathogen activity largely by forming homo-oligomers, paired NLRs encoded by head-to-head genes where one features an integrated domain have consistently been observed to function together. Clusters, even when not highly conserved, may lead to partnering activities between different members. These patterns of behavior can and should be used as guides as we continue to dissect plant defense signaling. The analysis of the interactions between *NLR*s within a cluster might reveal subtleties about immune sensing not previously observed, perhaps aiding in the effective application of plant immune knowledge to important crop species, which is the end goal of most plant immunity research.

The scope of this chapter was broad and intended more to raise questions than answers. It does not test functionality and is not intended to accurately predict the functional mechanism of unstudied NLR clusters. Future careful mechanistic investigations of these clusters are needed to identify more unique mechanisms of immune sensing associated with gene clustering, and to confirm whether these trends continue to hold true. Related questions that could be investigated include whether genomic arrangement comparisons of different species and ecotypes would

reveal NLR pairings influencing resistance to specific pathogen threats, or if utilizing clusters with multiple functional pairings could help to eliminate some of the fitness costs generally associated with plant immunity? It has been observed that woody plants tend to have different R gene arrangements in general than non-woody plants, which is likely to be due to their longer lifespan; could similar differences in clustering trends be observed among plants of different backgrounds or environments? Gene clustering has also been observed for other predicted immune receptor genes, such as those encoding receptor-like proteins and receptor-like kinases. Does the proximity–function correlation observed in many NLRs hold true for other plant immune receptors?

3.2 Chapter 3 Conclusions and future perspectives

I studied a pool of *camta1/2/3* suppressors in the hopes of finding a new regulator of plant immunity. While I found new alleles of many known suppressors, the evidence to date suggests that I also found mutations in novel regulators.

The only suppressor with a mutation in a gene known to encode a protein involved in defense which had not already been found in a previous screen was the *ALD1* mutant. *ALD1* mutants were already known to suppress *camta1/2/3*. However, mutations in neither *SARD4* nor *PBS3* were found, as well as only one *ald1* allele, so the screen still has not reached saturation. That does not mean that there are still novel mutants left to be found, of course, though I do think that a larger screen examining only partial suppressors could catch additional novel mutants. The drawback of this approach, in addition to the extensive complementation analysis needed to test each new suppressor, is that the partial mutants are hard to distinguish from *camta1/2/3*, and are less healthy and fertile. While growing the plants in hot chambers does increase *camta1/2/3* mutant size, it does not seem to increase the likelihood of crosses being successful.

In addition, because of the difficulty faced in crossing mutant lines, not all the mutants that failed to complement were conclusively shown to be recessive, meaning they could have dominant mutations in novel genes, though this is usually less common. Some of the mutants shown to be allelic also had phenotypes beyond what is expected of a mutation in that gene. For example, we noted that while 13-9#1 is allelic with *pad4 camta1/2/3*, but is both larger than that quadruple mutant, and has a distinctly different leaf shape that was not present in the F1 of the complementation cross. This could suggest that multiple genes are responsible for the suppression and morphological phenotype, which could be found by phenotyping the F2 from a cross between 13-9#1 and *pad4 camta1/2/3*, and sequencing the mutants with the more rounded, larger leaf shape.

Whilst this project has not yet identified additional proteins needed to regulate immunity and defense hormone synthesis, I do hope that the potentially novel mutants will yield new insight into plant immune regulation. I think that similar screens are still needed. The hormone crosstalk and feedback loops suggested by the suppression of *camta1/2/3* by mutations in genes encoding proteins involved in both SA and NHP biosynthesis raises questions that have yet to be satisfactorily answered. A fellow lab mate will continue working on the six mutants remaining, as well as 13-9#1, in order to identify if any of them contain mutations in novel genes.

If any of my remaining suppressors do turn out to not be allelic to any known *camta1/2/3* suppressors, then they can be backcrossed with *camta1/2/3* and the larger F2 can be used for whole genome sequencing in order to find the causal gene. I would not expect any novel genes at this point to be directly involved in hormone biosynthesis, but perhaps the novel gene could represent a hormone sensitive transcription factor or another more immune specific mediator or chaperone protein. If the gene appears to be a transcription factor, then the next steps would be to determine its gene targets, likely by using ChIP-Seq, as well as attempting to determine if there are other elements directly upstream of it, besides the CAMTAs. If it is not a transcription factor, then looking into which proteins it directly or indirectly interacts with would likely be the next step. In both cases, examining how the activity of the protein/gene changes in response to SA or NHP deficiency, and how its loss affects SA and NHP levels in reciprocal mutants, could indicate whether it plays a role in the crosstalk between these two pathways. If it does, then we could likely use its interactions to help form a more complete picture of the defense activations which occur downstream of CAMTA1/2/3.

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