CHARACTERIZATION OF THE MOS4-ASSOCIATED COMPLEX IN PLANT DEFENSE SIGNALING

by

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Abstract

The plant immune system is governed in part by Resistance (R) proteins that recognize pathogenic microorganisms and initiate enduring defense responses. While the terminal outputs of R protein activation are fairly well understood, information about signaling components involved in plant immunity is scarce. We previously showed that MODIFIER OF SNC1, 4 (MOS4) associates with the transcription factor AtCDC5 and the WD-40 protein PRL1 \textit{in vivo}, forming the MOS4-Associated Complex (MAC). The MAC is required for plant defense responses, including those activated in the autoimmune mutant snc1, in which the R protein SNC1 is constitutively active. To identify additional MAC proteins, hemagglutinin-tagged MOS4 was purified by affinity chromatography and over 20 associated proteins were subsequently identified by mass spectrometry. In addition to MOS4, AtCDC5, and PRL1, we identified two homologous U-box proteins as well as several nucleic-acid binding proteins and snRNP subunits predicted to be integral components of the spliceosome. This thesis describes the characterization of selected MAC proteins in plant defense as well as \textit{EDS17}, a gene unrelated to the MAC but that is likewise required for innate immunity in \textit{Arabidopsis}.

\textit{MAC3A} and \textit{MAC3B} encode highly similar E3 ubiquitin ligases with homology to the yeast and human protein Prp19. Through the analysis of loss-of-function mutants, we found that these loci are genetically redundant and are required for innate immunity in plants. \textit{MAC5A} and \textit{MAC5B} encode highly homologous putative RNA-binding proteins similar to the human protein RBM22. Analysis of these loci by reverse genetics revealed that they are partially redundant in a dosage-dependent manner and that both genes are essential for viability in \textit{Arabidopsis}. Importantly, the loss of either \textit{MAC3A/3B} or \textit{MAC5A} suppresses \textit{snc1}-associated autoimmune responses, indicating that these loci function in the \textit{snc1} pathway similar to \textit{MOS4}. Even though the MAC is closely associated with the spliceosome, we could not detect obvious splicing defects in MAC mutants, indicating that this protein complex is probably not required for general splicing in plants. Together, our data suggest that the MAC likely functions as a transcriptional regulator to fine-tune plant immune responses.
# Table of contents

Abstract .......................................................................................................................... ii  
Table of contents ............................................................................................................. iii  
List of tables .................................................................................................................. vii  
List of figures ................................................................................................................ viii  
List of abbreviations ..................................................................................................... x  
Acknowledgements ........................................................................................................ xiv  
Dedication ....................................................................................................................... xv  
Co-authorship statement ................................................................................................. xvi  

1 Plant innate immunity ................................................................................................. 1  
  1.1 Summary ............................................................................................................... 2  
  1.2 Introduction ......................................................................................................... 2  
  1.3 Recognition and response at the plant cell surface .......................................... 5  
    1.3.1 Microbe-associated molecular patterns and pattern recognition receptors ... 5  
    1.3.2 Signaling downstream of PRR activation ......................................................... 7  
  1.4 Immune responses mediated by plant resistance proteins ............................... 9  
    1.4.1 Pathogen virulence through the delivery of effectors .................................... 9  
    1.4.2 Resistance proteins ....................................................................................... 10  
    1.4.3 Recognition of pathogen effectors ................................................................. 12  
    1.4.4 R protein activation ....................................................................................... 13  
    1.4.5 R protein-mediated signaling ...................................................................... 15  
  1.5 Concluding remarks ............................................................................................ 17  
  1.6 Manuscript acknowledgements .......................................................................... 18  
  1.7 References ......................................................................................................... 20  

2 Dissecting plant defense signal transduction: modifiers of snc1 in Arabidopsis ... 27  
  2.1 Summary ......................................................................................................... 28
2.2 Introduction............................................................................................................ 28
  2.2.1 The snc1 suppressor screen.................................................................................. 30
2.3 Modifiers of snc1........................................................................................................ 32
  2.3.1 Nucleo-cytoplasmic trafficking machinery: MOS6, MOS3, and MOS7................. 32
  2.3.2 RNA processing proteins: MOS2 and MOS4....................................................... 34
  2.3.3 Protein modifying enzymes: MOS5 and MOS8.................................................... 35
2.4 Outlook....................................................................................................................... 37
2.5 Thesis objectives......................................................................................................... 38
2.6 References................................................................................................................ 41
3 Two Prp19-like U-box proteins in the MOS4-associated complex play redundant roles in plant innate immunity........ 45
  3.1 Summary.................................................................................................................. 46
  3.2 Introduction.............................................................................................................. 46
  3.3 Results...................................................................................................................... 49
    3.3.1 Identification of MAC proteins............................................................................ 49
    3.3.2 Isolation of mac3a and mac3b loss-of-function mutants...................................... 50
    3.3.3 MAC3A and MAC3B function redundantly in plant defense............................... 51
    3.3.4 mac3a mac3b displays defects in R protein-mediated defense pathways.............. 52
    3.3.5 mac3a mac3b suppresses autoimmune phenotypes associated with snc1.................. 53
    3.3.6 MAC3 localizes to the nucleus............................................................................ 54
    3.3.7 MAC3 associates with AtCDC5 in planta............................................................ 55
  3.4 Discussion................................................................................................................. 55
  3.5 Materials and methods............................................................................................. 62
    3.5.1 Plant growth, mutant isolation, pathology assays and phenotypic characterization..... 62
    3.5.2 Double and triple mutant construction............................................................... 63
    3.5.3 Molecular cloning............................................................................................... 63
4

The putative RNA-binding protein MOS4-ASSOCIATED COMPLEX5 functions in immunity mediated by suppressor of npr1-1, constitutive 1

4.1 Summary

4.2 Introduction

4.3 Results

4.3.1 Isolation and phenotypic characterization of mac5a loss-of-function mutants

4.3.2 MAC5A localizes to the nucleus and associates with AtCDC5 in planta

4.3.3 MAC5A and MAC5B are partially redundant

4.3.4 mac5a-1 suppresses snc1 autoimmunity but does not exhibit enhanced disease susceptibility

4.3.5 Genetic interaction between MAC5A and MOS4, AtCDC5, PRL1 and MAC3A/3B

4.4 Discussion

4.5 Methods

4.5.1 Plant growth and pathology assays

4.5.2 Mutant isolation and genetic crosses

4.5.3 Molecular cloning and expression analysis

4.5.4 Nuclear protein extraction and immunoprecipitation

4.6 Manuscript acknowledgements

4.7 References

5

The HEAT-repeat protein ENHANCED DISEASE SUSCEPTIBILITY17 functions in plant immunity

5.1 Summary

5.2 Introduction
5.3 Results ........................................................................................................... 124
  5.3.1 The loss of EDS17 causes pleiotropic defects in Arabidopsis ............ 124
  5.3.2 EDS17 is required for basal defense ..................................................... 126
  5.3.3 EDS17 is required for defense mediated by a subset of R proteins... 128
  5.3.4 EDS17 does not function in snc1-mediated immunity .................... 128
  5.3.5 EDS17 is required for SAR................................................................. 129
5.4 Discussion ................................................................................................... 130
5.5 Methods .................................................................................................... 132
  5.5.1 Plant growth, mutant isolation, and pathogen infection assays....... 132
  5.5.2 Genetic crosses .................................................................................. 133
  5.5.3 Quantification of chlorophyll .............................................................. 133
  5.5.4 Expression analysis ........................................................................... 133
5.6 Manuscript acknowledgements ................................................................. 134
5.7 References ............................................................................................... 142
6 Final discussion and perspectives .................................................................. 146
  6.1 The MOS4-associated complex and plant defense ............................. 147
  6.2 References ............................................................................................. 153
Appendices ..................................................................................................... 156
  A1 Transient expression of MAC3A-CFP and CFP-MAC3B in onion epidermal cells .......................................................... 157
  A2 F3 analysis of higher-order MAC mutants ........................................... 158
  A3 Heterologous expression of MAC3A is not able to complement the prp19-1 mutation in yeast ................................................. 159
  A4 Site-directed mutagenesis of a conserved cysteine MAC3A and MAC3B U-box domains ............................................................... 160
  A5 List of publications .................................................................................. 161
List of tables

Table 2.1  Fully characterized modifier of snc1 mutants identified in the snc1 and snc1 npr1 suppressor screens. 39

Table 3.1  MOS4-associated proteins identified by mass spectrometry 70

Table 3.2  Predicted sub-cellular localization of MAC components and details from mass spectrometry analysis 72

Table 4.1  Summary of phenotypes reported for MAC mutants in Arabidopsis 105
List of figures

Figure 1.1 Signaling events involved in plant innate immunity.......................... 19
Figure 2.1 Modifiers of snc1 in Arabidopsis.................................................. 40
Figure 3.1 MOS4-HA associated proteins...................................................... 68
Figure 3.2 Protein sequence alignment of MAC3A and MAC3B with homologs in other eukaryotes................................................................. 73
Figure 3.3 MAC3A and MAC3B function redundantly in basal defense.......... 74
Figure 3.4 Morphology of the mac3a mac3b double mutant and enhanced susceptibility to P.s.m. ES4326.............................................................. 75
Figure 3.5 Transgenic complementation of mac3a mac3b and snc1 mac3a mac3b by MAC3A and MAC3B................................................................. 76
Figure 3.6 MAC3A and MAC3B function redundantly in specific R-protein mediated resistance pathways.............................................................. 77
Figure 3.7 Suppression of snc1-associated phenotypes by mac3a mac3b....... 78
Figure 3.8 Sub-cellular localization of MAC3A.............................................. 79
Figure 3.9 Sub-cellular localization of MAC3B.............................................. 80
Figure 3.10 MAC3B cDNA sequence analysis............................................. 81
Figure 3.11 MAC3A-HA associates with AtCDC5 in planta.......................... 82
Figure 3.12 MAC3A-CFP associates with AtCDC5 in planta.......................... 83
Figure 4.1 Isolation of mac5a T-DNA insertion mutants............................... 106
Figure 4.2 Sub-cellular localization of MAC5A.......................................... 107
Figure 4.3 MAC5A-HA and AtCDC5 co-immunoprecipitate in planta......... 108
Figure 4.4 Multiple sequence alignment of MAC5A homologs in Arabidopsis, human, and budding yeast......................................................... 109
Figure 4.5 MAC5A and MAC5B are partially redundant............................... 110
Figure 4.6 PCNA2 expression is not affected in the mac5b-1 mutant.......... 111
Figure 4.7 mac5a-1 suppresses snc1 auto-immune phenotypes................... 112
Figure 4.8  Infection assays on MAC5A and MAC5B loss-of-function alleles........ 113
Figure 4.9  Double or triple mutant analysis between mac5a-1 and other MAC mutants................................................................. 114
Figure 4.10 General splicing is not affected in MAC combination mutants........... 115
Figure 5.1  EDS17-like proteins are found in eukaryotes in different kingdoms.... 135
Figure 5.2  Phenotypic analysis of eds17 T-DNA insertion mutants.................... 136
Figure 5.3  eds17 mutants exhibit a decrease in leaf chlorophyll ....................... 137
Figure 5.4  EDS17 is required for basal defense against Pseudomonas syringae. 138
Figure 5.5  EDS17 is required for resistance conditioned by specific R proteins... 139
Figure 5.6  Loss of EDS17 does not suppress snc1-associated phenotypes....... 140
Figure 5.7  EDS17 is required for SAR..................................................... 141
List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
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<tr>
<td>ABRC</td>
<td><em>Arabidopsis</em> biological resource center</td>
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<td>ACRE</td>
<td>Avr9/Cf9 rapidly elicited</td>
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<td><em>Arabidopsis</em> dynamin-like protein</td>
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<td>Avr</td>
<td>avirulence</td>
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<td>AvrB</td>
<td>avirulence gene from <em>Pseudomonas syringae</em> pv. <em>glycinea</em></td>
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<td>EDR</td>
<td>enhanced disease resistance</td>
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<td>MAMP</td>
<td>microbe associated molecular pattern</td>
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<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>MBO</td>
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<td>MEE</td>
<td>maternal effect embryo arrest</td>
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<td>MLA</td>
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<td>MOS</td>
<td>modifier of snc1</td>
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<td>MPK</td>
<td>mitogen-activated protein kinase</td>
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<td>MS</td>
<td>mass spectrometry</td>
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<td><em>Nicotiana</em> (as in N protein in tobacco)</td>
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<td>NASC</td>
<td>European <em>Arabidopsis</em> stock center</td>
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NB-LRR  nucleotide-binding leucine-rich repeat
NDR1  non-race specific disease resistance 1
NES  nuclear-export sequence
NLR  Nod-like receptor
NLS  nuclear-localization sequence
Noco2  *H.a.* isolate identified in Norwich on Col-0
NPR1  non-expressor of PR genes
NTC  nineteen complex (Prp19 complex)
Nup  nucleoporin
OD  optical density
*P.s.m.*  *Pseudomonas syringae* pv. *maculicola*
*P.s.t.*  *Pseudomonas syringae* pv. *tomato*
P35S  constitutive promoter from cauliflower mosaic virus (CaMV)
PABP  poly(A)-binding protein
PAD4  phytoalexin deficient 4
PAMP  pathogen associated molecular pattern
PCNA2  proliferating cell nuclear antigen 2
PCR  polymerase chain reaction
PLDα  phospholipase D alpha
PLRG  pleiotropic regulating locus gene
PP2A  protein phosphatase type 2A
PR  pathogenesis-related
Prf  *Pseudomonas* resistance and fenthion sensitivity
PRL1  pleiotropic regulatory locus 1
PRP  precursor mRNA processing
Prp19  precursor RNA processing 19
PRR  pattern recognition receptor
Pto  *Pseudomonas syringae* pv. *tomato*
PUB  plant U-Box proteins
R  resistance
RAR1  required for MLA12 resistance 1
RBM  RNA binding motif
RCN  roots curl in NPA
RIN4  RPM1-interacting protein 4
RLK  receptor-like kinase
RNA  ribonucleic acid
ROS  reactive oxygen species
RPM1  resistance to *Pseudomonas syringae* pv. *maculicola* 1
RPP  resistance to *Peronospora parasitica*
RPS  resistance to *Pseudomonas syringae*
RPW8  resistance to powdery mildew 8
RRS1  resistance to *Ralstonia solanacearum* 1
RSW  radial swelling
RT  reverse transcription
SA  salicylic acid
SAG101 senescence associated gene 101
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<td>SAR</td>
<td>systemic acquired resistance</td>
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<td>SGT1</td>
<td>suppressor of the G2 allele of skp1</td>
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<td>SID</td>
<td>salicylic acid deficient (as in SID1, SID2)</td>
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<td>SKIP</td>
<td>Ski-interacting protein</td>
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<td>SNC1</td>
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<td>SNEV</td>
<td>senescence evasion factor</td>
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<td>snRNP</td>
<td>small nuclear ribonucleoprotein</td>
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<td>abnormal suspensor</td>
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<td>SYF</td>
<td>synthetic lethality with Cdc40</td>
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<td>T3SS</td>
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<td>X-gluc</td>
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</table>
Acknowledgements

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Dedication

For Jeff
Co-authorship statement

The work described in this thesis is the culmination of research from September 2005 through December 2009. Below is a list of manuscripts (published, in press, under revision, or in preparation) that comprise this thesis, and the contribution made by the candidate.


- The candidate conceived of and wrote the manuscript. T. Weihmann drew Figure 1.1 based on the text written by the candidate, and X. Li supervised the preparation of the manuscript.


- The candidate conceived of and wrote the majority of the manuscript. H. Germain created Table 2.1 and Figure 2.1, T. Weihmann wrote introduction section 2.2 (but the candidate wrote section 2.2.1). X. Li supervised the preparation of the manuscript.


- The candidate performed most of the experiments and wrote the manuscript. F. Xu performed IP-Western experiments using transgenics generated by the candidate, wrote methods section 3.5.4, and conducted confocal microscopy using transgenic lines generated by the candidate (the candidate also did microscopy but F. Xu’s images were used in the manuscript). M. Gao performed yeast two-hybrid and bimolecular fluorescence experiments that were not shown in the manuscript using plasmids created by the candidate. Q. Zhao immuno-purified MOS4-HA and associated proteins and prepared samples for mass spectrometry. K. Palma generated the MOS4-HA transgenic lines and did the mac3a mac3b and snc1 mac3a mac3b genetic crosses. C. Long assisted in mass spectrometry under the supervision of S. Chen. S. Chen wrote the mass spectrometry methods section 3.5.5, and generated the data presented in Table 3.2. Y. Zhang (NIBS, Beijing) supervised the work done by F. Xu, M. Gao, Q. Zhao, and C. Long. X. Li (UBC, Vancouver) supervised the work done by the candidate and K. Palma, and supervised the preparation of the manuscript.

- The candidate performed most of the experiments and wrote the manuscript. F. Xu performed IP-Western experiments shown in Figure 4.3 and conducted confocal microscopy in Figure 4.2, and S. Xu generated the MAC5A-GFP and MAC5A-HA transgenic lines used to create these figures. Y. Zhang (NIBS, Beijing) supervised the work done by F. Xu and S. Xu. X. Li (UBC, Vancouver) supervised the work done by the candidate and supervised the preparation of the manuscript.


- The candidate performed all of the experiments and wrote the manuscript. X. Li supervised the work and the preparation of the manuscript.
1 Plant innate immunity¹

1.1 Summary

Plants possess an elaborate multi-layered defense system that relies on the intrinsic ability of plant cells to perceive the presence of pathogens and trigger local and systemic responses. Transmembrane receptors detect highly conserved microbial features and activate signaling cascades that induce defense gene expression. Pathogens deliver effector proteins into plant cells that suppress these responses by interfering with signaling components. Plants, in turn, evolved intracellular receptors called Resistance (R) proteins to recognize these effector proteins or their activities in the plant cell. Activated R proteins trigger a series of physiological changes in the infected cell that restrict pathogen growth locally and resonate systemically to enhance immunity throughout the plant. In this chapter we summarize our current understanding of defense responses employed by plants during pathogen infection.

1.2 Introduction

There are numerous examples of human suffering caused by the failure of crops due to plant disease. One of the most often cited examples is the great potato famine that hit Ireland in the middle of the 19th century as the result of potato late blight caused by Phytophthora infestans. This disease not only caused the death of an estimated one million people, but it also led to a mass emigration out of Ireland into North America, and has been credited as the linchpin that sparked a real interest in plant pathology as a scientific discipline (Holub, 2001; Judelson and Blanco, 2005). Plant diseases cost farmers billions of dollars each year due to crop loss or disease prevention strategies. Just one of many recent examples is the rice blast fungus, Magnaporthe grisea, which affects most rice-producing areas in the world and is estimated to ruin enough crop to feed 60 million mouths each year (Dean et al., 2005). The ability of plant pathogens to spread rapidly through crop fields and cause so much damage is exacerbated by the
modern practice of monoculture farming, where single cultivars are continually planted over large areas of land. However, most plants are resistant to most potential pathogens, and there has been a world-wide effort to understand the innate mechanisms that underlie this ability. A clear understanding of the interplay between plants and their pathogens is fundamental to the environmentally friendly management of plant disease and the development of disease-resistant cultivars of crop plants.

Even though plants are host to every type of microbial pathogen (including fungi, oomycetes, bacteria, and viruses), they are not infected easily. Plants present microbes with a number of obstacles to overcome before they can successfully infect plant cells. Examples include cuticular waxes, antimicrobial enzymes and other specialized metabolites, as well as plant cell walls (Thordal-Christensen, 2003). Microbes that have adapted to certain plants have found ways to circumvent these barriers and cause disease, whereas non-adapted microbes are unable to overcome these defenses. Plant species that can be colonized by a pathogen become ‘hosts’ for that pathogen whereas resistant species are ‘nonhosts’. However, individual plant cultivars within a host species can be resistant to pathogen infection if they express specific defense genes. The genetic relationship between host plants and their pathogens was first described in detail by Harold Flor in the 1940s and 50s. Flor meticulously studied the genetic relationship between races of flax rust fungus and a number of flax varieties with respect to host susceptibility and resistance (Flor 1971). Based on his work, Flor hypothesized that resistance is the consequence of the correct combination of single genetic loci in the host and the pathogen. He proposed that the gene products of plant Resistance (R) genes interact with pathogenic Avirulence (Avr) gene products in a corresponding gene-for-gene manner. These pathogenic proteins are called ‘avirulent’ because, instead of contributing to virulence, their recognition by R proteins leads to plant resistance. Rather than existing solely to reveal their identity, many Avr proteins contribute to virulence in susceptible plants (Jones and Dangl, 2006).

The advent of molecular biology and the use of genetic models have led to a number of recent discoveries outlining signaling components in plant defense pathways. Arabidopsis thaliana, which emerged as a model for plant molecular biology in the mid-1980s, has been widely adopted by plant pathologists studying defense signaling
networks. The labs of Brian Staskawicz, Jeffery Dangl, and Frederick Ausubel characterized the interaction between Arabidopsis and the leaf-spot causing bacterial pathogen Pseudomonas syringae and revealed a highly amendable plant-pathogen system that is now widely used to dissect genetic components of plant defense (Dong et al., 1991; Whalen et al., 1991; Katagiri et al., 2002). This system in particular has been instrumental to the field, and both genomes are now fully sequenced (Buell et al., 2003; The Arabidopsis Genome Initiative 2000). Arabidopsis is also host to the water mold Hyaloperonospora arabidopsidis which causes downy mildew on leaves (Slusarenko and Schlaich 2003), and this system, established largely by Eric Holub, Jonathan Jones, and Jane Parker, has been extremely useful in the study of plant defense. In addition to these Arabidopsis systems, agriculturally important plant-pathogen systems are also widely studied as models, such as powdery mildew of barley led by Paul Schulze-Lefert’s group, bacterial blight of rice pioneered by Pamela Ronald’s team, bacterial spot of tomato and pepper largely studied by Ulla Bonas’ group, leaf rust of flax by Jeff Ellis’ team, and leaf mold of tomato led by Jonathan Jones’ and Pierre de Wit’s groups. Together, the establishment of these model systems has enabled researchers to identify key players in host immune responses and pathogen virulence at the molecular level.

We now know that signaling in plant disease resistance shares many conceptual features with mammalian ‘innate’ immunity (Nurnberger et al., 2004), although there are several lines of evidence to suggest that these pathways evolved convergently (Ausubel, 2005). Though plants lack an ‘adaptive’ immune system like that found in vertebrates, plant cells are equipped with a number of extra- and intra-cellular immune receptors that detect the presence of pathogenic microbes and activate defense responses. Plants have a set of receptors that detect highly conserved and slowly evolving features of whole groups of microbes such as flagellin, the major protein found in bacterial flagella (Gomez-Gomez and Boller, 2002). The activation of these receptors induces defense gene expression, ion fluxes, and the production of reactive oxygen species in the plant cell that limit microbial growth. Successful pathogens have either adapted to evade recognition by plants, or have evolved ways of interfering with or suppressing defense signaling, mostly through the expression of effectors delivered into host cells during an infection (Jones and Dangl, 2006). In an elegant example of co-evolution, plants have, in
turn, evolved intracellular R proteins to recognize specific pathogenic effectors and activate signaling cascades leading to massive cellular re-programming that eventually restricts pathogen growth (Dangl and Jones, 2001; Jones and Dangl, 2006). Pathogens have evolved a number of effectors to suppress additional components of plant immune signaling, and thus, the ‘arms race’ between host and pathogen is revealed.

This chapter will outline some of the signaling events triggered in plant cells following the recognition of pathogens that feed on living plant tissue. This defense response is distinct from that used to combat herbivory and necrotizing pathogens, which will not be covered here. Interested readers are referred to the following excellent reviews: Farmer et al. (2003); Schilmiller and Howe (2005). We start with a discussion on the recognition of highly conserved microbial patterns at the plant cell surface and some of the defense responses that follow. Certain ways that pathogen effectors have adapted to suppress these events are briefly elaborated. Intracellular R proteins that respond to the presence of these effectors and some of the signaling components involved in R protein-mediated defenses form the remainder and bulk of the chapter.

1.3 Recognition and response at the plant cell surface

1.3.1 Microbe-associated molecular patterns and pattern recognition receptors

Like animals, plants are able to recognize highly conserved features of microbes known as microbe-associated molecular patterns (MAMPs). MAMPs are typically necessary for and integral to microbial lifestyles and are therefore not easily lost or mutated, making them ideal candidates as targets for detection by immune receptors. For example, both plants and animals can detect the presence of Gram-negative bacteria through the perception of lipopolysaccharides (LPSs) found in their outer membrane (Dow et al., 2000). Plants respond to other MAMPs including peptides or motifs characteristic to bacterial proteins such as flagellin, elongation factor Tu (EF-Tu), and cold shock proteins, as well as to sugars found in bacterial and fungal cell walls (peptidoglycan and
chitin, respectively; reviewed in Nurnberger et al., (2004). Thus, plants have evolved the ability to differentiate between self and non-self as part of an early warning system against potential pathogen infection.

MAMPs are recognized in mammals by transmembrane Toll-like receptors (TLRs) and cytosolic Nod proteins (Akira et al., 2006), collectively referred to as pattern or pathogen recognition receptors (PRRs). In plants, transmembrane receptor-like kinases (RLKs) play an integral role in MAMP perception and signal relay. Two PRRs that have been well characterized in plants include FLAGELLIN SENSITIVE 2 (FLS2; Gomez-Gomez and Boller, 2000), and EF-Tu RECEPTOR (EFR; Zipfel et al., 2006), which recognize bacterial flagellin and EF-Tu, respectively. FLS2 and EFR have an extracellular leucine-rich repeat (LRR) domain and a cytosolic serine/threonine kinase domain, and likely represent members of a larger group of RLKs involved in MAMP perception (Zipfel, 2008). Plants respond to MAMPs rapidly with pronounced changes in gene expression, cell wall alterations, accumulation of antimicrobial proteins and compounds, and changes in apoplastic pH levels that hinder the growth of microbial populations to some extent but are only slightly effective at preventing the growth of virulent pathogens (Gomez-Gomez and Boller, 2000).

Bacterial pathogenesis is largely reliant on the ability to move into and within the plant apoplast, and this motility is provided by flagella. FLS2 proteins in Arabidopsis, tomato, and tobacco recognize and respond to bacterial flagellin, indicating that this recognition module is conserved across plant species (Zipfel, 2008). FLS2 in Arabidopsis binds a small but highly conserved 22-amino acid epitope, flg22, from the N-terminus of the flagellin protein (Chinchilla et al., 2006). Arabidopsis plants treated with flg22 one day prior to infection with virulent bacteria exhibit a reduction in bacterial growth compared to plants that are not pre-treated; conversely, fls2 mutants are unable to perceive and respond to the flg22 elicitor, reflected in the higher susceptibility of these plants to bacterial infection (Zipfel et al., 2004). The same phenomenon has been observed using the EF-Tu elf18 epitope as an elicitor (Zipfel et al., 2006), demonstrating that the detection of single MAMPs can prime cells against further attack.

PRR activation and downstream signaling are tightly controlled. FLS2 is negatively regulated by the kinase-associated protein phosphatase KAPP (Gomez-Gomez et al., 2003).
et al., 2001) at the plasma membrane, and is internalized following flg22-binding by vesicle-mediated endocytosis as part of a negative feedback regulation scheme (Robatzek et al., 2006). Both FLS2 and EFR are positively regulated by another RLK, BRASSINOSTEROID ASSOCIATED KINASE 1 (BAK1; Chinchilla et al., 2007; Heese et al., 2007). Interestingly, both tobacco and Arabidopsis mutants with compromised FLS2 activity become susceptible to non-adapted pathogens (Zipfel, 2008), suggesting that PRRs are integral to both host and non-host resistance. Flagellin from the legume-associated nitrogen-fixing symbiont Rhizobium is not recognized in Arabidopsis by FLS2, nor is flagellin from the plant pathogen Agrobacterium (Felix et al., 1999), indicating that microbes are under evolutionary pressure to alter MAMPs to avoid recognition by the host PRR surveillance system.

1.3.2 Signaling downstream of PRR activation

The perception of MAMPs is converted into a defense response through finely tuned mitogen-activated protein kinase (MAPK) signaling cascades. MAPKs are used as signal transducers in all eukaryotes, and are an integral part of both mammalian and plant immunity (Nurnberger et al., 2004; Nakagami et al., 2005). These cascades are composed of at least a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK, activated by phosphorylation in that order. MAPK cascades that act both positively and negatively on resistance are activated following PRR activation. The Arabidopsis MAPKs MPK3, MPK4, and MPK6 are activated early in the FLS2-mediated pathway (Nakagami et al., 2005). Interestingly, whereas the phosphorylation cascade leading to MPK3 and MPK6 activation promotes resistance, the cascade involved in MPK4 activation plays an inhibitory role (Suarez-Rodriguez et al., 2007). This has been supported genetically, as mpk4 knock-out mutants constitutively activate defense markers and have enhanced resistance to pathogen infection (Petersen et al., 2000), whereas silencing MPK6 causes heightened susceptibility to pathogens (Menke et al., 2004). The details of these pathways have not yet been fully elucidated, but it is presumed that the simultaneous activation of both positive and negative regulators allows resistance outputs
to be carefully balanced according to the nature of the signal (Suarez-Rodriguez et al., 2007).

The activation of MPK3 and MPK6 induces the transcription of defense related genes via WRKY transcription factors (Asai et al., 2002). The WRKY family comprises a large group of stress response transcription factors (TFs) with a WRKY DNA-binding domain that binds W-box (C/TTGACC/T) promoter elements (Ulker and Somssich, 2004). W-boxes are found in the promoters of many defense related genes, and WKRYs have been implicated in transcriptional reprogramming in response to biotic stresses such as MAMP perception and pathogen infection (Eulgem and Somssich, 2007). There are over 70 WRKY TFs encoded in the Arabidopsis genome, and 90 in rice (Ulker and Somssich, 2004), and there is a high level of functional redundancy among the members of this large gene family (Eulgem and Somssich, 2007). The functional homologs WRKY22 and WRKY29 have been shown to be downstream targets of MPK3 and MPK6 activated in response to bacterial and fungal pathogens (Asai et al., 2002).

It is clear that plants are able to sense and respond to the presence of potential pathogenic microbes in their immediate environment. For pathogens to successfully colonize and exploit plant cells, they must avoid detection by their host. Phytopathogens (and animal pathogens; Finlay and McFadden, 2006) employ a number of strategies to evade host surveillance that, for the most part, interfere with or suppress host defense signaling in one way or other (Gohre and Robatzek, 2008; Zhou and Chai, 2008). Evasion and/or virulence are accomplished through the expression and delivery of pathogenic effector proteins into host cells during an infection. Thus, in addition to transmembrane PRRs, plants are also equipped with intracellular surveillance elements known collectively as R proteins to sense and respond to the activities of effectors.
1.4 Immune responses mediated by plant resistance proteins

1.4.1 Pathogen virulence through the delivery of effectors

Phytopathogens require access to plant cells to acquire photosynthate and other metabolites, and to accomplish this they have evolved various mechanisms to deliver effectors into the apoplast and/or directly into plant cells. The most widely studied bacterial delivery system used during plant infection is the type three secretion system (T3SS) employed by many Gram-negative bacteria to gain access to plant tissue. This secretion system is characterized by an assembled protein pilus that extends from the bacterium and punctures the cell membrane in a syringe-like manner, releasing a battery of effectors directly into the host cell (Jin and He, 2001). The pilus is essential to pathogenicity, as bacterial mutants lacking pilus components lose virulence and cannot cause disease on normally susceptible host plants (Alfano and Collmer, 1996). In addition to bacterial effectors, some fungal and oomycete effectors have been detected intracellularly (Birch et al., 2008). There is accumulating evidence to suggest that oomycetes secrete and translocate effectors into plant cells by hijacking the host endocytic pathway, a mechanism similar to that used by the human malaria parasite (Birch et al., 2008).

Several effectors in plant pathogens have been cloned (most of which are bacterial and delivered via the T3SS pilus), and their virulence functions are now being characterized. The role of most effectors has been elusive, as their primary amino acid sequences provide few clues regarding protein function. Recent structural and functional analysis, however, has revealed that some effectors mimic eukaryotic host signaling proteins including transcription factors, proteases and phosphatases to alter immune responses (Kay et al., 2007; Gohre and Robatzek, 2008). The structure of the \textit{P. syringae} effector AvrPtoB was recently shown to bear a striking resemblance to E3 ubiquitin ligases (Janjusevic et al., 2006), and was also found to have intrinsic E3 enzymatic activity (Abramovitch et al., 2006). As AvrPtoB requires this enzymatic function for virulence on susceptible plants, it is thought to suppress positive regulators of immunity
via protein degradation (Abramovitch et al., 2006; Janjusevic et al., 2006). Another *P. syringae* effector, AvrPto, was recently shown to bind the PRRs FLS2 and EFR, preventing their phosphorylation and thus suppressing downstream MAPK signaling and defense outputs in susceptible plants (He et al., 2006; Xiang et al., 2008). AvrPto also inhibits another kinase, the R protein Pto, contributing to virulence in susceptible plants (Xing et al., 2007). In addition, defense related MAPK cascades can be directly targeted by pathogenic effectors (Shan et al., 2007). Together, these examples demonstrate that successful pathogens use stealth and manipulation to evade host perception and suppress host defense responses.

### 1.4.2 Resistance proteins

Although used by pathogens to promote virulence in susceptible plants, some effector proteins can render infections avirulent if they are recognized in resistant plants by R proteins. The activation of R proteins triggers immune responses that are far more effective than those triggered by PRRs. The activation of R proteins leads to substantial ion fluxes, the induction of *PATHOGENESIS-RELATED (PR)* genes, the accumulation of the signaling molecule salicylic acid (SA), and an oxidative burst that leads to the accumulation of reactive oxygen species. Not only do these physiological changes create an unfavourable environment for pathogen growth, they are also often associated with a form of localized programmed cell death known as the hypersensitive response (HR), in which threatened cells commit suicide to restrict pathogen growth. The HR is particularly effective against pathogens requiring living tissue, as it confines them to dead cells where they are deprived of essential nutrients.

There are several classes of R proteins in plants. By far the most prominent class comprises intracellular NB-LRR proteins, which possess a central nucleotide-binding site (NB), and a C-terminal LRR domain. This group can be further subdivided based on two structural variations at the N-terminus; CC-NB-LRRs possess a putative coiled-coil (CC) domain and TIR-NB-LRRs possess a region similar to the Toll and Interleukin 1 receptor domain (TIR) found in mammalian immune receptors (Takeda et al., 2003). There are
an estimated 125 NB-LRR proteins encoded in the *Arabidopsis* genome (Jones and Dangl, 2006), although most of these genes have not yet been shown to function in resistance. Proteins with LRRs function in a diverse array of cellular processes in addition to plant immunity. LRRs have been shown to mediate protein-protein interactions in eukaryotes (Kobe and Deisenhofer, 1995), and, in the case of R proteins, are thought to determine Avr recognition specificity (Martin et al., 2003). NB domains are found in a number of proteins including ATPases, G-proteins, and, notably, in animal apoptosis regulators and proteins involved in innate immunity (Takken et al., 2006). This domain is thought to regulate the activity of R protein activation through the binding and hydrolysis of ATP (Tameling et al., 2006). The CC and TIR domains likely function in signaling, as CC- and TIR-NB-LRRs signal through distinct downstream pathways (Aarts et al., 1998), although it is also possible that these domains function in recognition specificity, as is the case with the R protein N in tobacco (Burch-Smith et al., 2007).

In addition to NB-LRRs, there are other classes of R proteins in plants. A large class of R proteins in tomato includes the Cf proteins, effective against infection by the leaf mold *Cladisporium fulvum* (Rivas and Thomas, 2005). These proteins span the plasma membrane and have an extracellular LRR domain and a small cytosolic domain of unknown function. The R protein Xa-21 in rice encodes an RLK similar to FLS2 and EFR that confers resistance against bacterial *Xanthomonas* species (Song et al., 1995), and tomato Pto is a cytosolic serine/threonine kinase required for resistance to *P. syringae pv. tomato* (Martin et al., 1993). Interestingly, no cloned *Arabidopsis* R genes encode proteins that clearly resemble Pto, Xa-21 or the Cf proteins, highlighting the importance of studying resistance mechanisms in a number of species (Martin et al., 2003). There are also some rather unusual R proteins found in *Arabidopsis*.

RESISTANCE TO R. SOLANASCEARUM 1 (RRS1), required for resistance to *Ralstonia solanascearum*, is a TIR-NB-LRR with a C-terminal nuclear localization sequence (NLS) and a WRKY domain, merging a defense receptor with a transcriptional regulator (Deslandes et al., 2002). RESISTANCE TO POWDERY MILDEW 8 (RPW8) confers resistance to a broad-range of powdery mildew strains and encodes a protein with a predicted N-terminal transmembrane domain and a CC domain (Xiao et al., 2001).
1.4.3 Recognition of pathogen effectors

Although several cognate R-Avr pairs have been identified, the relationship between these pairs is not always well understood at the molecular level. The simplest model predicts that R proteins are receptors for Avr ligands. For example, the R protein Pto has been shown to interact directly with its cognate effector AvrPto, and that this interaction is necessary for resistance (Tang et al., 1996). Although there are a few other cases, most attempts to show direct interactions between R and Avr proteins have not been fruitful, suggesting that additional host proteins are involved in effector recognition. In 1998, Eric Van der Biezen and Jonathan Jones introduced the idea that, as opposed to directly interacting with effector proteins, R proteins might guard or monitor the integrity of effector targets (Van der Biezen and Jones, 1998); an idea that was later articulated as the ‘guard hypothesis’ (Dangl and Jones, 2001). In this model, R proteins screen for pathogen-induced modifications in host proteins to trigger immune signaling.

A well established example of such a pathogen-modified protein in plants is RPM1-INTERACTING PROTEIN 4 (RIN4). RIN4 is localized to the plasma membrane, and is monitored by the likewise localized CC-NB-LRR R proteins RESISTANCE TO P. SYRINGAE PV. MACULICULA 1 (RPM1) and RESISTANT TO P. SYRINGAE 2 (RPS2). During infection, P. syringae pathovars release effectors into plant cells, including AvrRpm1, AvrB, and AvrRpt2, which are thought to target a number of host proteins as part of a virulence strategy. AvrRpt2, for example, is a cysteine protease (Coaker et al., 2005) that modifies plant auxin levels to promote virulence and pathogen growth (Chen et al., 2007). Although most virulence targets of these effectors have not been identified, it has been shown that AvrRpm1, AvrB, and AvrRpt2 interact with and modify RIN4 either by phosphorylation or cleavage (Mackey et al., 2002; Axtell et al., 2003). Intriguingly, these interactions with RIN4 do not promote virulence and are not required for successful infection (Belkhadir et al., 2004). Instead, RIN4 phosphorylation is monitored by RPM1 and its cleavage is monitored by RPS2, and either event leads to plant resistance (Mackey et al., 2002; Kim et al., 2005). RIN4 physically interacts with and represses both RPM1 and RPS2 (Mackey et al., 2002; Mackey et al., 2003). The inhibitory function of RIN4 has been shown genetically, as
partial loss-of-function *rin4* mutant plants have heightened resistance to virulent pathogens, suggesting a negative role in immunity (Mackey et al., 2002). Also, *rin4* phenotypes are fully suppressed in *rin4 rpm1 rps2* triple mutants, indicating that RIN4 is indeed a negative regulator of these R proteins (Belkhadir et al., 2004). Another example is AvrPto which, as mentioned before, targets the PRRs FLS2 and EFR to suppress plant immunity. AvrPto also binds and inhibits the kinase Pto (Xing et al., 2007), but unlike binding FLS2 and EFR, this interaction activates the NB-LRR protein PSEUDOMONAS RESISTANCE AND FENTHION SENSITIVITY (Prf) and leads to resistance (Mucyn et al., 2006). Thus, Pto might have evolved to compete with FLS2 and EFR binding to initiate defense (Zipfel and Rathjen, 2008). The guard hypothesis predicts that R proteins evolved to keep a watchful eye on a subset of proteins that are modified by pathogen effectors (including some plant proteins that may mimic virulence targets; Xing et al., 2007). It is likely that most effector modifications augment virulence in some way; however, the detection of even one of these events in a plant expressing the appropriate R protein can lead to an immune response and render the pathogen avirulent (Belkhadir et al., 2004).

### 1.4.4 R protein activation

There are significant physiological and metabolic consequences associated with deregulated R proteins in plants. For example, a point mutation in the predicted TIR-NB-LRR type *R* gene *SUPPRESSOR OF NPR1, CONSTITUTIVE 1 (SNC1)* renders the protein constitutively active, causing immune signaling components to be turned on in the absence of pathogen infection (Zhang et al., 2003a). Although *snc1* plants display enhanced resistance to pathogens, there are considerable costs to fitness. As the result of increased levels of SA, *snc1* plants exhibit severe dwarfism (Li et al., 2001), as do many mutants in which SA-mediated resistance signaling is overactive (Durrant and Dong, 2004). Other mutations associated with deregulated disease resistance pathways result in spontaneous HR-like lesion formation, as is the case with a gain-of-function mutation in the TIR-NB-LRR R protein SUPPRESSOR OF SA INSENSITIVITY OF NPR1-5, 4
(SSI4; Shirano et al., 2002). To avoid these extreme costs to plant health, defense pathways are tightly regulated.

R proteins are thought to exist in a repressed form in the absence of pathogens, either through inhibitory folding or interaction with negative regulators (Marathe and Dinesh-Kumar, 2003). Analysis of a potato CC-NB-LRR type R protein indicated that the CC and NB-LRR protein domains physically interact with each other in a non-threatening environment, but that these interactions dissipate in the presence of the cognate pathogen effector (Moffett et al., 2002). It is reasonable to expect that other NB-LRR R proteins undergo conformational changes in response to pathogen infection, and that they normally exist in an inhibitory conformation to avoid unwarranted activation. In addition, a number of NB-LRR R proteins associate with cytosolic heat-shock protein 90 (HSP90) and its co-chaperones REQUIRED FOR MLA12 RESISTANCE (RAR1), SUPPRESSOR OF THE G2 ALLELE OF SKP1 (SGT1), and cytosolic heat shock cognate 70 (HSC70; Shirasu and Schulze-Lefert, 2003; Noel et al., 2007). It is thought that this association facilitates the formation of R protein complexes and/or helps maintain R protein stability during the transition from a signal-incompetent to a signal-competent state (Shirasu and Schulze-Lefert, 2003).

This chaperone complex might also mediate the localization and movement of R proteins within the cell (Seo et al. 2008). Recent convincing evidence indicates that some NB-LRR R proteins likely shuttle from the cytoplasm to the nucleus. This finding was somewhat unexpected, as many NB-LRR R proteins are predicted to be cytosolic (Dangl and Jones, 2001). However, some pathogen effectors are thought to be targeted to the nucleus, so it is conceivable that R proteins might also be present in the nucleus to monitor their activities. The R proteins MILDEW A 10 (MLA10) in barley, N in tobacco, and RPS4 in Arabidopsis were shown to localize to both the cytoplasm and the nucleus, and that their nuclear localization and accumulation is necessary for downstream signaling and immunity to avirulent pathogens (Burch-Smith et al., 2007; Shen et al., 2007; Wirthmueller et al., 2007). In this regard, it is not surprising that certain mutations in components of the nucleo-cytoplasmic trafficking machinery have detrimental effects on defense responses (Wieter et al., 2007). In the nucleus, MLA10 interacts directly with a subset of WRKY TFs that repress MAMP-mediated gene expression, suggesting
that this R protein induces the expression of defense genes by sequestering negative regulators (Shen et al., 2007). Importantly, this finding also provides direct evidence that plants respond to MAMPs and effectors using some of the same resistance programs. The ability of R proteins to shuttle into the nucleus might afford plants an alternative and more direct route to modulate defense outputs when threatened by avirulent pathogens (Shen et al., 2007).

1.4.5 R protein-mediated signaling

R protein signaling channels through several pathways that later converge and activate a common suite of defense outputs (summarized in Figure 1.1B). NB-LRR proteins of the CC-type signal through the plasma membrane-associated protein NONSPECIFIC DISEASE RESISTANCE 1 (NDR1), whereas those of the TIR-type signal through the lipase-like protein ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and its interacting partners PHYTOALEXIN-DEFICIENT 4 (PAD4) and SENESCENCE ASSOCIATED GENE 101 (SAG101; Aarts et al., 1998; Feys et al., 2005). Importantly, there are two known R genes, RESISTANCE TO P. PARASITICA 7 (RPP7) and RPP8, that do not require NDR1 or EDS1 for downstream signaling, suggesting that additional transduction modules exist in defense signaling (McDowell et al., 2000). Aside from the fact that NDR1 works cooperatively with RIN4 to activate CC-NB-LRR R proteins such as RPM1 and RPS2 (Day et al., 2006), the molecular function of NDR1 and its specific downstream signaling components remain elusive. EDS1 interacts with PAD4 and SAG101 in distinct protein complexes in the cytosol and the nucleus (Feys et al., 2005), and is essential for the accumulation of SA and the transduction of signals derived from reactive oxygen species during infection (Wiermer et al., 2005). There are a number of additional negative regulators that suppress EDS1 induction, suggesting that EDS1-activated pathways are strictly controlled (Glazebrook, 2001). For example, ENHANCED DISEASE RESISTANCE 1 (EDR1) encodes a MAPKKK that functions upstream of EDS1 to suppress downstream signaling (Frye et al., 2001). Similarly,
MPK4, one of the MAPKs induced following FLS2 activation, negatively regulates EDS1-activated SA signaling (Petersen et al., 2000).

Whereas jasmonic acid (JA) and ethylene are integral to resistance against herbivores and necrotrophic pathogens in plants, SA has long been associated with resistance to biotrophic pathogens. JA and SA signaling networks generally antagonize one another, but there is some cross-talk between the two pathways. Infection by avirulent biotrophic pathogens leads to local accumulation of SA which is thought to mobilize a long-distance signal. In response to this mobile signal systemic cells accumulate SA and express defense genes, effectively guarding themselves against potential further attack by a broad range of virulent pathogens. This phenomenon is known as systemic acquired resistance (SAR; Durrant and Dong, 2004). SA production induced by infection is synthesized from chorismate by the enzyme isochorismate synthase (ICS1, also known as SA INDUCTION-DEFICIENT 2; SID2; Nawrath and Metraux, 1999; Wildermuth et al., 2001). The protein EDS5 (also known as SID1) is also required for SA accumulation, although the specific function of this protein has not yet been shown (Nawrath and Metraux, 1999; Nawrath et al., 2002). Mutants unable to synthesize or accumulate SA become more susceptible to pathogen infection (Nawrath and Metraux, 1999), highlighting the importance of this molecule in plant defense.

SA accumulation is associated with a buildup of reactive oxygen species that causes significant changes in cellular redox levels. These redox changes are sensed in the cytosol by the key defense protein NON-EXPRESSOR OF PR GENES 1 (NPR1; Dong, 2004). NPR1 is thought to exist in an inactive state as an oligomer that responds to redox alterations by monomerizing and relocating to the nucleus where it interacts with multiple basic leucine zipper TGA transcription factors to induce the expression of the defense gene PR1 (Mou et al., 2003). The transcription factors TGA2, TGA5, and TGA6 have overlapping functions and were shown to play both positive and negative roles in the regulation of PR1 gene expression and SAR (Zhang et al., 2003b). The molecular function of PR1 is unknown, but it is clear that local PR1 expression is associated with the onset of SAR. The activation of PR1 also requires that the suppressive effects of its negative regulator, SUPPRESSOR OF NPR1, INDUCIBLE 1 (SNI1), are lifted (Li et al., 1999). In addition to PR1 there are several other PR genes activated during defense.
These include chitinases, glucanases, proteinases, and RNases that degrade components of bacteria, fungi, and viruses to hinder their growth and thus their spread.

EDS1 activates additional pathways in an infected cell that are independent of SA accumulation and NPR1 expression. While *PR1* is down-regulated in *eds5* mutants, expression of another *PR* gene, the β-1,3-glucanase *PR2*, appears to be unaffected (Nawrath and Metraux, 1999). Also, *PR2* is constitutively expressed in *snc1* gain-of-function plants and is not suppressed by *npr1* and is only partially suppressed by *eds5* (Zhang et al., 2003a). Together, these data reveal that SA- and NPR1-independent defense signaling pathways are activated downstream of R proteins. A handful of components have been shown to function in these pathways, including a recently characterized multi-protein nuclear complex called the MOS4-ASSOCIATED COMPLEX (MAC; Palma et al., 2007). SA accumulation following pathogen infection is unaffected in MAC mutants, and epistasis analysis with *npr1* suggests that the MAC functions separately from NPR1 (Palma et al., 2007). Interestingly, the MAC seems to be required for resistance conditioned by both CC- and TIR-NB-LRR R proteins, representing a possible convergence point between the NDR1 and EDS1-activated pathways (Palma et al., 2007). Loss-of-function mutations in any of the MAC components lead to higher susceptibility to virulent pathogen infection (Palma et al., 2007), suggesting that the SA-independent pathway is necessary for both basal and R protein-mediated defenses.

### 1.5 Concluding remarks

The past decade has seen great advances in our understanding of the plant immune system. Plants, which are under constant threat of pathogen infection, rely on an intricate network of signaling components to effectively fend off colonization. The first level of defense is carried out at the plant cell surface where PRRs detect highly conserved MAMPs and activate low-level resistance responses. The detection of menacing effector proteins then activates R proteins that trigger more effective defense responses often
ending in an HR to inhibit the growth of biotrophic pathogens. The overall theme of an evolutionary arms race between plants and pathogens is presented in Figure 1.1A.

However, there are still many probing questions currently left unanswered: (1) What interplay occurs between MAMP- and R-mediated resistance? (2) How, mechanistically, and in which sub-cellular compartments, do R proteins recognize their cognate effectors? (3) How are multiple signaling pathways coordinated and what cross-talk exists between the distinct signaling pathways? Future work in these areas will truly enlighten our knowledge of plant immunity to microbial infection.

1.6 Manuscript acknowledgements

We thank Marcel Wiermer for helpful discussions and critical reading of the manuscript, and we are grateful to Yuti Cheng for research assistance.
(A) Plants have evolved the ability to perceive highly conserved microbe-associated molecular patterns (MAMPs) via transmembrane pattern recognition receptors (PRRs). PRR activation triggers mitogen-activated protein kinase (MAPK) signaling cascades that induce defense gene expression and hinder the growth of some microbial populations. During infection, pathogenic microbes deliver effector proteins into host cells where they function to suppress MAMP-triggered immunity and other defense responses. In resistant plants, cytoplasmic and membrane-associated resistance (R) proteins recognize effectors either directly or indirectly through the surveillance of guarded plant proteins and trigger effector-triggered immunity. Activated R proteins result in genetic reprogramming and pronounced physiological changes in the infected plant cell that ultimately result in resistance. (B) Genetic representation of some key signaling components activated during CC- and TIR-NB-LRR R protein-mediated resistance. Please see text for more details.
1.7 References


Belkhadir, Y., Nimchuk, Z., Hubert, D.A., Mackey, D., and Dangl, J.L. (2004). Arabidopsis RIN4 negatively regulates disease resistance mediated by RPS2 and RPM1 downstream or independent of the NDR1 signal modulator and is not required for the virulence functions of bacterial type III effectors AvrRpt2 or AvrRpm1. Plant Cell 16, 2822-2835.


stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY complex in plant innate immunity. Plant Cell 17, 2601-2613.


2 Dissecting plant defense signal transduction: modifiers of \textit{snc1} in \textit{Arabidopsis}\textsuperscript{1}

\textsuperscript{1} A version of this chapter has been published. Jacqueline Monaghan, Hugo Germain, Tabea Weihmann and Xin Li. (2010) \textit{Canadian Journal of Plant Pathology} 32 (1): 35-42.
2.1 Summary

Plants have evolved sophisticated defense mechanisms against pathogen infection, during which Resistance (R) proteins play a central role in recognizing pathogens and initiating downstream defense cascades. In *Arabidopsis*, the dominant mutant *snc1* was previously identified that constitutively expresses pathogenesis-related genes and displays enhanced resistance against both bacterial and oomycete pathogens. A point mutation in this gene renders the encoded TIR-NB-LRR type R protein constitutively active without pathogen interaction. Both suppressor screen and activation tagging approaches were used to identify regulators downstream of *snc1*. In a screen for suppressors of the constitutive defense responses in *snc1*, 15 complementation groups of modifier of *snc1* (*mos*) mutants have been identified. We have cloned eleven of the mutations (*mos1* to *mos11*) using map-based approaches, and have fully characterized *mos2-8*. Analysis of the corresponding *MOS* genes suggests a complicated signaling network downstream of R protein activation that involves nucleo-cytoplasmic trafficking, transcriptional reprogramming, RNA processing and protein modification.

2.2 Introduction

Plants are often exposed to pathogenic microorganisms. To protect themselves from infection, plants possess layered defense systems starting with preformed barriers including a waxy epidermis and rigid cell walls that are difficult to penetrate. Plant cells are also equipped with transmembrane receptors that recognize conserved features of microbes known as microbe associated molecular patterns (MAMPs) such as bacterial flagellin or fungal chitin (Zipfel and Felix, 2005). Successful pathogens have developed strategies to circumvent these broad host defenses. Bacterial, fungal and oomycete pathogens deliver effector molecules into host cells to interfere with or suppress immune responses, resulting in rapid colonization and plant disease (Jones and Dangl, 2006).
However, plants have evolved a specialized class of immune receptors encoded by *Resistance* (*R*) genes which can recognize specific pathogen effectors and trigger inducible plant defense responses. *R* proteins serve as surveillance proteins that sense and respond to the presence of pathogen effectors, either through direct interaction or indirectly through the recognition of effector activity (Van der Biezen and Jones, 1998; van der Hoorn and Kamoun, 2008).

The majority of cloned *R* genes encode proteins containing a nucleotide binding (NB) site and leucine rich repeats (LRRs). This group of predicted intracellular proteins can be categorized further according to an N-terminal coiled-coil (CC) or a Toll/Interleukin-1 receptor (TIR) domain (Jones and Dangl, 2006). Following perception of an effector by an NB-LRR *R* protein, the signal is transmitted through one of at least two pathways. CC-NB-LRR proteins tend to signal through NON RACE-SPECIFIC DISEASE RESISTANCE 1 (NDR1), whereas TIR-class NB-LRRs usually utilize ENHANCED DISEASE SUSEPTIBILITY 1 (EDS1), PHYTOALEXIN DEFICIENT 4 (PAD4) and SENESCENCE ASSOCIATED GENE 101 (SAG101; Aarts et al., 1998; Feys et al., 2005). *R* protein-mediated effector-triggered immunity is characterized by a rapid burst of reactive oxygen species, the induction of *PATHOGENESIS-RELATED* (*PR*) genes, and the accumulation of salicylic acid (SA). These responses generally end in a form of programmed cell death at the site of infection referred to as the hypersensitive response (HR; Jones and Dangl, 2006). In many cases, these local responses effectively prevent further invasion by biotrophic pathogens. In addition, SA accumulation and the HR trigger a systemic response conferring long-lasting broad range enhanced resistance known as systemic acquired resistance (SAR; Durrant and Dong, 2004). NON-EXPRESSOR OF PR GENES 1 (NPR1) is an essential positive regulator of SAR. The loss of *NPR1* renders plants extremely susceptible to pathogen infection, as systemic *PR* gene expression and SA-dependent disease resistance are abolished (Dong, 2004).

A genetic screen to identify suppressors of *npr1* resulted in the isolation of a unique gain-of-function mutant, *suppressor of npr1-1, constitutive 1 (snc1;* Li et al., 2001). *SNC1* encodes a TIR-NB-LRR-type *R* protein highly similar to RESISTANCE TO *PERONOSPORA PARASITICA* 4 (RPP4) and RPP5 (Zhang et al., 2003). Remarkably,
the base-pair mutation in \textit{snc1} that causes the conversion from glutamate (E) to lysine (K) in the linker region between the NB and LRR, produces an R protein that is constitutively active. Because \textit{snc1} plants display high endogenous levels of SA and constitutively express \textit{PR} genes, they also display enhanced resistance to pathogen infection. In addition, \textit{snc1} plants exhibit dwarfism and curly leaves, morphological features commonly found among mutants with high levels of SA, such as the \textit{constitutive expressor of PR genes (epr)} mutants (Bowling et al., 1994; Clarke et al., 1998).

Intriguingly, the morphological phenotypes of \textit{snc1} are dependent on temperature, humidity, and light conditions (Yang and Hua, 2004). Under lower temperatures (16°C-22°C), the ‘sncy’ phenotypes become more drastic, while at higher temperatures (22°C-28°C), \textit{snc1} plants grow to a nearly wild-type size (Li lab, unpublished data). Although constitutive R protein activation usually results in HR (Shirano et al., 2002), \textit{snc1} plants do not exhibit macroscopic cell death under normal growth conditions. \textit{snc1} plants are quite fertile and are capable of producing many seeds, a critical feature for conducting genetic analyses. Importantly, epistasis analysis between \textit{snc1} and loss-of-function mutants in defense regulators defined the presence of both SA- and NPR1-independent pathways involved in plant immune signaling. For example, \textit{ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5)}, which encodes a protein required for SA accumulation (Nawrath et al., 2002), is only partially required for \textit{snc1} signaling (Figure 2.1B).

### 2.2.1 The \textit{snc1} suppressor screen

The unique phenotypes of \textit{snc1} enabled us to hunt for regulatory components downstream of R protein activation using genetic means. Our lab employed multiple mutagenesis strategies that successfully identified a number of \textit{modifier of snc1 (mos)} mutants. The M2 generations of T-DNA, fast-neutron, or EMS mutagenized \textit{snc1} and \textit{snc1 npr1} populations were preliminarily examined for loss of \textit{snc1} morphology under normal growth conditions (i.e., 22°C, 16 hr day, <50% humidity). To weed out potential false positives from this primary screen, a secondary screen that examined loss of constitutive \textit{PR2} expression, facilitated by the presence of a \textit{PR2 promoter-GUS
construct in the sncl and sncl npr1 backgrounds, was also conducted (Li et al., 2001; Zhang et al., 2003). Because sncl morphological and defense phenotypes are very pronounced, suppressor mutants were relatively easily recovered. To thoroughly characterize the sncl mos mutants, several sncl phenotypes were assayed for suppression in addition to morphology and PR2 expression. These included endogenous SA levels, PRI expression, and resistance to the virulent pathogens Pseudomonas syringae pv. maculicola (P.s.m.) ES4326 and Hylaoperonospora arabidopsis (H.a.) strain Noco2. In total, fifteen independent complementation groups of mutants were identified from all efforts.

To facilitate mapping the mos mutations, sncl (originally in Col-0) was introgressed into Lansberg erecta (Ler) by repeated backcrossing. This Ler-snc1 line was crossed with each sncl mos mutant, that, after selfing, generated a segregating F2 population suitable for conducting linkage analysis between molecular markers and sncl-suppressing morphology (Zhang et al., 2007). Since a large number of DNA samples need to be processed for fine mapping, we utilized a quick-and-easy DNA extraction protocol (Zhang et al., 2007). DNA for genotyping was isolated by pressing leaf tissue onto FTA Classic Cards (Whatman) followed by incubation in extraction buffer and washing to remove residual buffer. In this way, 96 PCR reactions could be performed at once (representing 48 samples with 2 flanking markers), making it possible to map the mutations fairly quickly (Zhang et al., 2007). Once mapped to a small region (usually <50 kb), overlapping fragments spanning this area were PCR-amplified and sequenced. Once the mutations were identified, MOS genes were confirmed by transgenic complementation and, if available, alternate alleles were obtained from the Arabidopsis Biological Resource Center (ABRC). These additional alleles were used to verify the phenotypes observed and to test for allelism and sncl suppression. Single mos mutants were isolated by backcrossing with Col-0 and, together with the ABRC alleles, were further assayed for aberrations in basal and R protein-mediated defense signaling.

Many alleles of PAD4, which was previously shown to be fully required for sncl signaling (Zhang et al., 2003), were recovered. The other fourteen complementation groups contained mutations in loci previously unknown to encode components of resistance signaling. To date, eleven mos mutations have been cloned, seven of which
have been characterized in detail (Table 2.1). MOS6 encodes an α-importin involved in nuclear protein import. MOS3 and MOS7 encode proteins in the nuclear pore complex (NPC) that are involved in nuclear mRNA export and protein retention, respectively. MOS2 and MOS4 encode nuclear proteins with predicted functions in RNA processing, while MOS5 and MOS8 encode enzymes involved in protein modification. The identification and characterization of these MOS genes reveals the requirement of nucleocytoplasmic trafficking, transcriptional regulation/RNA processing, and protein modification in the signaling processes downstream of R protein activation (Figure 2.1).

2.3 Modifiers of snc1

2.3.1 Nucleo-cytoplasmic trafficking machinery: MOS6, MOS3, and MOS7

In eukaryotes, macromolecules constantly shuttle between the cytoplasmic and nuclear compartments. This process is essential to signal transduction cascades that culminate in gene activation, such as those associated with plant defense. A well-known example is NPR1, which senses pathogen-induced redox changes in the cytoplasm and responds by translocating to the nucleus where it interacts with TGA transcription factors to activate defense gene expression (Zhang et al., 1999; Zhou et al., 2000; Mou et al., 2003). EDS1 and PAD4 are also well known defense components that shuttle between the nucleus and the cytoplasm (Figure 2.1A). EDS1 and SAG101 form a protein complex in the nuclear compartment, whereas EDS1 and PAD4 form complexes both in the cytoplasm and the nucleus (Feys et al., 2005). In addition, recent reports have shown that several activated NB-LRR R proteins localize to the nucleus (Burch-Smith et al., 2007; Shen et al., 2007; Wirthmueller et al., 2007). The movement of proteins into and out of the nucleus depends on functional nuclear pores and mobile carriers that transport proteins across the nuclear envelope. Thus, the finding that three MOS genes encode components of nucleocytoplasmic trafficking machinery is of significant relevance.
Generally speaking, proteins larger than 40 kDa cannot cross the nuclear membrane without the aid of a group of mobile receptors known as importins and exportins (Terry et al., 2007). MOS6 encodes importin α-3 (Palma et al., 2005; Figure 2.1A), one of eight putative α-importins in Arabidopsis that shuttle nuclear-localization signal (NLS) containing proteins, together with β-importins, across the nuclear pore (Goldfarb et al., 2004). Mutant alleles of mos6 partially suppress enhanced resistance in snc1 and are more susceptible than wild-type to infection by the oomycete pathogen H.a. Noco2. However, MOS6 is almost identical to another importin in Arabidopsis, importin α-6 (IMPα6). It is therefore possible that loss of both MOS6 and IMPα6, if not lethal, would lead to enhanced susceptibility to other pathogens and may suppress snc1 phenotypes more completely (Palma et al., 2005). MOS6, and possibly also IMPα6, could be specifically required for the nuclear import of regulatory proteins such as the NLS-containing proteins NPR1, EDS1, PAD4, and/or SNC1, but experimental evidence still awaits.

Nucleoporins (Nups) are also employed downstream of snc1 and are required for innate immunity in plants (Figure 2.1A). Mutations in MOS3 or MOS7 suppress snc1-related constitutive resistance and result in impaired defense responses to virulent and avirulent pathogens (Zhang and Li, 2005; Cheng et al., 2009). Moreover, MOS7 is required for SAR (Cheng et al., 2009). MOS3 is homologous to human Nup96 and yeast C-Nup145p, components of the Nup107-160 subcomplex involved in nuclear pore stability and the export of mature mRNA transcripts (Wiermer et al., 2007). MOS7 shares homology with mammalian Nup88 and Drosophila MEMBERS ONLY (MBO), selectively required for nuclear protein retention (Roth et al., 2003). Matching their predicted functions, both MOS3 and MOS7 localize to the nuclear envelope (Zhang and Li, 2005; Cheng et al., 2009). Interestingly, these Nups also appear to be required for defense responses in animals, as a decrease in their expression or activity leads to immune deficiencies in mice and Drosophila (Uv et al., 2000; Enninga et al., 2002; Faria et al., 2006). In the case of Nup96, this is likely due to the nuclear retention of key defense transcripts that cannot subsequently be translated in the cytoplasm (Enninga et al., 2002; Faria et al., 2006). It is not clear whether specific transcripts are retained in mos3 mutant nuclei, but it has been demonstrated that the overall mRNA pool increases
in nuclei of plants defective for both MOS3 and AtNup160 (Parry et al., 2006). Conversely, partial loss of function of Drosophila mbo and mammalian nup88 mutant nuclei exhibit increased protein export and as such do not accumulate NFκB, a key transcriptional regulator necessary for defense against infection (Roth et al., 2003; Bernad et al., 2004; Xylourgidis et al., 2006). Similarly, although a null mutation in MOS7 causes lethality, the partial loss of function mos7-1 allele affects the retention of specific defense regulatory proteins, including snc1, NPR1, and EDS1 (Cheng et al., 2009). These data suggest that MOS7, Nup88, and MBO contribute to the nuclear retention of select proteins involved in innate immunity. Collectively, the characterization of MOS6, MOS3, and MOS7 reveals that movement of macromolecules across the nuclear envelope is an integral component of plant pathogen defense signaling.

2.3.2 RNA processing proteins: MOS2 and MOS4

Microarray studies have shown that many gene transcripts are up-regulated following pathogen perception (Navarro et al., 2004; Thilmony et al., 2006). Prior to being transported through the nuclear pore into the cytoplasm for translation, nascent pre-mRNA transcripts require processing. 5’capping, splicing, and 3’ poly-adenylation are major RNA processing events that are integral to gene expression. In addition, microRNAs have also been shown to regulate plant immunity (Jin, 2008). As such, functional RNA processing machinery must be a necessary component of the massive genetic reprogramming that occurs as the result of R protein activation.

MOS2 and MOS4 are nuclear proteins predicted to have roles in RNA processing (Zhang et al., 2005; Palma et al., 2007). Loss of MOS2 or MOS4 function results in enhanced susceptibility to infection by P.s.m. ES4326. In addition, MOS2 and MOS4 are required for signaling activated by certain TIR- and CC-NB-LRR R proteins, possibly representing a convergence point downstream of the initially streamlined pathways. Epistasis analyses between npr1 and mos2 or mos4 revealed that both MOS2 and MOS4 likely function independently of SA (Figure 2.1B). MOS2 encodes a protein that contains one G-patch and two KOW repeats (Zhang et al., 2005), conserved domains found in
several RNA-binding proteins. For example, the 45 kDa splicing factor (SPF45) contains a G-patch domain (Aravind and Koonin, 1999), and the transcriptional regulator NusG contains a KOW motif that binds both RNA and protein (Steiner et al., 2002). The relationship, if any, between MOS2 and MOS4 remains to be elucidated.

*MOS4* encodes a nuclear protein with sequence homology to human Breast Carcinoma Sequence 2 (BCAS2) that directly binds the atypical R2R3-MYB transcription factor CELL DIVISION CYCLE 5 (AtCDC5) in a yeast two-hybrid system and *in planta* (Palma et al., 2007). Further analysis revealed that MOS4 and AtCDC5 form a complex with the WD-40 protein PLEIOTROPIC REGULATORY LOCUS 1 (PRL1) and the E3-Ubiquitin Ligases MAC3A and MAC3B (Monaghan et al., 2009) called the MOS4-Associated Complex (MAC; Figure 2.1A). *MOS4*, *AtCDC5*, *PRL1*, and *MAC3A/3B* are all essential components of plant immunity, as knock-out mutations in any of these genes render plants more susceptible than wild-type to virulent and avirulent pathogens (Palma et al., 2007; Monaghan et al., 2009). Importantly, homologs of MOS4, AtCDC5 and PRL1 have been isolated several times as components of a spliceosome-associated protein complex in yeast and human known as the Nineteen Complex (NTC), named after the E3 ubiquitin ligase Precursor RNA Processing 19 (Prp19; Ajuh et al., 2000; Ohi et al., 2002). In addition to AtCDC5, PRL1, and MAC3A/3B, MOS4 associates with more than twenty other proteins *in planta* (Monaghan et al., 2009), many of which have predicted roles in RNA processing and/or splicing. We hypothesize that the MAC acts as a transcriptional modulator that functions closely with the spliceosome to regulate certain, as yet unidentified, defense-related genes.

### 2.3.3 Protein modifying enzymes: MOS5 and MOS8

Once properly processed, mRNA transcripts are transported to the cytoplasm to be translated into proteins. Post-translational modifications such as the addition of sugar or phosphate molecules can influence protein activation, degradation, and localization. As in probably every signal transduction pathway, protein modification also plays a key role
in plant defense signaling. For example, several lines of evidence suggest that R proteins are activated as the consequence of pathogen-induced modifications in host proteins. The prototypical example of an effector-modified protein in plants is RPM1-INTERACTING PROTEIN 4 (RIN4), whose modification by phosphorylation or cleavage is monitored by two CC-NB-LRR R proteins in *Arabidopsis* that, once activated, trigger plant resistance (Mackey et al., 2002; Axtell and Staskawicz, 2003; Kim et al., 2005).

The identification of *MOS5* and *MOS8* further highlights the role of protein modification in plant defense. *MOS5* encodes one of two ubiquitin-activating (UBA; also known as E1) enzymes in *Arabidopsis* (Goritschnig et al., 2007). UBA/E1 enzymes activate ubiquitin moieties for their subsequent conjugation (by E2 enzymes) and ligation (by E3 enzymes) to target proteins that are destined, in most cases, for degradation by the proteasome (Figure 2.1A). In *mos5*, the loss of *MOS5/UBA1* function leads to minor defects in basal defense against virulent *P.s.m. ES4326* and causes impaired signaling mediated by at least one CC-NB-LRR R protein. Furthermore, the *mos5* mutation partially suppresses *snc1* phenotypes. Interestingly, the other UBA/E1 enzyme, UBA2, is not involved in plant immunity responses and a loss-of-function *uba2* allele is unable to suppress *snc1*, suggesting that MOS5 may be preferentially recruited in defense signaling. However, a double *mos5 uba2* mutant is lethal, indicating that there is some level of redundancy between *MOS5* and *UBA2* (Goritschnig et al., 2007). In addition to UBA1/MOS5, several E3 ubiquitin ligase enzymes are selectively required for disease resistance (Craig et al., 2009), including two recently characterized and redundant MAC proteins, MAC3A and MAC3B (Monaghan et al., 2009). These and other data support an integral role for the ubiquitin pathway in plant defense.

Aside from ubiquitination, other protein modifications such as phosphorylation, glycosylation, and lipidation are often required in signal transduction cascades. The most common type of protein lipidation is prenylation, in which hydrophobic farnesyl- or geranylgeranyl moieties are covalently bound to target proteins, likely to facilitate their tethering to cellular membranes (Galichet and Gruissem, 2003). *MOS8* encodes the β-subunit of farnesyltransferase (Figure 2.1A), previously identified as *ENHANCED RESISTANCE TO ABA 1 (ERA1)* (Goritschnig et al., 2008). *ERA1* is involved in abisic acid (ABA) signaling (Cutler et al., 1996), and loss of *ERA1* function leads to guard cell
alterations that cause era1 mutants to display enhanced tolerance to drought stress (Pei et al., 1998). Importantly, in addition to this drought-resistant phenotype, several era1 alleles, including mos8, exhibit enhanced susceptibility to P.s.m. ES4326 and H.a. Noco2, and show slight aberrations in R protein signaling (Goritschnig et al., 2008). To investigate the significance of ABA in era1 defense signaling, epistasis analysis between era1 and two ABA biosynthesis mutants showed that ABA is only partly responsible for enhanced pathogen susceptibility observed in era1 mutants. Further genetic analysis with npr1 revealed additive contributions of era1 and npr1 in defense signaling, indicating that ERA1 and NPR1 function independently (Figure 2.1B). In constrast to farnesyltransferase, geranylgeranyltransferase 1, an enzyme used in an alternative prenylation pathway (Galichet and Gruissem, 2003), was not found to have altered defense responses like those of era1 (Goritschnig et al., 2008). This suggests specificity for protein farnesylation in plant immunity, although it remains to be determined whether other prenylation enzymes are involved in immunity responses. It is currently unknown what defense-related target proteins are modified by ERA1/MOS8.

### 2.4 Outlook

During the past eight years, our lab has focused on dissecting plant defense signal transduction by studying mutants that suppress snc1 autoimmune phenotypes. This short review summarizes the first seven MOS genes that have been characterized in detail. These MOSes provide insights on the involvement of nucleo-cytoplasmic trafficking machinery, RNA processing, and protein modification as key components of the defense signaling network. Future mechanistic investigations will uncover how these and other regulatory proteins work together to achieve plant immunity.
2.5 Thesis objectives

The primary aim of this thesis research was to uncover novel signaling components of the plant immune system. Our lab previously showed that a loss-of-function mutation in \textit{MOS4} suppresses the autoimmunity phenotypes of \textit{snc1}, and that MOS4 is part of a nuclear complex with the transcription factor AtCDC5 and the WD-40 protein PRL1 called the MAC. Homologs of MOS4, AtCDC5, and PRL1 are part of a large spliceosome-associated complex in yeast and human known as the NTC. We hypothesized that the MAC is homologous to the NTC and that additional MAC proteins, if they existed, may function in plant immunity. We initiated a large-scale reverse-genetics screen using sequence-indexed T-DNA insertion mutants from the ABRC representing lesions in NTC homologs (i.e., potential MAC components) in \textit{Arabidopsis}. We screened these mutants for enhanced disease susceptibility to \textit{Pseudomonas syringae} and other phenotypes common to \textit{mos4}, \textit{Atcdc5} and \textit{prl1} mutants. At the same time, our collaborators at NIBS in Beijing successfully affinity purified the MAC using HA-tagged MOS4 expressed in \textit{Arabidopsis}. This thesis describes several loci identified by these efforts that function in plant immunity.
Table 2.1  Fully characterized *modifier of snc1* mutants identified in the *snc1* and *snc1 npr1* suppressor screens.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>At number</th>
<th>Shares homology with</th>
<th>Suppression of <em>snc1</em> resistance</th>
<th>Type of mutation</th>
<th>Localization</th>
<th>Special features</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOS2</td>
<td>At1g33520</td>
<td>RNA-binding protein</td>
<td>Partial</td>
<td>2 bp insertion in <em>mos2-1</em></td>
<td>Nuclear</td>
<td>Has an NLS, G-patch, and KOW motifs.</td>
</tr>
<tr>
<td>MOS3</td>
<td>At1g80680</td>
<td>Nucleoporin 96</td>
<td>Complete</td>
<td>52 bp deletion in <em>mos3-1</em></td>
<td>Nuclear envelope</td>
<td>Part of the Nup 107-160 subcomplex required for mRNA export.</td>
</tr>
<tr>
<td>MOS4</td>
<td>At3g18165</td>
<td>Human BCAS2</td>
<td>Complete</td>
<td>2.2 kb insertion in <em>mos4-1</em></td>
<td>Nuclear</td>
<td>Part of the MAC multi-protein complex with AtCDC5, PRL1, and others.</td>
</tr>
<tr>
<td>MOS5</td>
<td>At2g30110</td>
<td>E1 ubiquitin activating enzyme UBA1</td>
<td>Complete</td>
<td>15 bp deletion in <em>mos5-1</em></td>
<td>Currently unknown</td>
<td>Double mutant with uba2, the other <em>Arabidopsis</em> E1 enzyme, is lethal.</td>
</tr>
<tr>
<td>MOS6</td>
<td>At4g02150</td>
<td>Importin α-3</td>
<td>Partial</td>
<td>5.3 kb chromosomal rearrangement in <em>mos6-1</em></td>
<td>Mostly nuclear</td>
<td>Has 7 homologs in <em>Arabidopsis</em>. Part of NLS protein nuclear import machinery.</td>
</tr>
<tr>
<td>MOS7</td>
<td>At5g05680</td>
<td>Nucleoporin 88</td>
<td>Complete</td>
<td>12 bp deletion in <em>mos7-1</em></td>
<td>Nuclear envelope</td>
<td>Required for nuclear protein retention/export. A null <em>mos7</em> allele is lethal.</td>
</tr>
<tr>
<td>MOS8</td>
<td>At5g40280</td>
<td>β-subunit of farnesyltransferase</td>
<td>Partial</td>
<td>G to C substitution in <em>mos8-1</em></td>
<td>Currently unknown</td>
<td>Is a loss-of-function allele of <em>ERA1</em>.</td>
</tr>
<tr>
<td>PAD4</td>
<td>At3g52430</td>
<td>Triacylglycerol lipase</td>
<td>Full</td>
<td>Many alleles</td>
<td>Cytosolic/ nuclear</td>
<td>Interacts with EDS1. Required for TIR-NB-LRR R protein signaling.</td>
</tr>
</tbody>
</table>
Figure 2.1: Modifiers of snc1 in Arabidopsis.

(A) Subcellular localization of snc1 signaling components. Nucleo-cytoplasmic transport machinery and the NPC are detailed in the inset. NLS-containing cargo proteins bind importin-α, which binds importin-β. This protein complex moves through the nuclear pore and dissociates when it reaches the nucleus. Nuclear export signal (NES) containing proteins can exit the nucleus by binding exportins. Exportin- or importin-containing cargo proteins travel through the nuclear pore. MOS3 and MOS7 are both integral components of the NPC. In the cytosol, MOS5/UBA1, a functional E1, promotes the ubiquitination of currently unknown target proteins. MOS8/ERA1 is also involved in protein modification; it is represented here farnesylating an unknown target protein. PAD4 was found multiple times from the snc1 modifier screen. It can bind EDS1 in the cytosol or the nucleus, whereas EDS1 can bind SAG101 only in the nucleus. In the nucleus, the MAC is comprised of MOS4, AtCDC5, PRL1 and several other proteins including the E3 ubiquitin ligases MAC3A/B. MOS2 is represented here physically bound to an unknown RNA molecule in the nucleus. Several NB-LRR proteins, including snc1, are observed in the cytosol and nucleus. (B) Defense pathways activated in snc1. EDS1 and PAD4 function directly downstream of snc1. In the SA-dependent pathway, EDS5 and isochoismate synthase, the SA synthesis enzyme encoded by SA INDUCTION-DEFICIENT 2 (SID2) are required for SA accumulation upstream of NPR1. MOS2, MOS4, MOS5, and MOS8 likely act in the SA-independent pathway.
2.6 References


Nup96 is required for proper expression of interferon-regulated proteins and functions. Immunity 24, 295-304.


Two Prp19-like U-box proteins in the MOS4-associated complex play redundant roles in plant innate immunity

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1 A version of this chapter has been published. Jacqueline Monaghan, Fang Xu, Minghui Gao, Qingguo Zhao, Kristoffer Palma, Chengzu Long, She Chen, Yuelin Zhang and Xin Li. (2009) *PLoS Pathogens* 5 (7): e1000526.
3.1 Summary

Plant Resistance (R) proteins play an integral role in defense against pathogen infection. A unique gain-of-function mutation in the R gene SNC1, snc1, results in constitutive activation of plant immune pathways and enhanced resistance against pathogen infection. We previously found that mutations in MOS4 suppress the autoimmune phenotypes of snc1, and that MOS4 is part of a nuclear complex called the MOS4-Associated Complex (MAC) along with the transcription factor AtCDC5 and the WD-40 protein PRL1. Here we report the immuno-affinity purification of the MAC using HA-tagged MOS4 followed by protein sequence analysis by mass spectrometry. A total of twenty-four MAC proteins were identified, nineteen of which have predicted roles in RNA processing based on their homology to proteins in the Prp19 Complex, an evolutionarily conserved spliceosome-associated complex containing homologs of MOS4, AtCDC5 and PRL1. Among these were two highly similar U-box proteins with homology to the yeast and human E3 ubiquitin ligase Prp19, which we named MAC3A and MAC3B. MAC3B was recently shown to exhibit E3 ligase activity in vitro. Through reverse genetics analysis we show that MAC3A and MAC3B are functionally redundant and are required for basal and R protein-mediated resistance in Arabidopsis. Like mos4-1 and Atcdc5-1, mac3a mac3b suppresses snc1-mediated autoimmunity. MAC3 localizes to the nucleus and interacts with AtCDC5 in planta. Our results suggest that MAC3A and MAC3B are members of the MAC that function redundantly in the regulation of plant innate immunity.

3.2 Introduction

Plants possess multi-layered defense systems against microbial pathogens. The first layer is governed by a collection of pattern recognition receptors (PRRs) that detect highly conserved features of whole groups of microbes known as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs; Zipfel, 2008). The second layer is
mediated by Resistance (R) proteins, which recognize specific pathogen effectors deployed during an infection. Recognition of effectors or their functions leads to resistance against that pathogen. The majority of cloned R genes encode intracellular NB-LRR proteins that contain a central nucleotide-binding site (NB), C-terminal leucine-rich repeats (LRR), and either a Toll/Interleukin-1-receptor-like (TIR) or a coiled-coil (CC) domain at the N-terminus (Jones and Dangl, 2006). Signaling through TIR- and CC-NB-LRR proteins is generally streamlined into two pathways (Aarts et al., 1998); TIR-NB-LRRs signal through ENHANCED DISEASE RESISTANCE 1 (EDS1), PYTOALEXIN DEFICIENT 4 (PAD4) and SENESCENCE ASSOCIATED GENE 101 (SAG101; Feys et al., 2005), whereas CC-NB-LRRs signal through NON-RACE SPECIFIC DISEASE RESISTANCE 1 (NDR1; Aarts et al., 1998). These pathways later converge and lead to common defense outputs in the infected cells to restrict pathogen growth, including defense gene expression, accumulation of the defense hormone salicylic acid (SA), cell wall strengthening and ion leakage, which in many cases culminate in a form of programmed cell death known as the hypersensitive response (HR) (Jones and Dangl, 2006). SA-dependent defense responses are mediated by the protein NON-EXPRESSOR OF PATHOGENESIS RELATED GENES 1 (NPR1; Cao et al., 1997). Mutations in NPR1 abolish SA-dependent resistance and lead to enhanced susceptibility to pathogen infection (Dong, 2004).

The unique gain-of-function mutant suppressor of npr1-1, constitutive 1 (snc1) suppresses npr1-related susceptibility to pathogens in Arabidopsis (Li et al., 2001). Both the double snc1 npr1 mutant and the single snc1 mutant constitutively express PATHOGENESIS-RELATED (PR) genes and accumulate high endogenous levels of SA, leading to enhanced pathogen resistance. SNC1 encodes a TIR-NB-LRR R protein homologous to RESISTANCE TO PERONOSPORA PARASITICA 4 (RPP4; Zhang et al., 2003). The snc1 point mutation causes a glutamate to lysine substitution in the linker region between the NB and LRR domains, leading to constitutive SNC1 activation and the constant stimulation of resistance responses even in the absence of pathogens (Li et al., 2001; Zhang et al., 2003). A suppressor screen to search for novel downstream components of the snc1-mediated signaling network led to the identification and characterization of several MODIFIER OF snc1 (MOS) genes as key players in plant
immunity (Palma et al., 2005; Zhang et al., 2005b; Zhang and Li, 2005; Goritschnig et al., 2007; Palma et al., 2007; Goritschnig et al., 2008). Like other mos mutants described to date, mos4 alleles suppress all phenotypes associated with snc1, including small stature, enhanced resistance to virulent pathogens, constitutive expression of PR genes and heightened endogenous levels of SA (Palma et al., 2007).

MOS4 is the founding member of the MOS4-Associated Complex (MAC), a nuclear protein complex containing the Myb-transcription factor CELL DIVISION CYCLE 5 (AtCDC5/MAC1) and the WD-40 repeat protein PLEIOTROPIC REGULATORY LOCUS 1 (PRL1/MAC2; Palma et al., 2007). MOS4, AtCDC5 and PRL1 are essential components of plant disease resistance signaling, as knockout mutations in any of these genes render plants more susceptible than wild-type to virulent and avirulent pathogens (Palma et al., 2007). AtCDC5 interacts with MOS4 and PRL1 in planta (Palma et al., 2007). Direct interaction between yeast and human homologs of these proteins has also been shown (Ohi and Gould, 2002; Ajuh and Lamond, 2003), indicating that the interactions are conserved across kingdoms. Importantly, homologs of AtCDC5, MOS4, and PRL1 have been isolated several times as components of a protein complex in yeast and human known as the Nineteen Complex (NTC; Tarn et al., 1993a; Tarn et al., 1994; Ajuh et al., 2000; Chen et al., 2002; Ohi et al., 2002; Zhou et al., 2002; Deckert et al., 2006; Gavin et al., 2006; Bessonov et al., 2008). This complex, named after the E3 ubiquitin ligase Precursor RNA Processing 19 (Prp19; Tarn et al., 1993a; Tarn et al., 1994), may facilitate spliceosome assembly (Chan et al., 2003), in addition to having roles in DNA repair (Grey et al., 1996; Mahajan and Mitchell, 2003; Beck et al., 2008) and cell-cycle progression (Ohi et al., 1994; Bernstein and Coughlin, 1997).

Proteomic analyses in yeast and human consistently identify Prp19, CDC5, Spf27/hMOS4 and PRL1 together, suggesting that these proteins form the core of the NTC. Several other proteins, including small nuclear ribonucleoproteins (snRNPs) and RNA-binding proteins, also associate with this core (Ajuh et al., 2000; Ohi et al., 2002; Deckert et al., 2006; Bessonov et al., 2008). Based on this, we hypothesized that the Arabidopsis MAC must contain more components. Here we report the immuno-affinity purification of the MAC using mos4-1 complementing transgenic lines expressing HA-tagged MOS4, followed by identification of its components through mass spectrometry
(MS). Two of the identified proteins are 82% identical to each other at the amino acid level and share sequence homology with Prp19, which we named MAC3A and MAC3B. Like Prp19, these proteins contain a highly conserved U-box domain (Azevedo et al., 2001), and MAC3B was recently shown to exhibit E3 ubiquitin ligase activity in vitro (Wiborg et al., 2008). Immunoprecipitation (IP) of MAC3A followed by western blot analysis using an anti-AtCDC5 antibody confirmed that MAC3 is indeed part of the MAC. Reverse genetics analysis revealed that while loss-of-function mac3a and mac3b single mutants do not display any aberrant phenotypes, double mutant mac3a mac3b plants are compromised in basal and R-mediated signaling, and are able to suppress the autoimmune phenotypes associated with snc1 to the same level as mos4-1. This suggests that MAC3A and MAC3B function redundantly in basal and R-mediated defense. Our findings reveal the conserved nature of the MAC and the redundant roles of MAC3A and MAC3B in the regulation of immune responses in plants.

3.3 Results

3.3.1 Identification of MAC proteins

To affinity purify the MAC, full-length MOS4 containing a C-terminal triple hemagglutinin (HA) epitope tag was expressed in mos4-1 under the control of its native promoter. MOS4-HA transformed into mos4-1 fully complements all mos4-1 associated phenotypes, including morphology (Figure 3.1A) and enhanced susceptibility to the virulent pathogen Pseudomonas syringae p.v. maculicula (P.s.m.) strain ES4326 (Figure 3.1B). This indicates that the MOS4-HA fusion protein functions the same as wild-type MOS4 and thus was used in affinity purification using anti-HA microbeads. As shown in Figure 3.1C, multiple protein bands were found to specifically associate with MOS4-HA isolated from nuclear extracts. Any bands in the MOS4-HA lane that were also present in the Col-0 lane were not excised to avoid false positives. MS sequencing of the excised bands identified a total of twenty-four proteins (Table 3.1, Table 3.2), including the
previously known MAC components MOS4, AtCDC5 and PRL1. Nineteen of the isolated proteins share homology to human and yeast proteins that have been characterized as NTC or NTC-associated proteins (Table 3.1) (Wang and Brendel, 2004), indicating that this protein complex is highly conserved throughout eukaryotic kingdoms.

Most of these proteins have not been studied in Arabidopsis but are predicted to be involved in pre-mRNA splicing or RNA processing based on their relatedness to NTC proteins. Among others, four predicted subunits of the U5 snRNP, one subunit of the U2 snRNP, and several RNA binding proteins and helicases were identified. In addition, five unrelated proteins were also revealed as potential MAC components (Table 3.1). These could be novel MAC proteins or they could represent experimental contaminants. Importantly, we identified two proteins, encoded by At1g04510 and At2g33340, with homology (23-24% identity, 41% similarity) to the E3 ubiquitin ligase Prp19, the founding member of the NTC. These proteins, which we named MAC3A and MAC3B, are 82% identical to each other at the amino acid level. An alignment of the amino acid sequences of Arabidopsis MAC3A and MAC3B with homologous sequences in several eukaryotes reveals a highly conserved U-box domain at the N-terminus (Figure 3.2). U-box domains have been shown to exhibit E3 ubiquitin ligase activity in several eukaryotic proteins. In a recent survey of plant U-box proteins, MAC3B was shown to exhibit E3 ubiquitin ligase activity in vitro (Wiborg et al., 2008), an enzymatic activity that has also been demonstrated in yeast and human Prp19 proteins (Hatakeyama et al., 2001; Ohi et al., 2003). In addition, these proteins contain a number of C-terminal WD-40 repeats predicted to engage in protein-protein interactions (Neer et al., 1994).

### 3.3.2 Isolation of mac3a and mac3b loss-of-function mutants

Since the MAC3 homolog in yeast and human is an integral member of the NTC and MAC3A and MAC3B were identified as potential MAC components in this study (Table 3.1), we were interested in testing if the biological function of MAC3 is related to MOS4 through the analysis of knockout mutants. T-DNA insertion mutants were obtained from the Arabidopsis Biological Resource Center (ABRC; Alonso et al., 2003). Salk_089300
(mac3a) carries an insertion in the intron between exons 10 and 11 of MAC3A, and Salk_050811 (mac3b) carries an insertion in the intron between exons 16 and 17 of MAC3B (Figure 3.3A). Homozygous lines were identified by PCR-based genotyping. To determine whether the T-DNA insertions affect MAC3A and MAC3B expression, semi-quantitative RT-PCR was performed using cDNA-specific primers flanking the insertions. As shown in Figure 3.3B, mac3a and mac3b both exhibit significantly reduced mRNA expression. Given that MAC3A and MAC3B are 82% identical and single mac3a and mac3b mutants showed no aberrant morphological phenotypes (Figure 3.4A), we hypothesized that MAC3A and MAC3B may function redundantly. When we crossed mac3a and mac3b to create a double mac3a mac3b mutant, plants with a distinct morphology similar to mos4-1 were observed. These plants are slightly smaller than Col-0 wild type (Figure 3.4A), have darker leaves, and they flower late (data not shown). Genotyping of the F2 progeny confirmed that only these plants were homozygous for both mac3a and mac3b alleles, indicating that the observed mutant phenotypes co-segregate with both T-DNA insertions and that mutations in both genes are the likely cause of the observed phenotypes.

### 3.3.3 MAC3A and MAC3B function redundantly in basal defense

Since mutations in any of the three previously characterized MAC genes MOS4, AtCDC5 and PRL1 result in enhanced disease susceptibility, and since the mac3a mac3b mutant phenocopies mos4-1 morphologically, we tested if MAC3 is likewise required for plant immunity against virulent pathogens. To test whether MAC3 is required for basal defense responses, mac3a and mac3b single mutants and mac3a mac3b double mutants were infected with a low dose (OD₆₀₀=0.0001) of the bacterial pathogens *P. syringae* p.v. *tomato* (P.s.t.) DC3000 and P.s.m. ES4236 and pathogen growth was assayed after three days. Resistance to P.s.t. DC3000 and P.s.m. ES4326 was similar in Col-0, mac3a and mac3b single mutants but was compromised in mac3a mac3b double mutant plants, which harboured an over 10-fold higher titer of bacteria compared to Col-0 in both cases (Figure 3.3C-D). This is similar to bacterial growth in mos4-1 and Atcdc5-2 (Figure...
Importantly, transgenic expression of either *MAC3A* or *MAC3B* cDNA driven by the constitutive 35S promoter complements *mac3a mac3b* morphology (Figure 3.5A) and susceptibility to *P. s.m. ES4326* (Figure 3.3D). Thus, the enhanced susceptibility phenotype observed in *mac3a mac3b* double mutants is due to the mutations in *MAC3A* and *MAC3B*. Together, these data suggest that *MAC3A* and *MAC3B* play redundant roles in basal defense against virulent pathogens.

### 3.3.4 *mac3a mac3b* displays defects in R protein-mediated defense pathways

To test whether different R protein-mediated signaling pathways rely on *MAC3*, we challenged *mac3a*, *mac3b*, and *mac3a mac3b* mutants with pathogens that express specific effectors recognized by distinct R proteins in *Arabidopsis*. Resistance against *P. s.t. DC3000* expressing either *avrRps4* (recognized by the TIR-NB-LRR R protein RESISTANT TO P. SYRINGAE 4; RPS4) or *avrPphB* (recognized by the CC-NB-LRR R protein RPS5) is compromised in *mac3a mac3b* double mutants but is unaffected in *mac3a* or *mac3b* single mutants, as indicated by an approximate 10-fold increase in bacterial growth in *mac3a mac3b* plants three days after infection compared to Col-0 (Figure 3.6A-B). Conversely, resistance against *avrRpm1* (conditioned by the CC-NB-LRR R protein RESISTANCE TO P.S.M. 1; RPM1) is not impaired in *mac3a mac3b* double mutants, as bacterial growth is comparable to Col-0 (Figure 3.6C). Resistance against *Hyaloperonospora arabidopsidis* (*H. a.*, formerly *H. parasitica*) isolate Cala2 (recognized by the TIR-NB-LRR R protein RESISTANT TO PERONOSPORA PARASITICA 2; RPP2) is also unaffected in *mac3a*, *mac3b*, or *mac3a mac3b*, as indicated by the level of conidiospores collected one week after infection, which was similar to Col-0 (Figure 3.6D). Together, these data further support redundancy between *MAC3A* and *MAC3B*, and suggest that these proteins are required for signaling pathways mediated by specific TIR- and CC-NB-LRR R proteins.
3.3.5 *mac3a mac3b* suppresses autoimmune phenotypes associated with *snc1*

Mutations in *MOS4* or *AtCDC5* suppress *snc1*, indicating that these MAC proteins contribute to *snc1*-mediated resistance (Palma et al., 2007). To test if *MAC3* is also part of the *snc1* pathway, we crossed *snc1* with *mac3a mac3b* to obtain a *snc1 mac3a mac3b* triple mutant. Whereas *snc1* plants are of small stature and have dark, curly leaves, the triple mutant does not exhibit *snc1*-like morphology and resembles the *mac3a mac3b* double mutant (Figure 3.7A). The *snc1* mutant exhibits enhanced resistance to the virulent pathogens *P.s.m.* ES4326 and *H.a.* isolate Noco2 (Li et al., 2001). To test if *snc1*-mediated enhanced resistance is impaired in *snc1 mac3a mac3b* plants, we infected plants with these two pathogens. As shown in Figure 3.7B, *snc1 mac3a mac3b* triple mutants sustain an approximate 100-fold higher titer of *P.s.m.* ES4326 compared to *snc1* three days after infection, to a level similar to *mac3a mac3b* (Figure 3.7B).

*Agrobacterium*-mediated transformation of *P35S-MAC3A* or *P35S-MAC3B* into *snc1 mac3a mac3b* restored *snc1* morphology (Figure 3.5B) and resistance to *P.s.m.* ES4326 (Figure 3.5C). Furthermore, *snc1 mac3a mac3b* mutants are as susceptible to infection by *H.a.* isolate Noco2 as Col-0 plants (Figure 3.7C), indicating that *mac3a mac3b* completely suppresses *snc1*-related enhanced resistance to these pathogens. In addition to increased resistance, *snc1* plants also accumulate high levels of the defense signaling molecule SA. High performance liquid chromatography (HPLC) analysis of SA extracts collected from Col-0, *snc1*, and *snc1 mac3a mac3b* plants revealed a marked reduction in endogenous free and total SA levels in the triple mutant comparable to the levels in Col-0 (Figure 3.7D). Semi-quantitative RT-PCR analysis indicated that *mac3a mac3b* also suppresses the expression of *PR-1* and *PR-2*, which are constitutively up-regulated in *snc1* plants (Figure 3.7E). Together, these data demonstrate that, similar to *mos4-1*, *mac3a mac3b* completely suppresses *snc1*-mediated autoimmune phenotypes.
3.3.6 MAC3 localizes to the nucleus

Several lines of evidence already suggest that MAC3 localizes to the nuclear compartment. First, both MAC3A and MAC3B proteins were isolated by affinity purification from nuclear protein extracts as described above (Table 3.1). In addition, MAC3A and MAC3B have nuclear localization signals in their protein sequence, as predicted by PSORT (version 6.4; Figure 3.2). To confirm that MAC3 is nuclear, we created $P35S\text{-}MAC3A\text{-}CFP$ and $P35S\text{-}CFP\text{-}MAC3B$ fusion constructs and transiently transformed onion cells by particle bombardment. In both cases, transformed cells showed exclusive nuclear localization (Appendix 1), as observed by a fluorescence microscope. To corroborate this data, we created stable $P35S\text{-}MAC3A\text{-}CFP$ transgenic lines in the $mac3a \ mac3b$ background using Agrobacterium-mediated transformation. The majority (10/11) of transgenic plants complemented $mac3a \ mac3b$ morphological phenotypes, and all complementing lines showed exclusive nuclear localization of $P35S\text{-}MAC3A\text{-}CFP$ under a confocal microscope. Importantly, resistance to $P.s.m.\ ES4326$ was restored in $mac3a \ mac3b$ $P35S\text{-}MAC3A\text{-}CFP$ plants (Figure 3.8A), indicating proper localization of over-expressed $MAC3A\text{-}CFP$. Guard cells from one of these lines are shown in Figure 3.8B, however nuclear localization was also observed in other tissues including roots (data not shown). Likewise, expression of $P35S\text{-}CFP\text{-}MAC3B$ in complementing $mac3a \ mac3b$ lines (data not shown) also showed distinct nuclear localization (Figure 3.9). Expression of $P35S\text{-}MAC3A\text{-}CFP$ or $P35S\text{-}CFP\text{-}MAC3B$ in $snc1 \ mac3a \ mac3b$ also displayed nuclear localization (data not shown), indicating that MAC3A and MAC3B localization is not altered in $snc1$. Together, these data suggest that MAC3 localizes to the nucleus.

While cloning $MAC3B$, we noticed a discrepancy at the 3’ end of the $MAC3B$ cDNA sequence. Sequence analysis of both strands of two individually cloned full-length $MAC3B$ cDNA constructs confirmed the presence of two cytosines at a position previously shown to contain only one cytosine (Figure 3.10). This extra cytosine causes a shift in frame and results in an earlier stop codon than the one predicted by The Arabidopsis Information Resource (TAIR8), making the protein 525 amino acids as opposed to the predicted 563 amino acids in length. The corrected cDNA sequence
translates into a polypeptide that better aligns at the C-terminal end with MAC3A and Prp19 (Figure 3.2). This corrected MAC3B cDNA sequence has been deposited to GenBank under accession number FJ820118.

3.3.7 MAC3 associates with AtCDC5 in planta

Prp19 interacts with CDC5 in yeast and human cells (Tsai et al., 1999; Ohi and Gould, 2002; Grillari et al., 2005). Since the MAC seems to be conserved across eukaryotes, we hypothesized that this interaction might also be conserved in Arabidopsis. To confirm that MAC3 associates with the MAC, and to corroborate our MS data, we tested whether MAC3 interacts with AtCDC5. To do this, P35S-MAC3A-HA tagged fusion constructs were stably transformed into mac3a mac3b by Agrobacterium-mediated transformation. This fusion protein fully complements mac3a mac3b mutant phenotypes and susceptibility to P.s.m. ES4326 (Figure 3.8A) suggesting that it functions the same as the native protein. Total nuclear protein was extracted from transgenic plants and MAC3A-HA was immunoprecipitated using anti-HA microbeads. Western blot analysis using an anti-AtCDC5 antibody revealed that AtCDC5 is present only in the eluant isolated from transgenic plants expressing MAC3A-HA and not from Col-0 control plants (Figure 3.11). A similar result was obtained when complementing P35S-MAC3A-CFP transgenic plants in the mac3a mac3b background were used in an independent co-IP experiment using anti-GFP microbeads (Figure 3.12). Thus, as in human and yeast, AtCDC5 and MAC3 associate with each other as components of the MAC in Arabidopsis.

3.4 Discussion

Precursor mRNA processing is central to gene expression in eukaryotes. The three pre-mRNA processing events take place in the nucleus and include 5’ capping, 3’ polyadenylation and splicing. Splicing is orchestrated by a large ribonucleoprotein
(RNP) complex called the spliceosome that produces protein-coding mRNA transcripts by splicing out introns and joining together exons. Spliceosome complexes are highly conserved and have been isolated and analyzed by proteomics approaches in budding yeast, human and Drosophila (Zhou et al., 2002; Deckert et al., 2006; Bessonov et al., 2008; Herold et al., 2009). Common proteins found in or associated with the spliceosome are uridine-rich snRNPs, RNA-binding proteins, RNA helicases, and serine/arginine-rich (SR) proteins. Plant spliceosome complexes have not yet been isolated, but a survey of genes in Arabidopsis indicates that homologs of most spliceosome proteins are conserved (Wang and Brendel, 2004).

Several sub-complexes have been isolated along with spliceosome proteins in yeast, human, and Drosophila, suggesting that there are peripheral complexes that work with the core splicing machinery, perhaps to affect transcript levels and/or alternative splicing in response to environmental cues. One protein complex that associates with the spliceosome is the NTC, which was shown to act simultaneously with or just after the dissociation of the U4/U6.U5 tri-snRNP during spliceosome assembly (Tarn et al., 1993b). The NTC core complex in higher eukaryotes consists of at least four proteins: SNEV/Prp19, CDC5/Cef1p, PLRG1/Prp46, and Spf27 (Ajuh et al., 2000; Chen et al., 2002; Ohi et al., 2002; Zhou et al., 2002; Zhang et al., 2005a; Deckert et al., 2006; Gavin et al., 2006; Bessonov et al., 2008). It is of interest to note that S. cerevisae does not encode a protein with homology to MOS4/Spf27, suggesting that higher eukaryotes have evolved additional NTC proteins. Homologs of all NTC core components exist in Arabidopsis (Wang and Brendel, 2004), but, like the spliceosome, this protein complex is not well understood in plants. We previously found that MOS4 (AtSpf27), AtCDC5, and PRL1 (AtPLRG1) play essential roles in plant immunity and form a nuclear protein complex called the MAC (Palma et al., 2007). Based on what is known in other eukaryotes, we hypothesized that this protein complex corresponds to the NTC in Arabidopsis and likely involves more binding partners. Here we present the first effort to isolate the MAC/NTC in a plant species.

We affinity purified MOS4-HA associated proteins and isolated MAC3A and MAC3B, two Arabidopsis proteins with sequence homology to Prp19 (Table 3.1; Figure 3.2). This association was confirmed by co-immunoprecipitation of MAC3A-HA with
AtCDC5 (Figure 3.11). Also found associated with MOS4-HA were AtCDC5 and PRL1, indicating that this core complex is indeed conserved in *Arabidopsis* and corroborating our previous findings (Palma et al., 2007). A number of other predicted NTC-associated proteins were identified as well, including homologs of RBM22/Ecm2, SKIP/Prp45, Isy1, XAB2, and CRN/Clf1 (Table 3.1). These proteins are predicted to have roles in RNA processing in plants based on their similarity to proteins in other eukaryotes where such roles have been demonstrated (Wang and Brendel, 2004). However, the biological functions of these proteins have not been studied in *Arabidopsis*.

We do not yet know all the detailed protein-protein interactions within the MAC. It appears that AtCDC5 represents an interaction hub, as it physically interacts with MOS4, PRL1, and MAC3 to form the MAC core complex. We were not able to detect direct protein-protein interactions between MOS4 and MAC3A or MAC3B using either bimolecular fluorescence complementation (BiFC) or yeast-2-hybrid (data not shown). Thus, it appears that core MAC proteins form a complex through their association with AtCDC5. Tetramerization is required for Prp19 and SNEV functionality in human and yeast cells, respectively (Grillari et al., 2005; Ohi et al., 2005). This may also be the case in *Arabidopsis*, although we do not have experimental evidence for this. In yeast two-hybrid experiments, we observed neither interaction between MAC3A and MAC3B to suggest hetero-tetramerization, nor interaction between MAC3A and MAC3A or MAC3B and MAC3B to suggest homo-tetramerization (data not shown). However, these data do not rule out the possibility of MAC3 still forming homo- or hetero-tetramers. Genetic redundancy between *MAC3A* and *MAC3B* supports the idea that these proteins probably do not interact with one another to form hetero-tetramers in plants, as the loss of one or the other protein does not result in a visible phenotype. Furthermore, immunoprecipitation of MAC3A-HA followed by protein sequencing using MS did not identify MAC3B, although other core proteins such as AtCDC5 and PRL1 were identified (data not shown). We could not distinguish MAC3A from MAC3A-HA in the MS data to indicate homo-dimerization. Nonetheless, if MAC3 tetramerization is required for its function similar to Prp19 and SNEV, it is most likely that MAC3A and MAC3B form homo-tetramers in plants. Additionally, it appears that there is no
preferential incorporation of MAC3A or MAC3B in the MAC, as both proteins were found to associate with MOS4-HA.

Proteins with predicted functions in splicing were also identified as MOS4-HA associated proteins, including several subunits of the U5 snRNP and one subunit of the U2 snRNP (Table 3.1). This is not unexpected since the NTC is known to closely associate with the spliceosome in yeast, human and Drosophila. The 220kD subunit of the U5 snRNP in yeast, Prp8, has recently been called ‘the heart of the spliceosome’ (Grainger and Beggs, 2005; Hacker et al., 2008; Ritchie et al., 2008), and forms a tri-snRNP with the ATPase Snu114p and the GTPase Brr2 (the 116kD and 200kD subunits, respectively), which are necessary for spliceosome activation. These subunits were identified as MAC components (ABNORMAL SUSPENDOR 2 (SUS2), CLOTHO (CLO), and EMBRYO DEFECTIVE 1507 (EMB1507), respectively; Table 3.1). Loss-of-function mutations in these genes have previously been reported to cause embryo lethality (Schwartz et al., 1994; Tzafrir et al., 2004; Moll et al., 2008), agreeing with their predicted functions in splicing. However, the detailed biochemical function of these proteins has yet to be demonstrated in Arabidopsis.

The fact that predicted snRNP and RNA-processing proteins associate with MOS4 points to a potential role for the MAC in splicing. However, when we previously tested the fidelity of splicing machinery in mos4-1, Atcdc5-1, and prl1-1 plants compared to Col-0 wild type, no difference in splicing efficiency was found for several alternatively spliced transcripts (Palma et al., 2007), suggesting that general splicing machinery is not affected by single mutations in genes encoding MAC core proteins. Also, it is unlikely that single MAC core proteins are essential for splicing because mos4, Atcdc5, prl1, and mac3a mac3b mutants are viable and have only minor morphological defects. However, when we tested genetic interactions between mos4-1, Atcdc5-1, and prl1-1 by analyzing double mos4-1 Atcdc5-1 and mos4-1 prl1-1 mutants, we found the interactions to be synthetically lethal (Palma et al., 2007). Synthetic lethality between MAC mutants was further confirmed when we tested the genetic interaction of mac3a mac3b with mos4-1 or prl1-1. These triple mutants are lethal as well (Appendix 2). Together, these data suggest that the MAC as a whole may be required for an essential process such as
spliceosome assembly, as reported for NTC components (McDonald et al., 1999; Chan et al., 2003), but that individual core MAC proteins are expendable for this process.

The yeast prp19-1 mutant is sensitive to high temperatures and exhibits splicing defects (Cheng et al., 1993). SNEV is likewise required for spliceosome assembly in human cells (Grillari et al., 2005). Transgenic expression of full-length SNEV in prp19-1 is unable to complement temperature sensitivity (Grillari et al., 2005), suggesting that SNEV and Prp19 are not completely orthologous even though they share sequence homology and both exhibit E3 ligase activity (Hatakeyama et al., 2001; Ohi et al., 2003). Similarly, we attempted to rescue prp19-1 temperature sensitivity by expressing full-length MAC3A cDNA in prp19-1. Like SNEV, we found that expression of this Arabidopsis protein was unable to complement prp19-1 in yeast (Appendix 3). This might indicate that yeast, human and Arabidopsis Prp19 may have evolved divergent biological functions, although the binding partners and enzymatic E3 ubiquitin ligase activity of these proteins seem to be conserved.

NTC proteins have been reported to be involved in many cellular processes in addition to spliceosome assembly, including DNA repair (Grey et al., 1996; Mahajan and Mitchell, 2003; Beck et al., 2008), cell-cycle progression (Ohi et al., 1994), and protein degradation (Loscher et al., 2005; Sihn et al., 2007). We previously established a novel role for MAC proteins in plant immune signaling (Palma et al., 2007), and the data we present here for MAC3 further highlights this. Loss-of-function mac3a and mac3b single mutant plants are not compromised in basal defense against P. s. m. ES4326 and P. s. t. DC3000, whereas mac3a mac3b double mutant plants exhibit enhanced susceptibility to pathogen infection, suggesting that MAC3A and MAC3B play redundant roles in plant immunity. Like mos4-1 and Atcdc5-1, mac3a mac3b suppresses constitutive expression of PR-1 and PR-2, high levels of endogenous SA, and enhanced resistance to virulent pathogens caused by snc1. Also, MAC3A and MAC3B seem to be required for responses mediated by specific TIR- and CC-NB-LRR R proteins. Intriguingly, MAC components appear to contribute differently to plant defense. For example, prl1 mutants regularly sustain higher pathogen growth than mos4, Atcdc5, and mac3a mac3b mutants (Palma et al., 2007), indicating that PRL1 may play a more prominent role in defense responses.
The *snc1* signaling network involves nucleo-cytoplasmic trafficking, RNA processing and protein modification (Monaghan et al., 2009). Epistasis analysis between *snc1* and loss-of-function mutants of defense regulators defined the presence of both SA- and NPR1-independent pathways activated in *snc1* (Li et al., 2001; Zhang et al., 2003). Accordingly, many *MOS* genes have been shown to function in the SA-independent pathway (Monaghan et al., 2009). SA accumulation following avirulent pathogen infection is unaffected in *mos4-1, Atcdc5-1, or prl1-1* mutants compared to Col-0 (Palma et al., 2007). Furthermore, a double *mos4-1 npr1-1* mutant shows quantitative bacterial growth compared to *mos4-1* and *npr1-1* single mutants (Palma et al., 2007). *MAC3A* and *MAC3B* also function in an SA-independent manner, as deduced from epistasis analysis between *eds5-3 npr1-1* and *mac3a mac3b* (data not shown). Together, these data suggest that the MAC functions independently of both SA and *NPR1*.

Plant defense responses are intimately controlled and precisely regulated in many ways including post-translational modifications resulting in protein activation, inhibition, or degradation. Targeted protein degradation in eukaryotes is mediated by a multi-protein complex called the ubiquitin proteasome (Smalle and Vierstra, 2004). This degradation is initiated by the sequential activation, conjugation, and ligation of a ubiquitin moiety to a target protein by E1, E2 and E3 enzymes, respectively (Ciechanover, 1998). One class of E3 ligase is defined by the presence of a ~70 amino acid U-box domain (Hatakeyama and Nakayama, 2003). U-box proteins bind both the E2 ubiquitin conjugating enzyme and the substrate to facilitate ubiquitin transfer and ligation to the target (Hatakeyama et al., 2001). There are 64 predicted plant U-box (PUB) proteins in *Arabidopsis* and 77 in rice (Yee and Goring, 2009), whereas Prp19 is one of only two U-box proteins encoded in the *S. cerevisae* genome. *MAC3A* and *MAC3B* contain U-box domains (representing AtPUB59 and AtPUB60), and, like Prp19, *MAC3B* was recently shown to have *in vitro* E3 ubiquitin ligase activity (Wiborg et al., 2008), indicating that this function is conserved in plants.

The ubiquitin pathway is an integral component of *snc1* signaling. It was previously shown that the loss of the ubiquitin activating (E1) enzyme encoded by *UBA1/MOS5* (but not *UBA2*, the only other E1 enzyme in *Arabidopsis*) suppresses *snc1* autoimmune phenotypes (Goritschnig et al., 2007). Our finding that the loss of E3
ubiquitin ligases MAC3A and MAC3B also suppresses snc1-mediated responses further supports a key role for the ubiquitin pathway in snc1 signaling. Interestingly, MAC3B appears to specifically work with two ubiquitin conjugating (E2) enzymes of the UBC4/5 class in in vitro ubiquitination assays (Wiborg et al., 2008). This suggests that UBA1/MOS5, UBC4/5 and MAC3A/3B could potentially function together in snc1-mediated resistance, however this remains to be shown specifically. Although U-box proteins bind both the E2 conjugating enzyme and the substrate, we did not identify UBC4/5 (or other E2 enzymes) in the MOS4-HA pull-down. This is probably due to the transient nature of E2-E3 interactions in signal transduction pathways.

It is tempting to hypothesize that MAC3 regulates defense responses by targeting defense repressors for degradation, some of which could be identified MAC members. Several E3 ligases, including PUBs, play important roles in plant defense. The PUB E3 ligase Avr9/Cf-9 RAPIDLY ELICITED 74 (ACRE74) is required for the establishment of an HR following pathogen infection in tobacco (Gonzalez-Lamothe et al., 2006), as is ACRE276 (Yang et al., 2006). AtPUB20 and AtPUB17, the Arabidopsis homologs of ACRE74 and ACRE276, respectively, are up-regulated upon recognition of pathogen-derived molecules (Heise et al., 2002; Navarro et al., 2004). Also, three redundant pathogen-induced U-box E3 ligases AtPUB22, AtPUB23 and AtPUB24 negatively regulate PAMP-triggered immunity (Trujillo et al., 2008). Interestingly, the bacterial effector protein AvrPtoB mimics a eukaryotic U-box structure and has been shown to possess E3 ligase activity (Janjusevic et al., 2006), highlighting the importance of protein degradation in plant defense signaling. Like MAC3, PRL1 is also implicated in proteasomal pathways. PRL1 inhibits the activity of AKIN10 and AKIN11, two redundant protein kinases involved in sugar signaling that interact with the α4 subunit of the 20S proteasome in Arabidopsis and are part of a CUL1-based E3 ubiquitin ligase complex (Farras et al., 2001). Moreover, PRL1 was recently found to function as a substrate receptor in a CUL4-based E3 ubiquitin ligase complex (Lee et al., 2008), indicating that PRL1 plays regulatory roles in two Cullin-based E3 ligase complexes in addition to its role in the MAC.

Pathogen recognition and the activation of defense responses involves massive transcriptional reprogramming (Jones and Dangl, 2006; Wise et al., 2007). The MAC
core component AtCDC5 is an atypical R2R3-Myb transcription factor that binds double-stranded DNA with specificity for the element CTCAGCG (Hirayama and Shinozaki, 1996), which is present in many Arabidopsis gene promoters. MOS4 is a small protein that likely serves a scaffolding function. The association between MOS4, AtCDC5, PRL1 and MAC3 suggests that both protein ubiquitination and transcriptional activation are regulatory functions employed by the MAC. As transcription is often coupled with splicing (Pandit et al., 2008), it is possible that the MAC is a transcriptional modulator that functions closely with the spliceosome to regulate defense-related genes. Future studies will uncover more details about how these and other MAC proteins work together and function in plant immunity.

3.5 Materials and methods

3.5.1 Plant growth, mutant isolation, pathology assays and phenotypic characterization

For most experiments, plants were grown on soil in a 16h light / 8h dark regime. T-DNA mutants were obtained from the ABRC and genotyped by PCR using the insertion-flanking primers 089300-F (5’-CGGAAGTTCTTTAACTTGCGC-3’) and 089300-R (5’-GTGTTAECTGCTTCATCCGAC-3’) for mac3a, or 050811-F (5’-ACGGAATACATAAGCAGACCAC-3’) and 050811-R (5’-TGTTGTGCAGTGAGTTTGATC-3’) for mac3b. Isolation of the mos4-1 mutant was previously described (Palma et al., 2007). Bacterial and oomycete infections were performed as described in Li et al., (2001). Briefly, bacterial pathogens were inoculated on the abaxial leaf surfaces of four-week old plants using a needless syringe. Leaf discs (with an area of 0.38cm²) were collected on the day of infection (Day 0) and three days later (Day 3) from different plants. H.a. isolates were spray-inoculated onto adaxial leaf surfaces of two-week old seedlings and counted using a hemocytometer seven to ten days later. RNA was extracted from 20 day old seedlings grown on Murashige and Skoog (MS) medium using the Totally RNA Kit (Ambion). Reverse transcription was
performed using Superscript II reverse transcriptase (Invitrogen). The primers used to amplify PR-1, PR-2 and Actin 1 have been described previously (Zhang et al., 2003). *MAC3A* and *MAC3B* cDNA was amplified for expression analysis using the primers 089300RT-F (5’-CGTTGGTGACACTGATCTTG-3’), 089300RT-R (5’GCAGCAGCCGTGTAATTTC-3’), 050811RT-F (5’-ATCTGCAGATGCGAACTCTG-3’), and 050811RT-R (5’-CCATTGCTGCAAATACCTCT-3’). SA was extracted and measured by HPLC from three-week old plants using a procedure described in Li et al., (1999).

### 3.5.2 Double and triple mutant construction

To obtain the mac3a mac3b double mutant, homozygous mac3a and mac3b plants were crossed and the double was identified in the F2 using PCR-based genotyping. The sncl mac3a mac3b triple mutant was obtained by crossing a homozygous sncl single mutant plant with a homozygous mac3a mac3b double mutant. sncl was fixed in the F2 first by phenotype and later confirmed by PCR using primers described previously (Li et al., 2001). The other two loci were confirmed by PCR to be heterozygous, and the triple mutant was identified in the F3. A backcross with sncl confirmed the genotype of the triple as all F1 individuals showed typical sncl morphology. A similar procedure was used to create the mos4-1 mac3a mac3b and prl1-1 mac3a mac3b triple mutants. All genotypes were confirmed by PCR.

### 3.5.3 Molecular cloning

A genomic fragment spanning the full-length MOS4 gene (gMOS4), without the stop codon but including a 1.5 kb sequence upstream of the translation initiation codon, was amplified from Col-0 DNA using Phusion Taq (Finnzymes) with the primers 5’-CACCACACTGTAGAGGTCTTGG-3’, and 5’-TTGCATTGTGGCATTGCTAGAC-3’. Similarly, the open reading frames (without the stop codons) of MAC3A and MAC3B
were amplified from cDNA using the primers 5’-CACCATGAATTGTGCAATTTCCGGC-3’, and 5’-TGAATCTTGTGCTGAATCTTC-3’ for MAC3A, and 5’-CACCATGAACTGTGCAATTTCAGGAG-3’, and 5’-TGAAATTCTCCCCCATTGCTG-3’ for MAC3B. These Gateway-adapted PCR fragments were cloned into pENTR using the Gateway pENTR/D-Topo kit (Invitrogen). Entry vectors were confirmed by sequencing using the M13F and M13R primers. For gMOS4, recombination into the pGWB13 binary destination vector with a C-terminal 3xHA tag (Nakagawa et al., 2007) was done using Gateway LR Clonase (Invitrogen). Transgenic seedlings were selected on MS plates containing 50 µg/ml Kanamycin and 50 µg/ml Hygromycin and confirmed by PCR. For MAC3A and MAC3B, recombination into destination binary vectors with a constitutive 35S promoter for C-terminal and N-terminal fusion protein expression analysis in Arabidopsis and onion was done using Gateway LR Clonase (Invitrogen). Destination vectors used were either N-terminal cCFP, C-terminal cCFP, or C-terminal 3xHA-StrepII. Transgenic seedlings were selected on soil with the herbicide Basta and confirmed by PCR. Cells of transgenic seedlings grown on MS medium containing 10 mg/ml Basta were examined under the confocal microscope for CFP fluorescence as described in Zhang et al., (2005b).

**3.5.4 Nuclear extraction and immunoprecipitation**

Approximately 100g of leaf tissue from complementing MOS4-HA in mos4-1 was frozen in liquid nitrogen, ground into a fine powder using a pestle and mortar and homogenized in lysis buffer (20 mM Tris-HCl, pH 7.4, 25% glycerol, 20 mM KCl, 2 mM EDTA, 2.5 mM MgCl₂, 250 mM sucrose) at 4°C. The homogenate was filtered through four layers of cheesecloth and then sequentially filtered through 100-, 70-, and 30-µm mesh nylon netting. The nuclei were pelleted by centrifugation at 1500g for 10 minutes and washed twice with nuclei resuspension buffer (NRBT; 20mM Tris-HCl, pH 7.4, 25% glycerol, 2.5 mM MgCl₂, 0.5% Triton X-100). The nuclear fraction was resuspended gently in NRB and centrifuged at 100g for 5 minutes to remove cell debris. Nuclei were pelleted by spinning the supernatant at 1500g for 10 minutes and were resuspended in ice-cold
buffer NE-2 (20 mM HEPES-KOH, pH 7.9, 2.5 mM MgCl₂, 0.42 M NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, with protease inhibitors). Nuclei were sonicated for 2 minutes in an ice-bath, incubated on ice for 30 minutes, and spun for 30 minutes at 25000xg at 4°C, releasing the nuclear proteins into the supernatant. The nuclear fraction was dialyzed in buffer NE-3 (20 mM HEPES-KOH, pH 7.9, 2.5 mM MgCl₂, 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, with protease inhibitors) for 5 hours before adding Anti-HA microbeads (Miltenyi Biotec Inc.). After gentle mixing for 30 minutes on ice, the microbeads were magnetically precipitated on columns according to the manufacturer’s instructions (µMACS). The microbeads were washed eight times before elution. For identification of MOS4-HA interacting proteins, immunoprecipitated proteins were eluted and loaded onto a 12% SDS-PAGE gel and stained with a mass spectrometry-compatible silver staining kit (Invitrogen). The MOS4-HA IP lane was cut into eight pieces and digested with trypsin, and the resulting proteins were analyzed by MS (see below). To test the in planta interaction between MAC3A-HA and AtCDC5, a similar protein extraction procedure was employed. Approximately 15g of leaf tissue from complementing P35S-MAC3A-HA in mac3a mac3b was frozen in liquid nitrogen and homogenized in lysis buffer as above. The homogenate was filtered through two layers of cheesecloth and sequentially filtered through 95- and 37- µm mesh nylon netting. The nuclei were pelleted as before and washed 3 times in NRB buffer with 0.2% Triton X-100. The nuclei were then washed with NRB buffer without Triton X-100 once. Nuclei were pelleted as above and resuspended in ice-cold NE-2 buffer with the following modifications: 100 mM NaCl, 1 mM DTT, 0.2% Triton X-100. Nuclei were sonicated and pelleted as above but were not dialyzed prior to the addition of anti-HA microbeads, which were precipitated according to the manufacturer’s instructions, as above. The microbeads were washed eight times prior to elution with NE-2 buffer with the following modifications: 150mM NaCl, 1 mM DTT, 0.2% Triton X-100. The eluted fraction was loaded on a 12% SDS-PAGE gel followed by Western Blot analysis using anti-HA (Roche) or anti-AtCDC5 (Palma et al., 2007) antibodies.
3.5.5 Mass spectrometry analysis

Protein bands of interest were sliced from the gel and cut into small pieces. After de-staining with ProteoSilver Distainer A and B (ProteoSilver Plus Silver Stain Kit, Sigma), the gel pieces were washed twice with ultra pure water and dehydrated in acetonitrile. The gel pieces were further dried in a CentriVap concentrator (Labconco) and then digested in-gel with 10 ng/μL sequencing grade modified trypsin (Promega) in 50 mM NH4HCO3 (pH 7.8) at 37°C overnight. The resulting tryptic peptides were extracted with 50-100 μl of 5% formic acid/50% acetonitrile and 0.1% formic acid/75% acetonitrile sequentially. The two extracts were combined and concentrated to around 10 μL in the CentriVap concentrator.

The tryptic peptides were loaded to a pre-column (75 μm × 8 cm) packed with 5-15 μm spherical C18 reverse phase particles (YMC). The pre-column was connected to a homemade analytical column (50 μm × 10cm) packed with YMC 5 μm spherical C18 reverse phase particles. The tip size of the analytical column was around 2 μm and the flow rate was controlled at 20-50 nl/min. To elute peptides from the column, an Agilent 1100 series binary pumps system (Agilent Technologies) was used to generate the following HPLC gradient: 0-5% B in 5 min, 5-40% B in 25 min, 40-100% B in 10 min (A = 0.1 M acetic acid in water, B = 0.1 M acetic acid /70% acetonitrile). The eluted peptides were sprayed directly into a QSTAR XL mass spectrometer (MDS SCIEX) equipped with a nano-ESI ion source under 2.1 kV spray voltage. The mass spectra were collected in information-dependent acquisition (IDA) mode cycling with one MS scan followed by three MS/MS scans. The mass range of the MS scan was 400-2000 Da, and 100-2000 Da for MS/MS scans. For each MS scan, the top 3 most abundant precursor ions were selected for MS/MS scans using low resolution for precursor ion isolation with enhance all mode. Database searches were performed on an in-house Mascot server (version 2.1, Matrix Science Ltd.) against the NCBI non-redundant protein database.
3.6 Manuscript acknowledgements

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Figure 3.1: MOS4-HA associated proteins.

(A) Full-length genomic MOS4-HA driven by its native promoter complements mos4-1 morphology. Soil-grown plants were photographed 4 weeks after germination. Size bar represents 1 cm. (B) MOS4-HA complements mos4-1 related enhanced susceptibility to P.s.m. ES4326, as shown by bacterial growth at 0 and 3 days post-inoculation. Values represent an average of four replicates ± SD. An unpaired Student’s t-test was used to analyze the statistical significance of bacterial growth compared to Col-0. Asterisk indicates P<0.0001. (C) MOS4-HA associated proteins were isolated by affinity purification from total nuclear extracts collected from 100g of leaf tissue from 3 week old transgenic and Col-0 seedlings. Interacting proteins were eluted, separated by SDS-PAGE on a 12% gel, and silver-stained.
Table 3.1: MOS4-associated proteins identified by mass-spectrometry.

Nuclear extracts from complementing mos4-1 plants expressing PMOS4-MOS4-HA were immunoprecipitated using anti-HA beads, separated by SDS-PAGE and silver-stained (Fig. 3.1C). The MOS4-HA IP lane was cut into eight pieces, digested, and the proteins contained were analyzed by mass-spectrometry. Sequenced peptides were used as queries in BLAST searches against the 2007 version of the Arabidopsis genome. Proteins with strong or partial homology to Arabidopsis proteins from other eukaryotes are listed in the columns to the right. Proteins are divided into sections according to their sequence homology to known NTC- or NTC-associated proteins, based on a previous computational organization (Wang and Brendel, 2004). Dashes indicate that no proteins with significant homology were identified by BLAST.

**Abbreviations:** ADL: Arabidopsis dynamin-like protein; BCAS: Breast carcinoma sequence; BRR: Bad response to refrigeration; CDC: Cell division cycle; CEF1: S. cerevisiae homolog of cdc5(+) ; CLF: Crooked neck-like factor; CLO: Clotho; CRN: Crooked neck pre-mRNA splicing factor-like; CWC: Complexed with Cef1p; CWF: Complexed with Cdc5p; DHX: DEAH-box; DNM: Dynamin; DRP: Dynamin-related protein; EDR: Enhanced disease resistance; EMB: Embryo lethal; ESP: Enhanced silencing phenotype; GFA: Gametophytic factor; HSH: Human Sap homolog; ISY: Interactor of Syf; LRR: Leucine-rich repeat; MAC: MOS4-associated complex; MEE: Maternal effect embryo arrest; MOS: Modifier of snc1; NTC: Nineteen complex; PLDα: Phospholipase D alpha; PP2A: Protein phosphatase type 2A; PRL/PLRG: Pleiotropic regulating locus; PRP: Precursor mRNA processing; RBM: RNA binding motif; RCN: Roots curl in NPA; RSW: Radial swelling; SAP: Splicing associated protein; SKIP: Ski-interacting protein; SNEV: Senescence evasion factor; SNU: Small nuclear ribonucleoprotein associated; SNW: “SNW” domain; SPF: Spliceosome-associated protein; SUS: Abnormal suspensor; SYF: Synthetic lethality with Cdc40; XAB: XPA-binding protein.

**References:** (1) Ajuh et al., 2000; (2) Ohi et al., 2002; (3) Chen et al., 2002; (4) Gavin et al., 2006; (5) Deckert et al., 2006; (6) Bessonov et al., 2008; (7) Zhou et al., 2002.
Table 3.1: MOS4-associated proteins identified by mass-spectrometry.

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Table 3.2: Predicted sub-cellular localization of MAC components and details from mass spectrometry analysis.

MAC proteins are organized as in Table 3.1, based on protein homology to NTC proteins in yeast and human. Predicted sub-cellular localization data is inferred mostly from organelle proteomics datasets, with the exception of the MAC/NTC core proteins, where localization has been shown with complementing transgenic lines expressing fluorescent protein translational fusions. Although the predicted sub-cellular localization for most MAC proteins is ambiguous, their predicted functions in spliceosome-mediated RNA processing suggest that they are nuclear. Mass spectrometry details including the number of unique peptides and sequence coverage, as well as Mascot scores, for all identified proteins, are included in the columns to the right. The 2007 version of the Arabidopsis genome was used for protein identification. Mascot calculates protein score as -10*LOG_{10}(P), where P is the absolute probability that the match is a random event. References: (1) Lin et al., 2007; (2) Palma et al., 2007; (3) Pendle et al., 2004; (4) Bae et al., 2003; (5) Kleffmann et al., 2004; (6) Benschop et al., 2007; (7) Bayer et al., 2005; (8) Mitra et al., 2007; (9) Zyballov et al., 2008; (10) Boudart et al., 2005; (11) Bindschedler et al., 2008; (12) Carter et al., 2004; (13) Jaquinod et al., 2006; (14) Borderies et al., 2003.
Table 3.2: Predicted sub-cellular localization of MAC components and details from mass spectrometry analysis.

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### Amino Acid Sequences from Other Eukaryotes

#### Figure 3.2: Protein Sequence Alignment of MAC3A and MAC3B with Homologs in Various Eukaryotes

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**Box and Predicted Nuclear Localization Signal (NLS)**

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#### Protein Sequence Alignment

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**U-box**

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**NLS**

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**Figure 3.2: Protein Sequence Alignment of MAC3A and MAC3B with Homologs in Other Eukaryotes.**

Amino acid sequences from *Homo sapiens* (Hs; human) Prp19/SNEV (accession number NP_055317); *Mus musculus* (Mm; mouse) Prp19/SNEV (accession number NP_598890); *Danio rerio* (Dr; zebrafish) Prp19 (accession number AAH45954); *Arabidopsis thaliana* (At; thale cress) MAC3A (accession number AAN13133) and MAC3B (accession number FJ820118); *Shizosaccharomyces pombe* (Sp; fission yeast) Prp19/Cwf8p (accession number CAB10135); and *Saccharomyces cerevisiae* (Sc; baker’s yeast) Prp19 (accession number CA97487), are compared. Identical amino acids are coloured black, and similar amino acids are coloured grey. Alignment was generated using ClustalW2. Boxshade version 3.21 was used to colour identical and similar amino acids. The conserved U-box and predicted nuclear localization signal (NLS) are indicated.
Figure 3.3: MAC3A and MAC3B function redundantly in basal defense.

(A) Gene structures of MAC3A (At1g04510) and MAC3B (At2g33340) showing the position of T-DNA insertions Salk_089300 (mac3a) and Salk_050811 (mac3b). Lines indicate introns and boxes indicate exons. The location of translation start (ATG) and stop (TGA) codons are found in the first and last exons, as indicated. (B) Expression levels of MAC3A and MAC3B in Col-0, mac3a, mac3b, and mac3a mac3b mutants as indicated by semi-quantitative RT-PCR using cDNA-specific primers flanking the T-DNA insertion sites. (C) Growth of P.s.t. DC3000 at 0 and 3 days post-inoculation. Values represent an average of four replicates ± SD. An unpaired Student’s t-test was used to analyze the statistical significance of bacterial growth compared to Col-0. Asterisk indicates P<0.001. (D) Growth of P.s.m. ES4326 at 0 and 3 days post-inoculation. P35S-MAC3A-CFP and P35S-MAC3B are expressed in the mac3a mac3b background. Values represent an average of four replicates ± SD. An unpaired Student’s t-test was used to analyze the statistical significance of bacterial growth compared to Col-0. Asterisk indicates P<0.0001. Experiments were repeated at least three times with similar results.
Figure 3.4: Morphology of the *mac3a mac3b* double mutant and enhanced susceptibility to *P.s.m.* ES4326.

(A) Morphology of Col-0, *mac3a*, *mac3b*, and *mac3a mac3b* plants. Soil-grown plants were photographed 4 weeks after planting. Size bar represents 1 cm. (B) Growth of *P.s.m.* ES4326 at 0 and 3 days post-inoculation. Values represent an average of four replicates ± SD. This trend is apparent in several repeated experiments. These plants were grown in 16h light and low humidity, in a cooler growth chamber than is usually used for pathogen infections. An unpaired Student’s *t*-test was used to analyze the statistical significance of bacterial growth compared to Col-0. Asterisks indicate *P*<0.02.
Figure 3.5: Transgenic complementation of *mac3a mac3b* and *snc1 mac3a mac3b* by *MAC3A* and *MAC3B*.

(A) Morphology of Col-0, *mac3a mac3b*, and *mac3a mac3b* plants expressing P35S-MAC3A-CFP or P35S-MAC3B. Soil-grown plants were photographed 4 weeks after planting. Size bar represents 1 cm. (B) Morphology of Col-0, *snc1, snc1 mac3a mac3b*, and *snc1 mac3a mac3b* plants expressing P35S-MAC3A-CFP or P35S-MAC3B. Soil-grown plants were photographed 4 weeks after planting. Bar represents 1 cm. (C) Growth of *P. s.m.* ES4326 at 0 and 3 days post-inoculation. Values represent an average of four replicates ± SD. Experiment was repeated twice with similar results. P values were calculated using an unpaired Student’s *t*-test comparing bacterial growth in *snc1 mac3a mac3b* with the transgenic lines. Asterisks indicate *P*<0.001.
Figure 3.6: MAC3A and MAC3B function redundantly in specific R-protein mediated resistance pathways.

(A-C) Growth of the bacterial pathogens *P.s.t. avrRps4*, *P.s.t. avrPphB*, and *P.s.t. avrRpm1* at 0 and 3 days post-inoculation. Values represent an average of four replicates ± SD. An unpaired Student’s *t*-test was used to analyze the statistical significance of bacterial growth compared to Col-0. Asterisks indicate *P*<0.0005. (D) Growth of avirulent *H.a.* isolate Cala2 seven days post-inoculation on 2-week old seedlings. *eds1-2* introgressed in Col-0 was used as a positive control for pathogen growth. Values represent an average of two replicates of at least 20 plants per genotype ± SD. All experiments were repeated at least three times with similar results.
Figure 3.7: Suppression of snc1-associated phenotypes by mac3a mac3b.

(A) Morphology of Col-0, mac3a mac3b, snc1, and snc1 mac3a mac3b plants. Soil-grown plants were photographed 4 weeks after germination. Size bar represents 1 cm. (B) Growth of P. s. m. ES4326 at 0 and 3 days post-inoculation. Values represent an average of four replicates ± SD. P values were calculated using an unpaired Student’s t-test comparing the mean bacterial growth in snc1 to that in snc1 mac3a mac3b. Asterisk indicates P<0.0005. (C) Growth of the H. a. isolate Noco2 seven days post-inoculation on 2-week old seedlings. Values represent an average of 4 replicates of at least 20 plants per genotype ± SD. P values were calculated using an unpaired Student’s t-test comparing oomycete growth in snc1 compared to that in snc1 mac3a mac3b. Asterisk indicates P<0.01. (D) Free and total SA was extracted from leaf tissue from 3-week old soil-grown plants and analyzed by HPLC. Values represent the average of 4 replicates ± SD. (E) Semi-quantitative RT-PCR of the pathogenesis-related genes PR-1 and PR-2. Actin1 was included as a control. All experiments were repeated at least twice with similar results.
Figure 3.8: Sub-cellular localization of MAC3A.

(A) Complementation of $P_{35S}$-MAC3A-CFP and $P_{35S}$-MAC3A-HA in mac3a mac3b. Growth of virulent $P.s.m.$ ES4326 at 0 and 3 days post-inoculation. Values represent an average of four replicates ± SD. This experiment was repeated twice with similar results. An unpaired Student’s t-test was used to analyze the statistical significance of bacterial growth compared to Col-0. Asterisk indicates P<0.0005. (B) Confocal microscopy was used to examine the localization of $P_{35S}$-MAC3A-CFP in transgenic mac3a mac3b plants. Guard cells from a representative line are shown. DAPI was used as a control for nuclear localization.
Figure 3.9: Sub-cellular localization of MAC3B.

Confocal microscopy was used to examine the localization of \textit{P35S-CFP-MAC3B} in complementing transgenic \textit{mac3a mac3b} plants. Root cells are shown. DAPI was used as a nuclear marker.
Figure 3.10: **MAC3B** cDNA sequence analysis.

A comparison of *MAC3B* cDNA sequences from this study (marked with an asterisk) and from TAIR8. The error in the annotated sequence is shaded grey, as is the corrected stop codon.
Figure 3.11: MAC3A-HA associates with AtCDC5 in planta.

Total nuclear extracts were isolated from a complementing mac3a mac3b transgenic line expressing P35S-MAC3A-HA (+) and Col-0 (-). MAC3A-HA was immunoprecipitated using anti-HA microbeads. MAC3A-HA and AtCDC5 were detected in the eluted fractions by Western blot analysis using antibodies against HA or AtCDC5.
Figure 3.12: MAC3A-CFP associates with AtCDC5 *in planta*.

Total nuclear extracts were isolated from a complementing *mac3a mac3b* transgenic line expressing *P35S-MAC3A-CFP* (+) and Col-0 (-). MAC3A-CFP was immunoprecipitated using anti-GFP microbeads. MAC3A-CFP and AtCDC5 were detected in the eluted fractions by Western blot analysis using antibodies against GFP or AtCDC5. In this experiment, less input was observed in the MAC3A-CFP sample due to poor recovery of nuclei. Since less protein was present in the IP experiment, reduction in MAC3A-CFP and AtCDC5 in the flow-through is observed.
3.7 References


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4 The putative RNA-binding protein MOS4-ASSOCIATED COMPLEX5 functions in immunity mediated by suppressor of npr1-1, constitutive 1

1 A version of this chapter is in preparation for publication. Jacqueline Monaghan, Fang Xu, Shaohua Xu, Yuelin Zhang and Xin Li.
4.1 Summary

Immunity in plants is mediated in part by Resistance (R) proteins that recognize pathogen effectors and trigger enduring defense responses. The MOS4-Associated Complex (MAC), an evolutionarily conserved and spliceosome-associated nuclear protein complex, was previously shown to participate in plant immunity. We recently purified the MAC from *Arabidopsis* nuclei, identified its potential components by mass spectrometry, and showed that at least four proteins in the MAC are required for defense responses in *Arabidopsis*. Here we report the functional analysis of a putative RNA-binding protein identified in the MAC named MAC5A. We confirmed that MAC5A is indeed a component of the MAC through co-immunoprecipitation with the previously described MAC protein AtCDC5. Like all other MAC proteins studied so far, MAC5A-GFP localizes to the nucleus. Double mutant analysis demonstrated that MAC5A and its close homolog MAC5B are partially redundant in a dosage-dependent manner, and that a double *mac5a mac5b* mutant results in lethality. Probably due to this partial redundancy, *mac5a* and *mac5b* single mutants do not exhibit enhanced susceptibility to virulent or avirulent pathogen infection. However, like other MAC mutations, *mac5a-1* partially suppresses the autoimmune phenotypes of *snc1*, a gain-of-function mutant that expresses a deregulated R protein. Our results suggest that MAC5 is another component of the MAC that regulates immunity in plants.

4.2 Introduction

Higher plants have evolved a sophisticated immune system against microbial pathogens. Major players include pattern recognition receptors (PRRs) and Resistance (R) proteins, two classes of receptors that recognize the presence of pathogens and trigger cell-autonomous defense responses (Jones and Dangl, 2006). Many PRRs are membrane-associated receptor-like kinases (RLKs) that contain extracellular leucine-rich repeats (LRRs) involved in the perception of non-self epitopes common to whole groups of microbes (Zipfel, 2009). Proteins with LRR domains play key roles in microbial defense in both animals and plants. For example, in addition to PRRs,
plant R proteins and animal Toll-like receptors (TLRs) and Nod-like receptors (NLRs) all contain LRR domains (Padmanabhan et al., 2009). Most cloned R genes encode intracellular proteins containing a nucleotide binding (NB) site in addition to C-terminal LRRs, and are known collectively as NB-LRR proteins. Conceptually similar to mammalian NLRs, NB-LRRs recognize effectors deployed by pathogens during an infection. Activated PRRs and R proteins transduce largely overlapping signaling cascades that differ mostly in the amplitude of the signal (Navarro et al., 2004). For example, whereas the activation of PRRs merely warns or primes the cell that infection may take place, the activation of NB-LRRs is often associated with the onset of a cell-death program known as the hypersensitive response (HR), in which the plant cell kills itself to restrict pathogen growth. Most published mutants with deregulated NB-LRR proteins exhibit seedling lethality, indicating that NB-LRRs must be under strict negative regulation to avoid unnecessary cell death (Lukasik and Takken, 2009). SUPPRESSOR OF NPR1-1, CONSTITUTIVE, 1 (SNC1) encodes a TIR-NB-LRR protein (Li et al., 2001b; Zhang et al., 2003). Interestingly, the partial gain-of-function mutant snc1 exhibits constitutive defense responses in the absence of cell death, representing an exploitable autoimmune model amendable to genetic analysis. A forward genetic screen for suppressors of snc1 revealed that snc1-mediated defense includes components involved in nucleo-cytoplasmic partitioning (Palma et al., 2005; Zhang and Li, 2005; Cheng et al., 2009), protein modification (Goritschnig et al., 2007; Goritschnig et al., 2008), and RNA processing (Zhang et al., 2005; Palma et al., 2007).

MODIFIER OF SNC1, 4 (MOS4) is the founding member of the MOS4-Associated Complex (MAC; Palma et al., 2007), a highly conserved spliceosome-associated complex required for plant defense and snc1 signaling. The MAC is composed of over 20 proteins, many of which have predicted roles in RNA processing (Monaghan et al., 2009). We previously demonstrated that the MAC core proteins MOS4, AtCDC5, PRL1, and MAC3A/3B associate in vivo and are all necessary components of plant immunity, as loss-of-function mutations in the genes encoding these proteins exhibit enhanced susceptibility to pathogen infection (Palma et al., 2007; Monaghan et al., 2009). Here we characterize MAC5A, a putative RNA binding protein with sequence similarity to RBM22/Ecm2, that we previously isolated as a component of the MAC. We demonstrate that MAC5A localizes to the nucleus and interacts with AtCDC5 in planta, confirming its association with the MAC. In addition, we show that MAC5A and its close homolog MAC5B are partially redundant in a dosage-dependent manner, and that a double
mac5a mac5b mutant is lethal. Although single mac5a and mac5b mutants do not exhibit obvious enhanced susceptibility to pathogens, mac5a-1 suppresses snc1-associated phenotypes. Overall, our results indicate that MAC5 is component of the MAC that functions in snc1-mediated immunity.

4.3 Results

4.3.1 Isolation and phenotypic characterization of mac5a loss-of-function mutants

We previously affinity purified the MAC and analyzed its components using mass spectrometry (Monaghan et al., 2009). Not surprisingly, we found that the MAC bears remarkable similarity to the spliceosome-associated Prp19 complex (NTC) in yeast and the CDC5L complex in human. We classified the MAC proteins into four groups based on a previous annotation done by Wang and Brendel (2004). These groups were as follows: (A) NTC/MAC core proteins, (B) Other NTC/MAC associated proteins, (C) Predicted splicing-related proteins, and (D) Unrelated proteins/possible contaminants. We previously established that components in group A, MOS4, AtCDC5, PRL1, and MAC3A/3B, function in plant innate immunity (Palma et al., 2007; Monaghan et al., 2009). In addition to enhanced susceptibility to pathogen infection, loss-of-function mos4, Atcdc5, prl1 and mac3a mac3b mutants display pleiotropic defects including altered flowering time, low fertility, aberrant leaf morphology, in addition to hormone and sugar sensitivities (Nemeth et al., 1998; Palma et al., 2007; Monaghan et al., 2009; JM and XL, unpublished data).

To investigate whether the remaining MAC components also function in plant immunity, we obtained T-DNA insertion mutants representing loss-of-function alleles in these loci (not shown). We were most interested in characterizing putative MAC components found in group B, as those in group C are predicted to be integral to the spliceosome and are therefore unlikely to be specifically involved in plant defense. Furthermore, loss-of-function mutations in several group C genes have been shown to be embryo lethal, suggesting that any efforts to study the role
of these loci by reverse genetics could be confounded by lethality (Table 4.1). To our knowledge, only two group B loci have been previously studied by reverse-genetics. Like many group C loci, MAC6/AtSKIP and MAC7/EMB2765 are required for embryo development, as null mutants are embryo lethal (Tzafrir et al., 2004; Lim et al., 2009; Table 4.1). However, partial loss-of-function Atskip mutants generated by antisense technology exhibited abnormal developmental phenotypes including reduced inflorescence stems, small rosette leaves, reduced seed yield, and sensitivity to abscisic acid (Lim et al., 2009). The other four components in group B (MAC5A, MAC8/AtISY1, MAC9/AtSYF1, and MAC10/AtCRN1C) have not yet been characterized.

To study the biological function of MAC5A, we obtained three T-DNA insertion alleles from the Arabidopsis Biological Resource Center (ABRC). Salk_132881 (mac5a-1) carries an insertion in the second exon of MAC5A (At1g07360), whereas Salk_142085 (mac5a-2) and Salk_072670 (mac5a-3) carry insertions in the first intron of MAC5A (Figure 4.1A). Only plants homozygous for mac5a displayed the same morphological phenotypes, indicating that the alleles are recessive and that the phenotypes co-segregate with the mutations (not shown). We observed that the leaves of these mutants were slightly serrated, twisty, and often exhibited elongated petioles (Figure 4.1B). In addition, mac5a mutants display shortened roots compared to Col-0 plants (Figure 4.1C), are early-flowering and have reduced seed yield (not shown). Importantly, these mutant alleles do not complement each other, confirming that they are allelic (Figure 4.1B). We chose to further characterize MAC5A because mac5a plants share phenotypes with some group A mutants, namely: serrated leaves, altered flowering time, and reduced fertility.

4.3.2 MAC5A localizes to the nucleus and associates with AtCDC5 in planta

To confirm that MAC5A is indeed a MAC component, we first tested whether MAC5A localizes to the nucleus and if it is capable of associating with another MAC protein, AtCDC5, in a co-immunoprecipitation assay. For this, we stably transformed either PMAC5A-MAC5A-GFP or PMAC5A-MAC5A-HA into mac5a-1 plants by Agrobacterium-mediated transformation. The GFP lines were used for nuclear localization, and the HA lines were used for co-immunoprecipitation. The majority of transgenic lines (24/25 independent GFP lines; 21/22
independent HA lines) complemented *mac5a-1* phenotypes such as morphology and root length (Figure 4.2A-B, and data not shown). Because the fusion proteins complement *mac5a-1* phenotypes, they should function the same as endogenous *MAC5A*. All *MAC5A-GFP* complementing lines showed clear nuclear localization when viewed under a fluorescence microscope. Root cells from one representative line are shown in Figure 4.2C, but nuclear localization was also observed in other tissues including leaves (not shown). This localization was not unexpected, as the MAC5A homolog in zebrafish, RBM22, also localizes to the nucleus (Montaville et al., 2006), as do other MAC core proteins. To test if MAC5A associates with AtCDC5, we isolated total nuclear protein and immunoprecipitated MAC5A-HA using anti-HA microbeads. Western blot analysis using an anti-AtCDC5 antibody revealed that AtCDC5 is present only in the eluted fraction from transgenic plants and not from control Col-0 plants (Figure 4.3). Association between MAC5A and AtCDC5 has been shown in other eukaryotes as well (McDonald et al., 1999; Ohi et al., 2002; Deckert et al., 2006; Gavin et al., 2006; Bessonov et al., 2008). Together, these data indicate that, like all other core MAC proteins so far characterized, MAC5A localizes to the nucleus and associates with AtCDC5 *in planta*. Thus, MAC5A is indeed a member of the MAC.

### 4.3.3 MAC5A and MAC5B are partially redundant

There are two proteins encoded in the *Arabidopsis* genome with homology to MAC5A: MAC5B (At1g29580) and MAC5C (At5g07060). MAC5A and MAC5B are proteins of approximately 480 amino acids in length that are 82% identical and contain a CCCH-type zinc-finger domain and an RNA recognition motif (RRM). Conversely, MAC5C encodes a truncated protein of 363 amino acids that contains only a zinc-finger domain and no RRM (Figure 4.4). The phylogenetic relationship between these proteins indicates that MAC5A and MAC5B are more closely related to each other than to MAC5C (Addepalli and Hunt, 2008; Wang et al., 2008; Figure 4.4A). MAC5A, MAC5B, and MAC5C share homology with the human protein RBM22/hECM2/fSAP47 (42-50% identity at the amino-acid level), and share very weak homology to the yeast protein Ecm2p/Slt11p (13-16% identity). These proteins have been repeatedly isolated as components of the NTC/MAC in several eukaryotes (Ohi et al., 2002;
According to publically available microarray data (Winter et al., 2007), *MAC5A* and *MAC5B* are expressed in most plant tissues although *MAC5A* is expressed at a much higher level. We confirmed that *MAC5A* is more highly expressed than *MAC5B* in Col-0 rosette leaf tissue using semi-quantitative RT-PCR (Figure 4.5B). Conversely, *MAC5C* is expressed at low levels in dry seeds, senescent leaves and floral organs but not in leaf tissue (Winter et al., 2007). Because of the closer relationship between *MAC5A* and *MAC5B*, we were interested to see if there was some functional redundancy between these proteins. To do this, we first isolated a *mac5b* T-DNA insertion line from the European *Arabidopsis* Stock Center (NASC). GK419F11 (*mac5b-1*) carries an insertion in the first exon of At2g29580/*MAC5B* (Figure 4.5A). This particular T-DNA insertion line is annotated as also being in the promoter region of At2g29570/*PCNA2*, a locus that neighbours *MAC5B* in the opposite orientation on chromosome 2. Because of this, we examined the expression levels of both genes in *mac5b-1* compared to Col-0 using semi-quantitative RT-PCR (Figure 4.6B-C). We found that expression of *MAC5B* was undetectable in *mac5b-1*, whereas expression of *PCNA2* was not affected, indicating that *mac5b-1* is a null allele of *MAC5B*. Plants homozygous for the insertion mutation did not exhibit any aberrant phenotypes and appeared morphologically similar to Col-0 plants (Figure 4.5C).

We next attempted to create a double *mac5a-1 mac5b-1* mutant by crossing *mac5a-1* with *mac5b-1*. Out of 127 randomly chosen progeny from a parent with the genotype *MAC5A/mac5a-1; mac5b-1/mac5b-1*, 52 were wild-type for *MAC5A*, 75 were heterozygous for *mac5a-1*, and none were homozygous for *mac5a-1*, indicating a 1:2:0 ratio rather than a normal 1:2:1 ratio. A chi-squared test using these values suggests that a combination of *mac5a-1* and *mac5b-1* is lethal ($x^2=36.86$ with 2 degrees of freedom; P<0.0001). From another segregating population in which the parent’s genotype was *MAC5A/mac5a-1; MAC5B/mac5b-1*, we recovered very few plants (typically 1 or 2 out of ~100 plants) with the genotype *mac5a-1/mac5a-1; MAC5B/mac5b-1*. These plants displayed severe developmental defects including dwarfism, delayed growth, abnormal floral organs and sterility (Figure 4.5C). Terminal outgrowths known as enations were also observed extending from the leaf margins of these plants (Figure 4.5C inset). Because of the difference in expression level between these genes, we hypothesized that over-expression of *MAC5B* may be able to compensate for the loss of *MAC5A*. 
To test this, we transformed \textit{P35S-MAC5B} into homozygous \textit{mac5a-1} plants. Indeed, over-expression of \textit{MAC5B} is able to complement \textit{mac5a-1} (Figure 4.5). Together, these data indicate that \textit{MAC5A} and \textit{MAC5B} are partially redundant in a dosage-dependent manner, and that both genes are essential for viability in \textit{Arabidopsis}.

### 4.3.4 \textit{mac5a-1} suppresses \textit{snc1} autoimmunity but does not exhibit enhanced disease susceptibility

\textit{MOS4}, \textit{AtCDC5}, and \textit{MAC3A/3B} are components of the \textit{snc1} signaling pathway, as deduced by double mutant analysis between \textit{snc1} and \textit{mos4}, \textit{Atcdc5}, or \textit{mac3a mac3b} alleles (Palma et al., 2007; Monaghan et al., 2009). To test if \textit{MAC5A} is likewise required for \textit{snc1} signaling, we created a \textit{snc1 mac5a-1} double mutant and conducted suppression analysis of \textit{snc1} related phenotypes. As shown in Figure 4.7A, the loss of \textit{MAC5A} suppresses \textit{snc1}-associated dwarfism, as \textit{snc1 mac5a-1} plants are more than twice the size of \textit{snc1}. Heightened resistance to \textit{Hyaloperonospora arabidopsidis (H.a.)} isolate Noco2 and \textit{Pseudomonas syringae} pathovar \textit{tomato (P.s.t.)} strain DC3000 was also suppressed in \textit{snc1 mac5a-1}, as shown by quantification of pathogen growth in these genotypes compared to Col-0 wild-type. As shown in Figure 4.7B, whereas \textit{H.a.} Noco2 was unable to grow on \textit{snc1} leaves, \textit{snc1 mac5a-1} plants are as susceptible as Col-0 to this pathogen. Infection with \textit{P.s.t.} DC3000 indicated that \textit{snc1 mac5a-1} plants sustained 100-fold higher bacterial growth over three days compared to \textit{snc1} (Figure 4.7C). Furthermore, the constitutive expression of \textit{PR-1} and \textit{PR-2} in \textit{snc1} was partially suppressed by \textit{mac5a-1}, as shown using semi-quantitative RT-PCR (Figure 4.7D), which was also confirmed using quantitative real-time RT-PCR (not shown). Together, these data indicate that \textit{MAC5A} is partially required for \textit{snc1} mediated immunity in \textit{Arabidopsis}, which is expected given that there is some functional redundancy between \textit{MAC5A} and \textit{MAC5B}.

Mutations in \textit{MOS4}, \textit{AtCDC5}, \textit{PRL1}, and \textit{MAC3A/3B} lead to enhanced susceptibility to pathogen infection (Palma et al., 2007; Monaghan et al., 2009). To test if \textit{MAC5A} is necessary for the full expression of defense responses, \textit{mac5a} mutants were challenged with a variety of pathogens. When we infected plants with \textit{P.s.t.} DC3000 we observed similar bacterial growth in Col-0 and the \textit{mac5a} alleles after three days, whereas the well-known enhanced susceptibility mutant \textit{npr1} (Cao et al., 1994) harboured an over 10-fold higher titre of bacteria (Figure 4.8A).
To test if R-protein mediated defenses other than \textit{snc1} autoimmunity require \textit{MAC5A}, we also infected plants with pathogens that express avirulence effectors specifically recognized by R-proteins in \textit{Arabidopsis}. \textit{P.s.t. avrRps4} and \textit{P.s.t. avrPphB} (recognized by RPS4 and RPS5, respectively) grew to similar levels in Col-0 and the \textit{mac5a} mutants (Figure 4.8B-C). In addition, the \textit{H.a.} isolates Emwa1 and Cala2, expressing avirulence effectors recognized by RPP4 and RPP2, respectively, grew to similar levels in Col-0 and \textit{mac5a-1} (not shown), and both genotypes were able to mount an HR at infection sites as indicated by lactophenol trypan blue staining on leaf tissue seven days after inoculation (Figure 4.8D-E). Moreover, \textit{mac5b-1} mutant plants also did not exhibit enhanced susceptibility in the same assays (Figure 4.8A-C). Together, these data reveal that \textit{MAC5A} is required for \textit{snc1}-mediated defense, but may not be required for other immune pathways in plants. However, the involvement of \textit{MAC5A/5B} in these other pathways is likely to be masked by the partial redundancy between the two loci.

### 4.3.5 Genetic interaction between \textit{MAC5A} and \textit{MOS4, AtCDC5, PRL1} and \textit{MAC3A/3B}

All double MAC mutant combinations so far described (\textit{mos4-1 Atcdc5-1, mos4-1 prl1-1, mos4-1 mac3a mac3b, and mac3a mac3b prl1-1}) result in lethality (Palma et al., 2007; Monaghan et al., 2009). However, we were able to successfully isolate double mutants when we crossed \textit{mos4-1, Atcdc5-1, prl1-2, or mac3a mac3b} with \textit{mac5a-1}, likely due to partial redundancy between \textit{MAC5A} and \textit{MAC5B}. The morphological phenotypes of the double mutants looked like a combination of phenotypes between the respective loci (Figure 4.9A). The most striking phenotype was observed with \textit{mac5a-1 prl1-2} double mutant plants which exhibited severe developmental defects and often displayed leaf enations similar to the \textit{mac5a-1/mac5a-1 MAC5B/mac5b-1} mutant. When we infected plants with \textit{P.s.t.} DC3000 and monitored growth three days later, we did not observe a further increase in bacterial titer in any of the combination mutants (Figure 4.9B), indicating that there is no functional overlap between these loci with respect to disease resistance which is to be expected for proteins in the same complex. But again, the contribution of \textit{MAC5} in immunity in these double and triple mutants could be masked by the redundancy between \textit{MAC5A} and \textit{MAC5B}. 

98
4.4 Discussion

We previously discovered a protein complex called the MAC that is required for plant immune responses including those activated in the constitutive defense mutant *snc1* (Palma et al., 2007; Monaghan et al., 2009). We recently showed that the MAC is composed of over 20 proteins and is highly similar to the spliceosome-associated NTC conserved throughout eukaryotic kingdoms (Monaghan et al., 2009). The MAC contains proteins with diverse functions, including the scaffold MOS4, the transcription factor AtCDC5, the WD-40 protein PRL1, the E3 ubiquitin ligases MAC3A and MAC3B, as well as several nucleic-acid binding proteins and snRNP subunits that are predicted to be integral components of the spliceosome. Here we present evidence suggesting that the putative RNA-binding protein MAC5A is partially redundant with its close homolog MAC5B, and functions in *snc1*-mediated autoimmunity as a component of the MAC.

MAC5A and MAC5B are predicted to be RNA-binding proteins by virtue of containing both an RRM and a CCCH type zinc-finger motif. The most abundant RNA binding domain in eukaryotes is the RRM (Lorkovic and Barta, 2002), often found in proteins also containing CCCH motifs, such as in MAC5A/5B. There are 196 RRM-containing proteins encoded in the *Arabidopsis* genome, including several predicted poly(A)-binding proteins (PABPs), arginine-rich (SR) proteins, and small nuclear ribonucleoproteins (snRNPs; Lorkovic and Barta, 2002). Importantly, it has been demonstrated that the RRM is necessary and sufficient for RNA binding (Jessen et al., 1991; Burd and Dreyfuss, 1994). In addition to the RRM, there are several other RNA-binding domains such as the CCCH zinc-finger motif. This family is also fairly large in *Arabidopsis*, with 68 proteins encoded in the genome (Wang et al., 2008). Whereas most zinc-fingers are involved in DNA binding, several proteins containing CCCH motifs have been shown to function post-transcriptionally and have RNA binding capability (Wang et al., 2008). For example, the *Arabidopsis* protein ENHANCER OF AG-4,1 (HUA1) contains 6 tandem CCCH motifs and binds RNA *in vitro* (Li et al., 2001a). Likewise, the *Arabidopsis* cleavage and polyadenylation specificity factor AtCPSF30 contains 3 tandem CCCH motifs and has also been shown to bind RNA (Delaney et al., 2006; Wang et al., 2008). Interestingly, a recent study demonstrated that five CCCH zinc finger proteins in *Arabidopsis*, representing proteins from
diverse subfamilies, are capable of degrading RNA in vitro, uncovering the intriguing possibility that ribonuclease activity may be a property of CCCH proteins in *Arabidopsis* (Addepalli and Hunt, 2008).

It is not hard to imagine roles for RNA-binding proteins and/or ribonucleases in plant defense. A huge number of genes are differentially regulated following the perception of pathogens, and this massive change in gene expression must be correlated with subsequent RNA processing and/or degradation events. A relevant example is the glycine-rich RRM-containing protein AtGRP7 which was recently shown to be targeted by HopU1, a *Pseudomonas* type-III effector with mono-ADP-ribosyltransferase activity, to suppress the plant immune response and augment virulence (Fu et al., 2007). Loss-of-function *Atgrp7* mutants were shown to display enhanced susceptibility to infection by *Pseudomonas* in the same study. In addition, MOS2, a protein containing RNA-binding G-patch and KOW motifs, is likewise required for innate immunity in *Arabidopsis* (Zhang et al., 2005). Our finding that MAC5A/5B is partly required for *snc1*-mediated autoimmunity points to a role for this RNA-binding protein as a positive regulator of plant defense. However, we were unable to observe enhanced susceptibility in *mac5a* or *mac5b* mutants when we infected plants with a variety of pathogens, probably because of the partial redundancy between *MAC5A* and *MAC5B*.

The loss of *MOS4*, *AtCDC5*, *PRL1*, or *MAC3A/3B* result in relatively minor developmental defects in *Arabidopsis*. However, the loss of both *MAC5A* and *MAC5B* is lethal, indicating that *MAC5A/5B* performs an essential function, such as splicing. Because of the close association between the MAC and the spliceosome, we previously tested if general splicing was impaired in the MAC mutants. For this, we monitored the presence of several known alternative transcripts in MAC mutants compared to Col-0 (Palma et al., 2007). We did not observe obvious differences in the presence or abundance of *U1-70K*, *AtSRp34/SR1*, or *AtSRp30* alternative transcript variants (Palma et al., 2007). Likewise, we did not observe any changes in the presence or abundance of these alternative transcripts in *mac5a-1* or in the *mac5a-1* *mos4-1*, *mac5a-1* *mac3a* *mac3b*, *mac5a-1* *Atcdc5-1*, or *mac5a-1* *prl1-2* combination mutants (Figure 4.10). We also did not observe any changes in these transcripts in the *mac5a-1*; *MAC5B/mac5b-1* mutant (not shown). These data further support that *MOS4*, *AtCDC5*, *PRL1*, *MAC3A/3B*, and *MAC5A/5B* are not required for general splicing even though the MAC is closely associated with proteins that form the spliceosome.
So then, what is the relationship between the MAC and the spliceosome? We can only speculate that this association has to do with transcriptional co-regulation or alternative splicing of important defense genes. Uncovering what genes are regulated by AtCDC5 and what RNA species are bound by MAC5A/5B is therefore of particular interest. These remain major questions driving future research into the molecular function of the MAC.

4.5 Methods

4.5.1 Plant growth and pathology assays

Plants were grown on soil in a 16h light / 8h dark regime or on Murashige and Skoog (MS) medium supplemented with 0.5% sucrose and 0.3% phytigel, depending on the experiment. Bacterial and oomycete infections were performed as described in Li et al. (2001b). Briefly, bacterial pathogens were inoculated on the abaxial leaf surfaces of four-week old plants using a needless syringe. Leaf discs (with an area of 0.38cm²) were collected on the day of infection (Day 0) and three days later (Day 3) from different plants. H.a. isolates were spray-inoculated onto adaxial leaf surfaces of two-week old seedlings and stained using lactophenol trypan blue seven days later using a protocol described in Koch and Slusarenko (1990).

4.5.2 Mutant isolation and genetic crosses

T-DNA mutants were obtained from the ABRC or NASC and genotyped by PCR using primers flanking the insertions. mac5a-1 was isolated and genotyped using the primers 5’-CACTCCTTAGGGGAGGTATC-3’ and 5’-GGTGTTTAGGTGGCGACCTGG-3’. The other two alleles, mac5a-2 and mac5a-3, were identified first by phenotypic comparison to mac5a-1 and then confirmed by crossing to test for allelism. For this, homozygous mac5a-1 females were crossed with males homozygous for either the mac5a-2 or mac5a-3 alleles and the F1 was analyzed for complementation. mac5b-1 was isolated and genotyped using the primers 5’-CAGCTTCAACACTAAGAAAC-3’ and 5’-TAGAGTGTGGATCGAAACGG-3’. Isolation of
snc1 (Li et al., 2001b; Zhang et al., 2003), mos4-1, Atdc5-1, prl1-2 (Palma et al., 2007), and mac3a mac3b (Monaghan et al., 2009) have been previously described, as has isolation of npr1-1 (Cao et al., 1994), eds1-2 (Col) (Bartsch et al., 2006), and ndr1 (Century et al., 1995). The snc1 mac5a-1 double mutant was obtained by crossing a homozygous snc1 single mutant plant with a homozygous mac5a-1 plant. The F1 was allowed to self and the double was isolated in the F2 using a combination of phenotyping and PCR-based genotyping. A similar procedure was used to create the mac5a-1 mos4-1, mac5a-1 Atdc5-1, mac5a-1 mac3a mac3b and mac5a-1 prl1-2 double and triple mutants. All genotypes were confirmed by genotyping with mutation-specific primers.

4.5.3 Molecular cloning and expression analysis

A genomic fragment spanning the full-length MAC5A gene including its native promoter was amplified from Col-0 DNA using the primers 5’-
CGGGGTACC CGGTTCCAATGTCACCGGCAG-3’ (Kpn1) and 5’-
AAAACTGCAG CTGAGACGAACCAGTAGCTGT-3’ (Pst1) and cloned into pGreen0229 in-frame with a C-terminal HA or GFP tag (Hellens et al., 2000), and confirmed by sequencing. The open-reading frame of MAC5B was amplified from Col-0 cDNA using the primers 5’-
CACC ATGGCGCATAGAATACTGAG-3’ and 5’-TTGAGACGAACCAGTAGTAAC-3’. This Gateway-adapted PCR fragment was cloned into pENTR using the Gateway pENTR/D-Topo kit (Invitrogen). Entry vectors were confirmed by sequencing using the M13F and M13R primers. Recombination into a destination binary vector containing a constitutive 35S promoter was carried out with Gateway LR Clonase (Invitrogen). Transgenic seedlings were selected on soil with the herbicide Basta and confirmed by PCR. For mRNA expression analysis, RNA was extracted from 20 day old seedlings grown on MS medium using the Totally RNA Kit (Ambion). Reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen). The primers used to monitor MAC5A expression were 5’- TGCAAGATATGTACACGACC-3’ and 5’-AGTGCCCATCTCACCAGCTT-3’. The primers used for MAC5B expression were 5’-
GCAAATCTGCTCTTCAAGGT-3’ and 5’-AGTGCCCATCTCACCAGCTT-3’. The primers used to monitor the expression of PCNA2 were 5’-GATGGTAGCGACACTGTTAC-3’ and 5’-
CCGATATCACCTGCTGTTGA-3’. The primers used to amplify *PR-1*, *PR-2* and *Actin 1* have been described previously (Zhang et al., 2003). Primers used to detect alternative transcript variants for *U1-70K*, *AtSRp34/SR1* and *AtSRp30* are described in (Savaldi-Goldstein et al., 2003). *TUBULIN* was amplified using the primers 5’-ACGTATCGATGTCTATTTCAACG-3’ and 5’-ATATCGTAGAGACCCCTATTGTCC-3’.

### 4.5.4 Nuclear protein extraction and immunoprecipitation

Approximately 15g of leaf tissue from complementing *PMAC5A-MAC5A-HA* in *mac5a-1* was used to isolate the nuclear protein fraction with a procedure described in (Monaghan et al., 2009). Immunoprecipitation was carried out using anti-HA microbeads (Miltenyi Biotec Inc.) as described in (Monaghan et al., 2009). The eluted fraction was loaded on a 12% SDS-PAGE gel followed by Western Blot analysis using anti-HA (Roche) or anti-AtCDC5 (Palma et al., 2007) antibodies.

### 4.6 Manuscript acknowledgements

We thank Mark Tessaro (UBC, Vancouver) for his assistance isolating T-DNA insertion lines provided by the *Arabidopsis* Biological Resource Center (ABRC; Ohio State University). This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Canadian Foundation for Innovation to XL and by a grant from the National Science Foundation of the People’s Republic of China to YZ. JM is supported by an NSERC post-graduate scholarship (PGS).
Table 4.1: Summary of phenotypes reported for MAC mutants in *Arabidopsis*.

A comprehensive literature search identified several reported loss-of-function alleles in MAC components.

**References:** (1) Palma et al., 2007; (2) Monaghan et al., 2009; (3) Nemeth et al., 1998; (4) This work; (5) Lim et al., 2009; (6) Tzafrir et al., 2004; (7) Moll et al., 2008; (8) Liu et al., 2008; (9) Yagi et al., 2009; (10) Pagnussat et al., 2004; (11) Herr et al., 2006.
Table 4.1: Summary of phenotypes reported for MAC mutants in *Arabidopsis*.

<table>
<thead>
<tr>
<th>MAC #</th>
<th>Protein</th>
<th>AGI</th>
<th>Molecular Function</th>
<th>Polymorphisms</th>
<th>Allele</th>
<th>Reported Phenotypes</th>
<th>Ref</th>
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<td><strong>MAC Core Proteins</strong></td>
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<td>MAC1</td>
<td>AtCDC5</td>
<td>At1g09770</td>
<td>R2R3 Myb Transcription Factor</td>
<td>Atcdc5-1 (Sail_207_F03)</td>
<td>null</td>
<td>serrated leaves, late flowering, EDS, male sterile</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Atcdc5-2 (GK_276800)</td>
<td>null</td>
<td>serrated leaves, late flowering, EDS</td>
<td>1</td>
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<tr>
<td>MAC3A</td>
<td>MAC3A</td>
<td>At1g04510</td>
<td>E3 ligase</td>
<td>mac3a (Sail_089300)</td>
<td>partial</td>
<td>single mutant is indistinguishable from Col-0; double mac3a mac3b mutant displays flattened leaves, late flowering, EDS</td>
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<td></td>
<td></td>
<td></td>
<td><em>Redundancy with MAC3B</em></td>
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<td>MAC3B</td>
<td>MAC3B</td>
<td>At2g33340</td>
<td>E3 ligase</td>
<td>mac3b (Sail_050811)</td>
<td>null</td>
<td>single mutant is indistinguishable from Col-0; double mac3a mac3b mutant displays flattened leaves, late flowering, EDS</td>
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<td></td>
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<td><em>Redundancy with MAC3A</em></td>
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<td>MOS4</td>
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<td>mos4-1</td>
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<td></td>
<td></td>
<td>mos4-2 (Sail_019535)</td>
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<td><strong>MAC2</strong></td>
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<td>At4g15900</td>
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<td>prl1-1 (Sail_008644)</td>
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<td>prl1-2 (Sail_039427)</td>
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<td>prl1-3 (Sail_096289)</td>
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<td><em>Partial redundancy with At2g29580/MAC5B</em></td>
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<td></td>
<td>mac5a-3 (Sail_072670)</td>
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<td>MAC6</td>
<td>AtISKIP</td>
<td>At1g77180</td>
<td>Transcriptional activation</td>
<td>Atiskip antisense lines</td>
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<td>dwarfism, severe developmental defects</td>
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<td>At2g38770</td>
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<td>emb2765-1 (SK147287)</td>
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<td>embryo lethal; embryos terminate at the globular stage</td>
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<td>emb2765-2 (SK141518)</td>
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<td>At1g06220</td>
<td>U5 snRNP; translation elongation; nucleic acid binding</td>
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<td>GFA1</td>
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<td>Similar to At1g561140/AtU5-200-4, At2g42270/AtU5-200-3</td>
<td>sgt13018</td>
<td>partial</td>
<td>embryo and gametophyte defects</td>
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<td>similar to At2g61140/AtU5-200-1, At3g27739/AtU5-200-4</td>
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<td>defects in the directional control of floral organ development</td>
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<td>ME5</td>
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<td>Similar to At1g56070/AtU5-116-2, At3g22980/AtU5-116-3</td>
<td>vaj-2 (Sail_143877)</td>
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<td>VAJ</td>
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<td>embryo lethal; embryos arrest at the globular stage</td>
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<td>Similar to At5g61140/AtU5-200-4, At2g42270/AtU5-200-3, At5g27739/AtU5-200-4</td>
<td>emb1507-2 (CS16091)</td>
<td>NT</td>
<td>embryo lethal; embryos arrest at the globular stage</td>
<td>6</td>
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<td>ESP3</td>
<td>At1g32490</td>
<td>DEAH RNA helicase</td>
<td>(C24) esp3-1 through 7</td>
<td>partial</td>
<td>serrated leaves, early flowering, altered RNA processing and silencing</td>
<td>11</td>
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<td>EMB2733</td>
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<td>Similar to At2g35340/MEE29/AtPRP-2,1B, At1g16680/AtPRP-2</td>
<td>emb2733 (CS16184)</td>
<td>NT</td>
<td>embryos terminate at the globular stage; embryo lethal</td>
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<td>MAC16</td>
<td>SUS2/EMB177</td>
<td>At1g80070</td>
<td>U5 snRNP</td>
<td>sus2+ (CS16072)</td>
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<td>embryo lethal; embryos arrest at the globular stage and exhibit abnormal suspensors</td>
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<td>Similar to At4g38780/AtPRP3B</td>
<td>sus2-5 (CS16073)</td>
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<td>embryo lethal; embryos arrest at the globular stage and exhibit abnormal suspensors</td>
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<td>AaSAP155</td>
<td>At5g64270</td>
<td>U2 snRNP; protein binding</td>
<td>not reported</td>
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Figure 4.1: Isolation of \textit{mac5a} T-DNA insertion mutants.

(A) Gene structure of \textit{MAC5A} (At1g07360) showing the position of T-DNA insertions Salk\_132881 (\textit{mac5a-1}), Salk\_142085 (\textit{mac5a-2}), and Salk\_072670 (\textit{mac5a-3}). Lines indicate introns and boxes indicate exons. The location of translation start (ATG) and stop (TGA) codons are found in the first and last exons, as indicated. (B) Morphology of Col-0, \textit{mac5a-1}, \textit{mac5a-2}, and \textit{mac5a-3} plants, as well as representative individuals from allelic crosses. Soil-grown plants were photographed 3 weeks after planting. Size bar represents 1 cm. (C) \textit{mac5a} mutants exhibit short roots compared to Col-0. Plants were grown on Murashige and Skoog (MS) media supplemented with 0.5\% sucrose and 0.3\% phytigel for 8 days. Values represent the mean of 10 replicates ± SD. An unpaired Student’s \textit{t}-test was used to analyze the statistical significance of root length compared to Col-0. Asterisks indicate P<0.005. This experiment was repeated three times with similar results.
Figure 4.2: Sub-cellular localization of MAC5A.

(A) Full-length genomic \textit{PMAC5A-MAC5A-GFP} driven by its native promoter complements \textit{mac5a-1} morphology. Soil-grown plants were photographed 4 weeks after germination. Size bar represents 1 cm. (B) Root length assay demonstrating functional complementation of \textit{mac5a-1} by the \textit{MAC5A-GFP} transgene. Plants were grown on MS media supplemented with 0.5% sucrose and 0.3% phytagel for 8 days. Values represent the mean of 10 replicates ± SD. An unpaired Student’s \textit{t}-test was used to analyze the statistical significance of root length compared to Col-0. Asterisk indicates P<0.0001. This experiment was repeated three times with similar results. (C) \textit{MAC5A-GFP} localizes to the nucleus. Confocal microscopy was used to examine the localization of \textit{MAC5A-GFP} in the transgenic line shown in A. Root cells from a representative line are shown. DAPI was used as a control for nuclear localization.
Figure 4.3: MAC5A-HA and AtCDC5 co-immunoprecipitate \textit{in planta}.

Total nuclear extracts were isolated from a complementing \textit{mac5a-1} transgenic line expressing \textit{PMAC5A-MAC5A-HA} (+) and Col-0 (-). MAC5A-HA was immunoprecipitated using anti-HA microbeads. MAC5A-HA and AtCDC5 were detected in the eluted fractions by Western blot analysis using antibodies against HA or AtCDC5.
Figure 4.4: Multiple sequence alignment of MAC5A homologs in *Arabidopsis*, human, and budding yeast.

(A) Comparison of MAC5A, MAC5B, and MAC5C gene structures and encoded protein domains as adapted from Wang et al. (2008). (B) Amino acid sequences from *Arabidopsis thaliana* MAC5A (accession number NP_563788), MAC5B (accession number NP_180518) and MAC5C (accession number NP_196323), *Homo sapiens* HsRBM22 (accession number NP_060517), and *Saccharomyces cerevisiae* ScEcm2 (accession number NP_009621) are compared. Identical amino acids are coloured black, and similar amino acids are coloured grey. Alignment was generated using ClustalW2. Boxshade version 3.21 was used to colour identical and similar amino acids. The location of the CCCH zinc-finger (signature CCCH residues are marked with asterisks) and the RNA recognition motif are indicated.
Figure 4.5: *MAC5A* and *MAC5B* are partially redundant.

(A) Gene structure of *MAC5B* (At2g29580) showing the position of T-DNA insertion in GK419F11 (*mac5b-1*). Lines indicate introns and boxes indicate exons. The location of translation start (ATG) and stop (TGA) codons are found in the first and last exons, as indicated. (B) Expression analysis of *MAC5A* and *MAC5B* compared to *ACTIN* in the respective genotypes, as indicated by equal cycles of semi-quantitative RT-PCR. This experiment was repeated using cDNA isolated from four independent experiments with similar results. (C) Morphology of soil-grown plants, photographed 4 weeks after planting. Arrows in the enlarged photo on the right indicate leaf enations. Size bar represents 1 cm.
Figure 4.6: PCNA2 expression is not affected in the mac5b-1 mutant.

(A) Schematic diagram representing the neighbouring genes At2g29570/PCNA2 and At2g29580/MAC5B in the opposite orientation on chromosome 2 in Arabidopsis. Arrows represent the genes and their direction indicates orientation. The location of the T-DNA insertion GK419F11 is indicated. Semi-quantitative RT-PCR analysis of At2g29570/PCNA2 (B) and At2g29580/MAC5B (C) in GK419F11 compared to Col-0 indicates that this insertion mutant is a MAC5B null and does not affect the expression of the neighbouring gene. ACTIN1 is included as a control. This experiment was repeated twice with the same results.
Figure 4.7: mac5a-1 suppresses snc1 auto-immune phenotypes.

(A) Morphology of soil-grown plants, photographed 3 weeks after planting. Size bar represents 1 cm. (B) Growth of H.a. isolate Noco2 seven days after infection with 4 x 10^4 spores/ml in water. This experiment was repeated three times with similar results. (C) Growth of P.s.t. DC3000 at 0 and 3 days after infection. Values represent an average of four replicates ± SD. This experiment was repeated six times with similar results. An unpaired Student’s t-test was used to analyze the statistical significance of pathogen growth compared to snc1 in (B) and (C); asterisks indicate P<0.0001 in both graphs. (D) Expression analysis of PR-1 and PR-2 compared to ACTIN1 using semi-quantitative RT-PCR. This experiment was repeated using cDNA isolated from five independent experiments with similar results.
Figure 4.8: Infection assays on MAC5A and MAC5B loss-of-function alleles.

(A-C) Growth of the bacterial pathogens *P.s.t.* DC3000, *P.s.t. avrRps4*, and *P.s.t. avrPphB*, at 0 and 3 days post-inoculation. Values represent an average of four replicates ± SD. An unpaired Student's *t*-test was used to analyze the statistical significance of bacterial growth compared to Col-0. Asterisks indicate P<0.0001 in all graphs. These experiments were repeated several times with similar results. (D-E) Lactophenol trypan blue staining of leaves from plants infected with *H.a.* isolates Emwa1 (D) and Cala2 (E), seven days after infection. HR: hypersensitive response; M: mycelia. *eds1-2 (Col-0)* is included as an experimental control. This experiment was repeated twice with similar results.
Figure 4.9: Double or triple mutant analysis between *mac5a-1* and other MAC mutants.

(A) Morphology of soil-grown plants, photographed 4 weeks after planting. Size bar represents 1 cm. (B) Growth of the bacterial pathogen *P.s.t.* DC3000 at 0 and 3 days post-inoculation. Values represent an average of four replicates ± SD. This experiment was repeated five times with similar results.
Figure 4.10: General splicing is not affected in MAC single, double, or triple mutants.

The presence of alternative transcript variants for the genes UI-70K, AtSRp34/SR1, and AtSRp30 were monitored in Col-0 compared with the MAC mutants mos4-1, Atcdc5-1, prl1-2, mac3a mac3b, mac5a-1 and the combination mutants. An equal amount of RNA from all genotypes was used to make cDNA for subsequent analysis by semi-quantitative RT-PCR. TUBULIN is included as a control. This experiment was repeated four times from independent trials. The schematic to the right represents the nature of the alternative transcript variants as adapted from Palma et al. (2007).
4.7 References


117


The HEAT-repeat protein ENHANCED DISEASE SUSCEPTIBILITY17 functions in plant immunity\textsuperscript{1,2}


\textsuperscript{2} Please note that *EDS17* was renamed *ILITHYIA* in accordance with Johnston et al., 2007 Genome Biol 8:R204, a paper we were previously unaware of, in which At1g64790/*ILA*/*EDS17* is very briefly characterized as a locus required for embryogenesis.
5.1 Summary

Plant innate immunity is mediated in part by Resistance (R) proteins that detect pathogens and mount a robust defense response to fight against infection. We previously characterized proteins in the MOS4-Associated Complex (MAC) that function in the regulation of plant immune responses downstream of the auto-activated R protein snc1. The MAC is a highly conserved spliceosome-associated complex homologous to the Nineteen Complex (NTC) in yeast and human. The availability of proteomics datasets in these organisms allowed us to systematically test the biological function of additional putative MAC proteins based on protein sequence homology and reverse genetics. In this study, we investigate the role of the GENERAL CONTROL NONDEREPRESSIBLE1 (GCN1) homolog in Arabidopsis. GCN1 was previously isolated as a novel component of the NTC in one proteomics study of the human spliceosome. We identified the GCN1 homolog in Arabidopsis and partially characterized its function using available T-DNA insertion mutants. This protein, which we named ENHANCED DISEASE SUSCEPTIBILITY17 (EDS17), is an integral component of basal resistance against Pseudomonas syringae as well as resistance conditioned by specific NB-LRR R proteins. Furthermore, EDS17 is required for systemic acquired resistance (SAR). Previous proteomic identification of MAC components in Arabidopsis did not identify EDS17, and epistasis analysis between snc1 and eds17 revealed that EDS17 does not function in snc1-mediated resistance. Thus, EDS17 is probably not a MAC component. However, our results indicate that EDS17 plays a vital function in plant immunity.

5.2 Introduction

Plants possess a multi-layered immune system to fight against pathogen infection. Basal-level defenses are mediated in part by pattern recognition receptors (PRRs) that reside in the plant cell membrane and detect highly conserved features of microbes known as microbe-associated molecular patterns (MAMPs). PRR activation leads to MAPK
signaling and the activation of several defense genes (Zipfel, 2008). In addition to PRRs, plants are also equipped with intracellular Resistance (R) proteins that recognize specific pathogen virulence factors known as effectors (Van der Biezen and Jones, 1998; van der Hoorn and Kamoun, 2008). Most functionally characterized R proteins are predicted to be cytosolic and belong to the nucleotide-binding, leucine-rich repeat (NB-LRR) family (Jones and Dangl, 2006). These proteins can be subdivided into two main groups based on their amino-terminal end, consisting of either a coiled-coil (CC; also known as a leucine zipper, LZ) or a Toll/Interleukin-1 receptor-like (TIR) domain. It has now been well established that CC- and TIR-type NB-LRR proteins generally signal through two independent pathways that later converge upon a common set of resistance responses such as the expression of PATHOGENESIS RELATED (PR) genes. CC-NB-LRR proteins tend to signal through the membrane-associated protein NON-RACE SPECIFIC DISEASE RESISTANCE1 (NDR1), while TIR-NB-LRR proteins utilize a signaling complex consisting of ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1), PHYTOALEXIN DEFICIENT4 (PAD4), and SENESCENCE ASSOCIATED GENE101 (SAG101; Aarts et al., 1998; Feys et al., 2005). R protein activation is associated with a robust defense response characterized by a burst of reactive oxygen species (ROS), a change in apoplastic pH, the induction of PR genes, and the accumulation of the phytohormone salicylic acid (SA). These events signal the onset of a programmed cell death cascade known as the hypersensitive response (HR) to restrict the growth of biotrophic pathogens. The accumulation of SA and the HR are often associated with the induction of a long-lasting defense response known as systemic acquired resistance (SAR), in which tissues distal to the site of infection are primed against further infection from a broad range of pathogens (Durrant and Dong, 2004).

A gain-of-function mutation in the gene encoding the TIR-NB-LRR R protein SUPPRESSOR OF NPR1-1, CONSTITUTIVE1 (SNC1) results in the accumulation of SA, the constitutive expression of PR defense markers, and enhanced resistance against pathogen infection (Li et al., 2001; Zhang et al., 2003). In a screen for suppressors of the constitutive defense responses in snc1, a number of modifier of snc1 (mos) mutants were identified. Analysis of the corresponding MOS genes revealed a complicated signaling network downstream of R protein activation involving nucleo-cytoplasmic trafficking.
(Palma et al., 2005; Zhang and Li, 2005; Cheng et al., 2009), RNA processing (Zhang et al., 2005b; Palma et al., 2007) and protein modification (Goritschnig et al., 2007; Goritschnig et al., 2008). MOS4 encodes a nuclear protein with a C-terminal amphipathic α-helix (also called a CC domain) predicted to engage in protein-protein interactions. In a yeast two-hybrid screen for potential MOS4 interacting proteins, it was found that MOS4 directly binds CELL DIVISION CYCLE5 (AtCDC5), an atypical R2R3-Myb transcription factor (Palma et al., 2007). Interestingly, homologs of MOS4 and AtCDC5 are components of the highly conserved spliceosome-associated Nineteen Complex (NTC) in yeast and the CDC5L-complex in human (Ohi et al., 2002; Zhou et al., 2002). Proteomics datasets from several studies on the protein components of the spliceosome (Zhou et al., 2002; Deckert et al., 2006; Bessonov et al., 2008; Herold et al., 2009), NTC (Chen et al., 2002; Ohi and Gould, 2002; Ohi et al., 2002; Gavin et al., 2006), and the CDC5L complex (Ajuh et al., 2000; Zhang et al., 2005a) allowed us to hypothesize what proteins may be part of the MOS4-Associated Complex (MAC) in Arabidopsis. Based on these data, we shortlisted putative core components of the MAC including homologs of CDC5L/Cef1p (AtCDC5), SPF27 (MOS4), PRL1/Prp46, and Prp19. We recently purified the MAC by affinity chromatography and identified its components by mass spectrometry (Monaghan et al., 2009). Using genetics and biochemical approaches, we demonstrated that AtCDC5, MOS4, PRL1, MAC3A, and MAC3B (AtPrp19) do in fact comprise the MAC core complex in plants (Palma et al., 2007; Monaghan et al., 2009). Loss-of-function mutations in MOS4, AtCDC5, or MAC3A/3B suppress the autoimmune phenotypes of snc1 and lead to enhanced pathogen susceptibility (Palma et al., 2007; Monaghan et al., 2009), indicating that the MAC plays an important role in immune signaling.

Interestingly, one human proteomics effort identified a protein in the core complex that had not been isolated in any other study (Ajuh et al., 2000). This protein, called CDC5L-COMPLEX ASSOCIATED PROTEIN6 (CCAP6; also known as GENERAL CONTROL NONDEREPRESSIBLE1, GCN1), contains a series of HEAT repeats and likely functions as a protein-protein interaction module. We used the human GCN1 protein sequence to identify homologous sequences in Arabidopsis and found that there is a single GCN1-like protein encoded in the Arabidopsis genome. We named this
gene **ENHANCED DISEASE SUSCEPTIBILITY17 (EDS17)** based on its role in plant immune responses (below). This was also to avoid confusion with the gene **AtGCN1** (At5g60790), which encodes an ABC transporter and is unrelated to **EDS17**. We obtained three T-DNA insertion alleles from the *Arabidopsis* Biological Resource Center (ABRC) and found that **eds17** mutants display enhanced susceptibility to pathogen infection. However, we did not identify EDS17 as a potential MAC component in our proteomics analysis (Monaghan et al., 2009). Furthermore, epistasis analysis revealed that **EDS17** does not function downstream of **snc1** like other MAC mutants, providing further evidence that this protein is probably not a MAC component. EDS17 is, however, an integral component of plant defense signaling, as loss-of-function **eds17** mutants display enhanced susceptibility to virulent and avirulent pathogens as well as defects in SAR. Our results suggest that EDS17 plays an important role in plant defense signaling.

### 5.3 Results

#### 5.3.1 The loss of **EDS17** causes pleiotropic defects in *Arabidopsis*

To identify proteins with similarity to GCN1 in *Arabidopsis*, we used the human and yeast proteins as queries in the Basic Local Alignment Search Tool (BLAST). One protein, encoded by At1g64790, is homologous to human GCN1 (35% identity, 54% similarity) and yeast GCN1 (33% identity, 53% similarity). The phylogenetic relationship between GCN1-like proteins in several species is shown in Figure 5.1. EDS17 and related proteins in other species contain multiple helical HEAT-repeats predicted to facilitate protein-protein interaction (Andrade and Bork, 1995). HEAT-repeats are found in several very large proteins, including those that define the domain: Huntingtin, Elongation Factor 3 (EF3), A subunit of Protein Phosphatase 2A (PP2A), and TOR1 (Andrade and Bork, 1995).

EDS17 is encoded by a 17 kb gene with 59 introns that are spliced out to yield a 7.6 kb transcript and a predicted protein of 2441 amino acids. To investigate the role of
using a reverse-genetics approach, we obtained three T-DNA insertion alleles from the ABRC. Salk_149084 (eds17-1) and Salk_119854 (eds17-2) carry insertions in the 22\textsuperscript{nd} and 24\textsuperscript{th} exons, respectively, whereas Salk_041123 (eds17-3) carries an insertion in the 54\textsuperscript{th} exon of EDS17 (Figure 5.2A). Homozygous lines were identified by PCR-based genotyping using primers flanking the insertion region. The eds17-1 and eds17-2 mutants are much smaller in size than Col-0 wild-type and they have serrated leaves that are yellow to light green in colour, especially in emerging leaves (Figure 5.2B). This colouration extends to the inflorescence stems and cauline leaves of these plants as well (not shown). In addition, eds17-1 and eds17-2 mutant plants often display terminal outgrowths on the abaxial leaf margins known as enations (Figure 5.2C; Eshed et al., 2004; Dinneny et al., 2006). Conversely, eds17-3 has only slightly serrated leaves, is greener in colour than the other alleles, and does not exhibit leaf enations. EDS17 is also required for plant fertility, as eds17-1 and eds17-2 mutants are male-sterile. Reciprocal crosses with Col-0 indicated that sterility is the result of a pollen defect, because eds17-1 and eds17-2 female organs are easily fertilized by Col-0 pollen (not shown). Siliques from the eds17-1 and eds17-2 mutants are unfertilized and much smaller compared to Col-0 and eds17-3, as shown in Figure 5.2D. Based on the severity of phenotypes observed in the three mutant alleles, it is likely that eds17-1 and eds17-2 represent complete loss-of-function alleles, whereas eds17-3 could be a partial loss-of-function allele. Importantly, co-segregation analysis revealed that these phenotypes are only present in plants homozygous for the insertions, indicating that the alleles are recessive and that the observed phenotypes are caused by the insertion mutations. We tested for allelism between eds17-3 and eds17-1 or eds17-2 by crossing eds17-1 or eds17-2 females with eds17-3 males. The F\textsubscript{1} plants retained eds17 phenotypes confirming that these mutations are indeed allelic (Figure 5.2B).

Pale or yellow leaves are commonly associated with chlorophyll deficiencies in plants. To test if the yellow leaves observed in eds17 mutants (Figure 5.3A) correlate with a decrease in chlorophyll, we measured the levels of chlorophylls A and B in 3-week old soil-grown plants. As shown in Figure 5.3B, eds17-1 and eds17-2 show drastically lower levels of chlorophyll compared to Col-0 wild-type. eds17-3 plants also have less chlorophyll than Col-0, but these levels are markedly higher than the eds17-1 and eds17-
2 mutants which have visibly yellower leaves. Chlorophyll deficiencies can be correlated with a decrease in expression of \textit{LIGHT HARVESTING COMPLEX B1} (\textit{LHCB1}; Lopez-Juez et al., 1998). To test if this was the case with \textit{eds17} mutants, we examined the steady-state transcript levels of \textit{LHCB1} using semi-quantitative RT-PCR (Figure 5.3C). The \textit{eds17-1} and \textit{eds17-2} alleles exhibited a clear decrease in \textit{LHCB1} expression compared to Col-0, while no obvious decrease in expression in the \textit{eds17-3} allele could be observed. These data show that the yellow-leaf phenotype associated with \textit{eds17} mutants is related to a decrease in chlorophyll levels and expression of \textit{LHCB1}.

\subsection*{5.3.2 \textit{EDS17} is required for basal defense}

In plants, the perception of general pathogen elicitors such as MAMPs activates basal-level defense responses (da Cunha et al., 2007). Basal defense is typically studied in \textit{Arabidopsis} by infecting plants with a low dose (OD$_{600}$=0.0001) of a virulent pathogen and monitoring \textit{in planta} growth over time. In this way, plant genotypes with enhanced disease susceptibility (EDS) compared to wild-type can be identified. To test if the basal resistance response relies on \textit{EDS17}, we infected plants with the virulent pathogen \textit{Pseudomonas syringae pv. tomato} (\textit{P.s.t.}) strain DC3000 and bacterial growth was assayed after three days. As shown in Figure 5.4A, bacteria grew approximately 60-fold higher in the \textit{eds17-1} and \textit{eds17-2} mutants and 12-fold higher in the \textit{eds17-3} mutant compared to Col-0 wild-type. Comparatively, bacteria grew about 100-fold higher in the well characterized EDS mutant \textit{nonexpressor of PR genes1} (\textit{npr1}).

There are several defense marker genes such as \textit{PR-1} that are up-regulated upon pathogen infection (Cao et al., 1994). To test if \textit{EDS17} expression is likewise regulated, we infected plants with either 10mM MgCl$_2$ (mock), or \textit{P.s.t.} DC3000 (OD$_{600}$=0.005) and monitored mRNA accumulation using semi-quantitative RT-PCR 24 hours later. As shown in Figure 5.4B, while \textit{PR-1} expression is up-regulated following infection with \textit{P.s.t. DC3000}, \textit{EDS17} is expressed at the same level in both treatments – a trend that can be corroborated by publically available \textit{Arabidopsis} microarray data (Winter et al., 2007). Thus, \textit{EDS17} is not likely regulated at the transcriptional level by pathogen infection.
Additionally, we tested whether EDS17 is required for basal defense against Hyaloperonospora arabidopsidis (H.a.) isolate Noco2 (Figure 5.4C), an isolate which is virulent on Col-0 plants. For this, we inoculated eds17-3 plants with H.a. Noco2 spores at a concentration of $2.5 \times 10^4$ spores/ml in water and compared oomycete growth a week later. Unlike npr1-1, eds17-3 plants were not significantly compromised in defense against this pathogen as oomycete growth was similar to Col-0 in several independent experiments (Figure 5.4C). These data suggest that EDS17 is required for basal defense against P.s.t. DC3000 but may not be required for defense against H.a. Noco2. We could not test Noco2 susceptibility in the stronger eds17-1 and eds17-2 alleles due to the fact that homozygotes need identified from a segregating population. Noco2 infections are done on young 2-week old seedlings and it is not possible to correctly identify eds17-1 and eds17-2 homozygous plants until they are approximately 3 weeks old, as they are slow to germinate compared to Col-0. Thus, we cannot say for certain whether EDS17 is required for immunity against H.a. Noco2.

Many pathogens are unable to cause disease on an entire plant species as the result of nonhost resistance (da Cunha et al., 2007). Nonhost resistance is studied by inoculating plants with non-virulent or non-adapted pathogens and monitoring pathogen growth over time. We chose to test this response in eds17 mutants using a P.s.t. DC3000 strain lacking HrpA, a component of the type-three secretion system (T3SS). P.s.t. relies on the TTSS to deliver bacterial effectors into Arabidopsis cells as part of its virulence strategy (da Cunha et al., 2007). Thus, by deleting HrpA, this P.s.t. strain effectively becomes non-virulent. Although P.s.t. DC3000 HrpA- grew to similar levels in Col-0 and the eds17-3 mutant three days after infection (Figure 5.4D), we repeatedly observed 5-fold higher bacterial growth in the eds17-1 and eds17-2 mutants. These data indicate that EDS17 is also required for nonhost resistance in Arabidopsis. This also suggests that the very high growth of P.s.t. DC3000 in these alleles is likely the result of combined defects in basal and nonhost resistance.
5.3.3 *EDS17* is required for defense mediated by a subset of R proteins

To test if R proteins require EDS17, we infected plants with avirulent pathogens carrying effectors specifically recognized by CC- and TIR-NB-LRR R proteins. Plants were infected with *P.s.t.* DC3000 expressing either *avrPphB* (recognized by the CC-NB-LRR R protein RESISTANT TO PSEUDOMONAS SYRINGAE5; RPS5) or *avrRps4* (recognized by the TIR-NB-LRR R protein RPS4), and bacterial growth was assayed after three days. Whereas Col-0 plants are highly resistant to these avirulent pathogens, as indicated by very little growth after 3 days, *eds17-3* plants sustain an over 10-fold higher titer of both avirulent pathogens (Figure 5.5A-B). This can be compared to growth in either the *ndr1* or *eds1-2* (Col-0) mutants which are fully required for CC- or TIR-NB-LRR mediated defense responses, respectively. To test if the TIR-NB-LRRs RESISTANT TO PERONOSPORA PARASITICA 4 (RPP4) and RPP2 signal through *EDS17*, we also infected plants with *H.a.* isolates Emwa1 and Cala2. Although we did not observe an increase in oomycete growth between Col-0 and *eds17-3* (Figure 5.5C-D), we occasionally observed trailing necrosis in *eds17-3* plants infected with *H.a*. Emwa1 (Figure 5.5C). This suggests that RPS4, RPS5, and to a lesser extent RPP4, signal through *EDS17*, but that this locus is not required for RPP2 signaling.

5.3.4 *EDS17* does not function in *snc1*-mediated immunity

To test whether *EDS17* functions in the *snc1* pathway, we crossed *eds17-3* with *snc1* to create a *snc1 eds17-3* double mutant for suppression analysis. We found that this mutation in *EDS17* does not, even partially, suppress any of the *snc1* phenotypes we assayed. The *snc1 eds17-3* double mutant looks morphologically like *snc1*, however they retain the virescent phenotype associated with *eds17-3* (Figure 5.6A). Also, constitutive expression of *PR2* in *snc1* is not suppressed by *eds17-3*, as shown using the *PR2promoter-GUS* reporter present in the *snc1* background, whereas *PR2* expression is virtually absent in Col-0 plants carrying *PR2pro-GUS* (Figure 5.6B). To test if *snc1*-associated enhanced resistance against *H.a*. Noco2 is maintained in *snc1 eds17-3*
mutants, we infected plants with $1 \times 10^6$ spores/ml in water and monitored growth seven days later. As shown in Figure 5.6C, oomycete growth was similar in snc1 and snc1 eds17-3, whereas Col-0 sustained much higher pathogen growth. Together, these data indicate that snc1 does not signal through EDS17.

### 5.3.5 EDS17 is required for SAR

Systemic acquired resistance (SAR) is a phenomenon whereby attempted infection by an avirulent pathogen primes systemic tissues against further infection by a broad range of pathogens (Durrant and Dong, 2004). To test if EDS17 is required for this systemic response, we pre-inoculated plants with an HR-causing dose (OD$_{600}$=0.2) of P.s.t. DC3000 expressing avrRps4 or 10 mM MgCl$_2$, and then infected systemic leaves 24 hours later with P.s.t. DC3000. Three days later we quantified bacterial growth. As shown in Figure 5.7A, eds17-3 is impaired in SAR, as bacterial growth (Figure 5.7A) and symptom development (Figure 5.7B) were similar in plants pre-treated with avrRps4 or MgCl$_2$. Conversely, Col-0 plants exhibit a decrease in bacterial growth and did not show disease symptoms when pre-treated with avrRps4. This indicates that SAR requires EDS17.

The establishment of SAR is associated in many cases with the induction of PR-1 in systemic leaves. To test if the SAR defect in eds17-3 is the consequence of the inability to induce systemic PR-1 expression, we infected plants with either 10mM MgCl$_2$ or an HR-causing dose (OD$_{600}$=0.2) of P.s.t. avrRps4 and collected infected (local) and uninfected (systemic) tissue from the same plants 24 hours later. Semi-quantitative RT-PCR was conducted on cDNA isolated from these samples. Interestingly, we found that eds17-3 mutant plants maintain the ability to induce PR-1 expression in systemic leaves (Figure 5.7B), which can be compared to npr1 tissues that do not systemically express PR-1. These data indicate that EDS17 is required for SAR independent of systemic PR-1 expression.
5.4 Discussion

In this study we show that the HEAT-repeat protein EDS17 plays a critical role in several plant immune responses. Loss of function eds17 alleles display increased susceptibility to the virulent bacterial pathogen P.s.t. DC3000, indicating a role for EDS17 in basal defense against Pseudomonas syringae. Also, the eds17-1 and eds17-2 mutants displayed enhanced susceptibility to P.s.t. DC3000 HrpA-, suggesting a role for this locus in nonhost resistance as well. We also found that EDS17 is required for defense responses conditioned by specific CC- and TIR-type NB-LRR R proteins, and is also required for avrRps4-induced SAR. Taken together, these data show that EDS17 represents a key component of plant disease resistance. Furthermore, eds17 mutants display aberrant leaf shape, are male sterile, and exhibit a deficiency in chlorophyll leading to a characteristic yellow-leaf phenotype. Our results show that EDS17 defines a locus that is required for several biological processes in plants, including leaf development, pollen fertility, chlorophyll biosynthesis, and bacterial defense signaling.

Homologs of EDS17 are found in a variety of eukaryotes, but these proteins have not been well characterized in any organism except for baker’s yeast. Yeast GCN1 contains a domain similar to translation elongation factor 3 (EF3; Marton et al., 1997) that associates with cytosolic ribosomes and plays a key role in protein translation during amino acid starvation (Sattlegger and Hinnebusch, 2005). There does not appear to be any EF3-like domain in EDS17, and, unlike GCN1, EDS17 has not been isolated with cytosolic ribosomes (Chang et al., 2005; Giavalisco et al., 2005). Moreover, the phylogenetic relationship between yeast GCN1 and Arabidopsis EDS17 is fairly divergent overall, suggesting that these proteins may have evolved different functions. A human protein with homology to yeast GCN1, CCAP6, was isolated as a core component of the CDC5L complex in one study (Ajuh et al., 2000). This led us to investigate whether the Arabidopsis GCN1 homolog is a component of the MAC, a protein complex with homology to the NTC/CDC5L complex. However, several lines of evidence suggest that EDS17 is probably not part of the MAC in Arabidopsis. The recently purified MAC did not contain EDS17 in the MOS4-HA fraction (Monaghan et al., 2009). Moreover,
while the MAC mutants mos4, Atcdc5, and mac3a mac3b suppress snc1 auto-immunity (Palma et al., 2007; Monaghan et al., 2009), eds17-3 failed to suppress snc1 phenotypes and is therefore not part of the snc1 signaling pathway. Together, these data suggest that EDS17 functions in a defense pathway separately from the MAC.

Our data indicate that EDS17 is required for resistance mediated by a subset of TIR-NB-LRR proteins (namely: RPS4 and RPP4, but not snc1 or RPP2), suggesting that there is some differentiation downstream of TIR-NB-LRR activation. This differentiation has been observed for other defense components as well (Cheng et al., 2009; Monaghan et al., 2009). We also show that EDS17 is required for SAR but maintains systemic expression of PR-1. Similarly, the Arabidopsis detached9 (dth9) mutant is defective in SAR, but retains expression of PR-1 in systemic leaves and accumulates normal levels of SA in response to pathogen infection (Mayda et al., 2000). Additionally, exogenous application of SA is not capable of inducing SAR in dth9 plants like it is in Col-0 plants. Our data and those presented by Mayda et al. (2000) indicate that there are factors other than systemic PR-1 expression that mark the onset of SAR in plants.

A key piece of data that may help us understand the molecular function of EDS17 in Arabidopsis is its sub-cellular localization. The phenotype of eds17 mutants and the availability of organelle proteomics datasets suggest that EDS17 may localize to the chloroplast. Although EDS17 does not contain a standard chloroplast targeting peptide (cTP), as predicted by the localization software ChloroP (version 1.1; Emanuelsson et al., 1999), it was recently identified in the stromal proteome of the Arabidopsis clpr4 mutant (Kim et al., 2009). However, EDS17 has not been identified in chloroplasts in three other proteomics studies (Kleffmann et al., 2004; Yu et al., 2008; Zybailov et al., 2008), so it remains to be resolved whether EDS17 is indeed chloroplastic. Nevertheless, the chlorotic phenotype of eds17 mutants suggests that EDS17 is required for chloroplast biology in some way. We found that mutations in EDS17 result in low chlorophyll levels and a decrease in expression of LHCBI. LHCBI encodes the light-harvesting chlorophyll A/B binding protein of photosystem II, and its expression is often used as a marker for chloroplast development (Lopez-Juez et al., 1998). The Arabidopsis chlorophyll a/b binding protein under-expressed (cue) mutants express LHCBI at low levels and have pale or yellow leaves that look very similar to eds17 (Lopez-Juez et al., 1998). Analysis
of the cue mutants revealed a correlation between reduced leaf greening and a delay in chloroplast development (Lopez-Juez et al., 1998; Vinti et al., 2005). This suggests that the low levels of chlorophyll observed in eds17 mutants may be associated with poorly developed chloroplasts. The chloroplast is the site of biosynthesis of molecules with important signaling functions in the plant immune response. For example, SA (Wildermuth et al., 2001) and ROS (Fernandez and Strand, 2008) are produced in the chloroplast, both of which play integral roles in the establishment of the HR (Mur et al., 2008). Further characterization of EDS17 is needed to fully elucidate the molecular function of this protein in chloroplast biology and plant immunity.

5.5 Methods

5.5.1 Plant growth, mutant isolation, and pathogen infection assays

For most experiments, plants were grown on soil in a 16h light / 8h dark regime. In other experiments, seedlings were grown on Murashige and Skoog (MS) media supplemented with 0.5% sucrose and 0.3% phytigel. T-DNA mutants were obtained from the ABRC and genotyped by PCR using the insertion-flanking primers 5'-TGTTAGCTCAGTCAAGTAC-3' and 5'-ATAGCCAGCTTCTTTTCTC-3' for eds17-1, 5'-CAGATGTTCGTGGAAAGATG-3' and 5'-CTTAAGCTTCACCAAACCGAC-3' for eds17-2, and 5'-CACAAGGACTAACCTTGTAG-3' and 5'-GAAGTTACTAGCGAGCAAGC-3' for eds17-3. The snc1 mutant has previously been described (Li et al., 2001). Bacterial and oomycete infections were performed as described in Li et al. (2001). Briefly, bacterial pathogens were inoculated on the abaxial leaf surfaces of four-week old plants using a needless syringe. Leaf discs (with an area of 0.38cm²) were collected on the day of infection (Day 0) and three days later (Day 3) from different plants. H.a. isolates were spray-inoculated onto adaxial leaf surfaces of two-week old seedlings and oomycete sporulation was counted using a hemocytometer seven days later.
5.5.2 Genetic crosses

For allelism, \textit{eds17-3} homozygous males were crossed with \textit{eds17-1} or \textit{eds17-2} females and the F\textsubscript{1} was examined for complementation. To generate the \textit{snc1 eds17-3} double mutant, homozygous \textit{snc1} plants were crossed with homozygous \textit{eds17-3} plants. The F\textsubscript{1} was allowed to self, and the double was identified in the segregating F\textsubscript{2} population by PCR.

5.5.3 Quantification of chlorophyll

To measure leaf chlorophyll, approximately 50 mg of leaf tissue was frozen in liquid nitrogen and extracted using 80\% acetone as described in Porra et al. (1989). Absorption at 663nm and 645nm was used to calculate chlorophyll levels.

5.5.4 Expression analysis

RNA was extracted using the Totally RNA Kit (Ambion), and reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen). Primers used for \textit{LHCB1} expression analysis were 5'-CGTGTGACAATGAGGAAGA-3' and 5'-CTCTGAGCGTGAAACCAAGCT-3'. These primers do not distinguish \textit{LHCB1.1} (At1g29920) from \textit{LHCB1.2} (At1g29910). The primers for \textit{ACTIN1} have been described previously (Zhang et al., 2003). \textit{EDS17} expression analysis was conducted using the primers 5'- GCGCTCTATGGAGTCTATTCA-3' and 5'-CACATATTACCGACAATTTGAG-3', and \textit{TUBULIN} was monitored using 5'-ACGTATCGATGTCTATTTCAACG-3' and 5'-ATATCGTAGAGCCTCATTTGTCC-3'. Expression of the \textit{PR2pro-GUS} fusion was analyzed by vacuum infiltration of whole 2-week old seedlings in X-gluc solution.
followed by incubation at 37°C overnight and were de-stained with ethanol as described in (Bowling et al., 1994).

5.6 Manuscript acknowledgements

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Figure 5.1: EDS17-like proteins are found in eukaryotes in different kingdoms.

Phylogenetic tree showing the relationship between EDS17 and similar proteins in other eukaryotes. Alignment was generated with MUSCLE using the following protein sequences: Arabidopsis thaliana (NP_176659.3), Homo sapiens (NP_006827.1), Mus musculus (NP_766307.2), Drosophila melanogaster (NP_001015316.1), Schizosaccharomyces pombe (NP_593669.1), Saccharomyces cerevisiae (NP_011320.1), Vitis vinifera (CAO38767.1), Ricinus communis (EEF40421.1), Physcomitrella patens sp. patens (XP_001754159.1), and Oryza sativa (japonica; AAT77858.1). The tree was generated with GARLI using the Dayhoff substitution model, and viewed using Fig Tree. There are six copies of EDS17 in O. Sativa; only one is shown in the tree.
Figure 5.2: Phenotypic analysis of eds17 T-DNA insertion mutants.

(A) Gene structure of EDS17 (At1g64790) showing the position of T-DNA insertions Salk_149084 (eds17-1), Salk_119854 (eds17-2), and Salk_041123 (eds17-3). Lines indicate introns and boxes indicate exons. The location of translation start (ATG) and stop (TGA) codons are found in the first and last exons, as indicated. (B) Morphology of Col-0, eds17-1, eds17-2, and eds17-3 mutants. Representative F1 plants from allelic crosses are shown to the right; eds17-3 was used as the female in both crosses. Soil-grown plants were photographed 4 weeks after germination. (C) eds17-1 and eds17-2 plants exhibit characteristic outgrowths extending from leaf margins. A representative leaf from eds17-2 is shown compared to Col-0. (D) Siliques from Col-0 and the eds17 mutant alleles. Size bar represents 1 cm.
Figure 5.3: eds17 mutants exhibit a decrease in leaf chlorophyll.

(A) Leaves from Col-0 compared to the eds17 mutant alleles. Plants were grown on soil and photographed 4 weeks after germination. (B) Levels of chlorophyll A, chlorophyll B and total chlorophyll extracted from 4 week old soil-grown plants. Values represent an average of three replicates ± SD. An unpaired Student’s t-test was used to analyze the statistical significance of chlorophyll levels compared to Col-0. Asterisks indicate P<0.01. (C) Expression of LHCB1 in the eds17 mutants compared to Col-0 using semi-quantitative RT-PCR. ACTIN1 is included as a loading control. Experiments were repeated twice with similar results.
Figure 5.4: EDS17 is required for basal defense against Pseudomonas syringae.

(A) Growth of *P. s.t.* DC3000 at 0 and 3 days post-inoculation. Values represent an average of four replicates ± SD. Statistically significant groups were calculated using a one-way ANOVA comparing the means of all samples; a represents the control Col-0, b represents *P<0.0001*, c represents *P<0.005*. This experiment was repeated several times with similar results. (B) Expression of *EDS17* and *PR-1* 24 hours after inoculation with either 10 mM MgCl₂ or *P. s.t.* DC3000 (OD₆₀₀=0.005), as monitored by semi-quantitative RT-PCR conducted on equal amounts of cDNA. *TUBULIN* is included as a control. This experiment was repeated three times with the same results. (C) Growth of *H. a.* isolate Noco2 at 7 days post-inoculation with 2.5x10⁴ spores/ml in water. Values represent an average of two replicates of 20 seedlings each ± SD. Statistical significance was calculated using an unpaired Student’s *t*-test compared to Col-0; asterisk indicates *P<0.008*. This experiment was repeated four times with similar results. (D) Growth of *P. s.t.* DC3000 *HrpA-* at 0 and 3 days post-inoculation. Values represent an average of four replicates ± SD. This experiment was repeated three times with similar results.
Figure 5.5: EDS17 is required for resistance conditioned by specific R proteins.

Growth of the bacterial pathogens P.s.t. avrRps4 (A) and P.s.t. avrPphB (B) at 0 and 3 days post-inoculation. Values represent an average of four replicates ± SD. An unpaired Student’s t-test was used to analyze the statistical significance of bacterial growth compared to Col-0; P<0.0001 for both graphs. These experiments were repeated at least three times with similar results. (C) Growth of H.a. isolate Emwa1 eight days after infection with 7.5x10⁴ spores/ml in water. Values represent an average of two replicates of 20 seedlings each ± SD. An unpaired Student’s t-test was used to analyze the statistical significance of growth compared to Col-0; P<0.005. Infection phenotypes of Col-0, eds17-3, and eds1-2 (Col-0), visualized by lactophenol trypan blue staining is shown underneath the infection graph. HR; hypersensitive response, TR; trailing necrosis, M; mycelium. (D) Growth of H.a. isolate Cala2 eight days after infection with 1x10⁵ spores/ml in water. Values represent an average of two replicates of 20 seedlings each ± SD. An unpaired Student’s t-test was used to analyze the statistical significance of growth compared to Col-0; P<0.05. Infection phenotypes of Col-0, eds17-3, and eds1-2 (Col-0), visualized by lactophenol trypan blue staining is shown underneath the infection graph. HR; hypersensitive response, M; mycelium.
Figure 5.6: Loss of EDS17 does not suppress snc1-associated phenotypes.

(A) Morphology of Col-0, snc1, and snc1 eds17-3. Soil-grown plants were photographed 4 weeks after germination. Size bar represents 1 cm. (B) PR-2 expression is not suppressed in snc1 eds17-3 plants, as shown using the PR2pro-GUS fusion present in the snc1 mutant background (and also present in Col-0). Plants were grown on MS media for 20 days. (C) Growth of H.a. Noco2 in Col-0, snc1, and snc1 eds17-3 mutants 7 days after infection with 1x10^6 spores/ml in water. Values represent an average of three replicates ± SD. A Student’s t-test was used to calculate statistical significance compared to Col-0; P<0.0005. This experiment was repeated twice with similar results.
Figure 5.7: EDS17 is required for SAR.

(A) Growth of P.s.t. DC3000 in plants that had been pre-inoculated with either 10mM MgCl₂ or an HR-causing dose (OD₆₀₀=0.2, resuspended in 10mM MgCl₂) of P.s.t. DC3000 expressing avrRps4 24 hours earlier. Values represent an average of three replicates ± SD. A Student’s t-test was used to calculate statistical significance compared to Col-0; P<0.02. This experiment was repeated several times with similar results. (B) Symptom development in Col-0 and eds17-3 plants in mock and avrRps4 pre-treated leaves. Leaves were photographed 3 days following infection with P.s.t. DC3000. (C) PR-1 expression analysis by semi-quantitative RT-PCR on mock (10mM MgCl₂) or P.s.t. avrRps4 (OD₆₀₀=0.2) treated plants in infected (local) and uninfected (systemic) leaves. This experiment was repeated twice with similar results.
5.7 References


function on elongating ribosomes in activation of eIF2alpha kinase GCN2. Mol Cell Biol 17, 4474-4489.


Yu, Q.B., Li, G., Wang, G., Sun, J.C., Wang, P.C., Wang, C., Mi, H.L., Ma, W.M., Cui, J., Cui, Y.L., Chong, K., Li, Y.X., Li, Y.H., Zhao, Z., Shi, T.L., and Yang, Z.N.


6 Final discussion and perspectives
6.1 The MOS4-associated complex and plant defense

R proteins govern a major branch of the plant immune system. *SNC1* encodes a protein with similarity to a class of R proteins known as NB-LRRs by virtue of containing both nucleotide-binding (NB) and leucine-rich repeat (LRR) domains (Zhang et al., 2003). R proteins of this class recognize intracellular pathogen effectors deployed during an infection and trigger the onset of a largely unknown signaling cascade that, in many cases, culminates in programmed cell death. Importantly, the pathogen effector recognized by SNC1 has not yet been identified so it cannot be said with absolute certainty that this locus encodes a bone fide R protein. However, the autoimmune phenotypes associated with *snc1*, coupled with their absolute dependence on *EDS1* and *PAD4*, strongly suggest that SNC1 is likely an R protein. In any case, the *snc1* mutant has provided us with an autoimmune model that is truly unique in our field. Most autoimmune mutants result in global cell death and lethality, whereas *snc1* does not. This is likely due to the partial activation of this NB-LRR, as over-expression of *SNC1* can result in seedling lethality (Li Lab, unpublished data). This gain-of-function mutation causes quantifiable and measurable autoimmune phenotypes, making *snc1* an ideal mutant for genetic analysis. Forward genetic screens using *snc1* have successfully revealed several aspects of the plant immune system. These MOS loci are outlined in Chapter 2 and encode proteins involved in nucleo-cytoplasmic partitioning (MOS3, MOS6, MOS7), protein modification (MOS5, MOS8) and RNA processing (MOS2, MOS4), in addition to others that have not yet been published.

A mutation in *MOS4* was identified by map-based cloning as a *snc1* suppressing locus (Palma et al., 2007). MOS4 was later found to interact with AtCDC5 *in vitro* and *in vivo* - a discovery that led to the hypothesis that these proteins belong to a larger protein complex based on what was known about AtCDC5 homologs in other eukaryotes. Elucidating the composition and function of this complex, called the MAC, has been a major focus of research in our lab. We now know that the MAC is composed of over 20 proteins in *Arabidopsis* and shares significant homology with the NTC in yeast and human (Monaghan et al., 2009). The MAC core complex contains MOS4, AtCDC5,
PRL1, and MAC3A/3B, and is required for innate immunity in plants. Chapter 3 outlines the characterization of MAC3A and MAC3B, functionally redundant Prp19-like E3 ubiquitin ligases. Chapter 4 describes work on MAC5A and MAC5B, partially redundant RBM22-like RNA-binding proteins. Lastly, Chapter 5 describes work on EDS17, a protein that is not part of the MAC but that functions in plant innate immunity. Importantly, the work presented in this thesis represents the first effort to study all five of these loci in *Arabidopsis* and adds significantly to our understanding of plant defense.

Like the NTC, the MAC is associated with components of the spliceosome and as such is the first spliceosome-associated complex to have a demonstrated role in plant immunity. That said, the relevance of this association has yet to be determined, as MOS4, AtCDC5, PRL1, MAC3A/3B, and MAC5A/5B do not appear to be required for general splicing in plants. However, we have not been able to directly test a defect in splicing due to the lack of an established protocol to recapitulate splicing reactions *in vitro* using plant proteins (Lorkovic et al., 2000). In addition, we cannot rule out the possibility that the MAC functions in alternative splicing. Eukaryotes use alternative splicing to regulate gene expression and/or increase the functional diversity of genes such that they can encode more than one protein. At least 42% of intron-containing genes undergo alternative splicing in *Arabidopsis* (Filichkin et al., 2009), and these events can be regulated by environmental stresses (Ali and Reddy, 2008) and developmental cues (Macknight et al., 2002). This indicates that plants actively employ alternative splicing to regulate gene expression and protein function in inducible signal transduction cascades.

Of interest, several NB-LRRs are known to be alternatively spliced (Jordan et al., 2002), although the function of these variants is not known in most cases. As one example, the transcript encoding the TIR-NB-LRR protein RPS4 is alternatively spliced – an event that is influenced by bacterial infection (Zhang and Gassmann, 2003, 2007). Alternative splicing of immune receptors plays an important role in mammalian innate immunity as well. For example, the mouse Toll-Like Receptor TLR4, involved in the perception of bacterial lipopolysaccharide, is alternatively spliced as part of a regulatory feedback mechanism (Iwami et al., 2000). Conceivably, alternative transcripts coding for immune receptor isoforms could influence defense activation and signaling (Gassmann, 2008). Importantly, it has been reported that, like *RPS4*, *SNC1* is also
alternatively spliced (Yi and Richards, 2007), however the biological significance of these transcript variants is not yet understood. Indeed, we have also detected such transcript variants in our own studies, but could not detect any changes in SNC1 alternative splicing in mos4 or snc1 mos4 compared to Col-0 (J.M. and X.L., unpublished data), nor could we detect any changes in RPS4 transcript variants in mos4-1, Atcdc5, or prl1 compared to Col-0 (Palma et al., 2007). We have not yet conducted a thorough investigation of possible alternative transcript variations following pathogen infection. This is largely because genome-wide alternative splicing events in response to biotic stresses have not yet been elucidated in plants, making it difficult to predict which transcripts to investigate. A recent study on genome-wide alternative transcript variants in human T and B cells showed that differential gene expression and alternative splicing are associated with the activation of the mammalian immune response (Grigoryev et al., 2009). The availability of this sort of resource in Arabidopsis will be extremely helpful for identifying MAC-regulated alternative splicing events specific to defense, if they exist.

Alternatively, it is possible that the association between the MAC and the spliceosome facilitates co-transcriptional regulation of important defense genes. This hypothesis is based on the presence of AtCDC5, a Myb transcription factor, in the MAC. To identify potential AtCDC5-regulated genes, we used Promomer (Toufighi et al., 2005) to scan the Arabidopsis genome for promoters containing the AtCDC5-binding site previously identified by Hirayama and Shinozaki (1996). We found over 200 promoters with this consensus sequence and cross-referenced these against pathogen-series e-Northern microarray data available through the University of Toronto’s Botany Array Resource (Toufighi et al., 2005). In this way, we shortlisted 15 candidate genes that are up-regulated following pathogen infection and which contain the AtCDC5-binding site in their promoter regions. The most promising lead identified from this search was FLAVIN MONOOXYGENASE 1 (FMO1), a gene that has been shown experimentally to be induced following Pseudomonas infection in an SA-independent but EDS1-dependent manner (Bartsch et al., 2006; Koch et al., 2006; Mishina and Zeier, 2006). In addition, we found that FMO1 is constitutively expressed similar to PR-1 and PR-2 in snc1, and that this expression is suppressed in snc1 mos4, snc1 Atcdc5-1, and snc1 mac3a mac3b
(J.M. and X.L., unpublished data). These and other data suggested that FMO1 induction following pathogen infection could be regulated at the transcriptional level by the MAC. To test this, we infected mos4-1, Atcdc5-1, prl1-1, mac3a mac3b, and mac5a-1 with a virulent Pseudomonas strain and monitored FMO1 expression compared to Col-0 using quantitative RT-PCR. Interestingly, FMO1 was induced to similar levels in all mutants and Col-0 (J.M. and X.L., unpublished data), indicating that although snc1-mediated FMO1 expression relies on the MAC, pathogen-induced FMO1 expression does not. These data suggest that factors other than the MAC are required for FMO1 induction following pathogen perception, and that the MAC is not required for the expression of this gene in a biologically relevant manner. Whether AtCDC5 regulates any of the other 14 candidate genes awaits further testing. Future work using chromatin immuno-precipitation followed by DNA sequencing with untreated leaf tissue compared to pathogen-infected tissue should identify relevant AtCDC5-regulated genes and could lead to a better understanding of the MAC’s function in plant immune signaling.

MAC3A and MAC3B are functionally redundant U-Box containing E3 ligases. This suggests that, in addition to direct transcriptional regulation via AtCDC5, protein ubiquitination is also a function of the MAC. A simple model predicts that MAC3A/3B targets a transcriptional repressor for degradation, resulting in positive regulation of defense genes by AtCDC5 to activate an immune response. Site-directed mutagenesis of a highly conserved cysteine to an alanine (C3A) in the U-box domain of either MAC3A or MAC3B results in non-functional proteins, as evidenced by trans-complementation experiments (Appendix 4). This indicates that E3 ligase activity is probably required for the function of MAC3A and MAC3B in Arabidopsis. U-box containing E3 enzymes are capable of binding both the E2 conjugating enzyme and the substrate (Hatakeyama et al., 2001), raising the possibility that MAC3A/3B interacting proteins could be MAC targets. Immuno-purification of MAC3A-HA using quantitative proteomics identified only AtCDC5, PRL1, and MAC9/AtSYF1 as MAC3A-associated proteins (Xu et al., 2009). We have not yet tested whether MAC3A/3B ubiquitinates these proteins in vitro, however, since our evidence suggests that AtCDC5 and PRL1 are positive regulators of plant defense they are unlikely to be targeted by MAC3A/3B for degradation.
MAC9/AtSYF1 encodes a protein that contains tetratricopeptide repeat regions but has an otherwise unknown function. The human homolog of MAC9, Syf1/XAB2, is involved in transcription and transcription-coupled DNA repair (Nakatsu et al., 2000), and has been isolated as a component of the NTC in several studies (Chen et al., 2002; Ohi et al., 2002; Deckert et al., 2006; Gavin et al., 2006; Bessonov et al., 2008). Furthermore, the purification and analysis of proteins associated with Syf1/XAB2 in human cell culture identified Prp19, hAquarius/KIAA0560, and Isy1 (Kuraoka et al., 2008) – all of which were also found in the MAC (MAC3A/3B, MAC7/EMB2765, and MAC8/AtISY1, respectively). In addition to the NTC, Syf1/XAB2 was shown to transiently interact with RNA Polymerase II, indicating a potentially direct link between splicing and transcription (Nakatsu et al., 2000). To test the biological function of MAC9/AtSYF1 in Arabidopsis, we have obtained two mac9 T-DNA insertion alleles from the ABRC, and are currently assaying these mutants with respect to pathogen defense and snc1 suppression. Identification of other MAC3A/3B interacting proteins using additional proteomics tools or through a yeast 2-hyrbid screen would also be useful to identify potential MAC substrates.

MAC5A and MAC5B are partially redundant putative RNA-binding proteins that contain both a CCCH-type zinc-finger and an RRM. Although the intrinsic ability of MAC5A and/or MAC5B to bind RNA species has yet to be shown experimentally, proteins containing CCCH zinc-fingers or RRMs have been demonstrated to bind RNA in vitro (Jessen et al., 1991; Burd and Dreyfuss, 1994; Wang et al., 2008). Importantly, whereas MOS4, AtCDC5, PRL1, and MAC3A/3B are not essential genes in Arabidopsis, both MAC5A and MAC5B are required for plant viability and are therefore essential in plants. Our original hypothesis predicted that MAC5A/5B could represent a direct link between splicing and the MAC, but this idea could not be supported experimentally. That is, the presence and abundance of transcript variants for three known alternatively spliced genes, U1-70K, AtSRp34/SR1, and AtSRp30, was not affected in mac5a-1 or mac5a-1; MAC5B/mac5b-1 mutants – indicating that the spliceosome is functional despite these lesions. Thus, it appears that MAC5A/5B is not essential for general splicing in plants. That said, identification of the RNA species bound by MAC5A/5B could lead to a better understanding of the MAC in general and its connection to RNA
processing/splicing, and could provide new leads about the role of the MAC in plant defense.

The identification of MAC substrates and downstream signaling components is of particular interest. Uncovering what genes are regulated by AtCDC5, what proteins are ubiquitinated by MAC3A/3B, and what RNA species are bound by MAC5A/5B will truly allow us to elucidate the molecular function of the MAC. A major hurdle, however, will be deciphering which downstream components are specific to the plant defense response. It is clear that MAC proteins are employed by several signaling networks, as indicated by the pleiotropic phenotypes observed for MAC mutants described to date. All MAC mutants, to one extent or another, show defects in flowering time, leaf development, fertility, root length, hormone signaling, sugar signaling, and plant defense (Nemeth et al., 1998; Palma et al., 2007; Monaghan et al., 2009; J.M. and X. L., unpublished data). Accumulating evidence suggests that cross-talk between signaling pathways is common in plants. In particular, cross-talk and coordination between hormone, development, and defense signaling seems to be especially important for a successful immune response (Chung et al., 2008; Spoel and Dong, 2008; Bari and Jones, 2009). Based on these observations, it is possible that the MAC acts as a key regulatory node utilized by many signaling pathways in plants.

Comparative studies between the NTC in yeast, human, and Drosophila, and the MAC in Arabidopsis, could uncover novel perspectives regarding the function of this complex across kingdoms. It appears that the NTC has a more essential function in yeast and mouse than in Arabidopsis. Whereas loss-of-function mutations in any gene encoding a NTC core protein results in lethality in yeast and mouse, the loss of homologous MAC genes does not result in lethality in plants. This indicates that, although the protein composition of the MAC/NTC is conserved, the function of this complex may differ between these organisms. A joint effort to study the molecular function of this complex across kingdoms could shed light on the role of the MAC/NTC in signal transduction relay and may lead to a better understanding of how plants utilize this complex to achieve immunity.
6.2 References


Appendices
Appendix 1:

Transient expression of MAC3A-CFP and CFP-MAC3B in onion epidermal cells

A single layer of fresh onion was cut and placed on MS media supplemented with 1% sucrose and 100 mg/ml ampicillin. Gold particles (BioRad) were coated with 5 µg plasmid DNA and delivered into the onion cells at 1100 psi. Transformed onion peels were incubated at room temperature in the dark for at least 16 hours prior to visualization under a confocal microscope. Light microscopy was used to examine the localization of P35S-MAC3A-cCFP and P35S-cCFP-MAC3B (cloning described in section 3.5.3) in onion cells transformed by particle bombardment. P35S-YFP was used as a control. Single transformed cells are marked with arrows in the bright field image.
Appendix 2:

F3 analysis of higher-order MAC mutants

<table>
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<th>Genotype</th>
<th># of plants with this genotype at the segregating locus</th>
<th>% Het</th>
<th>P value ($\chi^2$)</th>
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<td>mac3a/mac3a; mac3b/mac3b; MOS4-1/mos4-1</td>
<td>17 24 0</td>
<td>58</td>
<td>0.1&lt;P&gt;0.05</td>
</tr>
<tr>
<td>prl1-1/prl1-1; mac3b/mac3b; MAC3A/mac3a</td>
<td>30 40 0</td>
<td>57</td>
<td>0.5&lt;P&gt;0.1</td>
</tr>
</tbody>
</table>

To identify higher-order MAC mutants, homozygous mac3a mac3b mutants were crossed with homozygous mos4-1 or prl1-1 mutants. The F1s were allowed to self. In the F2s, individuals that were homozygous at two loci and segregating at the other (confirmed by PCR genotyping) were identified and allowed to self. The F3 was examined as above. Because the segregating ratio follows a 1:2 (WT:Het) pattern as opposed to a 1:2:1 (WT:Het:Hom), we conclude that the triple mutants are lethal. This could be supported by statistics analysis using a standard $\chi^2$ analysis.
Appendix 3:

Heterologous expression of MAC3A is not able to complement the prp19-1 mutation in yeast

To determine if heterologous expression of MAC3A is able to complement the temperature-sensitivity of the prp19-1 mutant in Saccharomyces cerevisiae, MAC3A and PRP19 were cloned into yeast expression vectors under the control of the strong PGAL4 promoter and C-terminally tagged with a V5 epitope. The primers used for cloning were as follows:

ScPrp19-F  5’-ggggACAAGTTTGTACAAAAAAGCAGGCTATGCTTTTGCTATTAGTGGA-3’
ScPrp19-R  5’-ggggACCACTTTGTACAAGAAAGCTGGGTcGGGTGTCAATGCAACAATAT-3’
MAC3A-F  5’-ggggACCAAGTTTGTACAAAAAAGCAGGCTATGACTGTGCAATTTCAAGG-3’
MAC3A-R  5’-ggggACCACTTTGTACAAGAAAGCTGGGTcTGAATCTTGTGCTGAATCTT-3’

The amplified Gateway compatible fragments were first recombined into an entry pDONR vector and confirmed by sequencing. Fragments were recombined into the destination pYES-DEST52 vector using LR Clonase (Invitrogen). These plasmids were transformed into the prp19-1 mutant strain using a standard lithium acetate protocol. Expression of PGAL4-PRP19-V5 and PGAL4-MAC3A-V5 was confirmed by Western blot using an anti-V5 antibody. When grown on galactose media at the permissive temperature of 30°C, all strains grew well. When grown on galactose media at the non-permissive temperature of 37°C, only the prp19-1 mutant expressing PRP19 could be complemented.
Appendix 4:

Site-directed mutagenesis of a conserved cysteine in MAC3A and MAC3B U-box domains

To determine if the function of MAC3A and MAC3B relies on E3 ubiquitination, a site-directed mutagenesis approach was undertaken. A highly conserved cysteine residue located in the U-Box (shown in A) was mutated to an alanine (C3A) using the primers:

MAC3A-C3A-F  5'-CACC\_ATG\_AATGCT\_GCAATTTCC-3'
MAC3A-C3A-R  5'-CGCCGGAAATTGCAGCATTCAT-3'
MAC3B-C3A-F  5'-CACC\_ATG\_AACGCT\_GCAATTTC-3'
MAC3B-C3A-R  5'-CCTGAAATTGCAGCGTTCATCG-3'

PCR was conducted to amplify the resulting Gateway-compatible fragments from Arabidopsis Col-0 cDNA. The fragments were recombined into Gateway entry vectors using the pENTR/D-Topo kit (Invitrogen). Entry vectors were confirmed by sequencing using the M13F and M13R primers. Recombination into the destination binary vector pXCSG was done using LR Clonase (Invitrogen). Arabidopsis genotype mac3a mac3b plants were transformed with Agrobacterium carrying these vectors and transgenics were selected using the herbicide Basta and confirmed by PCR. T1 plants were allowed to self and were examined for phenotypic suppression of mac3a mac3b phenotypes in the T2. None of the obtained transgenic lines showed morphological complementation, indicating that these residues are necessary for MAC3A and MAC3B function in plants. Representative individuals are shown in B. Plants were grown on soil for approximately 3 weeks prior to photography. Size bar indicates 1 cm.
Appendix 5:

List of publications

Research Articles


Reviews and Communications


Articles in preparation