REGULATING THE PLANT INNATE IMMUNE SYSTEM: THE ROLES OF THREE ARABIDOPSIS MUSE PROTEINS

by

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Abstract

The plant innate immune system is highly effective in impeding infection by a broad spectrum of microbial pathogens. Strict regulation of immune signaling in plants is required to both facilitate rapid defense response induction upon pathogen detection and prevent the precocious activation of immunity, the latter of which has associated fitness costs. Despite their significance, the regulatory mechanisms governing plant immunity have only been partially characterized. Previously, members of the Li research group employed a forward genetic screen to identify positive regulators of innate immunity. This suppressor screen was performed using the unique Arabidopsis autoimmune mutant *snc1* (*suppressor of npr1, constitutive 1*), which contains a gain-of-function mutation in an NLR (NOD-LIKE RECEPTOR) protein. The identified *MOS* (*MODIFIER OF SNC1*) genes highlighted the importance of diverse biological processes in the regulation of disease resistance. More recently, a *snc1* enhancer screen was conducted to identify negative regulators of plant immune signaling. This thesis describes the cloning and characterization of three mutants isolated from this *MUSE* (*MUTANT, SNC1-ENHANCING*) screen.

The *muse9* mutant carries a molecular lesion in the gene encoding the chromatin remodeler *SPLAYED* (*SYD*). Molecular analyses showed that SYD negatively regulates *SNC1* expression and thus functions antagonistically to MOS1 and MOS9, both of which were previously shown to positively regulate *SNC1* transcription. This study emphasizes the importance of finely-tuned transcriptional control in NLR-mediated immunity.

The *muse4* mutant contains a partial loss-of-function mutation in *NRPC7*, which encodes an RNA polymerase III (Pol III) subunit. This is the first reported viable Pol III mutant. Using

RT-PCR, it was established that the mutation in *NRPC7* affects the expression of a diverse suite of genes and results in distortions in alternative splicing.

The mutation responsible for the *muse7* phenotypes is in a gene that encodes a protein of unknown function. MUSE7 negatively regulates SNC1 at the protein level, although no interactions were detected between MUSE7 and other known regulators of NLR protein turnover. This suggests that MUSE7 either regulates protein synthesis or is involved in an alternate degradation pathway.

Taken together, these characterizations underscore the complexity inherent in the molecular mechanisms that control plant immune signaling.

Preface

The research comprising this thesis is the result of work performed between September 2010 and April 2016. Section 1.5 of Chapter 1 as well as Chapters 2 and 3 have been previously published and a manuscript corresponding to Chapter 4 is currently in preparation for publication. The details of these manuscripts and the contributions of the candidate are as follows:

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- The candidate wrote and edited the majority of the manuscript. O.X. Dong wrote Section 1.5.5 and created Figure 1.1. Y. Huang contributed to Sections 1.5.1 and wrote Sections 1.5.2 and 1.5.7. X. Li supervised the preparation of the manuscript.

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- The candidate performed most of the experiments and wrote the manuscript. S. Xia performed the positional cloning of the *muse9* mutant. X. Feng (JIC, Norwich) conducted DNA methylation analyses. X. Li (UBC, Vancouver) supervised the work performed by S. Xia and the candidate, as well as the preparation of the manuscript.

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- The candidate performed most of the experiments and wrote the manuscript. Y. Yu performed small RNA library preparations and northern blotting. L. Gao conducted small RNA library analyses. R.C. Eng performed confocal microscopy using transgenic lines generated by the candidate. X. Chen (UCR, Riverside) supervised work performed by Y. Yu, L. Gao, and the candidate. G.O. Wasteneys (UBC, Vancouver) supervised work performed by R.C. Eng. X. Li (UBC, Vancouver) supervised work performed by the candidate and the preparation of the manuscript.

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- The candidate performed most of the experiments and wrote the manuscript. J. Zhao performed the positional cloning of the *muse7* mutant. C. Roth conducted confocal microscopy using transgenic lines generated by the candidate. M. Wiermer (GAU, Göttingen) supervised the work performed by C. Roth. X. Li (UBC, Vancouver) supervised the work performed by J. Zhao and the candidate, as well as the preparation of the manuscript.

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List of Abbreviations

ABA	abscisic acid
ABRC	*Arabidopsis* biological resource center
ADR	activated disease resistance
ATXR7	*Arabidopsis* trithorax-related 7
Avr	avirulence
AvrB	avirulence gene from *Pseudomonas syringae* pv. *glycinea*
AvrPphB	avirulence gene from *Pseudomonas syringae* pv. *phaseolicola*
AvrRpm1	avirulence gene from *Pseudomonas syringae* pv. *syringae*
AvrRps4	avirulence gene from *Pseudomonas syringae* pv. *pisi*
AvrRpt2	avirulence gene from *Pseudomonas syringae* pv. *tomato*
BAK1	BRI1-associated receptor kinase 1
BAT2	HLA-B associated transcript 2
bHLH84	basic helix-loop-helix 84
BIK1	*Botrytis*-induced kinase 1
BRM	brahma
CC	coiled-coil
cDNA	complementary DNA
CERK1	chitin elicitor receptor kinase 1
CESA3	cellulose synthase 3
CHS3	chilling sensitive 3
CNL	CC-NB-LRR
Col-0	Columbia ecotype of *Arabidopsis thaliana*
CPR	constitutive expresser of PR genes
CSA1	constitutive shade-avoidance 1
CUC	cup-shaped cotyledons
DAMP	damage-associated molecular pattern
DDM1	decrease in DNA methylation 1
DNA	deoxyribonucleic acid
DND	defense no death
EDS1	enhanced disease susceptibility 1
EMS	ethyl methanesulfonate
ERA1	enhanced response to abscisic acid 1
ET	ethylene
ETI	effector-triggered immunity
FLAG	epitope tag with the amino acid sequence DYKDDDDK
FLC	flowering locus C
FLS2	flagellin-sensitive 2
GFP	green fluorescence protein
GUS	β-glucuronidase
H.a.	*Hyaloperonospora arabidopsidis*
HA	hemagglutinin

HDA19	histone deacetylase 19
HD-ZIP	homeodomain leucine zipper
HopA1	avirulence gene from *Pseudomonas syringae* pv. *syringae*
HopF2	avirulence gene from *Pseudomonas syringae* pv. *tomato*
HR	hypersensitive response
HSP	heat shock protein
JA	jasmonate
LAZ5	lazarus 5
L*er*	Landsberg *erecta* ecotype of *Arabidopsis thaliana*
LIM	"Lin11, Isl-1, Mec3" domain
LRR	leucine-rich repeat
MAC	MOS4-associated complex
MAMP	microbe-associated molecular pattern
miRNA	microRNA
MLA10	mildew A 10
MOS	modifier of snc1
mRNA	messenger RNA
MS	Murashige and Skoog
MUSE	mutant, snc1-enhancer
MYC	epitope tag derived from the human myelocytomatosis viral oncogene
N	*Nicotiana* (N protein in tobacco)
NB	nucleotide-binding
NDR1	non race-specific disease resistance 1
NLR	NOD-like receptor
NLS	nuclear localization signal
NOD	nucleotide-binding oligomerization domain
NPC	nuclear pore complex
NPR1	non-expresser of PR genes 1
NRPC7	nuclear RNA polymerase C subunit 7
Nup	nucleoporin
P.s.m.	*Pseudomonas syringae* pv. *maculicola*
P.s.t.	*Pseudomonas syringae* pv. *tomato*
PAD4	phytoalexin-deficient 4
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBS1	AvrPphB susceptible 1
PDAP1	PDGF-associated protein 1
PDGF	platelet-derived growth factor
PEPR	PEP1 receptor
PHB	phabulosa
Pol	RNA polymerase
PopP2	avirulence gene from *Ralstonia solanacearum*
PR	pathogenesis-related
PRP	pre-mRNA processing
PRR	pattern recognition receptor

PTI	PAMP-triggered immunity
PTM	post-translational modification
qPCR	quantitative polymerase chain reaction
R	resistance
REV	revulota
RGA	resistance gene analog
RIN4	RPM1-interacting protein 4
RLK	receptor-like kinase
RLP	receptor-like protein
RNA	ribonucleic acid
ROS	reactive oxygen species
Rpc	RNA polymerase core protein
RPL18	ribosomal protein L18
RPM1	resistance to *Pseudomonas syringae* pv. *maculicola*
RPP	recognition of *Peronospora parasitica*
RPS	resistant to *Pseudomonas syringae*
rRNA	ribosomal RNA
RRS1	resistant to *Ralstonia solanacearum* 1
SA	salicylic acid
SAG101	senescence-associated gene 101
SAR	systemic acquired resistance
SCF	SKP1-CULLIN-F-box complex
SDG8	set domain group 8
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
SNC1	suppressor of npr1, constitutive 1
snRNA	small nuclear RNA
SPL6	squamosa promoter binding protein-domain transcription factor 6
SPP2	spliceosomal protein 2
SR	serine-arginine rich protein
STAND	signal transduction ATPases with numerous domains
SWI/SNF	switch/sucrose non-fermentable
SWR1	SWI2/SNF2 related 1 complex
SYD	splayed
T-DNA	transfer-DNA
TAIR	the *Arabidopsis* information resource
TIR	Toll Interleukin receptor
TNL	TIR-NB-LRR
TPL	topless
TPR1	topless-related 1
TRN	transportin
tRNA	transfer RNA
UBQ	ubiquitin
WRKY	“WRKY” domain
Ws	Wassilewskija ecotype of *Arabidopsis thaliana*

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And finally, thanks to my partner Mike Scott – words do not suffice.

Dedication

To my mother,

Robin Minion.

Chapter 1: An introduction to plant innate immunity[1]

1.1 Significance

Plants associate with numerous microbes in their surrounding environment, the vast majority of which do not elicit symptoms of disease. This is because plants possess a layered innate immune system that is tightly regulated, enabling appropriate and specific responses to most pathogenic threats without spurious immune activation that can result in fitness costs. However, the relationship between plants and potentially pathogenic microbes is a dynamic one, with both sides evolving rapidly in an effort to detect and avoid detection, respectively. The ability of plants to defend themselves against infection has a direct societal impact as plant diseases cause significant reductions in crop yields; estimates vary, but global food production is thought to incur a loss of approximately 10% annually due to plant pathogens (Scott & Strange 2005). In addition to potentially limiting food availability, crop losses can destabilize the economy in regions that are highly dependent on food production as a source of employment. Climate change is likely to exacerbate this issue, as studies have demonstrated that predicted changes in carbon dioxide levels, temperature, and drought conditions may increase both disease prevalence and severity (reviewed in Gregory et al. 2009). Current agricultural practices involve the use of pesticides and fungicides to constrain pathogen proliferation. However, concerns have been raised over the long-term, intensive use of agrichemicals based on potential risks to human health and the environment. Additionally, over time these strategies may lead to the development

[1] A version of Section 1.5 of this chapter has been published. Kaeli C. M. Johnson, Oliver X. Dong, Yan Huang and Xin Li. (2012) *Cold Spring Harbor Symposia on Quantitative Biology* 77: 259-68.

of chemical-resistant pathogens that are even more difficult to control. One of the aims of studying the endogenous defense mechanisms employed by plants is to provide insights that may aid in the development of sustainable solutions to modern agricultural challenges. The tiers of the plant immune system will be discussed in this chapter, with a particular emphasis on immunity mediated by NOD-like receptor (NLR) proteins.

1.2 Physical and chemical barriers

The first line of defense against pathogen attack includes physical and chemical barriers to infection. One proposed physical barrier is the plant cuticle, which coats the aerial tissues of land plants and is composed of a matrix of cutin and associated cuticular waxes. Although the primary role of the cuticle is to reduce water loss, a number of reports indicate that it also functions to limit microbial infection. Enhanced susceptibility to the fungal pathogen *Exserohilium turcicum* was observed in *Sorghum bicolor* mutants with altered cuticular structures (Jenks et al. 1994). Similarly, tomato mutants with severe cutin deficiencies in the fruit cuticle were shown to be more susceptible to infection by the necrotrophic pathogen *Botrytis cinerea* (Isaacson et al. 2009). The plant cell wall presents another physical barrier to infection, as the network of cellulose, hemicellulose, and pectin that forms the cell wall is resistant to physical penetration.

Counter-intuitively, mutant studies in Arabidopsis have revealed that defects in these physical barriers can lead to enhanced disease resistance. A leaky mutation in the cellulose synthase gene *CESA3* (*CELLULOSE SYNTHASE 3*) results in enhanced resistance to powdery mildew which also correlates with a constitutive enhancement of jasmonate signaling (Ellis et al. 2002). From this work, it has been proposed that the cell wall is part of a mechanosensitive

signal transduction pathway and that modifications resulting in altered cell turgor may lead to defense response activation (Vorwerk et al. 2004). Relatedly, a number of Arabidopsis mutants with deficiencies in cutin biosynthesis display enhanced resistance to *B. cinerea* (Bessire et al. 2007; Chassot et al. 2007; Tang et al. 2007). The authors of these studies hypothesize that plants with more permeable cuticles are able to export antifungal compounds more readily, which is supported by the finding that leaf diffusates from mutants with altered cuticular structures have enhanced antifungal activity (Bessire et al. 2007). However, these findings may be an artifact resulting from the use of Potato Dextrose Broth in pathogen inoculation (Nawrath et al. 2013), as a study that performed water-based *B. cinerea* inoculations found that the cuticle development mutant *glabra1* displayed enhanced susceptibility to this pathogen (Xia et al. 2010). Together, these findings highlight the complicated interplay between seemingly passive resistance structures and active, inducible immune responses; the latter will be discussed in more detail in the subsequent sections of this chapter.

Chemical defense barriers include the production of anti-microbial enzymes and secondary metabolites. Plants produce a diverse array of defense-related chemicals with antibiotic activities through a variety of metabolic pathways. These compounds can be divided into two broad categories: phytoanticipins, which are constitutively produced, and phytoalexins, which are generated following pathogen detection (Van Etten et al. 1994).

Together these physical and chemical barriers block many attempts at infection, although some pathogens are able to circumvent these defenses. For example, upon detecting cutin the fungal pathogen *Fusarium solani f.sp. pisi* produces and secretes cutinase (Lin & Kolattukudy 1978). Many pathogenic fungi species possess a large number of cellulases, xylanases, and other cell wall degrading enzymes, the optimum activities of which are specific to their respective host

plants (King et al. 2011). Many microbes have evolved the ability to enzymatically detoxify certain plant defense compounds; *Colletotrichum coccodes* and *Septoria lycopersici* are able to degrade the toxic steroidal glycoalkaloid α-tomatine produced by *Lycopersicum* species, and this contributes to their ability to successfully parasitize the host plant (Sandrock & Van Etten 2001). When the physical and chemical barriers are breached, plants must rely upon their inducible defense responses to halt the progress of infection. The current conceptual understanding of the inducible plant immune system is based on the types of receptors and the resistance signaling pathways they initiate.

1.3 Immunity triggered by pathogen-associated molecular patterns

For the innate immune system to be effective, plants must have a sensitive, difficult to evade mechanism for detecting pathogen presence. They possess a number of immune receptors with varied extracellular recognition motifs that localize to the cell surface; these proteins are termed pattern recognition receptors (PRRs), and can be classified as either receptor-like kinases (RLKs) or receptor-like proteins (RLPs) depending on whether or not they possess an intracellular serine/threonine kinase domain.

Many PRRs recognize pathogen-associated molecular patterns (PAMPs), which are conserved features that are essential for the microbial lifestyle and include such things as the bacterial motility organ component flagellin and the fungal cell wall constituent chitin, amongst others (Boller & Felix 2009). Other PRRs detect damage-associated molecular patterns (DAMPs), which are signals indicative of pathogen-induced damage to the host cell and may include fragments of the plant cell wall or plant-derived peptides (Krol et al. 2010; Monaghan et

al 2012). Following ligand perception some PRRs associate with BAK1, an RLK first identified as a key component in brassinosteroid signaling (Li et al. 2002). The flagellin receptor FLS2 (FLAGELLIN SENSITIVE 2) and the DAMP receptors PEPR1 (PEP1 RECEPTOR 1) and PEPR2 are among the PRRs that form ligand-induced associations with BAK1 that are essential for downstream signal enhancement (Roux et al. 2011); however, other PRRs such as the chitin receptor CERK1 (CHITIN ELICITOR RECEPTOR KINASE 1) have downstream signaling pathways that are BAK1-independent (Gimenez-Ibanez et al. 2009).

Ligand perception by PRRs leads to the activation of PAMP-triggered immunity (PTI), which is initially characterized by an influx of calcium (Ma & Berkowitz 2007) and an increase in the production of reactive oxygen species (ROS) (O'Brien et al. 2012), which are early steps in the signaling pathways that lead to defense response outputs. PTI also leads to the activation of mitogen-activated protein kinase signaling cascades, resulting in large-scale transcriptional reprogramming that increases the expression of defense-related genes, the accumulation of phytoalexins, and the synthesis of defense-related phytohormones (Meng & Zhang 2013).

While PTI is sufficient to halt the advances of most potential pathogens, some are able to overcome this type of immunity through the use of effector proteins which disrupt PTI signal transduction. Our understanding of the mechanisms underlying effector biology are largely based on studies performed using bacterial pathogens, which employ a syringe-like structure known as a type-III secretion system to inject effector molecules into the host cytoplasm (Cunnac et al. 2009). Pathogenic bacterial strains typically have a complement of 20-30 effector proteins that can vary widely between species and often have redundant functions within the plant cell. For example, *Pseudomonas syringae* encodes at least four effectors (AvrB, AvrRpm1, AvrRpt2, and HopF2) that target the Arabidopsis protein RIN4 (RPM1-INTERACTION PROTEIN 4) (Axtell

et al. 2003; Mackey et al. 2003; Wilton et al. 2010). Eukaryotic pathogens, including fungi and oomycetes, also secrete effector molecules that inhibit PTI into the cytoplasm of the cells of the host plant, but the method of delivery is still poorly understood (Rafiqi et al. 2012). Oomycete effectors typically contain a conserved RXLR motif that is necessary for translocation into the plant cell (Whisson et al. 2007); no such conserved motifs have been identified for fungal effector proteins.

1.4 Immunity triggered by effector recognition

1.4.1 Overview

In an escalation of the "arms race" between plants and pathogens, plants have evolved an assemblage of intracellular receptor proteins that are able to recognize effector molecules either through direct protein-protein interactions or by perceiving effector activities within the cytosol. Effector proteins diverge extensively between species meaning that effector-triggered immunity (ETI) has an inherent degree of specificity that is unachievable in PTI, which relies upon the detection of features that typify entire classes of organisms.

1.4.2 The structure of NOD-like receptor (NLR) proteins

The receptors that recognize pathogen effectors are called resistance (R) proteins, and can be divided into five classes based on structure and subcellular localization (Dangl & Jones 2001). The largest class is comprised of STAND (SIGNAL TRANSDUCTION ATPASES WITH NUMEROUS DOMAINS) P-loop ATPases that belong to the AAA+ superfamily (Leipe et al. 2004). These intracellular proteins possess a central nucleotide binding (NB) domain and a C-

terminal leucine rich repeat (LRR) domain. In recent plant pathology literature, NB-LRR proteins are typically referred to as NLR proteins based on their structural similarity to mammalian NOD (NUCLEOTIDE-BINDING OLIGOMERIZATION DOMAIN)–like receptor (NLR) proteins. Despite aspects of structural conservation, the function of mammalian NLR proteins is actually more similar to that of plant PRRs, in that they recognize conserved microbial features rather than effector proteins (Li et al. 2015). Another striking difference is that NLR families in plants are hugely expanded compared to metazoans (Jacob et al. 2013).

NLR proteins can be further subdivided based on their N-terminal domains. Some possess a TIR (TOLL INTERLEUKIN RECEPTOR) domain and are thus referred to as TNLs, while others possess a CC (COILED-COIL) domain and are termed CNLs. While immune signaling in dicots employs both types of NLRs, monocots only possess CNLs. The reason for this difference is poorly understood, and is only one aspect of the extensive diversity observed in NLR complements both between and within plant species.

Some plant NLR proteins possess additional, non-canonical domains. For example, the Arabidopsis TNL protein CHS3 (CHILLING SENSITIVE 3) contains a C-terminal LIM domain (Yang et al. 2010). Another Arabidopsis TNL protein (RESISTANT TO RALSTONIA SOLANACEARUM 1; RRS1) has a C-terminal WRKY domain (Deslandes et al. 2002). These supplementary domains have been demonstrated to play unique roles in effector recognition.

1.4.3 Effector detection

A number of models have been proposed to explain the varied mechanisms by which NLR proteins recognize their cognate effector molecules (Khan et al. 2016). In the direct interaction model, NLR proteins bind effectors through direct protein-protein interactions. These

interactions seem to occur via the LRR domain of the NLR, as indicated by mutational analyses of the Arabidopsis TNL RPP1 and the flax TNLs L5 and L6 (Krasileva et al. 2010; Ravensdale et al. 2012).

In most cases, an indirect interaction model provides a better fit with the experimental data. Two variants of such a model have been proposed: the guard model and the decoy model. In the guard model, an NLR protein detects changes in the abundance or modifications of a host protein that is targeted by pathogenic effectors. A specific and well-characterized example can be found in the relationship between RIN4 and the CNL proteins RPS2 (RESISTANT TO PSEUDOMONAS SYRINGAE 2) and RPM1 (RESISTANCE TO PSEUDOMONAS SYRINGAE PV. MACULICOLA) in Arabidopsis. RIN4 is a regulator of basal resistance (Kim et al. 2005; Liu et al. 2009), and is targeted by effectors from a variety of pathogens (Axtell et al. 2003; Mackey et al. 2003; Wilton et al. 2010). These modifications to RIN4 are perceived by RPS2 and RPM1, which subsequently initiate ETI.

The decoy model is similar, except that the host protein targeted by effectors and monitored by a(n) NLR protein(s) has no immune function other than to aid in triggering ETI (van der Hoorn & Kamoun 2008). Decoy proteins are structurally similar to virulence targets in the basal defense pathway. The Arabidopsis protein kinase PBS1 (AVRPPHB SUSCEPTIBLE 1) is an example of a decoy. The CNL protein RPS5 (RESISTANT TO PSEUDOMONAS SYRINGAE 5) associates with PBS1, and is activated upon its cleavage by the *P. syringae* effector AvrPphB (Ade et al. 2007). In keeping with the definition of a decoy protein, PBS1 has not been implicated in basal defense although the related protein kinase BIK1 (BOTRYTIS-INDUCED KINASE 1) does have a role in PTI and is also cleaved by AvrPphB (Zhang et al. 2010).

As an extension of the decoy model, evidence suggests that the non-canonical domains possessed by some NLR proteins may function as built-in decoys. The WRKY domain of the TNL protein RRS1 is acetylated by the *R. solanacearum* effector PopP2 and associates with the *P. syringae* effector AvrRps4 (Le Roux et al. 2015; Sarris et al. 2015). It remains to be shown whether this is a trend that extends to other non-canonical NLR proteins.

An emerging trend in the field of plant pathology is the importance of NLR protein pairs in mediating ETI (Griebel et al. 2014). A classic example is the genetic and molecular relationship between RRS1 and its partner NLR protein RPS4 (Narusaka et al. 2009). In rice, the CNL proteins RGA4 (RESISTANCE GENE ANALOG 4) and RGA5 (RESISTANCE GENE ANALOG 5) physically and functionally interact in mediating resistance to *M. oryzae* (Cesari et al. 2014), and the Arabidopsis TNL protein CSA1 (CONSTITUTIVE SHADE-AVOIDANCE 1) is required for immunity mediated by its TNL protein partner CHS3 (Xu et al. 2015). Of note, in all three examples the NLR protein pairs are located genomically adjacent to one another, suggesting that the transcription of genes encoding paired NLR proteins may be intrinsically linked.

The mechanisms by which effectors are perceived are varied and complex. However, all modes of recognition result in the same phenomenon: NLR protein activation and subsequent induction of immune signaling.

1.4.4 NLR protein activation

It has been proposed that ETI signaling depends upon NLR proteins switching into an activated conformation. In the uninduced state the NB domain preferentially binds ADP (Maekawa et al. 2011), and the LRR domain forms intramolecular bonds with the rest of the protein, thereby

impeding intermolecular interactions necessary for signal transduction (Takken et al. 2006). The current model for protein activation states that following effector detection, the NLR protein undergoes a molecular shift that releases the inhibitory action of the LRR domain. This shift allows for nucleotide exchange at the NB domain such that ADP is replaced with ATP, and this is thought to result in an additional conformational change that allows for downstream signaling (Bonardi & Dangl 2012). However, the exact biochemical mechanism of plant NLR activation is unclear.

1.4.5 Downstream signaling

The signaling pathways downstream of NLR protein activation are only partially characterized. Some NLR proteins localize to the nucleus and modulate defense-related gene expression. The barley CNL protein MLA10 (MILDEW A 10) associates with WRKY transcription factors in the nucleus to positively regulate immunity (Shen et al. 2007). This stands in contrast to the observed trend for many CNL proteins, which tend to localize to the plasma membrane and transduce immune signaling via the membrane-localized protein NDR1 (NON RACE-SPECIFIC DISEASE RESISTANCE 1) (Aarts et al. 1998; Takemoto et al. 2012). The tobacco TNL protein N associates with the transcription factor SPL6 (SQUAMOSA PROMOTER BINDING PROTEIN-DOMAIN TRANSCRIPTION FACTOR 6), and this association is necessary for resistance to tobacco mosaic virus (Padmanabhan et al. 2013). The Arabidopsis TNL protein SNC1 (SUPRESSOR OF NPR1, CONSTITUTIVE 1) associates with the transcription factor bHLH84 (BASIC HELIX-LOOP-HELIX 84) and its paralogs, which are positive regulators of immunity (Xu et al. 2014).

Signaling mediated by TNL proteins converges on the lipase-like protein EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1) and its interacting partners PAD4 (PHYTOALEXIN-DEFICIENT 4) and SAG101 (SENESCENCE-ASSOCIATED GENE 101) (Feys et al. 2001; Feys et al. 2005). EDS1 forms two distinct complexes with PAD4 and SAG101, respectively (Wagner et al. 2013), but the details of signaling through this node are still unclear.

The defense outputs of ETI are similar to those observed for PTI, although the induction of these responses is stronger, more rapid, and more robust during ETI (Tao et al. 2003; Tsuda & Katagiri 2010). Additionally, ETI is classically associated with a type of localized programmed cell death referred to as the hypersensitive response (HR), although a number of PAMPs have also been demonstrated to elicit HR (Bailey et al. 1990; Wei et al. 1992; Naito et al. 2008). The functional role of HR in plant immunity is somewhat contentious. It was previously posited that HR aided in containing the spread of pathogenic microbes. However, numerous studies have reported an uncoupling of cell death and resistance during immune signaling (Cole et al. 2001; Gassmann 2005; Hafez et al. 2012). As such, it is likely that the cell death associated with HR is a result (as opposed to a cause) of highly activated immune responses, as was first suggested over forty years ago (Kiraly et al. 1972).

1.5 Positive regulation of SNC1-mediated immunity

1.5.1 Overview

In the *snc1* gain-of-function mutant, a glutamic acid to lysine substitution in the linker region between the NB and LRR domains results in constitutive activation of defense without pathogen

perception. The autoimmune phenotypes of *snc1* include dwarfed stature, increased levels of SA, constitutive expression of *PR* genes, and increased resistance against virulent pathogens such as the bacteria *Pseudomonas syringae* pv. *maculicola (P.s.m)* ES4326 and the oomycete *Hyaloperonospora arabidopsidis (H.a.)* Noco2 (Zhang et al. 2003). Like most TNLs, SNC1 signals through PAD4 and EDS1.

SNC1 resides in the *RPP4* (*RECOGNITION OF PERONOSPORA PARASITICA 4*) cluster on chromosome 4 in the Columbia (Col-0) ecotype. SNC1 is homologous to RPP4 and RPP5, with over 70% similarity at the amino acid level. The majority of the sequence dissimilarity occurs within the LRR domain, as the TIR-NB domain of SNC1 is almost identical to that of RPP4 and RPP5. . In support of the hypothesis that *SNC1* encodes a *bona fide* NLR protein, loss-of-function *snc1* alleles do not exhibit enhanced disease susceptibility, unlike what is observed in the loss-of-function *activated disease resistance* (*adr*) mutants which encode "helper" NLRs (Bonardi et al. 2011). However, the cognate effector protein for SNC1 remains to be discovered.

As the *snc1* mutation activates resistance signaling pathways without producing HR lesions, this unique autoimmune mutant is a useful tool for dissecting signaling events surrounding TNL activation. Multiple mutagenesis strategies have been employed in *snc1* suppressor screens, yielding a number of *modifier of snc1* (*mos*) mutants. We originally hypothesized that the activated snc1 protein needs sophisticated downstream signaling components such as EDS1 and PAD4 to transduce the "danger alarm" in order to induce defense outputs. The suppressor screen should yield mostly positive regulators of the signaling pathway.

In the primary screen, the M2 generations of ethyl methanesulfonate (EMS), T-DNA, or fast-neutron mutagenized *snc1* and *snc1 npr1* populations were screened for loss of *snc1* dwarf

morphology under normal lab growth conditions (i.e. 22°C, 16-hr day/8-hr night, ~50% humidity). To confirm that mutants isolated from the primary screen are indeed *snc1* suppressors and are involved in plant immunity, defense-related phenotypes including *PR* gene expression, endogenous SA levels and resistance to *P.s.m.* ES4326 and *H.a.* Noco2 were assessed in the M3 populations as part of the secondary screen. Consistent with the prediction, the *snc1 mos* mutants exhibit wild type-like morphology and have decreased levels of all examined defense outputs. In total, fifteen independent complementation groups of mutants exhibited varying degrees of suppression of the *snc1* autoimmune phenotypes.

During the secondary screen, one of the challenges we faced was to identify and discard mutants containing intragenic *snc1* mutations, which constituted the majority of the mutants isolated from the primary screen. Fortunately, loss-of-function *snc1* mutations typically result in a dominant wild type-like morphological phenotype when heterozygous with the gain-of-function *snc1* mutation. Using this criterion, we were able to focus on the <10% of the recessive mutants carrying mutations in second-site genes.

Positional cloning was used to clone 11 of the 13 novel *MOS* genes. To facilitate mapping, *snc1*, originally in the Col background, was introgressed into L*er* through six backcrosses. Crosses between *snc1 mos* mutants and the L*er-snc1* line were generated. The F_1 plants, which displayed *snc1*-like morphology due to the recessive nature of the *mos* genes, were allowed to self-fertilize to generate a segregating F_2 population suitable for conducting linkage analysis between the suppression of *snc1* morphology and molecular markers (Zhang and Li 2005). Typically, a population of 600 – 1000 F2 plants would enable us to narrow the region containing each mutation to less than 100 kb, and genes within that region were directly

sequenced to identify the putative molecular lesions. Once the mutations were identified, *MOS* gene cloning was confirmed by transgenic complementation and allelism tests if T-DNA alleles were available from the Arabidopsis Biological Resource Center (ABRC).

As expected, several mutant alleles of *PAD4* were identified. To date 13 novel *MOS* genes have been cloned using positional cloning (11) and T-DNA tagging (2) approaches. These encode proteins involved in RNA processing, nucleocytoplasmic trafficking, protein modification and epigenetic control of gene expression. The diverse MOS functions suggest that the activation of NLR-mediated resistance is highly complex (Palma et al. 2005; Zhang and Li 2005; Zhang et al. 2005; Goritschnig et al. 2007; Palma et al. 2007; Wiermer et al. 2007; Goritschnig et al. 2008; Cheng et al. 2009; Germain et al. 2010; Li et al. 2010; Xu et al. 2011; Xu et al. 2012).

1.5.2 Regulators of *SNC1* gene expression levels: MOS1 and MOS9

The *snc1* mutant displays morphological dwarfism together with constitutive defense activation, suggesting that maintaining NLR protein-mediated defense requires sacrifices to plant fitness. Accordingly, *NLR* gene expression must be tightly controlled under normal conditions. However, the mechanisms regulating *NLR* gene transcriptional control are largely unknown. In addition to conventional transcriptional regulation by transcription factors, epigenetic regulation via chromatin or histone modification has emerged as important for fine-tuning transcriptional control. For example, expression of the TNL-encoding gene *LAZ5* (*LAZARUS 5*) requires trimethylation of H3K36 (Histone 3, Lysine 36) by the histone lysine methyltransferase SDG8 (SET DOMAIN GROUP 8) in order to maintain a transcriptionally active chromatin state (Palma et al. 2010).

The identification of *MOS1* and *MOS9* has provided insights into the mechanisms regulating TNL-encoding gene expression. Like other *mos* mutants, loss of either *MOS1* or *MOS9* function suppresses all *snc1*-related phenotypes. *MOS1* encodes a protein containing a HLA-B ASSOCIATED TRANSCRIPT 2 (BAT2) domain that is conserved in both plants and animals (Li et al. 2010). Although an alteration of DNA methylation in a region upstream of *SNC1* is observed in the *mos1* mutant, it is not correlated with the repression of *snc1* expression level, thus *MOS1* likely does not function directly in DNA methylation. Furthermore, transgenic *snc1* expression is not altered in *mos1*, indicating that *MOS1* may regulate *SNC1* expression at its specific chromosomal location. Interestingly, the repression of *snc1* expression level can be mitigated by introducing *ddm1* (*decrease in dna methylation 1*), suggesting that MOS1 and DDM1 may function antagonistically to regulate the expression of *SNC1* at the chromatin level.

MOS9 encodes a plant-specific protein of unknown function (Xia et al. 2013). In the loss-of-function *mos9* mutant, the expression levels of *SNC1* and another TNL-encoding gene, *RPP4*, are reduced. Immunoprecipitation of MOS9 followed by mass spectrum analysis identified ATXR7 (ARABIDOPSIS TRITHORAX-RELATED 7) as a MOS9-associated protein. ATXR7 is a SET domain-containing H3K4 (Histone 3, Lysine 4) methylase required for the proper transcriptional activation of *FLC* (*FLOWERING LOCUS C*) via bulk methylation (Tamada et al. 2009). Identification of ATXR7 as a protein associated with MOS9 suggests that MOS9 likely regulates *NLR* gene transcription through H3K4me3 chromatin modification.

1.5.3 Components of the RNA processing machinery: MOS2, MOS4, and MOS12

To ensure appropriate functionality, stability and localization, nascent pre-mRNA transcripts are subject to a number of processing steps including 5’ capping, 3’ polyadenylation, and splicing,

followed by nuclear export. From our *MOS* screens, three genes were identified as important elements in RNA processing.

MOS2 is required for both CNL- and TNL-mediated resistance (Zhang et al. 2005). It is also required for basal resistance against *P.s.m.* ES4326. These results suggest that MOS2 acts as a point of convergence for a number of immune signaling pathways.

MOS2 is a nuclear protein containing one G-patch and two KOW (Kyrpides, Ouzounis, Woese) motifs, which are conserved among the MOS2 homologs in higher plants and animals. The glycine-rich G-patch motif is often found proximate to RS and RGG amino acid repeats, which have been implicated in non-specific protein-RNA interactions (Aravind and Koonin 1999). Similarly, the KOW motif from the bacterial elongation factor NusG has demonstrated nucleic acid binding activity and shares structural homology with the tudor protein-protein interaction motif (Steiner et al. 2002). While MOS2 has not been experimentally shown to bind RNA, the human MOS2 homolog GPKOW binds RNA in a manner dependent on its phosphorylation by protein kinase A (Aksaas et al. 2011). Furthermore, the remote yeast homolog SPP2 (SPLICEOSOMAL PROTEIN 2) is an essential protein required for the first RNA cleavage step in pre-mRNA splicing as the G-patch domain of SPP2 associates with Prp2 (PRE-mRNA PROCESSING 2), an RNA-dependent ATPase that activates the spliceosome (Roy et al. 1995; Silverman et al. 2004; Yeh et al. 2011). Therefore, MOS2 is predicted to bind RNA, appears to function in splicing, and may also interact with other proteins via its KOW motif. However, the precise role of MOS2 in RNA processing, particularly within an immunity context, remains unclear. Interestingly, the involvement of the yeast MOS2 homolog in pre-mRNA splicing provides evidence that a connection may exist between the roles of MOS2 and MOS4 in regulating plant immunity.

Like MOS2, MOS4 is a nuclear protein that is required for basal resistance against *P.s.m.* ES4326, as well as for both CNL- and TNL-mediated resistance (Palma et al. 2007). Along with 23 other proteins it forms a highly conserved spliceosome-associated complex known as the MOS4-associated complex (MAC) in Arabidopsis (Monaghan et al. 2009). All examined MAC component single mutants are viable but display pleiotropic defects, while all double mutant combinations are lethal (Nemeth et al. 1998; Palma et al. 2007; Monaghan et al. 2009; Monaghan et al. 2010). This indicates that while individual MAC components may be involved in regulating a number of different biological processes, the complex as a whole is required for some essential function, such as general mRNA splicing.

The yeast and human orthologous complexes have been implicated in spliceosome assembly and pre-mRNA splicing (Tarn et al. 1993; Ajuh et al. 2000; Ohi et al. 2002; Deckert et al. 2006), and it is expected that the MAC plays a similar role in plants due to the conserved nature of the complex. Several MAC components, including MOS4, were recently shown to be necessary for the proper splicing of *RPS4* and *SNC1* (Xu et al. 2012). It is tempting to hypothesize that defense-related gene transcripts may be differentially processed upon pathogen detection through modulation of the MAC.

While MOS2 has not been shown to directly associate with the MAC, the human and yeast homologs of MOS2 have been implicated as components of the conserved spliceosome-associated complex (Roy et al. 1995; Bessonov et al. 2010; Aksaas et al. 2011). Additionally, MOS2 has been shown to be required for proper splicing of *SNC1* (S. Xu and Y. Zhang, unpublished data). This provides further support that MOS2 is also involved in RNA processing, possibly in conjunction with the MAC.

MOS12 is required for basal resistance against *P.s.m.* ES4326, as well as for resistance mediated by a subset of NLR proteins, primarily those belonging to the TNL class (Xu et al. 2012). It encodes a nuclear arginine-rich protein with two cyclin domains at the N-terminus. Its closest homolog is human cyclin L, which is predicted to be involved in mRNA splicing due to its association with splicing factors and ability to stimulate splicing *in vitro* (Dickinson et al. 2002; de Graaf et al. 2004).

The *mos12* allele isolated from the *MOS* screens (*mos12-1*) contains a point mutation at an intron-exon splice junction that causes a reading frame shift, resulting in a truncated protein (Xu et al. 2012). However, the truncated protein is likely still partially functional as the null *mos12-2* T-DNA insertion allele is lethal, indicating that this gene plays an essential role in plant growth and development.

In *mos12-1* plants, the splicing patterns of both *SNC1* and *RPS4* are altered from those observed in wild type plants (Xu et al. 2012). In addition, MOS12 co-immunoprecipitates with MOS4. Together, these results indicate that MOS12 plays a critical role in the splicing of *NLR* gene transcripts, likely in association with the MAC through the spliceosome.

Plant defense responses may be regulated in part through alternative splicing of *NLR* gene transcripts, as pathogen detection has been shown to elicit the production of splice variants of a number of TNL-encoding genes, including tobacco *N* and Arabidopsis *RPS4* (Dinesh-Kumar and Baker 2000; Zhang and Gassmann 2003). In tobacco, transcripts of *N* are alternatively spliced following pathogen attack, and a specific ratio of full-length and truncated N proteins is thought to be required for complete resistance against tobacco mosaic virus as neither splice variant is able to individually induce defense response outputs (Dinesh-Kumar and Baker 2000). Similarly, alternative splicing of *RPS4* is induced in Arabidopsis following pathogen detection, and

alternatively spliced transcripts are required for complete *RPS4*-mediated immunity (Zhang and Gassmann 2003). Increased expression of the *RPS4* transcript is induced by the recognition of avrRps4, HopA1, or avrRpt2 effector molecules, of which only avrRps4 is specifically recognized by RPS4 (Zhang and Gassmann 2007). Additionally, detection of avrRps4 resulted in the altered splicing of not only *RPS4* but also of two other Arabidopsis genes known to have alternatively spliced forms, only one of which is thought to potentially function in defense response. Therefore, it has been proposed that increased TNL transcript production and alternative splicing may constitute a general response used to prime plants for resistance. While alternative splicing of *R* genes has been predominantly observed for TNL transcripts, there are reports of CNL gene transcript alternative splicing as well (Halterman et al. 2003; Peart et al. 2005).

1.5.4 Nuclear proteins important for mRNA export: The Nup107-160 complex and MOS11

Cellular compartmentalization in eukaryotic cells requires that processed transcripts be delivered into the cytoplasm before translation can occur. This provides an additional tier of regulation by controlling the nuclear export of mature mRNA molecules. Export of transcripts that are successfully processed requires RNA export proteins such as nuclear export factors, nucleoporins, and RNA chaperones. Although much insight has been gained using human and yeast models, RNA nucleocytoplasmic trafficking is still poorly understood in plants.

Common features exist in the *mos3* and *mos11* mutants, as they both suppress the constitutive autoimmune phenotypes of *snc1* (Zhang and Li 2005; Germain et al. 2010). In addition, the *mos3* single mutant exhibits enhanced disease susceptibility to both virulent and

avirulent pathogens, suggesting the role of MOS3 in both basal defense and NLR protein-mediated defense. However, such increased susceptibility was not observed in the *mos11* single mutant. *In situ* hybridization of total mRNA revealed a dramatic accumulation of transcripts in the nucleus of each mutant, which suggests that MOS3 and MOS11 are both required for successful mRNA export (Parry et al. 2006; Germain et al. 2010).

MOS3 localizes to the nuclear rim while MOS11 is present in the nuclear matrix, suggesting that MOS11 may function before MOS3 in the mRNA export process (Zhang and Li 2005; Germain et al. 2010). *MOS11* encodes a homolog of human CIP29, an RNA co-chaperone enhancing the activity of RNA helicase DDX39 (Sugiura et al. 2007; Dufu et al. 2010). MOS11 may function as part of a similar complex during the mRNA export process in plants.

MOS3 is homologous to NUCLEOPORIN (Nup) 96 in mammals and Nup145 in yeast, both of which have been reported to be involved in mRNA export (Fabre et al. 1994; Vasu et al. 2001). Nup96 functions as part of the Nup107-160 nuclear pore sub-complex. In mice, the loss-of-function *nup96* allele is lethal when homozygous and the immune systems of heterozygotes are severely impaired, indicating that Nup96 functions in both innate and adaptive mammalian immunity (Faria et al. 2006). MOS3 is thought to function as part of a homologous complex in Arabidopsis, and other putative complex components, including Nup160 and Seh1, were recently shown to be required for basal and TNL-mediated resistance (Wiermer et al. 2012). While it is uncertain how a general defect in non-specific mRNA export impairs NLR-mediated immunity without serious developmental consequences, one possibility is that plants have evolved to be more resilient, and loss of only one component of the Nup107-160 complex does not lead to lethality. However, the double mutant of *nup96 nup160* is seedling lethal (Parry et al. 2006).

1.5.5 Components involved in nucleocytoplasmic protein trafficking: MOS6, MOS7, and MOS14

The *mos6* mutant alleles were identified in the suppressor screens either in *snc1* or *snc1 npr1* backgrounds (Palma et al. 2005). In all cases, *mos6* alleles partially suppress the autoimmune phenotypes of *snc1*. Interestingly, the *mos6* single mutant exhibits enhanced disease susceptibility to *H.a.* Noco2, but not to *P.s.m.* ES4326, indicating that MOS6 may play a specific role in basal defense against oomycete infection. Positional cloning of *MOS6* showed that it encodes importin α3. The MOS6 protein has the typical features of importin α proteins, including an importin β-binding domain and nuclear localization signal (NLS)-binding pockets. GFP-localization of MOS6 showed that it is concentrated in the nucleus, which supports the idea that it is a functional importin α (Palma et al. 2005). Within the Arabidopsis genome, there are eight genes encoding homologs of importin α, including *MOS6*. This large number of importin homologs in Arabidopsis is unsurprising, given the essential role of protein import in every aspect of plant growth and development. Whether a subset of these importin α homologs is specifically involved in plant defense response is not known. Since all importin α proteins bind NLS, there are likely protein-protein interaction domains defining specificity.

Besides its involvement in *snc1*-mediated defense, MOS7 has also been suggested to play roles in basal defense and defense mediated by other NLR proteins. Furthermore, SAR is compromised in the *mos7* single mutant, as pre-treatment of the *mos7* plants with an avirulent bacterial pathogen failed to trigger subsequent immunity in distal leaves (Cheng et al. 2009). *MOS7* encodes a protein with homology to Nup88 in animals, which is involved in nuclear protein export (Uv et al. 2000). MOS7 localizes to the nuclear rim, indicating its potential role as a nucleoporin in Arabidopsis. Intriguingly, the nuclear accumulations of NPR1, EDS1 and SNC1

are reduced in the *mos7-1* single mutant as compared to wild type, while the nuclear distributions of HDA19, CDC5 and TGA2 remain unchanged (Cheng et al. 2009). This suggests that there is specificity in the protein export pathways affected by the *mos7-1* mutation. However, since MOS7 is a single-copy gene in the Arabidopsis genome and the *mos7-2* null T-DNA insertion allele is lethal, wild type MOS7 is likely required for general nuclear protein retention. Further work is required to determine how the *mos7-1* mutation leads to the specific enhancement of nuclear export activity of immunity-related proteins such as SNC1, EDS1, and NPR1.

MOS14 is a required intermediate in both basal resistance and some TNL-mediated resistance signaling pathways (Xu et al. 2011). It is a single copy gene in Arabidopsis encoding a nuclear protein with homology to metazoan transportin-SR (TRN-SR) proteins, which are nuclear import receptors. TRN-SR proteins belong to the importin-β super-family, members of which mediate the import of protein cargo through the nuclear pore complex (NPC) upon recognition of an NLS. TRN-SR proteins specifically transport serine-arginine rich (SR) proteins, which function in both constitutive and alternative splicing through their roles in pre-mRNA splice site recognition and spliceosome assembly (Long and Caceres 2009).

The MOS14 protein was shown to interact with four different SR proteins through its C terminus and AtRAN1, which is required for release of the cargo into the nucleus, through its N terminus (Xu et al. 2011). In the homozygous *mos14-1* mutant, nuclear localization of SR proteins is impaired. In keeping with this mislocalization of known splicing factors, the splicing patterns of *SNC1* and *RPS4* are altered in *mos14-1* plants, and resistance mediated by these proteins is attenuated.

Studies of MOS6, MOS7, and MOS14 have revealed the importance of nucleocytoplasmic protein trafficking in the regulation of plant defense. In addition to the mRNA

export process discussed in previously, fine-tuned nuclear import and export of defense regulators seem to play a key role in mounting effective immunity in plants.

1.5.6 Transcriptional co-repression with SNC1: MOS10 (TPR1)

MOS10 encodes a nuclear protein with high sequence similarity to *TOPLESS* (*TPL*) (Zhu et al. 2010), a transcriptional corepressor that functions during embryogenesis in an auxin-dependent manner (Szemenyei et al. 2008). As such, *MOS10* was renamed *TOPLESS RELATED 1* (*TPR1*). Like TPL, TPR1 is a transcriptional corepressor, which is a unique biological function amongst the identified MOS proteins.

Both *TPR1* and *TPL* are required for basal immunity, as well as resistance mediated by a number of TNL proteins (Zhu et al. 2010). Overexpression of *TPR1* results in the constitutive activation of defense phenotypes similar to those observed in the *snc1* mutant, including increased SA accumulation, constitutive *PR* gene expression, and enhanced resistance to *H.a.* Noco2. These responses in the *TPR1* overexpression lines require EDS1, PAD4, and SNC1. Co-immunoprecipitation experiments showed that TPR1 and SNC1 associate with one another *in planta*, likely through the TIR domain of SNC1.

The homozygous *tpl* mutant displays phenotypes similar to those observed in *histone deacetylase 19* (*hda19*), including apical shoot defects (Long et al. 2006). Additionally, the *hda19* single mutant exhibits compromised pathogen resistance (Kim et al. 2008). Co-immunoprecipitation experiments indicate that TPR1 associates with HDA19 *in vivo* (Zhu et al. 2010). Histone deacetylases remove acetyl groups from a histone lysine residue, thereby enhancing DNA condensing which in turn inhibits transcription. TPR1 has been shown to associate with the promoters of DEFENSE NO DEATH 1 (DND1) and DND2, two known

negative regulators of immunity (Yu et al. 1998; Yu et al. 2000), and may act together with HDA19 to regulate their transcription. SNC1 and other NLR proteins may activate downstream defense responses in part by modulating the transcriptional repression activity of TPR1.

1.5.7 Protein modifying enzymes: MOS5 and MOS8

In most eukaryotes, post-translational modifications (PTMs) modulate protein function by influencing their activity, stability and localization. PTMs are needed to regulate a diverse range of cellular functions. Increasing evidence indicates that PTMs, such as ubiquitylation and phosphorylation, play an important role in plant defense signaling.

The identification of *MOS5* and *MOS8* indicates that PTMs are crucial in regulating NLR protein activation. *MOS5* encodes one of two ubiquitin-activating (E1) enzymes in Arabidopsis (Goritschnig et al. 2007). Along with E2 (Ubiquitin conjugating enzyme) and E3 (Ubiquitin ligase) enzymes, E1s are involved in labeling protein substrates with ubiquitin moieties, typically to mark a substrate for degradation by the 26S proteasome. The loss of *MOS5* function partially suppresses *snc1* phenotypes and leads to both impaired basal and NLR--mediated defense activity. The single mutant of the other Arabidopsis E1 enzyme, *uba2*, displays no obvious phenotypic defects, but the *mos5 uba2* double mutant is lethal, indicating that a large degree of redundancy exists between these two E1 enzymes.

The *mos5* mutant contains a molecular lesion in the putative ubiquitin-fold domain, which likely disrupts the ubiquitylation process. The enhanced susceptibility phenotypes observed in the *mos5* mutant may result from the increased stability of negative defense regulators, the degradation of which might be essential in *snc1*-mediated defense resistance.

Alternatively, the *mos5* mutation may disrupt the function of positive defense regulators, which may require mono-ubiquitination for activation. Recent work has shown that the F-box protein CPR1, which belongs to the SKP1-CULLIN1-F-box (SCF) E3 complex, targets SNC1 and other NLRs for degradation, highlighting the importance of ubiquitylation in regulating NLR protein levels and preventing autoimmunity (Cheng et al. 2011). Surprisingly, while *mos5* suppresses the dwarf phenotype of *snc1*, it enhances the stunted growth morphology observed in *cpr1-2* (Gou et al. 2012). The complex *mos5* phenotype is likely a result of the mutant allele's impact on multiple E3 enzyme activities (Cheng and Li 2012).

Other PTMs that modify protein localization, solubility, and protein-protein interactions are also required in defense signal transduction. The common lipid modification, prenylation, involves the covalent binding of hydrophobic farnesyl- or geranylgeranyl- moieties to the target proteins, likely facilitating their binding to cellular membranes (Galichet and Gruissem 2003). *mos8* is an allele of *ERA1* (*ENHANCED RESPONSE TO ABSCISIC ACID 1*), which encodes the β-subunit of farnesyltransferase (Goritschnig et al. 2008). Like other *era1* alleles, *mos8* displays enhanced susceptibility to *P.s.m.* ES4326 and *H.a.* Noco2, implying a role for farnesylation in basal immunity. It also exhibits impaired defense responses mediated by several NLR proteins. Furthermore, epistatic analyses using *era1* and several abscisic acid (ABA) biosynthesis mutants indicates that enhanced susceptibility of *era1* is only partially dependent on ABA. The *era1 npr1* double mutant displays enhanced *era1* phenotypes, indicating that *ERA1* functions in an *NPR1*-independent pathway. Even though the target proteins modified by ERA1 are currently unknown, lipid modification is likely playing an important role in disease resistance signaling by targeting substrate proteins to cellular membranes and altering their activities.

1.5.8 Integration of the *MOS* genes

The results of the *MOS* screens were somewhat surprising in that they primarily resulted in the identification of novel factors regulating *SNC1* expression, RNA processing, nucleocytoplasmic trafficking, and protein localization and activity of *NLR* genes and their encoded products. We propose a model that brings together these seemingly disparate functions within a resistance signaling context, centering on NLR protein activation (Figure 2.1).

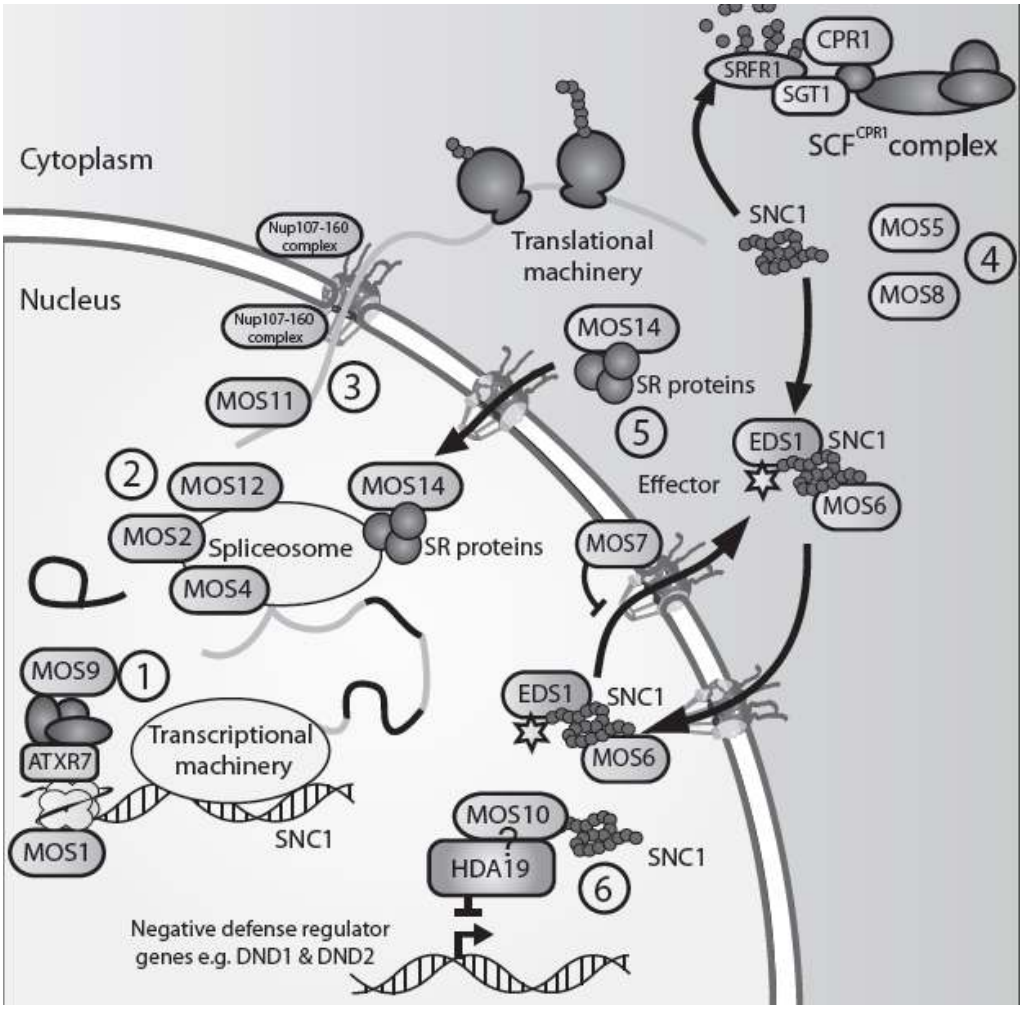

Figure 1.1. A model depicting the involvement of the MOS proteins in NLR protein-mediated defense signaling pathways in Arabidopsis, using SNC1 as an example of the journey of TNL proteins.
1. At chromosomal level, MOS1, ATXR7 and MOS9 up-regulate the transcription of *SNC1* through chromatin remodeling. **2.** MOS2, MOS4, and MOS12 are required for the proper

splicing of the transcripts of *SNC1*. **3.** The Nup107-160 complex and MOS11 play key roles in the export of total mRNA (including mature mRNA of *SNC1*), which is required for effective defense. **4.** MOS5 is an E1 ubiquitin-activating enzyme, an essential component of the ubiquitination cascade, required for the regulation of defense signaling components. As an example, the SCF^{CPR1} E3 ubiquitin ligase complex targets SNC1 for degradation which prevents autoimmunity caused by over-accumulation of NLR proteins. MOS8 positively regulates plant defense, possibly through prenylation that affects the targeting of defense regulators. **5.** MOS6 and MOS7 are involved in the nucleocytoplasmic shuttling of defense signaling molecules such as SNC1, EDS1, and NPR1. Like with RPS4, EDS1 is probably required for the nuclear localization and activation of SNC1 upon the recognition of its corresponding effector (Bhattacharjee et al. 2011; Heidrich et al. 2011). MOS14 is required for the nuclear import of splicing factors that may affect defense regulator RNA processing. **6.** MOS10 activates the SNC1-mediated defense through transcriptional repression of negative regulators of defense such as *DND1* and *DND2*.

Our model suggests that NLR protein-mediated signaling may not consist of a long, linear signaling pathway possessing numerous intermediates as we imagined earlier. Rather, the primary regulatory steps exist at the gene and protein processing levels. Collectively, the characterized *MOS* genes provide new insights into the complex regulatory mechanisms governing NLR-mediated immunity.

In the absence of pathogens, low *NLR* gene expression maintains a small reservoir of NLR proteins which putatively act as a surveillance system, protecting against pathogen attack. Upon pathogen detection, a number of factors including MOS1, ATXR7 and MOS9 likely alter chromatin structure or histone codes in order to up-regulate *NLR* gene transcription, thus enhancing pathogen detection capabilities and amplifying defense response signaling (Li et al. 2010; Y. Cheng and X. Li, unpubli.).

The processing of *NLR* gene transcripts is another regulatory node. MOS2, MOS4, MOS12 and MOS14 are all required for correct *NLR* transcript splicing (Xu et al. 2011; Xu et al. 2012; F. Xu and Y. Zhang, unpubl.). Following pathogen detection, these proteins may aid in recruiting the spliceosome preferentially to *NLR* gene transcripts in order to increase the speed,

specificity and strength of defense response activation. Alternatively, these proteins may play a role in *NLR* gene transcript alternative splicing, which is thought to provide specificity in the type and strength of immune response activated.

After splicing, transcripts must be exported to the cytosol to be translated. The Nup107-160 complex, which includes MOS3, functions in the same mRNA export pathway as MOS11 (Zhang and Li 2005; Germain et al. 2010; Wiermer et al. 2012). The mRNA export process could be another regulatory step modulating the final defense response outputs from *NLR* genes.

After translation in the cytosol, protein activity and localization may be altered by PTMs. MOS5 functions as an essential part of the ubiquitination pathway, which results in the addition of ubiquitin moieties to target proteins (Goritschnig et al. 2007). This may mark negative defense regulators for proteasomal degradation or, alternatively, activate positive regulators. MOS5 likely has a general role in ubiquitination. MOS8 is required for prenylation (Goritschnig et al. 2008), a type of PTM that regulates protein membrane targeting (Resh 2006). MOS8 may direct defense regulators to their correct locations thereby allowing signal transduction to proceed, although its targets have not yet been identified.

Following protein synthesis and modifications, many defense regulators are transported back to the nucleus in order for defense activation to occur. MOS6 and MOS7 are likely involved in the nuclear import and retention of defense-related proteins, although the degree of specificity in the activity of these proteins is unclear (Palma et al. 2005; Cheng et al. 2009). MOS14 is required for the nuclear import of SR proteins, most of which are splicing factors (Xu et al. 2011). While MOS14 is required for precise *NLR* gene splicing, specificity has not been demonstrated. Thus, NLR-mediated immunity may also be regulated by factors involved in

nuclear import and export of proteins. These events affect the nuclear retention of key defense regulators and in turn determine the final levels of defense outputs.

MOS10/TPR1 is unique among the MOS proteins. It represses the transcription of known negative regulators of defense and associates with SNC1 in the nucleus, likely as part of a complex (Zhu et al. 2010). The association of SNC1 with MOS10/TPR1 appears to be required for the strong and rapid repression of negative defense regulators in order to mount an effective defense response.

During the past decade, studies of the *MOS* genes in our laboratory have provided insight into the molecular details surrounding NLR-mediated immunity. However, the full picture of the regulation of *NLR* genes and their encoded products has not yet emerged.

The improved next generation sequencing strategies and biochemical approaches guarantee an impending revolution in molecular studies of plant biology.

1.6 Thesis objectives

The primary objective of the research presented in this thesis was to identify novel regulators of plant innate immunity. As the *MOS* screen previously conducted in the Li laboratory was highly successful in identifying positive regulators of NLR-mediated immunity by looking for suppressors of the autoimmune mutant *snc1*, an enhancer screen was utilized to identify negative regulators. This thesis describes the identification and characterization of three *MUTANT, SNC1-ENHANCHING* (*MUSE*) mutants isolated from this screen: *muse9*, *muse4*, and *muse7*. The aim of performing functional studies of these *MUSE* genes and the proteins they encode is to improve our understanding of the mechanisms that underlie plants' endogenous defense responses.

Chapter 2: The chromatin remodeler SPLAYED negatively regulates SNC1-mediated immunity[2]

2.1 Summary

SNC1 (SUPPRESSOR OF NPR1, CONSTITUTIVE 1) is one of a suite of intracellular Arabidopsis NOD-like receptor (NLR) proteins which, upon activation, result in the induction of defense responses. However, the molecular mechanisms underlying NLR activation and the subsequent provocation of immune responses are only partially characterized. To identify negative regulators of NLR-mediated immunity, a forward genetic screen was undertaken to search for enhancers of the dwarf, autoimmune gain-of-function *snc1* mutant. To avoid lethality resulting from severe dwarfism, the screen was conducted using *mos4* (*modifier of snc1, 4*) *snc1* plants, which display wild-type-like morphology and resistance. M2 progeny were screened for *mutant, snc1-enhancing* (*muse*) mutants displaying a reversion to *snc1*-like phenotypes. The *muse9 mos4 snc1* triple mutant was found to exhibit dwarf morphology, elevated expression of the *pPR2-GUS* defense marker reporter gene, and enhanced resistance to the oomycete pathogen *Hyaloperonospora arabidopsidis* Noco2. Via map-based cloning and Illumina sequencing, it was determined that the *muse9* mutation is in the gene encoding the SWI/SNF chromatin remodeler SYD (SPLAYED), and was thus renamed *syd-10*. The *syd-10* single mutant has no observable alteration from wild-type-like resistance, although the *syd-4* T-DNA insertion allele displays enhanced resistance to the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* ES4326.

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Transcription of *SNC1* is increased in both *syd-4* and *syd-10*. These data suggest that SYD plays a subtle, specific role in the regulation of *SNC1* expression and SNC1-mediated immunity. SYD may work with other proteins at the chromatin level to repress SNC1 transcription; such regulation is important for fine-tuning the expression of NLR-encoding genes to prevent unpropitious autoimmunity.

2.2 Introduction

To compensate for the vulnerability inherent in being sessile organisms, plants must maintain a tightly regulated innate immune system to ward off pathogenic infection (Dangl et al. 2013). As part of this system, the detection of conserved microbial features by receptors on the plant cell surface induces relatively mild defense responses (Macho and Zipfel 2014). However, successful pathogens are able to deliver effector molecules into the host cell to suppress this immune response and promote infection.

As an additional line of defense, plants possess a suite of intracellular receptors termed RESISTANCE (R) proteins which recognize effectors in a specific manner either directly or through their effects upon other host proteins (Chisholm et al. 2006; Dangl and Jones 2001). Although there are several classes of R proteins, the majority belong to the nucleotide-binding and leucine-rich repeat domain-containing/NOD-like receptor (NLR) class. Upon effector detection, NLR proteins become activated and strong, robust defense responses are induced. NLR protein-mediated immunity is characterized by an accumulation of the defense hormone salicylic acid (SA), increased expression of *PATHOGENESIS-RELATED* (*PR*) defense marker genes, and often a programmed cell death event known as the hypersensitive response (HR)

(Hammond-Kosack and Jones 1996). While NLR-mediated immunity is a well-documented phenomenon in higher plants, the molecular mechanisms underlying its regulation are only marginally understood.

In the absence of pathogen attack, NLR protein levels must be kept under stringent control in order to prevent growth defects and potential lethality resulting from unwanted activation of autoimmune responses. Upon infection, however, the repression of NLR protein-mediated signaling pathways must be released in order to allow the rapid induction of defense responses. The regulation of NLR-mediated immunity occurs at the transcriptional, translational, and post-translational levels. At the transcriptional level, a number of positive regulators of *NLR* gene expression have been identified. The histone lysine methyl transferase SDG8 trimethylates H3K36 (Histone 3, Lysine 36) at the NLR-encoding *LAZ5* locus, and this activity is required for the perpetuation of a transcriptionally active chromatin state (Palma et al. 2010). Similarly, MOS9 was shown to function together with the methyl transferase ATXR7 in the methylation of H3K4 at the NLR-encoding *SNC1* and *RPP4* loci, and this methylation is required for the full expression of these genes (Xia et al. 2013). The MOS1 protein, which contains an HLA-B ASSOCIATED TRANSCRIPT 2 domain, is required for full *SNC1* expression and functions antagonistically with the chromatin remodeling factor DECREASED DNA METHYLATION 1 (DDM1) (Li et al. 2010; Li et al. 2011). Although the mechanism of this regulation is not well understood, it is thought to occur at the chromatin level as the expression of transgenic *SNC1* does not require MOS1.

MOS1 and MOS9 were both identified from a forward genetic screen designed to isolate positive regulators of NLR-mediated immunity. The *MODIFIERS OF SNC1* (*MOS*) screen was designed to identify suppressors of the autoimmune mutant *snc1* (*suppressor of npr1,*

constitutive 1), which contains a gain-of-function mutation in an NLR-encoding gene (Li et al. 2001; Zhang et al. 2003). Mutant *snc1* plants display a dwarfed, dark green, curled-leaf morphology, accumulate SA, and exhibit constitutively activated defense responses, although lesions typically associated with HR fail to form. As such, the *snc1* mutant has become a useful genetic background in which to conduct forward genetic screens for regulators of immunity. From the MOS screens, mutants exhibiting a suppression of *snc1*-mediated defense responses were selected and many *mos* mutations were cloned.

As the MOS screens were successful in identifying positive regulators of NLR-mediated immunity (summarized in Johnson et al. 2012), we proceeded to design enhancer screens in the *snc1* background in order to identify negative regulators of immunity. To avoid lethality resulting from dramatic dwarfism the forward genetic screens were conducted by mutagenizing seeds from *mos4 snc1* plants, which are wild-type-like in terms of morphology and resistance levels. As part of the MUTANT, SNC1-ENHANCING (MUSE) screen a number of mutants displaying a reversion back to *snc1*-like morphology and defense outputs were isolated, several of which have been recently published (Huang et al. 2013; Huang et al. 2014a; Huang et al. 2014b; Xu et al. 2015, in press).

This study focuses on the isolation, identification, and characterization of *muse9*. The *muse9 mos4 snc1* triple mutant is dwarfed and displays elevated expression of the *pPR2-GUS* reporter gene. An elevation in resistance against the virulent oomycete strain *Hyaloperonospora arabidopsidis* (*H.a.*) Noco2 was observed in the triple mutant. The *muse9* mutation was found to be a novel allele of *splayed* (*syd-10*), which encodes a SWI/SNF chromatin remodeler. The *syd-10* single mutant exhibits wild-type-like resistance, but the *syd-4* T-DNA insertion allele exhibits enhanced resistance to *Pseudomonas syringae* pv. *maculicola* (*P.s.m.*) ES4326. Double mutant

analysis showed that mutations in the *SYD* locus enhance the dwarfism of *snc1*, and SYD is required for modulating transcription at the *SNC1* locus. Thus, we establish that SYD plays a subtle but specific role in repressing *SNC1* expression.

2.3 Results

2.3.1 Isolation of *muse9 snc1 mos4*

The *muse9* mutation was isolated from the MUSE forward genetic screen described previously (Huang et al. 2013), which was conducted in the *mos4 snc1* mutant background with ethyl methanesulfonate (EMS) as a mutagen. Mutant lines displaying a reversion to *snc1*-like phenotypes were selected as putative *snc1* enhancers. The *muse9 mos4 snc1* triple mutant displays *snc1*-like morphological phenotypes (Figure 2.1A).

In *snc1*, a number of *PATHOGENESIS-RELATED* (*PR*) defense marker genes are constitutively expressed. All mutants from the MUSE screens contain a reporter gene construct in which the promoter of *PR2* is fused to the coding region of β-glucuronidase (GUS), allowing for a rapid visualization of defense gene expression. In the wild-type Columbia (Col-0) background no GUS expression is observed (Figure 2.1B). The *muse9* mutation partially rescues the constitutive expression of the *pPR2-GUS* reporter gene observed in *snc1* but suppressed in *mos4 snc1* (Figure 2.1B).

The *snc1* mutation confers enhanced resistance against the virulent oomycete pathogen *H.a.* Noco2 (Zhang et al. 2003). Consistent with the observed rescue of *pPR2-GUS* constitutive expression noted above, the *muse9 mos4 snc1* triple mutant showed a moderate but significant enhancement in resistance against *H.a.* Noco2 as compared to the *mos4 snc1* double mutant

(Figure 2.1C). Together, these data indicate that the *muse9* mutation is able to partially enhance *snc1* phenotypes in the *mos4 snc1* background.

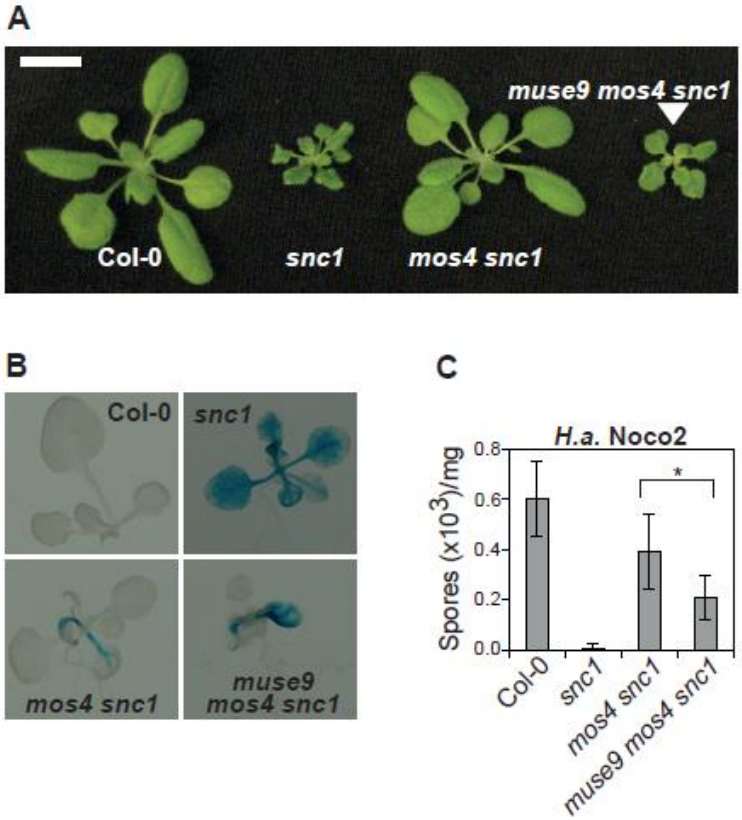

Figure 2.1. Phenotypic analysis of the *muse9 mos4 snc1* triple mutant.
(A) Morphology of soil-grown Col-0, *snc1*, *mos4 snc1*, and *muse9 mos4 snc1* plants. Photographs were taken 3 weeks post-germination. Scale bar indicates 1 cm.
(B) *PR2* gene expression depicted using the *pPR2-GUS* fusion construct present in all shown genetic backgrounds. Plants were grown for 10 d on MS media.
(C) Growth of *H.a.* Noco2 on indicated genotypes 7 d post-inoculation with $1x10^5$ spores/mL. Values represent the average of 4 replicates of 5 plants each ± SD. Significant difference between *mos4 snc1* and *muse9 mos4 snc1* indicated by * (P-value < 0.05). The experiment was repeated three times with similar results.

2.3.2 Phenotypes associated with *muse9* result from a point mutation in *SYD*

To determine the molecular lesion responsible for the *snc1*-enhancing phenotypes associated with *muse9*, a positional cloning strategy was employed. The *muse9 mos4 snc1* triple mutant in

the Col-0 ecotype was crossed with Landsberg *erecta* (L*er*) to generate the mapping population. Linkage analysis was performed using 24 F2 plants displaying *snc1*-like phenotypes, which revealed that *muse9* showed linkage with markers located between 9.2MB and 13.2MB on chromosome 2 (Figure 2.2A).

Fine mapping using >1000 F3 plants from F2 progeny that were homozygous for *snc1* and *MOS4*, but heterozygous for *muse9*, further narrowed down *muse9* to a region between 10.8MB and 12.4MB on chromosome 2.

To identify the exact mutation responsible for *muse9*, Illumina whole genome sequencing was performed. Comparisons between the mutant sequence and the reference Col-0 Arabidopsis genome indicated that five genes located within this mapped region contained mutations consistent with EMS mutagenesis (Figure 2.2B). However, four of these are either silent or intronic mutations. The other mutation is in *At2g28290*, and results in an amino acid change; therefore, it was selected as the most likely candidate for *muse9*. *At2g28290* encodes SPLAYED (SYD), a SWI/SNF chromatin remodeling ATPase previously implicated in development as well as jasmonate (JA) and ethylene (ET) signaling pathways (Wagner and Meyerowitz 2002; Walley et al. 2008). The C to T substitution in *muse9* occurs in the last exon of *SYD* in a region of the protein that does not contain any known conserved domains (Figure 2.2C-D), and results in the substitution of Ala2224 with Val (Figure 2.2E).

Transgene complementation is commonly employed in verifying positional cloning results. However, the large size of the *SYD* locus (>16 kb) precludes straightforward molecular cloning in binary plasmid vectors. Instead, to verify that the mutation in *SYD* is responsible for the *muse9* phenotypes, an allelism test was carried out between the *muse9* single mutant and *syd-*

4, a previously published T-DNA insertion allele (Zhu et al. 2013) that contains an insertion in the conserved helicase domain of SYD (Figure 2.2D).

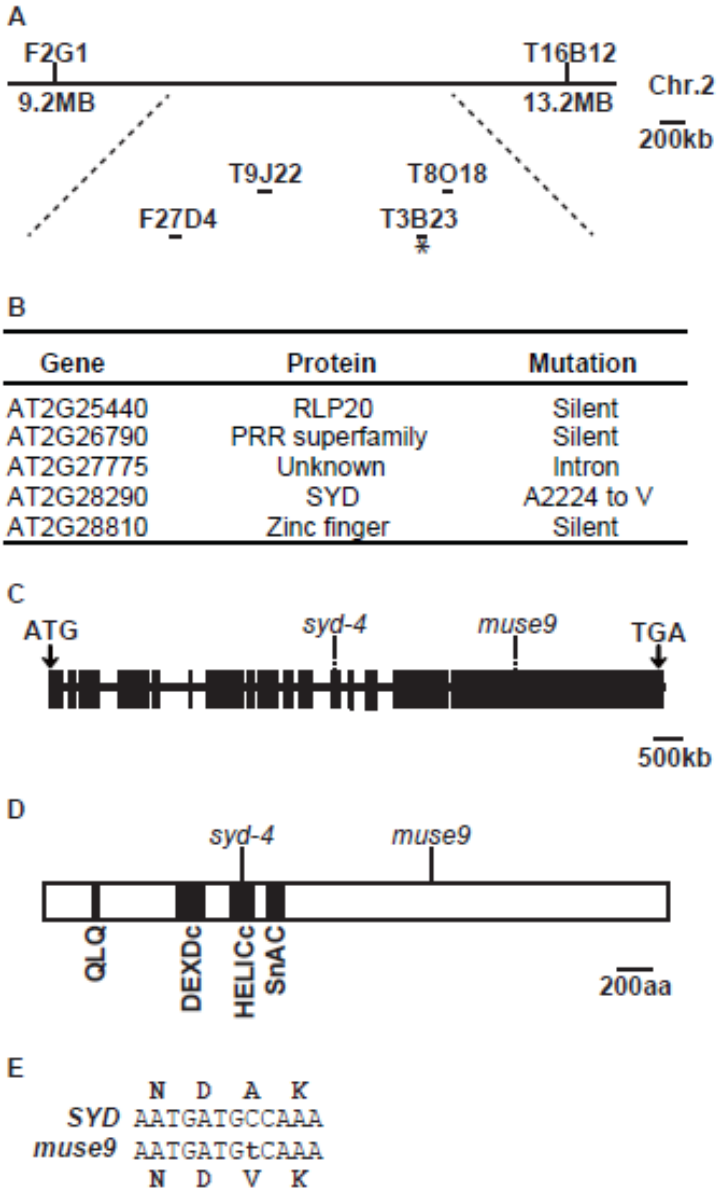

Figure 2.2. Positional cloning of the *MUSE9* locus on chromosome 2.
(A) A genetic map depicting the region of chromosome 2 that contains the *MUSE9* locus, with markers used for mapping indicated.
(B) Mutations identified within the mapping region of *muse9* using Illumina sequencing.
(C) The gene structure of *SYD*, with the locations of the *syd-4* and *muse9* (*syd-10*) mutations indicated. Boxes and lines represent exons and introns, respectively.

(D) The conserved domain structure of the SYD protein, with the sites of the *syd-4* and *muse9* mutations denoted. Domains were identified using the NCBI Conserved Domain Database.
(E) Sequence comparison between wild-type *SYD* and *muse9*. A nucleotide substitution, indicated by the lower-case bolded 't', results in an A2224V amino acid substitution.

The *muse9* single mutant was obtained by backcrossing *muse9 mos4 snc1* to Col-0 and selecting F2 lines that were homozygous for the *muse9* mutation and wild-type at the *MOS4* and *SNC1* loci. Both the *muse9* and *syd-4* mutations result in slightly crinkled leaves and a small reduction in stature as compared to wild-type. The F1 progeny resulting from a cross of these two genotypes retain these characteristics (Figure 2.3A), indicating that *muse9* failed to complement *syd-4* and therefore that *MUSE9* is *SYD*.

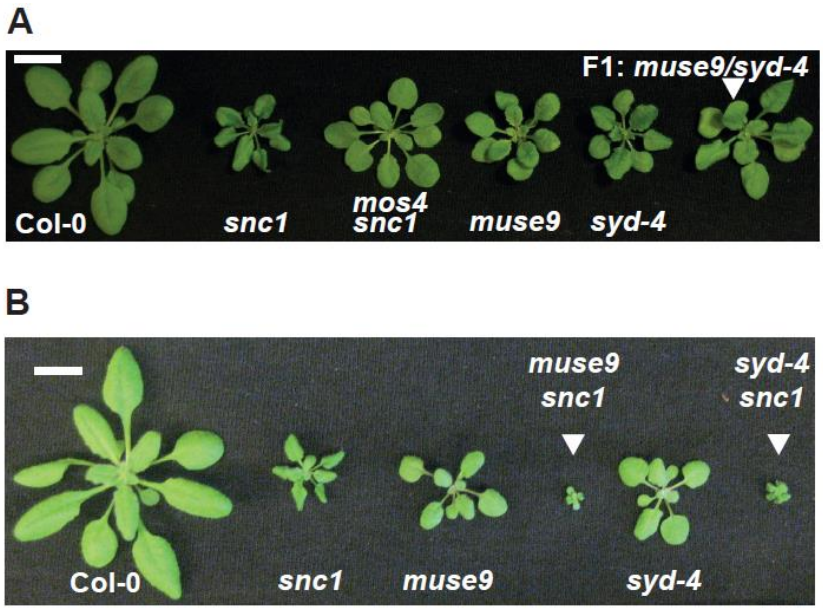

Figure 2.3. *MUSE9* encodes SPLAYED (SYD), an ATP-dependent chromatin remodeler.
(A) Complementation test between *muse9* and *syd-4*. Morphology of soil-grown Col-0, *snc1*, *mos4 snc1*, *muse9*, *syd-4*, and an F1 plant from a cross between *muse9* and *syd-4*. Photograph was taken 3 weeks post-germination. Scale bar indicates 1 cm.
(B) Morphology of soil-grown Col-0, *snc1*, *muse9*, *muse9 snc1*, *syd-4*, and *syd-4 snc1* plants. Photograph was taken 3 weeks post-germination. Scale bar indicates 1 cm.

As an additional method of verification, the *snc1*-enhancing effects of the two *syd* alleles were compared. The *muse9 snc1* double mutant was isolated from the F2 progeny of the backcross described above, and the *syd-4* mutant was crossed with *snc1* to generate the *syd-4 snc1* double mutant. Both double mutants show a dramatic reduction in size compared to either *muse9* or *syd-4* and *snc1* (Figure 2.3B). Taken together, we conclude that the phenotypes associated with *muse9* are a result of a mutation in *SYD*; therefore, we renamed *muse9* as *syd-10*.

2.3.3 The *syd-4* single mutant displays enhanced disease resistance

As demonstrated above, *syd-10* was found to enhance *snc1*-associated morphological and disease resistance phenotypes in the *snc1* and *mos4 snc1* genetic backgrounds. As growth of the *syd-10* single mutant is slightly stunted (Figure 2.3A), and fitness costs including diminished stature and reduced seed production are commonly associated with constitutive activation of NLR-mediated defense responses, it was hypothesized that the single mutant may show enhanced disease resistance independent of the presence of the *snc1* mutation. This hypothesis was tested using a number of infection assays with virulent pathogens.

As noted above, *snc1* displays enhanced resistance to the oomycete *H.a.* Noco2; however, resistance to this pathogen was found to be wild-type-like in both *syd-4* and *syd-10* (Figure 2.4A). *snc1* also displays enhanced resistance to the virulent bacterial strain *P.s.m.* ES4326 (Zhang et al. 2003). When *syd-10* and *syd-4* plants were challenged with this pathogen the *syd-10* single mutant was again found to display wild-type-like resistance, but enhanced resistance was consistently observed in the *syd-4* single mutant (Figure 2.4B). We found that *PR1* and *PR2* are upregulated in both *syd* alleles (Figures 2.4C-D), although expression was enhanced to a greater degree in the *syd-4* mutant. Consistent with a previous report which found

that expression of the defensin *PDF1.2a*, a marker of intact ET and JA signaling pathways, was reduced in the *syd-2* mutant (Walley et al. 2008), we also observed lower *PDF1.2a* expression in the *syd-4* and *syd-10* mutants (Figure 2.4E). Since *syd-4* contains an insertion in the conserved helicase domain of SYD while *syd-10* carries a point mutation in the weakly conserved N terminal region of the protein (Figure 2.2D; Figure 2.5), it is possible that *syd-10* is a weaker allele and therefore exhibits more subtle phenotypes.

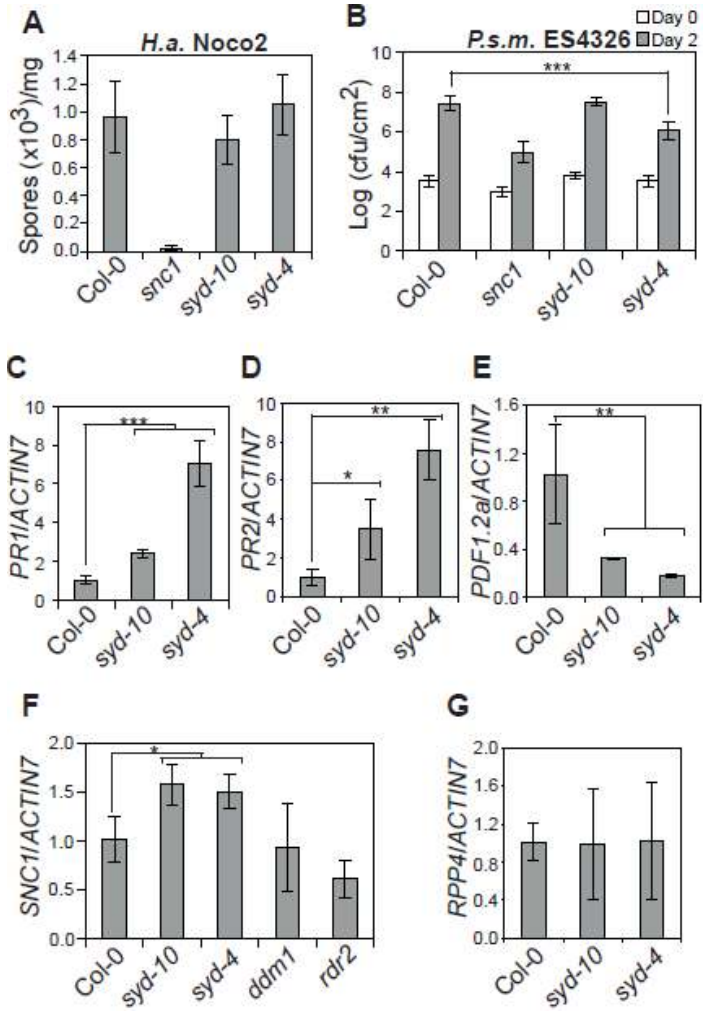

Figure 2.4. The *syd-10* single mutant does not display enhanced disease resistance.
(A) Growth of *H.a*. Noco2 on indicated genotypes 7 d post-inoculation with $1x10^5$ spores/mL. Values represent the average of 4 replicates of 5 plants each ± SD.
(B) Growth of *P.s.m.* ES4326 on indicated genotypes 2 d post-infiltration. Values represent the average of 5 replicates ± SD. Significant difference between Col-0 and *syd-4* indicated by *** (P-value < 0.001).
(C-G) Real-time qRT-PCR analysis of (C) *PR1*, (D) *PR2*, (E) *PDF1.2a*, (F) *SNC1*, and (G) *RPP4* expression in the indicated genotypes. Total RNA was extracted from seedlings grown for 12 d on MS media. Significant differences are indicated by asterisks (* P-value < 0.05, ** P-value < 0.01, *** P-value < 0.001). All experiments were repeated at least once with similar results.

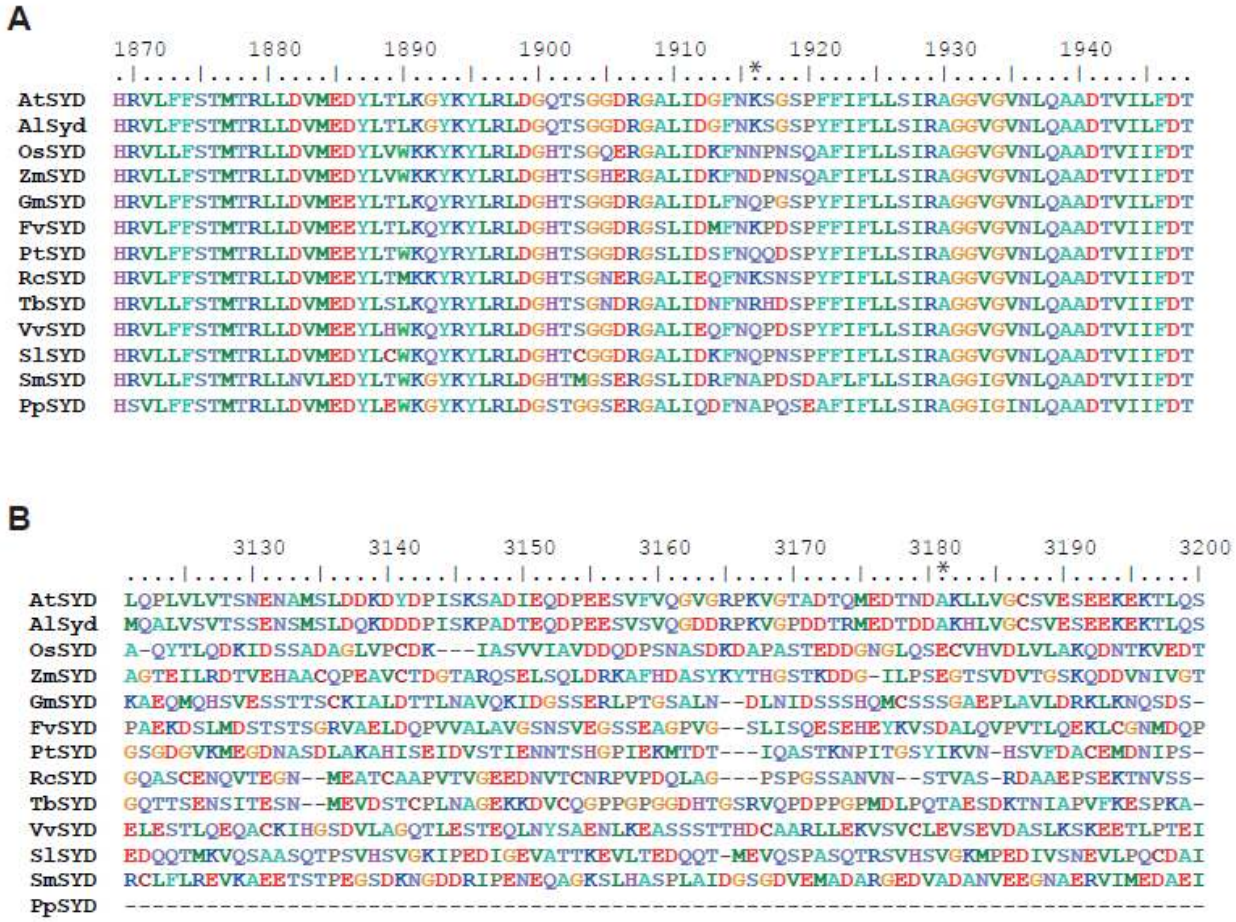

Figure 2.5. Alignment of SYD proteins from a number of plant species. BLAST searches using the AtSYD amino acid sequence were performed, and the first hit for each of the indicated species was included in the alignment. A multiple sequence alignment was performed in BioEdit using ClustalW. The regions of the alignment containing the (A) *syd-4* T-DNA insertion and (B) the *syd-10* mutation are shown, with the sites of the mutations indicated by an asterisk (*).The numbers above the alignment correspond to the amino acid positions of the *Arabidopsis thaliana* sequence. At – *Arabidopsis thaliana*; Al – *Arabidopsis lyrata*; Os – *Oryza sativa*; Zm – *Zea mays*; Gm – *Glycine max*; Fv – *Fragaria vesca*; Pt – *Populus trichocarpa*; Rc – *Ricinus communis*; Tc – *Theobroma cacao*; Vv – *Vitis vinifera*; Sl – *Solanum lycopersicum*; Sm – *Selaginella moelendorffii*; Pp – *Physcomitrella patens*.

2.3.4 Mutations in *SYD* result in elevated transcription of *SNC1*

One mechanism to enhance disease resistance in plants is to increase steady-state levels of NLR proteins through transcriptional up-regulation. As *SYD* encodes an ATP-dependent chromatin

remodeler, it was hypothesized that the enhancement of *snc1*-like phenotypes associated with mutations in *SYD* may be a result of altered SYD function and subsequent changes in transcriptional activity at the *SNC1* locus. Using real-time qRT-PCR, it was found that *SNC1* expression is moderately but significantly elevated in both the *syd-10* and *syd-4* single mutants (Figure 2.4F). However, expression of *RPP4*, another NLR-encoding gene that resides within the same gene cluster as *SNC1*, was unaltered in the *syd* mutants (Figure 2.4G). These data suggest that *SYD* is responsible for maintaining proper transcript levels of *SNC1* specifically. However, no obvious increase in SNC1 protein was observed in the *syd* single mutants (Figure 2.6). Without SYD function *SNC1* transcription is up-regulated, which can be amplified in the *snc1* mutant background and result in an enhancement of *snc1*-mediated autoimmunity.

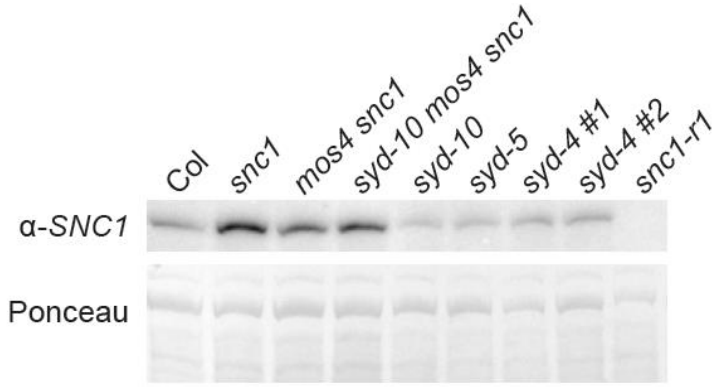

Figure 2.6. SNC1 protein levels in the indicated genotypes. *syd-5* is a T-DNA insertion allele (Salk_023209), and *snc1-r1* is a null *SNC1* allele which serves as a negative control. Two biological replicates of *syd-4* are included. Signals detected using Ponceau staining served as internal loading controls.

2.4 Discussion

Eukaryotic ATP-dependent chromatin remodeling complexes contain a DNA-dependent ATPase subunit which utilizes the energy derived from the hydrolysis of ATP to alter the positions of nucleosomes along the DNA strand (Clapier and Cairns, 2009). The resultant changes to

chromatin structure potentially modify the transcriptional activity at affected loci. One extensively studied ATPase in Arabidopsis is SYD, which belongs to the evolutionarily conserved SWI/SNF class of chromatin remodelers and was first identified as a regulator of reproductive development (Wagner and Meyerowitz 2002). Plant SWI/SNF chromatin remodeling complexes have been implicated in many biological processes in addition to development, including hormone signaling and RNA-mediated gene silencing (reviewed in Reyes 2014). In this study, we have determined a novel role for SYD in negatively regulating SNC1-mediated resistance.

SYD was previously shown to be a regulator of JA- and ET-mediated stress signaling pathways and is required for resistance against *Botrytis cinerea*, a necrotrophic fungus with a broad host range (Walley et al. 2008). The same study reported that two mutant alleles of *SYD* conferred wild-type-like resistance to the biotrophic bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000, resistance against which is primarily mediated by SA. These results suggested that SYD is specifically involved in the regulation of disease resistance mediated by JA and ET signaling pathways, but not involved in SA-mediated immunity. Consistent with the previously published data, we found that the novel *syd-10* allele also displays wild-type-like resistance to a different *Pseudomonas syringae* strain, *P.s.m.* ES4326 (Figure 2.4B). However, the *syd-4* single mutant displays enhanced resistance to this pathogen. Differences in the immune phenotypes of *syd-10* and *syd-4* may be a result of the differing strengths of the mutations: *syd-10* contains a point mutation in the weakly conserved N terminal region of SYD, while *syd-4* carries a T-DNA insertion in the conserved helicase domain (Figure 2.2D). Additionally, the *syd-10* allele confers enhanced resistance to the obligate biotrophic oomycete *H.a.* Noco2 in the *mos4 snc1* genetic background (Figure 2.1C). The finding that SYD plays a role in mediating

resistance to biotrophic pathogens is not wholly unprecedented, as the SA-responsive defense marker gene *PR1* was shown to be upregulated in *syd-2*, although none of the genes upstream in the SA signaling pathway were observed to have altered expression in the mutant (Walley et al. 2008). This supports our postulation that while SYD positively regulates JA- and ET-mediated defense against necrotrophs, it plays a role in the negative regulation of SA-mediated immunity.

From the phenotypic analysis of *syd* mutants, the role SYD plays in regulating SA-mediated defense responses appears to be quite subtle. This study has demonstrated that *syd-10* enhances morphological and resistance phenotypes associated with *snc1*; however, the degree of the enhancement is not as strong as observed for other published *muse* mutants. The presence of the *syd-10* mutation in the *mos4 snc1* background only partially rescues the *H.a.* Noco2 resistance associated with *snc1* (Figure 2.1C), and the immune phenotypes of the single mutant are almost indistinguishable from wild-type (Figure 2.4A-B), except for the enhanced resistance phenotype of the *syd-4* single mutant. While SNC1 protein levels appear to be elevated in *syd-10 mos4 snc1* as compared to *mos4 snc1*, SNC1 does not obviously accumulate in the *syd* single mutants (Figure 2.6). As *SNC1* gene expression is only slightly increased in the *syd* mutants (Figure 2.4F), the consequent minute protein level change is likely below the detection limit of the western blot method. Given these results, it is unsurprising that *syd* alleles were not identified from any prior known screens for regulators of SA-mediated immunity. The sensitized genetic background used in the MUSE screen has enabled the identification of *syd-10* and other novel components of immune signaling (Huang et al. 2013; Huang et al. 2014a; Huang et al. 2014b; Xu et al. 2015). One possible explanation as to why the defense phenotypes associated with the *syd-10* mutation are only observable in the *snc1* background is that in this background defense responses are constitutively activated; therefore, knocking out negative regulators of this

pathway results in a stronger, more quantifiable defense induction. In the wild-type genetic background, knocking out a minor negative immune regulator is insufficient to activate immune responses by itself; perhaps the threshold level of defense gene induction required to confer enhanced resistance cannot be reached.

The mild effects of mutations in *SYD* upon SA-mediated signaling may also be partially explained by redundancy with its close homolog *BRAHMA* (*BRM*). These two ATPases have been demonstrated to act on both shared and unique target genes, and elevated expression of a number of SA-dependent defense response genes including *PR1* has been observed in *brm-101* mutants (Bezhani et al. 2007; Wu et al. 2012).

Other ATP-dependent chromatin remodeling complexes have been shown to repress SA-dependent defense gene expression. Mutations in subunits of the Arabidopsis SWR1 chromatin remodeling complex result in enhanced resistance to *P.s.t.* DC3000 and constitutive expression of genes associated with systemic acquired resistance (SAR), a long-lasting broad spectrum defense mechanism that protects against future infection and requires SA (March-Diaz et al. 2008). Such differential gene expression is caused by the loss of H2A.Z (March-Diaz et al. 2008), a histone variant important for regulating gene expression deposited by SWR1 complexes in plants, yeasts and mammals (Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004; Ruhl et al. 2006; Deal et al. 2007). In Arabidopsis, H2A.Z is enriched at genes responsive to environmental and developmental stimuli, such as genes involved in immune and temperature responses, and plays an essential role in controlling their expression (Coleman-Derr and Zilberman 2012; Kumar and Wigge 2010). Knocking out another chromatin-remodeling ATPase, DDM1, has been shown to release the suppression of *SNC1* expression caused by the *mos1* mutation, although expression of *SNC1* in the *ddm1* mutant is comparable to levels observed in

wild-type (Li et al. 2010). Taken together, these reports highlight the contribution of chromatin remodeling in defense gene regulation.

ATP-dependent chromatin remodelers are also known to affect DNA methylation, a type of epigenetic mark that can result in modified chromatin accessibility and gene transcription. As such, an examination of the DNA methylation status around the *SNC1* locus was undertaken in *syd* plants. A slight decrease of DNA methylation in the asymmetric CHH (H = A, T or C) context was observed in *syd* at a transposon approximately 3 kb upstream of *SNC1*, as compared to wild type (Figure 2.7). To investigate if this is the cause of *SNC1* transcriptional elevation in *syd*, we took advantage of mutants that exhibit reduced CHH methylation in this transposon (*ddm1* and *rdr2*). No significant alteration of *SNC1* expression was observed in either mutant (Figure 2.4F), indicating the suppression of *SNC1* by SYD is unlikely to be mediated by DNA methylation at the *SNC1* locus.

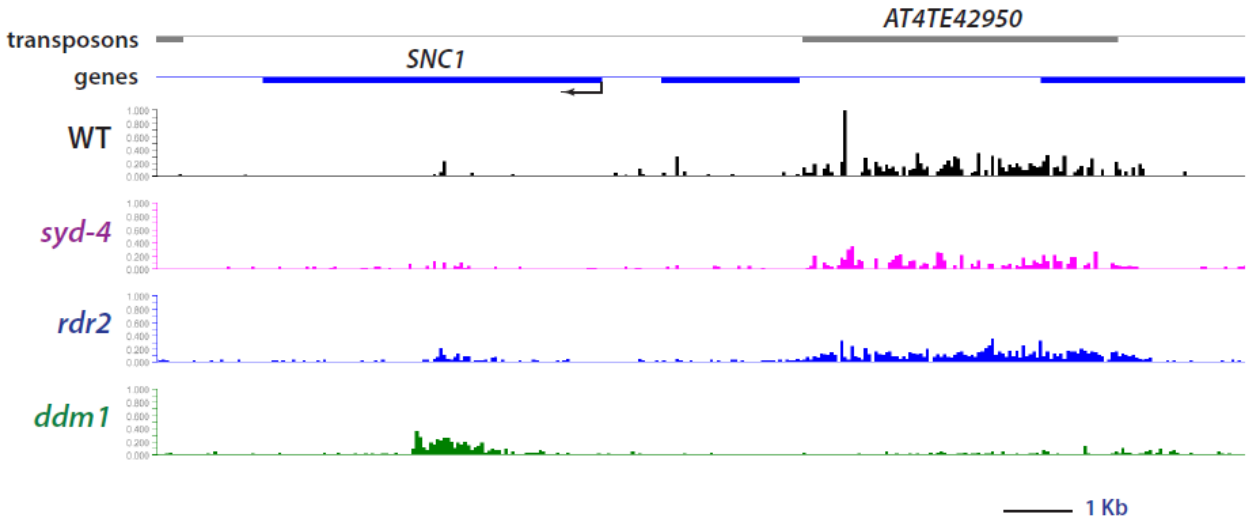

Figure 2.7. CHH methylation in wild type, *syd-4*, *rdr2*, and *ddm1* plants around the *SNC1* locus. DNA methylation was measured by bisulfite sequencing of genomic DNA from *syd-4* and wild-type 3-week-old seedlings, and analyzed as previously described (Ibarra et al. 2012). *rdr2-1* and *ddm1-2* mutant data were obtained from Zemach et al. 2013. H = A, C, or T.

A graphic representation of the potential role of SYD in regulating SNC1-mediated immunity is illustrated in Figure 2.8.

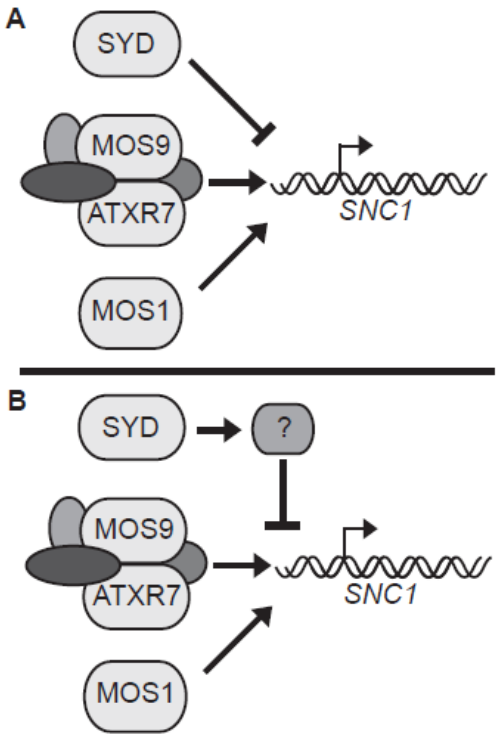

Figure 2.8. SYD functions antagonistically with MOS1 and MOS9 to regulate SNC1 transcription. The chromatin remodeler SYD is a negative regulator of SNC1-mediated immunity. It may exert its regulatory effects by directly modifying chromatin at the *SNC1* locus, thereby repressing *SNC1* transcription (A). Alternatively, SYD may affect *SNC1* transcription indirectly, by remodeling chromatin at a locus (or loci) elsewhere in the genome, thus affecting the expression of other regulators of *SNC1* expression (B). SYD acts in opposition to previously reported MOS1, ATXR7 and MOS9, which function as positive regulators of endogenous *SNC1* transcription.

SYD acts antagonistically to MOS1 and MOS9, and is required for negatively modulating transcription at the *SNC1* locus. As part of the SWI/SNF complex, SYD may directly affect the *SNC1* locus (Fig 2.8A). Alternatively, SYD may alter the chromatin at another locus (or loci), which indirectly results in the down-regulation of *SNC1* transcription (Figure 2.8B).

Although MOS1 and MOS9 also affect *RPP4* transcription (Li et al. 2010; Xia et al. 2013), SYD does not (Figure 2.4G), indicating that its effects on *SNC1* are more specific.

In summary, we have shown that mutations in the ATPase-encoding gene *SYD* enhance the morphological and resistance phenotypes associated with the gain-of-function *snc1* mutant and result in increased expression of *SNC1*. However, gaining comprehensive insight into the mechanism by which SYD regulates SNC1-mediated immunity requires further investigation.

2.5 Materials and methods

2.5.1 Plant growth conditions and mutant isolation

Soil-grown plants were kept in climate-controlled growth rooms at 22ºC on a 16h light/8h dark cycle. Plate-grown plants were propagated on ½ Murashige and Skoog medium supplemented with 0.5% sucrose and 0.3% phytagel and grown under the above conditions. The MUSE screen was conducted using EMS as described previously (Huang et al. 2013). The *syd-4* (Salk_149549) mutant was obtained from the Arabidopsis Biological Resource Center and genotyped by PCR using the following primers: 5'-TGAAGCTCTGACTTGCTCCTC-3' and 5'-TCAAAGCAACAGACCATCGG-3'.

2.5.2 Expression analysis

Approximately 0.1 g total plant tissue was collected from plate-grown 2-week-old seedlings. RNA was extracted using the Totally RNA Kit (Ambion, now Invitrogen), and Reverse Transcriptase M-MLV (Takara) was used to reverse transcribe 0.4 μg RNA. Primers used for

amplification of *SNC1* and *ACTIN7* were previously described (Zhang et al. 2003; Cheng et al. 2009).

2.5.3 Positional cloning

Positional cloning of *muse9* was performed using markers derived from insertion/deletion and single nucleotide polymorphisms between the Col-0 and L*er* Arabidopsis ecotypes, identified using sequence information available from TAIR (Jander et al. 2002; http://www.arabidopsis.org). After narrowing down the location of the molecular lesion to between 10.8 MB and 12.4 MB, extracted DNA from *muse9 mos4 snc1* was sequenced using the Illumina sequencing platform.

2.5.4 Pathogen assays

Bacterial and oomycete infection assays were performed as previously described (Li et al. 2001). In brief, bacterial infections were conducting using a needleless syringe to infiltrate the abaxial leaf surfaces of 4-week-old soil-grown plants with *P.s.m.* ES4326 (OD_{600} = 0.001). Bacterial growth was quantified using leaf discs (area = 0.38cm^2) collected on the day of infection (day 0) and 2 d later. Oomycete infections were conducted by spray-inoculating 2-week-old soil-grown seedlings with *H.a.* Noco2 ($1x10^5$ spores mL^{-1}). Sporulation was quantified 7 d post-infection. Total aerial plant tissue was used in the assay. For each genotype, 5 replicates of 5 plants were each suspended in 1mL ddH_20 and vortexed gently, and spores were counted using a hemocytometer. Spore counts were normalized to fresh weight (mg).

2.5.5 Genetic crosses

The *muse9* single mutant was generated by back-crossing *muse9 mos4 snc1* with Col-0 containing the *pPR2-GUS* reporter gene. The F1 progeny were allowed to self-fertilize, and *muse9* single mutants were identified among the F2 progeny by genotyping.

Chapter 3: A partial loss-of-function mutation in an Arabidopsis RNA polymerase III subunit leads to pleiotropic defects[3]

3.1 Summary

Plants employ five DNA-dependent RNA polymerases (Pols) in transcription. One of these polymerases, Pol III, has previously been reported to transcribe 5S rRNA, tRNAs, and a number of small RNAs. However, in-depth functional analysis is complicated by the fact that knockout mutations in Pol subunits are typically lethal. Here, we report the characterization of the first known viable Pol III subunit mutant, *nrpc7-1*. This mutant was originally isolated from a forward genetic screen designed to identify enhancers of the autoimmune mutant *snc1*, which contains a gain-of-function mutation in a nucleotide-binding leucine rich-repeat (NLR) immune receptor-encoding gene. The *nrpc7-1* mutation occurs in an intron/exon splice site and results in intron retention in some *NRPC7* transcripts. There is a global disruption in RNA equilibrium in *nrpc7-1*, exemplified by the altered expression of a number of RNA molecules, some of which are not reported to be transcribed by Pol III. There are developmental defects associated with the mutation, as homozygous mutants are dwarf, have stunted roots and siliques, and possess serrated leaves. These defects are possibly due to altered small RNA stability or activity. Additionally, the *nrpc7-1* mutation confers an *NLR*-specific alternative splicing defect that correlates with enhanced disease resistance, highlighting the importance of alternative splicing in regulating NLR activity. Altogether, these results reveal novel roles for Pol III in maintaining

[3] A version of this chapter has been published. Kaeli C. M. Johnson, Yu Yu, Lei Gao, Ryan C. Eng, Geoffrey O. Wasteneys, Xuemei Chen and Xin Li. (2016) *Journal of Experimental Botany* 67(8):2219-30.

RNA homeostasis, adjusting the expression of a diverse suite of genes, and indirectly modulating gene splicing. Future analyses using the *nrpc7-1* mutant will be instrumental in examining other unknown Pol III functions.

3.2 Introduction

Transcription under both static and dynamic conditions requires the action of evolutionarily conserved multi-subunit enzymes known as DNA-dependent RNA polymerases. All eukaryotes possess three distinct RNA polymerases (Pols I, II, and III), each of which transcribes specific suites of genes (Cramer *et al.*, 2008).

Pol I transcribes 45S rRNA, which is the precursor to 5.8S, 18S and 25S rRNAs. Pol II transcribes mRNA as well as most small nuclear (sn)RNAs and micro (mi)RNAs. Pol III was previously thought to be primarily required for the transcription of "housekeeping" genes such as those encoding 5S rRNA and tRNAs. However, recent reports indicate that the Pol III transcriptome is more diverse than formerly assumed (Dieci *et al.*, 2007). There are two additional plant-specific RNA polymerases, Pol IV and Pol V, which are required for the biogenesis and functional activity of small interfering (si)RNAs (Haag and Pikaard, 2011). As knockout mutations in the genes encoding the subunits of Pols I, II, and III are lethal, there is a dearth of functional analysis of plant Pols.

While there are a number of published studies examining global transcriptomic changes in plants under various conditions (e.g. Nagano *et al.*, 2012, Woo *et al.*, 2012, Zhu *et al.*, 2012), the literature to date has largely focussed on the roles played by Pol II-transcribed RNAs in regulating plants' responses to stimuli. Stimulus-induced alteration of expression of protein-

coding genes has been extensively documented. Numerous recent reports have highlighted the importance of miRNAs in regulating a broad spectrum of biological processes including development (Wu, 2013), flowering time (Spanudakis and Jackson, 2014), drought stress (Ding *et al.*, 2013), metal toxicity (Gupta *et al.*, 2014), immunity (Staiger *et al.*, 2013), and phytohormone crosstalk (Curaba *et al.*, 2014), among others. Furthermore, the biosynthesis, functional mechanisms, and degradation pathways of miRNAs have been well-studied (Rogers and Chen, 2013).

Comparatively little is known about Pol III-transcribed RNAs and how they aid plants in responding to intrinsic and extrinsic signals. An RNA molecule with significant sequence and structural similarity to 5S rRNA was found to regulate alternative splicing of certain pre-mRNAs in Arabidopsis (Hammond *et al.*, 2009). Intriguingly, studies in a variety of eukaryotes indicate that Pol III-transcribed non-coding RNAs may play regulatory roles in addition to their housekeeping functions (Hu *et al.*, 2012).

Among the various stimuli to which plants are subjected, biotic stress in the form of pathogenic infection requires that plants be able to respond rapidly and initiate signaling cascades specific to the type of pathogen being encountered. While plants possess physical barriers and broad spectrum resistance that is activated by conserved features of pathogenic microbes, many pathogens are able to inject infection-promoting effector molecules into the plant cell, thereby bypassing this layer of plant immunity (Bigeard *et al.*, 2015). However, the plant genome contains a large number of genes encoding nucleotide-binding leucine rich-repeat proteins (NLRs; also referred to as Nod-like receptors due to their structural similarity to mammalian proteins of the same name), which either directly bind to pathogenic effectors or detect their activities within the plant cell in a highly specific manner (Li *et al.*, 2015). Upon

recognition of its cognate effector, NLR activation results in rapidly induced and robust defense responses. Plant NLRs can be sorted into two classes based on their N-termini: some possess a Toll-Interleukin 1 receptor (TIR) domain and are thus termed TNLs, while others contain a coiled-coil (CC) domain and are referred to as CNLs.

NLR-mediated signaling must be tightly controlled under both resting and induced conditions, as improper signaling through this pathway may lead to either enhanced disease susceptibility or autoimmunity. However, the regulatory mechanisms underlying NLR-mediated signaling are only partially understood. A successful forward genetic suppressor screen previously conducted in our lab used the gain-of-function autoimmune TNL mutant *snc1* (*suppressor of npr1, constitutive 1*; Li *et al.*, 2001; Zhang *et al.*, 2003) to search for positive regulators of immunity (Johnson *et al.*, 2012). More recently, we have undertaken a forward genetic screen to identify negative regulators of NLR-mediated immunity.

Here, we report the characterization of *nrpc7-1*, a partial loss-of-function allele of the gene encoding the Arabidopsis ortholog of yeast Rpc25, a Pol III subunit. This mutant was isolated from our MUSE (MUTANT, *snc1*-ENHANCING) forward genetic screen conducted in the *mos4* (*modifier of snc1 4*) *snc1* double mutant background. A null mutation in *NRPC7* is lethal, while a mutation in an intron/exon splice site junction gives rise to intronic retention in some *NRPC7* transcripts, resulting in viable mutant plants. While the *nrpc7-1 mos4 snc1* triple mutant displays enhanced resistance against the virulent oomycete pathogen *Hyaloperonospora arabidopsidis* Noco2, the *nrpc7-1* single mutant exhibits wild type-level resistance. This correlates with the altered splicing of *SNC1* observed in the triple mutant but not in the single mutant. Morphologically, the *nrpc7-1* mutant is dwarf and has serrated leaves, short roots, and stunted siliques, although flowering time does not appear to be affected. The expression and

potentially activity of a number of RNAs are distorted in *nrpc7-1*, contributing to its developmental defects. In keeping with its known function, we observed that the NRPC7 protein localizes to the nucleus. This is the first reported viable Pol III subunit mutant in Arabidopsis.

3.3 Results

3.3.1 The isolation, characterization, and identification of the *muse4/nrpc7-1* mutant

The MUSE screen was designed to identify enhancers of the dwarf autoimmune mutant *snc1* and has been described previously (Huang *et al.*, 2013). To avoid potential lethality resulting from dramatically enhanced autoimmunity, the *snc1* suppressor *mos4* was included in the genetic background of the screen. Seeds from the wild type-like *mos4 snc1* plants were mutagenized with ethyl methanesulfonate, and the M2 population was screened for plants displaying a reversion back to *snc1*-like morphology and resistance. A number of mutant lines were isolated, one of which (*muse4*) was selected for further characterization. When the triple mutant was backcrossed to *mos4 snc1*, all progeny appeared wild type-like, indicating that *muse4* is a recessive mutation.

As shown in Figure 3.1A, the *muse4 mos4 snc1* plants exhibit dwarf, curled leaf morphology similar to that observed for *snc1* plants. In addition, the *muse4 mos4 snc1* plants have serrated and slightly chlorotic leaves. The *muse4* mutation also re-establishes the constitutive expression of the defense marker *PR* (*PATHOGENESIS-RELATED*) genes observed in *snc1* but absent in *mos4 snc1*. A *pPR2-GUS* reporter gene construct was used to visualize *PR2* gene expression in seedlings, and GUS staining was much stronger in the triple mutant than in

mos4 snc1 (Figure 3.1B). Consistent with this observation, qPCR demonstrated that expression of *PR1* and *PR2* is elevated in the triple mutant (Figure 3.1C).

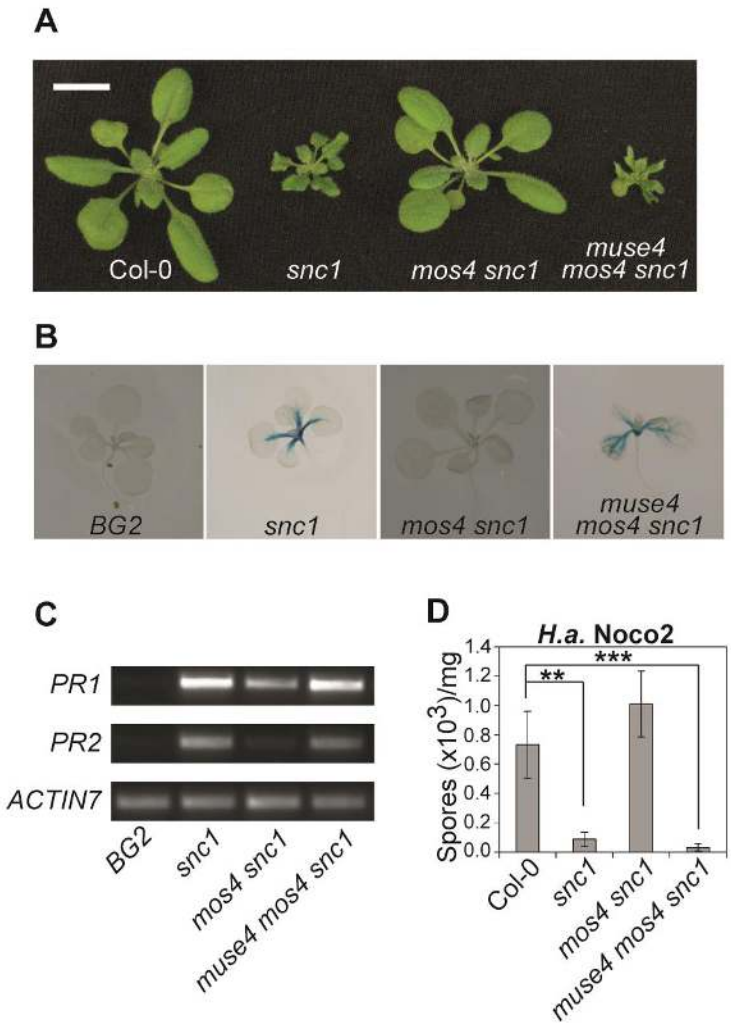

Figure 3.1. Characterization of the *muse4 mos4 snc1* triple mutant.
(A) Morphology of soil-grown plants of the indicated genotypes, photographed four weeks post-germination. Scale bar represents 1cm.
(B) *pPR2-GUS* expression in seedlings of the indicated genotypes grown on MS media for 10 d.
(C) *PR1* and *PR2* gene expression in the noted genotypes, as determined by qPCR. *ACTIN7* expression serves as a loading control.
(D) Growth of *H.a*. Noco2 on indicated genotypes 7 d post-inoculation with $1x10^5$ spores/mL. Values represent the average of 4 replicates of 5 plants each ± SD. **: p-value ≤ 0.01; ***: p-value ≤ 0.001.

To examine whether the *muse4* mutation alters resistance to the virulent oomycete strain *Hyaloperonospora arabidopsidis* Noco2, two-week-old triple mutant seedlings were spray-inoculated with this pathogen. The enhanced resistance observed in *snc1* but lost in *mos4 snc1* was found to be reconstituted in the triple mutant (Figure 3.1D). Together, these data indicate that *muse4* restores all examined *snc1*-like phenotypes in the *mos4 snc1* background.

A positional cloning strategy was employed to determine the molecular lesion responsible for the observed phenotypes. The *muse4 mos4 snc1* mutant, which was generated in the Col-0 ecotype, was crossed to Landsberg *erecta* (L*er*). From the F2 population, 24 plants displaying the triple mutant morphology were selected for crude mapping, which identified a linkage to the top of chromosome 1. Several F2 plants heterozygous at the top of chromosome 1 (but homozygous for *snc1* and *mos4* to prevent interference by these loci) were used to generate a fine mapping population of approximately 500 plants. The mutation was narrowed down to between the markers T7A14 (1.4 MB) and F22O13 (2.75 MB). Genomic DNA was extracted from *muse4 mos4 snc1* triple mutant plants and sequenced using the Illumina whole-genome sequencing platform. The sequencing results were compared with the Arabidopsis reference genome, and five genes in this region were found to contain mutations (Figure 3.2A). The mutations in three of these genes are located in introns and the mutation in one gene was found to be silent, therefore the mutation in the remaining gene (*At1g06790*) was selected as the most likely candidate for *muse4*. This gene encodes the Arabidopsis ortholog of the yeast Pol III subunit Rpc25, NRPC7 (NUCLEAR RNA POLYMERASE C, SUBUNIT 7; Ream *et al.*, 2015), and the *muse4* mutation is at the intron/exon junction just before the sixth exon (Figure 3.2B).

A

Gene	Protein	Mutation
AT1G05570	Callose synthase 1	Intron
AT1G05700	LRR protein kinase	Intron
AT1G06790	RNA polymerase Rpb7 domain-containing protein	Splice site
AT1G08130	DNA ligase 1	Silent
AT1G08190	Vacuolar sorting protein 41	Intron

B

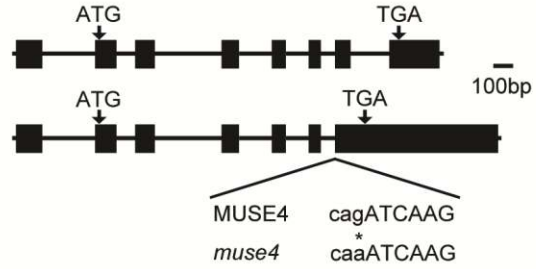

Figure 3.2. Map-based cloning of the *muse4* locus on chromosome 1.
(A) Point mutations identified within the mapping region from Illumina sequencing of the *muse4-1 snc1 mos4* triple mutant. Two independent mutations in introns were identified in AT1G05570.
(B) The two alternatively spliced variants of *MUSE4* and the position of the molecular lesion in *muse4-1* (*nrpc7-1*) and *muse4-2* (*nrpc7-2*). Boxes and lines represent exons and introns, respectively.

3.3.2 The mutation at an intron/exon junction of *NRPC7* results in intron retention and is responsible for the *muse4* phenotypes

To verify that the mutation in *NRPC7* is responsible for the *muse4* phenotypes, a full-length wild type copy of the gene driven by its native promoter and fused to the *GFP*-encoding gene at its 3' end was transformed into the single mutant, which was generated by backcrossing the triple mutant to Col-0 and selecting plants homozygous for wild type *SNC1* and *MOS4* that retained the serrated leaf phenotype and dwarf size. Eight independent T2 lines displayed wild type

morphology, and one representative line can be seen in Figure 3.3A. These data suggest that *NRPC7* can fully complement the *muse4* phenotypes, and therefore that *MUSE4* is indeed *NRPC7*.

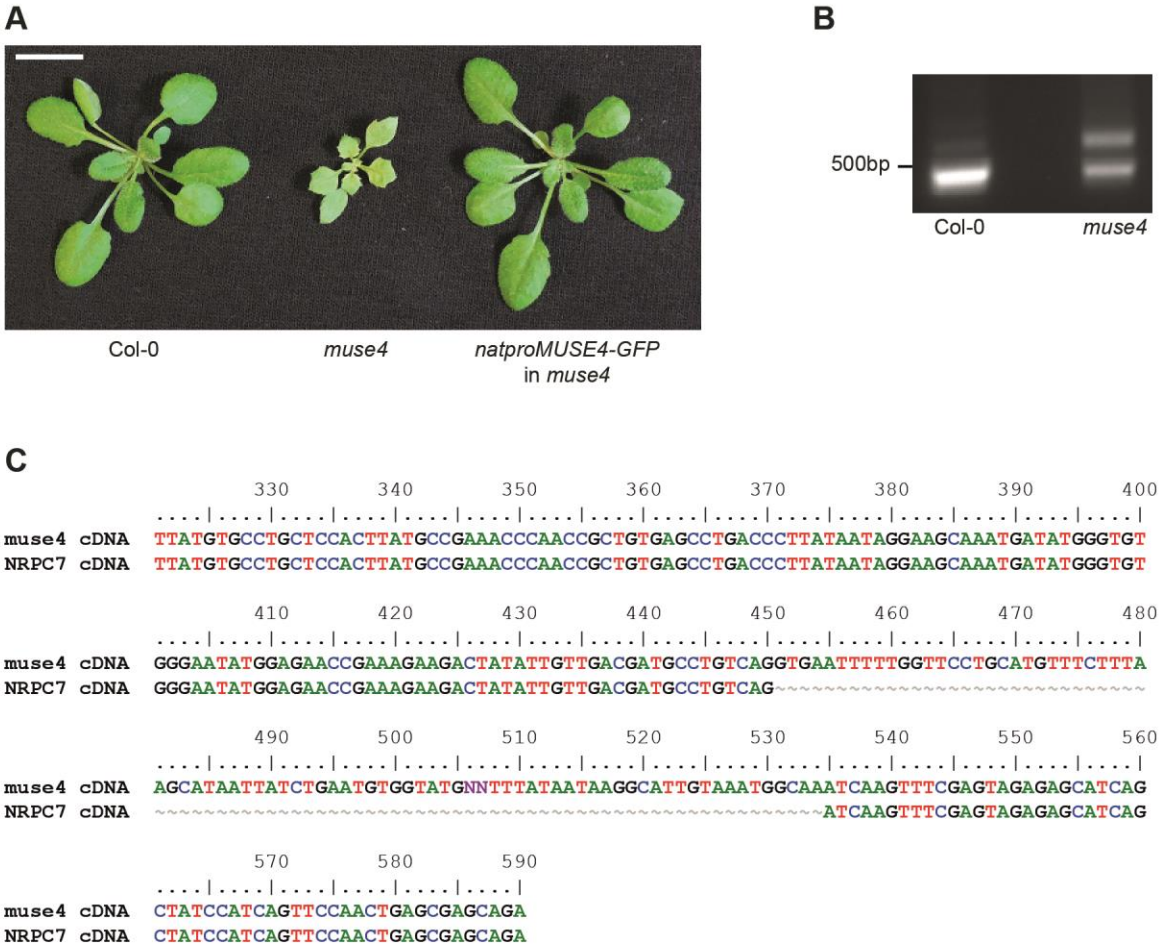

Figure 3.3. *MUSE4* is *NRPC7*.
(A) MUSE4 tagged with GFP and expressed under the control of its native promoter is able to complement the *muse4* single mutant defects. Plants were grown on soil for three weeks.
(B) The size of the *MUSE4* transcript in wild type and *muse4* was examined using cDNA reverse-transcribed from total RNA.
(C) The larger *muse4* band in (B) was excised, purified, and sequenced, and found to retain the intron preceding the intron/exon splice site mutation in *muse4*.

We hypothesized that the *muse4* mutation in the intron/exon junction of *NRPC7* results in retention of the preceding intron. To test this, we designed primers flanking the intron of interest and amplified cDNA from wild type and *muse4*. A strong band of the expected size (465 bp) was observed in wild type while in *muse4* two bands were observed, one of the expected size and one slightly larger (Figure 3.3B). The larger band was excised from the gel and the PCR product was purified and sequenced. As predicted, sequencing revealed that the larger band corresponded to a transcript in which the intron preceding the *muse4* mutation had been retained (Figure 3.3C).

Despite strong sequence similarity between NRPC7 and known Rpc25 proteins in other species (Figure 3.4), the *NRPC7* gene failed to complement a temperature sensitive *rpc25* yeast knockout line (Figure 3.5), suggesting divergence between the plant and yeast NRPC7.

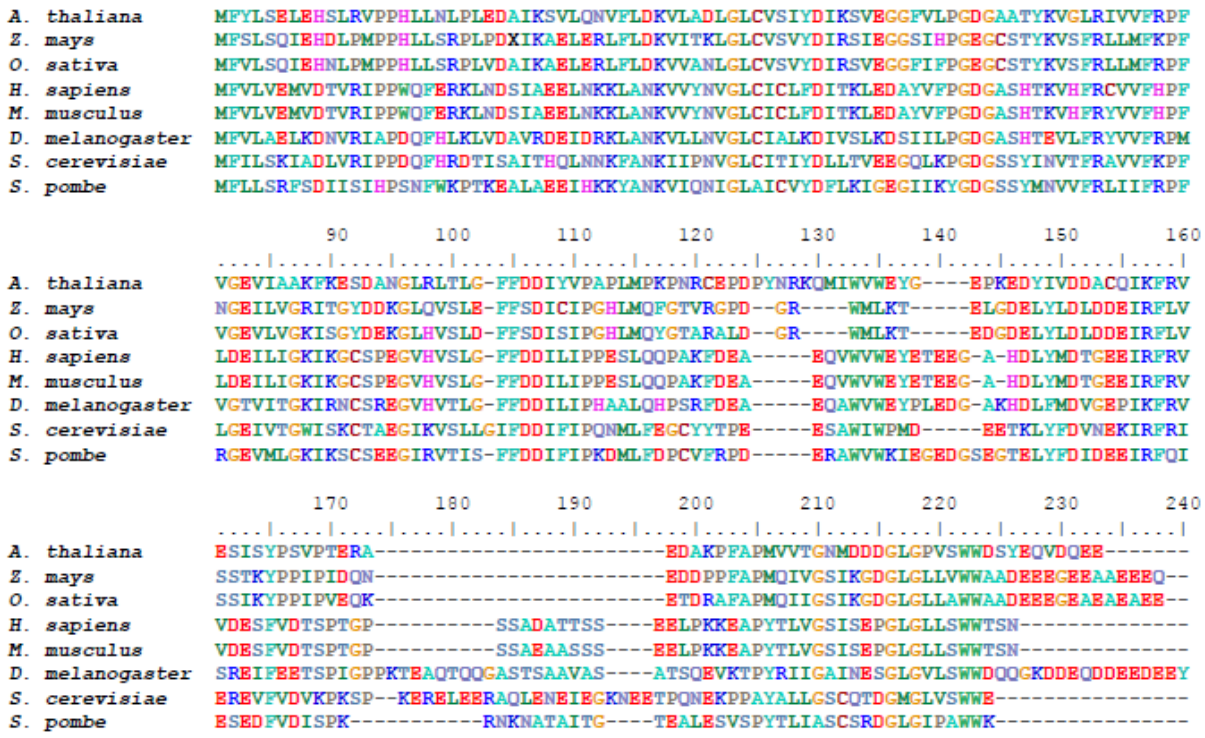

Figure 3.4. Sequence alignment of RPC25 from a broad range of species, based on BLAST analysis.

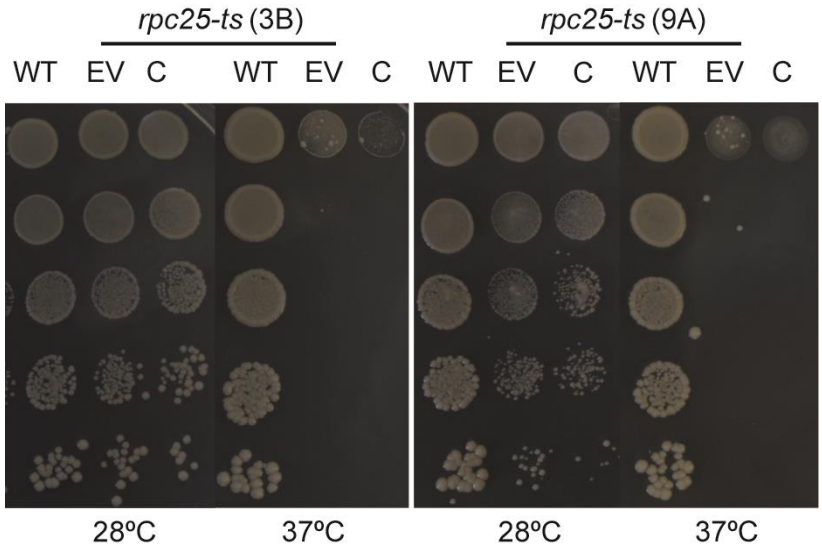

Figure 3.5. Yeast complementation with *NRPC7*. *NRPC7* CDS was introduced into two independent temperature-sensitive *rpc25* knockout yeast lines. WT: yeast rpc25 strain with no transgene; EV: rpc25 transformed with an empty vector; C: rpc25 transformed with the NRPC7-containing construct.

It is expected that a knockout mutation in *NRPC7* would be embryo lethal, as a previous study showed that loss-of-function mutations in RNA polymerase subunits are not transmitted maternally (Onodera *et al.* 2008). Indeed, when we let the heterozygous *nrpc7* T-DNA insertion line CS1001213 self-fertilize and then planted the progeny, we identified 23 wild type plants lacking the insertion, 46 heterozygotes, and 0 plants that were homozygous for the insertion, matching the expected 1:2:0 (wild type:heterozygote:homozygote) ratio for a lethal mutation. We also performed reciprocal crosses between this heterozygous T-DNA insertion line and *muse4* and found that none of the F1 progeny contained the T-DNA insertion, indicating that the T-DNA/*muse4* heterozygotes are not viable. These results, combined with the data in Figure 3.3B showing that *muse4* still produces some properly spliced transcripts without intron retention, as well as the fact that *muse4* is a recessive mutation, suggest that *muse4* is a partial loss-of-

function allele of *NRPC7*. Therefore, we renamed *muse4* as *nrpc7-1* and the T-DNA allele as *nrpc7-2*.

3.3.3 Splicing of *SNC1* is altered in the *nrpc7-1 mos4 snc1* background

In yeast, Rpc25 is required for Pol III transcription initiation (Zaros and Thuriaux, 2005). To assess whether Pol III function is affected by the *nrpc7-1* mutation, we used real-time qPCR to determine whether expression of U6, a snRNA component of the spliceosome that is known to be transcribed by Pol III (Waibel and Filipowicz, 1990), is different in *nrpc7-1* than in wild type. Relative to the expression levels of the Pol II-transcribed "housekeeping" gene *UBQ5*, U6 expression is significantly lower in *nrpc7-1* (Figure 3.6A). To examine whether the *nrpc7-1* mutation has a general effect on spliceosomal snRNA biosynthesis, the accumulations of Pol II-transcribed U1 and U2 snRNAs were also examined. While U1 accumulation is wild type-like, U2 expression is significantly reduced in *nrpc7-1*. This is likely due to an indirect effect of altered Pol III function on Pol II-transcribed genes.

The reduced expression of the spliceosome components U6 and U2 lead us to hypothesize that pre-mRNA splicing might be affected by the *nrpc7-1* mutation. Specifically, as *nrpc7-1* was isolated in our screen for *snc1* enhancers, we hypothesized that the mutation may affect the excision of introns from the *SNC1* pre-mRNA transcript. The alternative splicing of a number of plant NLR-encoding genes, including *SNC1*, is known to affect their function in plant immunity (Yi and Richards, 2007; Xu *et al.*, 2011). For *SNC1*, the second and third introns may be either retained or removed; therefore we used primers spanning these two introns to amplify the *SNC1* transcript variants. A dramatic accumulation of the largest transcript variant (with both introns retained) was observed in *nrpc7-1 mos4 snc1*, although the *SNC1* splicing pattern in the

nrpc7-1 single mutant was indistinguishable from that observed in wild type (Figure 3.6B; Figure 3.7C).

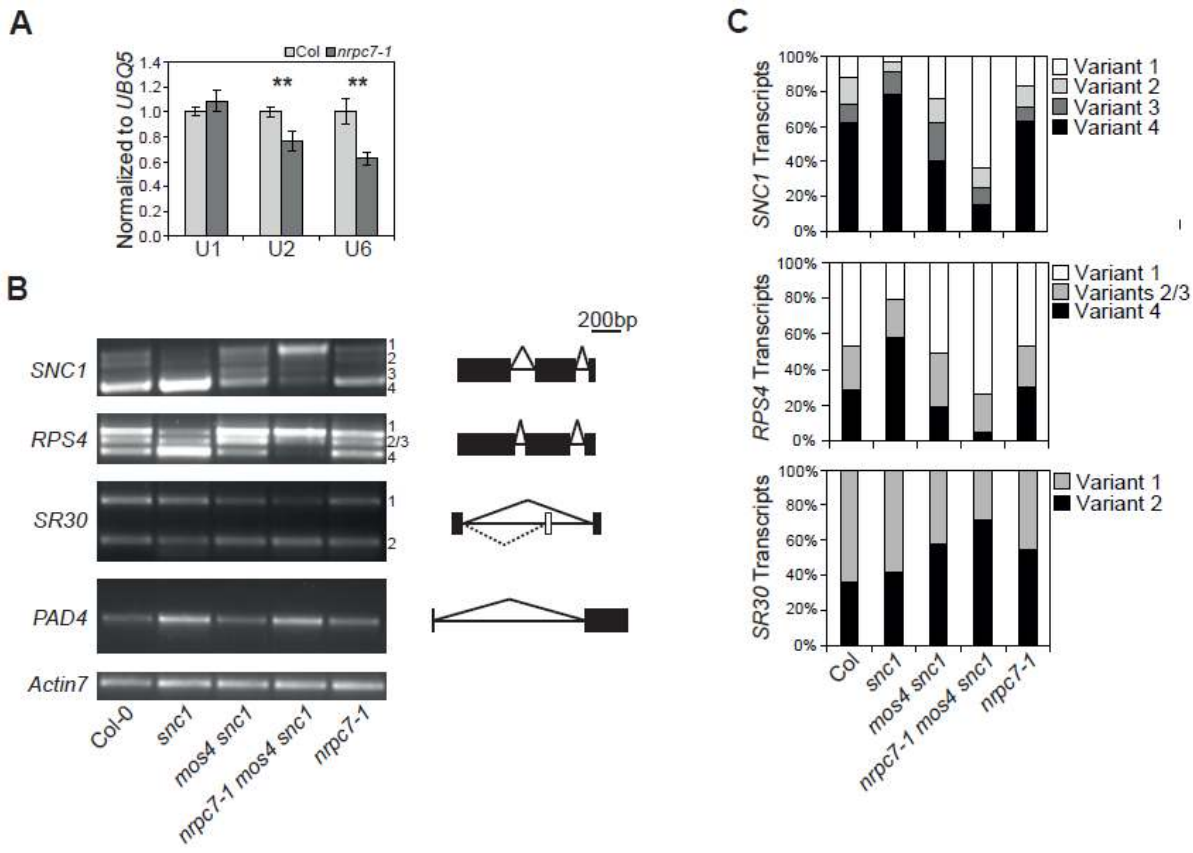

Figure 3.6. Splicing defects in *nrpc7-1*.
(A) Quantitative real-time qPCR was used to determine the expression of Pol II-transcribed U1 and U2 snRNAs, as well as Pol III-transcribed U6, relative to UBQ5. Bars represent the averages of three technical replicates of two biological replicates ± SD. **: p-value ≤ 0.01.
(B) An analysis of *SNC1*, *RPS4*, *SR30*, and *PAD4* splicing patterns in the indicated genotypes was performed using RT-PCR. Transcripts were amplified using 40 cycles. Numbers indicate transcript variants from largest to smallest. Schematic diagrams of the expected splicing events are shown to the right, with horizontal lines representing introns, black boxes representing exons, and white boxes representing alternatively retained exons that result in a premature stop codon.
(C) Quantification of the alternative transcript variants in (B) across genotypes. Band intensities were quantified using ImageJ.

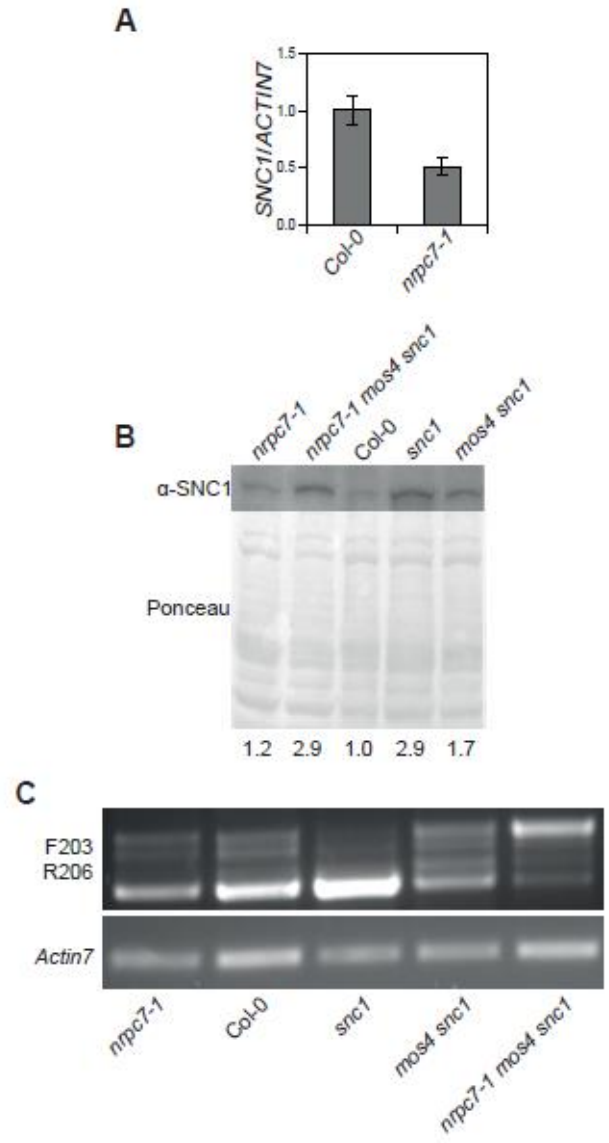

Figure 3.7. *SNC1* gene and protein expression in *nrpc7*.
(A) Real-time qPCR analysis of *SNC1* expression in the indicated genotypes. Total RNA was extracted from seedlings grown for 12d on MS media.
(B) Western blot analysis of SNC1 levels. Protein was extracted from seedlings grown on MS media for 12d.
(C) The original *SNC1* splicing pattern gel image. An altered version of this image was used in Figure 4.6B.

Alternative splicing defects were also observed in *nrpc7-1 mos4 snc1* for *RPS4*

(*RESISTANT TO PSEUDOMONAS SYRINGAE 4*), another NLR-encoding gene, as well as for

SR30, which encodes a serine/arginine-rich RNA-binding protein and is known to be alternatively spliced (Figure 3.6B). The relative proportions of the transcript variants in the genotypes examined are shown in Figure 3.6C. These data reveal significant alternative splicing defects caused by the Pol III subunit mutation.

To determine whether this splicing defect occurs at the level of basal splicing, transcripts of a gene that is not alternatively spliced (*PAD4*; *PHYTOALEXIN-DEFICIENT 4*) were also examined. No difference from the wild type splicing pattern was detected (Figure 3.6B). Both *snc1* and *nprc7-1 mos4 snc1* accumulated higher levels of *PAD4* compared to wild type, which is consistent with previous reports that *PAD4* is a defense-induced gene (Glazebrook 2001), whose expression is expected to be upregulated in autoimmune mutants.

NLR-mediated signaling is often regulated by modulating transcription and/or translation of NLRs. As such, we examined whether *SNC1* expression and protein accumulation are affected by the *nrpc7-1* mutation. *SNC1* expression was found to be slightly reduced in *nrpc7-1* as compared to wild type, while SNC1 protein levels were wild type-like (Figure 3.7). Similarly, the accumulation of SNC1 in *nrpc7-1 mos4 snc1* was not dramatically higher than that observed in *mos4 snc1*. Taken together, these data indicate that *SNC1* alternative splicing, but not overall gene expression or translation, is affected by the *nrpc7-1* mutation.

3.3.4 The *nrpc7-1* single mutant does not have altered immune responses

Since alterations in the splicing of *SNC1* were observed in the *nrpc7-1 mos4 snc1* triple mutant but not the *nrpc7-1* single mutant, we predicted that *nrpc7-1* may not exhibit the enhanced disease resistance observed in the triple mutant (Figure 3.1D). We challenged *nrpc7-1* with the oomycete pathogen *H.a*. Noco2 and the bacterial pathogen *Pseudomonas syringae* pv.

maculicola ES4326, and found no statistically significant difference in response from that observed in wild type plants (Figure 3.8). These data support our hypothesis that the retention of introns in the *SNC1* transcript in the *nrpc7-1 mos4 snc1* confers enhanced disease resistance, and is the reason why this mutation was isolated from our *snc1* enhancer screen.

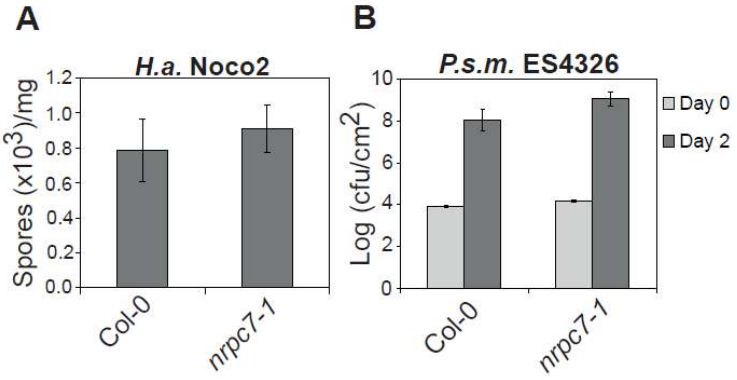

Figure 3.8. Immune characterization of *nrpc7-1* single mutant plants.
(A) Growth of *H.a*. Noco2 on indicated genotypes 7 d post-inoculation with $1x10^5$ spores/mL inoculum. Values represent the average of 4 replicates of 5 plants each ± SD.
(B) Growth of *P.s.m.* ES4326 on indicated genotypes 2 d post-infiltration. Values represent the average of 5 replicates ± SD.

3.3.5 *nrpc7-1* has global defects in RNA levels

Pol III transcribes tRNA, 5S rRNA, and assorted other non-coding RNAs. To further explore how Pol III function is affected by the *nrpc7-1* mutation, we examined the expression of a variety of RNAs by qPCR (Figure 3.9A). Relative to *UBQ5*, 5S rRNA and three representative tRNAs (coding for Gln, Gly, and Leu, respectively) showed significantly reduced accumulation in *nrpc7-1* compared to wild type.

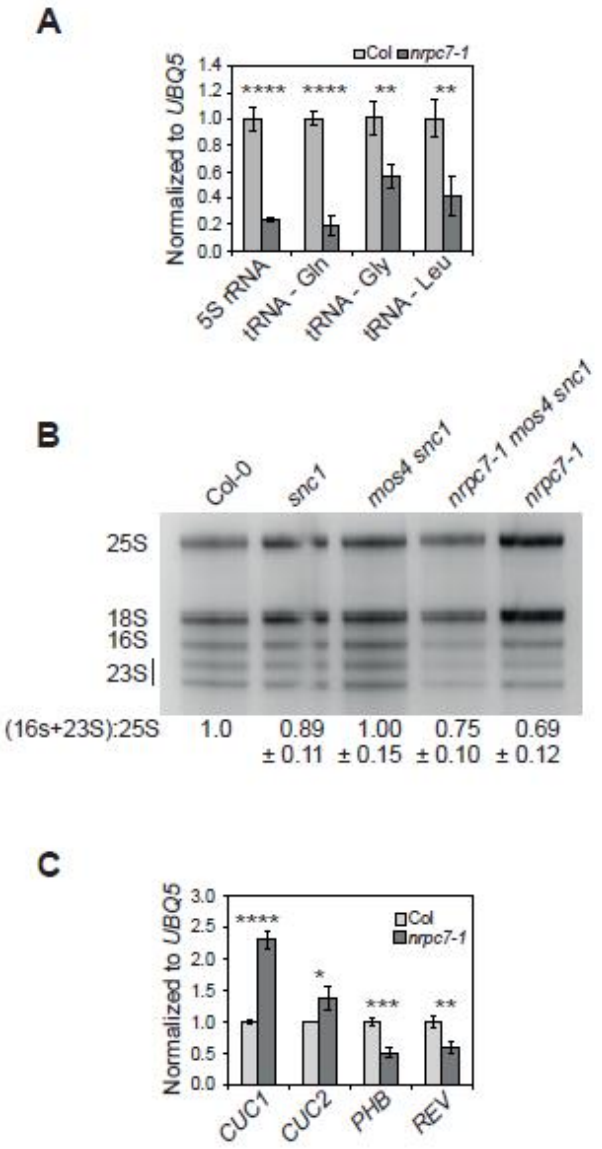

Figure 3.9. Global RNA defects in *nrpc7-1*.
(A) Expression of several representative Pol III-transcribed RNAs was examined in Col-0 and *nrpc7-1* using qPCR. Bars represent the averages of three technical replicates of two biological repeats ± SD.
(B) The proportions of rRNAs in the noted genotypes were compared by running total RNA extracted from seedlings grown on MS media for 12d on a 2% agarose gel. Band intensities were quantified using ImageJ, and the intensities of chloroplast rRNA (16S and 23S) relative to Pol I-transcribed rRNA (25S) were compared between genotypes for three biological replicates ± SD.
(C) The accumulation of *CUC1*, *CUC2*, *PHB*, and *REV* transcripts was examined using quantitative real-time qPCR. Bars represent the averages of three technical replicates of two biological replicates ± SD. *: p-value ≤ 0.05; **: p-value ≤ 0.01; ***: p-value ≤ 0.001; ****: p-value ≤ 0.0001.

In addition, when total RNA was run on a 2% agarose gel, altered relative proportions of the various rRNAs were consistently observable in association with the *nrpc7-1* allele (Figure 3.9B). Relative to Pol I-transcribed 25S rRNA, there appears to be a lower abundance of chloroplast 16S and 23S rRNA associated with the *nrpc7-1* allele, although as chloroplast numbers were not examined in the mutant we cannot rule out the possibility that nrpc7-1 affects chloroplast abundance. These data suggest that in addition to Pol III transcribed genes, the *nrpc7-1* mutation also affects abundance of other RNAs, likely through indirect mechanisms.

Small RNA libraries were then prepared from *BG2* plants (Col-0 with the *pPR2-GUS* reporter gene construct that is present in the *nrpc7-1* background) and two independently isolated *nrpc7-1* single mutant lines. Analysis of these small RNA libraries indicated that a number of miRNAs are differentially expressed in the mutant. Those miRNAs that exhibited a two-fold or greater change in expression are shown in Figure 3.10.

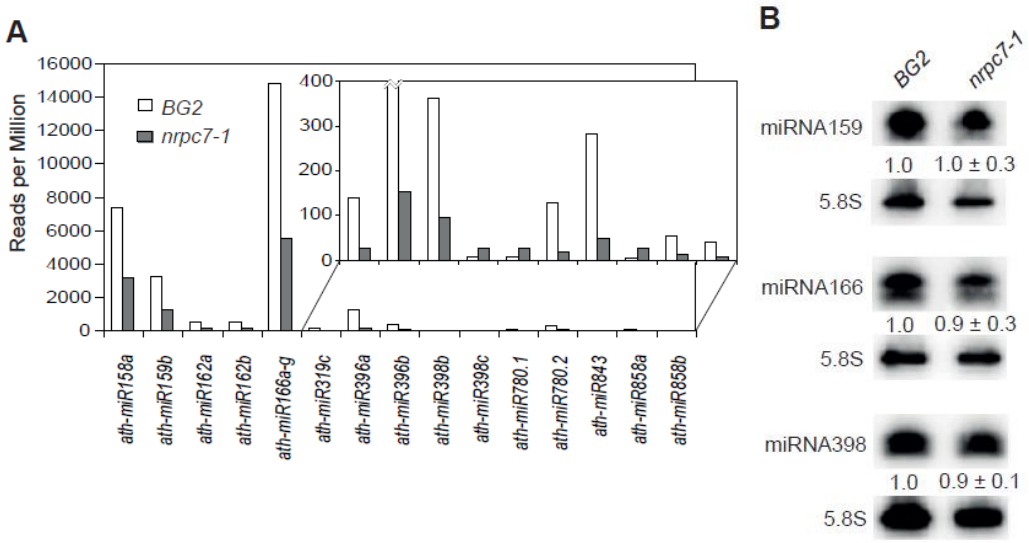

Figure 3.10. RNA defects in *nrpc7-1*.
(A) The expression of a number of miRNAs, quantified as reads per million, based on an analysis of small RNA libraries. Only miRNAs that had a fold change ≥ 2 between wild type and *nrpc7-1* and an FDR < 0.05 are included. Bars are representative of two biological replicates.

(B) Northern blot analysis of a select number of miRNAs that small RNA library analysis indicated were differentially expressed in *nrpc7-1*. Numbers indicate the average and standard deviation of two technical replicates of two biological replicates.

To validate these results, three representative miRNAs were selected for northern blot analysis. Although the data from the small RNA libraries indicated that expression of both *miR159* and *miR166* is reduced in *nrpc7-1* while *miR398* expression is increased, no significant alterations in the levels of these miRNAs were consistently observed via northern blotting (Figure 3.10B), suggesting that any differences that exist between the mutant and wild type are too subtle to be detected by this method.

The general disruption in RNA equilibrium combined with the striking serrated leaf phenotype and dwarf morphology of the *nrpc7-1* mutant lead us to hypothesize that the expression of (i) the *CUC* (*CUP-SHAPED COTYLEDONS*) genes, which are targeted by *miR164* (Mallory *et al.*, 2004), and (ii) the HD-ZIP (HOMEODOMAIN-LEUCINE ZIPPER) genes, which are targeted by *miR165/166* (Rhoades *et al.*, 2002), might be altered in the mutant. Expression of a *CUC2* transcript resistant to *miR164* cleavage was previously shown to result in enhanced leaf serration; the same morphological phenotype was observed in plants containing loss-of-function mutations in the Pol II-transcribed *MIR164* (Nikovics *et al.*, 2006). Overexpression of either *miR165* or *miR166* results in reduced expression of the HD-ZIP genes, which corresponds with dwarf morphology and altered rosette leaf morphology that is similar to that observed in *nrpc7-1* (Jung *et al*. 2007).

Real-time qPCR was used to determine that *CUC1* and *CUC2* accumulation is elevated in *nrpc7-1* (Figure 3.9C), although no alteration in *miR164* levels were observed in *nrpc7-1* based on our small RNA library data. Expression of the HD-ZIP genes *PHB* (*PHABULOSA*) and *REV*

(*REVULOTA*) was found to be decreased in *nrpc7-1* (Figure 3.9C). No changes in *miR165* levels were observed in *nrpc7-1*, and although *miR166* expression was elevated in the mutant according to the small RNA library sequencing data (Figure 3.10A) no detectable change in *miR166* levels was consistently measurable by northern blotting (Figure 3.10B). The altered expressions of *CUC1*, *CUC2*, *PHB*, and *REV* in the absence of detectable changes in *miR164*, *miR165*, and *miR166* abundances suggests that in *nrpc7-1* the activity of a number of small RNAs may be affected; alternatively, the *nrpc7-1* mutation may indirectly affect Pol II-mediated transcription of certain genes, including *CUC1*, *CUC2*, *PHB*, and *REV*, although the mechanism behind this specificity is unclear. Many of the RNAs that seem to be differentially expressed in *nrpc7-1* are not transcribed by Pol III (Figure 3.9), suggesting that this mutation results in a disruption of the global RNA equilibrium and homeostasis.

3.3.6 NRPC7 localizes to the nucleus

As part of the Pol III complex, NRPC7 is predicted to localize to the nucleus. To examine its localization, we used the complementing *nrpc7-1* lines containing the transgene with *NRPC7* fused to *GFP* under the control of the native promoter, described above. We analyzed cotyledon and root tissue using confocal microscopy, and GFP fluorescence was visible throughout the nucleus as it co-localized with nuclei stained with propidium iodide (Figure 3.11). Additionally, there appeared to be intense fluorescent foci within the nucleus and along the plasma membrane.

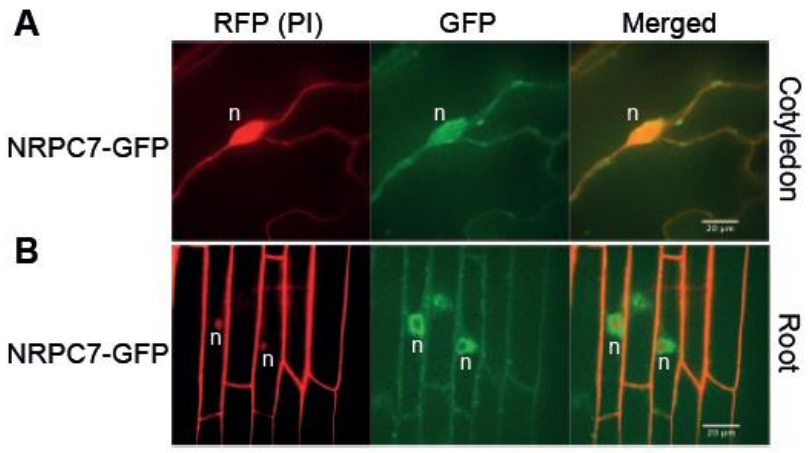

Figure 3.11. Subcellular localization of NRPC7-GFP.
NRPC7-GFP as observed by confocal microscopy in cells from the cotyledon (A) and root (B) from Arabidopsis seedlings grown for 12d on MS media. Propidium iodide (red) was used to stain the cell wall and nuclei. Scale bars represent 20 µm. n = nucleus.

3.3.7 *nrpc7-1* has pleiotropic developmental defects

The roles various small RNAs play in the regulation of plant development have been well studied. As *nrpc7-1* has large impacts on small RNA levels and, potentially, RNA activities, we examined the developmental phenotypes of the mutant. As described earlier, *nrpc7-1* has serrated leaves (Figure 3.3A; Figure 3.12A), and its growth is stunted (Figure 3.12B). When grown on half-strength MS media, *nrpc7-1* plants also have significantly shorter roots than wild type plants (Figure 3.12C). The siliques of *nrpc7-1* were consistently found to be smaller (Figure 3.12D). Flowering time was measured using several different assays, but a significant difference between *nrpc7-1* and wild type was only observed when measuring the number of days until the primary stalk reached 6 cm (Figure 3.12E), which is likely a reflection of the restricted growth of the mutant rather than an actual delay in flowering time. While the number and arrangement of the floral organs are wild type-like, the texture of the sepals is bumpy and irregular (Figure

3.12F). These results show that the *nrpc7-1* mutation is associated with a number of pleiotropic developmental defects.

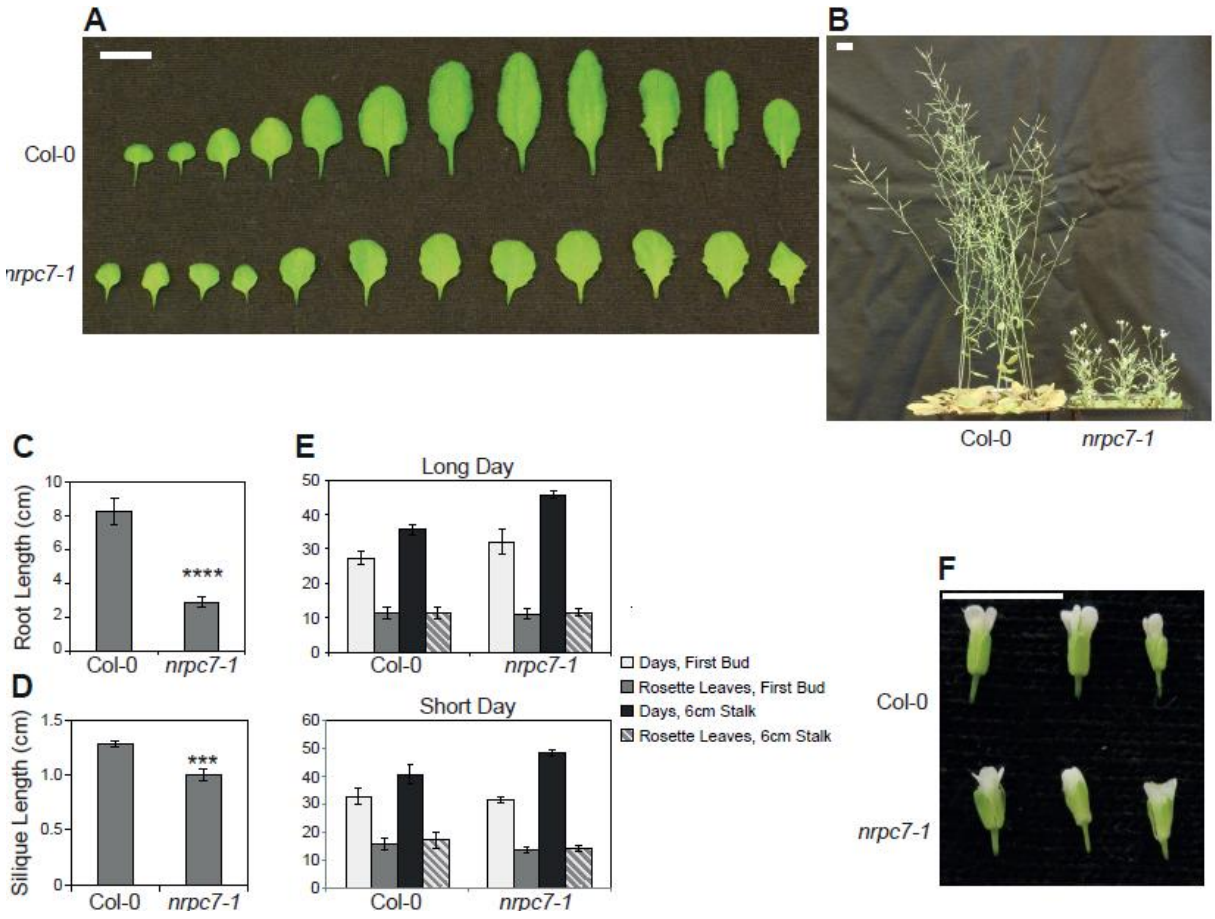

Figure 3.12. Developmental defects of the *nrpc7-1* mutant.
(A) Rosette leaf morphology from wild type and *nrpc7-1* plants at bolting. Bar indicates 1 cm.
(B) Morphology of soil-grown plants eight weeks post-germination. Scale bar represents 1cm.
(C) Comparison of root length in cm between wild type and *nrpc7-1*. Seedlings were grown vertically on ½ MS for 14d. Bars represent five replicates ± SD. ****: p-value ≤ 0.0001.
(D) Comparison of silique length between wild type and *nrpc7-1*. Siliques were harvested from mid-level of the primary stem for each plant. Bars represent three replicates of five siliques per plant ± SD. ***: p-value ≤ 0.001.
(E) Four different approaches were used to investigate flowering time in wild type and *nrpc7-1* plants under both short day and long day conditions.
(F) Morphology of wild type and *nrpc7-1* flowers. Scale bar indicates 0.5cm.

3.4 Discussion

We demonstrated that Pol III function is altered by the partial loss-of-function mutation *nrpc7-1* by showing that the expression of Pol III-transcribed U6 snRNA, 5S rRNA, and a number of tRNAs are reduced in the mutant (Figure 3.6A; Figure 3.9A) Pol II-transcribed U2 snRNA, but not U1 snRNA, also had reduced expression in *nrpc7-1* (Figure 3.6A), indicating that the transcriptional defects in the mutant extend to genes not directly transcribed by Pol III. The decreased accumulation of U6 and U2 snRNA lead us to hypothesize that spliceosome functionality is impaired in *nrpc7-1* and that alternative splicing of *SNC1* is consequently affected, thereby explaining why this mutation was isolated from a screen for enhancers of the autoimmune mutant *snc1*. Indeed, *SNC1* splicing is defective in the *nrpc7-1 mos4 snc1* triple mutant background, in that there is a dramatic accretion of a transcript variant that retains both the second and third introns (Figure 3.6B-C). A similar pattern was observed for the NLR-encoding gene *RPS4*. The second intron of *SNC1* contains an in-frame premature stop codon, thus retention of this intron should yield a truncated version of the protein. Previous reports have shown that an accumulation of the N termini of TNL proteins is sufficient to activate cell death and immunity (Weaver *et al.*, 2006; Swiderski *et al.*, 2009). This finding, combined with our data showing that transcription and translation of *SNC1* are not enhanced by the *nrpc7-1* mutation (Figure 3.7A-B), suggests that the modification in *SNC1* splicing could be the primary cause of the *snc1* enhancing effects of the *nrpc7-1* mutation in the *mos4 snc1* background (Figure 3.1).

It is notable that the *nrpc7-1* single mutant does not differ from wild type in either alternative splicing (Figure 3.6B-C) or disease resistance (Figure 3.8). This suggests that the *mos4* mutation is required in the genetic background for the *nrpc7-1*-associated splicing defects

to become obvious. MOS4 is an integral component of the evolutionarily conserved MOS4-associated complex (MAC) that functions together with the spliceosome to regulate pre-mRNA splicing (Johnson *et al.*, 2011). Mutations in *mos4* and other MAC components have previously been shown to affect the alternative splicing of both *SNC1* and *RPS4* (Xu *et al.*, 2012), and are associated with a suppression of *SNC1*-dependent immune signaling (Palma *et al.*, 2010). In this study we demonstrated that there is an increased accumulation of the intron-retaining *SNC1* transcripts in *mos4 snc1* compared with *snc1* (Figure 3.6C). However, this accumulation is radically enhanced in the *nrpc7-1* triple mutant. One possible explanation for these results is that the *mos4* mutation and, to a lesser extent the *nrpc7-1* mutation, individually disrupt splicing efficiency, reducing the pool of the functional full-length *SNC1* transcript variant with both introns excised but not increasing the production of alternative variants beyond the threshold required for immune activation. However, when these two mutations are combined in the *snc1* background, spliceosome activity is markedly disturbed and the accumulation of intron-retaining *SNC1* transcript variants is sufficiently high to yield enough truncated SNC1 to activate defense responses.

In addition to the splicing defects observed in *nrpc7-1*, the accumulations of rRNAs and tRNAs appear to be considerably distorted (Figure 3.9A-B). Ribosomal protein gene dosage was recently found to have an effect on embryonic stem cell differentiation in mice (Fortier *et al.*, 2015), indicating that alterations in the abundance of ribosome components can dramatically alter developmental progression. Homozygous *nrpc7-1* plants exhibit certain phenotypes that may be associated with impaired stem cell differentiation including short roots (Figure 3.12C) and delayed emergence of the first true leaves. This suggests that the sensitivity of ribosome function to changes in its subunit levels, as well as its role in regulating stem cell differentiation,

may be conserved in plants, although the data in support of this is preliminary and additional experiments are required to fully explore this hypothesis.

We also detected a reduction in the accumulation of the chloroplast 16S and 23S rRNAs relative to Pol I-transcribed 25S rRNA (Figure 3.9B). A similar rRNA abundance pattern was recently reported for *atybeY-1*, a mutant allele of an endoribonuclease required for chloroplast rRNA processing and development that also exhibits pale green leaves and delayed development (Liu *et al.*, 2015). Although the mechanism by which alterations in a Pol III subunit result in changes to the transcriptional regulation of Pol I-transcribed genes and the chloroplast genome is unclear, the light green colour of the *nrpc7-1* single mutant (Figure 3.3A) further suggests that this mutation may be associated with impaired chloroplast function.

The serrated leaf phenotype observed in *nrpc7-1* is likely linked to its elevated expression of *CUC1* and *CUC2* (Figure 3.9C), which could be a result of reduced *miR164* activity in the mutant background or an indirect effect of the *nrpc7-1* mutation on Pol II function. There may also be a link between the *nrpc7-1* mutant morphology and the decreased expression of the HD-ZIP genes (Figure 3.9C). Other studies have demonstrated tentative links between the transcriptional activities of Pol II and Pol III. One study identified areas of the genome where protein-coding genes on one DNA strand overlapped with tRNA-encoding genes on the opposite strand, and that their rates of transcription by Pol II and Pol III, respectively, were negatively correlated (Lukoszek *et al.*, 2013). Another study found that human *RPPH1* is transcribed by both Pol II and Pol III, and identified a number of transcriptional activators that associate with both Pols (Faresse *et al.*, 2012). Our data show that disturbances in Pol III function affects the expression of non-Pol III-transcribed RNAs, indicating that the role of Arabidopsis Pol III in transcriptional regulation is more complex than previously assumed.

There are twelve core subunits of Arabidopsis Pol III, each of which has a homolog or is itself also a component in Pols I, II, IV, and V (Haag and Pikaard, 2011; Ream *et al.*, 2015). There are also subunits specific to individual Pols. *NRPC7* encodes a core Pol III subunit with homologs in each of the other Pols, and shares significant sequence similarity with Rpc25 proteins from other model organisms (Figure 3.4). However, Arabidopsis *NRPC7* failed to complement a temperature-sensitive *rpc25* yeast knockout line (Figure 3.5), suggesting that the functional conservation of this protein by itself between yeast and plants is limited. This is not entirely unprecedented. Rpc25 is known to form a dimer with Rpc17 within the Pol III complex (Siaut *et al.*, 2003). The protein-protein interaction surface of NRPC7 may be sufficiently evolutionarily divergent so as to prohibit it from dimerizing with yeast Rpc17. Although the function of the protein complex is conserved, an individual component of the complex may still be divergent enough that it fails to complement a knockout of its ortholog in a distant organism.

In summary, we have demonstrated that a perturbation in Pol III function results in modified gene splicing as well as alterations in the abundances and potentially activities of a number of RNA molecules. These effects extend to several RNAs reported to be transcribed by other polymerases, revealing a novel role for Pol III in modulating the expression of a larger complement of genes than previously described. Moving forward, the partial loss-of-function *nrpc7-1* mutant provides a unique tool for performing other functional analyses of Pol III.

3.5 Materials and methods

3.5.1 Plant growth conditions and mutant isolation

Plants were grown either on soil or on half-strength Murashige and Skoog (MS) media supplemented with 1% sucrose and 0.3% phytagel. All plants were grown under long day conditions (16 h light/8 h dark) at 22ºC in climate-controlled chambers. The *muse4* mutant was isolated from the MUSE screen, described previously (Huang *et al.*, 2013).

3.5.2 Total RNA extraction and analysis

Approximately 0.1 g tissue was collected from 2-week-old seedlings grown on ½ MS, and the Totally RNA Kit (Ambion, now Invitrogen) was used to extract total RNA. For the comparison of rRNA levels, total RNA was run on a 2% agarose gel. To reverse transcribe 0.4 µg RNA to cDNA, the Reverse Transcriptase M-MLV (Takara) was used after treating the RNA with DNaseI (Promega). The sequences of primers used were: 4F 5'-AATCTCCCTCTCGAAGATGC-3' and 4R 5'-AAAGGCTTTGCGTCCTCTGC-3' for *MUSE4*/*NRPC7*; U1F 5'-TACCTGGACGGGGTCAAC-3' and U1R 5'-CCCTCTGCCACAAATAATGAC-3' for *U1*; U2F 5'-TCGGCCCACACGATATTAAC-3' and U2R 5'-GCAGTAGTGCAACGCATAGG-3' for *U2*; 5SF 5'-GGATGCGATCATACCAGC-3' and 5SR 5'-GAGGGATGCAACACGAGG-3' for *5S* rRNA; 7SLF 5'-CAAATCAAGTGGTTCAACCC-3' and 7SLR 5'-CTTCGACGTTATCATCTGCG-3' for *7SL* RNA; GlnF 5'-GGTTCTATGGTGTAGTGGTTAGC-3' and GlnR 5'-TACCGGGAGTCGAACCCAG-3' for *tRNA-Gln*; GlyF 5'-GCACCAGTGGTCTAGTGGTA-3' and GlyR 5'-TGCACCAGCCGGGAATCGAA-3' for *tRNA-Gly*; and LeuF 5'-

TGTCAGAAGTGGGGTTTGAACC-3' and LeuR 5'-TCAGGATGGCCGAGTGGTCTAA-3' for *tRNA-Leu*. Primers used for amplification of *SNC1*, *RPS4*, *SR30*, *PAD4*, and *ACTIN7* were previously described (Zhang *et al.*, 2003; Cheng *et al.*, 2009; Xu *et al.*, 2012).

3.5.3 Infection assays

H.a. Noco2 infection was performed by spraying 2-week-old soil-grown seedlings with a spore suspension with a concentration of 10^5 spores per mL of water. Inoculated seedlings were grown for 7 d at 18ºC in a growth chamber with ~80% humidity and a 12 h light/12 h dark cycle. Sporulation was then quantified using a hemocytometer to count the number of spores from five plants shaken in 1 mL of water. Five replicates were performed for each of three independent trials. *P.s.m.* ES4326 infection was performed by infiltrating the abaxial leaf surface of 4-week-old soil-grown seedlings with bacteria suspended in 10 mM $MgCl_2$ (OD_{600}=0.0005). Leaf punches were collected at day 0 and day 3, and serial dilutions were performed and plated on LB media. Plates were incubated at 28ºC for 24 h before colony forming units were measured.

3.5.4 Positional cloning and Illumina whole-genome sequencing

Positional cloning of *muse4* was performed by crossing the *muse4 mos4 snc1* triple mutant (generated in the Col-0 ecotype) with wild type Landsberg *erecta*. 24 F2 plants homozygous for all three mutations were used for crude mapping, and approximately 500 F3 plants homozygous for *mos4* and *snc1* and heterozygous for *muse4* were used for fine mapping. The markers used in mapping were derived from insertion/deletion polymorphisms between the Col-0 and L*er* Arabidopsis ecotypes (Jander *et al.*, 2002; http://www.arabidopsis.org). After determining that the mutation must be located on the top of chromosome 1 between 1.4 MB and 2.75 MB,

extracted genomic DNA from *muse4 mos4 snc1* was sequenced using the Illumina sequencing platform.

3.5.5 Preparation of transgenic plants and confocal microscopy

Full length *At1g06790* genomic DNA, including 766 bp upstream of the start codon, was amplified via PCR, cloned into the pCambia1305 vector, and transformed into *muse4 mos4 snc1* using the floral dip method (Clough and Bent, 1998). The full length genomic fragment was also cloned into a pCambia1305 vector containing a GFP tag. Transgenic plants were selected for on ½ MS plates containing 50 mg/mL hygromycin. Confocal images of wild type (negative control), 35S::X-GFP (positive control), and *NRPC7-GFP* transgenic seedlings were obtained using a Perkin Elmer Ultraview VoX Spinning Disc Confocal system (Perkin-Elmer) mounted on a Leica DM16000 B inverted microscope and equipped with a Hamamatsu 9100-02 electron multiplier CCD camera (Hamamatsu). An argon 488 nm laser line with a complementary (522/36) emission band-pass filter to detect GFP or a 561 nm laser with a complementary (595/50) emission band-pass filter to detect propidium iodide was used. Images were acquired with a 63x (water) objective lens. To stain the nuclei and the cell wall, seedlings were incubated in a 10 µg/mL solution of propidium iodide (Calbiochem) for 1 min, rinsed with water, and mounted on a slide and coverslip prior to imaging.

3.5.6 Yeast complementation

Full length *MUSE4* cDNA was cloned into the yeast expression vector p425-GPD with primers 5'-CGCggatccATGTTTTATCTTAGCGAGC-3'and 5'-ACGCgtcgacTCACTCTTCTTGATCAACC-3', using *Bam*HI and *Sal*I digestion sites. *MUSE4*

and empty vector control plasmids were introduced into the yeast *rpc25-ts* strain using a standard polyethylene glycol/lithium acetate yeast transformation protocol (http://labs.fhcrc.org/gottschling/Yeast%20Protocols/ytrans.html). Yeast transformants were grown overnight, serially diluted, and plated onto SD-Leu plates grown under either 28ºC or 37ºC to assay for growth.

3.5.7 Small RNA library construction and sequencing

Small RNAs within the size range of 15nt to 40nt were fractionated from total RNAs by 15% polyacrylamide gel electrophoresis. These small RNAs were then ligated with the 3' and 5' adapters sequentially using the Small RNA Sample Preparation Kit (Illumina) according to the manufacturer's instructions. A reverse transcription reaction followed by a low cycle PCR was performed to obtain final products for deep sequencing. The wild type and *muse4* libraries were barcoded and sequenced in one channel on an Illumina Hiseq2000.

3.5.8 Analysis of small RNA high throughput sequencing data

PERL scripts were used to process small RNA raw reads as per Lertpanyasampatha *et al.* (2012). To summarize, reads were passed through Illumina's quality control filter before being sorted into bins based on their barcodes and having their adaptor sequences removed. SOAP2 was used to map reads within the size range of 20–24 nt to the Tair10 Arabidopsis genome (Li *et al.*, 2009). Differential small RNA regions were identified as previously described (Dinh *et al.*, 2014). For analysis of differentially expressed miRNAs, all known Arabidopsis miRNAs were downloaded from miRBase (Release 20 from www.mirbase.org; Griffiths-Jones *et al.*, 2008). PERL scripts were used to determine the expression level of known miRNAs in the small RNA

libraries and then normalize these counts to RPM (reads per million). miRNAs with < 10 RPMs in both *nrpc7-1* and wild type libraries were removed. The differentially expressed miRNAs were identified by comparing expression in the *nrpc7-1* library with wild type. The Audic-Claverie method was used to calculate P-values (Audic *and* Claverie, 1997), which were subsequently adjusted as described by Benjamini *and* Hochberg (1995) to determine the false discovery rate (FDR). To qualify as a differentially expressed miRNA, both a fold change ≥ 2 between wild type and *nrpc7-1* and an FDR < 0.05 were necessary.

Chapter 4: The putative kinase substrate MUSE7 negatively regulates the accumulation of SNC1

4.1 Summary

The strict regulation of immune signaling in plants is required in order to enable rapid response to pathogen attack as well as to prevent spurious activation of defense responses that may be associated with fitness costs. However, these regulatory mechanisms are only partially understood. To identify novel negative regulators of plant immunity, a forward genetic screen was designed to look for enhancers of the dwarf autoimmune *snc1* (*suppressor of npr1, constitutive 1*) mutant. The screen was conducted using wild-type-like *mos4* (*modifier of snc1, 4*) *snc1* plants, and mutants were screened for a reversion to *snc1*-like phenotypes. The isolated *muse7* (*mutant, snc1-enhancing, 7)* mutant was shown to confer dwarf morphology, elevated expression of *PATHOGENESIS-RELATED* genes, and enhanced resistance to the virulent oomycete pathogen *Hyaloperonospora arabidopsidis* Noco2 when present in the *mos4 snc1* background. Map-based cloning and Illumina whole genome sequencing revealed that the *muse7* phenotypes are associated with a mutation in *At5g46020*, which encodes a protein of unknown function. This protein is conserved across most eukaryotes but is not present in *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*. Both the *muse7-1* allele isolated from this screen and the *muse7-2* exonic T-DNA insertion allele displayed enhanced resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000, but not to *P.s.t.* DC3000 expressing the effector proteins AvrRpm1, AvrRpt2, or AvrRps4. While transcription of *SNC1* is not elevated in the *muse7* mutants, SNC1 protein accumulates in both alleles. Although proteasome-mediated

degradation is a well-studied event in immune regulation, no interactions were detected between MUSE7 and known components of this pathway, suggesting that MUSE7 may regulate SNC1 at the translational level. This study has demonstrated a novel role for MUSE7 in modulating plant immune responses, and may benefit future studies of MUSE7 homologs in other species.

4.2 Introduction

The plant immune system is subject to tight regulation, enabling these sessile organisms to ward off infection by most pathogenic microorganisms. As an initial line of defense, plants possess many physical and chemical barriers that hinder microbial access to plant cells; these barriers include the cuticle, the cell wall, and anti-microbial enzymes. However, the relationship between plants and phytopathogens is highly dynamic, and these defenses are sometimes breached. In case of such events, plants also possess a sensitive surveillance system that detects the presence of invading pathogens. Receptors on the cell surface are able to perceive conserved pathogen-associated molecular patterns (PAMPs) (Macho & Zipfel 2014). Via complex signaling pathways, this recognition leads to the induction of PAMP-triggered immunity (PTI) (Bigeard et al. 2015), which is mediated by a mitogen-activated protein kinase signaling cascade and results in a number of defense response outputs, including callose deposition to strengthen the cell wall, production of reactive oxygen species (ROS), and accumulation of the defense hormone salicylic acid (SA) (Hammond-Kosack & Jones 1996). In turn, however, many successful pathogens are able to deliver molecules (termed effectors) into the plant cell to inhibit PTI and promote infection. Escalating this “arms race”, higher plants have evolved a suite of intracellular immune receptor proteins that are able to detect effector molecules, either through direct protein-protein

interactions or indirectly through the perception of effector activities within the plant cell. These plant proteins are referred to as NOD-like receptors (NLRs) due to their resemblance to metazoan nucleotide-binding oligomerization domain (NOD)-containing proteins, which function as PAMP receptors in animals (Li et al. 2015).

The majority of plant NLR proteins possess a central nucleotide-binding (NB) domain and a C-terminal leucine rich-repeat (LRR) domain. They can be subdivided into two classes based upon their different N-termini: some possess a Toll-interleukin1 receptor (TIR) domain and are thus termed TNLs, and others have a coiled-coil (CC) domain and are therefore referred to as CNLs. In the absence of pathogen detection, NLR proteins are expressed at low levels. Following effector recognition, they become activated and initiate a downstream signaling cascade that results in effector-triggered immunity (ETI). This type of immunity is typified by a stronger, faster, and more robust induction of the defense outputs that characterize PTI (Cui et al. 2015). ETI may also lead to a type of localized cell death referred to as the hypersensitive response (HR). Additionally, the immunity mediated by NLR proteins is subject to a positive transcriptional feedback defense amplification, whereby NLR protein activation results in transcriptional reprogramming that subsequently upregulates the expression of a number of defense-related genes, including many that encode NLR proteins themselves (Tsuda & Somssich 2015). These processes must be finely tuned, as there is a trade-off between plant growth and plant immunity; dwarfism and reduced viability are associated with precocious activation of immune responses.

The *snc1* autoimmune mutant has proven to be a useful tool in disentangling the regulatory events that govern NLR-mediated immunity. This mutant contains a gain-of-function mutation in the linker region between the NB and LRR domains of SNC1, a TNL protein (Li et

al. 2001; Zhang et al. 2003). Plants with this mutation are dwarf and have a distinct twisted leaf morphological phenotype. A previous screen for suppressors of *snc1* yielded a number of novel positive regulators of plant immunity (reviewed in Johnson et al. 2012). Results from the *MODIFIERS OF SNC1* (*MOS*) screen highlighted the importance of modulated transcription, RNA processing, nucleocytoplasmic trafficking, and protein modifications as key regulatory events in immunity.

Based on the success of the *MOS* screen, a screen for enhancers of *snc1* was performed in order to search for novel negative regulators of NLR-mediated immunity. The *MUTANT, SNC1-ENHANCING* (*MUSE*) screen was conducted using seeds from *mos4 snc1*, which is approximately wild-type-like in terms of both morphology and resistance (Palma et al. 2007). This mutant background was utilized in order to avoid potential lethality resulting from severe dwarfism caused by enhancer mutations. Mutants were screened for a reversion back to *snc1*-like phenotypes, and twelve mutant lines were selected for further characterization. A number of reports on *muse* mutants have been recently published. Approximately half of the characterized MUSE proteins were shown to play essential roles in NLR protein turnover (Huang et al. 2014a; Huang et al. 2014b; Xu et al. 2015; Huang et al. 2016). One *MUSE* gene was found to encode AtPAM16, a component of the protein import motor in the mitochondrial inner membrane that is required for the negative regulation of ROS production (Huang et al. 2013). Another *MUSE* gene encodes SPLAYED, a SWI/SNF chromatin remodeler that had previously been found to positively regulate immunity to necrotrophic pathogens (Johnson et al. 2015). Together, these reports demonstrate the efficacy of the *MUSE* screen in identifying molecular events in plant immunity. In this study, we report the characterization of *muse7*, which fully restores *snc1*-like resistance in the *mos4 snc1* background.

4.3 Results

4.3.1 The *muse7* mutation re-establishes *snc1*-like phenotypes in the *mos4 snc1* background

The *muse7* mutant line was originally isolated from a forward genetic screen designed to identify *snc1* enhancers in the *mos4 snc1* genetic background, which was previously described (Huang et al. 2013). Morphologically, *muse7 mos4 snc1* plants are similar to *snc1* plants in that they are dwarf and have slightly twisted leaves (Figure 4.1A). The triple mutant also displays enhanced defense marker *PATHOGENESIS-RELATED* (*PR*) gene expression as compared to *mos4 snc1* (Figures 4.1B-C), suggesting that the immune responses are partially activated in *muse7 mos4 snc1* even in the absence of pathogens. Additionally, the triple mutant exhibits enhanced resistance to the virulent oomycete pathogen *Hyaloperonospora arabidopsidis* (*H.a.*) Noco2 as compared to *mos4 snc1* (Figure 4.1D). Altogether these data show that the *muse7* mutation reconstitutes *snc1*-like phenotypes in the *mos4 snc1* background, indicating that *muse7* is an enhancer of *snc1*.

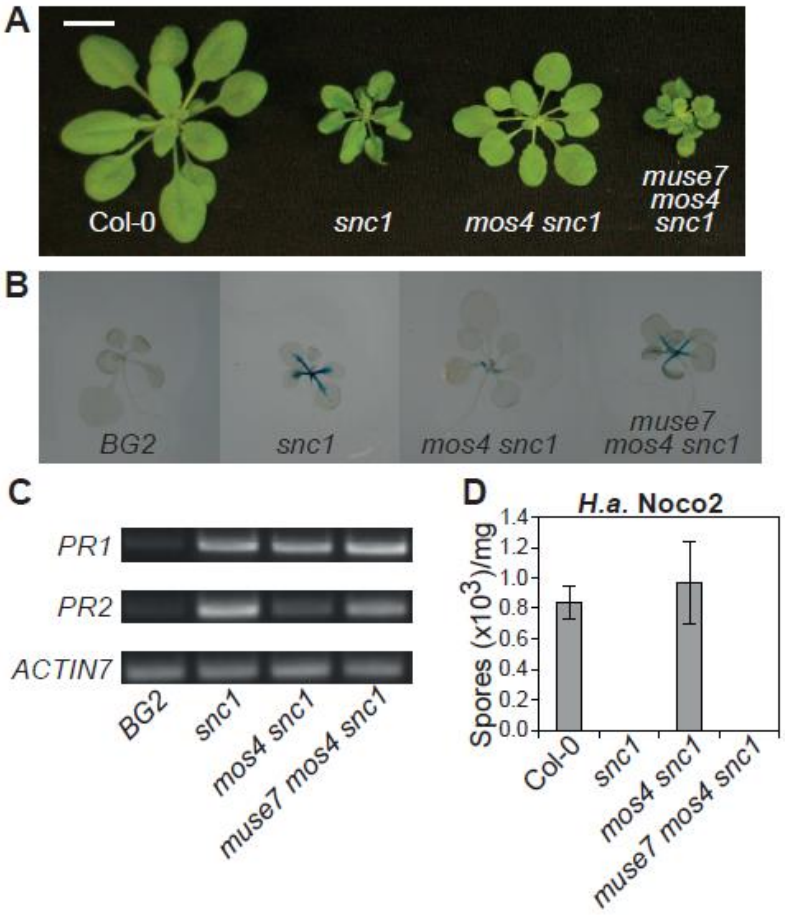

Figure 4.1. Phenotypic characterization of the *muse7 mos4 snc1*.
(A) Morphology of soil-grown wild-type, *snc1*, *mos4 snc1*, and *muse7 mos4 snc1* plants. Photographs were taken four weeks post-germination.
(B) *PR2* gene expression, visualized using the GUS reporter gene assay. All genotypes contain a construct in which the promoter region of *PR2* is fused to the coding sequence of β-glucuronidase (GUS), and following incubation with the substrate X-Gluc the presence and intensity of blue staining provides an indication of gene expression. Plants were grown for 10 days on MS media.
(C) Endogenous expression of *PR1* and *PR2* relative to *ACTIN7* as determined by reverse-transcription quantitative PCR using 30 cycles.
(D) Growth of *H.a*. Noco2 on indicated genotypes seven days post-inoculation with $1x10^5$ spores/mL. Values represent the average of four replicates of five plants each ± SD.

4.3.2 *MUSE7* encodes an uncharacterized protein conserved amongst eukaryotes

To identify the molecular lesion responsible for the phenotypes associated with *muse7*, a positional cloning strategy was employed. The *muse7 mos4 snc1* triple mutant, which was

generated in the Col-0 background, was crossed with wild-type Landsberg *erecta* (L*er*). 24 F2 plants displaying the original triple mutant morphology were selected for linkage analysis. Insertion/deletion DNA markers generated using known polymorphisms between the Col-0 and L*er* ecotypes were used to search for genomic regions with a strong linkage to the Col-0 genotype. Course mapping indicated that the *muse7* mutation is located on chromosome 5 between 17.3 and 19.9 MB (Figure 4.2A). Fine mapping using approximately 500 segregating F3 plants narrowed down the location of the *muse7* mutation to the region between 18.6 and 18.7 MB, at which point Illumina whole genome sequencing was performed to identify genes within this region containing mutations consistent with EMS mutagenesis. The only candidate gene identified was *At5g46020*, which contains a C to T point mutation resulting in a premature stop codon at Q121 (Figure 4.2B-C).

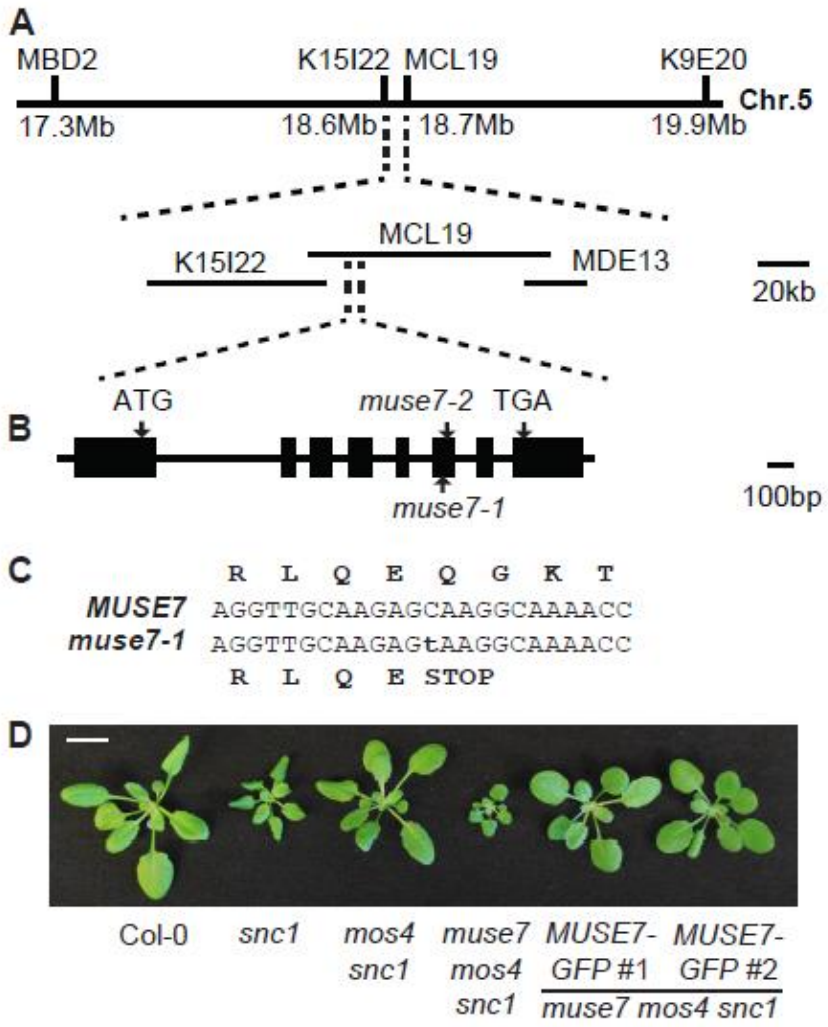

Figure 4.2. Positional cloning of *MUSE7*.
(A) A genetic map of the region of chromosome 5 containing the *MUSE7* locus, with markers used for mapping indicated.
(B) Gene structure of *MUSE7* and the position of the molecular lesions in *muse7-1* (an EMS allele) and *muse7-2* (an exonic T-DNA insertion allele). Boxes and lines represent exons and introns, respectively. Start and stop codons are indicated.
(C) Sequence comparison between wild-type *MUSE7* and *muse7-1*. Nucleotide substitution, indicated with a lower case 't', results in a change from Q121 to a stop codon.
(D) Four-week-old soil-grown plants of the genotypes noted. MUSE7-GFP#1 and MUSE7-GFP#2 are from two independent transgenic complementing lines, where MUSE7 tagged with GFP and under the control of its native promoter was expressed in the *muse7 mos4 snc1* background. Bar indicates 1 cm.

To verify that this mutation in *At5g46020* is responsible for the *muse7* phenotypes, transgene complementation was performed. A wild-type copy of the gene under the control of its native promoter was transformed into the *muse7 snc1 mos4* triple mutant, and a reversion back to *mos4 snc1*-like morphology was observed (Figure 4.2D), indicating that the correct gene was cloned. As *AT5G46020* has not been previously characterized, we have designated this locus as *MUSE7* and the mutant allele identified in our screen as *muse7-1*.

MUSE7 is a single copy gene in Arabidopsis, and orthologs are present in all examined land plants in low copy number (Table 4.1). The protein encoded by *MUSE7* possesses a conserved phosphoprotein PP28 domain (Figure 4.3). Using amino acid sequences for BLAST analysis, it was determined that MUSE7 homologs exist in all examined plants and animals as well as in many fungi, but not in either *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*. Overall, this suggests that MUSE7 is a highly conserved protein particularly among multicellular organisms, and that it likely functions in a conserved biological process.

Table 4.1. MUSE7 homologs are present in low copy number across all examined land plants. USEARCH was employed to identify all potential homologs.

Organism	Number of MUSE7 Homologs	Sequence IDs
Arabidopsis thaliana	1	AT5G46020 (MUSE7)
Arabidopsis lyrata	1	AL8G02320
Fragaria vesca	3	FV3G13410, FV3G17820, FV7G16180
Lotus japonicus	1	LJ0G410950
Glycine max	2	GM10G07980, GM13G21760
Ricinus communis	1	RC29900G00130
Theobroma cacao	2	TB06G015070, TB07G008980
Populus trichocarpa	3	PT01G37500, PT04G04860, PT11G05770
Carica papaya	1	CP00841G00030
Vitis vinifera	2	VV10G01620, VV18G10470
Solanum lycopersicum	1	SL460376108
Zea mays	4	ZM03G32110, ZM03G32130, ZM08G19110, ZM08G34850
Oryza sativa	2	OS01G54920, OS05G43970
Selaginella moelendorffii	1	SM00059G02030
Physcomitrella patens	2	PP00037G00560, PP00149G00280

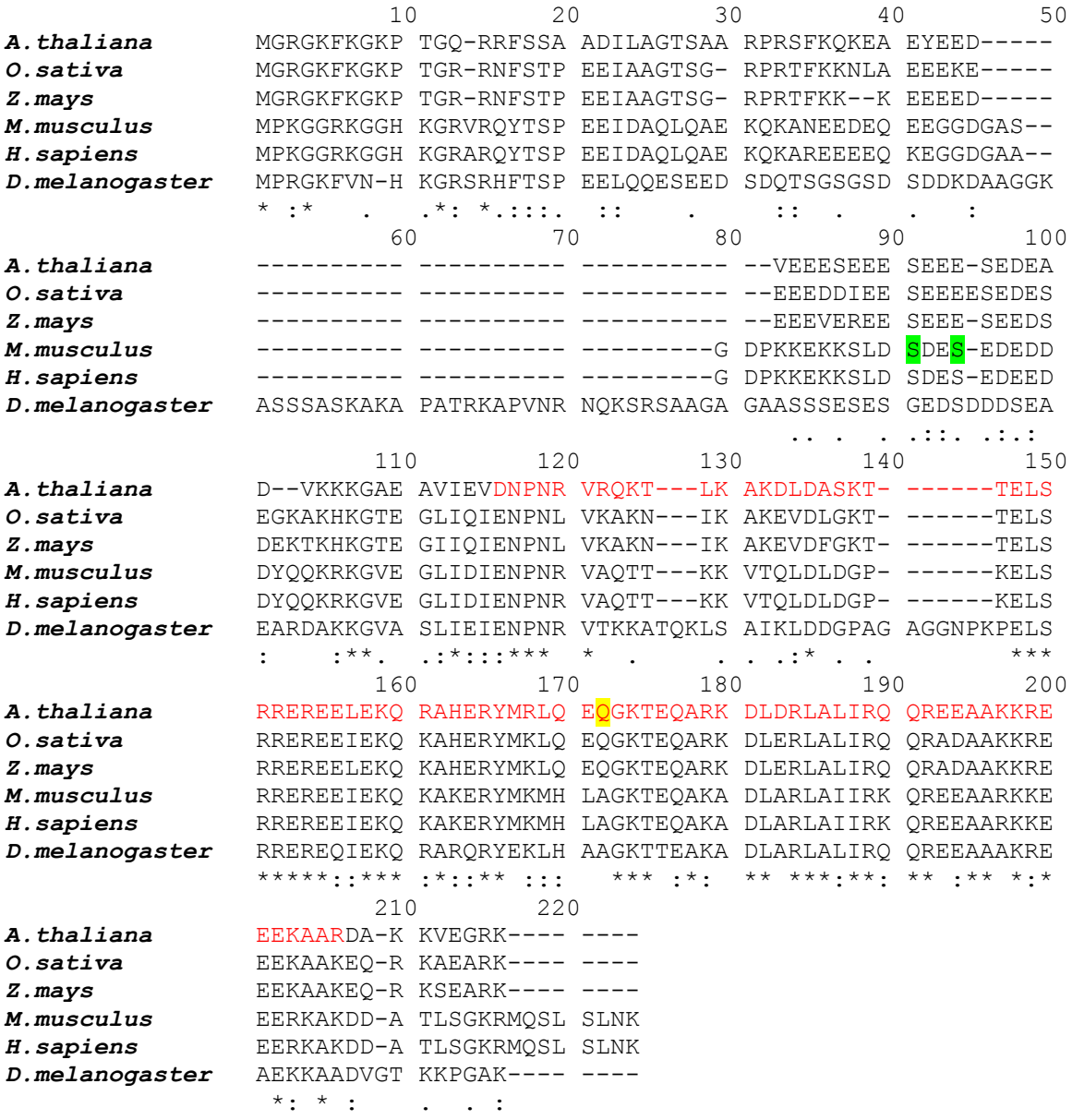

Figure 4.3. Multiple alignment of MUSE7 homolog amino acid sequences. MUSE7 is highly conserved in higher plants and animals. Dashes indicate alignment gaps; "*" indicates identical residues; ":" indicates conserved substitutions; "." indicates semi-conserved substitutions. Red font indicates location of casein kinase substrate, phosphoprotein PP28 domain. Highlighted locations: green – known phosphorylated serines in *Rattus norvegicus*, shown in the *M. musculus* homolog (Shen et al. 1996); yellow – *muse7-1* mutation.

4.3.3 Two independent *muse7* single mutant lines exhibit enhanced disease resistance

To assess how immune responses are altered in the *muse7* single mutant, *muse7-1* and *muse7-2* (an exonic T-DNA insertion allele) were characterized. Apart from the same slightly dwarf, rounded-leaf morphology (Figure 4.4A), both mutant lines were developmentally wild-type-like (Figure 4.5). Upon infection by the virulent bacterial strain *Pseudomonas syringae* pv. *tomato* (*P.s.t.*) DC3000, both *muse7* alleles exhibit enhanced resistance compared with wild-type (Figures 5.4B), although the resistance displayed by *muse7-2* is significantly stronger than that observed for *muse7-1*. When treated with the virulent oomycete *H.a.* Noco2 both alleles showed a general trend of enhanced resistance but only *muse7-2* was consistently different from wild-type (Figure 4.4C). When these two mutant alleles were challenged with the avirulent bacteria *P.s.t.* DC3000 expressing the effector proteins AvrRpt2, AvrRpt4, or AvrRpm1, respectively, no significant difference in resistance compared with wild-type was observed (Figures 4.4D-F). These data suggest that MUSE7 serves as a negative regulator of immunity.

4.3.4 MUSE7 localizes to both the nucleus and the cytoplasm

To gather clues as to the potential function of MUSE7, its subcellular localization was examined by transforming *muse7 mos4 snc1* plants with a construct containing *MUSE7* expressed using the native promoter and possessing a C-terminal GFP tag. The leaves and roots of two independent lines homozygous for single-copy transgene insertion were examined using confocal microscopy. GFP fluorescence was observed in both the nucleus and the cytoplasm in both tissue types (Figure 4.6), indicating that MUSE7 has a broad subcellular distribution. However, the MUSE7 protein has a predicted size of 18.9 kDa, thus we cannot exclude the possibility that it may freely diffuse into the nucleus.

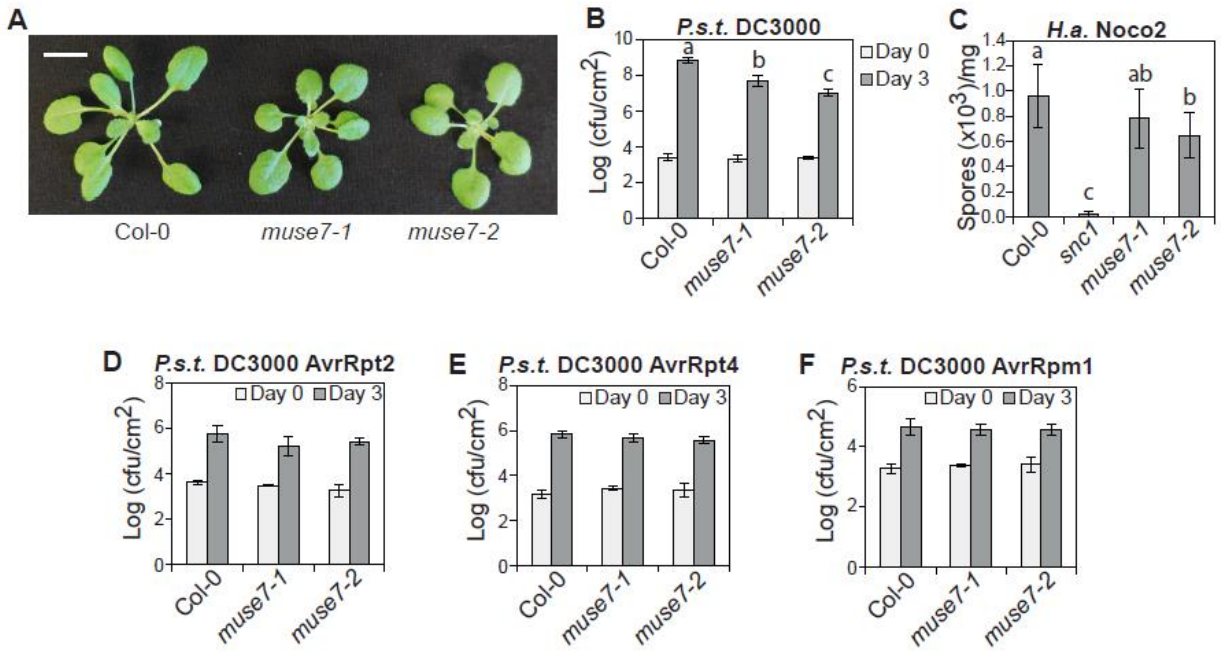

Figure 4.4. Characterization of two independent *muse7* single mutant alleles.
(A) Morphological phenotypes of four-week-old soil-grown wild-type, *muse7-1*, and *muse7-2* plants. Bar represents 1 cm.
(B) Growth of *P.s.t.* DC3000 on wild-type, *muse7-1*, and *muse7-2* plants three days post-infiltration. Values represent the average of five replicates ± SD. Letters indicate statistical difference (single-factor ANOVA and Tukey-Kramer post-hoc analysis).
(C) Growth of *H.a.* Noco2 on wild-type, *snc1*, *muse7-1*, and *muse7-2* plants seven days post-infection. Values presented are averages of three replicates ± standard deviation. Letters indicate statistical difference (single-factor ANOVA and Tukey-Kramer post-hoc analysis).
(D-F) Growth of (D) *P.s.t.* DC3000 AvrRpt2 (E) *P.s.t.* DC3000 AvrRpt4, and (F) *P.s.t.* DC3000 AvrRptm1 on indicated genotypes three days post-infiltration. Values represent the average of five replicates ± SD.

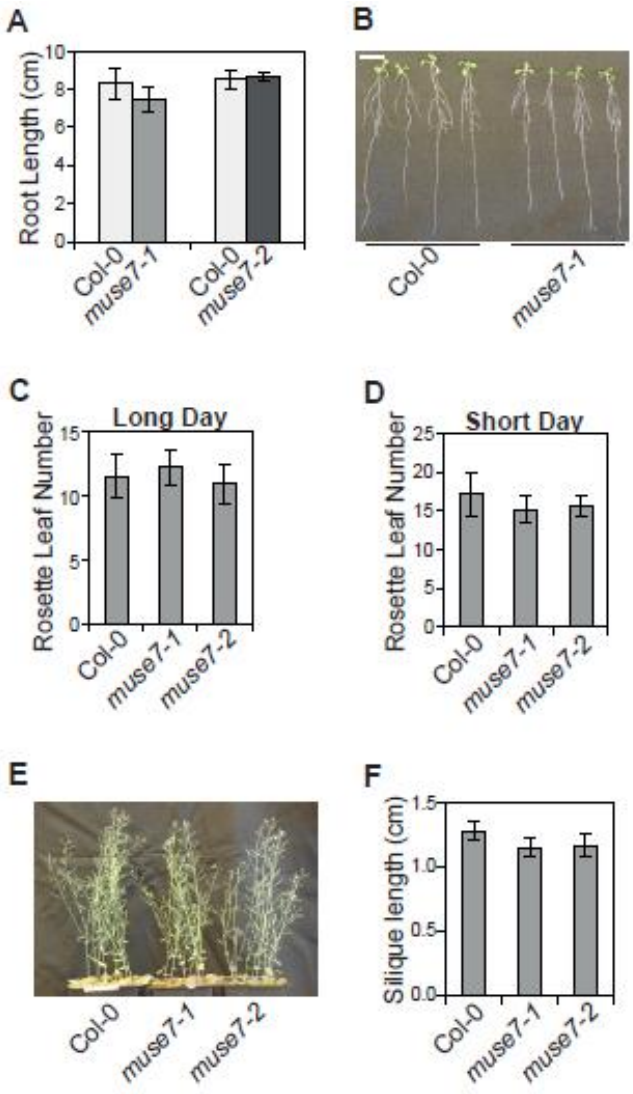

Figure 4.5. Characterization of *muse7* developmental phenotypes.
(A) Primary root lengths of wild-type, *muse7-1*, and *muse7-2* seedlings were measured to the nearest millimeter 10 DAG. Values presented are averages of four seedlings per genotype ± standard deviation, and are representative of values obtained in three biological replicates. Four wild-type seeds and four mutant seeds of one of the two *muse7* genotypes (eight seeds total) were sown on ½ MS media plates, and seedlings were grown vertically.
(B) Wild-type and *muse7-1* seedlings described in (A).
(C, D) Rosette leaves were counted for each genotype when the shoot was 6 - 10 cm. Plants were grown under (C) long day (16h light/8h dark) or (D) short day (8h light/16h dark) conditions. Values presented are the averages of 12 plants per genotype ± standard deviation.
(E) Approximately six-week-old wild-type and *muse7* plants grown under long day conditions. No obvious differences in height or silique distribution are apparent.
(F) Five siliques selected from mid-shoot were collected from three plants per genotype and measured to the nearest millimeter. Values presented are averages ± standard deviation.

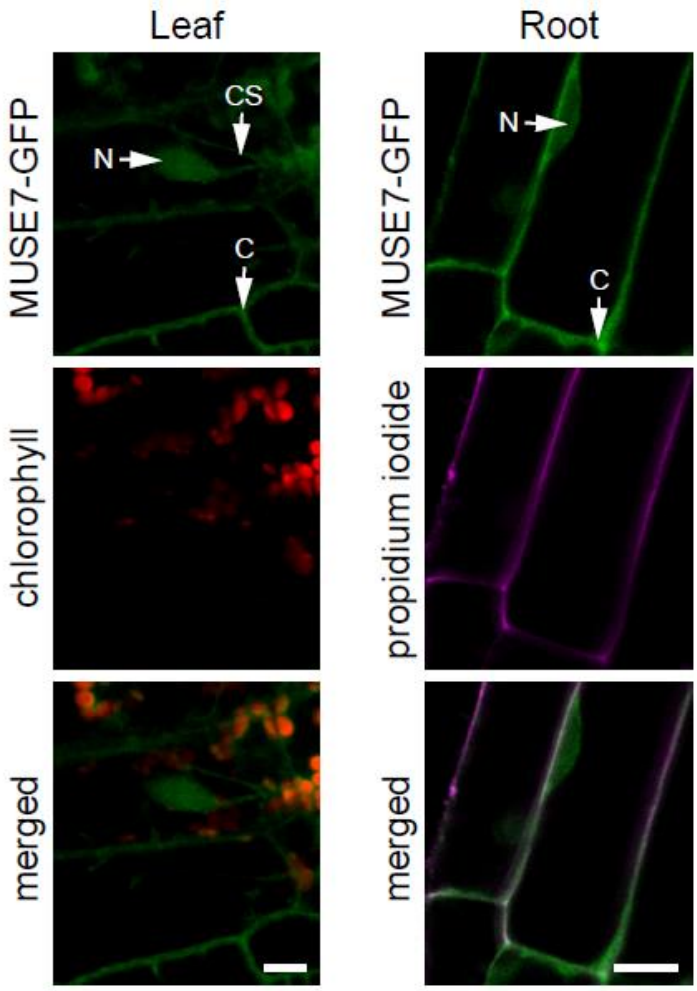

Figure 4.6. Subcellular localization of MUSE7-GFP.
MUSE7-GFP fluorescence in Arabidopsis leaf and root cells as observed by confocal microscopy. Experiments were repeated with multiple cells from independent *MUSE7::MUSE7-GFP* complementing lines (Figure 5.2D). Scale bars represent 10 µm. C: cytoplasm; CS: cytoplasmic strand; N: nucleus.

4.3.5 Mutations in *MUSE7* affect SNC1 accumulation

As the genesis of this project was a screen for enhancers of *snc1*, *muse7 snc1* double mutants were generated to examine the enhancing effect of *muse7* in the absence of *mos4*. Strikingly, both *muse7 snc1* lines exhibit severe dwarfism (Figure 4.7A). This enhancement of *snc1*-like phenotypes in the double mutants is likely the result of misregulation of *SNC1* at either (i) the

transcriptional level, or (ii) the protein level. Using qPCR, it was determined that *SNC1* expression is similar in *muse7-2 snc1* as compared to *snc1* (Figure 4.7B), suggesting that *MUSE7* does not regulate *SNC1* at the transcriptional level. SNC1 protein levels were assessed using western blotting and while there was no observable difference between wild-type and *muse7-2*, a significant accumulation of the protein was observed in *muse7-2 snc1* as compared to *snc1* (Figure 4.7C-E). These data reveal that MUSE7 is involved in the negative regulation of SNC1 protein accumulation, as the loss of MUSE7 results in a higher level of SNC1 protein.

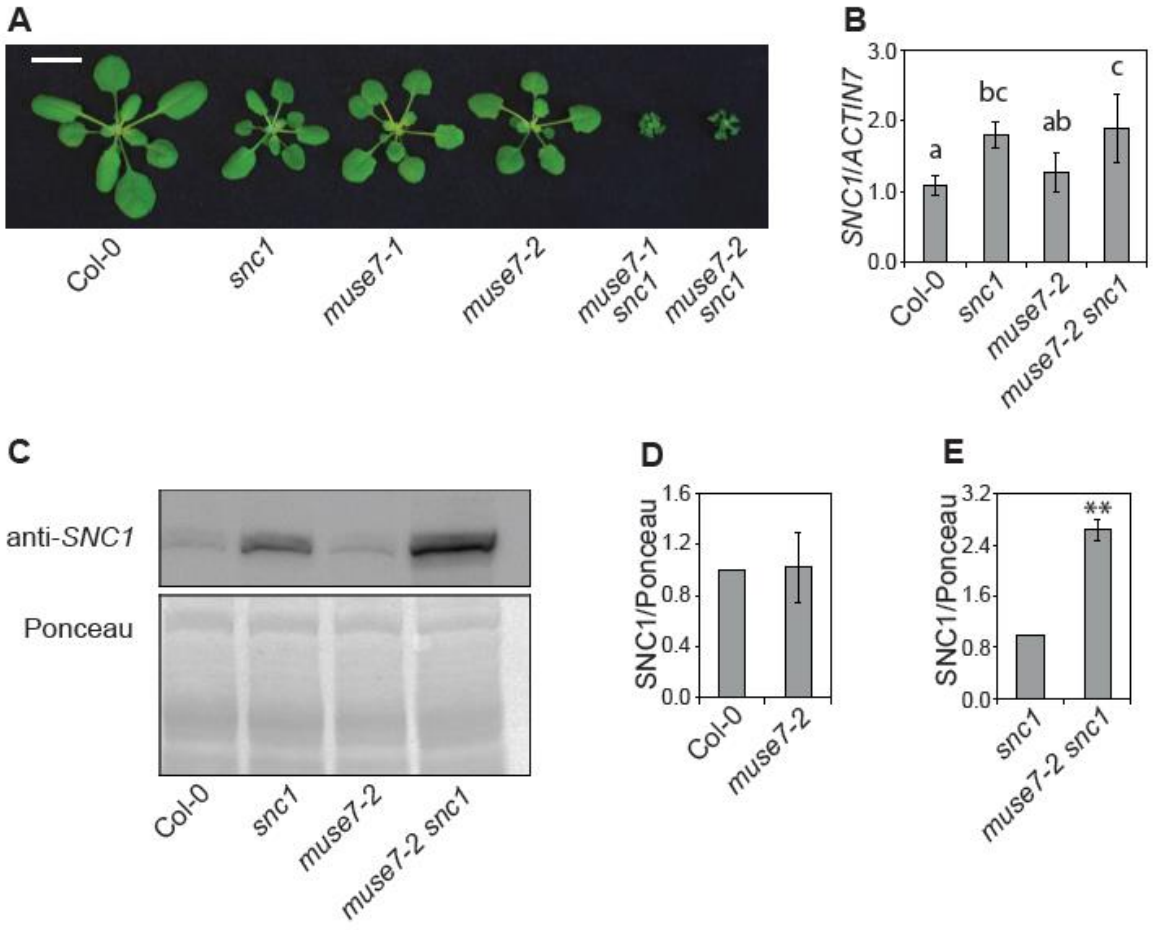

Figure 4.7. Regulation of SNC1 by MUSE7.
(A) Four-week-old wild-type, *snc1*, *muse7-1*, *muse7-2*, *muse7-1 snc1*, and *muse7-2 snc1* soil-grown plants. Bar indicates 1 cm.

(B) Endogenous expression of *SNC1* relative to *ACTIN7* in 21-day-old wild-type, *snc1*, *muse7-2*, and *muse7-2 snc1* soil-grown seedlings. Values presented are averages of three replicates ± standard deviation. Letters indicate statistical difference (Each pair, Student's t test, p-value < 0.05).
(C) Western blot analysis of SNC1 protein levels in total protein extracts from 21-day-old wild-type, *snc1*, *muse7-2*, and *muse7-2 snc1* soil-grown seedlings.
(D) Image J analysis of SNC1 protein levels in wild-type and *muse7-2*. Values presented are the average of five blots ± SD.
(E) Image J analysis of SNC1 protein levels in *snc1* and *muse7-2 snc1*. Values presented are the average of three blots ± SD. Significant differences are indicated by asterisks (** p-value < 0.01).

4.3.6 MUSE7 does not appear to interact with known regulators of SNC1 turnover

While the previous experiments indicate that MUSE7 regulates SNC1 at the protein level, it is unclear whether this regulation occurs at the stage of protein biosynthesis or through protein degradation. Recent reports on other MUSEs have identified a number of proteins that are involved in 26S-proteosome-mediated turnover of NLR proteins, highlighting the importance of this biological process in facilitating immune regulation. Co-immunoprecipitation experiments were therefore performed in *Nicotiana benthamiana* in order to determine if MUSE7 directly interacts with proteins known to play a role in NLR degradation (including HSP90.3 and CPR1) or with SNC1. However, no interactions were detected (Figure 4.8). These results indicate that MUSE7 likely regulates SNC1 protein levels either at the point of protein biosynthesis or via a previously uncharacterized degradation event.

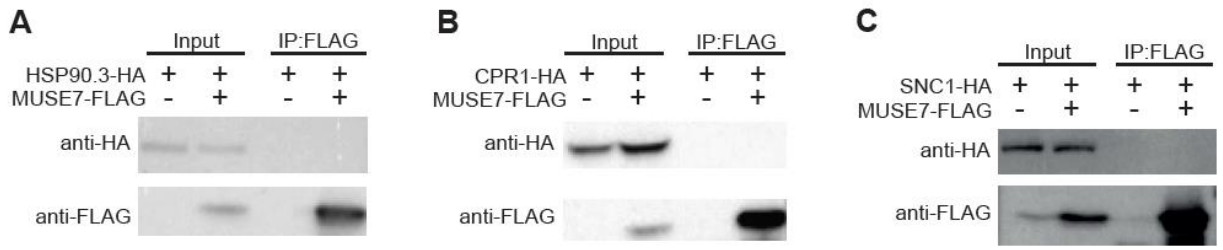

Figure 4.8. MUSE7 does not coimmunoprecipitate with HSP90.3, CPR1, or SNC1 following transient co-expression in *Nicotiana benthamiana*.
Co-immunoprecipitation assays were conducted using MUSE7-FLAG and (A) HSP90.3-HA, (B) CPR1-HA, and (C) SNC1-HA. All immunoprecipitations were performed 48 h following transient co-expression in *N. benthamiana* using anti-FLAG beads, and immunoblotting was performed using antibodies against HA and FLAG, respectively.

4.4 Discussion

In this study, the previously uncharacterized Arabidopsis protein MUSE7 was shown to be a negative regulator of NLR protein accumulation. Little can be inferred about the function of MUSE7 based on its homologs in other eukaryotes. The closest human homolog to MUSE7 is PDAP1 (PLATELET-DERIVED GROWTH FACTOR (PDGF)-ASSOCIATED PROTEIN 1), which binds to two different isoforms of PDGF and modulates their mitogenic activities (Fischer & Schubert 1996). However, the Arabidopsis genome does not encode any PDGF homologs, thus this function does not seem to be conserved across kingdoms. The *Rattus norvegicus* homolog of MUSE7 was previously shown to be phosphorylated by casein kinase II at S62 and S59, sequentially (Shen et al. 1996; Figure 4.3). This suggests that MUSE7 function may be modulated by phosphorylation. Although there is currently no empirical evidence to support this postulation, future experiments using phosphomimetic mutant versions of the MUSE7 protein may be useful in testing this hypothesis. The results presented in this study provide the first indication as to the biological role of MUSE7 in plants.

MUSE7 was isolated from a screen for enhancers of the autoimmune mutant *snc1*, suggesting that it is involved in the negative regulation of plant innate immunity (Figure 4.1). Consistently, both *muse7* mutant alleles showed enhanced resistance to *P.s.t.* DC3000 (Figure 4.4). However, both mutant lines show wild-type-like resistance to *P.s.t.* DC3000 that has been modified to express only AvrRpt2, AvrRpt4, or AvrRpm1 effectors, respectively. This result indicates that the resistance in *muse7* single mutants is not predominantly mediated by RPS2, RPS4, or RPM1. The NLR protein SNC1 was found to accumulate in the *muse7 snc1* double mutant as compared to *snc1* (Figure 4.7). An examination of the accumulation of other NLR proteins in *muse7* plants would provide insight into whether the effects of MUSE7 on protein levels are specific to SNC1.

The increased level of SNC1 protein in *muse7 snc1* relative to *snc1* did not correlate with elevated *SNC1* transcription (Figure 4.7). This implies that MUSE7 likely functions to negatively regulate either the synthesis or degradation of NLR proteins. The degradation of NLR proteins via the 26S proteasome is a key regulatory step in plant immunity, and recent reports have implicated a number of proteins in this process. To determine whether MUSE7 is also part of the 26S proteasome-mediated degradation pathway, co-immunoprecipitation assays were performed to ascertain whether it interacts with known components of this process. However, no interaction was detected between MUSE7 and CPR1, HSP90.3, or SNC1 (Figure 4.8). This is not an exhaustive list of the components that function in proteasome-mediated NLR protein degradation, and it is possible that MUSE7 interacts specifically and exclusively with untested protein(s) (e.g. SGT1, SRFR1, CUL1, etc.). Further experimentation is required to definitively rule out this possibility. It is also conceivable that MUSE7 may contribute to the degradation of

NLR proteins through a novel, uncharacterized proteasome-independent pathway, although this is unlikely and would be difficult to verify.

A more likely prospect is that MUSE7 functions to suppress NLR protein biosynthesis. In a transcriptional analysis of leaf-expressed genes in Arabidopsis, the expression profile of *MUSE7* was found to correlate closely with a number of genes involved in protein biosynthesis (Street et al. 2008). Also of note, human PDAP1 was identified as a candidate RNA-binding protein as part of a large-scale quantitative proteomics analysis (Baltz et al. 2012). It is conceivable that MUSE7 binds to mRNA transcripts and affects translation by the protein biosynthesis machinery. Future experiments assaying whether MUSE7 interacts with ribosomal proteins will be useful in testing this hypothesis. If MUSE7 is found to function as a regulator of protein synthesis, two models for its function are conceivable: (i) MUSE7 binds to transcripts encoding NLRs and represses their ability to be translated, or (ii) MUSE7 binds to non-NLR-encoding transcripts and actively recruits the protein synthesis machinery, such that these other transcripts are preferentially translated over NLR-encoding mRNAs. RNA immunoprecipitation and sequencing (RIP-seq) could be performed to determine if MUSE7 has RNA-binding activity, and if so, which sequences are bound.

Overall, our results show that we have identified a novel protein involved in plant immunity. This protein negatively regulates NLR protein levels, either in terms of protein biosynthesis or as part of an uncharacterized degradation pathway. The work presented here furthers our understanding of the regulation of NLR homeostasis and may serve to inform studies in other species, as the MUSE7 homologs across eukaryotes have not been well-characterized.

4.5 Methods and materials

4.5.1 Plant growth conditions and mutant isolation

Plants were grown either on soil or on half-strength Murashige and Skoog (MS) media supplemented with 1% sucrose and 0.3% phytagel. Plants were grown under long day conditions (16 h light/8 h dark) at 22ºC in climate-controlled chambers unless short day conditions are indicated, in which case 12 h light/12 h dark settings were used.

4.5.2 Positional cloning

The *muse7 mos4 snc1* triple mutant, which was generated in the Col-0 background, was crossed with wild-type Landsberg *erecta* (L*er*). Approximately 300 F2 seeds were planted and DNA was extracted from 24 mutant plants displaying the original triple mutant morphology. Insertion/deletion DNA markers were generated using the Monsanto Arabidopsis polymorphisms and Landsberg sequence collections (Jander et al. 2002) obtained from The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). These markers were used to search for genomic regions showing a strong linkage with the Col-0 genotype. Once linkage was established, approximately 500 F3 plants segregating for the *muse7* mutation but homozygous for *mos4* and *snc1* were used for fine mapping. After narrowing down the location of the mutation to between 18.6 and 18.7 MB on chromosome 5, genomic DNA extracted from plants homozygous Col-0 for the region containing the *muse7* mutation was analyzed using the Illumina sequencing platform.

4.5.3 Total RNA extraction and analysis

Approximately 0.1 g tissue was collected from 2-week-old seedlings grown on ½ MS. Total RNA was extracted using the Totally RNA Kit (Invitrogen), and 0.4 µg RNA was reverse transcribed to cDNA using the Reverse Transcriptase M-MLV (Takara). Primers used for amplification of *SNC1* and *ACTIN7* were previously described (Zhang *et al.*, 2003; Xu *et al.*, 2012).

4.5.4 Infection assays

Spray inoculation of *H.a*. Noco2 was performed using a solution with a concentration of 10^5 spores per mL of water. Seedlings were grown for 10 d prior to inoculation, and afterwards were grown at 18ºC in a growth chamber with ~80% humidity and a 12 h light/12 h dark cycle. After 7 d, five plants were shaken in 1 mL of water and a hemocytometer was used to quantify spore growth. Three trials were performed, and for each trial five replicates were included. For bacterial infections, a needle-less syringe was used to infiltrate the leaves of 4-week-old soil-grown plants. Bacterial suspensions (OD_{600}=0.001) in 10 mM $MgCl_2$ were used. Leaf discs of uniform size were harvested from infected leaves at day 0 and day 3 and were ground in 10 mM $MgCl_2$ (200µL on day 0; 500µL on day 3), and serial dilutions were performed and plated on LB media with streptomycin selection. Colony forming units were quantified after incubating plates at 28ºC for 24 h.

4.5.5 Preparation of transgene constructs and plant transformation

The full length *At5g46020* genomic sequence, including 952 bp upstream of the start codon, was amplified via PCR, cloned into the pCambia1305 vector (versions both with and without a C-terminal GFP tag were used), and transformed into *muse7 mos4 snc1* plants using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected for on ½ MS plates containing 50 mg/mL hygromycin. For transient protein expression in *Nicotiana benthamiana*, the coding sequence of MUSE7 was cloned into a modified pCambia1305 vector containing a 35S promoter region and a C-terminal FLAG tag. For transformation of 3-week-old *N. benthamiana*, construct-containing *Agrobacterium* was cultured overnight at 28°C in liquid LB media containing 50 µg/mL kanamycin. The overnight culture was then diluted (1:50) in resuspension media [10.5 g/L K2HPO4, 4.5 g/L KH2PO4, 0.5 g/L sodium citrate, 1.0 g/L (NH4)2SO4, 0.2% glucose, 0.5% glycerol, 1 mM MgSO4, 50 µM acetosyringone and 10 mM N-morpholino-ethanesulfonic acid (MES) pH 5.6], and incubated at 28°C for another 5-6 h. The bacteria were then pelleted by centrifugation at 4,000 RPM for 10 min and resuspended in MS buffer (4.4 g/L MS, 10 mM MES, 150 µM acetosyringone). For infiltration, each bacterial strain was diluted as follows: *35S::HSP90.3-HA* (OD_{600}=0.4), *MUSE13::MUSE13-GFP* (OD_{600}=0.4), *35S::SNC1-HA* (OD_{600}=0.2), *35S::MUSE7-FLAG* (OD_{600}=0.3), and *35S::CPR1-FLAG* (OD_{600}=0.3).

4.5.6 Protein extraction and co-immunoprecipitation

For extracting total protein from Arabidopsis plants, the protocol outlined in Tsugama *et al.* 2011 was employed. Briefly, 50 mg of aerial tissue was harvested from 3-week-old soil-grown plants. Tissue was then boiled in SDS buffer [0.1M EDTA, pH 8.0; 0.12M Tris-HCL, pH 6.8; 4% w/v

SDS; 10% v/v β-mercaptoethanol; 5% v/v glycerol; 0.005% w/v bromophenol blue] for 10 min before separating samples on SDS-PAGE gels. A SNC1-specific peptide (KAKSEDEKQS) was used to generate an anti-SNC1 antibody.

Co-immunoprecipitation of proteins transiently expressed in *N. benthamiana* was performed at 4°C. Approximately 1.5 g of leaf tissue was used for both the control (leaves only transformed with the prey) and the sample (leaves transformed with both bait and prey), respectively. Tissue was ground into fine power using liquid nitrogen and a pre-chilled mortar and pestle, and 2.5 w/v extraction buffer [50 mM Tris-HCl pH 7.5; 150 mM NaCl; 10 mM EDTA; 1 mM EGTA; 0.15% Nonidet P-40 substitute; 10% glycerol; 1 mM DTT; 2 mM NaF; 1 mM $Na_2MoO_4 \cdot 2H_2O$; 2% w/v polyvinylpolypyrrolidone; 1 mM PMSF; 1× protease inhibitor cocktail] was added to each sample. Samples were centrifuged (14,000 RPM, 10 min) and the supernatant was collected. Proteins were immunoprecipitated using 30 µL anti-FLAG M2 beads (Sigma; Cat. #A2220); samples were incubated for 3 h at 4°C. Beads were then washed three times using wash buffer [50 mM Tris-HCl pH 7.5; 150 mM NaCl; 10 mM EDTA; 1 mM EGTA; 0.15% Nonidet P-40 substitute; 10% glycerol; 1 mM DTT; 2 mM NaF; 1 mM $Na_2MoO_4 \cdot 2H_2O$; 1 mM PMSF; 1× protease inhibitor cocktail]. Beads were next incubated with 3XFLAG peptide for 1 h at 4°C, then centrifuged (4,000 RPM, 2 min). The supernatant was combined with 2X SDS loading buffer, and samples were incubated at 95°C for 10 min before separation on 10% SDS-PAGE gels.

Chapter 5: Final perspectives

5.1 Overview

An important layer of the plant immune system is constituted by a suite of intracellular NOD-like receptor (NLR) proteins that recognize pathogenic effector molecules and subsequently initiate signaling cascades that lead to the induction of defense responses (Li et al. 2015). While the outputs of immune signaling are fairly well-characterized, the means by which these signaling pathways are adjusted and controlled are less defined. By using the autoimmune gain-of-function mutant *snc1* (*suppressor of npr1, constitutive 1*) (Li et al. 2001; Zhang et al. 2003), the previously reported MODIFIER OF SNC1 (MOS) screen was able to successfully isolate a number of novel positive regulators of plant immunity. The MOS proteins were shown to regulate SNC1-mediated immunity via epigenetic regulation (MOS1 [Li et al. 2010; Li et al. 2011]; MOS9 [Xia et al. 2013]), transcriptional repression (MOS10 [Zhu et al. 2010]), RNA processing (MOS2 [Zhang et al. 2005]; MOS4 [Palma et al. 2007]; MOS12 [Xu et al. 2012]), mRNA export (MOS3 [Zhang & Li 2005]; MOS14 [Xu et al. 2011]), nucleocytoplasmic protein trafficking (MOS6 [Palma et al. 2005]; MOS7 [Cheng et al. 2009]; MOS11 [Germain et al. 2010]), and post-translational protein modifications (MOS5 [Goritschnig et al. 2007]; MOS8 [Goritschnig et al. 2008]).

Based on the success of the MOS screen, a MUTANT, *snc1*-ENHANCING (MUSE) screen was conducted to identify negative regulators of innate immunity. Recently, the characterizations of several *MUSE* genes have been published. The encoded proteins have demonstrated roles in NLR protein turnover (MUSE3 [Huang et al. 2014]; MUSE6 [Xu et al.

2015]; MUSE10 and MUSE12 [HSP90s; Huang et al. 2014]; MUSE13 and MUSE14 [Huang et al. 2016]), as well as regulating the production of reactive oxygen species (MUSE5 [Huang et al. 2013]). Together, these reports clearly demonstrate that the strict control of NLR protein levels is crucial in the modulation of immune responses; however, the negative regulatory mechanisms underlying the plant immune system are expected to extend to other biological processes as well. The overarching objective of the research that constitutes this thesis was to identify novel regulators of NLR-mediated immune signaling, with the ultimate goal of furthering the collective understanding of the molecular mechanisms that control the timing and amplitude of immune response activation. For this thesis research, three uncharacterized *muse* mutant lines were selected for further study. The molecular lesions responsible for the observed mutant phenotypes were cloned, and the roles of these three genes in regulating immunity were examined. Based on the experimental findings described in this dissertation, the three MUSE proteins (MUSE4, MUSE7, and MUSE9) can be incorporated into the SNC1 regulatory model (Figure 5.1). The studies reported here have provided new insights into three different regulatory steps that are essential for immune response modulation.

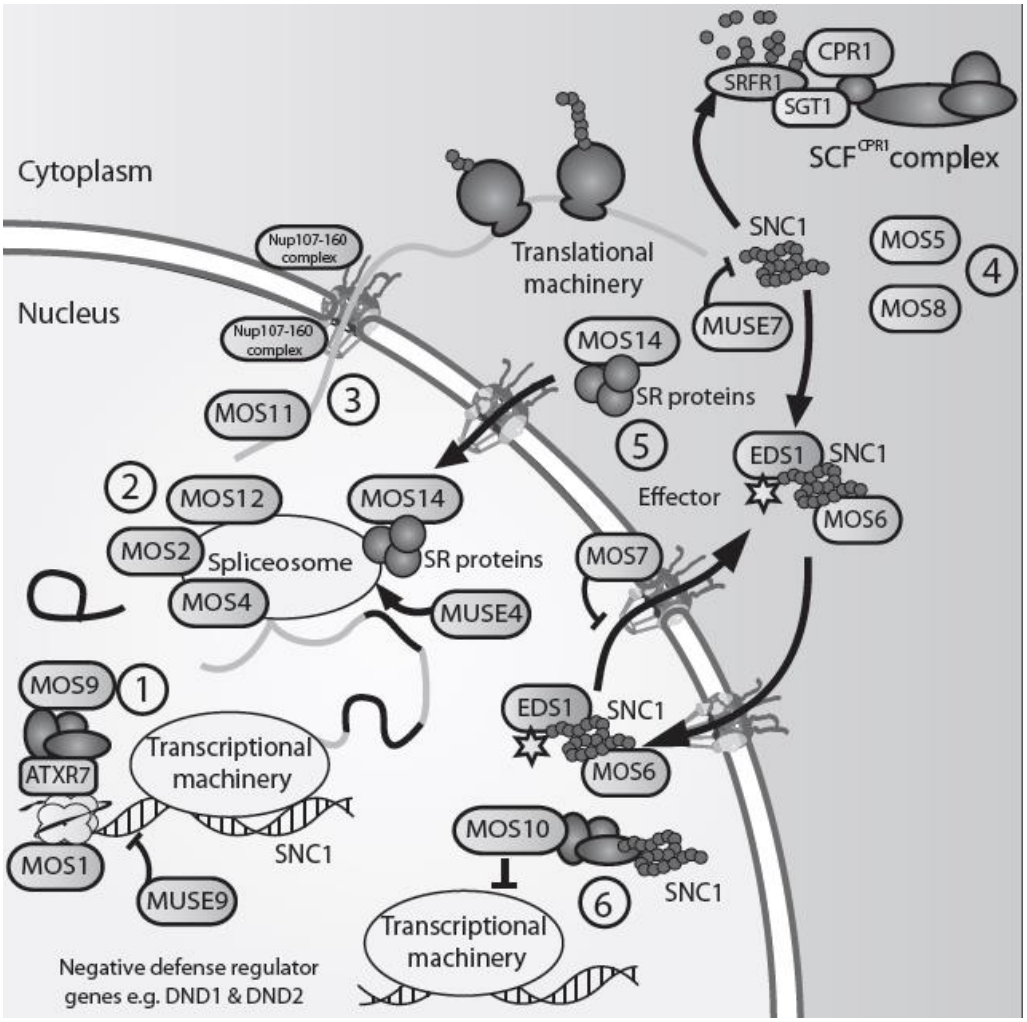

Figure 5.1. A modified model depicting the involvement of the MOS proteins and three MUSE proteins in NLR protein-mediated defense signaling pathways in Arabidopsis, using SNC1 as an example of the journey of TNL proteins.

1. At chromosomal level, MOS1, ATXR7 and MOS9 up-regulate the transcription of *SNC1* through chromatin remodeling, while MUSE9 (SPLAYED) negatively affects *SNC1* transcription. **2.** MOS2, MOS4, MOS12, and MUSE4 (NRPC7; an RNA Polymerase III subunit) are required for the proper splicing of the transcripts of *SNC1*. **3.** The Nup107-160 complex and MOS11 play key roles in the export of total mRNA (including mature mRNA of *SNC1*), which is required for effective defense. **4.** MOS5 is an E1 ubiquitin-activating enzyme, an essential component of the ubiquitination cascade, required for the regulation of defense signaling components. As an example, the SCF^{CPR1} E3 ubiquitin ligase complex targets SNC1 for degradation which prevents autoimmunity caused by over-accumulation of NLR proteins. MOS8 positively regulates plant defense, possibly through prenylation that affects the targeting of defense regulators. MUSE7 negatively regulates SNC1 protein accumulation. **5.** MOS6 and MOS7 are involved in the nucleocytoplasmic shuttling of defense signaling molecules such as SNC1, EDS1, and NPR1. Like with RPS4, EDS1 is probably required for the nuclear localization and activation of SNC1 upon the recognition of its corresponding effector

(Bhattacharjee et al. 2011; Heidrich et al. 2011). MOS14 is required for the nuclear import of splicing factors that may affect defense regulator RNA processing. **6.** MOS10 activates the SNC1-mediated defense through transcriptional repression of negative regulators of defense such as *DND1* and *DND2*.

5.2 Immunoregulatory mechanisms examined in this thesis

5.2.1 Chromatin architecture and transcriptional modulation

5.2.1.1 Findings from the MUSE9/SPLAYED study

The *muse9* mutation enhances all examined *snc1*-associated phenotypes in the *snc1 mos4* genetic background (Figure 2.1). The mutant was found to possess a point mutation in *At2G28290* (Figure 2.2), which encodes the SWI/SNF chromatin remodeler SPLAYED (SYD). While the *muse9*/*syd-10* single mutant did not display enhanced disease resistance against virulent pathogens, plants homozygous for the *syd-4* mutant allele were significantly more resistant to the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* (*P.s.m.*) ES4326 (Figure 2.4B). Both alleles are associated with the enhanced expression of *PATHOGENESIS-RELATED* (*PR*) genes and *SNC1* but not *RPP4*, which is an NLR-encoding gene located proximal to *SNC1* (Figure 2.4A, C).

As chromatin remodelers are known to affect DNA methylation profiles, which in turn can affect gene expression, the methylation status surrounding the *SNC1* locus was examined in the *syd-4* mutant (Figure 2.7). While an overall decrease in CHH methylation was observed in the mutant background, analyses of global methylation mutants indicated that a decrease in methylation around the *SNC1* locus does not always correspond with a decrease in *SNC1*

expression. This indicates that the increased *SNC1* expression observed in the *syd* mutants is unlikely to be a result of the observed changes in methylation status at this locus.

SYD was previously characterized as a positive regulator of jasmonate- and ethylene-mediated defense responses against necrotrophic pathogens (Walley et al. 2008). The study of *muse9*/*syd-10* demonstrated a novel role for SYD in negatively regulating salicylic acid-mediated defense responses via modulation of *SNC1* transcription. Therefore, it seems that SYD functions antagonistically to MOS1 and MOS9 in regulating transcription at this locus. This underscores the importance of fine-tuned transcriptional control of NLR protein-encoding loci in mediating immune responses.

5.2.1.2 Future directions

Studies of SYD are limited by the large size of the gene (16,870 bp), which makes its cloning into a binary vector a difficult task and thus precludes most biochemical analyses. To date, none of the laboratories that work with this gene have overcome this obstacle. Further studies are required to more thoroughly examine the immunoregulatory role played by SYD. Specifically of interest is whether SYD directly represses transcription of *SNC1*, perhaps through interactions with other transcriptional activators or repressors that act at this locus, or whether the effect is more indirect (for example, SYD may regulate the transcription of a gene encoding a protein that in turn affects *SNC1* transcription). Chromatin immunoprecipitation (ChIP) assays may be performed to determine whether SYD directly binds the *SNC1* locus; this would require the use of a SYD-specific antibody which has been developed by the Wagner research group (Walley et al. 2008). Alternatively, if SYD was not found to associate with the SNC1 locus, a large-scale ChIP-sequencing approach could be employed. Using this technique, all DNA fragments bound

by SYD would be sequenced. This may yield a large number of results, as SYD is known to bind multiple loci (Kwon et al. 2005; Walley et al. 2008). After developing a catalogue of putative SYD-bound loci, single and double mutant analyses using candidate genes together with the *snc1* mutant could be employed to determine if the candidate genes affect *SNC1* transcription.

5.2.2 Alternative splicing of genes encoding NLR proteins

5.2.2.1 Findings from the MUSE4/NRPC7 study

The *muse4* mutant allele confers an enhancement of *snc1*-like phenotypes in the *mos4 snc1* background (Figure 3.1). The mutation responsible for these observed phenotypes is located in an intron/exon splice site junction in *AT1G06790*, which encodes the RNA Polymerase (Pol) III subunit NRPC7 (Figure 3.2). The *muse4*/*nrpc7-1* mutation results in the retention of an intron in some (but not all) transcripts produced from this locus (Figure 3.4), and is likely a partial loss-of-function allele as all previously reported Pol I, II, and III mutants are lethal.

The *nrpc7-1* mutation was likely isolated in our screen for *snc1* enhancers due to the pleiotropic phenotypes of the mutant. The single mutant does not display increased *SNC1* expression or protein accumulation (Figure 3.5), nor does it display enhanced resistance to virulent pathogens (Figure 3.6). However, the *nrpc7-1 mos4 snc1* triple mutant shows an alteration in the alternative splicing patterns of both *SNC1* and *RPP4* (Figure 3.6). This may be due to the cumulative splicing defects associated with mutations in *nrpc7-1* and *mos4*: NRPC7 is required for the transcription of the spliceosome component U6 (Figure 3.6), and MOS4 is an important component of a spliceosome-associated complex (Johnson et al. 2011).

Alternative splicing of the genes encoding NLR proteins has previously been shown to affect their function and is posited to be a means of honing immune responses (Jordan et al. 2002). The characterization of *nrpc7-1* supports this hypothesis.

5.2.2.2 Future directions

The defects associated with *nrpc7-1* are broad, and the immune phenotypes observed in *nrpc7-1 mos4 snc1* are likely a result of broad changes in transcription and splicing. As such, the results of this study do not suggest that Pol III plays a direct, targeted role in immune regulation, thus the applications of the *nrpc7-1* mutant to examining plant immunity are limited.

By virtue of being the first reported viable Pol III mutant, *nrpc7-1* is likely to be of interest to researchers in the fields of transcription and RNA biology. For example, future studies could focus on using this mutant to examine the biogenesis of weakly characterized small RNAs. Although the suites of RNA molecules transcribed by the various Pols are largely known, there are some RNA molecules for which the biogenesis pathways are unclear. By looking at the accumulation of these RNAs in the *nrpc7-1* background, some insight may be gained as to whether their transcription requires fully functional Pol III.

5.2.3 NLR protein accumulation

5.2.3.1 Findings from the MUSE7 study

Similar to what was observed for *muse9* and *muse4*, the *muse7* mutation enhances *snc1*-like morphology and resistance in the *mos4 snc1* genetic background (Figure 4.1). The corresponding molecular lesion is located in *AT5G46020*, which encodes a protein of unknown function (Figure

4.2). The annotation of this gene in TAIR (arabidopsis.org) states that it contains a casein kinase substrate phosphoprotein PP28 domain; this is based on its homology to a protein studied in *Rattus norvegicus* that is phosphorylated by casein kinase II (Shen et al. 1996). *MUSE7* is conserved in higher plants and does not have any close homologs in the Arabidopsis thaliana genome (Figure 4.3).

While mutations in *muse7* do not result in an elevation of *SNC1* transcription, they do cause an increase in SNC1 protein levels (Figure 4.7). This indicates that MUSE7 either (i) contributes to the targeted degradation of NLR proteins, or (ii) negatively regulates their biosynthesis. In recent years, proteasome-mediated degradation of NLR proteins has emerged as a critical component of immune response regulation (Cheng et al. 2011; Huang et al. 2014a; Huang et al. 2014b; Huang et al. 2016). No interaction was detected between MUSE7 and CPR1 or HSP90.3, both of which are known regulators of NLR protein turnover (Figure 4.8). There was also no detectable interaction between MUSE7 and SNC1. These data, when taken together with a recent report that revealed that MUSE7 co-expresses with a number of protein biosynthesis genes (Street et al. 2008), indicate that MUSE7 may regulate SNC1 protein synthesis rather than degradation.

The study of *MUSE7* has resulted in the identification of a novel regulator of NLR protein accumulation, and is the most thorough characterization of this gene in any species. However, the lack of functional domains in the MUSE7 protein has made analyses of this protein somewhat difficult.

5.2.3.2 Future directions

Many potential avenues of future MUSE7 research exist. It will be important to determine whether MUSE7 affects the accumulation of NLR proteins other than SNC1, which can be done by examining the expression of tagged NLR proteins in the *muse7* background as compared to wild type. It will also be of interest to determine whether MUSE7 is phosphorylated and if so whether this phosphorylation affects its function in immune regulation. To examine these possibilities, phosphomimetic mutant versions of the MUSE7 protein can be generated and their resistance phenotypes can be assayed. To investigate whether the phosphorylation status of MUSE7 is altered during infection, a band mobility shift assay could be employed.

Importantly, future studies of MUSE7 should focus on identifying interactor protein(s) in order to clarify the functional role this protein plays in regulating NLR protein accumulation. Co-immunoprecipitation assays in *N. benthamiana* may be used to determine whether MUSE7 interacts with ribosomal proteins, as might be expected if MUSE7 does impact NLR protein synthesis. If no interactions are detected using a candidate-based approach, an immunoprecipitation/mass spectrometry (IP/MS) method may be employed. However, a potential disadvantage of this technique is the relatively high rate of false positives. Also, MUSE7 is expressed at a low level, and previous IP/MS attempts in our laboratory using weakly expressed proteins have been unsuccessful. A yeast two-hybrid screen may be an effective tool in identifying interacting proteins; however, MUSE7 has already been screened against a library of 8000 cDNAs, and no interactions were detected. Additionally, no candidate interactors are currently listed in the *Arabidopsis thaliana* CCSB Interactome Database (interactome.dfci.harvard.edu/A_thaliana/). Identifying interacting proteins will likely continue to be a challenge in future studies of this protein.

5.3 Summary

Together, these studies further our understanding of the regulatory mechanisms that underpin the plant immune system and provide a foundation upon which future research endeavors may be constructed. They also clearly demonstrate the value of a well-designed forward genetic screen in uncovering novel and unanticipated mutant alleles which may provide unique perspectives on plant biology.

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