Identification and characterization of two enhancers of the *suppressor of npr1, constitutive 1* (*snc1*)

by

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Abstract

Out of necessity, plants have evolved robust, multi-layered defence responses to combat pathogen attack. Resistance (R) protein-mediated defence is one such layer that provides resistance to pathogens that have successfully overcome plant cell surface defences. R proteins, such as SNC1 (SUPPRESSOR OF NPR1, CONSTITUTIVE 1), recognize pathogenic molecules within the plant cell and initiate down-stream defence responses. A gain-of-function mutation in SNC1, *snc1*, results in constitutive defence responses, dwarf stature and dark green, deformed leaves. Using the *snc1* mutant, many *MODIFIER OF SNC1* (*MOS*) genes were found through a *snc1* suppressor screen, which identified mutants based on increased size and decreased resistance responses. The wild-type-like phenotype of *mos snc1* mutants, allowed another screen to identify enhancers of *snc1* termed *MUTANT, SNC1 ENHANCING* (*MUSE*) genes. Here I describe the discovery of *snc1-4d*, an allele of *snc1,* and *muse8*, a partial loss-of-function mutant of *AtCDC48A*. *snc1-4d* is a unique allele of *snc1* in that it possesses a mutation in the Nucleotide-Binding (NB) region of this R-like protein. The enhanced *snc1* phenotype observed in this mutant is thought to be due to an increase in SNC1 activity as western blot analysis revealed no increase in *snc1* protein level. This novel allele of *SNC1* provides a unique opportunity to investigate the nature of the NB region of R proteins and how it may contribute to R protein activation. The partial loss-of-function allele of *AtCDC48A, muse8*, is also distinctive, as unlike previously characterized alleles, it remains viable when homozygous. This novel allele will be instrumental in the study of AtCDC48A's many functions and provides the first evidence of AtCDC48's involvement in plant innate immunity.

List of publications

Virginia Woloshen, Shuai Huang and Xin Li (2011) RNA-binding proteins in plant immunity. *Journal of Pathogens* Article ID: 278697.

A portion of this article has been incorporated into Chapter 1 of this thesis.

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ATP – Adenosine Tri-Phosphate

Avr – Avirulence

BAK1 – BRASSINOSTEROID ASSOCIATED KINASE 1

BAP1 – BON1 ASSOCIATED PROTEIN 1

BIR1 – BAK1 INTERACTING RECEPTOR-LIKE KINASE 1

BON1 – BONZAI 1

CC – Coiled Coil

CDC – CELL DIVISION CYCLE

Cf – CLADOSPORIUM FULVUM RESISTANT

CIP29 – CYTOKINE INDUCIBLE PROTEIN 29

Col-0 – Columbia-0

CPR1 – CONSTITUTIVE EXPRESSER OF PR GENES 1

E – Glutamic Acid

EDS1 – ENHANCED DISEASE SUSCEPTIBILITY 1

EFR – *EF*-TU RECEPTOR

EMS – Ethyl Methanesulfonate

ERAD – Endoplasmic-Reticulum-Associated Degradation

ETI – Effector-Triggered Immunity

ETS – Effector-Triggered Susceptibility

flg22 – flagellin conserved domain 22

FLS2 – FLAGELLIN SENSITIVE 2

GPKOW/T54 – human MOS2 homolog

GUS – beta-Glucuronidase

H.a. Noco2 – *Hyaloperonospora arabidopsidis* Noco2

HR – Hypersensitive Response

K - Lysine

L*er* – Landsberg *erecta*

MAC – MOS4 ASSOCIATED COMPLEX

MAPK – MAP-KINASE

MKP1 – MAP KINASE PHOSPHATASE 1

MLA10 – MILDEW A 10

MOS – MODIFIER OF SNC1

MUSE – MUTANT SNC1 ENHANCING

N - NICOTIANA

N terminal – amino terminal

NAHG – NAPHTHALENE/SALICYLATE HYDROXYLASE G

NB-LRR – Nucleotide-Binding Leucine Rich Repeat

NDR1 – NON-RACE SPECIFIC DISEASE RESISTANCE 1

NIM1 – NONINDUCIBLE IMMUNITY 1

NOD – Nucleotide-binding Oligomerization Domain

Npl4 – NUCLEAR PROTEIN LOCALIZATION 4

NPR1 – NONEXPRESOR OF PATHOGENESIS RELATED 1

Nup – NUCLEAR PORE PROTEIN

PAD4 – PHYTOALEXIN DEFICIENT 4

PAMP – Pathogen Associated Molecular Pattern

PKA – PROTEIN KINASE A

PR – PATHOGENESIS RELATED

Prf – PSEUDOMONAS RESISTANCE AND FENTHION SENSITIVITY

PRR – Pathogen Recognition Receptors

PRL1 – PLEIOTROPIC REGULATORY LOCUS 1

P.s.m. – Pseudomonas syringae pv. *maculicola*

PTI – PAMP-TRIGGERED IMMUNITY

R proteins – Resistance proteins

RAD23 – RADIATION SENSITIVE 23

RIN4 – RPM1 INTERACTING PROTEIN 4

RLK – Receptor-Like Kinase

RPM1 – RESISTANCE TO *PSEUDOMONAS SYRINGAE PV. MACULICOLA*

RPS4 – RESISTANCE TO *PSEUDOMONAS SYRINGAE*

SA – Salicylic Acid

SAG101 – SENESCENCE ASSOCIATED GENE 101

SAI – SA INSENSITIVE

SAR – Systemic Acquired Resistance

SCF – SKP1-CULLIN-F-BOX

SGT1 – SUPPRESSOR OF THE G2 ALLELE OF SKP1

SNC1 – SUPPRESSOR OF NPR1, CONSTITUTIVE 1

SRFR1 – SUPPRESSOR OF rps4-RLD)

STAND – Signal Transduction ATPases with Numerous Domains

T3SS – Type 3 Secretion System

T4SS – Type 4 Secretion System

TIR – Toll/Interleukin-1 Receptor

Ub – Ubiquitin

UBA – Ubiquitin Associating

UFD – UBIQUITIN FUSION DEGRADATION

VCP – VALOSIN CONTAINING PROTEIN

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Dedication

for My Family

1 Introduction to plant innate immunity[1]

1.1 Summary

Each cell in a plant is capable of mounting a defence in response to pathogen attack. Recognition of pathogens at the plant cell surface elicits a signalling cascade that leads to expression of defence genes. However, some pathogens have evolved mechanisms to circumvent this initial response through the injection of effector molecules that inhibit such pathways. Plants, in turn, have evolved Resistance (R) proteins that act as internal surveillance systems that recognize these effectors and again initiate a down-stream defence response. In this chapter I will discuss our current understanding of the plant innate immune system, primarily focused on R protein mediated defence. I also discuss a specific R-like protein SNC1 and its regulation in plant innate immunity.

1.2 Plant innate immunity

The robust immune system in plants has evolved out of necessity due to their sessile nature. The first barrier a pathogen must overcome is the plant surface. This is comprised of rigid cell walls, epicuticular waxes, and trichomes that serve as deterrents and prevent some pathogens from entering the cell. Some pathogens can circumvent these primary defences; however, they then must overcome plant innate immunity present in each plant cell. Conserved features of pathogens or Pathogen Associated Molecular Patterns (PAMPs) such as chitin or flagellin, of fungi and bacteria respectively, are recognized by Pathogen Recognition Receptors (PRRs), transmembrane proteins present on the plant cell surface (Jones and Dangl, 2006). Many PRRs are of the Receptor-Like Kinase (RLK) variety, they possess a transmembrane domain, a

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cytoplasmic Serine/Threonine protein kinase domain and variable amino (N) terminal domains on the cell surface that confer PAMP specificity. FLS2 (FLAGELLIN SENSITIVE 2), for example, recognizes flagellin from bacteria and can also be induced by flg22 alone, the conserved domain found in flagellin (Felix et al., 1999; Gómez-Gómez and Boller, 2000). EFR (*EF*-TU RECEPTOR), another PRR, recognizes the bacterial PAMP EF-Tu (Kunze et al., 2004; Zipfel et al., 2006). Subsequent to pathogen detection, map-kinase (MAPK) signalling cascades lead to defence responses, such as callose deposition at the cell walls, stomata closure, production of reactive oxygen species and other defence compounds, such as salicylic acid (SA) and transcription of *PATHOGENESIS RELATED* (*PR*) genes (Nürnberger et al., 2004). This initial response is termed PAMP-Triggered Immunity or PTI (Jones and Dangl, 2006).

Successful pathogens have evolved effector molecules, which are secreted by the pathogen into the apoplast to overcome PTI. In some cases effectors can be released directly into the plant cell. Bacterial pathogens often use the Type Three Secretion System (T3SS) or the Type Four Secretion System (T4SS) to accomplish this. The T3SS is a syringe-like structure encoded by the bacteria, which is assembled on the surface of the plant and subsequently injects effectors into the hosts cytoplasm (Jin and He, 2001). The T4SS delivers effectors through bacterial membrane-associated proteins (Backert and Meyer, 2006; Cascales and Christie, 2003). Nematodes employ the use of a stylet, a different syringe-like structure that injects effectors into the host (Grundler et al., 1998). Effectors of oomycetes and fungi, on the other hand, contain translocation signals and are recognized by receptor proteins on the plant cell surface, or directly bind the plant cell surface, and enter the plant cell through endocytosis (Whisson et al., 2007). Once inside the plant cell, these effectors serve to inhibit PTI (Jones and Dangl, 2006).

Inhibition of PTI via effectors can occur in a multitude of ways. AvrPtoB of *Pseudomonas syringae* (*P.s.*), for example, is similar to plant E3 ligases and possesses E3 ligase activity (Abramovitch et al., 2006; Janjusevic et al., 2006). As an E3 ligase it is hypothesized that AvrPtoB targets positive regulators of resistance for degradation. Another *P.s.* effector AvrPto has been shown to prevent the phosphorylation of FLS2 and EFR by binding their respective phosphorylation sites thus preventing downstream signalling cascades (Xiang et al., 2008). Both AvrPto and AvrPtoB target BAK1 (BRASSINOSTEROID ASSOCIATED KINASE 1), the co-receptor to various PRRs. They also inhibit chitin signalling, which is not associated with BAK1 function, suggesting multiple targets for these effectors (Shan et al., 2008). MAPKs can also be directly targeted by effector molecules, for example, Hop1A of *P.s.* targets both MPK3 and MPK6 (Zhang et al., 2007). Some effectors can mimic natural plant hormones that inhibit the defence response. For example, various pathovars of *P.s.* produce coronatine, an analog of jasmonic acid. The plant perceives this chemical as jasmonic acid and uses the jasmonic acid wound pathway to halt the production of SA (Zhao et al., 2003; Brooks et al., 2005). Introduction of coronatine to the host plant also leads to stomata opening, facilitating the entry of the bacteria (Melotto et al., 2006). Therefore PTI can be inhibited at various steps in the signalling cascade and ultimately lead to Effector-Triggered Susceptibility (ETS) (Jones and Dangl, 2006).

Plants have in turn developed mechanisms to combat ETS through Resistance (R) proteins that recognize pathogen effectors and initiate downstream defence responses. The most abundant R proteins belong to the NB-LRR cohort named for their nucleotide-binding (NB) and leucine-rich-repeat (LRR) motifs. They are similar to mammalian proteins used in inflammatory and immune responses termed NOD (Nucleotide-binding Oligomerization Domain) LRRs

(Inohara et al., 2005). The NB domain contains motifs reminiscent of Signal Transduction ATPases with Numerous Domains (STAND), which are often used in activation (Tameling et al., 2002). The LRR domain is involved in the regulation of intramolecular interactions and recognition specificity (Moffett et al., 2002; Dodds et al., 2001). In plants there are two main categories of NB-LRR R-proteins based on their N-terminal domain. TIR (Toll/Interleukin-1 Receptor) NB-LRR and CC (coiled-coil) NB-LRRs, which are both involved in the defence response following effector recognition; however, they have distinct downstream signalling pathways. CC-NB-LRRs use the NON-RACE SPECIFIC DISEASE RESISTANCE 1 (NDR1) pathway following effector recognition (Aarts et al., 1998). TIR-NB-LRRs, on the other hand, use the ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) paired with either PHYTOALEXIN DEFICIENT 4 (PAD4) or SENESCENCE ASSOCIATED GENE 101 (SAG101) (Aarts et al., 1998; Feys et al., 2005).

Effectors that are recognized by R proteins are termed Avirulence (Avr) proteins. NB-LRR R proteins recognize the presence of effectors in various ways. One widely accepted hypothesis is the guard hypothesis in which the R protein senses the effector indirectly by monitoring an endogenous plant protein that is targeted by the effector (Van der Biezen and Jones, 1998). For example, RPM1 (RESISTANCE TO *PSEUDOMONAS SYRINGAE* PV. *MACULICOLA*) is a CC-NB-LRR R protein that interacts with RIN4 (RPM1 INTERACTING PROTEIN 4), a protein that localizes to the plasma membrane. Various Avr proteins target RIN4. AvrRPM1 and AvrB are examples of effectors secreted by *P.s.* using the T3SS. Upon release into the host plant cell, these Avr proteins are acetylated and targeted to the plasma membrane (Nimchuk et al., 2000). Effectors then interact with RIN4, which is subsequently phosphorylated (Boyes et al., 1998; Mackey et al., 2002). RPM1 also localizes to the plasma

membrane and has been found to interact directly with RIN4 (Boyes et al., 1998). Upon RIN4 phosphorylation RPM1 is activated and leads to downstream defence responses. Another Avr protein that targets RIN4 is AvrRpt2. This effector molecule cleaves the C-terminal of RIN4 targeting it for protein degradation (Kim et al., 2005). The absence of RIN4 at the plasma membrane is sensed by RESISTANCE TO PSEUDOMONAS SYRINGAE 2 (RPS2), another R protein that is indirectly activated by pathogen effectors and initiates a defence response (Axtell and Staskawicz, 2003).

R protein mediated defence responses are characterized by a type of programmed cell death termed the Hypersensitive Response (HR). HR begins by a sudden burst of reactive oxygen species. These reactive oxygen species can interact with other molecules/organelles within the cell, which can induce their degradation (Berlett and Stadtman, 1997; Foyer and Noctor, 2005; Breusegem and Dat, 2006). The accumulation of SA is yet another response. This has been shown to bind catalases, which convert reactive oxygen species such as H_2O_2 to harmless forms like H_2O and O_2, and prevent their action (Wendehenne et al., 1998; Conrath et al., 1995). SA also induces certain MAPKs involved in plant innate immunity (Zhang and Klessig, 1997; Romeis et al., 1999). *PR* gene expression is also affected by elevated levels of SA. *NPR1* (*NONEXPRESSOR OF PATHOGENESIS RELATED 1*), *NIM1* (*NONINDUCIBLE IMMUNITY 1*), and *SAI* (*SA INSENSITIVE*) have been shown to require SA to induce downstream *PR* gene expression and mount a defence response (Cao et al., 1994; Glazebrook et al., 1996; Delaney et al., 1995; Shah et al., 1997). If successfully induced, this type of cell death can halt the invasion of pathogens. By killing infected cells, the plant prevents pathogen spread to neighbouring cells: the sacrifice of some to preserve the whole. Once HR has been

established, the plant attains Systemic Acquired Resistance (SAR), which increases whole plant resistance to future pathogen attacks.

The innate immunity in plants must be highly regulated. Mis-regulation can result in a decrease in plant fitness. For example, a gain-of-function mutation in the R-like protein SUPPRESSOR OF NPR1, CONSTITUTIVE 1 (SNC1), results in heightened disease resistance; however, it also results in dwarf stature and deformed leaves (Li et al., 2001; Zhang et al., 2003). Various mechanisms have been suggested for such regulation. In the cytosol, R proteins are thought to be present in their folded, inactive form (Ade et al., 2007). A CC-NB-LRR protein of potato indicated that the CC domain interacts with the NB-LRR domains in the absence of pathogens; however, upon infection these interactions cease (Moffett et al., 2002). Conversion of R proteins from the folded, inactive form to their less folded, active form is thought to be ATP-dependant. Following this conformational change, R proteins dimerize and it is this dimerization that activates downstream defence responses (Bernoux et al., 2011; Maekawa et al., 2011). In certain cases, R proteins must be shuttled to the nucleus for successful defence responses. The nuclear localization of RPS4 of *Arabidopsis*, NICOTIANA (N) of tobacco and MILDEW A 10 (MLA10) of barley is required for successful resistance to their respective pathogens (Shen et al., 2007; Burch-Smith et al., 2007; Wirthmueller et al., 2007). Lack of tight regulation can have detrimental effects that affect plant fitness, as we see in mis-regulated *snc1* (Zhang et al., 2003; Li et al., 2001).

1.3 SNC1, an R-like protein

SNC1 belongs to the TIR-NB-LRR class of R proteins; however, it is sometimes called an R-like proteins since its corresponding effector molecule or molecules have not yet been identified. It was isolated during a suppressor screen designed to identify mutants with enhanced

disease resistance (Li et al., 2001). *Arabidopsis thaliana* plants, of the Columbia (Col-0) ecotype, containing a loss-of-function mutation in *NPR1* were used as a background for this screen as they display enhanced disease susceptibility due to the absence of *PR* gene expression typically induced by functional NPR1 proteins (Cao et al., 1997). The mutation found in *snc1 npr1* plants caused a gain-of-function in that double and single mutant plants had increased and constitutive resistance to pathogens, *PR* gene expression and SA accumulation; however, unlike the wild-type R-mediated defence response, no HR was observed (Li et al., 2001). This increase in defence responses however, also causes a dwarf, dark green, curly-leaved phenotype exemplifying the consequences of mis-regulation in R-protein mediated defence responses.

Many regulators of *SNC1* have been identified (Gou and Hua, 2012; Johnson et al., 2012). BON1 (BONZAI 1) along with its functional partner BAP1 (BON1 ASSOCIATED PROTEIN 1) negatively regulates SNC1. *bon1* and *bap1* single mutants display a *snc1*-like phenotype. *SNC1* transcript was found to be significantly higher in *bon1* mutants and over-expression of this protein has been shown to exhibit auto-immune phenotypes (Li, et al., 2010b). Since BON1 and BAP1 are plasma membrane localized, they are thought to accomplish such regulation indirectly (Yang et al., 2006; Yang and Hua, 2004). Typically, SNC1 activation eventually leads to the accumulation of SA, which in turn up-regulates defence gene expression, including *SNC1* gene expression creating a feedback loop. When *bon1* mutants contain a mutation in *naphthalene/salicylate hydroxylase G* (*nahG*) or *pad4*, mutants that inhibit SA synthesis or accumulation, the increase in *SNC1* transcript is abolished (Yang and Hua, 2004). Therefore, negative regulation of SNC1 by BON1 is achieved indirectly through the SA feedback loop, which affects the transcription of *SNC1*. BON1 interacts with both BIR1 (BAK1 INTERACTING RECEPTOR-LIKE KINASE 1) and BAK1 (Wang et al., 2011). These proteins

are thought to be guarded by multiple R proteins that recognize changes in BIR1 or BAK1 and induce defence responses. Therefore, when mutated in *BAK1* or *BIR1*, mutant plants display an autoimmune phenotype. This can be partially rescued by introducing a loss-of-function mutation in *snc1*, confirming BAK1 and BIR1 are also indirect, negative regulators of SNC1 (Wang et al., 2011). Another indirect negative regulator of SNC1 is MKP1 (MAP KINASE PHOSPHATASE 1). Again, *mkp1* mutants display a *snc1*-like phenotype and can be partially rescued with the introduction of a *snc1* loss of function allele, suggesting involvement in plant innate immunity. Mutant *mkp1* phenotypes are dependent on MPK3 and MPK6, crucial components of PTI (Asai et al., 2002). The *mkp1* phenotype was also dependent on SA accumulation; however, no change in *SNC1* transcript was detectible suggesting MKP1 may negatively regulate SNC1 post-transcriptionally (Bartels et al., 2009).

CONSTITUTIVE EXPRESSOR OF PR GENES 1 (CPR1) is yet another negative regulator of SNC1. This protein is an F-box protein associated with the SCF (SKP1-CULLIN-F-BOX) protein complex, which directs target proteins to the 26S proteasome for degradation, and has been implicated in R protein regulation (Cheng et al., 2011). Similar to *bon1, bap1, bir1* and *bak1*, *cpr1* mutants display constitutive defence responses. A lack of this protein in the SCF complex prevents degradation of certain target proteins, one of which is thought to be SNC1, since *cpr1* mutants have increased SNC1 protein accumulation. Over-expression of CPR1 rescues the mutant phenotypes of the gain-of-function *snc1* mutant as well as *bon1,* and decreases the amount of SNC1 protein (Gou et al., 2012). Altogether these results indicate CPR1 is a negative regulator of SNC1, which is accomplished through SNC1 degradation by the 26S proteasome.

SNC1 accumulation is also regulated by SRFR1 (SUPPRESSOR OF rps4-RLD). Mutant *srfr1* show an increase in SNC1 protein and therefore display the same autoimmune phenotype previously discussed. However, there is no observable change in *snc1* transcript levels, therefore it is thought that SRFR1 regulates SNC1 by stabilizing the protein. Autoimmunity in *srfr1* mutants can be suppressed by loss of function mutations in *snc1* further confirming the role of SRFR1 in SNC1 regulation (Li, et al., 2010b). A similar role is proposed for SGT1 (SUPPRESSOR OF THE G2 ALLELE OF SKP1), which interacts with SRFR1, indicating that SRFR1 and SGT1 may act in a complex to regulate SNC1 stability (Li, et al., 2010b).

SNC1 is regulated at the DNA level by MOS1 (MODIFIER OF SNC1, 1). Mutant *mos1* plants were found to repress the transcription of *snc1*, the gain-of-function mutant allele, which suppressed the constitutive phenotype of *snc1*. Upon further investigation, *mos1* mutants possessed a change in DNA methylation upstream of the *SNC1* gene, indicating MOS1 is crucial for transcriptional regulation of the *SNC1* gene (Li et al., 2010a).

1.4 *Modifiers of the suppressor of npr1, constitutive 1* (*MOS*)

In an attempt to identify novel components of the SNC1-mediated defence pathway, a mutant screen was conducted using *snc1* gain-of-function mutants. This screen revealed fifteen possible *MOS* genes contributing via various ways to plant defence (Monaghan et al., 2010). MOS1, as previously discussed, was found to be involved in DNA methylation, and therefore transcription of *SNC1* (Li et al., 2010a). Following transcription, RNA must be processed prior to export into the cytoplasm and translated. MOS2 and MOS4 have both been implicated in RNA processing. Mutations in *MOS2* suppress the *snc1* phenotype, *PR* gene expression is completely abolished, as is constitutive resistance to pathogens. SA accumulation; however, is only partially suppressed, suggesting that MOS2 may be involved in the SA-independent pathway. The MOS2

protein localizes to the nucleus and contains one G-patch and two KOW domains, which function in nucleotide and protein binding. Altogether, these results suggest that MOS2 plays a role in RNA processing (Zhang et al., 2005). Recently a study was conducted using a MOS2 homolog, GPKOW/T54, from humans. This study revealed that T54 interacts *in vitro* with PROTEIN KINASE A (PKA), and confirmed the putative binding of RNA (Aksaas et al., 2011). This finding along with previous studies implicating PKA in RNA splicing, suggests that T54 also seems to be contributing to RNA splicing (Kvissel et al., 2007). Therefore, MOS2 could be an RNA-binding protein putatively involved in pre-mRNA splicing.

MOS4 is another nuclear protein involved in RNA processing. It was found to interact with several other components, transcription factor AtCDC5 (CELL DIVISION CYCLE 5), a WD-40 protein PRL1 (PLEIOTROPIC REGULATORY LOCUS 1), and E3 ubiquitin ligases MAC3A and MAC3b (MOS4-ASSOCIATED COMPLEX) (Palma et al., 2007; Monaghan et al., 2009). Mutations in all components display defects in plant defence. Together these proteins comprise the MOS4-Associated Complex (MAC). This complex is homologous to the Nineteen Complex found in humans and yeast (Ajuh et al., 2000; Ohi and Gould, 2002). This complex associates with the spliceosome and it is therefore thought that its *Arabidopsis* homolog, MAC is also involved in splicing (Hogg et al., 2010; Monaghan et al., 2009).

Subsequent to transcription and RNA splicing, mature RNA must then be transported to the cytoplasm. This is accomplished through shuttling mRNA to the nuclear envelope and exit through a nuclear pore complex. MOS3 and MOS7 both suppress the *snc1* phenotype and resistance when mutated and both localize to the nuclear envelope (Zhang and Li, 2005; Cheng et al., 2009). MOS3 is a homolog of Nup96 of human and C-Nup145p of yeast, which contribute to the Nup107-160 sub-complex involved in nuclear pore stability and export (Wiermer et al.,

2007). MOS7, on the other hand, is homologous to Nup88, which has been implicated in nuclear protein retention (Roth et al., 2003). These studies suggest MOS3 and MOS7 are associated with the nuclear pore complex and therefore, protein export required for plant innate immunity.

Shuttling of mature mRNA to nuclear pores must occur for export to take place. Recently, MOS11 was identified as an export protein with homology to the RNA binding protein CYTOKINE INDUCIBLE PROTEIN 29 (CIP29) of humans, which binds RNA (Lemieux and Bachand, 2009). *mos11* mutants have impaired resistance to pathogens in the *snc1* background and only partially suppress the *snc1* phenotype. Mutants also exhibit accumulation of polyA mRNA in the nucleus, indicating that export is impaired in *mos11* mutants (Germain et al., 2010). MOS11 is, therefore, implicated in mRNA shuttling to the nuclear envelope for export.

Nucleo-cytoplasmic trafficking also takes place in the other direction. Transcription factors and co-factors must be imported into the nucleus for function. MOS6 encodes an α-3 importin, which functions with β importins in transporting proteins with nuclear localization signals (Goldfarb et al., 2004). The suppression of *snc1* phenotypes is only partial in *mos6* mutants; this is most likely due to functional redundancy as MOS6 is strikingly similar to another importin IMPα6 (Palma et al., 2005).

Once exported, mRNA is translated and undergoes post-translational modifications to result in a fully functional protein. MOS5 and MOS8 are involved in such processes. MOS5 encodes a ubiquitin (Ub) activating (UBA) enzyme or E1 enzyme (Goritschnig et al., 2007). These enzymes activate ubiquitin, a ubiquitous protein typically used in marking proteins for degradation. Activated Ub is then transferred to an E2 enzyme, which brings the activated Ub to an E3 ligase that determines target specificity and, finally, transfers the Ub to the target (Chau et al., 1989; Hershkos et al., 1983; Reiss et al., 1989). Poly-ubiquitination results in the degradation

by the proteasome and has been shown to be important in plant defence (Cheng and Li, 2012). Mutant *mos5 snc1* plants suppress the *snc1* phenotype and impair disease resistance indicating the importance of MOS5 in innate immunity (Goritschnig et al., 2007). Another post-transcriptional modification is farnesylation. This modification is implicated in protein trafficking and membrane targeting (Resh, 2006). MOS8 was found to encode the β subunit of farnesyltransferase, which when mutated, displays enhanced susceptibility and impaired R protein mediated signalling (Goritschnig et al., 2008). Although the target of MOS8 is unknown, it exemplifies the importance of protein modification in plant innate immunity.

1.5 Concluding remarks and thesis objective

Plants and their pathogens have been co-evolving for millions of years. Successful infection of plants can be devastating not only to the plant, but also humans, who rely on plants for food, shelter, revenue and oxygen. We have already witnessed the catastrophic effects of when pathogenic disease overcomes plant immunity. The Irish potato famine caused the death of an estimated 1 million people, and the emigration of countless more in the mid-19th century. It was caused by the potato blight fungus *Phytophthora infestans*, which remains a problem to this day (Haverkort et al., 2009). Currently, we are facing new challenges. Our monoculture farming practices make our crop species more vulnerable, as one infection may destroy the entire crop due to low genetic diversity. Furthermore, with the onset of climate change, habitats of pathogens will expand. Plants that have never been exposed to certain pathogens and therefore never evolved resistance to such pathogens, will become their new hosts (Olesen and Bindi, 2002; Roos et al., 2010). It is therefore in our best interest to investigate the nature of plant innate immunity to avoid, or better respond, to future plant pathogen outbreaks. Although plant

innate immunity has been the focus of many studies, there are still considerable gaps in our knowledge.

Our lab has sought to utilize molecular genetic analyses in the *snc1* background to dissect the plant immune systmes. The objective of my thesis was to identify novel regulators in the defence against pathogens using the *snc1* background to further our understanding of the complex plant innate immune system. More specifically, I aimed to identify novel negative regulators of plant immunity using a genetic approach by identifying enhancers of *snc1*.

2 *Mutant snc1-enhancing* (*MUSE*) screen revealed a novel dominant allele of *snc1*

2.1 Summary

Each plant cell possesses a multi-layered innate immune system, which serves to protect it from pathogen attack. Some pathogens have evolved mechanisms to overcome initial plant defence responses through the injection of pathogen effector molecules into the host plant cell. However, plants have evolved R proteins, which recognize these effectors, and again mount a defence response. SNC1 is one such R protein tha,t when mis-regulated, exhibits constitutive defence responses. To elucidate the SNC1 defence pathway a genetic screen was conducted, which identified genes that, when mutated, either fully, or partially, suppress the *snc1* phenotype called *MOS* genes. *MOS2* was one such gene found during this screen that fully suppresses the dominant *snc1* phenotype. The *mos2* mutation was then used in the *snc1* background to screen for enhancers of *snc1*. During this screen, several alleles of *snc1* were found, one of which, *snc1-4d* had a mutation in the NB domain of the SNC1 protein. This allele has increased *PR* gene expression and heightened resistance to pathogens. Unlike other *snc1* alleles that enhance *snc1, snc1-4d* does not increase SNC1 protein stability. Therefore, it is thought that the increased resistance observed in this allele is due to self-activation of SNC1.

2.2 Introduction

Like humans, plants have evolved complex inducible mechanisms to combat pathogen attack. Typically, plant innate immunity begins with the recognition of PAMPs, conserved features of pathogens, by PRRs located on the plant cell surface (Zipfel, 2008). This PTI may prevent pathogenesis. However, the pathogen may be capable of circumventing this defence by releasing effector molecules into the host-plant cell, which lead to ETS allowing pathogenesis to

continue. Plants however, have evolved mechanisms to counteract ETS via R proteins, which can recognize pathogen effectors and lead to ETI (Jones and Dangl, 2006). Recognition of effectors can induce a signal transduction pathway, which leads to *PR* gene expression, accumulation of SA, production of reactive oxygen species and initiation of localized cell death or HR, which prevents the spread of pathogens to neighbouring cells (Nürnberger et al., 2004; Jones and Dangl, 2006).

Elucidating R-protein-mediated defence has been the focus of the research in our Lab for the past decade. One R-like protein studied extensively in our lab is *SNC1* gene, which was identified in the mutant *npr1* background (Zhang et al., 2003; Li et al., 2001). *NPR1* is induced during pathogen attack and essential for the SA-dependent pathway of plant defence (Cao et al., 1994). Mutant *npr1* plants are more susceptible to pathogen attack due to the inactive SA-dependent defence pathway upon infection. The point mutation found in *snc1* suppresses the *npr1* phenotype regardless of the presence of pathogens, and results in increased SA accumulation, dwarf plant stature and dark green, curly leaves. This gain-of-function mutant has become a useful resource in conducting further genetic screens due to its obvious phenotypes. *snc1* also illustrates the importance of regulating defence pathways, as a lack of regulation can result in unfavourable phenotypes, and reduce plant fitness possibly due to the reallocation of resources.

Recently, a group of fifteen genes, termed *MOS* genes, has been identified through mutant screens in our lab using *snc1* or *snc1 npr1* mutant plants (Monaghan et al., 2010). These suppressor screens were conducted in an attempt to identify genes that modify the *snc1* phenotype and play regulatory roles in ETI. Over ten of these *MOS* genes were identified by their either full, or partial, suppression of *snc1* phenotypes, and found to be involved in various

processes of the plant defence response, including epigenetic gene regulation, RNA processing, protein modification, and nucleocytoplasmic trafficking (Zhang et al., 2005; Zhang and Li, 2005; Goritschnig et al., 2007, 2008; Wiermer et al., 2007; Cheng et al., 2009; Germain et al., 2010; Palma et al., 2005).

MOS2 was identified in the *snc1 npr1* screen. The *mos2* mutation suppresses the dominant *snc1* phenotype and completely abolishes disease resistance in *mos2 snc1 npr1* mutants. Triple mutants are susceptible to both virulent pathogens, *Hyaloperonospora arabidopsidis* Noco2 (*H.a.* Noco2) and *Pseudomonas syringae* pv. *maculicola* ES4326 (*P.s.m*. ES4326). *mos2 snc1 npr1* triple mutants also restore wild-type like plant morphology. GUS staining was used to assess the level of *PR2* gene expression, which is positively associated with plant resistance against pathogens, and indicative of a functional SA-independent plant defence pathway. *PR2* expression is high in *snc1* plants; however, *mos2* abolishes *PR2* gene expression. SA accumulation was also assessed to determine if the high level of SA found in *snc1* is also suppressed in *mos2 snc1 npr1* triple mutants, and was indeed found to be suppressed; however, only partially. *MOS2* is therefore thought to be involved in the SA-independent plant defence pathway (Zhang et al., 2005). The restoration of wild-type-like phenotypes associated with the introduction of the *mos2* mutation makes *mos2 snc1 npr1* plants useful in further genetic screens to elucidate *snc1*-mediated plant defence responses and regulation.

Due to the pronounced dwarfism of *snc1*, constitutive resistance to pathogens and increased *PR* gene expression, it is difficult to identify genes that enhance *snc1*. The difference in size, resistance and *PR* gene expression would be difficult to score between *snc1* and *mutant-snc1 enhancer* (*muse*) *snc1* double mutants. Therefore, maintaining the *mos2* mutation, which suppresses *snc1* phenotypes, in the mutant background would facilitate successful identification

and characterization of *muse mos2 snc1 npr1* mutant plants. We hypothesize that the introduction of a mutation in a *muse* gene will enhance the *snc1* phenotype and effectively abolish *mos2* wild-type-like phenotypes. Therefore, *muse mos2 snc1 npr1* mutant plants should resemble *snc1* mutant plants and allow the identification and characterization of negative regulators of plant innate immunity.

2.3 Materials and methods

2.3.1 Plant growth, mutagenesis, primary and secondary screens

Plants for most experiments were grown on soil with a 16 hour light/8 hour dark cycle with a light intensity of 80 μE m^{-2} sec^{-1}. The *mos2 snc1 npr1* triple mutant was previously isolated (Zhang et al., 2005). Ethyl methanesulfonate (EMS) mutagenesis was carried out using 0.2% EMS for 18 hours on 40,000 *Arabidopsis thaliana* seeds homozygous for the *mos2, snc1,* and *npr1* mutations as well as the *BGL2-GUS* reporter gene as previously described (Bowling et al., 1994). For the first primary screen, potential mutants chosen based on their *snc1*-like morphology. The second primary screen identified potential mutants based on increased *PR2* expression visualized by GUS-staining as previously described (Zhang et al., 2003). Mutants obtained from the primary screens were subjected to a secondary screen testing resistance to the oomycete pathogen *H.a.* Noco2 as previously described (Li et al., 2001). Dwarf, *snc1*-like plants were chosen for further analysis.

2.3.2 Genetic mapping

Selected quadruple mutant plants in the Columbia-0 background were crossed with the Landsberg *erecta* (L*er*) ecotype. The F_2 generations were grown on soil and *snc1*-like but smaller than *snc1,* plants were selected for linkage analysis. Crude mapping was conducted on 24-48 plants depending on the mutant. Mutant *119* was narrowed down to a region on chromosome 4 near *snc1*. *119* DNA was Sanger sequenced by Nucleic Acid Protein Service Unit (NAPS) to determine the identity of any additional mutations in *snc1*.

2.3.3 Oomycete infection assay

snc1-4d mos2 snc1 npr1 quadruple mutants and the controls *mos2 snc1 npr1*, *snc1 npr1*, and wild-type plants were grown on soil. Seedlings were spray-inoculated onto adaxial leafe

surfaces with *Hyaloperonospora arabidopsidis* Noco2 using spray bottle containing a cell suspension of 50,000 spores/mL (Li et al., 2001). Inoculum was obtained from infected wild-type Col-0 plants. Inoculated plants were covered with a clear plastic dome and sealed. Infection was assessed through spore counting 7-14 days post-inoculation by cutting 20 randomly selected plants from each genotype and placed in 2 mL eppendorf tubes (5 plants per tube). 1 mL of ddH_2O was added and the tube vortexed to dislodge oomycete spores. Spores were counted using a hemocytometer, and by scoring the average percent spore coverage per plant of twenty randomly chosen plants from each genotype. Percent spore coverage was scored using the following: 0 spores per leaf = 0, less than 5 spores per leaf = 1, more than 5 spores per leaf = 2, less than 5 spores per 2 leaves = 3, more than 5 spores per 2 leaves = 4, and more than 5 spores per more than 2 leaves = 5.

2.3.4 Protein extraction and western blot analysis

Protein extraction and western blots were conducted by Yan Huang and Xuejin Chen. Total protein was extracted from 100mg of plant tissue from 10-14 day old plate-grown plants as previously described (Cheng et al., 2011). Briefly, collected plants were placed into 2mL tubes along with two 5mm glass beads and frozen in liquid nitrogen. Tissue was ground into a fine powder and placed on ice. Tissue was suspended in extraction buffer (100mM Tris-HCl pH8, 0.1% SDS, 2% β-mercaptoethonol) and spun for 5 mins at 5000 RPM in a 4°C microcentrifuge. The supernatant was placed in a 1.5 mL tube, boiled at 100°C for 5 mins then 4 x SDS loading buffer added. Samples were separated with SDS-PAGE and electro-blotted onto a nitrocellulose membrane. SNC1 protein was detected using an anti-SNC1 antibody followed by an anti-rabbit secondary antibody conjugated to horse radish peroxidase. The membrane was then treated with

SuperSignal West Chemiluminescent substrate (Thermo) and horse radish peroxidase substrate, then exposed to photographic film to visualize the SNC1 protein (Li, et al., 2010b).

2.3.5 Phylogenetic analysis

The SNC1 protein sequence was obtained from PubMed Protein and used to conduct a BLASTP search for homologs in *Arabidopsis thaliana.* Homologous sequences were chosen based on low e-values and the quality of the reference sequences. Protein alignment was achieved using Muscle (Edgar, 2004). The alignment sequence was then manually adjusted using Se-Al (Rambaut, 2002). Conserved amino acid residues were found using JalView (Waterhouse et al., 2009).

2.4 Results

In an attempt to find negative regulators of plant immunity, *mos2 snc1 npr1* plants were mutagenized and screened. Two primary screens based on morphology and *PR2* gene expression, as well as a secondary screen based on susceptibility to an oomycete pathogen, *H.a.* Noco2, identified over 40 putative mutants. Some have been identified and previously characterized as negative regulators of plant innate immunity. However, other putative mutants had yet to be identified and characterized such as *119 mos2 snc1 npr1*.

2.4.1 Mutant *119 mos2 snc1 npr1* carries an intragenic mutation in *snc1*

The morphology of the quadruple mutant *119 mos2 snc1 npr1* was evaluated and the mutant was found to be significantly smaller than *snc1* gain-of-function mutants (Fig. 2.1A). The quadruple mutant appears to restore the *snc1*-like morphology of dark green, curly leaves, which was fully suppressed by the *mos2* mutation. *119* quadruple mutants are sterile. Some homozygous mutants do not survive to reproductive age; however, they are highly resistant to the oomycete pathogen *H.a.* Noco2 (Fig. 2.1B). The mutation in *119* fully restores plant disease resistance, comparable to that of the *snc1* gain-of-function mutant, which is suppressed by *mos2*.

To determine the location of the mutation in mutant *119*, a map-based approach was used. It revealed that the mutation in *119* was located on chromosome 4 near *snc1*. We therefore used Sanger sequencing to sequence the *SNC1* gene in the *119 mos2 snc1 npr1* mutant. We found an additional mutation in the *SNC1* gene. The original *snc1* gain-of-function mutation, glutamic acid (E) 552 mutated to lysine (K), is located in the linker region of this R-like protein (Fig. 2.1C). The mutation in *119* is in to the NB region of SNC1, which is the only *snc1* allele with a mutation in this region. This mutation causes an amino acid change at residue 203 from E to K. Both E and K residues often correspond to protein activation sites, or protein binding sites.

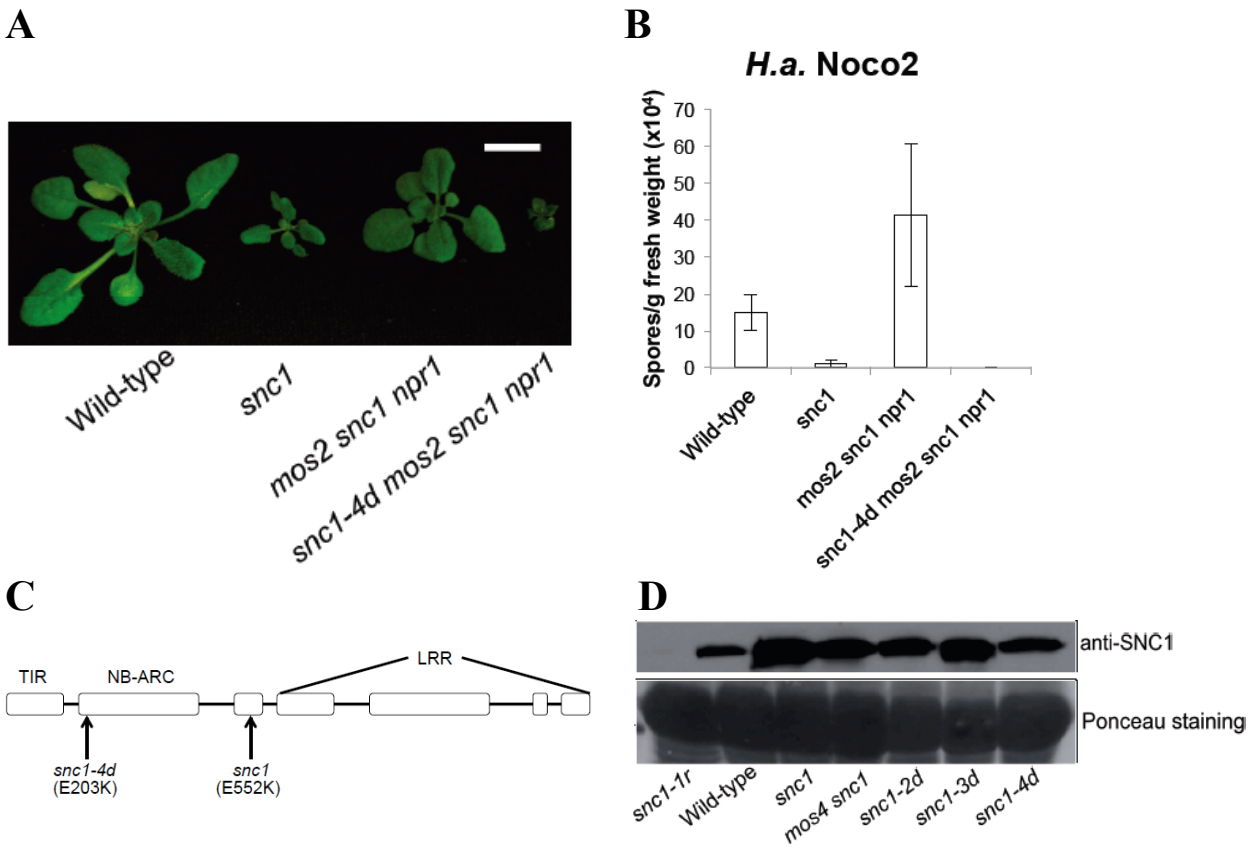
snc1 is exemplary in the constitutive activation that can occur when E is substituted by K. As an allele of *snc1*, *119* was renamed *snc1-4d* (*suppressor of npr1, constitutive 1-4, dominant*). Other alleles of *snc1* found in these screens are also indicated in Figure 2.1C.

2.4.2 *snc1-4d* protein stability

SNC1 protein stability was then assessed to determine if the *snc1-4d* mutation enhances SNC1 function through increased stability. A western blot analysis was carried out (by Yan Huang and Xuejin Chen) and, unlike the *snc1-1d* allele that enhances SNC1 stability, *snc1-4d* does not result in increased SNC1 protein stability (Fig. 2.1D).

2.4.3 Conservation of the NB domain in R proteins

A protein alignment was also carried out to determine if the corresponding amino acid residue mutated in *snc1-4d* is highly conserved among other similar R proteins. TIR-NB-LRR proteins of *Arabidopsis*, RESISTANCE TO PERONOSPORA PARASITICA (RPP) 4, RPP5 and RESISTANCE TO PSEUDOMONAS SYRINGAE 6 (RPS6) as well as a CC-NB-LRR of *Arabidopsis*, RPS2, shared high sequence homology in the NB domain. Amino acid residue 203 in SNC1, the location of the mutation was indeed highly conserved among all *Arabidopsis* NB-LRR proteins tested (Fig. 2.1E). However, alignments including R proteins from other plant species such as NICOTIANA (N) from tobacco and LINUM 6 (L6) from flax, do not show the same degree of conservation at this amino acid residue (Fig. 2.1F). Therefore, the residue corresponding to the *snc1-4d* mutation, E203K, seems only highly conserved among *Arabidopsis* R proteins.



E

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SNC1  195 SVLCLESKEARIMVGIWGQSGIGKSTIGRALYSKLSIQFHHRAFI------239
RPS2  168 ---FLSEEEERGIIGVYGPGGVGKTTLMQSINNELITKGHQYDVLIWVQMS 215
RPS6  199 SLLHLESEEVR-MVGIWGPSGIGKTTIARALFSRLSCQFQSSVFIDKVFIS 248
RPP4  197 SILCLESKEARIMVGIWGQSGIGKSTIGRALFSQLSSQFHHRAFI------241
RPP5  200 SVLCLESKEAR-MVGIWGQSGIGKSTIGRALFSQLSIQFPLRAFL------243
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F

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SNC1  203 EARIMVGIWGQSGIGKSTIGRALYSKL------SIQFHHRAFI-------TYK 242
RPS2  173 EERGIIGVYGPGGVGKTTLMQSINNELITK---GHQYDVLIWV---QMSREFG 219
RPS6  207 EVR-MVGIWGPSGIGKTTIARALFSRL------SCQFQSSVFIDKVFISKSME 252
RPP4  205 EARIMVGIWGQSGIGKSTIGRALFSQL------SSQFHHRAFI-------TYK 244
RPP5  208 EAR-MVGIWGQSGIGKSTIGRALFSQL------SIQFPLRAFL-------TYK 246
L6    257 NVT-MVGLYGMGGIGKTTTAKAVYNKI------SSCFDCCCFI------DNIR 296
N     208 GVR-IMGIWGMGGVGKTTIARAIFDTLLGRMDSSYQFDGACFL------KDIK 253
          *
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Figure 2.1 Analysis of mutant *snc1-4d*.

(A) Morphological analysis of *snc1-4d mos2 snc1 npr1* mutants grown on soil and photographed 3 weeks after planting. Size bar represents 1 cm. (B) Growth of oomycete pathogen *H.a.* Noco2. (C) Schematic diagram representing SNC1 protein structure. Residue change and location of *snc1* alleles are indicated with arrows. (D) Relative amount of SNC1 protein in alleles of *snc1* detected by western blot analysis using anti-SNC1 antibodies. (E) Amino acid sequence alignment with NB-LRR proteins homologous to SNC1 in *Arabidopsis*. *snc1-4d* mutation indicated with a star. (F) Amino acid alignment with NB-LRR proteins including L6 (flax) and N (tobacco). *snc1-4d* mutation indicated with a star.

2.5 Discussion

R proteins are typically composed of a TIR or CC N-terminal domain. They also possess a NB and a LRR region. In their inactive form, R-proteins are folded; however, upon effector recognition by the LRR domain, the protein unfolds and dimerizes with another R-protein, which can be either another copy of itself or another R-protein altogether (Moffett et al., 2002).

Previous studies have analysed the importance of each R protein domain to determine the function and necessity of each. The LRR domain possesses ligand binding activity and recognizes effector molecules and therefore determines effector-R protein specificity (Dodds et al., 2001; Wulff et al., 2001). Effector recognition by the LRR domain leads to ATP-dependant conformational change of the NB-LRR protein resulting in activation. Despite the importance of these domains they do not appear to be essential for defence activity, merely specificity. A study conducted on RPM1, a CC-NB-LRR, mutated several components of this R gene and found that if the protein was over-produced, the LRR was dispensable whereas the CC and NB domains were crucial for the induction of downstream defence responses (Tao et al., 2000).

The variable N-terminal domains of R proteins have been implicated in protein-protein interactions and it is thought that these domains are primarily responsible for dimerization and signalling. RPM1 for example, is a CC-NB-LRR type R protein and has been found to interact with RIN4 at its CC domain to negatively regulate resistance. When inactive, RIN4 and RPM1 are bound at the plasma membrane; however, the *P.s* effector AvrRpm1 may phosphorylate RIN4, which is perceived by RPM1, which is then activated and leads to a signalling cascade terminating in a defence response (Mackey et al., 2002).

The NB domain of NB-LRR proteins appears to be crucial for R protein activation possibly due to its ATP-binding capability. The NB domain of the flax R protein, P, is essential

for resistance against flax-rust. Certain mutations in this region render the plant susceptible to this fungus (Dodds et al., 2001). Furthermore, the NB domains of I-2 and Mi-1, two R-proteins of tomato, were found to be capable of and necessary in ATP binding, which is required to change R-protein conformation to the active form (Tameling et al., 2002, 2006).

The quadruple mutant *snc1-4d mos2 snc1 npr1* was initially identified based on its severe *snc1*-like morphology and its enhanced disease resistance, which abolishes the suppression imposed by *mos2*. Using a map-based approach I cloned the mutated gene containing a mutation resulting in an amino acid change in the NB domain of the SNC1 protein. The original gain-of-function *snc1* mutation causes an increase in SNC1 protein stability, and therefore possesses constitutive resistance against pathogens. However, western blot analysis indicated that *snc1-4d* has comparable SNC1 protein stability to that of wild-type plants. Therefore, the increased resistance observed in *snc1-4d* mutants is not due to increased protein stability, unlike other *snc1* alleles, and must be another mechanism that causes the enhanced *snc1* phenotype. One possibility may be that the mutation in the NB domain of *snc1* increases its affinity for ATP, thus increasing the rate of protein unfolding, and therefore its activation. Another possibility is that the mutation in *snc1-4d* prevents proper folding of SNC1 allowing it to remain in its active, less folded form. Further analyses must be conducted to determine if the mutation in *snc1-4d* is due to auto-activation of SNC1, or another mechanism altogether.

3 *AtCDC48A* negatively regulates plant innate immunity

3.1 Summary

Due to their sessile nature, plants had to evolve multi-layered immune systems to defend themselves against pathogens. In addition, plants have evolved regulatory mechanisms to keep the immune response in check. Much research has focused on the production of antimicrobial compounds and proteins; however, it is becoming increasingly evident that regulation at the level of protein degradation plays a key role in plant innate immunity. Through a genetic screen we discovered a novel negative regulator of plant innate immunity, *AtCDC48A*. Previously this protein has been implicated in the cell division cycle and proteasome-mediated proteolysis. However, here we find that mutant *atcdc48a* plants have heightened *PR* gene expression and increased resistance to the oomycete pathogen *H.a.* Noco2. These results implicate *AtCDC48A* as a negative regulator of plant innate immunity.

3.2 Introduction

Plants have evolved complex immune systems to protect and defend themselves against pathogens. Trans-membrane PRRs present on the plant cell surface, serve to recognize conserved PAMPs present on the surface of pathogens. This recognition leads to downstream defence responses. However, some pathogens have evolved mechanisms to inject effector molecules into the host plant cell. These effectors inhibit the defence responses of the plant and lead to successful infection of the host. In turn, plants have evolved a secondary layer of resistance through R proteins that act as internal surveillance molecules that recognize pathogen effector molecules and mount a defence response (Jones and Dangl, 2006).

R protein mediated defence responses lead to the expression of *PR* genes, accumulation of SA, and programmed cell death referred to as the HR. *snc1* encodes an R-like protein with a

gain-of-function mutation. It exhibits constitutive expression of *PR* genes, SA accumulation and has increased resistance to virulent pathogens. However, the gain-of-function mutation also results in extremely dwarf stature, and dark, deformed leaves (Li et al., 2001; Zhang et al., 2003). Due to the complex layered nature of the plant innate immune system and the detrimental phenotypes observed when these pathways are mis-regulated, as we see in *snc1* mutants, plants require stringent regulation of such processes. Much of the research in this field has primarily focused on the production of defence compounds and proteins; however, it is becoming increasingly evident that protein degradation and turnover also plays a significant role in the regulation of plant innate immunity.

The 26S proteasome pathway is the primary method of protein degradation in plants (Schubert et al., 2000). This complex is responsible for the turnover of many housekeeping proteins; however, it also plays a significant role in targeted protein degradation in response to internal or external cues. Degradation in this pathway is initiated by poly-ubiquitination of target proteins. Affixation of Ub, one of the most highly conserved and abundant proteins, marks the protein for degradation (Callis et al., 1995). Ubiquitination is accomplished through an E1, E2, E3 cascade in which an E1 enzyme activates Ub, which is subsequently transferred to an E2 enzyme by transesterification (Ciechanover et al., 1982; Hershkos et al., 1983). E2 then transports the activated Ub to an E3 ligase enzyme, which determines target specificity and the final transfer of Ub to the target protein typically at a lysine (K) residue (Hershkos et al., 1983; Reiss et al., 1989; Chau et al., 1989). This process is repeated until the correct length of ubiquitin chain is achieved for its degradation.

Once poly-ubiquitinated, target proteins require transfer to the proteasome for degradation. Complexes are formed that specifically recognize and recruit ubiquitinated proteins,

some of which have also been found to be associated with Cell Division Cycle 48 (CDC48) complex, a highly conserved protein complex that has been implicated in protein extraction from membranes and proteasome-mediated degradation (Richly et al., 2005; Ye et al., 2001; Baek et al., 2011). However, the exact role of CDC48 in targeted proteolysis remains unclear.

Here we report the identification of a *mutant snc1-enhancing 8* (*muse8*) gene that negatively regulates plant innate immunity as evidenced by increased *PR* gene expression and resistant to infection by the oomycete pathogen *H.a.* Noco2. Map-based cloning indicates the *muse8* phenotype is due to a mutation in *AtCDC48A*, a CDC48 homolog in *Arabidopsis thaliana.* Solexa sequencing revealed that the *muse8* mutation causes an amino acid change in a highly conserved residue of AtCDC48A, which forms a hexameric protein complex. This protein complex is involved in the cell division cycle and also implicated in proteasome-mediated proteolysis; however, here we observe a clear indication of its involvement in regulating plant innate immunity.

3.3 Materials and methods

3.3.1 Plant growth and mutant isolation

For most experiments, plants were grown on soil with a 16 hour light/8 hour dark cycle. Isolation of the *mos2 snc1 npr1* triple mutant was previously described (Zhang et al., 2005). The *muse8 mos2 snc1 npr1* was created by mutating 40,000 *mos2 snc1 npr1 Arabidopsis thaliana* seeds also carrying a *BGL2*-GUS construct with 0.2% EMS (ethylmethanesulfonate) for 8 hours (Bowling et al., 1994). Mutants were isolated by screening based on *snc1*-like morphology and *PR2* gene expression, which was assessed using a *GUS*-reporter construct that was previously introduced (Cao et al., 1997). Mutants obtained from these primary screens were subjected to a disease resistance screen where mutants were spray-inoculated with *H.a.* Noco2 as described previously (Li et al., 2001).

3.3.2 *muse8 mos2 snc1 npr1* characterization

muse8 mos2 snc1 npr1 morphology was characterized based on plant size, leaf shape and colour, sterility, and other deviations from wild-type or *snc1* mutant phenotypes. Photographs were taken using a digital camera and size bars added using Adobe Photoshop.

Total RNA was extracted from 14 day-old plants using the Totally RNA Kit (Ambion). Total RNA was then reverse transcribed using M-MLV Rerverse Transcriptase (Takara). *PR1*, *PR2*, and *Actin7* were amplified separately as previously described (Zhang et al., 2003). PCR products were then run on a 1% agarose gel containing ethidium bromide and imaged using an AlphaImager HG (AlphaInnotech).

Disease resistance was tested using *muse8 mos2 snc1 npr1*, *mos2 snc1 npr1*, *snc1*, and *BGL2* plants grown on soil. Two-week-old plants were spray inoculated with 50,000 spores/mL of *H.a.* Noco2 as previously described (Li et al., 2001). Infection was assessed precisely through

spore counting on twenty randomly chosen plants from each genotype 10-14 days post-inoculation using a hemocytometer.

3.3.3 Genetic mapping

muse8 mos2 snc1 npr1 mutants in the Columbia-0 ecotype of *Arabidopsis thaliana* were crossed with the Landsberg *erecta* (L*er*) ecotype. The F_2 generation was grown on soil and 48 plants smaller than *snc1* were selected for linkage analysis. The *muse8* mutation was narrowed down to a region on chromosome 3 between markers F3L34 (1.4) MB and MGH6 (4.19 MB). The fine mapping population (F_3) was obtained from three F_2 lines that were heterozygous in the aforementioned region. Fine mapping further narrowed the region to between T16O11 (2.7 MB) and MGH6 (4.19 MB). Homozygous quadruple mutant plant DNA was isolated from quadruple mutant plants grown on soil and sent to the National Institute of Biological Sciences in Beijing, China for Solexa sequencing.

3.3.4 Molecular cloning

T-DNA insertion lines for *At3G06660, At3G08950,* and *At3G09840* were obtained from Arabidopsis Biological Resource Center (ABRC). Complementation tests were done by crossing T-DNA mutant lines with *muse8 snc1* double mutant lines and examining the F1 for evidence of complementation by the presence of a wild-type phenotype. The F2 generation for each cross was also grown to confirm complementation or a lack of complementation. Further cloning confirmation was obtained by crossing T-DNA lines with *snc1* mutants and examining the subsequent F1 and F2 generations for *snc1*-enhancing phenotypes of the double mutants of *snc1* and the T-DNA insertion.

3.3.5 Phylogenetic analyses

The AtCDC48A protein sequence was obtained from PubMed online and used to conduct a BLASTP search. Homologous sequences from each kingdom were chosen based on low e-values and high quality reference sequences. Protein alignment was achieved using Muscle (Edgar, 2004). The alignment was then manually adjusted using Se-Al (Rambaut, 2002). Conserved amino acid residues were found using Jalview (Waterhouse et al., 2009). The optimal evolutionary model was chosen using ProtTest; the output form the AICc and BIC trials were compared and the most appropriate model was found to be LG+I+G (Darriba et al., 2011; Guindon and Gascuel, 2003). Heuristic searches and bootstrapping with 5000 replicates was conducted using SeaView (Gouy et al., 2010).

For the *Arabidopsis thaliana* CDC48 tree, paralogs were found using a BLASTP search. Protein sequences of AtCDC48A-E were obtained from TAIR. All sequences were aligned using Muscle (Edgar, 2004). Using ProtTest, comparing the output from AICc and BIC, the optimal evolutionary model was found to be LG+I (Darriba et al., 2011; Guindon and Gascuel, 2003). Heuristic searches and bootstrapping was conducted using 5000 replicates with SeaView (Gouy et al., 2010).

3.3.6 Microarray gene expression analysis

Expression data for each *AtCDC48* paralog was obtained using the AtGenExpress Visualization Tool (http://jsp.weigelworld.org/expviz/expviz.jsp). All AtGenExpress data is quantile-normalized using GeneChip Robust Multiarray Averaging (gcRMA) and absolute values provided by this database are linerarized gcRMA values. Data from various treatments was obtained by submitting the *Arabidopsis* gene number (ie. *At3G09840*), and selecting pathogen, development, or hormone from the experiment drop-down menu. Absolute values

from individual data points from the resulting expression graph in response to various treatments of pathogens, chemicals or various tissues were used in creating bar charts to determine if expression patterns of *AtCDC48A* are similar to those of the other *AtCDC48* paralogs. For developmental expression data, tissues were chosen based on their relative exposure to pathogens or due to the previous implication of *AtCDC48A* within that tissue (Feiler et al., 1995). For hormone expression data in each gene, MG132 was chosen due to its proteasome inhibition activity and cyclohexamide was chosen due to its involvement in protein synthesis inhibition to determine the involvement of *AtCDC48A* in proteasome-mediated protein degradation as previously hypothesized (Richly et al., 2005; Ghislain et al., 1996; Schuberth et al., 2004).

3.4 Results

3.4.1 *muse8 mos2 snc1 npr1* characterization

muse8 mos2 snc1 npr1 mutant was obtained from a primary screen based on *PR2* gene expression using a GUS construct that was fused to the endogenous *PR2* promoter. Expression of *GUS* indicates the *PR2* promoter is being bound and transcription is being initiated; therefore, *PR2* should also be expressed. A secondary screen based on resistance to *H.a.* Noco2 tested the resistance of putative mutants obtained from the primary screen. The quadruple mutant displays enhanced *snc1*-like morphology, which is dwarf, dark green and curly leaved; however, it is slightly smaller than *snc1* (Fig. 3.1A).

PR gene expression was assessed using RT-PCR. *muse8 mos2 snc1 npr1* restores the *PR1* and *PR2* gene expression levels similar to that of *snc1*, which were abolished by the *mos2* mutation (Fig. 3.1B). To determine if this *PR* gene expression confers increased disease resistance, *muse8* quadruple mutants as well as controls were infected with the oomycete pathogen *H.a.* Noco2. Spores were counted 10-14 days post-inoculation. Quadruple mutants indeed had increased disease resistance comparable to that of *snc1* plants (Fig. 3.1C).

3.4.2 *muse8* contains a mutation in *AtCDC48A*

The mutation responsible for the observed phenotype of *muse8 mos2 snc1 npr1* mutants was crude-mapped to the top of chromosome 3, near marker MGH6 at 4.2 MB. Fine-mapping narrowed down the region to between indel markers T16O11 (2.7MB) and MGH6 (4.2 MB) (Fig. 3.1E). Further mapping did not narrow down the region any further; therefore, nuclear DNA was extracted from quadruple mutants and sent to the National Institute of Biological Sciences in Beijing for Solexa sequencing. This next generation sequencing revealed mutations in many candidate genes within the 2MB on chromosome 3 (Table 3.1). Two mutations occurred

in a pseudogene of nodulin (*At3G06433*); however, as a pseudogene, it is also an unlikely candidate for the *muse8* mutation. Similarly, two amino acid changes were found in MOV34 (*At3G11270*), which is a PAD-1 family protein. One mutation resulted in a valine to methionine subtitution, both of which are hydrophobic amino acids and serve similar functions; therefore this mutation would most likely not change the function of the protein. The second mutation in this gene caused a threonine to isoleucine substitution. *At3G11402*, a cysteine/histidine-rich C1 domain family protein, *At3G11560* a LETM1-like protein, and *At3G12800* encoding a short-chain dehydrogenase-reductase B protein also contained point mutations; however, the recombination frequency seen in the F_2 and F_3 population indicated the mutation should be in a gene closer to 3MB.

Three genes seemed to be the most likely candidates due to either their protein function or the observed recombination frequency in the F_2 and F_3 generations. *At3G06660* encodes a PAPA-1-like family protein, a transcription factor located in the chloroplast. The location of the point mutation of this gene did not correspond to a functional domain, which makes it an unlikely candidate; however, due to its location in the genome it was considered further. *At3G08950* encodes a phosphoglycerate mutase found in mitochondria and used in copper ion binding and cytochrome c synthesis. The location of the mutation in this gene is within the last ten amino acid residues and does not correspond to a known functional domain. Due to its location in the genome and the recombination frequency observed, it was considered a possible candidate for the location of the *muse8* mutation. *At3G09840* or *AtCDC48A* seemed like the most likely candidate. This protein is involved in the conversion of ATP to ADP through two P-loop AAA ATPase domains. This protein forms a homohexamer creating a cylindrical structure. The location of the mutation, which causes a glycine to glutamic acid substitution at residue 274

is in the first P-loop AAA ATPase domain (Fig. 3.2A). Glycine is the smallest amino acid and as such is often found in the centre of tight complexes; changing this amino acid may alter the structure of this complex and therefore affect its function. Glycine is also the only amino acid that can bind phosphates from ATP and therefore may be crucial to the function of this complex.

To determine which of these three genes is responsible for the *muse8* phenotype, an allelism test was conducted. T-DNA insertion lines for each gene were obtained from ABRC and crossed to *muse8 snc1* double mutants obtained in the F_2 generation. A T-DNA insertion mutant of *At3G06660*, the transcription factor in the chloroplast, complemented the *muse8 snc1* double mutant phenotype, as did T-DNA insertion mutants of *At3G08950*, the phosphoglycerate mutase found in mitochondria (Fig. 3.2B). However, when the T-DNA mutant of *At3G09840* (*AtCDC48A*) was crossed with the *muse8 snc1* double mutant, it failed to complement in the F_1 generation in that plants heterozygous for the T-DNA insertion in *AtCDC48A* and heterozygous for *snc1,* displayed a *snc1*-like phenotype, indicating one non-functional copy of *atcdc48a* is sufficient to cause a *snc1*-like phenotype when heterozygous for *snc1*. Homozygous T-DNA insertion mutant, *atcdc48a*, is lethal; however, plants homozygous for *muse8* are partly sterile, in that they produce very small siliques with no seeds. In the F_1 generation, plants heterozygous for the T-DNA insertion and heterozygous for *muse8* were also sterile, which further supports that *AtCDC48A* is the gene responsible for the *muse8* quadruple mutant phenotype.

To confirm that *AtCDC48A* is *MUSE8*, T-DNA insertion lines were crossed with *snc1* and allowed to self to obtain double mutants homozygous for *snc1* and heterozygous for the T-DNA insertion. Since the *muse8* quadruple mutant enhances the *snc1* phenotype, the loss of function T-DNA insertion line corresponding to the *muse8* gene should also enhance the *snc1* phenotype. The PAPA-1 transcription factor mutant did not enhance *snc1* (Fig. 3.3A). Similar

results were obtained for the phosphoglycerate mutase T-DNA insertion line (Fig. 3.3B). Conversely, plants homozygous for the *snc1* mutation but heterozygous for the *AtCDC48* T-DNA insertion exhibit *snc1*-enhancing morphology (Fig. 3.3C). This again confirms that we have cloned the correct gene.

3.4.3 Phylogenetic analysis of AtCDC48A protein

To determine the level of conservation of AtCDC48A, a phylogenetic tree was constructed (Fig. 3.4A). CDC48A is highly conserved across kingdoms. The amino acid residue altered in *muse8* was also highly conserved in all organisms (Fig. 3.4B). In *Arabidopis thaliana* there are 4 paralogs of *AtCDC48A*. Examining their gene structure indicates that *AtCDC48A* is most closely related to *AtCDC48D* and *AtCDC48E* (Fig. 3.5A). Indeed this seems to be true based on the phylogenetic tree of the *AtCDC48* paralogs (Fig. 3.5B).

3.4.4 Expression analysis of the *AtCDC48* gene family

Although paralogous, little investigation into the *AtCDC48* genes has taken place. Microarray analysis revealed that all *AtCDC48* genes appear to be up-regulated upon infection with various strains of *P.s.*; however, *AtCDC48A* is by far the most highly expressed (Fig. 3.6A-E). Similar results were obtained by treating plants with *Phytophthora infestans*, another virulent pathogen, and well-known pathogen elicitors (data not shown). A similar trend is seen among various tissues (Fig. 3.7A). *AtCDC48A* expression is highest in roots and senescing leaves, relatively high in leaves, stamens, carpels and seeds and relatively low in mature pollen. Similar tissue specificity of expression is seen with *AtCDC48D* and *AtCDC48E*, which further support the observed phylogenetic relationship. Since *AtCDC48A* has previously been implicated in proteasome-mediated protein degradation, *AtCDC48A* expression was examined after treatment with MG132, a proteasome inhibitor, and was found to increase (Fig. 3.7B). Treatment with

MG132 had a similar effect on *AtCDC48D* and *E* expression but little to no change in *AtCDC48B* and *C* expression. Following treatment with cyclohexamide, a protein synthesis inhibitor, *AtCDC48A* expression decreased significantly as did *AtCDC48D* and *E* expression to a lesser extent (Fig. 3.7B). Alternatively, *AtCDC48B* and *C* expression again showed little to no change.

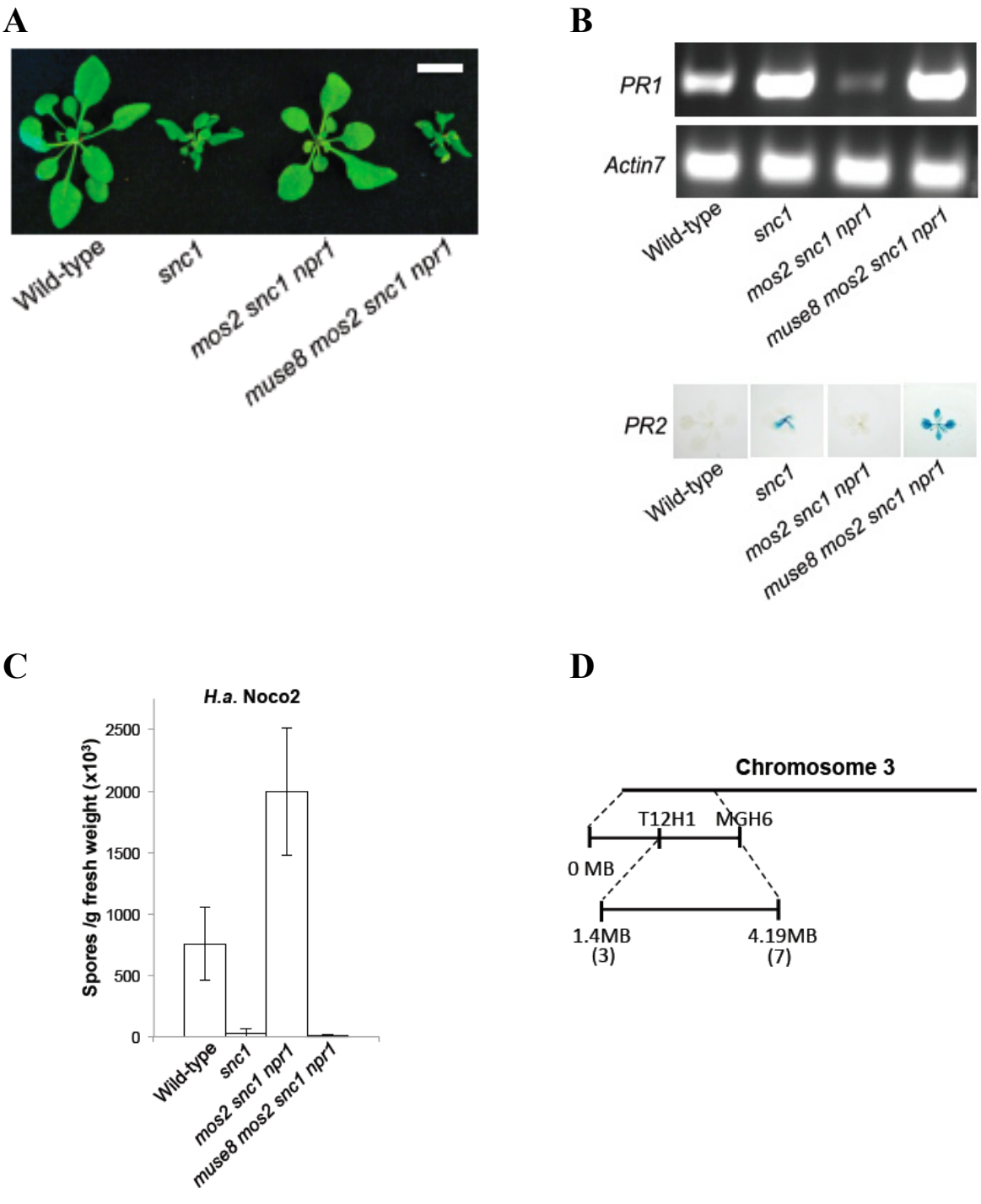


Figure 3.1: Characterization of *muse8 mos2 snc1 npr1*.
(A) Morphological analysis of *muse8 mos2 snc1 npr1* in comparison to *mos2 snc1 npr1*, *snc1*, and wild-type. (B) *PR1* (RT-PCR) and *PR2* (GUS) gene expression of aforementioned genotypes. (C) Growth of the oomycete pathogen *H.a.* Noco2 on each of the aforementioned genotypes. (D) Schematic diagram of chromosome 3 indicating region containing *muse8* narrowed down by a map-based approach.

Table 3.1: Next generation sequence analysis: list of potential candidate mutations in the mapped *muse8* region.

Gene	Predicted Protein	Number of Reads	Nucleotide Change	Amino Acid Change
At3G06433	Pseudogene of nodulin MtN3 family protein	6/8	C to T	R396*
At3G06433	Pseudogene of nodulin MtN3 family protein	3/4	G to A	R973H
At3G06437	Pseudogene, hypothetical protein	21/22	C to T	None
At3G06660	PAPA-1-like family protein/zinc finger	5/5	G to A	G113S
At3G07425	Unknown protein	3/6	C to T	None
At3G07670	Rubisco methyltranferase	2/3	C to T	(intron)
At3G08590	Phosphoglycerate mutates	2/2	G to A	A329S
At3G08930	LMBR1-like membrane protein	3/3	C to T	None
At3G09840	Cell Division Cycle 48	4/3	G to A	G274E
At3G10195	Putative membrane lipoprotein	2/3	C to T	(intron)
At3G10585	Homeodomain-like superfamily protein	2/4	G to A	(intron)
At3G10690	DNA GYRASE A	5/5	G to A	(intron)
At3G11260	WUSCHEL related homeobox 5	2/4	G to T or A	(intron)
At3G11270	Mov34/MPN/PAD-1 family protein	3/5	G to A	V127M
At3G11270	Mov34/MPN/PAD-1 family protein	2/2	C to T	T142I

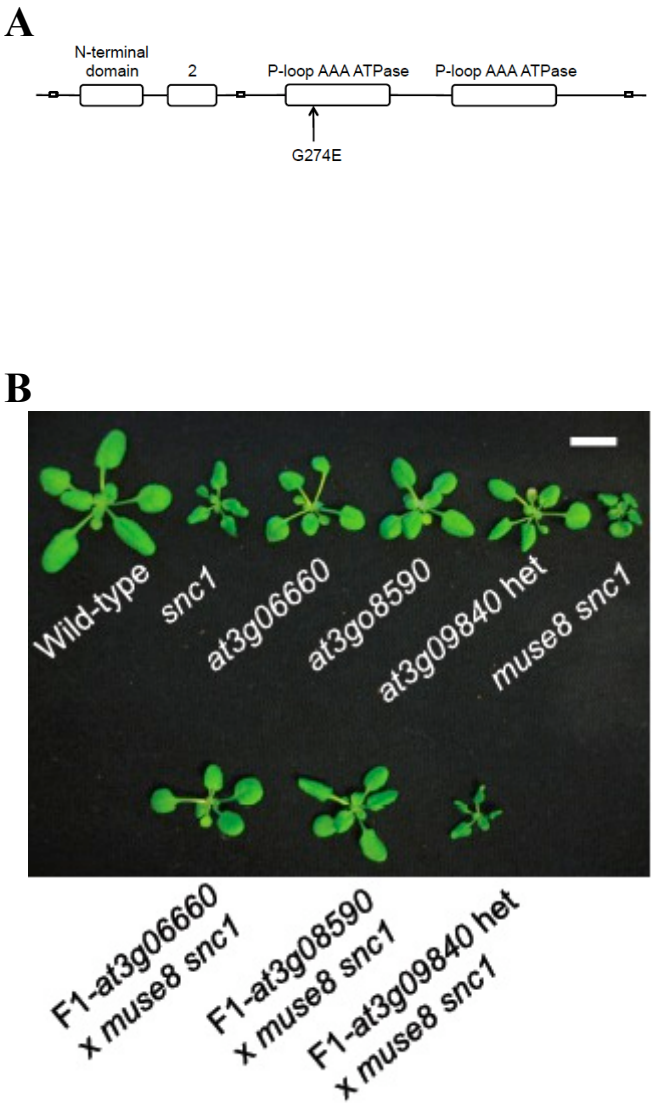


Figure 3.2: Cloning of *AtCDC48A*

(A) Schematic diagram of AtCDC48A indicating location of the mutation in *muse8*. (B) Complementation. *at3g06660* and *at3g08950* T-DNA insertion lines (CS860752 and SALK_119825c) complemented *muse8 snc1* mutants whereas heterozygous *atcdc48a* (*at3g09840* het) T-DNA insertion lines (SALK_064573) failed to compliment *muse8 snc1* plants. Wild-type, *snc1*, *muse8 snc1*, and T-DNA insertion line control plants are shown.

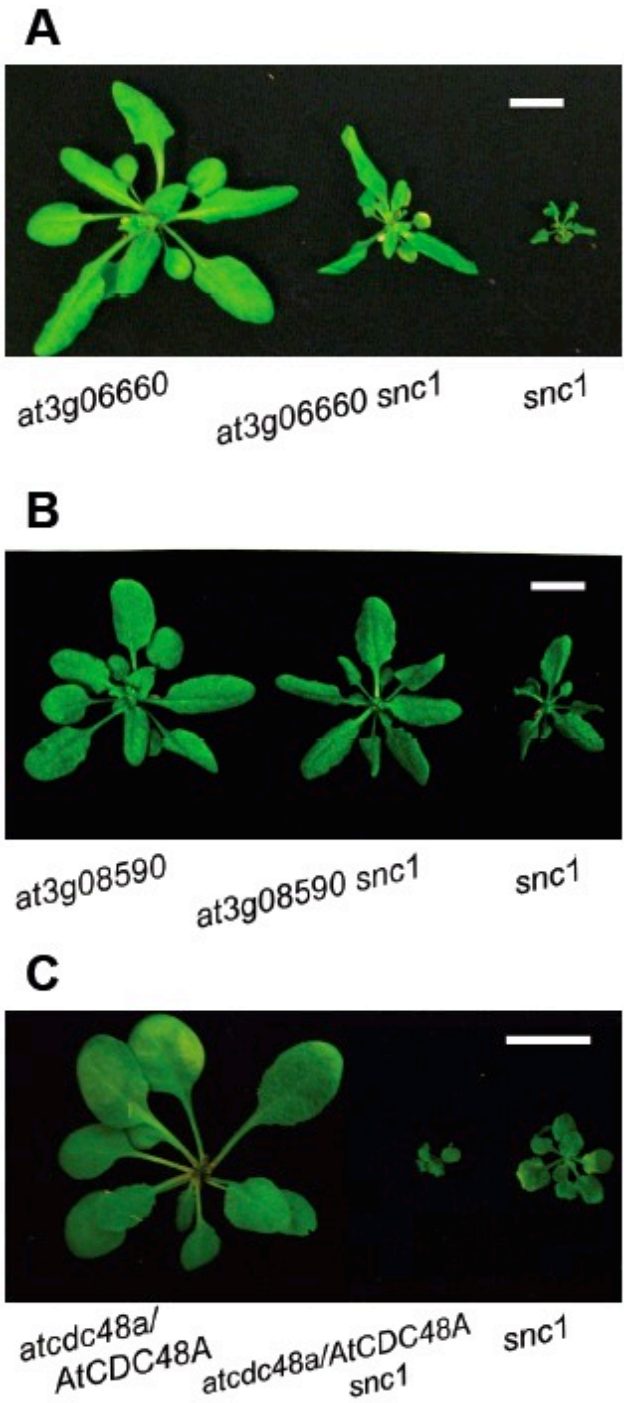
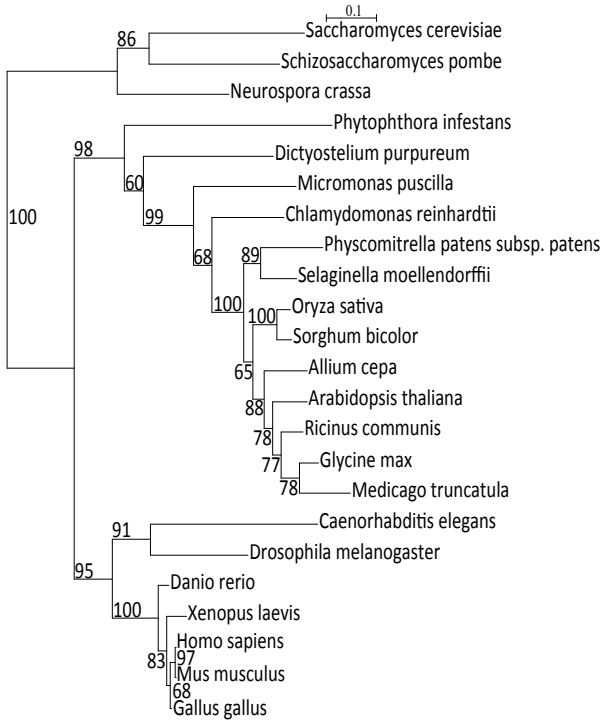


Figure 3.3: Crosses of T-DNA lines of candidate genes with *snc1*.
F_2 plants were grown on soil, genotyped and photographed 4 weeks post-planting. (A) F_2 progeny of a cross between *at3g06660* T-DNA insertion line (CS860752) and *snc1*. *at3g06660 snc1* double mutants do not display an enhanced *snc1*-like phenotype. (B) F_2 progeny of a cross between *at3g08590* T-DNA insertion line (SALK_119825c) and *snc1*. *at3g08950 snc1* double mutants do not display an enhanced *snc1*-like phenotype. (C) F_1 progeny of *at3g09840* T-DNA insertion line (SALK_064573) and *snc1*. Heterozygous *at3g09840 snc1* double mutants display an enhanced *snc1*-like phenotype in comparison to *snc1* and the T-DNA insertion line.

A



B

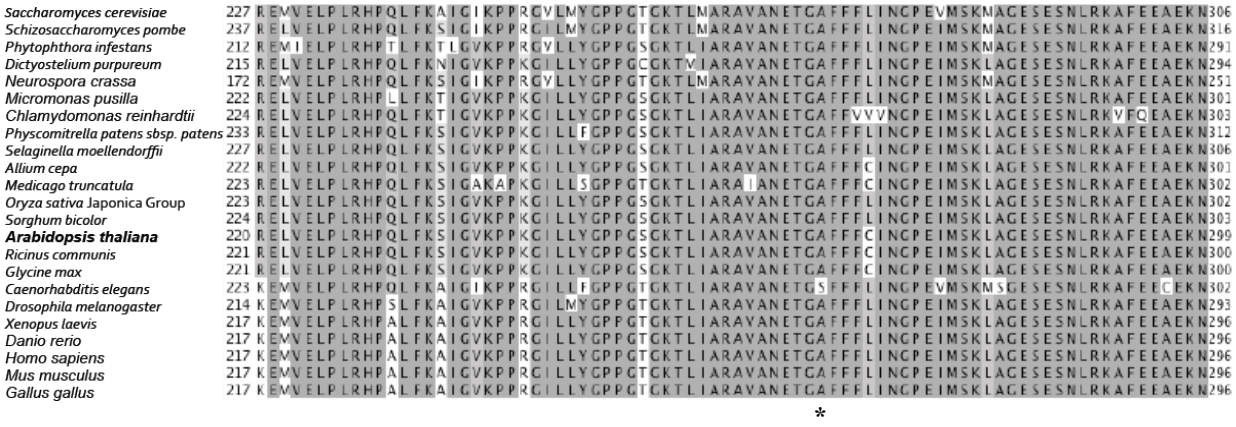


Figure 3.4: Phylogenetic analysis of CDC48A across kingdoms.
(A) Phylogenetic tree of AtCDC48A and representative homologs from other kingdoms. (B) Amino acid alignment of a portion of AtCDC48A and representative homologs from other kingdoms.

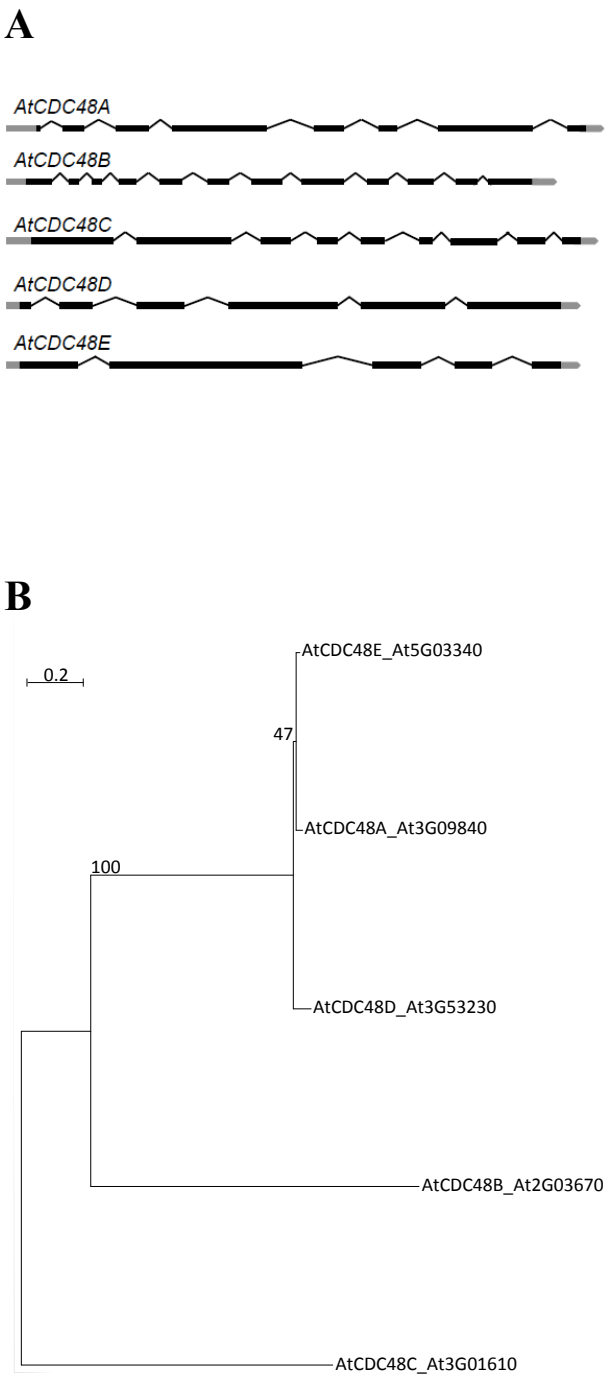


Figure 3.5: Phylogenetic analysis of *AtCDC48 Arabidopsis* orthologs.
(A) Schematic diagram representing the gene structure of *AtCDC48* orthologs. (B) Phylogenetic tree of *AtCDC48* orthologs indicating high homology between *AtCDC48A*, *AtCDC48D*, and *AtCDC48E*.

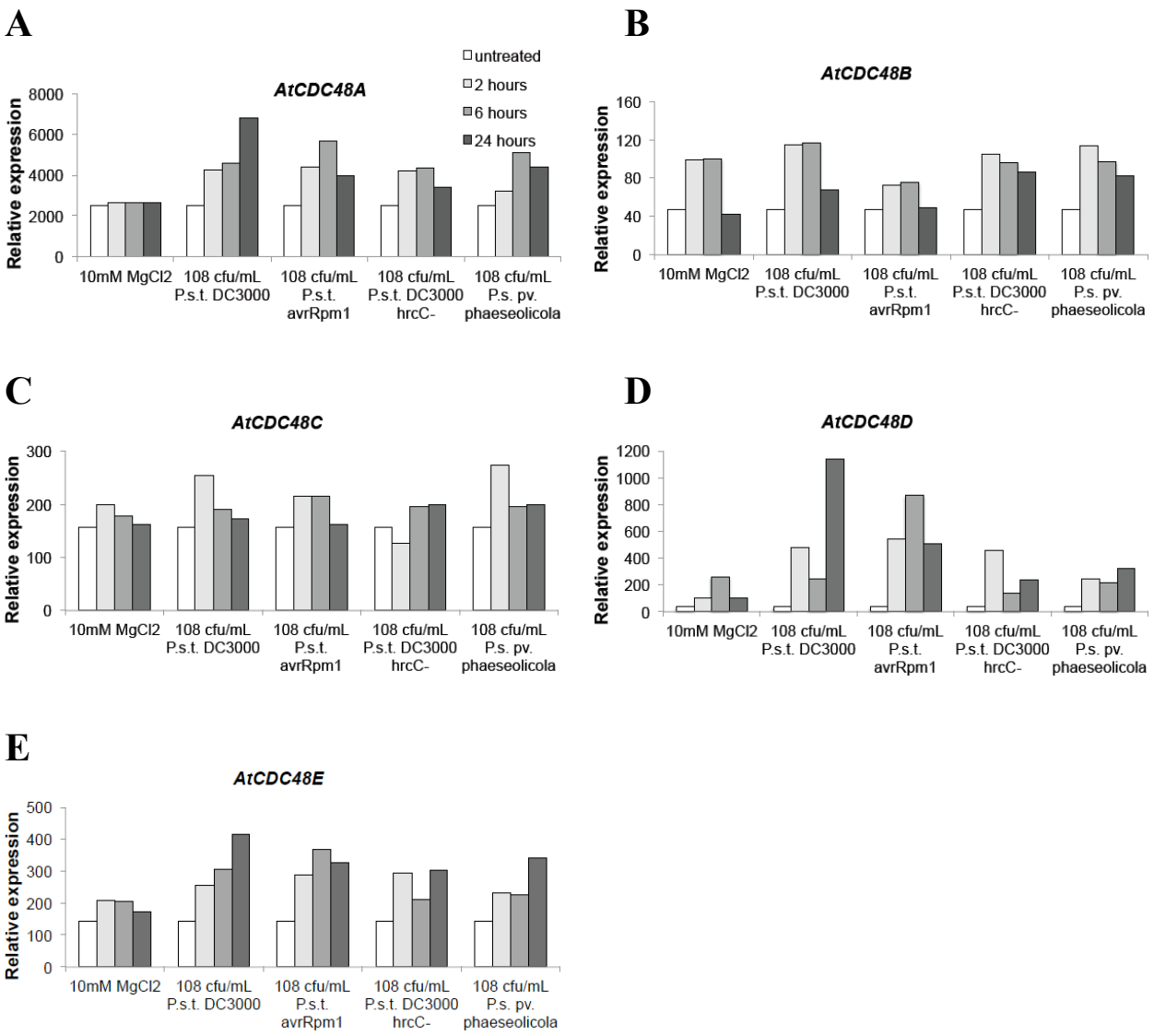


Figure 3.6: Microarray expression analysis of *AtCDC48* orthologs post-infection.

Expression data was obtained from AtGenExpress Visualization Tool. (A) Relative expression of *AtCDC48A* after treatments with various strains of *Pseudomonas syringae* pv. *tomato.* (B) Relative expression of *AtCDC48B* after treatments with various strains of *Pseudomonas syringae* pv. *tomato.* (C) Relative expression of *AtCDC48C* after treatments with various strains of *Pseudomonas syringae* pv. *tomato.*(D) Relative expression of *AtCDC48D* after treatments with various strains of *Pseudomonas syringae* pv. *tomato.* (E) Relative expression of *AtCDC48E* after treatments with various strains of *Pseudomonas syringae* pv. *tomato.*

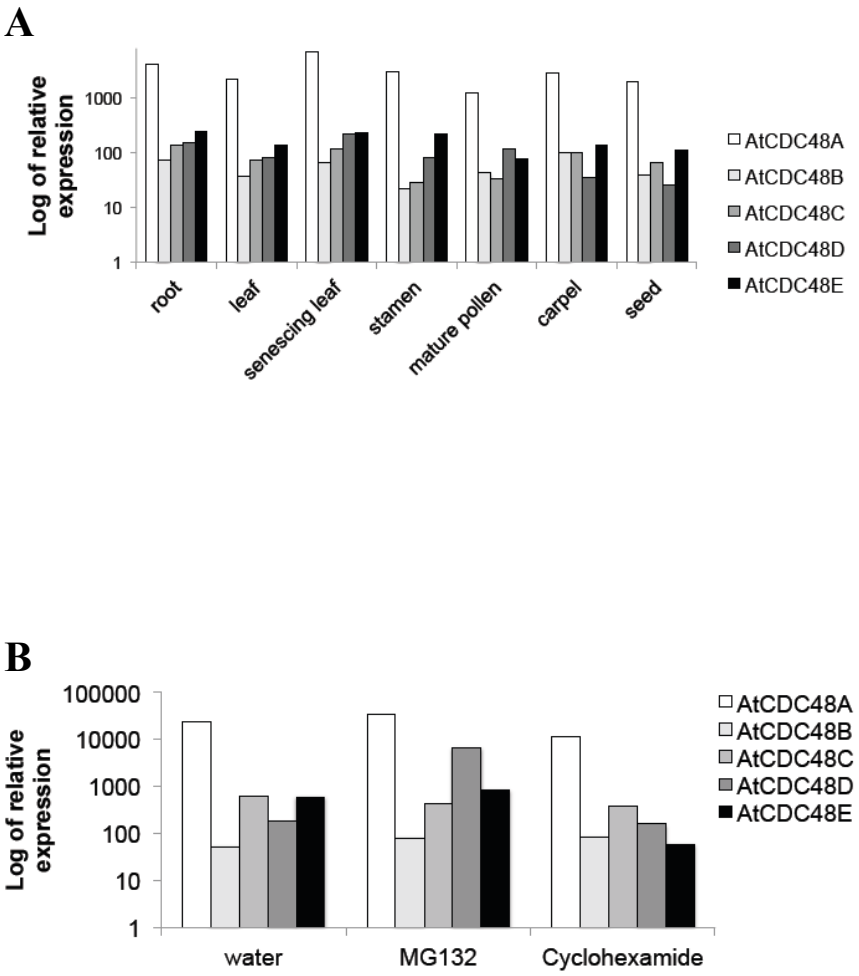


Figure 3.7: Microarray expression analysis of *AtCDC48* orthologs in tissues and in response to chemical treatment.

Expression data was obtained from AtGenExpress Visualization Tool. (A) Microarray expression of AtCDC48 orthologs in root, leaf, senescing leaf, stamen, mature pollen, carpel and seed. (B) Microarray expression of AtCDC48 orthologs in response to the proteasome inhibitor MG132 and the protein synthesis inhibitor cyclohexamide.

3.5 Discussion

Protein degradation is needed to mount a controlled, yet robust defence response. The 26S proteasome-mediated proteolysis is the most common type of protein degradation in plants, and requires ubiquitination of target proteins through E1, E2 and E3 enzymes (Schubert et al., 2000; Hershkos et al., 1983; Reiss et al., 1989; Chau et al., 1989). Targeted proteins are then transferred to the 26S proteasome for degradation.

Several E3 ligases have been implicated in plant defence (Cheng and Li, 2012). CPR1 of *Arabidopsis thaliana* is a negative regulator of plant innate immunity (Bowling et al., 1994). When mutated, *cpr1* mutants have elevated levels of SNC1 protein; however, when *CPR1* is over-expressed, SNC1 protein accumulation is abolished (Cheng et al., 2011; Gou et al., 2012). Therefore the E3 ligase CPR1 negatively regulates plant innate immunity by regulating SNC1 protein stability. Other examples of E3 ligases involved in plant defence are ACIF1 from tobacco, or ACRE189 of tomato. These E3s positively regulate HR mediated by various *R* genes such as CLADOSPORIUM FULVUM RESISTANCE 4 (CF-4), Cf-9, N, and PSEUDOMONAS RESISTANCE AND FENTHION SENSITIVITY (Prf) (Van den Burg et al., 2008). During infection, these F-box protein are up-regulated and associate with the SCF complex, which ubiquitinates target proteins to be degraded. Loss-of-function or silencing mutations in ACIF1 decrease the HR. Therefore, the role of ACIF1 in immunity is to target a negative regulator of defence for ubiquitination. Once degraded, the defence response can occur, and HR is observed.

Transfer of target proteins to the proteasome is thought to be accomplished via protein complexes that associate with both the ubiquitinated substrate, as well as the proteasome (Hartmann-Petersen et al., 2003). Some of these proteins are Ub-receptor proteins, which contain both a Ub-like (UBL) domain at the N ternimus that is recognized by the proteasome as well one

or multiple Ub-Associating (UBA) domains that function in Ub binding (Wilkinson et al., 2001). These UBL-UBA containing proteins bind target proteins via their Ub-chains and bind the proteasome, essentially delivering the target protein to the proteasome (Elsasser and Finley, 2005). These events have been shown to be crucial to plant innate immunity. A UBA-containing protein MOS5 has been previously implicated in plant defence (Goritschnig et al., 2007). The *mos5* loss-of-function mutant prevents the accumulation of SNC1 protein and therefore inhibits disease resistance. Therefore, MOS5 is essential in disease resistance mediated by the R-like protein, SNC1.

Another family of Ub-associated proteins, is the UBX family, also important in proteasome-mediated degradation as well as plant innate immunity. UBX proteins contain UBA domains and UBX domains, which are similar to UBL domains in that they resemble Ub itself. In *Arabidopsis*, microarray analysis of wild-type controls and plants infected with the powdery mildew *Golovinomyces orontii* revealed an up-regulation of a number of UBX proteins implicating them in plant defence (Chandran et al., 2009).

UBX proteins are often associated with the CDC48 complex (Orme and Bogan, 2012). This complex is comprised of six monomers of the CDC48 protein and forms a ring-like structure. Each monomer contains two AAA (ATPase Associated with different cellular Activities) ATPase domains, which use the hydrolysis of ATP to ADP to perform various cellular activities through conformational changes. Each monomer contains N-terminal domains, which allow binding of substrates. Since CDC48 is a homohexameric complex, it possesses six N-terminal domains, each capable of binding a different co-factor allowing the CDC48 complex to have a multitude of functions depending on the cofactors with which it is associated.

As its name suggests, CDC48 has been implicated in the cell division cycle and cell proliferation (Moir and Botstein, 1982; Peters et al., 1990; Park et al., 2008). However, CDC48, as well as its homologs p97 in mouse and frog, and VALOCIN CONTAINING PROTEIN (VCP) in humans, have been implicated in the proper assembly of endoplasmic reticulum and Golgi body (Kondo et al., 1997; Yuan et al., 2001; Roy et al., 2000), nuclear envelope formation (Hetzer et al., 2001), and ER-associated protein degradation (ERAD) (Alzayady et al., 2005; Schuberth and Buchberger, 2005; Romisch, 2006). In yeast, CDC48 is thought to act in the ERAD pathway through the extraction of proteins from the cytosolic side of the ER membrane via their interaction with CDC48 cofactors, UBIQUITIN FUSION DEGRADATION 1 (UFD1) and NUCLEAR PROTEIN LOCALIZATION 4 (Npl4) (Braun et al., 2002; Jarosch et al., 2002; Rabinovich et al., 2002; Ye et al., 2001, 2003). These protein substrates are subsequently poly-Ub by UFD2, an E4 ligase that has been found to bind CDC48 and utilize the E1, E2, E3 cascade to poly-ubiquitinate its target (Richly et al., 2005). This poly-ubiquitination recruits RADIATION SENSITIVE 23 (RAD23), a protein that recognizes poly-ubiquitinated substrates that binds both CDC48 and the proteasome, essentially escorting the target substrate to the proteasome for degradation (Richly et al., 2005). RAD23 binding of UFD2 and the proteasome is mutually exclusive. Recently, it has been shown that disassembly of the UFD2/RAD23 complex is facilitated by CDC48 in an ATP/UFD2 dependant manner, allowing the release of the poly-Ub subtrate/RAD23 complex and subsequent binding to the proteasome complex (Baek et al., 2011).

Despite the implication of various components involved in proteasome-mediated degradation such as various E3 ligases and UBX containing proteins in plant immunity and the association of this type of protein with CDC48, this multifunctional chaperone has yet to be implicated in plant innate immunity. Using a map-based cloning approach along with genetic

complementation, the *muse8* mutation was confirmed to be in *AtCDC48A*, the homolog of CDC48/p97/VCP found in other eukaryotes. *atcdc48a mos2 snc1 npr1* quadruple mutants display *snc1*-like phenotypes of dwarf stature, dark green, curly leaves as well as constitutive *PR* gene expression and resistance to *H.a.* Noco2.

Phylogenetic analysis confirmed the conserved nature of this protein. The *muse8* mutation was a single nucleotide polymorphism located in the first AAA ATPase domain of AtCDC48A, which, when folded in complex, resides inside the core of the cylindrical AtCDC48A homohexamer. This mutation results in an amino acid change from glycine, the smallest of amino acids often found in the centre of tight conformations and the only amino acid capable of binding phosphates of ATP, to glutamic acid and may hinder AtCDC48A activity. This mutation does however appear to be leaky. Previous studies regarding AtCDC48A have been difficult as all previous *atcdc48a* mutant lines described are lethal (Park et al., 2008). The *muse8* allele of *atcdc48a* is unique in that homozygous mutants, although sterile, do survive and may be useful in further characterization of this protein complex and its many functions.

Microarray expression data further confirmed *muse8* as *AtCDC48*. Quadruple mutant plants homozygous for *muse8* are female sterile, which is reflected in the tissue specific expression analysis. *AtCDC48A* is lowly expressed in mature pollen; however, it is highly expressed in carpels, as well as in developing embryos supporting the role of AtCDC48A in female fertility and embryogenesis as various *atcdc48a* mutants display female infertility and arrest at embryogenesis. Interestingly, *AtCDC48A* is also highly expressed in tissues that may interface with pathogens such as the roots, cauline leaves, and senescing leaves. Expression data of *AtCDC48A* also supports the role of AtCDC48A in proteasome-mediated proteolysis as in response to a proteasome inhibitor, MG132; without a functional proteasome, need for protein

degradation would increase, and the concentration of all positive regulators of proteasome-mediated protein degradation would be expected to increase in an attempt to compensate for this lack of degradation, which is what we observed for *AtCDC48A*. Similarly, a lack of protein synthesis would decrease the need for protein degradation; therefore, we would expect expression of positive regulators of protein degradation to increase, which is observed in *AtCDC48A* when exposed to cyclohexamide, a protein synthesis inhibitor.

AtCDC48A does appear to be involved in the regulation of plant innate immunity through proteasome-mediated protein degradation. This may be accomplished in a variety of ways; however, we propose one possible model (Fig. 3.8). Perhaps AtCDC48A regulates plant innate immunity through the degradation of positive regulators of defence. For example, it is known that SNC1 is negatively regulated by CPR1 in association with the SCF complex, which in turn poly-ubiquitinates SNC1, marking it for degradation (Cheng and Li, 2012). AtCDC48A may bind an E2 enzyme that recruits SNC1 and facilitate its association with the SCF complex. Once SNC1 is poly-ubiquitinated, AtCDC48A may disassemble the E2/SCF complex allowing the SCF complex to bring SNC1 to the proteasome for degradation, thus negatively regulating SNC1-mediated defence. Future studies must be conducted to find binding partners of this protein complex to elucidate which proteins are degraded to negatively regulate the immune response. The partial loss-of-function allele described here may prove to be an essential tool in discovering the many functions of the AtCDC48 complex.

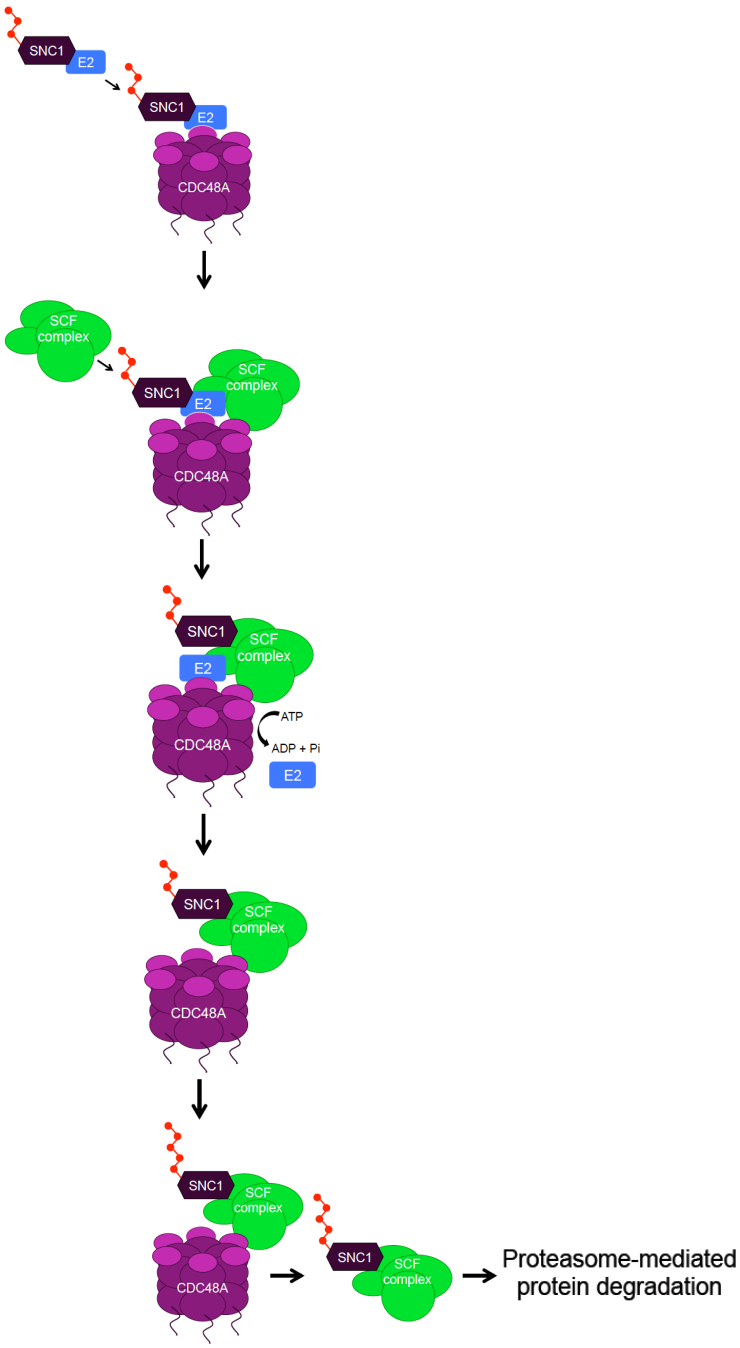


Figure 3.8: Proposed model of AtCDC48A in protein degradation: regulation of plant immunity.

SNC1 is recruited by an E2 enzyme (blue), which then binds with AtCDC48A (purple). An E3 ligase/complex such as the SCF complex (green) is also recruited to AtCDC48A. The target protein, SNC1 is then transferred to the SCF complex. AtCDC48A uses energy from ATP to dissociate E2 from the SCF complex. SNC1 is poly-ubiquitinated (red) by the SCF and subsequently, poly-ubiquitinated SNC1/SCF complex dissociates from AtCDC48A. The SCF complex then brings SNC1 to the proteasome for degradation.

4 Future directions and concluding remarks

4.1 *snc1-4d*

Several alleles of the *snc1* gain-of-function mutant have been found previously. In this study we have described the discovery of yet another allele of *snc1, snc1-4d*. Unlike other alleles of *snc1*, *snc1-4d* contains a mutation in the NB region of this R-like protein. *snc1-4d* mutants show enhanced disease resistance and *PR* gene expression. These mutants are much smaller than *snc1* original mutants and are sterile. SNC1 protein level was shown to be unchanged in these mutants in comparison to wild-type Col-0 plants.

Future experiments will be conducted to determine the effects of the *snc1-4d* allele on the SNC1 protein. Since SNC1 protein level was not altered in this allele, the observed enhanced *snc1* phenotype is not due to an increase in SNC1 stability. The mutation in the NB domain of this R-like protein may cause an increase in SNC1 activation. Since the NB domain has been implicated in ATP-binding and the conversion of ATP to ADP is required for R protein conformational change and activation, investigation into the ability of *snc1-4d* to bind ATP may be conducted. This allele may prove instrumental in the discovery of the activation mechanism of SNC1.

4.2 AtCDC48A

Here I have shown that a mutation in *AtCDC48A* is responsible for the enhanced *snc1*-like phenotype observed in *muse8 mos2 snc1 npr1* mutant plants. This mutation causes increased disease resistance, as well as the restoration of constitutive *PR* gene expression and is the first example of AtCDC48A's involvement in plant disease resistance. Expression data analysis supports the notion that *AtCDC48A* is involved in proteasome-mediated protein degradation, since it is up-regulated when the proteasome is inhibited but down-regulated when protein

synthesis is inhibited. Not only is AtCDC48A homologous to many other proteins in other eukaryotes, it is also homologous to other *Arabidopsis* proteins. The AtCDC48 family is composed of five members, A-E. AtCDC48A is by far the most highly expressed in all tissues and after pathogen attack. Previous studies on AtCDC48A revealed that homozygous *atcdc48a* null mutants are lethal; however, in this study we have found a unique allele of *AtCDC48A*. This partial loss-of-function allele is not lethal when homozygous and will therefore be an essential tool in future studies of AtCDC48A.

There are many questions about the roles of AtCDC48A. Previously this protein, which forms a homohexameric complex, has been shown to bind proteins associated with ubiquitination and proteasome degradation. Future studies will be conducted to determine if AtCDC48A binds proteins that are not only associated with ubiquitination and degradation but also plant innate immunity. CPR1, for example, seems a likely candidate as it negatively regulates the R-like protein SNC1 through association with the SCF complex, which is involved in protein degradation (Gou et al., 2012). This can be achieved by first examining the level of SNC1 protein in *atcdc48a* mutants. Second, direct interaction between AtCDC48A and CPR1 could be determined through a yeast-two-hybrid or Bimolecular Fluorescence Complementation (BiFC). Another possible candidate is the *Arabidopsis* homolog of yeast UFD2 protein, which is an E4 ligase that has been shown to bind CDC48 in yeast (Baek et al., 2011).

Since this is the first report of AtCDC48A being involved in plant defence, future studies will be conducted to determine where it acts in the plant immunity pathway. Crosses will be made with *pad4/eds1* mutants, which are defective in TIR-NB-LRR signal transduction. This will determine whether AtCDC48A acts prior to TIR-NB-LRR action or after signal transduction

has commenced. Crosses will also be done between *atcdc48a* mutants and loss-of-function *snc1* mutants to determine if *atcdc48a* relies on SNC1.

Analyses will also be conducted with the AtCDC48 homologs to determine if they share similar functions as AtCDC48A and in similar pathways. Morphology, pathogen resistance and defence responses will be assessed in loss of function T-DNA insertion lines for each AtCDC48 homolog. Subsequent experiments will be designed based on the findings from these studies.

4.3 Concluding statements

This thesis work provides tools for investigating the regulation of plant innate immunity. The *snc1-4d* allele provides a unique opportunity to study the highly conserved residue in the NB domain and perhaps lead to a more concrete model of R protein dimerization and activation. The allele of *AtCDC48A*, *muse8*, may also prove to be indispensible. This allele is unique in that it is currently the only allele of *AtCDC48A* that is not lethal when homozygous. Previous studies of AtCDC48A functions were carried out using mutants of AtCDC48A's binding partners due to difficulty in working with the available homozygous lethal *atcdc48a* mutants. Now, we can utilize the *muse8* allele to study the many binding partners and therefore functions of this complex, which is truly a 'jack of all trades.'

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