DIVERSE ROLES OF THE UBIQUITIN PROTEASOME SYSTEM IN REGULATION
OF PLANT IMMUNITY

by

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B.Sc., University of Alberta, 2012

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
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THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

Diverse roles of the ubiquitin proteasome system in regulation of plant immunity

Submitted by  Charles Copeland in partial fulfillment of the requirements for the degree of  Doctor of Philosophy in  Botany

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Abstract

Plants rely on a variety of immune signaling pathways to recognize pathogens and defend against pathogen attacks. In order to effectively recognize pathogens without causing autoimmunity, homeostasis of the proteins in these pathways must be maintained at optimal levels. The ubiquitin proteasome system (UPS) is an important mechanism by which the turnover of proteins, including immune system components, is achieved. This thesis describes novel roles for three UPS-related proteins in the regulation of plant immunity. The conserved E3 ligase C-TERMINUS OF HSP70 INTERACTING PROTEIN (AtCHIP) is a positive regulator of immunity in Arabidopsis, as overexpression of AtCHIP causes enhanced disease resistance. Loss of AtCHIP function causes enhanced susceptibility to virulent pathogens, but does not affect resistance mediated by Nucleotide-binding domain and leucine-rich repeat (NLR) receptors. The stability of the chaperone HEAT SHOCK PROTEIN 90.3 (HSP90.3) is also normal in atchip plants.

Previous studies have shown that UPS also functions as a negative regulator of immunity, by preventing excessive accumulation of NLR proteins such as SUPPRESSOR OF NPR1, CONSTITUTIVE 1 (SNC1). CELLCYCLE DIVISION CYCLE 48A (AtCDC48A) was identified as a negative regulator of immunity in a forward genetic screen for enhancers of autoimmunity caused by a gain-of-function allele of snc1. atcdc48A-4 plants exhibit dwarf morphology and enhanced disease resistance to the oomycete pathogen Hyaloperonospora arabidopsidis (H.a.) Noco2. SNC1 protein level is increased in atcdc48A plants, and AtCDC48A interacts with the E4 enzyme MUTANT, SNC1 ENHANCING 3 (MUSE3) in co-immunoprecipitation experiments, supporting a role for
AtCDC48A in NLR turnover. Loss of PROTEASOME REGULATOR 1 (PTRE1), a positive regulator of protein turnover through the UPS, also causes enhanced disease resistance and increases SNC1 protein stability, further supporting the importance of proteasomal degradation in NLR homeostasis. Thus, the UPS plays both positive and negative roles in immune regulation.
Lay Summar

Plants are exposed to a huge diversity of microbes in their environment, some of which have the potential to cause disease. Therefore, plants have evolved a sophisticated immune system to recognize these potential pathogens and defend against them. However, excessive activation of these immune responses is very detrimental to plant growth, so the immune system must be delicately regulated.

The objective of this thesis was the identification and characterization of genes involved in immune regulation through targeted degradation of immune-related proteins. Both positive and negative roles in immune regulation are described. One gene, *AtCHIP*, was shown to be important for disease resistance through an unknown mechanism. In contrast, two genes *AtCDC48A* and *PTRE1*, are negative regulators of immunity, by promoting the degradation of an immune receptor. Together, this thesis contributes to the current knowledge of the role of protein degradation in plant immune regulation.
Preface

The research contained in this thesis was performed between September 2012 and January 2018. Chapters 2 and 3 have previously been published, and a manuscript for publication has been prepared from the material in Chapter 4. The details of my personal contributions are as follows:

Chapter 2 has been published as:


- I performed most of the experiments. M. Tong and Y. Huang isolated the homozygous *atchip* mutant lines, and K. Ao, Y. Huang, and M. Tong performed some of the infection experiments. I wrote the manuscript together with K. Ao. X. Li supervised the work and the preparation of the manuscript.

Chapter 3 has been published as:

**Copeland, C.**, Woloshen, V.*, Huang, Y., Li, X. (2016) AtCDC48A is involved in the turnover of an NLR immune receptor. Plant J 88: 294-305 (*Co-first authors)

- I performed most of the experiments except for the cloning of *AtCDC48A*. V. Woloshen cloned *AtCDC48A* and produced the AtCDC48A-GFP construct. Y. Huang produced the MUSE3-flag construct and assisted with the co-immunoprecipitation. X. Li supervised the work and the preparation of the manuscript.
A manuscript has been prepared from Chapter 4 as:

Copeland, C., Li, X. The proteasome regulator PTre1 positively contributes to NLR degradation in Arabidopsis. Manuscript in preparation.

- I performed the experiments and wrote the manuscript. X. Li supervised the work and the preparation of the manuscript.
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<tbody>
<tr>
<td>AAA</td>
<td>ATPase associated with diverse cellular activities</td>
</tr>
<tr>
<td>ABRC</td>
<td>Arabidopsis Biological Resource Center</td>
</tr>
<tr>
<td>Avr</td>
<td>Avirulence; refers to pathogen effectors</td>
</tr>
<tr>
<td>Bgh</td>
<td><em>Blumeria graminis f.sp. hordei</em></td>
</tr>
<tr>
<td>CC</td>
<td>Coiled-coil domain</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CPK</td>
<td>Ca²⁺-dependent protein kinase</td>
</tr>
<tr>
<td>CP</td>
<td>Core particle</td>
</tr>
<tr>
<td>CRL</td>
<td>Cullin-RING ligase</td>
</tr>
<tr>
<td>ERAD</td>
<td>endoplasmic reticulum-associated degradation</td>
</tr>
<tr>
<td>ETI</td>
<td>Effector-triggered immunity</td>
</tr>
<tr>
<td>flg22</td>
<td>An epitope of bacterial flagellin</td>
</tr>
<tr>
<td>H.a.</td>
<td><em>Hyaloperonospora arabidopsidis</em></td>
</tr>
<tr>
<td>HR</td>
<td>Hypersensitive response</td>
</tr>
<tr>
<td>MAMP</td>
<td>Microbe-associated molecular pattern</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MOS</td>
<td>Modifier of SNC1</td>
</tr>
<tr>
<td>Ler</td>
<td>Landsberg <em>erecta</em></td>
</tr>
<tr>
<td>Nat</td>
<td>N-terminal acetyltransferase complex</td>
</tr>
<tr>
<td>NEL</td>
<td>Novel E3 ligase</td>
</tr>
<tr>
<td>NLR</td>
<td>A protein with nucleotide-binding, leucine-rich repeat domains, also referred to as NOD-like receptors</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PR</td>
<td>Pathogenesis Related</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PTI</td>
<td>PAMP-triggered immunity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td><em>P.s.m.</em></td>
<td><em>Pseudomonas syringae pv maculicola</em></td>
</tr>
<tr>
<td><em>P.s.t.</em></td>
<td><em>Pseudomonas syringae pv tomato</em></td>
</tr>
<tr>
<td>R</td>
<td>Resistance; refers to genes or proteins that recognize pathogen effectors</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>RhoGAP</td>
<td>RhoGTPase activating protein</td>
</tr>
<tr>
<td>RLK</td>
<td>Receptor-like kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RP</td>
<td>Regulatory particle</td>
</tr>
<tr>
<td>SA</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>SCF</td>
<td>Skp1-culling-F-box</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/Interleukin1 receptor homology domain</td>
</tr>
<tr>
<td>TPR</td>
<td>Tetratrichopeptide repeat</td>
</tr>
<tr>
<td>UBC</td>
<td>Ubiquitin conjugating</td>
</tr>
<tr>
<td>TNL</td>
<td>TIR-type NLR protein</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin-proteasome system</td>
</tr>
<tr>
<td><em>Xoo</em></td>
<td><em>Xanthomonas oryzae pv oryzae</em></td>
</tr>
</tbody>
</table>
### List of Gene and Protein Names

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
<th>Function and relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABI1</td>
<td>Abscisic acid Insensitive 1</td>
<td>Protein phosphatase that functions as a negative regulator of abscisic acid signalling; reported to be a substrate of PUB12/13</td>
</tr>
<tr>
<td>ACTIN7</td>
<td>Actin 7</td>
<td>A component of the cytoskeleton; <em>ACTIN7</em> is often used as a reference gene when analyzing transcript levels</td>
</tr>
<tr>
<td>APIP6</td>
<td>AvrPiz-t Interacting Protein 6</td>
<td>A RING-type E3 ligase in rice that positively regulates immunity, possibly by targeting OsELF3 for degradation. The <em>Magnaporthe oryzae</em> effector AvrPiz-t destabilizes APIP6</td>
</tr>
<tr>
<td>APIP10</td>
<td>AvrPiz-t Interacting Protein 10</td>
<td>A RING-type E3 ligase in rice that positively regulates immunity through an unknown mechanism. The <em>Magnaporthe oryzae</em> effector AvrPiz-t destabilizes APIP10 and may inhibit its E3 ligase activity. APIP10 also targets the NLR Piz-t</td>
</tr>
<tr>
<td>ATE</td>
<td>Argenyl Transferase</td>
<td>Enzymes that catalyze the addition of an arginyl group to the N-terminus of nascent peptides, which can be recognized by PRT E3 ligases</td>
</tr>
<tr>
<td>AvrPtoB</td>
<td><em>Pseudomonas syringae</em> pv. <em>tomato</em> effector that acts as an E3 ligase, targeting multiple Arabidopsis proteins in order to disrupt PTI and ETI</td>
<td></td>
</tr>
<tr>
<td>BAK1</td>
<td>BRI1-Associated Kinase 1</td>
<td>Co-receptor for signalling by many RLKs, including FLS2 in flagelling sensing</td>
</tr>
<tr>
<td>BTB</td>
<td>Broad-Complex, Tramtrack and Bric a brac</td>
<td>Components of BTB E3 ligases complexes involved in targeted protein degradation</td>
</tr>
<tr>
<td><strong>CDC48</strong></td>
<td><em>Arabidopsis thaliana</em> Cell Division Cycle 48</td>
<td>AAA ATPase family; AtCDC48A is characterized in this dissertation as a negative regulator of SNC1 stability in Arabidopsis</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>CERK1</strong></td>
<td>Chitin Elicitor Receptor Kinase 1</td>
<td>Receptor kinase that works together with LYK5 to mediate chitin perception and downstream signaling</td>
</tr>
<tr>
<td><strong>CHIP</strong></td>
<td>C-terminus of HSP70 Interacting Protein</td>
<td>U-box type E3 ligase; AtCHIP is characterized in this dissertation as a positive regulator of immunity in Arabidopsis</td>
</tr>
<tr>
<td><strong>CLP</strong></td>
<td>Caseinolytic protease</td>
<td>Chloroplastic protease complex; one of the subunits ClpP4 is a substrate of AtCHIP in Arabidopsis</td>
</tr>
<tr>
<td><strong>CPR1</strong></td>
<td>Constitutive expressor of PR genes 1</td>
<td>F-box protein found in the SCF complex that targets a subset of NLRs including SNC1. <em>cpr1</em> mutants show increased SNC1 stability and autoimmune phenotypes</td>
</tr>
<tr>
<td><strong>CUL</strong></td>
<td>Cullin</td>
<td>Components of CRL E3 ligases complexes involved in targeted protein degradation</td>
</tr>
<tr>
<td><strong>EBR1</strong></td>
<td>Enhanced Blight and Blast Resistance 1</td>
<td>A rice E3 ligase that negative regulates cell death and immunity by targeting OsBAG1 for degradation</td>
</tr>
<tr>
<td><strong>EDS1</strong></td>
<td>Enhanced Disease Susceptibility 1</td>
<td>Important immune component required for NLR signalling and other immune-signalling pathways. <em>eds1</em> mutants are very susceptible to infection and are often used as positive controls in infection assays</td>
</tr>
<tr>
<td><strong>EIRP1</strong></td>
<td><em>Erysiphe necator</em>-induced RING finger protein 1</td>
<td>RING-type E3 ligase induced during <em>Erysiphe necator</em> infection that positively regulates immunity by targeting the transcription factor WRKY11</td>
</tr>
<tr>
<td><strong>Fen</strong></td>
<td>Fenthion (sensitive)</td>
<td>Intracellular kinase</td>
</tr>
<tr>
<td>Gene/Protein</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>FLS2</td>
<td>Flagellin Sensing 2 - Membrane-localized receptor for bacterial flagellin</td>
<td></td>
</tr>
<tr>
<td>FtsH</td>
<td>Chloroplastic protease complexes; FtsH subunits are substrates of AtCHIP</td>
<td></td>
</tr>
<tr>
<td>HopM1</td>
<td>An effector from <em>Pseudomonas</em> that interacts with Rad23 and proteasome subunits, and may cause increased degradation of immunity-related Arabidopsis proteins</td>
<td></td>
</tr>
<tr>
<td>HvRACB</td>
<td>A Rac-type GTPase from barley that is a susceptibility factor for <em>Bgh</em> infection, and which is likely targeted by a CRL complex containing SKP1</td>
<td></td>
</tr>
<tr>
<td>HSP</td>
<td>Heat Shock Protein - Chaperone proteins that aid in folding and stability of client proteins, and facilitate degradation of misfolded proteins; HSP90 has both positive and negative roles in immune regulation</td>
<td></td>
</tr>
<tr>
<td>LYK5</td>
<td>Lysin motif receptor Kinase 5 - Receptor-like kinase involved in perception of the PAMP chitin in Arabidopsis, reported to be a substrate of PUB13</td>
<td></td>
</tr>
<tr>
<td>MIN7</td>
<td>HopM1 Interacting Protein 7 - A positive regulator of immunity in Arabidopsis that interacts with the effector HopM1, which reduces the stability of MIN7</td>
<td></td>
</tr>
<tr>
<td>MIR1</td>
<td>MLA Interacting RING-type E3 ligase - E3 ligases that interacts with the NRLs MLA1 and MLA10 and targets them for degradation</td>
<td></td>
</tr>
<tr>
<td>MLA</td>
<td>Mildew A - a group of allelic NLRs from barley that mediate resistance to specific isolates <em>Blumeria graminis</em>, which causes of powdery mildew</td>
<td></td>
</tr>
<tr>
<td>MOS2</td>
<td>Modifier of SNC1 2 - An RNA binding protein that positively regulates SNC1 function though its involvement in proper SNC1 splicing</td>
<td></td>
</tr>
<tr>
<td><strong>MUSE3</strong></td>
<td>Mutant, SNC1 Enhancing 3</td>
<td>The only identified E4 ligase in Arabidopsis; MUSE3 negatively regulates immunity by facilitating SNC1 turnover</td>
</tr>
<tr>
<td><strong>MUSE13/14</strong></td>
<td>Mutant, SNC1 Enhancing 13/14</td>
<td>TRAF-domain containing proteins that interact with the SCF$^{CPR1}$ complexe and negatively regulate immunity by facilitating SNC1 turnover</td>
</tr>
<tr>
<td><strong>NAA</strong></td>
<td>N-acetyl transferase</td>
<td>Subunits of the Nat complexes, which transfer acetyl groups to the N-termini of proteins. The substrates of each Nat complex and the effect of acetylation on its target protein are dependent on the N-terminal amino acids. In Arabidopsis, NatA and NatB antagonistically control the stability of SNC1 protein. NAA15 was identified in the <em>MUSE</em> screen as MUSE6</td>
</tr>
<tr>
<td><strong>NPR1</strong></td>
<td>Non-expressor of PR genes 1</td>
<td>One of the SA receptors in Arabidopsis; after perception of SA, NPR1 localizes to the nucleus and interacts with TGA transcription factors to induce transcription of immunity-related genes</td>
</tr>
<tr>
<td><strong>OsBAG1</strong></td>
<td>Bcl-2- Associated Athanogene</td>
<td>A rice protein that contributes to immunity and cell death through an unknown mechanism, and is targeted for degradation by EBR1. BAG proteins in mammals function in diverse processes including apoptosis</td>
</tr>
<tr>
<td><strong>OsPUB15</strong></td>
<td>Plant U-box 15</td>
<td>U-box type E3 ligase in rice that positively regulates immunity; it is phosphorylated and possibly activated by the RLK PID2</td>
</tr>
<tr>
<td><strong>OsRac1</strong></td>
<td></td>
<td>A Rac-type GTPase in rice that contributes to ROS production and cell death</td>
</tr>
<tr>
<td><strong>PAD4</strong></td>
<td>Phytoalexin-deficient 1</td>
<td>Works together with EDS1 in NLR and other immune signalling</td>
</tr>
<tr>
<td><strong>PID2</strong></td>
<td></td>
<td>an RLK in rice that mediates resistance against specific strains of <em>Magnaporthe oryzae</em>, the</td>
</tr>
</tbody>
</table>
causal agent of rice blast

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>POB1</td>
<td>POZ/BTB-containing G-protein 1</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>PR</td>
<td>Pathogenesis-Related</td>
</tr>
<tr>
<td>PRT</td>
<td>Proteolysis</td>
</tr>
<tr>
<td>PTRE1</td>
<td>Proteasome Regulator 1</td>
</tr>
<tr>
<td>PUB12/13</td>
<td>Plant U-box 12/13</td>
</tr>
<tr>
<td>PUB17</td>
<td>Plant U-box 17</td>
</tr>
<tr>
<td>PUB43</td>
<td>Plant U-box 43</td>
</tr>
<tr>
<td>RAD23</td>
<td>Radiation Sensitive</td>
</tr>
<tr>
<td>RAR1</td>
<td>Required for MLA12 Resistance 1</td>
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<td>Gene</td>
<td>Description</td>
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<td>-----------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>RBOHD</td>
<td>Respiratory Burst Oxidase Homolog D</td>
</tr>
<tr>
<td>RGA1</td>
<td></td>
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<tr>
<td>RPM1</td>
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<td>RPN</td>
<td>Regulatory Particle Non-ATPase</td>
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<td>RPP5</td>
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Dedication

To adventure
Chapter 1: Introduction

The plant immune system

Plants are exposed to a huge diversity of microbes in their environment, many of which are potentially pathogenic. If allowed to grow unrestricted, these pathogens have a devastating effect on plant health, including destruction of crops that are vitally important to humans (Dangl et al., 2013b). However, plants have evolved a sophisticated innate immune system to recognize microbial threats and prevent the establishment of infection by most microbes (Dangl and Jones, 2001).

The most general mechanism of pathogen perception involves recognition of Pathogen-Associated Molecular Patterns (PAMPs, also called Microbe-Associated Molecular Patterns or MAMPs), which are often conserved molecules that play important functions for the survival of the microbes (Jones and Dangl, 2006). PAMPs are recognized by plasma membrane-localized pattern recognition receptors (PRRs), which either contain a kinase domain, or associate with functional kinases after PAMP binding (Macho and Zipfel, 2014; Zipfel, 2014). Many PRRs utilize a common receptor-like kinase (RLK) BIR1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) as co-receptor for signaling. The most thoroughly studied example of PAMP perception is the recognition of flg22, an epitope of bacterial flagellin, by FLAGELLIN SENSING 2 (FLS2) (Gómez-Gómez and Boller, 2000; Chinchilla et al., 2006). Other examples of PAMPs include elongation factor Tu and peptidoglycan from bacteria, and chitin from fungi (Zipfel, 2014).

PAMP recognition results in an immune response known as PAMP-triggered immunity (PTI). Early events in PTI signaling involve phosphorylation cascades.
Activated PRRs and their co-receptors phosphorylate receptor-like cytoplasmic kinases, which in turn initiate mitogen-activated protein kinase (MAPK) cascades and phosphorylation of Ca$^{2+}$-dependent protein kinases (CPKs). The NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOG D (RbohD) can be phosphorylated by cytoplasmic kinases and CPKs, and produces a Reactive Oxygen Species (ROS) burst. The important defense hormone salicylic acid (SA) is also produced during PTI, although it is not required for many aspects of PTI signaling (Nürnberger and Kemmerling, 2009; Seyfferth and Tsuda, 2014). These signaling events lead to transcriptional changes, in which the WRKY family of transcription factors plays a major role. The defense outputs from PTI are quite effective in curtailing pathogen growth.

Because PAMPs are often indispensable for microorganisms, they tend to evolve slowly; therefore, a relatively small set of PRRs is capable of recognizing PAMPs from most microbes. However, host-adapted pathogens avoid triggering a PTI response by deploying effector molecules into the plant cell, which suppress PTI signaling (Jones and Dangl, 2006). Effectors are generally small diverged proteins with extremely varied biochemical activities, which may target plant proteins in all steps of PTI signaling (Büttner, 2016). By preventing PTI, these virulent pathogens are able to avoid detection and establish a successful infection.

In order to recognize and respond to adapted pathogens, plants have evolved Resistance (R) proteins that can be activated in the presence of the effector molecules and initiate a rapid and strong immune response known as Effector Triggered Immunity (ETI) (Jones and Dangl, 2006). In contrast to the conserved nature of PAMPs, the effector repertoires of pathogens are very specific, often differing between isolates of the same
pathogen species. ETI is activated and virulence overcome only if the plant contains a cognate R protein for one of these effectors.

Most R proteins belong to the NOD-like receptor (NLR) family, named for the presence of nucleotide-binding and leucine-rich repeat domains. In higher plants, they often contain other diverse domains (Li et al., 2015). A few R proteins are known to interact directly with the effector, while others guard host proteins such as components of the PTI signaling pathway and are activated if these components are perturbed.

The mechanisms of R protein activation are still unclear, and are dependent on the type of R protein. The presence of the effector may enact a conformational change in the NLR, releasing the signaling domain from auto-inhibition (Bernoux et al., 2011). Several NLRs have recently been shown to form dimers or oligomers which are important for signaling, although the nature of these complexes differs between NLRs (Li et al., 2015). The downstream signaling components required for ETI are also largely unknown. One major group of NLRs, which is distinguished by the presence of an N-terminal Toll/Interleukin1 receptor homology (TIR) domain, require the lipase-like proteins ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and PHYTOALEXIN DEFICIENT 4 (PAD4) or SENESCENCE ASSOCIATED GENE (SAG101) for their signaling (Wiermer et al., 2005). SA is also important in R protein signaling, although its role is again complex. ETI and PTI appear to share a number of defense outputs. However, ETI often results in stronger outcomes that result in a type of localized programmed cell death known as the hypersensitive response.

While PTI and ETI are crucial to preventing disease establishment, they can be detrimental to plant growth if uncontrolled. Mutations that cause constitutive immune
signaling usually also result in a dwarf stature, and may be lethal (Li et al., 2001; Liu et al., 2016). Thus, the immune system must be tightly regulated to ensure that immune signaling occurs rapidly and robustly, but only when pathogens become a threat. Production of immune components is regulated at many levels, including transcription, splicing, and translation (Johnson et al., 2013; Johnson et al., 2016). In addition, existing immune proteins are degraded either to prevent excess accumulation, or to rapidly remove negative regulators during immune signaling. Much of this degradation occurs through the ubiquitin proteasome system.

**The ubiquitin-proteasome system**

Targeted protein degradation by the ubiquitin-proteasome system (UPS) is conserved throughout the eukaryotes, and provides a mechanism to eliminate particular proteins from the cell in a rapid and controlled manner. Plants in particular rely on the involvement of the UPS for myriad signaling pathways, which is reflected by the fact that more than 5% of Arabidopsis genes are thought to encode UPS components (Vierstra, 2009). Higher plant genomes also encode much more expanded E3 families than in mammalian genomes.

Protein targeting in the UPS is accomplished by the addition of the small peptide ubiquitin onto substrate proteins. In order to be conjugated, the ubiquitin is first activated by an E1 ubiquitin activating enzyme, which consumes ATP to form a thioester bond between itself and a ubiquitin molecule. The ubiquitin is then transferred to an E2 ubiquitin conjugating enzyme, again via a thioester bond.
The E2-ubiquitin conjugates are brought into association with specific target proteins through binding with E3 ligases. While a large majority of E3 ligases interact with the E2 through a REALLY INTERESTING NEW GENE (RING)-like domain, the structure of the enzyme is quite variable. Some E3s have their E2 binding domain and the substrate-binding domain together in a single peptide. These simple E3s include RING as well as U-box E3 ligases, which have a related E2 binding domain. In contrast, other complex E3s have a multimeric structure, with the substrate-binding and RING domain on different subunits. Such E3s are referred to as Cullin-RING ubiquitin ligases (CRLs), as a Cullin protein serves as a scaffold between the RING-containing subunit and the substrate recognition domain.

CRL ligases can be further divided into different types. S-phase kinase-associated protein (Skp1)-cullin-F-box (SCF) E3s contain cullin1 (CUL1) as a scaffold, binding to a homolog of Skp1. Skp1 also binds to proteins with an F-box domain and various substrate recognition domains. Other types of CRL ligases differ in which CUL subunit forms the scaffold, and how the substrate-recognition subunit interacts with the rest of the complex. CUL3 often connects proteins with a Broad-Complex, Tramtrack and Bric a brac (BTB) domain to the RING-containing subunit, without a corresponding Skp-like adapter. In higher plants, SCF seems to be the most expanded E3 group, with over 700 encoded in the Arabidopsis genome, in contrast with approximately 70 found in human (Nakayama and Nakayama, 2006). This reflects the importance of SCFs in controlling diverse biological processes in plants.

When an E3 ligase enzyme interacts the E2-ubiquitin conjugate and its substrate, the ubiquitin is transferred to a lysine residue of the substrate with an
isopeptide bond (Zheng and Shabek, 2017). E3s can mediate the addition of ubiquitin onto the substrate itself, or onto one of the lysine residues on a previously transferred ubiquitin, creating a ubiquitin chain. The lysine residue of ubiquitin that is used for this addition has important ramifications for the fate of the protein. Proteolysis by the UPS, the most well-known common outcome of ubiquitination, generally relies on chains formed by linkages at lysine 48 (K48) (Yu and Matouschek, 2017). The role of other chain topologies is less clear, but both mono-ubiquitination or K63-linked chains may trigger formation of protein complexes and subsequent protein activation, or affect protein localization through endocytosis (Chen and Sun, 2009; Tomanov et al., 2014).

Poly-ubiquitinated substrates are most often recognized and degraded by the 26S proteasome, a large protein complex with diverse subunits. The proteolytic activity of the proteasome occurs in the 20S core particle (CP), which is composed of four heptameric rings arranged in a tube-like structure. The inner rings contain the proteolytic active sites, which are oriented towards the inside of the tube, while the N-termini of subunits of the outer rings form a gate that blocks protein entry (Schmidt and Finley, 2014). Therefore, the 20S core particle shows very minor proteasome activity without the 19S regulatory particle (RP), which binds to the ends of the 20S CP and mediates gate opening. Opening of the gate is dependent on a hexameric ring of AAA ATPases which form the base of the 19S RP (Bar-Nun and Glickman, 2012). ATP hydrolysis by these ATPases additionally provides the mechanical force necessary to unfold substrates before they enter the 20S CP. A number of non-ATPase subunits perform other functions required for proteasome activity (Schmidt and Finley, 2014). For example, the ubiquitin receptors RPN10 and RPN13 facilitate substrate recognition by
binding to poly-ubiquitinated proteins. Alternately, the poly-ubiquitinated substrates may be shuttled to the proteasome by substrate adaptor proteins (Yu and Matouschek, 2017). The deubiquitination activity of RPN11, which removes the ubiquitin peptides and allows them to be reused, is also required for substrate degradation. Once degradation is initiated, the substrate is cleaved by the protease subunits into very small peptides.

**E3 ligases with immune-related functions**

Because of the importance of E3 ligases in specifying targets of the UPS, the study of E3 ligases and their substrates has been a focus of research in plant biology (Vierstra, 2009). Because the discovery of immune-related E3 ligases has been summarized in several excellent reviews (Duplan and Rivas, 2014; Furniss and Spoel, 2015), this review will focus on recent reports of E3s that affect immune signaling.

**E3s involved in the turnover of immune receptors**

While immune receptors must be constitutively expressed in preparation for pathogen perception, excessively high levels of the receptors can lead to auto-activation, resulting in autoimmunity. Therefore, the abundance of many immune receptors is negatively regulated through degradation by the UPS, and a number of the responsible E3 ligases have been identified. PUB12 and PUB13, which encode a pair of U-box E3 ligases, have been reported to be important for degradation of FLS2 (Lu et al., 2011). While PUB12 and PUB13 were shown to ubiquitinate FLS2 in vitro, constitutive levels of FLS2 were not increased in the pub12 pub13 double mutant plants. Rather, the pub12
*pub13* mutation abolished the flg22-mediated reduction in FLS2 levels. These PUBs are phosphorylated by BAK1 after flg22 treatment, leading to the hypothesis that they negatively regulate the level of FLS2 specifically during flg22 signaling. However, Li et al. (2012) reported constitutive ROS production and cell death in the *pub13* mutant which was dependent on the SA signaling pathway. Recently PUB13 has also been shown to ubiquitinate the chitin receptor LYK5, and in this case LYK5 levels were constitutively increased in the *pub13* mutant (Liao et al., 2017). Adding to the complexity is the report that PUB12 and PUB13 ubiquitinate ABI1, a negative regulator in the signaling pathway of the hormone abscisic acid (ABA) (Kong et al., 2015). FLS2 and LYK5 are membrane-localized receptor-like kinases, while ABI1 is an intracellular protein phosphatase. Although some E3 ligases may target multiple substrates, such reports are rare in plants (Vierstra, 2009). Further research is required to disentangle the biological functions of PUB12 and PUB13.

Degradation of NLR proteins is also critical in avoiding autoimmunity. For example, the F-box protein CPR1 was shown to target the NLR SNC1 (Cheng et al., 2011 and Figure 1.1). Loss of CPR1 results in increased SNC1 protein stability and constitutive immune signaling, while CPR1 overexpression reduces SNC1 protein level and suppresses the phenotype of the gain-of-function *snc1* mutant. In another example, MLA1 and MLA10, NLRs from barley, are targeted by the RING E3 ligase MIR1 (Wang et al., 2016). While a *mir1* loss-of-function phenotype was not reported in this case, overexpression of MIR1 reduces the MLA protein level, which is associated with attenuated resistance to powdery mildew containing the cognate effectors for the MLA proteins.
Figure 1.1. A model of a SNC1 degradation pathway through the UPS before the current study.

(A) Nascent SNC1 polypeptides are acetylated by the NatA complex with NAA15/MUSE6 as a subunit, which serves as a degron.

(B) SNC1 protein is ubiquitinated by the SCF\textsuperscript{CPR1} E3 complex. A number of chaperones and other proteins are important for the E3 ligase activity, including HSP90/MUSE10/MUSE12, MUSE13/14, SGT1 and SRFR1.

(C) SNC1 is poly-ubiquitinated by the E4 enzyme MUSE3.

(D) Poly-ubiquitinated SNC1 is recognized by the 26S proteasome and degraded.

Pathogens can also employ effectors with E3 ligase activity to degrade immune receptors, allowing them to evade detection. One of the best studied examples of this is the effector AvrPtoB, which targets the receptor-like kinases FLS2 and CERK1 to
suppress PTI, and the cytoplasmic kinase Fen to suppress ETI (Rosebrock et al., 2007; Göhre et al., 2008; Gimenez-Ibanez et al., 2009). Although the primary sequence of AvrPtoB does not show similarity to known E3 ligase domains, analysis of the crystal structure of the C terminus revealed strong similarity to RING or U-box domains (Janjusevic et al., 2006). Many bacterial effectors have also been identified which possess E3 ligase activity through a Novel E3 Ligase (NEL) domain (Hicks and Galán, 2010). Unlike the C-terminus of AvrPtoB, the NEL domain is not structurally similar to other known E3 ligase domains. Although their specific targets are unknown, NEL-containing effectors from plant pathogens and symbionts have been shown to affect immune signaling and allow improved colonization by the bacteria. Transient expression of the NopM effector from *Rhizobium* reduces ROS production in response to flg22 treatment in tobacco, and this is dependent on a functional NEL domain (Xin et al., 2012). Mutation or deletion of NopM also impairs the ability of the *Rhizobium* to form nodules on a host legume. The *Ralstonia solanacearum* effectors RipAW and RipAR similarly inhibit the flg22-mediated ROS burst in tobacco leaves, causing enhanced disease susceptibility in Arabidopsis (Nakano et al., 2017).

**E3 ligases involved in downstream immune signaling**

In addition to receptors, the homeostasis of downstream immune signaling components also need to be controlled to avoid unnecessary and damaging immune responses without pathogens. For example, the rice U-box protein SPL11 is a negative regulator of immunity, and *spl11* mutants display spontaneous cell death and induction of
defense gene expression (Zeng et al., 2004). SPL11 is orthologous to Arabidopsis PUB13; however, unlike PUB13 it has not been reported to target an immune receptor. One potential target of SPL11 has been identified as SPIN6, a RhoGTPase activating protein (RhoGAP) (Liu et al., 2015). The two proteins directly interact, and co-expression of SPL11 and SPIN6 causes a dramatic reduction in SPIN6 protein level which is dependent on SPL11 E3 ligase activity. Unexpectedly, silencing or knocking out SPIN6 causes enhanced disease resistance, indicating that SPIN6 is itself a negative regulator of immunity. SPIN6 interacts with the GTPase OsRac1 and promotes GTP hydrolysis, so loss of SPIN6 would result in an increased proportion of active, GTP-bound OsRac1. Since OsRac1 has previously been shown to contribute to ROS production and cell death, this may explain the effect of SPIN6 on immunity (Kawano et al., 2010; Liu et al., 2015). However, further study is needed to reveal how SPL11 and SPIN6 both negatively regulate immunity.

Loss of the rice RING-type E3 ligase EBR1 also results in spontaneous cell death and resistance to the bacterial and fungal pathogens (You et al., 2016). EBR1 targets OsBAG4, and the increased levels of OsBAG4 in the ebr1 mutant are responsible for the resistant phenotype. In this case, it is unclear what molecular mechanism is responsible for the effect OsBAG4 on immunity.

E3 ligases themselves can be targeted for ubiquitination and degradation by the UPS. Many E3s can also self-ubiquitinate to control its own levels. In Nicotiana benthamiana, accumulation of PUB17 is reduced by overexpression of POB1, while silencing of POB1 increases PUB17 stability (Orosa et al., 2017). PUB17 was previously shown to promote immune-related cell death, and consistent with this, POB1
overexpression also reduced cell death triggered by a number of effectors. POB1 contains a BTB domain, which suggests that it forms part of a CRL complex together with CUL3. The increased turnover of PUB17 in POB1-overexpression plants was blocked by MG132, showing that PUB17 degradation is facilitated by the proteasome. However, the ubiquitination activity of POB1 has not been demonstrated.

After infection, proteasomal degradation plays an important positive role in immune signaling. A number of E3 ligases from rice have been reported to function downstream of specific receptors. The RING-type E3 Xb3 interacts with the Xa21, an RLK that confers resistance against the bacterial pathogen Xanthomonas oryzae pv oryzae (Xoo) (Wang et al., 2006). Xb3 can be phosphorylated by Xa21 in vitro, and silencing of Xb3 compromises Xa21-mediated resistance to Xoo. This suggests that following Xa21 activation during Xoo infection, Xb3 is phosphorylated, activating its E3 ligase function. Similarly, the U-box protein OsPUB15 interacts with the kinase domain of the RLK PID2 (Wang et al., 2015). The kinase domain of PID2 can phosphorylate OsPUB15 in vitro, and the E3 ligase activity of OsPUB15 seems to depend on this phosphorylation, providing another possible example of an E3 ligase becoming activated by phosphorylation and transducing further downstream signaling.

While the targets of Xb3 and OsPUB15 are expected to be negative regulators of immunity, their identities are unknown. However, both overexpression and silencing of OsPUB15 has been reported to cause spontaneous lesion formation, indicating a more complex role for OsPUB15 (Wang et al., 2015). A similar phenomenon has been observed with the Arabidopsis U-box protein SAUL1. Overexpression of SAUL1 leads to an autoimmune phenotype including constitutive defense gene expression and enhanced
disease resistance, indicating that \textit{SAUL1} plays a positive role in immunity (Tong et al., 2017). However, the \textit{saul1} mutant shows cell death and seedling lethality that is dependent on \textit{PAD4} (Salt et al., 2011; Vogelmann et al., 2012). This apparent contradiction was resolved by Tong et al. (2017), who found that the cell death in the \textit{saul1} mutant is due to activation of SOC3, an NLR that guards SAUL1. Mutations in \textit{soc3} suppress the cell death phenotype of \textit{saul1}. Moreover, \textit{saul1 soc3 pub43} triple mutants are more susceptible to the bacterial pathogen \textit{Pseudomonas syringae pv tomato} (\textit{P.s.t.}) \textit{hrcC}, a strain which lacks the ability to inject effectors, indicating that \textit{SAUL1} and its homolog \textit{PUB43} redundantly play a positive role in PTI signaling.

Another example of rice E3 ligases with a role in downstream immune signaling is that of \textit{APIP6} and \textit{APIP10} (Park et al., 2012). These RING-type E3 ligases were identified in a yeast-2-hybrid screen for interactors of AvrPiz-t, an effector protein from the rice blast fungus \textit{Magnaporthe oryzae}. Silencing of either \textit{APIP6} or \textit{APIP10} reduces PTI signaling after PAMP treatment, and causes enhanced susceptibility to virulent \textit{M. oryzae} isolates (Park et al., 2012; Park et al., 2016). AvrPiz-t destabilizes \textit{APIP6} and \textit{APIP10} proteins, and may also inhibit the E3 ligase activity of \textit{APIP10}, which may be responsible for the suppression of PTI by AvrPiz-t. A likely substrate of \textit{APIP6} is \textit{OsELF3-2}, as the two proteins directly interact in a yeast-2-hybrid assay and expression of \textit{APIP6} reduces \textit{OsELF3-2} stability \textit{in vivo} (Ning et al., 2015). Furthermore, \textit{Osself3-2} mutants are more resistant to \textit{M. oryzae} infection. It remains to be determined what role \textit{OsELF3-2} plays in immunity. An \textit{ELF3} homolog in Arabidopsis is involved in control of circadian rhythm and flowering time (Zhao et al., 2012). However, the rice genome
contains another paralog, OsELF3-1, which appears to have retained this function, while the molecular function of OsELF3-2 has diverged and is unknown.

The target of APIP10 in PTI signaling has not yet been identified. However, APIP10 has been found to target the NLR Piz-t, the cognate R protein for AvrPiz-t (Park et al., 2016). In plants transformed with Piz-t, APIP10 is required to maintain a low level of Piz-t protein, and silencing of APIP10 in these plants leads to severe and often lethal spontaneous cell death. Thus, Park et al. (2016) hypothesized that by reducing the activity of APIP10, AvrPiz-t causes Piz-t to accumulate and initiate ETI, functionally linking the effector with the R protein.

The UPS often targets transcription factors, thereby affecting transcriptional changes during the immune response. For example, the RING E3 ligase EIRP1 causes degradation of the WRKY11, a negative regulator of immunity. Expression of EIRP1 is induced by powdery mildew inoculation in wild grapevine Vitis pseudoreticulata, and overexpression of EIRP1 causes enhanced resistance to virulent pathogens in both V. pseudoreticulata and Arabidopsis (Yu et al., 2013). Thus, targets of the UPS include proteins involved in all steps of immune signaling.

**The role of general proteasome components in plant immunity**

In addition to the role of E3 ligases targeting specific proteins for degradation, regulation of general UPS components is also vital for proper immune regulation. Thus, perturbation of the UPS can lead to either enhanced or reduced immune signaling, depending on the nature of the disruption. These components include proteins...
involved in stability and function of E3 ligase complexes, as well as proteins responsible for recognition and degradation of poly-ubiquitinated substrates.

**Proteins involved in ubiquitination and E3 ligase function**

The function of E3 ligases relies on upstream ubiquitin activating (E1) and ubiquitin conjugating (E2) enzymes. Compared to the diversity of E3s, plant genomes contain much fewer genes that encode E1 and E2 enzymes, suggesting their ubiquitous usage which limits their substrate specificity (Vierstra, 2009). However, some specific roles have been identified for these components. For example, UBA1, one of only two E1 enzymes identified in Arabidopsis, is required for signaling downstream of the NLR SNC1 and for resistance to virulent pathogens (Goritschnig et al., 2007). Although UBA2 shares high similarity with UBA1, UBA2 does not seem to play a similar role in immunity. However, mutations in both *uba1* and *uba2* lead to lethality, indicating that these E1s are partially redundant.

Plant E2 gene families are slightly larger than E1 gene families, although still insignificant compared to the diversity of E3s. Based on the presence of a ubiquitin conjugating (UBC) domain, 37 E2-encoding genes have been identified in Arabidopsis and 40 in tomato and tobacco, suggesting that E2s may contribute to substrate specificity. Many of these have been confirmed to form thioester bonds with ubiquitin *in vitro*, validating their likely E2 activity (Kraft, 2005; Zhou et al., 2017; Zhou and Zeng, 2017). In one example, silencing one clade of tomato E2s, referred to as group III E2s, caused developmental changes and reduced responses to flg22 treatment, suggesting some
specialized function of these E2 enzymes (Zhou et al., 2017). However, a high degree of functional redundancy evidently exists among the E2s, as silencing a subset of the tobacco group III E2s did not affect the flg22 response (Zhou and Zeng, 2017). Silencing of the wheat E2 TaU4 in plants infected with Mycosphaerella graminicola delays appearance of symptoms and reduces sporulation of the fungus, indicating that this E2 likely also plays a positive role in immune signaling (Millyard et al., 2016).

Further study is needed to elucidate how the specificity of E2 enzymes arises. One possibility is that immune-related E3 ligases interact preferentially with group III E2s in the example of Zhou et al., 2017. In addition, since different E2 enzymes may use different lysine residues to initiate a polyubiquitin chain (Walsh and Sadanandom, 2014), it is possible that the topology of the chains arising from group III E2s may be important in controlling the fate of immune-related targets. For example, the tomato group IX E2 enzymes SIUBC13-1 (Sni3) and SIUBC13-2 have also been shown to positively regulate cell death mediated by the kinase Fen (Mural et al., 2013). However, these E2 enzymes specifically direct formation of K63-linked ubiquitin chains, and thus likely do not mark proteins for degradation by the 26S proteasome complex.

Although plants produce a huge number of different CRL-type E3 ligases, the variation of these protein complexes is largely due to the specific protein subunits that directly bind to their specific substrates, while other subunits in the complex are relatively more general (Vierstra, 2009). Nevertheless, disruption of these general subunits can lead to altered immune phenotypes. In Arabidopsis, the partial loss-of-function allele cul1-7 increases SNC1 stability and similarly results in higher PR gene expression (Cheng et al., 2011). In contrast, a barley SKP1 homolog HvSKP1-like
contributes positively to immune signaling, as HvSKP1-like silenced plants display enhanced disease susceptibility to powdery mildew caused by *Blumeria graminis f.sp. hordei* (*Bgh*) (Reiner et al., 2016). The enhanced susceptibility is likely due to increased abundance of HvRACB, a known susceptibility factor for *Bgh*, after HvSKP1-like silencing. However, the F-box subunit expected to target HvRACB has not yet been identified.

Because CRL E3 ligases separate E2-binding and substrate-binding domains into different subunits, proper assembly of the complex is essential for their ubiquitination function. Thus, molecular chaperones that affect the stability of CRLs with immune-related targets also have an effect on immunity. A number of chaperones and co-chaperones are associated with SCF complexes that target NLR proteins, and disruption of these proteins can perturb NLR turnover (Figure 1.1). For example, loss of SGT1b, which interacts with SKP1 homologs and acts as a co-chaperone together with HSP90, causes increased stability of the NLR RPS5 (Holt et al., 2005). Similarly, certain mutant alleles of the isoforms HSP90.2 and HSP90.3 increase SNC1 and RPS2 protein accumulation and enhance the phenotype of *snc1* (Huang et al., 2014a). SRFR1, which interacts with SGT1b and is therefore likely part of the chaperone complex, is also required for proper turnover of NLRs such as SNC1 and RPS2 (Li et al., 2010). However, interpreting the roles of these chaperones is complicated by the fact that NLRs also require chaperones for their activation and stability (Kadota and Shirasu, 2012).

Besides ubiquitination, additional layer of regulation can be provided by other post-translational modifications of the same substrate proteins, which may affect the ability of the cognate E3 to interact with or ubiquitinate them (He et al., 2017). For
example, mutations in the SUMO-ligase SIZ1 result in increased SNC1 protein accumulation and an autoimmune phenotype that is partially dependent on SNC1 (Gou et al., 2017). SUMO is a small peptide similar to ubiquitin, and since SNC1 is sumoylated in planta, an attractive hypothesis is that this sumoylation is required for efficient SNC1 turnover, although contribution from increased SNC1 transcription cannot be excluded.

A specialized branch of the UPS that often involves post-translational modifications is the N-end-rule pathway (Graciet and Wellmer, 2010). In this pathway, proteins are stabilized or destabilized based on the identity of their N-terminal amino acid residues. For example, arginine is a destabilizing residue, and arginylation at the N-terminus marks proteins for degradation. Arginyl groups are added to certain N-terminal residues by the arginyltransferases ATE1 and ATE2, allowing the peptide to be recognized by the E3 ligases PRT1 and PRT6. *ate1 ate2* double mutants show reduced glucosinolate production and enhanced susceptibility to a variety of pathogens, indicating that arginlyation branch of the N-end rule pathway regulates the stability of immune components (de Marchi et al., 2016).

Acetylation of N-terminal residues can also alter protein stability, and the identity of the N-terminal residue determines whether the effect is positive or negative. N-terminal acetylation events of SNC1 were shown to be important for regulating its stability (Xu et al., 2015 and Figure 1.1). Translation of SNC1 may begin at one of two consecutive methionine codons at the beginning of the open reading frame. Interestingly, the N-terminal acetyltransferase A (NatA) complex acetylates nascent SNC1 peptides that begin translation on the first methionine (Met) residue, and this acetylated Met acts as a degron, resulting in degradation of the protein. However, polypeptides starting at the
second Met are acetylated by NatB, which appears to stabilize the protein. Consequently, Arabidopsis naa15 mutants, which contain a mutation in the auxiliary subunit of NatA, contain higher SNC1 protein levels and display an autoimmune phenotype, while natb mutants accumulate less SNC1. The competitive relationship between the Nat complexes ensures a balanced production of the immune receptor.

While specific ubiquitination of substrates is performed by E3 enzymes in collaboration with activated E2 enzymes, this mechanism often only results in the addition of very short ubiquitin chains that are not efficiently recognized by the proteasome (Hoppe, 2005). Additional enzymes called E4 enzymes may be required to elongate the ubiquitin chain (Koegl et al., 1999). In Arabidopsis, the only E4 enzyme MUSE3 facilitates the degradation of SNC1, as muse3 mutations enhance the snc1 phenotype by affecting snc1 turnover (Huang et al., 2014b and Figure 1.1). Overexpression of MUSE3 leads to less SNC1 accumulation only when CPR1 is also overexpressed, suggesting that MUSE3 facilitates the degradation of SNC1 that has already been ubiquitinated to some extent.

**Proteins downstream of ubiquitination steps**

Although ubiquitination can mark proteins for a variety of fates, K48-linked poly-ubiquitination most often leads to recognition and degradation by the 26S proteasome complex, which requires the many subunits of 19S RP. Infection with *P.s.t. hrcC* causes an increase in proteasome activity, suggesting that the UPS contributes to defense signaling (Üstün et al., 2016). Consistent with this idea, partial loss-of-function mutants
in the regulatory particle subunits *rpt2a-2* and *rpn12a-1* were more susceptible to bacterial infection. In order to suppress immunity, pathogens may deploy effectors to interfere with proteasome activity (Banfield, 2015). The *Xanthomonas campestris* effector XopJ functions as a protease, cleaving the 19S RP ATPase subunit RPT6 and reducing protein turnover by the proteasome (Üstün and Börnke, 2015). Similarly, infection with virulent *P.s.t. DC3000* causes a decrease in proteasome activity, dependent on the effector HopM1 (Üstün et al., 2016). Immunoprecipitation of HopM1 followed by mass spectrometry (IP-MS) revealed that it interacts with a number of 19S RP subunits and with Rad23, an adaptor protein that shuttles substrates to the proteasome (Nomura et al., 2006). Interestingly, HopM1 facilitates the proteasomal degradation of AtMIN7 and possibly a 14-3-3 protein, which are involved in plant immunity (Nomura et al., 2006; Lozano-Durán et al., 2014). Thus, HopM1 seems to modify the specificity of the proteasome to favor degradation of positive immune regulators over other substrates. It will be interesting to identify plant proteins that modulate the degradation of proteins involved in specific processes, including defense.

**Thesis objectives**

**Forward and reverse genetic strategies to identify novel UPS components involved in immunity**

The role of the UPS in regulating plant immunity is complex, and many of the proteins and processes involved are still unknown. Our lab has carried out a number of successful genetic screens to identify positive and negative regulators of immunity, using
the gain-of-function snc1 NLR mutant. In particular, mutants recovered from the MODIFIER OF SNC1 (MOS) screen and the MUTANT, SNC1 ENHANCING (MUSE) screen have revealed novel aspects of NLR regulation, including the important role played by the UPS (Huang et al., 2013; Johnson et al., 2013). The major objective of this thesis was the identification of novel genes involved in the regulation of plant immunity.

In addition to the forward genetic MUSE screen, a reverse genetic approach was also undertaken to identify immune regulators specifically involved in the UPS pathway. The Arabidopsis genome contains a large number of genes encoding E3 ligases which have been poorly characterized. The U-box E3 ligase AtCHIP interacts with chaperones including HSP90, which has been shown to be involved in both positive and negative immune regulation (Kadota et al., 2010; Huang et al., 2014a). Thus, AtCHIP was examined for a potential role in immune regulation.

The degradation of ubiquitinated substrates at the proteasome is also known to involve a number of adaptor proteins, which have been most thoroughly characterized in yeast and mammalian cells (Yu and Matouschek, 2017). While poly-ubiquitin conjugates may interact directly with the proteasome, a number of substrates are shuttled to the proteasome by adaptor proteins (Figure 1.2). CDC48 (also named p97 or valosin containing protein in animals) contains a number of domains which interact with these adaptors. CDC48 also interacts with the proteasome regulator PTRE1 (PI31 or PSMF1 in animals) (Clemen et al., 2015). Because an allele of AtCDC48A was identified in the MUSE screen, we also chose to examine PTRE1 for an immunity-related function.
**Characterization of immune regulators**

In accordance with the thesis objective, this thesis aims to characterize three genes whose protein products have a potential role in regulation of immunity through the UPS, namely *AtCHIP*, *AtCDC48A*, and *PTRE1*. These genes were identified through a combination of forward and reverse genetic strategies. The results of these studies revealed a positive role for *AtCHIP* in immunity, through a currently unknown mechanism, and a negative role for *AtCDC48A* and *PTRE1*, through promotion of SNC1 turnover.

![Figure 1.2. A model illustrating the variety of proteins that influence substrate degradation at the proteasome.](image)

Poly-ubiquitinated substrates (purple) bind to the proteasome directly, or through adaptor proteins (red). CDC48 interacts with adaptor proteins and with components of the ubiquitination machinery such as E4 enzymes. CDC48 also interacts with the proteasome regulator PTRE1 and may affect proteasome activity.
Chapter 2: The evolutionarily conserved E3 ubiquitin ligase AtCHIP contributes to plant immunity

Summary

Plants possess a sophisticated immune system to recognize and respond to microbial threats in their environment. The level of immune signaling must be tightly regulated so that immune responses can be quickly activated in the presence of pathogens, while avoiding autoimmunity. HSP90s, along with their diverse array of co-chaperones, forms chaperone complexes that have been shown to play both positive and negative roles in regulating the accumulation of immune receptors and regulators. In this study, we examined the role of AtCHIP, an evolutionarily conserved E3 ligase that was known to interact with chaperones including HSP90s in multicellular organisms including fruit fly, C. elegans, plants and human. atchip knockout mutants display enhanced disease susceptibility to a virulent oomycete pathogen, and overexpression of AtCHIP causes enhanced disease resistance at low temperature. Although CHIP was reported to target HSP90 for ubiquitination and degradation, accumulation of HSP90.3 was not affected in atchip plants. In addition, protein accumulation of nucleotide-binding, leucine-rich repeat domain immune receptor (NLR) SNC1 is not altered in atchip mutant. Thus, while AtCHIP plays a role in immunity, it does not seem to regulate the turnover of HSP90 or SNC1. Further investigation is needed in order to determine the exact mechanism behind AtCHIP’s role in regulating plant immune responses.
Introduction

Plant immunity

Plants have evolved sophisticated immune systems to recognize and defend against infections by diverse microbial pathogens (Dangl et al., 2013b). Receptor-like kinases on the cell surface can recognize conserved microbial features termed pathogen associated molecular patterns (PAMPs) from microbes, and trigger a relatively weak PAMP-triggered immunity (PTI) response (Macho and Zipfel, 2014). While PTI is effective at preventing infection by many microbes, successful pathogens are able to deliver effector molecules into the plant cell to suppress PTI and promote virulence (Dangl et al., 2013b). An additional layer of the plant immune system involves resistance (R) proteins, which can recognize specific pathogen effectors and trigger a more rapid and robust effector-triggered immunity (ETI).

Plant genomes contain a large number of R genes, most encoding proteins with nucleotide-binding, leucine-rich repeat domains (NLRs; or Nod-like receptors). Typical NLRs in plants contain Toll-interleukin-1 receptor (TIR) or coiled-coil (CC) domains at their N termini (Jones and Dangl, 2006; Li et al., 2015). Immune responses mediated by TIR-type NLR (TNL) proteins often require the immune signaling module EDS1/PAD4/SAG101, but the detailed molecular events surrounding NLR activation are largely unclear (Wiermer et al., 2005). Rapid and strong ETI induction is important for preventing pathogen infections (Jones and Dangl, 2006). However, ETI signalling must be suppressed in healthy wild-type plants, as mutants with constitutive ETI can be small
and often show spontaneous cell death (Li et al., 2001; Yang et al., 2010). For example, a point mutation in Suppressor of npr1, constitutive 1 (SNC1), a TNL protein in *Arabidopsis thaliana*, results in the autoimmune snc1 mutant, which exhibits dwarfism, curled-leaf morphology, and enhanced resistance against virulent pathogens (Li et al., 2001; Zhang et al., 2003). More recent studies have shown that SNC1 and other NLRs are regulated post translationally, through degradation by the 26S proteasome pathway (Cheng et al., 2011; Huang et al., 2014b). E3 ubiquitin ligase complex containing the F-box protein CPR1 targets SNC1 for ubiquitination and subsequent degradation (Cheng et al., 2011). The point mutation in snc1 plants stabilizes the snc1 protein, increasing its steady state level and resulting in the autoimmune phenotype (Cheng et al., 2011). The sensitized background of snc1 has been used as an efficient tool to elucidate further components of plant immunity, as it can be used to search for both enhancers and suppressors.

**HSP90-containing chaperone complexes play positive and negative roles in immunity**

Chaperone complexes containing HSP90s play important roles in ensuring R protein stability and function (Kadota and Shirasu, 2012). The contribution of HSP90s is not straightforward, as mutations in different isoforms or different alleles of the same isoform often have differing, or even opposite phenotypic effects. For example, mutations in *HPS90.2* leads to reduced *RPM1* levels and function (Hubert et al., 2003; Takahashi et al., 2003). However, different alleles of *hsp90.2* and *hsp90.3* can result in increased NLR
stability leading to autoimmunity, such as with SNC1 and RPS2 (Huang et al., 2014b). This could be explained by HSP90’s differential chaperone roles in different protein complexes. They could serve in NLR activation complex, and at the same time, be involved in the NLR degradation complex as SCF (Skp1, Cullin 1 and F-box) E3 ligase complex members.

Adding to the complexity of HSP90’s role in NLR function are the many co-chaperones that function with HSP90, which are also presumably crucial for proper NLR folding and function. Mutations in \( SGT1b \), or alleles of \( HSP90 \) that abolish the interaction between \( SGT1b \) and HSP90, cause a reduction in R protein levels (Hubert et al., 2003; Zhang et al., 2008; Shirasu, 2009; Kadota and Shirasu, 2012). Mutations in \( SGT1b \) and \( RAR1 \) both partially compromise resistance mediated by RPP5, but these phenotypes are additive, indicating that they likely function independently in RPP5 signalling (Austin et al., 2002). The interaction between HSP90 and its co-chaperones must be important, as specific alleles of \( hsp90.2 \) can partially restore NLR signalling that is abolished in \( rar1 \) mutants (Hubert et al., 2003).

Like HSP90s, in addition to SGT1’s positive roles in plant immune receptor stability and activation, SGT1 also serves in SCF-mediated NLR protein turnover. SGT1 interacts directly with Skp1, a common component of SCF E3 ligase complexes (Kitagawa et al., 1999). In \( Arabidopsis \), mutations in \( SGT1b \) and \( SRFR1 \), encoding an interactor of SGT1, lead to higher accumulation of NLR proteins RPS5, SNC1, and RPS2 (Holt et al., 2005; Li et al., 2010). These diverse roles of SGT1 mirror those of HSP90s.
CHIP is a conserved E3 ligase that interacts with HSP chaperones

Given the importance of chaperone complexes in both positive and negative regulation of NLR accumulation, it is likely that other chaperone-interacting proteins also play roles in regulating NLRs. One evolutionarily conserved candidate protein with well-characterized interactions with HSP90 and HSP70 in animals is C-terminus of Hsc70 Interacting Protein (CHIP; AtCHIP in Arabidopsis), an E3 ligase that ubiquitinates unfolded client proteins bound by the chaperone complexes (Connell et al., 2001; Murata et al., 2003). Thus, CHIP is a unique E3 ligase that links heat-shock chaperone complexes with ubiquitination and proteosomal degradation. Work by Qian et al. (2006) suggests that the target of the E3 ligase activity depends on the amount of unfolded client proteins present, where CHIP preferentially ubiquitinates chaperone-bound substrates before ubiquitinating the chaperones themselves. Interestingly, CHIP does not seem to target a specific substrate protein like most other E3s, but rather, relies on the selectivity of its associated chaperones for client substrates. The crystal structure of mammalian CHIP, both alone and in interaction with the chaperone HSC70, has been solved (Zhang et al., 2005a; Zhang et al., 2015). CHIP protein contains a C-terminal U-box domain and an N-terminal tetratricopeptide repeat (TPR). Forms of CHIP with mutations in the TPR region retain self-ubiquitination activity, indicating that the U-box domain is sufficient for E3 ligase activity (Zhang et al., 2015). However, the TPR domain is required for the interaction with Hsc70 and ubiquitination of misfolded client proteins, emphasizing the importance of protein-protein interactions for CHIP function.
Previous studies on *AtCHIP* in Arabidopsis have identified a role for *AtCHIP* in response to abiotic stress. Yan et al. (2003) confirmed the E3 ligase activity of *AtCHIP*, and found that the expression of *AtCHIP* is up-regulated by osmotic and temperature stresses; however, constitutive overexpression of *AtCHIP* results in increased susceptibility to heat and chilling. Like animal CHIP, *AtCHIP* also interacts with HSC70, facilitating the degradation of plastid-targeted precursor proteins, preventing them from building up in the cytosol (Lee et al., 2009). In addition, *AtCHIP* was shown to interact with, ubiquitinate, and reduce the cellular levels of choloroplast proteins Clp4, a subunit of the chloroplast Clp proteolytic complex, and FtsH1/2, two subunits of the chloroplast Fts protease complex (Shen et al., 2007a; Shen et al., 2007b). These results indicate a role for *AtCHIP* in degradation of multiple protein targets through the 26S proteasome pathway. Additionally, *AtCHIP* interacts with and ubiquitinates PP2A (protein phosphatase 2A), which is involved in the response to low-temperature. However, overexpression of *AtCHIP* does not affect the steady-state levels of PP2A isoforms, and PP2A activity is increased in *AtCHIP-OE* plants under low-temperature conditions, indicating that ubiquitination by *AtCHIP* may play regulatory roles beyond proteasomal degradation (Luo et al., 2006).

**AtCHIP and plant immunity**

The function of *AtCHIP* in immune signaling is largely unexplored. Given that animal CHIP interacts with HSP90s and HSP70, *AtCHIP* interacts with HSC70-4 in
*Arabidopsis*, and HSP90 is involved in the stability regulation of a number of NLR proteins, we hypothesized that AtCHIP might play a role in regulating NLRs during plant immune responses. We therefore investigated the potential roles of AtCHIP in plant immunity, in order to expand our perspectives on its function in plants. Using a reverse genetics approach, we examined the immune phenotypes of *atchip* loss-of-function mutants, as well as plants overexpressing AtCHIP (AtCHIP-OE).

**Results**

*atchip* mutants show increased susceptibility to virulent but not avirulent pathogens

CHIP-encoding genes are broadly found in eukaryotes including fungi, plants and animals (Figure 2.1A). Intriguingly, the conservation of CHIP sequences is higher amongst animals; plant and fungal CHIPS show relatively more divergence. It is worth mentioning that no CHIP homolog was identified in budding or fission yeast.
Figure 2.1. atchip knockout mutants exhibit enhanced susceptibility to virulent but not avirulent pathogens, while overexpression of AtCHIP leads to enhanced resistance only at lower temperature.
(A) Maximum-likelihood tree of CHIP sequences from representative eukaryotes. Node labels represent bootstrap values from 1000 replicates. The scale bar represents the average number of substitutions per site in each branch. Organisms shown in the tree are Arabidopsis thaliana, Brachypodium distachyon, Caenorhabditis elegans, Chlamydomonas reinhardtii, Danio rerio, Drosophila melanogaster, Glycine max, Homo sapiens, Micromonas sp. RCC299, Mus musculus, Neurospora crassa, Oryza sativa, Physcomitrella patens, Populus trichocarpa, Rhizopus microspores, and Zea mays.

(B) Growth of Hyaloperonospora arabidopsidis Noco2 on Col, atchip-1, and atchip-2 plants. Two-week-old seedlings were sprayed with a spore suspension at a concentration of 5x10^4 spores per mL, and oomycete spores grown on leaf surface were quantified 7 days later using a hemocytometer. Asterisks indicate a significant difference from Col (p < 0.05) as determined by t tests. The experiment was repeated more than 3 times with similar results.

(C) Growth of Pseudomonas syringae pv maculicola ES4326 on wild type Col, atchip-1, atchip-2, and eds1 plants (eds1 serves as a susceptibility control). Leaves of 4-week-old plants were infiltrated with a bacterial suspension in 10mM MgCl_2 at OD_600 = 0.0001. Leaf discs within the infected area were taken immediately after infiltration (Day 0) and 3d after infiltration (Day 3) to quantify bacterial colony-forming units (cfu). Bars represent mean values of three (Day 0) or five (Day 3) replicates ± SD. Asterisks indicate a significant difference from Col (p < 0.05) as determined by t tests.

(D) and (E) Growth of Pseudomonas syringae pv tomato DC3000 expressing AvrRpt2 (D) or AvrRps4 (E) on wild type Col, atchip-2, and ndr1 or eds1 plants. Leaves of 4-week-old plants were infiltrated with a bacterial suspension in
10mM MgCl$_2$ at OD$_{600}$ = 0.001. Leaf discs within the infected area were taken immediately after infiltration (Day 0) and 3d after infiltration (Day 3) to quantify bacterial colony-forming units (cfu). Bars represent mean values of three (Day 0) or five (Day 3) replicates ± SE. Asterisks indicate a significant difference from Col ($p < 0.05$) as determined by ANOVA followed by Tukey’s HSD test.

(F) Salicylic acid (SA) accumulation in Col, atchip-2, and eds1 induced with *Pseudomonas syringae* pv tomato DC3000 carrying AvrRps4. Plants were infiltrated with bacterial suspension in 10mM MgCl$_2$ at OD$_{600}$ = 0.2. Tissue was harvested after 24h for total SA extraction and quantification using an HPLC. Asterisks indicate a significant difference from Col ($p < 0.05$) as determined by ANOVA followed by Tukey’s HSD test.

(G) Growth of *Pseudomonas syringae* pv. *maculicola* ES4326 on wild type Col, C24, and AtCHIP-OE plants. Leaves of 4-week-old plants were infiltrated with a bacterial suspension in 10mM MgCl$_2$ at OD$_{600}$ = 0.001. Leaf discs within the infected area were taken 3d after infiltration (Day 3) to quantify bacterial colony-forming units (cfu). Bars represent mean values of five (Day 3) replicates ± SE. Asterisks indicate a significant difference from C24 as determined by ANOVA followed by Tukey’s HSD test.

(H) Growth of *Pseudomonas syringae* pv. *maculicola* ES4326 on wild-type Col, C24, and CHIP-OE plants under low temperature. Plants were transferred to 18°C for at least 1 week, and infiltrated as in (G). Asterisks indicate significant difference ($p < 0.05$) from C24, as determined by a one-way ANOVA followed by Tukey’s HSD test.
According to publically available microarray expression data found on Arabidopsis eFP browser, AtCHIP expression is indeed induced by infections with virulent *Pseudomonas syringae* pv. *maculicola* (*P.s.m.*) ES4326 or the same strain carrying the effector AvrRpt2, as well as by treatment with SA (Figure 2.2). In order to determine whether AtCHIP plays a role in immune regulation, we first obtained two exonic T-DNA knockout alleles of *atchip*, named *atchip*-1 (SALK_048371) and *atchip*-2 (SALK_059253), and examined their immune phenotypes against a variety of pathogens. *atchip* plants of both mutant alleles exhibit enhanced disease susceptibility against the oomycete pathogen *Hyaloperonospora arabidopsidis* (*H.a.*) Noco2 (Figure 2.1B). We also observed slight enhanced susceptibility against the virulent bacterial pathogen *Pseudomonas syringae* pv. *maculicola* (*P.s.m.*) ES4326, although this was not always significant (Figure 2.1C). In addition, *atchip*-2 plants showed wild-type levels of resistance to *Pseudomonas syringae* pv. *tomato* (*P.s.t.*) DC3000 strains carrying either *AvrRpt2* or *AvrRps4*, which are avirulent on the Columbia (Col) ecotype due to the presence of the NLRs *RPS2* and *RPS4*, respectively (Figures 2.1D, 2.1E). Furthermore, when the plants were infiltrated with a high dose of *P.s.t.* DC3000 *AvrRps4*, the defense hormone salicylic acid (SA) accumulated to the same level as wild-type (Figure 2.1F). Therefore, *atchip* positively contributes to basal immunity, but does not appear to affect NLR-mediated immunity or SA accumulation.
Figure 2.2. AtCHIP expression is induced by pathogens and SA.

(A) AtCHIP expression after mock treatment, or infiltration with the indicated strains of *P.s.m.*, at the indicated time points.

(B) AtCHIP expression 3h after mock treatment or treatment with 10µM SA. Data for (A) and (B) were taken from AtGenExpress.

Overexpression of *AtCHIP* causes enhanced disease resistance under low temperature conditions

Because *atchip* knockout mutants exhibit a mild enhanced disease susceptibility phenotype, we hypothesized that increased levels of *AtCHIP* might cause enhanced disease resistance. We obtained plants overexpressing *AtCHIP* (*AtCHIP-OE*), which were generated in the C24 ecotype background and described previously by Yan et al. (2003). Under normal growth conditions, *AtCHIP-OE* plants supported similar *P.s.m.* ES4326 growth as the C24 controls (Figure 2.1G). Note that C24 is known to exhibit enhanced resistance to virulent pathogens (Lapin et al., 2012).
While *AtCHIP-OE* was previously reported to show wild-type-like morphology under standard growth conditions, they are temperature sensitive, as exposure to 7°C results in severely stunted growth and electrolyte leakage (Yan et al., 2003), indicative of autoimmunity. Therefore, we predicted that overexpression of *AtCHIP* may result in enhanced disease resistance under low temperature conditions. When plants were grown at 20°C, and transferred to 18°C for at least 1 week before infection, we observed a significant reduction in bacterial growth following *P.s.m.* ES4326 infection (Figure 2.1H). Thus, consistent with the enhanced susceptibility in *atchip* knockout mutants, overexpression of *AtCHIP* causes enhanced disease resistance, supporting a positive role *AtCHIP* plays in immune regulation.

**The autoimmune phenotype of *snc1* mutants does not depend on *AtCHIP***

The *atchip* mutation alone causes only mild enhanced disease susceptibility. However, single mutants of many genes involved in NLR regulation do not show strong enhanced disease susceptibility, yet can dramatically suppress the autoimmune phenotypes of *snc1*, an autoimmune mutant carrying a gain-of-function mutation in a TNL (Li et al., 2001; Johnson et al., 2013). We therefore created a *atchip-*2 *snc1* double mutant to test whether the *atchip* mutation could suppress the autoimmune phenotypes of *snc1*. The *atchip-*2 *snc1* double mutants displayed the same dwarf, curled-leaf morphology as *snc1* and were not significantly larger than *snc1* single mutants as examined by plant fresh weight (Figure 2.3A and 2.2B). *H.a.* Noco2 infection further confirmed that the *atchip-*2 *snc1* plants retain the enhanced disease resistance of *snc1*.
(Figure 2.3C). Therefore, AtCHIP does not seem to contribute to SNC1-mediated immunity.

Figure 2.3. atchip-2 knockout does not suppress the snc1 phenotype, and SNC1 and HSP90 levels are not altered in atchip-2 plants.

(A) Morphology of 4-week-old soil-grown plants of the indicated genotypes.

(B) Fresh weights of plants of the indicated genotypes. Asterisks indicate significant differences from snc1 at \( p < 0.05 \), as determined by one-way ANOVA and Tukey's HSD test.

(C) Resistance against H.a. Noco2 in Col, atchip, snc1, and snc1 atchip-2 plants.

Two-week-old seedlings were sprayed with a spore suspension at a concentration of \( 10^5 \) spores per mL, and spores were quantified 7 days later.
using a hemocytometer. Asterisks indicate a significant difference (p < 0.05) from 
snc1, as determined by t tests.

(D) SNC1 protein levels in atchip-2 plants. Total protein from 3-week-old plants of 
Col and atchip genotypes was subjected to immunoblotting with an α-SNC1 
antibody (Li et al., 2010). Ponceau staining is shown as a loading control.

(E) HSP90.3-HA levels in Col and atchip-2 genotypes. Total protein was extracted 
from the aerial tissue of 2-week-old seedlings of the indicated genotypes. 
HSP90.3-HA levels were examined using immunoblotting with an α-HA antibody. 
Ponceau staining is shown as loading control.

AtCHIP does not affect SNC1 turnover

Since animal CHIPS were reported to associate with evolutionarily 
conserved chaperones SGT1 and HSP90s, which contribute to NLR SNC1 turnover in 
Arabidopsis, we examined whether AtCHIP is also involved in NLR turnover (Connell et 
al., 2001; Zhang et al., 2008). When we examined the SNC1 protein levels in the atchip-2 
backgrounds; however, no obvious alteration in SNC1 levels was observed compared to 
the wild-type controls (Figure 2.3D). These results are consistent with the inability of 
atchip to suppress the autoimmune phenotype of snc1.

HSP90.3-HA levels are not affected in atchip knockout plants

Expression of CHIP in human cell lines reduces the accumulation of 
HSP70 when the level of unfolded clients is low (Qian et al., 2006). Human CHIP protein 
can also ubiquitinate chaperones HSP90 and HSP70 in vitro, creating ubiquitin chains
that contain K48 linkages, which is predicted to mark the HSPs for degradation (Kundrat and Regan, 2010). These findings led to the hypothesis that AtCHIP may play a role in the regulation of HSP90 levels. To test this, a \textit{HSP90.3-HA} transgene was introduced into the \textit{atchip-2} background by crossing (Huang et al., 2014a). However, no difference in the HSP90.3-HA protein levels was observed in the \textit{atchip-2} background compared to the wild-type (Figure 2.3E). There AtCHIP is unlikely to target HSP90s for ubiquitination and degradation.

\textbf{Discussion}

Animal CHIPS have been shown to interact with HSP chaperones, and in some cases modulate their turnover. Chaperones and their interactors play both positive and negative roles in immune signaling. For example, the stability of many NLR proteins is dependent on chaperone HSP90 and co-chaperones SGT1 and RAR1 (Kadota and Shirasu, 2012; Li et al., 2015). Here, we examined whether CHIP, as a component of animal chaperone complexes, similarly contributes positively or negatively to plant immune signalling. We observed slightly enhanced disease susceptibility in \textit{atchip} mutants and enhanced resistance in \textit{AtCHIP} overexpression lines (Figure 2.1). However, we did not observe defects in effector-triggered immunity mediated by specific NLR proteins in \textit{atchip} mutant plants. \textit{atchip} mutants retain resistance to avirulent \textit{P.s.t.} DC3000 expressing \textit{AvrRps4} and \textit{AvrRpt2} (Figure 2.1D and 2.1E), and \textit{atchip snc1} double mutants displayed the same level of autoimmunity as \textit{snc1} alone (Figures 2.2A-2.2C). SNC1 protein levels were also unaffected in the \textit{atchip} knockout (Figure 2.3D).
The lack of ETI phenotypes in *atchip* mutant argues against its predicted roles in chaperone-assisted NLR functions. However, as *atchip* does exhibit slight susceptibility against a virulent oomycete pathogen (Figure 2.1), it must contribute to plant immune regulation through a yet-to-be-identified mechanism. One alternative explanation could be that AtCHIP is perhaps only involved in the regulation of other untested NLRs besides SNC1, RPS2, or RPS4.

Multiple studies have shown that CHIP can ubiquitinate the chaperones HSP70 and HSP90, which often results in a reduction in the chaperone levels (Qian et al., 2006; Kundrat and Regan, 2010). While we found no difference in the levels of HSP90.3-HA in the *atchip* mutant compared to wild-type, we cannot rule out that *At*CHIP may play a role in immunity by affecting HSP90 function in some other way. Overexpression of CHIP does not reduce the stability of HSP70 and HSP90 in all cases, and ubiquitination of these chaperones may have other roles (Jiang et al., 2001; Morales and Perdew, 2007; Zhou et al., 2014; Edkins, 2015). Additionally, CHIP may affect the function of chaperone complexes by competing for protein-protein interaction sites on HSP90s. For example, the co-chaperone Hop facilitates the transfer of client proteins from HSP70 to HSP90, by simultaneously binding to both complexes (Edkins, 2015). However, binding of CHIP to HSP90 reduces the ability of Hop to bind to HSP90, interfering with the protein transfer and reducing the activity of HSP90 on these clients. CHIP may also target other proteins in the complex for degradation.

Typically, E3 ligases are thought to provide specificity to the ubiquitin-proteasome system, by targeting one or a small number of similar proteins (Deshais and Joazeiro, 2009). However, CHIP appears to be unusual for an E3 ligase, as it has been
shown to ubiquitinate many different substrate proteins and target them for degradation (Murata et al., 2003). While majority of CHIP’s known substrates have been identified in animal systems, the role of plant AtCHIP for regulating protein accumulation both under steady state and in response to heat stress suggests that AtCHIP has a similar function as animal CHIP (Murata et al., 2001; Zhou et al., 2014; Tillmann et al., 2015). The promiscuity of AtCHIP makes it difficult to identify the molecular mechanism underpinning its role in immunity. It is possible that one of the targets of AtCHIP is a negative regulator of immunity, and that this protein accumulates in atchip plants, causing the enhanced disease susceptibility. However, it is equally plausible that loss of AtCHIP function causes an abnormal increase of many proteins, which together contribute to a cellular environment that reduces immune signaling.

In summary, here we provide a new perspective on the potential regulation of HSP90 and NLRs through evolutionarily conserved AtCHIP. Additional investigation is needed to elucidate the function of AtCHIP in immunity. Because mutations in HSP complexes differentially affect signaling mediated by different NLRs, experiments need to be completed to test whether atchip mutants show defects in resistance from other untested NLRs. Greater insights into the dynamics of HSP90 complexes or detailed proteomic studies using atchip mutants or overexpression lines may be required to identify the exact mechanism by which AtCHIP contributes to plant immunity.

Materials and Methods
Plant Material

Soil-grown *Arabidopsis thaliana* plants were maintained in a growth chamber under 18h light / 6h dark, 22°C growth conditions. Plate-grown *A. thaliana* seedlings were grown on ½ strength MS medium in sealed plates. The *atchip-1* and *atchip-2* mutants were obtained from the Arabidopsis Biological Resource Center. *AtCHIP-OE* plants were obtained from Dr. Hong Zhang, and express the full-length *AtCHIP* gene under the control of the CaMV 35S promoter.

Phylogenetic analysis

The deduced amino acid sequence of AtCHIP was used as a query in BLAST searches to identify related sequences in model eukaryotic organisms and crops. Fungal and animal sequences are from the NCBI Protein Database, and plant and algal sequences are from the PLAZA comparative plant genomics database (Van Bel et al., 2012). Mesquite was used to align sequences with MUSCLE, along with the sequence of AT2G42810, which served as an outgroup. A maximum-likelihood tree was constructed with RaxML, using the JTT model with 1000 bootstrap replicates.

Pathogen infections

The methods used for infection experiments involving *H.a. Noco2* and *Pseudomonas syringae* were previously described (Li et al., 2001). For *H.a. Noco2* infections, 2-week-old seedlings were sprayed with a condiospore suspension at a concentration noted in the figure legends. Inoculated plants were kept at 18°C at 80%
humidity under a 12h light/12h dark cycle. The level of infection was quantified after 7 days by counting the number of condiospores present per gram of tissue using a hemocytometer. For *P.s.t.* DC3000 infections, plants were grown at 22° under a 12h light/12h dark cycle. Bacteria grown in LB and diluted to the indicated concentrations with 10mM MgCl2, was used to infiltrate leaves of 4-week-old plants. Leaf discs of the infected area were taken at 0d and 3d after infiltration to quantify bacterial colony-forming units (cfu) on agar plates with proper antibiotic selection. For low temperature treatment, plants were transferred to a chamber at 18°C under a 12h light/ 12h dark cycle for at least 1 week prior to infiltration.

**Protein level analysis**

Total protein was extracted as in (Huang et al., 2014b). Briefly, finely ground plant tissue was homogenized in extraction buffer and centrifuged, and the supernatant was transferred to new tubes containing loading buffer. Protein was separated using SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting with specific antibodies.

**SA Quantification**

SA was extracted using the protocol described by Li et al. (1999), and quantified by HPLC.
Chapter 3: AtCDC48A is involved in the turnover of an NLR immune receptor

Summary

Plants rely on different immune receptors to recognize pathogens and defend against pathogen attacks. Nucleotide binding domain and leucine rich repeat (NLR) proteins play a major role as intracellular immune receptors. Their homeostasis must be maintained at optimal levels in order to effectively recognize pathogens without causing autoimmunity. Previous studies have shown that the activity of the ubiquitin-proteasome system is essential to prevent excessive accumulation of NLR proteins such as Suppressor of NPR1, Constitutive 1 (SNC1). Attenuation of the ubiquitin E3 ligase SCF\textsuperscript{CPR1} (Constitutive expressor of PR genes 1) or the E4 enzyme MUSE3 (Mutant, SNC1-Enhancing 3) leads to NLR accumulation and autoimmunity. In the current study, we report the identification of AtCDC48A as a negative regulator of NLR-mediated immunity. Plants of \textit{atcdc48A-4}, a partial loss-of-function allele of \textit{AtCDC48A}, exhibit dwarf morphology and enhanced disease resistance to the oomycete pathogen \textit{Hyaloperonospora arabidopsidis} Noco2. SNC1 level is increased in \textit{atcdc48A-4} plants and AtCDC48A interacts with MUSE3 in co-immunoprecipitation experiments, supporting a role for AtCDC48A in NLR turnover. While \textit{Arabidopsis} contains four other paralogs of AtCDC48A, knockout mutants of these genes do not show obvious immunity-related phenotypes, suggesting functional divergence within this family. As an
AAA ATPase, AtCDC48A likely serves to process the polyubiquitinated NLR substrate for final protein degradation by the 26S proteasome.

Introduction

In order to defend against infections by pathogenic microbes, plants rely on a multi-layered innate immune system (Chisholm et al., 2006; Jones and Dangl, 2006; Dangl et al., 2013a). The first layer involves recognition of conserved molecular patterns from large groups of microbes, such as bacterial flagellin, which results in an induced defense response (Boller and Felix, 2009). To overcome this basal level of immunity, successful plant pathogens produce effector proteins, which can be delivered into the plant cell and function in a variety of ways to presumably inhibit immune signaling and promote virulence. However, higher plants have evolved resistance proteins (R proteins), which are activated upon recognition of their cognate effectors (also termed Avirulence, or Avr proteins) and trigger a robust immune response (Dodds and Rathjen, 2010). Most R proteins belong to the nucleotide-binding, leucine-rich repeat (NLR; or Nod-like receptors) family, and have either a Toll-interleukin-1 receptor (TIR) or coiled-coil (CC) domain at their N terminus (Maekawa et al., 2011). Although the signaling events surrounding NLR protein activation are not well understood, TIR-type NLR proteins (TNLs) often require Enhanced Disease Susceptibility 1 (EDS1) and Phytoalexin Deficient 4 (PAD4) in order to trigger a defense response (Falk et al., 1999; Jirage et al., 1999).
The effective NLR-mediated immunity involves rapid accumulation of reactive oxygen species (ROS), induction of synthesis of the defense hormone salicylic acid (SA), and up-regulation of defence marker Pathogenesis Related (PR) genes, and is often associated with a programmed cell death phenomena termed the hypersensitive response (HR), which is hypothesized to restrict pathogen growth at the site of infection (Jones and Dangl, 2006). Although these local responses are effective in preventing further growth of the pathogen, constitutive activation of the immune system can be detrimental to normal plant growth and development. For example, higher accumulation of TNL SNC1 leads to autoimmune phenotypes and stunted growth (Cheng et al., 2011). Therefore, the accumulation and activity of NLR proteins must be tightly regulated to ensure a balanced immune response (Li et al., 2015).

One important mechanism by which plants regulate the abundance of NLR proteins is through the 26S proteasome, where proteins are targeted for degradation through ubiquitination (Vierstra, 2009). The E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme and the E3 ubiquitin ligase activities are all essential for the initial ubiquitination of protein substrates, where E3s mostly determine their substrate specificity. Ubiquitinated substrates can be further processed by E4 enzymes, which catalyze the addition of more ubiquitin molecules to the growing ubiquitin chain (Hoppe, 2005), as polyubiquitinated proteins are preferred substrates for the 26S proteosome. The importance of this pathway in regulating NLR protein abundance is demonstrated by the striking autoimmune phenotypes of mutants with increased NLR protein abundance. The \textit{snc1} mutation encodes a TNL protein that accumulates to high levels, causing a characteristic dwarf, curled-leaf phenotype and enhanced disease resistance (Li et al.,
Overexpression of *CPR1*, which encodes SCF\textsuperscript{CPR1} that targets SNC1, reduces SNC1 levels and suppress the *snc1* phenotype, while *cpr1* mutations cause an increase in SNC1 levels and result in autoimmunity (Cheng et al., 2011). Mutations in MUTANT, *snc1*-ENHANCING 3 (MUSE3), an E4 enzyme that associates with and polyubiquitinates SNC1, similarly increase SNC1 protein levels and exhibit an autoimmune phenotype (Huang et al., 2014a). However, the full NLR degradation pathway by which NLR protein abundance is maintained at an optimal level is still unclear.

To search for negative regulators of NLR-mediated immunity, we carried out a Mutant, *snc1* Enhancing (MUSE) forward genetic screen (Huang et al., 2013). From the screen, many components functioning in the NLR degradation pathway were recovered, including CPR1, HSP90, MUSE6, MUSE13/14 and MUSE3 (Huang et al., 2013; Huang et al., 2014a; Huang et al., 2014b; Li et al., 2015; Xu et al., 2015; Huang et al., 2016). Here we report the identification of MUSE8, which encodes AtCDC48A, from this screen. AtCDC48A facilitates the turnover of the NLR protein SNC1 through its role in the 26S proteolytic pathway.

**Results**

**Identification and characterization of the muse8 mos2 snc1 npr1 mutant**

A previous forward genetic screen for suppressors of *snc1*, called the Modifier of *snc1* (MOS) screen, identified a number of positive regulators of NLR-mediated
immunity, including MOS2. Through affecting the splicing pattern of SNC1, the mos2 mutation suppresses the autoimmune phenotype of snc1, resulting in larger plant size, reduced defense marker Pathogenesis Related (PR) gene expression, and disease susceptibility to the oomycete pathogen Hyaloperonospora arabidopsidis (H.a.) Noco2 (Zhang et al., 2005b; Copeland et al., 2013). In order to identify novel negative regulators of NLR-mediated immunity, modified snc1 enhancer screens were carried out in mos snc1 backgrounds (Huang et al., 2013). The presence of snc1 provided a sensitized background for genetic enhancers of snc1, while the larger size caused by the mos mutations facilitated screening and avoided potential lethality due to extreme autoimmunity. The muse8 mutant was recovered in the mos2 snc1 npr1 background, initially based on its extreme dwarf phenotype (Figure 3.1A). Further analysis confirmed that the muse8 mutation enhances other immune aspects of the snc1 phenotype, as we observed strong PR2 gene expression (Figure 3.1B) and enhanced resistance to H.a. Noco2 (Figure 3.1C), even in the mos2 background. Therefore, muse8 is an immune enhancer of snc1.

**muse8 contains a mutation in AtCDC48A**

To map muse8, the muse8 mos2 snc1 npr1 mutant was crossed with Ler. Through linkage analysis of the F2 progeny, the mutation responsible for the observed snc1-enhancing phenotype of muse8 was mapped to the top arm of chromosome 3, close to marker MGH6 at 4.2 MB. Fine mapping narrowed down the mutation between indel markers T16O11 (2.7MB) and MGH6 (4.2 MB) (Figure 3.2A). To identify the potential
molecular lesion of *muse8*, nuclear DNA was extracted from the original quadruple mutant plants and sequenced by Illumina next-generation re-sequencing. Comparison of the obtained sequence with the wild-type *Arabidopsis* reference genome revealed a number of point mutations in many candidate genes within the 2MB region on chromosome 3 (Figure 3.2B). *At3g11270* was excluded as a *muse8* candidate based on mapping data since it is too close to the flanking marker. Other mutations in pseudogenes, introns, or not causing amino acid changes were also excluded. Eventually three mutations seemed to be the most likely candidates after the sequence analysis, which are the ones in *At3g06660, At3g08590* and *At3g09840*. 
Figure 3.1. Characterization of the muse8 mos2 snc1 npr1 quadruple mutant.

(A) Morphology of wild-type (Col), snc1, mos2 snc1 npr1, and muse8 mos2 snc1 npr1 plants. Plants were grown on soil, under 16 hr light/8 hr night regime.
(B) *PR2* gene expression in Col, *snc1, mos2 snc1 npr1*, and *muse8 mos2 snc1 npr1* plants. Plants containing the *PR2:GUS* reporter gene were stained for GUS activity.

(C) Growth of the oomycete pathogen *Hyaloperonospora arabidopsidis* (*H.a.*) Noco2 on the indicated genotypes. Spores were counted one week after inoculation using a hemocytometer.

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 Arabidopsis Biological Resource Center (ABRC) and crossed to *muse8 snc1mos2 npr1* quadruple mutants. A T-DNA insertion mutant of *At3G06660* (SAIL_365_F03), encoding a transcription factor in the chloroplast, complemented the *muse8* phenotype, as did T-DNA mutant of *At3G08590* (SALK_119825c), a phosphoglycerate mutase gene (Figure 2c). However, when the heterozygous T-DNA mutant of *At3G09840* (*atcdc48A-1*, SALK_064573; previously reported to be lethal when homozygous) was crossed with *muse8 snc1 mos2 npr1*, plants heterozygous for *atcdc48A-1* and *muse8* displayed a *snc1*-like phenotype, even when heterozygous for *snc1*. The *snc1*-like phenotype is therefore enhanced in these plants, since the dwarfism and curled leaf phenotype of *snc1* is recessive in the wild-type background (Li et al., 2001). Thus the *atcdc48A-1* mutation fails to complement the *snc1*-enhancing phenotype of *muse8*, demonstrating that *SALK_064537* and *muse8* are alleles of the same gene, *AtCDC48A*.

The homozygous *atcdc48A-1* (*SALK_064537*) is lethal, agreeing with previous published data on *atcdc48A-1* null mutants (Park et al., 2008). However, plants
homozygous for *muse8* are partly sterile, in that they produce very small siliques with few seeds. In the F$_1$ generation, plants heterozygous for the T-DNA insertion and *muse8* were also partly sterile, which further supports that *AtCDC48A* is the gene responsible for the *muse8* quadruple mutant phenotype.

Figure 3.2. Positional cloning of *MUSE8*.

(A) Schematic diagram of chromosome 3 indicating the region containing *muse8* narrowed down by mapping.

(B) Next generation re-sequencing analysis. List of potential candidate mutations in the mapped *muse8* region. The three likely candidates are bolded.
(C) Allelism test between $muse8$ and T-DNA alleles of candidate loci. F1 plants from the crosses between the T-DNA lines and $muse8\ snc1\ mos2\ npr1$ are shown at the bottom. Wild-type, $snc1$, $muse8\ snc1\ mos2\ npr1$, and T-DNA insertion line plants are shown as controls.

Transgenic complementation was further conducted to confirm that the $muse8$ mutation in $AtCDC48A$ is responsible for the phenotype of the $muse8$ mutant. The $muse8$ single mutant was identified in the F2 generation of a cross between $muse8\ mos2\ snc1\ npr1$ and wild-type plants containing the $PR2::GUS$ reporter gene. Transformation of $AtCDC48A$-$GFP$ fusion gene into the $muse8$ plants abolished the dwarf, curled-leaf phenotype of the mutant (Figure 3.3A). Taken together, we concluded that $MUSE8$ is $AtCDC48A$, and renamed $muse8$ as $atcdc48A-4$. Since $atcdc48A-1$ is lethal, $atcdc48A-4$ must be a partial loss-of-function allele of $atcdc48A$.

**CDC48A is an ATPase conserved among eukaryotes**

In order to determine the conservation of $AtCDC48A$ amongst plants and organisms in other kingdoms, the deduced amino acid sequences of its homologs were aligned and used to construct a phylogenetic tree. $AtCDC48A$ is widely conserved amongst eukaryotes, as homologs with a high degree of sequence similarity were identified in plants, fungi, and animals (Figures 3.4A and 3.4B). The site of the glycine affected by the G274E mutation in the $atcdc48A-4$ allele is a highly conserved residue in all of the examined species (Figure 3.4B).
AtCDC48A encodes a protein in the ATPase Associated with diverse cellular activities (AAA) family (Baek et al., 2013). AtCDC48A protein contains two AAA ATPase domains, which bind and hydrolyze ATP (Figure 3.4C). The N terminus of AtCDC48 also contains an N domain, which is involved in protein-protein interactions. Since the mutated residue is located in the annotated D1 ATPase domain, and a glycine to glutamate substitution would cause a major increase in size and negative charge at that site, it is likely that the atcdc48A-4 mutation would affect the protein function (Figure 3.4C).
Figure 3.3. Characterization of the \textit{atcdc48A-4} mutant phenotype.

(A) Morphology of 3-week-old soil grown plants of the indicated genotypes.

(B) \textit{PR2} gene expression. qRT-PCR was performed on RNA extracted from 4-week-old plants of the indicated genotypes. \textit{PR2} expression was normalized to that of \textit{ACTIN7}. Asterisks indicate a significant difference ($p < 0.05$) from Col as determined by ANOVA followed by a Tukey HSD test. Error bars represent the standard errors of the means.

(C) Growth of \textit{H.a. Noco2} on seedlings of the indicated genotypes. Spores were counted one week after inoculation. Asterisks indicate a significant difference ($p$...
< 0.05) from Col, as determined by ANOVA followed by a Tukey HSD test. Error bars represent the standard deviations.

(D) Growth of the bacterial pathogen *Pseudomonas syringae pv. maculicola* ES4326 in wild-type, *atdc48A-4*, and *snc1* leaves. Plants were infected by infiltration with bacteria at OD$_{600}$=0.005, and bacterial titer was quantified at 0 and 3 days post infection. Error bars represent the standard deviations.

(E) *AtCDC48A* gene expression. qRT-PCR was performed on RNA extracted from the 4-week-old plants of the indicated genotypes. *AtCDC48A* expression was normalized to that of *ACTIN7*. Asterisks indicate a significant difference (p < 0.05) from Col as determined by ANOVA followed by a Tukey HSD test. Error bars represent the standard errors of the means.
Figure 3.4. Phylogenetic analysis of CDC48A among eukaryotes

(A) Maximum likelihood tree of deduced amino acid sequences from different species. Branch labels are bootstrap values based on 5000 replicates.

(B) Alignment of deduced amino acid sequences indicating residues conserved in at least 50% of sequences (light grey) and 80% of sequences (dark grey). Asterisk indicates the position of the G to E substitution in atcdc48A-4.
Characterization of the *atcdc48A-4* single mutant

Single mutant *atcdc48A-4* plants exhibit a mildly dwarf phenotype and slightly curled leaves, suggesting a constitutive immune response (Figure 3.3A). qRT-PCR analysis showed that the *PR2* gene expression is indeed constitutively higher in *atcdc48A-4* than in wild-type (Figure 3.3B). *atcdc48A-4* plants also showed significantly enhanced disease resistance to *H. a. Noco2* (Figure 3.3C), but no observable difference in their immune response to the virulent bacterial pathogen *Pseudomonas syringae pv maculicola (P.s.m.) ES4326* (Figure 3.3D). Expression of *CDC48A-GFP* in the *atcdc48A-4* background rescued the increased *PR* gene expression and disease resistance of the single mutant, confirming that the fusion protein is functional (Figures 3.3B and 3.3C). We also examined the expression of *AtCDC48A* in the *atcdc48A-4* and complementing lines. No reduction in *AtCDC48A* transcript levels was observed between wild-type and *atcdc48A-4* plants (Figure 3.3E). As expected, however, both of the complementing lines showed significantly higher *AtCDC48A* expression. Taken together, these data indicate that the single *atcdc48A-4* mutant has a weak enhanced resistance phenotype.

Protein degradation through the 26S proteasome pathway has been shown as an important mechanism by which NLR proteins, including SNC1, are negatively regulated (Cheng *et al.*, 2011; Huang *et al.*, 2014a). In yeast and human, AtCDC48 orthologs are involved in the processing of ubiquitinated protein substrates for proteasomal degradation.
(Meyer et al., 2012). Because \textit{atcdc48A-4} was identified as an enhancer of the \textit{snc1} phenotype, and many of the MUSE proteins are involved in SNC1 turnover, we further examined whether this mutation affects the protein level of SNC1. Compared with wild-type, the \textit{atcdc48A-4} plants indeed showed a consistent increase in SNC1 protein levels (Figure 3.5A). However, qRT-PCR showed no increase in \textit{SNC1} transcripts (Figure 3.5B), suggesting that AtCDC48A contributes to the homeostasis of SNC1 through affecting its turnover rather than transcription. In agreement with their complemented morphological and disease-resistance phenotypes, the \textit{AtCDC48A-GFP} lines showed reduced SNC1 levels compared to \textit{atcdc48A-4}.

Much higher SNC1 protein level was also observed in the extremely autoimmune \textit{snc1 atcdc48A-4} plants compared with \textit{snc1}, while \textit{SNC1} transcript levels were not increased (Figures 3.5C – 3.5E), confirming the contribution of AtCDC48A in SNC1 turnover.

\textbf{Epistasis analysis of \textit{atcdc48A-4}}

Since the \textit{atcdc48A-4} mutant exhibits elevated SNC1 protein and enhanced resistance against \textit{H.a. Noco2}, we tested whether the enhanced immunity relies on SNC1 by crossing the \textit{atcdc48A-4} mutant with \textit{snc1-r1}, a loss-of-function null allele of \textit{snc1}. The morphology of the double mutant plants was similar to \textit{atcdc48A-4} (Figure 3.6A). However, \textit{snc1-r1 cdc48A-4} plants showed reduced \textit{PR2} gene expression and increased susceptibility to \textit{H.a. Noco2} than the \textit{cdc48A-4} single mutant (Figures 3.6B and 3.6C).
This suggests that the autoimmune phenotype of *atcdc48A-4* is largely dependent on SNC1, although AtCDC48A may also regulate NLRs other than SNC1.

**Figure 3.5. SNC1 protein abundance is increased in cdc48A-4 mutants.**

(A) SNC1 protein level in the indicated genotypes. Total protein was extracted from 4-week-old plants grown under 12-h-light/12-h-dark conditions. Protein was subjected to immunoblot analysis using a SNC1-specific antibody (Li et al., 2010). Ponceau staining was used as a loading control.

(B) SNC1 gene expression in the indicated genotypes. qRT-PCR was performed on RNA extracted from 4-week-old plants. SNC1 expression was normalized to that
of ACTIN7. No significant differences (p<0.05) were detected by ANOVA. Error bars represent the standard errors of the means.

(C) Morphology of 3-week-old soil grown plants of the indicated genotypes.

(D) SNC1 protein level in the indicated genotypes. Total protein was extracted from 4-week-old plants and subjected to immunoblot analysis using a SNC1-specific antibody (Li et al., 2010). Ponceau staining was used as a loading control.

(E) SNC1 gene expression in the indicated genotypes. qRT-PCR was performed on RNA extracted from the plants in I. SNC1 expression was normalized to that of ACTIN7. Error bars represent the standard errors of the means.

To further explore the specificity of atcdc48A-4 in immune signaling, an epistasis analysis was performed by crossing atcdc48A-4 plants with eds1, which blocks TNL signaling. Loss of EDS1 function restored a wild-type morphology in the atcdc48A-4 eds1-2 double mutant (Figure 3.6D). The plant fresh weight was further measured to determine the extent of this suppression of the atcdc48A-4 phenotype. As shown in Figure 3.6E, the weight of atcdc48A-4 eds1-2 plants was similar to eds1-2 single mutants, suggesting a complete suppression of the atcdc48A-4 phenotype. The atcdc48A-4 eds1-2 double mutant also showed enhanced disease susceptibility to H. a. Noco2 to similar levels as eds1-2 (Figure 3.6F). These data indicate that the autoimmune phenotype of atcdc48A-4 is fully dependent on EDS1 function. Besides SNC1, AtCDC48A may also contribute to the turnover of other TNLs.
Figure 3.6. Epistasis analysis of atcdc48A-4.

(A) Morphology of 4-week-old soil-grown plants of the indicated genotypes.

(B) PR2 gene expression of plants of the indicated genotypes. qRT-PCR was performed on RNA extracted from 4-week-old plants. PR2 expression was
normalized to that of ACTIN7. Asterisks indicate significant difference (p < 0.05) from snc1-r1 cdc48A-4 as determined by t-test. Error bars represent SEM.

(C) Growth of H. a. Noco2 on the indicated genotypes. Spores were counted one week after infection. Error bars represent SEM.

(D) Morphology of 3-week-old soil-grown plants of the indicated genotypes.

(E) Fresh weight of plants as in (A) The average of 6 plants is shown. Error bars represent SEM.

(F) Growth of H. a. Noco2 on the indicated genotypes. Spores were counted 1 week after infection. Error bars represent SEM.

**AtCDC48A interacts with E4 ligase MUSE3**

In order to examine how AtCDC48A regulates the turnover of NLR proteins, we tested for protein-protein interactions between AtCDC48A and known promoters of NLR protein degradation. In other organisms, CDC48 facilitates protein degradation through its interaction with many components of the ubiquitin proteasome pathway, including E3 ligases, E4 enzymes, and components of the proteasome itself (Meyer et al., 2012). Yeast Cdc48 interacts with the E4 enzyme Ufd2, and may play a role in shuttling polyubiquitinated substrates to the proteasome (Jentsch and Rumpf, 2007; Baek et al., 2011). Since MUSE3 encodes an Arabidopsis E4 ligase orthologous to Ufd2 and it was also identified in the MUSE screen to be responsible for polyubiquitination of NLRs (Huang et al., 2014a), the interaction between Arabidopsis AtCDC48A and MUSE3 was tested. Co-immunoprecipitation of transiently expressed proteins from tobacco leaves revealed that AtCDC48A-GFP indeed precipitated with MUSE3-flag (Figure 3.7A).
CPR1 and SNC1 were also tested as potential interactors of AtCDC48A. However, in co-immunoprecipitation experiments, AtCDC48A-GFP was not found to precipitate with either CPR1-flag or SNC1-flag (Figure 3.7B and 3.7C). This suggests that AtCDC48A does not interact with CPR1 or SNC1 directly, or the interaction is too weak to be detected.

![Table A](image)

**Figure 3.7.** AtCDC48A-GFP co-immunoprecipitates with MUSE3-flag.

(A) Proteins were transiently coexpressed in leaves of *Nicotiana benthamiana* by infiltration of *Agrobacterium*. Total protein was extracted, immunoprecipitated with α-flag antibodies, and analyzed via western blot. Immunoprecipitation with Protein A antibodies was used as a negative control.

(B) AtCDC48A-GFP does not co-immunoprecipitate with CPR1-flag. The immunoprecipitation was carried out as in (A).

(C) ATCDC48A-4 does not co-immunoprecipitate with SNC1-flag-2z. The immunoprecipitation was carried out as in (A).
AtCDC48A and its paralogs have different functions

When the AtCDC48A deduced amino acid sequence was used as a query in a BLAST search to search for closely related paralogous sequences, two genes, 

*AT3G53230* and *AT3G03340* were identified. They are very similar to AtCDC48A, sharing 91% and 96% sequence identity respectively (Figure 3.8A). We refer to 

*AT3G53230* as *AtCDC48B*, and *AT3G03340* as *AtCDC48C*, after previous reports on this gene family (Rancour et al., 2002; Park et al., 2008). However, the naming of the other AtCDC48A paralogs is inconsistent, as we also identified two more distantly related sequences, *AT2G03670* and *AT3G01610*, which are also annotated in NCBI and The Arabidopsis Information Resource as *AtCDC48B* and *AtCDC48C*, respectively. Based on its increased phylogenetic distance from *AtCDC48A*, we rename *AT2G03670* as

*AtCDC48D* (Figure 3.8A). Similarly, *AT3G01610* is renamed as *AtCDC48E*. The gene structure of the AtCDC48 family members is consistent with the evolutionary relationship determined from the phylogeny. *AtCDC48A*, *AtCDC48B*, and *AtCDC48C* have relatively fewer, longer exons, whereas *AtCDC48D* and *AtCDC48E* contain many short exons (Figure 3.8B).

In order to examine the possible function of *AtCDC48B* – *AtCDC48E* in NLR-mediated immunity, we obtained T-DNA knockout alleles of these genes that contain T-DNAs in their exons. In contrast to the *atcdc48A-1* knockout, *atcdc48B-2* (SAIL_566_E09) and *atcdc48C-1* (SAIL_1182_E09) were viable and had wild-type morphology (Figure 3.8C). To examine possible redundancy between the paralogs, we
generated the *Atcd48B-2 atcdc48C-1* double mutant, which also appeared wild-type (Figure 3.8C).

**Figure 3.8.** Mutants of AtCDC48A paralogs do not show obvious immune phenotypes.

(A) Maximum likelihood tree of AtCDC48A-E.
(B) Gene structure of AtCDC48A-E, showing exons (black boxes) and introns (thin lines). The insertion positions of T-DNA insertion alleles are indicated by arrowheads.

(C) and (d) Morphology of soil-grown plants of the indicated genotypes. Plants are approximately 4 weeks old (c, upper panel) and (d), or 5 weeks old (c, lower panel).

(D) Growth of H.a. Noco2 on the indicated genotypes. Spores were counted one week after infection. Error bars represent the standard deviations.

The T-DNA allele obtained for AtCDC48D (SALK_074372) was named atcdc48D-1, carrying a T-DNA insertion in the first exon (Figure 3.8B). atcdc48D-1 plants displayed no apparent morphological defects compared to wild-type (Figure 3.8C). Two alleles were also obtained for AtCDC48E. SALK_024462 (atcdc48E-1) contains an insertion in the first exon (Figure 3.8B). Among the offspring of heterozygous plants, no homozygous mutant plants were isolated, and the presence of the T-DNA insertion was observed in 28 out of 46 plants. This ratio supports a 2:1 T-DNA presence: absence ratio ($\chi^2 = 0.696, p > 0.05$), suggesting that this T-DNA allele results in lethality. In contrast, the insertion in WiscDsLox8G10 (atcdc48E-2) is located near the end of the penultimate exon. Plants homozygous for atcdc48E-2 displayed a slight morphological phenotype consisting of slightly flattened and serrated leaves (Figure 3.8C). This allele likely is a partial loss-of-function allele of AtCDC48E.
If the paralogs of AtCDC48A have only a minor function in regulating NLR protein-mediated immunity, or if they are positive regulators of plant immunity, the mutants may not display a visible morphological phenotype. We therefore crossed the T-DNA alleles of atcdc48B, atcdc48C, and atcdc48E into snc1, which provides a sensitive background to observe minor changes in TNL-mediated signaling through enhancement or suppression of the snc1 autoimmune phenotype. However, no difference in plant morphology was observed in the atcdc48B snc1, atcdc48C snc1, or atcdc48E snc1 double mutants compared to snc1 alone (Figure 3.8D).

To confirm that the AtCDC48 paralogs do not play a strong role in immunity, we challenged the T-DNA mutants with H.a. Noco2. No significant enhancement of disease resistance or susceptibility was observed in any of these genotypes (Figure 3.8E). We thus concluded that the other CDC48 paralogs have divergent functions and do not seem to participate in NLR function.

Discussion

AtCDC48A contributes to the degradation of NLR proteins

Emerging evidence indicates that regulated degradation of NLRs is needed to mount a controlled, yet robust defense response response (Cheng et al., 2011; Kadota and Shirasu, 2012; Huang et al., 2014a; Huang et al., 2014b). The 26S proteasome-mediated proteolysis is the most common type of protein degradation in plants, and requires ubiquitination of target proteins through E1, E2, E3, and likely E4 enzymes enzymes (Smalle and Vierstra, 2004; Hoppe, 2005). The target proteins are then transferred to the
26S proteasome for degradation. AtCDC48A facilitates the degradation of NLR proteins such as SNC1, likely through its interaction with the E4 enzyme MUSE3 (Figure 3.9).

Through studies in yeast and human, Cdc48/p97 is known to play roles in several biochemical processes, including membrane fusion, endoplasmic reticulum associated degradation (ERAD), and the ubiquitin-proteasome system for protein degradation, depending on the myriad cofactors that interact with CDC48 at its N domain or C terminus (Meyer et al., 2012; Baek et al., 2013). In yeast, a Cdc48-Ufd1-Npl14 complex binds ubiquitinated substrate proteins (Richly et al., 2005). The complex may be involved in recruiting the E4 ligase Ufd2 to further ubiquitinate the substrates, because mutations in Ufd1 or Npl14 reduce affinity of both Ufd2 and Cdc48 for ubiquitin conjugates.

![Diagram](image)

**Figure 3.9. Working model of the role of AtCDC48A in NLR protein degradation, using SNC1 as an example substrate.**

The NLR protein is first ubiquitinated by an E3 ubiquitin ligase, such as CPR1. The ubiquitinated protein is then recognized by E4 MUSE3, which adds additional ubiquitin molecules onto the chain in association with an E2 and possibly an E3 enzyme. As an AAA ATPase, AtCDC48A then interacts with MUSE3 to provide energy and facilitate transport of the substrate to the proteasome for degradation.
In addition, Cdc48 has a post-ubiquitination role in protein degradation, as loss of Cdc48 function can lead to accumulation of poly-ubiquitinated targets (Baek et al., 2011; Verma et al., 2011). Baek et al., (2011) identified mutant forms of Ufd2 that retain their E4 activity but lose their interaction with Cdc48. In cells containing these ufd2 mutations, degradation of artificial ubiquitin-fusion proteins was disrupted, but the proteins that accumulated were poly-ubiquitinated, indicating that the mutant ufd2 was still functional. Cdc48 also modulates the interaction between Ufd2 and Rad23, a ubiquitin-binding protein that interacts with the proteasome. Thus, AtCDC48 as an AAA ATPase may interact with MUSE3 and provide energy to facilitate shuttling of poly-ubiquitinated NLR substrates to the proteasome for degradation.

CDC48 has also been proposed to function with the proteasome directly. Archaeal Cdc48 physically interacts with the archaeal 20S core particle to form a functional proteasomal complex (Barthelme et al., 2014). Although no direct physical interaction has been observed for the eukaryotic orthologs of these proteins, mouse Cdc48 can stimulate polypeptide degradation by the mouse 20S core particle in vitro (Barthelme and Sauer, 2013). Therefore, it is possible that eukaryotic Cdc48 may play a role parallel to that of the 19S regulatory particle for some proteins, unfolding and feeding them into the 20S core particle for degradation (Pick and Berman, 2013).

Taken together, the evidence from other organisms suggests that AtCDC48A likely functions to shuttle ubiquitinated NLR targets through the 26S proteasome degradation pathway (Figure 3.9). To do so, it must interact sequentially with different components of the pathway, including E4 enzyme MUSE3, proteasome components, and
regulatory cofactors that control the activity of these other components. Further examination of the dynamic protein-protein interactions between AtCDC48A, SNC1 and ubiquitin-proteasome system components is required in order to determine which role AtCDC48 may be playing in NLR protein turnover.

\textit{atcdc48A-4/muse8} is a partial loss-of-function allele of \textit{AtCDC48A}

Although orthologs of \textit{AtCDC48A} are essential genes conserved among the eukaryotes and archaea, less is known about its specific roles in plants. Fluorescently tagged AtCDC48A protein localizes to the nucleus and cytoplasm, and may also be associated with the membrane (Aker et al., 2007). During cell division, it is observed at the spindle and division plane (Rancour et al., 2002; Park et al., 2008). AtCDC48A protein forms hexamers with ATPase activity, and rescues temperature sensitive yeast \textit{cdc48} mutants at the non-permissive temperature, indicating that the \textit{Arabidopsis} protein indeed functions as an AAA ATPase orthologous to yeast Cdc48 (Feiler et al., 1995; Rancour et al., 2004). Furthermore, specific mutations in the D1 and D2 ATPase domains of both the AtCDC48A and human p97 produce dominant negative forms that are defective in ATP hydrolysis (Dalal et al., 2004; Park et al., 2008). Such inducible dominant negative constructs, as well as virus-induced gene silencing, have been used to examine the role of CDC48 in plant development, demonstrating its crucial importance for development of vegetative and reproductive organs, and for protein quality control through ERAD (Muller et al., 2005b; Park et al., 2008; Bae et al., 2009). However, these techniques also lead to growth arrest or sterility, hampering detailed genetic analysis.
Knockout alleles of \textit{AtCDC48A} also cause defects in cell division and expansion, reducing pollen viability and causing lethality at or before the early seedling (Park et al., 2008; Mérai et al., 2014). In contrast, the partial loss-of-function \textit{atcdc48A-4} allele identified in the \textit{MUSE} screen is viable and shows only a modest decrease in fertility, which allows for a more detailed examination of the mutant phenotypes.

We do not know how the mutation in \textit{atcdc48A-4/muse8} affects the biochemical activity of the \textit{atcdc48A-4} protein, causing a partial loss-of-function. The mutation in \textit{atcdc48A-4} is a G to A transition that causes a glycine to glutamate substitution in the D1 ATPase domain (Figure 3.4). The large difference in size and charge between these two amino acids suggests that the activity of the D1 domain would be strongly affected. ATP hydrolysis by CDC48 changes the physical conformation of the hexamer, which allows the complex to remodel target proteins or to remove them from membranes (Meyer et al., 2012). Although the D2 ATPase domain has been thought to be the main driver of ATP hydrolysis, the D1 domain also possesses catalytic activity (Chou et al., 2014). Because the various CDC48 domains interact, the ATP binding state at the D1 domain can also modulate ATPase activity at the D2 domain, or cofactor binding at the N domain (Meyer et al., 2012; Chou et al., 2014). More thorough genetic and biochemical studies of \textit{atcdc48A-4} are required to determine how the activity of the protein is affected, and how specific the effect is on the many cellular functions of \textit{AtCDC48}. 

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The *Arabidopsis* **AtCDC48** paralogs have diversified functions

Four paralogs of *AtCDC48*, namely *AtCDC48B-E*, have been identified in *Arabidopsis* based on sequence homology. Among this family, *AtCDC48A* is the highest and most ubiquitously expressed (Figure 3.10). Despite their high amino acid sequence similarity, the two closest *AtCDC48A* paralogs do not appear to modulate NLR protein-mediated immunity, as the *atcdc48B atcdc48C* double mutant plants are still viable and do not show an altered morphological or disease resistance phenotype (Figure 3.8). *atcdc48D* T-DNA knockout mutant is also morphologically indistinguishable from wild-type.

![Figure 3.10. AtCDC48A shows higher expression than other AtCDC48 paralogs.](image)

Microarray expression of AtCDC48 orthologs in root, leaf, senescing leaf, stamen, mature pollen, carpel and seed. Expression data was obtained from AtGenExpress Visualization Tool.

Although one allele of *atcdc48E*, *atcdc48E-2*, causes a slight morphological difference from wild-type, with slightly serrated leaves, it does not cause a characteristic autoimmune phenotype and does not affect the size of *snc1* plants, suggesting a separate
role in development. As the null allele of atcdc48E-1 is lethal, AtCDC48E likely is essential for normal plant growth and development, with no redundancy with AtCDC48D. The distinct role for this protein is also supported by its subcellular localization. Unlike AtCDC48A, which is found in both the nucleus and cytoplasm, AtCDC48E has a nuclear localization (Aker et al., 2006). Taken together with the lack of an obvious disease resistance phenotype in T-DNA knockout alleles, these data indicate that AtCDC48B-E do not share the immune-regulatory function of AtCDC48A.

Given the broad role of AtCDC48A orthologs in other species, and the lethality of the knockout atcdc48A-1 and atcdc48E knockout alleles, it seems likely that this protein is involved in many other biological processes in Arabidopsis besides immunity. Further in-depth phenotypic studies of the atcdc48A-4 mutant may reveal additional protein targets whose degradation is facilitated by AtCDC48A.

Materials and Methods

Mutant genotyping

The muse screen was carried out as previously described by Huang et al. (2013), using ethyl methansulfonate mutagenized mos2 snc1 npr1 plants. The AtCDC48 mutants were genotyped using the following primers: atcdc48A-4-WT-F:

CTTTTTCTTCTGTATCAAGGG

atcdc48A-4-mutant-F: CTTTTTCTTCTGTATCAAGGA, AtCDC48A-R:

CTTCAGCCAGCTTCATGTTC, SAIL_1182E09_FP:
CGAGGCGATACAATTCTCAT, SAIL_1182E09_RP:
CCAATTCCTAATCTGAGCC,
    SAIL_566E10_FP: GAGATCATCATCATCGCTG, SAIL_566E10_RP:
GAGATATTCGACAGGCACG, SALK_005957_FP:
CTAATAGGAAAGATCGCC, SALK_005957_RP:
ACGAAGAAGGATTTTAGC, SALK_024462_FP:
    GAAAGAGGAAATTTGCAGC, SALK_024462_RP:
CTTCTTGAACCAATCGCATC, SALK_074372_FP:
ACCCCTGTAAAGCCAATAAC, SALK_074372_RP:
GCGAAAGCTTCCTTAAGAC, WiscDsLox8G10_FP:
ACTCGAGGGTAAGAGGTC, WiscDsLox8G10_RP:
TTGAGACCGGGTAGATTGG

**Genetic mapping**

*muse8 mos2 snc1 npr1* mutants in the Columbia-0 ecotype of *Arabidopsis thaliana* were crossed with the Landsberg erecta (Ler) 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.

**Molecular cloning**

T-DNA insertion lines for *At3G06660*, *At3G08950*, and *At3G09840* were obtained from ABRC. Complementation tests were done by crossing T-DNA mutant lines with *muse8 mos2 snc1 npr1* quadruple 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.

**Gene Expression Analysis**

For qRT-PCR, total RNA was extracted from soil-grown plants and reverse transcribed into cDNA using a SuperScript III kit (Invitrogen). *PR2*, *AtCDC48A*, *SNC1* and *ACTIN7* gene expression were measured by quantitative PCR.

**Pathogen infection**

Infection with *H.a. Noco2* and *P.s.m ES4326* infection was carried out as described by Li et al., (2001). For *H.a. Noco2*, spore solution was sprayed on two week old soil grown seedlings, which were then grown at 18°C and 80% relative humidity,
under 12h-light/12h-dark conditions. After 7 days, a hemocytometer was used to quantify the conidiospores.

**Protein level**

Leaf tissue was weighed and immediately frozen in liquid nitrogen for total protein extraction. Extraction buffer (100mM Tris-HCL pH 8.0, 0.1% SDS, 2% β-mercaptoethanol) was added and mixed with a vortex mixer, and samples were centrifuged at 4°C for 10 min at 15 000 rpm. The supernatant was transferred to a new tube with ⅛ volume of 4X Laemmli loading buffer (60 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 5% b-mercaptoethanol, 0.01% bromophenol blue).

Levels of specific proteins were assayed by separating the total protein using SDS-PAGE followed by Western blotting.

**Immunoprecipitation**

Transient protein expression in *N. benthamiana* and co-immunoprecipitation was performed as described in Huang et al., (2014a). For the SNC1-flag immunoprecipitation, approximately 1g of tissue was used, and the α-flag beads were washed 3 times after precipitation.
Chapter 4: The proteasome regulator PTRE1 contributes to NLR turnover in Arabidopsis

Introduction

Like all multicellular organisms, plants face constant challenge from pathogens, such as bacteria and fungi, in their environment. However, they have evolved a sophisticated innate immune system which enables them to recognize most potential pathogens and mount an immune response, preventing disease. Receptors on the plant cell surface bind to elicitor molecules, which are widely conserved among major groups of microbes (Jones and Dangl, 2006). Recognition of these elicitors, termed Pathogen Associated Molecular Patterns (PAMPs), activates an immune signaling pathway known as PAMP-Triggered Immunity (PTI). However, many plant pathogens produce effector proteins which can disrupt PTI signaling, allowing an infection to become established. In response, plants employ Resistance (R) proteins, which provide a second layer of pathogen detection by perceiving the intracellular effector proteins or their effects in the cell. Most R proteins belong to the nucleotide-binding, leucine-rich repeat (NLR) family (Maekawa et al., 2011).

R protein activation leads to a rapid and strong immune response, including the production of reactive oxygen species, production of antimicrobial compounds and induction of a type of programmed cell death known as the hypersensitive response (HR). While these defense outputs are quite effective at preventing pathogen spread, they are detrimental to plant growth. Such negative effects are obvious in mutants with mutations
that cause constitutive defense signaling which results in severe dwarfism (Li et al., 2001; Yang et al., 2010; Wang et al., 2013). Thus, in the absence of pathogen attack, negative regulation of \( R \) genes and their downstream signaling components is essential for optimal plant growth.

One important regulatory mechanism in eukaryotes is through targeted protein degradation by the ubiquitin proteasome system (UPS). In the UPS pathway, substrates are marked for degradation by the addition of the small peptide ubiquitin (Vierstra, 2009). Ubiquitin can be conjugated onto lysine residues within the substrate itself, or onto previously added ubiquitin moiety, forming a poly-ubiquitin chain. Poly-ubiquitinated substrates are subsequently recognized by the 26S proteasome, a large protein complex with proteolytic function. The 26S proteasome is made up of the 19S regulatory particle, which recognizes the poly-ubiquitin conjugates and unfolds them, and the 20S core particle (CP), the proteolytic core that possesses multiple subunits with protease activity.

The Mutant, snc1 enhancing (MUSE) screen was a forward genetic screen for mutants that enhance the autoimmunity mediated by a gain-of-function NLR mutant \( snc1 \), with the aim of identifying novel negative regulators of immunity (Huang et al., 2013). A number of the \( muse \) mutants identified in this screen are impaired in the turnover of the SNC1 protein by the ubiquitin proteasome system (UPS) (Cheng et al., 2011; Huang et al., 2014a; Huang et al., 2014b; Xu et al., 2015; Copeland et al., 2016; Huang et al., 2016). For example, a partial loss-of-function mutant of the ATPase AtCDC48A causes increased SNC1 protein accumulation which is associated with enhanced disease resistance and dwarf, curly-leaf morphology (Copeland et al., 2016).
*CDC48* homologs function in UPS-mediated degradation of diverse substrates, are essential genes in eukaryotes, including Arabidopsis, yeast, animals, and (Dai and Li, 2001; Rancour et al., 2002; Meyer et al., 2012).

The most well-characterized function of CDC48 in the UPS is promoting the degradation of substrates by facilitating their recognition by the proteasome. A number of other proteins also assist in UPS-mediated degradation by shuttling poly-ubiquitinated substrates to the proteasome, modulating proteasome activity (Yu and Matouschek, 2017). One proteasome regulator, named PI31 or PSMF1 in animals, interacts with the mammalian CDC48 and the proteasome, and inhibits 20S CP activity *in vitro* (Li et al., 2014; Clemen et al., 2015). However, overexpression of PI31 in the cell appears to increase the activity of the assembled 26S proteasome (Li et al., 2014). In Arabidopsis, the PI31 homolog PTRE1 also positively regulates cellular proteasome activity (Yang et al., 2016). Upon perception of the plant hormone auxin, proteasome activity in wild-type plants is reduced, while *ptre1* mutants have constitutively lower proteasome activity that is unaffected by auxin. This suggests that a reduction in PTRE1 function is responsible for auxin-mediated proteasome suppression.

*ptre1* mutant plants also display a dwarf, curly-leaf morphology similar to many mutants with constitutive immune signaling, including *atcdc48A-4* (Copeland et al., 2016; Yang et al., 2016). This prompted us to examine whether PTRE1 also functions in NLR degradation by the proteasome. Here we report that the *ptre1* mutation causes immune phenotypes such as increased *PR2* marker gene expression and enhanced disease resistance to a virulent pathogen. The stability of SNC1 is also increased, indicating that PTRE1 indeed promotes NLR turnover.
Results

**PTRE1 is a negative regulator of immunity**

Arabidopsis PTRE1 was recently identified based on homology to be orthologous to human proteasome regulator PI31. It seems to stimulate 26S proteasome activity (Yang et al., 2016). Interestingly, ptre1 loss-of-function mutants display a dwarf, curled-leaf phenotype similar to cdc48A-4 and other autoimmune mutants (Yang et al., 2016; Figure 4.1A). In order to determine whether this morphological phenotype is associated with increased immune signaling, we first examined expression of the defense marker gene PATHOGENESIS RELATED 2 (PR2). Plants of ptre1 T-DNA mutant indeed showed increased PR2 expression (Figure 4.1B). Enhanced disease resistance was also observed when ptre1 mutants were challenged with the virulent pathogen **Hyaloperonospora arabidopsidis** (H.a.) Noco2 (Figure 4.1C). The growth of **Psuedomonas syringae** pv **maculicola** (P.s.m.) ES4326 also tended to be lower in ptre1 plants, but this was not always statistically significant (Figure 4.1D). Taken together, knocking out PTRE1 leads to slight autoimmunity similar to that in atcdc48A-4.

The ptre1 mutant has increased SNC1 stability

atcdc48A-4 and a number of other muse mutants have reduced turnover of NLR proteins such as SNC1, which causes constitutive immune signaling. Since PTRE1 is thought to promote protein turnover by the proteasome, and may interact with
AtCDC48A, we tested whether \textit{ptre1} mutants also have reduced SNC1 turnover. SNC1 protein abundance was significantly higher in \textit{ptre1} plants than in the wild-type (Figures 4.2A, 4.2B). We did not observe increased \textit{SNC1} gene expression in the \textit{ptre1} plants, suggesting that the increased SNC1 protein abundance is due to increased stability of the protein (Figure 4.2C). To further confirm that the increase in SNC1 protein level is not increased due to indirect effects of increased immunity in the \textit{ptre1} mutant, we introduced the \textit{ptre1} mutation into the \textit{eds1} background, which is impaired in basal immunity and in signalling mediated by many NLRs (Wiermer et al., 2005). The \textit{ptre1 eds1} double mutant was morphologically similar to the \textit{ptre1} single mutant (Figure 4.2D), likely due to pleiotropic effects such as the auxin-related phenotypes reported by (Yang et al., 2016). A significant increase in SNC1 protein abundance was also observed in the \textit{ptre1 eds1} double mutant plants, further supporting our hypothesis that \textit{PTRE1} promotes SNC1 turnover (Figures 4.2E and 4.2F).
Figure 4.1. PTRE1 negatively regulates immunity.

(A) Morphology of 5-week-old plants of the indicated genotypes grown on soil.

(B) PR2 gene expression in the indicated genotypes. qRT-PCR was performed using RNA extracted from 4-week-old plants grown on soil.

(C) Growth of H.a. Noco2 on plants of the indicated genotypes. Plants were approximately 2.5 weeks old when inoculated, and spores were counted one week later. Error bars represent standard error of the mean (SEM).

(D) Growth of the bacterial pathogen P.s.m. ES4326 on plants of the indicated genotypes. Plants were infiltrated with bacterial suspension (O.D.600 = 0.001),
and bacterial titer was measured after 0 and 3 days. Error bars represent standard deviation.

**Figure 4.2.** A mutant of ptre1 displays increased SNC1 protein stability.
(A) Representative SNC1 protein level in the indicated genotypes. Total protein was extracted from 3-week-old plants grown on soil, and immunoblot analysis was performed using a SNC1-specific antibody (Li et al., 2010). Ponceau staining is shown as a loading control.

(B) Quantification of SNC1 protein level from (A). For each sample, the intensity of the SNC1 band was normalized to the Ponceau, then Col was set to 1. Error bars represent standard error of the mean. Asterisks indicate a significant difference (p < 0.05) from Col as determined by a t-test. The experiment was repeated at least 3 times with similar results.

(C) SNC1 gene expression in the indicated genotypes. qPCR was performed on RNA extracted from plants in (A). SNC1 expression was normalized to ACTIN7. Error bars represent standard error of the mean.

(D) Morphology of 4-week-old plants of the indicated genotypes grown on soil.

(E) Representative SNC1 protein level in the indicated genotypes. Experiment was performed as in (A).

(F) Quantification of SNC1 protein level from the plants in (E). Experiment was performed as in (B).

**PTRE1h is not redundant with PTRE1**

A BLAST search of the Arabidopsis genome using *PTRE1* as a query revealed a similar sequence, AT1G48530, which we named *PTRE1h*. Based on an alignment of *PTRE1* and *PTRE1h*, *PTRE1h* is a truncated sequence, containing homology to the first 3 exons of *PTRE1* (Figure 4A). However, the deduced amino acid sequences of both genes within this region share 56% identity and 70% similarity, prompting us to test whether
PTRE1h may share redundant function with PTRE1. A ptre1h mutant containing an exonic T-DNA insertion was morphologically indistinguishable from Col (Figure 4.3B). Although a statistically significant increase in PR2 gene expression was observed in the ptre1h mutant, this difference was only approximately 2-fold (Figure 4.3C). ptre1h plants were also as susceptible to H.a. Noco2 infection as Col plants (Figure 4.3D). To test for possible redundancy between PTRE1 and PTRE1h, we produced a ptre1 ptre1h double mutant by crossing. The morphology of the double mutant was similar to the ptre1 single mutant (Figure 4.3B). Thus, PTRE1h does not seem to have redundant function with PTRE1.
Figure 4.3. PTRE1h is not redundant with PTRE1.

(A) Gene model of PTRE1 and PTRE1h. Exons are represented as boxes and introns are represented as lines. Arrowheads indicate the positions of the T-DNA insertions in the mutants described.

(B) Morphology of 5-week-old plants of the indicated genotypes grown on soil.

(C) PR2 gene expression in the indicated genotypes. qRT-PCR was performed using RNA extracted from 5-week-old plants grown on soil.

(D) Growth of H.a. Noco2 on plants of the indicated genotypes. Plants were 2 weeks old when inoculated, and spores were counted 1 week later. Error bars represent standard error of the mean (SEM).

Discussion

NLR turnover through the UPS is crucial to limit NLR over-accumulation and prevent autoimmunity (Holt et al., 2005; Li et al., 2015). In this study, we showed that the proteasome regulator PTRE1 is a negative regulator of immunity and promotes NLR turnover. ptre1 mutants display a dwarf phenotype, increased PR2 gene expression, and enhanced resistance to virulent pathogens (Figure 4.1). SNC1 protein abundance is also increased in the ptre1 mutant. The Arabidopsis genome contains a truncated homolog of PTRE1, PTRE1h, that shares high homology in the N-terminal region (Figure 4.3). However, PTRE1h does not share redundant function with PTRE1.

The exact function of PTRE1 in proteasome regulation is still unclear (Fort et al., 2015). In vitro, mammalian homologs of PTRE1 interact with the 20S CP and inhibit its proteolytic activity (Li et al., 2014; Clemen et al., 2015). Some genetic and biochemical
evidence indicates that PTRE1 homologs stimulate 26S proteasome activity in vivo. Overexpression of Drosophila PI31 suppresses phenotypes caused by mutant proteasome subunits with impaired function (Bader et al., 2011). However, overexpression of PI31 in mammalian cells did not appear to affect the proteasome activity (Li et al., 2014). In Arabidopsis, ptre1 mutants showed reduced in vivo proteasome activity and the accumulation of ubiquitin conjugates (Yang et al., 2016). Our finding that the ptre1 mutation increases the stability of SNC1, a protein known to be degraded by the UPS, also suggests that PTRE1 activates proteasome activity, at least for some substrates.

The molecular mechanism of PTRE1 function also remains to be elucidated. PI31 interacts with F-box proteins in both Drosophila and human (Kirk et al., 2008; Bader et al., 2011). However, at least in Drosophila this interaction seems to stabilize PI31 but is not strictly required for its proteasome-regulatory function. PSMF1 from mouse interacts with VCP, the homolog of Arabidopsis AtCDC48A (Clemen et al., 2015). In vitro, VCP and PSMF1 were both shown to interact with purified 20S CP and play antagonistic roles in regulating its proteolytic activity, with VCP activating the 20CP and PSMF1 inhibiting it. However, given that PTRE1 likely promotes the activity of the 26S proteasome, the most common and active proteasome complex in vivo, it is possible that PTRE1 may work together with AtCDC48A, which is also known to promote SNC1 degradation (Copeland et al., 2016). However, the interaction between AtCDC48A and PTRE1 in Arabidopsis remains to be tested. In addition, AtCDC48A is thought to interact with a large diversity of proteins involved in multiple aspects UPS function, making it difficult to interpret the precise molecular mechanism by which PTRE1 and AtCDC48A may function together.
The *ptre1* mutant displays a range of altered phenotypes, including dwarf morphology, reduced fertility, and a defective phototrophic response in seedlings, which have been suggested to be related to auxin responses (Yang et al., 2016). Yang et al. (2016) also found that the *ptre1* mutant shows increased sensitivity to the hormones abscisic acid and brassinosteroids. The wide range of phenotypes displayed by the *ptre1* mutant suggest that PTRE1 promotes the turnover of many different substrates. **PTRE1** promoter activity was also widely detected throughout the plant, including root, leaf, and flower tissue (Yang et al., 2016). However, RGA1, a transcriptional repressor known to be degraded during gibberellin signaling, did not show increased stability in the *ptre1* mutant, indicating that some degree of specificity in PTRE1 function (Dill et al., 2004; Yang et al., 2016). Our finding that PTRE1 is involved in SNC1 turnover adds to the current knowledge of PTRE1 function. A more thorough characterization of protein stability in the *ptre1* mutant is required to determine the specificity of substrates affected by PTRE1.

**Materials and Methods**

**Mutant genotyping**

Except where otherwise noted, plants were grown at 22°C under 16h light/ 8 h dark light regime. Mutants were genotyped through PCR using the following primers:

SALK_034353_FP: TGCTTTCTCAGCATAATCC, SALK_034353_RP: TCAGGTTGGCTAGACCTCC, SAIL.seq_860F03_FP:
GTCCGGTTGAAGTGAGAATG, SAILseq_860F03_RP:
TTCCCTTTATCGTTATGGG.

Gene expression analysis

Total RNA was extracted from soil-grown plants using an EZ-10 Spin Column kit (BioBasic), and reverse transcribed into cDNA using reverse transcriptase (ABI). PR2, SNC1, and ACTIN7 gene expression were measured by quantitative PCR using specific primers.

Pathogen infection

Protocols for infection with *H. a. Noco2* and *P. s. m* ES4326 infection have been previously described by Li et al., (2001). Briefly, *H. a. Noco2*, spore suspension at a concentration of 100,000 spores/mL was sprayed on two week old soil grown seedlings, which were then grown at 18°C and 80% relative humidity, under 12h-light/12h-dark conditions. After 7 days, a hemocytometer was used to quantify the conidiospores. For *P. s. m* ES4326 infection, plants were grown at 22°C under 12h-light/12h-dark conditions.

Protein level

Protein was extracted and subjected to immunoblot as described in Copeland et al., (2016).
Chapter 5: Final summary and future perspectives

Positive regulation of immunity by AtCHIP

Findings from the AtCHIP study

Much of the study of the UPS focuses on the E3 ligase enzymes, as plant genomes encode a huge diversity of E3s and they are thought to be the determinants for substrate specificity (Vierstra, 2009). Because the biochemical reactions involved in ubiquitin transfer are well characterized, proteins with E3 ligase activity can be identified in silico with relative certainty based on the presence of domains that bind to E2 enzymes or CRL complexes. For monomeric E3 ligases, protocols have also been established to verify the ubiquitin ligase activity in vitro. Thus, E3 ligases may be tested for their role in plant immunity. The U-box containing E3 ligase AtCHIP was found to positively regulate immunity, as AtCHIP overexpression resulted in enhanced disease resistance to virulent pathogens, while atchip mutants displayed enhanced disease susceptibility. We were unable to ascertain which immune pathway AtCHIP functions in; however, since atchip mutants retained resistance mediated by two NLRs RPS4 and RPS2, atchip seems unlikely to be involved in NLR signaling. Further, although AtCHIP had been reported to ubiquitinate HSP90, a known regulator of immunity, we did not observe a difference in HSP90.3 protein level in either the atchip mutants or the AtCHIP-OX lines.
Possible mechanisms for the role of AtCHIP in immunity

Identifying the targets of E3 ligases remains a significant challenge. Finding protein-protein interactions with yeast-2-hybrid screens or IP-MS has been successful with some E3s; however, the interactions between E3s and their substrates are often transient and result in the degradation of the substrate, which may cause difficulty in identifying the substrate (Iconomou and Saunders, 2016). AtCHIP is also somewhat unique in that multiple substrates have already been reported, although of these only HSP90 has a known function in immunity (Kundrat and Regan, 2010; Huang et al., 2014a). Other degradation targets of AtCHIP include the chloroplast proteins Clp4 and FtsH1 (Shen et al., 2007a; Shen et al., 2007b). Since the immune phenotype of atchip mutants is relatively subtle, it is possible that it is caused indirectly from improper degradation of these substrates. Chloroplasts are the site of production of several plant hormones, including SA, and also contribute ROS and Ca\(^{2+}\) ions during defense signaling (Serrano et al., 2016). Thus, impairment of chloroplast function may also affect immunity.

Based on studies of AtCHIP homologs in mammalian cells, it may also function more generally to eliminate misfolded proteins that cannot be refolded by HSP70 and HSP90 chaperones (Connell et al., 2001; Murata et al., 2003). It is therefore possible that disruption of protein homeostasis may have a pleiotropic effect on immune signaling. This hypothesis is supported by the involvement of AtCHIP in response to temperature stress, including our observation that AtCHIP-OE plants show enhanced disease resistance specifically at low temperature (Yan et al., 2003).
Positive regulation of NLR turnover

Findings from the AtCDC48A study

The MUSE screen was a forward genetic screen for enhancers of the autoimmune phenotype of snc1. As with other muse mutants, muse8 was identified based on its extremely dwarf morphology. The causal mutation was initially identified by Virginia Woloshen and found to encode an allele of AtCDC48A; thus it was renamed atcdc48A-4.

AtCDC48A and its homologs in other species have been associated with many diverse cellular processes; however, the involvement of several muse mutants in NLR degradation prompted me to test whether AtCDC48A also contributes to this process. Indeed, atcdc48A-4 mutants do show increased SNC1 protein stability, and the autoimmune phenotype of atcdc48A-4 is largely dependent on SNC1. AtCDC48A also interacts with MUSE3, an E4 enzyme required for proper SNC1 degradation, leading to the conclusion that AtCDC48A facilitates poly-ubiquitination of SNC1 or recognition of the ubiquitinated SNC1 by the proteasome.

Possible roles of AtCDC48A in the UPS

Recognition of ubiquitinated substrates by the 26S proteasome complex is a possible source of regulation and specificity in UPS function that has not been well studied. While a chain of four ubiquitin peptides joined by K48 linkages has been accepted as the canonical signal for protein degradation, other characteristics of the protein, including interactors, influence the efficiency of degradation (Yu and
Matouschek, 2017). The hexameric ring of AtCDC48A may bind to substrates and use
the energy derived from ATP hydrolysis to extract them from membranes or protein
complexes, making them available for recognition and degradation by the UPS (Jentsch
and Rumpf, 2007).

Besides being recognized by the ubiquitin receptors in the 19S RP, ubiquitin
conjugates may also be recognized by adaptor proteins, which then shuttle the substrates
to the proteasome. These adaptors show preferences for specific substrates. For example,
in yeast, deletion of \textit{rad23} causes strong defects in proteasome-mediated degradation
only of specific substrates (Verma et al., 2004). CDC48 interacts with many adaptor
proteins and aids in their shuttling function (Baek et al., 2013). The interaction between
Rad23 and the yeast E4 Ufd2, for example, is mediated by simultaneous binding of both
proteins with Cdc48, which likely allows Rad23 to rapidly identify the poly-ubiquitinated
products of Ufd2.

Plant RAD23 homologs are targeted by multiple pathogen effectors and are
thought to be coopted to promote degradation of specific host immune proteins (Banfield,
2015; Üstün et al., 2016). It would thus be interesting to determine whether Arabidopsis
RAD23 or other cofactors function with AtCDC48A to promote NLR turnover.

\textbf{Findings from the PTRE1 study}

Based on the role of \textit{AtCDC48A} in promoting SNC1 turnover, we wondered
whether other proteasome-associated proteins affect immunity through affecting NLR
stability. Yang et al. (2016) recently reported that PTRE1 promotes proteasome activity,
and that \textit{ptre1} mutants exhibit a dwarf, curly-leaf morphology reminiscent of autoimmune mutants such as \textit{atcdc48}, which led us to hypothesize that PTRE1 may facilitate NLR turnover. Indeed, \textit{ptre1} mutants showed increased SNC1 protein level and immune-related phenotypes such as constitutive \textit{PR2} gene expression and mildly enhanced disease resistance.

**Future directions for studying PTRE1**

Although PTRE1 (PI31 or PSMF1 in animals) was identified as a proteasome regulator nearly two decades ago, its biochemical role remains unclear. PI31 was first shown to inhibit the activity of the 20S CP, which likely occurs when PI31 interacts with the 20S CP and prevents assembly of the 19S RP (Chu-Ping et al., 1992; Li et al., 2014). However, overexpression of PI31 does not inhibit overall proteasome activity, and actually seems to stimulate activity of the 26S proteasome, which is the functional form \textit{in vivo} (Bader et al., 2011; Li et al., 2014). PI31 was also found to interfere with the maturation of the immunoproteasome (Zaiss et al., 2002; Schmidt and Finley, 2014). The 20S CP of the immunoproteasomes incorporate specialized isoforms of several subunits, and are produced in animal cells following immune stimulation. Although immunoproteasomes have not been found in plants, plant genomes do contain multiple isoforms of many 20S CP subunits, raising the possibility of functional specialization (Smalle and Vierstra, 2004). It would be interesting to determine whether PTRE1 affects assembly of proteasomes containing different subunits.
A murine PTRE1 homolog has also been shown to interact with valosin containing protein (VCP), the homolog of CDC48 in mouse (Clemen et al., 2015). *In vitro*, the two proteins have an antagonistic effect: VCP promotes 20S proteasome activity and PTRE1 inhibits this effect. However, it is possible that *in vivo*, PTRE1 functions together with CDC48 to facilitate recognition and degradation of poly-ubiquitinated substrates by the 26S proteasome. It remains to be determined whether Arabidopsis PTRE1 and AtCDC48A interact. It would also be valuable to identify additional proteins whose degradation is facilitated by PTRE1 and AtCDC48A, and to determine how extensively these groups overlap. Overall, a more thorough examination of the genetic and biochemical interaction network of PTRE1 is required to elucidate its molecular function in the UPS.

**Summary**

Taken together, these studies demonstrate both positive and negative roles of the UPS in the regulation of plant immunity. The study of AtCHIP revealed its positive role in plant immune regulation. In contrast, the UPS-mediated turnover of NLR proteins, exemplified by SNC1, is a crucial mechanism to negatively regulate immunity. Many proteins involved in this process at the level of SNC1 ubiquitination had previously been identified, and this thesis adds AtCDC48A and PTRE1 as proteins likely involved further downstream, at the junction with the 26S proteasome itself. Overall, the studies described here contribute to our knowledge of immune regulation by the UPS.
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