CHARACTERIZATION AND CLONING OF

SUPPRESORS OF chs3-2D IN ARABIDOPSIS

by

Chipan Zhu

B.Sc., Huazhong Agricultural University, 2012

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Botany)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

February 2015

© Chipan Zhu, 2015
Abstract

Plant innate immunity depends on the function of a large number of intracellular immune receptor proteins, the majority of which are structurally similar to mammalian NUCLEOTIDE-BINDING OLIGOMERIZATION DOMAIN (NOD)-LIKE RECEPTOR (NLR) proteins. CHILLING SENSITIVE 3 (CHS3) encodes an atypical Toll/Interleukin 1 Receptor (TIR)-type NLR protein with an additional Lin-11, Isl-1 and Mec-3 (LIM) domain at its C terminus. The gain-of-function mutant allele chs3-2D exhibits severe dwarfism and constitutively activated defense responses, including enhanced resistance to virulent pathogens, high PATHOGENESIS-RELATED (PR) gene expression, and salicylic acid accumulation. To search for novel regulators involved in the CHS3-mediated immune signaling pathway, we conducted suppressor screens in the chs3-2D and chs3-2D pad4-1 genetic backgrounds. Alleles of sag101 and eds1-90 were isolated as complete suppressors of chs3-2D, and alleles of sgt1b were isolated as partial suppressors of chs3-2D pad4-1. This suggests that SAG101, EDS1-90, and SGT1b are all positive regulators of CHS3-mediated defense signaling. Additionally, the TIR-type NLR-encoding CSA1 locus located in the Arabidopsis genome adjacent to CHS3 was found to be required for CHS3-mediated signaling. CSA1 is located 3.9kb upstream of CHS3 and is transcribed in the opposite direction. Altogether, these data illustrate the distinct genetic requirements for CHS3-mediated defense signaling.
Preface

The work described in this thesis is the culmination of research from September 2012 through December 2014. This project benefited greatly from the contributions made by Dr. Fang Xu and Ms. Yanan Liu.

Chapter 2 is a manuscript that is under review, titled “Autoimmunity conferred by chs3-2D relies on CSA1, its adjacent TIR-NB-LRR-encoding neighbor” authored by Fang Xu, Chipan Zhu, Volkan Cevik, Kaeli Johnson, Yanan Liu, Kee Sohn, Jonathan D. Jones, Eric B. Holub and Xin Li. The first 3 authors are co-first authors who contributed equally to the project.

Dr. Xu started the suppressor screen of chs3-2D in 2012. She successfully isolated eight independent soch (suppressor of chs3-2D pad4-1) mutants with second-site mutations which fell into three complementation groups. Based on the Illumina next generation sequencing and map-based cloning data, Dr. Xu identified one sag101 allele and three eds1 alleles at that time. She also determined that one of the soch chs3-2D mutants carried a mutation in CSA1 by Illumina next generation sequencing; I cloned an additional 3 csa1 alleles by direct Sanger sequencing. Additionally, Yanan Liu conducted the mutagenesis of chs3-2D pad4-1 and identified plants with altered morphology. I continued this project for screening those plants for alterations in defense signaling and subsequently cloned two sgt1b alleles. Volkan Cevik, Kee Sohn, Jonathan D. Jones and Eric B. Holub contributed to the cloning of CSA1 and manuscript revision.

Since the ultimate goal of both the chs3-2D and chs3-2D pad-4 screens is to determine the CHS3-mediated defense pathway, I summarized the results from both screens and developed it into a full research story.
# Table of contents

Abstract ........................................................................................................................... ii

Preface ................................................................................................................................ iii

Table of contents ......................................................................................................... iv

List of figures ............................................................................................................... vi

List of abbreviations ........................................................................................................ vii

Acknowledgements ...................................................................................................... x

Chapter 1 Introduction and literature review ........................................................................... 1

1.1 Plant innate immunity ................................................................................................. 1

1.2 Multi-domain architecture of R proteins ....................................................................... 4

1.3 R protein regulation ..................................................................................................... 5

1.4 R protein signaling ...................................................................................................... 6

1.5 Dissecting plant immunity using \textit{snc1} ..................................................................... 8

1.5.1 \textit{snc1} and \textit{snc1} suppressor screens ................................................................. 8

1.5.2 \textit{Modifier of snc1 (MOS)} ...................................................................................... 9

1.6 Thesis objective ......................................................................................................... 13

Chapter 2 Autoimmunity conferred by \textit{chs3-2D} relies on SAG101, EDS1-90, SGT1 and CSA1, its adjacent TIR-NB-LRR neighbour ........................................................................................................... 14

2.1 Introduction ................................................................................................................. 14

2.2 Results ....................................................................................................................... 15

2.2.1 Identification and characterization of \textit{suppressors of chs3-2D} .............................. 15

2.2.2 \textit{chs3-2D} is only marginally suppressed by \textit{pad4-1} ........................................... 19
2.2.3 Identification and characterization of *suppressors of chs3-2D pad4-1* ........................................ 21

2.2.4 CHS3-mediated defense responses are completely dependent on EDS1-90 and SAG101 ...... 23

2.2.5 PAD4-independent CHS3-mediated defense signaling is partially dependent on SGT1b ...... 23

2.2.6 Immune signaling mediated by CHS3 requires its neighbor TNL protein CSA1 ................... 26

2.3 Discussion ........................................................................................................................................ 29

2.4 Materials and methods .................................................................................................................. 34

2.4.1 Plant growth .............................................................................................................................. 34

2.4.2 Oomycete infection assay ......................................................................................................... 34

2.4.3 *PR1* and *PR2* gene expression analysis ............................................................................... 34

2.4.4 *CSA1* gene expression ............................................................................................................ 35

2.4.5 Map-based cloning and Sanger sequencing .............................................................................. 35

Chapter 3 Discussion and future perspectives .................................................................................. 36

3.1 Insights to the LIM functionality possessed by CHS3 ................................................................. 36

3.2 CHS3 reliance on EDS1-90/SAG101 module ................................................................................ 38

3.3 Insights to SGT1b functionality .................................................................................................... 39

3.4 From “gene-for-gene” to “gene-for-genes” .................................................................................. 40

References ........................................................................................................................................... 42
List of figures

Figure 1.1: Signaling components downstream of TIR-type and CC-type NB-LRR R proteins ............... 7

Figure 2.1. Characterization of soch chs3-2D mutants ........................................................................ 17

Figure 2.2 Autoimmunity conferred by chs3-2D is only marginally attenuated by pad4-1 ................. 20

Figure 2.3 Characterization of socp chs3-2D pad4-1 mutants .............................................................. 22

Figure 2.4 Summary of the mutations in all the soch and socp mutants .............................................. 25

Figure 2.5 Complementation test of four csa1 alleles and gene arrangements of CHS3 and CSA1 ....... 27

Figure 2.6 CSA1 gene expression in wild type, chs3-2D, chs3-2D pad4-1, chs3-2D eds1-90-10, and chs3- 2D eds1-90-11 ........................................................................................................................................ 28

Figure 2.7 Proposed working model for the CHS3-mediated defense pathway .................................... 33
List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>APAF-1</td>
<td>Apoptotic peptidase activating factor 1</td>
</tr>
<tr>
<td>AtCDC5</td>
<td>Cell Division Cycle 5</td>
</tr>
<tr>
<td>Avr</td>
<td>Avirulent or Avirulence</td>
</tr>
<tr>
<td>AvrPphB</td>
<td>Avirulence protein from <em>Pseudomonas syringae</em> pv. <em>phaseolicola</em></td>
</tr>
<tr>
<td>Avr-Pita</td>
<td>Avirulence protein from <em>Magnaporthe grisea</em></td>
</tr>
<tr>
<td>AvrPto</td>
<td>Avirulence protein from <em>Pseudomonas syringae</em> Pv. <em>tomato</em></td>
</tr>
<tr>
<td>AvrPtoB</td>
<td>Avirulence protein from <em>Pseudomonas syringae</em> pv. <em>tomato</em></td>
</tr>
<tr>
<td>AvrRpm1</td>
<td>Avirulence protein from <em>Pseudomonas syringae</em> pv. <em>maculicola</em></td>
</tr>
<tr>
<td>AvrRps4</td>
<td>Avirulence protein from <em>Pseudomonas syringae</em> pv. <em>pisi</em></td>
</tr>
<tr>
<td>AvrRpt2</td>
<td>Avirulence protein from <em>Pseudomonas syringae</em> pv. <em>tomato</em></td>
</tr>
<tr>
<td>BAK1</td>
<td>BR11-Associated Receptor Kinase</td>
</tr>
<tr>
<td>BAT2</td>
<td>HLA-B Associated Transcripts 2</td>
</tr>
<tr>
<td>BCAS2</td>
<td>Breast Carcinoma Sequence 2</td>
</tr>
<tr>
<td>BIK1</td>
<td>Botrytis-Induced Kinase 1</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>CC</td>
<td>Coiled-Coil</td>
</tr>
<tr>
<td>CED-4</td>
<td>Cell Death Protein 4</td>
</tr>
<tr>
<td>CERK1</td>
<td>Chitin Elicitor Receptor Kinase 1</td>
</tr>
<tr>
<td>CEBiP</td>
<td>Chitin Elicitor-Binding Protein</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>Col-0 or Col</td>
<td>Columbia, an Arabidopsis ecotype</td>
</tr>
<tr>
<td>CPR1</td>
<td>Constitutive Expresser of <em>PR</em> Genes 1</td>
</tr>
<tr>
<td>CSA1</td>
<td>Constitutive shade avoidance 1</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxyl terminal</td>
</tr>
<tr>
<td>DND1</td>
<td>Defense no Death 1</td>
</tr>
<tr>
<td>DND2</td>
<td>Defense no Death 2</td>
</tr>
<tr>
<td>E1</td>
<td>Ubiquitin-activating enzyme</td>
</tr>
<tr>
<td>E2</td>
<td>Ubiquitin-conjugating enzyme</td>
</tr>
<tr>
<td>E3</td>
<td>Ubiquitin ligase</td>
</tr>
<tr>
<td>EDS1</td>
<td>Enhanced Disease Susceptibility 1</td>
</tr>
<tr>
<td>EFR</td>
<td>EF-Tu receptor</td>
</tr>
<tr>
<td>EF-Tu</td>
<td>Bacterial Elongation Factor Tu</td>
</tr>
<tr>
<td>elf18</td>
<td>An N-acetylated peptide comprising the first 18 amino acids of EF-Tu</td>
</tr>
<tr>
<td>EMS</td>
<td>Ethyl methanesulfonate</td>
</tr>
</tbody>
</table>
ETI  Effector-Triggered Immunity
ETS  Effector-Triggered Susceptibility
flg22 Flagellin conserved domain 22
FLS2 Flagellin-Sensitive 2
G-patch Glycine-rich nucleic acid binding domain
H.a. Hyaloperonospora arabidopsidis
HR Hypersensitive Response
HSP90 Heat Shock Protein 90
Ler Landsberg erecta; an Arabidopsis ecotype
LIM Lin11, Isl-1 & Mec-3
MAC MOS4-Associated Complex
MOS Modifier of snc1
MS medium Murashige and Skoog medium
NB-LRR Nucleotide Binding-Leucine Rich Repeat
NLR NOD-LIKE-RECEPTOR
NOD Nucleotide-binding Oligomerization
NPC Domain
NRG1 Nuclear Pore Complex
NPR1 Nonexpressor of PR genes
N-terminal Amino terminal
PAD4 Phytoalexin Deficient 4
PAMP Pathogen Associated Molecular Pattern
PBS1 AvrPphB susceptible 1
PR Pathogenesis-Related
PRL1 Pleiotropic Regulatory Locus 1
PRR Pattern Recognition Receptor
PTI PAMP-Triggered Immunity
pv Pathovar
R Resistance
RIN4 RPM1-Interacting Protein 4
ROS Reactive Oxygen Species
RPP4 RECOGNITION OF PERONOSPORA PARASITICA 4
RPS2 Resistant to P. Syringae 2
RPS4 Resistant to P. Syringae 4
RPS5 Resistant to P. Syringae 5
RRS1 Resistant to Ralstonia Solanacearum
SA Salicylic Acid
SAG101 Senescence-associated protein 101
SERK Somatic-Embryogenesis- Receptor-Like Kinase
SGT1 Suppressor of G2 Allele of SKP1
SKP1 Suppressor of Kinetochore Protein 1
SNC1 Suppressor of npr1, constitutive, 1
SRFR1 Suppressor of rps4-RLD 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-DNA</td>
<td>Transfer DNA</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/interleukin-1 Receptor</td>
</tr>
<tr>
<td>TPL</td>
<td>TOPELESS</td>
</tr>
<tr>
<td>TPR1</td>
<td>TOPLESS-RELATED 1</td>
</tr>
<tr>
<td>TTSS</td>
<td>Type III secretion system</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
Acknowledgements

It has been more than two years since I came to UBC, I was only able to finish my Master’s degree through the help of many people. First and foremost, I would like to thank Dr. Xin Li, who is an excellent and smart scientist; she provided me with countless valuable suggestions, not only academically, but also philosophically. Her enthusiasm towards science impresses me the most; she always works hard and energetically to try to solve problems in our field. It is really my great honor to have done my graduate studies under her supervision. Special thank goes to Dr. Yuelin Zhang for his sparkling ideas and thought-provoking suggestions for my projects. I would also like to thank my committee members, Dr. George Haughn and Dr. Carl Douglas for their suggestions and guidance during my research. I really appreciated George’s precious suggestions when I felt confused.

I would also like to thank every member in the Li lab, a place full of love and understanding. Many outstanding and helpful people helped advance my progress in academic research. Thanks to Fang Xu, Shuai Huang and Yuti Cheng for their patience and guidance on my projects; Thanks to Kaeli Johnson for her help with revision of my manuscript and editing of other writing; Thanks to Charles Coperland, Tongmei and Oliver Dong for valuable discussions; Thanks to Paul Kapos, Xionghui Zhong for their emotional support all the time; many thanks to previous lab mates, Yan Huang and Lily Li for suggestions. Also, I would like to thank Yanan Liu for starting the suppressor screen of chs3-2D pad4.

Thanks to the Botany Department for their financial support through teaching assistantships. I would like to give special thanks to Karen Smith and Sunita Chowrira for being inspirational teaching instructors. Moreover, I really appreciate the financial support from China Scholarship Council, which helps me focus on the research.

Finally, I could not find enough words to express my love and gratitude to my dear family members and friends in China. Your love and support, always drives me to continue and be strong during the tough time when I am abroad.
Chapter 1 Introduction and literature review

1.1 Plant innate immunity

Plants are under constant attack from various pathogens in their complex living environment, such as bacteria, fungi, viruses and nematodes. These pathogens use different methods to infect plants and absorb the plant’s nutrients for their own survival. However, plants are healthy most of the time; the diseased state is the exception. In addition to their rigid cell wall and cuticular waxes which can prevent pathogen entry (Thordal-Christensen, 2003), plants possess a multilayered immune system to protect themselves from microbial challenge.

The first layer of the plant surveillance mechanism relies on the recognition of PATHOGEN-ASSOCIATED-MOLECULAR-PATTERNS (PAMPs) via cell-surface localized PATTERN-RECOGNITION-RECEPTORS (PRR). PAMP collectively refers to evolutionarily conserved molecular features of pathogens that are integral to microbial lifestyles, including bacterial flagellin, ELONGATION FACTOR TU (EF-TU) and fungal chitin (Nürnberger et al., 2004). The recognition of PAMPs by transmembrane PRRs often leads to PAMP-TRIGGERED IMMUNITY (PTI) responses, which include MITOGEN-ACTIVATED PROTEIN (MAP) kinase cascade activation, reactive oxygen burst, callose deposition and accumulation of the plant defense hormone SALICYLIC ACID (SA) (Zipfel, 2014). As an example of PTI, flg22 is a conserved 22 amino acid epitope in the N terminus of flagellin and is sufficient to trigger defense responses (Gómez-Gómez et al., 1999). It can be recognized by FLAGELLIN-SENSITIVE 2 (FLS2), which contains an extracellular LEUCINE-RICH-REPEAT (LRR) domain, a transmembrane domain and an intracellular Ser/Thr kinase domain. fls2 mutants are insensitive to flg22 treatment and more susceptible to bacterial spray infection (Chinchilla et al., 2006).
Other well-studied PAMP/PRR pairs include EF-Tu and its receptor EF-TU RECEPTOR (EFR) (Zipfel et al., 2006) and chitin and CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) in Arabidopsis (Petutschnig et al., 2010), called CHITIN ELICITOR-BINDING PROTEIN in rice (Kaku et al., 2006). FLS2 and EFR can form ligand-induced complexes with BRI1-ASSOCIATED RECEPTOR KINASE 1, a LRR receptor-like kinases that belong to the SOMATIC-EMBRYOGENESIS-RECEPTOR-LIKE KINASE family (Roux et al., 2011). The heterodimerization of PRRs and BAK1 results in transphosphorylation and phosphorylation of BOTRYTIS-INDUCED KINASE 1, and the sequential phosphorylation of the complex results in the activation of downstream signaling (Lu et al., 2010).

In the molecular arms race between host and infectious microbe, successful pathogens are able to secrete effectors into the plant cell to perturb defense responses which leads to EFFECTOR-TRIGGERED-SUSCEPTIBILITY (Jones and Dangl, 2006). Gram-negative bacteria employ a TYPE III SECRETION SYSTEM during plant infection to release a battery of effectors into the host cell (Jin and He, 2001). Additionally, fungal and oomycete pathogens secrete and translocate effectors through haustoria structures which are also primarily responsible for nutritional intake (Koeck et al., 2011). Most effectors possess enzymatic activities and are responsible to enhance virulence in plants (Göhre and Robatzek, 2008). The Pseudomonas syringae pv tomato DC3000 effector AvrPto, for example, is shown to bind the PRRs FLS2 and EFR, inhibiting their auto-phosphorylation thus blocking defense outputs (Zong et al., 2008). Another P. syringae effector, AvrPtoB, exhibits similarity to E3 ligases and ubiquitinates FLS2 and CERK1 (Göhre et al., 2008) (Gimenez-Ibanez et al., 2009), thus promoting their degradation via the 26S-proteasome pathway.
Although effectors can suppress host defense in order to promote virulence in susceptible plants, defenses can instead be triggered if the effectors are recognized by corresponding RESISTANCE (R) proteins in the plant. Through specific recognition of effectors, R protein activation leads to the induction of the second layer of the plant immune system, EFFECTOR-TRIGGERED IMMUNITY (ETI). A cascade of immune responses includes induced PATHOGENESIS-RELATED (PR) gene expression and SA accumulation. ETI is more rapid and robust, often culminating in the hypersensitive response, a programmed cell death that retards pathogen growth (Jones and Dangl, 2006). R proteins perceive effectors through either direct or indirect protein-protein interaction (Van der Biezen and Jones, 1998). One example of direct effector recognition includes the perception of AvrPita by the R protein Pita in rice. Pita directly interacts with AvrPita in a yeast-two hybrid assay (Jia et al., 2000). Often, R proteins detect effector invasion in an indirect manner. For example, AvrRpm1 and AvrRpt2, two unrelated effectors from Pseudomonas syringae, can target the same host protein RPM1 INTERACTING PROTEIN 4 (RIN4) for phosphorylation and cleavage, respectively (Mackey et al., 2002). RIN4 is guarded by two R proteins, RPM1 and RPS2. They can detect the modifications of RIN4 by effectors and trigger defense responses to halt pathogen magnification (Axtell and Staskawicz, 2003) (Kim et al., 2005). Another well-studied example is AvrPphB, a cysteine protease from the plant pathogen Pseudomonas syringae (Zhu et al., 2004). Once AvrPphB entered the plant cell through the TYPE III SECRETION SYSTEM, it cleaves host protein PBS1. The cleavage of PBS1 (AVRPPhB SUSCEPTIBLE 1) can be perceived by RPS5 and thus defenses are triggered (Shao et al., 2003).
1.2 Multi-domain architecture of R proteins

In plants, R protein-mediated defense is a rapid and robust resistance to pathogens. R genes have been widely used in breeding agriculturally important plants and contribute greatly to reduce crop loss (Dangl et al., 2013). In Arabidopsis, there are five major classes of R proteins. Genome-wide analysis reveals that 150 R proteins belong to the NB-LRR protein family with a central NUCLEOTIDE-BINDING (NB) domain and a C-terminal LRR domain (Meyers et al., 2003). They are structurally similar to animal NUCLEOTIDE OLIGOMERIZATION DOMAIN (NOD) immune receptors, and thus referred to as NOD-LIKE RECEPTORS (NLR) (Inohara et al., 2005). The characteristic NB domain includes a binding site for ATP or ADP and is thought to function as switch for R protein activation (Tameling et al., 2006). With the presence of effectors, the NB domain may be involved in ATP binding or hydrolysis, which can result in R protein conformational changes (Tameling and Baulcombe, 2007). The LRR domain is variable in the proteins. The tertiary structure of a single LRR domain is horseshoe-shaped (Sela et al., 2012). The LRR domain is suggested to contribute to the intra-molecular regulation of R protein that inhibits the receptor activity without effector presence. After perceiving effectors, conformational change leads to the dissociation of the LRR domain from the NB domain and the R protein gets activated (Moffett et al., 2002). Most importantly, the LRR domain is thought to determine effector specificity (Dodds et al., 2001).

Based on the N-terminus divergence, the NLR protein family can be further divided into two subgroups: TIR-type NLRs (TNLs) whose N-terminal TIR domain has significant similarity to the Toll receptor in Drosophila and Interlekin-1 Receptors in human, and CC-type NLRs (CNLs) which possess a rod-shaped Coiled-Coil motif in the amino acid terminus (Jebanathirajah et al.,
From the study of the TIR domain of flax L6 and CC domain of Barley MLA10, we know that overexpression of N terminal domains alone is sufficient to trigger defense responses. Moreover, the homo-dimerization of those domains is required to activate defense events (Maekawa et al., 2011) (Bernoux et al., 2011).

1.3 R protein regulation

R proteins are under tight regulation at both the transcriptional and post-transcriptional levels as uncontrolled activation of defense responses is detrimental to plant development. Besides the intramolecular regulation conferred by individual domains, many regulatory mechanisms are involved in the inhibition of R protein activities.

It has been reported that some R genes undergo alternative splicing, which indicates that a single pre-mRNA produces multiple transcripts isoforms. *RPS4* gene, for example, which confers resistance to *Pseudomonas syringae pv tomato* strain DC 3000 expressing *AvrRPS4*, has six transcript isoforms due to retention of intron 2 and/or intron 3 (Zhang and Gassmann, 2003). Another TNL R protein in tobacco, N, which can specifically recognize helicase protein of tobacco mosaic virus, is also alternatively spliced (Whitham et al., 1994). One of the alternative transcripts yields a N protein lacking 13 of 14 LRR repeats (Dinesh-Kumar and Baker, 2000). Based on the functional consequences of alternative splicing events of Arabidopsis RPS4 and tobacco N, it is hypothesized that alternative transcripts are produced to limit function of R proteins in their native state, by competing with full length R protein in interactions with negative regulators upon effector recognition (Gassmann, 2008).

At the posttranscriptional level, SGT1 (SUPPRESSOR OF THE G2 ALLELE OF SKP1), RAR1
(REQUIRED FOR MLA12 RESISTANCE 1), AND HSP90 (HEAT SHOCK PROTEIN 90) are suggested to form a chaperone complex, which is critical for the proper folding and stabilization of NLR proteins (Shirasu, 2009). Some R proteins, such as MLA1, MLA6 from barley (Bieri et al., 2004) and RPS5 (Holt et al., 2005) in Arabidopsis, were shown to depend on RAR1 for protein accumulation. Besides associating with chaperones, SGT1 was shown to function together with SRFR1 (SUPPRESSOR OF rps4-RLD) to negatively regulate SNC1, RPS2 and RPS4 accumulation. Recently, the ubiquitination pathway has begun to emerge as an important mechanism to regulate NLR protein stability. CPR1, an F-box E3 ligase, can form a SCF (SKP1-CUL1-F-BOX PROTEIN) E3 ubiquitin ligase complex. The SCF^{cpr1} complex specifically targets SNC1 and RPS2 for ubiquitination and leads to protein degradation via 26S proteasome degradation. SNC1 protein accumulates in cpr1 and over-expression of CPR1 can abolish autoimmunity in snc1 (Cheng et al., 2011).

1.4 R protein signaling

How R protein activation leads to downstream signalling is still poorly understood. Through previous genetic studies, we know that TNL and CNL immune receptors have different requirements for downstream signaling regulators. TNL proteins rely on the ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1)/ PHYTOALEXIN DEFICIENT 4 (PAD4)/SENESCENCE-ASSOCIATED GENE101 (SAG101) complex (Aarts et al., 1998; Feys et al., 2001; Feys et al., 2005; Wiermer et al., 2005; Rietz et al., 2011). EDS1, PAD4 and SAG101 share sequence similarity to eukaryotic lipases (Falk et al., 1999). EDS1 was previously shown to be essential for both basal and TNL protein-mediated defense pathway (Falk et al., 1999). It can form a heterodimer with SAG101 in the nucleus and also interact with PAD4 in both the cytosol and nucleus (Feys et al., 2001; Feys et al., 2005). Genetic and biochemical evidence revealed that
the two distinct heterodimers were both essential for TNL-mediated defense signaling (Zhu et al., 2011). However, CNL immune receptors are more likely to pass signaling through the membrane-bound protein NONRACE SPECIFIC DISEASE RESISTANCE 1 (NDR1) (Aarts et al., 1998; McDowell et al., 2000) (Figure 1.1). ndrl was isolated as a component in resistance against the virulent pathogen *Pseudomonas syringae pv tomato* DC3000 (Century et al., 1995). The exact mechanism of how NDR1 regulates the CNL-mediated defense pathway remains obscure.

Figure 1.1: Signaling components downstream of TIR-type and CC-type NB-LRR R proteins.
1.5 Dissecting plant immunity using \textit{snc1}

1.5.1 \textit{snc1} and \textit{snc1} suppressor screens

A genetic suppressor screen aimed to identify negative regulators of plant immunity led to the isolation of a unique gain-of-function mutant, \textit{snc1 (suppressor of npr1-1, constitutive 1)}, which exhibits reduced growth, high \textit{PR} gene expression and elevated SA accumulation in the absence of pathogens (Li et al., 2001). Positional cloning revealed that one amino acid substitution in the linker region between the NB domain and LRR domain of this TIR-type NLR results in the autoimmunity of \textit{snc1} (Zhang et al., 2003). Due to the distinct morphological phenotype of \textit{snc1} and constitutive activation of defense responses, \textit{snc1} was thought to be an extraordinary tool for dissecting downstream components of TIR-type NLR R protein mediated defense pathways.

To search for components that are essential for SNC1 activation, our lab conducted suppressor screens in the background of \textit{snc1} by using various mutagenesis methods; including utilizing ETHYL METHANE SULFONATE (EMS) for chemical mutagenesis, Agrobacteria-mediated T-DNA insertional mutagenesis and fast neutron bombardment. The mutants were named \textit{modifier of snc1 (mos)} mutants and they fall into fifteen complementation groups. Functional studies of these MOS proteins reveal that RNA processing, nucleo-cytoplasmic trafficking and protein modifications are crucial downstream events involved in NB-LRR R protein mediated defense pathways (Johnson et al., 2012).
1.5.2 Modifier of snc1 (MOS)

1.5.2.1 Transcription regulation: MOS1, MOS9 and MOS10

*MOS1* (*modifier of snc1*) was shown to regulate *SNC1* expression by influencing chromatin structure. *MOS1* encodes a protein with an HLA-B ASSOCIATED TRANSCRIPT 2 (BAT2) domain that is conserved in both plants and animals. The gene expression of *SNC1* is reduced in the *mos1* mutant (Li et al., 2010a). Also, MOS9, which is a plant-specific protein with unknown function, was shown to function together with the ARABIDOPSIS TRITHORAX-RELATED 7 (ATXR7) to regulate *SNC1* and *RPP4* expression through H3K4 methylation (Xia et al., 2013). Moreover, MOS10, known as TOPLESS-RELATED 1 (TPR1) functions as transcriptional corepressor to target *DEFENSE NO DEATH 1* (*DND1*) and *DEFENSE NO DEATH 2* (*DND2*), which are two known negative regulators of immunity. Over-expression of *TPR1* constitutively activates *SNC1*-mediated defense responses, while knocking out *TPR1* and its homolog attenuates immunity conferred by *SNC1* and several other TNL proteins (Zhu et al., 2010).

1.5.2.2 Post-transcriptional regulation: MOS2, MOS3, MOS4, MOS11 and MOS12

Nascent RNA usually goes through RNA processing including 5’ capping, 3’ polyadenylation, and RNA splicing, followed by nuclear transport to ensure appropriate protein function. The isolation of several *mos* mutants involved in these processes indicates that RNA processing plays a crucial role in plant immunity.

*mos2* was identified as partial suppressor of *snc1* and was required for the basal defense against a virulent bacterial pathogen, *P.s.m* ES4326. Positional cloning revealed that MOS2 encodes a nuclear protein with a central G-patch domain and two KOW motifs at the C terminus. Based on
the sequence analysis between MOS2 and its homologs in Arabidopsis, human, mouse, and C. elegans, it is clear that the G-patch and KOW motifs are clearly conserved among these proteins (Zhang et al., 2005). The G-patch motif is predicted to mediate RNA-protein binding (Aravind and Koonin, 1999) and the KOW motif could bind to RNA according to the crystal structure microbial transcriptional modulator NusG (Steiner et al., 2002). Although the exact mechanism of MOS2 regulation of plant immunity remains elusive, recent study has shown that MOS2 is required for the proper splicing of SNC1 (Copeland et al., 2013).

mos4 can completely suppress enhanced disease resistance to virulent pathogen in snc1. MOS4 encodes a conserved nuclear protein which is homologous to human Breast Carcinoma Sequence 2 (Palma et al., 2007). MOS4 interacts with CELL DIVISION CYCLE 5 (AtCDC5) and PLEIOTROPIC REGULATORY LOCUS 1 (PRL1) in planta and forms a complex named as MOS4 ASSOCIATED COMPLEX (MAC). These three components are all essential for not only basal defense, but also for RPM1 and RPS4 mediated resistance (Palma et al., 2007). The homologs of MAC components in humans are members of NINETEEN COMPLEX (NTC), which is an evolutionarily conserved protein complex and has been shown to be essential for splicing. MAC is thus proposed to be the ortholog to the NTC. Analysis of transcript variants in several alternative spliced genes in mos4, cdc5, and prl1 backgrounds revealed that the splicing pattern of two TIR-type NLR encoding genes, SNC1 and RPS4, is altered in these single mutants. MAC complex is thought to affect plant immunity via regulating the splicing pattern of defense related genes. Later studies found that MOS4 associates with MOS12, which contains two conserved cyclin domains at the N-terminus. MOS12 has a close homolog cyclin L in human, and cyclin L was reported to be responsible for pre-mRNA splicing (Xu et al., 2012).
In eukaryotes, transcription and translation occur in different cellular compartments. Once the transcript is successfully processed, it needs to be transported from nucleus to cytoplasm for translation. MOS3 and MOS11 were reported to regulate plant immunity via the mRNA export pathway. *mos3* can suppress the constitutive *PR* gene expression and enhanced disease resistance to virulent pathogens in *snc1*. *mos3* single mutant also exhibits compromised basal defense level and defects in RPP4- and RPM1- mediated resistance. *MOS3* encodes a nuclear envelop-localized protein that is homologous to human nucleoporin 96, suggesting that nuclear-cytoplasmic trafficking of RNA molecules, proteins and other micro-molecules is essential for plant immunity (Zhang and Li, 2005).

*mos11* was identified as a T-DNA insertional mutant which can partially suppress the distinct dwarfism and enhanced disease resistance phenotypes of *snc1*. *MOS11* encodes a conserved protein with homology to the human CIP29, which was shown to bind RNA. *in situ* Poly-(A) hybridization assay has shown that *mos11* accumulates more mRNA in the nucleus, indicating the reduced mRNA export. Epistasis analysis between *mos3* and *mos11* revealed that these two proteins function in the same mRNA export pathway (Germain et al., 2010).

1.5.2.3 Nucleo-cytoplasmic trafficking: MOS6

*mos6* was isolated from a fast-neutron mutagenesis population in *snc1* background. It suppresses the constitutive *PR2* expression and elevated SA accumulation in *snc1*. The *mos6* single mutant also exhibits enhanced disease susceptibility to virulent pathogen *P.s.m* ES4326 (Palma et al., 2005). *MOS6* encodes one of eight α-importins in Arabidopsis. Alpha-importins have been shown to form a trimeric complex with classical NUCLEAR LOCALIZATION SIGNAL
(cNLS)-containing proteins and β-importin; the translocation of cNLS-containing proteins across nuclear envelope through nuclear-pore complex (NPC) is facilitated by α-importins. (Lange et al., 2007). It was hypothesized that MOS6 may function in the translocation of NPR1 from the cytoplasm to the nucleus, as NPR1 is imported into nucleus with SA induction (Cao et al., 1997).

**1.5.2.4 Protein modification: MOS5 and MOS8**

Post-translational protein modifications are frequently used to alter protein solubility, compartmentalization or interaction with other proteins. The identification of MOS5 and MOS8 has revealed the importance of protein modification in plant immunity. mos5 carries a 15bp deletion in UBA1, which is one of the two ubiquitin-activating enzyme genes in Arabidopsis. mos5 single mutant exhibits enhanced disease susceptibility to bacteria carrying avrRpt2, while uba2 does not have any disease defects (Goritschnig et al., 2007). Ubiquitination is a common protein modification that involves attaching the conserved ubiquitin onto a substrate. The covalent attachment of ubiquitin is a three-step process that requires sequential activities of E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme) and E3 (ubiquitin ligase) (Trujillo and Shirasu, 2010). Polyubiquitination with more than four ubiquitin moieties on the substrate often leads to protein degradation through the 26S proteosome pathway (Duplan and Rivas, 2014).

Additionally, MOS8 encodes the β-subunit of protein farnesyltransferase, which adds a 15-carbon farnesyl group to target proteins. Mutations in MOS8 not only completely suppress the extreme dwarfism but also the constitutive resistance to *P.s.m* ES4326 and *H.a.Noco2 in snc1* (Goritschnig et al., 2008). Resistance to avirulent pathogens mediated by R proteins RPM1, RPS4
and RPP4, are partially compromised in mos8, indicating the significance of farnesylation in plant immunity.

1.6 Thesis objective

In addition to SNC1, CHS3 is a TIR-type NLR, identified from a suppressor screen of npr1-1. CHS3 has the following distinct features: firstly, it has a unique domain arrangement, with an extra LIM domain in the C terminus; secondly, chs3-2d autoimmunity is only marginally dependent on PAD4, which is a positive regulator of TNLs. Thus, the main objective of my master’s research was to clarify the details of CHS3-mediated defense signaling by conducting genetic suppressor screens.
Chapter 2 Autoimmunity conferred by chs3-2D relies on SAG101, EDS1-90, SGT1 and CSA1, its adjacent TIR-NB-LRR neighbour

2.1 Introduction

Plants have evolved multilayered immunity to fend off microbial pathogen infections. R proteins play an irreplaceable role in defense through recognizing pathogen effector molecules and triggering downstream resistance. Most R genes encode proteins with NB domain and LRR domains, sharing structural similarities with animal pattern recognition receptors such as Nod1 and Nod2, therefore together are classified as NLRs. The majority of plant NLRs carries either a TIR or a CC domain at their N-termini, which is believed to mediate downstream signaling events. TIR-type NLRs mostly rely on the lipase-like protein EDS1 and salicylic acid for defense signaling, whereas most CC-type NLRs (CNRs) tend to signal through non-EDS1 signaling modes (McDowell et al., 2000). The Arabidopsis ecotype Col-0 genome encodes two EDS1 isoforms, At3g48080, designated EDS1-80, and At3g48090, designated EDS1-90. EDS1-80 exhibits ~85% amino acid identity with EDS1-90. Only EDS1-90 has been functionally characterized, while EDS1-80 nonfunctional in many ecotypes (Zhu et al., 2011; Wang et al., 2014). Although many NLR-encoding R genes have been cloned from different plant species during the past twenty years, the molecular events surrounding their activation remain elusive.

Arabidopsis CHILLING SENSITIVE 3 (CHS3) encodes an atypical TNL protein with a Lin-11, Isl-1 and Mec-3 (LIM) domain at its C terminus (Yang et al., 2010). LIM domain-containing proteins are found across eukaryotes and have been implicated as regulators of a variety of biological processes, including but not limited to gene expression, and signal transduction (Kadrmas and Beckerle, 2004). It is hypothesized that the LIM domain may act as a repressor
domain in CHS3 (Yang et al., 2010). Whether this non-canonical TNL employs the same downstream regulators as typical TNLs has yet to be explored.

*chs3-2D* is a gain-of-function mutant isolated from a forward genetic screen designed to isolate defense regulators (Bi et al., 2011). In the *chs3-2D* mutant, a C1340 to Y1340 substitution close to the LIM domain of CHS3 leads to autoimmune phenotypes including increased PR gene expression, SA accumulation, and enhanced resistance to the virulent oomycete strain *Hyaloperonospora arabidopsis*(H.a.) Noco2 (Bi et al., 2011).

In this study two independent suppressor screens in the *chs3-2D* or *chs3-2D pad4-1* genetic backgrounds, respectively, were carried out to explore the regulatory and signaling components of CHS3-mediated defense. Multiple mutants that can suppress the *chs3-2D* or *chs3-2D pad4-1* autoimmune phenotypes were identified and characterized. Using map-based cloning and Sanger direct sequencing techniques, we were able to clone a number of genes, including novel alleles of known downstream regulators of TNL-mediated signaling, such as SAG101, EDS1, and SUPPRESSOR OF THE G2 ALLELE OF SKP1, b (SGT1b) (Shirasu, 2009). Most significantly, our study revealed that the autoimmunity of *chs3-2D* requires the genomically adjacent TNL gene *CONSTITUTIVE SHADE-AVOIDANCE 1 (CSA1)*, as four independent mutant alleles of *csa1* were found to suppress the autoimmunity of *chs3-2D*.

### 2.2 Results

#### 2.2.1 Identification and characterization of *suppressors of chs3-2D*

The *chs3-2D* autoimmune mutant exhibits severe dwarfism (Bi et al., 2011). In order to search for regulatory and signaling components required for CHS3-mediated defense response, Fang Xu
screened for mutants that can suppress the autoimmunity of \textit{chs3-2D}, using suppression of stunted growth as a proxy during the primary screen. \textit{chs3-2D} seeds were first mutagenized by EMS. The M\textsubscript{1} plants were grown at 28°C to harvest the M2 seeds as \textit{chs3-2D} autoimmunity and concomitant dwarfism are temperature sensitive; the mutant is seedling lethal at 23 °C, but fertile at 28°C. The M\textsubscript{2} population from approximately 2000 M\textsubscript{1} plants were initially screened for individuals that were significantly larger than the original mutant at 23 °C. Mutants exhibiting a morphological suppression of \textit{chs3-2D}-associated phenotypes were then subjected to a secondary screen, in which resistance to the virulent oomycete strain \textit{H.a. Noco2} was examined. Mutants that displayed enhanced susceptibility to \textit{H.a. Noco2} as compared to \textit{chs3-2D} were selected for further characterization. The genetic background of each mutant was verified by directly sequencing the \textit{CHS3} locus. Mutants carrying intragenic mutations in \textit{CHS3} as revealed by the sequencing were classified as intragenic suppressors and eliminated from further analysis (Bi et al., 2011). Mutants carrying intragenic mutations in \textit{CHS3} as revealed by the sequencing were classified as intragenic suppressors and eliminated from further analysis (Bi et al., 2011). In summary, eight independent \textit{soch (suppressor of chs3-2D)} lines with second-site mutations were isolated

As shown in Figure 2.1A, all eight \textit{soch chs3-2D} mutants can completely suppress the morphology of \textit{chs3-2D}. Consistent with the morphological suppression, all of them showed significantly enhanced susceptibility to \textit{H.a. Noco2} (Figure 2.1B). In addition, the expression of \textit{PR} genes was significantly reduced in all mutants as compared to \textit{chs3-2D} (Figure 2.1C). Taken together, these data suggest that all of the eight mutants contain mutations that suppress the autoimmune phenotypes of \textit{chs3-2D}. 
Figure 2.1. Characterization of soch chs3-2D mutants.

A. Morphology of soil-grown plants of the indicated genotypes at 23 °C. The photograph
was taken when the plants were 21 days old.

B. Quantification of H.a. Noco2 sporulation on the indicated genotypes following
inoculation with pathogen isolate H.a. Noco2. Two-week-old soil-grown seedlings were
sprayed with a spore suspension of H.a. Noco2 at a concentration of 100,000 spores/mL
of water. The plants were then covered and incubated for seven days in a high humidity
growth chamber. Spores were counted in water suspension using a hemocytometer (bars
represent means of n replicates ± SD, n=3 or 4 with 4 plants each).

C. PR1 and PR2 gene expression of the indicated genotypes as determined by RT-PCR.
Both PR1 and ACTIN were amplified with 28 cycles while PR2 was amplified with 30
cycles.
### 2.2.2 *chs3-2D* is only marginally suppressed by *pad4-1*

In addition to *chs3-2D*, another gain-of-function autoimmune mutant allele, *chs3-1*, was isolated from a forward genetic screen searching for chilling sensitive mutants (Yang et al., 2010). Epistasis analysis revealed that the constitutive activation of defense response in *chs3-1* fully depends on EDS1 and partially relies on PAD4, suggesting that a PAD4 independent pathway might play an important function in CHS3-mediated defense response. When *chs3-2D pad4-1* double mutant was generated, the presence of the PAD4-independent pathway in *chs3-2D* mediated defense response was further confirmed as *pad4-1* only marginally suppressed the *chs3-2D* autoimmune phenotypes. *chs3-2D pad4-1* only showed a slight morphology suppression of the *chs3-2D*-associated dwarfism (Figure 2.2A) and still exhibited constitutive resistance to *H.a. Noco2* (Figure 2.2B).
Figure 2.2 Autoimmunity conferred by *chs3-2D* is only marginally attenuated by *pad4-1*.

A. Morphology of three-week-old soil-grown plants of the noted genotypes.

B. Quantification of *H.a. Noco2* sporulation on the indicated genotypes. The same experimental procedure was carried out as described in Figure 1B.
2.2.3 Identification and characterization of *suppressors of chs3-2D pad4-1*

In order to identify PAD4-independent regulators involved in the *chs3-2D*-mediated signaling pathway, I conducted a suppressor screen in the *chs3-2D pad4-1* background. A similar screening strategy was used as described above for the *chs3-2D* suppressor screen. Two *socp* (*suppressor of chs3-2D pad4-1*) mutants were isolated from the screen. As shown in Figure 2.3A, *socp1 chs3-2D pad4-1* and *socp2 chs3-2D pad4-1* showed significant morphological suppression of *chs3-2D pad4-1*. When the two mutants were challenged with *H. a. Noco2*, they exhibited considerable susceptibility compared to *chs3-2D pad4-1* (Figure 2.3B). In addition, the constitutive expression of *PR* genes in *chs3-2D pad4-1* was suppressed in the *socp1* and *socp2* mutants to some extent (Figure 2.3C). Therefore, *socp1* and *socp2* were able to partially suppress the autoimmunity of *chs3-2D pad4-1*. 
Figure 2.3 Characterization of *socp chs3-2D pad4-1* mutants.

**A.** Morphology of soil-grown plants of the indicated genotypes. The picture was taken when the plants were 24 days old.

**B.** Quantification of *H. a. Noco2* sporulation on the indicated genotypes. The experimental procedure was carried out as described in Figure 1B.

**C.** *PR1* and *PR2* gene expression of the indicated genotypes as determined by RT-PCR.
2.2.4 CHS3-mediated defense responses are completely dependent on EDS1-90 and SAG101

To map the SOCH loci, the soch chs-2D mutants (which were generated in the Col-0 ecotype) were crossed with wild type Landsberg erecta plants. Crude mapping using insertion/deletion markers specific to the two ecotypes revealed that soch1 is linked with SAG101 on chromosome 5. Since SAG101 is a known downstream component of TNL-mediated immunity, Fang Xu hypothesized that soch1 might contain a mutation in SAG101. Indeed, direct Sanger sequencing revealed that soch1 carried a G to A mutation in the second exon of SAG101 (At5g14930) resulting in a G1458 to A1458 substitution (Figure 2.4A). By using similar mapping strategies, we mapped soch2, soch3, and soch4 to chromosome 3, close to EDS1 (At3g48090). Sanger sequencing indeed found that all three mutants carried mutations in EDS1-90. One eds1-90 allele had a G to A mutation leading to a G483 to R483 substitution, and the other two alleles contained mutations at intron-exon junctions, which result in splice pattern changes (Figure 2.4A). Complementation tests further confirmed that those three mutants were allelic to each other as the F1 progeny of crosses between these mutants had wild type morphology (Figure 2.4B).

2.2.5 PAD4-independent CHS3-mediated defense signaling is partially dependent on SGT1b

Crude mapping of the two socp mutants isolated in the chs3-2D pad4-1 suppressor screen indicated that they both displayed linkage at the top of chromosome 4, a region that contains the known defense regulator SGT1b. Sanger sequencing uncovered that socp1 had a G to A splice site mutation in the fifth intron of SGT1b, and socp2 contained a point mutation leading to a
G328 to E328 substitution (Figure 2.4A). These mutations in SGT1b are able to partially suppress the morphological and resistance phenotypes of chs3-2D pad4-1, suggesting that SGT1b positively regulates CHS3-mediated defense responses, which might function independently of PAD4.
Figure 2.4 Summary of the mutations in all the *soch* and *socp* mutants

A. Mutations identified in the indicated mutants by traditional mapping and Illumina next generation or direct Sanger sequencing.

B. Complementation test of *soch2 chs3-2D*, *soch3 chs3-2D* and *soch4 chs3-2D*. Morphology of three-week-old soil-grown plants of the indicated genotypes from the pair-wise allelism test. One representative F1 plant was shown for each cross.
2.2.6 Immune signaling mediated by CHS3 requires its neighbor TNL protein CSA1

Mapping of soch5, soch6, soch7 and soch8 revealed that these suppressor loci were all closely linked to chs3-2D. Genomic DNA from soch6 chs3-2D was then sequenced using Illumina next generation sequencing. Upon comparison with the Col-0 reference sequence, a point mutation causing a G233 to E233 amino acid substitution was identified in CSA1, a gene adjacent to CHS3. Direct Sanger sequencing of the remaining soch alleles revealed that they contain independent mutations in CSA1. This indicated that CSA1 is required for the autoimmune responses of chs3-2D. Complementation was not observed from pair-wise crosses among these mutants, confirming that they contain mutations in the same gene (Figure 2.5A). CSA1 is adjacent to and divergently transcribed from CHS3, with an approximate 3.9 kb genomic region between their start codons (Figure 2.5B). It encodes a typical TNL. When the CSA1 expression was examined in chs3-2D background, we observed over two-fold higher expression in both chs3-2D and chs3-2D pad4-1 (Figure 5C). However, eds1-90 alleles can completely abolish the up-regulation of CSA1 in chs3-2D, indicating that the induced expression of CSA1 in chs3-2D is mediated through EDS1 (Figure 2.6).
Figure 2.5 Complementation test of four csal alleles and gene arrangements of CHS3 and CSA1.

A. Morphology of three-week-old soil-grown plants of the indicated genotypes from the pair-wise allelism test. Two representative F1 plants were shown for each cross.

B. Boxes indicate exons while lines indicate introns. The encoded protein domains are denoted below the corresponding genomic regions. The directions of gene transcription are indicated as arrows.
Figure 2.6 *CSA1* gene expression in wild type, *chs3-2D*, *chs3-2D pad4-1*, *chs3-2D eds1-90-10*, and *chs3-2D eds1-90-11*. Total RNA was extracted from two-week-old, plate-grown seedlings. Relative *CSA1* expression levels were determined by real-time PCR. Values were normalized to the expression of *Actin*. Error bars represent SD from three replicates.
2.3 Discussion

CHS3 is an atypical TNL protein with an additional LIM domain at its C terminus. The exact function of the LIM domain is still unclear. It has been proposed that it inhibits the NLR protein in its native state (Yang et al., 2010). The gain-of-function chs3-2D allele results in extreme dwarfism and enhanced resistance to virulent pathogens (Bi et al., 2011). Epistasis analysis indicates that PAD4, which is thought to be a critical regulator downstream of many TNL immune receptors (Wiermer et al., 2005), is only partially required for the chs3-2D phenotypes. The distinctive features of chs3-2D provide us with an excellent background in which to conduct genetic suppressor screens in order to identify downstream components involved in CHS3-mediated defense pathway, which seems to differ from those involved in canonical TNL-mediated signaling.

In this study, we determined that CHS3-mediated signaling relies differently on defense-related lipase-like proteins (EDS1/PAD4/SAG101) than typical TNLs. Mutations in SAG101 can completely suppress the autoimmunity of chs3-2D (Figure 2.1 and Figure 2.4A), while the suppression by pad4-1 is marginal (Figure 2.2), suggesting that CHS3-mediated signaling relies more strongly on SAG101. Genetic redundancy between PAD4 and SAG101 was previously suggested (Falk et al., 1999; Feys et al., 2005). However, previous research provided evidence that EDS1 forms distinct complexes with PAD4 and SAG101 with non-redundant signaling roles (Rietz et al., 2011). Our findings potentially support this model. Although genetic redundancy between the two Col-0 EDS1 genes (EDS1-80 and EDS1-90) was demonstrated for immune signaling mediated by the CNL HYPERSENSITIVE RESPONSE TO TCV (Zhu et al., 2011), CHS3-mediated defense responses seem to rely more on EDS1-90, as three mutations in EDS1-
90, while none in EDS1-80, were found to completely abolish the autoimmunity in chs3-2D. It is therefore possible that CHS3 preferentially utilizes EDS1-90 and SAG101 for its defense activation, while EDS1-80 and PAD4 are marginally used.

Previous studies have shown that SGT1b negatively regulates defense responses mediated by the TNL SNC1 by altering its accumulation, as mutations in SGT1b lead to higher SNC1 levels (Li et al., 2010b; Cheng et al., 2011). However, SGT1b appears to positively regulate CHS3-mediated defense responses, as mutations in SGT1b can partially suppress the phenotypes of chs3-2D pad4-1 ((Yang et al., 2010) and current study). Together, these data further highlight the differential roles SGT1b plays in NLR-mediated immunity. In the case of CHS3, SGT1b may serve as a molecular chaperon for proper assembly of the TNL complex for defense activation. In contrast, for SNC1, SGT1b is likely more involved in the SCF\textsuperscript{CPR1} complex formation for ubiquitination and further degradation of this TNL (Cheng et al., 2011).

The results of our suppressor screens have shown that the autoimmunity of chs3-2D requires the TNL CSA1, as loss-of-function mutations in CSA1 can completely suppress the chs3-2D phenotypes. CSA1 is adjacent to and divergently transcribed from CHS3, sharing an approximate 3.9 kb genomic region upstream of their start codons. This genomic arrangement is reminiscent of the R gene pair RPS4 and RRS1, whose hetero-dimerization is required for effector recognition (Williams et al., 2014). They are in a head-in-head arrangement with a 264bp intergenic region, and are transcribed in opposite directions. The promoter regions probably overlap and this gene pair is likely under transcriptional co-regulation. RRS1, a TNL immune receptor with an extra WRKY domain at the C-terminus, confers recognition of the fungal pathogen Colletotrichum higginsianum and effector PopP2 from the bacterial pathogen Ralstonia.
solanacearum, while RPS4 is a typical TNL immune receptor that confers recognition of effector AvrRPS4 from Pseudomonas syringae pv. tomato (Gassmann et al., 1999; Deslandes et al., 2002; Narusaka et al., 2009). Interestingly, both RRS1 and RPS4 are required for resistance conferred by either of the protein pair, revealing a dual resistance gene system (Deslandes et al., 2002; Narusaka et al., 2009). Structural studies show that TIR domain hetero-dimerization is critical to form a functional RPS4/RRS1 effector interaction interface (Williams et al., 2014). It is proposed that upon effector perception, TIR domain hetero-dimerization is released to allow for RPS4 TIR domain homo-dimerization for defense activation (Williams et al., 2014). In addition to RPS4/RRS1 and CSA1/CHS3, there are another eight conserved TNL gene pairs in the Arabidopsis genome, which suggests the presence of several conserved dual resistance gene systems (Narusaka et al., 2009).

Curiously, CSA1 is the closest homolog of RPS4 and over-expression of RPS4 is able to complement the loss-of-function csa1 phenotype (Faigón-Soverna et al., 2006). Moreover, CHS3 is the closest homolog of RRS1 in the Col-0 ecotype. They have similar domain arrangements, and both the LIM and WRKY domains are proposed to be transcriptional repression domains (Noutoshi et al., 2005). In addition, RPS4 is required for the constitutive activation of defense responses conferred by a gain-of-function allele RRS1^{SLH1} which contains a single amino acid insertion in the WRKY DNA-binding domain (Sohn et al., 2014). Therefore we speculate that CHS3 may function similarly as RRS1. However, the biochemical interaction between CHS3 and CSA1 awaits further examination.
Proposed working model for CHS3-mediated defense pathway

From our suppressor screens, we isolated several downstream regulators of CHS3-mediated signaling. Based on our current genetic data, we propose a working model for the CHS3-mediated pathway. SGT1 may form a chaperone complex with RAR1 and HSP90 to properly assemble the CHS3 activation complex. The C-terminal LIM domain has been hypothesized to inhibit CHS3 protein activation in the absence of pathogens. Moreover, CHS3 needs its TNL protein neighbor, CSA1 to confer defense responses. The interaction between these two TNL proteins in planta will be investigated in the future. Instead of relying on the EDS1/PAD4/SAG101 module, CHS3 signaling seems to preferentially employ the EDS1-90/SAG101 complex (Figure 2.6).
Figure 2.7 Proposed working model for the CHS3-mediated defense pathway.

The LIM domain was proposed to repress CHS3 activities in the absence of pathogens. SGT1b, which can form a chaperone complex with HSP90 and RAR1, is required for the proper assembly of CHS3 activation complex. CHS3 activation relies on its TNL neighbor CSA1, and this signaling pathway appears to primarily rely on SAG101/EDS1-90.
2.4 Materials and methods

2.4.1 Plant growth

Seeds were sterilized by soaking them in a solution of 15% bleach and 0.1% Tween 20 followed by rinsing twice with sterile water. Seeds were cold treated in the dark at 4 °C for three days. Plate-grown plants were grown on ½ MS media at 22 °C and exposed to a 16h light and 8h dark regime.

2.4.2 Oomycete infection assay

Two-week-old seedlings were spray-inoculated with *H. a. Noco2* at a spore concentration of 1x 10^5 spores per mL. Oomycete growth was scored seven days later. The 16 plants from each genotype were divided into groups of four and placed in 1 mL of ddH2O in 15ml tubes (4 plants per tube). Spores were suspended in solution by vortexing and counted using a hemocytometer. Three independent replicates were performed.

2.4.3 *PR1* and *PR2* gene expression analysis

Total RNA was extracted from 13-day-old seedlings grown on ½ MS media using the RNA Mini-preps Kit (Bio Basic Incop). Total RNA was then reverse transcribed using Superscript II reverse transcriptase (Applied Biological Materials). The resulting cDNA was used as template for PCR. Both *PR1* and *ACTIN* were amplified with 28 cycles while *PR2* was amplified with 30 cycles. PCR products were then run on 1% agarose gel containing ethidium bromide and imaged using an Alphalmager HG (Alphalnnotech). The primers used to amplify *ACTIN* were 5’-CGATGAAGCTCAATCCAAACGA-3’ and 5’-CAGAGTCGAGCACAATACCG-3’, the primers used to amplify *PR1* were 5’-GTAGGTGCTCTTTGTCTTCCC-3’ and 5’-CACATAATTCCCACGAGGATC-3’, and the primers used to amplify *PR2* were 5’-GCTTCCTTCTTCAACCACACAGC-3’ and 5’-CGTTGATGTACCGGAATCTGAC-3’.
2.4.4 CSA1 gene expression

The cDNA of wild type, *chs3-2D*, *chs3-2D pad4-1*, *chs3-2D eds1-90-10*, and *chs3-2D eds1-90-11* were obtained as described before. Relative CSA1 expression levels were determined by real-time PCR. The primers used to amplify CSA1 were 5’-CAAAAAACAAGGGAGGTCTA-3’ and 5’-TTTGGGTGCATCCTTTGTTATC-3’.

2.4.5 Map-based cloning and Sanger sequencing

After the secondary screen, *soch chs3-2D* and *socp chs3-2D pad4-1* mutants in the Col-0 ecotype of *Arabidopsis thaliana* were crossed with the Landsberg erecta (Ler) ecotype. Plants from the F2 population were genotyped for the *chs3-2D* allele using insertion/deletion marker MPI7 (5’GTGAATTCCAATTAGACCGCA’ and 5’TCCTTGATACCGACGGTGTA3’). Since *chs3-2D* is in Col-0 ecotype background, plants were homozygous for Col-0 at the *CHS3* locus (homozygous for Col-0 mutant alleles of *chs3-2D*, and will not segregate), were used for further linkage analysis.
Chapter 3 Discussion and future perspectives

3.1 Insights to the LIM functionality possessed by CHS3

To isolate negative regulators in plant immune system, independent \textit{npr1-1} suppressor screens were conducted in different labs, which successfully identified a number of genes including \textit{SUPPRESSOR OF NPR1, INDUCIBLE 1 (SNI1)} (Li et al., 1999), \textit{SNC1} (Zhang et al., 2003), \textit{SNC2} (Zhang et al., 2010), \textit{SNC4} (Bi et al., 2010) and \textit{SNC5} (Li et al., 2010b). In addition, \textit{chs3-2D}, which exhibited dwarfism and curled leaf, was identified as a \textit{suppressor of npr1-1}. CHS3 encodes a TIR-type NLR with a LIM domain at the C terminus. A C1340 to Y1340 substitution close to the LIM domain in \textit{chs3-2D} leads to constitutively activated defense responses (Bi et al., 2011). As result, \textit{chs3-2D} shows significantly up-regulated expression of \textit{PR} genes and enhanced resistance to pathogens (Bi et al., 2011).

\textit{Chilling sensitive (chs)} mutants were first isolated from a forward genetic screen, which was done at 10 °C to 15 °C using EMS mutagenesis. The mutants were identified as they developed necrosis or chlorosis only at low temperature but appeared morphologically normal at 22°C. During the past 20 years, chilling sensitive mutants have been under extensive study to reveal the molecular regulators of plant responses to chilling stress.

\textit{chs1-2}, for example, exhibits leaf chlorosis at chilling temperatures. Also, it displays defense-associated phenotypes including induced \textit{PR} gene expression, SA accumulation and reactive oxygen species production. Map-based cloning showed that \textit{CHS1} encodes a TIR-NB-type protein (Wang et al., 2013). \textit{chs3-1}, another chilling sensitive allele, had arrested growth when shifted to 16 °C. Additionally, \textit{chs3-1} showed enhanced defense phenotypes in a temperature-
dependent manner. When grown at 22°C, chs3-1 had comparable PR gene expression and SA accumulation with wild type. However, chs3-1 had significantly enhanced disease resistance at 16 °C. Positional cloning revealed that the chs3-1 contained a G to A point mutation in the ninth intron-exon junction of CHS3, which may result in an abnormal splicing pattern and a truncated protein (Yang et al., 2010).

CHS3 encodes an atypical TNL protein that contains a C-terminal zinc-binding LIM domain (Yang et al., 2010). The LIM domain is a zinc-finger binding domain and mediates protein-protein interactions. LIM domains are widely present in proteins with diverse cellular roles as regulators of gene expression, cyto-architecture, cell adhesion, cell motility and signal transduction in animals (Kadrimas and Beckerle, 2004). In Arabidopsis, LIM domain-containing proteins are encoded by genes from two distinct subfamilies. The first subfamily encodes proteins which are structurally related to animal CYSTEINE-RICH-PROTEINS (CRPs) (Thomas et al., 2007). CRPs contain two LIM domains and were reported to directly bind actin filaments (Tran et al., 2005). The second subfamily, that includes CHS3, encodes plant-specific DA1 and seven DA1-Related (DAR) proteins (Li et al., 2008). CHS3 contains a single conserved LIM domain (Zhao et al., 2014). Curiously, both autoimmune mutants of CHS3, chs3-1 and chs3-2D, contain mutations in a region close to the C terminus LIM domain (Yang et al., 2010; Bi et al., 2011). The distribution of these mutations reveals the importance of C terminus domain in R protein function. Moreover, it is reported that overexpression of the LIM domain alone was able to suppress chs3-1 phenotypes (Yang et al., 2010). Thus, the LIM domain is hypothesized to work as a repressor of CHS3 activities in the absence of pathogenic effector molecules through intra-molecular interactions (Yang et al., 2010). To explore this possibility,
interaction between LIM domain and other N terminus domains of CHS3 can be further investigated by using yeast-two hybrid assay. In addition, the over-expression of N terminus of CHS3 alone could be analyzed to explore the function of CHS3 without the putative suppression of LIM domain.

**3.2 CHS3 reliance on EDS1-90/SAG101 module**

Previous genetic studies indicated that the two major types of R proteins in Arabidopsis, TNL and CNL, rely on different downstream signaling components. Lipase-like proteins (EDS1/PAD4/SAG101) are thought to be essential regulators in TNL proteins (Aarts et al., 1998; Feys et al., 2001; Feys et al., 2005; Wiermer et al., 2005; Rietz et al., 2011). EDS1 forms distinct complexes with PAD4 and SAG101 with non-redundant signaling roles (Rietz et al., 2011). Our findings further support this model as the sag101-10 allele which is isolated from our suppressor screen can completely suppress chs3-2D autoimmunity while pad4-1 only has marginal effect on it (Figure 2.1; Figure 2.2). A complementation test between soch1 and an available T-DNA knock out allele of sag101 will be conducted to confirm the suppression of chs3-2D by mutation in SAG101.

EDS1 has two isoforms in Arabidopsis Col-0 ecotype, EDS1-80 and EDS1-90, which share high amino acid identity and are encoded by two adjacent genes by DNA duplication. These two EDS1 genes were previously demonstrated to be redundantly required for immune responses mediated by the CNL HYPERSENSITIVE RESPONSE TO TCV (Zhu et al., 2011). However, CHS3-mediated defense responses seem to rely more on EDS1-90, as mutations in EDS1-90 was able to completely abolish the autoimmunity in chs3-2D (Figure 2.1; Figure 2.4). We thus hypothesize that CHS3 preferentially utilizes EDS1-90 and SAG101 for its defense activation,
while EDS1-80 and PAD4 are marginally used. To further test this hypothesis, an EDS1-80 T-DNA knock-out allele was also ordered from SALK institute. *chs3-2D eds1-80* double mutant will be generated in the near future by crossing *chs3-2D* with *EDS1-80* T-DNA allele. If *chs3-2D eds1-80* still showed unimpaired autoimmunity, our hypothesis will be supported.

### 3.3 Insights to SGT1b functionality

SGT1 is a highly conserved eukaryotic protein that functions in various biological processes. It contains a TETRATRICOPEPTIDE REPEAT DOMAIN, the CS motif (present in CHP and SGT1 proteins) and the SGT1 SPECIFIC SEQUENCE motif (Shirasu and Schulze-Lefert, 2003). In *Arabidopsis*, there are two SGT1 isoforms, SGT1a and SGT1b with 87% amino acid similarity. *SGT1b* was identified from several genetic screens as a component of some *R* gene mediated-defense responses. For example, SGT1b is reported to be required for RPP5-mediated defense to powdery mildew fungus (Austin et al., 2002). Also, *sgt1b* mutant is compromised in resistance to *Peronospora parasitica* isolates Hiks1 which is conferred by RPP7 (Tör et al., 2002). However, *SGT1a* was never isolated from any of those screens suggesting that SGT1b may be preferentially recruited to these pathways. By forming chaperone complex with RAR1 and HSP90, SGT1b was shown to regulate R protein accumulation, properly at the level of protein assembly (Shirasu, 2009). Interestingly, SGT1 was previously reported to negatively regulate R protein accumulation, mainly through formation of SCF E3 ligase complex for ubiquitination. For example, mutations in *SGT1b* lead to enhanced SNC1 accumulation (Cheng et al., 2011). In contrast, it appears to positively regulate CHS3-mediated defense responses, as mutations in *SGT1b* can partially suppress the phenotypes of *chs3-2D pad4-1* (Yang et al., 2010 and current study). Taken together, SGT1b may serve as a molecular chaperon for proper
assembly of the TNL complex for defense activation. Future study will explore whether knocking out *SGT1b* indeed leads to reduced CHS3 protein level by conducting western blot.

### 3.4 From “gene-for-gene” to “gene-for-genes”

Flor proposed Gene-for-Gene concept based on the studies on the inheritance of pathogenicity in the flax rust fungus, *Melampsora lini*. He suggested that disease responses can be activated only when AVIRULENCE (Avr) molecules from pathogens are specifically recognized by the corresponding R proteins from the host. However, as more plant-pathogen interactions were studied, Gene-for Gene concept needs to be developed to better fit our current understanding of plant pathogen interactions. Instead of recognizing the Avr gene product by single R protein from plants, emerging evidence indicates that the cooperative work between two R proteins are required (Eitas and Dangl, 2010). Several examples of dual resistance protein systems have been described in recent years. The TNL R protein N from *Nicotiana benthamiana* requires CNL protein NRG1 for function (Peart et al., 2005). Similarly, two linked TNL genes *RPP2A* and *RPP2B* together provide resistance to the *Hyaloperonospora arabidopsidis* isolate Cala2 (Sinapidou et al., 2004). Another well-studied R protein pair is RRS1/RPS4, two TNL R proteins, which are encoded by two adjacent genes. Both RRS1 and RPS4 are required for resistance to bacterial pathogen *P. syringae* and *Ralstonia solanacearum* and the fungal pathogen *Colletotrichum higginsianum* (Narusaka et al., 2009). Structural studies show that TIR domain mediated-hetero-dimerization is critical to form a functional RPS4/RRS1 effector interaction interface (Williams et al., 2014). It is proposed that upon effector perception, TIR domain mediated hetero-dimerization of RRS1/RPS4 is released to form RPS4 homo-dimerization mediated by TIR domain to activate defense (Williams et al., 2014).
Our studies further demonstrate a new pair of R protein in Arabidopsis, CHS3/CSA1, which might work together. CSA1 was previously shown to regulate plants’ response to neighbors. *csa1* mutants display a shade-avoidance phenotype including long stem, long petioles, erect and pale leaves (Faigón-Soverna et al., 2006). *CSA1* is adjacent to *CHS3* on the chromosome, but transcribed in the opposite direction, sharing approximately 3.9 kb with *CHS3* upstream of their start codons. In addition to the CHS3/CSA1 R gene pair, there are another 8 conserved TNL R protein pairs in head to head arrangement in the *Arabidopsis* genome.

From the suppressor screens of *chs3-2D* and *chs3-2D pad4-1*, we gained a better understanding of CHS3-mediated defense pathway. In addition to depending on potential downstream regulators of TIR-type NLR such as EDS1 and SAG101; CHS3-conferred resistance also genetically relies on its TNL neighbor CSA1.
References


conferred by the recessive RRS1-R gene, a member of a novel family of resistance genes. Proc Natl Acad Sci U S A 99, 2404-2409.


**Dodds, P.N., Lawrence, G.J., and Ellis, J.G.** (2001). Six amino acid changes confined to the leucine-rich repeat beta-strand/beta-turn motif determine the difference between the P and P2 rust resistance specificities in flax. Plant Cell 13, 163-178.


signaling through a plasma membrane receptor. Proc Natl Acad Sci U S A 103, 11086-11091.


**Li, X., Clarke, J.D., Zhang, Y., and Dong, X.** (2001). Activation of an EDS1-mediated R-gene pathway in the snc1 mutant leads to constitutive, NPR1-independent pathogen resistance. Mol Plant Microbe Interact 14, 1131-1139.


Narusaka, M., Shirasu, K., Noutoshi, Y., Kubo, Y., Shiraishi, T., Iwabuchi, M., and 
Narusaka, Y. (2009). RRS1 and RPS4 provide a dual Resistance-gene system against 
fungal and bacterial pathogens. Plant J 60, 218-226.

Noutoshi, Y., Ito, T., Seki, M., Nakashita, H., Yoshida, S., Marco, Y., Shirasu, K., and 
Shinozaki, K. (2005). A single amino acid insertion in the WRKY domain of the 
Arabidopsis TIR-NBS-LRR-WRKY-type disease resistance protein SLH1 (sensitive to 
low humidity 1) causes activation of defense responses and hypersensitive cell death. 
Plant J 43, 873-888.

plants and animals: striking similarities and obvious differences. Immunol Rev 198, 249- 
266.


Palma, K., Zhao, Q., Cheng, Y.T., Bi, D., Monaghan, J., Cheng, W., Zhang, Y., and Li, X. 
(2007). Regulation of plant innate immunity by three proteins in a complex conserved 
across the plant and animal kingdoms. Genes Dev 21, 1484-1493.

Peart, J.R., Mestre, P., Lu, R., Malcuit, I., and Baulcombe, D.C. (2005). NRG1, a CC-NB-
LRR protein, together with N, a TIR-NB-LRR protein, mediates resistance against 

lysin motif receptor-like kinase (LysM-RLK) CERK1 is a major chitin-binding protein in 
Arabidopsis thaliana and subject to chitin-induced phosphorylation. J Biol Chem 285, 
28902-28911.


Xia, S., Cheng, Y.T., Huang, S., Win, J., Soards, A., Jinn, T.L., Jones, J.D., Kamoun, S., Chen, S., Zhang, Y., and Li, X. (2013). Regulation of transcription of nucleotide-
binding leucine-rich repeat-encoding genes SNC1 and RPP4 via H3K4 trimethylation.

Plant Physiol 162, 1694-1705.

Xu, F., Xu, S., Wiermer, M., Zhang, Y., and Li, X. (2012). The cyclin L homolog MOS12 and the MOS4-associated complex are required for the proper splicing of plant resistance genes. Plant J 70, 916-928.


