USING CHEMICAL GENETICS TO DISCOVER
REGULATORS IN PLANT IMMUNITY

By

SHUAI HUANG

B.Sc., the Northwest A&F University, 2010

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

MASTER OF SCIENCE

In

THE FACULTY OF GRADUATE STUDIES
(BOTANY)

THE UNIVERSITY OF BRITISH COLUMBIA
(VANCOUVER)

March 2013
© SHUAI HUANG, 2013
ABSTRACT

Plants fend off pathogens by using Resistance (R) protein receptors to recognize pathogenic effectors. Classical genetic methods have been used to study R protein activation. However, the usage of classical genetics is limited by redundancy and lethality. To overcome these problems, I performed a high-throughput chemical genetics screen. I screened a chemical library containing 3,600 small-molecule compounds with known functions in animal systems. I identified one compound, 353D7, an NMDA (N-methyl-D-aspartate) subtype glutamate receptor antagonist in animals, that was able to completely suppress the autoimmune phenotypes of chs3-2D (chilling sensitive 3, 2D), including the arrested growth morphology and PR (pathogenesis related) gene expression. I also demonstrated that 353D7 suppressed the morphology of chs3-2D in a dosage dependent manner. In addition, 353D7 could partially suppress flg22–induced reactive oxygen species production. In Arabidopsis, there are twenty glutamate-like receptors (GLR)–encoding genes that share high homology with the animal ionotropic glutamate receptors (iGluRs). Activation of NMDA receptors triggers ion fluxes including Ca\(^{2+}\) influx across the plasma membrane (PM). Cytosolic Ca\(^{2+}\) is one of the most ubiquitous secondary messengers mediating numerous biological processes including plant defense. We hypothesize that 353D7 probably perturbs plant GLR–mediated Ca\(^{2+}\) influx. This work will potentially help us discover the long-sought Ca\(^{2+}\) channels on the plasma membrane necessary for plant defense.
TABLE OF CONTENTS

ABSTRACT .............................................................................................................................................. ii
TABLE OF CONTENTS ........................................................................................................................... iii
LIST OF TABLES ...................................................................................................................................... v
LIST OF FIGURES .................................................................................................................................... vi
LIST OF ABBREVIATIONS ....................................................................................................................... vii
AKNOWLEDGEMENTS ............................................................................................................................ x

Chapter 1 Introduction ............................................................................................................................... 1

Chapter 2 Establishing screening conditions to search for positive regulators of TIR-NB-LRR-mediated immunity ......................................................................................................................... 12

2.1 Introduction ...................................................................................................................................... 12

2.2 Plant growth ................................................................................................................................... 14

2.3 Fresh weight measurement ............................................................................................................... 14

2.4 Results ............................................................................................................................................ 14

2.4.1 Autoimmune mutants behave similarly on different types of plates ........................................ 15

2.4.2 Autoimmune mutants behave differently at different temperatures on both 24-well and 96-well plates ................................................................................................................................... 15

2.5 Conclusion and Discussion .............................................................................................................. 16

Chapter 3 Using chs3-2D to discover regulators of plant immunity ....................................................... 21

3.1 Chemical library .............................................................................................................................. 21

3.2 Materials and methods .................................................................................................................... 21

3.2.1 Plant growth .............................................................................................................................. 21

3.2.2 Assay for chemical screening .................................................................................................. 21

3.2.3 Phenotype collection ................................................................................................................ 22

3.2.4 GUS staining assay ................................................................................................................ 22

3.2.5 Reactive oxygen species (ROS) assay ...................................................................................... 23
3.3 Results .......................................................................................................................... 23

3.3.1 Isolation of 353D7 as a plant immunity inhibitor .................................................. 23

3.3.2 353D7 suppresses chs3-2D autoimmune morphology in a dosage dependent
manner .............................................................................................................................. 24

3.3.3 353D7 suppresses the constitutive PR gene expression of chs3-2D and flg22–induced
ROS production .............................................................................................................. 25

3.3.4 353D7 did not suppress the autoimmune phenotypes of other selected autoimmune
mutants .......................................................................................................................... 26

3.3.5 Testing the role of other agonists and antagonists on the morphology of chs3-2D ..... 26

3.4 Discussion .................................................................................................................. 27

3.4.1 Challenges of using chemical genetics in plant immunity ...................................... 28

3.4.2 Molecular targets of 353D7 .................................................................................. 29

3.4.2.1 CHS3-2D ........................................................................................................ 29

3.4.2.2 Arabidopsis glutamate-like receptor (AtGLR) .................................................. 30

3.4.2.3 Unknown targets ............................................................................................ 36

Chapter 4 Future directions and conclusions .................................................................. 53

REFERENCES ............................................................................................................... 54
LIST OF TABLES

Table 1-1 Fully characterized modifier of sncl mutants identified in the sncl and sncl npr1 suppressor screens ........................................................................................................11

Table 3-1 Various responses of chs3-2D to different chemicals........................................49

Table 3-2 Effect of other agonists and antagonists of animal glutamate receptors on the morphology of chs3-2D ........................................................................................................52
LIST OF FIGURES

Figure 1-1 Comparison of forward and reverse chemical genetics ......................................... 9

Figure 2-1 Growth of autoimmune mutants on 24-well screening plates................................. 17

Figure 2-2 Growth of autoimmune mutants on 96-well screening plates................................. 19

Figure 3-1 Schematic of primary and secondary screen.......................................................... 37

Figure 3-2 Effect of 353D7 on the morphology of chs3-2D. ................................................... 39

Figure 3-3 Analysis of different immune responses in 353D7-treated plants............................ 41

Figure 3-4 Analysis of the effect of 353D7 on selected autoimmune mutants......................... 43

Figure 3-5 Structure of glutamate receptor and ligand perception by NMDA receptor............. 45

Figure 3-6 Maximum likelyhood tree of Arabidopsis GLRs, human iGluRs, Drosophila, C.elegans, Physcomitrella and prokaryotic Synechocystis glutamate receptors......................... 47
LIST OF ABBREVIATIONS

AMP — α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ATD — N-terminal domain

BGL2 — β-1, 3-glucanase

\([\text{Ca}^{2+}]_{\text{cyt}}\) — cytosolic concentration of \(\text{Ca}^{2+}\)

CAM — Calmodulin

CBL — calcineurin B-like

CDPKs — calcium-dependent protein kinases

\(\text{chs3-2D} \) — chilling sensitive 3, 2D

CML — CAM-like

CNGC — cyclic nucleotide gated channel

CNL — CC-NB-LRR

CNS — central nervous systems

CPR1 — Constitutive \(PR\) gene expression, 1

DAS — 4,15-diacetoxyscirpenol

EDS1 — enhanced disease susceptibility, 1

EFR — EF-Tu Receptor

EF-Tu — elongation factor Tu

ETI — effector-triggered immunity

flg22 — flagellin 22

FLS2 — flagellin-sensing 2

FN — fast neutron
GLR — glutamate-like receptors

GUS — β-glucuronidase

H.a Noco2 — *Hyaloperonospora arabidopsidis* Noco2

HR — hypersensitive response

HTS — high-throughput screening

iGluRs — ionotropic glutamate receptors

KA — kainite

LBD — ligand binding domain

LIM — Lin-11, Isl-1, Mec-3

LPS — lipopolysaccharide

MAPK — MAP kinase

*mos* — *modifier of snc1*

MS — Murashige and Skoog

NB-LRR — Nucleotide binding-leucine-rich repeat

NDR1 — non-race-specific disease resistance

NEO — neosolaniol

NMDA — *N*-methyl-D-aspartate

NO — nitric oxide

NOS — nitric oxide synthase

*P.s.m* ES4326 — *Pseudomonas syringae pv. maculicola* ES4326

PAD4 — phytoalexin deficient 4

PAMPs — pathogen-associated molecular patterns
PM — plasma membrane

PR genes — Pathogenesis-Related genes

PRRs — pattern-recognition receptors

PTI — PAMP-triggered immunity

R proteins — Resistance proteins

RIN4 — RPM1-interacting protein 4

ROS — Reactive oxygen species

RPS6 — Resistance to Pseudomonas Syringae 6

SA — salicylic acid

SAG101 — Senescence associated gene 101

SCF — SKP1-CUL1-F-box

SNC1 — SUPPRESSOR OF npr1-1, CONSTITUTIVE 1

snc6-ID — suppressor of npr1-1, constitutive 6-ID

SRFR1 — SUPPRESSOR OF rps4-RLD1

TMD — transmembrane domain

TNL — TIR-NB-LRR

TTSS — type III secretion system

VIGS — virus-induced gene silencing
AKNOWLEDGEMENTS

This project would not have been possible without the assistance of many outstanding people. I would like to thank my supervisor, Dr. Xin Li for her patience, guidance and support. I would also like to thank my committee members, Dr. George Haughn and Dr. Lacey Samuels for their precious comments and suggestions during committee meetings.

I would like to acknowledge the China Scholar Council (CSC) for financial support.

There are several people I would like to acknowledge for training, technical assistance and discussion: Dr. Michel Roberge (UBC) for the chemical librarys creening, Aruna Balgi for help with the handling of the robot. Dr. Terry Snutch (UBC) and Dr. Yueling Zhang for discussions. Pingtao Ding for help with the ROS assay and discussion, Charles Copeland and Chipo Zhu for help with the primary screen, and Yuti Cheng, Fang Xu, Meixuezi Tong and Zhibin Zhang for discussions.

Finally, I would like to thank my family members and friends for their support and encouragement. Without them, I could not have completed this degree.
Using chemical genetics to discover regulators in plant immunity

Chapter 1 Introduction

Plants interact directly with many other species. Sometimes, the interaction between plants and microbes or pests can cause tremendous loss of crops in agriculture. Understanding how pathogens overcome plant defense systems and cause disease, how plants protect themselves and stop pathogen growth, and what is the molecular mechanism of plant-pathogen interactions are important for sustainable agriculture (Grierson et al. 2011).

Like animals, plants defend themselves from pathogen attack through sophisticated mechanisms (Chisholm et al. 2006; Jones and Dangl 2006). There are at least two layers of immune systems in plants. In the first layer, pattern-recognition receptors (PRRs) residing on the plasma membrane can recognize conserved pathogen-associated molecular patterns (PAMPs or MAMPs), e.g., bacterial lipopolysaccharide (LPS) and flagella, and fungal chitin and glucans (Boller and Felix 2009). Recognition of PAMPs by PRRs can trigger a relatively weak immune response, which is referred to as PAMP-triggered immunity, or PTI. Downstream signaling of PTI includes ion fluxes, activation of the MAP kinase (MAPK) cascade and transcriptional changes (Schwessinger and Zipfel 2008). Some well characterized examples of PTI signaling are flagellin 22 (flg22) recognition by FLS2 (FLAGELLIN-SENSING 2) and elongation factor Tu (EF-Tu) perception by EFR (EF-TU RECEPTOR) in Arabidopsis (Kunze et al. 2004; Zipfel et al. 2004; Zipfel 2008).
In response to PAMP-triggered plant defense, some pathogens evolved effectors which are often involved in subverting PTI and promoting virulence. Once secreted into the plant cell by means of type III secretion system (TTSS) of bacteria or haustoria of fungal pathogens, many effectors can be recognized by plant Resistance (R) proteins and trigger the second layer of plant defense: effector-triggered immunity, or ETI (Jones and Dangl 2006). ETI often leads to a hypersensitive response (HR), reminiscent of programmed cell death in animal systems (Coll et al. 2011). HR can often induce systemic acquired resistance (SAR), a long-lasting defense response that can protect plants from repeated pathogen infections. SAR requires the small molecule plant hormone salicylic acid (SA) (Chisholm et al. 2006). In ETI, different effectors are recognized by specific Resistance (R) gene products through either direct or indirect interactions (Jones and Dangl 2006). For example, the flax R protein L recognizes the corresponding flax rust fungal protein AvrL directly (Dodds et al. 2006). However, bacterial AvrRpt2, AvrRpm1 and AvrB are recognized indirectly by their corresponding R proteins. After injection into the plant cell, AvrRpt2 cleaves the Arabidopsis RPM1-interacting protein 4 (RIN4). The R protein RPS2 can detect the elimination of RIN4 and activate the RPS2 initiated-signaling pathway without direct recognition of AvrRpt2 (Mackey et al. 2003). AvrRpm1 and AvrB, two unrelated effectors from the bacteria Pseudomonas syringae, can cause auto-phosphorylation of RIN4, which can then be sensed by the R protein RPM1 and trigger ETI (Mackey et al. 2002).

In plants, there are five major classes of R genes (Dangl and Jones 2001). Nucleotide binding-leucine-rich repeat (NB-LRR)-type R proteins represent the largest class of R proteins. Based on their N-terminus, NB-LRR can be further classified into two
subgroups. The first group has homology with the intracellular components of *Drosophila* Toll and Interleukin-1 Receptor in animals. This type of NB-LRR is called TIR-NB-LRR, or TNL proteins. The other encodes an N-terminal coiled-coil motif and thus called CC-NB-LRR or CNL proteins (Meyers et al. 2003). Signaling downstream of NB-LRR proteins seems to depend on the structural types of particular R proteins. Mutations in EDS1 (ENHANCED DISEASE SUSCEPTIBILITY, 1), a positive regulator in basal defense, often suppress TIR-NB-LRR mediated defense, while mutations in NDR1 (NON-RACE-SPECIFIC DISEASE RESISTANCE) usually block signaling from CC-NB-LRRs. It was shown that EDS1 could recruit PAD4 (PHYTOALEXIN DEFICIENT 4) and SAG101 (SENESCENCE ASSOCIATED GENE 101) to form a protein complex to mediate plant defense (Aarts et al. 1998; Feys et al. 2005). EDS1 was shown to interact directly with three TIR-NB-LRR R proteins: RPS4 (RESISTANCE TO *Pseudomonas Syringae* 4), SNC1 (SUPPRESSOR OF npr1-1, CONSTITUTIVE 1) and RPS6 (RESISTANCE TO *Pseudomonas Syringae* 6), and with another negative regulator of plant defense SRFR1 (SUPPRESSOR OF rps4-RLD1) to mediate defense responses in *Arabidopsis*. This indicates that nucleo-cytoplasmic coordination of EDS1/RPS4/SRFR1 complex is essential for bacterial effector AvrRpt4 triggered plant defense (Bhattacharjee et al. 2011; Heidrich et al. 2011). However the molecular events surrounding R protein activation are still largely unknown.

Classical genetic methods have provided a powerful tool to help dissect biological pathways in model plant *Arabidopsis thaliana* and other model organisms (Forsburg 2001; Patton and Zon 2001; Jorgensen and Mango 2002; Page and Grossniklaus 2002; St Johnston 2002; Kile and Hilton 2005). Forward genetics entails randomly introducing
mutations into the whole organism and looking for mutants with phenotypes of interest, and identifying mutated genes responsible for the phenotype through map-based cloning. Classical reverse genetics entails mutating a specific gene of interest and studying the resulting phenotype (Page and Grossniklaus 2002).

**SNC1** (*SUPPRESSOR OF NPR1-I, CONSTITUTIVE 1*) encodes a TIR-NB-LRR R protein. *snc1* was identified as an autoimmune mutant in the *npr1-1* background due to a gain-of-function mutation causing constitutive expression of *Pathogenesis-Related (PR)* genes (Zhang et al. 2003). *snc1* mutant plants exhibit dwarfism, curly dark-colored leaves and enhanced disease resistance to virulent oomycete pathogen *Hyaloperonospora arabidopsidis* (*H.a*) Noco2 and the bacterial pathogen *Pseudomonas syringae pv. maculicola* ES4326 (Li et al. 2001; Zhang et al. 2003). SNC1 was shown to localize in both the cytosol and the nucleus. It interacts with transcriptional repressor TPR proteins to repress negative regulators of defense response (Zhu et al. 2010). Constitutive defense response is detrimental to the plants. To prevent auto-immunity, defense responses are under strict control, which is largely mediated by negative regulators. We have shown that one negative regulator, CPR1 (*Constitutive PR* gene expression, 1), an F-box protein which is a key component of the SCF (SKP1-CUL1-F-box protein) E3 ubiquitin ligase complex, targets SNC1 for degradation to prevent autoimmunity (Cheng et al. 2011). However, how exactly *snc1* activates defense responses still remains unclear.

To search for signaling components required for *snc1* activation, a forward genetic screen was carried out to search for suppressors of *snc1*. *snc1* or *snc1 npr1* seeds were treated with various mutagens, including fast neutron (FN), T-DNA and EMS mutagens. Suppressors of *snc1* were defined by reverting the *snc1* autoimmune phenotype to wild-
type, including the reduced plant size, enhanced PR gene expression and disease resistance against virulent pathogens. These suppressor genes were named mos (modifier of sncl) mutants. From the MOS screen, a total of 15 mos mutants were identified. Thirteen novel MOS genes were cloned and studied (Table 1-1). The functions of these MOS proteins are quite diverse, indicating an intricate signaling network surrounding R proteins activation (Palma et al. 2005; Zhang et al. 2005; Zhang and Li 2005; Goritschnig et al. 2007; Goritschnig et al. 2008; Cheng et al. 2009; Monaghan et al. 2009; Germain et al. 2010; Li et al. 2010; Monaghan et al. 2010; Wiermer et al. 2010; Zhu et al. 2010; Xu et al. 2011; Xu et al. 2012). However, forward genetics has its limitations in that mutations in highly redundant gene families may not result in distinguishable phenotypes, or mutations in housekeeping genes may not generate viable mutants.

Here I describe using chemical genetics to study plant defense pathways, particularly signaling downstream of TIR-NB-LRR type of R protein activation. Analogous to classical genetics, chemical genetics is an approach using diverse small-molecule compounds (<500 Daltons) to explore biological processes (Stockwell 2000). Forward chemical genetics (phenotype-based screening) entails screening a large number of diverse chemicals, looking for the phenotype of interest, and identifying the molecular target of the chemical responsible for the phenotype. Reverse chemical genetics (target-based screening) entails perturbing the function of the target of interest (e.g., a protein) by a small molecule binding partner, and studying the phenotype of the consequences (Figure 1-1). Compared with classical genetics, the major advantage of chemical genetics is that antagonists with low selectivity can inhibit the function of a whole protein family and therefore bypass redundancy. In addition, chemicals can be added or removed at will,
and high-throughput screening (HTS) method enables a large number of chemicals to be tested in a relative short time period.

However, chemical genetics also has its limitations in that it highly depends on the availability of the chemical compounds. Compared with the number of biomolecules in a cell, the number of small molecules available is still quite limited. Although the application of combinatorial chemistry can yield very large libraries, it is still in its infancy (Dolle et al. 2006). Another drawback of chemical genetics is that promiscuous specificity may render target identification difficult.

Despite these disadvantages, chemical genetics has shown its power in illustrating myriad biological processes in plants (Min et al. 1999; Hayashi et al. 2001; Zhao et al. 2003; Park et al. 2009). For example, the longly sought-after ABA receptor was identified from a forward chemical genetic screen (Park et al. 2009). Using chemical genetics to study plant-microbe interactions and dissect plant immune pathways has also emerged in recent years (Serrano et al. 2007; Serrano et al. 2010; Abdel-Hamid et al. 2011; Noutoshi et al. 2012).

Using submerged cultures of Arabidopsis seedlings, Serrano et al. (2007) screened a small chemical library containing 120 small molecules with known biological functions and looked for compounds that could interfere with early PAMP–responsive marker genes. Several small molecules were identified from this screen that could either induce or inhibit PAMP–triggered immune responses. One compound, triclosan, might inhibit PTI through targeting fatty-acid synthase II, indicating a role of lipid signaling in PTI.
Recognition of effectors by R proteins leads to programmed cell death (hypersensitive response, HR). It is believed to limit the propagation of pathogens (Lam et al. 2001; Greenberg and Yao 2004). However, the downstream components of HR are still unclear. Using cell death-based phenotype, Serrano et al. (2010) screened a library containing 6,800 natural compounds for molecules that could suppress AvrRpm1 induced cell death. Two mycotoxins, 4, 15-diacetoxyscirpenol (DAS) and neosolaniol (NEO), which are synthesized by certain fungi pathogens, could inhibit RPM1-avrRpm1 dependent cell death. DAS and NEO inhibit AvrRpm1 protein translational, indicating an inhibitory role of mycotoxins in HR through translation inhibition.

Plant activators, which could protect plants from pathogen invasion through activating plant defense responses, are agriculturally important. Through a high-throughput screening, Noutoshi et al. (2012) screened a library containing 10,000 synthetic compounds. Using Arabidopsis cell suspension cultures they identified five compounds that could promote Pst-avrRpm1 induced cell death. These compounds could prime immunity only in the presence of elicitors, while they had no toxic effect without avr-Rpm1 treatment. They named these compounds imprimatins. Through knockout mutant analysis, it was demonstrated that these imprimatins probably target SA glucosyltransferases to block the glucosylation of SA to SAG, a storage form of SA. SA, a putative plant defense hormone, accumulates during infection (Durrant and Dong 2004). Thus, more free SA is available in the cell when glucosyltransferases are inhibited by imprimatins, which is thought to be important to immunity priming (Noutoshi et al. 2012).

These studies demonstrated that the high-throughput screening (HTS) method is reliable and successful in dissecting biological processes, which could complement the
classical genetics approaches. However, nearly all the previous studies on plant defense pathways were based on single cell phenotype (cell death) or a particular pathway (PTI), which may not reflect the whole plant immune systems. I describe here our efforts using a whole organism-based phenotype to establish an HTS method to study plant immunity, particularly components downstream of TIR-NB-LRR R protein activation.

Autoimmune mutants (e.g., snc1, ssi1, chs3-2D and snc4) all exhibit constitutively activated defense response and arrested growth morphology (Shah et al. 1999; Zhang et al. 2003; Bi et al. 2010, 2011). They have been used as powerful tools to study plant immune pathways through suppressor screens that can revert or enhance the autoimmune phenotype. The success of the MOS screen reinforces the elegance of these auto-immune systems. Here we describe the use of chemical genetics to discover positive regulators of plant defense, taking advantage again of the unique auto-immune phenotypes of our mutants.

I screened a small molecule library containing 3,600 pure compounds with known functions in animal systems. I identified one compound, 353D7 that can completely suppress the autoimmune phenotypes of chs3-2D (chilling sensitive 3, 2D including constitutive activated PR gene expression, enhanced disease resistance to virulent pathogen Hyaloperonospora arabidopsidis (H.a) Noco2 and arrested growth morphology (Bi et al. 2011). 353D7 was previously reported to be a putative antagonist of glutamate receptors in animal systems. We hypothesized that 353D7 might suppress plant immunity by inhibiting glutamate-like receptors (GLR), which could play pivotal roles in regulating immune responses through calcium signaling.
A. Forward chemical genetics

Cells or whole organisms

One small molecule each well

Phenotype of interest

Choose the small molecule that contributes to the phenotype of interest

Small molecule

Target identification of the compound

B. Reverse chemical genetics

Protein of interest

Screen for small molecules that can bind to protein

Add small molecule to cells or whole organism

Study phenotype of the consequence

Modified from Brent R. Stockwell, 2000
Figure 1-1. Comparison of forward and reverse chemical genetics.

(A) Forward chemical genetics (phenotype-based screening) entails screening a large amount of diverse chemicals, looking for the phenotype of interest, and identifying the molecular target of the chemical responsible for the phenotype.

(B) Reverse chemical genetics (target-based screening) entails perturbing the function of the target of interest (e.g. a protein) by a small molecule binding partner, and studying the phenotype of the consequences.
Table 1-1. Fully characterized modifier of snc1 mutants identified in the snc1 and snc1 npr1 suppressor screens.

<table>
<thead>
<tr>
<th>At number</th>
<th>Gene name</th>
<th>Shares homology with</th>
<th>Mutagen</th>
<th>Suppression of snc1</th>
<th>Type of mutation over 4 alleles obtained</th>
<th>Subcellular localization</th>
<th>Special features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>At4g24680</td>
<td>MOS1</td>
<td>DNA methylation protein</td>
<td>FN</td>
<td>Complete</td>
<td>8-bp deletion in mos1-1</td>
<td>Nuclear</td>
<td>with HLA-B associated (BAT2) domain, may function in gene regulation</td>
<td>Li et al. 2011</td>
</tr>
<tr>
<td>At1g33520</td>
<td>MOS2</td>
<td>RNA-binding protein</td>
<td>FN</td>
<td>Partial</td>
<td>2 bp insertion in mos2-1</td>
<td>Nuclear</td>
<td>NLS, G-patch and KOW motifs that may be involved in RNA binding.</td>
<td>Zhang et al. 2005</td>
</tr>
<tr>
<td>At1g80680</td>
<td>MOS3</td>
<td>Nucleoporin 96</td>
<td>FN</td>
<td>Complete</td>
<td>52 bp deletion in mos3-1</td>
<td>Nuclear envelope</td>
<td>Part of the Nop 107–160 subcomplex required for mRNA export.</td>
<td>Zhang et al. 2005</td>
</tr>
<tr>
<td>At3g18165</td>
<td>MOS4</td>
<td>Human BCAS2</td>
<td>FN</td>
<td>Complete</td>
<td>2.2 kb insertion in mos4-1</td>
<td>Nuclear</td>
<td>Part of the MAC multi-protein complex with AICDC5, PRL1, MAC3A/3B and others.</td>
<td>Palma et al. 2007</td>
</tr>
<tr>
<td>At2g30110</td>
<td>MOS5</td>
<td>E1 ubiquitin activating enzyme</td>
<td>FN</td>
<td>Complete</td>
<td>15 bp deletion in mos5-1</td>
<td>N/A</td>
<td>Double mutant with uba2, the other Arabidopsis E1 is lethal.</td>
<td>Gerswitzsieg et al. 2007</td>
</tr>
<tr>
<td>At4g02150</td>
<td>MOS6</td>
<td>Importin α-3</td>
<td>FN</td>
<td>Partial</td>
<td>3 alleles, deletion/rearrangements</td>
<td>Mostly nuclear</td>
<td>Has 7 homologs in Arabidopsis. Part of NLS protein nuclear import machinery.</td>
<td>Palma et al. 2005</td>
</tr>
<tr>
<td>At5g05680</td>
<td>MOS7</td>
<td>Nucleoporin 88</td>
<td>FN</td>
<td>Complete</td>
<td>12 bp deletion in mos7-1 G to C substitution in mos8-1</td>
<td>Nuclear envelope</td>
<td>Required for nuclear protein retention/export. Null mos7 alleles are lethal.</td>
<td>Marcel et al. 2010</td>
</tr>
<tr>
<td>At5g40280</td>
<td>MOS8</td>
<td>β-subunit of farnesyltransferase</td>
<td>FN</td>
<td>Partial</td>
<td>Chromosome deletion and rearrangement</td>
<td>N/A</td>
<td>Is a loss-of-function allele of ERA1.</td>
<td>Gerswitzsieg et al. 2008</td>
</tr>
<tr>
<td>At1g12530</td>
<td>MOS9</td>
<td>Plant specific unknown protein</td>
<td>FN</td>
<td>Partial</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>At1g80490</td>
<td>MOS10</td>
<td>Transcriptional repressor</td>
<td>T-DNA</td>
<td>Partial</td>
<td>921 bp deletion in mos10-1</td>
<td>N/A</td>
<td>N-terminal LisH domain, C-terminal to LisH (CTLIH) domain and WD 40 repeats.</td>
<td>Zha et al. 2010</td>
</tr>
<tr>
<td>At5g02770</td>
<td>MOS11</td>
<td>human RNA binding protein CIP29</td>
<td>T-DNA</td>
<td>Partial</td>
<td>T-DNA insertion in mos11-1</td>
<td>Nuclear envelope</td>
<td>mRNA export.</td>
<td>Germains, et al. 2010</td>
</tr>
<tr>
<td>At2g06430</td>
<td>MOS12</td>
<td>human cyclin L</td>
<td>EMS</td>
<td>Complete</td>
<td>G to A mutation in mos12-1</td>
<td>Nuclear</td>
<td>Associated with MAC complex and function in alternative splicing. Homologous to TRN-SR in Drosophila, may function as transporter for SR proteins</td>
<td>Xu et al. 2012</td>
</tr>
<tr>
<td>At5g62600</td>
<td>MOS14</td>
<td>importin-β super-family</td>
<td>EMS</td>
<td>Partial</td>
<td>G to A mutation in mos14-1</td>
<td>Nuclear</td>
<td></td>
<td>Xu et al. 2011</td>
</tr>
<tr>
<td>MOS15</td>
<td>non-coding RNA</td>
<td></td>
<td>T-DNA</td>
<td>Partial</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT3G52430</td>
<td>PAD4</td>
<td>lipase like protein</td>
<td>FN</td>
<td>Complete</td>
<td>N/A</td>
<td>Nuclear &amp; cytosol</td>
<td>Modified from Jacqueline Moraghan</td>
<td></td>
</tr>
</tbody>
</table>

Note: N/A = not available
FN=fast neutron
Chapter 2 Establishing screening conditions to search for positive regulators of TIR-NB-LRR-mediated immunity

2.1 Introduction

The molecular mechanisms surrounding R protein activation is still obscure. Autoimmune mutants with gain-of-function mutations in R genes provide powerful tools to dissect plant immune pathways in that blocking components downstream of R proteins could suppress the autoimmune phenotypes. We took advantage of the established HTS method using small molecules to perturb biological pathways. Similar to the forward genetic screen discussed above, small molecules targeting defense positive regulators may either enhance or inhibit the defense pathways. Small molecule plant defense inhibitors are able to suppress the autoimmune phenotypes similar to the mos mutants and, different from the MOS genes, may also perturb the functions of highly redundant genes or lethal genes but generate viable “chemical-knock-down” mutants.

To search for proper screening conditions, I tested two unique autoimmune mutants: snc1 (suppressor of npr1-1, constitutive 1) and chs3-2D (chilling sensitive 3-2D) (Zhang et al. 2003; Bi et al. 2011). Both mutants show strikingly dwarf phenotypes caused by constitutively activated plant defense. The autoimmunity of snc1 was due to a gain-of-function mutation in the linker region between NB and LRR domains (Li et al. 2001; Zhang et al. 2003). chs3 was initially isolated as a chilling temperature sensitive mutant (Schneider et al. 1995). The mutant chs3-1 was indistinguishable from wild-type plants in room temperature (22°C), but showed arrested growth morphology and enhanced
defense responses when grown in low temperature (16°C), which probably due to enhanced chs3 protein level (Yang et al. 2010). chs3-2D, a gain-of-function allele of CHS3, was isolated in an independent screen for suppressors of npr1-1 and was previously named as snc6-1D (suppressor of npr1-1, constitutive 6-1D) (Bi et al. 2011). CHS3 encodes an atypical TIR-NB-LRR R protein with a C-terminal zinc-binding LIM (Lin-11, Isl-1, Mec-3) domain which may function in protein-protein interactions (Schmeichel and Beckerle 1994; Bi et al. 2011). The autoimmunity of chs3-2D was due to a point mutation after the LIM domain, rendering the chs3 protein auto-active. chs3-2D shows a more striking dwarf phenotype than snc1 mutant under either 22°C or 16°C growth conditions.

pSNC1::snc1-GFP, a snc1 overexpression line was also tested in this screen. The transgenic snc1-GFP plants accumulate a higher level of SNC1 protein than the snc1 single mutant, therefore the snc1-GFP plants show a stronger autoimmune phenotype including dwarfism, enhanced PR gene expression and resistance to bacterial pathogen P.s.m ES4326 and oomycete pathogen H.a Noco2.

We utilized these three unique autoimmune mutants to establish an optimum sterile screening condition under which the mutant plants are morphologically the most distinguishable from wild-type plants. We hypothesized that potential small molecule(s) being able to suppress plant defense could also suppress the arrested growth morphology of the selected mutants.
2.2 Plant growth

Arabidopsis Col-0, sncI, sncI-GFP and chs3-2D seeds were surface sterilized, re-suspended in 0.1% agar and stratified under 4°C for 48 h. I tested the effect of both 24-well and 96-well Costar deep-well sterile tissue culture plates on plant growth. Different volumes of ½ Murashige and Skoog (MS) media (0.5% sucrose, 0.3% phyto gel, pH 5.7) was manually added to 24-well or 96-well Costar sterile tissue culture plates using a RAININ 5 mL electronic pipette. After the media was solidified, six seeds were transferred to each well of the 24-well plate, or two to three seeds to each well of the 96-well plate. Plates were tightly sealed with parafilm and incubated in 16°C (12-h-light/12-h-dark cycles, 70% humidity) or 22°C growth chambers (16-h-light/8-h-dark cycles, 70% humidity) for the temperature test. Seedlings were grown for 14-16 days before fresh weight measurement was performed.

2.3 Fresh weight measurement

Whole seedlings were harvested from the growth medium. Agar and water attached to the plant surface was removed using paper towels. Since the weight of a single seedling is very light and difficult to measure, the fresh weight of a single plant was calculated by taking the average of the fresh weight of 3~10 plants as a group. At least three groups were measured.

2.4 Results

To search for an optimum screening condition for our proposed chemical genetics screen, I tested a collection of factors that could affect plant growth, including temperature, genotypes, type of screening plates and the volume of growth media.
2.4.1 Autoimmune mutants behave similarly on different types of plates

I observed similar results for plants growing on either 24-well or 96-well plates. On both types of screening plates, chs3-2D grew smallest under 16°C growth condition, while snc1-GFP plants were smallest under 22°C (Figure 2-1, 2-2). Media volume did not significantly affect the output, except that on 600 µL media of 24-well plates Col-0 grew obviously larger than snc1, snc1-GFP and chs3-2D in fresh weight (Figure 2-1A and C). This is probably because more growth resource was available in the medium, thus Col-0 plants absorb more water as it is morphologically similar as that grown on 400 µL media. No significant difference was observed among plants grown on different volume of media of 96-well screening plates (Fig 2-2).

2.4.2 Autoimmune mutants behave differently at different temperatures on both 24-well and 96-well plates

24–well plate

Under 16°C growth condition, chs3-2D shows a strikingly dwarf phenotype compared with Col-0, snc1 and snc1-GPF (Figures 2-1A). There is a significant difference in fresh weight between chs3-2D and wild-type control plants (Figures 2-1B). chs3-2D grew to 50% of the fresh weight of Col-0, while snc1 and snc1-GPF were comparable in fresh weight with wild-type plants.

At 22°C, on 400 µL medium chs3-2D and snc1 were comparable with Col-0 while snc1-GFP was slightly smaller than Col-0, snc1-GFP grew to about 85% of Col-0 in fresh weight (Figures 2-1C). On 600 µL medium, chs3-2D and snc1 were 50% of Col-0
in fresh weight, while snc1-GFP showed the most dwarf morphology and lowest fresh weight (30% of Col-0).

96-well plate

At 16°C, chs3-2D was the smallest on different volumes of medium. On 75 µL medium, chs3-2D was about 45% of Col-0 in fresh weight, while snc1 and snc1-GFP were 90% of Col-0 (Figure 2-2). On both 100 and 125 µL medium, compared with Col-0, the fresh weight of chs3-2D was about 30%, and snc1 and snc1-GFP were about 80%. At 22°C, both chs3-2D and snc1-GFP showed the most dwarf morphology, however, snc1-GFP was slightly smaller than chs3-2D on 75 and 125 µL medium. snc1 was smaller than Col-0 but larger than both chs3-2D and snc1-GFP.

2.5 Conclusion and Discussion

I tested several factors that could affect plant growth. For mutants’ growth phenotypes, screening plates and media volume did not significantly affect the results. Temperature significantly affects plant growth. At 16°C, plant growth was stunted with reduced plant size and fresh weight compared with plants grown at 22°C. Different autoimmune mutants showed dramatically different responses to temperature changes, indicating different chilling tolerance ability. As chs3-2D showed a most striking phenotypic difference under low temperature compared with wild-type plants, we decided to use chs3-2D as our screening genotype. For high throughput screening we decided to use 96-well tissue culture plates as screening plates since they are compatible with the liquid-handling robots. Since medium volume did not affect the phenotypes of the selected mutants, 100 µL ½ MS media was used in the future screen.
**Figure 2-1.** Growth of autoimmune mutants on 24-well screening plates.

(A) Growth morphology of plants grown at indicated temperatures on 24-well plates containing either 400 µL or 600 µL ½ strength MS medium, pictures were taken when plants were two weeks old.

(B) and (C) Fresh weight of plants shown in (A) grown at 16°C or 22°C on 400 µL or 600 µL media. Error bars represent mean ± SD (n=3 with 6 plants each). Stars indicate statistical difference (one-way ANOVA followed by Bonferroni post – test, **P < 0.01, ***P<0.001) between autoimmune mutants and wild-type Col-0 plants within each medium volume (not between medium volume).
A

<table>
<thead>
<tr>
<th>Temperature</th>
<th>75µL</th>
<th>100µL</th>
<th>125µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 °C</td>
<td><img src="https://example.com/image1.png" alt="Image" /></td>
<td><img src="https://example.com/image2.png" alt="Image" /></td>
<td><img src="https://example.com/image3.png" alt="Image" /></td>
</tr>
<tr>
<td>22 °C</td>
<td><img src="https://example.com/image4.png" alt="Image" /></td>
<td><img src="https://example.com/image5.png" alt="Image" /></td>
<td><img src="https://example.com/image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>

B

16 °C

<table>
<thead>
<tr>
<th>Medium volume (µL)</th>
<th>Col-0</th>
<th>chs3-2D</th>
<th>sncl</th>
<th>sncl-GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td><img src="https://example.com/chart1.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart2.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart3.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart4.png" alt="Bar Chart" /></td>
</tr>
<tr>
<td>100</td>
<td><img src="https://example.com/chart5.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart6.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart7.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart8.png" alt="Bar Chart" /></td>
</tr>
<tr>
<td>125</td>
<td><img src="https://example.com/chart9.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart10.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart11.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart12.png" alt="Bar Chart" /></td>
</tr>
</tbody>
</table>

C

22 °C

<table>
<thead>
<tr>
<th>Medium volume (µL)</th>
<th>Col-0</th>
<th>chs3-2D</th>
<th>sncl</th>
<th>sncl-GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td><img src="https://example.com/chart13.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart14.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart15.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart16.png" alt="Bar Chart" /></td>
</tr>
<tr>
<td>100</td>
<td><img src="https://example.com/chart17.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart18.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart19.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart20.png" alt="Bar Chart" /></td>
</tr>
<tr>
<td>125</td>
<td><img src="https://example.com/chart21.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart22.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart23.png" alt="Bar Chart" /></td>
<td><img src="https://example.com/chart24.png" alt="Bar Chart" /></td>
</tr>
</tbody>
</table>
**Figure 2-2.** Growth of autoimmune mutants on 96-well screening plates.

(A) Morphology of two-week-old plants grown at 16°C on 96-well plates containing different volumes of ½ strength MS medium for two weeks. 
(B) and (C) Fresh weight of two-week old plants grown at indicated conditions on 96-well screening plates. Error bars mean ± SE (n=3 with 10 plants each). Stars indicate statistical difference (one-way ANOVA followed by Bonferroni post - test; *P<0.01) within each medium volume (not between medium volume). The experiment was repeated once with similar results.
Chapter 3 Using *chs3-2D* to discover regulators of plant immunity

3.1 Chemical library

A collection of chemicals containing 3,600 pure bioactive compounds with known biological functions in animal systems was provided by Dr. Michel Roberge’s lab in the Department of Biochemistry and Molecular Biology at the University of British Columbia. This library contains small molecules from Prestwick, BioMol, Sigma and Microsource. Chemical stock concentration is 5mM dissolved in 100% DMSO.

3.2 Materials and methods

3.2.1 Plant growth

Col-0 and *chs3-2D* seeds were surface sterilized and resuspended in 0.1% agar. Seeds were stratified in 4°C for at least 48 h before further experimentation. Two to three *Arabidopsis* seeds were manually spotted onto 96-well screen plates containing 100 µL ½ MS (0.5% sucrose, 0.3% phytogel, pH 5.7) in each well (Figure 3-1A). Plates were tightly sealed with parafilm and stored at room temperature before chemicals was added within 12 h.

3.2.2 Assay for chemical screening

*Primary screen*

About 340 nano liters (nL) of different chemicals was added into each well of the screening plate using a robot-controlled 96-well pin tool to get a final chemical concentration of 15 µM. Row 1 and 12 contained no chemicals and serve as controls (Figure 3-1A). Plates were tightly sealed using parafilm. Seedlings were grown in a 16°C
growth chamber (12-h-light/12-h-dark cycles, 70% humidity) for 14 days before phenotyping. All obvious morphological changes due to the chemicals were recorded.

**Secondary screen**

Each candidate chemical was serially diluted into different concentrations (Figure 3-1B). DMSO was used as a negative control. Two to three *Arabidopsis* Col-0 or *chs3-2D* seeds were transferred to each screening well containing different concentrations of chemicals. Seedlings were grown at 16°C for 10 days before further analysis.

### 3.2.3 Phenotype recording

Phenotypes were recorded at 18 and 26 days after plating. *chs3-2D* mutant plants grown at 16°C showed severe growth defects after 4 weeks. Older leaves turned yellow and senesced early. To alleviate this chilling stress, 26 d old plants grown at 16°C were shifted to 22°C for 7-9 d before a final round of phenotype recording.

### 3.2.4 GUS staining assay

The GUS staining assay was performed as previously described (Zhang et al. 2003). Both *snc1* and *chs3-2D* carry a chimeric reporter gene containing the Arabidopsis *PR* gene β-1, 3-glucanase (*BGL2*) promoter fused with the coding sequence of β-glucuronidase (*GUS*) in the background. Besides the dwarf morphology of the selected autoimmune mutants, there is no visible phenotype associated with enhanced defense response. Therefore, the *BGL2-GUS* reporter system enables easy detection of the expression of defense marker genes. For the histochemical GUS staining assay, five to ten two-week-old seedlings were placed in 18-well Costar plates and 1.5 mL staining solution was added to cover the seedlings. The seedlings were drew a vacuum for 10 min to allow the staining solution to infiltrate the samples. Samples were then incubated at
37°C overnight. For de-staining, the staining solution was replaced by 1.5 mL 75% ethanol and washed for three times (1 h each).

### 3.2.5 Reactive oxygen species (ROS) assay

ROS assay was performed as previously described (Gomez-Gomez et al., 1999). Flg22, a 22-amino acid peptide derived from flagellin, was shown to be able to induce oxidative burst in leaf slices (Felix et al. 1999; Gomez-Gomez et al. 1999). Reactive oxygen species production was one of the earliest events during PTI (Blume et al. 2000). To test whether 353D7 could affect PTI, we performed the ROS assay using H\textsubscript{2}O\textsubscript{2}-dependent luminescence of luminol. Fresh leaves of four-week-old Arabidopsis thaliana plants grown at short day (12-h-day/12-h-night) condition were cut into approximately 0.3 mm × 3 mm slices using a sharp blade. Six leaf slices were floated in one well of a 96-well screening plate containing 200 μL H\textsubscript{2}O or 200 μL H\textsubscript{2}O with 100 μM 353D7 overnight. 1 μM flg22, 10 mg peroxidase and 20 μM luminol was added into each well before ROS was measured in 1-min intervals using a Tecan Infinite 200 Plate Reader.

### 3.3 Results

#### 3.3.1 Isolation of 353D7 as a plant immunity inhibitor

The arrested growth morphology of chs3-2D mutant plants was due to constitutively activated plant defense (Bi et al. 2011). By screening a chemical library containing 3,600 bioactive pure compounds, we aimed to identify small molecules that could suppress the autoimmune morphology of chs3-2D. From the primary screen, plants showed various responses to different chemical treatments (Table 3-1). Twenty-six compounds were shown to be able to suppress the arrested growth morphology of chs3-2D at different
growth stages (Table 3-1). Most of the chemicals showed suppression at a very late growth stage (4 weeks later), which could be purely due to environmental changes. A confirmation assay using the 26 chemical candidates with serial dilutions was performed (Figure 3-1B). Here we describe our only target compound, 353D7, a non-competitive NR2B selective antagonist of NMDA glutamate receptors in mammalian systems. The confirmation assay showed that 353D7 can completely suppress the arrested growth morphology of the chs3-2D autoimmune mutant at all tested concentrations (5 µM to 50 µM) (Figure 3-1B).

3.3.2 353D7 suppresses chs3-2D autoimmune morphology in a dosage dependent manner

Plant hormones and signal molecules usually function at extremely low concentrations (10^{-9} ~ 10^{-6} mol/L) within plant cells (Muller and Munne-Bosch 2011). I tested a series of 353D7 concentrations for inhibition of chs3-2D autoimmune morphology. As shown in Fig 3-2A, inhibition of the arrested growth morphology by 353D7 was dosage dependent. The inhibition of the dwarfism of chs3-2D was already saturated at about 5 µM (Figure 3-2B). Interestingly, a spraying assay showed that 353D7 (15 µM) was not able to suppress the arrested growth morphology of chs3-2D and other selected auto-immune mutants grown on soil, including snc1 (Zhang et al. 2003), snc2-1D eds5-3 npr1-1 (Zhang et al. 2010), snc4 (Bi et al. 2010) and bir1 pad4 (Gao et al. 2009). However, chemically treated chs3-2D plants were slightly larger than untreated plants, indicating that probably leaf tissues do not absorb the chemical as efficiently as roots. Also, the spraying assay showed that 353D7 could not effectively suppress the enhanced disease resistance of all selected autoimmune mutants to the virulent pathogen
Hyaloperonospora arabidopsidis Noco2. These results indicate that 353D7 may need to be effectively absorbed by the plant roots to show a suppression effect.

3.3.3 353D7 suppresses the constitutive PR gene expression of chs3-2D and flg22-induced ROS production

To exclude the possibility that 353D7 suppresses the chs3-2D morphology by promoting growth rather than by perturbing plant immunity, I examined the expression of defense marker gene PR2 (BGL2). 353D7-treated chs3-2D mutant plants completely abolished the constitutive expression of PR2, indicating that 353D7 suppresses the autoimmune morphology of chs3-2D probably by inhibiting plant defense (Figure 3-3A).

To examine whether 353D7 also affects other aspects of plant immunity, I tested whether 353D7 can affect flg22 induced reactive oxygen species (ROS) production in wild-type plants (Gomez-Gomez et al. 1999). 353D7 partially inhibited flg22-induced ROS production, suggesting that 353D7 also affects the PTI signaling pathway (Figure 3-3B). One of the earliest events during PTI is a rapid transient cytosolic calcium influx from the intracellular space (Blume et al. 2000). I tested whether removal of calcium from the apoplastic store could also suppress the growth morphology of chs3-2D. Intriguingly, using a calcium chelator EGTA, the fresh weight of chs3-2D was twice as big as untreated plants (Figure 3-3C). These data suggests that the molecular target(s) of 353D7 probably plays a general role in plant defense pathways and the inhibition of PAMP induced \([\text{Ca}^{2+}]_{\text{cyt}}\) increase by 353D7 probably contributes to the suppression of the chs3-2D autoimmune phenotype.
3.3.4 353D7 did not suppress the autoimmune phenotype of other selected autoimmune mutants

Although the spraying assay did not suppress the morphology of all the selected autoimmune mutants, this result could have occurred because leaf tissues do not have high efficiency in absorbing the chemical. I therefore planted the mutants on growth medium. However, 353D7 did not suppress the morphological defects of other autoimmune mutants tested (Figure 3-4A, B). I also checked whether 353D7 could suppress the PR gene expression of the selected autoimmune mutants. 353D7 can completely suppress the expression of PRI and PR2 of chs3-2D. However the PR gene expression of other autoimmune mutants did not show obvious differences (Figure 3-4C).

3.3.5 Testing the role other agonists and antagonists on the morphology of chs3-2D

I also tested whether other putative iGluRs antagonists or calcium chelators could suppress the chs3-2D autoimmune growth phenotype, including CNQX, DNQX, spermine, LaCl3 and EGTA (Table 3-2). These chemicals were shown to be able to inhibit the animal iGluR function. AMPA antagonists CNQX, DNQX and a pore blocker spermine did not suppress the chs3-2D phenotype. Both LaCl3 and EGTA showed toxic effect at high concentrations (≥ 1mM and ≥ 5mM, respectively) on seedlings grown on ½ MS medium. However, plants grown on ½ MS medium containing 2mM EGTA treated were viable and showed partial suppression of the chs3-2D morphology (Figure 3-3C).

353D7 was originally identified as a non-competitive, NR2B subunit selective, NMDA (N-methyl-D-aspartate) receptor antagonist in the animal systems. Evolutionally conserved glutamate receptors have been cloned from prokaryotes and eukaryotes
The Arabidopsis genome contains twenty glutamate-like receptor (GLR)-coding genes which share high homology to the mammalian ionotropic glutamate receptors (iGluRs). We hypothesized that 353D7 may target the plant AtGLR. Several amino acids have been reported to be able to activate plant AtGLRs, including Gly, Glu, L-serine, D-serine and Asn (Vincill et al. 2012). If any of the above amino acid functions as the natural ligand of AtGLR (Arabidopsis glutamate like receptor), it would be able to antagonize 353D7 and thus alleviate the suppression by 353D7. I tested several amino acids and amino acid combinations (Table 3-2). However, I did not observe significant difference of chs3-2D morphology, indicating that either plant AtGLRs do not use these amino acids as natural ligand or they use instead certain modified amino acids, such as pipecolic acid (Navarova et al. 2012).

3.4 Discussion

In this study, I established a chemical genetic screening condition using an autoimmune mutant chs3-2D as the screening material to look for small molecule compounds that can perturb plant immunity. In a chemical library containing 3,600 compounds, I identified one small molecule, 353D7, which can completely suppress the autoimmune phenotypes of chs3-2D. The success of the establishment of a screening condition and the identification of a repeatable positive result indicate that chemical genetics can be used to study plant defense pathways in whole plant tissue, particularly useful for dissecting biological pathways in model organisms, e.g., Arabidopsis thaliana. However, there are many challenges to be considered when performing a small molecule screen.
3.4.1 Challenges of using chemical genetics in plant immunity

One big challenge facing biologists performing a chemical genetic screen is how to identify and eliminate false positive results (Soderholm et al. 2006). Of the 26 candidate small molecules from the primary screen, only one compound, 353D7, showed reproducible result. The high number of false positive results in this screen is probably because of the less stringent screening criteria in the primary screen. Many other aspects can also lead to false positives, for example, the properties of the compounds and assay design. One example of the property of a compound generating false positives is the fluorescence-based screening, e.g., looking for compounds that are able to perturb protein-protein interactions using fluorescence resonance energy transfer (FRET). Fluorescent compounds will interfere with fluorescence polarization and energy transfer. This is more problematic when small molecules tend to absorb and emit at shorter wavelengths, e.g., blue and green regions. Assay design can also generate false positives. The screening criteria used will directly affect the screening output.

Another challenge is how to perform a relatively simple yet robust high throughput screening (HTS). In a typical forward chemical genetic screen, about 10,000 to 100,000 small molecules are tested (Kawasumi and Nghiem 2007). Hence, the assay must be miniaturized to adapt to 96-, 384- or 1536-well format. Compounds are generally dispensed into screening plates by a handling machine or a robot. Screening material can be added automatically or manually. I spotted the Arabidopsis seeds manually. This was the rate-limiting step of our screen and will probably get more tedious with the increase of the number of compounds. Choosing an appropriate type of screening plate is also important in that the phenotype of interest may vary due to the different type of screening.
plates. This becomes important when performing a chemical genetic screen using whole plant seedling. In this screen, seeds were spotted into 96-well screening plates. Plants show different growth morphology under different growth conditions. Compared with those grown on 24-well plates, the stunted growth morphology of *chs3-2D* was more obvious on 96-well plates.

### 3.4.2 Molecular targets of 353D7

Using forward chemical genetics, I identified 353D7, a putative NMDA glutamate receptor in animal systems that can completely suppress the autoimmune phenotypes of *chs3-2D*. However, 353D7 did not suppress the autoimmune phenotypes of other selected mutants, indicating that the suppression of autoimmunity by 353D7 might be *chs3-2D* specific. 353D7 can also suppress flg22 induced oxidative burst, this suggests that the target(s) of 353D7 might play a general role in plant defense. But the suppression of ROS by 353D7 could also be due to the possibility that the chemical consumes the ROS (in this case, hydrogen peroxide), rather than by inhibiting a specific molecular target. Therefore, it is necessary to identify and characterize the molecular target(s) of 353D7 in plants. Here I discuss several possible candidates.

#### 3.4.2.1 CHS3-2D

One likely candidate of the target of 353D7 is CHS3-2D protein itself. This could be possible because among all tested autoimmune mutants, 353D7 specifically suppresses the *chs3-2D* autoimmune phenotype. For example, 353D7 might inhibit the active form of *chs3-2D*, or it might interfere with the interaction between CHS3-2D with downstream components. However, the fact that 353D7 suppresses *chs3-2D* specifically, not other tested autoimmune mutants, could also be due to the possibility that different
autoimmune mutants utilize distinct defense pathways. Indeed, downstream signaling components of the selected autoimmune mutants are known to be different from that of chs3-2D. snc1, a typical TIR-NB-LRR, can be completely suppressed by pad4, which can only partially suppress the chs3-2D phenotype. SNC2 encodes a receptor-like protein (RLP). snc2 mutant shows constitutively activated plant defense, which could be suppressed by a transcription factor wrky70 and mutations in another membrane protein BDA1. The signaling downstream of SNC2 is also different from the typical NB-LRR type R proteins as the snc2 mutant phenotype is independent of the common genes known to be required for NB-LRR R protein pathway (Zhang et al. 2010; Yang et al. 2012). SNC4, a receptor like kinase, can be largely suppressed by ndr1. NDR1, but not EDS1, is usually required for many CC-NB-LRR type R proteins, indicating a unique pathway downstream of SNC4 (Bi et al. 2010). BIR1 (BAK1 interacting receptor-like kinase), a negative regulator of cell death, can be suppressed by sobir1 (suppressor of bir1). Interestingly, the bir1 phenotype can also be partially suppressed by pad4 (Gao et al. 2009). These data suggest that, the molecular target of 353D7 probably function as a major contributor in the chs3-2D-mediated immune pathway, but a minor component in other autoimmune systems (snc1, snc2, snc4, and bir1).

3.4.2.2 Arabidopsis glutamate-like receptor (AtGLR)

The animal iGluR homologs in plants, AtGLRs, could be another likely candidate of the molecular target of 353D7 (Lam et al. 1998). In the animal central nervous systems (CNS), ionotropic glutamate receptors (iGluRs) function as glutamate-activated ion channels in excitatory synaptic transmission, which play significant roles in learning and memory (Watkins and Evans 1981; Mayer and Westbrook 1987; Mayer 2006). Four
types of glutamate receptors have been found in mammalian cells based on their pharmacologically selective agonists — NMDA (N-methyl-d-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate), KA (kainate) and δ (no known ligands) (Traynelis et al. 2010). The latter three are also known as non-NMDA glutamate receptors. Tremendous progress has been made in understanding the functions and structures of mammalian glutamate receptors during the last two decades. Glutamate receptors are integral membrane spanning proteins composed of four modular domains (Figure 3-5A): the extracellular N-terminal domain (ATD), the transmembrane domain (TMD), the extracellular ligand binding domain (LBD) and a C-terminus tail which forms the intracellular C-terminal domain (CTD) (Traynelis et al. 2010). The transmembrane domain includes TM1, TM3 and TM4 and a re-entrant P-loop (TM2). Two extracellular globular domains (S1 and S2) cross over and form the ligand-binding pocket (LBD) stretched from TM1 and TM3-TM4 loop, respectively. Ligand binding to the LBD domain causes a conformational change, opening the ion channel pore formed mainly by TM1, TM2 and TM3. Activation of glutamate receptors triggers ion fluxes, especially calcium influx which functions as important intracellular messengers (Figure 3-5B). iGluRs are non-selective calcium channels. However, amino acid residues within the pore determine the selectivity of cations (Panchenko et al. 2001). Pharmacological and electrophysiological studies have identified a number of agonists and antagonists that can either induce or inhibit the functions of iGluRs, respectively. Agonists activate iGluRs in an analogous manner as natural ligands while antagonists perturb the function of iGluRs by either competing with the ligand by binding to ligand binding sites (e.g., CNQX and DNQX) or in an allosteric manner (e.g., 353D7).
Phylogenetic analysis reveals that the *Arabidopsis* genome contains twenty glutamate receptor-like (GLR)-encoding genes (Figure 3-6). The *Arabidopsis* GLRs fall into three clades (clade I, II and III). Plant GLRs and animal iGluRs share high homology in the transmembrane domains and the ligand binding pocket (Lam et al. 1998). However, whether plant GLR has the same functions as their animal counterparts is still controversial. Based on their highly homologous amino acid sequences, there is a possibility that plant GLRs are also amino acid gated calcium channels. In support of this idea, glutamate (Glu) treatment triggers a transient increase of cytosolic concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\text{cyt}) and membrane depolarization in *Arabidopsis*, indicating a role of glutamate in Ca\(^{2+}\) fluxes (Dennison and Spalding 2000). In animals, glycine (Gly), together with glutamate, regulate ion fluxes mediated by NMDA during neuron transmission. Glycine and glutamate were also shown to synergistically regulate hypocotyl elongation and transient increase of [Ca\(^{2+}\)]\text{cyt} in *Arabidopsis* (Dubos et al. 2003). Ala, Asn, Cys, Glu, Gly, and Ser can trigger membrane depolarization and transient increase of ([Ca\(^{2+}\)]\text{cyt}) dependent on the GLR3.3 (Qi et al. 2006). Although great effort was paid to hunt for the ligands of the plant GLRs in the past decade, the real ligand is still unknown. There is a possibility that plant GLRs may perceive certain kinds of modified amino acids, which increases the complexities of these receptors.

Animal iGluRs are calcium permeable ion channels. In *Arabidopsis*, the *AtGLR3.4* was shown to be able to form an amino acid (Asn, Gly and Ser) –gated ion channel which is permeable to calcium when expressed in animal cells (Vincill et al. 2012). Mutations in *AtGLR3.1* compromises long-term Ca\(^{2+}\) -mediated stomata closure (Cho et al. 2009). *AtGLR1.2* and *AtGLR3.7* were shown to form Ca\(^{2+}\) channels and function in pollen tube
growth and morphogenesis (Michard et al. 2011). These indicate that plant GLRs, like the animal iGluRs, respond to amino acids induction and, at least one member (AtGLR3.4), likely more, is permeable to calcium.

Calcium, which is probably the most ubiquitous secondary messenger, regulates numerous cellular processes (Berridge et al. 2000; Konrad et al. 2011). Ca\(^{2+}\) influxes into the cytosol from the apoplastic space across the plasma membrane (PM) play pivotal roles in plants immunity (Lecourieux et al. 2006). Knight et al. (1991) challenged tobacco seedlings with fungal elicitors and found that there was an increase of [Ca\(^{2+}\)]\(_\text{cyt}\). Of note, this was different from the increase of [Ca\(^{2+}\)]\(_\text{cyt}\) caused by touch or cold shock in terms of Ca\(^{2+}\) fluctuation duration, kinetics and intensity, indicating that calcium influx may function as an activated defense signal during pathogen invasion in a form different from other abiotic stresses. An early event during PTI was the increase of [Ca\(^{2+}\)]\(_\text{cyt}\), which leads to downstream defense responses (Ma and Berkowitz 2007). Ca\(^{2+}\) influx into the cytosol is hypothesized to be through Ca\(^{2+}\) channels residing on the PM. Two likely candidates of Ca\(^{2+}\) channels in plants are the 20-member CYCLIC NUCLEOTIDE GATED CHANNEL (CNGC) family and the 20-member GLR family (Ma and Berkowitz 2007). Previous studies have shown that cngc loss-of-function mutants showed compromised Ca\(^{2+}\) influx and HR response when challenged with avirulent pathogens (Clough et al. 2000; Jurkowski et al. 2004; Ali et al. 2007). PAMP induction activates CNGC mediated Ca\(^{2+}\) influx (Ali et al. 2007). However, this Ca\(^{2+}\) influx could also be controlled by GLRs. Using pharmacological methods, perturbing Arabidopsis GLRs with iGluRs antagonists inhibited MAMP triggered Ca\(^{2+}\) influx (Kwaaitaal et al. 2011). The transient increase of [Ca\(^{2+}\)]\(_\text{cyt}\) was also an early event during ETI. At the
homeostasis state, the $[\text{Ca}^{2+}]_{\text{cyt}}$ in both animal and plant cells is maintained at a relatively low level (McAinsh et al. 1992). Using transgenic Arabidopsis plants with cytosol localized $\text{Ca}^{2+}$ sensitive aequorin, Grant et al. reported that pathogen avr proteins caused a biphasic $\text{Ca}^{2+}$ elevation (Grant et al. 2000). They also showed that the second peak could only be observed in the presence of avr proteins. Processes downstream of $\text{Ca}^{2+}$ signaling are quite complex. Numerous MAMPs were shown to be able to trigger $\text{Ca}^{2+}$ signatures in the cell (Lecourieux et al. 2005; Gust et al. 2007). However, how $\text{Ca}^{2+}$ influx confers diverse defense responses and, more importantly, the identity of $\text{Ca}^{2+}$ channels across the PM, is unclear.

In plants, sensing of $\text{Ca}^{2+}$ signals was thought to be mediated mainly by three types of $\text{Ca}^{2+}$ sensors: calcineruin B-like proteins, CAMs and CAM-like proteins, and calcium-dependent protein kinases (CDPKs). In a study of potassium channel, Lee et al. (2007) showed that plants respond to low $\text{K}^+$ content in the environment by enhancing uptake of $\text{K}^+$ in a $\text{Ca}^{2+}$ dependent manner. They showed that two calcineurin B-like calcium sensors (CBL1 and CBL9) and their target (CIPK23) could activate a $\text{K}^+$ channel (AKT1). Interestingly, low $\text{K}^+$ uptake generates ROS and triggers HR, signatures of activated defense responses. In parallel with the $\text{Ca}^{2+}$ influx is often the $\text{K}^+$ efflux (Wendehenne et al. 2004), this indicates a possible self-balance capability of plants in response to biotic and abiotic stresses.

An early defense response during ETI is nitric oxide (NO) generation, which is important in initiating a HR (Wendehenne et al. 2004). Ma et al (2008) showed that the activation of nitric oxide synthase (NOS) during pathogen induction depends on CaM and/or a CaM-like protein (CML). Treatment of CaM antagonists abolished NO
generation. The loss-of-function mutant *cml24-4* delimited defense responses induced by avirulent pathogens. This suggests a role of CaMs and CaM-like proteins in perceiving Ca$^{2+}$ signatures.

The third type of Ca$^{2+}$ sensor is the CDPK. The *Arabidopsis* genome encodes 34 members of the CDPKs, which fall into four subgroups (Cheng et al. 2002). Pathogen Avr proteins are recognized by specific host R proteins (Dangl and Jones 2001). In a study of tomato *Cf-9/Avr9* gene-for-gene interaction, Romeis *et al.* (2000) showed that a CDPK was phosphorylated within 5min after addition of the elicitor Avr9. Avr9 caused a long-lasting activation of CDPKs, which was due to the phosphorylation event (Romeis *et al.* 2001). Using virus-induced gene silencing (VIGS) in *N.benthamiana*, they demonstrated that the race-specific defense responses were compromised in silenced plants. In a recent work, Boudsocq *et al.* (2010) demonstrated that during PTI, Ca$^{2+}$ signals were sensed by calcium-dependent protein kinases (CDPKs) and relayed to trigger early MAMP defense response. The activation of CDPKs was transient after flg22 induction, which was different from the pronounced activation of CDPK induced by Avr9. These indicate a complex regulation of Ca$^{2+}$ signaling during plant defense. Understanding how plants decipher the different Ca$^{2+}$ signatures during different defense stimuli is needed. Notably, identification of Ca$^{2+}$ channels during plant defense will promote tremendous progress in revealing the Ca$^{2+}$ pathways and their contributions to plant immunity.

We hypothesized that 353D7 might suppress the autoimmunity of *chs3-2D* by targeting some of the *Arabidopsis* glutamate-like receptors. However, some other putative iGlur antagonists present in this library we used, such as the channel blocker
dizocilpine (MK-801), the glycine site antagonist kynurenic acid, the AMPA and kainite antagonists CNQX and DNQX (Dingledine et al. 1999), did not show obvious suppression of \textit{chs3-2D} at tested concentrations. This indicates that 353D7 probably does not target AtGLR in plants. Although the animal iGluRs and plant AtGLRs share high homology, their functions may be fully divergent. However, we cannot exclude the possibility that the binding site of 353D7 in its animal target is conserved in plants, while the binding sites of other iGluR antagonists are absent.

### 3.4.2.3 Unknown targets

Apart from CHS-2D and AtGLRs as the possible targets, 353D7 might target some unknown cellular macromolecules(s). Looking for 353D7 insensitive mutants using a forward genetic screen will help discover these unknown targets. To examine these unknown targets, \textit{chs3-2D} seeds can be mutagenized to generate a mutant population. I will look for mutants that only abolish the sensitivity to 353D7 but do not compromise the defense flow through \textit{chs3-2D}. Potential mutants would be \textit{chs3-2D}-like in growth morphology in the presence of 353D7 at 16°C. Such mutants could carry mutation(s) in the target of 353D7, or some defects in development, or another mutation that constitutively activates plant defense analogous to \textit{chs3-2D} but does not require the target of 353D7. Potential mutants could be grown at 28°C. If the high temperature can rescue the growth phenotype of those mutants, it indicates that probably the defense pathway of \textit{chs3-2D} was not compromised, and these mutants can be pursued further.
Figure 3-1. Schematic of primary and secondary screen.

(A) Primary screen. Two to four seeds were manually spotted into each well of the screening plates. Row 1 (Col-0) and 12 (chs3-2D) are control rows containing no chemicals. Chemicals were added into rows 2 to 12 (containing chs3-2D seeds) using a handling robot. Each well contains different chemicals with the final chemical concentration of 15µM.

(B) Confirmation assay. Candidate chemicals were serial diluted into 6 different concentrations. Chemicals were added manually into each well. The same volume of DMSO was added as controls. Seeds were spotted into each well manually as indicated.
**Figure 3-2.** Effect of 353D7 on the morphology of *chs3-2D*.

(A) Morphology of Col-0 and *chs3-2D* grown on ½ strength MS media. Different concentrations of chemicals were added manually at day 1 and day 10. DMSO control was always tested alongside the experiment. The photograph shows 2-week-old plants.

(B) Fresh weights of plants shown in (B). The fresh weight of a single plant was calculated by taking the average of 3 plants as a group. Error bars represent mean ± SD (n=3 with 3 plants each).
A. Photographs of Arabidopsis thaliana plants under different conditions:

- Col-0
- chs3-2D

B. Graph showing relative luminescence units (RLU) over time:

- Red line: Mock
- Blue line: 100 μM 353D7

C. Bar graph comparing fresh weight per plant (mg):

- Col-0
- chs3-2D

Conditions:
- 1/2 MS
- 1/2 MS with 2mM EGTA
**Figure 3-3.** Analysis of different immune responses in 353D7- treated plants.

(A) Morphology and GUS-staining of *chs3-2D* (with the *BGL2::GUS* reporter gene) grown on ½ strength MS medium containing indicated chemicals. Photograph shows two-week-old plants grown at 16 °C. The experiment was performed twice with similar results.

(B) flg22-induced oxidative burst in Mock (H₂O) or chemical-treated Col-0 plants. Leaf slices were treated with H₂O or 100 μM 353D7 under light overnight. 1 μM flg22 was added before ROS was measured. Error bars represent mean ± SE (n=12, Student’s *T*-test, *P*<0.01). The experiment was performed at least three times with similar results.

(C) The effect of EGTA on the fresh weight of *chs3-2D*. Plants were grown at 16 °C for 3 weeks on ½ MS medium with or without 2 mM EGTA before the fresh weight was measured. Error bars represent mean ± SE (n=3 with 6 plants each). Stars indicate statistic difference between plants grown on different medium within each genotype (Student’s *T*-test, ***P*<0.001).
**Figure 3-4** Analysis of the effect of 353D7 on selected autoimmune mutants.

(A) Morphology of Col-0, chs3-2D, snc1, snc2 npr1-1 eds5-3, snc4 and bir1 pad4 grown on ½ strength MS medium ± 30 μM 353D7. The photograph shows 3-week-old plants.  
(B) Fresh weights of plants shown in (A). Error bars represent mean ± SE (n=3 with 3 plants each).  
(C) PR gene expression analysis. RNAs were prepared from 20-day-old plants grown at 16°C on ½ strength MS medium and reverse transcribed to obtain total cDNA. PRI, PR-2 and Actin1 in different mutant plants then were amplified by 28 cycles of PCR using equal amounts of total cDNA, and the PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.
Modified from Stephen F. Traynelis, 2009
Figure 3-5. Structure of glutamate receptor and ligand perception by NMDA receptor. (A) Modular structures of glutamate receptors. The glutamate receptors are composed of the extracellular N-terminal domain (ATD), the transmembrane domain (TMD), the extracellular ligand binding domain (LBD) and a C-terminal domain (CTD). The transmembrane domain includes TM1, TM3 and TM4 and a re-entrant P-loop (TM2). Two extracellular globular domains (S1 and S2) form the ligand-binding pocket. (B) Activation of NMDA by glutamate and glycine trigger ion fluxes.
**Figure 3-6.** Maximum likelyhood tree of *Arabidopsis* AtGLRs, human iGluRs, *Drosophila, C.elegans, Physcomitrella* and prokaryotic *Synechocystis* glutamate receptors.
Table 3-1. Various responses of *chs3-2D* to different chemicals.

<table>
<thead>
<tr>
<th>Response</th>
<th>Chemical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less sny, bigger</td>
<td>Dequalinium dichloride</td>
</tr>
<tr>
<td></td>
<td>Metanephrine HCl</td>
</tr>
<tr>
<td></td>
<td>Dapsone</td>
</tr>
<tr>
<td></td>
<td>Diphenhydramine HCl</td>
</tr>
<tr>
<td></td>
<td>Trimethoprim</td>
</tr>
<tr>
<td></td>
<td>Griseofulvin</td>
</tr>
<tr>
<td></td>
<td>Sulfamethoxypyridazine</td>
</tr>
<tr>
<td></td>
<td>Sulfadimethoxine</td>
</tr>
<tr>
<td></td>
<td>Nalbuphine HCl</td>
</tr>
<tr>
<td></td>
<td>Aristolochic acid</td>
</tr>
<tr>
<td></td>
<td>353D7</td>
</tr>
<tr>
<td></td>
<td>Florfenicol</td>
</tr>
<tr>
<td></td>
<td>Simvastatin</td>
</tr>
<tr>
<td></td>
<td>Aristolochic acid</td>
</tr>
<tr>
<td></td>
<td>Lasalocid Na salt</td>
</tr>
<tr>
<td></td>
<td>Nalidixic acid Na salt</td>
</tr>
<tr>
<td></td>
<td>Panaxadiol</td>
</tr>
<tr>
<td></td>
<td>Sedanolide</td>
</tr>
<tr>
<td></td>
<td>(-)-Bicuculline methbromide, 1(S), 9(R)</td>
</tr>
<tr>
<td></td>
<td>Hydroquinone</td>
</tr>
<tr>
<td></td>
<td>GW7647</td>
</tr>
<tr>
<td></td>
<td>Flumazenil</td>
</tr>
<tr>
<td></td>
<td>Formoterol</td>
</tr>
<tr>
<td></td>
<td>Hemicholinium-3</td>
</tr>
<tr>
<td></td>
<td>Oligomycin</td>
</tr>
<tr>
<td></td>
<td>2,4-Dinitrophenol</td>
</tr>
<tr>
<td>No germination</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td></td>
<td>Trifluridine</td>
</tr>
<tr>
<td></td>
<td>Gliotoxin</td>
</tr>
<tr>
<td></td>
<td>Silybine</td>
</tr>
<tr>
<td></td>
<td>Evodiamine</td>
</tr>
<tr>
<td></td>
<td>Senecionine</td>
</tr>
<tr>
<td></td>
<td>1-Aminocyclopropanecarboxylic acid hydrochloride</td>
</tr>
<tr>
<td></td>
<td>Acetarsol</td>
</tr>
<tr>
<td></td>
<td>Thimerosal</td>
</tr>
<tr>
<td></td>
<td>Phenylmercuric acetate</td>
</tr>
<tr>
<td></td>
<td>Ezetimibe</td>
</tr>
<tr>
<td></td>
<td>Deacetylgedunin</td>
</tr>
<tr>
<td></td>
<td>Pentachlorophenol</td>
</tr>
<tr>
<td></td>
<td>Elymoclavine</td>
</tr>
</tbody>
</table>
Table 3-1. Continued

<table>
<thead>
<tr>
<th>Response</th>
<th>Chemical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early bolting</td>
<td>Neomycin sulphate</td>
</tr>
<tr>
<td></td>
<td>Methylergometrine maleate</td>
</tr>
<tr>
<td></td>
<td>Maprotiline hydrochloride</td>
</tr>
<tr>
<td></td>
<td>Fusidic acid sodium salt</td>
</tr>
<tr>
<td></td>
<td>Lactobionic acid</td>
</tr>
<tr>
<td></td>
<td>Iohexol</td>
</tr>
<tr>
<td></td>
<td>Conessine</td>
</tr>
<tr>
<td></td>
<td>Vitexin</td>
</tr>
<tr>
<td></td>
<td>Nadolol</td>
</tr>
<tr>
<td></td>
<td>ARL 67156 trisodium salt</td>
</tr>
<tr>
<td></td>
<td>5-Bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td></td>
<td>3,4-Didesmethyl-5-Deshydroxy-3'-Ethoxyscleroin</td>
</tr>
<tr>
<td></td>
<td>Estriol methyl ether</td>
</tr>
<tr>
<td></td>
<td>Ginkgolic acid</td>
</tr>
<tr>
<td></td>
<td>Telithromycin</td>
</tr>
<tr>
<td></td>
<td>Carbadox</td>
</tr>
<tr>
<td></td>
<td>3-Chloro-8beta-Hydroxycarapin, 3,8-Hemiacetal</td>
</tr>
<tr>
<td></td>
<td>Brazilein</td>
</tr>
<tr>
<td></td>
<td>Leoidin</td>
</tr>
<tr>
<td></td>
<td>Asarylaldehyde</td>
</tr>
<tr>
<td>Dead</td>
<td>Lovastatin</td>
</tr>
<tr>
<td></td>
<td>Cinchonidine</td>
</tr>
<tr>
<td></td>
<td>Mevastatin</td>
</tr>
<tr>
<td></td>
<td>Camptothecin</td>
</tr>
<tr>
<td></td>
<td>2,4-dinitrophenol</td>
</tr>
<tr>
<td></td>
<td>Sulfacetamide</td>
</tr>
<tr>
<td></td>
<td>Cetylpyridinium chloride</td>
</tr>
<tr>
<td></td>
<td>Tioxolone</td>
</tr>
<tr>
<td></td>
<td>Ciprofloxacin</td>
</tr>
<tr>
<td></td>
<td>Floxuridine</td>
</tr>
<tr>
<td></td>
<td>Sulfadoxine</td>
</tr>
<tr>
<td></td>
<td>2-thiouracil</td>
</tr>
<tr>
<td></td>
<td>Sedanolide</td>
</tr>
<tr>
<td></td>
<td>Salinomycin</td>
</tr>
<tr>
<td></td>
<td>Oxiconazole nitrate</td>
</tr>
<tr>
<td></td>
<td>Moxifloxacin hydrochloride</td>
</tr>
<tr>
<td></td>
<td>Clarithromycin</td>
</tr>
<tr>
<td></td>
<td>Gatifloxacin</td>
</tr>
<tr>
<td></td>
<td>Abscisic acid</td>
</tr>
<tr>
<td></td>
<td>Metrifudil</td>
</tr>
<tr>
<td></td>
<td>Tunicamycin B</td>
</tr>
<tr>
<td></td>
<td>Acivicin</td>
</tr>
<tr>
<td>Response</td>
<td>Chemical</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Smaller</td>
<td>Lysergol</td>
</tr>
<tr>
<td></td>
<td>Carbinoxamine maleate salt</td>
</tr>
<tr>
<td></td>
<td>Roxarsone</td>
</tr>
<tr>
<td></td>
<td>Mesalamine</td>
</tr>
<tr>
<td>Grey, yellowing leaf</td>
<td>Levofloxacin</td>
</tr>
<tr>
<td></td>
<td>Tomatidine hydrochloride</td>
</tr>
<tr>
<td></td>
<td>Sulfquainoxaline sodium salt</td>
</tr>
<tr>
<td></td>
<td>Rosamicin</td>
</tr>
<tr>
<td></td>
<td>Hippeastrine hydrobromide</td>
</tr>
<tr>
<td></td>
<td>Beta-Escin</td>
</tr>
<tr>
<td></td>
<td>Gossypol</td>
</tr>
<tr>
<td></td>
<td>Karakoline</td>
</tr>
</tbody>
</table>
Table 3-2. Effect of other agonists and antagonists of animal glutamate receptors on the morphology of chs3-2D.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Role in animal iGLRs</th>
<th>Result (suppression↓, enhancement↑ or no effect -)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>agonist</td>
<td>-</td>
<td>Vincill et al., 2012</td>
</tr>
<tr>
<td>Glutamate</td>
<td>agonist</td>
<td>-</td>
<td>Vincill et al., 2012</td>
</tr>
<tr>
<td>Glutamate+glycine</td>
<td>agonist</td>
<td>-</td>
<td>Vincill et al., 2012</td>
</tr>
<tr>
<td>Serine</td>
<td>agonist</td>
<td>-</td>
<td>Vincill et al., 2012; Michard et al., 2011</td>
</tr>
<tr>
<td>Spermine</td>
<td>antagonist</td>
<td>-</td>
<td>Traynelis et al., 2010</td>
</tr>
<tr>
<td>MgCL₂</td>
<td>antagonist</td>
<td>-</td>
<td>Traynelis et al., 2010</td>
</tr>
<tr>
<td>LaCL₃</td>
<td>Calcium chelator</td>
<td>toxic</td>
<td>Traynelis et al., 2010</td>
</tr>
<tr>
<td>EGTA</td>
<td>Calcium chelator</td>
<td>↓↓</td>
<td>Traynelis et al., 2010</td>
</tr>
<tr>
<td>CNQX</td>
<td>antagonist</td>
<td>-</td>
<td>Imamachi et al., 1999</td>
</tr>
<tr>
<td>DNQX</td>
<td>antagonist</td>
<td>-</td>
<td>Paul et al., 2005</td>
</tr>
</tbody>
</table>
Chapter 4 **Future directions and conclusions**

Plant pathogens cause tremendous crop losses worldwide each year. Understanding the mechanisms of how plants defend themselves against pathogens attach is important agriculturally, economically and scientifically. Model organism *Arabidopsis thaliana* provides a powerful tool to study plant-pathogen interactions. Using a unique autoimmune mutant *chs3-2D*, I performed a forward chemical genetic screen in whole plant tissue to search for small molecules that can rescue the autoimmune morphology of *chs3-2D*. In this screen I identified 353D7, a putative NMDA glutamate receptor in animal systems that can completely suppress the autoimmune phenotypes of *chs3-2D*. How 353D7 suppresses the autoimmunity of *chs3-2D* remains unclear. Key to answering this question relies on the identification of the molecular target(s) of 353D7. Future work will focus on searching for the molecular target(s) of 353D7 and functional study of the potential target(s). I also developed methodology on using forward chemical genetic screen with whole seedlings to study plant immunity.
References


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


