GENETIC ANALYSIS OF RECEPTOR-LIKE PROTEIN SNC2-MEDIATED PLANT RESISTANCE IN *ARABIDOPSIS*

by

Yuli Ding

B.Sc., China Agricultural University, 2012

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate and Postdoctoral Studies

(Botany)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

March 2018

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

GENETIC ANALYSIS OF RECEPTOR-LIKE PROTEIN SNC2-MEDIATED PLANT RESISTANCE IN *ARABIDOPSIS*

submitted by Yuli Ding in partial fulfillment of the requirements for

the degree of Doctor of Philosophy

in Botany

Examining Committee:

Dr. Yuelin Zhang, Botany

Supervisor

Dr. James Kronstad, Plant Science

Supervisory Committee Member

Dr. George Haughn, Botany

University Examiner

Dr. Richard Hamelin, Forestry

University Examiner

Additional Supervisory Committee Members:

Dr. Carl Douglas, Botany

Supervisory Committee Member

Dr. Xin Li, Botany

Supervisory Committee Member

Abstract

Plant immunity is usually governed by two types of immune receptors: 1) pattern recognition receptors (PRRs) recognize the conserved molecular features of pathogens (pathogen-associated molecular patterns, PAMPs) and trigger PTI (PAMP-triggered immunity) and 2) nucleotide-binding and leucine-rich repeats-containing proteins (NLRs) serve as intracellular immune receptors to recognize the presence of relatively diverse pathogen effectors and trigger ETI (effector-triggered immunity). The *Arabidopsis thaliana* mutant *snc2-1D* (*suppressor of npr1-1, constitutive 2*) contains a gain-of-function mutation in a receptor-like protein (RLP) and displays a dwarf morphology. Here I report the characterization of *bda4-1D* (*bian da 4-1D*), which was identified as a complete suppressor of *snc2-1D* dwarf morphology. Positional cloning showed *bda4-1D* contains a gain-of-function mutation in *Non-Expressor of Pathogenesis-Related Proteins 4* (renamed *npr4-4D*). Functional analysis indicated NPR4, as well as its close homolog NPR3 (Non-Expressor of Pathogenesis-Related Proteins 3), function as transcriptional repressors. They function downstream of SNC2, independent of NPR1 (Non-Expressor of Pathogenesis-Related Proteins 1). In addition, salicylic acid (SA) was shown to inhibit the transcriptional activities of NPR3/4 and promote the expression of key immune regulators. The *npr4-4D* mutation leads to constitutive repression of SA-induced immune responses, indicating that the mutant protein can no longer respond to SA. On the other hand, the equivalent mutation in NPR1 also abolishes its ability to bind SA and renders reduced SA-induced defence gene expression. My results demonstrated that both NPR1 and NPR3/NPR4 are *bona fide* SA receptors, but play opposite roles in transcriptional regulation of SA-induced defence gene expression.
In the independent *eds5-3 snc2-1D npr1-1* suppressor screen, I report the identification and characterization of four more *bda* mutants, *bda3-1D*, *bda5-1*, *bda6* and *bda7*. Cloning of *BDA6* and *BDA7* showed that they encode FMO1 and ALD1 respectively, which are involved in biosynthesis of N-Hydroxypipecolic Acid (NHP) and pipecolic acid. My results indicate that enzymes involved in Lysine metabolism are also important for signaling in SNC2-mediated immune pathway.

Overall, the studies I completed in my Ph.D. thesis expand our knowledge in understanding of the signaling pathways downstream of SNC2 as well as the general regulatory mechanisms of SA receptors in plant innate immunity.

Lay summary

This work aims at providing knowledge of how to protect plants from serious diseases. Using *Arabidopsis thaliana* as a model system, the main goal of this work is to understand how immune gene regulators, especially one of the cell-surface receptors, work at the molecular level, how they are activated or repressed, and how the positive or negative effects consequently influence the amplitude of immune responses. Part of this work represents a major breakthrough in the understanding of the perception and molecular signaling of salicylic acid, one of the most important plant immune-related phytohormones. Together with others, findings from this work will largely contribute to a better understanding of plant immune system. In addition, molecular mechanisms revealed by this work can provide sustainable solutions to crop diseases by engineering plant resistance.

Preface

The chapters reported in this Ph.D. thesis describe the research results collected from September 2012 through January 2018. Below is a list of manuscripts (published or in revision) and the author contributions that comprise this thesis.

Chapter 2- Opposite roles of salicylic acid receptors NPR1 and NPR3/NPR4 in transcriptional regulation of plant immunity was modified from the manuscript: Ding, Y. *; Sun, T.*; Ao, K.; Peng, Y.; Zhang, Y.X.; Li, X.; Zhang Y. Opposite roles of salicylic acid receptors NPR1 and NPR3/NPR4 in transcriptional regulation of plant immunity. (* Co-first authors)

- The candidate performed most of the experiments under the supervision of Y.Z. and wrote the relevant results and methods sections. T. S. performed the following experiments and wrote the methods: [^{3}H]SA-binding assays; generation of *pSARD1::Luc*, *pSARD1*(mt)*::Luc*, and pUC19-35S-RLUC constructs; chromatin immunoprecipitation; generation of NPR3-related transgenic lines; yeast two-hybrid analysis (with the help of the candidate); RNA sample preparation for RNA-seq; qRT-PCR analysis on *MC2*, *NAC004*, *RLP23* and *WRKY51*; generation of *npr1-7 npr4-4D* and qRT-PCR analysis. K. A. analyzed the RNA-seq data. Y. P. performed the co-immunoprecipitaton in *N. benthamiana* with constructs generated by the candidate and T. S.. Y.X. Z. carried out the *snc2-1D npr1-1* suppressor screen and isolated the *bda4-1D snc2-1D npr1-1*. Y.Z. wrote the abstract, introduction and discussion parts of the manuscript. X.L. and Y.Z. revised the manuscript drafts.

Chapter 3- A forward genetic screen to identify novel components in the SNC2-mediated plant resistance pathway

- The candidate performed all the experiments under the supervision of Y. Z..

Table of contents

List of tables

List of figures

List of abbreviations

35S	a strong constitutive promoter from Cauliflower mosaic virus (CaMV)
ACD6	Accelerated Cell Death 6
ALD1	AGD2-Like Defence Response Protein 1
ANOVA	Analysis of Variance
Avr	Avirulence
AvrB	Avirulence protein B; an avirulence effector from *Pseudomonas syringae* pv. *glycinea*
AvrPita	Avirulence protein Pita; an avirulence effector from *Magnaporthe grisea*
AvrPto	Avirulence protein Pto; an avirulence protein from *Pseudomonas syringae*
AvrRpm1	Avirulence protein Rpm1; an avirulence protein isolated from *Pseudomonas syringae* pv. *maculicola* strain M2
AvrRpt2	Avirulence protein Rpt2; an avirulence protein isolated from *Pseudomonas syringae* pv. *tomato*
AzA	Azelaic Acid
BAK1	BRI1- Associated Receptor Kinase 1
BIK1	Botrytis-Induced Kinase 1
BDA	Bian Da; “becoming big” in Chinese
BSK1	BR-Signaling Kinase 1
BTB/POZ	Broad-Complex, Tramtrack, Bric-à-brac/Poxvirus, Zinc-finger
bZIP	basic leucine zipper
Cas9	CRISPR-associated 9
CBP60	Calmodulin Binding Protein 60
CC	Coiled-Coil
CERK1	Chitin Elicitor Receptor Kinase 1
CEBiP	Chitin Elicitor Binding Protein
CFU	Colony-Forming Unit
ChIP	Chromatin Immunoprecipitation
CHS	Chilling Sensitive
CLV2	Clavata 2
CME	Clathrin-Mediated Endocytosis
Col-0	Columbia-0, an *Arabidopsis* ecotype; it is also referred as wild type in

	this thesis work
CPSF	Cleavage and Polyadenylation Specificity Factor
CRCK3	Calmodulin-binding Receptor-like Cytoplasmic Kinase 3
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSPR	Receptor-like protein Required for CSP22 Responsiveness
C terminal	Carboxyl terminal
Cul	Cullin
DA	Dehydroabietinal
E. coli	*Escherichia coli*
EAR	Ethylene-responsive element binding factor-associated Amphiphilic Repression
ECD	Ectodomain
EDS	Enhanced Disease Susceptibility
EF-Tu	Elongation Factor Tu
EFR	EF-TU Receptor
EGF	Epidermal Growth Factor
elf18	a conserved N-terminal epitope of the bacterial elongation factor Tu
EMS	Ethyl Methane Sulfonate; a chemical mutagen
EMSA	Electrophoretic Mobility Shift Assay
ERF	Ethylene Response Factor
ETI	Effector-Triggered Immunity
ETS	Effector-Triggered Susceptibility
FLAG	An epitope protein tag composed of a single or repeated DYKDDDDK sequence
flg22	a conserved 22-amino acid epitope of the N terminus of the bacterial flagellin
FLS2	Flagellin Sensing 2
FMO1	Flavin-Dependent Monooxygenase 1
G3P	Glycerol-3-Phosphate
GD	Gal4 DNA-binding domain
GO	Gene Ontology
GST	Glutathione S-Transferase
GUS	β-glucuronidase
H.a.	*Hyaloperonospora arabidopsidis*
HA	Hemagglutinin; an epitope protein tag composed of a single or repeated YPYDVPDYA sequence

His	Histidine
ICS	Isochorismate Synthases
INA	2,6-dichloroisonicotinic acid
LD-VP16	LexA DNA-binding domain-VP16 activation domain
Ler	*Landsberg erecta*; an *Arabidopsis* ecotype
LRR	Leucine-Rich Repeat
Luc	firefly luciferase
LysM	Lysine motif
MAPK	Mitogen-Associated Protein Kinase
MBP	Maltose-Binding Protein
MeSA	Methyl salicylate
mRNA	messenger RNA
MS	Murashige and Skoog
NADPH	Nicotinamide adenine dinucleotide phosphate
NB-LRR	Nucleotide Binding-Leucine Rich Repeat
NDR1	Non-race-specific Disease Resistance 1
NHP	N-Hydroxypipecolic Acid
NIMIN1	NIM1-Interacting 1
NLP	Necrosis and ethylene-inducing peptide1-like protein
NLR	Nucleotide-binding/leucine-rich-repeat
N terminal	Amino Terminal
NPR	Non-Expressor of Pathogenesis-Related Proteins
OD	Optical Density
P2C	Δ1-piperideine-2-carboxylic acid
PAD4	Phytoalexin Deficient 4
PAL	Phenylalanine ammonia-lyase
PAMP	Pathogen-associated molecular pattern
PBL	PBS1-LIKE
PCRK	PTI Compromised Receptor-like Cytoplasmic Kinase
PEPR1	PEP receptor 1
Pip	Pipecolic acid
PR	Pathogenesis-related
PRR	Pattern recognition receptor
P.s.m.	*Pseudomonas syringae* pv. *maculicola*
P.s.t.	*Pseudomonas* syringae pv. *tomato*

PTI	PAMP-triggered immunity
pv	Pathovar
qRT-PCR	Quantitative Reverse Transcriptase PCR
R	Resistance
RbohD	Respiratory Burst Oxidase Homolog D
ReMax	Receptor of eMax
RIN4	RPM1-Interacting Protein 4
RLCK	Receptor-like cytoplasmic kinase
RLK	Receptor-like kinase
RLP	Receptor-like protein
RLUC	Renilla luciferase
ROS	Reactive oxygen species
RPM1	Resistance to *P. syringae* pv. *maculicola* 1
RPS2	Resistant to *P. syringae* 2
RPS4	Resistant to *P. syringae* 4
RRS1	Resistance to *R. solanacearum* 1
SA	Salicylic acid
SAG	SA O-β-glucoside
SAG101	Senescence Associated Gene 101
SAR	Systemic acquired resistance
SARD	Systemic acquired resistance deficient
SERK	Somatic embryogenesis receptor kinase
SNC	Suppressor of *npr1-1*, constitutive
SOBIR1	Suppressor of BIR1
SRFR1	Suppressor of *rps4-RLD* 1
SsE1	*Sclerotinia sclerotiorum* Elicitor-1
SUMM2	Suppressor of *mkk1 mkk2* 2
T-DNA	Transfer DNA
TIR	Toll/interleukin-1 Receptor
TMM	Too Many Mouths
TMV	Tobacco mosaic virus
TNV	Tobacco necrosis virus
TTSS	Type III secretion systems

Acknowledgements

Many people have contributed to this research project and I feel immensely fortunate and grateful for the scientific and personal support I have received throughout my degree.

First and foremost, I would like to thank my supervisor, Dr. Yuelin Zhang. His diligence to work has strongly motivated me to work through all the difficulties in science during my Ph.D.. His availability in the lab allowed me to discuss questions or to explore new puzzles efficiently with him whenever they arose. My projects would not have gone so smoothly without his patient supervision. As a bonus, a five-year training on lab management has also helped me build up some very useful skills and connections.

Thanks to my committee members, Dr. Xin Li, Dr. James Kronstad and Dr. Carl Douglas, for their advice and guidance during the course of my degree. Thanks to my graduate advisor, Dr. Ljerka Kunst, for her enthusiasm and for the many ways she has encouraged and supported me.

Thanks to all the past and current lab members. The Zhang lab has been a wonderful place to work, from China to Canada. Without exception, I am grateful to everyone I have met. I would like to give special thanks to Dr. Yaxi Zhang, for all the previous work she has done for my project and her mentorship in genetic screening. Thanks to Tongjun Sun, Yujun Peng, Kevin Ao and Weijie Huang for their contributions to my projects. I really appreciate it. Lastly, I would also like to thank all the Li Lab members who provided me with lots of support and help that allowed me to finish my degree successfully.

I feel extremely lucky to be involved in the wonderful PRoTECT (Plant Responses To Eliminate Critical Threats) program with Georg-August-University in Goettingen, Germany. Many thanks to Prof. Dr. Ivo Feussner and Mr. Dmitrij Rekhter. The SARD4 project would not be accomplished so nicely without this fantastic collaboration. Thanks to all the staff, faculty members and students in the PRoTECT program, as well as all the Feussner lab members, for being such terrific hosts. I am pleased that we had the opportunity to work together and discuss science.

Thanks to Biol112 teaching team, especially to Ms. Karen Smith, for showing me what great leadership is like and being a role model for teaching. I am very happy to see my growth in my teaching abilities and I am really thankful for the freedom and supportive environment this course provided.

I am grateful to the Chinese Scholarship Council (CSC) for the major financial support during my Ph.D. as well as other resources including TAship, Dewar Cooper Doctoral Scholarship and Natural Sciences and Engineering Research Council of Canada (NSERC).

Thank you to my parents, for their understanding and support throughout my degree. I would not have thought about studying aboard without their initial encouragements.

Thanks to all my friends, especially the new ones I met in Vancouver, for your kindness and for great times. Your friendship made my life so colourful in the past five years.

Last but not least, I would like to thank Dr. Pingtao Ding, not only for his mentorship during my Ph.D., but mostly for his love and huge mental support during some really difficult periods. Thanks for having faith in me all the time.

1 Introduction

1.1 Plant disease and plant defense systems

Plant diseases contribute greatly to annual crop losses and pose a real threat to food security worldwide. One of the most often cited examples is the Great Irish Famine in the 19th century as the result of potato late blight epidemic caused by *Phytophthora infestans*. This disease not only caused the deaths of over one million people, but it also led to a mass emigration out of Ireland into North America. The Irish potato famine is of specific importance because disputes about the cause of the rotted potatoes over decades finally gave birth to the science of plant pathology (Holub 2001; Judelson and Blanco 2005). Nevertheless, many food-and cash-crops, such as wheat, rice, maize, soybean, barley, potato, cotton, canola, and others are still under threat of many different types of diseases.

Even though plants are host to every type of microbial pathogen (including fungi, oomycetes, bacteria, and viruses), plants have evolved complicated immune systems to combat pathogen infections. Physical barriers on the plant surface, such as epidermal hairs, wax layers and the cell wall, can prevent the initial establishment of pathogens (Thordal-Christensen 2003). Additionally, anti-microbial enzymes and other specialized metabolites present in the apoplast compose a chemical barrier to limit pathogen invasion (Heath, 2000). However, adapted pathogens can bypass those barriers to colonize host plants. When these pathogens are recognized by plant immune receptors, a two-branched innate immune system is activated (Jones and Dangl, 2006).

1.2 Recognition and response at the plant cell surface

1.2.1 Microbial patterns and plant pattern recognition receptors

The first active line of plant defence is governed by the recognition of evolutionarily conserved pathogen–associated molecular patterns (PAMPs), such as

fungal chitin or flagellin from bacteria. PAMPs are usually essential for microbial lifecycles, making them ideal targets for detection by immune receptors. PAMPs are recognized by pattern recognition receptors (PRRs) and trigger profound physiological changes in plant cells resulting in PAMP-triggered immunity (PTI) (Boller and Felix, 2009).

Plant PRRs are typically trans-membrane receptor-like kinases (RLKs) or receptor-like proteins (RLPs) (Boller and Felix, 2009). Both RLKs and RLPs comprise an extracellular ectodomain (ECD) and a transmembrane domain, but RLPs lack a C-terminal intracellular kinase domain. According to domains or motifs in the ECDs, PRRs can be classified into different subfamilies: leucine-rich repeat (LRR) domain, lysine motifs (LysM), lectin domain, or epidermal growth factor (EGF)-like domain (Dangl and Jones 2001; Couto and Zipfel 2016; Tang et al. 2017). All known LRR-containing PRRs bind proteins or peptides. For example, the *Arabidopsis* bacterial flagellin receptor, LRR-RLK FLAGELLIN SENSING2 (FLS2) binds a conserved 22-amino acid epitope (flg22) of the N terminus of the bacterial flagellin (Chinchilla et al. 2006). EF-TU RECEPTOR (EFR) recognizes a conserved N-terminal epitope (elf18) of the bacterial elongation factor Tu (EF-Tu) (Zipfel et al. 2006).

Several LRR-RLPs have been shown to recognize proteinaceous patterns. *Arabidopsis* RLP23 specifically binds and recognizes nlp20, a conserved 20-amino-acid fragment from necrosis and ethylene-inducing peptide1-like proteins (NLPs), which are widely produced by multiple prokaryotic (bacterial) and eukaryotic (fungal, oomycete) species (Albert et al., 2015). In tobacco (*Nicotiana benthamiana*), the LRR-RLP RECEPTOR-LIKE PROTEIN REQUIRED FOR CSP22 RESPONSIVENESS (CSPR) confers resistance to the epitope csp22 derived from bacterial cold shock protein (Saur et al., 2016).

1.2.2 PRR activation complex

Ligand-induced dynamic rearrangement of PRR complexes with co-receptors and other regulatory proteins ensures prompt signaling activation and attenuation. Upon ligand binding, PRRs of the LRR-RLK class recruit BRI1- ASSOCIATED RECEPTOR

KINASE (BAK1), a member of SOMATIC EMBRYOGENESIS RECEPTOR KINASES (SERKs) family (Couto and Zipfel 2016). For example, FLS2 and BAK1 form heterodimers in the presence of flg22, which results in rapid phosphorylation of both FLS2 and BAK1 and activation of downstream signaling events (Chinchilla et al. 2007; Schulze et al. 2010). Molecular and genetic studies showed that SERKs are also required for signaling mediated by EFR and XA21 receptor in rice, which recognizes conserved protein in many *Xanthomonas* species (Schulze et al. 2010; Chen et al. 2014; Song et al. 1995).

As LRR-RLPs do not carry a cytoplasmic kinase domain. They associate with RLKs to transmit the signal to downstream components. In rice, chitin binding to LysM-RLP CEBiP recruits CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) to form a heterocomplex for signaling (Shimizu et al. 2010). The LRR-RLK SUPPRESOR of BIR1 (SOBIR1) has been shown to function as a common adaptor for a number of LRR-RLP-type PRRs (Gust and Felix 2014). SOBIR1 constitutively associates with tomato Ve1 and Cf4 as well as *Arabidopsis* RLP23 in a ligand-independent manner (Liebrand et al. 2013; Albert et al. 2015). In addition, SOBIR1 also associates with RLP30, which is involved in the perception of elicitor SCLEROTINIA SCLEROTIORUM ELICITOR-1 (SsE1) from *Sclerotinia sclerotiorum* (Zhang et al. 2013). Therefore, LRR-RLPs form a complex with adaptor RLK before ligand binding and then recruit SERK family members to form an active receptor complex upon ligand binding.

A number of receptor-like cytoplasmic kinases (RLCKs) have emerged as essential components linking PRRs to downstream defence. The best studied BOTRYTIS-INDUCED KINASE1 (BIK1) associates with FLS2 and BAK1 in the absence of ligand. Upon flg22 elicitation, BIK1 is phosphorylated and then dissociates from the PRR complex to activate downstream signaling (Lu et al. 2010; J. Zhang et al. 2010). Additional *Arabidopsis* RLCKs, including PBS1-LIKE1 (PBL1), PBS1-LIKE27 (PBL27), PCRKs, and BR-signaling kinase 1 (BSK1), have also been shown to play important roles in pattern-triggered immunity by directly interacting with PRRs (J. Zhang et al. 2010; Shinya et al. 2014; Kong et al. 2016; Shi et al. 2013).

1.2.3 PRR downstream signaling

Upon PAMP recognition, a series of cellular events are triggered in minutes, including production of reactive oxygen species (ROS), activation of mitogen-associated protein kinases (MAPK) cascade, increase calcium influx and anion effluxes as well as extracellular alkalization (Boller and Felix 2009). Extracellular ROS is proposed to act as a cross-linker of plant cell wall components as well as a secondary messenger to trigger downstream immune responses (Lamb and Dixon 1997). In *Arabidopsis*, the plasma-membrane localized NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOG D (RbohD) is essential for pattern-triggered ROS production (Torres et al. 2005). RbohD is constitutively associated with the PRR complex at the plasma membrane. Within this complex, the plasma-membrane-associated BIK1 directly binds and rapidly phosphorylates RbohD upon PAMP perception (Kadota et al. 2014; Li et al. 2014).

MAPK cascades are conserved modules in all eukaryotes. They are composed of three sequentially activated kinases, a MAPK kinase kinase (MAPKKK or MEKK), a MAPK kinase (MAPKK or MKK) and a MAPK (MPK). Two canonical MAPK cascades have been shown to play crucial roles downstream of PTI in regulating defence gene expression and phytoalexin biosynthesis (Meng and Zhang 2013). One is known to positively regulate plant defence, with an unknown MAPKKK, MKK4/MKK5 (two redundant MAPKKs) and MPK3/MPK6 (two partially redundant MAPKs). Upon activation, MPK3 and MPK6 further induce massive transcriptional programming via phosphorylation of different transcription factors, such as WRKY33 and ETHYLENE RESPONSE FACTOR6 (ERF6) (Asai et al. 2002). Both have been identified as direct substrates of MPK3 and MPK6 to promote biosynthesis of camalexin and indole glucosinolates respectively (Ren et al. 2008; Mao et al. 2011; Xu et al. 2016).

The other cascade, MEKK1-MKK1/MKK2-MPK4 was originally considered to negatively regulate plant immune responses as loss of function mutants of MEKK1, MKK1/MKK2, and MPK4 all exhibit constitutive defence responses (Petersen et al. 2000; Gao et al. 2008). Analysis of *mkk1 mkk2* suppressor mutants revealed the autoimmune phenotypes in the mutants of this cascade are actually caused by activation of defence responses mediated by the intracellular nucleotide-binding/leucine-rich-repeat (NLR)

protein SUMM2 (SUPPRESSOR OF *mkk1 mkk2* 2)(Zhang et al. 2012). Further studies showed that the MEKK1-MKK1/MKK2-MPK4 cascade promotes basal resistance against pathogens and is guarded by SUMM2, which monitors the phosphorylation status of MPK4 substrate CRCK3 (Calmodulin-binding receptor-like cytoplasmic kinase 3) (Zhang et al. 2017).

1.3 Pathogen effectors perturbing plant immunity

Adapted pathogens usually deliver a suite of effectors into the plants, which promotes pathogen virulence and results in effector-triggered susceptibility (ETS) in host plants (Jones and Dangl 2006). Plant pathogenic bacteria deliver effectors into host cells using type III secretion systems (TTSS). Some fungal and oomycete effectors have also been detected intracellularly.

A large number of effectors in plant pathogens have been cloned. Many of them contribute to virulence by targeting different components of the PTI pathways to suppress plant defence response. For example, *Pseudomonas syringae* effector AvrPto directly targets and inhibits the kinase activities of PRRs, such as FLS2 and EFR, thus blocking PAMP-induced immunity in *Arabidopsis* (Xiang et al. 2008). In addition, MAPK cascades are directly targeted by pathogenic effectors. *Pseudomonas* HopAI1 effector protein inactivates MPK3 and MPK6 to promote virulence (Zhang et al. 2007) and MPK4 was shown to be an additional virulence target of HopAI1 (Zhang et al. 2012). Together, these examples demonstrate that by secreting effectors, pathogens have employed various mechanisms to evade host perception and suppress host defence responses.

1.4 Effector-triggered immunity (ETI)

1.4.1 Nucleotide-binding/leucine-rich-repeat (NLR) proteins

Pathogen effectors are recognized by specific disease resistance (*R*) genes. Most *R* genes were found to encode NLR proteins. Genome-wide analysis revealed that

there are around 150 NLR coding genes in *Arabidopsis*, which mainly fall into two distinct groups: TIR-NB-LRR (TNL) group with an N-terminal Toll and interleukin-1 (TIR)-like domain, and CC-NB-LRR (CNL) group with an N-terminal coiled-coil domain (Meyers et al. 2003).

TNLs, such as SNC1 (Suppressor of *npr1-1*, constitutive 1) and RPS4 (RESISTANT TO *P. syringae* 4)/RRS1 (Resistance to *R. solanacearum* 1), require the lipase-like family proteins EDS1 (Enhanced Disease Susceptibility 1) / PAD4 (Phytoalexin Deficient 4) and SAG101 (Senescence Associated Gene 101) complex for signaling (Aarts et al. 1998; Feys et al. 2005). Several TNLs appear to act in the nucleus, but some well-characterized CNLs, such as RPM1 (RESISTANCE TO *P. syringae* pv. *maculicola* 1) and RPS2 (RESISTANT TO *P. syringae* 2), are associated with the cell membrane and require NDR1 (Non-race-specific Disease Resistance 1) for their functions (Aarts et al. 1998).

1.4.2 Recognition of pathogen effectors by NLRs

Harold Flor's studies on the genetic relationships between races of flax rust fungus and a number of flax varieties in 1940s raised the gene for gene hypothesis: the resistant variant of the plant has a gene for resistance in correspondence to the avirulence (Avr) gene of pathogens (Flor 1971). This classic gene-for-gene model was supported by various studies showing that plant NLRs directly interact with the products of *Avr* genes. For example, rice NLR protein Pita detects effector AvrPita from rice blast fungus, *Magnaporthe grisea* by direct protein-protein interaction (Jia et al. 2000). However, a number of cases indicated the perception of pathogen effectors by NLRs is mostly indirect as physical interactions cannot be detected between various R-Avr combinations.

In 1998, Eric Van der Biezen and Jonathan Jones proposed the guard model. It predicts that NLRs "guard" (ie monitor the integrity of) the virulence target (guardee) of the effector to activate defence after detection of effector-induced modifications (Van der Biezen and Jones 1998; Dangl and Jones 2001). A well-established example of such a pathogen-modified protein in plants is RIN4 (RPM1-INTERACTING PROTEIN 4).

RIN4 is localized to the plasma membrane, and is monitored by the likewise localized CNLs, RPM1 and RPS2. *P. syringae* effectors AvrB and AvrRpm1, target RIN4 and lead to its phosphorylation which triggers the activation of RPM1 (Chung et al. 2011; Liu et al. 2011). Another *P. syringe* effector AvrRpt2 cleaves RIN4, activating RPS2-mediated immunity (Axtell and Staskawicz 2003; Chung et al. 2011; Mackey et al. 2003).

In an elaboration of the guard model, the newly proposed decoy model implies that the plants could evolve guarded decoys that had lost their original functionality and now only functioned as "effector baits" (van der Hoorn and Kamoun 2008). As an example, the *Xanthomonas campestris* pv *vesicatoria* effector AvrBs3 functions as a transcription factor and binds to the promoter of the resistance gene *Bs3* (*pBs3*) in resistant pepper plants. *Bs3* encodes a flavin monooxygenase but the expression of *Bs3* has not been detected in the absence of AvrBs3 (Römer et al. 2007). These data suggested that effector target, such as *pBs3*, is a decoy which only functions in the detection of the effector by the NLRs and itself has no critical role during the development of disease or resistance (Zhou and Chai 2008; van der Hoorn and Kamoun 2008). Altogether, the guard and decoy models describe efficient mechanisms by which a plant can use a limited repertoire of NLRs to recognise a multitude of pathogens via specifically guarding a limited number of host proteins.

1.5 Systemic acquired resistance (SAR)

After the defence response is activated locally, a secondary immune response is activated in distal tissue of plants, named systemic acquired resistance. The history of SAR can be retraced back to early 20th century. In 1901, Beauverie and Ray independently realized that plants previously infected by a pathogen could better resist further infection (Beaunerie 1901; Ray 1901). In 1933, Chester reviewed over 200 published studies and raised the theory of physiological acquired immunity (Chester 1933). In the 1960s, Ross showed that tobacco plants challenged with tobacco mosaic virus (TMV) developed increased resistance to secondary infection in distal tissues. Moreover, the infected tobacco plants also showed resistance against tobacco necrosis virus (TNV) and some other bacterial pathogens (Ross 1961). This spread of resistance

throughout the plant's tissues was later termed systemic acquired resistance. The resistance conferred is long-lasting and effective against a broad-spectrum of pathogens including viruses, bacteria, fungi, and oomycetes (Ryals et al. 1996; Sticher et al. 1997).

Associated with SAR is the expression of a set of genes called SAR genes. Most of the SAR genes encode proteins whose presence or activity is tightly correlated with maintenance of the resistance state. Analysis of SAR proteins showed that many belong to the class of pathogenesis-related (PR) proteins (Van Loon and Van Strien 1999). PR proteins were originally identified as novel proteins accumulating after TMV infection of tobacco leaves (Van Loon and Van Kammen 1970). Although many PR proteins have antimicrobial properties *in vitro* (Van Loon and Van Strien 1999), the role of each PR protein in establishing SAR has not been clearly defined. Nevertheless, *PR* genes still serve as useful molecular markers for the onset of SAR. In *Arabidopsis*, the widely-used marker genes are *PR1*, *PR2*, and *PR5* (Uknes et al. 1992).

1.5.1 SAR signal molecules

For SAR to be activated in the systemic tissue, a signal must be generated in the inoculated tissue and transported systemically via the vascular system, generally the phloem (Vlot et al. 2008; Shah 2009). Early grafting experiments have supported this idea, showing that a primary infected leaf of a plant can produce a systemic signal that is graft transmissible from the rootstock to scion (Dean and Kuć 1986; Jenns and Kuc 1979). While this signal is not species specific, the nature of the mobile signal has been a subject of controversy for many years.

1.5.1.1 Salicylic acid (SA)

SA was proposed as the first candidate of mobile signal for SAR as significant amounts of SA was detected in the phloem and systemic leaves (Métraux et al. 1990; Yalpani et al. 1991). Compelling evidence supporting this idea also comes from the labeling studies in TMV-infected tobacco, which showed that 69% of the SA

accumulated systemically was made and exported from the inoculated leaf (Shulaev et al. 1995; Molders et al. 1996).

However, there is clear evidence arguing against SA being the mobile signal. The strongest evidence comes from the grafting experiment in tobacco between wild-type scions and *nahG*-expressing rootstocks. The bacterial gene *nahG*, encoding salicylate hydroxylase, removes SA by conversion to catechol (Friedrich et al. 1995). Although the *nahG*-expressing rootstock is not able to accumulate SA, the chimeric plants containing a wild type scion grafted onto this SA-deficient rootstock was still able to develop SAR (Vernooij et al. 1994). This result suggests that either SA is not the long-distance signal or very small amount of SA in infected leaves are sufficient for full SAR induction.

1.5.1.2 Other putative long-distance signals

Continued efforts to identify the phloem-mobile SAR signal have implicated more candidates, including a methylated derivative of SA (MeSA), a glycerol-3-phosphate (G3P)-dependent signal, a lipid-based signal molecule, the dicarboxylic acid, azelaic acid (AzA), the abietane diterpenoid, dehydroabietinal (DA), and the amino acid-derivative pipecolic acid (Pip) (Park et al. 2007; Chanda et al. 2011; Maldonado et al. 2002; Jung et al. 2009; Chaturvedi et al. 2012; Návarová et al. 2012). Some of these signals work cooperatively to activate SAR and/or regulate MeSA metabolism (Dempsey and Klessig 2012). However, Pip, a product of lysine derivative, appears to activate SAR via an independent pathway in the systemic tissue (Bernsdorff et al. 2016) .

Pip accumulates in local and systemic leaves after pathogen infection in *Arabidopsis*. AGD2-Like Defence Response Protein 1 (ALD1), which is required for SAR, was shown to be also required for pathogen-induced Pip accumulation. ALD1 functions as an aminotransferase, which converts lysine to the precursor of Pip, Δ1-piperideine-2-carboxylic acid (P2C). P2C is further reduced by the reductase SARD4 (SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 4) to produce Pip (Ding et al. 2016; Hartmann et al. 2017).

Arabidopsis FMO1 (Flavin-Dependent Monooxygenase 1) is also required for SAR (Koch et al. 2006; Mishina and Zeier 2006). Overexpression of *FMO1* results in constitutive defence responses, which requires both ALD1 and SARD4 (Koch et al. 2006; Ding et al. 2016). Interestingly, the pathogen-induced level of is increased in the *fmo1* mutant (Návarová et al. 2012; Ding et al. 2016), suggesting it may be involved in the synthesis of a defence signal molecule derived from Pip. A very recent study showed that FMO1 functions as a pipecolate N-hydroxylase, catalyzing the biochemical conversion of Pip to N-Hydroxypipecolic Acid (NHP) (Hartmann et al. 2018)

1.5.2 The role of SA in SAR

Despite that fact that it is unlikely that the mobile signal for SAR is SA, SA plays key roles in both local defence and SAR signaling. Exogenous SA can induce SAR and SAR gene expression (White 1979; Ward et al. 1991; Uknes et al. 1992) while mutants with defects in SA accumulation are compromised in SAR, indicating that SA accumulation is required for SAR induction (Wildermuth et al. 2001; Cao et al. 1994).

1.5.2.1 SA synthesis

SA in plants can be generated via two distinct pathways, the isochorismate (IC) and the phenylalanine ammonia-lyase (PAL) pathways. Both pathways require the primary metabolite chorismate, the end product of the shikimate pathway, to produce SA (Dempsey et al. 2011). Chorismate-derived L-phenylalanine can be converted into SA via either benzoate intermediates or coumaric acid via a series of enzymatic reactions initially catalyzed by PAL enzymes. Chorismate can also be converted into SA via isochorismate catalyzed by isochorismate synthases (ICS) (Lee et al. 1995; Wildermuth et al. 2001; Strawn et al. 2007; Garcion et al. 2008). Homologs of *ICS* and *PAL* genes are present throughout the plant kingdom, including *Arabidopsis*, tobacco, tomato, poplar, sunflower, and pepper (Wildermuth et al. 2001; Cochrane et al. 2004; Uppalapati et al. 2007; Catinot et al. 2008; Yuan et al. 2009; Sadeghi et al. 2013; Kim and Hwang 2014), suggesting that these two SA biosynthesis pathways are

evolutionary conserved. *Arabidopsis* quadruple PAL mutants, in which PAL activity is reduced to 10%, show lower SA accumulation (50%) compared to the wild type upon pathogen infection (Huang et al. 2010). On the other hand, *Arabidopsis* encodes two ICS enzymes. Mutations in *ICS1* lead to an approximately 90% loss of SA accumulation induced by pathogens or UV light (Wildermuth et al. 2001). The appearance of residual SA in an *ics1 ics2* double mutant confirms that the ICS pathway is not the only source of SA in *Arabidopsis* (Garcion et al. 2008). Therefore, the ICS pathway is the major route for SA biosynthesis during plant immunity although contribution of the PAL pathway is still evident.

In chloroplasts, ICS catalyzes the conversion of chorismate into isochorismate, which is further converted to SA (Wildermuth et al. 2001; Strawn et al. 2007; Garcion et al. 2008; Dempsey et al. 2011). SA export from chloroplasts is likely to be mediated by the MATE-transporter EDS5 (ENHANCED DISEASE SUSCEPTIBILITY 5) (Serrano et al. 2013). Since SA accumulation is compromised in *eds5* mutants, this export seems to be important for SA accumulation and distribution in the cell (Nawrath et al. 2002; Ishihara et al. 2008).

Most of the SA produced in planta is converted into SA *O*-*β*-glucoside (SAG) by a pathogen-inducible SA glucosyltransferase (Lee and Raskin 1998; Lee and Raskin 1999; Song 2006). SAG is actively transported from the cytosol into the vacuole (Dean and Mills 2004; Dean et al. 2005), where it may function as an inactive storage form that can be converted back to SA.

1.5.2.2 Regulation of SA biosynthesis

Salicylic acid biosynthesis is tightly regulated since constitutive SA accumulation has a detrimental effect on plant fitness. The CaM-binding transcription factor CBP60g (CALMODULIN BINDING PROTEIN 60g) and its homolog SARD1 (SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1) were found to promote pathogen-induced SA synthesis by regulating *ICS1* transcript (Wang et al. 2009; Y. Zhang, Xu, et al. 2010; Wang et al. 2011; Wan et al. 2012). CaM-binding is required for CBP60g function, whereas SARD1 does not appear to be a CaM-binding protein (Wang et al. 2009).

Despite this difference, CBP60g and SARD1 are partially redundant in regulating *ICS1* expression and SA accumulation during immunity (Y. Zhang, Xu, et al. 2010; Wang et al. 2011). Another close homolog of CBP60g, CBP60a, negatively regulates *ICS1* expression upon CaM-binding (Truman et al. 2013). Therefore, regulation of SA synthesis involves multiple level of control. In the absence of pathogen, CBP60a is repressing immunity while CBP60g and SARD1 have low activity. Upon pathogen infection, CBP60g and SARD1 bind to the *ICS1* promoter and activate its expression and release the negative regulation by CBP60a.

1.5.2.3 SA-mediated signaling

Signaling downstream of SA is largely regulated via NON-EXPRESSOR OF PATHGENESIS-RELATED PROTEINS 1 (NPR1). Mutations in *NPR1* lead to an almost complete loss of SA-induced *PR* gene expression and enhanced susceptibility to biotrophic pathogens (Cao et al. 1994; Shah et al. 1997; Volko et al. 1998; Dong 2004). NPR1 contains a BTB/POZ (*Broad-Complex*, *Tramtrack*, *Bric-à-brac*/Poxvirus, Zinc-finger) domain, an ankyrin-repeat domain and a nuclear localization signal (Cao et al. 1997; Ryals et al. 1997). Functional studies have shown that accumulation of NPR1 in the nucleus after treatment with SAR inducers is essential for *PR* gene induction (Mou et al. 2003).

Yeast two-hybrid screens have revealed direct interactions between NPR1 and several members of the TGA family of basic leucine zipper (bZIP) transcription factors. In *Arabidopsis*, NPR1 interacts specificity for TGA2, TGA3, TGA5, and TGA6 (Zhang et al. 1999; Zhou et al. 2000; Kim and Delaney 2002). Reverse genetic analysis revealed that the *tga2 tga5 tga6* triple mutant has phenotypes similar to *npr1*, showing compromised SAR and decreased tolerance to high concentrations of SA (Zhang, Tessaro, et al. 2003). All three genes must be inactivated to observe the phenotype, indicating that TGA2, TGA5, and TGA6 play essential and redundant roles in the induction of SAR. As transcription factors, TGA proteins bind to the consensus DNA sequence TGACG, which is found in promoters of genes activated during defence, such as *Arabidopsis PR1* (Katagiri et al. 1989; Lebel et al. 1998). Electrophoretic mobility

shift assays (EMSA) confirmed that TGA2 binds to the promoter of *PR1* (Zhang et al. 1999; Després et al. 2000). Furthermore, binding of TGA2 was enhanced by the addition of NPR1, suggesting that NPR1 functions as a transcriptional activator (Després et al. 2000).

1.6 Suppressors of *npr1*

To identify other components of SAR signaling, several genetic screens in *Arabidopsis* have been conducted to look for suppressors of *npr1*. One screen used a transgenic line expressing the *GUS* (β-glucuronidase) reporter gene driven by the promoter of *PR2* in the null allele of *NPR1*, *npr1-1* (Li et al. 2001). Unlike *npr1-1*, the suppressor mutants showed constitutive or SA-inducible GUS activity. Interestingly, a number of autoimmune mutants were isolated from the screen. They generally exhibit phenotypes including dwarfism, elevated SA levels, constitutive expression of defence genes and enhanced disease resistance to pathogens, and in some cases with spontaneous lesion formation (van Wersch et al. 2016).

To date, four *snc* (suppressor of *npr1-1*, constitutive) mutations have been cloned and further characterized. *snc1* contains one single amino acid change in a TNL, which leads to over-accumulation of the SNC1 protein and activation of defence responses (Zhang, Goritschnig, et al. 2003; Cheng et al. 2011). Similarly, *snc6-1D* contains a gain-of-function mutation in an atypical TNL, CHILLING SENSITIVE 3 (CHS3), with an extra LIM domain on its C terminus (Bi et al. 2011). *snc2-1D* contains a gain-of-function mutation in a LRR-RLP (Y. Zhang, Yang, et al. 2010). Besides the gain-of-function mutations in plant immune receptors, SNC5/SRFR1 (SUPPRESSOR OF RPS4-RLD 1) was identified as a negative regulator involved in regulating SNC1 protein levels (Li et al. 2010). Overall, the studies of *snc* mutants provided new knowledge input in plant immunity. More importantly, the distinct morphological phenotypes caused by autoimmunity serves as a nice tool for genetic analysis or screens.

1.7 SNC2-mediated immune pathway

SNC2 encodes a LRR-RLP with an extracellular LRR domain, a transmembrane domain and a short cytoplasmic tail with only four amino acids. The *snc2-1D* mutation (G412R) in the conserved GXXXG motif of the trans-membrane domain leads to a constitutively activated defence response. Loss of function of SNC2 results in enhanced susceptibility to virulent bacteria strain *Pseudomonas syringae* pv *tomato* (*P.s.t.*) DC3000 and the type III secretion deficient bacteria strain *P.s.t.* DC3000 *hrcC*$^{-}$, indicating that SNC2 plays an important role in basal resistance and PTI (Y. Zhang, Yang, et al. 2010).

To dissect signal transduction pathways downstream of SNC2, a suppressor screen was performed in the *snc2-1D npr1-1* background. *BDA1* (for *Bian Da*; "becoming big" in Chinese) encodes a novel protein with N-terminal ankyrin-repeat and domain and C-terminal trans-membrane domains. Loss-of-function mutations in *BDA1* suppress the dwarf morphology and constitutive defence responses in *snc2-1D npr1-1* and result in enhanced susceptibility to pathogens. By contrast, a gain-of-function allele of *BDA1, bda1-17D,* constitutively activates cell death and defence responses, suggesting that BDA1 is a critical regulator of plant immunity. However, the biochemical function of BDA1 as well as the mechanism of how BDA1 regulates plant defence response is still largely unknown (Y. Zhang, Yang, et al. 2010; Yang et al. 2012).

BDA2 encodes the transcription factor WRKY70. WRKY70 was shown to play complex roles in modulating defence responses and senescence (Li et al. 2004; Knoth et al. 2007; Besseau et al. 2012). Interestingly, free SA levels in *wrky70 snc2-1D npr1-1* are comparable to those in *snc2-1D npr1-1*, suggesting that WRKY70 functions in an SA-independent pathway downstream of SNC2. Additionally, the partial suppression of the autoimmune phenotype of *snc2-1D npr1-1* by *eds5-3* mutation also supports the presence of SA-independent pathway downstream of SNC2 (Y. Zhang, Yang, et al. 2010).

1.8 Thesis objectives

As newly discovered PRRs, the signaling pathways mediated by LRR-RLPs are still largely unknown compared with LRR-RLKs. The autoimmune RLP mutant in *Arabidopsis*, *snc2-1D*, provides a nice platform to conduct genetic analysis. The reported studies of *bda* mutants showed the characterization of these mutants are of great use in dissecting signaling pathways downstream of SNC2.

The primary aim of this research is to further dissect signaling pathways downstream of SNC2. The specific objectives of my research were: (1) to screen for novel suppressors of *snc2-1D* to identify signaling components involved in the SNC2-mediated resistance pathway and (2) to characterize the isolated suppressor mutants, identify mutated genes and decipher the mechanism of how these proteins regulate plant defence responses downstream of SNC2.

In chapter 2, I describe the characterization of *bda4-1D snc2-1D npr1-1*. Positional cloning showed that *bda4-1D* contains a gain-of-function mutation in *NPR4* (renamed *npr4-4D*). Functional analysis indicated that NPR4, as well as its close homolog NPR3, function as transcriptional repressors. They function downstream of SNC2, independent of NPR1. In addition, SA was shown to inhibit the transcriptional activities of NPR3/4 and promote the expression of key immune regulators. The *npr4-4D* mutation leads to constitutively repression of SA-induced immune responses, indicating that the mutant protein can no longer respond to SA. On the other hand, the equivalent mutation in NPR1 also abolishes its ability to bind SA and renders reduced SA-induced defence gene expression. My results demonstrated that both NPR1 and NPR3/NPR4 are *bona fide* SA receptors, but play opposite roles in transcriptional regulation of SA-induced defence gene expression.

In chapter 3, I describe another suppressor screen of *snc2-1D* in the *eds5-3 snc2-1D npr1-1* background. I isolated 66 mutant lines with restored morphological phenotype. After Sanger sequencing analysis, I chose to focus on four novel *bda* mutants, *bda3-1D*, *bda5*, *bda6* and *bda7*. Cloning of *BDA6* and *BDA7* showed that they encode FMO1 and ALD1 respectively, which are both essential components in SAR. My

results indicate that enzymes involved in secondary metabolite synthesis in SAR, are also important for signaling in SNC2-mediated immune pathway.

In chapter 4, I summarize key results and conclusions of my work and discuss their significance in a broader context. I also highlight some of the questions that arose from my research that could be addressed in the future.

2 Opposite roles of salicylic acid receptors NPR1 and NPR3/NPR4 in transcriptional regulation of plant immunity

2.1 Summary

Salicylic acid (SA) is a plant defence hormone required for immunity. *Arabidopsis* NPR1 and NPR3/NPR4 were previously shown to bind SA and all three proteins were proposed as SA receptors. NPR1 functions as a transcriptional activator, whereas NPR3/NPR4 were suggested to function as E3 ligases that promote NPR1 degradation. Here we report that NPR3/NPR4 function as transcriptional repressors and SA inhibits their activities to promote the expression of downstream immune regulators. *npr4-4D*, a newly identified gain-of-function *npr4* allele that renders NPR4 unable to bind SA, constitutively represses SA-induced immune responses. In contrast, the equivalent mutation in *NPR1* abolishes its ability to bind SA and promotes SA-induced defence gene expression. Further analysis revealed that NPR3/NPR4 and NPR1 function independently to regulate SA-induced immune responses. Our study indicates that both NPR1 and NPR3/NPR4 are *bona fide* SA receptors, but play opposite roles in transcriptional regulation of SA-induced defence gene expression.

2.2 Introduction

Salicylic acid (SA) is a phytohormone important for plant defence against pathogens (Vlot et al., 2009). Following pathogen infections, SA accumulates in both infected and systemic tissue, and it is required for both local and systemic acquired resistance (SAR) (Delaney et al. 1994; Gaffney et al. 1993). Exogenous application of SA or SA analogs induces immunity to pathogens (Görlach et al. 1996; Metraux Ahl-Goy, P., Staub, T., Speich, J., Steinemann, A., Ryals, J., and Ward, E. 1991), whereas reducing SA accumulation by expressing the bacterial salicylate hydroxylase gene *NahG* in transgenic plants results in SAR deficiency (Gaffney et al. 1993). Similarly, SA-deficient mutants such as *sid2* and *eds5* in *Arabidopsis* exhibit defects in basal

resistance and SAR (Nawrath et al. 2002; Nawrath and Métraux 1999). *SID2* encodes an isochorismate synthase that converts chorismate to isochorismate (Wildermuth et al. 2001), which is further converted to SA through an unknown mechanism. *EDS5* encodes a MATE transporter that is likely involved in exporting SA from chloroplast to cytoplasm (Nawrath et al. 2002; Serrano et al. 2013).

In *Arabidopsis*, pathogen-induced SA is mainly synthesized through Isochorismate Synthase 1 (ICS1/SID2) (Wildermuth et al. 2001). Two plant-specific transcription factors SARD1 and CBP60g promote pathogen-induced SA synthesis by regulating the expression of *ICS1* (Wang et al. 2009; Wang et al. 2011; Y. Zhang, Xu, et al. 2010). In addition to *ICS1*, SARD1 and CBP60g also bind to the promoter regions of a large number of genes including those that encode positive regulators of SAR as well as signaling components for effector-triggered immunity and pathogen-associated molecular pattern (PAMP)-triggered immunity, suggesting that these two transcription factors play broad roles in regulating plant immunity (Sun et al. 2015).

Arabidopsis NPR1 is required for SA-induced *PR* gene expression and resistance against pathogens (Cao et al. 1994; Delaney et al. 1995; Shah et al. 1997). Loss of NPR1 results in SA-insensitivity, leading to enhanced disease susceptibility and compromised SAR. NPR1 contains an N-terminal BTB/POZ domain, a central ankyrin-repeat domain and a C-terminal transactivation domain (Cao et al. 1997; Rochon et al. 2006). NPR3 and NPR4 are two paralogs of NPR1 with very similar domain structures as NPR1 (Liu et al. 2005). Loss of NPR3 and NPR4 does not affect the induction of *PR* gene by SA. Instead it results in elevated *PR* gene expression and enhanced disease resistance in the *npr3 npr4* double mutants (Zhang et al. 2006). The constitutive disease resistance phenotype of *npr3 npr4* can be complemented by NPR3 as well as NPR4, suggesting that NPR3 and NPR4 play redundant roles in negative regulation of immunity.

Intriguingly, NPR1 and NPR3/NPR4 all interact with TGA transcription factors (Després et al. 2000; Zhang et al. 2006; Zhang et al. 1999; Zhou et al. 2000). NPR1 has been shown to serve as a transcriptional activator (Fan and Dong 2002; Rochon et al. 2006) and NPR3/NPR4 were suspected to also function in transcription regulation (Kuai et al. 2015; Zhang et al. 2006). Among the TGA transcription factors that interact with

NPR1/NPR3/NPR4, TGA2, TGA5 and TGA6 function redundantly in positive regulation of SA-induced *PR* gene expression and pathogen resistance (Zhang, Tessaro, et al. 2003). However, basal *PR* gene expression levels are elevated in the *tga2 tga5 tga6* triple knockout mutant, suggesting that TGA2/TGA5/TGA6 are also involved in negative regulation of defence responses (Zhang, Tessaro, et al. 2003).

A large number of SA-binding proteins with different affinity to SA have been identified in plants (Klessig et al. 2016), but how SA is perceived as a defence hormone remains controversial. In one study, NPR3 was suggested as a low-affinity and NPR4 as a high-affinity SA receptor, whereas NPR1 was ruled out as an SA receptor based on its lack of SA-binding activity (Fu et al. 2012). On the other hand, NPR1 was shown to bind SA with high affinity in two separate studies (Manohar et al. 2014; Wu et al. 2012), and two Cysteine residues (C521 and C529) in the C-terminal domain of NPR1 are required for the binding of SA and SA-induced *PR1* expression (Rochon et al. 2006; Wu et al. 2012). NPR3 and NPR4 were proposed to function as E3 ligases that mediate the degradation of NPR1 (Fu et al. 2012). It was hypothesized that low levels of SA inhibit the interaction between NPR4 and NPR1 to allow for NPR1 accumulation, whereas high levels of SA during pathogen infection promote the association between NPR3 and NPR1 and degradation of NPR1. As previously discussed by Kuai *et al*., this model is inconsistent with some of the biochemical and genetic data observed from the *npr3*, *npr4* and *npr3 npr4* mutant plants and cannot explain the apparent genetic redundancy between *NPR3* and *NPR4* (Kuai et al. 2015). As NPR1 and NPR3/NPR4 belong to the same gene family, share similar domain structures and have high sequence similarity, it is surprising that NPR1 functions as a transcriptional activator, but NPR3/NPR4 are proposed to work as E3 ligases.

Here we report that NPR3/NPR4 serve as transcriptional repressors for SA-responsive genes. Multiple lines of evidences suggest NPR4 and NPR1 function separately to regulate SA-induced immune responses. By inhibiting the transcriptional repression activity of NPR4 and promoting the transcriptional activation activity of NPR1, SA activates the expression of key immune regulators. A gain-of-function *npr4-4D* mutant that is unable to bind SA constitutively represses SA-induced immune responses, whereas the equivalent mutation in NPR1 abolishes its SA-binding activity

and its ability to promote SA-induced defence gene expression, indicating that NPR1 and NPR3/NPR4 are all *bona fide* SA receptors despite their opposite roles in transcriptional regulation of SA-induced defence gene expression.

2.3 Results

2.3.1 Identification and characterization of *bda4-1D snc2-1D npr1-1*

Arabidopsis SNC2 encodes a receptor-like protein required for basal resistance against bacterial pathogens (Y. Zhang, Yang, et al. 2010). A dominant mutation in *SNC2* leads to constitutive activation of immune responses and dwarfism in the *snc2-1D npr1-1* double mutant (Y. Zhang, Yang, et al. 2010). From a suppressor screen of *snc2-1D npr1-1* to search for NPR1-independent immune regulators, we identified the *bda4-1 snc2-1D npr1-1* triple mutant (*BDA*: *Bian DA*; becoming bigger in Chinese) (Y. Zhang, Yang, et al. 2010). When backcrossed with the *snc2-1D npr1-1* parent, the F1 plants exhibited similar size and morphology as *bda4-1 snc2-1D npr1-1* (Figure 2.1B), indicating that the *bda4-1* mutation is dominant. Therefore, the mutant was renamed as *bda4-1D snc2-1D npr1-1*. In *bda4-1D snc2-1D npr1-1*, the dwarf morphology of *snc2-1D npr1-1* was almost fully suppressed (Figure 2.1A). Real-time RT-PCR (qRT-PCR) analysis showed that the constitutive expression of defence marker genes *PR1* (Figure 2.1C) and *PR2* (Figure 2.1D) in *snc2-1D npr1-1* is completely suppressed in the *bda4-1D snc2-1D npr1-1* triple mutant. In addition, the enhanced resistance to the virulent oomycete pathogen *Hyaloperonospora arabidopsidis* (*H.a.*) Noco2 in *snc2-1D npr1-1* is also suppressed in *bda4-1D snc2-1D npr1-1* (Figure 2.1E). Taken together, *bda4-1D* suppresses the dwarf morphology as well as constitutive defence responses in *snc2-1D npr1-1*.

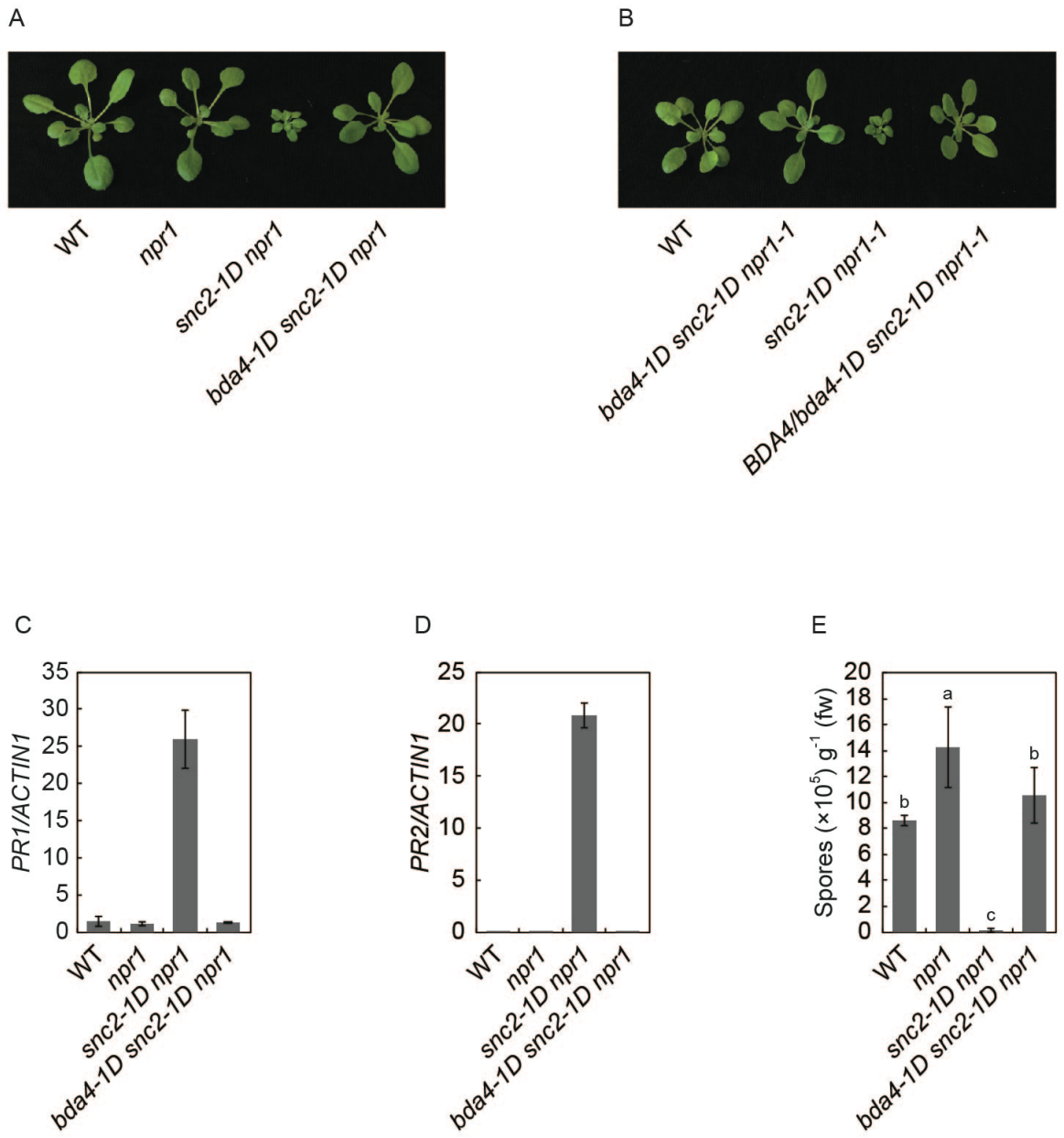

Figure 2.1 *bad4-1D*/*npr4-4D* suppresses the constitutive defence responses in *snc2-1D npr1-1*.

(A) Morphology of wild type (WT), *bda4-1D snc2-1D npr1-1*, *snc2-1D npr1-1* and *BDA4*/*bda4-1D snc2-1D npr1-1* heterozygous plants. Plants were grown on soil and photographed four weeks after planting.

(B) Morphology of wild type (WT), *npr1-1*, *snc2-1D npr1-1* and *bda4-1D snc2-1D npr1-1* plants. The photo was taken four weeks after planting.

(C-D) Expression of *PR1* (C) and *PR2* (D) in the indicated genotypes. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.
(E) Growth of *H.a.* Noco2 on the indicated genotypes. Two-week-old seedlings were sprayed with spores of *H.a.* Noco2 (5×10^4 spores/ml). Infection was scored seven days after inoculation by counting the numbers of spores per gram of leaf samples. Statistical differences among different genotypes are labeled with different letters ($P< 0.01$, One-way ANOVA/Tukey's test, $n = 4$).

2.3.2 *bda4-1D* carries a gain-of-function mutation in *NPR4*

The *bda4-1D* mutation was mapped to a region between markers 10.6 Mb and 10.9 Mb on chromosome 4. A single G-to-A mutation in *NPR4* (*AT4G19660*) was identified in this region by whole genome re-sequencing. This mutation results in an amino acid change (Arg-419 to Gln-419) located in the C-terminal domain of NPR4 (Figure 2.2A). To confirm that this mutation in *NPR4* is responsible for the suppression of the autoimmune phenotype of *snc2-1D npr1-1*, a genomic clone containing the mutant *NPR4* gene was transformed into *snc2-1D npr1-1*. As shown Figure 2.2B, the transgenic plants displayed *bda4-1D snc2-1D npr1-1*-like morphology (Figure 2.2B). Analysis of three representative transgenic lines showed that constitutive expression of *PR1* and *PR2* and enhanced resistance to *H.a.* Noco2 in *snc2-1D npr1-1* were completely suppressed in these lines (Figure 2.2C-E), suggesting that the Arg-419 to Gln-419 mutation in NPR4 is responsible for the suppression of *snc2-1D npr1-1* mutant phenotypes by *bda4-1D*. Thus, we conclude that *bda4-1D* is a dominant allele of *NPR4* and renamed *bda4-1D* as *npr4-4D*.

Loss of both *NPR4* and *NPR3* results in elevated *PR* gene expression and enhanced disease resistance (Zhang et al. 2006). To determine whether *npr4-4D* is a gain-of-function or dominant-negative mutation, we transformed the *npr4-4D* mutant gene under the control of its native promoter into *npr3-2 npr4-2* background. As shown in Figure 2.2 F-G, elevated *PR1* and *PR2* expression in *npr3-2 npr4-2* was suppressed in three independent transgenic lines, indicating that *npr4-4D* is a gain-of-function

mutation of *NPR4* that suppresses the constitutive defence responses in *snc2-1D npr1-1* as well as in *npr3-2 npr4-2*.

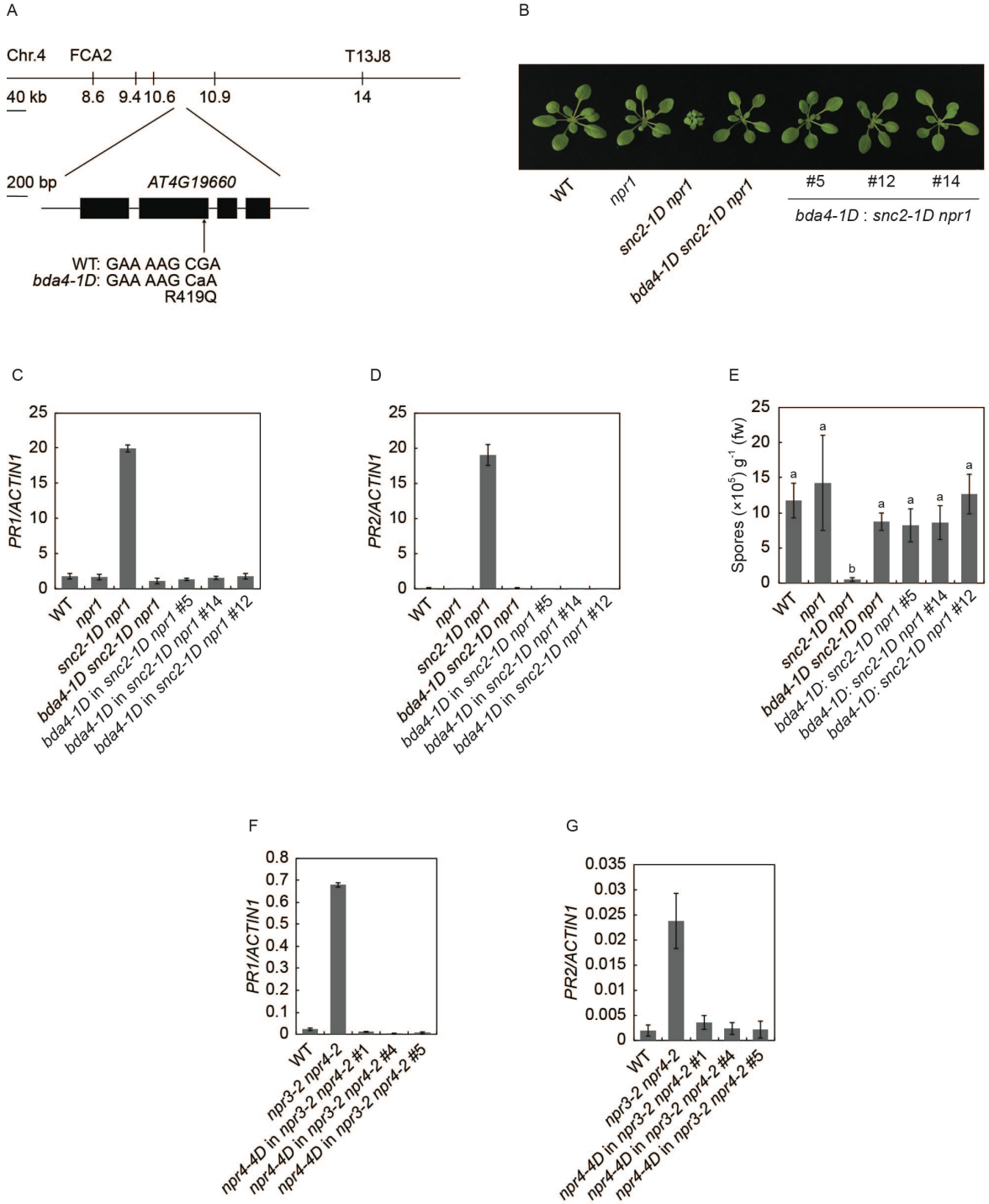

Figure 2.2 *bda4-1D* carries a gain-of-function mutation in *NPR4*.

(A) Map position and the mutation in *bda4-1D*.

(B) Morphology of four-week-old transgenic lines expressing the *bda4-1D* mutant gene in the *snc2-1D npr1-1* background.
(C-D) Expression of *PR1*(C) and *PR2*(D) in wild type (WT), *npr1-1*, *snc2-1D npr1-1*, *bda4-1D snc2-1D npr1-1* and transgenic lines expressing the *bda4-1D* mutant gene in *snc2-1D npr1-1* background. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.
(E) Growth of *H.a.* Noco2 on the indicated genotypes. Two-week-old seedlings were sprayed with spores of *H.a.* Noco2 (5×10^4 spores/ml). Infection was scored seven days after inoculation. Statistical differences among different genotypes are labeled with different letters ($P< 0.05$, One-way ANOVA/Tukey's test, $n = 4$).
(F-G) Expression of *PR1*(F) and *PR2*(G) in wild type (WT), *npr3-2 npr4-2* and transgenic lines expressing the *npr4-4D* mutant gene in *npr3-2 npr4-2* background. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.

2.3.3 Arg-419 residue in NPR4 is conserved in plants

Interestingly, the Arg-419 residue in NPR4 is conserved not only in NPR1 and NPR3, but also in their homologs of other plants (Figure 2.3A). To test whether NPR3 functions similarly as NPR4, we mutated the corresponding residue Arg-428 in NPR3 to Gln and expressed $NPR3^{R428Q}$ under the 35S promoter in *snc2-1D npr1-1*. As shown in Figure 2.3B and 2.3C, the dwarf morphology of *snc2-1D npr1-1* was suppressed by $NPR3^{R428Q}$, but not the wild type NPR3, confirming that NPR3 and NPR4 have redundant functions.

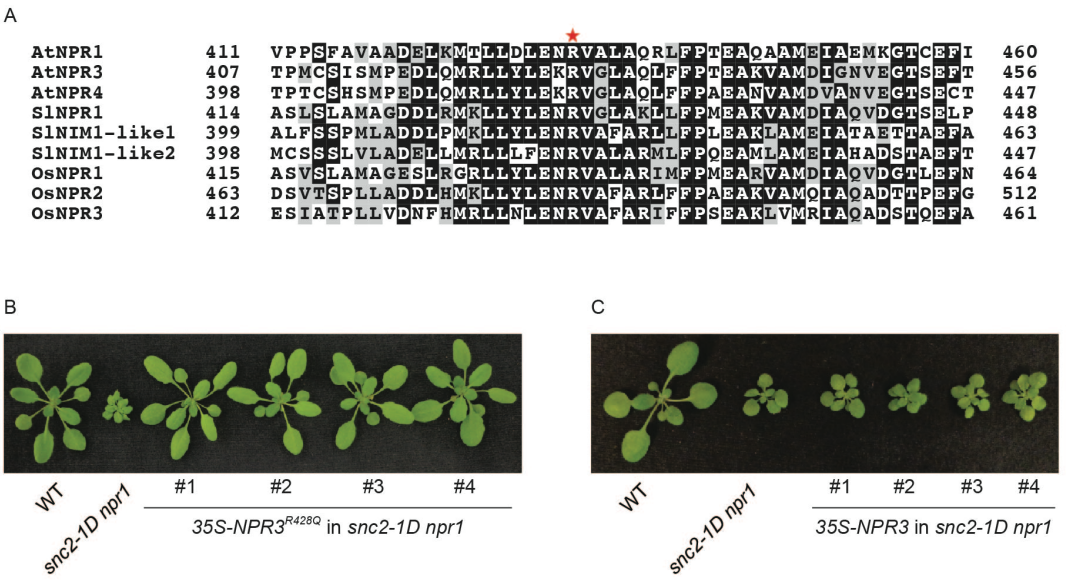

Figure 2.3 Suppression of the dwarf morphology of *snc2-1D npr1-1* by *NPR3^{R428Q}*

(A) Alignment of the conserved C-terminal regions of NPR1/NPR3/NPR4. At: *Arabidopsis thaliana;* Sl: Tomato, *Solanum lycopersicum*; Os: Rice, *Oryza sativa.* * indicates the mutation site in *npr4-4D*.

(B-C) Morphology of four-week-old soil-grown wild type (WT), *snc2-1D npr1-1* and transgenic lines expressing the 35S: *NPR3^{R428Q}* (B) or *35S: NPR3* (C) in the *snc2-1D npr1-1* background.

2.3.4 *npr4-4D* suppresses the expression of *SARD1*, *CBP60g* and *WRKY70*

Several transcription factors including SARD1, CBP60g and WRKY70 are required for the autoimmunity of *snc2-1D npr1-1* (Sun et al. 2015; Y. Zhang, Yang, et al. 2010). qRT-PCR analysis revealed that the expression of *SARD1*, *CBP60g* and *WRKY70* is much higher in *snc2-1D npr1-1* than in wild-type and *npr1-1*, but the increased expression of these genes is completely blocked in *npr4-4D snc2-1D npr1-1* (Figure 2.4A-C). To test whether *npr4-4D* affects the induction of *SARD1*, *CBP60g* and *WRKY70* by pathogens, we crossed *npr4-4D snc2-1D npr1-1* with wild-type Col-0 and isolated the *npr4-4D* single mutant. As shown in Figure 2.4 D-F, the expression of these three genes is strongly induced by the type III secretion deficient bacteria strain *Pseudomonas syringae* pv. *tomato* (*P.s.t.*) DC3000 *hrcC*$^{-}$ in wild type plants, but the

induction is dramatically reduced in *npr4-4D*. Similarly, the induction of *SARD1*, *CBP60g* and *WRKY70* by the virulent bacterial strain *Pseudomonas syringae* pv. *maculicola* (*P.s.m.*) ES4326 is also greatly reduced in *npr4-4D* (Figure 2.4 G-I). These data suggest that NPR4 negatively regulates the expression of *SARD1*, *CBP60g* and *WRKY70*.

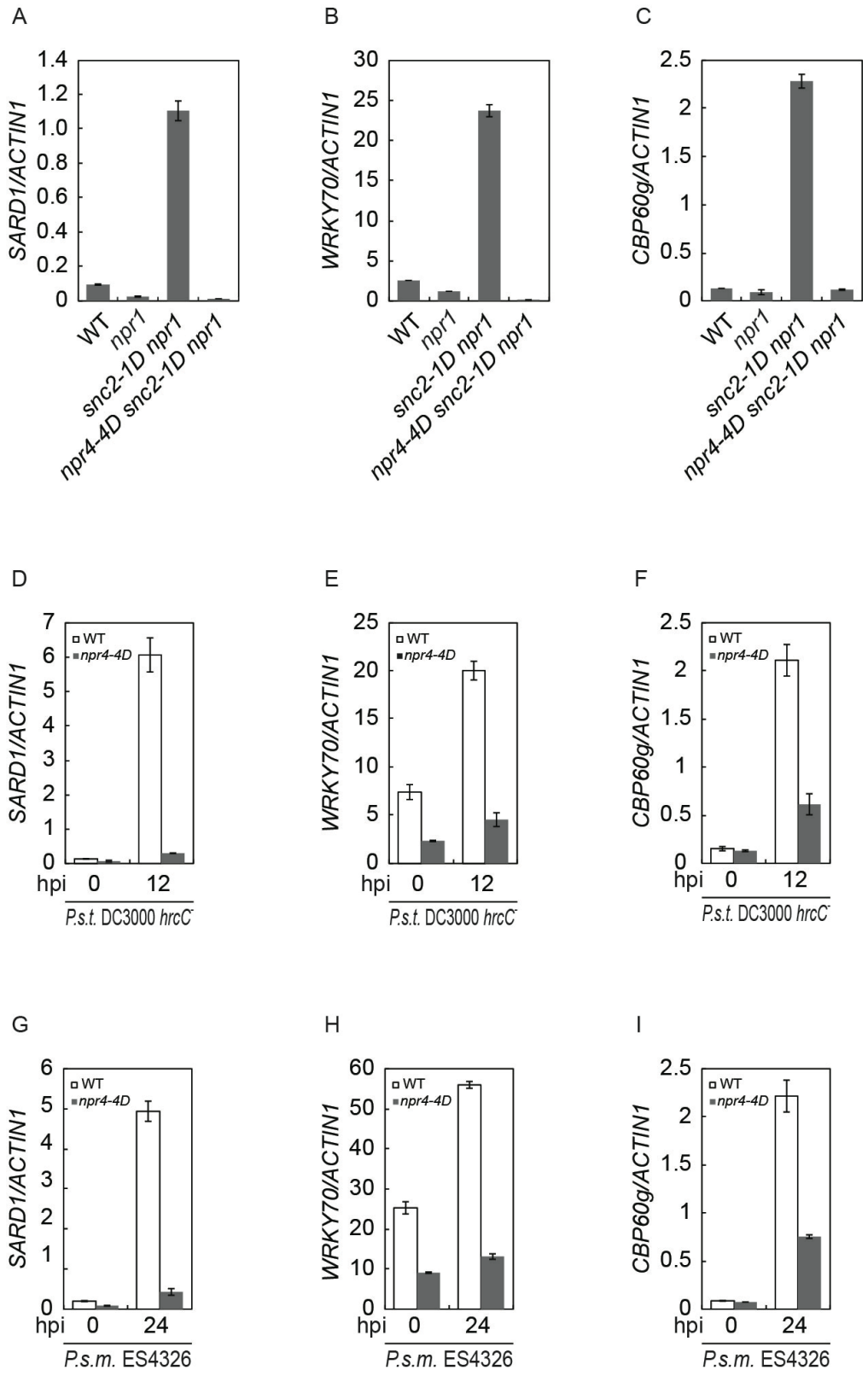

Figure 2.4 Repression of the expression of *SARD1*, *CBP60g* and *WRKY70* by *npr4-4D*.

(A-C) Expression of *SARD1* (A), *WRKY70* (B) and *CBP60g* (C) in wild type (WT), *npr1-1*, *snc2-1D npr1-1* and *npr4-4D snc2-1D npr1-1* plants. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.
(D-F) Induction of *SARD1* (D), *WRKY70* (E) and *CBP60g* (F) by *P.s.t.* DC3000 *hrcC*$^-$ in plants of WT and *npr4-4D*. Leaves of three-week-old plants grown in short-day conditions were infiltrated with *P.s.t.* DC3000 *hrcC*$^-$ at a dose of OD_{600} = 0.05. hpi: hours post inoculation. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.
(G-I) Induction of *SARD1* (G), *WRKY70* (H) and *CBP60g* (I) by *P.s.m.* ES4326 in plants of wild type (WT) and *npr4-4D*. Leave of three-week-old plants grown in short-day conditions were infiltrated with *P.s.m.* ES4326 at a dose of OD_{600} = 0.001. hpi: hours post inoculation. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.

2.3.5 The *npr4-4D* mutation results in compromised basal defence

Next we tested whether *npr4-4D* affects basal resistance against pathogens. Similar to the positive control (*agb1-2*), *npr4-4D* supported considerably higher growth of *P.s.t.* DC3000 *hrcC*$^-$ compared with the wild type (Figure 2.5A). When *npr4-4D* was challenged with the virulent bacteria *P.s.m.* ES4326, similar to *npr1*, *npr4-4D* plants also supported significantly higher growth of the pathogen than the wild type (Figure 2.5B), suggesting that *npr4-4D* suppresses basal resistance.

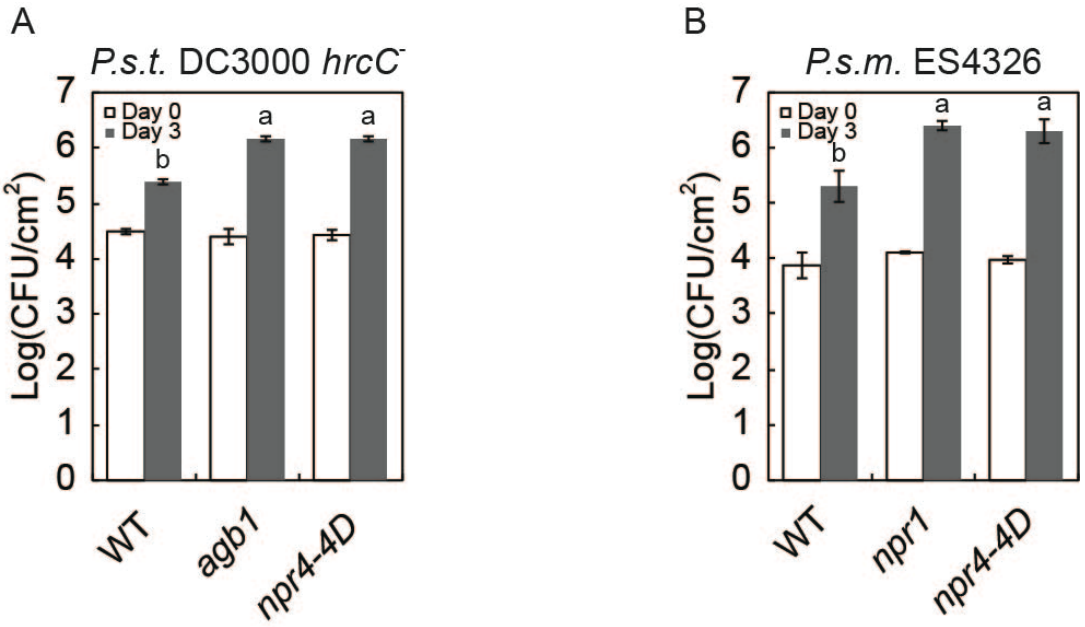

Figure 2.5 *npr4-4D* mutation leads to compromised basal defence and PTI.

(A) Growth of *P.s.t.* DC3000 *hrcC*⁻ on WT, *agb1-2*, and *npr4-4D* plants. Leaves of four-week-old plants were infiltrated with a bacterial suspension at a dose of OD_{600} = 0.002. cfu, Colony-forming units. Statistical differences among different genotypes are labeled with different letters (P< 0.01, One-way ANOVA/Tukey's test, n = 6).

(B) Growth of *P.s.m.* ES4326 on plants of WT, *npr1-1* and *npr4-4D*. Leaves of four-week-old plants were infiltrated with a bacterial suspension at a dose of OD_{600} = 0.0002. cfu, Colony-forming units. Statistical differences among different genotypes are labeled with different letters (P< 0.01, One-way ANOVA/Tukey's test, n = 6).

2.3.6 Loss of both NPR3 and NPR4 results in elevated *SARD1* and *WRKY70* expression

To test whether the expression of *SARD1*, *CBP60g* and *WRKY70* is affected in loss-of-function mutants of *NPR3* and *NPR4*, we compared their expression levels in wild type and *npr3 npr4* double mutants. As shown in Figure 2.6A-B, *SARD1* and *WRKY70* expression is dramatically elevated in the *npr3-2 npr4-2* double mutant, whereas the *CBP60g* expression level is only modestly increased in *npr3-2 npr4-2* (Figure 2.6C). A slight increase of *SARD1* expression was also observed in the *npr3-2*

and *npr4-2* single mutants (Figure 2.6A). Similar to *npr3-2 npr4-2*, the *npr3-1 npr4-3* double mutant also exhibit elevated basal *SARD1* and *WRKY70* expression (Figure 2.6D-E). These data suggest that NPR3 and NPR4 function redundantly in negative regulation of *SARD1* and *WRKY70* expression.

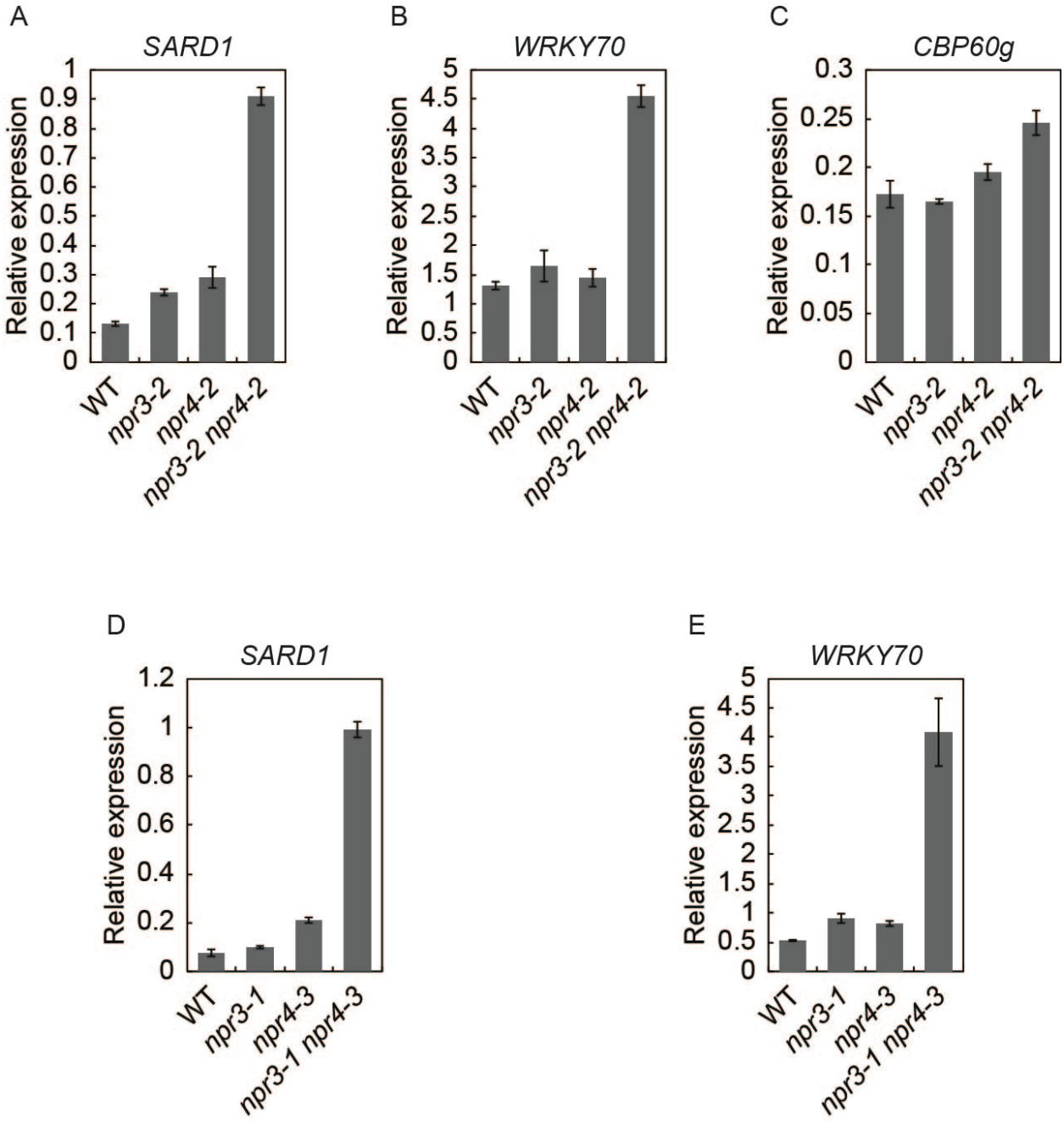

Figure 2.6 Loss of both NPR3 and NPR4 results in elevated *SARD1* and *WRKY70* expression.

(A-C) Expression of *SARD1* (A), *WRKY70* (B) and *CBP60g* (C) in wild type (WT), *npr3-2*, *npr4-2* and *npr3-2 npr4-2* plants. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.
(D-E) Expression levels of *SARD1* (D) and *WRKY70* (E) in plants of wild type (WT), *npr3-1*, *npr4-3* and *npr3-1 npr4-3* plants. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.

2.3.7 NPR3 and NPR4 function as transcriptional repressors that negatively regulate the expression of *SARD1* and *WRKY70*

NPR4 was previously shown to interact with TGA transcription factors (Zhang et al. 2006). To test whether NPR3/NPR4 serve as transcriptional repressors to negatively regulate *SARD1* and *WRKY70* expression, we made constructs expressing a luciferase reporter gene under the control of the promoters of *SARD1* or *WRKY70*. As shown in Figure 2.7A, when the *pSARD1::Luc* reporter gene was co-transformed with plasmids over-expressing *NPR3* or *NPR4* into protoplasts, the expression of luciferase is significantly reduced compared with the empty vector control. Co-transformation of plasmids over-expressing *NPR3* or *NPR4* with the *pWRKY70::Luc* reporter gene also results in reduced reporter gene expression (Figure 2.7B). These data suggest that overexpression of *NPR3* or *NPR4* in *Arabidopsis* protoplasts represses the expression of *SARD1* and *WRKY70*, and they are likely transcriptional repressors.

At the C-terminus of NPR3 and NPR4 but not NPR1, there is a conserved motif (VDLNETP) that has high similarity to the ethylene-responsive element binding factor-associated amphipathic repression motif (EAR; L/FDLNL/F(x)P) (Ohta et al. 2001). To determine whether this motif is required for the transcriptional repression activity of NPR4, we mutated the conserved amino acid sequence “DLN” in NPR4 to “GVK”, the corresponding amino acid sequence in NPR1. The NPR4^{GVK} mutant protein can still interact with TGA2 in the yeast two-hybrid assay (Figure 2.7C), but it no longer represses the expression of *SARD1* and *WRKY70* when expressed in protoplasts (Figure 2.7D-E).

To further test the transcriptional repression activity of NPR3/NPR4, we made constructs expressing NPR3 or NPR4 fused to the Gal4 DNA-binding domain (GD). Co-transformation of these constructs with a Renilla luciferase reporter gene driven by a promoter containing 2×Gal4 DNA-binding sites in protoplasts resulted in suppression of the expression of the reporter gene (Figure 2.7F), confirming that NPR3/NPR4 function as transcriptional repressors. Transforming a construct expressing GD fused with the NPR4 C-terminal domain (NPR4C) together with the Renilla luciferase reporter gene also results in suppression of the reporter gene (Figure 2.7G), suggesting that the C-terminal domain of NPR4 serves as a transcriptional repression domain.

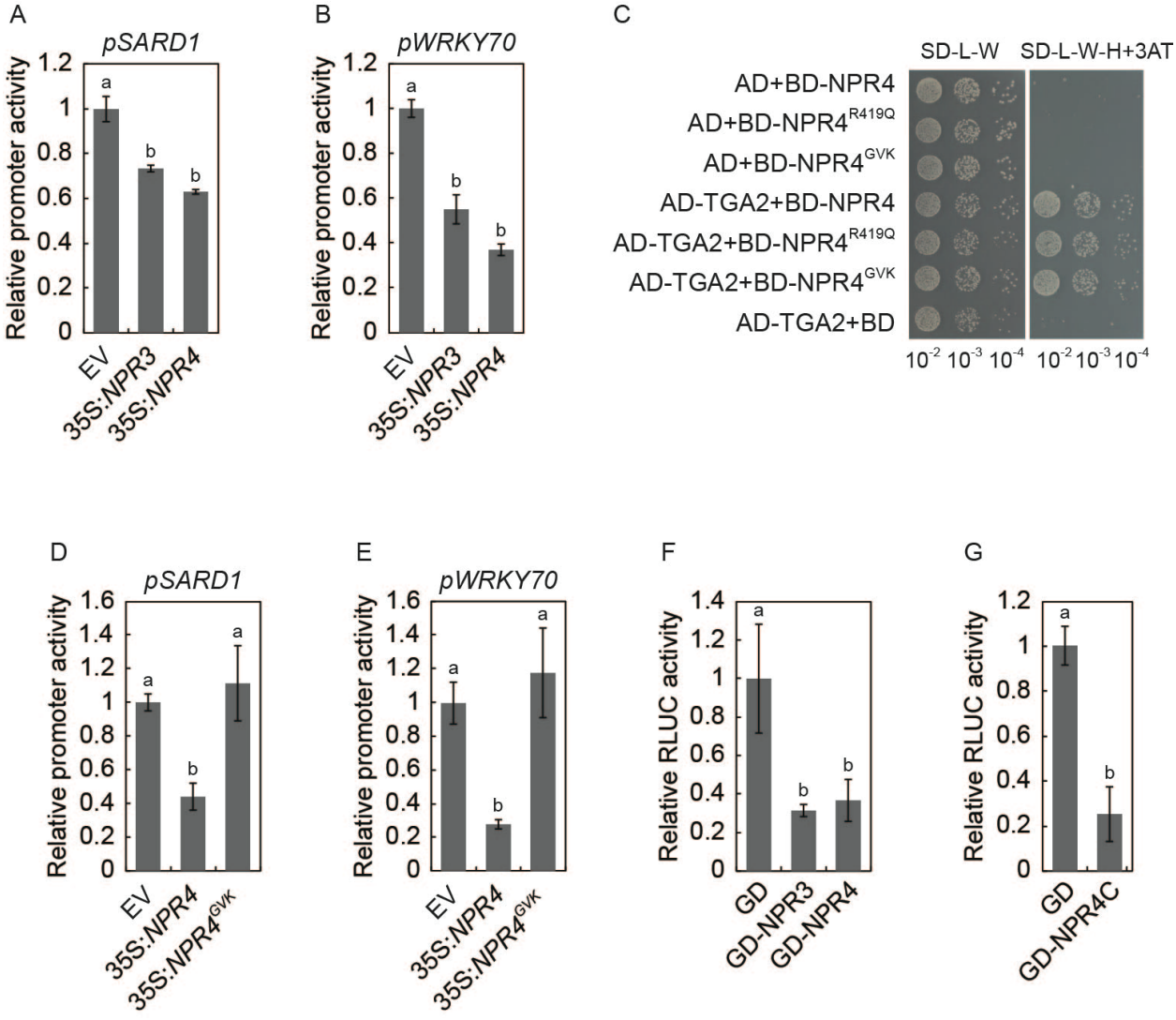

Figure 2.7 NPR3 and NPR4 function as transcriptional repressors that negatively regulate the expression of *SARD1* and *WRKY70*.

(A-B) Firefly luciferase activities in *Arabidopsis* protoplasts co-transformed with effector constructs [empty vector (EV), *35S:NPR3* or 35S:*NPR4*] and the *pSARD1-Luc* (A) or *pWRKY70-Luc* (B) reporter constructs. Statistical differences are labeled with different letters (P< 0.05, One-way ANOVA/Tukey's test, n = 3).

(C) Yeast two-hybrid analysis of interactions between the NPR4 mutants and TGA2. Yeast strains were serially diluted and 10 µl of each dilution (OD_{600}=10^{-2}, 10^{-3}, 10^{-4}) was plated on synthetic drop media without Leu and Trp (SD-L-W) plate or synthetic drop media without Leu, Trp and His (SD-L-W-H) plus 4 mM 3-aminotriazole (3AT).

(D-E) Firefly luciferase activities in *Arabidopsis* protoplasts co-transformed with effector constructs [EV, *35S*:*NPR4* or *35S*:*NPR4*(*GVK*)] and the *pSARD1-Luc* (D) or *pWRKY70-Luc* (E) reporter constructs. Statistical differences are labeled with different letters (P< 0.01, One-way ANOVA/Tukey's test, n = 3).

(F) Relative Renilla luciferase activities in *Arabidopsis* protoplasts co-transformed with a Renilla luciferase reporter gene and constructs expressing GAL4 DNA-binding domain (GD), GD-NPR3, GD-NPR4 were shown. Statistical differences are labeled with different letters (P< 0.01, One-way ANOVA/Tukey's test, n = 3).

(G) Relative Renilla luciferase activities in *Arabidopsis* protoplasts co-transformed with a Renilla luciferase reporter gene and constructs expressing GAL4 DNA-binding domain (GD), or GD fused with the C terminal domain of NPR4 (GD-NPR4C) were shown. Statistical differences are labeled with different letters (P< 0.01, One-way ANOVA/Tukey's test, n = 3).

For (A-B) and (D-E), a Renilla luciferase reporter under the control the promoter of *UBQ1* was included as the internal transfection control. The transformed protoplasts were incubated for 16-20 h before the luciferase activities were measured using a Dual-Luciferase Reporter Assay (Promega). The ratio of firefly luciferase/Renilla luciferase was used to calculate the relative luciferase activities. The value was compared with empty vector control, which was set as 1.

For (F-G), A construct expressing the LexA DNA-binding domain-VP16 activation domain (LD-VP16) fusion protein was included in all the assays for the activation of the reporter gene. A 35S promoter-driven firefly luciferase reporter was included as internal control. The transformed protoplasts were incubated for 16-20 h before the luciferase

activities were measured using a Dual-Luciferase Reporter Assay (Promega). The ratio of Renilla luciferase/firefly luciferase was used to calculate the relative luciferase activities. Values were compared with the GD control, which was set as 1.

2.3.8 NPR4 functions together with TGA transcription factors to repress the expression of *SARD1* and *WRKY70*

SARD1 and *WRKY70* each contain two TGACG motifs in their promoter region. To test whether the TGA-binding motifs are required for the repression of *SARD1* and *WRKY70* by NPR4, we mutated these motifs in the *pSARD1::Luc* and *pWRKY70::Luc* luciferase reporter genes (Figure 2.8A). As shown in Figure 2.8B-C, overexpression of *NPR4* in *Arabidopsis* protoplasts does not lead to repression of the mutant *pSARD1::Luc* and *pWRKY70::Luc* luciferase reporter genes. These data suggest that the TGA factors are likely necessary for transcriptional repression of *SARD1* and *WRKY70*.

Similar to *npr3 npr4* double mutants, the *tga6-1 tga2-1 tga5-1* (*tga256*) triple knockout mutant also has elevated *PR* gene expression (Zhang, Tessaro, et al. 2003). To test whether TGA2/TGA5/TGA6 also regulate the expression of *SARD1* and *WRKY70*, we compared the basal expression levels of *SARD1* and *WRKY70* in wild type and *tga256*. As shown in Figure 2.8D-E, the expression of *SARD1* and *WRKY70* is much higher in the *tga256* triple mutant and modestly increased in the *tga25* double mutant compared to the wild type. These data suggest that TGA2/TGA5/TGA6 are also required for negative regulation of the basal expression of *SARD1* and *WRKY70*.

To determine whether *SARD1* and *WRKY70* are direct targets of the TGA transcription factors, ChIP-qPCR experiments were carried out on wild type and *tga256* plants using anti-TGA2 antibodies (Figure 2.8F). As shown in Figure 2.8G-I, DNA in the promoter regions of *SARD1* and *WRKY70*, but not *CBP60g*, is clearly enriched in the immuno-precipitated samples from the wild type, but not the *tga256* mutant plants, suggesting that *SARD1* and *WRKY70* are both direct targets of TGA2.

Since NPR3/NPR4 and TGA2/TGA5/TGA6 interact with each other and are both required for the negative regulation of *SARD1* and *WRKY70* expression, we further

determined whether TGA2/TGA5/TGA6 are required for the repression of *SARD1* or *WRKY70* by NPR4. First we checked whether the repression of defence responses in *snc2-1D npr1-1* by *npr4-4D* requires TGA transcription factors. We crossed *npr4-4D snc2-1D npr1-1* with the *tga256* triple mutant to obtain the *npr4-4D snc2-1D npr1-1 tga6-1*, *npr4-4D snc2-1D npr1-1 tga25* and *npr4-4D snc2-1D npr1-1 tga256* mutant lines. As shown in Figure 2.8J, while *npr4-4D snc2-1D npr1-1 tga6-1* and *npr4-4D snc2-1D npr1-1 tga25* plants have a similar morphology to *npr4-4D snc2-1D npr1-1*, the sextuple mutant *npr4-4D snc2-1D npr1-1 tga256* shows extreme dwarf morphology similar to *snc2-1D npr1-1*. Consistently, the constitutive expression of *SARD1* and *WRKY70* is restored in the sextuple mutant (Figure 2.8K-L). We further tested whether NPR4 can repress the expression of the *pSARD1::Luc* and *pWRKY70::Luc* luciferase reporter genes in the *tga256* protoplasts. As shown in Figure 2.8M-N, overexpression of *NPR4* reduces the expression of both reporter genes in wild type, but not in the *tga256* mutant protoplasts. These data provide strong genetic evidence that NPR3/NPR4 work together with TGA2/TGA5/TGA6 to repress the expression of *SARD1* and *WRKY70*.

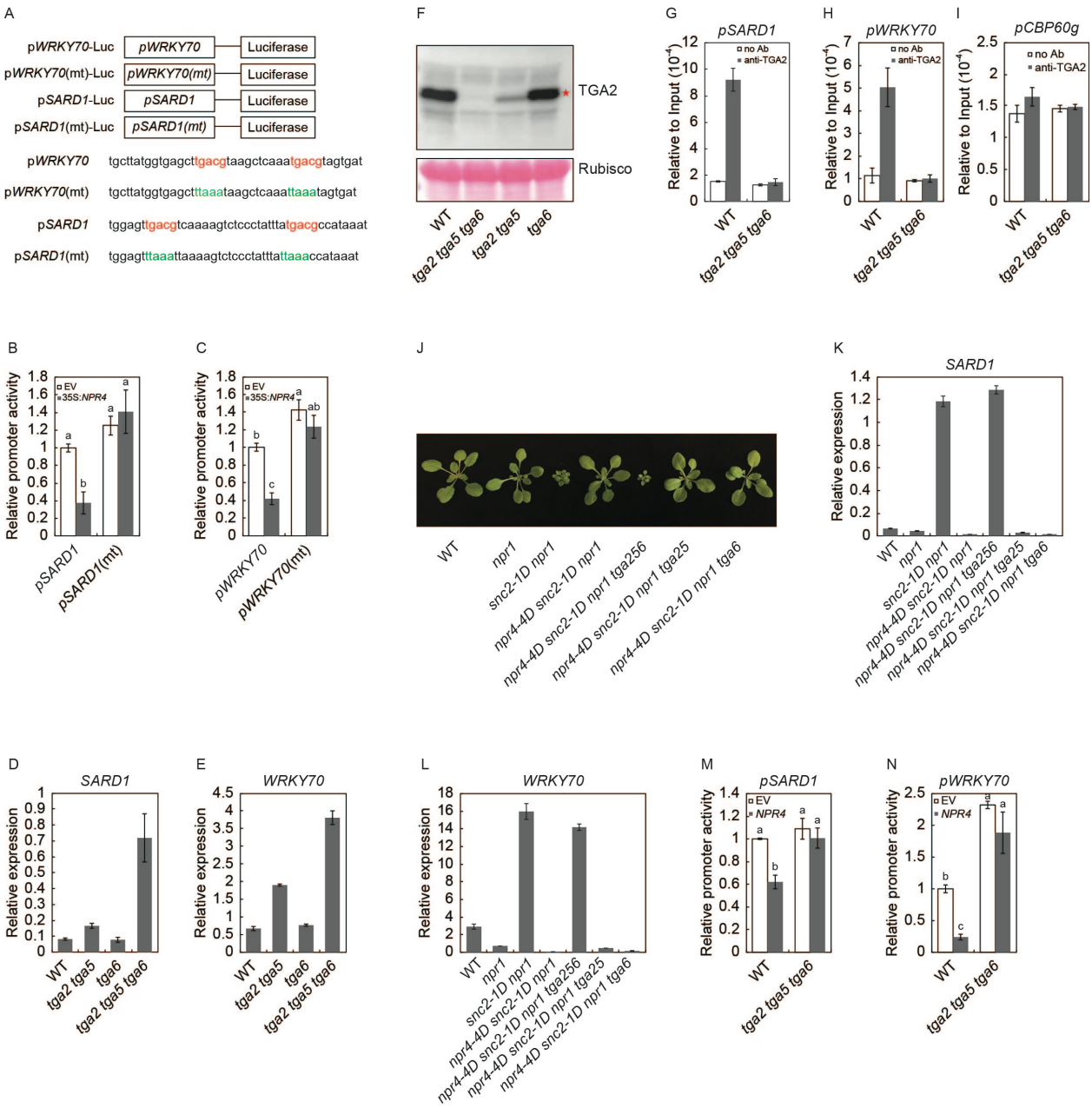

Figure 2.8 NPR4 functions together with TGA transcription factors to repress the expression of *SARD1* and *WRKY70*.

(A) Reporter constructs used in the promoter activity assay. The original TGACG motif sequence and ttaaa mutant sequences are colored.

(B-C) Firefly luciferase activities in *Arabidopsis* protoplasts transformed with empty vector (EV) or *35S:NPR4* effector constructs together with a luciferase reporter driven by wild type or mutant *SARD1*(B)/*WRKY70*(C) promoters with mutation in the “TGACG”

motifs. Statistical differences are labeled with different letters (P< 0.01, One-way ANOVA/Tukey's test, n = 3).

(D-E) Expression levels of *SARD1*(D) and *WRKY70*(E) in wild type (WT), *tga2-1 tga5-1*, *tga6-1* and *tga2-1 tga5-1 tga6-1* plants. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.

(F) Characterization of the TGA2 antibody. Western blot analysis was carried out on total proteins extracted from wild type (WT), *tga2-1 tga5-1*, *tga6-1* and *tga2-1 tga5-1 tga6-1* using the anti-TGA2 antibody.

(G-I) Binding of TGA2 to promoter regions of *SARD1*(G), *WRKY70*(H) and *CBP60g*(I) as revealed by chromatin immunoprecipitation assay. Twelve-day-old seedlings were collected and cross-linked with 1% formaldehyde. TGA2 chromatin complexes were immunoprecipitated with anti-TGA2 antibodies and protein A-agarose beads. Control reactions were performed in parallel using non-immunized serum (no Ab). The bound DNA was quantified by qPCR. ChIP results are presented as 10^{-4} of signal relative to input. Bars represent means ± s.d. (n = 3).

(J) Morphology of the indicated genotypes. Plants were grown on soil and photographed four weeks after planting.

(K-L) Expression levels of *SARD1*(K) and *WRKY70*(L) in plants of wild type (WT), *npr1-1*, *snc2-1D npr1-1*, *snc2-1D npr1-1 npr4-4D, snc2-1D npr1-1 npr4-4D tga2-1 tga5-1 tga6-1, snc2-1D npr1-1 npr4-4D tga2-1 tga5-1* and *snc2-1D npr1-1 npr4-4D tga6-1*. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.

(M-N) Firefly luciferase activities in *Arabidopsis* wild type (WT) and *tga2-1 tga5-1 tga6-1* protoplasts transformed with empty vector (EV) or *35S:NPR4* effector constructs together with the *pSARD1-Luc* (M) or *pWRKY70-Luc* (N) reporter constructs. Statistical differences are labeled with different letters (P< 0.01, One-way ANOVA/Tukey's test, n = 3).

For (B-C) and (M-N), a Renilla luciferase reporter under the control the promoter of *UBQ1* was included as the internal transfection control. The transformed protoplasts were incubated for 16-20 h before the luciferase activities were measured using a Dual-Luciferase Reporter Assay (Promega). The ratio of firefly luciferase/Renilla luciferase

was used to calculate the relative luciferase activities. The value was compared with empty vector control, which was set as 1.

2.3.9 SA inhibits the transcriptional repression activity of NPR4

Following SA treatment, the expression of both *SARD1* and *WRKY70* is rapidly induced and the induction is greatly reduced in *npr4-4D* (Figure 2.9A-B). Since SA can bind to NPR4, we tested whether the transcriptional repression activity of NPR4 is affected by SA. We treated wild type *Arabidopsis* protoplasts co-transformed with the *35S:NPR4* plasmid and the *pSARD1::Luc* or *pWRKY70::Luc* reporter gene with SA and examined the expression of luciferase 3h later. As shown in Figure 2.9C-D, overexpression of *NPR4* represses the expression of both reporter genes, and the repression is released by SA treatment. In contrast, repression of the reporter genes by *35S:npr4-4D* was not affected by SA treatment. These data suggest that SA inhibits the transcriptional repression activity of NPR4 and the *npr4-4D* mutant protein no longer responds to SA treatment.

To test whether SA affects the recruitment of NPR4 to the promoters of *SARD1* and *WRKY70*, we carried out ChIP-qPCR experiments using transgenic plants expressing NPR4-3HA protein. As shown in Figure 2.9E-G, NPR4-3HA was recruited to the promoters of *SARD1* and *WRKY70* but not *CBP60g*, and treatment with SA did not affect the association of NPR4-3HA with *SARD1* and *WRKY70* promoters. ChIP-qPCR experiments using transgenic plants expressing NPR3-3HA protein showed similar results where NPR3-3HA was also recruited to the promoters of *SARD1* and *WRKY70* (Figure 2.9H-I) and the interactions between NPR3-3HA and the promoters are not affected by SA treatment. Consistent with the data from ChIP-qPCR experiments, SA does not disrupt the interactions between NPR3/NPR4 and TGA2 in the yeast two-hybrid assay (Figure 2.9J). Interestingly, treatment of SA abolishes the repression of the Renilla luciferase reporter gene under the promoter with 2×Gal4 DNA-binding sites by GD-NPR3 and GD-NPR4 (Figure 2.9K), indicating a negative effect of SA on the transcriptional repression activities of NPR3/NPR4.

Next we tested whether SA-induced disease resistance is affected in the *npr4-4D* mutant. We treated wild type and *npr4-4D* seedlings with the SA analog INA (2,6-dichloroisonicotinic acid) and challenged the plants with *H.a.* Noco2. As shown in Figure 2.9L, exogenous application of INA renders the wild type plants resistant to the pathogen. Like in *npr1-1*, INA-induced resistance against *H.a.* Noco2 is largely blocked in *npr4-4D*, confirming that *npr4-4D* is an SA-insensitive mutant.

Previously GST-tagged NPR3 and NPR4 recombinant proteins were shown to bind SA with different affinities (Fu et al. 2012). To confirm the binding of SA to NPR3 and NPR4 and determine whether the *npr4-4D* mutation affects SA binding, we expressed His_6-MBP-tagged NPR3, NPR4 and NPR4-4D ($NPR4^{R419Q}$) proteins in *Escherichia coli* (*E. coli*) and purified the recombinant proteins for SA binding assays. The His_6-MBP tag was used because the previously reported GST-NPR3 and GST-NPR4 fusion proteins did not express well under our experimental conditions (Fu et al. 2012). As shown in Figure 2.9M-N, both NPR3 and NPR4 have high binding affinity to [^{3}H]-SA. The dissociation constants (K_d) for NPR3 and NPR4 were 176.7 ± 28.31 nM and 23.54 ± 2.743 nM respectively. The $NPR4^{R419Q}$ mutant protein can still interact with TGA2 (Figure 2.7C) and form homodimers (Figure 2.9O). However, it has hardly detectable binding affinity with [^{3}H]-SA (Figure 2.9P and Figure 2.9N), exhibiting an estimated K_d of about 250-fold lower than the wild type protein, suggesting that the Arg-419 residue in NPR4 is essential for its SA-binding activity.

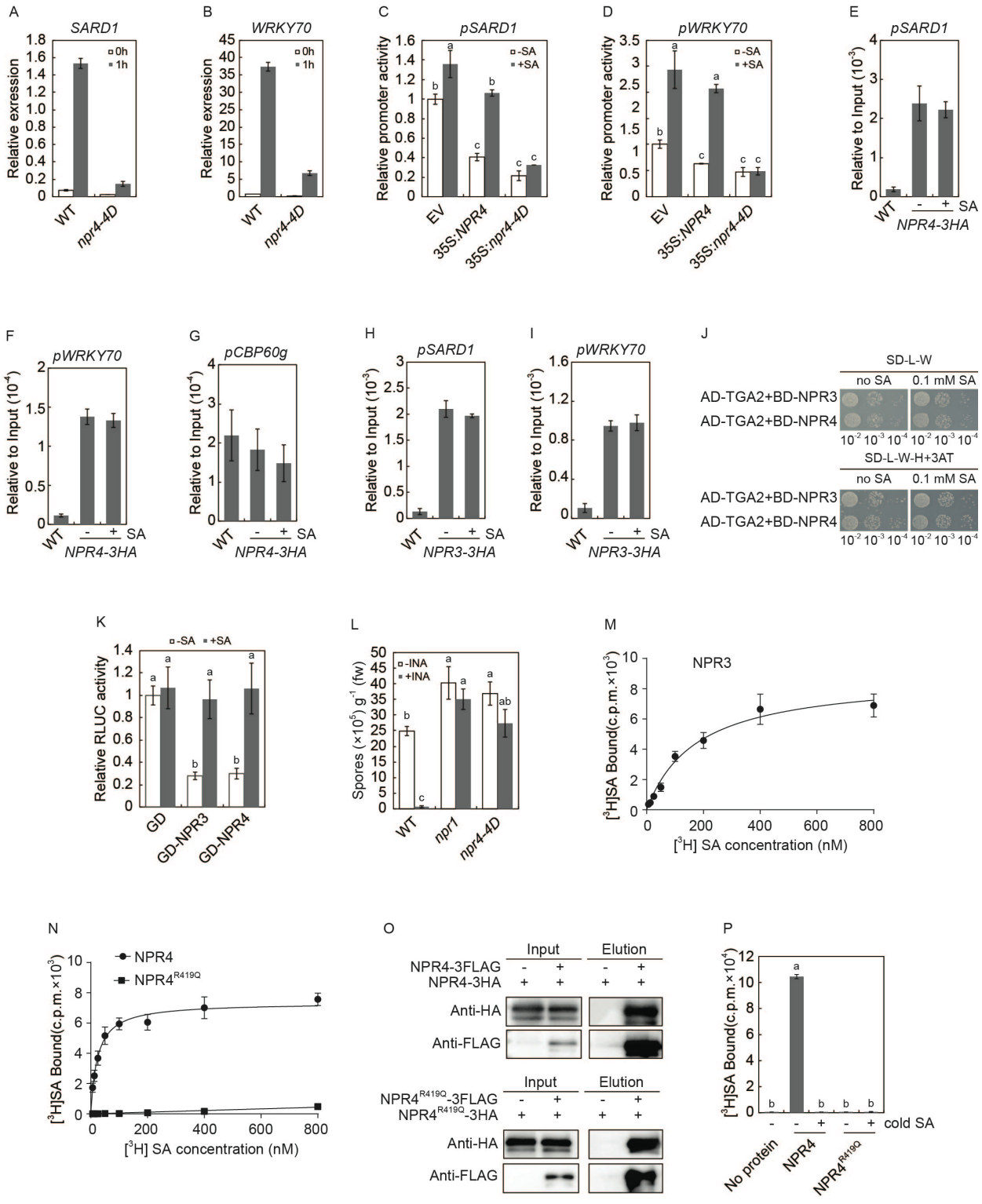

Figure 2.9 SA inhibits the transcriptional repression activity of NPR4 and the *npr4-4D* mutation abolishes SA-binding and renders SA insensitivity.

(A-B) Induction of *SARD1*(A) and *WRKY70*(B) gene expression by SA in plants of wild type (WT) and *npr4-4D*. Two-week-old seedlings grown on MS media were sprayed with 0.2 mM SA for quantitative RT-PCR analysis. Samples were collected at 0 and 1 h after treatment. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.

(C-D) Firefly luciferase activities in *Arabidopsis* wild type protoplasts co-transformed with effector constructs (empty vector, *35S:NPR4* or *35S*: *npr4-4D*) and the *pSARD1-Luc* (C) or *pWRKY70-Luc* (D) reporter constructs. After overnight incubation, an aliquot of the cells was treated with 0.2 mM SA for three hours before the luciferase activities were measured. The value was compared with empty vector transfection, which was set as 1. Statistical differences are labeled with different letters (P< 0.01, One-way ANOVA/Tukey's test, n = 3).

(E-G) Chromatin immunoprecipitation (ChIP)-PCR analysis of the effect of SA on the binding of NPR4-3HA to the promoter regions of *SARD1*(E), *WRKY70* (F) and *CBP60g*(G). Twelve-day-old seedlings were sprayed with or without 50 μM SA one hour before cross-linking with 1% formaldehyde. Chromatin complexes were immunoprecipitated with an anti-HA antibody. Control reactions were performed on non-transgenic plants (WT). The immunoprecipitated DNA was quantified by qPCR. ChIP-PCR results are presented as 10^{-3} of signal relative to input. Bars represent means ± s.d. (n = 3).

(H-I) Chromatin immunoprecipitation-PCR analysis of the effect of SA on binding of NPR3-3HA to the promoter regions of *SARD1*(H) and *WRKY70* (I). Twelve-day-old seedlings were sprayed with or without 50 μM SA one hour before cross-linking with 1% formaldehyde. Chromatin complexes were immunoprecipitated with an anti-HA antibody. Control reactions were performed on non-transgenic plants (WT). The immunoprecipitated DNA was quantified by qPCR. ChIP-PCR results are presented as % of signal relative to input. Bars represent means ± s.d. (n = 3).

(J) Yeast two-hybrid analysis of interactions between NPR3/NPR4 and TGA2 with or without the presence of SA (0.1mM). Yeast strains were serially diluted and 10 μl of each dilution (OD_{600}=10^{-2}, 10^{-3}, 10^{-4}) was plated on synthetic drop media without Leu

and Trp (SD-L-W) plate or synthetic drop media without Leu, Trp and His (SD-L-W-H) plus 4 mM 3-aminotriazole (3AT).

(K) Relative Renilla luciferase activities in *Arabidopsis* protoplasts co-transformed with a Renilla reporter gene and constructs expressing GAL4 DNA-binding domain (GD), GD-NPR3 or GD-NPR4. A construct expressing the LexA DNA-binding domain-VP16 activation domain (LD-VP16) fusion protein was included in all the assays for activation of the reporter gene. After overnight incubation, an aliquot of the cells was treated with 0.2 mM SA for three hours before the luciferase activities were measured. The values were compared with the GD control, which was set as 1. Statistical differences among treatments/genotypes are labeled with different letters (P< 0.01, One-way ANOVA/Tukey's test, n = 3).

(L) Growth of *H.a.* Noco2 on wild type (WT), *npr1-1* and *npr4-4D* plants. Two-week-old seedlings were sprayed with water or 0.1 mM INA. *H.a.* Noco2 spores (5×10^4 spores/ml) were sprayed one day after INA treatment. Infection was scored seven days after inoculation. Statistical differences among different genotypes are labeled with different letters (P< 0.01, One-way ANOVA/Tukey's test, n = 4).

(M) Saturation SA-binding assay of NPR3 using size exclusion chromatography. 1.5 µg of His_6-MBP-NPR3 protein was incubated with [^{3}H] SA at different concentrations (from 6.25 to 800 nM). Three replicates in a single experiment were used to calculate the K_d of NPR3 (176.7 ±28.31 nM). The experiment was repeated twice with similar results. Bars represent means ± s.d. (n = 3). CPM, count per minute.

(N) Saturation SA-binding assay of NPR4 and NPR4^{R419Q} using size exclusion chromatography. 1.5 µg of His_6-MBP-NPR4 or His_6-MBP-NPR4^{R419Q} protein was incubated with [^{3}H] SA at different concentrations (from 6.25 to 800 nM). Three replicates in a single experiment were used to calculate the K_d for NPR4 (23.54 ± 2.74 nM). The experiment was repeated twice with similar results. Bars represent means ± s.d. (n = 3).

(O) Analysis of homodimerization of NPR4 and NPR4^{R419Q} by co-immunoprecipitation. The proteins were transiently expressed in *N. benthamiana* using *Agrobacteria* strains carrying constructs expressing NPR4-3HA, NPR4^{R419Q}-3HA, NPR4-3FLAG or

NPR4^{R419Q}-3FLAG under a 35S promoter. IP was carried out using anti-FLAG beads. Western blot analysis was carried out using anti-FLAG or anti-HA antibodies.
(P) Binding of NPR4 protein to [^{3}H] SA as revealed by size exclusion chromatography. 0.4 μg/μl of HIS$_6$-MBP-NPR4 or HIS$_6$-MPB-NPR4^{R419Q} protein was incubated with 200 nM [^{3}H] SA in 50 μl of PBS buffer with or without 10,000-fold excess of unlabeled SA (cold SA). The reaction without protein (No protein) was used as negative control. Bars represent means ± s.d. (n = 4). CPM, count per minute.

2.3.10 NPR1 promotes the transcription of *SARD1* and *WRKY70* in response to SA

Since the Arg-419 residue in NPR4 is conserved in NPR1 (Figure 2.3A), we tested whether the corresponding Arg-432 in the C-terminal domain of NPR1 is also required for binding SA. We expressed His$_6$-MBP-tagged NPR1 and NPR1^{R432Q} proteins in *E. coli* and purified them for testing SA binding activities. As shown in Figure 2.10A, the His$_6$-MBP-tagged NPR1 has high binding affinity for [^{3}H]-SA, with a K_d of 223.1 ± 38.85 nM. The NPR1^{R432Q} mutant protein exhibits very low binding affinity for [^{3}H]-SA (Figure 2.10A), with a K_d estimated to be about 50-fold lower than the wild type protein, suggesting that Arg-432 plays an important role in SA binding. To determine whether the R432Q mutation affects the other functions of NPR1, we tested interactions of NPR1^{R432Q} with TGA2 and NIMIN1 (NIM1-INTERACTING 1), which interact with the ankyrin repeats and the C-terminal domain of NPR1, respectively (Weigel et al. 2001; Zhang et al. 1999). As shown in Figure 2.10B, NPR1^{R432Q} still interacts with both TGA2 and NIMIN1 in yeast two-hybrid assays.

NPR1 was previously shown to function as a transcriptional activator for *PR1* expression in response to SA (Fan and Dong 2002; Rochon et al. 2006). It is partially required for SA-induced *WRKY70* expression (Figure 2.10D) (Li et al. 2004). Induction of *SARD1* by SA is also partially dependent on NPR1 (Figure 2.10C). To determine whether the NPR1^{R432Q} mutation affects the function of NPR1 in the induction of *SARD1* and *WRKY70* by SA, we made transgenic lines expressing HA-tagged NPR1 or NPR1^{R432Q} in the *npr1-1* background (Figure 2.10E). As shown in Figure 2.10F and

2.10G, transgenic lines expressing NPR1-HA in the *npr1-1* background showed similar expression levels of *SARD1* and *WRKY70* as wild type after SA treatment. INA-induced resistance to *H.a.* Noco2 was also restored in the NPR1-HA transgenic lines (Figure 2.10H). In contrast, in the transgenic lines expressing NPR1^{R432Q}-HA, the expression levels of *SARD1* and *WRKY70* after SA treatment are similar in *npr1-1*. In addition, INA-induced resistance to *H.a.* Noco2 was not restored in the *NPR1^{R432Q}-HA* transgenic lines either. These data suggest that NPR1^{R432Q} cannot complement the defect of *npr1-1* in SA-induced defence responses.

We further tested whether the NPR1^{R432Q} mutation affects SA-induced *pSARD1::Luc* reporter gene expression. When a construct expressing wild type *NPR1* was co-transformed with the *pSARD1::Luc* reporter gene construct into *npr1-1* protoplasts, SA treatment induces the expression of luciferase (Figure 2.10I). In contrast, when the NPR1^{R432Q} construct was co-transformed with the reporter gene construct into *npr1-1* protoplasts, the expression of luciferase is not induced by SA, confirming that the NPR1^{R432Q} mutation renders NPR1 insensitive to SA. SA treatment did not induce the expression of the *pSARD1::Luc* reporter gene with mutations in the “TGACG” motifs (Figure 2.10J), suggesting that the induction of *pSARD1::Luc* expression by SA is dependent on the “TGACG” motifs in the *SARD1* promoter.

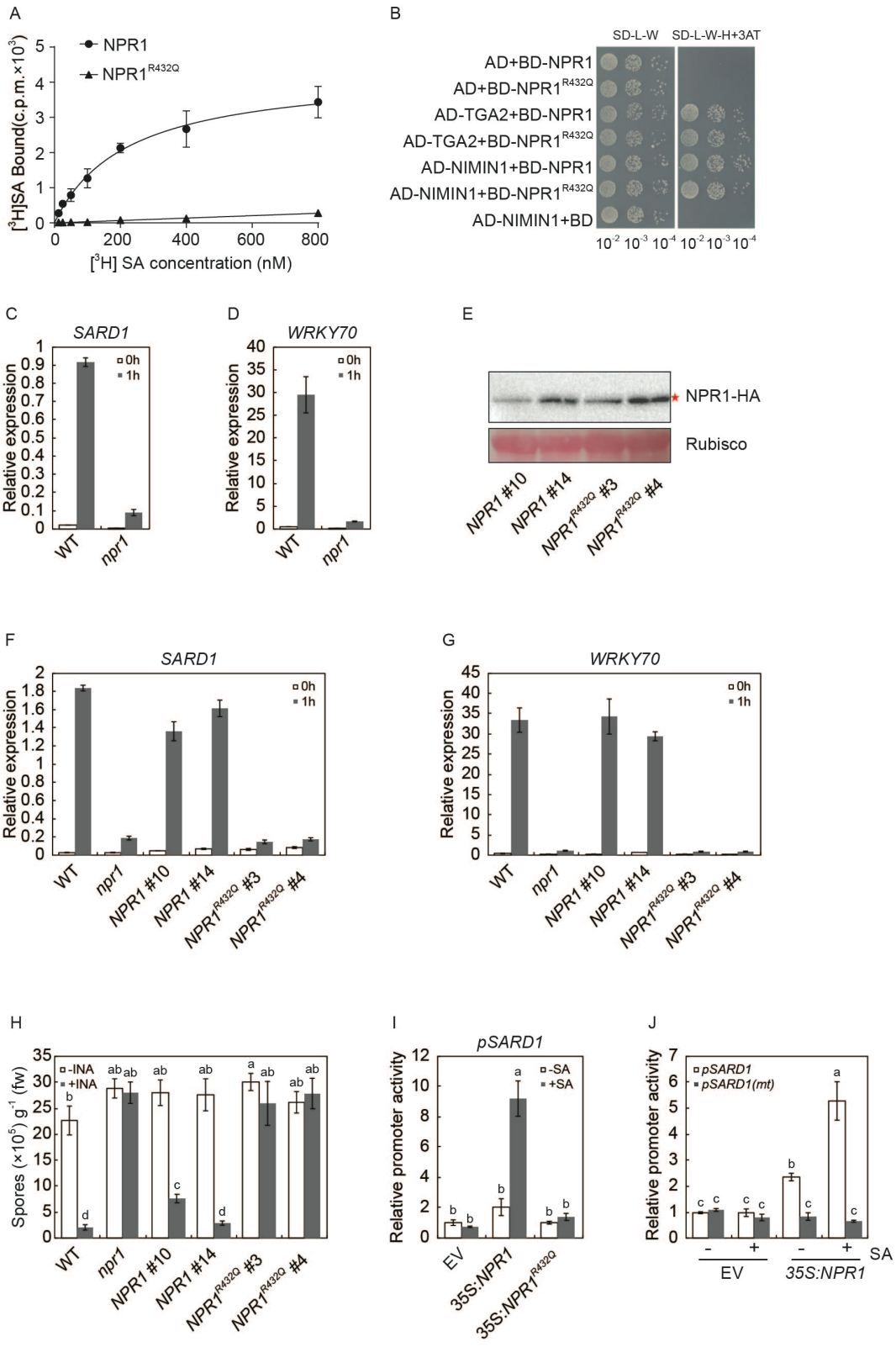

Figure 2.10 NPR1 promotes the expression of *SARD1* and *WRKY70* upon SA induction.

(A) Saturation binding assay of NPR1 and NPR1^{R432Q} using size exclusion chromatography. 5 µg of His$_6$-MBP-NPR1 or His$_6$-MBP-NPR1^{R432Q} protein was incubated with [^{3}H] SA at different concentrations (from 12.5 to 800 nM). Three replicates in a single experiment were used to calculate the K_d of NPR1 (221.3 ±38.85 nM). The experiment was repeated twice with similar results. Bars represent means ± s.d. (n = 3). CPM, count per minute.

(B) Yeast two-hybrid analysis of interactions between NPR1^{R432Q} and TGA2 or NIMIN1. Yeast strains were serially diluted and 10 µl of each dilution (OD_{600}=10^{-2}, 10^{-3}, 10^{-4}) was plated on synthetic drop media without Leu and Trp (SD-L-W) plate or synthetic drop media without Leu, Trp and His (SD-L-W-H) plus 4 mM 3-aminotriazole (3AT).

(C-D) Induction of *SARD1* (C) and *WRKY70* (D) expression by SA in plants of wild type (WT) and *npr1-1*. Error bars represent the standard deviation of three repeats.

(E) NPR1-HA and NPR1^{R432Q}-HA protein levels in transgenic lines in the *npr1-1* background. Western blot analysis was carried out on total plant proteins using an anti-HA antibody.

(F-G) Induction of *SARD1* (F) and *WRKY70* (G) by SA in WT, *npr1-1* and the *NPR1-HA* or *NPR1^{R432Q}-HA* transgenic lines in the *npr1-1* background. Error bars represent the standard deviation of three repeats.

(H) Growth of *H.a.* Noco2 on WT, *npr1-1* and the *NPR1-HA* or *NPR1^{R432Q}-HA* transgenic lines in the *npr1-1* background. Two-week-old seedlings were sprayed with water or 0.1 mM INA one day before spraying with *H.a.* Noco2 spores (5×10^4 spores/ml). Infection was scored seven days later. Statistical differences among different genotypes are labeled with different letters (P< 0.01, One-way ANOVA/Tukey's test, n = 4).

(I) Luciferase activities in *npr1-1* protoplasts co-transformed with effector constructs (empty vector, *35S:NPR1* or *35S:NPR1^{R432Q}*) and the *pSARD1-LUC* reporter construct. Statistical differences are labeled with different letters (P< 0.05, One-way ANOVA/Tukey's test, n = 3).

(J) Luciferase activities in *npr1-1* protoplasts co-transformed with effector constructs (empty vector or *35S:NPR1*) and the wild type or mutant *pSARD1-LUC* reporter construct with mutations in the TGACG motifs. Statistical differences are labeled with different letters (P< 0.01, One-way ANOVA/Tukey's test, n = 3).

For (C-D and F-G), two-week-old seedlings grown on MS media were sprayed with 0.2 mM SA. Samples were collected 0 and 1 h after treatment for qRT-PCR analysis. Values were normalized to the expression of *ACTIN1*. For (I-J), Samples were collected three hours after 0.2 mM SA treatment. The value was compared with empty vector control, which was set as 1.

2.3.11 NPR4 functions independently of NPR1

NPR3/NPR4 were previously reported to interact with NPR1 and function as E3 ligases for degrading NPR1 (Fu et al. 2012). However, we were not able to confirm the interactions between NPR3/NPR4 and NPR1 in yeast two-hybrid assays (Figure 2.11A). We also failed to detect interactions between NPR3/NPR4 and Cul3A in co-immunoprecipitation assays using epitope-tagged proteins transiently expressed in *Nicotiana benthamiana* (Figure 2.11B and 2.11C). To further determine the relationship between NPR3/NPR4 and NPR1, we analyzed the expression of *SARD1* and *WRKY70* in the *npr1-1 npr3-2 npr4-2* triple mutant. As shown in Figure 2.11D and 2.11E, elevated *SARD1* and *WRKY70* expression in *npr3-2 npr4-2* is not affected by *npr1-1*, suggesting that activation of *SARD1* and *WRKY70* in *npr3-2 npr4-2* is independent on NPR1. Next we performed promoter-luciferase assays in *npr1-1* protoplasts by transforming the *pSARD1-Luc* or *pWRKY70-Luc* reporter gene together with the 35S:NPR4 construct. As shown in Figure 2.11F and 2.11G, NPR4 can still repress the expression of the *pSARD1::Luc* and *pWRKY70::Luc* reporter genes in *npr1-1* protoplasts, suggesting that NPR4 regulates *SARD1* and *WRKY70* expression independent of NPR1.

To test whether NPR1 and NPR4 function in parallel in SA-induced gene expression, we compared SA-induced *SARD1* expression in the *npr1-1* and *npr4-4D* single mutants and the *npr1-1 npr4-4D* double mutant. As shown in Figure 2.11H, induction of *SARD1* by SA is partially blocked in *npr4-4D* and *npr1-1*, but it is completely blocked in the double mutant, suggesting that NPR1 and NPR4 function independently to regulate SA-induced *SARD1* expression. Analysis of the induction of *SARD1* and the defence marker gene *PR2* by *P.s.m.* ES4326 further showed that their induction is only

partially affected in the *npr1-1* and *npr4-4D* single mutants, but completely blocked in the *npr1-1 npr4-4D* double mutant (Figure 2.11I-J).

In addition, we analyzed the contribution of *npr1-1* and *npr4-4D* to the suppression of *snc2-1D*. As shown in Figure 2.11K, *snc2-1D npr1-1* and *snc2-1D npr4-4D* plants are only slightly bigger than *snc2-1D*, whereas the *snc2-1D npr1-1 npr4-4D* triple mutant has similar size as the wild type. The expression of *SARD1* and *WRKY70* in *snc2-1D* is lower in *snc2-1D npr1-1* and *snc2-1D npr4-4D*, and further reduced in *snc2-1D npr1-1 npr4-4D* (Figure 2.11L-M). Similarly, the enhanced resistance against *H.a.* Noco2 in *snc2-1D* is not significantly affected in *snc2-1D npr1-1* and *snc2-1D npr4-4D*, but completely lost in *snc2-1D npr1-1 npr4-4D* (Figure 2.11N). These data suggest that *npr4-4D* and *npr1-1* have additive effects on the suppression of the autoimmune phenotype of *snc2-1D*, further supporting that NPR1 and NPR4 function independently to regulate SA responses.

We further tested the effects of *npr1-1* and *npr4-4D* on basal resistance against pathogens. As shown in Figure 2.11O and 2.11P, *npr1-1* and *npr4-4D* supported significantly higher growth of *H.a.* Noco2 and *P.s.t.* DC3000. The *npr1-1 npr4-4D* double mutant supported even higher growth of these two pathogens than the single mutants. When *npr1-1*, *npr4-4D* and *npr1-1 npr4-4D* were challenged with the non-pathogenic *P.s.t.* DC3000*hrcC*$^{-}$, growth of the bacteria was also significantly higher in the single mutants and further increased in the *npr1-1 npr4-4D* double mutant (Figure 2.11Q). All these data indicate that NPR1 and NPR3/NPR4 function separately.

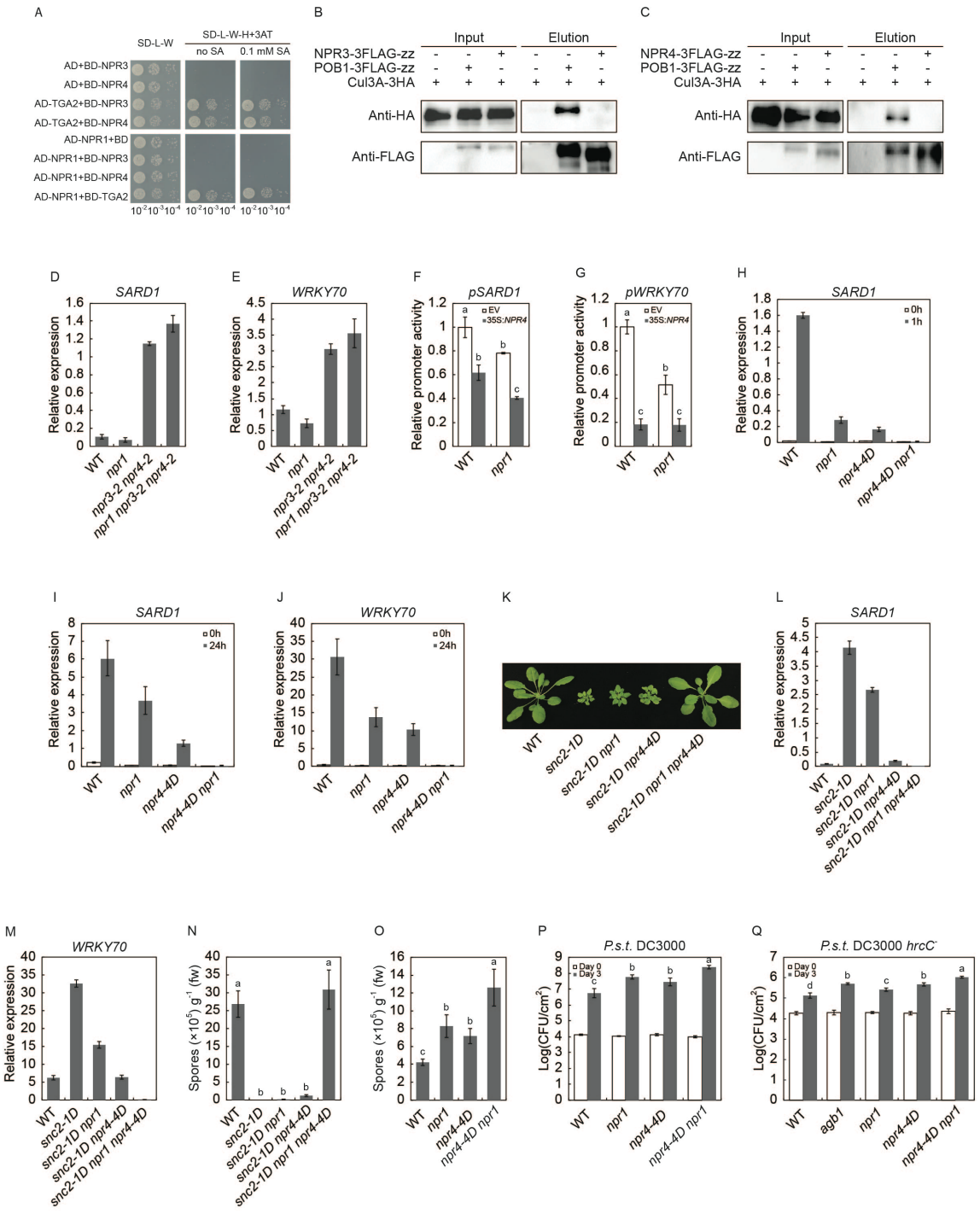

Figure 2.11 NPR3 and NPR4 function independently of NPR1.

(A) Yeast two-hybrid analysis of interactions between NPR3/NPR4 and NPR1 in the presence or absence of SA (0.1mM). Yeast strains were serially diluted and 10 μl of each dilution (OD_{600}=10^{-2}, 10^{-3}, 10^{-4}) was plated on synthetic drop media without Leu

and Trp (SD-L-W) plate or synthetic drop media without Leu, Trp and His (SD-L-W-H) plus 4 mM 3-aminotriazole (3AT).

(B-C) Analysis of interactions between NPR3 (B)/NPR4 (C) and Cul3A by co-immunoprecipitation. The E3 ligase BTB-POZ-CONTAINING PROTEIN 1 (POB1)/ LIGHT-RESPONSE BTB 2 (LRB2) was used as a positive control. The Cul3A-3HA and FLAG-ZZ-tagged NPR3/NPR4/POB1 proteins were transiently expressed in *N. benthamiana* by infiltrating leaves of 4-week-old plants with *Agrobacterium* (OD_{600} = 0.5) carrying plasmids expressing the Cul3A or NPR3/NPR4/POB1 fusion proteins. Samples were harvested 48 h post-inoculation. Immunoprecipitation was carried out on the total protein extracts using anti-FLAG conjugated beads. Cul3A-3HA was detected by immunoblot using an anti-HA antibody.

(D-E) Expression levels of *SARD1* (D) and *WRKY70* (E) in wild type (WT), *npr1-1*, *npr3-2 npr4-2* and *npr1-1 npr3-2 npr4-2* plants. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.

(F-G) Luciferase activities in *Arabidopsis* wild type (WT) and *npr1-1* protoplasts transformed with empty vector (EV) or *35S:NPR4* effector constructs, together with the *pSARD1-Luc* (F) or *pWRKY70-Luc* (G) reporter constructs. Statistical differences are labeled with different letters (P< 0.01, One-way ANOVA/Tukey's test, n = 3).

(H) Induction of *SARD1* by SA in wild type (WT), *npr1-1*, *npr4-4D* and *npr1-1 npr4-4D* double mutant plants. Two-week-old seedlings grown on MS media were sprayed with 0.2 mM SA. Samples were collected 0 and 1 h after treatment for qRT-PCR analysis. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.

(I-J) Induction of *SARD1* (I) and *PR2* (J) by *P.s.m.* ES4326 in the indicated genotypes. Leaves of three-week-old plants were infiltrated with *P.s.m.* ES4326 at a dose of OD_{600} = 0.001. Samples were collected at 0 and 24 h for qRT-PCR analysis. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.

(K) Morphology of plants of wild type (WT), *snc2-1D*, *snc2-1D npr1-1*, *snc2-1D npr4-4D* and *snc2-1D npr1-1 npr4-4D* plants. The picture was photographed four weeks after planting.

(L-M) Expression of *SARD1* (L) and *WRKY70* (M) in wild type (WT), *snc2-1D*, *snc2-1D npr1-1*, *snc2-1D npr4-4D* and *snc2-1D npr1-1 npr4-4D* plants. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.

(N) Growth of *H.a.* Noco2 on wild type (WT), *snc2-1D*, *snc2-1D npr1-1*, *snc2-1D npr4-4D* and *snc2-1D npr1-1 npr4-4D*. Two-week-old seedlings were sprayed with spores of *H.a.* Noco2 [5×10^4 spores/ml]. Infection was scored seven days after inoculation by counting the numbers of spores per gram of leaf samples. Statistical differences among different genotypes are labeled with different letters ($P< 0.01$, One-way ANOVA/Tukey's test, $n = 4$).

(O) Growth of *H.a.* Noco2 on wild type (WT), *npr1-1*, *npr4-4D* and *npr1-1 npr4-4D* double mutant plants. Two-week-old seedlings were sprayed with spores of *H.a.* Noco2 [1×10^4 spores/ml]. Infection was scored seven days after inoculation by counting the numbers of spores per gram of leaf samples. Statistical differences among different genotypes are labeled with different letters ($P< 0.01$, One-way ANOVA/Tukey's test, $n = 4$).

(P-Q) Growth of *P.s.t.* DC3000 (P) or *P.s.t.* DC3000 *hrcC*$^-$ (Q) on the indicated genotypes. Leaves of four-week-old plants were infiltrated with *P.s.t.* DC3000 (OD_{600} = 0.0002) or *P.s.t.* DC3000 *hrcC*$^-$ (OD_{600} = 0.002). cfu, Colony-forming units. Statistical differences among different genotypes are labeled with different letters ($P< 0.01$, One-way ANOVA/Tukey's test, $n = 6$).

2.3.12 Opposite roles of NPR1 and NPR4 in early defence gene expression in response to SA

To assess the contribution of NPR1 and NPR4 to early SA-induced gene expression, we carried out RNA-sequencing (RNA-seq) analysis on wild-type, *npr1-1* and *npr4-4D* plants before and after SA treatment. Two-week-old seedlings were treated with SA for one hour prior to sample collection. In the wild type plants, 2455 genes were found to be differentially expressed upon SA treatment (fold change ≥ 2 and false discovery rate (FDR) < 0.05), including 1543 induced genes and 912 repressed

genes. Gene ontology enrichment analysis showed that genes involved in defence responses were highly enriched among SA-induced genes (Figure 2.12A). Consistent with the involvement of TGA transcription factors in SA-induced defence gene expression, the preferred TGA2-binding sequence “TGACTT” is overrepresented in the promoters (1 kb upstream of the translation start sites) of the 1543 SA-induced genes (P <10^{-9}). Surprisingly, many key regulators of plant immunity were induced within one hour after SA treatment. Consistent with the antagonistic interactions between SA and JA, genes involved in JA-related processes are enriched among genes down-regulated in response to SA treatment (Figure 2.12A).

Among the 1543 genes induced by SA, the induction of 1107 and 286 genes is attenuated in *npr1-1* and *npr4-4D* respectively (log fold change ≥ 0.5 and FDR <0.05). Most genes affected by *npr4-4D* were also affected by *npr1-1* (Figure 2.12B and 2.12C), which is not surprising considering that regulation of defence gene expression by NPR1 and NPR4 is mediated by the same TGA transcription factors. Further analysis showed that 588 out of the 1107 genes affected by *npr1-1* and 252 out of the 286 genes affected by *npr4-4D* can still be partially induced by SA. To determine whether *npr1-1* and *npr4-1D* have additive effect on the induction of these genes, we carried out additional RNA-seq analysis on the *npr1-1 npr4-4D* double mutant before and after SA treatment. The induction of 331 genes partially affected in *npr1-1* and 181 gene partially affected in *npr4-4*D is completely blocked in the double mutant (FDR <0.05), confirming the additive effect of *npr1* and *npr4-4D* mutants in SA-induced immunity.

The expression of five representative genes regulated by both NPR1 and NPR4 (*WRKY70*, *MC2*, *NAC004*, *RLP23*, and *WRKY51*) was validated by qRT-PCR analysis. As shown in Figure 2.12D and 2.12E, the induction of these genes by SA is lower in *npr1-1* and *npr4-4D* than in the wild type, and further reduced in *npr1-1 npr4-4D* compared with the single mutants. We also examined the induction of *SARD1*, *MC2*, *NAC004*, and *WRKY51* in *npr1-7*, a deletion mutant lacking the translation start codon and most of the coding region of *NPR1*. Similarly, induction of these four genes by SA is partially blocked in *npr1-7* and completely blocked in the *npr1-7 npr4-4D* double mutant (Figure 2.12F). Together these data support that NPR1 and NPR4 act independently in the regulation of SA-induced gene expression.

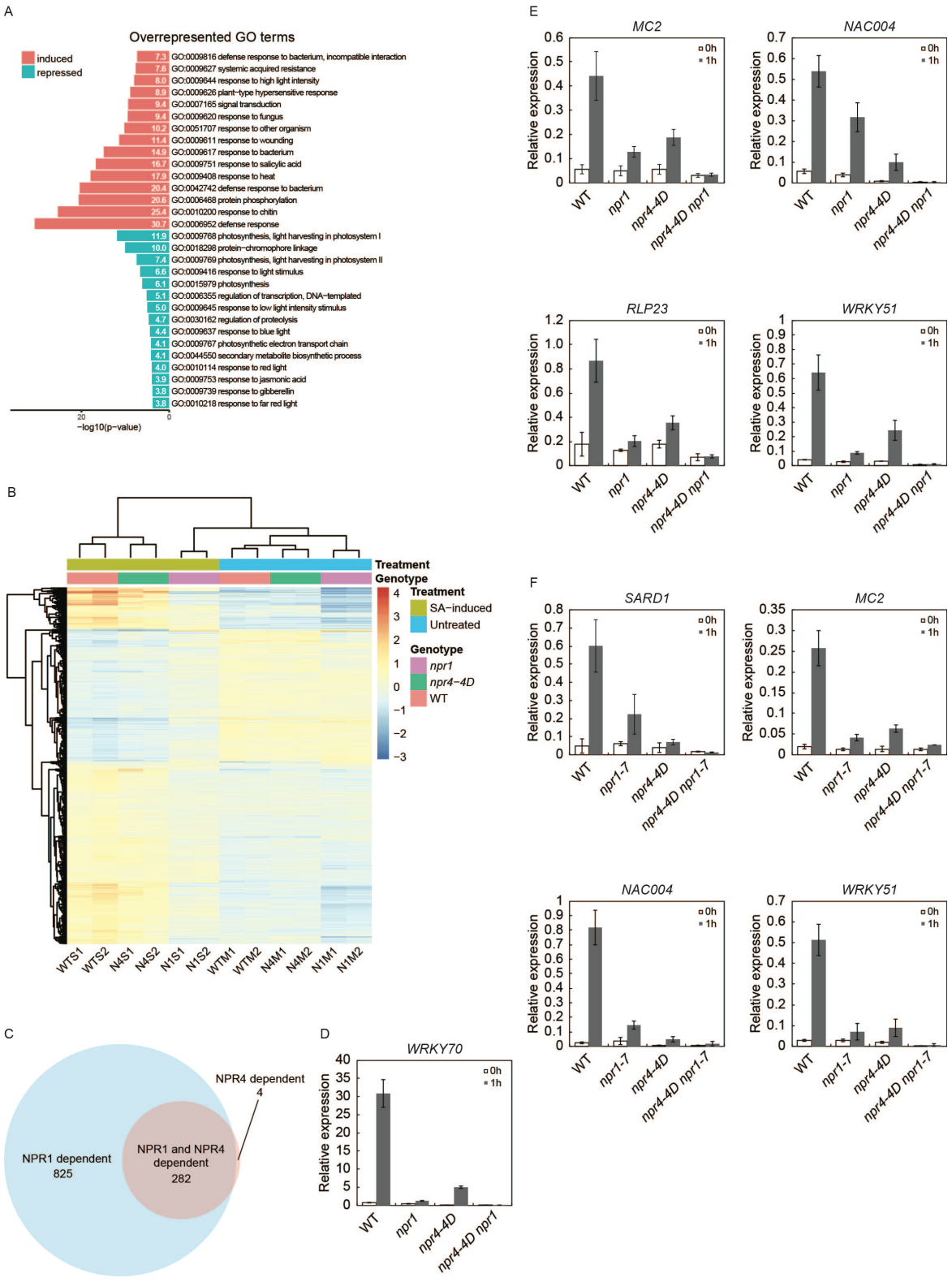

Figure 2.12 Opposite roles of NPR1 and NPR4 in early defence gene expression in response to SA.

(A) Gene ontology (GO) enrichment analysis of SA-induced and SA-repressed genes. The x-axis indicates the enrichment scores for each of the biological process GO terms. Up to the top 15 significantly enriched GO terms are shown. Red = GO-term enrichment of SA-induced genes, Green = GO-term enrichment of SA-repressed genes.
(B) Clustering analysis of RNA-seq samples. Raw counts were rlog transformed and compared using R package pheatmap. The y-axis represents SA-induced and SA-repressed genes, the x-axis represents the independent samples, and the fill represents the rlog normalized expression relative to the mean of the expression across all samples. WTS1, SA-treated wild type sample 1; WTS2, SA-treated wild type sample 2; N4S1: SA-treated *npr4-4D* sample 1; N4S2: SA-treated *npr4-4D* sample 2; N1S1: SA-treated *npr1-1* sample 1; N1S2: SA-treated *npr1-1* sample 2; WTM1, mock-treated wild type sample 1; WTM2, mock-treated wild type sample 2; N4M1, mock-treated *npr4-4D* sample 1; N4M2, mock-treated *npr4-4D* sample 2; N1M1, mock-treated *npr1-1* sample 1; N1M2, mock-treated *npr1-1* sample 2.
(C) SA-induced genes dependent on NPR1 or NPR4. Among genes induced by SA, the induction of 1107 genes is attenuated in *npr1-1* and the induction of 286 genes is attenuated in *npr4-4D* (log fold change ≥ 0.5 and FDR <0.05).
(D-E) Induction of *WRKY70* gene expression by SA in wild type (WT), *npr1-1*, *npr4-4D* and *npr1-1 npr4-4D* plants. Two-week-old seedlings were sprayed with 50 μM SA. Samples were collected 0 and 1 h after treatment for qRT-PCR analysis. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.
(E) Induction of *MC2*, *NAC004*, *RLP23* and *WRKY51* gene expression by SA in wild type (WT), *npr1-1*, *npr4-4D* and *npr1-1 npr4-4D* plants. Two-week-old seedlings were sprayed with 50 μM SA. Samples were collected 0 and 1 h after treatment for qRT-PCR analysis. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.
(F) Induction of *SARD1*, *MC2*, *NAC004* and *WRKY51* by SA in wild type (WT), *npr1-7*, *npr4-4D* and *npr1-7 npr4-4D*. Two-week-old seedlings grown on MS media were sprayed with 0.2 mM SA. Samples were collected 0 and 1 h after treatment for qRT-

PCR analysis. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.

2.4 Discussion

Previously we showed that NPR3 and NPR4 function redundantly as negative regulators of plant immunity (Zhang et al. 2006), but the mechanism of how they regulate plant defence responses was unclear. Here we show that NPR3/NPR4 serve as transcriptional repressors of key immune regulators such as SARD1 and WRKY70 and repression of *SARD1* and *WRKY70* expression by NPR3/NPR4 is facilitated by their interacting transcription factors TGA2/TGA5/TGA6. When tethered to the Gal4 DNA-binding domain, NPR3/NPR4 repress the transcription of a reporter gene under the control of a promoter with Gal4 DNA-binding sites, further supporting that NPR3/NPR4 function as transcriptional repressors. Surprisingly, SA serves as an inhibitor of NPR3/NPR4. In the SA-insensitive *npr4-4D* mutant, SA-induced defence gene expression is attenuated. In addition, treatment with SA abolishes the repression of the *pSARD1::Luc* and *pWRKY70::Luc* reporter genes by NPR4, but not the SA-insensitive NPR4^{R419Q} mutant.

Multiple lines of evidence suggest that SA-induced de-repression of defence genes is critical in activating plant immunity, despite that the number of genes affected by the *npr4-4D* mutation is much less than those affected in *npr1-1*. Similar to *npr1-1*, *npr4-4D* displayed enhanced susceptibility to *H.a.* Noco2 and INA-induced resistance to the pathogen is completely blocked in *npr4-4D*. *npr4-4D* is also more susceptible to *P.s.t.* DC3000 and *P.s.t.* DC3000 *hrcC*$^{-}$. In addition, the constitutive defence responses in *snc2-1D npr1-1* are almost completely suppressed by *npr4-4D*. The effects of *npr4-4D* and *npr1-1* on plant defence are almost always additive, suggesting that both de-repression and activation of SA-responsive genes are important to activating plant immunity.

Our study confirms NPR1 as a high-affinity SA-binding protein and provides strong evidence that the SA-binding activity of NPR1 is required for it function in SA-induced immunity. Previously two evolutionarily unconserved Cys residues

(Cys521/Cys529) in NPR1 were shown to be required for SA-binding and SA-induced *PR1* expression (Rochon et al. 2006; Wu et al. 2012). Whether they are required for the induction of other defence genes and resistance to pathogens by SA is unclear. Unlike Cys521/Cys529, the Arg-432 residue in NPR1 and the corresponding Arg-419 in NPR4 are highly conserved among NPR1/NPR3/NPR4 and their orthologs in other plants. The NPR1 R432Q mutation, which disrupts SA-binding but not its interactions with TGA2 and NIMIN1, abolishes its function in promoting SA-induced defence gene expression and pathogen resistance. Together these data strongly support that NPR1 is a *bona fide* SA receptor.

Our data do not support the previous hypothesis that NPR3/NPR4 regulate plant immunity by controlling NPR1 protein levels (Fu et al. 2012). Multiple lines of evidence from our study suggest that NPR3/NPR4 function independently of NPR1 in plant immunity. First, the *npr4-4D* mutant was isolated in a background containing the *npr1-1* mutation, a null allele of *NPR1* that was previously shown to completely abolish its interaction with the TGA transcription factors and SA-induced *PR* gene expression (Cao et al. 1994; Zhang et al. 1999), and the *npr4-4D* and *npr1-1* mutations have additive effects on the suppression of the autoimmune phenotypes of *snc2-1D*. Second, the *npr1-1* mutation has no effect on the increased *SARD1* and *WRKY70* expression in *npr3 npr4* mutant plants. Third, repression of the *pSARD1::Luc* and *pWRKY70::Luc* reporter genes by NPR4 is not affected by *npr1-1*. Finally, the induction of a large number of genes by SA is partially affected in the *npr4-4D* and *npr1-1* single mutants, but completely blocked in the *npr4-4D npr1-1* double mutant. Furthermore, previously reported interactions between NPR3/NPR4 and NPR1 cannot be independently confirmed under our experimental conditions. Whether NPR3/NPR4 really function as E3 ligases for degrading NPR1 needs to be further evaluated.

SA has been known as an inducer of plant defence responses for many years, but how SA treatment results in enhanced resistance against pathogens was unclear. Our RNA-seq analysis revealed that SA treatment results in rapid induction of a large number of genes within one hour. Among the early SA-induced genes, many encode key regulators required for plant immunity. Overexpression of some of these immune regulators such as SARD1, WRKY70, SOBIR1, ALD1, ADR1 and EDS1/PAD4 has

previously been shown to result in enhanced pathogen resistance (Cecchini et al. 2015; Cui et al. 2017; Gao et al. 2009; Grant et al. 2003; Li et al. 2004; Y. Zhang, Xu, et al. 2010), suggesting that their induction by SA contributes to SA-induced immunity. Interestingly, a number of known negative regulators of plant immunity are also rapidly up-regulated following SA treatment. The induction of these genes might play important roles in negative feedback regulation of defence responses.

Our SA-binding data suggest that both NPR3 and NPR4 are high-affinity SA receptors. The SA-binding affinities for NPR3 (K_d = 176.7 ± 28.31 nM) and NPR1 (K_d = 223.1 ± 38.85) are comparable, whereas the affinity of NPR4 to SA (K_d = 23.54 ± 2.743 nM) is considerably higher. The K_ds for the MBP-tagged NPR1 and NPR4 protein in our study are similar to the previously reported K_ds for NPR1 and NPR4 (Fu et al. 2012; Manohar et al. 2014; Wu et al. 2012), but the K_d for the MBP-tagged NPR3 is much lower than the previously reported K_d for the GST-tagged NPR3, which could be due to low activity of the GST-NPR3 recombinant protein used in the assay. In the absence of pathogen infection, the basal level of SA in *Arabidopsis* leaf tissue is around 1.4 μM (0.2 μg per g of tissue) (Kong et al. 2016), which is much higher than the K_ds for NPR1 and NPR3/NPR4. As defence genes are not strongly induced by the basal level of SA, the SA-binding affinities for endogenous NPR1 and NPR3/NPR4 proteins might be considerably lower than what is observed with the recombinant proteins due to potential post-translational modifications in the plant cells. Alternatively, the concentration of SA in the nucleus could be lower than the average SA level in case of uneven distribution of SA in different subcellular compartments.

NPR1 was previously shown to interact with the promoter of *PR1* before and after SA treatment (Rochon et al. 2006). SA induces a conformational change in the C-terminal transactivation domain of NPR1, which results in the release of the inhibitory effect of the N-terminal BTB/POZ domain and activation of NPR1 (Wu et al. 2012). Interestingly, SA was also shown to promote the interaction between NPR1 and TGA2 in transient expression assays using tobacco and potato protoplasts (Subramaniam et al. 2001). Our ChIP-PCR data showed that NPR3/NPR4 also interact with the promoters of defence genes. SA treatment has no effect on these interactions, consistent with the observation that SA does not block the interactions between TGA2

and NPR3/NPR4. As SA abolishes GD-NPR3 and GD-NPR4-mediated repression of the luciferase reporter gene driven by a promoter with Gal4 DNA-binding sites, it is likely that binding of SA directly affects the transcriptional repression activities of NPR3/NPR4.

In summary, NPR1 functions as a transcriptional activator and NPR3/NPR4 serve as redundant transcriptional repressors for SA-responsive defence genes. NPR1 and NPR3/NPR4 all interact with and are dependent on TGA transcription factors for their activities. We propose a model where there is an equilibrium of NPR:TGA:promoter complexes in the plant cells, with dynamic exchange of specific NPR and TGA proteins (Figure 2.13). Binding of SA to NPR3/NPR4 inhibits their transcriptional repression activity, whereas perception of SA by NPR1 enhances its transcriptional activation activity, both contribute to induction of defence gene expression.

Although SA is the first case in plants where one hormone is perceived by multiple non-redundant receptors, such examples do exist among neurohormones such as epinephrine, dopamine and histamine. The evolution and maintenance of different receptors for SA is most likely due to the requirement for intricate control of the SA responses. When the SA levels are low, NPR3/NPR4 repress defence gene expression, which prevents autoimmunity. Increased SA accumulation removes the repression and allows further induction of defence gene expression through the transcription activator NPR1.

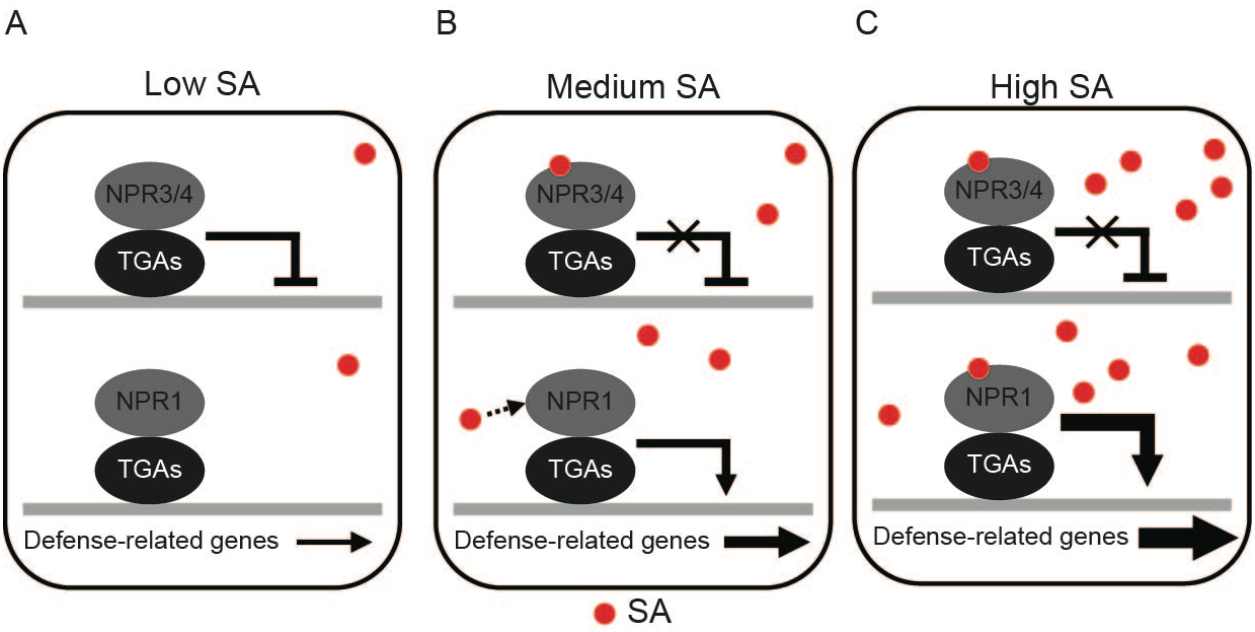

Figure 2.13 A working model of NPR1/NPR3/NPR4 in SA-induced defence activation.

(A) When the SA level is low under uninfected state, NPR3/NPR4 interacts with TGA2/TGA5/TGA6 to inhibit the expression of defence-related gene expression.

(B) As the SA level increases during pathogen infection, SA binds to NPR3/NPR4 to release the transcriptional repression of defence genes.

(C) Meanwhile, binding of SA to NPR1 promotes activation of the transcription of the defence genes.

2.5 Material and methods

2.5.1 Plant Material and Growth Condition

All *Arabidopsis* mutants used are in the Columbia (Col-0) ecotype. The *npr1-1*, *agb1-2*, *snc2-1D*, *snc2-1D npr1-1*, *npr3-1 npr4-3*, *npr3-1 npr4-3 npr1-1*, *tga2-1 tga5-1* (*tga25*), *tga6-1* and *tga2-1 tga5-1 tga6-1* (*tga256*) mutants were reported previously (Cao et al. 1994; Sun et al. 2015; Ullah et al. 2003; Zhang et al. 2006; Zhang, Tessaro, et al. 2003; Y. Zhang, Yang, et al. 2010). The *npr3-2 npr4-2 npr1-1* triple mutant was obtained by crossing *npr1-1* with *npr3-2 npr4-2*. The *bda4-1D* (*npr4-4D*) *snc2-1D npr1-1*

mutant was identified from an EMS-mutagenized *snc2-1D npr1-1* mutant population (Y. Zhang, Yang, et al. 2010). The *npr4-4D* single and *snc2-1D npr4-4D* double mutant were obtained by crossing *npr4-4D snc2-1D npr1-1* with Col-0 wild type plants. The *npr4-4D npr1-1* double mutant was obtained by crossing *npr1-1* with *npr4-4D*. The sextuple mutant *snc2-1D npr1-1 npr4-4D tga256* was obtained by crossing *snc2-1D npr1-1 npr4-4D* with *tga256*. *snc2-1D npr1-1 npr4-4D tga25* and *snc2-1D npr1-1 npr4-4D tga6-1* were isolated from the same population. The *npr1-7* and *npr4-4D npr1-7* mutants were generated by transforming a CRISPR-Cas9 construct expressing two guide RNAs targeting the *NPR1* locus into wild type and *npr4-4D* background. The deletion in *npr1-7* was confirmed by Sanger sequencing. The *NPR1-HA* and *NPR1*^{R432Q}*-HA* transgenic lines were generated by transforming *npr1-1* plants with *Agrobacteria* strains carrying pCambia1305-NPR1-3HA or pCambia1305-NPR1^{R432Q}-3HA constructs, which contain the wild type or mutant *NPR1* gene driven by its own promoter. Plants were grown under 16 h light at 23℃ and 8 h dark at 19℃ for long day conditions and 12 h light at 23℃ and 12 h dark at 19℃ for short day conditions.

2.5.2 Mutant characterization

For gene expression analysis, RNA was isolated from two-week-old seedlings grown on ½ MS media and used for subsequent quantitative reverse transcription PCR (qRT-PCR) analysis. Each experiment was repeated three times with independently grown plants. Briefly, RNA was extracted using the EZ-10 Spin Column Plant RNA Mini-Preps Kit from Biobasic (Canada) and treated with RQ1 RNase-Free DNase (Promega, USA) to remove the genomic DNA contaminations. Reverse transcription was carried out using the EasyScript™ Reverse Transcriptase (ABM, Canada). qPCR was performed using the Takara SYBR Premix Ex (Clontech, USA). Primers for qPCR were described previously (Sun et al. 2015; Zhang, Tessaro, et al. 2003) or listed in Table 2.1.

Analysis of resistance to *H.a.* Noco2 was carried out by spraying two-week-old seedlings with *H.a.* Noco2 spores at a concentration of 5×10^4 spores/mL. Growth of *H.a.* Noco2 was quantified as previously described (Bi et al. 2010). Bacterial infection assays

were carried out by infiltrating two fully grown leaves of four-week-old plants grown under short day conditions.

2.5.3 Genetic mapping of *npr4-4D*

Crude mapping of the *npr4-4D* mutation was carried out using the F2 population of a cross between *npr4-4D snc2-1D npr1-1* (in Col-0 ecotype background) and Landsberg *erecta* (L*er*). The genome of *npr4-4D snc2-1D npr1-1* was re-sequenced using Illumina sequencing to identify single nucleotide polymorphisms between the mutant and wild type. Fine mapping was carried out using F2 population of a cross between *npr4-4D snc2-1D npr1-1* and *snc2-1D npr1-1* using single nucleotide polymorphisms identified by the whole genome sequencing.

To confirm that the *npr4-4D* mutation is responsible for the suppression of the autoimmunity in *snc2-1D npr1-1*, a genomic fragment of *NPR4* was amplified from *npr4-4D* genomic DNA using primers NPR4-KpnI-F and NPR4-SalI-R and cloned into the binary vector pCambia1305. The construct was transformed into *Agrobacteria* strain GV3101 and used to transform *snc2-1D npr1-1* and *npr3-2 npr4-2* plants. A genomic fragment of *NPR3* was amplified using primers NPR3-BamHI-F and NPR3-PstI-R and cloned into binary vector pCambia1305-35S. The $NPR3^{R428Q}$ mutant was generated by overlapping PCR using primers NPR3-RQ-R and NPR3-RQ-F. The resulting constructs were used to transform *snc2-1D npr1-1* plants. The sequence of primers used for cloning is listed in Table 2.1.

2.5.4 Promoter-luciferase Assay

An 1887 bp fragment upstream of *SARD1* coding sequence or a 1075 bp fragment upstream of *WRKY70* coding sequence was cloned into pGreenII0229-LUC-nos vector. Promoter with mutations in the TGACG motif was generated by overlapping PCR. The *35S-NPR3* (pCambia1300-35S-NPR3-3HA) and *35S-NPR4* (pCambia1300-35S-NPR4-3HA) constructs were generated by inserting PCR fragments containing the coding regions of *NPR3* or *NPR4* into pCambia1300-35S-3HA. The $NPR4^{GVK}$ mutation

was generated by overlapping PCR and introduced into the *35S-NPR4* construct. The constructs used in the transcriptional repressor assays were described previously (Tiwari et al. 2006) except that the GUS reporter gene was replaced with a PCR fragment containing the Renilla luciferase reporter gene amplified using primers Rluc-XhoI-F and Rlus-SacI-R. The coding regions of *NPR3*, *NPR4* and the C-terminus region of *NPR4* was amplified from the wild type cDNA and cloned in to pUC19-35S-GD. Primers used for the PCR amplification are listed in Table 2.1 and All constructs were confirmed by Sanger sequencing.

Promoter activity assays were performed in *Arabidopsis* protoplasts by transforming the reporter constructs together with the different effector constructs. Protoplasts were prepared as previously described (Wu et al. 2009). A pUBQ1-driven Renilla luciferase reporter was included in the firefly luciferase assays as internal transfection control. A 35S-driven firefly luciferase reporter was included in the Renilla luciferase assays as internal transfection control. After 16 h incubation, protoplasts were collected and the dual-luciferase assay system (Promega) was used to measure the activity of firefly luciferase and renilla luciferase sequentially using a BioTek™ Synergy™ 2 Multi-Mode Microplate Reader.

2.5.5 Yeast two-hybrid assay

The yeast two-hybrid vectors pBI880 (BD vector) and pBI881 (AD vector) and the constructs pBI880-NPR3 (BD-NPR3), pBI880-NPR4 (BD-NPR4) and pBI881-TGA2 (AD-TGA2) were described previously (Kohalmi Nowak, J., and Crosby, W.L. 1997; Zhang et al. 2006). *TGA2*, *NIMIN1*, *NPR3* and *NPR4* fragments were subcloned into pBI881 or pBI880 to obtain pBI881-NIMIN1 (AD-NIMIN1), pBI881-NPR3 (AD-NPR3), pBI881-NPR4 (AD-NPR4) and pBI880-TGA2 (BD-TGA2). The $NPR4^{R419Q}$ coding sequence was amplified from total cDNA of *npr4-4D* seedlings and the $NPR4^{GVK}$ mutant gene was generated by overlapping PCR. The DNA fragments were inserted into pBI880 to obtain pBI880-$NPR4^{R419Q}$ (BD-$NPR4^{R419Q}$) and pBI880-$NPR4^{GVK}$ (BD-$NPR4^{GVK}$). The *NPR1* coding sequence was amplified by PCR and inserted into modified pBI880/pBI881 vectors with two Sfi I sites. The $NPR1^{R432Q}$ mutation was

introduced by overlapping PCR. All the constructs were confirmed by sequencing and the sequences of primers used for cloning are listed in Table 2.1.

Different combinations of the yeast two-hybrid constructs were co-transformed into the yeast strain YPH1347. Colonies grown on synthetic drop media without Leu and Trp (SD-L-W) were cultured for 20 hr in SD-L-W liquid media. The cultures were then serially diluted and plated on synthetic drop media without Leu, Trp and His (SD-L-W-H) containing 4 mM 3-aminotriazole (3AT). Plates were kept at 30℃ for 2 days before taking photos.

2.5.6 ChIP analysis

ChIP-PCR assays were performed as previously described (Sun et al. 2015). The chromatin complex containing TGA2/5/6 proteins were pulled down using anti-TGA2 antibodies and Protein A Agarose beads (GE). The anti-TGA2 antibody was purified form the serum of Rabbit immunized with recombinant TGA2 protein. The specificity of the TGA2 antibodies was confirmed by western blot using total proteins from wild type and *tga256* mutant plants. The *NPR3-3HA* and *NPR4-3HA* transgenic plants used for ChIP assays were generated by transforming wild type plants with *Agrobacteria* strains carrying pCambia1300-35S-NPR3-3HA or pCambia1300-35S-NPR4-3HA. Twelve-day-old seedlings were sprayed with 50 μM SA in H_2O (plus 0.01% silwet L-77) or H_2O one hour before crosslinking. The chromatin complexes containing NPR3-3HA or NPR4-3HA fusion protein were immunoprecipitated using an anti-HA antibody (Roche) and Protein A/G Agarose beads (GE). The immunoprecipitated DNA was analyzed by qPCR using gene specific primers which were listed in the Table 2.1.

2.5.7 Co-immunoprecipitation

The pCambia1300-35S-NPR4-3FLAG construct was generated by inserting a genomic fragment of NPR4 amplified by PCR using primers NPR4cds-KpnI-F and NPR4cds-BamHI-R into pCambia1300-35S-3flag. The pCambia1300-35S-NPR4^{R419Q}-3FLAG construct was generated similarly using PCR fragments amplified from *npr4-4D*

genomic DNA. Constructs expressing NPR3-FLAG-ZZ and NPR4-FLAG-ZZ fusion proteins were generated by subcloning *NPR3* and *NPR4* genomic fragments into a modified pCambia1305 vector pBASTA-35S-FLAG-ZZ. The coding sequence of Cul3A was amplified from WT cDNA by PCR and cloned into pCambia1300-35S-3HA to obtain pCambia1300-35S-Cul3A-3HA. All the constructs were confirmed by sequencing and the sequences of primers used for cloning are listed in Table 2.1. The constructs were transformed into *Agrobacteria* strain GV3101.

For transient expression of the epitope tagged proteins in *N. benthamiana*, leaves of about four-week-old plants were infiltrated with *Agrobacteria* suspension (OD_{600} = 0.5). Two days later, about 2 g of tissue from the infiltrated area was collected and frozen with liquid nitrogen. The tissue was grinded into powder using a mortar and a pestle. All subsequent steps were carried out on ice or in a 4°C cold room. Briefly, about two volumes of extraction buffer (10% glycerol, 25 mM Tris–HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.15% NP-40, 1mM NaF, 1mM PMSF, 10 mM DTT, 2% PVPP, 1× protease inhibitor cocktail from Roche) were added to each sample to homogenize the powder. The resuspended samples were centrifuged at 14,000 rpm for 10 min and the supernatant was subsequently transferred to 2 ml microcentrifuge tubes. The supernatant was centrifuged again to remove additional debris. Afterwards it was transferred to a new tube containing anti-FLAG-conjugated beads (Sigma) and incubated for 2 h. The beads were collected by centrifugation and washed four times with the extraction buffer. Protein bound to the beads were eluted by adding 1× SDS loading buffer (preheated to 95°C) followed with 5-min incubation at room temperature. The eluted proteins were analyzed by western blot using an anti-FLAG antibody (Sigma) or an anti-HA antibody (Roche).

2.5.8 Recombinant protein expression and purification

The coding sequences of *NPR1* and *NPR4* were amplified by PCR and cloned into a modified pMAL-c2x (NEB) vector to express the His_6-MBP fusion proteins. $NPR4^{R419Q}$ was amplified from the cDNA prepared from *npr4-4D* total RNA. The $NPR1^{R432Q}$ mutation was introduced by overlapping PCR. All the constructs were

confirmed by sequencing and the sequences of primers used for cloning are listed in Table 2.1. For protein expression, the constructs were transformed into the *E. coli* Rosetta2 (DE3) strain. The bacteria were cultured in LB media containing 100 µg/ml Ampicillin and 34 µg/ml chloramphenicol to an OD_{600} of 0.4 at 37°C and then switch to 18°C. One hour after switching, IPTG was added to a final concentration of 0.2 mM to induce protein expression. After incubation at 18°C for 20 hr, the bacteria were collected by centrifugation and stored at -80°C until use.

The recombinant proteins were purified following the procedure described previously (Manohar et al. 2014). The bacteria were resuspended in lysis buffer (50 mM tris pH 7.4, 500 mM NaCl, 10% glycerol, 20 mM Imidazole, 0.1% triton X-100 and 1 mM PMSF) and lysed by sonication. After spinning at 15000 g for 30 min at 4°C, the clear supernatant was applied to an Ni-NTA column and washed with about 40× bed volumes of lysis buffer containing increasing concentrations (20, 30, and 40 mM) of imidazole. Proteins were eluted by adding lysis buffer containing 250 mM of imidazole. The eluted His_6-MBP-NPR1 protein was dialyzed three times with PBS buffer containing 10% glycerol and 0.1% Triton X100 at 4°C. The eluted His_6-MBP-NPR4 protein was treated with 200 mM DTT for 30 min on ice before dialysis against PBS buffer with 10% glycerol, 2mM DTT and 0.1% Triton X100 at 4°C. The protein after dialysis was aliquoted and stored at −80°C until use.

2.5.9 [^{3}H]SA-binding assay

Size exclusion chromatography was used for [^{3}H] SA binding assays as described previously (Manohar et al. 2014). Size exclusion columns were prepared by adding 0.1g of sephadex™ G-25 (GE healthcare) to QIAGEN shredder columns. The columns were pre-equilibrated with PBS buffer containing 0.1% Tween-20 overnight at 4°C, and excess buffer was removed by spinning at 735×g for 2 min. The binding reactions were carried out with 200 nM [^{3}H] SA (American Radiolabelled Chemicals, specific activity 30 Ci/mmol) with or without the presence of unlabeled SA (10,000-fold excess) in 50 µl of PBS buffer. The reaction mixtures were incubated on ice for 1 h, and then loaded to the columns and centrifuged immediately as above. The flow through

was collected and the radioactivity was measured by a scintillation counter (LS6500; Beckman Coulter). The saturation binding experiments were performed using [^{3}H] SA concentration from 6.25 to 800 nM and the dissociation constant (K_d) was calculated by fitting the specific binding data into non-linear model of Michaelis-Menten equation using GraphPad Prism4.

2.5.10 RNA-Seq analysis

For RNA-seq analysis, two-week-old seedlings of *npr1-1*, *npr4-4D*, *npr1-1 npr4-4D* and wild- type plants grown on ½ MS media were sprayed with 50 μM SA and samples were collected 0 or 1 h after treatment with SA. RNA was extracted using RNeasy Mini Kit (Qiagen) with on-column DNase digestion, following the manufacturer's instructions.

Library preparation and RNA-seq were performed by BGI America or Novogene using an Illumina HiSeq 2000 resulting in ~21-25 million reads per sample. Raw RNA-seq reads were subjected to quality checking and trimming to remove adaptor sequences, contamination and low-quality reads. The trimmed reads of each sample were aligned to the publicly available reference genome of *Arabidopsis* (TAIR10, https://support.illumina.com/sequencing/sequencing_software/igenome.html) using HISAT2 version 2.0.4 on default parameters (Kim et al. 2015). SAMtools version 0.1.12 was used to convert SAM files, sort and index BAM files (Li et al. 2009). Read counts were generated for each gene using *summarizeOverlaps* (R package GenomicAlignments) with the following settings: mode = "Union", ignore.strand = TRUE, inter.feature = FALSE, singleEnd = TRUE (Lawrence et al. 2013). R package DESeq2 version 1.16.1 was used to determine differentially expressed genes (Love et al. 2014). Gene Ontology (GO) analysis was performed to search for significantly over- or under-represented GO terms using the R package goseq version 1.28.0 (Young et al. 2010) with TAIR10 GO annotations. Clustering was performed using R package pheatmap version 1.0.8 using rlog transformed counts. Finally, plots were created using R package ggplot2 version 2.2.1.

Table 2.1 Primer used in chapter 2

Primer	5'-3' sequence	Purpose	Vector
NPR4-KpnI-F	ccggGGTACCCATGAGTTTTGCTACTCGTG	Cloning	pCAM1305
NPR4-SalI-R	gcggcgGTCGACtccagagtctgttacaggtt	Cloning	pCAM1305
NPR3-BamHI-F	CGCGGATCCATGGCTACTTTGACTGAGC	Cloning	pCAM1305-35S
NPR3-PstI-R	AAAACTGCAGTGTTGTGTTGTGCAGGTCAT	Cloning	pCAM1305-35S
WRKY701kbpro-KpnI-F	ccggGGTACCtttccgggtgaaagaaaatac	Cloning	pG229-Luc-Nos
WRKY701kbpro-EcoRI-R	ccgGAATTCttgttagttttgaggaagttt	Cloning	pG229-Luc-Nos
W70pro-MT-F	atttaatttgagcttatttaaagctcaccataagcaaaa	Cloning	pG229-Luc-Nos
W70pro-MT-NR	gtgagctttaaataagctcaaattaaatagtgatgaatg	Cloning	pG229-Luc-Nos
NPR4cds-KpnI-F	ccggGGTACCATGGCTGCAACTGCAATAGA	Cloning	pCAM1300-35S-3HA
NPR4cds-StuI-R	gagaAGGCCTTGTTGGATTCTCTAAGGCTTC	Cloning	pCAM1300-35S-3HA
NPR3cds-KpnI-F	ccggGGTACCATGGCTACTTTGACTGAGCCA	Cloning	pCAM1300-35S-3HA
NPR4cds-BamHI-R	cgccgcGGATCCTGTTGGATTCTCTAAGGCTTC	Cloning	pCAM1300-35S-3flag
NPR3-SpeI-R	cccACTAGTTGTTGTGTTGTGCAGGTCATC	Cloning	pCAM1300-35S-3HA
NPR4-GVK-F	GGTAAAGTCGgTgTAAAgGAAACGCCTTATG	Cloning	pCAM1300-35S-3HA
NPR4-GVK-R	CATAAGGCGTTTCcTTTAcAcCGACTTTACC	Cloning	pCAM1300-35S-3HA
NPR3-RQ-R	cagtcaattacCTTGCTTTTCTAGGTACA	Cloning	pCAM1305-35S
NPR3-RQ-F	TGTACCTAGAAAAGCAAGgtaattgactg	Cloning	pCAM1305-35S
NPR1pro-KpnI-F	ccggGGTACCtttatacaatatatgtacgg	Cloning	pCAM1305-3HA
NPR1-BamHI-R	CGCCGCGGATCCCCGACGACGATGAGAGAGTT	Cloning	pCAM1305-3HA
SARD1-PF	AACACCGCTCGAGGGAGATGACTCGAGCTCATA	Cloning	pG229-Luc-Nos
SARD1-PR	CGCGGATCCGGAATTGTTCTGGTGAGTTGT	Cloning	pG229-Luc-Nos
SARD1pro-mutF	tttaaattaaaagtctccctatttattaaaccataaatagattattcg	Cloning	pG229-Luc-Nos
SARD1pro-mutR	Ggtttaataaatagggagacttttaatttaaactccaatttagaaagc	Cloning	pG229-Luc-Nos
pAtUBQ1-HindIII-F	tgcAAGCTTcccgggatatttcacaaatt	Cloning	pUC19
pAtUBQ1-BamHI-R	ggcGGATCCtttgtgtttcgtcttctctc	Cloning	pUC19
Rluc-BamHI-F	ggcGGATCCATGACTTCGAAAGTTTAT	Cloning	pUC19
Rluc-sacI-R	cggGAGCTCTTATTGTTCATTTTTGAG	Cloning	pUC19
NPR1-RQ-F(genomic)	TCGATCTTGAAAATCAAGGTATCTATCAAG	Cloning	pCAM1305-3HA

Table 2.1 Primer used in chapter 2

Primer	5'-3' sequence	Purpose	Vector
NPR1-RQ-R(genomic)	CTTGATAGATACCTTGATTTTCAAGATCGA	Cloning	pCAM1305-3HA
NPR1-NdeISfi1A-F	cggaattcCATATG aGGCCGTCAAGGCCa ATGGAC ACCACCATTGATGG	Cloning	pBI880
NPR1-Sfi1BSacI-R	cgggatccGAGCTC GGCCCATGAGGCCTCACCGAC GACGATGAGAGA	Cloning	pBI880
NPR1CDS-RQ-F	TCGATCTTGAAAATcaaGTTGCACTTGCTC	Cloning	
NPR1CDS-RQ-R	GAGCAAGTGCAACttgATTTTCAAGATCGA	Cloning	
Rluc-Xho1-F	GGATTCCTCGAGATGACTTCGAAAGTTTATGA	Cloning	
Rluc-sacI-R	cggGAGCTCTTATTGTTCATTTTTGAG	Cloning	
NPR4-Sfi1A-F	cgcggatccGGCCGTCAAGGCCaATGGCTGCAACTG CAATAGA	Cloning	pUC19-GD
NPR4-Sfi1B-R	cgcggatccGGCCCATGAGGCCTCATGTTGGATTCT CTAAGG	Cloning	pUC19-GD
NPR4Cter-Sfi1A-F	cgcggatccGGCCGTCAAGGCCaATGTGTAGGAGA CTCACTAG	Cloning	pUC19-GD
NPR3-Sfi1A-F	cgcggatccGGCCGTCAAGGCCaATGGCTACTTTGA CTGAGCC	Cloning	pUC19-GD
NPR3-Sfi1B-R	cgcgaattcGGCCCATGAGGCCTCATGTTGTGTTGT GCAGGTC	Cloning	pUC19-GD
CUL3A-KpnI-F	cggGGTACCtttgttttggattcaggtttcaaaat	cloning	pCAM1300-35S-3HA
CUL3A-StuI-R	gccAGGCCTGGCTAGATAGCGGTAAAGTT	cloning	pCAM1300-35S-3HA
AtPOB1-KpnI-F	cggGGTACCATGAGAGGTACTACTGAGAA	cloning	pBasta-35s-Flag-zz
AtPOB1-SpeI-F	aaggACTAGTAGGATCTGTAGACCTTTTGAT	cloning	pBasta-35s-Flag-zz
WRKY70RT-F	GCCAAATTCCCAAGAAGTTAC	RT	
WRKY70RT-R	CTTGTGATCTTCGGAATCCAT	RT	
NAC004-RT-F	CGATTGAGGAGGAATGGAAA	RT	
NAC004-RT-R	GGACCTTGGCTCACCTCTT	RT	
RLP23-RT-F	ATCAAGGTCCTCTCGGGTTT	RT	
RLP23-RT-R	TATAACCATAGCCGCCTTCG	RT	
MC2-RT-F	GATGAGGAAGAGGAAGTAAACC	RT	
MC2-RT-R	GCTCAACTGTGGTTCCTGAGT	RT	
WRKY51-RT-F	TGGAGGAAGTATGGCAAGAAA	RT	
WRKY51-RT-R	TAAGCTGCATCGTCACCATC	RT	

Table 2.1 Primer used in chapter 2

Primer	5'-3' sequence	Purpose	Vector
FCA2-F	GTTGATGGAACCATCCGAGGATCC	Mapping	
FCA2-R	GGAGCATGGTGCACTCCTCCTAG	Mapping	
T13J8-F	ATGTTCCCAGGCTCCTTCCA	Mapping	
T13J8-R	GAGATGTGGGACAAGTGACC	Mapping	
NPR4-F	gcttcgtaactatgttgagaag	Genotyping	
NPR4-R	atctttcggcctagtgagtc	Genotyping	
NPR3-F	ctccagatgagactgttgtacc	Genotyping	
NPR3-R	cgcggatcctggtgcagtttcatgttgtg	Genotyping	
NPR1_gR1_BsF	ATATATGGTCTCGATTGATTCATCGGAACCTGTTGAGTT	Cloning	pHEE401E
NPR1_gR1_F0	TGATTCATCGGAACCTGTTGAGTTTTAGAGCTAGAAATAGC	Cloning	pHEE401E
NPR1_gR2_R0	AACCAAGCCAGTTGAGTCAAGTCAATCTCTTAGTCGACTCTAC	Cloning	pHEE401E
NPR1_gR2_BsR	ATTATTGGTCTCGAAACCAAGCCAGTTGAGTCAAGTC	Cloning	pHEE401E
SARD1pro0.3kb-chipF	ggaaccgtccatttgtcaac	ChIP-PCR	
SARD1pro0.3kb-chipR	ttcgaagaacgacaaaggaaa	ChIP-PCR	
CBP60Gpro0.15kb-chipF	gtttcactgctgcttcgtca	ChIP-PCR	
CBP60Gpro0.15kb-chipR	GGCTGTTCCGAATCTTCATt	ChIP-PCR	
WRKY70-P-FP	AAGCAAAAGAAATGGGTGGA	ChIP-PCR	
WRKY70-P-RP	TTTCCTCTTGGTGTGGTTTG	ChIP-PCR	

3 A forward genetic screen to identify novel components in the SNC2-mediated plant resistance pathway

3.1 Summary

Plants utilize a large number of immune receptors to recognize pathogens and activate defence responses. A small number of these receptors belong to the receptor-like protein family. Previously, we showed that a gain-of-function mutation in the receptor-like protein SNC2 leads to constitutive activation of defence responses in *snc2-1D* mutant plants. To identify additional defence signaling components downstream of SNC2, we carried out a suppressor screen in the *Arabidopsis eds5-3 snc2-1D npr1-1* mutant background. Four new mutants were identified from this screen. Map-based cloning of two of the suppressor genes, *BDA5* and *BDA6*, showed that they encode FMO1 and ALD1 respectively, which are involved in biosynthesis of N-Hydroxypipecolic Acid (NHP) and Pip. Loss-of-function mutations in *FMO1* or *ALD1* can suppress the dwarf morphology and constitutive defence responses in *eds5-3 snc2-1D npr1-1* and also result in enhanced susceptibility to virulent oomycete pathogens. These data suggest that FMO1 and ALD1 are positive regulators functioning downstream of SNC2 to regulate plant immunity.

3.2 Introduction

RLPs are plasma-membrane-localized receptors that typically consist of an extracellular leucine-rich repeat domain, a transmembrane domain, and a short cytoplasmatic tail (Dangl and Jones 2001). In *Arabidopsis thaliana*, there are 57 putative RLP-encoding genes (Wang et al. 2008). CLV2 and TMM are the first two well-studied RLPs involved in plant development. CLAVATA2 (CLV2) was found to be crucial for maintaining a balanced meristematic stem cell population (Jeong et al. 1999). TOO MANY MOUTHS (TMM), is involved in regulation of stomatal distribution across the epidermis (Nadeau and Sack 2002).

In several plant species, RLPs have also been found to play important roles in disease resistance. *Cf-9*, the first RLP gene identified from tomato (*Solanum lycopersicum*) mediates resistance against strains of the leaf mold fungus *Cladosporium fulvum* (Jones et al. 1994). Several other *Cf* resistance genes have been cloned from tomato that all to belong to the RLP gene family (Dixon et al. 1996; Dixon et al. 1998; Thomas et al. 1997; Takken et al. 1999). In apple (*Malus domestica*), the RLP HcrVf-2 confers resistance against the apple scab fungus *Venturia inaequalis* (Belfanti et al. 2004). Emerging studies came out in recent years with newly discovered RLPs involved in plant immunity, including *Arabidopsis* RLP23, RLP30, RLP1/ReMax (Receptor of eMax) and tobacco NbCSPR (Jehle et al. 2013; Zhang et al. 2013; Albert et al. 2015; Saur et al. 2016).

Unlike most RLPs, SNC2 is highly conserved in plants (Fritz-Laylin et al. 2005). A gain-of-function mutation in *SNC2* (*snc2-1D*) leads to autoimmunity (Y. Zhang, Yang, et al. 2010). The *snc2-1D* mutant provides a unique system to perform genetic analysis of RLP-mediated immunity in *Arabidopsis*. Epistasis analysis showed that SNC2-mediated defence responses do not require common signaling components in NLR-mediated signaling, such as EDS1, PAD4 and NDR1 (Y. Zhang, Yang, et al. 2010). This suggests SNC2-mediated resistance pathways are distinct from the NLRs. In addition, mutation in the SA-transporter EDS5 (*eds5-3*) only partially blocks the expression of the defence marker gene *PR2* and has limited effects on the *snc2-1D* dwarf morphology, suggesting that both SA-dependent and SA-independent resistance pathways are activated downstream of SNC2 (Y. Zhang, Yang, et al. 2010).

On the other hand, the partial suppression of dwarfism by the *eds5-3* mutation largely recovers the sterile phenotype of the original *snc2-1D npr1-1* double mutant. The *eds5-3 snc2-1D npr1-1* triple mutant sets a large number of seeds, which makes it a useful genetic material to perform a more saturated suppressor screen.

3.3 Results

3.3.1 *eds5-3 snc2-1D npr1-1* suppressor screen

To identify suppressors of *eds5-3 snc2-1D npr1-1*, approximately 10,000 *eds5-3 snc2-1D npr1-1* seeds were treated with ethyl methane sulfonate (EMS). Roughly 4,000 M1 plants were allowed to self-fertilize and harvested into 250 pools with 16 plants per pool. The primary screen was carried out using 500 M2 plants per pool to look for mutants displaying wild-type like morphology. In total, 158 putative *eds5-3 snc2-1D npr1-1* suppressors were isolated from the primary screen.

Among the 158 M2 mutant lines that were identified based on morphology, one of the largest plants from each pool was picked and checked for heritability. In the M3 generation, 71 lines showed heritable suppression of the dwarf morphology. To exclude the possibility of wild type contamination, DNA was extracted from each line and subjected to sanger sequencing analysis on *SNC2*. Among the 71 lines, 5 lines do not contain the original *snc2-1D* mutation and therefore were excluded from further analysis. Meanwhile, 13 lines of intragenic suppressors were identified by sequencing of *SNC2*. The mutations are clustered in the LRR domains and a region of 60 amino acids before the LRR domain of the SNC2 protein (Figure 3.1A). As mutations in *BDA1* or *WRKY70* were previously reported to suppress the autoimmunity in *snc2-1D npr1-1* plants (Y. Zhang, Yang, et al. 2010; Yang et al. 2012), the remaining 53 lines were subjected to additional sequencing of *BDA1* and *WRKY70*. 19 mutant lines were found to contain mutations in BDA1, with mutations mostly occuring in the ankyrin repeat domain and the linker between ankyrin repeat domain and transmembrane domain (Figure 3.1B). 2 mutant lines contain mutations in WRKY70 (Figure 3.1 C-D).

Excluding mutants with mutations in *SNC2*, *BDA1* or *WRKY70*, 31 lines emerged as potential novel suppressors. Among them, 7 wild-type like lines were chosen to perform further genetic analysis.

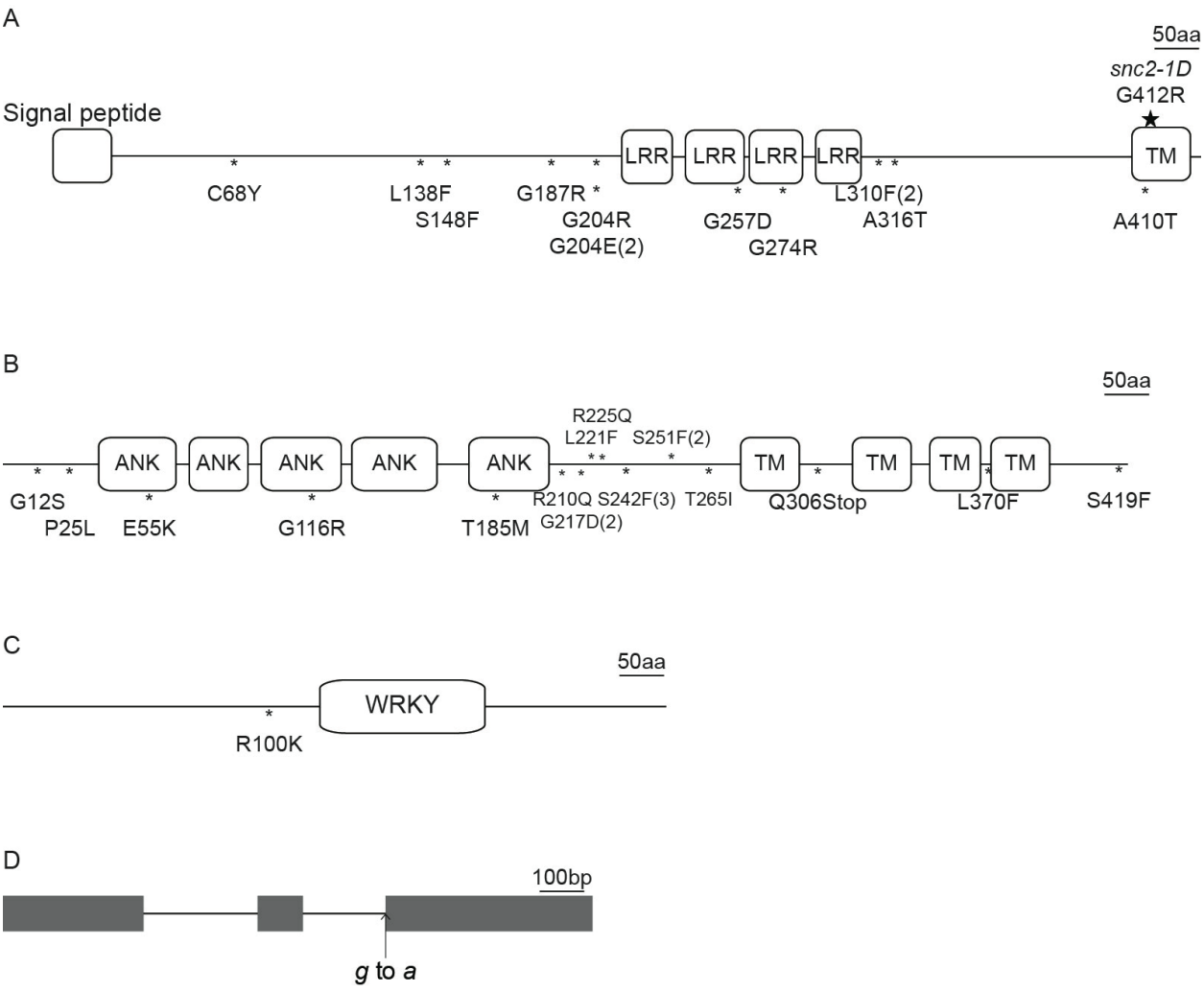

Figure 3.1 Map of known gene mutations.

(A) Map of thirteen intragenic *SNC2* mutations. LRR, leucine rich repeat; TM, transmembrane motif. The G204E and L310F mutations were found twice in mutants from different M1 pool.

(B) Map of nineteen *BDA1* mutations. ANK, ankyrin repeat. TM, transmembrane motif. The G217D and S251F mutations were found twice and S242F mutation was found three times in mutants from different M1 pool.

(C-D) Map of the *WRKY70* mutations. Mutation in (D) occurs in the junction of the second intron and the third exon.

3.3.2 Four novel *bda* mutants suppress autoimmunity in *eds5-3 snc2-1D npr1-1* plants

To determine whether the mutation in each mutant is dominant or recessive, backcrosses were performed between each mutant and *eds5-3 snc2-1D npr1-1*. F1 plants are homozygous for the background mutations (*eds5-3 snc2-1D npr1-1*) but heterozygous for the mutation of the suppressor. Therefore, a wild-type like morphology observed in the F1 progeny indicates dominant mutations, while a dwarf morphology indicates recessive mutations. Among the 7 *bda* mutants, only one mutant contains a dominant mutation (Figure 3.2A). Allelism tests and crude mapping revealed that the 7 mutants fall into four complementation groups, named *bda3-1D*, *bda5*, *bda6*, and *bda7* (Figure 3.2 B-E).

To further characterize these *bda* mutants, defence-related phenotypes including *PR* gene expression and resistance to *H.a.* Noco2 were assessed. All the mutants showed almost complete suppression of elevated *PR2* gene expression except that the *bda5-1 eds5-3 snc2-1D npr1-1* plants displayed a partial reduction (Figure 3.2F). Consistently, the enhanced resistance to the virulent oomycete pathogen *H.a.* Noco2 in *eds5-3 snc2-1D npr1-1* plants is fully suppressed by *bda3-1D*, *bda6*, and *bda7* mutations, and partially suppressed by *bda5-1* mutation (Figure 3.2 I-K). Taken together, the *bda3-1D*, *bda5-1*, *bda6*, and *bda7* mutations suppress the dwarf morphology as well as constitutive defence responses in the *eds5-3 snc2-1D npr1-1* background.

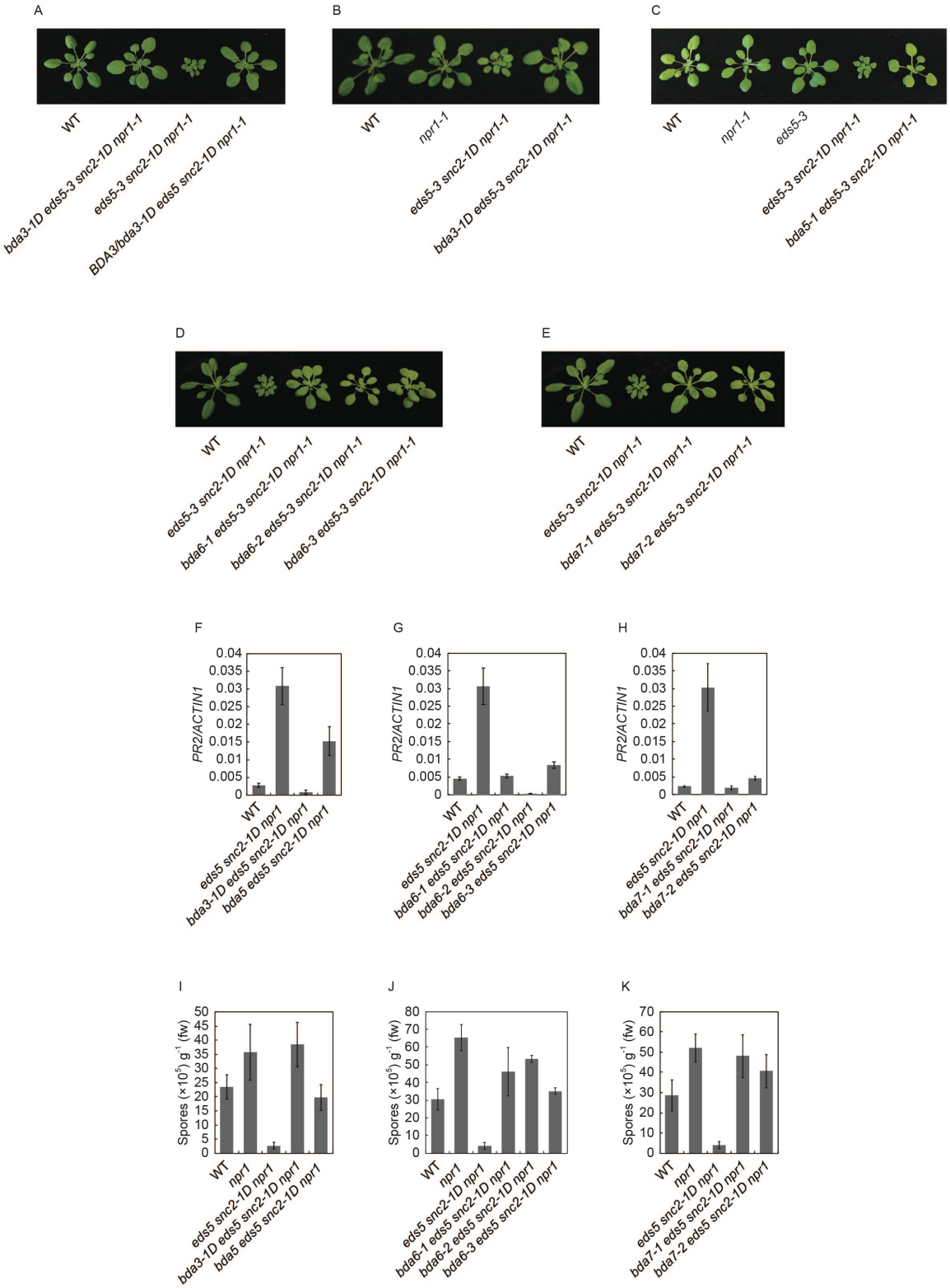

Figure 3.2 *bad3-1D*, *bda5-1*, *bda6* and *bda7* suppress the constitutive defense responses in *eds5-3 snc2-1D npr1-1*.

(A) Morphology of wild type (WT), *bda3-1D eds5-3 snc2-1D npr1-1*, *eds5-3 snc2-1D npr1-1* and *BDA3*/*bda3-1D eds5-3 snc2-1D npr1-1* heterozygous plants. Plants were grown on soil and photographed four weeks after planting.
(B-E) Morphology of *bda3-1D eds5-3 snc2-1D npr1-1* (B), *bda5-1 eds5-3 snc2-1D npr1-1*(C), *bda6 eds5-3 snc2-1D npr1-1* (D), and *bda7 eds5-3 snc2-1D npr1-1* (E) and control genotypes. Plants were grown on soil and photographed four weeks after planting.
(F-H) Expression of *PR2* in wild type (WT), *eds5-3 snc2-1D npr1-1* and *bda3-1D eds5-3 snc2-1D npr1-1* (F), *bda5-1 eds5-3 snc2-1D npr1-1*(F), *bda6 eds5-3 snc2-1D npr1-1* (G), and *bda7 eds5-3 snc2-1D npr1-1* (H). Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.
(I-K) Growth of *H.a.* Noco2 on wild type (WT), *npr1-1*, *eds5-3 snc2-1D npr1-1* and *bda3-1D eds5-3 snc2-1D npr1-1* (I), *bda5-1 eds5-3 snc2-1D npr1-1*(I), *bda6 eds5-3 snc2-1D npr1-1* (J), and *bda7 eds5-3 snc2-1D npr1-1* (K). Two-week-old seedlings were sprayed with spores of *H.a.* Noco2 (5×10^4 spores/ml). Infection was scored seven days after inoculation.

3.3.3 *BDA6* and *BDA7* encode essential enzymes involved in SAR

Crude mapping revealed that three alleles of *bda6* all showed genetic linkage with the *FMO1* locus on Chromosome 1, while two alleles of *bda7* showed linkage on Chromosome 2 where *ALD1* is located. Sequencing analysis of *FMO1* showed that *bda6-1* and *bda6-2* mutants contain missense mutations in FMO1 and *bda6-3* contains a G to A mutation in the junction of the third exon and intron of *FMO1* (Figure 3.3A), which probably affects the intron splicing of FMO1. To confirm that loss of function of FMO1 results in suppression of the *snc2-1D* mutant phenotype, a T-DNA allele with an insertion in the fourth exon of FMO1 (Figure 3.3A) was crossed into *eds5-3 snc2-1D npr1-1* plants. The *fmo1 eds5-3 snc2-1D npr1-1* quadruple mutant showed a similar morphology as the three *bda6* alleles, with almost complete suppression of the dwarf morphology of *eds5-3 snc2-1D npr1-1* plants (Figure 3.3B). Additionally, the elevated *PR2* gene expression and enhanced disease resistance against *H.a.* Noco2 were also suppressed by the T-DNA insertion mutation (Figure 3.3C-D).

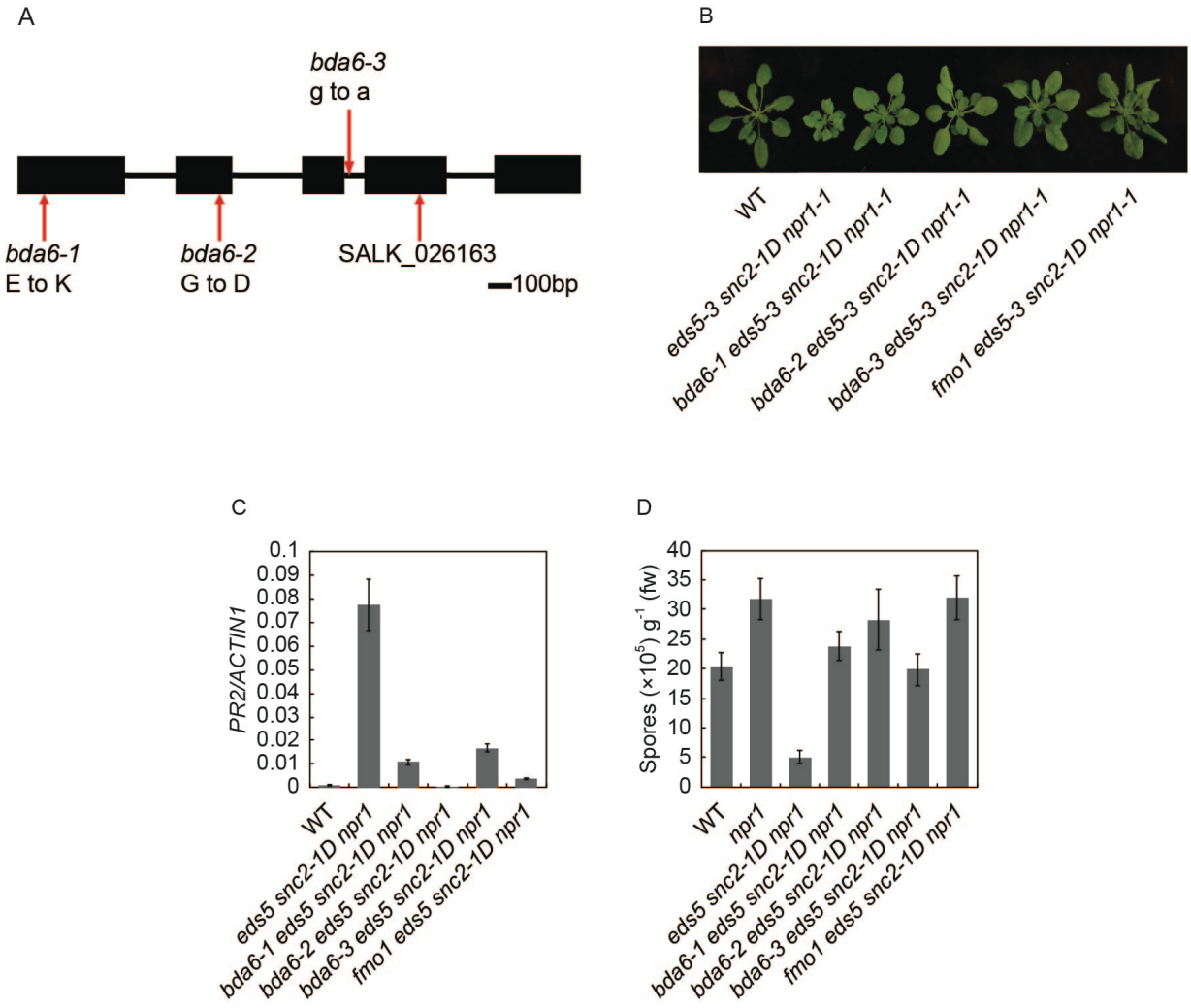

Figure 3.3 *BDA6* encodes FMO1.

(A) Map of the *bda6* mutations and the T-DNA insertion position.

(B) Morphology of wild-type (WT), *eds5-3 snc2-1D npr1-1*, three *bda6 eds5-3 snc2-1D npr1-1* alleles and *fmo1 eds5-3 snc2-1D npr1-1*. Plants were grown on soil and photographed four weeks after planting.

(C) Expression of *PR2* in wild type (WT), *eds5-3 snc2-1D npr1-1*, three *bda6 eds5-3 snc2-1D npr1-1* alleles and *fmo1 eds5-3 snc2-1D npr1-1*. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.

(D) Growth of *H.a.* Noco2 on wild type (WT), *npr1-1*, *eds5-3 snc2-1D npr1-1*, three *bda6 eds5-3 snc2-1D npr1-1* alleles and *fmo1 eds5-3 snc2-1D npr1-1*. Two-week-old

seedlings were sprayed with spores of *H.a.* Noco2 (5×10^4 spores/ml). Infection was scored seven days after inoculation.

In parallel, sequencing analysis performed on *ALD1* showed that *bda7-1* and *bda7-2* plants contain missense mutations in ALD1 (Figure 3.4A). To confirm that *BDA7* encodes ALD1, a T-DNA allele with an insertion in the first exon of ALD1 was crossed into *eds5-3 snc2-1D npr1-1* plants. The *ald1 eds5-3 snc2-1D npr1-1* quadruple mutant showed similar morphology as the plants with the two *bda7* alleles, with almost complete suppression of the dwarf morphology of *eds5-3 snc2-1D npr1-1* plants (Figure 3.4B). Consistently, the elevated *PR2* gene expression and enhanced disease resistance against *H.a.* Noco2 were also suppressed by introducing the *ald1* mutation (Figure 3.4 C-D).

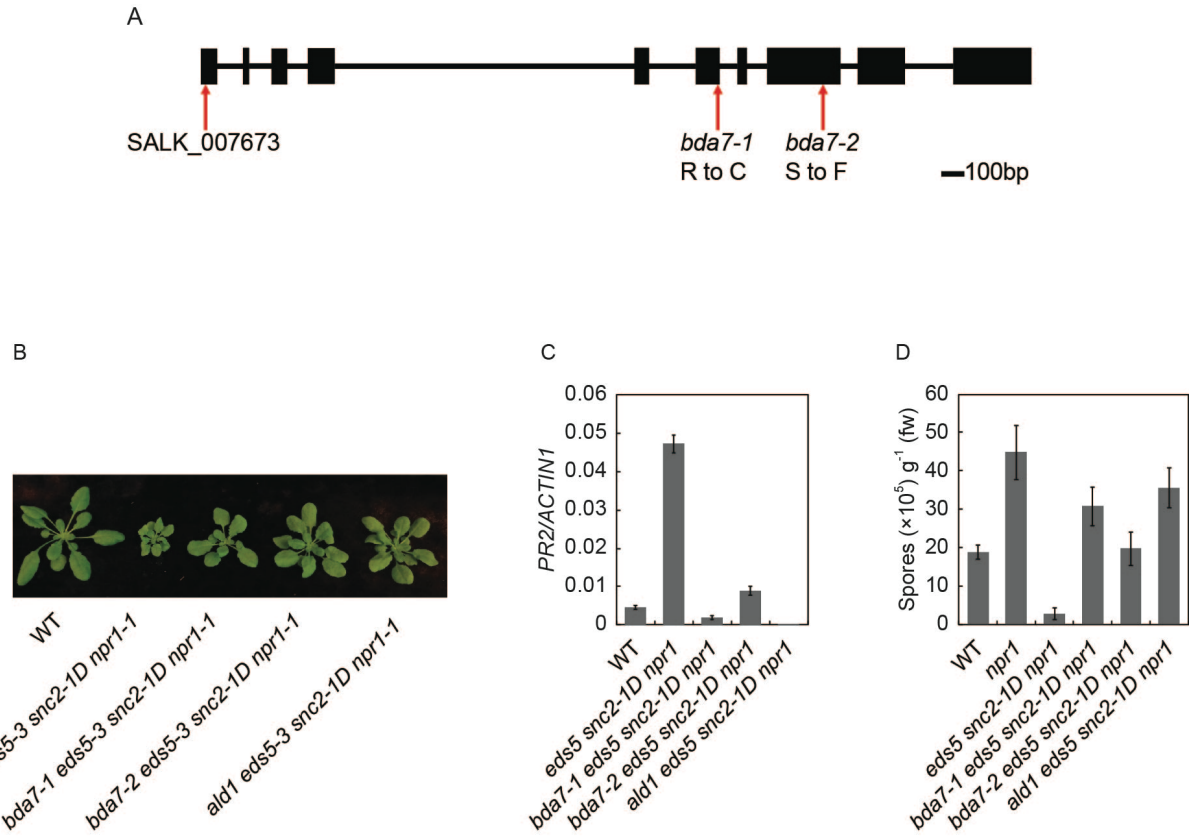

Figure 3.4 *BDA7* encodes ALD1.

(A) Map of the *bda7* mutations and the T-DNA insertion position.

(B) Morphology of wild-type (WT), *eds5-3 snc2-1D npr1-1*, two *bda7 eds5-3 snc2-1D npr1-1* alleles and *fmo1 eds5-3 snc2-1D npr1-1*. Plants were grown on soil and photographed four weeks after planting.
(C) Expression of *PR2* in wild type (WT), *eds5-3 snc2-1D npr1-1*, two *bda7 eds5-3 snc2-1D npr1-1* alleles and *ald1 eds5-3 snc2-1D npr1-1*. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three repeats.
(D) Growth of *H.a.* Noco2 on wild type (WT), *npr1-1*, *eds5-3 snc2-1D npr1-1*, two *bda6 eds5-3 snc2-1D npr1-1* alleles and *ald1 eds5-3 snc2-1D npr1-1*. Two-week-old seedlings were sprayed with spores of *H.a.* Noco2 (5×10^4 spores/ml). Infection was scored seven days after inoculation.

3.4 Discussion

Here we report the suppressor screen of *eds5-3 snc2-1D npr1-1* plants to identify novel components involved in resistance pathways downstream of SNC2. From the screen, we identified four novel *bda* mutants showing various degree of suppression of autoimmunity in *eds5-3 snc2-1D npr1-1* plants. *bda3-1D* completely suppresses and *bda5* only partially suppresses the phenotypes of the *eds5-3 snc2-1D npr1-1* triple mutant. Another 5 mutants fall into two complementation groups, *bda6* and *bda7*. Sequencing analysis showed that they contain mutations in *FMO1* and *ALD1* respectively. Further studies with T-DNA insertion alleles confirmed that FMO1 and ALD1 are positive regulators downstream of SNC2.

ALD1 has been known to be an essential component in basal resistance and SAR (Song et al. 2004; Jing et al. 2011; Cecchini et al. 2015). ALD1 has been shown to function as an aminotransferase, converting lysine to the precursor of Pip, Δ1-piperideine-2-carboxylic acid (P2C) (Ding et al. 2016; Hartmann et al. 2017).

Suppression of *eds5-3 snc2-1D npr1*-1 by *ald1* suggests that Pip plays an important role in SNC2-mediated defence responses, which is consistent with a previous report that SA and Pip act both independently and synergistically in *Arabidopsis* (Bernsdorff et al. 2016).

FMO1 is also known to play key roles in basal resistance and SAR (Koch et al. 2006; Mishina and Zeier 2006). A very recent study showed that FMO1 functions as a pipecolate N-hydroxylase, catalyzing the biochemical conversion of Pipecolic acid to NHP (Hartmann et al. 2018). Epitasis analysis indicated that *fmo1* and *eds5-3* mutations have additive effect on the suppression of *snc2-1D* autoimmunity (Figure 3.5). This is consistent with a previous report that mutations in *SID2* and *FMO1* have additive effect on RPP2-mediated resistance against *H.a.* Cala2 (Bartsch et al. 2006). These data suggest that in general FMO1 functions in a defence pathway in parallel with SA. Previously it was shown that the pathogen-induced Pip level is significantly higher in a *fmo1* mutant compared to WT (Bernsdorff et al. 2016; Ding et al. 2016). This suggests that FMO1 may be involved in the synthesis of a defence signal molecule derived from Pip.

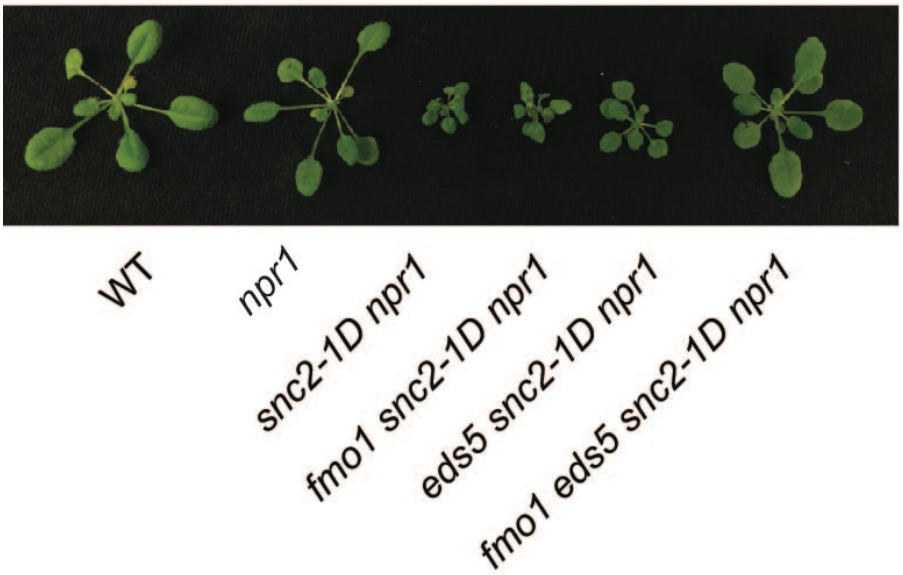

Figure 3.5 *fmo1* and *eds5-3* have additive effects on the suppression of the autoimmune phenotypes of *snc2-1D*.
Morphology of indicated genotypes. Plants were grown on soil and photographed four weeks after planting.

The mechanism by which FMO1 and ALD1 contribute to SNC2-mediated signaling remains to be explored. One possibility is they are regulated by transcription factors since the transcripts of *ALD1* and *FMO1* are both highly upregulated upon pathogen treatment. ChIP-seq analysis identified both *ALD1* and *FMO1* as direct targets of transcription factors SARD1 and CBP60g. This is further supported by the suppression of the autoimmunity in *snc2-1D* by a *sard1 cbp60g* double mutant (Sun et al. 2015), indicating that SARD1 and CBP60g are also positive regulators downstream of SNC2.

The cloning of *BDA3* and *BDA5* indicate that they both encode novel components in plant immunity. *BDA3* encodes a clathrin assembly protein-like protein while BDA5 is potentially involved in post-transcriptional modification. Similar to FMO1 and ALD1, BDA5 also acts additively with EDS5 in suppression of the autoimmunity of *snc2-1D npr1-1*. However, the suppression of *bda3-1D* is independent of EDS5 (Figure 3.6). These preliminary genetic data could guide future analyses of these two proteins to determine how they're involved in SNC2-mediated resistance or plant immunity in general.

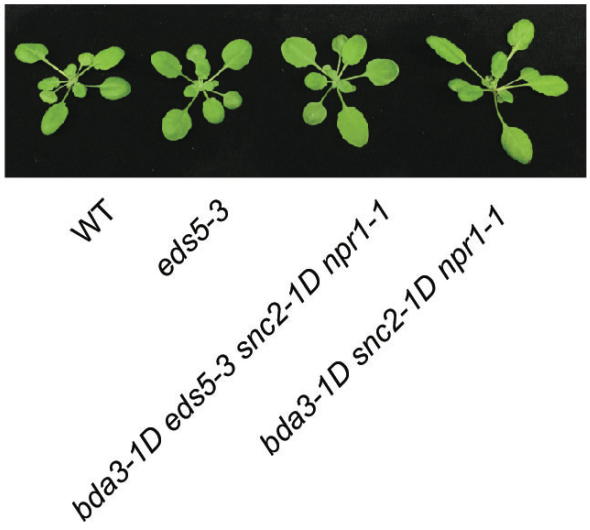

Figure 3.6 BDA3 functions independent of EDS5 downstream of SNC2.
Morphology of wild-type (WT), eds5-3, *bda3-1D eds5-3 snc2-1D npr1-1*, and *bda3-1D snc2-1D npr1-1*. Plants were grown on soil and photographed four weeks after planting.

3.5 Material and methods

3.5.1 Plant materials and growth conditions

All *Arabidopsis thaliana* mutants used are in the Columbia (Col-0) ecotype. The *npr1-1*, *eds5-3*, *snc2-1D*, *snc2-1D npr1-1*, *eds5-3 snc2-1D npr1-1* mutants were reported previously (Cao et al. 1994; Nawrath et al. 2002; Sun et al. 2015; Y. Zhang, Yang, et al. 2010). *fmo1* (salk_026163) and *ald1-T2* (SALK_007673) were obtained from the Arabidopsis Biological Resource Center. The *fmo1 eds5-3 snc2-1D npr1-1* and *ald1-T2 eds5-3 snc2-1D npr1-1* quadruple mutant was obtained by crossing *eds5-3 snc2-1D npr1-1* with *fmo1* (salk_026163) or *ald1-T2* respectively. The *snc2-1D fmo1* double mutant and *snc2-1D npr1-1 fmo1* triple mutant were isolated from the same population as *fmo1 eds5-3 snc2-1D npr1-1*. The *bda3-1D snc2-1D npr1-1* triple mutant was obtained by crossing *bda3-1D eds5-3 snc2-1D npr1-1* with Col-0 wild type plants. Plants were grown under 16 h light at 23℃ and 8 h dark at 19℃.

3.5.2 Mutant Characterization

For gene expression analysis, RNA was isolated from two-week-old seedlings grown on ½ MS media and used for subsequent quantitative reverse transcription PCR (qRT-PCR) analysis. Each experiment was repeated three times with independently grown plants. Briefly, RNA was extracted using the EZ-10 Spin Column Plant RNA Mini-Preps Kit from Biobasic (Canada) and treated with RQ1 RNase-Free DNase (Promega, USA) to remove the genomic DNA contaminations. Reverse transcription was carried out using the EasyScript™ Reverse Transcriptase (ABM, Canada). qPCR was performed using the Takara SYBR Premix Ex (Clontech, USA).

Analysis of resistance to *H.a.* Noco2 was carried out by spraying two-week-old seedlings with *H.a.* Noco2 spores at a concentration of 5×10^4 spores/ml. Growth of *H.a.* Noco2 was quantified as previously described (Bi et al. 2010). Bacterial infection assays were carried out by infiltrating two fully grown leaves of four-week-old plants grown under short day conditions.

3.5.3 Cloning of *bda* mutants

Crude mapping of the *bda* mutations was carried out using the F2 population of a cross between *bda3-1D/bda5/bda6/bda7 eds5-3 snc2-1D npr1-1* (in Col-0 ecotype background) and Landsberg *erecta* (L*er*).

Fine mapping was carried out on *bda3-1D* and *bda5* using F3 population from F2 lines which are heterozygous for the mutation (i.e. heterozygous at both flanking markers) and are homozygous at the *SNC2* locus (*snc2-1D*) and *EDS5* locus. When the mutation is narrowed down to 1Mb, the genome of *bda3-1D eds5-3 snc2-1D npr1-1* and *bda5-1 eds5-3 snc2-1D npr1-1* was re-sequenced using Illumina sequencing to identify single nucleotide polymorphisms between the mutant and wild type.

Based on chromosome linkage identified in crude mapping, *bda6* and *bda7* were subjected to sequencing analysis of *FMO1* and *ALD1* respectively.

The sequences of primers used for crude mapping and sequencing analysis are listed in

Table 3.1 Primer used in chapter 3

Primer	5'-3' sequence	Purpose
SNC2-F	GAACCGGTTCGGTTATTCTC	sequencing
SNC2-R	CAACTGTCACATGACCCATC	sequencing
BDA1-F1	CATAACCTTAAGCACCTACAG	sequencing
BDA1-F2	TACCACCGGACATTTGTATG	sequencing
BDA1-R1	GTCAATAGACTCACTACTCAG	sequencing
WRKY70-F1	ACAGTACATACACTCATTAGAG	sequencing
WRKY70-R2	CACACACTTCTCTTCTTTCC	sequencing
WRKY70-F3	AGCTCAGACCACATTTATGG	sequencing
FMO1-F4	ATCCTTGACCAAGGTCATAC	sequencing
FMO1-F6	CCACTGAGGAGAGTAGAAGC	sequencing
FMO1-R1	GACGTTCCAAGAATACCAGC	sequencing
FMO1-R5	CCATTCCTCTCCTC	sequencing
ALD1-F3	GGTTATTGGTACTTACTTGGAG	sequencing genotyping
ALD1-F5	TGGTCATAGCAAATGCATCG	sequencing
ALD1-F4	GTATCAGATGGTGCACAAAG	sequencing

Table 3.1 Primer used in chapter 3

Primer	5'-3' sequence	Purpose
ALD1-R1	GGGTTTAGGTCGGATGAATA	sequencing
ALD1-R2	GGTAGGATCTTGCACAGCAA	sequencing
ALD1-R6	ATTATGGTACAAGAGGTGGAAG	sequencing
ALD1-R8	GAAGAAATACTCTATCCGGG	sequencing genotyping
F13K23IND-F	TTTATTTCACACATAGTGCAG	crude mapping
F13K23IND-R	GGAGATTTAGGGGATTACGAGATCG	crude mapping
F14M2-F	CGCATACGTGTCACCGTGAG	crude mapping
F14M2-R	TGTCCGGGACTGCCTTTAGC	crude mapping
T2E12-F	TGGTGTTATAATCATGAAGC	crude mapping
T2E12-R	GTGTTCCATTTTGGTACTTAG	crude mapping
T12J2-F	TGAACCCTTATAATATGGCTGGC	crude mapping
T12J2-R	GGTAAGCAAGGAAAGGAACAATTC	crude mapping
F27D4-F	AGAGTCTTAAGAGTCTCAAGAAGC	crude mapping
F27D4-R	TAGAATCGCAAGAAGAGTACG	crude mapping
T16B12-F	CGAACTAAAGCAATCGATCAG	crude mapping
T16B12-R	GCTAGGGTGACTAACACATG	crude mapping
MIE1-F	CTAAGTTCTTCCACCATCTG	crude mapping
MIE1-R	CAAGGAGCATCTAGCCAGAG	crude mapping
T13J10-F	ATTCGGACAAGATCGGTGC	crude mapping
T13J10-R	TGATTCTTCTGAGCATAGAG	crude mapping
F24B22-F	GTGTTGTGTATGTCCTGAGC	crude mapping
F24B22-R	CCTAAAGTACAATGCCAAGACG	crude mapping
T13D4-F	CATACCAAGCCTACGTCAAC	crude mapping
T13D4-R	AAACTCCCTGGATCAGGCAG	crude mapping
FCA5-F	AATGCGGTGTTACCCATGGC	crude mapping
FCA5-R	ACTCTTCCGATAAACTTCCTC	crude mapping
T13J8-F	ATGTTCCCAGGCTCCTTCCA	crude mapping
T13J8-R	GAGATGTGGGACAAGTGACC	crude mapping
F19H22-F	ATGACGAGGCTAGAAGGTGG	crude mapping
F19H22-R	GGGTTCAATCTTCTCATCCG	crude mapping
T9L3-F	GTAACGTATGCATGGTTTG	crude mapping
T9L3-R	AAGTTTTGGTTAGATTACAC	crude mapping
F3F24-F	CTAAATGCACCATCACCGTG	crude mapping
F3F24-R	CTTGCGATTTGAAATCTGTTACC	crude mapping
K19E20-F	GACAAGAACCACATGAGAGC	crude mapping
K19E20-R	GTTATGTGTACACTTCAGGTC	crude mapping

Table 3.1 Primer used in chapter 3

Primer	5'-3' sequence	Purpose
MUB3-F	AATAGATCAAAGCCTGGCTG	crude mapping
MUB3-R	GATTCCTTTGCTTACCACAC	crude mapping
F3N11-F	ATGTAAGTACCAAGATCACC	crude mapping
F3N11-R	AATCAGATACTGTCGCCATC	crude mapping
T9J22-F	GGACACACCTCACATAAGTC	fine mapping
T9J22-R	ACTCCTACATGgtttgtgac	fine mapping
F13M23-F	gtgtgtggtttttacgcttg	fine mapping
F13M23-R	tgtcggtaaaccctagacac	fine mapping
M4I22-F	atttccaccactttcatcgg	fine mapping
M4I22-R	acacatttcgtgaactttgac	fine mapping
F28A21-F	aagcacattcaaacaaaatctcc	fine mapping
F28A21-R	gtttcttgatatggccaagc	fine mapping
FMO1-TDNA-F	CTCTCTTCTGGTTAGTCATC	genotyping
FMO1-TDNA-R	GGCTTCCACTTGTACCACTG	genotyping

4. Conclusions and future directions

The main goal of my Ph.D. project was to further dissect the signaling pathways downstream of the *Arabidopsis* immune receptor SNC2. The gain-of-function *snc2-1D* mutant displays autoimmune phenotypes including enhanced *PR* gene expression, elevated levels of salicylic acid and reduced pathogen growth. Additionally, the constitutive defence responses in *snc2-1D* plants lead to a dwarf morphology with dark green and curly leaves. These phenotypes provide a unique system to perform genetic analyses to study SNC2-mediated immunity in *Arabidopsis*. For example, a previous *snc2-1D npr1-1* suppressor screen resulted in successful identification of BDA1 and WRKY70 as positive regulators downstream of SNC2.

In my Ph.D. study, I fully characterized another suppressor mutant isolated from the *snc2-1D npr1-1* screen, *bda4-1D*. Map-based cloning revealed that *bda4-1D* contains a gain-of-function mutation in *NPR4*. NPR4, as well as its close homolog NPR3, were previously identified as redundant negative regulators in plant immunity. The *npr3-1 npr4-3* knockout mutant shows elevated *PR* gene expression and enhanced disease resistance against *P.s.m.* ES4326 and *H.a.* Noco2 (Zhang et al. 2006). In contrast, the *npr4-4D* single mutant exhibits enhanced disease susceptibility, further supporting the idea that NPR4 functions as a negative regulator downstream of SNC2.

Epistasis analysis showed that *npr1-1* and *npr4-4D* have additive effects on the suppression of the autoimmune phenotypes of *snc2-1D*, indicating NPR1 and NPR3/4 function independently downstream of SNC2. This is further confirmed by the analysis of the *npr1-1 npr4-4D* double mutant, which is always more susceptible to pathogens than the single mutants. NPR1 and NPR3/NPR4 have all been shown to interact with TGA transcription factors (Zhang et al. 1999; Liu et al. 2005; Zhang et al. 2006). NPR1 functions as a transcriptional activator whereas NPR3/4 serve as transcriptional repressors downstream of SNC2 to repress *SARD1* and *WRKY70*, both encoding essential positive regulators downstream of SNC2. Furthermore, the repression activity is fully dependent on TGA transcription factors. Consistently, repression of defence responses in *snc2-1D npr1-1* plants by *npr4-4D* also requires TGA transcription factors.

NPR1 and NPR3/NPR4 were previously shown to bind SA and proposed as SA receptors (Wu et al. 2012; Fu et al. 2012; Klessig et al. 2016). Interestingly, SA treatment releases the repression activity of NPR3 and NPR4. The *npr4-4D* mutation results in loss of SA-binding activity and leads to insensitivity of SA and its analog INA. RNA-seq analysis revealed that NPR1 and NPR4 act independently in the regulation of SA-induced gene expression. The complete suppression of the autoimmune phenotypes of *snc2-1D* by *npr1-1* and *npr4-4D* indicates that SA perception is essential in SNC2-mediated resistance pathways. Collectively, our data showed that both de-repression and activation of SA-responsive genes are important to plant immunity.

On the other hand, overexpression of *NPR1* has been shown to enhance broad-spectrum disease resistance in *Arabidopsis*, rice and wheat, suggesting the importance of NPR1-mediated defense mechanism during the course of evolution (Cao et al. 1998; Chern et al. 2005; Makandar et al. 2006). These studies have led to strategies of engineering resistant crops through ectopic transcription of *NPR1*. However, enhanced resistance obtained through such strategies is often associated with substantial penalties to fitness. For example, the overexpression of *OsNPR1*/*NH1* in rice spontaneously activated resistant genes and resulted in a lesion-mimic phenotype (Chern et al. 2005).

In contrast to NPR1, NPR3 and NPR4 function as negative regulators in plant immunity. Knockout mutants of *NPR3* and *NPR4* in *Arabidopsis* showed enhanced disease resistance against pathogens but without any significant morphology change, such as the size of the plants or reproductions. As NPR3 and NPR4 are also conserved in different plant species (Wang et al. 2015), generation of knockout mutants or conditional knockdown of *NPR3* and *NPR4* in crop plants might enable us to engineer plant resistance with reduced fitness costs.

To further decipher the signaling pathways activated by SNC2, I sought to identify novel components downstream of SNC2 by performing a forward genetic screen in the *eds5-3 snc2-1D npr1-1* background. This screen resulted in the isolation of 71 putative suppressors. Seven suppressor mutants, which fell into four complementation groups, designated *bda3-1D*, *bda5*, *bda6*, and *bda7*, were further analyzed.

The *bda7* alleles contain mutations in *ALD1*, which could largely suppress the autoimmunity in *eds5-3 snc2-1D npr1-1* plants. *ALD1* encodes an aminotransferase, converting lysine to the precursor of Pip, Δ1-piperideine-2-carboxylic acid (P2C) (Ding et al. 2016; Hartmann et al. 2017). As one of the enzymes involved in Pip biosynthesis pathway, ALD1 is required for both local and systemic accumulation of Pip (Návarová et al. 2012; Ding et al. 2016). The isolation of *ald1* alleles in the *eds5-3 snc2-1D npr1*-1 suppressor screen suggests Pip is also required for SNC2-mediated defence responses. The additive effect of the *eds5-3* and *ald1* mutations on the suppression of *snc2-1D* mutant phenotype indicates that SA and Pip act independently downstream of SNC2.

SARD4 was recently identified as another critical enzyme involved in biosynthesis of Pip in systemic leaves. Unlike *ald1*, the *sard4* mutant still shows a significant amount of Pip accumulation in local tissue (Ding et al. 2016). Interestingly, loss of *SARD4* does not show significant suppression of autoimmunity in *eds5-3 snc2-1D npr1-1* plants (data not shown). This indicates that additional components are involved in Pip biosynthesis downstream of SNC2.

The *bda6* mutants were found to be alleles of *fmo1*. Similar to ALD1, FMO1 also plays critical roles in both basal and systemic resistance. Overexpression of *FMO1* leads to increased resistance against virulent pathogens, whereas loss of function of FMO1 leads to enhanced susceptibility to pathogens and complete loss of SAR (Koch et al. 2006; Mishina and Zeier 2006). Consistent with previous studies, the suppression of *snc2-1D* autoimmunity depends on mutation in both *EDS5* and *FMO1*, further validating that FMO1 functions independently of SA. Although pathogen-induced Pip accumulation is not reduced in *fmo1* plants, mutations in *ALD1* or *SARD4* can fully suppress the enhanced resistance conferred by overexpression of *FMO1*, suggesting that FMO1 may be involved in the synthesis of a defense signal molecule derived from Pip (Ding et al. 2016). A very recent study showed that FMO1 functions as a pipecolate N-hydroxylase, catalyzing the biochemical conversion of Pipecolic acid to NHP (Hartmann et al. 2018). The role of NHP in SNC2-mediated signaling pathway could be further analyzed.

While my Ph.D. thesis study has led to further understanding of the signaling pathways downstream of SNC2, there are still some missing links that remain to be

identified. Multiple studies have shown that RLPs associate with RLKs to transduce signal (Liebrand et al. 2014; Albert et al. 2015; Couto and Zipfel 2016). However, no such RLK(s) were found to mediate SNC2-mediated resistance in two independent suppressor screens. This might be due to genetic redundancy. Biochemical approaches could be utilized to look for potential interactors of SNC2, which may lead to identification of the RLK(s) working together with SNC2.

Apart from the unidentified RLKs which function together with SNC2 as the core receptor complex, it is also unknown if other common RLK co-receptors, such as the SERK family members (Liebrand et al. 2014), are involved. Preliminary data indicated that BAK1 and SER4/BKK1 (BAK1-LIKE 1) are not involved in SNC2-mediated signaling pathways. The carboxyl terminal tail (CT) of BAK1 was shown to be required for PTI but dispensable for brassinosteroid responses and BAK1/BKK1-inhibited cell death signaling (Wu et al. 2017). Mutants of other LRR-RLK homologs with this unique CT structure can be tested in order to identify additional RLK co-receptors.

Further studies are required regarding BDA1 and WRKY70 and their roles in plant immunity. *BDA1* encodes a protein with ankyrin repeats and transmembrane domains (Yang et al. 2012). In *Arabidopsis*, there are 37 predicted ankyrin-repeat transmembrane proteins (Becerra et al. 2004). Among them, the ACCELERATED CELL DEATH 6 (ACD6) is involved in positive regulation of SA signaling in local defence (Lu et al. 2003). ACD6 interacts with PRRs, including FLS2 and CERK1, and positively regulates the abundance of the PRRs (Zhang et al. 2014; Tateda et al. 2014). Since BDA1 also interacts with SNC2 (data not shown), it might be similarly involved in the regulation of SNC2 protein turn over. In addition, a gain-of-function mutation in the second transmembrane domain of BDA1 (*bda1-17D*) leads to constitutively activate cell death and defence responses (Yang et al. 2012). Interestingly, the gain-of-function mutation in *acd6-1* also occurs in a predicted transmembrane helix (Lu et al. 2003). These data suggest that the transmembrane domains of BDA1 and ACD6 may play critical roles in their self-inhibitions. Another plausible explanation could be that these transmembrane domains interact with their negative regulators and that the mutations in *bda1-17D* and *acd6-1* disrupt these interactions.

WRKY70 was shown to function in modulating defence responses and senescence (Li et al. 2004; Knoth et al. 2007; Besseau et al. 2012). *WRKY70* is a direct target of transcription factor SARD1 (Sun et al. 2015). However, a more recent study showed WRKY70 can also bind to the promoter of *SARD1 in vitro* (Zhou et al. 2018), suggesting a feedback regulation is potentially involved. However, *snc2-1D npr1-1 sard1* plants do not show significant suppression of the dwarf morphology compared to *snc2-1D npr1-1 wrky70* plants (data not shown). This could be explained by the redundant roles between *SARD1* and *CBP60g*, so it is worthy of testing if WRKY70 can regulate *CBP60g* gene expression through its binding to the promoter of *CBP60g*. In addition, performing ChIP-seq analysis for WRKY70 protein will reveal genes specifically regulated by WRKY70 but not SARD1, which can also be used to explain the different phenotypes between *snc2-1D npr1-1 wrky70* and *snc2-1D npr1-1 sard1*.

Further studies on *BDA3* and *BDA5* would provide new insights into how SNC2-mediated signaling pathways are regulated. *BDA3* encodes a clathrin assembly protein-like protein. Clathrin protein, composed of light and heavy chains, is one of the coat proteins involved in vesicle budding in multiple pathways (Robinson and Bonifacino 2001; Hwang and Robinson 2009). In plants, clathrin-mediated endocytosis (CME) is the predominant endocytic mechanism. It has been shown that three different cell-surface immune receptors FLS2, EFR and PEPR1 (PEP receptor 1) are all removed from the cell surface via CME during immune activation. Given that CME occurred on the plasma membrane, further analysis on the subcellular localization of BDA3 could validate its role in CME. Identification of the targets of BDA3 might provide potential leads to the missing RLK(s) in the pathway.

Preliminary studies suggest that *BDA5* encodes a potential cleavage and polyadenylation specificity factor (CPSF) in *Arabidopsis*. CPSFs play important roles in the cleavage of the 3' signaling region from a newly synthesized pre-messenger RNA (pre-mRNA) molecule in the process of gene transcription (Mandel et al. 2006). It remains to be determined whether BDA5 functions as a CPSF and, if it does, how the CPSF function plays a role in the immune signaling mediated by SNC2.

In summary, I have identified several new components involved in the regulation of SNC2-mediated defence responses through the genetic screens. Analysis of these

components has helped to better understand NPR1/SA-independent defence responses. Further studies need to be carried out to dissect the missing links in the SNC2 mediated immune pathways.

References

Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J. and Parker, J.E. 1998. Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* 95(17), pp. 10306–10311.

Albert, I., Böhm, H., Albert, M., Feiler, C.E., Imkampe, J., Wallmeroth, N., Brancato, C., Raaymakers, T.M., Oome, S., Zhang, H., Krol, E., Grefen, C., Gust, A.A., Chai, J., Hedrich, R., Van den Ackerveken, G. and Nürnberger, T. 2015. An RLP23-SOBIR1-BAK1 complex mediates NLP-triggered immunity. *Nature Plants* 1, p. 15140.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.-L., Gomez-Gomez, L., Boller, T., Ausubel, F.M. and Sheen, J. 2002. MAP kinase signalling cascade in *Arabidopsis* innate immunity. *Nature* 415(6875), pp. 977–983.

Axtell, M.J. and Staskawicz, B.J. 2003. Initiation of RPS2-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. *Cell* 112(3), pp. 369–377.

Bartsch, M., Gobbato, E., Bednarek, P., Debey, S., Schultze, J.L., Bautor, J. and Parker, J.E. 2006. Salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 signaling in *Arabidopsis* immunity and cell death is regulated by the monooxygenase FMO1 and the Nudix hydrolase NUDT7. *The Plant Cell* 18(4), pp. 1038–1051.

Beaunerie, J. 1901. Essais d'immunisation des végétaux contre les maladies cryptogamiques. *C R Acad Sci Ser* 133, pp. 107–110.

Becerra, C., Jahrmann, T., Puigdomènech, P. and Vicient, C.M. 2004. Ankyrin repeat-containing proteins in *Arabidopsis*: characterization of a novel and abundant group of genes coding ankyrin-transmembrane proteins. *Gene* 340(1), pp. 111–121.

Belfanti, E., Silfverberg-Dilworth, E., Tartarini, S., Patocchi, A., Barbieri, M., Zhu, J., Vinatzer, B.A., Gianfranceschi, L., Gessler, C. and Sansavini, S. 2004. The HcrVf2 gene from a wild apple confers scab resistance to a transgenic cultivated variety.

Proceedings of the National Academy of Sciences of the United States of America 101(3), pp. 886–890.

Bernsdorff, F., Döring, A.-C., Gruner, K., Schuck, S., Bräutigam, A. and Zeier, J. 2016. Pipecolic Acid Orchestrates Plant Systemic Acquired Resistance and Defense Priming via Salicylic Acid-Dependent and -Independent Pathways. *The Plant Cell* 28(1), pp. 102–129.

Besseau, S., Li, J. and Palva, E.T. 2012. WRKY54 and WRKY70 co-operate as negative regulators of leaf senescence in Arabidopsis thaliana. *Journal of Experimental Botany* 63(7), pp. 2667–2679.

Bi, D., Cheng, Y.T., Li, X. and Zhang, Y. 2010. Activation of plant immune responses by a gain-of-function mutation in an atypical receptor-like kinase. *Plant Physiology* 153(4), pp. 1771–1779.

Bi, D., Johnson, K.C.M., Zhu, Z., Huang, Y., Chen, F., Zhang, Y. and Li, X. 2011. Mutations in an Atypical TIR-NB-LRR-LIM Resistance Protein Confer Autoimmunity. *Frontiers in plant science* 2, p. 71.

Boller, T. and Felix, G. 2009. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual review of plant biology* 60, pp. 379–406.

Cao, H., Bowling, S.A., Gordon, A.S. and Dong, X. 1994. Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. *The Plant Cell* 6(11), pp. 1583–1592.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S. and Dong, X. 1997. The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* 88(1), pp. 57–63.

Cao, H., Li, X. and Dong, X. 1998. Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. *Proceedings of the National Academy of Sciences of the United States of America* 95(11), pp. 6531–6536.

Catinot, J., Buchala, A., Abou-Mansour, E. and Métraux, J.-P. 2008. Salicylic acid production in response to biotic and abiotic stress depends on isochorismate in Nicotiana benthamiana. *FEBS Letters* 582(4), pp. 473–478.

Cecchini, N.M., Jung, H.W., Engle, N.L., Tschaplinski, T.J. and Greenberg, J.T. 2015. ALD1 regulates basal immune components and early inducible defense responses in arabidopsis. *Molecular Plant-Microbe Interactions* 28(4), pp. 455–466.

Chanda, B., Xia, Y., Mandal, M.K., Yu, K., Sekine, K.-T., Gao, Q., Selote, D., Hu, Y., Stromberg, A., Navarre, D., Kachroo, A. and Kachroo, P. 2011. Glycerol-3-phosphate is a critical mobile inducer of systemic immunity in plants. *Nature Genetics* 43(5), pp. 421–427.

Chaturvedi, R., Venables, B., Petros, R.A., Nalam, V., Li, M., Wang, X., Takemoto, L.J. and Shah, J. 2012. An abietane diterpenoid is a potent activator of systemic acquired resistance. *The Plant Journal: for Cell and Molecular Biology* 71(1), pp. 161–172.

Chen, X., Zuo, S., Schwessinger, B., Chern, M., Canlas, P.E., Ruan, D., Zhou, X., Wang, J., Daudi, A., Petzold, C.J., Heazlewood, J.L. and Ronald, P.C. 2014. An XA21-associated kinase (OsSERK2) regulates immunity mediated by the XA21 and XA3 immune receptors. *Molecular Plant* 7(5), pp. 874–892.

Cheng, Y.T., Li, Y., Huang, S., Huang, Y., Dong, X., Zhang, Y. and Li, X. 2011. Stability of plant immune-receptor resistance proteins is controlled by SKP1-CULLIN1-F-box (SCF)-mediated protein degradation. *Proceedings of the National Academy of Sciences of the United States of America* 108(35), pp. 14694–14699.

Chern, M., Fitzgerald, H.A., Canlas, P.E., Navarre, D.A. and Ronald, P.C. 2005. Overexpression of a rice NPR1 homolog leads to constitutive activation of defense response and hypersensitivity to light. *Molecular Plant-Microbe Interactions* 18(6), pp. 511–520.

Chester, K.S. 1933. The Problem of Acquired Physiological Immunity in Plants. *The Quarterly Review of Biology* 8(3), pp. 275–324.

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T. and Felix, G. 2006. The *Arabidopsis* receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. *The Plant Cell* 18(2), pp. 465–476.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J.D.G., Felix, G. and Boller, T. 2007. A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448(7152), pp. 497–500.

Chung, E.-H., da Cunha, L., Wu, A.-J., Gao, Z., Cherkis, K., Afzal, A.J., Mackey, D. and Dangl, J.L. 2011. Specific threonine phosphorylation of a host target by two unrelated type III effectors activates a host innate immune receptor in plants. *Cell Host & Microbe* 9(2), pp. 125–136.

Cochrane, F.C., Davin, L.B. and Lewis, N.G. 2004. The Arabidopsis phenylalanine ammonia lyase gene family: kinetic characterization of the four PAL isoforms. *Phytochemistry* 65(11), pp. 1557–1564.

Couto, D. and Zipfel, C. 2016. Regulation of pattern recognition receptor signalling in plants. *Nature Reviews. Immunology* 16(9), pp. 537–552.

Cui, H., Gobbato, E., Kracher, B., Qiu, J., Bautor, J. and Parker, J.E. 2017. A core function of EDS1 with PAD4 is to protect the salicylic acid defense sector in Arabidopsis immunity. *The New Phytologist* 213(4), pp. 1802–1817.

Dangl, J.L. and Jones, J.D. 2001. Plant pathogens and integrated defence responses to infection. *Nature* 411(6839), pp. 826–833.

Dean, J.V. and Mills, J.D. 2004. Uptake of salicylic acid 2-O-beta-D-glucose into soybean tonoplast vesicles by an ATP-binding cassette transporter-type mechanism. *Physiologia Plantarum* 120(4), pp. 603–612.

Dean, J.V., Mohammed, L.A. and Fitzpatrick, T. 2005. The formation, vacuolar localization, and tonoplast transport of salicylic acid glucose conjugates in tobacco cell suspension cultures. *Planta* 221(2), pp. 287–296.

Dean, R.A. and Kuć, J. 1986. Induced systemic protection in cucumbers: the source of the “signal.” *Physiological and Molecular Plant Pathology* 28(2), pp. 227–233.

Delaney, T.P., Friedrich, L. and Ryals, J.A. 1995. Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. *Proceedings of the National Academy of Sciences of the United States of America* 92(14), pp. 6602–6606.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E. and Ryals, J. 1994. A central role of salicylic Acid in plant disease resistance. *Science* 266(5188), pp. 1247–1250.

Dempsey, D.A. and Klessig, D.F. 2012. SOS - too many signals for systemic acquired resistance? *Trends in Plant Science* 17(9), pp. 538–545.

Dempsey, D.A., Vlot, A.C., Wildermuth, M.C. and Klessig, D.F. 2011. Salicylic Acid biosynthesis and metabolism. *The Arabidopsis book / American Society of Plant Biologists* 9, p. e0156.

Després, C., DeLong, C., Glaze, S., Liu, E. and Fobert, P.R. 2000. The Arabidopsis NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. *The Plant Cell* 12(2), pp. 279–290.

Ding, P., Rekhter, D., Ding, Y., Feussner, K., Busta, L., Haroth, S., Xu, S., Li, X., Jetter, R., Feussner, I. and Zhang, Y. 2016. Characterization of a pipecolic acid biosynthesis pathway required for systemic acquired resistance. *The Plant Cell* 28(10), pp. 2603–2615.

Dixon, M.S., Hatzixanthis, K., Jones, D.A., Harrison, K. and Jones, J.D. 1998. The tomato Cf-5 disease resistance gene and six homologs show pronounced allelic variation in leucine-rich repeat copy number. *The Plant Cell* 10(11), pp. 1915–1925.

Dixon, M.S., Jones, D.A., Keddie, J.S., Thomas, C.M., Harrison, K. and Jones, J.D. 1996. The tomato Cf-2 disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. *Cell* 84(3), pp. 451–459.

Dong, X. 2004. NPR1, all things considered. *Current Opinion in Plant Biology* 7(5), pp. 547–552.

Fan, W. and Dong, X. 2002. In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis. *The Plant Cell* 14(6), pp. 1377–1389.

Feys, B.J., Wiermer, M., Bhat, R.A., Moisan, L.J., Medina-Escobar, N., Neu, C., Cabral, A. and Parker, J.E. 2005. Arabidopsis SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. *The Plant Cell* 17(9), pp. 2601–2613.

Flor, H.H. 1971. Current status of the gene-for-gene concept. *Annual Review of Phytopathology* 9(1), pp. 275–296.

Friedrich, L., Vernooij, B., Gaffney, T., Morse, A. and Ryals, J. 1995. Characterization of tobacco plants expressing a bacterial salicylate hydroxylase gene. *Plant Molecular Biology* 29(5), pp. 959–968.

Fritz-Laylin, L.K., Krishnamurthy, N., Tör, M., Sjölander, K.V. and Jones, J.D.G. 2005. Phylogenomic analysis of the receptor-like proteins of rice and Arabidopsis. *Plant Physiology* 138(2), pp. 611–623.

Fu, Z.Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., Mohan, R., Spoel, S.H., Tada, Y., Zheng, N. and Dong, X. 2012. NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486(7402), pp. 228–232.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. 1993. Requirement of salicylic Acid for the induction of systemic acquired resistance. *Science* 261(5122), pp. 754–756.

Gao, M., Liu, J., Bi, D., Zhang, Z., Cheng, F., Chen, S. and Zhang, Y. 2008. MEKK1, MKK1/MKK2 and MPK4 function together in a mitogen-activated protein kinase cascade to regulate innate immunity in plants. *Cell Research* 18(12), pp. 1190–1198.

Gao, M., Wang, X., Wang, D., Xu, F., Ding, X., Zhang, Z., Bi, D., Cheng, Y.T., Chen, S., Li, X. and Zhang, Y. 2009. Regulation of cell death and innate immunity by two receptor-like kinases in *Arabidopsis*. *Cell Host & Microbe* 6(1), pp. 34–44.

Garcion, C., Lohmann, A., Lamodière, E., Catinot, J., Buchala, A., Doermann, P. and Métraux, J.-P. 2008. Characterization and biological function of the ISOCHORISMATE SYNTHASE2 gene of Arabidopsis. *Plant Physiology* 147(3), pp. 1279–1287.

Görlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K.H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H. and Ryals, J. 1996. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *The Plant Cell* 8(4), pp. 629–643.

Grant, J.J., Chini, A., Basu, D. and Loake, G.J. 2003. Targeted activation tagging of the Arabidopsis NBS-LRR gene, ADR1, conveys resistance to virulent pathogens. *Molecular Plant-Microbe Interactions* 16(8), pp. 669–680.

Gust, A.A. and Felix, G. 2014. Receptor like proteins associate with SOBIR1-type of adaptors to form bimolecular receptor kinases. *Current Opinion in Plant Biology* 21, pp. 104–111.

Hartmann, M., Kim, D., Bernsdorff, F., Ajami-Rashidi, Z., Scholten, N., Schreiber, S., Zeier, T., Schuck, S., Reichel-Deland, V. and Zeier, J. 2017. Biochemical principles and functional aspects of pipecolic acid biosynthesis in plant immunity. *Plant Physiology* 174(1), pp. 124–153.

Hartmann, M., Zeier, T., Bernsdorff, F., Reichel-Deland, V., Kim, D., Hohmann, M., Scholten, N., Schuck, S., Bräutigam, A., Hölzel, T., Ganter, C. and Zeier, J. 2018. Flavin Monooxygenase-Generated N-Hydroxypipecolic Acid Is a Critical Element of Plant Systemic Immunity. *Cell*.

Holub, E.B. 2001. The arms race is ancient history in Arabidopsis, the wildflower. *Nature Reviews. Genetics* 2(7), pp. 516–527.

van der Hoorn, R.A.L. and Kamoun, S. 2008. From Guard to Decoy: a new model for perception of plant pathogen effectors. *The Plant Cell* 20(8), pp. 2009–2017.

Huang, J., Gu, M., Lai, Z., Fan, B., Shi, K., Zhou, Y.-H., Yu, J.-Q. and Chen, Z. 2010. Functional analysis of the Arabidopsis PAL gene family in plant growth, development, and response to environmental stress. *Plant Physiology* 153(4), pp. 1526–1538.

Hwang, I. and Robinson, D.G. 2009. Transport vesicle formation in plant cells. *Current Opinion in Plant Biology* 12(6), pp. 660–669.

Ishihara, T., Sekine, K.T., Hase, S., Kanayama, Y., Seo, S., Ohashi, Y., Kusano, T., Shibata, D., Shah, J. and Takahashi, H. 2008. Overexpression of the Arabidopsis thaliana EDS5 gene enhances resistance to viruses. *Plant Biology* 10(4), pp. 451–461.

Jehle, A.K., Lipschis, M., Albert, M., Fallahzadeh-Mamaghani, V., Fürst, U., Mueller, K. and Felix, G. 2013. The receptor-like protein ReMAX of *Arabidopsis* detects the microbe-associated molecular pattern eMax from *Xanthomonas*. *The Plant Cell* 25(6), pp. 2330–2340.

Jenns, A.E. and Kuc, J. 1979. Graft transmission of systemic resistance of cucumber to anthracnose induced by Colletotrichum lagenarium and tobacco necrosis virus. *Phytopathology* 7, pp. 753–56.

Jeong, S., Trotochaud, A.E. and Clark, S.E. 1999. The Arabidopsis CLAVATA2 gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. *The Plant Cell* 11(10), pp. 1925–1934.

Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P. and Valent, B. 2000. Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *The EMBO Journal* 19(15), pp. 4004–4014.

Jing, B., Xu, S., Xu, M., Li, Y., Li, S., Ding, J. and Zhang, Y. 2011. Brush and spray: a high-throughput systemic acquired resistance assay suitable for large-scale genetic screening. *Plant Physiology* 157(3), pp. 973–980.

Jones, D.A., Thomas, C.M., Hammond-Kosack, K.E., Balint-Kurti, P.J. and Jones, J.D.G. 1994. Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science* 266(5186), pp. 789–793.

Jones, J.D.G. and Dangl, J.L. 2006. The plant immune system. *Nature* 444(7117), pp. 323–329.

Judelson, H.S. and Blanco, F.A. 2005. The spores of Phytophthora: weapons of the plant destroyer. *Nature Reviews. Microbiology* 3(1), pp. 47–58.

Jung, H.W., Tschaplinski, T.J., Wang, L., Glazebrook, J. and Greenberg, J.T. 2009. Priming in systemic plant immunity. *Science* 324(5923), pp. 89–91.

Kadota, Y., Sklenar, J., Derbyshire, P., Stransfeld, L., Asai, S., Ntoukakis, V., Jones, J.D.G., Shirasu, K., Menke, F., Jones, A. and Zipfel, C. 2014. Direct regulation of the NADPH oxidase RBOHD by the PRR-associated kinase BIK1 during plant immunity. *Molecular Cell* 54(1), pp. 43–55.

Katagiri, F., Lam, E. and Chua, N.H. 1989. Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. *Nature* 340(6236), pp. 727–730.

Kim, D., Langmead, B. and Salzberg, S.L. 2015. HISAT: a fast spliced aligner with low memory requirements. *Nature Methods* 12(4), pp. 357–360.

Kim, D.S. and Hwang, B.K. 2014. An important role of the pepper phenylalanine ammonia-lyase gene (PAL1) in salicylic acid-dependent signalling of the defence response to microbial pathogens. *Journal of Experimental Botany* 65(9), pp. 2295–2306.

Kim, H.S. and Delaney, T.P. 2002. Over-expression of TGA5, which encodes a bZIP transcription factor that interacts with NIM1/NPR1, confers SAR-independent resistance in Arabidopsis thaliana to Peronospora parasitica. *The Plant Journal: for Cell and Molecular Biology* 32(2), pp. 151–163.

Klessig, D.F., Tian, M. and Choi, H.W. 2016. Multiple targets of salicylic acid and its derivatives in plants and animals. *Frontiers in immunology* 7, p. 206.

Knoth, C., Ringler, J., Dangl, J.L. and Eulgem, T. 2007. Arabidopsis WRKY70 is required for full RPP4-mediated disease resistance and basal defense against Hyaloperonospora parasitica. *Molecular Plant-Microbe Interactions* 20(2), pp. 120–128.

Koch, M., Vorwerk, S., Masur, C., Sharifi-Sirchi, G., Olivieri, N. and Schlaich, N.L. 2006. A role for a flavin-containing mono-oxygenase in resistance against microbial pathogens in Arabidopsis. *The Plant Journal: for Cell and Molecular Biology* 47(4), pp. 629–639.

Kohalmi Nowak, J., and Crosby, W.L., S.E. 1997. The yeast two-hybrid system. In: Hansen, E. and Harper, G. eds. *Differentially Expressed Genes in Plants: A Bench Manual*. London: Taylor and Francis, p. 63?2.

Kong, Q., Sun, T., Qu, N., Ma, J., Li, M., Cheng, Y.-T., Zhang, Q., Wu, D., Zhang, Z. and Zhang, Y. 2016. Two Redundant Receptor-Like Cytoplasmic Kinases Function Downstream of Pattern Recognition Receptors to Regulate Activation of SA Biosynthesis. *Plant Physiology* 171(2), pp. 1344–1354.

Kuai, X., MacLeod, B.J. and Després, C. 2015. Integrating data on the Arabidopsis NPR1/NPR3/NPR4 salicylic acid receptors; a differentiating argument. *Frontiers in plant science* 6, p. 235.

Lamb, C. and Dixon, R.A. 1997. The oxidative burst in plant disease resistance. *Annual review of plant physiology and plant molecular biology* 48, pp. 251–275.

Lawrence, M., Huber, W., Pagès, H., Aboyoun, P., Carlson, M., Gentleman, R., Morgan, M.T. and Carey, V.J. 2013. Software for computing and annotating genomic ranges. *PLoS Computational Biology* 9(8), p. e1003118.

Lebel, E., Heifetz, P., Thorne, L., Uknes, S., Ryals, J. and Ward, E. 1998. Functional analysis of regulatory sequences controlling PR-1 gene expression in Arabidopsis. *The Plant Journal: for Cell and Molecular Biology* 16(2), pp. 223–233.

Lee, H.I., León, J. and Raskin, I. 1995. Biosynthesis and metabolism of salicylic acid. *Proceedings of the National Academy of Sciences of the United States of America* 92(10), pp. 4076–4079.

Lee, H.I. and Raskin, I. 1998. Glucosylation of Salicylic Acid in Nicotiana tabacum Cv. Xanthi-nc. *Phytopathology* 88(7), pp. 692–697.

Lee, H.I. and Raskin, I. 1999. Purification, cloning, and expression of a pathogen inducible UDP-glucose:Salicylic acid glucosyltransferase from tobacco. *The Journal of Biological Chemistry* 274(51), pp. 36637–36642.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R. and 1000 Genome Project Data Processing Subgroup 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25(16), pp. 2078–2079.

Li, J., Brader, G. and Palva, E.T. 2004. The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *The Plant Cell* 16(2), pp. 319–331.

Li, L., Li, M., Yu, L., Zhou, Z., Liang, X., Liu, Z., Cai, G., Gao, L., Zhang, X., Wang, Y., Chen, S. and Zhou, J.-M. 2014. The FLS2-associated kinase BIK1 directly phosphorylates the NADPH oxidase RbohD to control plant immunity. *Cell Host & Microbe* 15(3), pp. 329–338.

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. *Molecular Plant-Microbe Interactions* 14(10), pp. 1131–1139.

Li, Y., Li, S., Bi, D., Cheng, Y.T., Li, X. and Zhang, Y. 2010. SRFR1 negatively regulates plant NB-LRR resistance protein accumulation to prevent autoimmunity. *PLoS Pathogens* 6(9), p. e1001111.

Liebrand, T.W.H., van den Berg, G.C.M., Zhang, Z., Smit, P., Cordewener, J.H.G., America, A.H.P., Sklenar, J., Jones, A.M.E., Tameling, W.I.L., Robatzek, S., Thomma, B.P.H.J. and Joosten, M.H.A.J. 2013. Receptor-like kinase SOBIR1/EVR interacts with receptor-like proteins in plant immunity against fungal infection. *Proceedings of the National Academy of Sciences of the United States of America* 110(24), pp. 10010–10015.

Liebrand, T.W.H., van den Burg, H.A. and Joosten, M.H.A.J. 2014. Two for all: receptor-associated kinases SOBIR1 and BAK1. *Trends in Plant Science* 19(2), pp. 123–132.

Liu, G., Holub, E.B., Alonso, J.M., Ecker, J.R. and Fobert, P.R. 2005. An Arabidopsis NPR1-like gene, NPR4, is required for disease resistance. *The Plant Journal: for Cell and Molecular Biology* 41(2), pp. 304–318.

Liu, J., Elmore, J.M., Lin, Z.-J.D. and Coaker, G. 2011. A receptor-like cytoplasmic kinase phosphorylates the host target RIN4, leading to the activation of a plant innate immune receptor. *Cell Host & Microbe* 9(2), pp. 137–146.

Love, M.I., Huber, W. and Anders, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* 15(12), p. 550.

Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L. and He, P. 2010. A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. *Proceedings of the National Academy of Sciences of the United States of America* 107(1), pp. 496–501.

Lu, H., Rate, D.N., Song, J.T. and Greenberg, J.T. 2003. ACD6, a novel ankyrin protein, is a regulator and an effector of salicylic acid signaling in the Arabidopsis defense response. *The Plant Cell* 15(10), pp. 2408–2420.

Mackey, D., Belkhadir, Y., Alonso, J.M., Ecker, J.R. and Dangl, J.L. 2003. Arabidopsis RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell* 112(3), pp. 379–389.

Makandar, R., Essig, J.S., Schapaugh, M.A., Trick, H.N. and Shah, J. 2006. Genetically engineered resistance to Fusarium head blight in wheat by expression of Arabidopsis NPR1. *Molecular Plant-Microbe Interactions* 19(2), pp. 123–129.

Maldonado, A.M., Doerner, P., Dixon, R.A., Lamb, C.J. and Cameron, R.K. 2002. A putative lipid transfer protein involved in systemic resistance signalling in Arabidopsis. *Nature* 419(6905), pp. 399–403.

Mandel, C.R., Kaneko, S., Zhang, H., Gebauer, D., Vethantham, V., Manley, J.L. and Tong, L. 2006. Polyadenylation factor CPSF-73 is the pre-mRNA 3’-end-processing endonuclease. *Nature* 444(7121), pp. 953–956.

Manohar, M., Tian, M., Moreau, M., Park, S.-W., Choi, H.W., Fei, Z., Friso, G., Asif, M., Manosalva, P., von Dahl, C.C., Shi, K., Ma, S., Dinesh-Kumar, S.P., O’Doherty, I., Schroeder, F.C., van Wijk, K.J. and Klessig, D.F. 2014. Identification of multiple salicylic acid-binding proteins using two high throughput screens. *Frontiers in plant science* 5, p. 777.

Mao, G., Meng, X., Liu, Y., Zheng, Z., Chen, Z. and Zhang, S. 2011. Phosphorylation of a WRKY transcription factor by two pathogen-responsive MAPKs drives phytoalexin biosynthesis in Arabidopsis. *The Plant Cell* 23(4), pp. 1639–1653.

Meng, X. and Zhang, S. 2013. MAPK cascades in plant disease resistance signaling. *Annual Review of Phytopathology* 51, pp. 245–266.

Metraux Ahl-Goy, P., Staub, T., Speich, J., Steinemann, A., Ryals, J., and Ward, E., J.P. 1991. Induced resistance in cucumber in response to 2,6-dichloroisonicotinic acid and pathogens. In: Hennecke, H. and Verma, D. P. S. eds. *Advances in Molecular Genetics of Plant-Microbe Interactions*. Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 432–439.

Métraux, J.P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W. and Inverardi, B. 1990. Increase in salicylic Acid at the onset of systemic acquired resistance in cucumber. *Science* 250(4983), pp. 1004–1006.

Meyers, B.C., Kozik, A., Griego, A., Kuang, H. and Michelmore, R.W. 2003. Genome-wide analysis of NBS-LRR-encoding genes in Arabidopsis. *The Plant Cell* 15(4), pp. 809–834.

Mishina, T.E. and Zeier, J. 2006. The Arabidopsis flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance. *Plant Physiology* 141(4), pp. 1666–1675.

Molders, W., Buchala, A. and Metraux, J.P. 1996. Transport of Salicylic Acid in Tobacco Necrosis Virus-Infected Cucumber Plants. *Plant Physiology* 112(2), pp. 787–792.

Mou, Z., Fan, W. and Dong, X. 2003. Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113(7), pp. 935–944.

Nadeau, J.A. and Sack, F.D. 2002. Control of stomatal distribution on the Arabidopsis leaf surface. *Science* 296(5573), pp. 1697–1700.

Návarová, H., Bernsdorff, F., Döring, A.-C. and Zeier, J. 2012. Pipecolic acid, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity. *The Plant Cell* 24(12), pp. 5123–5141.

Nawrath, C., Heck, S., Parinthawong, N. and Métraux, J.-P. 2002. EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in Arabidopsis, is a member of the MATE transporter family. *The Plant Cell* 14(1), pp. 275–286.

Nawrath, C. and Métraux, J.P. 1999. Salicylic acid induction-deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *The Plant Cell* 11(8), pp. 1393–1404.

Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H. and Ohme-Takagi, M. 2001. Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *The Plant Cell* 13(8), pp. 1959–1968.

Park, S.-W., Kaimoyo, E., Kumar, D., Mosher, S. and Klessig, D.F. 2007. Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science* 318(5847), pp. 113–116.

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., Sharma, S.B., Klessig, D.F., Martienssen, R., Mattsson, O., Jensen, A.B. and Mundy, J. 2000. Arabidopsis map kinase 4 negatively regulates systemic acquired resistance. *Cell* 103(7), pp. 1111–1120.

Ray, J. 1901. Les maladies cryptogamiques des végétaux. *Revue générale de Botanique* 13, pp. 145–151.

Ren, D., Liu, Y., Yang, K.-Y., Han, L., Mao, G., Glazebrook, J. and Zhang, S. 2008. A fungal-responsive MAPK cascade regulates phytoalexin biosynthesis in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* 105(14), pp. 5638–5643.

Robinson, M.S. and Bonifacino, J.S. 2001. Adaptor-related proteins. *Current Opinion in Cell Biology* 13(4), pp. 444–453.

Rochon, A., Boyle, P., Wignes, T., Fobert, P.R. and Després, C. 2006. The coactivator function of Arabidopsis NPR1 requires the core of its BTB/POZ domain and the oxidation of C-terminal cysteines. *The Plant Cell* 18(12), pp. 3670–3685.

Römer, P., Hahn, S., Jordan, T., Strauss, T., Bonas, U. and Lahaye, T. 2007. Plant pathogen recognition mediated by promoter activation of the pepper Bs3 resistance gene. *Science* 318(5850), pp. 645–648.

Ross, A.F. 1961. Systemic acquired resistance induced by localized virus infections in plants. *Virology* 14, pp. 340–358.

Ryals, J., Weymann, K., Lawton, K., Friedrich, L., Ellis, D., Steiner, H.Y., Johnson, J., Delaney, T.P., Jesse, T., Vos, P. and Uknes, S. 1997. The Arabidopsis NIM1 protein shows homology to the mammalian transcription factor inhibitor I kappa B. *The Plant Cell* 9(3), pp. 425–439.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y. and Hunt, M.D. 1996. Systemic Acquired Resistance. *The Plant Cell* 8(10), pp. 1809–1819.

Sadeghi, M., Dehghan, S., Fischer, R., Wenzel, U., Vilcinskas, A., Kavousi, H.R. and Rahnamaeian, M. 2013. Isolation and characterization of isochorismate synthase and cinnamate 4-hydroxylase during salinity stress, wounding, and salicylic acid treatment in Carthamus tinctorius. *Plant Signaling & Behavior* 8(11), p. e27335.

Saur, I.M.L., Kadota, Y., Sklenar, J., Holton, N.J., Smakowska, E., Belkhadir, Y., Zipfel, C. and Rathjen, J.P. 2016. NbCSPR underlies age-dependent immune responses to bacterial cold shock protein in *Nicotiana benthamiana*. *Proceedings of the National Academy of Sciences of the United States of America* 113(12), pp. 3389–3394.

Schulze, B., Mentzel, T., Jehle, A.K., Mueller, K., Beeler, S., Boller, T., Felix, G. and Chinchilla, D. 2010. Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. *The Journal of Biological Chemistry* 285(13), pp. 9444–9451.

Serrano, M., Wang, B., Aryal, B., Garcion, C., Abou-Mansour, E., Heck, S., Geisler, M., Mauch, F., Nawrath, C. and Métraux, J.-P. 2013. Export of salicylic acid from the chloroplast requires the multidrug and toxin extrusion-like transporter EDS5. *Plant Physiology* 162(4), pp. 1815–1821.

Shah, J. 2009. Plants under attack: systemic signals in defence. *Current Opinion in Plant Biology* 12(4), pp. 459–464.

Shah, J., Tsui, F. and Klessig, D.F. 1997. Characterization of a salicylic acid-insensitive mutant (sai1) of Arabidopsis thaliana, identified in a selective screen utilizing the SA-

inducible expression of the tms2 gene. *Molecular Plant-Microbe Interactions* 10(1), pp. 69–78.

Shi, H., Shen, Q., Qi, Y., Yan, H., Nie, H., Chen, Y., Zhao, T., Katagiri, F. and Tang, D. 2013. BR-SIGNALING KINASE1 physically associates with FLAGELLIN SENSING2 and regulates plant innate immunity in *Arabidopsis*. *The Plant Cell* 25(3), pp. 1143–1157.

Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., Minami, E., Okada, K., Yamane, H., Kaku, H. and Shibuya, N. 2010. Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. *The Plant Journal: for Cell and Molecular Biology* 64(2), pp. 204–214.

Shinya, T., Yamaguchi, K., Desaki, Y., Yamada, K., Narisawa, T., Kobayashi, Y., Maeda, K., Suzuki, M., Tanimoto, T., Takeda, J., Nakashima, M., Funama, R., Narusaka, M., Narusaka, Y., Kaku, H., Kawasaki, T. and Shibuya, N. 2014. Selective regulation of the chitin-induced defense response by the Arabidopsis receptor-like cytoplasmic kinase PBL27. *The Plant Journal: for Cell and Molecular Biology* 79(1), pp. 56–66.

Shulaev, V., Leon, J. and Raskin, I. 1995. Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? *The Plant Cell* 7(10), pp. 1691–1701.

Song, J.T. 2006. Induction of a salicylic acid glucosyltransferase, AtSGT1, is an early disease response in Arabidopsis thaliana. *Molecules and Cells* 22(2), pp. 233–238.

Song, J.T., Lu, H., McDowell, J.M. and Greenberg, J.T. 2004. A key role for ALD1 in activation of local and systemic defenses in Arabidopsis. *The Plant Journal: for Cell and Molecular Biology* 40(2), pp. 200–212.

Song, W.Y., Wang, G.L., Chen, L.L., Kim, H.S., Pi, L.Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.X., Zhu, L.H., Fauquet, C. and Ronald, P. 1995. A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. *Science* 270(5243), pp. 1804–1806.

Sticher, L., Mauch-Mani, B. and Métraux, J.P. 1997. Systemic acquired resistance. *Annual Review of Phytopathology* 35, pp. 235–270.

Strawn, M.A., Marr, S.K., Inoue, K., Inada, N., Zubieta, C. and Wildermuth, M.C. 2007. Arabidopsis isochorismate synthase functional in pathogen-induced salicylate biosynthesis exhibits properties consistent with a role in diverse stress responses. *The Journal of Biological Chemistry* 282(8), pp. 5919–5933.

Subramaniam, R., Desveaux, D., Spickler, C., Michnick, S.W. and Brisson, N. 2001. Direct visualization of protein interactions in plant cells. *Nature Biotechnology* 19(8), pp. 769–772.

Sun, T., Zhang, Yaxi, Li, Y., Zhang, Q., Ding, Y. and Zhang, Yuelin 2015. ChIP-seq reveals broad roles of SARD1 and CBP60g in regulating plant immunity. *Nature Communications* 6, p. 10159.

Takken, F.L., Thomas, C.M., Joosten, M.H., Golstein, C., Westerink, N., Hille, J., Nijkamp, H.J., De Wit PJ and Jones, J.D. 1999. A second gene at the tomato Cf-4 locus confers resistance to cladosporium fulvum through recognition of a novel avirulence determinant. *The Plant Journal: for Cell and Molecular Biology* 20(3), pp. 279–288.

Tang, D., Wang, G. and Zhou, J.-M. 2017. Receptor Kinases in Plant-Pathogen Interactions: More Than Pattern Recognition. *The Plant Cell* 29(4), pp. 618–637.

Tateda, C., Zhang, Z., Shrestha, J., Jelenska, J., Chinchilla, D. and Greenberg, J.T. 2014. Salicylic acid regulates Arabidopsis microbial pattern receptor kinase levels and signaling. *The Plant Cell* 26(10), pp. 4171–4187.

Thomas, C.M., Jones, D.A., Parniske, M., Harrison, K., Balint-Kurti, P.J., Hatzixanthis, K. and Jones, J.D. 1997. Characterization of the tomato Cf-4 gene for resistance to Cladosporium fulvum identifies sequences that determine recognitional specificity in Cf-4 and Cf-9. *The Plant Cell* 9(12), pp. 2209–2224.

Thordal-Christensen, H. 2003. Fresh insights into processes of nonhost resistance. *Current Opinion in Plant Biology* 6(4), pp. 351–357.

Tiwari, S., Wang, S., Hagen, G. and Guilfoyle, T.J. 2006. Transfection assays with protoplasts containing integrated reporter genes. *Methods in Molecular Biology* 323, pp. 237–244.

Torres, M.A., Jones, J.D.G. and Dangl, J.L. 2005. Pathogen-induced, NADPH oxidase-derived reactive oxygen intermediates suppress spread of cell death in Arabidopsis thaliana. *Nature Genetics* 37(10), pp. 1130–1134.

Truman, W., Sreekanta, S., Lu, Y., Bethke, G., Tsuda, K., Katagiri, F. and Glazebrook, J. 2013. The CALMODULIN-BINDING PROTEIN60 family includes both negative and positive regulators of plant immunity. *Plant Physiology* 163(4), pp. 1741–1751.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E. and Ryals, J. 1992. Acquired resistance in Arabidopsis. *The Plant Cell* 4(6), pp. 645–656.

Ullah, H., Chen, J.-G., Temple, B., Boyes, D.C., Alonso, J.M., Davis, K.R., Ecker, J.R. and Jones, A.M. 2003. The β-subunit of the Arabidopsis G protein negatively regulates auxin-induced cell division and affects multiple developmental processes. *The Plant Cell* 15(2), pp. 393–409.

Uppalapati, S.R., Ishiga, Y., Wangdi, T., Kunkel, B.N., Anand, A., Mysore, K.S. and Bender, C.L. 2007. The phytotoxin coronatine contributes to pathogen fitness and is required for suppression of salicylic acid accumulation in tomato inoculated with Pseudomonas syringae pv. tomato DC3000. *Molecular Plant-Microbe Interactions* 20(8), pp. 955–965.

Van der Biezen, E.A. and Jones, J.D. 1998. Plant disease-resistance proteins and the gene-for-gene concept. *Trends in Biochemical Sciences* 23(12), pp. 454–456.

Van Loon, L.C. and Van Kammen, A. 1970. Polyacrylamide disc electrophoresis of the soluble leaf proteins from Nicotiana tabacum var. ‘Samsun’ and ‘Samsun NN.’ *Virology* 40(2), pp. 199–211.

Van Loon, L.C. and Van Strien, E.A. 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiological and Molecular Plant Pathology* 55(2), pp. 85–97.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H. and Ryals, J. 1994. Salicylic acid is not the translocated signal

responsible for inducing systemic acquired resistance but is required in signal transduction. *The Plant Cell* 6(7), pp. 959–965.

Vlot, A.C., Klessig, D.F. and Park, S.-W. 2008. Systemic acquired resistance: the elusive signal(s). *Current Opinion in Plant Biology* 11(4), pp. 436–442.

Volko, S.M., Boller, T. and Ausubel, F.M. 1998. Isolation of new Arabidopsis mutants with enhanced disease susceptibility to Pseudomonas syringae by direct screening. *Genetics* 149(2), pp. 537–548.

Wan, D., Li, R., Zou, B., Zhang, X., Cong, J., Wang, R., Xia, Y. and Li, G. 2012. Calmodulin-binding protein CBP60g is a positive regulator of both disease resistance and drought tolerance in Arabidopsis. *Plant Cell Reports* 31(7), pp. 1269–1281.

Wang, C., Liu, Y., Li, S.-S. and Han, G.-Z. 2015. Insights into the origin and evolution of the plant hormone signaling machinery. *Plant Physiology* 167(3), pp. 872–886.

Wang, G., Ellendorff, U., Kemp, B., Mansfield, J.W., Forsyth, A., Mitchell, K., Bastas, K., Liu, C.M., Woods-Tör, A., Zipfel, C., de Wit, P.J.G.M., Jones, J.D.G., Tör, M. and Thomma, B.P.H.J. 2008. A genome-wide functional investigation into the roles of receptor-like proteins in *Arabidopsis*. *Plant Physiology* 147(2), pp. 503–517.

Wang, L., Tsuda, K., Sato, M., Cohen, J.D., Katagiri, F. and Glazebrook, J. 2009. Arabidopsis CaM binding protein CBP60g contributes to MAMP-induced SA accumulation and is involved in disease resistance against Pseudomonas syringae. *PLoS Pathogens* 5(2), p. e1000301.

Wang, L., Tsuda, K., Truman, W., Sato, M., Nguyen, L.V., Katagiri, F. and Glazebrook, J. 2011. CBP60g and SARD1 play partially redundant critical roles in salicylic acid signaling. *The Plant Journal: for Cell and Molecular Biology* 67(6), pp. 1029–1041.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Metraux, J.P. and Ryals, J.A. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. *The Plant Cell* 3(10), pp. 1085–1094.

Weigel, R.R., Bäuscher, C., Pfitzner, A.J. and Pfitzner, U.M. 2001. NIMIN-1, NIMIN-2 and NIMIN-3, members of a novel family of proteins from Arabidopsis that interact with NPR1/NIM1, a key regulator of systemic acquired resistance in plants. *Plant Molecular Biology* 46(2), pp. 143–160.

van Wersch, R., Li, X. and Zhang, Y. 2016. Mighty dwarfs: arabidopsis autoimmune mutants and their usages in genetic dissection of plant immunity. *Frontiers in plant science* 7, p. 1717.

White, R.F. 1979. Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. *Virology* 99(2), pp. 410–412.

Wildermuth, M.C., Dewdney, J., Wu, G. and Ausubel, F.M. 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414(6863), pp. 562–565.

Wu, D., Liu, Y., Xu, F. and Zhang, Y. 2017. Differential requirement of BAK1 C-terminal tail in development and immunity. *Journal of integrative plant biology*.

Wu, F.-H., Shen, S.-C., Lee, L.-Y., Lee, S.-H., Chan, M.-T. and Lin, C.-S. 2009. Tape-Arabidopsis Sandwich - a simpler Arabidopsis protoplast isolation method. *Plant Methods* 5, p. 16.

Wu, Y., Zhang, D., Chu, J.Y., Boyle, P., Wang, Y., Brindle, I.D., De Luca, V. and Després, C. 2012. The Arabidopsis NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell reports* 1(6), pp. 639–647.

Xiang, T., Zong, N., Zou, Y., Wu, Y., Zhang, J., Xing, W., Li, Y., Tang, X., Zhu, L., Chai, J. and Zhou, J.-M. 2008. Pseudomonas syringae effector AvrPto blocks innate immunity by targeting receptor kinases. *Current Biology* 18(1), pp. 74–80.

Xu, J., Meng, J., Meng, X., Zhao, Y., Liu, J., Sun, T., Liu, Y., Wang, Q. and Zhang, S. 2016. Pathogen-Responsive MPK3 and MPK6 Reprogram the Biosynthesis of Indole Glucosinolates and Their Derivatives in Arabidopsis Immunity. *The Plant Cell* 28(5), pp. 1144–1162.

Yalpani, N., Silverman, P., Wilson, T.M., Kleier, D.A. and Raskin, I. 1991. Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. *The Plant Cell* 3(8), pp. 809–818.

Yang, Y., Zhang, Yaxi, Ding, P., Johnson, K., Li, X. and Zhang, Yuelin 2012. The ankyrin-repeat transmembrane protein BDA1 functions downstream of the receptor-like protein SNC2 to regulate plant immunity. *Plant Physiology* 159(4), pp. 1857–1865.

Young, M.D., Wakefield, M.J., Smyth, G.K. and Oshlack, A. 2010. Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biology* 11(2), p. R14.

Yuan, Y., Chung, J.-D., Fu, X., Johnson, V.E., Ranjan, P., Booth, S.L., Harding, S.A. and Tsai, C.-J. 2009. Alternative splicing and gene duplication differentially shaped the regulation of isochorismate synthase in Populus and Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* 106(51), pp. 22020–22025.

Zhang, J., Li, W., Xiang, T., Liu, Z., Laluk, K., Ding, X., Zou, Y., Gao, M., Zhang, X., Chen, S., Mengiste, T., Zhang, Y. and Zhou, J.-M. 2010. Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a Pseudomonas syringae effector. *Cell Host & Microbe* 7(4), pp. 290–301.

Zhang, J., Shao, F., Li, Y., Cui, H., Chen, L., Li, H., Zou, Y., Long, C., Lan, L., Chai, J., Chen, S., Tang, X. and Zhou, J.-M. 2007. A Pseudomonas syringae effector inactivates MAPKs to suppress PAMP-induced immunity in plants. *Cell Host & Microbe* 1(3), pp. 175–185.

Zhang, W., Fraiture, M., Kolb, D., Löffelhardt, B., Desaki, Y., Boutrot, F.F.G., Tör, M., Zipfel, C., Gust, A.A. and Brunner, F. 2013. Arabidopsis receptor-like protein30 and receptor-like kinase suppressor of BIR1-1/EVERSHED mediate innate immunity to necrotrophic fungi. *The Plant Cell* 25(10), pp. 4227–4241.

Zhang, Y., Cheng, Y.T., Qu, N., Zhao, Q., Bi, D. and Li, X. 2006. Negative regulation of defense responses in Arabidopsis by two NPR1 paralogs. *The Plant Journal: for Cell and Molecular Biology* 48(5), pp. 647–656.

Zhang, Y., Fan, W., Kinkema, M., Li, X. and Dong, X. 1999. Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. *Proceedings of the National Academy of Sciences of the United States of America* 96(11), pp. 6523–6528.

Zhang, Y., Goritschnig, S., Dong, X. and Li, X. 2003. A gain-of-function mutation in a plant disease resistance gene leads to constitutive activation of downstream signal transduction pathways in suppressor of npr1-1, constitutive 1. *The Plant Cell* 15(11), pp. 2636–2646.

Zhang, Y., Tessaro, M.J., Lassner, M. and Li, X. 2003. Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. *The Plant Cell* 15(11), pp. 2647–2653.

Zhang, Y., Xu, S., Ding, P., Wang, D., Cheng, Y.T., He, J., Gao, M., Xu, F., Li, Y., Zhu, Z., Li, X. and Zhang, Yuelin 2010. Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors. *Proceedings of the National Academy of Sciences of the United States of America* 107(42), pp. 18220–18225.

Zhang, Y., Yang, Y., Fang, B., Gannon, P., Ding, P., Li, X. and Zhang, Yuelin 2010. Arabidopsis snc2-1D activates receptor-like protein-mediated immunity transduced through WRKY70. *The Plant Cell* 22(9), pp. 3153–3163.

Zhang, Z., Liu, Y., Huang, H., Gao, M., Wu, D., Kong, Q. and Zhang, Y. 2017. The NLR protein SUMM2 senses the disruption of an immune signaling MAP kinase cascade via CRCK3. *EMBO Reports* 18(2), pp. 292–302.

Zhang, Z., Shrestha, J., Tateda, C. and Greenberg, J.T. 2014. Salicylic acid signaling controls the maturation and localization of the arabidopsis defense protein ACCELERATED CELL DEATH6. *Molecular Plant* 7(8), pp. 1365–1383.

Zhang, Z., Wu, Y., Gao, M., Zhang, J., Kong, Q., Liu, Y., Ba, H., Zhou, J. and Zhang, Y. 2012. Disruption of PAMP-induced MAP kinase cascade by a Pseudomonas syringae effector activates plant immunity mediated by the NB-LRR protein SUMM2. *Cell Host & Microbe* 11(3), pp. 253–263.

Zhou, J.-M. and Chai, J. 2008. Plant pathogenic bacterial type III effectors subdue host responses. *Current Opinion in Microbiology* 11(2), pp. 179–185.

Zhou, J.M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J. and Klessig, D.F. 2000. NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the PR-1 gene required for induction by salicylic acid. *Molecular Plant-Microbe Interactions* 13(2), pp. 191–202.

Zhou, M., Lu, Y., Bethke, G., Harrison, B.T., Hatsugai, N., Katagiri, F. and Glazebrook, J. 2018. WRKY70 prevents axenic activation of plant immunity by direct repression of SARD1. *The New Phytologist* 217(2), pp. 700–712.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D.G., Boller, T. and Felix, G. 2006. Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell* 125(4), pp. 749–760.