PLANT IMMUNITY REGULATION BY TRANSCRIPTION FACTORS SARD1 AND CBP60G: DOWNSTREAM, UPSTREAM AND THE AMPLIFICATION LOOP

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Abstract

Activated plant defense responses consist of PAMP (pathogen associated molecular pattern)-triggered immunity (PTI) and effector-triggered immunity (ETI) at infected sites and a secondary immune response in distal parts of the host plant, termed systemic acquired resistance (SAR). Salicylic acid (SA) plays critical roles in plant immunity and its level increases upon pathogen infection. Pathogen-induced SA biosynthesis predominantly relies on *ICS1* (*ISOCHORISMATE SYNTHASE 1*), whose induction mainly depends on transcription factors SARD1 (SAR DEFICIENT 1) and CBP60g (CALMODULIN BINDING PROTEIN 60 g). Meanwhile, the expression of *SARD1* and *CBP60g* is also highly induced by pathogens. My Ph.D. research focuses on identification of immune regulators that function upstream and downstream of SARD1 and CBP60g.

First, we performed chromatin immunoprecipitation-sequencing experiments to identify candidate targets of SARD1. We found that SARD1 and CBP60g directly control the expression of a large number of key regulators involved in PTI, ETI and SAR. Among them, two genes essential for SAR, *ALD1* (*AGD2-LIKE DEFENSE RESPONSE PROTEIN 1*) and *SARD4*, are involved in the biosynthesis of pipecolic acid (Pip), a plant secondary metabolite required for SAR. Consistently, the *sard1cbp60g* double mutant accumulates less Pip than wild type, suggesting that SARD1 and CBP60g regulate Pip biosynthesis in addition to SA. Secondly, we showed that transcription factors TGA1 and TGA4 act upstream of *SARD1* and *CBP60g* and thus regulate the biosynthesis of SA and Pip. Lastly, we revealed a novel mechanism of SA perception by its receptors NPR3 (NPR1-LIKE PROTEIN 3) and NPR4. NPR3/NPR4 interact with transcription factors TGA2, TGA5 and TGA6, and act as transcriptional repressors. SA inhibits the transcriptional repression activities of NPR3/NPR4 and promotes the transcriptional activation activity of NPR1 (NONEXPRESSER OF *PR* GENES 1); both contribute to SA-induced defense gene expression. We also found that SA induces *SARD1* expression, revealing a feedback amplification loop between SA and SARD1, where SARD1 promotes SA biosynthesis via directly activating *ICS1* expression and SA induces *SARD1* expression by regulating the activities of NPR/TGA complexes.

Altogether studies in this dissertation provide new insights on the functions of SARD1 and CBP60g in plant immunity and the mechanism of SA perception and signaling.

Lay Summary

Like humans, plants have an immune system that recognizes foreign invaders and fights against them. Recognition of invaders triggers production of two molecules, salicylic acid and pipecolic acid, which function as signals to activate further immune responses. Proper regulation of immunity is important because over-activation can lead to defects in growth and development, while under-activation leaves plants vulnerable to disease. The studies in this dissertation revealed two proteins that function as master regulators controlling activation of defense responses as well as levels of salicylic acid and pipecolic acid. Meanwhile, a new mechanism of salicylic acid perception by plants was discovered, and salicylic acid was found to induce the accumulation of the two master regulators we had previously identified. Altogether these discoveries suggest a positive feedback loop between salicylic acid and the two master regulators that eventually leads to a powerful immune response.

Preface

The work presented in this thesis is the result of research performed between September 2012 and April 2018. Below is a list of publications that comprise this thesis and the contribution made by authors.

Chapter 2: ChIP-seq reveals broad roles of SARD1 and CBP60g in regulating plant immunity was modified from the manuscript:

Sun, T., Zhang, YX., Li, Y., Zhang, Q., Ding, Y. and Zhang, Y. 2015. "ChIP-seq reveals broad roles of SARD1 and CBP60g in regulating plant immunity." *Nat Commun* no. 6:10159. doi: 10.1038/ncomms10159.

- The candidate performed most of the experiments. YX Zhang carried out the ChIP-seq experiment and Y. Li performed the ChIP-seq data analysis. Q. Zhang helped with characterization of *sard1 cbp60g snc2-1D* and Y. Ding provided the *snc2-1D* seed. Y. Zhang supervised the research and wrote the manuscript together with the candidate.

Chapter 3: TGA1 and TGA4 regulate salicylic acid and pipecolic acid biosynthesis by modulating the expression of *SARD1* and *CBP60g* was modified from the manuscript:

Sun, T., Busta, L., Zhang, Q., Ding, P., Jetter, R. and Zhang, Y. 2018. "TGACG-BINDING FACTOR 1 (TGA1) and TGA4 regulate salicylic acid and pipecolic acid biosynthesis by modulating the expression of *SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1* (*SARD1*) and *CALMODULIN-BINDING PROTEIN 60g* (*CBP60g*)." *New Phytol* no. 217 (1):344-354. doi: 10.1111/nph.14780.

- The candidate and Y. Zhang designed the experiments and analyzed the data. The candidate performed most of the experiments. L. Busta set up and conducted the GC-MS measurement of pipecolic acid. Q. Zhang helped with pathogen infection assay. P. Ding provided information on SARD4's function before publication. R. Jetter supervised work performed by L.Busta. Y. Zhang wrote the manuscript together with the candidate.

Chapter 4: 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, YX., Li, X. and Zhang, Y. 2018. "Opposite Roles of Salicylic Acid Receptors NPR1 and NPR3/NPR4 in Transcriptional Regulation of Plant Immunity." *Cell*. doi: 10.1016/j.cell.2018.03.044. (*Co-first authorship)

- Y. Ding and the candidate conducted most of the experiments. The candidate performed the following experiments: ChIP experiment, purification of recombinant NPR1, NPR3 and NPR4 proteins, and SA binding experiments, preparation of RNA samples for RNA-seq analysis, RT-qPCR analysis for confirming RNA-seq data, generation of NPR3-related transgenic lines, yeast two-hybrid assays and NPR1 transcription activation assay in protoplasts. The candidate discovered the elevated basal transcript level of *SARD1* in *npr3 npr4* and *tga2 tga5 tga6* mutants and induction of *SARD1* by SA, and Y. Ding repeated the results and collected the data for figures. Y. Ding characterized *bda4-4D snc2-1D npr1-1* and *bda4-4D*/*npr4-4D* single mutant and identified *bda4-4D* as a gain-of-function allele of *NPR4*. The candidate identified the EAR-like transcription repression motif in NPR4 and Y. Ding performed the transcription repression assay for NPR3 and NPR4 in protoplasts. Y. Ding isolated *npr1-1 npr4-4D* double mutant and performed the phenotypic analysis of *npr1-1 npr4-4D*. Y. Ding generated and analyzed NPR1-related transgenic lines as well as *snc2 npr1 npr4-4D tga2 tga5 tga6* sextuple mutant. K. Ao performed RNA-seq data analysis. Y. Peng conducted Co-IP experiments in *N. benthamiana*. YX Zhang carried out the *bda* screen and isolated *bda4-4D snc2-1D npr1-1* mutant. Y. Zhang and X. Li supervised the research and wrote the manuscript together with Y. Ding, the candidate and K. Ao.

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List of Abbreviations

35S	A strong promoter from Cauliflower mosaic virus (CaMV)
AD	GAL4 activation domain
ADR1	Activated disease resistance 1
ADR-L1	ADR1-like 1
AGB1	*Arabidopsis* GTP binding protein beta 1
AGP5	Arabinogalactan-protein 5
ALD1	AGD2 (Aberrant growth and death 2)-like defense response protein 1
ATR1	Arabidopsis thaliana recognized 1
AvrE	An avirulence gene from *Pseudomonas syringae pv. tomato* DC3000
AVR-Pita	An avirulence gene from *Magnaporthe grisea*
AvrPphB	An avirulence gene from *Pseudomonas syringae pv. tomato* DC3000
AvrPtoB	An avirulence gene from *Pseudomonas syringae pv. tomato* DC3000
AvrRpt2	An avirulence gene from *Pseudomonas syringae pv. tomato* DC3000
BAK1	BRI1-associated receptor kinase 1
BAP1	BON1-associated protein 1
BD	GAL4 DNA binding domain
BDA	Bian Da; “becoming big” in Chinese
BIK1	*Botrytis*-induced kinase 1
BIR1	BAK1-interacting receptor-like kinase
BKK1	BAK1- like 1
BON1	BONZAI 1
BSK1	BR-signaling kinase 1
BTB/POZ	Broad-Complex, Tramtrack, Bric-à-brac/Poxvirus, Zinc-finger
CAMTA1	Calmodulin binding transcription activator 1
Cas9	CRISPR-associated 9
CBP60g	Calmodulin binding protein 60 g
CC	Coiled-coil domain
CERK1	Chitin elicitor receptor kinase 1
CHE	CCA1 (Circadian clock associated 1) hiking expedition
ChIP	Chromatin immunoprecipitation
ChIP-seq	ChIP-sequencing
CML46	Calmodulin like 46
CNL	Coiled-coil type NLR
CPK4	Calcium-dependent protein kinase

CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DIR1	Defective in induced resistance 1
EDS1	Enhanced disease susceptibility 1
EDS5	Enhanced disease susceptibility 5
EFR	EF-Tu receptor
EF-Tu	Elongation factor thermo unstable
ETI	Effector-triggered immunity
FLS2	FLAGELLIN SENSING 2
FMO1	Flavin-dependent monooxygenase 1
H.a. Noco2	*Hyaloperonospora arabidopsidis* Noco2
HA	An epitope protein tag composed of YPYDVPDYA from Hemagglutinin
His6-MBP	Hexahistidine-tagged maltose-binding protein
HopM1	An avirulence gene from *Pseudomonas syringae pv. tomato* DC3000
HR	Hypersensitive response
ICS1	Isochorismate synthase 1
K_d	Dissociation constant
LD-VP16	LexA DNA-binding domain-VP16 activation domain
LRR	Leucine rich repeat domain
LUC	Firefly luciferase
MAPKKK	Mitogen-associated protein kinase kinase kinase, also called MEKK (MAPK/ERK kinase kinase)
MC2	Metacaspase 2
MKK	Mitogen-associated protein kinase kinase
MLO2	Mildew resistance locus O 2
MPK	Mitogen-associated protein kinase, also called MAPK
N. benthamiana	*Nicotiana benthamiana*
NAC004	NAC (NAM, ATAF1/2 and CUC2) domain containing protein 4
nahG	A bacterial gene encoding a salicylate hydroxylase
NB-LRR	Nucleotide-binding and leucine-rich repeat domains
NDR1	Non-race disease resistance 1
NHP	N-hydroxypipecolic acid
NIM1	Non-inducible immunity 1
NLR	NOD (Nucleotide-binding oligomerization domain)-like receptor
NOD	Nucleotide-binding oligomerization domain
NOS101	A basal promoter of the nopaline synthase gene (-101 to +4)
NPR1	Nonexpressor of *PR* genes 1

NPR3	NPR1-like protein 3
NPR4	NPR1-like protein 4
NTL9	NTM1 (NAC with transmembrane motif 1)-like 9
NUDT6	Nucleoside diphosphate linked to some moiety X 6
OCS	Octopine synthase gene
P.s.m. ES4326	*Pseudomonas syringae pv. maculicola* ES4326
PAD4	Phytoalexin-deficient 4
PAL	Phenylalanine ammonia lyase
PAMP	pathogen-associated molecular pattern
PBL1	avrPphB susceptible 1 (PBS1) -like 1
PBS3	avrPphB SUSCEPTIBLE3
PCRK1	PTI compromised receptor-like kinase 1
Pip	Pipecolic acid
Pi-ta	An R protein from rice
PR gene	*Pathogenesis-related* gene
PRR	Pattern recognition receptor
Pst DC3000	Bacterial pathogen *Pseudomonas syringe* pv. *tomato* DC3000
Pst DC3000 *hrcC*	*Pst* DC3000 lacking *hrcC* gene, defective in Type III secretion system
PTI	PAMP-triggered immunity
PTMs	Post-translational modifications
PUB25	Plant U-box protein 25
RBOHD	Respiratory burst oxidase homolog D
REN	Renilla luciferase
RIN4	RPM1 interacting protein 4
RLCK	Receptor-like cytoplasmic kinase
RLK	Receptor like kinase
RLP	Receptor-like protein
ROS	Reactive oxygen species
RPM1	Resistance to *Pseudomonas syringe pv. maculicola*
RPP1	Recognition of *Peronospora parasitica* 1
RPP2	Recognition of *Peronospora parasitica* 2
RPP4	Recognition of *Peronospora parasitica* 4
RPS2	Resistance to *Pseudomonas syringe* 2
RPS5	Resistance to *Pseudomonas syringe* 5
RT-PCR	Real time-polymerase chain reaction
SA	Salicylic acid

SABPs	SA-binding proteins
SAG101	Senescence-associated gene 101
SAI1	Salicylic acid insensitive 1
SAR	Systemic acquired resistance
SARD1	Systemic acquired resistance deficient 1
SARD4	Systemic acquired resistance deficient 4
SID2	Salicylic acid induction deficient 2
SNC1	Suppressor of *npr1-1*, constitutive 1
SNC2	Suppressor of *npr1-1*, constitutive 2
SOBIR1	Suppressor of bir1 (BAK1-interacting receptor kinase 1), 1
T3SS	Bacterial type III secretion system
T-DNA	Transfer DNA
TGA1	TGACG sequence-specific binding protein 1
TIR	Toll interleukin receptor domain
TNL	TIR type NLR
UTR	Untranslated regions
WRKY70	WRKY DNA- binding protein 70
ZZ	Two immunoglobulin-binding domains of protein A from *Staphylococcus aureus*

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And finally, I would like to thank my friendly and lovely family, my parents, my older brothers and sisters, my nephews, my partner Qian Zhang, my mother-in-law and my children for their limitless love and support.

Dedication

To my father and mother,

Xixiu Sun and Jiqin Ji

Chapter 1: Introduction

1.1 The plant immune systems

Plants provide. They generate oxygen and are essential components of any food chain and ecosystem. In their living environment, plants face challenges from various microbial pathogens including bacteria, fungi, oomycetes and viruses. Most plants are healthy most of the time, as they have evolved sophisticated mechanisms that provide immunity to pathogens. At the very front line, plants use physical barriers such as waxy cuticles and cell walls as well as antimicrobial enzymes and secondary metabolites to prevent pathogen invasion (Thordal-Christensen 2003). Adapted pathogens can overcome these preformed defense systems to infect plants. For example, the well-studied foliar bacterial pathogen *Pseudomonas syringe* pv. *tomato* (*Pst*) DC3000 can enter the subepidermal space in leaves via stomata. The bacteria are perceived by guard cells and this triggers closure of stomata to prevent bacterial entry. However, *Pst* DC3000 secretes the toxin coronatine to interfere with the stomatal closing response and make stomata re-open (Melotto et al. 2006), allowing more bacteria to enter into the apoplast where they take nutrients from host plants and cause disease. Compared with the more passive physical barriers, the activated plant defense responses are mediated by immune receptors, which are conceptually defined into two types to sense pathogen infection and activate immunity.

1.2 PAMP-triggered immunity (PTI)

1.2.1 Overview

The first type of plant immune receptor is pattern recognition receptors (PRRs), which are localized on plasma membrane and recognize pathogen-associated molecular patterns (PAMPs). Plant PRRs are typically receptor like kinases (RLKs) or receptor-like proteins (RLPs) (Zipfel 2014). RLKs comprise a ligand-binding ectodomain, a transmembrane motif and an intracellular kinase domain. RLPs are architecturally similar to RLKs but lack the intracellular kinase domain. PAMPs are conserved in evolution and are usually important for microbial fitness. Bacterial flagellin and elongation factor Tu (EF-Tu), and fungal chitin are the best studied examples of PAMPs (Zipfel 2014). Recognition of PAMPs by their cognate PRRs transduces signals from the apopalst into the cytosol and activates a series of immune responses,

collectively called PAMP-triggered immunity (Boller and Felix 2009), which is critical to restrict pathogen invasion.

1.2.2 Signaling events within pattern recognition receptor (PRR) complexes

An increasing amount of evidence suggests PRRs form complexes with various interacting proteins, including co-receptors for perception, regulatory proteins for controlling signal amplitude and attenuation, and receptor-like cytoplasmic kinases (RLCKs) for signal transduction (Liang and Zhou 2018). One of the well-studied plant PRRs is the *Arabidopsis* receptor kinase FLS2 (FLAGELLIN SENSING 2) (Gomez-Gomez and Boller 2000), which recognizes bacterial flagellin through perception of flg22, a conserved peptide of 22 amino acids within its N terminus (Felix et al. 1999). The FLS2 protein contains 28 leucine rich repeats (LRRs) in its ectodomain, which directly binds flg22 (Chinchilla et al. 2006). The leucine-rich repeat (LRR) receptor-like kinase BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE 1) rapidly forms a complex with FLS2 upon flg22 perception and is required for flg22-triggered signaling (Chinchilla et al. 2007). Structural analysis further revealed the molecular mechanisms underlying flg22 perception by FLS2-BAK1 complex (Sun et al. 2013). flg22-induced FLS2-BAK1 complex is mediated by their ectodomains. flg22 binds to the concave surface spanning LRR3 to LRR16 of the FLS2 superhelical ectodomain. The C-terminal segment of flg22 acts as a "molecular glue" and bridges LRRs of FLS2 and BAK1 to stabilize the heterodimerization. Heterodimerization of FLS2 and BAK1 leads to rapid phosphorylation of their cytoplasmic kinase domains and subsequent activation of downstream signaling events (Schulze et al. 2010).

Several RLPs were shown to function as PRRs. As RLPs lack a cytoplasmic kinase domain, they rely on associated RLKs to transduce the signal. *Arabidopsis* LRR-RLK SOBIR1 (SUPPRESSOR OF BIR1 1) has been shown to serve as a common adaptor kinase for LRR-RLP type PRRs (Gust and Felix 2014). For example, RLP23, the receptor for nlp20 (a conserved 20-amino-acid peptide found in many Necrosis and Ethylene-Inducing Peptide 1-Like Proteins), constitutively interacts with SOBIR1 (Albert et al. 2015). Upon binding of nlp20, RLP23 recruits BAK1 to form a tripartite complex, which is essential for nlp20 perception and subsequent signaling activation (Albert et al. 2015).

Surfaced-localized PRRs require cytoplasmic partners such as receptor-like cytoplasmic kinases (RLCKs) to activate downstream intracellular signaling. Several RLCKs, including BIK1 (BOTRYTIS-INDUCED KINASE 1), PBL1 (avrPphB SUSCEPTIBLE 1 (PBS1) -LIKE 1), BSK1 (BR-SIGNALING KINASE 1), PCRK1 (PTI COMPROMISED RECEPTOR LIKE KINASE 1) and PCRK2, have been shown to directly associate with PRR complexes and function as positive regulators of PTI signaling (Liang and Zhou 2018). Among them, BIK1 is the best studied RLCK in PRR complexes. It associates with FLS2 and BAK1 in the absence of flg22 and becomes phosphorylated by BAK1 upon flg22 perception. Phosphorylated BIK1 subsequently dissociates from the PRR complex to activate downstream signaling (Zhang, Li, et al. 2010, Lu et al. 2010). BIK1 also associates with other PRRs such as EFR (EF-TU RECEPTOR) and CERK1 (CHITIN ELICITOR RECEPTOR KINASE 1) and plays important roles in response to efl18 and chitin, suggesting that BIK1 acts as a common signaling component downstream of multiple PRRs to regulate PTI (Liang and Zhou 2018). BIK1 accumulation is under tight controls by positive regulation via heterotrimeric G proteins (Liang et al. 2016) and negative regulation via calcium-dependent protein kinase CPK28 (CALCIUM-DEPENDENT PROTEIN KINASE 28) (Monaghan et al. 2014). A recent study showed that two homologous E3 ligases PUB25 (PLANT U-BOX PROTEIN 25) and PUB26 target BIK1 for degradation and that the E3 ligase activity of PUB25/26 is inhibited by heterotrimeric G proteins and enhanced by CPK28-mediated phosphorylation of conserved residues in PUB25/26. Thus, a multiprotein regulatory module present in PRR complexes tightly regulates BIK1-mediated immune responses (Wang et al. 2018).

1.2.3 Signaling downstream of PRR complexes

A number of cellular responses are activated upon PAMP perception, including very early responses within minutes [Ion fluxes, production of ROS (Reactive oxygen species) and activation of MAPKs (Mitogen-associated protein kinases, also called MPKs)], early responses within an hour (Ethylene production and activation of gene expression) and later responses (Callose deposition, seedling growth inhibition and production of salicylic acid (SA) (Boller and Felix 2009). These responses are involved in either transducing signal further downstream or preparing cells physiologically for defense against pathogens. For example, ROS can directly kill

a pathogen (Levine et al. 1994) or strengthen cell walls by crosslinking cell wall glycoproteins to inhibit pathogen invasion (Bradley, Kjellbom, and Lamb 1992). ROS also acts as a signal to induce further responses, including activation of stomatal closure (Kwak et al. 2003). In *Arabidopsis*, apoplastic ROS production in defense responses is mainly catalyzed by the NADPH oxidase, RBOHD (RESPIRATORY BURST OXIDASE HOMOLOG D) (Torres, Dangl, and Jones 2002). RBOHD has been shown to be part of the PRR complex and is a direct substrate of BIK1. Upon PAMP perception, RBOHD is rapidly phosphorylated by BIK1 and phosphorylation of RBOHD is required for PAMP-induced ROS burst and stomatal immunity (Li et al. 2014, Kadota et al. 2014).

MAPK cascades are involved in transducing signals from upstream transmembrane receptors and play crucial roles in controlling many biological processes, such as stomata development and plant immunity (Meng and Zhang 2013). Generally, activation of plasma membrane receptors such as RLKs lead to the activation of MAP kinase kinase kinases (MAPKKKs), which sequentially activate downstream MAP kinase kinases (MAPKKs or MKKs) that in turn activate MPKs. Active MPKs target various substrates including other protein kinases, enzymes, or transcription factors. For example, perception of flg22 by FLS2 activates the MEKK1-MKK1/MKK2-MPK4 cascade (Qiu, Zhou, et al. 2008, Gao et al. 2008). Active MPK4 then phosphorylates its substrate MKS1 (MAP KINASE SUBSTRATE 1). Subsequently, the MKS1 and WRKY33 (WRKY DNA-BINDING PROTEIN 33) complex is released from MPK4, allowing WRKY33 to regulate target gene expression in the nucleus (Qiu, Fiil, et al. 2008).

The MKK4/MKK5-MPK3/MPK6 module can assemble with different MAPKKKs and transduce different input signals to activate various downstream output responses. For example, the YODA-MKK4/MKK5-MPK3/MPK6 cascade plays crucial role in regulating the stomata development and patterning pathway. Loss-of-function of this MAPK cascade disrupts the epidermal cell fate coordination and results in clustered stomata (Wang et al. 2007). This module can also be activated by pathogen infection and then induces defense gene expression and confers resistance to pathogens (Ren et al. 2008, Meng and Zhang 2013). A recent study identified two redundant MAPKKKKs, MAPKKK3 and MPKKK5, that function upstream of MKK4/MKK5-MPK3/MPK6 to positively regulate plant immunity (Sun et al. 2018). PAMP-

triggered MPK activation and basal resistance to bacterial pathogen are compromised in *mapkkk3 mapkkk5* double mutant (Sun et al. 2018). A very recent research has revealed that RLCKs play direct roles in transducing signals from PRR complexes to the MAPK cascade (Bi, et al 2018).

1.3 Effectors-triggered susceptibility

Adapted pathogens are able to secrete effectors that interfere with PTI and facilitate pathogen colonization (Jones and Dangl 2006). *Pseudomonas syringe* bacteria deliver effectors into plant cells via type III secretion system (T3SS) (Galan and Collmer 1999). There are a large number of reports on identifying host targets of type-III effectors, which have revealed two main mechanisms of how effectors contribute to pathogenesis, either by suppressing host immune response or by causing an aqueous apoplastic environment to facilitate bacteria growth (Xin, Kvitko, and He 2018). Many type-III effectors suppress plant immune response by targeting various PTI components including PRR complexes and downstream RLCKs and MAPK cascades (Dou and Zhou 2012, Macho and Zipfel 2015). For example, AvrPtoB functions as an E3 ligase for the degradation of FLS2 (Gohre et al. 2008). Recently, AvrPtoB was also shown to facilitate the degradation of the SA receptor NPR1 to block SA signaling (Chen et al. 2017). Another effector AvrPphB functions as a cysteine protease that cleaves BIK1 and other PBLs to inhibit PTI (Zhang, Li, et al. 2010). Moreover, HopAI1 is a phosphothreinine lyase that interacts with and inactivates MPK3 and MPK6 by removing the phosphate group from phosphothreonine (Zhang et al. 2007). Lastly, HopM1 and AvrE can trigger a “water soaking” symptom in infected leaves where liquid is accumulated in the apoplast (Xin et al. 2016). This aqueous apoplast is critical for bacterial virulence, as the *Pst* DC3000 mutant lacking both HopM1 and AvrE effectors shows greatly reduced virulence and poorly infects its host plants. This compromised virulence can be restored by supplementation of water to the apoplast (Xin et al. 2016).

1.4 Effector-triggered immunity (ETI)

1.4.1 Overview

In addition to PRRs, plants have evolved another type of immune receptors to perceive secreted effectors directly or indirectly and activate effector-triggered immunity (ETI). Recognition of pathogen effectors by their cognate receptors often results in rapid cell death known as the hypersensitive response (HR) (Jones and Dangl 2006). Most immune receptors involved in perception of pathogen effectors are intracellular and possess nucleotide-binding (NB) and leucine-rich repeat (LRR) domains. They are structurally similar to mammalian NOD (Nucleotide-Binding Oligomerization Domain)-like receptors and are thus called plant NOD-like receptors (NLRs).

1.4.2 Plant NLRs

Typical plant NLRs can be subdivided into two groups depending on the structure of their N-terminal domains. Those possessing a TIR (Toll interleukin receptor) domain are designated as TIR-NB-LRR proteins or TNLs, and the ones that possess a CC (Coiled-coil) domain are termed CC-NB-LRR proteins or CNLs. Many CNLs requires membrane-localized protein NDR1 (NON RACE-SPECIFIC DISEASE RESISTANCE 1) for their function (Aarts et al. 1998). Signaling activated by TNLs converges on the lipase-like proteins EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1), PAD4 (PHYTOALEXIN DEFICIENT 4), and SAG101 (SENESCENCE-ASSOCIATED GENE 101) (Aarts et al. 1998, Feys et al. 2001, Feys et al. 2005).

1.4.3 Perception of effectors by NLRs.

Three different models, namely the direct interaction model, guard model and decoy model, have been proposed to explain how NLRs perceive their cognate effectors (van der Hoorn and Kamoun 2008). In the direct interaction model, the NLRs directly bind to and recognize effector proteins. For example, *Arabidopsis* TNL RPP1 (RECOGNITION OF PERONOSPORA PARASITICA 1), which recognizes oomycete effector ATR1 and causes HR, directly interacts with ATR1 via its LRR domain (Krasileva, Dahlbeck, and Staskawicz 2010). Another example is

the effector AVR-Pita from rice blast fungus *Magnaporthe grisea*, which is directly recognized by rice NLR Pi-ta. *AVR-Pita* is predicted to encode a metalloprotease with N-terminal secretory and pro-protein sequences. Transient expression of *AVR-Pita*$_{176}$, which lacks the secretory and pro-protein sequences, inside plant cells triggered a Pi-ta-dependent resistance response. AVR-Pita$_{176}$ protein was shown to physically interact with LRR domain of Pi-ta and this physical interaction was implicated in initiating Pi-ta-mediated defense response (Jia et al. 2000).

In the guard model, the NLR protein monitors alteration of a host protein (also called guardee) that is targeted by the effector protein(van der Hoorn and Kamoun 2008). One well-studied example of guardee is RIN4 (RPM1 INTERACTING PROTEIN 4), which is monitored by two CNLs RPS2 (RESISTANT TO P. SYRINGAE 2) and RPM1 (RESISTANCE TO P. SYRINGAE PV MACULICOLA 1). RIN4 plays a role in basal resistance in *Arabidopsis* and is targeted by effectors AvrRpt2 and AvrRpm1 for degradation and phosphorylation, respectively. The alteration of RIN4 by AvrRpt2 and AvrRpm1 is perceived by the cognate NLRs RPS2 and RPM1, and triggers activation of ETI (Kim et al. 2005).

In the decoy model, the host proteins (also called decoys) that are targeted by pathogen effectors and monitored by cognate NLRs have no immune function in the absence of cognate NLRs (van der Hoorn and Kamoun 2008). As mentioned above, PBS1 and PBLs are targets of the effector protein AvrPphB, which functions as a cysteine protease to cleave these kinases. Cleavage of PBS1 is monitored by the CNL RPS5 and triggers activation of ETI (Ade et al. 2007). While PBLs are positive regulators of PTI and cleavage of PBLs by AvrPphB inhibits PTI and benefits bacterial colonization in plants that lack RPS5, PBS1 doesn't play a role in PTI itself and functions as a "decoy" for these PBLs (Zhang, Li, et al. 2010, Ade et al. 2007). Both guard and decoy models expand the recognition capacity of the NLRs, allowing limited number of plant NLRs to recognize large number of effectors from different pathogens.

1.4.4 Immune responses activated by NLRs

Perception of effector's activities by cognate NLR leads to the activation of NLRs. NLR activation has been proposed to involve NLR protein conformational change and/or oligomerization (Bonardi and Dangl 2012). However, the exact molecular mechanisms of NLR activation remain unclear. The immune responses activated in ETI and PTI are similar, including

ROS burst, activation of MAPKs, transcriptional reprogramming, plant metabolite changes, etc.; but the responses in ETI are generally more prolonged and stronger compared to those in PTI (Jones and Dangl 2006). How NLRs transduce defense signals to activate downstream responses is still unclear.

1.5 Systemic acquired resistance (SAR)

1.5.1 Overview

Both PTI and ETI occur at the infection sites. These local defense responses tend to activate secondary immune responses in systemic parts of the host plant, conferring enhanced resistance against subsequent pathogen attack, a phenomenon called systemic acquired resistance (Ross 1961, Durrant and Dong 2004, Mishina and Zeier 2007). Description of the SAR phenomenon can date from the beginning of 20th century as reviewed in detail by Conrath U. (Conrath 2006). Early studies on SAR made two main discoveries on SAR signaling, namely, *PATHOGENESIS-RELATED* (*PR*) genes as SAR marker genes (van Loon 1985) and salicylic acid (SA) as a SAR signal inducer (White 1979). SA was shown to be an essential signaling molecule but not the mobile signal in SAR (Gaffney 1993, Delaney 1994, Vernooij, Friedrich, et al. 1994). *PR* genes have been widely used as markers for studying SAR signaling (Ryals et al. 1996). There are several common features in SAR (Durrant and Dong 2004). First, it is a broad-spectrum resistance against various pathogens. Second, it is long-lasting and can remain effective for as long as several months. Third, it involves physiological changes such as SA accumulation and *PR* gene expression.

1.5.2 Signaling molecules in SAR

Early grafting experiments suggest that the infected tissue is the source of the signal(s) for SAR and the signal(s) is/are transmissible from infected leaf to systemic tissues (White 1979, Dean and Kuc 1986). Since then, a large number of signaling molecules have been implicated in SAR signaling, including salicylic acid (Vernooij, Uknes, et al. 1994), methyl salicylate (Park et al. 2007), the lipid-transfer protein DIR1 (Defective in induced resistance 1) (Maldonado et al. 2002), glycerol-3-phosphate (Chanda et al. 2011), azelaic acid (Jung et al. 2009),

dehydroabietinal (Chaturvedi et al. 2012), the amino acid-derivative pipecolic acid (Pip) (Navarova et al. 2012) and its derivative N-hydroxypipecolic acid (Hartmann et al. 2018, Chen et al. 2018). The roles of those signals in SAR have been discussed extensively in reviews (Dempsey and Klessig 2012, Shah and Zeier 2013, Adam et al. 2018). Whether one or more of these signals is/are the mobile signal(s) for SAR remains to be clarified.

1.5.3 Signaling pathways downstream of SAR

The signaling pathways activated in systemic tissues after perception of the mobile signal(s) probably overlap with those observed in both PTI and ETI, as most mutants that are defective in SAR also show different levels of compromise in PTI and/or ETI. For example, as mentioned above, EDS1 and PAD4 are required for TNL-activated ETI responses; *Arabidopsis* mutants with mutations in EDS1 or PAD4 also exhibit severely compromised SAR (Jirage et al. 1999, Falk et al. 1999). The components involved in SA signaling were implicated in both SAR and local responses (Wildermuth et al. 2001, Nawrath et al. 2002, Cao et al. 1997, Gao et al. 2015). In addition, the Pip signaling pathway was also shown to play critical roles in both SAR and local responses (Song et al. 2004, Koch et al. 2006, Mishina and Zeier 2006, Navarova et al. 2012).

1.6 Salicylic acid (SA) signaling in plant immunity

1.6.1 Overview

As mentioned above, SA was found to act as a SAR inducer in early studies by White et al, which demonstrated that application of SA or aspirin (acetylsalicylic acid) could induce PR protein accumulation and enhanced resistance against tobacco mosaic virus (TMV) infection (White 1979). Two reports in 1990 suggest that SA could function as an endogenous signal whose levels increase in both infected leaves and systemic leaves (Malamy 1990, Metraux 1990). This idea was subsequently supported by analysis of transgenic tobacco or *Arabidopsis* plants expressing the bacterial *nahG* gene, which encodes a salicylate hydroxylase that converts SA to catechol (Gaffney 1993). These plants failed to accumulate SA and develop SAR, but also exhibited increased susceptibility to various pathogens, demonstrating that SA is an essential

signal in plant immunity (Gaffney 1993, Delaney 1994). Since then, SA signaling in plant immunity has been extensively examined, including SA biosynthesis pathways as well as upstream and downstream signaling components.

1.6.2 SA biosynthesis pathways

The main SA biosynthesis pathway was revealed by characterizing *Arabidopsis sid2* (*salicylic acid induction deficient 2*) mutant and cloning of *SID2* gene, which encodes Isochorismate Synthase 1 (ICS1) (Wildermuth et al. 2001). *Arabidopsis* genome carries two *ICS* genes, *ICS1* and *ICS2* (Garcion et al. 2008). Only the expression of *ICS1* is induced by pathogens. SA production as well as both local resistance and SAR are dramatically compromised in mutants lacking a functional ICS1, which contributes to about 90% of total SA production induced by pathogens (Wildermuth et al. 2001). Thus, pathogen-induced SA biosynthesis in *Arabidopsis* is predominantly dependent on the ICS pathway, where the primary metabolite chorismate is converted into isochorismate through ICS1 and then the isochorismate is further lysed into SA and pyruvate through unidentified enzyme(s) (Wildermuth et al. 2001). The SA level is further reduced but still detectable in *ics1 ics2* double mutant, indicating that the ICS pathway is not the sole source of SA in *Arabidopsis* (Garcion et al. 2008).

The ICS1 and ICS2 are both localized in chloroplasts, suggesting SA synthesis starts in chloroplasts (Strawn et al. 2007, Garcion et al. 2008). As SA functions in the cytosol and the nucleus, SA might require a transporter to be exported from chloroplasts to the cytosol. Characterization of the *sid1*/*eds5* (*Enhanced disease susceptibility 5*) mutant and cloning of *EDS5* revealed that *SID1*/*EDS5* encodes a multidrug and toxin extrusion-like transporter, which localizes to the chloroplast envelope and has been suggested to mediate the specific transport of SA (Nawrath and Metraux 1999, Nawrath et al. 2002, Serrano et al. 2013). This transport process seems to be critical for SA signaling as both SA accumulation and disease resistance are greatly compromised in *eds5* mutant (Nawrath and Metraux 1999, Nawrath et al. 2002).

Another reported SA biosynthesis pathway is the PAL (PHENYLALANINE AMMONIA LYASE) pathway, which has been reviewed in detail by Dempsey *et al.* (Dempsey et al. 2011). *Arabidopsis* genome carries four PAL genes (PAL1-4). The quadruple knockout mutants, which possess only 10% of PAL activity, accumulate reduced level (50%) of total SA compared to wild

type after pathogen infection (Huang et al. 2010), confirming that the PAL pathway also contributes to SA biosynthesis in *Arabidopsis*.

1.6.3 Defense signaling upstream of SA

SA signaling plays important roles in ETI as revealed by studies using plants defective in SA accumulation. It was shown that transgenic plants expressing *nahG* gene exhibited enhanced susceptibility to avirulent pathogens (Delaney 1994) and delayed hypersensitive response (HR) (Mur et al. 1997). In addition, the *sid2* mutant that is unable to accumulate SA in response to avirulent pathogen *Pst*. DC3000 *AvrRpt2* exhibited compromised resistance against this avriulent pathogen (Ranf et al. 2011). Several studies have shown that SA functions downstream of the ETI regulators EDS1/PAD4 and NDR1, as reviewed in detail by Vlot *et al.* (Vlot, Dempsey, and Klessig 2009). However, how NLR activation during ETI leads to increased SA levels remains largely unknown.

SA signaling was also shown as an integral part of PTI. Treatment with PAMPs such as flg22 or bacterial lipopolysaccharide (LPS) activates SA accumulation as well as SAR response (Mishina and Zeier 2007). flg22 treatment induces the expression of genes involved in SA signaling (Denoux et al. 2008). SA levels also increase significantly in plants challenged with *Pst* DC3000 *hrcC*, a strain defective in Type III secretion system that is unable to secrete effectors and serve as an elicitor of the PTI response (Tsuda et al. 2008, Wei et al. 2000). The *sid2* mutant fails to accumulate SA in response to *Pst* DC3000 *hrcC* and exhibits enhanced susceptibility to this bacterium. In addition, resistance against *Pst* DC3000 induced by flg22 treatment was compromised in *sid2* mutant plants, further demonstrating that SA is important for PTI (Tsuda et al. 2008).

Since the expression of *ICS1* is highly induced upon pathogen infection, identifying upstream transcription factors that regulate *ICS1* expression is critical to understand the regulation of SA accumulation. Two closely related transcription factors (TFs), SARD1 (SAR DEFICIENT 1) and CBP60g (CALMODULIN BINDING PROTEIN 60 g) were found to be required for *ICS1* expression and SA accumulation during plant immunity, and their expression is also highly induced upon pathogen infection (Zhang, et al. 2010, Wang, et al. 2011). SARD1 and CBP60g were shown to directly bind to the promoter region of *ICS1* to regulate its

expression and SA biosynthesis during pathogen infection (Zhang, et al. 2010). SARD1 and CBP60g carry a DNA binding domain in their conserved central regions (Zhang, et al. 2010), but they have diverse N- and C- terminal regions, implying that their activities may be regulated differently. Indeed, overexpression of *SARD1*, but not *CBP60g*, leads to constitutive activation of immune responses (Zhang, et al. 2010). Unlike CBP60g, which binds calmodulin through its N-terminus, SARD1 is unable to bind calmodulin, suggesting that CBP60g, but not SARD1, requires calmodulin binding for its activity (Zhang, et al. 2010, Wang, et al 2009). As the expression of *ICS1* and SA accumulation during plant defense was slightly affected in *sard1* and *cbp60g* single mutants, but almost completely blocked *in sard1 cbp60g* double mutant, SARD1 and CBP60g have been proposed to function in two parallel pathways to regulate the expression of *ICS1* and SA biosynthesis in plant immunity (Zhang, et al. 2010).

Besides SARD1 and CBP60g, other transcription factors that directly regulate *ICS1* expression have also been identified. Among them, EIN3 (ETHYLENE-INSENSITIVE 3) and its homolog EIL1 (EIN3-LIKE 1), and three Arabidopsis NAC (NAM, ATAF1/2 and CUC2) domain-containing proteins (ANAC019, ANAC055, and ANAC072) were identified as transcriptional repressors that negatively regulate *ICS1* expression and SA levels (Chen et al. 2009, Zheng et al. 2012). On the other hand, TFs TCP8 (TCP DOMAIN PROTEIN 8), TCP9, NTL9 (NAC TRANSCRIPTION FACTOR-LIKE 9) and CHE (CCA1 HIKING EXPEDITION) have been shown to function as positive regulators for *ICS1* induction and SA accumulation during plant immune responses (Wang et al. 2015, Zheng et al. 2015). Notably, unlike SARD1 and CBP60g, which are the two major TFs that control *ICS1* expression and SA accumulation during plant defense (Zhang, et al. 2010, Wang, et al. 2011), TCP8 and TCP9 play a minor role in regulating pathogen-induced *ICS1* expression and SA accumulation (Wang, et al. 2015). NTL9 was reported to be responsible for flg22-induced *ICS1* expression in guard cells and CHE was shown to regulate the circadian oscillations of *ICS1* expression and SA levels (Zheng et al. 2015). Together these findings suggest that *ICS1* expression is regulated in a delicate and complicated manner.

1.6.4 Defense signaling downstream of SA

Signaling downstream of SA in plant immunity has been extensively studied. Several genetic screens aiming for SA insensitive mutants resulted in multiple alleles of one gene *NPR1* (*NONEXPRESSOR OF PR GNENS 1*) /*NIM1* (*NON-INDUCIBLE IMMUNITY 1*) /*SAI1* (*SALICYLIC ACID INSENSITIVE 1*) (Cao et al. 1997, Ryals et al. 1997, Shah, Tsui, and Klessig 1997). These *npr1* alleles are insensitive to SA and its analogue INA and BTH, and are compromised in both SAR and basal resistance, indicating an essential role of NPR1 downstream of SA.

NPR1 contains an N-terminal BTB domain, a central ankyrin repeat-containing domain and a C- terminal domain. Lacking a canonical DNA- binding domain, NPR1 interacts with and relies on TGA transcription factors to regulate *PR* gene expression and SAR (Cao et al. 1997, Zhang et al. 1999, Despres et al. 2000, Zhou et al. 2000, Zhang et al. 2003). Recent studies showed that NPR1 is an SA-binding protein (Wu et al. 2012, Manohar, et al. 2014). Binding of SA causes a conformational change in NPR1 and enhances its activity in transcriptional activation, suggesting that NPR1 is an SA receptor (Wu et al. 2012). Two closely related NPR1 paralogues NPR3 and NPR4, which serve as negative regulators in plant immunity (Zhang et al. 2006), were also shown to bind SA with different affinities and were proposed to function as adaptors for a cullin3 E3 ligase complex to regulate NPR1 degradation in response to SA (Fu et al. 2012). However, this model is not always consistent with the experimental data observed from *npr3*, *npr4*, *npr3 npr4* mutant plants (Kuai, et al. 2015). For example, this model proposed that binding of SA to NPR4 inhibits its association with NPR1, whereas binding of SA to NPR3 promotes its interaction with NPR1 and degradation of NPR1, suggesting that NPR3 and NPR4 function as independent SA-receptors in response to low and high SA concentrations, respectively. This is somehow contrary to the previous finding that NPR3 and NPR4 were shown to function redundantly in negative regulation of plant immunity (Zhang et al. 2006). In addition, based on this model in which NPR4 is a CUL3 substrate adaptor only in the absence of SA (Fu et al. 2012), it would be expected that the *npr3* single mutant would be as resistant as the *npr3 npr4* double mutant in response to pathogen infection during which SA level increased considerably. However, it was observed that *npr3 npr4* mutant was more resistant than the single *npr3* mutant (Zhang et al. 2006, Fu et al. 2012). Furthermore, NPR1 protein levels still increased in *npr3*

npr4 double mutant after SA treatment as shown in the *in vivo* NPR1 degradation experiment (Fu et al. 2012), indicating that the *npr3 npr4* double mutant was still responsive to SA, suggesting that there is(are) other SA receptor(s) which contribute to NPR1 accumulation. A different model has been proposed based on new data from a gain-of-function allele of *NPR4* (Ding et al. 2018), also see details in chapter 4.

Besides its critical roles in plant immunity, SA is also involved in other biological processes such as thermogenesis. SA might regulate these processes by targeting different proteins. Using various biochemical approaches, about 30 SA-binding proteins (SABPs) have been identified as SA-binding partners (Klessig, et al. 2016). The functions of some of these SABPs have been characterized. SABP1 is a catalase involved in the production of H_2O_2, whose activity is inhibited by SA (Chen, Ricigliano, and Klessig 1993). SABP2, a lipase protein in tobacco was implicated in plant innate immunity, whose lipase activity is enhanced by SA (Kumar and Klessig 2003). The tobacco SABP3 is a chloroplast carbonic anhydrase (CA) with antioxidant activity, which contributes to the hypersensitive defense response (Slaymaker et al. 2002). Thus, SA could exert its effects via different SABPs and functional analysis of SABPs may provide a better understanding of the functions of SA.

1.7 Pipecolic acid (Pip) and N-hydroxypipecolic acid in plant immunity

Pathogen infection induces transcription reprogramming in both local and systemic tissues. Two SAR regulators, *ALD1* (*AGD2-LIKE DEFENSE RESPONSE PROTEIN 1*) and *FMO1* (*FLAVIN-DEPENDENT MONOOXYGENASE 1*), are induced locally and systemically upon pathogen infection. Phenotypic analysis of *ald1* and *fmo1* mutants demonstrated that both genes are required for systemic SA accumulation and SAR as well as full activation of local responses (Song et al. 2004, Mishina and Zeier 2006, Koch et al. 2006). Another SAR regulator SARD4 (SAR DEFICIENT 4) was found to be required for enhanced disease resistance in a *FMO1* activation-tagging line (Ding et al. 2016). The expression of *SARD4* is also induced during pathogen infection and *sard4* mutant plants exhibit compromised SAR (Ding et al. 2016, Hartmann et al. 2017). Further biochemical analysis showed that ALD1 and SARD4 are involved in the biosynthesis of pipecolic acid (Pip), a plant secondary metabolite required for both local resistance and SAR (Navarova et al. 2012, Ding et al. 2016, Hartmann et al. 2017).

ALD1 encodes an aminotransferase that removes α-amino group from L-Lys to form 1-piperideine-2-carboxylic acid (P2C) and *SARD4* encodes a reductase that reduces P2C to Pip (Ding et al. 2016, Hartmann et al. 2017). Consistently, exogenous application of Pip overrides compromised local resistance and loss of SAR in *ald1* and *sard4* plants (Navarova et al. 2012, Ding et al. 2016, Hartmann et al. 2017). However, exogenous Pip is unable to restore *fmo1* defects in SAR and local responses, suggesting that FMO1 may function downstream of Pip (Navarova et al. 2012).

Two very recent studies revealed that FMO1 monooxygenates Pip to form N-hydroxypipecolic acid (NHP) (Hartmann et al. 2018, Chen et al. 2018). NHP accumulates in both local and systemic tissues in SAR-induced wild type plants, but not in *fmo1* mutant plants. Exogenous NHP enhances disease resistance in wild-type plants and, more importantly, overrides the defects of *fmo1* in SAR and local responses, demonstrating that NHP is a critical molecule in plant immune response (Hartmann et al. 2018, Chen et al. 2018). Thus, a plant secondary metabolic pathway consisting of ALD1, SARD4 and FMO1 converts L-Lys into NHP to positively regulate plant immunity. However, how NHP enhances plant resistance is currently unclear.

1.8 Thesis objectives

As SARD1 and CBP60g regulate pathogen-induced SA accumulation and the expression of both genes is also highly induced upon pathogen infection, one objective of this dissertation is to identify signaling components that regulate the expression of *SARD1* and *CBP60g* in plant immunity. Another objective is to identify downstream targets of SARD1 and CBP60g to better understand their roles in plant immunity.

Chapter 2: ChIP-seq reveals broad roles of SARD1 and CBP60g in regulating plant immunity

2.1 Summary

Recognition of pathogens by host plants leads to rapid transcriptional reprograming and activation of defense responses. The expression of many defense regulators is induced in this process, but the mechanisms of how they are controlled transcriptionally are largely unknown. Chromatin immunoprecipitation -sequencing analysis showed that transcription factors SARD1 and CBP60g control the expression of a large number of key regulators of plant immunity. Among them are positive regulators of systemic immunity and signaling components for effector-triggered immunity and PAMP-triggered immunity, which is consistent with the critical roles of SARD1 and CBP60g in these processes. In addition, SARD1 and CBP60g target a number of negative regulators of plant immunity, suggesting that they are also involved in negative feedback regulation of defense responses. Our study revealed that SARD1 and CBP60g function as master regulators of plant immune responses.

2.2 Introduction

Plants use a multilayered defense system to combat microbial pathogens. At the front line, pattern recognition receptors on the plasma membrane recognize conserved features of microbes, collectively known as microbe-associated molecular patterns or pathogen-associated molecular patterns (PAMPs), to activate PAMP-triggered immunity (PTI) (Monaghan and Zipfel 2012). Most PAMP receptors belong to the receptor-like kinase (RLK) and the receptor-like protein (RLP) families. A second line of plant defense called effector-triggered immunity (ETI) relies on resistance (R) proteins that detect effector proteins secreted by pathogens to inhibit PTI (Jones and Dangl 2006). The majority of plant R proteins belong to the intracellular nucleotide-binding site (NB) leucine-rich repeats (LRR) protein family. Recognition of pathogens and activation of local defense responses further induce a secondary immune response in the distal part of plants termed systemic acquired resistance (SAR) (Durrant and Dong 2004).

Salicylic acid (SA) is a signal molecule that plays key roles in local defense as well as SAR (Vlot, Dempsey, and Klessig 2009). *SALICYLIC ACID INDUCTION–DEFICIENT 2* (*SID2*) and *ENHANCED DISEASE SUSCEPTIBILITY 5* (*EDS5*) are required for pathogen-induced SA accumulation (Nawrath and Metraux 1999, Wildermuth et al. 2001). Mutations in *SID2* or *EDS5* block the accumulation of SA, resulting in enhanced susceptibility to pathogens and loss of SAR (Nawrath and Metraux 1999, Wildermuth et al. 2001, Rogers and Ausubel 1997). *SID2* encodes Isochorismate Synthase 1 (ICS1), which is a key enzyme in pathogen-induced SA synthesis (Wildermuth et al. 2001). *EDS5* encodes a transporter involved in exporting SA from chloroplast to cytoplasm (Nawrath et al. 2002, Serrano et al. 2013). Activation of defense gene expression and pathogen resistance by SA depends on the downstream component NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) (Dong 2004). Recent studies showed that NPR1 and its paralogs, NPR3 and NPR4, bind to SA and may function as SA receptors (Wu et al. 2012, Fu et al. 2012).

Several genes encoding enzymes implicated in the synthesis of secondary metabolites have also been identified to be essential for SAR. Among them, *FLAVIN-DEPENDENT MONOOXYGENASE1* (*FMO1*) encodes a putative monooxygenase (Bartsch et al. 2006, Koch et al. 2006, Mishina and Zeier 2006), *AGD2-LIKE DEFENSE RESPONSE PROTEIN1* (*ALD1*) encodes an aminotransferase (Song, Lu, and Greenberg 2004), and *avrPphB SUSCEPTIBLE3* (*PBS3*) encodes a member of the firefly luciferase superfamily (Jagadeeswaran et al. 2007, Lee et al. 2007, Nobuta et al. 2007). In *fmo1*, *ald1* and *pbs3* mutants, SAR is severely compromised (Mishina and Zeier 2006, Song et al. 2004, Jing et al. 2011). ALD1 is involved in the synthesis of pipecolic acid, which contributes to the induction of SAR (Navarova et al. 2012), while the chemicals synthesized by FMO1 and PBS3 remain to be determined.

Two pathogen-induced transcription factors, SAR DEFICIENT1 (SARD1) and CAM-BINDING PROTEIN 60 G (CBP60g), regulate the expression of *ICS1* and are required for pathogen-induction of SA synthesis (Wang et al. 2009, Zhang, Xu, et al. 2010, Wang, Tsuda, Truman, Sato, Nguyen le, et al. 2011). Following pathogen infection, SARD1 and CBP60g are recruited to the promoter of *ICS1* (Zhang, Xu, et al. 2010). In the *sard1 cbp60g* double mutant, induction of *ICS1* expression and SA synthesis is blocked (Zhang, Xu, et al. 2010, Wang, Tsuda, Truman, Sato, Nguyen le, et al. 2011). SARD1 and CBP60g belong to the same protein family

but are regulated differently, suggesting that they function in two parallel pathways to activate *ICS1* expression (Wang et al. 2009, Zhang, Xu, et al. 2010). CBP60g, but not SARD1, can bind camodulin (CaM). On the other hand, over-expression of *SARD1* but not *CBP60g* leads to constitutive activation of defense responses.

Arabidopsis *SNC2* encodes an RLP that is required for resistance against pathogenic bacteria *Pseudomonas syringae* pv *tomato* (*P.s.t.*) DC3000 as well as non-pathogenic bacteria *P.s.t.* DC3000 *hrcC* (Zhang, Yang, et al. 2010, Yang et al. 2012). A gain-of-function mutation in *snc2-1D* leads to constitutive activation of both SA-dependent and SA-independent defense pathways (Zhang, Yang, et al. 2010). The *snc2-1D* mutant has small stature, accumulates high levels of salicylic acid, constitutively expresses *PATHOGENESIS-RELATED* (*PR*) genes, and exhibits enhanced pathogen resistance. From a suppressor screen of *snc2-1D npr1-1*, WRKY DNA-BINDING PROTEIN 70 (WRKY70) was identified as an essential regulator of the SA-independent pathway downstream of *snc2-1D* (Zhang, Yang, et al. 2010).

Here we report that SARD1 and CBP60g regulate not only the expression of *ICS1* and SA synthesis, but also the expression of *WRKY70* and the SA-independent defense pathway in *snc2-1D*. Chromatin immunoprecipitation (ChIP) analysis revealed that a large number of plant defense regulators including *WRKY70* are direct target genes of SARD1 and CBP60g, suggesting that SARD1 and CBP60g function as master regulators of plant defense responses.

2.3 Material and method

2.3.1 Plant materials and growth conditions

Arabidopsis sard1-1, *cpb60g-1*, *sard1-1 cbp60g-1* and *snc2-1D npr1-1* mutants and *SARD1-HA* and *CBP60g-HA* transgenic plants were described previously (Zhang, Xu, et al. 2010, Zhang, Yang, et al. 2010). The *snc2-1D* single mutant was identified from the F2 population of a cross between Col-0 and *snc2-1D npr1-1*. *sard1-1 snc2-1D*, *cpb60g-1 snc2-1D* and *sard1 cpb60g snc2-1D* mutants were isolated from the F2 population of a cross between *sard1-1 cpb60g-1* and *snc2-1D npr1-1*. Primers used for genotyping are listed in Supplementary Table 2 (Sun et al. 2015). Plants were grown under long day conditions (16 h light/ 8 h dark cycle) at 23°C unless otherwise specified.

2.3.2 Gene expression analysis

To analyze gene expression in *snc2-1D, sard1-1 snc2-1D, cbp60g-1 snc2-1D* and *sard1 cbp60g snc2-1D,* 50 mg of leaves were collected from three-week-old soil-grown plants for RNA isolation. To analyze gene expression after *P.s.m.* ES4326 infection, leaves of 25-day-old plants grown under short day conditions (12 h light/ 12 h dark cycle) were infiltrated with *P.s.m.* ES4326 or 10 mM $MgCl_2$ 12 h before sample collection. Four leaves from four individual plants were mixed as one sample. RNA was isolated using EZ-10 Spin Column Plant RNA Mini-Preps Kit (BIO BASIC CANADA). M-MuLV reverse transcriptase was used for reverse transcription according to the manufacturer's instructions (New England Biolabs). Real-time PCR was performed using the SYBR Premix Ex Taq II (TAKARA). Primers used for real-time PCR were reported in Supplementary Table 2 (Sun et al. 2015). *ACTIN1* was used as an internal control.

2.3.3 Pathogen infection assays

H.a. Noco2 infection assays were carried out on three-week-old soil-grown plants by spraying plants with *H.a.* Noco2 spore suspension at a concentration of 50,000 per ml water. Afterwards, plants were covered with a clean dome and grown at 18°C under 12 h light/ 12 h dark cycle in a growth chamber. *H.a.* Noco2 sporulation was scored seven days later as previously described (Bi et al. 2010).

To assay for flg22-induced pathogen resistance, leaves of four-week-old plants were infiltrated with 1 μM of flg22 or ddH_2O as control. After 24 hrs, the same leaves were inoculated with *P.s.t.* DC3000 (OD_{600} = 0.001) in 10 mM $MgCl_2$. Three days post inoculation, a leaf disc was taken from each infected leaf and two leaf discs from the same plant were collected as one sample. The samples were ground, diluted serially in 10 mM $MgCl_2$, and plated on Lysogeny broth (LB) agar plates with 25 μg/ml rifampicin and 50 μg/ml kanamycin. After incubation at 28°C for 36 hrs, bacterial colonies were counted from selected dilutions and the colony numbers were used to calculate colony forming units (CFU).

2.3.4 ChIP Analysis.

For ChIP experiments, two to three fully expanded leaves of 25-day-old plants grown under short day condition were infiltrated with *P.s.m.* ES4326 (OD_{600} = 0.001). The inoculated leaves were collected after 24 hrs. About four grams of leaf tissue was cross-linked in 75 ml of 1% formaldehyde solution plus 0.01% silwet L-77 under vacuum for 20 minutes. 2 M glycine was added to a final concentration of 0.125 M and the sample was vacuumed for an additional 5 minutes to stop cross-linking. The tissue was rinsed three times with 60 ml of cold ddH_20 and dried with blotting paper. The nuclei were prepared as previously described (Cheng et al. 2009) and resuspended in 300 µl of nuclei lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, 0.1 mM PMSF, 1xPI). The nuclei suspensions were subsequently sonicated to shear the DNA to an average size of 0.3 to 1 kb.

The sonicated chromatin suspension was spun at 12,000 g for 5 min at 4°C to pellet debris. The supernatant was moved to a new 15 ml tube. An aliquot of 5µl from each sample was moved into a clean 1.5 ml Eppendorf tube and set aside at –20°C as "input". 3 ml of ChIP dilution buffer (1.1% Triton X-10, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl) was then added to the 15 ml tube. For pre-clearing, 100 µl of Protein A agarose beads balanced with the ChIP dilution buffer was added to the chromatin samples and kept at 4 °C for 1 hour with rotation. The beads were pelleted at 4500 rpm for 2 min and the supernatants were divided equally into two samples. One sample was added with 5 µl of anti-HA antibody for immunoprecipitation and the other sample was added with immunoglobin G as control. The samples were incubated overnight at 4°C with gentle agitation. Subsequently 100 µl of Protein A agarose beads balanced with ChIP dilution buffer was added to each sample and kept at 4°C for 2 h with gentle agitation.

The Protein A beads were then pelleted by centrifugation at 2400 g for 10 sec at 4°C. The beads were washed with Low salt wash buffer (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0), high salt wash buffer (500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0), LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and TE buffer sequentially. For each buffer, a quick wash by spinning at 2400 g for 10 s and a second wash with 5 min agitation were performed. 1ml of buffer was used in each wash.

After the final wash, the samples were pelleted for an additional 2 min at 2400 g rpm in order to remove the supernatant thoroughly. To elute the immune complexes, 250µl of Elution Buffer (1% SDS, 0.1 M $NaHCO_3$) was added to the beads. The samples were vortexed briefly and incubated at 65°C for 15 min with gentle agitation. After spinning at 3800 g for 2 min, the supernatant was carefully transferred to a fresh tube. The pellet was eluted one more time with 250µl of Elution Buffer and the two eluates were combined (a total of about 500 µl). At the same time, 500 µl of Elution Buffer was added to the input samples collected before immunoprecipitation. 1 µl of 10 mg/ml DNase-free RNase A was added to each sample. After incubatation at 37°C for 1 hour, 10 µl of 0.5 M EDTA, 20 µl 1 M Tris-HCl (pH 6.5) and 2 µl of 10 mg/ml proteinase K were added to each sample. The samples were incubated at 45°C for 1 h and extracted with the same volume of Tris saturated phenol:chloroform:isoamyl alcohol (25:24:1 v/v) twice. DNA was then precipitated by adding 0.7 volume isoproponal, 1/10 volume of 3 M NaOAc and 1µl 2M glycogen and incubating at room temperature for 30 min. DNA was pelleted by spinning for 20 min at 12000 rpm. The DNA pellets were washed with 80% ethanol, dried at room temperature, resuspended in 50 µl TE buffer, and stored at -20 °C for further use.

For ChIP-sequencing, DNA sequencing libraries were prepared from chromatin immunoprecipitated DNA and sequenced using an Illumina Genome Analyzer. Tissue from untreated SARD1-HA transgenic plants was used as the negative control. Sequence reads were mapped to Arabidopsis genome sequence using Bowtie 0.12.8 (Langmead et al. 2009). Sequence coverage at each position on the genome was scored by Samtools (Li, Handsaker, Wysoker, Fennell, Ruan, Homer, Marth, Abecasis, Durbin, et al. 2009) and used to identify peaks in the genome. The 500 bp sequences centered on the peak summits of genes shown in Supplementary Table 1 (Sun et al. 2015) were used to identify conserved SARD-binding motifs using DREME (Bailey 2011). DREME was run with default settings and sequences from the promoter regions of randomly chosen genes were used as background control. Confirmation of ChIP-seq results was carried out with independent ChIP experiments and immunoprecipitated DNA was quantified by real-time PCR using gene-specific primers. The primers used to amplify the promoter regions of the target genes are reported in Supplementary Table 2 (Sun et al. 2015). Real-time PCR was performed in 96-well format using Bio-Rad CFX connect Real-Time PCR systems and the SYBR Premix Ex Taq II (TAKARA).

2.3.5 Promoter activity assay

The *NOS101-Luciferase* reporter vector was created by modifying pGreen0229 to include a firefly luciferase gene driven by a basal promoter of the nopaline synthase gene (-101 to +4, designated NOS101). The wild type and mutant versions of the 56 bp promoter fragment of *ICS1* were synthesized and inserted upstream of the NOS101 basal promoter in the reporter vector. Promoter activity assays were performed by expressing the reporter constructs with the 35S-SARD1 construct or empty vector in *Arabidopsis* protoplasts. A 35S-driven Renilla luciferase reporter was included in the assays as internal transfection controls. Transformed protoplasts were incubated for 16-20 hrs before the activities of the luciferases were measured using a Dual-Luciferase Reporter Assay (Promega).

2.4 Results

2.4.1 SARD1 and CBP60g are required for the activation of SA synthesis and SA-independent defense responses in *snc2-1D*

To determine whether the increased SA synthesis in *snc2-1D* mutant plants is dependent on SARD1 and CBP60g, we crossed *sard1-1* and *cbp60g-1* into *snc2-1D* to obtain the *sard1-1 snc2-1D* and *cbp60g-1 snc2-1D* double mutants and the *sard1-1 cbp60g-1 snc2-1D* triple mutant. Quantitative RT-PCR analysis showed that the expression of *ICS1* in *snc2-1D* is much higher than in wild type, but the increased expression of *ICS1* is blocked in the *sard1-1 cbp60g-1 snc2-1D* triple mutant (Figure 2.1A). Consistent with the expression levels of *ICS1*, increased accumulation of SA in *snc2-1D* is also suppressed in the triple mutant (Figure 2.1B).

The *sard1-1 snc2-1D* and *cbp60g-1 snc2-1D* double mutants have similar morphology as *snc2-1D* and are only slightly bigger than *snc2-1D* (Figure 2.1C). Surprisingly, the mutant morphology of *snc2-1D* is almost completely suppressed in the *sard1-1 cbp60g-1 snc2-1D* triple mutant (Figure 2.1C). Quantitative RT-PCR analysis showed that the expression levels of defense marker genes *PR1* and *PR2* are slightly lower in the double mutants but are dramatically reduced in the triple mutant compared to *snc2-1D* (Figure 2.1D and E). In addition, the enhanced resistance to *Hyaloperonospora arabidopsidis* (*H.a.*) Noco2 in *snc2-1D* is partially reduced in the double mutants and almost completely lost in the triple mutant (Figure 2.1F). As blocking SA

accumulation by *eds5-3* has very little effect on the morphology, *PR2* expression and resistance to *H.a.* Noco2 in *snc2-1D* (Zhang, Yang, et al. 2010), these data suggest that SARD1 and CBP60g also regulate SA-independent pathways in *snc2-1D*.

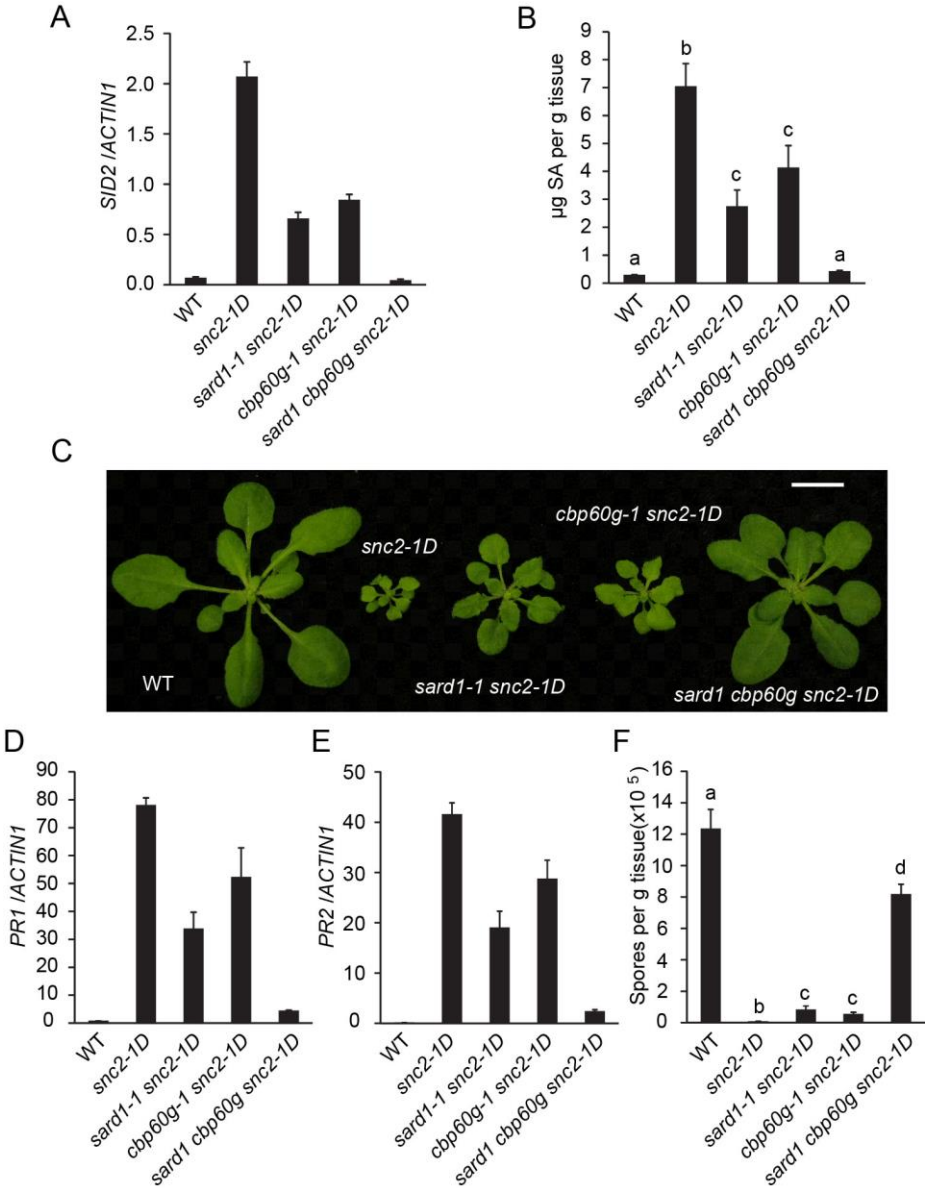

Figure 2.1 Mutations in *SARD1* and *CBP60g* largely suppress *snc2-1D* mediated autoimmunity.

(A) *SID2* expression in wild type Col-0 (WT), *snc2-1D*, *sard1-1 snc2-1D*, *cbp60g-1 snc2-1D* and *sard1-1 cpb60g-1 snc2-1D* mutant plants. The expression was normalized with *ACTIN1*. Bars represent means ± standard deviations (s.d.) (n = 3).

(B) Free SA levels in the indicated genotypes. Bars represent means ± s.d. (n = 4). Statistical differences among the samples are labeled with different letters (ANOVA, $P < 0.01$).

(C) Morphology of three-week-old soil-grown plants of the indicated genotypes. Bar = 1 cm.

(D-E) Expression levels of *PR1* (D) and *PR2* (E) in the indicated genotypes as normalized by *ACTIN1*. Bars represent means ± s.d. (n = 3).
(F) Quantification of *H.a.*Noco2 sporulation on the indicated genotypes. Bars represent means ± s.d. (n = 4). Statistical differences among the samples are labeled with different letters ($P < 0.01$, one-way ANOVA; n=3).
Plants were grown on soil at 23°C and assayed three weeks after planting.

2.4.2 The expression of *WRKY70* is directly controlled by SARD1 and CBP60g

In *sard1 cpb60g* mutant plants expressing the SARD1-HA fusion protein under its native promoter, pathogen-induced *ICS1* expression was restored to similar level as in the *cbp60g* single mutant, suggesting that SARD1-HA functions similarly as wild type SARD1 protein (Figure 2.2). To identify genes targeted by SARD1, chromatin immunoprecipitation (ChIP) was carried out on transgenic plants expressing a SARD1-HA fusion protein under its own promoter using an anti-HA antibody. The immunoprecipitated DNA was sequenced by Illumina sequencing. Analysis of the ChIP-sequencing (ChIP-seq) data showed a 20x genome coverage.

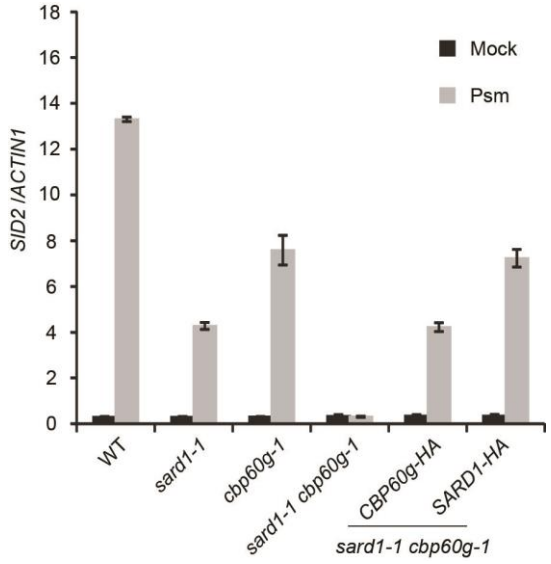

Figure 2.2 Expression levels of *SID2* in the indicated genotypes.
Samples were collected from plants of indicated genotypes 24 hours after inoculation with *P.s.m.* ES4326 (OD_{600} = 0.001) or 10mM $MgCl_2$ (mock). *SARD1-HA* and *CBP60g-HA* transgenic lines were generated by transforming *sard1-1 cbp60g-1* with constructs expressing SARD1-HA or CBP60g-HA under their own promoters. Expression levels of *SID2* were determined by quantitative RT-PCR and normalized with *ACTIN1*.
Bars represent means ± s.d. (n = 3).

Sequence coverage at each position on the genome was plotted to identify peaks in the Arabidopsis genome. Analysis of peaks in the genic region showed that most sequence peaks are located in the 1.5 kb region upstream of the translation start site which includes the 5'-UTRs and promoter regions. After removing genes that showed similar sequence peaks in the negative control, peaks with heights of 90 or greater were found in the introns of 84 genes, the 3'-UTRs of

60 genes and the 1.5 kb region upstream of the translation start sites of 1902 genes. We focused our analysis on the group containing peaks with heights of 90 or greater in the 1.5 kb region upstream of the translation start sites, as shown in Supplementary Table 1 (Sun et al. 2015), because it contains many genes encoding known regulators of plant defense that are strongly induced by pathogen infection (Table 2.1).

Table 2.1 Known defence regulators identified as candidate target genes of SARD1 by ChIP-sequencing.

Locus	Protein Name	Peak Height
AT3G56400	WRKY DNA-BINDING PROTEIN 70 (WRKY70)	214
AT1G74710	ISOCHORISMATE SYNTHASE 1 (ICS1)	125
AT4G39030	ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5)	110
AT1G64280	NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1)	163
AT1G19250	FLAVIN-DEPENDENT MONOOXYGENASE1 (FMO1)	99
AT2G13810	AGD2-LIKE DEFENSE RESPONSE PROTEIN1 (ALD1)	138
AT5G13320	avrPphB SUSCEPTIBLE3 (PBS3)	199
AT3G48090	ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1)	258
AT3G52430	PHYTOALEXIN DEFICIENT 4 (PAD4)	137
AT1G33560	ACTIVATED DISEASE RESISTANCE 1 (ADR1)	117
AT4G33300	ADR1-LIKE 1 (ADR1-L1)	324
AT5G04720	ADR1-LIKE 2 (ADR1-L2)	230
AT4G33430	BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1)	134
AT2G13790	BAK1-LIKE 1 (BKK1)	190
AT4G34460	ARABIDOPSIS G PROTEIN β-SUBUNIT1 (AGB1)	200
AT2G39660	BOTRYTIS-INDUCED KINASE1 (BIK1)	99
AT4G08500	MAPK/ERK KINASE KINASE 1 (MEKK1)	135
AT1G51660	MITOGEN-ACTIVATED PROTEIN KINASE KINASE 4 (MKK4)	141
AT3G45640	MITOGEN-ACTIVATED PROTEIN KINASE3 (MPK3)	264
AT4G09570	CALCIUM-DEPENDENT PROTEIN KINASE 4 (CPK4)	104
AT3G46510	PLANT U-BOX 13 (PUB13)	207
AT1G80840	WRKY DNA-BINDING PROTEIN 40 (WRKY40)	97
AT2G25000	WRKY DNA-BINDING PROTEIN 60 (WRKY60)	138
AT2G04450	NUCLEOSIDE DIPHOSPHATE LINKED TO SOME MOIETY X 6 (NUDT6)	107
AT4G12720	NUCLEOSIDE DIPHOSPHATE LINKED TO SOME MOIETY X 7 (NUDT7)	169
AT1G11310	MILDEW RESISTANCE LOCUS O 2 (MLO2)	100
AT5G61900	BONZAI 1 (BON1)	151
AT3G61190	BON ASSOCIATION PROTEIN 1 (BAP1)	215
AT2G45760	BON ASSOCIATION PROTEIN 2 (BAP2)	314

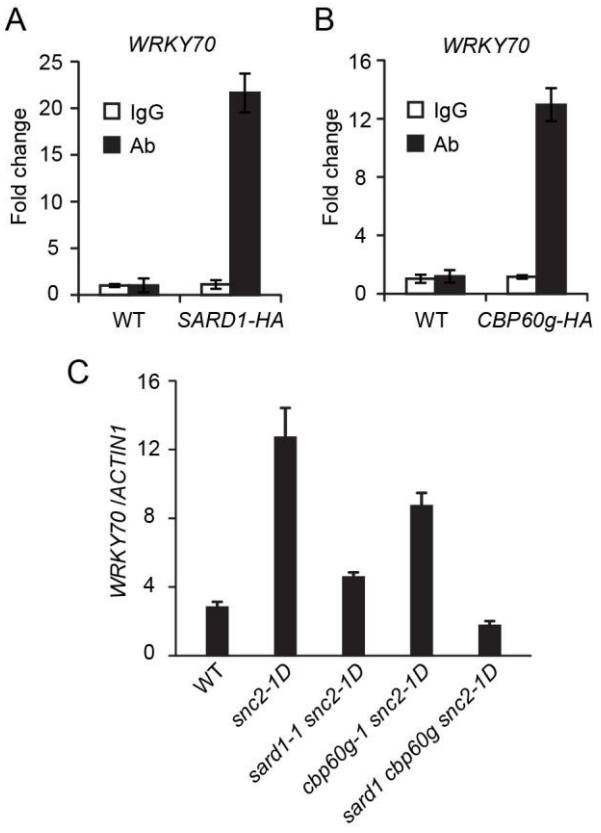

Figure 2.3 SARD1 and CBP60g directly regulate the expression of *WRKY70*.
(A-B) Binding of SARD1 (A) and CBP60g (B) to the promoter of *WRKY70* as determined by ChIP-PCR. Leaves of 25-day-old wild type plants and *SARD1-HA* (A) or *CBP60g-HA* (B) transgenic plants were infiltrated with *P.s.m.* ES4326 (OD_{600} = 0.001) 24 hours before collecting and cross-linking with 1% formaldehyde. SARD1-HA and CBP60g-HA chromatin complexes were immunoprecipitated with an anti-HA antibody and protein A-agarose beads. Negative control reactions were performed in parallel using immunoglobin G (IgG). Immunoprecipitated DNA samples were quantified by real-time qPCR using primers specific to *WRKY70* promoter. ChIP results are presented as fold changes by dividing signals from ChIP with the anti-HA antibody by IgG controls, which are set as one. Bars represent means ± s.d. (n = 3).
(C) *WRKY70* expression in the indicated genotypes as normalized with *ACTIN1*. Bars represent means ± s.d. (n = 3).

One of the candidate target genes of SARD1 identified by ChIP-seq is *WRKY70* (Table 2.1), which is known to regulate SA-independent defense responses in *snc2-1D* (Zhang, Yang, et al. 2010). Quantitative PCR analysis of the DNA immuno-precipitated by the anti-HA antibody confirmed that *WRKY70* is a target gene of SARD1 (Figure 2.3A). In *sard1 cpb60g* mutant plants expressing the CBP60g-HA fusion protein under its native promoter, pathogen-induced *ICS1* expression was restored to similar level as in the *sard1* single mutant, suggesting that CBP60g-HA functions similarly as wild type protein (Figure 2.2). To determine whether *WRKY70* is also a target gene of CBP60g, we carried out ChIP-PCR experiments on transgenic plants expressing a CBP60g-HA fusion protein under its own promoter using the anti-HA antibody. As shown in Figure 2.3B, CBP60g is also targeted to the promoter region of *WRKY70*.

Next we analyzed the expression of *WRKY70* in *snc2-1D*, *sard1-1 snc2-1D*, *cbp60g-1 snc2-1D* and *sard1-1 cbp60g-1 snc2-1D* mutant plants. As shown in Figure 2.3C, WRKY70 is

expressed at a considerably higher level in *snc2-1D* than in wild type. The expression of *WRKY70* is slightly lower in *cbp60g-1 snc2-1D* and clearly reduced in *sard1-1 snc2-1D* compared to *snc2-D*. However, it is further reduced to below wild type level in the *sard1-1 cbp60g-1 snc2-1D* triple mutant (Figure 2.3C). These data suggest that SARD1 and CBP60g have overlapping functions in regulating the expression of *WRKY70* and that reduced expression of WRKY70 is at least partly responsible for the suppression of the *snc2-1D*-mediated SA-independent constitutive defense responses in the *sard1-1 cbp60g-1 snc2-1D* triple mutant.

2.4.3 SARD1 and CBP60g directly regulate the expression of *EDS5* and *NPR1*

EDS5 is involved in pathogen-induced SA synthesis (Rogers and Ausubel 1997, Nawrath and Metraux 1999). Analysis of the SARD1 ChIP-seq data revealed that *EDS5* is a potential target gene of SARD1 as well (Table 2.1). A peak with a height of 110 was identified about 700 bp upstream of the translation start site of *EDS5*. ChIP-PCR experiments confirmed that SARD1 is targeted to the promoter region of *EDS5* (Figure 2.4A). Further ChIP-PCR analysis showed that CBP60g also binds to the promoter region of *EDS5* (Figure 2.4B). To determine whether SARD1 and CBP60g are required for the induction of *EDS5* by *P.s.m.* ES4326, we compared the expression levels of *EDS5* in wild type and *sard1-1 cbp60g-1* plants. As shown in Figure 2.4C, induction of *EDS5* by *P.s.m.* ES4326 is greatly reduced in the *sard1-1 cbp60g-1* double mutant. These data suggest that SARD1 and CBP60g directly regulate pathogen-induced expression of *EDS5*.

Another candidate target gene of SARD1 identified by ChIP-seq is *NPR1*, which encodes a putative SA receptor (Wu et al. 2012). A peak with a height of 163 was identified about 100 bp upstream of the translation start site of *NPR1* (Table 2.1). Binding of SARD1 to the promoter region of *NPR1* was confirmed by ChIP-PCR (Figure 2.4D). As shown in Figure 2.4E, CBP60g is also targeted to the promoter region of *NPR1*. Analysis of the expression levels of *NPR1* in wild type and *sard1-1 cbp60g-1* plants showed that induction of *NPR1* by *P.s.m.* ES4326 is compromised in the *sard1-1 cbp60g-1* double mutant (Figure 2.4F). These data suggest that SARD1 and CBP60g also regulate pathogen-induced expression of *NPR1*.

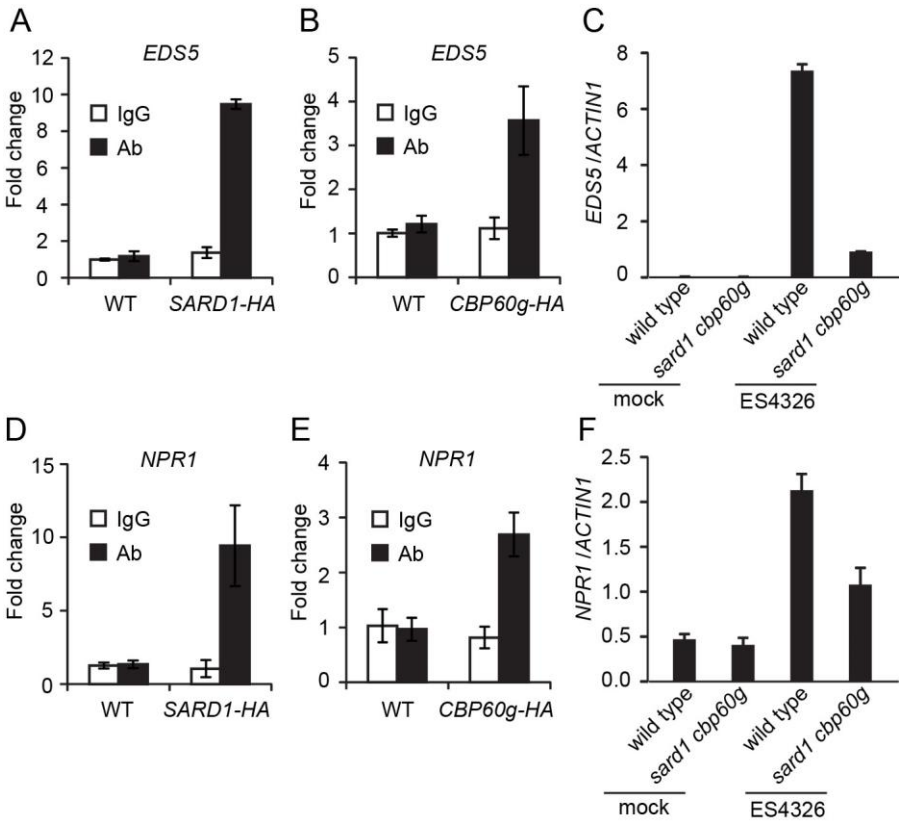

Figure 2.4 *EDS5* and *NPR1* are direct target genes of SARD1 and CBP60g.
(A-B) Recruitment of SARD1-HA (A) and CBP60g-HA (B) to *EDS5* promoter after *P.s.m.* ES4326 infection as determined by ChIP-PCR. ChIP was performed as described in Figure 2. Real-time PCR was carried out using primers specific to *EDS5* promoter. ChIP results are presented as fold changes by dividing signals from ChIP with the anti-HA antibody by those of IgG controls, which are set as one. Bars represent means ± s.d. (n = 3).
(C) Induction of *EDS5* expression in wild type and *sard1-1 cpb60g-1* by *P.s.m.* ES4326. Leaves of 25-day-old plants were infiltrated with *P.s.m.* ES4326 (OD_{600} = 0.001) or 10mM $MgCl_2$ (mock) 12 hours before collection for RT-PCR analysis. Bars represent means ± s.d. (n = 3).
(D-E) Recruitment of SARD1-HA (D) and CBP60g-HA (E) to the promoter of *NPR1* after treatment with *P.s.m.* ES4326. ChIP and data analysis were carried out similarly as in (A-B). Bars represent means ± s.d. (n = 3).
(F) Induction of *NPR1* expression by *P.s.m.* ES4326 in wild-type and *sard1-1 cpb60g-1* double mutant plants. Samples were collected 12 hours after infiltration with *P.s.m.* ES4326 (OD_{600} = 0.001) or 10mM $MgCl_2$ (mock). Bars represent means ± s.d. (n = 3).

2.4.4 SAR regulators *FMO1*, *ALD1* and *PBS3* are target genes of SARD1 and CBP60g

In addition to *EDS5* and *NPR1*, three other genes required for SAR, *FMO1*, *ALD1* and *PBS3*, were identified as candidate target genes of SARD1 from the ChIP-seq data. The height of the peaks identified in the promoter regions of *FMO1*, *ALD1* and *PBS3* are 99, 138 and 199,

respectively (Table 2.1). Binding of SARD1 to the promoters of these three genes was confirmed by ChIP-PCR experiments (Figure 2.5A). Further ChIP-PCR analysis showed that CBP60g also binds to the promoters of these genes (Figure 2.5B). Consistent with data from previous gene expression studies (Wang, Tsuda, Truman, Sato, Nguyen le, et al. 2011, Truman and Glazebrook 2012), we also observed dramatic reduction in bacteria-induced expression of *FMO1*, *ALD1* and *PBS3* in the *sard1-1 cbp60g-1* double mutant (Figure 2.5C). Taken together, SARD1 and CBP60g directly regulate the expression of *FMO1*, *ALD1* and *PBS3* in plant defense responses.

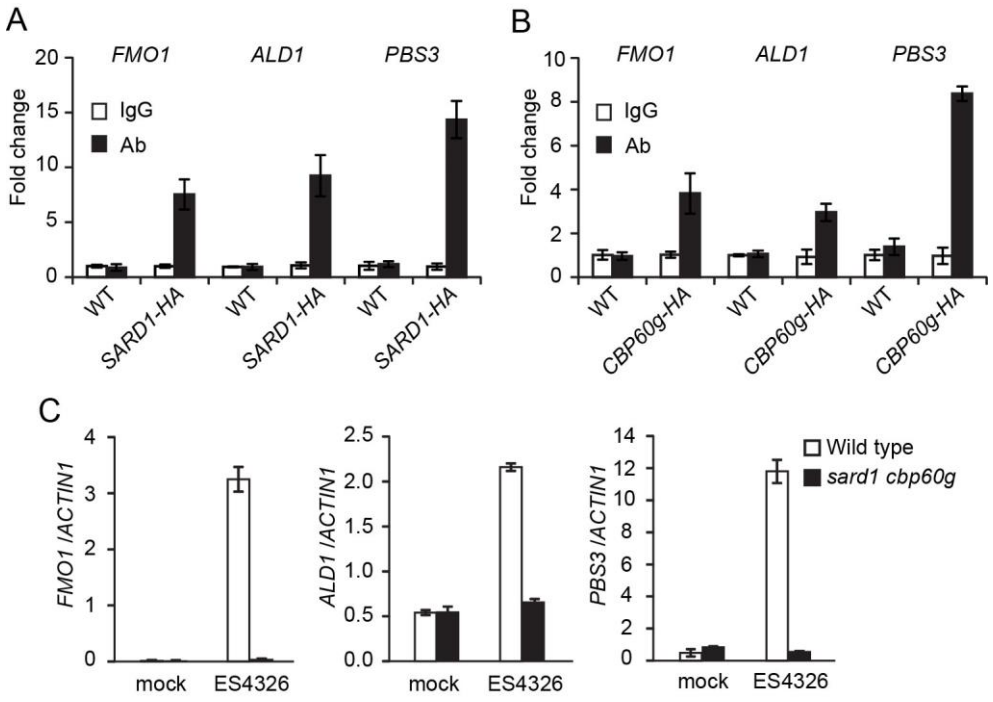

Figure 2.5 *FMO1, ALD1* and *PBS3* are direct targets of SARD1 and CBP60g.
(A-B) Binding of SARD1-HA (A) and CBP60g-HA (B) to the promoter regions of *FMO1*, *ALD1* and *PBS3* following infection by *P.s.m.* ES4326 as determined by ChIP-PCR. ChIP was performed as described in Figure 2. Real-time PCR was carried out using gene-specific primers. ChIP results are presented as fold changes by dividing signals from ChIP with the anti-HA antibody by those of the IgG control, which are set as one. Bars represent means ± s.d. (n = 3).
(C) Induction of the expression of *FMO1*, *ALD1* and *PBS3* by *P.s.m.* ES4326 infection. Samples were collected 12 hours after inoculation with *P.s.m.* ES4326 (OD_{600} = 0.001) or 10mM $MgCl_2$ (mock). Expression levels were normalized with *ACTIN1*. Bars represent means ± s.d. (n = 3).

2.4.5 SARD1 and CBP60g regulate the expression of positive regulators of R protein mediated immunity

ENHANCED DISEASE SUSCEPTIBILITY 1 (*EDS1*) and *PHYTOALEXIN DEFICIENT 4* (*PAD4*) encode positive regulators of defense responses activated by TIR-NB-LRR R proteins (Aarts et al. 1998, Parker et al. 1996, Glazebrook, Rogers, and Ausubel 1996, Jirage et al. 1999, Falk et al. 1999). *NDR1* is required for defense responses activated by CC-NB-LRR R proteins (Century et al. 1997, Aarts et al. 1998). *EDS1* and *PAD4*, but not *NDR1*, were identified as the candidate target genes of SARD1 by ChIP-seq (Table 2.1). The height of the peaks identified in the promoter regions of *EDS1* and *PAD4* are 258 and 137, respectively. ChIP-PCR experiments showed that SARD1 was targeted to the promoter regions of *EDS1* and *PAD4*, but not *NDR1* (Figure 2.6A). In addition, CBP60g is also targeted to the promoters of *EDS1* and *PAD4*, but not *NDR1* (Figure 2.6B). Quantitative RT-PCR was subsequently carried out to determine whether induction of the expression of *EDS1* and *PAD4* by bacterial infections is dependent on SARD1 and CBP60g. As shown in Figure 2.6C, induction of *EDS1* and *PAD4* by *P.s.m.* ES4326 is dramatically reduced in the *sard1-1 cbp60g-1* double mutant. These data suggest that induction of *EDS1* and *PAD4* following pathogen infection is directly regulated by SARD1 and CBP60g.

ADR1, *ADR-L1* and *ADR-L2* encode three closely related CC-NB-LRR proteins required for immunity mediated by TIR-NB-LRR R proteins RPP2 and RPP4 (Bonardi et al. 2011). They were also identified as candidate target genes of SARD1 by ChIP-seq (Table 2.1). The heights of the peaks identified in the promoter regions of *ADR1*, *ADR-L1* and *ADR-L2* are 117, 324 and 230, respectively. ChIP-PCR analysis confirmed that SARD1 binds to the promoter regions of these three genes (Figure 2.6D). ChIP-PCR experiments showed that CBP60g is also targeted to the promoter regions of *ADR1*, *ADR-L1* and *ADR-L2* (Figure 2.6E). As shown in Figure 2.6F, the expression of *ADR1*, *ADR-L1* and *ADR-L2* is induced by *P.s.m.* ES4326 and the induction is partially dependent on SARD1 and CBP60g. Taken together, SARD1 and CBP60g directly regulates the expression of *ADR1*, *ADR-L1* and *ADR-L2* in plant defense.

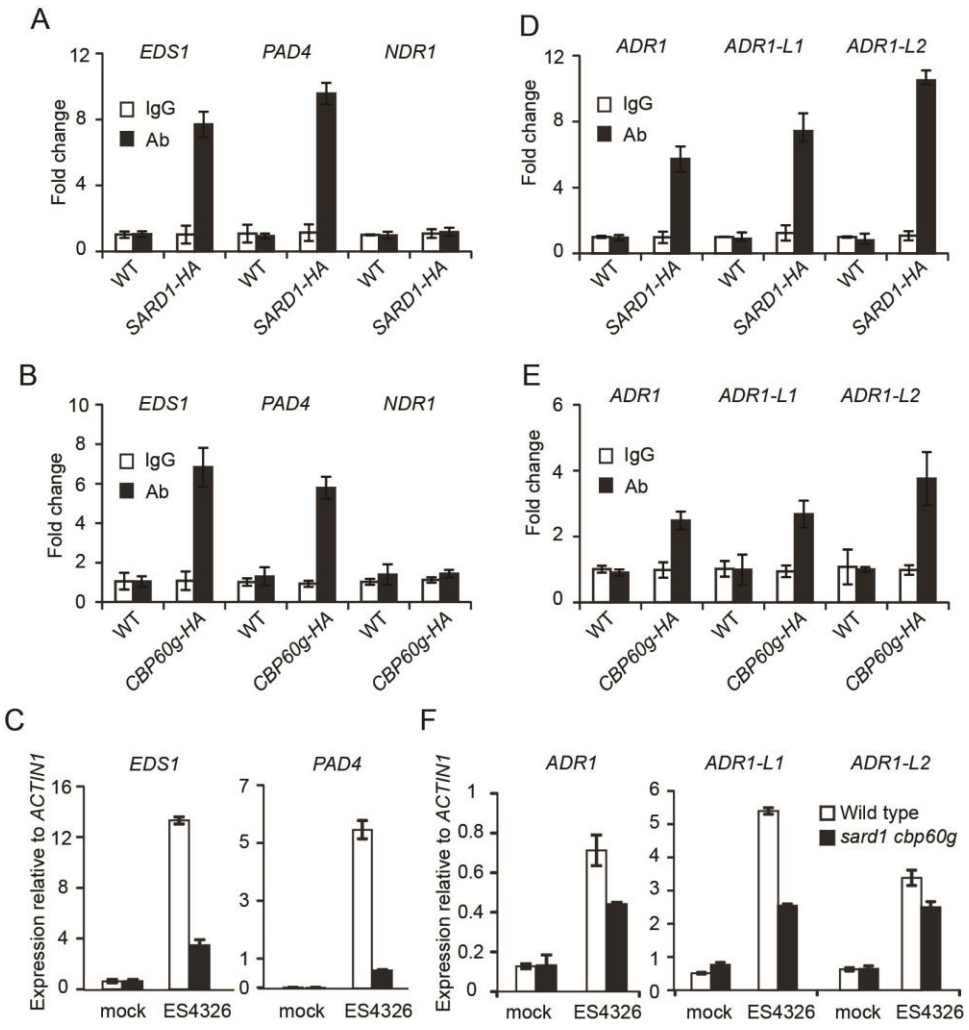

Figure 2.6 SARD1 and CBP60g directly regulate the expression of *EDS1*, *PAD4*, *ADR1*, *ADR-L1* and *ADR1-L2*.

(A-B) Binding of SARD1-HA (A) and CBP60g-HA (B) to promoter regions of *EDS1* and *PAD4* following infection by *P.s.m.* ES4326 as determined by ChIP-qPCR. *NDR1* is not a direct target of SARD1 or CPB60g and is used as negative control. ChIP was performed as described in Figure 2. Real-time PCR was carried out using primers specific to *EDS1*, *PAD4* and *NDR1* promoters. ChIP results are presented as fold changes by dividing signals from ChIP with the anti-HA antibody by those of the IgG controls, which are set as one. Bars represent means ± s.d. (n = 3). (C) Induction of *EDS1* and *PAD4* expression in wild-type and *sard1-1 cpb60g-1* plants after *P.s.m.* ES4326 infection. Expression levels were normalized with *ACTIN1*. Bars represent means ± s.d. (n = 3). (D-E) Binding of SARD1-HA (D) and CBP60g-HA (E) to the promoter regions of *ADR1*, *ADR1-L1* and *ADR1-L2* following infection by *P.s.m.* ES4326 as determined by ChIP-PCR. ChIP and data analysis were carried out similarly as in (A-B). Bars represent means ± s.d. (n = 3).(F) Induction of *ADR1*, *ADR1-L1* and *ADR1-L2* expression by *P.s.m.* ES4326 as determined by real-time RT-PCR. Samples were collected 12 hours after inoculation with *P.s.m.* ES4326 (OD_{600} = 0.001) or 10mM $MgCl_2$ (mock). Expression levels were normalized with *ACTIN1*. Bars represent means ± s.d. (n = 3).

2.4.6 SARD1 and CBP60g regulate the expression of signaling components downstream of PAMP receptors and contribute to PAMP-triggered immunity

Among the candidate target genes of SARD1 identified by ChIP-seq, eight genes including *BAK1*, *BKK1*, *AGB1*, *BIK1*, *MEKK1*, *MKK4*, *MPK3* and *CPK4* (Table 2.1) were previously shown to encode positive regulators of PAMP-triggered immunity (Roux et al. 2011, Chinchilla et al. 2007, Heese et al. 2007, Liu et al. 2013, Torres et al. 2013, Ishikawa 2009, Kadota et al. 2014, Li et al. 2014, Lu et al. 2010, Zhang, Li, et al. 2010, Zhang et al. 2012, Asai et al. 2002, Boudsocq et al. 2010). Binding of SARD1 to the promoter regions of these genes was further confirmed by ChIP-PCR (Figure 2.7A). In addition, CBP60g is also targeted to the promoter regions of these genes (Figure 2.7B). As shown in Figure 2.7C, expression of *BAK1*, *BKK1*, *AGB1*, *BIK1*, *MEKK1*, *MKK4*, *MPK3* and *CPK4* is induced *P.s.m.* ES4326 and the induction is reduced in the *sard1-1 cbp60g-1* double mutant, suggesting that SARD1 and CBP60g directly regulates their expression in plant defense responses.

To test whether SARD1 and CBP60g are required for PAMP-triggered immunity, we analyzed bacterial growth in wild type, *sard1-1*, *cbp60g-1* and *sard1-1 cbp60g-1* plants pretreated with flg22, a peptide from bacterial flagellin that is recognized by FLAGELLIN-SENSITIVE2 (FLS2) (Gomez-Gomez and Boller 2000). As shown in Figure 2.7D, flg22-induced resistance to *Pseudomonas syringae* pv. *tomato* (*P.s.t.*) DC3000 is not obviously affected in the *sard1-1* and *cbp60g-1* single mutants, but clearly reduced in the *sard1-1 cbp60g-1* double mutant, suggesting that SARD1 and CBP60g contribute to PAMP-triggered immunity.

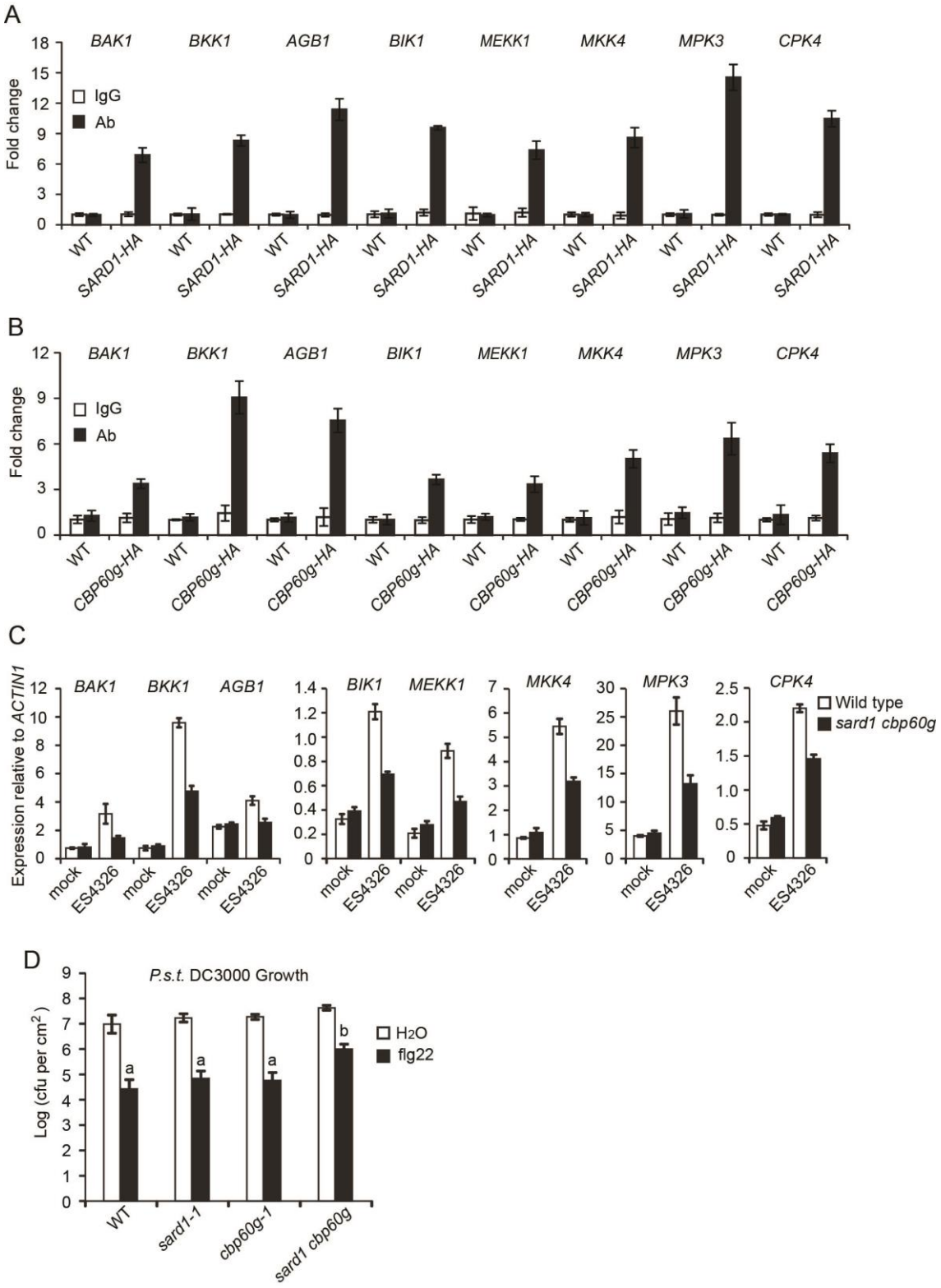

Figure 2.7 SARD1 and CBP60g directly control expression of signaling components of PAMP-triggered immunity and contribute to flg22-induced resistance to *P.s.t.* DC3000.

(A-B) Binding of SARD1-HA (A) and CPB60g-HA (b) to the promoter regions of *BAK1*, *BKK1*, *AGB1*, *BIK1*, *MEKK1*, *MKK4*, *MPK3* and *CPK4* following *P.s.m.* ES4326 infection as determined by ChIP-PCR. ChIP was performed as described in Figure 2. Real-time PCR was carried out using gene-specific primers. ChIP results are presented as fold changes by dividing signals from ChIP with the anti-HA antibody by those of the IgG controls, which are set as one. Bars represent means ± s.d. (n = 3).
(C) Induction of *BAK1*, *BKK1*, *AGB1*, *BIK1*, *MEKK1*, *MKK4*, *MPK3* and *CPK4* expression by *P.s.m.* ES4326 as determined by real-time RT-PCR. Samples were collected 12 hours after inoculation with *P.s.m.* ES4326 (OD_{600} = 0.001) or 10mM $MgCl_2$ (mock). Expression levels of the genes were normalized with *ACTIN1*. Bars represent means ± s.d. (n = 3).
(D) flg22-induced resistance to *Pseudomonas syringae* pv. tomato (*P.s.t*) DC3000 on the indicated genotypes. Four-week-old plants were infiltrated with 1 μM flg22 or H_2O one day before inoculation with *P.s.t.* DC3000 (OD_{600} = 0.001). Bacterial growth was determined three days post inoculation. cfu, colony forming unit. Bars represent means ± s.d. (n = 5). Statistical differences among the samples are labeled with different letters (ANOVA, $P < 0.001$).

2.4.7 SARD1 and CBP60g regulate the expression of a large number of negative regulators of plant immunity

Analysis of the SARD1 ChIP-seq data also identified a number of negative regulators of plant immunity including *PUB13*, *WRKY40*, *WRKY60*, *NUDT6*, *NUDT7*, *MLO2*, *BON1*, *BAP1* and *BAP2* as candidate target genes of SARD1 (Table 2.1). Binding of SARD1 to the promoter regions of *PUB13*, *WRKY40*, *WRKY60*, *NUDT6*, *NUDT7*, *MLO2*, *BON1*, *BAP1* and *BAP2* was confirmed by ChIP-PCR analysis (Figure 2.8A). In addition, CBP60g was also found to target the promoter regions of these nine genes (Figure 2.8B). Quantitative RT-PCR analysis showed that the expression of *PUB13*, *WRKY40*, *WRKY60*, *NUDT6*, *NUDT7*, *MLO2*, *BON1*, *BAP1* and *BAP2* are all induced by *P.s.m.* ES4326 and the induction is either reduced or blocked in the *sard1-1 cbp60g-1* double mutant (Figure 2.8C). These data suggest that SARD1 and CBP60g regulate the expression of these negative regulators of plant immunity during plant defense.

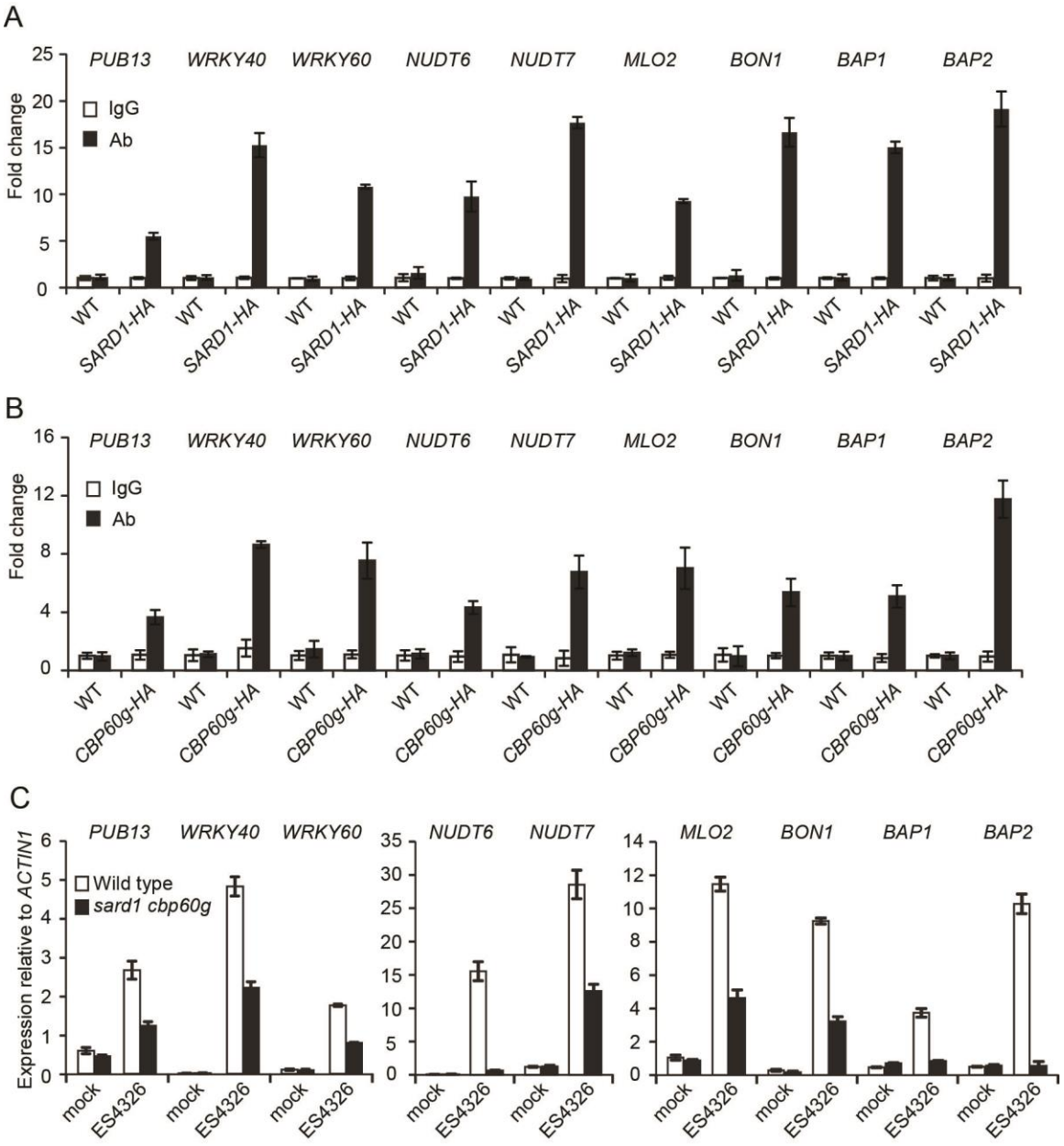

Figure 2.8 SARD1 and CBP60g target genes encoding negative regulators of plant defense.
(A-B) Recruitment of SARD1-HA (A) and CBP60g-HA (B) to the promoter regions of *PUB13, WRKY40, WRKY60, NUDT6, NUDT7, MLO2, BON1, BAP1* and *BAP2* following *P.s.m.* ES4326 infection as determined by ChIP-PCR. ChIP was performed as described in Figure 2. Real-time PCR was carried out using gene-specific primers. ChIP results are presented as fold changes by dividing signals from ChIP with the anti-HA antibody by those of the IgG controls, which are set as one. Bars represent means ± s.d. (n = 3).
(C) Induction of *PUB13, WRKY40, WRKY60, NUDT6, NUDT7, MLO2, BON1, BAP1* and *BAP2* genes in wild type and *sard1-1 cpb60g-1* by *P.s.m.* ES4326. Samples were collected 12 hours after inoculation with *P.s.m.* ES4326 (OD_{600} = 0.001) or 10mM $MgCl_2$ (mock). Expression levels of the genes were normalized with *ACTIN1*. Bars represent means ± s.d. (n = 3).

2.4.8 SARD1 activates target gene expression through the GAAATTT element

Previously we showed that SARD1 and CBP60g bind preferentially to the oligonucleotide probe GAAATTTTGG (Zhang, Xu, et al. 2010). Bioinformatics analysis showed that the GAAATTT motif within this probe is over-represented in the promoters of the genes with SARD1 and CBP60g-dependent expression (Wang, Tsuda, Truman, Sato, Nguyen le, et al. 2011) . Analysis of the 1,902 candidate target genes of SARD1 showed that the GAAATTT motif is also over-represented in the promoter regions of this group of genes ($P < 10^{-15}$). This motif is over-represented in the promoter regions of 29 confirmed target genes of SARD1 and CBP60g listed in Table 2.1 ($P < 0.005$) as well. However, not every gene in this group contains this motif in their promoter region. It is likely SARD1 and CBP60g can also bind to certain variants of the GAAATTT motif. Interestingly, a closely related sequence motif, G(A/T)AATT(T/G), was identified as a conserved motif ($P < 10^{-25}$) among the sequence peaks of the 1,902 candidate target genes of SARD1 using the motif discovery algorithm DREME.

To test whether SARD1 activates its target gene expression through the GAAATTT motif, we made a construct expressing the luciferase reporter gene under the control of a 56 bp fragment from the ChIP-Seq peak region in the promoter of *ICS1*, which contains a GAAATTT and a related GAAATT motif (Figure 2.9A). Two additional constructs containing mutations in these two motifs were also created to determine whether they are required for activation of reporter gene expression by the 56 bp fragment. These reporter gene constructs were transformed into Arabidopsis protoplasts to examine the luciferase reporter expression levels. As shown in Figure 2.9B, all three constructs expressed similar levels of luciferase as the original NOS101-Luc vector, suggesting that the 56 bp promoter fragment cannot activate luciferase expression on its own in protoplast transient assays. However, when the luciferase reporter gene constructs were co-transformed together with a plasmid expressing the SARD1 protein into protoplasts, luciferase expression was much higher in samples transformed with the construct containing the wild type 56 bp promoter fragment compared to samples transformed with the NOS101-Luc vector (Figure 2.9C). In comparison, samples transformed with the construct carrying mutations in the GAAATTT motif exhibited significantly reduced luciferase activity. When both the GAAATTT and GAAATT motifs were mutated, the luciferase activity was further reduced to a

level similar to that in the NOS101-Luc vector control. Together, these data suggest that SARD1 activates gene expression through GAAATTT or similar DNA sequence elements.

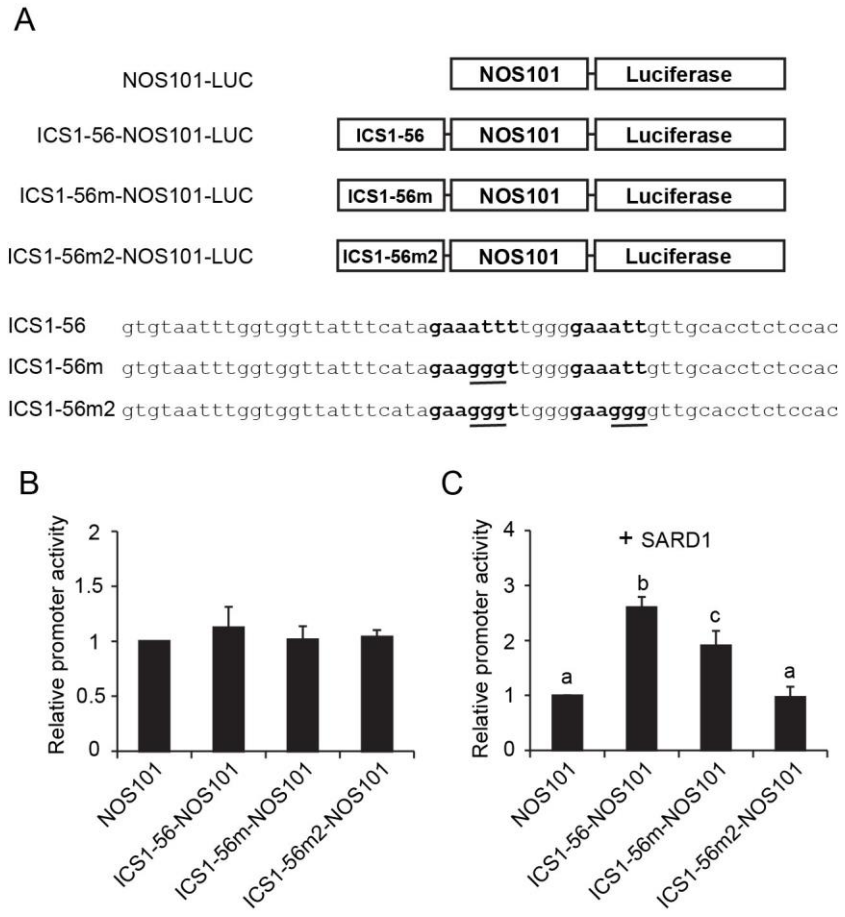

Figure 2.9 SARD1 activates reporter gene expression through the GAAATTT motif.
(A) Reporter constructs used in the promoter activity assay. NOS101, a basal promoter of the nopaline synthase gene (-101 to +4); ICS1-56, a 56 bp fragment from the ChIP-Seq peak region in *ICS1* promoter; ICS1-56m and ICS1-56m2, mutant versions of ICS1-56 with mutations (underlined) in the GAAATTT or GAAATT motif. The GAAATTT and GAAATT sequences are bolded.
(B) Luciferase activities in protoplasts transformed with individual reporter constructs.
(C) Luciferase activities in protoplasts transformed with individual reporter constructs together with a *35S-SARD1* plasmid.
A *35S-Renilla Luciferase* construct was included in all assays as an internal transformation efficiency control. The activities of luciferase were normalized with the expression of the Renilla luciferase and compared to the value obtained from protoplasts transfected with *NOS101-LUC* construct, which was set as 1. The error bars represent means ± s.d. from three biological replicates.

2.5 Discussion

SA functions as a key signaling molecule in SAR. SARD1 and CBP60g have previously been shown to regulate pathogen-induced SA synthesis (Wang et al. 2009, Zhang, Xu, et al. 2010, Wang, Tsuda, Truman, Sato, Nguyen le, et al. 2011). In this study, we showed that, in addition to *ICS1*, the expression of another regulator of SA synthesis, *EDS5*, is also controlled by SARD1 and CBP60g. *NPR1*, a gene required for the perception of SA by plants, is a target gene of SARD1 and CBP60g as well. Moreover, SARD1 and CBP60g also regulate pathogen-induced expression of several other genes required for SAR. Both SARD1 and CBP60g are targeted to the promoter regions of *FMO1*, *ALD1* and *PBS3* and induction of these genes by *P.s.m.* ES4326 is dramatically reduced in the *sard1 cbp60g* double mutant. These data suggest that SARD1 and CBP60g function in coordinating the induction of SAR regulators during plant defense.

Several defense regulators that function upstream of SA synthesis are also regulated by SARD1 and CBP60g. Both PAD4 and EDS1 are required for pathogen-induced SA synthesis (Jirage et al. 1999, Feys et al. 2001). SARD1 and CBP60g are targeted to their promoters and are required for their induction by *P.s.m.* ES4326. In addition, *ADR1*, *ADR1-L1* and *ADR1-L2*, three helper R genes required for pathogen-induced SA synthesis (Bonardi et al. 2011), are also target genes of SARD1 and CBP60g. Regulation of the induction of *PAD4*, *EDS1*, *ADR1*, *ADR1-L1* and *ADR1-L2* by SARD1 and CBP60g may play critical roles in promoting SA synthesis during pathogen infection.

We also found that SARD1 and CBP60g function downstream of the receptor-like protein SNC2 to regulate both SA-dependent and SA-independent defense pathways. SARD1 and CBP60g are required for the increased expression of *ICS1* and SA synthesis in *snc2-1D*. Regulation of the SA-independent defense pathway by SARD1 and CBP60g is at least partly through their control of the expression of *WRKY70*, a key regulator of the SA-independent defense responses in *snc2-1D* (Zhang, Yang, et al. 2010), as both SARD1 and CBP60g are targeted to the promoter of *WRKY70* and are required for the induction of WRKY70 in *snc2-1D*.

Furthermore, a large number of genes encoding regulatory components of PAMP-triggered immunity are targets of SARD1 and CBP60g and require SARD1 and CBP60g for induction by bacterial infection. Among them, BAK1 and BKK1 serve as co-receptors of FLS2 and EF-TU RECEPTOR (EFR) (Roux et al. 2011, Chinchilla et al. 2007, Heese et al. 2007).

BIK1 and AGB1 function downstream of multiple PAMP receptors to regulate ROS production and defense against pathogen infection (Liu et al. 2013, Torres et al. 2013, Ishikawa 2009, Kadota et al. 2014, Li et al. 2014, Lu et al. 2010, Zhang, Li, et al. 2010). MEKK1, MKK4, and MPK3 are components of MAP kinase cascades downstream of PAMP receptors (Gao et al. 2008, Ichimura et al. 2006, Nakagami et al. 2006, Suarez-Rodriguez et al. 2007, Asai et al. 2002). CPK4 was identified as a calcium dependent protein kinase downstream of FLS2 (Boudsocq et al. 2010). The critical role of SARD1 and CBP60g in PAMP-triggered immunity was further confirmed by the attenuation of flg22-induced pathogen resistance in the *sard1 cbp60g* double mutant.

In addition to up-regulation of positive regulators, negative regulators are often induced during plant defense as well. Induction of negative regulators is critical for feedback inhibition of defense responses to prevent uncontrolled activation, which may lead to autoimmunity. We showed that a number of negative regulators of plant immunity including *PUB13*, *WRKY40*, *WRKY60*, *NUDT6*, *NUDT7*, *MLO2*, *BON1*, *BAP1* and *BAP2* are also target genes of SARD1 and CBP60g. Among them, PUB13 is a U-box/ARM E3 ubiquitin ligase that regulates cell death as well as degradation of FLS2 after flagellin induction (Li et al. 2012, Lu et al. 2011); WRKY40 and WRKY60 function redundantly with their close homolog, WRKY18, to repress basal defense (Xu et al. 2006, Shen et al. 2007); NUDT6 and NUDT7 are two Nudix domain-containing proteins that negatively regulate EDS1-dependent immune responses (Bartsch et al. 2006, Ge et al. 2007, Wang et al. 2012); MLO2 functions as a negative regulator of resistance to powdery mildew (Consonni et al. 2006); BON1 functions as a negative regulator of immunity mediated by the TIR-NB-LRR R protein SNC1 (Yang and Hua 2004); BAP1 and BAP2 encode two C2 domain-containing proteins that negatively regulate programed cell death (Yang, Li, and Hua 2006, Yang et al. 2007). All these genes are induced following infection by *P.s.m.* ES4326 and their induction requires SARD1 and CBP60g, suggesting that SARD1 and CBP60g also play an important role in the negative feedback regulation of plant defense.

Bioinformatics analysis has previously been used to analyze genes that are co-expressed with a group of SARD1/CBP60g-dependent genes (Truman and Glazebrook 2012). Four genes including *AGP5*, *At5g52760*, *CML46* and *CML47* that form a small cluster with *SARD1* and *ICS1* were identified as candidate target genes of SARD1 and CBP60g. These genes are also

identified as targets of SARD1 in our ChIP-seq data. *EDS1* and *PAD4* were also found to cluster with *ICS1* in the co-expression analysis. They were placed upstream of SARD1 and CBP60g. Interestingly, both *EDS1* and *PAD4* have been shown to be targets of SARD1 and CBP60g in our ChIP studies. The commonly used defense marker genes *PR1* and *PR2* were also found in one of the clusters co-expressed with SARD1/CBP60g-dependent genes. However, both of them were not identified as target genes of SARD1 in our ChIP-seq data, suggesting that they are not directly regulated by SARD1 and CBP60g. It is likely that genes co-expressed with SARD1/CBP60g-dependent genes include genes that are either directly or indirectly regulated by SARD1 and CBP60g.

In summary, the expression of a large number of genes encoding key regulators of plant immunity is directly controlled by SARD1 and CBP60g during plant defense. This is consistent with the functions of these two transcription factors in PTI, ETI and SAR. Our study revealed that SARD1 and CBP60g orchestrate the induction of plant defense regulators in plant immunity (Figure 2.10).

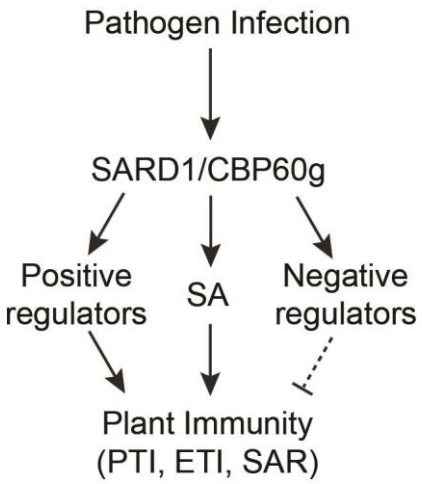

Figure 2.10 Proposed scheme for regulation of plant defence responses bySARD1 and CBP60g.

Following pathogen infection, SARD1 and CBP60g are activated. Subsequently, the expression of a large number of their target genes is turned on. Upregulation of positive regulators of PTI, ETI and SAR and increased SA synthesis lead to enhanced plant immunity against pathogens. Meanwhile, negative regulators of plant immunity are turned on to attenuate plant defence responses

Chapter 3: TGA1 and TGA4 regulate SA and Pip biosynthesis by modulating the expression of SARD1 and CBP60g

3.1 Summary

Salicylic acid (SA) and pipecolic acid (Pip) play important roles in plant immunity. Here we analyzed the roles of transcription factors TGA1 and TGA4 in regulating SA and Pip biosynthesis in Arabidopsis. We quantified the expression levels of *SARD1* and *CBP60g*, which encode two master transcription factors of plant immunity, and the accumulation of SA and Pip in *tga1 tga4* mutant plants. We tested whether *SARD1* and *CBP60g* are direct targets of TGA1 by chromatin immunoprecipitation-PCR (ChIP-PCR). In addition to promoting pathogen-induced SA biosynthesis, we found that SARD1 and CBP60g also positively regulate Pip biosynthesis by targeting genes encoding key biosynthesis enzymes of Pip. TGA1/TGA4 are required for full induction of SARD1 and CBP60g in plant defense. ChIP-PCR analysis showed that *SARD1*is a direct target of TGA1. In *tga1 tga4* mutant plants, the expression levels of *SARD1* and *CBP60g* along with SA and Pip accumulation following pathogen infection are dramatically reduced compared to those in wild type plants. Consistent with reduced expression of *SARD1* and *CBP60g*, PAMP-induced pathogen resistance and systemic acquired resistance is compromised in *tga1 tga4*. Our study showed that TGA1/TGA4 regulate Pip and SA biosynthesis by modulating the expression of *SARD1* and *CBP60g*.

3.2 Introduction

Salicylic acid (SA) is an important signal molecule in plant immunity (Vlot, Dempsey, and Klessig 2009). Following infection by pathogens, SA levels increase in both local and distal parts of plants (Malamy 1990, Metraux 1990, Rasmussen 1991). Blocking SA accumulation by expressing the SA-degrading enzyme salicylate hydroxylase leads to enhanced disease susceptibility and loss of systemic acquired resistance (SAR) (Gaffney 1993). *Arabidopsis thaliana* mutants with reduced pathogen-induced SA biosynthesis also exhibit enhanced susceptibility to pathogens and compromised SAR (Nawrath and Metraux 1999). SA signaling is regulated both at the level of SA production and at the level of SA perception.

In *Arabidopsis*, pathogen-induced SA accumulation is mainly synthesized from chorismate by Isochorismate Synthase 1 (ICS1) (Wildermuth et al. 2001). *ICS1* expression is strongly induced by pathogen infection. Two plant-specific transcription factors, SAR DEFICIENT 1 (SARD1) and CALMODULIN BINDING PROTEIN 60 G (CBP60g), are involved in this process (Zhang, Xu, et al. 2010, Wang, Tsuda, Truman, Sato, Nguyen le, et al. 2011). Chromatin-immunoprecipitation (ChIP) sequencing analysis revealed that SARD1 and CBP60g target many other important regulators of plant defense as well, showing that they play broad roles in regulating plant immunity in addition to SA biosynthesis (Sun et al. 2015).

Arabidopsis NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1), NPR3 and NPR4 bind SA *in vitro* and have been suggested to function as the receptors for SA (Wu et al. 2012, Fu et al. 2012). NPR1 is required for SA-induced *Pathogenesis-related* (*PR*) gene expression and pathogen resistance (Dong 2004). In contrast, NPR3 and NPR4 negatively regulate *PR* gene expression and pathogen resistance (Zhang et al. 2006). NPR1, NPR3 and NPR4 interact with a subgroup of basic leucine zipper transcription factors (Zhang et al. 1999, Zhou et al. 2000, Despres et al. 2000, Zhang et al. 2006). Among them, TGACG-BINDING FACTOR2 (TGA2), TGA5 and TGA6 function redundantly in SA-induced *PR* gene expression and pathogen resistance (Zhang et al. 2003). In the absence of SA treatment, TGA2, TGA5 and TGA6 function as negative regulators of plant immunity to repress the expression of *PR* genes (Zhang et al. 2003).

A second plant metabolite, pipecolic acid (Pip), is also involved in the amplification of defense responses (Navarova et al. 2012). A recent study showed that Pip is biosynthesized from L-lysine via the intermediate Δ^1-piperideine-2-carboxylic acid (P2C) in Arabidopsis (Ding et al. 2016). The aminotransferase ALD1 transforms L-lysine into P2C, which the reductase SARD4 subsequently converts into Pip. Loss of function of ALD1 blocks Pip biosynthesis and leads to reduced pathogen resistance and complete loss of SAR. The immune defects in *ald1* mutants can be complemented by exogenous application of Pip (Navarova et al. 2012). Loss of function of SARD4 also results in reduced pathogen-induced Pip accumulation in the local tissue and complete block of Pip biosynthesis in distal leaves (Ding et al. 2016). However, it is currently not clear how plants regulate Pip responses during pathogen infection.

Two closely related Arabidopsis transcription factors, TGA1 and TGA4, belong to the same subgroup of basic leucine zipper transcription factors as TGA2, TGA5 and TGA6. TGA1 and TGA4 are involved in basal resistance against pathogens, but are not required for SA-induced *PR* gene expression (Shearer et al. 2012, Kesarwani, Yoo, and Dong 2007). Analysis of the *tga1 tga4 npr1* triple mutant revealed that it is more susceptible to pathogens than the *tga1 tga4* double mutant and *npr1* single mutant. In addition, the enhanced pathogen resistance in the autoimmune *snc1 npr1* mutant is partially dependent on TGA1 and TGA4. These data suggest that TGA1 and TGA4 regulate plant defense in an NPR1-independent fashion (Shearer et al. 2012). However, the mechanism of how TGA1 and TGA4 regulate plant defense responses is currently unknown, and the goal of the present study was to test whether TGA1 and TGA4 promote pathogen-induced biosynthesis of SA and Pip.

3.3 Material and method

3.3.1 Plant materials and growth conditions

Arabidopsis thaliana snc1, *sard1-1 cbp60-1*, *tga1-1*, *tga4-1*, *tga1-1 tga4-1*, *TGA1* complementation lines, the SARD1-HA lines and CBP60g-HA lines were described previously (Zhang, Xu, et al. 2010, Li et al. 2001, Shearer et al. 2012). The *snc1 tga1 tga4* triple mutant was identified from the F2 population of a cross between *snc1* and *tga1-1 tga4-1*. Plants used in all assays (unless specified) were grown under 12 h white light at 23°C/12 h dark at 19°C in a plant growth room.

3.3.2 Gene expression analysis

For pathogen-induced gene expression analysis, leaves of four-week-old plants were infiltrated with *Pseudomonas syringae pv. maculicola* (*P.s.m.*) ES4326 or *Pseudomonas syringae pv. tomato* (*P.s.t.*) DC300 *hrcC*, and samples were collected 12 h later. Each sample consisted of four leaves from four individual plants. RNA was isolated using an EZ-10 Spin Column Plant RNA Mini-Preps Kit (Bio Basic, CANADA). After DNA removal using DNase (Promega) treatment, the RNA samples were reverse transcribed into cDNA using M-MuLV reverse transcriptase (Applied Biological Materials, Canada). Quantitative PCR was performed on the

cDNA using SYBR Premix Ex Taq II (TAKARA). Sequences of primers used for quantitative PCR are shown in Table 3.1. All real-time RT-PCR experiments were repeated three times using independent grown plants.

Table 3.1 Primers used in this study.

Primers for real time RT-PCR		
Gene ID	primer name	primer sequence (5'-->3')
AT2G37620	ACTIN1-F	CGATGAAGCTCAATCCAAACGA
	ACTIN1-R	CAGAGTCGAGCACAATACCG
AT1G73805	SARD1-RTF	TCAAGGCGTTGTGGTTTGTG
	SARD1-RTR	CGTCAACGACGGATAGTTTC
AT5G26920	CBP60g-RTF	GATGACATGACCTCAAGCTG
	CBP60g-RTR	TTAACCTTACACCACCTGGC
AT1G74710	SID2-F101-RT	GTCGTTCGGTTACAGGTTCC
	SID2-R102-RT	ATTAAACTCAACCTGAGGGAC
AT2G13810	ALD1-F101-RT	TTCCCAAGGCTAGTTTGGAC
	ALD1-R102-RT	GCCTAAGAGTAGCTGAAGACG
AT5G52810	SARD4-RTF	GCGAAACCAAGCTTGAGAAG
	SARD4-RTR	TCCGGGTTTCAAGAACTCAC
Primers for ChIP-PCR		
Gene ID	primer name	primer sequence (5'-->3')
AT1G73805	SARD1pro0.3kb-chipF	ggaaccgtccatttgtcaac
	SARD1pro0.3kb-chipR	ttcgaagaacgacaaaggaaa
AT5G26920	CBP60Gpro0.15kb-chipF	gtttcactgctgcttcgtca
	CBP60Gpro0.15kb-chipR	GGCTGTTCCGAATCTTCATt
AT5G26920	CBP60Gpro1.1kb-chipF	tcacctaagcgtggcttttt
	CBP60Gpro1.1kb-chipR	tcttggtctaattaggtgaatgaat
AT5G52810	SARD4pro-chipF	aagctttggctcacagGAAA
	SARD4pro-chipR	acgaacccagattggtcttg

3.3.3 Quantification of SA and Pip levels

To analyze pathogen-induced SA and Pip accumulation, four-week-old plants were infiltrated with *P.s.m.* ES4326 or *P.s.t.* DC300 *hrcC*, and the infiltrated leaves were collected 12 h after inoculation. For SA analysis, each sample consisted of about 100 mg of leaf tissue from five to six individual plants. SA was extracted and measured by high performance liquid chromatography as previously described (Sun et al. 2015). For Pip measurement, each sample

consisted of about 50 mg of tissue from four independent plants. Pip was extracted and quantified by GC-MS using the EZ:faast free amino acid analysis kit (Phenomenex) following a previously described procedure (Navarova et al. 2012).

3.3.4 Bacterial infection assays

To assay for resistance against *P.s.t.* DC3000 *hrc*C, leaves of four-week-old plants were infiltrated with the bacteria at a dose of OD_{600} = 0.002. Samples were collected at 0 and 3 days after inoculation, with each sample consisting of two leaf discs from two infected leaves of the same plant. Bacterial titres were determined by plating the ground samples on Lysogeny broth (LB) agar plates. For the analysis of flg22-induced pathogen resistance, leaves of four-week-old plants were infiltrated with 1 μM flg22 or ddH_2O 24 h before the same leaves were infiltrated with *P.s.t.* DC3000 (OD_{600} = 0.001). Samples were collected three days after inoculation, and bacterial titres were determined by plating the ground samples on LB agar plates.

3.3.5 ChIP-PCR Analysis.

To express the 3xHA-TGA1 protein in protoplasts, the genomic DNA of TGA1 was amplified by PCR and cloned into a modified pBluescript vector containing a 35S promoter, the coding sequence of an N-terminal 3xHA tag and an OCS terminator. The pBS-35S-3xHA-TGA1 plasmid was transformed into Arabidopsis mesophyll protoplasts (250 μg of plasmid was transformed into $1.5\sim2x10^6$ protoplasts). 20 h after incubation, the protoplasts were cross-linked in 8 ml of ES buffer (0.6 M mannitol, 5 mM MES pH5.7, 10 mM KCl) containing 1% formaldehyde for 10 min at room temperature and stopped by adding 0.1 M glycine. After washing once with ES buffer, cross-linked protoplasts were re-suspended in Nuclei lysis buffer (50 Mm Tris-HCl pH8, 10 mM EDTA, 1% SDS, 1x protease inhibitors cocktail (Roche)) and sonicated to shear the DNA to an average size of 0.3 to 1 kb. ChIP was subsequently carried out using anti-HA antibody (Roche) as previously described (Sun et al. 2015). For ChIP experiments using transgenic plants expressing SARD1-HA and CBP60g-HA under their own promoters, leaves of 25-day-old plants were infiltrated with *P.s.m.* ES4326 (OD_{600} = 0.001), and the infiltrated leaves were collected 24 h later. ChIP was carried out using anti-HA antibody as

previously described (Sun et al. 2015). The immunoprecipitated DNA was quantified by real-time PCR using gene-specific primers (Table 3.1). Real-time PCR was performed using the SYBR Premix Ex Taq II (TAKARA).

3.4 Results

3.4.1 TGA1 and TGA4 positively regulate SA biosynthesis

Arabidopsis displays a basal resistance against *P.s.m.* ES4326 (Glazebrook, et al. 1996), which is probably due to activation of weak effector-triggered immunity in addition to PAMP-triggered immunity (PTI). The *tga1-1 tga4-1* double mutant was previously shown to exhibit compromised resistance against *P.s.m.* ES4326. To test whether TGA1 and TGA4 are involved in regulating *P.s.m.* ES4326-induced SA biosynthesis, we compared SA levels in wild type (Col-0), *tga1-1*, *tga4-1* and *tga1-1 tga4-1* plants. Following treatment with *P.s.m.* ES4326, SA levels increased in both the wild type and the mutants, but significantly less in *tga1-1* and even less in the *tga1-1 tga4-1* double mutant (Figure 3.1A and B). Consistent with the SA levels, expression of *ICS1* was also much lower in *tga1-1 tga4-1* than in wild type (Figure 3.1C), suggesting that TGA1 and TGA4 positively regulate SA biosynthesis in plant defense. In two transgenic lines expressing *TGA1* in the *tga1-1 tga4-1* background, both the SA levels and *ICS1* expression were similar to those in the wild type (Figure 1), showing that the SA deficiency phenotype of the mutant can be rescued by the *TGA1* transgene.

Constitutive defense responses in the autoimmune mutant *snc1* are partially dependent on TGA1 and TGA4 (Shearer et al. 2012), prompting us to test whether TGA1 and TGA4 are also involved in promoting SA biosynthesis in *snc1* mutant plants. Both free SA and total SA levels were significantly lower in *snc1 tga1-1 tga4-1* than in *snc1* (Figure 3.2A and B). Similarly, the expression level of *ICS1* was also much lower in *snc1 tga1-1 tga4-1* than in *snc1* (Figure 3.2C), further underlining that TGA1 and TGA4 function as positive regulators of SA biosynthesis in plant immunity.

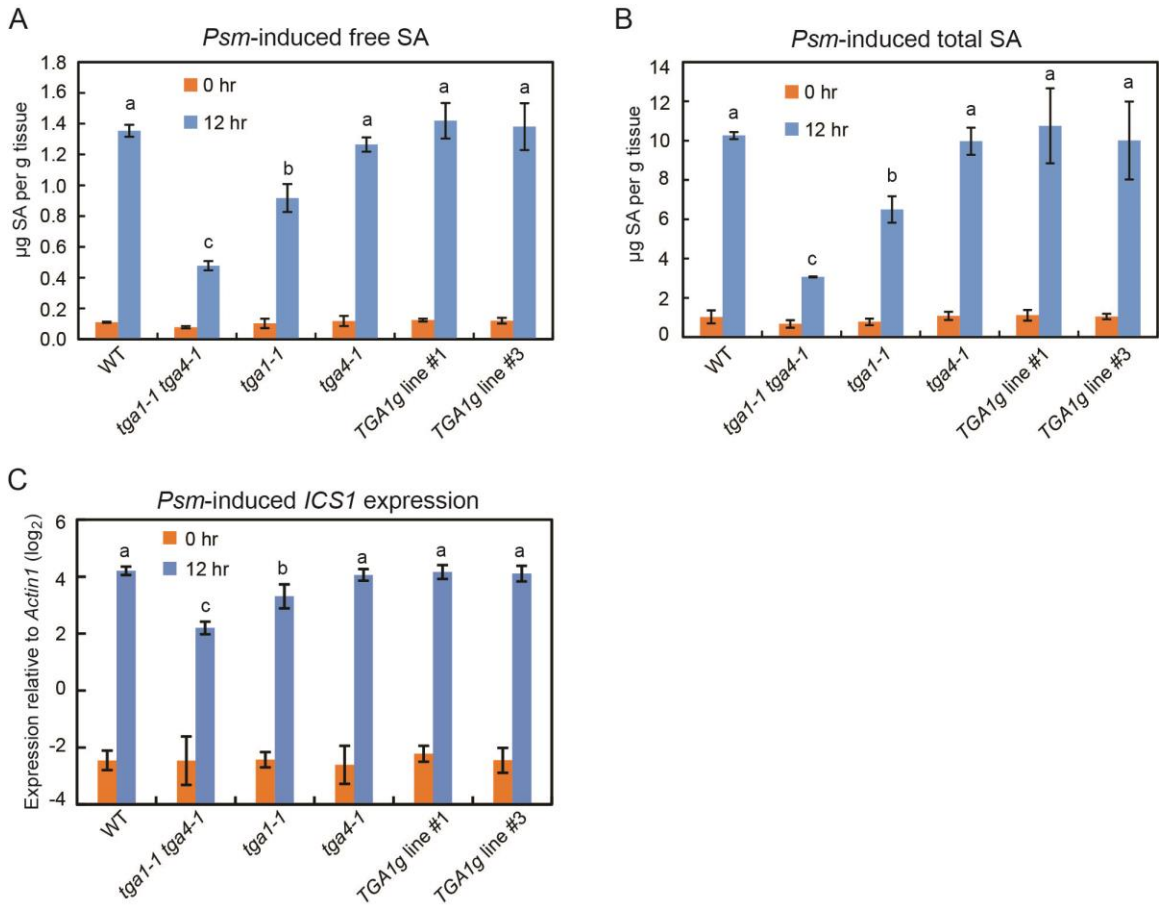

Figure 3.1 TGA1 and TGA4 positively regulate SA biosynthesis.
(A-B) Free SA (A) and total SA (B) levels in the indicated *Arabidopsis thaliana* genotypes. Wild type, WT. *TGA1g* lines #1 and #3 are two transgenic lines expressing *TGA1* in the *tga1-1 tga4-1* background. Four-week-old plants were infiltrated with *P.s.m.* ES4326 at a dose of OD_{600}=0.01. Samples were harvested at 0 h (untreated) and 12 h after infiltration. Error bars represent the standard deviation of three independent biological samples. Statistical differences among different genotypes are labeled with different letters (P< 0.05, One-way ANOVA/Tukey's test, n = 3). The experiment was repeated twice, each yielding similar results.
(C) Induction of *ICS1* by *P.s.m.* ES4326 in the indicated genotypes. Leaves of four-week-old plants were infiltrated with *P.s.m.* ES4326 at a dose of OD_{600}=0.001. Samples were harvested at 0 h (untreated) and 12 h after infiltration. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three independent biological replicates. Statistical differences among different genotypes are labeled with different letters (P< 0.05, One-way ANOVA/Tukey's test, n = 3).

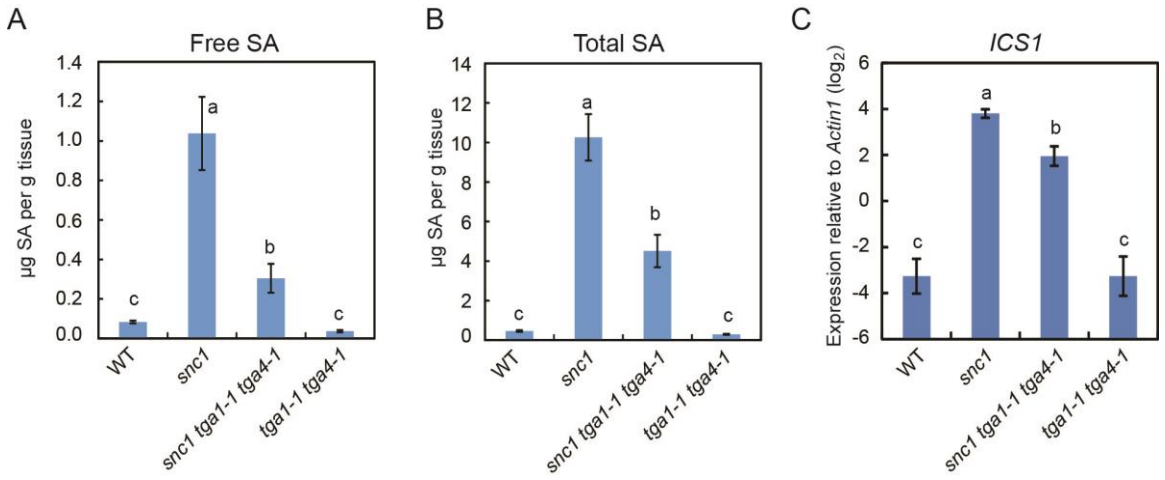

Figure 3.2 TGA1 and TGA4 positively regulate SA biosynthesis in *snc1*.
(A-B) Free SA (A) and total SA (B) levels in *Arabidopsis thaliana* wild type (WT), *snc1*, *snc1 tga1-1 tga4-1* and *tga1-1 tga4-1*. Error bars represent the standard deviation of three independent biological samples. Statistical differences among different genotypes are labeled with different letters ($P < 0.01$, One-way ANOVA, $n = 3$). The experiment was repeated twice, each yielding similar results.
(C) *ICS1* expression levels in wild type (WT), *snc1*, *snc1 tga1-1tga4-1* and *tga1-1 tga4-1*. Values were normalized to the expression of *ACTIN1*. Error bar represent the standard deviation of three independent biological replicates. Statistical differences among different genotypes are labeled with different letters ($P < 0.01$, One-way ANOVA, $n = 3$). Leaves of four-week old plants grown under long day conditions were collected for SA and RNA analysis.

3.4.2 TGA1 and TGA4 are required for induction of *SARD1* and *CBP60g* in plant defense

Both SARD1 and CBP60g function as critical regulators of pathogen-induced SA biosynthesis (Zhang, Xu, et al. 2010, Wang, Tsuda, Truman, Sato, Nguyen le, et al. 2011). To determine whether TGA1 and TGA4 are required for induction of *SARD1* and *CBP60g*, we analyzed the expression levels of both genes in various mutant lines. The expression levels of *SARD1* and *CBP60g* were comparable between *P.s.m.* ES4326-treated wild type and the *TGA1* complementation lines, but significantly lower in *tga1-1* and further reduced in *tga1-1 tga4-1* (Figure 3.3A and B). In the *snc1 tga1-1 tga4-1* mutant, the expression of *SARD1* and *CBP60g* was also considerably lower than in *snc1* (Figure 3.4A and B). Together, these data suggest that TGA1 and TGA4 are required for full induction of *SARD1* and *CBP60g* in plant defense.

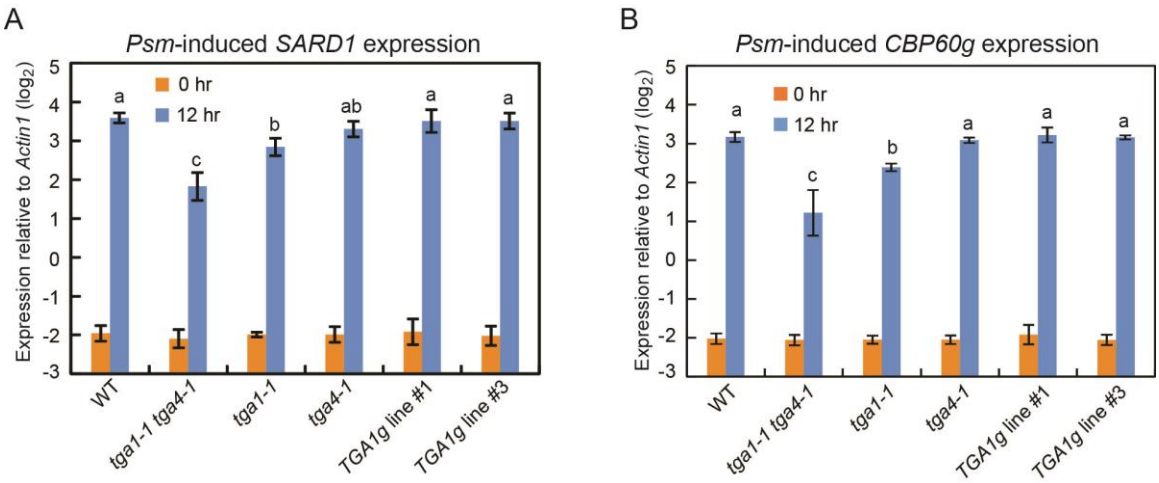

Figure 3.3 TGA1 and TGA4 are required for the full induction of *SARD1* and *CBP60g*.
P.s.m. ES4326-induced *SARD1* (A) and *CBP60g* (B) expression was analyzed in the indicated *Arabidopsis thaliana* genotypes. Leaves of four-week-old plants were infiltrated with *P.s.m.* ES4326 at a dose of OD_{600}=0.001. Samples were harvested at 0 h (untreated) and 12 h after infiltration. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three independent biological replicates. Statistical differences among different genotypes are labeled with different letters (P< 0.05, One-way ANOVA/Tukey's test, n = 3).

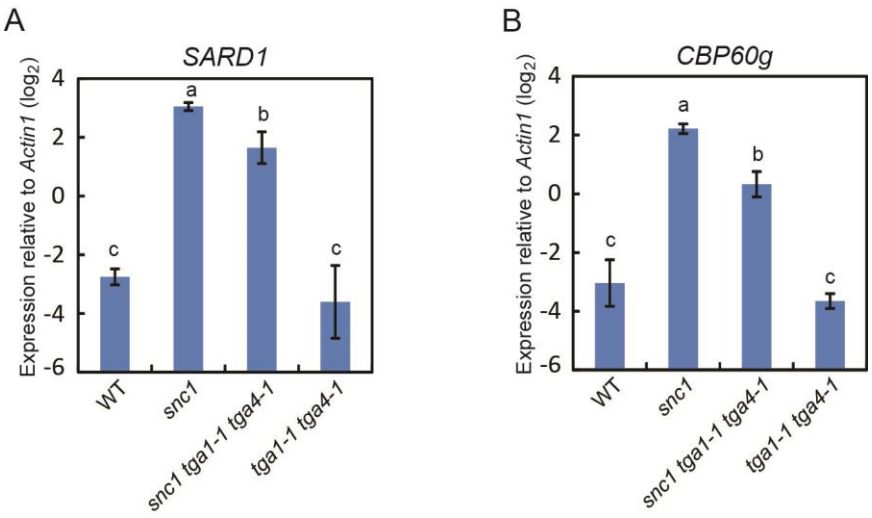

Figure 3.4 Expression levels of *SARD1* and *CBP60g* in wild type (WT), *snc1*, *snc1 tga1-1 tga4-1* and *tga1-1 tga4-1*.
Leaves of four-week-old *Arabidopsis thaliana* plants grown under long day conditions were collected for RNA analysis. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three independent biological replicates. Statistical differences among different genotypes are labeled with different letters (P< 0.01, One-way ANOVA, n = 3).

3.4.3 *SARD1* is a target gene of TGA1

Analysis of the DNA sequence upstream of the *SARD1* coding region revealed that there are three predicted TGA transcription factor binding sites in the promoter region of this gene (Figure 3.5A). In the promoter region of *CBP60g*, there are also two predicted TGA transcription factor binding sites. To determine whether *SARD1* and *CBP60g* are direct target genes of TGA1, we carried out ChIP experiments on Arabidopsis protoplasts transiently expressing a 3×HA-TGA1 fusion protein using an anti-HA antibody. Quantitative PCR on the immunoprecipitated DNA template, showed clear enrichment of the DNA surrounding the putative TGA1-binding sites in the promoter region of *SARD1* (Figure 3.5B), but not of *CBP60g* (Figure 3.5C and D), suggesting that TGA1 binds to the promoter region of *SARD1*.

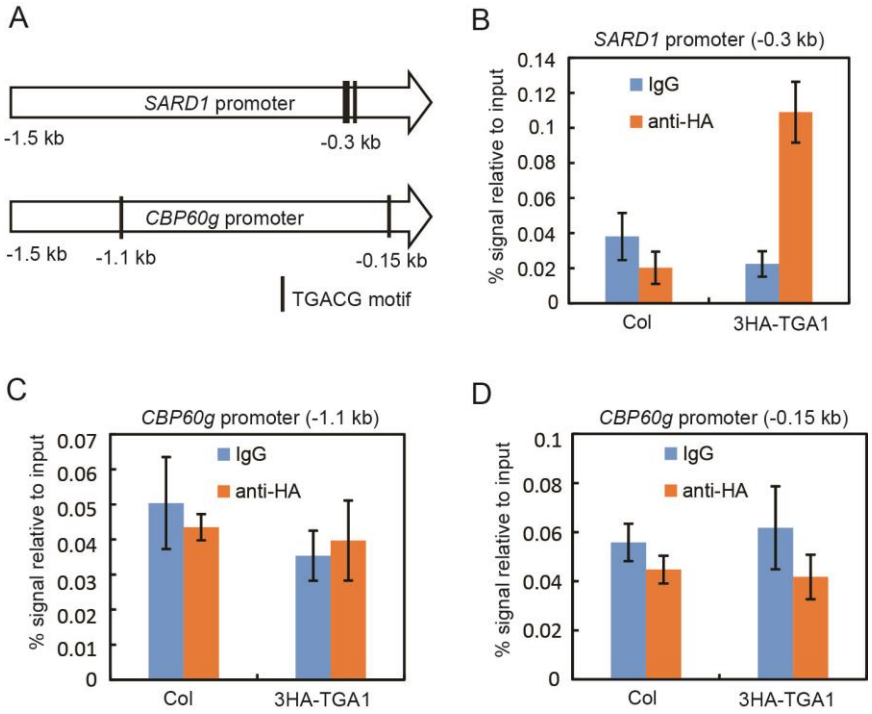

Figure 3.5 TGA1 binds to the promoter region of *SARD1*.
(A) Locations of the TGACG motifs in the promoter regions of *SARD1* and *CBP60g* (1.5 kb upstream of the start codons). The open arrow represents the promoter regions.
(B-D) ChIP-PCR analysis of TGA1 binding to the promoter regions of *SARD1* and *CBP60g*. Wild type *Arabidopsis thaliana* Col-0 protoplasts were transfected with a plasmid expressing 3xHA-TGA1 under control of the 35S promoter. Un-transformed protoplasts were used as the control. The 3xHA-TGA1 chromatin complex was immunoprecipitated with an anti-HA antibody. Negative control reactions were performed using immunoglobin G (IgG).

Immunoprecitated DNA samples were quantified by real-time PCR (qPCR) using primers specific to the *SARD1* and *CBP60g* promoters. ChIP results are presented as signals from immunoprecipitated samples relative to input. Error bars represent the standard deviation of three technical repeats. Experiments were repeated twice with independently grown plants, each yielding similar results.

3.4.4 TGA1 and TGA4 are required for PAMP-triggered immunity

As SARD1 and CBP60g play important roles in PAMP-triggered immunity (Sun et al. 2015), we next tested whether PTI is affected by mutations in TGA1 and TGA4. We infiltrated the wild type, *tga1-1*, *tga4-1*, *tga1-1 tga4-1* and the *TGA1* complementing lines in *tga1-1 tga4-1* background with *P.s.t.* DC3000 *hrcC*, a bacterial strain deficient in delivery of type-III effectors and inducing PTI responses, and quantified the expression levels of *SARD1* and *CBP60g*. As shown in Figure 3.6A and B, *SARD1* and *CBP60g* were induced to similar levels in wild type, *tga4-1* and the TGA1 complementation lines, but their expression was significantly lower in *tga1-1* and further reduced in *tga1-1 tga4-1* plants. Similarly, the expression level of *ICS1* after *P.s.t.* DC3000 *hrcC* treatment was also much lower in *tga1-1 tga4-1* (Figure 3.6C). Consistent with the reduced *ICS1* expression, *tga1-1 tga4-1* also accumulated less SA than wild type and the TGA1 complementation lines following treatment with *P.s.t.* DC3000 *hrcC* (Figure 3.6D and E).

Next we quantified the growth of *P.s.t.* DC3000 *hrcC* in the plants. The *tga1-1 tga4-1* mutant supported significantly higher growth of the bacteria than the wild type and the TGA1 complementation lines (Figure 3.6F). We also analyzed flg22-induced resistance to *P.s.t.* DC3000 in the wild type, *tga1-1*, *tga4-1*, *tga1-1 tga4-1* and the *TGA1* complementation lines. Treatment with flg22 suppressed bacterial growth in the wild type and the TGA1 complementation lines to circa 1% of the untreated controls, but only to approximately 3% and 10% in *tga1-1* and *tga1-1 tga4-1*, respectively (Figure 3.6G). These results suggest that both TGA1 and TGA4 contribute to PAMP-triggered immunity.

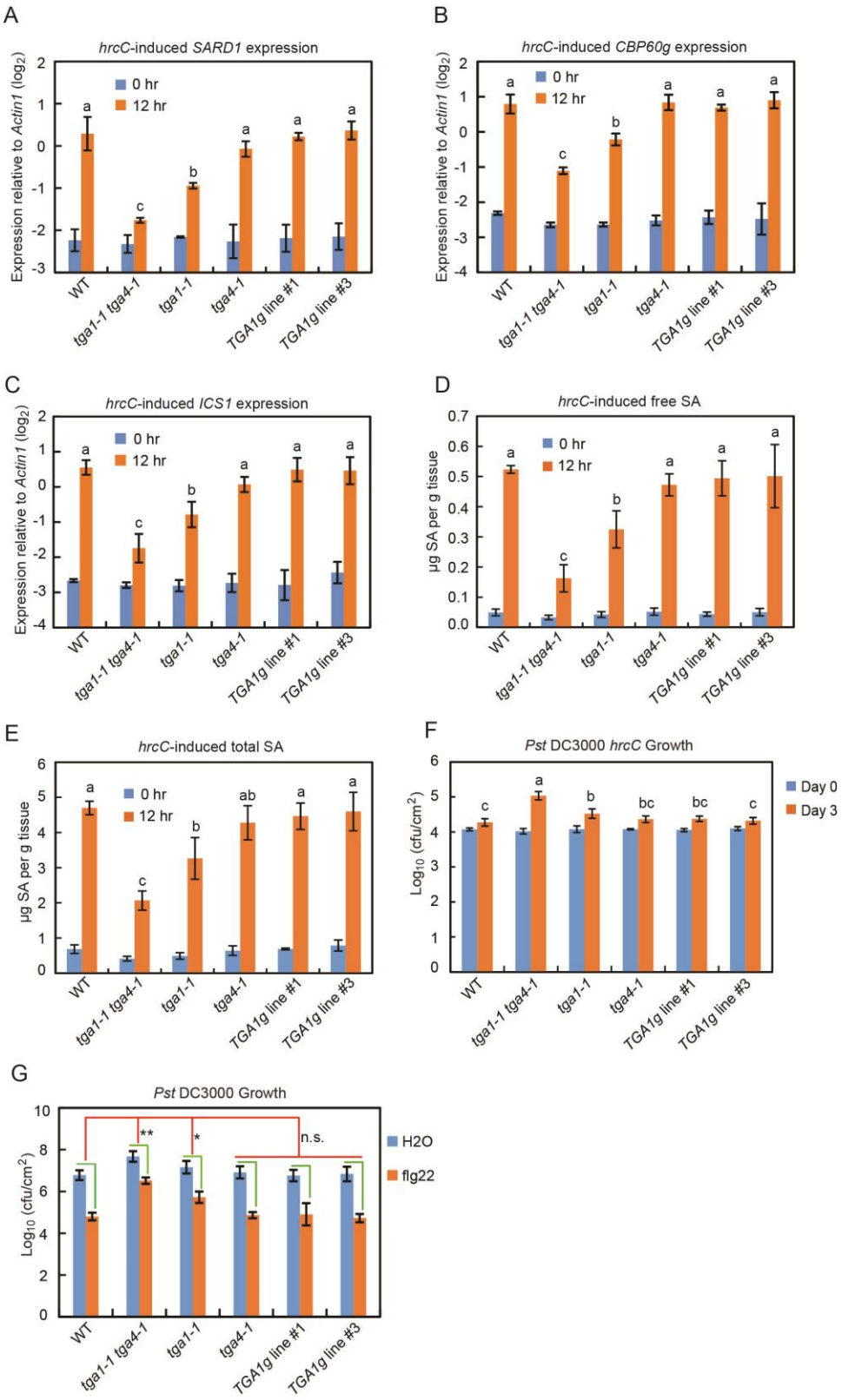

Figure 3.6 TGA1 and TGA4 are required for PAMP-triggered immunity.

(A-C) Induction of *SARD1* (A), *CBP60g* (B) and *ICS1* (C) by *P.s.t.* DC3000 *hrcC* in the indicated *Arabidopsis thaliana* genotypes. Leaves of four-week-old plants were infiltrated with *P.s.t.* DC3000 *hrcC* at a dose of OD_{600}=0.05. Samples were harvested at 0 h and 12 h after infiltration. Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three independent biological replicates. Statistical differences among different genotypes are labeled with different letters (P< 0.05, One-way ANOVA/Tukey's test, n = 3).
(D-E) Free SA (D) and total SA (E) levels in the indicated genotypes. Four-week-old plants were infiltrated with *P.s.t.* DC3000 *hrcC* at a dose of OD_{600}=0.05. Samples were harvested at 0 h (untreated) and 12 h after infiltration. Error bars represent the standard deviation of three independent biological samples. Statistical differences among different genotypes are labeled with different letters (P< 0.05, One-way ANOVA/Tukey's test, n = 3). The experiment was repeated twice, each yielding similar results.
(F) Growth of *P.s.t.* DC3000 *hrcC* on the indicated genotypes. Four-week-old plants were infiltrated with *P.s.t.* DC3000 *hrcC* at a dose of OD_{600} = 0.002. Bacterial titers were quantified at 0 and 3 days after infiltration. Error bars represent the standard deviation of five replicates. Statistical differences among different genotypes are labeled with different letters (P< 0.01, One-way ANOVA/Tukey's test, n = 5). The experiment was repeated three times, each yielding similar results.
(G) Growth of *P.s.t.* DC3000 on the indicated genotypes after H_2O or flg22 treatment. Leaves of four-week-old plants were infiltrated with H_2O or 1 μM flg22. After 24 h, the treated leaves were infiltrated with *P.s.t.* DC3000 at a dose of OD_{600} = 0.001. Samples were taken three days after *P.s.t.* DC3000 inoculation. Error bars represent the standard deviation of five replicates. The green lines represent flg22-induced protection or the reduction of bacteria titer after flg22 treatment in each genotype. The flg22-induced protection in the mutant lines was compared to that in WT: ** P<0.001, * P<0.05 and n.s., not significant (two-way ANOVA/Tukey's test, n = 5). The experiment was repeated twice, each yielding similar results.

3.4.5 SARD1 and CBP60g positively regulate Pip biosynthesis

SARD1 and CBP60g regulate the expression of a large number of key regulators of plant immunity (Sun et al. 2015). One of the target genes of SARD1 and CBP60g is *ALD1*, which is involved in the biosynthesis of Pip. *SARD4*, another gene involved in Pip biosynthesis, is also among the candidate target genes of SARD1 identified by ChIP-sequencing (Sun et al. 2015). Here, we confirmed the ChIP-sequencing result using ChIP-PCR on transgenic lines expressing SARD1-HA and CBP60g-HA proteins, revealing that both SARD1 and CBP60g are targeted to the promoter region of *SARD4* (Figure 3.7A and B).

Previously it had been shown that pathogen induction of *ALD1* is dependent on SARD1 and CBP60g (Sun et al. 2015). To test whether SARD1 and CBP60g are also required for

inducing expression of *SARD4*, we compared the expression levels of *SARD4* in wild type and *sard1 cbp60g* following treatment with *P.s.m.* ES4326. The expression levels of *SARD4* before induction were comparable between wild type and *sard1 cbp60g*, but after infection by *P.s.m.* ES4326 *SARD4* levels were much lower in *sard1 cbp60g* than in wild type (Figure 3.7C).

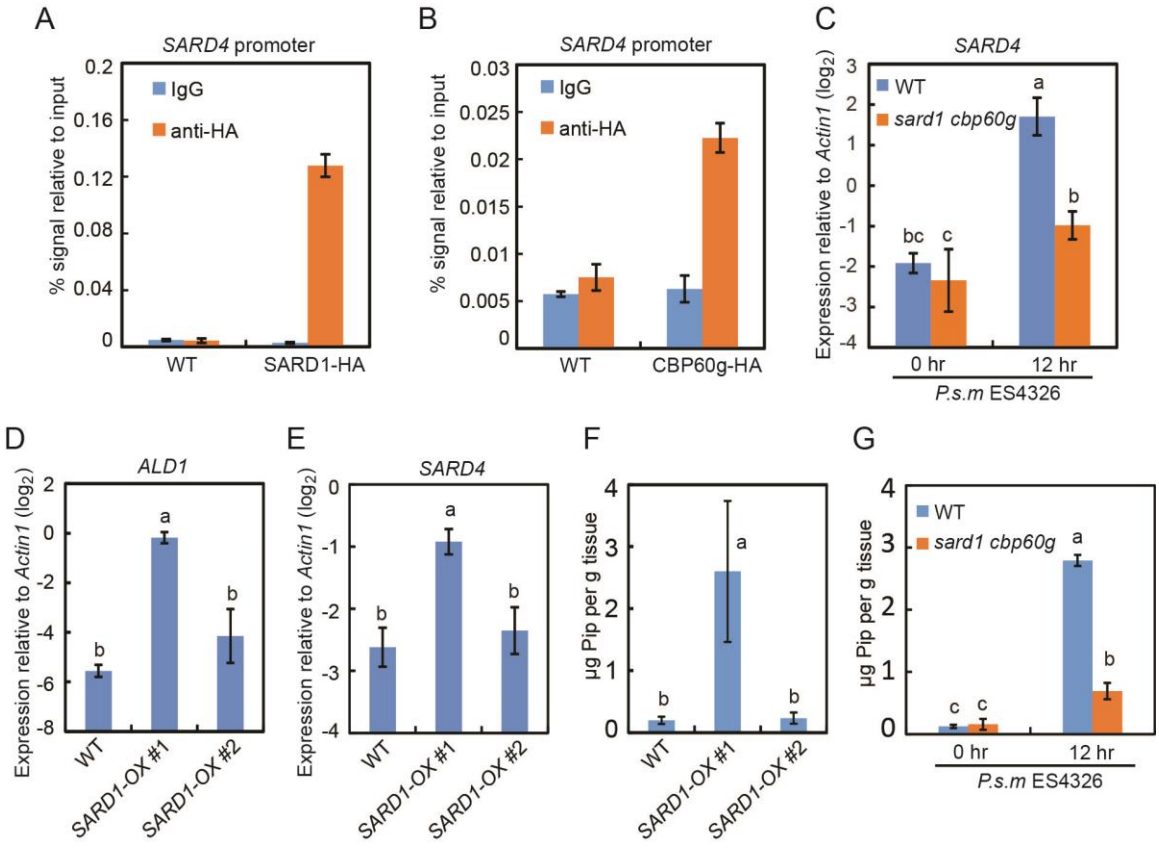

Figure 3.7 SARD1 and CBP60g positively regulate Pip biosynthesis.
(A-B) Binding of SARD1-HA (A) and CBP60g-HA (B) to the promoter region of *SARD4* as determined by ChIP-PCR. Leaves of four-week old *Arabidopsis thaliana* wild type and transgenic plants expressing *SARD1-HA* (A) or *CBP60g-HA* (B) were infiltrated with *P.s.m* ES4326 (OD_{600}=0.001). Samples were collected 24 h later. Chromatin complexes were immunoprecipated using an anti-HA antibody. IgG was used as the negative control. The immunoprecipitated samples were analyzed by qPCR using primers specific to the *SARD4* promoter. ChIP results are presented as signals from immunoprecipitated samples relative to input. Error bars represent the standard deviation of three technical repeats. The experiments were repeated twice with independently grown plants, each yielding similar results.
(C) *Psm*-induced *SARD4* expression in wild type (WT) and *sard1-1 cbp60g-1*. Leaves of four-week-old wild type and *sard1-1cbp60g-1* plants were collected 0 h and 12 h after infiltration with *P.s.m* ES4326 (OD_{600} = 0.001).
(D-E) Expression levels of *ALD1* (D) and *SARD4* (E) in WT and *SARD1* overexpression lines.
(F) Pipecolic acid accumulation in wild type and *SARD1* overexpression lines. The experiments were repeated twice, each yielding similar results.

(G) *Psm*-induced Pip accumulation in wild type and *sard1-1 cbp60g-1*. Leaves of four-week-old wild type and *sard1-1 cbp60g-1* plants were collected at 0 h and 12 h after infiltration with *P.s.m* ES4326 (OD_{600} = 0.01). The experiments were repeated twice, each yielding similar results.

Values of gene expression were normalized to *ACTIN1* (C-E). Error bars represent the standard deviation of three independent biological replicates. Statistical differences among different genotypes are labeled with different letters (P< 0.01, One-way ANOVA/Tukey's test, n = 3).

Overexpression of *SARD1* is known to cause enhanced resistance to pathogens (Zhang, Xu, et al. 2010), leading us to test whether increased levels of *SARD1* enhance Pip biosynthesis by increasing the expression levels of *ALD1* and *SARD4*, which encode two key enzymes for Pip formation. Two previously characterized transgenic lines expressing *SARD1-HA* at different levels (Zhang, Xu, et al. 2010) were analyzed, and expression of *ALD1* and *SARD4* was greatly enhanced in the strong *SARD1-HA* expressing line (line 1), but not in the weaker *SARD1-HA* expressing line (line 2, Figure 3.7D and E). Consistent with the *ALD1* and *SARD4* expression levels, Pip also accumulated to a considerably higher level in the strong *SARD1* expressing line compared to wild type and the other transgenic line (Figure 3.7F).

To test whether SARD1 and CBP60g are required for pathogen-induced Pip accumulation, we measured Pip levels in the wild type and the *sard1 cbp60g* double mutant. Pip levels increased upon treatment with *P.s.m.* ES4326 to a much lesser degree in the double mutant than in wild type (Figure 3.7G), suggesting that the increase in Pip levels during pathogen infection is partially dependent on SARD1 and CBP60g.

3.4.6 TGA1 and TGA4 are required for the induction of Pip biosynthesis during pathogen infection

Since TGA1 and TGA4 are required for the induction of *SARD1* and *CBP60g* during pathogen infection, we tested whether they are also required for the induction of Pip biosynthesis. The expression levels of *ALD1* and *SARD4* were comparable between *P.s.m.* ES4326-treated wild type and *tga4-1*, but significantly lower in *tga1-1* and further reduced in *tga1-1 tga4-1* plants (Figure 3.8A and B). Consistent with the expression of *ALD1* and *SARD4*, the Pip level was much lower in *P.s.m.* ES4326-treated *tga1-1 tga4-1* plants than in wild type (Figure 3.8C). In the transgenic lines expressing *TGA1* in the *tga1-1 tga4-1* background, Pip levels and the expression

of *ALD1* and *SARD4* were similar to those in the wild type (Figure 3.8A-C), indicating that the Pip deficiency phenotype in *tga1-1 tga4-1* can be complemented by the *TGA1* transgene.

3.4.7 TGA1 and TGA4 are required for SAR

Since TGA1 and TGA4 are required for *P.s.m.* ES4326-induced SA and Pip biosynthesis, we tested whether they are required for SAR. We infiltrated the local leaves of wild type, *sard1 cbp60g* and *tga1-1 tga4-1* plants with *P.s.m.* ES4326 and subsequently challenged the distal leaves with *Hyaloperonospora arabidopsidis* Noco2 (*H.a.* Noco2) to assay for SAR. As shown in Figure 3.8D, following primary infection by *P.s.m.* ES4326, wild type plants developed strong systemic resistance, but SAR is greatly reduced in *tga1-1 tga4-1* and completely abolished in *sard1 cbp60g*, suggesting that loss of TGA1 and TGA4 results in compromised SAR.

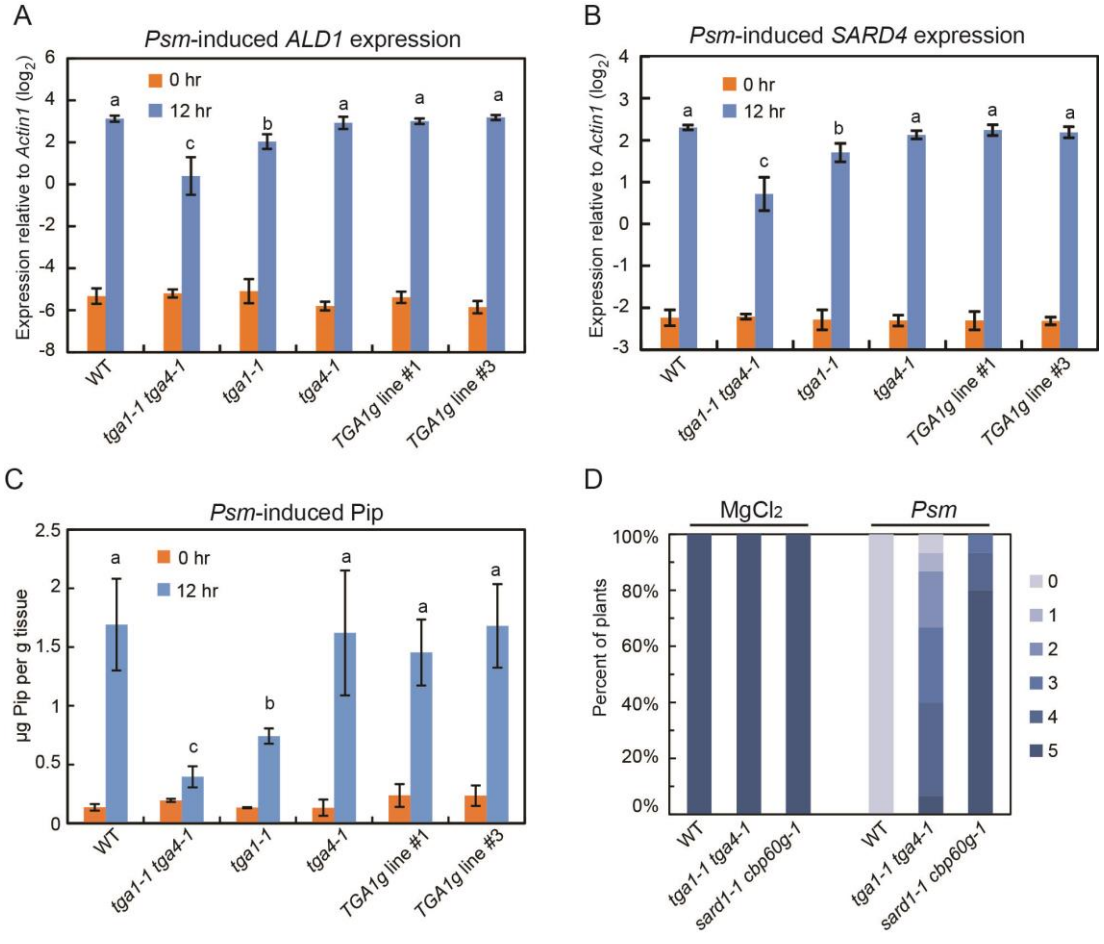

Figure 3.8 TGA1 and TGA4 are required for the induction of Pip biosynthesis during pathogen infection and SAR.

(A-B) Expression levels of *ALD1* (A) and *SARD4* (B) in the indicated *Arabidopsis thaliana* genotypes following pathogen infection. Leaves of four-week old plants of indicated genotypes were collected at 0 and 12 h after infiltration with *P.s.m* ES4326 (OD_{600} = 0.001). Values were normalized to the expression of *ACTIN1*. Error bars represent the standard deviation of three independent biological replicates. Statistical differences among different genotypes are labeled with different letters ($P< 0.05$, One-way ANOVA/Tukey's test, $n = 3$).
(C) *Psm*-induced Pip accumulation in the indicated genotypes. Leaves of four-week old plants of indicated genotypes were collected at 0 and 12 h after infiltration with *P.s.m* ES4326 (OD_{600} = 0.01) for free amino acid analysis. Error bars represent the standard deviation of three independent biological replicates. Statistical differences among different genotypes are labeled with different letters ($P< 0.05$, Student's t-test, $n = 3$). The experiments were repeated twice, each yielding similar results.
(D) Growth of *H.a.* Noco2 on the distal leaves of the indicated genotypes. The SAR assay was carried out as previously described (Zhang *et al.*, 2010). Two days after infiltrating two primary leaves with *P.s.m.* ES4326 (OD_{600} = 0.001) or 10 mM $MgCl_2$, plants were sprayed with *H. a.* Noco2 spores at a concentration of 5×10^4 spores per ml water. Infection was scored seven days later by counting the number of conidiophores on the distal leaves. A total of 15 plants were scored for each treatment. Disease rating scores are as follows: 0, no conidiophores on the plants; 1, one leaf was infected with no more than 5 conidiophores; 2, one leaf was infected with more than 5 conidiophores; 3, two leaves were infected but no more than 5 conidiophores on each infected leaf; 4, two leaves were infected with more than 5 conidiophores on each infected leaf; 5 more than two leaves were infected with more than 5 conidiophores. Experiments were repeated twice with independently grown plants, each yielding similar results.

3.5 Discussion

The plant metabolite Pip plays an important role in the amplification of defense responses against pathogens (Navarova et al. 2012, Zeier et al. 2015). Recently, it was shown that in Arabidopsis Pip is biosynthesized from L-lysine via a pathway consisting of the enzymes ALD1 and SARD4 (Ding et al. 2016), but how Pip biosynthesis is regulated is largely unknown. In this study, we showed that SARD1 and CBP60g function as key transcription factors in promoting pathogen-induced Pip biosynthesis. Both SARD1 and CBP60g target *ALD1* and *SARD4*. Induction of *ALD1* and *SARD4* by pathogen infection is dramatically reduced in the *sard1 cbp60g* double mutant, whereas overexpression of *SARD1* leads to elevated expression levels of both *ALD1* and *SARD4*. Consistent with the expression levels of *ALD1* and *SARD4*, pathogen-induced Pip accumulation is dramatically reduced in *sard1 cbp60g* and overexpression of *SARD1* results in elevated Pip levels. These findings suggest that SARD1 and CBP60g promote Pip biosynthesis by regulating *ALD1* and *SARD4* expression (Figure 3.9).

Plant defense against pathogen infection is activated through rapid transcriptional reprogramming, and SARD1 and CBP60g are known to be involved in the induction of a large number of key immune regulators in this process (Sun et al. 2015). The expression of *SARD1* and *CBP60g* is also rapidly up-regulated following pathogen infection (Zhang, Xu, et al. 2010, Wang et al. 2009). Unlike CBP60g, whose activity is modulated by Ca^{2+} (Wang et al. 2009), *SARD1* is mainly regulated at the transcription level (Zhang, Xu, et al. 2010), as elevated SARD1 expression is sufficient to activate SA and Pip biosynthesis and defense against pathogens. How the transcription of *SARD1* and *CBP60g* is regulated in plant defense was largely unknown. However, the dramatic reduction of pathogen-induced *SARD1* and *CBP60g* expression in the *tga1 tga4* double mutant we report here indicates that TGA1 and TGA4 are required for the induction of *SARD1* and *CBP60g* by pathogens (Figure 3.9). Furthermore, the binding of TGA1 to the promoter region of *SARD1* shows that *SARD1* is a direct target gene of TGA1. Conversely, our ChIP-PCR finding that TGA1 does not bind to the promoter region of *CBP60g* suggests that TGA1 regulates CBP60g expression indirectly through another transcription factor. However, it cannot be ruled out that the interaction between TGA1 and the promoter of *CBP60g* was too weak to detect under our experimental conditions.

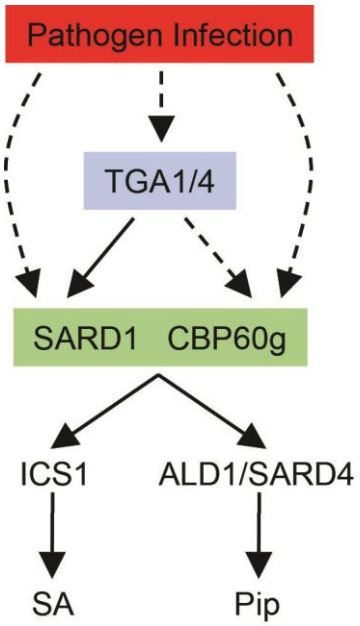

Figure 3.9 A working model for regulation of SA and Pip biosynthesis by transcription factors TGA1/TGA4 and SARD1/CBP60g.
Upon pathogen infection, *Arabidopsis thaliana* TGA1 andTGA4 are activated and contribute to the induction of *SARD1* and *CBP60g*. SARD1 and CBP60g subsequently activate the expression of *ICS1*, *ALD1* and *SARD4*, which leads to increased SA and Pip biosynthesis. Solid lines represent direct regulation and dashed lines represent indirect regulation.

Previously, SARD1 and CBP60g have been shown to play important roles in PTI (Sun et al. 2015). Consistent with the reduction in *SARD1* and *CBP60g* expression and SA level, the *tga1 tga4* double mutant supports about seven-fold higher growth of *P.s.t.* DC3000 *hrcC* and exhibits compromised flg22-induced resistance against *P.s.t.* DC3000, suggesting that TGA1 and TGA4 play an indispensable role in PTI by promoting the expression of *SARD1* and *CBP60g*.

Several target genes of SARD1 and CBP60g including *ICS1*, *ALD1* and *SARD4* that are involved in pathogen-induced SA and Pip biosynthesis were expressed at lower levels in *tga1 tga4* than in wild type. Consistently, accumulation of SA and Pip following pathogen infection was significantly reduced in *tga1 tga4*, suggesting that TGA1 and TGA4 play critical roles in promoting SA and Pip biosynthesis in plant defense (Figure 3.9). Previously it was shown SA-induced *PR* gene expression was not affected by loss of TGA1 and TGA4 (Shearer et al. 2012), which is consistent with that they function upstream rather than downstream of SA in plant defense. Since both SA and Pip play important roles in resistance against pathogens, we conclude that the enhanced disease susceptibility and compromised *P.s.m.* ES4326-induced SAR in *tga1 tga4* is at least partially caused by reduced pathogen-induced SA and Pip biosynthesis.

In the *tga1 tga4* double mutant, induction of *SARD1* and *CBP60g* expression and biosynthesis of SA and Pip is reduced but not completely blocked, suggesting that there may be additional, currently unknown transcription factors that play roles in the up-regulation of *SARD1* and *CBP60g* in parallel with TGA1 and TGA4 (Figure 3.9). Previously it was shown that *PR* gene expression and SAR induced by *P.s.t.* DC3000 *AvrRpt2* are not affected in the *tga1 tga4* double mutant (Shearer et al. 2012). It is likely the loss of TGA1 and TGA4 is compensated by other transcription factors in these processes. It will be interesting to determine whether the induction of SA and Pip production by avirulent pathogens such as *P.s.t.* DC3000 *AvrRpt2* is affected in the *tga1 tga4* double mutant. How the activities of TGA1 and TGA4 are regulated during pathogen infection is still unclear. It was previously shown that the redox status of Cys residues in TGA1 affect its interaction with NPR1 (Despres et al. 2003), suggesting that TGA1 and TGA4 might be activated by changes in cellular redox potential during pathogen infection.

Chapter 4: Opposite roles of salicylic acid receptors NPR1 and NPR3/NPR4 in transcriptional regulation of plant immunity

4.1 Summary

Salicylic acid (SA) is a plant defense 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 co-activator, whereas NPR3/NPR4 were suggested to function as E3 ligases that promote NPR1 degradation. Here we report that NPR3/NPR4 function as transcriptional co-repressors and SA inhibits their activities to promote the expression of downstream immune regulators. *npr4-4D*, a 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 promote SA-induced defense 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 defense gene expression.

4.2 Introduction

Salicylic acid (SA) is a phytohormone required for plant defense against pathogens (Vlot, Dempsey, and Klessig 2009). Pathogen infection induces SA accumulation in both infected and systemic tissue. Blocking SA accumulation results in compromised plant immunity (Gaffney 1993), whereas exogenous application of SA or SA analogs induces immunity to pathogens (Gorlach et al. 1996, Metraux 1991). In *Arabidopsis*, pathogen-induced SA is mainly synthesized through Isochorismate Synthase 1 (ICS1/SID2) (Wildermuth et al. 2001). SARD1 and CBP60g promote pathogen-induced SA synthesis by regulating the expression of *ICS1* (Wang, Tsuda, Truman, Sato, Nguyen le, et al. 2011, Zhang, Xu, et al. 2010). In addition to *ICS1*, SARD1 and CBP60g also regulate the expression of a large number of other immune regulators, suggesting that these two transcription factors play broad roles in 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, Friedrich, and Ryals 1995). NPR1 contains an N-terminal BTB/POZ domain, a central ankyrin-repeat domain and a C-terminal transactivation domain (Cao et al. 1997, Rochon, Boyle, Wignes, Fobert, and Després 2006). NPR3 and NPR4 are two paralogs of NPR1 with very similar domain structures as NPR1. 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 defense 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 (Zhang et al. 2006, Zhou et al. 2000, Zhang et al. 1999, Despres et al. 2000). NPR1 were shown to serve as a transcriptional co-activator (Rochon, Boyle, Wignes, Fobert, and Després 2006, Fan and Dong 2002) and NPR3/NPR4 were suspected to also function in transcriptional regulation (Zhang et al. 2006, Kuai, MacLeod, and Després 2015). Three TGA transcription factors, TGA2, TGA5 and TGA6, function redundantly in positive regulation of SA-induced *PR* gene expression and pathogen resistance (Zhang 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 defense responses (Zhang et al. 2003).

A large number of SA-binding proteins with different affinity to SA have been identified in plants (Klessig, Tian, and Choi 2016), but how SA is perceived as a defense 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 (Wu et al. 2012, Manohar et al. 2015). 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. 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, MacLeod, and

Després 2015). As NPR1 and NPR3/NPR4 share similar domain structures and have high sequence similarity, it is surprising that NPR1 functions as a transcriptional co-activator, but NPR3/NPR4 are proposed to work as E3 ligases.

In this chapter, the candidate showed that basal expression level of *SARD1* is elevated in *npr3 npr4* double mutant, suggesting that NPR3 and NPR4 negatively regulate basal level of *SARD1*. The candidate further discovered that C-terminus of NPR3 and NPR4 but not NPR1, possess a conserved motif (VDLNETP) with high similarity to the ethylene-responsive element binding factor-associated amphipathic repression motif (EAR; L/FDLNL/F(x)P) (Ohta et al. 2001), implying that they may function as transcriptional repressors, which was confirmed by Y. Ding using the transcriptional repressor assay. YX. Zhang isolated the *bda4-4D snc2-1D npr1-1* triple mutant. Y. Ding identified *BDA4* as a gain-of-function allele of *NPR4* (*npr4-4D*) and characterized the *npr4-4D* single mutant. The candidate showed that the NPR4-4D mutant protein, like NPR4 protein, can still interact with transcription factor TGA2 in yeast two-hybrid assay. Through *in vitro* SA-binding assay using recombinant NPR4 and NPR4-4D proteins, the candidate found that the NPR4-4D protein fails to bind to SA. Y. Ding provided evidence that *npr4-4D* plants are insensitive to SA. Together these data revealed that *npr4-4D* is insensitive to SA. The candidate generated equivalent mutation in *NPR3* and showed that this equivalent mutation in *NPR3 (NPR3^{R428Q})*, like *npr4-4D*, is able to suppress *snc2-1D npr1-1*, confirming that NPR3 and NPR4 function redundantly. Y. Ding found that SA-binding inhibits the transcription repression activity of NPR3 and NPR4, but not NPR4-4D in the transcriptional repressor assay. The candidate further showed that SA binding did not prevent the association between NPR3/ NPR4 and TGA2 in Y2H assays, or the association of NPR3/ NPR4 with defense gene promoters in ChIP experiments, indicating that SA releases the repression of defense gene expression by NPR3 and NPR4 by blocking their transcription repression activity. The candidate also found that the equivalent mutation in *NPR1 (NPR1^{R432Q})* dampens its ability to bind SA, but still interacts with TGA2 and NIMIN1 in Y2H assays, and Y. Ding showed that SA-induced defense gene expression and INA-induced immunity in *npr1-1* could be complemented by expressing *NPR1* but not *NPR1^{R432Q}*, suggesting that the R432 in NPR1 is essential for SA binding and signaling. Y. Ding showed that *npr1-1* and *npr4-4D* have additive effects on regulating disease resistance as well as suppression of *snc2-1D*. The candidate carried

out the RNA-seq experiment to analyze the SA-induced gene expression in Col, *npr1-1*, *npr4-4D* and *npr1-1 npr4-4D* and confirmed the RNA-seq data by RT-qPCR. K. Ao carried out data analysis for the RNA-seq experiment. Data from the RNA-seq experiment identified a large number of genes regulated by SA and confirmed that *npr1-1* and *npr4-4D* have additive effects on regulating SA-induced defense gene expression, suggesting that NPR1 and NPR4 function independently to regulate SA singling. Taken together, our study indicates that that NPR1 and NPR3/NPR4 are all *bona fide* SA receptors despite their opposite roles in transcriptional regulation of SA-induced defense gene expression.

4.3 Material and method

4.3.1 Plant materials and growth conditions

Arabidopsis plants were grown on soil in a growth chamber at 23℃/19℃ day/night and ~70% relative humidity. 16h/8h light/dark photoperiod is used for long day conditions and 12 h light at 23℃ and 12h/12h light/dark photoperiod is used for short day conditions. To grow *Arabidopsis* seedlings on MS medium, seeds were firstly surface-sterilized with 15% (vol/vol) bleach and washed thoroughly in sterile water for 2 times, and then germinated on the sterile ½ MS solid medium (pH 5.7) supplemented with 1% sucrose and 0.6% agar with appropriate antibiotic. Plated seedlings were grown in a growth chamber at 23℃/19℃ day/night with 16/8h light/dark photoperiod.

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* (*tga2/5*), *tga6-1* and *tga2-1 tga5-1 tga6-1* (*tga2/5/6*) mutants were reported previously (Cao et al. 1994, Ullah et al. 2003, Zhang, Yang, et al. 2010, Sun et al. 2015, Zhang et al. 2006, Zhang et al. 2003).

4.3.2 Mutant generation and genetic mapping of *npr4-4D*

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 (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 tga2/5/6* was obtained by crossing *snc2-1D npr1-1 npr4-4D* with *tga2/5/6*. *snc2-1D npr1-1 npr4-4D tga2/5* 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 the CRISPR-Cas9 construct pHEE2A-NPR1 targeting the *NPR1* locus into wild type and *npr4-4D* background.

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 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 genome sequencing.

4.3.3 Constructs and Transgenic Plants

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.

To generate constructs for promoter-luciferase assay, a 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 pCABMIA1300-35S-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.

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 1997, Zhang et al. 2006). *TGA2*, *NPR3* and *NPR4* fragments were subcloned into pBI881 or pBI880 to obtain 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 SfiI sites. The $NPR1^{R432Q}$ mutation was introduced by overlapping PCR.

To generate the NPR3-3HA and NPR4-3HA transgenic plants for ChIP assays, wild type plants were transformed with *Agrobacteria* strains carrying pCambia1300-35S-NPR3-3HA or pCambia1300-35S-NPR4-3HA.To generate constructs for co-immunoprecipitation assay, 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 region of *POB1* was amplified from genomic DNA and cloned into the same vector to obtain pBASTA-35S-POB1-FLAG-ZZ. The coding region of *Cul3A* was amplified from genomic DNA by PCR and cloned into pCambia1300-35S-3HA to obtain pCambia1300-35S-Cul3A-3HA. The constructs were transformed into *Agrobacteria* strain GV3101 and used for transient expression of the epitope-tagged proteins in *N. benthamiana*.

To generate constructs used for expressing the His_6-MBP-tagged recombinant proteins, the coding sequences of wild type and mutant *NPR1* and *NPR4* were amplified by PCR and

cloned into a modified pMAL-c2x (NEB) vector. *NPR4*^{R419Q} was amplified from the cDNA prepared from *npr4-4D* plant RNA. The NPR1^{R432Q} mutation was introduced by overlapping PCR.

To generate the *NPR1-HA* transgenic lines, coding sequence driven by its native promoter was PCR-amplified from wild-type genomic DNA and cloned into pCambia1305-3HA plasmid. The pCambia1305-NPR1^{R432Q}-HA construct was generated by site-directed mutagenesis. The resulting constructs were introduced into *npr1-1* plants by *Agrobacterium*-mediated transformation using standard protocol. The CRISPR-Cas9 construct expressing two guide RNAs targeting the *NPR1* locus (pHEE2A-NPR1) was generated by replacing the gRNA sequences in the pHEE401 vector by PCR (Wang, Xing, et al. 2015). Primers used for the cloning are reported in Table S4 (Ding et al. 2018) and all constructs were confirmed by sequencing.

4.3.4 Quantitative PCR

RNA was extracted from three independent samples 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). Expression values were normalized to the expression of *ACTIN1*. Primers for qPCR were described previously (Sun et al. 2015; Zhang et al., 2003) or reported in Table S4 (Ding et al. 2018).

4.3.5 Pathogen infection assay

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 seven days later as previously described (Bi et al. 2010). For bacterial infection, two full-grown leaves of four-week-old plants grown under short day conditions were inoculated by syringe infiltration with different *Pseudomonas* strains. Bacterial growth (Colony forming units per cm^2) was determined 3 days post inoculation, day 0 counts were analyzed in infiltrated leaves to ensure that no statistical difference was present at inoculation and that day 3 showed positive

growth. The experiments were repeated in at least three individual biological replicates, each with six technical replicates.

4.3.6 Promoter-luciferase Assay

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. A construct expressing the LexA DNA-binding domain-VP16 activation domain (LD-VP16) fusion protein was included in the transcriptional repression assays for the activation of the reporter gene. 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^{TM} Synergy^{TM} 2 Multi-Mode Microplate Reader. The ratio of firefly luciferase/renilla luciferase was used to calculate the relative luciferase activities.

4.3.7 Yeast two-hybrid assay

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.

4.3.8 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 antibody 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 TGA2 antibody was confirmed by western blot using total proteins from wild type and *tga2/5/6* 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 or H_2O one hour before crosslinking. The chromatin complexes containing NPR3-3HA or NPR4-3HA fusion protein were immunoprecipitated using an anit-HA antibody (Roche) and Protein A/G Agarose beads (GE). The immunoprecipitated DNA was analyzed by qPCR using gene specific primers which were reported in the Table S4 (Ding et al. 2018).

4.3.9 Co-immunoprecipitation

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 re-suspended 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).

4.3.10 Recombinant protein expression and purification

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, Tian, Moreau, Park, Choi, Fei, Friso, Asif, Manosalva, and von Dahl 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 a Ni-NTA column and then the column was washed three times with 10 ml of lysis buffer supplemented with imidazole (20, 30 and 40 mM). Proteins were eluted by adding lysis buffer containing 250 mM of imidazole. The eluted His_6-MBP-NPR1 and His_6-MBP-NPR1^{R432Q} proteins were dialyzed three times with PBS buffer containing 10% glycerol and 0.1% Triton X100 at 4°C. The eluted His_6-MBP-NPR3, His_6-MBP-NPR4 and His_6-MBP-NPR4^{R419Q} proteins were 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.

4.3.11 [^{3}H]SA-binding assay

Size exclusion chromatography was used for [^{3}H] SA binding assays as described previously (Manohar, Tian, Moreau, Park, Choi, Fei, Friso, Asif, Manosalva, and von Dahl 2014). Size exclusion columns were prepared by adding 0.13 g 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 Prism7.

4.3.12 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 before (0 h) 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://www.arabidopsis.org) using HISAT2 version 2.0.4 on default parameters (Kim, Langmead, and Salzberg 2015). SAMtools version 0.1.12 was used to convert SAM files, sort and index BAM files (Li, Handsaker, Wysoker, Fennell, Ruan, Homer, Marth, Abecasis, and Durbin 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, Huber, and Anders 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 (Wickham 2016).

4.3.13 Quantification and statistical analysis

Error bars in all of the figures represent a standard deviation. Number of replicates is reported in the figure legends. Statistical comparison among different samples is carried out by one-way ANOVA with Tukey's HSD (honest significant difference) test. Samples with statistically significant differences ($P< 0.05$ or $P< 0.01$ as indicated in the figure legends) are marked with different letters (a, b, c *etc*.); whereas samples with no statistically significant difference are labeled with the same letter. "ab" is used to mark samples with no statistical difference to two separate statistically different groups marked with "a" or "b".

4.4 Results

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

Arabidopsis SNC2 encodes a receptor-like protein required for basal resistance against bacterial pathogens (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 (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) (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 4.1A), 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 4.1A and B). Quantitative RT-PCR (qRT-PCR) analysis showed that constitutive expression of *PR1* and *PR2* is completely suppressed in *bda4-1D snc2-1D npr1-1* (Figure 4.1C and D). In addition, the enhanced resistance to the oomycete pathogen *Hyaloperonospora arabidopsidis* (*H.a.*) Noco2 is also suppressed in *bda4-1D snc2-1D npr1-1* (Figure 4.1E). Taken together, *bda4-1D* suppresses the constitutive defense responses in *snc2-1D npr1-1*.

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

bda4-1D was mapped to a small region on chromosome 4. A single G-to-A mutation was identified in this region, which results in an amino acid change (R419 to Q419) located in the C-terminal domain of NPR4 (Figure 4.2A). To confirm this mutation 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*. The transgenic plants displayed wild type morphology (Figure 4.2B), and constitutive expression of *PR1* and *PR2* and enhanced resistance to *H.a.* Noco2 are abolished in the transgenic lines (Figure 4.2C-E), suggesting that the mutation in NPR4 is responsible for the suppression of *snc2-1D npr1-1* mutant phenotypes. Thus we renamed *bda4-1D* as *npr4-4D*.

To determine whether *npr4-4D* is a gain-of-function or dominant-negative mutation, we transformed the *npr4-4D* mutant gene under its own promoter into *npr3-2 npr4-2*. As shown in Figure 4.2F and G, elevated *PR1* and *PR2* expression in *npr3-2 npr4-2* was suppressed in the transgenic lines, indicating that *npr4-4D* is a gain-of-function mutation.

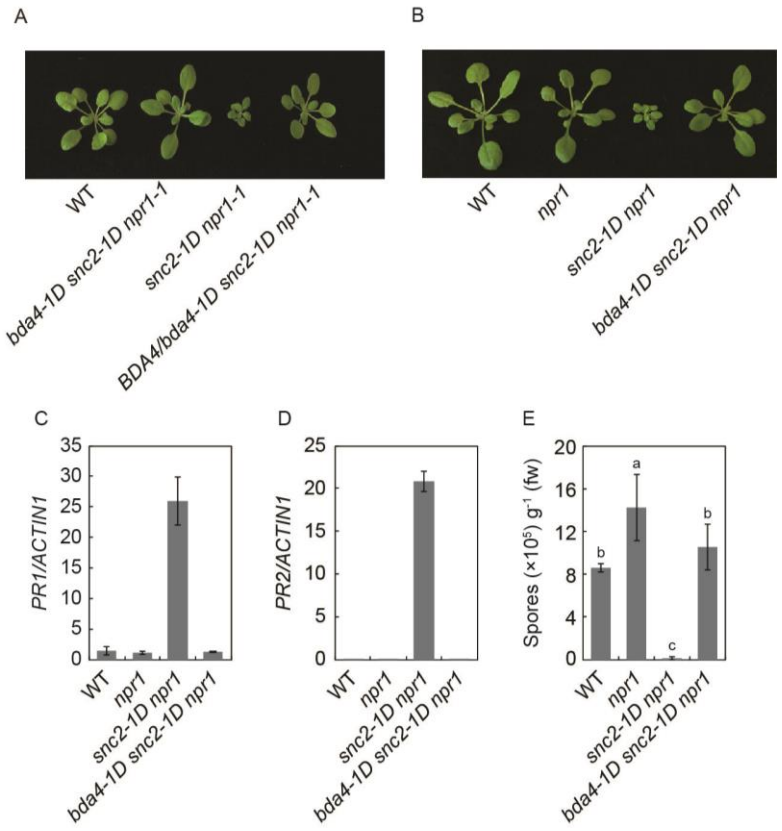

Figure 4.1 *bda4-1D* suppresses the constitutive defense responses in *snc2-1D npr1-1*.

(A-B) 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 (A) and morphology of wild type Col-0 (WT), *npr1-1*, *snc2-1D npr1-1* and *bda4-1D snc2-1D npr1-1* plants (B). Plants were grown on soil and photographed four weeks after planting.

(C-D) Expression of *PR1* (C) and *PR2* (D) in the indicated genotypes. Bars represent means ± s.d. ($n = 3$).

(E) Growth of *H.a.* Noco2 on the indicated genotypes. Different letters (a, b or c) are used to label genotypes with statistical differences ($P < 0.01$, $n = 4$).

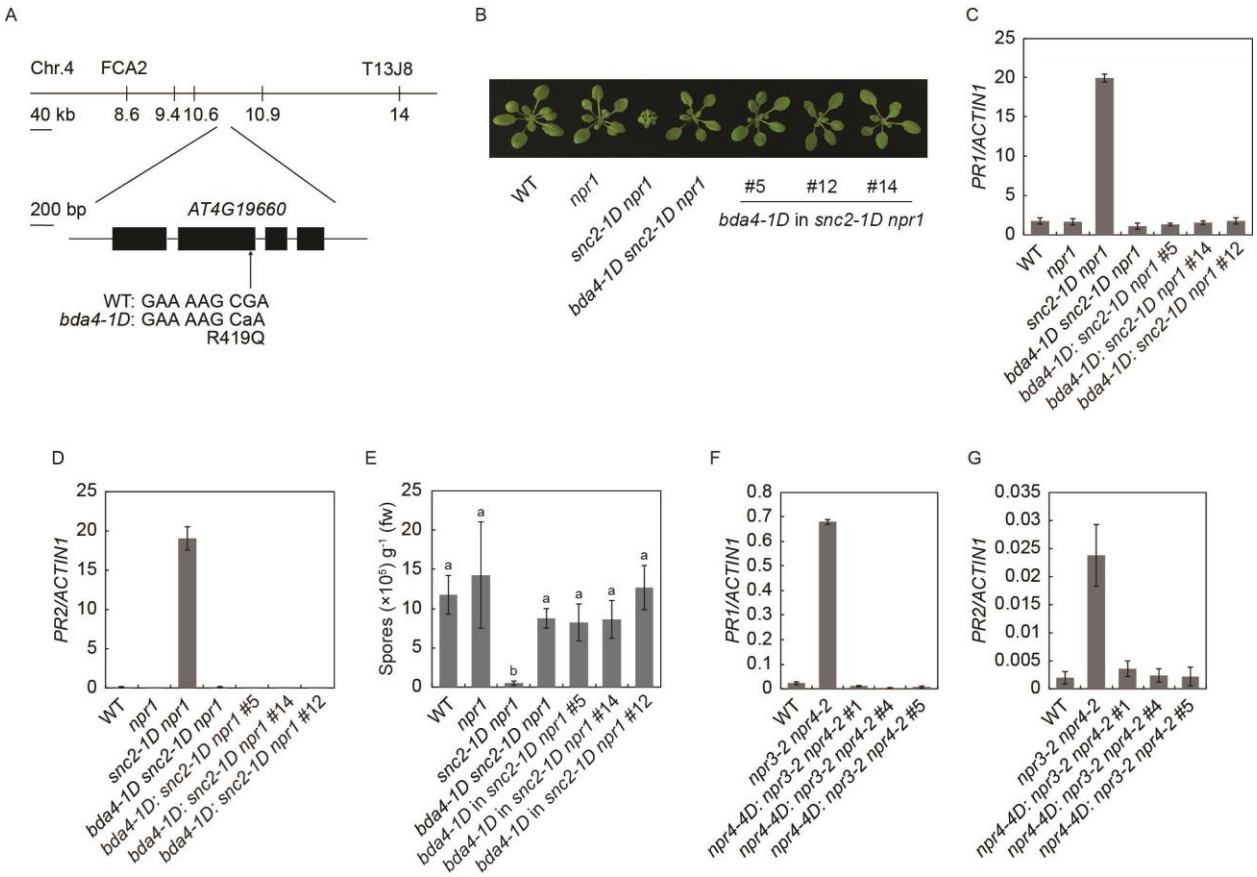

Figure 4.2 *bda4-1D* carries a gain-of-function mutation in *NPR4*.

(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* and *PR2* in the indicated genotypes. Values were normalized to the expression of *ACTIN1*. Bars represent means ± s.d. (n = 3).
(E) Growth of *H.a.* Noco2 on the indicated genotypes. Different letters (a or b) are used to label genotypes with statistical differences (P< 0.05, n = 4).
(F-G) Expression of *PR1* and *PR2* 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*. Bars represent means ± s.d. (n = 3).

The R419 residue in NPR4 is conserved in NPR1 and NPR3 as well as their homologs in other plants (Figure 4.3A). To test whether NPR3 functions similarly as NPR4, we mutated the corresponding residue R428 in NPR3 to Q428 and expressed $NPR3^{R428Q}$ in *snc2-1D npr1-1*. As shown in Figure 4.3B, the dwarf morphology of *snc2-1D npr1-1* was suppressed by $NPR3^{R428Q}$, confirming that NPR3 and NPR4 have redundant functions.

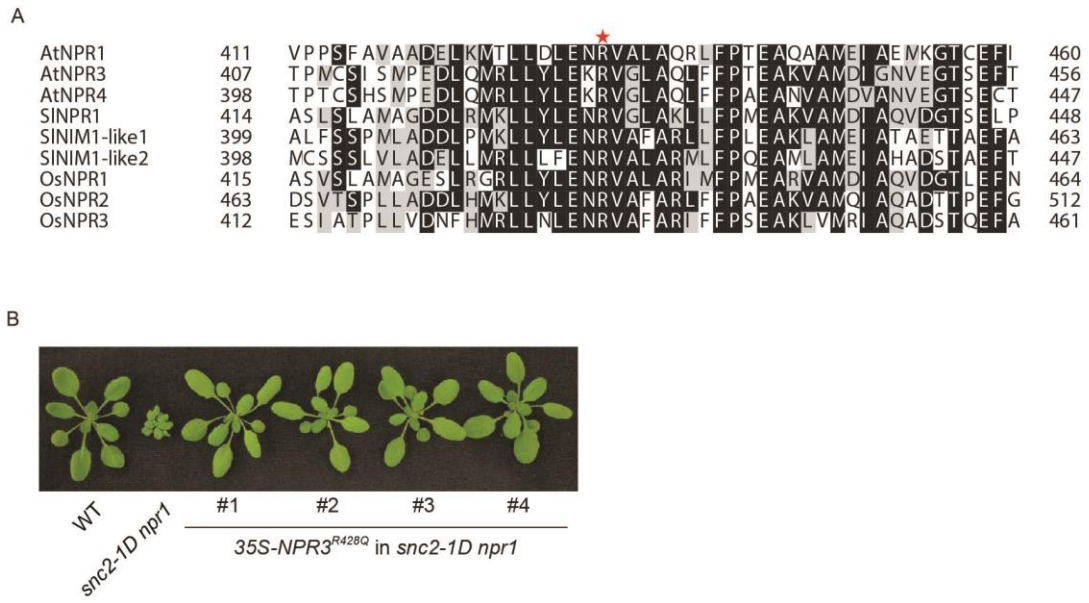

Figure 4.3 Suppression of the dwarf morphology of *snc2-1D npr1-1* by *NPR3^{R428Q}*.
(A) Alignment of the conserved C-terminal regions of NPR1/NPR3/NPR4. At: *Arabidopsis thaliana;* Sl: Tomato, *Solanum lycopersicum*; Os: Rice, *Oryza sativa*. * indicates the mutation site in *npr4-4D*.
(B) Morphology of four-week-old soil-grown wild type (WT), *snc2-1D npr1-1* and transgenic lines expressing the 35S: *NPR3^{R428Q}* in the *snc2-1D npr1-1* background.

4.4.3 *npr4-4D* suppresses the expression of *SARD1*, *CBP60g* and *WRKY70* and results in compromised basal defense

Several transcription factors including SARD1, CBP60g and WRKY70 are required for the autoimmunity of *snc2-1D npr1-1* (Sun et al. 2015, Zhang, Yang, et al. 2010). Their expression is much higher in *snc2-1D npr1-1* than in *npr1-1*, but the increased expression is completely blocked by *npr4-4D* (Figure 4.4A-C). Similarly, their induction by the type III secretion deficient bacteria *Pseudomonas syringae* pv. *tomato (P.s.t.)* DC3000 *hrcC*$^-$ and the virulent bacteria *Pseudomonas syringae* pv. *maculicola* (*P.s.m.*) ES4326 is also greatly reduced in *npr4-4D* (Figure 4.4D-I). These data suggest that NPR4 negatively regulates the expression of *SARD1*, *CBP60g* and *WRKY70*.

We further 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 4.4J). When challenged with *P.s.m.* ES4326, *npr4-4D*

plants also supported significantly higher growth of the pathogen than the wild type (Figure 4.4K), suggesting that *npr4-4D* supresses basal resistance.

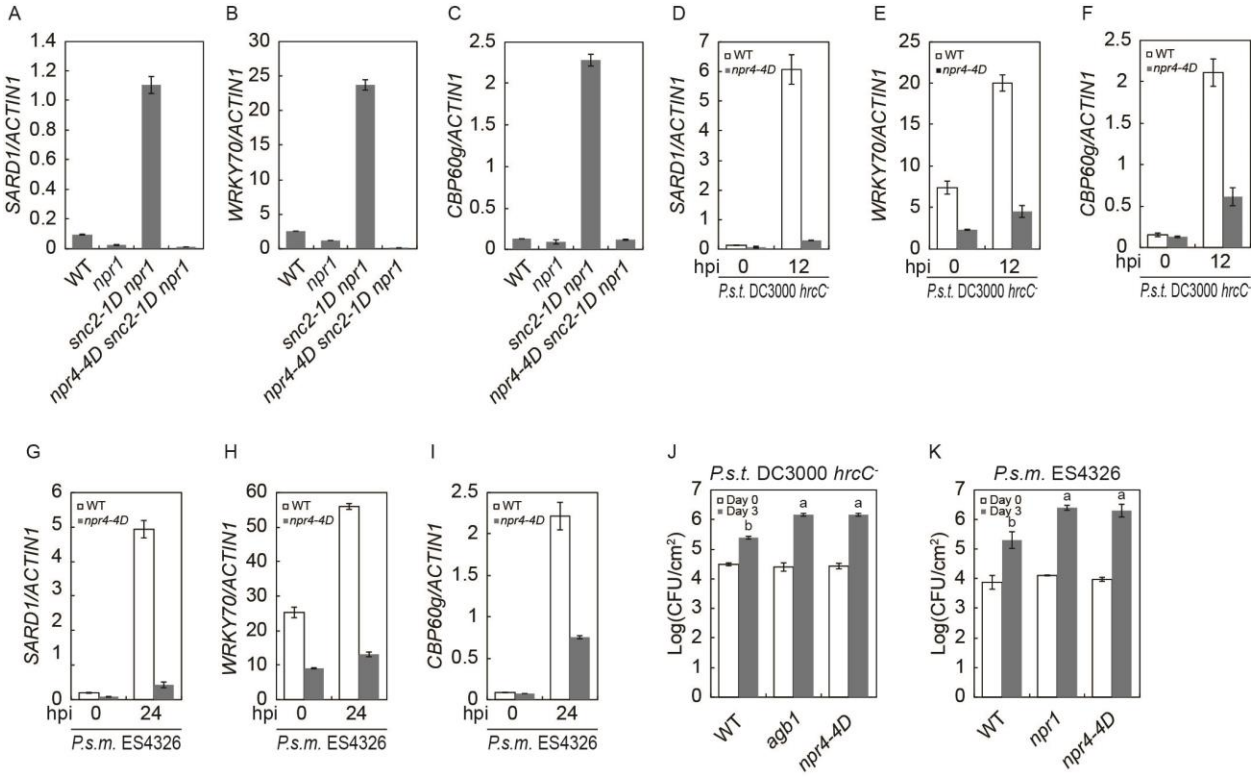

Figure 4.4 *npr4-4D* suppresses defense gene expression and disease resistance.

(A-C) Expression of *SARD1* (A), *WRKY70* (B) and *CBP60g* (C) in the indicated genotypes. Bars represent means ± s.d. (n = 3).

(D-F) Induction of *SARD1* (D), *WRKY70* (E) and *CBP60g* (F) by *P.s.t.* DC3000 *hrcC*⁻ in wild type (WT) and *npr4-4D*. Three-week-old plants were infiltrated with *P.s.t.* DC3000 *hrcC*⁻ (OD_{600} = 0.05). hpi: hours post inoculation. Bars represent means ± s.d. (n = 3).

(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*. Bars represent means ± s.d. (n = 3).

(J) Growth of *P.s.t.* DC3000 *hrcC*⁻ on WT, *agb1-2*, and *npr4-4D*. Four-week-old plants were infiltrated with *P.s.t.* DC3000 *hrcC*⁻ (OD_{600} = 0.002). cfu, Colony-forming units. Different letters (a or b) are used to label genotypes with statistical differences (P< 0.01, n = 6).

(K) Growth of *P.s.m.* ES4326 on WT, *npr1-1* and *npr4-4D*. Four-week-old plants were infiltrated with *P.s.m.* ES4326 (OD_{600} = 0.0002). Different letters (a or b) are used to label genotypes with statistical differences (P< 0.01, n = 6).

4.4.4 Loss of 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. In *npr3-2 npr4-2*, *SARD1* and *WRKY70* expression is dramatically elevated (Figure 4.5A and B), but *CBP60g* expression is only modestly increased (Figure 4.5C). Similarly, *npr3-1 npr4-3* also exhibits elevated basal *SARD1* and *WRKY70* expression (Figure 4.5D and E). These data suggest that NPR3 and NPR4 negatively regulate the expression of *SARD1* and *WRKY70*.

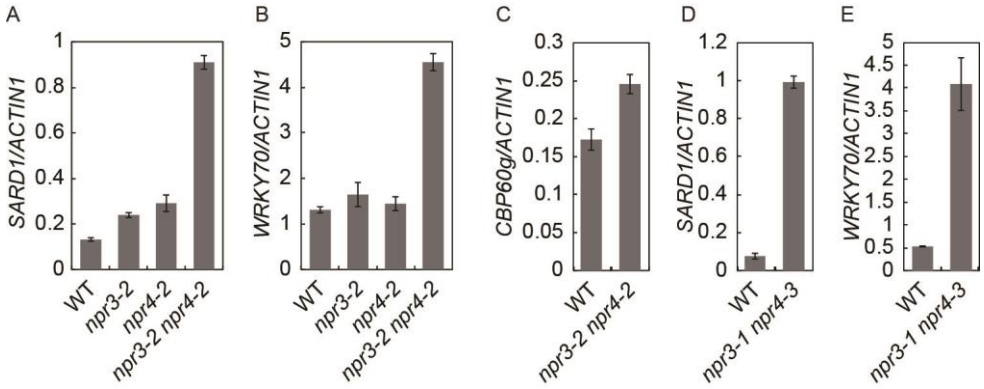

Figure 4.5 Elevated expression of *SARD1* and *WRKY70* in *npr3 npr4*.

(A-B) Expression levels of *SARD1* (A) and *WRKY70* (B) in wild type (WT), *npr3-2*, *npr4-2* and *npr3-2 npr4-2*.
(C) Expression levels of *CBP60g* in WT and *npr3-2 npr4-2*.
(D-E) Expression levels of *SARD1* (D) and *WRKY70* (E) in WT and *npr3-1 npr4-3*.
(A-E)Values were normalized to the expression of *ACTIN1*. Bars represent means ± s.d. (n = 3).

4.4.5 NPR3 and NPR4 function as transcriptional co-repressors regulating the expression of *SARD1* and *WRKY70*

To test whether NPR3/NPR4 serve as transcriptional co-repressors regulating *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 4.6A, 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. These data suggest that overexpression of

NPR3 or *NPR4* in *Arabidopsis* protoplasts represses the expression of *SARD1* and *WRKY70*, and they are likely to function as transcriptional co-repressors.

At the C-terminus of NPR3 and NPR4 but not NPR1, there is a conserved motif (VDLNETP) with 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 acids "DLN" in NPR4 to "GVK", the corresponding amino acids in NPR1. The NPR4^{GVK} mutant protein can still interact with TGA2 in the yeast two-hybrid assay (Figure 4.6B), but it no longer represses the expression of *SARD1* and *WRKY70* (Figure 4.6C and D).

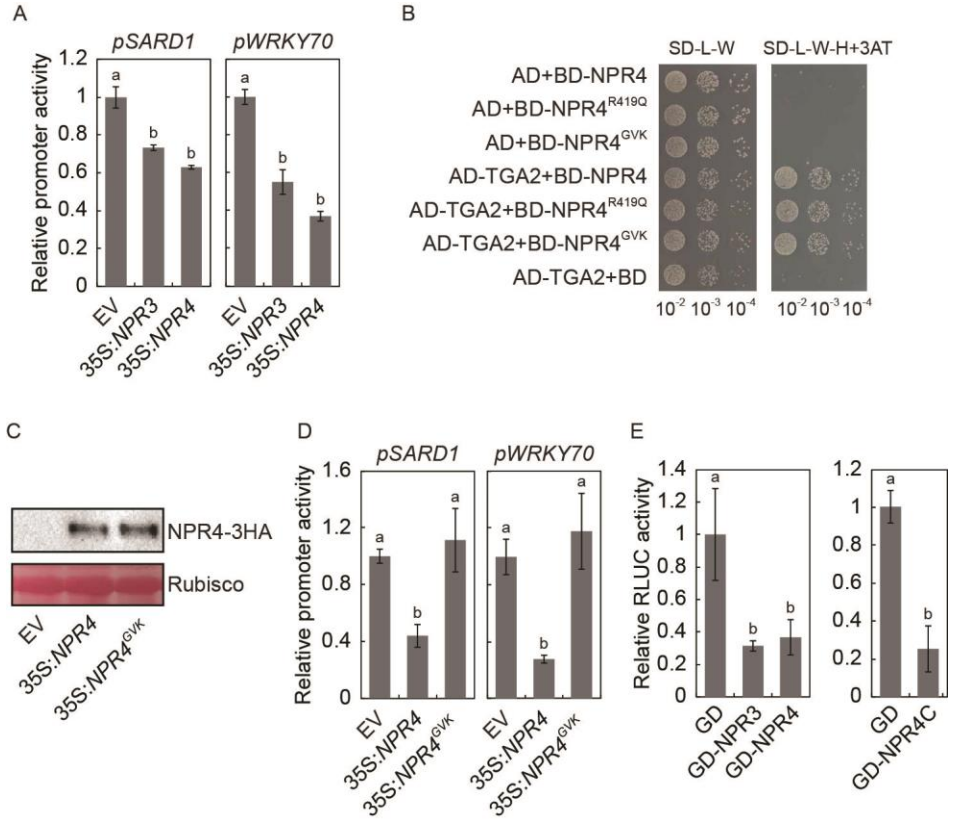

Figure 4.6 NPR3 and NPR4 act as transcriptional repressors.

(A) Firefly luciferase activities in *Arabidopsis* protoplasts co-transformed with the indicated constructs.

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

(C) Expression of NPR4-3HA and NPR4^{GVK}-3HA proteins in *Arabidopsis* protoplasts transformed with empty vector (EV), 35S:NPR4-3HA or 35S:NPR4 ^{GVK}-3HA constructs. Cells were harvested after overnight incubation. Western blot analysis was carried out on the total protein extracts using an anti-HA antibody.
(D) Firefly luciferase activities in *Arabidopsis* protoplasts co-transformed with the indicated constructs.
(E) Relative Renilla luciferase activities in *Arabidopsis* protoplasts co-transformed with a Renilla luciferase reporter gene driven by a promoter containing 2 x LexA DNA-binding sites and 2 x Gal4 DNA-binding sites, a construct expressing LD-VP16 (LexA DNA binding domain fused with VP16 transcriptional activation domain) and constructs expressing GAL4 DNA-binding domain (GD), GD-NPR3, GD-NPR4 or GD fused with the C terminal domain of NPR4 (GD-NPR4C).
For (A, D and E), a Renilla luciferase reporter (in A and D) or firefly luciferase reporter (in E) under the control the promoter of *UBQ1* was included as the internal transfection control. Values were compared with the EV or GD control, which was set as 1. Different letters (a or b) are used to label samples with statistical differences ($P< 0.01$, $n = 3$).

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). Transformation of these constructs with a construct expressing LD-VP16 (LexA DNA binding domain fused with VP16 transcriptional activation domain) and a Renilla luciferase reporter gene driven by a promoter containing 2 x LexA DNA-binding sites and 2×Gal4 DNA-binding sites in protoplasts resulted in suppression of the reporter gene (Figure 4.6E), confirming that NPR3/NPR4 function as transcriptional co-repressors. Co-expression of GD fused with the NPR4 C-terminal domain (NPR4C) with the Renilla luciferase reporter gene also results in suppression of the reporter gene (Figure 4.6E), suggesting that the C-terminal domain of NPR4 serves as a transcriptional repression domain.

4.4.6 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* reporter genes (Figure 4.7A). As shown in Figure 4.7B, overexpression of *NPR4* in protoplasts does not lead to repression of the

mutant *pSARD1::Luc* and *pWRKY70::Luc* reporter genes, suggesting that TGA factors are likely required for the repression of *SARD1* and *WRKY70*.

To test whether TGA2/TGA5/TGA6 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 4.7C, *SARD1* and *WRKY70* have much higher expression in *tga256* than in wild type, suggesting that TGA2/TGA5/TGA6 are involved in negative regulation of basal expression of *SARD1* and *WRKY70*. To determine whether *SARD1* and *WRKY70* are direct targets of the TGA factors, ChIP-qPCR experiments were carried out on wild type and *tga256* plants using anti-TGA2 antibodies (Figure 4.7D). As shown in Figure 4.7E, 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 *tga256* , suggesting that *SARD1* and *WRKY70* are both direct targets of TGA2.

Since NPR3/NPR4 and TGA2/TGA5/TGA6 interact with each other, 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 defense responses in *snc2-1D npr1-1* by *npr4-4D* requires TGA transcription factors. As shown in Figure 4.7F, the sextuple mutant *npr4-4D snc2-1D npr1-1 tga256* displayed dwarf morphology similar to *snc2-1D npr1-1*. Constitutive expression of *SARD1* and *WRKY70* is also restored in the sextuple mutant (Figure 4.7G). Furthermore, overexpression of *NPR4* reduces the expression of *pSARD1::Luc* and *pWRKY70::Luc* reporter genes in wild type, but not the *tga256* mutant protoplasts (Figure 4.7H). These data suggest that NPR3/NPR4 work together with TGA2/TGA5/TGA6 to repress the expression of *SARD1* and *WRKY70*.

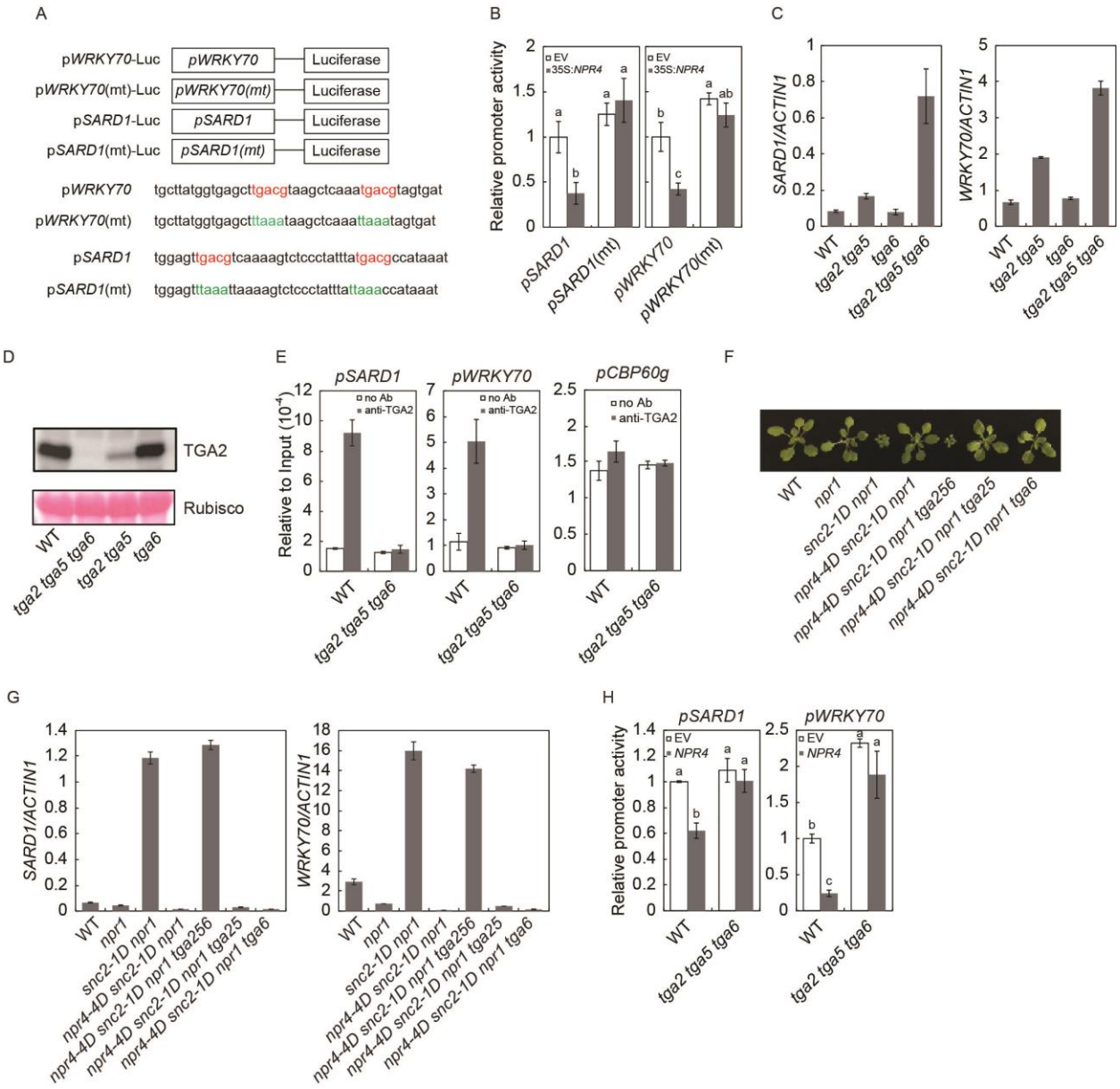

Figure 4.7 NPR4 functions together with TGA2/TGA5/TGA6 to repress the expression of *SARD1* and *WRKY70*.

(A) Reporter constructs used in the promoter activity assay. The sequence of *SARD1* or *WRKY70* promoter regions harboring the TGACG motifs is shown and the original TGACG motif sequence and ttaaa mutant sequences are colored.

(B) Firefly luciferase activities in *Arabidopsis* protoplasts transformed with empty vector (EV) or *35S:NPR4* constructs together with a luciferase reporter driven by wild type or mutant *SARD1/WRKY70* promoters with mutations in the "TGACG" motifs. Different letters (a, b or c) are used to label samples with statistical differences and "ab" is used to label the sample with no statistical difference with samples labeled with "a" or "b" ($P < 0.01$, $n = 3$).

(C) Expression levels of *SARD1* and *WRKY70* in wild type (WT), *tga2-1 tga5-1*, *tga6-1* and *tga2-1 tga5-1 tga6-1*. Bars represent means ± s.d. ($n = 3$).

(D) 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.
(E) Binding of TGA2 to promoter regions of *SARD1*, *WRKY70* and *CBP60g*. TGA2 chromatin complexes were immunoprecipitated with anti-TGA2 antibodies and protein A-agarose beads. 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).
(F) Morphology 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*. Plants were grown on soil and photographed four weeks after planting.
(G) Expression levels of *SARD1* and *WRKY70* in plants of the indicated genotypes. Values were normalized to the expression of *ACTIN1*. Bars represent means ± s.d. (n = 3).
(H) Firefly luciferase activities in *Arabidopsis* wild type (WT) and *tga2-1 tga5-1 tga6-1* protoplasts transformed with empty vector (EV) or *35S:NPR4* constructs together with the indicated reporter constructs. Different letters (a, b or c) are used to label samples with statistical differences (P< 0.01, n = 3).
For (B and H), a Renilla luciferase reporter under the control the promoter of *UBQ1* was included as the internal transfection control. The values were compared with empty vector controls, which were set as 1.

4.4.7 SA inhibits the transcriptional repression activity of NPR4

Following SA treatment, *SARD1* and *WRKY70* are rapidly induced in wild type, but the induction is greatly reduced in *npr4-4D* (Figure 4.8A). Since SA can bind to NPR4, we tested whether the transcriptional repression activity of NPR4 is affected by SA. We treated wild type protoplasts co-transformed with *35S:NPR4* and *pSARD1::Luc* or *pWRKY70::Luc* constructs with SA and examined the expression of luciferase. As shown in Figure 4.8B, overexpression of *NPR4* represses the expression of the 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 4.8C and D, NPR4-3HA was recruited to the promoters of *SARD1* and *WRKY70* but not *CBP60g*, and treatment of SA did not affect the association of NPR4-3HA with *SARD1* and *WRKY70* promoters. Similarly, NPR3-3HA was also recruited to the promoters of *SARD1* and *WRKY70* and the interactions between NPR3-3HA and the promoters were not

affected by SA treatment (Figure 4.8E). Consistent with the ChIP-qPCR experiments, SA does not disrupt the interactions between NPR3/NPR4 and TGA2 in the yeast two-hybrid assay (Figure 4.8F). 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 4.8G), 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 *npr4-4D*. Wild type and *npr4-4D* seedlings pre-treated with the SA analog INA were challenged with *H.a.* Noco2. As shown in Figure 4.8H, exogenous application of INA renders the wild type plants resistant to the pathogen. Like in *npr1-1*, the INA-induced resistance 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* and purified the recombinant proteins for SA binding assays. The His_6-MBP tag was used because the GST-NPR3 and GST-NPR4 fusion proteins did not express well under our experimental conditions. As shown in Figure 4.8I and J, 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 still interacts with TGA2 (Figure 4.6B) and forms homodimers (Figure 4.8K). However, it has very low affinity for [^{3}H]-SA (Figure 4.8J and L), exhibiting an estimated K_d of about 250-fold higher than the wild type protein, suggesting that the R419 residue in NPR4 is essential for its SA-binding activity.

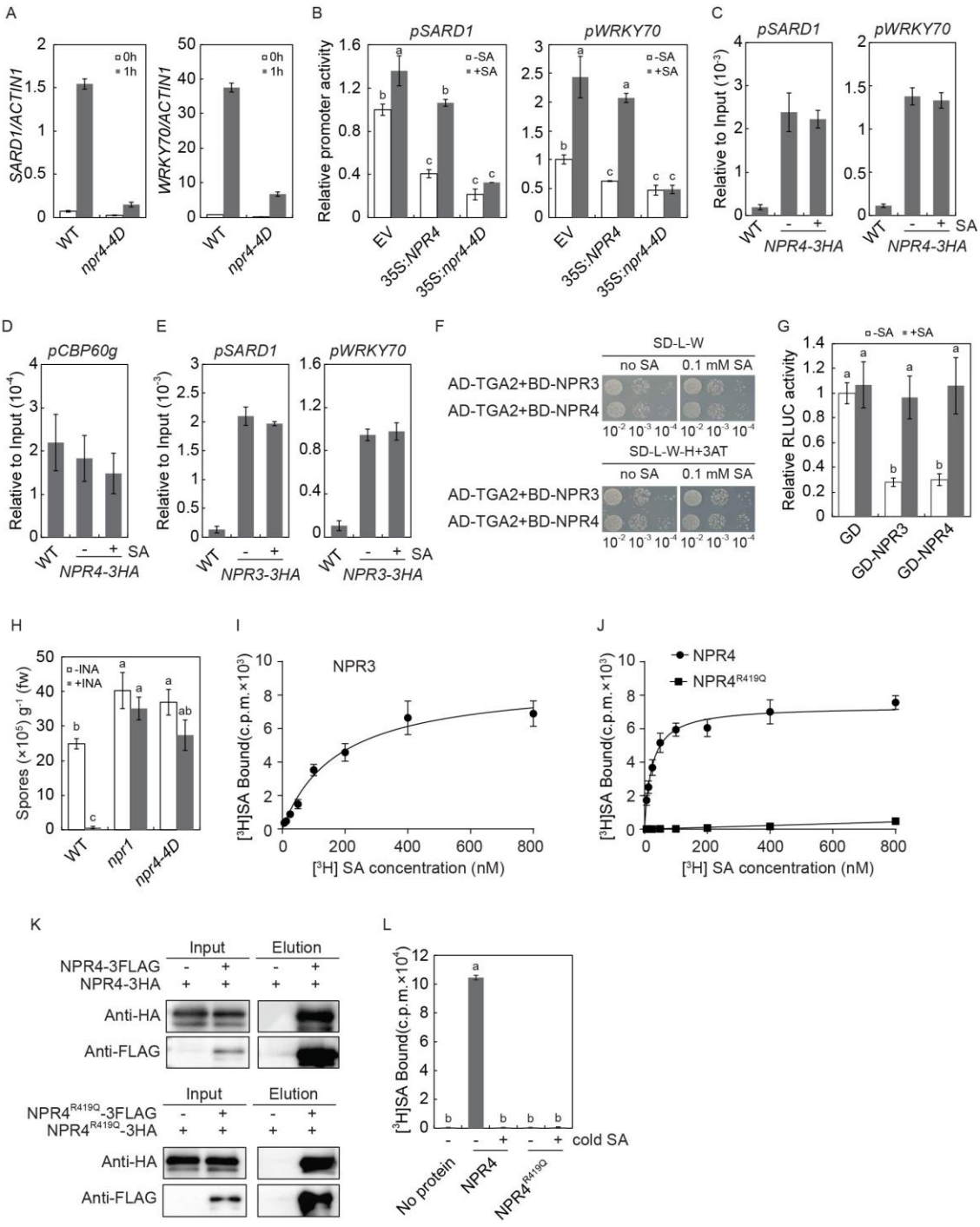

Figure 4.8 SA inhibits the transcriptional repression activity of NPR4 and the *npr4-4D* mutation abolishes SA-binding and renders SA insensitivity.

(A) Induction of *SARD1* and *WRKY70* by SA in wild type (WT) and *npr4-4D*. Two-week-old seedlings were sprayed with 0.2 mM SA. Samples were collected at 0 and 1 h after treatment. Bars represent means ± s.d. (n = 3).

(B) Firefly luciferase activities in *Arabidopsis* protoplasts co-transformed with the indicated constructs together with the *pSARD1::Luc* or *pWRKY70::Luc* 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 empty vector control, which was set as 1. Different letters (a, b or c) are used to label samples with statistical differences ($P < 0.01$, $n = 3$).
(C-D) ChIP-qPCR analysis of the effect of SA on the binding of NPR4-3HA to the promoter regions of *SARD1*, *WRKY70* and *CBP60g*. 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. The immunoprecipitated DNA was quantified by qPCR. ChIP-PCR results are presented as 10^{-3} (C) or 10^{-4} (D) of signal relative to input. Bars represent means ± s.d. ($n = 3$).
(E) Chromatin immunoprecipitation-PCR analysis of the effect of SA on binding of NPR3-3HA to the promoter regions of *SARD1* and *WRKY70*. 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. ($n = 3$).
(F) 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 dropout media without Leu and Trp (SD-L-W) plate or synthetic dropout media without Leu, Trp and His (SD-L-W-H) plus 4 mM 3-aminotriazole (3AT).
(G) Relative Renilla luciferase activities in *Arabidopsis* protoplasts co-transformed with a Renilla reporter gene (same as figure 4.6E), a construct expressing LD-VP16 and constructs expressing GAL4 DNA-binding domain (GD), GD-NPR3 or GD-NPR4. 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. Different letters (a or b) are used to label samples with statistical differences ($P < 0.01$, $n = 3$).
(H) Growth of *H.a.* Noco2 on the indicated genotypes with or without INA treatment. Different letters (a, b, c) are used to label samples with statistical differences and "ab" is used to label the sample with no statistical difference with samples labeled with "a" or "b" ($P < 0.01$, $n = 4$).
(I) Saturation SA-binding assay of NPR3. 1.5 μg of His_6-MBP-NPR3 protein was incubated with [^{3}H] SA at different concentrations. Three replicates in a single experiment were used to calculate the K_d of NPR3 (176.7 ±28.31 nM). Bars represent means ± s.d. ($n = 3$). CPM, count per minute.
(J) Saturation SA-binding assay of NPR4 and NPR4^{R419Q}. 1.5 μg of His_6-MBP-NPR4 or His_6-MBP-NPR4^{R419Q} protein was incubated with [^{3}H] SA at different concentrations. Three replicates in a single experiment were used to calculate the K_d for NPR4 (23.54 ± 2.74 nM). Bars represent means ± s.d. (n = 3). See also Fig. S4.
(K) 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.
(L) 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-MBP-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. Different letters (a or b) are used to label samples with statistical differences ($P< 0.01$, $n = 4$). CPM, count per minute.

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

Since the R419 residue in NPR4 is conserved in NPR1 (Figure 4.3A), we tested whether the corresponding R432 in the C-terminal domain of NPR1 is also required for binding SA. We expressed His_6-MBP-tagged NPR1 and NPR1^{R432Q} proteins and purified them for SA-binding assays. As shown in Figure 4.9A, 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, with a K_d estimated to be about 50-fold higher than the wild type protein, suggesting that R432 plays an important role in SA binding. Further analysis showed that NPR1^{R432Q} can still interact with TGA2 and NIMIN1 in yeast two-hybrid assays (Figure 4.9B).

NPR1 is partially required for the induction of *SARD1* and *WRKY70* by SA (Figure 4.9C). To determine whether the NPR1^{R432Q} mutation affects the induction of *SARD1* and *WRKY70*, we made transgenic lines expressing HA-tagged NPR1 or NPR1^{R432Q} in the *npr1-1* background (Figure 4.9D). Following SA treatment, plants expressing NPR1-HA in the *npr1-1* background showed similar expression levels of *SARD1* and *WRKY70* as wild type (Figure 4.9E and F). In addition, INA-induced resistance to *H.a.* Noco2 was also restored in the NPR1-HA transgenic lines (Figure 4.9G). In contrast, in the transgenic lines expressing NPR1^{R432Q}-HA, the expression levels of *SARD1* and *WRKY70* after SA treatment are similar as in *npr1-1* and INA-induced resistance to *H.a.* Noco2 was not restored either (Figure 4.9E-G), suggesting that *NPR1^{R432Q}* cannot complement the *npr1-1* mutant phenotype.

We further tested whether the NPR1^{R432Q} mutation affects SA-induced *pSARD1::Luc* reporter expression. When a construct expressing wild type *NPR1* was co-transformed with *pSARD1::Luc* into *npr1-1* protoplasts, SA treatment induces the expression of luciferase (Figure 4.9H). In contrast, when the NPR1^{R432Q} construct was co-transformed with the reporter gene 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 *pSARD1::Luc* reporter gene with mutations in the "TGACG" motifs (Figure 4.9I), suggesting that the induction of *pSARD1::Luc* expression by SA is dependent on the "TGACG" motifs.

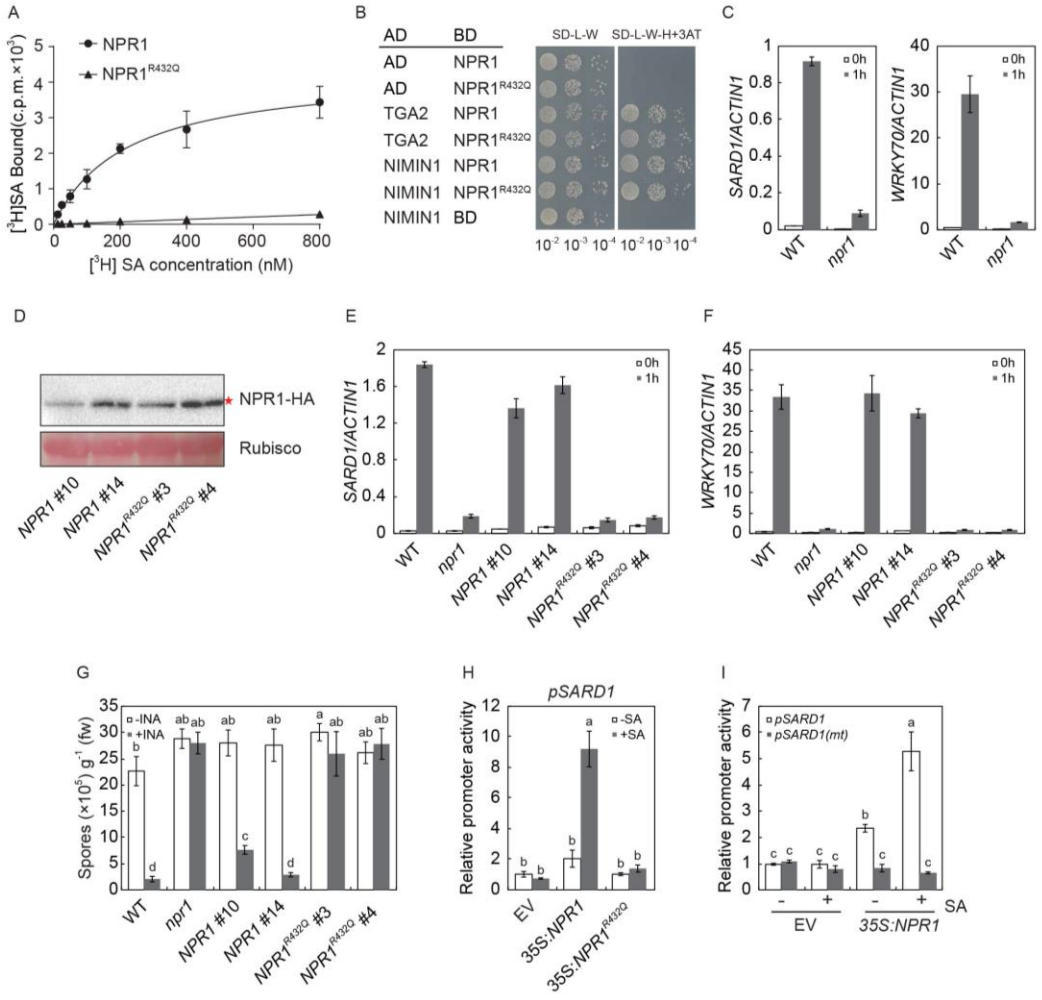

Figure 4.9 NPR1 promotes the expression of *SARD1* and *WRKY70* upon SA induction.

(A) Saturation binding assay of NPR1 and NPR1^{R432Q}. 5 μg of His$_6$-MBP-NPR1 or His$_6$-MBP-NPR1^{R432Q} protein was incubated with [^{3}H] SA at different concentrations. Three replicates in a single experiment were used to calculate the K_d of NPR1 (221.3 ±38.85 nM). 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 was plated on synthetic dropout media without Leu and Trp (SD-L-W) plate or synthetic dropout media without Leu, Trp and His (SD-L-W-H) plus 4 mM 3-aminotriazole (3AT).

(C) Induction of *SARD1* and *WRKY70* expression by SA in wild type and *npr1-1*. Bars represent means ± s.d. (n = 3).

(D) NPR1-HA and NPR1^{R432Q}-HA protein levels in transgenic lines in the *npr1-1* background.

(E-F) Induction of *SARD1* (E) and *WRKY70* (F) by SA in WT, *npr1-1* and the *NPR1-HA* or *NPR1*^{R432Q}*-HA* transgenic lines in the *npr1-1* background. Bars represent means ± s.d. (n = 3).
(G) Growth of *H.a.* Noco2 on the indicated genotypes. Different letters (a, b, c, or d) are used to label samples with statistical differences and "ab" is used to label samples with no statistical difference with samples labeled with "a" or "b" (P< 0.01, n = 4).
(H) Luciferase activities in *npr1-1* protoplasts co-transformed with the indicated constructs together with the *pSARD1-LUC* reporter gene. Different letters (a or b) are used to label samples with statistical differences (P< 0.05, n = 3).
(I) Luciferase activities in *npr1-1* protoplasts co-transformed with the indicated constructs together with the wild type (*pSARD1*) or mutant *pSARD1-LUC* [*pSARD1(mt)*] reporter gene containing mutations in the TGACG motifs. Different letters (a, b or c) are used to label samples with statistical differences (P< 0.01, n = 3).
For (C, E and F), two-week-old seedlings were sprayed with 0.2 mM SA. Samples were collected 0 and 1 h after treatment. For (H-I), Samples were collected three hours after 0.2 mM SA treatment. The values were compared with empty vector controls, which were set as 1.

4.4.9 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 4.10A). 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 4.10B and C).

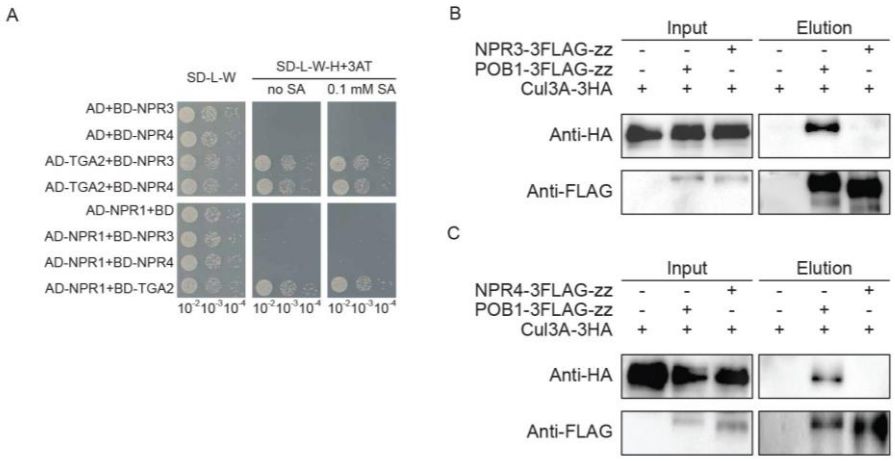

Figure 4.10 Analysis of interactions between NPR3/NPR4 and NPR1 or Cul3A.

(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 dropout media without Leu and Trp (SD-L-W)

plate or synthetic dropout 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.

To further determine the relationship between NPR3/NPR4 and NPR1, we analyzed the expression of *SARD1* and *WRKY70* in *npr1-1 npr3-2 npr4-2*. As shown in Figure 4.11A, the 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 not dependent on NPR1. In addition, NPR4 can still repress the expression of the *pSARD1::Luc* and *pWRKY70::Luc* reporter genes in *npr1-1* protoplasts (Figure 4.11B), suggesting that NPR4 regulates *SARD1* and *WRKY70* independent of NPR1.

To test whether NPR1 and NPR4 function in parallel in SA-induced gene expression, we compared the induction of *SARD1* by SA in *npr1-1*, *npr4-4D* and *npr1-1 npr4-4D*. As shown in Figure 4.11C, induction of *SARD1* by SA is reduced in *npr4-4D* and *npr1-1*, and 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 *PR2* by *P.s.m.* ES4326 also showed that their induction is only partially affected in *npr1-1* and *npr4-4D*, but completely blocked in *npr1-1 npr4-4D* (Figure 4.11D).

Next we analyzed the contribution of *npr1-1* and *npr4-4D* to the suppression of *snc2-1D*. As shown in Figure 4.11E, *snc2-1D npr1-1* and *snc2-1D npr4-4D* plants are only slightly bigger than *snc2-1D*, but *snc2-1D npr1-1 npr4-4D* 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 4.11F). 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 4.11G). These data suggest that *npr4-4D* and *npr1-1* have additive effects on the suppression of the autoimmune phenotype of *snc2-1D*.

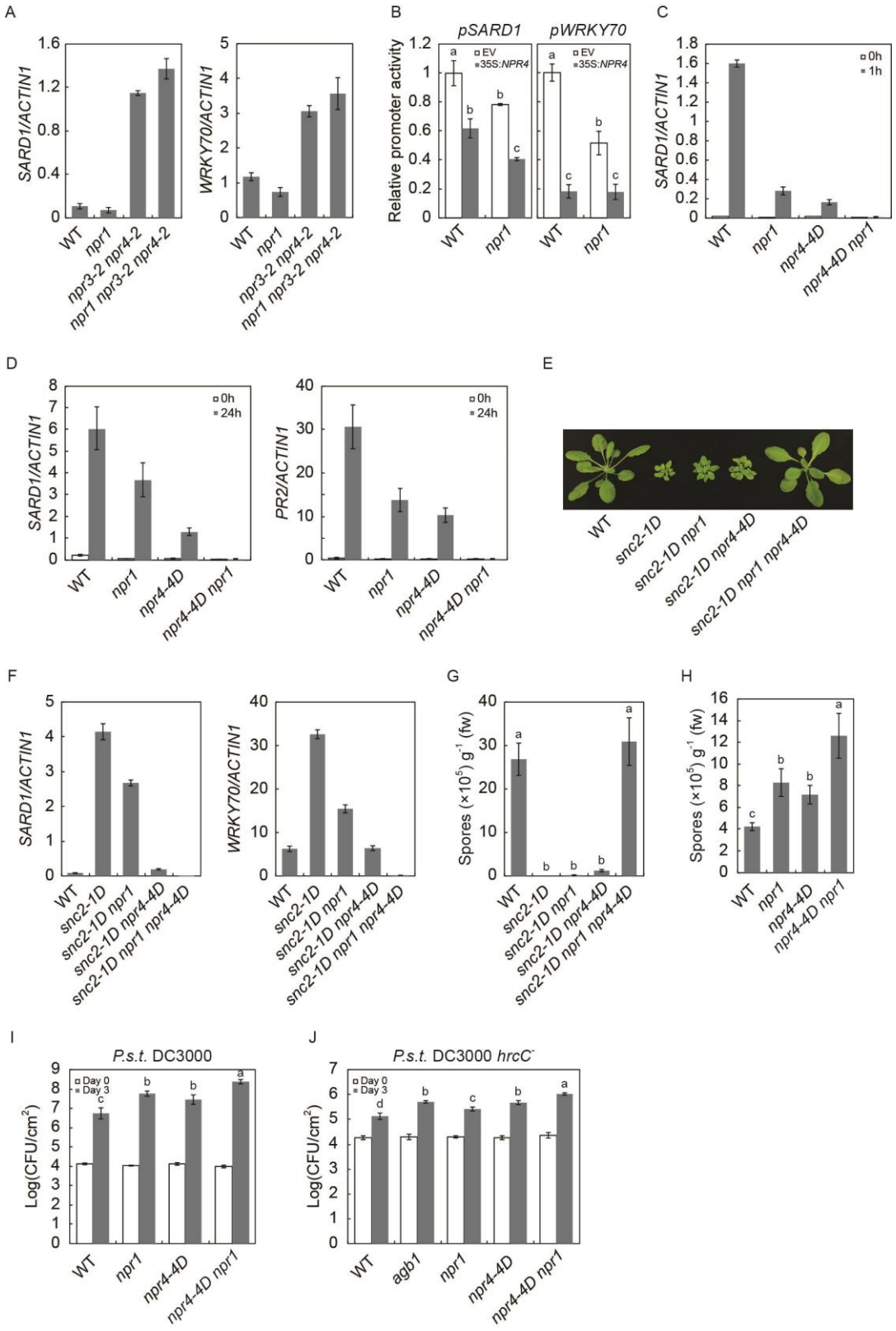

Figure 4.11 NPR3 and NPR4 function independently of NPR1.

(A) Expression levels of *SARD1* and *WRKY70* in the indicated genotypes. Bars represent means ± s.d. (n = 3).
(B) Luciferase activities in wild type (WT) and *npr1-1* protoplasts transformed with empty vector (EV) or *35S:NPR4* effector constructs, together with the indicated reporter gene. Different letters (a, b or c) are used to label samples with statistical differences (P< 0.01, n = 3).
(C) Induction of *SARD1* by SA in the indicated genotypes. Two-week-old seedlings were sprayed with 0.2 mM SA. Samples were collected 0 and 1 h after treatment. Bars represent means ± s.d. (n = 3).
(D) Induction of *SARD1* and *PR2* by *P.s.m.* ES4326 in the indicated genotypes. Leaves of three-week-old plants were infiltrated with *P.s.m.* ES4326 (OD_{600} = 0.001). Samples were collected at 0 and 24 h. Bars represent means ± s.d. (n = 3).
(E) Morphology of plants of the indicated genotypes. The picture was photographed four weeks after planting.
(F) Expression of *SARD1* and *WRKY70* in the indicated genotypes. Bars represent means ± s.d. (n = 3).
(G-H) 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 in (G) and 1×10^4 spores/ml in (H)]. Different letters (a, b, or c) are used to label genotypes with statistical differences (P< 0.01, n = 4).
(I-J) Growth of *P.s.t.* DC3000 (I) or *P.s.t.* DC3000 *hrcC*$^-$ (J) 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). Different letters (a, b, c, or d) are used to label genotypes with statistical differences (P< 0.01, n = 6).

We further tested the effects of *npr1-1* and *npr4-4D* on basal resistance against pathogens. As shown in Figure 4.11H and I, *npr1-1* and *npr4-4D* supported significantly higher growth of *H.a.* Noco2 and *P.s.t.* DC3000, and *npr1-1 npr4-4D* supported even higher growth of these two pathogens. When *npr1-1*, *npr4-4D* and *npr1-1 npr4-4D* were challenged with *P.s.t.* DC3000 *hrcC*, growth of the bacteria was also significantly higher in the single mutants and further increased in the double mutant (Figure 4.11J). All these data indicate that NPR1 and NPR3/NPR4 function separately.

4.4.10 Opposite roles of NPR1 and NPR4 in SA-induced early defense gene expression

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*. Two-week-old seedlings were treated with SA for one hour prior to sample collection. In 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 (Table S1 in Ding *et al*. 2018) and

912 repressed genes (Table S2 in Ding *et al*. 2018). Gene ontology enrichment analysis showed that genes involved in defense responses were highly enriched among SA-induced genes (Figure 4.12A). Consistent with the involvement of TGA transcription factors in SA-induced defense gene expression, the preferred TGA2-binding sequence "TGACGT" 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 (Table 4.1). 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 4.12A).

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 4.12B), which is not surprising considering that regulation of defense 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. Additional RNA-seq analysis on *npr1-1 npr4-4D* revealed that 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 *npr1-1* 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 4.12C-G, 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*. 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 4.13A-D). Together these data support that NPR1 and NPR4 act independently in the regulation of SA-induced gene expression.

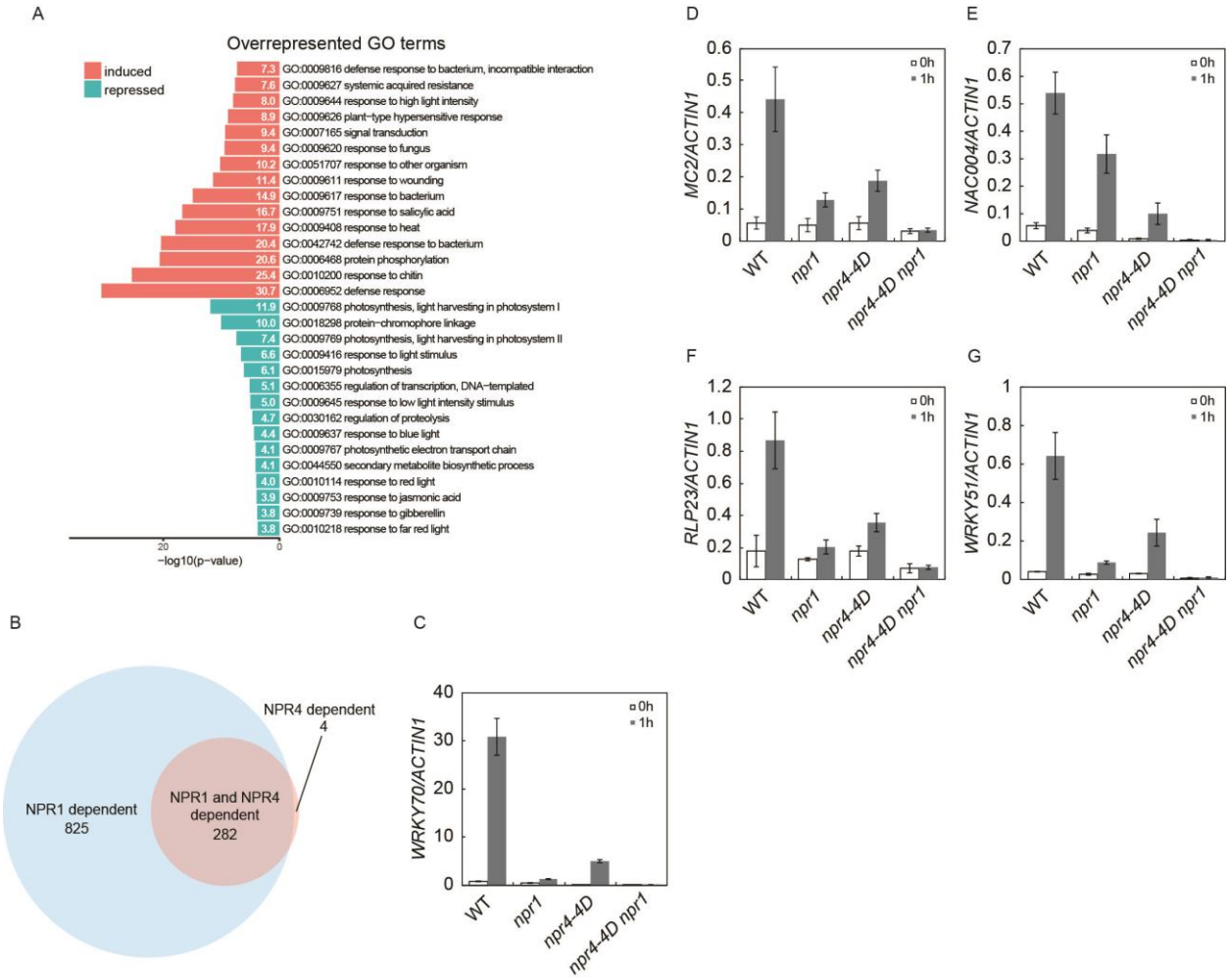

Figure 4.12 Opposite roles of NPR1 and NPR4 in early defense gene expression in response to SA.

(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) 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).

(C-G) Induction of *WRKY70* (B), *MC2* (C), *NAC004* (D), *RLP23* (E) and *WRKY51* (F) expression by SA in the indicated genotypes. Two-week-old seedlings were sprayed with 50 µM SA. Samples were collected 0 and 1 h after treatment. Bars represent means ± s.d. (n = 3).

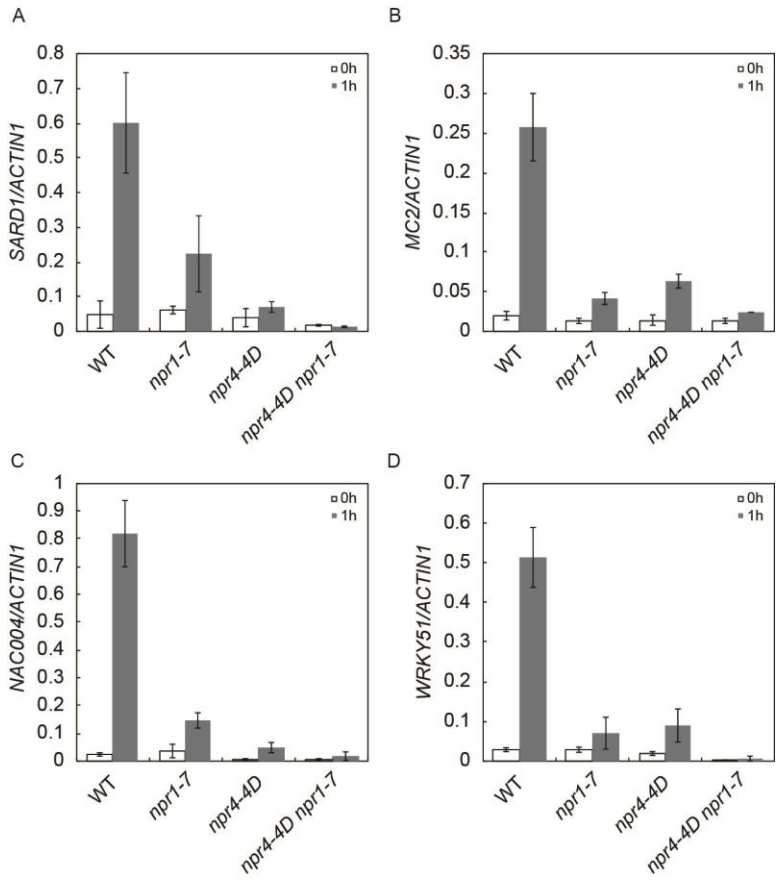

Figure 4.13 Analysis of genes regulated by NPR1 and NPR4.

(A-D) 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*. Bars represent means ± s.d. (n = 3).

Table 4.1 Known defense regulators induced in early SA response in wild type plants. Information extracted from RNAseq data (Table S1 in Ding *et al*. 2018). Fold change indicates comparison (50 μM SA 1hr vs 0hr treatment).

Locus	Name	Fold change	False discovery rate
AT4G14400	ACD6	3.408739836	7.37E-21
AT4G33300	ADR1-L1	2.542157086	1.91E-31
AT3G63420	AGG1	2.465959418	3.88E-17
AT1G31280	AGO2	3.434752746	4.25E-30
AT2G13810	ALD1	7.348887241	4.84E-04
AT5G54610	ANK	9.997954811	2.30E-24
AT3G61190	BAP1	3.142206148	4.24E-05
AT5G48380	BIR1	3.514611062	1.35E-44
AT5G61900	BON	2.888950552	1.26E-22
AT5G26920	CBP60G	2.615342697	6.61E-05
AT1G18890	CDPK1	2.108990072	3.52E-13
AT2G17290	CDPK3	2.550681529	9.85E-27
AT3G21630	CERK1	2.434476038	3.37E-20
AT1G17610	CHS1	2.400860792	2.72E-06
AT1G73965	CLE13	3.702349651	1.82E-02
AT5G04870	CPK1	2.543866586	4.34E-28
AT4G09570	CPK4	2.335062362	6.39E-24
AT5G24530	DMR6	14.22639628	3.63E-153
AT5G05190	EDR4	3.871767602	2.81E-11
AT3G48090	EDS1	2.850502002	1.71E-14
AT4G39030	EDS5	4.384191417	5.89E-24
AT5G20480	EFR	3.506614909	8.58E-13
AT2G31880	EVR	3.484817338	1.91E-33
AT1G07000	EXO70B2	4.448122014	9.02E-22
AT5G46330	FLS2	3.111467143	1.23E-04
AT1G74710	ICS1	3.058022959	2.67E-16
AT1G51800	IOS1	17.23465206	1.08E-18
AT2G33580	LYK5	4.170208686	4.04E-21
AT5G66850	MAPKKK5	2.069932897	4.85E-10
AT4G25110	MC2	8.7845288	1.61E-47
AT4G26070	MKK1	3.793201292	1.03E-33
AT4G29810	MKK2	2.706068672	1.86E-36
AT1G51660	MKK4	3.932599195	9.36E-26
AT3G21220	MKK5	2.931704437	3.06E-23
AT1G01560	MPK11	8.212830309	1.81E-12
AT3G20600	NDR1	2.465965196	8.09E-14
AT1G02450	NIMIN-1	33.99114315	1.01E-41
AT5G45110	NPR3	3.862070959	1.54E-32
AT4G19660	NPR4	2.417051313	3.72E-13

Locus	Name	Fold change	False discovery rate
AT2G04450	NUDT6	5.246614959	5.37E-14
AT4G12720	NUDT7	5.799464351	6.17E-63
AT3G52430	PAD4	3.786038995	1.15E-36
AT5G35580	PBL13	2.275654815	2.12E-09
AT3G09830	PCRK1	3.837876895	1.04E-21
AT5G64890	PROPEP2	2.610067479	3.50E-02
AT3G52450	PUB22	6.012189013	3.34E-18
AT2G35930	PUB23	3.735573342	5.62E-14
AT3G11840	PUB24	3.044336935	4.61E-10
AT1G20780	PUB44	2.462590393	4.88E-11
AT4G16990	RLM3	2.34690924	5.02E-11
AT2G32680	RLP23	27.73865517	9.53E-55
AT3G05360	RLP30	2.593753444	2.51E-08
AT3G07040	RPM1	2.641919848	4.00E-20
AT5G46470	RPS6	2.006931121	1.38E-13
AT5G14930	SAG101	2.900357561	4.33E-12
AT1G73805	SARD1	10.02872941	1.46E-37
AT5G52810	SARD4	4.107984577	1.04E-42
AT2G13790	SERK4	7.345325604	1.46E-98
AT3G11820	SYP121	3.083286964	8.78E-24
AT3G52400	SYP122	2.247440128	7.43E-08
AT2G24570	WRKY17	2.148429361	4.95E-05
AT4G31800	WRKY18	11.31801374	7.85E-18
AT1G80840	WRKY40	9.73429394	2.65E-06
AT5G64810	WRKY51	23.68232289	2.41E-52
AT3G56400	WRKY70	6.120910995	1.96E-27
AT4G34390	XLG2	2.572303118	6.95E-18
AT3G50950	ZAR1	2.333415281	1.45E-18

4.5 Discussion

Previously we showed that NPR3/NPR4 function redundantly as negative regulators of plant immunity (Zhang et al. 2006), but the mechanism of how they regulate plant defense 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 co-repressors.

Surprisingly, SA serves as an inhibitor of NPR3/NPR4 to release the repression of defense genes. Multiple lines of evidence suggest that SA-induced de-repression of defense genes is critical in plant immunity. The SA-insensitive *npr4-4D* mutant not only displays enhanced disease susceptibility but also completely blocks INA-induced pathogen resistance. In addition, the constitutive defense responses in *snc2-1D npr1-1* are almost completely suppressed by *npr4-4D*. The effects of *npr4-4D* and *npr1-1* on plant defense are almost always additive, suggesting that both de-repression and activation of SA-responsive genes are important to 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 its 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 (Wu et al. 2012, Rochon, Boyle, Wignes, Fobert, and Després 2006). Whether they are required for the induction of other defense 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 defense gene expression and pathogen resistance. Together these data strongly support NPR1 as a *bona fide* SA receptor.

Our data do not support the 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, *npr4-4D* was isolated in a background containing the *npr1-1* mutation, a null allele of *NPR1* that completely abolishes its interaction with the TGA transcription factors and SA-induced *PR* gene expression (Zhang et al. 1999, Cao et al. 1994), and the *npr4-4D* and *npr1-1* mutations have additive effects on the suppression of *snc2-1D*. Second, *npr1-1* has no effect on the increased *SARD1* and *WRKY70* expression in *npr3 npr4*. 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 defense 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 (Table 4.1). 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 (Zhang, Xu, et al. 2010, Li, Brader, and Palva 2004, Gao et al. 2009, Cecchini et al. 2015, Grant et al. 2003, Cui et al. 2017), 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 defense 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 (Wu et al. 2012, Manohar et al. 2015, Fu 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 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 defense 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, Boyle, Wignes, Fobert, and Després 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). Our ChIP-qPCR data showed that NPR3/NPR4 also interact with the promoters of defense 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 co-activator and NPR3/NPR4 serve as redundant transcriptional co-repressors for SA-responsive defense 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 4.14). 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 defense gene expression.

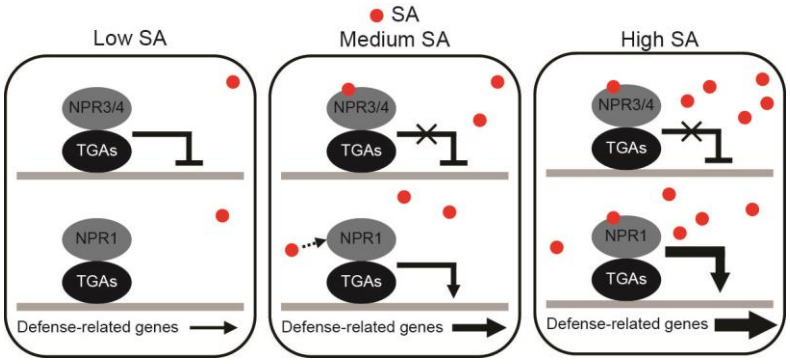

Figure 4.14 A working model of NPR1/NPR3/NPR4 in SA-induced defense activation.

When SA level is low under uninfected state, NPR3/NPR4 interacts with TGA2/TGA5/TGA6 to inhibit the expression of defense-related gene expression. As the SA level increases during pathogen infection, SA binds to NPR3/NPR4 to release the transcriptional repression of defense genes. Meanwhile, binding of SA to NPR1 promotes activation of the transcription of defense genes.

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 defense gene expression, which prevents autoimmunity. Increased SA accumulation removes the repression and allows further induction of defense gene expression through the transcription co-activator NPR1.

Chapter 5: Conclusion and future directions

5.1 Conclusion

The goal of this dissertation is to understand signal transduction mediated by transcription factors SARD1 and CBP60g, including upstream regulators and downstream targets. Studies in chapter 2 demonstrated that SARD1 and CBP60g function as master regulators of plant immunity by targeting a large number of genes encoding key signaling components in PTI, ETI and SAR. In addition to promoting pathogen-induced SA biosynthesis, SARD1 and CBP60g regulate Pip accumulation through direct control of the expression of genes encoding key enzymes involved in Pip biosynthesis. Studies in chapter 2 further showed that induction of *FMO1*, which encodes a monooxygenase that converts Pip to N-hydroxypipecolic acid (NHP) (Hartmann et al. 2018, Chen et al. 2018), is also regulated by SARD1 and CBP60g, suggesting that SARD1 and CBP60g are also involved in regulating NHP level in plant defense.

Research in chapter 3 uncovered the roles of TGA1 and TGA4 in plant immunity. We demonstrated that TGA1 and TGA4 regulate SA and Pip accumulation through modulating the expression of *SARD1* and *CBP60g*. Compromised accumulation of SA and Pip seen in *tga1 tga4* double mutant at least partially explains its enhanced disease susceptibility phenotype. In addition, pathogen-induced expression of *SARD1* and *CBP60g* in the *tga1 tga4* double mutant is greatly reduced, but not blocked, suggesting that there is a TGA1/TGA4 independent pathway that regulates the expression of *SARD1* and *CBP60g*.

Studies in chapter 4 lead to the proposal of a model on SA perception by its receptors NPR1, NPR3 and NPR4. NPR1 functions as a transcriptional activator and NPR3/NPR4 serve as redundant transcriptional repressors for SA-responsive defense genes. NPR1 and NPR3/NPR4 all interact with and rely on TGA2, TGA5 and TGA6 for their activities. Binding of SA to NPR3/NPR4 inhibits their transcriptional repression activity, whereas perception of SA by NPR1 enhances its transcriptional activation activity, both contributing to the induction of defense gene expression.

Furthermore, we found that SA induces the expression of *SARD1* and the induction of *SARD1* by SA is blocked in *npr1-1 npr4-4D* double mutant, suggesting that there is a feedback amplification loop between SA and SARD1, in which SARD1 directly activates *ICS1* expression

and SA accumulation upon pathogen infection, and SA in turn promotes *SARD1* expression through differentially regulating the activities of NPR1/TGA and NPR3/4 /TGA transcriptional factor complexes. Increased SARD1 level would further elevate *ICS1* expression, resulting in higher levels of SA that fuels further amplification of *SARD1* expression. Since SARD1 targets a large number of positive regulators of plant immunity, induction of *SARD1* by SA would activate robust defense responses via upregulation of these targets.

Our RNA-seq analysis in chapter 4 revealed that SA treatment induces the expression of a large number of genes within one hour. Many of these early-induced genes encode key regulators in plant immunity such as SARD1, WRKY70, ALD1, SARD4 and EDS1/PAD4 (Table 4.1). Induction of *ALD1* and *SARD4* by SA indicates that SA promotes biosynthesis of Pip. Notably, beside *SARD1*, SA also induces expression of other genes involved in SA signaling and biosynthesis such as *CBP60g*, *ICS1* and *EDS5*. Since SARD1, but not CBP60g, was identified as a target of TGA2/TGA5/TGA6 in ChIP-PCR experiments, CBP60g is probably not a direct target in the SA amplification loop.

In summary, studies in this dissertation revealed broad roles of SARD1 and CBP60g in plant immunity, the functions of TGA1/TGA4 in regulating *SARD1* and *CBP60g* expression, and new insights on the mechanism of perception of SA by its receptors NPR1/NPR3/NPR4.

5.2 Future directions

While studies in this dissertation have made significant progresses in transcriptional regulation of plant immunity by SARD1 and CBP60g as well as mechanisms of SA perception, a number of questions remain to be addressed as discussed below.

5.2.1 Signaling upstream of SARD1 and CBP60g

One of the questions that remain to be answered is how the activity of TGA1/TGA4 is regulated. It was previously reported that two cysteine residues in TGA1/TGA4 are important for their interaction with NPR1 (Despres et al. 2003). However, we found that compromised immunity in *tga1 tga4* can be fully complemented by introducing a *TGA1* variant with mutations in both cysteine residues (data not shown), indicating that these two cysteine residues in TGA1 are not

essential for its function in plant immunity. We also noticed that TGA1/TGA4 carry two TP residues, which are potential target sizes for MPKs (Clark-Lewis, Sanghera, and Pelech 1991), suggesting that MAPK cascade might acts upstream of TGA1/TGA4. However, a *TGA1* variant with phospho-dead mutations (TP to AP) can still complement compromised immunity in *tga1 tga4* (data not shown), suggesting that these potential MPK target sites are not required for its function. How the activity of TGA1/TGA4 is regulated in plant immunity remains to be determined.

Another question to be addressed is what are the components involved in the TGA1/TGA4 independent pathway(s) that regulate pathogen- induced expression of *SARD1* and *CBP60g*. When transgenic plants carrying a reporter gene driven by a mutated *SARD1* promoter with mutations in the TGACG motifs were challenged with *Pst* DC3000 *hrcC*, the reporter gene expression can still be induced (data not shown), suggesting that the TGA1/TGA4 independent pathway does not rely on any TGA transcription factors. Sequence analysis showed that there are putative CAMTA binding sites in the promoter regions of *SARD1* and *CBP60g*. Search of DNA affinity purification sequencing (DAP-seq) data also showed that *SARD1* and *CBP60g* promoter regions contain binding sites for CAMTA1 (O'Malley et al. 2016). Consistently, the expression of *SARD1* and *CBP60g* is elevated in *camta3*, *camta2/camta3* and *camta1/camta2/camta3* triple mutant (Kim et al. 2013). Whether CAMTA TFs directly regulate the expression of *SARD1* and *CBP60g* needs to be confirmed by further analysis such as by ChIP-qPCR experiments. It is always possible that other TFs are also involved in regulating the expression of *SARD1* and *CBP60g* via not yet identified *cis* elements in promoter regions of *SARD1* and *CBP60g*.

Previously, two redundant RLCKs PCRK1 and PCRK2 were shown to function downstream of PRRs in regulating pathogen-induced expression of *SARD1*, *CBP60g* and *ICS1* as well as SA accumulation (Kong et al. 2016). A recent study showed that BIK1, a RLCK closely related to PCRK1/PCRK2, localizes to the nucleus and targets WRKY transcription factors to regulate the immune responses (Lal et al. 2018). It remains to be examined whether PCRK1/PCRK2 can localize to the nucleus and whether they can interact with TGA1/TGA4 and SARD1/CBP60g.

SARD1 and CBP60g have been proposed to function in parallel and define two independent pathways that regulate *ICS1* expression and SA accumulation (Zhang, Xu, et al.

2010). Moreover, overexpression of *SARD1* can activate plant immunity, but overexpression of *CBP60g* cannot (Zhang, Xu, et al. 2010). SARD1 may be regulated mainly through control of its transcription while CBP60g may be regulated both transcriptionally and post-transcriptionally. There could be regulatory components specific to the SARD1 pathway or the CBP60g pathway. A *SAR-deficient* enhancer screen has been carried in *cbp60g* mutant background (data not shown). As *sard1* and *cbp60g* single mutants have subtle *SAR deficiency* phenotypes, but *sard1 cbp60g* double mutant exhibits complete loss of SAR (Zhang, Xu, et al. 2010), any mutants affecting *SARD1* expression in a *cbp60g* background should show an enhanced *SAR-deficient* phenotype. Analysis of the enhancers will facilitate the identification of upstream regulators of *SARD1*. Similarly, identification and characterization of *SAR-deficient* enhancers in *sard1* mutant background will also help to identify regulatory components involved in the CBP60g pathway.

5.2.2 Perspective on SA perception and signaling

Currently it is unclear how exactly SA binds to its receptors NPR1, NPR3 and NPR4. Two cysteine residues (Cys521/529) in NPR1 were previously implicated in SA binding via a transition metal copper (Wu et al. 2012). Our studies in chapter 4 showed that the arginine residue (R419 in NPR4 and R432 in NPR1) is critical for SA binding. Consistent with our finding that the residue R432 in NPR1 is important for its function in promoting SA-induced pathogen resistance, *nim1-4*, one of the SA insensitive *NPR1* mutant alleles, contains a G to A mutation that results in a R432 to K change (Ryals et al. 1997). Whether these residues directly interact with SA remains to be determined. Future solution of crystal structures of NPRs with and without SA would be critical to address these questions.

Both NPR1 and NPR4 carry an N-terminal BTB/POZ domain, a central ankyrin repeat containing domain and a C-terminal domain. The C-terminal domain in NPR1 was shown to harbor a transactivation domain (Rochon, Boyle, Wignes, Fobert, and Despres 2006). It was proposed that binding of SA causes a conformational change in NPR1 that release the inhibitory effect of BTB/POZ domain on the C-terminal transactivation domain (Wu et al. 2012). Our data revealed that the C-terminal domain of NPR4 possesses transcriptional repression activity and that binding of SA to NPR4 inhibits this activity. It is still unclear how SA binding leads to the

repression of NPR4. One possibility is that NPR4 interacts with other transcription corepressor(s) and binding of SA disrupts their interactions, resulting in release of NPR4-mediated transcriptional repression. Alternatively, binding of SA could recruit an unknown inhibitor of NPR4 that blocks its transcriptional repression activity.

Another puzzle is that defense genes are not strongly induced by the basal level of SA, even though the basal SA level in *Arabidopsis* leaf tissue is much higher than the K_ds for NPR1 and NPR3/NPR4 (as mentioned in chapter 4). It is possible that SA-binding affinities of endogenous NPR1, NPR3 and NPR4 is much lower than those of recombinant NPRs due to potential post-translational modifications (PTMs) in the plant cells. Indeed, several studies have shown that NPR1 has multiple PTMs including phosphorylation, sumoylation and oxidation, and that these PTMs were implicated in regulation of NPR1 activity (Withers and Dong 2016). Whether these PTMs in NPR1 affect its SA binding activity needs further investigation. Structural analysis again will be critical in solving these questions regarding NPR1 PTM sites. It will be also interesting to determine whether NPR3 and NPR4 carry any PTMs and whether these PTMs affect their activities.

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