Regulation of the Defense-related *Sl*NAC1 Transcription Factor During Defense Signaling in Tomato

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Authorization to Submit Dissertation

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Abstract

As sessile organisms, plants are constantly exposed to diverse pathogens. Mounting a successful defense response depends on the early recognition of a pathogen's presence, followed by a rapidly transmitted signal that is decoded and translated into appropriate cellular events by transcription factors (TFs). Thus, plants have evolved a sophisticated and deeply interconnected signaling network with defense-related TFs tuning plant immunity in a precise spatial-temporal manner.

The main goal of my doctoral research is to study the molecular basis of defense signaling in tomato in response to *Pseudomonas syringae pv. tomato* (*Pst*) infection, with particular focus on the defense-related *Sl*NAC1 TF. In tomato, resistance to *Pst* is achieved by two proteins, the Pto Ser/Thr kinase and the Prf resistance protein, which work together to initiate Hypersensitive Response (HR) cell death upon detection of *Pst*-secreted effectors, AvrPtoB and AvrPto. Although the role of Pto and Prf in tomato immunity to *Pst* were described almost two decades ago, direct downstream signaling components that translate pathogen detection into an appropriate cellular defense events remain elusive.

It was previously reported that NAC1-silenced *Nicotiana benthamiana* plants show compromised immunity to *Pst.* The steady-state level of *Sl*NAC1 is controlled by the ubiquitin-proteasome system. Here, the tomato *Sl*SINA3 was identified as a cognate E3 ubiquitin ligase specifically binding to and ubiquitinating *Sl*NAC1 to promote its degradation in the absence of *Pst*. Upon AvrPtoB/AvrPto detection by the Pto kinase, the Prf resistance protein is activated to interact with and stabilize *Sl*NAC1 to stimulate its transcriptional activity. The presented data suggest a model that Prf interferes with *Sl*NAC1 ubiquitination

by sequestering *Sl*NAC1 away from *Sl*SINA3 to prevent its degradation, thereby, enabling robust transcriptional reprograming. Significantly, *Sl*NAC1 is a positive regulator of HR cell death and its overexpression in transgenic tomato is associated with the elevated level of SA, and reduced expression of JA, -marker genes.

Together, my dissertation studies uncover the dynamic post-transcriptional regulation of *Sl*NAC1, in which the interplay between *Sl*SINA3 and activated Prf allows for a delicate balance between preventing costly autoimmunity in the absence of the pathogen and rapidly activating defenses once the pathogen is detected.

Acknowledgements

Standing at this finishing line of my doctoral study and looking back at the past six years, I would like to take this opportunity to express my appreciation to all the people who helped me along this journey.

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Thank you very much, everyone!

Dedication

I dedicate this dissertation to my family, especially my mom (Urszula), my dad (Kazimierz), and my husband (Ahmed) as an appreciation for their endless love, support and encouragement to dream big, work hard and become a better person at every step of this journey. This is for all of you….

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List of Abbreviations

4-MUG	4-methyl-umbelliferyl-β-D-glucuronide
ABA	abscisic acid
ACC	1-aminocyclopropane-1- carboxylic acid
ACO	1-aminocyclopropane-1- carboxylic acid oxidase
ACS	1-aminocyclopropane-1- carboxylic acid synthase
AP2/ERF	APETALA2/Ethylene-Responsive Factor
ARC	APAF-1, R proteins, and CED-4 domain
ATAF	ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR
Avr	avirulence
AzA	acid azelaic acid
AZI1	AzA insensitive 1
BAK1	BRI1-ASSOCIATED KINASE 1
Bgh	*Blumeria graminis f. sp. Hordei*
bHLH	Basic helix-loop-helix
BiFC	Bimolecular fluorescence complementation
BSMT1	BENZOATE/SALICYLATE CARBOXYL METHYLTRANSFERASE 1
BTB	Broad complex Tramtrack Bric-a-Bric protein
bZIP	Basic Leucine Zipper
CaM	calmodulin
CAMTA	Calmodulin binding transcriptional activator

CaMV	cauliflower mosaic virus
CAT	catalase
CBP60g	CaM-binding protein 60g
CC	coiled-coil domain
CDPK	calcium-dependent protein kinase
CERK1	Chitin elicitor receptor kinase 1
CF	culture filtrate
ChIP	chromatin immunoprecipitation
CML	calmodulin-like kinase
COI1	coronatine-insensitive 1
CoIP	co-immunoprecipitaion
COR	coronatine
CP	capsid protein
CRL	CUL-RING ligase
CTR1	CONSTITUTIVE TRIPLE RESPONSE 1
CUC	CUP-SHAPED COTYLEDONS
CUL	Cullin
DA	diterpenoid dehydroabietinal
DBD	DNA binding domain
DDB1	DNA-Binding Domain 1
DEFL	Defensin-like protein

DIR1	Defective in Induced Resistance 1
DND	DEFENSE NO DEATH
dpi	days post infection
DTT	Dithiothreitol
DWD 40	DDB1-binding WD 40
EDTA	Ethylenediaminetetraacetic acid
EBF1	EIN3 BINDING F-BOX1
EF-Tu	elongation factor Tu
EIL1	EIN3- LIKE 1
EIN2	ETHYLENE INSENSITIVE 2
EIRP1	Erysiphe nectar-induced RING finger protein 1
ER	endoplasmic reticulum
ERD1	EARLY RESPONSE TO DEHYDRATION 1
ERF	Ethylene-Responsive Element Binding Protein
ET	ethylene
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
ETR1	ETHYLENE RESPONSE 1
FLS2	FLAGELLIN-SENSING 2
FOXO4	Forkhead boxO 4
G3P	glycerol-3-phosphate

GFP	green fluorescent protein
GST	Glutathione S-transferase
GTF	general transcription factor
GUS	β-glucuronidase reporter system
His	histidine
HA	hemagglutinin
HAUSP	Herpesvirus-associated ubiquitin-specific protease
HECT	Homologous to E6-associated protein Carboxyl Terminus
HR	hypersensitive response
ICS1	Isochorismate Synthase 1
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JA	jasmonic acid
JA2	jasmonic acid 2
JA2L	JA2-like
JAZ	jasmonate ZIM domain
LHY	LATE ELONGATED HYPOCOTYL 1
LRR	Leucine-rich repeat domain
LRR-RK	leucine-rich repeat receptor kinases
LTP	lipid transfer protein
MAPK	mitogen-associated protein kinase
MAPKK	mitogen-associated protein kinase kinase

MAPKKK mitogen-associated protein kinase kinase kinase

MBP Maltose-Binding Protein

MetJAq methyl-jasmonic acid

MetSA methyl-salicylic acid

MIEL1 MYB30-Interacting E3 Ligase 1

MLA10 Mildew A 10

MU 4-Methylumbelliferone

MYB myeloblastosis

NAC *Petunia* NAM, *Arabidopsis* ATAF1&2, and CUC2

NACRS NAC recognition sequence

NAM NO APICAL MERISTEM

NB nucleotide-binding domain

NCED 9-cis-epoxycarotenoid dioxygenase

NES nuclear export signal

NINJA NOVEL INTERACTOR OF JAZ

NIT2 nitrilase 2

NLR nucleotide binding leucine-rich repeat protein

NLS nuclear localization signal

NO nitric oxide

NPR1 non-expresser of PR genes 1

NRS nitrogen reactive specious

NTP	NAC Targeted by *Phytophthora*
oe	overexpression
PAMP	pathogen associated molecular pattern
PAO3	Polyamine Oxidase 3
Pb1	Panicle blast 1
PCD	programmed cell death
PCR	polymerase chain reaction
Pip	pipecolic acid
PMSF	phenylmethylsulfonyl fluoride
Prf	*Pseudomonas* resistance and fenthion sensitivity
PRR	PAMP recognition receptor
PR	pathogenesis-related
Pst	*Pseudomonas syringae pv. tomato*
PTI	pattern-triggered immunity
PTM	post-translational modification
Pto	resistance to *Pseudomonas syringae pv. tomato*
qRT-PCR	quantitative Real-Time PCR
R	resistance
RBOHD	Respiratory burst oxidase homolog D
REn	geminiviral replication enhancer
RIN4	RPM1-interacting protein 4

RING	really interesting new gene
ROS	reactive oxygen species
RPM1	RESISTANCE TO P. SYRINGAE PV MACULICOLA 1
RPS2	RESISTANCE TO PSEUDOMONAS SYRINGAE 2
SA	salicylic acid
SAMT1	SALICYLIC ACID METHYL TRANSFERASE 1
SBD	substrate-binding and dimerization
SBP	SQUAMOSA PROMOTER BINDING PROTEIN
SCF	Skp1/Cullin/F-box
SINA	SEVEN IN ABSENTIA
SNC1	SUPPRESSOR OF NPR1-1, CONSTITUTIVE 1
SNI1	SUPPRESSOR OF NONEXPRESSOR OF PR GENES INDUCIBLE 1
SOD	superoxide dismutase
SPL6	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 6
SRN1	Stress-related NAC1
STAND	signal transduction ATPases with numerous domains
TCV	turnip crinkle virus
TF	transcription factor
TIP	TCV-interacting protein
TIR	Toll/interleukin-1 receptor
TLCV	*Tomato leaf curl virus*

TMV	*Tobacco mosaic virus*
TOPLESS	Groucho/Tup1-type co-repressor TPL
TPR1	Topless related 1
TRD	transcription regulation domain
TTSS	type III secretion systems
Ub	ubiquitin
UPS	ubiquitin-proteasome system
VLCFA	Very-Long-Chain-Fatty-Acid
VSP1	Vegetative Storage Protein 1
WB	Western blotting
WT	wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Y2H	yeast two-hybrid
YFP	yellow fluorescence protein

CHAPTER 1 Introduction

As sessile organisms, plants are constantly exposed to both abiotic and biotic threats, therefore, they need to be successful in protecting themselves from all kinds of environmental stresses to maintain their fitness. Like animals, plants can be infected by pathogens including viruses, bacteria, fungi, oomycetes and nematodes. Regardless of the type of pathogen, as in warfare, there are three crucial steps for effective immunity: 1) the early recognition of enemy's presence, 2) rapid information transfer, and 3) activation of the appropriate defensive mechanisms together with their tightly controlled attenuation once the situation is under control. To achieve this goal, plants have evolved a sophisticated multilayer innate immune system that, in a precise spatial-temporal manner, successfully protects them from pathogen threats.

Plant Innate Immunity

Step 1. Pathogen detection and three tires of plant innate immunity

Plants lack the circulatory system and mobile immune cells typical for animals. Instead, they rely entirely on innate immune responses assembled independently by each individual cell that detects invaders. The constant evolutionary pressure, on both plants and pathogens, results in a multilevel relationship that can be conceptually divided into three types of plant immunity against pathogens: pattern-triggered immunity (PTI) relying on recognition of pathogen-associated molecular patterns (PAMPs), effector-triggered immunity (ETI) activated by pathogen-secreted effectors and systemic acquired resistance (SAR) caused by plant-generated systemic signals. The following section briefly describes how those three

levels of plant immunity are activated and consequently what corresponding defense mechanisms are implemented.

Pattern-triggered immunity (PTI)

Our current understanding of plant innate immunity focuses on two types of host immune receptors that recognize non-self molecules originating from the invading pathogens. The first type of immune receptors (pattern recognition receptors - PRRs) are membrane-localized leucine-rich repeat receptor kinases (LRR-RK) sensing PAMPs at the early infection stage to activate PTI, which is also called basal defense (Jones and Dangl, 2006). PAMPs are defined as conserved molecules critical for pathogen viability and are usually associated with a particular class of microbes, such as bacterium-specific flagellin and elongation factor Tu (EF-Tu), chitin typical for fungi and heptaglucans characteristic for oomycetes. In the case of PTI to bacteria, upon stimulation by corresponding PAMPs, PRRs activate the signaling pathway that controls stomata closure to limit entry of bacteria (Melotto et al., 2008; Sawinski et al., 2013) and restricts nutrient transfer from the cytosol to the apoplast to limit bacterial multiplication (Wang et al., 2012). Another defense responses include rapid production and secretion of toxic reactive oxygen species (ROS) and antimicrobial compounds (such as phytoalexins) to the apoplast to inhibit pathogen growth (O'Brien et al., 2012, Ahuja et al., 2012), and deposition of callose to reinforce cell walls (Bigeard et al., 2015). Interestingly, the activation of PTI also results in plant growth inhibition. This phenotype was used to identify the first PRR, FLAGELLIN-SENSING 2 (FLS2) receptor able to recognize the first 22 N-terminal amino acids of flagellin (Gomez-Gomez and Boller, 2000).

Effector-triggered immunity (ETI)

To counteract PTI, pathogens have developed an arsenal of quickly evolving effector proteins, which are delivered to the cytoplasm of plant cells through a syringe-like structure called a type III secretion system (TTSS) (Staskawicz et al., 2001), to actively suppress PTI, resulting in effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). For instance, the *Pseudomonas syringa*e effector AvrPtoB was found to directly target at least two PRRs, FLS2 and the chitin elicitor receptor kinase 1 (CERK1, recognizing the chitin oligosaccharide), to inhibit PTI by promoting their degradation (Gimenez-Ibanez et al., 2009; Göhre et al., 2008). To counteract the breaching of this basal defense by pathogens, plants developed the second type of immune receptors called resistance proteins (R) as a part of ETI to specifically recognize effectors, which, in this case, function as avirulence (*Avr*) proteins (Jones and Dangl, 2006). This type of plant-pathogen interaction was previously described as the gene-for-gene resistance, where a specific gene in a pathogen and a host gene confer resistance (Flor, 1971). R proteins belong to a class of cytoplasmic nucleotide-binding, leucine-rich receptors (NLRs) that are present in both plants and animals. Interestingly, due to the lack of adaptive immunity in plants, *NLR* genes in higher plants are more abundant and diverse than those in animals to keep up with rapidly changing pathogen effectors, making *NLRs* some of the fastest evolving genes in plants. For example, there are 159, 188 and 464 NLRs in the genome of *Arabidopsis thaliana*, tomato and rice, respectively (Seo et al., 2016), whereas there are only ~20 NLRs in vertebrate genomes (Lange et al., 2011). The initial gene-for-gene model proposed that NLRs recognize pathogen effectors through direct interactions such as occurs in the case of the rice resistance protein Pi-ta interacting with the *Magnaporthe grisea* effector AvrPita (Jia et al., 2000). However, few other examples of direct recognition have

been reported. Although the number of R proteins encoded by the plant genome is large, it still cannot explain the broad immune specificities against pathogens (Cesari et al., 2013; Deslandes et al., 2003; Dodds et al., 2006; Jia et al., 2000; Krasileva et al., 2010). Alternative, indirect-interaction, models of effector recognition emerged with an accessory protein that is either a virulence target of the pathogen effector - the Guard hypothesis (Van Der Biezen and Jones, 1998), or a structural mimic of the virulence target – the Decoy/Bait hypothesis (Collier and Moffett, 2009; van der Hoorn and Kamoun, 2008). In both cases, the effector-triggered changes in the accessory protein are further sensed by the corresponding R proteins to transduce the defense signal. The interactions of the accessory protein RPM1-interacting protein 4 (RIN4) with the RESISTANCE TO P. SYRINGAE PV MACULICOLA 1 (RPM1) and RESISTANCE TO P. SYRINGAE 2 (RPS2) is the classical example of the Guard model, in which the plant immune response is activated when either RPS2 detects RIN4 cleavage by the cysteine protease AvrRpt2 effector (Axtell et al., 2003; Axtell and Staskawicz, 2003; Mackey et al., 2003) or RPM1 senses the AvrRpm1 effector-triggered phosphorylation of RIN4 (Mackey et al., 2002). Large-scale protein network analysis showed that both pathogen effectors and plant R proteins interacted with an overlapping set of host "hub" proteins essential for plant defense (Mukhtar et al., 2011). Therefore, the limited R protein repertoire effectively monitors the integrity of crucial defense components to protect plants from a broad spectrum of pathogens (Jones and Dangl, 2006). The Decoy/Bait model represents an alternative indirect recognition hypothesis that has been based on studies of the receptor complex containing the *Pseudomonas* resistance and fenthion sensitivity (Prf) NLR protein and the *Pseudomonas syringae pv. tomato* (Pto) Ser/Thr kinase, which work together to recognize the bacterial AvrPtoB effector. In this example, Pto mimics closely related LRR-

RKs, FLS2 and CERK1, which are virulence targets of AvrPtoB (Gimenez-Ibanez et al., 2009; Göhre et al., 2008). Thus, Pto behaves as a decoy and provides the recognition signal to Prf (Balmuth and Rathjen, 2007; Mucyn et al., 2009). Although each recognition of an *Avr* effector by its cognate R protein distinctively activates ETI, at some point all R-mediated defense signaling pathways converge and lead to the final outcome called the hypersensitive response (HR). The HR is a tightly controlled, local programed cell death (PCD) occurring at the site of pathogen recognition, which is characterized by cytoplasmic shrinkage, mitochondrial swelling, chromatin condensation, vacuolization and chloroplast disruption (Mur et al., 2008).

Systemic acquired resistance (SAR)

Besides PTI and ETI, pathogen infection can also trigger a long-lasting SAR that is defined as "whole-plant" resistance achieved by generation of a systemic signal at the primary infection site that is then transported through the phloem to uninfected, distal systemic tissues to induce broad-spectrum resistance against secondary infections for a period of weeks to months (Fu and Dong, 2013). SAR can even be passed onto progenies through epigenetic regulation (Luna et al., 2012). Although the typical hallmark of SAR establishment is accumulation of the plant phytohormone salicylic acid (SA) in the systemic tissues to render extensive transcriptional reprogramming (Delaney et al., 1994; Gaffney et al., 1993), SA itself is not the initial SAR signal (Vernooij et al., 1994). Several candidates for this long-distance signal have been proposed, including methyl-SA (MetSA), the diterpenoid dehydroabietinal (DA), azelaic acid (AzA), pipecolic acid (Pip), auxin, glycerol-3-phosphate (G3P), nitric oxide (NO), ROS, galactolipids, factors contributing to cuticle formation, the lipid transfer proteins (LTPs), Defective in Induced Resistance 1 (DIR1) and AzA insensitive 1 (AZI1)

(Chanda et al., 2011; Chaturvedi et al., 2012; El-Shetehy et al., 2015; Gao et al., 2014a; Jung et al., 2009; Maldonado et al., 2002; Mandal et al., 2012; Návarová et al., 2012; Park et al., 2007; Truman et al., 2010; Wang et al., 2014; Xia et al., 2009, 2010, 2012; Yu et al., 2013a). All of these putative SAR signals likely function coordinately to achieve long-lasting non-specific resistance (Dempsey and Klessig, 2012). The most important consequence of SAR is enhanced biosynthesis of pathogenesis-related proteins (PRs) and endoplasmic reticulum (ER)-resident proteins. As executors of SAR, PRs are peptides with a wide range of antimicrobial activities which inhibit pathogen growth upon secretion into apoplast through the vacuolar system with the help of ER-resident proteins (Pajerowska-Mukhtar et al., 2012).

Step 2. Signal transduction downstream of activated immune receptors

Pathogen recognition and initiation of PTI/ETI/SAR are just the first two steps in the complex immune signaling network. As described above, the defense responses are specific to each tier of plant immunity with PTI generally effective against non-adapted pathogens, ETI acting against adapted pathogens, and SAR priming plants for secondary infections. Interestingly, although PTI, ETI and SAR utilize overlapping sets of signaling molecules, due to quantitative differences in the strength, duration and activation kinetics of those signal messengers, the implemented defenses are qualitatively different (Cui et al., 2014; Tsuda and Katagiri, 2010). The following paragraphs highlight the most important classes of signaling molecules involved in translating the activation of immune receptors into appropriate defense responses.

Ca^{2+} influx and Ca^{2+}-dependent kinases

The rapid, but rather transient, extracellular Ca^{2+} influx into the cytosol is the first known physiological response in PTI and it occurs within ~30 seconds from PRR activation

(Jeworutzki et al., 2010). On the other hand, a prolonged and sustained increase of cytosolic Ca^{2+} concentration is required for the induction of ETI-specific HR cell death (Grant et al., 2000). The Ca^{2+} signaling plays a crucial role in controlling diverse aspects of plant biotic interactions through differential Ca^{2+} sensory machineries (Gao et al., 2014b; Reddy et al., 2011). The increased cytosolic concentration of Ca^{2+} is detected by calcium-dependent protein kinases (CDPKs), calmodulins (CaMs) and calmodulin-like kinases (CMLs). Through phosphorylation of their substrates, they not only regulate biosynthesis of ROS, NO and ethylene (ET), but also directly control defense-related transcriptional reprograming by manipulating transcription factors (TFs) of diverse families such as Calmodulin binding transcriptional activators (CAMTAs), myeloblastosis (MYB), WRKY, TGA and NAM, ATAF1&2, CUC2 (NAC) (Kim et al., 2007b; Park et al., 2005; Popescu et al., 2007; Reddy et al., 2000; Yang and Poovaiah, 2000, 2002; Yoo et al., 2005).

ROS and NO

Although extracellular ROS produced at the plasma membrane by respiratory burst oxidase homolog D (*At*RBOHD) directly inhibits pathogen growth due to its toxicity (Lambeth, 2004), ROS also functions as an important early signaling molecule involved in stomatal closure, HR cell death development and long-distance systemic signaling in SAR establishment (Macho et al., 2012; Matika and Loake, 2014; Miller et al., 2009; Torres and Dangl, 2005). Likewise, nitrogen reactive species (NRS), such as NO and its derivatives, are also recognized as a key signaling molecule. The production of ROS and NRS occurs in many organelles including chloroplasts, peroxisomes, mitochondria and at the plasma membrane; and this process is coordinated in a complex spatial-temporal manner (Baxter et al., 2014; Gross et al., 2013). It appears that there is a tight relationship and extensive crosstalk between

NO and ROS singling pathways (Gross et al., 2013; Scheler et al., 2013). ROS and NO are both able to directly mediate post-translational modifications (PTMs) of target proteins to manipulate their functions. For example, NO acts as a key regulator in SAR by controlling the oligomeric state of the non-expresser of PR genes 1 (*At*NPR1), a master regulator of defense gene expression, through cysteine S-nitrosylation (Tada et al., 2009). Through the same PTM, NO regulates activity of RBOHD responsible for ROS production (Yun et al., 2011). Moreover, due to their nature, ROS and NO can perturb the cellular redox homeostasis, thereby further generating highly specific cellular signals through redox-sensing proteins that provide the cell with a relevant defense response (Foyer and Noctor, 2005).

Mitogen-associated protein kinase (MAPK)

Protein phosphorylation mediated by kinases is probably the most common PTM in eukaryotes which controls protein stability, enzyme activity, and/or subcellular localization (Minguez et al., 2012). Besides previously described CDPKs, mitogen-associated protein kinase (MAPK) cascades represent the second major family of kinases essential for plant immunity. MAPKs are evolutionarily conserved enzymes which have been considered as a general signal transduction mechanism that connects different receptors to their cellular targets (Sewelam et al., 2016). MAPK cascade signaling pathways consist of three tiers of sequential phosphorylation events at the Tyr (Y) and Thr (T) residues in the TXY motif. The first level phosphorylation of the MAP kinase kinase kinase (MAPKKK) is usually initiated by the activated LRR-RK or LRR-RK interacting protein(s). MAPKKK can then phosphorylate and activate the second level MAP kinase kinase (MAPKK) that further phosphorylates the third level MAP kinase (MAPK) (Rodriguez et al., 2010). Phosphorylation of MAPK stimulates its kinase activity which leads to phosphorylation/activation of

transcription factors and other signaling nodes (Meng and Zhang, 2013). Because one MAPKK can activate more than one MAPK, the MAPK cascades are key signaling elements regulating a large spectrum of substrates and they act as divergence points in signaling pathways. Almost half of immune MAPK targets are transcription factors including members of the WRKY, Basic Leucine Zipper (bZIP), and ET response TF families (Andreasson et al., 2005; Bethke et al., 2009; Djamei et al., 2007; Mao et al., 2011). MAPK cascades were shown to play a pivotal role in HR cell death, ROS bursts, stomatal closure and biosynthesis of phytoalexins (del Pozo et al., 2004; Mao et al., 2011; Melech-Bonfil and Sessa, 2010; Pitzschke and Hirt, 2009; Segonzac et al., 2011). Moreover, MAPKs have been implicated in signaling events downstream of jasmonic acid (JA), SA and ET (Beckers et al., 2009; Han et al., 2010; Liu and Zhang, 2004; Ouaked et al., 2003; Seo et al., 2007; Yoo et al., 2008; Zhang et al., 2007).

Phytohormones

Phytopathogens range in their infection and feeding strategies. Biotrophs, like powdery mildew, seek food on living host cells. By contrast, necrotrophic fungus like *Botrytis cinereal*, kill plant cells to access their nutritional content. Hemi-biotrophic pathogens like *P. syringae* pursue a third strategy. Depending on the type of infection, plants activate different defense responses which are accomplished both locally and systemically by a blend of signaling hormones, with SA, JA ad ET playing the most prominent roles (Pieterse et al., 2012). SA-triggered signaling is engaged against biotrophs or hemi-biotrophs, whereas JA and ET regulate defense responses during necrotrophic infections (Glazebrook, 2005). SA and JA signaling pathways are often considered as antagonistic to each other. For instance, elevated SA signaling in response to biotrophic pathogens is often associated with attenuation

of JA signaling and reduced resistance to necrotrophic pathogens, whereas ET can significantly impair the suppressive effect of SA on the JA pathway (Leon-Reyes et al., 2010; Spoel and Dong, 2008). Thus, a growing body of evidence has indicated that extensive multi-level cross-talk among signaling hormones occurs frequently, allowing plants to fine-tune the defense response against pathogens (Pieterse et al., 2012).

Step 3. Transcriptional reprogramming – decoding defense signaling into immune responses

After pathogen perception by immune receptors and signal amplification through transducers and secondary massagers, an endpoint in each signaling cascade is the activation or inhibition of specific TFs, which consequently modulate expression of a specific set of genes. To efficiently fend off pathogen attack, the detection of invaders is followed by rapid and massive transcriptional reprogramming of up to 1/3 of all plant genes to redirect plant metabolism from routine cellular processes to the defensive mode. The highly dynamic and tightly controlled plant transcriptional machinery is governed by TFs and co-regulatory proteins which act coordinately in the intricate and deeply interconnected signaling network (Moore et al., 2011). TFs, as the major receiver and decoders of defense signals, can activate different biochemical pathways depending on the nature of the signal and what other pathways are activated at the same time. In fact, some pathogens can hijack host transcriptional machinery to enhance their virulence, further suggesting the significance of transcriptional regulation for host defense responses (Kazan and Lyons, 2014).

Regulation of the Defense-Related Transcriptional Reprograming

Unlike prokaryotic RNA polymerases, eukaryotic Pol II is unable to initiate promoter-specific transcription on its own. It requires interactions with the general transcription factors (GTFs) that engage it at the core promoter elements. However, the recruitment of Pol II and GTFs to the particular fragment of chromatin to facilitate both initiation and elongation of transcription is contingent on the action of gene-specific TFs that regulate the expression rate of target genes. TFs contain two characteristic domains: a DNA Binding Domain (DBD) that recognizes specific cis-acting element in the promoter region of target gene and a Transcription Regulatory Domain (TRD) that facilitates or inhibits assembly of the transcription initiation complex, respectively. Therefore, by binding to a given promoter region, TFs can either repress or induce expression of that particular gene(s). While other domains of the proteins may vary, DBDs define TFs into specific families (Hernandez-Garcia and Finer, 2014). Due to the sessile lifestyle and lack of an adaptive immune system in plants, the repertoire of defense-related TFs has expanded during evolution, and now includes both common and plant-specific TF families, such as the Basic helix-loop-helix (bHLH), APETALA2/Ethylene-Responsive Factors (AP2/ERF), bZIP, MYB, NAC, and WRKY. Given the importance of TFs in mounting successful transcriptional reprogramming and immunity, it is not surprising that plants are equipped with sophisticated mechanisms to fine-tune their function. On one hand, the abundance of mRNA is controlled at the transcriptional level; on the other hand, protein-protein interactions and PTMs are utilized in multiple ways to tightly control TFs' stability, localization and activity (Moore et al., 2011). The following section summarizes the diverse mechanisms used by plants during their defense response to regulate the function of different TF families.

Regulation by R and PRR proteins

Given the fact that activation of NLRs leads to intensive transcriptional reprograming and that the nuclear partition of at least some NLRs is indispensable for their function in ETI, it becomes evident that NLRs are capable of directly manipulating transcriptional machinery.

In barley, the *Hv*MLA10 NLR protein re-localizes from cytoplasm to nucleus upon recognition of fungus *Blumeria graminis f. sp. Hordei (Bgh).* The nuclear pool of *Hv*MLA10 is then able to interact with two WRKY transcriptional repressors, *Hv*WRKY1 and *Hv*WRKY2, which negatively regulate immunity against powdery mildew fungus, and the MYB transcriptional activator *Hv*MYB6 that functions as a positive regulator of immunity (Chang et al., 2013; Shen et al., 2007). In normal conditions, *Hv*WRKY1 binds to *Hv*MYB6 thereby suppressing its function. However, the *Bgh*-induced nuclear distribution of *Hv*MLA10 enhances *Hv*MYB6 DNA binding activity and releases *Hv*MYB6 from *Hv*WRKY1 suppression (Chang et al., 2013). In *Arabidopsis*, the resistance protein *At*SNC1 confers immunity to *P. syringae* through modulating transcriptional activity of at least two TFs from two different families, Topless related 1 (*At*TPR1) and *At*bHLH84. *At*TPR1 functions as a transcriptional co-repressor of two well-known negative defense regulators, DEFENSE NO DEATH (*At*DND1) and *At*DND2, (Zhu et al., 2010), whereas *At*bHLH84 is a transcriptional activator important for the *At*SNC1-mediated immunity (Xu et al., 2014). In *Nicotiana benthamiana*, the *Nb*N protein specifically recognizes the *Tobacco mosaic virus* (TMV) replicase to trigger defense responses (Burch-Smith et al., 2007). Significantly, the nuclear interaction between *Nb*N and SQUAMOSA PROMOTER BINDING PROTEIN (SBP)-domain TF, *Nb*SPL6, is indispensable for the *Nb*N-mediated immunity (Padmanabhan et al., 2013). In rice, the nucleocytoplasmic *Os*Pb1 (Panicle blast1) protein interacts with

the *Os*WRKY45 transcription factor to confer immunity against the rice blast fungus *Magnaporthe oryzae* (Inoue et al., 2013; Matsushita et al., 2013). In unchallenged rice cells, *Os*WRKY45 is subjected to ubiquitin-proteasome system (UPS)-mediated degradation (Matsushita et al., 2013). Upon fungal detection, the nuclear *Os*Pb1 protects *Os*WRKY45 from degradation which leads to enhanced *Os*WRKY45 accumulation and stimulation of its transcriptional activity (Inoue et al., 2013).

Additionally, at least one rice PRR has been shown to regulate transcriptional reprogramming. The plasma membrane localized *Os*XA21 confers resistance against the rice blight pathogen *Xanthomonas oryzae pv. oryzae* (Song et al., 1995). Upon activation, the intracellular kinase domain of *Os*XA21 is cleaved and re-localized to the nucleus to interact with the *Os*WRKY62 transcription factor. Although details of this interaction remain unknown, cleavage of *Os*XA21 followed by partitioning of the kinase domain to the nucleus is essential for the *Os*XA21-mediated immunity (Park et al., 2012).

Taken together, the nature of interactions between a protein's NLR/PRR and a downstream interacting TF is specific to each case. R proteins can protect TFs from the UPS-mediated degradation, enhance their DNA binding activity or release them from suppression by other proteins. Even though some molecular mechanisms driving NLR/TF interactions have been elicited, how activated NLRs modulate transcriptional reprogramming remains an unresolved and worthwhile research topic.

Regulation by Ca^{2+}

The calcium ion is an important secondary messenger that impacts the function of many TFs. For instance, the transcription of the SA biosynthesis gene Isochorismate Synthase

1 (*At*ICS1) is tightly controlled by multiple CaM-binding TFs. While some of them are negative regulators, such as *At*CAMTA3/SR and CaM-binding protein 60g (*At*CBP60g), others, such as *At*CBP60a, positively regulate SA accumulation (Kim et al., 2013; Truman et al., 2013; Wang et al., 2011; Zhang et al., 2010). Besides controlling SA biosynthesis, CaM-binding TFs also regulate SA-mediated defense responses. Interaction between CaM and *At*TGA3, the key bZIP type TF in the SA-signaling pathway, enhances the DNA-binding activity of *At*TGA3 (Szymanski et al., 1996). On the other hand, the CaM-binding NAC TF, *At*CBNAC1, functions as a transcranial suppressor by directly binding to the promoter of the SA marker gene *At*PR1 (Kim et al., 2012a).

Unlike CaMs, CDPKs not only act as Ca^{2+} sensors, but also phosphorylate their downstream target TFs to control defense transcriptional responses. For example, upon pathogen-triggered activation, *At*CPK4/5/6/11 re-localize to the nucleus to interact with, and phosphorylate, the WRKY factors *At*WRKY8/28/48. This phosphorylation is indispensable in regulating the expression of another WRKY gene, *AtWRKY46*, during ETI signaling (Gao et al., 2013). Additionally, phosphorylated *At*WRKY28 directly controls SA accumulation by regulating the expression of *AtICS1* (van Verk et al., 2011).

Collectively, upon detection of altered Ca^{2+} concentrations, activated CDPKs and CaMs bind to TFs to modulate SA biosynthesis and SA-mediated transcriptional reprogramming. CaMs control their downstream interacting TF through protein-protein interactions, whereas CDPKs bind to and phosphorylate their targets.

Regulation by MAPKs-mediated phosphorylation

MAPK cascades represent another class of signaling pathways that link stimuli to downstream responses via phosphorylate on of substrate TFs. In *Arabidopsis*, *At*MPK3 and *At*MPK6 are activated by the upstream MAPK kinases *At*MKK4 and *At*MKK5, which are activated by the upstream MAPK kinase kinase *At*MEKK1(Asai et al., 2002).

*At*MPK3 and *At*MPK6 phosphorylate *At*WRKY33 to induce expression of its target genes required for biosynthesis of the phytoalexin camalexin that confers immunity against the necrotrophic fungus *B. cinerea* (Mao et al., 2011; Stefanato et al., 2009). Phosphorylation of *At*ERF6, another target of *At*MPK3 and *At*MPK6, results in increased stability of *At*ERF6, thereby enhancing expression of *At*ERF6 target genes such as the defensin gene, *At*PDF1.2 (Meng and Zhang, 2013). *At*MPK3 also phosphorylates the VirE2 interacting protein 1 (*At*VIP1) to initiate its distribution from cytoplasm to nucleus where *At*VIP1 regulates expression of MYB and WRKY TFs responsible for SA- and JA-mediated transcriptional reprograming (Djamei et al., 2007; Li et al., 2004; Pitzschke et al., 2009; Shim et al., 2013).

In summary, although the mechanisms by which these MAPK cascades are activated during plant defenses remain to be determined, activated MAPKs control many different TFs through phosphorylation, thereby influencing their activity, stability, or localization.

Regulation by phytohormones

Given the fact that SA is a major component of PTI and SAR as well as an important signaling molecule in some R-mediated immunity, SA is proposed to be an essential intermediate signal that integrates inputs from different initial defense activation signals to initiate an appropriate defense response (Tsuda et al., 2009; Tsuda and Katagiri, 2010).

*At*NPR1 and its paralogs, *At*NPR3 and *At*NPR4, act as SA receptors (Attaran and He, 2012; Fu et al., 2012; Wu et al., 2012). Under normal conditions, *At*NPR1 proteins oligomerize in the cytosol (Mou et al., 2003). However, in response to pathogen-triggered SA accumulation, NPR1 monomerizes and enters the nucleus to activate defense gene expression through a transcriptional cascade, which includes transcription activators (e.g., TGA3) and repressors (e.g., WRKY70) (Fu and Dong, 2013; Johnson et al., 2003; Mou et al., 2003; Zhou et al., 2000).

Like *At*NPR1 in SA-mediated defense signaling, *At*MYC2 TF has emerged as a master regulator of the JA signaling pathway. Under normal conditions, jasmonate ZIM domain (JAZ) proteins, together with NOVEL INTERACTOR OF JAZ (*At*NINJAs) and the Groucho/Tup1-type co-repressor TPL (*At*TOPLESS) (Pauwels et al., 2010), form a repression complex with *At*MYC2, *At*MYC3, and *At*MYC4, three key transcriptional activators of JA responses (Chini et al., 2007; Fernández-Calvo et al., 2011). Upon wounding, herbivore or necrotrophic pathogen attack, the signaling molecule jasmonoyl isoleucine (JA-Ile) is rapidly synthesized in both local and distal tissues (Fonseca et al., 2009; Staswick and Tiryaki, 2004). The accumulation of JA-Ile promotes physical interaction between CORONATINE-INSENSITIVE 1 (COI1), an F-box protein that is part of the SCF^{COI1} (Skp1/Cullin/F-box^{COI1}) E3 ubiquitin ligase complex, and JAZ proteins (Xu et al., 2002). COI1 then ubiquitinates JAZs promoting its degradation through the UPS, leading to de-repression of MYC transcription factors and initiating the expression of JA-responsive genes (Chini et al., 2007; Katsir et al., 2008; Thines et al., 2007).

In the ethylene signaling pathway, ETHYLENE RESPONSE 1 (*At*ETR1), localizes to the membrane of the endoplasmic reticulum, and functions as a receptor for this gaseous

hormone. In the absence of ethylene, *At*ETR1 negatively regulates the key signaling component ETHYLENE INSENSITIVE 2 (*At*EIN2) through phosphorylation mediated by the protein kinase CONSTITUTIVE TRIPLE RESPONSE 1 (*At*CTR1) (Merchante et al., 2013). Upon ethylene perception, *At*EIN2 is released from negative regulation by *At*ETR1/*At*CTR1, which triggers cleavage of the C-terminal part of *At*EIN2 and consequently translocation to the nucleus to initiate expression of ethylene-responsive genes such as *At*PDF1.2 (Wen et al., 2012). Transcriptional reprograming is achieved by manipulation of two key TFs, *At*EIN3 and EIN3- LIKE 1 (*At*EIL1). In normal conditions *At*EIN3 and *At*EIL1 are constantly degraded through UPS, which is mediated by two F-box proteins, EIN3 BINDING F-BOX1 (*At*EBF1) and *At*EBF2 (Gagne et al., 2004; Guo and Ecker, 2003; Potuschak et al., 2003). The activated *At*EIN2 stabilizes *At*EIN3 and *At*EIL1 as well as induces degradation of *At*EBF1 and *At*EBF2, which, in turn, further stabilizes *At*EIN3 and *At*EIL1 (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012).

In summary, TFs involved in phytohormone signaling are abundantly expressed. However, they are kept inactive by default in the absence of pathogen threat. The mechanisms responsible for regulating their transcriptional activity relies on sequestering master regulator TFs away from nucleus where their cis acting elements and other TFs reside.

Regulation by redox

Pathogen attack is frequently associated with changes in host cell redox state (Koornneef et al., 2008; Mateo et al., 2006; Spoel and Loake, 2011). Redox changes are then sensed by intrinsically reactive Cys residues in regulatory proteins. Several Cys-containing transcriptional regulators have been shown to translate pathogen-induced cellular redox changes into transcriptional reprogramming during immune responses. For example, Cys

residues in NPR1 form disulfide bonding, resulting in a stable oligomer that is confined to the cytoplasm. Upon pathogen challenge, the accumulation of SA triggers transient cellular redox changes, which reduces NPR1 disulfide bonds to release NPR1 monomer that translocate to the nucleus to activate gene transcription (Kinkema et al., 2000; Mou et al., 2003; Tada et al., 2009). Besides NPR1, TGAs that physically interact with NPR1 to form a transcription activating complex are also redox-regulated (Boyle et al., 2009; Rochon et al., 2006; Zhang et al., 1999; Zhou et al., 2000). SA-induced redox changes are necessary to reduce disulfide bonds in TGAs to induce their interaction with NPR1 (Després et al., 2003). Thus, TFs can be directly regulated by redox changes that impact their molecular conformation and oligomerization state to control their localization and transcriptional activity.

Regulation by ubiquitination

The major function of ubiquitination in the plant immunity is targeted degradation of (co)repressors and TFs to control both timing and amplitude of defense responses upon pathogen perception. Ubiquitin (Ub) is a 76 amino acid-long highly conserved protein modifier (8.5kDa) that, upon covalent attachment to a target protein, can lead to UPS-mediated degradation or other signaling-related destiny such as re-localization or endocytosis. The fate of modified proteins is dependent on the length of its poly-Ub chain and its topology for generation of structurally diverse Ub-Ub linkages (Ikeda and Dikic, 2008). For example, protein tagged with at least four Ubs linked at the Lys48 residue is recognized by the 26S proteasome for proteolysis-based degradation (Ravid and Hochstrasser, 2008), whereas mono-ubiquitination and other linkages, such as ones linked at the Lys63, are recognized by Ub-binding receptors for non-proteolytic outcomes (Ikeda and Dikic, 2008). The 26S proteasome is a complex proteolytic barrel-like structure containing a 20S proteasome core

structure made of four stacked rings to form a central catalytic pore and two 19S regulatory lids attached at both ends of the 20S core enzymatic cylinder to recognize the Ub-marked proteins (Smalle and Vierstra, 2004). Covalent attachment of Ub to target protein requires the sequential action of three enzymes, namely E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin ligase) (Vierstra, 2009). In the first step, Ub is activated by E1 in an ATP-dependent manner, followed by Ub transfer to a Cys residue in E2 as a second step. In the last step, a member of a highly diverse class of enzymes known as E3 ubiquitin ligases recognizes the specific substrates and ubiquitinate them by either catalyzing Ub transfer from the Ub-E2 intermediate or serving as a Ub donor by itself. Single E3 ubiquitin ligases are classified into three main subfamilies depending on structural and functional characteristics such as Homologous to E6-associated protein Carboxyl Terminus (HECT), Really Interesting New Gene (RING) and U-box (Vierstra, 2009). The HECT proteins contain the N-terminal domain with an E2-Ub binding site and the C-terminal domain with the Ub accepting Cys residues, making HECTs the only type of E3 ubiquitin ligases that form a thioester intermediate with Ub before ubiquitinating their targets (Downes et al., 2003). On the contrary, RING and U-box proteins transfer Ub from the E2-Ub intermediate to the substrate using zinc chelation or hydrogen bonds/salt bridges, respectively (Stone et al., 2005; Yee and Goring, 2009). The typical RING domain is an octet of Cys (C) and His (H) amino acids coordinated by metal ion ligands. Depending on the amino acid composition, RING domains can be classified as one of two canonical structures (C3HC4, RING-HC or C3H2C3, RING-H2) or one of six modified structures with single amino acid substitution. The U-box domain is similar to the RING structure; however, the entire domain is stabilized by salt bridges or hydrogen bonds instead of chelating ions. Besides acting independently, RING type

proteins can also be a part of multisubunit Cullin(CUL)-RING ligase complexes (CRLs). Three types of CRLs have been described in plants based on the different cullin subunits (CUL1, CUL3a/3b, or CUL4) that provide scaffold for the entire complex. The second CRL subunit is one of eight possible RING structures participating in E2-Ub intermediate binding. The last subunit of CRL is a substrate-recruiting protein representing one of the following families: F-box proteins, Broad complex Tramtrack Bric-a-Bric (BTB) proteins, or DNA-Binding Domain 1(DDB1)-binding WD 40 (DWD 40) proteins (Vierstra, 2009).

Arabidopsis has only two E1s and thirty-seven E2s but possesses over 1400 E3s or components of CRL (Callis, 2014). This divergence in the number of particular type of enzymes is associated with their function. E1s mediate the conserved process of Ub activation, while E2s have more impact to the ubiquitination outcome by governing the processivity and affecting Ub chain topology (David et al., 2010; Wijk and Timmers, 2017; Windheim et al., 2008). Nevertheless, E3s are essential enzymes during ubiquitination due to their ability to specifically recognize substrate proteins and bind to their regulatory sequence termed degron that determines the degradation rate (Ravid and Hochstrasser, 2008; Schrader et al., 2009). E3 ubiquitin ligases are characterized as either positive or negative regulators of plant defense signaling depending on their target proteins. For example, in wild grapevine (*Vitis pseudoreticulata*), a RING-type E3 ubiquitin ligase, Erysiphe nectar-induced RING finger protein 1 (*Vp*EIRP1), plays a positive role in resistance against fungal and bacterial pathogens by targeting the *Vp*WRKY11 TF, a negative regulator of basal defense (Yu et al., 2013b). On the other hand, the *Arabidopsis* RING-type MYB30-Interacting E3 Ligase1 (*At*MIEL1) attenuates defense-related cell death by ubiquitinating *At*MYB30 that activates HR responses

through regulation of Very-Long-Chain-Fatty-Acids (VLCFAs) biosynthesis (Marino et al., 2013; Raffaele et al., 2008).

UPS provides plants with different mechanisms to efficiently regulate transcriptional reprograming. First, since *de novo* synthesis of transcriptional activators in response to pathogen perception can be time consuming, the proteasomal degradation of (co)repressor which keeps major transcriptional regulators inactive, ensures immediate activation of transcription. This strategy appears to be a frequent theme in phytohormonal signaling pathways. For example, the JA-Ile mediated degradation of JAZs de-represses the MYC TFs to activate JA-responsive genes (Chini et al., 2007; Katsir et al., 2008; Thines et al., 2007). Second, UPS fine-tunes the steady-state levels of transcription (co)activators before infection and destroys them when their function is no longer needed. For instance, the cytoplasmic *At*NPR1 is continuously subjected to degradation mediated by *At*NPR3 and *At*NPR4, which serve as a substrate adapter of the Cullin 3-based E3 ubiquitin ligase, to fine-tune a steady-state level of *At*NPR1 in the absence of pathogen attack. On the other hand, the pathogen/SA-induced turnover of nuclear *At*NPR1 plays an essential role in controlling the rate and termination of transcriptional reprogramming after bacterial challenge. In the presence of pathogens, plants can also indirectly stabilize defense-related TFs by reducing transcript levels of the corresponding E3 ubiquitin ligases. One example of this response is repression of *AtMIEL1* expression that release *At*MYB30 from negative regulation during plant defense responses (Marino et al., 2013). Third, some (co)activators paradoxically require UPS-mediated degradation for the full-scale activation of their transcriptional potential which is described as the activation-by-destruction mechanism. The emerging evidence of a striking overlap between activation domains and degrons further support this notion (Geng et al.,

2012). According this model, gene-specific activators initiate transcription by recruiting GTFs and PolII to the target cis-acting element in a highly ordered manner. Once transcription has successfully initiated, the re-initiation of another round of transcription cycle requires promoter clearance. Thus, to maintain a high rate of gene expression, cyclical proteasomal degradation of the "spent" activators is necessary. This type of transcriptional regulation was observed for the nuclear pool of *At*NPR1 where the turnover of transcriptional activators stimulates target gene expression during SAR (Spoel et al., 2009). The molecular mechanism that governs coupling of protein activation and turnover is linked to the sequential PTMs that cooperate with one another: one PTM either promotes (or inhibits) the establishment of a second-site PTM within the same protein. Indeed, the detailed analysis of *At*NPR1 activity revealed that sequentially linked individual PTMs generate robust and dynamic *At*NPR1-mediated transcriptional responses. Saleh et al. showed that, in the resting state, *At*NPR1 is phosphorylated at Ser55/Ser59, which prevents sumoylation of *At*NPR1 and promotes its interaction with *At*WRKY70 to repress the PR1 gene. Pathogen-induced SA accumulation leads to dephosphorylation of Ser55/Ser59 which permits sumoylation of *At*NPR1, resulting in its dissociation from *At*WRKY70. Sumoylation of *At*NPR1 is indispensable for its phosphorylation at Ser11/Ser15, which in turn leads to *At*NPR1 interaction with the *At*TGA3 to induce *AtPR1* gene expression. In this case, phosphorylation at Ser11/Ser15 both activates *At*NPR1 and marks it as "spent" to promote ubiquitination and consequent proteasomal degradation mediated by *At*NPR3, which in turn facilitates the recruitment of non-marked *At*NPR1 to the promoter of *PR1* for additional rounds of transcription. Taken together, the transient nature of *At*NPR1 activation is determined by ubiquitination sequentially linked to other PTMs (Saleh et al., 2015).

In animal systems, mono-ubiquitination of some TFs leads to re-localization and stimulation of transcriptional activity. For example, the attachment of single Ub to the Forkhead box O 4 (FOXO4), a TF involved in oxidative stress response and cell death, promotes its entry into the nucleus and enhances its transcriptional potential, whereas deubiquitination of FOXO4 by the USP7/herpesvirus-associated ubiquitin-specific protease (HAUSP) reverses this process to ensure tight balance of FOXO4-driven transcription in the response of environmental cues (Horst et al., 2006). Alternatively, the "molecular clock" model has been proposed for the regulation of the SRC-3 co-activator, where its activation by mono-ubiquitination is followed by polyubiquitination leading to destruction of this TF as a self-limiting mechanism (Wu et al., 2007).

In summary, ubiquitination modulates transcriptional reprograming by targeting both transcriptional (co)activators and (co)repressors to alter their stability, activity, and localization. Furthermore, ubiquitination acts in concert with other highly dynamic and largely reversible PTMs to orchestrate appropriate transcriptional responses.

NAC Transcription Factors

NAC genes constitute one of the largest transcription factor families in the plant kingdom with an estimated 101 members in tomato, 117 in *A. thaliana*, 151 in rice and 153 in *Nicotiana benthamiana* (Jin et al., 2017; Nuruzzaman et al., 2010; Rushton et al., 2008). The main characteristic of these plant-specific TFs is the presence of the NAC domain, a specific motif originally identified based on a common sequence in the petunia NO APICAL MERISTEM (NAM) protein (Souer et al., 1996) and in the ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR (ATAF) protein as well as Arabidopsis

CUP-SHAPED COTYLEDONS (CUC) proteins (Aida et al., 1997). The NAC domain consists of five subdomains (designated as A-E) localized to the N-terminal region. Among them, domains A, C and D are highly conserved while B and E show more diversity (Ooka et al., 2003). Subdomains C and D have been shown to be required for nuclear localization (Kikuchi et al., 2000), whereas subdomains D and E function as the DNA binding sequence. The NAC domain is accompanied by a C-terminal activation domain that is functionally linked to transcriptional activity. Additionally, some NACs have transmembrane motifs in the C-terminal region for anchoring to the plasma membrane (PM) or ER (Liu et al., 2014; Seo et al., 2008).

Role of NAC TFs in plant immunity

As a widespread plant-specific group of TFs, NACs have been implicated in a wide range of developmental processes, including seed development (Sperotto et al., 2009), embryo development (Duval et al., 2002), shoot apical meristem formation (Kim et al., 2007a), lateral root development (Xie et al., 2000), fiber development (Ko et al., 2007), cell division (Kim et al., 2006), leaflet boundaries in compound leaf (Berger et al., 2009), leaf senescence (Breeze et al., 2011; Guo et al., 2005), flower-boundary morphogenesis (Hendelman et al., 2013), and fruit maturation (Ma et al., 2014; Zhu et al., 2014). Additionally, the rapid induction of *NAC* genes in response to environmental cues makes them appreciated for their roles in both abiotic and biotic stress responses (Nuruzzaman et al., 2012). For example, it has been reported that tolerance to drought, cold and salinity stress is tightly controlled by NACs in many plant species, and transgenic plants overexpressing these NACs showed significantly improved tolerance to environmental stresses (Nakashima et al., 2012; Nuruzzaman et al., 2013; Puranik et al., 2012; Tweneboah and Oh, 2017). Furthermore, given the fact that treatment with

exogenous phytohormones, such as abscisic acid (ABA), JA, SA, and ET, results in the upregulation of *NAC* genes expression, NACs function as important nodes of signaling convergence and divergence in the molecular and biochemical regulation of plant in response to both abiotic and biotic stresses (Chen et al., 2017; Delessert et al., 2005; Feng et al., 2013; He et al., 2016; Hénanff et al., 2013; Kim et al., 2012a; Ma et al., 2013; Nakashima et al., 2007; Nuruzzaman et al., 2012; Oh et al., 2005; Sun et al., 2013; Tran et al., 2004; Wang et al., 2015, 2016a; Yoshii et al., 2010; Zhu et al., 2012). In agreement with experimental results, the promoter region of *NAC* genes is usually rich in stress-responsive cis-acting elements, such as Dehydration-responsive elements, ROS-responsive elements, and ABA-/JA-/SA- and ET-responsive elements (Nakashima et al., 2007).

Several lines of evidence support the critical role of NACs in regulation of plant defense responses against different types of pathogens. *Arabidopsis turnip crinkle virus*(TCV)-interacting protein (*At*TIP) was the first identified NAC family member linked to a plant defense response (Ren et al., 2000). *At*TIP specifically binds to the capsid protein (CP) of TCV and this interaction is crucial for induction of HR and resistance to TCV in *Arabidopsis* (Ren et al., 2000). Further genetic studies identified a number of *NAC* genes essential for regulation of defense responses in different plant species against infection of a wide range of pathogens (Table 1.1).

Table 1.1 NAC transcription factors involved in plant immunity

Plant	Gene	Regulator	Resistance to	Reference
Tomato	*Sl*NAC1	Positive	*P. syringae*	(Huang et al., 2013; Selth et al., 2005)
	*Sl*SRN1	Positive	*B. cinereal* and *P. syringae*	(Liu et al., 2014)
	*Sl*NAC35	Positive	*Pseudomonas solanacearum*	(Wang et al., 2016a)
	*Sl*JA2	Positive	*P. syringae*	(Du et al., 2014)
	*Sl*JA2L	Negative	*P. syringae*	(Du et al., 2014)
Potato	*St*NAC	Positive	*P. infestans*	(Collinge and Boller, 2001)
	NTP1/NTP2	Positive	*P. infestans*	(McLellan et al., 2013)
A. thaliana	*At*TIP	Positive	*Turnip Crinkle Virus*	(Ren et al., 2000)
	ATAF1	Negative	*B. cinerea* and *P. syringae*	(Jensen et al., 2008; Wang et al., 2009a; Wu et al., 2009)
		Positive	*Blumeria graminis f. sp. Hordei*	
	ATAF2	Negative	*Fusarium oxysporum*	(Delessert et al., 2005)
		Positive	*Tobacco Mosaic Virus*	(Wang et al., 2009b; Wang and Culver, 2012)
	ANAC019	Negative	*P. syringae* and *B. cinerea*	(Bu et al., 2008; Zheng et al., 2012)
	ANAC042	Positive	*Alternaria brassicicola*	(Saga et al., 2012)
	ANAC055	Negative	*P. syringae* and *B. cinerea*	(Bu et al., 2008; Zheng et al., 2012)
	ANAC072	Negative	*P. syringae*	(Zheng et al., 2012)
	*At*CBNAC1	Negative	*P. syringae*	(Kim et al., 2012a)
	*At*NTL6	Positive	*P. syringae*	(Seo et al., 2010)
Rice	*Os*NAC4	Positive	*Acidovorax avenae*	(Kaneda et al., 2009)
	*Os*NAC6	Positive	*Magnaporthe grisea*	(Nakashima et al., 2007)
	*Os*RIM1	Positive	*Rice dwarf virus*	(Yoshii et al., 2009, 2010)
	ONAC122/ ONAC131	Positive	*Magnaporthe grisea*	(Sun et al., 2013)
Barley	*Hv*NAC6	Positive	*Blumeria graminis f. sp. hordei*	(Jensen et al., 2007)
Grapevine	*Vv*NAC1	Positive	*B. cinerea* and *Hyaloperonospora arabidopsidis*	(Le Hénanff et al., 2013)
	*Vp*NAC1	Positive	*Erysiphe cichoracearum* and *Phytophthora parasitica*	(Zhu et al., 2012)
Chili pepper	*Ca*NAC			(Oh et al., 2005)
Egg plant	*Sm*NAC	Negative	*Ralstonia solanacearum*	(Na et al., 2016)
Bread wheat	*Ta*NAC1	Negative	*Puccinia striiformis f. sp. tritici* and *P. syringae*	(Wang et al., 2015)
Cotton	*Gb*NAC1	Positive	*Verticillium dahlia*	(Wang et al., 2016b)
	*Gh*ATAF1	Negative	*Verticillium dahliae* and *B. cinereal*	(He et al., 2016)
Canola	*Bna*NAC56	Positive	*Sclerotinia sclerotiorum*	(Chen et al., 2017)

Genetic analyses indicate that either reduced or elevated levels of defense-related NAC protein in transgenic plants results in altered expression of *PR* genes, which may explain the importance of NACs in plant immunity (Hénanff et al., 2013; Kim et al., 2012a; Liu et al., 2014; Nakashima et al., 2007; Sun et al., 2013; Wang et al., 2009b, 2016a; Zhu et al., 2012). Furthermore, some NACs were shown to directly bind to the promoter region of *PR* genes. For example, a CaM-regulated *Arabidopsis* CBNAC negatively regulates *PR1* by binding to the E0-1-1 element of its promoter. In the absence of pathogens, *At*CBNAC interacts with the protein SUPPRESSOR OF NONEXPRESSOR OF PR GENES INDUCIBLE 1 (SNI1) to enhance the DNA-binding activity of CBNAC, consequently enhancing repression of the *PR1* gene. Upon pathogen detection, the SNI1/CBNAC repressor protein complex can be disassembled by pathogen-activated NPR1, CaM or other unknown mechanisms (Kim et al., 2012a), resulting in induction of the *PR1* gene.

ROS plays an important role in plant defense signaling. All forms of ROS are viewed as highly reactive and toxic molecule. Thus, if over-accumulated, ROS can lead to oxidative stress and PCD consequently. The equilibrium in redox homeostasis is maintained by the production and scavenging of ROS (Mullineaux and Baker, 2010). Recent evidence has revealed that some NACs play a role in controlling oxidative stress. For instance, ATAF2 binds to the promoter of *Polyamine Oxidase 3* (*PAO3*) that is involved in the production of ROS from the catalysis of polyamines to activate defense responses (Moschou et al., 2008; Wang and Culver, 2012). Additionally, other studies have shown the overexpression of ATAF1, *Bna*NAC56 or *Sl*NAC35 results in enhanced accumulation of ROS and increased ROS-mediated cell death, while overexpression of *Os*NAC6 or *Sl*NAC1 leads to up-regulation

of peroxidases involved in H_2O_2 scavenging (Chen et al., 2017; Ma et al., 2013; Nakashima et al., 2007; Wang et al., 2016a; Wu et al., 2009).

Because NACs act as hubs for responses to both biotic and abiotic stresses, crosstalk between the two types of stress responses is likely. The first evidence for this phenomena was described in rye where *PR* genes are strongly induced and disease resistance is significantly enhanced after exposure to low temperatures (Hon et al., 1995; Pihakaski-Maunsbach et al., 2001). This adaptive process ensures fast physiological responses in plants to incoming pathogens that frequently invade during cold weather. The molecular link that incorporates cold signals into pathogen resistance responses was in *Arabidopsis*. Cold stimulates proteolytic activation of NTL6, which allows NTL6 to enter the nucleus to induce expression of *PR* genes by directly binding to cis-acting elements in the promoters of cold-responsive *PR* genes, including *PR1*, *PR2*, and *PR5* (Seo et al., 2010).

So far, only a few NAC TFs have been shown to be regulated by PTMs. *Os*NAC4, a key positive regulator of HR cell death in rice, is translocated into the nucleus in a phosphorylation-dependent manner upon recognition of an avirulent pathogen (Kaneda et al., 2009). *Os*RIM1 has been proposed to function as a negative regulator of JA signaling pathway, where JA accumulation results in UPS-mediated degradation of *Os*RIM1 to activate plant defense, resembling the mechanism of regulation of *At*JAZ repressors in *Arabidopsis* (Yoshii et al., 2010). In addition, the abundance of a nuclear pool of potato *St*NTP1 and *St*NTP2 is tightly controlled by UPS to attenuate defense signaling (McLellan et al., 2013).

A growing body of evidence has suggested that pathogens can directly manipulate defense mechanisms involving NACs to promote virulence. In *Arabidopsis*, *ATAF2* gene is

highly induced in response to TMV infection and the overexpression of *ATAF2* leads to the induction of defense-related genes. However, biochemical analysis reveals that ATAF2 protein is targeted for UPS-mediated degradation during TMV attack suggesting an anti-defense countermeasure (Wang et al., 2009b). Another example of pathogen countermeasures against host defenses is the use of coronatine (COR), a Ja-Ile mimicking toxin produced by *P. syringae*. COR promotes the opening of stomata for bacterial entry through manipulation of three homologous *Arabidopsis* NAC TFs ANAC019, ANAC055, and ANAC072 (Zheng et al., 2012). At the same time, COR inhibits the accumulation of the immune signaling hormone SA (Zheng et al., 2012) by inducing *At*MYC2, a master regulator of JA-signaling pathway to induce ANAC019, ANAC055, and ANAC072 which then repress ICS1 and activate BENZOATE/SALICYLATE CARBOXYL METHYLTRANSFERASE (BSMT1), two enzymes that are involved in SA biosynthesis and metabolism, respectively (Bu et al., 2008; Zheng et al., 2012). Thus, ANAC019, ANAC055, and ANAC072 are hijacked by COR to promote virulence by exploiting antagonistic cross-talk between JA and SA (Bostock, 2005). In tomato, the pathogen-triggered stomatal movement is antagonistically regulated by two most closely related NAC TFs, jasmonic acid 2 (*Sl*JA2) and JA2-like (*Sl*JA2L). While *Sl*JA2 acts as a positive regulator of *Pst*-mediated stomatal closure, *Sl*JA2L regulates stomatal reopening by controlling SA-metabolism through binding to the promoter region of the *SALICYLIC ACID METHYL TRANSFERASE 1* (*SAMT1*) gene encoding an enzyme that converts SA into the inactive MetSA (Tieman et al., 2010). As a virulent strategy, *Pst* induces expression of the *SlJA2L* gene through COR to hijack *Sl*JA2L-SAMT1 pathway, which leads to reduced accumulation of SA otherwise necessary for stomata closure (Du et al., 2014). Furthermore, the geminiviral replication enhancer (REn) protein from *Tomato leaf curl virus*

(TLCV) interacts with and induces expression of *Sl*NAC1 to increase viral DNA accumulation. Selth et al. proposed that *Sl*NAC1 is hijacked by geminiviruses, through the action of REn proteins, to promote virulence (Selth et al., 2005). In potato, an RxLR effector Pi03192 from *P. infestans* was shown to interact with two NAC Targeted by *Phytophthora* TFs (*St*NTP1 and *St*NTP2). Upon *P. infestans* culture filtrate (CF) treatment, *St*NTP1 and *St*NTP2 are released from the ER membrane to re-localize to the nucleus. The interference with CF-triggered re-localization of *St*NTP1/*St*NTP2 was suggested as Pi03192 mode-of-action to promote disease progression (McLellan et al., 2013). Interestingly, Pi03192 is not the only pathogen effector reported to alter NAC localization to interfere with plant immunity. The interaction between the CP of TCV and *At*TIP results in exclusion of *At*TIP from the nucleus, compromising its ability to regulate defense response to TCV. Remarkably, the localization of *At*TIP is monitored by a HYPERSENSITIVE RESPONSE PROTEIN (termed HRT), which guards plant cells against such virulent strategies to activate HR defense (Ren et al., 2000).

Cis-acting elements recognized by NAC TFs and their downstream target genes

Currently, only a few cis-acting elements have been described for NAC TFs (Table 1.2). The NAC recognition sequence (NACRS) (containing CATGT and harboring CACG) in the regulatory region of the drought inducible *EARLY RESPONSE TO DEHYDRATION 1* (*ERD1*) gene was identified as the core DNA binding site for three *Arabidopsis* NACs ANAC019, ANAC055, and ANAC072 (Simpson et al., 2003; Tran et al., 2004); and the same NAC core motif was further confirmed in the *Vegetative Storage Protein 1* (*VSP1*) promoter (Bu et al., 2008). Furthermore, ANAC019 was shown to recognize a CACG core binding site in the promoter of the SA synthesis gene *ICS1* and SA metabolism genes *SAGT1*/*BSMT1* to

repress or stimulate their expression, respectively, in response to *Pseudomonas* infection (Zheng et al., 2012). Taken together, studies on ANAC019 show that a single NAC TF often responds to diverse stimuli, and a NAC protein may participate in the positive or negative regulation of several seemingly disparate processes (Nuruzzaman et al., 2013). Thus, although the core site in the promoter of different target genes is the same, the flanking sequences may define both binding affinity and specificity of different NACs, allowing for the recognition of a vast array of DNA-Binding sequences and regulation of multiple downstream target genes (Shao et al., 2015).

Table 1.2 Cis-acting elements for NACs

NAC	Cis-acting sequence	Target	Method	Reference
ANAC019 ANAC055 ANAC072	5'- TCNNNNNNNACACGCATGT-3'	ERD1	Y1H	(Tran et al., 2004)
ANAC019	CACG core binding site	ICS1/SMAT1/BSMT1	ChIP	(Zheng et al., 2012)
ANAC019	5'-CATGTCCACG-3'	VSP1	EMSA	(Bu et al., 2008)
ATAF1	5'-TTGCGTA-3'	NCED3	ChIP	(Jensen et al., 2013)
ATAF2	5'-AAATAAGAAGGCAAATATAA TTTGATAACAACTATT-3'	NIT2	EMSA	(Huh et al., 2012)
ATAF2	5'-TCAGAAGAGCAATCAAATTA AAACACATAT-3'	DEFL	EMSA	(Wang and Culver, 2012)
*At*NTL6	5'-TGGACCATGTATTTACAAAA ACGTGAGATC-3'	PR1	ChIP	(Seo et al., 2010)
*At*CBNAC	5'-TAATAATGCTTAGTTATAAA TTACT-3'	PR1	EMSA/ ChIP	(Kim et al., 2007b, 2012a)
*Sl*JA2	CACG core binding site	NCED1	EMSA/ ChIP	(Du et al., 2014)
*Sl*JA2L	CACG core binding site	SAMT1 SAMT2	ChIP	(Du et al., 2014)
*Sm*NAC	CACG core binding site	ICS1	Y1H	(Na et al., 2016)

Y1H -yeast one hybrid, **ChIP** – chromatin immunoprecipitation, **EMS** - electrophoretic mobility shift assay

It is worthwhile to note that tomato has two NACs, *Sl*JA2 and *Sl*JA2L, that recognize the CACG core binding site to initiate the expression of two target genes encoding a rate-limiting enzyme of ABA biosynthesis, 9-cis-epoxycarotenoid dioxygenase 1 (NCED1) and SA metabolism enzymes SMAT1/2, respectively (Du et al., 2014). Similarly, the eggplant *ICS1* gene was identified as a direct target of *Sm*NAC based on the presence of CACG core binding site, suggesting that the recognition sequence for stress-inducible NAC TFs might be conserved across plant specious (Na et al., 2016).

Interestingly, *Arabidopsis* ATAF1 and ATAF2 were originally isolated based on their ability to activate the *Cauliflower mosaic virus* (CaMV) 35S promoter in yeast (Souer et al., 1996). Further studies have identified the consensus motif of TTGCGTA in the cis-acting element of the *NCED3* promoter as a recognition site for ATAF1 (Jensen et al., 2013) and two unrelated recognition sites for ATAF2 in the promoter of the *Defensin-like* (*DEFL*) family gene and the *nitrilase 2* (*NIT2*) (Table 1.2) (Huh et al., 2012; Wang and Culver, 2012). These data suggest that a single NAC can recognize more than one cis-acting element.

Additionally, the expression of *PR1* gene is distinctly regulated by two different NACs recognizing unrelated sites in the *PR1* promoter. While *At*NTL6 binds to the NAC core motif, a novel DNA binding sequence consisting of a GCTT motif has been identified for *At*CBNAC (Kim et al., 2007b, 2012a; Seo et al., 2010).

Tomato NAC1 TF

The tomato (*Solanum lycopersicum*) NAC1 (*Sl*NAC1) is highly induced by abiotic stresses such as chilling stress, heat stress, high salinity, osmotic stress and mechanical wounding. Consistently, transgenic tomato plants overexpressing *Sl*NAC1 (*Sl*NAC1-*oe*) show

the elevated chilling tolerance (Ma et al., 2013). *Sl*NAC1 is also up-regulated to different extents in response to ectopic application of primary defense-related hormones methyl-JA (MetJA), SA and ET, suggesting its involvement in phytohormonal signaling in response to stresses. In fact, Ma and colleagues have showed that the emission of ET in the *Sl*NAC1-*oe* tomato plants is reduced due to suppressed expression of ET biosynthesis genes *1-aminocyclopropane-1- carboxylic acid* (*ACC*) *synthase 2* (*SlACS2), SlACS4* and *ACC oxidase 1* (*SlACO1)* (Ma et al., 2013). Conversely, the opposite phenotype was observed in *Sl*NAC1-RNAi plants (Meng et al., 2016). Furthermore, the yeast one-hybrid assay provided evidence that *Sl*NAC1 can bind to the promoter region of *SlACS2,* and *SlACO1* genes, therefore, the authors concluded that the *Sl*NAC1 is a negative regulator of ET biosynthesis (Ma et al., 2014).

Moreover, given the fact that the *Sl*NAC1 transcript is also elevated in response to ROS hydrogen peroxide (H_2O_2) treatment and *Sl*NAC1-*oe* plants show enhanced activity of enzymes responsible for reduction of ROS such as superoxide dismutase (SOD) and catalase (CAT), *Sl*NAC1 is proposed to be involved in ROS scavenging (Ma et al., 2013).

The involvement of tomato *Sl*NAC1 in biotic stress first become apparent when it was found the REn protein of TLCV hijacked it during the course of viral infection (Selth et al., 2005). Interestingly, the *SlNAC1* gene is also strongly up-regulated by the non-pathogenic *PstDC3000 hrcC* strain and the avirulent *PstDC3000* strain (Huang et al., 2013; Mysore et al., 2002), suggesting that *Sl*NAC1 functions in both basal defense and in gene-for-gene resistance. However, *Sl*NAC1 appears to be a positive regulator of immunity to *Pst,* given the fact that silencing of the *NAC1* ortholog in *N. benthamiana* results in enhanced susceptibility to *Pst* (Huang et al., 2013).

As an important defense-related TF, *Sl*NAC1 is fine-tuned at both transcriptional and post-translational levels. The current body of evidence indicates that in the absence of stress, *Sl*NAC1 accumulates at a very low level due to rapid polyubiquitination and tightly controlled protein turn-over via the 26S-dependent pathway to prevent autoactivation of stress response signaling (Huang et al., 2013). Nevertheless, the dynamic regulation of *Sl*NAC1 protein stability during stress responses remains elusive.

Dissertation Outline

My doctoral research focuses on studying the molecular basis of defense signaling in tomato *(Solanum lycopersicum*) upon *Pseudomonas syringae* infection, with particular interest in regulation of the defense-related *Sl*NAC1 transcription factor. In tomato, resistance to *P. syringae* is conferred by two proteins, Pto Ser/Thr kinase and Prf NLR receptor, which work together to detect bacterial effectors AvrPtoB and AvrPto. Although Pto and Prf were identified almost two decades ago, direct downstream signaling components which translate pathogen detection into appropriate cellular defense events remain unknown.

Chapter 2 of this dissertation characterizes the *Sl*SINA3 E3 ubiquitin ligase responsible for post-translational control of *Sl*NAC1 stability. *Sl*SINA3 specifically binds to and ubiquitinates *Sl*NAC1 to promote its UPS-dependent degradation to fine tune the protein level of this important defense-related TF.

Chapter 3 of this dissertation explores the role of *Sl*NAC1 in the Prf-mediated defense response and the molecular mechanisms underlying regulation of *Sl*NAC1 stability and activity upon *P. syringae* infection. The Prf activation, upon AvrPto/AvrPtoB recognition,

results in direct interaction with and stabilization of *Sl*NAC1 through sequestering *Sl*NAC1 away from *Sl*SINA3, thereby inhibiting *Sl*NAC1 ubiquitination.

Finally, Chapter 4 summarizes all my findings in the perspective of our current knowledge about direct manipulation of transcription reprograming by pathogen-activated NLRs. In addition, this part highlights some interesting questions for future studies.

CHAPTER 2 The defense-related tomato *Sl*NAC1 transcription factor is ubiquitinated by the E3 ubiquitin ligase SEVEN IN ABSENTIA 3 (*Sl*SINA3) for proteasome-mediated degradation

Modified from:

"The ubiquitin ligase SEVEN IN ABSENTIA (SINA) ubiquitinates a defense-related NAC transcription factor and is involved in defense signaling".

New Phytol. 2016 Jul;211(1):138-48.

Abstract

We have recently identified a defense-related tomato *Sl*NAC1 (from *Petunia* NAM, *Arabidopsis* ATAF1&2, and CUC2) transcription factor that is subjected to the ubiquitin-proteasome system (UPS)-dependent degradation in plant cells. In this study, we reported a cognate tomato E3 ubiquitin ligase, SEVEN IN ABSENTIA 3 (*Sl*SINA3), that controls *Sl*NAC1 turn-over. Yeast two hybrid (Y2H), co-immunoprecipitation (CoIP) and bimolecular fluorescence complementation (BiFC) assays were used to determine the specific interaction between *Sl*NAC1 and *Sl*SINA3 *in vivo*. Furthermore, our ubiquitination assay showed that *Sl*SINA3 polyubiquitinates *Sl*NAC1 *in vitro* and promotes its degradation *in vivo*. Using quantitative Real-Time PCR analysis (qRT-PCR), we also found that, in contrast to previously reported rapid induction of *SlNAC1* expression upon *Pseudomonas* infection, the expression of *SlSINA3* is repressed. Moreover, *Agrobacterium*-mediated transient co-expression of *Sl*SINA3 with the auto-active resistance (R) proteins, including Prf^{D1416V}, Rx^{D460V} and Rpi^{D475V}, showed that *Sl*SINA3 interferes with defense-related hypersensitive reaction (HR)

cell death. Taken together, our results suggest that *Sl*SINA3 ubiquitinates the defense-related *Sl*NAC1 transcription factor for degradation and plays a negative role in the plant immune signaling.

Key words: NAC, transcription factor, E3 ligase, SINA, ubiquitination

Introduction

The tomato *Sl*NAC1 transcription factor was first described as a host protein that interacts with the geminivirus replication enhancer (REn) protein of tomato leaf curl virus, facilitating viral replication (Selth et al., 2005). Interestingly, our recent findings suggest that the *SlNAC1* gene is rapidly induced upon *Pseudomonas* infection. However, in this case *Sl*NAC1 acts as a positive regulator of plant defense, as demonstrated by the fact that silencing of *NAC1* homologs in *N. benthamiana* resulted in enhanced susceptibility to *Pseudomonas* (Huang et al., 2013). Moreover, our previous results indicate that not only is the *SlNAC1* gene tightly controlled at the transcriptional level, but also the *Sl*NAC1 protein is fine-tuned through post-translational ubiquitination resulting in protein degradation (Huang et al., 2013). The UPS-mediated protein degradation is an important strategy used by plants to maintain homeostasis of the regulatory proteins, especially those involved in stress responses, such as transcription factors and signaling kinases. Given the significant role of *Sl*NAC1 in plant defense, we hypothesized that tomato plants have developed a mechanism to regulate the abundance of *Sl*NAC1 in a spatial-temporal manner through UPS.

The UPS pathway relies on the coordinated function of three enzyme types: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) working together to covalently link ubiquitin to the substrate. Among them, the ubiquitin E1s

and E2s are relatively conserved, whereas the E3s are highly diverse and determine the substrate specificity (Harper and Schulman, 2006). The SEVEN IN ABSENTIA (SINA) ubiquitin ligases represents one class of single subunit REALLY INTERESTING NEW GENE (RING)-type E3 enzymes that directly bind to both E2 and specific substrate to facilitate ubiquitin transfer. The first SINA ubiquitin ligase was described in *Drosophila melanogaster* as an essential protein for eye development (Carthew and Rubin, 1990). SINA proteins share a characteristic multi-domain structure with a conserved N-terminal cysteine-rich C3H4 RING domain (House et al., 2003), a central section of two zinc finger motifs and a C-terminal domain responsible for substrate-binding and dimerization (SBD) (Hu and Fearon, 1999). In plants, a genome-wide analysis revealed that *SINA* genes belong to multiple-member gene families. However, only five SINA proteins have been described in detail (Den Herder et al., 2008, 2012; Ning et al., 2011; Park et al., 2010; Welsch et al., 2007; Xie et al., 2002). In *Arabidopsis*, *At*SINAT5 regulates lateral root growth (Xie et al., 2002) and floral development (Park et al., 2010). Another *Arabidopsis* protein, *At*SINAT2, was linked to carotenogenesis (Welsch et al., 2007). *Os*DIS1 form rice negatively regulates the drought stress response (Ning et al., 2011), while the *Lotus japonicas Lj*SINA4 and *Medicago truncatula*, *Mt*SINAT5 positively affect nodulation (Den Herder et al., 2008, 2012). Significantly, our knowledge about SINA's ubiquitination substrates is still limited, although a few SINA-interacting proteins have been identified. The SINA-interacting proteins include the *Arabidopsis* transcription factors *At*NAC1 (Xie et al., 2002), *At*LHY (Park et al., 2010), and *At*RAP2.2 (Welsch et al., 2007), the *Lotus symbiosis* receptor-like kinase *Lj*SYMRK (Den Herder et al., 2012) and the rice tubulin complex-related serine-threonine protein kinase *Os*Nek6 (Ning et al., 2011), but only *At*NAC1 and *At*LHY have been shown to be directly

ubiquitinated by *At*SINAT5 (Park et al., 2010; Xie et al., 2002). Given the multiple types of their putative target proteins, SINA ubiquitin ligases may regulate diverse cellular processes through ubiquitinating signaling molecules such as transcription factors and/or signaling kinases for turn-over.

Results

*Sl*NAC1 interactions with the E3 ubiquitin ligase *Sl*SINA3 in nucleus

Although the accumulation of the *Sl*NAC1 transcription factor has been shown to be regulated by UPS (Huang et al., 2013), no corresponding E3 ubiquitin ligase has been yet described. Given the fact that *At*NAC1 is targeted by *At*SINAT5 for ubiquitination and consequent degradation (Xie et al., 2000, 2002), it is possible that *Sl*NAC1 can be regulated via similar SINA-like RING-finger E3 ubiquitin ligase, even though *Sl*NAC1 is not orthologous with *At*NAC1. We BLAST-searched for *At*SINAT5 homologs in the tomato genome database (http://solgenomics.net/) and found six tomato homologs sharing 78.6% identity at the amino acid level with *At*SINAT5 and we named them *Sl*SINA1-6. The *Sl*SINA1-6 proteins contain a highly conserved RING domain, a typical SINA-specific Zn-finger domain and a substrate-binding and dimerization (SBD) domain (Hu and Fearon, 1999). To investigate the possible direct interaction between *Sl*SINA1-6 and *Sl*NAC1, which reflects an enzyme-substrate relationship, we performed a Y2H assay using *Sl*SINA1-6 as prey and *Sl*NAC1$_{1\text{-}260}$ (containing the N-terminal 260 amino acids of *Sl*NAC1; the full-length *Sl*NAC1 exhibits autoactivation in yeast (Selth et al., 2005)) as bait. The growth of yeast clones on X-gal media identified the protein-protein interaction occurring only in yeast cells containing *Sl*SINA3 and *Sl*NAC1$_{1\text{-}260}$ (Figure 2.1A). Equal protein accumulation for all

combinations of *Sl*NAC1$_{1\text{-}260}$ and *Sl*SINAs was verified by Western blotting (WB) analysis (Figure 2.1B).

To further verify specific interactions between *Sl*NAC1 and *Sl*SINA3 in plant cells, we carried out a Co-IP assay. In short, *Agrobacterium* containing constructs with the combination of epitope-tagged full-length *Sl*NAC1 (*Sl*NAC1-FLAG) with *Sl*SINA1-6-HA or a vector control were infiltrated into *N. benthamiana* leaves for transient expression. All constructs were expressed from the cauliflower mosaic virus (CaMV) 35S promoter and the proteasome inhibitor MG132 was added to the *Agrobacterium* inoculum to prevent *Sl*NAC1 degradation. After protein extraction and immunoprecipitation with the α-HA antibody matrix, the immunoprecipitated protein complex was assayed by WB using the α-FLAG or α-HA antibody.

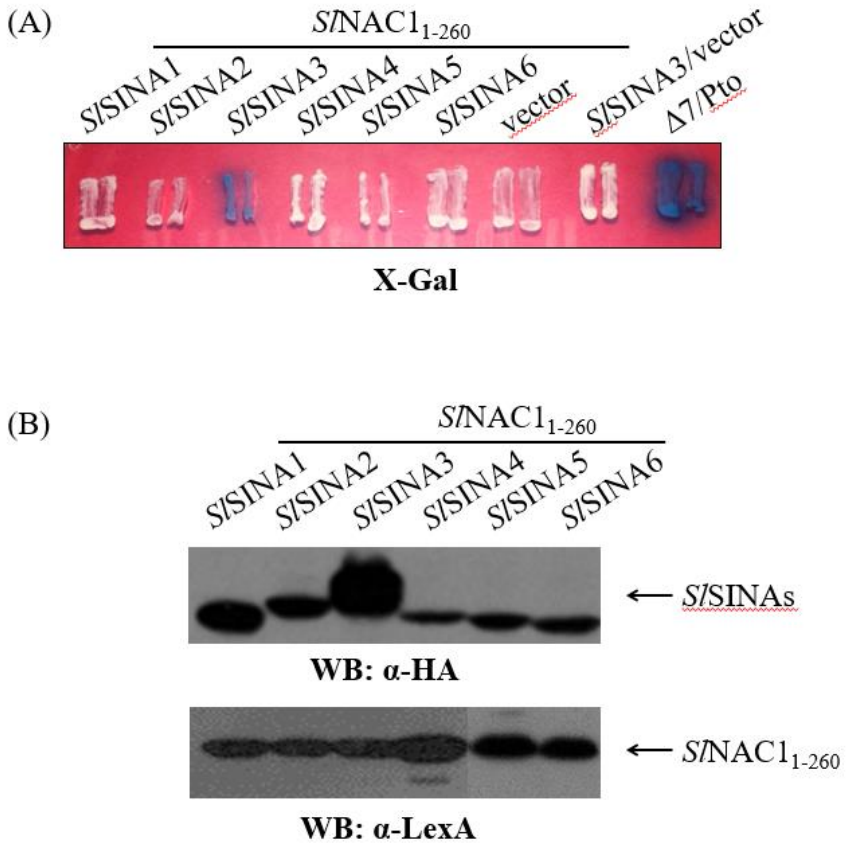

Figure 2.1 *Sl*NAC1 interacts with *Sl*SINA3 in Y2H

Interaction between *Sl*SINA (cloned into the prey vector pJG4-5) and *Sl*NAC1$_{1-260}$ (cloned into the bait vector pEG202) was examined using the LexA-based Y2H assay. **(A) Interaction between *Sl*NAC1$_{1-260}$ and *Sl*SINA3 in yeast**. Blue yeast colonies on X-gal media indicated protein-protein interaction. Yeast cells co-transformed with *Sl*SINA3 and the empty vector served as a negative control while AvrPtoBΔ7 and Pto as a positive control **(B) The accumulation of tested proteins.** WB from yeast cells co-transformed with *Sl*NAC1$_{1-260}$ and *Sl*SINAs showed accumulation of all tested proteins.

As shown in Figure 2.2, the *Sl*NAC1-FLAG protein was detected in the α-HA complex immunoprecipitated from the leaf tissue expressing *Sl*NAC1-FLAG and *Sl*SINA3-HA, but not in the immunoprecipitated complex from the leaf tissue expressing *Sl*NAC1-FLAG and any other *Sl*SINA-HAs or the vector control. These results indicate that *Sl*NAC1 specifically interacts with *Sl*SINA3 in plant cells, further suggesting that *Sl*SINA3 could be the corresponding E3 ubiquitin ligase responsible for *Sl*NAC1 degradation.

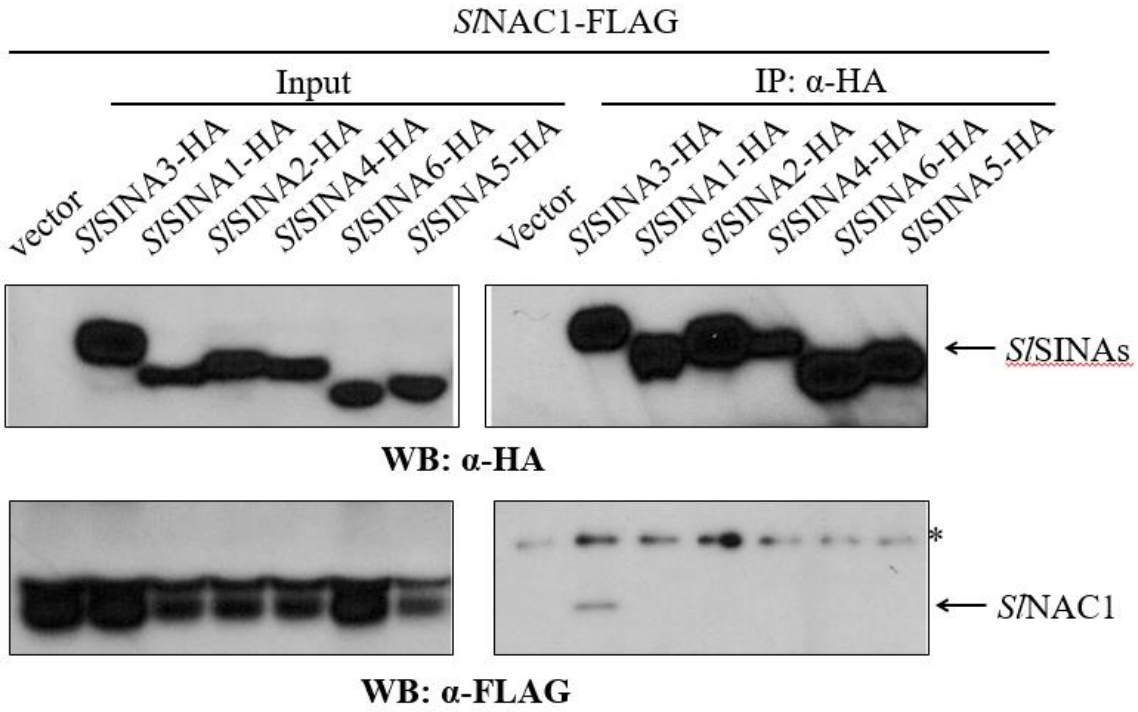

Figure 2.2 *Sl*NAC1 interacts with *Sl*SINA3 in vivo

HA-tagged *Sl*SINAs and FLAG-tagged *Sl*NAC1 were transiently co-expressed in *N. benthamiana* leaves as indicated. 34h post infiltration, leaf tissues were collected for protein extraction and IP with α-HA beads. WB with α-FLAG antibody revealed a specific interaction between *Sl*NAC1 and *Sl*SINA3. The asterisk indicates an unspecific band detected by α-FLAG antibody in all tested samples.

Next, we used BiFC (Walter et al., 2004; Kanaoka et al., 2008) to determinate where within plant cells the *Sl*NAC1-*Sl*SINA3 complex resides. The confocal microscopy results indicate that, in the presence of MG132 that prevents *Sl*NAC1 degradation, *Sl*NAC1-green fluorescence protein (GFP) was exclusively localized in the nucleus, whereas *Sl*SINA3-GFP was observed in both cytoplasm and nucleus. To determine the localization of *Sl*NAC1-*Sl*SINA3 interaction, *Sl*NAC1 was transiently co-expressed with either *Sl*SINA3 or *Sl*SINA3$_{1\text{-}181}$ (the N-terminal 181-amino-acid region of *Sl*SINA3 lacking the SBD) in BiFC vectors (Walter et al., 2004) and protein accumulation was assessed 2 days after agroinfiltration on *N. benthamiana* leaves. A strong YFP signal was observed when *Sl*NAC1-NYFP was co-expressed with *Sl*SINA3-CYFP, but not with the control *Sl*SINA3$_{1\text{-}181}$-CYFP, which lacks the SBD. Significantly, this specific interaction was limited to the nucleus of the *N. benthamiana* cells, suggesting that *Sl*NAC1 interacts with *Sl*SINA3 in nucleus (Figure 2.3).

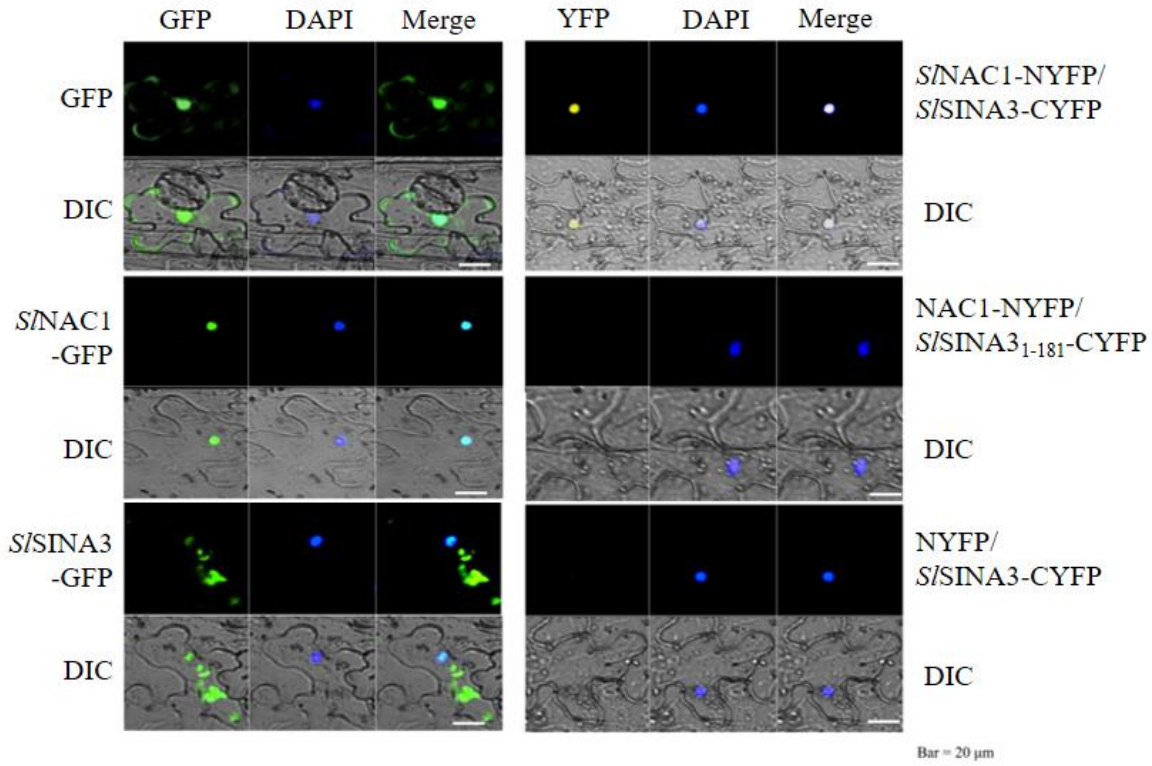

Figure 2.3 *Sl*NAC1 interacts with *Sl*SINA3 in nucleus

The localization of tested proteins was examined using transient expression of the fluorescence protein-fusion constructs in *N. benthamiana* leaves, followed by confocal microscopy of epidermal cell layers two days after agroinfiltration. MG132 (100μM) was added to prevent *Sl*NAC1 degradation. 40,6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus. **(A) Subcellular localization of *Sl*NAC1 and *Sl*SINA3**. *Sl*NAC1-GFP was co-localized in the DAPI-stained nucleus, whereas the *Sl*SINA3-GFP was localized in both cytoplasm and nucleus. Differential interference contrast (DIC) images of the same view are aligned underneath the GFP signal images. Free GFP served as a control. **(B) *Sl*NAC1 interacts with *Sl*SINA3 in nucleus.** The co-expression of *Sl*NAC1-NYFP and *Sl*SINA3-CYFP constructs resulted the YFP signal indicating interaction between *Sl*NAC1 and *Sl*SINA3, while co-expression of *Sl*NAC1-NYFP with *Sl*SINA3$_{1-181}$-CYFP or NYFP with *Sl*SINA3-CYFP did not render production of YFP signal and served as negative controls. DIC images of the same view are aligned underneath the YFP signal images.

*Sl*SINA3 possesses ubiquitin ligase activity and ubiquitinates *Sl*NAC1 *in vitro*

Given the nature of SINA-like proteins and the specific *in planta* interaction of *Sl*NAC1 with *Sl*SINA3, we next tested if *Sl*NAC1 is a substrate of *Sl*SINA3 for ubiquitination. To verify this, we first determined the self-ubiquitination capacity of *Sl*SINA3 in the presence of ubiquitin E1 and E2 enzymes. The *in vitro* ubiquitination assay was carried out as described before (Abramovitch et al., 2006) using recombinant E1 (glutathione S-transferase (GST)-AtUBA1) and E2 (GST-AtUBC8) enzymes, FLAG-tagged ubiquitin (FLAG-Ub), and maltose-binding protein (MBP)- *Sl*SINA3. The characteristic self-ubiquitination signal of *Sl*SINA3 was detected in lane 1 of Figure 2.4A, unlike the 2-5 control lanes where different essential components were missing in the ubiquitination reaction mixtures (Figure 2.4A, upper panel). With data indicating that *Sl*SINA3 is a functional E3 ubiquitin ligase, we next tested if *Sl*NAC1 is a substrate of *Sl*SINA3. To this end, the recombinant MBP-*Sl*NAC1-HA protein was added to the *in vitro* ubiquitination reaction mixture and immunoprecipitated with the α-HA antibody matrix after incubation. WB using α-FLAG antibody revealed a ubiquitin-associated ladder-like smear, indicating the presence of polyubiquitination of both MBP-*Sl*SINA3 (self-ubiquitination) and MBP-*Sl*NAC1-HA (Figure 2.4B, upper panel). Further WB analysis using α-HA antibody specifically identified MBP-*Sl*NAC1-HA in 2-5 control lanes and polyubiquitinated MBP-*Sl*NAC1-HA in lane 1 (Figure 2.4B, lower panel). Taken together, we concluded that *Sl*SINA3 ubiquitinates *Sl*NAC1 *in vitro*.

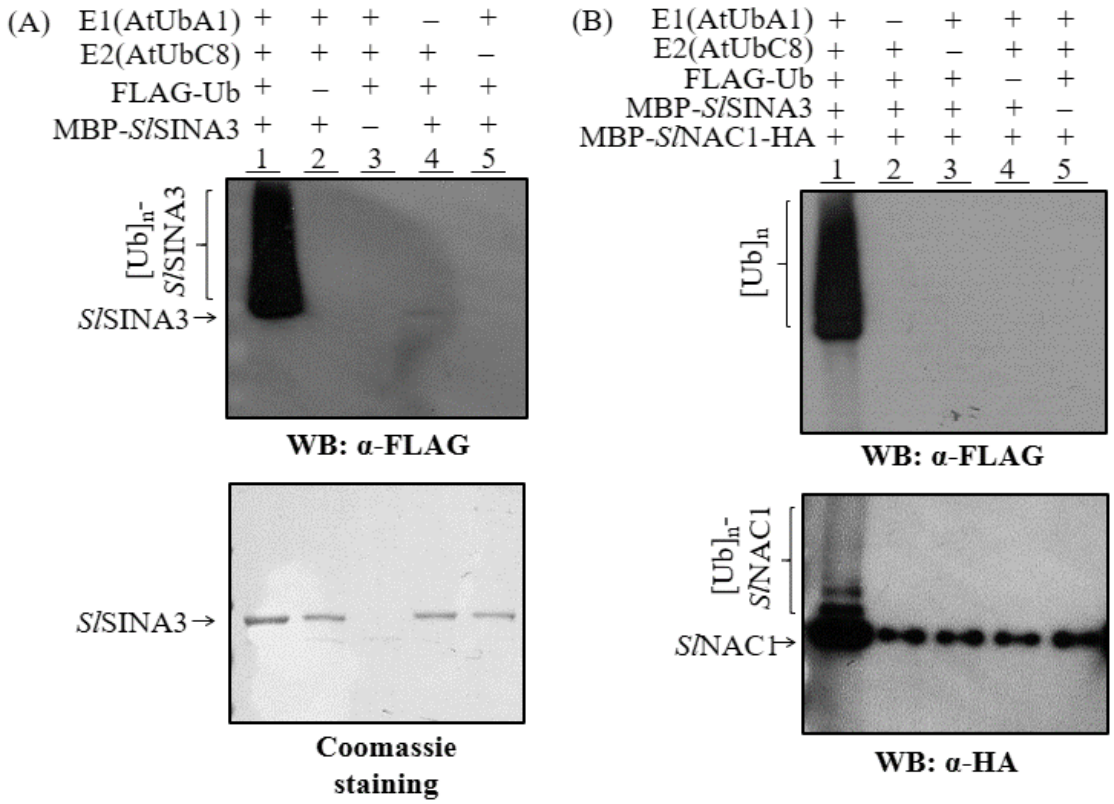

Figure 2.4 *Sl*SINA3 ubiquitinates *Sl*NAC1 *in vitro*

In vitro ubiquitination reactions were carried out using recombinant E1, E2, MBP-*Sl*SINA3, MBP-*Sl*NAC1-HA and FLAG-Ub as indicated in the Figure. **(A) The E3 ubiquitin ligase activity of *Sl*SINA3**. Polyubiquitination of *Sl*SINA3 was only observed in the presence of recombinant E1, E2 and FLAG-Ub (lane 1), but not in any control reactions in which any of the necessary components was missing (lanes 2– 5). Coomassie staining of WB membrane indicates an equal amount of *Sl*SINA3 present in the reactions (lower panel). **(B) Polyubiquitination of *Sl*NAC1 by *Sl*SINA3**. The *in vitro* ubiquitination reaction mixture was immunoprecipitated with α-HA antibody matrix to purify the MBP-*Sl*NAC1-HA substrate, followed by WB using α-FLAG antibody (top panel) or α-HA antibody (lower panel) to determine all polyubiquitination forms (including self-ubiquitination of *Sl*SINA3, which represents the majority of the polyubiquitinated protein) and the presence of polyubiquitinated MBP-*Sl*NAC1-HA protein, respectively.

*Sl*NAC1 degradation *in vivo* by *Sl*SINA3 is RING domain-dependent

Protein degradation imposed through the proteasome is the usual consequence of polyubiquitination. Therefore, we sought to determinate whether *Sl*SINA3 can promote *Sl*NAC1 degradation *in vivo*. In tomato, *Sl*NAC1 protein can be only detected by WB in the presence of proteasome-specific inhibitor MG132 (Huang et al., 2013), thus, we utilized a heterogenous *N. benthamiana* system where *Sl*NAC1 is transiently expressed using *Agrobacterium* and able to accumulate to a certain detectible level. As shown in Figure 2.5A, the level of *Sl*NAC1 was dramatically attenuated when co-expressed with *Sl*SINA3 but not with the vector control. To determine that *Sl*NAC1 degradation is dependent on enzymatic activity of *Sl*SINA3, we also included the E3 ligase deficient mutant *Sl*SINA3^{C72S}, where the conservative Cys was substituted with a Ser in the RING domain (Den Herder et al., 2012). The non-functional *Sl*SINA3^{C72S} did not significantly affect *Sl*NAC1 accumulation (Figure 2.5A), indicating that *Sl*SINA3 promotes *Sl*NAC1 degradation *in vivo*.

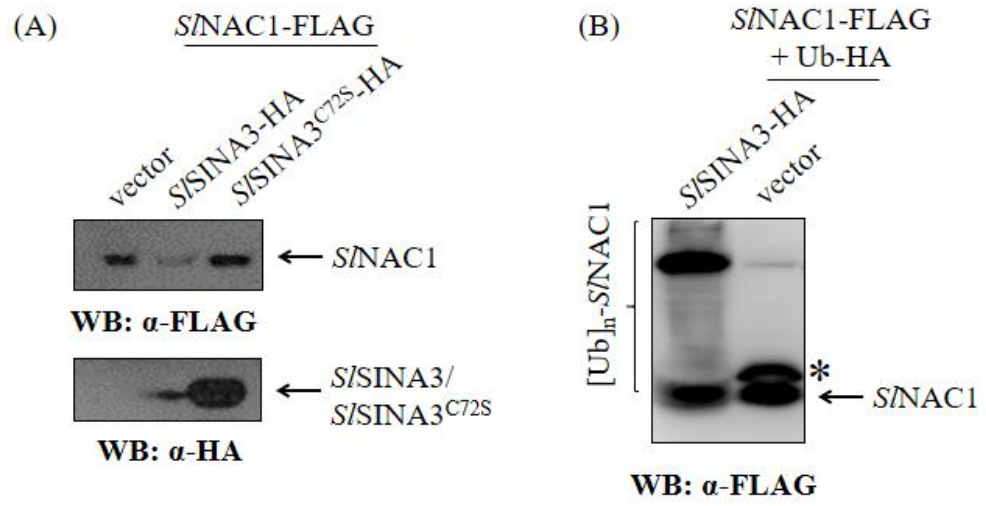

Figure 2.5 *Sl*SINA3 promotes the ubiquitination-mediated degradation of *Sl*NAC1 *in vivo*

(A) *Sl*SINA3 promotes *Sl*NAC1 degradation. *Sl*NAC1-FLAG in combination with either *Sl*SINA3-HA, *Sl*SINA3^{C72S}-HA mutant, or an empty vector were transiently expressed in *N. benthamiana*. 32h after infiltration leaf tissues were harvested for WB using the α-FLAG or α-HA antibody. *Sl*NAC1 accumulated at much lower level in presence of *Sl*SINA3, whereas *Sl*SINA3^{C72S} mutant did not interfere with *Sl*NAC1 abundance. (**B) *Sl*SINA3 ubiquitinates *Sl*NAC1 *in vivo*.** HA-Ub and *Sl*NAC1-FLAG were co-infiltrated into *N. benthamiana* leaves together with either *Sl*SINA3-HA or the empty vector as a control. MG132 (100 μM) was added to the *Agrobacterium* suspension to prevent the degradation of *Sl*NAC1 protein. 32h after infiltration, leaf tissues were harvested for WB using the α-FLAG to determine *Sl*NAC1-associated polyubiquitin chain, which appears as a smear. The asterisk indicates an unidentified modification form of *Sl*NAC1.

We next sought to examine the potential ubiquitination of *Sl*NAC1 by *Sl*SINA3 *in vivo*. *Sl*NAC1-FLAG and HA-Ub were co-expressed in *N. benthamiana* leaves with either *Sl*SINA3-HA or vector control in the presence of MG132 to prevent *Sl*NAC1 deration. The polyubiquitinated *Sl*NAC1-HA moieties, indicated as the smear banding pattern above the *Sl*NAC1-HA, were detected in the presence of *Sl*SINA3 but not the vector control. Surprisingly, in the absence of *Sl*SINA3, a second slower-migrating *Sl*NAC1 form was detected (indicated by the asterisk in Figure 2.5B), however, the identity of this *Sl*NAC1 band was not further determined. Taken together, these data suggest that *Sl*SINA3 is an E3 ubiquitin ligase responsible for the ubiquitination-mediated degradation of *Sl*NAC1 in plant cells.

In contrast to up-regulation of the *SlNAC1* gene, the expression of the *SlSINA3* gene is down-regulated during the defense response to *Pseudomonas* infection

We have recently shown that *SlNAC1* gene expression is highly induced during *Pseudomonas* infection, pointing out a role of *Sl*NAC1 in defense signaling. Increased synthesis and/or accumulation of the *SlNAC1* transcript is one of the mechanisms used by plants to produce more *Sl*NAC1 protein to compensate for its degradation by the ubiquitin ligase (Huang et al., 2013). However, plants might also adopt other strategies to interfere with the ubiquitination and ensure accumulation of *Sl*NAC1 protein by repressing transcription of the gene encoding the cognate E3 ubiquitin ligase. To test that hypothesis, we measured the abundance of *SlSINA3* transcript side by side with *SlNAC1* transcript during the response to *Pseudomonas syringae pv. tomato DC3000* (*PstDC3000*) infection. Three different interactions between tomato and *PstDC3000* were examined: the PTI interaction of susceptible RG-*prf3* plants inoculated with the non-pathogenic *PstDC3000 hrcC* mutant strain (Deng et al., 1998); the disease interaction of susceptible RG-*prf3* plants (containing a 1 kb

deletion in the *Prf* gene) (Salmeron et al., 1996) inoculated with *PstDC3000*; the ETI interaction of resistant RG-*PtoR* plants (expressing the resistance gene *Prf*) inoculated with *PstDC3000*. RG-*prf3* plants infiltrated with 10 mM $MgCl_2$ served as a mock control. To determine the correlation between the expression patterns of *SlNAC1* and *SlSINA3* genes, we monitored the transcript levels of both genes at 0h, 2h, 4h and 8h after bacterial infiltration by qRT-PCR analysis. As expected, the *SlNAC1* mRNA was induced in all three interactions (Figure 2.6A). Significantly, the *SlSINA3* mRNA was reciprocally repressed and the down-regulation pattern of *SlSINA3* gene was inversely correlated with the up-regulation pattern of the *SlNAC1* gene in the tested tomato-*PstDC3000* interactions (Figure 2.6B). Together with our previously findings (Huang et al., 2013), these results suggest that tomato has evolved a complex mechanism to tightly regulate the abundance of *Sl*NAC1 protein in response to *Pseudomonas* infection by not only up-regulation of the *SlNAC1* gene to presumably produce more *Sl*NAC1 protein but also down-regulation of the *SlSINA3* gene encoding the E3 ubiquitin ligase to compensate for *Sl*NAC1 degradation.

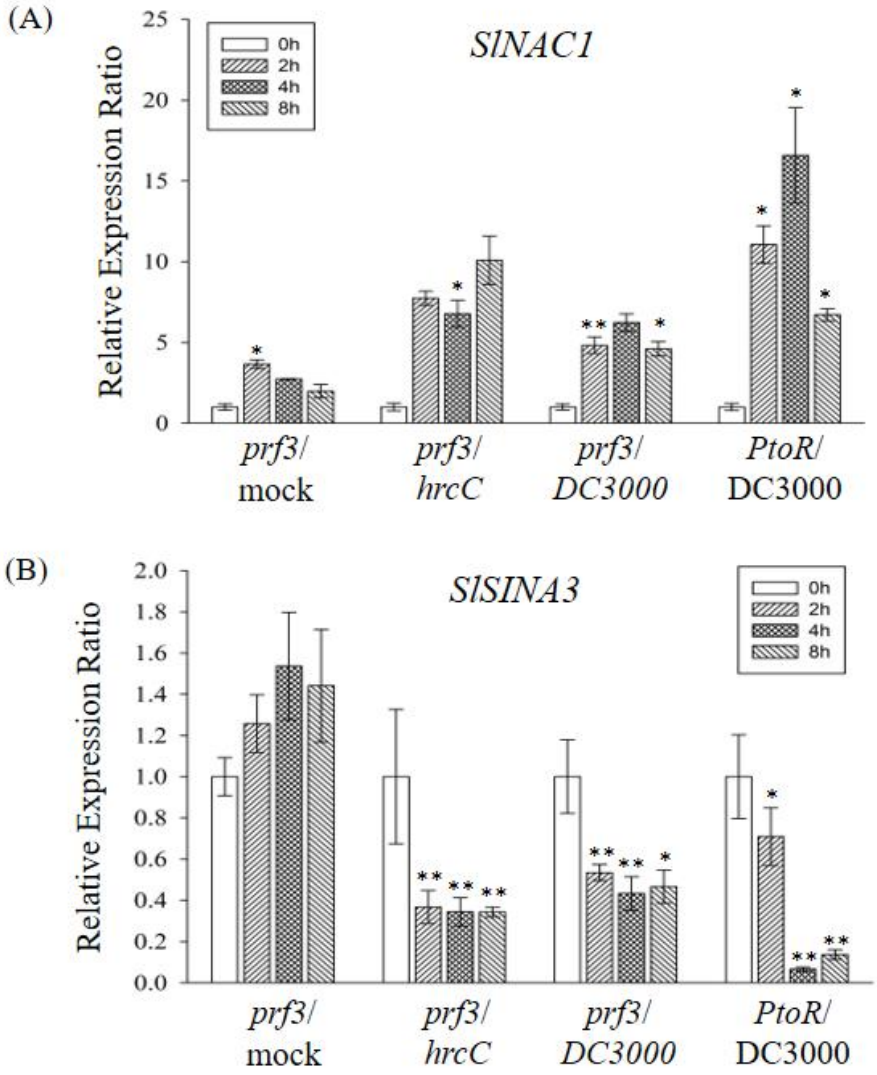

Figure 2.6 The inverse correlation between *SlNAC1* and *SlSINA3* expression during *Pst*DC3000 infection

Resistant RG-*PtoR* or susceptible RG-*prf3* tomato plants were inoculated with appropriate *Pst*DC3000 strains as indicated at an inoculum of $2x10^7$ colony forming units (CFU)/ml or mock solution (10mM $MgCl_2$). Total RNA was isolated at different time-points after *Pst* infiltration and were used to generate cDNA. The relative expression level of **(A) *SlNAC1*** gene and **(B) *SlSINA3*** was measured by qRT-PCR using gene-specific primers with the tomato *EF1α* gene as an internal control for normalization. The presented values are means ± SE of three replicates. The experiment was repeated three times with similar results. (*P < 0.01; Student's t test).

Overexpression of *Sl*SINA3 represses R protein-mediated HR cell death in *N. benthamiana*

To determinate the biological significance of the *Sl*SINA3 E3 ubiquitin ligase in plant defense responses, we examined the effect of *Sl*SINA3 on HR signaling mediated by the tomato resistance protein Prf, which confers resistance to *PstDC3000* (Salmeron et al., 1996). We carried out the HR cell death suppression assay using the auto-active mutant form of Prf, Prf^{D1416V}, which triggers defense-related cell death in the absence of *PstDC3000* when transiently expressed in *N. benthamiana* leaves (Du et al., 2012). Prf^{D1416V}-FLAG was co-expressed with *Sl*SINA3-HA or a vector control in *N. benthamiana* leaves via *Agrobacterium*-mediated expression at a 4:1 (*Sl*SINA3:Prf^{D1416V}) inoculum ratio. As shown in Figure 2.7A, the HR cell death triggered by Prf^{D1416V} was abolished in the presence of *Sl*SINA3, suggesting that *Sl*SINA3 negatively regulates the plant defense signaling. Given the E3 ubiquitin ligase nature of *Sl*SINA3, we sought to test a possibility that *Sl*SINA3 suppresses HR cell death by promoting Prf^{D1416V} ubiquitination and consequent degradation. To this end, we co-expressed Prf^{D1416V} with *Sl*SINA3 at a slightly higher concentration of inoculum, OD_{600} = 0.3, and assessed protein accumulation by WB. As shown in Figure 2.7A, our result demonstrate *Sl*SINA3 does not trigger Prf^{D1416V} degradation. We next asked whether the cell death suppression activity of *Sl*SINA3 is specific to Prf-mediated HR cell death signaling, or whether *Sl*SINA3 acts as a general negative regulator for HR cell death. We assessed the ability of *Sl*SINA3 to interfere with HR cell death triggered by two other autoactive R proteins, Rpi-blb1^{D475V} and Rx^{D460V}, both of which cause HR cell death when overexpressed in *N. benthamiana* leaves (Bendahmane et al., 2002; van Ooijen et al., 2008). We found that Rpi-blb1^{D475V}- or Rx^{D460V}-triggered cell death was suppressed by overexpression of *Sl*SINA3, and

*Sl*SINA3 did not trigger Rpi-blb1^{D475V} or Rx^{D460V} degradation (Figure 2.7B, C). The Rx^{D460V} protein was not detected when expressed with the empty vector control (Figure 2.7C), presumably as a result of the nonspecific protein degradation caused by extremely strong cell death.

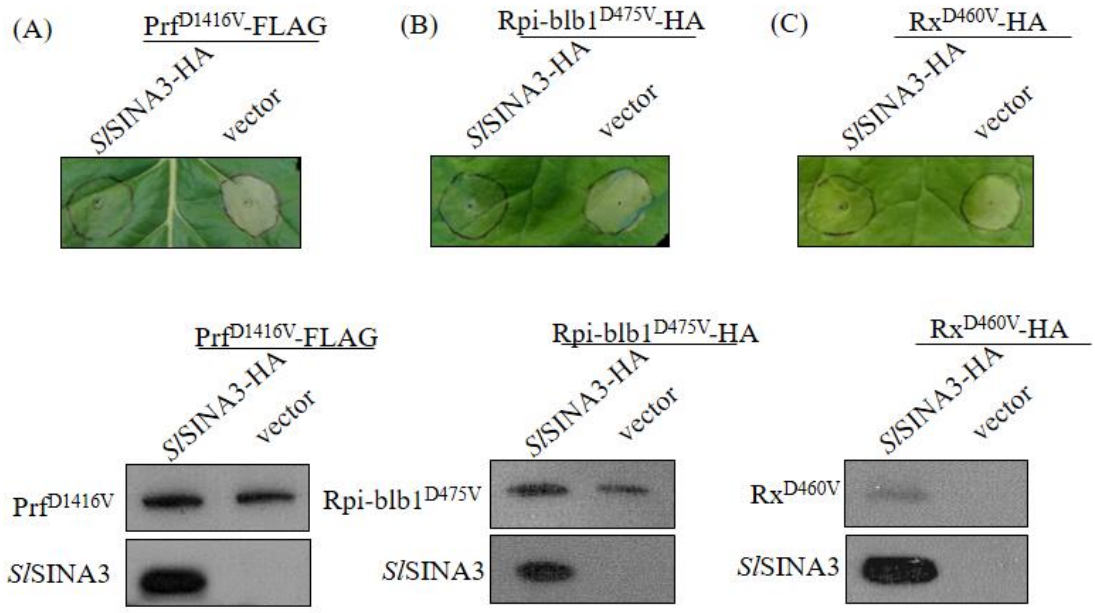

Figure 2.7 *Sl*SINA3 represses HR type cell death mediated by multiple auto-active resistance proteins

N. benthamiana leaves were transiently co-expressed with **(A) Prf^{D1416V}-FLAG, (B) Rpi-blb1^{D475V}-HA** or **(C) Rx^{D460V}-HA** and either *Sl*SINA3-HA or vector as a control. Photographs showing HR type cell death suppression by *Sl*SINA3 but not vector control were taken 3 days after agroinfiltration. WB results indicated that *Sl*SINA3 does not trigger degradation of Prf^{D1416V}, Rpi-blb1^{D475V} or Rx^{D460V}.

Discussion

Given the diverse roles of NACs in physiological processes including development and stress responses, a complex multi-level regulation of those transcription factors is

essential for plant cell homeostasis. NACs are controlled at the transcriptional level by either induction or suppression upon internal or external stimuli (Olsen et al., 2005; Puranik et al., 2012). Some NACs, like CUC1/2 and *At*NAC1, are also regulated post-transcriptionally via miRNA-mediated cleavage to control abundance of already present transcripts (Olsen et al., 2005; Puranik et al., 2012). The third mechanism of regulation is achieved through post-translational modifications of NACs. For example, *At*NTL6 and *Os*NAC2 are phosphorylated by the SnRK2.8 kinase and an as-yet-unidentified kinase (Kaneda et al., 2009; Kim et al., 2012b), respectively, and *At*NAC1 is ubiquitinated by the SINAT5 ubiquitin ligase (Xie et al., 2002).

We previously reported the transcriptional and post-translational regulation of the *Sl*NAC1 transcription factor, as shown by up-regulation of *SlNAC1* expression in response to *Pseudomonas* infection and the ubiquitination of *Sl*NAC1 protein (Huang et al., 2013). In this work, we sought to identify the E3 ubiquitin ligase that regulates *Sl*NAC1 protein stability. We found six tomato SINA ubiquitin ligases (*Sl*SINA1-6) and verified that only *Sl*SINA3 specifically interacts with *Sl*NAC1 in nucleus. We further demonstrated that *Sl*SINA3 ubiquitinates *Sl*NAC1 *in vitro* and promotes *Sl*NAC1 degradation *in vivo* in an E3-ligase dependent manner. Moreover, the expression pattern of the *SlSINA3* gene was inversely correlated with the expression pattern of the *SlNAC1* gene. Finally, we showed that *Sl*SINA3 acts as a negative regulator of HR cell death by interfering indirectly with defense signaling mediated by multiple resistant proteins.

Considering the importance of *Sl*NAC1 for plant disease resistance, it is not surprising that this transcription factor is fine-tuned at both transcriptional and post-translational levels (Huang et al., 2013). We speculate that, under normal conditions, plants tightly regulate TFs

like *Sl*NAC1 to prevent auto-activation of defense signaling. According this hypothesis, the *SlNAC1* gene is expressed at a low basal level to produce a limited amount of *Sl*NAC1 protein, and at the same time, rapid protein turnover of *Sl*NAC1 driven by *Sl*SINA3-mediated ubiquitination helps to maintain the signaling balance. When *Pst* infects, the expression of *SlNAC1* is rapidly induced to compensate for the degradation of *Sl*NAC1 protein. Moreover, *SlSINA3* gene is down-regulated to facilitate *Sl*NAC1 accumulation by interfering with its ubiquitination.

It is interesting to note that poly-ubiquitination may not be the only PTM of *Sl*NAC1. *Sl*NAC1 protein was detected in two different forms by WB in our *in vivo* ubiquitination experiment. An additional slow-migrating form of *Sl*NAC1 in the absence of *Sl*SINA3 (Figure 2.5B) indicates an as-yet-unidentified modification of *Sl*NAC1 protein. Significantly, this modification was abolished when *Sl*SINA3 was co-expressed *in planta*. We speculate that this might be a mono-ubiquitinated and/or phosphorylated form of *Sl*NAC1, a topic to be explored in future experiments.

So far, all the SINA E3 ubiquitin ligases identified from different plant species seem to function as negative regulators of certain physiological processes. For example, *At*SINAT5 targets the *At*NAC1 transcription factor which is essential for the auxin-mediated lateral root development, to negatively control lateral root growth (Xie et al., 2002). *Lj*SINA4 plays a role in response to *Sinorhizobium* infection and is speculated to negatively affect the nodulation process by targeting the symbiosis receptor-like kinase (SYMRK), a positive regulator in symbiotic signal transduction, for degradation (Den Herder et al., 2012). *Os*DIS1 plays a negative role in drought stress tolerance, presumably by targeting the tubulin complex-related kinase *Os*Nek6, although the evidence for the ubiquitination of *Os*Nsk6 by *Os*DIS1 is

still lacking (Ning et al., 2011). Our data indicate that *Sl*SINA3 also functions as a negative regulator in plant defense responses by targeting *Sl*NAC1, a positive defense-related regulator, for degradation (Figure 2.5A). This notion is further supported by interference of overexpressed *Sl*SINA3 with defense-related HR cell death triggered by different resistance proteins (Figure 2.7).

Although no other *Sl*SINA3-interacting proteins have been yet identified, it is possible that, besides the *Sl*NAC1 transcription factor, *Sl*SINA3 also ubiquitinates other positive regulators of HR cell death signaling to facilitate their degradation. In fact, the *Sl*SINA3-*Sl*NAC1 interaction is restricted to the nucleus (Figure 2.3), suggesting that *Sl*SINA3 ubiquitinates *Sl*NAC1 for proteasome-mediated degradation in this cellular compartment. Given that *Sl*NAC1 exclusively accumulates in nucleus, whereas *Sl*SINA3 can be detected in both the nucleus and cytoplasm, it is likely that *Sl*SINA3 has other cytoplasm-localized targets. Thus, identification of additional *Sl*SINA3 substrates will help to elucidate the mechanistic basis by which *Sl*SINA3 regulates defense responses. Interestingly, *Sl*SINA3 interferes with HR cell death triggered by multiple R proteins without affecting their accumulation, suggesting the target(s) of *Sl*SINA3 my play a common role downstream of different R proteins at a convergence point of HR cell death signaling.

Acknowledgement

I would like to thank Dr. Min Miao for performing BiFC assay and *in vivo* ubiquitination experiments. I would like to also thank Dr. Xianlgi Niu for performing CoIP and *in vitro* ubiquitination assays.

Materials and Methods

Yeast Two Hybrid (Y2H)

A LexA yeast two-hybrid (Y2H) assay was used to detect protein-protein interactions. *Sl*NAC1$_{1\text{-}260}$ was cloned into the bait vector pEG202 (-His selection) at the *Eco*RI and *Sal*I sites, whereas the *Sl*SINA genes were cloned into the prey vector pJG4-5 (-Trp selection) at *Eco*RI and *Xho*I sites. The bait and prey constructs were then co-introduced into yeast (*Saccharomyces cerevisiae*) EGY48 containing pSH18-34 LacZ reporter plasmid (-Ura selection) using LiAc-mediated transformation (Gietz and Woods, 2002) and selected on -Ura/-His/-Trp media. To evaluate protein-protein interactions, transformed yeast cells were streaked onto YPD medium with X-Gal. Photographs were taken at two days after incubation at 30°C.

Agrobacterium-mediated transient assay

Agrobacterium-mediated transient expression was carried out as described previously (Xiao et al., 2007). *Agrobacterium tumefaciens* GV2260 strains expressing FLAG-tagged, HA-tagged or Ub-tagged protein were syringe-infiltrated into *N. benthamiana* leaves. The concentrations of bacterial inocula were depended on the construct and the type of experiment ranging from OD_{600} 0.05 to 0.4. In certain experiments, MG132 was added to *Agrobacterium* inoculum (final concertation of 100μM), to inhibit proteasomal degradation of transiently expressed proteins.

Western blotting (WB), co-immunoprecipitaion (CoIP) and *in vivo* ubiquitination assay from plant tissue

Agrobacterium-infected *N. benthamiana* leaf tissues were collected at 28-36h after infiltration and ground with liquid nitrogen. The fine tissue powder was resuspended with 300 µl of protein extraction buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 5mM EDTA, 2mM DTT, 10% glycerol, 1% polyvinylpolypyrolidone, 1mM PMSF, plant protease inhibitor cocktail (Sigma-Aldrich, USA)) and centrifuged at 13,000g/4°C for 15 minutes. For the immunoprecipitation assay, one-tenth of each protein extract (v:v) was saved as the input sample, and the rest of the of the protein solution was used for precipitation with either an anti-HA affinity matrix (Roche Applied Sciences) or an α-FLAG affinity matrix (Sigma-Aldrich) for 2h at 4°C. The immunoprecipitated protein complex was washed four times with washing buffer (50mM Tris-HCl, pH 7.5, 250mM NaCl, 5mM EDTA, 10% glycerol, 1mM PMSF). Protein samples were separated by SDS-PAGE and detected using the α-HA or α-FLAG antibody.

Western blotting (WB) form yeast cells

Yeast cells co-transformed with pEG202::*Sl*NAC1$_{1\text{-}260}$ and pJG4-5:: *Sl*SINA1-6 were grown overnight with YPD media, then spun down at 12,000 rpm for 1 min and resuspended in 200 µl of lysis buffer (50mM Tris-HCL pH 7.5, 150mM NaCl, 1% Triton X100, 1mM EDTA, 5mM DTT and protease inhibitor cocktail for yeast extracts (Sigma-Aldrich, USA)). An equal volume of glass beads was added to the yeast suspension and the tubes were vortexed at the 13,000 rpm for 1 min followed by 1 min incubation on ice -repeated five times. Samples were then centrifuged at 13,000 rpm/4ºC for 10 min, 5xSDS PAGE buffer was added to the

supernatant and boiled for 5 min. Proteins were separated by SDS-PAGE and detected using α-HA antibody for *Sl*SINAs or α-LexA antibody for *Sl*NAC1$_{1-260}$.

In vitro ubiquitination assay

*Sl*SINA3 and *Sl*NAC1-HA were cloned into the pMAL-c2 vector using *EcoRI* and *SalI* to generate the MBP-fusion proteins. The recombinant proteins were expressed in *Escherichia coli* BL21 using 0.5μM IPTG for induction. The *in vitro* ubiquitination assay was performed as described previously (Abramovitch et al., 2006) with few adjustments. 40ng GST-E1 (*At*UBA1), 100ng GST-E2 (*At*UBC8), 1μg MBP *Sl*SINA3, 2μg FLAG-Ub (Boston Biochem, USA) were combined together with the ubiquitination buffer (50mM Tris HCl, pH7.5, 2mM ATP, 5mM $MgCl_2$, 30mM creatine phosphate (Sigma-Aldrich) and 50ng/μl creatine phosphokinase (Sigma-Aldrich, USA)). The 30μl reaction mixture was incubated for 2h at 30^{o}C. For *Sl*NAC1 *in vitro* ubiquitination by *Sl*SINA3, 500ng MBP-*Sl*NAC1-HA was used as a substrate. To immunoprecipitate the ubiquitinated MBP-*Sl*NAC1-HA protein, 15μl anti-HA affinity matrix (Roche Applied Science, USA) was added to the reaction mixture and incubated for another 2h at 4^{o}C. Beads were then washed three times with the washing buffer (20mM Tris HCl, pH7.5, 0.1M NaCl, 0.1mM EDTA, 0.05% Tween 20). Proteins were separated with 7.5% SDS-PAGE and identified by WB using the α-FLAG or α-HA antibody (Sigma-Aldrich, USA).

Site-directed mutagenesis

The C72S substitution in the RING domain of *Sl*SINA3 was introduced using PfuUltra polymerase-driven PCR and primers containing the desired mutation in the center with correct *Sl*SINA3 sequences on both sides (F 5'-CTTGAATGCCCTGTTAGTACTAATTCAATGTAT-3', R 5'-

ATACATTGAATTAGTACTAACAGGGCATTCAAG-3' – the mutation site underlined), and *Sl*SINA3 cloned into a small vector pTEX::HA as a PCR template. DpnI restriction enzyme was added to digest original template DNA after mutation-introducing PCR and DNA was transformed into DH5α *E.coli* cells. C72S substitution was verified by sequencing and *Sl*SINA3 C72S with the entire expression cassette (including 35S promoter and NOS terminator) was next re-cloned into final pBTEX binary vector for transient expression in plants using EcoRI and HindIII restriction sites.

qRT-PCR

Total RNA from tomato plants was isolated with TRIzol reagent (Invitrogen). One microgram of total RNA was treated with DNase I (Invitrogen), followed by reverse transcription using a Super Script II reverse transcriptase (Invitrogen). qRT-PCR analysis was performed on an ABI Prism 7100 sequence detection system using Power SYBR Green reagents (Life Technologies, Carlsbad, CA, USA). The tomato Actin gene was used as an internal control for normalization. Relative expression ratios were determined based on the comparative CT method (ΔΔCT) using the StepOne Software.

Table 2.1 Primer sequence for qRT-PCR

Primer name	Primer sequence
***Sl*EF1_RTF**	5’-ATTGGAAATGGATATGCTCCA-3’
***Sl*EF1_RTR**	5’-TCCTTACCTGAACGCCTGTCA-3’
***Sl*NAC1_RTF**	5’-ATGGGACGAAGACCACAGAAAC-3’
***Sl*NAC1_RTR**	5’-GTCTTGGAAATTGTTGAACTGGTC-3’
***Sl*SINA3_RTF**	5’-CTTTCCTCCAAGCAGAAGCTTAAA-3’
***Sl*SINA3_RTR**	5’-CACTCTCTGTCTTCAGATGTGATG-3’

CHAPTER 3 The mechanistic basis by which the Prf resistance protein manipulates the defense-related *Sl*NAC1 transcription factor during defense signaling

Abstract

In tomato, resistance to bacterial speck disease (caused by *Pseudomonas syringae pv. tomato (Pst)*) is determined by Prf, a nucleotide binding domain and Leucine-rich repeat (NLR)-type resistance protein that remains inactive in the absence of *Pst.* The activation of Prf, triggered by recognition of the *Pst* effectors AvrPto and AvrPtoB, has been well-investigated, but the transmission of a defense signal from the activated Prf to downstream cellular defense events is poorly understood. We have recently reported that a defense-related NAM, ATAF1.2, CUC2 1 (*Sl*NAC1) transcription factor is a positive regulator of the Prf-mediated defense signaling and its stability is tightly controlled by the *Sl*SINA3 E3 ubiquitin ligase that ubiquitinates *Sl*NAC1 for proteasome-mediated degradation. Here, we investigate the molecular mechanism underlying *Sl*NAC1 regulation in the Prf-mediated disease resistance. We found that activated Prf, in its signaling-competent form, can interact with and stabilize *Sl*NAC1 *in vivo*. *Pst* infection rendered *Sl*NAC1 stabilization only in the resistant RG-*PtoR* plants containing the functional *Prf* gene. Significantly, *Sl*NAC1 was a positive regulator of the Prf-mediated hypersensitive response (HR) and signaling-competent Prf stimulated *Sl*NAC1 transcriptional activity. Moreover, upon challenge with *Pst*, transgenic RG-*PtoR* tomato plants overexpressing *Sl*NAC1 exhibited enhanced resistance to the avirulent *PstDC3000* strain and partial resistance to virulent *PstDC3000ΔAvrPtoΔAvrPtoB* strain, which was associated with elevated level of SA-marker genes and reduced expression of

JA-marker genes. Our ubiquitination analysis and *in vitro* binding assay suggest the mechanism of action where the signaling-competent Prf interferes with *Sl*NAC1 ubiquitination by sequestering *Sl*NAC1 away from *Sl*SINA3 to prevent *Sl*NAC1 degradation, thereby, enabling robust transcriptional reprograming upon pathogen perception.

Key words: resistance protein, NAC, transcription factor, E3 ligase, RING

Introduction

NLR receptors possess a modular structure with an amino-terminal Toll/Interleukin-1 receptor (TIR) or coiled-coil (CC) domain, a central nucleotide-binding (NB)-APAF-1, R proteins, and CED-4 (ARC) domain and a carboxyl-terminal Leucine-rich repeat (LRR) domain. NLRs are classified as members of the signal transduction ATPases with numerous domains (STAND) family of P-loop NTPases, in which the NB-ARC domain functions as an ATP-hydrolyzing switch. The reversible intra- and inter-molecular interactions among domains make NLRs a dynamic signaling molecule that determines whether or not a plant activates its defense responses (Takken et al., 2006; Takken and Goverse, 2012). According this model, in the resting state, the LRR and CC/TIR domain interact with the NB-ARC domain to form a closed auto-inhibited conformation. When the corresponding pathogen infects, an ATP-dependent conformational change occurs resulting in an open, signaling-competent protein state that initiates further defense signaling and activation of defense responses (Takken et al., 2006; Takken and Goverse, 2012).

The evidence is mounting that pathogen-activated NLRs may control reprograming machinery by directly manipulating defense-related transcription factors (TFs). Recent discoveries on barley Mildew A 10 (*Hv*MLA10), *Arabidopsis* SUPPRESSOR OF NPR1-1,

CONSTITUTIVE 1 (*At*SNC1), rice Panicle blast 1 (*Os*Pb1), and tobacco N proteins shed light on the activation mechanism underlying defense signaling in nucleus. The pathogen-activated *Hv*MLA10 shuttles to the nucleus to interact with two WRKY TFs, *Hv*WRKY1/2, consequently interfering with their repression activity on defense signaling (Shen et al., 2007). Moreover, in the absence of pathogen, *Hv*WRKY1 binds to *Hv*MYB6 to sequester it so that it cannot bind the cognate cis-acting elements of defense-related genes. Upon activation, *Hv*MLA10 also releases *Hv*MYB6 from *Hv*WRKY1 repression and stimulates its binding to the target DNA (Chang et al., 2013). *Arabidopsis* SNC1 is another NLR protein that interacts with both transcriptional repressors and activators during defense responses. *At*SNC1 activates plant immunity by associating with transcriptional co-repressor Topless related 1 (*At*TPR1), which in turn blocks the expression of its target genes, DEFENSE NO DEATH 1 (*At*DND1) and *At*DND2, two well-known negative defense regulators (Zhu et al., 2010). *At*SNC1 also binds to the *At*bHLH84 transcriptional activator that constitutively activates plant defense responses when overexpressed (Xu et al., 2014). In rice, *Os*Pb1 was found to directly interact with the *Os*WRKY45 transcription factor to protect it from the UPS-mediated degradation in the nucleus during defense signaling (Inoue et al., 2013). In tobacco, the activated *Nb*N associates with SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 6 (*Nb*SPL6), a positive regulator of defense genes (Padmanabhan et al., 2013).

In tomato, resistance to *Pst* is conferred by *Pseudomonas* resistance and fenthion sensitivity (Prf) NLR resistance protein. However, Prf alone is unable to confer immunity and requires an accessory protein, resistance to *Pseudomonas syringae pv. tomato* kinase (Pto), to detect the two unrelated *Pst* effector proteins AvrPto and AvrPtoB. AvrPto and AvrPtoB are secreted by *Pst* directly into the plant cell where they target multiple proteins important for

basal defense, including Flagellin sensing 2 (FLS2) (Göhre et al., 2008), BRI1-ASSOCIATED KINASE 1 (BAK1) (Shan et al., 2008) and Chitin elicitor receptor kinase 1 (CERK1) (Gimenez-Ibanez et al., 2009) to abolish the activation of PAMP-triggered immunity (PTI). In the absence of pathogen, Prf exists in the inactive Prf/Pto complex containing at least two Prf and two Pto molecules, where a unique N-terminal domain of Prf serves as a dimerization platform and interaction site for Pto. The oligomeric Prf/Pto complex works as a molecular trap with one Pto molecule acting as a decoy by mimicking virulence targets of effectors and the second helper Pto detecting conformational changes of the Pto-decoy upon binding of either AvrPto or AvrPtoB. As a consequence of the effector detection, the trans-phosphorylation and conformational changes of Pto proteins are further sensed by the N-terminal domain of Prf and cause the complex activation, consequently leading to effector-triggered immunity (ETI), as manifested by a hypersensitive response (HR) (Ntoukakis et al., 2013). Although more than 25 genes have been implicated in the Pto/Prf-triggered immunity, including genes encoding molecular chaperones, proteins involved in hormone signaling, TFs and mitogen-activated protein kinases (MAPKs), the molecular mechanism that governs defense signaling remains unclear (Oh and Martin, 2011).

We have previously identified a defense-related tomato transcription factor *Sl*NAC1 that is highly induced during *Pst* infection. Moreover, *Sl*NAC1 appears to be a positive regulator of disease resistance since the *NAC1*-silenced *N. benthamiana* plants showed enhanced susceptibility to *Pst* (Huang et al., 2013). Besides transcriptional control, the abundance of *Sl*NAC1 protein is regulated at the post-translational level through a cognate E3 ubiquitin ligase, SEVEN IN ABSENTIA 3 (*Sl*SINA3), which specifically ubiquitinates *Sl*NAC1 to promote its degradation. The inverse correlation of *SlNAC1* and *SlSINA3*

expression upon pathogen infection adds yet another layer of regulation employed by plants (Miao et al., 2016). Our previous results indicate that *Sl*NAC1 is an important defense-related transcription factor tightly controlled during *Pst* infection. Nevertheless, the function of *Sl*NAC1 and its regulation in the Prf-mediated defense signaling remain to be determined.

Here we report the role and the molecular mechanism underlying regulation of *Sl*NAC1 transcription factor in Prf-mediated defense signaling. We show that upon activation by *Pst*, Prf directly interacts with *Sl*NAC1 to sequester it away from its cognate E3 ubiquitin ligase, *Sl*SINA3. Due to the enhanced accumulation and transcriptional activity in the presence of signaling-competent Prf, *Sl*NAC1 positively regulates Prf-mediated HR cell death and expression of defense-relevant genes involved in resistance to *Pst*.

Results

Activation of Prf is indispensable for interaction with *Sl*NAC1

Several resistance proteins have been shown to directly interact with TFs. Thus, we first sought to explore the possibility that *Sl*NAC1 is an interacting partner of Prf in the defense signaling cascade. The NB-ARC domain of Prf contains two conserved motifs: the P-loop and the IHD (isoleucine-histidine-aspartic acid) motif, which are important for the ATPase activity. The single amino acid substitution of D to V in the IHD motif of Prf results in the effector-independent autoactivity that is manifested by elicitation of HR cell death when Prf^{D1416V} is transiently expressed in *N. benthamiana* (Du et al., 2012). To confirm that the autoactivity of Prf^{D1416V} mimics the P-loop-dependent activation of the wild-type (WT) Prf triggered by effector AvrPto or AvrPtoB, we introduced the secondary loss-of-function mutation G1128E in the P-loop motif of Prf^{D1416V}. As expected, the $Prf^{G1128E/D1416V}$-HA mutant

no longer triggered HR when transiently expressed through *Agrobacterium* in *N. benthamiana* leaves (Figure 3.1A), despite the Prf$^{G1128E/D1416V}$-HA mutant protein accumulated at the same level as Prf^{D1416V}-HA when tested by Western blotting (WB) (Figure 3.1B). Thus, we concluded that the autoactivity of Prf^{D1416V} mutant resembles a similar mechanism of activation of NLR protein by effectors. To simplify our analysis of Prf-mediated defense signaling, we used the Prf^{D1416V} gain-of-function mutant as a defense signaling-competent form of Prf for further experiments.

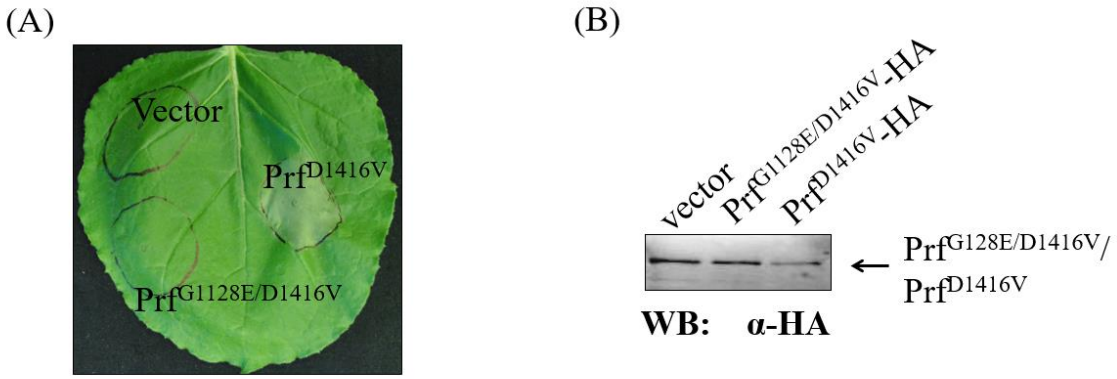

Figure 3.1 Auto-activity of Prf^{D1416V} is P-loop dependent

HA-tagged Prf^{D1416V}, Prf$^{G1128E/D1416V}$ and vector were transiently expressed in *N. benthamiana* leaf. 48h post-infiltration the photo was taken to record HR cell death and samples were collected for WB. **(A) HR cell death triggered by Prf^{D1416V} is p-loop dependent**. Unlike Prf$^{G1128E/D1416V}$ and vector control, only Prf^{D1416V} with an intact p-loop induces HR cell death. (B) **Both mutant proteins, Prf^{D1416V} and Prf$^{G1128E/D1416V}$, accumulate to similar levels as the WT Prf when transiently expressed in *N. benthamiana*.** Proteins were detected by WB with α-HA antibody.

To explore possible interaction between *Sl*NAC1 and Prf, we transiently co-expressed *Sl*NAC1-FLAG with Prf-HA, Prf^{D1416V}-HA or green fluorescence protein (GFP)-HA in *N. benthamiana* leaves. The proteasomal inhibitor MG132 was added to the infiltration buffer (100 µM final concertation) to prevent degradation of the unstable *Sl*NAC1 protein (Huang et al., 2013). WB (using α-FLAG) analysis on the immunoprecipitation (IP) complex obtained with α-HA agarose beads showed that the Prf resistance protein needs to be activated prior to interaction with the *Sl*NAC1, since only the signaling-competent Prf^{D1416V}mutant, but not the WT Prf, immunoprecipitated with *Sl*NAC1 (Figure 3.2).

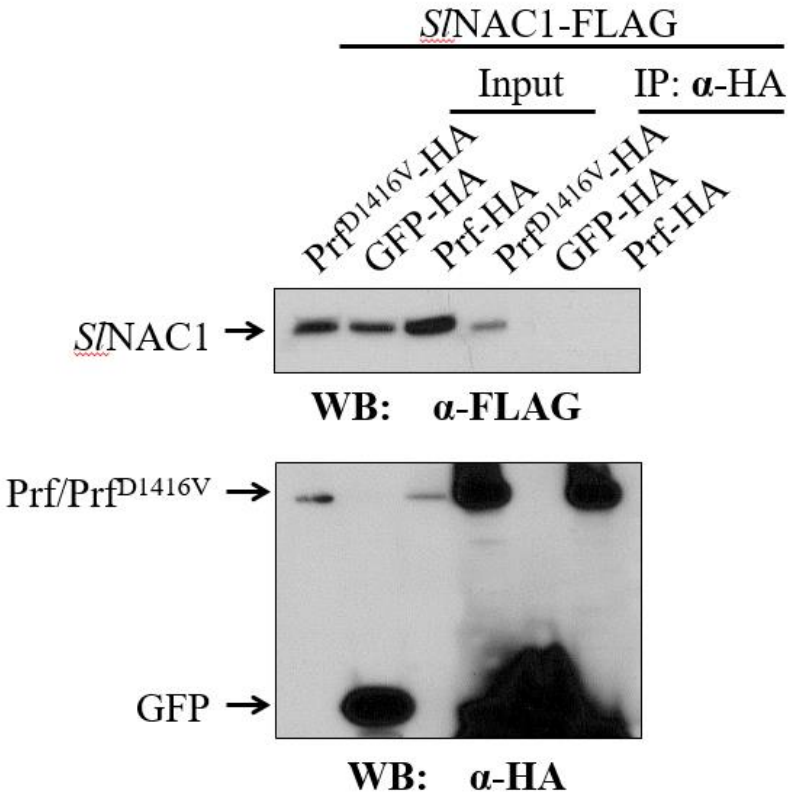

Figure 3.2 Activation of Prf is required for interaction with *Sl*NAC1

FLAG-tagged *Sl*NAC1 and either HA-tagged Prf, Prf^{D1416V} or GFP were transiently co-expressed in *N. benthamiana* leaves in the presence of MG132 (100 µM final concertation) as indicated in the Figure. 36h post infiltration, leaf tissues were collected for protein extraction and samples were subjected for IP with α-HA agarose beads. WB with α-FLAG antibody showed specific interaction between *Sl*NAC1 and Prf^{D1416V}, undetected in the case of WT Prf or GFP control.

The *Sl*NAC1 protein is stabilized by the activate Prf^{D1416V} form, but not the WT Prf form

Given the fact that *Sl*NAC1 undergoes rapid degradation in tomato (Huang et al., 2013), we next asked whether *Sl*NAC1 stabilization is attributed to its interaction with Prf^{D1416V}. To address this possibility, we simply co-expressed *Sl*NAC1-FLAG with Prf-FLAG, Prf^{D1416V}-FLAG, or GFP-FLAG as a control in the heterogeneous *N. benthamiana* system (without application of MG132), where stable *Sl*NAC1-FLAG can accumulate to a detectable level (Miao et al., 2016). To detect the difference in accumulation of *Sl*NAC1-FLAG, we used a relatively low inoculum (OD_{600} = 0.1) for *Agrobacterium* carrying the *Sl*NAC1-FLAG construct. At this inoculum, the *Sl*NAC1-FLAG signal was barely detectable when co-expressed with the unrelated GFP protein. Consistent with the co-IP result showing its interaction exclusively with the activated Prf, *Sl*NAC1 accumulated to the significantly higher level in the presence of the signaling-competent Prf^{D1416V} form but not the WT Prf form (Figure 3.3).

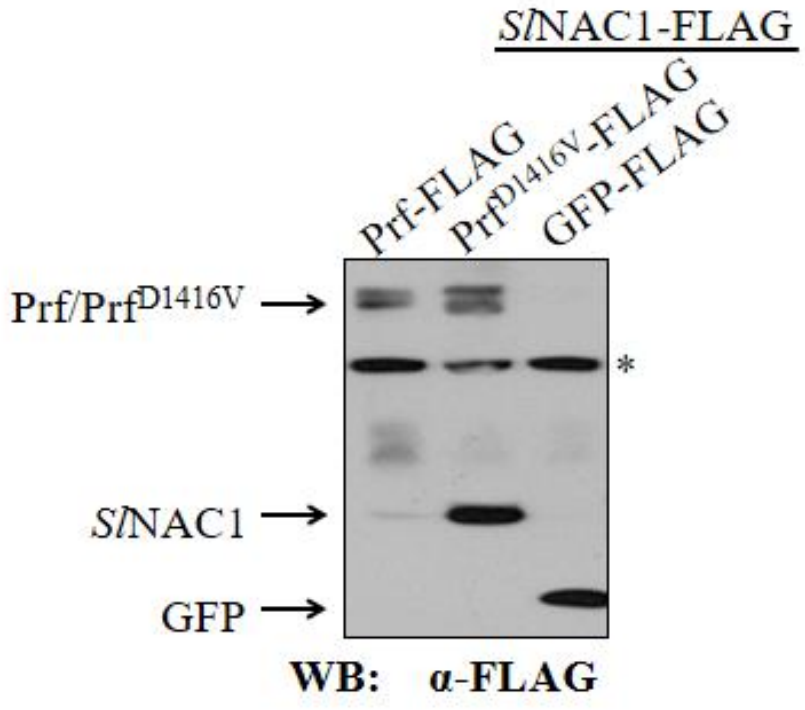

Figure 3.3 Auto-active Prf^{D1416V} stabilizes *Sl*NAC1

FLAG-tagged *Sl*NAC1 was transiently co-expressed with either Prf-FLAG, Prf^{D1416V}-FLAG or GFP-FLAG in *N. benthamiana* leaves as indicated in the Figure. 30h post infiltration, leaf tissue was collected for protein extraction and samples were subjected for WB. Protein detection with α-FLAG showed enhanced accumulation of *Sl*NAC1 in the presence of Prf^{D1416V}. The asterisk indicates an unspecific band detected by α-FLAG antibody in all tested samples.

The *Sl*NAC1 protein is stabilized in tomato during the Prf-mediated resistance to *Pst*

To further verify the data obtained from the heterogeneous *N. benthamiana* system, we next examined the dynamics of *Sl*NAC1 accumulation during the Prf-mediated defense responses in the native tomato-*Pst* interaction system. To this end, resistant RG-*PtoR* tomato plants (containing the functional *Prf* gene) and susceptible RG-*prf3* tomato plants (with a 1-kb deletion in the *Prf* gene) were challenged with *PstDC3000* expressing AvrPto/AvrPtoB to assess *Sl*NAC1 accumulation using the *Sl*NAC1-specific antibody. Significantly, although we previously reported that *SlNAC1* gene is induced in both RG-*PtoR* and RG-*prf3* plants by *Pst* (Huang et al., 2013), the stabilization effect on *Sl*NAC1 protein was only observed in the case of *PstDC3000*/RG-*PtoR* interaction (Figure 3.4). Taken together, our findings gave rise to a hypothesis that the Prf resistance protein, only upon activation by effectors, associates and stabilizes *Sl*NAC1 TF during defense signaling in tomato.

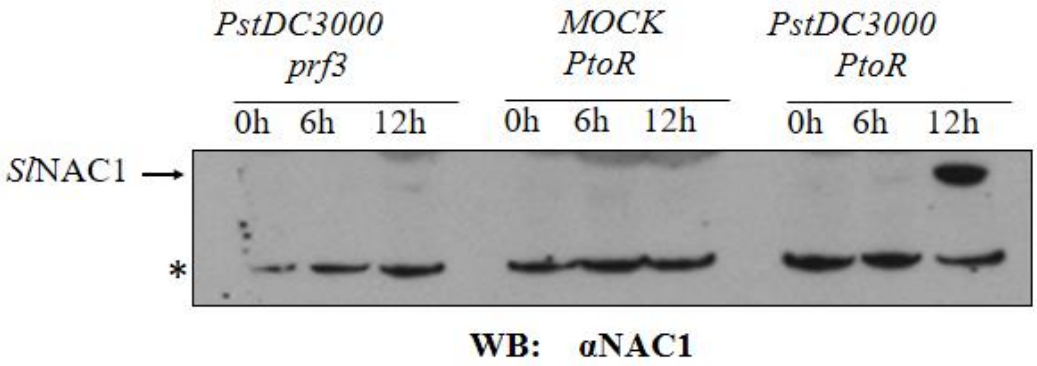

Figure 3.4 Dynamic *Sl*NAC1 stability during *PstDC3000* infection

Resistant RG-*PtoR* tomato plants and susceptible RG-*prf3* tomato plants were challenged with *PstDC3000* ($2x10^5$cfu/ml). MOCK inoculation on RG-*PtoR* plants was used as a control. As indicated, leaf tissue was collected for WB at 0h, 6h and 12h post-infection. Protein detection with α-NAC1 antibody showed the dynamic change in the accumulation of *Sl*NAC1 in RG-*PtoR* plants challenged with *PstDC3000*, whereas no *Sl*NAC1 was detected in other two tested scenarios. The asterisk indicates an unspecific band detected by the α-NAC1 antibody in all tested samples.

The signaling-competent Prf^{D1416V} stimulates the transcriptional activity of *Sl*NAC1

To obtain more details about the mechanism by which activated Prf controls *Sl*NAC1-mediated transcriptional reprograming during plant defenses, we examined whether the transcriptional activity of *Sl*NAC1 can be influenced by Prf. To this end, we used a previously developed β-glucuronidase reporter system (GUS) driven by four tandem repeats of the 30-bp cis-regulatory sequence of the Arabidopsis *ATAF2* gene fused to the minimal 35S CaMV promoter (Wang and Culver, 2012). Since ATAF2 belongs to the NAC transcription factor family and is the *Arabidopsis* ortholog of tomato *Sl*NAC1, we expected that this reporter system developed for ATAF2 would likely work for tomato *Sl*NAC1. The GUS reporter construct and *Sl*NAC1-HA were agroinfiltrated into *N. benthamiana* leaves in combination with an empty vector, or vectors encoding the WT Prf-FLAG or the autoactive Prf^{D1416V}-FLAG genes. As predicted, *Sl*NAC1 was able to activate the GUS reporter gene. Although the WT Prf enhanced *Sl*NAC1's transcriptional activity on the GUS reporter gene, the *Sl*NAC1-activated GUS reporter gene expression was strengthened to a much greater level by the autoactive Prf^{D1416V} (Figure 3.6A and B). Regardless whether the observed increase in the overall transcription activity of *Sl*NAC1 was simply a consequence of the elevated accumulation of *Sl*NAC1 protein or the enhanced transcriptional potential of *Sl*NAC1 during Prf-mediated defense signaling, or both, the data from GUS assay further support our model that activated Prf manipulates *Sl*NAC1.

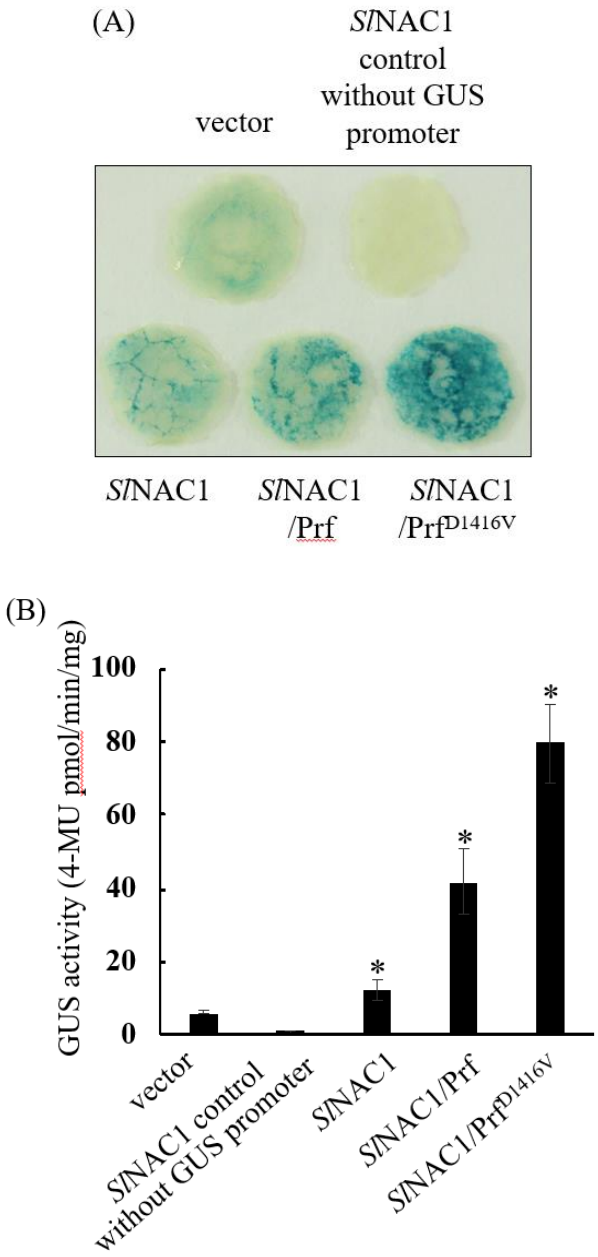

Figure 3.5 Prf^{D1416V} enhances *Sl*NAC1 transcriptional activity

*Sl*NAC1 transcriptional activity was evaluate as described previously for ATAF2 (Wang and Culver, 2012). *N. benthamiana* leaves were agroinfiltrated with the β-glucuronidase reporter system (GUS) reporter construct in combination with either an empty vector, *Sl*NAC1-HA itself or together with WT Prf-FLAG and autoactive Prf^{D1416V}-FLAG. **GUS activity was evaluated (A) visually** by histochemical staining with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) or **(B) quantitatively** measuring fluorescence of GUS product, MUG. Co-expression of *Sl*NAC1 and the autoactive Prf^{D1416V} yielded a significant increase of GUS activity relative to *Sl*NAC1 control, whereas, the WT Prf only partially affected *Sl*NAC1 transcriptional activity.

The activated Prf^{D1416V} interferes with *in vivo Sl*NAC1 ubiquitination to prevent its degradation

Our next objective was to determinate the mechanistic basis by which the Prf resistance protein manipulates *Sl*NAC1. To answer the question how activated Prf protects *Sl*NAC1 form degradation, we sought to examine the possibility that the *Sl*NAC1 ubiquitination might be attenuated during the Prf-mediated defense signaling. To test the *in planta Sl*NAC1 ubiquitination, NAC-FLAG and Ub-HA were co-expressed with Prf-FLAG, Prf^{D1416V}-FLAG or an empty vector in *N. benthamiana* leaves. MG132 was included to prevent *Sl*NAC1 degradation. The immunoprecipitation using α-HA agarose beads, followed by WB using α-FLAG antibody revealed the characteristic smear banding of *Sl*NAC1 representing the ubiquitin-associated *Sl*NAC1 protein, indicating the *in vivo* ubiquitination of *Sl*NAC1, as seen previously (Huang et al., 2013). Significantly, as speculated, the ubiquitination signal was reduced in the presence of Prf^{D1416V}, but not WT Prf, suggesting that activated Prf^{D1416V} interferes with *Sl*NAC1 ubiquitination *in planta* (Figure 3.6).

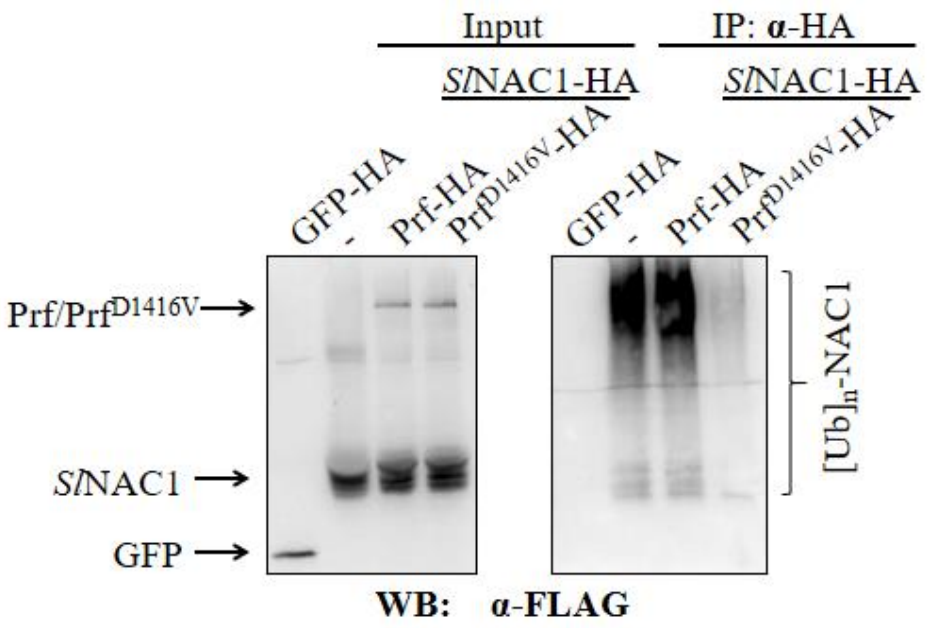

Figure 3.6 Activated Prf protects *Sl*NAC1 from degradation by interfering with its ubiquitination *in vivo*

N. benthamiana leaves were co-injected with *Agrobacterium* carrying NAC-FLAG and Ub-HA alone as a control and with either Prf-FLAG or Prf^{D1416V}-FLAG to test possible differences in ubiquitination level of *Sl*NAC1. MG132 was added to prevent *Sl*NAC1 degradation. 36h post-infiltration, leaf tissues were collected for protein extraction 36h post-agroinfiltration and samples were subjected for IP with α-HA agarose beads. WB with α-FLAG antibody against the IP fraction showed a specific smear of *Sl*NAC1-associated polyubiquitin in the control lane and in the presence of WT Prf, whereas the ubiquitination of *Sl*NAC1 was attenuated in the presence of Prf^{D1416V}.

The *Sl*NAC1-Prf^{D1416V} interaction sequesters *Sl*NAC1 away from *Sl*SINA3

The next logic question needed to be addressed was: How does the interaction between *Sl*NAC1 and Prf^{D1416V} affect the ubiquitination of *Sl*NAC1? Since *Sl*SINA3 is the ubiquitin ligase responsible for *Sl*NAC1 ubiquitination, this phenomenon can be explained by a sequestration-based mechanism whereby the activated Prf binds to *Sl*NAC1, and displacing it from *Sl*SINA3. To test this hypothesis, we conducted an *in vitro* binding assay to examine the impact of Prf^{D1416V} on the *in vitro Sl*SINA3-*Sl*NAC1 interaction. GST-*Sl*NAC1 was used to pull down MBP-*Sl*SINA3 in the absence or presence of Prf-FLAG and Prf^{D1416V}-FLAG. As presented in Figure 3.7, in the absence of Prf-FLAG or Prf^{D1416V}-FLAG, *Sl*SINA3 bound strongly to *Sl*NAC1 *in vitro*, which is consistent with previously reported substrate-enzyme specificity between these two proteins. The addition of Prf^{D1416V} protein to the binding reaction gradually decreased the amount of *Sl*SINA3 bound to *Sl*NAC1. In contrast, addition of the WT Prf protein failed to disrupt such interaction. Therefore, we conclude that the activated Prf^{D1416V} binds to *Sl*NAC1 thereby displacing *Sl*NAC1 from *Sl*SINA3, which results in attenuation of *Sl*SINA3-mediated ubiquitination of *Sl*NAC1 to prevent its degradation.

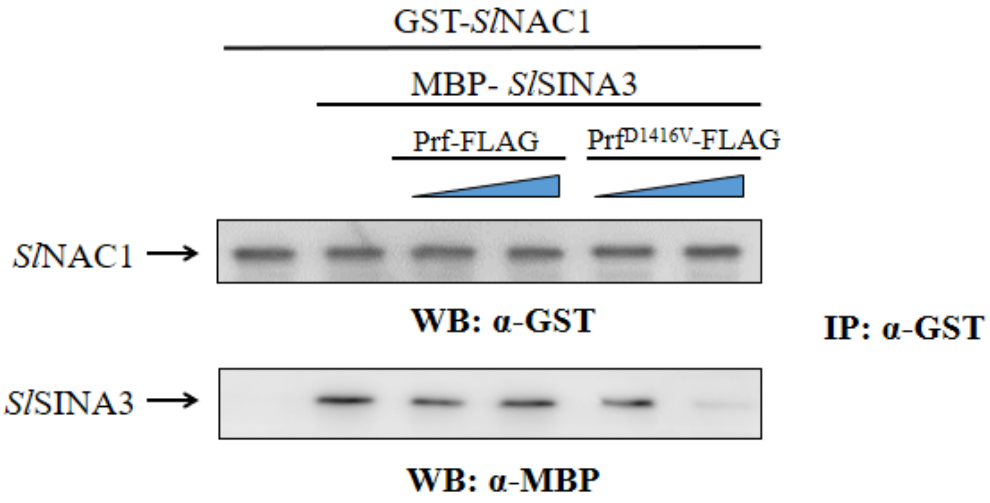

Figure 3.7 *In vitro* binding assay between *Sl*NAC1 and *Sl*SINA3 abolished by Prf^{D1416V}

IP was carried out using α-GST agarose beads which specifically recognizes GST-*Sl*NAC1 to pull down MBP-*Sl*SINA3 in the absence or presence of Prf-FLAG and Prf^{D1416V}-FLAG. WB with α-MBP antibody confirmed that *Sl*SINA3 strongly interacted with *Sl*NAC1.This interaction was not disrupted in the presence of WT Prf. However, the addition of increasing amounts of Prf^{D1416V} into the binding reaction gradually decreased the levels of *Sl*SINA3 bound to *Sl*NAC1.

*Sl*NAC1 is a positive regulator of the Prf-triggered HR cell death

Development of HR cell death around the infection site, as a consequence of R protein activation, is an important strategy employed by plants to prevent invading pathogens from spreading to surrounding tissues. Thus, we next verified the involvement of *Sl*NAC1 in the Prf-mediated defense signaling by examining its effect on HR cell death triggered by the autoactive Prf^{D1416V} mutant when transiently expressed in *N. benthamiana* leaves. We found that the co-expression of *Sl*NAC1-FLAG with Prf^{D1416V}-HA resulted in an earlier and stronger HR cell death compared with the co-expression of Prf^{D1416V}-HA with vector control (Figure 3.8). This result supports the notion that *Sl*NAC1 plays a positive role in the Prf-mediated defense signaling.

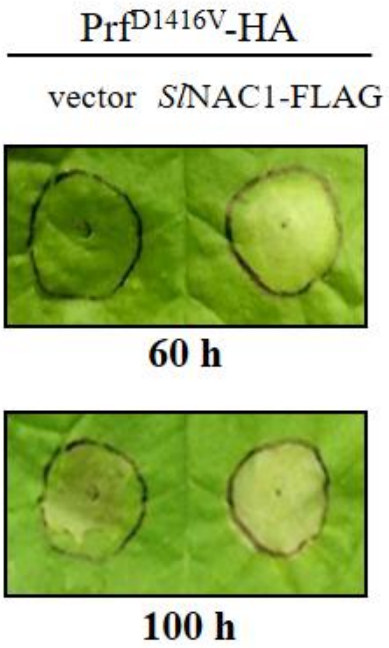

Figure 3.8 *Sl*NAC1 enhances HR cell death triggered by Prf ^{D1416V}

N. benthamiana leaves were transiently co-expressed with Prf^{D1416V}-HA and either *Sl*NAC1-FLAG or vector. Photos showing faster development of HR cell death in presence of *Sl*NAC1 were taken 60h and 100h after agroinfiltration.

Overexpression of *Sl*NAC1 leads to constitutive activation of defense responses and enhanced resistance to *Pst*

We next examined the role of *Sl*NAC1 in tomato disease resistance by gain-of-function approach. To this end, we generated tomato plants overexpressing 35S::*Sl*NAC1-HA (*Sl*NAC1-*oe*) in a RG-*PtoR* background. Interestingly, *Sl*NAC1-*oe* plants exhibited dwarf morphology (Figure 3.9A). Quantitative Real-Time PCR (qRT-PCR) analysis confirmed that 250 times more abundant *Sl*NAC1 transcript was found in *Sl*NAC1-*oe* plants than in the WT plants (Figure 3.9B).

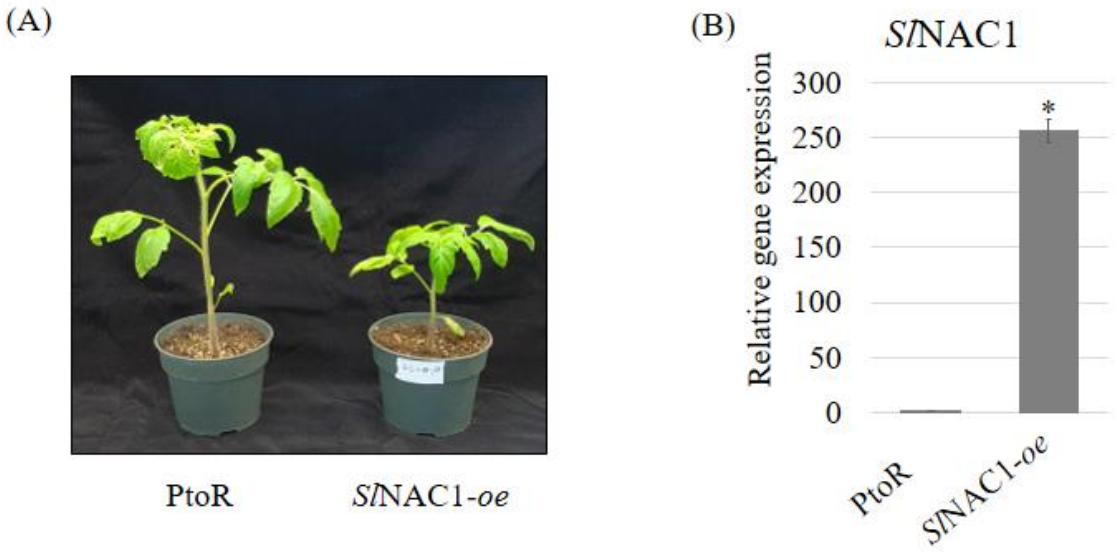

Figure 3.9 Characterization of *Sl*NAC1-*oe* transgenic plants in a PtoR background

(A) Phenotype of *Sl*NAC1-*oe* plants. *Sl*NAC1 overexpression results in dwarf phenotype associated with smaller fruit compared with WT RG-*PtoR* plants. **(B) Relative *SlNAC1* gene expression in *Sl*NAC1-*oe* plants**. Total RNA was isolated from plants to generate cDNA. The relative level of *Sl*NAC1 transcript was measured by qRT-PCR using gene-specific primers with the *Actin* gene as an internal control for normalization. The presented values are means ± SE of three replicates. (*P < 0.01; Student's t test).

Since RG-*PtoR* is already resistant to avirulent *PstDC3000*, to evaluate potential enhanced resistance in *Sl*NAC1-*oe* transgenic plants, we infected them with the avirulent *PstDC3000* strain or the virulent *PstDC3000 ΔAvrPtoΔAvrPtoB* strain (both *AvrPto* and *AvrPtoB* genes have been removed). As expected, no disease symptoms were observed in any plants challenged with avirulatent *PstDC3000*, however, the bacterial growth was 7-fold reduced in the *Sl*NAC1-*oe* plants (Figure 3.10A) compared to the non-transgenic PtoR tomato plants. In addition, disease symptoms caused by the virulent *PstDC3000ΔAvrPtoΔAvrPtoB* strain were much less severe in the *Sl*NAC1-*oe* plants than in the non-transgenic PtoR plants, even though there was no significant difference in *Pst* growth in these two genotypes (Figure 3.10B), suggesting that overexpression of *Sl*NAC1 enhanced resistance to *Pst*.

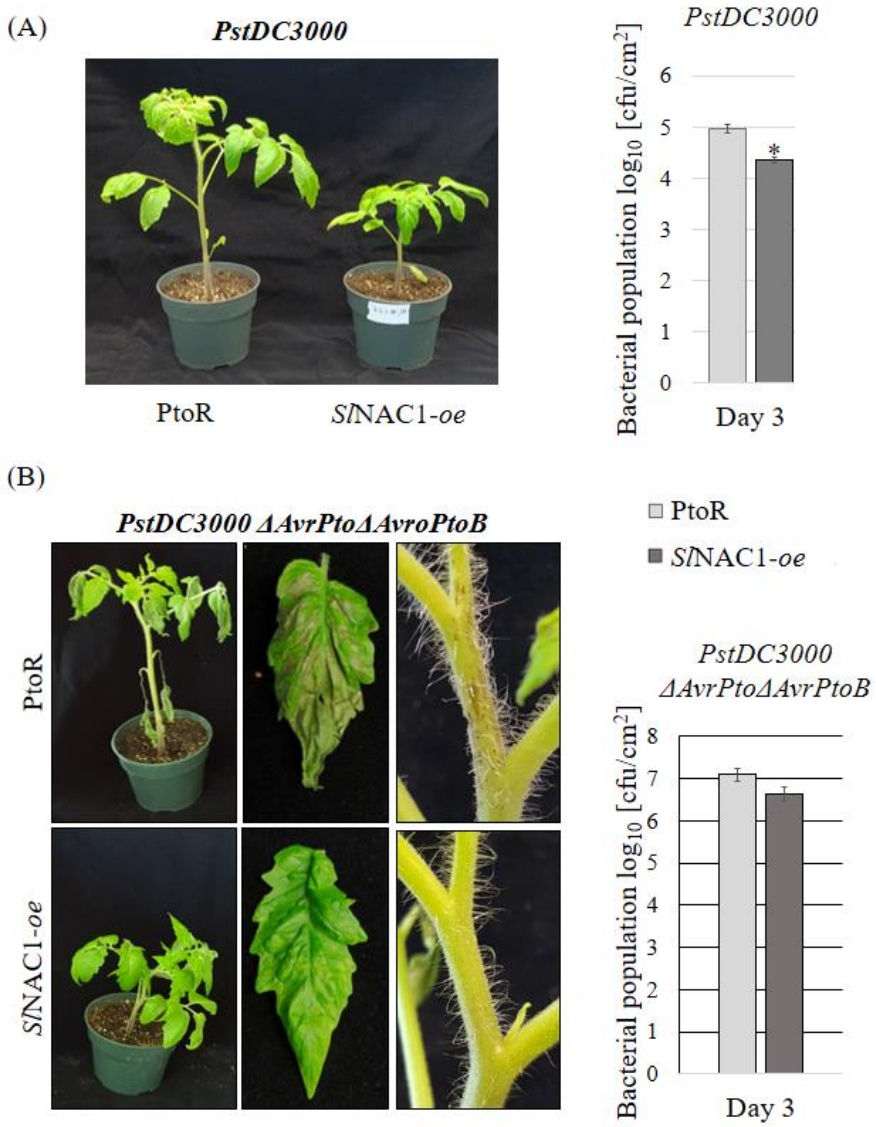

Figure 3.10 Pathogen assay on *Sl*NAC1-*oe* transgenic plants in PtoR background

Resistant RG-*PtoR* or *Sl*NAC1-*oe* plants in RG-*PtoR* background were vacuum-infiltrated with **(A) avirulent *PstDC3000* strain** using an inoculum of $2x10^5$ colony forming units (CFU)/ml or **(B) virulent *PstDC3000ΔAvrPtoΔAvrPtoB* strain** at an inoculum of $4x10^5$ CFU/ml. The bacterial population in leaves was assessed 3 days after *Pst* infiltration, whereas, pictures for disease symptoms were taken at day 5. The presented values are means ± SE of three replicates (*P < 0.01; Student's t test). No disease symptoms were observed in plants challenged with avirulent, *PstDC3000*. However, the bacterial growth was significantly reduced in *Sl*NAC1-*oe* plants compared with WT RG-*PtoR* tomato. Plants infected with virulent *PstDC3000ΔAvrPtoΔAvrPtoB* showed a significant difference in *Pst*-triggered necrotic lesions between transgenic and WT plants, although, both type of plants exhibited similar bacterial growth.

To further understand how overexpression of *Sl*NAC1 enhances resistance to *Pst*, we examined the expression of salicylic acid (SA)- and jasmonic acid (JA)-induced pathogenesis-related (PR) genes in *Sl*NAC1-*oe* transgenic plants. Consistent with the positive role of *Sl*NAC1 in defense signaling, SA-inducible defense genes such as PR1 and PR7 were up-regulated in *Sl*NAC1-*oe* transgenic plants, whereas JA responsive genes (PI I and PI II) were strongly suppressed (Figure 3.11A). As shown in Figure 3.11B, the up-regulated expression of the SA marker genes was even greater and more prolonged in *Sl*NAC1-*oe* transgenic plants upon infected with *PstDC3000*. These findings suggest that *Sl*NAC1 contributes to the transcriptional regulation of these defense-related genes and its overexpression leads to constitutive activation of defense responses.

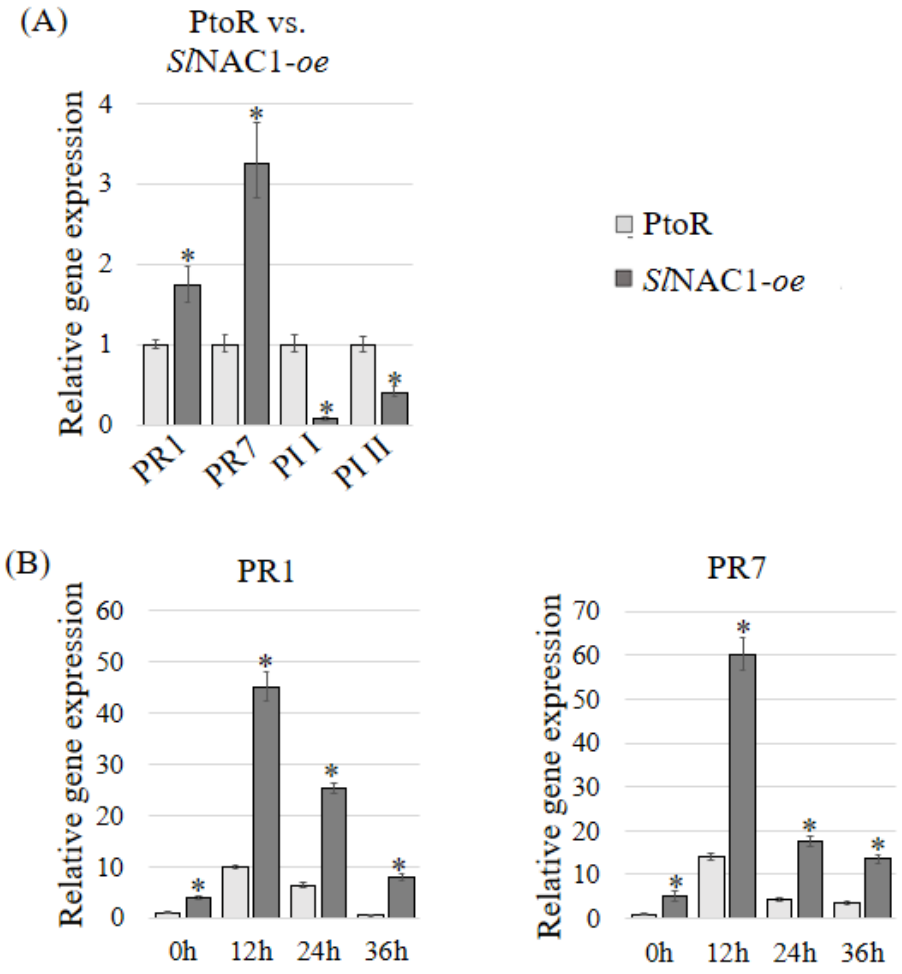

Figure 3.11 Expression of marker genes in WT PtoR tomato and *Sl*NAC1-*oe* transgenic plants.

Total RNA was isolated from both WT RG-*PtoR* and *Sl*NAC1-*oe* plants in RG-*PtoR* background to generate cDNA. The relative expression level of SA- and JA-marker genes was measured by qRT-PCR using gene-specific primers (included in Materials and Methods) with *Actin* as an internal control for normalization. The presented values are means ± SE of three replicates (*P < 0.01; Student's t test). **(A) The expression pattern of SA- and JA-marker genes in WT RG-*PtoR* and *Sl*NAC1-*oe* plants.** SA-inducible *PR1* and *PR7* marker genes were more abundant in *Sl*NAC1-*oe* plants, whereas, JA-marker genes, PI I and PI II, were strongly suppressed. **(B) The dynamic expression pattern of SA-marker genes during an incompatible *Pst*-tomato interaction in WT RG-*PtoR* and *Sl*NAC1-*oe* plants.** Both types of plants were challenged with *PstDC3000* at an inoculum of $2x10^7$ CFU/ml prior to leaf tissue collection. Total RNA was isolated at four timepoints: 0h, 12h, 24h, 36h post infection. The difference in expression level of SA-marker genes was greater and more prolonged after challenge with *PstDC3000* compared with samples collected from uninfected plants.

Discussion

The successful defense against pathogenic invaders in plants relies on quick recognition of attackers and robust activation of defense responses. To this end, the activated NLRs may directly manipulate transcriptional reprograming through binding to TFs to ensure immediate implementation of defense responses (Chang et al., 2013; Padmanabhan et al., 2013; Shen et al., 2007; Xu et al., 2014; Zhu et al., 2010). It became evident that NLRs employ different strategies to modulate action of both transcriptional activators and repressors belonging to diverse transcription factor families. For example, to activate plant defenses, both *Hv*MLA10 and *Os*Pb1 bind plant-specific WRKY type TFs. However, *Hv*WRKY1/2 function as transcriptional repressors, whereas *Os*WRKY45 is a transcriptional activator. *Os*Pb1 protects *Os*WRKY45 from UPS-mediated degradation through physical interaction. The activated *Hv*MLA10 also interacts with a member of MYB TF family, the transcriptional activator *Hv*MYB6, to release it from *Hv*WRKY1 suppression and stimulate MYB6-dependent gene expression. *At*SNC1 is another example of an R protein capable of regulating both transcriptional activation and repression during a plant immune response through interactions with *At*bHLH84 and *At*TPR1, respectively. Here, we provide molecular and genetic evidence that *Sl*NAC1, as a transcriptional activator, is a direct interacting partner of the activated Prf and is required for the Prf-mediated resistance to *Pst.* Our data indicate that *Sl*NAC1 is a positive regulator of the Prf-mediated HR cell death and its overexpression leads to constitutive activation of defense-related genes. Finally, we showed that activated Prf, in its signaling-competent state, modulates *Sl*NAC1 stability and activity by sequestering it away from *Sl*SINA3 to inhibit *Sl*SINA3-induced polyubiquitination and consequent degradation of

*Sl*NAC1. Significantly, our results, for the first time, shed light on the mechanistic basis by which an NLR protein manipulates a TF to control defense gene expression.

JA and SA act antagonistically to fine-tune plant defenses against different types of pathogens depending on their lifestyle. For the hemibiotrophic pathogens such as *Pst*, SA is an essential signaling phytohormone activating plant immunity and at the same time suppressing the JA-mediated defense response through signaling crosstalk (Bostock, 2005). Although genes directly regulated by *Sl*NAC1 during plant defense responses remain to be identified, the overexpression of *Sl*NAC1 in tomato resulted in elevated transcript accumulation of SA marker genes, *PR1* and *PR7*, while the mRNA level of JA-marker genes, *PI I* and *PI II*, was significantly reduced. The difference of expression levels of SA defense-related genes in WT PtoR and *Sl*NAC1-*oe* tomato plants was even greater when measured 12h and 24h after challenging with *Pst*, which explains why *Sl*NAC1-*oe* plants exhibited enhanced resistance to the avirulent *PstDC3000* strain and partial resistance to the virulent *PstDC3000ΔAvrPtoΔAvrPtoB* strain. Moreover, we have shown previously that strong overexpression of *Sl*SINA3 abolishes Prf-mediated HR cell death without interfering with the Prf accumulation *in planta* (Miao et al., 2016), suggesting that *Sl*SINA3 negatively regulates signaling components downstream of the activated Prf. In the current study, we found that *Sl*NAC1 enhanced HR cell death when co-expressed with auto-active Prf^{D1416V}. Since *Sl*NAC1 is ubiquitinated and degraded by *Sl*SINA3, this supports the notion that *Sl*SINA3 functions as a negative regulator of the Prf-triggered HR cell death.

The constitutive expression of defense-related gene is usually achieved at the expense of plant growth and development, thus, the undesirable dwarf phenotype of *Sl*NAC1-*oe* plants (Figure 4.9A) can be attributed to growth-defense tradeoff (Heidel et al., 2004; Heil et al.,

2002; Huot et al., 2014). To prevent costly *Sl*NAC1-mediated autoimmunity, *Sl*NAC1 is highly unstable in tomato due to constant degradation by *Sl*SINA3 so that plant growth is not compromised in the absence of pathogen (Miao et al., 2016). To initiate robust transcriptional reprogramming, Prf manipulates *Sl*NAC1 abundance in at least two different ways. Firstly, as shown by enhanced accumulation of *Sl*NAC1 in *N. benthamiana* by the auto-active Prf^{D1416V} mutant and in the resistant RG-*PtoR* tomato infected by the avirulent *Pst*DC3000 strain, tomato has developed a strategy to immediately initiate defense signaling through stabilization of *Sl*NAC1 by the activated resistance protein Prf upon pathogen perception instead of maintaining the elevated level of *Sl*NAC1 protein constitutively. Secondly, to ensure sufficient *Sl*NAC1 transcript accumulation, the expression of *SlNAC1* gene is up-regulated whereas the *SlSINA3* gene is down-regulated as a part of the positive feedback loop during Prf-mediated resistance to *Pst* (Miao et al., 2016).

Our study not only identified *Sl*NAC1 as interacting partner of Prf resistance protein, but more importantly, gave rise to a hypothesis for the molecular mechanism by which the activated Prf manipulates transcriptional reprograming by controlling the stability and activity of *Sl*NAC1. In the resting state, the Prf/Pto complex is kept in a closed and auto-inhibited state through domain–domain interactions, in which the Prf LRR and the N-terminal end fold back on the NB-ARC core and around the Pto molecule (Saur et al., 2015). Intra- and intermolecular conformational changes in this receptor complex following pathogen perception render Prf an open structure, in which previously hidden interfaces are now exposed to activate defense signaling. Several lines of evidence support the activation of Prf as a requirement for initiating *Sl*NAC1-medaited signaling. In this study, we used the P-loop-dependent auto-active Prf^{D1416V} that mimics activation of WT Prf *in planta*. Ectopic co-expression of *Sl*NAC1 and either

Prf^{D1416V} or Prf in *N. benthamiana* revealed that only signaling-competent Prf^{D1416V} binds to *Sl*NAC1 and this interaction leads to its increased accumulation. Moreover, Prf^{D1416V} dramatically enhanced *Sl*NAC1 transcriptional activity. We proposed that the binding between *Sl*NAC1 and activated Prf sequesters *Sl*NAC1 away from *Sl*SINA3 thereby inhibiting ubiquitination of *Sl*NAC1 by *Sl*SINA3. We provided two pieces of evidence to support this claim. First, our *in vivo* ubiquitination data showed that activated Prf significantly reduces the amount of ubiquitination of *Sl*NAC1. Second, the *in vitro* binding assay on *Sl*NAC1 and *Sl*SINA3 demonstrated the disruption of *Sl*NAC1-*Sl*SINA3 binding by the singling-competent Prf^{D1416V} but not the WT Prf.

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Materials and Methods

Agrobacterium-mediated transient assay

Agrobacterium-mediated transient expression was carried out as described previously (Xiao et al., 2007). *Agrobacterium tumefaciens* GV2260 strains expressing FLAG-tagged, HA-tagged or Ub-tagged protein were syringe-infiltrated into *N. benthamiana* leaves.

The concentration of bacterial inoculum was dependent on the construct and the type of experiment. In certain experiments, MG132 was added to *Agrobacterium* inoculum (final concertation of 100µM), to inhibit proteasomal degradation of transiently expressed proteins as indicated in the figure legends.

Western blotting (WB), co-immunoprecipitaion (CoIP) and *in vivo* ubiquitination assay from plant tissue

Agrobacterium-infiltrated *N. benthamiana* leaf tissues were collected at 28-36h after infiltration and ground with liquid nitrogen. The fine tissue powder was resuspended with 300 µl of protein extraction buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 5mM EDTA, 2mM DTT, 10% glycerol, 1% polyvinylpolypyrolidone, 1mM PMSF, plant protease inhibitor cocktail (Sigma-Aldrich, USA)) and centrifuged at 13,000g/4°C for 15 minutes. For immunoprecipitation assay, one-tenth of each protein extract (v:v) was saved as the input sample, and the rest of the of the protein solution was used for precipitation with anti-HA affinity matrix (Roche Applied Sciences) or α-FLAG affinity matrix (Sigma-Aldrich) for 2h at 4°C. The immunoprecipitated protein complex was washed four times with washing buffer (50mM Tris-HCl, pH 7.5, 250mM NaCl, 5mM EDTA, 10% glycerol, 1mM PMSF). Protein samples were separated by SDS-PAGE and detected using the α-HA or α-FLAG antibody.

In vitro ubiquitination assay

*Sl*SINA3 and *Sl*NAC1-HA were cloned into the pMAL-c2 vector using *EcoRI* and *SalI* to generate the MBP-fusion proteins. The recombinant proteins were expressed in *Escherichia coli* BL21 using 0.5µM IPTG for induction. The *in vitro* ubiquitination assay was performed as described previously (Abramovitch et al., 2006) with a few adjustments. 40ng GST-E1 (*At*UBA1), 100ng GST-E2 (*At*UBC8), 1µg MBP *Sl*SINA3, 2µg FLAG-Ub (Boston Biochem,

USA) were combined together with the ubiquitination buffer (50mM Tris HCl, pH7.5, 2mM ATP, 5mM $MgCl_2$, 30mM creatine phosphate (Sigma-Aldrich) and 50ng/µl creatine phosphokinase (Sigma-Aldrich, USA)). The reaction mixture of total 30µl was incubated for 2h at 30^{o}C. For *Sl*NAC1 *in vitro* ubiquitination by *Sl*SINA3, 500ng MBP-*Sl*NAC1-HA was used as a substrate. To immunoprecipitate the ubiquitinated MBP-*Sl*NAC1-HA protein, 15µl anti-HA affinity matrix (Roche Applied Science, USA) was added to the reaction mixture and incubated for another 2h at 4^{o}C. Beads were then washed three times with the washing buffer (20mM Tris HCl, pH7.5, 0.1M NaCl, 0.1mM EDTA, 0.05% Tween 20). Proteins were separated with 7.5% SDS-PAGE and identified by WB using the α-FLAG or α-HA antibody (Sigma-Aldrich, USA). To test the influence of Prf^{D1416V} and Prf on *Sl*NAC1 *in vitro* ubiquitination by *Sl*SINA3, purified Prf^{D1416V}-FLAG and Prf-FLAG were added to reaction mixture at different concentrations. Prf^{D1416V}-FLAG and Prf-FLAG were obtained by transient expression in *N. benthamiana* leaves followed by proteins extraction and immunoprecipitation using α-FLAG affinity matrix (Sigma-Aldrich, USA). For elution, 100 µl of 150 ng/µl 3xFLAG peptide solution in TBS was incubated with samples for 30 min at 4°C and supernatant containing purified Prf^{D1416V}-FLAG and Prf-FLAG was collected after centrifugation at 5000 rpm for 30 seconds.

Pathogen assay

All *P. syringae pv tomato* strains used in this work (*Pst DC3000* WT, *Pst DC3000* Δ*AvrPto*Δ*AvrPtoB* (Lin and Martin, 2005) and *Pst DC3000* Δ*hrcC* (Deng et al., 1998) were prepared as described previously (Anderson et al., 2006). Bacteria were grown on the agar media for 3 days, then they were collected and resuspended in 10mM $MgCl_2$ solution containing 0.003% Silwet-77. The bacteria culture concentration was adjusted depending on

strain and type of experiment, as indicated in the figure legends. Five-week-old tomato plants were vacuum-infiltrated for 3 min. For measurement of bacterial growth, three 1 cm^2 leaf disks were collected and ground in 1 ml of 10mM $MgCl_2$. Bacterial numbers were determined by plating 10-fold serial dilutions and counting the resulting colonies two days later.

GUS assay

GUS activity was measured using the previously developed reporter system for ATAF2 (Wang and Culver, 2012). Briefly, a GUS reporter construct, containing a 30-bp segment from the promoter region of the defensin-like protein At1G68907 functioning as a cis-regulatory binding sequence for ATAF2 (5' TCAGAAGAGCAATCAAATTAAAACACATAT 3') cloned in front of the 35S minimal promoter, was agroinfiltrated with either ATAF2 or *Sl*NAC1 alone, with Prf and with Prf^{D1416V}. For quantitative measurements of GUS activity, two days after injection plant tissues were grounded in extraction buffer (150 mM sodium phosphate, pH 7.0, 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton X-100, 0.1% sarcosyl, 140 μM PMSF) and centrifuged at 20,000g/4°C for 15 minutes. Fluorometric substrate 4-methyl-umbelliferyl-β-D-glucuronide (4-MUG) was added to the supernatant to a final concentration of 1.0 mM and the sample was incubated in darkness at 37°C for 20 mins. 10 μl aliquots from each reaction were mixed with 190 μl stop buffer (0.2M Na_2CO_3) in a black 96-well plate and resulting samples were then subjected to the fluorescent 4-Methylumbelliferone (MU) produced detection using a SpectraMax M2 microplate reader (MTX Lab systems, Vienna, VA) with excitation at 365 nm and emission at 455 mm. For visual measurements of GUS activity, the histochemical staining with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) of the agroinfiltrated plant tissue was used as described previously (Wang et al., 2009b).

In vitro competitive binding assay

The recombinant GST-*Sl*NAC1 and MBP-*Sl*SINA3 proteins were expressed and purified from BL21 *E. coli* strain using either 0.5μM IPTG for induction. Prf-FLAG and Prf^{D1416V}–FLAG were obtained through transient expression in *N. benthamiana* leaves as described in previous paragraph. For the *in vitro* assay, GST-*Sl*NAC1, MBP-*Sl*SINA3 with either Prf-FLAG or Prf^{D1416V}–FLAG were mixed together in reaction buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 5mM EDTA, 2mM DTT, 10% glycerol, 1mM PMSF, plant protease inhibitor cocktail (Sigma-Aldrich, USA)). IP was carried out using α-GST agarose beads (Sigma-Aldrich, USA) which specifically recognizes GST-*Sl*NAC1 to pull down MBP-*Sl*SINA3 in the absence or presence of Prf-FLAG and Prf^{D1416V}–FLAG at different concentrations. WB with α-MBP and α-GST antibody was used to detect *Sl*SINA3-*Sl*NAC1 interaction.

qRT-PCR

Total RNA from tomato plants was isolated with TRIzol reagent (Invitrogen). One microgram of total RNA was treated with DNase I (Invitrogen), followed by reverse transcription using a Super Script II reverse transcriptase (Invitrogen). qRT-PCR analysis was performed on an ABI Prism 7100 sequence detection system using Power SYBR Green reagents (Life Technologies, Carlsbad, CA, USA). The tomato Actin gene was used as an internal control for normalization. Relative expression ratios were determined based on the comparative CT method (ΔΔCT) using the StepOne Software. Values are means ± SE (standard error) of three technical replicates. Statistical significance was determined by Student's t-test. Asterisk represents significant results for $p<0.05$

Table 3.1 Primer sequence for qRT-PCR

Primer name	Primer sequence
***Sl*Actin_RTF**	5’-GCTCTTGACTATGAACAGGAAC-3’
***Sl*Actin_RTR**	5’-AAGGACCTCAGGACACCG-3’
***Sl*NAC1_RTF**	5’-ATGGGACGAAGACCACAGAAAC-3’
***Sl*NAC1_RTR**	5’-GTCTTGGAAATTGTTGAACTGGTC-3’
***Sl*PR1_RTF**	5’-TCACTTGTCTCATGGTATTAGCC-3’
***Sl*PR1_RTR**	5’-GCATCGTTATGAACCGCAAG-3’
***Sl*PR7_RTF**	5’-GGTCCAATCCCTTTCCATAGAG-3’
***Sl*PR7_RTR**	5’-CACCTACCGTAAGAATCCAAGG-3’
***Sl*PI I_RTF**	5’-CCTATTCAAGATGTCCCCGTTC-3’
***Sl*PI I_RTR**	5’-GGTTCATCACTCTCTCCTTCAC-3’
***Sl*PI II_RTF**	5’-GGAGTCAAAGTTTGCTCACATC-3’
***Sl*PI II_RTR**	5’-TCCTTCGCACATCAAGTTAGAG-3’

CHAPTER 4 Conclusions and future directions

Over last two decades, great progress has been made in uncovering the molecular mechanisms underlying the activation of plant defense signaling. Although early events, such as pathogen recognition by NLR type R proteins, are well characterized, the signaling pathways downstream of activated R proteins remain elusive due to functional redundancy and complexity of the signaling network. My doctoral research focused on such a signaling pathway with a dynamic network of interactions between the Prf resistance protein, the defense-related *Sl*NAC1 transcription factor and the *Sl*SINA3 E3 ubiquitin ligase.

The data presented in this dissertation supports the following model that explains the molecular events from pathogen recognition to transcriptional reprogramming. Under normal conditions, *Sl*NAC1 is a short-lived protein rapidly degraded by *Sl*SINA3 (Figure 4.1A). Upon *Pst* challenge, the activation of the Prf/Pto complex by avirulent effectors AvrPto and AvrPtoB leads to conformational changes in Prf protein, allowing for its binding to downstream signaling molecules such as *Sl*NAC1. Enhanced stability and transcriptional activity of *Sl*NAC1 is a direct consequence of Prf protection on *Sl*NAC1 from degradation, presumably through binding to *Sl*NAC1 to sequester it away from *Sl*SINA3, thereby inhibiting *Sl*NAC1 ubiquitination. More stable and active *Sl*NAC1 positively regulates defense-related genes and HR cell death. To ensure prolonged and sufficient accumulation of *Sl*NAC1 protein, *SlNAC1* gene is highly up-regulated in a Prf-dependent manner, whereas, *SlSINA3* transcript is significantly suppressed (Figure 4.1B).

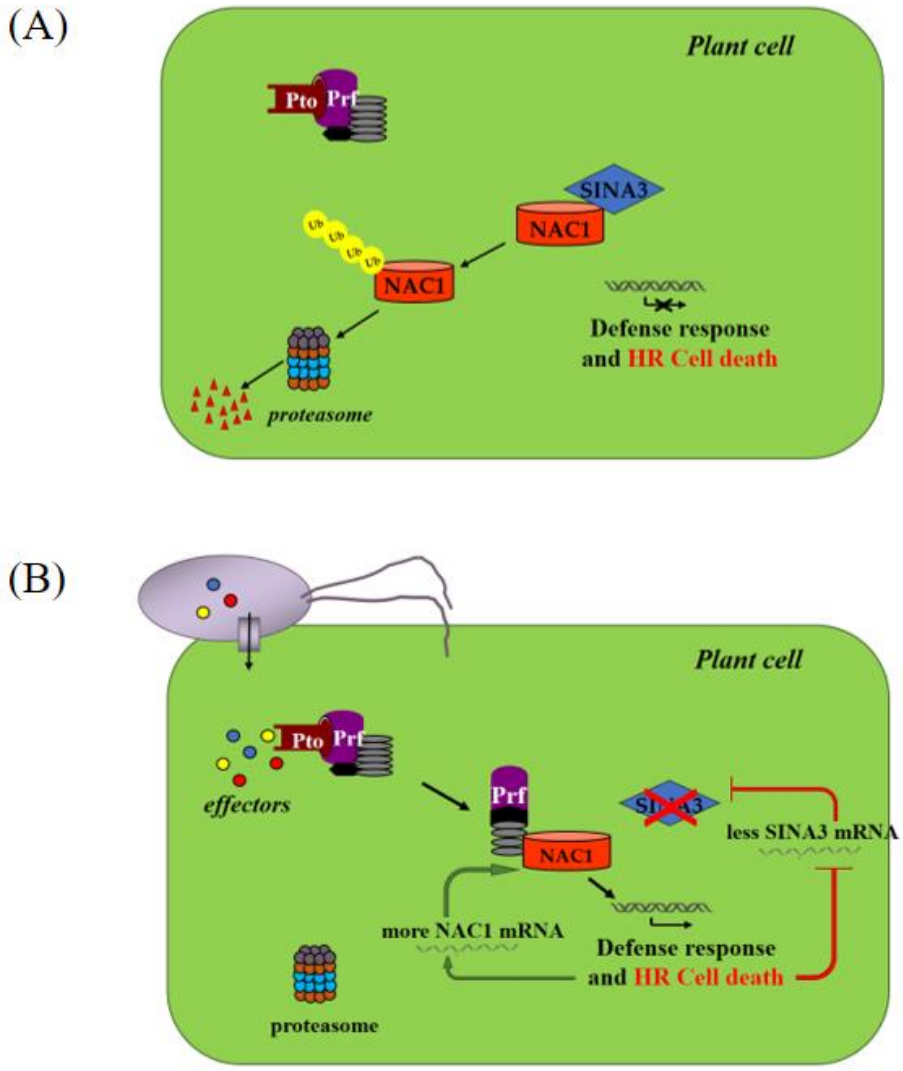

Figure 4.1 Proposed model

(A) Unchallenged plants. Prf exists in the inactive Prf/Pto complex, whereas *Sl*NAC1 is a short-lived protein rapidly ubiquitinated by *Sl*SINA3 and degraded through the 26S-proteasome. **(B) Plants challenged with *PstDC3000*.** Upon detection of avirulent effectors AvrPto/AvrPtoB injected by *Pst* into cytoplasm, the Prf/Pto complex undergoes intra- and intermolecular conformational changes, which allow the signaling-competent Prf to interact with downstream signaling molecules such as *Sl*NAC1. The enhanced stability and transcriptional activity of *Sl*NAC1 by the activated Prf is a direct consequence of Prf protection on *Sl*NAC1 from degradation, presumably through sequestering it away from *Sl*SINA3, thereby inhibiting *Sl*NAC1 ubiquitination. In turn, the highly abundant and transcriptionally active *Sl*NAC1 positively regulates expression of defense-related genes and HR cell death. To ensure prolonged and sufficient accumulation of *Sl*NAC1 protein, *SlNAC1* gene is strongly up-regulated in a Prf-dependent manner, whereas, *SlSINA3* transcript is significantly suppressed.

This proposed model sheds light on the mechanism by which the activation of Prf translates a defense signal into transcriptional reprograming during defense responses, however, there are still questions that need to be addressed in order to fill gaps in our understanding of the Prf signaling network: where in the cell does *Sl*NAC1/Prf interaction occur? does pathogen recognition leads to changes in Prf localization? does Prf manipulate other TFs? does Pto actively participate in Prf-mediated signaling? is ubiquitination the only PTM controlling *Sl*NAC1 activity? what are the target genes directly regulated by *Sl*NAC1?

NLRs tend to co-localize with their cognate effectors. To link receptor function with transcriptional reprogramming, upon activation NLRs undergo nucleocytoplasmic trafficking that allow them to physically associate with target TFs in nucleus. For instance, the nucleocytoplasmic partitioning has been shown to play an important role in the *Hv*MLA10-mediated defense against *Blumeria graminis f. sp. Hordei (Bgh)*. Shen et al., showed that, in unchallenged plants, the majority of MLA10 is localized in the cytoplasm and the nuclear pool represents only small fraction of the total protein. However, upon pathogen perception, *Hv*MLA10 is re-localized to the nucleus (Shen et al., 2007) and this trafficking is essential to restrict *Bgh* growth. Interestingly, some of NLRs, such as *At*RPS4 (resistance to *Pseudomonas syringae* 4) and *At*SNC1, possess a predicted nuclear localization signal (NLS), while others, like *Hv*MLA and *Os*Pb1, do not. Nevertheless, their nuclear localization is indispensable for conferring resistance against corresponding pathogens (Cheng et al., 2009; Inoue et al., 2013; Shen et al., 2007; Wirthmueller et al., 2007). Meanwhile, the growing body of evidence points to a distinct bifurcation of HR cell death and disease resistance signaling. For example, while a nuclear pool of *Hv*MLA10 is associated with defense signaling and resistance, the cytoplasmic localization is indispensable to induce HR cell death (Bai et al., 2012). Similarly,

the nucleocytoplasmic trafficking of *At*RPS4 protein recognizing AvrRps4 effector from *P. syringae* confirms that activation of HR cell death can be separated from activation of resistance, which most likely is due to association with different downstream signaling pathways. Although Prf does not have a predicted NLS, it is reasonable to envisage that Prf/*Sl*NAC1 interaction takes place in the nucleus since *Sl*NAC1 is a transcriptional activator exclusively localized to this compartment. In the future, BiFC and protein fractionation should be performed to investigate the subcellular distribution of Prf and how this protein accumulation pattern changes upon *Pst* infection. Additionally, the careful evaluation of HR cell death and resistance against *Pst* with the artificial cytoplasmic and nuclear distribution of Prf by fusion with the NLS and nuclear export signal (NES) can help reveal how those two defense-related activities contribute to the disease resistance.

In the Prf/Pto complex, prior to recognition of AvrPto/AvrPtoB, the two Prf N-terminal domains dimerize, fold around two Pto molecules and interact with the C-terminal LRR domain to create closed and auto-inhibited structures (Saur et al., 2015). Although AvrPto/AvrPtoB recognition results in an open, signaling-competent state of Prf, the association of Pto with Prf is not completely abolished (Saur et al., 2015). Given the fact that Pto has been earlier shown to specifically interact with three TFs (Pti4/5/6) and phosphorylate at least one serine/threonine kinase Pti1 (Zhou et al., 1995, 1997), it is possible that Pto also plays a role in the *Sl*NAC1-mediated defense signaling. For example, *Sl*NAC1 can be brought into close proximity to Pto, through interaction with the activated Prf, which can lead to *Sl*NAC1 phosphorylation by Pto. In fact, in Chapter 2 we conclude that polyubiquitination may not be the only one PTM of *Sl*NAC1. We speculate that the second slow-migrating band of *Sl*NAC1 detected by WB may represent either a mono-ubiquitinated

and/or phosphorylated form of *Sl*NAC1. To asses this possibility, both forms of *Sl*NAC1 protein can be recovered from SDS-PAGE gel and analyzed by mass spectrometry to determine their identity. Furthermore, previous research on *Hv*MLA10 and *At*SNC1 indicate that some NLRs, which manipulate transcriptional reprograming during plant responses to pathogens, are capable to spontaneously interact with multiple TFs belonging to different transcription factor families (Chang et al., 2013; Shen et al., 2007; Xu et al., 2014; Zhu et al., 2010). Thus, it would be interesting to test if Prf associates with the Pto-interacting TFs: Pti4, Pti5 and Pti6 (Zhou et al., 1997).

Overall, our current knowledge suggests that *Sl*NAC1 functions as a positive regulator of defense responses against *Pst*. However, the molecular targets of *Sl*NAC1 remain unknown. Given the fact *Sl*NAC1 is involved in regulation of biological processes ranging from fruit ripening to plant immunity, it is possible that there are different mechanisms controlling the specificity of its transcriptional activity towards distinct physiological processes. Interestingly, *Sl*NAC1 possesses two transcription activation domains (TADs), TAD1 (*Sl*NAC1$_{191\text{-}270}$) and TAD2 (*Sl*NAC1$_{271\text{-}301}$). TAD1 resides in the region of aa191 to aa270, whereas TAD2 resides in the region of aa271 to aa301. It is possible that these two TADs can act independently by recruiting different transcription activators and/or repressors to control expression of a wide range of target genes involved in diverse physiological processes. Furthermore, the 'molecular behavior' of *Sl*NAC1 can be controlled by highly dynamic and reversible PTMs. PTMs may modulate action of TFs in several non-exclusive ways, such as controlling DNA-binding specificities and affinities as well as ability to interact with protein partners, including (co)activators and (co)repressors that serve as adaptor(s) in the 'decoding' stress signals. It is apparent that the array of PTMs may act independently or cooperatively to

orchestrate the activity of TFs. The comprehensive identification of Prf-*Sl*NAC1-regulated genes can be accomplished by combined systems approaches including RNA sequencing (RNA-seq) and chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) analysis. The RNA-seq-based expression profiling using the WT RG-*PtoR* and *Sl*NAC1-knockout transgenic lines challenged with *Pst* will help answer the question which genes are differentially regulated in the presence and absence of *Sl*NAC1. To identify genes directly regulated *Sl*NAC1, *Pst*-challenged tomato plants can be used to immunoprecipitate the cross-linked genomic DNA-*Sl*NAC1 complexes using the anti-NAC1 antibody, followed by the massively parallel sequencing to determine the promoter region of the *Sl*NAC1 target genes.

Moreover, it is worthwhile noting that chimeric R proteins equipped with a TF domain appear to have evolved multiple times in the plant kingdom (Rinerson et al., 2015). For example, RRS1 is an atypical TIR-NB-LRR protein which possesses an additional C-terminal plant-specific WRKY TF domain that has been shown to bind to the W-box *in vitro* (Noutoshi et al., 2005). The function of the WRKY domain in RRS1 has been investigated for years, and recently it has been shown that this domain serves as a decoy that structurally mimics the pathogen's virulence targets, the defense-related WRKY TFs, to monitor the attempted host immunosuppression (Le Roux et al., 2015; Sarris et al., 2015). Interestingly, given the fact that the W-box is enriched in the promoters of RRS1-regulated genes (Heidrich et al., 2013), it is possible that RRS1 also contributes to transcriptional reprogramming triggered by pathogen perception. Therefore, it was suggested that R-TF chimeric proteins combine different components of signaling pathways to accelerate defense signal transduction by short-

circuiting signaling pathways (Rinerson et al., 2015). In this light, it would be interesting to search genomes of different plants for R-NAC1 gene fusions.

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