BIOLOGICAL FUNCTIONS OF ARABIDOPSIS TGA1 AND TGA4 TRANSCRIPTION FACTORS

A Thesis Submitted to the College of

Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

In the Department of Biology

University of Saskatchewan

Saskatoon, Canada

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ABSTRACT

Plant immune system comprises three main layers. Recognition of conserved microbe-associated molecular patterns (MAMPs) initiates the first layer of immune response, MAMP-triggered immunity (MTI). However successful pathogens utilize virulent strategies, such as effector proteins, to suppress MTI and facilitate their growth, resulting into effector-triggered susceptibility (ETS). In response to ETS, plants acquire resistance proteins to monitor the presence of the effector proteins, leading to stronger immune response, effector-triggered immunity (ETI). During the plant immune response, large-scale transcriptional reprogramming is activated by numerous transcription (co)factors. The Arabidopsis TGA factors have been shown to be required for resistance to disease. In the present study, the biological functions of clade I TGA factors (TGA1 and TGA4) during plant immune responses were investigated in depth.

My results demonstrate that TGA1 and TGA4 are positive regulators in disease resistance against virulent pathogens, such as bacterial pathogen *Pseudomonas syringae* and fungal pathogen *Colletotrichum higginsianum*. In addition, TGA1 and TGA4 positively contribute to disease resistance against a nonpathogenic strain, *P. syringae* pv. *tomato hrcC*$^{-}$, an avirulent strain, *P.s.t. AvrRpt2* and a nonhost pathogen, *P. syringae* pv. *phaseolicola* 1448a.

Loss of resistance in the *tga1-1 tga4-1* double mutant was shown to be associated with defects in cell wall-based defence responses, including callose deposition, apoplastic oxidative burst and extracellular PATHOGENESIS-RELATED 1 (PR-1) protein accumulation. Interestingly, transcript levels of *PR-1*, callose synthase and other genes encoding defence proteins accumulated at, or above, wild-type levels in the mutants. Furthermore, the double mutant is more sensitive to the glycosilation inhibitor, tunicamycin, indicative of a compromised endoplasmic reticulum (ER) stress response. These results suggest that clade I TGA factors control defence-related secretion events that are required for cell wall-associated defence responses.

Analysis of the *non-expressor of pathogenesis-related genes 1* (*npr1-1*) mutant and an *tga1-1 tga4-1 npr1-1* triple mutant indicate that clade I TGA factors act substantially independent of NPR1 during plant immune responses. Moreover, mutation of clade I TGA factors also results in developmental changes, including curly leaves and late flowering. Together, these results demonstrate that clade I TGA factors play a unique role in mediating both defence responses and developmental processes.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my supervisor and mentor, Dr. Pierre Fobert, for giving me the great opportunity to study in Canada. During the past five years, your immense knowledge and scientific attitude always pushed me forward; your kindness and patience always warmed me; your enthusiasm and motivation always encouraged me. I could not have imagined having a better advisor and mentor for my Ph.D study.

I would like to thank my supervisory committee members: Dr. Isobel Parkin, Dr. Yangdou Wei and Dr. Jitao Zou. Your advice and help were invaluable. A special thank to Dr. Yangdou Wei for your useful comments on plant pathology and kindly providing the fungal pathogen *Colletotrichum higginsianum*. And I would also like to thank Dr. Fouad Daayf for serving as my external examiner.

I am very grateful to Dr. Heather Shearer for many years of help and advice, especially teaching me the most critical lab technique for my Ph.D. study, "bacterial-disease test". I would like to send many thanks to Maureen Anderson, Dr. Nirmala Sharma and Catherine DeLong for their great technical support and useful comments. I would also like to thank other members of the Fobert lab, who cheerfully answered my questions and maximally endured my habits in the lab. My thanks would also be sent to all the individuals or units who provided me with research materials and technical services. I am glad to thank NSERC, NRC-PBI, and U of S for their financial support.

Finally and most importantly, I would like to express my great appreciations to my family: my parents, who gave me life and brought me up; my parents-in-law, who provided financial support and full understanding during my Ph.D. study; my wife, Ms. Shu Long, who selflessly supports my study and life; and my two sons, Zixuan and Ziyang, who made me understand the true meaning of life.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

°C	degree Celsius
%	percentage
µ	micro-
µM	micromolar
ABA	abscisic acid
ABRC	Arabidopsis Biological Resources Center
agd2	*aberrant growth and death2*
ANOVA	Analysis of Variance
AP	Alkaline phosphatase
ARF-GEF	ADP ribosylation factor guanine nucleotide exchange factor
as-1	*activating sequence-1*
ATP	adenosine triphosphate
A. tumefaciens	*Agrobacterium tumefaciens*
Avr	Avirulent
B. cinerea	*Botrytis cinerea*
BiP	BINDING PROTEIN
BLAST	basic local alignment search tool
BOP	BLADE-ON-PETIOLE
bp	base pair
BTB/POZ	broad-complex, tramtrack, and bric-a-brac/pox virus and zinc finger
BTH	benzo (1,2,3) thiadiazole-7-cabothionic acid S-methyl ester
bZIP	basic region/leucine zipper
CalS	*callose synthase* gene
CaMV35S	Cauliflower Mosaic Virus 35S promoter
CC	coiled-coil
cDNA	complementary DNA
CEL	conserved effector locus
cfu	colony forming units
C. higginsianum	*Colletotrichum higginsianum*
ChIP	chromatin immunoprecipitation

CNX	calnexin
COI1	CORONATINE INSENSITVE1
Col-0	*Arabidopsis thaliana* ecotype Columbia
COR	coronatine
CRT	calreticulin
CSP	cold shock protein
Cys	cysteine residues
DAD1	DEFENDER AGAINST APOPTOTIC DEATH 1
DAMPs	damage-associated molecular patterns
DNA	deoxyribonucleic acid
dpi	days post inoculation
E	Einstein units
edr1	*enhanced disease resistance 1*
EDS	enhanced disease susceptibility
EFR	EF-Tu receptor
EF-Tu	elongation factor Tu
Eil-0	*Arabidopsis thaliana* ecotype Eilenburg
elf18	conserved 18 amino acid peptide from elongation factor Tu
EMS	Ethylmethane Sulphonate
EMSA	electrophoretic mobility shift assay
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ERF1	ETHYLENE RESPONSE FACTOR 1
ERQC	ER quality control
ERSEs	ER stress-response elements
ET	ethylene
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
FLC	*FLOWERING LOCUS C*
flg22	conserved 22 amino acid peptide from flagelin
FLS2	FLAGELLIN SENSING 2

FRK1	*FLG22-INDUCED RECEPTOR-LIKE KINASE 1*
FT	*FLOWER LOCUS T*
g	gram(s)
GA	gibberellins
GFP	green fluorescent protein
GRP94	GLUCOSE REGULATED PROTEIN 94
GRX	glutaredoxins
GSNO	S-nitroglutathione
h	hour(s)
HEL	*HEVEIN-LIKE*
H_2O_2	hydrogen peroxide
Hop	Hrp outer protein
H. arabipodsidis	*Hyaloperonospora arabipodsidis* (formerly *Peronospora parasitica*)
HR	hypersensitive response
hrc	hypersensitive response and conserved
hrp	hypersensitive response and pathogenicity
HRP	horseradish peroxidase
HSP	heat shock protein
IF	intercellular fluid
INA	2,6-dichloroisonicotinic acid
ISR	induced systemic resistance
JA	jasmonic acid
JA-Ile	jasmonoyl-isoleucine
JAZ	JASMONATE ZIM-DOMAIN
JIN1	JASMONATE-INSENSITIVE1
k-RT-PCR	Kinetic reverse-transcriptase PCR
l	litre(s)
LB	left T-DNA border repeats
LD	long day condition
Ler-0	*Arabidopsis thaliana* ecotype Landsberg erecta
LOX2	*LIPOXYGENASE2*

LPS	lipopolysaccharide
LRRs	leucine rich repeats
LS	linker scan
lsd6	*lesions simulating diesease6*
M	molar
MAMPs	microbe-associated molecular patterns
MAPK	mitogen activated protein kinase
$MgCl_2$	magnesium chloride
Min	minute(s)
ml	milliliter
mm	millimeter
mM	millimolar
mol	moles
mRNA	messenger RNA
MS	Murashige and Skoog
MTI	MAMP-triggered immunity
NahG	*salicylate hydroxylase*
NB	nucleotide binding
NHO1	*NONHOST RESISTANCE 1*
NHR	non-host resistance
NIM1	*NON-INDUCIBLE IMMUNITY 1*
NIMIN	*NIM1 INTERACTING PROTEIN 1*
NO	nitric oxide
Nö	*Arabidopsis thaliana* ecotype Nössen
NPR1	*NON-EXPRESSER OF PATHOGENESIS-RELATED GENES 1*
NPTII	*neomycin phosphotransferase II*
ocs	*octopine synthase*
ODx	absorbance at wavelength (x)
OE	overexpression
PAD4	*PHYTOALEXIN DEFICIENT 4*
PAL	*PHENYLALANINE AMMONIA LYASE*

PAMPs	pathogen-associated molecular patterns
PAN	PERIANTHIA
PAGE	polyacrylamide gel electrophoresis
PCD	programmed cell death
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDF1.2	*PLANT DEFENSIN 1.2*
PDIs	protein disulfide isomerases
PEN2	*PENETRATION2*
PM	plasma membrane
PMR4	*POWDERY MILDEW RESISTANCE 4*
PMSF	phenylmethylsulfonyl fluoride
PR	pathogenesis-related
PRRs	pattern recognition receptors
P. syringae	*Pseudomonas syringae*
P.s.m.	*Pseudomonas syringae* pv. *maculicola*
P.s.t.	*Pseudomonas syringae* pv. *tomato*
pv	pathovar
PVDF	polyvinylidene difluoride
RB	right T-DNA border repeats
RBOH	RESPIRATORY BURST OXIDASE HOMOLOG
RCH	recognition of *C. higginsianum*
R-gene	*Resistance*-gene
RLKs	Receptor-like kinases
RLPs	receptor-like proteins
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RPL29	*RIBOSOMAL PROTEIN L29*
RPS4	*RESISTANCE TO PSEUDOMONAS SYRINGAE 4*
RRS1	*RESISTANCE TO RALSTONIA SOLANACEARUM 1*

SA	salicylic acid
SAI1	*SALICYLIC ACID INSENSITIVE 1* (aka *npr1-5*)
SAR	systemic acquired resistance
SCL14	SCARECROW-like 14
SD	short day condition
SDS	sodium dodecyl sulphate
Sec	second(s)
SID2	*SALICYLIC ACID DEFICIENT2*
SIGnAL	Salk Institute Genome Analysis Laboratory
SNAP	synaptosomal-associated protein
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNC1	*SUPPRESSOR OF npr1-1, CONSTITUTIVE 1*
SGI	seedling growth inhibition
sum1 sum2	*small ubiquitin-like modifier1* and *2*
SYP	syntaxin proteins,
T3SS	type III secretion system
T3SE	T3SS effectors
TAT3	*TYROSINE AMINOTRANSFERASE3*
T-DNA	*Agrobacterim tumefaciens* transfer-DNA
TE	Tris buffer and EDTA
Ti	tumour-inducing
TIR	Toll-interleukin-1 receptor
TM	tunicamycin
Tween-20	polyoxyethylene sorbitan monolaurate
UBQ5	*UBIQUITIN5*
UPR	unfolded protein response
UTR	untranslated region
VAMP	vesicle-associated membrane protein
VIGS	virus-induced gene silencing
VSP2	*VEGETATIVE STORAGE PROTEIN 2*
v/v	volume/volume

WIR	wound-induced resistance
WS	*Arabidopsis thaliana* ecotype Wassilewskija
Wt	wild type
w/v	weight/volume

CHAPTER 1 LITERATURE REVIEW

To live or die: this is an everlasting and common theme between plants and microbes since the establishment of the first land plants. Microbes have evolved to plunder plant nutrients and energy which they cannot produce by themselves, whereas plants have developed a powerful defence system to protect themselves from microbial attacks. The plant defence system is similar to immune systems described in animals. Both consist of biological structures and processes responsible for protecting against disease and avoiding unwanted biological inasions. These similarities extend to the molecular level, where the plant defence system has been found to share many common features with animal innate immunity, including defined receptors for microbe-associated molecules, conserved mitogen-associated protein kinase signaling cascades, transcriptional reprogramming and the production of antimicrobial compounds (Ausubel, 2005). Therefore, the plant defence system is also referred to as the plant immune system, which relies on the innate immunity of each cell and on systemic signals spreading from infection sites.

1.1 Plant pathogens

Many plant-microbe interactions can be described as parasitism. A microbe is called a parasite if it lives on or in a plant and obtains its nutrients and energy from the latter (Agrios, 2005). Similarly, the plant that produces nutrients and food for the parasite is referred to as the host. In some cases of parasitism, both the plant and the microbe benefit from the interaction. This phenomenon is known as symbiosis. However, in most cases the removal of nutrients and water by the parasite from the host plant usually interrupts the normal growth of the plant and becomes detrimental to further development and reproduction of the host. Such parasites are considered as pathogens, which cause disease on the host plants. The qualitative ability of a pathogen to invade and multiply in the host and finally cause disease is termed pathogenicity, while the degree of the pathogen to cause disease is termed virulence. Moreover the process of pathogen infection, colonization, and pathogen reproduction, or the mechanism by which the disease is caused, is known as pathogenesis (Agrios, 2005).

According to their lifestyles, plant pathogens can be classified as necrotrophs or biotrophs (Glazebrook, 2005). Necrotrophs kill plant tissues immediately after entry into the host and derive nutrients from the resulting dead material. Necrotrophs can synthesize chemical toxins to poison plant cells or can produce hydrolytic enzymes to break down the polymers of the plant cell wall. For example, the fungal pathogen *Botrytis cinerea* and bacteria of the genus *Erwinia* are considered necrotrophs (van Kan, 2006). In contrast, biotrophic pathogens, including mildew and rust fungi, must grow on living tissue to complete their normal life cycle. Biotrophs usually plunder metabolites from hosts and alter the balance of host phytohormones involved in growth and development (Jones, 2009). These infections can result in delayed senescence of host tissues as well as stunting and abnormal growth patterns. Many pathogens display both lifestyles during their life cycle. These pathogens initially deploy a biotrophic strategy, and then switch to a necrotrophic mode at later stages of the infection (Glazebrook, 2005). The switch is usually triggered by increasing nutritional demands as the pathogen biomass increases. Such pathogens are called hemibiotrophs.

The bacterial pathogen *Pseudomonas syringae* can be best considered as a hemibiotrophic pathogen (Katagiri et al., 2002). After entering through wounds and natural openings, *P. syringae* multiplies in intercellular spaces without host cell death. During the late stage of pathogenesis, however, host cells are killed and infected tissues show water-soaked patches, which eventually become chlorotic and necrotic (Agrios, 2005). It is noteworthy that there are also numerous references in the literature of *P. syringae* as a biotrophic pathogen (Alfano and Collmer, 1996).

Like many bacterial pathogens of animals, *P. syringae* uses the type III secretion system (T3SS) as its major virulence strategy (Grant et al., 2006). The T3SS is structurally similar to the bacterial flagellum, forming a pilus between the bacteria and the host membrane to establish cell-to-cell contact, through which is injected a diverse group of bacterial proteins, known as T3SS effectors (T3SE), into host plant cells. Components of the T3SS are encoded by a suite of *hrp* (hypersensitive response and pathogenicity) and *hrc* (hypersensitive response and conserved) genes (Alfano and Collmer, 2004). Mutations in *hrp/hrc* genes result in malfunctioning of the T3SS and prevent bacterial colonization within plant tissue, highlighting the critical role of T3SS and T3SE for bacterial pathogenesis. Effectors display high specificity both within and among

bacterial species, and their presence is highly polymorphic within a species (Chisholm et al., 2006). Effectors delivered by the T3SS possess diverse enzymatic activities, such as cystein protein protease, ubiquitin-like protease, E3 ubiquitin ligase, transcriptional activity and protein phosphatase activity (Abramovitch et al., 2006; Block and Alfano, 2011; Zhou and Chai, 2008). These effectors can modify host proteins to suppress host defence responses and create a more favorable niche for the microbe, thereby promoting proliferation (Grant et al., 2006; Mudgett, 2005). More than thirty effectors are delivered by *P. syringae* pathovar (pv.) *tomato* (*P.s.t.*) into the plant cell (Chang et al., 2005; Petnicki-Ocwieja et al., 2002).

In addition to effector proteins, bacterial pathogens may also produce phytotoxins, such as coronatine (COR), to promote disease. Plant hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play important signaling roles in the plant defence response (see section 1.4). In general, SA-dependent signaling is responsible for resistance against biotrophic pathogens, whereas JA/ET-mediated signaling is important for resistance against necrotrophs and chewing insects (Glazebrook, 2005; Vlot et al., 2009). These two pathways act antagonistically to some extent. *P. syringae* exploits this fact to secrete COR into host cell, which is structurally similar to JA and suppresses SA-mediated defence responses (He et al., 2004; Reymond and Farmer, 1998). Furthermore, *P. syringae* uses COR to induce opening of plant stomata to permit entry of the pathogen (Melotto et al., 2006).

As a plant pathogen, *P. syringae* can infect a wide variety of plant species and cause disease symptoms ranging from leaf spots to stem cankers (Agrios, 2005). Over 50 different pathovars exist in the species of *P. syingae* (Hirano and Upper, 2000). *P. syringae* had already been proven to be an excellent genetically tractable pathogen of many crop plants (Keen, 1990). The interaction between *P. syringae* and *Arabidopsis thaliana* (herein referred to as Arabidopsis) was characterized in detail following the identification of several strains of *P. syringae* which can infect this model dicot plant in the 1980's (Katagiri et al., 2002). Owing to availability of both genome sequences (Arabidopsis Genome Initiative, 2000; O'Brien et al., 2011), the Arabidopsis-*P. syringae* pathosystem has emerged as an important model system for experimental characterization of the molecular mechanism underlying plant-pathogen interactions.

1.2 Plant defence responses

In nature, several physical structures and preexisting chemicals form the first line of defence when microbes come into contact with plant surfaces through different media (Agrios, 2005). Water-repellent waxes and a thick mat of hairs on the plant surface can prevent the formation of a water film where microbes might be deposited, germinate and multiply. The thick cuticle and epidermal cell wall form hard physical barriers against microbial penetration. Moreover, plants possess a large variety of secondary metabolites which have antimicrobial properties and are sequestered in vacuoles or organelles in the outer cell layers of plant tissues. These are sometimes referred to as phytoanticipins (Morrissey and Osbourn, 1999).

In addition to preexisting physical and chemical barriers, plants also employ an active immune system to detect and repel invading microbial pathogens (Figure 1.1). The first and most ancient layer of this inducible system relies on the recognition of conserved microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) and is known as MAMP-triggered immunity (MTI) (Boller and Felix, 2009; Chisholm et al., 2006; Jones and Dangl, 2006). MAMPs play an essential role in microbial lifestyle, are widely distributed among different microbes, and are absent in the host (Nurnberger et al., 2004). Examples of MAMPs include cell-surface components of Gram-negative bacteria, such as flagellin, the protein subunit of the flagellum, and lipopolysaccharide (LPS), a major constituent of the outer membrane, as well as chitin and ergosterol in the cell wall of fungi (Baureithel et al., 1994; Gomez-Gomez and Boller, 2002; Granado et al., 1995; Newman et al., 2002). Intracellular proteins, such as the bacterial cold shock protein (CSP) (Felix and Boller, 2003) and the translation elongation factor Tu (EF-Tu) are also recognized by plant hosts as MAMPs (Kunze et al., 2004). Besides these non-self molecules, plants also can sense non-self activity through damage-associated molecular patterns (DAMPs), sometimes also referred to as danger-associated molecular patterns (Boller and Felix, 2009). For example, cutin derivatives degraded by fungal cutinases are able to elicit plant defence responses and prime plants for further responsiveness to MAMPs (Fauth et al., 1998; Hückelhoven, 2007).

Perception of MAMPs is carried out by plant pattern recognition receptors (PRRs) located on the plasma membrane (Boller and Felix, 2009; Chisholm et al., 2006; Jones and Dangl,

2006). Plant PRRs can be divided into two classes: Receptor-like kinases (RLKs) containing an intracellular serine/threonine kinase domain, and receptor-like proteins (RLPs) with a short cytoplasmic tail on the intracellular side. The extracellular domains of PPRs can contain leucine-rich repeats (LRRs) or LysM-motifs, which are responsible for MAMPs perception (Boller and Felix, 2009). FLAGELLIN SENSING 2 (FLS2) and EF-Tu receptor (EFR) are the two best characterized PRRs in Arabidopsis, and recognize bacterial flagellin and EF-Tu, respectively (Gomez-Gomez and Boller, 2000; Zipfel et al., 2006). Loss-of-function of FLS2 or EFR results in enhanced susceptibility to bacterial strains in Arabidopsis, providing evidence for a key role of both PRRs in host immunity (Zipfel et al., 2004; Zipfel et al., 2006).

Downstream of MAMP perception, multiple defence responses are activated that restrict the growth of attacking pathogens. For example, some early defence events occur within minutes after perception, including changes in ion-fluxes across the plasma membrane, increased intracellular Ca^{2+} concentration, production of reactive oxygen species (ROS) and activation of MAPK (mitogen activated protein kinase) signaling (Schwessinger and Zipfel, 2008). Later on, within 30 min and up to several days after recognition, global transcriptional changes are induced to produce antimicrobial products, such as PR (pathogenesis related) proteins and phytoalexins. At this stage, MTI can efficiently prevent pathogen proliferation in the apoplast and no or very few macroscopic disease symptoms occur (Göhre and Robatzek, 2008).

However, successful pathogens have evolved to overcome MTI by different means. As discussed in section 1.1, pathogenic Gram-negative bacteria deliver T3SEs into the host cells (Staskawicz et al., 2001). Also, biotrophic fungal phytopathogens can form a specialized infection structure, called haustorium, to deliver effectors into the intercellular space of the host (Chisholm et al., 2006). Fungal and bacterial effectors can suppress MTI and alter plant physiology in susceptible hosts to benefit pathogen colonization. As a consequence, plant defences are compromised in their ability to stop pathogen propagation and can only limit its level of virulence: This is called effector-triggered susceptibility (ETS) (Chisholm et al., 2006; Jones and Dangl, 2006). The identification of mutants hyper-susceptible to virulent pathogens, sometimes referred to as an enhanced disease susceptibility (EDS) phenotype (Glazebrook et al., 1996; Rogers and Ausubel, 1997) has provided genetic evidence for the existence of defence responses operating during ETS.

Plants, in turn, have acquired resistance (R) proteins to detect pathogen effectors or their effects on host targets, resulting in a strong immune response known as effector-triggered immunity (ETI) (Chisholm et al., 2006; Jones and Dangl, 2006). Thus, effectors function as virulence factors to suppress MTI and facilitate pathogen growth in plants where the corresponding R protein is absent. Otherwise, effectors can be recognized by corresponding R proteins as avirulent factors (Avr) to trigger ETI. Plant-pathogen interactions where effectors are not recognized by R proteins and result in disease are said to be compatible, while those where one or more effector is recognized as an Avr factor, triggering ETI, are called incompatible. These pairwise associations have been characterized genetically as gene-for-gene resistance (Flor, 1971).

Most characterized R proteins are receptor-like proteins which contain a nucleotide binding (NB) site and LRR domains (Collier and Moffett, 2009). According to their N-terminal domain, many NB-LRR R proteins can be further divided into coiled-coil (CC) NB-LRR or Toll-interleukin-1 receptor (TIR) NB-LRR. R proteins can act as receptors that directly interact with the pathogen effectors. This ligand-receptor model is supported by a few R-Avr combinations, such as the interaction between the rice Pi-ta R protein and the *Magnaporthe grisea* effector AvrPita (Jia et al., 2000). However, in most cases R proteins recognize effectors indirectly. Based on experimental data, two conceptual models have been proposed to understand the indirect effector perception mechanism. The ‘guard’ model predicts that R proteins guard a host protein(s) (or guardee) that is targeted and modified by pathogen effectors (Dangl and Jones, 2001). Modification of its target(s) by the effector activates the corresponding R protein, leading to ETI in the host. According to the model, this guarded protein is required for the virulence function of the effector protein in the compatible host. However, new data have shown that guardee proteins are often dispensable for the virulence activities of effectors in plants lacking the R protein. To resolve this discrepancy, the ‘decoy model’ was proposed, in which a host protein, termed ‘decoy’, mimics effector targets to trap the pathogen into a recognition event (van der Hoorn and Kamoun, 2008). Different from the guardee in the guard model, the decoy only functions in perception of pathogen effectors without contribution either in the development of disease or resistance.

ETI will accelerate and reinforce the defence response of a host having compromised MTI. Two levels of ETI have been observed, weak and strong. Weak ETI usually does not cause any macroscopic symptoms, while strong ETI is often associated with a hypersensitive response (HR), a rapid form of programmed cell death (PCD), localized at the site of infection to limit the access of pathogen to water and nutrients (Göhre and Robatzek, 2008).

During the co-evolutionary "arms race" between pathogens and their host plants, selection pressure drives pathogens to avoid ETI by altering or eliminating the effectors that are recognized or to suppress the ETI response by acquiring novel effectors (Jones and Dangl, 2006; Mudgett, 2005). Ultimately, this dynamic co-evolution continuously selects novel pathogen races that overcome ETI and new plant genotypes that resurrect ETI.

The above "zig-zag, zig" model provides a useful framework to describe various types of plant-pathogen interactions. For example, non-host resistance (NHR), wherein an entire plant species displays resistance against all members of a pathogen species (Heath, 2000; Lipka et al., 2008), has been proposed to result from a combination of MTI and ETI, with the relative contribution of either form of immunity varying depending on the specific interaction (Schulze-Lefert and Panstruga, 2011). Host-compatible interactions, in which plants display what is sometimes referred to as basal resistance, have been described as "MTI plus weak ETI, minus ETS" (Jones and Dangl, 2006; Nishimura and Dangl, 2010). In these interactions, the level of MTI suppression by ETS is sufficient to cause disease and residual defences can only limit the extent of disease. Finally, resistance in host-incompatible interactions is mediated by *R*-genes according to the gene-for-gene hypothesis, and is largely attributed to ETI.

Although the zig-zag, zig model has been widely accepted by molecular plant pathologists, increasing evidence argues that the conceptual and artificial dichotomies between MAMPs and effectors, PRRs and R proteins, and ETI and MTI are often difficult to resolve in nature (Thomma et al., 2011). For example, some effectors are widely distributed and qualify to be designated as MAMPs, while some MAMPs are only narrowly conserved or contribute to pathogen virulence. Furthermore, ETI and MTI seem to share many, common downstream signaling and responses, such as the ROS burst (Torres et al., 2006), activation of MAPKs (Pitzschke et al., 2009), hormonal changes (Tsuda and Katagiri, 2010), transcriptome

reprogramming (Navarro et al., 2004), callose deposition (Ham et al., 2007; Tang et al., 1999) and the HR (Thomma et al., 2011). Therefore, MTI and ETI should instead be considered as opposite ends of a continuum in which plant immune receptors recognize appropriate ligands to activate defence responses, the amplitude of which is likely determined by the level required for effective immunity (Thomma et al., 2011).

1.3 Systemic immunity

Besides the local and primary defence responses discussed above, plants can mount long lasting and systemically induced resistance against a broad spectrum of microbes. Depending on the microbes that interact with the plant, there are three well known types of induced resistances. Systemic acquired resistance (SAR) is triggered by pathogens causing limited infection such as HR or necrosis (Durrant and Dong, 2004). Rhizobacteria-induced systemic resistance (ISR) is activated upon colonization of roots by selected strains of non-pathogenic rhizobacteria (van Loon et al., 1998), and wound-induced resistance (WIR) is typically elicited upon tissue damage, such as that caused by insect feeding (Kessler and Baldwin, 2002).

1.4 Defence-related plant hormones

Plant defence in response to pathogen challenge is regulated through a complex network of signaling pathways involving plant hormones. The importance of SA, JA, and ET as key defence-related hormones is well established (Broekaert et al., 2006; Browse, 2009; Vlot et al., 2009). In addition, other plant hormones, including abscisic acid, auxin, gibberellins, cytokinin and brassinosteroids have been implicated in plant defence against microbial pathogens (Robert-Seilaniantz et al., 2007; Robert-Seilaniantz et al., 2011). In general, SA-dependent signaling is responsible for resistance against biotrophic pathogens, whereas JA/ET-mediated signaling is important for resistance against necrotrophs (Glazebrook, 2005). These two pathways are mostly antagonistic: elevated SA-dependent resistance against biotrophs is often correlated with increased susceptibility to necrotrophs, and vice versa (Pieterse et al., 2009; Robert-Seilaniantz et al., 2011). Besides the antagonistic crosstalk between SA and JA/ET pathways, synergistic interactions also have been reported (Pieterse et al., 2009). Analysis of a quadruple mutant blocking three signaling pathways revealed that JA, ET and SA signaling all contribute

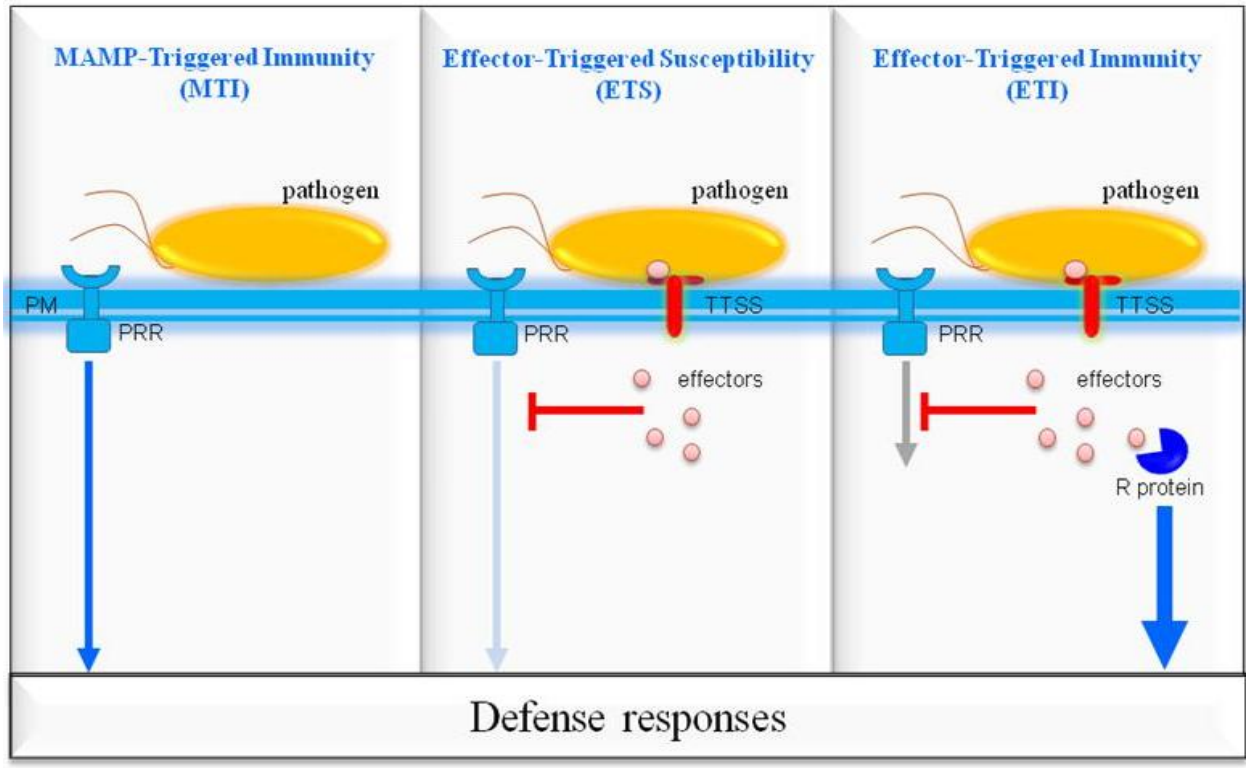


Figure 1.1 Model for the plant immune system.
Recognition of MAMPs by PRR initiates MAMP-triggered immunity (MTI), which prevents infection of most microbes in the apoplast. However successful pathogens have evolved mechanisms to suppress MTI and promote disease. Specifically, the bacterial pathogen *Pseudomonas syringae* uses a type III secretion system (T3SS) to deliver effectors into the plant cytoplasm, resulting in effector-triggered susceptibility (ETS). In response to ETS, plants have acquired resistance (R) proteins to detect pathogen effectors or their effects on host targets, resulting in a stronger immune response, known as effector-triggered immunity (ETI). (Modified from Chisholm et al., 2006.)

positively to MTI and ETI (Tsuda et al., 2009). Several key regulators in SA-JA crosstalk have been identified, such as NPR1/NIM1 (section 1.7) (Spoel et al., 2003), clade II TGA factors (section 1.8), glutaredoxin GRX480 (Ndamukong et al., 2007) and transcription factor WRKY70 (Li et al., 2004).

1.5 The JA/ET signaling pathway

A key regulator in the JA signaling pathway is an F-box protein CORONATINE INSENSITVE1 (COI1) (Feys et al., 1994; Xie et al., 1998; Yan et al., 2009). Upon stimulation of the JA response, jasmonoyl-isoleucine (JA-Ile), the active form of JA, promotes COI1 to interact with the JASMONATE ZIM-DOMAIN (JAZ) proteins, which negatively regulate JA signaling by inactivating transcription factor AtMYC2/JIN1 (JASMONATE-INSENSITIVE1) (Chini et al., 2007; Thines et al., 2007). After COI1-mediated ubiquitinylation and subsequent degradation of JAZ proteins, AtMYC2 can activate JA-responsive genes specific to wounding and insect attack, such as *VSP2* (*VEGETATIVE STORAGE PROTEIN 2*) and *LOX2* (*LIPOXYGENASE2)* (Lorenzo et al., 2004; Lorenzo and Solano, 2005). At the same time, AtMYC2 is a negative regulator of *PDF1.2 (PLANT DEFENSIN 1.2)* and *HEL* (*HEVEIN-LIKE*), which are ET/JA-co-responsive genes induced by necrotrophic pathogens (Lorenzo et al., 2004). The cooperation of the ET and JA signals relies on transcriptional factors, such as ORA59 and ERF1 (ETHYLENE RESPONSE FACTOR 1) (Lorenzo et al., 2004).

1.6 The SA signaling pathway

The plant hormone salicylic acid (SA) has been shown to play a critical signaling role in the various plant defence responses against pathogen infection. MTI- and ETI-mediated pathogen recognition can both trigger endogenous SA accumulation which correlates with the induction of *PR* genes and the activation of disease resistance (Malamy et al., 1990; Metraux et al., 1990; Mishina and Zeier, 2007a; Mishina and Zeier, 2007b; Tsuda et al., 2008). In addition, exogenous treatment with SA or its synthetic functional analogs, such as benzo (1,2,3) thiadiazole-7- carbothioic acid S-methyl ester (BTH) and 2,6-dichloroisonicotinic acid (INA), can stimulate *PR* gene expression and/or enhance disease resistance in many plant species (Vlot et al., 2009).

Conclusive evidence supporting the importance of SA in plant defence comes from the studies of transgenic and mutant plants with altered levels of this phenolic metabolite. For example, transgenic tobacco or Arabidopsis plants expressing the bacterial *NahG* gene, encoding salicylate hydroxylase that converts SA to catechol, fail to accumulate high SA levels and express *PR* genes, resulting in enhanced susceptibility to a variety of pathogens (Delaney et al., 1994; Gaffney et al., 1993; Kachroo et al., 2000). Mutation in a gene responsible for SA biosynthesis, *SALICYLIC ACID DEFICIENT2/ENHANCED DISEASE SUSCEPTIBILITY16* (*SID2/EDS16*), abolishes *PR* gene activation and disease resistance after biotrophic pathogen infection (Durrant and Dong, 2004). Furthermore, a lipase-like protein ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and its sequence-related interacting partner PHYTOALEXIN DEFICIENT 4 (PAD4) are also required for activation of SA accumulation in basal resistance against biotrophic pathogens as well as in ETI initiated by TIR-NB-LRR R proteins (Wiermer et al., 2005). Recently, EDS1 has been shown to behave as an effector target and guarded by R proteins during ETI (Bhattacharjee et al., 2011; Heidrich et al., 2011).

As a marker gene in the SA signaling, the positive regulation of *PR-1* (At2g14610) expression largely relies on NPR1/NIM1 (section 1.7), TGA factors (section 1.8) and other SA-signaling components mentioned above. In addition, the mechanisms of negative regulation of *PR-1* gene expression have been also well studied. *PR-1* promoter contains two negative *cis*-elements: WRKY-binding site *LS4* and TGA factor-binding site *LS5* (Lebel et al., 1998). Mutations in *LS4* enhanced promoter activity (Lebel et al., 1998), suggesting that WRKY transcription factors binding to *LS4* negatively regulate *PR-1* expresion. Due to the repression of basal *PR-1* expression by clade II TGA factors, it was proposed that they bind to *LS5* (Boyle et al., 2009; Kesarwani et al., 2007; Zhang et al., 2003b). In addition, the NIMIN1 (NIM-INTERACTING 1) protein, negatively regulates *PR-1* expression through interaction with NPR1 and TGA factors (Weigel et al., 2005). The two NPR1 paralogs NPR3 and NPR4 have been shown to interact with TGA factors (Liu et al., 2005; Zhang et al., 2006). The *npr3 npr4* double mutant displayed elevated basal *PR-1* expression, suggesting a role for NPR3 and NPR4 in repressing *PR-1* transcription in the absence of pathogen challenge (Zhang et al., 2006).

Another, indirect, mechanism of *PR-1* regulation relies on signal transduction events. For example, mutation in a TIR-NB-LRR-type *R* gene, *SNC1* (*SUPPRESSOR OF npr1-1,*

CONSTITUTIVE 1; At4g16890), results in constitutive *PR-1* gene expression and enhanced resistance to several pathogens (Li et al., 2001; Zhang et al., 2003a). It has been proposed that enhanced resistance in *snc1* mutants relies on ETI (Germain et al., 2010). In addition, some mutants defective in the secretion pathway and cell wall biosynthesis also constitutively activate *PR-1* expression level. For example, mutation in two secretion-related syntaxin proteins, SYP121/PEN1 (At3g11820) and SYP122 (At3g52400), result in the elevated *PR-1* expression (Zhang et al., 2007). Loss of function of callose synthase gene *CalS12* (also known as *POWDERY MILDEW RESISTANCE 4, PMR4;* At4g03550) also results in constitutively elevated *PR-1* transcript levels (Nishimura et al., 2003).

1.7 NPR1

Signaling transduction from SA to activated *PR* gene expression and disease resistance is largely regulated by NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1; also known as NO-INDUCED IMMUNITY1 or NIM1), a key regulator of plant immunity (Dong, 2004; Durrant and Dong, 2004; Pieterse and van Loon, 2004). While plants with mutation in *NPR1* continue to accumulate high levels of SA following pathogen challenge, they are compromised in SA-induced *PR* gene expression and fail to develop SAR (Cao et al., 1994). The *npr1* mutants also show enhanced disease susceptibility to virulent pathogens and are impaired in ETI against avirulent pathogens (Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). However, NPR1 is not required for NHR against *P. syringae* pv. *phaseolicola* (*P.s.p.*) (van Wees and Glazebrook, 2003) or MAMP-induced resistance (Zipfel et al., 2004).

NPR1 contains two protein-protein interaction domains, a BTB/POZ (Broad-Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger) domain at the N-terminus and an ankyrin repeat domain in the central region (Cao et al., 1997; Rochon et al., 2006; Ryals et al., 1997), as well as a nuclear localization signal and a transactivation domain containing two oxidized cysteine residues at the C-terminus (Kinkema et al., 2000; Rochon et al., 2006).

In resting cells of wild type Arabidopsis, endogenous NPR1 was detected in both the cytoplasm and the nucleus (Després et al., 2000). In the cytoplasm, NPR1 has been postulated to predominantly exist in the form of oligomer complex through analysis of an NPR1:GFP fusion

protein (Mou et al., 2003). Cytosolic NPR1:GFP oligomers are held together by redox-sensitive intermolecular disulfide bonds between conserved cysteine residues. Upon pathogen infection, accumulation of SA induces a change in cellular redox state, resulting in partial reduction of NPR1 oligomer to monomer (Mou et al., 2003; Tada et al., 2008). NPR1 monomer is subsequently translocated into the nucleus, where it functions as a coactivator of gene transcription (Kinkema et al., 2000). The NPR1 protein does not possess a canonical DNA binding domain and has never been shown to bind DNA directly. Therefore, NPR1 was proposed to regulate *PR* gene expression through interactions with other transcription factors, such as WRKY and TGA transcription factors (Eulgem, 2005).

1.8 TGA transcription factors

TGA factors are members of the basic region/leucine zipper (bZIP) class of transcription factors originally isolated based on their ability to bind to the SA-, JA-, and auxin-inducible *activating sequence-1* (*as-1*) element found in the cauliflower mosaic virus (CaMV) 35S promoter (Katagiri et al., 1989) or a similar element in the *octopine synthase* (*ocs*) promoter from the *Agrobacterium tumefaciens* tumour-inducing (Ti) plasmid (Fromm et al., 1989).

The bZIP domain in TGA factors contains two structural features located on a contiguous α-helix: the N-terminal basic region responsible for DNA binding activity, and the C-terminal region containing a heptad repeat of leucines or other bulky hydrophobic amino acids which mediates dimerization by forming a parallel coiled coil called the leucine zipper (Jakoby et al., 2002). TGA factors typically function as homodimers and/or heterodimers when bound to DNA (Fobert, 2007).

The as-1 element is a 21 base pair (bp) sequence containing two TGACG motifs which is recognized by TGA factors. This has been demonstrated *in vitro* using gel mobility shift and *in vivo* by chromatin immunoprecipitation (ChIP) assays (Johnson et al., 2003; Jupin and Chua, 1996; Lam and Lam, 1995; Miao and Lam, 1995; Rochon et al., 2006). The *as-1* or *as-1*-like elements have been found to be over-represented in the promoters of genes differentially expressed under abiotic stress and pathogen infection (Mahalingam et al., 2003) and also appear on the promoters of many SA-responsible genes, such as *PR-1* gene (Lebel et al., 1998). Linker-

scanning (LS) mutagenesis of the *PR-1* promoter revealed two TGACG motifs (*LS5* and *LS7*), which have opposite effects on *PR-1* expression in response to SA and INA (Lebel et al., 1998). TGA factors have been shown to bind to the *PR-1* promoter *in planta*, presumably on these same TGACG motifs (Johnson et al., 2003; Rochon et al., 2006).

The Arabidopsis TGA family consists of 10 members (Jakoby et al., 2002). Seven TGA factors (TGA1 to TGA7) have been shown to interact with NPR1 (Després et al., 2000; Després et al., 2003; Zhang et al., 1999; Zhou et al., 2000) and play overlapping roles in plant disease resistance and stress responses (Kesarwani et al., 2007; Shearer et al., 2012; Zhang et al., 2003b). Based on sequence similarity, Arabidopsis TGA factors can be divided into three subclasses: clade I (TGA1 (At5g65210) and TGA4 (At5g10030)); clade II (TGA2 (At5g06950), TGA5 (At5g06960) and TGA6 (At3g12250)); and clade III (TGA3 (At1g22070) and TGA7 (At1g77920)) (Figure 1.2) (Hepworth et al., 2005; Xiang et al., 1997). In addition, TGA9/bZIP21 (At1g08320) and TGA10/bZIP65 (At5g06839) form a distinct subclade and PERIANTHIA (PAN, At1g68640) is a unique member in the phylogenetic tree (Figure 1.2). These last three TGA factors have been shown to contribute developmental process (Chuang et al., 1999; Murmu et al., 2010; Running and Meyerowitz, 1996).

Emerging data indicate that the Arabidopsis TGA factors regulate the expression of *PR* genes and are required for resistance to disease. Mutation in all three members of clade II TGA factors compromise SA-induced *PR-1* expression and SAR against virulent strains of *P. syringae* and the NOCO2 isolate of the biotrophic oomycete *Hyaloperonospora arabipodsidis* (formerly known as *Peronospora parasitica*) (Zhang et al., 2003b). However, the *tga2-1 tga5-1 tga6-1* triple mutant retained wild type levels of basal resistance to virulent strains of these pathogens and displayed elevated basal levels of *PR-1* in the absence of SA or pathogen elicitation (Zhang et al., 2003b).

Further studies have revealed a dual role of clade II TGA factors on *PR-1* induction. In fact, TGA2 is a transcriptional repressor which constitutively binds to the *PR-1* promoter (Kesarwani et al., 2007; Rochon et al., 2006). After stimulation with SA, TGA2 is incorporated into a transactivating complex with NPR1, forming an enhanceosome in which the C terminus of NPR1 functions as a transcriptional transactivator (Boyle et al., 2009; Rochon et al., 2006). In

contrast to this negative role of TGA2 on *PR* gene expression, TGA5 and TGA6 may have positive roles. An activation-tagged mutant of *TGA6* displayed elevated basal as well as induced *PR-1* expression (Kesarwani et al., 2007), while overexpression of TGA5 in Arabidopsis enhanced disease resistance against a virulent strain of *H. arabipodsidis* NOCO2 without altering *PR* gene expression (Kim and Delaney, 2002). The dual activity of clade II TGA factors is consistent with the results of the *PR-1* promoter study showing that the two TGA-binding elements have opposite effects on transcription (Fobert, 2007; Lebel et al., 1998).

In addition to regulating *PR-1* expression with NPR1, clade II TGA factors have been found to interact with other proteins to regulate *as-1*-containing genes which are expressed in an NPR1-independent manner (Blanco et al., 2009; Thurow et al., 2005). For example, microarray analyses revealed that clade II TGA factors regulate transcriptional responses to xenobiotic stress through interaction with a regulatory protein, SCARECROW-like 14 (SCL14) (Fode et al., 2008; Mueller et al., 2008).

More recently, clade II TGA factors have been implicated in JA/ET-dependent defence mechanisms. Clade II TGA factors can interact with glutaredoxin-like proteins, including AtGRXC9 (At1g28480, also named GRX480), which is involved in the suppression of *PDF1.2* during SA-JA crosstalk (Ndamukong et al., 2007). In addition, the *tga2-1 tga5-1 tga6-1* triple mutant is impaired in JA/ET-dependent defence responses against the necrotroph *B. cinerea* (Zander et al., 2010). Clade II TGA factors have been shown to repress the expression of the *AtGRXS13* (At1g03850) gene, which encodes a glutaredoxin-like protein required for a successful colonization of Arabidopsis by *B. cinerea* (La Camera et al., 2011).

Members of Clade III TGA factors also interact with NPR1 (Després et al., 2000; Shearer et al., 2009; Zhou et al., 2000). Transcripts of clade III TGA factors accumulate following treatment with SA or pathogen challenge (Shearer et al., 2009), suggesting that they may also contribute to disease resistance. Genetic analysis has shown that a loss-of-function (knockout) mutant, *tga3-1*, is impaired in SA-induced *PR* gene expression and compromised in basal resistance against virulent pathogen *P. syringae* pv. *maculicola* (*P.s.m.*) ES4326 (Kesarwani et al., 2007). However, by testing a different allele, the knockdown mutant $tga3^{kd}$, an independent

study argued that TGA3 is only involved in a novel form of cytokinin-induced resistance, but not basal resistance (Choi et al., 2010).

Members of clade I (TGA1 and TGA4) are particularly interesting because their interaction with NPR1 is regulated by SA-induced redox changes (Després et al., 2003; Fobert and Després, 2005). This property has been reported to depend on two conserved cysteine residues (Cys-260 and Cys-266) located in TGA1 and TGA4. Under oxidizing conditions, these two Cysteines form an intramolecular disulfide bridge in TGA1 to preclude interaction with NPR1 (Després et al., 2003). Site-directed mutagenesis of these cysteine residues, which mimics the reduced state of cysteine residues, permits interaction with NPR1 in yeast (Després et al., 2003). Analysis in plants has revealed that NPR1 preferentially interacts with wild type TGA1 in the presence of increased levels of SA; however, the interaction between NPR1 and mutated TGA1 is equally strong in the present or absence of SA (Després et al., 2003). Using a novel labeling strategy that distinguishes between protein sulfhydryls and disulfides, Després et al. (2003) demonstrated that the redox status of cysteines in TGA1 and /or TGA4 shifted following SA treatment to become predominantly reduced (note that the antibody used in these studies did not distinguish between members of clade I TGA). Thus, strong interaction of TGA1 and/or TGA4 with NPR1 is correlated with the reduced state of their cysteines. In addition to Cys-260 and Cys-266, the other two cysteines (Cys-172 and Cys-287) of TGA1 also have been shown to be involved in the formation of intramolecular structures (Lindermayr et al., 2010). Interestingly, transforming a TGA1 site-directed mutant of these two cysteines into *tga1-1 tga4-1* knockout plants resulted in hyperexpression of *PR* genes, suggesting that reduction of these Cys residues is important for TGA1 activity, since the mutations mimic their reduced status (Lindermayr et al., 2010).

Redox regulation of TGA1 and NPR1 has been proposed to be regulated by nitric oxide (NO) (Lindermayr et al., 2010). Both proteins can be S-nitrosylated *in vitro* following treatment with S-nitroglutathione (GSNO) (Lindermayr et al., 2010; Tada et al., 2008), which is a general physiological transport and storage form of NO in plants and animals. This S-nitrosoglutathione protects TGA1 from oxygen-mediated modification and enhances DNA binding activity of TGA1 towards its cognate target in the presence of NPR1.

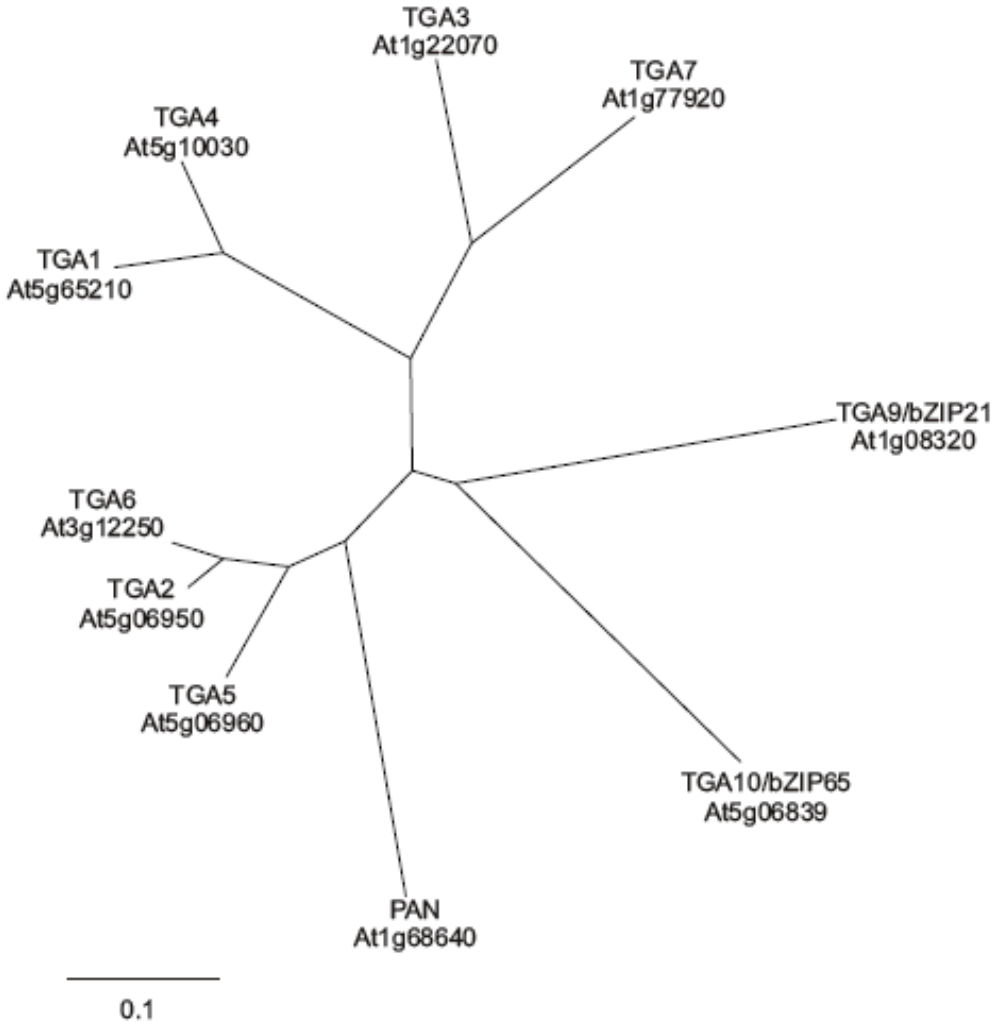


Figure 1.2 Phylogenic tree of the ten Arabidopsis TGA factor proteins.
This tree was drawn using Clustal W as implemented in the DNAStart-Laser Gene MegAlign module (v6) using default settings. The bar represents an evolutionary distance of 0.1 nucleotide substitutions per site. Arabidopsis Genome Initiative numbers are shown under each member.

Transgenic hairpin RNA interference (RNAi) studies demonstrated that Arabidopsis TGA4 has a negative role in regulating reporter gene expression under the control of the *as-1*-like element *ocs* (Foley and Singh, 2004), while virus-induced gene silencing (VIGS) indicated that a tomato TGA1 homologue is required for *AvrPto*-mediated resistance against *P.s.t.* (Ekengren et al., 2003). Analysis of Arabidopsis T-DNA insertion alleles indicated that clade I TGA factors contribute to basal resistance against virulent *P. syringae*, but not to SAR against virulent *P. syringae* (Kesarwani et al., 2007; Lindermayr et al., 2010; Shearer et al., 2012). Unexpectly, *tga1 tga4* double mutant plants accumulated elevated levels of basal *PR-1* transcripts (Lindermayr et al., 2010; Shearer et al., 2012). Introducing cDNA clones of wild type *TGA1* into *tga1-1 tga4-1* did not complement this hyperexpression of *PR* genes and enhanced disease susceptility to *P. syringae* (Lindermayr et al., 2010).

1.9 Cell wall associated defence reponse

Unlike mammalian pathogens, most plant pathogens do not have an intracellular life style and usually colonize tissues intercellularly. Therefore, cell wall-associated defence responses play an important role in plant disease resistance. One well-characterized and very rapid response following recognition of bacterial phytopathogens is a transient apoplastic burst of ROS (Torres et al., 2006). This oxidative burst relies on the plasma membrane resident RBOH (RESPIRATORY BURST OXIDASE HOMOLOG) and can function as an antibiotic agent directly, or contribute indirectly to defence by causing cell wall cross-linking and acting as a secondary stress signal to induce defence responses (Boller and Felix, 2009). At a later time following pathogen detection, the plant cell wall in regions of pathogen attack is reinforced with several polymers. Most commonly observed is the deposition of papillae containing the β-D 1,3 glucan callose, lignin-like polymers, phenolics, and structural proteins (Hematy et al., 2009). As a marker response of the papillae formation, pathogen-induced callose deposition has been well studied. Callose is synthesized by a series of callose synthase enzymes located on the plasma membrane. In Arabidopsis, twelve genes encoding putative callose synthases have been identified (Richmond and Somerville, 2000; Verma and Hong, 2001). *CalS1* (At1g05570) and *CalS12* are highly induced by SA and pathogens (Dong et al., 2008) with *CalS12* being required for callose deposition in response to fungal and bacterial pathogens (Jacobs et al., 2003; Kim et al., 2005; Nishimura et al., 2003). In addition to these physical barriers, plant cells secrete toxic

cocktails of antimicrobial compounds in response to challenge by bacterial pathogens. Genes encoding many of these secreted proteins are activated following pathogen detection as part of massive transcriptional reprogramming of the genome (Hauck et al., 2003; Thilmony et al., 2006).

The success of cell wall-associated defence is dependent on effective secretion processes**.** Newly synthesized defence-related proteins have to be translocated into endoplasmic reticulum (ER)/Golgi apparatus for proper folding and assembly, and then delivered by vesicles to the plasma membrane (PM) or extracellular space.

The quality of folding and assembly is monitored by a mechanism, called ER quality control (ERQC) (Liu and Howell, 2010b). ERQC dictates transportation of properly folded proteins to their functional sites and elimination of misfolded proteins through ER-associated degradation (ERAD). Three main systems in the ERQC have been well characterized in yeast and mammals (Sitia and Braakman, 2003). Primarily, protein folding in the ER is aided by chaperones and cochaperones that bind directly to client proteins and help their folding. This group of protein includes BINDING PROTEIN (BiP) from heat shock protein (HSP70) family, ERdj proteins from HSP40 family and GLUCOSE REGULATED PROTEIN 94 (GRP94) from HSP90 family. Secondly, proteins containing free thiol groups are thought to form disulfide bonds by protein disulfide isomerases (PDIs) and oxidoreductases. Finally, the best studied system is specific to glycoproteins which are modified by the addition of N-linked oligosaccharides and then folded through calnexin/calreticulin (CNX/CRT) cycle.

However, when protein folding is inhibited because of mutations or unbalanced subunit synthesis, or when the folding machinery is overloaded under stressful conditions, unfolded or misfolded proteins accumulate in the ER in an event called ER stress (Liu and Howell, 2010b; Schröder and Kaufman, 2005). Consequently, cells activate the unfolded protein response (UPR), which alleviates ER stress by increasing the capacity of folding and degradation or by attenuating translation (Rutkowski and Kaufman, 2004). The major activity for UPR is the transcriptional upregulation of genes related to protein folding and degradation.

Ttranscripts encoding components of the ER machinery were shown to accumulate at higher levels during environmental stress, including pathogen attack, drought, heat, cold, and salinity, reflecting the need for cells to accommodate a massive buildup of secreted proteins under these conditions (Anderson et al., 1994; Gao et al., 2008; Jelitto-Van Dooren et al., 1999; Liu et al., 2007c). Although the importance of the secretory machinery has been well documented, less is known on the mechanism of transcriptional regulation of these genes during stress responses. Pathogen-induced upregulation of these genes is likely to be directly and coordinately controlled by NPR1 (Wang et al., 2005). Due to lack of DNA-binding domain in NPR1, this regulation needs other unknown transcription factor(s), which act through a common *cis*-acting element present in the promoters of these genes (Wang et al., 2005). In addition, abiotic stress-induced transcriptional upregulation of these genes relies on three ER membrane-associated bZIP transcription factors, bZIP17 (At2g40950), bZIP28 (At3g10800), and bZIP60 (At1g42990) (Iwata et al., 2008; Liu et al., 2007b; Liu et al., 2007c; Liu and Howell, 2010a).

Cargo proteins that pass the ERQC are then delivered to the Golgi apparatus, where additional modifications can occur (Bassham et al., 2008). Vesicles containing the final products fuse to the plasma membrane or other cellular destinations. Vesicle trafficking at each stage is mediated by specific SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complexes. Each SNARE complex comprises a vesicle membrane-localized VAMP (vesicle-associated membrane protein), a soluble SNAP (synaptosomal-associated protein) protein, and a target membrane-localized syntaxin protein (Kwon et al., 2008b).

The importance of protein secretion to resistance against bacterial pathogens has been recently confirmed by several reports showing that mutation of genes encoding components of the ER machinery responsible for folding and processing of nascent polypeptides compromise defence responses and disease resistance. For example, the Arabidopsis EF-Tu receptor, EFR, is a PRR and transmembrane protein which has to mature in the ER (Zipfel et al., 2006). Mutations in several components of ERQC affect the biogenesis of EFR and defence responses induced by efl18, a peptide derived from EF-Tu (Li et al., 2009a; Lu et al., 2009; Nekrasov et al., 2009; Saijo et al., 2009). However, unexpectedly, the mutant plants were not impaired in FLS2 accumulation, indicating the existence of different genetic requirements for these two PPRs (Saijo, 2010).

In addition, secretion of extracellular PR-1 protein is impaired in the plants lacking functional ER chaperone proteins, such as BiP2 (At5g42020) and DEFENDER AGAINST APOPTOTIC DEATH 1 (DAD1; At1g32210) (Wang et al., 2005). These reductions in PR-1 secretion impair SA-induced resistance against bacterial pathogens. Moreover, silencing a Syntaxin protein (NbSYP132) in *Nicotiana benthamiana,* resulted in a delay of PR protein accumulation in the cell wall after inoculation with *P. syringae* pv *tabacina* (Kalde et al., 2007).

To counteract cell wall-based defences, virulent pathogens suppress the accumulation of extracellular proteins either by blocking the transcriptional activation of genes encoding putatively secreted cell wall and defence proteins (Hauck et al., 2003) or by inhibiting the host secretion system (Nomura et al., 2006). For example, the conserved *P. syringae* effector HopM1 (Hrp outer protein M1; formerly hopPtoM) interferes with the plant secretion system by degredation of the host protein, AtMIN7 (HopM interactors 7; At3g43300), an ADP ribosylation factor guanine nucleotide exchange factor (ARF-GEF), which is key component involved in vesicle trafficking (Nomura et al., 2006).

1.10 *Arabidopsis-Colletotrichum* pathosystem

Colletotrichum higginsianum is an economically important fungal pathogen, causing anthracnose diseases in a wide range of cruciferous plants (Agrios, 2005). Under laboratory conditions, *C. higginsianum* can also infect Arabidopsis through a hemibiotrophic infection process (Narusaka et al., 2004; O'Connell et al., 2004). During interactions with its hosts, *C. higginsianum* develops a series of specialized infection structures, including germ tubes, appressoria, biotrophic primary hyphae and secondary necrotrophic hyphae. Arabidopsis accessions display variation in their susceptibility to *C. higginsianum* (Narusaka et al., 2004; O'Connell et al., 2004). For example, ecotype Eilenburg (Eil-0) and Wassilewskija (Ws-0) appear to be resistant, whereas Landsberg *erecta* (Ler-0) is more susceptible and Columbia (Col-0) is intermediate (Birker et al., 2009; Narusaka et al., 2004).

By inoculating different Arabidopsis accessions with isolates of *C. higginsianum,* Narusaka et al. (2004) found that Arabidopsis resistance to *C. higginsianum* is controlled primarily by two dominant *R* gene loci, designated *RCH1* and *RCH2* (RECOGNITION OF *C.*

higginsianum). *RCH1* is a single dominant gene identified in the accession Eil-0 and mapped to Arabidopsis chromosome 4 (Narusaka et al., 2004). The *RCH2* locus contains two TIR-NB-LRR type *R* genes, *RPS4* (*RESISTANCE TO PSEUDOMONAS SYRINGAE 4*; At5g45250) and *RRS1* (*RESISTANCE TO RALSTONIA SOLANACEARUM 1*; At5g45260), which are both required for resistance to *C. higginsianum* in Ws-0 (Narusaka et al., 2009). In addition, EDS1, which acts downstream of RPS4/AvrRps4 recognition, is also required for resistance to *C. higginsianum* in Ws-0 (Birker et al., 2009). In ecotype Col-0, however, the intermediate resistance to *C. higginsianum* does not rely much on RPS4 or RRS1 (Birker et al., 2009).

Defence-related hormone signaling pathways have been shown to play an important role during defence responses to *C. higginsianum.* Genetic analysis of each signaling pathway indicated a requirement for the SA- and ET-dependent signaling pathways (Liu et al., 2007a; O'Connell et al., 2004). In contrast, microarray analysis showed that signaling by JA and ET was more important than SA (Narusaka et al., 2004). In addition, disease resistance to *C. higginsianum* is compromised in the *edr1* (*enhanced disease resistance 1*) mutant (Hiruma et al., 2011), which was previously shown to activate SA signaling and enhance resistance to the biotrophic pathogen *Erysiphe cichoracearum*, the causal agent of powdery mildew in Arabidopsis (Frye et al., 2001; Frye and Innes, 1998). Microarray analysis also revealed that JA-responsive genes, such as plant defensin (PDF) genes, were severely impaired in *edr1* plants (Hiruma et al., 2011).

1.11 Link between defence response and flowering time

The transition to flowering in plants is tightly controlled by a fine regulatory network that requires the perception of proper endogenous developmental stage and favorable environmental conditions (Boss et al., 2004). However, when plants are exposed to stressful conditions, such as pathogen infection, the floral transition can be accelerated as a means to survive a transient threatening condition. For example, infection with *P. syringae* has been shown to accelerate reproductive development of Arabidopsis plants and to alter their shoot architecture (Korves and Bergelson, 2003). The effects of pathogen infection on plant flowering have been shown to be mediated, at least in part, through plant hormones (Davis, 2009).

As a major defence related hormone, SA has been proposed to regulate flowering in plants (Rivas-San and Plasencia, 2011; Vlot et al., 2009). Exogenous SA treatment accelerates the transition to flowering (Martínez et al., 2004). SA-deficient plants, including NahG, *eds5/sid1* and *sid2* mutants, exhibit a late-flowering phenotype (Martínez et al., 2004). Correlating with this phenotype, these plants expressed higher mRNA levels of the floral repressor gene *FLOWERING LOCUS C (FLC)*, while decreased levels of genes that promote flowering such as *FLOWER LOCUS T (FT)* (Martínez et al., 2004). Furthermore, TGA4 was shown to physically interact with CONSTANS, a positive regulator of floral induction (Song et al., 2008), suggesting that clade I TGA factors may also be involved in flowering transition.

1.12 Research goals

The primary goal of this thesis is to characterize the biological functions of clade I TGA factors during plant immune responses. Specific objectives addressed in this thesis were:

Objective 1: Confirm that the phenotypes reported for the *tga1-1* and *tga4-1* mutants are specifically due to loss of clade I TGA factors. Prior to initiating this project, the Fobert lab had identified one T-DNA insertional mutant in each of *TGA1* and *TGA4* (*tga1-1* and *tga4-1*) and generated the double mutant (*tga1-1 tga4-1*). Subsequently, the phenotype of these single or double mutants has been published by the Fobert lab and others (Kesarwani et al., 2007; Lindermayr et al., 2010; Shearer et al., 2012). However, all reports have relied on single mutant alleles, and an attempt to demonstrate specificity by complementation through transgenic expression of TGA1 was not successful (Lindermayr et al., 2010). Thus, to demonstrate specificity, additional T-DNA alleles and transgenic lines were generated and analyzed.

Objective 2: Characterize the contribution of clade I TGA factors to different types of plant immunity, as defined by the zig-zag model. Most analysis of clade I TGA factors has focused on interactions with virulent strains of *P. syringae*, which trigger ETS in the plant host. To better ascertain the contribution of clade I TGA factors to plant immunity, I analyzed Arabidopsis-*P. syringae* interactions in which effective defence responses, such as MTI and ETI, are fully deployed. I also tested the interaction of Arabidopsis clade I TGA factor mutants with the hemibiotrophic fungus *C. higginsianum.*

Objective 3: Investigate defence responses regulated by clade I TGA factors. After pathogen perception, multiple defence response events occur, such as *PR* gene expression, callose deposition and ROS burst (section 1.2). I employed different assays to investigate the involvement of clade I TGA factors in these responses.

Objective 4: Determine the requirements of NPR1 towards clade I TGA factor function. Clade I TGA factors interact with NPR1 in leaves after SA treatment (Després et al., 2003) and *in vitro* S-nitrosylation enhances DNA-binding activity of TGA1 in the presence of NPR1 (Lindermayr et al., 2010). This suggests that clade I TGA factors may share a common pathway with NPR1 during defence responses. However, epistasis and microarray analyses indicate that a substantial portion of clade I TGA function is independent of NPR1 (Shearer et al., 2012). To ascertain their relationship during plant defence responses, a *tga1-1 tga4-1 npr1-1* triple mutant was analyzed.

Objective 5: Characterize the role of clade I TGA factors in developmental processes. During the course of this study, developmental abnormalities, such as curly leaves and altered flowering time, were observed in the clade I TGA mutant plants. The involvement of clade I TGA factors in development is also suggested by the observation that clade I TGA factors have been shown to interact with several developmental regulators, such as NPR1 paralogs, BOP1 (BLADE-ON-PETIOLE1; At3g57130) and BOP2 (At2g41370) (Hepworth et al., 2005), the glutaredoxin-like protein, ROXY1 (At3g02000) (Li et al., 2009b), the floral regulator CONSTANS (Song et al., 2008) and that other members of TGA factor family have been shown to play a role in development (Chuang et al., 1999; Murmu et al., 2010; Running and Meyerowitz, 1996).

CHAPTER 2 MATERIAL AND METHODS

2.1 Plant material and growth conditions

Arabidopsis thaliana L., herein referred to as Arabidopsis, was used throughout this study. The *npr1-1*, *npr1-3*, *tga1-1*, and *tga4-1*, single mutants, *tga1-1 tga4-1* double mutant and *tga1-1 tga4-1 npr1-1* triple mutant are in the ecotype Columbia (Col-0) genetic background and have been previously described (Cao et al., 1994; Shearer et al., 2012). The *npr1-1* mutant was isolated in a screen for plants that failed to activate SA-inducible *PR* genes after exposure to INA (Cao et al., 1994). The *npr1-1* allele contains an EMS (Ethylmethane Sulphonate) -induced point mutation altering a highly conserved histidine (residue 334) in the third ankyrin-repeat consensus sequence to a tyrosine (Cao et al., 1997). The *npr1-3* mutant was isolated in a different genetic screen aimed at identifying genes involved in resistance against virulent *P. syringae* pv. *maculicola* (*P.s.m.*) ES4326 (Glazebrook et al., 1996). The *npr1-3* allele contains a nonsense codon (residue 400) that results in a truncated protein lacking the last 194 amino acids. The *npr1-5* mutant in the Nössen background, earlier known as *SA insensitive1* (*sai1*), was identified in a different genetic screen for genes involved in SA-induced *PR* gene expression (Shah et al., 1997). The *tga1-1* and *tga4-1* single mutants (Salk_028212 and Salk_127923, respectively) are T-DNA insertion mutants generated by the Salk Institute Genome Analysis Laboratory (SIGnAL) project (Alonso et al., 2003) and obtained from the Arabidopsis Biological Resource Center (ABRC; Columbus, Ohio, USA). The T-DNA in *tga1-1* is located 77 base pair (bp) downstream of the start codon in the first exon of *TGA1*, resulting in a truncated product which only has 32 amino acids. The *tga4-1* allele contains a T-DNA 550 bp downstream of the start codon in the second exon of *TGA4*. The product of *tga4-1* is predicted to encode a protein of 108 amino acids containing the basic domain, but lacking an intact leucine zipper.

Additional insertional mutant lines were obtained from the corresponding stock centers. The *tga1-2* allele (FLAG_027G07), in the Wassilewskija (WS) background, was obtained from the Versailles Center of the National Institute for Agronomical Research (INRA); *tga1-3* (RATM15-2760-1), in Nössen (Nö), is from the RIKEN BioResource; and *tga4-2* (WiscDsLox441E10) in Col-0 is from the ABRC. The *tga1-2* and *tga4-2* contain T-DNA insertions while *tga1-3* contains an insertion of the maize *Ds* transposable element.

For surface sterilization, seeds were first treated with 70% ethanol for 2 min, then with 30% bleach (5.25% sodium hypochloride) containing 0.09% Tween-20 for 10 min, followed by five rinses with sterile water. Seeds were resuspended in 0.1% agarose, and stratified at 4˚C for 2 days prior to sowing on 1 x Murashigue and Skoog (MS) medium (M-5519, Sigma, St. Louis, MO) and 2% sucrose supplemented with 4.5g l^{-1} phytagel (P-8169, Sigma, St. Louis, MO) in 100 x 25 mm Petri dishes and sealed with micropore tape (3M, St. Paul, MN). Seven days post-germination, seedlings were transplanted to 72-cell flats (4 x 4 cm per cell) of Sunshine mix #4 (Sun Gro, Bellvue, WA). Plants, in soil or on plates, were grown in controlled environmental chambers maintained at 70% relative humidity, at an irradiance of 150 µE (cool white fluorescent), with a 10 h, 21˚C light period, and a 19˚C, 14 h dark period. For seed production purposes, plants were grown on soil at 22˚C with 16 h photoperiods. Plants were fertilized once a week (3g l^{-1} of 20-20-20). For seedling assays, seeds were poured on 1/2 x MS supplemented with 1% (w/v) sucrose and 8g l^{-1} phytagar (A1296, Sigma, St. Louis, MO) and grown at 22˚C with a 16-h-light/8-h-dark cycle.

2.2 Isolation of T-DNA insertion mutants

T-DNA insertion Information was obtained from the SIGnAL website at http://signal.salk.edu (Alonso et al., 2003). The location of the T-DNA insertions were confirmed by sequencing of PCR fragments using the T-DNA border primers (T-DNA_LB and T-DNA_RB) and gene-specific primers (RP and LP) (Table 2.1). Plants homozygous for the T-DNA insertion were identified by screening self-fertilized progeny from the mutant using PCR amplification. The presence of the T-DNA was confirmed using LB/RP and RB/LP and subsequently, homozygous plants were identified by the lack of PCR products using gene-specific primers only. The number of T-DNA insertion loci in the mutants was estimated by the segregation ratio of kanamycin-resistant:kanamycin-sensitive plants in the T_1 and T_2 selfed progeny and analyzed using the chi-squared test (Witte, 1989). Third or fourth generation self-pollinated generations (T_3 or T_4), homozygous for T-DNA insertions were used throughout this study.

Table 2.1 Information of PCR oligonucleotides for T-DNA identification

mutant	Transformation vector	Primer name	5’ to 3’ sequence
tga1-2	pGKB5 (Bouchez et al., 1993)	tga1-2:T-DNA-RB	TTTCTACAGGACGTAACATAAGG
		tga1-2:T-DNA-LB	CGTGTGCCAGGTGCCCACGGAATAGT
		tga1-2:TGA1-RP	GTTCCTCTACGAAAAATCGCC
		tga1-2:TGA1-LP	TTGCCACATTTTGATGTCTTG
tga1-3	pCGN (Fedoroff and Smith, 1993)	tga1-3: Ds3-4	CCGTCCCGCAAGTTAAATATG
		tga1-3: Ds5-3	TACCTCGGGTTCGAAATCGAT
		tga1-3:TGA1-RP	AGGGAATCTCCGTGTCCCCTCTGG
		tga1-3:TGA1-LP	AACCTGTTGTCTAGCACGATCGAG
tga4-2	pDs-Lox (Woody et al., 2007)	tga4-2:T-DNA-RB	AGATCCGTCGACCTGCAGATCG
		tga4-2:T-DNA-LB	AACGTCCGCAATGTGTTATTAAGTTGTC
		tga4-2:TGA4-RP	TTAACCACGCGACAAGCGGCTAG
		tga4-2:TGA4-LP	AGCCGTTGATCTGACCGATTGGG

2.3 Plasmid constructions

For TGA1 overexpression in the *tga1-1 tga4-1* background, the full-length coding region from the TGA1 cDNA, under the control of the enhanced 35S Cauliflower Mosaic Virus (CaMV) promoter, was cloned into binary vector pCAMBIA2300 by Ms Catherine DeLong (NRC-PBI) using a strategy similar to the one described in Liu et al. (2005).

To determine which gene is responsible for the *tga1-2* phenotype, genomic DNA corresponding to *TGA1* and the adjacent gene *RPL29* (*RIBOSOMAL PROTEIN L29*; At5g65220) were separately cloned into transformation vectors. For *TGA1*, BAC MQN23 (ABRC) was digested with *Sac*II and *Sal*I, yielding a 7,274-bp genomic fragment containing the TGA1 gene. This fragment was gel-purified and redigested with *Spe*I and *Apa*I to create a fragment containing TGA1 genomic coding sequence as well as 2000 nucleotides upstream of the start codon and 700 nucleotides downstream of the stop codon. The resulting digestion product was cloned into the modified pH2GW7 vector (Karimi et al., 2002), in which the 35S CaMV promoter/terminator were removed. For *RPL29*, 1.2-kb of genomic DNA was amplified from the BAC MQN23 template by PCR using EX Taq DNA polymerase (Takara Bio Inc., Madison, WI) with the primers at5g65220-5'-SpeI: 5’-CTTCTTCCTCCGCCACTAGTCTACAATGCTTAG-3’ and at5g65220-3'-XhoI: 5’-AATAACAGAGATTAACAACATCTCGAGGAAAC-3’, and cloned into the modified pER330 vector (Teerawanichpan et al., 2007). All plasmid constructs were verified by sequencing.

2.4 Arabidopsis transformation

Transformation of Arabidopsis plants was achieved by the Agrobacterium-mediated floral dip method (Clough and Bent, 1998). The vectors were separately transformed into *Agrobacterium tumefaciens* strain GV3101 (pMP90) (Koncz and Schell, 1986). Flowering plants of the *tga1-1 tga4-1* double mutant were dipped into infiltration medium with *A. tumefaciens* containing the TGA1 cDNA vector. The *tga1-2* plants were separately dipped into medium with *A. tumefaciens* containing TGA1 and RPL29 genomic DNA. Seeds (T_1) were surface-sterilized and plated on ½ MS medium supplemented with kanamycin (50 µg ml^{-1}) or hygromycin (25 µg ml^{-1}). Two-week-old healthy seedlings were transferred to soil and two leaves from each plant were collected to test transgene expression levels two weeks later. Plants with high transgene expression level were kept for seed harvest. The number of T-DNA insertion was estimated by the segregation ratio of antibiotic-resistant: antibiotic-sensitive plants in the T_1 and T_2 selfed progeny and analyzed using the chi-squared test (Witte, 1989). Third or fourth generation self-pollinated generations (T_3 or T_4), homozygous for T-DNA insertions were used throughout this study.

2.5 Bacterial pathogen infections

All *Pseudomonas* strains were propagated at 28˚C on King's B medium (Difco, Becton Dickinson, Sparks, MD, USA) containing streptomycin (300 µg ml^{-1}) or rifampicin (100 µg ml^{-1}). Overnight cultures having OD_{600} between 0.3 and 0.6 were pelleted and resuspended in a volume of 10 mM $MgCl_2$ equivalent to the original culture. The OD_{600} were measured on a spectrophotometer blanked with 10 mM $MgCl_2$. The bacterial suspension was diluted to an appropriate concentration and syringe-infiltrated into 4-week-old leaves. Virulent *P.s.m.* ES4326, at 1 x 10^6 colony forming units (cfu) ml^{-1} (OD_{600}=0.001 in 10 mM $MgCl_2$ equaling to 1 x 10^6 cfu ml^{-1}), was used for basal resistance tests. The T3SS-deficient mutant *P.s.t. hrcC*$^-$ (1 x 10^5 cfu ml^{-1}) was used for MAMP-triggered immunity (MTI) tests. Avirulent *P.s.t. AvrRpt2*, at 1 x 10^5 cfu ml^{-1}, was used for effector-triggered immunity (ETI) tests. *P. syringae* pv. *phaseolicola* (*P.s.p.*) 1448a, at 1 x 10^6 cfu ml^{-1}, was used for non-host resistance (NHR) tests. The *P.s.t.* ΔCEL mutant (1 x 10^5 cfu ml^{-1}) was used for testing the contribution of effectors implicated in vesicle transport. Leaf bacterial titres were measured at 0 and 3 or 4 days post-inoculation (dpi). Four leaves per

plant were inoculated and two discs were cut from each leaf by using a cork borer. Eight leaf discs from one plant were collected as one replicate and 6 or 8 replicates were analyzed per experiment. Leaf discs were then homogenized in 10 mM $MgCl_2$, serially diluted and plated onto King's B medium supplemented with appropriate antibiotics. The massive multiplication of the virulent bacteria (*P.s.t.* DC3000) correlates well with symptom development (Katagiri et al., 2002). However, the nonpathogenic (*P.s.t. hrcC*$^-$), avirulent (*P.s.t. AvrRpt2*), and non-host strains (*P.s.p.* 1448a) do not multiply to high titres and do not produce disease symptoms (Katagiri et al., 2002). For these reasons, and because of its quantitative aspect, emphasis was placed on bacterial counts rather than visual disease symptoms. The pathogens *P.s.t.* DC3000 and *P.s.t. AvrRpt2* were a generous gift from Dr. Robin Cameron (McMaster University, Hamilton, ON) and *P.s.m.* ES4326, *P.s.t. hrcC*$^-$, and *P.s.p.* 1448a were kindly provided by Dr. Darrell Desveaux (University of Toronto, Toronto, ON). *P.s.t.* ΔCEL mutant was kindly provided by Dr. M. Hossein Borhan (Agriculture and Agri-Food Canada, Saskatoon, SK).

2.6 Fungal pathogen infections

The fungal pathogen, *Colletotrichum higginsianum* Sacc., kindly provided by Dr. Yangdou Wei (University of Saskatchewan, Saskatoon, SK), was maintained on potato dextrose agar (PDA) plates (Difco Laboratories, Detroit, MI) at 24°C in the dark (Wei et al., 2004). Conidia were obtained by gentle scraping of cultures incubated for 7 to 10 days and filtered through two layers of sterile cheesecloth. The spore concentration was determined by using a hemacytometer (Cambridge Instruments Inc., Buffalo, NY). Four-week-old plants were either sprayed with conidial suspensions (1×10^6 spores ml^{-1} in distilled water) or spotted with 5 µl droplets on the leaf surface on either side of the leaf mid-vein. After inoculation, plants were covered with a dome and kept in a 100% humidity chamber.

Infected leaves of the same developmental stage were harvested at different time points and fixed in a solution of 60% methanol, 30% chloroform and 10% acetic acid. After rehydration, the material was examined by light microscopy. Disease symptoms were also scored at 4 dpi. A Vernier caliper (Manostat, Switzerland) was used to measure lesion sizes in spot-inoculated leaves. Each experiment was repeated at least twice and each included 30 to 50 individual leaves.

2.7 RNA isolation and kinetic reverse-transcriptase PCR analysis

Total RNA was extracted from leaves or seedlings using the RNeasy plant mini kit (Qiagen, Mississauga, ON) according to the supplier's instructions. After treatment with DNase I (Invitrogen, Carlsbad, CA), first strand cDNA synthesis was generated using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), and the $(dT)_{17}VN$ oligo in the presence of 0.4 U RNasin (Fisher Scientific, Pittsburg, PA). The newly-synthesized cDNA was diluted 1/200 to reflect a concentration of 10 ng μL^{-1} input total RNA.

Kinetic RT-PCR was performed on an MX3000 spectrofluorometric thermal cycler (Stratagene, LaJolla, CA) using a two temperature cycling regime initiated with a 15 min activation at 95˚C, followed by 40 cycles of 2 min of annealing and extension at 66 ˚C and 10 sec denaturation at 95 ˚C. Each assay contained 0.5 pmol oligonucleotides, 5 ng cDNA, and 1 X SYBR Green® (Quantitech; Qiagen, Mississauga, ON), prepared as described in Rutledge and Stewart (2008). The fluorescence data collected at the end of each PCR cycle was analyzed by absolute quantification via the C_t method (Rutledge and Stewart, 2008). Values were normalized against *UBIQUITIN5*. The primer sets used in the different experiments are listed in Table 2.2.

2.8 Callose deposition

Four-week-old leaves were infiltrated with 1 x 10^8 cfu ml^{-1} of *P.s.t. hrcC*$^-$ or *P.s.p.*1448a, 5 µM flg22, a peptide derived from the MAMP flagellin (Boller and Felix, 2009) and 10 mM $MgCl_2$. After 12h or 13h, whole leaves were harvested and stained with 0.01% aniline blue (Kim et al., 2005). Callose depositions were observed with a Leica FluoIII (Leica Microsystems, Wetzlar, Germany) epifluorescence microscopy. The numbers of callose depositions were counted by the GENETOOLS software (Syngene, Frederick, MD) and verified by manual counts. Three plants for each genotype were analyzed. Four leaves were collected from each plant and the average of four areas on each leaf was photographed for counting callose deposits. Flg22 was kindly provided by Dr. Darrell Desveaux (University of Toronto, Toronto, ON).

Table 2.2 PCR oligonucleotides for k-RT-PCR

Target	Primer name	5’ to 3’ sequence	Efficiency (%)	Amplicon Size (bp)	T_m (˚C)
Oligos for Figure 3.1 and Figure 3.2					
TGA1 At5g65210	TGA1-P1	AAATGAAGAAAATGGAAAGACCTCAAAAAT	100.9	109	62
	TGA1-P2	GCAGAGTAAAAATAAATATCAGCAGCAACC			
TGA4 At5g10030	TGA4-P1′	AGAAACTGCAACATACATTAGCAGAGAGTG	96.5	215	62
	TGA4-P2	ATACTCCCCTAATGCTAACAAACCTCTAGC			
Oligos for Figure 3.2					
TGA4-T-DNA	TGA4-RP	TTAACCACGCGACAAGCGGCTAG	108.9	455	62
	T-DNA-LB	AACGTCCGCAATGTGTTATTAAGTTGTC			
Oligos for Figure 3.3					
RPL29	RPL29-P1	ACCCGCTATTGATTTATGGTCTCTTCCTTGTT	107.8	163	66
	RPL29-P2	GAATCCCTTAAAGATTGAAATCGTTCCTTCA			
Oligos for Figure 3.4					
TGA1-HA	HA-5’	CCTTATGATGTCCCTGATTATGCTTCTCTG	99.4	207	66
	HA-TGA1	CACGTTGTTGTCTAGTTTCTGATTATTCGGTATTAT			
Oligos for Figure 3.13;3.14;3.15					
PR1 At2g14610	PR1F	GCTCTTGTAGGTGCTCTTGTTCTTCC	104.4	173	66
	PR1R	AGTCTGCAGTTGCCTCTTAGTTGTTC			
Oligos for Figure 3.16					
PAL1 At2g37040	PAL1-Q5’	GAGCTGCAGCGGAGCAAATGAAAGGTAGCC	97.7	137	66
	PAL1-Q3’	ACCAATAGTTGAGATCGCAGCCACTTGTCC			
FRK1 At2g19190	FRK1-Q5’	ATTAGATGCAGCGCAAGGACTAGAGTATCTT	101.6	124	62
	FRK1-Q3’	GAAGTCCGCCATCTTCGCTTGGAGCTTCTC			

Table 2.2 continued

NHO1 At1g80460	NHO1-Q5'	CTTCTGATGCAGATTCAGGCTGATCTGATGG	101.6	95	66
	NHO1-Q3'	CCAGCTGCATAGGCTGCTCCTAATGCTGTTGT			
PEN2 At2g44490	PEN2-Q5’	GACTTCATGCTTGGTTGGCATCAGCATCC	105.5	148	66
	PEN2-Q3’	AGTTTATACCAACGTAATCGCAAGAGCCTA			
CalS1 At1g05570	CalS1-Q5’	TCAAAAAACAAGGAGTGACATGTACACATG	98.6	173	66
	CalS1-Q3’	CTTTCTATTATAATGGACGCGAACTTATGG			
CalS12 At4g03550	CalS12-Q5’	TGAAGAATTACACTATTTTCTGGAATGCTG	107.2	199	62
	CalS12-Q3’	GATTTCTTCCCAGTGACAATCTGCATGATG			
Oligos for Figure 3.17					
PR2 At3g57260	At3g57260-5′a	TAAGCGTATTTAAAAATTGGGAACTTGTTG	90.5	119	66
	At3g57260-3′a	TTTCCTTATTTATGCTTGCAGCTCATTTAT			
PR5 At1g75040	At1g75040-5′a	TAACTACGAAATCACTTTCTGCCCTTAAAA	105.9	124	66
	At1g75040-3′a	TCATCAGCTTTCCTTATTTATCTTTCCGTA			
WRKY70 At3g56400	At3g56400-5′a	CATGGATTCCGAAGATCACAAGAGTCCTAGTT	101.0	186	66
	At3g56400-3′a	TCGATAGATGTACTCGTTTTCCCATTGACGTA			
At3g29240	At3g29240-F1	TATAGGAGTCATCCTAAACCGTCCATCTCT	104.8	103	66
	At3g29240-R1	AAAGAAGAGTCTTTTGTCTGAAAACGTTCC			
NIMIN1 At1g02450	At1g02450-5b’	TCTGGCGTCGTGAGGAGGAAATCTAAC	94.8	121	66
	At1g02450-3b’	AAACAAACATCAATGGCGGCTTCAAAC			
WRKY54 At2g40750	At2g40750-5’	GTGGAGATTCCGGTGAGAGTAAGAAGAAGAGA	104.3	118	66
	At2g40750-3’	GTGGAGATTCCGGTGAGAGTAAGAAGAAGAGA			

Table 2.2 continued

TAT3 At2g24850	At2g24850Q1	TTGTCTCTTTTGTCCCAAGAAACCCGAATC	110.4	189	66
	At2g24850Q2	TCGGTTCCTATCGATATCCTCACCCAATTC			
CxxS9 At2g30540	CxxS9Q1	CCACGCCGGTCCCAGCCATCTTTGTGGGTG	96.9	210	66
	CxxS9Q2	CTACATTACAAAGGTTGCATAGGCTATAAG			
At4g21830	At4g21830F1	CTATGATCTGTGTGAGCTTGCTAGGTTTCT	107.9	120	62
	At4g21830R1	GTACATCGAACGAAGTGGAATCAATATCAT			
At1g22550	At1g22550Q1	GTTCCAACTTATGAGCGTGTTTTCCTTCCG	98.9	169	62
	At1g22550Q2	CATGTTCTTTAGCCGTCTCGAGCCGTTTCA			
At5g52390	At5g52390Q1	AGTGCATTAAAGCGTGTGGTCTAGACCGGA	100.3	148	66
	At5g52390Q2	GCAGCAAGGTTGAAGTAGAGATCGACTACG			
Reference gene for all experiments					
Ubiquitin 5 At3g62250	Ubiquitin5 5′a	ACCTACGTTTACCAGAAAGAAGGAGTTGAA	102.9	102	66
	Ubiquitin5 3′a	AGCTTACAAAATTCCCAAATAGAAATGCAG			

2.9 Measurement of ROS generation

The production of ROS was measured by the H_2O_2-dependent luminescence of luminal (Gomez-Gomez et al., 1999). Leaf discs, cut from four-week-old leaves, were floated on water overnight and treated with 2µM flg22 in 200 µl of buffer containing 400 µM luminal (A8511, Sigma, St. Louis, MO) and 20 µg ml^{-1} horseradish peroxidase (HRP; P8125, Sigma, St. Louis, MO). The flg22-induced H_2O_2 combined with HRP oxidized luminal to generate chemiluminescence, which was detected by a luminometer (VICTOR^3 multilabel readers; PerkinElmer, Waltham, MA) at 30 s intervals for 20 min.

2.10 MAMP-inhibited seedling growth

Seedlings grown for 5 days after germination on MS agar plates were transferred to liquid MS medium supplied with flg22, or elf18, a peptide derived from the MAMP EF-Tu (Boller and Felix, 2009) (two seedlings per 400 µl of medium in wells of 24-well-plates). The effect of treatment with the different peptides on seedling growth was analyzed after 7-14 days by weighing (fresh weight).

2.11 Protein extraction and western blot

Four-week-old leaves were syringe-infiltrated with bacterial suspensions of *P.s.t. hrcC*$^-$ or *P.s.t.* DC3000 at a high concentration (1 x 10^8 cfu ml^{-1}). Leaves were harvested at 1 dpi and 2 dpi. Fresh leaf tissues were immersed in ice-cold extraction buffer (100 mM Tris-HCl pH7.8, 500 mM sucrose, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM β-mercaptoethanol; Wang et al., 2006) and vacuum-infiltrated for three periods of 1-min each. The tissues were then gently blotted dry, packed in a syringe and placed in a 50 ml conical-bottom tube and centrifuged at 3000 rpm, for 10 min at 4 °C. The fluid collected in the conical-bottom tube was designated 'intercellular fluid' (IF). The protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Fifty µg IF protein were run on 15% SDS-PAGE gels, transferred to PVDF (polyvinylidene difluoride) membrane (Bio-Rad, Hercules, CA), and probed with antibodies specific to the PR-1 protein. After primary antibody hybridization, the blot was hybridized with AP (Alkaline phosphatase)-conjugated secondary antibodies (A2306, Sigma, St. Louis, MO) and then incubated with a chemiluminescent AP

substrate (Millipore, Billerica, MA). The blot was exposed to X-ray film (Agfa-Gevaert N.V., Mortsel, Belgium) for an appropriate duration and the X-ray film was manually developed (White Mountain imaging, Webster, NH). Two different PR-1 antisera were used and kindly provided by Dr. Daniel J. Kliebenstein (University of California, Davis, CA) (Kliebenstein et al., 1999) and Dr. Darrell Desveaux (University of Toronto, Toronto, ON) (unpublished), respectively.

2.12 Tunicamycin assays

Plants were grown in the presence of this glycosilation inhibitor to assess for potential defects in ER secretion pathways. Two assays were used in this study. In the first, seeds were poured on ½ MS medium containing tunicamycin (TM) (T7765, Sigma, St. Louis, MO) at different concentration. At 5 days after germination, seedlings were transferred to TM-free MS medium and grown for another five days.

In the second assay, seeds were poured on ½ MS and 1% sucrose medium without TM. Five days after germination, seedlings were immersed in ½ MS liquid with or without 0.8 µg ml^{-1} TM for 6 h. After treatment, seedlings were rinsed three times with TM-free ½ MS liquid, and grown for a further 5 days on TM-free ½ MS agar. Fresh weight of TM-treated or untreated seedlings were measured.

2.13 Flowering time measurement

Plants were grown on soil under long day (16h light) conditions. Flowering time was measured by counting the total number of leaves (rosette plus cauline), excluding the cotyledons, once the bolt was 5 cm tall. The number of days from sowing to the bolting was also recorded. Twenty four plants were analyzed for every genotype and the mean value ± standard error was calculated.

2.14 Statistical analysis

Data were analyzed statistically using unpaired Student's *t*-test ($P<0.05$) (Witte, 1989) or Analysis of Variance (ANOVA) at $\alpha = 0.05$, General Linear Model, as implemented in the SAS

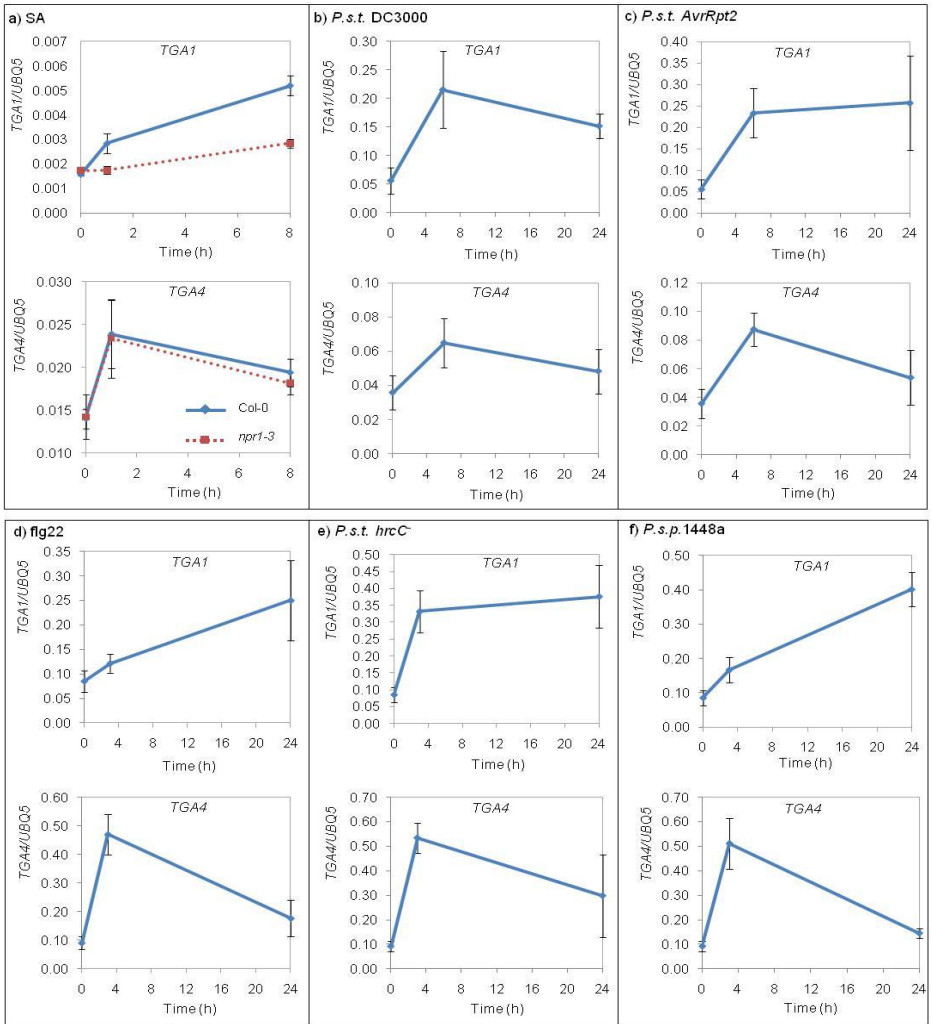
software package (SAS Institute Inc., Cary, NC). Detail information on number of replicates and repeats performed is shown in each figure legend.

CHAPTER 3 RESULTS

3.1 Clade I TGA factor gene expression is induced by SA, pathogen and flg22

In planta, Clade I TGA factors interact with NPR1 following SA treatment of leaves (Després et al., 2003). To determine whether *TGA1* and *TGA4* gene expression is regulated by SA or NPR1, four-week-old leaves of Col-0 and *npr1-3* mutants were sprayed with 1 mM SA and transcript levels of clade I TGA factors were measured by k-RT-PCR (Figure 3.1a). In Col-0 leaves, *TGA1* transcripts gradually accumulated and reached their highest level (3.25-fold induction) relatively late (8 h) after SA treatment. In contrast, *TGA4* transcripts rapidly increased, peaking (1.70-fold induction) at an early time point (1 h) following treatment with SA. In addition, levels of SA-induced *TGA1*, but not *TGA4*, transcripts, were reduced in *npr1-3* mutant plants (Figure 3.1a).

The SA signaling pathway is a major component for disease resistance against the bacterial pathogen *Pseudomonas syringae* (Katagiri et al., 2002). As the first step in investigating the potential involvement of *TGA1* and *TGA4* in disease resistance, levels of their transcripts were measured by k-RT-PCR following pathogen challenge. Leaves of Col-0 plants were infiltrated with four different *Pseudomonas* strains which elicit different immune responses in Arabidopsis. Virulent strain *P.syringae* pv *tomato* (*P.s.t.*) DC3000 triggers basal resistance (ETS); *P.s.t.* DC3000 harboring the avirulence gene *AvrRpt2* is avirulent on Col-0 and activates gene-for-gene resistance or ETI; *P.s.t. hrcC*$^{-}$ harbours a mutation in the *hrcC* gene and does not produce a functional T3SS, triggering MTI; Finally, isolates of *P. syringae* pv. *phaseolicola* (*P.s.p.*) do not cause disease and activate non-host resistance (NHR), likely a combination of MTI and ETI, on Arabidopsis. As shown in Figure 3.1, *TGA1* and *TGA4* transcripts accumulated to higher levels after inoculation with all these four strains, with the fold change for *TGA1* ranging from 2.13 to 5 and for *TGA4* from 1.35 to 5.89. Consistent with expression patterns after SA treatment, *TGA1* transcripts started to increase after inoculation and remained elevated at the later time point. In contrast, *TGA4* transcripts increased to its highest level early and then decreased at the later time point. Treatment of Arabidopsis plants with purified MAMPs, such as flg22 and elf18, cause a large number of transcriptional changes (Boller and Felix, 2009). *TGA1* (3.13-fold induction at 24 h) and *TGA4* (5.22-fold induction at 3 h) transcripts also accumulated



Fold Induction	TGA1		TGA4	
a) SA treatment	1 h	8h	1h	8h
Col-0	1.75	3.25	1.70	1.38
npr1-3	1	1.64	1.64	1.28

Fold Induction	TGA1		TGA4	
Treatment	6 h	24h	6h	24h
b) *P.s.t.* DC3000	3.84	2.71	1.82	1.35
c) *P.s.t. AvrRpt2*	4.16	4.59	2.46	1.51
	3h	24h	3h	24h
d) flg22	1.5	3.13	5.22	2
e) *P.s.t. hrcC-*	4.13	4.75	5.89	3.33
f) *P.s.p.1448a*	2.13	5	5.67	1.56

Figure 3.1 Expression of *TGA1* and *TGA4* in response to SA, pathogen infection and flg22 treatment.

Four-week-old leaves of Col-0 and *npr1-3* were sprayed with 1 mM SA or H_2O as control (a). Col-0 leaves were separately syringe-infiltrated with 10 mM $MgCl_2$ as control or with *P.s.t.* DC3000 (1 x 10^6 cfu ml^{-1}) (b), *P.s.t. AvrRpt2* (1 x 10^6 cfu ml^{-1}) (c), 5 µM flg22 (d), *P.s.t. hrcC*$^-$ (1 x 10^8 cfu ml^{-1}) (e), and *P.s.p.* 1448a (1 x 10^8 cfu ml^{-1}) (f). Values were normalized to the expression of *UBIQUITIN5* and represent the average of 3 biological replicates ± standard error. cDNA templates for analysis presented in b and c were generated by Dr. Heather Shearer. Fold induction is the ratio of normalized mRNA values in treated relative to untreated plants.

to higher levels following treatment with 5µM flg22 (Figure 3.1d). Taken together, the data demonstrate that clade I TGA factors transcripts accumulate to higher levels after SA, pathogen and MAMPs treatment, suggesting that clade I TGA factors could mediate disease responses during biotic stress.

3.2 Identification and characterization of T-DNA insertion mutants and transgenic overexpression lines for clade I TGA factors

Previously, the Fobert lab had identified one T-DNA knockout allele in each of *TGA1* and *TGA4* (*tga1-1* and *tga4-1*), and generated the double mutant (*tga1-1 tga4-1*). Their results indicated that both single mutants, as well as the double mutant, are more susceptible to virulent strains of *P. syringae* (Shearer et al., 2012). These results are consistent with data published on *tga1-1* and *tga4-1* by Kesarwani et al. (2007) and suggest partial redundancy in gene function between clade I TGA factors. They also indicate that clade I TGA factors act as positive regulators of disease resistance. However, the mutants were not compromised in SAR against virulent *P. syringae* (Shearer et al., 2012).

To confirm the specificity of the reported *tga1-1* and *tga4-1* phenotypes, I employed two different approaches; the analysis of additional, independent T-DNA insertion lines and complementation assays by introducing the TGA1 cDNA into the double mutant background. First, I identified additional T-DNA insertions in or near the *TGA1* and *TGA4* coding regions (Figure 3.2a). The FLAG_027G07 line in the Wassilewskija (WS) background was named *tga1-2*, The RATM15-2760-1 line in the Nössen (Nö) background was named *tga1-3*, while the WiscDsLox441E10 line in Col-0 was named *tga4-2*. In addition to demonstrating specificity, these new alleles could provide information about TGA function in diverse Arabidopsis genetic backgrounds. This is relevant in light of findings that SA signaling differs substantially between Arabidopsis ecotypes (van Leeuwen et al., 2007).

Homozygous individuals were identified using a PCR-based strategy. Sequencing of PCR products confirmed the presence and specific locations of T-DNA insertions (Figure 3.2a). The insertion in *tga1-2* is located 1,853 bp downstream of the ATG start codon in the 3' untranslated region (3'UTR) of *TGA1* and the insertion in *tga1-3* is located 70 bp downstream of the ATG

start codon in the first exon of *TGA1*. The *tga4-2* allele contains a T-DNA insertion towards the end of the *TGA4* 3' UTR.

Kinetic-RT-PCR analysis was employed to determine whether transcript levels were altered by the T-DNA insertions in the new alleles. For *TGA1*, one pair of primers (TGA1-P1 and P2) was designed after or overlapping the T-DNA insertion site (Figure 3.2a). Large reductions of *TGA1* transcripts were observed in leaves of the *tga1-2* and *tga1-3* mutants (Figure 3.2b and c). This decrease in transcript abundance, together with a T-DNA insertion that disrupts the reading frame after 23 amino acids, strongly suggests that *tga1-3* is a loss-of-function allele (knockout). Because *tga1-2* is not affected in the protein coding region of TGA1, it can be considered a knock-down allele. The T-DNA insertion site in *tga4-2* is close to the end of 3'UTR of *TGA4*. Accordingly, gene-specific primers (TGA4-P1 and P2) could only be designed before the T-DNA insertion site (Figure 3.2a). Analysis with this primer pair detected near wild type transcript levels of *TGA4* in the *tga4-2* mutant (Figure 3.2d). To resolve whether chimeric transcripts are produced in this mutant, a primer pair consisting of one TGA4-specific oligo (TGA4-RP) and one T-DNA-specific oligo (T-DNA-LB) was designed. These detected the presence of a chimeric TGA4-T-DNA transcript in the *tga4-2* mutant, but not in the wild type (Figure 3.2e). We speculate that this chimeric structure may disrupt 3' UTR functions, such as forming secondary structure, interacting with proteins and regulating transport, translation, and stability of the mRNA (Mazumder et al., 2003), thereby compromising TGA4 function.

The T-DNA insertion in *tga1-2* locates in the 3'UTR region of *TGA1*, which also corresponds to the promoter region of the closest neighbouring gene (At5g65220; Figure 3.3Figure 3.3a). The transcript level of At5g65220 was also found to be reduced in *tga1-2*, but not in the other *tga1* mutants available in the Col-0 background (Figure 3.3b), indicating that the *tga1-2* T-DNA reduces expression of both *TGA1* and At5g65220. In light of this observation, it is noteworthy that *tga1-2* plants are dwarfed and have a light green leaf phenotype, which is not observed with other *tga1* alleles or other *tga* mutants (Figure 3.3c; Shearer et al., 2012). To determine which of the two affected genes (*TGA1* or At5g65220) is responsible for the dwarf phenotype, the genomic DNA of both genes were isolated by PCR, separately cloned into transformation vectors and introduced into *tga1-2* plants. Twenty successful transformants for each construct were identified by the selective antibiotic resistance marker. Only At5g65220,

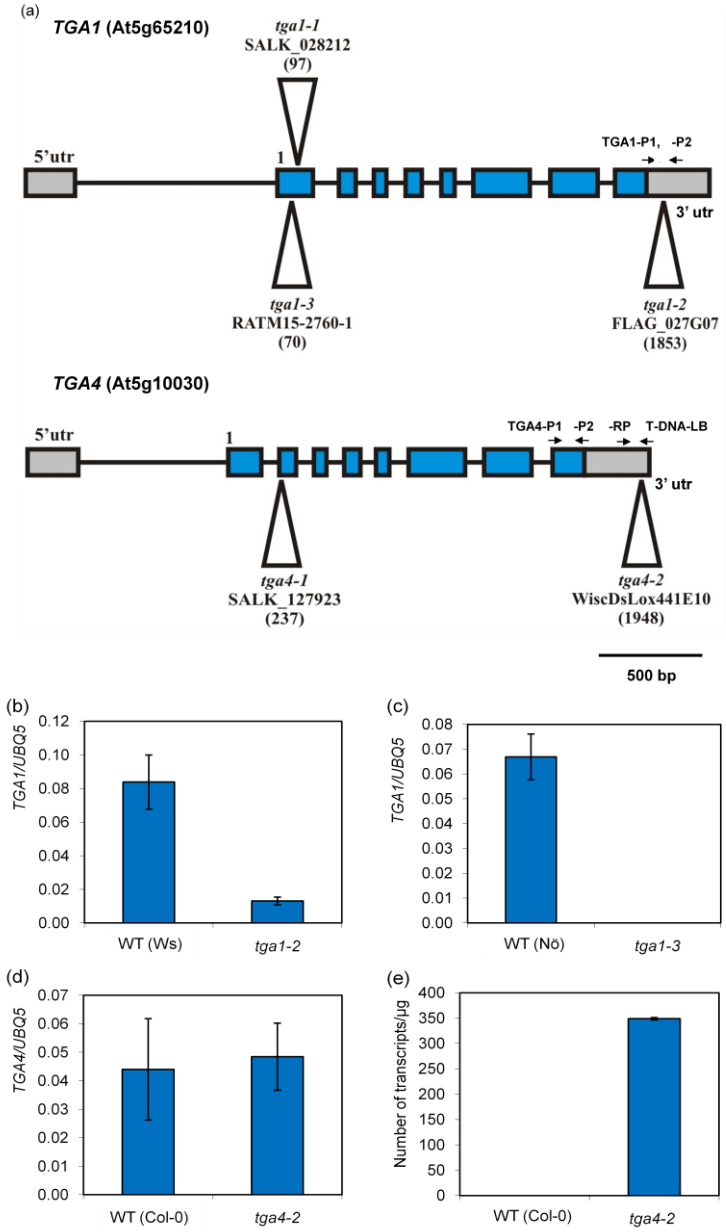


Figure 3.2 Characterization of clade I TGA factor T-DNA insertion mutants.
Schematic representations of the *tga1* and *tga4* genomic DNA showing the location of the T-DNA insertion sites. Blue boxes represent exons; interconnecting lines represent the introns; grey boxes represent the 5' and 3' untranslated regions (utr). The locations of T-DNA insertion sites, represented as triangles, are relative to the first nucleotide of the coding region and were confirmed by DNA sequencing. The arrows represent primers for k-RT-PCR. With the exception of primer and T-DNA sizes, the diagram is to scale. b), c), d) and e) K-RT-PCR analysis of *TGA* gene expression in the mutants. Total RNA was extracted from two-week-old seedlings grown on MS plates. The bars in b) to d) show the relative amount of the transcript of the corresponding *TGA* gene in wild type and mutants normalized to the levels of the *UBIQUITIN5* (*UBQ5*) gene. The bar in e) shows the number of chimeric TGA4-T-DNA transcripts per µg RNA in wild type and *tga4-2* mutant. All values represent the average ± standard error of four biological samples, each analyzed twice (technical replicates).

which encodes ribosomal protein L29, RPL29, was found to complement the mutant phenotype (Figure 3.3d and e). Many studies show that mutation in other RPL members have a similar phenotype as *tga1-2* (Imai et al., 2008; Nishimura et al., 2005). Given the nonspecific nature of *tga1-2*, this allele was not be used in the following study.

To further verify the function of clade I TGA factors, molecular complementation experiments were also performed. The *tga1-1 tga4-1* double mutant was transformed with a transgene containing the wild type *TGA1* cDNA under the control of strong constitutive CaMV 35S promoter (Figure 3.4a). Transformants were screened from MS medium with kanamycin. *TGA1* transcript levels of 70 primary transgenic plants were analyzed by k-RT-PCR. Among them, three independent transgenic lines expressing high transgene levels were selected for further studies (Figure 3.4b). This material is referred to as TGA1OE/*tga1x4* lines.

3.3 Clade I TGA factors contribute to basal resistance against virulent *Pseudomonas syringae*

At the onset of this study, preliminary data from our lab indicated that loss of function in *TGA1* and *TGA4*, in the form of the *tga1-1* and *tga1-4* alleles, resulted in enhanced disease susceptibility (EDS) to virulent *P.s.m.* ES4326 and *P.s.t.* DC3000 (Shearer et al., 2012). To confirm that these phenotypes were specifically attributed to loss of clade I TGA factor function, EDS tests were carried out on the *tga1-3* and *tga4-2* mutants as well as TGA1OE/*tga1x4* lines.

Three- or four-week-old leaves were infiltrated with virulent *P.s.m.* ES4326. As shown in Figure 3.5a and b, *tga1-3* and *tga4-2* mutant plants harboured statistically more bacterial growth than wild type 3 dpi, confirming previous findings that loss of function of either gene results in enhanced disease susceptibility to *P.s.m.* ES4326.

Analysis of the three independent TGA1 transgenic lines indicated that levels of bacterial growth were statistically lower than those observed in the double mutant plants, but similar to that in the wild type, indicating that re-introducing *TGA1* into this genetic background is able to complement the EDS phenotype of the *tga1-1 tga1-4* double mutant (Figure 3.5c). Taken together, both lines of evidence confirm the specificity of the disease phenotype attributed to clade I TGA factors. Having demonstrated specificity of *TGA1* and *TGA4* insertion alleles, and

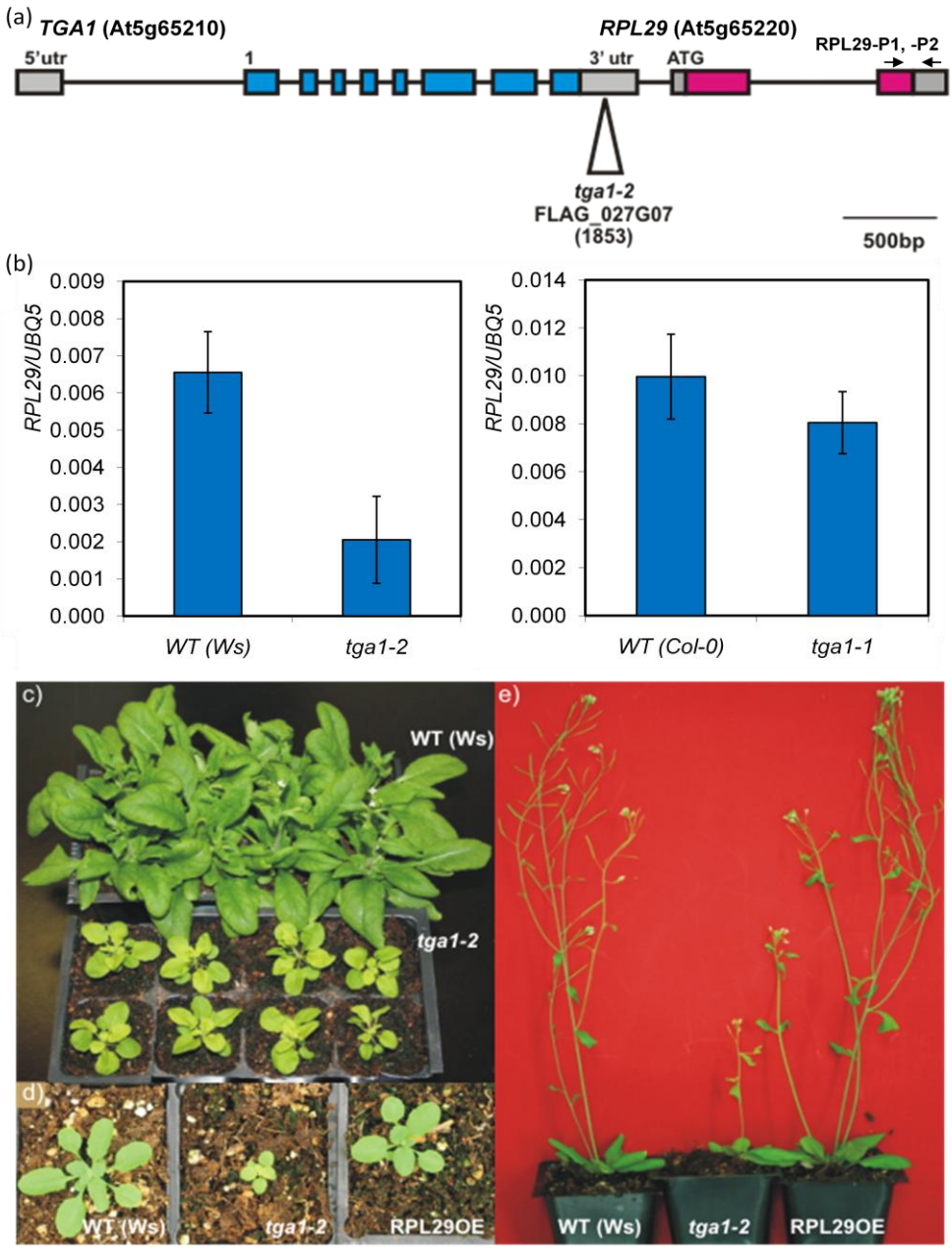


Figure 3.3 Characterization of *tga1-2* mutant plants.
Schematic representation of the T-DNA insertion site in *tga1-2* showing the location of both *TGA1* (At5g65210) and *RPL29* (At5g65220). The arrows represent primers for k-RT-PCR of *RPL29*. b) k-RT-PCR analysis of *RPL29* gene expression in different *tga1* mutants. Total RNA was extracted from two-week-old seedlings grown on MS plates. The bars show the relative amount of *RPL29* transcripts in wild type and mutants normalized to the levels of the *UBIQUITIN5* (*UBQ5*) gene. All values represent the average ± standard error of four biological samples, each analyzed twice (technical replicates). c) Morphological phenotypes observed in *tga1-2* plants. Four-week-old plants were grown in long day conditions (16h light photoperiod). d) and e) the phenotype of transgenic plants expressing the *RPL29* cDNA in *tga1-2* (RPL29OE line). The photos were taken at 2 and 8 weeks after germination, respectively.

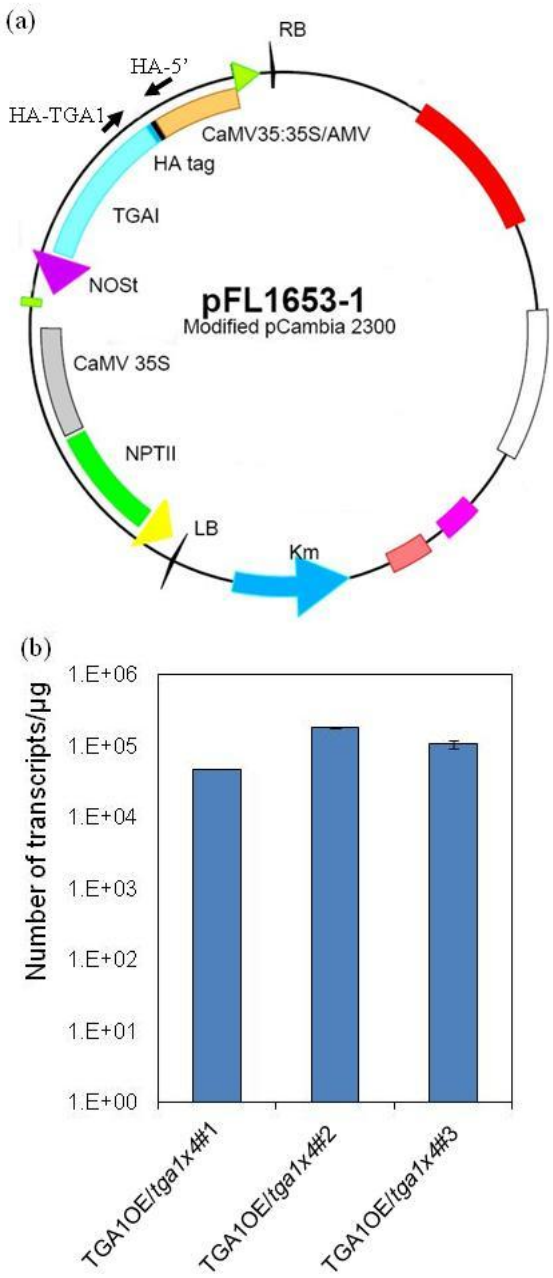


Figure 3.4 Analysis of *TGA1* transgenic plants.
a) Diagram showing the expression vector for transformation. RB and LB, right and left T-DNA border repeats; CaMV35:35S/AMV, double Cauliflower Mosaic Virus 35S promoter with Alfalfa Mosaic Virus translational enhancer; HA tag, TGA1, Arabidopsis TGA1 cDNA; NOSt, Nopaline synthase terminator; NPTII, Neomycin phosphotransferase II, a gene coding for kanamycin resistance. HA-TGA1 and HA-5' were used for k-RT-PCR to detect transgene expression level. b) k-RT-PCR for transgenic plants. Total RNA was extracted from four-week-old plants which were selected on kanamycin. Seventy (70) individual plants were analyzed and three lines with highest transgene level, shown here, were used for further analysis. Values represent the average ± standard error of four technical replicates per sample.

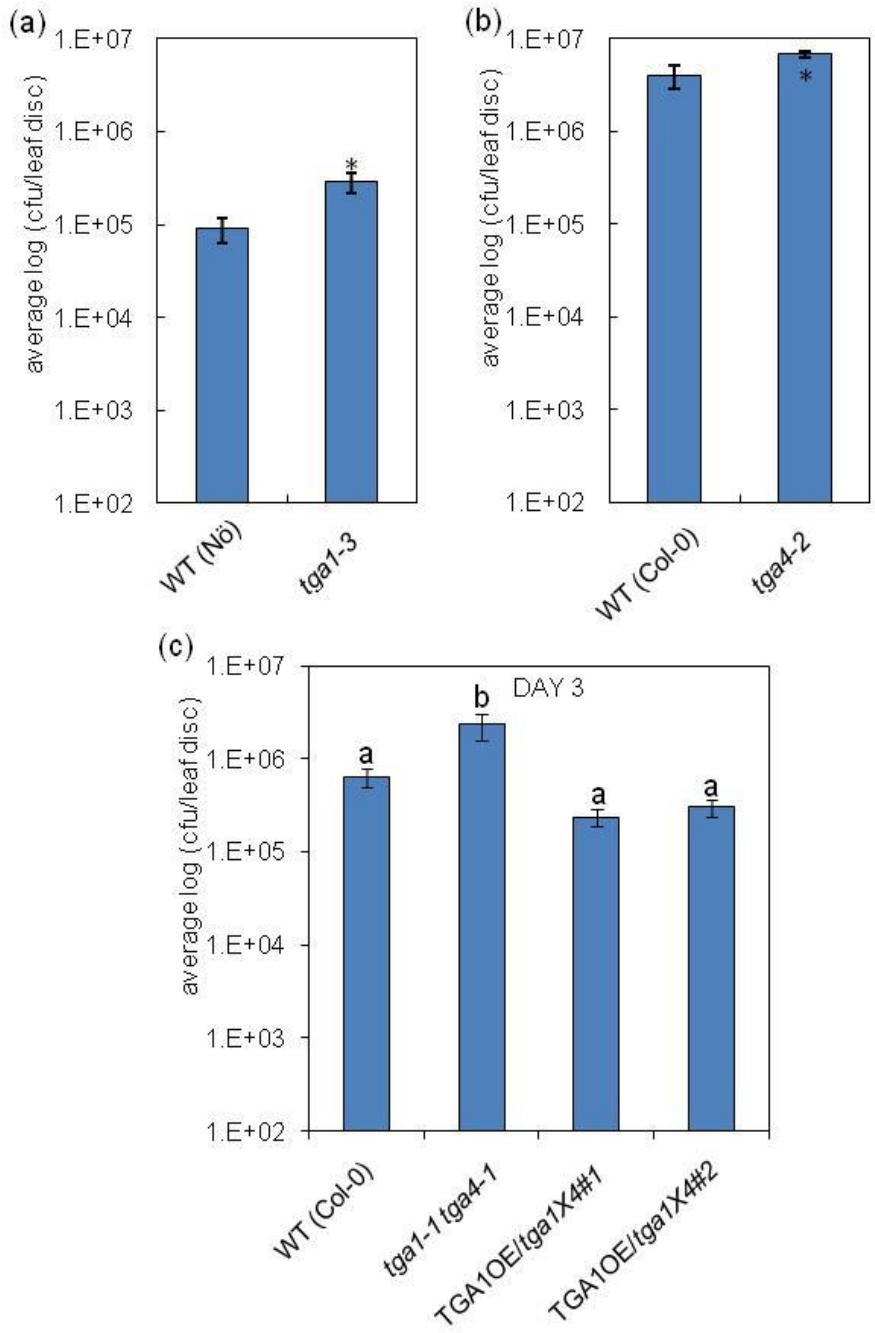


Figure 3.5 Growth of *P.s.m.* ES4326 in Col-0, *tga1-3*, Nö, *tga4-2*, mutants, and TGA1OE/*tga1x4* lines.

Four leaves were syringe-infiltrated with 1 x 10^6 cfu ml^{-1} *P.s.m.* ES4326. Bacteria were extracted from leaf discs 3 days later. Error bars represent the standard error of 8 replicates. a) and b) An asterisk indicates a statistically significant difference compared with WT ($p<0.05$, Student's *t*-test). c) An ANOVA of the log-transformed data was performed at $\alpha = 0.05$; treatments with common letters over the error bars are not significantly different from each other. The experiments in b) and c) were repeated twice with similar results. Experiment in c) was performed once.

due to functional redundancy within clade I TGA factors, all subsequent studies involving bacterial pathogens were performed with the *tga1-1 tga4-1* double mutant together with corresponding single mutants.

3.4 Clade I TGA factors contribute to MTI against the *hrcC* mutant of *P. syringae* pv. *tomato* DC3000

All functional analyses of clade I TGA factor mutants against *P. syringae* reported to date have focused on interactions with virulent strains of this bacterial pathogen (Kesarwani et al., 2007; Lindermayr et al., 2010; Shearer et al., 2012), which suppress MTI (Chisholm et al., 2006; Jones and Dangl, 2006). To ascertain the possible role of clade I TGA factors in MTI and ETI, loss-of-function mutants in these transcription factors were challenged with isolates of *P. syringae* previously reported to elicit predominantly one or both of these immune responses in the Col-0 ecotype.

I first tested *P.s.t. hrcC*$^-$, which does not produce a functional T3SS, and accordingly, is incapable of delivering T3SEs into the plant cell (Hauck et al., 2003). In the absence of T3SEs, MTI is the predominant immunity response limiting bacterial growth and disease symptoms of Col-0 against *P.s.t. hrcC*$^-$ (Hauck et al., 2003).

Leaves from four-week-old plants were infiltrated with *P.s.t. hrcC*$^-$ and bacterial growth monitored for several days. To confirm that similar amounts of bacteria were originally infiltrated into leaves, bacterial titres were measured immediately after inoculation. As shown in Figure 3.6b and c, titres in different genotypes were indeed similar on the day of infiltration (day 0). Four days after infection (day 4), bacterial titres measured in leaves of the *tga1-1* single mutants were similar to those in Col-0 (Figure 3.6a). However, leaves of the *tga4-1* single mutant or the *tga1-1 tga4-1* double mutant harboured significantly higher titres of *P.s.t. hrcC*$^-$ than Col-0 at 4 dpi (Figure 3.6). Analysis of TGA1OE/*tga1x4* lines indicated that leaf bacterial titres at 4 dpi were slightly lower than those in the double mutant but still higher than those in Col-0 (Figure 3.6c). While bacterial titres in none of the three lines were statistically different from the mutant at this time point, two lines were also no different from the Col-0. Results that overexpression of *TGA1* cannot completely rescue the double mutant phenotype suggest possible

unique functions for each member of clade I TGA factors and that the relative contribution of TGA4 to resistance against *P.s.t. hrcC*⁻ may be more important than it is against virulent *P.s.t.* DC3000. This notion is supported by the observation that slight increases in *P.s.t. hrcC*⁻ growth were observed in *tga4-1* but not *tga1-1*, although differences were not statistically different. Additional repeats of the experiments will be required to confirm these results.

Microarray and genetic studies have shown that clade I TGA factors act in both NPR1-dependent and -independent defence pathways against virulent pathogens (Shearer et al., 2012). To determine the relationship between clade I TGA factors and NPR1 during defence against *P.s.t. hrcC*⁻, this strain was also infiltrated into leaves of *npr1-1* and the *tga1-1 tga4-1 npr1-1* triple mutant. Unlike the *tga1-1 tga4-1* mutant, *npr1-1* did not support higher titres of *P.s.t. hrcC*⁻ at 4 dpi (Figure 3.6b). Furthermore, *P.s.t. hrcC*⁻ multiplied to similar titres in leaves of the triple *tga1-1 tga4-1 npr1-1* and the double *tga1-1 tga4-1*mutants. Together these results suggest that clade I TGA factors act mainly in an NPR1-independent fashion with respect to resistance against *P.s.t. hrcC*⁻.

3.5 Clade I TGA factors contribute to non-host resistance against the bacterial pathogen *P. syringae* pv. *phaseolicola*

Isolates of *P.s.p.* do not cause disease on Arabidopsis. These interactions are classified as type 1 NHR and occur in the absence of an hypersensitive response (HR) (Mysore and Ryu, 2004)**.** Non-host resistance of *P.s.p.* strain NPS3121 on Col-0 was shown to involve both MTI and ETI (Ham et al., 2007), although a recent study attributed resistance predominantly to MTI, with possible weak ETI (Zhang et al., 2010).

Leaves of different genotypes were infiltrated with *P.s.p.* 1448a and bacterial growth monitored for several days post-infection. In either single clade I TGA factor mutant, bacterial titres were similar to those in Col-0 at 4 dpi (Figure 3.7a). However, leaves of the *tga1-1 tga4-1* double mutant harboured significantly higher titres of *P.s.p.* 1448a than Col-0 at this time (Figure 3.7). Bacterial titres in the double mutant were similar to those measured in Col-0 at 0 dpi (Figure 3.7b and c). The three TGA1OE/*tga1x4* lines partially rescued the double mutant

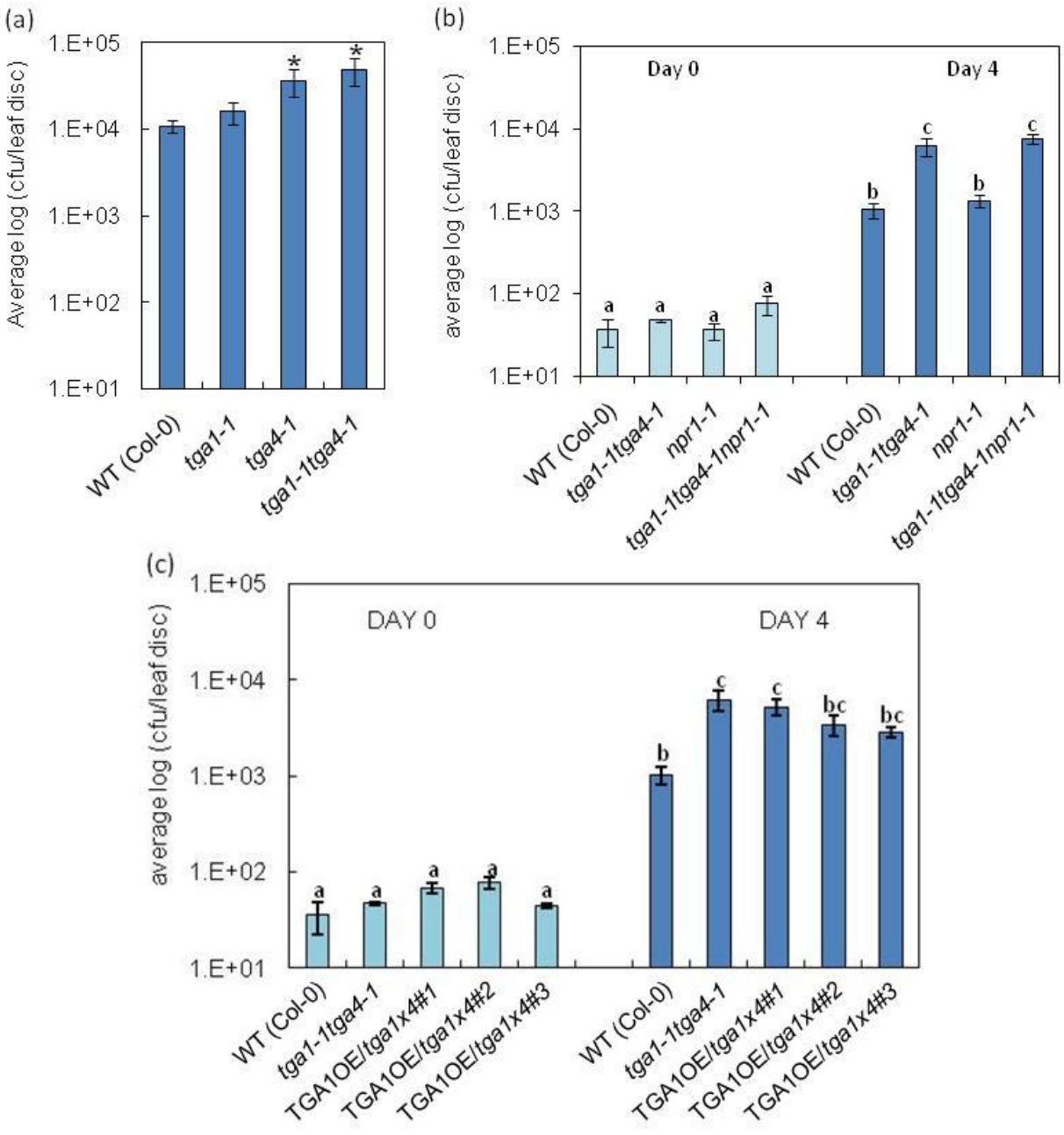


Figure 3.6 Clade I TGA factors are required for disease resistance against *P.s.t. hrcC*⁻.
Four-week-old leaves were syringe-infiltrated with 1 x 10^5 cfu ml^{-1} *P.s.t. hrcC*. Leaf bacterial titres were measured at 0 and 4 dpi. The error bars represent the standard error of 6 replicates. a) An asterisk indicates a statistically significant difference compared with Col-0 ($p<0.05$, Student's *t*-test). This experiment was performed once. b) and c) An ANOVA of the log-transformed data was performed at $\alpha = 0.05$; treatments with common letters over the error bars are not significantly different from each other. The experiments in b) and c) were repeated twice with similar results.

disease resistance phenotype; titres in one OE lines were statistically lower than those in the double mutant (Figure 3.7c). These findings suggest that TGA1 and TGA4 perform overlapping functions with respect to resistance against the non-host pathogen *P.s.p*.

Previous studies demonstrated that NPR1 plays a limited role in NHR against *P.s.p.* (Ham et al., 2007; van Wees and Glazebrook, 2003). Consistent with these studies, bacterial growth in the *npr1-1* mutant was similar to that in wild type (Figure 3.7b). However, when combined with mutations in clade I TGA factors, the *tga1-1 tga4-1 npr1-1* triple mutant supported significantly higher bacterial titres than wild type and similar to that in the *tga1-1 tga4-1* double mutant (Figure 3.7b), suggesting that the higher bacterial titres observed in the triple mutant is primarily due to loss of function in clade I TGA factors.

3.6 Clade I TGA factors contribute to ETI against *P.s.t AvrRpt2*

As a model for ETI, we infiltrated leaves of the *tga1-1 tga4-1* double mutant with *P.s.t.* harboring the avirulence gene *AvrRpt2*. In this host-incompatible interaction, resistance ensues following the recognition of *AvrRpt2* by the *R*-gene *RPS2* (Axtell and Staskawicz, 2003; Mackey et al., 2003). In order to better assess bacterial growth *in planta*, leaves were infiltrated with a low dose of *P.s.t. AvrRpt2* that does not elicit an HR. As shown in Figure 3.8, titres of *P.s.t. AvrRpt2* were similar in leaves of Col-0 and *tga1-1 tga4-1* at day 0, but the double mutant harboured significantly more bacterial growth at 4 dpi. Thus, clade I TGA factors are positive regulators of ETI against *P.s.t. AvrRpt2*. The response of single *TGA1* or *TGA4* mutants was not determined.

NPR1 has been reported to be required for ETI against a subset of *R* genes (Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). Our analysis that bacterial titres in *npr1-1* mutant are higher than those in Col-0 and similar to *tga1-1 tga4-1*, confirms a requirement of NPR1 for ETI against *P.s.t. AvrRpt2* (Figure 3.8b). Interestingly, the triple *tga1-1 tga4-1 npr1-1* mutant supported higher bacterial growth than the *npr1-1* single mutant and the *tga1-1 tga4-1* double mutant (Figure 3.8b), indicating that the function of *TGA1* and *TGA4* in ETI against *P.s.t. AvrRpt2* is at least partly independent of *NPR1*.

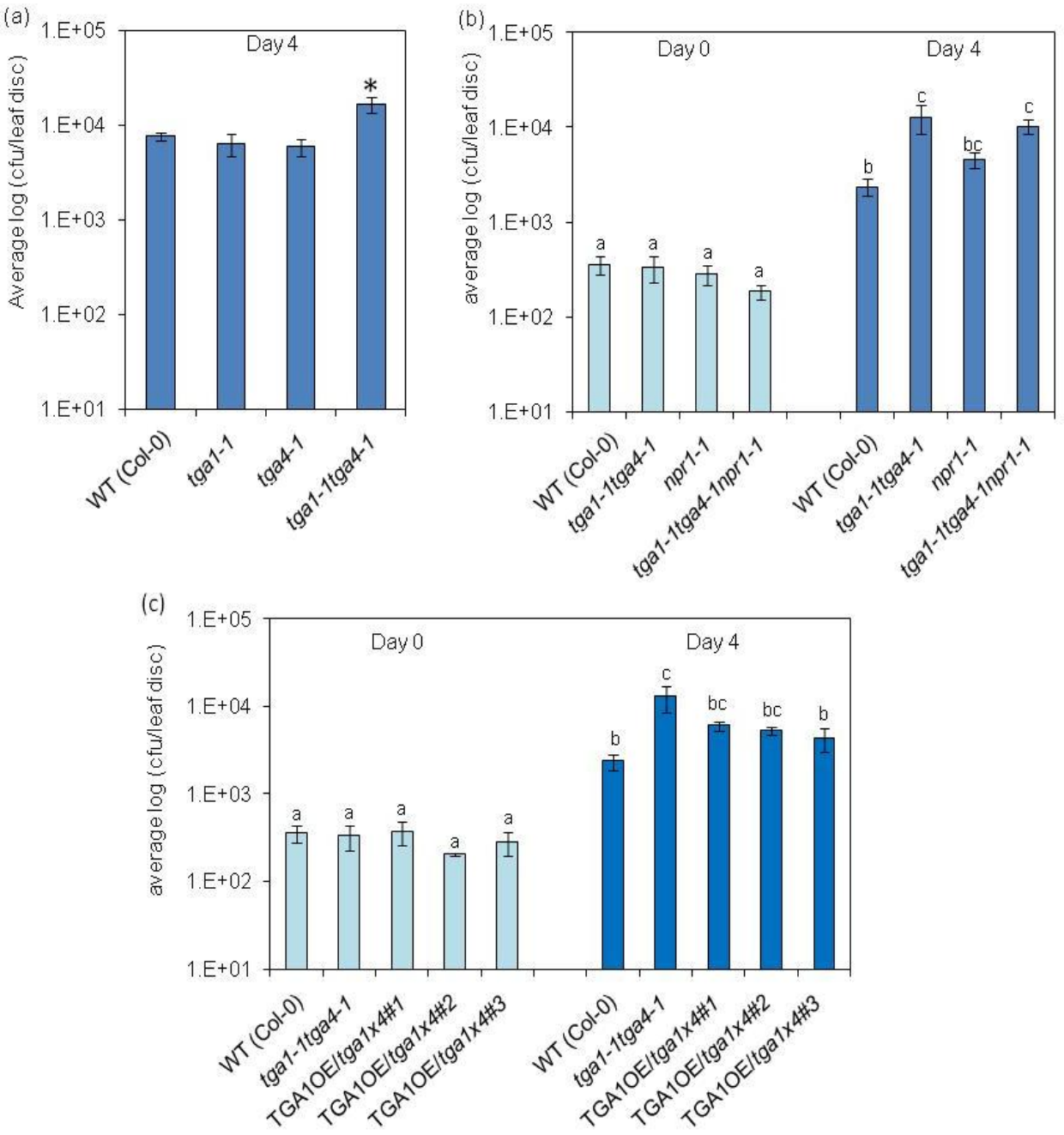


Figure 3.7 Clade I TGA factors are required for non-host resistance against *P.s.p.* 1448a. Four-week-old leaves were syringe-infiltrated with 1 x 10^6 cfu ml^{-1} *P.s.p.* 1448a. Leaf bacterial titres were measured at 0 and 4 dpi. The error bars represent the standard error of 6 replicates. a) An asterisk indicates a statistically significant difference compared with wild type ($P<0.05$, Student's *t*-test). b) and c) An ANOVA of the log-transformed data was performed at $\alpha = 0.05$; treatments with common letters over the error bars are not significantly different from each other. The experiments in a) and c) were performed once. The experiment in b) was repeated twice with similar results.

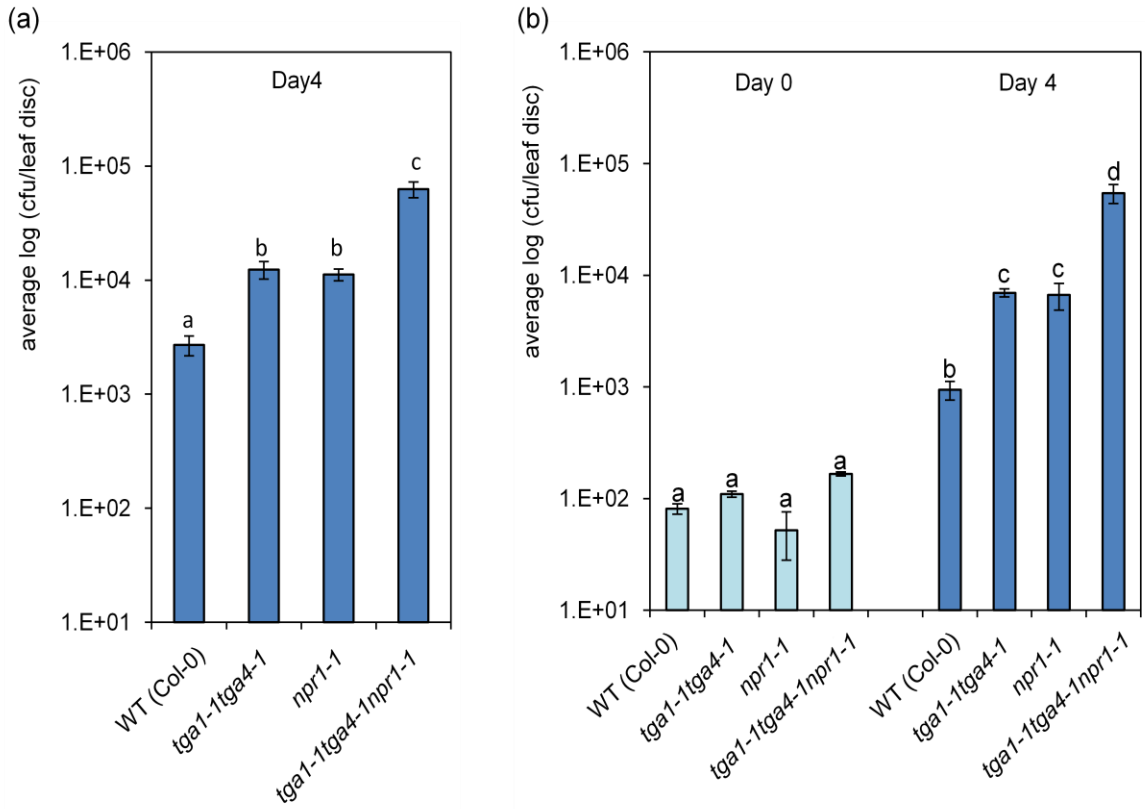


Figure 3.8 Clade I TGA factors are required for resistance against *P.s.t. AvrRpt2*.
a) and b) Bacterial growth in leaves of Col-0, *tga1-1 tga4-1*, *npr1-1*, and *tga1-1 tga4-1 npr1-1* plants. Leaf bacteria were quantified at day 0 and day 4 after inoculation of 4-week-old plants. Error bars represent the standard error of 8 replicates. An ANOVA of the log-transformed data was performed at $\alpha = 0.05$; treatments with common letters over the error bars are not significantly different from each other. Data in a) and b) are from two separate repeats.

3.7 The *tga1-1 tga4-1* double mutant is impaired in pathogen- and MAMP-induced callose deposition

To ascertain which defence responses may be compromised in clade I TGA factor mutants, leading to higher bacterial titres, I first measured callose deposition as an example of a typical cell wall-associated defence response induced by MAMPs or non-infectious pathogens (Nicaise et al., 2009). The number and size of callose deposits were measured in wild type and mutant leaves following challenge with various pathogens and after staining with aniline blue. In wild type Col-0 leaves, visible callose cell wall modifications were observed after inoculation with *P.s.t. hrcC*$^-$ (Figure 3.9). In contrast, the number of callose foci following challenge with virulent, wild type *P.s.t.* DC3000 was no higher than observed in buffer controls (Figure 3.9). This is likely due to an inability of the *hrcC*$^-$ mutant to suppress host defence responses through the secretion and action of T3SEs, such as AvrPto, AvrRpt2 and AvrRpm1 (Hauck et al., 2003; Kim et al., 2005).

A substantially lower number of callose depositions was observed in the *tga1-1* and *tga4-1* single mutants after challenge with *P.s.t. hrcC*$^-$, indicating that *TGA1* and *TGA4* both contribute to this cell wall-based modification against *P.s.t. hrcC*$^-$ (Figure 3.9a). Moreover, the number of callose deposits in the double mutant is lower than either of the single mutant, indicating that *TGA1* and *TGA4* perform overlapping functions during the *P.s.t. hrcC*$^-$-induced callose deposition (Figure 3.9a).

Callose numbers in the TGA1 overexpressing line challenged with *P.s.t. hrcC*$^-$ was significantly higher than that in the double mutant, and reached the level in Col-0 (Figure 3.9a), indicating that TGA1 can complement this phenotype in the double mutant. Interestingly, when challenged with *P.s.t.* DC3000, the overexpressing line also supported higher callose numbers, similar to those after *P.s.t. hrcC*$^-$ challenge (Figure 3.9a). This suggests that the abundant transgenic *TGA1* may enhance the signal(s) activating callose deposition and that T3SEs fail to suppress this stronger signal in the overexpression line.

To directly monitor the response of clade I TGA factor mutants to MAMPs, plants were also treated with a purified MAMP, the flg22. A large number of callose deposits were observed

after flg22 treatment in leaves of Col-0 plants (Figure 3.9b). In leaves of *tga1-1 tga4-1*, the number of deposits was reduced by 50% following flg22 treatment. These reductions were found to be statistically significant ($p<0.05$). The intensity of aniline blue staining was also weaker in the *tga1-1 tga4-1* mutants. Together, these results indicate that clade I TGA factors are required for flg22-induced callose deposition.

The non-host pathogen *P.s.p.* NPS3121 induces two morphologically different types of callose deposits (small and big) in Col-0 (Ham et al., 2007). These two types of callose are separately triggered by MAMPs and T3SEs of *P.s.p.* As shown in Figure 3.9b, *P.s.p.* 1448a infiltration also triggered both types of callose deposits. A lower number of both big and small callose deposits were induced in the *tga1-1 tga4-1* mutant, indicating that clade I TGA factors are potentially involved in both MTI- and ETI-mediated callose productions.

3.8 The *tga1-1 tga4-1* double mutant is impaired in the MAMP-induced oxidative burst response

An oxidative burst is an early defence response triggered upon pathogen perception (Boller and Felix, 2009). The availability of purified MAMPs such as flg22 and elf18 provide convenient reagents for testing this response. To ascertain whether clade I TGA factors are involved in this early MAMP-induced response, we measured ROS production in leaves of the double *tga1-1 tga4-1* mutant after treatment with flg22. An oxidative burst was rapidly induced in Col-0 plants, peaking after 4 min (Figure 3.10). This response was clearly reduced in the double mutant, reaching only about half the intensity of Col-0. This indicates that clade I TGA factors are positive regulators of the oxidative burst during MTI.

3.9 The *tga1-1 tga4-1* double mutant is more susceptible to *P.s.t.* ΔCEL mutant

In *P. syringae* strains, type III secretion–associated *hrp*/*hrc* genes and a conserved effector locus (CEL) are located on a common genomic pathogenicity island (Alfano et al., 2000). A partial deletion of the CEL region in the ΔCEL mutant of *P.s.t.* DC3000 results in the notable reduction of bacterial virulence (Alfano et al., 2000; Debroy et al., 2004). The severe loss of virulence in the ΔCEL mutant bacteria is primarily caused by the deletion of the effector genes *hopM1* (Debroy et al., 2004). HopM1 interrupts the plant secretion system by inducing degradation of host protein AtMIN7 important for vesicle trafficking to the cell surface

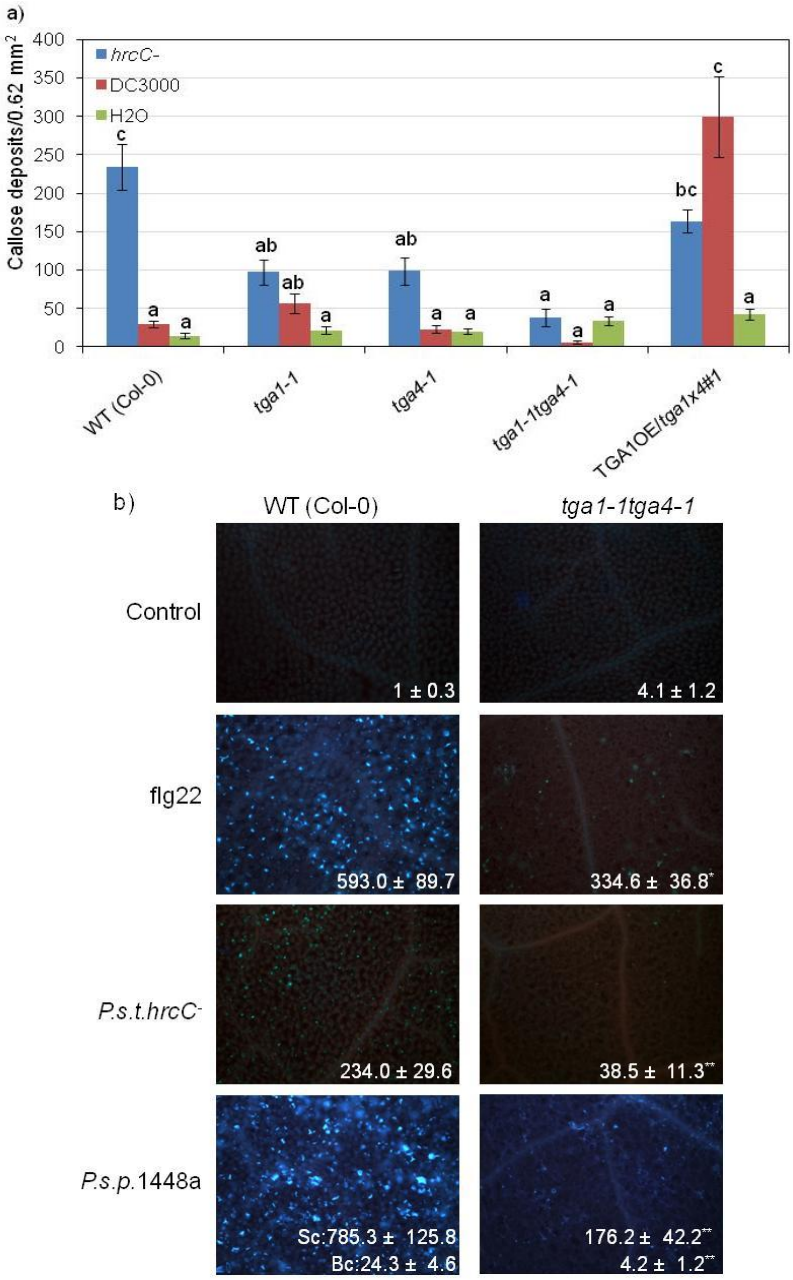


Figure 3.9 Clade I TGA factors are required for pathogen- and MAMP-induced callose deposition.

Four-week old leaves were syringe-infiltrated with 1 x 10^8 cfu ml^{-1} of *P.s.t. hrcC*$^-$, *P.s.t.* DC3000, *P.s.p.* 1448a, 5µM flg22, or 10mM $MgCl_2$ as control. Leaves were stained with aniline blue and observed under florescent microscopy 12h after treatment. a) An ANOVA of data was performed at $\alpha = 0.05$; treatments with common letters over the error bars are not significantly different from each other. b) Microscopic photographs of callose deposits are shown with the corresponding number of callose deposits indicated below each photograph. An asterisk indicates a statistically significant difference compared with Col-0 ($p<0.05$, Student's *t*-test), and two asterisk indicate $p<0.01$. Each treatment was repeated three times with similar results. For both a) and b) error bars indicate standard error. For control and *hrcC*$^-$ treatments, the same data are shown in a) and b).

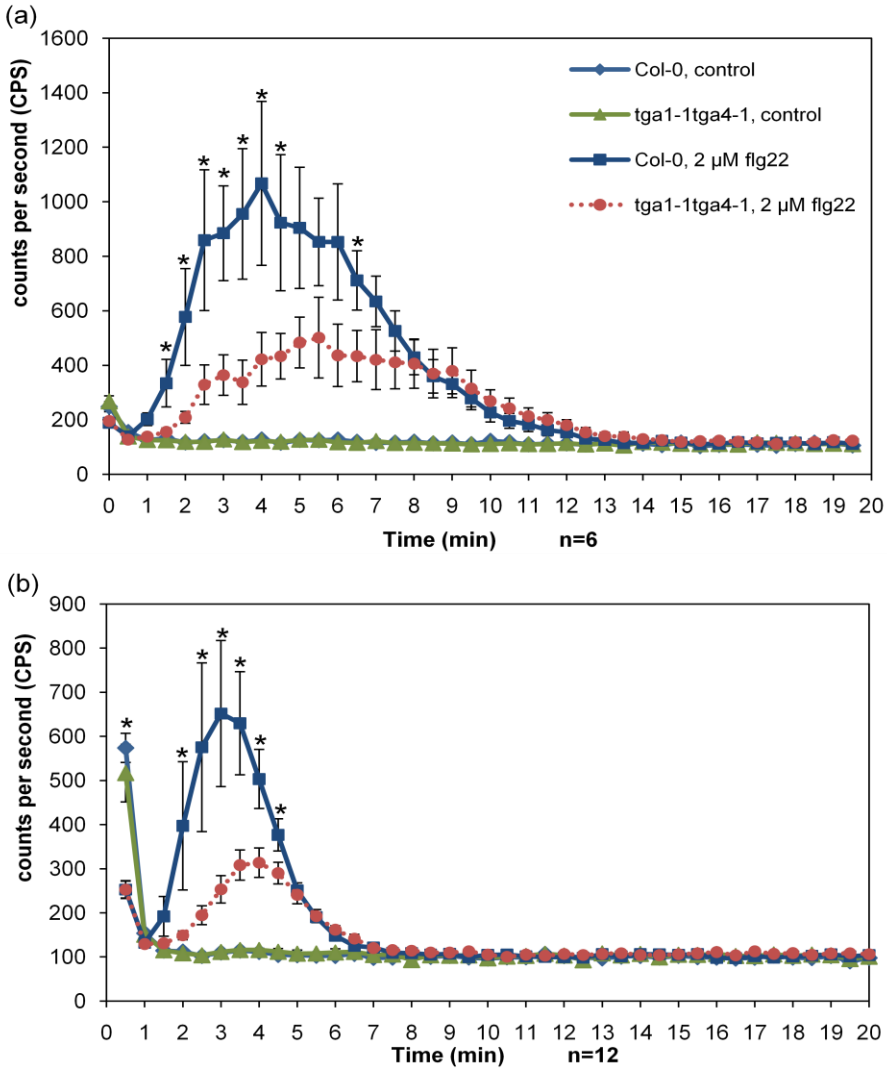


Figure 3.10 MAMP-induced oxidative burst in wild type Col-0 and *tga1-1 tga4-1* plants. Four-week-old leaf discs were treated with or without 2 µM flg22 in presence of luminol, and the H_2O_2 generated was measured at every 30 seconds after treatment for 20 min. Results are means ± standard error. Statistical differences were analyzed between genotypes at each time point and an asterisk indicates treatments different from Col-0 ($p<0.05$, student's test). The experiment was repeated five times with similar results. Data for two repeats are shown above.

(Nomura et al., 2006). The virulence of ΔCEL mutant can be restored by mutation in the *AtMin7* or by treatment of Col-0 with brefeldin A, an inhibitor of vesicle trafficking (Nomura et al., 2006). Because of the inability to suppress the host secretion pathway, the ΔCEL mutant induces a SA-dependent cell wall defence response in Col-0 (Debroy et al., 2004). Taken together, the disease resistance against ΔCEL mutant is mainly dependent on the vesicle-related secretion pathway and cell wall associated defence responses.

Results from my study presented above demonstrate that clade I TGA factors regulate cell wall-associated defence response, such as callose deposition and apoplastic ROS production, during plant immunity. To examine whether clade I TGA factors are involved in disease resistance against ΔCEL mutant, the *tga1-1 tga4-1* double mutant and Col-0 plants were inoculated with ΔCEL mutant strain. As shown in Figure 3.11, the double mutant plants supported a significantly higher bacterial growth than Col-0, suggesting that clade I TGA factors play a role in disease resistance against the ΔCEL mutant.

3.10 Clade I TGA factors are not involved in MAMP-induced seedling growth inhibition (SGI)

MAMPs such as flg22 and elf18 cause a strong inhibition of seedling growth in Arabidopsis (Gomez-Gomez et al., 1999; Zipfel et al., 2006). This response may reflect a physiological change from growth to a defence program (Boller and Felix, 2009). To determine whether clade I TGA factors are required for this MAMP-induced seedling growth inhibition (SGI), five-day-old seedlings of Col-0 and *tga1-1 tga4-1* were transferred to liquid 1 x MS medium containing 1 % sucrose supplemented with flg22 or efl18 peptide. The fresh weight of seedlings was measured one week after treatment. Similar to Col-0 seedlings, double mutant seedlings show dose-dependent inhibition with increasing flg22 concentration (Figure 3.12a). The fresh weights between Col-0 and double mutant did not show statistical differences at any given flg22 concentration.

The flg22-induced SGI signaling pathway in Arabidopsis is different from the elf18-induced. Some mutant plants are impaired in elf18-induced SGI, but retain flg22-dependent SGI (Li et al., 2009a; Lu et al., 2009; Nekrasov et al., 2009; Saijo et al., 2009). Therefore, seedlings were also treated with elf18. Similar to the result obtained with flg22 treatment, the fresh weight

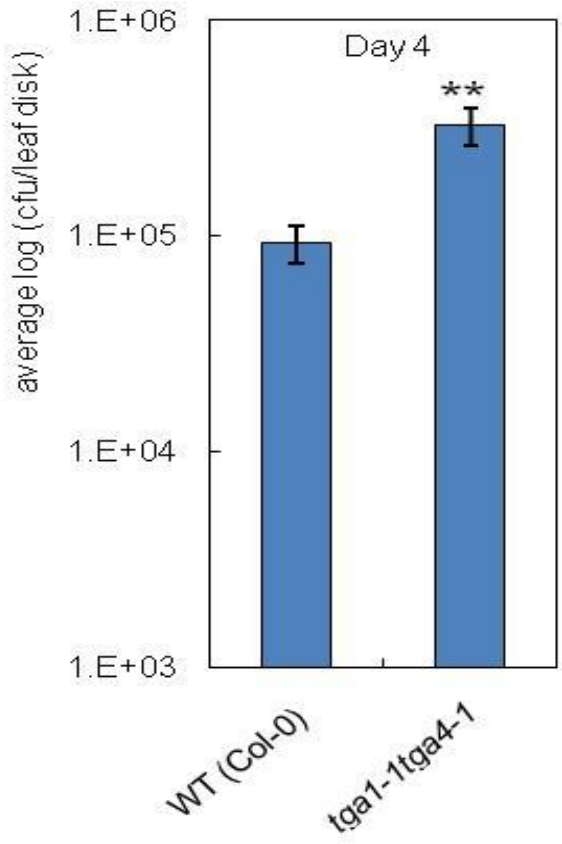


Figure 3.11 Growth of *P.s.t.* ΔCEL mutant in wild type and *tga1-1 tga4-1* plants.
Four-week old leaves were infiltrated with a bacterial suspension (1 x 10^6 cfu ml^{-1}). Bacterial titres were measured at 4 dpi. Error bars represent the standard error of 6 replicates. Two asterisks indicate a statistically significant difference compared with Col-0 ($p<0.01$, Student's *t*-test). This experiment was repeated twice with similar results.

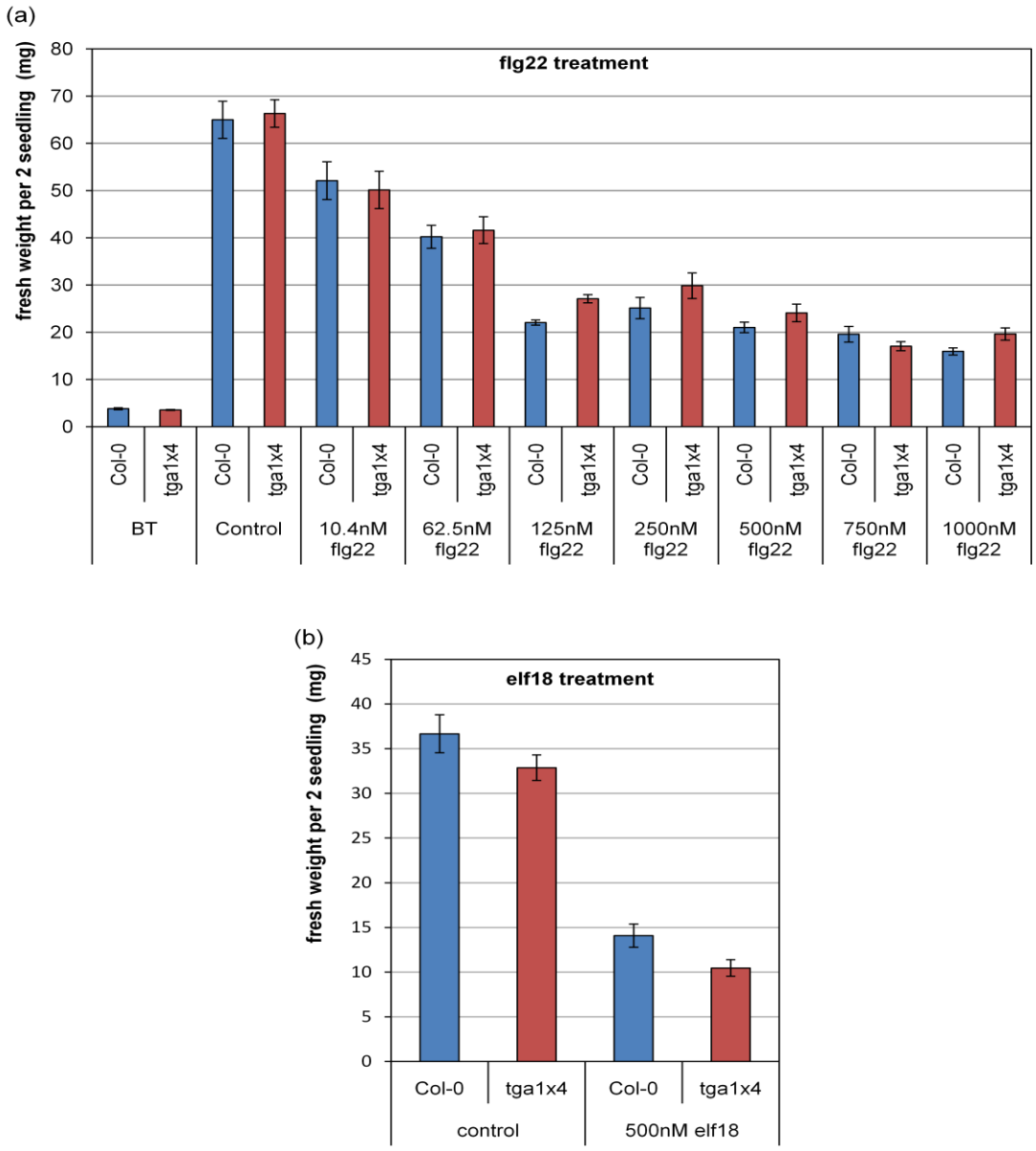


Figure 3.12 MAMP-induced seedling growth inhibition in wild type and *tga1-1 tga4-1* mutants.

Five-day-old seedlings were transferred to liquid MS medium containing 1% sucrose supplemented with the indicated concentrations of peptides. BT indicates fresh weight of seedling before treatment. Fresh weight of seedlings was measured one week after treatment. Two seedlings were counted as one sample for measurement and 6 samples were measured for each genotype. Results are means ± standard error (n=6). Student's *t*-test were performed between Col-0 and double mutant at each concentration ($p<0.05$). These experiments were repeated three times with similar results.

of double mutant seedlings are comparable to those of wild type (Figure 3.12b). Taken together, loss-of-function in clade I TGA factors did not affect flg22- or elf18-induced SGI.

3.11 *PR-1* gene expression is not reduced in clade I TGA factor mutants

PR-1 is a marker gene for SA-mediated defence against biotrophic pathogens (Durrant and Dong, 2004). Application of SA or the SA analog, benzo (1,2,3) thiadiazole-7- carbothioic acid S-methyl ester (BTH), results in higher steady-state levels of *PR-1* transcipt in Arabidopsis. To evaluate the role of clade I TGA factors in regulating *PR-1* gene expression, we analyzed the steady-state transcript levels of *PR-1* in *tga* mutants following different inducing conditions. First, two-week-old seedlings grown on MS medium containing 75 mg l^{-1} BTH were collected for RNA isolation and k-RT-PCR analysis. Compared with Col-0 seedlings, all available clade I TGA factor mutants showed higher *PR-1* expression (from 1.4- to 20.8-fold) under this chronic exposure to BTH (Figure 3.13), suggesting the negative role for clade I TGA factors in regulating SA-mediated *PR-1* gene expression. This is consistent with results previously obtained in the Fobert lab (Shearer et al., 2012) and another recent study (Lindermayr et al., 2010) who analyzed *PR-1* transcript levels in plants shortly after spraying with SA.

To expand on these results, two TGA1OE/*tga1x4* transgenic lines were analyzed to examine whether they can rescue the higher *PR-1* expression levels in the double mutant. Four-week-old plants grown in soil were sprayed with water or 1 mM SA. One hour after treatment, leaf tissue was harvested for RNA isolation. This time point was chosen because previous trials indicated that it is when wild type and *tga* mutants showed the greatest difference in *PR-1* expression (Shearer et al., 2012). Untransformed Col-0 plants possessed very low steady-state *PR-1* mRNA 1 h after SA treatment (Figure 3.14a). However, substantially higher levels of transcript were detected in *tga1-1 tga4-1* leaves, regardless of whether plants were treated with water or SA (Figure 3.14b). Plants overexpressing the *TGA1* transgene possessed levels of *PR-1* intermediate between the wild type and *tga1-1 tga4-1* mutant (Figure 3.14b). Although these results suggest that *TGA1* may be able to partially rescue this aspect of the *tga1-1 tga4-1* phenotype, the large variation in *PR-1* levels observed in the double mutant makes it difficult to reach any firm conclusions.

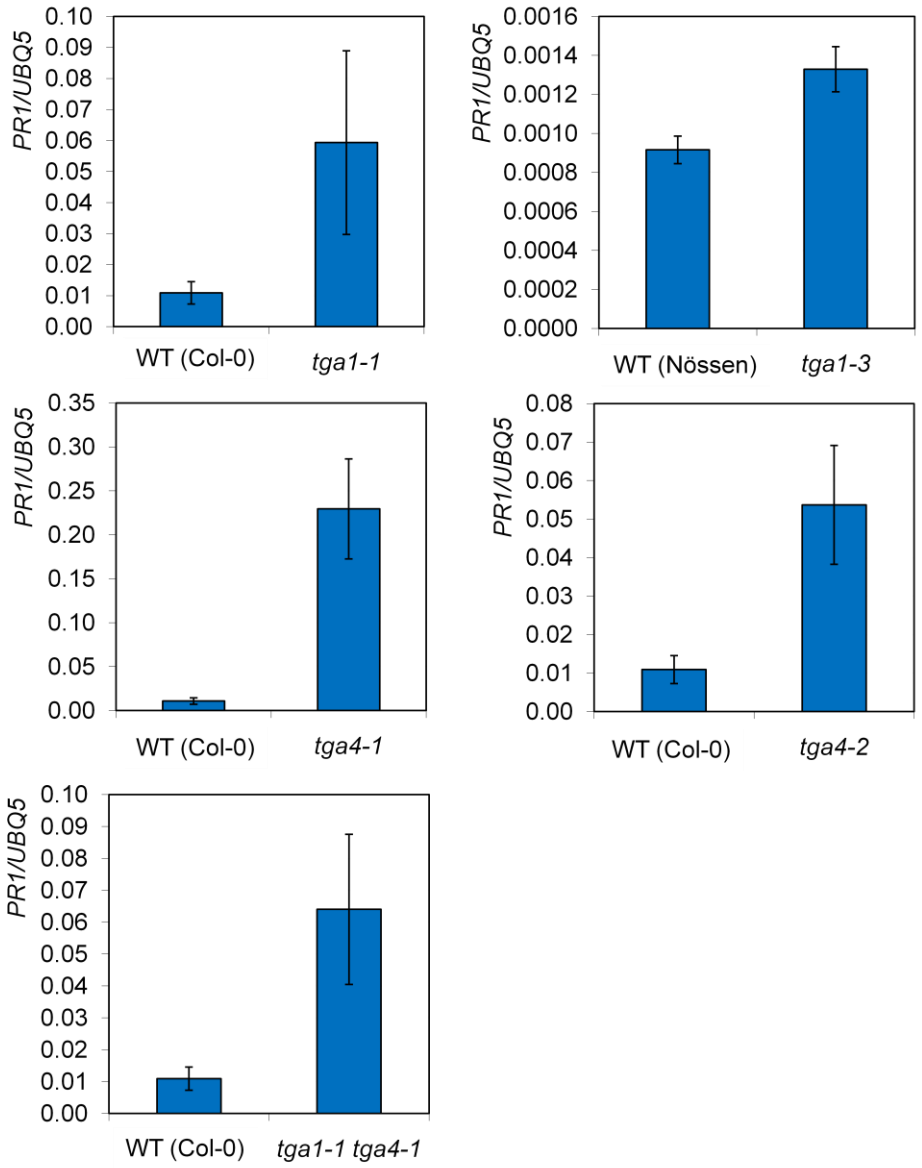


Figure 3.13 BTH-induced *PR-1* gene expression in wild type and clade I TGA factor mutants.

RNA was extracted from two-week-old seedlings grown on MS plates containing 75 mg/L BTH. The bars show the relative amount of steady-state transcript for the *PR-1* gene in wild type and mutants normalized to the levels of the *UBIQUITIN5* (*UBQ5*) gene. All values represent the average ± standard error of four biological samples, each analyzed twice (technical replicates).

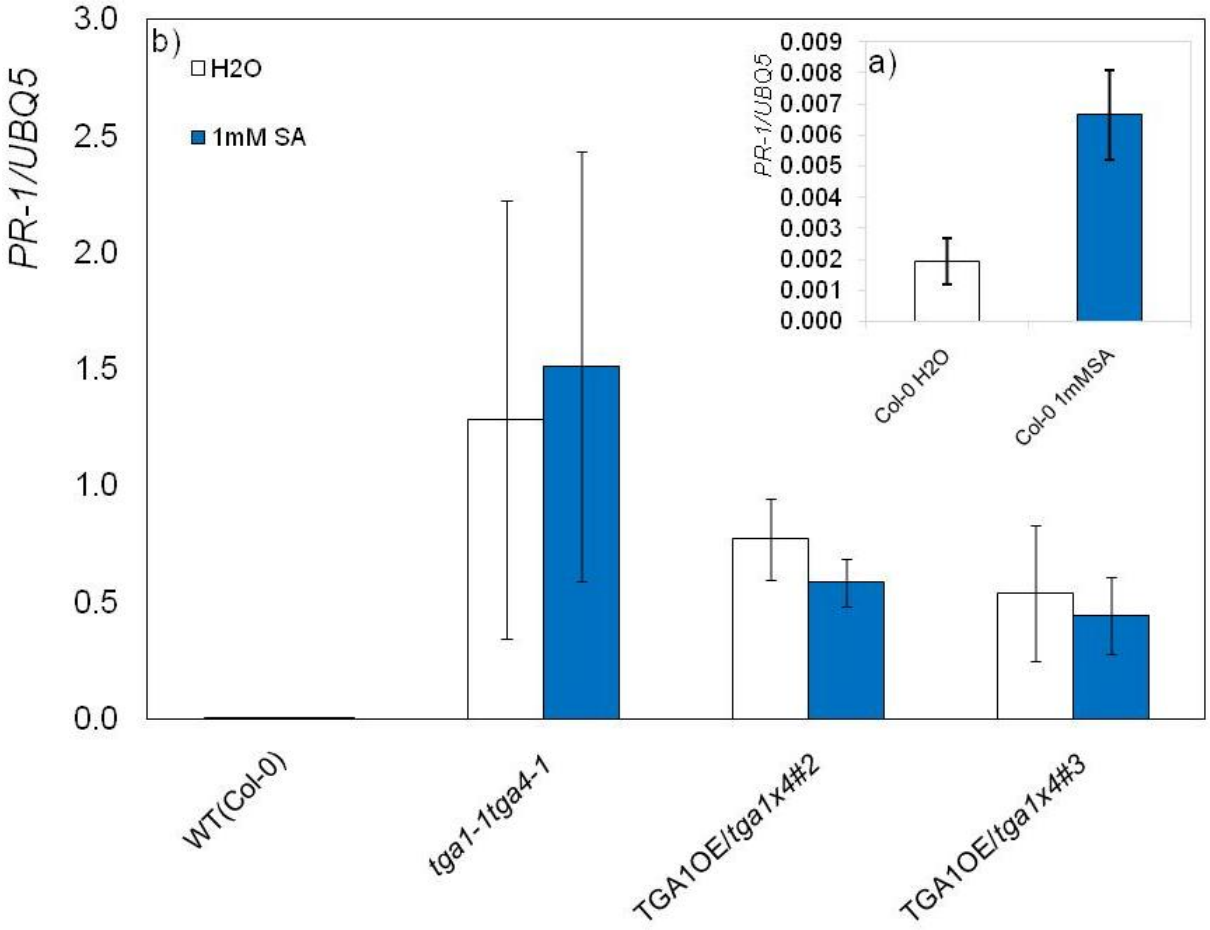


Figure 3.14 SA-induced *PR-1* gene expression in wild type, *tga1-1 tga4-1* and TGA1OE/*tga1x4* lines.

Four-week-old leaves were sprayed with water or 1mM SA. Leaf tissues were collected for RNA isolation at 1h after treatment. Three plants were pooled as one biological replicate and three biological replicates per genotype were analyzed. Values were normalized to the expression of *UBIQUITIN5*. The error bars represent standard error. a) fine-scale representation of wild type data shown in b).

Furthermore, I examined the *PR-1* expression levels in double mutant plants following pathogen challenge. The Fobert lab has previously reported that *PR-1* transcript levels in the *tga1-1 tga4-1* double mutant continues to accumulate at or above wild type levels following infection with virulent or avirulent *P.s.t.* DC3000 (Shearer et al., 2012). In this study, *PR-1* transcript levels were analyzed in leaves of Col-0 and the double mutant plants infiltrated with T3SS-deficient mutant *P.s.t. hrcC*$^-$ and the non-host pathogen *P.s.p.* 1448a (Figure 3.15). At the early time points, the levels of *PR-1* transcripts in the double mutant were comparable to those in wild type; however, the double mutant accumulated higher *PR-1* at later time points (Figure 3.15). Although they display enhanced susceptibility to these pathogens, the double mutant plants continued to accumulate higher pathogen-induced *PR-1* transcripts, which usually correlate with disease resistance. These results suggest that clade I TGA factors do not contribute to disease resistance by increasing *PR-1* expression.

3.12 Defence-related gene expression is not impaired in the *tga1-1 tga4-1* double mutant

Pathogen challenge involves massive changes in plant gene expression (Eulgem, 2005). To investigate whether clade I TGA factors play a role in regulating genes other than *PR-1* during defence responses, transcript levels of some well-known defence-related markers were quantified in leaves of Col-0 and *tga1-1 tga4-1* after challenge with *P.s.t. hrcC*$^-$. Phenylalanine ammonia lyase 1 (PAL1; At2g37040) is a key enzyme of phenylpropanoid biosynthesis and is involved in lignification during cell wall fortifications at the inoculation site (Rohde et al., 2004). Its transcripts are rapidly upregulated by *P. syringae hrp* mutants, avirulent *P.s.t.*, or non-host bacteria, but supressed by virulent *P.s.t.* bacteria (Mishina and Zeier, 2007a). *FLG22-INDUCED RECEPTOR-LIKE KINASE 1* (*FRK1*; At2g19190) is a MAMP-induced marker gene (Asai et al., 2002). *NONHOST RESISTANCE 1* (*NHO1*; At1g80460) encodes a glycerol kinase, which is required for NHR against *P.s.p.* (Kang et al., 2003; Lu et al., 2001). *PENETRATION2* (*PEN2*; At2g44490) encodes a glycosyl hydrolase that localizes to peroxisomes and acts as a component of preinvasion resistance mechanism (Lipka et al., 2005). As shown in Figure 3.16a, transcripts for all these marker genes were up-regulated 5 h after infiltration with *P.s.t. hrcC*$^-$. However, levels of transcripts measured in leaves of the *tga1-1 tga4-1* mutant following challenge with *P.s.t. hrcC*$^-$ were comparable to those found in Col-0. In several instances, levels in the mutant were slightly higher than in Col-0, but in no case were levels reduced.

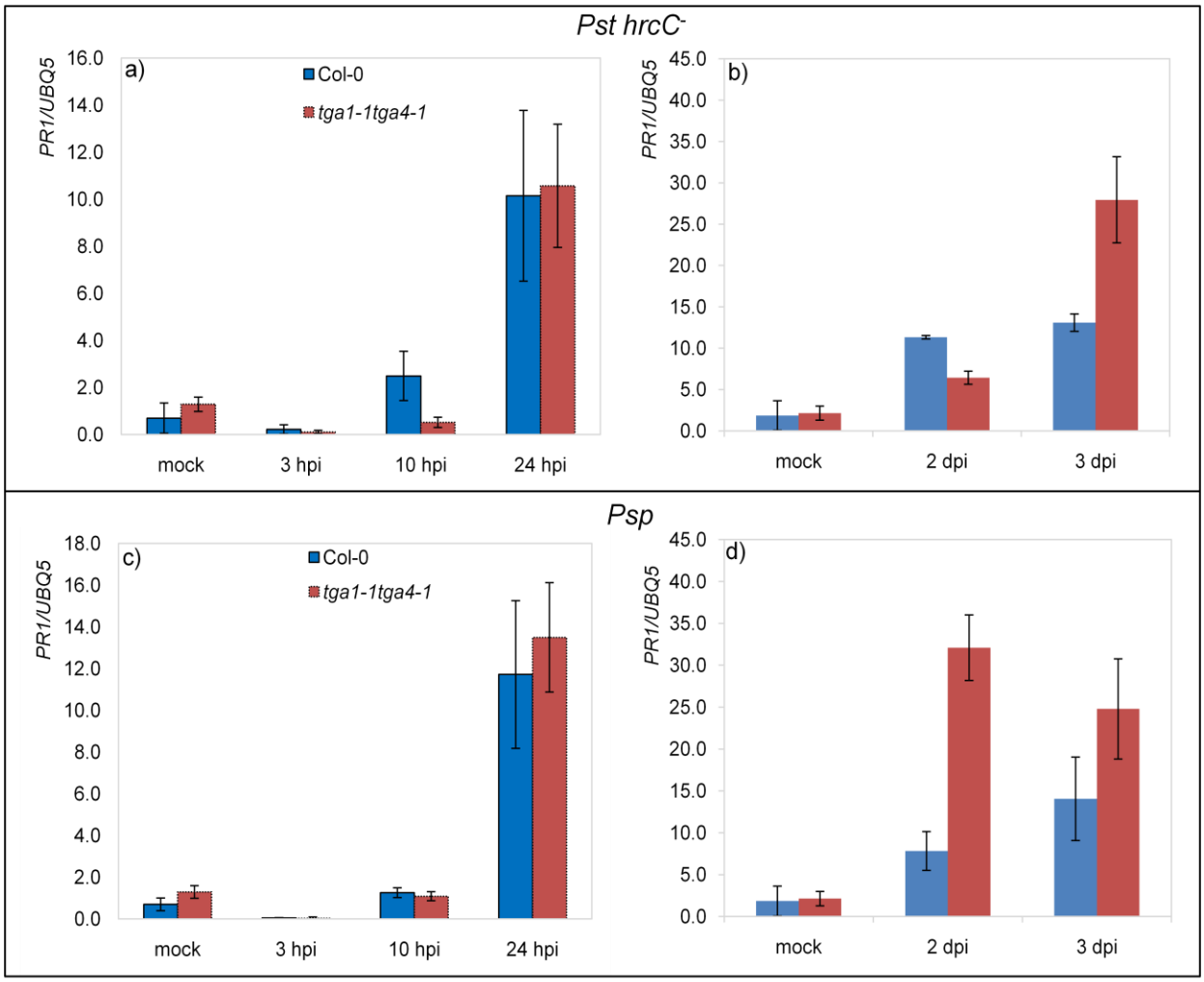


Figure 3.15 Pathogen-induced *PR-1* gene expression in wild type and the *tga1-1 tga4-1* mutant.

Four-week-old leaves were syringe-infiltrated with *P.s.t. hrcC*$^-$ (1 x 10^8 cfu ml^{-1}) or *P.s.p.* 1448a (1 x 10^8 cfu ml^{-1}). Values were normalized to the expression of *UBIQUITIN5*. The error bars represent standard error. a) and c) are short time-course experiments. b) and d) are long time-course experiments. These two time-course experiments were independently performed at different times.

Because pathogen-induced callose deposition was impaired in the *tga1-1 tga4-1* mutant, I next examined whether this phenomenon was associated with reduced transcripts of callose synthase genes in the double mutant. There are 12 callose synthase genes in Arabidopsis (Verma and Hong, 2001). *CALLOSE SYNTHASE 1* (*CalS1;* At1g05570) and *CALLOSE SYNTHASE 12* (*CalS12;* At4g03550) are highly induced by SA and pathogens (Dong et al., 2008). *CalS12* (also known as *POWDERY MILDEW RESISTANCE 4*, *PMR4*) is required for callose deposition in response to fungal and bacterial pathogens (Jacobs et al., 2003; Kim et al., 2005; Nishimura et al., 2003). As shown in Figure 3.16, transcripts of both *CalS* genes in the double mutant plants were similar to those in Col-0.

3.13 Clade I TGA factors regulate NPR1-dependent and NPR1-independent genes

Physical interaction between clade I TGA factors and NPR1 after SA treatment (Després et al., 2003) suggests that they may function together. However, the results obtained in the genetic studies presented above and by Shearer et al. (2012) also suggest that clade I TGA factors have NPR1-independent functions. To further understand the relationship between NPR1 and clade I TGA factors in regulating gene expression, microarray analysis was performed on wild type, *tga1-1 tga4-1* and *npr1-3* mutants following SA treatment (Shearer et al., 2012). Consistent with other studies (Blanco et al., 2009; Pan et al., 2004; Wang et al., 2005), NPR1 was found to regulate a large number of SA-induced genes (960) (Shearer et al., 2012). In contrast, only a small number of genes (93) were found to be differentially regulated in the *tga1-1 tga4-1* mutant (Shearer et al., 2012). Based on different expression patterns in *npr1-3* and *tga1-1 tga4-1* mutants, these genes can be classified into three different groups.

The biggest group contains genes (867) that are differentially regulated by *npr1-3*, but not *tga1-1 tga4-1*. Four representative genes from this group were selected and their expression patterns in the different mutants were validated by k-RT-PCR (Figure 3.17a). Some of these genes have been well studied. For example, *WRKY70* (At3g56400) is a NPR1-dependent and SA-induced gene which play a role in SA-JA crosstalk during plant immunity (Li et al., 2004). NIMIN1 (At1g02450) encodes an NPR1-interacting protein that negatively regulates *PR-1* gene expression and disease resistance against pathogens (Weigel et al., 2005). The gene expression of *WRKY54* (At2g40750) has previously been shown to be regulated by NPR1 (Wang et al., 2006).

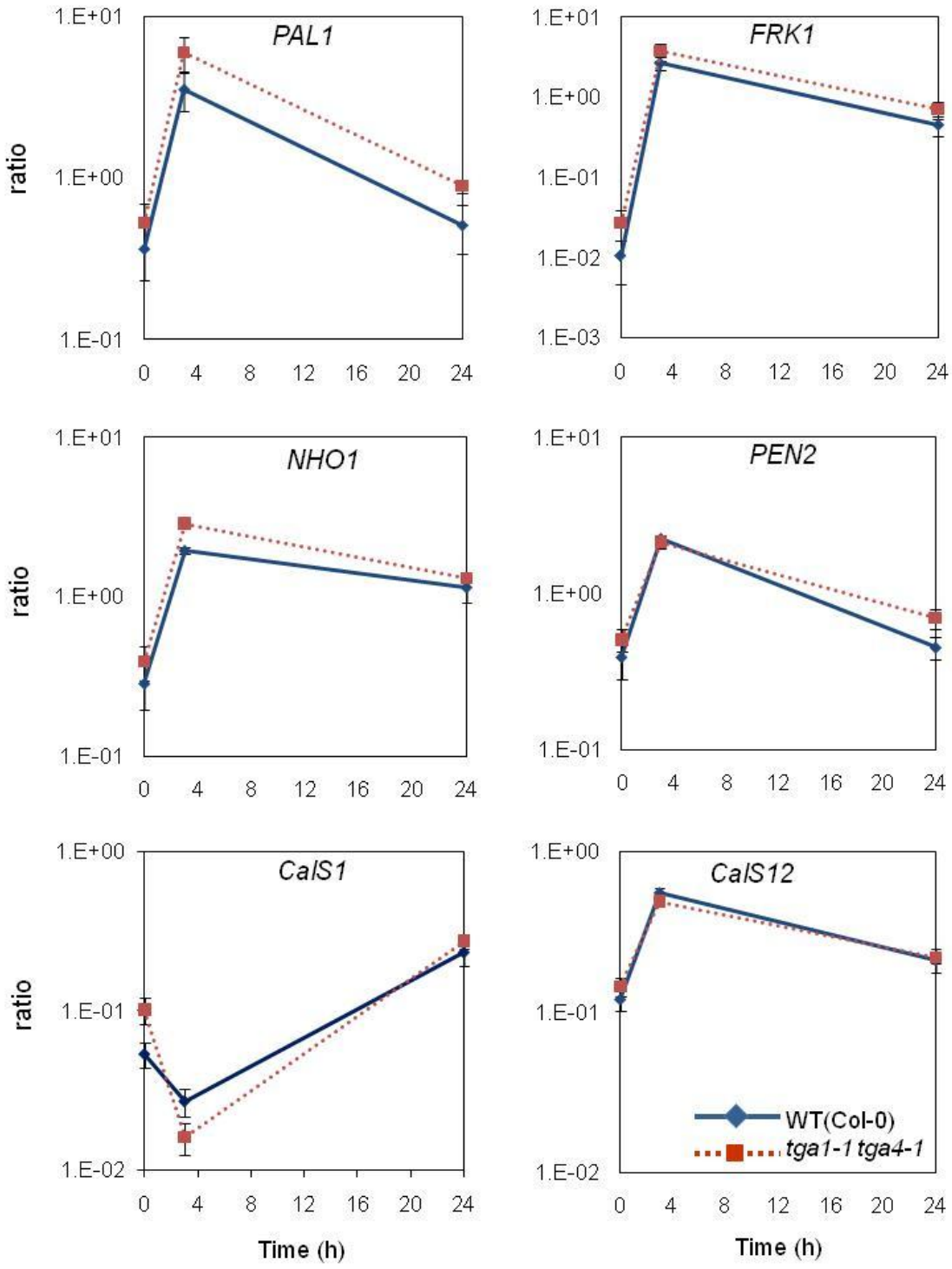


Figure 3.16 Defence-related gene expression in wild type and *tga1-1 tga4-1* mutant plants. Four-week-old leaves were syringe-infiltrated with *P.s.t. hrcC*$^-$ (1 x 10^8 cfu ml^{-1}) or *P.s.p.* 1448a (1 x 10^8 cfu ml^{-1}). Values were normalized to the expression of *UBIQUITIN5*. All values represent the average ± standard error of three biological samples, each analyzed twice (technical replicates).

The second group is most interesting, because these genes (45) are differentially regulated in both *npr1-3* and *tga1-1 tga4-1*, but in all cases, the direction of change was opposite in the two mutants. Four representative genes from this group validated by k-RT-PCR include the SA marker genes, *PR-1*, *PR-2*, *PR-5* and a JA-responsive gene *TAT3* (*TYROSINE AMINOTRANSFERASE3*; At2g24850) (Figure 3.17b). As shown in Figure 3.17b, these genes are up-regulated in *tga1-1 tga4-1* relative to wild type and were only affected at the early time points (0 h and 1 h); by 8 h after the SA treatment, the expression levels in wild type and *tga1-1 tga4-1* were similar. However, the transcripts of these genes failed to be induced in the *npr1-3* mutant through the entire time series (Figure 3.17b).

The third group is the genes (48) are differentially regulated by *tga1-1 tga4-1* but not in *npr1-3*. The representative genes in this group were also analyzed by k-RT-PCR (Figure 3.17c). These data confirmed the conclusion that clade I TGA factors regulated both NPR1-dependent and NPR1-independent genes.

3.14 Clade I TGA factors are required for the extracellular accumulation of PR-1

An important defence response against intercellular bacterial pathogens is the production of extracellular proteins and metabolites that reinforce cell walls or have antimicrobial activities (Kwon et al., 2008a). To study defence-related production of extracellular proteins in the *tga1-1 tga4-1* mutant, I monitored the accumulation of PR-1 in the apoplastic fluids following challenge with *P.s.t. hrcC*$^{-}$ and *P.s.t.* DC3000 by immunoblotting (Figure 3.18a and b). PR-1 protein accumulated in the extracellular space of Col-0 leaves two days after pathogen inoculation. However, this extracellular accumulation was reduced in the double mutant, indicating that clade I TGA factors contribute to pathogen-induced PR-1 secretion.

3.15 Clade I TGA factors are involved in ER secretion pathway

The observations that loss of clade I TGA factors affected callose deposition and extracellular PR-1 accumulation (Figure 3.10 and 3.18), but not the steady-state levels of either *callose synthase* or *PR-1* transcripts (Figure 3.15 and 3.16), suggested that the mutant may be compromised in some aspect of protein secretion. Examination of genes differentially expressed between leaves of Col-0 and *tga1-1 tga4-1* treated with SA revealed an enrichment for gene

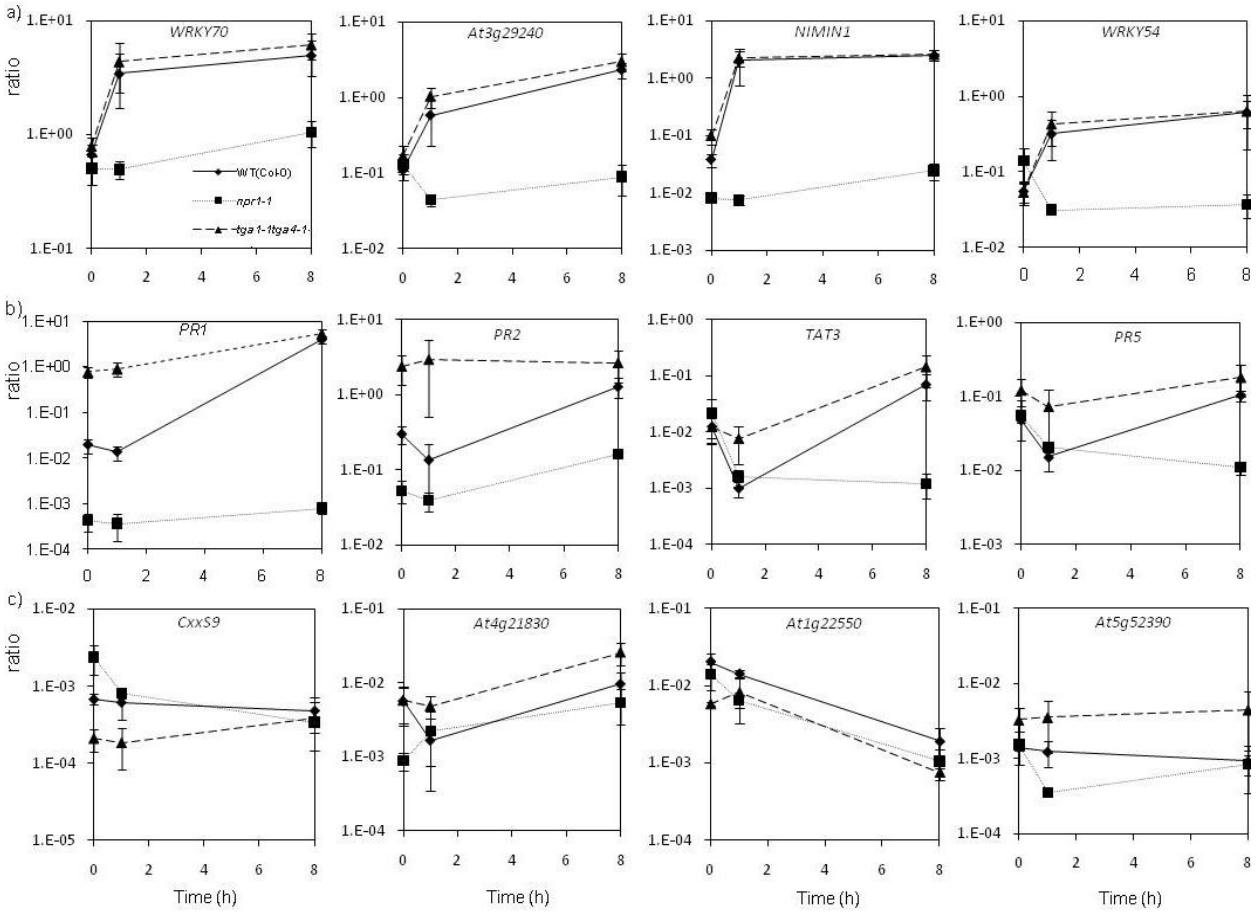


Figure 3.17 SA-induced gene expressions in wild type, *npr1-3* and *tga1-1 tga4-1*.
a) Genes differentially regulated by *npr1-3* but not *tga1-1 tga4-1*; b) genes differentially regulated in both *npr1-3* and *tga1-1 tga4-1*; c) genes differentially regulated by *tga1-1 tga4-1* but not *npr1-3*. Values were normalized to the expression of *UBIQUITIN5* and represent the average of 3 biological replicates ± standard error. Depending on specific genes analyzed, analyses were repeated at least twice with similar results.

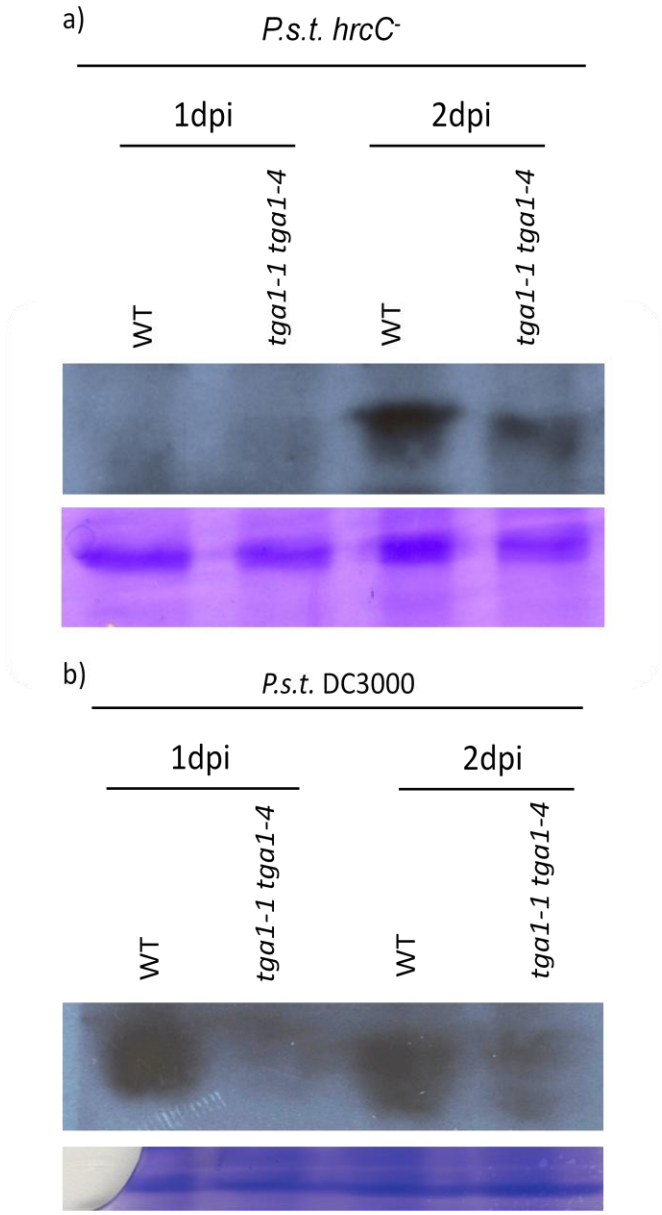


Figure 3.18 Western blot analysis of accumulation of extracellular PR-1 protein in wild type and *tga1-1 tga4-1* plants after pathogen challenge.
Four-week-old plant leaves were syringe-infiltrated with of *P.s.t. hrcC*$^-$ (1 x 10^8 cfu ml^{-1}) (a), or *P.s.t.* DC3000 (1 x 10^6 cfu ml^{-1}) (b). Intercellular fluid (IF) was collected at day 1 and 2 post inoculation and separated on 15% SDS-PAGE gels. PR-1 protein (16 kDa) was detected with PR-1 antisera kindly provided by Dr. Darrell Desveaux (unpublished). These experiments were repeated three times with similar results. Additionally, similar results were obtained with a different PR-1 antiserum, described in (Kliebenstein et al., 1999).

ontology classifications related to the ER, other membranes, extracellular, and protein binding (Shearer et al., 2012). Of note, eight genes known to be involved in ER quality control were impaired in *tga1-1 tga4-1* (Shearer et al., 2012; Table 3.1). In all cases, transcript levels of these genes in the double mutant were higher than those in Col-0 prior to SA treatment or shortly thereafter (1 h), and by 8 h post-treatment, expression levels were similar to or slightly lower than those in Col-0 (Table 3.1). Among this group of genes, seven were also regulated by NPR1, which has been shown to regulate ER-related gene expression during plant defence (Shearer et al., 2012; Wang et al., 2005; Table 3.1). However, in all cases, the direction of change in *tga1-1 tga4-1* and *npr1-3* mutants is always opposite, while expression following treatment of *npr1-1* with dexamethasone, which resulted in nuclear localization of a NPR1-glutacorticoid receptor transgene, paralleled that in the *tga1-1 tga4-1* mutant.

However, as summarized in the Table 3.1, the larger number of NPR1-dependent genes (29) identified by microarray analysis suggests that other transcription factors may work with NPR1 (Table 3.1). There are three ER membrane-associated bZIP transcription factors, bZIP17, bZIP28, and bZIP60, which are responsible for transcriptional upregulation of these ER-related genes during ER stress (Iwata et al., 2008; Liu et al., 2007b; Liu et al., 2007c; Liu and Howell, 2010a). Through comparision between four microarray datasets, NPR1-dependent genes largely overlap with the genes dependent on bZIP28 (12 out of 15) or bZIP60 (14 out of 16) (Table 3.1). In addition, seven out of eight TGA1/4-dependent genes are also regulated by bZIP28 or bZIP60, although, once again, the direction of change is opposite (Table 3.1). Taken together, these comparisions suggest that clade I TGA factors and NPR1 may play a role in response to ER stress by regulating ER-related gene expression, although with opposing effects.

To further explore the possible role of clade I TGA factors in ER stress, the seeds of Col-0 and the *tga1-1 tga4-1* double mutant were germinated on MS plates containing different concentrations of tunicamycin (TM), an inhibititor of asparagine N-linked glycosylation that can trigger ER stress (Liu and Howell, 2010b). Five days after treatment, seedlings were transferred to TM-free MS plates for 10 days. In the seedlings transferred from MS without TM, there were no differences in growth between wild type and the double mutant, indicating that seedling growth is normal in the double mutant (Figure 3.19a). However, seedlings transferred from different concentrations of TM displayed substantial differences between genotypes, with double

Table 3.1 Comparisons among microarray datasets of ER-related gene expression in different mutants

		Shearer et al., 2012								Wang et al., 2005				Liu and Howell, 2010a		Iwata et al., 2008	
Microarray method	Plant material	Four-week-old rosette leaves								Four-week-old rosette leaves				1-week-old seedlings		10-day-old seedlings	
	Treatment	Spray with 1mM SA, and harvest at 0h, 1h, and 8h after treatment								Spray with 0.5mM SA for 24h, followed by 5μM Chx for 2h, and then 5μM Dex for 8h				Treated with 5μg ml^{-1} TM or DMSO (mock) for 2h		Treated with or without 5 μg ml^{-1} TM for 5h	
	Microarray	Affymetrix *Arabidopsis* gene chips (ATH1)								Affymetrix ATH1				Affymetrix ATH1		*Arabidospsis* 2 oligo Microarrays (Agilent Technologies)	
	Genotype	*tga1-1tga4-1*/Wt				*npr1-3*/Wt				NPR1-GR/*npr1-3*				Wt/*bzip28*		Wt/*bzip60*	
AGI ID	gene description	0h	1h	8h	P-value	0h	1h	8h	P-value	R1 F.C	P-value	R2 F.C	P-value	D.o .D	P-value	FI/FI	P-value
Chaperones/Co-chaperones																	
At5g28540 / At5g42020	Bip1/Bip2; ATP binding	1.4	2.8	0.8	7.3E-03	0.9	1.0	0.2	8.6E-04	3.2	7.0E-06	2.5	0				
At1g09080	Bip3; ATP binding	3.3	4.3	1.0	6.2E-02	1.1	1.0	0.1	6.8E-04					8.9	0.07	4.6	0.008
At4g24190	SHD (SHEPHERD)/ GRP94	1.7	2.0	0.9	2.0E-02	0.9	0.8	0.2	4.1E-05	7.5	0	2.3	0	4.5	0.03	1.5	0.002
At3g62600	ERdj3B	1.6	2.4	0.9	1.0E-02									2.3	0.00		
At3g08970	ERdj3A					0.9	0.9	0.1	2.0E-07							1.5	0.004
At4g21180	ERdj2B					1.1	1.1	0.3	5.1E-03							1.5	0.024
At2g25110	SDF2	1.3	2.1	0.9	3.5E-03					2.4	4.0E-03	2.1	4.0E-03	2.7	0.06		
At4g16660	heat shock protein 70 (HSP70)	1.6	2.1	0.8	8.4E-03	0.8	1.0	0.3	6.3E-05					1.7	0.08	1.4	0.039
Disulfide bond formation (PDI)																	
At1g21750	PDI-LIKE 1-1					1.0	1.0	0.2	5.1E-06	3.6	5.0E-03	2.1	5.0E-03	1.6	0.03	1.4	0.021
At1g77510	PDI-LIKE 1-2	1.8	4.5	0.8	3.7E-03	0.8	1.1	0.1	2.5E-07							1.5	0.007
At3g54960	PDI-LIKE 1-3					0.6	0.7	0.1	1.3E-02	1.6	8.0E-04	3.0	1.0E-06			1.8	0.012
At2g47470	PDI					1.0	1.0	0.3	1.0E-04	3.2	3.1E-05	2.0	1.0E-06	1.3	0.01	1.2	0.022
At1g04980	PDI-LIKE 2-2					0.8	1.3	0.2	1.7E-05					1.0	0.06		
At2g32920	PDI, putative					0.9	1.1	0.5	3.6E-03							2.2	0.018
At1g72280	ER oxido-reductin (ERO1)															2.1	0.001
N-glycosylation																	
At1g32210	DAD1					1.0	1.0	0.4	1.9E-04	3.0	1.0E-06	2.0	0				
At1g56340	Calreticulin 1 (CRT1)					1.1	1.3	0.4	3.0E-05							1.4	0.037
At1g09210	Calreticulin 2 (CRT2)					1.1	1.2	0.3	2.0E-04	4.3	1.0E-06	3.1	0			1.2	0.006

Table 3.1 continued

At1g08450	Calreticulin 3 (CRT3)									4.0	0	2.3	0				
At5g61790	Calnexin 1 (CNX1)	1.5	2.2	0.8	1.4E-03	0.9	1.1	0.3	7.6E-05	6.1	4.3E-05	2.6	0	3.6	0.03		
At5g07340	Calnexin 2 (CNX2)					1.0	1.0	0.4	2.2E-03	1.4	5.0E-06	2.1	1.2E-05	1.5	0.09	1.1	0.032
Translocation																	
At2g34250	Sec61α subunit					1.0	1.0	0.4	2.9E-03	2.1	5.4E-05	1.5	0				
At2g45070	Sec61β subunit									1.4	4.0E-02	1.3	1.0E-02				
Secretion pathway																	
At2g45770	Signal recognition particle receptor									4.9	3.1E-05	6.1	0				
At2g01720	Ribophorin I									3.7	8.0E-06	1.5	1.0E-06				
At4g22670	Tetratrico-redoxin					0.9	0.9	0.3	2.3E-04	3.2	0	1.9	0				
At2g47320	Cyclophilin									4.0	2.0E-06	1.4	7.9E-05				
At1g10730	Clathrin-coat assembly protein									3.7	5.8E-02	2.0	4.7E-05				
At1g14010	Trans-membrane trafficking protein									4.3	2.2E-04	2.0	1.0E-06				
At1g30900	Vacuolar sorting receptor					0.5	0.4	0.0	1.8E-02	7.0	1.0E-05	3.5	0				
At2g03120	signal peptide peptidase family protein					1.0	1.0	0.4	4.5E-04	2.4	2.0E-02	1.8	2.0E-02	1.5	0.08	1.3	0.032
At3g07680	emp24/ gp25L/p24 family protein					0.9	0.9	0.4	5.4E-03					1.2	0.06		
others																	
At4g29520	unknown protein					0.8	1.5	0.2	6.2E-03					2.0	0.05		
At1g61780	Post-synaptic protein-related													1.1	0.10		
At5g35630	GS2													1.0	0.01		
At3g51980	SIL1, putative															1.6	0.035

SDF2 (STROMAL CELL-DERIVED FACTOR 2-LIKE PROTEIN PRECURSOR); GS2 (GLUTAMINE SYNTHETASE 2); glutamate-ammonia ligase; F.C. Fold Change; D.o.D. difference of differences means that the difference in TM-treated versus untreated wild type less the difference in TM-treated versus untreated zip28-2 mutnat; FI/FI fold induction of wild type/fold induction of *bzip60* mutant

mutant seedlings being more sensitive to growth inhibition by TM than Col-0. Most Col-0 seedlings recovered from TM treatment after transplanting, whereas most *tga1-1 tga4-1* mutant seedlings did not (Figure 3.19a). In addition, increasing the TM concentration resulted in fewer seedlings being recovered, indicating that growth inhibition is determined by TM. At the highest TM concentration tested (0.7 µg ml^{-1}), none of the double mutant seedlings recovered from TM inhibition (Figure 3.19a). These results further implicate clade I TGA factors in the proper functioning of the ER secretion pathway.

To examine whether NPR1 is involved in TM-induced ER stress, the *npr1-1* single and *tga1-1 tga4-1 npr1-1* triple mutant were added to the anlaysis. In addition, to confirm the results in Figure 3.19a, two TGA1 overexpression lines were also analyzed. To do quantitative comparisons between different genotypes, different TM assays were employed. Five-day-old seedlings grown on TM-free solid MS medium were submerged in MS liquid with or without 0.8 µg ml^{-1} TM for 6 h, and were allowed to recover for 5 days in the absence of TM. The fresh weight of TM-treated seedlings were measured and normalized by the fresh weight of untreated seedlings.

Compared to the untreated seedlings, fresh weight of wild type was reduced by 30% after TM treatment, indicating that TM efficiently inhibited the seedling growth in this assay (Figure 3.19b and c). In addition, the reduction of fresh weight in *tga1-1 tga4-1* (60% of non-treated) was statitistically siginificant, compared with that in wild type. However, two TGA1OE/*tga1x4* lines displayed similar fresh weight reductions to wild type. These results confirm the above conclusion that clade I TGA factors are involved in the ER secretion pathway.

The seedling growth of *npr1-1* is also inhibited by TM and the TM sensitivity in *npr1-1* is similar to that in the *tga1-1 tga4-1* (Figure 3.19b and c). The combination of *npr1-1* and *tga1-1tga4-1* did not increase sensitivity to TM, suggesting that clade I TGA factors and NPR1 function in the same pathway during TM-induced ER stress.

3.16 The *tga1-1 tga4-1* double mutant is more susceptible to *Colletotrichum higginsianum*

Clade I TGA factors have been shown to positively contribute to disease resistance against the bacterial pathogen *P. syringae* (see sections 3.3-3.15; Shearer et al., 2012) and the

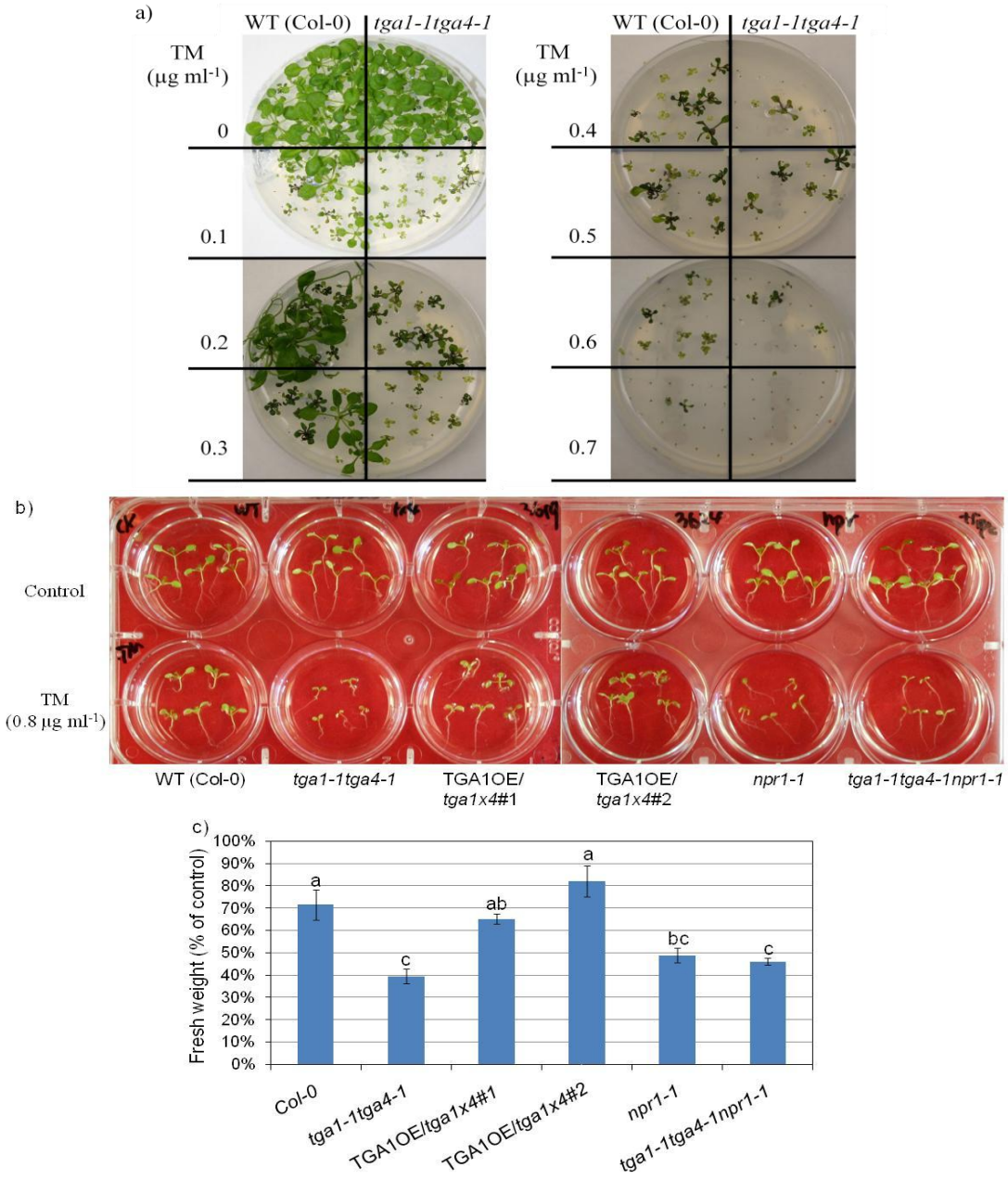


Figure 3.19 Seedlings of the *tga1-1 tga4-1* mutant are hypersensitive to tunicamycin.
a) Five-day-old seedlings of wild type (Col-0) and the *tga1-1 tga4-1* double mutant grown on MS with different TM concentration were transplanted to TM-free MS agar and grown for a further 5 days prior to photography. This experiment was repeated three times with similar results. b) Five-day-old seedlings of Col-0, *tga1-1 tga4-1*, *npr1*, *tga1-1 tga4-1 npr1-1* and two TGA1OE/*tga1x4* lines grown on TM-free MS were submerged in MS liquid with or without 0.8 µg ml^{-1} TM for 6 h, and were allowed to recover for 5 days without TM. c) Fresh weight of seedlings in b) was quantified. Each fresh weight of TM-treated seedlings was divided by the average fresh weight of 5 untreated seedlings to generate percentage of control. The results are averages +/- standard error (n=5) An ANOVA of data was performed at $\alpha = 0.05$; treatments with common letters over the error bars are not significantly different from each other. This experiment was repeated twice with similar results.

obligate biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis* (Shearer et al., 2012). However, little is known on the involvement of clade I TGA factors in disease resistance against other pathogens, such as fungi. To address this question, hemibiotrophic fungal pathogen *Colletotrichum higginsianum* was inoculated onto *tga1-1 tga4-1* and Col-0 leaves. *C. higginsianum* induced larger lesions on the double mutant plants compared to Col-0 (Figure 3.20b and c). Microscopic examination revealed that the fungal development in the double mutant leaves was faster than wild type (Figure.3.20a). These data indicate that clade I TGA factors function in basal resistance responses against this fungal pathogen.

To determine the contribution of each member of clade I TGA factors to disease resistance against *C. higginsianum,* single mutants of clade I TGA factors were inoculated by droplet of conidial suspension. The infection lesion sizes in *tga4-1* and *tga4-2* mutants are statistically similar to that in wild type (Figure 3.21a). However, *tga1-1* and *tga1-3* displayed enhanced susceptibility to *C. higginsianum* (Figure 3.21). In addition, the level of disease susceptibility in *tga1-1* is the same as that in the double mutant (Figure 3.21a), suggesting that the increased susceptibility observed in the double mutant is primarily caused by mutation in TGA1. These results suggest that TGA1 may be more important than TGA4 for disease resistance against C. *higginsianum.*

Arabidopsis accessions display variation in their susceptibility to *C. higginsianum* (Narusaka et al., 2004; O'Connell et al., 2004). As shown in Figure 3.21b, Nössen plants are more resistance than Col-0 to *C. higginsianum*. In addition, *npr1-5* is more susceptible than wild type Nössen plants and reaches the susceptible level of Col-0 (Figure 3.21b), suggesting that NPR1 is important for disease resistance in Nössen background.

3.17 The *tga1-1 tga4-1* mutant displays morphological and developmental abnormalities

In addition to the important roles in the defence responses, clade I TGA factors are also involved in developmental processes, such as leaf curling and late flowering. Leaves of *tga1-1 tga4-1* double mutant displayed right-handed petiole torsions (Figure 3.22a). Neither single mutant had this phenotype, suggesting that TGA1 and TGA4 function redundantly to regulate this phenotype (Figure 3.22a). In addition, although the curly leaves were not observed in the

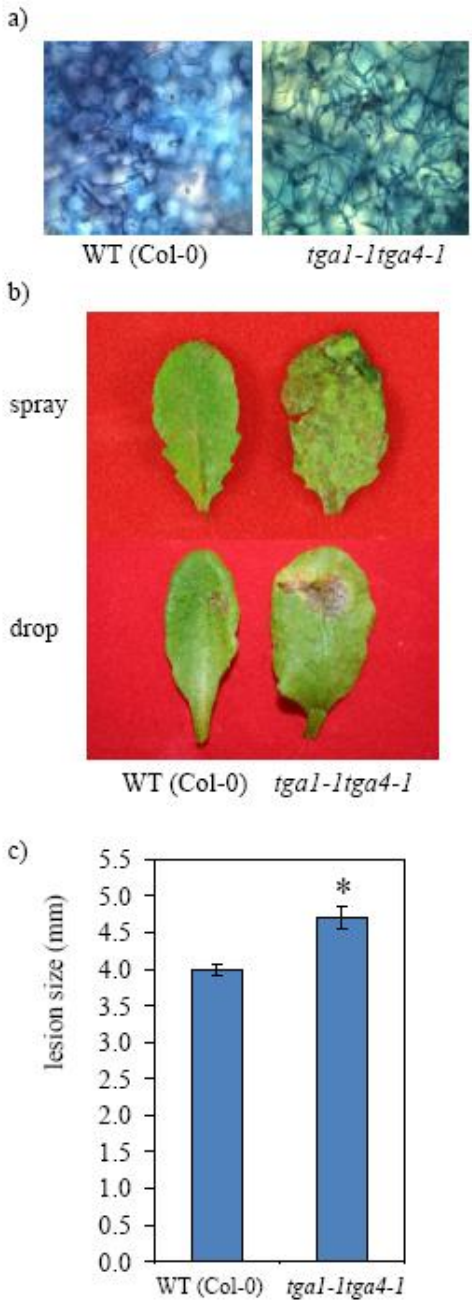


Figure 3.20 The *tga1-1 tga4-1* double mutant plants show enhanced disease susceptibility to *C. higginsianum*.

a) Microscopic examination of fungal development in wild type (Col-0) and *tga1-1 tga4-1* double mutant plants. Four-week-old leaves were sprayed with *C. higginsianum* conidial suspension (1 x 10^6 spores ml^{-1}) and stained with trypan blue at 3 dpi. b) Representative disease symptoms. Leaves either sprayed with *C. higginsianum* conidial suspension (upper panel), or spotted with 5 µl droplets of conidial suspension (lower panel) were photographed at 4 dpi. c) Lesion size in spot-inoculated leaves. The lesion size was measured from 20 to 30 independent leaves per genotypes at 4 dpi. An asterisk indicates a statistically significant difference compared with Col-0 ($p<0.05$, Student's *t*-test). This is one of three independent experiments with similar results. Error bars indicate standard error.

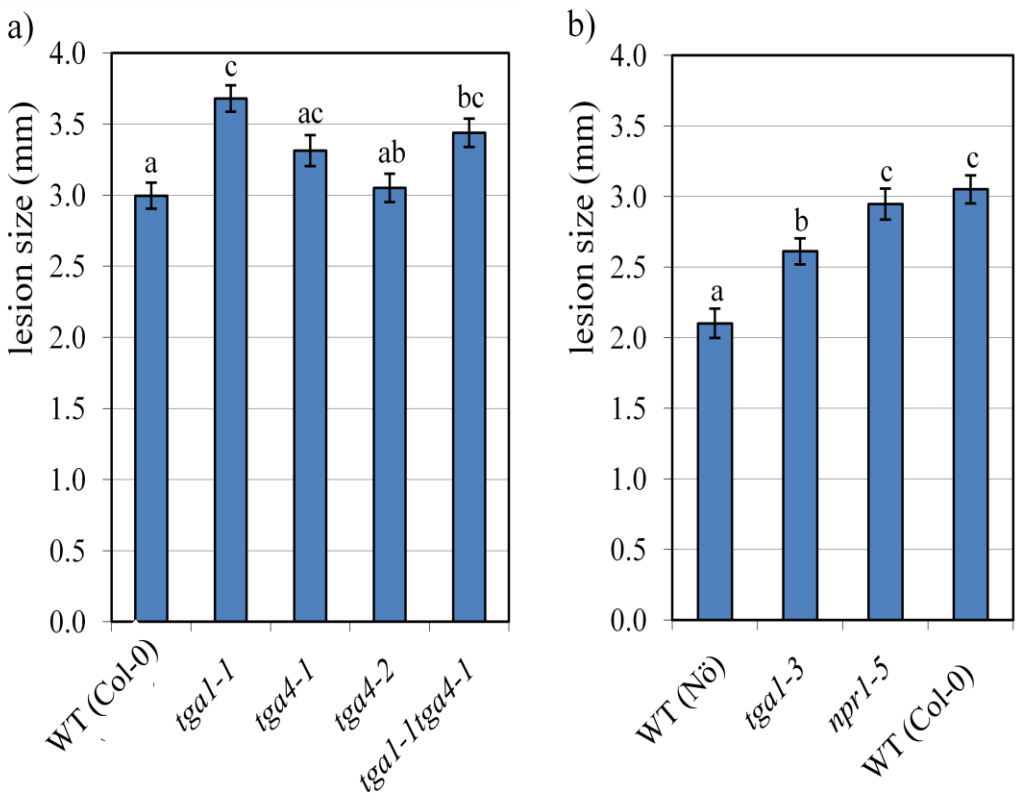


Figure 3.21 Lesion size in leaves spot-inoculated with *C. higginsianum*.
Four-week-old leaves were spotted with 5 µl droplets of a *C. higginsianum* conidial suspension (1 x 10^6 spores ml^{-1}). The lesion size was measured from 20 to 30 independent leaves per genotypes at 4 dpi. An ANOVA of data was performed at $\alpha = 0.05$; treatments with common letters over the error bars are not significantly different from each other. This is one of two independent experiments with similar results. Error bars indicate standard error.

npr1-1 mutant plants, the *tga1-1 tga4-1 npr1-1* triple mutant plants displayed the phenotype (Figure 3.22a).

To quantitatively measure flowering time, at least 24 plants per genotype were grown under long day (LD, 16 h light and 8 h dark) growth conditions. The total leaf number and the day number at bolting were recorded. As shown in Figure 3.22d and e, Col-0 plants have on average 16 leaves and are 25 days-old when their primary inflorescence apex is first visible. This result is similar to most published results on flowering time of Col-0 under LD conditions (Koornneef et al., 1991), indicating that plant growth was not under environmental stress which alters the flowering time. Compared to Col-0, the *tga1-1 tga4-1* double mutant plants flowered later (Figure 3.22d and e). The late flowering phenotype was also observed in *tga1-1* single mutant, but not in *tga4-1* single mutant plants when the primary inflorescence apex appeared. The leaf number and day number required for *tga1-1* to flower is significantly higher than Col-0, but similar to the double mutant, suggesting that the late flowering phenotype observed in the double mutant is primarily caused by mutation in *TGA1*.At a late stage (6 weeks old) after bolting, both single mutant plants are similar to Col-0, while the double mutant displayed shorter primary inflorescence stems and fewer secondary inflorescences (Figure 3.22b). This suggests that TGA1 and TGA4 are both involved in controlling development at this stage and function redundantly.

Although SA signaling has been shown to be involved in regulating flowering (Martínez et al., 2004), the role of NPR1 has not been reported yet. As shown in Figure 3.22d and e, the *npr1-1* mutant displayed an early flowering phenotype. However, the *tga1-1 tga4-1 npr1-1* triple mutant plants are similar to the *tga1-1 tga4-1* double mutant in flowering late (Figure 3.22d and e). Furthermore, like the double mutant, the triple mutant plants have shorter primary inflorescence stems fewer secondary inflorescences at late stage after bolting (Figure 3.22c).

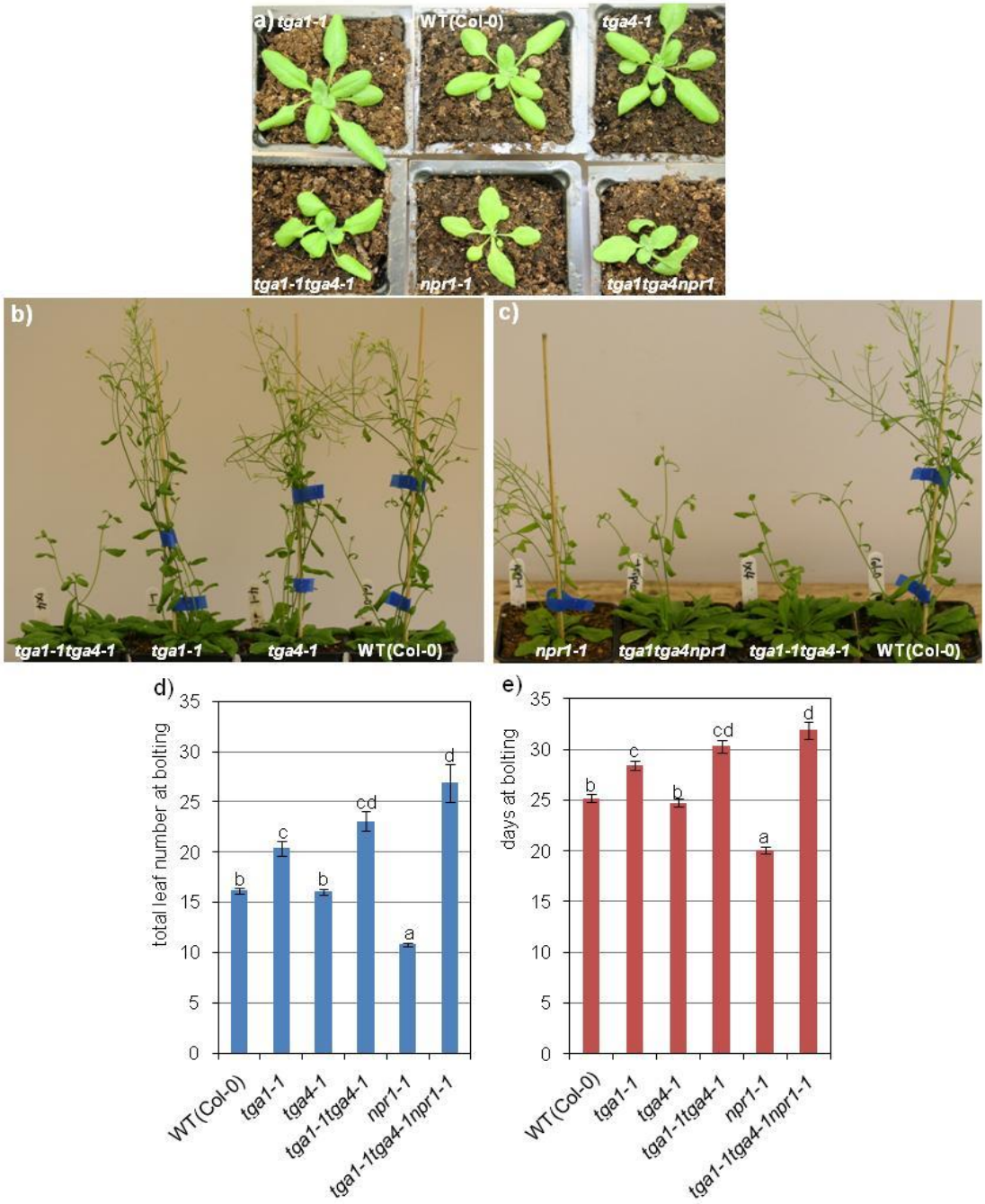


Figure 3.22 Morphological and developmental phenotypes of wild type and clade I TGA mutants.

All plants were grown under long day (16 h light/8 h dark) conditions. Photographs were taken at 3 weeks (a) and 6 weeks (b and c) after germination. d) and e) An ANOVA of data was performed at $\alpha = 0.05$; treatments with common letters over the error bars are not significantly different from each other. The total leaf number (blue bar) and day number (red bar) at bolting were counted. Values represent the means ± standard errors. A representative experiment of four independent repeats with n=24 each is shown.

CHAPTER 4 DISCUSSION

4.1 The role of clade I TGA factors in response against the bacterial pathogen *P. syringae*

Large-scale transcriptional reprogramming is an important aspect of plant defence in response to pathogen infection. The regulation of the defence transcriptome requires members of several groups of transcription factors, such as WRKY, ERF, TGA, Whirly and Myb factors (Eulgem, 2005; Fobert, 2007). The Arabidopsis TGA factors have been shown to regulate the expression of *PR* genes and are required for resistance to disease. In the present study, the biological functions of clade I TGA factors during plant immune response were investigated in depth.

Disease-testing results on additional mutant alleles and overexpression lines (Figure 3.5) confirmed previous findings (Kesarwani et al., 2007; Lindermayr et al., 2010; Shearer et al., 2012) that clade I TGA factors positively contribute to basal resistance against virulent isolates of the bacterial pathogen *P. syringae*. According to the "zig-zag, zig" model, basal resistance has been described as "MTI plus weak ETI, minus ETS". MAMP-induced defence responses are suppressed by virulence effectors, meanwhile other effectors are recognized by R proteins to trigger ETI defences (Jones and Dangl, 2006). Accordingly, compatible interactions involving virulent pathogens, wherein plant defence responses are suppressed, may not be the most appropriate to study the contribution of clade I TGA factors to disease resistance. This prompted me to investigate the role of these transcription factors towards different immune responses.

Transcripts of clade I TGA factors accumulate to higher levels after treatment with the MAMP flg22 or inoculation with *P.s.t. hrcC*$^{-}$ that cannot transfer T3SE to the plant cytoplasm (Figure 3.1d and e), suggesting that clade I TGA factors may play a role in MTI. This notion is supported by disease-testing results that the *tga1-1 tga4-1* double mutant was compromised in resistance against *P.s.t. hrcC*$^{-}$ (Figure 3.6). The involvement of clade I TGA factors in MTI is also supported by the results that the *tga1-1 tga4-1* double mutant was impaired in two responses, callose deposition and the ROS burst, following treatment with a purified MAMP (Figure 3.9 and 3.10).

The requirement of clade I TGA factors for another type of immunity, ETI, was also examined in this study. The *tga1-1 tga4-1* double mutant was found to be defective in ETI-mediated resistance against the avirulent pathogen *P.s.t. AvrRpt2* (Figure 3.8). Furthermore, *P.s.t. AvrRpt2* inoculation induced high transcript levels of clade I TGA in the Col-0 plants (Figure 3.1c). The involvement of clade I TGA factors in ETI is also supported by findings that enhanced resistance triggered by constitutive activation of SNC1, an NB-LRR-type of R protein, is compromised in the *tga1-1 tga4-1 snc1 npr1-1* quadruple mutant (Shearer et al., 2012). Together, these results indicate that clade I TGA factors act as positive regulators of ETI.

Since clade I TGA factors are involved in both types of immunity (MTI and ETI), I further asked whether clade I TGA factors contribute to NHR which requires MTI and ETI at the same time (Mysore and Ryu, 2004). Non-host resistance against *P.s.p*. is compromised in the *tga1-1 tga4-1* double mutant (Figure 3.7). Furthermore, not only the MAMP-induced small callose, but also the effector-induced big callose was reduced in the double mutant, suggesting that clade I TGA factors are involved in both MTI and ETI during the interaction with *P.s.p* (Figure 3.9b).

The enhanced disease susceptibility (EDS) phenotype observed in the double mutant (Figure 3.5) suggests that TGA1/4-dependent immune response was not completely eliminated following challenge with virulent *P.s.m.*, but attenuated by virulence effectors. Compared to the fold induction after *P.s.t. hrcC*$^{-}$ inoculation, the fold induction of clade I TGA factors is relatively small after *P.s.t.* DC3000 inoculation (Figure 3.1b and e), suggesting that effectors may suppress the function of clade I TGA factors at the transcriptional level. In addition, virulence effectors may also interrupt clade I TGA factors at the posttranscriptional level or the signaling pathway acting downstream of these transcription factors. However, if and how effectors suppress TGA1/4-dependent immunity remains to be determined.

Taken together, the broad TGA1/4-dependent disease resistance suggests that clade I TGA factors may regulate a universal and basal mechanism to control disease resistance. Although a growing number of studies suggest that MTI and ETI share common signaling pathways and defence responses (Dodds and Rathjen, 2010; Thomma et al., 2011), less is known on the common regulators of both types of immunity. It has been recently proposed that MTI

and ETI should be considered as opposite ends of a continuum in which plant immune receptors recognize appropriate ligands to activate defence responses, the amplitude of which is likely determined by the level required for effective immunity (Thomma et al., 2011). Despite this possibility, MTI and ETI show differential requirements for other known regulators of plant defence responses, including NPR1 (Delaney et al., 1995; Shah et al., 1997; van Wees and Glazebrook, 2003; Zipfel et al., 2004). My results suggest that clade I TGA factors are common regulators which contribute to both MTI and ETI.

4.2 Clade I TGA factors regulate multiple defence signaling pathways

Plant defence in response to pathogen challenge is regulated through a complex network of signaling pathways involving plant hormones, such as SA, JA, and ET (Broekaert et al., 2006; Browse, 2009; Vlot et al., 2009). Many lines of evidence suggest that clade I TGA factors are involved in the SA signaling pathway, which plays an important role in disease resistance against virulent *P. syringae.* The involvement of clade I TGA factors in SA-meditated signaling was originally based on two lines of evidence: their ability to bind to the SA-, JA-, and auxin-inducible *as-1* DNA element (Fromm et al., 1989; Katagiri et al., 1989), and interaction with NPR1 upon SA-induced redox changes (Després et al., 2003). The observation that transcript levels of clade I TGA factors increased following SA treatment (Figure 3.1a) further suggests that clade I TGA factors may play a role in SA signaling. The most convincing evidence for a role of clade I TGA factors in SA-dependent defence comes from reverse genetic studies revealing that mutant plants of clade I TGA factors are impaired in resistance against virulent *P. syringae* (Kesarwani et al., 2007; Lindermayr et al., 2010; Shearer et al., 2012; Figure 3.5).

However, the contribution of clade I TGA factors to SA signaling is limited, because they are not required for SA-dependent SAR against virulent pathogen *P.s.m.* ES4326 (Shearer et al., 2012). Microarray analysis further demonstrated that clade I TGA factors are not major regulators for SA-induced genes expression (Shearer et al., 2012). Compared to hundreds of genes controlled by NPR1 (Shearer et al., 2012; Wang et al., 2006), relatively few genes are regulated by clade I TGA factors after treatment with SA. Moreover, most genes differentially expressed between Col-0 and the *tga1-1 tga4-1* double mutant only appeared at early time points after SA treatment (Shearer et al., 2012; Figure 3.17). Those genes, including SA-marker gene

PR-1, were up-regulated in *tga1-1 tga4-1* at 0 h and 1 h, and by 8 h after SA treatment, the expression levels were similar to those in Col-0. These results indicate that clade I TGA factors may act as repressors of SA-responsive gene expression in the absence of elicitation. The repressive action of clade I TGA factors on SA-inducible genes is minor and gradually disappears. It is possible that upon stress stimulation, either TGA1/4 (Després et al., 2003; Lindermayr et al., 2010) and/or NPR1 (Mou et al., 2003; Spoel et al., 2009) undergo redox-mediated posttranslational modifications to facilitate TGA1/4-NPR1 interaction , relieving transcriptional repression of TGA1/4.

No well-known SA-inducible gene was downregulated in *tga1-1 tga4-1* at the 8 h after SA treatment, suggesting that clade I TGA1 factors are not important activators of SA-regulated gene expression. This function appears to be attributed to other transcription (co)factors, such as NPR1, TGA2, and WRKY (Eulgem, 2005; Fobert, 2007).

Disease-testing results also suggest that clade I TGA factors may regulate defence responses other than those mediated by the SA signaling pathway. Although continuing to express SA pathway marker genes at or above wild type levels following challenge with different types of pathogen (Shearer et al., 2012; Figure 3.15), the *tga1-1 tga4-1* double mutant is more susceptible to the corresponding pathogens than Col-0 plants (Shearer et al., 2012; Figure 3.5, 3.6 and 3.7). In addition, NHR against *P.s.p.* relies on multiple defence-signaling pathways, such as SA-induced PR-1 protein accumulation and morphologically distinct types of callose deposition (Ham et al., 2007). Disrupting the SA signaling pathway with mutations does not affect callose deposition and is not enough to compromise NHR against *P.s.p.* (Ham et al., 2007; van Wees and Glazebrook, 2003; Figure 3.7b). However, loss of function in the clade I TGA factors caused defects in callose deposition and compromised NHR against *P.s.p*. (Figure 3.7 and 3.9b), indicating that clade I TGA factors contribute to additional defence pathways during the plant immune response.

Although SA-dependent signaling is a major pathway for disease resistance against virulent *P. syringae*, other signaling pathways, such as JA/ET-mediated signaling have been reported to be important (Robert-Seilaniantz et al., 2007; Robert-Seilaniantz et al., 2011). My prelimimary results suggest that levels of JA/ET mediated markers such as *PDF1.2, VSP* and

LOX2 continue to be expressed at levels comparable or higher than wild type in the *tga1-1 tga4-1* double mutant (Wang, Chatur and Fobert, unpublished result). Considering the elevated *PR-1* expression in the double mutant after pathogen challenge (Figure 3.15), clade I TGA factors may be involved in SA-JA/ET crosstalk during immunity. Of note, clade II TGA factors have been also shown to be involved in SA-JA/ET crosstalk (Ndamukong et al., 2007).

Signal transduction events downstream of pathogen recognition rely heavily on mitogen activated kinases (MAPK) (Asai et al., 2002). Accordingly, transcript levels of some representive markers for these pathways were quantified in Col-0 and *tga1-1 tga4-1* after challenge with *P.s.t. hrcC*$^-$ (Figure 3.16). Although the expression of these genes was not affected in the double mutant following a short time course (24 h), some genes were expressed differentially in the double mutant and wild type following longer exposures (2 dpi and 3 dpi). For example, the MAPK signaling pathway marker *FRK1* is induced early and levels of its transcripts decrease at later times after pathogen challenge (Asai et al., 2002). However, *FRK1* expression remained high in the double mutant at 3 day after *P.s.t. hrcC*$^-$ challenge (Wang, Chatur and Fobert, unpublished result). Furthermore, microarray data also show that *FRK1* was upregulated in the seedling of the double mutant (Wang and Fobert, unpublished result). These results demonstrate that clade I TGA factors may play a role in regulating genes implicated in the MAPK signaling pathway. As was the case with markers for the SA and JA/ET pathways, clade I TGA appear to be negative regulators of gene expression for MAPK markers.

4.3 Clade I TGA factors are involved in cell wall-associated defence responses

Cell wall associated defences are critical for disease resistance against bacterial pathogens. Impairment of pathogen-induced callose deposition and oxidative burst in the *tga1-1 tga4-1* double mutant (Figure 3.9 and 3.10), indicate that clade I TGA factors also contribute to cell wall associated defence responses.

Moreover, clade I TGA factors are involved not only in MTI-type small callose deposition induced by *P.s.t. hrcC*$^-$, flg22 or MAMPs of *P.s.p.*, but also in ETI-type big callose deposition induced by T3SEs of *P.s.p.* (Figure 3.9). This suggests that these two different types of callose deposition rely to some extent on common signaling pathways which require clade I

TGA factors. To date, only mutation of the callose synthase gene, *CalS12/PMR4*, has been shown to affect all of the above types of callose deposition (Ham et al., 2007; Kim et al., 2005). Therefore, we asked whether the contribution of clade I TGA factors to pathogen-induced callose deposition is due to their transcriptional regulation of the callose synthase genes. However, pathogen-induced gene expression level of *CalS12* and another callose synthase gene, *CalS1*, in the double mutant are similar to those in the Col-0 (Figure 3.16), suggesting that the pathogen-induced callose synthase gene expression does not require clade I TGA factors. Interestingly, despite loss of pathogen-induced callose deposition, the *pmr4* mutant, impaired in *CalS12*, still retained NHR against *P.s.p.* (Ham et al., 2007). Considering the requirement of clade I TGA factors for NHR against *P.s.p.*, we further speculated that clade I TGA factors may regulate other cell wall defence responses in addition to callose deposition to account for loss of resistance.

4.4 Clade I TGA factors play a role in the defence-related secretory pathway

The observation that loss of clade I TGA factors affected cell wall defence responses and disease resistance against pathogens, but not the steady-state levels of either callose synthase or *PR-1* transcripts, suggested that the mutant may be compromised in some aspect of posttranscriptional regulation required for plant immunity. The success of cell wall-associated defence is dependent on effective secretion processes**.** Disrupting the secretion pathway results in seriously reduced extracellular accumulation of PR-1 protein (Kalde et al., 2007; Wang et al., 2005). Callose deposition in the papillae is delayed in mutant plants defective in vesicle-associated secretion processes (Kwon et al., 2008b), implicating callose precursors and/or the callose synthase protein as one potential defence component delivered to infection sites by the vesicle-related secretion pathway.

Therefore, we tracked the extracellular accumulation of PR-1 protein in the double mutant after pathogen challenge. Less extracellular PR-1 protein was detected in the double mutant, compared to that in Col-0 (Figure 3.18), consistent with the existence of an abnormal secretion pathway in the double mutant affecting the secretion of defence-related proteins following pathogen challenge. In addition, since MTI and NHR rely on multiple defence pathways, I speculated that a TGA1/4-dependent secretion pathway may also regulate the secretion of defence-related proteins other than PR1, such as callose synthase proteins and the

enzymes responsible for apoplastic ROS production. This would account for the observed defects of multiple cell wall-associated defence responses in the double mutant plants, including PR-1 protein accumulation, callose deposition and the ROS burst.

Microarray analysis has revealed that clade I TGA factors may regulate ER-related gene expression after SA treatment (Shearer et al., 2012; Table 3.1). These ER-related genes are upregulated under ER stress and contain conservered *cis*-elements in their promoters, called ER stress-response elements (ERSEs) (Yoshida et al., 1998). One of the ERSEs is UPRE, corresponding to TGACGGTGG, which contains a TGA binding motif, suggesting that clade I TGA factors may bind on this element to directly regulate the expression of these genes. The involvement of clade I TGA factors in response to ER stress is also supported by the result that *tga1-1 tga4-1* seedlings are more sensitive to TM treatment than Col-0 (Figure 3.19). Although experiments have shown that the motif TGACG is sufficient for TGA factor binding *in vitro* (Lam et al., 1989), it is necessary to prove the binding ability of clade I TGA factors to ERSEs in promoters of these genes *in vitro* by EMSA (electrophoretic mobility shift assay) or *in vivo* by ChIP (chromatin immunoprecipitation) (Després and Fobert, 2006).

In Arabidopsis, three bZIP membrane-bound transcription factors (bZIP17, bZIP28 and bZIP 60) have been identified that regulate gene expression in response to ER stress (Liu and Howell, 2010b). Under ER stress, these ER membrane-located transcription factors are activated and translocated into the nucleus where they activate gene expression (Liu and Howell, 2010b). Comparisions of microarray data demonstrate that many TGA1/4-dependent genes are also regulated by bZIP28 or bZIP60 (Table 3.1). Clade I TGA factors do not have a transmembrane domain and haven't been shown to locate on ER membranes (Stonehouse, 2002). Therefore, it is speculated that clade I TGA factors may interact with these transcription factors to form heterodimers to activate gene expression together during ER stress. However, this idea is speculative and needs to be tested.

NPR1 is another potential candidate which co-regulates ER-related gene expression with clade I TGA factors. Microarray analysis (Shearer et al., 2012; Wang et al., 2005) and the hypersensitivity of the *npr1-1* mutant to TM (Figure 3.19) indicate that NPR1 also plays a role in the response to ER stress. Furthermore, *tga1-1 tga4-1 npr1-1* triple mutant seedlings displayed

the same sensitivity level as either the *tga1-1 tga4-1* double mutant or the *npr1-1* single mutant (Figure 3.19b), suggesting that clade I TGA factors and NPR1 function in the same pathway during TM-induced ER stress. Microarray data also revealed that many ER-related genes are regulated by both clade I TGA factors and NPR1, although in all cases, the direction of change was opposite in the two mutants (Shearer et al., 2012; Table 3.1). If this is in the case, several questions need to be addressed. For example, cytosolic NPR1 is translocated into the nucleus upon pathogen infection or SA treatment (Mou et al., 2003; Spoel et al., 2009). Can this translocation occur under ER stress conditions, such as TM treatment? The interaction between NPR1 and TGA1/4 is mediated by SA-induced redox changes (Després et al., 2003). Can this interaction occur under ER stress conditions? It is noteworthy that ER stress in mammalian cells causes changes in the redox status, which further activate redox sensitive transcription factors, such as NF-κB (Fedoroff, 2006; Schröder and Kaufman, 2005). Finally, since NPR1 does not locate on the ER membrane, NPR1 needs to be activated by additional ER-located protein which sense the ER stress. What is this ER-stress sensor?

In addition to contributing to ER-related secretion, clade I TGA factors may also play a role in the vesicle-related secretion pathway. The *tga1-1 tga4-1* double mutant displayed enhanced disease susceptibility to the ΔCEL mutant of *P.s.t.* which lacks virulence on wild type Arabidopsis due to an inability to suppress the host vesicle-related secretion pathway (Figure 3.11).

The TGA1/4-dependent secretion pathway may not regulate all defence-related protein secretion. For example, biogenesis of receptor proteins, such as EFR, requires the functional ER secretion pathway (Saijo, 2010). Due to an inability to accumulate functional EFR on the plasma membrane, mutants in ER components loose seedling growth inhibition (SGI) in response to the MAMP elf18 (Li et al., 2009a; Lu et al., 2009; Nekrasov et al., 2009; Saijo et al., 2009). The *tga1-1 tga4-1* double mutant still retains the SGI response to efl18 and flg22 (Figure 3.12), suggesting that secretion pathways responsible for EFR or FLS2 are normal in the double mutant plant.

4.5 Clade I TGA factors act in NPR1-dependent and -independent pathways

NPR1-dependency of TGA1 and TGA4 is consistent with the observation that they physically interact with NPR1 *in planta* following SA treatment (Després et al., 2003). However, the involvement of clade I TGA factors in NPR1-independent pathways is largely uncharacterized. Based on my findings and recent work from the laboratory, NPR1-independence of clade I TGA factors can be summarized in four points.

First, clade I TGA factors and NPR1 can function in parallel and have additive effects. This was demonstrated by focusing on well-known NPR1-dependent responses. NPR1 is required for ETS-mediated resistance against the virulent pathogen *P. syringae* and ETI-mediated resistance against *P.s.t. AvrRpt2* and *H.a.* (Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). The single and double mutants of clade I TGA factors are compromised in resistance against these pathogens, suggesting a requirement for these transcription factors (Shearer et al., 2012; Figure 3.5 and 3.8). Furthermore, combining the *npr1-1* mutation with *tga1-1 tga4-1* resulted in increased susceptibility to these pathogens (Shearer et al., 2012; Figure 3.8), indicating that the enhanced disease susceptibility phenotype in *tga1-1 tga4-1* and *npr1-1* is additive and the function of TGA1 and TGA4 in disease resistance is at least partially independent of NPR1.

Secondly, clade I TGA factors may function in the same pathway as NPR1, but have opposite effects. This is best exemplified by pathogen- and SA-induced *PR-1* expression which is dramatically reduced in *npr1* mutants (Cao et al., 1994; Liu et al., 2005), but not in any mutants of clade I TGA factors. Instead, clade I TGA mutants showed higher expression of *PR-1* after treatment with BTH or SA, or pathogen inoculations (Figure 3.13; 3.14 and 3.15), which was abolished in the *tga1-1 tga4-1 npr1-1* triple mutant (Shearer, Wang and Fobert, unpublished data). Increased expression of *PR-1* in clade I mutants is consistent with results previously obtained in the Fobert lab (Shearer et al., 2012) and another recent study (Lindermayr et al., 2010) who analyzed *PR-1* transcript levels in plants shortly after spraying with SA. The same expression pattern of *PR-1* also appeared in double mutant after inoculation with different *P. syringae* strains (Shearer et al., 2012; Figure 3.15). In contrast, Kesarwani et al. (2007) reported no increase, and a possible reduction, in *PR-1* expression in single mutants of *tga1-1* and *tga4-1*.

These authors did not analyze *PR-1* expression shortly after treatment with SA but rather germinated and grew seedlings in the presence of the SA analog INA. Since SAR-inducing chemicals, such as BTH, SA or INA, induce *PR-1* transcript accumulation in both a temporal and dose-dependent manner (Kesarwani et al., 2007; Lawton et al., 1996), I germinated and grew seedlings under conditions similar to those reported by Kesarwani et al. (2007) except that the SA analog BTH was used instead of INA. Under these conditions, *PR-1* levels continued to be elevated in clade I TGA mutants (Figure 3.13). The reasons for these discrepencies in results between this thesis and Kesarwani et al. (2007) remain to be determined.

Thirdly, clade I TGA factors have functions that are independent of NPR1. This was ascertained by asking whether clade I TGA factors can function in defence responses which do not require NPR1. Data shown in Figure 3.6 and 3.7 indicte that clade I TGA factors contribute to MAMP-mediated resistance against *P.s.t. hrcC*$^{-}$ and NHR against *P.s.p.*, which do not required NPR1 (van Wees and Glazebrook, 2003; Zipfel et al., 2004; Figure 3.6 and 3.7). Furthermore, the *snc1* mutation has been reported to constitutively activate an NPR1-independent defence response (Li et al., 2001; Zhang et al., 2003a). The observation that this activated defense response is blocked by mutation in *TGA1* and *TGA4* (Shearer et al., 2012), suggests that clade I TGA factors are involved in an NPR1-independent pathway during immunity.

In addition to clade I TGA factors, NPR1-independent function has been reported for other clades of TGA factors. In tobacco, clade II factor TGA2.2 regulates NPR1- and NPR1-independent promoters, indicating the existence of the two different signaling networks regulated by this TGA factor (Butterbrodt et al., 2006; Thurow et al., 2005). Arabidopsis TGA2 can interact with a transcriptional regulatory protein, SCARECROW-like 14 (SCL14) to activate NPR1-independent, SA- and 2,4-D-inducbile genes which are involved in the detoxification of xenobiotics and possibly endogenous harmful metabolites (Fode et al., 2008). Furthermore, TGA2 can interact with a glutaredoxin-like protein to regulate JA/SA crosstalk which shows partial independence from NPR1 (Ndamukong et al., 2007). The clade III factor TGA3 has also been suggested to function in a partially NPR1-independent fashion based on the analysis of a *tga3 npr1* double mutant (Kesarwani et al., 2007). Thus, it appears that interactions with NPR1 are required to mediate only some of the functions of TGA factors. It is possible that NPR1-

independent functions rely on NPR1 paralogues, such as NPR3 and NPR4, which have been implicated in mediating defense responses (Zhang et al., 2006). Alternatively, they may require other transcriptional cofactors.

Lastly, previous literatures also suggest that some NPR1-dependent phenomena phenotypes did not require clade I TGA factors. For example, the *npr1* mutant is impaired in SAR against virulent pathogen, but clade I TGA factors are not required for SAR (Shearer et al., 2012). Seedlings lacking NPR1 are hypersensitive to the toxic effects of high concentrations of SA (Cao et al., 1997). In contrast, TGA1 and TGA4 are not required for the regulation of tolerance to SA (Shearer et al., 2012).

4.6 Clade I TGA factors play a role in developmental processes

Besides the role in plant immunity, clade I TGA factors are involved in several developmental processes, including leaf shape, floral transition and branch development (Figure3.22). Abnormal growth phenotypes have been observed in mutants with altered SA level (Dong, 2001; Lorrain et al., 2003; Rivas-San and Plasencia, 2011). For example, altered leaf shape was always observed in consititutive defence mutants with high SA levels and disease resistance, such as *agd2* (*aberrant growth and death2*) (Rate and Greenberg, 2001), *lsd6* (*lesions simulating diesease6*) (Weymann et al., 1995), and *sum1 sum2* (*small ubiquitin-like modifier1* and *2*) (van den Burg et al., 2010). Moreover, a late flowering phenotype was observed in SA-deficient plants, such as NahG, *eds5* and *sid2* (Martínez et al., 2004). Therefore, considering their involvement in SA pathway during immunity, I speculate that clade I TGA factors may regulate these development processes through an SA-mediated pathway. If this is the case, it is worth testing whether the *tga1-1 tga4-1* double mutant plants have altered SA levels.

The involvement of clade I TGA factors in floral transition also has been suggested in other studies. In a microarray analysis, seedlings of the *tga1-1 tga4-1* double mutant expressed higher transcript levels of *FLC,* a key repressor of flowering time (Wang and Fobert, unpublished results). TGA4 was shown to physically interact with CONSTANS (CO), a positive regulator of floral induction, and to bind to the promoter of the *FLOWERING LOCUS T* (*FT*), a direct target of CO (Song et al., 2008). In addition, like CO, the abundance of *TGA4* mRNA

oscillates diurnally in both LDs and SDs (Song et al., 2008). However, *tga1 tga4* single mutant plants do not show a late flowering phenotype (Figure 3.22d and e). This may be explained if TGA4 functions redundantly with CO. Considering the multiple pathways regulating floral transition, one possibility is that TGA4 plays a role in the signaling pathway other than the photoperiod pathway under long day conditions. In contrast to TGA4, TGA1 was not shown to interact with CO (Song et al., 2008), but is involved in regulating flowering time (Figure 3.22d and e), suggesting that TGA1 may play a role upstream or downstream of CO to regulate floral transition.

After floral transition, plants develop flowers and branches. The *tga1-1 tga4-1* double mutant, but not single mutants, displayed shorter primary inflorescences and fewer secondary inflorescences (Figure 3.22b), suggesting TGA1 and TGA4 function redundantly to regulate developmental processes at later stages after floral transition. Consistent with this idea, TGA1 and TGA4 have been shown to interact with two NPR1-like proteins, BOP1 and BOP2, which control growth asymmetry, an important aspect of patterning in leaves and flowers (Hepworth et al., 2005).

In addition, clade I TGA factors interact with the glutaredoxin (GRX)-like protein, ROXY1, which functions redundantly with its closest homolog ROXY2 to regulate anther development and microspore formation (Li et al., 2009b; Xing et al., 2005; Xing and Zachgo, 2008). GRXs are small, ubiquitous oxidoreductases that mediate the reversible reduction of intracellular disulfide bonds (Buchanan and Balmer, 2005). GRXs have been shown to be involved in many cellular processes and play an important role in the response to oxidative stress (Rouhier et al., 2004). Considering the redox regulation of clade I TGA factors in immunity, the redox regulation of clade I TGA factors through GRXs proteins may also occur during plant development.

A dual role in development and immunity is unique to clade I TGA factors within the TGA family. For example, clade II and clade III TGA factors have been reported to play a role only in immunity (Kesarwani et al., 2007; Zhang et al., 2003b), while TGA9, TGA10 and PAN are specifically involved in flower developmental processes (Chuang et al., 1999; Murmu et al., 2010; Running and Meyerowitz, 1996). Interestingly, this distinct function in the TGA family is

coincident with their ability to interact with NPR1. Clade II and clade III TGA factors constitutively interact with NPR1 (Després et al., 2000; Després et al., 2003; Zhang et al., 1999; Zhou et al., 2000), while TGA9, TGA10 and PAN have not been shown to interact with NPR1. Other than these TGA factors, the interaction between clade I TGA factors and NPR1 does not occur under non-stressed conditions, but do occur after SA treatment (Després et al., 2003).

CHAPTER 5 CONCLUSIONS AND FUTURE STUDIES

5.1 The specificity of the reported *tga1-1* and *tga4-1* phenotypes

Additional T-DNA alleles (*tga1-3* and *tga4-2*) (Figure 3.2) and TGA1 overexpressing lines in the *tga1-1 tga4-1* double mutant background (TGA1OE/*tga1x4* lines) (Figure 3.4) were identified and analyzed to confirm the specificity of the reported *tga1-1* and *tga4-1* phenotypes. Like *tga1-1* and *tga4-1*, *tga1-3* and *tga4-2* displayed enhanced disease susceptibility (EDS) to virulent pathogen *P.s.m.* ES4326 (Figure 3.5a and b), and expressed elevated *PR-1* transcripts after BTH treatment (Figure 3.12). In addition, TGA1OE/*tga1x4* lines complemented the EDS phenotype (Figure 3.5c) and partially rescued the high *PR-1* expression level in the *tga1-1 tga4-1* double mutant (Figure 3.13). These data provide greater confidence that the phenotypes associated with *tga1-1* and *tga4-1* are indeed specifically due to loss of gene function. Of note, while I was able to demonstrate complementation of the *tga1-1 tga4-1* double mutant using an overexpression transgene, Lindermayr et al. (2010) could not rescue the mutant phenotype with either wild type or mutant versions of TGA1.

5.2 Clade I TGA factors are important regulators in plant immunity

Results presented in this thesis demonstrate a broad role for clade I TGA factors in disease resistance against various pathogens. First, clade I TGA factors contribute positively to basal resistance against virulent bacterial pathogen *P.m.s.* ES4326 (Figure 3.6) and the fungal pathogen *Colletotrichum higginsianum* (Figure 3.20 and 3.21). Second, clade I TGA factors act as positive regulators in MTI-mediated disease resistance against *P.s.t. hrcC*$^-$ (Figure 3.6) and ETI-mediated disease resistance against *P.s.t. AvrRpt2* (Figure 3.8). Finally, clade I TGA factors are required for non-host resistance against *P.s.p.* 1448a (Figure 3.7). My results suggest that clade I TGA factors are common regulators which contribute to both MTI and ETI.

5.3 Clade I TGA factors are involved in cell wall-associated defence responses

The observation that loss of resistance in mutants of clade I TGA factors was not associated with reduced levels of PR gene transcripts was unexpected. However, I was able to

demonstrate that the *tga1-1 tga4-1* double mutant is impaired in well-known cell wall-associated defence responses including callose deposition (Figure 3.9), the oxidative burst (Figure 3.10), and the accumulation of extracellular PR-1 protein (Figure 3.18). Thus, I conclude that loss of resistance is attributed to defects in these, and possibly other cell wall-associated defences. These responses appear to be compromised at the post-transcriptional level, since mRNA levels for key defence genes are not affected in a negative fashion in clade I TGA factor mutants.

5.4 Clade I TGA factors are involved in defence-related secretion pathways

The success of cell wall-associated defence is dependent on effective secretion processes. The *tga1-1 tga4-1* double mutant displayed hypersensitivity to the ER stress inducer, tunicamycin (Figure 3.19), implicating clade I TGA factors in the proper functioning of the ER secretion pathway. Comparisons between different microarray datasets revealed that clade I TGA factors regulate many ER-related genes which have been shown to be regulated by well-known ER related transcription factors (Table 3.1). In addition, the *tga1-1 tga4-1* double mutant displayed enhanced disease susceptibility to the *P.s.t.* ΔCEL which lacks virulence on wild type Arabidopsis due to an inability to suppress the host vesicle-related secretion pathway (Figure 3.11). Taken together, these results suggest that clade I TGA factors function in the secretion pathway, possibly through transcriptional regulation to ER related gene expression. Furthermore, the abnormal secretion pathway in the double mutant affects the defence-related protein secretion, eventually resulting in defects in cell wall-associated defence responses and loss in disease resistance against pathogens. In summary, the contributions of clade I TGA factors in plant immunity are described in Figure 5.1.

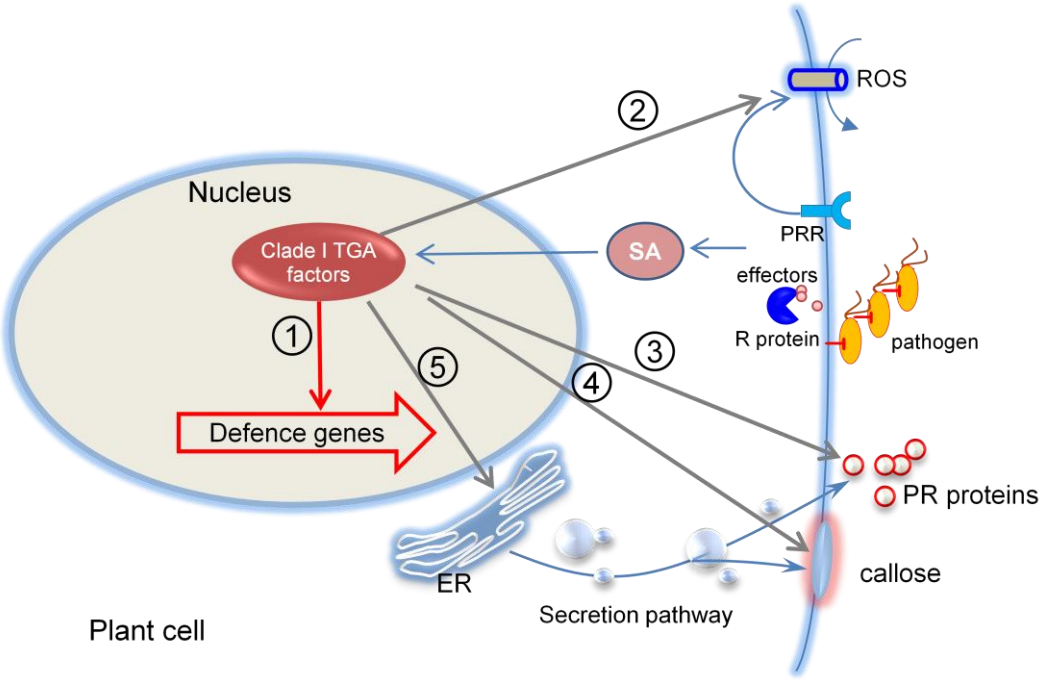


Figure 5.1 A proposed working model for clade I TGA factors during plant immunity. After pathogen recognition through PRR (pattern recognition receptors) or R proteins, SA-activated clade I TGA factors regulate defence-related genes expression (indicated with red arrow and circled 1). These clade I TGA factors-dependent genes encode proteins responsible for different defence events, including ROS burst (2), extracellular PR protein accumulation (3), callose deposition (4), and ER-related secretion (5). Some genes directly function in certain event. For example, callose synthases are directly responsible for callose synthesis. And other genes indirectly affect defence events. For example, ER components are responsible for secretion of defence-related proteins, such as PR proteins. Since clade I TGA factors affect these defence events indirectly through transcriptional regulation on related genes, the relationships between them are indicated with gray arrows.

5.5 Clade I TGA factors function NPR1-independently

Analysis of the *npr1-1* mutant and a *tga1-1 tga4-1 npr1-1* triple mutant indicate that clade I TGA factors act substantially independent of NPR1 during plant immune responses. Taken together, a working model for the relationship between clade I TGA factors and NPR1 during plant immunity is presented in Figure 5.2. After pathogen recognition, SA accumulation triggers the change in cellular oxido-reduction (ΔRedox) balance, which results in post translation modification of clade I TGA factors and NPR1 (Després et al., 2003; Mou et al., 2003). Clade I TGA factors independently regulate a group of genes, which may contribute to MTI and non-host resistance. Later on, activated NPR1 translocate into nucleus and interact with clade I TGA factors to co-regulate second group of genes (type II), which may contribute to ETS and ETI. Meanwhile NPR1 also independently regulate another group of genes (type III) to contribute to NPR1-dependent resistance.

5.6 Clade I TGA factors are involved in development processes

Besides the role in plant immunity, clade I TGA factors are involved in several developmental processes, including leaf shape, floral transition and branch development (Figure3.22). These results indicate that clade I TGA factors are uniqe among the TGA factors in having dual functions in regulating development and immunity.

5.7 Futere work

Clade I TGA factors have been shown to be required for disease resistance against three different pathogen species, the bacterial pathogen *P. syringae,* the fungal pathogen *C. higginsianum,* and the oomycete pathogen *H. arabipodsidis*. In the case of *P. syringae,* use of different isolates has shown that clade I TGA factors are broadly involved in different types of disease resistance responses, such as basal resistance, MTI, ETI and NHR. All tested pathogens are biotrophs or hemibiotrophs, and it is still worth testing the response of the mutants to necrotrophs. Of note, preliminary results revealed that the *tga1-1 tga4-1* double mutant is impaired in JA/ET mediated marker gene expression after pathogen challenge, such as *PDF1.2*, *VSP* and *LOX2* (Wang, Chatur and Fobert, unpublished result), suggesting that response to

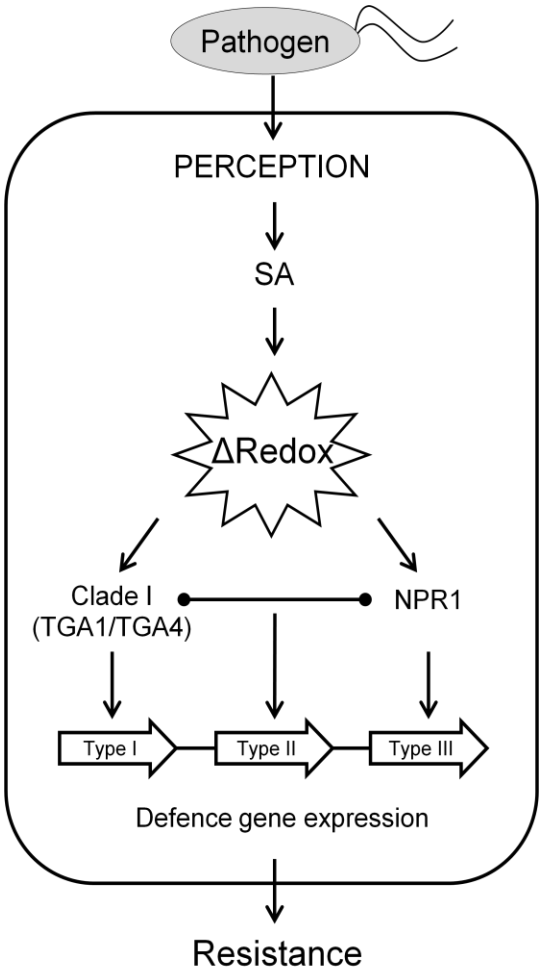


Figure 5.2 A proposed working model for the relationship between clade I TGA factors and NPR1 during plant immunity.
After pathogen recognition, SA-mediated redox changes activate clade I TGA factors and NPR1. Three types of genes are regulated by clade I TGA factors and NPR1. Expression of type I genes is only dependent on clade I TGA factors; expression of type II genes relies on clade I TGA factors and NPR1 through their interaction. Expression of type III genes is only dependent on NPR1.

necrotrophic pathogens, such as *Botrytis*, may also be compromised. This would establish clade I TGA factors among the relatively few genes that have been shown to be required for resistance to both biotrophs and necrotrophs.

The *tga1-1 tga4-1* mutant plants were impaired in cell wall associated defence responses such as callose deposition and the ROS burst. To futher study the role of clade I TGA factors in cell wall associated defense responses, it would be desirable to perform disease tests on the double mutant by using biotrophic fungal pathogen *Erysiphe.* This pathogen is an excellent experimental system to test cell wall defense responses because successful infection relies mostly on cell wall penetration.

The results obtained in this study suggest that clade I TGA factors are involved in regulating the plant secretion pathway. However, the molecular mechanisms involved are still unknown. To better understand these, several experiments should be performed in the future. First of all, it is necessary to perform microarray analysis on the double mutant and wild type under ER stress induced by TM. This would identify the transcriptome regulated by clade I TGA factors. Meanwhile, the binding ability of clade I TGA factors to ERSEs can be examined *in vitro* by EMSA (electrophoretic mobility shift assay) or *in vivo* by ChIP (chromatin immunoprecipitation). The relationship between clade I TGA factors and other ER-related bZIP transcription factors can be characterized by several experiments, such as protein interaction assays, epistasis analysis with mutants in controlling key ER stress processes, and gene expression analysis. In addition, the function of NPR1 in the ER secretion pathway should also be addressed. For example, can the nuclear translocation and interaction of NPR1 with clade I TGA factors be activated by ER stress?

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