Addendum

For this thesis independent batches of *Arabidopsis* seeds and flg22-peptide were used to achieve comparable results. Nevertheless, it has to be mentioned that the phenotype of the *tga2,5,6* mutant is inconsistent. In experiments done after finishing this thesis the *tga2,5,6* mutant did not show significant difference to the Columbia wild type regarding to flg22-inducible *FRK1* expression, callose deposition or stomata closure.

The Role of

Arabidopsis Class-II TGA Transcription Factors

in PAMP-mediated Defense Responses

Dissertation

zur Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultäten

der Georg-August-Universität zu Göttingen

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Göttingen 2010

D7

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Tag der mündlichen Prüfung:	28. April 2010

„Gib jedem Tag die Chance, der schönste deines Lebens zu werden“

(MarkTwain)

Contents

Abbreviations

aa	amino acids
A	Ampere
A	adenosine
ABA	abscisic acid
ACS	1-aminocyclopropane-1-carboxylic acid synthase
APS	ammoniumpersulfate
as-1	activating sequence 1
AT	marks a protein from *Arabidopsis thaliana*
A. thaliana	*Arabidopsis thaliana*
avr	avirulence
B. cinerea	*Botrytis cinerea*
BHA	Butylated hydroxyanisole
bp	base pairs
BSA	bovine serum albumine
bZIP	basic leucine zipper
C	cytosine
CalS	callose synthase
CaMV	cauliflower mosaic virus
cDNA	copy DNA
ChIP	chromatin immunoprecipitation
COI1	CORONATINE INSENSITIVE 1
Col-0	*Arabidopsis thaliana* ecotype Columbia
COR	coronatine
CT	threshold cycle
C-terminal	carboxy-terminal
CTR1	CONSTITUTIVE TRIPLE RESPONSE 1

CYP	cytochrome P450
Da	Dalton
ddNTPs	didesoxy nucleotides
DMSO	dimethylsulfoxide
DMTU	Dimethylthiourea
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	desoxyribonucleotides
dpi	days past infection
DPI	diphenyleneiodonium chloride
DOC	sodium deoxycholate
DTT	dithiotreitol
ECL (kit)	enhanced chemoluminescence (kit for western detection)
E. coli	*Escherichia coli*
EDS1	ENHANCED DISEASE SYMPTOMS 1
EDTA	ethylenediaminetetraacetic acid
EF-Tu	translation elongation factor Tu
EIN	ETHYLENE INSENSITIVE
EIL1	ETHYLENE INSENSITIVE3-LIKE1
elf18/elf26	Petides derived from bacterial EF-Tu
ERF	ETHYLENE RESPONSE FACTOR
ET	ethylene
et al.	et alii (and others)
ETI	effector-triggered immunity
EtOH	ethanol
ETR1	ETHYLENE RESISTANT 1
ETS	effector-triggered susceptibility
F	Farad
flg22	peptide derived from bacterial flagellin
FLS2	FLAGELLIN-SENSING 2

FRK1	FLG22-INDUCED RECEPTORKINASE 1
g	gravitation
g	gram
G	guanine
GA	gibberellic acid
GB	gradient buffer
GC	guard cell
GFP	green fluorescence protein
GRX	glutaredoxin
GST (U)	glutathione-S-transferase
GUS	glucuronidase
h	hours
H_2O_2	hydrogen peroxide
hpi	hours past infection
HPLC	high presure liquid chromatography
HR	hypersensitive response
HSP	herring sperm DNA
IAA	auxin
ICS	isochorismate synthase
IGS	4-methoxy-indol3-ylmethl glucosinolate
Ile	isoleucine
INA	2,6-dichloroisonicotinic acid
JA	jasmonic acid
JAZ	Jasmonate ZIM-domain
k	kilo (103)
L	litre
LOX	lipoxygenase
LPS	lipopolysaccharide
LRR	leucine rich repeat
µ	micro (10^{-6})

m	mili (10^{-3})
m	meter
M	molarity [mol/L]
MAMP	microbial-associated molecular patterns
MAP	Mitogen activated-protein
MAPK	MAP kinase
Me-SA	methyl salicylate
min	minutes
mRNA	messenger RNA
MS	Murahige and Skoog
n	nano (10^{-9})
NBS	nuclear binding site
NLS	nuclear localization sequence
NPR1	NON-EXPRESSOR of *PR*-GENES 1
N-terminal	amino-terminal
Ω	Ohm
OD	optical density
OE	over expressing
OGA	oligogalacturonic acid
o/n	over night
p	pico (10^{-12})
PAA	polyacrylamide
PAD4	PHYTOALEXIN DEFICIENT 4
PAGE	polyacrylamide gelelectrophoresis
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PCD	programmed cell death
PDF1.2	Plant defensin 1.2
pH	negative $\log_{10}$ of proton concentration
PMSF	phenyl-methyl-sulfonyl-fluoride

PR	*Pathogenesis related*
PRR	pattern recognition receptor
Pst	*Pseudomonas syringae pv. tomato*
PTI	PAMP-triggered immunity
pv.	pathovar
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative real time PCR
Rboh	RESPIRATORY BURST OXIDASE HOMOLOGUE
REN	restriction endonucleases
RES	reactive electrophile species
RK	receptor kinase
RLK	receptor like kinase
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
rpm	rotations per minute
RPS4	RESISTANCE TO PSEUDOMONAS SYRINGAE 4
RT	room temperature
s	second
SA	salicylic acid
SAG	SA 2-o-ß-D-glucoside
SAR	systemic acquired resistance
SB	sonic buffer
S. cerevisiae	*Saccharomyces cerevisiae (bakers yeast)*
SCL14	SCARECROW-LIKE 14
SCF	skip-cullin-F-box
SD	standard deviation
SDS	sodium dodecylsulfate
SEM	Standard error mean
SID2	SA INDUCTION-DEFICIENT 2

SOD	superoxide dismutase
T	thymine
TE	tris-EDTA buffer
TEMED	N,N,N',N'-tetraethylenediamine
TGA	*as-1* (TGACG motive) binding bZIP transcription factors
TMV	tobacco mosaic virus
Tris	tris-hydroxymethylamino methane
TTSS	type three secretion system
u	unit (quantity for enzyme activity)
U	uracil
UV	ultra violet
V	Volt
VSP2	VEGETATIVE STORAGE PROTEIN 2
v/v	volume per volume
W	Watt
Ws-0	Arabidopsis thaliana ecotype Wassilewskija
WT	wildtype
w/v	weight per volume

1 Summary

The primary immune response in plants is induced upon recognition of invariant microbial structures like flagellin, chitin, glycoproteins and lipopolysaccharides. These pathogen-associated molecular patterns (PAMPs) are recognized by specific receptors, which in turn initiate diverse downstream signaling events leading to the synthesis of the stress signaling hormone salicylic acid (SA) and to the activation of basal defense.

Transcriptional reprogramming is essential in plant defense responses. The redundant class-II TGA transcription factors TGA2, TGA5 and TGA6 are well known as important activators of SA-induced expression of *PATHOGENESIS-RELATED* (*PR*) genes and systemic acquired resistance illustrating their role in innate immunity.

In this thesis, the influence of class-II TGA factors in PAMP-induced early defense reactions was investigated using the *tga2,5,6* mutant. This mutant shows hyper-induced responses to flg22 as revealed by enhanced root growth inhibition, hyper-induced expression of early defense genes including WRKY transcription factors and increased callose deposition. Hyperinduction of flg22-induced root growth inhibition still occurs in the *tga2,5,6/sid2-2* which is deficient in flg22-induced SA synthesis. Thus, TGA factors dampen PAMP -triggered immune responses in an SA-independent manner. Inhibition of flg22-induced stomatal closure, a process that is dependent on SA, by the jasmonic acid-isoleucine (JA-Ile) mimic coronatine (COR) depends on the presence of class-II TGA factors, illustrating a role of TGA factors in coronatine-mediated processes. Likewise, wound-induced callose deposition depends on the presence of class-II TGA factors, substantiating their role in JA-induced processes. In contrast, SA-independent flg22-induced *FRK1* expression is antagonized by COR in a TGA-independent manner.

After flg22 pretreatment, the *tga2,5,6/sid2-2* quadruple mutant is more resistant to the hemibiotrophic bacterial pathogen *Pseudomonas syringae* than the *sid2-2* single mutant. However, *tga2,5,6* mutants showed higher susceptibility than the wild type, suggesting an SA-dependent positive function and an SA-independent negative function of class-II TGA factors in defense responses.

2 Introduction

2.1 Principles of innate immunity

Recognition of non-self structures and activation of defense against the attacking pathogen is known from all multi-cellular organisms. Although plants lack an adaptive immune system, they effectively deploy a series of preformed and induced defenses to combat microbial invasion. We can distinguish two branches of the inducible plant immune system. One uses transmembrane pattern recognition receptors (PRRs) that respond to slowly evolving microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs). The second acts inside the cell, using NBS-LRR proteins encoded by disease resistance (*R*) genes. They are named after their characteristic nucleotide binding site (NBS) and leucine rich repeat (LRR) domains (Nimchuk et al., 2003). The current view of the plant immune system can be represented as a 'zigzag' model (Figure 2-1; Jones and Dangl, 2006).

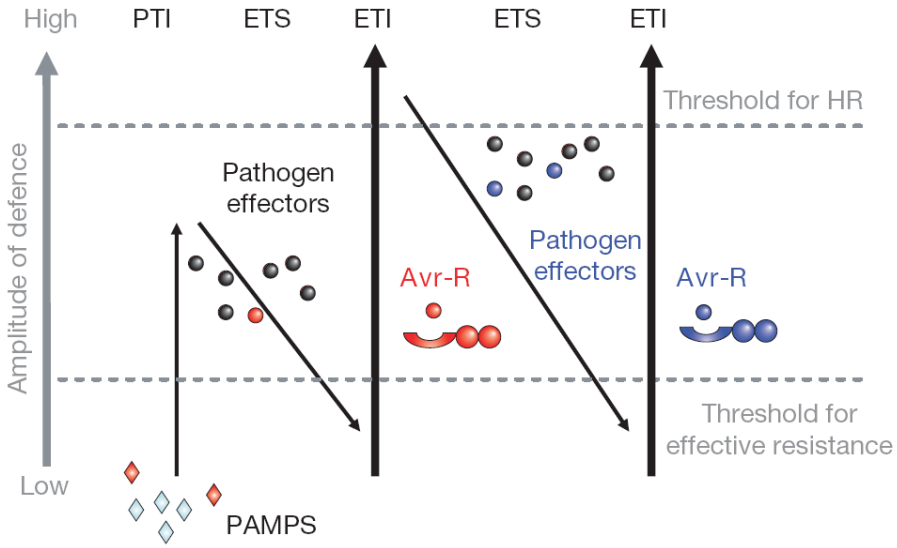

Figure 2-1 | Zigzag model of the plant immune system. Phase 1: Recognition of PAMPs resulting in PTI. Phase 2: Release of effectors by the pathogen disturbs PTI causing ETS. Phase 3: Effector recognition by R proteins leads to ETI (Jones and Dangl 2006)

PAMPs are recognized by PRRs, resulting in basal defense responses, also called PAMP-triggered immunity (PTI) that can stop further colonization by the pathogen. Adapted pathogens release effectors that contribute to pathogen virulence and disturb PTI. This leads to effector-triggered susceptibility (ETS). A given effector is specifically recognized by one of the NBS-LRR proteins, causing effector-triggered immunity (ETI). ETI is a faster and stronger PTI response, resulting in disease resistance and, mostly, a hypersensitive cell death response (HR) at the infection site.

2.1.1 Basal defense responses (PTI)

The first line of inducible defense relies on the recognition of PAMPs by PRRs of the host. PAMPs are conserved structures essential for the microorganism, widely distributed among different microbes and absent in the host. Typical PAMPs are bacterial derived lipopolysaccharides (LPS) and flagellin or fungal structures like chitin (Newman et al., 2002; Felix et al., 1999; Gust et al., 2007).

Flagellum-based motility is important for bacterial pathogenicity in plants (Felix et al., 1999). The highly conserved 22 amino acid N-terminal domain of flagellin (flg22) is an extracellular PAMP which is recognized by most plant species. Interestingly, not all PAMPs are extracellular components of the microbe. For example, the translation elongation factor Tu (EF-Tu), which also function as a PAMP, is an intracellular protein (Zipfel and Felix, 2005). In *Arabidopsis*, flg22 induces callose formation, accumulation of defense proteins like *PATHOGENESIS-RELATED 1* (*PR-1*), and strong inhibition of seedling growth (Gómez-Gómez et al., 1999). Growth inhibition was used in a mutant screen that identified a number of flg22-insensitive mutants. One of the mutated loci, *FLAGELLIN-SENSING 2* (*FLS2*), encodes a LRR receptor kinase (LRR-RK) (Gómez-Gómez and Boller, 2000). FLS2 acts together with the kinase BAK1, but FLS2 alone carries the specificity towards flg22. BAK1 is also involved in cell death control and brassinosteroid signaling in *Arabidopsis* and appears to be a general heterodimerisation partner of LRR-RLKs (Kemmerling et al., 2007).

fls2 mutants exhibit enhanced sensitivity to spray application of the bacterial phytopathogen *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000), but not to syringe infiltration into the leaf apoplast (Zipfel et al., 2004). Under natural conditions, *Pst* DC3000 enters host plants through wounds or natural openings such as stomata, and then spreads

and multiplies in intercellular spaces (Katagiri et al., 2002). Thus, the infiltration of bacteria with a syringe seems to bypass the first steps of the natural defense that rely on PAMP-induced stomatal closure (Melotto et al., 2006).

The second known receptor like kinase (RLK) in *Arabidopsis* involved in PAMP perception is EFR which recognizes the elongation factor EF-Tu, one of the most abundant and most conserved proteins of bacteria. Peptides corresponding to the acetylated N terminus of EF-Tu, called elf18 and elf26, trigger PAMP responses in *Arabidopsis* at subnanomolar concentrations. Responsiveness to elf18/elf26 was found in various *Brassicaceae* species but not in members of other plant families tested, indicating that perception of EF-Tu as a PAMP has specifically developed in *Brassicaceae* (Kunze et al., 2004).

Fragments of chitin, the main building component of fungal cell walls, are classical PAMPs (Felix et al., 1993). The rice chitin elicitor-binding protein, CEBiP, contains two LysM motifs that are involved in the recognition of chitin oligosaccharides (Kaku et al., 2006). On the basis of this finding, two groups examined *Arabidopsis* insertion mutants encoding LysM-domain-containing proteins, and found one of them to be completely nonresponsive to chitin fragments (Miya et al., 2007; Wan et al., 2008). The gene affected could be allocated to an RLK, called CERK1 (Miya et al., 2007) or LysM RLK1b (Wan et al., 2008). As it has proven difficult so far to detect chitin binding in *Arabidopsis*, it remains elusive if LysM RLK1 (CERK1) directly binds chitin or acts via cooperation with another protein (Miya et al., 2007; Wan et al., 2008).

PAMP perception initiates intracellular signaling that results in a number of responses thought to contribute to defense against the invading microbe. Depending on their first appearance these responses are separated in early and late signaling responses. Smooth transition exists between them, whereas early responses occur few minutes until 1 hour after PAMP perception mostly in a transient way, late responses are more long-lasting and starts after one hour or later.

2.1.1.1 Early signaling responses

Ion fluxes and oxidative burst. In soybean roots, seconds to minutes after PAMP treatment an alkalinization of the growth medium is detectable due to changes of the ion flux across the plasma membrane causing depolarization (Mithöfer et al., 2005). Also, in other plant species, including *Arabidopsis thaliana,* a flagellin-induced alkalinization response in suspension-cultured cells takes place (Felix et al., 1999). An influx of Ca^{2+} from the apoplast

occurs and causes a rapid increase in cytoplasmic Ca^{2+} concentrations, which might serve as second messengers to promote the opening of other membrane channels in *Arabidopsis* (Ali et al., 2007), or to activate calcium-dependent protein kinases in tobacco (Ludwig et al., 2005). Simultaneously, an oxidative burst takes place with extracellular generation of reactive oxygen species (O_2^- and its dismutation product H_2O_2) by membrane localized NADPH oxidases RESPIRATORY BURST OXIDASE HOMOLOGUE D and F (RbohD and RbohF) (Torres et al., 2002).

Localized extracellular ROS have been detected during plant-pathogen interactions (Thordal-Christensen et al., 1997), and has been found to have an antimicrobial effect on phytopathogens (Lamb and Dixon, 1997). A moderate concentration of ROS activates the cellular defense response (Levine et al., 1994). Tobacco plants inoculated with the tobacco mosaic virus (TMV) developed systemic acquired resistance (SAR) that was mediated by a burst of ROS (Lamb et al., 1997) and a rapid production of ROS could also inhibit pathogen growth by restricting pathogen penetration via cross-linking of cell wall glycoproteins (Bradley et al., 1992) or by induction of defense-related genes (Desikan et al., 2001).

Activation of MAPKs. Another early PAMP triggered response is the activation of Mitogen activated-protein (MAP) kinases (MAPK). In *Arabidopsis*, two MAPK cascades containing MAP kinase (MPK3)/MPK6 or MPK4 are activated by flg22 and other PAMPs (Mészáros et al., 2006). This activation occurs within 5 min after treatment, even in the presence of cycloheximide, which inhibits translation, indicating a direct link between receptors and the initiation of the MAPK signaling pathways. The MPK3/MPK6-cascade activates the early flg22-induced expression of the defense-related genes *WRKY29 (WRKY DNA-BINDING PROTEIN 29)* and *FRK1 (FLG22-INDUCED RECEPTORKINASE 1)* in protoplasts (Asai et al., 2002), whereas the MPK4-cascade acts negatively on the same responses (Suarez-Rodriguez et al., 2007; Petersen et al., 2000). Activation of MAPK is accompanied by changes in protein phosphorylation. It could be shown that WRKY25 and WRKY33 are substrates of MPK4 (Andreasson et al., 2005), which are likely to serve as the first WRKY proteins activated in response to PAMP-triggered MAPK signaling. Furthermore, a number of membrane proteins that display flg22-responsive phosphorylation in *Arabidopsis* cells could be identified. Interestingly, RbohD, the NADPH oxidase that mediates the oxidative burst, is among these proteins (Benschop et al., 2007; Nühse et al., 2007).

Receptor endocytosis. Within 20–40 min of treatment, flg22 was found to specifically trigger accumulation of the normally plasma membrane-resident FLS2 into intracellular

vesicles (Robatzek et al., 2006). FLS2 contains a PEST-like motif which is reported to mediate receptor endocytosis via mono-ubiquitination in yeast and mammals (Hammond et al., 2001). FLS2 mutants with a point mutation in this motif were not only defective in endocytosis but also affected in flg22 responses. These findings strongly support the notion that FLS2 endocytosis contributes to flg22 signaling (Robatzek et al., 2006).

Gene activation. Treatment of *Arabidopsis* plants with flg22 causes the induction of nearly 1000 genes and the downregulation of about 200 genes within 30 min (Zipfel et al., 2004). Other PAMPs such as elf26 and fungal chitin seem to induce a similar set of genes (Zipfel et al., 2006; Libault et al., 2007). An extensive number of the flg22-upregulated genes can be classified as being involved in signal perception, signal transduction, transcriptional regulation and potential antimicrobial action (Zipfel et al., 2004).

2.1.1.2 Late defense responses

Ethylene synthesis. Ethylene (ET) modulates developmental and defense events in the plant. The synthesis of this simple hydrocarbon gas is regulated by diverse plant hormones like auxin and cytokinin and greatly enhanced by diverse abiotic and biotic stresses. Infiltration of *Arabidopsis* leaves with flg22 or elf18 leads to a transient ET release (Kunze et al., 2004; Li et al., 2009). The flg22-activatable MPK6 is involved in ET biosynthesis by stabilization of 1-aminocyclopropane-1-carboxylic acid synthase (ACS), the rate-limiting enzyme of ethylene biosynthesis (Liu and Zhang, 2004). The *Arabidopsis* transcription factors ETHYLENE INSENSITIVE3 (EIN3) and ETHYLENE INSENSITIVE3-LIKE1 (EIL1) mediate ethylene signaling. Furthermore, the *ein3-1/eil1-1* double mutant display enhanced PAMP-related defense responses in the absence of ET integrating EIN3 and EIL1 as negative regulators in PTI defenses (Chen et al., 2009). More downstream components of the ethylene cascade are transcription factors of the Ethylene Response factor Family. One member of this family, ERF104, was recently identified to interact with MPK6. This interaction is disrupted after flg22 perception and requires ET biosynthesis and signaling. Many defense-related genes are up-regulated in ERF104 over expressing plants and it is supposable that ERF104 is a positive regulator for PAMP-triggered immunity activated by ET (Bethke et al., 2009).

Callose deposition. Deposition of callosic plugs or papillae at sites of fungal penetration or bacterial entry is a widely recognized response of host plants to microbial attack. Biosynthesis and deposition of the polyglucan callose in the extracellular space is a relative late defense-associated response. The callose synthase isoform CalS12 (Hong et al., 2001)/GSL5 (Jacobs et al., 2003)/PMR4 (Nishimura et al., 2003) plays a crucial role in inducible callose accumulation upon wounding and during biotic stress.

Flg22-induced callose requires induction of multiple pathways, including an ethylene/MYB51-dependent indole-3-glucosinolate (I3G) biosynthesis pathway and a cytochrome CYP81F2 monooxygenase pathway, essential for pathogen-induced accumulation of 4-methoxy-indol3-ylmethl glucosinolate (IGS). Glucosinolates are secondary metabolites with potential antimicrobial effect. Mutants involved in both pathways failed in PAMP-triggered callose deposition (Clay et al., 2009).

Stomatal closure. To gain access to the intercellular spaces and internal leaf tissues, microbial pathogens must cross the cuticle and epidermis. Bacteria and many fungi cannot directly penetrate the leaf epidermis and must enter leaf tissues through natural openings or wounds on the leaf surface. Stomata are small pores on the surface of plant leaves that are composed of a pair of specialized epidermal cells referred to as guard cells. Through stomata, plants conduct gas exchange necessary for photosynthesis and control water loss by regulating the width of the pore. Plants regulate the opening and closing of these pores through changes in turgor pressure within the guard cells. The plant hormone abscisic acid (ABA) plays a major role in guard cell signaling leading to stomatal closure. Stomatal movements are influenced by numerous environmental cues including light intensity, air humidity, CO_2 concentration and drought stress (Underwood et al., 2007). In addition to abiotic stresses, stomata have also been found to respond to various microbe-derived compounds. The fungal elicitors oligogalacturonic acid (OGA) and chitosan both induce stomatal closure in tomato (Lee et al., 1999).

More recently, *Arabidopsis* stomata were found to respond to the presence of living bacteria or PAMPs (Figure 2-2). Perception of flg22 induces closure of stomata in epidermal peels of *Arabidopsis* leaves in a FLS2 dependent manner (Melotto et al., 2006), demonstrating that recognition of bacterial PAMPs through PRRs leads to stomatal closure. This suggests a potential role for stomata in the plant innate immune response against bacteria. Consistently, stomata respond to suspensions of *Pseudomonas syringae* and the human pathogen *Escherichia coli* (Melotto et al., 2006).

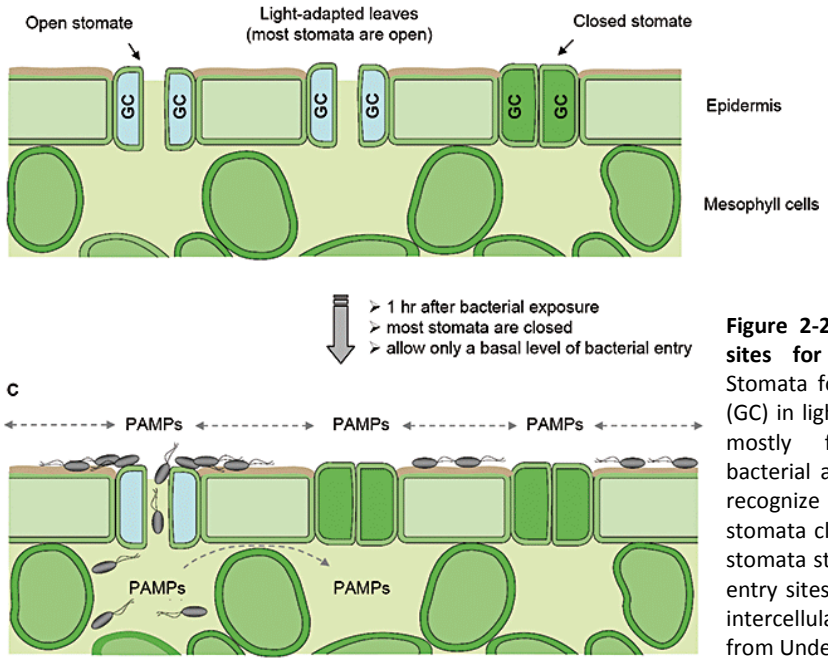

Figure 2-2 | Stomata as entry sites for bacterial invasion. Stomata formed by guard cells (GC) in light adapted leaves are mostly fully open. Upon bacterial attack the guard cells recognize PAMPs and many stomata close within 1 h. Some stomata stay open and serve as entry sites for bacteria into the intercellular space (adapted from Underwood et al. 2007)

2.1.2 *R*-gene-mediated resistance (ETI)

Plants have developed a defense strategy against bacterial derived effectors based on disease *R*-genes. *R*-genes are only effective if a specific avirulence (*avr*) gene is present in the pathogen. This gene-for-gene hypothesis was introduced by Flor in the 1940s, and dozens of *R*-*avr* -gene combinations have since been characterized (Dangl and Jones, 2001). R-protein-mediated defenses include the hypersensitive response (HR), a rapidly induced programmed cell death. The cell death is localized near the site of recognition, and kills both the plant cell and the attacking pathogen with the aim of limiting pathogen spread (Lam, 2004).

Most R-proteins are intracellular located and have a NBS-LRR, with either a coiled-coil domain or a Toll-interleukin-1-like domain at the N terminus. As most bacterial *avr* genes encode cytoplasmic type III effectors, it has been postulated that R-proteins function as intracellular receptors that directly interact with type III effectors after they are released into the host cell. Surprisingly, a direct interaction between an R-protein and a type III effector has been identified only in few cases (Tang et al., 1996; Deslandes et al., 2003).

2.2 Bacterial weapons to counter-act PTI

To successfully colonize plants, *P. syringae* and other plant pathogenic bacteria have evolved a variety of virulence factors to subvert host defenses and to obtain nutrients. The type three secretion system (TTSS) is structurally related to the bacterial flagellum and forms a pilus to inject a large number of virulence effector proteins into the host cell (Abramovitch et al., 2006). Over thirty effectors are secreted by *Pst*. Some of these effectors have diverse enzymatic activities but most effectors have no sequence similarity to known proteins and their functions have remained unknown. Two secreted effectors, AvrPto and AvrPtoB physically interact with the kinase domains of FLS2, EFR or BAK1 (Xiang et al., 2008; Göhre et al., 2008; Shan et al., 2008). AvrPtoB contains a E3 ubiquitin ligase initiating the degradation of PRRs (Göhre et al., 2008; Gimenez-Ibanez et al., 2009). This observation explains that this effector suppresses a variety of responses of PTI, including callose deposition, activation of kinase cascades and expression of PAMP-responsive genes (Hauck et al., 2003; He et al., 2006).

2.3 Phytohormones coordinating plant defense responses

The regulation of the defense network which translates the pathogen-induced early signaling events into the activation of long lasting defense responses depends on the action of phytohormones. The importance of salicylic acid (SA) (Loake and Grant, 2007), jasmonates (JAs) (Katsir et al., 2008) and ET (van Loon, Geraats, et al., 2006) as signals in the regulation of the plant's immune response is well known. Also other phytohormones like abscisic acid (ABA) (Mauch-Mani and Mauch, 2005), auxins (Navarro et al., 2006) and cytokinins (Walters and McRoberts, 2006) are involved, but their significance is less well understood. Pathogen infection stimulates the plant to synthesize one or more hormonal signals depending on the type of attacker (De Vos et al., 2005). According to their lifestyles, plant pathogens are generally divided into biotrophs and necrotrophs. Biotrophs take nutrients from living host tissues without disrupting it, whereas necrotrophs first destroy host cells, often through the production of phytotoxins, after which they feed on the contents. Many plant pathogens, like *Pseudomonas syringae* display both lifestyles, depending on the stage of their life cycle, and are called hemibiotrophs. To examine the

role of the different phytohormones in plant immune response, different mutants and transgenic lines of *Arabidopsis* and tobacco impaired in hormone biosynthesis, recognition or signaling were generated. In general, biotrophic pathogens are generally sensitive to SA-induced defense responses, whereas pathogens with a necrotrophic lifestyle are opposable by defenses that are controlled by JAs and ET (Glazebrook, 2005). The wound response, which is effective against insect herbivores, is also regulated by the JA signaling pathway (León et al., 2001).

Jasmonic acid. The oxylipin JA is produced via the oxidative metabolism of polyunsaturated fatty acids by enzymes of the octadecanoid pathway. The COI1 (CORONATINE INSENSITIVE 1) protein is required for all known JA-dependent signaling events and serves as a receptor of the JA conjugate JA-isoleucine (JA-Ile) (Katsir et al., 2008). *coi1-1* mutants exhibit increased susceptibility to necrotrophic fungi and herbivores and the induction of JA-responsive marker genes like *LIPOXYGENASE2* (*LOX2*) and *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) or *PDF1.2*, a plant defensin, is completely abolished (Reymond et al., 2004; Stintzi et al., 2001). The COI1 protein is an E3-ligase that forms the multi protein complex SCF^{COI1} (skip-cullin-F-box) to target proteins of the JAZ (Jasmonate ZIM-domain) family for ubiquitination and subsequent degradation by the 26S proteasome (Katsir et al., 2008). The JAZ proteins analyzed so far are negative regulators of the JA response and bind to activators of JA-dependent genes. Degradation of these repressors is required for the activation of JA responses (Fonseca et al., 2009).

Ethylene. Beside its function in basal defense, ET signaling also contributes to resistance against necrotrophic pathogens (Thomma et al., 1999). Central regulators of this pathway are the ET receptor ETR1 (ETHYLENE RESISTANT 1), the suppressor CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1), the membrane-located positive regulator EIN2 (ETHYLENE INSENSITIVE 2) and the transcription factors EIN3 and EIL1. CTR1 suppresses ET signaling in the absence of the hormone and EIN3 is targeted constantly for degradation through the 26S proteasome by the two EIN3-binding F-box proteins EBF1 and EBF2. CTR1 is inactivated upon binding of ET to the ETR1 receptor, which subsequently leads to a MAP-kinase (mitogen-activated protein)-mediated phosphorylation cascade and a stabilization of EIN3 (Guo and Ecker, 2003). EIN3 and EIL1 activate *ETHYLENE RESPONSE FACTOR1* (*ERF1*) and other primary responsive genes containing EIN3-binding sites in their promoter regions (Yoo et al., 2009).

Salicylic acid. The SA pathway is crucial for basal and *R*-gene mediated resistance against biotrophic pathogens (Tsuda et al., 2008). After pathogen attack, SA is synthesized from chorismate, derived from the shikimate pathway, by the enzyme isochorismate synthase (ICS1) localized in the stroma of chloroplasts (Wildermuth et al., 2001). A mutation of the *ICS1* gene (*sid2;* SA INDUCTION-DEFICIENT 2) causes a reduction of SA accumulation after infection to only 5-10% of the wild-type level and a decrease in PTI and ETI (Wildermuth et al., 2001). In case of resistance mediated via the *R*-gene *RPS4* (RESISTANCE TO PSEUDOMONAS SYRINGAE 4), key regulatory proteins upstream of ICS1 are the two lipase-like proteins EDS1 (ENHANCED DISEASE SYMPTOMS 1) and PAD4 (PHYTOALEXIN DEFICIENT 4), which function in a positive feedback loop to increase SA biosynthesis and their own expression (Feys et al., 2001). Treatment of *Arabidopsis* plants with the SA analog 2,6-dichloroisonicotinic acid (INA) produces a biphasic change of cellular redox potential. First a pro-oxidative effect and then an antioxidant effect of INA takes place (Mou et al., 2003). These changes of the redox status lead to the activation of different sets of target genes. Early SA-responsive genes play a role in detoxifying oxidative stress, like glutathione-S-transferases or glucosyltransferases (Blanco et al., 2009). Later, expression of *pathogenesis related* (*PR*)-genes like *PATHOGENESIS RELATED-1* (*PR-1*) takes place (Lebel et al., 1998; van Loon, Rep, et al., 2006).

SA is an electrophilic compound and high concentrations can cause harmful effects due to xenobiotic stress. To avoid this, plants are able to form the bioinactive SA conjugate SA 2-o-ß-D-glucoside (SAG), which can be stored in the vacuole and serves as a hydrolysable source for SA. In *Arabidopsis*, the enzymes responsible for this conversion are the UDP-glucosyltransferases UGT74F1 and UGT74F2 (Dean and Delaney, 2008).

The establishment of systemic acquired resistance (SAR) generates an increased protection against a wide range of pathogens not only at the local site of infection, but also in the whole host plant to prevent a subsequent invasion of the pathogen. SAR is usually described as a phenomenon whereas localized inoculation with a pathogen renders a plant more resistant to subsequent pathogen infection. Localized application of PAMPs or the SA analogon INA also causes local and systemic induction of endogenous SA levels and defense gene expression, similar to SAR-like disease resistance (Mishina and Zeier, 2007; Zhang et al., 2003). SAR is associated with an activation of signal transduction pathways, the accumulation of PR proteins and increase of SA in local and systemic tissues (Uknes et al., 1992; Van Loon, 1997; Durrant and Dong, 2004). This enhanced capacity to mobilize infection-induced cellular defense responses is called “priming” (Conrath et al., 2002).

Plants which are impaired in SA signaling are not able to develop SAR and do not show *PR* gene activation upon pathogen infection, which indicates that SA is a necessary signal molecule for the initiation of SAR (Mauch-Mani and Metraux, 1998; Durrant et al., 2004). At least in *Arabidopsis*, the mobile signal that travels from the site of infection through the plant to develop SAR in distal tissues has remained unknown. Reciprocal grafting experiments with *NahG* rootstocks and wildtypic scions in tobacco demonstrate that SA is not important for the generation of the mobile signal (Vernooij et al., 1994). More recently, it was shown that the SA derivative methyl salicylate (MeSA) acts as a long-distance mobile signal for SAR in tobacco (Park et al., 2007). Other studies suggest a lipid-based molecule to be one of the key mobile signals in SAR. The *dir1* (DEFECTIVE IN INDUCED RESISTANCE 1) mutant carries a mutation in a gene similar to lipid transfer proteins (LTPs) and displays normal local resistance to pathogens, while the generation of SAR and induction of *PR*-genes in systemic tissues fails (Maldonado et al., 2002).

Moreover, it could be shown that an interaction between PAMP- and SA-signaling exists. PAMPS induce SA accumulation in a SID2-dependent manner and expression profiling discovered that some PAMP-induced genes are SA independent, whereas other genes become SA dependent at later time points. Furthermore, SA signaling is required for PAMP-triggered resistance to *Pseudomonas syringae*, but a part of the response is SA-independent (Tsuda et al., 2008). SA is also involved in other basal defense responses. Defense through stomatal closure (Melotto et al., 2006) and some branches of PAMP-triggered callose deposition are SA-dependent (Clay et al., 2009; Adams-Phillips et al., 2010).

So far, the receptor for SA could not be identified, still many compounds downstream in the signaling cascade are known, as NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) and TGA transcription factors, which function as key regulators of SAR and are necessary for activation of *PR* gene expression (Zhang et al., 2003).

2.3.1 Hormonal networks to finetune plant defense

Due to the different kinds of defense responses required against biotrophic, hemibiotrophic and necrotrophic pathogens as well as herbivorous insects, a tightly regulated fine tuning of the hormonal pathways is essential for the fitness of the plant. Activation of the SA signaling cascade causes a negative cross-talk on JA signaling, as revealed by a lack of *PDF1.2* induction after combined exogenous application of SA and JA (Kunkel and Brooks, 2002). This negative regulation depends on NPR1 (Spoel et al., 2003), but the NPR1

dependency is lost when ET signaling modulates the SA/JA cross-talk (Leon-Reyes et al., 2009).

2.3.2 Suppression of host defense by the bacterial toxin coronatine

Pathogens exploit the complex interplay between hormonal signaling pathways and evolved strategies to manipulate the immune response of the plant to increase pathogenicity. The phytotoxin coronatine (COR) is produced by different pathovars of *P. syringae* (Bender et al., 1999). COR is required for full virulence on several host species. COR– mutants of *Pst* DC3000 do not grow to wild-type levels or induce typical disease symptoms on either dip-inoculated *Arabidopsis thaliana* or tomato (Brooks et al., 2004; Penaloza-Vazquez et al., 2000). Recently, it was shown that COR binds to COI1 (Yan et al., 2009) and consequently acts as a molecular mimic of JA-Ile with a high biological activity to activate JA signaling (Feys et al., 1994; Weiler et al., 1994; Bender et al., 1999) and finally suppresses SA-dependent defenses, thereby promoting susceptibility of the plant to this pathogen (Brooks et al., 2005; Uppalapati et al., 2007).

In addition to the described observations, COR is able to suppress innate immune responses. COR was found to overcome PAMP- and bacteria-induced stomatal closure downstream of ABA in a COI1-dependent way (Melotto et al., 2006). Interestingly, a COR-defective mutant could not cause disease when inoculated onto the leaf surface but caused wild-type infection if infiltrated directly into the apoplast, bypassing the epidermis (Mittal and Davis, 1995). These results suggest that suppression of stomatal defense is the primary function of COR in local leaves and that the COR-mediated suppression of stomatal defense is critical for *Pst* DC3000 infection of host plants (Figure 2-3).

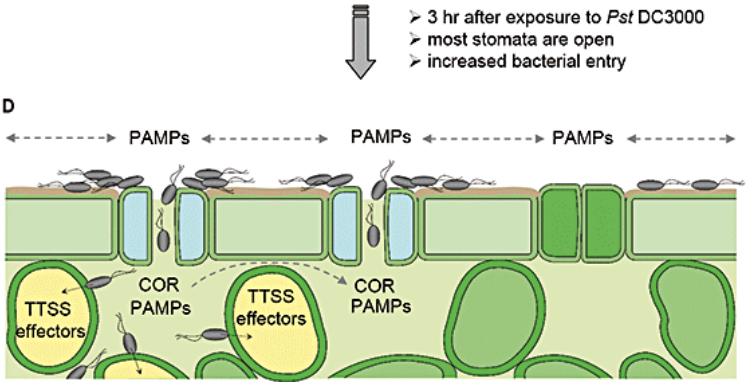

Figure 2-3 | Virulent *Pst* DC3000 produce coronatine (COR) to overcome PAMP induced stomatal closure. 3h after infection bacteria produce COR to re-open closed stomata, thereby increasing the number of entry sites for bacterial invasion. In addition, bacteria inject TTSS effectors to suppress host defenses (adapted from Underwood et al. 2007)

2.4 TGA transcription factors as regulators of defense responses

As mentioned above, the current model of the SA signal transduction chain needs NPR1 and TGA transcription factors for activation of *PR*-gene expression. NPR1 does not contain any known DNA interaction domains. Several yeast two hybrid screens identified TGA transcription factors, a subgroup of the large family of *bZIP* transcription factors, as interaction partners of NPR1 (Jakoby et al., 2002). The name of the TGA family is derived from their ability to bind TGACG motifs in regulatory promoter regions (Katagiri et al., 1989). They were first characterized in tobacco by their ability to bind the *activating sequence 1* (*as-1*) element of the CaMV 35S promoter, a 20-bp element containing two TGACG boxes, and to promote transcription (Katagiri et al., 1989). The consensus *as-1* element is TGACGTCAg---TGACGTCA, where the central bases are not conserved and the spacing between the palindromes in late-regulated promoters such as *PR-1* is larger and more variable (Krawczyk et al., 2002). In vitro, the TGACG motif is sufficient for TGA factor binding (Lam et al., 1989).

In *Arabidopsis*, ten closely related group members of TGA factors exist (Figure 2-4). TGA2, TGA3, TGA5, TGA6 and TGA7 are able to interact with NPR1 (Després et al., 2000; Kim and Delaney, 2002; Zhang et al., 2003; Zhou et al., 2000), whereas TGA1 and TGA4 show only weak interaction in yeast unless two cystein residues are reduced (Després et al., 2003). Based on sequence similarities, the TGA factors are grouped into different classes (Miao et al., 1994). TGA1 and TGA4 form class I, TGA2, TGA5 and TGA6 build class II, and TGA3 and TGA7 represent class III.

TGA4 together with the second class I member TGA1 are sensors for changes in the cellular redox state. Although both proteins show no interaction with NPR1 in yeast, the interaction takes place *in planta* after SA induction, linking the two TGA factors to defense gene expression and resistance. In both proteins, two conserved cysteine residues (Cys260 and Cys266) form a disulfide bond under non-induced conditions. If the redox potential changes to a more reducing environment, the disulfide bond dissociates and the TGA factors are able to interact with NPR1 (Després et al., 2003). Analysis of the *tga1* and *tga4* double mutants revealed a partially redundant role in regulation of basal resistance, whereas the single mutants have only moderate effects on *PR*-gene expression (Kesarwani et al., 2007).

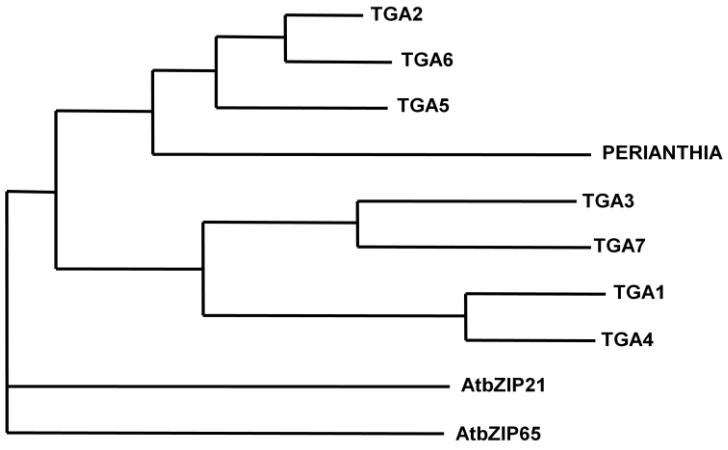

Figure 2-4 | Phylogenetic tree of the TGA transcription factor family of *Arabidopsis thaliana*.

The class-II TGA transcription factors are redundant regulators of *PR*-gene expression and SAR. *tga2 tga5 tga6* triple mutants (*tga2,5,6*) show a similar phenotype like *npr1-1* regarding a compromised SAR and an increased sensitivity to SA (Zhang et al., 2003) but are not impaired in basal defense (Kesarwani et al., 2007). Furthermore, the basal expression of *PR*-genes is increased, demonstrating a dual function for TGA factors as transcriptional activators and repressors (Kesarwani et al., 2007). Conflicting data have been reported about their role for induction of *PR-1*, ranging from a complete loss of *PR-1* induction (Zhang et al., 2003) to delayed induction kinetics upon treatment with SA or INA (Blanco et al., 2009). Class-II TGA transcription factors are also involved in NPR1-independent detoxification processes induced by oxylipins, formed in consequence of accumulation of xenobiotic compounds or ROS (Fode et al., 2008; Mueller et al., 2008). The NPR1-independent induction of detoxification related stress responses uses the GRAS protein SCL14 (SCARECROW-LIKE 14) as co-regulator of TGA2, TGA5 and TGA6 (Fode et al., 2008).

Recently it could be shown that TGA2, TGA5 and TGA6 are essential for the activation of JA- and ET-dependent defense mechanisms which counteract necrotrophic pathogens. In this case, the TGA factors act antagonistically to JIN1/AtMYC2 which functions as a negative regulator on JA/ET signaling and is necessary to install SA-mediated suppression of JA/ET-induced defense responses (Zander et al., 2009).

Further proteins known to interact with members of class-II TGAs belong to the glutaredoxin (GRX) family. GRXs catalyze thiol disulfide reductions and therefore are implicated in regulatory processes regarding the redox state of the cell (Lemaire, 2004). The

expression of *GRX480* is induced by SA with induction requiring a subset of TGA factors and NPR1 (Ndamukong et al. 2007).

The *tga3-1* mutant of subclass III TGA factor TGA3 was found to be defective in basal defense. Additionally, the increased *PR-1* background expression observed in the *tga2,5,6* mutant is normalized in the *tga2,3,5,6* quadruple mutant, once more displaying the complex interconnection of the TGA family members (Kesarwani et al., 2007). The function of the TGA factors *AtbZIP21* and *AtbZIP65* is still unclear.

2.5 WRKY transcription factors: key players of plant immunity

The 74 members of the WRKY family represent plant-specific transcription factors most of them involved in SAR or basal defense. Common to these proteins is a DNA-binding region with the conserved sequence motif WRKYGQK neighboring to a zinc-finger motif. WRKY factors have been implicated in the regulation of many plant processes like pathogen defense, wound response and senescence (Eulgem et al., 2000; Robatzek and Somssich, 2002; Dong et al., 2003). The regulation of gene expression by WRKY factors occurs with recognition of W-box sequences (C/T)TGAC(T/C) in the promoters of target genes (Eulgem et al., 2000).

In *Arabidopsis*, 49 out of 72 tested WRKY genes respond to bacterial infection or SA treatment. A considerable enrichment of W boxes in the promoter regions of these defense regulated WRKY genes could be examined. These results strongly suggest that WRKY proteins themselves play an important role in the differential regulation of their own expression during the activation of plant defense responses (Dong et al., 2003).

On the one hand, some of the pathogen-induced WRKY proteins function as important positive regulators of plant disease resistance. As mentioned above, *WRKY22* and *WRKY29* are induced by a MAPK pathway that confers resistance to both bacterial and fungal pathogens and expression of *WRKY29* in transiently transformed leaves led to reduced disease symptoms (Asai et al., 2002; Andreasson et al., 2005). Furthermore, mutations of *WRKY70* enhances plant susceptibility to both biotrophic and necrotrophic pathogens (Li et al., 2004; AbuQamar et al., 2006; Li et al., 2006).

On the other hand, many WRKY proteins can function as negative regulators of plant defense. For example, mutations of *Arabidopsis WRKY7*, *WRKY11*, *WRKY17* and *WRKY48*

enhance basal plant resistance to virulent *P. syringae* strains (Journot-Catalino et al., 2006; Kim et al., 2006). Likewise, mutations of *Arabidopsis WRKY25* enhance tolerance to *P. syringae* and overexpression of either *WRKY25* or closely related *WRKY33* enhances susceptibility to the bacterial pathogen and suppresses SA-regulated *PR1* gene expression (Zheng et al., 2006, 2007). The structurally related WRKY18, WRKY40, and WRKY60 also function partially redundant as negative regulators in plant resistance against *P. syringae* (Xu et al., 2006) and the fungal biotrophic pathogen *Golovinomyces orontii* (Shen et al., 2007). Among these, *WRKY18* was described as direct target of NPR1 and TGA transcription factors (Wang et al., 2006).

2.6 Aim of the study

PTI is a very important mechanism to attack pathogens and assures the survival of plants living in a stressful environment. To understand the signal transduction-pathways behind this defense reaction, it is necessary to identify the involved components and their function. It could be shown that PAMPs are able to induce an SAR-like defense with activation of *PR* genes in local and systemic tissue indicating an interaction between PAMP-triggered and SA-mediated signaling (Mishina et al., 2007). PAMP-triggered resistance is partially SA-dependent and microarray analysis discovered a group of genes induced by PAMPs in a SID2-dependent manner (Tsuda et al., 2008).

Class-II TGA transcription factors play important roles to regulate different branches of defense responses. In several studies, it could be shown that they are included in SA-dependent (Zhang et al., 2003; Kesarwani et al., 2007) as well as JA/ET dependent pathways (Zander et al., 2009). As revealed by mutant analysis, class-II TGA factors together with NPR1 are also important for the SA–JA crosstalk (Spoel et al., 2003; Ndamukong et al., 2007). So far, a function of TGA factors in PAMP signaling is unknown.

In a root growth assay it could be shown that the *tga2,5,6* mutant is more sensitive to growth inhibition induced by flg22. Based on these observations, the aim of this study was to investigate a possible involvement of class-II TGA factors in basal defense responses based on PAMP-signaling. For this work, the *tga2,5,6* mutant, impaired in all three members of the class-II family, should be investigated in different assays to test basal defense responses. Different assays should be used to analyze root growth inhibition, ROS-burst, gene expression, callose deposition and stomata closure. Beside flg22, other PAMPs

should be used to find out, if the phenoptype of the *tga2,5,6* mutant is flg22 specific. Moreover, treatment with SA or the JA mimic coronatine should elucidate, if phytohormones are involved.

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

Device	Model	Source
Autoclave	3870 ELV	Tuttnauer
Automatic pipettes	pipetman	Gilson
Balance	SPO52; SAC62; 1207MP2	Scaltec; Satorius
Bio imager	BAS 1000	Fuji
Blotting device		University of Göttingen
Chambers for gel electrophoreses		University of Göttingen
Chemiluminescence plate reader	Tecan infinite 200	Tecan
Cooling centrifuge	Sorvall RC 5B Plus	DuPont
Cooling centrifuge with overhung rotor	Rotina 35A	Hettich
Digital camera	Powershot A510	Canon
Gel documentation device		MWG Biotech
Heat block		Boekel Scientific
Heated stirrer	RCT basic	IKA Labortechnik
Heated shaker	Thermomixer 5436	Eppendorf
homogenizer	Miccra-D8	ART Labortechnik
Ice machine	Af20	Scotsman
Locker for incubations		WTC binder; Memmert

Device	Model	Source
Microscope	DM 5000B + CTR 5000	Leica
PCR cycler	iCycler	BioRad
pH-Meter	HI 9321	Hanna Instruments
Photometer	Unikon 720 LC	Kontron
Photometer for microtiter plates	MRX Dynex Plate Reader	Dynex
Radiation monitor	Contamat	Eberline
Realtime PCR cycler	MyiQ + iCycler	BioRad
RNA-/DNA-Calculator	GeneQuant II	Pharmacia
RNA-/DNA-Calculator (Spectrophotmeter)	NanoVue	GE Healthcare
Rotary mixer	IntelliMixer RM-2L	ELMI
Scanner	GT 9600	Epson
Sequencer	ABI PRISM 3100	Perkin-Elmer
Shaker	ST5M	Zipperer GmbH
Sterile bench	Microflow Laminar	Nunc
Sterile bench	Microflow Biohazard	Nunc
Table-top micro centrifuge	Biofuge pico	Heraeus Christ
Table-top micro centrifuge, cooled	Micro 200 R	Hettich
Ultrasonic homogenisator	Labsonic M	Satorius
UV transilluminator	FLX-20 M	Vilber Lourmat
Water deionization device	Option 4, Maxima	ELGA
Vacuum pump	MD-1C	Vaccuumbrand
Vortex	L46	Labinco BV, Niederlande
Water bath	1086	GFL

3.1.2 Consumables

Product	Source
Filter paper Miracloth	Calbiochem
Flow paper 3MM	Whatman
96well microtiter- plates, white, flat- bottom	Greiner bio-one
Microtiter plates	Roth
nylon membrane Hybond N+	Amersham
Para-film M	American National Can™
Plastics one-way material	Biozym; Eppendorf; Greiner; Roth; Sarstedt
pump aerosol can	Roth
PVDF membrane Immobilon™-P	Millipore
X-ray film Cronex 5	Agfa, Belgium
Ultra clear adhesive tape	TESA

3.1.3 Chemicals

Chemical	Source
30 % (w/v) Acrylamide: N,N´-Methylenebisacrylamide (37,5:1)	Roth
Agarose SeaKem LE	Biozym
Aniline blue diammonium salt	Sigma
Ampicillin	AGS
APS (Ammonium persulfate)	Biometra
Bradford-Reagent	Roth
Bromophenol blue	Roth
BSA	Serva

Chemical	Source
BHA (Butylated hydroxyanisole)	Sigma
Chitin from crab shells	Sigma-Aldrich
Chitosan from crab shells	Sigma-Aldrich
Coomassie brilliant blue G-250	BioRad
[α-^{32}P]dATP; 800 Ci/mmol	Hartmann Analytic
Dimethyl sulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
DMTU (Dimethylthiourea)	Sigma
dNTPs	MBI
DPI	diphenyleneiodonium chloride
Ethylene diaminetetraacetate (EDTA)	AppliChem
Ethidiumbromide	Roth
Fat-free milk powder	Glücksklee
Fluoresceine	BioRad
Gelrite	Duchefa
Glycogen G-8751	Sigma-Aldrich
HEPES	Roth
Horse radish peroxidase	Sigma
Hydrogen peroxide	Roth
Herring sperm DNA (HSP)	Sigma
Kanamycine	Sigma
Luminol	Sigma
β-Mercaptoethanol	Roth
MES	Roth
Murashige and Skoog (MS) medium	Duchefa
Orange G	Sigma
Percoll	Sigma

Chemical	Source
Phenol	Sigma
Phenylmethane sulfonylchloride (PMSF)	Fluka
PIPES	Roth
Ponceau S	Sigma
Protein A agarose beads	Sigma
Proteose Peptone No. 3	BD
Rifampicine	Duchefa
X-ray developer LX24	Kodak
X-ray fixer AL4	Kodak
Salicylic acid (SA)	Merck
Select Agar	Life Technologies
Select Yeast Extract	GIBCO BRL
Sucrose	Roth
SYBR Green I	Cambrex
TEMED	Roth
Triton X-100	Roth
Trypton	Oxoid
Tween20	Roth

Further standard chemicals were purchased from the following companies: Boehringer, Fluka, Merck, Serva, Sigma and Roth

3.1.4 Enzymes and Kits

Enzyme/Kit	Source
BCA Protein Assay Kit	Thermo Scientific
BigDye™ Terminator Cycle Sequencing Ready Reaction Kit v.3.1	Perkin-Elmer Corporation
BioTaq DNA polymerase	Bioline
desoxyribonuclease I (DNaseI) RNase-free	MBI Fermentas
Enhanced Chemiluminescence Plus™ Kit (ECL+)	GE Healthcare
HiDi-Mix	ABI PRISM
Image-iT LIVE Green Reactive Oxigen Species Detection Kit	Molecular Probes
Immolase DNA polymerase	Bioline
iProof high fidelity DNA polymerase	BioRad
Klenow DNA polymerase exo$^-$	MBI Fermentas
Megaprime DNA labeling system	Amersham
Nucleo Spin® Extract II	Macherey-Nagel
Nucleo Spin® Plasmid	Macherey-Nagel
Reverse transcriptase H-	MBI Fermentas
Restriction enzymes	MBI Fermentas, New England Biolabs
RNase A (DNase-free)	Qiagen
RNase inhibitor	MBI Fermentas
T4 DNA-ligase	MBI Fermentas
T4 DNA-polymerase	MBI Fermentas

3.1.5 Standards

Standard	Source
GeneRuler DNA Ladder Mix	MBI Fermentas
Prestained Protein Ladder	MBI Fermentas

3.1.6 Antibodies

Antibody	Specificity	Properties	Reference
αTGA2/5 (serum) (SA 4364)	TGA2 and TGA5 C-terminal region including the zipper domain	polyclonal from rabbit (final bleeding) (1:1000)	(Fode et al., 2008)

3.1.7 Peptides

The following peptides were synthesized by EZBiolab, Westfield USA:

Peptide	aa sequence	Purity	Reference
elf18	Ac-SKEKFERTKPHVNVGTIG	95%	(Kunze et al., 2004)
flg22	QRLSTGSRINSAKDDAAGLQIA	85%	(Felix et al., 1999)

3.1.8 Nucleotides

3.1.8.1 Plasmids

Plasmid	Description	Reference
pSK-T	Cloning and sequenicing vector; pBluescriptII SK (Stratagene, Cedar Cree, Texas) was restricted with *Eco*RV and treated with terminal transferase in presence of ddTTP; *lac*Zα, *amp*^{r}	Guido Kriete, unpublished
pSK-T_QPR1	pSK-T vector with amplificat derived from PCR with Quantitect PR-1 primer assay	this thesis

3.1.8.2 Primers and Oligos

Primers were synthesized by Invitrogen. QuantiTect Primer Assays from Qiagen contain both, forward and reverse primer. They are indicated as "QPA" and are described on: http://www1.qiagen.com/Products/Pcr/QuantiTect/PrimerAssays.aspx
False QuantiTect primers (FQ) are designed on basis of sequence analysis of the PCR fragment after amplification with the original primer assays. PCR-product was ligated in vector pSK-T and DNA sequencing was done with UNI and REV primers. The design of PCR-primers was done with respect to avoid secondary structures and that an annealing temperature of 55°C was obtained. These primers were diluted and mixed to 4 µM stock solution containing forward and reverse primer.

Gene	Primer	Sequence 5´→3´	Source
	qRT-PCR		
FRK1 (At2g19190)	QT00752444	QPA	Qiagen
ICS1 (At1g74710)	QT00893473	QPA	Qiagen
PMR4 (At4g03550)	QT00798077	QPA	Qiagen
PR-1 (At2g14610)	QPR1 forw QPR1 rev	CTG ACT TTC TCC AAA CAA CTT G GCG AGA AGG CTA ACT ACA ACT AC	FQ, this work
RbohD (At5g47910)	QT00741104	QPA	Qiagen
CYP79B2 (At4g39950)	RT CYP79B2 forw RT CYP79B2 rev	GTA ACT TCG GAG CAT TCG T TCG CCG GAT ATC ACA TCC	(Clay et al., 2009)
CYP81F2 (At5g57220)	RT CYP81F2 forw RT CYP81F2 rev	CTC ATG CTC AGT ATG ATG C CTC CAA TCT TCT CGT CTA TC	(Clay et al., 2009)
UBQ5 (At3g62250)	UBQ5 fwd. RT UBQ5 rev RT	GAC GCT TCA TCT CGT CC GTA AAC GTA GGT GAG TCC A	(Kesarwani et al., 2007)
WRKY22 (At4g01250)	QT00809886	QPA	Qiagen
WRKY29 (At4g23550)	QT00813645	QPA	Qiagen
	genotyping		
PMR4 (At4g03550)	pmr4-1-NheI-F pmr4-1-NheI-R	TTA CCA GCC CAA CCA ATT TC AGA TCA GGG ACA TGG GAC AG	(Nishimura et al., 2003)
ICS1 (At1g74710)	sid2-2 HindIII forw sid2-2 DraI rev	CTC AAT TAG GTG TCT GCA GTG AAG C GTT GTA GCA AAA ACC GTA ATG ATC G	(Wildermuth et al., 2001)

Gene	Primer	Sequence 5´→3´	Source
TGA2 (At5g06950) TGA5 (At5g06960)	TGA25genom forw TGA25wtrev TGA25genom rev	GTC AAT CCG GTT TCA TAT TCT CCT C CCG CAT AAA CAA TAA ACC AAG AGA G GAG CGA CAA CTC CTT TCA ACT CAT C	this thesis
TGA6 (At3g12250)	TGA6genom forw TGA6genom rev	TTC TCA CTT TGT GAT TTG CCT TTG G TGG GCA ATC TTG CTA TGA TTT CAA G	this thesis
	northern probe		
FRK1 (At2g19190)	FRK1cDNA forw FRK1cDNA rev	TCT TTC ATC GAT TTT ATT CAC AAG C TAG TTT TCC TGA TCA GTC ACT ATG CC	this thesis
	sequencing		
	UNI	ACG ACG TTG TAA AAC GAC GGC CAG	
	REV	TTC ACA CAG GAA ACA GCT ATG ACC	
	cDNA synthesis		
	oligodT	TTT TTT TTT TTT TTT TTT TT	
	random nonamere	NNN NNN NNN	

3.1.9 Organisms

3.1.9.1 Plant genotypes

Genotype	Description	Reference
Columbia, Col-0	wild type	NASC Stock Nr. N1092, NASC 2002
cpr5	constitutive expressor of *PR* genes	(Bowling et al., 1997)
NahG	Col-0 carrying *NahG*-transgene	(Delaney et al., 1994)
npr1-1	point-mutation in *NPR-1* gene	(Cao et al., 1997)
pmr4-1	Callose synthase CalS12 deficient	(Nishimura et al., 2003)
sid2-2	SA-induced deficient	(Wildermuth et al., 2001)

Genotype	Description	Reference
tga1,4	TGA1 and TGA4 double mutant	Y. Zhang, (Kesarwani et al., 2007)
tga3-1	Single knock out of TGA3	Y. Zhang, (Kesarwani et al., 2007)
tga6-1	Single knock out of TGA6	Y. Zhang, (Zhang et al., 2003)
tga2,5	TGA2 and TGA5 double mutant	Y. Zhang, (Zhang et al., 2003)
tga2,5,6	Knock out line lacking all three class II TGA transcription factors, impaired in SAR	X. Dong, (Zhang et al., 2003)
tga2 ,3,5 ,6	Knock out line lacking all three class II TGA transcription factors and TGA3	X. Dong, (Zhang et al., 2003)
tga2,5,6/pmr4		this work
tga2,5,6/sid2		this work
tga2,5,6:: 35S:TGA2	Over-expression line, expressing the *TGA2* gene under control of the CaMV *35S* promoter	M. Zander , (Zander et al., 2009)
tga2,5,6:: 35S:TGA5	Over-expression line, expressing the *TGA5* gene under control of the CaMV *35S* promoter	M. Zander , (Zander et al., 2009)
tga2,5,6:: 35S:TGA6	Over-expression line, expressing the *TGA6* gene under control of the CaMV *35S* promoter	M. Zander , (Zander et al., 2009)

3.1.9.2 Bacteria

Species	Properties	Reference
Escherichia coli DH5α	F-, *gyr*A96 (Nalr), *rec*A1, *end*A1, *thi*-1, *hsd*R17 (rkmk+), *gln*V44, *deo*R, D (*lac*ZYA-*arg*F) U169 [p80dD(*lac*Z)M15]	(Hanahan, 1983)

Species	Properties	Reference
Pseudomonas syringae pv. *tomato* DC3000	pLAFR3; rif^r	(Innes et al., 1993)
Pseudomonas syringae pv. *tomato* DC3000 COR-	Coronatine deficient; rif^r; kan^r	J. Zeier; (Zhao et al., 2003)
Pseudomonas syringae pv. *tomato* DC3000 hrpA-	Deficient in TTSS; rif^r; kan^r	J. Zeier; (Mishina and Zeier, 2007)
Pseudomonas syringae pv. *maculicola* ES4326	pLAFR3; rif^r	(Whalen et al., 1991)

3.1.10 Standard buffers

Buffer	Content
PBS (10 x)	1.4 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.3
PBS-T (1 x)	1 x PBS with 0.05 % (v/v) Tween-20
TAE (20 x)	0.8 M Tris, 2.3 % (v/v) acetic acid, 20 mM EDTA
TE	10 mM Tris, 1 mM EDTA, pH 7.5
Buffer O+	50 mM Tris-HCl (pH 7.5 at 37°C); 10 mM $MgCl_2$; 100 mM NaCl; 0.1 mg/ml BSA
Buffer B+	10 mM Tris-HCl (pH 7.5 at 37°C); 10 mM $MgCl_2$; 0.1 mg/ml BSA
Buffer G+	10 mM Tris-HCl (pH 7.5 at 37°C); 10 mM $MgCl_2$; 50 mM NaCl; 0.1 mg/ml BSA
Buffer R+	10 mM Tris-HCl (pH 8.5 at 37°C); 10 mM $MgCl_2$; 100 mM KCl; 0.1 mg/ml BSA
Buffer Y+	33 mM Tris-Acetat (pH 7.9 at 37°C); 10 mM magnesium acetat; 66 mM potassium acetat; 0.1 mg/ml BSA
10 x DNA-sample buffer	67 % succrose; 50 mM EDTA, pH 8.0; 0.42 % (w/v) Orange G
SSC (20 x)	2 M NaCl, 0.3 M sodiumcitrat, pH 7.0 with HCl

3.1.11 Growing media

All media and heat-stable solutions were sterilized by autoclaving for 20 min at 121°C. The heat sensitive solutions were sterilized by filtering through a 0.2 µm membrane filter (Heinemann Labortechnik GmbH, Germany). To solidify the media 8 g/L select agar or 5 g/L Gelrite were added.

Medium	Content
King's B	10 g/L Proteose-Pepton No 3; 1,5 g/L K_2HPO_4; 15 g/L glycerol; pH 7 with HCl; add after autoclaving 5 ml/l 1M $MgSO_4$
1MS+MES	1 % sucrose,.4 g/L MS medium; 1g/L MES; pH 5.7 with KOH
LB	10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0 (NaOH)

3.1.12 Software

Software	Manufacturer
AIDA	Raytest
GraphPad Prism 5	GraphPad
i-control	TECAN
ImageJ	W. Rasband
iQ5	BioRad
Leica Application Suite	Leica

3.2 Methods

3.2.1 Plant growth conditions

3.2.1.1 Grow conditions on soil

Plants were grown on steamed soil supplemented with Confidor (50 mg/l) and fertilizer (0.5 ml/l Wuxal) under short day conditions (22°C/18°C, ~70 µmol/m^2/s Par, 8 h light/16 h dark, 60% humidity). During germination, a transparent hood was used to reach a high humidity. After 5-6 weeks the plats can be used for investigations.
For infections with *P. syringae* 10-20 seed were spread out on peat balls (Jiffy Products International AS, Norway).

3.2.1.2 Axenic growth conditions

Approximately 200 seeds were surface sterilized (chapter 3.2.3) and sown on sterile 1x MS-plates comprise 1g/L MES, pH 5.7. After stratification for approximately two days the plants were grown for 10-14 days at 22°C under long day conditions (16h light/ 8h dark) and 60% humidity in a climate chamber.

3.2.2 Generation of quadruple mutants

To obtain *tga2-1 tga5-1 tga6-1 pmr4-1* and *tga2-1 tga5-1 tga6-1 sid1-2* quadruple mutant, *tga2,5,6* was crossed with *pmr4-1* and *sid2-2*, respectively. F1 plants were allowed to self-fertilize and mutants were screened from the F2 population using PCR. TGA2 and TGA5 are located next to each other. *tga6-1 tga2-1 tga5-1* were identified using primers within the deletions to confirm homozygosity at both loci (Zhang et al., 2003). The pmr4-1 allele was confirmed by PCR using the cleaved-amplified polymorphic sequence marker primers described in 3.1.8.1, followed by digestion with NheI (Nishimura et al., 2003). The *sid2-2* allele was also identified by PCR, using primers located in the big deletion site (Wildermuth et al., 2001).

3.2.3 Surface sterilization of *A. thaliana* seeds

E-cups with open lid containing *Arabidopsis* seeds (up to a volume of approx. 100 µl) were placed in an exsiccator together with 100 ml hypochloric solution and 5 ml hydrochloric acid. To close the exsiccator firmly, a weak vacuum was used. After 5 h in chloric acid atmosphere the seeds are sterile to spread out.

3.2.4 Root length determination

Approx. 40 seeds were placed with the help of a toothpick in a horizontal line on 1MS-MES medium plus GELRITE (5g/L), in square petri dishes. For every assay one control plate and a second containing flg22 peptide were used. After stratification, seedlings were grown for 10 days in vertical position under long day conditions. Pictures were taken with a digital camera. The computer software ImageJ was used to quantify the root length. In case of using a ROS inhibitor, the length of seven day old roots were measured five days after transfer to DPI, BHA or DMTU containing plates (concentrations as indicated).

3.2.5 ROS-staining in roots

Seedlings of *A. thaliana* were grown as described for the root inhibition assays (chapter 3.2.3). 7-day-old seedlings were spray-treated with H_2O, 1 mM SA or 1 µM flg22 for 60 min and then transferred to staining buffer (10 mM MES, 0.1 mM KCl, 0.1 mM $CaCl_2$, pH 6.0) plus 0.001 % DMSO (unstained control) or staining solution (10 mM H_2DCFDA in staining buffer) plus 1 µM SA or 1 µM flg22 (and control). After 30 min of incubation in the dark, seedlings were washed with staining buffer. Root tips were immediately observed under a microscope (Leica DM 5000B + CTR 5000, fluorescence cube GFP) with 100x magnification. Quantification of the staining was performed with Leica application suite. The intensity of staining was calculated as average fluorescent intensity per mm^2 root area.

3.2.6 ROS-burst assay

To analyse the production of reactive oxygen species after chitin and flagellin application a luminol based assay was used. When PAMPs are added, the leaves release reactive oxygen species and the horseradish peroxidase catalyses the oxidation of luminol to 3-aminophthalate via several intermediates. The reaction is accompanied by emission of low intensity light at 428 nm. This chemiluminescence can be quantified in a plate reader. Leaf discs from 4-6 weeks old soil-grown plants cultivated under short day conditions were used. Every well of a white 96-well microtiter plate was filled with 100µl water. For each mutant/background line 16-24 leaf discs were harvested and incubated in water, until the wound response has worn off (o/n). The luminol solution (10 mM Tris/HCl, pH 9.5; 10 µg/ml horse radish peroxidase, 17 µg/ml luminol) was splitted off: to one half the designated PAMP was added to a final concentration of 100 µg/ml chitin or chitosan or 1 µM flg22. The water was removed from the wells of the microtiter plate without wounding the leaf discs. To one half of the leaf discs 100µl of luminol solution without PAMP and to the other half 100 µl luminol solution with PAMP was added with a multichannel pipette to be fast. The reaction starts immediately. The measurement of the chemiluminescence in a plate reader has to be done promptly for 60 min with measurement intervals of 1 min.

3.2.7 Callose staining in seedlings and soil grown plants

Seedlings were grown like described for the root length measurement (3.2.4). Callose deposition was induced by spray treatment with 1 µM flg22 for 24h. The leaves were separated from the roots with the help of a scalpel blade and incubated in 80% EtOH for several days for fixation and de-staining. For determination of callose deposition after pathogen infection, leaves of 4-6 weeks old soil grown plants were syringe infiltrated with the bacterial solution (OD_{600}=0.02). To accelerate the de-staining the solution can be exchanged several times until it becomes clear and the leaves are completely colorless. For rehydration the leaves were first incubated in 50% EtOH and afterwards in H_2O. The get the tissue transparent 2 h incubation at 37°C with 10% NaOH was conducted. Afterwards, several washing steps with H2O were done until the pH value gets neutral. The water was changed with 150 mM KH_2PO_4 (pH 12). The staining with aniline blue (0.01 % in 150 mM KH_2PO_4, pH 12) occurs on a shaking platform o/n. For analysis, the leaves were transferred on object slides with staining solution + 50% glycerol and observed under the microscope with UV light (filtercube A; Leica) and 25x magnification. For quantification of the fluorescent spots the software AIDA was used. The intensity of staining was calculated for each image as the index of stained pixels of the leaf area normalized to the average of three points representing unstained area from all pictures taken in one experiment.

3.2.8 Measurement of stomatal response to different treatments

To assure that most stomata were open before beginning experiments, plants were kept under light for at least 3 hr. Fully expanded young leaves of 4-6 week old soil-grown plants cultivated under short day conditions were immersed in tab water, 5 µM flg22 or flg22 in combination with 0.5 ng/µl COR. Alternatively, a bacterial solution of *Psm* ES4326 (OD_{600}=0.2) was used. 3 h after treatment with chemical compounds, and 1h and 3h after bacterial inoculation, epidermal peels of three leaves was peeled off by applying the leaf lower surface on a piece of ultraclear adhesive tape and observed under a microscope (Leica DM 5000B). Pictures were taken of random regions. The width of the stomatal aperture was measured using the software ImageJ.

3.2.9 Induction of gene expression in *A. thaliana*

10-14 days old, sterile grown seedlings were treated by spraying with a pump aerosol can producing a fine drizzle until the seedings are equally moistened. The solutions were prepared freshly for every treatment with deionized water. The following solutions were used:

- 1 mM salicylic acid
- 100 nM or 1 µM flg22 peptide as indicated
- 1 µM elf18 peptide
- 100 µg/ml chitosan
- 100 µg/ml chitin
- 5 µM coronatine

Chitosan and chitin are not soluble in water. Therefore, grinding to fine powder was done before water was added.

3.2.10 Bacterial growth assay

The measurement of bacterial growth was done in collaboration with Jane Glazebrook (University of Minnesota, St Paul, USA) as previously described in (Tsuda et al., 2008). *Pst* DC3000 (OD_{600} = 0.0001, 1x 10^5 CFU/ml) bacterial suspensions were infiltrated into 5-week-old plants 1 day after treatment with water or 1 µM flg22. Each sample consisted of two leaf discs (total surface 0.57 cm^2) taken from a single leaf. Leaf discs were pulverized in 400 µl of 5 mM $MgSO_4$ and dilution series were made. Of each dilution, 10 µl was streaked on King's B plates containing 25 µg/ml of rifampicin. The leaf bacterial titer was measured at 0 and 2 dpi for *Pst*DC3000. From this data, the colony-forming units (CFU) per cm^2 leaf surface area were calculated.

3.2.11 Standard molecular methods

3.2.11.1 Transformation of chemical competent *E. coli* DH5α

The transformation procedure was followed as described in Hanahan (1983). In brief, 200 µl competent *E. coli* cells were thawed on ice for 20 min, 50 ng of plasmid DNA were added to the cells and mixed gently. The mixture was incubated on ice for 30 min. After a heat shock for 90 sec at 42°C the cells were placed immediately on ice for at least 3 min. 800 µl of LB medium were added to the tube and the suspension was mixed on a roller for 45-60 min at 37°C depending on selectable antibiotic resistance marker. Different volumes of the culture were spread on plates containing LB medium supplemented with antibiotics. The plates were incubated overnight at 37°C.

3.2.11.2 Cryoconservation of bacteria

For long-term storage, the overnight culture was supplemented with 20 % glycerol and stored at -80°C.

3.2.11.3 High-purity plasmid DNA isolation

For sequencing and transformation purposes, high-purity plasmid DNA was isolated using Nucleospin Mini kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Optional steps were always followed according to the manufacturer's recommendation. A 4 ml overnight culture was used to isolate plasmid and the isolated DNA was eluted with 50 µl (high copy) or 30 µl (low copy) EB buffer.

3.2.11.4 Determination of DNA/ RNA concentrations

The concentration of nucleic acids was estimated by measuring their absorption in a spectrophotometer at a wavelength of 260 nm (maximum nucleic acid absorption value; due to the π-electron systems of the heterocyclic nucleotides). At 10 mm path-length OD_{260} = 1.0 is equivalent to 50 µg/mL double-stranded DNA and 40 µg/mL RNA, respectively. Absorption at 280 nm (for the presence of aromatic rings from amino acids and phenol compounds) was used to give information about the purity of the DNA or RNA sample, where an optimal ratio OD_{260}/OD_{280} is in the range of 1.9-2.0 for RNA and 1.8 for DNA.

3.2.11.5 Ligation of DNA fragments

The conventional cloning of a DNA fragment into a selected plasmid was performed using the T4-DNA ligase enzyme, which is able to catalyze the formation of a phosphodiester chemical bond between free 5´-phosphate and 3´-OH groups of double stranded DNA fragments and vectors. The donor DNA fragment (10 fold higher concentrated compared to the vector) was incubated with the vector DNA, 2 µl of ligation buffer and 1 µl of T4-DNA ligase for 2 hours at room temperature. Ligase was inactivated by heating at 65°C for 10 min before using the ligated DNA for transformation.

3.2.11.6 DNA sequencing

The DNA sequencing was done using the BigDye Terminator RR Mix Cycle Sequencing kit (Perkin-Elmer Corporation, Massachusetts, USA). The principle of DNA sequencing is based on the chain-termination method described by (Sanger et al., 1977). In the chain-termination method, didesoxynucleotides (terminators) are incorporated into a newly synthesized complementary chain that will lead to stop its elongation in a PCR reaction. Each of didesoxynucleotides is labeled with a specific fluorescent dye and the terminated chains can be specifically detected using an ABI Prism 310 Capillary Sequencer (Applied Biosystems). The PCR sequencing reaction was performed using 500-1000 ng plasmid DNA, 5 pmol primer, 2 µl RR mix (ready reaction) and H_2O up to a total volume of 10 µl. The samples were subjected to 25 cycles of: 10 sec at 95°C, 5 sec at 50°C, 4 min at 60°C in a thermocycler. The DNA product was precipitated using 9.5 µl water and 30.5 µl of absolute ethanol and left for 1 hour. The DNA was collected by centrifugation for 20 min at 13000 rpm. The pellet was washed using 125 µl 70 % ethanol and then centrifuged for 10 minutes at 13000 rpm, than dried at 95°C for 1 min and resuspended in 15 µl of template-suppression reagent (TSR, Perkin-Elmer). After the final denaturing step at 95°C for 2 min tubes were directly put on ice. The reaction was loaded on an ABI-Prism 310 capillary electrophoresis sequencing station for analysis.

3.2.11.7 Separation of DNA on agarose gels

The electrophoretic separation of DNA for analytical and preparative purpose was done in a horizontal agarose gel device (10 cm x 7 cm x 0.3 cm, 16 lanes) with 1x TAE as running buffer. An agarose concentration of 1 % was used for separation of fragments bigger than 500 bp. For DNA fragments with lower size as 500 bp, a 2 % agarose gel was used. DNA samples were mixed with 1/10 volume of 10x DNA loading buffer, applicated in separate lanes and electrophoretically separated at 120 V for 40-45 min. Incubation in Ethidiumbromide solution (0.1 % w/v) for 10 min was used to visualize the DNA fragments. Before exposure with UV light, the gel was rinsed briefly in H_2O to reduce background staining. The detection of DNA was done with an UV-transilluminator and Gel

documentation device. The sizes and amount of the DNA fragments were determined using DNA standards.
The elution of DNA fragments from agarose gel was done using the QIAquick or Nucleospin Extract II Gel Extraction kit following the manufacturer's instructions. The eluted fragments were verified by electrophoresis as described above.

3.2.11.8 Restriction digestion of DNA

Type II endonucleases were used to digest a double stranded DNA molecule for analytical and cloning purposes. The enzymes cut the DNA either as 5´ or 3´ "sticky" overhangs or as blunt ends. The digestion reactions were incubated in a buffer system optimized for the used enzyme (see chapter 3.1.11; buffer B+; G+; O+; R+; Y+, MBI-Fermentas). In the case of a double digestion, a universal buffer system (1x or 2x Y+) was chosen. The activity of the restriction enzymes was determined in "units" (U), where 1 U was defined as the amount of enzyme cutting completely 1 µg of λ DNA (48.5 kb) in 60 minutes at optimal conditions. The minimal amount of enzyme necessary for each restriction was determined according to the following formula:

U = (bp[λ] x No. of restriction sites in target DNA) / (No. of restriction sites in [λ] x bp of target DNA)

The incubation temperature was 37°C unless otherwise indicated for special restriction enzymes. Due to the adverse effect of high glycerol concentration on enzyme activity, the total volume of restriction enzymes should not extend more than 10% in the restriction mix.

3.2.11.9 Isolation of genomic DNA from *Arabidopsis thaliana* leaves for genotyping

This qick and dirty method was used for PCR based genotyping of the F2 generation after crossing of different mutants. A leaf disc was cut out with the help of the lid of a microcentrifuge tube avoiding cross-contamination with foreign plant material. 100 µl extraction buffer was added (200 mM Tris-HCl, pH 7.5; 250 mM NaCl; 25 mM EDTA; 0.5% SDS) to grind the tissue with a pistil fitting in a 1.5 ml microcentrifuge tube. 300 µl extraction buffer was added and the tubes inverted before a centrifugation step (15000 rpm, 5 min, 4°C) occurs. 300 µl of the supernatant was transferred to a new microcentrifuge tube and supplemented with 300 µl 2-propanol. After inverting the tube several times the samples were centrifuged (13000 rpm, 5 min, RT). The supernatant has to be removed before the resulting pellet could be washed with 200 µl of 70% EtOH. After removing the supernatant the pellet should dry at 37°C for 10 min to be resolved in 100 µl water (ultra pure) for 10 min at 65°C. After a last centrifugation (13000 rpm, 5 min, RT), the supernatant containing the isolated genomic DNA was transferred to a new microcentrifugation tube and can be stored at -20°C.

3.2.11.10 Preparation of DNA-free cDNA for qRT-PCR

To analyse gene expression by PCR it is necessary to synthesize cDNA from RNA. DNaseI restriction was done before cDNA synthesis. 1 µg RNA template together with 1 µL of 10x DNaseI-reaction buffer and 1 µl DNaseI, RNase-free was added with water to a final reaction volume of 10 µL. The mixture was incubated at 37°C for 30 minutes. To deactivate the DNaseI enzyme1 µL 25 mM EDTA was added and incubated at 65°C for 10 minutes. cDNA synthesis was performed with 1 µg total RNA (DNA-free), 20 pmol of oligo-dT primer and 200 pmol of random nonamer oligonucleotides. Water was added to a final reaction

volume of 12.5 µL. For annealing of the primers, the mixture was heated to 70°C for 10 min and immediately cooled down on ice. Subsequently 20 nmol dNTPs, 4 µL RT - 5x first strand reaction buffer and 60 u reverse transcriptase H- and competed with H_2O to a final volume of 20 µl. The mixture was incubated at 42°C for 70 min and then heated to 70°C for 10 min.

3.2.11.11 Quantitative real time RT-PCR

This high sensitive method was used to investigate gene expression on RNA level. Prepaired cDNA-was diluted 1:10 with sterile water. The amplification mix consisted of 1x NH_4-reaction buffer; 2 mM $MgCl_2$; 100 µM dNTPs; 0.4 µM primers, 0.25 u BIOTaq DNA polymerase; 10 nM Fluoresceine; 100,000 times diluted SYBR Green I solution; 1 µL of the diluted cDNA as template and water (ultra pure) added to a total volume of 25 µL. PCR consisted of a 6 min initial denaturation step at 95°C followed by 40 cycles of 20 s at 95°C, 20 s at 55°C (annealing) and 40 s at 72°C (elongation). A final elongation step was done for 4 min at 72°C followed by a melt curve analysis. During the elongation and annealing phase measurement of the fluorescence occurs. As housekeeping gene *UBQ5* (At3G62250) was used. The data analysis was done with the help of the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008) to quantify the relative expression levels.

3.2.11.12 RNA extraction

The extraction method based on TRIZOL extraction can be used to extract RNA, DNA and proteins from plants (Chomczynski, 1993). This method uses a Phenol/ Chloroform (dichloromethane) extraction to solve RNA in the aqueous phase while other parts like proteins solved in the hydrophobic chloroform phase. The two thiocyanates in the extraction buffer inhibit RNAses. After grinding of the plant material under liquid nitrogen 1.3 mL extraction buffer (380 ml/l Phenol saturated with 0.1 M citrate buffer pH 4.3; 0.8 M guanidiniumthiocyanate; 0.4 M ammoniumthiocyanate; 33.4 ml Na-acetate, 3 M, pH 5.2; 5% glycerol) was added to ~150 mg plant material. After shaking for 15 min at RT, chloroform (260 µL) was added to each sample. After an additional shaking step (15 min, RT) and centrifugation (12000 rpm, 30-60 min, 4°C) the supernatant (900 µL) was transferred to new microcentrifuge tubes. Precipitation buffer (HSPB, 1.2 M NaCl1, 0.8 M Na-citrate) and 2-propanol (each 325 µL) were added, the tubes inverted several times and the samples were incubated for 10 min at RT and centrifuged (12000 rpm, 20 min, 4°C) followed by a washing step with 70% ethanol. After removing the supernatant, samples were dried and afterwards resolved in 50 – 100 µL water (ultra pure). Concentration was measured as described in 3.2.11.4.

3.2.11.13 Northern blot analysis

Total RNA was extracted from 100 mg plant tissue using the trizol method (chapter 3.2.10.12) and analyzed by Northern blot analysis (Heinekamp et al., 2002). A fragment representing the cDNA of FRK1 was amplified by PCR using the primers FRK1cDNA forw and FRK1cDNA rev. After separation on an agarose gel, the fragment was gel-eluated with the Nucleo Spin® Extract II kit. 50 ng PCR fragment was used as template for the northern probe. Probes were radioactively labeled using the random-priming method with the Megaprime DNA labeling system. Aliquots of total RNA were fractionated on denaturing agarose gels and transferred to nylon membranes. Hybridization was performed over night. Membranes were washed with 2x SSC / 0.1 % SDS at 65°C for 1 h and with 1x SSC / 0.1 % SDS at 65°C for 1 h. Quantification of RNA levels was performed using Bio-imager analysis.

3.2.12 Biochemical methods

3.2.12.1 Measurement of free SA

The measurement of free SA was done in collaboration with Ivo Feussner (Georg-August-University, Göttingen) as previously described in (Ochsenbein et al., 2006).

3.2.12.2 Whole cell protein extracts

The extractions of proteins were performed under denaturing conditions and on whole cell extract level. Extraction buffer containing urea (4 M urea, 16.6 % glycerol, 5 % SDS, 0.5 % ß-mercaptoethanol) was used to extract the proteins. After grinding the plant material in liquid nitrogen, extraction buffer (450 µL) was added to ~150 mg plant material. The samples were incubated at 65°C for 10 min and centrifuged (13000 rpm, 20 min, RT). The supernatant was transferred to new microcentrifuge tubes and used for SDS-PAGE.

3.2.12.3 Determination of concentrations of proteins

Protein concentration was estimated by two different methods. A colorimetric assay was used to determine the concentration from proteins extracted without detergent usage according to (Bradford and Williams, 1976). The assay was conducted by pipetting equal amounts of protein extract into a microtiter plate containing 200 µL of 5-fold diluted Bradford reagent and the OD_{595} was measured with a plate reader. Protein concentrations were calculated with the help of a standard curve derived from different BSA protein amounts (1, 3 and 6 µg) on the same plate. Proteins isolated using buffers containing detergents were either defined to equal amounts in a coomassie stained SDS gel (scanned and analysed with TINA2.0).

3.2.12.4 SDS-PAGE

In sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins are separated mostly on the basis of polypeptide length. The electrophoresis was done using a discontinuous buffer system, in which a non-restrictive large pore gel, called a stacking gel, is layered on top of a separating gel called a resolving gel. The recipe for the resolving gel was consisting of: 7-8 % (w/v) acrylamide/ bisacrylamide (37.5:1), 400 mM Tris-HCl pH 8.8, 0.1 % (w/v) SDS, 0.1 % (w/v) TEMED and 0.1 % (w/v) APS. The stacking gel was consisting of: 5 % (w/v) acrylamide/bisacrylamide (37.5:1), 125 mM Tris-HCl pH 6.8, 0.1 % (w/v) SDS, 0.2 % (w/v) TEMED and 0.1 % (w/v) APS. The denatured protein extract samples (each ~10 µL, or defined equal amounts after a first coomassie stained gel) were boiled with 15 µL 2x SDS sample buffer (0.09 M Tris, 20 % glycerol, 2 % SDS, 0.02 % bromophenol blue, 0.1 M DTT) at 95°C for 5 minutes and cooled on ice. The electrophoresis was performed at 120 V with 1x SDS running buffer (250 mM Tris, 2 M Glycine, 1 % SDS) until the bromophenol blue band reached the lower end of the gel. 6 µL pre-stained protein ladder were used for the estimation of the size of the separated proteins.

3.2.12.5 Coomassie staining of SDS gels

The Coomassie Brilliant Blue G-250 dye was used to detect proteins separated on SDS-PAGE. The gels were incubated with coomassie staining solution (colloidal coomassie) o/n. The gels were destained in water o/n.
Colloidal coomassie consists of 400 mL solution A (40 g ammonium sulphate and 8 mL phosphoric acid) and 10 mL solution B (0.5 g coomassie brilliant blue G250, this has to be solved shaking at least for 0.5 h). Each gel was stained in 40 mL colloidal coomassie complemented with 10 mL methanol.

3.2.12.6 Western blot

The proteins separated in the SDS-PAGE were blotted onto a PVDF membrane using semi-dry blotting method in an electric field between two graphite plates. The PVDF membrane was activated before blotting using MeOH. For the transfer of proteins from the gel to the membrane, the gel on top of the membrane was sandwiched between two times 3-layers of Whatman papers (pre-soaked with transfer buffer). The whole arrangement was placed within a blot apparatus and transfer was performed under amperage of one mA/cm^2 for 1.4 hours. (Optional: Ponceau S staining was done to observe the success of the transfer. De-staining was done using 1 x PBS.) After blotting the membrane was dried between two layers of Whatman paper. The standard was marked on the membrane with an iMark (pen containing pre-immune serum from rabbit) for later detection of standard bands with the second antibody and ECL kit to visualize them on the film. After 5 min the membrane was reactivated in MeOH and non-specific binding to the proteins on the membrane was prevented by blocking the membrane with non-fat dried milk powder (5 % in 1x PBST) o/n at 4°C on a shaking platform. The detection of specific proteins on the membrane was performed using an antiserum directed against the protein of interest in a 1:1000 dilution in 1x PBST (with 0.5 % milk powder). The membrane was therefore incubated with the respective antiserum for 2 h at RT on a shaking platform. The incubation with the second antibody (anti-rabbit 1:25000 in 1x PBST) was performed for 1 h at RT on a shaking platform. This second antibody is conjugated to horseradish peroxidase (HRP). The HRP can utilize the enhanced chemi-luminescent substrate (ECL, GE Healthcare, incubation of the membrane in ECL mix for 5 min) emitting luminescence, which allows visualization of the membrane bound proteins on autoradiography films. The films were exposed to the membrane in detection cassettes for 30 s up to 10 min depending upon the strength of chemi-luminescence signal generated by the respective amounts of bound protein.

3.2.12.7 Chromatin immunoprecipitation (ChIP)

ChIP allows the analysis of the *in vivo* binding status of transcription factors or other DNA-associated proteins to certain DNA sequences. Intact cells are treated with formaldehyde to crosslink promoter-associated proteins to the DNA. After isolation and shearing of the chromatin, protein-DNA complexes are immunoprecipitated with specific antibodies against the protein of interest. The precipitated DNA fragments are subsequently purified and analysed by PCR using primers flanking the (putative) binding site of the protein. The amount of PCR product obtained is indicative for the relative amount of protein bound to the DNA when the tissue was harvested. The procedure allows detecting of quantitative differences in the relative amount of protein-DNA complexes, so that stimulus-induced binding can be detected. Chromatin immunoprecipitations and subsequent real-time PCR analysis were done as specified by Fode et al., 2008.

3.2.12.7.1 Crosslinking

3-5 g of leaf material from plants grown for 6 weeks under short day conditions was placed into a plastic basket (15 cm diameter) that was put into 500 ml of 1% formaldehyde in 50 mM KH_2PO_4/K_2HPO_4, pH 5.8. Vacuum was applied twice for 5 min and samples were afterwards left for another 20 min in this buffer. Subsequently, the leaf material was placed into a buffer containing 0.3 M glycine in 50 mM KH_2PO_4/K_2HPO_4, pH 5.8. Vacuum was applied twice for 5 min with subsequent incubation for 5 min. Leaves were washed twice in water, dried with paper towels, frozen and stored in liquid nitrogen till further processing.

3.2.12.7.2 Isolation of nuclei

Nuclei were isolated according to a modified protocol (Folta and Kaufman, 2000). Briefly, the frozen tissue was ground with a pistil under liquid nitrogen, resuspended in 20 ml extraction buffer (1 M hexylene glycol, 50 mM PIPES KOH, pH 7.2, 10 mM $MgCl_2$, 5 mM ß-mercaptoethanol) and homogenized for 5 min using a Miccra-D8 homogenizer (14000 rpm). The homogenate was passed through a double layer of Miracloth. Triton X-100 (25%) was added dropwise to the resulting liquid fraction with constant stirring to a final concentration of 1% to lyse organelle membranes. The lysate was gently layered on top of a 6 ml 35% percoll cushion in gradient buffer (0.5 M hexylene glycol, 50 mM PIPES KOH pH 7.2, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1% Triton X-100). After centrifugation at 2100 x g for 30 min in a swinging bucket rotor, the nuclei were found as a pellet at the bottom of the tube. Nuclei were resuspended in 21 ml of gradient buffer and again gently layered on top of a 6 ml 35% percoll cushion. After centrifugation as above, the pellet was resuspended in 1 ml of gradient buffer and centrifuged at 2100 x g for 10 min.

3.2.12.7.3 Chromatin extraction and immunoprecipitation

Chromatin extraction and chromatin immunoprecipitation (ChIP) was performed as described previously (Turck et al., 2004) with slight modifications. Nuclei from 3-5 g of formaldehyde cross-linked leaf material were first resuspended in 1 ml sonication buffer (10 mM HEPES/NaOH, pH 7.4, 1 mM EDTA, 0.5% SDS and diluted with 1 ml sonication buffer without SDS. Chromatin was sheared to an average size of 500-1000 base pairs by repetitive sonication (4 times 20 s in an ethanol/ice bath, interrupted by 1 min cooling steps) at 12 amplitude microns. The final centrifugation was performed at 11200 x g for 20 min at 4°C. To normalize different samples for equal DNA content, the DNA concentration was measured after the following purification steps: 50 µl of the chromatin were brought to a volume of 450 µl with sonication buffer containing 0.25% SDS, incubated first in the presence of 10 µg of Proteinase K for 1 h at 37°C and subsequently at 65°C for 16 h for de-crosslinking. Free DNA was purified from the solution by phenol/chloroform/isoamylalcohol (25:24:1) extraction followed by chloroform/isoamylalcohol (24:1) extraction. DNA was precipitated by the addition of 10 µg glycogen, one-tenth volume of 3 M sodium acetate and 2.5 volumes of ethanol at -80°C for at least 3 h. After centrifugation, DNA was resuspended and used for OD_{260} measurements.
Equal amounts of chromatin as measured by DNA content (15 µg) were brought to a total volume of 200 µl with sonication buffer (10 mM HEPES/NaOH, pH 7.4, 1 mM EDTA, 0.25% SDS). After adding 300 µl RIPA buffer (50 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate), samples were incubated in the presence of preimmune serum (5 µl) for 1 h at 4°C on a rotary shaker. Next, 50 µl Protein A agarose beads (50% slurry in RIPA buffer supplemented with 0.1% SDS) were added and incubated for additional 1 h. Beads were pelleted and 50 µl of the supernatant was removed for the

input control (see below). The residual supernatant was incubated with 5 µl of the immune serum for 4 h at 4°C on a rotary shaker. Subsequently, 50 µl Protein A agarose beads (50% slurry in RIPA with 0.1% SDS) were added. After incubation for 2 h on a rotary shaker at 4°C, immunoprecipitates were washed three times in 1 ml of RIPA buffer with 0.1% SDS, followed by an additional wash with 800 µl and a transfer into a fresh tube. Immunocomplexes were then eluted from the beads by two sequential incubations in 150 µl of elution buffer (0.1 M glycine, 0.5 M NaCl, and 0.05% Tween 20, pH 2.5) followed by centrifugation and addition of 150 µl 1 M Tris/HCl, pH 8.0 to the combined eluates. As input control, 50 µl of the supernatant from preimmune incubations (10% of the sample) was brought to a volume of 450 µl with sonication buffer (10 mM HEPES/NaOH, pH 7.4, 1 mM EDTA, 0.25% SDS). Eluted DNA and DNA of the input control were treated with Proteinase K, heat treated and purified as described above. Precipitation of the DNA was done at –80°C for at least 3 h. DNA was resuspended in 35 µl (ChIP-DNA) or 175 µl (input control) of water for PCR analysis. Comparison of the amounts of PCR products yielded with immunoprecipitated and input DNA, respectively, allowed us to estimate that roughly 0.8% of the input promoters are precipitated.

3.2.12.7.4 Analysis by ChIP-on-chip array

The analysis of ChIP samples by the ChIP-on chip array was done in collaboration with Christopher Town (Institute for Genomic Research, Rockwille, USA).The experimental procedures are described in principle by Thibaud-Nissen et al., 2006. The used custom-made array contained the initial ~ 200 promoter targets (including *PR-1* and *GST6*) selected on the basis of their published association with SAR, plus 50 new targets identified from the first NimbleGen ChIP data (Thibaud-Nissen, 2006) and 67 targets identified based upon their co-expression with *PR-1* in analysis of ATH1 microarray expression data. In addition the array contains 89 sets of control spots that consist of a pool of 7 non-target amplicons for normalization purposes. Immunoprecipitated and control (raw chromatin) samples are labeled by incorporation of amino allyl nucleotides during PCR amplification, conjugated with Cydyes and hybridized as a flip dye pair.
The signal intensities of the IP samples were normalized to the signal intensities of raw chromatin. Probes with a values ≥ 1.0 were considered as enriched

3.2.12.7.5 Analysis by real-time PCR

The analysis of ChIP experiments by real-time PCR (qPCR, chapter 3.2.10.11) is recommended. The conditions for the PCR depend on the primers that are used for amplification of the promoter DNA sequence. It is recommended to use primers that amplify a fragment of about 250 bp. As template, use 2.5 µL of the purified IP-DNA and the input DNA, each. Calculation was done according to the $2^{-\Delta\Delta CT}$ method (Schmittgen et al., 2008), taking *ACTIN8* reference sequences for normalization.

4 Results

4.1 The growth inhibition effect of flg22 is strongly increased in the *tga2,5,6* mutant

The addition of the flagellin-derived peptide flg22 to the liquid medium of young *A. thaliana* seedlings causes a strong reduction in growth (Gómez-Gómez et al., 1999). Tsuda et al. (2008) demonstrated that an interplay between PAMP-triggered and SA-mediated defense responses exist. Therefore, mutants of the SA biosynthesis or signaling pathway were tested for an altered response to flg22 in a root growth assay. *Arabidopsis* seedlings were grown for 14 days on vertical axenic plates containing 100 nM flg22 peptide. Seedlings grown on plates without flg22 show no difference in root growth, but all of the tested mutants show increased flg22-induced root growth inhibition in comparison to the wild-type Col-0 plants (Figure 4-1).

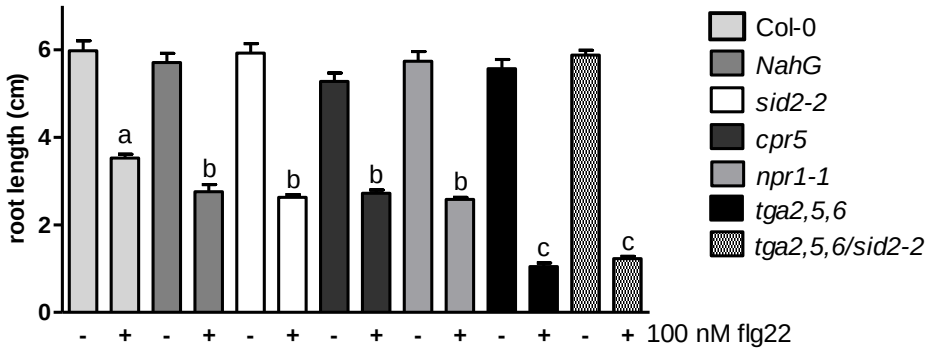

Figure 4-1| **Growth inhibition caused by flg22 in mutants impaired in SA biosynthesis and signaling.**
A. thaliana seedlings of ecotype Col-0, N*ahG*, *sid2-2*, *cpr5*, *npr1-1, tga2,5,6* and *tga2,5,6/sid2-2* were grown vertically for 10 days on 1MS-MES medium containing 100nM flg22. For quantification, root length of 30 seedlings per genotype was determined with ImageJ. Each bar represents the average ± SEM. Different letters indicate significant differences between genotypes within a treatment (1wayAnova, $P < 0,05$).

The roots of *NahG*, *sid2-2*, *cpr5* and *npr1-1* mutants grown in the presence of flg22 are about 25% shorter than the roots of Col-0 seedlings. *NahG* and *sid2-2* mutants are impaired in SA accumulation. *sid2-2* contains a point mutation in the *ISOCHORISMATE SYNTHASE 1 (ICS1)* gene, whereas salicylate hydroxylase converts SA to catechol in plants carrying the

NahG transgene (Delaney et al., 1994). *cpr5* mutations show pleiotropic phenotypes including enhanced constitutive expression of *PR*-genes and elevated SA and JA levels (Bowling et al., 1997). NPR1 together with the class-II TGA factors TGA2, TGA5 and TGA6 are important key regulators of SAR (Cao et al., 1997; Zhang et al., 2003).

The strongest flg22-induced growth inhibition was observed in the *tga2,5,6* mutant with over 70% shorter roots than the wild type. Hyper-induction of flg22-induced root growth inhibition still occurs in the *tga2,5,6/sid2-2* mutant. Thus, TGA factors enhance PAMP-triggered immune responses in an SA-independent way.

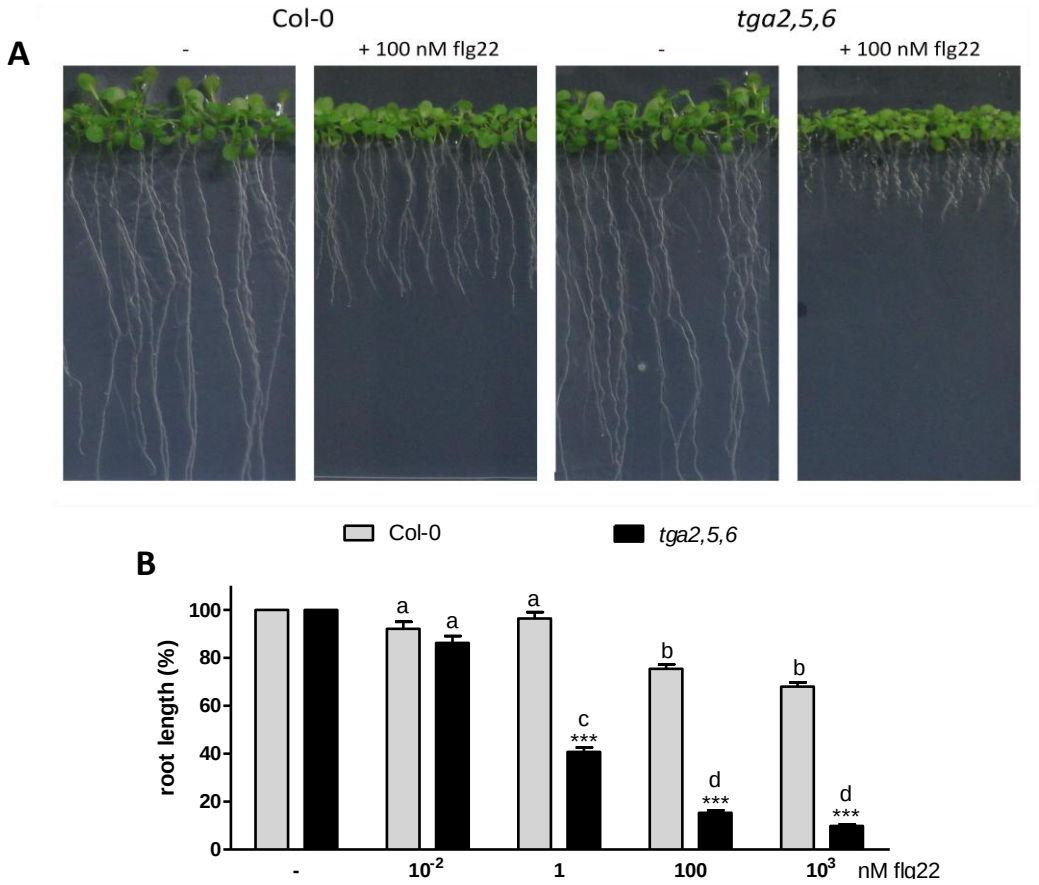

Figure 4-2| ***tga2,5,6* mutants show enhanced sensitivity to flg22 in root growth assay.**
(A) Representative pictures of ten-days-old wild-type (Col-0) and *tga2,5,6* seedlings grown vertically on 1MS-MES medium with or without 100 nM flg22 peptide.
(B) Dose-dependence of growth inhibition caused by flg22. Bars represent the average and SEM of n = 30 seedlings. The root length of each phenotype grown on control plates without flg22 was set to 100%. Asterisks represent significant differences between wild-type and mutant plants (2wayAnova; ***P<0.001). Different letters indicate significant differences between treatments within a genotype (1wayAnova; P< 0.01).

Flg22 not only has an inhibitory effect on roots. In addition, the aerial parts stay smaller on flg22-containing medium. This effect is more pronounced in the *tga2,5,6* mutant (Figure 4-2A). Next, the effect of different flg22-concentrations on root growth was tested in Col-0 and *tga2,5,6* seedlings (Figure 4-2B). The roots of wild-type Col-0 plants show a growth

reduction of about 30% at a concentration of 100 nM. The inhibition in the *tga2,5,6* mutant is much stronger; about 80% in the presence of 100 nM flg22. This effect already occurs at very low nanomolar concentrations of flg22. The *tga2,5,6* mutant is much more sensitive to flg22 than the wild-type. For further investigations an flg22 concentration of 100 nM was used based on the finding that this concentration is sufficient to induce a maximized root length inhibition.

To investigate whether other TGA transcription factors act as suppressors of flg22-induced root growth, several *tga* single and multiple mutants were tested (Figure 4-3). The quadruple mutant of *tga2, tga3, tga5 and tga6* (*tga2,3,5,6*) shows the same phenotype as the triple *tga2,5,6* mutant. The observation that the *tga3* single mutant shows also an increased root growth inhibition, leads to the assumption that the root growth inhibition in the *tga2,5,6* mutant reaches its maximum and is not further increased by a mutation of TGA3. The *tga6* single mutant shows no altered root growth. The increased root growth inhibition observed in the double *tga2,5* is not as severe as in the *tga2,5,6* mutant, indicating that TGA6 partially complement the root growth phenotype. These results point out that the class II TGA factors TGA2, TGA5 and TGA6 may operate together as negative regulators of PAMP triggered immunity. Mutants of other TGA factors show an increased root growth inhibition indicating an overlapping function of different members of this transcription factor family.

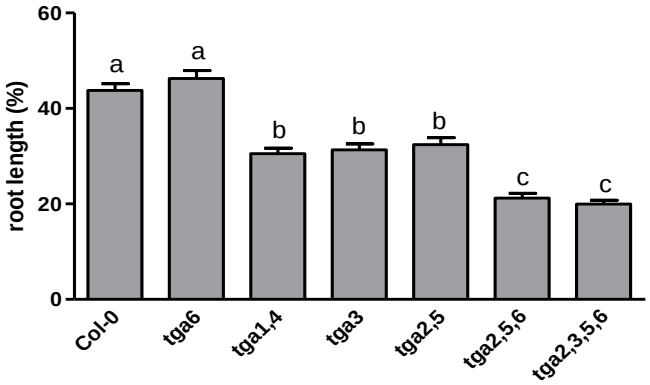

Figure 4-3| **Growth inhibition caused by flg22 in different *tga* mutants.**
Ten-days-old *A. thaliana* seedlings of ecotype Col-0 and different *tga* mutants were grown vertically on 1MS-MES medium containing 100 nM flg22. Bars represent the average and SEM of n = 30 seedlings. The root-length of each genotype grown on control plates without flg22 was set to 100%. Different letters indicate significant differences between genotypes (1wayAnova, $P < 0,05$)**.**

To determine whether TGA2, TGA5 or TGA6 can complement the mutant phenotypes of *tga2,5,6*, transgenic plants expressing the cDNAs of TGA2, TGA5 or TGA6 under the control of the *Cauliflower mosaic virus* (*CaMV*) *35S* promoter in the *tga2,5,6* background were used for root growth inhibition analysis (Figure 4-4A). Western blot analysis was done to verify the protein levels in roots and shoots (Figure 4-4B). The used serum, generated against the C-termini of TGA2 and TGA5, also detects TGA6 protein in roots and shoots. Roots contain higher class-II TGA-protein levels than shoots. The transgenic protein levels in the two lines tested for each construct are similar in roots, but differ in shoots of TGA5OE and TGA6OE transgenic lines.

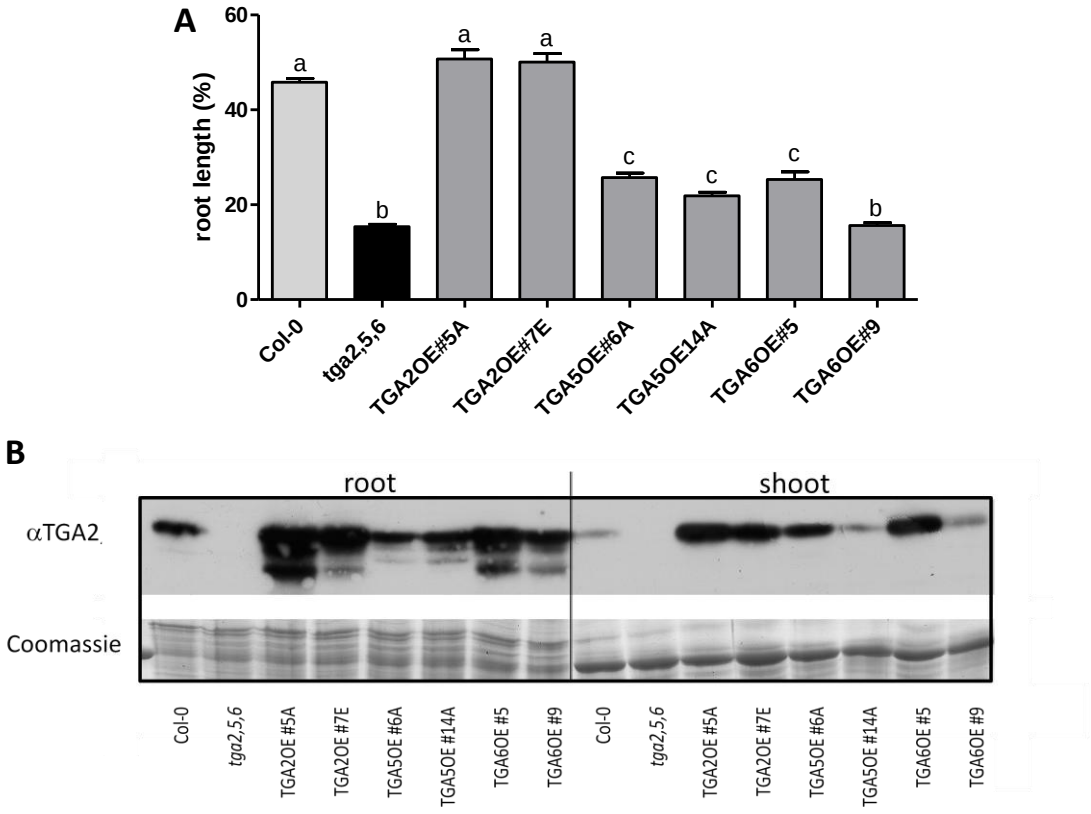

Figure 4-4| **Complementation of the growth phenoptype of *tga2,5,6* mutants on flg22.**
(A) Ten-days-old *A. thaliana* seedlings of ecotype Col-0, *tga2,5,6* or transgenic lines ectopically expressing *TGA2*, *TGA5* or *TGA6* in the *tga2,5,6* mutant background grown vertically on 1MS-MES medium containing 100 nM flg22. Bars represent the average and SEM of n = 30 seedlings. The root-length of seedlings grown on control plates without flg22 was set to 100%. Different letters indicate significant differences between genotypes (1wayAnova, $P < 0,05$).
(B) Western blot analysis of the transgenic lines analyzed in (a) using an antiserum (1:1000 dilution) generated against the C termini of TGA2 and TGA5 (Fode et al 2008). The samples for protein extraction were taken from untreated seedlings grown in the same experiment as for root-length measurement shown in (A). Crude protein extracts (10 µg) were separated by SDS-PAGE. Coomassie staining is shown as loading control.

The hyper-susceptibility of *tga2,5,6* roots to flg22 was only restored to wild-type levels in the *tga2,5,6* mutant plants overexpressing TGA2. The TGA5 transgene is able to partially rescue the root growth phenotype. Transgenic plants containing TGA6 show a non-consistent result. Only TGA6OE#5, containing higher protein levels than TGA6OE#9 is able to rescue the root growth phenotype partially. Obviously, TGA2 is sufficient to rescue this phenotype and TGA5 and TGA6 play a more marginal role in regulating these flg22-mediated response. Based on the intermediate root growth phenotype in the *tga2,5* double mutant and a wild-type like phenotype in the *tga6* single mutant (Figure 4-3), TGA6 seems to be involved in regulation of the signaling cascade leading to growth inhibition only if TGA2 and TGA5 are not present in the cell. TGA6 is not sufficient to completely replace TGA2 and TGA5, indicated by the intermediate phenotype of the *tga2,5* mutant and the fact that ectopic expression of TGA6 only partially rescues the root growth phenotype of the *tga2,5,6* mutant.

4.2 Roots of the *tga2,5,6* mutant contain higher ROS levels than the wild-type

It is widely accepted that ROS play a central role in many signaling pathways during stress perception, regulation of photosynthesis, pathogen response, hormonal action, and plant growth and development (Mittler et al., 2004; Apel and Hirt, 2004). ROS perception is also involved in root growth. The NADPH oxidase RHD2 regulates root development by producing ROS that stimulate plant cell expansion through the activation of Ca^{2+} channels, and the inhibition of ROS formation leads to short root hairs and stunted roots (Foreman et al. 2003). In contrast, high intracellular concentrations of ROS lead to cell damage or cell death. Therefore the level of ROS needs to be tightly regulated (Mittler et al., 2004). To determine whether TGAs restrain growth via a ROS-dependent mechanism, the contribution of ROS to flg22-mediated root growth inhibition was analyzed.

To visualize the generation of ROS, an assay based on 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA), a ROS-sensitive dye with good intracellular retention in growing roots, was used (Jiang et al., 2003). The non-fluorescent carboxy-H_2DCFDA permeates living cells and is deacetylated by nonspecific intracellular esterases. In the presence of nonspecific ROS (produced throughout the cell, particularly

during oxidative stress) the reduced fluoresceine compound is oxidized and emits bright green fluorescence. Seedlings of Col-0 and *tga2,5,6* plants were pretreated with flg22 or SA (positive control (Boursiac et al., 2008)) for 30 min, before the seedlings were transferred to the staining solution. As shown in Figure 4-5, roots of the *tga2,5,6* mutant exhibit higher levels of ROS than roots from wild-type seedlings. It was not possible to induce a ROS burst by flg22 or SA due to the fact that roots of mock treated plants show the same fluorescence signal as roots treated with flg22 or SA.

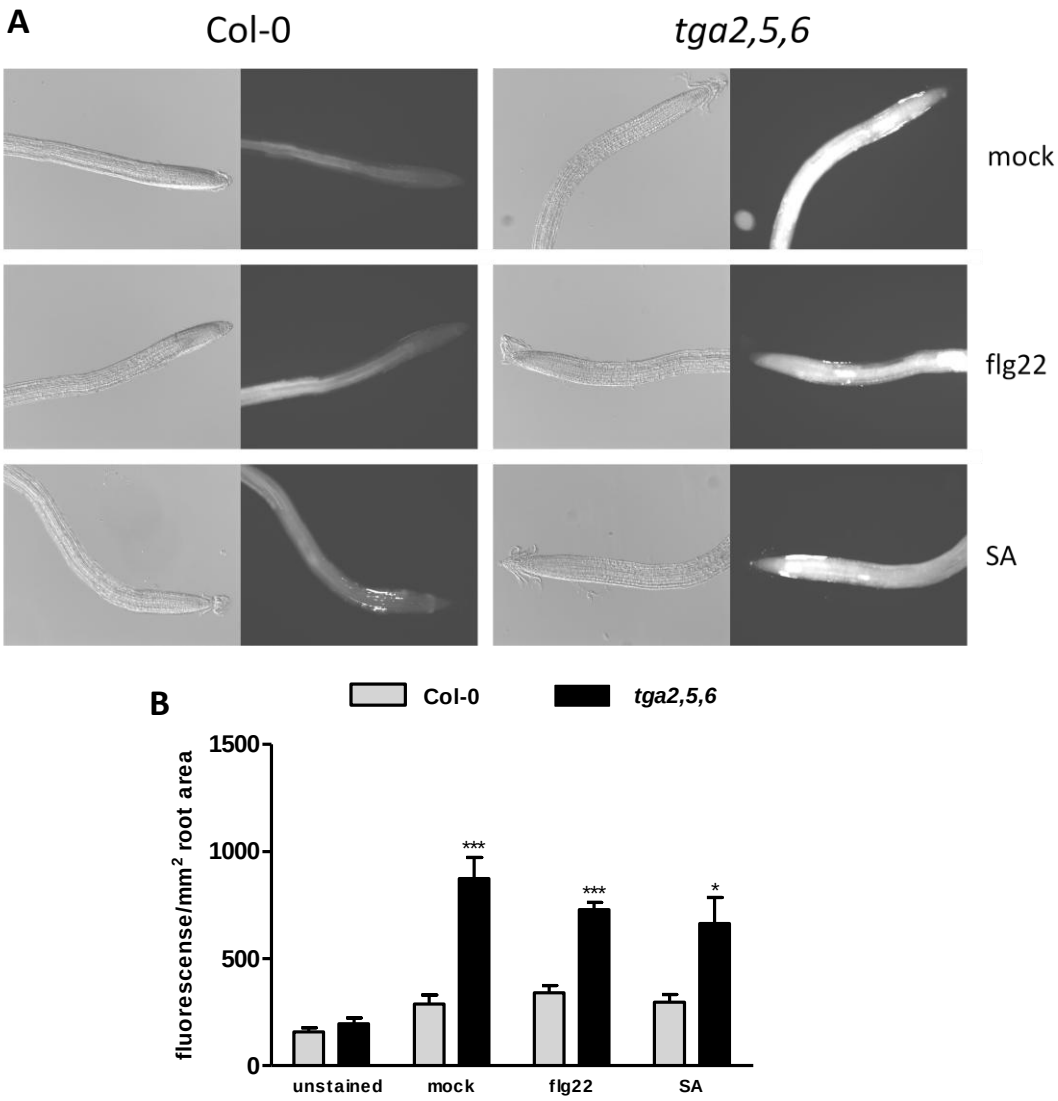

Figure 4-5| **ROS accumulation in *Arabidopsis* roots.**
(A) ROS accumulation (H_2DCFDA imaging) of WT (Col-0) and *tga2,5,6* mutant seedling roots after mock, flg22 or SA treatment. *A. thaliana* seedlings were grown vertically on 1MS+MES medium for 7 days. Seedlings were spray-treated with H_2O (mock), 1 µM flg22 and 1 mM SA for 30 min before transfer to staining solution or DMSO (unstained) for additional 30 min was performed. After a washing step, images of root tips were captured with a fluorescence microscope (GFP filter) and 100x magnification.
(B) Quantification of H_2DCFDA staining. Bars represent the average and SEM of at least n = 8 roots. Asterisks represent significant differences between wild-type and mutant plants (1wayAnova, ***P < 0.001; *P < 0.05).

Different chemical compounds are known to inhibit ROS production. Butylated hydroxyanisole (BHA) is a synthetic phenol which can scavenge reactive oxygen species by donating labile hydrogen to oxygen radicals. This lipophilic compound is generally used as antioxidant in food industries. Dimethylthiourea (DMTU) is a powerful scavenger of hydroxyl radicals (.OH) and diphenyleneiodonium chloride (DPI) is an inhibitor of nitric oxide synthases and NADPH oxidases like the ROS-producing Rboh enzymes. The root growth of *ga1-3* seedlings, which accumulate lower basal levels of ROS than the WT, was more resistant to the inhibitory effect of DPI than that of the WT (Achard et al., 2008).
These three inhibitors were used in a root growth assay to investigate, whether increased ROS levels in the roots of the *tga2,5,6* mutant lead to a higher sensitivity to ROS scavengers.

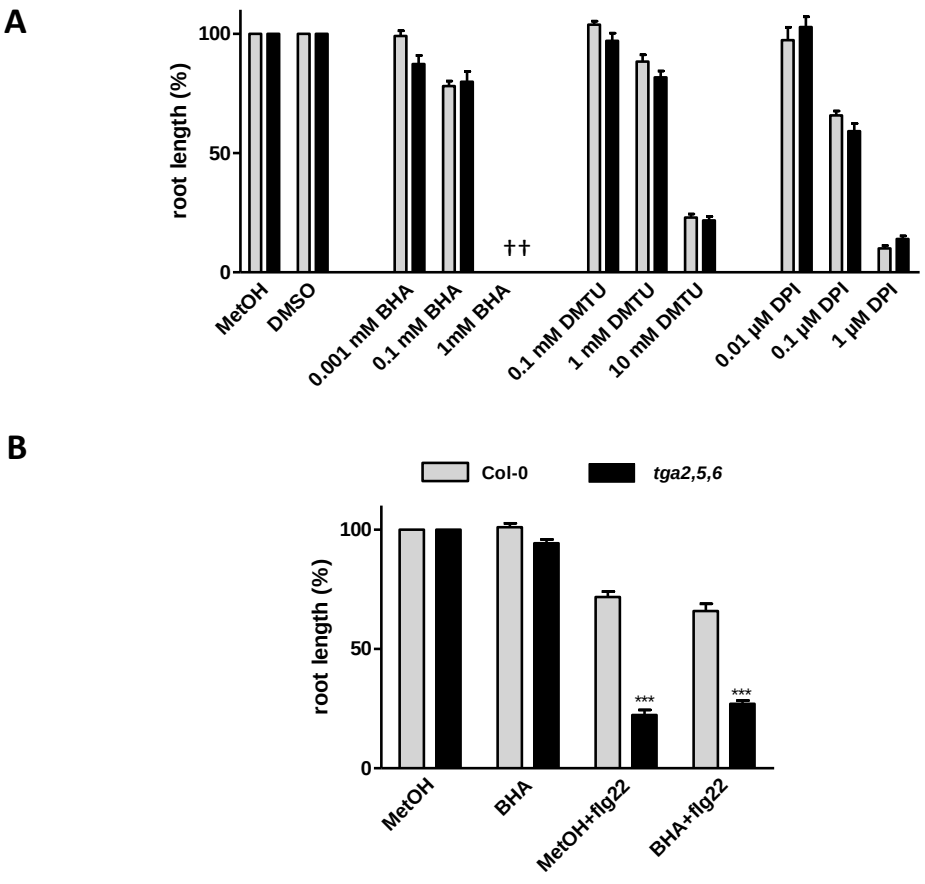

Figure 4-6| **Influence of different ROS inhibitors on root growth.**
(A) Two-days-old Col-0 and *tga2,5,6* seedlings were transferred to vertical plates containing 1MS-MES and different concentrations of the ROS inhibitors BHA, DMTU and DPI or the organic solvents MetOH and DMSO. Five days after transfer, the root length was measured.
(B) Influence of BHA on flg22 induced root growth inhibition. Two-days-old Col-0 and *tga2,5,6* seedlings were transferred to vertical plates containing MetOH, 100 nM flg22, 0,1 mM BHA or a combination of both. Five days after transfer, the root length was measured.
Bars represent the average and SEM of n = 15 seedlings. The root-length of seedlings grown on control plates (MetOH for BHA and DMTU; DMSO for DPI) was set to 100%. Crosses indicate no plant survived. Asterisks represent significant differences between wild-type and *tga2,5,6* plants (1wayAnova, ***P < 0.001).

In an initial experiment it was tested, how seedlings grow on media containing different concentrations of the used inhibitors. Increasing concentrations lead to a reduction of root growth with every tested inhibitor (Figure 4-6A). A concentration of 1 mM BHA is sufficient to inhibit the root growth completely.

Col-0 and *tga2,5,6* seedlings show no significant differences in the sensitivity to the different inhibitors. A concentration of 0.1 mM BHA leads only to a weak decrease in root elongation. This BHA concentration was used to investigate the root growth in presence of flg22 together with a ROS inhibitor. The presence of the ROS inhibitor has no influence on the growth inhibitory effect of flg22 (Figure 4-6B).

4.3 PAMP-induced ROS burst is not influenced in the *tga2,5,6* mutant

Few minutes after PAMP-perception, a transient ROS burst is initiated and displays one of the earliest responses of the plant. A luminol based chemiluminescence assay was used to detect the oxidative burst after treatment of leaf slices with different elicitors. Horseradish peroxidase catalyses the ROS-mediated oxidation of luminol to 3-aminophthalate via several intermediates. The reaction is accompanied by emission of low intensity light at 428 nm and the emitted chemiluminescence is proportional to the amount of accumulated ROS. Like flg22, elf18 is a peptide derived from a bacterial elicitor, called EF-Tu. EF-Tu is highly conserved in all bacteria and the N-acetylated peptide elf18 comprising the first 18 amino acids of the protein is fully active to induce defense responses (Kunze et al., 2004). It was possible to trigger a rapid release of ROS with both elicitors flg22 and elf18. In comparison to Col-0 plants, the *tga2,5,6* mutant shows slightly increased ROS burst after flg22 perception (Figure 4-7A). A treatment with elf18 has the opposite effect: the *tga2,5,6* mutant is less sensitive to elf18 as the wild-type (Figure 4-7B). These differences are not statistical significant and probably are due to the big variances between the samples.

Chitin and chitosan are fungal elicitors. Chitosan is a hydrophilic biopolymer and is obtained by *N*-deacetylation of chitin. It could be shown that chitosan induces various defense reactions in plants (Iriti and Faoro, 2009). For *Arabidopsis* it is only known that chitosan stimulates a NADPH-dependent, hyperpolarization-activated Ca^{2+} influx current in guard cells, necessary for stomata closure (Klüsener et al., 2002).

Chitin and chitosan induce only a very weak ROS burst in comparison to the treatments with flg22 and elf18 (Figure 4-7C+D). Like for flg22 and elf18 perception, no significant differences between Col-0 and *tga2,5,6* were detectable. Remarkably, the ROS burst released by chitosan is much stronger than the burst triggered by chitin.

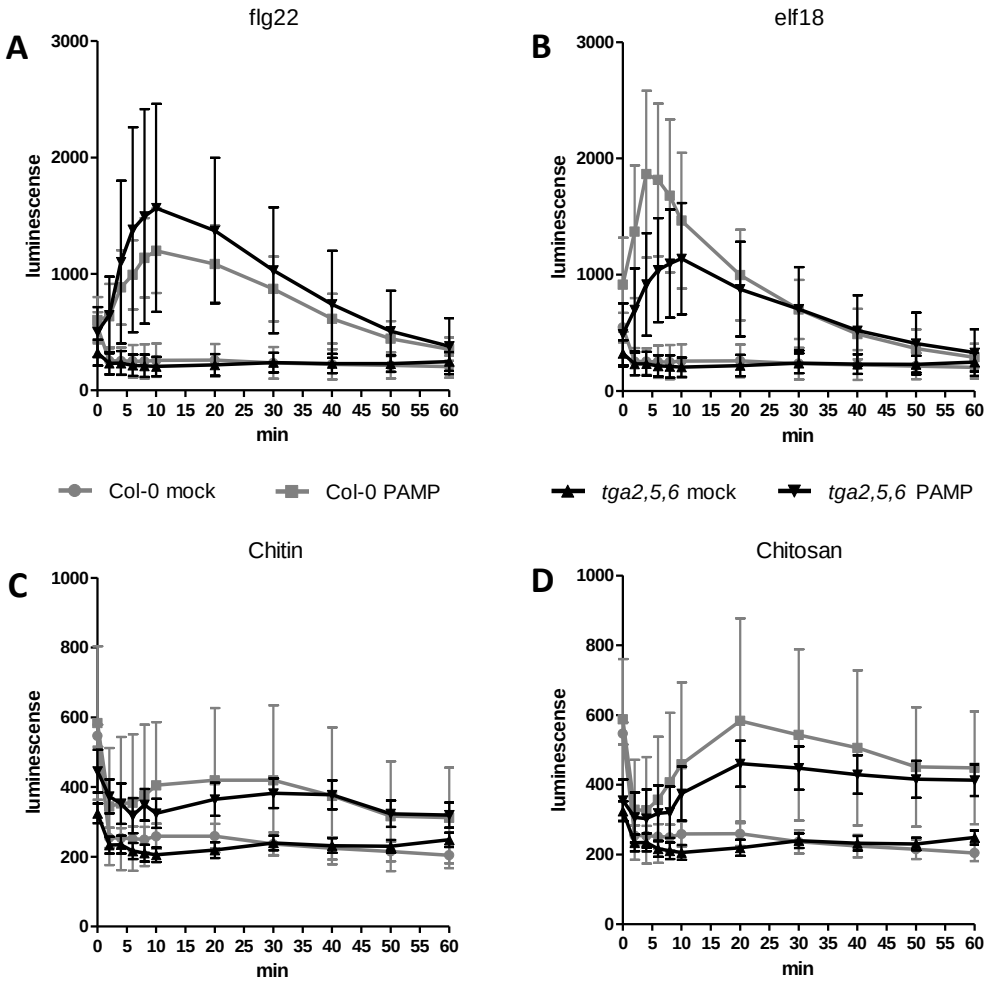

Figure 4-7| **Oxidative burst in the leaf tissues of Col-0 and *tga2,5,6*.**
Luminescence of *A. thaliana* leaf discs in a solution with luminol and peroxidase after treatment with different PAMPs and after control treatment (mock), as indicated. Light emission at the very beginning of the experiments is caused by phosphorescence of the green tissue. Every data point represents the average and SD of n = 12 leaf discs. Statistical analysis with 1wayAnova does not point out significant differences between Col-0 and *tga2,5,6* plants. This experiment was repeated once with similar results. (A) 1 µM flg22 (B) 1µM elf18 (C) 100 mg/L chitin (D) 100 mg/L chitosan

4.4 Gene expression analysis shows an enhanced activation of early flg22-inducible genes in the *tga2,5,6* mutant

Flg22 induces numerous defense related genes in *Arabidopsis thaliana.* Very early induced genes (after 60 min) mostly encode signaling components, such as transcription factors, protein kinases/phosphatases, and proteins that regulate protein turnover. Approximately 80% of these genes were also up-regulated by treatment with cycloheximide. This suggests that many early flg22-induced genes are negatively regulated by rapidly turned-over repressor proteins (Navarro et al., 2004). For TGA2 it is described that this transcription factor can act as a repressor for *PR*-gene expression (Kesarwani et al., 2007). The *tga2,5,6* mutant shows enhanced root growth inhibition after treatment with flg22 (e.g. Figure 4-1). In order to investigate, if the class-II TGA factors TGA2, TGA5 and TGA6 also act as negative regulators for early flg22-induced genes, a quantitative PCR (qRT-PCR) expression analysis was used.

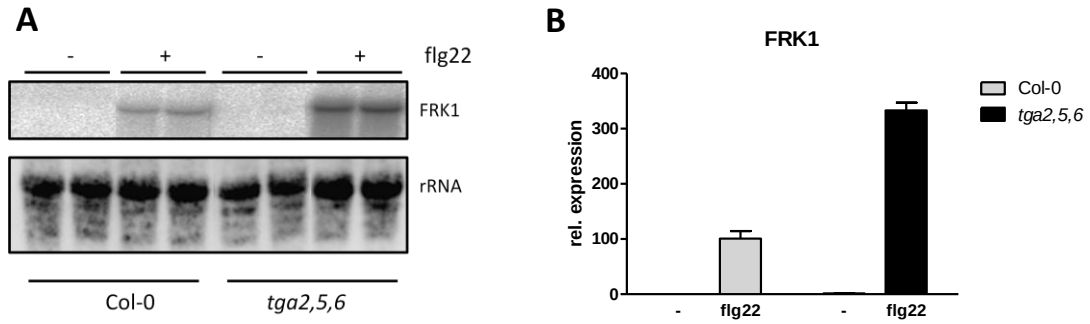

Figure 4-8| **Comparison of *FRK1* expression analyzed by northern blot and qRT-PCR.**
10-14-days-old wild-type and *tga2,5,6* mutant seedlings grown on 1MS+MES medium were spray-treated with H_2O (-) or 1 µM flg22. After 2 hours of treatment, approximately 50 seedlings were harvested for RNA extraction.
(A) Northern blot. 10 ng RNA per lane was loaded. The hybridization occurs with a specific probe against *FRK1*.
(B) qRT-PCR with specific primer against *FRK1*. The transcript levels are normalized to the house keeping gene *UBQ5*. The expression level in flg22-treated Col-0 seedlings was set to 100 %. The average ± SEM of n = 2 samples is shown.

The analysis of *FRK1* expression was selected because *FRK1* is well described as early transcriptional activated after flg22 perception (Asai et al., 2002) and serves as a marker gene for flg22 signaling. Total RNA of 14 days old seedlings spray-inoculated for two hours with 1 µM flg22 peptide was isolated and simultaneously analyzed by a northern blot or by qRT-PCR (Figure 4-8).

For northern blot analysis, a radioactive-labeled probe comprising the cDNA of *FRK1* was used. The northern blot analysis revealed that the *FRK1* expression was induced by flg22 in Col-0 and *tga2,5,6* seedlings, albeit with different intensities (Figure 4-8A). The *tga2,5,6* seedlings are much more sensitive to the flg22 stimulus as the wild-type. The same result could be obtained from the qRT-PCR (Figure 4-8B). This method is much faster and more sensitive than a northern blot and it is possible to analyze many genes with the same RNA preparation, so qRT-PCR was selected for further expression analysis.

To elucidate, if other *tga* mutants beside the *tga2,5,6* mutant show altered expression levels of *FRK1* after flg22 treatment, the same mutants as those used in the root growth assay were used for gene expression analysis (Figure 4-9). *FRK1* expression is not affected in flg22-treated *tga2,5* double mutant plants. The single mutants *tga3* and *tga6* as well as the double mutant *tga1,4* show only slightly increased expression levels of *FRK1*, which does not significantly differ from the wild-type expression. In turn, the *tga2,3,5,6* quadruple mutant shows the same elevated induction as the triple mutant *tga2,5,6* does.
Root growth assays showed that an ectopic expression of *TGA2* is sufficient to rescue the phenotype of enhanced root growth inhibition in the *tga2,5,6* mutant (Figure 4-4A). The transgenic line TGA2OE#7 was used for qRT-PCR analysis of *FRK1* expression (Figure 4-10). TGA2 is sufficient to repress the expression of *FRK1* to wild-type levels, indicating that TGA2 is an important regulator of early defense genes.

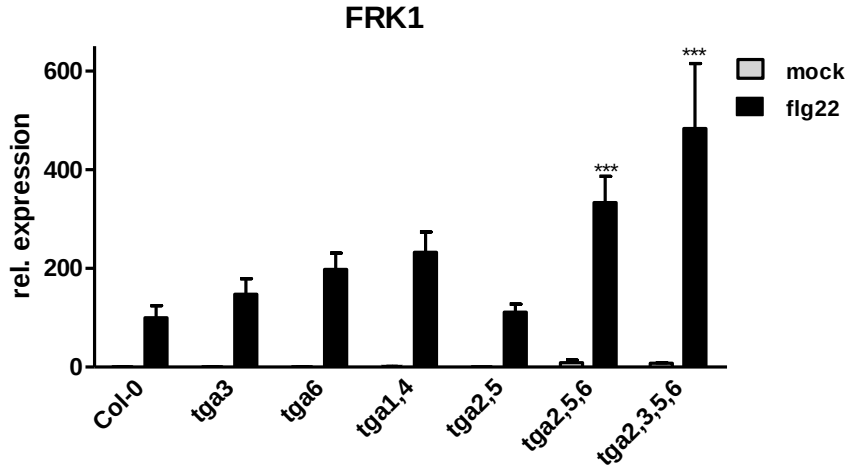

Figure 4-9| **flg22-induced *FRK1* expression in Col-0 and different *tga* mutants.**
10-14-days-old *A. thaliana* seedlings grown on 1MS+MES medium were spray treated with H_2O (mock) or 100 nM flg22. Two hours after treatment approximately 50 seedlings were harvested for RNA extraction. Transcript levels were quantified by qRT-PCR with specific primers against *FRK1* and normalized to relative expression in comparison to the house keeping gene *UBQ5*. The expression level in flg22-treated Col-0 seedlings was set to 100 %. The average ± SEM of n = 3 samples is shown. Asterisks represent significant differences between wild-type and *tga* mutant plants within a treatment (Student's t-test, ***P < 0.001).

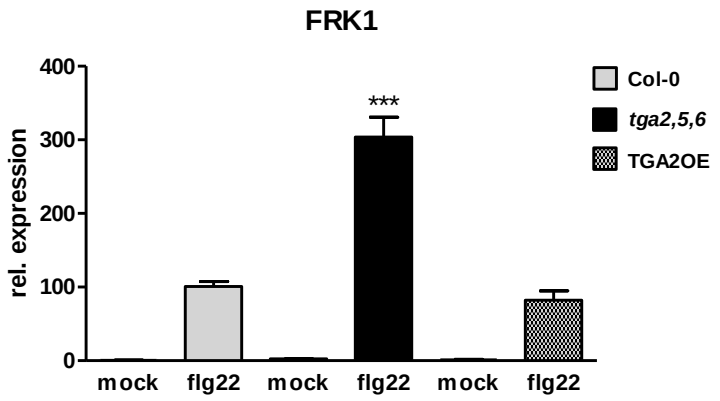

Figure 4-10| **flg22-induced *FRK1* expression in Col-0, *tga2,5,6* and plants ectopically expressing *TGA2*.**
10-14-days-old *A. thaliana* seedlings (Col-0, *tga2,5,6* and transgenic line TGA2OE#7E ectopically expressing *TGA2* in *tga2,5,6* background) grown on 1MS+MES medium were spray treated with H_2O (mock) or 100 nM flg22. Two hours after treatment approximately 50 seedlings were harvested for RNA extraction. Transcript levels were quantified by qRT-PCR with specific primers against *FRK1* and normalized to relative expression in comparison to the house keeping gene *UBQ5*. The expression level in flg22-treated Col-0 seedlings was set to 100 %. The average ± SEM of n = 3 samples is shown. Asterisks represent significant differences between wild-type, *tga2,5,6* and TGA2OE plants within a treatment (Student's t-test, ***P < 0.001).

For further expression analysis, 10 days old seedlings were treated with 100 nM flg22 for 15, 30, 60, 90 and 120 minutes or 2, 12 and 24 hours in two separate time course experiments. *RbohD*, *WRKY22*, *WRKY29* and *FRK1*, which are known to be transcriptionally activated by flg22, were chosen for the analysis. The *Arabidopsis* NADPH-oxidases RbohD and RbohF are known to produce ROS after pathogen attack (Torres et al., 2002). WRKY22 and WRKY29, members of the same subgroup of the WRKY transcription factor family are direct targets of the flg22 activated MAP kinase pathway regulating the expression of *FRK1* in protoplasts (Asai et al., 2002).

The *RbohD* gene is very fast inducible by flg22 (Figure 4-11A+B). 15 min after flg22 treatment, the expression of *RbohD* was strongly induced in Col-0 and *tga2,5,6* seedlings. The maximal expression was detected after 30 min and declines to almost background levels after one hour. No significant differences could be detected between the *RbohD* expression of wild-type and *tga2,5,6* mutant seedlings. The induction of *WRKY22* shows a similar kinetic as the expression of *RbohD*, with the difference that the maximal expression was detected after 60 min (Figure 4-11C+D). Furthermore, at early time points, *WRKY22* is significantly stronger expressed in the *tga2,5,6* mutant than in the wild-type. The closely related *WRKY22* and *WRKY29* are functionally redundant (Asai et al., 2002), but the expression kinetic differs.

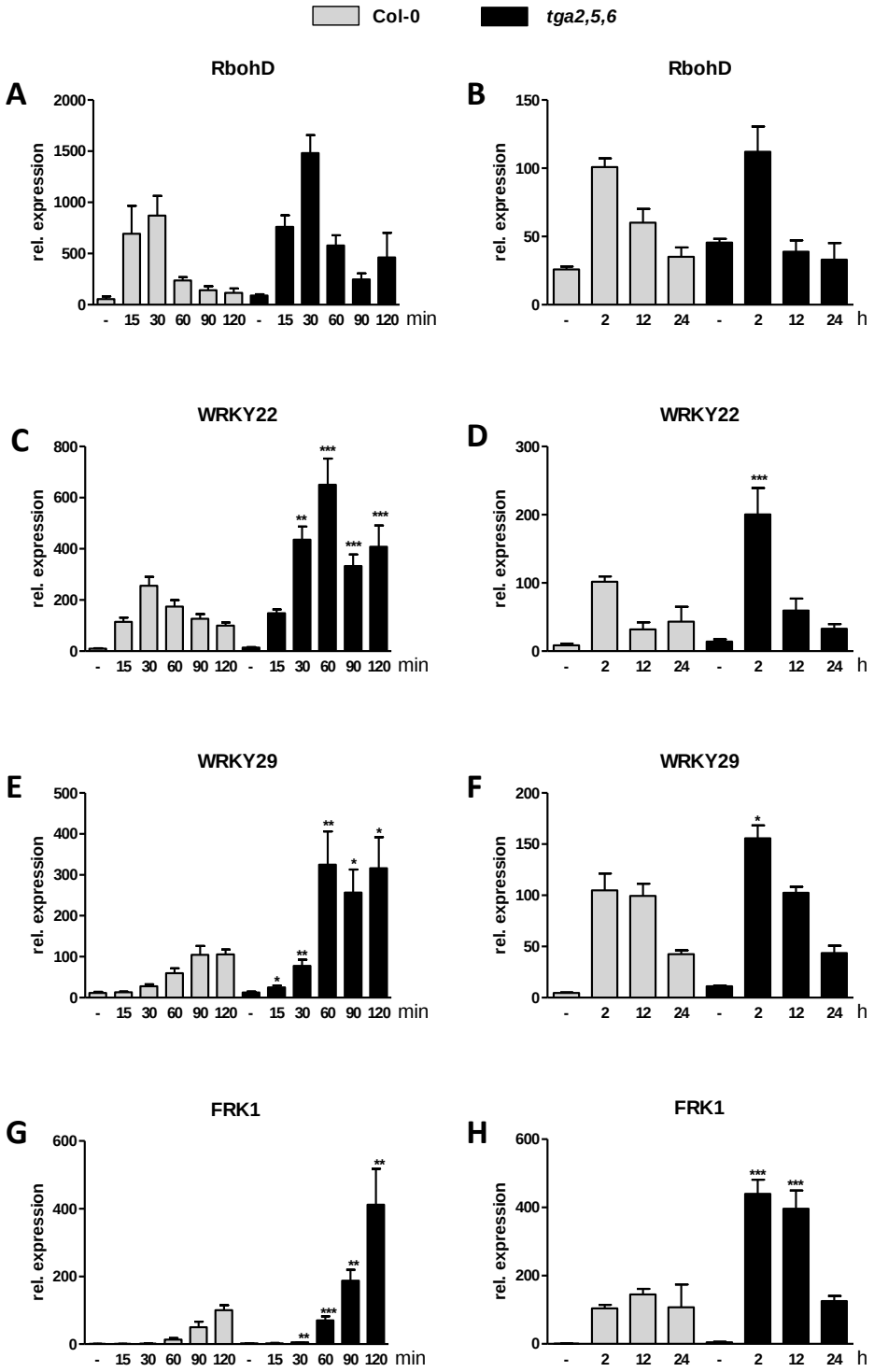

Figure 4-11| **Expression analysis of early flg22-inducible genes in Col-0 and *tga2,5,6* seedlings.** 10-14-days-old Col-0 (gray bars) and *tga2,5,6* (black bars) *A. thaliana* seedlings grown on 1MS+MES medium were spray treated with H_2O (mock) or 100 nM flg22 in two different time course experiments. Approximately 50 seedlings were harvested for RNA extraction after the indicated time points. Transcript levels were quantified by qRT-PCR with specific primers against *RbohD*, *WRKY22*, *WRKY29* and *FRK1* and normalized to the house keeping gene *UBQ5*. The expression level in Col-0 seedlings treated with flg22 for 2 h was set to 100 %. Every bar represents the average ± SEM of

(A), (C), (E), (G) n = 9 samples derived from three independent experiments

(B), (D), (F), (H) n = 6 samples derived from two independent experiments

Asterisks represent significant differences between Col-0 and *tga2,5,6* plants within a treatment (Student's t-test, ***P < 0.001; **P < 0.01; *P < 0.05)

The activation of *WRKY22* proceeds in a transient manner, whereas the expression of WRKY29 is a more long lasting effect (Figure 4-11E+F). *WRKY29* is also hyper-inducible in the *tga2,5,6* mutant and reaches wild-type levels after 12 hours. *WRKY22* and *WRKY29* are able to activate the *FRK1* promoter directly (Asai et al., 2002). The expression data confirms with this situation, since the transcriptional activation of *FRK1* starts after 60 min (Figure 4-11G+H) at a time point where *WRKY22* and *WRKY29* are still expressed. In the *tga2,5,6* mutant the *FRK1* expression is not only stronger than in Col-0 plants, in addition the expression starts earlier (after 30 min instead of 60 min in Col-0).

To investigate, wether other PAMPs induce an increased expression of flg22-induced genes in the *tga2,5,6* mutant, seedlings were treated with the bacterial PAMP elf18 and the fungal elicitor chitosan to analyze *FRK1* expression after 2h of treatment (Figure 4-12). Indeed, a treatment with each of the PAMPs tested induced a hyper-activation of *FRK1*-expression. Elf18 induces *FRK1*-expression to a level comparable with flg22-induction. A slightly enhanced response to chitosan was detected in *tga2,5,6* plants, while the *FRK1*-expression in Col-0 plants is close to background levels. This result corresponds to the weak ROS burst induced by chitosan (4-7D).

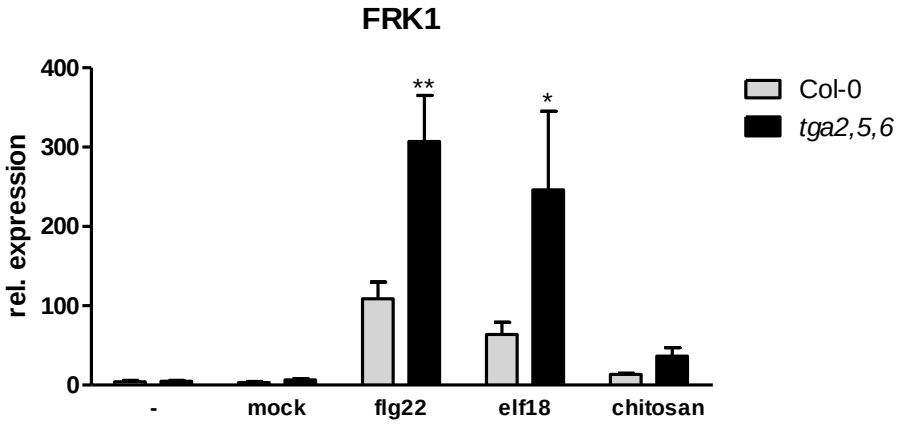

Figure 4-12| **Expression analysis of *FRK1* in Col-0 and *tga2,5,6* seedlings after PAMP treatment.** 10-14-days-old Col-0 (gray bars) and *tga2,5,6* (black bars) *A. thaliana* seedlings grown on 1MS+MES medium were spray treated with H_2O (mock), 1µM flg22, 1µM elf18 or 100 mg/L chitosan. After two hours of treatment, approximately 50 seedlings were harvested for RNA extraction. Transcript levels were quantified by qRT-PCR with specific primers against *FRK1* and normalized to relative expression in comparison to the house keeping gene *UBQ5*. The expression level in flg22-treated Col-0 seedlings was set to 100 %. The average ± SEM of n = 5 samples is shown. The experiment was replicated once with similar results. Asterisks represent significant differences between Col-0 and *tga2,5,6* plants within a treatment (2wayAnova, **P < 0.01; *P < 0.05)

4.5 The effect of flg22 on late cell wall-based defense responses is fortified in the *tga2,5,6* mutant

Another typical PAMP response is callose deposition. Flg22-induced callose responses were monitored in *Arabidopsis* Col-0 and *tga2,5,6* seedlings grown under the same conditions as described for the root growth assay. Callose is a ß(1,3) glucan polymer which is deposited at sites of fungal or bacterial entry and is described as a comparatively late defense-associated response. Staining with aniline blue was used to visualize callose (Figure 4-13). Fluorescent deposits on the cotyledons of Col-0 seedlings treated with 1 µM flg22 were observed that were absent in water treated plants. A mutant of the callose synthase encoded by the *PMR4* gene did not respond to flg22 treatment, demonstrating that the appearance of these fluorescent deposits depends on PMR4. In comparison to wild-type seedlings, the *tga2,5,6* mutant shows a much stronger callose deposition after flg22 treatment. Furthermore, non-induced *tga2,5,6* cotyledons exhibit significantly more callose spots. *The pmr4-1/tga2,5,6* quadruple mutant behaves like the *pmr4-1* single mutant, indicating that the enhanced callose deposition in the *tga2,5,6* mutant depends exclusively on PMR4.

QRT-PCR was performed, to test whether *PMR4*, *CYP81F2* and *CYP79B2* genes, involved in flg22-induced callose deposition, are transcriptionally hyper-activated like the early defense genes *FRK1*, *WRKY22* and *WRKY29*. Early expression of *PMR4* is not altered in the *tga2,5,6* mutant (Figure 4-14A). However, no clear results could be obtained for the two hours time point. Together with the significantly enhanced transcript levels 24 hours after flg22-treatment a slight hype-ractivation of *PMR4* in the *tga2,5,6* mutant could be observed (Figure 4-14B). The flg22-triggered callose response in *Arabidopsis* seedlings requires ET- and MYB51-dependent I3G biosynthesis by cytochrome CYP79B2 and CYP81F2-dependent 4-methoxylation of IGS (Clay et al., 2009). The transcript levels of *CYP79B2* and *CYP81F2* are elevated in the *tga2,5,6* mutant but display different kinetics. Whereas *CYP81F2* expression is most activated after one hour (Figure 4-14C+D), the expression of *CYP79B2* starts later with a maximum after 12 hours and is a more long lasting effect (Figure 4-14E+F). In conclusion, the fortified callose deposition might be due to transcriptional hyper-activation of *CYP81F2* and *CYP79B2*.

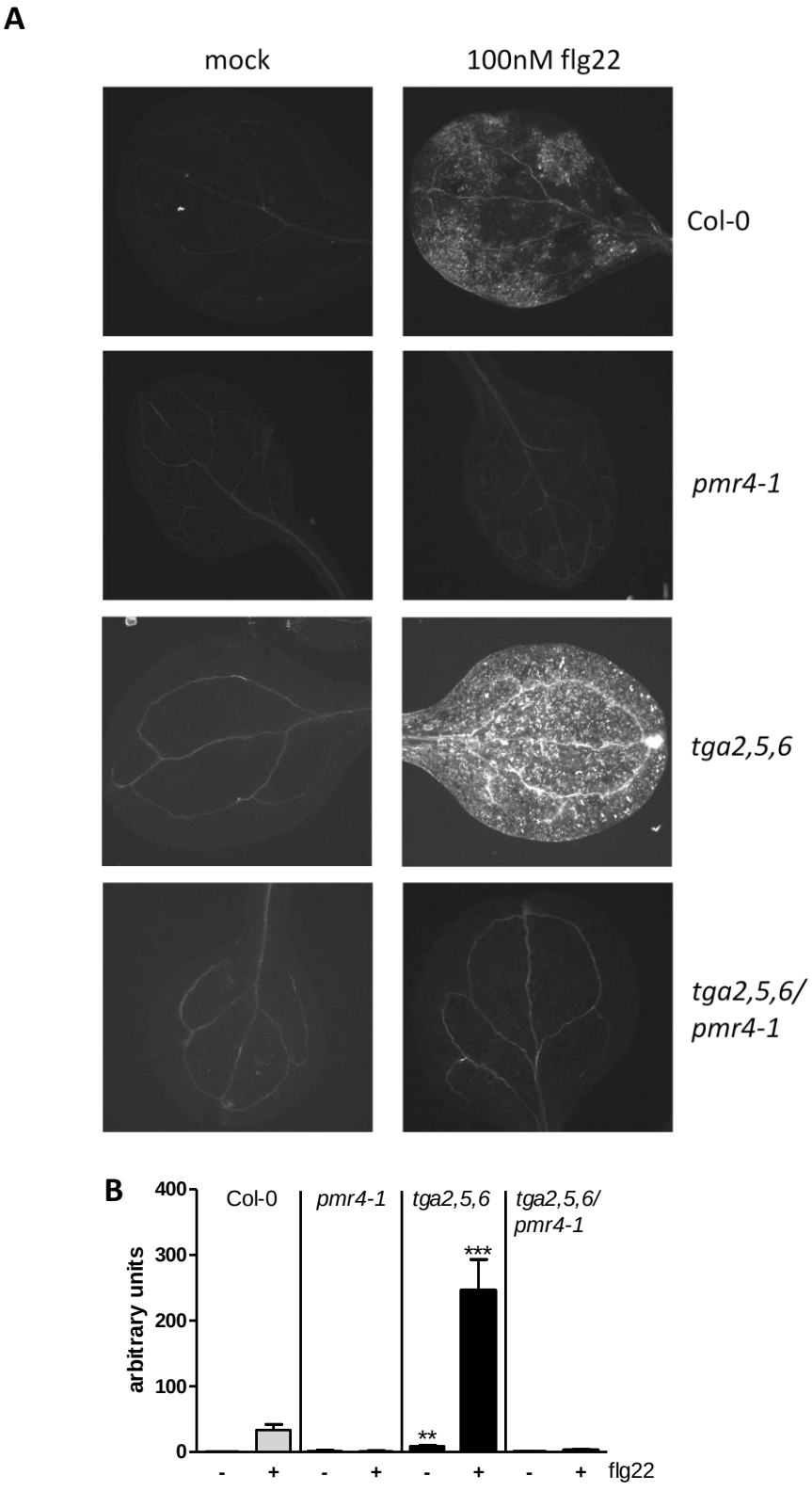

Figure 4-13| **Callose deposition in Col-0, *pmr4-1*, *tga2,5,6* and *tga2,5,6/pmr4-1* seedlings.**
(A) Aniline blue staining of cotyledons from 14 days old seedlings treated with 1 µM flg22. After 24h, leaves were stained for callose by aniline blue and fluorescence was detected under UV light (filtercube A; 25x magnification).
(B) Quantification of callose deposition. The average ± SEM of callose deposits from n = 10 independent cotyledons is shown. Asterisks represent significant differences between wild-type and *tga256* plants within a treatment (Student's t-test, ***P < 0.001; **P < 0.01).

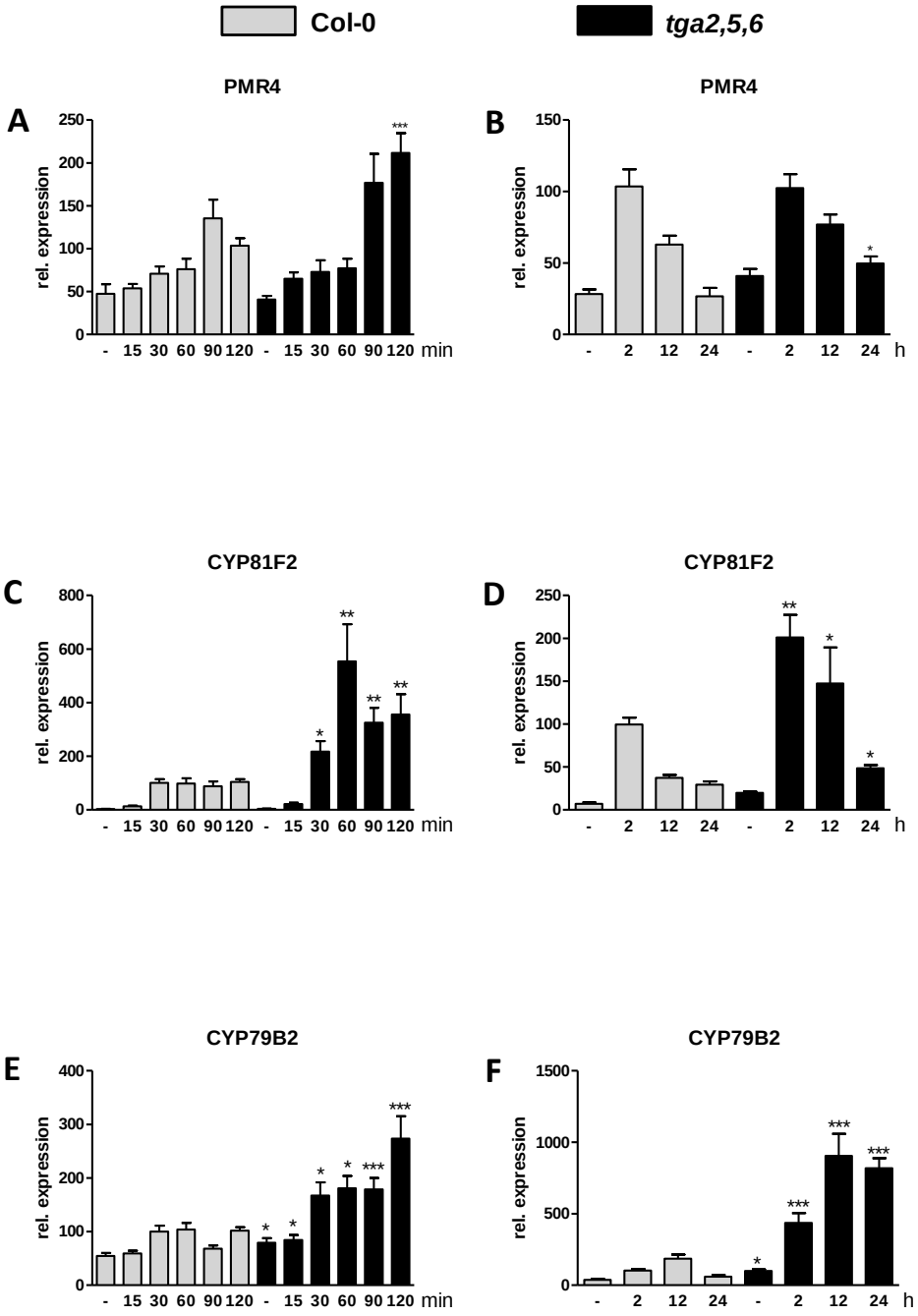

Figure 4-14| **Expression analysis of genes involved in flg22-induced callose deposition in Col-0 and *tga2,5,6* seedlings.**

10-14-days-old Col-0 (gray bars) and *tga2,5,6* (black bars) *A. thaliana* seedlings grown on 1MS+MES medium were spray treated with H_2O (mock) or 100 nM flg22 in two different time course experiments. Approximately 50 seedlings were harvested for RNA extraction after the indicated time points. Transcript levels were quantified by qRT-PCR with specific primers against *PMR4*, *CYP81F2* and *CYP79B2* and normalized to the house keeping gene *UBQ5*. The expression level in Col-0 seedlings treated for 2h with flg22 was set to 100 %.

Every bar represents the average ± SEM of

(A), (C), (E) n = 9 samples derived from three independent experiments

(B), (D), (F) n = 6 samples derived from two independent experiments

Asterisks represent significant differences between Col-0 and *tga2,5,6* plants within a treatment (Student's t-test, ***P < 0.001; **P < 0.01; *P < 0.05)

4.6 The *tga2,5,6* mutant fails to develop callose deposition after wounding

To exclude that the increased callose deposition in the *tga2,5,6* mutant is an unspecific effect, callose deposition was stimulated by wounding the leaves with a syringe or forceps in seedlings or soil grown plants (Figure 4-15). In wild type leaves, callose deposition develops around the wounding sites. In the *tga2,5,6* mutant, callose deposition after wounding is hardly detectable. The signal measured by the quantification with AIDA© (Figure 4-15C+D) shows callose deposition predominantly next to leaf veins. This result not only indicates that an increase of callose deposition in the *tga2,5,6* mutant is specific for PAMP induction, moreover the *tga2,5,6* mutant fails to develop callose deposits after wounding.

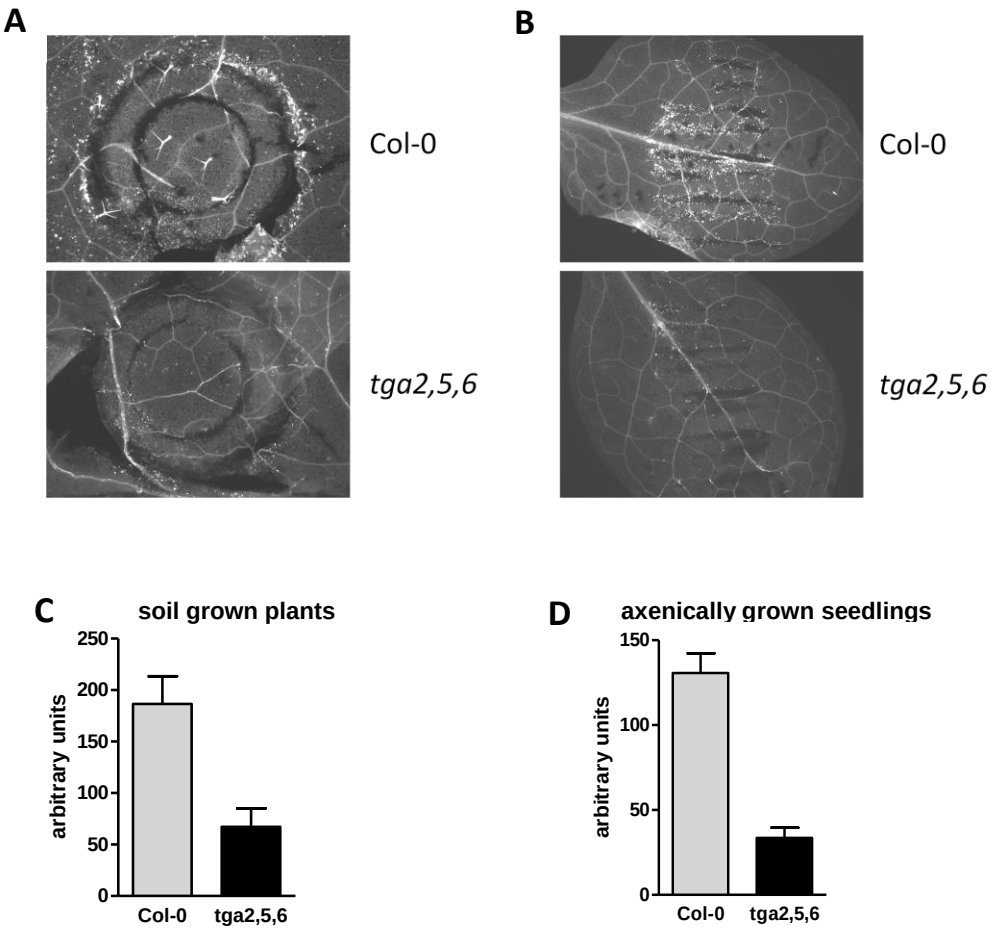

Figure 4-15| **Callose deposition in Col-0 and *tga2,5,6* leaves after wounding.**
(A) 4 week old soil grown plants were wounded by forceps or stamping with a syringe without needle or (B) 14 days old, axenically grown seedlings. After 24h, leaves were stained for callose by aniline blue and fluorescence was photographed under UV light (filtercube A; 25x magnification). (C) + (D) Quantification of callose deposition. The average ± SEM of fluorescence from n = 12 leaves is shown. The experiment was repeated twice with similar results.

4.7 *tga2,5,6* mutants show no altered callose deposition after bacterial infection

Bacterial plant pathogens do not only present PAMPs that are recognized by the host plant, furthermore, they secrete many effectors with different outcomes. In order to assess whether the increased callose deposition in the *tga2,5,6* mutant occurs after bacterial infection, soil grown plants were syringe infiltrated with different *Pseudomonas syringae* strains. The virulent *Pst* DC3000 strain and the COR- strain (missing the bacterial phytotoxin coronatine) induce a weak callose deposition (Figure 4-16). The hrpA-strain, impaired in TTSS induce a strong callose deposition in Col-0 and *tga2,5,6* leaves. This result fits the observation that elicitors secreted by the TTSS suppress callose deposition (Underwood et al., 2007). No significant difference could be observed between Col-0 and the *tga2,5,6* mutant.

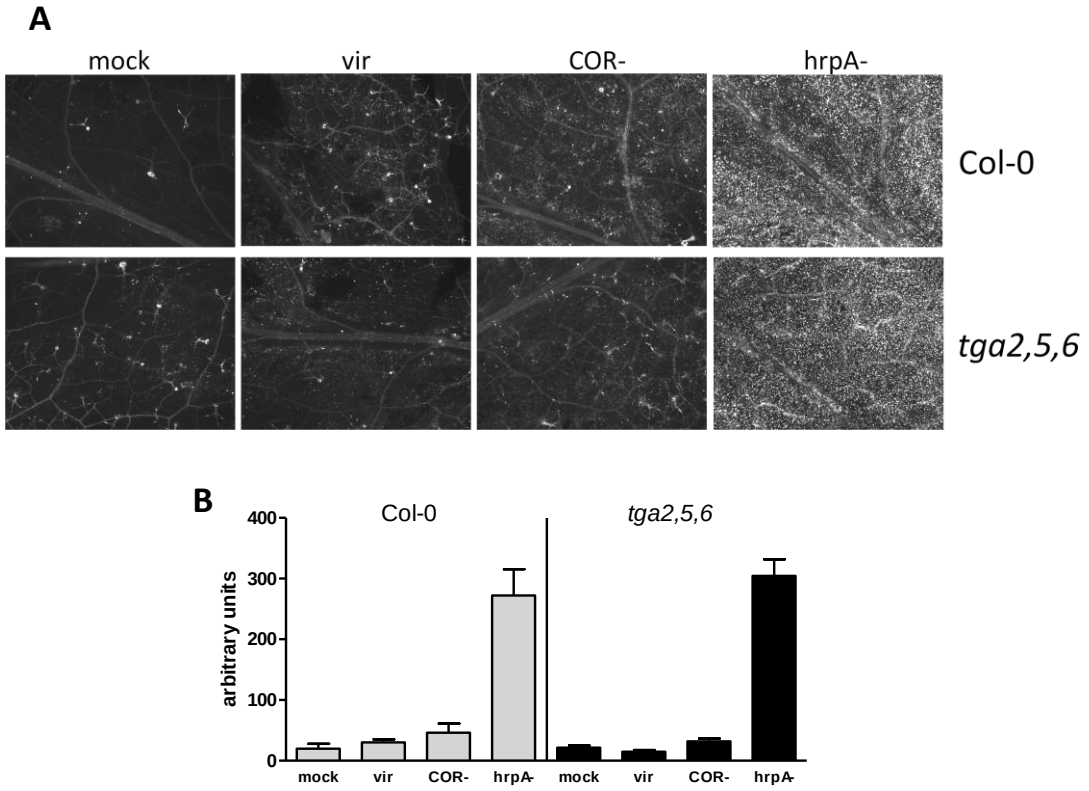

Figure 4-16| **Callose deposition in Col-0, *pmr4-1*, *tga2,5,6* and *tga2,5,6/pmr4-1* leaves after infection with different *Pseudomonas syringae* strains.**
(A) The leaf lower surface of 4-weeks-old soil grown plants were infiltrated with *Pst* DC3000 (vir) or *Pst* DC3000 COR$^-$ (cor-) or *Pst* DC3000 hrpA- (hrpA-) (OD_{600}= 0,02) using a syringe. After 24h, leaves were stained for callose by aniline blue and fluorescence was photographed under UV light (filtercube A; 25x magnification).
(B) Quantification of callose deposition. The average ± SEM of fluorescence from at least n = 6 leaves is shown.

4.8 *tga2,5,6* mutant plants are insensitive to coronatine (COR) triggered stomatal closure

During infection, stomata can serve as passive openings for bacterial entry. It was shown that stomatal guard cells are able to perceive bacterial PAMPs like flg22 leading to stomatal closure as a defense response (Melotto et al., 2006). In turn, the phytotoxin coronatine produced by several bacteria like *P. syringae*, is able to circumvent this innate immune response (Melotto et al., 2006). Also, MeJA-treatment completely suppresses the flg22-induced callose response (Clay et al., 2009). One hypothesis, considering the antagonism between JA and SA pathways, is that COR promotes susceptibility to *P. syringae* infection by stimulating JA signaling in plants, thereby inhibiting SA-mediated defenses that normally limit growth of *P. syringae* within host tissues. Class-II TGA factors are not only necessary for the establishment of SAR (Zhang et al., 2003); moreover they are important activators of JA/ET-induced responses (Zander et al., 2009).

To determine whether stomata of the *tga2,5,6* mutant show altered response to flg22 and COR, leaf slices of adult soil grown plant were incubated with flg22 and COR. After 3 hours, the stomatal apertures of epidermal peals were measured. Whereas in control treated leaves most stomata are open, a marked reduction of stomatal aperture can be observed after 3 hours of flg22-incubation (Figure 4-17). If COR was added to the incubation solution, no stomatal closure can be observed any more in wild-type plants. The stomata of *tga2,5,6* plants are insensitive to COR. Stomata from leaf slices incubated in a solution containing flg22 and COR are predominantly closed, similar to treatment with flg22 alone.

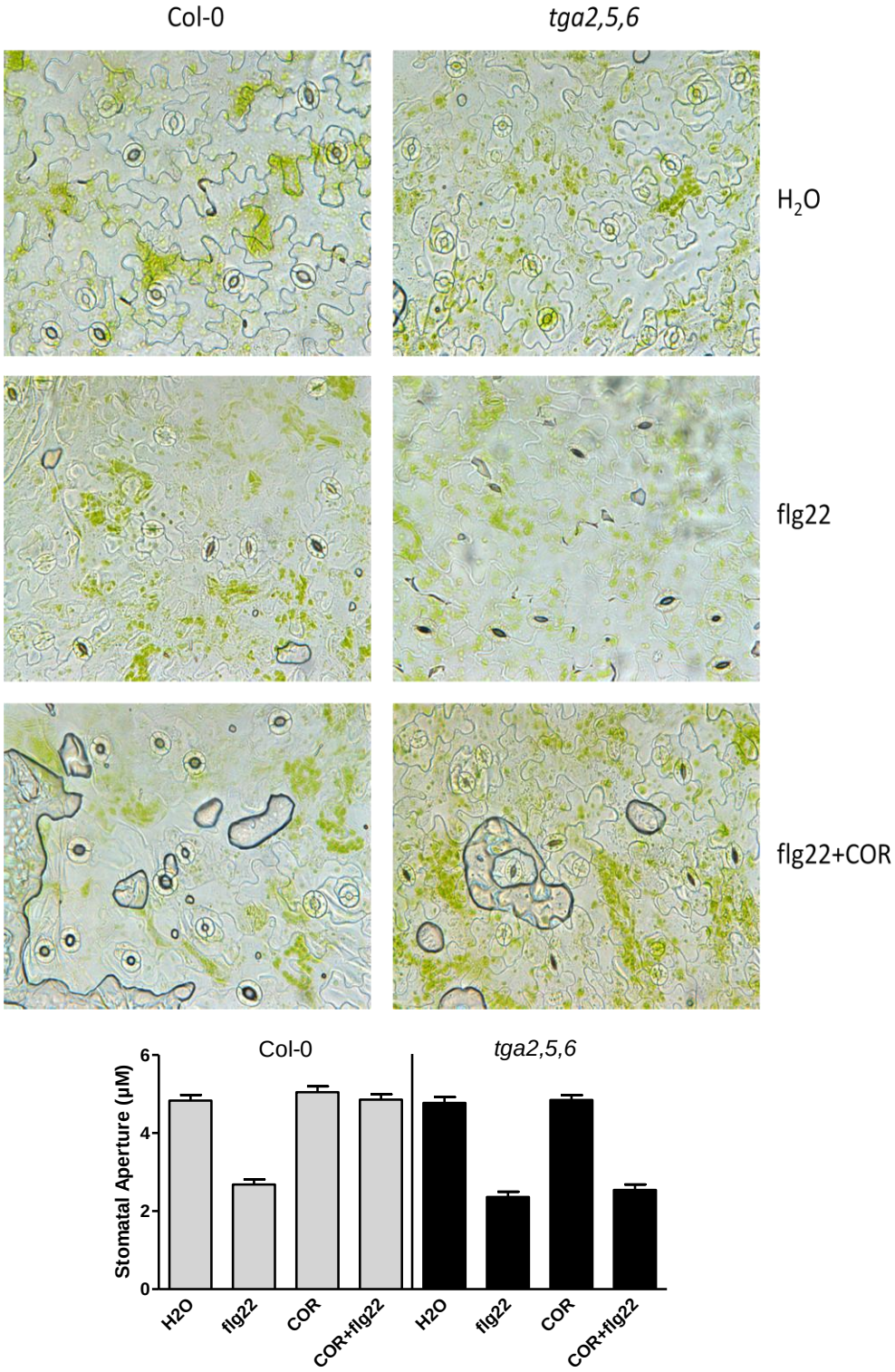

Figure 4-17| **Stomatal closure after treatment with flg22 and flg22/ COR in Col-0 and *tga2,5,6* plants.**

(A) Leaf slices of 6 week old soil grown plants were floated with H_2O, 5 µM flg22 or flg22 in combination with 0,5 ng/µl COR. After 3 h, epidermis was fixed by applying the leaf lower surface on ultra clear adhesive tape and observed under a microscope (bright field; 400x magnification). (B) Quantification of stomatal aperture shown in (A). The average ± SEM from n = 100 stomata derived from two independent experiments is shown.

To investigate, wether the stomatal closure also occurs in response to virulent bacteria, leaf slices were incubated with the virulent *Psm* ES4326. After one hour of incubation with *Psm* ES4326 a stomatal closure occurs in Col-0 and *tga2,5,6* plants to the same degree as after flg22 incubation (Figure 4-18). Interestingly, after three hours of incubation, when the bacteria had time to release COR, the stomata of wild-type plants are re-opened, whereas the stomata of *tga2,5,6* plants are still closed. The inhibitory effect of COR on PAMP induced stomatal closure is abolished in both experimental set ups, after treatment with the isolated substances or in the more biological system by infection with bacteria.

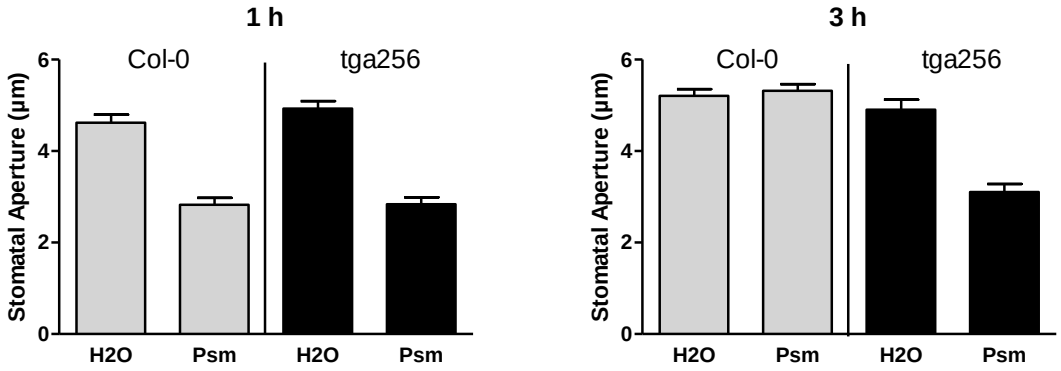

Figure 4-18| **Stomatal closure after treatment with *Psm* ES4326 in Col-0 and *tga2,5,6* plants.** Leaf slices were exposed to water or virulent *Psm* ES4326 (OD_{600}=0.2). After 1 h and 3 h, epidermis was fixed by applying the leaf lower surface on ultra clear adhesive tape and observed under a microscope (bright field; 400x magnification). The average ± SEM from n = 100 stomata derived from two independent experiments is shown.

Next, it was tested if the ectopic expression of one TGA transcription factor is sufficient to restore the wildtype-like stomatal response to flg22 and COR or exposition to virulent *P. syringae*. All plants accomplish a stomatal closure reaction 3h after treatment with flg22 or 1h after incubation in the presence of *Psm* ES4326 (Figure 4-19). Ectopic expression of TGA2 under the control of the *35S* promoter in the *tga2,5,6* mutant (here shown for line TGA2OE#7) leads to a wildtype-like response to COR+flg22 regarding stomatal re-opening (Figure 4-19A), demonstrating that TGA2 is sufficient to complement the phenotype in the *tga2,5,6* mutant. However, plants ectopically expressing TGA5 show only a partial complementation of the phenotype.

As shown for the pharmacological assay, ectopic expression of TGA2 is also sufficient to rescue the wild-type like stomatal re-opening 3h after treatment with *Psm* ES4326, while TGA5 is not able to fulfill this function (Figure 4-19B).

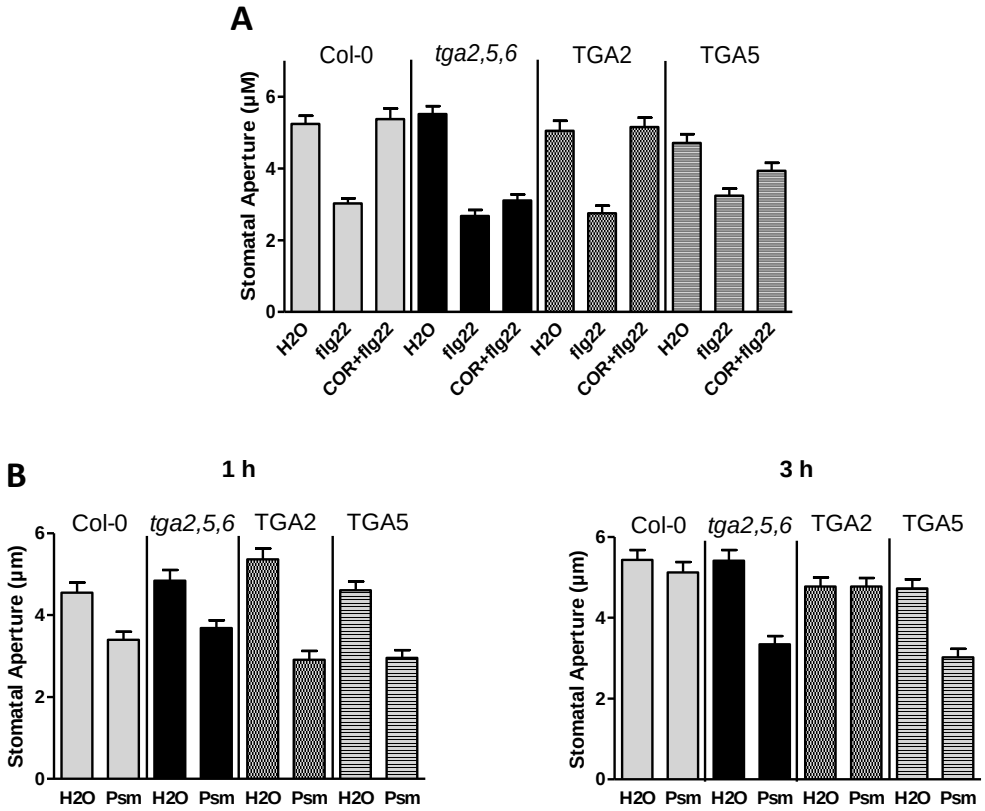

Figure 4-19 **Stomatal closure in Col-0, *tga2,5,6*, *TGA2OE* and *TGA5OE* plants.**
(A) Leaf slices of 6-weeks-old soil grown plants were floated with H_2O and 5 µM flg22 or flg22 in combination with 0,5 ng/µl COR. After 3 h, epidermis was fixed by applying the leaf lower surface on ultra clear adhesive tape and observed under a microscope (bright field; 400x magnification).
(B) Leaf slices of 6-weeks-old soil grown plants were floated with H_2O and virulent *Psm* ES4326 (OD_{600}=0.2). After 1 h and 3 h, epidermis was fixed by applying the leaf lower surface on ultra clear adhesive tape and observed under a microscope (bright field; 400x magnification). The average ± SEM from n = 50 stomata is shown.

4.9 Class-II TGA factors are involved in flg22-triggered defense against *Pst* DC3000

In this thesis, it could be pointed out that class-II TGA transcription factors act as negative regulators of PAMP-triggered responses. Furthermore, they are important positive regulators in SA-signaling leading to SAR. It was shown that interplay between PAMP-triggered and SA-mediated defense responses exists (Tsuda et al., 2008). Pre-treatment with flg22 induces an SAR-like defense in *Arabidopsis* (Zipfel et al., 2004) with SA accumulation in local and systemic leaves (Mishina and Zeier, 2007). Due to this fact, the bacterial titers in *tga2,5,6* and *tga2,5,6/sid2-2* mutants were determined. The use of the quadruple mutant *tga2,5,6*/*sid2-2* in comparison to *sid2-2* is necessary to investigate the

function of class-II TGA factors independently of SA to distinguish between PAMP- or SA-dependent effects.

The bacterial growth assay was performed in collaboration with Jane Glazebrook, (University of Minnesota). To induce PAMP-triggered defense, 5 week old plants were infiltrated with 1 µM flg22. Control plants were treated with water. After 1 day, leaves were infiltrated with a suspension of *Pst* DC3000 bacteria (OD_{600} = 0.0001). The bacterial titer was measured at 0 and 2 days after inoculation (dpi) (Figure 4-20). Comparison of bacterial growth between mock pre-treated and flg22 pre-treated wild-type plants at 2 dpi confirmed the previous observation that pre-treatment with flg22 confers resistance to *Pst* DC3000 (Zipfel et al., 2004). Bacterial titers in mock pre-treated *tga2,5,6* plants are similar to the wild type. *sid2-2* and *tga2,5,6*/*sid2-2* plants were slightly different from mock pre-treated Col-0 at 2 dpi, illustrating the enhanced susceptibility phenotypes of these mutants, founded in the absence of SA-dependent defense responses. The bacterial titers in flg22 pre-treated *tga2,5,6*, *sid2-2* and *tga2,5,6/sid2-2* plants were clearly higher than flg22 pre-treated Col-0 at 2 dpi. Importantly, *sid2-2* displays the most pronounced susceptibility to *Pst* DC3000, significantly higher than *tga2,5,6* and *tga2,5,6/sid2-2* plants. If SA biosynthesis and class-II TGA factors are absent, the plants become more resistant to *Pst* DC3000, confirming the hypothesis that in the absence of SA, class-II TGA factors negatively regulate PAMP-triggered response. However, there was still a large difference between mock and flg22 pre-treated plants 2 dpi, indicating that the effects of flg22 pre-treatment on resistance to *Pst* DC3000 are only partially dependent on SA signaling and class-II TGA factors.

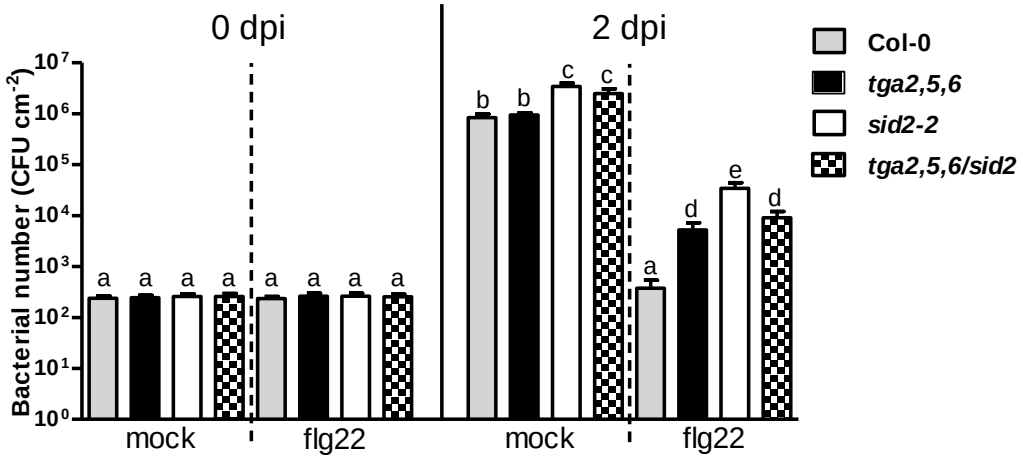

Figure 4-20| **Influence of flg22 on bacterial growth.**
Pst DC3000 bacterial suspension (OD_{600}= 0.0001) was infiltrated into Col-0, *tga2,5,6*, *sid2-2* and *tga2,5,6/sid2-2* plants 1 day after treatment with water (mock) or 1 µM flg22 (flg22). The average ±SEM from three independent experiments is shown. Significant differences are indicated by different letters (Student's t-test, P < 0.05).

4.10 COR suppresses flg22-induced *FRK1* expression in Col-0 and *tga2,5,6* mutant

As COR is not able to suppress flg22-induced stomatal closure in the *tga2,5,6* mutant, it was important to know, if COR has an inhibitory effect on PAMP induced expression of defense genes and if this effect takes place in the *tga2,5,6* mutant. To prove this hypothesis, seedlings were spray-induced with COR, flg22 and flg22/COR for 2 hours. The isolated RNA was used for qRT-PCR to analyze the expression levels of *FRK1* after the different treatments (Figure 4-21). COR alone is not able to induce *FRK1*. In comparison to flg22 treatment alone, simultaneous application of flg22 together with COR leads to a weaker *FRK1* expression in wild-type and *tga2,5,6* plants. This reduction of *FRK1* transcript levels by COR is eminently pronounced in the *tga2,5,6* mutant, only reaching flg22-induced wild-type expression levels.

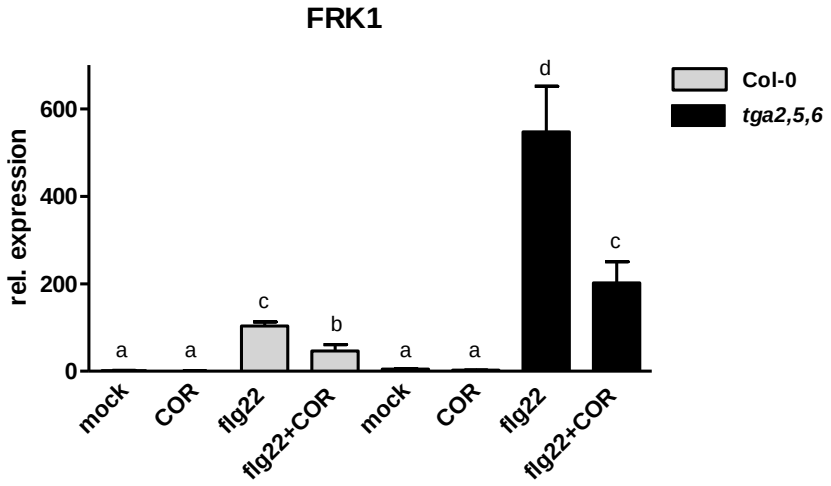

Figure 4-21| **Expression analysis of *FRK1* in Col-0 and *tga2,5,6* seedlings after treatment with flg22 and COR.**
RNA was extracted from Col-0 (gray bars) and *tga2,5,6* (black bars) seedlings harvested 2 h after treatment with 1 µM flg22, 5 µM COR or a combination of both as indicated. Transcript levels were quantified by qRT-PCR with specific primer against *FRK1* and normalized to the house keeping gene *UBQ5*. Expression in Col-0 seedlings that were flg22-treated for 2h was set to 100 %. The average ± SEM of n = 12 samples derived from four independent experiments is shown. Asterisks represent significant differences between two treatments in comparison to the wild-type (Student's t-test, $P^{***} < 0{,}001$)

4.11 SA and flg22 act synergistically on *FRK1*-expression in Col-0 seedlings

To prove wether application of SA has an opposite effect on FRK1 expression as described for COR, a similar experimental setup was chosen as used for the COR+flg22 treatments. The only remarkable difference is the application of SA 24 hours before flg22 treatment, to ensure that SA-dependent responses are activated, like it is the case in primed plants. SA alone activates *FRK1* expression only very slightly (Figure 4-22). However, a pre-treatment with SA in combination with flg22 leads to an enhanced expression level of *FRK1* in Col-0 seedlings. This difference is significant but transcript levels are not as high as in the *tga2,5,6* mutant. No effect of SA on *FRK1* expression was detectable in the mutant seedlings, assuming a constitutive primed status of the *tga2,5,6* mutant.

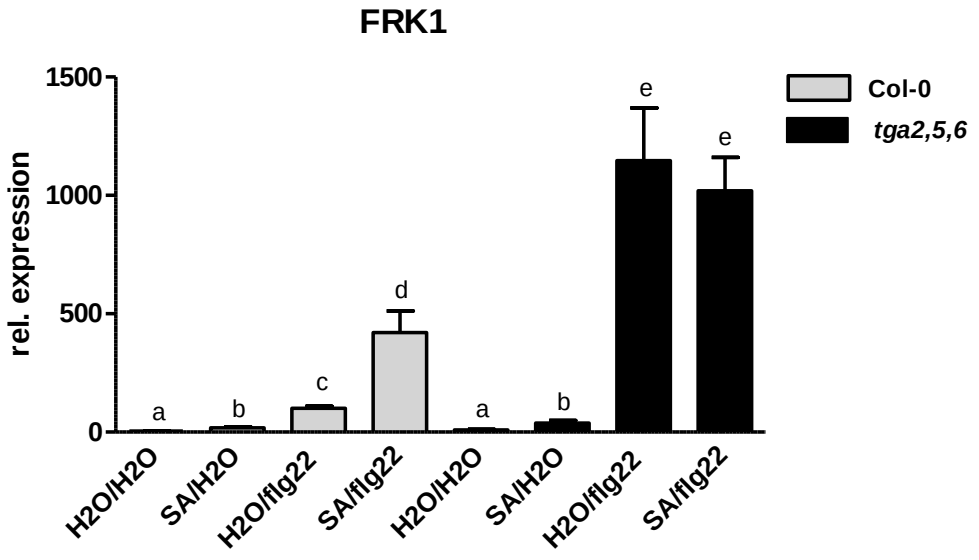

Figure 4-22| **Expression analysis of *FRK1* in Col-0 and *tga2,5,6* seedlings after treatment with SA and flg22.**

10-14-days-old Col-0 (gray bars) and *tga2,5,6* (black bars) seedlings were spray-inoculated with H_2O or 1 mM SA. After 24h, a second spray-inoculation with H_2O or 1 µM flg22 occurs. RNA was isolated two hours after the second treatment. Transcript levels were quantified by qRT-PCR with specific primer against *FRK1* and normalized to the house keeping gene *UBQ5*. Expression in Col-0 seedlings flg22-treated for 2h was set to 100 %. The average ± SEM of n = 8 samples derived from three independent experiments is shown. Similar letters indicate significant differences between treatments or genotypes (Student's t-test, $P < 0,01$).

Recently, it was demonstrated that PAMPs induce SA accumulation within 6 h in a SID2-dependent manner (Tsuda et al., 2008). SA levels were measured in Col-0 and *tga2,5,6* seedlings with and without flg22-treatment (Figure 4-23). Flg22 treatment leads to slightly enhanced SA levels. Treated and untreated *tga2,5,6* seedlings contain higher levels of SA as the wild-type, but no significant differences could be observed.

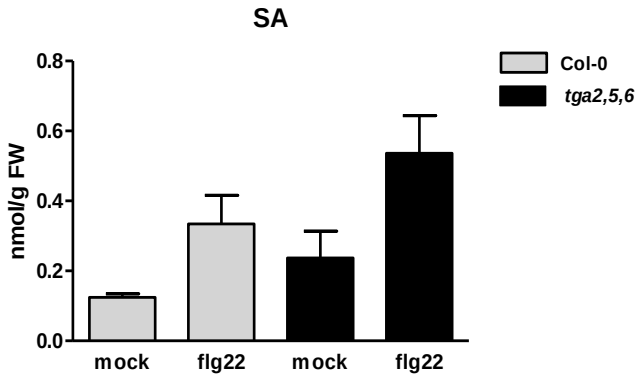

Figure 4-23| **Measurement of SA levels by HPLC.**
Col-0 (gray bars) and *tga2,5,6* (black bars) seedlings were spray-inoculated with 1 µM flg22 for 2 h. Every bar represents the average ± SEM of n = 6 samples derived from two independent experiments.

In the *tga2,5,6* mutant, increased basal transcript levels of the SA-inducible *PR-1* gene were known from the literature (Zhang et al., 2003; Blanco et al., 2009). To elucidate whether the *tga2,5,6* mutant also shows enhanced basal expression of *ICS1*, which is responsible for pathogen-induced SA biosynthesis, the expression levels of *ICS1* and *PR-1* were analyzed by qRT-PCR (Figure 4-24). The basal transcript levels of *ICS1* are 1.5 times enhanced, and after flg22-treatment, the *ICS1*-expression is hyperactivated in the *tga2,5,6* mutant (Figure 4-24A). The same result, but much more pronounced could be observed for *PR-1* (Figure 4-24B). The basal *PR-1* expression in *tga2,5,6* plants is 100-fold higher than in wild-type plants. Treatment with flg22 leads to an even stronger expression of *PR-1* up to around 700-fold higher than the wild type 24 hours after treatment suggesting that SA-dependent basal defense responses might also be enhanced in the *tga2,5,6* mutant.

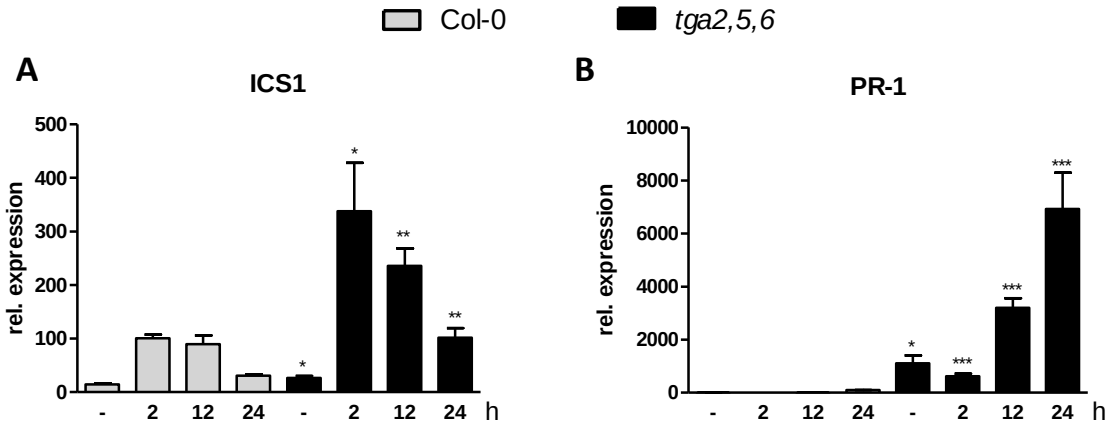

Figure 4-24| **Expression analysis of *ICS1* and *PR-1* in Col-0 and *tga2,5,6* seedlings.**
10-14-days-old Col-0 (gray bars) and *tga2,5,6* (black bars) *A. thaliana* seedlings grown on 1MS+MES medium were spray treated with H_2O (mock) or 100 nM flg22 in a time course experiment. Approximately 50 seedlings were harvested for RNA extraction after the indicated time points. Transcript levels were quantified by qRT-PCR with specific primers against *ICS1* (A) and *PR-1* (B) and normalized to the house keeping gene *UBQ5*.
(A) *ICS1*expression. Col-0 2h flg22 was set to 100%
(B) *PR-1* expression. Col-0 24h flg22 was set to 100%.
Every bar represents the average ± SEM of n = 6 samples derived from two independent experiments. Asterisks represent significant differences between Col-0 and *tga2,5,6* plants within a treatment (Student's t-test, ***P < 0.001; **P < 0.01; *P < 0.05).

4.12 Expression of *FRK1* is not influenced in mutants of the SA or JA pathway

To investigate if an imbalance of the phytohormones SA or JA is the reason for the enhanced basal defense responses in the *tga2,5,6* mutant, qRT-PCR analysis were done with mutants impaired in SA-biosynthesis (*sid2-2*), JA-biosynthesis (*aos*) or SA signaling and SA/JA cross-talk (*npr1-1*) (Figure 4-25). None of the tested mutants show altered *FRK1* transcript levels.

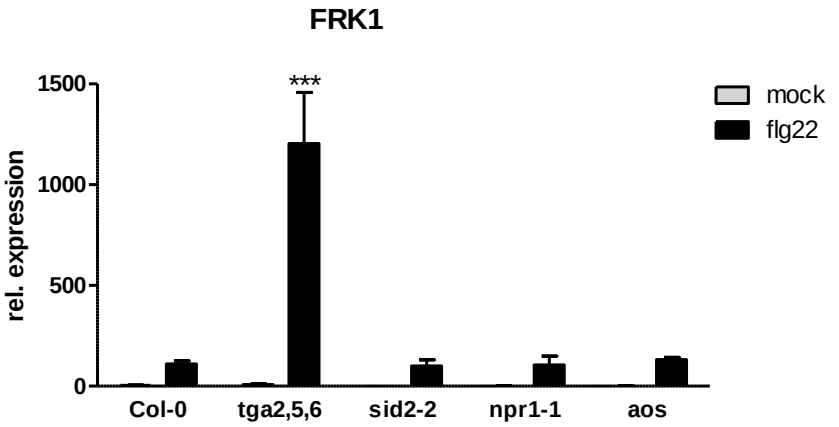

Figure 4-25| **flg22-induced *FRK1* expression in Col-0 and different mutants of the SA or JA pathways.**
10-14-days-old *A. thaliana* seedlings of ecotype Col-0, *tga2,5,6, sid2-2*, *npr1-1* and *aos* grown on 1MS-MES medium were spray inoculated with 1µM flg22. Two hours after treatment the seedlings were harvested for RNA extraction. Transcript levels were quantified by qRT-PCR with specific primers against *FRK1* and normalized to the house keeping gene *UBQ5*. Every bar represents the average ± SEM of n = 6 samples derived from two independent experiments. Asterisks represent significant differences between Col-0 and mutant within a treatment (Student's t-test, ***P < 0.001).

4.13 TGA-dependent suppression of early flg22-induced genes occurs indirectly

To investigate whether TGA2 or TGA5 are recruited directly to the promoter regions of the flg22-induced genes up-regulated in the *tga2,5,6* mutant, chromatin-immunoprecipitation (ChIP) analyses were done. ChIP is a powerful method to study the *in vivo* binding of transcription factors to target motives in the genome. For this, leaf tissue of Col-0 and *tga2,5,6* mutant plants was treated with formaldehyde to crosslink promoter-associated proteins to the DNA. After isolation and shearing of the chromatin, protein-DNA complexes were immunoprecipitated with antiserum generated against TGA2 and TGA5 (Fode et al., 2008).

In collaboration with Christopher Town it was possible to analyse Col-0 ChIP-DNA (untreated and treated with SA for 2 hours) with a mini array spotted with 200 putative target promoters for TGA2. From the flg22-inducible and in the *tga2,5,6* mutant up-regulated genes, only *PR-1*, *WRKY22* and *WRKY29* were spotted on the array (Table 4-1 and supplemental data). *PR-1* and *GSTU7* are depicted as positive controls, because it was shown previously that TGA2 and TGA5 bind to the related promoter regions (Fode et al., 2008). In comparison to raw chromatin, no increased binding of class-II TGAs to the analyzed promoter regions of *WRKY22* and *WRKY29* is detectable. A sequence analysis of

the promoter regions (2000 bps upstream of the translation start) reveals no putative *as-1-like* element but some TGACGTCA-motives. It was shown that members of the TGA family are able to bind to a single TGACGTCA motive *in vitro* but TGA2 is not included (Lam and Lam, 1995). Remarkably, several other WRKY transcription factors could be identified as putative targets for class-II TGA factors (Table 4-2).

Table 4-1| **Promoter analysis of *WRKY22* and *WRKY29* for putative TGA binding sites and signals from the ChIP-on-chip array.**

Gene	AGI code	*as-1 like* element	TGACGTCA motive	IP/RAW (avg)
WRKY22	At4g01250	-	-317 TGACGgat -310 -380 TGACGgat -373 -779 aatCGTCA -772	0.90 ± 0.03
WRKY29	At4g23550	-	- 95 TctCGTCA -88 -568 TGACGcCA -561	0.57 ± 0.16
GSTU7	At2g29420	+	- 64 gttCGTCActggTGACGTCA -45	1.83 ± 0,04
PR-1	At2g14610	+	-665 ctACGTCActattttacTtACGTCA -641	1.19 ±0,13

Chromatin immunoprecipitated with antiserum against TGA2,5 was hybridized to a mini array representing 200 putative target promoters for TGA2 (cooperation with C. Town, Institute for Genomic Research, Rockwille, USA). The average ± SD from three independent experiments, normalized to the signal derived from raw-chromatin, is shown. A detailed array analysis of putative target of class-II TGAs is listed in supplemental data.

Table 4-2| **Promoter analysis of WRKY factors for TGA binding sites enriched in the ChIP-on-chip array.**

Gene	AGI code	*as-1 like* element	TGACGTCA motive	IP/RAW (avg)
WRKY62	At5g01900	+	-658 TGACGTCA -650 -682 TGAgGTCAccgaaccctACGTCA -659	2.88
WRKY54	At2g40750	-	-1419 TGaCGTCA -1411	2.30
WRKY51	At5g64810	+	-286 TGACGTCAtaacagaTGACGTCA -263	2.01
WRKY66	At1g80590	-	-	1.64
WRKY28	At4g18170	+	-1398 ctACGTCA -1390	1.64
WRKY47	At4g01720	+	-798 TGcCGTCA -790 -745 TcACGTCgtctttctcCGTCA -724	1.64
WRKY70	At3g56400	+	-225 ctACGTCAtttgagcTtACGTCA -202	1.55
WRKY67	At1g66550	-	-640 TGACGatA -632	1.27
WRKY6	At1g62300	+	-153 TGACGcaggatcTGACGTaA -133	1.25
WRKY59	At2g21900	-	-	1.21
WRKY48	At5g49520	-	-1519 ttACGTCA -1511 -850 TGACGTaA -842	1.16
WRKY11	At4g31550	-	-	1.15
WRKY38	At5g22570	+	-1250 TGACGTCA -1242 -1213 ctACGTCAtggggcTGACGTCg -1191	1.09
WRKY4	At1g13960	-	-	1.06
WRKY42	At4g04450	+	-568 TGACGTCgacacTGACGaaA -548	1.03
WRKY75	At5g13080	-	-1913 TGACGTCg -1905 -1220 gGACGTCA -1212	1.02

To verify the results of the ChIP-on-chip-array, the ChIP-DNA was analysed with real-time PCR (Figure 4-26). The template ChIP-DNA used for the PCR derived from two independent experiments. The DNA-template derived from the first experiment was shown to be suitable for IP in PCR analysis against *PR-1* and *GSTU7* described in Fode et al. (2008). The second DNA-template derived from an independent ChIP. The chromatin preparation used in this experiment was also positively tested for TGA2,5-binding to the *PR-1* and *GSTU7* promoter. The amount of PCR product is indicative for the relative amount of protein bound to the DNA. For *WRKY22*, primers flanking the first both TGACGTCA-motives (-317, -380) were used. A second primer pair includes the third motive (-779). For normalization, a PCR was performed using primers against the coding region of *ACTIN8*, which does not contain putative TGA binding motives. Chromatin including the amplified regions of the WRKY22 promoter isolated from *tga2,5,6* plants was precipitated with the same efficiency as from Col-0 plants. This confirms the result from the ChIP-on-chip array, suggesting that TGA factors indirectly regulate the expression of early flg22-inducible genes.

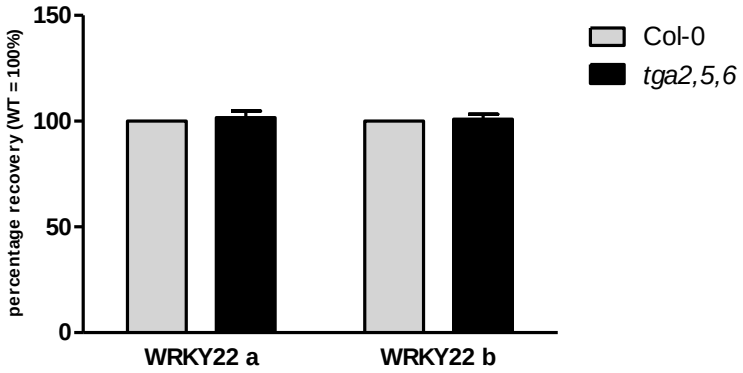

Figure 4-26| ***In vivo* TGA factor binding to the WRKY22 promoter region revealed by ChIP analysis**
Leaves from five-weeks-old (short-day) Col-0 plants and *tga2,5,6* mutants were incubated in 1% formaldehyde before chromatin preparation. Chromatin samples were subjected to immunoprecipitation using 5 µl of the αTGA2,5 antiserum. The DNA was recovered after reversal of the cross-links and analyzed for the enrichment of promoter sequences by quantitative real-time PCR using two different primer pairs including three putative TGA binding sites. *ACTIN8* was taken as reference for normalization. Bars represent the results from two independent experiments. Statistical analysis revealed no significant differences.

5 Discussion

TGA transcription factors TGA2, TGA5 and TGA6 are essential regulators of the SA-dependent defense response SAR (Zhang et al., 2003). Moreover, they are crucial for the activation of detoxification pathways upon chemical stress (Fode et al., 2008; Mueller et al., 2008) and indispensible for the induction of JA-inducible genes like *PDF1.2* and *b-Chi* under conditions of increased ET levels, and finally contribute to the defense against the necrotrophic pathogen *B. cinerea* (Zander et al., 2009). In this study, a combination of molecular, pharmacological, physiological and microscopic assays were used to determine a new role of class-II TGA transcription factors as negative regulators during plant basal defense responses. They act as important regulators for PTI responses like root growth inhibition, gene expression, callose deposition and stomatal closure. Furthermore, the *tga2,5,6* mutant is more resistant to *Pst* DC3000 in a *sid2-2* background.

5.1 flg22-induced growth inhibition is damped by class-II TGA factors

The effect of flg22 as a plant defense-activating elicitor was first described by Felix et al., 1999, who used alkalinization of tomato cell culture medium as a quantifiable defense response. The alkalinization response occurs also in other plant species, including *Arabidopsis thaliana* (Felix et al., 1999). Further studies revealed a negative effect of flg22 on plant growth. This growth inhibition depends on the presence of the FLS2 receptor indicating that it is due to the activation of the defense signaling cascade. Incubation of seedlings in the presence of flg22 resulted in dwarf plants, but the seedlings remained green and did not show necrosis (Gómez-Gómez et al., 1999).

Using root growth assays, a strong enhancement of flg22-mediated growth inhibition took place in the *tga2,5,6* triple mutant (e.g. Figure 4-2). This result establishes a so far unknown role for TGA factors in PAMP-mediated defense responses.

Little is known about mechanisms which are responsible for flg22-mediated growth inhibition. Previously, several mutants impaired in hormone signaling or biosynthesis were tested for their involvement in flg22-induced growth inhibition (Navarro et al., 2008). The signaling mutants *tir1-1* (auxin), *ein3-1* (ET) and *coi1-16* (JA) as well as the SA-biosynthesis

mutant *sid2-1* showed a wild type like growth inhibition. Exclusively, mutants involved in GA biosynthesis or signaling showed an altered growth inhibition (Navarro et al., 2008). In *Arabidopsis*, GA is perceived by AtGID1a/b/c GA receptors. Binding of GA to these receptors promotes AtGID1-DELLA protein-protein interactions, which in turn lead to polyubiquitination (through the E3 ubiquitin ligase SCF^{SLY1}) and subsequent degradation of DELLAs by the 26S proteasome (Jiang and Fu, 2007). Mutants that stabilize one or more DELLAs, including *sly1-10*, the dominant *gai*, and the GA-deficient *ga1-3*, showed a more pronounced flg22-induced growth inhibition, thus exhibiting the same phenotype as the *tga2,5,6* mutant. The loss of function mutant *gai-t6/rga-t2/rgl1-1/rgl2-1* (quadruple-DELLA mutant, deficient in GAI, RGA, RGL1, and RGL2 protein), was slightly less inhibited in growth by flg22 than the wild type controls (Navarro et al., 2008). Further, it was shown that flg22 treatment delayed GA-mediated degradation of a GFP-RGA fusion protein in roots. These results suggest that flg22-induced DELLA stabilization contributes to growth inhibition.

Interestingly, the GA deficient mutant *ga1-3* exhibits constitutively elevated transcript levels of defense related genes that are induced in the wild type only upon flg22 treatment (Navarro et al., 2008). This was shown by a comparison of microarray datasets generated from untreated *ga1-3* mutant (Cao et al., 2006) and wild-type *Arabidopsis* seedlings treated with flg22 (Zipfel et al., 2004). Approximately 30% of the transcripts were more elevated in the *ga1-3* mutant, as compared to La-er, were also upregulated by flg22 (Navarro et al., 2008).

To find out, if some of the putative downstream target genes of DELLAs in flg22-response are also putative target genes of TGA2, the genes identified by Navarro et al., 2008 were compared with microarray datasets generated from the *tga2,5,6* mutant seedlings (Mueller et al., 2008). Though it has to be taken account of the fact that different ecotypes were used for the microarrays, the picture emerges that TGA factors and DELLA proteins operate through distinct mechanisms, because *WRKY70* (At3g56400) and *WRKY53* (At4g23810) display the only genes fulfilling the requested conditions. WRKY70 was shown to act as an activator of SA-responsive genes and a repressor of JA- inducible genes, thereby functioning as a molecular switch between both pathways (Li et al., 2004). WRKY53 plays a regulatory role in the early events of leaf senescence (Miao et al., 2004) and is also involved in basal resistance against *Pseudomonas syringae* (Murray et al., 2007). In future work, it would be interesting to investigate, if TGAs together with DELLAs bind to the promoter region of WRKY70 or WRKY53 to negatively regulate PAMP-induced defense responses.

As reduced ROS inhibit root growth (Achard et al., 2008), we tested whether an altered flg22-induced ROS accumulation in the *tga2,5,6* mutant is responsible for the enhanced

root growth inhibition. Increased ROS levels were detected in the *tga2,5,6* mutant already in the absence of flg22 (Figure 4-5). Most likely, the known function of TGA factors in detoxification processes (Mueller et al., 2008; Fode et al., 2008) is responsible for the increased levels. However, in our hands, ROS levels were independent from SA- or flg22 treatment and thus cannot account for the increased flg22-responsiveness of the mutant. Consistently, the root growth of *tga2,5,6* shows no altered sensitivity to different ROS inhibitors (Figure 4-6).

5.2 Class-II TGA factors are not involved in PAMP-induced ROS burst

The ROS burst in leaves after treatment with different PAMPs was not influenced in the *tga2,5,6* mutant (Figure 4-7). It is not unlikely that very early defense responses, like the ROS burst, which occurs within the first minutes after PAMP perception, are regulated by protein destabilization, whereas later defense responses are regulated on the transcriptional level. Examples for negative regulators of PAMP-triggered immunity are the three homologous E3 ubiquitin ligases PUB22, PUB23 and PUB24. The triple mutant *pub22/pub23/pub24* is de-repressed in basal immune responses resulting in enhanced resistance against bacterial and oomycete pathogens (Trujillo et al., 2008). The mechanism, how the E3 ligases suppresses basal defense seems to be independent of TGA2, TGA5 and TGA6, because in the *pub22/pub23/pub24* mutant the oxidative burst is strongly enhanced and prolonged after treatment with different PAMPs, whereas the *tga2,5,6* mutant shows only a slightly fortified ROS burst after flg22 in comparison to the wild type. Furthermore, the set of marker genes with enhanced expression is different. In the *pub22/pub23/pub24* mutant, transcript levels of genes related to oxidative stress signaling (*RhbohD* and *OXIDATIVE SIGNAL-INDUCIBLE1* (*OXI1*)) are elevated, which is not the case in the *tga2,5,6* mutant. Moreover, *FRK1* and *WRKY22*, which show the strongest de-regulation in the *tga2,5,6* mutant are not up-regulated in the *pub22/pub23/pub24* mutant (Trujillo et al., 2008).

5.3 The *tga2,5,6* mutant shows enhanced sensitivity to many early defense responses

5.3.1 Gene expression

In qRT-PCR, it was shown that transcript levels of early flg22-inducible genes are elevated in the *tga2,5,6* mutant (Figure 4-11). The activation of *RbohD* in the flg22-mediated signaling cascade is independent of class-II TGA factors, because the transcript levels of this NADPH oxidase are not significantly enhanced in the *tga2,5,6* mutant. This conforms to the results obtained by the ROS burst assay (Figure 4-7). Genes (*WRKY22, WRKY29, FRK1*), which are located downstream of *RbohD*, have an increased expression in the *tga2,5,6* mutant.

Most of the TGA-regulated genes did not display constitutive changes but rather earlier or stronger induction by flg22, indicating that TGAs rather sensitize plants to PAMPs than function as a repressor of PAMP-mediated signaling. To proof this hypothesis, microarray datasets generated from untreated *tga2,5,6* mutant (Mueller et al., 2008) and flg22-induced (1h and 3h) Col-0 seedlings (Denoux et al., 2008) were compared, similar to the analysis done by Navarro et al., 2008. Whereas in the stabilized DELLA mutant *ga1-3* about over 100 genes were elevated compared with flg22-treated wild type plants, the transcript levels of only six genes are elevated in *tga2,5,6* mutants as well as flg22-treated Col-0 (Supplemental data; Table 6-3). Interestingly, three of them are members of the WRKY transcription factor family, all involved in defense responses. As mentioned before, WRKY53 was described as putative DELLA target. WRKY75 has been described to be involved in regulation of phosphate starvation responses (Devaiah et al., 2007). Furthermore, a role for WRKY75 in the activation of basal and *R*-mediated resistance against *P. syringae* in *Arabidopsis* could be demonstrated (Encinas-Villarejo et al., 2009). WRKY40 is structurally related to WRKY18 and WRKY60. All together, they have partially redundant roles as negative regulators in plant resistance against bacterial as well as fungal pathogens (Xu et al. 2006; Shen et al. 2007). *WRKY75*, *WRKY53* and *WRKY40* were spotted on a ChIP-on-chip array and binding of TGA2 to the related promoter regions was shown, at least after SA-treatment. *TAT3* transcripts are JA and wound-inducible (Titarenko et al., 1997), whereas *RLP35* and *BGLU17* are not further characterized. Thus, TGAs constitutively repress only a few plant defense related-genes, which in turn may act as upstream regulators of flg22-hyperinduced genes like *WRKY22, WRKY29* or *FRK1*.

5.3.2 Callose deposition

Pathogen attack, wounding and other stresses induce callose deposition. The *tga2,5,6* mutant showed an increased callose deposition after flg22, which is abolished in the *tga2,5,6/pmr4* quadruple mutant (Figure 4-13). In *Arabidopsis*, 12 callose synthase genes are known, which are expressed specifically in different tissues during plant development (Dong et al., 2008). Transcript levels of callose synthase *PMR4*, which is responsible for callose synthesis after pathogen attack, are inducible 6h after SA treatment or 4 dpi with the pathogen *Hyaloperonospora arabidopsis*. Five other callose synthases are also inducible by SA treatment and pathogen infection, whereas *PMR4* and *CalS1* are the only ones, which show an NPR1-dependent induction by SA (Dong et al., 2008). However, the expression of *PMR4* is only slightly inducible by flg22 and transcript levels are little elevated in the *tga2,5,6* mutant (Figure 4-14A+B). Some branches of PAMP-triggered callose deposition are SA dependent. Mutants impaired in SA biosynthesis or signaling show normal callose deposition (Clay et al., 2009; Adams-Phillips et al., 2010), but interestingly, SA is able to rescue flg22-induced callose deposition in *pen2*, *pcs1* and *vtc1* mutants impaired in glucosinolate hydrolysis (Clay et al., 2009). More recently, it was shown that inhibition of poly(ADP-ribosyl)ation, a posttranslational protein modification, blocks basal defense responses including callose deposition and again, pre-treatment with SA is able to rescue callose deposition (Adams-Phillips et al., 2010). Surprisingly, the *pmr4* mutant became more resistant to pathogens, rather than more susceptible (Jacobs et al., 2003; Nishimura et al., 2003; Flors et al., 2008). This resistance is based on an enhanced SA response, indicating a negative cross-talk between the callose response and SA signaling (Nishimura et al., 2003). These results indicate a kind of negative feedback loop for the regulation of *PMR4*. Perception of PAMPs induces SA biosynthesis that activates transcription of *PMR4*, which in turn suppresses the SA pathway. flg22 itself is only a very weak elicitor for transcriptional activation of *PMR4* leading to the presumption, that flg22-mediated callose deposition is induced by posttranslational modification independent of class-II TGA factors.

The PMR4 callose synthase is also responsible for wound-induced callose synthesis (Jacobs et al., 2003; Nishimura et al., 2003). In contrast to PAMP perception, the *tga2,5,6* mutant failed to develop callose deposition after wounding (Figure 4-15). Wound-induced responses depend on JA-signaling. This result indicates a role of class-II TGA factors as positive regulators not only involved in JA/ET signaling (Zander et al., 2009), but also for wound induced JA signaling.

Recently, it was shown that glucosinolate metabolites are involved in callose synthesis after PAMP treatment upstream of PMR4 (Clay et al., 2009). Mutants in the glucosinolate biosynthetic pathway, including cytochrome P450 monooxygenases CYP81F2 and CYP79B2, failed in PAMP-triggered callose deposition. Interestingly, both genes are up-regulated in the *tga2,5,6* mutant (Figure 4-14), and may display a target for transcriptional regulation by class-II TGA factors.

If an infection with *P. syringae* was used instead of the synthetic peptide flg22, no differences between wild type and *tga2,5,6* mutant could be observed (Figure 4-16). It seems likely that *P. syringae* present additional PAMPs, such as LPS or harpin (Livaja et al., 2008), which may act independently of class-II TGA factors to induce callose deposition. Another explanation is founded in differences in experimental setups. The flg22-mediated callose deposition was induced by spray-treatment on axenically grown seedlings, similar to the procedure to induce gene expression. The bacterial inoculation takes place by syringe infiltration of adult soil grown plants according to the infection for the bacterial growth assay.

5.3.3 Stomatal closure

Stomata serve as natural openings to provide bacterial pathogens entry into the apoplast. Stomatal closure occurs through changes in turgor pressure within the guard cells, a very specific cell type embedded in the epidermis of the leaf. Stimuli activating stomatal function exist in a large number. Hence, it is not surprising that beside the indispensable key regulator ABA also other phytohormones contribute to stomatal aperture regulation. The ethylene receptor ETR1 mediates H_2O_2 signaling in stomatal guard cells. Consequently, stomata in the loss-of-function *etr1-7* mutant do not close in response to H_2O_2 (Desikan et al., 2005). Independent of ABA, MeJA induces stomatal closure and promotes H_2O_2 production in guard cells (Suhita et al., 2004). Stomatal closure in response to bacterial pathogens is compromised in *NahG* plants and the SA biosynthetic mutant *eds16-2*, indicating that SA is required for stomatal defense (Melotto et al., 2006). Interestingly, the JA signaling mutant *coi1-20* is not impaired in stomatal defense against bacteria (Melotto et al., 2006) and is able to respond to ABA, but not to MeJA-induced stomatal closure (Munemasa et al., 2007). The JA mimic COR is able to inhibit PAMP-induced stomatal closure in a COI1 dependent manner (Melotto et al., 2006). Several studies have shown strong antagonistic interactions between JA signaling and SA- or ABA-mediated signaling

(e. g. Laurie-Berry et al., 2006; Anderson et al., 2004). Therefore, an attractive hypothesis is that COR exploits the endogenous antagonistic interactions between JA, SA, and ABA hormone signaling pathways in plants to affect stomatal response after pathogen attack. It could be shown that under abiotic stresses like drought or UV light, an accumulation of JA occurs in soybean and tobacco leaves (Creelman and Mullet, 1995; Demkura et al., 2009). These results suggest a potentially bimodal effect of JA and COR on stomatal response, depending on the trigger. Due to the fact that the *tga2,5,6* mutant is insensitive to COR-induced stomatal re-opening (Figure 4-17), class-II TGA factors function as regulators for this mechanism.

Recently, a novel function of the bimodal defense regulator RIN4 in stomata closure was elucidated. Whereas class-II TGAs act as negative regulators for PTI and positive regulators for *PR* gene expression, RIN4 acts as negative regulator for PTI and ETI. *rin4* knockout lines exhibit increased callose deposition after PAMP treatment and decreased *Pst* growth, consistent with enhanced PTI signaling (Kim et al., 2005). In addition, two R proteins, RPM1 and RPS2 monitor RIN4. In the absence of pathogen perception, RIN4 acts as a negative regulator of RPM1 and RPS2. When the *P. syringae* effectors AvrRpm1 or AvrB are delivered to the plant cell, RIN4 is hyper-phosphorylated, which in turn leads to the activation of RPM1-mediated resistance (Mackey et al., 2002). Another *P. syringae* effector, AvrRpt2, is a protease that directly targets RIN4, leading to the activation of RPS2-mediated resistance (Mackey et al., 2003).

Similar to the *tga2,5,6* mutant, stomata of the *rin4* knock out could not be re-opened by virulent *Pst.* RIN4 interacts with AHA1 and AHA2, two plasma membrane H^+-ATPases to regulate stomatal apertures during pathogen attack (Liu et al., 2009). RIN4 has no motifs predictive of enzyme function. So, it is supposed that it acts as an adaptor protein involved in negatively regulating PAMP signal transduction or ETI (Kim et al., 2005). It is supposable that class-II TGA factors may act together with RIN4 to negatively regulate PAMP-triggered responses. It would be of big interest to determine, if *RIN4* expression in mesophyll or guard cells is regulated by class-II TGA factors.

5.4 Ectopic expression of TGA2 is sufficient to restore the wild-type situation

Overexpression of TGA2 in the *tga2,5,6* mutant is sufficient to rescue the wild type phenotype of root growth, gene expression and stomatal closure. To investigate the function of single class-II TGA factors, an expression system driven by the *CaMV 35S* promoter was used. With this system, the creation of transgenic plants with a very high global expression of the transgene in all tissues is possible but also could cause pleiotropic effects. Gene regulation also occurs by tissue-specific expression of transcription factors. The main tissues involved in defense responses against pathogens are mesophyll and guard cells. Using the endogenous promoter instead of the *CaMV 35S* promoter would restore a more physiological situation and may lead to other results. Tissue specific promoters can be used to elucidate the tissue-specific function of TGA factors. For example, under the control of a promoter specifically expressed in guard cells, like *pGCI* (Yang et al. 2008) the function of class-II TGA factors in stomatal closure could be investigated.

5.5 The absence of class-II TGA factors partially rescues the enhanced susceptibility phenotype of the *sid2-2* mutant regarding flg22-triggered defense against *Pst* DC3000

Comparison of bacterial growth between mock-pretreated and flg22-pretreated plants also revealed a negative effect of class-II TGA factors on PAMP-triggered defense responses. A difficulty in obtaining the role of TGA2, TGA5 and TGA6 in basal or flg22-mediated resistance to *Pst* DC3000 is the circumstance that SA signaling is involved in this defense responses (Tsuda et al., 2008) and as consequence, the *tga2,5,6* mutant is abolished in SAR (Zhang et al., 2003). To be able to analyze the flg22 response in the absence of the SA-dependent defense pathway, a *tga2,5,6/sid2-2* mutant was used for the experiments.

After pretreatment with flg22, a significant difference in bacterial growth between the *sid2-2* and the *tga2,5,6/sid2-2* mutant was observed, indicating a negative regulation of flg22-induced defense by class-II TGA factors (Figure 4-20). This experiment shows that the negative effect of TGA factors on PAMP signaling has a consequence for bacterial growth. Still, the bacterial titer in the *tga2,5,6/sid2-2* mutant is not reduced to levels found in wild

type plants, indicating that the de-repression of the PAMP-pathway cannot fully complement for the lack of the SA-responses. The *tga2,5,6* mutant showed the same defense response as the *tga2,5,6/sid2-2* mutant indicating that all SA-dependent processes relevant for this resistance are dependent on class II TGA factors.

Unexpectedly, mock pretreated *tga,2,5,6* plants show no difference in bacterial growth compared to the wild type as well as *tga2,5,6/sid2-2* plants do not in comparison to *sid2-2*. Thus, class II TGA factors are mediators of flg22 induced resistance but do not play a role in the absence of preactivation by PTI. The bacterial growth experiments were obtained upon infiltration of the bacterial solution directly into the apoplast. Thus, the effect of class-II TGA factors in coronatine-induced stomatal re-opening was not assessed. The *tga2,5,6* mutant is insensitive to COR-induced stomatal re-opening. Due to this fact, it is possible that an infection by spray application or dipping may unravel an increased resistant phenotype of the *tga2,5,6* mutant despite its lack of major parts of the SA-signaling pathway. In this context, the *tga2,5,6/sid2-2* mutant cannot be used for direct comparison, because PAMP- or bacterial-mediated stomatal closure is SA-dependent (Melotto et al., 2006).

5.6 Influence of SA and JA on flg22-induced gene expression

Regarding activation of the basal defense marker gene *FRK1*, the activation of the SA-pathway acts synergistically to flg22 (Figure 4-22), whereas simultaneous application of coronatine had the opposite effect (Figure 4-21). The antagonism between SA and JA dependent pathways is well established and several observations indicate an involvement of TGA factors together with NPR1 or GRX480 in SA/JA crosstalk beside their function in establishment of SAR (Spoel et al., 2003; Ndamukong et al., 2007).

One hypothesis applying the antagonism between JA- and SA-pathways deals with the assumption that COR promotes susceptibility to *P. syringae* infection by stimulating JA-signaling in plants. The consequence is inhibition of SA-mediated defenses that normally limit *P. syringae* growth. In this context, the transcription factor MYC2 could be identified as important mediator of COR or JA-Ile dependent suppression of SA signaling. The MYC2 mutant *jin1* exhibit both reduced susceptibility to *Pst* DC3000 and reduced sensitivity to COR or MeJA. The reduced disease susceptibility in *jin1* mutants is correlated with elevated

expression of *PR-1* and is dependent on SA accumulation (Laurie-Berry et al., 2006). MYC2 acts as positive regulator for JA-responsive genes like *VSP2* and *LOX3*, but has a negative effect on JA/ET-inducible genes like *PDF1.2* (Lorenzo et al., 2004). In turn, class-II TGA factors are required to antagonize the negative effect of MYC2 on *PDF1.2* expression (Zander et al., 2009). The cross-talk mediated suppression of SA on JA-induced gene expression of *PDF1.2* occurs normally in the *tga2,5,6* mutant (Ndamukong et al., 2007), whereas class-II TGA factors are required to mediate the SA-JA/ET crosstalk in the *jin1-1* mutant background (Zander et al., 2009).

5.7 Functional analysis of class-II TGA factors in basal resistance

Binding sites for TGA factors exist in many genes, responding to diverse signals in pathogen defense and xenobiotic stresses (Thibaud-Nissen et al., 2006; Ndamukong et al., 2007; Fode et al., 2008). It is evident from these studies that the specific activity of a TGA factor is determined or modified by its interacting protein. Until now, three proteins interacting with class-II TGA transcription factors could be identified by yeast-two-hybrid screens (Després et al., 2000; Ndamukong et al., 2007; Fode et al., 2008). SCL14 is essential for induction of stress-inducible promoters (Fode et al., 2008), whereas the nuclear function of NPR1 plays a role for activation of SA-responsive genes and SA homeostasis, the cytosolic function of NPR1 is involved in SA/JA crosstalk (Glazebrook et al., 2003; Zhang et al., 2010; Leon-Reyes et al., 2009). The TGA-interacting GRX480, which is transcriptionally activated by SA, is a negative effector of JA-inducible expression of *PDF1.2* and therefore represents a potential regulatory component of the SA/JA antagonism (Ndamukong et al., 2007). TGA4, a class-I TGA factor interacts with AtEBP, which binds the ethylene response element present in many *PR* gene promoters and with OBP1, a protein belonging to the Dof family (Büttner and Singh, 1997; Zhang et al., 1995).

In several studies it was observed that TGA2 may act as a constitutive repressor, but its role in basal defense responses was never described before. By using an *in vivo* plant transcription assay, it could be shown that TGA2 behaves like a constitutive repressor, but forms an enhanceosome with NPR1 (Rochon et al., 2006). The analysis of several single and multiple knock outs revealed that the *tga2-2* knockout mutant, un-induced and grown on

plates containing the SA-analogon INA, showed higher expression of *PR-1* (Kesarwani et al., 2007).

By chromatin-immunoprecipitation (ChIP), it was shown that class-II TGA factors bind constitutively to the *PR-1* promoter, independently of SA and NPR1 (Rochon et al., 2006; Fode et al., 2008). More recently, it was demonstrated that purified TGA2 protein forms enormous oligomers containing 40 and more units of TGA2 (Boyle et al., 2009). This oligomer binds to TGACG motives to suppress transcription by an unknown mechanism. NPR1 negates the TGA2 repressor function by excluding TGA2 oligomers from the DNA (Boyle et al., 2009). A concentration-dependent regulation of gene expression by transcription factors is known for the Krüppel zinc-finger protein, which can act both as an activator and repressor on the same DNA element in *Drosophila* (Sauer and Jäckle, 1991). At low concentration, Krüppel binds DNA as a monomer and activates transcription, but at higher concentration, it forms a homodimer and acts as a repressor (Sauer and Jäckle, 1993).

However, it cannot be excluded that TGA transcription factors may function as repressors if they are associated with other TGA factors or regulators. TGA transcription factors belong to the bZIP family. For other bZIP-proteins it was documented that they differ in their function depending on the heterodimerisation partner (Weltmeier et al., 2009). A similar mechanism could be responsible for the observation that different TGA factors are involved in growth inhibition and gene expression. A further explanation display post-transcriptional mechanisms, which may determine the distribution of the TGA gene products in a particular tissue. In a study with transgenic tobacco expressing GFP-tagged TGA 1, TGA2 or TGA3 from *Arabidopsis*, TGA1 and TGA3 are rapidly degraded in mature tissues whereas TGA2 levels remained essentially constant. In contrast to this differential regulation in mature tissues, all three TGA factors were present in seedlings, which demonstrate the existence of a post-transcriptional mechanism controlling the presence of specific TGA factors in different tissues during development (Pontier et al., 2002).

ChIP analysis was performed to prove, whether class-II TGA factors bind directly to the promoters of the up-regulated genes (Figure 4-26). The promoter region of *WRKY22* shows no enhanced binding of class-II TGA factors. So it seems to be obvious that the influence of the TGA factors on basal defense responses occurs indirectly by regulating a regulator. Other members of the WRKY transcription factor family would present ideal candidates for this regulator because many WRKY genes are transcriptionally regulated and putative targets for the regulation by class-II TGA factors (Table 4-2). Many defense related genes

contain W-boxes in their promoter region, including WRKY genes themselves, like *WRKY22* and *WRKY29* (Dong et al., 2003), to build a big network (Eulgem and Somssich, 2007). In addition, it was shown that some defense related WRKYs are transcriptionally regulated by TGA factors and NPR1 (Wang et al., 2006). In order to differentiate the function of TGA2 and TGA5, it would be necessary to analyze single mutants. Unfortunately, the *tga2-2* mutant characterized by Kesarwani et al., 2007 is generated in the Ws-0 ecotype a natural *fls2* mutant insensitive to flg22 (Zipfel et al., 2004). So far, no single mutant for TGA5 is known. A screen of other T-DNA insertion lines or the creation of RNAi-lines would be useful for further characterizations.

5.8 Proposed model

Recognition of PAMPs activates a signaling cascade leading to different immune responses. Class-II TGA transcription factors are involved in some of these responses, like callose deposition, transcriptional activation or by suppression of an unknown factor. The bacterial toxin coronatine (COR) works together with the TGA factors against some of the observed immune responses. On the one hand, COR and class-II TGA factors suppress the transcription of flg22-responsive genes and on the other hand, they are necessary to stimulate stomatal re-opening to facilitate bacterial entry.

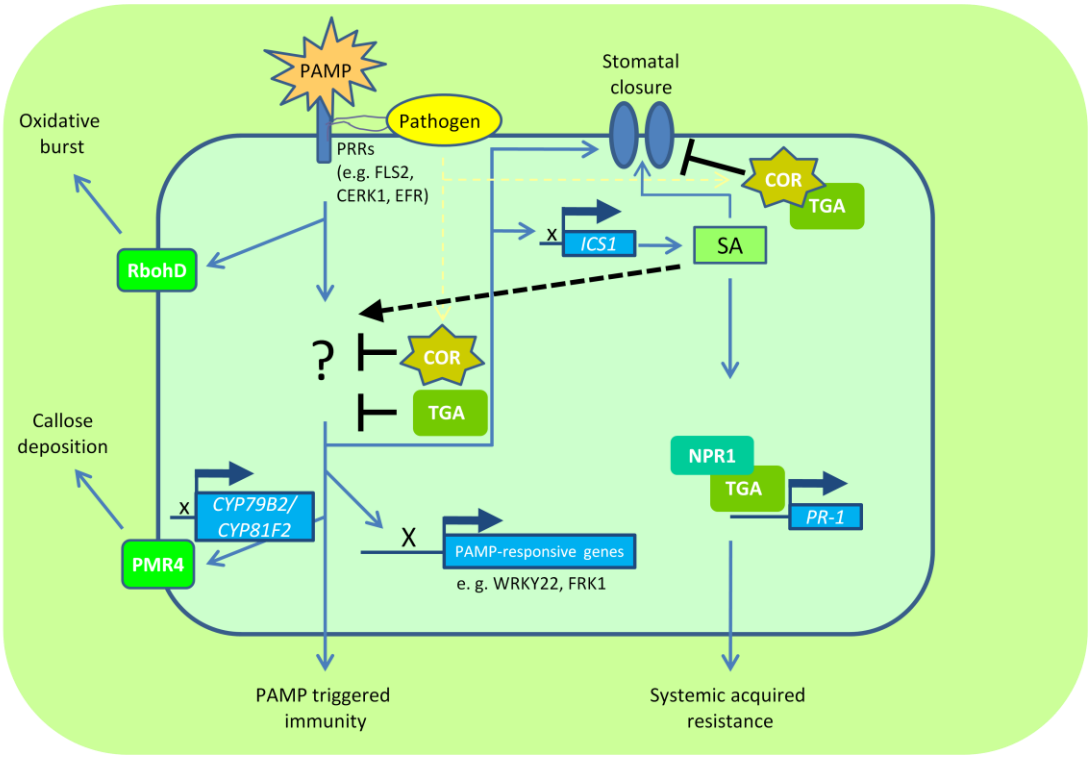

Figure 5-1| **Model for negative regulation of PAMP-induced defense responses by class-II TGA factors.**

Bacteria or fungal derived PAMPs are recognized by specific receptors (PRRs) located in the plasma membrane. Oxidative burst occurs by enzymatic activity of RbohD and is not regulated by class-II TGA factors. Callose deposition depending on callose synthase PMR4, and expression of PAMP-responsive genes are located more downstream and are negatively regulated by an unknown mechanism dependent on class-II TGA factors, prominently TGA2. Flg22-induced *ICS1* expression is also suppressed by class-II TGA factors. Downstream of SA, TGAs and NPR1 build an enhanceosome to activate *PR-1* gene expression and systemic acquired resistance. The bacterial toxin coronatine (COR) suppresses PAMP-signaling independent of class-II TGA factors, but acts together with them to activate stomatal re-opening.

6 Supplemental data

Table 6-1| **Putative class-II TGA target genes identified as enriched (IP/RAW) in ChIP-on-chip array. The genes are sorted according to their grade of enrichment.**

AGI code	Gene description	IP/RAW
At3g50850/60	unknown protein	3.03
At1g21670/80	unknown protein	2.97
At1g02450	NIMIN1	2.94
At1g03230	extracellular dermal glycoprotein, putative	2.90
At5g01900	WRKY62 WRKY Transcription Factor; Group III	2.88
At3g53400	unknown protein	2.86
At4g18020	pseudo-response regulator 2 (APRR2) (TOC2)	2.80
At5g51990	CBF4 member of the DREB subfamily A-1 of ERF/AP2 transcription factor family.	2.66
At4g34060	similar to HhH-GPD base excision DNA repair family protein (ROS1)	2.63
At5g54540/50	unknown protein	2.60
At3g11780/800/810	unknown protein	2.59
At1g76600	unknown protein	2.59
At2g44980/90	transcription regulatory protein SNF2	2.55
At5g25070/80	unknown protein	2.51
At5g13760	unknown protein	2.48
At5g05240/50	unknown protein	2.35
At3g13810/20	zinc finger (C2H2 type) family protein	2.31
At2g40750	WRKY54 WRKY Transcription Factor; Group III	2.30
At1g12460	leucine-rich repeat transmembrane protein kinase, putative	2.29
At2g28640/50	A member of EXO70 gene family	2.19
At5g66910/20	disease resistance protein (CC-NBS-LRR class)	2.06
At5g61990/62000	pentatricopeptide (PPR) repeat-containing protein	2.06
At4g00370	sugar transporter family protein	2.05
At5g22450	unknown protein	2.03
At5g64810	WRKY51 WRKY Transcription Factor; Group II-c	2.01
At3g13224	RNA recognition motif (RRM)-containing protein	2.00
At1g50060	pathogenesis-related protein, putative	1.99
At2g29420	ATGSTU7 glutathione transferase belonging to the tau class of GSTs	1.83
At3g03190	ATGSTF11 glutathione transferase belonging to the phi class of GSTs	1.77
At2g05940	protein kinase, putative	1.75
At2g47730	ATGSTF8 glutathione transferase belonging to the phi class of GSTs	1.71
At3g53020/30	RPL24A encodes ribosomal protein L24	1.70
At3g12690	AGC KINASE 1.5 putative serine/threonine kinase expressed specifically in pollen	1.65

At1g80590	WRKY66 WRKY Transcription Factor; Group III	1.64
At4g18170	WRKY28 WRKY Transcription Factor; Group II-c	1.64
At4g01720	WRKY47 WRKY Transcription Factor; Group II-b	1.64
At2g29480	ATGSTU2 glutathione transferase belonging to the tau class of GSTs.	1.59
At3g56400	WRKY70 WRKY Transcription Factor; Group III.	1.55
At1g69150/60	unknown protein	1.53
At3g52060/70	unknown protein	1.52
At1g78380	ATGSTU19 glutathione transferase that is a member of Tau GST gene family	1.51
At1g78340	TAU22 glutathione transferase belonging to the tau class of GSTs.	1.50
At2g29490	GST19 glutathione transferase belonging to the tau class of GSTs	1.45
At1g49860	ATGSTF14 glutathione transferase belonging to the phi class of GSTs.	1.42
At1g17180	ATGSTU25 gluthatione transferase belonging to the tau class of GSTs.	1.37
At1g18250	ATLP-1 thaumatin-like protein	1.37
At5g67040	unknown protein	1.35
At4g32020	unknown protein	1.32
At2g17860	pathogenesis-related thaumatin family protein	1.29
At4g36000	unknown protein	1.28
At1g78370	unknown protein	1.28
At1g66550	WRKY67 WRKY Transcription Factor; Group III	1.27
At1g62300	WRKY6 WRKY Transcription Factor; Group II-b	1.25
At1g78360	ATGSTU21 glutathione transferase belonging to the tau class of GSTs	1.21
At2g21900	WRKY59 WRKY Transcription Factor; Group II-c	1.21
At2g29470	ATGSTU3 glutathione transferase belonging to the tau class of GSTs	1.18
At5g49520	WRKY48 WRKY Transcription Factor; Group II-c	1.16
At4g31550	WRKY11 WRKY Transcription Factor; Group II-d	1.15
At5g10030	TGA4 member of basic leucine zipper transcription gene family	1.14
At2g29450	ATGSTU1/5 member of the TAU glutathione S-transferase gene family	1.14
At2g29470	ATGSTU3 glutathione transferase belonging to the tau class of GSTs	1.12
At4g38670	pathogenesis-related thaumatin family protein	1.11
At2g29460	ATGSTU4 glutathione transferase belonging to the tau class of GSTs	1.11
At1g77920	bZIP family transcription factor	1.11
At1g02920	ATGST11 glutathione transferase belonging to the phi class of GSTs	1.11
At3g09270	ATGSTU8 glutathione transferase belonging to the tau class of GSTs	1.11
At1g73620	thaumatin-like protein, putative / pathogenesis-related protein	1.10
At1g17170	ATGSTU24 glutathione transferase belonging to the tau	1.10

	class of GSTs	
At5g62480	ATGSTU9 glutathione transferase belonging to the tau class of GSTs	1.09
At5g22570	WRKY38 WRKY Transcription Factor; Group III	1.09
At5g02790	In2-1 protein, putative	1.09
At1g20030	pathogenesis-related thaumatin family protein	1.08
At2g14610	PR-1	1.08
At3g11010	ATRLP34 receptor like protein 34	1.07
At1g13960	WRKY4 WRKY Transcription Factor; Group I	1.06
At1g77290	tetrachloro-p-hydroquinone reductive dehalogenase-related	1.04
At4g04450	WRKY42 WRKY Transcription Factor; Group II-b	1.03
At4g29940	PRHA PATHOGENESIS RELATED HOMEODOMAIN PROTEIN A	1.03
At4g08555/60	unknown protein	1.03
At5g13080	WRKY75 WRKY Transcription Factor	1.02
At4g24180	unkown protein	1.01
At2g43570	CHI chitinase, putative	1.01

Chromatin immunoprecipitated with antiserum against TGA2,5 was hybridized to a mini array representing 200 putative target promoters for TGA2 (cooperation with C. Town, Institute for Genomic Research, Rockwille, USA). The average from three independent experiments, normalized to the signal derived from raw-chromatin, is shown.

Table 6-2| **Putative class-II TGA target genes identified as enriched (IP/RAW) in ChIP-on-chip array. The genes are sorted according to their increase of enrichment (fold induction) after 12h SA-treatment in comparison to mock treatment.**

AGI code	description	IP/RAW 0hSA	IP/RAW 12hSA	fold induction
At4g23810	WRKY53 WRKY Transcription Factor; Group III	0.84	1.45	1.73
At2g46400	WRKY46 WRKY Transcription Factor; Group III	0.98	1.64	1.69
At5g22570	WRKY38 WRKY Transcription Factor; Group III	1.09	1.76	1.61
At2g14610	PR-1	1.08	1.69	1.56
At1g80840	WRKY40 Transcription factor; Group II-a	0.73	1.11	1.53
At1g62300	WRKY6 WRKY Transcription Factor; Group II-b	1.25	1.73	1.38
At5g13080	WRKY75 WRKY Transcription Factor	1.02	1.36	1.33
At5g61990/62000	pentatricopeptide (PPR) repeat-containing protein	2.06	2.67	1.30
At2g21900	WRKY59 Transcription Factor; Group II-c	1.21	1.50	1.24
At1g76600	unkown protein	2.59	3.19	1.23
At1g78380	ATGSTU19 glutathione transferase that is a member of Tau GST gene family	1.51	1.85	1.22
At5g22450	unkown protein	2.03	2.48	1.22
At1g21670/80	unkown protein	2.97	3.61	1.21
At2g44980/90	transcription regulatory protein SNF2	2.55	3.09	1.21
At2g38470	WRKY33 Transcription Factor, Group I	0.94	1.13	1.21
At4g22070	WRKY31 WRKY Transcription Factor; Group II-b	0.87	1.01	1.16
At4g01250	WRKY22 Transcription Factor; Group II-e	0.90	1.04	1.15
At3g53020/30	RPL24A encodes ribosomal protein L24, homolog of cytosolic RPL24	1.70	1.94	1.14
At1g02450	NIMIN1	2.94	3.35	1.14
At3g56400	WRKY70 WRKY Transcription Factor; Group III	1.55	1.75	1.13
At1g50060	pathogenesis-related protein, putative	1.99	2.24	1.13
At1g13960	WRKY4 WRKY Transcription Factor; Group I	1.06	1.20	1.13
At2g28640/50	member of EXO70 gene family	2.19	2.45	1.12
At2g47730	ATGSTF8 glutathione transferase belonging to the phi class of GSTs	1.71	1.86	1.09
At4g01720	WRKY47 WRKY Transcription Factor; Group II-b	1.64	1.75	1.07
At4g18170	WRKY28 WRKY Transcription Factor; Group II-c	1.64	1.74	1.06

At4g36000	unknown protein	1.28	1.36	1.06
At5g66910/20	disease resistance protein (CC-NBS-LRR class), putative	2.06	2.18	1.06
At5g64810	WRKY51 WRKY Transcription Factor; Group II-c	2.01	2.11	1.05
At4g34060	similar to HhH-GPD base excision DNA repair family protein (ROS1)	2.63	2.74	1.04
At1g17180	ATGSTU25 glutathione transferase belonging to the tau class of GSTs	1.37	1.42	1.04
At1g17170	ATGSTU24 glutathione transferase belonging to the tau class of GSTs	1.10	1.13	1.03
At5g49520	WRKY48 WRKY Transcription Factor; Group II-c	1.16	1.18	1.02
At5g62480	ATGSTU9 glutathione transferase belonging to the tau class of GSTs	1.09	1.12	1.02
At2g29450	ATGSTU1/5 member of the TAU glutathione S-transferase gene family	1.14	1.16	1.02
At4g00370	sugar transporter family protein	2.05	2.09	1.02
At2g40750	WRKY54 WRKY Transcription Factor; Group III	2.30	2.33	1.01
At1g02920	ATGST11 glutathione transferase belonging to the phi class of GSTs	1.11	1.12	1.01
At2g29420	ATGSTU7 glutathione transferase belonging to the tau class of GSTs	1.83	1.84	1.01

Chromatin immunoprecipitated with antiserum against TGA2,5 was hybridized to a mini array representing 200 putative target promoters for TGA2 (cooperation with C. Town, Institute for Genomic Research, Rockwille, USA). The average ± SD from three independent experiments, normalized to the signal derived from raw-chromatin, is shown.

Table 6-3| **List of genes that are more elevated in *tga2,5,6* vs. Col-0 (cell culture, Mueller et al., 2008) and up-regulated 1h or 3h after flg22 treatment (Col-0 seedlings, Denoux et al., 2008).**
(+) means an ≥10 fold increased transcript level. (-) display transcript levels < 10 fold increased at the indicated time point. *WRKY40, WRKY53* and *WRKY75* are spotted on the ChIP-on-chip array and enrichment of IP in comparison to raw-chromatin is shown.

AGI code	description	1h flg22	3h flg22	IP/RAW 0h SA	IP/RAW 12h SA
AT1G80840	WRKY40	+	-	0.73	1.11
AT4G23810	WRKY53	+	+	0.84	1.45
AT5G13080	WRKY75	+	+	1.02	1.36
AT2G44480	BGLU17 (BETA GLUCOSIDASE 17)	-	+	/	/
AT2G24850	TAT3 (TYROSINE AMINOTRANSFERASE 3)	+	+	/	/
AT3G11080	ATRLP35 (RECEPTOR LIKE PROTEIN 35)	+	-	/	/

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8 Acknowledgement

Zunächst möchte ich mich ganz herzlich bei allen bedanken, die mir diese Arbeit ermöglicht haben.

Von all jenen hat natürlich Christiane Gatz den größten Anteil an dieser Arbeit. Durch viele anregende Diskussionen sind innovative Ideen entstanden, die meine Arbeit sehr bereichert haben.
Auch Wolfgang Dröge-Laser möchte ich sehr herzlich für die Übernahme des Korreferats. Trotz Umzugsstress danken. Wolfgang, viel Erfolg in Würzburg!
Ein ganz besonderer Dank geht an Corinna Thurow für die vielen hilfreichen Ratschläge zu meinem Projekt. Du warst mir immer eine Hilfe beim Versuch, dem Laborchaos Einhalt zu gebieten.
Ein riesiger Dank geht auch an Guido Kriete. Danke Guido, für deine stete Hilfestellung bei jedem noch so kleinen Problem, besonders, wenn die Computer mal wieder nicht so wollten, wie sie sollten. Heike Freundt danke ich für ihre Unterstützung in organisatorischen Dingen und ihre Hilfe bei der Auswertung von Callose-Färbungen und Wurzellängenmessungen.
Außerdem möchte ich mich auch bei der Abteilung Zellbiologie bedanken. Ganz besonderer Dank gebührt hierbei Christian Löfke für seine Tipps rum um das Thema Wurzel, Christine Klapprodt für den Exkurs in die Welt des Mehltaus und Elena Petutschnig für den Support bezüglich der ROS-Messungen.
Ein herzliches Dankeschön auch an Thomas Griebel (Uni Würzburg) für die Möglichkeit, beim Meister der SAR zu lernen und für das zur Verfügung stellen von *Pseudomonas syringae*-Stämmen.
Unseren TAs Anette Gunkel, Larissa Kunz, Ronald Scholz und Anna Hermann möchte ich für die zuverlässige und gewissenhafte Hilfe im Labor danken. Ihr habt mir meine Arbeit unglaublich erleichtert. Auch unseren Gärtnern Uwe Wedemeyer und Feli Glasenapp möchte ich vor allem für die Bereitstellung von Tabletts mit Erde gestopften Töpfen danken. Jenny Krüger möchte ich sehr dafür danken, dass sie mich in die Welt der Meeresaquaristik eingeführt hat, nichts entspannt mehr und ist zugleich so faszinierend.

Ein riesengroßes Dankeschön an meine einzigartigen Laborkollegen, die mir das Laborleben abwechslungsreich und angenehm gestaltet haben. Den „Jungs“ danke ich für die vielfältigen Ablenkungsmanöver in Zeiten des Trübsal-Blasens, die Teilnahme an Pokerrunden trotz: „ich muss jetzt gehen, mein Zug fährt“ und die kreative Gestaltung des Mittagessens. Julia danke ich besonders für ihre Bereitschaft, Wochenendschichten mit einem Eis zu versüßen und die unermüdliche Beschaffung von Literatur.

Auf jeden Fall sollen auch noch die „alten Hasen“ genannt werden:
Zu aller erst natürlich vielen, vielen Dank dir, meine liebe Ulli, du bist mir immer eine teure Freundin, zu jeder Tages- und Nachtzeit. Mit dir teile ich meine Vorliebe für Chai-Tee und

blasse Engländer. Gott sei Dank gibt es Telefon-Flat-Rate. Katrin und Ben, die Rollenspiel-Nachmittage mit euch werde ich nie vergessen, und Thula Bärentöter bestimmt auch nicht. Der modernen Technik sei gedankt, dass wir, trotz der riesen Entfernung über den großen Teich, doch Nachbarn sein können. Auch danke ich Hella für ihr offenes Ohr und Rat und Tat in jeder Lebenslage.

An dieser Stelle möchte ich mich ganz herzlich bei meiner Familie bedanken, die meine Leidenschaft bezüglich der Forschung zwar nicht immer nachvollziehen konnten, aber toleriert und mich unterstützt haben.

Zu guter Letzt möchte ich noch dem wichtigsten Menschen in meinem Leben danken, meinem Mann Mic. Danke, dass du mein Taxi gespielt hast, meine Haushaltshilfe und Koch warst, ohne groß zu murren meine exotischen Arbeitszeiten ertragen, und meinen Probevorträgen geduldig zugehört hast.

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