Regulation of the Cytochrome P450 Gene, *CYP81D11,* in *Arabidopsis thaliana,* Subjected to Chemical Stress

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Contents

Abbreviations

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
aa	amino acids
A	Ampere
A	adenosine
ABA	abscisic acid
AD	activation domain
amp	ampicilin
APS	ammoniumpersulfate
as-1	*activating sequence 1*
ASN1	*GLUTAMINE-DEPENDENT ASPARAGINE SYNTHASE 1*
AT	marks a protein from *Arabidopsis thaliana*
A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
B. cinerea	*Botrytis cinerea*
bp	base pairs
BSA	bovine serum albumine
bZIP	basic leucine zipper
C	cytosine
CaMV	cauliflower mosaic virus
cDNA	copy DNA
ChIP	chromatin immunoprecipitation
COI1	CORONATINE INSENSITIVE 1
COR	coronatine
COR78	*COLD REGULATED 78*
C_T	threshold cycle
C-terminal	carboxy-terminal
Da	Dalton
DIN11	*DARK INDUCIBLE 11*
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	desoxyribonucleotides
dpi	days past infection
EB	elusion buffer

ECL (kit)	enhanced chemoluminescence (kit for western detection)
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ET	ethylene
et al.	*et alii* (and others)
EtOH	ethanol
F	Farad
FF	Firefly
g	gravitation
g	gram
G	guanine
GA	gibberellic acid
GFP	green fluorescence protein
GST (U)	glutathione *S*-transferase (τ-class)
GUS	glucuronidase
h	hours
HSP	herring sperm DNA
IAA	auxin
ICS1	*ISOCHORISMATE SYNTHASE 1*
Ile	isoleucine
INA	isonicotinic acid
JA	jasmonic acid
JA-Ile	Jasmonic acid isoleucine
jar1	*jasmonate-resistante 1*
jin1	*jasmonate-insensitive 1*
JAZ	JASMONATE-ZIM-DOMAIN
k	kilo (10^3)
kan	Kanamycine
KWS	KWS SAAT AG
L	litre
LB	left border
Leu	leucine
LOX2	*LIPOXIGENASE 2*
LRR	Leucine rich repeat
LUC	Luciferase

µ	micro (10^{-6})
m	mili (10^{-3})
m	meter
M	molarity [mol/L]
MeJA	methyl jasmonate
min	minutes
MtN19-like	*MAC9.6*, *Medicago truncatula N19-like*
mRNA	messenger RNA
MS	Murahige and Skoog
n	nano (10^{-9})
NPR1	NON-EXPRESSOR of *PR*-GENES 1
N-terminal	amino-terminal
Ω	Ohm
OD	optical density
OE	over expressing
o/n	over night
OPDA	12-oxophytodienoic acid
p	pico (10^{-12})
P450	cytochrome P450
PAA	polyacrylamide
PAGE	polyacrylamide gelelectrophoresis
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
PDF1.2	*Plant defensin 1.2*
pH	negative $\log^{10}$ of proton concentration
PR	*Pathogenesis related*
qRT-PCR	quantitative real time PCR
RB	right border
RES	reactive electrophile species
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
rpm	rotations per minute
RT	room temperature

s	second
SA	salicylic acid
SCF	skip-cullin-F-box
SCL14	SCARECROW-LIKE 14
SDS	sodium dodecylsulfate
SEM	Standard error mean
T	thymine
TEMED	N,N,N',N'-tetraethylenediamine
TGA	*as-1* (TGACG motive) binding bZIP transcription factors
TIBA	2,3,5-triiodobenzoic acid
TNT	2,4,6-trinitrotoluene
Tris	tris-hydroxymethylamino methane
u	unit (quantity for enzyme activity)
U	uracil
UV	ultra violet
V	Volt
v/v	volume per volume
W	Watt
WT	wild-type
w/v	weight per volume

1 Summary

In higher plants, toxic chemicals induce the expression of a set of detoxification genes. In *Arabidopsis thaliana*, transcriptional activation of a subset of these depends on Class II TGA transcription factors and the TGA-interacting GRAS protein SARECROW-LIKE 14 (SCL14). The TGA2,5,6/SCL14-activated cytochrome P450 gene *CYP81D11* has been detected in a number of microarray analyses as being highly responsive to treatments with different reactive chemicals, like the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA), the allelocemical benzoxazolin-2(3H)-one (BOA), the explosive 2,4,6-trinitrotoluene (TNT) and phytoprostanes (highly reactive compounds generated by non-enzymatic lipid oxidation processes). In contrast to other known TGA2,5,6/SCL14-dependent genes, *CYP81D11* is inducible by the plant hormone methyl jasmonate (MeJA), which is a precursor of the active hormone jasonate-isoleucine (JA-Ile) that specifically binds to the receptor/co-activator complex CORONATINE INSENSITIVE1 (COI1)/JAZ.

In this thesis, we demonstrate that three distinct mechanisms of COI1 action merge on the *CYP81D11* promoter: (i) the well-established function that leads to the activation of MYC2 upon action of COI1 by elevated JA-Ile levels after MeJA and pathogen treatment; (ii) a novel function that requires basal JA-Ile levels, the transcriptional activator MYC2, a MYC2 binding site in the promoter and functional JAZ repressors; and (iii) as second novel function that is independent from all the known components of COI1-dependent signalling including the ligand JA-Ile. Whole genome microarray analysis of TIBA-treated wild-type and *coi1* plants revealed that 73 genes are induced only in the presence of COI1. Real-time RT-PCR and hierarchical cluster analysis indicated that the JA-Ile-independent COI1 function is likely to be unique for the *CYP81D11* promoter under these conditions. In contrast, COI1 is important for the expression of a large set of genes even although JA-Ile levels do not increase. *DARK IINDUCED11* (*DIN11*), for example, is expressed under extended night conditions in a COI1-dependent manner although increased JA-Ile levels cannot be detected. This novel COI1 function is constitutively activated in plant protoplasts leading to the expression of *CYP81D11* and *DIN11*.

While COI1 positively regulates *CYP81D11,* overexpression of the two NAC transcription factors ATAF1 and ANAC032 negatively effects *CYP81D11* expression after TIBA as well as after MeJA treatment.

2 Introduction

2.1 Function of COI1 in jasmonic acid signal transduction

The fatty acid-derived plant hormone jasmonic acid (JA) is involved in regulating plant growth (Staswick et al. 1992a), pollen ripening (McConn & Browse 1996a) and defense responses against herbivorous or necrotrophic pathogens (Farmer et al. 2003). JA and its derivative methyl-jasmonate (MeJA) were long considered as the active hormones. However, the discovery of the JA-insensitive mutant *jar1* (*jasmonate resistant 1*) gave the first hint that JA-Ile is the active component in JA signaling. The *jar1* mutant exhibits a defect in an enzyme converting JA to its amino acid conjugates, preferentially JA-isoleucine (JA-Ile) (Staswick et al. 1992a; Staswick & Tiryaki 2004). This was supported by studies complementing the *jar1* mutant by external JA-Ile application (Fonseca et al. 2009).

COI1 (CORONATINE INSENSITIVE 1) has been known for a long time as a key component of JA signaling. *coi1* was discovered in a mutant screen for individuals that are insensitive to the bacterial phytotoxin and JA-Ile analog coronatine (Feys et al. 1994). COI1, an F-box protein, exhibits sequence homology to the auxin receptor TIR1 (TRANSPORT INHIBITOR RESPONSE 1) (Xie et al. 1998; Dharmasiri et al. 2005). F-box proteins are known to determine the specificity of SKP-CULLIN-F-box (SCF) E3 ubiquitin ligase complexes, which ubiquitinylate other proteins and thereby mark them for degradation via the 26S proteasome (Moon et al. 2004). SCF^{TIR1} is activated by auxin and leads to the ubiquitinylation of AUX/IAA proteins, which function as repressors of auxin signaling (Tan et al. 2007). The sequence identity between COI1 and TIR1 gave the first hint for the involvement of proteasome-dependent protein degradation in JA signaling. Plants that carry defects in components of the SCF complex that are shared by SCF^{COI1} and SCF^{TIR1} are impaired in both JA and auxin signaling (Xu et al. 2002; Tiryaki & Staswick 2002).

The targets of the SCF^{COI1} complex remained unknown for a long time until a group of functionally redundant JA-inducible ZIM (zinc-finger protein expressed in plant inflorescence meristem) domain proteins were identified by genetic screens and microarray analysis (Chini et al. 2007; Thines et al. 2007). These JAZ (jasmonate-ZIM

domain) proteins show a high homology based on the conserved ZIM domain and a C-terminal Jas motive. JAZ proteins (JAZ1, JAZ3, and JAZ10) with either a mutated or a deleted Jas motif exhibit a dominant-negative effect on many JA-inducible genes (Thines et al. 2007; Chini et al. 2007). Yeast two-hybrid analysis and *in vitro* pull-down experiments showed an interaction of COI1 and JAZ proteins in the presence of JA-Ile or coronatine, but not in the presence of JA, MeJA (methyl jasmonate), or the JA precursor 12-oxophytodienoic acid (OPDA) (Thines et al. 2007).

Most recent interaction studies and the crystal structure of the COI1/JAZ1/coronatine complex demonstrated that JAZ proteins are essential for an efficient binding of coronatine to COI1. Furthermore, the crystal structure of this complex revealed that JAZ1 directly binds to the coronatine molecule, which is bound in the ligand binding pocket of COI1. By closing the ligand binding pocket like a clamp, JAZ1 contributes to stable ligand binding. This contribution to ligand recognition is carried out by seven amino acids located in the N-terminal part of the protein (Sheard et al. 2010). This is in contrast to the publication of Melotto et al. (2008) claiming that the C-terminal Jas domain is the COI1-interacting domain. For TIR1, it was known before that an inositol hexakisphosphate ($InsP_6$) molecule is bound in the center of the protein underneath the auxin binding pocket (Tan et al. 2007). Due to the high homology between TIR1 and COI1, inositol phosphates were considered as possible co-interactors of the COI1/JAZ/coronatine (JA-Ile) complex. In fact, inositol tetrakisphosphate ($InsP_4$) and inositol pentakisphosphate ($InsP_5$) were shown to promote the ligand binding of COI1 and JAZ1. As concluded from these data, a three-molecule complex consisting of COI1, a JAZ protein, and an inositol phosphate was identified as the JA-Ile (coronatine) receptor in *Arabidopsis* (Sheard et al. 2010).

JAZ proteins function as repressors of JA signaling by directly interacting with MYC2. In addition, a JA-Ile-independent interaction of JAZ1 and MYC2 has been shown. MYC2 is a positive transcriptional regulator of JA-responsive genes; it is involved in JA-mediated inhibition of root growth and in response to wounding. Mutants with a defect in the MYC2 transcription factor were named *jin1* (*jasmonate insensitive 1*). Yet, the *jin1* mutant carrying a mutated *MYC2* allele is fertile, which is in contrast to the *coi1* mutant and to plants expressing a dominant-negative JAZ protein. It has been concluded that MYC2 is not the only transcriptional activator downstream of the COI1-dependent JA signaling cascade (Lorenzo et al. 2004; Berger et al. 1996).

The presence of JA-Ile does not only promote the interaction of JAZ proteins and COI1 but also to the degradation of JAZ proteins via the 26S proteasome leading to the activation of JA-responsive gene expression by MYC2. Interestingly, the truncated proteins were not only resistant to proteasomal degradation but additionally prevented the degradation of other JAZ proteins. Until now, the reason for the dominant-negative effects has not been entirely deciphered. It is presumed that heterodimers of truncated and wild-type JAZ proteins are protected against degradation and are still able to repress transcriptional activation by MYC2 (Memelink 2009; Chini et al. 2007).

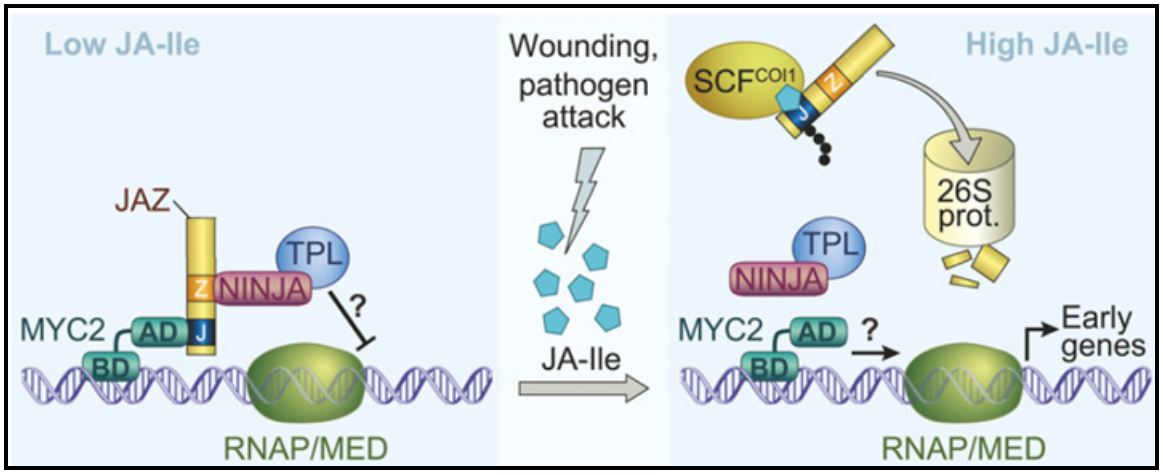

Figure 2.1: Current model of JA signal transduction in *Arabidopsis thaliana*

JAZ (J, Jas domain; Z, ZIM domain) proteins accumulate under conditions with low JA-Ile levels and bind to the transcription factor MYC2 (BD, DNA-binding domain; AD, activation domain). The subsequent repression of JA response genes is mediated by NINJA, which binds to the JAZ proteins and recruits the corepressor TOPLESS (TPL). The working mechanism of TPL remains unknown, so far.
In response to stress, which activates JA synthesis, high levels of JA-Ile promote the interaction of SCF^{COI} and JAZ proteins and thus ubiquitination and degradation of JAZs via the 26S proteasome (26S prot.). Upon JAZ degradation, the repression of MYC2 mediated by NINJA and TPL is removed and MYC2 can activate transcription by recruiting RNA polymerase II (RNAP), which possibly requires a mediator complex (MED). (Howe et al. 2010, modified)

A characteristic example for mediating the repression of gene expression was shown in AUX/IAA proteins (repressors of auxin-dependent genes). They comprise an EAR (ETHYLENE RESPONSE FACTOR (ERF)-associated amphiphilic repression) motif to recruit the corepressor TOPLESS (TPL), which leads to the repression of gene expression (Szemenyei et al. 2008). However, JAZ proteins lack this kind of motif. Recently, the adaptor protein NINJA (novel interactor of JAZ), which recruits the corepressor TOPLESS to the JAZ proteins was identified. NINJA interacts with the TIFY domain of JAZ proteins and binds TOPLESS via its EAR motif. This result demonstrates that auxin and JA signaling do not only share the ubiquitinylation of repressors by SCF E3 ubiquitin ligases and their degradation by the 26S proteasome, but

also the repression of their target genes by engagement of the corepressor TOPLESS (Pauwels et al. 2010).

2.2 JA-dependent gene regulation in plant stress responses

In plants, adaptation to stress is regulated by plant hormones (Feys & Parker 2000; Glazebrook 2005). JA is involved in the response to herbivorous insects and to necrotrophic pathogens. To protect the plant against herbivores, JA induces two distinct defense strategies: During direct defense, plants produce enzymes or secondary metabolites that act as feeding deterrents or toxins. Indirect defense, on the other hand, uses the production of volatiles to attract enemies of the herbivores (Dicke 1999; Kessler & Baldwin 2002). Consistently, mutants in JA synthesis or signaling were shown to be more susceptible to herbivores. Caterpillars of *Pieris rapae*, e.g., perform much better on *coi1* mutants than on wild-type plants (Reymond et al. 2000). Wounding- and herbivore-induced genes like *VSP2* (VEGETATIVE STORAGE PROTEIN 2) and *LOX2* (LIPOXYGENASE 2) are activated by JA (Lorenzo et al. 2004).

In contrast to this, genes induced in response to necrotrophic pathogens like the plant defensin gene *PDF1.2* require concomitant activation by JA and ethylene (ET). Consistently, *PDF1.2* is neither expressed in the *coi1* mutant nor in ET signaling mutants like *ein2* (*ethylene insensitive 2*). Furthermore, both functional JA signaling and functional ET signaling are required for resistance against necrotrophic pathogens like *Alternaria brassicicola* (Penninckx et al. 1998) or *Botrytis cinerea* (Thomma et al. 1999).

These two JA-responsive pathways act partly in an antagonistic way. Although ET, in addition to JA, leads to the activation of *PDF1.2*, *VSP2* expression is repressed under these conditions. These divergent branches of JA signaling are regulated by MYC2 on the one side and ERF1 (ET RESPONSE FACTOR 1) and ORA59 (OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59) on the other (Lorenzo et al. 2003; Lorenzo et al. 2004; Pré et al. 2008). MYC2 activates the expression of JA-inducible genes, but at the same time it represses those activated by JA/ET. In contrast to this, ERF1 attenuates the expression of JA-inducible genes while it activates JA/ET-inducible genes (Lorenzo et al. 2004). In addition to this, ERF1 overexpression was

shown to restore resistance against necrotrophic pathogens in the *coi1* and *ein2* mutants. Therefore, it was concluded that it acts downstream of both JA and ET signaling (Lorenzo et al. 2003).

Recently, the TGA class II transcription factors, which had been known before for their involvement in salicylic acid (SA) and xenobiotic responses (Zhang et al. 2003; Mueller et al. 2008; Fode et al. 2008), were identified as additional regulators of the JA/ET response. They are required for *PDF1* expression in response to JA/ET and *Botrytis cinerea*, but have no influence on *VSP2* or *LOX2* (Zander et al. 2010).

Since simultaneous infection with both types of pathogens can occur, cross-communication between the hormone pathways further fine-tunes the defense responses. SA, which regulates the defense response against biotrophs, suppresses JA and JA/ET signaling (Bostock 2005; Beckers & Spoel 2006). This mechanism requires TGA class II transcription factors. The NPR1 (NONEXPRESSOR OF PR-GENES 1) protein, a key regulator of SA-dependent signal transduction, is only required in case of low ET levels (Spoel et al. 2003; Ndamukong et al. 2007; Leon-Reyes et al. 2009).

Vice versa, JA is capable of repressing the SA response (Nomura et al. 2005). In contrast to this, ET has a positive effect on SA-dependent defense signaling and increases the accumulation of the marker gene *PR-1* (De Vos et al. 2005).

Cross-talk experiments using the biotroph *Pseudomonas syringae* and the necrotroph *Alternaria brassicicola* demonstrated the biological relevance of the negative influence of SA on the JA response. Due to the different lifestyles of these pathogens, plants are forced to compromise between the induced defense pathways (Spoel et al. 2007). This enables pathogens to manipulate plant defense responses: The phytotoxin coronatine, which is produced by some *Pseudomonas syringae* strains, mimics JA-Ile (Sheard et al. 2010) to activate JA signaling and thereby suppresses SA-mediated defense responses. The *coi1* mutant was more resistant in these experiments (Nomura et al. 2005).

In addition, abscisic acid (ABA), a plant hormone predominantly involved in abiotic stress, was shown to influence JA responses. In contrast to SA, which represses JA as well as JA/ET signaling, ABA represses JA/ET-mediated gene expression but promotes the expression of the JA-inducible gene *VSP2* (Anderson et al. 2004).

2.3 Detoxification of xenobiotics in plants

Xenobiotics ("life-foreign substances") are compounds that do not occur naturally in the respective organisms or at least occur in much lower concentrations. Plants are challenged by different kinds of xenobiotics from diverse sources. Characteristic examples are pathogens producing toxins to harm the plant and gain nutrients, neighboring plants producing allelochemicals to restrict germination or growth of competitors, and humans using agrochemicals or releasing industrial chemicals into the environment. Xenobiotics can cause severe damage to the plants, either after direct application or after being taken up from the soil.

Since the evolutionary arms race between plants or between plants and pathogens created a nearly ubiquitous threat, plants developed pathways to detoxify a variety of harmful substances. In general, the plant process to detoxify a xenobiotic is divided into three phases: phase I, activation; phase II, conjugation; and phase III, compartmentation.

During the first phase, xenobiotics are activated by oxidation, hydrolysis or reduction via enzymes such as cytochrome P450 monooxygenases, esterases, amidases, peroxidases and reductases. The activated metabolites provided by phase I are more reactive, and therefore may be sometimes even more dangerous to the cell (Sandermann Jr. 1992; Cole 1994; Dohn & Krieger 1981). Their toxicity decreases upon conjugation to hydrophilic endogenous metabolites, mainly to sugars like glucose and malonate or to glutathione. Which conjugates are produced often depends on the reactive chemical groups of the xenobiotics. It is not necessary that all xenobiotics be activated by first-phase reactions; molecules already possessing suitable residues can directly be metabolized in phase II. For example, electrophilic substances can be directly conjugated to glutathione, with the reaction being catalyzed by glutathione *S*-transferases (Armstrong 1991; Dixon et al. 1998). The conjugation of UDP-glucose (uridine diphospho-glucose) to hydroxyl, sulfhydryl, amino and carboxyl groups of xenobiotics by glycosyltransferases represents another conjugation reaction. Although glucose is the most commonly used sugar in this kind of conjugation, various monosaccharides, disaccharides and amino acids may also be conjugated to xenobiotics (Cole 1994; Coleman et al. 1997). The products obtained from these chemical transformations are usually less toxic and more hydrophilic than the parent components. Yet, the conjugates produced are not accumulated in the cytoplasm, because they may inhibit the action of conjugation enzymes by product inhibition. After phase II reactions,

the xenobiotic conjugates are transported either to the vacuole or to the apoplastic space by ABC-transporters. In the cell wall, xenobiotic conjugates may be further conjugated to lignin or cellulose (Sandermann 1994; Coleman et al. 1997). As detoxification of xenobiotics in plants proceeds analogous to the human liver mechanism, the term "green liver" has been proposed to describe phytotransformation.

2.4 Gene regulation in response to xenobiotic stress in plants

The metabolization of diverse xenobiotics has been studied extensively in plants and in mammals, but little is known about the transcriptional regulation of the enzymes involved in this process in plants. In contrast, different ways of transcriptional regulation of genes that are involved in detoxification have been elucidated in mammals. Some of the predominant examples of xenobiotic receptors are the aryl hydrocarbon nuclear receptor (AhR), the pregnane X receptor (PXR), and the constitutive androstane receptor (CAR), which bind promiscuously to structurally diverse xenobiotics. Upon xenobiotic ligand binding, the cytosolic transcription factor AhR is translocated into the nucleus; there, it dimerizes with its co-activator Arnt (AhR nuclear translocator) to activate the transcription of its target genes (Denison & Nagy 2003). In a similar way, PXR and CAR dimerize with the 9-*cis*-retinoic acid receptor upon ligand binding and subsequently lead to transcriptional activation (Kliewer et al. 2002).

Another mammalian system to sense xenobiotics is redox regulated. Since many xenobiotics are electrophilic, they are able to oxidize biomolecules. This leads to redox changes, which are sensed by receptors. For instance, Nrf2 (NF-E2-related factor 2) is tethered in the cytoplasm by binding to reduced Keap1 (Kelch-like ECH-associated protein 1). Electrophilic stress causes the oxidation of two critical cysteine residues of Keap1, resulting in Nrf2 release, which then translocates into the nucleus. In the nucleus, it heterodimerizes with a small Maf protein and binds to the antioxidant response element/electrophile response element (ARE/EpRE) to initiate the transcription of its target genes (Nguyen et al. 2004).

The mechanisms by which xenobiotic stress leads to transcriptional activation in plants remain relatively unknown. Previous studies have reported the involvement of TGA (TGACG motif-binding) transcription factors in this process (Mueller et al. 2008; Fode et al. 2008; Baerson et al. 2005). TGA transcription factors are a group of basic-leucine-

zipper (bZIP) transcriptional regulators, named after their ability to bind TGACG motifs (Katagiri et al. 1989).

After treatment of plants with phytoprostanes (highly reactive compounds generated by non-enzymatic lipid oxidation processes), a subset of putative detoxification genes was induced. The expression of 60 % of the genes is reduced in the *tga2,5,6* mutant (Mueller et al. 2008). Furthermore, TGACG motif enrichment was demonstrated in the promoter regions of genes that are responsive to the allelochemical compound benzoxazolin-2(3*H*)-one (BOA) (Baerson et al. 2005) and the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) (Fode et al. 2008).

TGA transcription factors were initially reported as mediators of the SA response (Zhang et al. 2003). In addition, many genes exhibiting TGA binding sites in their upstream region, among them many *GST* genes (Wagner et al. 2002) and synthetic promoters containing TGA binding motifs (Redman et al. 2002) are additionally activated by the auxin analog 2,4-dichlorophenoxyacetic acid (2,4-D). While the only SA-inducible genes require the known TGA interacting ankyrin repeat protein NPR1 (NONEXPRESSOR OF PR) (Zhang et al. 2003), the SA- and 2,4-D-inducible promoters are NPR1 independent (Butterbrodt et al. 2006). Since the induction of these promoters by SA and 2,4-D requires unphysiologically high concentrations (Pascuzzi et al. 1998), a general response to xenobiotic stress rather than specific SA or auxin signaling was postulated (Zhang & Singh 1994).

Previous studies performing yeast two-hybrid assays in order to find proteins interacting with TGA2 identified SCL14 (SCARECROW-LIKE 14) (Siemsen 2005, Fode 2008). SCL14 was demonstrated to be an additional component in the regulation of detoxification genes (Fode et al. 2008). Microarray analysis comparing the transcriptome of *scl14* knockout plants with those of Col-0 wild-type plants and plants ectopically expressing SCL14 revealed that SCL14 proteins activate the expression of several genes that are putatively involved in the detoxification of xenobiotics, such as glutathione *S*-transferases, (UDP-)glycosyltransferases and cytochrome P450 proteins (Siemsen 2005; Fode 2008).

SCL14 is a member of the GRAS protein family, named after the founding members GIBBERELLIN ACID INSENSITIVE (GAI), REPRESSOR of GA1 (RGA), and SCARECROW (SCR) (Pysh et al. 1999). The GRAS family of proteins, which is unique to plants, includes 33 members in *Arabidopsis*. They exhibit diverse functions in plant growth and development (Bolle 2004). GRAS proteins contain a unique

N terminus and a conserved C-terminal GRAS domain, which is defined by two leucine-rich regions (LHRI and LHRII) and three characteristic amino acid signatures: VHIID, PFYRE, and SAW (Bolle 2004). Although GRAS proteins were classified as transcriptional regulators, direct DNA binding has not been shown for any of them (Tian et al. 2004). DELLA proteins are a well-investigated group of GRAS proteins, which act as inhibitors of GA signaling (Hartweck 2008). Recently, it was shown that they regulate gene expression via the binding of transcriptional activators, the PIF (PHYTOCHROME-INTERACTING FACTOR) proteins, by preventing their binding to DNA (de Lucas et al. 2008).

Chromatin immunoprecipitation (ChIP) analysis revealed that recruitment of SCL14 to its target promoters of the *CYP81D11*, *MtN19-like* and *GSTU7* genes depended on TGA2,5,6. In contrast to this, binding of TGA class II transcription factors to TGACG motifs occurred in an SCL14-independent manner. This indicates that the SCL14/TGA complex binds to its target promoters via the DNA binding ability of the TGA factors. Especially *CYP81D11* shows strong induction in response to a variety of xenobiotics, such as TIBA, isonicotinic acid (INA), SA and 2,4-D. The involvement of SCL14 and TGA2,5,6 in xenobiotic detoxification is further supported by the ability of ectopically expressed SCL14 to mediate increased tolerance to these xenobiotics (TIBA, INA, SA and 2,4-D) compared to the wild-type, whereas *scl14* and *tga2,5,6* mutant plants exhibited higher susceptibility (Fode 2008).

Three studies investigating xenobiotic stress in response to TIBA, BOA and phytoprostanes (Mueller et al. 2008; Fode et al. 2008; Baerson et al. 2005) and a study analyzing the response to *cis*-jasmone (CJ) performed microarray analyses. All of them reveal *CYP81D11* as one of the most strongly up-regulated genes. Additionally, serial analysis of gene expression (SAGE) revealed that this gene was up-regulated in response to the explosive agent TNT (Ekman et al. 2003).

Up to now, the catalytic reaction of CYP81D11 has remained unknown. Furthermore, a function for CYP81D11 has only been reported for the attraction of insects after CJ treatment. CJ is a plant-derived volatile released as part of the floral volatile bouquet and in response to herbivore attacks. It attracts the aphid parasitoid *Aphidius ervi*, as an "indirect induced defense" response. CJ-treated *cyp81d11* knockout mutants were unable to attract these aphid parasitoids, while *A. ervi* spent twice as much time on CJ-treated wild-type plants compared to untreated ones. The above observation revealed the

importance of CYP81D11 in CJ-dependent indirect herbivore defense (Matthes et al. 2010; Bruce et al. 2008).

2.5 Aim of this study

This study is based on the thesis of Benjamin Fode who demonstrated the involvement of a TGA2,5,6/SCL14 complex in gene regulation after chemical-induced stress. As a direct target gene of this complex, he identified the cytochrome P450 gene *CYP81D11*. He further demonstrated a strong induction of this gene and its co-regulation with further SCL14 target genes in response to several chemicals.

A unique and unusual feature of the *CYP81D11* gene was reported by Mueller et al. (2008): In contrast to many xenobiotic-induced genes, *CYP81D11* is induced additionally by JA. Moreover, its response to reactive phytoprostanes was dependent on COI1. Based on this observation the following questions were addressed in this thesis:

- What is the role of JA and components of the JA signal transduction pathway in the regulation of the expression of *CYP81D11*?
- Do this mechanisms control other genes induced by xenobiotic stress?
- Which role plays the SCL14/TGA2,5,6 complex for the regulation of *CYP81D11* and other JA-responsive genes after JA treatment?
- Are the transcription factors ATAF1 and ANAC032 that are co-regulated with *CYP81D11* in response to xenobiotic, but are independent of COI1, involved in the regulation of *CYP81D11*?

3 Materials and methods

3.1 Materials

3.1.1 Equipement

Device	Model	Source
Autoclave	3870 ELV	Tuttnauer
Automatic pipettes		Gilson
Balance	SPO52; SAC62; 1207MP2	Scaltec; Satorius
Blotting device		University of Göttingen
Chambers for gel electrophoresis		University of Göttingen
Cooling centrifuge	Sorvall RC 5B Plus	DuPont
Cooling centrifuge	Rotina 35R	Hettich
Cooling microcentrifuge	Mikro 200R	Hettich
Counting chamber	Fuchs-Rosenthal	Brand
Counting chamber	Thoma	
Digital camera	Powershot A510	Canon
Electroporator	Gene Pulser® II	BioRad
Gel documentation device		MWG Biotech
Heating block		Boekel Scientific
Heated stirrer	RCT basic	IKA Labortechnik
Heated shaker	Thermomixer 5436	Eppendorf
Ice machine	Af20	Scotsman
Incubator		WTC binder; Memmert
Microcentrifuge	Biofuge pico	Heraeus Christ
Microcentrifuge, cooled	5403	Eppendorf
Microscope	DM 5000B + CTR 5000	Leica
PCR cycler	MiniCycler™ PTC-150, iCycler	MJ Research, Biorad
pH meter	HI 9321	Hanna Instruments
Photometer	Unikon 720 LC	Kontron
Photometer for microtiter plates	MRX Dynex Plate Reader	Dynex
Real-time RT-PCR cycler	iCycler	BioRad

Device	Model	Source
Photometer for the calculation of DNA/RNA concentrations	GeneQuant II, NanoVue	Pharmacia, GE Healthcare
Scanner	ScanJet 4c	Hewlett-Packard
Sequencer	ABI PRISM 3100	Perkin-Elmer
Sonication device	Soniprep 150	MSE
Clean bench	Microflow Laminar	Nunc
Clean bench	Microflow Biohazard	Nunc
UV transilluminator	FLX-20 M	Vilber Lourmat
Water deionization device	Option 4, Maxima	ELGA
Vacuum pump	Cyclo 1	Roth
Vortex	L46	Labinco BV, The Netherlands

3.1.2 Consumables

Product	Source
Blotting paper 3MM	Whatman
Filter paper Miracloth	Calbiochem
96-well microtiter plates, white, flat-bottom	Greiner bio-one
Microtiter plates	Roth
Parafilm 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

3.1.3 Chemicals

Chemical	Source
30 % (w/v) acrylamide/N,N'-methylenebisacrylamide (37.5:1)	Roth
Agarose SeaKem LE	Biozym
Ampicillin	AGS
APS (ammonium persulfate)	Biometra
Benoxacor	Sigma
BOA	Sigma
Bradford reagent	Roth
Bromophenol blue	Roth
BSA	Serva
Coomassie Brilliant Blue G-250	BioRad
Dimethyl sulfoxide (DMSO)	Sigma
dNTPs	MBI; Roth
Ethylene diaminetetraacetate (EDTA)	AppliChem
Ethidiumbromide	Roth
Fat-free milk powder	Glücksklee
Fluoresceine	BioRad
Gentamycin	Duchefa
IAA	Roth
Jasplakinolide	Invitrogen
Kanamycin	Sigma
β-Mercaptoethanol	Roth
Methyl jasmonate	Sigma-Aldrich
Murashige and Skoog medium	Duchefa
Orange G	Sigma
Phenol	Sigma
Rifampicin	Duchefa
X-ray developer LX24	Kodak
X-ray fixer AL4	Kodak
Select Agar Life	Technologies
Select Yeast Extract	GIBCO BRL
SYBR Green I	Cambrex

Chemical	Source
TEMED	Roth
TIBA	Sigma
Triton X-100	Roth

3.1.4 Kits

Enzyme/Kit	Source
BigDye Terminator Cycle Sequencing Ready Reaction Kit v.3.1	Perkin-Elmer Corporation
BioTaq DNA polymerase	Bioline
Desoxyribonuclease I (DNase I) RNase free	MBI Fermentas
Dual-luciferase reporter assay system	Promega
Enhanced Chemiluminescence Plus Kit (ECL+)	GE Healthcare
HiDi-Mix	ABI PRISM
Immolase DNA polymerase	Bioline
iProof High-Fidelity DNA Polymerase	BioRad
Nucleo Spin Extract II	Macherey-Nagel
Nucleo Spin Plasmid	Macherey-Nagel
Pierce 660nm Protein Assay Kit	Thermo Scientific
Reverse transcriptase H–	MBI Fermentas
Restriction enzymes	MBI Fermentas, New England Biolabs
RNeasy	Qiagen
RNase A (DNase free)	Qiagen
T4-DNA ligase	MBI Fermentas

3.1.5 Standards

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

3.1.6 Antibodies

Antibody	Source
HA-tag antibody ChIP grade	Abcam

3.1.7 DNAs

3.1.7.1 Plasmids

Plasmid	Description	Reference
pAlligator2	gateway™ vector for plant transformation, contains the *CaMV 35S* promoter, the *nos* terminator, a 3× HA-tag (N-terminal), and a GFP selection marker under control of the seed-specific promoter *At2S3*, *spn*^{r}	http://www.isv.cnrs-gif.fr/JG/alligator/intro.html
pAlligator2-COI1	pAlligator2 derivative containing the *COI1* coding sequence	Sonja Schöttle, personal communication
pAllogator2-COI1-85	pAlligator2 derivative containing the coding sequence for a COI1 protein with an amino acid substitution of aa 85–88 from RAAM to HFAD	Sonja Schöttle, personal communication
pAlligator2-COI1-G98D	pAlligator2 derivative containing the coding sequence for a COI1 protein with an amino acid exchange of aa 98 from G to D	Sonja Schöttle, personal communication
pB2GW7	gateway™ vector for plant transformation, contains the *CaMV 35S* promoter and a BASTA resistance gene as selection marker, *spn*^{r}	(Karimi et al. 2002)
pB2GW7-HA	gateway™ vector for plant transformation, contains the *CaMV 35S* promoter, a 3× HA-tag (N-terminal), and a BASTA resistance gene as selection marker, *spn*^{r}	C. Thurow, personal communication
pB2GW7-HA-ANAC032	pB2GW7-HA derivative containing the *ANAC032* coding sequence	this thesis
pB2GW7-HA-ATAF1	pB2GW7-HA derivative containing the *ATAF1* coding sequence	this thesis
pBGWFS7	gateway™ vector for plant transformation, contains the *GUS* reporter gene and a BASTA resistance gene as selection marker, *spn*^{r}	(Karimi et al. 2002)

Plasmid	Description	Reference
pBGWL7	gateway™ vector for plant transformation, contains the firefly (ff) luciferase reporter gene and a BASTA resistance gene as selection marker, *spn*^{r}	(Karimi et al. 2002)
pBGWFS-cyp-prom	pBGWFS7 derivative containing an 894-bp *CYP81D11* promoter fragment and the *CYP81D11* 5' UTR upstream of the *GUS* reporter gene, *spn*^{r}	this thesis
pBGWFS-cyp-mas1	pBGWFS7 derivative containing an 894-bp *CYP81D11* promoter fragment with mutated *as1*-like element and the *CYP81D11* 5' UTR upstream of the *GUS* reporter gene, *spn*^{r}	this thesis
pBGWFS-cyp-prom-mG-box	pBGWFS7 derivative containing an 894-bp *CYP81D11* promoter fragment with mutated G-boxes and the *CYP81D11* 5' UTR upstream of the *GUS* reporter gene, *spn*^{r}	this thesis
pBGWFS-cyp-mas1-mG-box	pBGWFS7 derivative containing an 894-bp *CYP81D11* promoter fragment with mutated *as1*-like element and G-boxes and the *CYP81D11* 5' UTR upstream of the *GUS* reporter gene, *spn*^{r}	this thesis
pBGWL-cyp-prom	pBGWL7 derivative containing an 894-bp *CYP81D11* promoter fragment and the *CYP81D11* 5' UTR upstream of the *GUS* reporter gene, *spn*^{r}	this thesis
pBGWL-cyp-mas1	pBGWL7 derivative containing an 894-bp *CYP81D11* promoter fragment with mutated *as1*-like element and the *CYP81D11* 5' UTR upstream of the *ff luciferase* reporter gene, *spn*^{r}	this thesis
pBGWL-cyp-prom-mG-box	pBGWL7 derivative containing an 894-bp *CYP81D11* promoter fragment with mutated G-boxes and the *CYP81D11* 5' UTR upstream of the *ff luciferase* reporter gene, *spn*^{r}	this thesis
pBGWL-cyp-mas1-mG-box	pBGWL7 derivative containing an 894-bp *CYP81D11* promoter fragment with mutated *as1*-like element and G-boxes and the *CYP81D11* 5' UTR upstream of the *ff luciferase* reporter gene, *spn*^{r}	this thesis
pDONOR201	gateway™ entry vector for cloning of PCR fragments, *km*^{r}	Invitrogen
pDONOR201-cyp-prom	pDONOR201 derivative containing an 894-bp *CYP81D11* promoter fragment and the *CYP81D11* 5' UTR, *km*^{r}	this thesis

Plasmid	Description	Reference
pDONOR201-cyp-mas1	pDONOR201 derivative containing an 894-bp *CYP81D11* promoter fragment with mutated *as1*-like element and the *CYP81D11* 5' UTR, km^r	this thesis
pDONOR201-cyp-prom-mG-box	pDONOR201 derivative containing an 894-bp *CYP81D11* promoter fragment with mutated G-boxes and the *CYP81D11* 5' UTR, km^r	this thesis
pDONOR201-cyp-mas1-mG-box	pDONOR201 derivative containing an 894-bp *CYP81D11* promoter fragment with mutated *as1*-like element and G-boxes and the *CYP81D11* 5' UTR, km^r	this thesis
p70SRUC	Plasmid containing the Renilla luciferase gene controlled by the 70S promoter, amp^r	KWS

3.1.7.2 Oligonucleotides

Oligonucleotides were synthesized by Invitrogen. QuantiTect Primer Assays from Qiagen contain both forward and reverse primers. They are indicated as "QPA" and are described at http://www1.qiagen.com/Products/Pcr/QuantiTect/PrimerAssays.aspx.
Oftern used QuantiTect primers were imitated on the basis of sequence analysis of a cloned PCR fragment after amplification with the original assay primers. These primers were diluted and mixed to result in a 4 µM stock solution containing forward and reverse primers.

Gene	Oligonucleotide	Sequence 5'→3'	Source
Quantitative real-time RT-PCR primers			
AACT1	QT00846762	QPA	Qiagen
ANAC032	QT00743561	QPA	Qiagen
ASN1	Q_ASN1_forw Q_ASN1_rev	TTCTTGAGCTTTCTCGCAGAT CCGTTCTGATATAAGCCACTCC	Invitrogen
ATAF1	QT00866439	QPA	Qiagen
ATSIP2	QT00793912	QPA	Qiagen
COR78	QT00840406	QPA	Qiagen
CYP81D11	Q_CYP81D11_forw Q_CYP81D11_rev	TTATGATACTTGCCGGGACTG CGATTTCGTCTTTGCC	Invitrogen
DIN11	QT00788424	QPA	Qiagen
GSTU1	QT00759423	QPA	Qiagen

Gene	Oligonucleotide	Sequence 5'→3'	Source
GSTU4	QT00759402	QPA	Qiagen
GSTU7	QT00759381	QPA	Qiagen
ICS1	QT00893473	QPA	Qiagen
JAZ10	QT00828401	QPA	Qiagen
LOX2	QT00785309	QPA	Qiagen
MYC2	QT00872333	QPA	Qiagen
PDF1.2	PDF1.2 RT fwd PDF1.2 RT rev	CTTGTTCTCTTTGCTGCTTTC CATGTTTGGCTCCTTCAAG	Invitrogen
VSP2	VSP2 fwd RT VSP2 rev RT	CAAACTAAACAATAAACCATACC ATAA GCCAAGAGCAAGAGAAGTGA	Invitrogen
at3g23550	QT00777994	QPA	Qiagen
Primers for genotyping			
dde2-2	*dde2-2*_up *dde2-2*_rp	AATCGTAGGACCAATCAAAGACC G GGTGGTAGACTAAATGTATGGAT GAGAGG	Invitrogen
jar1-1	*jar1-1*_up *jar1-1*_rp	CGGATAAGAGATGGCAATACAA GG AAACTGTGGTCTCAATGGAAACG	Invitrogen
ataf1-1/-2	ATAF1_UP ATAF1_RP	CGCCAAGTTTCAGAGGTAGAGAG AG TAAAACGGTCTCGTGTTGCCATA A	Invitrogen
SALK_012253	SALK_012253_UP SALK_012253_RP	TTTTTAATTACGGCGGAAAGAGA ATAG CTTAATACCAACCGGTTTAGGAC G	Invitrogen
SALK_132588	SALK_132588_UP SALK_132588_RP	ACCGGTTTACAATTTACAGACAT GGC TTGCTTCCTGAAAATAACAACAC AATACAG	Invitrogen
JAZ1Δ3A	JAZ1_up_GW OCG_42	GGGGACAAGTTTGTACAAAAAA GCAGGCTTCATGTCGAGTTCTAT GGAATGTTCTG TAGCGATCCAGACTGAATGCCAC A	Invitrogen
Primers for cloning			
ANAC032	ANAC032_GW_UP ANAC032_GW_RP	GGGGACAAGTTTGTACAAAAAA GCAGGCTTCATGATGAAATCTGG GGCTGATTTG GGGGACCACTTTGTACAAGAAAG CTGGGTCTCAGAAAGTTCCCTGC CTAACCAC	Invitrogen

Gene	Oligonucleotide	Sequence 5'→3'	Source
ATAF1	ATAF1_GW_UP	GGGGACAAGTTTGTACAAAAAA GCAGGCTTCATGTCAGAATTATT ACAGTTGCCTCCAG	Invitrogen
	ATAF1_GW_UP	GGGGACCACTTTGTACAAGAAAG CTGGGTCCTAGTAAGGCTTCTGC ATGTACATGAAC	
CYP81D11 promoter	CYP81D11as1m-low	GTGATTTACTATGTAATCTTGCAT CTAGAATTGTTTTTTTCTTTCTTT GTTTTG	Invitrogen
	CYP81D11as1m-up	CAATTCTAGATGCAAGATTACAT AGTAAATCACATAATGTTCACGT GTC	
	CYP81D11Prom_724up	CAATCACGAAATCAATAATCAAT AATATCC	
	CYP81D11Prom_up	GGGGACAAGTTTGTACAAAAAA GCAGGCTAAGGGTAATTTGGTCT TAACAATCTCC	
	CYP81D11Prom-low	GGGGACCACTTTGTACAAGAAAG CTGGGTGACATTGATTAAAAACA TGTGAGTTATAGCTG	
	CYPP-Δmyc_UP	TATGCAATGACGACAAGTAAATC ACATAATGTTTTCAAGTTTCAAA GAT	
	CYPP-Δmyc_RP	ATCTTTGAAACTTGAAAACATTA TGTGATTTACTTGTCGTCATTGCA	
	CYPP-Δ*as1*-Δmyc_UP	CTAGATGCAAGATTACATAGTAA ATCACATAATGTTTTCAAGTTTCA AAGAT	
	CYPP–Δ*as1*-Δmyc_RP	ATCTTTGAAACTTGAAAACATTA TGTGATTTACTATGTAATCTTGCA T	
Primers for Sequencing			
GUS	OCG_42	TAGCGATCCAGACTGAATGCCAC A	Invitrogen
LUC	OCG_43	ATGCAGTTGCTCTCCAGCGGTTC C	Invitrogen
pDonor201	Seq-L1	TCGCGTTAACGCTAGCATGGATC TC	Invitrogen
	Seq-L2	GTAACATCAGAGATTTTGAGACA C	
pB2GW7(-HA)	pB2GW7fwd pB2GW7rev	CACAATCCCACTATCCTTCGCA CATGAGCGAAACCCTATAAGAAC C	Invitrogen

3.1.8 Organisms

3.1.8.1 Plant genotypes

Genotype	Description	Reference
Columbia, Col-0	Wild type	NASC stock no. N1092, NASC 2002
as-1:GUS/35S:SCL14-HA	Overexpression line, expressing the *SCL14* gene fused to an HA-tag (N-terminal) under control of the *CaMV* 35S promoter, expresses *GFP* in seeds for selection, line #5 was used in this work	(Siemsen 2005)
coi1-1	Knockout line lacking COI1, impaired in most JA-dependent responses	(Feys et al. 1994; Xie et al. 1998)
coi1-t	T-DNA insertion within the *COI1* gene	(Mosblech et al. 2010)
dde2-2	Mutant with a defect in JA biosynthesis	(Park et al. 2002)
DR5:GUS	Transgenic plant containing the *GUS* reporter gene under the control of the synthetic auxin-responsive promoter *DR5*	(Ulmasov et al. 1997)
HS:AXR3-1	Transgenic plant containing a mutated AXR3 protein under the control of a heat shock promoter	(Knox et al. 2003)
jar1-1	Mutant deficient in JA-Ile biosynthesis	(Staswick et al. 1992b)
JAZ1Δ3A-GUS	Transgenic plant containing a JAZ1 protein with a deletion in domain 3, exhibiting a JA-insensitive phenotype	(Thines et al. 2007); Browse (Washington State University, USA)
jin1-1	Mutant with a defect within MYC2	(Berger et al. 1996)
scl14 (SALK_126931)	Knockout line containing a T-DNA insertion 55 bp upstream of the ATG of the *SCL14* gene, km^r	(Fode et al., 2008)

Genotype	Description	Reference
tga2,5,6	Knockout line lacking all three class II TGA transcription factors, impaired in systemic acquired resistance	(Zhang et al., 2003); X. Dong (Duke University, Durham, USA)

3.1.8.2 Bacteria

Genotype	Description	Reference
Agrobacterium tumefaciens GV3101	PMP90RK *rif*^{r}, *gm*^{r}	(Koncz and Schell, 1986)
Escherichia coli DB3.1	F$^-$, *gyr*A 462, *end*A1, D(*sr*1-*rec*A), *mcr*B, *mrr*, *hsd*S20 (r_B^- m_B^-), *sup*E44, *ara*-14, *gal*K2, *lac*Y1, *pro*A2, *rps*L20(*Sm*^{r}), *xyl*-S, λ-leu, *mtl*-1	(Bernard et al. 1993)
Escherichia coli DH5α	F$^-$, *gyr*A 96 (*Nal*^{r}), *rec*A1, *end*A1, *thi*-1, *hsd*R17 (rk-mk+), *gln*V44, *deo*R, D (*lac*ZYA-*arg*F) U169 [p80dD(*lac*Z)M15]	(Hanahan 1983)

3.1.8.3 Fungal cultivars

Genotype	Reference
Botrytis cinerea BMM	provided by Brigitte Mauch-Mani (University of Neuchatel, Switzerland)

3.1.9 Standard media

Media	Content/Source
dYT medium for bacteria	20 g/L tryptone, 10 g/L yeast extract, 10 g/L NaCl
LB medium for bacteria	10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl
YEB medium for bacteria	10g/L beef extract, 2g/L yeast extract, 5g/L peptone, 5g/L sucrose, pH 7.0 with NaOH → autoclave 2mM $MgSO_4$ (sterile)

Media	Content/Source
MS medium for plants	4.4 g/L MS medium, pH 5.7 with KOH, 6.8 g/L select agar
PDA for fungi	Merck
PDB for fungi	Fulda

3.1.10 Standard buffers

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 O+	50 mM Tris-HCl (pH 7.5 at 37°C), 10 mM $MgCl_2$, 100 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-acetate (pH 7.9 at 37°C), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL BSA
TAE (20×)	0.8 M Tris, 2.3 % (v/v) acetic acid, 20 mM EDTA
TBS (10×)	24.2 g Tris, 80 g NaCl, pH 7.6
TBS-T (1×)	1× TBS + 0.1 % Tween 20
GUS extraction buffer	50 mM $NaPO_4$ buffer pH7.5 (80.95 ml Na_2HPO_4(0.5M) + 19.05 ml NaH_2PO_4 (0.5M)), 10 mM EDTA, 0.1 % Triton-X100, 0.1 % N-lauryl-sarkosine

3.2 Methods

3.2.1 Standard molecular methods

3.2.1.1 Isolation of plasmid DNA from *E. coli*

3.2.1.1.1 Alkaline lysis

Plasmid DNA for was isolated from *E. coli* using a modified alkaline lysis method. First, 1.5 ml of *E. coli* overnight culture (stationary phase) were collected by

centrifugation at 13,000 rpm for 1 min. The supernatant was removed and the cells were resuspended in 100 µL buffer I (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/µL RNase A). Next, 200 µL buffer II (0.2 M NaOH, 1 % (w/v) SDS) were added to the cell suspension and incubated for 5 min on ice. The suspension was neutralized by adding 150 µL buffer III (29.4 g potassium acetate, 5 mL formic acid and water to 100 mL) and inverting 6–8 times. The suspension was centrifuged for 10 min at 13,000 rpm at room temperature, and the aqueous solution (~400 µL) was transferred into a new microcentrifuge tube containing 1 mL 96 % (v/v) ethanol. The DNA was precipitated from the solution by incubating for 20 min at –20°C. Plasmid DNA was collected by centrifugation for 10 min at 13,000 rpm and 4°C. The pellet was washed with 70 % (v/v) ethanol and air-dried for 10 min at 37°C. The DNA was dissolved in 20 µL EB buffer (10 mM Tris-HCl pH 8.5).

3.2.1.1.2 Isolation of high-quality plasmid DNA

For sequencing and gateway® cloning purposes, high-purity plasmid DNA was isolated using the Nucleospin Mini Kit (Macherey-Nagel), following the manufacturer's instructions. Optional steps were always performed according to the manufacturer's recommendation. A 5-mL overnight culture was used to isolate plasmids and the isolated DNA was eluted with 50 µL (high-copy plasmids) or 30 µL (low-copy plasmids) EB buffer or water (ultra-pure).
To isolate larger amounts of plasmid DNA from *E. coli*, Macherey-Nagel Midi or Maxi Kit were used. The manufacturer's protocol, including the optional recommendations, was followed.

3.2.1.2 Determination of DNA and RNA concentrations

The concentration of nucleic acids was determined 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 nucleotide heterocycles). An OD_{260} reading of 1 in a cuvette with 10 mm path length corresponds to 50 and 40 µg/mL double-stranded DNA and RNA, respectively. Absorption at 280 nm (due to the presence of aromatic rings from amino acids and phenol compounds) was used to give information on the purity of the DNA or RNA sample, with the optimal ratio of OD_{260}/OD_{280} being in the range of 1.9–2.0 for RNA and 1.8 for DNA. DNA concentrations less than 100 ng/µL were measured on an agarose gel using the Gene Ruler Ladder Mix as a standard.

3.2.1.3 Restriction digestion of DNA

Type II endonucleases were used to digest double-stranded DNA molecules for analytical and cloning purposes. The enzymes cleave the DNA resulting in either 5' or 3' "sticky" overhangs or in blunt ends. The digestion reactions were incubated in the buffer system optimized for the enzyme used or, in the case of double digestion, a universal buffer was used. The activity of the restriction enzymes was given in "units"

(U), where 1 U is defined as the amount of enzyme completely cutting 1 µg of λ DNA in 60 min under optimal conditions. The minimal amount of enzyme necessary for each restriction digest was determined according to the following formula: U = (bp[λ] × number of restriction sites in target DNA)/(number of restriction sites in [λ] × bp of target DNA), with λ = 48,500 bp.
The incubation temperature was 37°C unless otherwise mentioned for a particular restriction enzyme. Due to the adverse effects of high glycerol concentrations, the total volume of the restriction enzymes did not exceed 10 % of the restriction mix.

3.2.1.4 Separation of DNA on agarose gels

The electrophoretic separation of DNA for analytical and preparative purpose was done in a horizontal agarose gel (10 cm × 7 cm × 0.3 cm, 16 lanes) with 1× TAE as running buffer. DNA fragments ranging between 500 bp and 14 kb were run on a 1 % agarose gel, whereas DNA fragments of lower size were run on a 2 % agarose gel. DNA samples were mixed with 1/10 the volume of 10× DNA loading buffer, loaded into separate lanes and run at 120 V for 40–45 min. Ethidium bromide solution (0.1 % w/v) was used to stain the DNA fragments. The detection of DNA was done under UV light (260 nm). When a preparative gel was run and DNA fragments of a particular band had to be cut out, e.g. for cloning purposes, detection was done using larger-wavelength UV light (320 nm). Before exposure to UV light, the gel was rinsed briefly in H_2O to reduce the background staining. In a gel documentation station, the gels were visualized on a UV-transilluminator. The sizes and amounts of the DNA fragments were determined using DNA standards.
Elution of DNA fragments from an agarose gel was done using the Nucleospin Extract II Gel Extraction Kit following the manufacturer's instructions. The eluted fragments were verified by electrophoresis as described above.

3.2.1.5 Hybridization of complementary DNA fragments

For hybridization 1 nmol of complementary DNA oligonucleotides (in a total volume of 20 µL) was added into a screw-cap reaction tube and heated for 10 min in a 100°C water bath. The samples were then allowed to cool down to room temperature overnight.

3.2.1.6 Ligation of DNA fragments

Conventional cloning of a DNA fragment into a selected vector 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× excess over the vector) was incubated with the vector DNA, 2 µL of ligation buffer and 1 µL of T4-DNA ligase, for 2 h at room temperature. Ligation of blunt ended DNA fragments was performed in the ligation mixture descibed above supplemented with 5 % (w/v) PEG 4000. Ligase activity was destroyed by incubating at 65°C for 10 min.

3.2.1.7 Gateway® cloning

The cloning of binary vectors for transient and stable plant transformation purposes was performed with the gateway® cloning system from Invitrogen. The gateway® technology is based on the site-specific recombination of bacteriophage λ and thereby provides a fast method to move DNA sequences between multiple vector systems without the use of restriction enzymes (Landy 1989; Hartley et al. 2000). All cloning steps were performed as described in the Invitrogen manual, Version E, September 22, 2003.

3.2.1.8 DNA sequencing

DNA sequencing was done using the BigDye Terminator RR Mix Cycle Sequencing Kit. The principle of DNA sequencing is based on the chain termination method (Sanger et al. 1977). In the chain termination method, dideoxynucleotides (terminators) are incorporated into a newly synthesized complementary chain, thus stopping its elongation in a PCR reaction. Each kind of dideoxynucleotide is labeled with a specific fluorescent dye, and the terminated chains can be specifically detected using an ABI Prism 3100 Capillary Sequencer (Applied Biosystems). The PCR sequencing reaction was performed using 500–1000 ng plasmid DNA, 5 pmol primer, 2 µL ready reaction (RR) mix and H_2O up to 10 µL. The samples were subjected to 25 cycles of 10 s at 95°C, 5 s at 50°C, and 4 min at 60°C in a thermocycler. The DNA product was precipitated using 9.5 µL water and 30.5 µL absolute ethanol and left for 1 h. The DNA was collected by centrifugation for 20 min at 13,000 rpm. The pellet was washed using 125 µL 70 % ethanol and then centrifuged for 10 min at 13,000 rpm. The pellet was dried at 95°C for 1 min and resuspended in 10 µL of HiDi reagent. The samples were placed on ice. The reactions were loaded onto an ABIPrism 3100 capillary electrophoresis sequencing station for analysis.

3.2.1.9 Cloning procedures

Vector	Construction
pDONOR201-ANAC032	*ANAC032* CDS was amplified by PCR from cDNA using the iProof™ High-Fidelity PCR Kit from Bio-Rad and ANAC032_GW_UP and ANAC032_GW_RP primers, using the standard protocol and PCR cycler program recommended by the manufacturer; the PCR product was cloned into pDONOR201 by BP reaction.
pDONOR201-ATAF1	*ATAF1* CDS was amplified by PCR from cDNA using the iProof™ High-Fidelity PCR Kit from Bio-Rad and ATAF1_GW_UP and ATAF1_GW_UP primers, using the standard protocol and PCR cycler program recommended by the manufacturer; the PCR product was cloned into pDONOR201 by BP reaction.

Vector	Construction
pDONOR201-cyp-prom	The *CYP81D11* promoter fragment was amplified from gDNA using the iProof™ High-Fidelity PCR Kit from Bio-Rad and the CYP81D11Prom_up and CYP81D11Prom_low primers, using the standard protocol and PCR cycler program recommended by the manufacturer; the PCR product was cloned into pDONOR201 by BP reaction.
pDONOR201-cyp-mas1	The substitution of the *as-1*-like element was obtained via overlap extension PCR. For this, two different fragments were utilized seperately in two iProof™ PCRs, using the standard protocol and PCR cycler program recommended by the manufacturer (primers: fragment 1: CYP81D11Prom_724up and CYP81D11as1m-low; fragment 2: CYP81D11as1m-up and Seq-L2). The two fragments yielded from the first PCR were purified after separation on a 1 % agarose gel and diluted 1:200. The two diluted fragments were used as templates in a second PCR (primers: CYP81D11Prom_724up and Seq-L2). The mutated fragment was cloned into pDONOR201-cyp-prom cleaved by *Bcu*I and *Pst*I.
pDONOR201-cyp-prom-mG-box	Oligonucleotides CYPP-mmyc_UP and CYPP-mmyc_RP containing the *CYP81D11* promoter fragment with the mutated G-box were hybridized and cloned into pDONOR201-cyp-prom using the *Nde*I and *Eco*RV restriction sites.
pDONOR201-cyp-mas1-mG-box	Oligonucleotides CYPP-mas1-mmyc_UP and CYPP—mas1-mmyc_RP containing the *CYP81D11* promoter fragment with the mutated G-box were hybridized and cloned into pDONOR201-cyp-prom using the *Xba*I and *Eco*RV restriction sites.
pBGWFS-cyp-prom	LR reaction was performed using pBGWFS7 and pDONOR201-cyp-prom.
pBGWFS-cyp-mas1	LR reaction was performed using pBGWFS7 and pDONOR201-cyp-mas1.
pBGWFS-cyp-prom-mG-box	LR reaction was performed using pBGWFS7 and pDONOR201-cyp-prom-mG-box.
pBGWFS-cyp-mas1-mG-box	LR reaction was performed using pBGWFS7 and pDONOR201-cyp-mas1-mG-box.
pBGWL-cyp-prom	LR reaction was performed using pBGWL7 and pDONOR201-cyp-prom.
pBGWL-cyp-mas1	LR reaction was performed using pBGWL7 and pDONOR201-cyp-mas1.
pBGWL-cyp-prom-mG-box	LR reaction was performed using pBGWL7 and pDONOR201-cyp-prom-mG-box.
pBGWL-cyp-mas1-mG-box	LR reaction was performed using pBGWL7 and pDONOR201-cyp-mas1-mG-box.
pB2GW7-HA	3× HA-tag was inserted into pB2GW7 by three-fragment ligation of pB2GW7 *Eco*147I/*Eco*RI, pB2GW7 *Eco*147I/*Sal*I, and pE-HA *Xho*I/*Eco*RI.
pB2GW7-HA-ANAC032	LR reaction was performed using pB2GW7-HA and pDONOR201-ANAC032.

Vector	Construction
pB2GW7-HA-ATAF1	LR reaction was performed using pB2GW7-HA and pDONOR201-ATAF1.

3.2.1.10 Gene transfer into *E. coli*

E. coli cells are not naturally competent, i.e. they are not able to accept foreign DNA molecules from the environment. To enable the bacterial cells to take up circular vector DNA, they have to be made competent using special treatments. For transformation of *E. coli* bacteria the heat shock method was used (Hanahan 1983).
To preduce chemical competent *E. coli* cells for heat shock transformation, 5 ml of liquid culture were incubated over night (37°C, 250 rpm) and transferred to 300 ml SOK media. Absorbance at 600 nm was monitored, and when the OD_{600} reached 0.22 the culturing temperature was decreased to 18°C. When the OD_{600} reached 0.4 - 0.5 the cells were harvested by centrifugation (10 min, 4°C, 4000 rpm) in six 50 ml tubes. All following steps were performed on ice. Cells were resuspended in with 15 ml of transformation buffer, incubated for 15 min and again centrifuged (two tubes with 45 ml each). Pellets were resuspended in 15 ml of transformation buffer and subsequently 2 x 525 µl of DMSO were added with 5 min of incubation in between. Cell suspension was portioned into 1.5 ml tubes (200µl each) and frozen in liquid nitrogen before storing at -70°C
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. The cells were heat shocked for 90 s at 42°C. Next, 700 µL LB medium were added to the tube and the suspension was incubated for 45–60 min at 37°C. The cultures were spread on plates containing LB medium supplemented with antibiotics. The plates were incubated overnight at 37°C.

SOK media (300 ml)	2 % Trypton, 0.5 % g Yeastextract, 10 mM NaCl, 2.5 mM KCl → autoclaved 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose (separately autoclaved)
Transformation buffer	10 mM PIPES, 15 mM, 250 mM KCl, → pH 6,7 55 mM $MnCl_2$ → sterile filtration

3.2.1.11 Gene transfer into *Agrobacterium tumefaciens*

A. tumefaciens GV3101 cells were transformed by electroporation. To generate electrocompetent *A. tumefaciens* cells, 10 ml of liqid culture (YEB medium) were grown over night (28°C, 250 rpm), transferred to 250 ml of YEB medium and again cultivated over night. When the OD_{600} reached 0.5 the culture was cooled on ice and subsequently centrifuged (5 min 5000 rpm, 4°C). The supernatant was discarded and the pellet was washed twice using 50 ml of sterile H_2O and twice using 10 ml of 15 % glycerine. Next the pellet was resuspended in 1 ml of 15 % of glycerine and transferred

to 1.5 ml tubes with 40 µl per tube, before tubes were frozen in liquid nitrogen and stored at -70°C.
For transformation, cells were thawed on ice, mixed with the respective plasmid DNA (ca. 100 ng) and transferred into an electroporation cuvette. An electric pulse (2.5 kV, 25 µF, 400 Ω) was applied for ~5 s. Subsequently, the cells were incubated with 1 mL LB medium for 2 h at 30°C and spread on selective YEB plates. Incubation of plates was performed for 2–3 days at 30°C. Transformed cells from the plates were grown in 25 mL selective YEB liquid medium o/n at 30°C. From 5 mL of this pre-culture, plasmid DNA was extracted (QIAprep Kit, Qiagen) to check the transformed cells. The rest of the pre-culture was transferred into 400 mL selective YEB liquid medium and incubated o/n at 30°C. Cells were harvested by centrifugation (2500 rpm, 30 min) and suspended in 5 % sucrose solution to an OD_{600} of 0.8. Silvet-L77 (0.05 %) was added to this solution prior to *Arabidopsis thaliana* transformation by floral dip transformation.

3.2.1.12 Gene Transfer into *Arabidopsis thaliana* protoplasts

Protoplast isolation and transformation was performed according to the method described by (Sheen 2001). Rosette leaves of 4- to 6-week-old plants were harvested (1 leaf / 4 transformation reactions) and the lower surface was lightly scored with a razor blade. The scored leaves were placed in a Petri dish containing 5 ml of enzyme solution and incubated over night. Filtration (75-µm mesh) of the digested leaves was used to separate protoplasts from undigested material. To wash the protoplasts they were pelleted by centrifugation (2 min, 780 rpm, soft start (Rotina 35R, Hettich)) and the pellet was resuspended in 10 ml solution W5. This process was repeated twice and the protoplasts were subsequently incubated for at least 4 h on ice.
Solution W5 was removed and the protoplasts were resuspended in solution MMg (250 µl / transformation reaction). Next, 200 µl protoplast suspension were added to 11 µg plasmid DNA (10 µg of promoter:*firefly luciferase* plasmids (pBGW7L derivatives) and for standardization 1 µg of 35S:*Renilla luciferase* (p70SRUC, provided by D. Stahl of the KWS SAAT AG (Einbeck, Germany)) in a 2 ml reaction tube and inverted gently before addition of 220 µl of PEG solution. After 30 min of incubation, the transfection mixture was carefully diluted with 800 µl of solution W5. The protoplasts were sedimented stepwise by 3 x centrifugation (1 min, 780 rpm, soft start) and the supernatant was removed, before they were immediately resuspended in 200 µl of solution WI. After over night incubation, the WI solution was removed and protoplasts were frozen in liquid nitrogen. Whenever protoplasts were pipetted, cut tips were used.

Buffers used for protoplast transformation:

Solution	**Content**
Enzyme solution	1.3 % cellulase R10 (w/v) 0.3 % macerozyme R10 (w/v) 0.4 M mannitol 20 mM KCl 20 mM MES (pH 5.7) 10 mM $CaCl_2$

Solution	Content
PEG solution	40 % PEG4000 (w/v) 0.8 M mannitol 1 M $CaCl_2$
Incubation solution WI	0.5 M mannitol 4 mM MES (pH 5.7) 20 mM KCl
Washing solution W5	154 mM NaCl 125 mM $CaCl_2$ 5 mM KCl 2 mM MES (pH 5.7)
Transformation solution MMg	0.4 M mannitol 15 mM $MgCl_2$ 4 mM MES (pH 5.7)

3.2.1.13 *Agrobacterium*-mediated gene transfer into *Arabidopsis thaliana*

Transformation of *A. thaliana* with *Agrobacterium* was performed according to Clough (2005). Flowering plants were dipped into an *Agrobacterium* solution (OD_{600} = 0.8). The plants were subsequently cultured until seed development. Selection of the primary transformants was performed using the appropriate selection markers.

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

This "quick and dirty" method was used for PCR-based genotyping of the F2 generation plants. A leaf disc was cut out using the lid of a microcentrifuge tube, making sure to avoid cross-contamination with foreign plant material. The tissue was ground in 100 µL of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS) using a small pestle (Roth) that fits within a 1.5-mL centrifuge tube. An additional 300 µL of extraction buffer were added and mixed by inversion before centrifugation (15,000 rpm, 5 min, 4°C). Of the supernatant, 300 µL were transferred into a new microcentrifuge tube and supplemented with 300 µL 2-propanol. After inverting the tube several times, the samples were centrifuged (13,000 rpm, 5 min, RT). The supernatant was removed and the pellet was washed with 200 µL 70 % EtOH. After removing the supernatant, the pellet was dried at 37°C for 10 min and subsequently dissolved in 100 µL of water (ultra-pure) by incubating for 10 min at 65°C. After a last centrifugation step (13,000 rpm, 5 min, RT), the supernatant containing the isolated genomic DNA was transferred to a new microcentrifugation tube and stored at –20°C.

3.2.1.15 Plant growth conditions

3.2.1.15.1 Growth of transgenic reporter gene lines

Approximately 100 F2 seeds of selected reporter gene lines were surface sterilized and sown on sterile MS plates. The plants were grown for 16 days at 22°C under long-day conditions (14 h light/10 h dark, 60 µmol photons/m^2 x sec) and at 60 % humidity.

3.2.1.15.2 Plant growth conditions for transient expression studies and stress induction

Plants for protoplast transformation and stress induction were grown on steamed soil (Archut, Fruhstorfer Erde, T25, Str1 fein) under 12 h light/12 h dark low-light conditions (37-45 µmol photons/m^2 x sec) over a period of 6–7 weeks at 22°C and 60 % humidity. For each square pot (8.5 cm x 8.5 cm), for four seeds were sown.

3.2.2 Stress induction in *A. thaliana*

3.2.2.1 TIBA

Plants were sprayed with 0.1 mM TIBA (0.1% DMSO) and incubated for 8 h under conditions. Control plants were sprayed with water + DMSO (0.1 %) and incubated for the same time span or harvested before treatment (time course).

3.2.2.2 BOA

Plants were sprayed with 2 mM BOA (0.2% EtOH) and incubated for 8 h under normal growth conditions. Control plants were sprayed with water + EtOH (0.2 %) and incubated for the same time span.

3.2.2.3 Benoxacor

Plants were sprayed with 0.05 mM benoxacor (0.1% DMSO) and incubated for 8 h under normal growth conditions. Control plants were sprayed with water + DMSO (0.1 %) and incubated for the same time span.

3.2.2.4 IAA

Plants were sprayed with 0.01 mM IAA and incubated for 8 h under normal growth conditions. Control plants were sprayed with water and incubated for the same time span.

3.2.2.5 Jasplakinolide

Leaves of 6–7-week-old plants were cut which a scalpel, and the petioles were submerged in 10 µM jasplakinolide (1% DMSO) and incubated under normal growth conditions for 24 h. Control leaves were treated with H_2O + 1 % DMSO and incubated for the same time span.

3.2.2.6 MeJA

Plants were transferred into a gas-proof tank, and 1 µL MeJA/$L_{air\ volume}$ was added to ten filter paper pieces sticking to the inner glass surface. The tank was immediately sealed with grease. The plants were incubated for 8 or 24 h. Control plants were incubated for the same time span in a sealed tank without MeJA.

3.2.2.7 Extended darkness

Plants were covered at the beginning of the dark phase and kept in the dark for 36 h. Control plants stayed in the normal light cycle (12 h light/12 h dark). The plants were harvested at the beginning of the light phase.

3.2.2.8 Infection of *A. thaliana* with *Botrytis cinerea*

3.2.2.8.1 Cultivation

Pieces of mycelium from a PDA plate (growing plate) or spores from a glycerol stock (-80°C) were transferred to fresh malt extract plates (sporulation plate). Growing of the fungi was carried out in the dark at 20–24°C for about 7–14 days, until full sporulation. The collected spores (see below) were frozen as glycerol stocks (30 %) in 50-µL aliquots at –80°C.

3.2.2.8.2 Collection of spores

Of quarter-strength PDB medium, 5 mL were dropped onto a plate with fungi in full sporulation. The fungal hyphae were scraped off with a Drygalski spatula and the medium containing the hyphae and the spores was filtered through three layers of gauze bandage. The spores were counted under a microscope in a Thoma counting chamber and diluted with quarter-strength PDB medium to 2×10^5 spores/mL.

3.2.2.8.3 Infection of plants

The collected spores in quarter-strength PDB medium were incubated in the light for 2–3 h, at RT. Plants with fully expanded leaves were inoculated with 6 µL of spore solution (2×10^6 spores/mL) or 6 µL of quarter-strength PDB (mock) on each leaf. The plants were transferred to a gas-proof tank, which was sealed with exsiccator grease to obtain high humidity, and incubated for 3 days before the infected leaves were harvested.

3.2.2.9 Protoplast isolation

Protoplast isolation and transformation was performed according to the method described by Sheen (2001). Instead of transformation, the protoplasts were only incubated for 30 min in buffer MMG (see also Section 3.2.1.12: Gene Transfer into *Arabidopsis thaliana* protoplasts). For RNA isolation, six protoplast samples were pooled.

3.2.3 Transcript analysis

3.2.3.1 RNA extraction

The TRIZOL extraction method can be used to extract RNA, DNA, and proteins from plants (Chomczynski 1993). This method uses phenol/chloroform (dichloromethane) extraction to dissolve RNA in the aqueous phase while other compounds like proteins are dissolved in the hydrophobic chloroform phase. The two thiocyanate compounds in the extraction buffer inhibit RNAse activity. Plant tissue was ground to fine powder in liquid nitrogenusing using pre cooled mortar and pestle. To ~150 mg ground plant material 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 3 M Na-acetate pH 5.2, 5 % glycerol) was added. 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 (12,000 rpm, 30–60 min, 4°C), the supernatant (900 µL) was transferred into new microcentrifuge tubes. Precipitation buffer (HSPB, 1.2 M NaCl, 0.8 M Na-citrate) and 2-propanol (325 µL each) were added, the tubes were inverted several times and the samples were incubated for 10 min at RT and centrifuged (12,000 rpm, 20 min,

4°C). The pellets were washed with with 70 % ethanol. After removing the supernatant, the samples were dried at room temperature and afterwards dissolved in 50–100 µL water (ultra-pure). The concentration was measured as described in Section 3.2.1.2.

3.2.3.2 Preparation of gDNA-free cDNA for qRT-PCR

To analyze gene expression by PCR, it is necessary to synthesize cDNA from RNA. In order to prevent gDNA contamination, DNase I digestion was done before cDNA synthesis. First, 1 µg RNA template together with 1 µL 10× DNase I reaction buffer and 1 µL DNase I (RNase free) was added with water to a final reaction volume of 10 µL. The mixture was incubated at 37°C for 30 min. To deactivate the DNase I enzyme, 1 µL 25 mM EDTA was added and incubated at 65°C for 10 min. cDNA synthesis was performed with 1 µg total RNA (DNA free), 20 pmol oligo-dT primer, and 200 pmol 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 5× first-strand reaction buffer and 60 U reverse transcriptase H– were added and brought to a final volume of 20 µL with H_2O. The mixture was incubated at 42°C for 70 min and then heated to 70°C for 10 min.

3.2.3.3 Quantitative real-time RT-PCR

This highly sensitive method was used to investigate gene expression on the RNA level. cDNA was diluted 1:10 with sterile water. The amplification mix consisted of 1× 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× 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. The 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 melting curve analysis. During the elongation and annealing phases, measurement of the fluorescence intensity was performed in the Biorad icycler. The housekeeping gene *UBQ5* (At3G62250) was used as control. Data analysis was done with the help of the 2-ΔΔCT method (Schmittgen and Livak 2008) to quantify the relative expression levels.

3.2.3.4 Microarray analyses

The transcriptomes of Col-0 and *coi1-t* plants after mock and TIBA treatment was compared by microarray analyses. Plants were grown and treated according to Sections 3.2.1.15.2 and 3.2.2.1. Eight plants of each kind of treatment and genotype were combined. Total RNA was extracted according to the TRIZOL method (Section 3.2.3.1) and purified using the RNeasy Kit (Qiagen). Quality control and hybridizations were performed by the NASC's International Affymetrix Service (Nottingham Arabidopsis

Stock Centre, University of Nottingham, UK), using "Affymetrix Arabidopsis ATH1 Genome Array" chips.
Raw data analysis and data quality control were performed using Robin (Lohse et al. 2010). Normalization of the raw data and an estimation of signal intensities were carried out using the Robust Multichip Average (RMA) methodology. Genes that showed an absolute log 2 fold-change value of at least 1 and a P value lower than 0.05 were considered as significantly differentially expressed. Subsequently, criteria for "TIBA inducible" were defined as "Col-0 TIBA/Col-0 mock ≥ 3" and for "COI1 dependent" as "*coi1-t* TIBA/Col-0 TIBA ≤ 0.3".

3.2.4 Protein analysis

3.2.4.1 Whole-cell protein extracts

Extraction of proteins was performed under denaturing conditions. An extraction buffer containing urea (4 M urea, 16.6 % glycerol, 5 % SDS, 0.5 % β-mercaptoethanol) was used to extract the proteins. After grinding the plant material under liquid nitrogen, 450 µL of extraction buffer were added to ~150 mg plant material. The samples were incubated at 65°C for 10 min and centrifuged (13,000 rpm, 20 min, RT). The supernatant was transferred into new microcentrifuge tubes and used for SDS-PAGE.

3.2.4.2 Determination of protein concentrations

Protein concentrations were determined by two different methods. A colorimetric assay was used to determine the concentration of 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 using a standard curve derived from different BSA protein amounts (1, 3 and 6 µg).
Proteins isolated using buffers containing detergents were measured with the Pierce 660 nm protein assay kit (Thermo Scientific) according to the instruction manual.

3.2.4.3 SDS PAGE

In sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins are separated mostly on the basis of their polypeptide length. Electrophoresis was performed 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 is as follows: 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 consisted 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 (~10 µL each, or equal amounts of protein defined after a first Coomassie-stained gel) were boiled with 15 µL 2× SDS sample buffer (0.09 M Tris, 20 % glycerol, 2 % SDS, 0.02 % bromophenol blue, 0.1 M DTT) at 95°C for 5 min, then cooled on ice. Electrophoresis was performed at 120 V with 1× SDS running buffer (250 mM Tris, 2 M glycine, 1 % SDS) until the bromophenol blue band reached the lower end of the gel. A pre-stained protein ladder (6 µL) was used for estimating the size of the separated proteins.

3.2.4.4 Coomassie staining of SDS gels

The Coomassie Brilliant Blue G-250 dye was used to detect proteins separated by SDS-PAGE. The gels were incubated with 40 ml of Coomassie staining solution (colloidal Coomassie) and 10 mL of methanol overnight. Colloidal Coomassie consists of 400 mL solution A (40 g ammonium sulfate and 8 mL phosphoric acid) and 10 mL solution B (0.5 g Coomassie Brilliant Blue G-250; this has to be dissolved by shaking for at least 0.5 h). The gels were destained in water overnight.

3.2.4.5 Western blot

The proteins separated by SDS-PAGE were blotted onto a PVDF membrane using the semi-dry blotting method, applying an electric field between two graphite plates. The PVDF membrane was activated by MeOH before blotting. For the transfer of proteins from the gel to the membrane, the gel on top of the membrane was sandwiched between three layers of Whatman paper (pre-soaked with transfer buffer). The whole arrangement was placed within a blotting apparatus, and the transfer was performed at an amperage of 1 mA/cm^2 for 1.4 h. (Optional: Ponceau S staining was done to observe the success of the transfer (2 g Ponceau S, 30 g trichloroacetic acid and 30 g sulfosalicylic acid in 100 mL H_2O)) After blotting, the membrane was dried between two layers of Whatman paper. The standard was marked on the membrane with an iMark (pen containing rabbit pre-immune serum) for later detection of standard bands with the second antibody and an ECL kit to visualize them on the film. After 5 min, the membrane was reactivated in MeOH and nonspecific binding to proteins on the membrane was prevented by blocking the membrane with non-fat dried milk powder (5 % in 1× TBST) o/n at 4°C on a shaking platform. The detection of specific proteins on the membrane was performed using an antibody directed against the protein of interest at 1:4000 dilution in 1× TBST (with 0.5 % milk powder). The membrane was incubated with the respective antibody for 2 h at RT on a shaking platform. Incubation with the second antibody (anti-rabbit 1:25,000 in 1× TBST) was performed for 1 h at RT on a shaking platform. This second antibody was conjugated to horseradish peroxidase (HRP). HRP can utilize the enhanced chemiluminescent substrate (ECL, GE Healthcare; incubation of the membrane in ECL mix for 5 min) to generate a luminescence-emitting product, which allows visualization of the membrane-bound proteins on autoradiography films. The films were exposed to the membrane in detection cassettes between 30 s to 10 min, depending on the strength of the chemiluminescence signal generated by the respective amount of bound protein.

3.2.4.6 Preparation of protein extracts for enzymatic GUS assays

Extraction of proteins for GUS assays was conducted by the addition of 700 µL GUS extraction buffer pH 7.5 (supplemented with 0.05 % β-mercaptoethanol, see section 3.1.10) to frozen (liquid nitrogen) and ground plant material (300–500 mg). Subsequently, the samples were centrifuged (10 min, 12,000 rpm at 4°C), and the supernatant was transferred to a new 1.5-ml reaction tube. The samples were stored at –80°C.

3.2.4.7 Measurement of relative GUS activities

For the determination of GUS activities from protein extracts, 1 µL of a 1:100 dilution was analysed in a GUS assay using a flat-bottom multititer plate. After addition of 99 µL MUG extraction buffer pH 7.5 + β-mercaptoethanol, 100 µL of MUG solution were added to each well and the plate was placed in a 37°C incubator to start the enzymatic reaction. After 10 min, a t_0 value was taken by pipetting 100 µL of the sample to 100 µL of GUS-stop buffer (200 mM Na_2CO_3). The remaining reaction was stopped 60 min later to gain a t_{60} value. The t_0 and t_{60} values were used to calculate the relative GUS activities of the samples.

3.2.4.8 Histochemical GUS staining

GUS staining was used to examine the tissue-specific expression of transcriptional promoter:GUS (β-glucuronidase) fusions. GUS activity was assayed using X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide). After hydrolysis by the GUS enzyme, oxidation of the indole derivative causes dimerization and the production of an insoluble indigo dye.
Whole seedlings were incubated for 20 min in 90 % precooled acetone (–20°C) at room temperature. The acetone was discarded and the plant material was washed three times with GUS staining buffer (50 mM Na_2HPO_4/NaH_2PO_4 pH 7.2, 0.2 % Triton X-100, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$). Subsequently, GUS staining solution (2 mM X-Gluc, 50 mM Na_2HPO_4/NaH_2PO_4 pH 7.2, 0.2 % Triton X-100, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$) was vacuum-infiltrated for 20 min. The plant material was incubated o/n at 37°C and washed with increasing concentrations of EtOH (20, 35, and 50 %).
Root tips were analyzed by microscopy (DM 5000B + CTR 5000, Leica).

3.2.4.9 Measurement of relative luciferase activities

Transfected and frozen protoplasts (see 3.2.1.12) were resolved in 50 µl of cell lysis buffer (Promega) and subsequently luciferase activity tests were performed using the "Dual luciferase kit" (Promega) according to the instruction manual.

3.2.5 Hormone analysis

3.2.5.1 Determination of JA and JA-Ile contents in plant material

Determination of JA and JA-Ile concentations was performed by the Department of Plant Biochemistry (Prof. Dr. I. Feußner) of the University of Göttingen (Germany), by GC-MS/MS analyses (Luo et al. 2009).

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4 Results

4.1 *CYP81D11* expression depends on the TGA/SCL14 complex in response to jasmonic acid and *Botrytis cinerea*

Expression of *CYP81D11* in response to the auxin transport inhibitor TIBA strongly depends on the TGA class II transcription factors TGA2, TGA5 and TGA6 (TGA2,5,6) as well as on the GRAS family protein SCL14 (Fode et al. 2008). In addition, *CYP81D11* is inducible by MeJA in a TGA2,5,6-dependent manner (Mueller et al. 2008), although TGA2,5,6 are not part of the known JA signaling cascade. To elucidate if SCL14 plays a role for *CYP81D11* expression in response to MeJA, Col-0 wild-type and *tga2,5,6* and *scl14* mutant plants were treated with MeJA. The effects on the *CYP81D11* expression elicited by the mutations were analyzed by quantitative real-time RT-PCR (Figure 4. 1). TIBA treatment was performed under the same conditions in order to reproduce the results published previously. As a control, *VSP2* expression in response to MeJA and TIBA was investigated. *VSP2* (*VEGETATIVE STORAGE PROTEIN 2)* is a well-investigated JA-inducible gene, often used as a marker gene for response to JA, e.g. in pharmacological treatments, wounding, or herbivore attack (McConn et al. 1997a; Lorenzo et al. 2004).

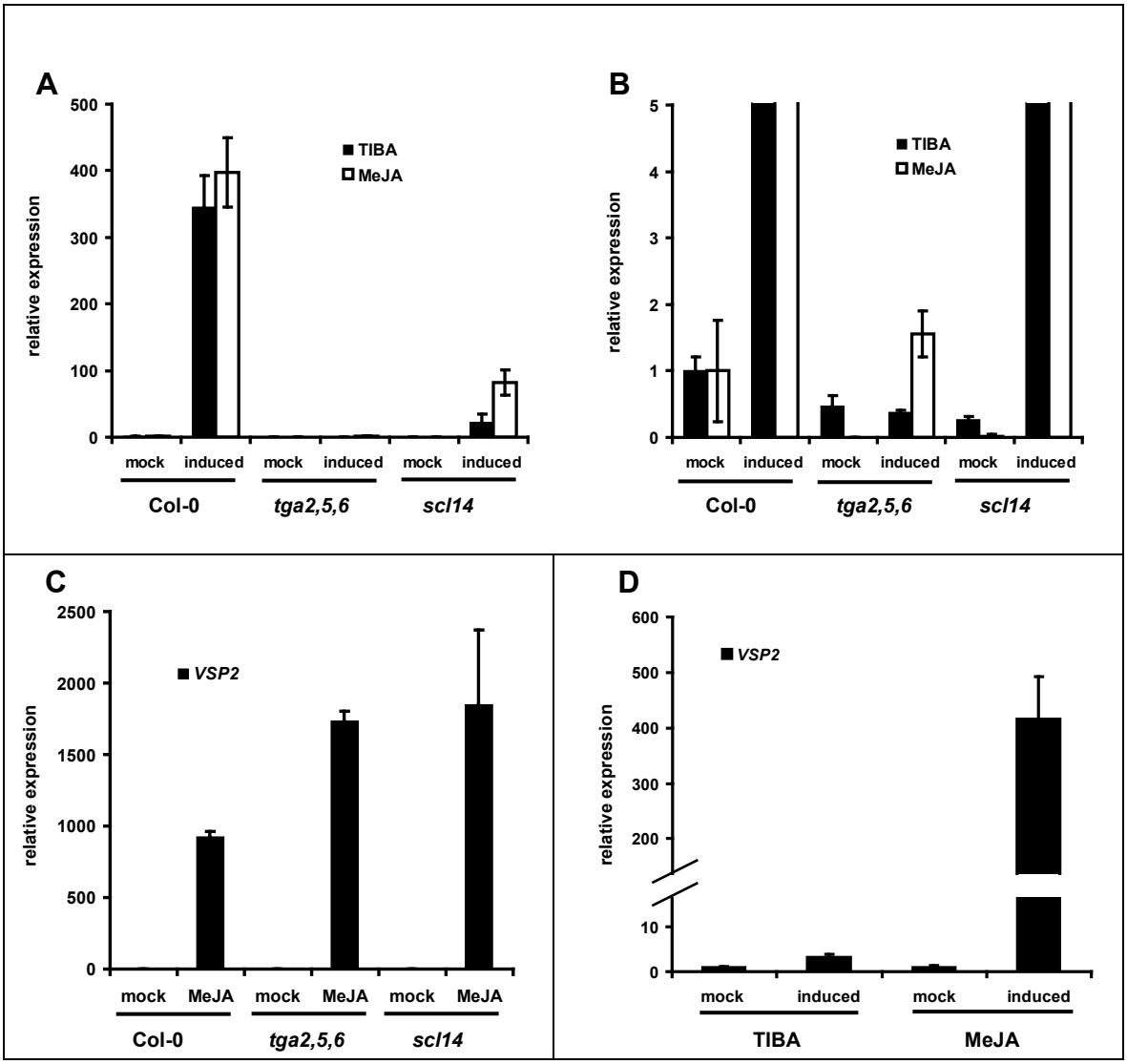

Figure 4. 1: Expression of *CYP81D11* and *VSP2* in the *tga2,5,6* and *scl14* mutants in response to TIBA and MeJA

Quantitative real-time RT-PCR analysis of *CYP81D11* and *VSP2* transcript levels (normalized to the housekeeping gene *UBQ5*) in Col-0 wild-type and *tga2,5,6* and *scl14* mutant plants. 6–7-week-old soil-grown plants were either sprayed with 100 µm TIBA for 8 h or treated with MeJA (1 µL/L air) for 24 h. During the MeJA treatment, plants were kept in a gas-proof tank; liquid MeJA was applied to Whatman paper that was attached to the inner glass surface. Whole rosettes were harvested for RNA isolation. Transcript values in mock-treated Col-0 plants were set to 1.
(A, B) *CYP81D11* expression in Col-0 wild-type, *tga2,5,6* and *scl14* plants. No transcript could be detected in mock-treated (MeJA) *tga2,5,6* samples. Each bar represents the average ± SEM of three (MeJA) or four (TIBA) biological replicates. In (B) the scale was changed to visualize low values.
(C) *VSP2* expression in Col-0, *tga2,5,6* and *scl14* plants. Each bar represents the average ± SEM of three biological replicates.
(D) *VSP2* expression in response to TIBA and MeJA treatment in Col-0 plants. Bars represent the average ± SEM of eight (TIBA), five (MeJA mock) or nine (MeJA induced) biological replicates.

CYP81D11 expression in the *tga2,5,6* mutant was nearly abolished in response to MeJA as well as in response to TIBA. For many mock-treated samples, the transcript levels

were even below detection levels. Nevertheless, in response to MeJA, an induction was achieved. The *scl14* mutant shows less stringent effects. After treatment with TIBA or MeJA, increased *CYP81D11* expression levels were observed. Nevertheless, MeJA leads to a stronger *CYP81D11* induction in the *scl14* mutant than TIBA, indicating a less stringent SCL14 dependency in response to MeJA than in response to TIBA.
VSP2 expression in response to MeJA shows no reduction in the *tga2,5,6* and *scl14* mutants compared to the Col-0 wild type. These mutants rather exhibit a two times stronger *VSP2* induction. This demonstrates that TGA class II transcription factors and SCL14 have distinct functions in regulating *CYP81D11* and *VSP2* transcription in response to JA and that they have no general function in JA signal transduction.
In TIBA-treated wild-type plants, *VSP2* transcript levels were only slightly increased compared to mock-treated plants (about 3-fold). In contrast, MeJA treatment induced *VSP2* transcript levels by about 400-fold. Thus, JA-inducible genes are not in general inducible by TIBA, as confirmed by the Genevestigator database (www.genevestigator.com).

In addition to pharmacological treatments, *CYP81D11* expression is also inducible by pathogens like the necrotrophic fungal pathogen *Botrytis cinerea*. To investigate a more natural situation, *A. thaliana* wild-type, *scl14* and *tga2,5,6* plants were drop-inoculated with *B. cinerea*. At 3 days post infection, inoculated leaves were harvested and four leaves (of different plants) were pooled as one sample. *CYP81D11* transcript levels were investigated by quantitative real-time RT-PCR (Figure 4. 2).

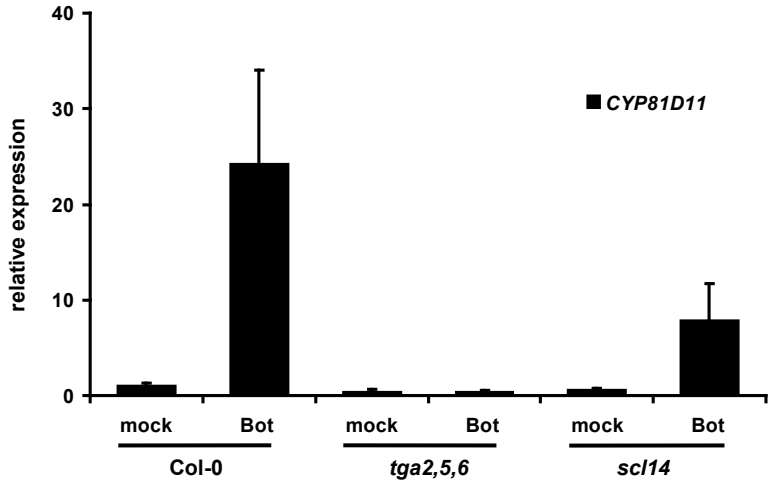

Figure 4. 2: Expression analysis of *CYP81D11* after infection with *Botrytis cinerea*

Quantitative real-time RT-PCR analysis of relative *CYP81D11* transcript levels (normalized to the housekeeping gene *UBQ5*) in wild-type, *tga2,5,6* mutant and *scl14* mutant plants 3 days after inoculation with *B. cinerea*. 6-week-old soil-grown plants were drop-inoculated with 6 µL of a *B. cinerea* spore solution (2×10^5 spores/mL) (Bot) or with 6 µL of quarter-strength potato dextrose broth (mock). Four inoculated leaves were pooled as one sample for RNA extraction. Each bar represents the average ± SEM of two to three (mock) or four to seven samples (Bot).

After *B. cinerea* infection, *CYP81D11* expression was clearly induced, although the transcript levels were notably weaker than in response to the chemical treatments. Nevertheless, the *tga2,5,6* and *scl14* mutants show similar behavior compared to the treatment with TIBA and MeJA (Figure 4. 1). The *tga2,5,6* mutant displayed a reduced expression background in mock-treated leaves that was no longer inducible by *B. cinerea* infection. The *scl14* mutant on the other hand exhibited inducible *CYP81D11* expression; however, it only reached about 30 % of the wild-type level. These results indicate that infection with *Botrytis cinerea* is more closely reflected by MeJA than by TIBA treatment.

Fode et al. (2008) demonstrated that SCL14 binds to the *CYP81D11* promoter via the TGA2,5,6 transcription factors, which themselves bind to the *as-1*-like element contained in the promoter of *CYP81D11* and other SCL14 target genes. Considering the extraordinary importance of TGA2,5,6 for *CYP81D11* expression, the *as-1*-like element was expected to be indispensable for the transcription of *CYP81D11*.

To elucidate the role of the *as-1*-like element, *CYP81D11 promoter:GUS* reporter gene constructs were generated. The 894-bp fragment upstream of the *CYP81D11* transcription start and the 5' UTR were isolated. The *as-1*-like element (position –243 to –225) was modified by overlapping PCR. The wild-type promoter fragment (WT) and the one with the mutated *as-1*-like element (m*as-1*) were inserted by gateway® cloning

into an *A. thaliana* expression vector upstream of the GUS reporter gene. Transgenic *A. thaliana* plants containing these constructs were generated by *Agrobacterium tumefaciens*-mediated gene transfer and primary transformants were selected by the herbicide BASTA. F1 generation plants, representing a pool of homozygous and heterozygous transformants as well as wild-type plants, were analyzed after TIBA and MeJA treatment (Figure 4. 3).

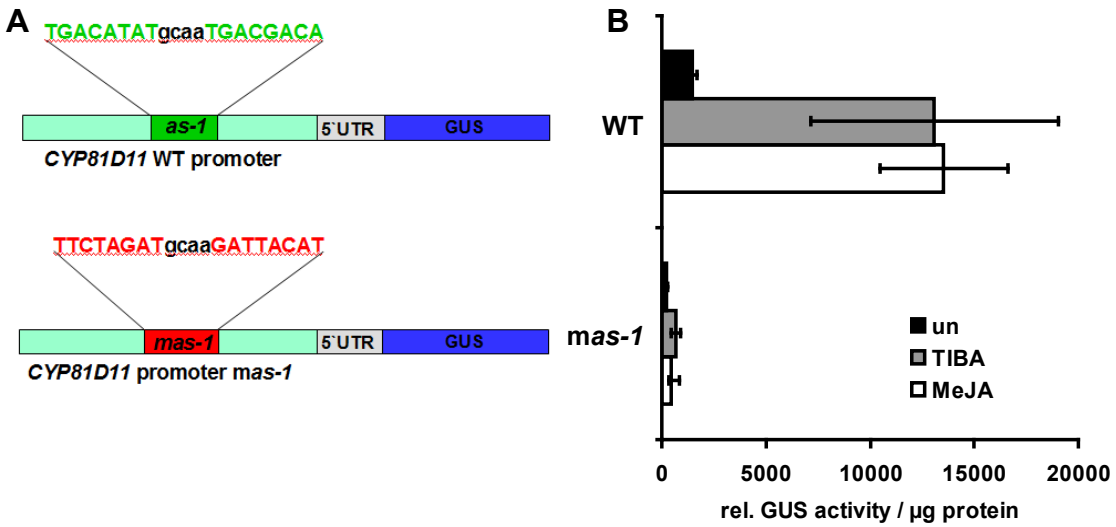

Figure 4. 3: Average activities of *CYP81D11* WT promoter constructs and a construct lacking the *as-1-like* element in response to TIBA and MeJA

(A) Scheme of the *CYP81D11 promoter:GUS* reporter gene constructs, which were used to generate the transgenic lines. The light green box indicates the *CYP81D11* promoter fragment 894 bp upstream of the transcription start; the *as-1*-like element (position –243 to –225) is marked in green while the mutated sequence is marked in red. The grey box depicts the 5' UTR of the *CYP81D11* gene and the GUS reporter gene is indicated in blue.
(B) GUS activities obtained from transgenic plants containing the *CYP81D11 promoter:GUS* constructs depicted in (A), in response to TIBA and MeJA treatment. Seedlings were grown for 16 days on MS agar under long-day conditions (14 h light, 10 h dark) and either sprayed with 100 µm TIBA for 8 h or treated with gaseous MeJA (1 µL/L air) for 24 h. Control plants remained untreated. Whole seedlings were harvested for protein extraction. Each bar represents the average ± SEM of 17 (m*as-1*) or 18 (WT) transgenic lines.

While the WT *CYP81D11 promoter:GUS* construct was well inducible by TIBA and MeJA, the m*as-1* construct led to GUS activities that were even lower than those of the uninduced WT construct. It is concluded that the *as-1*-like element is of essential importance for the expression of *CYP81D11*.

4.2 *CYP81D11* shares common properties with the JA marker gene *VSP2*

Mutation of the JA-Ile receptor COI1 leads to a very strong JA-insensitive phenotype and strongly reduces the expression of JA-inducible genes (Ellis & Turner 2002; Devoto et al. 2005; Feys et al. 1994). *CYP81D11* expression was previously reported to be dependent on COI1 in response to MeJA treatment. This indicates that *CYP81D11* is regulated by the known JA signaling pathway. This pathway activates two groups of JA-inducible genes: only JA-inducible genes like *VSP2* and JA/ET-inducible genes like *PDF1.2*. To classify *CYP81D11*, Col-0 wild-type plants were treated with the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) in addition to MeJA. Transcription levels of *CYP81D11*, *VSP2* and *PDF1.2* were determined by quantitative real-time RT-PCR (Figure 4. 4). To reproduce the previously published result that MeJA induction of *CYP81D11* is COI1 dependent (Mueller et al. 2008), also *CYP81D11* and *VSP2* expression in the *coi1-1* (Xie et al. 1998) mutant in response to MeJA were investigated. The *coi1-1* mutant exhibits an additional mutation in the *GLABROUS* gene, which leads to a defect in trichome development (Larkin et al. 1994). Therefore, *gl1* plants carrying the same mutation were used as control.

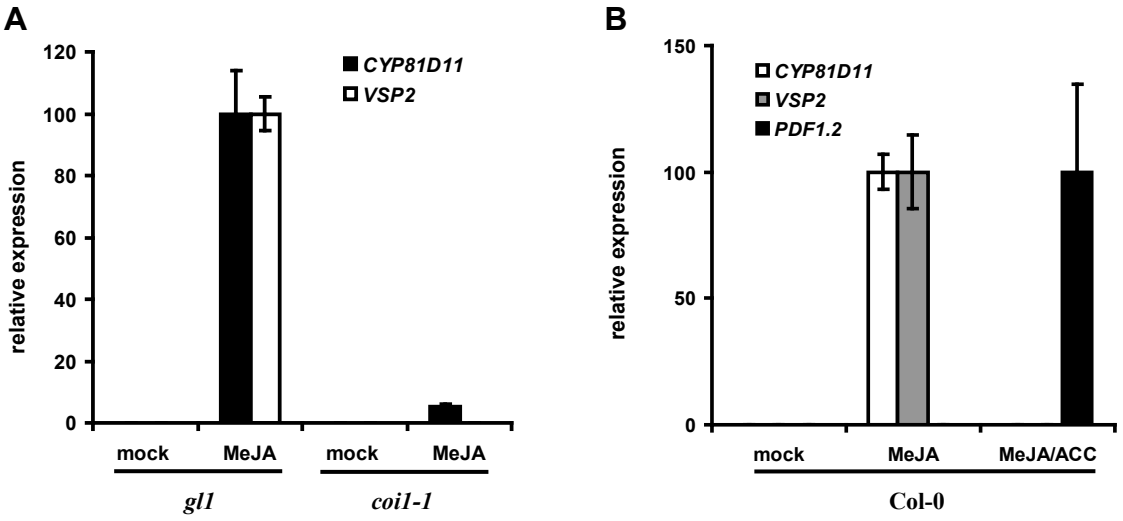

Figure 4. 4 : Expression analyses of *CYP81D11*, *VSP2* and *PDF1.2* after treatment with MeJA and the ethylene precursor ACC

Quantitative real-time RT-PCR analyses of *CYP81D11*, *VSP2* and *PDF1.2* transcript levels (normalized to the housekeeping gene *UBQ5*).
(A) *gl1* and *coi1-1* plants were grown on MS agar under short-day conditions; for *coi1-1*, the MS medium contained 50 µM MeJA to select homogeneous individuals. After 3 weeks, they were transferred to soil and grown for another 4 weeks under 12-h day and 12-h night conditions. The plants were treated with gaseous MeJA (1 µL/L air) for 24 h. During MeJA treatment, the plants were kept in a gas-proof tank; liquid MeJA was applied to Whatman paper that was attached to the inner glass surface, and evaporated into the gas phase. Whole rosettes were harvested for RNA isolation. Transcript values of MeJA-treated Col-0 plants were set to 100. Each bar represents the average ± SEM of four biological replicates.
(B) 6–7-week-old soil-grown Col-0 wild-type plants were treated with gaseous MeJA (1 µL/L air) for 24 h and sprayed with 5 mM ACC. Whole rosettes were harvested for RNA isolation. For each gene, the highest transcript level was set to 100. Each bar represents the average ± SEM of four biological replicates.

In the *coi1-1* mutant, the expression of *CYP81D11* in response to MeJA is reduced to 5 % compared to the *gl1* plants, indicating that JA-induced *CYP81D11* expression depends on the known JA signaling pathway. Nevertheless, *VSP2* transcript levels were reduced to about 0.1 % of those of MeJA-treated *gl1* plants, both in mock- and MeJA-treated *coi1-1* plants. This indicates an additional way of JA induction for *CYP81D11* expression.

VSP2 as well as *CYP81D11* expression are well inducible by application of JA alone, while this treatment only leads to a very slight increase in *PDF1.2* expression. In contrast to this, JA/ACC treatment abolishes the increased transcription levels of *VSP2* and *CYP81D11* obtained by JA, but strongly induces the transcription of *PDF1.2*. Thus, *CYP81D11* is classified as an only JA-inducible gene.

4.3 TIBA-induced *CYP81D11* expression depends on COI1 in the absence of increased JA-Ile levels

CYP81D11 expression requires COI1 not only in response to MeJA, but also in response to phytoprostanes (Mueller et al. 2008). This contradicts the current model of COI1 function as JA-Ile receptor (Memelink 2009). To investigate the COI1 dependency in response to TIBA, *gl1* and *coi1-1* plants were treated with 100 µM TIBA; *CYP81D11* expression levels were monitored by quantitative real-time RT-PCR and compared to those of three other TIBA-inducible SCL14 target genes (Figure 4. 5).

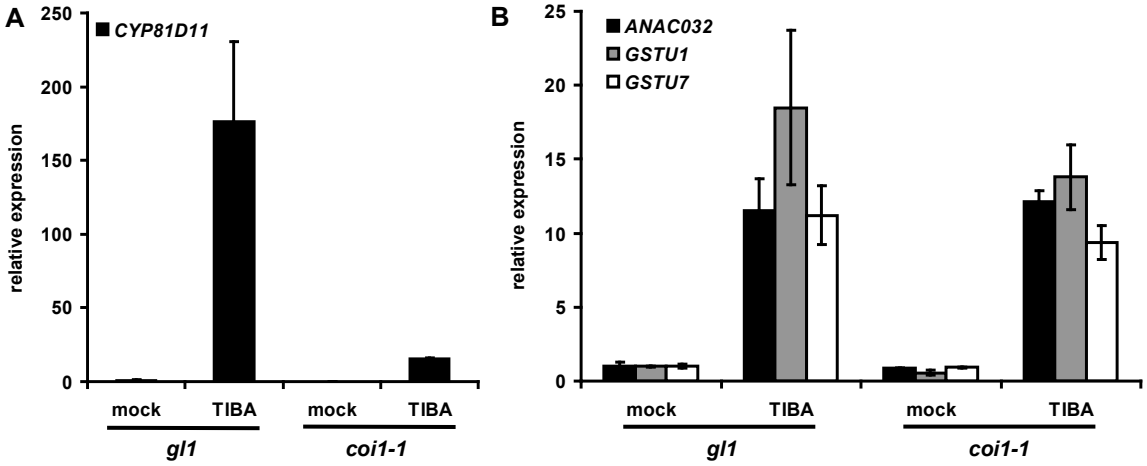

Figure 4. 5: Expression analysis of SCL14 target genes in response to TIBA

Quantitative real-time RT-PCR analysis of relative *CYP81D11*, *ANAC032*, *GSTU1* and *GSTU7* transcript levels (normalized to the housekeeping gene *UBQ5*) in *gl1* and *coi1-1* mutant plants. 6–7-week-old plants were sprayed with 100 µM TIBA. Whole rosettes were harvested for RNA isolation. Transcript values in mock-treated Col-0 plants were set to 1. Each bar represents the average ± SEM of two (mock) or three (TIBA) biological replicates.

In TIBA-treated *coi1-1* plants, *CYP81D11* expression is reduced to about 10 % compared to the corresponding "wild-type" *gl1*. This result is consistent with the COI1-dependent *CYP81D11* expression in response to phytoprostanes. Except for this result, the COI1 protein has only been reported to be involved in JA signaling, and no reports about its involvement in the xenobiotic response are known. Furthermore, the transcript of three other SCL14 target genes, *ANAC032*, *GSTU1* and *GSTU7*, were not reduced in the *coi1-1* mutant. Therefore, a general involvement of COI1 in the xenobiotic response seems unlikely.

An explanation for the involvement of COI1 in *CYP81D11* expression in response to TIBA would be an accumulation of JA-Ile after TIBA treatment. Thus, JA-Ile levels in

mock- and TIBA-treated soil-grown plants were determined by HPLC-MS/MS (Figure 4.6).

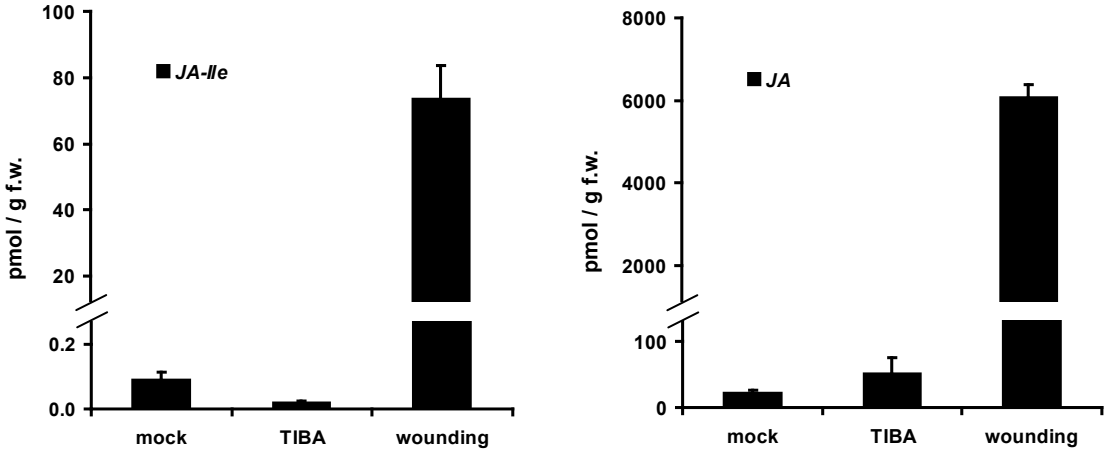

Figure 4. 6: JA-Ile and JA levels in mock- and TIBA-treated *A. thaliana*

Determination of JA-Ile and JA levels by HPLC-MS/MS was performed in Col-0 wild-type plants grown on soil for 6–7 weeks under 12-h light/12-h dark conditions. The plants were sprayed with either 100 µM TIBA or 0.1 % DMSO and incubated for 8 h. Whole rosettes were harvested. As a control, leaves were wounded by squeezing with forceps and incubated for 2 h. In this case, only the wounded leaves were harvested. For JA (mock and TIBA), each bar represents the average ± SEM of seven biological replicates from two independent experiments. In case of JA-Ile (mock and TIBA), each bar represents the average ± SEM of four biological replicates from one experiment. As in the second experiment, the JA-Ile levels were below detectable levels. Wounding experiments were only performed once with two biological replicates. JA-Ile and JA amounts are depicted as pmol/g fresh weight.

JA-Ile levels are not increased in TIBA-treated compared to mock-treated plants; in contrast, they are even slightly decreased. Under comparable conditions, wounding leads to a high accumulation of JA-Ile. TIBA also does not elicit accumulation of JA, although for JA the variation among the samples was higher. Still, in comparison to wounded plants, JA levels in mock-induced as well as in TIBA-induced plants were very low. This finally proves that JA-Ile accumulation is not the reason for the COI1 dependency of *CYP81D11* expression in response to TIBA.

The described function of COI1 in response to JA-Ile accumulation is to mark JAZ proteins for degradation. To trace this process, fusion proteins of JAZ proteins and the beta-glucuronidase protein (GUS) or the green fluorescing protein (GFP) have been used successfully (Thines et al. 2007; Chini et al. 2007). JAZ stability in response to TIBA was monitored in transgenic plants expressing a JAZ1-GUS fusion protein (Thines et al. 2007). The concentration of JAZ1-GUS was determined by quantitative measurements of GUS activity in protein extracts prepared from *A. thaliana* seedlings 8 h after TIBA treatment. MeJA-treated JAZ1-GUS seedlings were used as positive control.

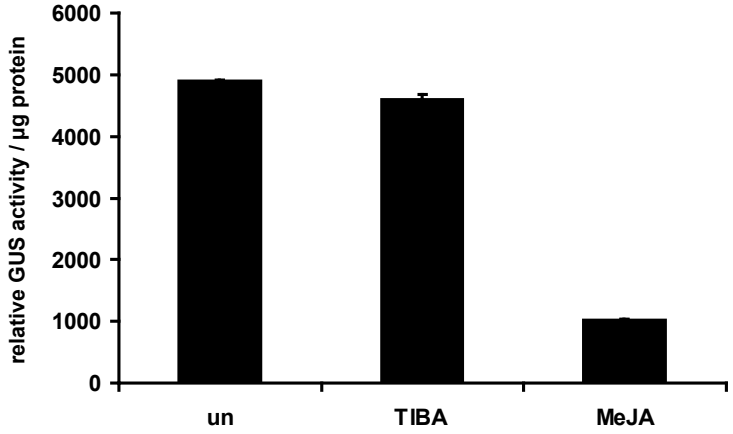

Figure 4. 7: Relative JAZ1-GUS levels after treatment with TIBA and MeJA

16-day-old seedlings ectopically expressing JAZ1-GUS fusion proteins, grown on MS agar, were either sprayed with 100 µm TIBA for 8 h or treated with gaseous MeJA (1 µL/L air) for 8 h. About 50 seedlings grown on the same agar plate were pooled for protein extraction and GUS activity tests. The GUS activity was normalized to the protein concentration. Each bar represents the average ± SEM of two independent seedling pools.

TIBA treatment of JAZ1-GUS seedlings did not lead to a decrease in GUS activity, demonstrating that no increased JAZ1 degradation occurs. Still, this assay only reflects the JAZ1:GUS protein concentrations; a possible constant turnover of JAZ proteins cannot be monitored.

As positive control, protein extracts of the MeJA-treated JAZ1-GUS plants show a clear reduction in GUS activity, thereby providing evidence that increased JA-Ile levels indeed reduced the JAZ1 levels in this experiment (Figure 4. 7).

4.4 *CYP81D11* expression in response to xenobiotic stress requires a JA-Ile-independent COI1 function

JA-Ile measurements demonstrated that no JA-Ile accumulation occurs in response to TIBA. Nevertheless, *CYP81D11* expression is strongly dependent on the JA-Ile receptor COI1. To answer the question whether JA-Ile is required, the JA synthesis mutant *dde2-2* was used. The *DDE2* gene encodes the enzyme allene oxide synthase (AOS) which converts 13(S)-hydroperoxylinolenic acid to 12,13-epoxylinolenic acid (Park et al. 2002). The *dde2-2* mutant was used to assess the impact of the basal JA levels in mock- and TIBA-treated plants on the TIBA-induced *CYP81D11* expression. Soil-grown plants were treated with TIBA and incubated for 8 h. RNA was isolated to allow profiling of the *CYP81D11* transcript by quantitative real-time RT-PCR (Figure 4. 8).

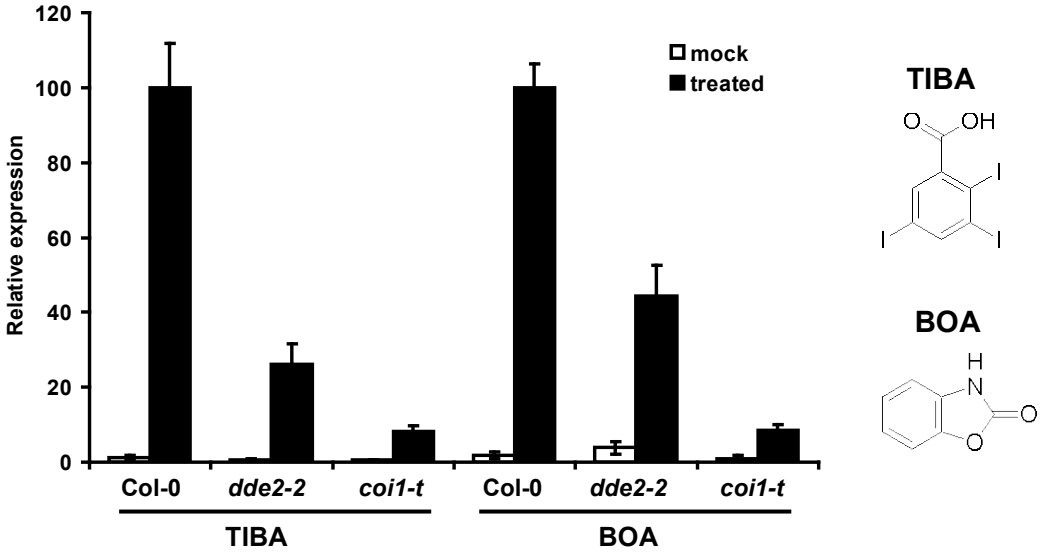

Figure 4. 8: *CYP81D11* transcript levels after TIBA and BOA treatment in the *dde2-2* and *coi1-t* mutants

Quantitative real-time RT-PCR analysis of relative *CYP81D11* transcript levels (normalized to the housekeeping gene *UBQ5*) in Col-0 (wild-type), *dde2-2* and *coi1-t* (Mosblech et al. 2010) plants. 6–7-week-old plants were sprayed with 100 µM TIBA or 2 mM BOA and, 8 h after induction, whole rosettes were harvested for RNA isolation. Transcript values of treated Col-0 plants were set to 100. Each bar represents the average ± SEM of three to eight biological replicates.
The chemical structures of TIBA and BOA are indicated.

CYP81D11 transcript levels in TIBA-treated *dde2-2* plants were reduced to about 30 % of the Col-0 wild type. This indicates that basal JA-Ile levels have an important influence on TIBA induction of *CYP81D11* expression. Nevertheless, *CYP81D11* transcript levels in the *dde2-2* mutant are still three times higher than in the *coi1-t* mutant. Comparable to the previous experiment using the *coi1-1* mutant (Figure 4. 5), *CYP81D11* levels are reduced to about 10 %. Based on these data, it is concluded that the absence of JA has less severe consequences for *CYP81D11* expression than the absence of the F-box protein COI1. This result was unexpected as it implicates that the COI1 protein, which so far has only been known to function as a JA-Ile receptor in JA signal transduction, exhibits a function independent of JA.

To determine whether this effect is specific for TIBA, the allelochemical benzoxazolin-2(3*H*)-one (BOA) was used for the induction of *CYP81D11* expression. BOA has previously been identified as an inducer for *CYP81D11* (Baerson et al. 2005), but has no similarity to TIBA regarding the chemical structure. Comparable effects concerning the different strengths of *CYP81D11* expression in the *dde2-2* and *coi1-t* mutants were observed in response to BOA and TIBA. This demonstrates that the differential *CYP81D11* expression in the *dde2-2* and *coi1-t* mutants in response to xenobiotic treatment is not specific to TIBA.

To support the result that the absence of JA-Ile affects *CYP81D11* expression less severely than the absence of COI1, another JA-Ile-deficient *A. thaliana* mutant, *jar1-1* (*jasmonate resistant 1*), was used for TIBA treatment (Figure 4. 9). The *jar1-1* mutant exhibits a defect in an enzyme converting JA to its amino acid conjugate JA-Ile. Additionally, it should be excluded that minor JA levels, which might still be produced in the *dde2-2* mutant, are responsible for the observed *CYP81D11* expression. Therefore, a *dde2-2*/*jar1-1* double mutant, in which potential minor JA levels will not be converted to JA-Ile, was created. By quantitative real-time RT-PCR, *CYP81D11* transcript levels were determined in the *dde2-2 and jar1-1* single mutants and the *dde2-2/jar1-1* double mutant, in response to TIBA (Figure 4. 9).

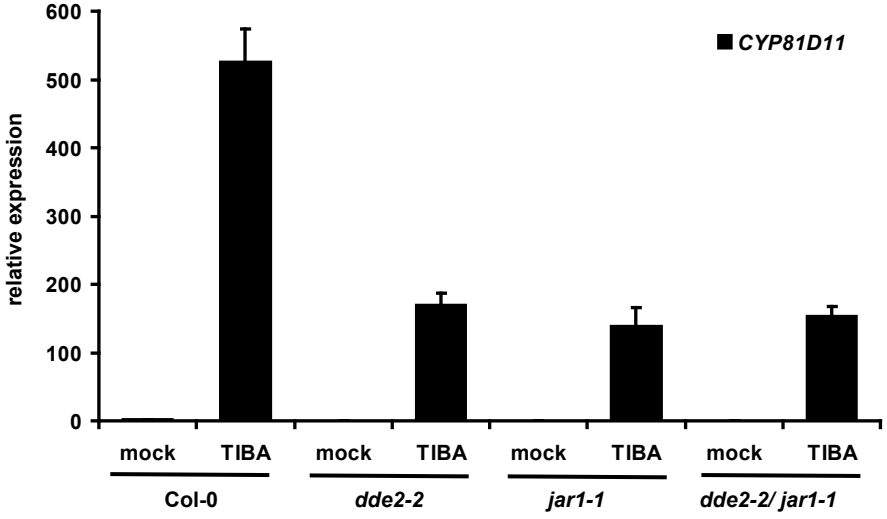

Figure 4. 9: Expression analysis of *CYP81D11* in JA synthesis mutants after TIBA

Quantitative real-time RT-PCR analysis of relative *CYP81D11* transcript levels (normalized to the housekeeping gene *UBQ5*) in Col-0 (wild-type), *dde2-2, jar1-1* and *dde2-2/jar1-1* mutant plants. 6–7-week-old plants were sprayed with 100 µM TIBA. Whole rosettes were harvested for RNA isolation. Transcript values in mock-treated Col-0 plants were set to 1. Each bar represents the average ± SEM of four (Col-0, *dde2-2* and *jar1-1*) or eight (*dde2-2/jar1-1*) biological replicates.

The *jar1-1* mutant exhibits reduced *CYP81D11* expression to the same extent as the *dde2-2* mutant, underlining the importance of basal JA-Ile levels for *CYP81D11* expression and supporting the result that JA-Ile deficiency reduces *CYP81D11* expression less severely than the absence of COI1. The idea that the differences in *CYP81D11* expression in JA-Ile synthesis mutants and the JA signaling mutant *coi1-t* might be due to an induction by minor residual JA-Ile levels synthesized by alternative reactions was excluded by the *dde2-2/jar1-1* mutant. The *CYP81D11* expression level in

this double mutant resembles the expression levels in the single mutants and was not further reduced.

4.5 Maximum *CYP81D11* expression in response to TIBA requires basal JA-Ile levels and JA signaling components

As the previous experiments have indicated a JA-Ile-independent COI1 function in response to TIBA, it was investigated if this depends on JA signaling downstream of COI1, which is mediated by JAZ proteins and the transcription factor MYC2. For MYC2, the mutant line *jin1-1* (*jasmonate insensitive 1*) is available. The degradation of JAZ proteins is abolished in transgenic plants expressing a JAZ protein with a deletion in the C-terminal Jas domain (e.g. JAZ1Δ3A (Thines et al. 2007)). On the one hand, these proteins themselves were shown to be resistant to degradation; on the other hand, they exhibited a dominant-negative effect that also blocks the degradation of other JAZ proteins, leading to a strong JA-insensitive phenotype. TIBA treatment of *jin1-1* mutants and JAZ1Δ3A plants and subsequent quantitative real -time RT-PCR revealed the effect of JA-Ile signaling components downstream of COI1 on the TIBA-dependent expression of *CYP81D11* (Figure 4. 10).

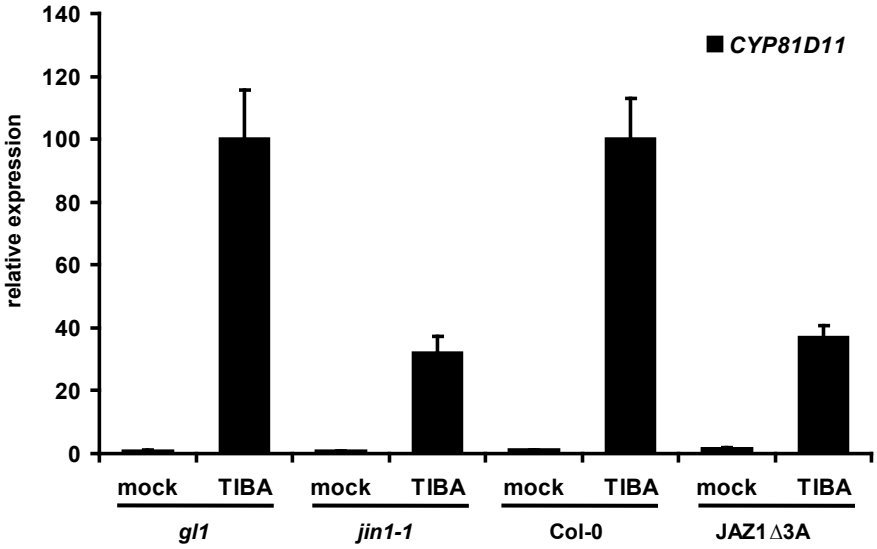

Figure 4. 10: Expression analysis of *CYP81D11* in plants deficient in JA signal transduction after TIBA

Quantitative real-time RT-PCR analysis of relative *CYP81D11* transcript levels (normalized to the housekeeping gene *UBQ5*) in *gl1* ("wild-type") and *jin1-1* mutant plants as well as Col-0 (wild-type) plants ectopically expressing JAZΔ3A, a JAZ1 protein lacking the Jas domain. 6–7-week-old plants were sprayed with 100 µM TIBA and, after 8 h, whole rosettes were harvested for RNA isolation. Transcript values in mock-treated Col-0 plants were set to 1. Each bar represents the average ± SEM of four (*gl1* and *jin1-1*), five (Col-0 mock), six (Col-0 TIBA and JAZ1Δ3A TIBA), or seven (JAZ1Δ3A mock) biological replicates.

The *jin1-1* mutant and the JAZ1Δ3A plants exhibit *CYP81D11* transcript levels in response to TIBA that are reduced to about one-third of those of the corresponding wild type (Figure 4. 10). This is consistent with the reduction in JA-Ile-deficient mutants (Figure 4. 9), indicating that MYC2, together with degradation of the JAZ proteins, is involved in the signaling response to basal JA levels, but is not involved in the signaling downstream of the new, JA-Ile-independent COI1 function.

4.6 Mutation of the MYC2 binding site leads to a JA-insensitive but COI1-dependent *CYP81D11* promoter

MYC2 transcription factors bind to sequence motifs called G-boxes (Abe et al. 2003; Abe et al. 1997; Dombrecht et al. 2007). To investigate the role of the G-boxes in the *CYP81D11* promoter, reporter gene constructs with mutated G-boxes were designed. The *CYP81D11* promoter contains two neighboring G-boxes (position –206 to –193) separated by only one base pair; one G-box exhibits the sequence CACGTG and the other is represented by CACATG. They were replaced by TTCAAG and TTCAAA (Abe et al. 1997) as replacement with these sequences creates no other known

transcription factor binding sites (http://www.dna.affrc.go.jp/PLACE/). *CYP81D11 promoter:GUS* constructs in binary plant vectors were created, either using an 894-bp promoter fragment exhibiting the wild-type sequence or the promoter sequence with mutated G-boxes. Transgenic plants were produced by *A. tumefaciens*-mediated gene transfer into *A. thaliana* Col-0 plants. The seedlings of the F1 generation grown on agar plates were used for TIBA and MeJA treatment (Figure 4. 11).

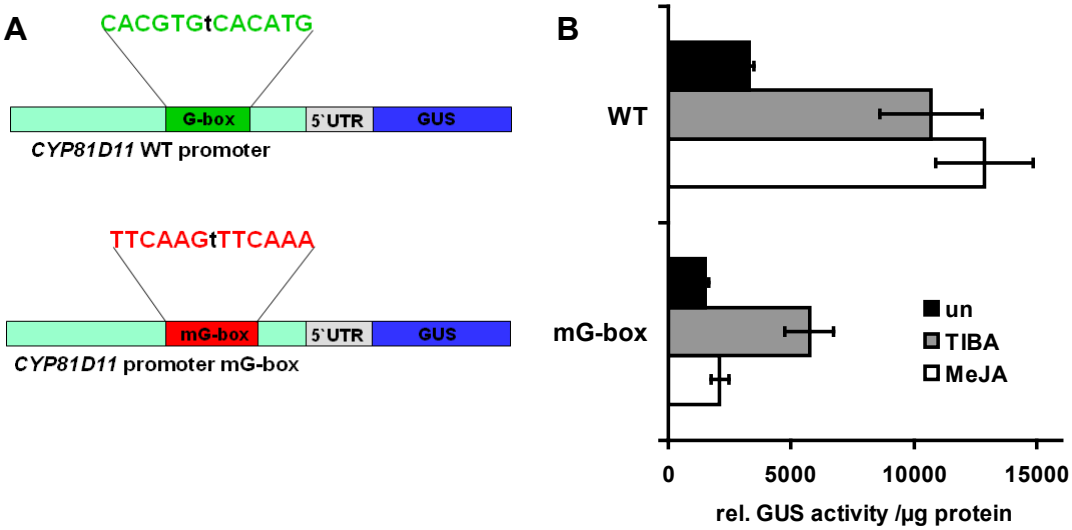

Figure 4. 11: Average activities of a *CYP81D11* WT promoter construct and a construct lacking the G-boxes in response to TIBA and MeJA

(A) Scheme of the distinct *CYP81D11 promoter:GUS* reporter gene constructs that were used to generate the transgenic lines employed in this experiment. The light green box indicates an 894-bp fragment upstream of the *CYP81D11* transcription start. The double G-box (position –206 to –193) is marked in green, while the altered sequence is marked in red; both sequences are indicated. The grey box depicts the 5' UTR of the *CYP81D11* gene and the *GUS* reporter gene is indicated in blue.
(B) GUS activities in response to TIBA and MeJA treatment obtained from transgenic plants containing the *CYP81D11 promoter*:*GUS* constructs depicted in (A). Seedlings were grown for 16 days on MS agar and either sprayed with 100 µm TIBA for 8 h or treated with gaseous MeJA (1 µL/L air) for 24 h. Control plants remained untreated. Whole seedlings were harvested for protein extraction. Each bar represents the average ± SEM of two experiments, each carried out with 19–20 transgenic lines.

The GUS activity in transgenic plants encoding the *CYP81D11 WT promoter:GUS* construct was inducible to similar extents in response to TIBA and MeJA. Plants containing the *CYP81D11 mG-box promoter:GUS* construct were not inducible by MeJA. This result indicates that the double G-box is absolutely essential for JA-induced *CYP81D11* expression. The non-induced and the TIBA-induced levels were reduced about two times. This leads to the suggestion that the G-boxes play a role as elements for constitutive activation, but not as activated elements in response to TIBA.

The substitution of the G-boxes in the *CYP81D11* promoter generated a promoter construct insensitive to JA. To investigate the COI1 dependency of this promoter

construct, transfection experiments in Col-0 and *coi1-t* protoplasts were performed (Figure 4. 12).

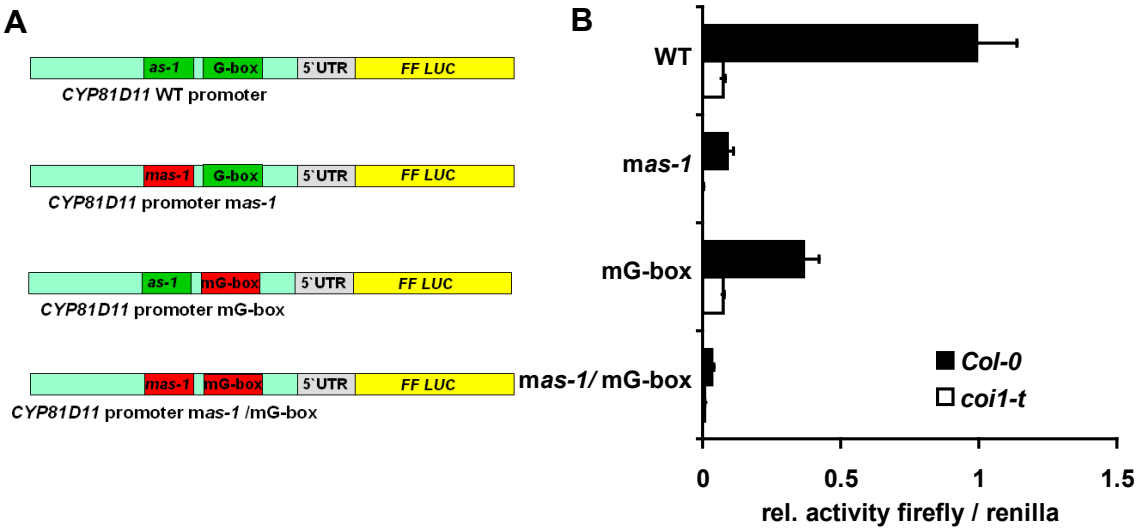

Figure 4. 12: Transient expression analysis of distinct *CYP81D11* promoter constructs carrying sequence alterations at the site of the *as-1*-like element and the site of the G-boxes

(A) Schematic illustration of the distinct *CYP81D11 promoter:firefly luciferase* constructs used for protoplast transfection. The light blue box indicates an 894-bp fragment upstream of the *CYP81D11* transcription start site; wild-type sequences of the *as-1* element (position –243 to –225) and the double G-box (position –206 to –193) are marked in green while the altered sequences are depicted by red boxes. The grey box marks the 5' UTR of the *CYP81D11* gene and the yellow box indicates the *firefly luciferase* (*FF LUC*) reporter gene. The construct referred to as WT contains the 894-bp fragment upstream of the *CYP81D11* transcription start site and the 5' UTR of the *CYP81D11* gene. The m*as-1* construct contains sequence alterations within the *as-1* element (position –243 to –225) while the mG-box construct contains alterations in the sequence of the double G-box (position –206 to –193). The m*as-1*/mG-box construct exhibits alterations in both elements.
(B) Luciferase activities obtained from transfection of distinct *promoter:firefly luciferase* constructs in *A. thaliana* Col-0 and *coi1-t* protoplasts. Leaves of 7-week-old soil-grown and non-induced plants were used for protoplast isolation. The x-coordinate demonstrates the ratio of the firefly luciferase activity to the internal *Renilla* luciferase standard.

In protoplasts *CYP81D11* is constitutively expressed. Therefore they allow expression analyses in the absence of additional treatments. In *coi1-t* protoplasts, the activity of the WT promoter construct is strongly reduced in comparison to the Col-0 protoplasts. This demonstrates a similar COI1 dependency in protoplasts as in whole plants. Additionally, the reduced activity of the mG-box construct compared to the WT construct in wild-type protoplasts also reflects the results obtained by TIBA treatments in whole plants.
A comparison of the reporter gene expression of the mG-box construct in Col-0 and *coi1-t* protoplasts demonstrated the COI1 dependency of this construct. This result leads to the assumption that COI1 carries out its JA-Ile-independent function via another promoter element than the G-boxes which are responsible for JA-Ile-dependent *CYP81D11* expression.

Nevertheless, the fact that in *coi1-t* protoplasts the WT construct and the mG-box construct have the same activity demonstrates that the G-boxes only function in the presence of COI1.

The activities of the m*as-1* and the m*as-1*/mG-box constructs are still strongly reduced in the *coi1-t* mutant compared to the wild-type protoplasts. Thus, it was assumed that COI1 regulates the *CYP81D11* promoter not via the TGA binding site.

4.7 A large group of genes depends on COI1 and basal JA-Ile levels in response to TIBA

In response to TIBA, COI1-dependent regulation of *CYP81D11* expression depends on basal JA-Ile levels and on a novel JA-Ile-independent COI1 function. In order to identify other genes showing this type of regulation, mock- and TIBA-treated Col-0 and *coi1-t* plants were compared by transcriptome analyses (Figure 4. 13). As a control, *CYP81D11* expression was investigated by quantitative real-time RT-PCR.

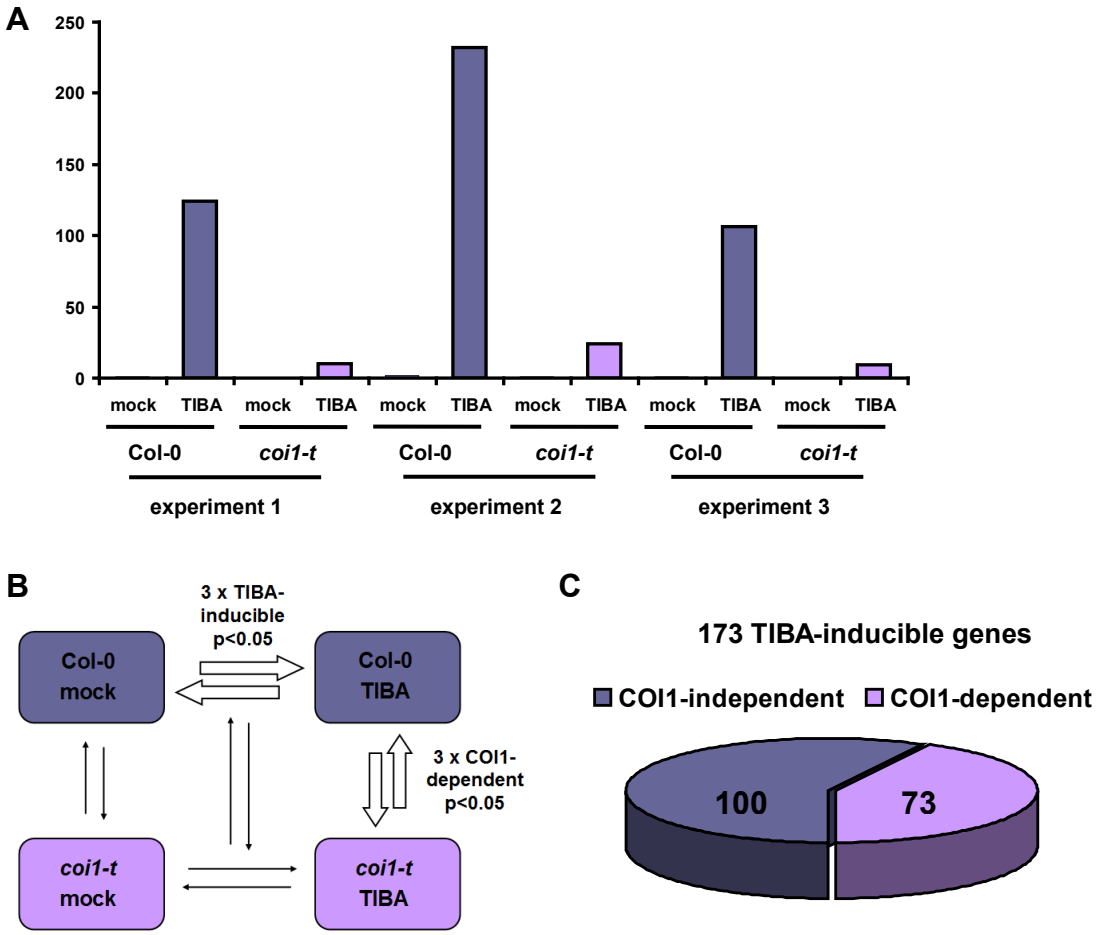

Figure 4. 13: Microarray analyses of TIBA-treated Col-0 and *coi1-t* plants

Plants for the microarray analyses are grown on MS agar under short-day conditions; to select homozygous *coi1-t* plants, the MS medium contained 50 µM MeJA. After 3 weeks, JA-insensitive plants were transferred to soil and grown for another 4 weeks under 12-h light/12-h dark conditions. Plants were either sprayed with 100 µM TIBA or 0.1 % DMSO (mock) and were harvested after 8 h as pools of eight plants. Three independent experiments were performed. Hybridizations were performed by NASC's International Affymetrix Service using "Arabidopsis ATH1 Genome Array" chips. Raw data were analyzed by Robin (Lohse et al. 2010).
(A) Quantitative real-time RT-PCR analysis of relative *CYP81D11* transcript levels (normalized to the housekeeping gene *UBQ5*).
Scheme (B) is indicating the investigated samples and the comparison made between the different mutants and treatments. All further results were obtained by comparing Col-0 mock and Col-0 TIBA to define genes inducible by TIBA, and by comparing Col-0 TIBA and *coi1-t* TIBA to define COI1-dependent genes.
Diagramm (C) is indicating the numbers genes which are at least 3-fold TIBA-inducible and are COI1 dependent or independent. Genes with expression levels in the *coi1-t* mutant after TIBA treatment reaching less than 30 % of the Col-0 expression level (P-value <0.05) were defined as COI1 dependent.

Although *CYP81D11* expression varied among the independent experiments, the ratio between Col-0 and *coi1-t* is consistent within each experiment.

By comparing the transcript levels of mock-treated and TIBA-treated Col-0 plants, 173 genes were identified whose expression is at least 3-fold induced by TIBA (P-value <0.05). These were classified into COI1-dependent and COI1-independent genes by comparing TIBA-treated wild-type and TIBA-treated *coi1-t* plants. Genes whose

expression in the *coi1-t* mutant after TIBA treatment reached less the 30 % of the Col-0 expression (P-value <0.05) were defined as COI1 dependent. This sorting resulted in 100 COI1-independent and 73 COI1-dependent genes.

Table 4. 1: Compolation of those ten COI1-dependent genes, which show the strongest expression after TIBA treatment

Gene code	Symbol	Description	Col-0 TIBA/ Col-0 WT	P-value	*coi1-t* TIBA/ Col-0 TIBA	P-value
at3g28740	*CYP81D11*	monooxygenase	55.64	8.6E-09	0.12	1.0E-05
at1g43160	*RAP2.6*	transcription factor	30.96	1.1E-12	0.03	9.3E-13
at5g13220	*JAZ10*	jasmonate-ZIM-domain protein	28.62	2.0E-10	0.02	4.8E-11
at3g49620	*DIN11*	oxidoreductase	23.70	3.5E-07	0.01	1.2E-08
at1g10585		transcription factor	18.92	1.4E-10	0.06	3.4E-10
at5g63450	*CYP94B1*	monooxygenase	16.61	7.1E-12	0.06	7.0E-12
at2g34600	*JAZ7*	jasmonate-ZIM-domain protein	12.90	7.2E-09	0.07	5.1E-09
at4g21680		proton-dependent oligopeptide transport (POT) family protein	10.93	2.9E-10	0.12	1.4E-09
at3g23550		MATE efflux family protein	9.95	3.1E-10	0.10	3.1E-10
at3g09940	*ATMDAR3*	monodehydroascorbate reductase	8.92	1.4E-07	0.07	1.8E-08

With more than 55-fold induction in wild-type plants, *CYP81D11* is the most strongly TIBA-inducible gene identified in this array. In the *coi1-t* mutant it shows 12 % of the wild-type transcript levels after TIBA treatment. In contrast to this, the genes that are next strongly induced by TIBA are very strictly dependent on COI1 (Table 4. 1). Therefore, those genes were selected for further analyses, which resemble *CYP81D11* either for their strong inducibility by TIBA or for their less strict COI1 dependency (Table 4. 2).

Table 4. 2: COI1-dependent TIBA-inducible genes that are either very strongly induced by TIBA or show only weak COI1 dependency

Gene code	Symbol	Description	Col-0 TIBA/ Col-0 WT	P-value	*coi1-t* TIBA/ Col-0 TIBA	P-value
at3g28740	*CYP81D11*	monooxygenase	55.64	8.6E-09	0.12	1.0E-05
at5g13220	*JAZ10*	jasmonate-ZIM-domain protein	28.62	2.0E-10	0.02	4.8E-11
at3g49620	*DIN11*	oxidoreductase	23.70	3.5E-07	0.01	1.2E-08
at3g23550		MATE efflux family protein	9.95	3.1E-10	0.10	3.1E-10
at3g57520	*AtSIP2*	hydrolase	7.63	1.1E-06	0.28	1.2E-04
at3g47340	*ASN1*	asparagine synthase	6.75	9.0E-05	0.24	1.1E-03
at2g29460	*ATGSTU4*	glutathione transferase	6.71	1.3E-05	0.22	1.1E-04
at5g61160	*AACT1*	acyltransferase	6.61	1.2E-06	0.07	2.4E-08

In order to distinguish whether basal JA-Ile levels or the JA-Ile-independent COI1 function contributes to the induction of the COI1-dependent genes, the transcription levels of a selection of COI1-dependent genes were monitored in Col-0 wild-type, *coi1-t* and *dde2-2* plants after TIBA treatment by quantitative real-time RT-PCR in an experiment independent of that used for the microarray (Figure 4. 14).

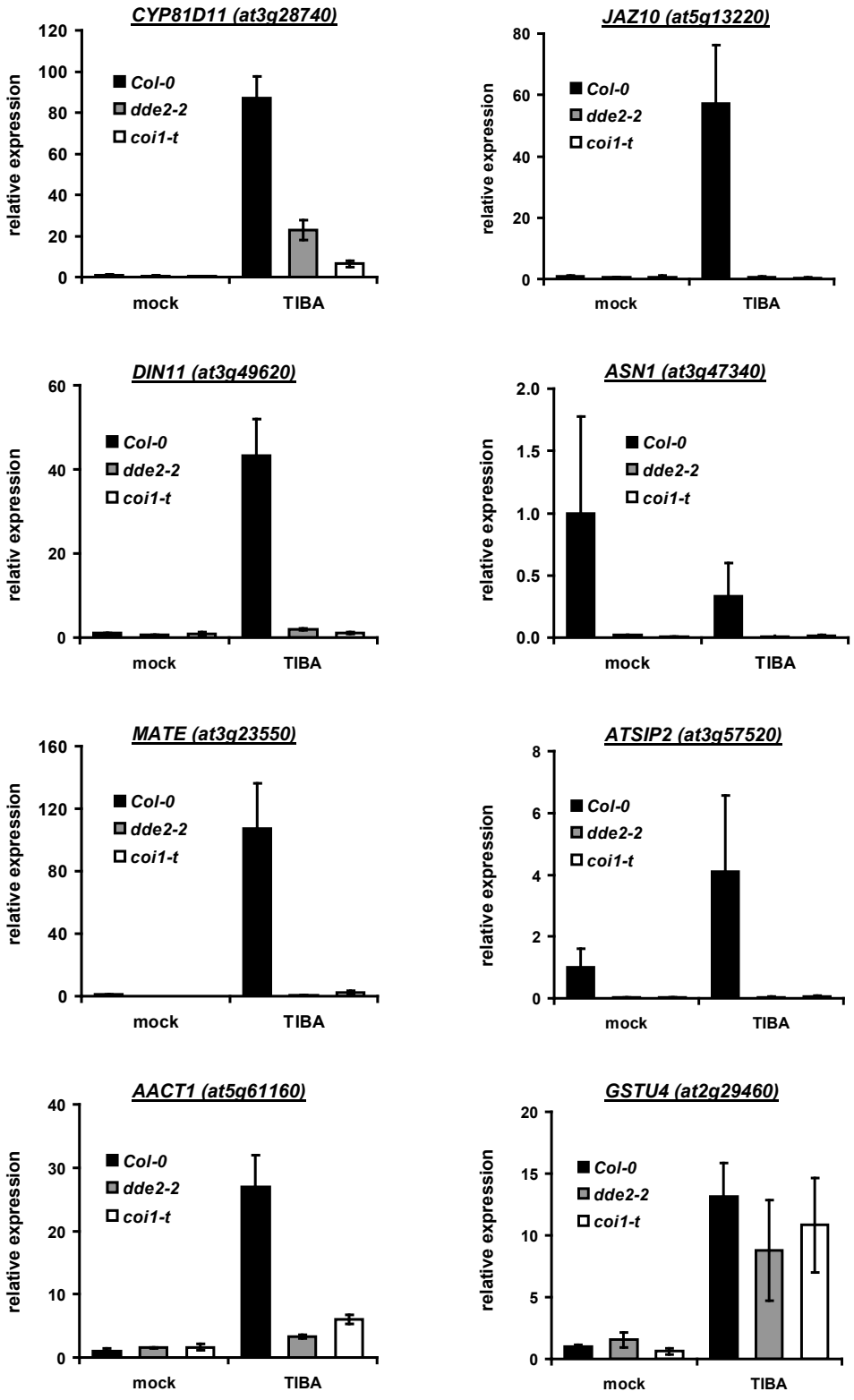

Figure 4. 14: Transcript levels of TIBA-inducible genes in Col-0, *dde2-2* and *coi1-t plants*

Quantitative real-time RT-PCR analysis of the relative *CYP81D11*, *JAZ10*, *DIN11*, *ASN1*, *MATE* (*at3g23550*, gene coding for a MATE family protein), *ATSIP1*, *AACT1* and *GSTU4* transcript levels (normalized to the housekeeping gene *UBQ5*) in Col-0 (wild-type), *dde2-2* and *coi1-t* plants. 6–7-week-old plants were sprayed with 100 µM TIBA and, 8 h after induction, whole rosettes were harvested for RNA isolation. The average transcript level of mock-treated Col-0 plants was set to 1. Each bar represents the average ± SEM of four (*coi1-t* mock, three) independent plants, if all samples exhibited detectable transcript levels. In some samples, like all the mock-treated *dde2-2* and *coi1-t* samples of *MATE* (*at3g2350*), no transcript could be detected.

CYP81D11, *JAZ10*, *DIN11*, *ASN1*, *MATE* (*at3g23550*), *ATSIP1*, *AACT1* and *GSTU4* transcript levels were analyzed via quantitative real-time RT-PCR in Col-0, *dde2-2* and *coi1-t* plants in response to TIBA treatment. For *CYP81D11*, *JAZ10*, *DIN11*, *MATE* (*at3g23550*), *ATSIP1* and *AACT1*, the TIBA inducibility and the COI1 dependency shown in the microarray analyses were reproduced. However, except for *CYP81D11* and *AACT1*, all show a very strict COI1 dependency, without notable differences between the expression levels in the *dde2-2* and *coi1-t* mutants. *AACT1* expression depends less stringently on COI1; still, no stronger expression in the *dde2-2* mutant than in the *coi1-t* mutant is observed. These results indicate that *JAZ10*, *DIN11*, *MATE* (*at3g23550*), *ATSIP1* and *AACT1* expression in response to TIBA require *COI1* activity and basal JA-Ile levels, but none of them is regulated by a JA-Ile-independent COI1 function. So far, this regulation mode remains unique for *CYP81D11*.

For *ASN1* and *GSTU4*, the results obtained from the microarray experiments were not reproduced: *ASN1* expression is strongly dependent on COI1, but in this experiment it is not induced by TIBA. In contrast to this, *GSTU4* expression is induced by TIBA, but no dependency on COI1 or JA-Ile is shown.

4.8 *CYP81D11* is more closely co-regulated with COI1-independent than with COI1-dependent TIBA-inducible genes

Although a JA-Ile-independent COI1 function is not required for the regulation of the more closely investigated COI1-dependent TIBA-inducible genes except for *CYP81D11*, most of them shared the dependency on the basal JA-Ile levels. Hierarchical cluster analysis was performed (https://www.genevestigator.ethz.ch/) to identify the genes most closely co-regulated with *CYP81D11*, as these could be candidate gens, which might be under the same control.

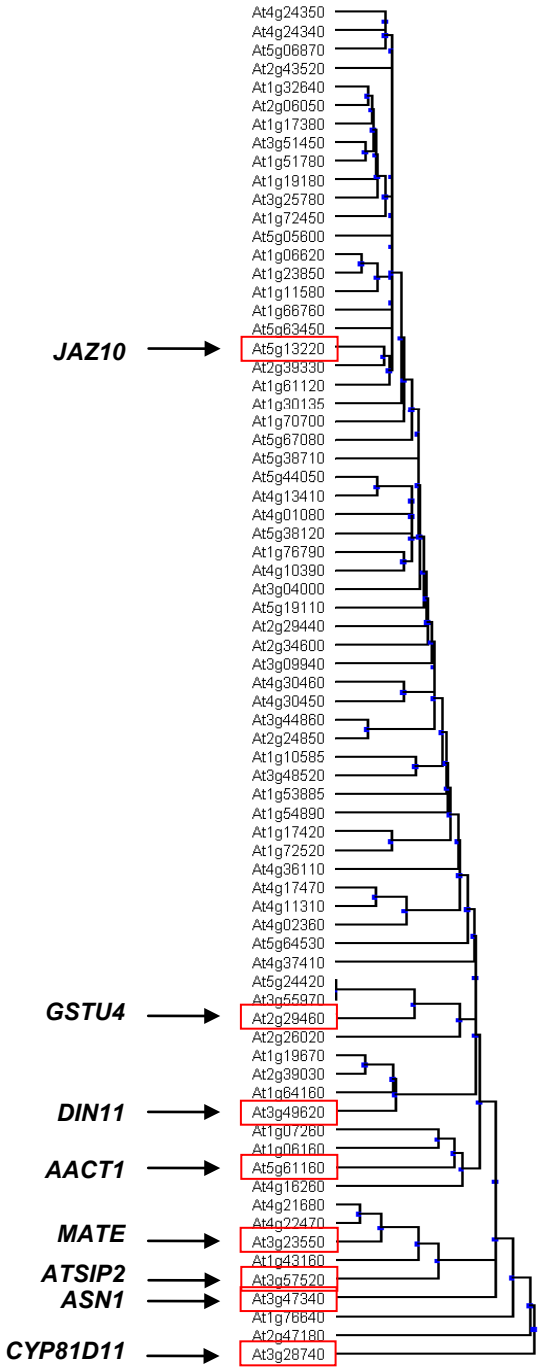

Figure 4. 15: Co-regulation of COI1-dependent TIBA-inducible genes after various stimuli

Hierarchical cluster analysis of all COI1-dependent TIBA-inducible genes identified by microarray analysis. The analysis was performed by the Genevestigator cluster analysis tool, choosing the adjustment "stimulus". Results are depicted as tree model.
Genes that were more closely investigated in the *dde2-2* and *coi1-t* mutants by quantitative real-time RT-PCR are highlighted by a red box. Gene symbols of the highlighted genes are indicated.

The tree model depicted in Figure 4. 15 was calculated based on hierarchical cluster analyses investigating the co-regulation of single genes within the group of COI1-dependent TIBA-inducible genes after diverse stimuli. *CYP81D11* is placed at the outmost edge of the tree, without being grouped to any other genes. This indicates a low correlation of regulation in comparison to all other COI1-dependent TIBA-inducible

genes. Closer examination of the induction patterns of the COI1-dependent genes revealed no co-regulation under conditions not related to JA.

Since *CYP81D11* shares the feature to be co-regulated with TGA-dependent genes that are independent of COI1, we performed Hierarchical cluster analysis with *CYP81D11* and the COI1-independent genes (Figure 4.16).

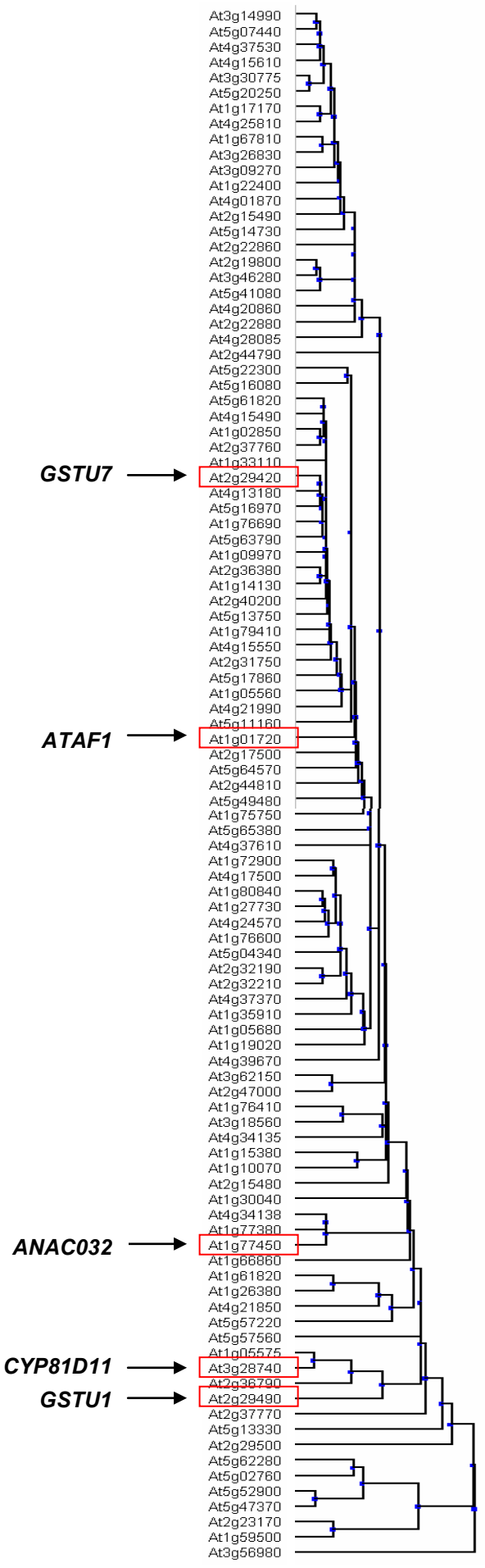

Figure 4. 16: **Co-regulation of COI1-independent TIBA-inducible genes with *CYP81D11***
Hierarchical cluster analysis of *CYP81D11* and all COI1-independent TIBA-inducible genes identified by microarray analysis. The hierarchical cluster analysis was performed by the Genevestigator cluster analysis tool, choosing the adjustment "stimulus". Gene symbols are indicated if possible.

By comparison of *CYP81D11* expression with the expression of COI1-independent TIBA-inducible genes, *CYP81D11* was indeed grouped to four other genes. The one most closely co-regulated with *CYP81D11* encodes an unknown protein. Two other genes in this group code for known proteins: *UGT73C6* encodes a UDP-glycosyl transferase, while *ATGSTU1* codes for a glutathione *S*-transferase. Both of these proteins are putatively involved in detoxification (Poppenberger et al. 2006; Fode et al. 2008), which is consistent with the induction of *CYP81D11* in response to a variety of xenobiotics. Furthermore, *ATGSTU1* was identified as an SCL14 target gene by Fode et al. (2008), and also the genes *UGT73C6* and *At1g05575* exhibit putative TGA binding (TGACG) motifs in their promoters.

In a time course experiment, *CYP81D11* expression was compared with COI1-dependent and COI1-independent genes. 6–7-week-old soil-grown Col-0 plants were sprayed with 100 µM TIBA and rosettes were harvested 1–24 h later. *CYP81D11*, *DIN11*, *JAZ10*, *ANAC032*, *GSTU1* and *GSTU7* transcript levels were elucidated by quantitative real-time RT-PCR (Figure 4. 17).

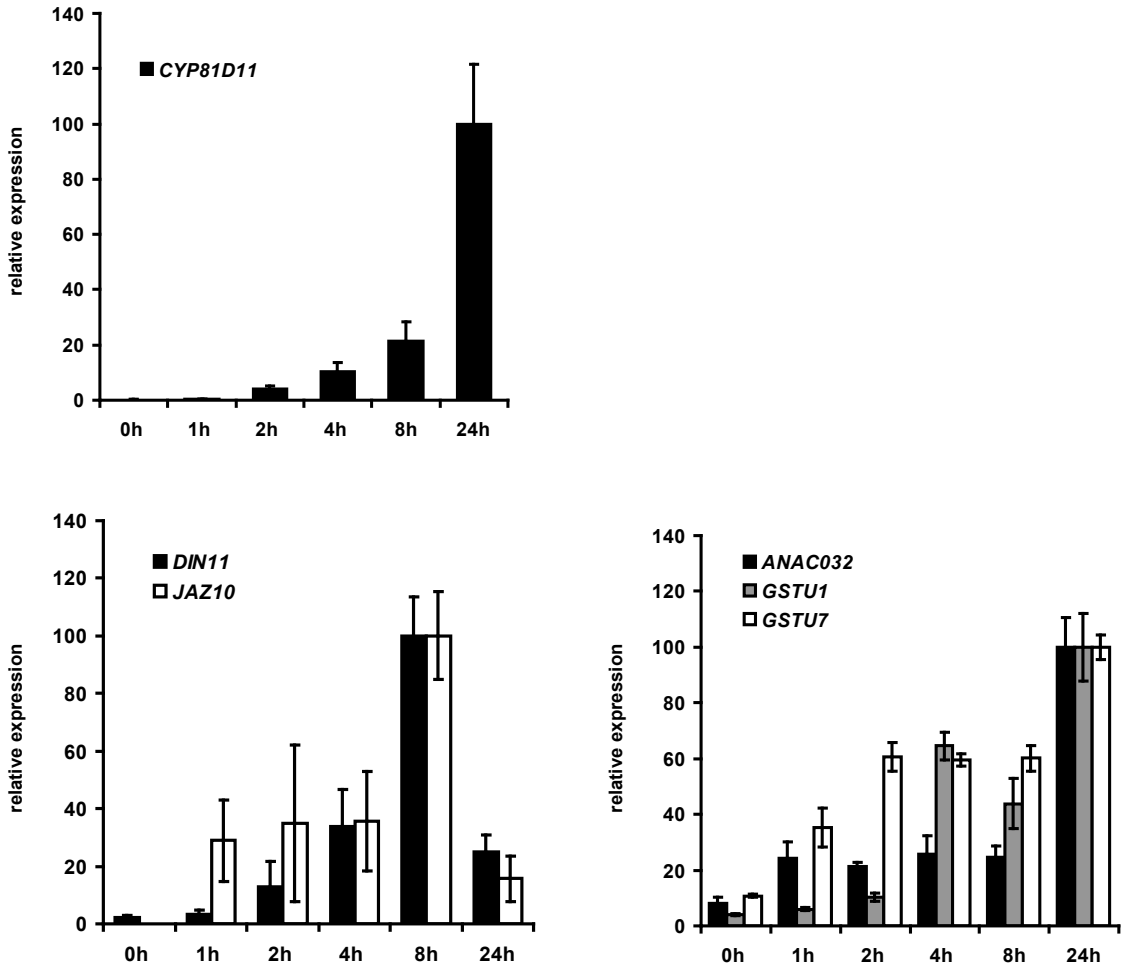

Figure 4. 17: Time course experiment of TIBA treatment in Col-0 plants

Quantitative real-time RT-PCR analysis of relative *CYP81D11*, *DIN11*, *JAZ10*, *ANAC032*, *GSTU1* and *GSTU7* transcript levels (normalized to the housekeeping gene *UBQ5*) in Col-0 wild-type plants. 6–7-week-old plants were sprayed with 100 µM TIBA and whole rosettes were harvested for RNA isolation at the indicated time points; for the 0-h time point, the plants remained untreated. Highest transcript levels were set to 100. Each bar represents the average ± SEM of four independent plants.

CYP81D11 transcript levels rise continuously after treatment with TIBA and show their maximum at 24 h after treatment. In contrast, *DIN11* and *JAZ10* transcript levels reach a peak at 8 h and transcription decreases again between 8 and 24 h. Although the time-dependent development of expression varies among the COI1-independent TIBA-inducible and TGA2,5,6/SCL14-dependent genes, *ANAC032*, *GSTU1* and *GSTU7* exhibit the highest transcript levels during the investigated time span of 24 h after treatment. Thus, expression of *CYP81D11* shows a similar time dependent behavior as that of the COI1-independent genes.

To investigate the TGA and SCL14 dependency of a TIBA-inducible and COI1-dependent gene, *DIN11* expression was monitored in TIBA-treated plants ectopically

expressing SCL14 and in *tga2,5,6* mutant plants, in comparison to expression of *CYP81D11* (Figure 4. 18).

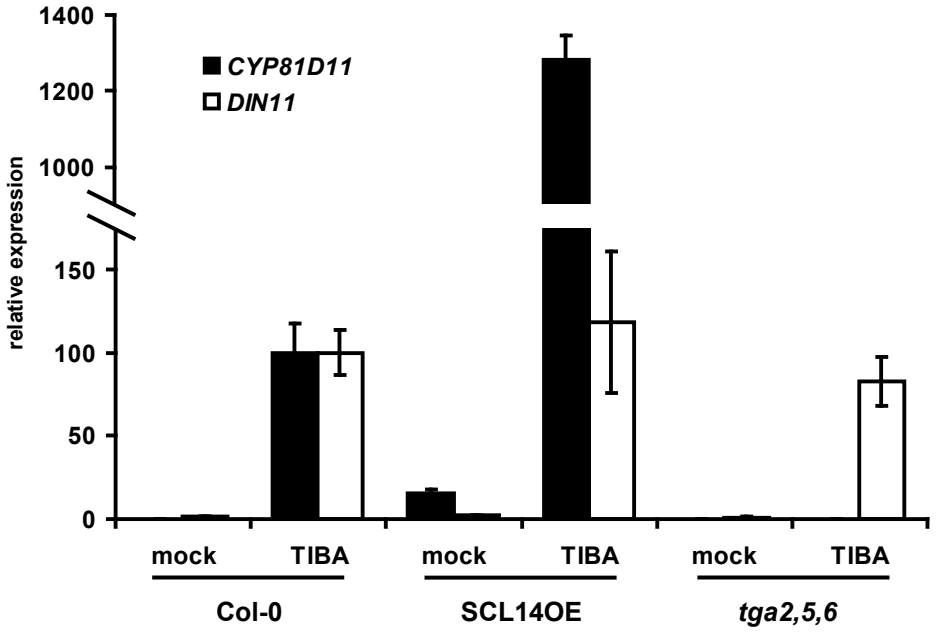

Figure 4. 18: *CYP81D11* and *DIN11* transcript levels in SCL14-overexpressing and *tga2,5,6* mutant plants

Quantitative real-time RT-PCR analysis of relative *CYP81D11* transcript levels (normalized to the housekeeping gene *UBQ5*) in Col-0 (wild-type) plants, plants ectopically expressing SCL14 (SCL14OE), and *tga2,5,6* mutant plants. 6–7-week-old plants were sprayed with 100 µM TIBA and, 8 h after induction, whole rosettes were harvested for RNA isolation. Transcript levels of TIBA-treated Col-0 plants were set to 100. Each bar represents the average ± SEM of four independent plants.

The SCL14-overexpressing plants exhibit increased *CYP81D11* expression even in mock-treated plants and also after TIBA treatment. *CYP81D11* transcript levels are about 13 times higher than in Col-0 wild-type plants. As shown before (Figure 4. 1), *CYP81D11* expression is nearly abolished in the *tga2,5,6* mutant both after mock and TIBA treatment. *DIN11* expression is influenced neither in the SCL14-overexpressing plants nor in the *tga256* plants compared to wild-type.

In order to obtain more information about the functions of COI1-dependent, TIBA-inducible genes, functional categorizations were performed and compared to the functional distribution of all genes in the *A. thaliana* genome (Figure 4. 19).

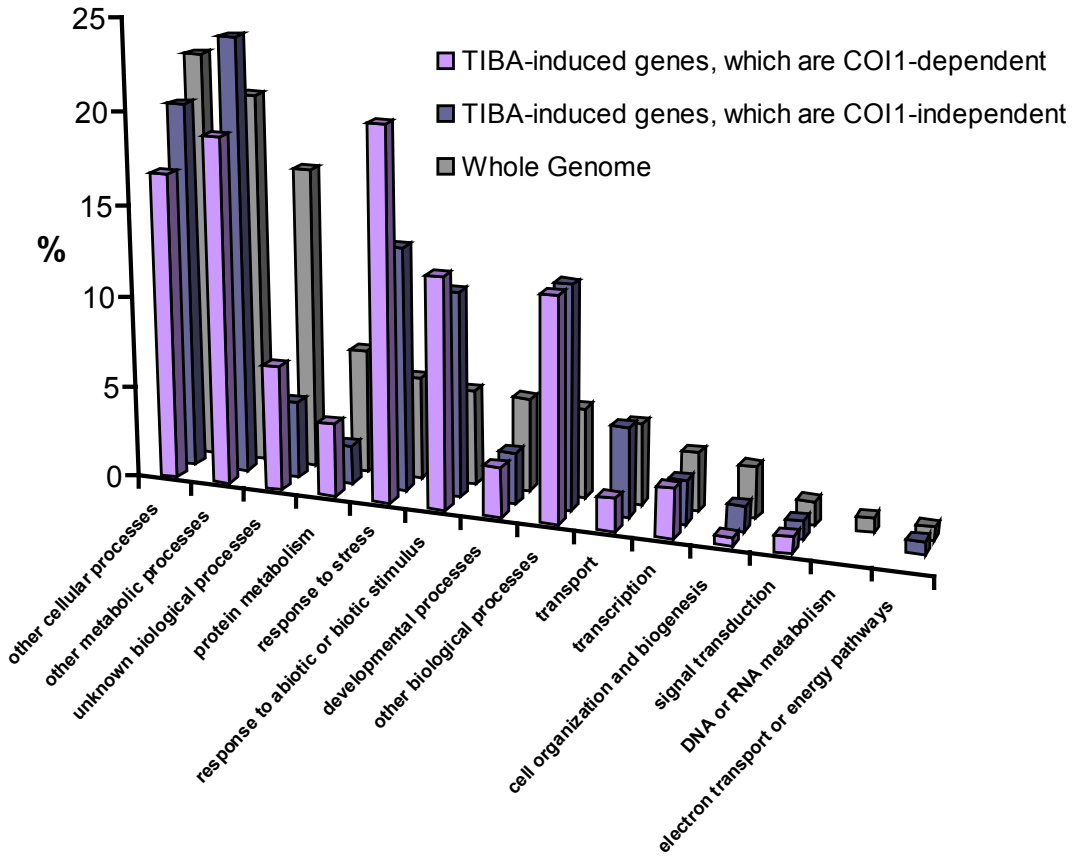

Figure 4. 19: Functional categorization of COI1-dependent and COI1-independent TIBA-inducible genes

The functional distribution of genes that are TIBA-induced in a COI1-dependent manner as compared to all genes of the *A. thaliana* genome. The classification was performed with the TAIR GO classification database tool.

Genes up-regulated in a COI1-dependent and COI1-independent manner show a relatively similar functional distribution. In both groups, genes involved in "response to stress", in "response to abiotic and biotic stimulus", and in "other biological processes" are overrepresented. In case of the COI1-dependent genes, the list of genes "responsive to stress" includes the genes "responsive to abiotic and biotic stimulus" (Table 6. 5). Interestingly, both lists include mainly JA-responsive genes, genes involved in JA signaling, and even some involved in JA biosynthesis. This result demonstrates that JA-induced stress-responsive genes represent an important part of the group of COI1-dependent TIBA-induced genes.

4.9 *CYP81D11* and *DIN11* show COI1-dependent and JA-Ile-independent expression in protoplasts

A large number of TIBA-inducible genes are induced in protoplasts, irrespective of whether their expression depents on COI1 (https://www.genevestigator.ethz.ch/). This is also the case for *CYP81D11* whose expression in protoplasts is COI1 dependent (Figure 4. 12). To elucidate if the requirement for COI1 of *CYP81D11* corresponds to a requirement JA-Ile, protoplasts were isolated from Col-0, *dde2-2* and *coi1-t* plants (Figure 4. 20). After overnight incubation, RNA was isolated to perform quantitative real-time RT-PCR analyses.

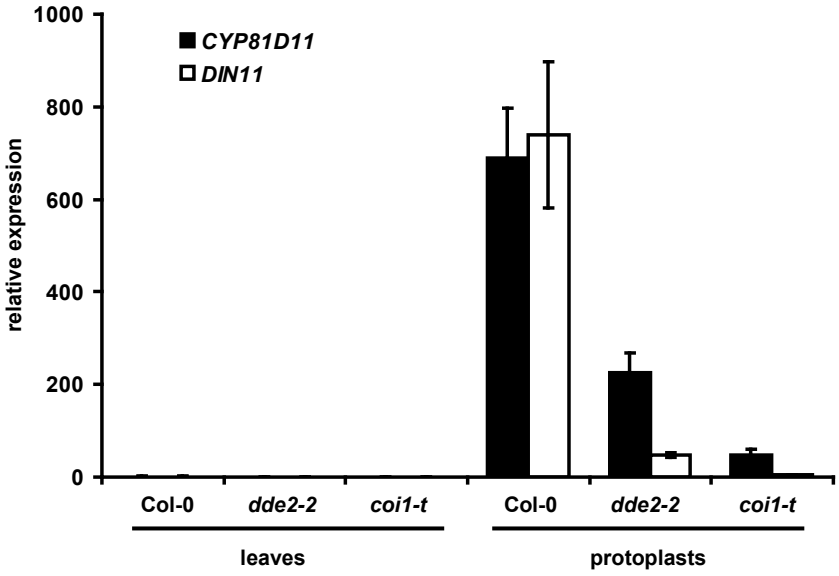

Figure 4. 20: Expression analysis of *CYP81D11* and *DIN11* in leaf protoplasts of Col-0, *dde2-2* and *coi1-t* plants

Quantitative real-time RT-PCR analysis of relative *CYP81D11* and *DIN11* transcript levels (normalized to the housekeeping gene *UBQ5*) in whole leaves and leaf protoplasts of Col-0 (wild-type), *dde2-2* and *coi1-t* mutant plants. Leaves of 6–7-week-old plants were used for protoplast preparation. The leaves were incubated overnight in a cell wall-degrading enzyme solution, extracted, washed and again incubated for another 16 h. Whole leaves harvested before protoplast extraction served as control. Transcript levels in whole Col-0 leaves were set to 1. Each bar represents the average ± SEM of five replicates from two independent experiments.

CYP81D11 was about 700–800-fold induced in protoplasts compared to intact leaves in Col-0 plants. *CYP81D11* expression in the *dde2-2* mutant is reduced to about 33 %, while in the *coi1-t* mutant *CYP81D11* expression is reduced to 7 %. This indicates that also in protoplasts the JA-Ile-independent COI1 function is involved in the induction of *CYP81D11* expression.

In comparison, also *DIN11* expression in protoplasts was analyzed and showed similar expression as *CYP81D11* in the wild-type. Interestingly, also *DIN11* expression is more severely reduced in the *coi1-t* mutant (0.6 %) than in the *dde2-2* mutant (6 %). In contrast to *CYP81D11*, this was not the case after TIBA treatment. However, *DIN11* transcript levels are more strongly compromised in comparison to *CYP81D11* levels in both the *dde2-2* and *coi1-t* mutants.

4.10 The expression of many JA-inducible genes is not increased in protoplasts

The reduction of *CYP81D11* and *DIN11* transcript levels in protoplasts derived from *dde2-2* mutant plants demonstrates that either basal or increased JA-Ile levels contribute to maximal expression. It was tested whether JA-inducible genes like *MYC2*, *LOX2*, *VSP2* and *PDF1.2* are induced in response to protoplasting (Figure 4. 21).

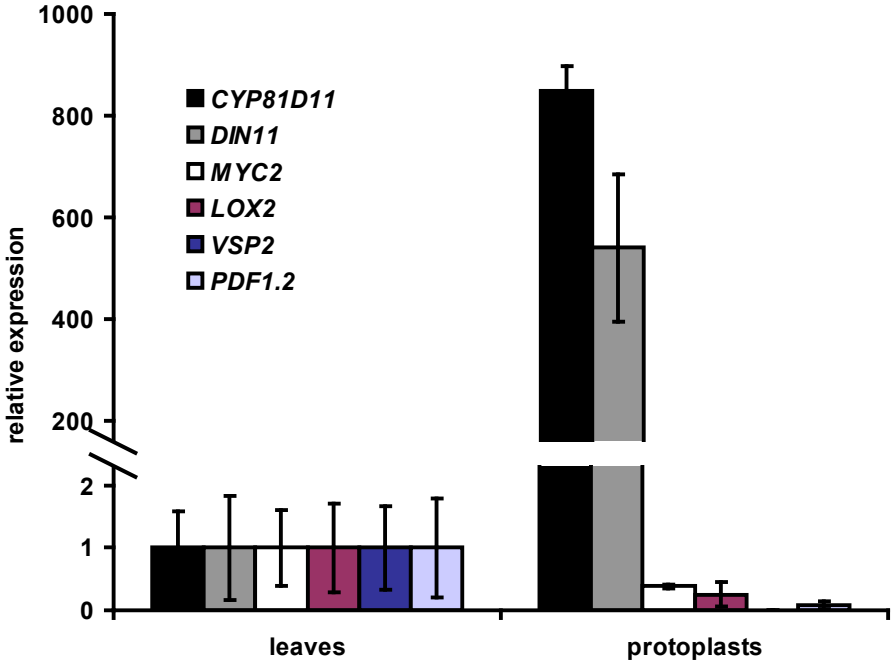

Figure 4. 21: Expression analysis of JA-inducible genes in leaves and protoplasts

Quantitative real-time RT-PCR analysis of relative *CYP81D11*, *DIN11*, *MYC2*, *LOX2*, *VSP2* and *PDF1.2* transcript levels (normalized to the housekeeping gene *UBQ5*) in whole leaves and leaf protoplasts of Col-0 (wild type). Leaves of 6–7-week-old plants were either harvested and immediately frozen as a control or used for protoplast preparation. The leaves were incubated overnight in a cell wall-degrading enzyme solution, extracted, washed and again incubated for another 16 h. Transcript levels in Col-0 leaves were set to 1. Each bar represents the average ± SEM of three replicates. For the *LOX2* gene, only two protoplast samples showed detectable transcript values, while for the *VSP2* gene transcript levels of all protoplast samples were below the limit of detection.

As shown before, *CYP81D11* and *DIN11* are well induced in protoplasts. In contrast, *MYC2*, *LOX2*, *VSP2* and *PDF1.2* expression levels were reduced to different extents. In case of *VSP2*, no transcript was detectable in protoplasts.

Since the severe wounding during protoplast isolation strongly suggests increased contents of JA-Ile in protoplasts, JA and JA-Ile levels were determined by HPLC-MS/MS. After digestion of the cell wall (overnight) and washing, Col-0 protoplasts were incubated for another 16 h (Figure 4. 22).

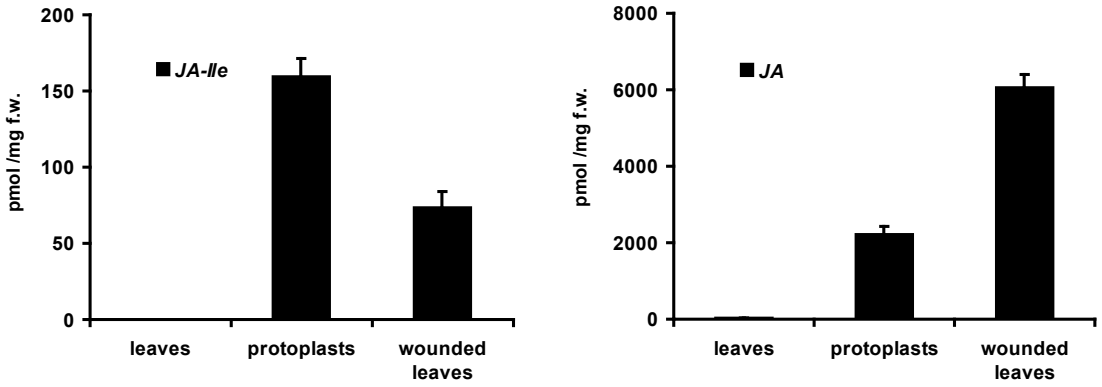

Figure 4. 22: JA-Ile and JA levels in *A. thaliana* protoplasts

HPLC-MS/MS analyses for JA-Ile and JA levels were performed in whole leaves and leaf protoplasts of Col-0 wild-type plants. Leaves of 6–7-week-old plants were used for protoplast preparation. The leaves were incubated overnight in a cell wall-degrading enzyme solution, extracted, washed and again incubated for another 16 h. Whole leaves harvested before protoplast extraction served as control. As additional control, leaves wounded by squeezing and incubated for 2 h were used. Each bar represents the average ± SEM of four (leaves), three (protoplasts), and two (wounded leaves) biological replicates. JA-Ile and JA amounts are depicted as pmol/g fresh weight. For the protoplasts, the weight of whole leaves was determined before protoplast isolation, and 40 % loss during the washing steps was estimated.

Protoplasts accumulate two times higher JA-Ile levels than leaves wounded for 2 h. Consistently, the levels of free JA were also heavily increased, but were more than two times lower than in wounded leaves.

The high endogenous JA-Ile levels in protoplasts do not induce expression of JA marker genes. To elucidate if JA signaling is generally functional, the exogenous application of the JA-Ile mimic coronatine was tested. Coronatine was added to the buffer used for overnight incubation of the protoplasts. Protoplasts of transgenic JAZ1-GUS plants were used to monitor JAZ1 stability by quantitative GUS activity tests. Therefore, fractions of the protoplast samples were used for RNA isolation and protein extraction (Figure 4. 23).

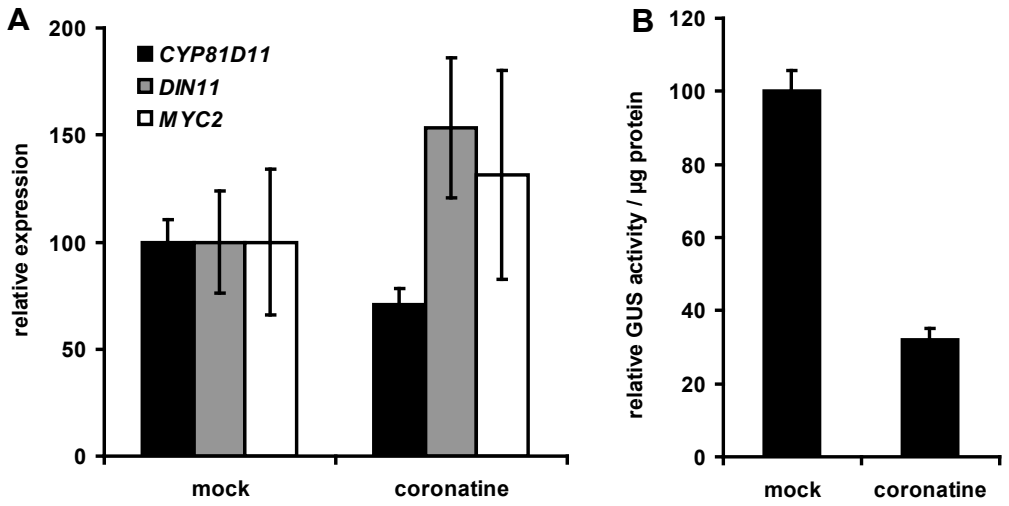

Figure 4. 23: JAZ1-GUS amounts and *CYP81D11*, *DIN11* and *MYC2* transcript levels in coronatine-treated protoplasts

Protoplasts were isolated from 4-week-old JAZ1-GUS plants, washed and incubated overnight (16 h) in buffer containing 10 µM coronatine. Mock-treated protoplasts were incubated in buffer without any additives.
(A) *CYP81D11*, *DIN11 and MYC2* transcript levels (normalized to the housekeeping gene *UBQ5*) in mock- and coronatine-treated protoplasts obtained by quantitative real-time RT-PCR. Each bar represents the average ± SEM of four samples from two independent experiments. The average transcript level of the mock-treated samples was set to 100.
(B) GUS activity of protein extracts of these protoplasts normalized to protein concentration. Each bar represents the average ± SEM of eight samples from three independent experiments. The average GUS activity of the mock-treated samples was set to 100.

The decrease in GUS activity in the JAZ1-GUS protoplasts after coronatine treatment demonstrates the functional response to coronatine. Thus the enhanced JA-Ile levels in the protoplasts are not sufficient for COI1 mediated protein degradation (Figure 4. 23A). Neither the genes *CYP81D11* and *DIN11*, which are already induced in protoplasts without any additional treatment, nor *MYC2* show increased induction after coronatine treatment.

4.11 Proteasome-dependent protein degradation is essential for *CYP81D11* and *DIN11* expression in protoplasts

The effects of inhibition of the 26S proteasome and subsequent protein stabilization were investigated by addition of the proteasome inhibitor MG132 to JAZ1-GUS protoplasts before overnight incubation. In the morning, material was harvested and *CYP81D11* and *DIN11* transcription was investigated by quantitative real-time RT-PCR. Transcript levels of both *CYP81D11* and *DIN11* are strongly decreased (*CYP81D11*: 10 %; *DIN11*: 4 %) after treatment with MG132, which indicates the

importance of proteasome-dependent protein degradation for their induction. Since the reduction of *CYP81D11* and *DIN11* expression after MG132 treatment is stronger than in the *dde2-2* mutant, it is concluded that both COI1 functions that drive the *CYP81D11* and *DIN11* genes may lead to the degradation of negative regulators.

Increased GUS activity in protein extracts obtained from these MG132-treated JAZ1-GUS protoplasts demonstrated the stabilization of JAZ proteins and thus the functional inhibition of proteasome-dependent protein degradation (Figure 4. 24).

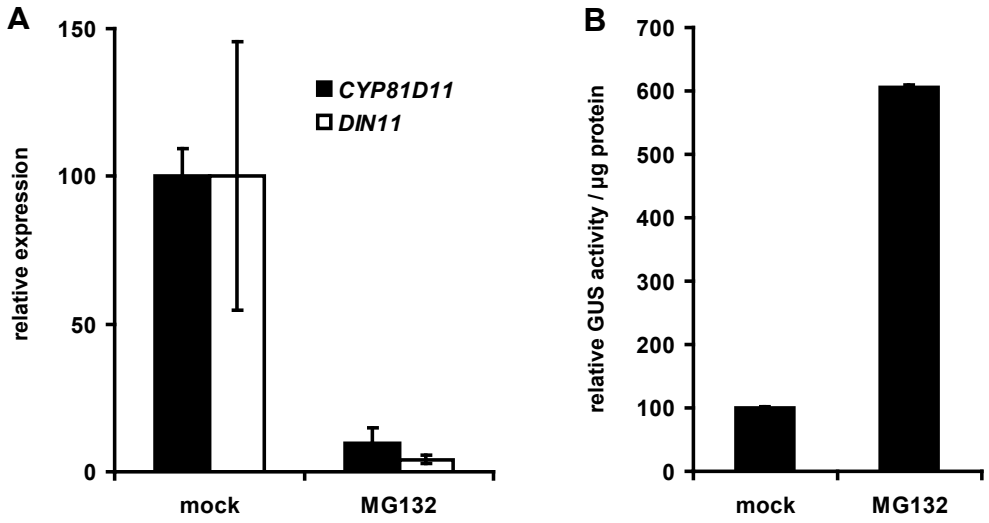

Figure 4. 24: **Transcription levels of *CYP81D11* and *DIN11* and JAZ1-GUS levels in protoplasts after treatment with proteasome inhibitor**

Transgenic JAZ1-GUS plants were grown for 4 weeks under low-light and long-day conditions. Protoplast were isolated, washed and incubated overnight (16 h) with or without 50 µM of the proteasome inhibitor MG132.
(A) Transcript levels of *CYP81D11* and *DIN11* (normalized to the housekeeping gene *UBQ5*) investigated by quantitative real-time RT-PCR. Each bar represents the average ± SEM of two samples.
(B) GUS activity of total protein extracts of the JAZ1-GUS protoplasts. GUS activity is taken as a measure for the degree of JAZ1-GUS levels. Each bar represents the average ± SEM of two samples.

The reduction of *CYP81D11* expression in protoplasts obtained from *dde2-2* and *coi1-t* mutants demonstrates that both a COI1 function in response to JA-Ile (basal or elevated levels) and the JA-Ile-independent COI1 function take place.

4.12 ATAF1 and ANAC032 repress basal expression of *CYP81D11*

Apart from *CYP81D11*, the two closely related NAC transcription factor genes *ATAF1* and *ANAC032* were found to be regulated by the TGA2,5,6/SCL14 complex. ATAF1 was shown to be involved in the response to abiotic and biotic stress, like drought and abscisic acid (ABA) treatment (Lu et al. 2007; Jensen et al. 2008; Wu et al. 2009), and upon pathogen attack (Wu et al. 2009; Wang et al. 2009; Jensen et al. 2008). Less is known about ANA032. Since it is the closest relative to ATAF1 and since it is co-regulated with ATAF1, functional redundancy has been proposed (Kleinow et al. 2009). A function in the xenobiotic stress response has not been reported for either of them. Expression of both genes is up-regulated by TIBA in a COI1-independent manner.

In order to assess whether ATAF1 and ANAC032 regulate the expression of SCL14-dependent, TIBA-inducible genes, transgenic plants ectopically expressing HA-ATAF1 or HA-ANAC032 controlled by the CMV35S promoter were generated.

Transformation of Col-0 plants was carried out by *A. tumefaciens*-mediated gene transfer and primary transformants were selected by spraying the plants with the herbicide BASTA.

Primary transformants expressing HA-ATAF1 were either indistinguishable from Col-0 wild-type plants or exhibited a strong dwarf phenotype with strongly upwards-bent leaves or short, heavily branched inflorescences, which developed only late (Figure 4. 25). All of these dwarf plants were sterile and exhibited an extended lifetime.

HA-ANAC032 primary transformants showed similar effects as the HA-ATAF1 plants. Many primary transformants selected by BASTA treatment showed the wild-type phenotype, but also here dwarfism occurred. Dwarf plants exhibited less severe upwards-bending of the leaves, but the leaves were crinkled and showed early yellowing patches. Again, the inflorescences developed late and were short and the plants exhibited extended lifetimes. The described phenotype was observed in different degrees of severity. Most of the dwarf plants were sterile, but some individuals with less severe symptoms produced seeds.

CYP81D11 expression was investigated in the pimary transformants. Two to three leaves were harvested from six plants exhibiting a wild-type phenotype (3 weeks old) and from six plants exhibiting dwarfism (6 weeks old). As the dwarf plants grew much slower, leaf material had to be harvested at different time points to obtain material from similarly sized plants.

Quantitative real-time RT-PCR was performed to determine the transcript levels of the overexpressed genes and of *CYP81D11* (Figure 4. 25).

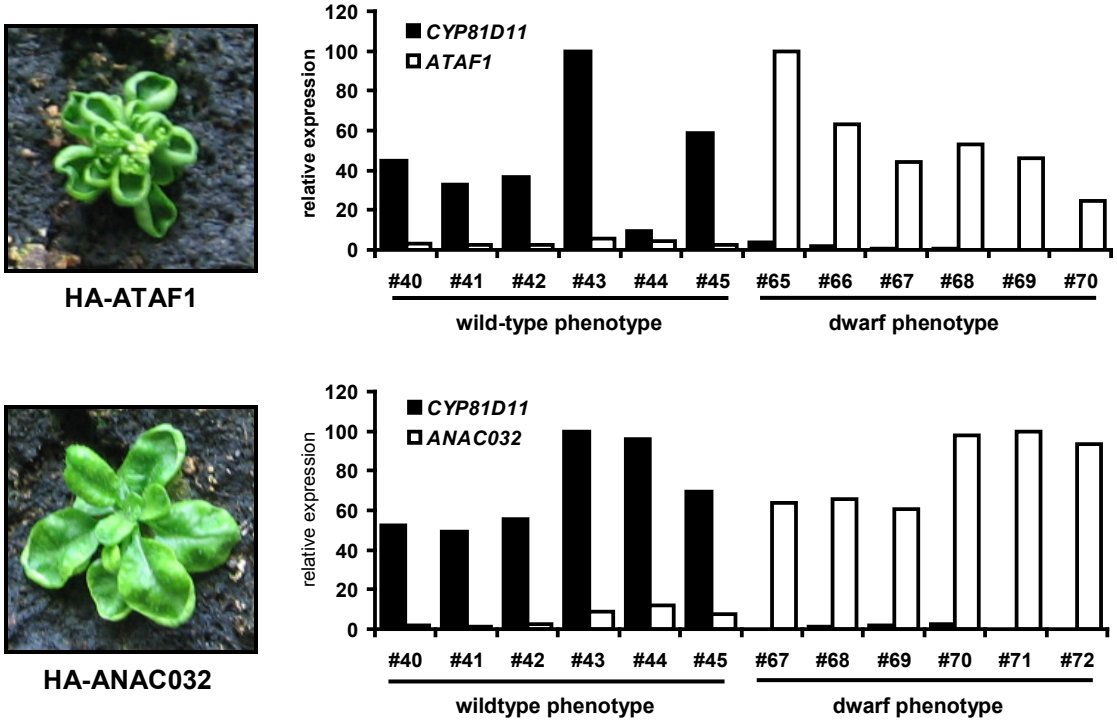

Figure 4. 25: Phenotype and transcript levels of *CYP81D11*, *ATAF1* and *ANAC032* of primary transformants ectopically expressing HA-ATAF1 or HA-ANAC032

Primary transformants exhibiting HA-ATAF1 or HA-ANAC032 were grown under long-day conditions and selected by treatment with BASTA. Plants surviving BASTA treatment showed either wild-type growth or dwarfism, curly leaves, an extended lifetime, and late flowering. RNA was isolated from six plants exhibiting the wild-type phenotype (3 weeks old) and from six plants exhibiting dwarfism (six weeks old) from each transformation and quantitative real-time RT-PCR analysis was performed using primers specific for *ATAF1*, *ANAC032* and *CYP81D11*. Transcript level values were normalized to the housekeeping gene *UBQ5*. Each bar represents the transcription level of one primary transformant. For each gene, the highest transcript level was set to 100.

Transcript levels of the corresponding overexpressed genes in HA-ATAF1 and HA-ANAC032 primary transformants strictly correlated with the phenotype. All plants exhibiting wild-type growth showed relatively low *ATAF1* or *ANAC032* expression. In contrast to this, all dwarf individuals exhibited high *ATAF1* or *ANAC032* transcript levels.

Interestingly, the *CYP81D11* expression behavior was opposite to the expression of *ATAF1* and *ANAC032*. Dwarf plants containing high levels of *ATAF1* and *ANAC032* transcript levels showed strongly repressed levels of *CYP81D11* compared to the individuals looking like the wild type. It is concluded that ATAF1 and ANAC032 act as negative regulators at least of the basal *CYP81D11* expression.

4.13 HA-ANAC032 F1 generation exhibits increased ANAC032 transcript levels and expresses the HA-ANAC032 protein

Although most of the HA-ANAC032 primary transformants were sterile, seeds from some plants showing a less severe dwarf phenotype could be obtained. The F1 generation represents a pool composed of wild-type individuals, heterozygous transformants, and homozygous transformants. The genotype of the individuals was estimated based on their phenotype. Homozygous HA-ANAC032 plants showed dwarfism and crinkling as well as early yellowing at the leaf edges and between the leaf vessels. Heterozygous individuals exhibited wild-type-like growth and were mainly identified by the crinkling and early yellowing of the leaves, which was again mainly present at the leaf edges and between the vessels (Figure 4. 26). Due to their wild-type growth and thus good comparability of heterozygous individuals to the wild type, all following results were obtained from heterozygous HA-ANAC032 plants.

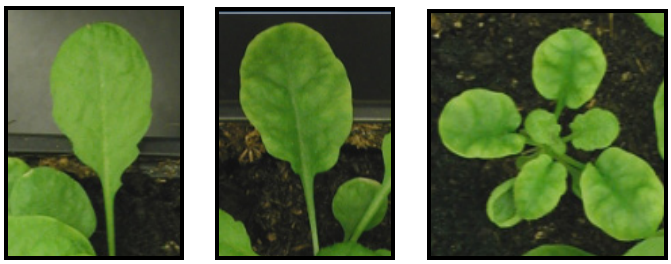

Figure 4. 26: Phenotype of HA-ANAC032-overexpressing plants

Col-0 and HA-ANAC032#68 (F1 generation) plants were grown for 6 weeks under 12-h light/12-h dark conditions. Homozygous and heterozygous pants were distinguished by the severity of dwarfism and leaf yellowing.

Untreated plants of three independent HA-ANAC032 lines (#47, #67, and #68) grown on soil for 6 weeks were investigated by quantitative real-time RT-PCR, to compare their ANAC032 expression to that of Col-0 wild-type plants.

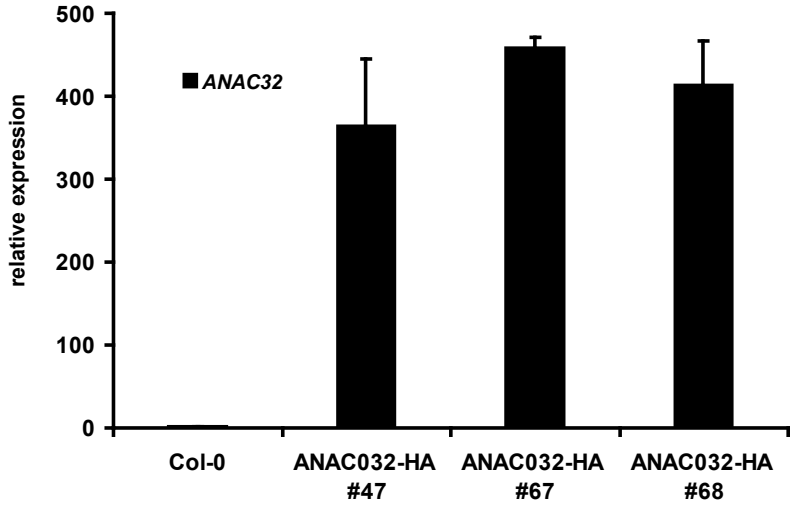

Figure 4. 27: **Transcript levels of *ANAC032* in plants overexpressing HA-ANAC032**

Quantitative real-time RT-PCR to assess *ANAC032* transcript levels in transgenic plants ectopically expressing HA-ANAC032 controlled by the CMV35S promoter. Transcript levels were normalized to the housekeeping gene *UBQ5*. Each bar represents the average ± SEM of two to four independent plants. The average transcript level of the Col-0 plants was set to 1.

All three investigated HA-ANAC032 lines exhibit similar amounts of *ANAC032* transcript, which are about 400-fold higher than in the wild-type.

In order to prove that not only the *HA-ANAC032* transcript but also the protein is produced, Western blot analyses were performed. 6–7-week old HA-ANAC032 plants of three independent lines were sprayed with TIBA and incubated for 8 h. Whole protein extracts were prepared and the proteins were separated by SDS-PAGE.

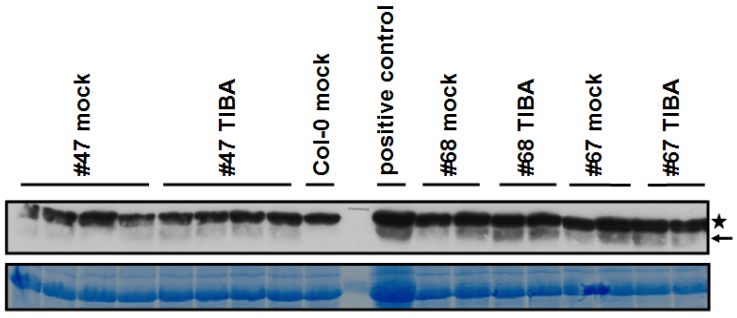

Figure 4. 28: **Western blot analyses of HA-ANAC032-overexpressing plants**

Three independent lines (#47, #67, and #68) ectopically expressing ANAC032 were treated with 100 µM TIBA for 8 h and whole protein extracts were prepared. An antibody against the HA-tag was used for Western blot analyses. The specific band is indicated by an arrow; an unspecific band is marked by an asterisk. As positive control, plant material of a sterile primary transformant with a strong dwarf phenotype was used.
The Coomassie blue-stained SDS-PAGE gel (12 % acrylamide) is shown as loading control.

Western blot analyses revealed that the HA-ANAC032 protein is produced in all the tested transgenic lines (Figure 4. 28), although protein amounts differ. Lines #67 and #68 exhibit similar protein amounts while the HA-ANAC032 signal is weaker in line

#47. Col-0 protein extracts were used as negative control; this line also showed the strong unspecific band. The specific band is detected below the unspecific one and is not contained in the Col-0 control sample. As positive control, plant material of a sterile primary ANAC032 individual was used. The strengths of the HA-ANAC032 signals differ slightly between individuals within one transgenic line, but these differences do not correlate with the treatment of the individuals. Thus, TIBA treatment does not influence the protein stability of ANAC032.

4.14 ANAC032 negatively regulates TIBA induced *CYP81D11* expression

In the primary transformants, only basal *CYP81D11* expression could be investigated. This was strongly reduced in HA-ANAC032-overexpressing plants (Figure 4. 25). Next, *CYP81D11* expression was investigated under inducing conditions. Heterozygous HA-ANAC032 plants from independent lines were grown on soil for 6–7 weeks, sprayed with TIBA and incubated for 8 h. Quantitative real-time RT-PCR revealed reduced transcript levels of *CYP81D11* in the HA-ANAC032 lines #47, #67, and #68 (Figure 4. 29).

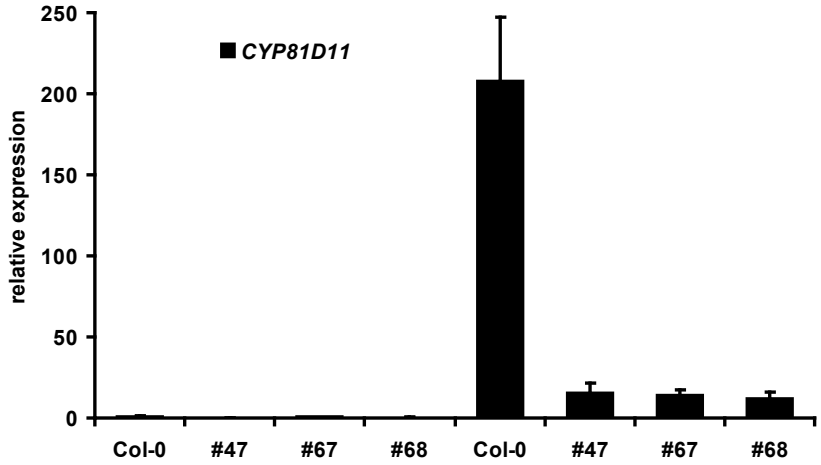

Figure 4. 29: *CYP81D11* transcript levels in HA-ANAC032-overexpressing plants in response to TIBA

Quantitative real-time RT-PCR analysis of *CYP81D11* transcript levels in transgenic plants ectopically expressing HA-ANAC032 after treatment with 100 µM TIBA. 8 h after induction, whole rosettes were harvested for RNA isolation. The numbers (#47, #67, and #68) symbolize independent transgenic lines; only heterozygous individuals were analyzed in this experiment. Each bar, except that of #67 mock, represents the average ± SEM of two to six independent plants; for line #67, only one mock-treated plant was investigated. The average transcript level of the mock-treated Col-0 plants was set to 1. The transcript levels were normalized to the housekeeping gene *UBQ5*.

CYP81D11 transcript levels were strongly reduced in untreated and TIBA-treated HA-ANAC032-overexpressing plants. It is concluded that ANAC032 negatively regulates *CYP81D11* expression in untreated plants as well as in response to xenobiotic stress.

The regulation of *CYP81D11* differs from that of other SCL14-dependent TIBA-inducible genes with respect to its COI1 dependency. Thus, *GSTU1*, *GSTU7* and *ATAF1* expression, but also the expression of the COI1-dependent and SCL14-independent gene *DIN11*, were investigated by quantitative real-time RT-PCR in TIBA-treated HA-ANAC032 plants. cDNA from the same experiment as shown in Figure 4. 29 was used (Figure 4. 30).

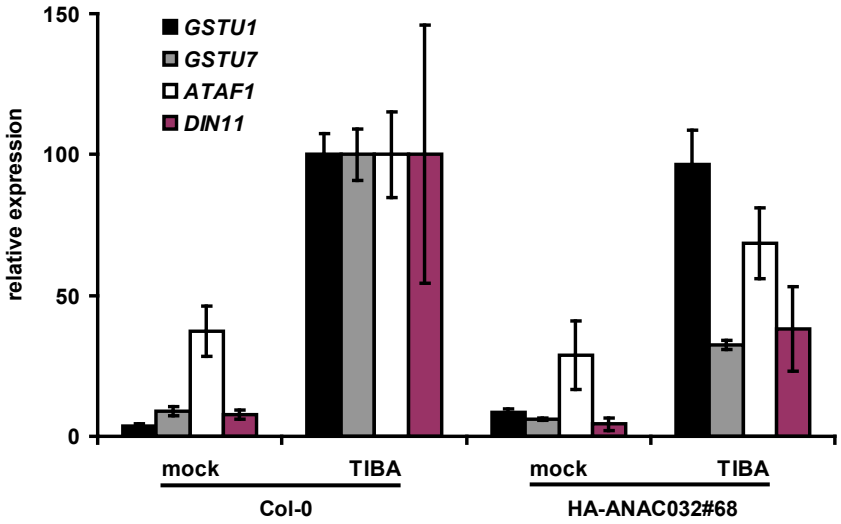

Figure 4. 30: **Transcript levels of *GSTU1, GSTU7*, *ATAF1* and *DIN11* in HA-ANAC032-overexpressing plants in response to TIBA**

Quantitative real-time RT-PCR analysis of *GSTU1, GSTU7*, *ATAF1* and *DIN11* transcript levels in transgenic plants ectopically expressing HA-ANAC032 after treatment with 100 µM TIBA. 8 h after induction, whole rosettes were harvested for RNA isolation. Transcript levels were normalized to the housekeeping gene *UBQ5*. Each bar represents the average ± SEM of two to four independent plants. The average transcript level of the TIBA-treated Col-0 plants was set to 100.

While *GSTU1* transcription was not influenced in ANAC032#68 plants, neither in mock-treated nor in TIBA-treated plants, *GSTU7* transcript levels were slightly reduced in mock-treated ANAC032#68 plants and reduced to about 40 % after TIBA treatment. *ATAF1* transcript levels varied only slightly between HA-ANA032 overexpressing and wild-type plants. These findings indicate that ANAC032-dependent regulation does not influence all SCL14 target genes. The COI1-dependent but SCL14-independent gene *DIN11* also shows a reduction in transcript levels to about 40 % in the HA-ANAC032-overexpressing plants. Still, in comparison to *CYP81D11*, this effect is relatively weak,

demonstrating that also among COI1-dependent TIBA-inducible genes the repression by ANAC032 is not homogeneous.

4.15 ANAC032 strongly represses *CYP81D11*, *VSP2* and *PDF1.2* transcript levels in response to MeJA

As *CYP81D11* is not only activated by xenobiotic stress but also by MeJA (Mueller et al. 2008) (Figure 4. 1), the impact of ANAC032 overexpression on MeJA-mediated *CYP81D11* expression was investigated. In addition, expression of the JA marker gene *VSP2* and the JA/ET-induced gene *PDF1.2*, as well as *DIN11*, was monitored. The HA-ANAC032-overexpressing lines #47 and #67 were grown for 6–7 weeks on soil and treated with gaseous MeJA for 24 h; subsequently, quantitative real-time RT-PCR was performed.

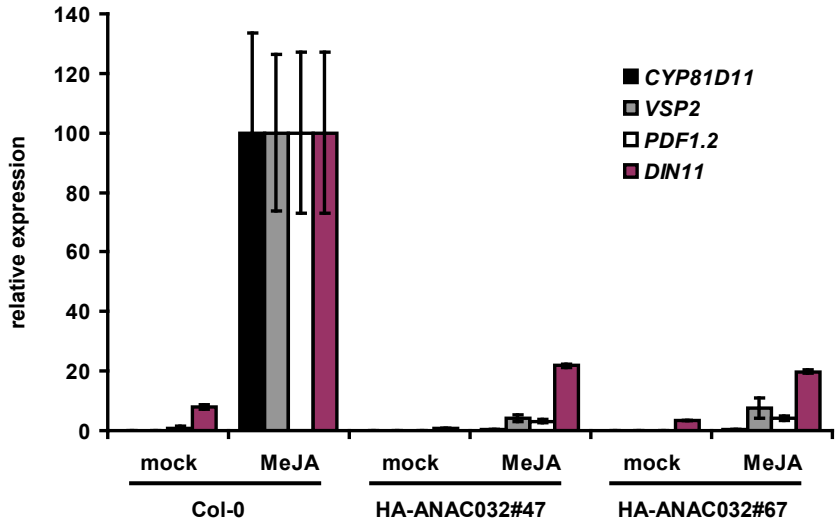

Figure 4. 31: **Expression analysis of *CYP81D11*, *VSP2* and *PDF1.2* after MeJA treatment in plants overexpressing HA-ANAC032**

Quantitative real-time RT-PCR analysis of *CYP81D11*, *VSP2*, *PDF1.2* and *DIN11* transcript levels (normalized to the housekeeping gene *UBQ5*) in Col-0 wild-type plants and plants ectopically expressing HA-ANAC032 controlled by the CMV35S promoter. 6–7-week-old soil-grown plants were treated with gaseous MeJA (1 µL/L air) for 24 h. During MeJA treatment, the plants were kept in a gas-proof tank; liquid MeJA was applied to Whatman paper that was attached to the inner glass surface, and evaporated into the gas phase. Whole rosettes were harvested for RNA isolation. Transcript values of MeJA-treated Col-0 plants were set to 100. Each bar represents the average ± SEM of two to four biological replicates. In case of the *VSP2* gene, no transcript levels could be detected in mock-treated plants.

CYP81D11 expression in response to MeJA was most strongly reduced (<0.4 %) in HA-ANAC032 plants compared to the wild type. The expression of *VSP2* and *PDF1.2* was also strongly reduced by ANAC032 overexpression, although the effect was not as

striking (VSP: >8 %; PDF: >5 %) as for *CYP81D11*. *DIN11* expression in response to MeJA was less severely affected in the ANAC032-overexpressing plants. Transcript levels remained at about 20 % of the wild-type levels. The transcript levels in mock-treated samples were reduced for all of the genes (*VSP2* could not be detected), although this occurred to different extents. These results lead to the conclusion that JA-dependent gene expression might in general be reduced by ANAC032.

4.16 *ataf1*/*anac032* double-knockout mutants do not affect *CYP81D11* expression

Overexpression of ATAF1 and ANAC032 strongly represses *CYP81D11* expression. Therefore, the question should be answered whether knockout mutants of ATAF1 and ANAC032 exhibit increased *CYP81D11* expression, as it would be expected in the absence of a negative regulator. For ATAF1, T-DNA insertion lines, which do not contain the intact ATAF1 mRNA, have already been published: *ataf1-1* and *ataf1-2* (Lu et al. 2007). Both lines contain a T-DNA insertion in the third exon. For ANAC032, T-DNA insertion lines obtained from the Salk Institute were used: *salk_012253*, which contains a T-DNA insertion in the first exon, and *salk_132588*, which is interrupted within exon 3 (Figure 4. 32).

To ensure that no intact mRNA is produced in any of the used T-DNA insertion mutants, homozygous individuals were treated with TIBA to induce ATAF1 and ANAC032 expression, RNA was extracted for cDNA preparation, and PCR analyses were performed using primers specific for the ATAF1 and ANAC032 transcripts (Figure 4. 32C).

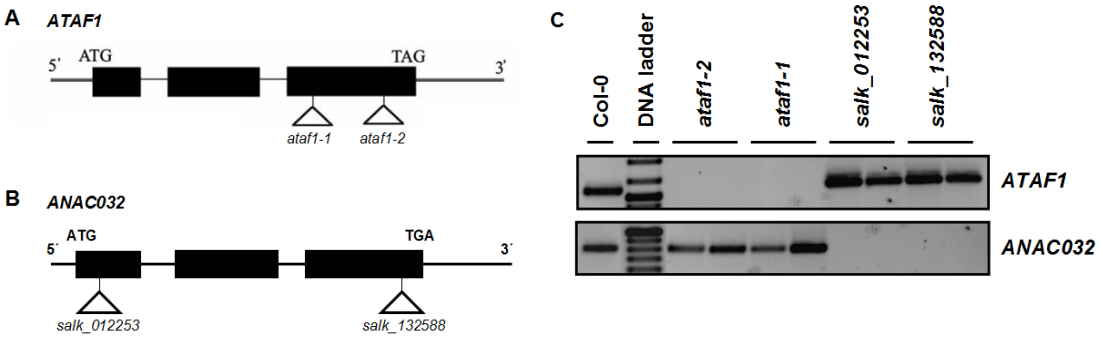

Figure 4. 32: Localization of T-DNA insertions in ATAF1 and *ANAC032*

(A, B): Schematic illustration of the *ATAF1* and *NAC032* genes. Black boxes indicate exons while UTRs and introns are depicted by thin lines. Triangles demonstrate the positions of T-DNA insertions; the designations of the mutants are indicated. (A) The *ATAF1* gene and the T-DNA positions in the mutants *ATAF1-1* and *ATAF1-2* (Lu et al. 2007). (B) The *ANAC032* gene and the T-DNA positions in the mutant lines *salk_012253* and *salk_132588.*
(C) PCR was performed with *ATAF1*- and *ANAC032*-specific primers using cDNA obtained from 6–7-week-old TIBA-treated Col-0, *ataf1-1*, *ataf1-2*, *salk_012253*, and *salk_132588* plants. Two independent individuals per mutant line were tested. The PCR products were analyzed by electrophoresis and stained by ethidium bromide. The fat band of the DNA ladder exhibits a size of 1 kb.

Figure 4. 32C demonstrates that the T-DNA insertion lines contain insertions in the *ATAF1* and *ANAC032* genes. These T-DNA insertion lines indeed do not produce the corresponding transcripts and thus are real knockout lines.

Since functional homology between ATAF1 and ANAC032 is assumed, double mutants expressing neither ATAF1 nor ANAC032 were generated by crossing the above-described T-DNA insertion lines. The *ataf1-1*/*salk_132588 and ataf1-2/salk_012253* double mutants were grown on soil for 6–7 weeks and treated which TIBA for 8 h, or with MeJA for 24 h. *CYP81D11* transcript levels were monitored by quantitative real-time RT-PCR (Figure 4. 33).

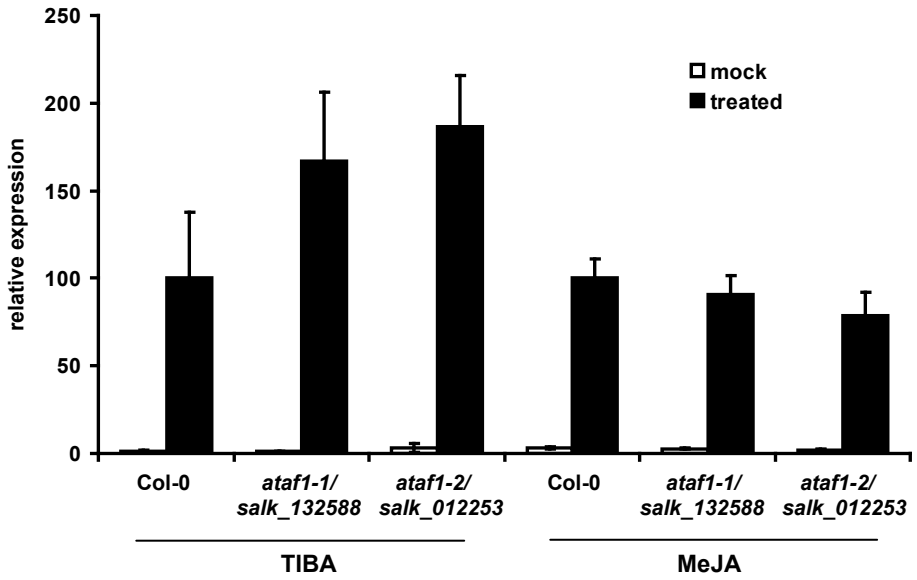

Figure 4. 33: **Expression of *CYP81D11* in *ataf1/anac032* mutants in response to TIBA and MeJA**
Quantitative real-time RT-PCR analysis of *CYP81D11* transcript levels (normalized to the housekeeping gene *UBQ5*) in Col-0 wild-type and *ataf1/anac032* mutant plants (*ataf1-1*/*salk_132588* and *ataf1-2/salk_012253*). 6–7-week-old soil-grown plants were either sprayed with 100 µm TIBA for 8 h or treated with gaseous MeJA (1 µL/L air) for 24 h. Whole rosettes were harvested for RNA isolation and cDNA synthesis. Transcript values in treated Col-0 plants were set to 100. Each bar represents the average ± SEM of three or four biological replicates.

The *ataf1/anac032* double mutants show no altered *CYP81D11* transcript levels, neither in response to TIBA nor to MeJA. This indicates that further functional redundancy among ATAF1, ANAC032 and other NAC transcription factors might exist. The most likely candidates that might have overlapping functions are the two other ATAF family members, ATAF2 and ANAC102.

5 Discussion

CYP81D11 belongs to the most strongly induced genes in response to different chemical stimuli. Moreover, two kinds of regulatory proteins, namely the TGA class II transcription factors and the GRAS protein SCL14, are already known to regulate this gene, establishing it as a marker gene for the response to xenobiotics. In contrast to other TGA/SCL14 target genes, *CYP81D11* is activated by JA in a COI1-dependent manner. Interestingly, COI1 is also required for the induction by the xenobiotics TIBA and BOA. This finding pompted us to investigate the role of COI1 in response to xenobiotics.

5.1 TIBA as an inducer of xenobiotic stress

TIBA was chosen for the experiments as it is listed as a strong inducer of *CYP81D11*. Since it also functions as an auxin transport inhibitor, we tested whether it can be replaced by other chemicals. *Cis*-jasmone (CJ) induces *CYP81D11* expression very strongly, but this induction was reported to be independent of the COI1 protein (Bruce et al. 2008). This was also the case for the herbicide safener benoxacor (Figure 6. 1). Phytoprostanes, which are also very strong and COI1-dependent inducers of *CYP81D11* (Mueller et al. 2008), are not commercially available.

When the allelochemical BOA (Baerson et al. 2005) was tested, it was shown that in the *coi1-t* mutant *CYP81D11* expression was more severely reduced than in the *dde2-2* mutant (Figure 4. **8**). These results are consistent with the effects observed after TIBA treatment, although the chemical structures of TIBA and BOA are not related. In comparison to TIBA, the induction of *CYP81D11* achieved by BOA treatment was rather weak; thus, our experiments were continued with TIBA.

It has previously been shown that a subset of JA-inducible genes is additionally induced by auxins. To further rule out that TIBA-induced activation is due to its potentially functional auxin-like structure, we tested several auxin-related conditions for their ability to influence *CYP81D11* expression (Thimann & Bonner 1948). In contrast to TIBA, the native auxin indole-3-acetic acid (IAA) showed no effect on *CYP81D11* expression (Figure 6. 3). Jasplakinolide, an actin stabilizer which leads to similar effects

like TIBA regarding growth and auxin transport (Dhonukshe et al. 2008), was also not efficient in inducing *CYP81D11* expression (Figure 6. 2). Transgenic plants overexpressing the dominant-negative AXR3 protein, a repressor of the auxin response, under the control of a heat shock promoter (Ouellet et al. 2001; Knox et al. 2003) were treated with TIBA. Expression of AXR3-1 had no effect on the TIBA-induced *CYP81D11* expression (Figure 6. 4). Also the tissue-specific expression patterns of *DR5:GUS*, a commonly used synthetic auxin marker (Ulmasov et al. 1997), and *CYP81D11:GUS* in transgenic plants were investigated. No similar expression in root tips, either untreated or treated with TIBA, was observed (Figure 6. 5). These observations led to the conclusion that TIBA does not interact with the auxin signaling pathway to induce *CYP81D11* expression. We rather assume that its electrophilic properties, exhibited by its halogen-substituted aromatic ring, lead to the activation of the detoxification program. This assumption is supported by the fact that TIBA treatment induces a large number of xenobiotic-responsive genes (including *CYP81D11*), which were also found to be expressed under a variety of oxidative stress conditions (Fode 2008). Thus, in this thesis, TIBA was used as an inducer of xenobiotic stress.

5.2 Basal JA levels and JA signalling are required for full induction of *CYP81D11* in response to TIBA

TIBA treatment does not lead to the accumulation of JA-Ile. Nevertheless, JA-Ile-deficient mutants (*dde2-2* and *jar1-1*) show a notable reduction in *CYP81D11* expression in response to TIBA (Figure 4. 9). Thus, it is concluded that the basal JA-Ile levels are important for maximal *CYP81D11* expression.

Another JA-deficient mutant often used to investigate JA-responsive processes is the *fad3,7,8* mutant, which is impaired in the production of the JA precursor linolenic acid (McConn & Browse 1996b; Nibbe et al. 2002; Cipollini et al. 2004; Jakob et al. 2007). Surprisingly, in response to TIBA, this mutant behaved like the wild type with respect to the expression of *CYP81D11* (Figure 6. 6). This finding contradicts the results obtained from the JA biosynthesis mutants *dde2-2* and *jar1-1*. Furthermore, no changes in *CYP81D11* transcript levels were observed in uninduced plants. As the expression of classical JA-responsive genes like *VSP* was impaired in the *fad3,7,8* plants (McConn et al. 1997b), it was concluded that indeed no JA-dependent gene regulation takes place.

FAD3,7,8 code for fatty acid desaturases, catalyzing the desaturation of dienoic fatty acids (16:2, 18:2) to trienoic fatty acids (16:3, 18:3) including linolenic acid. In the *fad3,7,8* triple mutant it has been shown that dienoic fatty acids are accumulated (McConn & Browse 1996b). A possible explanation may be a compensation of the reduced *CYP81D11* expression due to the lack of JA. This might occur if the accumulation of dienoic fatty acids is somehow capable of inducing *CYP81D11*. Under oxidative-stress conditions, unsaturated fatty acids are common targets for oxidation, and it was indeed shown that trienoic fatty acids are an important sink for reactive oxygen species. Thus, the *fad3,7,8* mutant accumulates H_2O_2 compared to the *dde2-2* mutant (Mène-Saffrané et al. 2009). As *CYP81D11* is inducible by H_2O_2 (Fode 2008), this might indeed mask the reduced *CYP81D11* expression that is expected due to the lack of JA.

In transgenic JAZΔ3A plants and in the *jin1-1* mutant (Figure 4. 10), the same reduction of *CYP81D11* expression was observed as in *dde2-2* and *jar1-1* (Figure 4. 9). This indicates that JAZ degradation and the MYC2 transcription factor are required for the TIBA-activated *CYP81D11* expression depending on basal JA-Ile levels.

In previous studies, JAZ degradation was only observed in response to accumulated JA levels. Nevertheless, since TIBA-induced plants contain basal levels of JA-Ile, it can be presumed that a constant turnover of JAZ proteins takes pace. Thus, an imaginable scenario may be that JAZ proteins constantly release MYC2 before being replaced, thus providing access for interactors mediating transcription. This co-activation might be an important step for the activation of JA-regulated genes by stimuli that do not lead to increased JA-levels, as for example the expression of *PDF1.2* in response to ET (Zander et al. 2010).

An alternative explanation could be that the ubiquitination of JAZ proteins induced by JA-Ile not only leads to the degradation of JAZ proteins but that ubiquitinated JAZ proteins act as transcriptional activators. In yeast, it has been reported that the VP16 transcription factor is activated by ubiquitination prior to subsequent degradation. Furthermore, the direct fusion of VP16 to ubiquitin led to transcriptional activation independent of protein degradation. Thus, it was demonstrated that ubiquitination can generate active but short-lived transcriptional activators (Salghetti et al. 2001). Assuming a comparable mechanism for JAZ proteins, basal JA-Ile levels would lead to a constant production of transcriptional activators that function as constitutive co-activators.

Microarray analysis revealed that the expression of 73 TIBA-inducible genes requires the COI1 protein (Figure 4. 13). Furthermore, none of the seven more closely investigated genes depends on a JA-Ile-independent COI1 function (Figure 4. 14). It is concluded that at least the majority of these genes require basal JA-Ile levels for TIBA-induced expression.

However, in contrast to *CYP81D11*, *DIN11* expression is not compromised in the *jin1-1* mutant (Figure 6. 7). This indicates that, although the induction of all of these genes needs basal JA-Ile levels, different mechanisms of gene activation may exist. Other transcription factors (possible homologs of MYC2) acting downstream of COI1 have been postulated before (Berger et al. 1996; Lorenzo et al. 2004). Thus, processes comparable to those speculated for the *CYP81D11* promoter may activate *DIN11* expression, although another transcriptional activator would be involved.

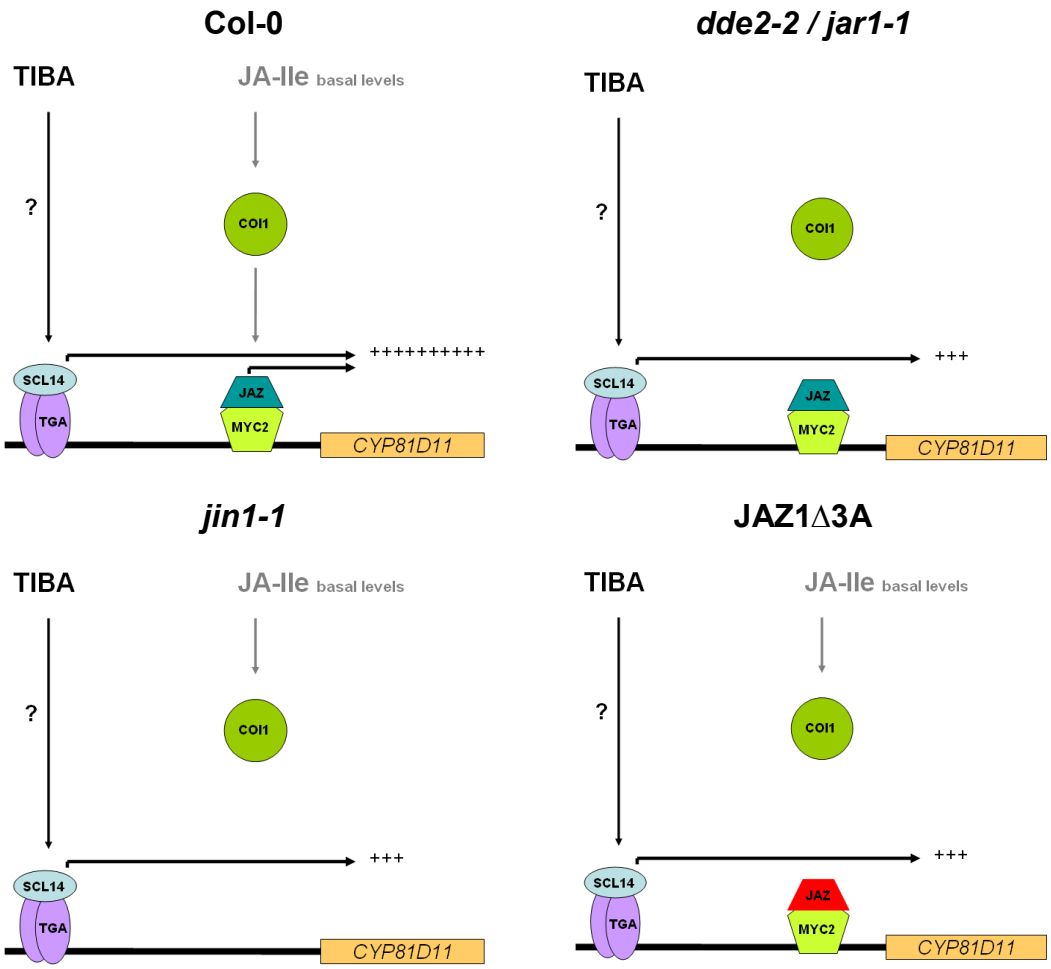

Figure 5. 1: Scheme of the *CYP81D11* regulation on different genetic backgrounds

The scheme shows the *CYP81D11* promoter in the wild type, the *dde2-2, jar1-1* or *jin1-1* mutant, and in transgenic JAZ1Δ3A plants. The number of + signs indicates the strength of induction.

5.3 *CYP81D11* is regulated by a JA-Ile-independent COI1 function

The common function so far associated with the COI1 protein is the degradation of JAZ proteins in response to accumulated JA-Ile levels. An additional function of COI1 as a regulator of a large group of JA-inducible genes requiring basal JA-Ile levels was discussed above. In response to TIBA, BOA, and protoplasting, a JA-Ile-independent function was shown for the regulation of *CYP81D11* (Figure 4. 8, Figure 4. 20). Recently, a JA-Ile-independent function of the COI1 protein has been reported for ET-induced inhibition of root growth (Adams & Turner 2010). While wild-type plants as well as JA synthesis and signaling mutants (*dde2*, *opr3* (*12-oxophytodienoic acid reductase*), *jar1* and *jin1*) exhibited strong root growth inhibition on ET (ACC)-supplemented media, this effect was reduced in the *coi1-16* mutant. The effects of *coi1-16* and ET-insensitive mutants on reducing root growth inhibition were additive. Thus, it was concluded that COI1 acts by a pathway independent of the known ET signal transduction.

Less severe inhibition of root growth was also observed in *coi1-16* mutants complemented with COI1 proteins with a mutated LRR domain, that contains the binding site for JA-Ile. In contrast to this, normal root growth inhibition occurred in *coi1-16* mutants complemented with a COI1 protein that was unable to assemble the SCF complex. These results indicate a COI1 function that requires its ligand binding site but not the SCF complex. An interaction of COI1 with ethylene-related components to regulate root growth without forming an SCF complex was postulated.

In this thesis, a more severe reduction of *CYP81D11* expression in response to TIBA, the allelochemical BOA (Figure 4. 8), and protoplasting (Figure 4. **20**) was observed for the *coi1-t* mutant compared to the JA-Ile synthesis mutants. In case of protoplasts, but not after TIBA treatment, this effect was also observed for *DIN11*. It is concluded that JA-Ile only contributes to part of the COI1 function in regulating *CYP81D11* expression, and that another part is independent of JA-Ile. Furthermore, this COI1 function is independent of known downstream signaling events, such as degradation of JAZ proteins and transcriptional activation via MYC2 (Figure 4. 10).

For the JA-Ile-independent COI1 function in response to ET, Adams and Turner (2010) speculated about the binding of ethylene-related components to the ligand binding site of the COI1 protein. To determine if in protoplasts COI1 exhibits a function independent of substrate binding (to this binding site), complementation of *coi1*

protoplasts with a COI1 protein mutated in the ligand binding site was performed (Figure 6. 10). Unfortunately, contradictory results were obtained in these experiments. The first attempt showed complementation of *CYP81D11* and *DIN11* expression by the wild-type COI1 and by a COI1 protein exhibiting alterations in the JA-Ile binding site. In contrast to this, repetition of this experiment showed no complementation by the mutated COI1. In a third experiment, *CYP81D11* but not *DIN11* expression was increased by the altered protein. Thus, no conclusion could be drawn regarding the requirement of the JA-Ile binding site and regarding a putative new COI1 ligand produced in protoplasts and in response to TIBA and BOA. Due to their different structures, and due to the fact that the JA-Ile-independent COI1 function was also observed in protoplasts without any chemical treatment, direct binding of TIBA or BOA to COI1 seems unlikely. To obtain further information about the kind of JA-Ile-independent function exhibited by COI1 in this case, transgenic plants carrying COI1 proteins will be used that are either unable to assemble the SCF complex or are altered within the LRR domain.

Inhibition of the proteasome-dependent protein degradation reduces the expression of *CYP81D11* in protoplasts (Figure 4. 24). The decrease caused by MG132 in wild-type plants is more severe than the reduction of *CYP81D11* expression in the *dde2-2* mutant (Figure 4. 20). This demonstrates that proteasomal degradation independent of JA-Ile-dependent JAZ degradation plays a role in *CYP81D11* activation. In this context, it may be possible to speculate about protein degradation in response to the JA-Ile-independent COI1 function. Still, as proteasome-dependent degradation is involved in a variety of regulatory pathways, COI1-independent processes have to be considered. Thus, no final conclusion about protein degradation in response to the JA-Ile-independent COI1 function can be made so far.

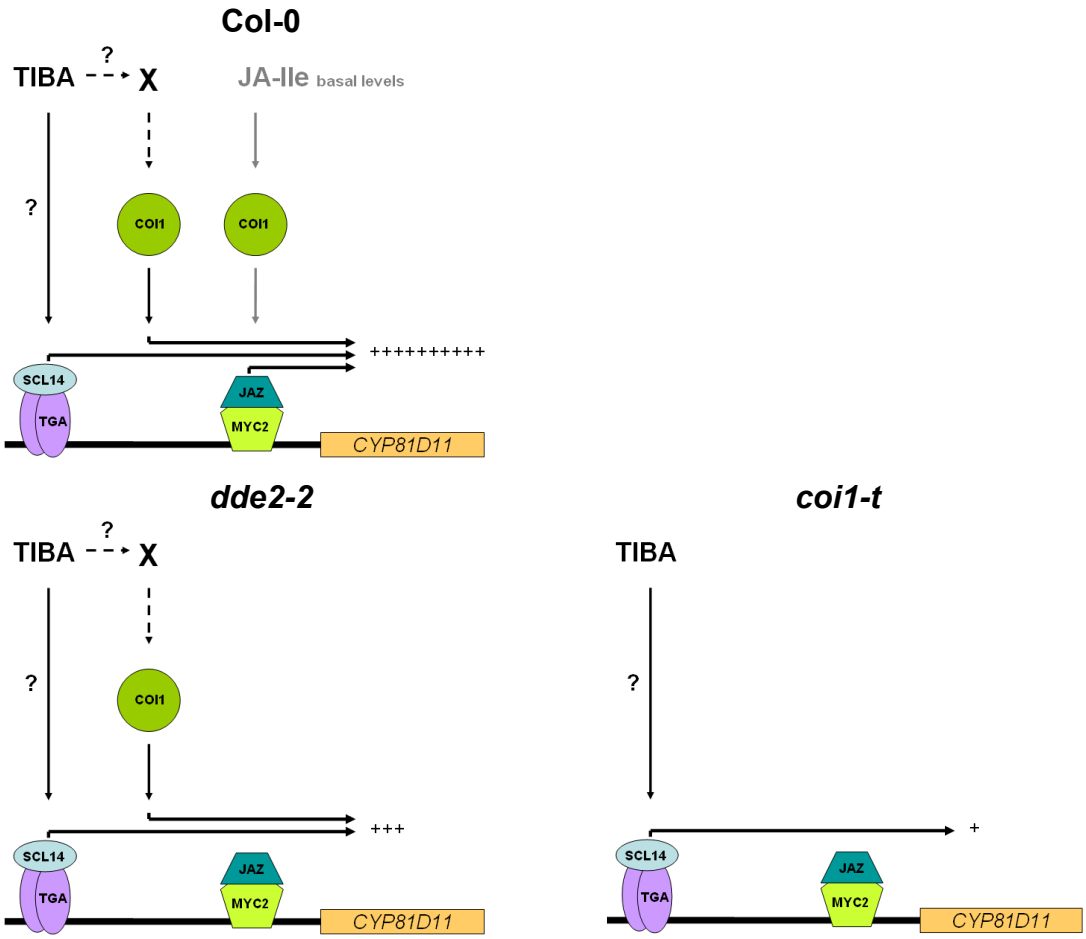

Figure 5. 2: Scheme of the *CYP81D11* regulation on different genetic backgrounds

The scheme shows the *CYP81D11* promoter in the wild-type, the *dde2-2* and *coi1-t* mutant. The number of + indicates the strength of the induction.

5.4 Function of the G-box for *CYP81D11* expression

Transgenic plants containing the *CYP81D11* promoter mG-box reporter construct showed no increased reporter gene expression in response to MeJA treatment (Figure 4. 11), demonstrating that the G-box is essential for activation of the *CYP81D11* promoter by JA.

In *coi1-t* protoplasts, the reporter gene activity of this MeJA-insensitive mG-box construct is further reduced in comparison to wild-type protoplasts (Figure 4. 12). It is concluded that the JA-Ile-independent COI1 function is mediated by another promoter element. This is consistent with the independency of this function from MYC2 and JAZ protein degradation in intact TIBA-treated plants (Figure 4. 10). The possibility that the *as-1*-like element is targeted by COI1 was excluded as the activity of the constructs

containing the mutated *as-1*-like element (m*as-1* and the m*as-1*/mG-box) is still strongly reduced in the *coi1-t* mutant (Figure 4. 12).

Also in *dde2-2* protoplasts, a reduced activity of the mG-box construct was observed (Figure 6. 8). This reduction was less severe than in the *coi1-t* protoplasts. Although these results exhibited higher variation than those obtained for *coi1-t* protoplasts, it was concluded that not only the JA-Ile-independent COI1 function acts via a promoter element independent of the double G-box, but also basal JA levels act partially via additional elements or possibly in an indirect manner.

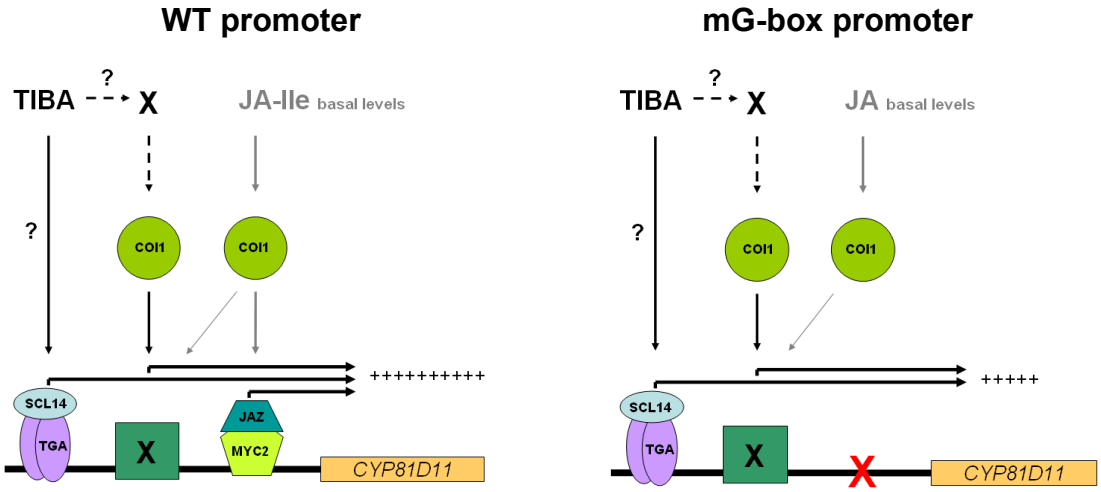

Figure 5. 3: Scheme of the *CYP81D11* wild-type and mG-box promoter regulation

The scheme shows the *CYP81D11* WT and mG-box promoter constructs in the wild type. The number of + signs indicates the strength of induction.

5.5 Expression of JA-responsive genes in protoplasts

In this study, high JA-Ile levels in protoplasts were demonstrated (Figure 4. 22). Still, compared to intact leaves, reduced expression of several well-investigated JA-responsive genes was shown (Figure 4. 21). In contrast to this, *CYP81D11* and *DIN11* were strongly induced in protoplasts compared to intact leaves (Figure 4. 20, Figure 4. 21), but neither *CYP81D11* and *DIN11* nor *MYC2* were induced by additional coronatine treatment (Figure 4. **23**). It is concluded that the activation of JA-inducible genes is not functional in protoplasts.

The JAZ proteins, which are degraded by the 26S proteasome in a SCF^{COI1}-dependent manner, are known repressors of JA signal transduction (Thines et al. 2007; Chini et al. 2007). Stabilization of JAZ proteins might either occur due to inhibition of their

degrading pathway or by expression of a dominant-negative JAZ protein lacking the Jas domain. These kinds of proteins have been shown to exist not only as truncated mutant versions but also endogenously as splice variants (Chung & Howe 2009). Nevertheless, a complete inhibition of JAZ degradation in protoplasts can be excluded, as coronatine treatment led to JAZ1-GUS degradation (Figure 4. 23) while the inhibition of the 26S proteasome by the proteasome inhibitor MG132 caused the accumulation of JAZ1-GUS (Figure 4. 24).

Although the coronatine treatment did not lead to increased *CYP81D11* and *DIN11* expression (Figure 4. 23), inhibition of the proteasome-dependent protein degradation reduced the expression of these genes in protoplasts (Figure 4. 24). This indicates that protein degradation is involved in *CYP81D11* and *DIN11* expression in protoplasts. The reduced levels of *CYP81D11* and *DIN11* expression in *dde2-2* protoplast compared with the wild type indicate that JA-Ile contributes to the activation of these genes in response to protoplasting (Figure 4. 20). Still, the reduced expression of JA marker genes demonstrates that activation of *CYP81D11* and *DIN11* occurs in an alternative manner. Furthermore, the more severe reduction in the *coi1-t* mutant compared with the *dde2-2* mutant shows the contribution of a JA-Ile-independent COI1 function.

5.6 A large group of genes is induced by TIBA in a COI1- and JA-Ile-dependent manner

Microarray analyses revealed that the expression of 73 TIBA-inducible genes depends on COI1 (Figure 4. 13). Furthermore, as none of the seven further investigated genes requires a JA-Ile-independent COI1 function, it is concluded that at least the majority of these genes require basal JA-Ile levels for TIBA-induced expression. Functional categorization of these genes revealed that a large part of them is involved in the JA-dependent stress response (Figure 4. 19), in many cases either as component of the JA synthesis cascade or as JA signaling component (Table 6. 5). Especially the up-regulation of the JA synthesis genes was rather unexpected as JA-Ile did not accumulate. Consistently, not all genes needed for JA biosynthesis are up-regulated.

Consistent with the high number of JA-responsive stress-inducible genes, analyses using the Genevestigator database revealed that nearly all of the COI1-dependent genes identified in response to TIBA are inducible by JA. Further conditions that are not

connected to JA accumulation but induced at least the majority of these genes were not identified.

In addition to TIBA, *DIN11* is up-regulated in response to extended darkness. Under these conditions, JA-Ile levels remain very low; nevertheless, also this induction depends on COI1 (Figure 6. 9). It is therefore concluded that basal JA-Ile levels contribute to full induction of COI1-dependent genes in response to different stimuli.

In contrast to *CYP81D11*, the other TIBA-inducible genes regulated in a COI1-dependent manner are not listed among the BOA-induced or phytoprostane-induced genes related to detoxification (Baerson et al. 2005; Mueller et al. 2008), with the exception of only one gene, *at3g04000* (coding for a short-chain dehydrogenase/reductase (SDR) family protein), which was listed among the detoxification-related BOA-responsive genes. In contrast to this, 30 % of the COI1-independent TIBA-inducible genes were included in at least one of those groups. Thus, it was concluded that the COI1-dependent TIBA-inducible genes are not part of the common, broad-specificity detoxification response postulated for phytoprostanes, BOA and TIBA (Baerson et al. 2005; Mueller et al. 2008; Fode 2008).

TIBA is an auxin transport inhibitor and potentially a functional auxin (Thimann & Bonner 1948; Dhonukshe et al. 2008); thus, induction of this group of genes due to an auxin-related function of TIBA is possible. As mentioned before, it was shown that a subset of JA-inducible genes (*LOX2*, *AtVSP* and *AOS*) is induced by auxin. Nevertheless, none of these genes is among the COI1-dependent, TIBA-induced genes found in this array.

Furthermore, the Genevestigator data indicate no induction of the most COI1-dependent genes in response to either the auxins IAA (indole-3-acetic acid) and NAA (1-naphthaleneacetic acid) or the auxin transport inhibitor NPA (*N*-1-naphthylphthalamic acid). MAPMAN analyses revealed four genes involved in JA metabolism, with only one taking part in the biosynthesis of auxin (and one in SA metabolism). Therefore, it seems unlikely that the activity of TIBA as putatively active auxin or as auxin transport inhibitor is the critical factor for the induction of these genes.

5.7 *CYP81D11* is co-regulated with COI1-independent genes

Cluster analyses were used to identify COI1-dependent TIBA-inducible genes co-regulated with *CYP81D11*. No COI1-dependent TIBA-inducible genes were identified that were co-regulated with *CYP81D11* in response to diverse stimuli (Figure 4. 15). In contrast to this, when *CYP81D11* expression was compared to the COI1-independent TIBA-inducible genes, it was grouped with three other genes (Figure 4. 16). Two of them, a gene coding for a UDP-glycosyltransferase (*UGT73C6*) and a gene coding for a glutathione *S*-transferase (*GSTU1*), are putatively involved in detoxification. This is consistent with the fact that *CYP81D11* is strongly induced by different xenobiotics. Furthermore, *GSTU1* has been identified as a TIBA-inducible SCL14-dependent gene (Fode 2008). The other two genes most closely co-regulated with *CYP81D11* have not been shown to depend on SCL14.

The co-regulation of *CYP81D11* with other TGA2,5,6/SCL14 target genes was supported by a time course experiment after TIBA treatment (Figure 4. 17). While the COI1-dependent TIBA-inducible genes *JAZ10* and *DIN11* reached their maximal expression at 8 h after TIBA treatment, the highest transcription levels (among the investigated time points) of *CYP81D11*, *GSTU1*, *GSTU7* and *ANAC032* were observed after 24 h. At this time point, the *JAZ10* and *DIN11* levels had already decreased again. The variations in the *JAZ10* and *DIN11* expression kinetics in comparison to those of *CYP81D11*, *GSTU1*, *GSTU7* and *ANAC032* imply that different regulatory mechanisms work for these genes in response to TIBA.

The direct TGA2,5,6 target gene, *GSTU7*, which is induced by TIBA, BOA, phytoprostanes and CJ, was expected to be co-regulated with *CYP81D11*. Nevertheless, this was not reflected by the hierarchical cluster analyses, indicating that the co-regulation of *CYP81D11* and *GSTU7* (Fode et al. 2008) is restricted to only a subset of stimuli.

The co-regulation with COI1-independent genes indicates a unique position of *CYP81D11* among the xenobiotic-inducible genes. Figure 5.4 summarizes the different induction patters for *CYP81D11*, other TGA/SCL14-dependent genes, and other COI1-dependent TIBA-inducible genes.

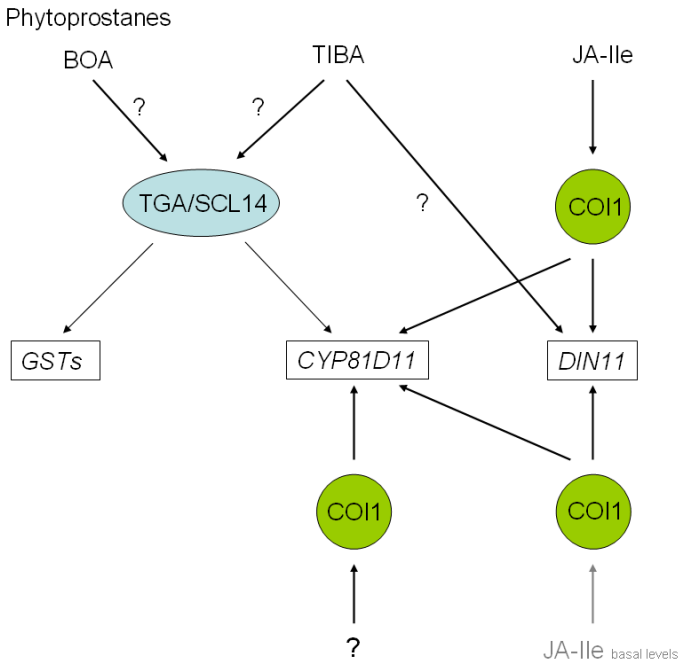

Figure 5. 4: Model of regulation of different classes of TIBA inducible genes

Mode of activation of TGA/SCL-dependent TIBA-inducible genes (represented by *GSTs*), of *CYP81D11* and of COI1-dependent TIBA-inducible genes (represented by *DIN11*). TIBA and phytoprostanes (and BOA) induce a subset of detoxification genes in a TGA2,5,6(/SCL14)-dependent manner. One of these genes is *CYP81D11*, but it is additionally activated by increased JA-Ile levels, e.g. after MeJA treatment. This induction requires COI1. Furthermore, also the induction by TIBA, BOA and phytoprostanes (Mueller et al. 2008) requires COI1. In this context, two new COI1 functions have been identified: (i) a COI1 function requiring basal JA-Ile levels, which is involved in the activation of a large group of TIBA-inducible genes including *CYP81D11* and *DIN11*; (ii) a JA-Ile-independent COI1 function, which co-activates *CYP81D11* expression in response TIBA and BOA. So far, it could not be elucidated whether a new COI1 ligand is involved in this function ("?").
Two more steps depicted in this scheme (also marked with "?") have not been deciphered so far: It is not yet clear (i) whether the TGA/SCL14 complex is activated upon chemical stress or whether it is a constitutive activator and regulatory steps are carried out by other yet unknown components; (ii) by which pathway *DIN11* (and other SCL14-independent genes) is activated in response to TIBA.

5.8 ANAC032 negatively regulates *CYP81D11* expression

ATAF1 and *ANAC032* are both SCL14-dependent and up-regulated genes in response to TIBA. Thus, their influence on other TIBA-inducible genes was investigated. NAC proteins represent a large family of transcription factors that are involved in developmental processes and defense and abiotic-stress responses (Olsen et al. 2005). In the last years, several studies investigating the role of ATAF1 in abiotic stress and defense responses were performed. Due to many contradictory results, a clear picture of ATAF1 function cannot be drawn. A consistent observation is the up-regulation of *ATAF1* expression in response to drought and ABA. The role of ATAF1 in the drought

stress response is controversial. In two studies, ABA hypersensitivity and a higher recovery rate after drought stress were shown for knockout mutants of ATAF1 compared to the wild type (Lu et al. 2007; Jensen et al. 2008). In another study, ATAF1 overexpressors mediated increased drought resistance and were hypersensitive to ABA, while no differences were observed for the same mutants compared to wild-type plants (Wu et al. 2009). At least for the drought tolerance experiments in two of the studies, the experimental setups were quite similar (Wu et al. 2009; Lu et al. 2007). In agreement with the role of ATAF1 as a negative regulator of the ABA response, an ATAF1-dependent decrease of ABA levels was observed in response to infection with the non-host pathogen *Blumeria graminis* f. sp. *hordei*. Thus, an ATAF1 function in crosstalk between abiotic stress and pathogen resistance was concluded (Jensen et al. 2008). Further evidence for an involvement of ATAF1 in pathogen resistance was provided by *Botrytis cinerea* infection experiments. Overexpressors of ATAF1 showed enhanced susceptibility to this necrotrophic fungus (Wu et al. 2009; Wang et al. 2009), while transgenic lines expressing an ATAF1 repressor domain fusion protein exhibited increased resistance (Wang et al. 2009). Nevertheless, the expression patterns of the pathogen-related genes *PDF1.2* and *PR1* differed among the studies. Wang et al. (2009) showed decreased *PDF1.2* and *PR1* levels in the overexpressor and increased levels in the repressor fusion lines compared to the wild type, while Wu et al. (2009) demonstrated unaffected *PDF1.2* levels either in the overexpressor lines or in the knockout plants. Hence, *PR1* expression was stronger in the overexpressor and weaker in the mutant. However, the differences in the expression of *PR* genes were relatively weak in both studies. Furthermore, one study used drop inoculation while in the other study spray inoculation was used.

Due to the sterility of the ATAF1-overexpressing plants used in this thesis, only uninduced levels of *CYP81D11* were investigated. Still, strong reduction of *CYP81D11* expression was observed (Figure 4. 25). The same effects were shown for ANAC032-overexpressing plants, but as here a few plants were fertile, further experiments were performed with those lines. Severe reduction of *CYP81D11* transcript levels was observed in response to TIBA (Figure 4. 29) and to JA (Figure 4. 31). Among the TIBA-inducible genes, the observed effects differed strongly. *CYP81D11* was the only investigated SCL14-dependent gene that showed these severe effects. While *GSTU1* was not influenced at all, *GSTU7* expression was reduced to about one-third and ATAF1 showed only a slight reduction (Figure 4. 30). Also the expression of the gene

DIN11, whose induction by TIBA is independent of TGA2,5,6 and SCL14, was reduced to about 40 % in the ANAC032-overexpressing plants. These results indicate that TGA class II transcription factor- and SCL14-dependent gene regulation is independent of regulation by ANAC032.

Even stronger suppression of *CYP81D11* expression (0.4 %) occurred in the ANAC032 overexpressors in response to MeJA, and also *VSP2* and *PDF1.2* expression were strongly compromised (to less than 10 %) (Figure 4. 31). *DIN11* was again less severely influenced, but still expression was reduced to about 20 %. All the genes tested in response to MeJA are COI1 dependent. Thus, it seems possible that ANAC032 somehow interferes with the COI1 function.

ATAF1 was shown to be involved in ABA signaling and in the crosstalk between the abiotic-stress and the pathogen response. As also ANAC032 is inducible by ABA, a negative effect of the ABA signaling response at least on JA-responsive genes seems feasible. For JA/ET-inducible genes, a negative effect of ABA has been shown before (Anderson et al. 2004). Although in the same experiments *VSP2* as an only JA-inducible gene was positively affected, it cannot be excluded that the repressing effect of ANAC032 is associated with ABA signaling.

Although in this thesis only genes that are negatively influenced by ANAC032 were shown, it is likely that ANAC032 is a positive transcriptional regulator. For ATAF1, transcriptional activation capacity was shown in yeast (Lu et al. 2007). Furthermore, ATAF1 repressor domain fusions were shown to exhibit contrary effects to the overexpressed wild-type protein, indicating an activating function of ATAF1 in plants (Wang et al. 2009). Thus, the transcriptional repression in the ATAF1- and ANAC032-overexpressing plants observed in this thesis was most certainly mediated by a repressor whose expression is activated directly or indirectly by ATAF1 and ANAC032.

ATAF1 and ANAC032 are the two closest homologues of the ATAF subfamily, but also ATAF2 and ANAC102, the two other members of this family, show high sequence identity. Furthermore, *ATAF1*, *ANAC032* and *ANAC102* group together in biclustering analyses in response to a broad range of treatments including *Botrytis cinerea* infection, H_2O_2, TIBA, or MeJA treatment, wounding, drought and ABA treatment (Kleinow et al. 2009). Additionally, no single mutant from the ATAF subfamily shows any developmental phenotype, which is in contrast to the overexpressing plants. Thus, it was concluded that the members of the ATAF subfamily exhibit redundant or at least

overlapping functions. Although phenotypes with resistance to drought or pathogens were observed for both the *ataf1* (Lu et al. 2007; Jensen et al. 2008) and the *ataf2* (Delessert et al. 2005) single mutants, the fact that a repressor fusion of ATAF1 showed more severe effects than the knockout mutant further supports this assumption.
In this thesis, *ataf1/anac032* double mutants neither show developmental defects nor do they affect *CYP81D11* expression (Figure 4. 33). These results indicate that, at least in response to TIBA and MeJA, functional redundancy exists not only between ATAF1 and ANAC032 but may also include ANAC102 and/or ATAF2.

5.9 ATAF1- and ANAC032-overexpressing plants exhibit severe growth and developmental phenotypes.

The ATAF1 and ANAC032 overexpressors generated in this thesis both show severe dwarfism, curly upwards-bent leaves exhibiting early yellowing mostly between the vessels and at the edges, late flowering, short inflorescences, sterility, and an extended lifetime (Figure 4. 25, Figure 4. 26). This phenotype was even a bit stronger in the ATAF1-overexpressing plants which showed increased branching; however, these phenotypes were largely similar. This observation further supports the hypothesis of functional redundancy.
To a certain extent, similar phenotypes were described for ATAF1 overexpressors in the literature (Wu et al. 2009), but also ATAF1-overexpressing plants with only more round leaves and no developmental phenotype have been described (Wang et al. 2009). Nevertheless, referring to the transcript levels, the overexpression in this case does not seem to be very strong. So far, none of the studies using ATAF1-overexpressing lines have offered a full explanation. Wu et al. (2009) reasoned that delayed flowering might be connected to an involvement of ATAF1 in the ABA response, as also the overexpression of other transcription factors involved in ABA signaling (ABI3 and ABF4) exhibited a late flowering phenotype (Kang et al. 2002; Zhang et al. 2005). Additionally, growth retardation was described for ABF4-overexpressing plants. As a reason for this phenotype, a constitutively activated ABA response was assumed. However, at least in the ANAC032-overexpressing plants, no alteration of the ABA-responsive gene COR78 was observed (Figure 6. 11).
In this thesis, many JA-responsive genes were repressed in the ANAC032-overexpressing plants. This may be a reason for their sterile phenotype, as JA synthesis

and some JA signaling mutants exhibit male sterility (Park et al. 2002; Xie et al. 1998; McConn & Browse 1996b). Nevertheless, none of the mutants involved in JA synthesis or signaling exhibited dwarfism or curly, early yellowing leaves. Thus, it is concluded that other developmental processes independent of ABA and JA are influenced as well.

Although examples for dwarf mutants have been described with the involvement of many pathways, dwarf phenotypes have most frequently been described for mutants with defects in the synthesis or signaling of either gibberellic acid (GA) (Schwechheimer 2008; Ueguchi-Tanaka et al. 2007) or brassinosteroids (BRs) (Choe et al. 1998; Choe et al. 2000; Kwon & Choe 2005). GA is essential for normal plant growth regulation. Mutants in GA signaling or synthesis exhibit a characteristic dwarf phenotype with darker leaves, decreased apical dominance, and late flowering. Additionally, seed germination is impaired in those mutants (Harberd et al. 2009; Richards et al. 2001). Mutants associated with brassinosteroids were reported to exhibit a short robust stature, reduced fertility, a prolonged life span, and leaves with darker green color and a round and curled shape (Kwon & Choe 2005). For many characteristics, ATAF1 and ANAC032 overexpressors seem to resemble mutants associated with BRs, still BR and GA mutants exhibit darker green leaves compared to the wild type, while leaves of ATAF1 and ANAC032 overexpressors are more yellowish. However, whether ATAF1 and ANAC032 influence GA or BR synthesis or signal transduction remains to be elucidated.

6 Supplemental data

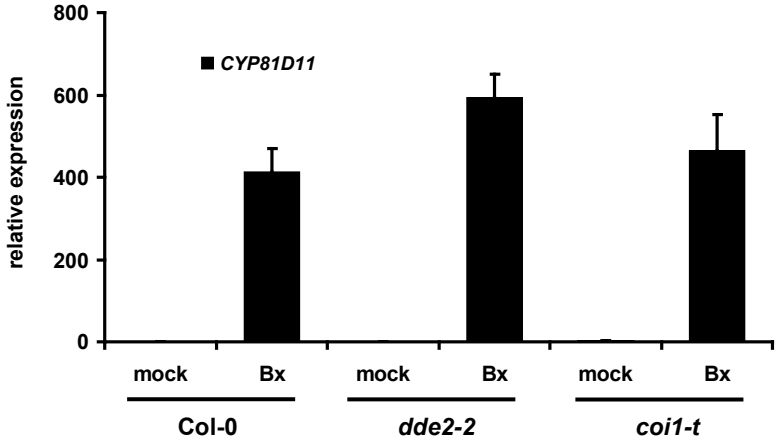

Figure 6. 1: Expression analysis of *CYP81D11* in the *dde2-2* and *coi1-t* mutants in response to benoxacor

Quantitative real-time RT-PCR analysis of relative *CYP81D11* transcript levels (normalized to the housekeeping gene *UBQ5*) in Col-0 (WT), *dde2-2* and *coi1-t* mutant plants. 6-7-week-old plants were sprayed with 50 µM benoxacor (Bx) or 0.1 % DMSO (mock) and incubated for 8 h. Whole rosettes were harvested for RNA isolation. Transcript values in mock treated Col-0 plants were set to 1. Every bar represents the average ± SEM of four (two for *coi1-t* mock) biological replicates.

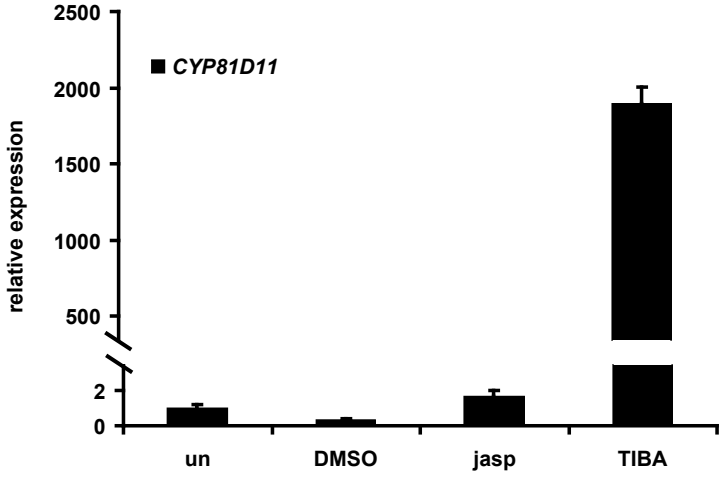

Figure 6. 2: *CYP81D11* transcript levels in Col-0 leaves in response to TIBA and jasplakinolide

Quantitative real time RT-PCR analyses of *CYP81D11* transcript levels (normalized to the housekeeping gene *UBQ5*). Plants were grown on soil under 12 h light/12 h dark for six weeks. Leaves were cut and petioles were submerged in H_2O (un), 1 % DMSO, in 10 µM jasplakinolide in 1 % DMSO (jasp) or 100 µM TIBA in 0,1 % DMSO for 24 h. Transcript values of untreated plants were set to 1. Every bar represents the average ± SEM of four biological replicates.

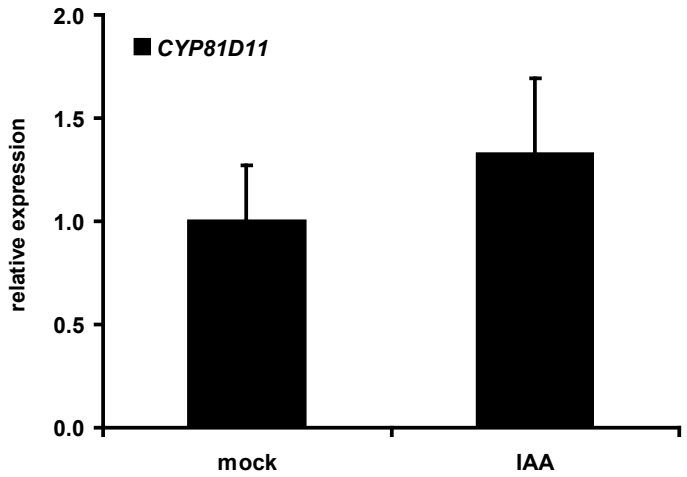

Figure 6. 3: Expression analysis of *CYP81D11* in response to IAA

Quantitative real-time RT-PCR analysis of relative *CYP81D11* transcript levels (normalized to the housekeeping gene *UBQ5*) in Col-0 plants. 6-7-week-old plants were sprayed with 10 µM IAA or H_2O (mock). Whole rosettes were harvested for RNA isolation. Transcript values in mock treated plants were set to 1. Every bar represents the average ± SEM of four biological replicates

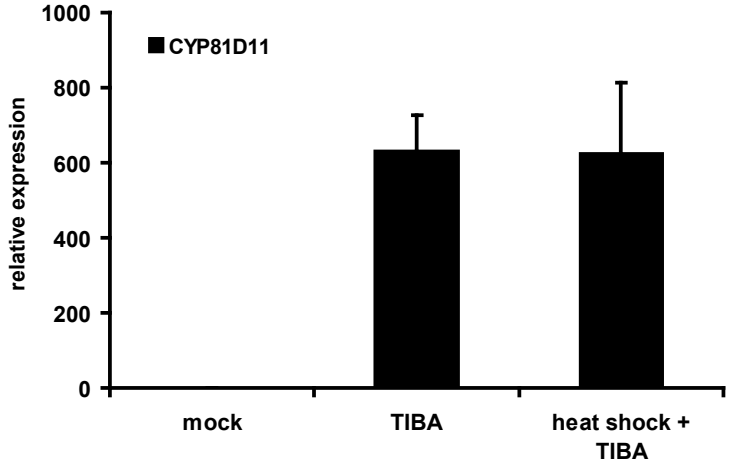

Figure 6. 4: Transcripti levels of *CYP81D11* in the transgenic *HS:AXR3-1* plants in response to TIBA

Quantitative real-time RT-PCR analysis of relative *CYP81D11* transcript levels (normalized to the housekeeping gene *UBQ5*) in transgenic *HS:AXR3-1* plants. 6-7-week-old plants were kept in the dark at 20°C (mock and TIBA) or in the dark at 37°C for 2 h and were subsequently sprayed with 100 µM TIBA and incubated for 8 h in the light. The heat shock was performed to induce the expression of *AXR3-1* which is regulated by a heat shock promoter. Whole rosettes were harvested for RNA isolation. Transcript values in mock treated Col-0 plants were set to 1. Every bar represents the average ± SEM of four biological replicates.

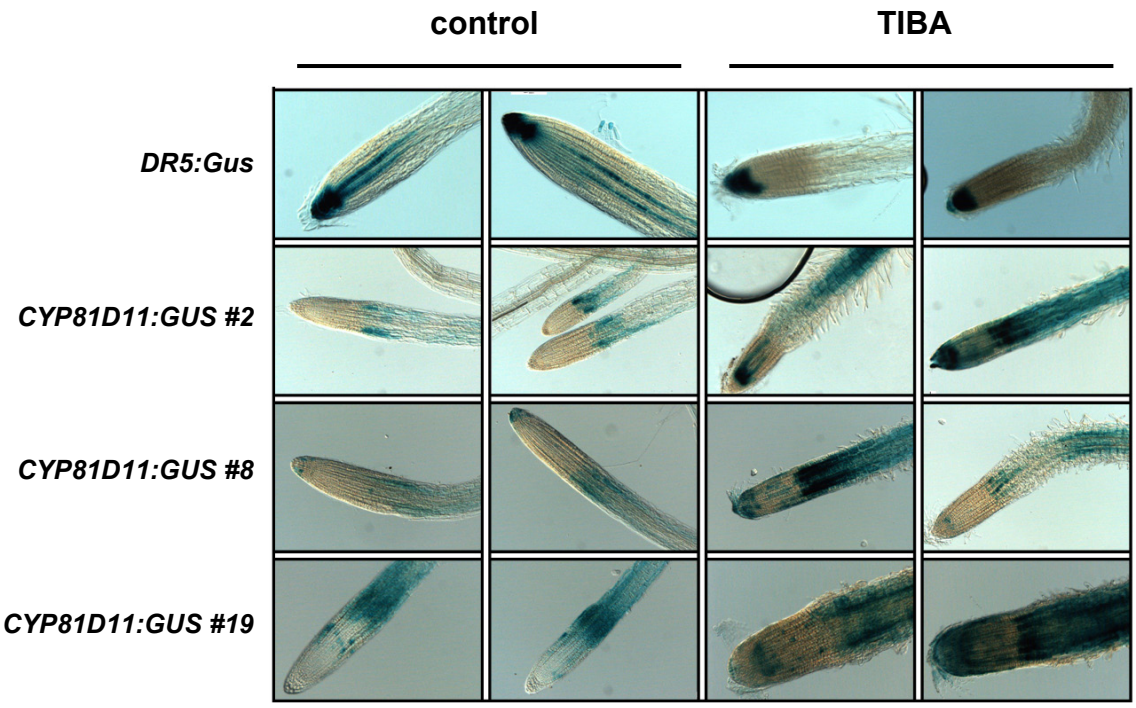

Figure 6. 5: Histochemical staining for GUS activity in root tips of transgenic *DR5:GUS* and *CYP81D11:GUS* plants

Root tips of transgenic plants plants containing *DR5:GUS* and *CYP81D11:GUS* transcriptional fusion constructs were staind with the GUS substate X-gluc (5-bromo, 4-chloro, 3-indoyl ß–glucuronide) to determine tissue specific GUS activity. Seadlings were grown for 14 days (under 14 h light/10 h dark) on MS agar either supplemented with 5 µM TIBA or without any additives. Representative pictures of three indenpendet *CYP81D11:GUS* lines are displayed.

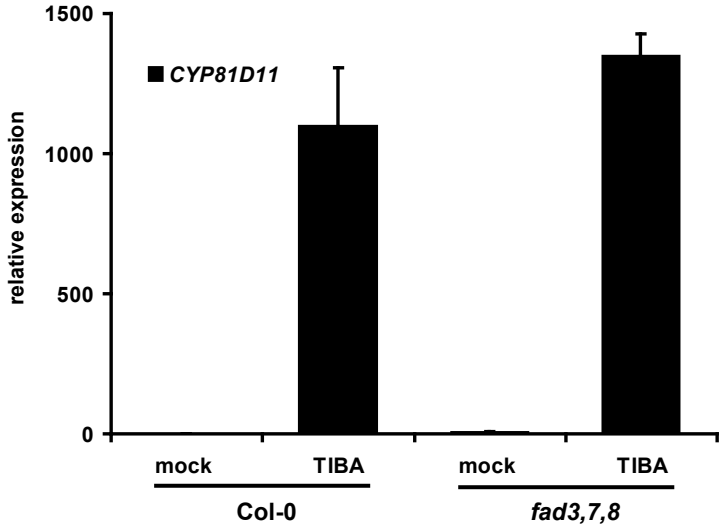

Figure 6. 6: **Expression analysis of *CYP81D11* in the *fad3,7,8* mutant in response to TIBA**

Quantitative real-time RT-PCR analysis of relative *CYP81D11* transcript levels (normalized to the housekeeping gene *UBQ5*) in Col-0 (WT) and *fad3,7,8* mutant plants. 6-7-week-old plants were sprayed with 100 µM TIBA or 0,1 % DMSO and incubated for 8 h. Whole rosettes were harvested for RNA isolation. Transcript values in mock treated Col-0 plants were set to 1. Every bar represents the average ± SEM of three (Col-0 TIBA) or four biological replicates.

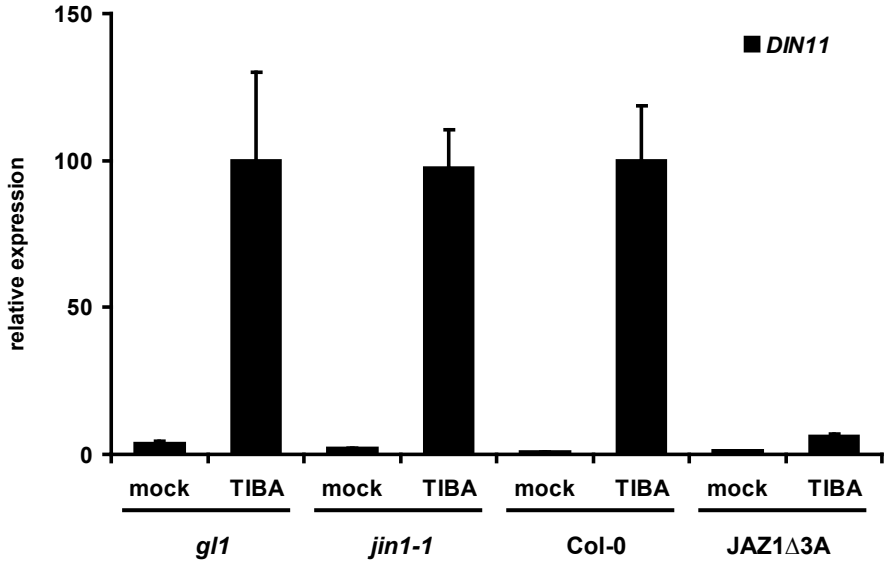

Figure 6. 7: Expression analysis of *DIN11* in plants deficient in JA signal transduction after TIBA

Quantitative real-time RT-PCR analysis of relative *DIN11* transcript levels (normalized to the housekeeping gene *UBQ5*) in *gl1* ("wild-type") and *jin1-1* mutant plants as well as Col-0 (wild-type) plants ectopically expressing JAZΔ3A, a JAZ1 protein lacking the Jas domain. 6-7-week-old plants were sprayed with 100 µM TIBA and whole rosettes were harvested for RNA isolation after 8 h. Transcript values in mock-treated Col-0 plants were set to 1. Every bar represents the average ± SEM of four (*gl1*and *jin1-1*), five (Col-0 mock) six (Col-0 TIBA and JAZ1Δ3A TIBA) or seven (JAZ1Δ3A mock) biological replicates.

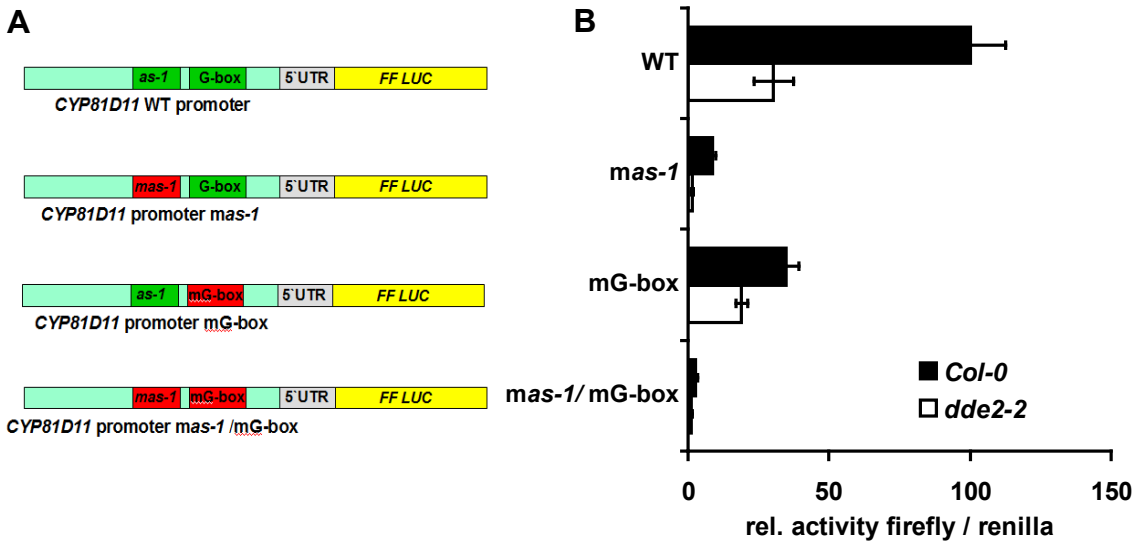

Figure 6. 8: Transient expression analysis of distinct *CYP81D11* promoter constructs carrying sequence alterations at the site of the *as-1*-like element and the site of the G-boxes

(A) Schematic illustration of the distinct *CYP81D11 promoter:firefly luciferase* constructs used for protoplast transfection. The light blue box indicates an 894 bp fragment upstream of the *CYP81D11* transcription start site, wild-type sequences of the *as-1* element (position -243 to -225) and the double G-box (position -206 to -193) are marked in green, while the altered sequences are depicted by red boxes. The grey box marks the 5´UTR of the *CYP81D11* gene and the yellow box indicates the *firefly luciferase* (*FF LUC*) reporter gene. The construct referred to as WT contains the 894 bp fragment upstream of the *CYP81D11* transcription start site and the 5´UTR of the *CYP81D11* gene. The m*as-1* construct contains sequence alteration within the *as-1* element (position -243 to -225), while the mG-box construct contains alterations in the sequence of the double G-box (position -206 to -193). The m*as-1*/mG-box construct exhibits alterations in both elements
(B) Luciferase activities obtained from transfection of distinct *promoter:firefly luciferase* constructs in *A. thaliana* Col-0 and *dde2-2* protoplasts. Leaves of 7-week-old soil-grown and non-induced plants were used for protoplast isolation. The x-coordinate demonstrate the ratio of the firefly luciferase activity to the internal *Renilla* luciferase standard.

Table 6. 1: Genes being TIBA-inducible in a COI1 dependent manner

COI1-dependent TIBA-inducible genes sorted due to their TIBA inducibility. COI1 dependency and P-values are indicated.

Gene code	Symbol	Description	Col-0 TIBA/ Col-0 mock	P-value	*coi1-t* TIBA/ Col-0 TIBA	P-value
at3g28740	*CYP81D11*	monooxygenase	55.64	8.6E-09	0.12	1.0E-05
at1g43160	*RAP2.6*	transcription factor	30.96	1.1E-12	0.03	9.3E-13
at5g13220	*JAZ10*	jasmonate-ZIM-domain protein	28.62	2.0E-10	0.02	4.8E-11
at3g49620	*DIN11*	oxidoreductase	23.70	3.5E-07	0.01	1.2E-08
at1g10585		transcription factor	18.92	1.4E-10	0.06	3.4E-10
at5g63450	*CYP94B1*	monooxygenase	16.61	7.1E-12	0.06	7.0E-12
at2g34600	*JAZ7*	jasmonate-ZIM-domain protein	12.90	7.2E-09	0.07	5.1E-09
at4g21680		proton-dependent oligopeptide transport (POT) family protein	10.93	2.9E-10	0.12	1.4E-09
at3g23550		MATE efflux family protein	9.95	3.1E-10	0.10	3.1E-10
at3g09940	*ATMDAR3*	monodehydroascorbate reductase	8.92	1.4E-07	0.07	1.8E-08
at3g48520	*CYP94B3*	monooxygenase	8.86	5.7E-09	0.11	5.7E-09

Gene code	Symbol	Description	Col-0 TIBA/ Col-0 mock	P-value	*coi1-t* TIBA/ Col-0 TIBA	P-value
at5g67080	*MAPKKK19*	protein kinase	8.39	1.1E-08	0.12	1.1E-08
at5g05600		oxidoreductase, 2OG-Fe(II) oxygenase family protein	8.03	4.8E-10	0.02	3.0E-13
at3g57520	*AtSIP2*	hydrolase	7.63	1.1E-06	0.28	1.2E-04
at4g37410	*CYP81F4*	monooxygenase	7.61	2.3E-09	0.04	1.0E-11
at1g06620		2-oxoglutarate-dependent dioxygenase, putative	7.05	1.2E-09	0.11	2.7E-10
at3g47340	*ASN1*	asparagine synthase	6.75	9.0E-05	0.24	1.1E-03
at2g29460	*ATGSTU4*	glutathione transferase	6.71	1.3E-05	0.22	1.1E-04
at5g61160	*AACT1*	acyltransferase	6.61	1.2E-06	0.07	2.4E-08
at5g44050		MATE efflux family protein	6.17	1.6E-07	0.12	3.5E-08
at1g30135	*JAZ8*	jasmonate-ZIM-domain protein	5.96	1.1E-05	0.14	3.9E-06
at1g70700	*JAZ9*	jasmonate-ZIM-domain protein	5.86	3.1E-10	0.04	1.3E-13
at1g17380	*JAZ5*	jasmonate-ZIM-domain protein	5.71	7.8E-09	0.10	2.2E-10
at5g19110		extracellular dermal glycoprotein-related	5.70	3.4E-09	0.11	2.0E-10
at5g38120		4-coumarate--CoA ligase family protein	5.26	5.9E-10	0.16	1.7E-10
at3g44860/ at3g44870	*FAMT* / -	S-adenosylmethionine-dependent methyltransferas S-adenosyl-L-methionine:carboxyl methyltransferase family protein	5.25	5.8E-10	0.04	1.0E-13
at1g51760/ at1g51780	*IAR3* / *ILL5*	IAA-Ala conjugate hydrolase / IAA-amino acid conjugate hydrolase	5.25	6.4E-09	0.12	3.7E-10
at4g16260		hydrolase, hydrolyzing O-glycosyl compounds	5.24	4.7E-06	0.08	3.4E-08
at3g04000		short-chain dehydrogenase/reductase (SDR) family protein	5.14	9.0E-07	0.24	4.3E-06
at2g39030		GCN5-related N-acetyltransferase (GNAT) family protein	5.11	5.1E-07	0.06	7.7E-10
at1g64160		disease resistance-responsive family protein	5.10	1.5E-09	0.22	3.6E-09
at4g24350		phosphorylase family protein	5.02	1.5E-07	0.09	1.6E-09
at1g17420	*LOX3*	lipoxygenase	4.97	1.9E-10	0.16	3.4E-11
at4g24340/ at4g24350		phosphorylase family protein / phosphorylase family protein	4.88	1.6E-07	0.10	2.0E-09
at2g26020	*PDF1.2b*	plant defensin	4.77	2.0E-05	0.06	3.6E-08
at4g30450		glycine-rich protein	4.71	5.1E-07	0.28	3.9E-06
at4g02360		unknown protein	4.68	3.9E-09	0.15	3.5E-10
at1g72520		lipoxygenase, putative	4.65	6.6E-10	0.27	4.2E-09
at3g25780	*AOC3*		4.54	4.6E-08	0.18	9.4E-09
at5g24420		glucosamine/galactosamine-6-phosphate isomerase-related	4.46	3.5E-06	0.03	2.9E-10
at5g64530	*ANAC104*	transcription factor	4.23	1.9E-08	0.28	9.3E-08
at4g30460		glycine-rich protein	4.06	7.6E-09	0.26	1.4E-08
at2g39330	*JAL23*		4.04	4.7E-07	0.06	1.3E-10
at2g43520	*ATTI2*	serine-type endopeptidase inhibitor	4.01	7.7E-07	0.29	2.9E-06
at3g55970		oxidoreductase, 2OG-Fe(II) oxygenase family protein	3.98	9.4E-06	0.08	1.3E-08
at5g06870	*PGIP2*	protein binding	3.96	2.6E-10	0.05	1.1E-14

Gene code	Symbol	Description	Col-0 TIBA/ Col-0 mock	P-value	*coi1-t* TIBA/ Col-0 TIBA	P-value
at2g29440	*ATGSTU6*	glutathione transferase	3.91	7.4E-08	0.21	1.7E-08
at1g54890		late embryogenesis abundant protein-related	3.89	7.0E-10	0.27	9.5E-10
at4g11320/ at4g11310		cysteine proteinase, putative / cysteine proteinase, putative	3.88	2.6E-06	0.06	6.2E-10
at1g76640		calmodulin-related protein, putative	3.85	1.4E-07	0.23	5.9E-08
at2g47180	*AtGolS1*	transferase, transferring glycosyl/ hexosyl groups	3.84	1.0E-05	0.16	3.7E-07
at1g76790		O-methyltransferase family 2 protein	3.82	3.3E-05	0.09	5.7E-08
at1g66760		MATE efflux family protein	3.80	4.0E-07	0.17	1.4E-08
at4g17470		palmitoyl protein thioesterase family protein	3.72	3.9E-03	0.03	4.7E-07
at1g19180	*JAZ1*	jasmonate-ZIM-domain protein	3.59	2.1E-05	0.05	1.4E-09
at1g53903/ at1g53885		- / senescence-associated protein-related	3.58	4.7E-04	0.03	1.1E-08
at1g11580	*ATPMEPCRA*	enzyme inhibitor, pectinesterase	3.57	3.4E-08	0.07	5.1E-12
at1g07260	*UGT71C3*	UDP-glycosyltransferase	3.57	1.2E-06	0.24	4.0E-07
at4g01080		unknown protein	3.55	1.9E-04	0.10	3.9E-07
at1g19670	*ATCLH1*	chlorophyllase	3.52	4.1E-05	0.04	1.2E-09
at1g06160	*ORA59*	transcription factor	3.52	7.5E-05	0.25	3.0E-05
at4g10390		protein kinase family protein	3.51	2.0E-08	0.23	2.9E-09
at1g23850		unknown protein	3.51	3.6E-07	0.24	9.1E-08
at4g22470		protease inhibitor, seed storage/lipid transfer protein (LTP) family protein	3.45	1.3E-05	0.29	1.2E-05
at5g38710		proline oxidase, putative	3.44	6.7E-07	0.24	1.6E-07
at3g51450		strictosidine synthase family protein	3.30	1.6E-06	0.05	2.7E-11
at1g61120	*TPS04*	(E,E)-geranyllinalool synthas	3.25	3.4E-06	0.20	9.3E-08
at1g32640	*ATMYC2*	transcription factor	3.24	2.8E-08	0.09	3.7E-12
at4g13410	*ATCSLA15*	cellulose synthase; transferase, transferring glycosyl groups	3.14	2.5E-06	0.19	4.6E-08
at1g72450	*JAZ6*	jasmonate-ZIM-domain protein	3.06	1.9E-05	0.11	1.1E-08
at2g06050	*OPR3*	12-oxophytodienoate reductase	3.04	4.3E-07	0.10	8.3E-11
at4g36110		auxin-responsive protein, putative	3.03	9.6E-07	0.24	5.1E-08
at2g24850	*TAT3*	L-tyrosine:2-oxoglutarate aminotransferaseauxin-responsive protein, putative; transaminase	3.02	9.4E-04	0.06	8.3E-08

Table 6. 2: Genes being TIBA-inducible in a COI1 dependent manner

COI1-dependent TIBA-inducible genes are sorted due to their bin numbers. TIBA inducibility and COI1 dependency are indicated.

Gene code	Symbol	Description	Col-0 TIBA/ Col-0 mock	*coi1-t* TIBA/ Col-0 TIBA
at4g13410	ATCSLA15	10.2 cell wall.cellulose synthesis	3.14	0.19
at5g06870	PGIP2	10.6.3 cell wall.degradation.pectate lyases and polygalacturonases	3.96	0.05
at1g11580	ATPMEPCRA	10.8.1 cell wall.pectin*esterases.PME	3.57	0.07
at3g47340	ASN1	13.1.3.1.1 amino acid metabolism.synthesis.aspartate family.asparagine.asparagine synthetase	6.75	0.24
at5g38710		13.2.2.2 amino acid metabolism.degradation.glutamate family.proline	3.44	0.24
at2g24850	TAT3	15.2 metal handling.binding, chelation and storage	3.02	0.06
at1g61120	TPS04	16.1.5 secondary metabolism.isoprenoids.terpenoids	3.25	0.20
at1g76790		16.10 secondary metabolism.simple phenols	3.82	0.09
at5g38120		16.2.1.3 secondary metabolism.phenylpropanoids.lignin biosynthesis.4CL	5.26	0.16
at3g51450		16.4.1 secondary metabolism.N misc.alkaloid-like	3.30	0.05
at5g05600		16.8.1 secondary metabolism.flavonoids.anthocyanins	8.03	0.02
at5g61160	AACT1	16.8.1 secondary metabolism.flavonoids.anthocyanins	6.61	0.07
at3g55970		16.8.1 secondary metabolism.flavonoids.anthocyanins	3.98	0.08
at1g51760, at1g51780	IAR3 / ILL5	17.2.1 hormone metabolism.auxin.synthesis-degradation	5.25	0.12
at4g36110		17.2.3 hormone metabolism.auxin.induced-regulated-responsive-activated	3.03	0.24
at1g06160	ORA59	17.5.2 hormone metabolism.ethylene.signal transduction	3.52	0.25
at1g17420	LOX3	17.7.1.2 hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	4.97	0.16
at1g72520		17.7.1.2 hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	4.65	0.27
at3g25780	AOC3	17.7.1.4 hormone metabolism.jasmonate.synthesis-degradation.allene oxidase cyclase	4.54	0.18
at2g06050	OPR3	17.7.1.5 hormone metabolism.jasmonate.synthesis-degradation.12-Oxo-PDA-reductase	3.04	0.10
at3g44860, at3g44870	FAMT / -	17.8.1 hormone metabolism.salicylic acid.synthesis-degradation AND 29.4 protein.postranslational modification	5.25	0.04
at1g19670	ATCLH1	20.1 stress.biotic AND 20.2.4 stress.abiotic.touch/wounding	3.52	0.04
at1g64160		20.1.7 stress.biotic.PR-proteins	5.10	0.22
at2g26020	PDF1.2b	20.1.7.12 stress.biotic.PR-proteins.plant defensins	4.77	0.06
at2g43520	ATTI2	20.1.7.12 stress.biotic.PR-proteins.plant defensins	4.01	0.29
at1g06620		21.2 redox.ascorbate and glutathione	7.05	0.11
at3g09940	ATMDAR3	21.2.1 redox.ascorbate and glutathione.ascorbate	8.92	0.07
at3g28740	CYP81D11	26.10 misc.cytochrome P450	55.64	0.12
at5g63450	CYP94B1	26.10 misc.cytochrome P450	16.61	0.06
at3g48520	CYP94B3	26.10 misc.cytochrome P450	8.86	0.11
at4g37410	CYP81F4	26.10 misc.cytochrome P450	7.61	0.04
at2g39330	JAL23	26.16 misc.myrosinases-lectin-jacalin	4.04	0.06
at1g07260	UGT71C3	26.2 misc.UDP glucosyl and glucoronyl transferases	3.57	0.24

Gene code	Symbol	Description	Col-0 TIBA/ Col-0 mock	*coi1-t* TIBA/ Col-0 TIBA
at4g22470		26.21 misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	3.45	0.29
at3g04000		26.22 misc.short chain dehydrogenase/reductase (SDR)	5.14	0.24
at2g39030		26.24 misc.GCN5-related N-acetyltransferase	5.11	0.06
at4g16260		26.4.1 misc.beta 1,3 glucan hydrolases.glucan endo-1,3-beta-glucosidase	5.24	0.08
at2g29460	ATGSTU4	26.9 misc.glutathione S transferases	6.71	0.22
at2g29440	ATGSTU6	26.9 misc.glutathione S transferases	3.91	0.21
at1g43160	RAP2.6	27.3.3 RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	30.96	0.03
at1g10585		27.3.6 RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	18.92	0.06
at5g67080	MAPKKK19	29.4 protein.postranslational modification	8.39	0.12
at4g17470		29.4 protein.postranslational modification	3.72	0.03
at1g32640	ATMYC2	29.4 protein.postranslational modification	3.24	0.09
at4g11320, at4g11310		29.5.3 protein.degradation.cysteine protease	3.88	0.06
at2g47180	AtGolS1	3.1.1.1 minor CHO metabolism.raffinose family.galactinol synthases.known	3.84	0.16
at3g57520	AtSIP2	3.1.2.2 minor CHO metabolism.raffinose family.raffinose synthases.putative	7.63	0.28
at4g10390		30.2.99 signalling.receptor kinases.misc	3.51	0.23
at1g76640		30.3 signalling.calcium	3.85	0.23
at1g54890		33.2 development.late embryogenesis abundant	3.89	0.27
at3g49620	DIN11	33.99 development.unspecified	23.70	0.01
at5g64530	ANAC104	33.99 development.unspecified	4.23	0.28
at1g53903, at1g53885		33.99 development.unspecified	3.58	0.03
at4g21680		34.13 transport.peptides and oligopeptides	10.93	0.12
at3g23550		34.99 transport.misc	9.95	0.10
at5g44050		34.99 transport.misc	6.17	0.12
at1g66760		34.99 transport.misc	3.80	0.17
at5g19110		35.1 not assigned.no ontology	5.70	0.11
at4g24350		35.1 not assigned.no ontology	5.02	0.09
at4g24340, at4g24350		35.1 not assigned.no ontology	4.88	0.10
at4g30450		35.1.40 not assigned.no ontology.glycine rich proteins	4.71	0.28
at4g30460		35.1.40 not assigned.no ontology.glycine rich proteins	4.06	0.26
at5g13220	JAZ10	35.2 not assigned.unknown	28.62	0.02
at2g34600	JAZ7	35.2 not assigned.unknown	12.90	0.07
at1g30135	JAZ8	35.2 not assigned.unknown	5.96	0.14
at1g70700	JAZ9	35.2 not assigned.unknown	5.86	0.04
at1g17380	JAZ5	35.2 not assigned.unknown	5.71	0.10
at4g02360		35.2 not assigned.unknown	4.68	0.15
at1g19180	JAZ1	35.2 not assigned.unknown	3.59	0.05
at4g01080		35.2 not assigned.unknown	3.55	0.10
at1g23850		35.2 not assigned.unknown	3.51	0.24

Gene code	Symbol	Description	Col-0 TIBA/ Col-0 mock	*coi1-t* TIBA/ Col-0 TIBA
at1g72450	JAZ6	35.2 not assigned.unknown	3.06	0.11
at5g24420		7.1.2 OPP.oxidative PP.6-phosphogluconolactonase	4.46	0.03

Table 6. 3: Genes being TIBA-inducible in a COI1-independent manner

Genes were sorted due to their TIBA indicibility, P-values are indicated

Gene code	Symbol	Description	Col-0 TIBA / Col-0 mock	p-value
at2g23170	GH3.3	indole-3-acetic acid amido synthetase	46.94	5.1E-12
at1g17170	ATGSTU24	glutathione transferase	28.98	7.2E-08
at1g05680		UDP-glucosyl transferase family protein	20.43	2.1E-05
at5g13330	Rap2.6L	transcription factor	17.94	7.0E-13
at2g29490	ATGSTU1	glutathione transferase	16.51	8.2E-08
at2g22860	ATPSK2	growth factor	14.17	2.2E-10
at5g49480	ATCP1	calcium ion binding	12.73	1.4E-10
at4g34131, at4g34135	UGT73B3 / UGT73B2	UDP-glycosyltransferase / UDP-glucosyltransferase	11.43	6.7E-10
at1g05560	UGT1	UDP-glucosyltransferase	11.39	1.9E-08
at5g52900		unknown protein	10.69	1.4E-11
at3g30775	ERD5	proline dehydrogenase	9.73	2.7E-06
at1g30040	ATGA2OX2	gibberellin 2-beta-dioxygenase	9.46	2.9E-11
at1g77450	ANAC032	transcription factor	9.10	4.8E-07
at1g76680, at1g76690	OPR1 / OPR2	12-oxophytodienoate reductase /	9.03	1.4E-09
at2g15480	UGT73B5	UDP-glucosyltransferase	8.39	4.9E-08
at2g29420	ATGSTU7	glutathione transferase	7.97	1.4E-08
at4g01870		tolB protein-related	7.61	1.5E-06
at4g13180		short-chain dehydrogenase/reductase	7.21	5.3E-08
at1g35910		trehalose-6-phosphate phosphatase, putative	6.79	2.4E-08
at1g15380		lactoylglutathione lyase family protein	6.45	4.0E-06
at2g17500		auxin efflux carrier family protein	6.25	2.5E-06
at3g14990		4-methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthesis protein, putative	6.01	1.5E-09
at5g62280		unknown protein	5.95	1.9E-05
at5g02760		protein phosphatase 2C family protein /	5.50	1.7E-07
at4g34138	UGT73B1	UDP-glycosyltransferase	5.34	1.1E-07
at1g59500, at4g37390	GH3.4 / GH3.2	indole-3-acetic acid amido synthetase / indole-3-acetic acid amido synthetase	5.31	2.0E-06
at1g67810	SUFE2	enzyme activator	5.24	7.9E-07
at1g01720	ATAF1	transcription factor	5.20	1.0E-08
at1g79410	AtOCT5	carbohydrate transmembrane transporter	5.11	1.2E-06
at1g02850	BGLU11	hydrolase	5.03	1.8E-06
at1g80840	WRKY40	transcription factor	5.02	5.9E-08

Gene code	Symbol	Description	Col-0 TIBA / Col-0 mock	p-value
at4g20860		FAD-binding domain-containing protein	4.87	2.2E-05
at5g61820		molecular function unknown	4.86	7.8E-08
at5g14730		unknown protein	4.85	7.1E-07
at1g10070	ATBCAT-2	branched-chain-amino-acid transaminase	4.81	4.5E-06
at4g34131, at4g34135	UGT73B3 / UGT73B2	UDP-glycosyltransferase / UDP-glucosyltransferase	4.71	6.7E-10
at5g07440	GDH2	glutamate dehydrogenase	4.71	3.0E-07
at2g36800, at2g36790	DOGT1 / UGT73C6	UDP-glycosyltransferase / UDP-glucosyltransferase	4.63	6.9E-06
at5g57560	TCH4	hydrolase	4.59	5.3E-06
at2g40200		basic helix-loop-helix (bHLH) family protein	4.56	4.9E-09
at5g63790	ANAC102	transcription factor	4.52	8.3E-08
at2g36380	PDR6	ATPase	4.52	1.1E-10
at3g26830	PAD3	dihydrocamalexic acid decarboxylase/ monooxygenase	4.31	5.2E-05
at1g72900		disease resistance protein (TIR-NBS class), putative	4.30	5.2E-09
at2g31750	UGT74D1	UDP-glycosyltransferase	4.27	8.7E-08
at2g19800	MIOX2	inositol oxygenase	4.20	7.3E-05
at1g76600		unknown protein	4.16	1.8E-09
at1g19020		unknown protein	4.06	1.0E-05
at4g21850	ATMSRB9	methionine sulfoxide reductase domain-containing protein	4.05	1.7E-05
at3g09270	ATGSTU8	glutathione transferase	4.04	5.7E-05
at1g76410	ATL8	protein binding	4.04	1.8E-07
at5g47370	HAT2	transcription factor	4.03	1.2E-07
at5g11160	APT5	adenine phosphoribosyltransferase	4.01	1.2E-07
at1g14130		2-oxoglutarate-dependent dioxygenase, putative	3.99	4.1E-08
at1g61820	BGLU46	hydrolase	3.94	3.0E-05
at1g22400	UGT85A1	UDP-glycosyltransferase	3.89	5.3E-06
at5g22300	NIT4	3-cyanoalanine hydratase/ cyanoalanine nitrilase	3.84	5.5E-05
at4g25810	XTR6	hydrolase	3.78	2.3E-06
at2g32190, at2g32210		unknown protein / unknown protein	3.75	1.9E-03
at3g56980	BHLH039	transcription factor	3.66	7.4E-04
at2g37760		aldo/keto reductase family protein	3.63	2.2E-05
at5g17860	CAX7	calcium:sodium antiporter	3.51	4.7E-04
at4g15490	UGT84A3	UDP-glycosyltransferase	3.50	5.8E-05
at4g15550	IAGLU	UDP-glycosyltransferase	3.50	1.1E-05
at2g44810	DAD1	phospholipase A1/ triacylglycerol lipase	3.50	3.6E-07
at4g37610	BT5	transcription regulator	3.49	6.8E-05
at1g33110		MATE efflux family protein	3.48	1.5E-05
at3g46280		protein kinase-related	3.46	4.7E-05
at1g75750	GASA1		3.45	4.7E-04
at2g15490	UGT73B4	UDP-glucosyltransferase	3.41	1.5E-04
at4g28085		unknown protein	3.40	2.6E-07
at4g37370	CYP81D8	monooxygenase	3.40	2.5E-06

Gene code	Symbol	Description	Col-0 TIBA / Col-0 mock	p-value
at5g65380		ripening-responsive protein, putative	3.36	3.8E-09
at4g15610		integral membrane family protein	3.35	1.5E-04
at3g18560		unknown protein	3.34	3.5E-07
at4g17500	ATERF-1	transcription activator	3.32	1.5E-06
at2g29500		17.6 kDa class I small heat shock protein	3.32	4.4E-05
at1g27730	ZAT10	transcription factor	3.31	4.4E-07
at5g41080		glycerophosphoryl diester phosphodiesterase family protein	3.30	9.0E-06
at5g57220	CYP81F2	monooxygenase	3.29	1.5E-07
at5g04340	C2H2	transcription factor	3.29	3.2E-07
at4g24570	DIC2	mitochondrial substrate carrier family protein	3.29	5.0E-09
at5g16970	AT-AER	2-alkenal reductase	3.29	1.3E-06
at2g37770		aldo/keto reductase family protein | chr2:15834867-15836881 FORWARD	3.28	9.9E-05
at1g26380		FAD-binding domain-containing protein	3.24	9.5E-06
at5g13750	ZIFL1	tetracycline:hydrogen antiporter	3.24	3.5E-07
at4g39670		glycolipid transporter	3.22	3.6E-05
at2g22880		VQ motif-containing protein	3.21	8.2E-07
at3g62150	PGP21	ATPase	3.21	1.1E-04
at5g64570	XYL4	hydrolase	3.20	8.5E-05
at5g20250	DIN10	hydrolase	3.19	3.5E-04
at1g66860		hydrolase	3.18	1.3E-04
at4g37530, at4g37520		peroxidase, putative / peroxidase 50	3.16	1.7E-05
at1g05575		unknown protein	3.15	1.0E-06
at2g44790	UCC2	electron carrier	3.10	8.7E-05
at1g77380	AAP3	amino acid transmembrane transporter	3.07	1.3E-08
at4g21990	APR3	adenylyl-sulfate reductase	3.04	1.2E-05
at5g16080	AtCXE17	hydrolase	3.02	1.3E-07
at2g47000	MDR4	ATPase / xenobiotic-transporting ATPase	3.02	2.6E-04
at1g09970	LRR XI-23	protein kinase	3.01	2.2E-05

Table 6. 4: Genes being TIBA-inducible in a COI1-independent manner

Genes were sorted due to their TIBA bin numbers, TIBA inducibility is indicated

Gene code	Symbol	Description	Col-0 TIBA / Col-0 mock
at5g64570	XYL4	10.6.2 cell wall.degradation.mannan-xylose-arabinose-fucose	3.20
at5g57560	TCH4	10.7 cell wall.modification	4.59
at4g25810	XTR6	10.7 cell wall.modification	3.78
at5g41080		11.9.3.3 lipid metabolism.lipid degradation .lysophospholipases. glycerophosphodiester phosphodiesterase	3.30
at5g07440	GDH2	12.3.1 N-metabolism.N-degradation.glutamate dehydrogenase	4.71
at1g10070	ATBCAT-2	13.1.4.1.4 amino acid metabolism.synthesis.branched chain group.common.branched-chain amino acid aminotransferase AND 18.4.1 Co-factor and vitamine metabolism. pantothenate.branched-chain amino acid aminotransferase	4.81
at3g30775	ERD5	13.2.2.2 amino acid metabolism.degradation.glutamate family.proline	9.73
at4g21990	APR3	14.2 S-assimilation.APR	3.04
at5g57220	CYP81F2	16.5.1.1.3.4 secondary metabolism.sulfur-containing.glucosinolates.synthesis.indole.synthesis.cytochrome P450 monooxygenase AND 26.10 misc.cytochrome P450	3.29
at5g22300	NIT4	16.5.1.3.3 secondary metabolism.sulfur-containing .glucosinolates.degradation.nitrilase AND 26.8 misc.nitrilases, *nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	3.84
at2g36800, at2g36790	DOGT1 / UGT73C6	16.8.4 secondary metabolism.flavonoids.flavonols AND 20.1 stress.biotic	4.63
at1g05560	UGT1	17.2.1 hormone metabolism.auxin.synthesis-degradation	11.39
at2g23170	GH3.3	17.2.3 hormone metabolism.auxin.induced-regulated-responsive-activated	46.94
at1g59500, at4g37390	GH3.4 / GH3.2	17.2.3 hormone metabolism.auxin.induced-regulated-responsive-activated	5.31
at1g22400	UGT85A1	17.4.1 hormone metabolism.cytokinin.synthesis-degradation	3.89
at4g17500	ATERF-1	17.5.2 hormone metabolism.ethylene.signal transduction	3.32
at1g30040	ATGA2OX2	17.6.1.13 hormone metabolism.gibberelin.synthesis-degradation.GA2 oxidase	9.46
at1g75750	GASA1	17.6.3 hormone metabolism.gibberelin.induced-regulated-responsive-activated	3.45
at2g44810	DAD1	17.7.1.1 hormone metabolism.jasmonate.synthesis-degradation.lipases	3.50
at1g76680, at1g76690	OPR1 / OPR2	17.7.1.5 hormone metabolism.jasmonate.synthesis-degradation.12-Oxo-PDA-reductase	9.03
at1g05680		17.8.1 hormone metabolism.salicylic acid.synthesis-degradation	20.43
at3g14990		18.2 Co-factor and vitamine metabolism.thiamine	6.01
at1g67810	SUFE2	18.7 Co-factor and vitamine metabolism.iron-sulphur clusters	5.24
at1g72900		20.1.7 stress.biotic.PR-proteins	4.30
at4g21850	ATMSRB9	20.2 stress.abiotic	4.05
at2g29500		20.2.1 stress.abiotic.heat	3.32
at5g11160	APT5	23.3.1.1 nucleotide.metabolism.salvage. phosphoribosyltransferases .apt	4.01
at5g16080	AtCXE17	24 Biodegradation of Xenobiotics	3.02
at1g15380		24.2 Biodegradation of Xenobiotics.lactoylglutathione.lyase	6.45
at3g26830	PAD3	26.10 misc.cytochrome P450	4.31
at4g37370	CYP81D8	26.10 misc.cytochrome P450	3.40
at4g37530,		26.12 misc.peroxidases	3.16

Gene code	Symbol	Description	Col-0 TIBA / Col-0 mock
at4g37520			
at2g44790	UCC2	26.19 misc.plastocyanin-like	3.10
at4g34131, at4g34135	UGT73B3 / UGT73B2	26.2 misc.UDP glucosyl and glucoronyl transferases	11.43
at2g15480	UGT73B5	26.2 misc.UDP glucosyl and glucoronyl transferases	8.39
at4g34138	UGT73B1	26.2 misc.UDP glucosyl and glucoronyl transferases	5.34
at2g31750	UGT74D1	26.2 misc.UDP glucosyl and glucoronyl transferases	4.27
at4g15490	UGT84A3	26.2 misc.UDP glucosyl and glucoronyl transferases	3.50
at4g15550	IAGLU	26.2 misc.UDP glucosyl and glucoronyl transferases	3.50
at2g15490	UGT73B4	26.2 misc.UDP glucosyl and glucoronyl transferases	3.41
at4g13180		26.22 misc.short chain dehydrogenase/reductase (SDR)	7.21
at1g02850	BGLU11	26.3 misc.gluco-, galacto- and mannosidases	5.03
at1g61820	BGLU46	26.3 misc.gluco-, galacto- and mannosidases	3.94
at1g14130		26.7 misc.oxidases - copper, flavone etc.	3.99
at5g16970	AT-AER	26.7 misc.oxidases - copper, flavone etc.	3.29
at4g20860		26.8 misc.nitrilases, *nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	4.87
at1g26380		26.8 misc.nitrilases, *nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	3.24
at1g17170	ATGSTU24	26.9 misc.glutathione S transferases	28.98
at2g29490	ATGSTU1	26.9 misc.glutathione S transferases	16.51
at2g29420	ATGSTU7	26.9 misc.glutathione S transferases	7.97
at3g09270	ATGSTU8	26.9 misc.glutathione S transferases	4.04
at1g27730	ZAT10	27.3.11 RNA.regulation of transcription.C2H2 zinc finger family	3.31
at5g04340	C2H2	27.3.11 RNA.regulation of transcription.C2H2 zinc finger family	3.29
at5g47370	HAT2	27.3.22 RNA.regulation of transcription.HB,Homeobox transcription factor family	4.03
at5g13330	Rap2.6L	27.3.3 RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	17.94
at1g80840	WRKY40	27.3.32 RNA.regulation of transcription.WRKY domain transcription factor family	5.02
at2g40200		27.3.6 RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	4.56
at3g56980	BHLH039	27.3.6 RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	3.66
at5g02760		29.4 protein.postranslational modification	5.50
at1g76410	ATL8	29.5.11.4.2 protein.degradation.ubiquitin.E3.RING	4.04
at4g37610	BT5	29.5.11.4.5.2 protein.degradation.ubiquitin.E3.BTB/POZ Cullin3.BTB/POZ	3.49
at5g20250	DIN10	3.1.2.2 minor CHO metabolism.raffinose family.raffinose synthases.putative	3.19
at1g35910		3.2.2 minor CHO metabolism.trehalose.TPP	6.79
at2g19800	MIOX2	3.4.4 minor CHO metabolism.myo-inositol.myo inositol oxygenases	4.20
at2g37760		3.5 minor CHO metabolism.others	3.63
at2g37770		3.5 minor CHO metabolism.others	3.28
at1g09970	LRR XI-23	30.2.11 signalling.receptor kinases.leucine rich repeat XI	3.01
at3g46280		30.2.99 signalling.receptor kinases.misc	3.46
at5g49480	ATCP1	30.3 signalling.calcium	12.73

Gene code	Symbol	Description	Col-0 TIBA / Col-0 mock
at2g22860	ATPSK2	33.99 development.unspecified	14.17
at1g77450	anac032	33.99 development.unspecified	9.10
at1g01720	ATAF1	33.99 development.unspecified	5.20
at5g63790	ANAC102	33.99 development.unspecified	4.52
at5g65380		33.99 development.unspecified	3.36
at2g36380	PDR6	34.16 transport.ABC transporters and multidrug resistance systems	4.52
at3g62150	PGP21	34.16 transport.ABC transporters and multidrug resistance systems	3.21
at2g47000	MDR4	34.16 transport.ABC transporters and multidrug resistance systems	3.02
at1g79410	AtOCT5	34.2 transporter.sugars	5.11
at5g17860	CAX7	34.21 transport.calcium	3.51
at1g77380	AAP3	34.3 transport.amino acids	3.07
at2g17500		34.99 transport.misc	6.25
at1g33110		34.99 transport.misc	3.48
at5g13750	ZIFL1	34.99 transport.misc	3.24
at4g01870		35.1 not assigned.no ontology	7.61
at4g15610		35.1 not assigned.no ontology	3.35
at2g22880		35.1 not assigned.no ontology	3.21
at5g52900		35.2 not assigned.unknown	10.69
at5g62280		35.2 not assigned.unknown	5.95
at5g61820		35.2 not assigned.unknown	4.86
at5g14730		35.2 not assigned.unknown	4.85
		35.2 not assigned.unknown	4.71
at1g76600		35.2 not assigned.unknown	4.16
at1g19020		35.2 not assigned.unknown	4.06
at2g32190, at2g32210		35.2 not assigned.unknown	3.75
at4g28085		35.2 not assigned.unknown	3.40
at3g18560		35.2 not assigned.unknown	3.34
at4g39670		35.2 not assigned.unknown	3.22
at1g66860		35.2 not assigned.unknown	3.18
at1g05575		35.2 not assigned.unknown	3.15
at4g24570	DIC2	9.8 mitochondrial electron transport / ATP synthesis.uncoupling protein	3.29

Table 6. 5: COI1-dependent TIBA-inducible genes classified as stress responsive

List of COI1-dependent TIBA-inducible genes classified as "stress responsive" by the TAIR GO classification database tool. Genes additionally classified as "responsive to abiotic or biotic stimuli" are marked in grey.

Gene code	Symbol	Description	Col-0 TIBA/ Col-0 mock	p-value	coi1-t TIBA/ Col-0 TIBA	p-value
at3g28740	*CYP81D11*	monooxygenase	55.64	8.6E-09	0.12	1.0E-05
at1g43160	*RAP2.6*	transcription factor	30.96	1.1E-12	0.03	9.3E-13
at5g13220	*JAZ10*	jasmonate-ZIM-domain protein	28.62	2.0E-10	0.02	4.8E-11
at3g49620	*DIN11*	oxidoreductase	23.70	3.5E-07	0.01	1.2E-08
at2g34600	*JAZ7*	jasmonate-ZIM-domain protein	12.90	7.2E-09	0.07	5.1E-09
at3g09940	*ATMDAR3*	monodehydroascorbate reductase	8.92	1.4E-07	0.07	1.8E-08
at3g47340	*ASN1*	asparagine synthase	6.75	9.0E-05	0.24	1.1E-03
at1g51760/ at1g51780	*IAR3 / ILL5*	IAA-Ala conjugate hydrolase / IAA-amino acid conjugate hydrolase	5.25	6.4E-09	0.12	3.7E-10
at4g16260		hydrolase, hydrolyzing O-glycosyl compounds	5.24	4.7E-06	0.08	3.4E-08
at1g64160		disease resistance-responsive family protein	5.10	1.5E-09	0.22	3.6E-09
at4g24350		phosphorylase family protein	5.02	1.5E-07	0.09	1.6E-09
at1g17420	*LOX3*	lipoxygenase	4.97	1.9E-10	0.16	3.4E-11
at2g26020	*PDF1.2b*	plant defensin	4.77	2.0E-05	0.06	3.6E-08
at1g72520		lipoxygenase, putative	4.65	6.6E-10	0.27	4.2E-09
at3g25780	*AOC3*		4.54	4.6E-08	0.18	9.4E-09
at2g43520	*ATTI2*	serine-type endopeptidase inhibitor	4.01	7.7E-07	0.29	2.9E-06
at5g06870	*PGIP2*	protein binding	3.96	2.6E-10	0.05	1.1E-14
at2g47180	*AtGolS1*	transferase, transferring glycosyl/ hexosyl groups	3.84	1.0E-05	0.16	3.7E-07
at1g66760		MATE efflux family protein	3.80	4.0E-07	0.17	1.4E-08
at1g19180	*JAZ1*	jasmonate-ZIM-domain protein	3.59	2.1E-05	0.05	1.4E-09
at1g19670	*ATCLH1*	chlorophyllase	3.52	4.1E-05	0.04	1.2E-09
at1g06160	*ORA59*	transcription factor	3.52	7.5E-05	0.25	3.0E-05
at4g10390		protein kinase family protein	3.51	2.0E-08	0.23	2.9E-09
at1g61120	*TPS04*	(E,E)-geranyllinalool synthas	3.25	3.4E-06	0.20	9.3E-08
at1g32640	*ATMYC2*	transcription factor	3.24	2.8E-08	0.09	3.7E-12
at1g72450	*JAZ6*	jasmonate-ZIM-domain protein	3.06	1.9E-05	0.11	1.1E-08
at2g06050	*OPR3*	12-oxophytodienoate reductase	3.04	4.3E-07	0.10	8.3E-11
at2g24850	*TAT3*	L-tyrosine:2-oxoglutarate aminotransferaseauxin-responsive protein, putative; transaminase	3.02	9.4E-04	0.06	8.3E-08

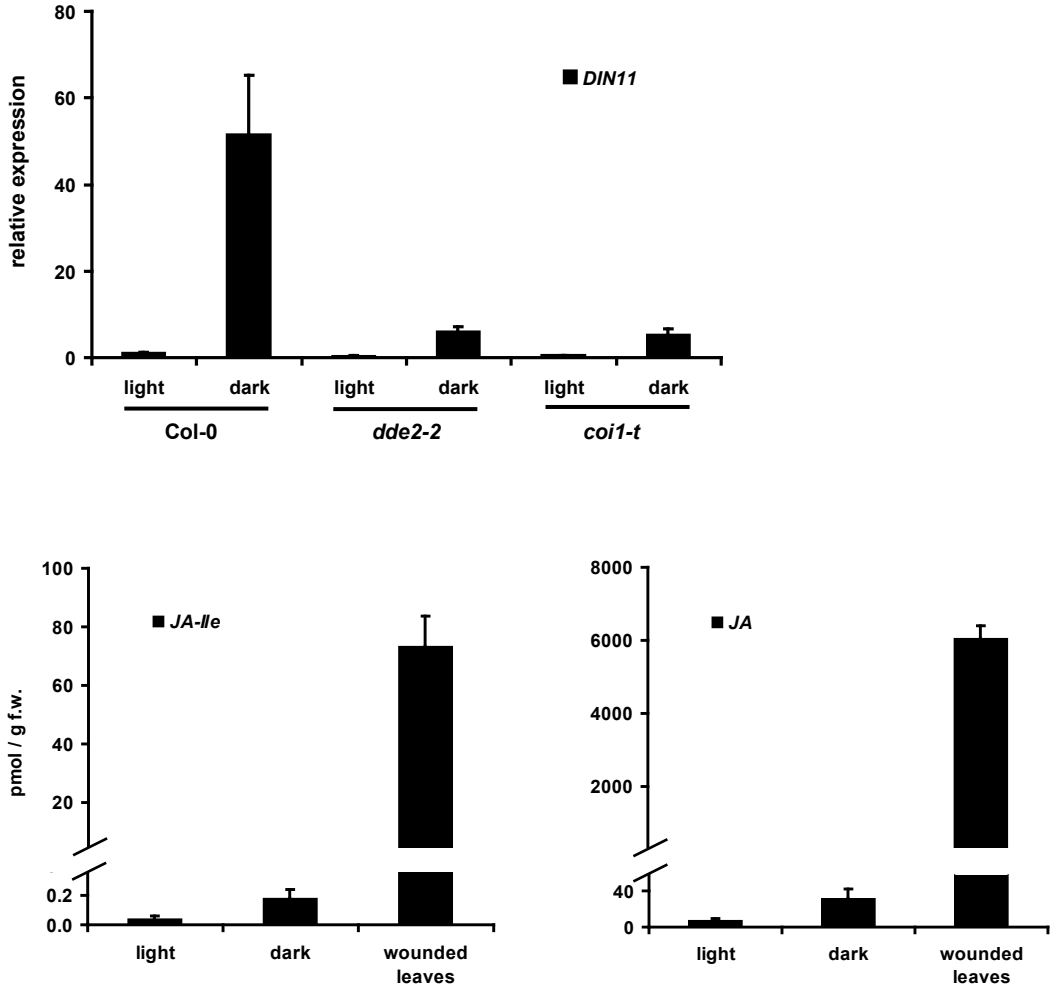

Figure 6. 9: *DIN11* expression and JA/JA-Ile levels in response to extended darkness

6-7-week-old soil grown plants (12 h light/12 h dark) were kept in the dark for 36 h (dark), while control plants remained under normal light cycle (light). Whole rosettes were harvested when the light period started.

(A) Quantitative real time PCR analysis of *DIN11* transcript levels (normalized to the housekeeping gene *UBQ5*). Every bar represents the average ± SEM of 4 biological replicates.

(B) Determination of JA-Ile and JA levels by HPLC-MS/MS was performed of Col-0 wild-type plants. As a control, leaves were wounded by squeezing with forceps and incubated for 2 h. In this case only wounded leaves were harvested. For light and dark every bar represents the average ± SEM of 4 biological replicates. Wounding experiment was only performed with 2 biological replicates. JA-Ile and JA amounts are depicted as pmol/g fresh weight.

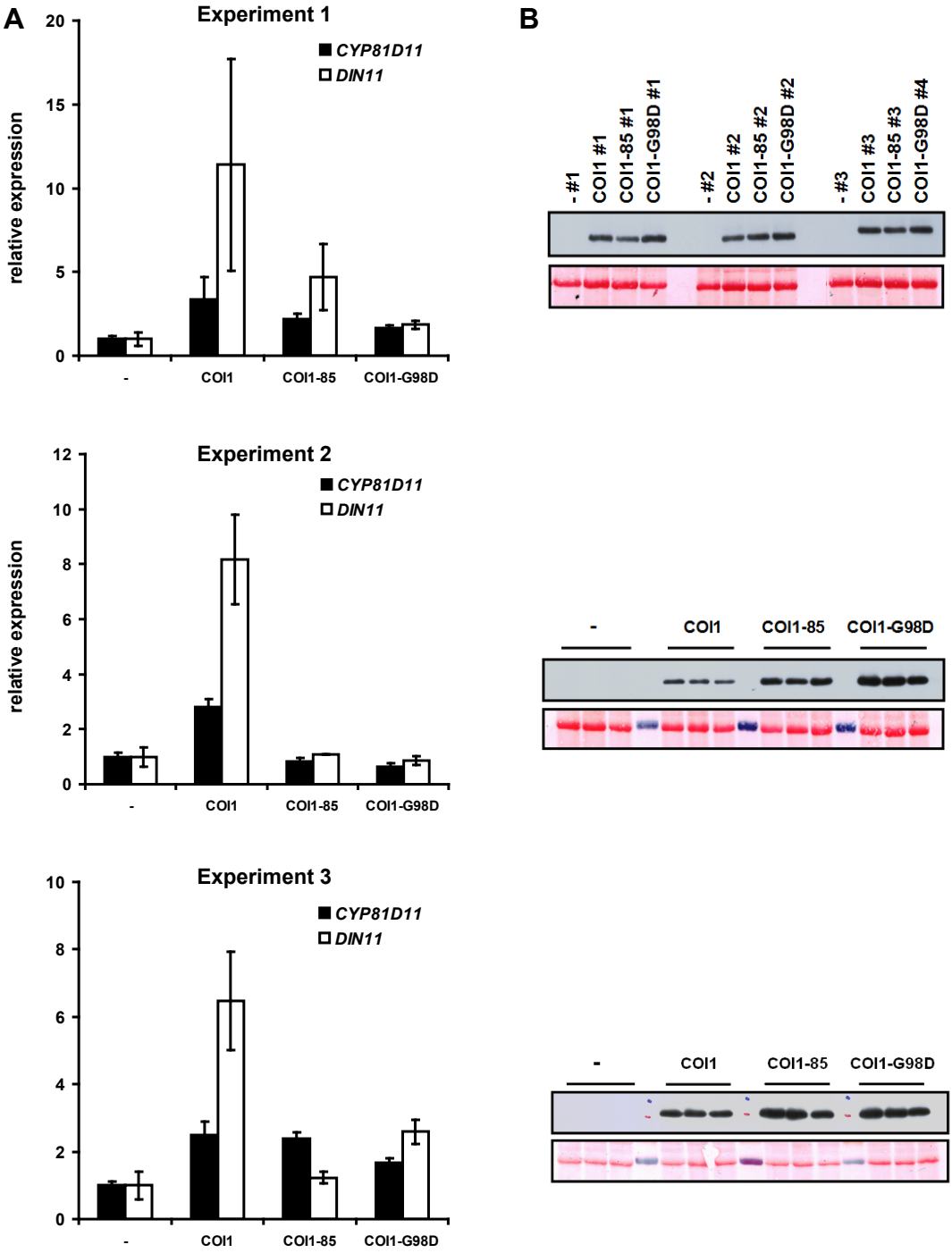

Figure 6. 10: Complementation of the *CYP81D11* and *DIN11* expression in the *coi1-t* mutant by distinct COI1 proteins

coi1-t protoplasts were transfected with vectors carrying the genes for COI1 as well as for two mutated COI1 proteins COI1-85, which exhibits amino acid substitution of aa85-88 from RAAM to HFAD, and COI1-G98D, which exhibits an amino acid exchange of aa98 from G to D (Yan et al. 2009) all COI1 proteins were fused to an HA tag. - indicates the empty vector control. Transfected protoplasts were incubated over night (16 h) before RNA was isolated or protein extracts were prepared. Three independent experimentes are depicted.

A: Quantitative real time PCR of *CYP81D11* and *DIN11* (normalized to the housekeeping gene *UBQ5*). Every bar represents the average ± SEM of three samples (for *CYP81D11* experiment 1 COI-85; for *DIN11* experiment 1 empty vector, COI-85 and experiment 3 empty vector only two replicates exhibited detectable transcript levels). Average of the transcript levels of the empty vector control samples was set to 1.

B: Western blot analyses using an HA antibody. Ponceau red staining of the membrane is indicated as a loading control.

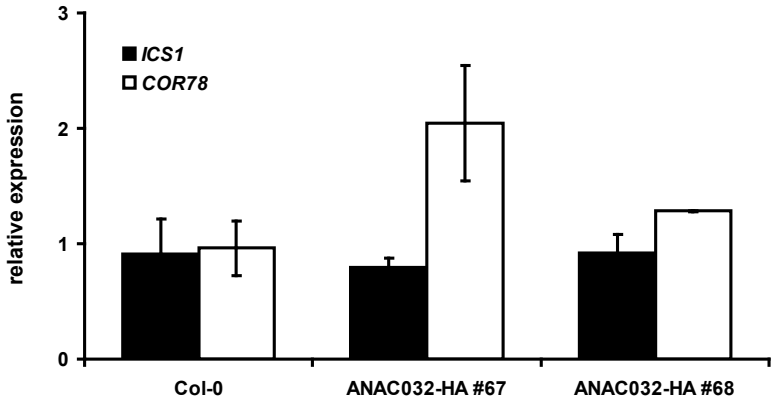

Figure 6. 11: *ICS1* and *COR78* transcript levels in ANAC032-overexpressing plants

Quantitative real-time RT-PCR analysis of *ICS1*and *COR78* transcript levels (normalized to the housekeeping gene *UBQ5*) in 6-7-week-old soil-grown Col-0 wild-type plants and plants ectopically expressing HA-ANAC032 controlled by the *CMV*35S promoter. Whole rosettes were harvested for RNA isolation. Transcript values Col-0 plants were set to 1. Every bar represents the average ± SEM of two (HA-ANAC032) or four (Col-0) biological replicates

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