The GRAS Protein SCL14 and TGA Transcription Factors Interact to Regulate Stress-Inducible Promoters

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Für dich, Joe

Contents

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
aa	amino acids
A	Ampere
A	adenosine
ABA	abscisic acid
(Bc)ActinA	*ActinA* gene from *B. cinerea*
AD	activation domain
ADE	marker gene for adenine prototrophy
ADH1	*alcohol dehydrogenase 1*
amp	ampicilin
APS	ammoniumpersulfate
APX	ascorbate peroxidase
as-1	*activating sequence 1*
AT	marks a protein from *Arabidopsis thaliana*
A. thaliana	*Arabidopsis thaliana*
(AT)MRP	multidrug resistance protein (from *A. thaliana*)
A. tumefaciens	*Agrobacterium tumefaciens*
B. cinerea	*Botrytis cinerea*
bp	base pairs
BSA	bovine serum albumine
BY-2	bright yellow-2
bZIP	basic leucine zipper
C	cytosine
CaMV	cauliflower mosaic virus
CAT	catalase
cDNA	copy DNA
ChIP	chromatin immunoprecipitation

CLB	crosslink buffer
COI1	CORONATINE INSENSITIVE 1
C_T	threshold cycle
C-terminal	carboxy-terminal
CYP	*cytochrome P450*
Da	Dalton
ddNTPs	didesoxy nucleotides
Dex	dexamethasone
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	desoxyribonucleotides
dpi	days past infection
DOC	sodium deoxycholate
DTT	dithiotreitol
EB	elusion buffer
ECL (kit)	enhanced chemoluminescence (kit for western detection)
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ERF1	ETHYLENE RESPONSE FACTOR 1
ET	ethylene
et al.	*et alii* (and others)
EtOH	ethanol
F	Farad
g	gravitation
g	gram
G	guanine
GA	gibberellic acid
β-Gal	β-galactosidase
GAL4-AD	activation domain of GAL4 transcription factor from yeast

GB	gradient buffer
GFP	green fluorescence protein
GPX	glutathione peroxidase
GR	glucocorticoid receptor
GST (U)	glutathione-S-transferase (τ-class)
GUS	glucuronidase
h	hours
HIS	marker gene for histidine prototrophy
H_2O_2	hydrogen peroxide
hpi	hours past infection
HR	hypersensitive response
HSP	herring sperm DNA
IAA	auxin
INA	isonicotinic acid
JA	jasmonic acid
k	kilo (10^3)
L	litre
lacZ	gene coding for β-galactosidase
LB	left border
LEU	marker gene for leucine prototrophy
LOX1	*lipoxygenase 1*
µ	micro (10^{-6})
m	mili (10^{-3})
m	meter
M	molarity [mol/L]
Me-SA	methyl salicylate
min	minutes
MtN19-like	*MAC9.6, Medicago truncatula N19-like*
mRNA	messenger RNA
MS	Murahige and Skoog

n	nano (10^{-9})
NEB	nuclei extraction buffer
NES	nuclear export sequence
NLS	nuclear localization sequence
NPR1	NON-EXPRESSOR of *PR*-GENES 1
N-terminal	amino-terminal
Ω	Ohm
OD	optical density
o/n	over night
oNPG	o-nitrophenyl-β-D-galactopyranoside
OPDA	12-oxophytodienoic acid
p	pico (10^{-12})
PAA	polyacrylamide
PAGE	polyacrylamide gelelectrophoresis
PCR	polymerase chain reaction
PCD	programmed cell death
PDA	potato dextrose agar
PDB	potato dextrose broth
PDF1.2	*Plant defensin 1.2*
pH	negative $\log_{10}$ of proton concentration
PMSF	phenyl-methyl-sulfonyl-fluoride
PR	*Pathogenesis related*
PUFA	poly unsaturated fatty acid
PVDF	polyvinylidene fluoride
RB	right border
REN	restriction endonucleases
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
SAR	systemic acquired resistance
SB	sonic buffer
S. cerevisiae	*Saccharomyces cerevisiae (bakers yeast)*
SCL14	SCARECROW-LIKE 14
SD	minimal yeast medium
SDS	sodium dodecylsulfate
SOD	superoxide dismutase
T	thymine
TE	tris-EDTA buffer
TEMED	N,N,N',N'-tetraethylenediamine
TGA	*as-1* (TGACG motive) binding bZIP transcription factors
TIBA	2,3,5-triiodobenzoic acid
Tris	tris-hydroxymethylamino methane
TRP	marker gene for tryptophane prototrophy
u	unit (quantity for enzyme activity)
U	uracil
UV	ultra violet
V	Volt
v/v	volume per volume
W	Watt
Wt, WT	wildtype plants
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1 Summary

TGA factors are members of the leucine zipper family of transcription factors and play a role in the regulation of various stress defence genes in Arabidopsis. TGA factors bind specifically to the *cis* element *as-1* (*activating sequence-1*). Previous studies have shown the inducibility of *as-1*-mediated transcription by salicylic acid (SA) and the synthetic auxin 2,4-D.

Tanja Siemsen has identified the GRAS protein family member Scarecrow-like 14 transcription factor (SCL14) as a TGA-interacting factor. SCL14 is able to activate transcription in yeast after complex formation with *as-1*-bound TGA2. In the absence of TGA2, no activation occurs, indicating that SCL14 is recruited to the promoter by its interaction with TGA2. In micro array experiments comparing wildtype and *scl14* mutant plants, Tanja Siemsen identified several SCL14-dependent genes.

In this work, three genes (*CYP81D11*, *MtN19-like* and *GSTU7*) were verified as direct targets of SCL14 and TGA2 by ChIP experiments. SCL14 is not recruited to these promoters in the *tga2,5,6* mutant. These results indicate that transcriptional regulators of the GRAS family can be recruited to specific promoters by DNA-bound transcription factors. Consistently, these genes are not expressed in the *scl14* and *tga2 tga5 tga6* mutants.

Micro array experiments comparing the RNA of wildtype and *scl14* mutant plants under xenobiotic-induced conditions revealed additional target genes for SCL14 involved in detoxification. When associated with TGA factors, SCL14 functions as transcriptional co-activator mediating specific responses of *Arabidopsis* plants to electrophiles and xenobiotic stress. Consistently, xenobiotics like TIBA induce target genes of SCL14 in a TGA dependent manner and *scl14* and *tga2 tga5 tga6* mutant plants are more susceptible to xenobiotic substances.

In addition, overexpression of SCL14 leads to a high resistance against the necrotrophic fungal pathogen *Botrytis cinerea*. However, scl14 mutants showed no higher susceptibility, suggesting a minor role for SCL14 target genes in response to necrotrophic pathogens.

2 Introduction

Plants have evolved a broad spectrum of defence responses in order to cope with stress conditions that might threaten their survival. Stress can be evoked either by abiotic changes in the environment like drought, high light intensities, salt, cold and heat or biotic stressors like bacteria, fungi, viruses and herbivores. In some cases the cell wall, the cuticula and the constitutive synthesis of basal levels of toxic compounds cannot provide sufficient defence. As the permanent establishment of a highly efficient defence status would require too many resources, plants have evolved inducible defence mechanisms (van Hulten et al., 2006).

2.1 The role of plant hormones in stress responses

Plant hormones (phytohormones) play a major role in defining the outcome and specificity of an inducible defence by activating signalling cascades to remodel gene expression. All phytohormones have distinct roles in stress adaptation but their signalling pathways can also influence each other either in a synergistic or an antagonistic manner. This crosstalk between the signalling pathways allows the plants to fine tune defence responses and thus react in a very specific way to different threatening situations. In particular, abscisic acid (ABA), ethylene (ET), jasmonic acid (JA) and salicylic acid (SA) have been shown to possess crucial functions in mediating or orchestrating responses to stress. When plants suffer from drought ABA regulates the water status of the plants by controlling stomatal closure and gene expression to adapt to low water conditions. Additionally, ABA plays a role in defence against different pathogens (Adie et al., 2007) e.g. by regulating callose deposition to prevent intrusion of necrotrophs (Ton and Mauch-Mani, 2004). The most dominant phytohormones in defence against pathogens are JA, ET and SA.

The enzymatically derived oxylipin JA is the key hormone for defence against necrotrophic pathogens and also against herbivores. The COI1 (CORONATINE INSENSITIVE 1) protein is required for all known JA dependent signalling events. The

coi1-1 mutant is JA-insensitive and fails to activate JA dependent defence responses against herbivoral attack. Larvae of *Pieris rapae* perform much better on *coi1-1* mutants due to the lack of JA-dependent defence (Reymond et al., 2004). The defence against necrotrophic pathogens like *Alternaria brassicicola* and *Botrytis cinerea* is also dramatically reduced in *coi1-1* mutants (Stintzi et al., 2001). COI1, an F-Box protein, is integrated in the multi protein SCF^{COI} (skip-cullin-F-box) complex which targets proteins for degradation. Proteins that are recognized by SCF^{COI} are poly-ubiquitinated and shuttled to the 26S proteasome. Most recent results show that COI1 binds the JA conjugate JA-isoleucine (JA-Ile). This interaction enables the SCF^{COI} complex to recognize JAZ (JASMONATE ZIM-DOMAIN) family proteins (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). The JAZ proteins analyzed so far are negative regulators of the JA response and bind to activators of JA-dependent genes. Degradation of these repressors is required for the activation of JA responses (Staswick, 2008).

ET is required for defence responses against herbivores and necrotrophic pathogens as well. ET-dependent gene expression requires two central components, the transcription factor EIN3 (ETHYLENE INSENSITIVE 3) and the important regulator EIN2 (ETHYLENE INSENSITIVE 2) (Alonso et al., 1999). Both mediate integrated ET signals from a MAP-kinase (mitogen-activated protein) cascade including the Raf-like kinase CTR1 (CONSTITUTIVE TRIPLE-RESPONSE 1). CTR1 actively suppresses ET signalling in the absence of the hormone and is inactivated upon binding of ET to the CTR1/ET receptor complex (Gao et al., 2003). Subsequently, phosphorylation of EIN3 by the MAP-kinase cascade renders it insensitive to SCF^{EBF} mediated proteasomal degradation, thus, allowing EIN3 to activate transcription. In addition, EIN2 is thought to influence the stability of EIN3 by interfering with the action of EBFs (Ethylene-dependent F-Box proteins).

In *Arabidopsis*, synergistic crosstalk between the JA and ET signalling pathway regulates expression of defence genes like o*smotins* or the plant *defensin PDF1.2*. These genes code for proteins which are key players in defence against necrotrophic pathogens (Thomma et al., 1998) and have direct anti-microbial properties (Verburg and Huynh, 1991; Capelli et al., 1997). Defensins directly attack certain lipid stretches (sphingolipids) on the surface of fungal cells causing membrane permeabilization (Aerts et al., 2007). A key regulator in the JA/ET crosstalk is the transcription factor ERF1

(ETHYLENE RESPONSE FACTOR1). Full expression of ERF1, which is of importance in resistance against necrotrophic fungal pathogens like *Botrytis cinerea* requires JA and ET activity. Overexpression of ERF1 can rescue resistance both in the *ein2-1* (*ethylene insensitive2*) and the JA signalling mutant *coi1-1* (Lorenzo et al., 2003) indicating that it acts downstream of these central regulators.

Beside JA and ET, other pathways like the SA-dependent gene expression (e.g. *PR-1*) are also active during necrotrophic pathogen attack pointing at a crosstalk between the SA and the JA/ET signalling pathways. During infection with *Botrytis cinerea*, systemic acquired resistance (SAR) is proposed to be important for restriction of fungal spread (Zimmerli et al., 2001). In addition, the production of the phytoalexin camalexin is absolutely required for resistance (Ferrari et al., 2007) and one of the camalexin synthesis genes *PAD3* (PHYTOALEXIN DEFICIENT 3) is activated by oligogalacturonides (OGs) independently of SA, JA and ET. Those OGs are released from plant cell walls by fungal polygalacturonases during attack contributing to basal defence of the plant.

SA is the key hormone for defence responses against biotrophic pathogens. Biotrophic pathogens, in contrast to necrotrophs, require living host cells to gain nutrients and water. Plants have developed defence programs to limit the spread of the pathogen. After recognition of a pathogen, plants mount a hypersensitive response (HR), including accumulation of reactive oxygen species (ROS, oxidative burst), an increase of salicylic acid (SA) and programmed cell death (PCD) of the infected cells (Lam et al., 2001). In addition, defence genes like *PR-1* (*Pathogenesis Related-1*) or the *glutathione-S-transferase GSTF8* are expressed. This local response can be followed by the establishment of a systemically heightened level of defence against secondary infections, called systemic acquired resistance (SAR). The SAR is efficient against a broad spectrum of pathogens. For tobacco it has been shown that systemic movement of methyl salicylate from the infected tissue is required to establish an SAR (Park et al., 2007). Methyl salicylate is converted into SA in the systemic leaves and subsequently induces expression of the defence-related genes including *PR-1*. One key regulator of SA-dependent defence including SAR is NPR1 (NON EXPRESSOR OF *PR*-GENES 1) which is translocated into the nucleus upon pathogen recognition (Mou et al., 2003). Mutants lacking NPR1 (*npr1-1*) are impaired in SAR. *npr1-1* mutants fail to activate *PR*

gene expression in the local and the systemic leaves (Dong, 2004). Lacking a DNA binding domain NPR1 was proposed to regulate *PR* gene expression as a cofactor of DNA-binding transcription factors. Interaction between NPR1 and TGA transcription factors could be demonstrated in yeast and *in planta* (Subramaniam et al., 2001). Consistently, several of the stress related genes contain a TGA factor binding motif in their promoter. This regulatory element is called *activating sequence-1* (*as-1*).

2.2 The regulatory promoter element *activating sequence 1*

The regulatory element *as-1* was originally described as a functional *cis* element in the *Cauliflower Mosaic Virus* (*CaMV*) *35S* promoter (Benfey and Chua, 1990). The consensus *as-1*-like element typically contains two copies of the TGAC/GTCA palindrome spaced by 12 bps (Qin et al., 1994). However, the stringency of the binding requirement is quite low and two sequences with only low homology can serve as a functional *as-1*-like sequences if the spacing between the palindromic centres is 12 bp (Krawczyk et al., 2002). Regulatory sequences related to the *as-1* element are found in a number of stress-responsive plant promoters including the pathogen-inducible *PR-1* gene promoter (Lebel *et al.*, 1998). Induction of this promoter by pathogens requires SA as well as one of the redundant *as-1*-binding proteins TGA2, TGA5 or TGA6 (Zhang *et al.*, 2003) and the TGA-interacting ankyrin repeat protein NPR1 (Cao *et al.*, 1997; Ryals *et al.*, 1997). Other SA-inducible genes that contain a functional *as-1*-like element (Chen and Singh, 1999) are expressed in an NPR1-independent manner (Uquillas et al., 2004b; Blanco et al., 2005). Many of these genes encode *glutathione S-transferases* (*GSTs*). In contrast to *PR-1*, most of the *GST* genes are not only activated by SA, but also by the auxin analogue 2,4-dichlorophenoxyacetic acid (2,4-D) (Wagner *et al.*, 2002). Likewise, synthetic promoters or promoter deletions that contain either one or multiple *as-1* elements as the only regulatory upstream sequences are 2,4-D- and SA-inducible (Redman *et al.*, 2002) in the absence of NPR1 (Butterbrodt *et al.*, 2006).

2.3 The role of TGA transcription factors in defence signalling

The TGA transcription factor family comprises ten members in *A. thaliana* (Jakoby et al., 2002). They are named after their ability to bind TGACG motifs (*as-1*-like elements) (Johnson et al., 2001; Rochon et al., 2006). TGA factors are basic leucine zipper transcription factors clustering into six subclasses based on their sequence homology. TGA1 and TGA4 belong to class I, TGA2, TGA5 and TGA6 to class II, TGA3 and AtbZIP50 to class III, PERIANTHIA to class IV, AtbZIP21 to class V and AtbZIP65 to class VI.

TGA factors are involved in several signalling pathways including the NPR1-dependent SA signal transduction. The ability to interact with NPR1 has been demonstrated for the members of the first three classes of TGA factors (Xiang et al., 1997; Kesarwani et al., 2007). TGA1 and TGA4 only interact with NPR1 after SA stimulus, whereas the others can interact with NPR1 under non-inducing conditions. It was shown that reduction of cysteine residues in TGA1 and TGA4 is required to allow interaction with NPR1 (Despres et al., 2003). Mutants lacking all class II TGA transcription factors (*tga2 tga5 tga6* mutants or *tga2,5,6* mutants) are compromised in establishment of SAR after pathogen attack showing the importance of the TGA factors for SA-dependent defence signalling (Zhang et al., 2003). Moreover, TGA factors can act as both negative and positive regulators of gene expression (Kesarwani et al., 2007).

TGA factors are also necessary for the SA-mediated inhibition of the JA/ET pathway influencing the repression of JA/ET induction of the *defensin PDF1.2* by SA (Ndamukong et al., 2007).

As unphysiological high concentrations of 2,4-D are required for TGA/*as-1* mediated activation of transcription in tobacco (Pascuzzi et al., 1998), TGA factors are believed to participate in stress response to so called xenobiotics. Consistent with this idea, *GSTs* are involved in the detoxification of harmful substances and radicals (Hayes and McLellan, 1999) and several of the *GSTs* contain TGA binding motifs in their promoters.

2.4 Detoxification of xenobiotic compounds

Xenobiotics ("life-foreign substances") are compounds which do not occur naturally in the respective organisms or at least occur in much lower concentrations. Chemical arms race between plants and pathogens or competing plants might have led to the establishment of efficient detoxification mechanisms in the plants. These detoxification processes are induced after recognition of xenobiotic substances and include alteration of gene expression. The influence of xenobiotic substances on gene expression was first described in animals (Prestera et al., 1993a). In plants, regulation of gene expression by xenobiotics occurs at least partly independent of possible hormone functions as xenobiotic-mediated induction of the *PR-4* gene (*HEL*) is insensitive to interruptions in ET, JA and SA hormone signalling pathways (Almeras et al., 2003). Likewise, 2,4-D promotes responses at least partially distinct from the auxin signalling pathway although 2,4-D is a functional analogue of IAA (Rahman et al., 2006). Actually, several xenobiotics exhibit electrophilic properties, suggesting that they activate electrophile-dependent signalling pathways leading to their detoxification.

Reactive electrophile species (RES) are formed in the cells under oxidative stress conditions. Oxidation caused by reactive oxygen species (ROS) can lead to formation of such RES. ROS like superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals are formed under biotic (oxidative burst during hypersensitive response) and abiotic stress conditions (Alvarez et al., 1998). They accumulate during pathogen attack due to the repression of ROS-scavenging enzyme activity by SA or nitric oxide (NO) (Klessig et al., 2000). The rising concentrations of hydrogen peroxide lead to activation of defence responses including programmed cell death (PCD) to restrict the spread of invading biotrophic pathogens. Thus, ROS act as secondary messengers for the plant during stress conditions leading to changes in gene expression but they also cause severe damage to cell compounds by denaturing proteins and oxidizing lipids. Because of this dual function (Bailey-Serres and Mittler, 2006) ROS scavenging is highly controlled by the cell. Nevertheless, interaction of ROS with proteins, nucleic acids and lipids cannot be totally avoided. In this case spreading damage of the respective cellular compounds must be restricted otherwise it leads to formation of further ROS and

harmful compounds with oxidative potential like RES (Montillet et al., 2005; Grun et al., 2007). Stress-induced oxylipins for example can exhibit reactive electrophile properties. Characteristically for many plant-pathogen interactions is the production of such oxylipins and other lipid-derived compounds in the plants (Howe and Schilmiller, 2002). Lipid peroxidation either enzymatically or non-enzymatically leads to the generation of these compounds in stressed or diseased plants (Imbusch and Mueller, 2000). Many of these lipid-derived products contain a carbonyl group in their molecular context (e.g. hexanal) contributing to an electrophilic character. In case of a subgroup of compounds containing an α/β-unsaturated carbonyl group the electrophilic properties are even increased. Several studies could show the ability of these highly reactive substances to alter gene expression in animals (Prestera et al., 1993b). In plants electrophiles mainly induce the expression of genes related to stress and detoxification like *glutathione-S-transferases*. Studies demonstrated the activation of the *GSTF6* gene by electrophiles like malonaldehyde (Vollenweider et al., 2000). The primary function of *GST*s is the detoxification of RES by catalysing the conjugation of glutathione to the electrophilic core of such lipophilic compounds.

Additionally, the precursor of JA, 12-oxo-phytodienoic acid (OPDA) is an enzymatically derived electrophilic oxylipin. Endogenous OPDA plays a role in plant-insect interaction and resistance to fungal pathogens (Stintzi et al., 2001). Additionally, gene expression induced by exogenously applied OPDA differs from JA-induced gene expression as most OPDA induced genes are COI1 independent. The differences in electrophilic properties between JA and OPDA contribute to this different induction pattern (Mueller et al., 2008). The OPDA-induced genes are predominantly coding for proteins involved in detoxification processes (*Cytochrome P450s* and *glutathione-S-transferases*). Likewise, some classes of plant isoprostanes (phytoprostanes) also alter gene expression with respect to their electrophilic potential. These phytoprostanes are derived from peroxidation of polyunsaturated fatty acids (PUFAs) (Sattler et al., 2006). During oxidation of PUFAs several lipid derived radicals are formed (lipid peroxyl radicals) which propagate their own formation by attacking neighbouring PUFAs. This chain reaction leads to the accumulation of lipid radicals, which spontaneously form phytoprostanes. Scavenging of those reactive radicals involves the reduction of lipid peroxyls by tocopherol (vitamine E). Thus, RES can activate or modulate plant defence

responses but are also harmful as oxidative stressors (Almeras et al., 2003). To reduce this oxidative stress, plants mainly activate detoxification pathways (induction of *glutathione-S-transferases*) after recognition of RES.

As many RES and xenobiotic induced *glutathione-S-transferase* genes contain *as-1* regulatory elements in their promoter regions, a role for *as-1* binding TGA transcription factors was assumed. In addition, gene expression induced by electrophilic phytoprostanes includes a variety of *GSTs* and this induction largely depended on TGA2, TGA5 and TGA6 (Mueller et al., 2008). However, most of the *GST*s are activated independently of the known TGA-interacting protein NPR1 (Uquillas et al., 2004a) suggesting an alternative TGA-dependent pathway for regulation of RES/ xenobiotic-detoxification associated genes.

2.5 The GRAS protein SCL14 interacts with TGA transcription factors

As compared to the NPR1-dependent pathway, the NPR1-independent activation of *as-1*-containing promoters is far less explored. Assuming that TGA2 interacts with a different regulatory protein for activation of NPR1-independent pathways, a yeast protein interaction screen was performed by Tanja Siemsen using *Arabidopsis* TGA2 as bait.

The yeast two hybrid (YTH) screen has successfully been applied to isolate protein interaction partners (Fields and Song, 1989). However, fusion of the bait protein with a heterologous DNA binding domain or fusion of the prey protein with an activation domain might hamper certain interactions. In the screen done by Tanja Siemsen (2002), this potential drawback was circumvented by cloning three copies of the *as-1* element (*3x as-1*) upstream of the *HIS3* selectable marker gene (Weigel *et al.*, 2005) and expressing TGA2 *in trans*. As *as-1*-bound TGA2 proteins do not activate transcription in yeast, growth on selective medium is compromised. A cDNA expression library (Minet *et al.*, 1992) was transformed into this strain and screening for prototrophic growth was performed. Five clones out of 1 x 10^6 yeast transformants were identified on selective media. One of the clones encoded an open reading frame of 769 amino acids

and was identical to the GRAS protein SCL14 (*At1g07530, AtGRAS-2*) (Bolle, 2004; Tian et al., 2004).

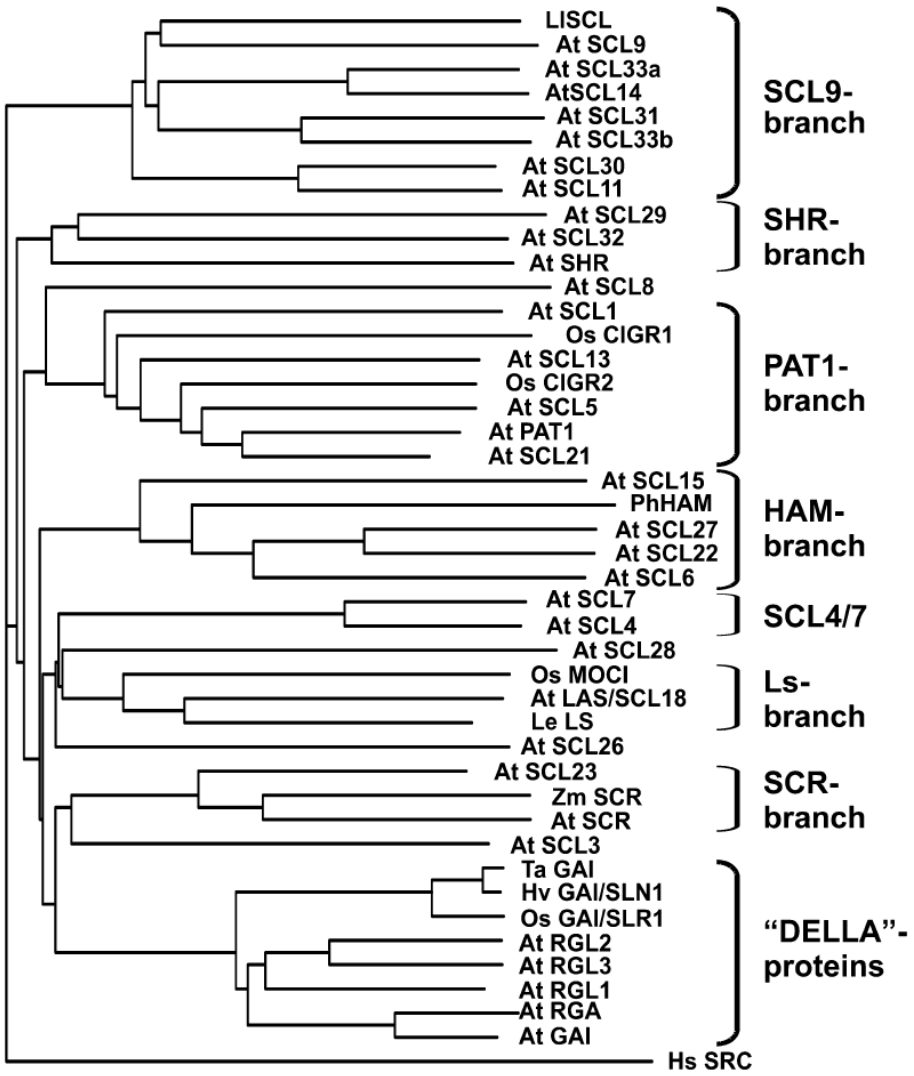

Figure 2-1 Phylogenetic tree of the GRAS protein family (Bolle, 2004)

The family of GRAS proteins is divided into 9 sub-classes including the DELLA proteins involved in GA signalling. SCL14 belongs to the "SCL9" branch and shows relatively high homology to LlSCL from Lily.

Based on sequence analysis, SCL14 belongs to the plant-specific GRAS protein family that comprises 33 members in *Arabidopsis* (Bolle, 2004). GRAS proteins have been shown to be involved in gibberellic acid (GA) and phytochrome signalling, root and axillary shoot development, and maintenance of the shoot apical meristem.

The acronym GRAS was coined after identification of the founding members GAI (GIBBERELLIC-ACID INSENSITIVE), RGA (REPRESSOR of GA1) and SCR (SCARECROW) (Pysh *et al.*, 1999). GAI and RGA have important roles in gibberellic acid dependent signal transduction processes (Silverstone *et al.*, 1998), whereas SCR was isolated in a screen for mutations that affect root development. GAI, RGA, SCR and SCL (SCARECROW-LIKE) proteins contain several conserved amino acid signatures in the so called GRAS domain at the C terminus. In contrast, the amino acid sequence of the N-terminal domain is more variable (Di Laurenzio *et al.*, 1996).

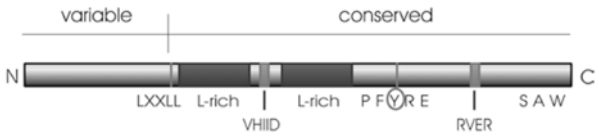

Figure 2-2 Domain structure of the GRAS proteins (Bolle, 2004)

GRAS proteins have a conserved C-terminal region with characteristic amino acid sequence stretches and two leucine rich repeat regions probably involved in protein-protein interaction. The N-terminal region is quite variable among the GRAS proteins probably contributing to their different functions.

Like all the other members of the family, SCL14 contains a unique N terminus and a conserved C-terminal GRAS domain. The roughly 380 amino acid long GRAS domain is characterized by two leucine-rich regions (LHRI and LHRII) and three separate conserved amino acid signatures: VHIID, PFYRE, and SAW (Pysh *et al.*, 1999). Only two studies have addressed its function. The VHIID domain of a GRAS protein from *Brassica napus* interacts with a histone deacetylase supporting the notion that GRAS proteins function in regulating gene expression at the level of transcription (Gao *et al.*, 2004). The GRAS domain of the regulator of GA signalling GAI contributes to the interaction with the F-box protein SLEEPY1 (Dill *et al.*, 2004), which is of major importance for the control of protein abundance as a function of GA. Though direct DNA binding to a specific target sequence has never been reported, GRAS proteins are classified as transcriptional regulators (Riechmann et al., 2000; Zentella et al., 2007).

The yeast data suggested an activating function for SCL14 as histidine prototrophy was restored only in the presence of TGA2, indicating that SCL14-mediated activation of the *3x as-1:HIS3* construct requires TGA2. These data were confirmed in a yeast strain

harbouring three copies of the *as-1* element upstream of the *ß-galactosidase* (*lacZ*) gene (Siemsen, 2005).

To obtain independent evidence for the formation of a TGA2/SCL14 complex, an *in vitro* pull-down assay with recombinant proteins was performed by Ulrike Süthoff (2006). Crude *E. coli* extracts containing either SCL14 fused to a glutathione *S*-transferase (GST-SCL14) or His-tagged TGA2 (His_6-TGA2) were combined and loaded onto glutathione-sepharose affinity beads. After washing and subsequent elution under denaturing conditions, eluates were analyzed for the presence of His_6-TGA2. Though GST-SCL14 was subject to protein degradation in *E. coli* and during subsequent processing steps, sufficient amounts of the protein were bound to the glutathione matrix to retain His_6-TGA2. In contrast, equivalent amounts of unfused GST protein were unable to interact with His_6-TGA2. This experiment supports the initial yeast data that implicate an interaction between SCL14 and TGA2.

To analyze the *in vivo* effect of SCL14 on *as-1*-mediated gene expression, the *SCL14* cDNA was cloned into a binary vector designed to express HA_3-tagged proteins under the control of the *Cauliflower Mosaic Virus (CaMV) 35S* promoter (pAlligator2-SCL14). *Arabidopsis* plants encoding the *ß-glucuronidase* gene (*GUS*) downstream of the "truncated" *Cauliflower Mosaic Virus (CaMV) 35S* promoter (*as-1:GUS*) (Redman *et al.*, 2002) were used for transformation. Transcription from this promoter fragment (+1 to -90), which contains *as-1* as the only regulatory sequence, can be induced by SA and 2,4-D. Out of 48 primary transformants, only two plants showed expression of HA_3-SCL14 as revealed by Western blot analysis of crude extracts performed with an αSCL14 antiserum (Siemsen, 2005; line #5 was used in this work). Expression of *35S:SCL14* led to an increased basal expression of the GUS gene and an increased induction after treatment with SA or 2,4-D.

The *Arabidopsis* mutant *SALK_126931*, carries a T-DNA insertion in the *5'UTR* of *SCL14*. Homozygous mutant plants showed no detectable *SCL14* mRNA and SCL14 protein levels (Figure 2-3) as revealed by Northern and Western blot analysis, respectively. Those plants were crossed with wildtype plants carrying the *as-1:GUS* construct and lines homozygous for the mutated *SCL14* gene were selected. In these transgenic mutants transcript of the *GUS* reporter gene was no longer inducible by 2,4-D or SA (Siemsen, 2005; line #14 was used in this work as *scl14* mutant). However,

transcript levels of the NPR1-dependent *PR-1* gene and the NPR1-independent *GSTF8* gene showed no difference to those found in wildtype plants after SA treatment and 2,4-D-induced expression of *GSTF8* was also not affected. The *HA_3-SCL14* line also responded like wildtype with respect to these genes, implicating that *PR-1* and *GSTF8* are not subject to SCL14-dependent regulation.

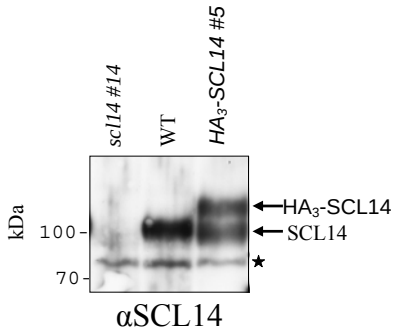

Figure 2-3 Western blot analysis of SCL14 protein in different genotypes

Five-wee-old wildtype plants (WT; encoding the wildtype *SCL14* allele and the *as-1:GUS* transgene), plants transformed with the *CaMV 35S:HA_3-SCL14* construct and *scl14* mutant plants were compared regarding their SCL14 protein levels. The αSCL14 antiserum was used in a 1:1000 dilution. The asterisk marks an unspecific band.

Additionally, *SCL14* transcription was found to be induced under abiotic stress conditions like ozone treatment or dark-induced senescence suggesting a role for SCL14 during oxidative stress. In addition, micro array analysis revealed a subset of endogenous genes, which requires SCL14 protein for full expression (Siemsen, 2005). Many of the identified genes (e.g. *CYP81D11* and *GSTU7*) are putatively involved in detoxification processes. Thus, SCL14 is a good candidate as a co-regulator of TGA-dependent gene expression mediating detoxification of electrophilic/ xenobiotic compounds.

2.6 Aim of the study

This study is based on results of the PhD thesis of Tanja Siemsen, who identified SCL14 as a TGA-interacting protein. Moreover, she identified putative SCL14 target genes by comparing the transcriptome of wildtype plants with that of *scl14* mutants and

transgenic plants ectopically expressing SCL14. Cluster analysis revealed induction of these genes under conditions of oxidative/ xenobiotic stress.

The aim of this study was to verify whether these candidate genes were induced in an SCL14- and TGA-dependent manner under different stress conditions. Moreover, chromatin immunoprecipitation experiments should answer the question, whether the TGA/ SCL14 complex is formed *in vivo* on promoters that contain an *as-1* element. In addition, the biological function of SCL14 should be elucidated by applying different forms of biotic and abiotic stress and subsequent monitoring of growth of different plant genotypes. As the previous transcriptome profiling experiment had been performed under non-inducing conditions, a second profiling experiment should be done to identify more SCL14-dependent genes likely to play a role in response to the stress applied.

3 Materials and Methods

3.1 Materials

3.1.1 Devices

Device	Model	Source
Autoclave	3870 ELV	Tuttnauer
Automatic pipettes		Gilson
Blotting device		University of Göttingen
Chambers for gel electrophoreses		University of Göttingen
Cooling centrifuge	Sorvall RC 5B Plus	DuPont
Cooling micro centrifuge with overhung rotor		Hettich
Counting chamber	Fuchs-Rosenthal	Brand
Counting chamber, spores	Thomma	
Electroporator	Gene Pulser® II	BioRad
Fluorescence microscope		
Fluorometer	CytoFluorII Plate Reader	PerSeptive
Gel documentation device		MWG Biotech
Heat block		Boekel Scientific
Heated stirrer	RCT basic	IKA Labortechnik
Heated shaker	Thermomixer 5436	Eppendorf
Locker for incubations		WTC binder; Memmert
Micro centrifuge	Biofuge pico	Heraeus Christ
Micro centrifuge, cooled	5403	Eppendorf
PCR cycler	MiniCycler™ PTC-150	MJ Research
pH-Meter	HI 9321	Hanna Instruments
Photometer	Unikon 720 LC	Kontron
Photometer for microtiter plates	MRX Dynex Plate Reader	Dynex
Realtime PCR cycler	iCycler	BioRad
RNA-/DNA-Calculator	GeneQuant II	Pharmacia
Rotation platform	IntelliMixer	
Scanner	ScanJet 4c	Hewlett Packard
Scanner for array slides	G2505B	Agilent Technologies
Sequencer	ABI PRISM 3100	Perkin-Elmer
Sonication device	Soniprep 150	MSE
Sterile bench	Microflow Laminar	Nunc

Device	Model	Source
Sterile 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, Niederlande

3.1.2 Materials

Product	Source
Electroporation cuvettes	BioRad
Filter paper Miracloth	Calbiochem
Flow paper 3MM	Whatman
Microtiter plates	Roth
Para-film M	American National Can™
Plastics one-way material	Biozym; Eppendorf; Greiner; Roth; Sarstedt
PVDF membrane Immobilon™-P	Millipore
X-ray films Cronex 4, Cronex 10T	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
Bradford-Reagent	Roth
Bromophenol blue	Roth
BSA	Serva
Coomassie Brilliant Blue G-250	BioRad
2,4-Dichlorophenoxyacetic acid (2,4-D)	Sigma
Diethylpyrocarbonat (DEPC)	Roth
N,N-Dimethyl formamide (DMF)	J.T. Baker Chemicals B.V.
Dimethyl sulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
dNTPs	MBI; Roth
Ethylene diaminetetraacetate (EDTA)	AppliChem
Ethidiumbromide	Roth
Gentamycine	Duchefa
PIPES	Roth
Hydrogen peroxide	Roth

Chemical	Source
Herring sperm DNA (HSP)	Sigma
Isonicotinic acid (INA)	ABCR
Isopropylthiogalactosid (IPTG)	Bio Tech Trade
Kanamycine	Sigma
Leptomycine B	LC Laboratories®
Powdered milk	Glücksklee
β-Mercaptoethanol	Roth
Murashige and Skoog medium	Duchefa
Orange G	Sigma
Percoll	Sigma-Aldritch
Phenol	Sigma
Phenylmethane sulfonylchloride (PMSF)	Fluka
Ponceau S	Sigma
Rifampicine	Duchefa
X-ray developer LX24	Kodak
X-ray fixer AL4	Kodak
Salicylic acid (SA)	Merck
Select Agar	Life Technologies
Select Yeast Extract	GIBCO BRL
TEMED	Roth
2,3,5-triiodobenzoic acid (TIBA)	Sigma
Triton X-100	Roth
Trypton	Oxoid
Tween20	Roth
X-Gal	Bio Tech Trade
X-Gluc	Roth
Xylene cyanol FF	Roth
Yeast Nitrogen Base w/o amino acids	Difco

3.1.4 Kits

Kit	Source
BCA Protein Assay Kit	Thermo Scientific
BigDye™ Terminator Cycle Sequencing Ready Reaction Kit v.3.1	Perkin-Elmer Corporation
Enhanced Chemiluminescence Plus™ Kit (ECL+)	GE Healthcare
HiDi-Mix	ABI PRISM™
Invisorb® Spin Plant RNA Mini Kit	Invitek

Megaprime™ DNA Labelling Systems	Amersham Life Science
Nucleo Spin® Extract II	Macherey-Nagel
Nucleo Spin® Plasmid	Macherey-Nagel
Qiagen Plasmid Preparation Kits (Midi, Maxi)	Qiagen
QIAprep Spin Miniprep Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen

3.1.5 Enzymes

Enzyme	**Source**
Biotaq DNA polymerase	Bioline
Cellulase "Onozuka R-10"	Serva
Immolase DNA polymerase	Bioline
iProof high fidelity DNA polymerase	BioRad
Klenow DNA polymerase exo$^-$	MBI Fermentas
Lyticase	Sigma
Macerozyme R-10	Serva
Reverse transcriptase H-	MBI Fermentas
Restriction enzymes	MBI Fermentas, New England Biolabs
RNase A (DNase-free)	Qiagen
RNase inhibitor	MBI Fermentas
T4 DNA-Ligase	MBI Fermentas

3.1.6 Standards

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

3.1.7 Antibodies

Antibody	Specificity	Properties	Reference
αHA-tag		polyclonal from rabbit (1:1000)	Santa Cruz Biotechnology
α–rabbit Ig	Rabbit-Immunoglobulin	polyclonal from donkey, coupled to peroxidase (1:25000)	Amersham
αSCL14 (serum) (SA 2495)	SCL14 full length	polyclonal from rabbit (final bleeding) (1:1000)	Tanja Siemsen, 2005
αTGA2/5 (serum) (SA 4364)	TGA2 and TGA5 C-terminal region including the zipper domain	polyclonal from rabbit (final bleeding) (1:1000)	This work

3.1.8 Nucleic acids

3.1.8.1 Primers

Primers were synthesized by Operon or Invitrogen as indicated in the last line. QuantiTect Primer Assays from Qiagen contain both, forward and reverse primer. They are indicated as "QPA" and are described on:
http://www1.qiagen.com/Products/Pcr/QuantiTect/PrimerAssays.aspx
False QuantiTect primers (FQ) are designed on basis of sequence analysis of a cloned PCR fragment after amplification with the original primer assays. These primers were diluted and mixed to 4 µM stock solution containing forward and reverse primer.

Primer	Sequence 5´→ 3´	Source/ Application
Actin8 forward	GGT TTT CCC CAG TGT TGT TG	Operon/ ChIP
Actin 8 reverse	CTC CAT GTC ATC CCA GTT GC	Operon/ ChIP
AlKeprsen	AGT TCT GTC TTC TGT GAT ACG TG	Operon/ ChIP
AlKeprant	ACT AGC TAT TAA AGG GTG AGA AG	Operon/ ChIP
At_At1g07530_1_SG	*SCL14*	QPA
At_ATGSTU7_1_SG	*GSTU7/ GST25*	QPA
At_At5g61820_2_SG	*MAC9.6/ MtN19-like*	QPA
At_At3g28740_1_SG	*CYP81D11*	QPA
At_At1g77450_1_SG	*NAC32*	QPA
At_ADH1_1_SG	*ADH1*	QPA
At_ATMRP2_1_SG	*ATMRP2*	QPA

At_LOX1_1_SG	*LOX1*	QPA
At_ATGSTU1_1_SG	*GSTU1*	QPA
At_ PR1_1_SG	*PR-1*	QPA
At_LCR70/PDF2.1_1_SG	*PDF1.2*	QPA
BcActAfwd	CTT CGT GTA GCA CCA GAG GA	Operon/ qRT PCR, (Gronover et al., 2001)
BcActArevalt	TCA ACA CGA GCA ATG GCG	Operon/ qRT PCR
Cyp81sen	AAA GTA GGT TTG TTG GTT TCA AG	Operon/ ChIP
Cyp81ant	CTG ATT TTA TAG TGC ATT TGG AAG	Operon/ ChIP
FQCypfwd	TTA TGA TAC TTG CCG GGA CTG	FQ (false QPA), Operon
FQCyprev	TCG ATT TCG GTC TTT GCC	FQ (false QPA), Operon
FQGst25sen	GAA GAC CGG AAA AGA GAG AG	FQ (false QPA), Operon
FQGst25ant	CAA AGT CGC CAC AAT ATC C	FQ (false QPA), Operon
FQMtn19sen	CGA TTC GTT CTC ACG AGA TG	FQ (false QPA), Operon
FQMtn19ant	TTG GCT CGG CGA TAT GC	FQ (false QPA), Operon
Gst25prsen	CTA AGA CCC CCA GTA ATT AAT TC	Operon/ ChIP
Gst25prant	TGG ACT AAG GTT AAT AGG TTA TG	Operon/ ChIP
Mtn19prsen	TGG TCG TCT ATC TAC TTT TGT TTG	Operon/ ChIP
Mtn19prant	ATT CGG GAG TTG TCT ATT TAA TAC	Operon/ ChIP
PP2a sense	AAG CAG CGT AAT CGG TAG G	FQ/ qRT PCR
PP2a anti	GCA CAG CAA TCG GGT ATA AAG	FQ/ qRT PCR
Rev23	TTC ACA CAG GAA ACA GCT ATG ACC	Invitrogen/ cloning
SCL14woHArev	ACC TGA ACA AGA ACC GTC AGG GCT ATC AC	Invitrogen/ cloning
SCL14woHAub	CGC TGA CAA GCT GAC TCT AGT AAA AAT GGG TTC TTA TCC GGA TGG ATT CC	Invitrogen/ cloning
TGA2anti	CCA ATG AAT TCT CAC TCT CTG GGTCGA GCA AGC	Invitrogen/ cloning

TGA2sense	CCT AAG GAT CCG CTT ATG TTC AGC AGC TAG AGA AC	Invitrogen/ cloning
Uni24	ACG ACG TTG TAA AAC GAC GGC CAG	Invitrogen/ cloning

3.1.8.2 Plasmids

Plasmid	Description	Reference
pAlligator2	Gateway™ vector for plant transformation, contains the CaMV 35S promoter, the *nos* terminator, a 3x HA-tag (N-terminal), and a GFP selection marker under control of a seed specific promoter At2S3, *spn*^{r}	Parcy, http://www.isv.cnrs-gif.fr/JG/alligator/intro.html
pAlligator2/SCL14	pAlligator2 derivative, contains the CDS of SCL14-cDNA under control of the CaMV 35S promoter, *spn*^{r}	This work
pAlligator2/SCL14-HA	pAlligator2 derivative, contains the CDS of SCL14-cDNA fused to the 3´-end of the 3x HA-tag under control of the CaMV 35S promoter, *spn*^{r}	Siemsen, 2005
pGAD424	Vector for expression of GAD fusion proteins under control of the *ADH1* promoter in yeast, $GAL4_{(768-881)}$ activation domain, *LEU2*, *amp*^{r}	Clontech
pGAD424/SCL14	pGAD424 derivative, contains the CDS of the *SCL14*-cDNA and 3'UTR fused to the 3´-end of the *GAL4-AD*, *LEU2*, *amp*^{r}	Siemsen, 2005
pGAD424/SCL14 1-161	pGAD424 derivative, contains a truncated CDS of the *SCL14*-cDNA (1-479) fused to the 3´-end of the *GAL4-AD*, *LEU2*, *amp*^{r}	This work
pGAD424/SCL14 1-381	pGAD424 derivative, contains a truncated CDS of the *SCL14*-cDNA (1-1139) fused to the 3´-end of the *GAL4-AD*, *LEU2*, *amp*^{r}	This work

pGBT9	Vector for expression of GBD fusion proteins under control of the *ADH1* promoter in yeast, $GAL4_{(1\text{-}147)}$ DNA binding domain, *TRP1*, amp^r	Clontech
pGBT9/TGA2	pGBT9 derivative, contains the CDS of the *TGA2*-cDNA fused to the 5´-end of the GAL4-DNA-BD under control of the *ADH1* promoter, *TRP*1, amp^r	(Weigel et al., 2001)
pGEX/2.1ct	pGEX-4T1 derivative, contains the truncated CDS of the *TGA2.1*-cDNA from tobacco (only the C-terminus), for expression of GST fusion proteins	
pGEX/TGA2ct	pGEX-4T1 derivative, contains the C-terminal part (plus zipper region) of the CDS of the *TGA2*-cDNA (817 bp)	This work
pGEX/TGA5ct	pGEX-4T1 derivative, contains the C-terminal part (plus zipper region) of the CDS of the *TGA5*-cDNA	This work
pHBT	Vector for transient expression in plant cells, pHBTL-sGFP derivative, deletion of sGFP gene by *Nco*I / *Not*I restriction, Klenow fill in and religation, amp^r	(Heinekamp et al., 2002)
pHBTL-sGFP	pHBT-sGFP derivative (Sheen, 1993), contains the sGFP-S65T gene between the *HBT* promoter and the *nos* terminator, additional *Kpn*I and *Eco*RI restriction sites in front of the *Bam*HI site, amp^r	(Nickolov, 2003)
pHBTL/SCL14-sGFP	pHBTL derivative, contains the CDS of the SCL14-cDNA fused to the 5´-end of the sGFP under control of the *HBT* promoter, amp^r	(Siemsen, 2005)
pSK-T	Vector for cloning and sequencing, *lacZ*α, amp^r	Kriete, unpublished

3.1.9 Organisms

3.1.9.1 Bacteria

Species	Properties	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 (Nalr), *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)

3.1.9.2 Yeast strains

Strain	Properties	Reference
PJ69-4a	MATα, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, GAL2-ADE2, LYS2 ::GAL1-HIS3, met2::GAL7-*lac*Z	(James et al., 1996)

3.1.9.3 Fungal cultivars

Strain	Properties	Reference
B. cinerea BH/1	Infects *A. thaliana* Col-0	Kindly provided by Brigitte Mauch-Mani, University of Neuchatel
B. cinerea B1.26	Infects *A. thaliana* Col-0	Kindly provided by Andreas von Tiedemann, University of Göttingen

3.1.9.4 Plant genotypes

Genotype	Description	Reference
Columbia, Col-0	Wildtype	NASC Stock Nr. N1092, NASC 2002
Col-0/ *as*-1:*GUS*	Plants expressing the *GUS* reporter gene under control of the truncated CaMV *35S* (-90) promoter, used as "wildtype" control in this work	(Redman et al., 2002)
coi1-1 mutant	Knock out line, lacking COI1, impaired in most JA dependent responses	(Feys et al., 1994) (Xie et al., 1998)
npr1-1 mutant	Knock out line lacking NPR1	(Cao et al., 1994)
as-1:GUS/ 35S:SCL14 expressing plants	Over-expression line, expressing the *SCL14* gene under control of the CaMV *35S* promoter, expresses GFP in seeds for selection	This work
as-1:GUS/ 35S:SCL14-HA expressing plants	Over-expression 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
scl14 mutant (SALK_126931)	Knock out line, expressing a T-DNA insertion 55 bp upstream of the ATG from *SCL14* gene, km^r	SALK Stock Centre, Nottingham
as-1:GUS/ scl14	*scl14* mutant, expressing *GUS* reporter gene under control of truncated CaMV *35S* promoter, line #14 (~5 *as-1:GUS* insertions) was used during this work, km^r	Siemsen, 2005
as-1:GUS/ scl14/ 35S:SCL14	*scl14* mutant (#14) complemented with *SCL14* under control of the CaMV *35S* promoter, expresses GFP in seeds for selection, km^r	This work

as-1:GUS/ scl14/ 35S:SCL14-HA	*scl14* mutant (#14) complemented with *SCL14* fused to a HA-tag under control of the CaMV *35S* promoter, expresses GFP in seeds for selection, km^r	This work
as-1:GUS/ scl14/ SCL31 RNAi	*scl14* mutant (#14) also expressing a RNAi construct targeting *SCL31* mRNA, km^r	Fode, unpublished
tga2 tga5 tga6 triple mutant (*tga2,5,6* mutant)	Knock out line lacking all three class II TGA transcription factors, impaired in SAR, kindly provided by Y. Zhang	Zhang et al., 2003

3.1.10 Growing media

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
Malt extract medium for fungi	48 g/L Malt extract agar (Merck), 2 g/L Yeast extract
MS medium for plants	4.4 g/L MS medium; pH 5.7 with KOH
PDA medium for fungi	4 g/L Potato dextrose agar (Fluka)
PDB medium for fungi	12 g/L Potato dextrose broth (Sigma)
SD medium for yeast	6.7 g/L Yeast nitrogen base without amino acids, pH 5.8; 100 mL/L DROP OUT-Stock (10x); 50 mL/L Glucose (40 %); amino acids (for solid medium: 14 g/L Select agar)
YPAD medium for yeast	20 g/L Tryptone / Peptone; 10 g/L Select Yeast extract; 50 mL/L Glucose (40 %); 20 mL/L Adenine (Hemi sulfate) (0.2 %); pH 5.8 (for solid medium: 14 g/L Select agar)

3.1.11 Standard buffers

PBS (10 x)	1.4 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.3
PBS-T (1 x)	1 x PBS with 0.05 % (v/v) Tween-20
TAE (20 x)	0.8 M Tris, 2.3 % (v/v) acetic acid, 20 mM EDTA
TE	10 mM Tris, 1 mM EDTA, pH 7.5

3.2 Methods

3.2.1 Standard molecular biology methods

3.2.1.1 Isolation of plasmid DNA from *E. coli*

3.2.1.1.1 Alkaline lysis

Small amounts of plasmid DNA for analytical purposes were isolated from *E. coli* using a modification of the alkaline lysis method. *E. coli* overnight culture (stationary phase) of 1.5 mL was collected by centrifugation at 13000 rpm for 1 minute. The supernatant was removed and the cells were resuspended in 100 µL of buffer I for plasmid DNA isolation (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 µg/ µL RNase A). The cell suspension was lysed for 5 minutes on ice using 200 µL of buffer II (0.2 M NaOH; 1 % (w/v) SDS). The suspension was neutralized with 150 µL of buffer III (29.4 g potassium acetate; 5 mL formic acid and water till 100 mL). The solution was mixed well by inverting 6-8 times and the suspension was centrifuged for 10 minutes at 13000 rpm at room temperature. The aqueous solution (~400 µL) was transferred into a new microcentrifuge tube containing 1 mL of 96 % (v/v) ethanol. The DNA was left to precipitate for 20 min at -20°C. Plasmid DNA was collected by centrifugation for 10 minutes at 13000 rpm and 4°C. The pellet was washed with 70 % (v/v) ethanol and air-dried for 10 minutes at 37°C. The DNA was dissolved in 20 µL of EB buffer (10 mM Tris-HCl, pH 8.5).

3.2.1.1.2 High-quality plasmid DNA isolation: Spinprep

For sequencing and yeast transformation purposes, high-purity plasmid DNA was isolated using QIAprep (Qiagen) or Nucleospin Mini kit (Machery&Nagel) following the manufacturer's instructions. Optional steps were always followed according to the manufacturer's recommendation. A 3 mL overnight culture was used to isolate plasmid and the isolated DNA was eluted with 50 µL (high copy) or 30 µL (low copy) EB buffer or water (ultra pure).
Larger amounts of plasmid DNA from E. coli with high purity were isolated using Qiagen or Macherey-Nagel Midi and Maxi kit depending upon the required end concentration. Manufacturer's protocol including the optional recommendations was followed and final elution volume depended on the plasmid copy number, size of the DNA pellet to be eluted and final concentration required.

3.2.1.2 Determination of DNA/ RNA concentrations

The concentration of nucleic acids was estimated by measuring their absorption in a spectrophotometer at a wavelength of 260 nm (maximum nucleic acid absorption value; due to the π-electron systems of the heterocycles of the nucleotides). In a cuvette having 10 mm path-length where OD_{260} reading is 1 corresponds to 50 and 40 µg/ mL double-stranded DNA and RNA, respectively. Absorption at 280 nm (for the presence of aromatic rings from amino acids and phenol compounds) was used to give information about the purity of the DNA or RNA sample, where an optimal ratio OD_{260}/OD_{280} is in the range of 1.9-2.0 for RNA and 1.8 for DNA. DNA concentrations lower than 100 ng/ µL were measured on an agarose gel using the Gene Ruler Ladder Mix.

3.2.1.3 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 x 7 cm x 0.3 cm, 16 lanes) with 1x TAE as running buffer. DNA fragments ranging between 500 bp and 14 kb were run in an agarose gel concentration of 1 % where DNA fragments with lower size were run in a 2 % agarose gel. DNA samples were mixed with 1/10 volume of 10x DNA loading buffer, loaded in separate lanes and run at 120 V for 40-45 min. Ethidiumbromide 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 particular band fragments were needed to cut out, detection was done using larger wavelength UV light (320 nm). Before exposure to the UV light, the gel was rinsed briefly in H_2O to reduce background staining. In a gel-documentation station, gels were visualized on a UV-transilluminator and documented. The sizes and amount of the DNA fragments were determined using DNA standards.
The elution of DNA fragments from agarose gel was done using the QIAquick or Nucleospin Extract II Gel Extraction kit following the manufacturer's instructions. The eluted fragments were verified by electrophoresis as described above.

3.2.1.4 Restriction digestion of DNA

Type II endonucleases were used to digest a double stranded DNA molecule for analytical and cloning purposes. The enzymes cut the DNA either as 5′ or 3′ "sticky" overhangs or as blunt ends. The digestion reactions were incubated in a buffer system optimized for the used enzyme and in the case of double digestion a universal buffer system was used. The activity of the restriction enzymes was estimated in "units" (U), where 1 U was defined as that amount of enzyme cutting completely 1 µg of λ DNA in 60 minutes at optimal conditions. The minimal amount of enzyme necessary for each restriction was determined according to the following formula:
U = (bp[λ] x No. of restriction sites in target DNA) / (No. of restriction sites in [λ] x bp of target DNA) with λ = 48500 bp
The incubation temperature was 37°C unless otherwise mentioned for particular restriction enzyme. Due to the adverse effect of high glycerol concentration, the total volume of restriction enzymes should not extend more than 10% in the restriction mix.

3.2.1.5 Ligation of DNA fragments

The conventional cloning of a DNA fragment into a selected plasmid was performed using the T4-DNA ligase enzyme, which is able to catalyze the formation of a phosphodiesther chemical bond between free 5′-phosphate and 3′-OH groups of double stranded DNA fragments and vectors. The donor DNA fragment (10x accesses over the vector) was incubated with the vector DNA, 2 µL of ligation buffer and 1 µL of T4-DNA ligase for 2 hours at room temperature. The ligation of DNA fragments with blunt ends was performed in the presence of 5 % (w/v) PEG 4000 with the ligation mix described above. Ligase activity was destroyed by heating at 65°C for 10 min before using the ligated DNA for transformation.

3.2.1.6 Sequencing of DNA

The 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 that will lead to stop its elongation in a PCR reaction. Each of dideoxynucleotides 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 RR mix (ready reaction) and H_2O up to 10 µL. The samples were subjected to 25 cycles of: 10 seconds at 95°C, 5 seconds at 50°C, 4 minutes at 60°C in a thermocycler. The DNA product was precipitated using 9.5 µL water and 30.5 µL of absolute ethanol and left for 1 hour. The DNA was collected by centrifugation for 20 minutes at 13000 rpm. The pellet was washed using 125 µL 70% ethanol and then centrifuged for 10 minutes at 13000 rpm. The pellet was dried at 95°C for one minute and resuspended in 10 µL of

HiDi-reagent. The samples were placed on ice. The reactions were loaded on an ABI-Prism 3100 capillary electrophoresis sequencing station for analysis.

3.2.1.7 Cloning of vectors

3.2.1.7.1 pAlligator2-SCL14

This vector was constructed by overlapping PCR from pAlligator2-SCL14-HA and pUCA7. Primers for first fragment: SCL14woHAub and SCL14woHArev on pAligator2-SCL14-HA. Primers for second fragment: Rev23 and Alligator-ohneHA on pUCA7 (provided by C. Thurow). The PCR on both fragments (15 ng) was performed with primers Rev23 and SCL14woHA antisense. This product was cut with EcoRV and BspHI and ligated into pAlligator-SCL14-HA cut with EcoRV and BspHI.

3.2.1.7.2 pGAD424-SCL14 1-161, 1-381

Deletion derivatives of SCL14 were created in the pGAD424-SCL14 plasmid.
SCL14 (aa 1-161): pGAD424-SCL14 plasmid was cut with REN NheI and religated without the cut-off (middle part) of SCL14 (bp 479 – bp 1996).
SCL14 (aa 1-381): pGAD424-SCL14 plasmid was cut with REN BcuI and religated without the cut-off (C terminus) of SCL14 (bp 1139 – bp 2716:3'-UTR).

3.2.1.7.3 pGEX-TGA2ct, TGA5ct

To express GST-fused TGA proteins for antibody generation the C-terminal parts (including zipper) of TGA2 and TGA5 were cloned into pGEX-2.1C-term plasmids.
TGA5: pGBT9-TGA5 was cut with RENs NdeI and SalI and the TGA5 fragment was ligated into the equally cut pGEX-2.1C-term.
TGA2: PCR with primers TGA2sense and TGA2anti produced a TGA2ct fragment with addition of a BamHI (5') and an EcoRI (3') restriction site. This product was cut with RENs BamHI and EcoRI and ligated into the equally cut vector pGEX-2.1C-term.
These vectors were used to produce TGA2ct-GST and TGA5ct-GST fusion proteins, which were sent to Eurogentec as antigens for antibody production.

3.2.1.8 Gene transfer to *E. coli*

E. coli cells have no competent nature, *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. Two transformation methods were used to transform competent bacteria cells: The heat shock and the electroporation. The heat shock method was used only to transform *E. coli* chemical

competent cells (Hanahan, 1983). In brief, 200 µL competent *E. coli* cells were thawed on ice for 20 min, 50 ng of plasmid DNA was added to the cells and mixed gently. The mixture was incubated on ice for 30 minutes. The cells were heat shocked for 90 s at 42°C. 700 µL of LB medium were added to the tube and the suspension was mixed on a roller for 45-60 min at 37°C depending on selectable antibiotic resistance marker. Different volumes of the culture were plated on plates containing LB medium supplemented with antibiotics. The plates were incubated overnight at 37°C.

The transformation using electroporation was done for *E. coli* cells according to a slight modification of (Dower et al., 1988). The electroporation was done using a Gene Pulser II. Bacterial competent cells were thawed on ice slowly before adding 2 µL of plasmid DNA. The mixture was transferred into an ice-cooled electroporation cuvette (2 mm electrode distance). The cuvette was subjected to electroporation at 25 µF, 2.5 kV, 200 Ω. The cells were suspended immediately with 1 mL LB medium and incubated for 45-60 min at 37°C. Different volumes of the culture were plated on LB media supplemented with antibiotics and incubated overnight (12-16 hours) at 37°C.

3.2.1.9 Gene transfer to *S. cerevisiae*

Competent yeast cells were used for transformation. For generation of competent cells a yeast colony (Strain PJ69-4a) from a plate was used to inoculate 10 mL of YPAD medium. Subsequently, the medium was incubated o/n at 30°C. Cells were harvested the next day by centrifugation (3000 rpm for 5 min). After removal of supernatant, cells were resuspended in residual medium and transferred to 200 mL YPAD medium. Growing of cells at 30°C was extended until the culture reached OD_{600} = 0.6. Cells were pelleted as 4 x 50 mL aliquots by centrifugation like before and supernatant was discarded. Subsequently, cells were resuspended in each 10 mL Solution A (10 mM Bicine pH 8.35, 1 mM sorbitol, 3 % ethylenglycol) and centrifuged again. After resuspension in each 1 mL Solution A the 4 samples were combined. Cells were frozen as 100 µL aliquots at -80°C.
1-2 µg plasmid DNA and 50 µg HSP DNA were added to frozen competent yeast cells (100 µL per sample). Cells were thawed for 3 min at 37°C. 1 mL of Solution B (200 mM Bicine pH 8.35, 40 % PEG 1000) was added to each sample. Samples were mixed, incubated for 60 min at 30 °C and centrifuged (10 s, 13000 rpm). After removal of supernatant cells were washed in 800 µL Solution C (10 mM Bicine pH 8.35, 150 mM NaCl) and centrifuged again. Subsequently, cells were resuspended in 100 µL Solution C and stroked out on selective SD plates.

3.2.1.10 Gene transfer to *A. tumefaciens*

Competent cells of *Agrobacterium tumefaciens* GV3101 were transformed by electroporation method. Cells were thawed on ice, mixed with respective plasmid DNA and transferred to an electroporation cuvette. Electric pulse (2.5 kV, 25 µF, 400 Ω) was applied for ~5 s. Subsequently, 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 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) to control 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 resolved in 5 % sucrose solution to an OD_{600} of 0.8. Silvet-L77 (0.05 %) was added to this solution prior to *A. thaliana* transformation.

3.2.1.11 *Agrobacterium* mediated gene transfer to *A. thaliana*

Transformation of *A. thaliana* with *Agrobacterium* was performed after (Clough, 2000). Therefore, flowering plants were dipped into an *Agrobacterium* solution. Plants were subsequently cultured to seed development. Selection was performed with respective selection markers on the integrated transgenic DNA.

3.2.2 Stress induction in *A. thaliana*

3.2.2.1 SA/ INA

Arabidopsis plants were grown on soil under short-day conditions (8 h light) for five to six weeks. Plantlets were sprayed with 1 mM SA (in water) or 1 mM INA (in EtOH) and incubated under same conditions for additional 10 to 12 hours. Control plants were sprayed with water + EtOH (0.1 %) and incubated for the same time spans or harvested before treatment. All controls are depicted as 0 h.

3.2.2.2 2,4-D

Arabidopsis plants were grown on soil under long-day conditions (16 h light) for three to four weeks. Plantlets were washed out from the soil and floated in 50 mM potassium phosphate buffer pH 5.8 containing either 0.1 mM 2,4-D (in DMSO) or 0.1 % DMSO. Incubation was performed under growing conditions for additional 10 hours. Control plants were floated in buffer + DMSO and incubated for the same time spans or harvested before treatment. All controls are depicted as 0 h.

3.2.2.3 TIBA

Arabidopsis plants were grown on MS medium under long-day conditions (16 h light) for three weeks. Plantlets were sprayed with 0.1 mM TIBA (in DMSO) and incubated under same conditions for additional 10 hours. Control plants were sprayed with water + DMSO (0.1 %) and incubated for the same time spans or harvested before treatment. All controls are depicted as 0 h.

3.2.2.4 Hydrogen peroxide

Arabidopsis plants were grown on MS medium under long-day conditions (16 h light) for three weeks. Plantlets were transferred to 50 mM potassium phosphate buffer pH 5.8 containing 16 mM hydrogen peroxide and floated under growing conditions for additional 8 hours. Control plants were floated on buffer only and incubated for the same time spans. All controls are depicted as 0 h.

3.2.2.4.1 Germination assays

Germination assays were performed under long-day conditions (16 h light). Seeds from all genotypes were sterilized and directly sowed on MS medium lacking sucrose. MS medium contained either nothing, 0.1 mM TIBA, 0.1 mM INA or 0.05 mM SA. Plants were grown for three weeks on these plates before pictures were taken and plantlets were weighted.

3.2.2.5 Infection of *Arabidopsis* with *B. cinerea*

Botrytis cinerea strains: BH/1 and B1.26

3.2.2.5.1 Culturing

Pieces of mycelium from a PDA plate (growing plate) or spores from glycerol stock (-80°C) were transferred to fresh malt extract plates (sporulation plate). Growing of the fungi was carried out in darkness at 20-24°C for about 7-12 days. Sporulation was initiated by UV irradiation of the plates (under a UV lamp) for 24 h. The plates were subsequently incubated in darkness till full sporulation occurs (4-7 days). Collected spores (see below) were frozen as glycerol stocks (30 %) in aliquots (50 µL) at -80°C.

3.2.2.5.2 Collection of spores

Five mL of PDB medium were dropped onto a plate with a full sporulating fungus. The fungal hyphes were scraped off with a Drygalski applicator and the medium containing the hyphes and spores was filtered through three layers of gauze bandage. Spores were counted under the microscope in a Thomma counting chamber (no. of spores in the whole chamber x 10,000 = spores/mL medium) and diluted with PDB medium to 1 x 10^6 spores/mL.

3.2.2.5.3 Infection of plants

The collected spores in the PDB medium were incubated in light at RT for 2-3 h. Plants with full expanded leaves were inoculated with 5 µL of spore solution (1 x 10^6 spores/mL) on each leaf. The inoculated plants were kept under long day conditions for 24 h. Optional: The inoculated plants were covered with plastic lids (24 hpi) to maintain a higher humidity to provide the fungi with optimized growing conditions. Pictures were taken and infected leaves were harvested during infection process every 24 h.

3.2.3 Transcriptome analysis

3.2.3.1 RNA extraction

The extraction method based on TRIZOL extraction can be used to extract RNA, DNA and proteins from plants (Chomczynski, 1993). This method uses a Phenol/ Chloroform (dichloromethane) extraction to solve RNA in the aqueous phase while other parts like chlorophyll is solved in the hydrophobic chloroform phase. The two thiocyanates in the extraction buffer inhibit RNAses. After grinding of the plant material under liquid nitrogen 1 mL extraction buffer was added to ~150 mg plant material. After shaking for 15 min at RT, chloroform (200 µL) was added to each sample. After an additional shaking step (15 min, RT) and centrifugation (12000 rpm, 35 min, 4°C) the supernatant (700 µL) was transferred to new microcentrifuge tubes. Precipitation buffer (HSPB) and 2-propanol (each 250 µL) were added and the samples were incubated for 10 min at RT and centrifuged (12000 rpm, 20 min, 4°C). After removing the supernatant, samples were dried at RT. The dried pellets were resolved in 50 – 100 µL water (ultra pure). Concentration was measured as described in 2.2.1.2.

3.2.3.2 Quantitative Realtime RT-PCR (qRT-PCR)

RNA extraction of plant leaf material was performed as described above. DNaseI restriction was done before cDNA synthesis. 1 µg of RNA, 1 µL of 10x reaction buffer with $MgCl_2$ (Fermentas, St. Leon-Roth, Germany) and desoxyribonuclease I (DNaseI), RNase-free was added with water to a final reaction volume of 10 µL. The mixture was incubated at 37°C for 30 minutes. To denatured the DNaseI 1 µL 25 mM EDTA was added and incubated at 65°C for 10 minutes. cDNA synthesis was performed with 1 µg total RNA (DNA-free), 20 pmol of oligo-dT primer and 200 pmol of random nonamer oligonucleotides. Water was added to a final reaction volume of 12.5 µL. The mixture was heated to 70°C for 10 min, 20 nmol dNTPs, 4 µL 5x reaction buffer (Fermentas, St. Leon-Roth, Germany) and 30 u ribonuclease inhibitor (Eppendorf, Hamburg, Germany) were added and the mixture was heated to 37°C for 10 min. 100 u of RevertAid™ H Minus M-MuLV reverse transcriptase (Fermentas, St. Leon-Roth, Germany) was added (final volume 20 µL) and the mixture was incubated at 42°C for 70 min, then heated to 70°C for 10 min. The iCycler System (Bio Rad, Hercules, CA, USA) was used for the

amplification and quantification of cDNA using QuantiTect Primer Assays (Qiagen, //www1.qiagen.com/Products/Pcr/QuantiTect/PrimerAssays.aspx) for the respective genes and for the *PDF2* subunit *PP2a* as reference gene. The amplification mix consisted of 1x NH_4-reaction buffer (Bioline, Luckenwalde, Germany); 2 mM $MgCl_2$; 100 µM of dNTPs; 0.4 µM of primers, 0.25 u BIOTaq DNA polymerase (Bioline Luckenwalde, Germany); 10 nM Fluoresceine (BioRad, Hercules, CA, USA); 100,000 times diluted SYBR Green I solution (Cambrex, Rockland, ME, USA); 1 µL of a 1:10 dilution of cDNA as template and water (ultra pure) added to a total volume of 25 µL. PCR consisted of a 6 min initial denaturation step at 95°C followed by 40 cycles of 20 s at 95°C, 20 s at 55°C and 40 s at 72°C.

3.2.3.3 Micro array analysis

Untreated plants were compared with TIBA-induced plants using pools of about 50 two week old plantlets in a square design comparing each possible condition and genotype combination (induced state comparing wildtype and *scl14* mutant was doubled) with ten micro arrays (Landgrebe et al., 2004). Leaf material from induced plants harvested at 10 hpi was frozen and ground in liquid nitrogen. Total RNA was extracted according to the TRIZOL method (Invitrogen GmbH, Karlsruhe, Germany) and purified using the RNeasyMini Kit (Qiagen, Hilden, Germany). Micro arrays spotted with the *Arabidopsis* Genome Oligo Set version 3.0 (Qiagen, Hilden, Germany) were obtained from D. Galbraith (University of Arizona, Tucson Arizona, USA). Slides were rehydrated at 60^{o}C and UV-cross linked according to the supplier's web page. The Amino Allyl MessageAmp^{TM} II aRNA Amplifcation Kit (Ambion, Darmstadt, Germany) was used for cDNA synthesis, *in vitro* transcription and Cy3/Cy5-labeling of the 5-(3-aminoallyl)-UTP-containing aRNAs with the following modifications: Purification and concentration of double-stranded cDNA was done using the DNAclear^{TM} Kit (Ambion, Darmstadt, Germany), and the large scale transcription reaction was purified with the MEGAclear^{TM} Kit (Ambion, Darmstadt, Germany). Hybridization and washing was done as recommended on the supplier's web page (http://www.ag.arizona.edu/microarray/). The slides were scanned with a G2505B Micro array Scanner (Agilent Technologies, Böblingen, Germany). Non-linear loess regression was used to adjust the micro array data for technical and biological effects. To increase comparison of all slides, each normalized dataset was scaled by division with its standard deviation. The differences in gene expression were computed using a mixed model statistics in references to the applied design (ANOVA (Langrebe et al., 2006) allowing estimation of biological and technical variance. To estimate the significance, *t*-tests were applied for each feature. Multiplicity adjustment of the resulting p-values was done by the Benjamini-Hochberg procedure (Bretz et al., 2005). Normalization and statistic computation was done independently for a high and a low gain dataset, allowing the recovery of lost data from saturated spots.

3.2.4 Proteome analysis

3.2.4.1 Whole cell protein extracts

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

3.2.4.2 Determination of concentrations of proteins

Protein concentration was estimated by two different methods. A colorimetric assay was used to determine the concentration from proteins extracted without detergent usage according to (Bradford and Williams, 1976). The assay was conducted by pipetting equal amounts of protein extract into a microtiter plate containing 200 µL of 5-fold diluted Bradford reagent. The OD595 was measured with a MRX plate reader (Dynex). Protein concentrations were calculated with the help of a standard curve derived from different BSA protein amounts (1, 3 and 6 µg) on the same plate. Proteins isolated using buffers containing detergents were either defined to equal amounts in a coomassie stained SDS gel (scanned and analysed with TINA2.0) or measured with the BCA protein assay kit (Thermo scientific) according to the instruction manual.

3.2.4.3 SDS-PAGE

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

µL pre-stained protein ladder was loaded on each gel for the estimation of 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 on SDS-PAGE. The gels were incubated with coomassie staining solution (colloidal coomassie) o/n. The gels were destained in water o/n.
Colloidal coomassie consists of 400 mL solution A (contain 40 g ammonium sulphate and 8 mL phosphoric acid) and 10 mL solution B (contain 0.5 g coomassie brilliant blue G250, this has to be solved shaking at least for 0.5 h). Each gel was stained in 40 mL colloidal coomassie complemented with 10 mL methanol.

3.2.4.5 Western blot

The proteins separated in the SDS-PAGE were blotted onto a PVDF membrane using semi-dry blotting method in an electric field between two graphite plates. The PVDF membrane was activated before blotting using MeOH. For the transfer of proteins from the gel to the membrane, the gel on top of the membrane was sandwiched between two 3- layers of Whatman papers (pre-soaked with transfer buffer). The whole arrangement was placed within a blot apparatus and transfer was performed under amperage of one mA/cm^2 for 1.4 hours. (Optional: Ponceau S staining was done to observe the success of the transfer. De-staining was done using 1 x PBS.) After blotting the membrane was dried between two layers of Whatman paper. The standard was marked on the membrane with an iMark (pen containing pre-immune serum from rabbit) for later detection of standard bands with the second antibody and ECL kit to visualize them on the film. After 5 min the membrane was reactivated in MeOH and non-specific binding to the proteins on the membrane was prevented by blocking the membrane with non-fat dried milk powder (5 % in 1x PBST) o/n at 4°C on a shaking platform. The detection of specific proteins on the membrane was performed using an antiserum directed against SCL14 or TGA2/5 in a 1:1000 dilution in 1x PBST (with 0.5 % milk powder). The membrane was therefore incubated with the respective antiserum for 2 h at RT on a shaking platform. The incubation with the second antibody (anti-rabbit 1:25000 in 1x PBST) was performed for 1 h at RT on a shaking platform. This second antibody is conjugated to horseradish peroxidase (HRP). The HRP can utilize the enhanced chemi-luminescent substrate (ECL, GE Healthcare, incubation of the membrane in ECL mix for 5 min) emitting luminescence, which allows visualization of the membrane bound proteins on autoradiography films. The films were exposed to the membrane in detection cassettes for 30 s up to 10 min depending upon the strength of chemi-luminescence signal generated by the respective amounts of bound protein.

3.2.4.6 ONPG assay

Determination of transcriptional activation of reporter gene *lacZ* was done from its coded product, β-galactosidase catalyzed breakdown of substrate *o*-nitrophenyl-β-D galactopyranoside (ONPG). The β-galactosidase catalyzed the colourless substrate ONPG into the fluorescent *o*-nitrophenyle substance, which could be quantified at 420 nm. Fresh clones were used to inoculate 2 ml SD media with appropriate selection for the plasmid and supplemented with respective amino acids grown overnight at 30°C with 200-220 rpm shaking. Cells were harvested from 2 mL of overnight culture by centrifugation (13000 rpm, 1 min, RT) and resuspended in 675 µL H buffer (100 mM HEPES/KOH, pH 7.0; 150 mM NaCl; 2 mM $MgCl_2$; 1 % (w/v) BSA) with one yeast-lacking sample as control. From each sample 10 µL aliquots were diluted with 190 µL in a round-bottom 96-well plate for measuring the cell density at OD_{595}. The rest 665 µL cells were lysed by addition of 55 µL chloroform and 55 µL 0.1 % (w/v) SDS along with vigorous vortexing for 1 min. 125 µL of ONPG substrate (4 mg/ml ONPG in H buffer) were added to the samples. All samples were incubated at 37°C until they turn yellow (15 min up to 2 h). Necessary time required for the solution to become yellow was recorded. The reaction was stopped upon appearance of yellow colour with 400 µL 1 M Na_2CO_3 and samples were centrifuged (13000 rpm, 5 min, RT). 200 µL of the supernatant was transferred into microtiter plate for quantification at 420 nm in a spectrophotometer. The calculation of β-galactosidase activity was done according to the following formula:

β-galactosidase activity = $(1000 \times OD_{420})/(V \times t \times OD_{600})$

Where, V was the volume (200 µL) and t was the reaction time. The β-galactosidase activity was expressed in Miller Units (MU).

3.2.4.7 Localization of proteins: GFP-fusions in BY-2 protoplasts

For the localization studies, BY-2 cells from tobacco liquid cell cultures were used. For each sample, 20 mL of the cell culture was transferred to a 50 mL centrifugation tube and centrifuged for 5 min (1550 rpm, RT, soft start and stop). After removal of the supernatant the cells were resuspended in the rest of the liquid phase. The cells were washed with 20 mL osmoticum (0.5 % BSA, 0.01 % β-mercaptoethanol, 0.05 M $CaCl_2$, 0.01 M sodium acetate, 0.25 M mannitol, set to pH 5.8 with HCl) by centrifugation for 5 min (1550 rpm, RT, soft start and stop). The volume of the supernatant in the tubes was reduced to 5 mL and the cells were resuspended. After addition of 40 mL enzyme solution (1 % cellulose onozuka RS, 0.5 % macerozyme, 0.1 % pectinase) the suspension was divided up to three Petri dishes. The dishes were closed with para-film and incubated in the dark o/n at 25°C. On the next day, protoplasts were transferred to centrifugation tubes and centrifuged (100 ***g***, 5 min, RT, soft start and stop). After removal of supernatant, protoplasts were resuspended in residual medium and washed in 20 mL osmoticum. Centrifugation and resuspension was done like before. Subsequently, 10 mL W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, set to pH 5.8 with HCl) were added and centrifugation was repeated. This step was repeated with 5 mL W5 solution and the three protoplast samples were combined.

Cell number was counted in a counting chamber (Fuchs-Rosenthal): counted cells per 4 squares x volume (µL) / 0,2 µL
Protoplasts were incubated at 4°C for 30-60 min in the dark and centrifuged like before. After complete removal of supernatant, MMM solution (15 mM $MgCl_2$, 0.1 % MES, 0.5 M mannitol, set to pH 5.8 with KOH) was added to dilute the protoplasts to 2 x 10^6 cells/ mL. 30 µg of DNA was added to 300 µL of protoplast suspension. These samples were mixed with 300 µL PEG solution (40 % PEG 4000, 0.4 M mannitol, 0.1 M $Ca(NO3)_2$, set pH to 8-9 with KOH) and incubated for 20 min at RT. Subsequently 10 mL W5 solution were added and samples were centrifuged like before. After removal of supernatant 700 µL MSF medium were added and samples were incubated o/n at 25°C in the dark. The next day protoplasts were analyzed under fluorescence microscope.

3.2.4.8 Chromatin Immunoprecipitation (ChIP)

An important method to study the regulation of transcription in living cells is the chromatin immunoprecipitation (ChIP) assay (Orlando, 2000). ChIP allows the analysis of the *in vivo* binding status of transcription factors or other DNA-associated proteins to certain DNA sequences (X-ChIP). Intact cells are treated with formaldehyde to crosslink promoter-associated proteins to the DNA. After isolation and shearing of the chromatin, protein-DNA complexes are immunoprecipitated with specific antibodies against the protein of interest. The precipitated DNA fragments are subsequently purified and analysed by polymerase chain reactions (PCR) using primers flanking the (putative) binding site of the protein. The amount of PCR product obtained is indicative for the relative amount of protein bound to the DNA when the tissue was harvested. The procedure allows detecting of quantitative differences in the relative amount of protein-DNA complexes, so that stimulus-induced binding can be detected.
If the binding site (*cis* element) of the transcription factor is not known, the promoters of putative target genes can be identified by ChIP followed by micro array analyses (ChIP-Chip, (Thibaud-Nissen et al., 2006)). In these experiments, new direct target genes can be identified, especially when whole genome tiling arrays are available (like for *Arabidopsis thaliana*). Alternatively, a library can be generated by cloning precipitated fragments after amplification by ligation mediated PCR (Wang et al., 2002).
In addition to the analysis of transcription factor binding, multi-protein complexes associated with the DNA can be studied using ChIP. As formaldehyde also crosslinks interacting proteins, multi-protein complexes (enhanceosome) can be mapped in vivo (Rochon et al., 2006). In this context, the so called SeqChIP can be used to address whether two proteins are simultaneously bound to a stretch of DNA (Geisberg and Struhl, 2004). As ChIP is also used to detect modifications at histones (for review see (Kuo and Allis, 1999)) a comprehensive snapshot of the events taking place during transcriptional activation can be obtained.
The following protocol describes a classical X-ChIP approach performed with leaves from *Arabidopsis thaliana*.

The protocol starts with infiltrating the material with a buffer containing formaldehyde. Formaldehyde is a very reactive bipolar compound that reacts with amino and imino groups of amino acids and DNA thus causing reversible crosslinks between proteins and

DNA or between proteins (Orlando, 2000). Crosslinking is terminated by adding 0.3 *M* glycine.
After the crosslinking, nuclei are prepared by two centrifugation steps of filtered whole cell extracts on Percoll® cushions. Afterwards, the nuclear envelope is solubilised with detergents. The chromatin is sheared into 500 to 1000 bp fragments by sonication. After spinning down the insoluble debris, the supernatant is directly used for the immunoprecipitation. Immunoprecipitations can be either performed with affinity-purified antibodies or with the complete antiserum. When using unprocessed antisera, control precipitations with the respective pre-immune serum have to be done. Mutant plants lacking the transcription factor and/or amplification of a fragment lacking the putative binding site are also valuable tools to prove the specificity of the precipitation. After the immunoprecipitation, the DNA has to be purified for PCR analysis. A combination of de-crosslinking by heat treatment, protease A digestion and phenol extractions serves to remove the protein moiety of the complex. Chromatin that is not subjected to the immunoprecipitation is purified in the same way and serves to demonstrate that the same amount of chromatin is used for each sample (input control). Quantitative analysis of the so called IP-DNA by real-time PCR provides information to what extent a given transcription factor is occupying its target sequence within a promoter.

3.2.4.8.1 Buffers and Materials

3.2.4.8.1.1 Preparation of Samples

Approximately 3-5 g of *Arabidopsis thaliana* leaf material is needed.

3.2.4.8.1.2 Crosslinking of Proteins to DNA

(1) CLB1 (crosslink buffer 1): 50 m*M* KH_2PO_4/K_2HPO_4, pH 5.8, 1% formaldehyde (see **Note 1**)
(2) CLB2 (crosslink buffer 2): 50 m*M* KH_2PO_4/K_2HPO_4, pH 5.8, 0.3 *M* glycine

3.2.4.8.1.3 Isolation of Nuclei

(1) 25% Triton X-100, stored at 4°C.
(2) NEB (nuclei extraction buffer): 1 *M* hexylene glycol (2-methyl-2,4-pentanediol), 50 m*M* PIPES-KOH, pH 7.2, 10 m*M* $MgCl_2$. Before use, ß-mercaptoethanol has to be added to a final concentration of 5 m*M* (see **Note 2**).
(3) GB (gradient buffer): 0.5 *M* hexylene glycol (ß-methyl-2.4-pentanediol), 50 m*M* PIPES-KOH, pH 7.2, 10 m*M* $MgCl_2$, 1% (w/v) Triton X-100. Before use, ß-mercaptoethanol has to be added to a final concentration of 5 m*M*.
(4) Percoll cushions: 75% and 35% (w/v) Percoll® in GB.
(5) Miracloth (Calbiochem, Merck Chemicals Ltd., Nottingham, UK).

(6) Nuclear stain: 300 n*M* DAPI (4,6-diamidino-2-phenylindole) in water, to be stored at -20°C.

3.2.4.8.1.4 Extraction of Chromatin

(1) SB (sonic buffer): 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA (ethylenediamine tetraacetic acid) containing either 0, 0.25 or 0.5% (w/v) sodium dodecyl sulfate (SDS). The buffer is stored at room temperature.
(2) Protease inhibitor mix for plants (P-9599, Sigma-Aldrich, St. Louis, MO).

3.2.4.8.1.5 Purification of DNA

(1) PCI-Mix: phenol (equilibrated, pH 7.6 - 8.0, AppliChem)/chloroform/isoamyl alcohol 25:24:1 (v/v/v). The mixture is stored at 4°C. All work involving phenol and chloroform should be done under a hood.
(2) CI-Mix: chloroform/isoamyl alcohol 24:1 (v/v).
(3) 3 *M* Na-Acetate
(4) Glycogen 10 mg/mL (G-8751, Sigma-Aldrich), store at 2 – 8°C.

3.2.4.8.1.6 Chromatin immunoprecipitation

(1) RIPA-F: 50 m*M* HEPES-NaOH, pH 7.4 (see **Note 3**), 140 m*M* NaCl, 1 m*M* EDTA, 1% Triton X-100, 0.1% sodium deoxycholate (DOC, minimum 97%, Sigma-Adrich), 0.1% SDS; RIPA-F buffer without SDS is required for sample dilution prior to immunoprecipitation (see below). RIPA-F buffer may be used for up to 4 weeks when stored at 4°C.
(2) Protein A Sepharose (from *Staphylococcus aureus*, Sigma-Aldrich), store at 2 – 8°C.
(3) EB (elution buffer): 0.1 *M* glycine (adjust with HCl to pH 2.5), 0.5 *M* NaCl, 0.05% Tween20. This buffer can be stored for up to 4 weeks at 4°C.
(4) 10 m*M* Tris-HCl, pH 7.4
(5) Proteinase K (20 mg/mL). Aliquots (100 µL) should be stored at -20°C.

3.2.4.8.2 Plant Growth

Grow *Arabidopsis* plants in a climate chamber under short day conditions (see **Note 4**) for about 4 to 5 weeks until they have developed enough biomass (about 3 - 5 g per sample).
Depending on the biological question to be asked, subject your plants to the appropriate treatment (e.g. pathogens, hormones, etc.).
Harvest 3 - 5 g of leaf material.

3.2.4.8.3 *In vivo* Crosslinking of Proteins to DNA

Note: All steps for the crosslinking procedure are carried out at room temperature if not noted otherwise.
Put the leaves in a suitable device for subsequent vacuum-infiltration (see **Note 5**). This device should prevent floating of the leaves to the surface in order to make sure that the buffer, but not the air, is sucked in when the vacuum is released.
Put the device containing the leaf material into a beaker filled with CB1 so that the leaves are submerged.
Put the beaker in a desiccator and apply vacuum for 5 min using an oil pump. Release the vacuum, re-apply vacuum for additional 5 min, release the vacuum and allow the crosslinking to proceed for 20 min (see **Note 6**).
Discard CB1 and wash the samples with CB2. Infiltrate CB2 into the leaves by applying vacuum for 5 min, release the vacuum and incubate the samples for another 5 min.
Discard CB2 and wash the samples twice with distilled water. After removing as much water as possible, the material can be frozen and stored in liquid nitrogen.

3.2.4.8.4 Isolation of Nuclei

Note: All steps are carried out at 4°C. Working in a cooling chamber is recommended.
Grind the plant material in liquid nitrogen.
Transfer the leaf powder into 50 mL tubes filled with 20 mL of NEB and mix with a glas pipette until the powder is completely submersed in the buffer.
Homogenize the sample with an electronic blender at low power (e.g. MiccraRT from Art Labortechnik, Müllheim, Germany) at 14 000 rev min^{-1} for 5 min. **Caution:** High power mixers are not recommended as they will heat up the samples.
Filter the suspension through two sheets of Miracloth and collect the filtrate in 100 mL beakers (a small spoon can be used for careful mixing to accelerate the flow-through process). Add NEB to a total volume of 24 mL.
Add 1 mL of Triton X-100 (25%) dropwise to the filtrate on a magnetic stirrer. This step serves to lyse the organelles while leaving the nuclei intact. **Caution:** Add the Triton slowly to avoid high local concentrations of the detergent which might lead to disruption of the nuclear envelope.
Continue stirring for at least 30 min (this step can be extended up to 2 h).
In the meantime, prepare the Percoll cushions in fresh 50 mL tubes. Overlay 6 mL of Percoll-cushion (75%) carefully with 6 mL of Percoll-cushion (35%).
Carefully place the resuspended nuclei on top of the Percoll cushions.
Centrifuge at 2100 ***g*** and 4°C for 30 min in a swinging bucket rotor (soft start and stop).
The nuclei should appear as a greyish layer at the interface between the cushions.
Recover the nuclei with a blue tip (1 mL), transfer them into a fresh 50 mL tube and add GB to a total volume of 20 mL. This step might work better when cut tips are used to recover the nuclei.
Add 6 mL Percoll-cushion (35%) to fresh tubes
Overlay the Percoll-cushion (35%) with the resuspended nuclei.
Centrifuge at 2 100 ***g*** and 4°C for 10 min in a swinging bucket rotor (soft start and stop).

Discard the supernatant, resuspend the nuclei in 1 mL GB and transfer them into 10 mL tubes. Take an aliquot to stain the nuclei with DAPI (see **Note 7**). The isolated nuclei appear under the fluorescent microscope as crescent shaped structures.
Centrifuge at 2100 ***g*** and 4°C for 10 min (soft start and stop) and discard the supernatant. **Note:** Nuclei can be stored for one day at -80°C.

3.2.4.8.5 Preparation of Chromatin

Note: All preparation steps should be carried out at 4°C.
Resuspend the nuclei in 1 mL of SB, 0.5% SDS and add the protease inhibitor mix (1:100). Incubate under gentle agitation for 20 min at 4°C. Dilute (1:1) with 1 mL SB lacking SDS to get a final concentration of 0.25% SDS.
Sonicate 4 times for 20 s with a power of 100 W when using the MSE Soniprep 150 ultrasonic disintegrator (Sany-Gallen-Kamp, Loughboro, Leicestershire, UK; see **Note 8**). Cool the samples in ice/EtOH during and between the sonication pulses. If foaming is a problem, reduce the percentage of SDS in the sample.
Centrifuge at 11 200 ***g*** and 4°C for 15 min to separate the soluble chromatin from the debris of the nuclei.
Freeze aliquots of 200 µl in liquid nitrogen and store at -80°C. Take one aliquot of 50 µL for the quantification of the DNA content.

3.2.4.8.6 Quantification of DNA in Chromatin Samples

Add 200 µL SB, 0.25% SDS and 5 µL of Proteinase K (from a 20 mg/mL stock solution) to 50 µl of chromatin.
Incubate samples for 1 h at 37°C and 16 h at 42°C in a heating block to reverse the crosslinks. **Note:** Proteinase K treatment can also be done after the reversion of the crosslinks.
Extract with 250 µL PCI, vortex rigorously for 30 s and separate the phases by centrifugation in a microcentrifuge (5 min at 13.000 rpm, RT). Transfer the supernatant to a fresh microcentrifuge tube.
Extract with 250 µL CI, vortex rigorously for 30 s and separate phases by centrifugation in a microcentrifuge (5 min at 13 000 rpm, RT). Transfer the supernatant to a fresh microcentrifuge tube.
Optional: Add 1 µL RNaseA (10 mg/mL) to the supernatant and incubate at RT for 15 min.
Add 1/10 volumes of 3 *M* Na-Acetate, 1 µL glycogen (10 mg/mL) and 2 volumes of absolute ethanol, mix by inverting the tubes for 6 - 8 times and precipitate for 2 - 4 h at -80 °C.
Pellet the DNA by centrifugation in a microcentrifuge (35 min at 13 000 rpm, 4°C) and wash with 800 µL 70% ethanol.
Pellet the DNA by centrifugation in a microcentrifuge (20 min at 13 000 rpm, RT) and discard the supernatant. Place the open microcentrifuge tube for 10 min at 37°C and resuspend the dried pellet in 50 µL water (ultra pure).

Measure the OD_{260} and analyze the size of the DNA fragments on a 1% agarose gel (see **Note 9**).

3.2.4.8.7 Immunoprecipitation of Protein-DNA Complexes

Thaw chromatin samples on ice (this will take some time).
Bring equal amounts of chromatin as measured by DNA content (15 µg) to a total volume of 200 µl with SB, 0.25% SDS and add 300 µl RIPA-F lacking SDS.
Incubate for 1 h with 5 µL PPI (pre-immune serum) with slow rotation at 4°C (e.g. on a rotation platform like an Intelli-Mixer, LTF Labortechnik, Wasserburg, Germany).
In the meantime, add 1 mL RIPA-F to Protein A-Sepharose beads. You will need 2 x 50 µl 50% Protein A-Sepharose beads per sample: first, the complexes associated with the pre-immuneserum are captured, and afterwards the specific complexes are enriched with the immuneserum. To equilibrate the beads, let them sit for 15 min with slow rotation at 4°C. Centrifuge in a microcentrifuge at 5000 rpm for 3 min, discard RIPA-F (works nicely with an insulin needle) and wash the beads once again with 1 mL RIPA-F (slowly rotate for 5 min at 4°C). Discard the supernatant like before and dissolve the beads with RIPA-F to get a 50% beads solution.
Add 50 µL of the equilibrated 50% Protein A-Sepharose beads to the samples and incubate them for 1 h at 4°C with slow rotation. This step serves to remove complexes from the chromatin that interact with the pre-immuneserum.
Centrifuge the samples in a microcentrifuge for 2 min at 13 000 rpm. Take 50 µl of the supernatant for later use as an input control (see 7) and use the remainder of the supernatant for the immunoprecipitation (see 8).
Add 400 µl of SB, 0.25% SDS to the input control samples.
Transfer the remainder of the supernatant into a fresh microcentrifuge tube, add the antibody (1 to 5 µL) and incubate for 2 h at 4°C with slow rotation (see **Note 10**).
Add 50 µl of the 50 % Protein A-Sepharose beads and let the samples rotate for additional 2 h at 4°C.
Centrifuge for 3 min at 5 000 rpm in a microcentrifuge and remove the supernatant with an insulin needle until the Sepharose is half-dry.
Wash the beads by adding 1 mL RIPA-F and let them rotate for 5 min at 4°C, centrifuge for 3 min at 5 000 rpm in a microcentrifuge and discard the supernatant with an insulin needle. Repeat this washing step for two more times.
Add 800 µl RIPA-F and transfer the samples into a fresh microcentrifuge tube. Incubate for 5 min at 4°C with slow rotation, centrifuge for 3 min at 5 000 rpm and discard the supernatant like before.
Add 150 µL EB to the beads, vortex rigorously for 30 s, centrifuge for 1 min at 13 000 rpm in a microcentrifuge, transfer the supernatant with a yellow tip and into a microcentrifuge tube filled with 150 µL 1 M Tris-base, repeat the elution and combine the samples to a final volume of 450 µL.
Add 5 µL Proteinase K (20 mg/mL; also to input controls set aside in 7) and incubate samples for 1 h at 42°C. Reverse the crosslinks for about 4 h at 65°C.

3.2.4.8.8 Purification of DNA for PCR

Extract with 450 µL PCI, vortex rigorously for 30 s and separate the phases by centrifugation in a microcentrifuge (5 min at 13 000 rpm, RT). Remove the supernatant with a blue tip while holding the microcentrifuge tube in an angle of about 45° and transfer the supernatant to a fresh microcentrifuge tube.
Add 450 µL CI, vortex rigorously for 30 s and separate phases by centrifugation in a microcentrifuge (5 min at 13 000 rpm, RT). Transfer the supernatant into a fresh microcentrifuge tube.
Optional: Add 1 µL RNaseA (10 mg/mL) and incubate at RT for 15 min.
Add 1/10 volumes of 3 M Na-Acetate, 1 µL glycogen (10 mg/mL) and 2 volumes of absolute ethanol, mix by inverting the tubes 6 - 8 times and precipitate for 2 - 4 h at -80°C.
Pellet the DNA by centrifugation in a microcentrifuge (35 min at 13 000 rpm, 4°C) and wash with 800 µL 70% ethanol.
Pellet the DNA by centrifugation in a microcentrifuge (20 min at 13 000 rpm, RT) and discard the supernatant completely. Dry the pellet for 10 min at 37°C in an open microcentrifuge tube. Dissolve IP DNA in 35 µL and input DNA in 175 µL of water (ultrapure)
Resuspend the DNA at 65°C for 15 min while shaking at low speed. The purified DNA should be stored at -20°C.

3.2.4.8.9 Analysis by PCR or real-time PCR

The analysis of ChIP experiments by real-time PCR (qPCR) is recommended. If the PCR products are analyzed by agarose gel electrophoresis, different numbers of cycles have to be run for each sample in order to ensure that the reaction is in the linear range. The conditions for the PCR depend on the primers that are used for amplification of the promoter DNA sequence. It is recommended to use primers that amplify a fragment of about 250 bp (see **Note 11**).
For qPCR, use the following protocol as a start, some optimizations might be necessary depending on the sequence of the primers: Use 2.5 µL of the purified IP-DNA and the input DNA, each. For a typical pipetting scheme *see* Fehler! Verweisquelle konnte nicht gefunden werden..
A typical qPCR program is:
6 min at 95°C (+2 min when no fluoresceine measurement is performed)
40 cycles of 20 s at 95°C, 20 s at 60°C, 28 s at 72°C
4 min at 72°C
1 min at 95°C
1 min at 55°C
This program should be followed by a melting curve measurement to make sure that the primers have amplified a specific product.
Note: The initial denaturation time (in this case 6 min) depends on the polymerase that is used. The annealing temperature has to be adapted to the primers used (see **Note 12**).
Threshold cycles, which define the beginning of the exponential phase of the PCR, are used as units to indicate the relative amount of DNA in the samples.

3.2.4.8.10 Notes

(1) Take special care when working with formaldehyde. It is highly toxic and has to be disposed separately. All work should be done under a hood. Wear protection clothes all the time.
(2) It is recommended to prepare NEB and GB freshly for each preparation. Add the ß-mercaptoethanol just before you start the experiment. Work carefully with ß-mercaptoethanol as it is harmful and causes damage to the environment. Waste should be disposed separately.
(3) The use of NaOH instead of KOH to adjust pH of the RIPA-F buffer is recommended as potassium forms precipitates with SDS in the buffer. RIPA-F contains a combination of denaturing and non-denaturing detergents (Triton, DOC, SDS) to solubilise the chromatin.
(4) Short day conditions are 8/16 h light/dark period, 22°C. Grow the plants on soil with ~20 plants per pot. It is also possible to grow the plants under long day conditions, but the fraction containing the nuclei might be less clean as the cells accumulate starch under long day conditions.
(5) One possibility is to use nylon stockings.
(6) The incubation time with formaldehyde depends on the proteins which should be crosslinked. Shorter periods (of about 10 minutes) are sufficient for nucleosomal proteins, for crosslinking other proteins, the tissue should be treated for 20 minutes to 1 hour. The crosslinking should not be extended to longer periods as proteins get denatured or masked by formaldehyde. If problems (low yield of chromatin, or low yield of IP-DNA) occur, a time-course experiment should be performed.
(7) As DAPI (4,6-diamidino-2-phenylindole) moves slowly through membranes, an incubation time of 1.5 h for the samples (at 4°C, in the dark) is recommended. The excitation wavelength is 358 nm, emitted light has a wavelength of 461 nm. Emission at 400 nm is due to binding of DAPI to RNA and should be subtracted by an appropriate filter.
(8) (9) The shearing of the chromatin by sonication should be controlled by gel electrophoresis on a 1% agarose gel after the precipitation of the DNA. The fragments should appear as a smear from 2 kb down to 300 bp. Depending on the sonication device, the power and the length and number of pulses need to be optimized.
(10) This so called “cleaning” step with the pre-immune serum is strongly recommended when an antiserum is used instead of a purified antibody.
(11) The performance of the ChIP largely depends on the antibody which is used. Some optimization is necessary to determine the best concentration for each antibody or antiserum.
(12) In this work real-time PCR quantification was performed using the SYBR Green technology in a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Munich, Germany). The SYBR Green stock is from Cambrex Bio Science, Rockland, USA and the Immolase DNA polymerase mix and the reaction buffer are from Bioline, Randolph, USA. Fluoresceine is added to calibrate for equal efficiency of fluorescence in each well.

Table 3-1 Standard pipetting scheme for quantitative realtime PCR analyzing ChIP-DNA

	stock	final conc.	µL
Water			X*
Buffer	10 x	1 x	2.5
dNTPs 1:10	10 mM	100 µM	0.25
1. Primer	10 µM	0.25 µM	0.625
2. Primer	10 µM	0.25 µM	0.625
SybrGreen 1:1000	100 x	1 x	0.25
$MgCl_2$	50 mM	X*	X*
Taq Polymerase	5 U/µl	1.25 U	0.25
Fluoresceine 1:1000	100 x	1 x	0.25
DNA			2.5
Final volume		25 µl	

*The $MgCl_2$ concentration to be used depends on the type of Taq polymerase. In some cases, $MgCl_2$ is included in the reaction buffer supplied with the enzyme or in commercially available master buffers for qPCR (which also contain SYBR Green or an alternative fluorophore and fluoresceine).

4 Results

4.1 The N terminus of SCL14 is required for TGA interaction

In order to define the domain of SCL14 that interacts with TGA factors, a classical yeast-two-hybrid (YTH) system using TGA2 fused to the GAL4 DNA binding domain (GBD-TGA2) and SCL14 derivatives fused to the GAL4 activation domain (GAD-SCL14) was used. Different GAD-SCL14 deletion derivatives were co-transformed into yeast with GBD-TGA2.

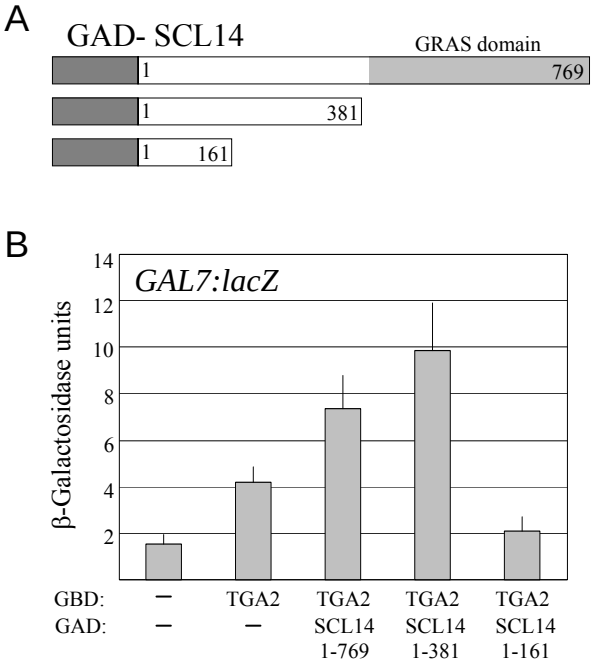

Figure 4-1Yeast two hybrid analysis of SCL14 deletion derivatives

(A) Schematic drawing of the tested SCL14 deletion derivatives. The GRAS domain (aa 391-765) is highlighted in grey. Numbers indicate the N-terminal and C-terminal amino acids of SCL14 in the respective constructs.

(B) Measurement of β-galactosidase activities in the yeast strain PJ69-4A, which contains the *lacZ* reporter gene under the control of the *GAL7* promoter. Transformed effector plasmids encode TGA2 fused to the GAL4 DNA binding domain (GBD), SCL14 and deletion derivatives fused to the GAL4 activation domain (GAD) or non-fused GBD (vector pGBT9) or GAD (vector pGAD424). Four independent clones were taken for each combination of constructs.

Complex formation between the two hybrid proteins was still possible, when only the variable N-terminal domain (aa 1-381) of SCL14 was used. However, the very first 161 N-terminal amino acids alone were not sufficient to support the interaction. Thus, the domain required for the interaction with TGA2 is located to the N-terminal region of SCL14 (Figure 4-1).

4.2 Intracellular localization of SCL14-GFP fusion proteins

The subcellular localization of SCL14 was assessed by microscopic analysis of a SCL14- green fluorescent protein (GFP) fusion protein that was transiently expressed in tobacco BY-2 protoplasts. As documented in Figure 4-2 (C), SCL14-GFP is localized both in the nucleus and in the cytosol.

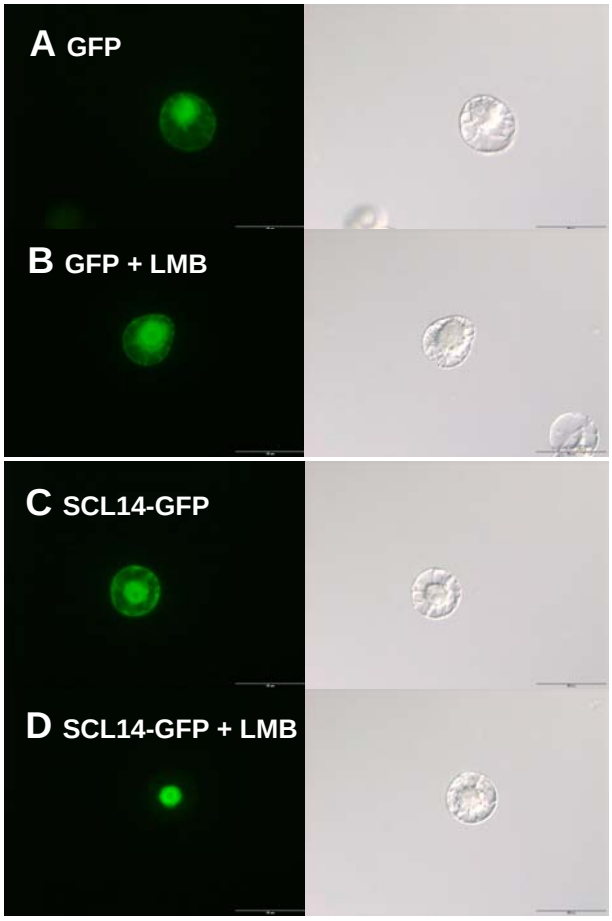

Figure 4-2 Localization of the SCL14-GFP fusion protein in protoplasts of tobacco BY-2 cells

Protoplasts were transfected with plasmids encoding GFP or SCL14-GFP and analyzed by fluorescence microscopy. In the right part bright field images are placed adjacent to the corresponding fluorescence pictures. BY-2 protoplasts transformed with GFP without LMB treatment (A) and with 2 µM LMB (B); Protoplasts transformed with SCL14-GFP without LMB treatment (C) show localisation of SCL14 to cytoplasm and nucleus whereas SCL14 localizes only to the nucleus after treatment with 2 µM LMB (D)

In order to assess whether the cytosolic localization of the protein is due to incomplete import or to active export, protoplasts were treated with Leptomycin B (LMB), which blocks the function of the exportin receptor XPO1 (Kudo *et al.*, 1999). The GFP alone is distributed equally in the cytosol and the nucleus (A). Treatment with LMB shows no influence on the localization of the unfused reporter (B). In contrast, the LMB treatment led to the accumulation of the majority of SCL14-GFP in the nucleus (the original experiment was performed by Tanja Siemsen; Figure 4-2 shows the reproduction during this work). Thus, the cytosolic localization of SCL14 in the absence of LMB is most likely due to the XPO1-dependent nuclear export.

4.3 Sequence analysis of endogenous genes activated by SCL14

Based on the results obtained in yeast the hypothesis was that SCL14 is recruited to the promoters of direct target genes by TGA factors bound to *as-1*-like elements. Therefore the promoter sequences of the genes identified by Tanja Siemsen were searched for putative *as-1*-like elements. Nine out of the 13 genes differentially regulated by SCL14 contain putative *as-1*-like elements within 250 bps from the transcriptional start site (Table 4-1).

Table 4-1 Differentially expressed genes in *as-1:GUS* plants transformed with a *CaMV 35S:HA$_3$-SCL14* construct (*HA$_3$-SCL14*) versus *scl14* mutant plants (log2 ratio >5.0)

Gene	AGI code	*HA$_3$-SCL14* vs. *scl14* *log2 ratio*	wildtype vs. *scl14* *log2 ratio*	*as-1-like* element (-1000 bp promoter region)
SCARECROW-like transcription factor 14 (SCL14)	*At1g07530*	11.2	7.5	
Cytochrome P450 family protein (CYP81D11)	*At3g28740*	10.8	8.4	-243 TGACATATgcaaTGACGACA -224
UDP-glucoronosyl/UDP-glucosyl transferase family protein	*At1g05680*	8.7	6.9	
Oxidoreductase	*At2g37770*	8.2	5.4	
MtN19-like protein	*At5g61820*	7.6	5.7	-121 TGACGAGAgcggTGACGTCA -102
NADP-dependent oxidoreductase	*At5g16980*	6.3	4.6	-240 TGACGTCAtactTGATAAGT -221 -120 AAGAATCAacgaTGACGACG -101
4-Methyl-5(B-hydroxyethyl)-thiazole monophosphate biosynthesis protein	*At3g14990*	6.1	3.9	-71 TTACGTCAtcgcACACGTCT -52
Monooxygenase (MO1)	*At4g15760*	6.1	4.8	-107 TGGCGTCAtaggcTTGCGTCA -87
Nitrilase 4 (NIT4)	*At5g22300*	5.8	4.0	
Glutathione S-transferase 25 (GSTU7)	*At2g29420*	5.5	3.3	-64 GTTCGTCActggTGACGTCA -45
Short-chain dehydrogenase/ reductase (SDR) family protein	*At4g13180*	5.5	3.4	-134 ACACGTCActgcTTACGAAA -115
Ubiquitin carboxyl-terminal hydrolase-related	*At5g61950*	5.3	3.5	
Arabidopsis NAC domain containing protein 2 (ANAC002, ATAF1)	*At1g01720*	5.2	3.9	-179 AGACGTAAgcaaTGACAACA -160
Arabidopsis NAC domain containing protein 32 (ANAC032)	*At1g77450*	5.1	3.1	-206 TGACGTAAgcaaTGACAAAA -187
as-1:GUS				-83 TGACGTAAgggaTGACGCAC -64
consensus				TGACGTCAnnnnTGACGTCA

On the right side of the table, sequences of *as-1*-like elements found in the respective promoter regions are shown. The numbers indicate their positions relative to the transcriptional start sites (+1). Nucleotides corresponding to the 8 bp palindromes within the *as-1* consensus sequence (last row) are highlighted in bold capitals and written in red colour if they are conserved. Half sites of the palindromes which contain at least 3 of the 4 conserved TGAC nucleotides are marked by arrows. Additionally, the sequence and the position of the *as-1* element within the *as-1:GUS* construct is shown.

4.4 Recruitment of SCL14 and TGA2 to target promoters

A powerful method to study the *in vivo* binding of transcription factors is the chromatin immunoprecipitation (ChIP) assay. Intact leaf tissue was treated with formaldehyde to crosslink promoter-associated proteins to the DNA. After isolation and shearing of the chromatin, protein-DNA complexes were immunoprecipitated with antiserum generated against SCL14 or TGA2,5. The precipitated DNA fragments were subsequently purified

and analysed by quantitative real-time PCR using primers flanking the TGA binding site (*as-1*-like element) of the promoters (marked in Table 4-1). The amount of PCR product obtained is indicative for the relative amount of protein bound to the DNA when the tissue was harvested. Thus, ChIP allows detection of quantitative differences in the relative amount of SCL14-DNA and TGA2,5-DNA complexes. To confirm binding of TGA factors to the promoters identified by micro array analysis, chromatin immunoprecipitations (ChIP) of selected promoter fragments were performed with the antiserum against TGA2,5. TGA2,5 is recruited to the target promoters as shown for *CYP81D11*, *MtN19-like* and *GSTU7* by ChIP analysis of chromatin from wildtype plants (Figure 4-3).

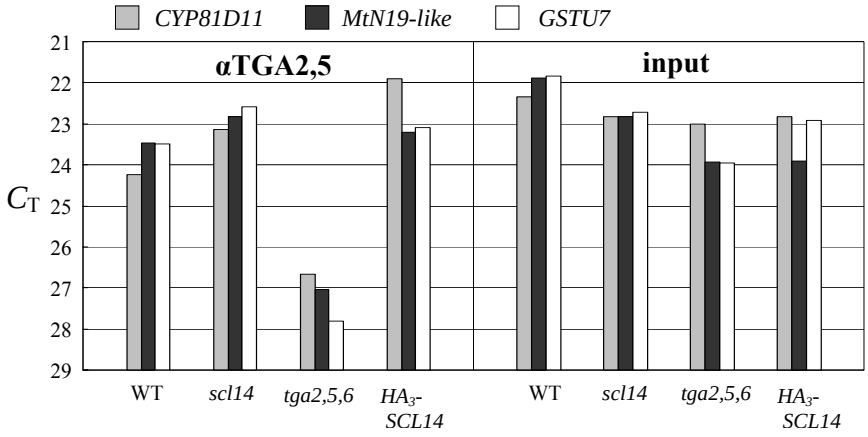

Figure 4-3 *In vivo* TGA factor binding to the promoters of *CYP81D11*, *MtN19-like* and *GSTU7* as revealed by chromatin immunoprecipitation analysis

Leaves from five-week-old (short-day) *as-1:GUS* (WT) plants, plants transformed with the *CaMV 35S:HA3-SCL14* construct (*HA3-SCL14*), and *scl14* and *tga2 tga5 tga6* (*tga2,5,6*) mutants were incubated in 1% formaldehyde before chromatin preparation. Chromatin samples were subjected to immunoprecipitation using 5 µl of the αTGA2,5 antiserum. The DNA was recovered after reversal of the cross-links and analyzed for the enrichment of promoter sequences by quantitative real-time PCR. C_T -values are given as a parameter to quantify the amount of PCR products. C_T -values obtained from the respective input controls are shown in the right part of the diagram. The amount of precipitated DNA in the *tga2,5,6* mutant is strongly reduced compared to wildtype chromatin showing the specificity of the antiserum.

As expected, chromatin from wildtype plants yielded more immunoprecipitated promoter fragments than chromatin from *tga2 tga5 tga6* plants, confirming the specificity of the antiserum. The amount of TGA factors at the three promoters tested was similar in wildtype, *scl14* and *HA3-SCL14* plants, which corresponds with the

amounts of protein detected by Western blot analysis in these plants (Figure 4-4). Thus, TGA factors bind to the *as-1*-like elements of target promoters independently of SCL14.

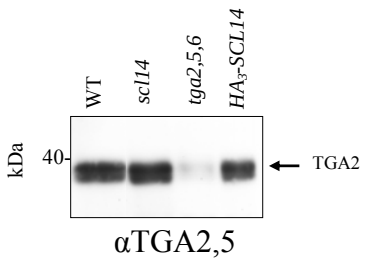

Figure 4-4 Western blot analysis of chromatin samples using the αTGA2,5 antiserum

Chromatin extracted from five-week-old plants grown under short-day conditions was used for Western blot analysis. 5 µg of proteins in each sample (determined with BCA Kit) were separated on an 8 % PAA gel. The genotype of the analyzed plants is indicated above the lanes. The αTGA2,5 antiserum was used in a 1:1000 dilution.

The same chromatin used for TGA2,5-ChIP was subjected to immunoprecipitation reactions with the αSCL14 antiserum. As crosslinking with formaldehyde prior to ChIP experiments can also crosslink interacting proteins, binding of SCL14 to TGA2-bound target promoters could be detected even though SCL14 has no own DNA binding ability. This ChIP experiment revealed recruitment of SCL14 to the promoters of *CYP81D11*, *MtN19-like* and *GSTU7* in chromatin from wildtype plants (Figure 4-5).

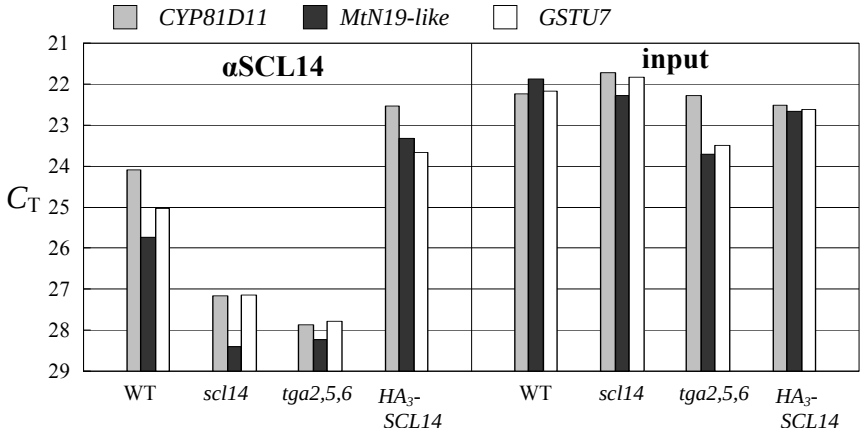

Figure 4-5 *In vivo* SCL14 binding to the promoters of *CYP81D11*, *MtN19-like* and *GSTU7* as revealed by chromatin immunoprecipitation analysis

Leaves from five week old (short-day) *as-1:GUS* (WT) plants, plants transformed with the *CaMV 35S:HA3-SCL14* construct (*HA3-SCL14*), and *scl14* and *tga2 tga5 tga6* (*tga2,5,6*) mutants were incubated in 1% formaldehyde before chromatin preparation. Chromatin samples were subjected to immunoprecipitation using 5 µl of the αSCL14 antiserum. The DNA was recovered after reversal of the cross-links and analyzed for the enrichment of promoter sequences by quantitative real-time PCR. C_T-values are given as a parameter to quantify the amount of PCR products. C_T -values obtained from the respective input controls are shown in the right part of the diagram. The amount of precipitated DNA in the *scl14* mutant is reduced compared to wildtype chromatin proving the specificity of the antiserum.

Chromatin prepared from the *scl14* mutant yielded reduced amounts of PCR fragments documenting the specificity of the antiserum. The promoters were also less efficiently immunoprecipitated with the αSCL14 antiserum when chromatin was prepared from the *tga2 tga5 tga6* triple mutant. As Western blot analysis proved wildtype levels of SCL14 in these plants (Figure 4-6), the conclusion can be made that TGA factors are required to recruit SCL14 to the target promoters analyzed here. When using the immunoprecipitated DNA from *HA3-SCL14* plants, slightly increased amounts of immunoprecipitated promoter fragments were obtained in this experiment, which is consistent with slightly increased protein levels (Figure 2-3) and increased expression of *as-1:GUS* reporter construct.

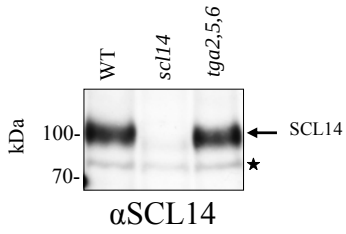

Figure 4-6 Western blot analysis of crude whole cell extracts using the αSCL14 antiserum

Five-week-old plants grown under short-day conditions were used. Amounts of proteins were normalized on a coomassie gel (scanned and quantified with TINA2.0) prior to Western blot analysis. The genotype of the analyzed plants is indicated above the lanes. The αSCL14 antiserum was used in a 1:1000 dilution. The asterisk marks a non-specific band.

4.5 Expression pattern of SCL14 target genes

The promoters of the three analyzed genes *CYP81D11*, *MtN19-like* and *GSTU7* were targets for SCL14 and TGA2,5 binding. This binding occurs independently of any stress stimulus. Investigation of the expression pattern of the three target genes should reveal their possible function and whether binding of SCL14 and TGA2,5 is sufficient to activate their transcription. Tanja Siemsen (2005) showed the SCL14-dependent inducibility of the transgenic *as-1:GUS* reporter gene by SA and 2,4-D whereas increased *GUS* gene expression occurs in the SCL14 over-expressing plants. In order to analyze whether the endogenous target genes of SCL14 follow the same expression pattern as *as-1:GUS*, Arabidopsis plants were treated with 2,4-D (Figure 4-7) and SA (Figure 4-8). RNA from three week old plants treated for 0 h or 10 h and 0 h, 4 h or 8 h with the respective substance was analyzed in quantitative real-time RT-PCR (qRT-PCR). This analysis revealed that expression of *CYP81D11*, *MtN19-like* and *GSTU7* was induced by both chemicals, albeit with different selectivity: whereas expression of *CYP81D11* was more responsive to SA than expression of *MtN19-like* and *GSTU7*, the latter two genes were more responsive to 2,4-D than *CYP81D11*.

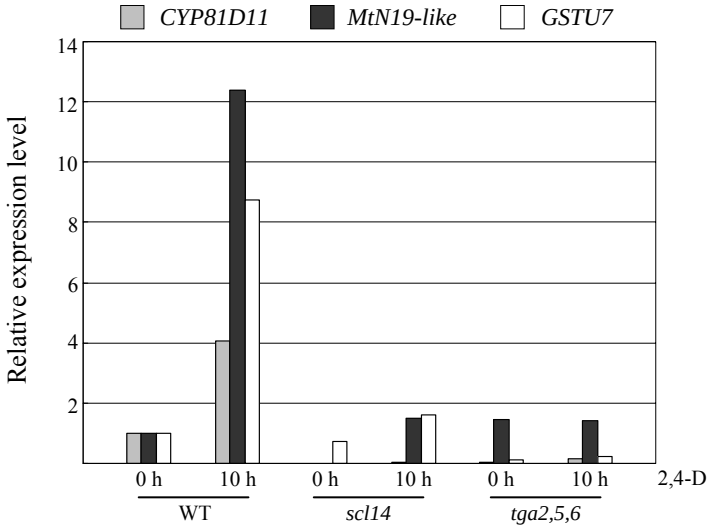

Figure 4-7 Expression of endogenous SCL14 target genes after 2,4-D treatment

Quantitative real-time RT-PCR analysis of relative *CYP81D11*-, *MtN19-like*- and *GSTU7* transcript levels in *as-1:GUS* (WT), and *scl14* and *tga2 tga5 tga6* (*tga2,5,6*) mutant plants. Three-week-old plants were floated in 50 mM potassium phosphate buffer pH 5.7, containing 0.1 mM 2,4-D for the indicated time spans. The values of the three genes in untreated *as-1:GUS* plants were set to "1".

Overall, expression levels were lower in the *scl14* and *tga2 tga5 tga6* mutant plants confirming that these genes were regulated at least in part in an SCL14- and TGA-dependent manner (Figure 4-7 and Figure 4-8).

Tanja Siemsen has shown previously that the NPR1-dependent *PR-1* gene expression was unaltered in *scl14* mutants and SCL14 over-expressing plants (Siemsen, 2005). In order to analyze the influence of NPR1 on the SCL14 target genes, their expression after SA stimulus in the *npr1-1* was investigated. Induction of all three tested genes by SA was more efficient in *npr1-1* mutants than in wildtype plants (Figure 4-8). This effect was most pronounced for *CYP81D11*, which correlates with its higher responsiveness to SA as compared to the two other genes.

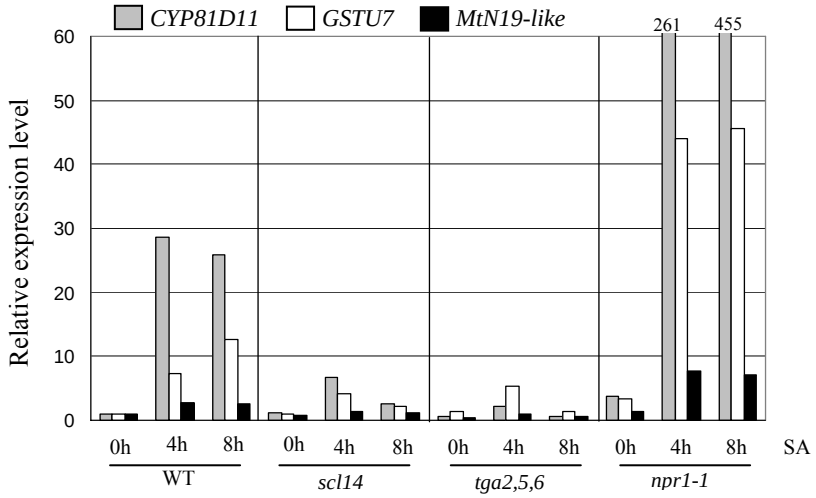

Figure 4-8 Expression of endogenous SCL14 target genes after treatment with SA

Quantitative real-time RT-PCR analysis of relative *CYP81D11-*, *MtN19-like-* and *GSTU7* transcript levels in *as-1:GUS* (WT), and *scl14*, *tga2 tga5 tga6* (*tga2,5,6*) and *npr1-1* mutant plants. Three-week-old plants were treated with 1 mM SA for the indicated time spans. The values of the three genes in untreated *as-1:GUS* plants were set to "1".

To further elucidate the role of SCL14 target genes and to identify additional putative inducing stimuli a Genevestigator V3 Clustering Analysis of the genes that showed the highest difference in the expression levels between transgenic *HA_3-SCL14* plants and *scl14* mutants was performed. This analysis revealed that seven of these genes – including *CYP81D11*, *MtN19-like* and *GSTU7* - belong to a cluster of genes that is up-regulated after infection with *Botrytis cinerea* and *Pseudomonas syringae* and a variety of different chemicals including hydrogen peroxide (H_2O_2), TIBA and 2,4-D (Figure 4-9).

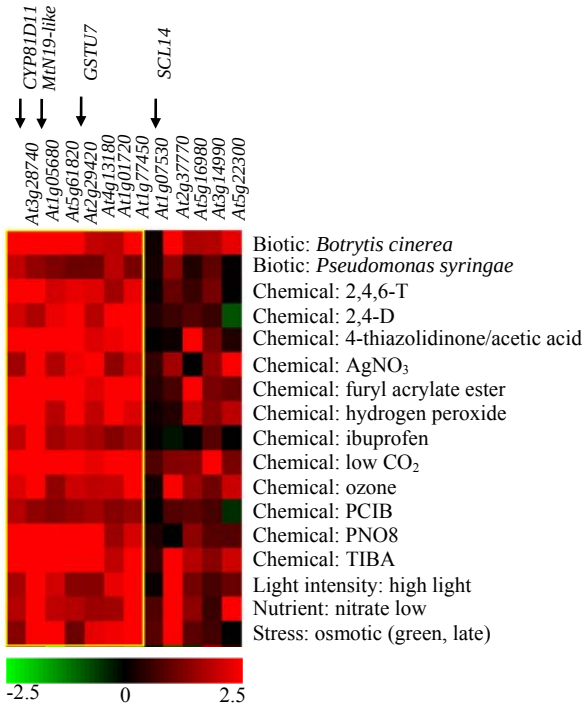

Figure 4-9 Genevestigator V3 Clustering Analysis of genes listed in Table 4-1

The Bicluster (BiMax algorithm) of genes and stimulus categories (at least 6 columns upregulated after stimulus) is marked by the yellow box. For the genes At4g15760 and At5g61950, no probe sets are available on the 22k Affymetrix array. 2,4,6-T, 2,4,6-trihydroxybenzamide (auxin inhibitor); 2,4-D, 2,4-dichlorophenoxyacetic acid; 4-thiazolidinone/acetic acid (auxin signaling inhibitor); $AgNO_3$ (ethylene inhibitor); furyl acrylate ester (auxin signaling inhibitor); ibuprofen (jasmonic acid biosynthesis inhibitor); PCIB: p-chlorophenoxyisobutyric acid (auxin inhibitor); PNO8, N-octyl-3-nitro-2,4,6-trihydroxybenzamide (photosystem II inhibitor); TIBA, 2,3,5-triiodobenzoic acid (auxin transport inhibitor).

4.5.1 The SA analogue 2,6-isonicotinic acid induces the SCL14 target genes in a TGA-dependent manner

In order to investigate more putative inducers of SCL14 dependent target genes, 5 week-old plants were treated with 1 mM 2,6-isonicotinic acid (INA) a functional homologue of SA often used to induce *PR* gene expression. The expectation that INA exhibits the same inducing potential compared to SA was confirmed by analyzing RNA of the INA treated plants by qRT-PCR. The expression of the target genes was activated by INA in a SCL14 and TGA dependent manner (Figure 4-10). Moreover, a hyper-inducibility of the genes in *HA_3-SCL14* expressing plants was observed. This in fact fits

with the heightened levels of the *as-1:GUS* reporter transcript in those plants (Siemsen, 2005) after SA or 2,4-D treatment.

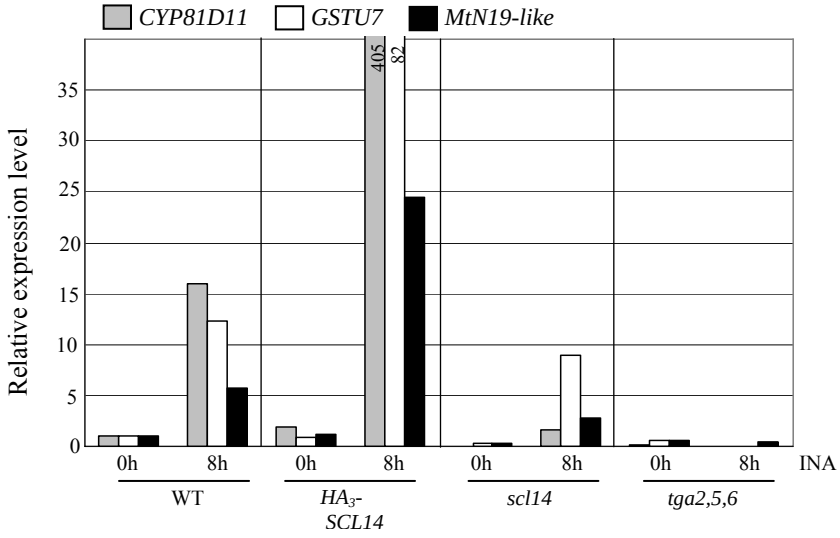

Figure 4-10 Expression of endogenous SCL14 target genes after INA treatment

Quantitative real-time RT-PCR analysis of relative *CYP81D11-*, *MtN19-like-* and *GSTU7* transcript levels in *as-1:GUS* (WT), *HA*$_3$*-SCL14* expressing plants, *scl14*and *tga2 tga5 tga6* (*tga2,5,6*) mutant plants. Five-week-old plants were sprayed with 1 mM INA and incubated for the indicated time spans. The values of the three genes in untreated *as-1:GUS* plants were set to "1".

However, these results could not clearly answer the question what kind of stimulus is needed for activation of SCL14 dependent target genes. The investigated substances SA, INA and 2,4-D all have phytohormonal (the latter one as auxin analogue) as well as xenobiotic character depending on their concentration and electrophilic character due to their reactive side groups.

4.5.2 Hydrogen peroxide triggers the SCL14-dependent activation of *CYP81D11*, *MtN19-like* and *GSTU7*

Next, hydrogen peroxide was analyzed for its inducing properties. RNA from plants floated 0 h or 8 h in potassium phosphate buffer containing 16 mM hydrogen peroxide was analyzed in qRT-PCR. The target genes *CYP81D11*, *MtN19-like* and *GSTU7* were induced by hydrogen peroxide in wildtype plants. This induction is independent of NPR1 (unaltered in *npr1-1* mutants). In contrast to the induction with SA (Figure 4-8),

transcript levels of target genes are not hyperinducible in the *npr1-1* mutants. In the SCL14 over-expressing plants expression levels of the three genes are increased even under non-inducing conditions but only *CYP81D11* transcript is hyperinducible by hydrogen peroxide in these plants. This constitutive expression was already observed for the GUS gene in those plants (Siemsen, 2005).

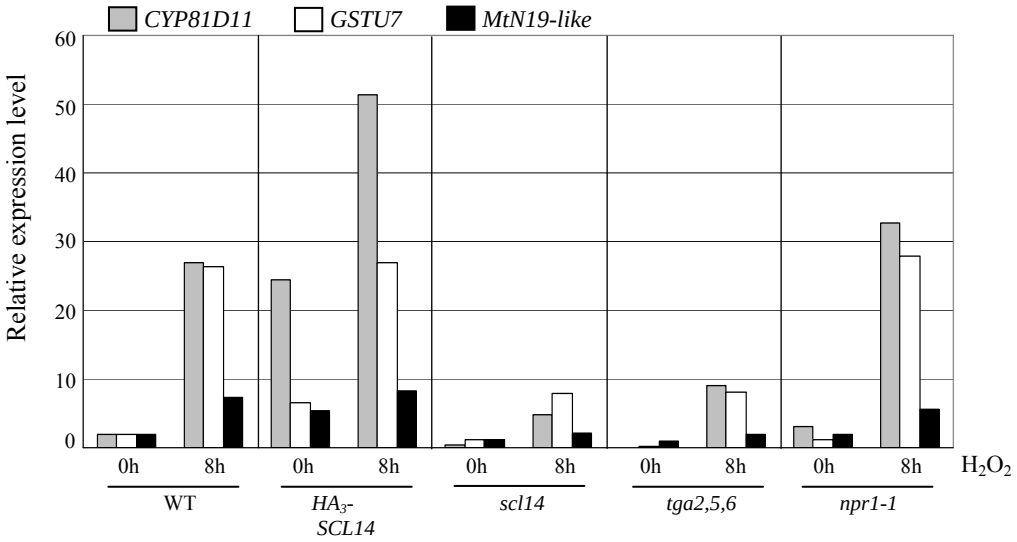

Figure 4-11 Expression of endogenous SCL14 target genes after treatment with hydrogen peroxide

Quantitative real-time RT-PCR analysis of relative *CYP81D11-*, *MtN19-like-* and *GSTU7* transcript levels in *as-1:GUS* (WT), *HA_3-SCL14*expressing plants, *scl14, tga2 tga5 tga6* (*tga2,5,6*) and *npr1-1* mutant plants. Three-week-old, MS-grown plants were floated in 50 mM potassium phosphate buffer (pH5,7) containing 16 mM H_2O_2 for the indicated time spans. The values of the three genes in untreated *as-1:GUS* plants were set to "1".

The induction of the SCL14 target genes by hydrogen peroxide treatment depends at least partly on SCL14 and the TGA factors as their expression is reduced in the *scl14* and the *tga2,5,6* mutant.

4.5.3 The chemical TIBA elicits SCL14 and TGA dependent expression of *CYP81D11*

2,3,5-triiodobezoic acid (TIBA), a compound known to inhibit auxin transport, was one of the strongest inducers of the genes identified in the micro array experiment as revealed by Genevestigator analysis (Figure 4-9). RNA from three week old plants

grown on MS-medium and treated for 0 h or 10 h with TIBA was analyzed by qRT-PCR analysis. The genes *CYP81D11*, *MtN19-like* and *GSTU7* were induced in wildtype plants. Similar to the results obtained with SA, INA and 2,4-D, induction of the three tested genes depends on SCL14 and TGA2, TGA5 or TGA6 (Figure 4-12).

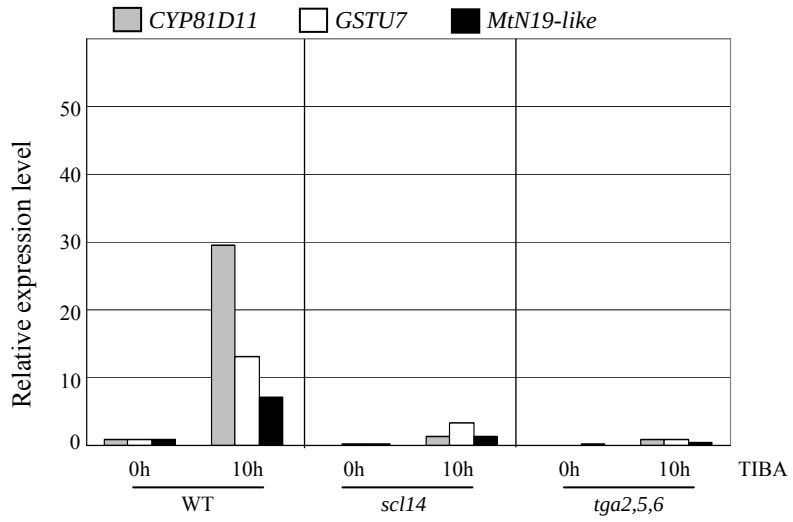

Figure 4-12 Expression of endogenous SCL14 target genes after treatment with TIBA

Quantitative real-time RT-PCR analysis of relative *CYP81D11*-, *MtN19-like*- and *GSTU7* transcript levels in *as-1:GUS* (WT), *scl14* and *tga2 tga5 tga6* (*tga2,5,6*) mutant plants. Three-week-old plants grown on MS plates were treated with 0.1 mM TIBA for 10 hours. The values of the three genes in untreated *as-1:GUS* plants were set to "1".

4.5.4 Complementation of the *scl14* mutant

Taken together, the three SCL14 target genes *CYP81D11*, *MtN19-like* and *GSTU7* are inducible by a variety of substances (SA, INA, 2,4-D, H_2O_2 and TIBA). To control whether the observed differences in the relative expression levels of the SCL14 target genes between wildtype and *scl14* mutants only refer to the loss of SCL14 protein, the *scl14* mutant was complemented with ectopically expressed SCL14.

Expression of SCL14 (untagged) under the control of the *CaMV 35S* promoter restored TIBA-dependent induction of *CYP81D11* transcript in the *scl14* mutants (Figure 4-13). Western blot analysis confirmed accumulation of SCL14 protein similar to wildtype levels in different transgenic lines (Figure 4-13). This experiment demonstrates that the defects of transcriptional activation in the *scl14* mutants are due to the lack of SCL14.

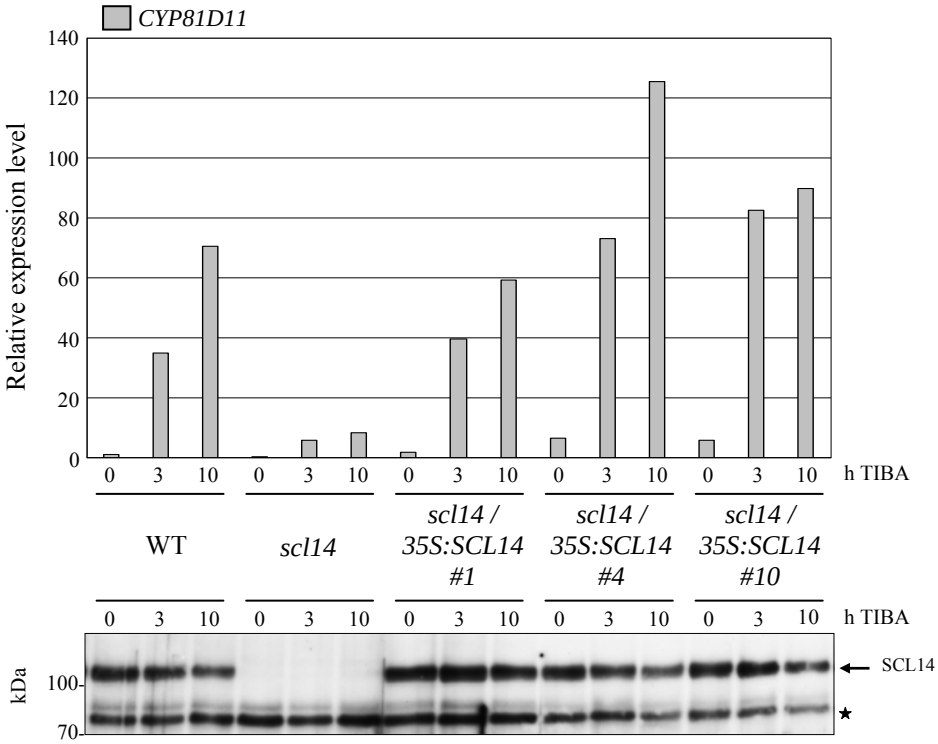

Figure 4-13 Complementation of the *scl14* mutant phenotype

Quantitative real-time RT-PCR analysis (upper panel) of relative *CYP81D11* transcript levels in *as-1:GUS* (WT), *as-1:GUS/scl14* (*scl14*), and three independent transgenic lines expressing SCL14 under the control of the *CaMV 35S* promoter in the *as-1:GUS/scl14* mutant background (*scl14/35S:SCL14*). Three-week-old plants grown on MS plates were treated with 0.1 mM 2,3,5-triiodobenzoic acid (TIBA) and incubated for further 10 hours. The expression value in untreated *as-1:GUS* plants was set to "1".

Crude protein extracts of the plants used for quantitative real-time RT-PCR analysis were subjected to Western blot analysis with the αSCL14 antiserum (lower panel). The antiserum was used in a 1:1000 dilution. The asterisk marks an unspecific band that serves as a loading control.

4.5.5 SCL14 binding to DNA is not influenced by TIBA

The auxin transport inhibitor TIBA efficiently increases the expression of SCL14-dependent target genes like *CYP81D11* (Figure 4-13). To study if this induction is reflected on the level of DNA binding of SCL14, ChIP experiments with chromatin from TIBA-treated wildtype plants were performed. The same wildtype plant material from the complementation experiment (Figure 4-13) was used in this experiment. The untreated *scl14* mutant serves as a control for the specificity of the αSCL14 antiserum.

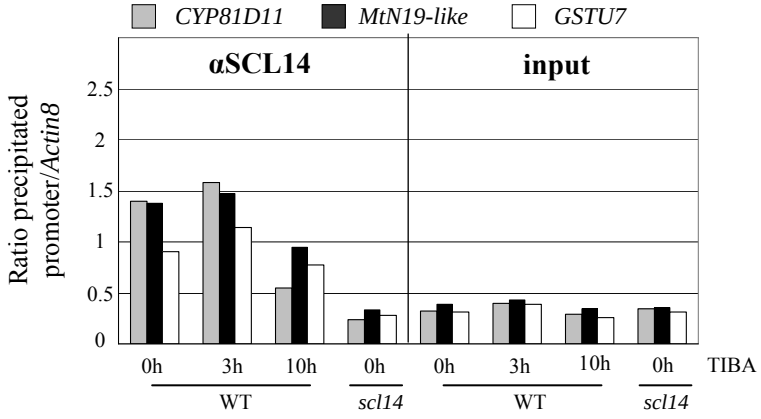

Figure 4-14 *In vivo* SCL14 binding to the promoters of *CYP81D11*, *MtN19-like* and *GSTU7* after treatment with TIBA as revealed by chromatin immunoprecipitation analysis

Leaves from *as-1:GUS* (WT) plants treated 3 or 10 hours with 0.1 mM TIBA and *scl14* mutants were incubated in 1% formaldehyde before chromatin preparation. Chromatin samples were subjected to immunoprecipitation using 5 µl of the αSCL14 antiserum. The DNA was recovered after reversal of the cross-links and analyzed for the enrichment of promoter sequences by quantitative real-time PCR. In this diagram the ratio between the respective precipitated promoter fragments and unspecifically precipitated *Actin 8*-DNA is depicted. Therefore, C_T-values obtained from the real-time PCR for the target promoters were set off with C_T -values for *Actin8* with the $2^{-\Delta C_T}$ method to normalize the amount of DNA in all preparations. Input controls are shown in the right part of the diagram.

The ratio between the specific promoter fragments and residual *Actin8* DNA (this "contamination" should be equal in all samples prepared with the same antiserum) as depicted in Figure 4-14 reflects the amount of precipitated target promoter DNA. An increased ratio in the samples compared to the respective input controls demonstrates enrichment of specific promoter fragments by immunoprecipitation. The ratio in the *scl14* mutant is equal to the input control, showing that αSCL14 antiserum failed to enrich specific promoter fragments in the mutants, thus showing the specificity of the antiserum. In contrast, the ratio of specific promoter fragments to *Actin8* fragments is increased in αSCL14-precipitated chromatin from wildtype plants, demonstrating recruitment of SCL14 to the target promoters in the uninduced wildtype plants. The binding of SCL14 remains nearly unaltered during treatment with TIBA. After 10 h of treatment the amounts of DNA-bound SCL14 are slightly decreased. This observation correlates with detected protein levels of SCL14 (Figure 4-15). Nevertheless, transcription of the target genes is activated after 10 h of treatment with TIBA (Figure 4-12) suggesting that binding of SCL14 alone is not sufficient to activate target gene promoters. Regulation of transcriptional activation might be achieved on another level,

e.g. modification of SCL14 or the TGA factors or removal of an additionally bound repressor protein.

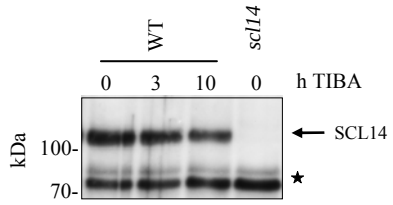

Figure 4-15 Abundance of SCL14 protein in TIBA-treated plants

Three-week-old plants grown on MS plates were treated with 0.1 mM 2,3,5-triiodobenzoic acid (TIBA) and incubated for further 10 hours. Crude protein extracts of the plants indicated above the lanes were subjected to Western blot analysis with the αSCL14 antiserum. The antiserum was used in a 1:1000 dilution. The asterisk marks an unspecific band that serves as a loading control.

4.6 Influence of SCL14 protein levels on tolerance of *Arabidopsis* to xenobiotic stress

The coding region of the SCL14 target genes imply that they might play a role in detoxification processes; this is consistent with the fact that transcription of these genes is efficiently induced by xenobiotic substances. To investigate whether this transcriptional activation can actually lead to resistance of plants to xenobiotics the performance of plants with different SCL14 protein levels and the *tga,2,5,6* mutants on medium containing different xenobiotic stressors was assayed. Therefore seeds of the different plant genotypes were directly germinated on MS-plates containing SA, INA or TIBA.

The general growth and development was strongly affected for all plants germinating on MS-plates containing 0.05 mM SA (Figure 4-16). But, consistent with the loss of transcriptional activation of SCL14 target genes, growth of *scl14* and *tga2,5,6* mutants are affected more strongly. In addition, SCL14 overexpressing plants (*HA_3-SCL14*) were more tolerant to SA, which correlates with hyper-inducibility of the SCL14/ TGA-dependent target genes (Figure 4-10, Figure 4-11).

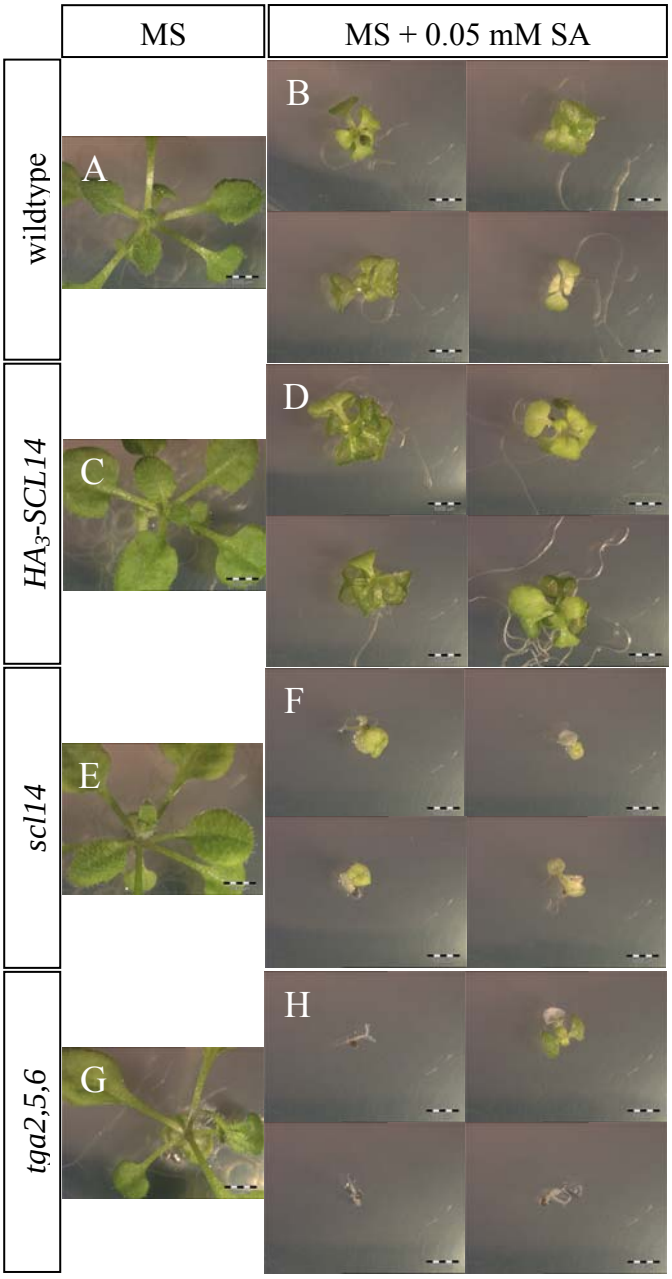

Figure 4-16 Seedling development on MS-plates containing SA

All plants on the right pictures were grown for 3 weeks on MS plates containing 0.05 mM salicylic acid (SA). The pictures on the left show the respective plants on MS plates without SA. Wildtype plants (*as-1:GUS*) show a reduction in growth (A, control; B, grown on SA), *35S:HA$_3$-SCL14* expressing plants are more resistant (C, control; D, grown on SA). Both mutants, *scl14* and *tga2,5,6* can hardly grow on plates containing SA (E,G, respective controls; F,H, respective mutants germinated on SA). The scale bar marks 2 mm.

The observed differences in tolerance to chemical stress are even more pronounced when plants germinate on MS plates containing 0.1 mM INA (Figure 4-17). Actually, tolerance to INA also correlates with the ability of the different plants to activate

transcription of SCL14/TGA dependent genes (Figure 4-8). The respective mutants fail to activate these genes and thus fail to tolerate the xenobiotic levels of INA.

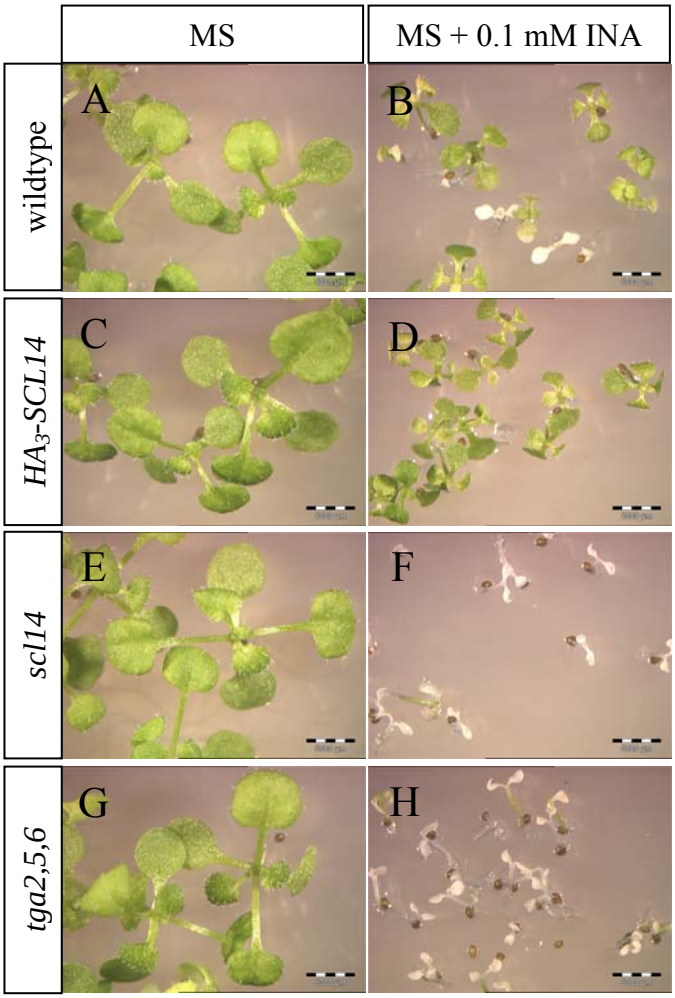

Figure 4-17 Seedling development on MS-plates containing INA

All plants on the right pictures were grown for 3 weeks on MS plates containing 0.1 mM isonicotinic acid (INA). The pictures on the left show the respective plants on MS plates without INA. Wildtype plants (*as-1:GUS*) show a reduction in growth (A, control; B, grown on INA), *35S:HA$_3$-SCL14* expressing plants are more resistant (C, control; D, grown on INA). Both mutants, *scl14* and *tga2,5,6* can not survive on INA (E,G, respective controls; F,H, respective mutants germinated on INA). The scale bar marks 5 mm.

4.6.1 Complementation of the growth defect of *scl14* mutants on xenobiotics

Consistent with the results for transcriptional activation, where TIBA elicits the strongest induction of SCL14 target genes, plants are most affected on MS plates containing 0.1 mM TIBA. Even wildtype plants can hardly survive. The restriction of

root elongation and general growth of the seedling only allows the plants to develop the first pair of leaves following the cotyledons (Figure 4-18 B). However, overexpression of SCL14 again leads to an increase in ability to cope with the xenobiotic stress, whereas, neither the *scl14* mutants nor the *tga2,5,6* mutants can survive on TIBA.

Ectopic expression of SCL14 (untagged) under the control of the *35S* promoter in the *scl14* mutant (Figure 4-18 J, here shown for *scl14*/ *35S:SCL14* line #10) led to a wildtype-like tolerance to TIBA, showing that SCL14 is sufficient to tolerate xenobiotic stress.

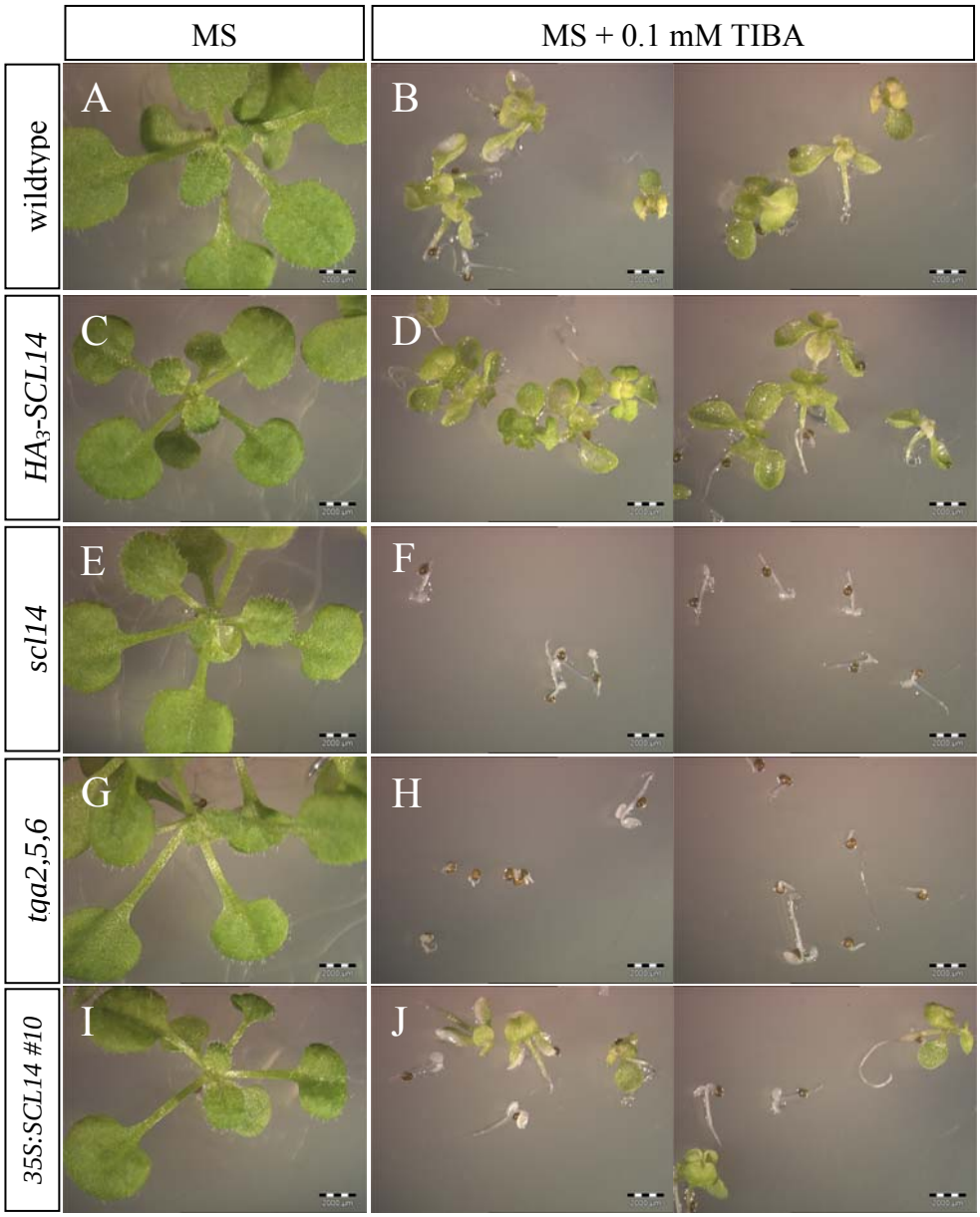

Figure 4-18 Seedling development on MS-plates containing TIBA

All plants on the right pictures (two plates are shown exemplarily for each genotype) were grown for 3 weeks on MS plates containing 0.1 mM 2,3,5-triiodobenzoic acid (TIBA). The pictures on the left show the respective plants on MS plates without TIBA. Wildtype plants (*as-1:GUS*) show a strong reduction in growth but can survive (A, control; B, grown on TIBA), *35S:HA*$_3$*-SCL14* expressing plants are more resistant to xenobiotics (C, control; D, grown on TIBA). Both mutants, *scl14* and *tga2,5,6* can not survive on TIBA (E,G, respective controls; F,H, respective mutants germinated on TIBA) but survival can be restored by expressing *35S:SCL14* in the *scl14* mutant background (I, control; J, *scl14/ 35S:SCL14* #10 grown on TIBA). The scale bar in the pictures marks 5 mm.

To quantify the observed differences in tolerance against xenobiotics 100 seeds per genotype were germinated on MS medium containing SA, INA or TIBA. Determination of the fresh weight of germinated plants after three weeks (not-germinated seeds were

counted as 0 mg) confirmed that the mutants accumulated less biomass than the wildtype (Figure 4-19). Increased accumulation of biomass exhibited by the *HA$_3$-SCL14* plants was detected only after TIBA treatment.

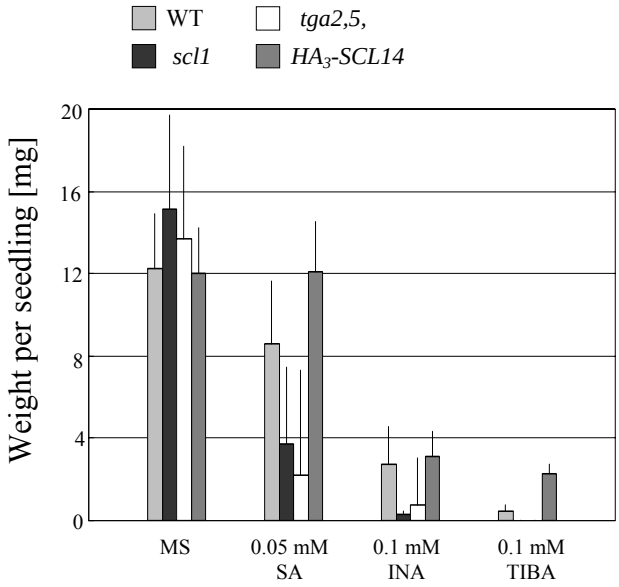

Figure 4-19 Determination of the fresh weight of seedlings grown on MS plates containing different xenobiotics

All indicated genotypes were grown for three weeks on MS plates containing either no xenobiotic (MS), 0.05 mM SA, 0.1 mM INA or 0.1 mM TIBA. For each treatment and genotype 100 seeds from five individual plates were weighted.

4.7 Identification of SCL14-dependent genes under inducing conditions

As SCL14 contributes to tolerance of plants to xenobiotics like TIBA, micro array experiments comparing *scl14* mutants and wildtype plants after TIBA treatment were performed to reveal additional target genes for SCL14 specifically regulated by xenobiotic stress.

RNA derived from about 50 plantlets of wildtype plants and *scl14* mutants grown aseptically for 21 days on MS medium induced with TIBA for 10 hours was hybridized with Arizona whole genome micro arrays.

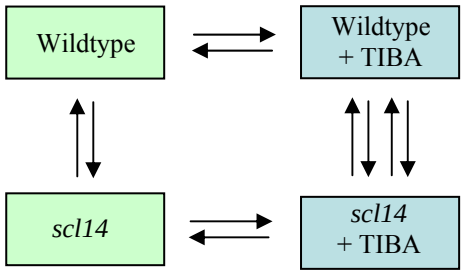

Figure 4-20 Scheme of the micro array analysis of TIBA-induced plants

RNA extracted from about 50 two week old, MS-grown plantlets for each genotype either uninduced or sprayed with 0.1 mM TIBA for 10 h was hybridized with Arizona whole genome array slides. Each arrow stands for an independent micro array slide.

84 genes including *SCL14* showed an over 2.5-fold (log_2) up-regulation in induced wildtype plants compared to *scl14* mutants induced with TIBA. 37 genes were regulated in a reciprocal manner (see supplemental data for the complete list comparing all conditions). The genes were clustered (Table 4-2) for their differential expression under non-inducing conditions and their altered TIBA-inducibility in the *scl14* mutants. Those marked in gold are the most promising candidates, because they are already reduced in the *scl14* mutant under non-inducing conditions and TIBA treatment fails to induce those genes in *scl14* mutants albeit strongly induces them if SCL14 is present.

Table 4-2 Short list of the up-regulated genes in micro array analysis comparing wildtype (WT) and *scl14* mutant (*scl14*) after TIBA treatment

The genes are sorted according to their differential expression after TIBA treatment comparing wildtype plants and *scl14* mutants (displayed as $\log_2$ fold induction). In this table all analysed combinations of treated and untreated plants are displayed allowing a detailed view on the expression pattern and SCL14 dependency of each gene. Red marked AGI codes indicate genes also found to be SCL14-dependent in the initial micro array experiment using only uninduced plants. The complete micro array analysis is listed in supplemental data.

AGI code	Gene Description	Wt (TIBA)/ scl14 (TIBA)	Wt/ scl14	scl14 (TIBA)/ scl14	Wt (TIBA)/ Wt
At3g59140	ATMRP14	4,57	1,69	0,04	2,92
At2g29490	ATGSTU1	4,39	2,05	1,96	4,3
At4g08555	unknown protein	4,33	0,99	2,51	5,85
At1g77120	ADH1	4,1	2,18	0,44	2,36
At3g28740	CYP81D11	4,07	2,96	4,22	5,34
At5g53990	glycosyl transferase	3,87	3,57	0,38	0,69
At3g43190	UDP-glycosyltransferase	3,73	3,66	-0,06	0,01
At1g77450	ANAC032	3,7	2,47	0,51	1,75
At4g37290	unknown protein, F6G17.7	3,59	0,07	0,05	3,57
At5g22140	disulfide oxidoreductase	3,52	1,81	2,72	4,42
At1g17170	ATGSTU24	3,45	2,6	3,43	4,28
At5g61820	MAC9.6 MtN19-like	3,42	2,06	0,98	2,33
At3g50970	XERO2	3,36	2,8	2,02	2,58
At4g34131	UDP-glycosyltransferase	3,33	1,46	2,31	4,19
At1g67810	Fe-S metabolism associated domain-containing protein	3,28	0,62	1,88	4,55
At1g05680	UDP-glycosyltransferase	3,26	1,01	3,3	5,54
At1g18970	GLP4	3,25	2,25	0,87	1,86
At5g06090	ATGPAT7/GPAT7	3,23	2,4	1,59	2,42
At1g17180	ATGSTU25	3,22	1,35	0,52	2,38
At2g29420	ATGSTU7	3,21	1,48	1,71	3,44
At1g07530	SCL14	3,2	3,51	-0,16	-0,46
At1g62570	disulfide oxidoreductase	3,19	2,34	0,06	0,92
At1g14130	oxidoreductase	3,18	0,7	1,04	3,52
At3g10880	unknown protein, T7M13.4	3,17	-0,02	0,87	4,07
At3g04890	unknown protein, T9J14.16	3,09	1,47	0,39	2,01

At2g34660	ATMRP2	3	0,93	-0,55	1,53
At1g55020	LOX1	2,98	1,76	0,22	1,44
At1g05560	UGT1	2,98	1,72	2,3	3,57
At2g29480	ATGSTU2	2,94	0,68	1,31	3,57
At1g78340	ATGSTU22	2,88	2,39	0,55	1,04
At4g14630	GLP9	2,86	1,05	0,11	1,92
At2g02120	LCR70/PDF2.1; protease inhibitor	2,81	1,22	1,64	3,24

	in *scl14* reduced, only inducible by TIBA in Wt
	in *scl14* reduced, stronger inducible by TIBA in Wt
	in *scl14* reduced, not inducible by TIBA (*SCL14*)
	in *scl14* unchanged

TAIR based analysis (http://www.arabidopsis.org) of the genes listed in Table 4-2 shows an over representation of transport related genes (Figure 4-21, 5), abiotic stimulus related genes (Figure 4-21, 8) and stress related genes (Figure 4-21, 11) in the array compared to the functional distribution of all genes in the *Arabidopsis* genome (Figure 4-21).

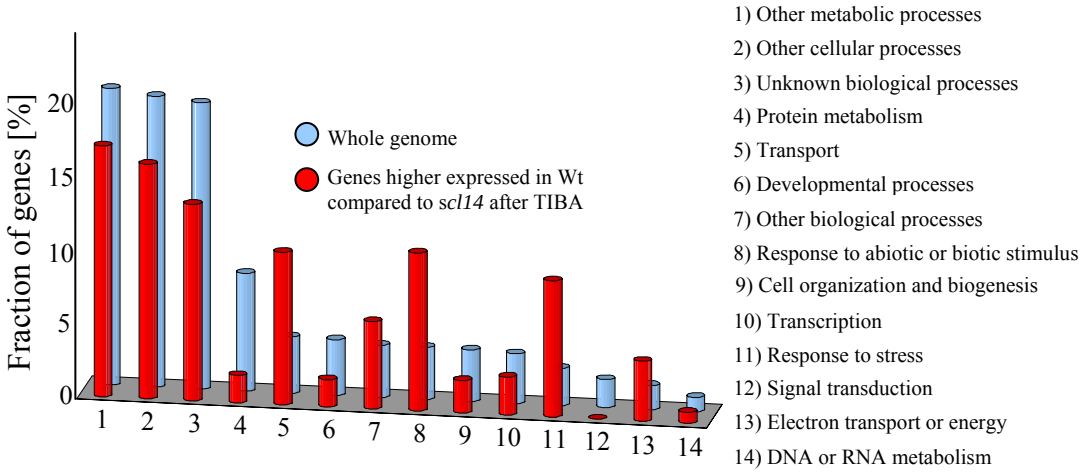

Figure 4-21 Functional distribution of genes higher expressed in wildtype plants compared to *scl14* mutants after TIBA treatment

The functional distribution of genes in the whole *Arabidopsis* genome (blue) and the performed micro array based comparison between wildtype and *scl14* mutants after TIBA treatment (red). Classification was performed with TAIR GO classification database tools.

Among these genes, a lot of stress related genes like *cytochrome P450* family members or *glutathione-S-transferases* are induced (Figure 4-22). All of them showed an over 2.7-fold ($\log_2$) difference when comparing the expression in wildtype plants versus expression in the *scl14* mutant after TIBA treatment. 7 out of the up-regulated genes were originally identified as SCL14 dependent genes in the initial micro array experiment comparing uninduced conditions of wildtype and *scl14* mutants (referring to the short list published by Tanja Siemsen, 2005).

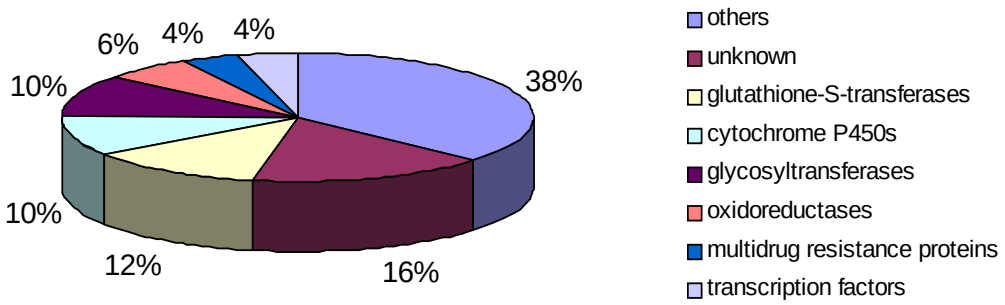

Figure 4-22 Distribution of up-regulated genes after TIBA induction comparing wildtype and *scl14* mutants

The functional classification was performed by TAIR database analysis.

4.7.1 Verification of TIBA-induced SCL14 target genes

From the genes listed in Table 4-2 putative target genes were selected for quantitative real-time RT-PCR analysis. *ATGSTU1* contains an *as-1*-like element in the promoter, thus represents a putative direct target gene for SCL14/ TGA dependent regulation. The *ADH1* gene showed the greatest differences (beside *ATMRP14* and *ANAC032*) in expression between wildtype and *scl14* mutant plants under induced and non-induced conditions. *ATMRP2* was also found to be induced by treatment with electrophile phytoprostanes PPA_1 (Mueller et al., 2008) demonstrating association of this gene to electrophile/ xenobiotic stress responses. These three genes were analysed in an independently performed TIBA induction experiment to reproduce the achieved data from the micro array analysis (Figure 4-23).

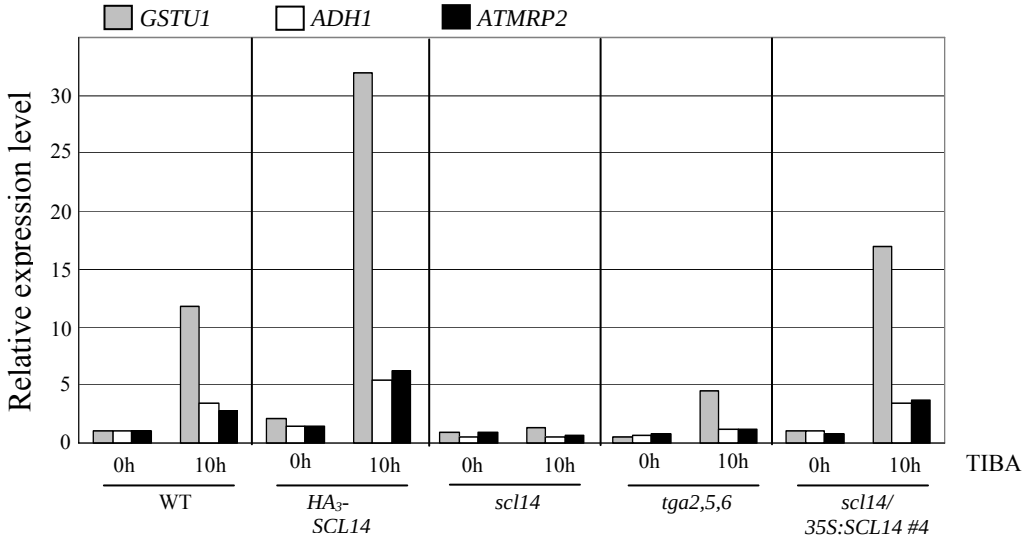

Figure 4-23 Expression of endogenous SCL14 target genes after TIBA treatment

Quantitative real-time RT-PCR analysis of relative *GSTU1-*, *ADH-* and *ATMRP2* transcript levels in *as-1:GUS* (WT), *35S:HA$_3$-SCL14* expressing plants, *scl14* mutants, *tga2 tga5 tga6* (*tga2,5,6*) mutants and *scl14* mutants complemented with *35S:SCL14*. Three-week-old plants grown on MS plates were treated with 0.1 mM TIBA for 10 hours. The values of the three genes in untreated *as-1:GUS* plants were set to "1".

All three genes are induced in an SCL14- and TGA-dependent manner confirming the results from the micro array. Their altered expression in the *scl14* mutant can be restored to wildtype levels by ectopic expression of SCL14 in the mutant background. Consistent with the results for *CYP81D11*, *MtN19-like* and *GSTU7*, the expression of the three genes is hyper-inducible in the *HA$_3$-SCL14* expressing plants.

Chromatin immunoprecipitation experiments might reveal the direct regulation of the *GSTU1* gene by SCL14 as it harbours a conserved *as-1*-like element in the promoter.

All data shown for the target genes propose a role for SCL14 in response to electrophilic/ xenobiotic substances. Whether this role also influences the tolerance of the plants against necrotrophic biological enemies like fungal pathogens, which elicit oxidative stress and the formation of RES, should be revealed by infection experiments with the fungus *Botrytis cinerea*, which also showed up as a strong inducer of SCL14 target genes in Genevestigator analysis (Figure 4-9).

4.8 Overexpression of SCL14 leads to resistance against the necrotrophic fungus *Botrytis cinerea*

The grey mould causing fungus *Botrytis cinerea* is a major pathogen for a wide range of plant species. In *Arabidopsis thaliana* resistance against this necrotrophic pathogen is mainly mediated by jasmonates and ethylene and mostly depends on a functional COI1 and EIN2 protein. Beside the JA/ET response, camalexin synthesis (PAD3-dependent) is required for resistance. In addition, salicylic acid dependent genes are activated (e.g. *PR-1*). In different microarray experiments the SCL14 target genes like *CYP81D11* (Figure 4-9) were also found to be activated after *B. cinerea* infection. To investigate whether the SCL14 target genes show an influence on defence reaction the different transgenic plants and mutants were inoculated with spores of *B. cinerea*. The infection was carried out using different strains of *Botrytis cinerea* showing the results of two of them. The strains used are B1.26 (Thiedemann, Figure 4-26) and BH/1 (B. Mauch-Mani, Figure 4-30). Leaves of three week old plants were inoculated with 5 µL of spores (1 x 10^6 spores/ mL PDB) from the respective *B. cinerea* cultivar. Wildtype plants and *coi1-1* mutants were inoculated with *Botrytis cinerea* BH/1 to investigate the degree of resistance against this fungal cultivar.

Figure 4-24 Infection of *coi1-1* mutants with *Botrytis cinerea* BH/1

Pictures showing leaves of (A) wildtype plants (*as-1:GUS*) and (B) *coi1-1* and the development of disease symptoms 96 hpi with *Botrytis cinerea* strain BH/1. Each leaf was inoculated with 5 µL of spore solution (1 x 10^6 spores/ mL PDB). As an uninfected control, the leftmost leaf was obtained from a non-infected plant grown side-by-side with the infected plants, respectively.

The F-box protein COI1 is essential for most of the aspects of JA dependent defence responses. As COI1 regulates activation of gene expression by targeting negative

regulators for degradation the mutants fail to activate sufficient defences against necrotrophic pathogens like *Botrytis cinerea*. Actually, the degree of resistance is remarkably lower in *coi1-1* mutants compared to wildtype plants (Figure 4-24). Compared to the *coi1-1* mutants, *tga2,5,6* mutants are more resistant to *B. cinerea* infection, although they show enhanced susceptibility when compared to wildtype plants (Figure 4-25).

Figure 4-25 Infection of plants with *Botrytis cinerea* BH/1

Pictures showing leaves of (A) wildtype plants (*as-1:GUS*) and (B) *tga2,5,6* mutants and the development of disease symptoms 96 hpi with *Botrytis cinerea* strain BH/1. Each leaf was inoculated with 5 µL of spore solution (1 x 10^6 spores/ mL PDB). As an uninfected control, the leftmost leaf was obtained from a non-infected plant grown side-by-side with the infected plants, respectively.

To investigate the influence of SCL14 on the resistance against *B. cinerea*, fungal propagation on wildtype plants was compared to propagation on *scl14* mutants and SCL14 overexpressing plants (*HA_3-SCL14*). This infection was performed using the *B. cinerea* cultivar B1.26 on four week old plants. As indication for a susceptible phenotype, *tga2,5,6* mutants were also infected with this cultivar (Figure 4-26).

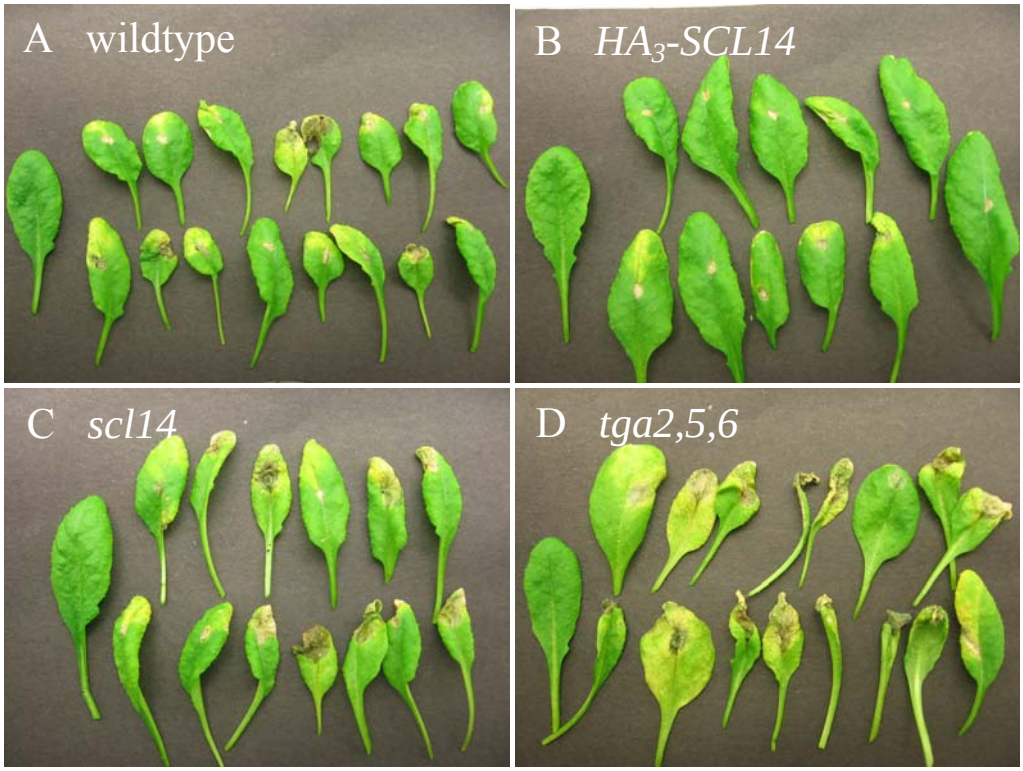

Figure 4-26 Infection of plants with *Botrytis cinerea* B1.26

Pictures showing leaves of (A) wildtype plants (*as-1:GUS*), (B) *35S:HA$_3$-SCL14* expressing plants, (C) *scl14* mutants and (D) *tga2,5,6* mutants and the development of disease symptoms 96 hpi with *Botrytis cinerea* strain 1.26. Each leaf was inoculated with 5 µL of spore solution (1 x 10^6 spores/ mL PDB). As an uninfected control, the leftmost leaf was obtained from a non-infected plant grown side-by-side with the infected plants, respectively.

The infected leaves of wildtype plants and the *scl14* mutants show no obvious differences in susceptibility to the fungus (Figure 4-26 A and C). The leaves of *tga2,5,6* mutants show a higher susceptibility to this *B. cinerea* cultivar in consistence with the observed phenotype after BH/1 infection. However, overexpression of SCL14 leads to a high degree of resistance against *Botrytis cinerea* (Figure 4-26 B). Leaves of *HA$_3$-SCL14* plants only show small lesions at the direct site of infection.

The propagation of the fungus was measured by amplification of *B. cinerea ActinA* gene by qRT-PCR analysis. The relative expression levels of *BcActinA* gene reflect the observed phenotypes from the infected leaves (Figure 4-27).

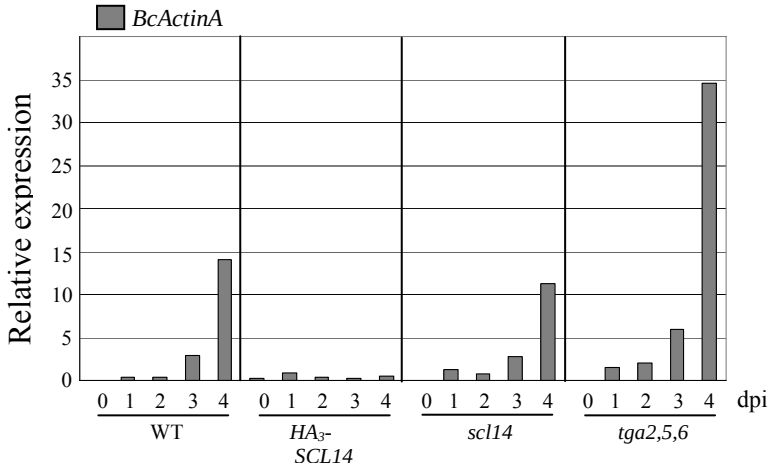

Figure 4-27 Quantitative RT-PCR analysis of fungal *ActinA* transcript levels in plants infected with *Botrytis cinerea* strain 1.26

The level of *ActinA* marks the degree of fungal propagation on the respective plants. Inoculated leaves were collected at the indicated time points (every 24 hours).

In *HA_3-SCL14* expressing plants the amount of detectable *ActinA* transcript is not increased during infection, reflecting the growth restriction of the fungus on these plants. In the wildtype and *scl14* mutant plants *ActinA* transcript increases during infection reflecting spread of the fungus on the leaves. The *tga2,5,6* mutants were more susceptible, thus, *ActinA* transcripts accumulate to higher levels especially in later stages of infection (96 hpi).

To elucidate whether the SA- and JA-dependent defence response pathways contribute to the observed phenotypes the expression of the *PR-1* and *PDF1.2* gene 96 h after *B. cinerea* infection was analyzed as markers for SA and JA signalling respectively.

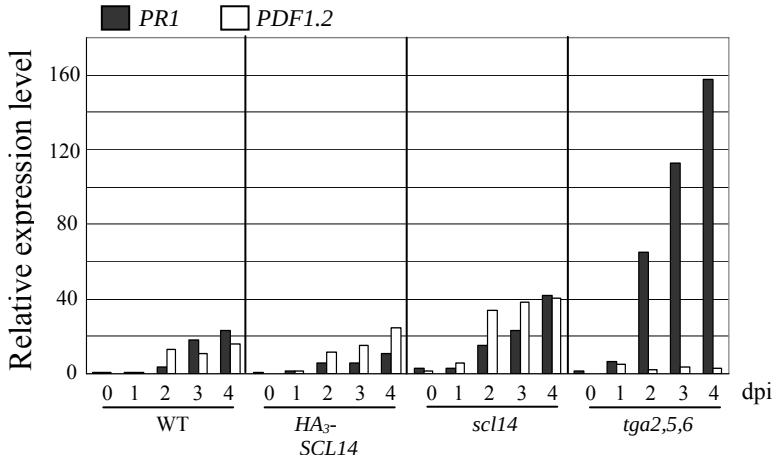

Figure 4-28 Quantitative RT-PCR analysis of *PR-1 and PDF1.2* transcript levels in plants infected with *Botrytis cinerea* strain 1.26

The expression of *PDF1.2* after *B. cinerea* infection depends on the TGA factors. In contrast expression of *PR-1* after infection with *Botrytis* is enhanced in the *tga2,5,6* mutant. Inoculated leaves were collected at the indicated time points (every 24 hours).

The *Botrytis* induced expression of the *PDF1.2* gene is reduced in the *tga2,5,6* mutants (Figure 4-28). Interestingly, the *PR-1* transcript levels are increased in the mutants after fungal attack, pointing at an influence of the TGA factors in SA/JA and ET crosstalk during *B. cinerea* infection. Further studies are necessary to determine the exact role of the TGA factors in this regulation of resistance against *Botrytis cinerea*.

However, PR-1 and PDF1.2 transcript levels are both slightly increased in scl14 mutants whereas PDF1.2 transcript levels are slightly lower in the SCL14 overexpressing plants. This might due to the infection of more cells in the *scl14* mutants in comparison with the SCL14 overexpressing plants.

In addition, Botrytis cinerea was found to be a good inducer of SCL14 target genes in Genevestigator analysis. However, induction of SCL14 target genes in *B. cinerea* infection experiments was not reproducible (here shown for *CYP81D11* (Figure 4-29)). All analyzed transcript levels (*CYP81D11*, *MtN19-like, GSTU7*, *GSTU1* and *ATMRP2* were unchanged during infection (data not shown).

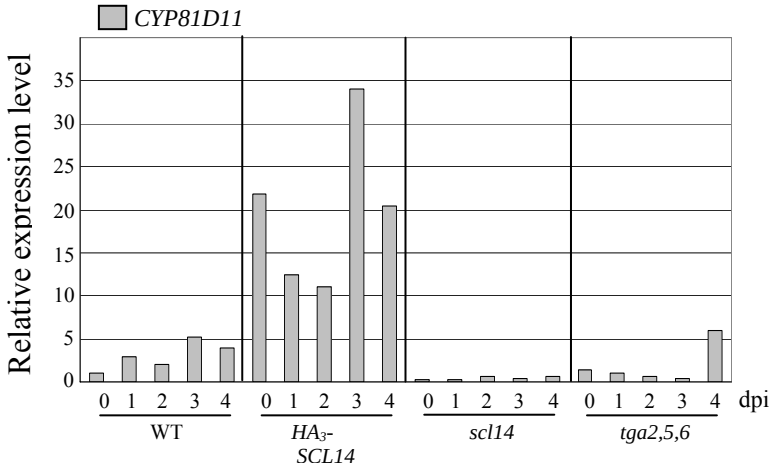

Figure 4-29 Quantitative RT-PCR analysis of *CYP81D11* transcript levels in plants infected with *Botrytis cinerea* strain 1.26

The level of *CYP81D11* depends on SCL14 and TGA factors. Expression in the *tga2,5,6* mutant at 96 hpi possibly due to total maceration of the tissue. Inoculated leaves were collected at the indicated time points (every 24 hours). The relative levels of *CYP81D11* transcript in uninfected wildtype plants were set to "1", all others are shown as "fold induction" over the uninfected wildtype.

Expression of *CYP81D11* transcript is nearly unaffected in wildtype plants. Minimal induction of the gene depends on SCL14 and TGA factors (although transcript accumulates in *tga2,5,6* mutants after 96 hpi). In contrast, transcript levels of *CYP81D11* are constitutively higher in SCL14 overexpressing plants. This leads to the hypothesis of a more generally resistant phenotype of the *HA_3-SCL14* expressing plants due to constitutive active expression of genes associated with detoxification like *CYP81D11* and *GSTU7*. This also resembles the phenotypes on MS-plates containing xenobiotic substances like TIBA, SA or INA (Figure 4-16, Figure 4-17 Figure 4-18).

To exclude an influence of the HA_3-tag, infection experiments with a plant line overexpressing *35S:SCL14* (untagged) were performed. These plants also show a high degree of resistance against fungal attack (Figure 4-30).

Figure 4-30 Infection of SCL14 overexpressing plants with *Botrytis cinerea* BH/1

Pictures showing leaves of (A) wildtype plants (*as-1:GUS*) and (B) *35S:SCL14* (untagged) expressing plants and their development of disease phenotypes 96 hpi with *Botrytis cinerea* strain BH/1. Each leaf was inoculated with 5 µL of spore solution (1 x 10^6 spores/ mL PDB). As an uninfected control, the leftmost leaf was obtained from a non-infected plant grown side-by-side with the infected plants, respectively.

5 Discussion

The TGA family of transcription factors and the corresponding target sequence *as-1* belong to the first experimental systems to be established for studying transcriptional control mechanisms in plants (Katagiri *et al.*, 1989). The *as-1* element was originally identified as the only regulatory *cis* element within the "truncated" *CaMV 35S* promoter (Lam *et al.*, 1989). This 90 bp promoter fragment confers transcriptional activation in response to high levels of SA and 2,4-D in tobacco (Liu and Lam, 1994; Qin et al., 1994) and Arabidopsis (Redman *et al.*, 2002). The activating pathway is independent from NPR1 (Butterbrodt et al., 2006), a regulatory protein that interacts with TGA factors to confer expression of genes involved in systemic acquired resistance (Fan and Dong, 2002). In this work, chromatin immunoprecipitation assays present evidence that *as-1*-bound TGA factors recruit the GRAS protein SCL14 to a number of endogenous promoters that are inducible by SA, 2,4-D and other chemicals. Activation of the corresponding genes helps to protect plants against at least certain types of xenobiotic stresses. In addition, overexpression of SCL14 leads to a high resistance against the necrotrophic fungus *Botrytis cinerea*.

5.1 Functional role of the intracellular transport of SCL14

In SCL14, the GRAS domain harbours a putative NES (aa 393 to 401: LRTLLVLC). This sequence might facilitate constant shuttling of the protein between the nucleus and the cytosol, which would explain its localization in both compartments (Figure 4-2). Studies from Thomas Merkle showed the interaction of SCL14 with the nuclear export receptor XPO1 in yeast, suggesting an active export of SCL14 from the nucleus. This active export can be disturbed by treatment with LMB. In fact, SCL14 only localizes to the nucleus after treatment of cells with LMB demonstrating that this export actually occurs in plant cells. Unfortunately, experiments with mutant versions of SCL14 protein with disturbed NES (L393A, L396A, V398A) were not successful as the expression of

those mutant proteins was lethal at least for protoplasts in transient assays. Thus, the exact role of the transport remains to be elucidated but one possible function might be that SCL14 may receive modifications in the cytosol or act as carrier to import or export other regulatory proteins.

5.2 Function of the N-terminal domain of SCL14

Direct DNA binding has never been reported for any of the GRAS proteins, but a recent study reported association of DELLA proteins with selected GA-responsive promoters (Zentella et al., 2007). SCL14 cannot be detected at its target promoters in the absence of TGA2, TGA5 or TGA6 (Figure 4-5). As the N terminus of SCL14 provides the interface for the interaction with TGA2 (Figure 4-1), the conclusion is that this interaction recruits the protein to the respective promoter regions. It may be speculated that the DELLA proteins are also associated with their target promoters through the action of sequence-specific DNA-binding proteins.

Based on the occurrence of conserved amino acid sequences in the more variable N-terminus of GRAS proteins, the GRAS gene family has been divided into eight subfamilies (Tian *et al.*, 2004). SCL14 belongs to the LlSCL/SCL9-branch. Its best characterized member is LlSCL (*Lilium longiflorum* SCARECROW-like) from lily, which is predominantly expressed in anthers during the premeiotic phase (Morohashi *et al.*, 2003). In contrast, SCL14 is weakly expressed in all tissues, and shows 10-fold higher levels of gene expression in dry seeds (https://www.genevestigator.ethz.ch/). When fused to the GAL4 DNA binding domain, the N terminus of the LlSCL protein confers transcriptional activation in yeast as well as in plant cells. The activation domain was mapped to a stretch of 19 residues containing 7 acidic amino acids (motif I). This motif as well as the sequence DEDED (motif II) is conserved in SCL14 at positions aa 83 to 101 and 309 to 313, respectively, and might be responsible for the transcriptional activation conferred by the TGA2/ SCL14 complex in yeast.

Screens in yeast for SCL14 interacting proteins failed until now because of its activation potential, which leads to auto activation in yeast, when SCL14 is fused to a DNA-binding domain (like GAL4-BD). Derivatives of SCL14 mutated in this possible

activation domain showed no strong reduction in auto activation potential in yeast suggesting that additional domains confer activation.

5.3 Genevestigator clustering analysis of SCL14 target genes

In order to identify the conditions leading to activation of SCL14-dependent genes a Genevestigator clustering analysis was performed. This analysis revealed a variety of conditions, which induce SCL14 target genes (Figure 4-9). One common characteristic of the treatments is that they might create some sort of oxidative stress. For instance, reactive oxygen species (ROS) are generated after infection of plants with *Botrytis cinerea* or *Pseudomonas syringae*, hydrogen peroxide and ozone are ROS themselves and low CO_2, treatment with the photosystem II inhibitor PNO8, and high light conditions enhance the ROS-forming potential of the chloroplasts (Apel and Hirt, 2004). Chemicals related to auxin action, like 2,4-D, 2,4,6-T, PCIB and TIBA, also induce SCL14 target genes. A common feature of these chemicals is that they consist of a halogen-substituted aromatic ring system that is likely to react with sulfhydryl groups. Therefore, they might activate the anti-xenobiotic genes by changing the redox state of the cell rather than by changing the auxin response. Consistently, the auxin transport inhibitor NPA (naphthylphthalamic acid), which does not contain strong electron-drawing substituents, does not induce expression of SCL14 target genes, supporting the hypothesis that the electrophilic character rather than the effects on auxin action are critical for eliciting the anti-xenobiotic stress response. Likewise, electrophilic phytoprostanes, which can damage cells by integrating into membranes and alter the selective permeability of the lipid bilayer (Mueller, 2004), are also capable of inducing an anti-xenobiotic stress response largely dependent on TGA2, TGA5 or TGA6 (Mueller et al., 2008). Thus, redox-dependent processes influencing the activity of bZIP transcription factors may constitute a common principle between mammals and plants.
Additionally, five of the genes listed as SCL14-dependent (*CYP81D11 (At3g28740)*, *UDP-glucosyl transferase (At1g05680)*, *NADPH-dependent oxidoreductase (At5g16980)*, *GSTU7 (At2g29420)*, *AtNAC002 (At1g01720)*) were identified in microarray experiments designed to monitor global changes in the *Arabidopsis*

transcriptome after treatment with the allelochemical benzooxazolinone (BOA) (Baerson et al., 2005). The chemical warfare between neighbouring plants or other organisms that produce toxic compounds might have led to the evolution of mechanisms that allow the detoxification of a wide spectrum of harmful chemicals, irrespective of whether they occur in nature or whether they are synthetic.

Induction of the genes *CYP81D11*, *MtN19-like* and *GSTU7* by these chemicals (TIBA, SA, INA, hydrogen peroxide) was strongly reduced in *scl14* and *tga2,5,6* mutants, showing the importance of both factors, SCL14 as well as the class II TGA transcription factors, for the regulation of the xenobiotic-induced genes. The lower performance of the *scl14* and *tga2,5,6* seedlings on medium containing harmful concentrations of SA, INA or TIBA (Figure 4-16, Figure 4-17, Figure 4-18) is consistent with the idea that these compounds are less efficiently detoxified because of insufficient induction of the corresponding genes. Actually, the levels of *SCL14* transcript are about ten-fold higher in dry seeds suggesting a role of SCL14 in detoxification processes during germination.

5.4 Functional role of SCL14 target genes

SCL14 target genes were identified by Tanja Siemsen (2005) by comparing the transcriptome of plants with higher or lower levels of SCL14 (*HA_3-SCL14* transgenic plants versus *scl14* mutant plants). Consistent with the idea that a subset of TGA-regulated genes is related to defence responses against xenobiotic stress (Zhang and Singh, 1994; Pascuzzi et al., 1998; Klinedinst et al., 2000), genes with putative functions in the inactivation of toxic compounds were found. In plants and animals, the detoxification process starts with the introduction of functional groups by enzymes like P450 monooxygenases (phase I), which are subsequently conjugated to glucose of glutathione by enzymes such as UDP-glucosyl transferases and GSTs (phase II). Such modifications result in less toxic and/or more water-soluble conjugates which are subsequently deposited either in the vacuole or the apoplast (phase III) (Sandermann, 1992). Some of the SCL14 target genes are potentially involved in phase I (*cytochrome P450 family protein CYP81D11 (At3g28740)*, *monooxygenase MO1 (At4g15760)*) or

phase II (*UDP-glucosyl transferase (AT1g05680)*, *oxidoreductase (At2g37770)*, *NADPH-dependent oxidoreductase (At5g16980)*, *GSTU7 (At2g29420))*.

Since the first micro array analysis only considers uninduced conditions a new micro array analysis was performed comparing the *scl14* mutant with wildtype plants after treatment with the electrophilic substance 2,3,5-triiodobenzoic acid (TIBA) in order to find SCL14 target genes directly connected to xenobiotic stress. The main analysis focussed on the up-regulated genes after TIBA treatment comparing transcript levels of the wildtype with those found in the *scl14* mutant (Table 4-2). Some of the previously found genes (*CYP81D11*, *MtN19*-like also named *Mac9.6*, *GSTU7*, one UDP-glycosyl transferase and the transcription factor *ANAC032*) also showed up in this second experiment. In addition, several new putative target genes for SCL14 were identified, reflecting the role of SCL14 for the regulation of genes associated with detoxification processes.

As expected, one major group of genes overrepresented in the "TIBA micro array" are involved in the response to general stress and abiotic stimuli including several *glutathione-S-transferases*. These genes putatively are involved in the detoxification of radicals and xenobiotics or in the balancing of the redox state of the cell.

Additionally, genes coding for proteins involved in transport are overrepresented in the micro array after TIBA. Among these transport related genes, two genes coding for transporters of the ABCC-type belonging to the family of multidrug-resistance associated proteins (MRPs) were induced (*ATMRP2* and *ATMRP14*). Those transporters are mainly involved in the deposition of toxic compounds into the vacuole (Klein et al., 2006), thus playing a role in the phase III of detoxification processes. Likewise, *ATMRP2* transcript was also found to be induced after treatment of plants with electrophilic phytoprostanes (Mueller et al., 2008).

To verify the genes identified in the micro array analysis being in fact SCL14-dependent, some of the most promising candidates were chosen and confirmed as target genes (*GSTU1*, *ADH1*, *ATMRP2,* Figure 4-23) or false positives (*LOX1*, data not shown) using quantitative real-time RT PCR. Transcripts of *GSTU1*, *ADH1* and *ATMRP2* also responded in a SCL14 and TGA dependent manner in this independent TIBA induction experiment. However, promoter sequence analysis revealed an *as-1*-like element only for *GSTU1* suggesting the others to be secondary SCL14-dependent genes. So far, ChIP

experiments confirmed *CYP81D11*, *GSTU7*, *MtN19-like* (FX), the transcription factor *ANAC032* and *monooxygenase MO1* (data not shown) as genes directly regulated by the TGA/SCL14 complex.

5.5 Regulation of genes involved in detoxification

In mammalian systems, transcriptional responses of genes involved in phase I, II and III detoxification steps can be mediated by at least three different pathways. The first two involve receptor-xenobiotic ligand interactions: (1) After binding of the ligand, the aryl hydrocarbon nuclear receptor (AhR) is translocated to the nucleus and subsequently dimerizes with the co-activator Arnt to stimulate transcription (Denison and Nagy, 2003). (2) The pregnane X receptor and the androstane receptor can bind promiscuously to structurally diverse xenobiotic ligands and activate gene expression after forming heterodimers with the 9-*cis*-retinoic acid receptor (Kliewer et al., 2002). (3) The third mammalian xenobiotic-sensing system involves the bZIP transcription factor Nrf2, which is retained in the cytosol by the protein Keap1. In the presence of a wide range of structurally diverse sulfhydryl-reactive electrophilic compounds, two critical cysteine residues in the Keap1 protein are oxidized. This releases Nrf2, which enters the nucleus and subsequently activates target promoters, possibly in association with small bZIP transcription factors of the Maf family (Nguyen et al., 2003).

In plants, the mechanisms leading to the activation of transcriptional responses to xenobiotic stress are far less elucidated. So far, only the TGA factors have been implicated in this response (Zhang and Singh, 1994; Pascuzzi et al., 1998; Klinedinst et al., 2000), and TGA1 has been shown recently to be redox-regulated after treatment of plants with SA (Fobert and Despres, 2005). As SCL14 target genes are potentially involved in detoxification steps, a role for TGA/ SCL14 in the regulation of detoxification steps was assumed.

5.6 Role of SCL14 during infection with *Botrytis cinerea*

Botrytis cinerea is a necrotrophic fungus infecting a broad spectrum of host plants causing grey mould not only on plant leaves or stems but also on fruits before and after harvest. During infection, hydrogen peroxide and electrophilic compounds like phytoprostanes are generated in the plant cells. Plants react to the infection by a variety of defence responses including gene expression remodelling to accumulate anti-fungal proteins like plant defensins (including PDF1.2). The SCL14 target genes are also induced after *B. cinerea* attack as revealed by Genevestigator analysis (Figure 4-9). Unfortunately, this induction was not reproducable in the *B. cinerea* experiments done in this work. However, *scl14* mutants showed no enhanced susceptibility (Figure 4-26), suggesting only a minor role for SCL14 target genes during infection. In addition, plants lacking SCL14 and the possibly redundant TGA-interacting SCL31 protein (Süthoff, 2006) are also not more susceptible to *Botrytis* infections than wildtype plants or *scl14* mutants (data not shown), indicating that other plant responses are more important in determining the outcome of the interaction. In wildtype situation resistance against necrotrophic pathogens mostly depends on camalexin synthesis via PAD3 (cytochrome P450 family protein) (Ferrari et al., 2007) and JA/ ET-dependent defence responses mediated by ERF1 (Lorenzo et al., 2003). These pathways provide dominant resistances where loss of SCL14 has no influence.

In contrast, SCL14 overexpressing plants show a heightened resistance against *B. cinerea* (Figure 4-26). A model for the *35S:SCL14* expressing plants propose, that the constitutive activation of genes coding for detoxifying enzymes (Figure 4-11, Figure 4-29) leads to the heightened resistance. Other studies pointed out that the HR during attack of the pathogen lead to enhancement of fungal spread (Govrin and Levine, 2000) as necrotrophs like *Botrytis* utilise nutrients from dead cells. In *35S:SCL14* expressing plants, fast and constant removal of radicals by constitutively expressed detoxification proteins possibly limits cell death and so the supply of nutrients for the fungus. However, whether the *35S:SCL14* mediated resistance might overcome *pad3*, *ein2* or *coi1* susceptibility has yet not been elucidated.

In contrast, *tga2,5,6* mutants are highly susceptible to *B. cinerea*, showing the importance of TGA factors during fungal attack. This lowered resistance probably due

to TGA-function in JA/ET signalling. As the *tga2,5,6* mutant fails to induce *PDF1.2* gene expression after JA/ET stimulus (Mark Zander, personal communication), the interrupted JA/ET signalling most likely also contributes to the lack of induction of *PDF1.2* transcript after *B. cinerea* infection (Figure 4-28). This lack of PDF1.2 correlates with susceptibility also in the bos3 (*Botrytis*-susceptible 3) mutant (Veronese et al., 2004). In addition, the JA signalling mutant *coi1-1* shows severely reduced resistance (Figure 4-24) demonstrating the importance of JA signalling pathways during fungal attack.

5.7 Direct regulation of target gene transcription by the TGA/ SCL14 complex at *as-1*-like elements

Western blot analysis revealed that TGA factors and SCL14 are pre-existent in the cell and ChIP analysis indicated that the TGA/ SCL14/ *as-1* complex is preformed at the promoter (Figure 4-5, Figure 4-3). However, this complex is less efficient to activate transcription under non-inducing conditions as compared to inducing conditions. Additionally, SCL14 protein levels and the degree of binding of SCL14 to the target promoters are only very slightly increased in *35S:SCL14* expressing plants (Figure 4-5) whereas transcription of target genes is constitutively active (Figure 4-11, Figure 4-29) even in uninduced situations. Moreover, under inducing conditions (TIBA treatment), complex formation of TGA/ SCL14 at *as-1*-like elements remains unchanged (Figure 4-14) suggesting another mechanism of transcriptional activation than recruitment of SCL14 to the respective promoters. It remains to be investigated how the different chemicals are perceived and how this leads to the activation of target promoters. The elucidation of this mechanism should also explain why even the three target genes (*CYP81D11*, *GSTU7*, *MtN19-like*) show a differential responsiveness towards SA versus 2,4-D (Figure 4-7, Figure 4-8). Whether the GRAS protein SCL14 is a direct target of the regulation or whether it is only needed for the activation process as soon as another compound is modified remains to be shown.

Another enigma that has to be solved is to define the characteristics that render *as-1*-containing regulated promoters SCL14-dependent, NPR1-dependent or even independent from both proteins like e.g. *GSTF8*. A recent study suggested that NPR1 is

recruited to the *PR-1* promoter by a yet unknown protein (Rochon et al., 2006). The binding site of this unknown protein might be missing in the endogenous promoters that require SCL14 for their expression. It also remains to be investigated, why SCL14 target promoters are hyperinducible in the *npr1* mutant after SA stimulus (Figure 4-8), a phenomenon that has been observed before for *GSTU7* (Blanco et al., 2005).

6 Supplemental data

Table 6-1 Up-regulated genes after treatment with TIBA comparing wildtype and *scl14* mutant

The genes are sorted according to their differential expression after TIBA treatment comparing wildtype plants and *scl14* mutants (displayed as $\log_2$ fold expression). In this table all analysed combinations of treated and untreated plants are displayed allowing a detailed view on the expression pattern and SCL14 dependency of each gene. Genes listed were expressed with a false discovery rate of 10 %.

AGI code	Gene Description	Wt/ *scl14*	*scl14* (TIBA)/ *scl14*	Wt (TIBA)/ Wt	Wt (TIBA)/ *scl14* (TIBA)
At3g44980	unknown protein	-0,1	-4,62	0,41	4,92
At4g08530	lipid binding	0,73	-3,04	0,95	4,72
At3g59140	ATMRP14	1,69	0,04	2,92	4,57
At2g29490	ATGSTU1 (GLUTATHIONE S-TRANSFERASE 19); glutathione transferase	2,05	1,96	4,3	4,39
At4g08555	-	0,99	2,51	5,85	4,33
At1g77120	ADH1 (ALCOHOL DEHYDROGENASE 1); alcohol dehydrogenase	2,18	0,44	2,36	4,1
At3g28740	CYP81D11	2,96	4,22	5,34	4,07
At3g44935	-	0,67	-6,39	-3	4,06
At5g53990	transferase, transferring glycosyl groups	3,57	0,38	0,69	3,87
At3g43190	UDP-glycosyltransferase/ sucrose synthase/ transferase, transferring glycosyl groups	3,66	-0,06	0,01	3,73
At4g31950	CYP82C3; heme binding / iron ion binding / monooxygenase/ oxygen binding	2,3	-4,15	-2,72	3,72
At1g77450	ANAC032; transcription factor	2,47	0,51	1,75	3,7
At3g57010	strictosidine synthase	3,21	-3,33	-2,9	3,63
At4g37290	unknown protein	0,07	0,05	3,57	3,59
At5g22140	disulfide oxidoreductase/ electron carrier	1,81	2,72	4,42	3,52
At5g10830	unknown protein	1,69	0,53	2,31	3,48
At4g23700	ATCHX17; monovalent cation:proton antiporter	2,81	0,74	1,38	3,45
At1g17170	-	2,6	3,43	4,28	3,45

At5g61820	unknown protein	2,06	0,98	2,33	3,42
At4g31970	CYP82C2; heme binding / iron ion binding / monooxygenase/ oxygen binding	-0,96	-0,77	3,58	3,4
At3g50970	XERO2	2,8	2,02	2,58	3,36
At5g62340	enzyme inhibitor/ pectinesterase/ pectinesterase inhibitor	1,24	-2,89	-0,78	3,34
At5g49480	ATCP1; calcium ion binding	1,06	-0,28	1,99	3,34
At4g34131	UDP-glycosyltransferase/ transferase, transferring hexosyl groups	1,46	2,31	4,19	3,33
At3g06170	unknown protein	2,84	-1,02	-0,55	3,31
At3g50640	unknown protein	2,35	-2,31	-1,37	3,29
At1g67810	unknown protein	0,62	1,88	4,55	3,28
At1g05680	UDP-glycosyltransferase/ transferase, transferring glycosyl groups / transferase	1,01	3,3	5,54	3,26
At1g18970	GLP4 (GERMIN-LIKE PROTEIN 4); nutrient reservoir	2,25	0,87	1,86	3,25
At5g06090	ATGPAT7/GPAT7; 1-acylglycerol-3-phosphate O-acyltransferase/ acyltransferase	2,4	1,59	2,42	3,23
At1g17180	ATGSTU25; glutathione transferase	1,35	0,52	2,38	3,22
At2g29420	ATGSTU7 (GLUTATHIONE S-TRANSFERASE 25); glutathione transferase	1,48	1,71	3,44	3,21
At1g07530	transcription factor	3,51	-0,16	-0,46	3,2
At1g62570	disulfide oxidoreductase/ monooxygenase/ oxidoreductase	2,34	0,06	0,92	3,19
At1g14130	oxidoreductase, acting on paired donors	0,7	1,04	3,52	3,18
At3g10880	unknown protein	-0,02	0,87	4,07	3,17
At3g25830	ATTPS-CIN; myrcene/(E)-beta-ocimene synthase	3,37	-2,43	-2,67	3,13
At3g04890	unknown protein	1,47	0,39	2,01	3,09
At4g22070	WRKY31; transcription factor	1,33	-0,89	0,81	3,04
At2g34660	ATMRP2 (MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 2); ATPase	0,93	-0,55	1,53	3
At2g30750	CYP71A12; heme binding / iron ion binding / monooxygenase/ oxygen binding	3,29	3,16	2,85	2,98
At1g55020	LOX1; lipoxygenase	1,76	0,22	1,44	2,98
At1g05560	UGT1; UDP-glycosyltransferase/ transferase, transferring glycosyl groups	1,72	2,3	3,57	2,98
At3g25820	-	3,42	-2,67	-3,13	2,96
At2g29480	ATGSTU2 (GLUTATHIONE S-TRANSFERASE 20); glutathione transferase	0,68	1,31	3,57	2,94
At1g78340	ATGSTU22; glutathione transferase	2,39	0,55	1,04	2,88
At4g14630	GLP9 (GERMIN-LIKE PROTEIN 9); nutrient reservoir	1,05	0,11	1,92	2,86
At2g02120	LCR70/PDF2.1; protease inhibitor	1,22	1,64	3,24	2,81
At4g37410	CYP81F4; heme binding / iron ion binding / monooxygenase/ oxygen binding	3,28	-0,72	-1,23	2,77
At3g54040	unknown protein	2,83	0,36	0,31	2,77
At5g63900	DNA binding / protein binding / zinc ion binding	1,91	2,53	3,39	2,77

At2g36380	ATPase, coupled to transmembrane movement of substances	1,28	0,29	1,77	2,76
At2g15490	UDP-glycosyltransferase/ transferase, transferring glycosyl groups	1,62	3,24	4,36	2,74
At2g33790	unknown protein	4,07	-0,86	-2,2	2,73
At5g05960	lipid binding	2,51	-1,68	-1,46	2,73
At3g01190	peroxidase	3,27	-1,04	-1,59	2,72
At2g39230	unknown protein	1,59	-0,97	0,16	2,72
At5g42180	-	3,16	-1,04	-1,5	2,7
At1g75270	glutathione dehydrogenase (ascorbate)	1,57	1,33	2,46	2,7
At4g19810	hydrolase, hydrolyzing O-glycosyl compounds	2,06	-0,41	0,23	2,69
At3g57510	ADPG1; polygalacturonase	-0,33	-2,69	0,33	2,69
At5g61950	cysteine-type endopeptidase/ ubiquitin thiolesterase	2,38	0,8	1,1	2,68
At4g29930	DNA binding / transcription factor	0,8	-3,78	-1,89	2,68
At3g13228	-	0,34	0,89	3,23	2,68
At5g45070	ATPP2-A8; transmembrane receptor	2,25	-0,56	-0,15	2,66
At3g66656	-	2,1	-1,23	-0,67	2,66
At2g31750	UDP-glycosyltransferase/ transferase, transferring glycosyl groups / transferase	0,38	-1,37	0,9	2,65
At2g15220	unknown protein	1,97	0,11	0,77	2,62
At2g35380	peroxidase	2,96	0,28	-0,07	2,61
At4g22235	-	1,85	-1,63	-0,88	2,6
At1g52060	unknown protein	3,3	-0,06	-0,79	2,58
At1g66700	S-adenosylmethionine-dependent methyltransferase	2,78	1,04	0,84	2,58
At3g28730	-	0,19	1,01	3,41	2,58
At3g05150	carbohydrate transporter/ sugar porter	2,45	1,11	1,24	2,57
At5g61070	histone deacetylase	1,76	-0,91	-0,1	2,57
At5g63790	protein binding / ubiquitin-protein ligase/ zinc ion binding	1,14	1,94	3,37	2,57
At5g40510	unknown protein	3,37	-2,01	-2,81	2,56
At5g38910	nutrient reservoir	0,64	-0,76	1,16	2,56
At4g15390	transferase	3,6	-0,99	-2,05	2,54
At4g13180	oxidoreductase	1,46	1,21	2,29	2,54
At4g05020	calcium ion binding / disulfide oxidoreductase	1,38	0,13	1,28	2,53
At1g74590	ATGSTU10; glutathione transferase	1,74	0,67	1,46	2,52
At5g59510	unknown protein	0,81	0,88	2,59	2,52

Table 6-2 Down-regulated genes after treatment with TIBA comparing wildtype and *scl14* mutant

The genes are sorted according to their differential expression after TIBA treatment comparing wildtype plants and scl14 mutants (displayed as $\log_2$ fold expression). In this table all analysed combinations of treated and untreated plants are displayed allowing a detailed view on the expression pattern and SCL14 dependency of each gene. Genes listed were expressed with a false discovery rate of 10 %.

AGI code	Gene Description	Wt/ *scl14*	*scl14* (TIBA)/ *scl14*	Wt (TIBA)/ Wt	Wt (TIBA)/ *scl14* (TIBA)
At5g44420	PDF1.2	-8,45	1,33	-0,21	-9,99
At5g37130	unknown protein	2,01	12,36	0,74	-9,61
At1g75830	LCR67/PDF1.1	-6,92	0,98	-0,39	-8,29
At5g44430	PDF1.2c	-7,07	0,61	-0,43	-8,11
At3g01345	-	-7,41	-0,85	0,04	-6,52
At2g26020	PDF1.2b	-5,14	1,13	-0,23	-6,5
At5g02940	unknown protein	-0,12	6,37	1,29	-5,2
At2g26010	PDF1.3	-2,89	1,13	-0,39	-4,41
At5g46730	unknown protein	-1,11	2,3	-0,92	-4,33
At5g54720	protein binding	0,65	3,1	-1,78	-4,23
At5g61160	AACT1; transferase	-3,29	4,15	3,29	-4,15
At2g14610	PR1 (PATHOGENESIS-RELATED GENE 1)	-5,84	-1,6	0,6	-3,64
At5g24110	WRKY30; transcription factor	-1,23	1,75	-0,42	-3,41
At1g72260	THI2.1 (THIONIN 2.1); toxin receptor binding	-2,55	-0,26	-1,05	-3,35
At3g49620	DIN11 (DARK INDUCIBLE 11); iron ion binding / isopenicillin-N synthase	-3,62	3,1	3,45	-3,27
At1g52400	BGL1 (BETA-GLUCOSIDASE HOMOLOG 1); hydrolase	-2,61	0,36	-0,25	-3,21
At1g08630	aldehyde-lyase	-1,42	2,4	0,65	-3,16
At3g59930	unknown protein	-1,56	1,21	-0,3	-3,08
At1g75750	GASA1	-1,51	0,84	-0,71	-3,07
At5g56550	unknown protein	-2,02	0,85	-0,08	-2,95
At1g52410	unknown protein	-1,75	0,98	-0,21	-2,94
At1g71270	-	-0,79	2,85	0,7	-2,93
At5g09610	RNA binding	-0,69	2,01	-0,21	-2,91
At2g39330	unknown protein	-1,79	0,28	-0,84	-2,91
At2g39320	cysteine-type peptidase	-0,14	0,39	-2,37	-2,9

At2g20670	unknown protein	-1,89	-0,1	-0,97	-2,76
At4g26260	MIOX4	-1,47	3,45	2,18	-2,74
At3g47340	-	-2,69	0,65	0,62	-2,73
At4g38680	GRP2; nucleic acid binding	-1,01	1,3	-0,4	-2,71
At5g57550	XTR3 (XYLOGLUCAN ENDOTRANSGLYCOSYLASE 3); hydrolase	-1,78	-0,07	-1,01	-2,71
At2g05530	unknown protein	-0,82	3,47	1,59	-2,7
At1g52040	MBP1 (MYROSINASE-BINDING PROTEIN 1)	-3,11	-0,23	0,19	-2,69
At5g33355	unknown protein	-1,12	1,06	-0,46	-2,64
At3g44970	heme binding / iron ion binding / monooxygenase/ oxygen binding	-1,49	-0,12	-1,22	-2,6
At3g28220	unknown protein	-1,68	-1,46	-2,37	-2,6
At2g21060	ATGRP2B (GLYCINE-RICH PROTEIN 2B); DNA binding / nucleic acid binding	-0,41	1,98	-0,21	-2,59
At3g26230	CYP71B24; heme binding / iron ion binding / monooxygenase/ oxygen binding	-1,36	-0,66	-1,88	-2,59
At2g05520	GRP-3 (GLYCINE-RICH PROTEIN 3)	-0,77	3,22	1,44	-2,56

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Curriculum Vitae:

Seit 01/2005	Dissertation "The GRAS Protein SCL14 and TGA Transcription Factors interact to regulate Stress-Inducible Promoters"
11/2003-11/2004:	Diplomarbeit "Analyse des Terpensynthase28-Promotors in transienten und stabilen Transformationen von *Arabidopsis thaliana*"
11/2003:	mündliche Diplomprüfung Botanik: Ausreichend, Mikrobiologie: Sehr Gut, Geologie: Gut
02/2001:	Vordiplom Gesamtnote: Befriedigend
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