”Studies on Scopolin and Scopoletin -

Stress-Induced Accumulation and Regulatory Elements"

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Abstract

Scopolin and its aglycone scopoletin are nearly ubiquitous coumarin derivatives synthesized in the phenylpropanoid pathway. They are heavily involved in pathogen- and oxidative stress defence and might have regulatory functions. Recently, a large medicinal potential was discovered. Despite their importance, some aspects of the late biosynthesis are still unclear and regulatory elements for their accumulation are virtually unknown. Feruloyl-CoA-6-Hydroxylase 1 (F6'H1) had been recently identified by Kai et al. (2008) as a key enzyme for scopoletin synthesis in *Arabidopsis thaliana*. An insertion mutant of F6'H1, which is scopolin-free, was used in this work for analyses of scopolin localisation and pathogen susceptibility. Phenylpropanoid profiling of *f6'h1* revealed that F6'H1 is essential for the synthesis of other fluorescing compounds. One of these was esculin, which was never thought to be dependent on F6'H1, giving new insights into coumarin biosynthesis. The highest accumulation of scopolin and scopoletin is usually found in roots, but its function in this organ is barely understood. This work shows that scopolin accumulates constitutively in the root endodermis and is sometimes also present in pericycle and cortex, the later especially after elicitation. It is absent in rhizodermis or stele. A reinforcement of the endodermis barrier and possible growth-regulating effects at the pericycle are discussed. The absence of scopolin and scopoletin during an infection of *Arabidopsis thaliana* with the root pathogen *Plasmodiophora brassicae* resulted in slightly higher infection rates and symptom severity, especially at a low pathogen pressure and under additional stress. Pathogen infection, high-sucrose, osmotic stress and cold application led to an increase of scopolin and scopoletin accumulation in roots, but also in leaves. Especially cold stress led to a dramatic increase of scopolin and scopoletin in leaves of *Arabidopsis*, which were hitherto regarded as poor accumulators of both coumarins. Three regulators of scopolin accumulation were identified. The gene AtTHO1 (HPR1), which is a member of the RNA processing and exporting THO/TREX complex, positively influences scopolin levels. A T-DNA insertion mutant of AtTHO1 had also changed root growth patterns under stress, but no defects in root tissue development. The THO/TREX complex is known to be involved in RNA silencing, probably as a mediator of the silencing RNA signal. A model is presented in which the THO/TREX-mediated signal acts as a switch which blocks competing processes and/or promotes scopolin accumulation under stress conditions. Furthermore, two transcription factors of *Arabidopsis* were found, BEE1 and BEE1-LIKE (At5g45580) that were necessary for the scopolin cold response in leaves. Insertion mutation lines of both transcription factors have strikingly identical phenylpropanoid profiles under cold stress, dissimilar from wild type, and might be members of the same signalling chain.

Abbreviations

°C	degree Celsius
µg	microgram
µl	micro litre
35S	CaMV 35S promoter
Å	angstrom
AA	amino acid(s)
ABA	abscisic acid
ABRC	Arabidopsis Biological Resource Center
ACT	ACTIN
ADP	Adenosine diphosphate
Affy ID	Affymetrix Identification number
AGO1	ARGONAUTE1
ANOVA	analysis of variance
AtPSK2	*Arabidopsis thaliana* phytosulphokine 2 precursor
AtTHO1	Arabidopsis homolog of the yeast gene THO1 (HPR); At5g09860
attho1	insertion mutation of AtTHO1
BASTA+	glufonsinate (PPT) -resistance
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BEE1	Brassinosteroid Enhanced Expression1
BEH	ethylene-bridged hybrid
BLAST (N)	Basic local Alignment Search Tool (nucleotide)
bp	base pairs
C3'H	*p*-coumaroyl shikimate/quinate 3'-hydroxylase
CAMV	Cauliflower Mosaic Virus
CA*O*MT1	Caffeic acid 3-*O*-methyltransferase 1
CCoA*O*MT 1	Caffeoyl-CoA-*o*-methyltransferase1
ccoaomt1	insertion mutation of CCoA*O*MT1
cDNA	complementary DNA
CDS	coding sequence (of a gene)
CESA5	Cellulose Synthase 5
CLSM	confocal laser scanning microscope
cm	centimetre
CMP	cytidine monophosphate
CoA	Coenzyme A
Col-0/7	Columbia 0/7, ecotypes of *Arabidopsis thaliana*
Comp	Complementation line
Cy	cyanine dye
DNA	desoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
em.	emission
EMS	ethylmethane sulfonate
Epi-UV	ultra-violet light from above
ER	endoplasmatic reticulum
ERK 1/2	extracellular-signal-regulated kinase 1/2
exc.	excitation
E-Z	cis-trans
F6'H1	Feruloyl-CoA-6-Hydroxylase 1 (a Fe(II)- and 2-oxoglutarate-dependent dioxygenase)
f6'h1	insertion mutation of F6'H1
FLD	Fluorescence detector
FW	fresh weight
g	gram
GFP	Green Fluorescent Protein
GK/GABI-KAT	Genom Analyse im biologischen System Pflanze - Kölner Arabidopsis T-DNA lines
glc	glucose
H	hydrogen
Heynh.	Gustav Heynhold 1800-1860
HPLC	High Performance Liquid Chromatography
HPR	hyper-recombination
HR	hypersensitive reaction
IAA	indole acetic acid
ICE1	INDUCER OF CBF EXPRESSION 1
IgG	immunoglobulin G
IL-6	Interleukin-6
kg	kilogram
KO	"knock-out" (loss-of-function mutation)
L.	Carl Linnaeus 1707-1778
LB	left border
LC-MS	Liquid Chromatography – Mass Spectrometry
LD50	lethal dose 50%
LDL	low density lipoproteins
M	1. molar 2. molar mass
w/v	weight-to-volume ratio
mA	milliampere
Maxim.	Carl Johann (Ivanovič)

	Maximowicz 1827-1891
MDCA	methylenedioxycinnamic acid
Me	methyl-
mg	milligram
min	minute
miRNA	microRNA
MJ	methyljasmonate
ml	millilitre
mM	millimolar
mRNP	messenger ribonucleoprotein
mV	millivolt
MYB	myeloblastosis family of transcription factors
MYB40	MYB DOMAIN PROTEIN 40
myb40	insertion mutation of MYB40
MΩ	megaohm
n	number of biological replicates
N.D.	not detected
n.s.	not significant
NAD+	Nicotinamide adenine dinucleotide
NASC	Nottingham Arabidopsis Stock Centre
NBT	nitro blue tetrazolium
NCBI	National Center for Biotechnology Information
NDR	nuclear Dbf2-related
nm	nanometre
NO	nitric oxide
o	*ortho* position
OE	over-expression
OPDA	12-oxo-phytodienoic acid
p	p-value ("probability")
p	*para*-position
p.	page
p. a.	pro analysi
PAL	phenylalanine ammonia-lyase
PCR	polymerase chain reaction
PDA	Photodiode Array
PGE(2)	Prostaglandin E2
pH	pondus Hydrogenii
PPAR gamma 2	Peroxisome proliferator-activated receptor gamma
p.p.m.	parts per million
PPT	phosphinotricin
puriss.	purissimum
pv.	patovar
PVDF	polyvinylidene fluoride
R2R3-MYB	Repeat2 Repeat3 MYB (myeloblastosis) transcription factor
RAV1	RELATED TO ABI3/VP1 1
RB	right border
rha	rhamnose
RIKEN	Rikagaku Kenkyūjo
RING	RING (Really Interesting New Gene) finger domain: a protein structural domain of the zink finger type
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RT	retention time
RT-PCR	reverse transcriptase – polymerase chain reaction
S2LS3	cell suspension culture of *Nicotiana tabacum* cv. havana
SA	salicylic acid
SAIL	Syngenta *Arabidopsis* Insertion Library
SALK	Salk Institute for biological studies
SAR	systemic acquired resistance
SDS	sodium dodecyl sulfate
sec	second
siRNA	small interfering RNA
SNN	Samsun NN
sRNA	small RNA
SD	standard deviation
STO	SALT TOLERANCE
T0	parent plant of transformation
T1	first generation of seeds and plants after transformation
T2	seeds and plants of (selfed) T1 plants
TAIR	The Arabidopsis Information Resource
T-DNA	Transfer-DNA
TF	transcription factor
THO	suppressors of the transcriptional defects of *hpr1*Δ by overexpression
TIGR	The Institute for Genomic Research (at J.Craig Venter Institute)
TMV	Tobacco Mosaic Virus
TNF-alpha	tumor necrosis factor-alpha
TOF-MS	Time-of-flight – mass spectrometry
TOF-MS ES+	Time-of-flight – mass spectrometry with electrospray ionization, positive mode
TREX	transcription and (mRNA)export
TRIS	tris(hydroxymethyl)aminometha

	ne
t-test	Student's *t* distribution test
U-Box	Ubiquitin-ligase domain
UGT	Uridine 5'-diphospho-glucuronosyltransferase
UPLC	Ultra Performance Liquid Chromatography
U-test	Mann–Whitney *U* test
UV	ultra-violet light
V	volt
v/v	volume-to-volume ratio
WT	wild type
x *g*	gravitation force
YEB	yeast extract broth

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Introduction

Plants have the remarkable ability to produce more than 100,000 low-molecular-mass compounds, which are called secondary metabolites. They are generally non-essential for the basic metabolic processes, but the development of such diversity was driven by their role in stress defence (Dixon, 2001). In unfavourable conditions like cold or intense light the accumulation of phenylpropanoids is a part of the protective measurements taken by the plant to survive. Phenylpropanoids are also a key factor in the defence against microbial, insect or predator attack. Secondary compounds might be specific for certain taxa, but others exert their defensive function across the plant kingdom, like some branches of the phenylpropanoid metabolism. Two phenylpropanoid compounds are the coumarins scopoletin and its glycosylated form scopolin (Figure 1). Scopolin is regarded as the less-reactive storage and transport form and scopoletin the active form (Chong et al., 1999). Scopolin accumulates in vacuoles (Taguchi et al., 2000). To restore the active form, scopolin is deglycosylated by β-glucosidases (Ahn et al., 2010). In plant cells, glucosides and the corresponding reactive aglycone-releasing β-glucosidases are generally located in different cellular compartments (Kojima et al., 1979). A source for β-glucosidases was found in the ER, which forms protein aggregates upon disruption, in which the activity of glucosidases increases (Yamada et al., 2011). Additionally, evidence was provided that extracellular scopolin may be hydrolyzed by a cell-wall-associated β-glucosidase leading to the release of scopoletin in the apoplast (Chong et al., 1999). Sargent and Skoog (1960) report a steady-state equilibrium with a scopolin to scopoletin ratio of 13:1 in healthy tissue, which shifts towards scopoletin under pathogen attack.

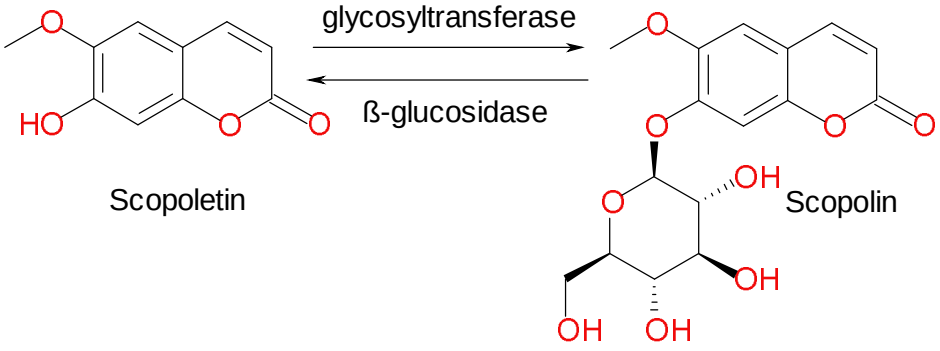


Figure 1: Structures and conversion of scopoletin and scopoletin.

History of scopolin and scopoletin research

More than 100 years ago, J.F. Eykman discovered scopolin and its aglycone scopoletin in the Japanese belladonna *Scopolia japonica* Maxim. (Solanaceae), a traditional Japanese medicinal plant. With preparations of this plant, chronic bronchitis, dysentery, inflammations and deep wounds were treated. Eykman isolated a nitrogen-free substance from its roots that formed slightly gray or yellowish crystals, which had a strong blue fluorescence in ethanolic or acidic solutions. He named it scopoletin after the plant it was

found in. In the same extract, he also found the glycoside of scopoletin, which he named scopolin, which formed white crystals. 0.1 g scopolin caused sleepiness for hours in a dog without being lethal. Eykman also postulated correctly, based on their chemical properties, that scopoletin is closely related to the other cinnamic acid derivatives known at that time, namely esculin, esculetin, daphnin, daphnetin, caffeic acid, ferulic acid and umbelliferone. He also suggested the correct sum formula for scopoletin, $C_{10}H_8O_4$, which he called a methyl-esculetin, which also was proven to be true. In addition, he gave the exact reaction equation for the deglycosylation of scopolin to glucose and scopoletin (Eykman, 1883).

However, scopoletin had been already discovered before Eykman. A substance with a striking blue fluorescence had been described for the first time by Geiger (1843) from *Atropa belladonna* (Solanaceae). Richter (1837) working at the same time with extracts of *Atropa belladonna* named it "Blauschillerstoff" (blue sparkling substance). Kunz (1885), after chemical analyses of the "Schillerstoff", renamed the fluorescing substance into chrysatropa acid. Henschke proved in 1888 that Eykman's scopoletin is identical with Kunz' chrysatropa acid. In the yellow jasmine *Gelsemium sempervirens* (Gelsemiaceae) scopoletin was found by Wormley (1870), who named it gelseminic acid. Moreover, in 1863, de Vry (1864) isolated a fluorescing substance he called Murrayin from the flowers of *Murraya exotica* L. (Orange Jessamine, Rutaceae) which turned out to be scopolin.

Distribution of scopolin and scopoletin in the plant kingdom

It is not well recognized that scopoletin and scopolin are not only present in the families they were discovered in during the 19th century and early 20th century, which are the Solanaceae, the Gelsemiaceae, the Rutaceae, the Convolvulaceae and the Asteraceae (Wehmer, 1931). Until now, they have been found in nearly 80 plant families (see appendix p. A1) evenly distributed over the magnolids, monocots and dicots. From the larger families, no references for only a number of monocot families (Piperaceae, Annonaceae, Amaryllidaceae, Iridaceae, Asparagaceae, Arecaceae, Bromeliaceae, Cyperaceae) could be found, as well as for only few of the dicot families (Begoniaceae, Cucurbitaceae, Myrtaceae, Melastomataceae, Gesneriaceae, Lamiaceae, Boraginaceae, Campanulaceae), although in most of these scopolin and scopoletin might also be present. Most interestingly, scopoletin and other coumarins were also found in fungi (Murray et al., 1982; Tirillini and Stoppini, 1996; Wang et al., 2007). Reports of occurrence in gymnosperms and early angiosperms do not exist until now (Figure 2).

Whereas the distribution of scopoletin and scopolin throughout the plant families is impressive, the ability to produce them is sometimes lost, too. As this is known especially from cultivars of crops (Santamour Jr and Riedel, 1994), this did probably happen during breeding, but an evolutionary loss in some taxa is also likely. Their ubiquity leads to the conclusion that the pathway towards them developed very early (or parallel?) in the phylogenetic history of plants and the fact that it was kept in so many taxa points strongly to a vital role in plants.

Early angiosperms			Amborellales, Nymphaeales, Austrobaileyales, Chloranthales
Magnolids			Canellales, Piperales, Laurales, Magnoliales
Monocots			Acorales, Alismatales, Petrosaviales, Dioscoreales, Pandanales, Liliales, Asparagales
	Commelinids		Arecales, Poales, Commelinales, Zingiberales
Eudicots			Ceratophyllales, Ranunculales, Sabiales, Proteales, Trochodendrales, Buxales, Gunnerales, Dilleniales, Saxifragales, Vitales
	Rosids	Fabids	Zygophyllales, Celastrales, Oxalidales, Malphigiales, Fabales, Rosales, Cucurbitales, Fagales
		Malvids	Geraniales, Myrtales, Crossomatales, Picramniales, Sapindales, Huerteales, Malvales, Brassicales
			Santalales, Berberidopsidales, Caryophyllales
	Asterids		Cornales, Ericales
		Lamids	Garryales, Gentianales, Lamiales, Solanales, Boraginales
		Campanulids	Aquifoliales, Asterales, Escalloniales, Bruniales, Apiales, Paracryphiales, Dipsacales

Figure 2: Phylogenetic distribution of scopolin and scopoletin in angiosperms. Taxa of Angiosperms down to orders, for which references for the presence of scopolin and/or scopoletin were found (blue). Phylogenetic tree simplified from Stevens (2001 onwards).

Functions of scopolin and scopoletin

Scopolin and scopoletin are involved in pathogen defence responses

Very early, scopoletin was found to be linked to pathogen infections: Rupert Best (1936, 1944, 1948) found scopoletin in a ring around necrotic lesions after an infection with tomato spotted wilt virus in *Nicotiana tabacum*, *N. glutinosa* (tobacco), *Lycopersicon esculentum* (tomato), *Petunia hybrida* and *Solanum tuberosum* (potato). Scopoletin was found to accumulate also in potato tubers infected with the leaf roll virus (Andreae, 1948). It was often reported that scopoletin levels rise also after infection with fungi, e.g. with *Plasmopara halstedii* (downy mildew) on sunflower (Spring et al., 1991), *Ceratocystis* on plane tree (Elmodafar et al., 1993) and *Microcyclus ulei* on rubber tree (Garcia et al., 1995). The glycoside scopolin was found in potato tubers infected with *Phytophtora infestans* (Hughes and Swain, 1960). A rise in scopolin also occurred during a bacterial infection (*Pseudomonas solanacearum*, Sequeira , 1967), especially adjacent to infected tissue, but with a systemic rise of scopolin levels as well. Interestingly, the colonization with an arbuscular mycorrhiza elicited no scopoletin accumulation, but also no repression (Maier et al., 2000; Schwob et al., 2000). Scopoletin was also found on the leaf surface of Apiaceae, Rutaceae and Lauraceae, where they have inhibiting effects on spore germination, and also in the stem bark and wood in the beech and rue family (Zobel et al., 1991; Somanabandhu et al., 1992; Neville and Bohm, 1994; Prats et al., 2002).

Numerous reports showed that *in vitro*, scopoletin is able to inhibit the growth of different species of bacteria and fungi (Goy et al., 1993). However, many authors doubt a direct action against the attacking pathogen because of too low concentrations *in planta* (e.g. Minamikawa et al. (1963)). Snook et al. (1991) found that resistance of tobacco cultivars towards black shank disease did not depend on the presence or absence of scopoletin in the cultivars, although scopoletin was highly active against the fungus *in vitro*. Breton et al. (1997) did not consider scopoletin a major defence mechanism for the rubber tree, mostly because the pathogens tested had the ability to detoxify scopoletin. In most cases, the effectiveness of scopoletin to prevent diseases seems to depend on the pathogen's ability to detoxify the coumarin. To support this theory, a study in sunflower showed that pathogenic and non-pathogenic fungi caused a considerable rise in scopolin and scopoletin levels, with the pathogenic fungus being able to rapidly degrade scopoletin and the non-pathogenic not (Tal and Robeson, 1986). El Oirdi et al. (2010) also correlated a reduced resistance of tobacco cultivars to *Botrytis cinerea* (grey mold) with the fungus' ability to metabolize scopoletin. Other case studies showed a direct connection between pathogen resistance and constitutively high amounts of scopoletin (Goy et al., 1993) or the ability to react fast with scopolin production. In the pathosystem plane tree (*Platanus spp.*) and *Ceratocystis fimbriata* f. sp. *platani*, an agent of cancer stain disease, scopoletin was part of a successful defence: a quick accumulation of fungitoxic levels of scopoletin and excretion on the leaf surface combined with a hypersensitive reaction prevented the disease in resistant plane tree species. Susceptible plane trees were characterized by large necroses and a slow and

low phytoalexin production (Elmodafar et al., 1995). In some cases, when scopoletin production was still too slow to inhibit fungal germination, it aided at least in the confinement of the pathogen by forming a ring around the infected area (Elmodafar et al., 1993). The resistance of elm species towards Dutch Elm disease (*Ophiostoma novo-ulmi/Ophiostoma ulmi* syn. *Ceratocystis ulmi)* was also tentatively linked to their ability to react very fast with scopoletin production towards the fungal attack (Valle et al., 1997). Likewise, in rubber tree, the degree of resistance towards the ascomycete *Microcyclus ulei* was related to the rapidity and intensity of scopoletin accumulation (Garcia et al., 1995; Garcia et al., 1999; Churngchow and Rattarasarn, 2001). Scopolin is usually regarded as the inactive form of scopoletin, but in head rot of sunflower it had the same inhibitory effects as scopoletin (Prats et al., 2006; Prats et al., 2007). Other authors are convinced of a specific action of scopoletin against fungi besides a general antifungal activity of phenolic compounds (Urdangarin et al., 1999): a synergistic effect with other phenylpropanoids was described by Carpinella et al. (2005). In that study, scopoletin on itself had only weak effects on the *Fusarium verticillioides* fungus *in vitro*. But combined with other phenylpropanoids, it enhanced dramatically their antifungal activities. De Rafael et al. (2001) postulated, that the presence of scopoletin during Dutch Elm Disease might be only a consequence of H_2O_2 production. However, the majority of authors confer to scopoletin in aerial parts as a phytoalexin, which is per definition *de-novo* synthesized at the site of a pathogen infection and acts antimicrobial. In roots, accumulation seems to be constitutively.

Additionally, scopoletin offers protection against feeding insects (Olson and Roseland, 1991; Adfa et al., 2010; Tripathi et al., 2011) and could decrease their survival (Westcott et al., 1992). There are also strong hints that scopolin-rich sunflower cultivars have an increased resistance towards the parasitic plant *Orobanche cernua* (Serghini et al., 2001; Sauerborn et al., 2002).

Scopoletin has antioxidative properties and is a scavenger of ROS

For scopoletin, a protection against oxidation and radical formation by reactive oxygen species is strongly assumed. The antioxidative properties of phenolics arise from their high reactivity as electron donors. As good reducing agents, they can either act as replacement targets for oxidative processes, or they function as scavengers of ROS: after collision with a free radical, the newly-formed phenolic radical can stabilize the unpaired electron in the delocalized electron cloud of its aromatic ring and thus stop the chain reaction. Some phenolics also chelate metal ions and thus terminate the radical producing Fenton reaction (Karuppanapandian et al., 2011). In plants, scopoletin and scopolin accumulation is strongly induced by processes leading to an increased production of ROS. There was only a weak direct activity observed against lipid oxidation (Thuong et al., 2010). However, scopoletin was found to enhance the activity of other antioxidants like superoxide dismutase, catalase and glutathione (Shaw et al., 2003; Panda and Kar, 2006). One special mode of oxidation protection could be the inhibition of peroxidases, which is most likely exerted not only by competitive inhibition (Andreae, 1952; Sirois and Miller, 1972). Kang et al. (1999) suggest a

system where scopoletin inhibits the production of nitric oxide (NO) by inhibiting an NO synthase and therefore the production of peroxynitrite ($ONOO^-$) out of NO and superoxide ($O2^{-*}$), which is much more toxic than both molecules alone.

Assays for determination of ROS-scavenging show also direct quenching activities for scopoletin. The EC_{50} (50% of quenching) of scopoletin for superoxide O_2^- was determined as 1.27 mg/ml, for singlet oxygen ($^1O_2$) 0.68 mg/ml, for hydroxyl radicals (•OH) > 4.00 mg/ml and for peroxynitrite (ONOO-) 0.042 mg/ml, respectively (Ikeda et al., 2009). This is a low to moderate activity towards hydroxyl radicals, superoxide and singlet oxygen (around 2% of the activity of ascorbic acid), but a high quenching value for peroxynitrite (Ikeda et al., 2009). Thuong et al. (2010) testify scopoletin a high activity against peroxyl radicals (ROO•), but no effectiveness against DPPH, also none for hydroxyl radicals (•OH), hyperoxid anions ($O_2\bullet^-$) and hypochlorid (HOCl). The antioxidant properties of scopoletin might have been under-evaluated, since no ROS-scavenging activity was shown in *in-vitro* tests with the commonly used artificial RO-species DPPH, see also Shaw et al. (2003).

The oxidation of scopoletin and the resulting loss of fluorescence have been used for an assay for measuring the production of reactive oxygen species (ROS), mostly for hydrogen peroxide (H_2O_2), in animal tissue systems (Shah and Walker (1992). There are hints that this reaction plays also an important role *in planta*: scopoletin quickly accumulates during the deterioration of cassava roots after the harvest in all parts of the root and coincides with a quick rotting process. Scopoletin and its oxidized form used to be made responsible for the quick deterioration of cassava under storage. But Reilly et al. (2003) finally developed a model where an H_20_2 burst occurs as a wounding reaction after harvest, which is not stopped as in an intact plant. The massive scopoletin production would then be a last measure of the plant to eliminate H_20_2 when other methods had failed (e.g. when no more catalase is present) and not the cause for the rotting process. In the reaction with H_2O_2, scopoletin obviously acts strongly as a replacement target for oxidation. Interestingly, the oxidized form of scopoletin was four times more effective against fungus germination than scopoletin itself (Gomez-Vasquez et al., 2004).

Scopoletin is part of hypersensitive responses

Connected to the close relationship between H_2O_2- and scopoletin formation, there are hints that scopoletin is directly involved in the hypersensitive response (HR). The HR is a very efficient mechanism of induced disease resistance in plants. During the HR, the infected tissue undergoes a local cell-death program and surrounding areas are fortified with mechanical and chemical barriers, which stops the pathogen spread. One of the earliest responses is the so-called oxidative burst, a massive increase in the production of reactive oxygen species (ROS), which are probably the key mediators of cell death. They also could act as diffusible signals for adjacent cells in order to activate a broad range of defence responses without those cells being destined to die (Chong et al., 2002). In tobacco, scopoletin was found in areas surrounding necrotic tissue, forming a characteristic, strongly fluorescing ring (Dorey et al., 1997). Dorey et al. (1999) also observed a consumption of

scopoletin during HR. Chong et al. (1999) developed a model, in which the pathogen elicitor leads to an early accumulation of a glycosyltransferase, which is followed by an accumulation of scopolin. In this model, H_2O_2 production causes a release of scopolin in the extracellular space as H_2O_2 scavenger after deglycosylation. Antisense plants of this glycosyltransferase with heavily depleted levels of scopoletin and scopolin were not able to contain the necrotic area and were more susceptible to the virus (Chong et al., 2002). The authors concluded a dual role for scopolin/scopoletin in the HR: First, to stop cell death by eliminating ROS in adjacent cells and secondly, to stop the virus by forming an antiviral barrier. A similar model was created for *Pseudomonas syringae* pv. *tomato* and *Arabidopsis thaliana* (L.) Heynh. by Simon et al. (2010). There, scopoletin is found in the infected area and scopolin in adjacent cells. Scopolin is thought to provide a source for free scopoletin to scavenge ROS. In addition, Massoud et al. (2012) showed that scopoletin accumulation might be a result, but not the cause for systemically acquired resistance (SAR) mediated by phloem-mobile phosphite (H_3PO_3).

Abiotic factors and phytohormones affect scopolin and scopoletin accumulation

Scopoletin and scopolin production is also induced by abiotic factors: Increased contents were reported after treatment with UV, cold and x- as well as gamma radiation (Koeppe et al., 1969; 1970a, 1970b; Riov et al., 1971). In these cases, the antioxidative properties of scopoletin might help to minimize damages resulting from radical formation. Nitrogen- (Armstrong et al., 1970), potassium- (Lehman and Rice, 1972) and boron deficiency also caused a dramatic increase of scopolin in tobacco, the latter presumably by inhibiting lignin biosynthesis and subsequent accumulation of coumarin precursors (Watanabe et al., 1961). Alkenale and alkanale also caused scopoletin increase and may act as volatile elicitors for plant defence (Zeringue, 1992). Gutierrez et al. (1995) tested several other abiotic stressors in sunflower like sucrose, shortwave UV, Triton-100 and $CuCl_2$, which elicited scopoletin production in the aerial parts, but not in the roots. Long wave UV, salicylic acid, dichloroisonicotinic acid or glutathione had no effect in their studies. The reactions were age-dependent. Elevated levels of CO_2 also caused scopolin/scopoletin levels to increase, leading to an increased virus resistance (Matros et al., 2006). Sargent and Skoog (1960) reported that scopoletin levels rose due to enzymatic hydrolysis of scopolin after wounding and also after the application of the auxin indole acetic acid (IAA). Skoog and Montaldi (1961) postulated a complex interaction between plant hormones and scopoletin, affecting plant growth. Many other phytohormones affect scopoletin and scopolin accumulation: salicylic acid increased scopolin and scopoletin accumulation in tobacco leaves (Maslak, 2002), but not in sunflower (Gutierrez et al., 1995). Methyljasmonate increases scopoletin accumulation in fruits (Zeringue, 2002). The synthetic auxin 2,4-dichlorophenoxyactic acid (2,4-D) increased both coumarins in shoots and roots (Dieterman et al., 1964). Grosskinsky et al. (2011) proved that scopoletin could substitute for the cytokinin signal in a salicylic acid-independent defence mechanism against *Pseudomonas syringae* pv. *tabaci*. Wounding also induced scopoletin accumulation (Cabello-Hurtado et al., 1998).

Scopoletin, a potential regulator of growth?

Andreae (1952) showed that externally applied scopoletin concentrations of 1 p.p.m. promoted root growth and 50 p.p.m. inhibited it. The inhibiting effect of scopoletin manifested itself *in vitro* as retarded elongation, inhibited root hair development, discoloration and blackening (at $3x10^{-4}$ M; Pollock et al., 1954). Pollock et al. (1954) showed an antimitotic activity of scopoletin. The growth-promoting effects of scopoletin at low concentrations were attributed to the inhibition of IAA degradation shown *in vitro,* in which scopoletin was an alternative substrate for an IAA oxidase (Andreae, 1952). IAA (indole acetic acid) is an auxin with a growth-inhibitory effect in higher doses and a stimulating effect in very low doses. Goodwin and Kavanagh (1949) and Goodwin and Pollock (1954) reported a scopoletin gradient in *Avena* roots: high concentrations in the non-growing parts, low concentrations at the root tip. Based on this gradient, the *in vitro* growth-effecting properties of scopoletin and its interaction with IAA, Pollock et al. (1954) and Avers and Goodwin (1956) proposed it to be an internal growth regulator in *Avena*. Supporting their theory, scopoletin concentrations at the root tip did not reach the inhibitory level, but scopoletin was indeed in the inhibitory range in the older portion of roots. Sirois and Miller (1972) further refined the IAA model by postulating a stabile scopoletin-peroxidase complex, which can be reverted by excess IAA concentrations. It is also known that scopolin and scopoletin levels itself are regulated by plant hormones (Skoog and Montaldi, 1961; Taguchi et al., 2001; see previous paragraph). It could play a regulatory role in growth and differentiation of roots. In animal systems, high scopoletin concentrations also could lead to apoptosis; as it was shown to stop the proliferation of tumour cells and to activate a signal cascade leading to programmed cell-death (Liu et al., 2001; Kim et al., 2005).

Other functions of scopolin and scopoletin

Phenylpropanoid molecules consist only of carbon, hydrogen and oxygen. Unlike alkaloids for example, they do not compete for nitrogen and do also not impact on the limiting element phosphorus. Instead they offer an “energy escape valve” for excess energy and assimilates from photosynthesis with the additional bonus of their antioxidative, light protective, antibiotic and predator-deterrent properties (Hernández and Van Breusegem, 2010). Scopolin and scopoletin, as one of the most common hydroxycoumarins besides umbelliferone and esculetin (Harborne, 1980), probably provide such a convenient carbon sink. There are hints that scopoletin and other non-conjugated phenylpropanoids are selectively enriched under excess carbon, as, at least under high CO_2, other nitrogen-free compounds like structural phenylpropanoids (lignins) and sesquiterpenoids are not enriched (Matros et al., 2006).

A. thaliana and tobacco produced scopoletin and scopolin upon infection with the thaxtomin A-producing bacterium *Streptomyces scabei.* The resulting programmed cell-death in tobacco was not induced by scopoletin, since injections of scopoletin did not cause apoptosis. The direct antimicrobial effect of scopoletin on *S. scabei* was only moderate, but it caused considerable decrease in toxin production by rapidly downregulating a known synthesis gene of thaxtomin (Lerat et al., 2009).

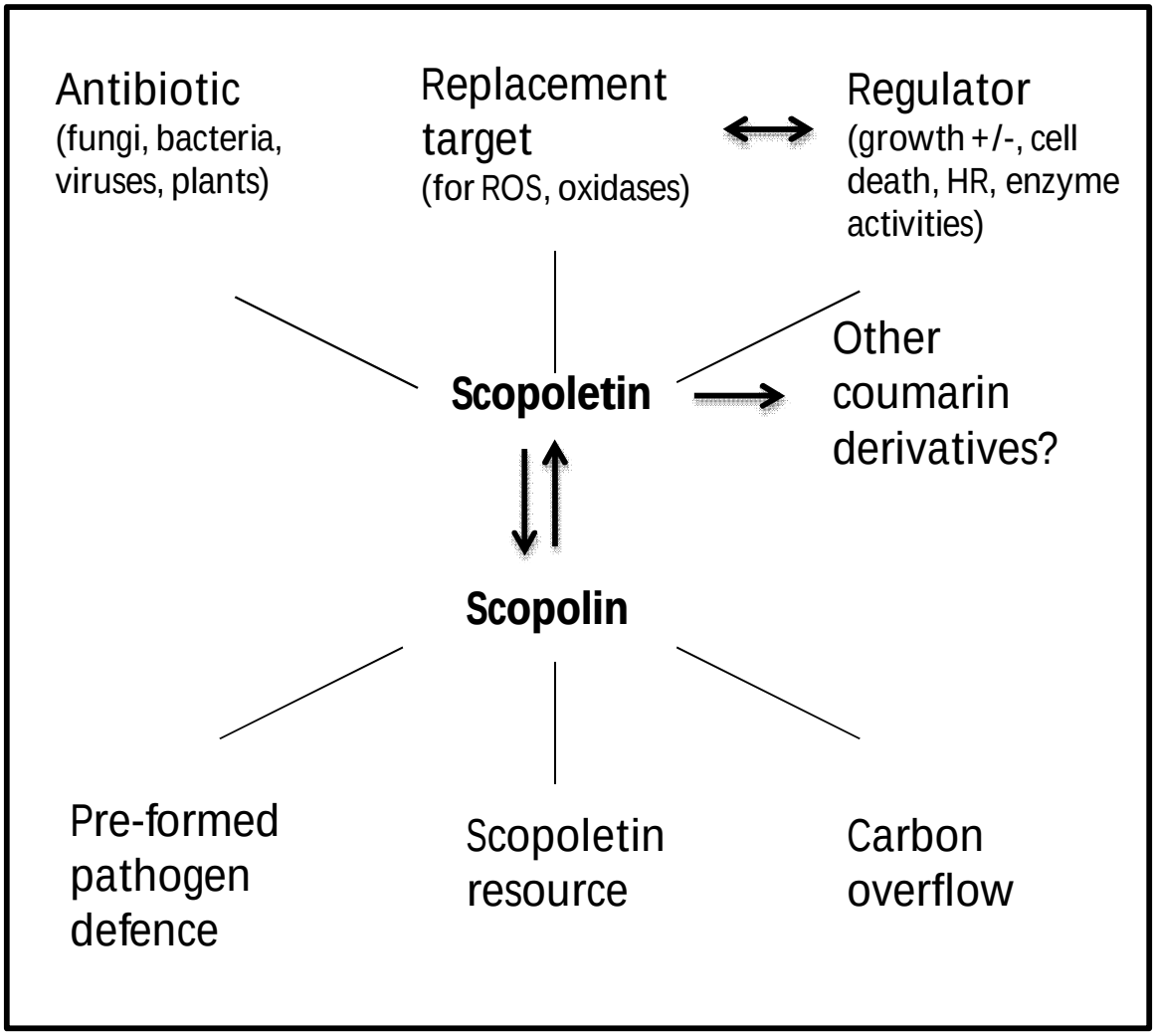


Figure 3: The multiple functions of scopoletin and scopolin in plants

Müller-Enoch (1994) assigned to scopoletin a role as a photoreceptor and phototransducer in cytochrome P450. Some studies indicated that scopoletin has allelopathic effects on the root growth of neighbouring plants when combined with other compounds like vanillic acid and *o*-coumaric acid (Fay and Duke, 1977; Perez and Ormenonunez, 1991; Baghestani et al., 1999; Kim and Lee, 2011). Furthermore, Ma et al. (2004) report a copper-dependent ability of scopoletin to cleave DNA. A function for this in plants is not known, but it was discussed as a possible antitumor agent in cancer therapy.

Medicinal properties of scopoletin

Quite recently, scopoletin was identified as one or the active compound in traditional medicinal plants all over the world. Interestingly, some medicinal properties resemble some proven or suspected properties in plants, like the growth-regulating effects in root tissue and the tumour-reducing effects of scopoletin. Here, the putative functions are summarized in the following table:

Table 1: Medicinal properties of scopoletin

Medicinal effect	Description and mechanisms	References
Antispasmodic and hypotensive	Scopoletin was determined as the active compound from smooth-muscle-relaxing traditional *Viburnum* preparations. The muscle-relaxing effects were attributed to its ability to inhibit the intracellular calcium mobilization. An additional reduction of blood pressure by dilatation of blood vessels was reported. It is also diuretic.	Jarboe et al. (1967); Oliveira et al. (2001); Ojewole and Adesina (1983); Schmedahirschmann et al. (1994)
Cardioprotective	Scopoletin also seems to activate lipoprotein lipase, which plays a central role in the removal of triglycerides from the blood stream. Scopoletin inhibits the oxidation of low density lipoproteins (LDL). Both are major factors causing arteriosclerosis. Scopoletin influences heart rate and - contraction.	Yang et al. (2007); Thuong et al. (2005) Guantai and Addae-Mensah (1999)
Anticoagulant	Scopoletin, like many coumarins, has an inhibitory effect on platelet aggregation (blood clotting).	Okada et al. (1995)
Analgetic, antinociceptive, antipyretic, antiphlogistic	Scopoletin is a prostaglandin synthesis inhibitor, which relieves pain and inflammation. It was five times more effective than aspirin. Scopoletin also suppresses pro-inflammatory cytokines and PGE(2) and other inflammatory mediators like IL-6 and TNF-alpha. It was also reported to reduce pain reception ("antinociceptive") caused by inhibition of glutamatergic transmission and cytokine inhibition. The healing effect for gastro-oesophageal inflammation by the traditional preparation of *Morinda citrifolia*, "Noni", could be recreated by the corresponding dose of pure scopoletin alone.	Farah and Samuelsson (1992); Kim et al. (2004; 2006); Moon et al. (2007); Choi et al. (2012); Meotti et al. (2006) ;Ribas et al. (2008); Tanaka et al. (1977); Mahattanadul et al. (2011)
Antitumoral	Scopoletin has a selective effect on tumour cells, shown by an antiproliferative effect on tumoral lymphoma cells. Artificial scopoletin derivatives even have a higher anti-tumour activity. Scopoletin is also a moderate inhibitor of xanthine oxidase, an enzyme reducing xanthin, but also producing ROS and uric acid in the human system, which may lead to cancer or gout, respectively. In addition it is uricosuric, which means it promotes excretion of uric acid. Scopoletin inhibited the mutagenicity of cigarette smoke condensate, but toxicity for the smoker himself could not be excluded.	Arcos et al. (2006); Adams et al. (2006); Manuele et al. (2006); Zhou et al. (2012);Chang and Chiang (1995); Ding et al. (2005); Romert et al. (1994).
Antiangiogenic	Scopoletin inhibits the formation of new blood vessels (angiogenesis) and not via cytotoxicity, but via downregulating ERK1/2 extracellular-signal-regulated kinases. Angiogenesis plays a role in cancer, arthritis and eye retina diseases. An anti-arthritic effect could be shown.	Pan et al. (2009; 2010; 2011)
Antibiotic	Scopoletin is active against some pathogenic fungi, moderate antibacterial towards *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Staphylococcus aureus, Klebsiella pneumoniae* and *Escherichia coli*. No action against some other bacterial pathogens and the malaria-causing protist *Plasmodium falciparum* was observed. Scopoletin, esculetin and umbelliferone (and also esculin when hydrolysed by the gut flora) have the ability to terminate enterohaemorrhagic *E.coli* (EHEC) in the gut, hence a coumarin-rich diet in animals and humans was suggested as a way to prevent the more and more frequent outbreaks of EHEC.	Jurd et al. (1971); Kayser and Kolodziej (1997); Ma et al. (2008); Duncan et al. (1998)
Neuroprotective	Scopoletin has an acetylcholinesterase inhibitor activity, which might help in treatment of Alzheimer's disease. Acetylcholinesterase degrades the neurotransmitter acetylcholine, which is lacking due to the neuron loss marking that disease. In a screen with 11000 natural compounds for potential acetylcholinesterase inhibitors, scopolin and scopoletin were selected and their effectiveness proofed *in vitro* and *in vivo*. Neuroprotectice properties during oxygen-glucose deprivation were described. An antidepressant effect of scopoletin which is connected to serotonin, noradrenalin and dopamine receptor systems, was reported.	Lee et al. (2004); Hornick et al. (2011) Rollinger et al. (2004) Orhan et al. (2008) Son et al. (2007) Capra et al. (2010)
Hepatoprotective	Scopoletin has hepatoprotective properties, as shown in cell culture. An inhibitory effect of scopoletin on inducible nitric oxide synthases whose induction may insert pro-inflammatory effects by accumulation of NO or a mechanism involving the inhibition of eicosanoid biosynthesis, cell influx and peroxidation were suggested as active principle. As a non-competitive inhibitor of β-glucoronidase, scopoletin has the potential to inhibit gall stone formation.	Kang et al.(1998; 1999); Ding et al. (2008); Schmedahirschmann et al. (1994)

Treatment of metabolic disorders	A superior performance of scopoletin than a standard thyroid drug against hyperthyroidism and hyperglycaemia without hepatotoxicity was reported. Scopoletin can ameliorate insulin resistance by upregulating PPAR gamma 2 expression, so it may be a useful candidate for managing metabolic disorders, including type 2 diabetes mellitus. In addition, scopoletin has aldose reductase inhibitor ability, an enzyme which causes complications during diabetes.	Panda and Kar (2006); Zhang et al. (2010); Park et al. (2011)

Side effects: Scopoletin is not classified as a toxic substance. The acute toxicity LD50 (oral, rat) is 3800mg/kg. For comparison, ethanol has an LD50 of 6200 mg/kg, acetylsalicylic acid (Aspirin) 1500 mg/kg. However, it is classified as irritating to eyes, respiratory system and skin. Sunlight aggravates skin reaction (Material Safety Data Sheets from Carl Roth GmbH, Karlsruhe, Sigma-Aldrich GmbH, München and Roth et al. (1994)). However, scopoletin was linked to a neuropathy seen in populations sustaining on a Cassava root diet, which contains excessive amounts of scopoletin (Ezeanyika et al., 1999). Yet, other authors attribute the detrimental effects of a Cassava diet solely to its cyanogenic compounds, which may not be adequately removed during food preparation (Blagbrough et al., 2010). Side effects of scopoletin administration were not reported (e.g. Santamaria et al. (2012)).

Until now, no drug containing scopoletin besides the traditional herbal preparations has been introduced.

The synthesis of scopolin and scopoletin within the phenylpropanoid pathway

Scopoletin and scopolin originate from the amino acid phenylalanine, which is first desaminated by the enzyme PAL (phenylalanine ammonia-lyase). The product is cinnamic acid. The next step is the introduction of a hydroxyl group onto the phenol ring, leading to *p*-coumaric acid. *P*-coumaric acid is then esterified to p-coumaroyl-CoA. Under loss of the CoA ester, shikimic acid is introduced to the carboxyl group yielding *p*-coumaroylshikimic acid. A second hydroxylation of the phenol ring leads to caffeoylshikimic acid. The loss of the shikimic acid group leads to caffeic acid, which is also present as its CoA ester (Bourgaud et al., 2006; Heldt and Piechulla, 2008). The next step in *Arabidopsis thaliana* is the methylation of the 3'-hydroxy group of caffeic acid or caffeoyl-CoA by the caffeic acid-*o*-methyltransferase CA*O*MT1 or the caffeoyl-CoA-*o*-methyl-transferase CCoA*O*MT1, respectively, leading to ferulic acid or its CoA ester (Kai et al., 2006; Kai et al., 2008).Then, a second hydroxyl group is introduced into the phenol ring of feruloyl-CoA, which can originate from caffeoyl-CoA or ferulic acid. This step is catalysed by the Fe(II)- and 2-oxoglutarate-dependent dioxygenase F6'H1, which is essential for scopoletin synthesis in *Arabidopsis*. The resulting 6-hydroxyferuloyl-CoA is the last ascertained step towards scopoletin. The theoretically following reactions towards scopoletin are the E-Z isomerisation of the carboxyl-group and the proton at the double bond, the closing of the second ring (lactonisation), which are still uncertain. Kai et al. (2008) favour an enzyme-free, light-dependent modus of lactonisation, whereas Bayoumi et al. (2008) provide evidence for a light-independent isomerase for the E-Z isomerisation step in Cassava. The final glycosylation towards scopolin is done by one or several of the 120 glycosyltransferases in *Arabidopsis*, from which many have scopoletin glycosyltransferase activity *in vitro* (Lim et al., 2003). Two alternative pathways for scopoletin synthesis are discussed, the first from caffeic acid via 2'hydroxy caffeic acid or their CoA esters and esculetin, the second from *p*-coumaric acid via umbelliferone and esculetin (Bourgaud et al., 2006, figure 4). In tobacco, both pathways do not seem to be involved in scopoletin formation (Fritig et al., 1970). In

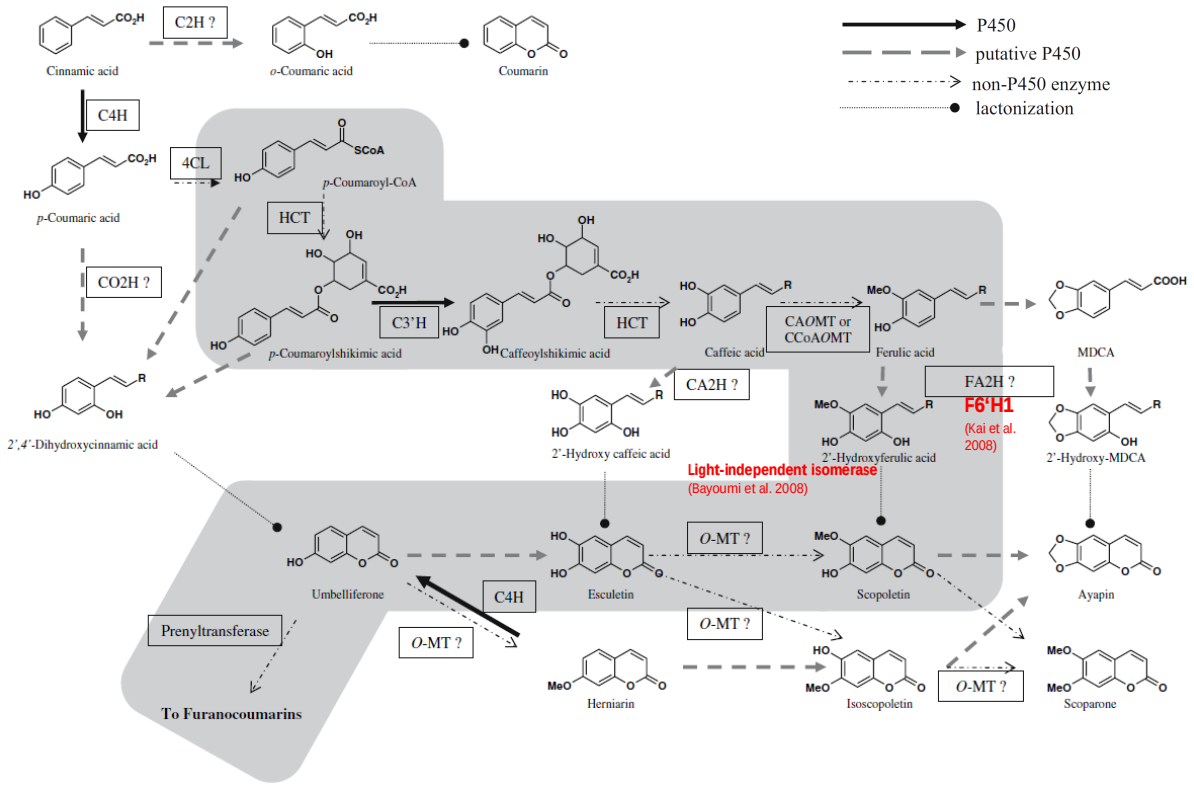


Figure 4: Phenylpropanoid pathway leading to coumarins adapted from Bourgaud et al. (2006): "Pathways in grey have been unequivocally established. Glycosylated compounds are not shown for clarity. Enzymes assigned by a question mark are hypothetical. R=CO_2H or CO-ScoA; C2H, cinnamic acid 2-hydroxylase; C4H cinnamic acid 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CO2H, 4-coumaric acid 2-hydroxylase; HCT, hydroxycinnamoyl-transferase; CAOMT, caffeic acid *O*-methyltransferase; CCoAOMT, caffeoyl CoA *O*-methyltransferase; CA2H, caffeic acid 2-hydroxylase; FA2H, ferulic acid 2-hydroxylase; MDCA2H, methylenedioxycinnamic acid 2-hydrolase; *O*-MT, *O*-methyltransferase". Kai et al (2008) found the hypothetical enzyme „FA2H?" in *Arabidopsis* and named it feryloyl CoA **6**-hydroxylase 1 (F6'H1). Bayoumi et al. (2008) established an enzymatic E-Z-isomerisation of hydroxyferulic acid in cassava (red annotations).

Arabidopsis, Kai et al. (2008) showed that only the pathway via feruloyl-CoA leads to scopoletin. However, Kai et al. give no explanation for the traces of esculetin they found earlier in *Arabidopsis* (Kai et al., 2006), which should not be present if the alternative pathways do not exist.

All authors agree that pathways may vary in different species. Substances, for which scopolin and scopoletin are the precursors, are not known, besides a non-fluorescing compound which is a product of peroxidation of scopoletin by a new-found peroxidase. This enzyme was made responsible for a decline of scopoletin 72 hours after elicitation (Edwards et al., 1997). Nevertheless, there might be other compounds with scopoletin or scopolin as precursors.

Regulation of scopolin and scopoletin synthesis and accumulation

The general principle of regulation in the phenylpropanoid pathway is that each branch is under separate control, which ensures that the appropriate compounds are produced when

and where required. The control takes largely place at the level of transcription of the regulators and of the corresponding biosynthetic genes (Quattrocchio et al., 2006). Until now, only a few cases of posttranslational control of phenylpropanoid metabolism are known, like the dependence of anthocyanin synthesis on one splicing variant of a synthesis gene in Black Mexican Sweet maize (Pairoba and Walbot, 2003). Translational regulation is exercised by transcription factors (TFs), proteins that recognise specific promoter sequences of the genes they control. They recruit RNA polymerase II to the gene for mRNA synthesis. TF binding is also dependent on chromatin structure and histone modification. Plants encode 2000 to 4000 transcription factors, but the number of characterized TFs is comparatively small. According to the current state of knowledge, plant TFs have a hierarchical structure resembling a pyramid, in which master TFs at the top control few central TFs (figure 5). Those control TFs on lower hierarchical levels and so on down to the TFs that control the biosynthetic enzymes (Grotewold, 2008).

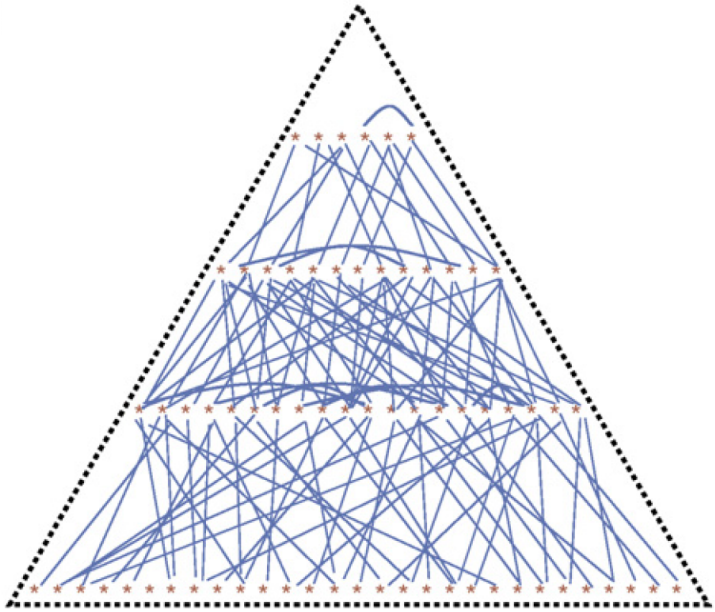


Figure 5: Diagram of a model gene regulatory network in plants showing TFs (red stars) at various levels in the pyramidal hierarchical arrangement. Blue lines indicate the direct interaction of one TF with another (Grotewold, 2008).

TFs can be positive or negative regulators of a pathway (Quattrocchio et al., 2006). Several studies revealed that phenylpropanoid biosynthesis is controlled by different types of TFs, from which the most prominent are the MYB factors. These TFs were described first in the avian myeloblastosis virus (AMV) by Klempnauer et al. (1982). A MYB domain consists of one to three imperfect repeats (R1, R2, R3) with about 52 amino acid residues forming a helix-turn-helix structure, which intercalates in the major groove of the DNA. Plant MYB proteins were classified into three major groups: R2R3-MYB, with two adjacent repeats; R1R2R3-MYB, with three adjacent repeats; and a heterogeneous group collectively referred to as the MYB-related proteins, which usually but not always contain a single MYB repeat (Yanhui et

al., 2006). MYB factors were shown to control anthocyanin biosynthesis (Borevitz et al., 2000) and flavonol accumulation (Mehrtens et al., 2005; Stracke et al., 2007). Other known families of TFs controlling phenylpropanoid synthesis are WDR (tryptophan-aspartic acid repeat protein) bHLH (basic helix-loop-helix protein), WIP zink finger , WRKY, HD-GL2 (homeodomain GLABRA2), ABS MADS (Arabidopsis B-Sister MADS) and DOF (DNA-binding-with-one-finger) transcription factors (Kubo et al., 1999; Johnson et al., 2002; Nesi et al., 2002; Sagasser et al., 2002; Baudry et al., 2004; Skirycz et al., 2007). TFs either directly control the synthetic pathways of the phenylpropanoids or they determine the cellular identity of a group of cells, and phenylpropanoid production is the consequence of a change in the developmental program of such cells (Grotewold, 2008). Regulation by transcription factors sometimes needs coactivators. TFs are also targets of miRNAs, which cause gene silencing and have been shown to mediate cell-to-cell and long-distance signalling (Grotewold, 2008; Molnar et al., 2011).

Knowledge about the regulation of the branch of the phenylpropanoid pathway leading specifically to scopolin and scopoletin is scarce. Elicitation is caused by various biotic and abiotic factors (see above). According to Sharan et al. (1998), scopoletin production is differentially controlled as it is common for the phenylpropanoid pathway: PAL, the key enzyme for phenylpropanoid metabolism, was activated both by methyl jasmonate (MJ) and a fungal elicitor (*Fusarium*). Scopolin and scopoletin levels rose only after MJ treatment. One of the very few works dealing with the regulation of scopolin production by transcription factors is about the antagonizing transcription factors MYB12 promoting flavonol synthesis and MYB4 suppressing flavonol synthesis and thus promoting scopoletin production (Schenke et al., 2011). The expression of glycosyltransferases performing the last synthesis step from scopoletin to scopolin does also effect scopolin and scopoletin accumulation: tobacco plants ectopically expressing a pathogen/SA-inducible glycosyltransferase had an enhanced capacity for scopolin formation and a higher resistance against potato virus X (Matros and Mock, 2004). In another case study, overexpression of a glycosyltransferase lead to doubled scopoletin/scopolin contents under TMV attack, but this increased only the necrotic areas and did not lead to increased virus resistance (Gachon et al., 2004).

Objectives

The first aim of this study was to find yet unknown regulators of scopolin accumulation. In *Arabidopsis thaliana,* scopoletin was discovered only quite recently (Rohde et al., 2004). This model plant is completely sequenced, all gene loci are known and many genes are annotated. There are also vast collections of mutant plants. This gave the possibility to screen for mutants in the accumulation of scopolin and pinpoint the mutation directly to a known locus of the *Arabidopsis* genome. To achieve this, two new screening systems with different elicitors of scopolin were developed and applied to *Arabidopsis* mutant collections. The first system was based on the application of sucrose to a collection of activation-tagging lines. Phenotyping was done by a pre-selection of fluorescing roots under UV-light and determination of scopolin contents by HPLC. With activation-tagging, the gene whose over-

expression caused the phenotype, had still to be identified from the genes surrounding the insertion site of the activation-tag. The second screening system used the induction of scopolin under cold stress in leaves. Here, insertion mutation collections with emphasis on putative transcription factors were screened. In both systems, candidates were found and characterized in the frame of this work.

The second aim was to elucidate the role of scopolin and scopoletin in roots in more detail. The function of scopoletin as a phytoalexin, whose production is initiated after pathogen attack in leaves and stems is relatively well characterised. However, it is much higher accumulated in roots, where its role is much less clear. Many authors observed constitutively high levels in roots, but hints towards a specific function in roots are rare: There are the early works postulating an influence of scopoletin on root growth, but nearly no published results about pathosystems investigating a possible protective function of scopolin or scopoletin. One exception is the action of scopoletin against the parasitic plant *Orobanche* in sunflower. This might be, because root pathogens were not relevant in the investigated species or because pathosystems for roots are not very common. To draw conclusions for a possible function, the distribution of scopolin in different root tissues under various conditions was determined. The effects of biotic and abiotic stresses on root scopolin content and on gene expression were monitored. The performance of a scopolin-free mutant in a root-pathogen-system using the protist *Plasmodiophora brassicae* was evaluated. Coumarin profiling of this mutant also gave new insights into coumarin biosynthesis in *Arabidopsis*.

Results

Scopolin is localized in endodermis and cortex of roots

High-sucrose conditions cause an accumulation of scopolin, which can be monitored by increasing root fluorescence

Roots of *Arabidopsis thaliana* fluoresce under UV light which can be considerably enhanced by growth on high-sucrose medium (Figure 6 A+B). HPLC analyses showed that scopolin accumulation in roots is dependent on the sucrose content of the medium. The chromatograms also showed that under the applied conditions, scopolin and scopoletin contribute the most to the fluorescence of the root extracts (e.g. at 3% sucrose the peaks of scopoletin and scopolin accounted for 80-95% of the total peak area of all fluorescing compounds; Figure 6 C-E). Provided that no fluorescence from insoluble cell wall components exceeded considerably the scopolin fluorescence, it was ascertained that the strong fluorescence of roots under high-sucrose is nearly exclusively caused by an accumulation of scopolin. These results were used to visualize the accumulation of scopolin in microscopic root images and to establish a screening procedure using a common gel documentation system to identify mutants from collections that are affected in scopolin accumulation. For that, the optimum fluorescence conditions of scopolin and scopoletin at vacuolar pH were determined as follows: excitation wavelength 328 nm or 336 nm for scopolin and for scopoletin 346 nm; best wavelength to detect emission for scopolin 438 nm and for scopoletin 456 nm.

Confocal laser scanning microscopy (CLSM) revealed a substance in vacuoles of root endodermis and cortex with a scopolin-like emission spectrum

CLSM was applied to localize scopolin and to distinguish between cell-wall fluorescence and the extractable compounds. Fluorescence images were taken from wild type *Arabidopsis* roots cultivated in 0.5% or 3% sucrose medium. Fluorescing areas with two different underlying spectra could be convoluted with the imaging analysis. One fluorescence spectrum which belonged to a strong fluorescence was found in the cortex and the endodermis. It had a maximum emission between 430nm and 440nm, like scopolin. The other spectrum was measured in the vascular tissue and in cell walls. It had two maxima at 420nm and 450nm (Figure 7).

To verify the putative localization of scopolin in endodermis and cortex, around 70 samples were analyzed: At 3% sucrose, the fluorescence with the spectrum of scopolin was always detected in the endodermis and in most samples also in the cortex. The intensity did vary, but was usually very strong in these areas. The pericycle, when it was visible in bright field might also fluoresce, but that was difficult to discern. The scopolin-like type of fluorescence was never visible in the vascular tissue, the rhizodermis or the root hairs. At 0.5% sucrose, that fluorescence was always detected in the endodermis, but seldom in the cortex (figure 8A). The intensity was weaker than in 3% samples. In the root tip, the fluorescence was differently distributed: in the outermost part of the tip, fluorescence was weak and evenly

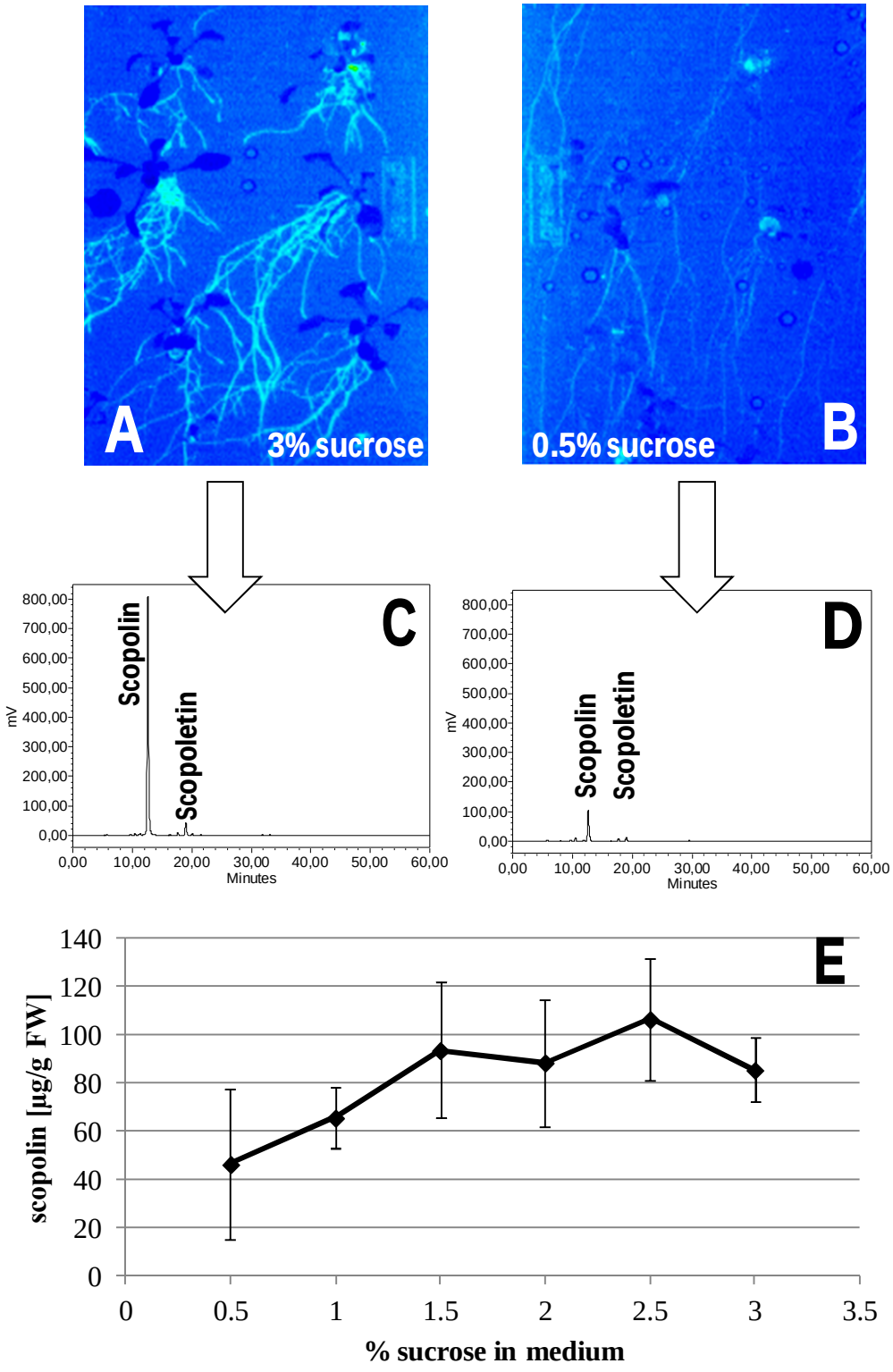


Figure 6: Root fluorescence of *Arabidopsis thaliana* and scopolin content on media with varying sucrose concentrations. The scopolin content of roots rises with increasing sucrose availability. Roots on high-sucrose medium have a strong fluorescence and high scopolin contents. A+B: False-colour fluorescence images of plants grown on 3% sucrose- **(A)** or 0.5% sucrose-containing agar plates **(B). C+D** Fluorescence chromatograms of root extracts from hydroponic culture with 3% sucrose- **(C)** or 0.5% sucrose medium **(D). E** Scopolin contents of root extracts from hydroponic culture with varying sucrose concentrations (mean and SD, n=5).

distributed among the undifferentiated cells. In the differentiation zone, before the root hairs are formed, fluorescence was usually very intense, while the root core lost its fluorescence. As soon as root hairs appeared, the fluorescence also vanished out of the outer layers and centred more and more on the cell layers surrounding the central cylinder (Figure 8 G). The fluorescence of the endodermis was visible in the whole length of the root, until endodermis and cortex became dissolved at the oldest parts of the root. Samples from soil showed the same distribution. Interestingly, samples with inclusions most likely from pathogens or symbionts (Twan Rutten[1], personal communication) had no effect on the accumulation of the putative scopolin fluorescence at the inclusion sites, but a third fluorescence spectrum surrounding the inclusion bodies was detected (Figure 8 C). Tobacco showed the same distribution of the putative scopolin fluorescence (Figure 8 D+F) as *Arabidopsis*.

Validation of the localization of scopolin with scopolin-free insertional mutants

According to Kai et al. (2008) *Arabidopsis thaliana* mutants defective in the methyltransferase CCoA*O*MT1 (*ccoaomt1)* produce less scopolin than wild type and hydroxylase F6'H1 mutants (*f6'h1)* contain virtually no scopolin. HPLC measurements confirmed the data of Kai et al. with line *ccoaomt1* having half the scopolin content of the wild type and no detectable scopolin in *f6'h1*. CLSM-fluorescence images at comparable settings revealed that roots of *ccoaomt1* had a medium fluorescence compared to a strong fluorescence of WT roots, and in *f6'h1* the fluorescence was weak (figure 9). Hence, without scopolin or scopoletin, there is no root fluorescence in endodermis and cortex, or any strong fluorescence at all, only a weak fluorescence of the cell walls.

The performance of the insertional mutant *f6'h1* in an imaging system using plants on agar plates under UV-light is shown in figure 10. The difference between *f6'h1* plants and WT plants was very obvious: Roots of the scopolin-free *f6'h1* produced barely any fluorescence signals.

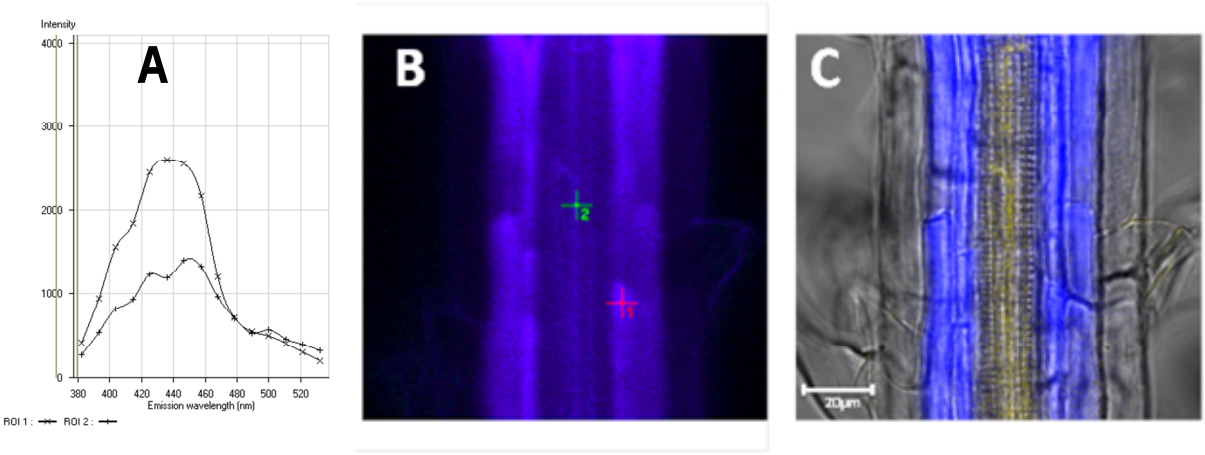
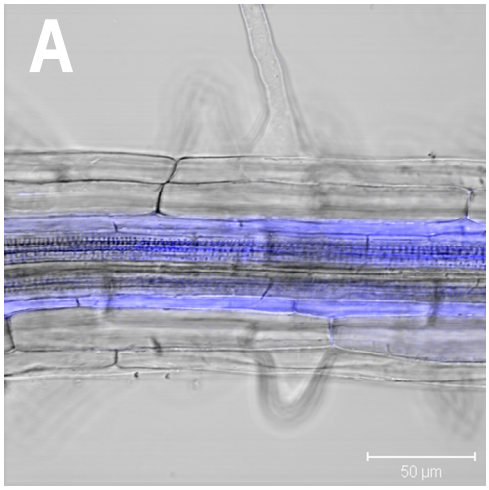
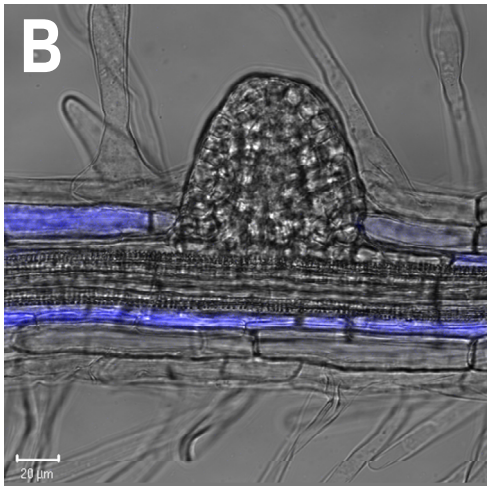
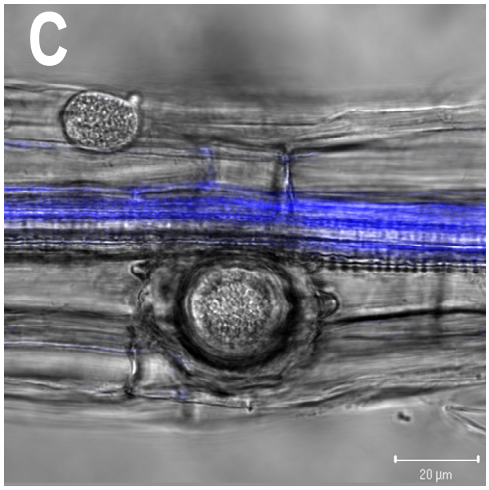


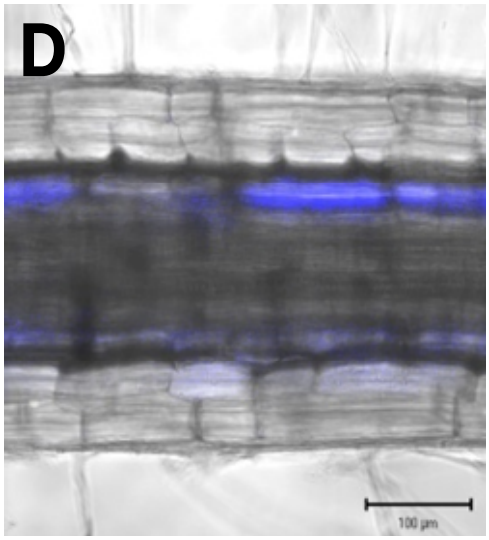
Figure 7: Longitudinal view of an *Arabidopsis* root from fluorescence microscopy. Cortex and endodermis had a strong fluorescence, which had the emission spectrum of scopolin. Two zones with different fluorescence spectra were detected. The first spectrum with an emission maximum at 430nm-440nm was detected in cortex and endodermis. The second spectrum with two maxima at 420nm and 450nm was detected in the area of the vascular tissue and in all cell walls. **A**: Emission spectra of the two selected areas 1 and 2 from B. **B**: Fluorescence image of a root with fluorescing areas. 1 (red cross) = endodermis, 2 (green cross) = vascular tissue. **C** Overlay of a bright field image and the two detected fluorescences unmixed: Blue: All pixels having spectrum 1. Yellow: All pixels with spectrum 2.

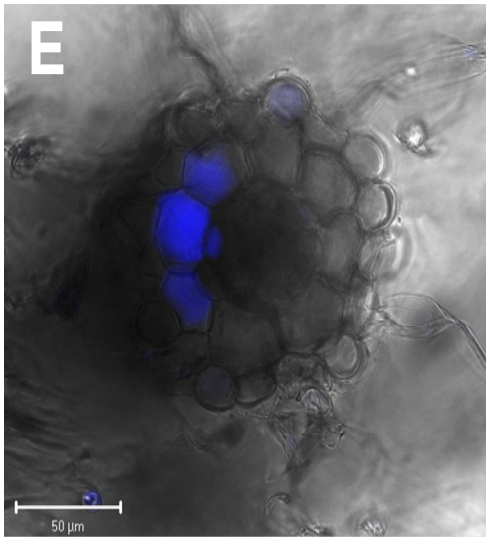
Figure 8: Examples having fluorescence with the emission maximum of scopolin (blue colour). All tested root samples have a strong fluorescence at the endodermis zone and sometimes also the cortex which has the emission spectrum of scopolin. A *Arabidopsis* root from hydroponic culture with 0.5% sucrose. **B** *Arabidopsis* root from hydroponic culture with 3% sucrose with the beginning formation of a lateral root. **C** *Arabidopsis* root from soil. Fluorescence unmixed. An inclusion of foreign bodies is visible. **D** Longitudinal view of a tobacco root**. E** Cross-section of an *Arabidopsis* root from 3% sucrose. **F** Cross-section of a tobacco root (unmixed) **G** *Arabidopsis* root tip from hydroponic culture with 3% (two images assembled). Images by Dr. Twan Rutten and Stefanie Döll. **(Next page.)**

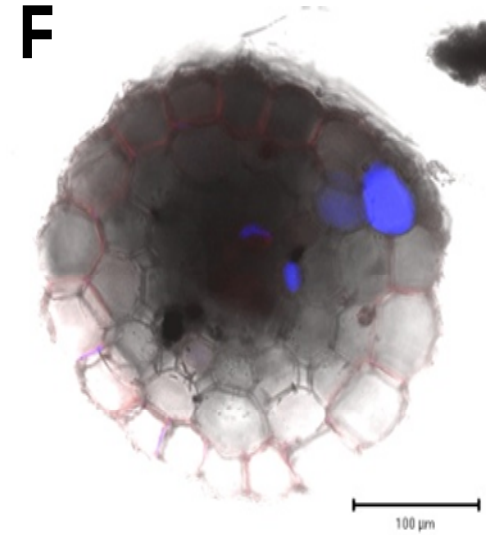

A
50 µm

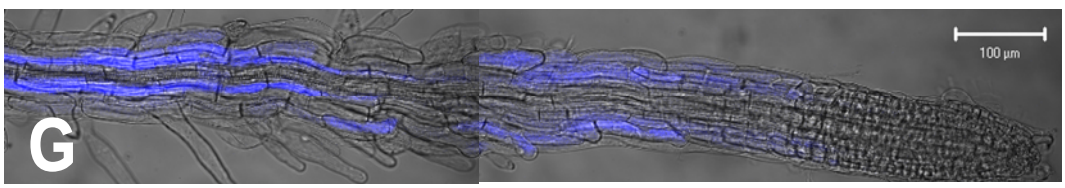

B
20 µm


C
20 µm


D
100 µm


E
50 µm


F
100 µm


G
100 µm

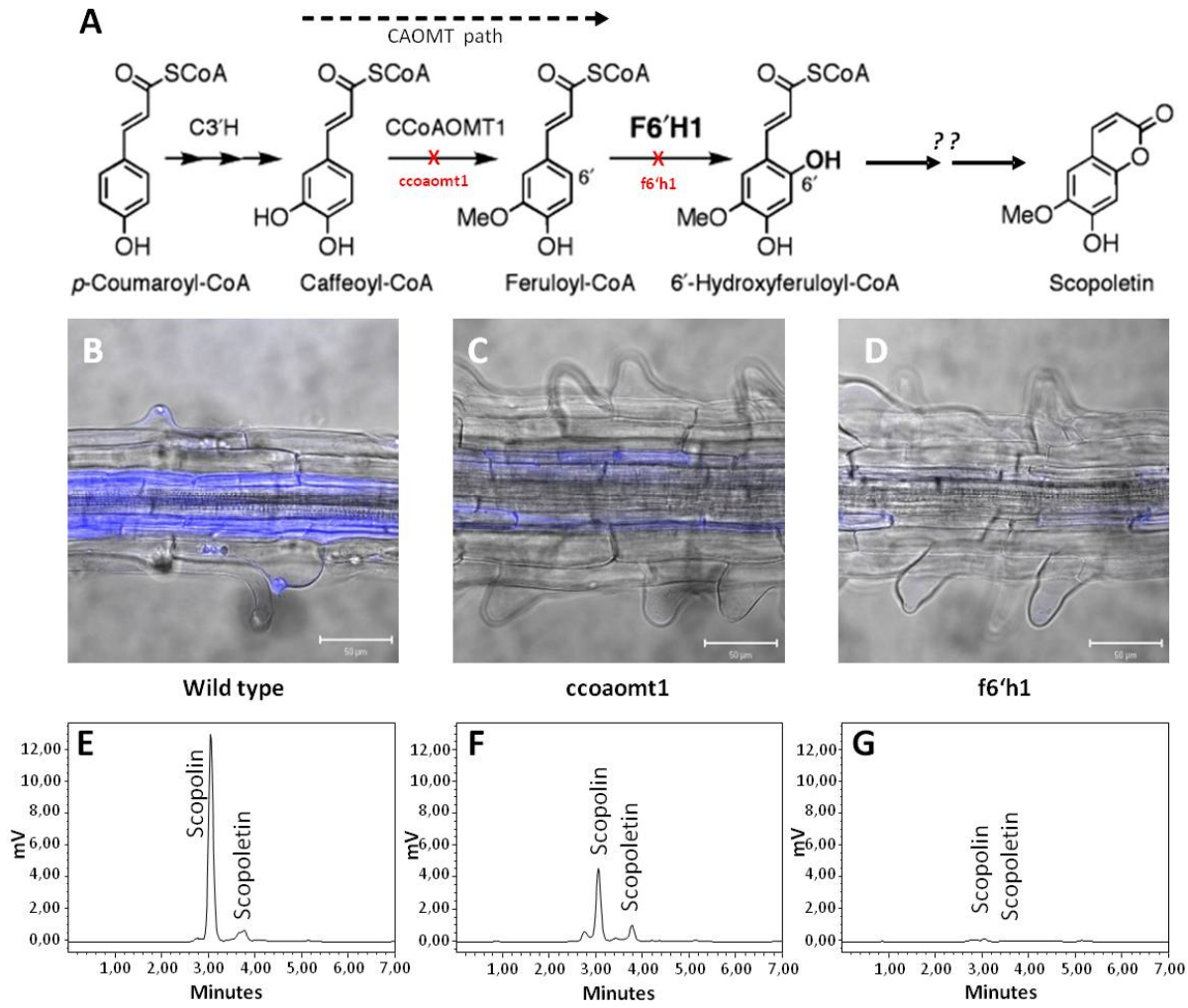


Figure 9: Root fluorescence of the insertional mutants *ccoaomt1* and *f6'h1* compared to WT. The strong fluorescence of endodermis and cortex under high-sucrose conditions depends on the presence of scopolin. Scopolin and scopoletin were the soluble compounds that contributed the most to the fluorescence of root extracts. A: The latest steps in the synthesis pathway towards scopoletin established by Kai et al (2008). (Markings in red show the missing steps in the mutants *ccoaomt1* and *f6'h1*.) **B-D** CLSM images of roots from WT and mutant *Arabidopsis* plants (exc.364nm; em. 385-450nm. Overlay of fluorescence and bright field images. Mutants with a deficiency in the scopolin pathway showed severely reduced root fluorescence. **E-G** Fluorescence chromatograms of root extracts from respective plants (exc. 340nm, em.430nm).

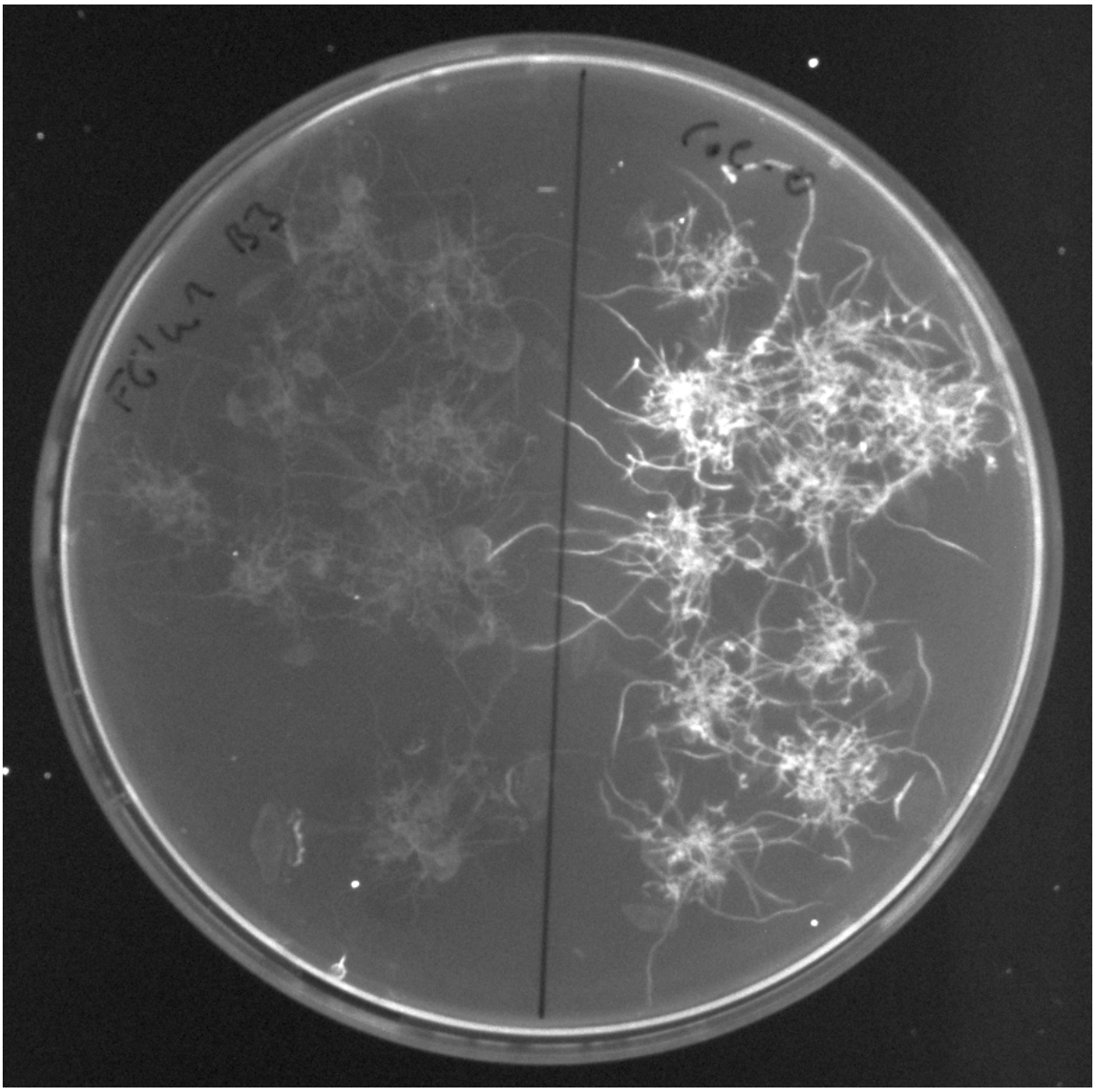


Figure 10: Fluorescence image of an agar plate (3% sucrose) with *f6'h1* plants (left) and wild type Col-0 plants (right). The plate was turned upside-down and Epi-UV (UV-light from above) was used, enabling a view on the roots without disturbance by the leaves.

F6'H1 is not only essential for the synthesis of scopolin and scopoletin, but also for the synthesis of other fluorescing compounds

As shown above, scopolin and scopoletin contributed the most to the fluorescence of roots in WT and their absence caused the non-fluorescing phenotype of *f6'h1*. However, a thorough inspection of fluorescence chromatograms of WT and *f6'h1* revealed that at least six substances were missing or severely reduced in *f6'h1* besides scopolin and scopoletin (Figure 11A), which was not mentioned in Kai et al. (2008). The UV chromatograms showed only minor reductions in some peaks of *f6'h1*, and no missing peaks (Figure 11B).

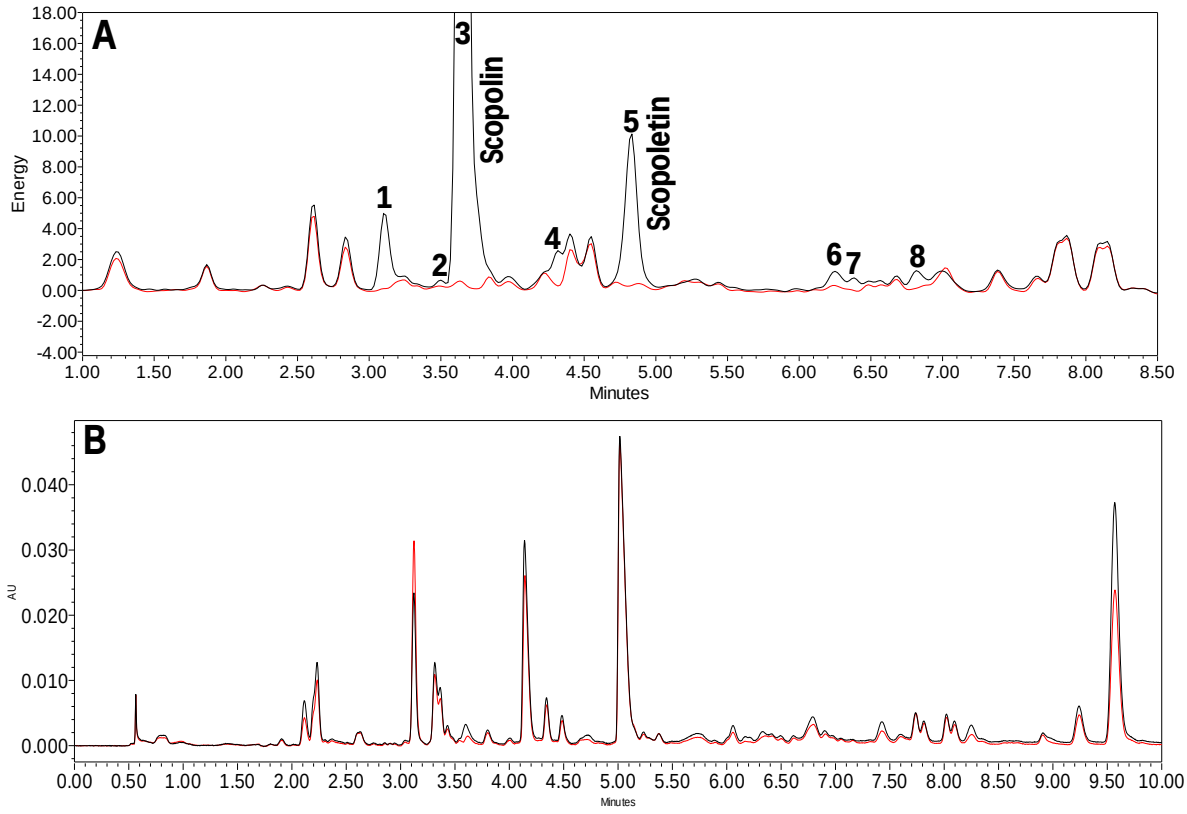


Figure 11: Overlays of root extracts from WT Col-0 (black) and *f6'h1* (red). A. Eight substances are missing or strongly reduced in the insertional mutant *f6'h1* in a fluorescence chromatogram. Number 3 and 5 had already been annotated by Kai et al. (2008) as scopolin and scopoletin. (Fluorescence detection at 336nm/438nm, gain1). **B.** Overlay of root extracts as UV chromatograms. No UV peak is missing, although some substances seem to be slightly reduced in *f6'h1*. Chromatograms were extracted at 280nm UV detection.

Peak 1 was identified as esculin by fluorescence, retention time and mass[1]. Isoscopolin matched the absent peak 2. A substance with the mass of 207 with an earlier retention time

[1] For peak identification, a combined fluorescence detection/LC-MS approach was applied, because fluorescence detection is still more sensitive for the very low amounts of coumarin derivatives in *Arabidopsis* than LC-MS. FL-detection also has the benefit to be very specific for coumarins when appropriate wavelengths are chosen. As against that, LC-MS detection suffered from the low abundance of coumarins and the signal overload of more than 800 mass signals detectable in *Arabidopsis* roots (von Roepenack-Lahaye et al., 2004).

was additionally found. Both were absent in *f6'h1*. Esculetin with the same RT as peak 2 was not found in WT, although the detection limit was determined as low as 0.1- 1µg/ml in LC-MS. (Esculetin fluorescence was quenched by the acidic solvents and could not be used for esculetin quantification.) Peak 3 was confirmed as scopolin. Peak 4 did not match any standard tested. However, two masses at the exact retention time were found in this fraction, 373 and 355, that were at least severely reduced in *f6'h1*. The respective fractions were also checked for the masses of ayapin, hydroxycaffeic acid/hydroxyconiferyl alcohol and hydroxyferulic acid. The only difference found was for the mass belonging to hydroxyferulic acid (M+H=211), eluting slightly too early to match peak 4. This mass was not present in the *f6'h1* fractions. This could be a fragment of 6-hydroxy feruloyl CoA, which was expected to be absent in *f6'h1*. A standard for hydroxyferulic acid was not available. Peak 5 was confirmed to be scopoletin. The retention time of peak 6 matched the retention time of the main mass of this fraction, 663, and peak 7 the mass 811, both were present in WT and nearly absent in *f6'h1*. Two other masses eluting at the same time, 343 and 503, were not different. Peak 8 matched exactly the retention time of 7-methoxycoumarin (herniarin) and 3-methoxycoumarin in the FLD chromatograms. Their mass (M+H) of 177 was found in WT at the exact retention time, but was closely surrounded by other peaks with the same mass. The main masses of the WT fraction were 321, 373, 517, 535 and 591. The mass of 321 was reduced in *f6'h1*. The mass 591 was not detected there, so this was another candidate for peak 8. Additional standards were also used as reference for the LC-MS analyses of the fractions. From these, sinapoylglucoside and sinapic acid were reduced in *f6'h1* and isoscopoletin was completely missing. Substances found in both lines were traces of *p*-coumaric acid, caffeic acid and ferulic acid from the core pathway, furthermore chlorogenic acid, catechin and weak traces of rutin. *Trans*-cinnamic acid, *o*-coumaric acid and the flavonoids quercitrin, hesperidin and kaempferol-3-*o*-rutinoside, MDCA, umbelliferone, scoparone, daphnetin and 3,4 dihydrocoumarin were not detected or uncertain already in WT. Umbelliferone determination was uncertain, as it had only an integrated area of 3 in both lines, which is close to background noise. Figure 12 shows comparative LC-MS runs of standards, wild type- and *f6'h1* extracts. For masses, retention times and compounds identified and present in WT and mutant, see appendix tables 1-3. Figure 13 shows comparative LC-MS runs of reference substances and concentrated fractions of wild type and *f6'h1* fractions.

Substances found missing or strongly reduced in *f6'h1* as determined by LC-MS:

Esculin (absent)
Isoscopolin (absent)
Scopolin (absent)
Unknown Compound 1 (M=207, absent)
Sinapoylglucoside (reduced)
Unknown compound 2 (M=211. absent)
Unknown Compound 3 (M1=373; M2=355, reduced)
Isoscopoletin (absent)
Sinapic acid (reduced)
Scopoletin (absent)
Unknown compound 4 (M=663) (reduced)
Unknown compound 5 (M=811) (reduced)
Unknown compound 6 (M=321) (reduced)
Unknown compound 7 (M=591) (absent)
Herniarin (?)

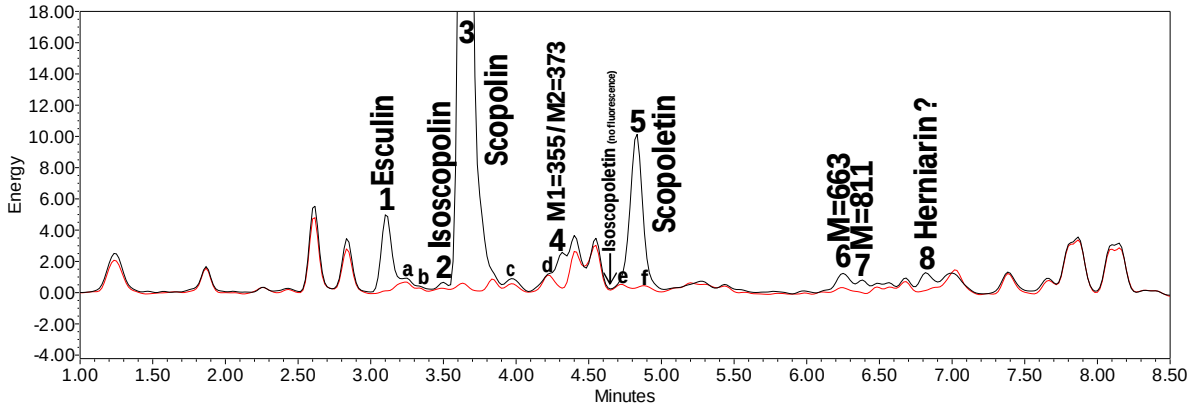
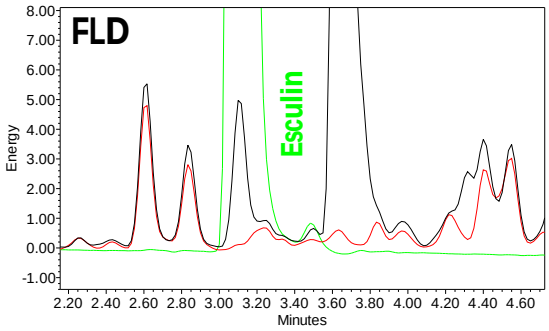


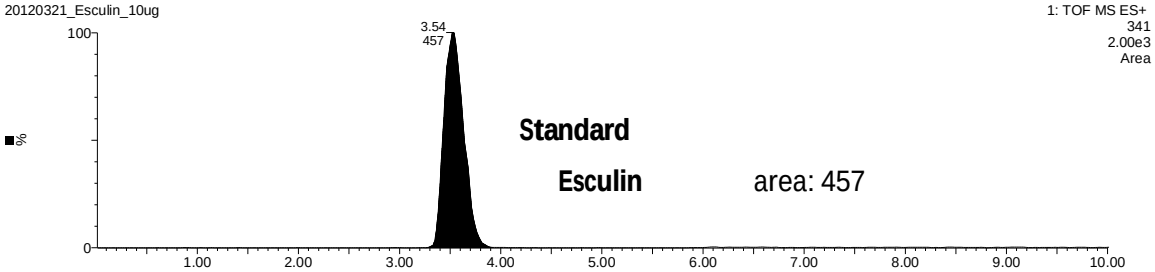
Figure 12: Annotation of fluorescence chromatograms of root extracts showing identified peaks and the difference between WT Col-0 and the mutant *f6'h1*. a) chlorogenic acid, b) caffeic acid, c) sinapoyl glucoside (reduced to 1/3), d) *p*-coumaric acid, e) ferulic acid, f) sinapic acid. Annotated peaks match exactly the mass and retention time of standards. Peak 8 was only tentatively annotated, because it matched also mass and retention time of its isoform 3-methoxycoumarin and because several peaks with the same mass ran closely together, from which the others were also present in *f6'h1*. Another candidate for peak 8 had the mass of 591.

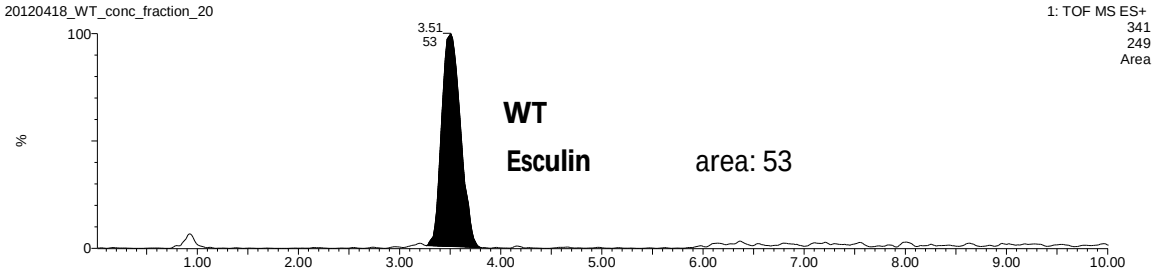
Figure 13 (Next pages): TOF-MS analyses of fractions corresponding to the 6 unknown absent peaks in *f6'h1*. Comparisons between reference substances, WT and *f6'h1* fractions of the respective retention times. FLD: The absent peak is shown in an overlay of root extracts from WT Col-0 (black), *f6'h1* (red) and reference substances (green). Fluorescence detection at 336nm/438nm, gain1. **TOF MS:** Mass chromatograms of reference substances and the corresponding fractions of WT and *f6'h1*. Each chromatogram shows a single mass extracted from the mass spectrum (number at the upper right corner of each chromatogram, number in the second line of the text field). Upper number at top of peak = retention time. Second number on top of peak = peak area. (The area can be used for comparison. Peak height (y-axis: %) is given in percent of the highest peak in each run and not suitable for quantification. Most compounds eluted in more than one fraction). **LC-MS spectra** at the retention time of the absent peak.

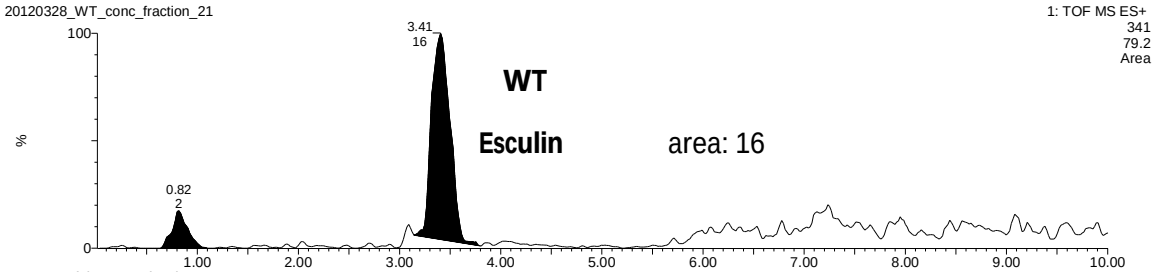
Peak 1: Esculin

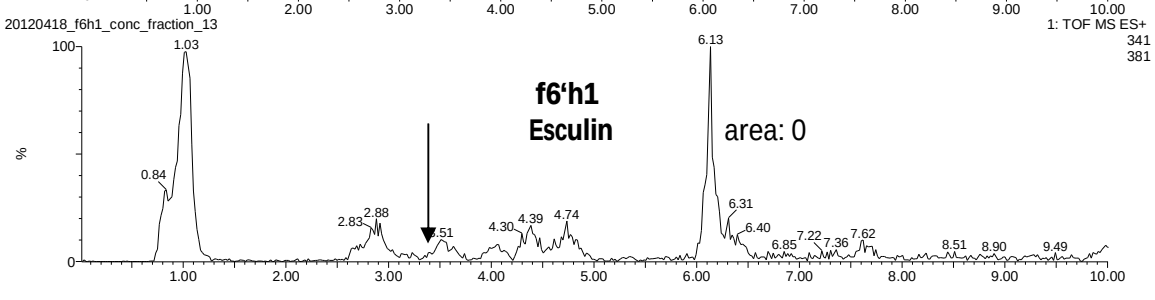


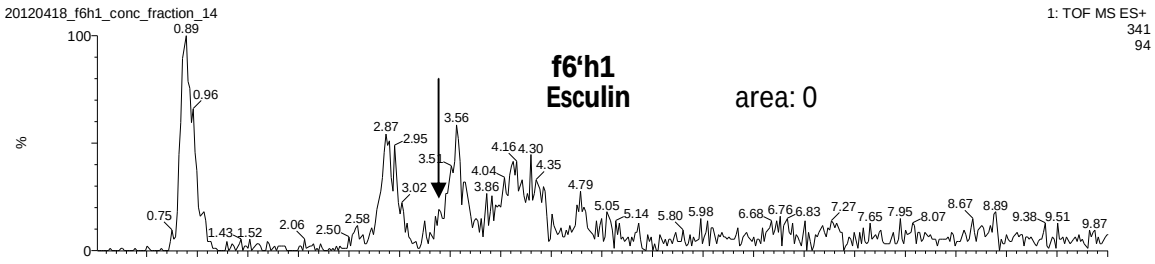
TOF MS





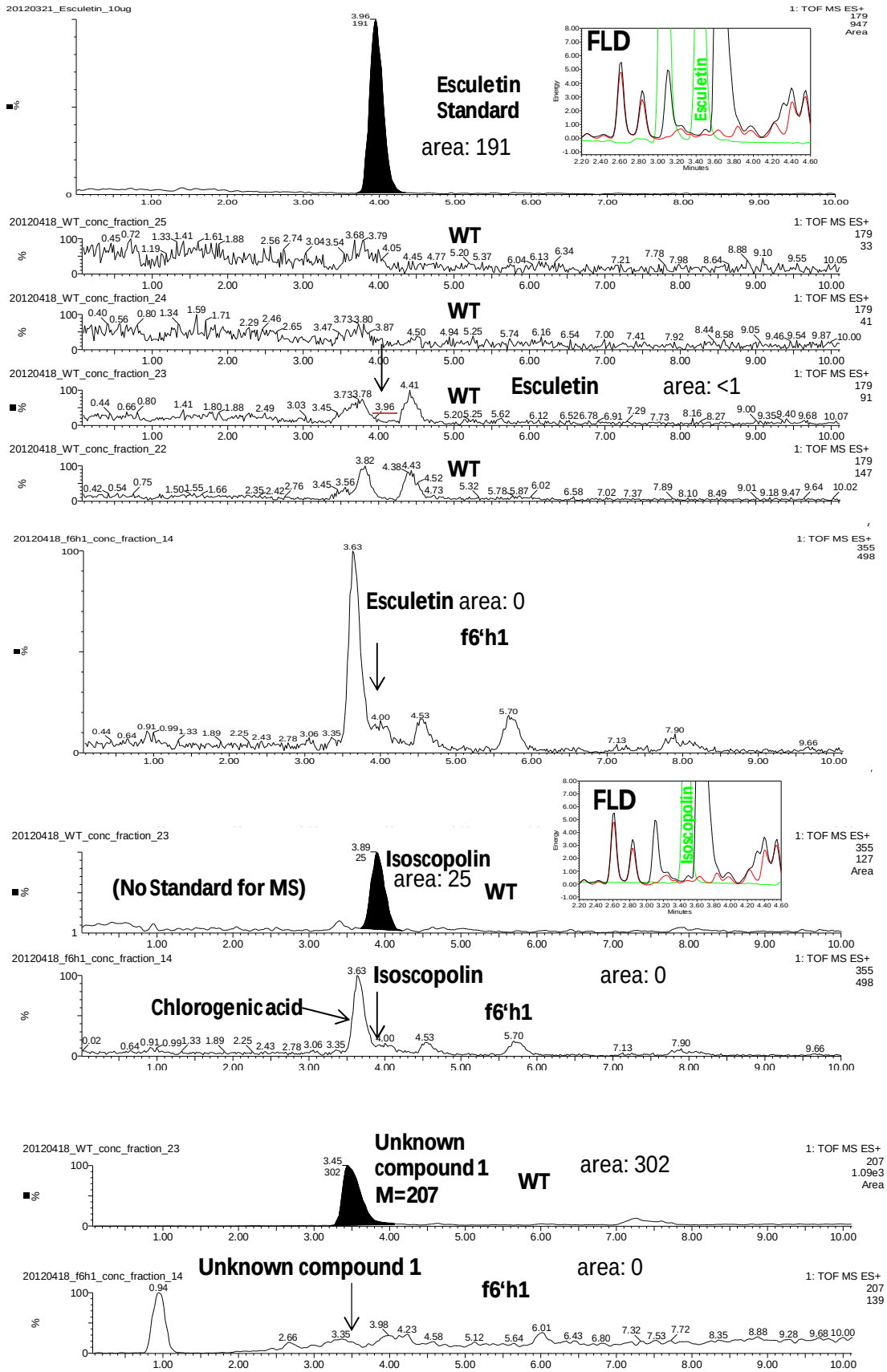






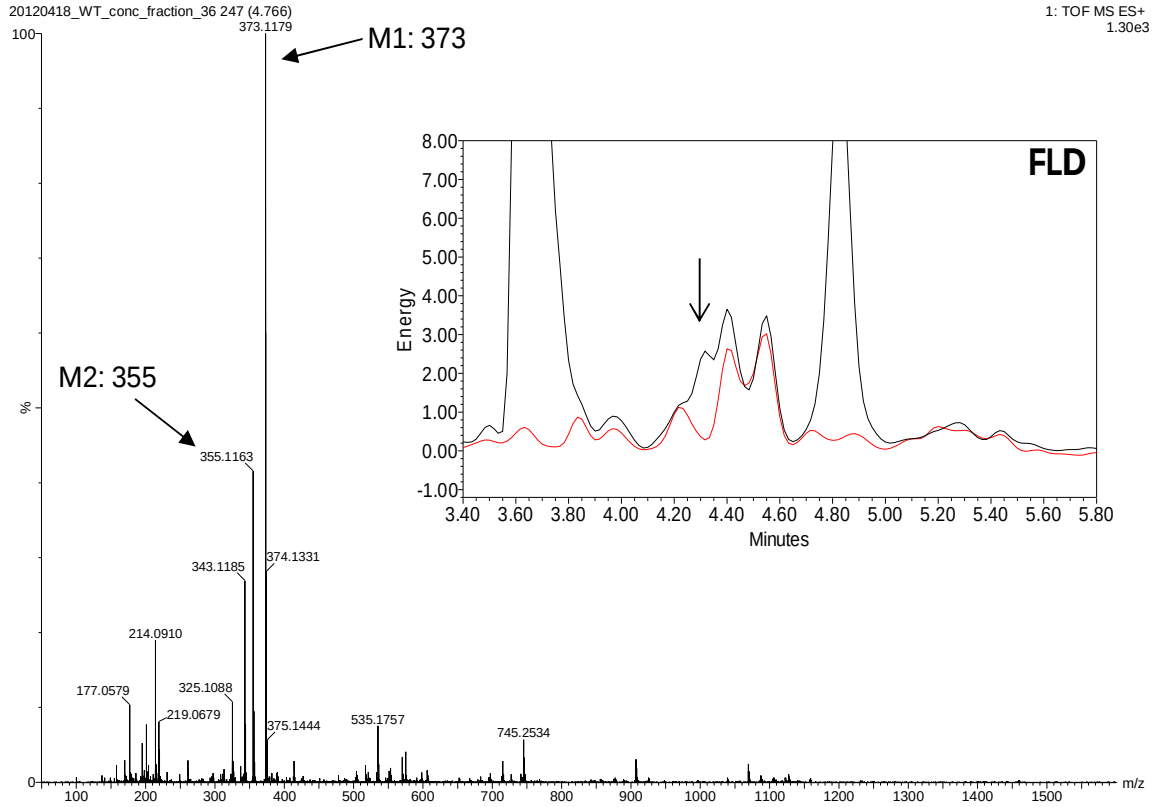
Peak 2:

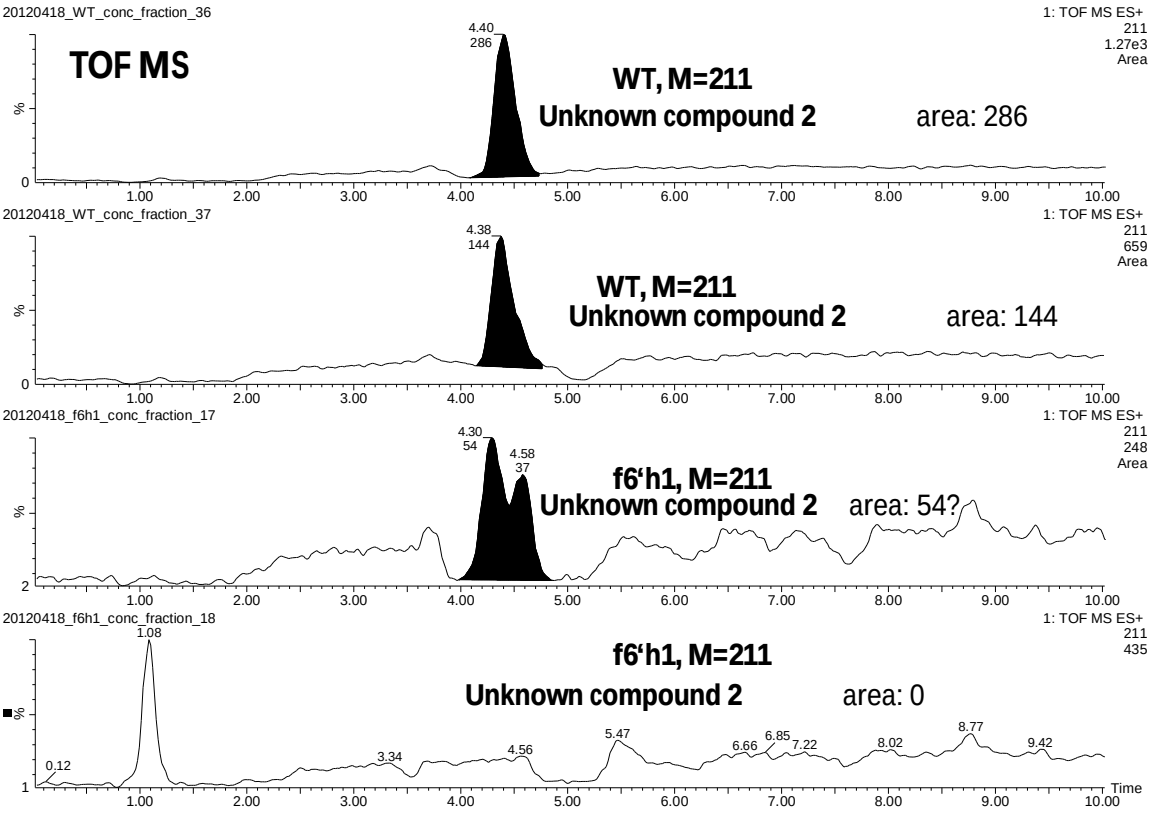
TOF MS


20120321_Esculetin_10ug
Esculetin Standard
area: 191
FLD
Esculetin
20120418_WT_conc_fraction_25
WT
20120418_WT_conc_fraction_24
WT
20120418_WT_conc_fraction_23
WT
Esculetin
area: <1
20120418_WT_conc_fraction_22
WT
20120418_f6h1_conc_fraction_14
Esculetin area: 0
f6'h1
20120418_WT_conc_fraction_23
(No Standard for MS)
Isoscopolin
area: 25
WT
FLD
Isoscopolin
20120418_f6h1_conc_fraction_14
Isoscopolin
area: 0
Chlorogenic acid
f6'h1
20120418_WT_conc_fraction_23
Unknown compound 1
M=207
WT
area: 302
20120418_f6h1_conc_fraction_14
Unknown compound 1
area: 0
f6'h1

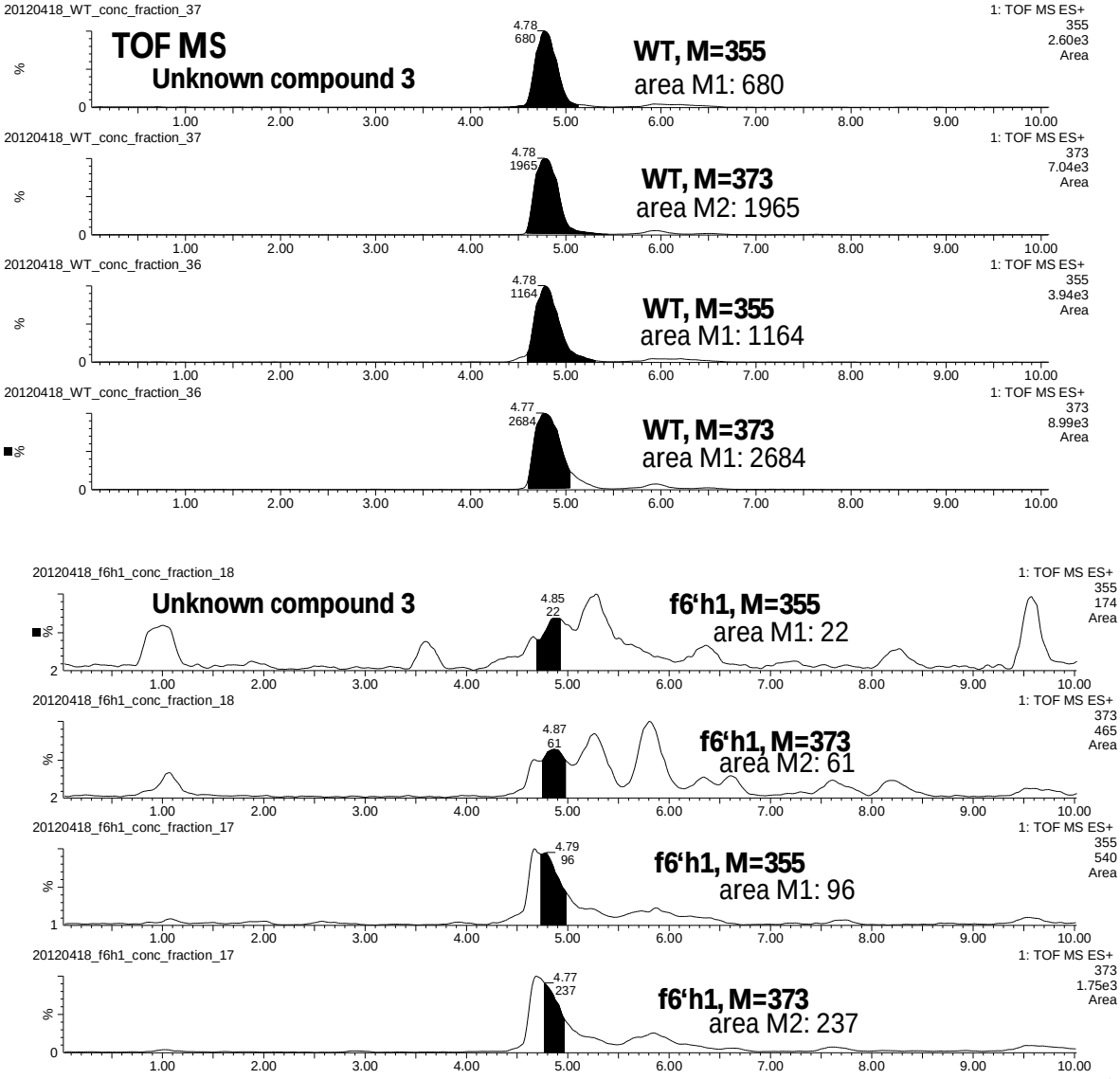
Peak 4

Mass spectrum of main MS peak

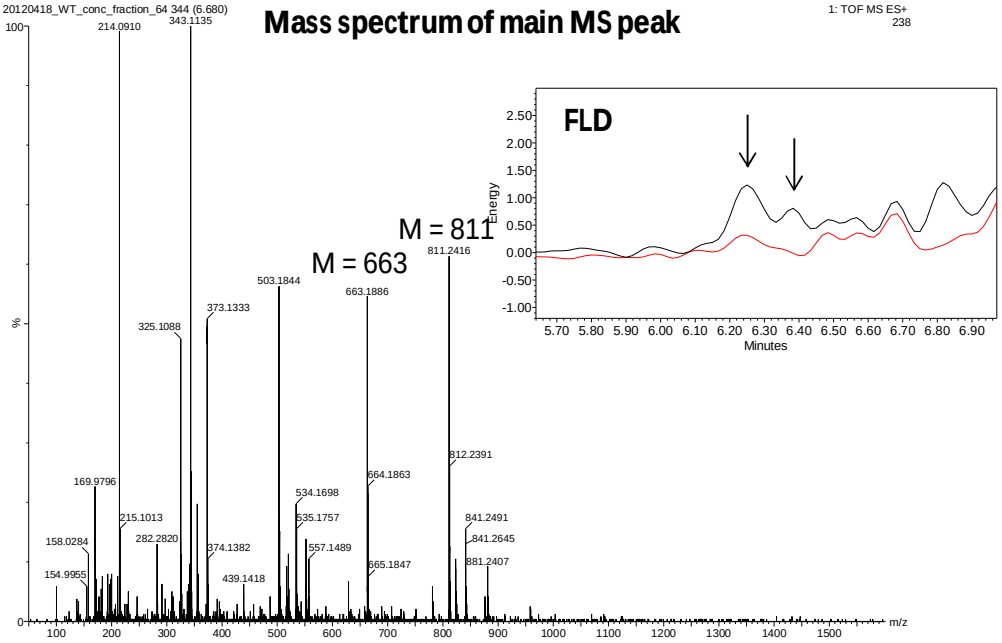


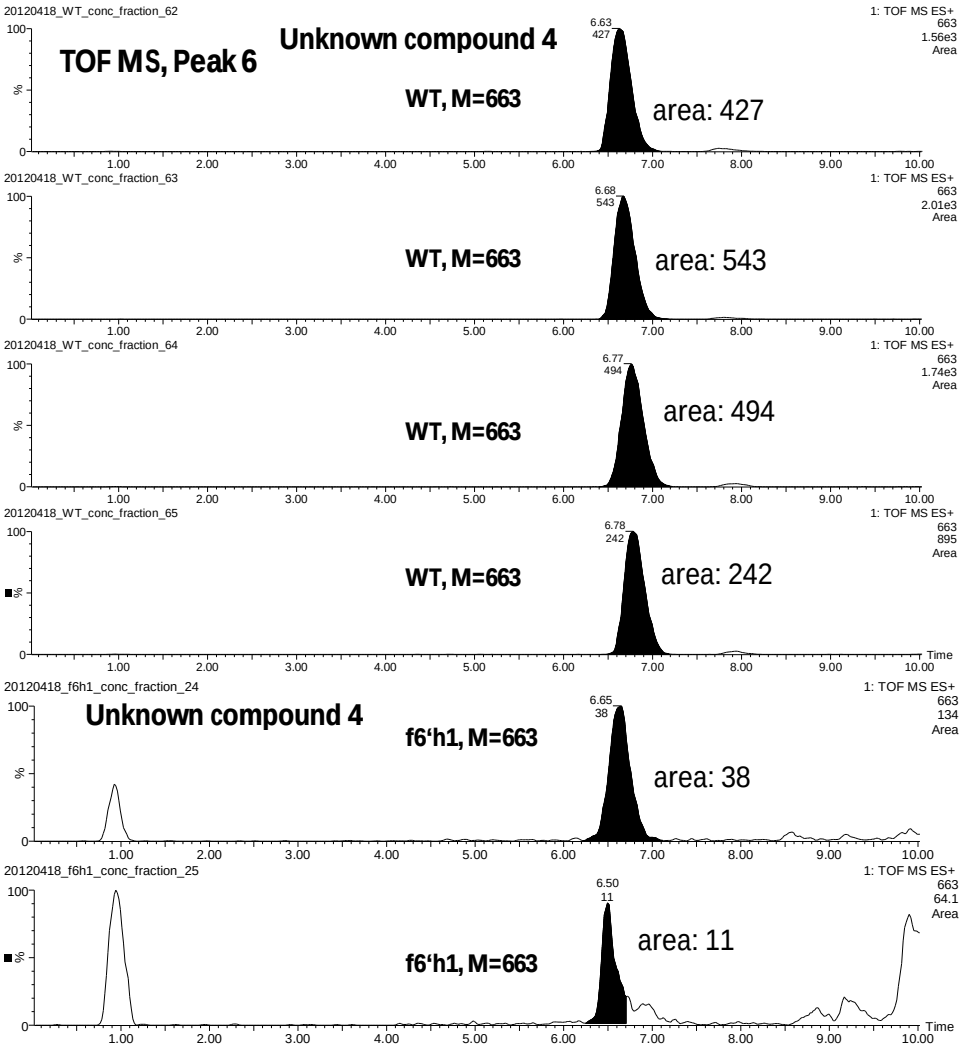


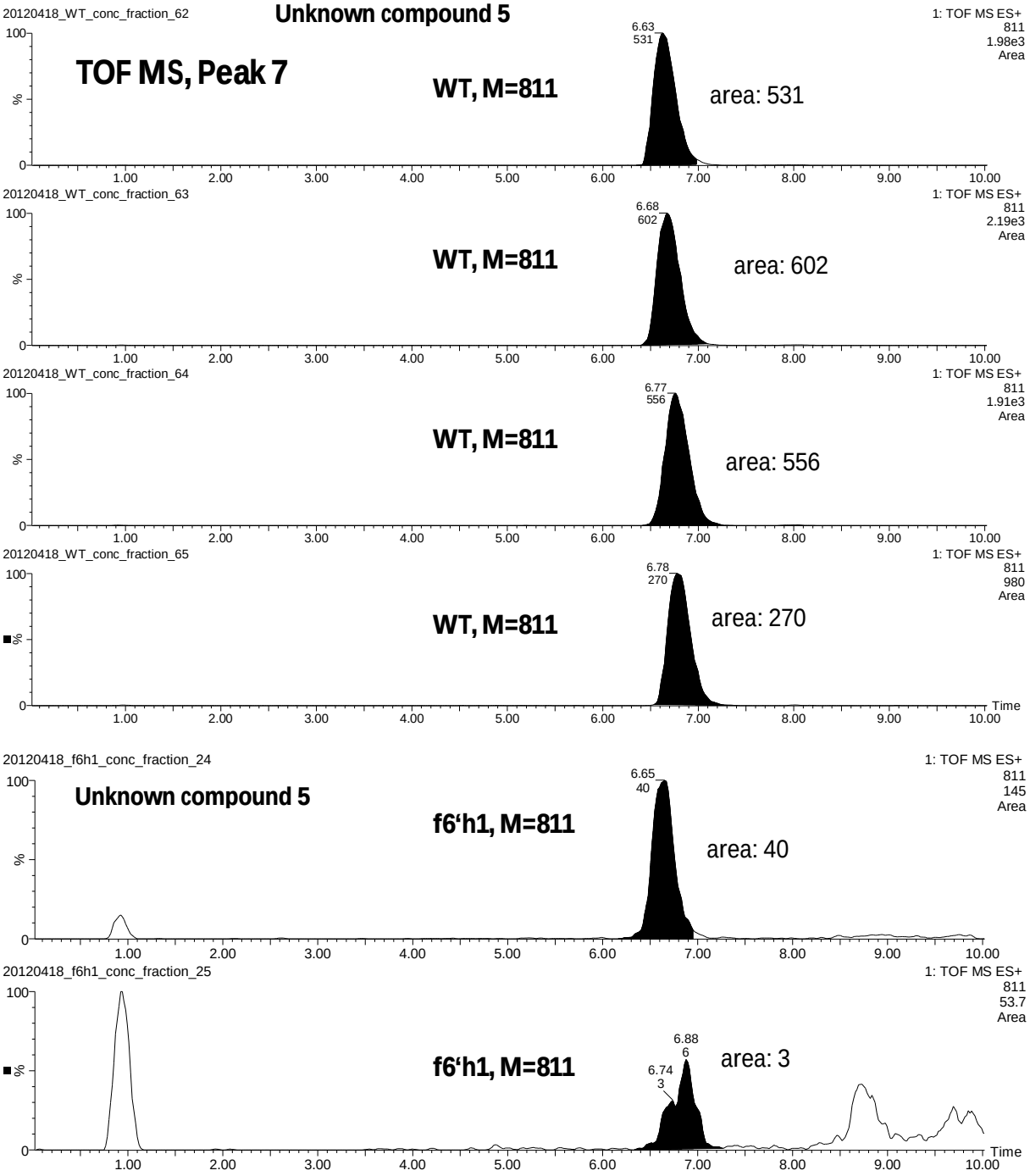
Peak 4, continued



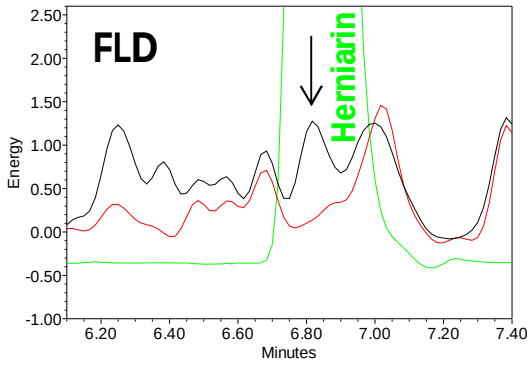
Peaks 6+7


Mass spectrum of main MS peak
FLD
M = 811
M = 663
Energy
Minutes
m/z

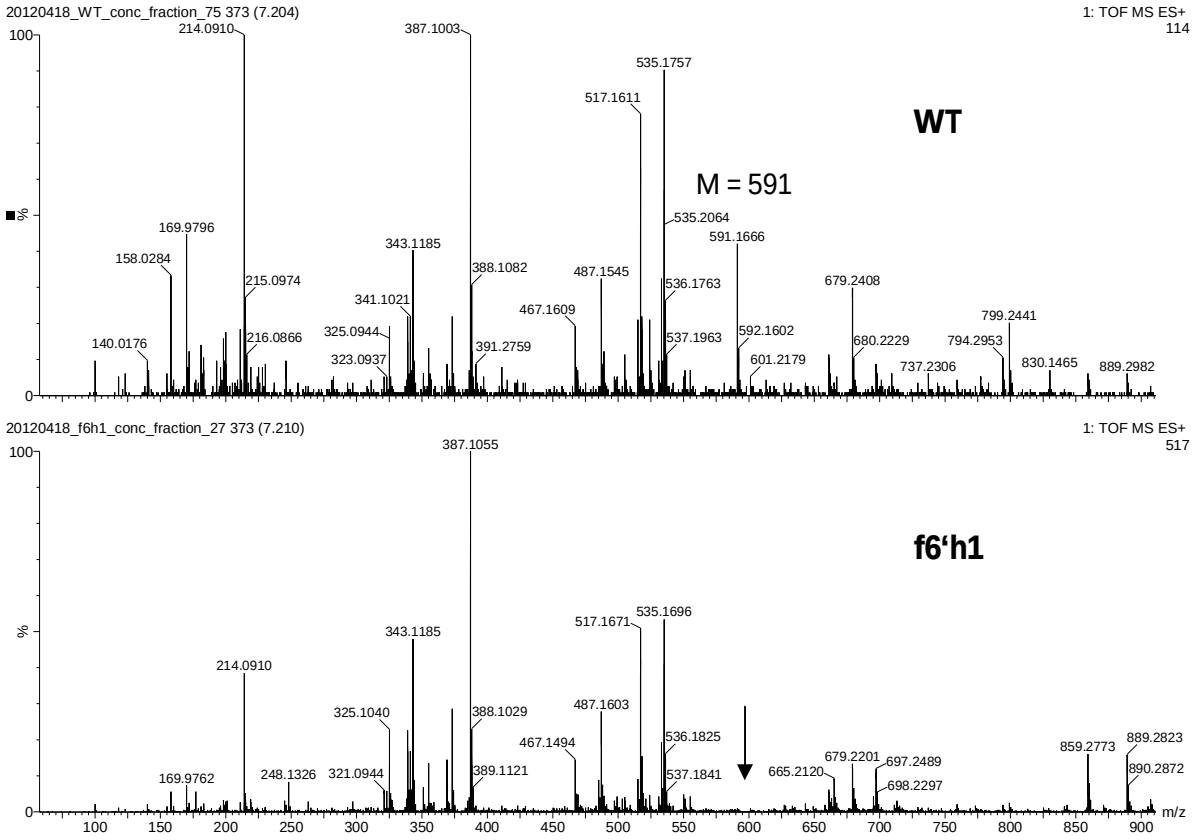

TOF MS, Peak 6
Unknown compound 4
WT, M=663
area: 427
WT, M=663
area: 543
WT, M=663
area: 494
WT, M=663
area: 242
Unknown compound 4
f6'h1, M=663
area: 38
f6'h1, M=663
area: 11
Time


20120418_WT_conc_fraction_62
Unknown compound 5
1: TOF MS ES+
811
1.98e3
Area
TOF MS, Peak 7
WT, M=811
area: 531
6.63
531
20120418_WT_conc_fraction_63
1: TOF MS ES+
811
2.19e3
Area
WT, M=811
area: 602
6.68
602
20120418_WT_conc_fraction_64
1: TOF MS ES+
811
1.91e3
Area
WT, M=811
area: 556
6.77
556
20120418_WT_conc_fraction_65
1: TOF MS ES+
811
980
Area
WT, M=811
area: 270
6.78
270
Time
20120418_f6h1_conc_fraction_24
1: TOF MS ES+
811
145
Area
Unknown compound 5
f6'h1, M=811
area: 40
6.65
40
20120418_f6h1_conc_fraction_25
1: TOF MS ES+
811
53.7
Area
f6'h1, M=811
area: 3
6.88
6
6.74
3
Time

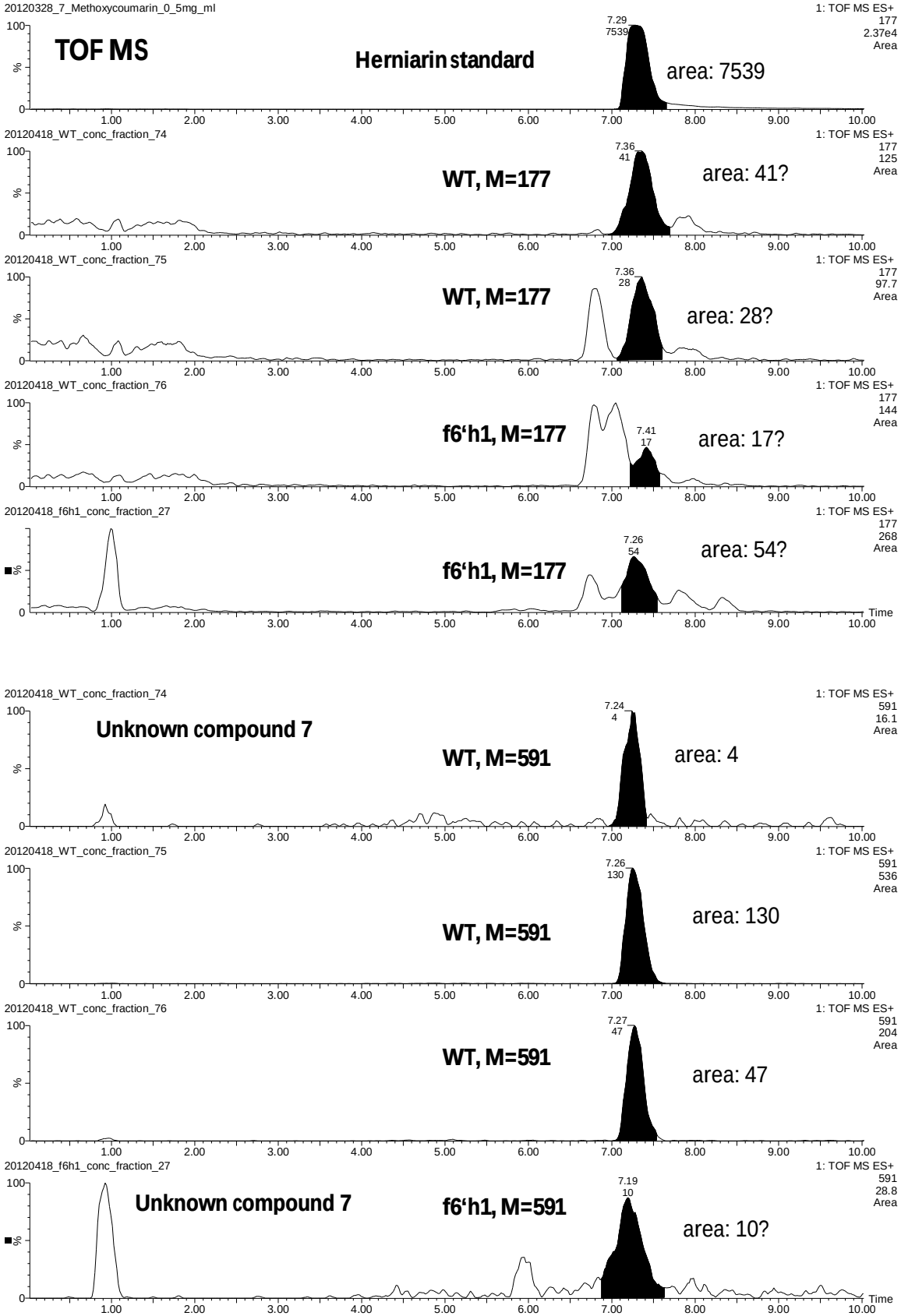
Peak 8



Mass spectrum at herniarin/3-methoxycoumarin RT



Peak 8, continued



Root growth of insertion mutation lines *ccoaomt1* and *f6'h1 differed from WT* - First results.

To investigate if the scopolin content had an influence on root growth, line *ccoamt1* with about half the scopolin contents of WT and *f6'h1,* which is scopolin-free, were cultivated vertically for 16 days on agar plates with 0.5% sucrose in a pilot experiment. To half of the set an additional stress factor was added (digital images of plants courtesy of Nicole Schmid[2]). The stressed groups had shorter roots and more lateral roots than the non-stressed groups. Within the non-stressed group, *ccoaomt1* had longer roots than WT and *f6'h1*, which were identical. But both *f6'h1* and *ccoaomt1* had less lateral roots than WT. In the stressed group, however, *ccoaomt1* had shorter roots even than WT, whereas *f6'h1* was again identical to WT. *Ccoaomt1* also had under stress conditions less lateral roots than WT, *f6'h1* did not differ from WT (ANOVA with Bonferroni correction, n=24-40).

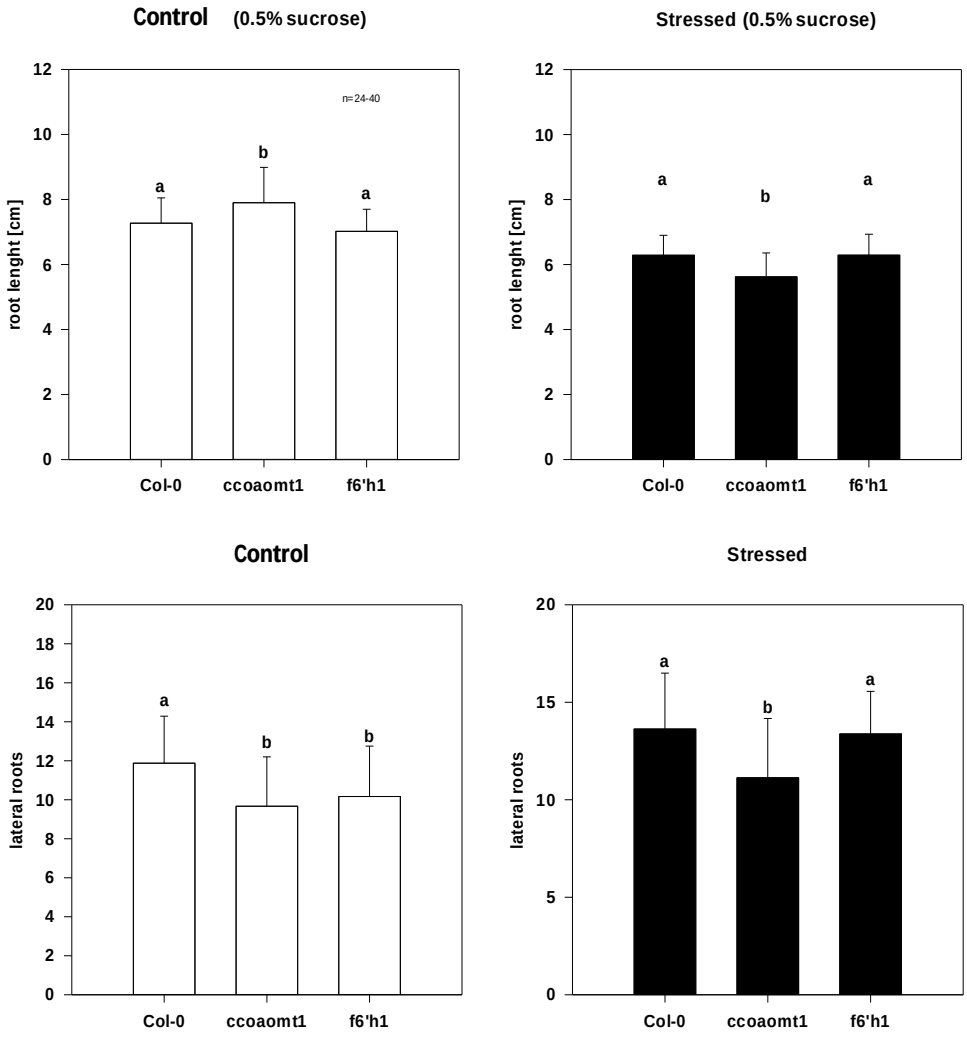


Figure 14: Root growth of WT Col-0 and two loss-of-function mutants towards the production of scopolin and scopoletin. Line *ccoamt1* has half the scopolin contents of WT, *f6'h1* is scopolin-free.

[2] Molecular Plant Nutrition, IPK Gatersleben, Germany

The presence of scopolin has minor effects on the severity of a *Plasmodiophora brassicae* infection

A pathogen test developed by Siemens et al. (2002) was applied to WT and *f6'h1* plants to test whether the presence of scopolin offers protection against this gall-forming root pathogen. The most relevant parameters measured in this test are the infection rate and the disease index. The disease index evaluates the severity of symptoms by sorting each plant into defined classes from 0 – symptom free to 4 – heavy deformations. Four independent experiments were carried out, however with varying spore virulence and environmental conditions. Scopolin levels in WT were measured for each symptom class in the last three experiments. For roots, symptom class 1 had the highest scopolin content in experiment 2 and 3, but in experiment 4 the scopolin peak was shifted to symptom class 2. The leaf scopolin content rose moderately from class 0 to class 4. Scopoletin levels were roughly the same in classes 0, 1 and 2 as the one of an untreated control, but they sank considerably in classes 3 and 4. In leaves, there was a slight increase towards class 4 in two experiments (Figure 15). In the first experiment, there was a tendency that *f6'h1* plants were more severely affected than WT plants, although results just failed to be significant (p = 0.11, Figure 16). The result from the first experiment could be recreated once (p=0.14). Two other experiments failed to produce any differences. Results are summarized in Table 2.

Table 2: Infection rates and disease indices for WT and *f6'h1* after an infection with *Plasmodiophora brassicae*.

Experiment	Spores per plant	WT Col-0 Infection rate (mean)	*f6'h1* Infection rate (mean)	p (t-test) Infection rate	WT Col-0 Disease Index (mean)	*f6'h1* Disease Index (mean)	p (t-test) Disease Index	n
1.	$2x10^6$	**38%**	**56%**	**0.11**	**19**	**28**	0.12	4
	$2x10^5$	8%	12%	0.24	3	6	0.69 (U-test)	4
2.	$2x10^7$	100%	100%	0.93	87	87	0.98	9
	$2x10^6$	97%	97%	0.75	82	81	0.5	9
3.	$2x10^6$	98%	91%	-	75	73	-	1
.	$2x10^5$	**77%**	**84%**	**0.14**	47	49	0.60	8
4.	$2x10^5$	94%	99%	0.045	64	67	0.39	2
	$2x10^4$	66%	67%	0.94	37	34	0.681	6

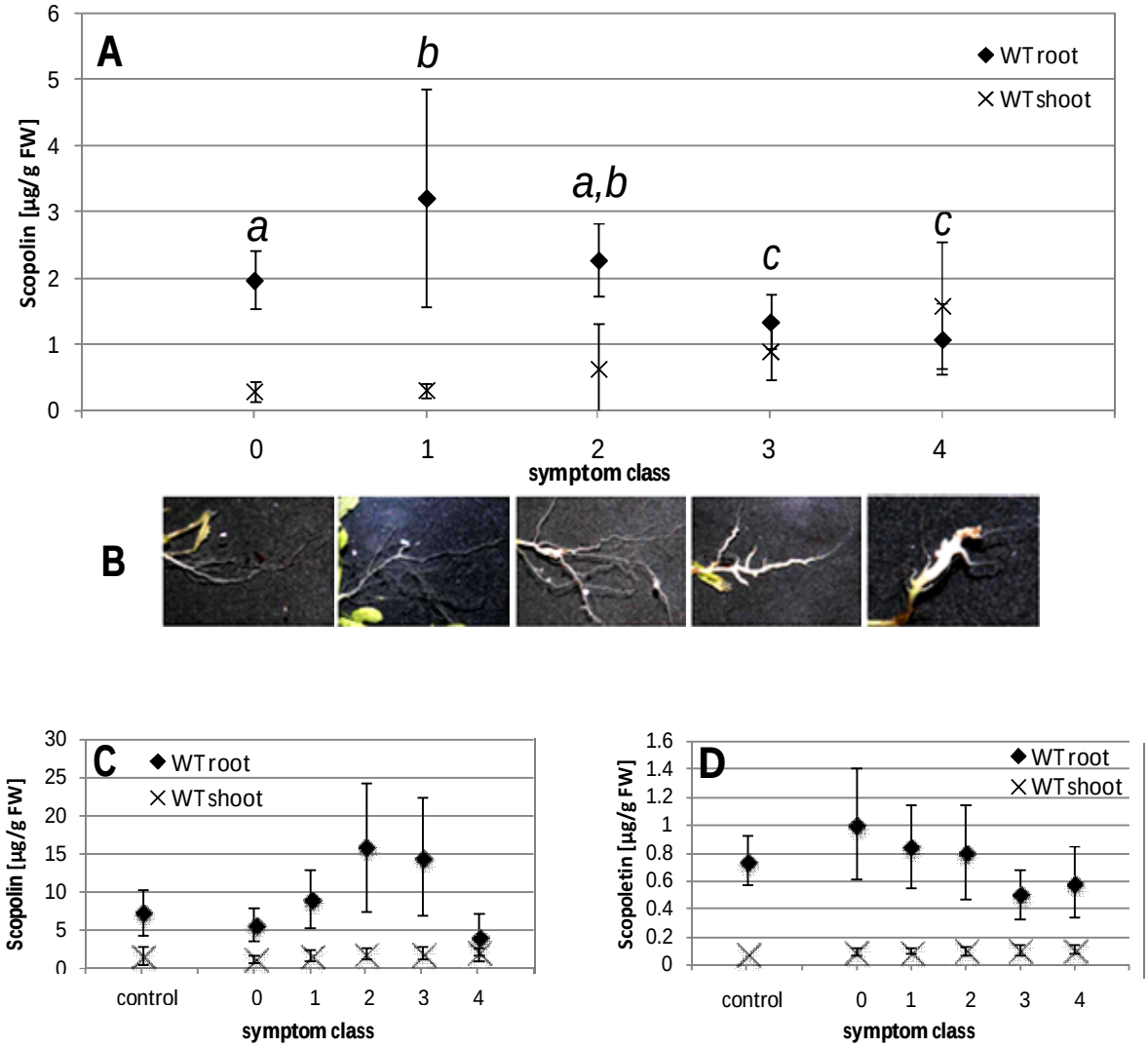
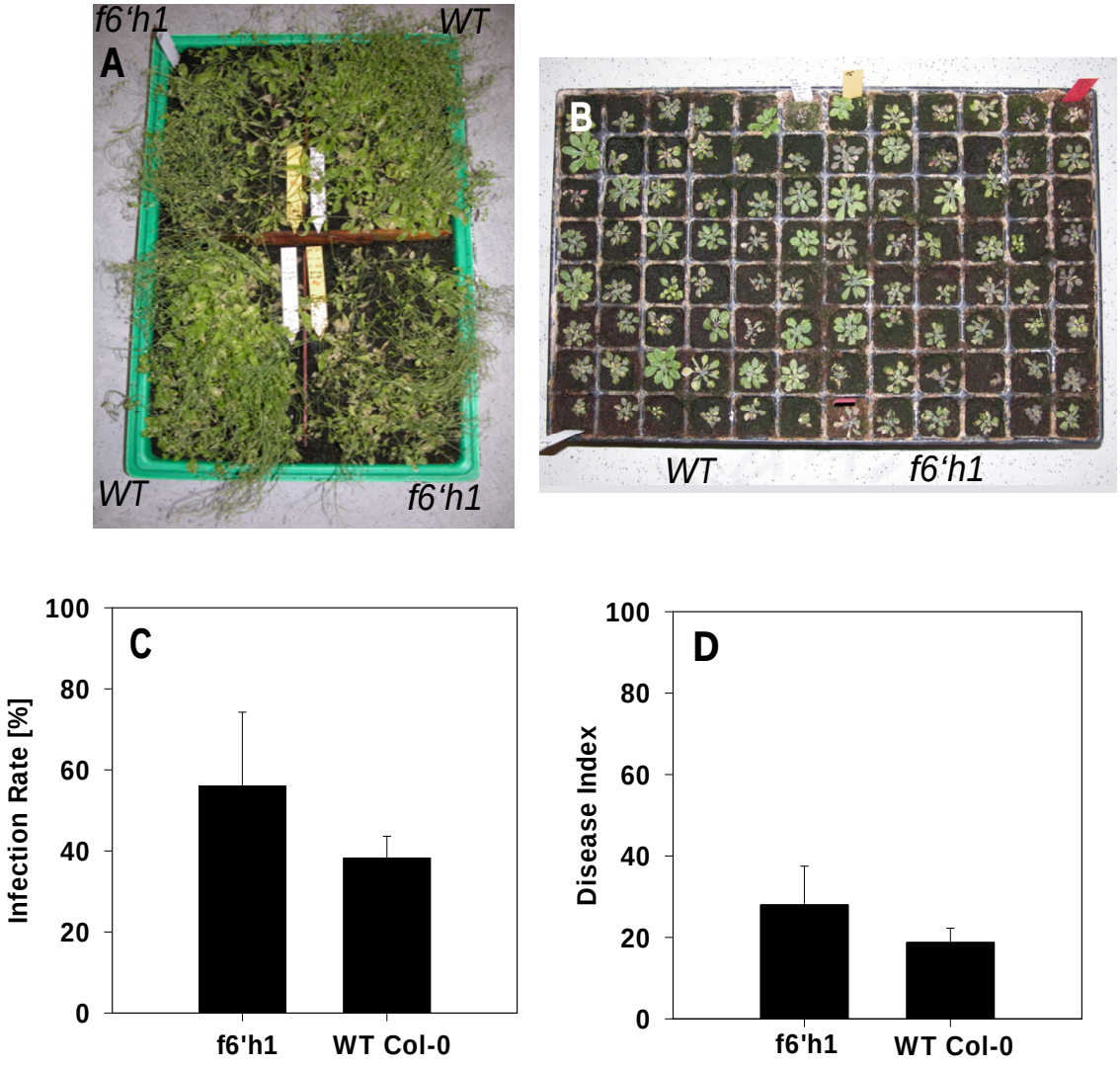


Figure 15: WT Col-0 after a *Plasmodiophora brassicae* infection: Scopolin accumulates highest in plant roots with only minor symptoms. The scopoletin content decreases in heavily affected plants. Leaf contents are not affected or rise slightly with the severity of the infection. A Second experiment: Scopolin content of roots and leaves of 8 pools per symptom class (mean value and standard deviation). Data points with different letters (a, b, c) are significantly different from each other ($p < 0.05$, ANOVA with Student-Newman-Keuls post-hoc test). **B:** Plants with typical manifestation of symptoms for each class. **C** Fourth experiment: Scopolin content of roots and leaves of 8 pools per symptom class and an untreated control. **D** Scopoletin content of the same pools as C (mean value and standard deviation).

Figure 16 (Next page) Experimental design and exemplary results of a *Plasmodiophora brassicae* experimental system. The scopolin-free mutant *f6'h1* is compared to WT Col-0. A First experiment: Same weights of seeds were sown on one quarter of a tray and 2ml of a spore suspension was pipetted to the roots of each plant. WT performed visually better than *f6'h1*. **B** Second to fourth experiment: Seedlings were transplanted to 96-well trays, where each single plant was separated. Mean values for infection rate **(C)** and severity of symptoms **(D)** were lower for *f6'h1*, however, differences failed to be significant (p=0.1, n=4).



Identification of genes involved in the accumulation of scopolin under high-sucrose

A shift from low-sucrose conditions to high-sucrose conditions alters the expression of more than 4000 genes in *A. thaliana*

In an *Arabidopsis* plant, a shift from low-sucrose medium to high-sucrose medium in a special sterile hydroponic culture system triggers the accumulation of the coumarin scopolin in roots and leaves (Figure 17, data for leaves not shown). An array expression analysis was performed to monitor the overall responses and to correlate changes in mRNA levels to the observed metabolic changes. It was also meant to find candidates that might be responsible for the enhanced scopolin accumulation. To determine the suitable time point for harvesting to gain relevant RNA populations, RNA extracts and protein extracts and from several time points after a shift were made. Scopolin accumulation in roots increased after 48 hours and had its highest value at 7 days. In leaves, the scopolin content increased until 72 hours, where it reached a plateau. Scopolin accumulation mainly took place in roots and therefore

roots were selected for analysis. The protein extracts were analysed for their ability to transform a scopoletin standard into scopolin. The protein extracts' conversion rates increased very slightly and with fluctuations until 36 hours. At 48 hours, they reached a peak and declined in later samples (Figure 17). In a Northern Blot, a probe for the glycosyltransferase UGT 73B3, which is known to have scopoletin as a substrate (Maslak, 2002) was applied to the RNA extracts. However, signals showed only minor differences between the time points and could not be used for the determination of the optimum time point for harvest. As the accumulation of enzyme precedes the accumulation of product, the RNA peak for the enzyme should precede the enzyme peak. Based on these results, it was clear that samples for RNA extraction had to be taken distinctly earlier than 48 hours (Figure 17), so the time point 24 hours after the shift was chosen. For the chip analysis itself, *Arabidopsis* Col-0 plants were grown in the hydroponic culture system on 0.5% sucrose. After four weeks cultivation, time point zero samples were harvested, as well as samples from plants 24 hours after the shift to high-sucrose and samples from a control group, which was shifted only to fresh 0.5% sucrose medium. Comparisons were done from time point zero to high sucrose and from time point zero to the control group. Fold changes, which occurred in the control group, were subtracted from the high-sucrose fold changes to eliminate changes which occurred only because of the shift to fresh medium.

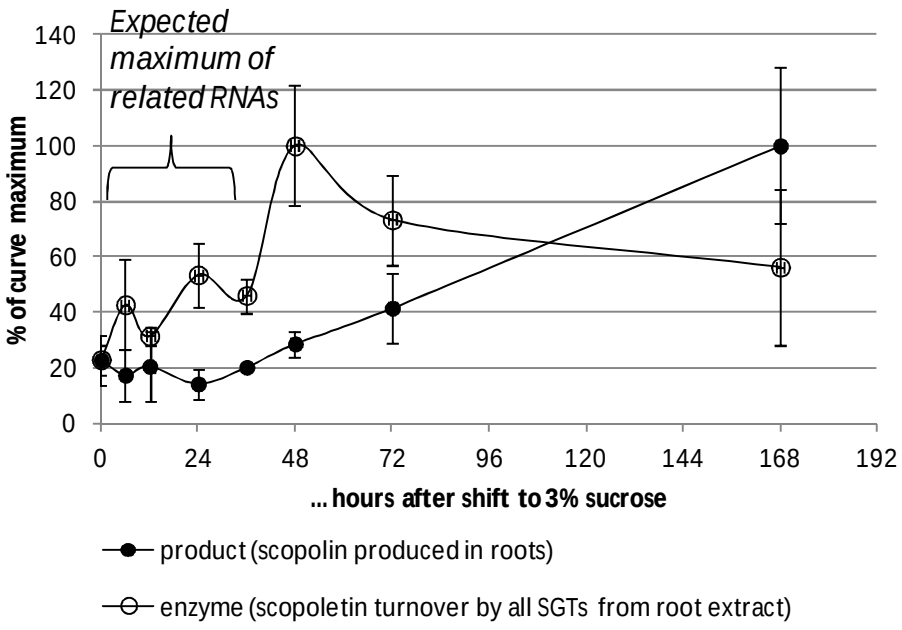


Figure 17: Determination of the optimum time point of harvest to gain sucrose-shift related RNAs. The product, scopolin, was measured at defined time points after the shift from 0.5% sucrose to 3% sucrose in root extracts [µg scopolin/g FW]. Total protein extracts from every time point were used to produce scopolin out of a scopoletin standard [µg scopolin/µg total protein]. The highest value of each curve was set as 100%. Mean values and standard deviation, n = 3. SGTs: scopoletin glucosyltransferases of *A. thaliana*.

Results were filtered by the parameters of a minimum fold change of 2 and a significance level of $p<0.01$. 1876 genes were upregulated and 2288 were downregulated. The Top 100

genes for each case are given in appendix 1. The highest upregulation of gene expression occurred for an NAD+ ADP-ribosyltransferase gene, followed by a gene coding for an unknown protein and a gene which is involved in anaerobic respiration. Upregulated genes from the top 100 that belonged to the primary metabolism were involved in carbohydrate synthesis and fatty acid metabolism. 31 genes from the top 100 were involved in stress response. They were associated with hypoxia, osmotic stress, oxidative stress, heat stress, wounding, detoxification or were involved in flavonoid biosynthesis. Three sucrose synthases were remarkable, which are activated under osmotic stress. Moreover, there was an activation of genes involved in transcription and translation, transport, ageing and cell wall modification (see appendix table A5).

The genes that were most highly downregulated coded for a dormancy/auxin related protein of unknown function, the peptidase SCPL13 (serine carboxypeptidase-like 13, involved in proteolysis) and a plant invertase/methylesterase inhibitor. Primary metabolism genes in the top 100 were associated mainly with protein degradation and modification or had nutrient reservoir activity. From the downregulated genes associated with stress, 12 were involved in pathogen defence. Others were related to osmotic stress, oxidative stress or responded to water deprivation. Two genes were involved in sugar transmembrane transport (SWEET 3, SWEET 11), probably accompanied by two other transporters from the same nodulin superfamily. Furthermore, some downregulated genes were involved in lignin/cell wall biosynthesis, root development and growth (see appendix table A6).

The number of genes that were regulated in the shifting experiment was too high to select suitable candidates responsible for the increase in scopolin. For that reason, a mutant collection was screened for their scopolin content to identify single genes influencing the regulation of scopolin accumulation.

Screening of activation-tagging lines revealed that AtTHO1 positively influenced scopolin accumulation

Overview:

First part: Screen

1. **Screen of a collection of activation-tagging lines for enhanced root fluorescence**
2. **Isolation of the activation-tagged line N21392 6/1 with enhanced scopolin levels**
3. **Determination of the T-DNA insertion site in line N21392 6/1**
4. **Selection and testing of candidate genes near the insertion site of the T-DNA in line N21392 6/1 - Identification of At5g09860 (AtTHO1) as the gene causing the phenotype in N21392 6/1**
5. **Verification of the influence of AtTHO1 by the construction of independent overexpression lines and the complementation of a deletion mutation line**

Second part: Characterisation of AtTHO1

6. ***In silico* analyses of AtTHO1**
7. ***In silico* analyses of genes associated with AtTHO1 (complex members) and phenotyping of deletion mutations of the complex**
8. **Gene expression analysis of a deletion mutation line of AtTHO1 (*attho1*)**
9. **Further phenotyping of *attho1:* Morphology, reaction to cold, high-sucrose and osmotic stress**

1. Screen of a collection of activation-tagging lines for enhanced root fluorescence

A collection of activation-tagged *Arabidopsis thaliana* lines was screened for increased scopolin content. These lines carried a T-DNA randomly inserted into the genome (Weigel et al., 2000). This T-DNA contained four 35S enhancer elements which activated the transcription of nearby genes up- or downstream of the insertion site. This collection had to be screened for mutants in scopolin accumulation. For an initial screen, enhanced root fluorescence was used as a marker for increased scopolin content. The screen by root fluorescence resulted in 36 candidate lines (Figure 18).

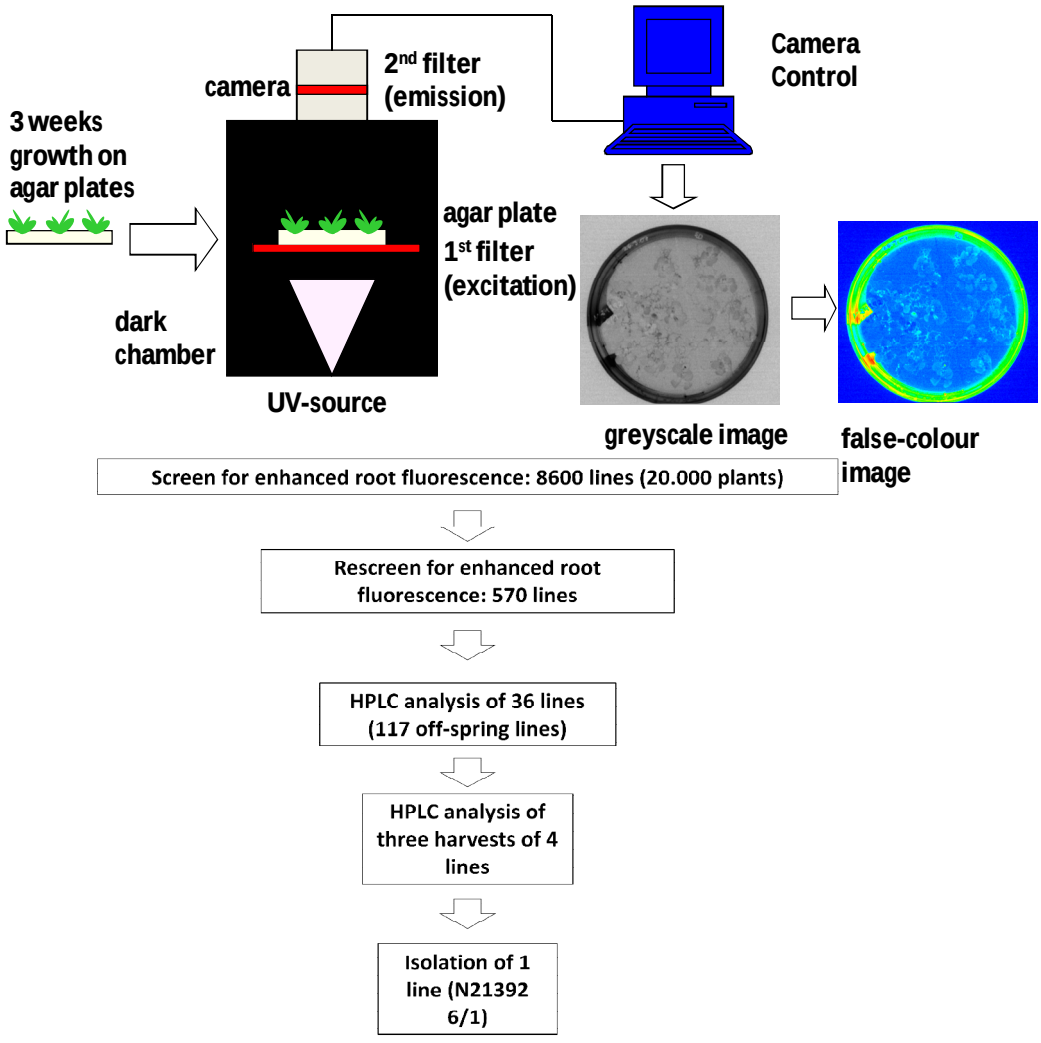


Figure 18 (previous page): System for the screen of the collection of activation-tagging lines for enhanced scopolin accumulation. For the first two rounds of screening, an imaging system was applied: The mutant plants were grown together with wild type plants on agar plates. A gel documentation system was fitted with appropriate excitation and emission filters and mutant plants compared to wild type plants for more strongly fluorescing roots on 0.5% sucrose. Selected plants were cultivated further for seed production. The final selection of candidates was achieved by HPLC analysis of root extracts from liquid culture.

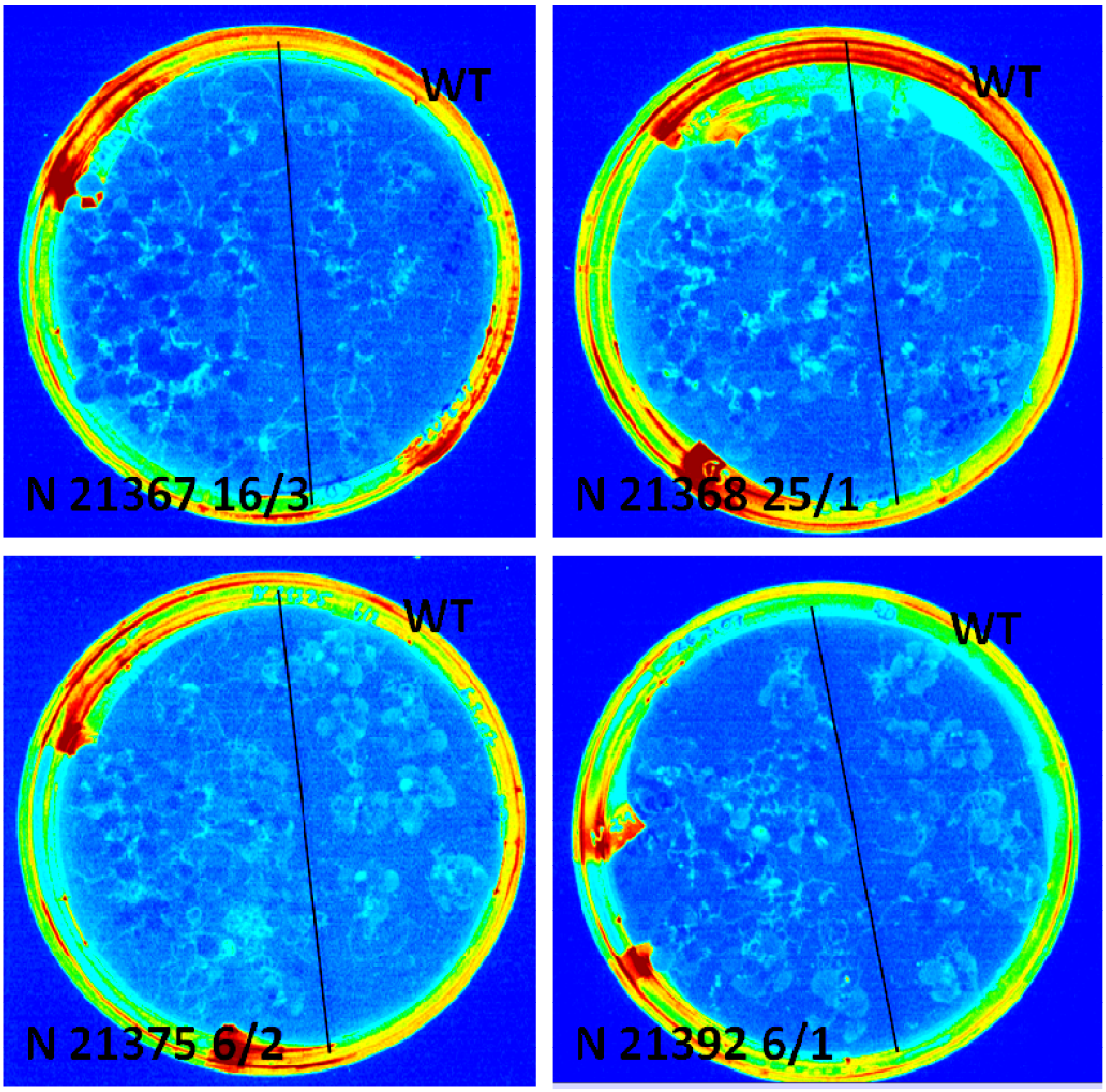


Figure 19: Four examples of the 36 activation-tagging lines that were selected for enhanced root fluorescence compared to wild type (WT). False-colour fluorescence pictures of plantlets grown on 0.5% sucrose agar plates. Mutant lines are on the left side of the plate, WT on the right. Roots of WT plants are nearly invisible, whereas most mutant plant roots show a bright fluorescence. The roots are visible as the white lines, partially covered by dark or light blue areas which are the leaves. Roots from the mutant lines appear brighter than the respective WT on the right.

2. Isolation of the activation-tagged line N21392 6/1 with enhanced scopolin levels

Several independent HPLC analyses confirmed the higher scopolin content of line N21392 6/1 in hydroponic and partially in soil culture (figure 20). Line N21368 25/1 also had a significantly higher scopolin content. In soil culture the scopolin content of N21368 25/1 was significantly lower than WT.

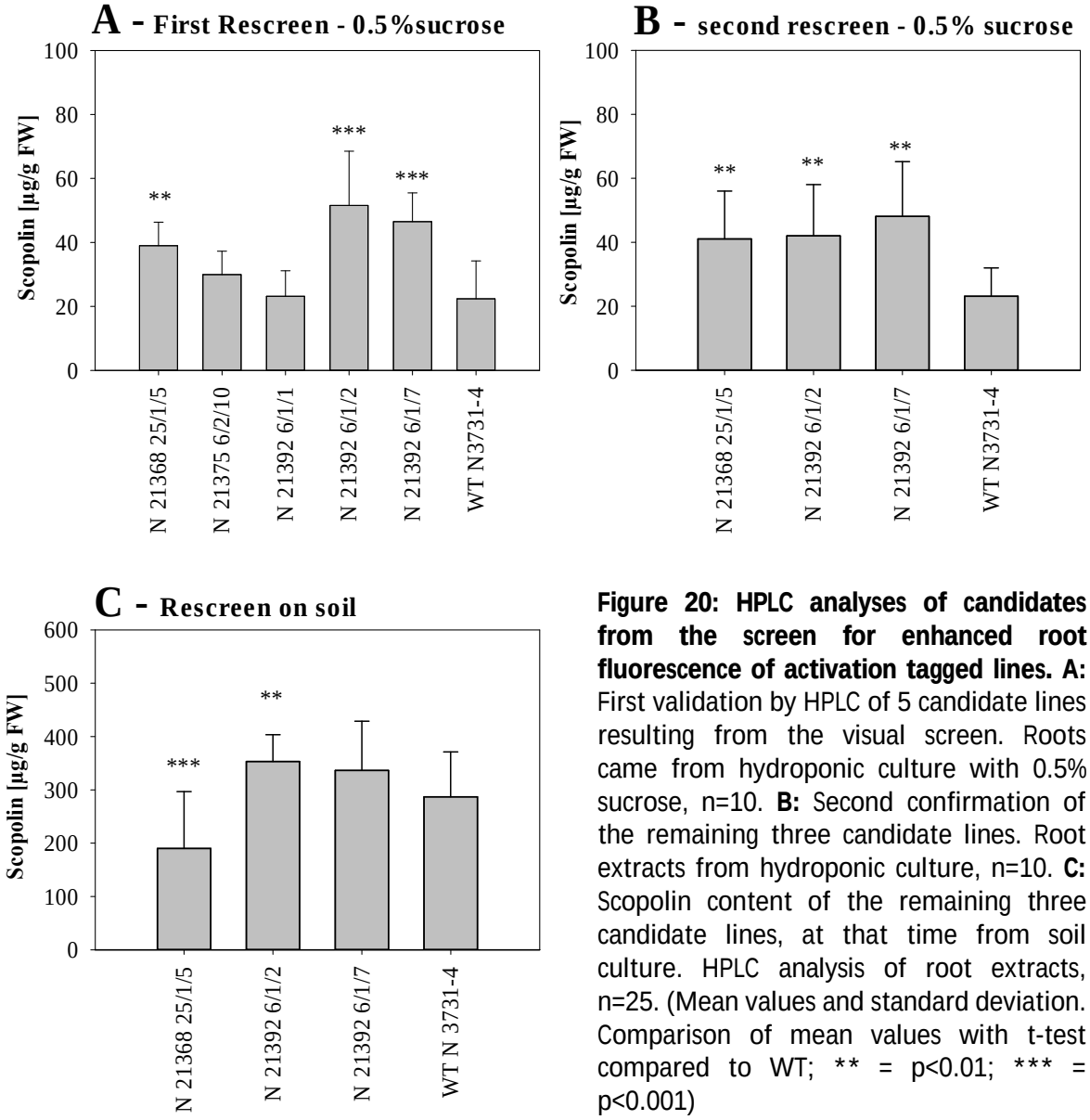


Figure 20: HPLC analyses of candidates from the screen for enhanced root fluorescence of activation tagged lines. A: First validation by HPLC of 5 candidate lines resulting from the visual screen. Roots came from hydroponic culture with 0.5% sucrose, n=10. **B:** Second confirmation of the remaining three candidate lines. Root extracts from hydroponic culture, n=10. **C:** Scopolin content of the remaining three candidate lines, at that time from soil culture. HPLC analysis of root extracts, n=25. (Mean values and standard deviation. Comparison of mean values with t-test compared to WT; ** = $p<0.01$; *** = $p<0.001$)

3. Determination of the T-DNA insertion site of the activation tag in line N21392 6/1

PCRs on the T-DNA sequence revealed that only 50-60% of the offspring lines N21392 6/1/2 and N21392 6/1/7 contained the T-DNA insertion. Plasmid rescue resulted in two plasmid-containing *E.coli* colonies from an EcoRI digest of line N21392 6/1/2-DNA. Sequencing was successful for the right border primer RB. The sequenced fragment was 724 bp long, however, the clipped sequence reached only from base pair 78 to 349 (=272bp) with a high degree of low to medium quality sequencing results. Sequencing and alignment with NCBI

Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) revealed that the insertion site of the T-DNA was most certainly within one of *Arabidopsis*' numerous actin genes. Best high scores and probability were reached for ACT8 and ACT7 (Table 3). Because of the similarity in the genomic sequence of the actin genes and the low quality of sequencing, the correct insertion site was uncertain and had to be confirmed with additional PCRs. For line N21368 25/1, gene identification brought no conclusive results (see appendix p. A20).

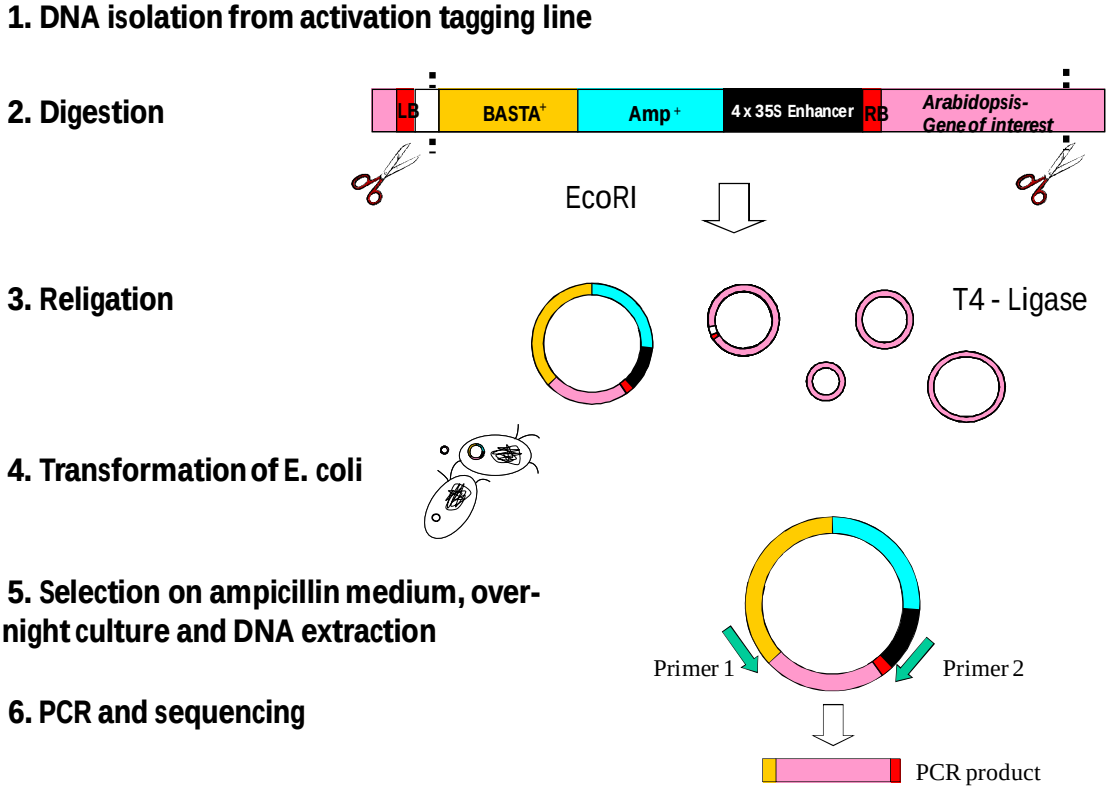


Figure 21: Principle of the identification of the T-DNA's insertion site: Genomic mutant DNA is digested with an enzyme that cuts once within the T-DNA and anywhere in the genomic DNA. With the religation, a new plasmid is formed which contains part of the T-DNA and a fragment of the genomic DNA that was interrupted by the T-DNA-insertion. With transforming in *E.coli* and antibiotica selection, the newly created plasmid with the desired fragment can be identified and amplified (plasmid rescue, Weigel et al., 2000).

Table 3: BLAST (Altschul et al., 1997) results of the DNA-fragment adjacent to the T-DNA insertion in line N21392 6/1/2

Sequences producing high scoring Segment Pairs	Highscore	Probability P
At1g49240 68414.t05050 actin 8 (ACT8)	*843*	*3.2e-39*
At5g09810 68418.t01028 actin 7 (ACT7)	*816*	*9.8e-39*
At3g18780 68416.t02127 actin 2 (ACT2)	*780*	*9.9e-38*
At3g12110 68416.t01337 actin 11 (ACT11)	*744*	*3.6e-36*
At3g46520 68416.t04694 actin 12 (ACT12)	*645*	*9.8e-31*
At5g59370 68418.t06891 actin 4 (ACT4)	*650*	*6.0e-30*
At2g37620 68415.t04258 actin 1 (ACT1)	*726*	*3.0e-27*
At3g53750 68416.t05511 actin 3 (ACT3)	713	*1.2e-26*

The clipped sequence of the 800bp-fragment was aligned to Actin 8. It partially matched the base pairs 1537-1817. Adding the unsequenced part of the fragment, the insertion site was assumed to be on position 1460 of Actin 8 or roughly in the same area, if one of the other actins was interrupted. Specific primers for Actin 7 and 8 were designed, located up- and downstream of the calculated insertion site. A PCR on the rescued plasmid with these specific primers resulted in a PCR product for ACT7. As against that, specific primers for ACT 8 produced no result (Figure 22). To exclude an insertion into another actin gene besides ACT7, a complementary PCR approach was used: A new set of ACT7 primers binding upstream and downstream of the calculated insertion site of the T-DNA was created. The template for the new PCR was the genomic DNA of 12 single plants from line N21392 6/1/2 that had been tested for the T-DNA insertion. In this approach, primers for ACT8 led to a PCR product in all plants tested. Therefore, at least one intact ACT8 was present in all plants. However, with primers for ACT7, for 5 out of 12 plants, there was no PCR product (Figure 23). In these plants, there was no intact ACT7, which meant they were homozygous for a T-DNA insertion in ACT7.

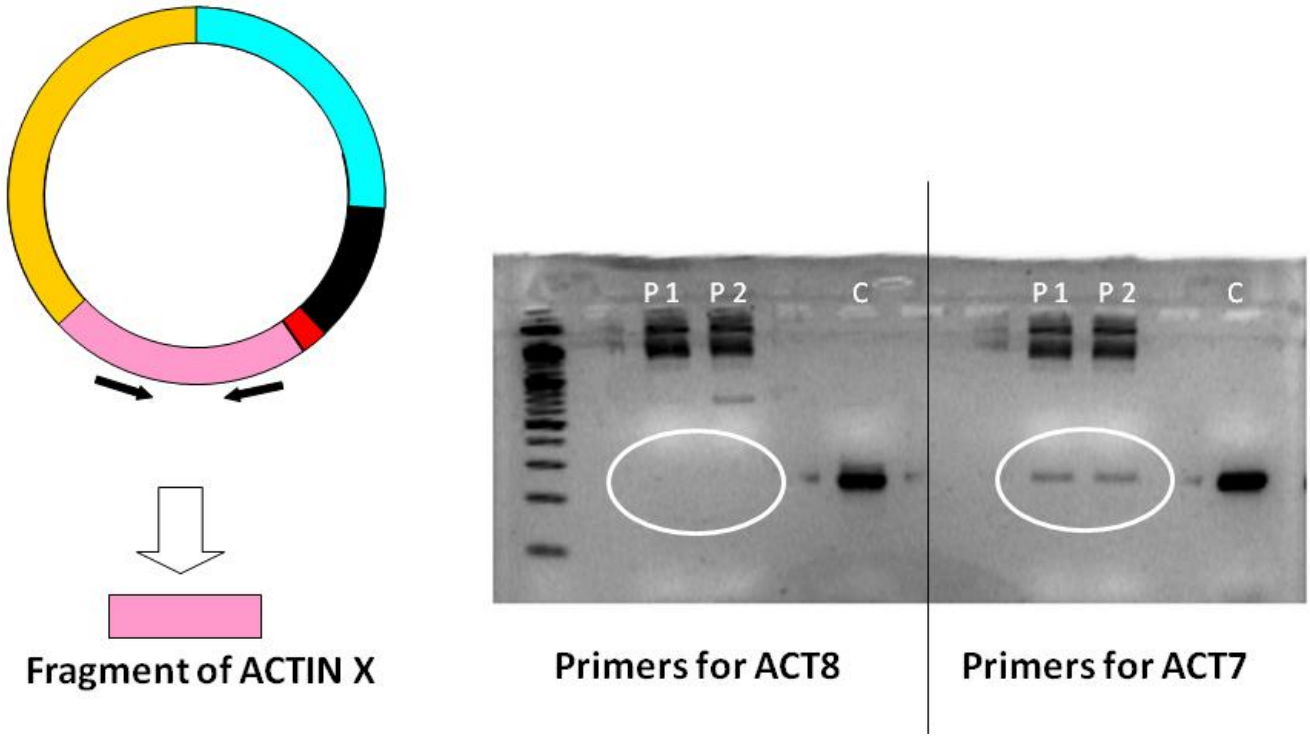


Figure 22: Identification of the insertion site of the T-DNA from N21392 6/1: Distinguishing between ACT8 and ACT7. PCRs with specific primers on the rescued plasmids P1 and P2. These plasmids were formed out of a fragment after a digest of mutant plant DNA from the activation-tagged line. It contained a part of the T-DNA and a fragment of adjacent plant DNA. Only primers for ACT7 bound to the genomic DNA closest to the T-DNA insertion. C (Control) = Col-7 wild type whole plant DNA.

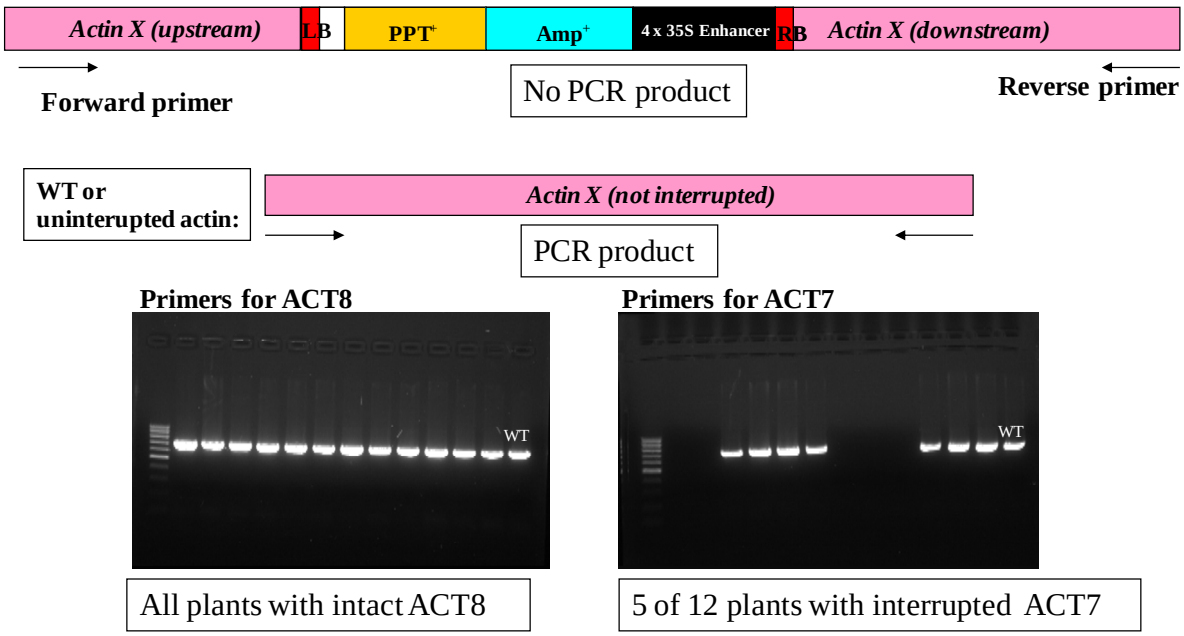


Figure 23: Identification of the insertion site of the T-DNA from line N21392 6/1, complementary approach. Confirmation of insertion into ACT7. Plants from the F2 generation of the original single plant. PCRs for amplification of an intact ACT8 produced fragments in every plant tested. PCRs for an intact ACT7 resulted in no fragments in 5 out of 12 plants.

4. Selection and testing of candidate genes near the insertion site of the T-DNA in line N21392 6/1 – Identification of At5g09860 (AtTHO1) as the gene causing the phenotype

The T-DNA insertion into Actin 7 (At5g09810) had probably destroyed its function and activated the transcription of nearby genes. Based on the annotation of the genes surrounding Actin7, a selection was made from these candidates, which genes were likely to cause a scopolin phenotype. (Appendix Table A7 shows the genes surrounding ACT7, their former and their current annotations.) Genes were selected that were close to the insertion site (approx. ten gens up- and downstream) and had annotations that suggested a possible influence on secondary metabolism (transcription factors, reactions to biotic stress) or that were annotated as "unknown". Excluded were genes with a distinct expression only in certain parts or organelles or at certain developmental stages like flowers/flowering, as well as structural-, cell-wall-organizing or house-keeping genes (like a cellulose synthase or the Actin 7).

The following genes were selected as possible candidates:

1. At5g09750 DNA binding/transcription factor
2. At5g09780 Transcriptional factor B3 family protein
3. At5g09805 Similar to unknown Protein At5g64667.1
4. At5g09830 BolA-like family protein

5. At5g09850 Transcription elongation factor

6. At5g09860 Nuclear matrix protein related

7. At5g09890 Protein kinase, putative

8. At5g09800 U-Box containing protein, involved in protein ubiquitination, response to chitin

In a double approach, over-expression lines of the selected candidate genes were created and insertional mutants acquired, since there was the possibility that one of the methods might fail to create a phenotype: A single "knock-out" might not be sufficient to reduce the scopolin level because of other genes with redundant functions or might only effect scopolin production under certain conditions. Recreating the phenotype of the original Weigel-line N21392 6/1 by over-expressing the gene might also fail, because too high expression levels may cause RNAi effects (Fire et al., 1998) or inhibit the formation of protein complexes („Squelching" (Cahill et al., 1994)).

Generation and analysis of over-expression lines (OE) of the candidate genes

Selection of T1 seeds on kanamycin after *Agrobacterium*-mediated transformation of Col-7 WT plants returned transgenic lines for At5g09750, At5g09805 and At5g09830. For At5g09860 and At5g09890 there were also kanamycin-resistant T1 plants, but the transformation was unstable and the T2 generation had lost its kanamycin resistance. None of the created over-expression lines had a significantly altered scopolin level (Figure 24).

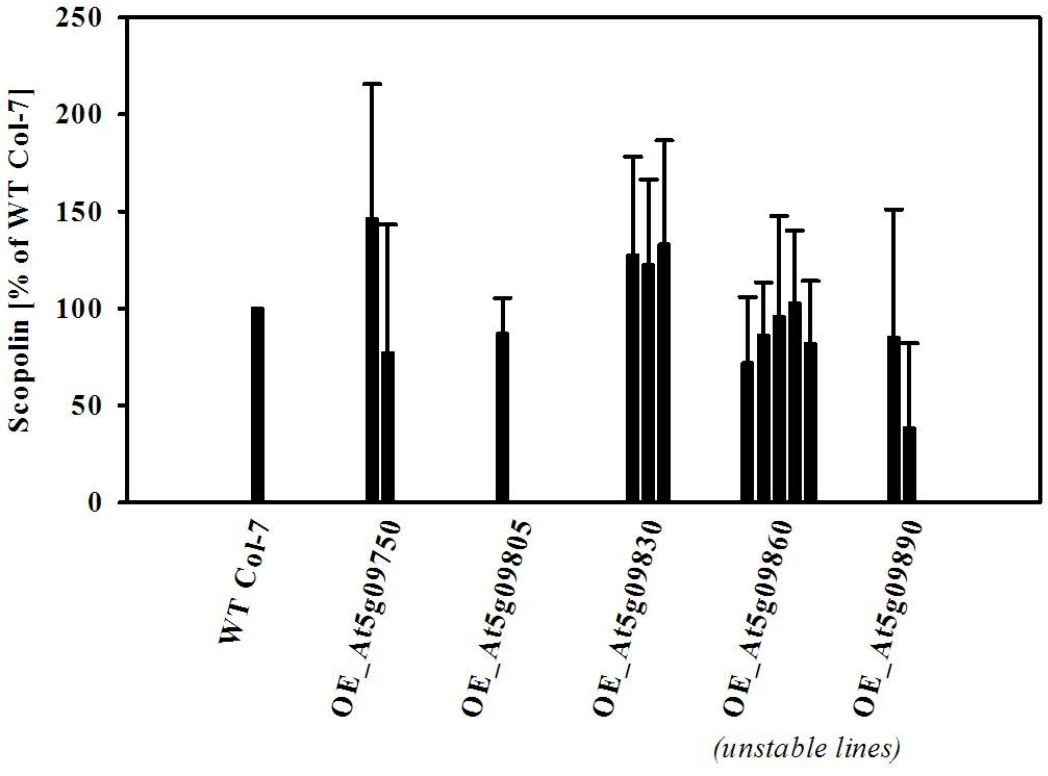
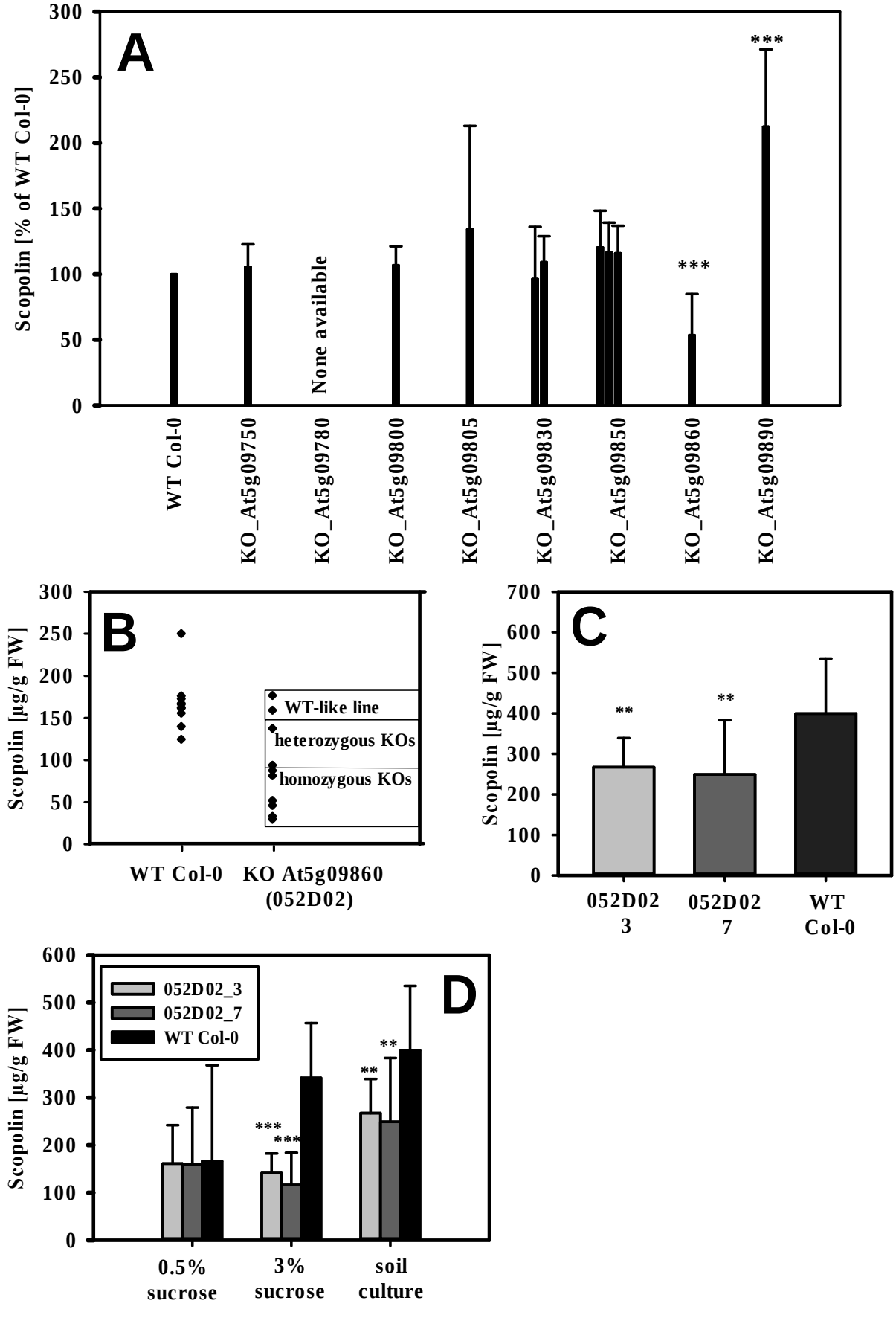


Figure 24 (previous page): Scopolin contents of lines in which candidate genes were overexpressed. No significant difference was found. This is a summary of several experiments. For each locus, one or more independent over-expression lines were cultivated on 0.5% sucrose. Values are given as percentage of the mean value of the wild type samples grown at the same time. Comparison of mean values was done with the numeric data (µg scopolin/g FW) to the according wild type cultivation (t-test or ANOVA).

Analysis of insertional mutants of the candidate genes

The off-spring of homozygous or segregating insertional mutants from the candidate genes At5g09750, At5g09800, At5g09805, At5g09830, At5g09850, At5g09860 and At5g09890 were cultivated on high-sucrose medium. As their scopolin content was compared to WT, it became apparent that many offspring lines of the GABI-KAT insertional mutant GK052D02 for the gene At5g09860 had severely or moderately reduced levels of the coumarin. Those offspring lines were analyzed by PCR for their segregation status, with the result that the lines with the severely reduced scopolin content were homozygous for the T-DNA insertion, those with the moderately reduced level were heterozygous and the lines with WT scopolin levels had no T-DNA insertion. Two homozygous offspring lines, GK052D02_3 and GK052D02_7, were regrown and analyzed several times and showed in all replicates a significantly reduced scopolin content, only around 40-60% of the wild type Col-0 (Figure 25). With that, the most likely candidate to influence scopolin accumulation was identified. The gene At5g09860 was 13 kbases upstream from the insertion site of the activation tag. The insertional mutant of At5g09890, however, had not a lower scopolin content, but a 2-3 times higher scopolin content than wild type in two independent experiments. This gene lies downstream of the insertion site on the anti-sense strain of the DNA. According to TAIR (Rhee et al., 2003), it codes for a protein kinase family protein. A BLAST N search (Altschul et al., 1997) identified with a probability of 0e+00 a catalytic domain of Nuclear Dbf2-Related kinase-like Protein Serine/Threonine Kinases. NDR kinases regulate mitosis, cell growth, embryonic development, neurological processes and they are also required for proper centrosome duplication (Hergovich et al., 2006; Marchler-Bauer et al., 2011). Therefore, this gene was very unlikely to have an influence on scopolin accumulation. However, it is known that an insertion mutation into a gene can alter the expression of neighbouring genes (Pekarik and Izpisua Belmonte, 2008), for example via 35S promoters which are often contained in the transfer DNA, as it is also the case in the SALK-vector pROK2. This also gives a second proof for the presence of a scopolin-influencing gene in the vicinity. An insertional mutant for the last candidate gene, At5g09780, is still not available.

Figure 25 (Next page): Scopolin content of insertional mutant lines from the candidate genes surrounding Actin7. HPLC analyses of root extracts. A: Scopolin content of all insertional mutants on 3% sucrose. From At5g09830 and At5g09850, several independent insertional mutants were available; n=10. **B** Scatterplot of the KO (insertion mutation) of At5g09860 (GK052D02) and wild type. The zygoty status of each sample is marked as determined by PCR. Lines determined as homozygous had the lowest scopolin contents. **C** Validation of scopolin contents of GK052D02 compared to WT on 3% sucrose. N=8. **D** Homozygous insertional mutants of At5g09860 compared to WT under different conditions. n=10; 10; 20. (Bar charts: Mean values and standard deviation, comparison of mean values with t-test compared to WT, *** = p<0.001; ** - p = <0.01)


A
Scopolin [% of WT Col-0]
300
250
200
150
100
50
0
None available
***
***
WT Col-0
KO_At5g09750
KO_At5g09780
KO_At5g09800
KO_At5g09805
KO_At5g09830
KO_At5g09850
KO_At5g09860
KO_At5g09890
B
Scopolin [µg/g FW]
300
250
200
150
100
50
0
WT-like line
heterozygous KOs
homozygous KOs
WT Col-0
KO At5g09860
(052D02)
C
Scopolin [µg/g FW]
700
600
500
400
300
200
100
0
**
**
052D02
3
052D02
7
WT
Col-0
D
Scopolin [µg/g FW]
600
500
400
300
200
100
0
052D02_3
052D02_7
WT Col-0
***
***
**
**
0.5%
sucrose
3%
sucrose
soil
culture

Analysis of the genetic background of GK052D02

According to GABI-KAT[3], line GK052D02 might have two other T-DNA insertions besides the insertion into At5g09860 (confirmation status listed as "unknown"): one unconfirmed insertion was in gene At1g07260 (UDP-glucosyl transferase 71C3), the other into At5g14340 (myb domain protein 40). The original stock of GK052D02 still contained insertional mutant plants of UGT71C3, as PCRs with start- and end primers for the gene showed. The offspring lines GK052D02-3 and GK052D02-7, which were used for all the experiments described in this work, contained only the intact gene: PCRs with start- and end primers for the gene gave a PCR signal for every single plant. The PCR product was sequenced and was 100% identical to the TAIR sequence (Rhee et al., 2003). In addition, PCRs gave no signal with one gene specific primer and the left- or right border primer of the T-DNA. Moreover, a SALK-insertional mutant line of At1g07260 had no reduced scopolin content compared to WT (Figure 26 A, C, D). So, it was valid to completely discount this part of the genetic background. Offspring lines GK052D02-3 and GK052D02-7 had in addition to the insertion into AtTHO1 also a homozygous insertion into MYB40 (as proven by PCR, Figure 26 B). The original line GK052D02 still contained a small proportion of plants with at least one intact copy of MYB40. An independent insertional mutant line of MYB40 was acquired. A first phenotyping of a few single plants of before bulking and determination of the segregation status was completed, showed a very slight reduction of scopolin in roots in *myb40*, but not as low as in GK052D02 upon sucrose feeding. The later cultivation of two homozygous *myb40* lines resulted in a slight reduction of the scopolin content for one line and a wild type-like phenotype for the other (see figure 33 and appendix figure A2). The additional insertion might have contributed to the low scopolin content, but was not the primary cause for it. Another line with an insertion only in At5g09860 was not available[4]. A segregation of both insertions via backcrossing was not an option because of the close proximity of the genes.

5. Verification of the influence of At5g09860 (AtTHO1) by the construction of independent over-expression lines and the complementation of a deletion mutation line

Construction of over-expression plants for At5g09860

The failed attempt to create a stable over-expression line for At5g09860 was repeated to have a second independent proof of the role of At5g09860 in the scopolin regulation. The cDNA clone G14437 used for the first attempt was found to have a non-silent point-mutation. Hence, the CDS region of At5g09860 was amplified by RT-PCR. Several reverse transcriptases and oligonucleotides were tested. An RT-PCR setup with the gene specific reverse primer and a high-yield reverse transcriptase finally brought the desired fragment. It was incorporated into Gateway vector pENTR-D-TOPO, sequenced and proved to be a

[3] http://www.gabi-kat.de/db/showseq.php?line=052D02&gene=At5g09860; Bielefeld University , Chair of Genome Research, Bielefeld, Germany

[4] Recently, the lines FLAG204H05 and FLAG191B10 with an insertion in At5g09860 became available.

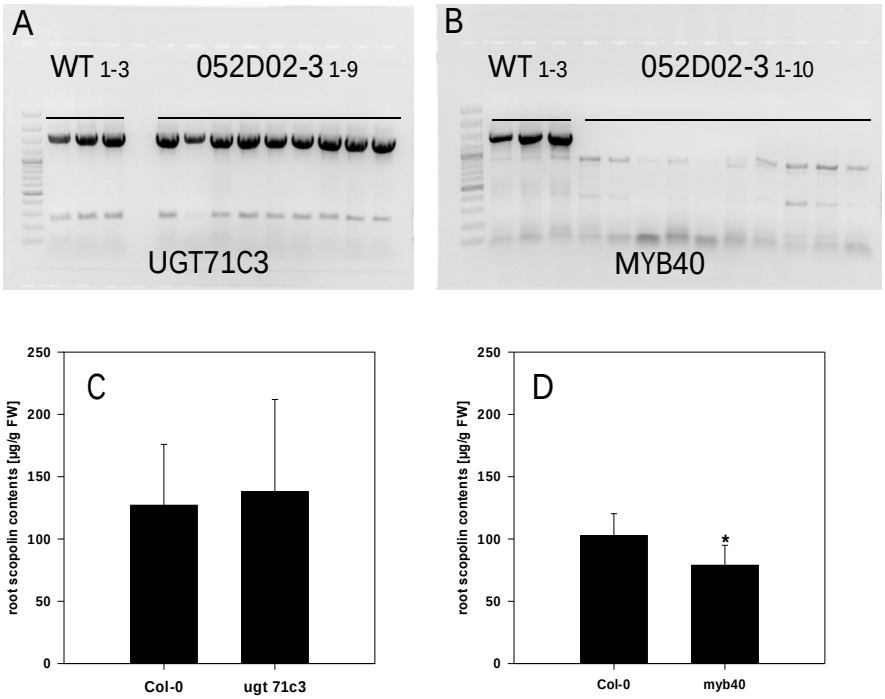
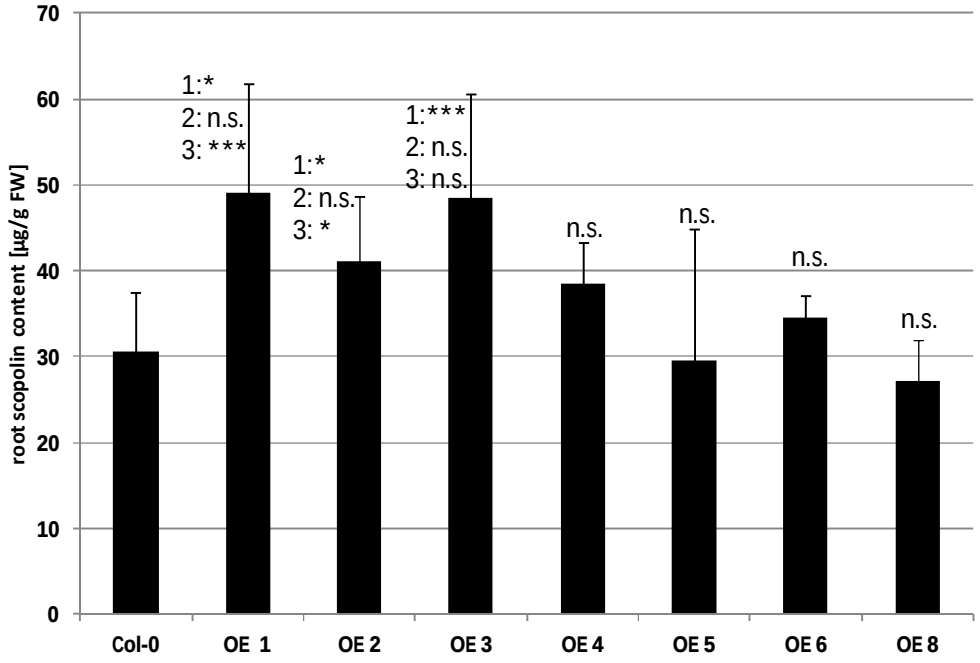


Figure 26: Determination of additional T-DNA insertions into the scopolin-reduced mutant line GK052D02 and phenotyping of possible background insertion mutations. A+ B PCRs on single plants of offspring line GK052D02-3 and WT for intact genes. **A:** There is an intact copy of At1g07260 (UGT71C3) present in all plants of GK052D02-3. B. GK052D02-3 is a homozygous insertional mutant for At5g14340 (MYB40). C: The root scopolin content of an insertional mutant of UGT71C3 does not have a scopolin phenotype other than WT (HPLC analysis of root extracts from hydroponic culture with 3% sucrose, t-test, n=10, p=0.701, mean and STD). D: Scopolin content of an insertional mutant line of *myb40* (pre-test): The scopolin content is slightly reduced (t-test, n=9, *-p<0.05).

faultless clone. *Arabidopsis* plants were transformed using the binary vector pB2GW7 and *Agrobacterium*-mediated plant transformation. 8 plants bearing the glufosinate resistance could be selected. Their T2 generation is segregating 3:1 for the BASTA resistance or fully resistant. From these lines, overexpression lines 1 and 2 accumulated significantly more scopolin than WT in two out of three independent harvests, line 3 in only one replicate. (However, the second harvest, in which no line had higher scopolin content than WT, had also unusually high scopolin contents in WT, Figure 27) Their scopolin contents resembled or surpassed the phenotype of line N21392 6/1 from the original activation tagging screen.

Figure 27 (next page): Root scopolin content of overexpression lines of At5g09860. Graphical summary of three independent harvests. Overexpression lines 1 and 2 accumulated significantly more scopolin than WT in two out of three replicates, line 3 in one replicate. Col-0 plants were transformed with the CDS-region of AtTHO1 in the overexpression vector pB2GW7. Mean value and standard deviation of the average scopolin contents from three harvests. Statistics were calculated individually for each harvest = inscriptions next to each column: 1: 1st harvest. 2: 2nd harvest. 3: 3rd harvest, n=10-15); * p<0.05; ** p<0.01: *** p<0.001, t-test compared to WT.



Complementation of the insertion mutation line GK052D02 of with the genomic and promoter sequence of At5g09860

To prove that the T-DNA insertion in At5g09860 caused the significant reduction of scopolin compared to wild type in GABI-KAT line GK052D02, that line was complemented with the intact sequence of At5g09860 and promoter region. For this, a 6113bp long fragment, consisting of the genomic region and 1810bp upstream as possible promoter sequence, had to be amplified accurately from genomic DNA, integrated into a cloning vector, a destination vector, and finally into the insertional mutant line. Because of the large fragment length, several adjustments to the manufacturers' standard protocols had to be made (see material and methods): The amplification from genomic DNA succeeded using a high fidelity polymerase and the closing of the plasmid ring was aided by a considerable elongation of the incubation time. Integrated into the entry vector and sequenced, a faultless clone could be identified. With A*grobacterium*-mediated plant transformation, twenty glufosinate-resistant T1 plants could be selected. Seeds from 17 lines were grown in hydroponic culture with 3% sucrose and compared to WT and GK052D02. Scopolin levels of the lines ranged from the value of the insertional mutant to the value of the wild type, and in one case, line 15, surpassing wild type level considerably. However, the scopolin content of the lines deviated considerably in different harvests. Yet, line 15 reached and surpassed WT level in all tests. Complemented line 7 was significantly wild type-like in 2 harvests and a bit lower than WT in the two other experiments. Protein extracts of the complemented lines were tested with the produced antibody against AtTHO1. A weak signal was gained for lines 1, 2, 4, 7, 9, 15, 16 and 18.

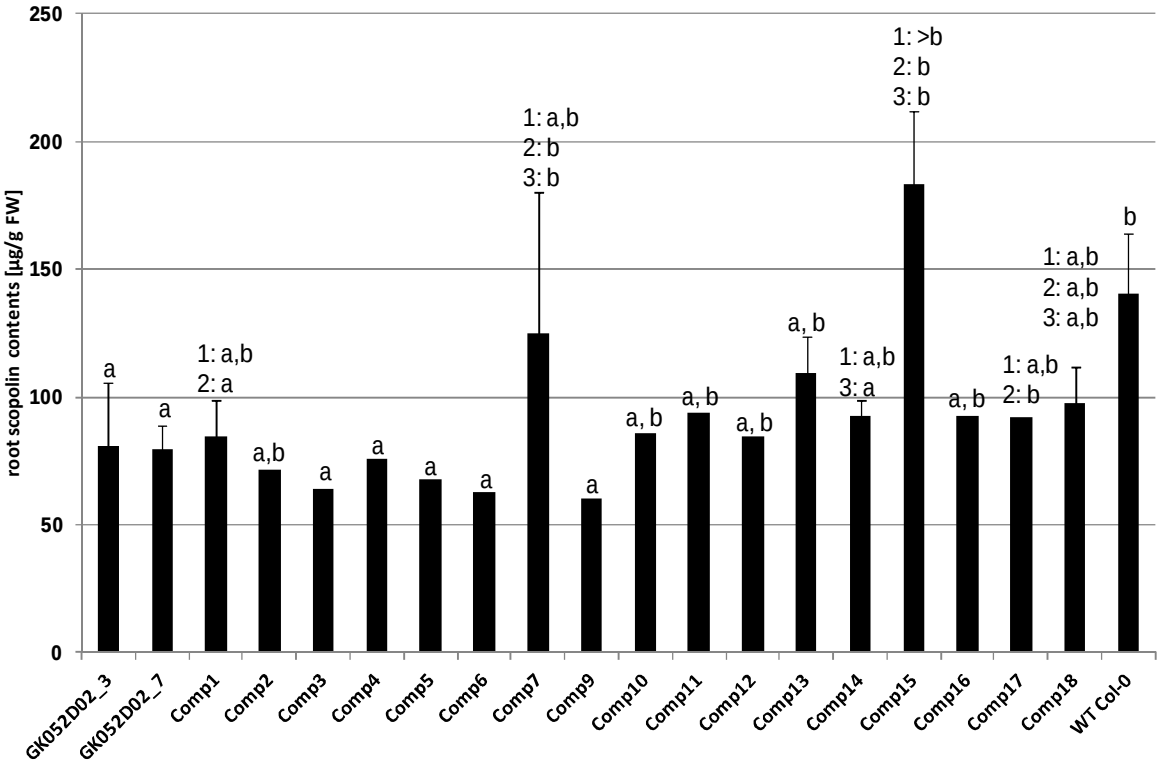


Figure 28: Scopolin contents of 17 independent complemented lines (Comp1-Comp18) of GABI-Kat line GKO52D02 with the promoter and genomic region of At5g09860 (AtTHO1). Graphical summary of three independent harvests. Mean value and standard deviation of the average scopolin contents from three harvests. Statistics were calculated individually for each harvest = inscriptions next to each column: 1: 1st harvest. 2: 2nd harvest. 3: 3rd harvest. Scopolin content of root extracts from hydroponic culture. Complementation lines with an "a" are not significantly different from the insertional mutant GK052D02; lines with a "b" are not significantly different from the WT line Col-0. (t-test or u-test, $p<0.05$). Lines Comp2, 3, 4, 5, 6, 9, 16 were only cultivated once, lines Comp1, 13, 14, 17 twice.

6. *In-silico* analyses of AtTHO1

Current annotations and publications referring to At5g09860 – The gene was annotated as AtTHO1, a member of the AtTHO/TREX complex involved in mRNA processing and export

In 2010, the previously as "nuclear matrix protein related" annotated gene was identified as a homologue to the yeast gene THO1 (HPR1) at the same time by Furumizu et al. (2010) and by Yelina et al. (2010). Yelina et al. referred to it as AtTHO1, which was assumed for this work. Furumizu et al. called AtTHO1 EMU for "*erecta mRNA under-expressed".* In the newest literature it is mostly referred to as HPR1. In yeast, this gene is part of THO/TREX complex, which processes and exports mRNAs (Chavez et al., 2000; Sträßer et al., 2002). In accordance with the yeast nomenclature, THO stands for "suppressors of the transcriptional defects of *hpr1*Δ by overexpression (Piruat and Aguilera, 1998) and TREX for "transcription" and "(mRNA)export"(Sträßer et al., 2002). In the array data collection of Genevestigator (Hruz et al., 2008) AtTHO1 has high expression levels in embryo and endosperm and a medium expression in all other tissues except for sperm cell culture. According to Genevestigator

(Hruz et al., 2008), AtTHO1 is more than two-fold downregulated in some and downregulated by trend in most experiments with the pathogen *Pseudomonas syringae* and other pathogens and the pathogen-attack simulating elicitor flagellin22. ABA causes a more than two-fold downregulation in one experiment and a downwards trend in the nearly all ABA treated samples. In all cold stress experiments included in Genevestigator, AtTHO1 was upregulated, in some experiments more than two-fold.

Promoter analysis of AtTHO1

The putative promoter region contains a number of binding sites which are related to hormonal signalling pathways. A number of motifs are related to floral and leaf development and cell growth, two of these, AtMYC2 and Bellringer are related to lateral growth. A number of MYB factor binding sites are related to dehydration. Three of its MYB sites are connected with phenylpropanoid metabolism. Other sites contain motifs known from light- or circadian-clock-responsive gens. The promoter also contains a recognition site for ICE1 (MYCCONSENSUSAT), the "Core of low temperature responsive element" (LTRECOREATCOR15) and the low temperature-responsive RAV1 (Chang et al., 2008).

Expression of AtTHO1 in roots

According to Birnbaum et al. (2003), AtTHO1 had a comparatively low expression in roots with a hybridisation signal from 20 to 90. (Results were gained on 10-day-old plants under high-sucrose by protoplast sorting. Maximum signal was 14600). In that setup, AtTHO1 had its highest expression at the root tip and the lowest in the differentiated root. The tissue with the highest expression is the stele, closely followed by endodermis and epidermis (figure 29).

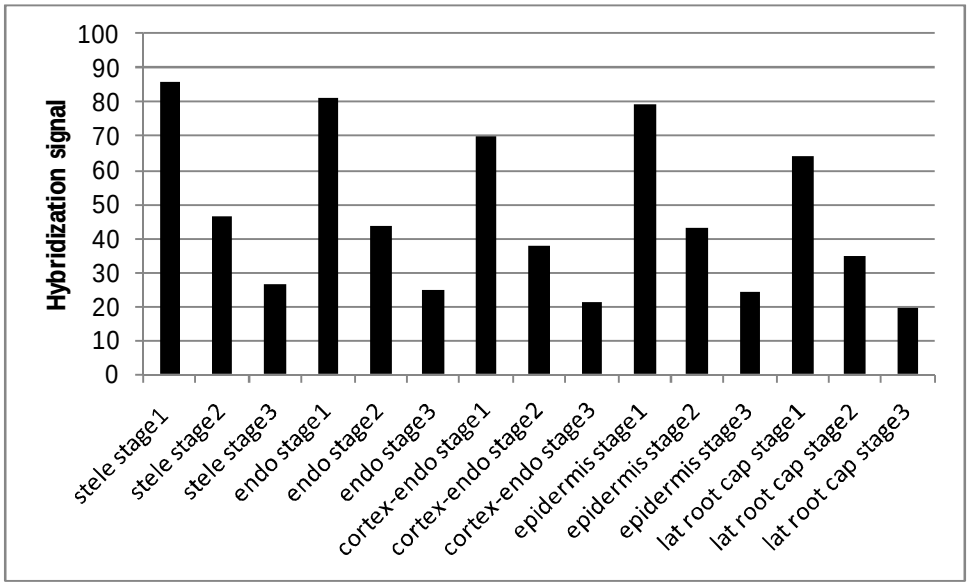


Figure 29: Relative gene expression of AtTHO1 (At5g09860) in different root tissues according to Birnbaum et al. (2003). Stage 1: Root tip. Stage 2: Differentiation zone Stage 3: Root hair zone.

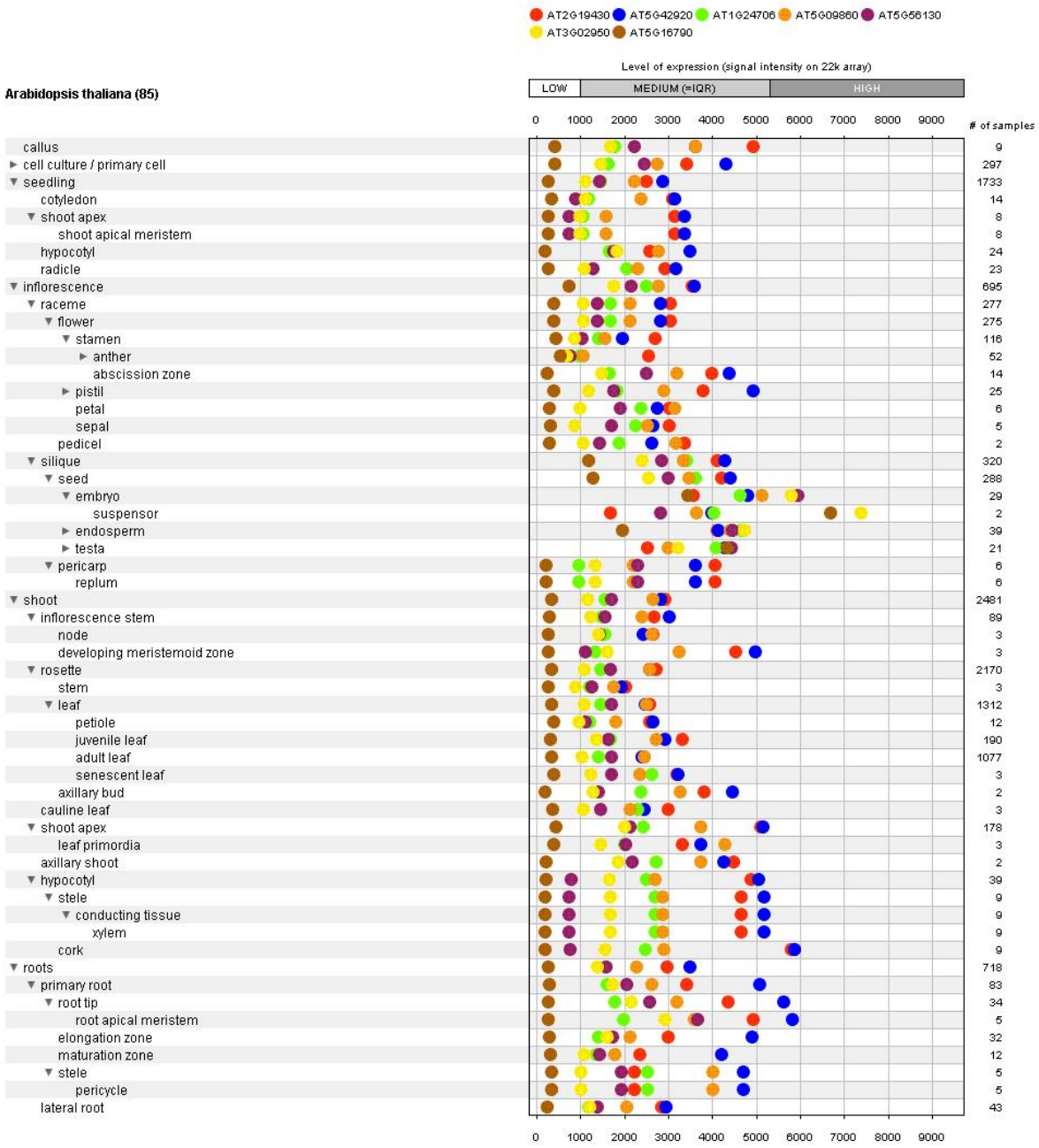


Figure 30 Expression pattern of the putative components of the predicted THO/TREX complex in *Arabidopsis thaliana.* The complex as a whole is only equally expressed in the developing seed (Genevestigator (Hruz et al., 2008) as of 2012/07/16. At5g09860 = AtTHO1 (orange); At1g24706= AtTHO2 (green), At5g56130= AtTHO3 (lilac); At5g42920= AtTHO5a (blue), At2g19430= AtTHO6 (red), At5g16790= AtTHO7a (brown), At3g02950 = AtTHO7b (yellow).

7. *In-silico* analyses of genes associated with AtTHO1 and phenotyping of deletion mutations in the complex

Expression levels of other members of the putative THO/TREX complex

Eight loci of *Arabidopsis* were identified as putative members of the predicted *Arabidopsis* THO/TREX complex (Yelina et al., 2010). Using Genevestigator data, expression levels of the complex members in different tissues were compared. (For THO5b (At1g45233), no

Genevestigator data was available.) In the developing seed, the predicted complex members are present in nearly equal amounts. In all other tissues, the mRNA detected differs considerably between the components: THO5a usually has the highest expression, followed by AtTHO6. AtTHO1, AtTHO2 and AtTHO3 have a medium, more or less matching expression (Figure 30). Looking at expression levels of the complex members under different conditions, there is in most cases no strong regulation or divergence between the components. For example, under continuous light the complex as a whole is down-regulated, and under cold stress there is a tendency to up-regulate all genes of the complex. During senescence, all complex members, except for 7A and 7B, are upregulated.

Other members of the THO/TREX complex also have altered scopolin levels

In three independent harvests, the scopolin content of insertional mutants for each complex member was compared to WT. Insertional mutants for AtTHO1, 5b, 6, 7a and 7b were homozygous. Line *attho3* (1) and *attho5a* were homozygous with few plants with a still intact gene copy. Lines *attho2* and *attho3* (2) were still segregating. None of the lines had the same low scopolin content as GK052D02. However, *attho2* had a significantly lower content than WT in two out of three harvests, line *attho3* (1) also in two out of three, as well as *attho5a* and *7b*. Lines *attho5b*, *6* and *7a* were significantly lower in one out of two experiments (Figure 31).

8. Gene expression analysis of GABI-KAT line GK052D02 -3 containing the insertional mutant of AtTHO1 compared to WT

To estimate, if or which transcript levels might be influenced by the probably RNA-processing and RNA-signal-producing AtTHO1 during elicitation of scopolin accumulation, GABI-KAT line GK052D02_3 containing a homozygous insertional mutant of AtTHO1 was analysed on an Affymetrix Gene expression chip. WT and GK052D02 were grown on low-sucrose medium, transferred to high-sucrose medium and their roots harvested after 24 hours. The analysis of the array data sets revealed 762 significantly differently expressed genes comparing WT and insertional mutant at a significance level of $p<0.05$. Rejected were all genes with changes smaller than two-fold to identify robust changes which left only 121 genes. Those were filtered again to eliminate probe sets with too weak signals. These generally accepted methods and parameters for array analysis. (Wodicka et al., 1997) revealed 3 induced and 3 repressed genes, as shown in Table 4 and Table 5.

Transcripts that were detected as increased in the presence of an intact AtTHO1 after the treatment were a RING/U-Box superfamily protein, the unknown protein of At3g05770 and the phytosulfokine precursor AtPSK2.

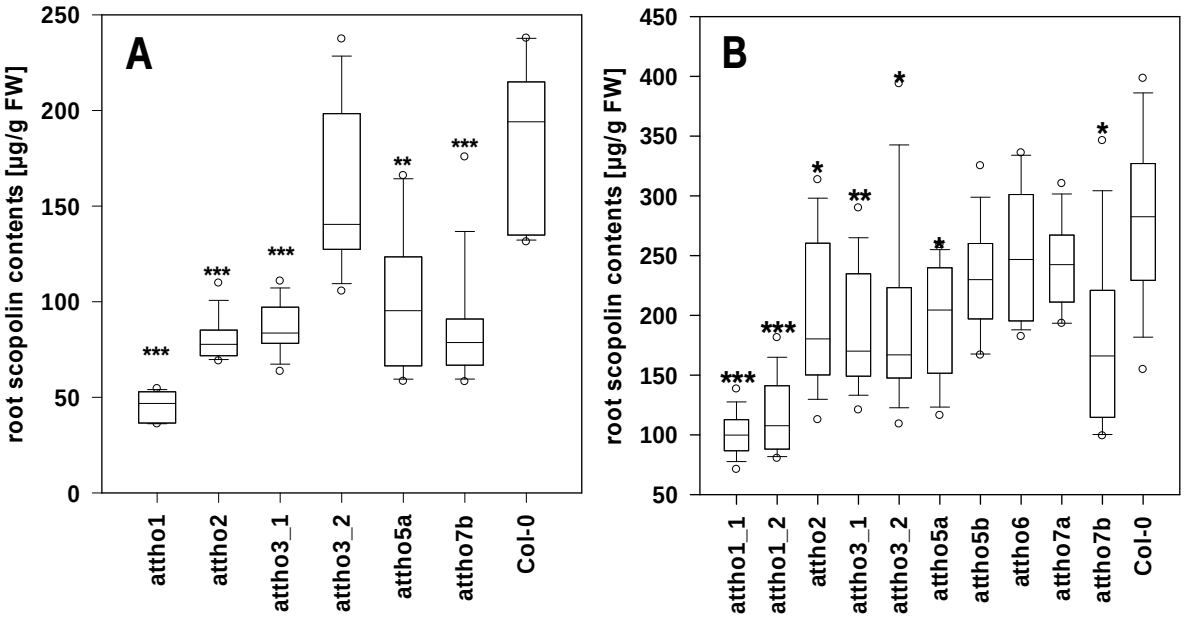


Figure 31: Root scopolin contents of insertional mutants of the members of the putative THO/TREX complex in *Arabidopsis,* harvest two and three (hydroponic culture; 3% sucrose). Insertional mutant lines for each member of the THO/TREX complex tend to have a lower scopolin content than WT. However, in some cases results failed to be significant or were not consistent. **A:** Second harvest of segregating lines *attho2*, *attho3* (1) + (2), *attho5a* and homozygous lines *attho1* and *7b*, n = 10: All lines tested were significantly lower than WT except for *attho3* (2). **B**: Third harvest: Lines *attho2*, *3*, and *5* were still segregating. All lines have a clear tendency to have lower scopolin content than WT (significant for *attho1*, *2*, *3*, *5a*, *7b*). Normally distributed data was compared to WT by t-test, not normally distributed data by Mann-Whitney-U-test. * p<0.05; ** p<0.01; *** p<0.001. Box Plots: Horizontal line, middle = median; upper and lower horizontal line = 25th and 75th percentile, "whiskers" = 5% and 95% percentile, circles = outliers.

Table 4 Genes higher expressed in wild type compared to GK052D02 (*attho1*/*myb40*). This table shows 3 upregulated genes, sorted by their absolute fold change. A fold change of e.g. 2 indicates that the gene is two-fold upregulated in the Col-0 group. The first column gives the rank of the gene among the regulated genes.

Rank	Affy ID	Gene Code	Gene symbol/Gene name	Short description	p-Value	Log(Fold change)	Fold change
3)	260973_at	At1g53490	[F22G10.16]	RING/U-box superfamily protein	3.676e-02	1.738	3.335
5)	258739_s_at	At3g05770	[F20D21.12]	unknown protein	2.360e-03	1.074	2.106
6)	266799_at	At2g22860	[ATPSK2]/ PHYTOSULFOKINE 2 PRECURSOR	Phytosulfokine 2 precursor, coding for a unique plant peptide growth factor	3.999e-03	1.050	2.070

Table 5 Genes lower expressed in wild type compared to GK052D02 (*attho1*/*myb40*). A fold change of e.g. -2 indicated that the gene is two-fold downregulated in the Col-0 group. The first column gives the rank of the gene among the regulated genes.

Rank	Affy ID	Gene Code	Gene symbol/Gene name	Short description	p-Value	Log(Fold change)	Fold change
1)	250505_at	At5g09870	[CESA5]/ CELLULOSE SYNTHASE 5	Encodes a cellulose synthase isomer, related to CESA6	1.449e-04	-3.080	-8.456
2)	262719_at	At1g43590		transposable element gene; similar to unknown protein	1.816e-02	-2.456	-5.488
4)	250173_at	At5g14340	[AtMYB40]/ MYB DOMAIN PROTEIN 40	Member of the R2R3 factor gene family	9.948e-04	-1.511	-2.851

In-silico analyses of the genes with an altered expression in GK052D02 (*attho1*)

The **RING/U-Box superfamily protein** with a DNA-binding function contains the conserved domains LOH1CR12 from the LOH1CR12 superfamily and F-BAR-Fes-Fer from the BAR superfamily. LOH1CR12 (Loss of heterocygosity 12) is a tumour suppressor protein of unknown exact function which was discovered in human. F-BAR domains are dimerization modules that bind and bend membranes and are found in proteins involved in membrane dynamics and actin reorganization. Fes (feline sarcoma), and Fer (Fes related) are cytoplasmic (or nonreceptor) tyrosine kinases that play roles in haematopoiesis, inflammation and immunity, growth factor signalling, cytoskeletal regulation, cell migration and adhesion, and the regulation of cell-cell interactions (Marchler-Bauer et al., 2011), at least in vertebrates. A function in plants is not known. The protein is present with 99-100% similarity in *Arabidopsis lyrata*, *Populus trichocarpa*, *Vitis vinifera*, *Sorghum bicolor* and *Oryza sativa*. The closest hit with 45% for a protein with a known function in the plant kingdom is the transcription factor bZIP113 from soybean which is a negative regulator of ABA and confers salt and freezing tolerance in *Arabidopsis*. At1g53490 is expressed in medium to high levels in the developing seed and in low to medium levels in all organs. In the array data collected by Genevestigator, it is more than two-fold regulated only by the chemicals 2,4-dichlorophenoxyacetic acid, CMP and fenclorim. For cold and drought stress, its regulation resembled strikingly the one of AtTHO1 (Figure 32).

At3g05770 is still annotated as **unknown**. It codes probably for a 410AA protein. Close similarities are only between unknown proteins of *A. thaliana*, *A. lyrata* and *Brassica napus subsp. pekinensis*. It has medium expression levels in anthers and pollen and low to medium expression in stamen, root cell culture, sperm cell culture, flower and root. It has a more than two-fold upregulation after contact with nematodes, benzyladenine, CMP, fenclorim, norflurazon and OPDA. Downregulation (in part more than two-fold) occurs after IAA

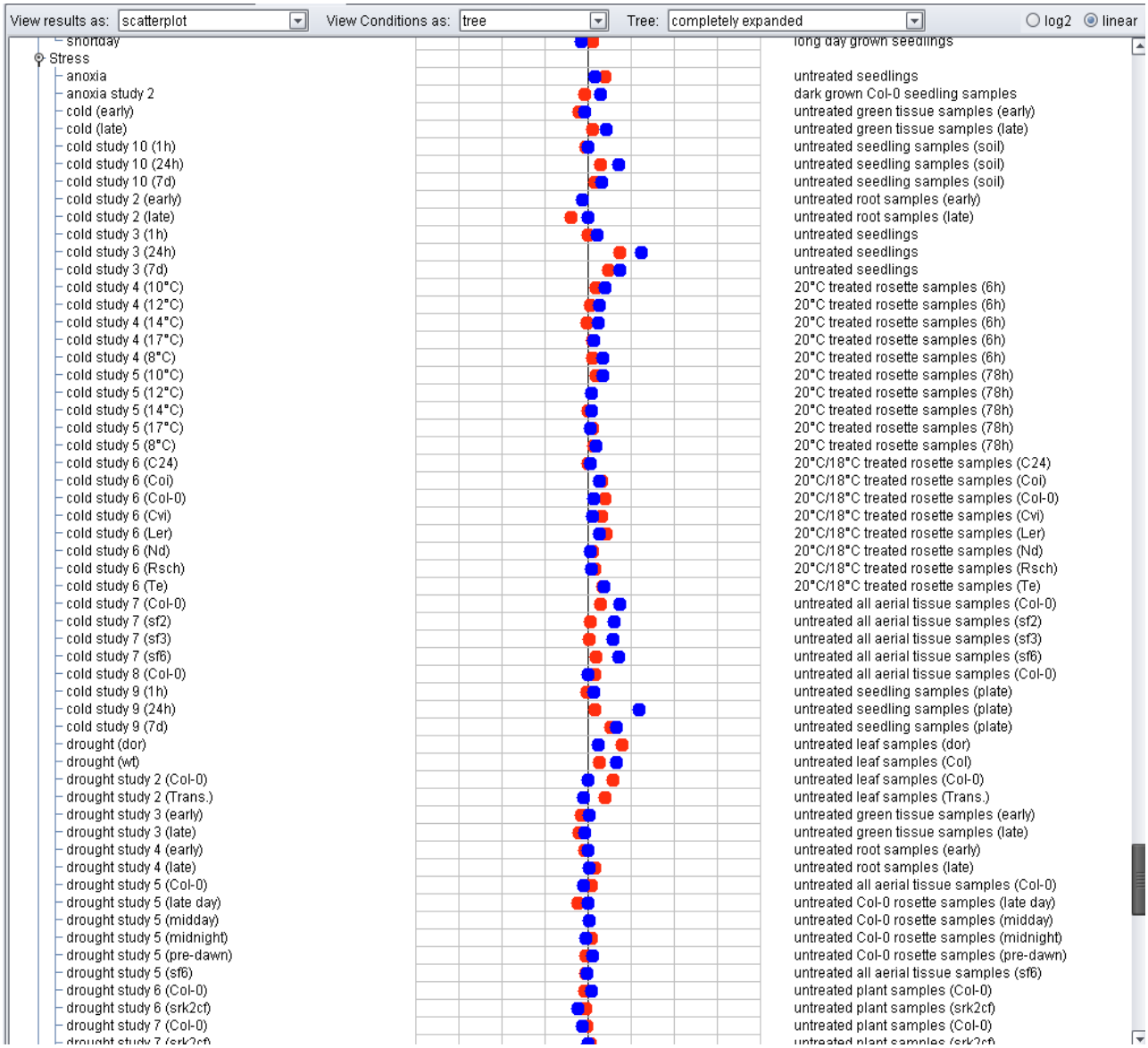


Figure 32: Genevestigator (Hruz et al., 2008) collection of array data for At1g53490 (RING/U-Box protein; red) and AtTHO1 (blue); sorted for experimental conditions; excerpt. The regulation of both genes under cold and draught stress has a striking resemblance to each other. One rectangle comprises a two-fold-change. Left column: condition, right column: control sample.

exposition, light, iron deficiency, nitrate starvation and hypoxia. No putative conserved domains were detected.

AtPSK2 is a phytosulfokine 2 precursor. It is modified into a signal peptide required for proliferation and differentiation of plant cells. Phytosulfokines are active as either a pentapeptide or a C-terminally truncated tetrapeptide. The cDNA of AtPSK2 is 544bp long; the coding region is only 264bp long. In WT, this gene is two-fold upregulated. The promoter of AtPSK2 was shown to be active in the central cylinder of the differentiated parts of the roots. It is particularly active in the sites where new lateral roots were formed, but not at all in the root tip (Kutschmar et al., 2009).

Transcripts that are higher abundant in GK052D02 were Cellulose Synthase 5, a transposable element gene and the Myb Domain Protein 40. **CESA 5** is a **cellulose synthase** isomer sorted in the group of five primary wall-type CesAs in *Arabidopsis*. It is required for the production of the murcilage coat of imbibed seeds (Sullivan et al., 2011). It also supports cell wall thickening in shoot trichomes (Betancur et al., 2010). It has the highest expression in root cortex cell culture, stem nodes, axillary buds and shoots and the seed coat, but expression is medium to high in all tissues except for anthers and pollen. It is probably negatively regulated by GLABRA2, which results in less cellulose in *Arabidopsis* roots (Tominaga-Wada et al., 2009). It has to be emphasised, that this gene lies directly upstream from AtTHO1; that means, its expression was 8 times higher, when the neighbouring gene was knocked out. So, the enhanced transcription might be an artefact of the deletion mutation nearby. As already mentioned above, such artefacts are known and can be caused by 35s CAMV promoters in the T-DNA (Pekarik and Izpisua Belmonte, 2008). (Actually, the T-DNA of vector pAC161 in line GK052D02 contains a 35s CAMV promoter.)

At1g43590 contains the conserved domain Plant-tran from the Plant-tran super family. This family contains **transposases** which are putative members of the PIF/Ping-Pong family. Those transposases are responsible for the amplification of *Tourist*-like MITES (miniature inverted-repeat transposable elements) (Zhang et al., 2004). Transposition is very probably activated by stress and might be controlled by the plant via RNA silencing (Mirouze and Paszkowski, 2011). It has medium expression levels in cell cultures and endosperm. It is upregulated more than two fold in experiments with *Golovinomyces cichoracearum*, ABA, night extension and cold. It is more than two-fold downregulated by *Pseudomonas syringae*, high CO_2, brassinolid/hydroborate, light, glucose, sucrose and drought (Genevestigator, Hruz et al.2008).

MYB 40 is a member of the R2R3-MYB gene family of transcription factors. Members of this family have been shown to regulate phenylpropanoid metabolism in *Arabidopsis thaliana*, but their function is not exclusively limited to secondary metabolism (Stracke et al., 2001). It is very root specific and has its highest expression in lateral roots and the elongation zone. It is downregulated more than two-fold by IAA and hypoxia, and upregulated by nitrate starvation, cell-sorting and protoplasting (Genevestigator, Hruz et al., 2008). Line GK052D02_3 was shown to have a second T-DNA insertion in this gene (See: "Analysis of the genetic background of GK052D02"), which probably caused the enhanced expression in the deletion mutation line.

Analyses of the scopolin contents of insertion mutation lines of genes with an altered expression in GK052D02

A T-DNA insertion in CESA5 or the transposon At1g43590, which were over-expressed in GK052D02 caused no scopolin/scopoletin phenotype different from WT. Insertion mutants of the RING-U-Box protein and the unknown protein, that were under-expressed in the mutant, had a slight decrease in the scopolin content which was significant for all but one lines tested. The phenotype of line GK052D02 was not reached (Figure 33).

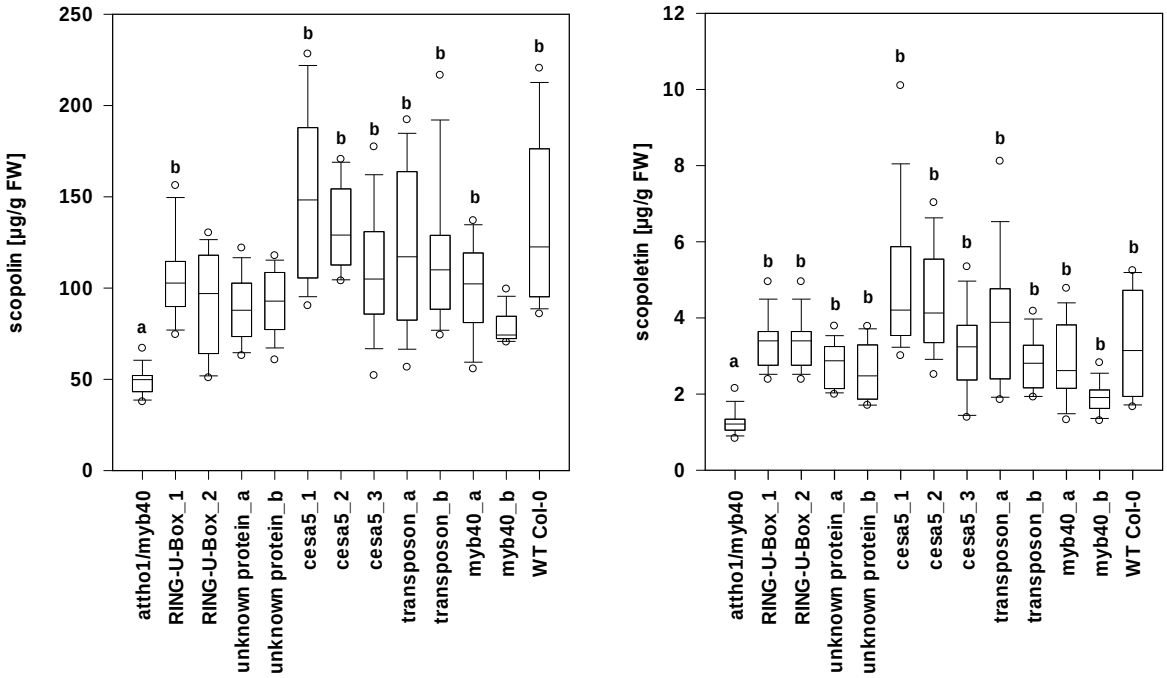


Figure 33: Root scopolin and scopoletin contents of T-DNA insertion lines of differentially expressed genes in GK052D02 (*attho1*/*myb40*). RING-U-Box and the unknown protein were higher expressed in WT, CESA5 and the transposon were higher expressed in GK052D02. A second T-DNA insertion in line GK052D02 was found in MYB40. None of the lines had scopolin contents as low as in GK052D02 (*attho1*/*myb40*). Cesa5 and the transposon deletion have WT-like scopolin/scopoletin content. The insertion into the unknown protein, MYB40 and probably also in RING-U-Box caused an intermediate phenotype. Boxes with the letter "a" are not significantly different from GK052D02, with a "b" not significantly different from WT, boxes with no letter are significantly different from both. (Mann-Whitney-U-test, n=10, $p<0.05$.)

9. Further phenotyping of *attho1*

Insertion mutation line GK052D02-3 has no other obvious changes in phenylpropanoid content expect for one other fluorescing compound

Grown at high-sucrose, scopoletin was strongly reduced and another fluorescing compound at 3.1 min was moderately reduced in the mutant GK052D02-3 (*attho1*/*myb40*) in addition to scopolin (figure 34). The masses to this peak could only be tentatively assigned because of low signal intensity. They were M1 = 369, M2 = 207 at 3.1 min. The difference between these masses (162) corresponds to the mass of a conjugated hexose, so this substance might be a compound conjugated to a sugar residue. A match to a known compound was not possible. Other differences were only minor.Overexpression lines of AtTHO1 had no visible difference in the peak at 3.1 min and in no other peak expect for scopolin and scopoletin. Homozygous *myb40* lines did not have a difference in this peak compared to WT.

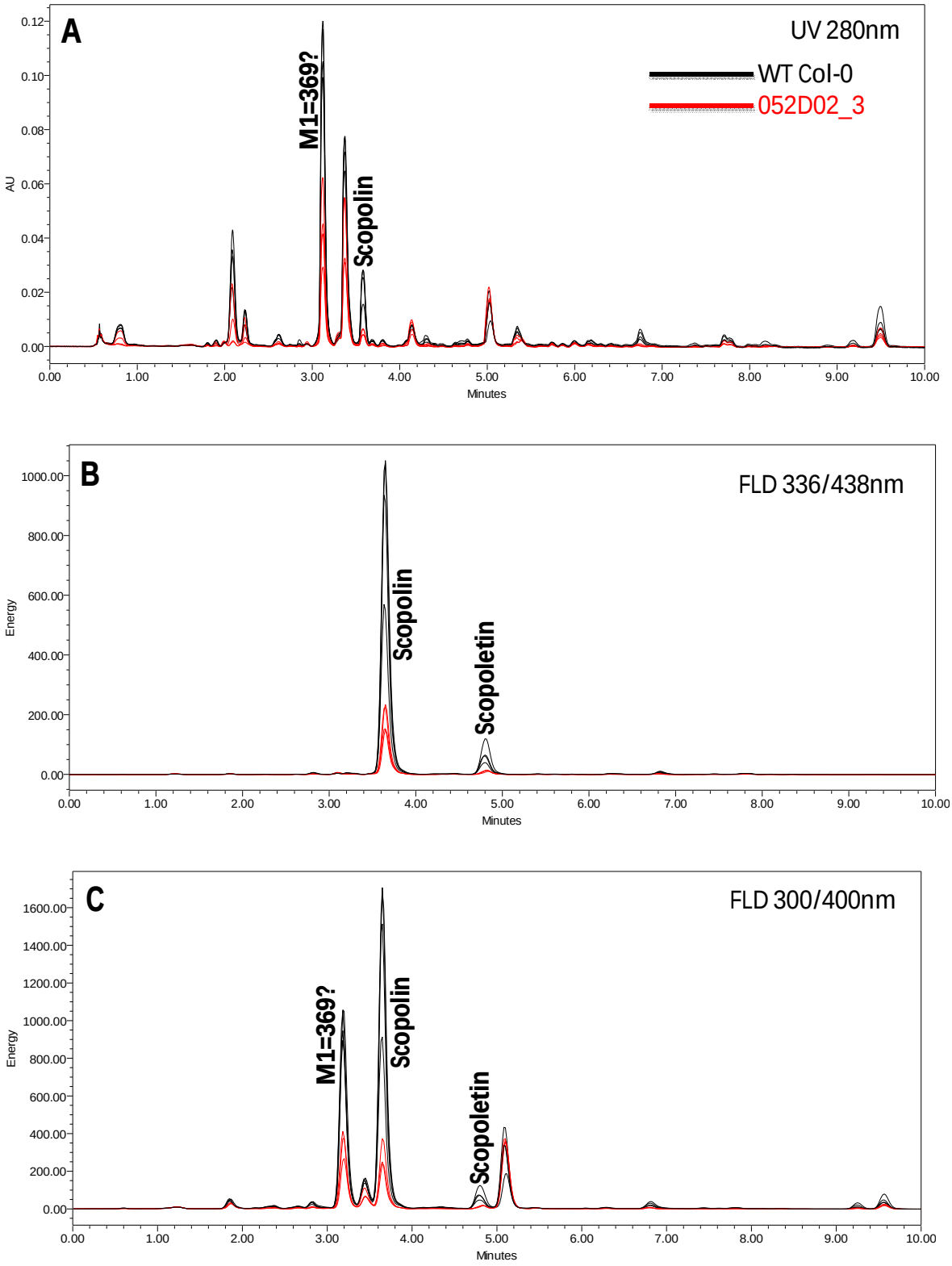


Figure 34: Chromatograms of root extracts from WT and GK052D02-3 (*attho1*). Scopolin and scopoletin are strongly reduced. Another substance with a fluorescence at 300/400nm at 3.1min is also reduced. A mass of M = 369 was tentatively assigned to it. Overlays of four pools of 10 plants for each line. **A:** UV detection at 280 nm. **B:** Fluorescence detection at 336/438 nm (= optimum conditions for scopolin detection). C Fluorescence detection at 300/400 nm (= conditions for detection of most fluorescing phenylpropanoids)

AtTHO1 influences the cold-induced accumulation of scopolin

In regard of the *in-silico* results, cold stress experiments with the insertional mutant of AtTHO1, GK052D02 and the wild type Col-0 were performed. Four-week-old plants were kept at 10°C for one week or remained in 20°C as the control. Comparing the root scopolin content: Wt in 10°C and 20° was significantly different from each other (t-test; p=0.004). GK052D02 in 10°C and 20°C was also significantly different (t-test; p<0.001). WT in 10°C and GK052D02 in 10°C were different with p=0.029 (Mann-Whitney U-test). WT in 20°C and GK052D02 in 20°C were not different from each other (U-test). As for the shoot (=leaf) scopolin content: In WT at 10°C, scopolin was close to the detection limit. In all other leaf samples tested, it was not detected (Figure 35). However, in previous experiments (see Cold stress experiments), scopolin was found to accumulate clearly under cold stress. When analyzing marker peaks for cold stress in chromatograms at 280nm UV detection, no difference in the reaction of the visible secondary compounds could be found.

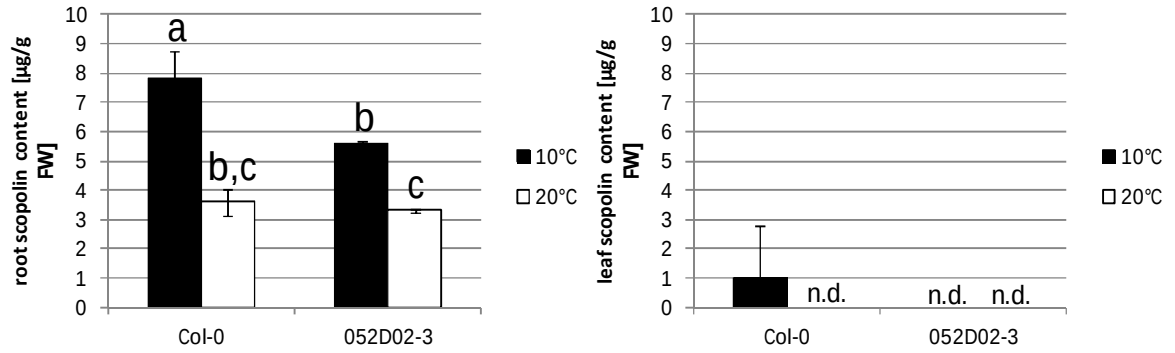


Figure 35: Scopolin contents of shoots and roots after cold treatment (10°C) and control conditions (20°C). Tested lines were the wild type Col-0 and the insertional mutant of AtTHO1, GK052D02_3. (Pair-wise t-tests, level of significance p<0.05, columns with the same letters (a, b, c) do not differ significantly from each other; 10°C: n=4 pools, 20°C: n=2 pools).

A defect in AtTHO1 reduces the reaction towards a carbon source and towards osmotic stress without additional carbon

As the screening had already revealed, GK052D02 had significantly reduced scopolin levels under high-sucrose conditions compared to WT. The reaction of the mutant in varying sucrose concentrations and in a shifting experiment from low-sucrose to high-sucrose or mannitol-medium had been observed during the diploma thesis of D. Berreth (Berreth, 2011). With rising sucrose concentration, the scopolin content in roots rose in WT. The curve of GK052D02 followed the WT curve, but did not reach its level. (Experiments were performed twice with similar results.) In another experiment for distinguishing between osmotic and feeding effects of sucrose, 4-week-old plants pre-cultured on 0.5%-sucrose medium were transferred to fresh media with different concentrations of sucrose or mannitol. Mannitol was applied as an osmolyte which cannot be metabolised by *Arabidopsis*. An addition of 0.5% sucrose to the mannitol media was necessary because growth on sucrose-free medium impeded plant growth considerably. Mannitol addition up to 1.5% mannitol (=2% osmoticum) caused an increase in scopolin production in WT, but not

as much as with sucrose as the only osmoticum. Mannitol in higher concentrations led to a decline in scopolin accumulation. In the mutant GK052D02, the initial increase in scopolin at lower mannitol concentrations was missing (figure 36).

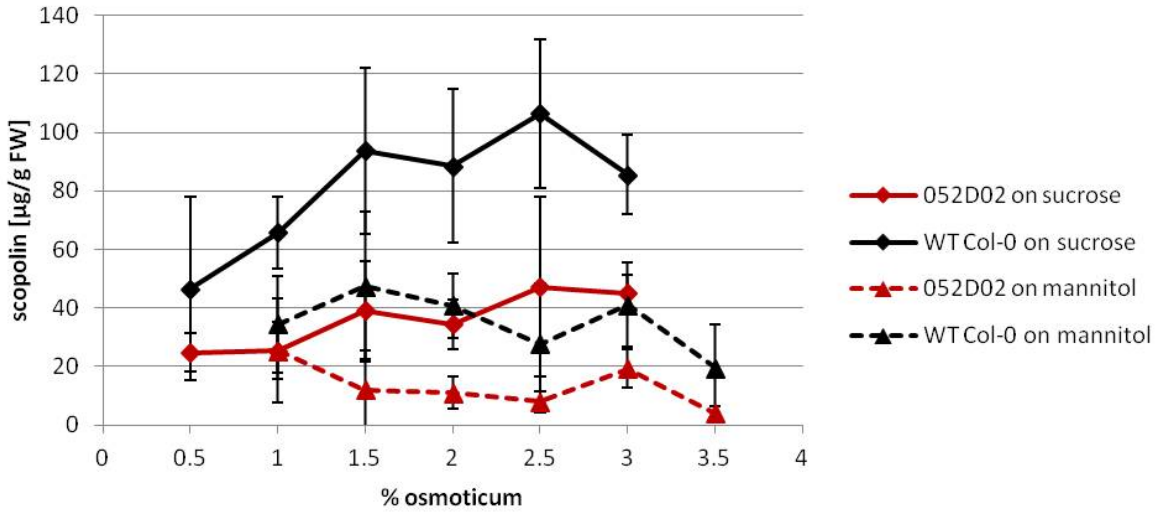


Figure 36 Reaction of scopolin in WT and GK052D02 roots towards different concentrations of osmotica (hydroponic culture) one week after transfer to the respective medium. N=5. Mannitol-media always contained 0.5% sucrose. For the mannitol curves, an x-value of e.g. 1.5% osmoticum stands for 1% mannitol and 0.5% sucrose. (Plant growth, sample preparation and HPLC measurements by D. Berreth, graphs by the author.)

Morphological characteristics of GK052D02 under standard and stress conditions

(The morphological characterisation and the behaviour of line GK052D02 under different stress conditions compared to wild type was part of the Diploma thesis of Dorothee-Carina Berreth (Berreth, 2011) supervised by the author and Dr. Hans-Peter Mock. Raw data and photographs were made available to the author to complete unfinished measurements. So, the following paragraphs contain data which was in parts already published in Berreth 2011, but is now presented in the complete version. The contributions of D. Berreth and the author to each result are explicitly stated in each paragraph.)

The *in-silico* analysis of the functions of AtTHO1 suggested an influence of this gene on developmental patterns via its effect on ERECTA, whose most prominent function is amongst others the influence on tissue development (Furumizu et al., 2010). For that reason, it had to be investigated if the observed decline in scopolin content in *attho1* was caused by deformations or loss of tissue identity. A thorough morphological characterisation of the mutant line should reveal abnormalities and, if not, a more direct influence of AtTHO1 on scopolin accumulation was to be assumed.

Cell layers and scopolin accumulation in different tissues; root organization and root tip formation in WT and GK052D02;

In *Arabidopsis*, all root tissues, (except the vascular bundle) consist of a single cell layer. In the differentiated root, all the cell layers present in WT were also present in GK052D02. Rhizodermis, cortex endodermis and pericycle were clearly visible and showed no abnormal formations. The anatomy of root tips also did not differ in an obvious way (figure 37).

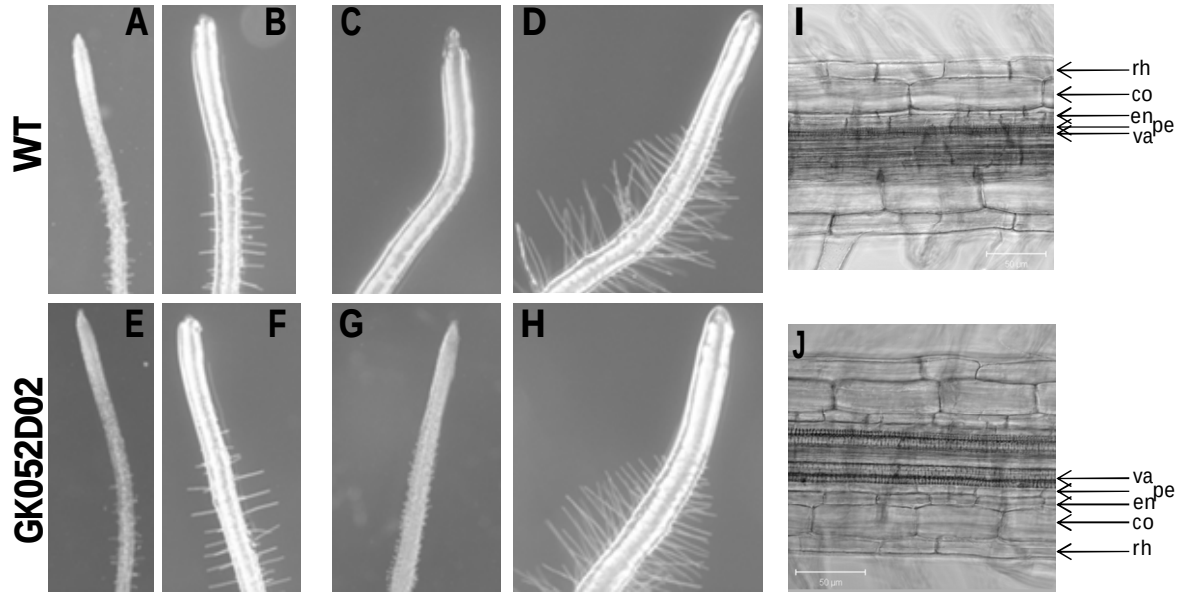


Figure 37: Comparative root morphology of WT and GK052D02. Root tips and cell layers. Root tips of WT and GK052D02 do not differ from another in an obvious way. The proportions are the same and no deformations occur. In the mutant, all cell layers are present, and tissues are not formed differently. A-H: root tips of *A. thaliana* (20x). **A+B** Two typical forms of root tips from WT on 0.5% sucrose. **C+D** Two typical forms of root tips from WT on 3% sucrose. **E+F** Two typical forms of root tips from GK052D02 on 0.5% sucrose. **G+H** Two typical forms of root tips from GK052D02 on 3% sucrose. **I+J** Confocal laser scanning images of differentiated roots of WT (I) and GK052D02 (J) at the root hair zone (va = vascular tissue, pe=pericycle, en=endodermis, co=cortex, rh=rhizodermis). (Binocular images by D. Berreth, CLSM -images by Dr. Twan Rutten, D. Berreth and Stefanie Döll.)

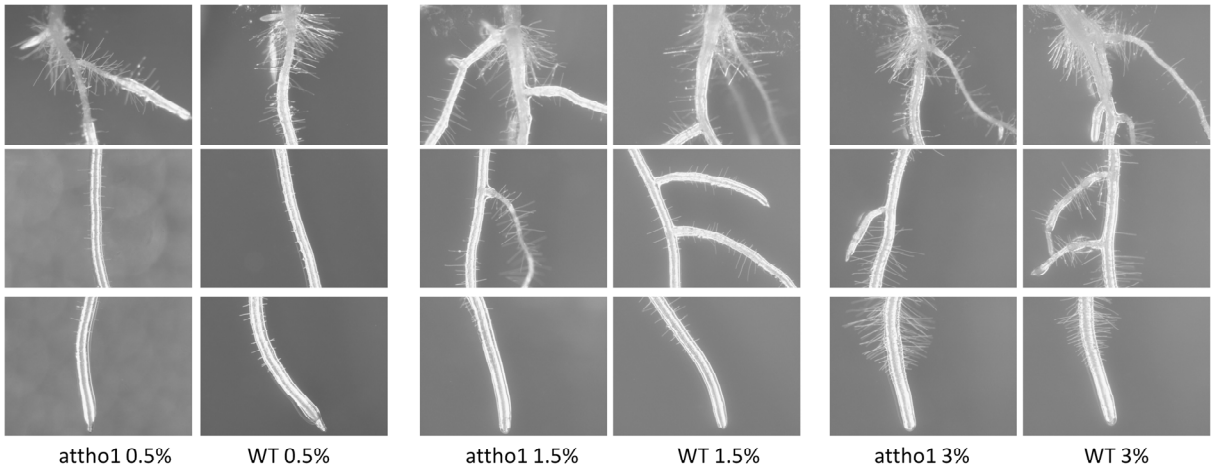


Figure 38: Comparative root morphology of WT and GK052D02. There are no deformations or obvious anomalies in the roots of GK052D02. Images of root base, middle section and root tips grown on solid media with varying sucrose concentrations (=percent values). (Binocular images by D. Berreth).

No obvious anomalies in macroscopic root images were found (figure 38). Root bases, root middle sections and root tips of GK052D02 were not deformed and not abnormally proportioned (Berreth, 2011). In particular, root tips of GK052D02 were not lengthened or

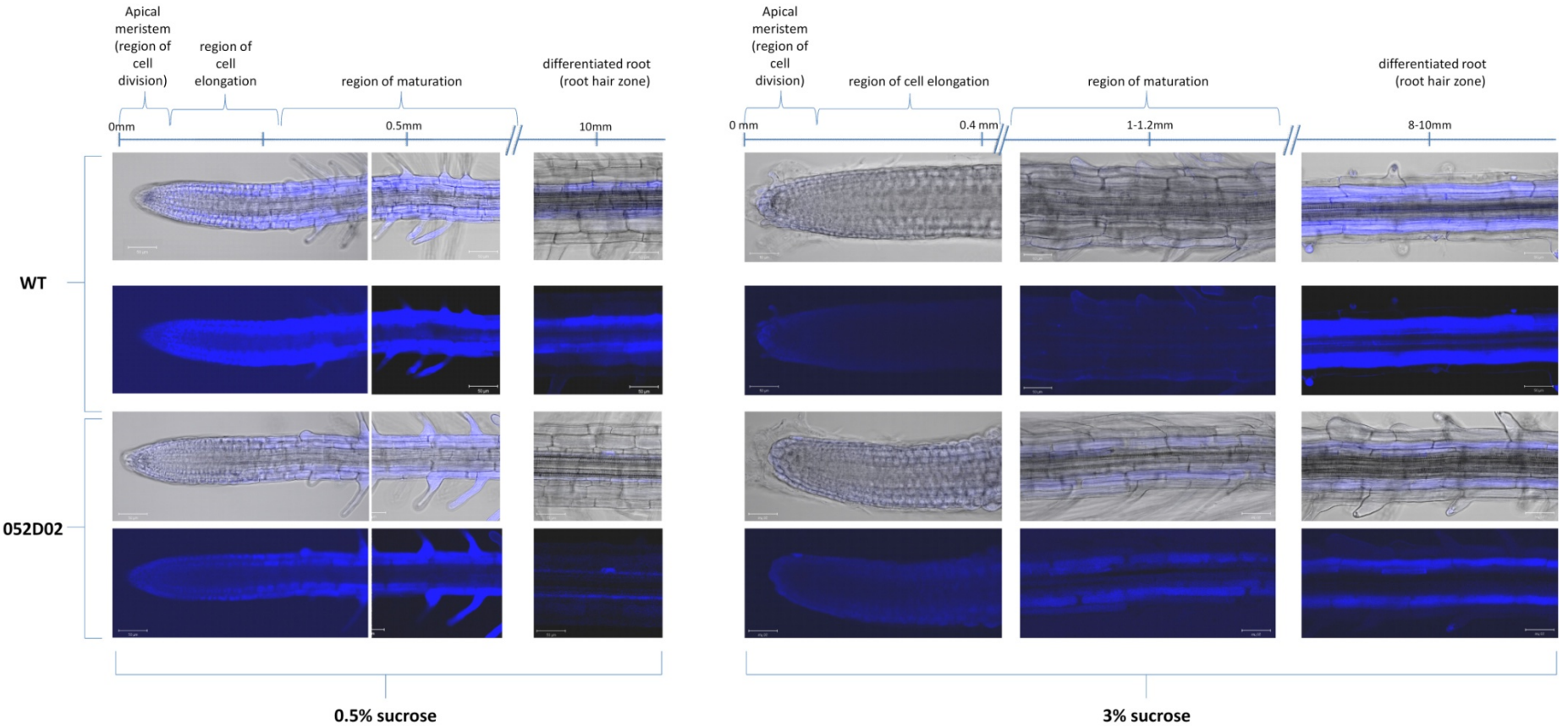


Figure 39: Root tips of *Arabidopsis thaliana* wild type Col-0 and the insertion mutation line GK052D02 from low-sucrose and high-sucrose medium. Scopolin accumulates in exactly the same tissues in GK052D02 as in WT under high- and low-sucrose conditions. The scopolin accumulation in the single cell is reduced. Overlays of bright-field images and fluorescence images at exc. 364nm/em. 385-450nm. Below each overlay is the original fluorescence image. CLSM -images by Dr. Twan Rutten, D. Berreth and Stefanie Döll.)

Table 6: Root morphology of GK052D02. Comparisons of biometrical data from WT and GK052D02: n.s. = not significant * p<0.05, **p<0.01, ***p<0.001, t-tests compared to the corresponding WT; - measurements not possible/not done.

Experiment	**1. Exp.** (n=10)	**2. Exp.** (old plants, n=48-87)	**3. Exp.** (n=115-149)	**4. Exp.** (n=77-108)
	Root length (longer)			
0.5% sucrose	n.s.	n.s.	n.s.	n.s.
1.5% sucrose	n.s.	***	n.s.	n.s.
3% sucrose	*	n.s.	n.s.	***
	Root hairs per mm (more)			
0.5% sucrose	n.s.	-	-	-
1.5% sucrose	n.s.	-	-	-
3% sucrose	**	-	-	-
	Lateral roots (less)			
0.5% sucrose	n.s.	-	**	***
1.5% sucrose	n.s.	-	n.s.	***
3% sucrose	*	-	***	n.s.
	Lateral roots per cm (less)			
0.5% sucrose	n.s.	-	***	***
1.5% sucrose	n.s.	-	n.s.	***
3% sucrose	n.s.	-	***	***

shortened or deformed and had no obviously different phenotype, both under standard (0.5% sucrose) and stress conditions (3%sucrose). Fluorescence images of root tips revealed that the accumulation of scopolin in WT and GK052D02 took place in exactly the same tissues (Figure 39): In the differentiated root from low-sucrose medium, scopolin accumulated in that experiment exclusively in the endodermis in both lines. The fluorescence of each single endodermis cell was just weaker in GK052D02. At 3% sucrose, scopolin fluorescence was visible in endodermis and cortex cells in both lines. Again, the fluorescence of the single cells in GK052D02 was weaker. The accumulation of scopolin in the undifferentiated root seems to be equal, maybe a bit more pronounced in WT at low-sucrose. Cell layers, root tip formation and the tissues that accumulated scopolin were identical in WT and GK052D02, both under stress and standard conditions. Only the amount of scopolin, which a single cell accumulated, differed slightly under standard conditions and considerably under high-sucrose conditions.

Root length, lateral root formation and root hairs in WT and GK052D02 under control and stress conditions

The primary root length and the number of lateral roots were determined in four independent experiments on agar plates with varying sucrose content. WT plants had the longest primary roots at 1.5% sucrose. The main root was shortened at 0.5% and 3%. The

number of lateral roots was equal in 1.5 and 3% sucrose and reduced in 0.5%. Line GK052D02-3 had a tendency to not shorten its roots under high sucrose. It also had a very strong tendency to produce less lateral roots, especially at 3% sucrose, which was highly significant for most comparisons (Table 6). During the first experiment, root hairs were counted on the middle section of the primary root. There was a significant difference between WT and GK052D02, but only at 3% sucrose. There, GK052D02 had more root hairs per mm. However, this seemed to be caused mainly by the reduced branching. A graphical summary for the first experiment is given in Figure 40. (The second experiment was done with plants in a later developmental stage where the counting of lateral roots became impossible.)

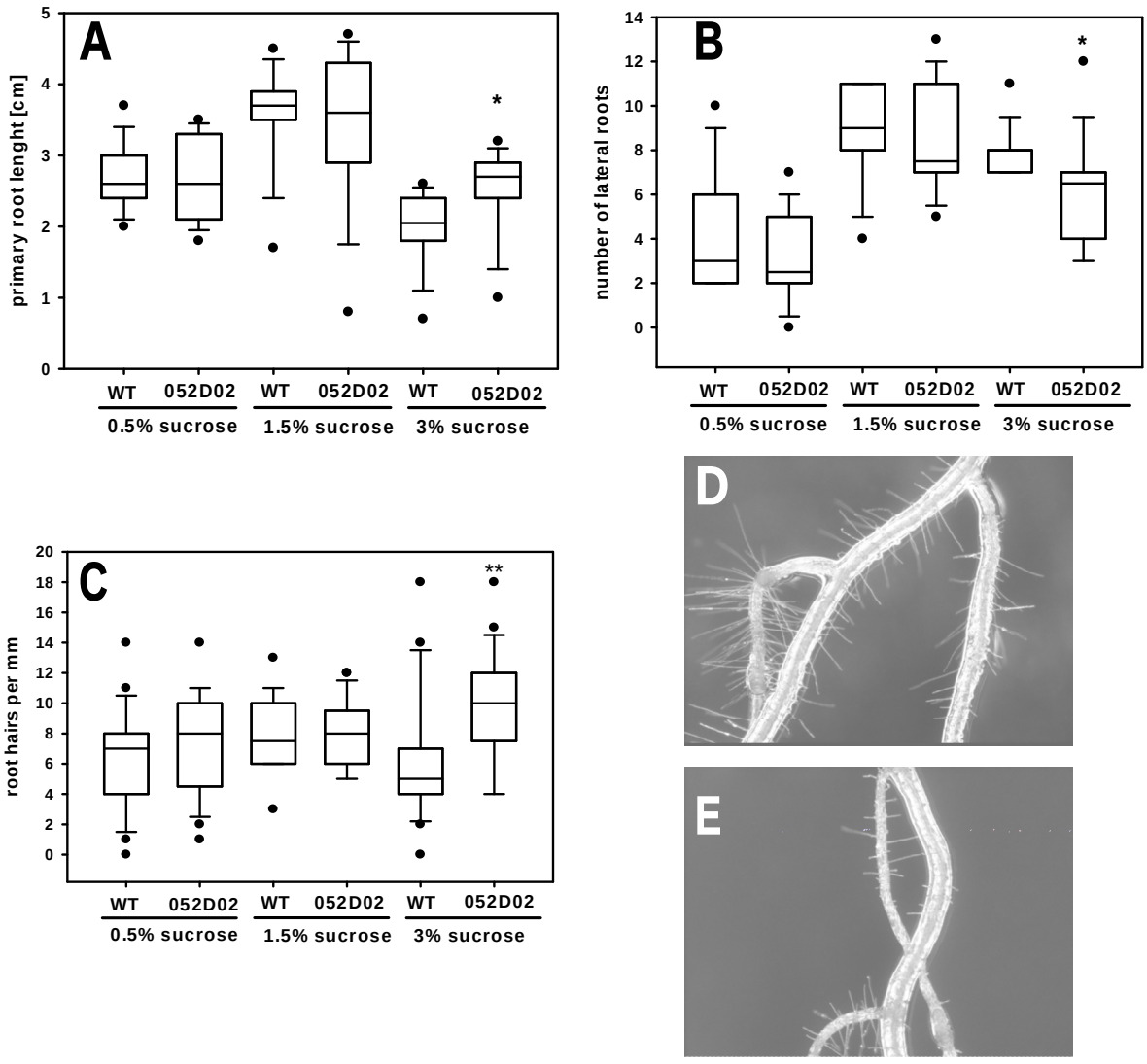


Figure 40 (previous page): Biometric data: GK052D02 compared to WT Col-0 from media with varying sucrose concentration. The mutant GK052D02 reacts different only at 3% sucrose. There, the primary roots are not as much shortened as the WT's, the number of lateral roots is lower and it has more root hairs at the middle section of the main root. A: Primary root length, n=10. **B:** Number of lateral roots per plant; n=10 **C:** Root hairs per mm on middle section of the main (primary) root; n=20 **D** Middle section with root hairs of a GK052D02 root. **E:** Middle section with root hairs of a WT Col-0 root **A-C:** Box Plots: Horizontal line in the middle = median; upper and lower horizontal line = 25th and 75th percentile, "whiskers" = 5th and 95th percentile, circles = outliers, comparison of GK052D02 values towards the wild type values of each group for significant differences (Mann-Whitney-U-test: * = p<0.05; **=p<0.01 (Binocular images by D. Berreth, measurements and statistical analysis by the author).

In summary, mutant line GABI-Kat GK052D02 has two distinct and consistently confirmed phenotypes:

1. A reduced scopolin accumulation per cell under stress conditions (high-carbon, osmotic, cold stress).
2. Less lateral roots than WT. Differences are more pronounced under less-than-optimum growth conditions (high-sucrose).

Cold stress induces the accumulation of scopolin in leaves

Arabidopsis thaliana reacts to cold stress with the production of phenylpropanoids and other secondary compounds in leaves (e.g. Dixon and Paiva (1995)). Under standard conditions, scopolin can only be found in traces in leaves of *Arabidopsis thaliana*. During a pilot experiment it became apparent that scopolin was strongly increased after cold stress. But not only scopolin accumulated, but a number of about 20 well-discernible peaks were increased, both in fluorescence and UV detection (figure 41).

Substance identification

The identity of the scopolin peak could be confirmed by MS data. The mass of scopolin was found at the retention time of a scopolin standard (mass 355 [M+H], figure 42). The three UV main peaks were identified by LC-MS as kaempferol-derivatives, following the earlier peak identification of *Arabidopsis* phenylpropanoids under cold stress of Korn et al. (2008), completed with additional information by Maruyama et al. (2009): Peak 1 was kaempferitrin (kaempferol 3-rhamnoside 7-rhamnoside, mass 579 (M+H) , peak 2 kaempferol+rhamnose+glucose (kaempferol-3-glucoside-7-rhamnoside, mass 595 (M+H)) and the third peak kaempferol+ glucose+ rhamnose +rhamnose (kaempferol 3-[6'-glucosyl) rhamnoside] 7-rhamnoside, mass 741 (M+H)). One fragment mass of all three peaks was 287, which is the mass of kaempferol [M+H]. Peak 1 also contained sinapoylmalate as a shoulder peak; however, the calculated area for this mass peak only comprised 1/10 of the area of kaempferitrin (figure 43).

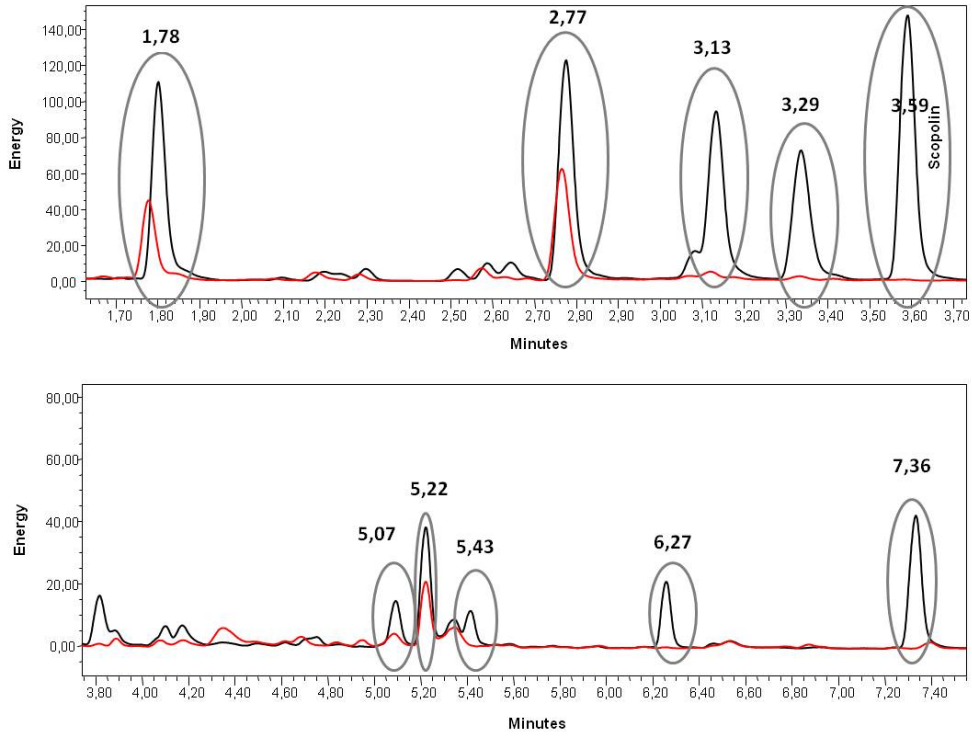


Figure 41: Effects of cold stress on fluorescing leaf compounds in *Arabidopsis thaliana*. Overlay of two fluorescence chromatograms at 300/400nm of leaf extracts from 36-day-old plants. Several peaks of fluorescing substances increase after cold stress. Black line: Plants exposed to 10°C for 1 week; red line: control plants kept at growing conditions (20°C).

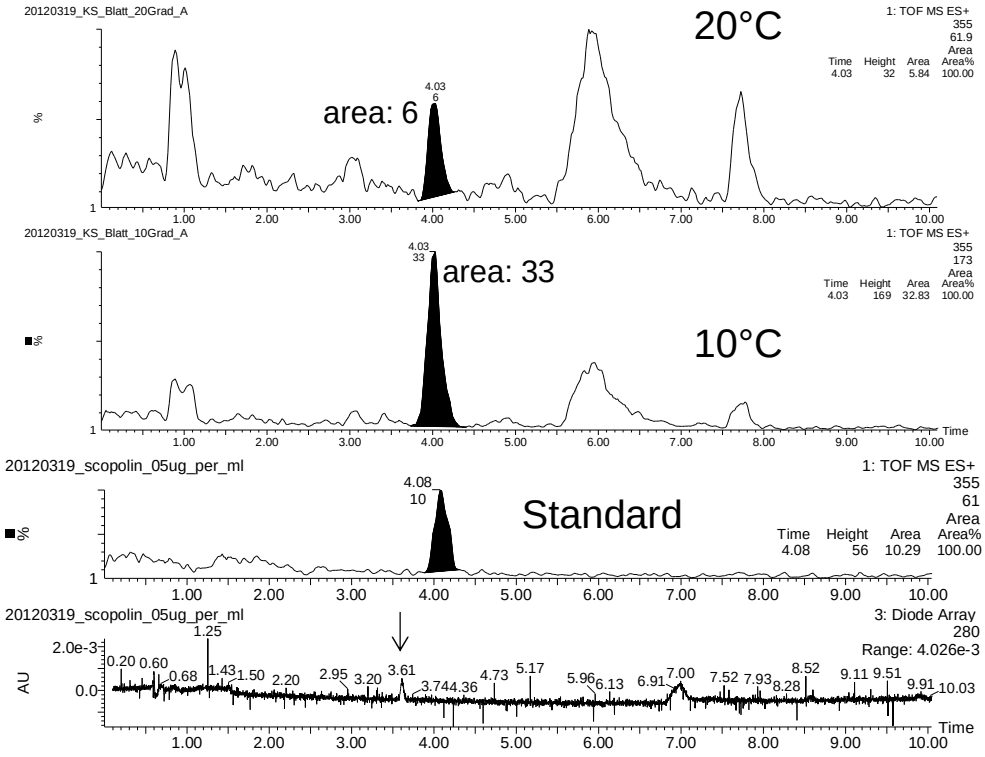


Figure 42: Confirmation of the identity of scopolin: the coumarin is the fluorescence peak (RT 3.59) which reacts strongly to cold stress. (LC-MS data. Leaf extracts of 6 week old plants, the 10°C chromatogram belongs to plants that were kept at 10°C for one week before harvesting.)

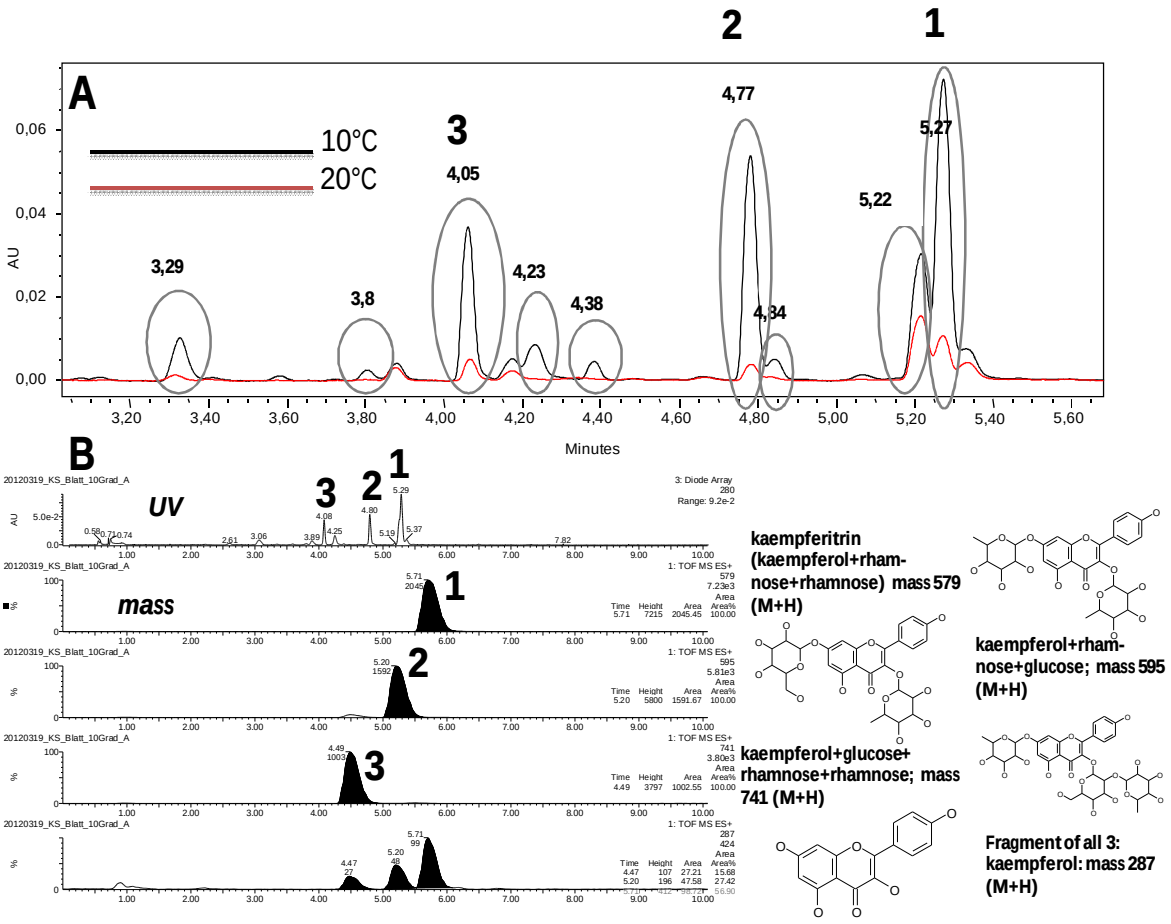


Figure 43: Identification of the three main UV peaks reacting to cold stress. A: Overlay of two chromatograms (280nm UV). Black line: Plants exposed to 10°C for 1 week; red line: control plants kept at growing conditions (20°C). **B:** The peaks were identified by their main mass. A fragment with the mass of kaempferol was found at the retention time of all three peaks (mass chromatograms on the left). Structures of kaempferol and its three most prominent cold-reacting derivatives (right): Peak 1: kaempferol 3-rhamnoside 7-rhamnoside. Peak 2: Kaempferol 3-glucoside 7-rhamnoside. Peak 3: kaempferol 3-[6'-glucosyl) rhamnoside] 7-rhamnoside. Structures according to Maruyama et al. (2009).

Development of a screening system for cold stress experiments with *Arabidopsis thaliana*

The hitherto unknown fact that scopolin accumulated distinctly in *Arabidopsis* leaves under cold stress was used to create a screening system for regulatory genes of scopolin accumulation. The chance to use leaf material instead of roots opened up the possibility to screen faster and easier for mutants in scopolin accumulation. In the previous screen with varying degrees of sucrose, roots had to be gained from sterile culture, which limited the number of screened lines considerably. Besides that, the new system should be used to screen simultaneously for mutants generally deficient in cold response. For that, the three main UV peaks and the scopolin peak were elected as marker peaks.

For maximum success in material and time consuming screens of large mutant collections, a robust and reliable screening system had to be carefully developed. At first, the technical aspects were resolved. For details see "Materials and Methods". The results of comparative runs were to use an HPLC separations method from Yonekura-Sakakibara et al. (2008), a

method which used a phenyl column and a gradient from 100% polar solvent to 60% polar solvent within 10minutes (HPLC method 4). The standard laboratory extraction method, which was developed for 100mg plant material, was tested for the minimum amount of starting material to accommodate for low sample amounts that were expected for young plants: 25mg of powdered plant material led to exactly the same peak heights as 100mg from the same material. However, 10mg were not sufficient. To determine optimum conditions for a screen under cold stress, HPLC chromatograms from plants grown at 20°C and plants transferred to 10°C for 1 week before harvest were compared to each other. The following growing conditions were varied during that experiment: age of plants, substrate and growing place. The age of the plants had the greatest influence: Differences between cold stress and control were most pronounced at the youngest age tested, in 5-week-old plants, although the total amounts of phenylpropanoids were the lowest there. At that age, peaks were up to 20 times higher under cold stress, whereas in the next age group peaks are only doubled. Plants of 9 weeks and older showed only small differences or none at all. The choice of substrate had a considerable effect on total amounts of detected substances; however, the effects were not consistent for all peaks. Some reacted to an increase in nutrients by increasing and some declined. The effect on the relations of peak areas gained under control or treatment conditions were minor and also not consistent. Slightly more peaks showed greater differences on nutrient-rich substrate. The effect increased with plant age. The two growing places tested had minor to no effect on the total amount and relative increase of substances. At the youngest age, the smaller plant growth chamber caused a higher increase in slightly more peaks than the large environmental room (see Table 7 and Figure 44). In addition, biometric data was gathered for each combination of conditions. The growing place had no influence on plant development. Plants grown in nutrient-rich substrate had a tendency to produce larger rosettes, more leaf biomass and larger leaves; the root weight was not affected. Differences became only prominent in the older age groups.

For scopolin, differences between cold treatment and control were up to 200times high, although the loss of difference was already severe in the second age group.

Table 7: Peaks selected as marker peaks for large screenings in cold stress experiments

Marker peak name	Peaks (retention time)	Positive difference control vs. treatment [%]	Peak area [mV*sec] after cold treatment (36-day-old plants)
Scopolin	3.59 (FLD 300/400nm)	1567-21669	3709-10294
3 (Kaempferol+glc+rha+rha)	4.05 (PDA 280nm)	504-625	63-117
2 (Kaempferol+glc+rha)	4.77 (PDA 280nm)	915-1747	120-133
1 (Kaempferitrin)	5.27.(PDA 280nm)	386-565	148-201

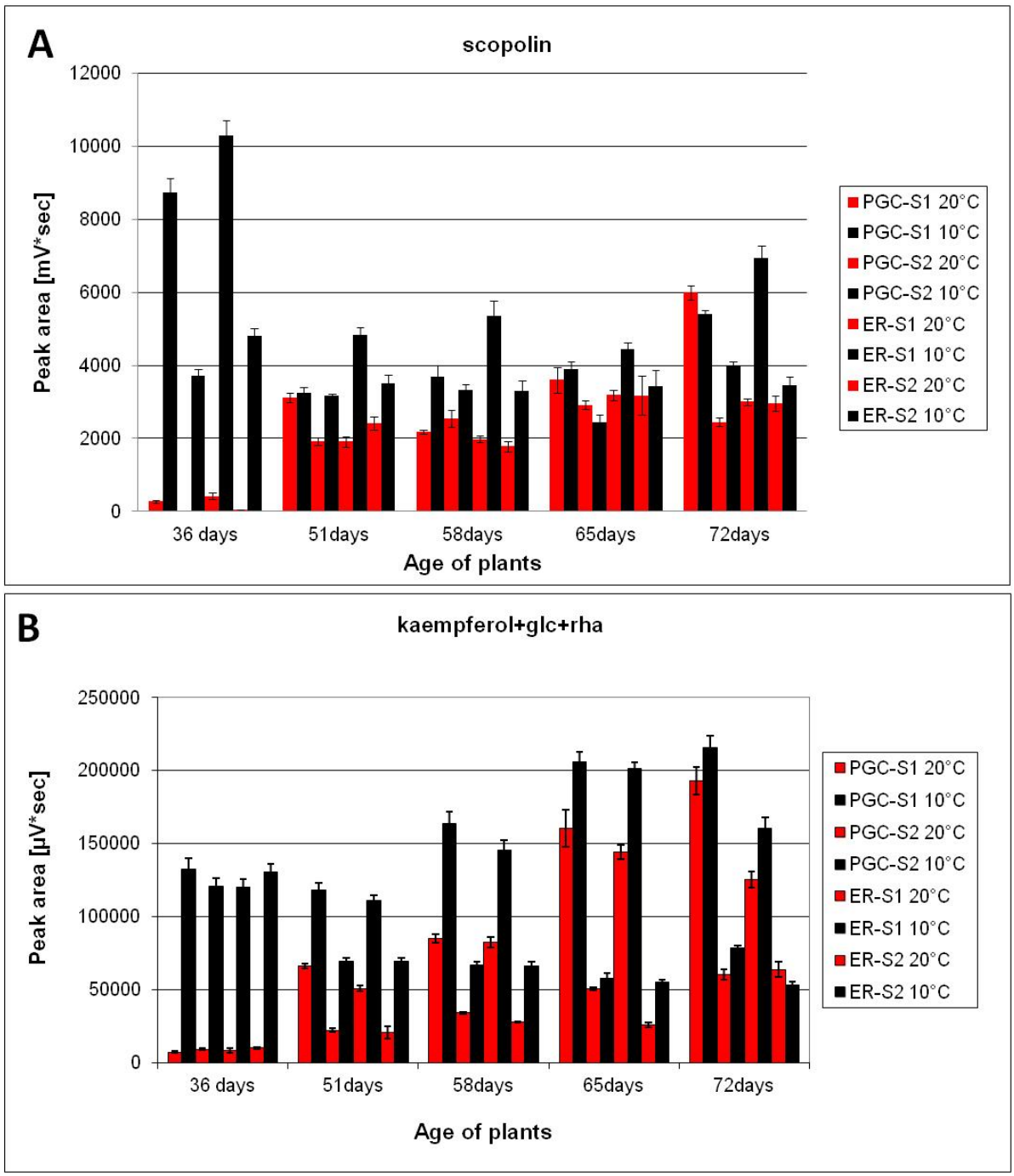


Figure 44: Test for optimum conditions for a cold stress screening with *Arabidopsis thaliana* to show maximum difference towards a 20°C control. Combination of plant age, substrate and growing place were evaluated. Peak areas gained for all conditions were compared as shown here for scopolin and the most prominent kaempferol derivative. Different harvests after 5, 7, 8, 9 and 10 weeks. The legend shows column names in each age group from left to right: PGC = plant growing chamber; S1 = low-nutrient substrate; S2 = nutrient-rich substrate; ER = environmental room.

Table 8: Peaks at 280nm UV detection from *Arabidopsis thaliana* leaf extracts which increased significantly after cold stress and the best conditions for maximum differences between control and treatment for each peak. PGC = plant growth chamber; ER = environmental room, S1 = low-nutrient substrate, S2 nutrient-rich substrate, - = indifferent

Peak (retention time)	Optimum plant age for maximum differences between control and cold treatment	Best combination of growing place and substrate in the group of the determined optimum plant age
3.29	36days	ER/S2
3.8	36days	ER/S2
4.05	36days	PGC/S1
4.23	36days, 51days	PGC/S1/S2
4.39	36days	-
4.77	36days	PGC/S1
4.84	36days, 51 days	-
5.22	36days	PGC/S2
5.27	36days	ER/S2
5.32	36days	-
6.07	36days	-
Best conditions	**36days**	**Plant growing chamber/Nutrient rich substrate**

Table 9 Peaks at 300/400nm fluorescence detection from *Arabidopsis thaliana* leaf extracts which increased significantly after cold stress treatment. Best conditions to cause maximum positive differences between treatment and control. PGC = plant growth chamber; ER = environmental room, S1 = low-nutrient substrate, S2 nutrient-rich substrate, - = indifferent

Peaks (retention time)	Optimum plant age for maximum differences between control and cold treatment	Best combination of growing place and substrate in the group of the determined optimum plant age
1.78	36days	ER/S2
2.77	36days	PGC/S1
3.13	36days	PGC/S2
3.29	36days	-
3.59 (Scopolin)	36days	PGC/S2
5.07	36days	ER/S2
5.22	36days	PGC/S2
5.43	36days	-
6.27	36days	-/S2
7.36	36days	-/S1
Best conditions	**36days**	**Plant growing chamber/Nutrient rich substrate**

Summary: Differences were most pronounced at the youngest age tested. The older the plants, the smaller became the difference between treatment and control until it was lost in the oldest plants. The substrate influenced peak heights, but not equally for all peaks. The growing place had no influence. The optimum conditions for a cold stress screening experiment for scopolin and other phenylpropanoids accumulating under cold stress were determined as: Cold treatment of four-week-old plants in nutrient-rich substrate.

Application of the newly established screening system for cold stress experiments

EMS mutants with deficiencies in cold stress reactions accumulated different amounts of scopolin

From 46 EMS-mutagenized candidate lines from an earlier cold stress screen (Peterek and Mock, unpublished) 3 lines were confirmed, which showed not the strong increase in some compounds after cold stress, as WT plants did. All compounds stayed exactly at the level as in the 20°C control which matched also the 20°C WT control (Figure 45). Only in line 8-73-22 the marker peaks were already reduced at 20°C. The three lines were analysed for their reaction in the scopolin peak. Line 10-294-5-5 did react to cold with accumulation of scopolin, but not as strong as WT. Line 8-73-22 had the same accumulation of scopolin like WT. In line 8-78-24, the 10°C fluorescence chromatogram was identical to a WT control at 20°C (Figure 46). Other lines from the rescreen showed minor differences to WT and were not analyzed further.

Transcription factors BEE1 and At5g45580 positively influence scopolin accumulation under cold stress

The seed collection *RIKEN Ds transposon lines* (Kuromori et al., 2004) contains lines with a single insertion mutation caused by a T-DNA insertion on a known location. A part of the collection comprises insertion mutations of 313 putative transcription factors. This part was screened with the newly developed system described above. In three independent experiments, three lines were isolated, which did not react to cold stress with scopolin accumulation. Two of these had nearly completely identical chromatograms, including an additional peak and the same reduction of kaempferol-derivatives. Those were the brassinosteroid synthesis regulator BEE1 and the line TF300 (locus At5g45580), which is from now on referred to as "BEE1-LIKE" (Figure 47). Additional lines with an altered reaction towards cold stress that were detected in the screens are given in Table 10.

Current annotation for BEE1 and BEE1-like

According to TAIR (Rhee et al., 2003), At5g45580 (BEE1-LIKE) codes for a myb-type transcriptional regulator. Next hit according to NCBI's alignment tool BLASTn (Altschul et al., 1997) with the whole genomic DNA is transcription factor APL (Altered Phloem Development, At1g79430) with a maximum identity of 80%. The predicted protein is 264 amino acids long. With NCBI's Protein BLAST (Altschul et al., 1997) three conserved domains were found: 1. SHAQKYF class MYB-like DNA-binding domain. This is a DNA-binding domain restricted to (but common in) plant proteins, many of which also contain a response regulator domain. 2. A MYB-CC type transfactor with a LHEQLE motif. This family is found towards the C-terminus of MYB-CC type transcription factors and carries a highly conserved LHEQLE sequence motif. 3. Domain PLN03162, annotated as "golden-2 like transcription factor domain (provisional)". Closest protein hits were the hypothetical protein ARALYDRAFT_356449 from *Arabidopsis lyrata subsp. lyrata*, a putative transcription factor

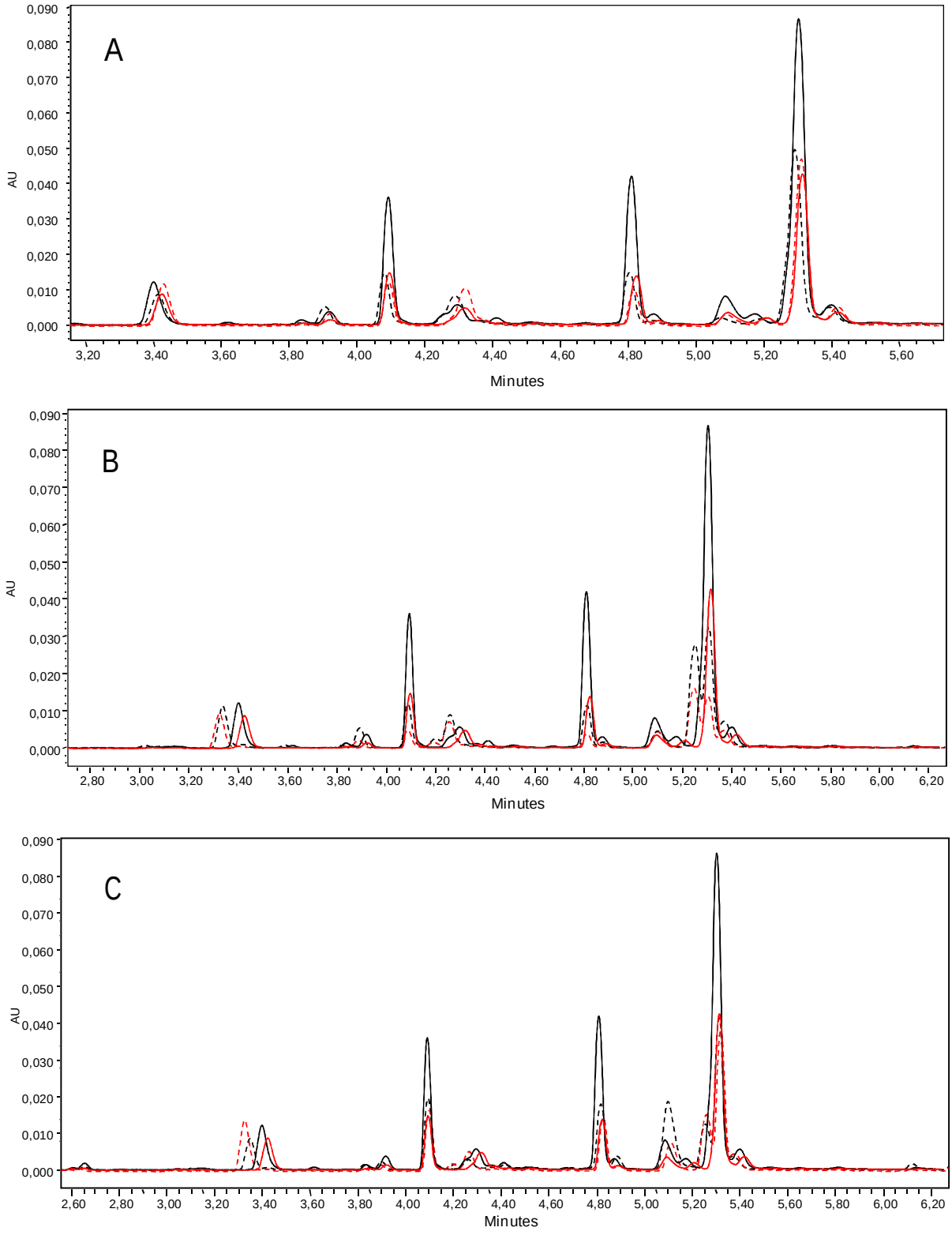


Figure 45: HPLC-UV profiles of candidates from a cold stress screen with *Arabidopsis thaliana* EMS mutants. EMS lines at 10°C, EMS lines at 20°C and WT at 20° have similar peak heights. UV detection at 280nm. Black solid line = WT at 10°C; red solid line = WT at 20°C; black dashed line=EMS line at 10°; red dashed line EMS line at 20°C. A: WT and EMS line 10-294-5-5. B: WT and EMS line 8-73-22. C WT and EMS line 8-78-24.

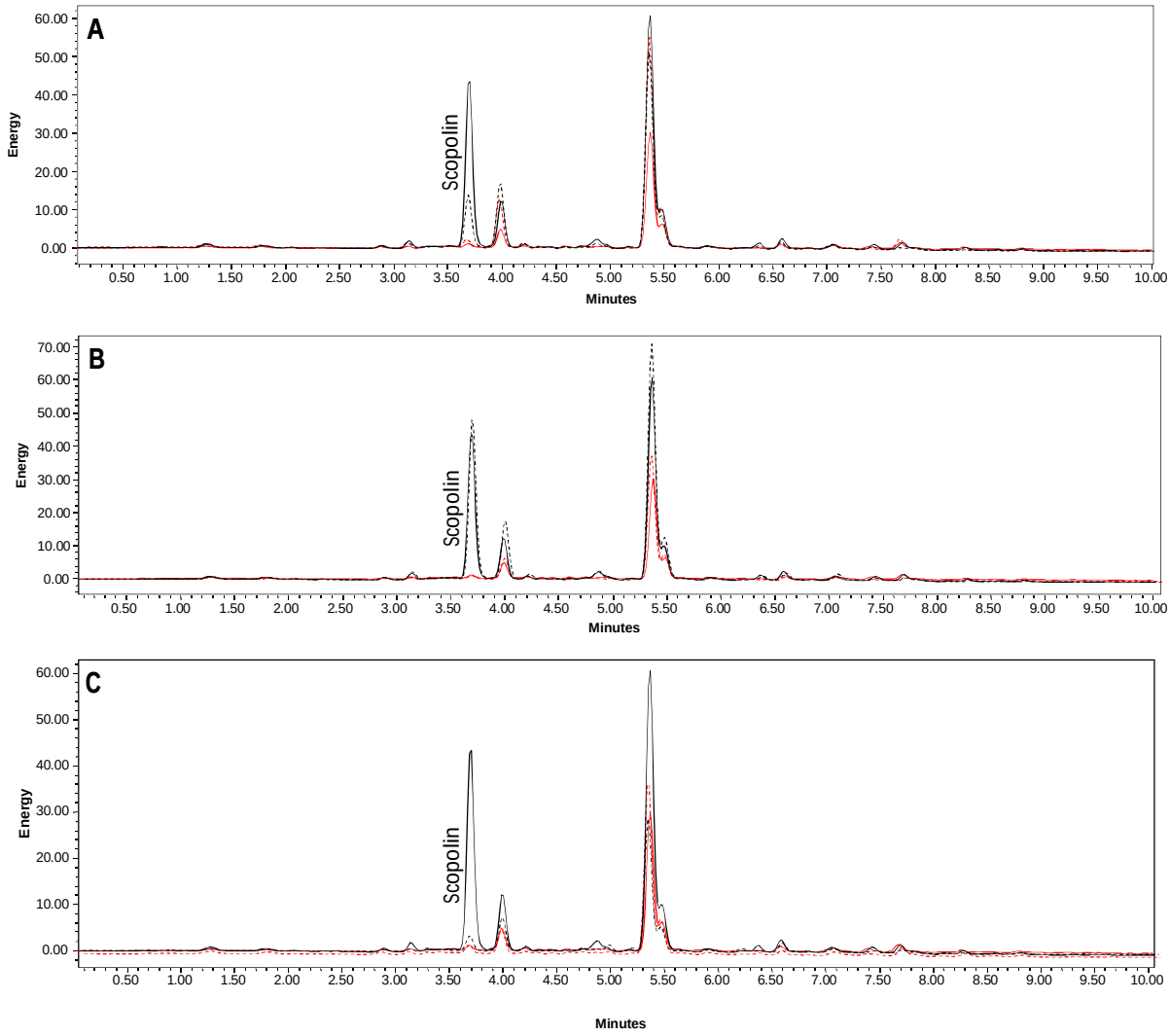


Figure 46: Fluorescence profiles of the candidates from the cold stress screen with *Arabidopsis thaliana* EMS mutants. The mutant lines did not react to cold stress with an accumulation of kaempferol derivatives (marker peaks). However, they differ in the scopolin accumulation after cold stress. A: WT and EMS line 10-294-5-5. The scopolin accumulation was reduced compared to WT. **B**: WT and EMS line 8-73-22. The line accumulated exactly as much scopolin as the WT under cold stress. **C** WT and EMS line 8-78-24. The EMS line behaved after cold stress exactly like a WT under control condition; there was no accumulation of scopolin. Fluorescence detection at exc.360/em.450nm. Black solid line = WT at 10°C; red solid line = WT at 20°C; black dashed line=EMS line at 10°; red dashed line EMS line at 20°C.

from *Ricinus communis* and a predicted APL-like myb family transcription factor from *Glycine max*. Closest hit in *Arabidopsis thaliana* is a HTH transcriptional regulator, MYB-type, with the locus AT3G24120. Gene expression analysis data from Genevestigator is nearly non-existent.

BEE1 is one of three basic helix-loop-helix (bHLH) transcription factors called Brassinosteroid Enhanced Expression (BEE) which were identified in Arabidopsis due to their rapid upregulation by brassinolide. Brassinolide is a brassinosteroid plant hormone active in numerous signalling pathways, like salt stress (El-Mashad and Mohamed, 2012), elongation (Kurepin et al., 2012) and drought resistance (Li and Feng, 2011). BEE1 transcription is

Table 10: Candidates of a cold stress experiment with insertional mutants of putative transcription factors in *Arabidopsis thaliana*. Their phenotype was confirmed in 2 independent experiments. Gene descriptions and references were acquired from TAIR (Rhee et al., 2003). The screen was conducted for three marker substances reacting to cold stress in WT: 1= kaempferol 3-rhamnoside 7-rhamnoside. 2: Kaempferol 3-glucoside 7-rhamnoside. 3: kaempferol 3-[6'-glucosyl) rhamnoside] 7-rhamnoside.

Locus	Line number	Description	Kaempferol derivatives at 10°C	Scopolin at 10°C
At1g06040	TF215	STO, plays a role in light signalling (Indorf et al., 2007)	1 enhanced, extra peak at 4.38min	Slightly reduced accumulation.
At1g18400	TF311	BEE1, regulates brassinosteroid synthesis (Poppenberger et al., 2011), controlled by ICE1 (Lee et al., 2005)	2+3 slightly reduced, extra peak at 4.62min	Not accumulating.
At2g45680	TF298	cold- and light-responsive (Vergnolle et al., 2005; Lopez-Juez et al., 2008)	3 slightly reduced	Reduced accumulation.
At2g48160	TF295	contains TUDOR-domain, contains RNA-polymerase II, large subunit domain	2+3 slightly reduced	WT-like.
At4g00940	TF296	cold-responsive (Vergnolle et al., 2005)	3 slightly reduced, extra peak at 4.62	Reduced accumulation.
At4g30980	TF292	LRL2, regulates root hair development (Karas et al., 2009), modulates circadian parameters (Hanano et al., 2008)	2+3 slightly reduced	WT-like.
At5g25830	TF287	GATA12, reacts to light stimulus and circadian rhythms (Manfield et al., 2007)	1+3 slightly reduced, extra peak at 4.62min	Not accumulating.
At5g45580	TF300	Homeodomain-like superfamily protein; contains SHAQKYF class myb-like DNA-binding domain, a MYB-CC type transfactor with a LHEQLE motif and domain PLN03162 (golden-2 like transcription factor)	2+3 slightly reduced, extra peak at 4.62min	Not accumulating.

upregulated by ICE1 signalling, one of the most prominent genes in Arabidopsis mediating early response towards cold stress (Lee et al., 2005). BEE1 is thought to be a controller of brassinosteroid synthesis (Friedrichsen et al., 2002).

Promoter analysis of BEE1-LIKE

Motifs of transcription factor binding sites found in 1851 bp upstream of the translation start, sorted by number of different motifs: They are involved in development, dehydration light, cold (RAV1, ICE1 binding sites), phenylpropanoid synthesis, ABA response, auxin response, anaerobic conditions and phosphate and sulphur starvation (Chang et al., 2008).

Intensity of scopolin accumulation under cold stress is dependent on light quality

In the first screen of the RIKEN collection, the wild type had the highest scopolin values measured during all the cold stress experiments. This experiment was conducted in a large walk-in environmental room instead in "Percival"-incubators as the previous experiments. Temperature and total amount of light were maintained as in the incubators. However, the light spectrum reached more into the UV range. In that screen, scopolin reached higher values as previously in the incubators. In the WT samples, absolute values for scopolin in the large chamber were five times higher than in the incubator under control conditions and three times higher at cold stress. The ratio between control and treatment however stayed

the same: Scopolin under cold stress was 10 times higher than under control conditions in the chamber as well as in the incubators.

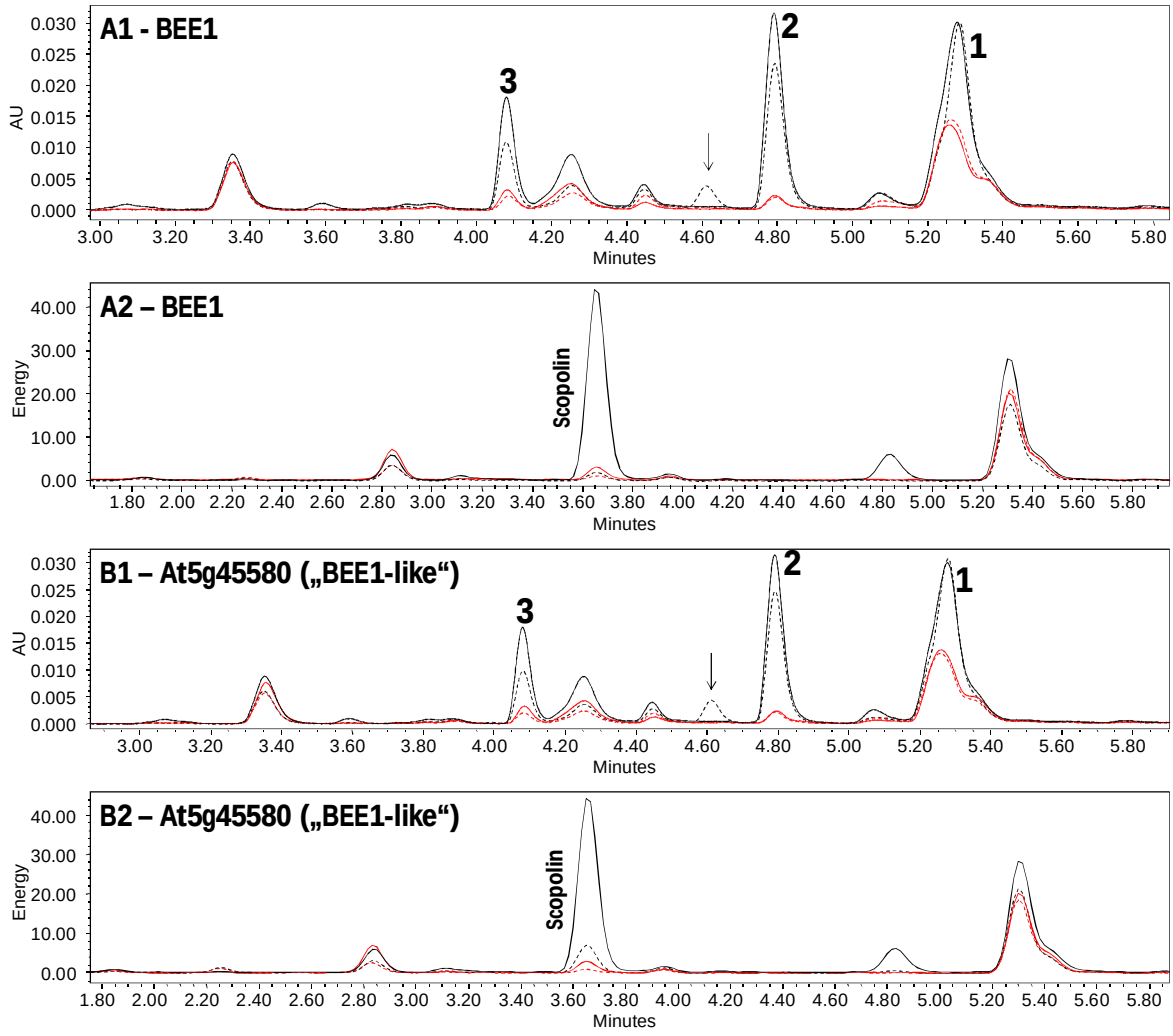


Figure 47: The MYB-like transcription factor At5g45580 had nearly identical profiles as the brassinosteroid synthesis regulator BEE1 at cold stress. A1: RIKEN line TF311 (At1g18400, BEE1) and WT at 280nm. Reaction to cold stress is reduced. There was one additional peak (black arrow). A2 TF311 (At1g18400, BEE1) and WT at 336/438nm fluorescence detection. In BEE1 the scopolin peaks remained at control level. B1 RIKEN line TF300 (At5g45580, BEE1-LIKE) and WT: Reduced reaction to cold stress and one additional peak (black arrow). B2: TF300 (At5g45580, BEE1-LIKE) and WT at 336/438nm fluorescence detection. (HPLC-chromatograms of leaf extracts. Black solid line = WT at 10°C. Red solid line = WT at 20°C. Black dashed line: RIKEN line at 10°C. Red dashed line: RIKEN line at 10°C.)

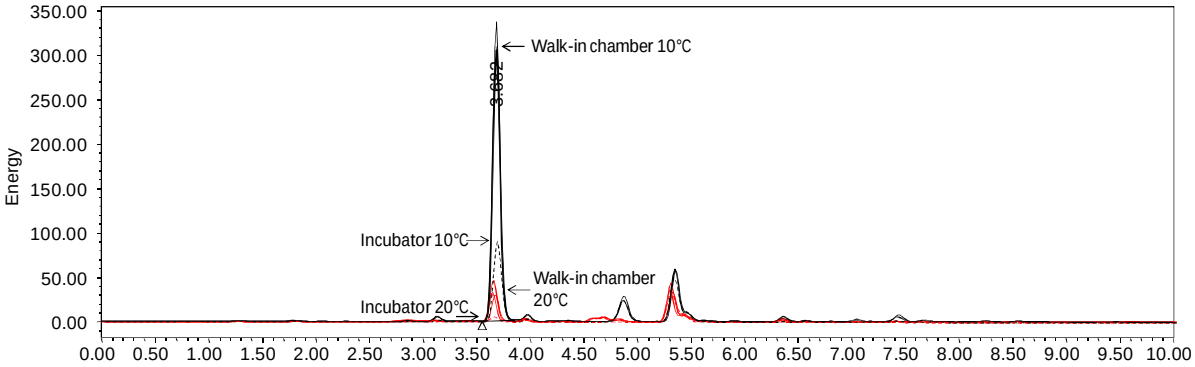


Figure 48: Amounts of scopolin in wild type (Nossen-0) differ in different growing facilities. HPLC-chromatograms of leaf extracts from all biological WT replicates. Black solid line = walk-in chamber at 10°C. Red solid line = walk-in chamber at 20°C. Black dashed line: incubator at 10°C. Red dashed line: incubator at 20°C. Fluorescence chromatograms at 360/450nm.

Discussion

F6'H1 is not only essential for the synthesis of scopolin, but also for the synthesis of other fluorescing compounds, one of these being esculin

In the mutant *f6'h1*, the enzyme F6'H1 catalysing the hydroxylation of feruloyl CoA towards 6'hydroxyferuloyl CoA is disabled. Kai et al. (2008) proofed that scopolin and scopoletin production in *Arabidopsis thaliana* is dependent on this step. However, the thorough analysis of the mutant revealed that six other fluorescing compounds and the non-fluorescing isoscopoletin are also not present without a functioning F6'H1. Identification of coumarins in Arabidopsis was rather challenging because of very low levels of most coumarin derivatives for LC-MS detection. Amongst others, scopolin, scopoletin, esculin, a substance with the mass and retention time of herniarin and traces of isoscopolin, isoscopoletin and umbelliferone, but no esculetin or scoparone were detectable in wild type. In comparison, Kai et al. 2006 also detected scopolin, scopoletin, esculin or cichoriin, but also traces of esculetin. They did not detect umbelliferone, herniarin and scoparone. The esculin contents of *Arabidopsis* wildtype plants was very low, at about 0.3 µg/g fresh weight compared to scopolin (10-600 µg/g FW) or scopoletin (1-10 µg/g FW). (Interestingly, these values resemble the contents of tobacco callus cultures of cultivars Samsun NN and S2LS3 (ee appendix, figure A4). Esculetin, if it is present in *Arabidopsis*, which should be a necessity for the formation of esculin, would be much lower than 0.1 µg/g FW. However, as I could show that at least esculin is definitely present in *Arabidopsis* WT (and probably also in tobacco), but not in the F6'H1 mutant, the current coumarin pathway must be modified (see Figure 49): Umbelliferone was detected in very small traces in *f6'h1* and WT, but esculin was not, so this hydroxylation step will not happen in *Arabidopsis*. Likewise, the methylation towards herniarin probably does not happen, if the absent peak is really herniarin. If the identification of umbelliferone and herniarin was wrong, and Kai et al. are right in claiming that they are not present in Arabidopsis, then were is still the problem of the origin of esculin and esculetin. Since F6'H1 was proven not to hydroxylate caffeic acid and its ester (Kai et al. 2008), the reactions from hydroxy caffeic acid also does not happen. This leaves only scopoletin as the precursor for esculetin/esculin, isoscopoletin/isoscopolin (and for herniarin, if the identification was correct). To form esculetin, scopoletin's methyl group must be exchanged for a hydroxylgroup. Such a reaction is mentioned in the review of Bourgaud et al. (2006) for the reaction of herniarin to umbelliferone. The amounts of scopolin/scopoletin and isoscopolin/isoscopoletin as well as esculin/esculetin differ that much from each other in favour of scopolin and scopoletin; that one could say that the production of the latter are not favoured in *Arabidopsis*. There might not be an enzyme which is specific for these reactions, and they might happen as a trace activity from enzymes from other pathways. Or they might be actively suppressed. There are hints that esculin and not scopolin accumulates in the root tip of *Avena* (Pollock et al., 1954; Avers and Goodwin, 1956). If this is also the case in *Arabidopsis*, this might be a site, where such an inhibition is stopped. Furthermore, recent discoveries indicate, that esculetin might be needed in roots

as a chelator under nutrient deficiency (Schmid[5], unpublished). This could be another incident, where the reaction from scopoletin to esculetin is promoted in *Arabidopsis*. As for the other substances affected in the mutant, sinapic acid and sinapoylglucose were found to be reduced. The sinapic acid derivatives originate from the same pathway as scopoletin including the methylation of caffeoyl-CoA by CCoAOMT towards feruloyl-CoA, but then followed by the reaction to coniferylaldehyde, to 5-hydroxyconiferylaldehyde, to sinapaldehyde, to sinapic acid (sinapate) and by glycosylation to sinapoylglucose (Milkowski and Strack, 2010). This main pathway should not be affected by a mutation of F6'H1. However, there might be two alternative routes (Kanehisa et al., 2010): The first via hydroxyferulic acid, without a CoA ester group. In scopoletin synthesis the pathway was strictly ferulic acid – feruloyl-CoA – hydroxyferuloyl-CoA (Kai et al., 2008), so it is probable, that free hydroxyferulic acid is not present. The second alternative route would be from feruloyl-CoA to hydroxyferuloyl-CoA which is exclusively catalyzed by F6'H1. From there, hydroxyconiferylalcohol or sinapoyl CoA could be formed, linking this alternative route back to the main pathway (Figure 50). If this alternative route is missing by a loss of F6'H1, the

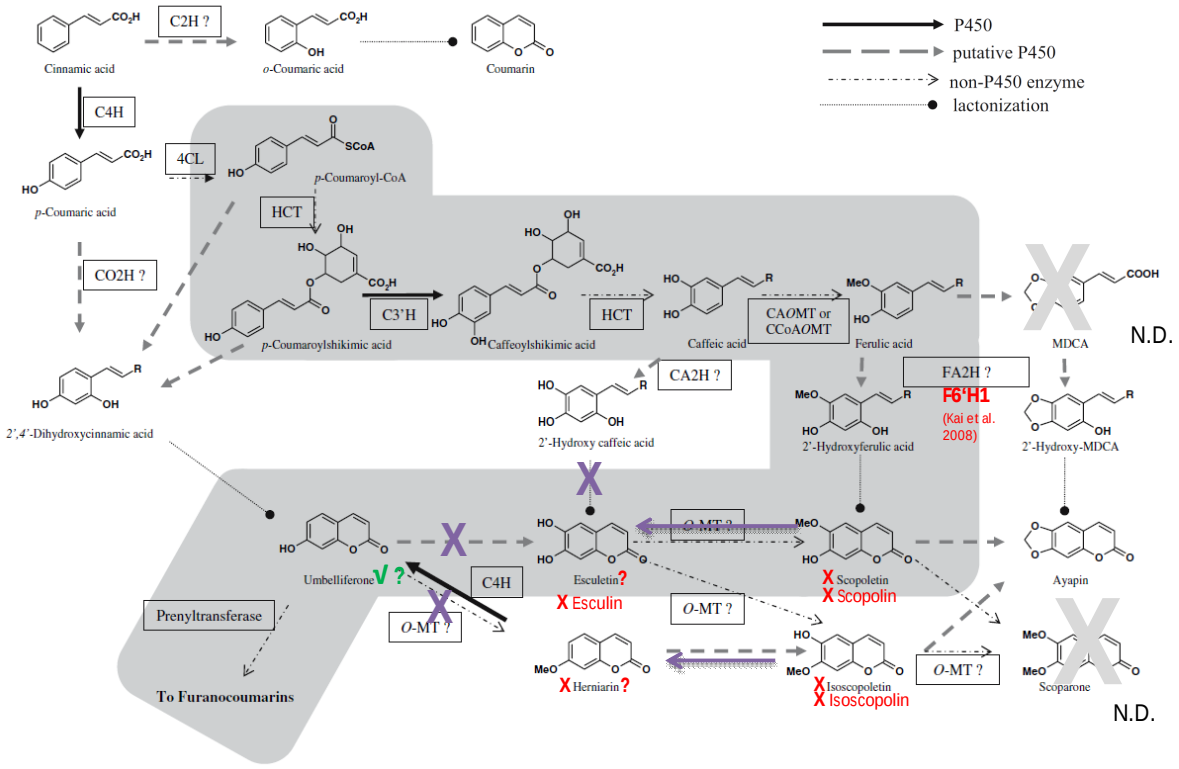


Figure 49: The phenylpropanoid pathway from Bourgaud et al. (2006), modified to accomodate the findings in *Arabidopsis thaliana* WT and the mutant *f6'h1* with the defective ferulic acid hydroxylase. Coumarins marked with a red cross were present in the WT and missing in *f6'h1*. Umbelliferone was found in traces in both WT and *f6'h1*. MDCA and Scoparone were not detected in WT. Markings in lilac are proposed changes to the pathway. 2-hydroxyferulic acid and/or scopoletin are necessary precursor for esculetin/esculin, isoscopoletin/isoscopolin, probably also herniarin.

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Levels of sinapic acid derivatives could very well be affected.

The substance underlying peak 4 had the two masses 373 and 355. 355 was also the mass gained for scopolin and the difference to 373 indicates the loss of one molecule of water. So, this peak might be a substance similar to scopolin. Peaks 6 and 7 belonged to compounds with a high molecular weight, 663 and 811: No further hints of the identity of these compounds could be found, except that von Roepenack-Lahaye et al. (2004) also listed a substance with the mass of 810.2553 as an unknown root compound. Also no substance was assigned to the mass of 591, which was found in peak 8.

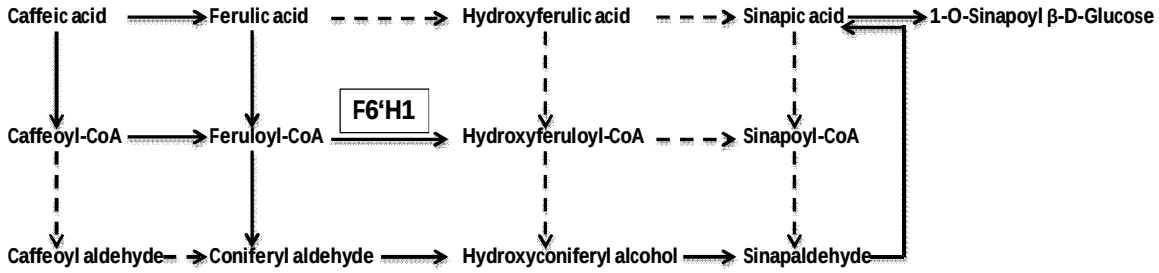

Figure 50: Established pathway and alternative routes towards sinapic acid in *Arabidopsis thaliana*. Solid arrows: established routes (Kai et al., 2008; Milkowski and Strack, 2010). Dashed arrows: alternative routes, (Kanehisa et al., 2010) that might or might not exist in *Arabidopsis.* The phenotype of an insertion mutant of F6'H1 suggests an alternative pathway via hydroxyferuloyl-CoA.

Scopolin accumulates specifically in the root endodermis – Does it support endodermis function?

In the developed root, scopolin was present in the endodermis. This was shown for all the samples from *Arabidopsis* and tobacco under all conditions tested. For this reason, it is valid to say that scopolin is constitutively accumulated in the endodermis of these species. At high-sucrose conditions, which increased scopolin accumulation considerably, it was also found in the cortex. When the pericycle was discernible, scopolin was sometimes also found in that tissue. It was never found in the stele, the rhizodermis or root hair cells. This very specific localisation was surprising, because if the functions of scopolin in roots resembled the assumed ones in the aerial parts (pathogen defence and protection against ROS, see introduction), one would have expected a less specific distribution.

The experiments with sucrose and mannitol showed that the osmotic effect of sucrose solutions only plays a minor role in the accumulation of scopolin, because lower concentrations of mannitol were less effective than sucrose solutions with the same osmotic potential. Higher concentrations of mannitol even had a negative effect on scopolin accumulation. Under stress conditions, where photosynthesis products cannot be utilised for growth, phenylpropanoids are assumed to be a carbon sink and energy dissipater, to remove excess photoassimilates and –energy. The plant also profits from their additional functions like herbivore deterrents, antioxidants, allelopathic compounds and their other assumed

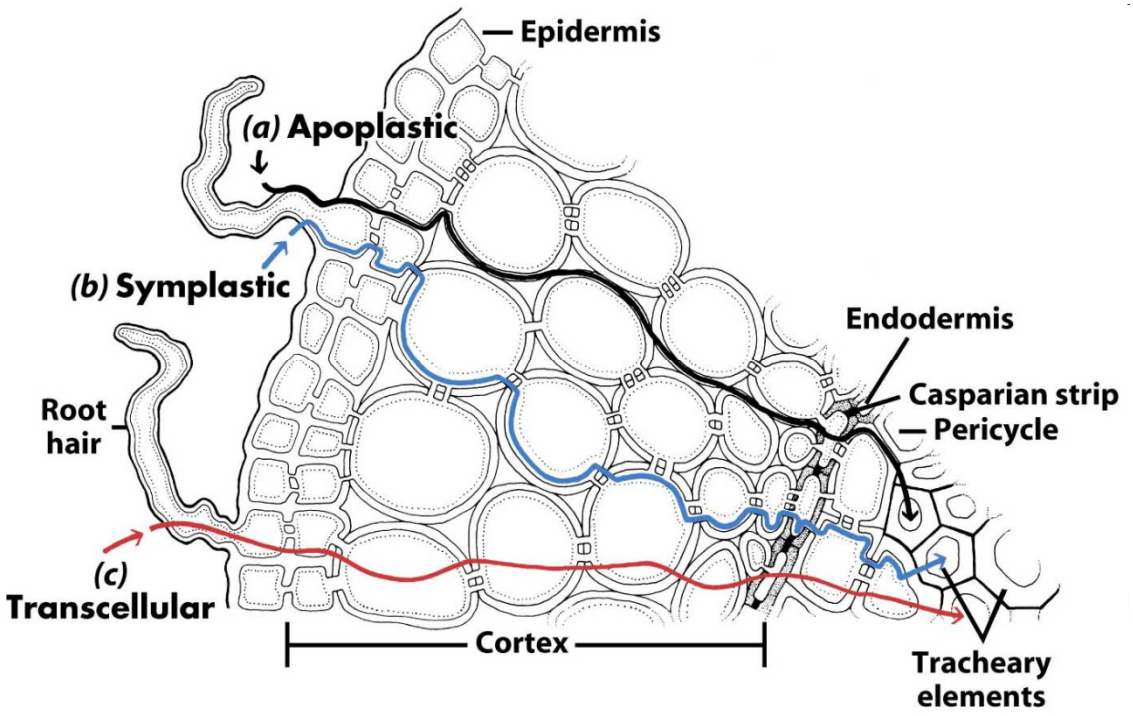

Figure 51: Ways of transport of water and solubles in the root. Apoplastic transport (a): By diffusion through cell walls and extracellular spaces outside of cells. The apoplastic transport ends at the endodermis, where it is taken up through pores into the lumen of endodermis cell, but can continue in the apoplast of the pericycle. Symplastic transport (b). Free diffusion in the cytoplasma of cells connected by plasmodesmata. Transcellular transport (c): Water and solubles are taken up in the vacuoles (Raven et al., 2007).

functions (Hernández and Van Breusegem, 2010). Scopolin might be one of the main phenylpropanoid compounds acting as carbon sinks. But as all the conditions that occur under high-sucrose (as indicated by the gene expression analysis), like hypoxia (Blokhina et al., 2003), osmotic stress and the activation of the metabolic processes itself (Karuppanapandian et al., 2011) also cause an increase in ROS, it is highly probable that scopoletin acts additionally as a scavenger of ROS.

The specific and distinctive accumulation of scopolin in the endodermis, even under low-stress conditions, points to a very special function in this tissue. The endodermis is distinguished from the other cell layers by forming the Casparian strip. This is a cell-wall modification which by incrustination of lignin and sometimes suberin makes the endodermis impenetrable for apoplastic ions and water (Figure 51). Any substance which is not part of the symplastic movement of solubles is prevented from entering the vascular bundle. By this, the water and ion uptake is tightly controlled and the ion gradient maintained, which powers the transpiration stream. But also a backflow of ions is prevented, when transpiration is low (Bonnett, 1968; Enstone et al., 2002). Nutrients are directionally and selectively transported through the endodermis by influx and efflux carriers (Alassimone et al., 2012). Alassimone et al. (2012) even call the endodermis the “plant’s inner skin”, as it is the structure which completely separates soil and plant. The easiest explanation for the presence of scopolin in endodermal cells would be a co-activation of its synthesis when

lignin production for the Casparian strip is activated. Guaiacyl lignin and syringyl lignin are derived from direct precursors of scopolin (e.g. hydroxyferuloyl-CoA, feruloyl-CoA or caffeoyl-CoA, Kanehisa et al. (2010)). However, the various properties of scopoletin make additional functions and even an independent regulation from lignin formation very likely: Another assumed function of the endodermis is to prevent a pathogen infection of the vascular tissue. This is probably more important than protecting other root tissues like cortex and epidermis, whose colonisation might even be beneficial during symbiotic mycorrhiza formation. As soon as a pathogen succeeds in entering the transport vessels, the plant usually dies quickly as it is known from wilting diseases caused by various pathogens like *Fusarium*, so a good defence at this site is crucial for plant survival. Evidence for a role of the endodermis in biotic stress defence is still lacking (Alassimone et al., 2012). But, it has been reported that a *Fusarium* infection was stopped at the endodermis (Bhalla et al., 1992) and that mycorrhizal fungi never penetrate this cell layer (Enstone et al., 2002). The Casparian strip is resistant to cell-wall-degrading enzymes used by pathogens attacking primary cell walls (Schreiber et al., 1999). Juvenile root knot nematodes avoid passing the endodermis, move apoplastically to the root tip and enter the vascular bundle through meristematic cells (Williamson and Gleason, 2003). In the defence against pathogens trying to enter the vascular tissue, scopolin might be a second barrier behind the Casparian strip, a pre-formed pathogen defence which pathogens encounter as soon as they enter the endodermis. Its localisation is strikingly similar to the localisation of homospermidine synthase (HS), the first specific enzyme for the synthesis of pyrrolizidine alkaloid (PA). In two plant species being able to produce this alkaloid in their roots (*Symphytum officinale*, *Cynoglossum officinale*), HS was exclusively localised in endodermis and pericycle. As PAs are key components for the chemical defence of these species, the activation of its synthesis pathway in the endodermis was also interpreted as a measurement to protect sensitive tissues like the reproductive pericycle (Niemüller et al., 2012).

During the presented study, three insertion mutation lines with reduced or no scopolin (*ccoaomt1*, *attho1*, *f6'h1*) deviated from wild type in lateral root formation and root length, which was most pronounced under stress conditions. Nicole Schmid[6] (personal communication), having found a deficiency in nutrient uptake in *ccoaomt1* and *f6'h1,* suggested an indirect influence of scopoletin or a closely related compound like esculetin on root growth resulting in a starvation phenotype. On the other hand, scopoletin and esculetin themselves have growth-influencing properties *in vitro* as shown in older studies (Andreae, 1952; Pollock et al., 1954; Avers and Goodwin, 1956; Sirois and Miller, 1972). Antiproliferatic effects of scopoletin on cells were confirmed by modern cancer research (Adams et al., 2006; Arcos et al., 2006; Manuele et al., 2006). The earlier authors suggested a concentration-dependent growth-promoting or -inhibiting effect of scopoletin in the root. Targets in the root for growth-regulating substances are the meristem at the root tip and the

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meristematic part of the pericycle. When meristematic pericycle cells undergo cell division, lateral root development is initiated (Charlton, 1996). Auxins, like indole acetic acid, have been established as the main signal for lateral root formation (Casimiro et al., 2003; De Smet et al., 2007). Supports for growth-influencing properties of scopoletin from this study are the strikingly specific accumulation next to a meristematic tissue and the reduction of lateral roots in scopolin-deficient mutants. The endodermis might release scopoletin into the apoplast towards the pericycle cells and thus prevent cell division. Scopolin and scopoletin have actually been found in the apoplast (cassava root, Buschmann et al., 2000). Lateral root formation might be promoted by enhancing the auxin signal, if scopoletin protected auxin molecules from degradation.

Scopolin and scopoletin offer minor protection against the biotrophic protist *Plasmodiophora brassicae*

Scopoletin has been shown to be a true phytoalexin in aerial parts of the plant with proven action against bacteria, viruses and fungi (see introduction). Therefore, a similar function in roots was expected. The breeding of plants which react fast towards pathogens with scopolin/scopoletin production or have constitutively high coumarin levels is regarded as a tool for plant breeders to provide crops with a high pathogen resistance. Initial trials are currently held with the rubber tree, which is threatened by the more and more fungicide-resistant South American leaf blight (*Microcyclus ulei*) or with head-rot-resistant sunflower cultivars (Garcia et al., 1999; Prats et al., 2006). *Plasmodiophora brassicae* is an obligate biotrophic pathogen belonging to the kingdom of Protista and there in the phylum Rhizaria. It was formerly classified as a slime mould. It causes the club root disease in Brassicaceae. In the first step of the infection, the zoospore infects the root hairs. The second stage occurs in cortex and stele, where sporulating plasmodia cause large gall formation at the roots or hypocotyl. The galls form a strong metabolic sink, leading to stunted growth and finally, in most cases, the death of the infected plant. With the decay of the infected root the resting spores are released. It is an economically important and currently spreading disease in Brassicaceae (Ludwig-Müller and Schuller, 2008).

In the presented study, Col-0 wild type plants had in two out of four experiments by trend lower infection rates than the scopolin-free mutant *f6'h1*. The experiment with the greatest differences between WT and mutant suffered from some extreme conditions (heat, dryness, and aphids in a non-conditioned green house; low infection potential of spores). For the following experiments, cultivation conditions were improved and a new batch of spores acquired. Infection rates increased dramatically and no differences could be detected between WT and mutant anymore, until spore density was further lowered. Results indicate that scopoletin has only a defence-adjuvant function in this patho-system, which offers only a moderate protection when the infection pressure is low and abiotic stresses have to be coped with at the same time.

Antimicrobial plant natural products have a relatively broad spectrum activity, as it was also shown for sopoletin *in-vitro*, and specificity is often determined by whether or not a

pathogen has the enzymatic machinery to detoxify a particular host product (Vanetten et al., 1989). Accumulation of inducible compounds is usually linked to the perception of the pathogen by receptors of the host (Dixon, 2001). However, Päsold et al. (2010) showed that F6'H1 gene expression is increased two-fold in an early state of a *P. brassicae*-infection alongside numerous other genes from the flavonoid pathway. In the present study, scopolin/scopoletin levels were increased, especially in plants with less severe symptoms. Obviously, there is a specific activation of scopoletin production after the attack with *P. brassicae, but* only a minor protective effect. As against that, in the study of Päsold et al. flavonoid levels also increased, but flavonoid-free mutants performed even better than WT.

It could be worthwhile to test the scopolin-free mutant against other root pathogens, ideally against microbes that try to enter the vascular system to test a possibly protective function of scopolin in the endodermis. But it would have to be a pathogen, against which *Arabidopsis* wild type plants have a relatively high resistance. Pathosystems for leaves are numerous, for example with *Pseudomonas* (Grosskinsky et al., 2011), *Peronospora* (Weigel and Glazebrook, 2001) or *Xanthomonas* (Feng et al., 2012), but pathosystems specific for *Arabidopsis* roots are rare besides the well-established *Plasmodiophora* system. *Verticillium* might be a good choice, but WT Col-0, the background of *f6'h1*, was reported to be rather susceptible to it (Ralhan et al., 2012), *Fusarium* might be another option, but its pathogenicity on *Arabidopsis* roots would have to be tested at first, which is also true for a *Phytophtora* system. Another possibility for a pathosytem might be the application of *Pseudomonas* to roots (Millet et al., 2010).

Cold stress induces high-level scopolin accumulation in *Arabidopsis* leaves

Arabidopsis leaves had been described as poor accumulators of scopolin (Kai et al., 2006), their contents being 180 times lower than in roots. Indeed, in leaves under standard conditions, scopolin was usually lower than 0.1 µg/g FW, whereas in roots the measured scopolin contents ranged from 10 µg to 600 µg under different conditions. However, the cold stress experiments presented above were marked by a dramatic increase in scopolin to 3-50 µg and more, which rivalled quite the lower levels of scopolin measured in roots. This new finding raises the question of a function of scopolin or its aglycone scopoletin under cold stress.

Cold stress application in *Arabidopsis* leads to changes in expression levels of several hundred genes and to dramatic alterations in plant metabolite profiles (e.g. (Hannah et al., 2005; Kaplan et al., 2007). These changes are adaptations to growth at low temperatures and preparations to survive freezing. Freezing damage control is achieved by keeping the water within the cell to prevent the formation of ice crystals between cell wall and cell membrane. The formation of large crystals causes tissue disruption. Membranes and proteins have to be stabilized to maintain their functions. The water is kept in the cell by the accumulation of osmotically active so-called compatible solutes like sugars, proline or betaine (Yancey et al., 1982).

But already at above-freezing temperatures the plant is prone to damage by cold: Most chemical reactions in plants follow the rule of van-'t Hoff, which says that they are slowed down at cold temperatures, but the light reaction in photosynthesis does not (Chance and Nishimura, 1960). Intermediates cannot be used by the slowed-down enzymes of the Calvin-Cycle and reactive oxygen species occur. These are highly detrimental to all molecules in the cell, but also thought to be an important signal for the cold-acclimation process. Also, by the destruction of photosystems by ROS the pressure of the accumulation of photosynthesis products will be attenuated. Yet, ROS have to be scavenged to stop the chain reaction and prevent too much damage. To protect the plant from excess light, phenylpropanoids are thought to be accumulated as UV screens and visible light absorbers or dissipaters (Havaux and Kloppstech, 2001). Scopoletin itself has reactive oxygen scavenging properties, its effectiveness depends on the molecule to be scavenged (see introduction). Additionally, it preserves the activity of GSH (gluthathion), SOD (superoxide dismutase), GST (gluthathion S-transferases) and prevents lipid peroxidation (Kang et al., 1998). Furthermore, scopoletin inhibits the formation of peroxynitrite (Kang et al., 1999). Therefore, it would be a good candidate to offer protection against ROS from photosynthesis.

Absolute scopolin accumulation in the presented experiments depended on the facility used for cultivation. The major difference between those was the quality of light. High UV seemed to increase the scopolin accumulation under cold stress. But in a UV-free incubator, there was still a reaction of scopolin. The kaempferol derivatives reacted more independently from the growing facilities. Korn et al. (2008) already suggested for those a UV-light-independent function under cold stress, like membrane stabilisation or ROS scavenging in the chloroplast. However, even if scopolin does only react to ROS production caused by excessive light during cold stress it would still be a component of cold reaction, as cold stress and high-light stress are usually related in natural habitats, when incoming light energy cannot be used due to slowed down metabolism.

AtTHO1, a member of the mRNA-processing THO/TREX complex, influences scopolin accumulation and root growth under stress conditions.

In the experiments presented in this work, the mutant GK052D02 containing a T-DNA-insertion in AtTHO1 failed to react towards cold stress with scopolin accumulation in leaves. Challenged with high-sucrose, wild type plants accumulated scopolin in roots, shortened their root growth and initiated the development of secondary roots. The mutant was impaired in all of these reactions. Summarized in a simple model, the WT plant percepted a stress, cold-, high-sucrose- or osmotic stress, and the intact AtTHO1 mediated a signal which finally led to enhanced scopolin accumulation.

By overexpression, deletion mutation and complementation of the deletion mutation with AtTHO1, a positive influence of this gene on scopolin and scopoletin accumulation was shown. This effect on scopolin accumulation could also be shown for other members of the THO/TREX complex in *Arabidopsis*. A deletion mutant of *attho1*, GK052D02-3 had the additional phenotype of altered root growth under stress conditions. In that mutant, four

other genes were differentially expressed. However, this mutant line had a second insertion into the gene MYB40, which might have influenced the expression of these genes and root growth. It also contributed very slightly to the scopolin phenotype. Further analyses of newly-available insertion mutants of AtTHO1 and MYB40 will clear up the additional traits. Focussing on the influence of AtTHO1 on the accumulation of scopolin/scopoletin, a model will be presented that combines the observed phenotype with the functions of the THO/TREX complex suggested in literature (table 11).

Table 11: Overview over publications about AtTHO1.

Authors	Title	Content	Reference
Furumizu, C. Tsukaya, H. Komeda, Y.	Characterization of EMU, the Arabidopsis homolog of the yeast THO complex member HPR1	AtTHO1 (EMU) was identified as homologous to a member of the yeast THO complex. The EMU mutant was found to accumulate unprocessed mRNA of the developmental regulator ERECTA and had a slightly increased occurrence of deformed seedlings.	Furumizu et al. (2010)
Yelina, N. E. Smith, L. M. Jones, A. M. Patel, K. Kelly, K. A. Baulcombe, D. C.	Putative Arabidopsis THO/TREX mRNA export complex is involved in transgene and endogenous siRNA biosynthesis	Eight loci in *Arabidopsis* for members of an AtTHO/TREX complex were identified, including AtTHO1. TEX1 was shown to interact with the predicted members. TEX1 mutants were defective in the processing of a long siRNA precursor. The authors postulate a function of the AtTHO/TREX complex in cell-to-cell and long-distance signalling by RNAi-silencing.	Yelina et al. (2010)
Jauvion, V. Elmayan, T. Vaucheret, H.	The conserved RNA trafficking proteins HPR1 and TEX1 are involved in the production of endogenous and exogenous small interfering RNA in *Arabidopsis*	The authors postulate the existence of a THO/TREX complex in plants. They identify AtTHO1 (HPR1) and AtTEX1 as homologs to members of the animal THO/TREX complex. They showed that both genes are involved in post-transcriptional gene silencing, because mutants accumulated siRNA precursors and had a decreased accumulation of mature siRNAs. They propose that both HPR1 and TEX1 participate in the trafficking of siRNA precursors to the ARGONAUTE catalytic center.	Jauvion et al. (2010)
Pan, H. Liu, S. Tang, D.	HPR1, a component of the THO/TREX complex, plays an important role in disease resistance and senescence in *Arabidopsis*	HPR1 mutants were shown to be more resistant to necrotrophic fungi and more susceptible to biotrophic pathogens. A function as a switch between two competing defence reactions was proposed. HPR1 was also shown to counteract ethylene induced senescence.	Pan et al. (2012)

AtTHO1 plays a role in the production of microRNAs (miRNAs), which are loaded on messenger ribonucleoprotein (mRNP) particles which mediate mRNA processing (Furumizu et al., 2010; Jauvion et al., 2010; Yelina et al., 2010). Furumizu et al. (2010) showed that the loss of AtTHO1 also led to a loss of alternative splicing variants. AtTHO1 is also involved in the production of small interfering RNAs (siRNAs). These siRNAs or their precursors can

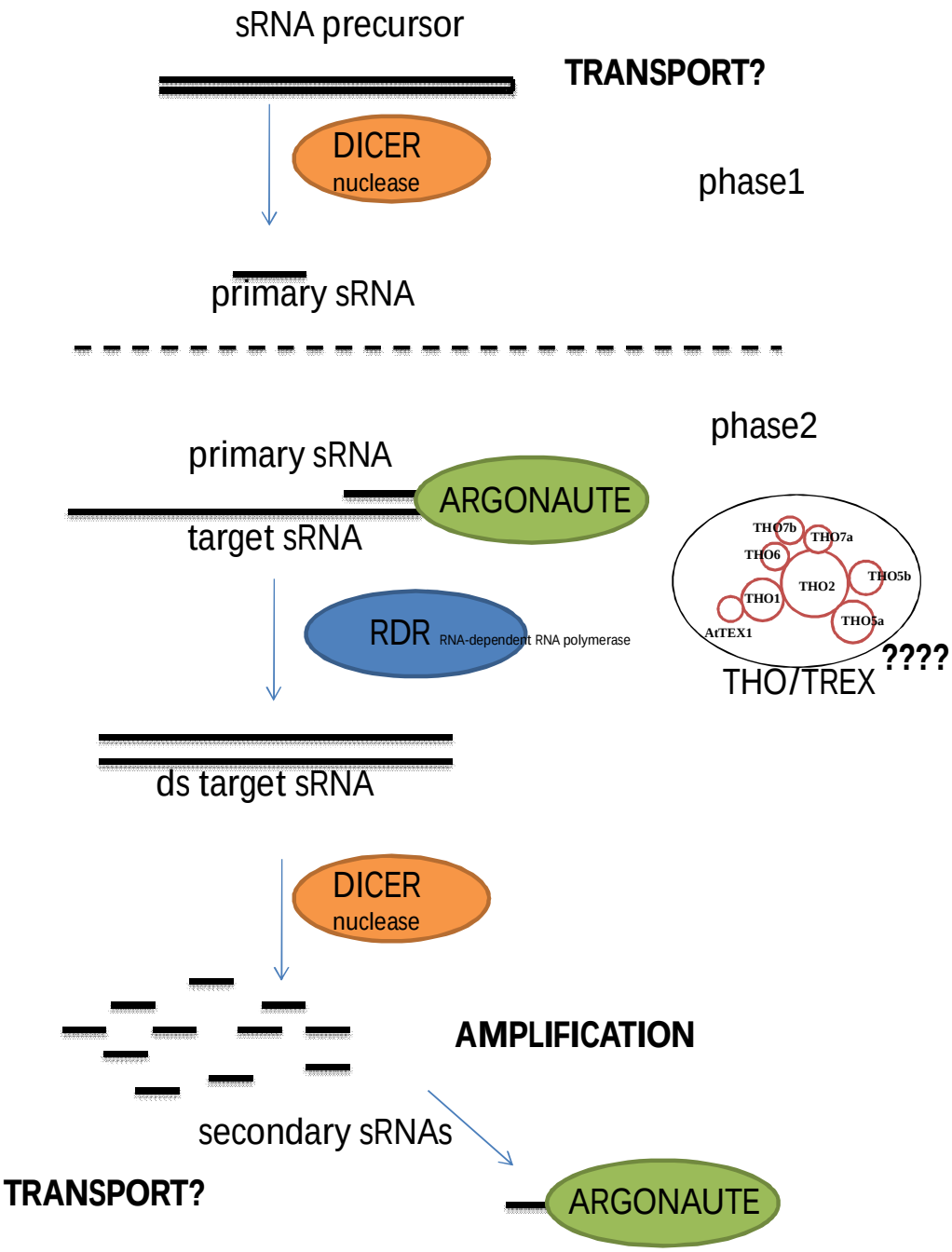

Figure 52: Intercellular and systemic RNAi-silencing in plants: Phase 1: A longer s(small)RNA-precursor molecule in a hairpin-structure is processed by DICER nuclease into primary sRNAs (miRNAs or siRNAs). Phase 2: The primary sRNA is bound to an ARGONAUTE protein and guided to a second target single-strand sRNA. An RNA-dependent RNA polymerase (RDR) synthesizes a second strand to form a double-strand sRNA. This is cut by DICER into many siRNAs. These are loaded on an ARGONATE molecule and guided to a complementary RNA or DNA. A targeted RNA molecule can be cleaved, repressed or destabilized (post transcriptional gene silencing). Targeted DNA or histones can be epigenetically modified (transcriptional gene silencing). Any of the small RNAs might be transported cell-to-cell or in the phloem. The suggested role of the THO/TREX complex is to affect the transport of a siRNA precursor from its place of synthesis to the place of processing (after Yelina et al. (2010) and Melnyk et al. (2011)). The size of the depicted components of the THO/TREX corresponds to their actual size in kDa.

move between cells via plasmodesmata, move to and cause silencing in a sink tissue (Jauvion et al., 2010; Yelina et al., 2010). Figure 52 shows the mechanism how a silencing signal is produced and amplified and the components involved in this process. There are 7179 loci in *Arabidopsis* for small RNAs, from which 35% are associated with mobile signalling (Molnar et al., 2010). The THO/TREX complex is not a general requirement for sRNA biosynthesis, but is selectively recruited to some precursor molecules. The cause for this selectivity is not known (Yelina et al., 2010).

AtTHO1 was shown to influence the developmental regulator ERECTA, a serine/threonine kinase, which is amongst other functions involved in determining organ specifity (Furumizu et al., 2010). For this reason, there was the possibility that cell identity in the analysed mutants was affected, leading to a deviating set of accumulated secondary metabolites. However, the presented results showed no abnormal tissue organisation, and scopolin was accumulated in the same tissue in wild type and mutant. An influence of AtTHO1 on scopolin accumulation via regulation of organ-development by ERECTA does not seem likely. However, ERECTA is regarded as a pleiotropic regulator of developmental and physiological processes, as well as a modulator of responses to environmental stimuli (van Zanten et al., 2009). A disease resistance related to ERECTA was shown, which was independent of its functions in organ development (Sanchez-Rodriguez et al., 2009). For this reason, an involvement of AtTHO1 and ERECTA on scopolin accumulation is possible. Interestingly, the *A. thaliana* ecotype variant Landsberg erecta carries a mutation in the ERECTA gene, and preliminary analyses of the scopolin contents of different wild types showed a tendency of Landsberg erecta to accumulate less scopolin than other ecotypes from the presented study (appendix figure A5).

In this study, phenotypic differences between wild type and *attho1* plants were only pronounced under stress conditions, an observation which was confirmed by Pan et al. (2012). For this reason, the importance of the THO/TREX complex must be specific for challenging conditions. Interestingly, Pan et al. (2012) report that AtTHO1 was necessary for resistance against the biotrophic powdery mildew *Golovinomyces cichoracearum*, *Pseudomonas syringae* (bacterium) and *Hyaloperonospora* (oomycete). In particular, it was connected to induced cell death and contributed to the production of hydrogen peroxide in the oxidative burst during a hypersensitive reaction. In their model, the gene ENHANCED DISEASE RESISTANCE1 (EDR1) promotes the defence against necrotrophic pathogens and blocks defensive measurements against biotrophic attacks. Consequently, the loss-of-function mutation edr1 was resistant against the abovementioned biotrophic pathogens and more susceptible towards the necrotrophic *Alternaria brassicicola* and the hemibiotrophic *Colletotrichum higginsianum*. The *edr1* resistance acts via the salicylic acid pathway and not the ethylene pathway. Within an *edr1*/*attho1* double mutant, the biotrophic resistance was lost. Furthermore, AtTHO1 influenced callose disposition and suppressed the expression of senescence-related genes after ethylene treatment. They showed also that AtTHO1 is expressed in all tissues and the protein is localized in the nucleus only. They see the role of

the THO/TREX complex in the control of the nucleo-cytoplasmatic trafficking of important regulators of defence. In their model, AtTHO1 plays the role of a switch between two competing defence reactions.

The phenotype observed in *attho1* in this study (reduced scopolin accumulation and lateral root formation) was most pronounced under stress conditions. In these conditions, the mutant resembled the WT phenotype under standard conditions. Therefore, this signalling chain in which AtTHO1 is involved is most likely a chain for stress perception or stress response.

The postulated "switch" from standard growth to stress response caused by AtTHO1 could be achieved by alternative splicing variants of mRNAs, the processing and transport of RNA signals as silencers for the "normal" metabolism or even the production of small RNAs as long-distance signals towards meristems and scopolin producing/accumulating tissues or ectopic regulators of gene expression. Targets for this post-translational control are consequently members (or a single member) of a signalling chain of transcription factors or signalling molecules (like the kinase ERECTA), leading finally to the observed "low-scopolin/longer-and-less-branched roots"-phenotype.

Expression analysis of the genes in the mutant line with an insertion into AtTHO1, GK052D02, resulted in some interesting candidates that might be downstream components of the signalling chain influenced by AtTHO1[7]. The unique growth factor ATPSK2, which is related to branching and was lower expressed in the mutant GK052D02, is very likely involved in the formation of lateral roots. The RING-U-Box protein At1g53490, also lower expressed in the mutant, had a strikingly similar regulation as AtTHO1. Another RING-protein with ubiquitin-ligase activity (KEEP ON GOING – KEG) is involved in the abovementioned edr1-resistance as well (Wawrzynska et al., 2008). The unknown protein At3g05770 may be another downstream component. Insertion mutations of both genes had lower scopolin contents than wild type, but not as low as GK052D02. The overabundance of the transposon RNA of At1g43590 in the mutant is an interesting find, since it is the current opinion, that transposon transcription is activated by stress and that they are controlled by RNA silencing (Mirouze and Paszkowski, 2011). Hence, the THO/TREX complex might be such a controller of potentially gene disrupting transposition. As an insertion mutation into the transposon DNA produced no phenotype, a connection to the observed phenotype is very unlikely. Figure 53 shows a model for the AtTHO1-mediated response causing the high-scopolin/branched root phenotype in WT as a reaction to stress.

It is highly probable, that AtTHO1 acts in an assembly variant of the THO/TREX complex, as the mutants of AtTHO2 (although still heterozygous) AtTHO3, 5a and 7b also had a reduced scopolin accumulation. (The difference in the scopolin contents between the members of these members of THO complex and the investigated mutant line GK052D02 might be

[7] Gene expression analysis had to be done on a mutant with an additional insertion mutation (*myb40*) and needs verification with another *attho1* and/or *myb40* line, which have become available only recently.

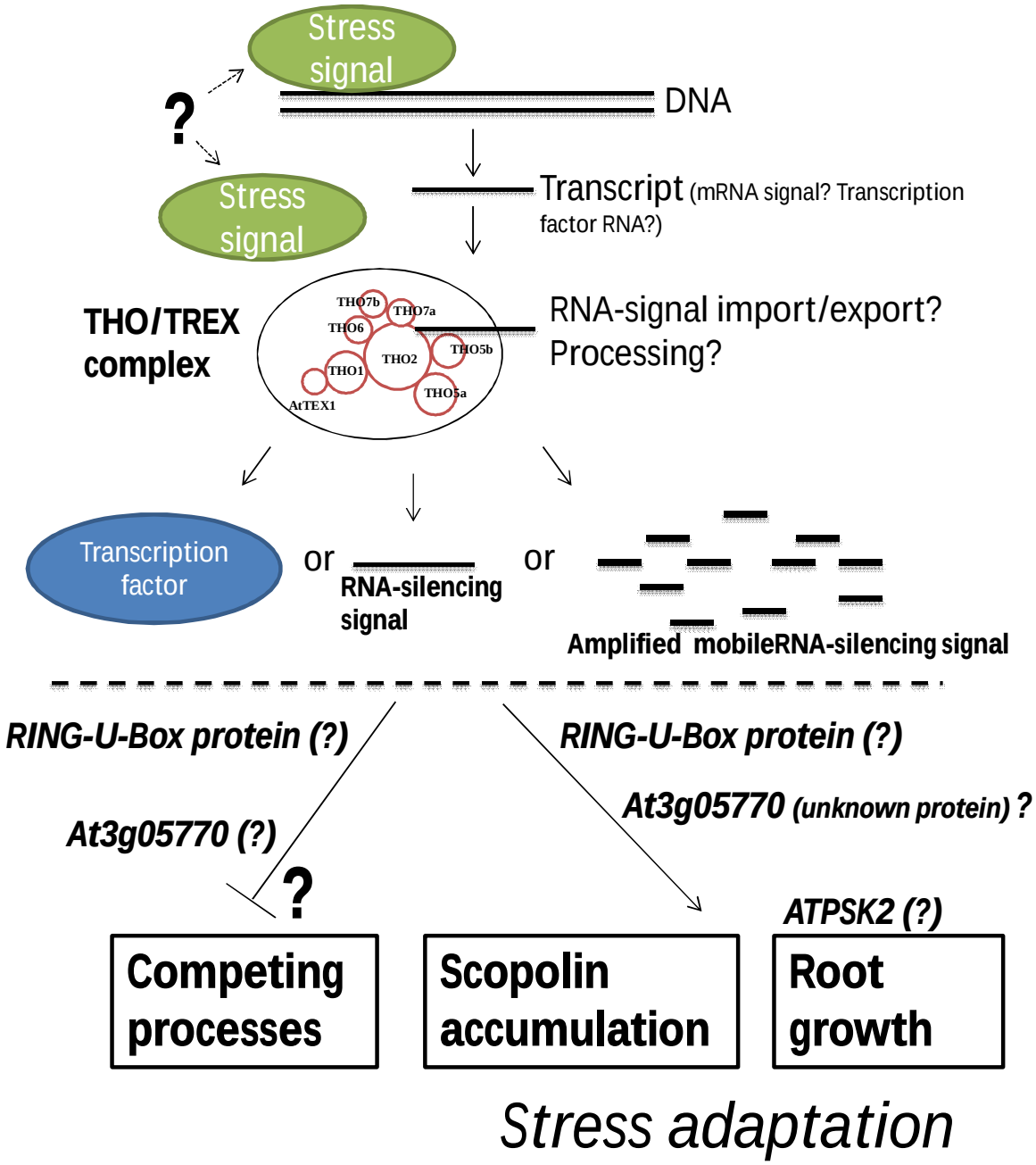

Figure 53: Working model for the role of AtTHO1 in scopolin accumulation and root growth under stress conditions: A stress signal activates the transcription of a DNA-locus or the processing of an already present RNA molecule by the THO/TREX complex. The complex might process the RNA or mediate the transport towards a processing or silencing site. The result is a signal which is able to affect scopolin accumulation and root growth. This can be an alternative splice variant of a transcription factor, a silencing RNA or a moving sRNA signal which might trigger a systemic reaction. The signal might be a negative regulator of "normal" processes (acting as a "switch") or an activator of stress defence. This model is based on the phenotype and expression analysis of the AtTHO1 mutant GK052D02. The genes coding for the RING-U-Box protein, At3g05770 and ATPSK2 were higher expressed in WT than in GK052D02. Insertion mutations of RING-U-Box and At3g05770 had reduced scopolin contents. ATPSK2, which is a precursor to a unique growth factor present at the branching sites of roots, but not at the root tip, is associated with secondary root formations. References: see text.

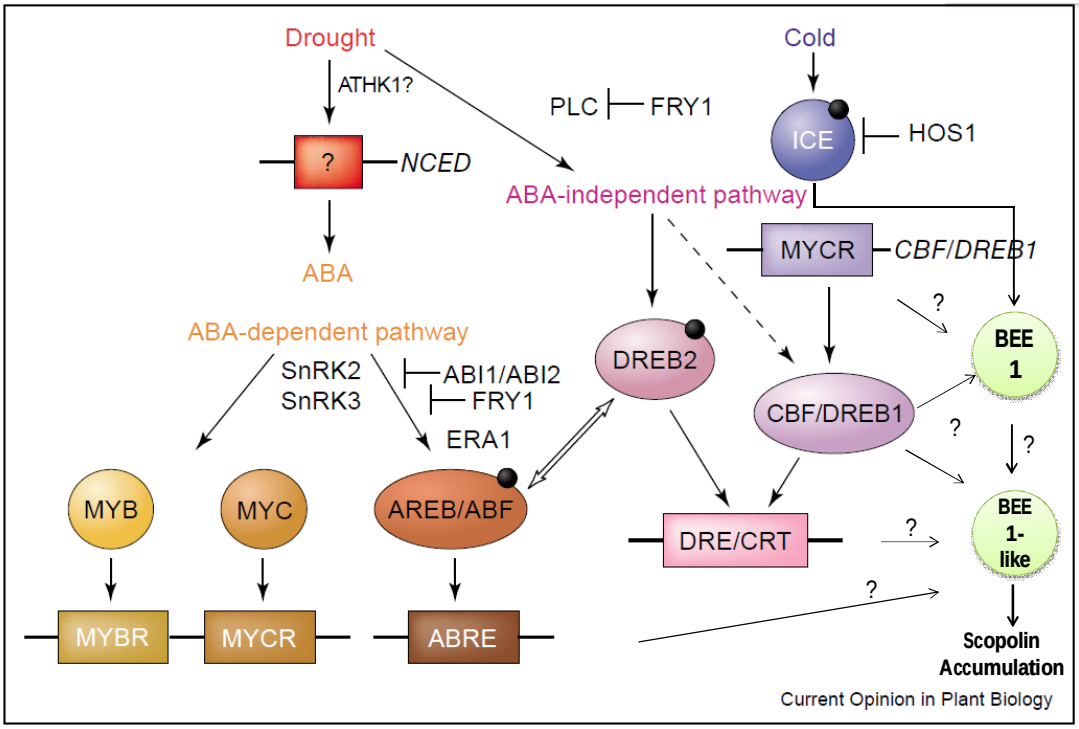

Figure 54: Cold stress signaling in *Arabidopsis thaliana* and its connection to drought stress signalling. Many genes are activated by both stresses, so a cross talk is postulated. CBF/DREB1 is involved in draught and cold stress signaling. DREB2's main function is draught resistance. It does affect cold resistance only slightly. ABA signalling is not involved in cold-stress signalling. BEE1 is activated by ICE1. Mutations in BEE1 and BEE1-like have similar effects on scopolin accumulation under cold stress. Boxes: cis-acting elements, ovals and circles: transcription factors (Shinozaki et al. (2003), with the putative scopolin signalling pathway added.)

explained by an additional effect of its second insertion into MYB40). Interestingly, the expression patterns in roots of the members of the THO/TREX complex with a scopolin phenotype in deletion mutations match quite well. The diverging expression patterns of the other members of the AtTHO complex suggest that it might be assembled differently in different tissues. This suggests that it could have various functions, for which not all members are necessary for each task, a view also held by Yelina et al. (2010).

Transcription factors BEE1 and At5g45580 ("BEE1-LIKE") are involved in scopolin accumulation under cold stress

Under cold stress, two insertion mutation lines were isolated with a strikingly identical phenylpropanoid profile. This profile diverged from wild type first and foremost, because no scopolin accumulated in leaves after exposure to 10°C. But there was also a minor reduction of kaempferol derivatives and a substance not accumulating in wild type. The function of At5g45580 ("BEE1-LIKE"), annotated as a MYB-like transcription factor, is completely unknown. Promoter analysis points to a role in dehydration besides cold stress. BEE1 is involved in brassinosteroid signalling and is activated by ICE1, the main cold-mediating transcription factor in *Arabidopsis*. Judging from the identical profile, BEE1 and BEE1-like might be members of the same signal transduction chain, leading to modulation of the

phenylpropanoid content under cold stress. Cold stress signalling is in some parts interlaced with drought stress signalling with or without ABA signal transduction (figure 55). To elucidate their role in the cold stress signalling chain, expression levels of BEE1 and BEE1-like, CBF1 (early responding cold stress gene), Cor15a (late responding cold stress gene) and F6'H1 (late synthesis gene) in WT and deletion mutations of BEE1, BEE1-LIKE, ICE1, CBF1 and an ABA-deficient line shall be analysed in a follow-up project to this work. This project will also analyze the reaction of BEE1 and BEE1-LIKE in various stresses like UV or high-sucrose.

Materials and Methods

General procedures

Sterilisation of seed material

A small aliquot of seeds was incubated in 1 ml of ethanol 70% (v/v) for 2 minutes. The liquid was removed and replaced by 500 µl sodium hypochlorite solution (NaOCl[1] 5% (v/v)) with 20 µl Tween 20[2]. Incubation time was 8 min. The bleach was removed and seeds were rinsed trice with sterile water. For easier distribution, seeds were suspended in 0.1% (w/v) sterile agarose[3] (modified after Schlesier et al., 2003).

Cultivation of *Arabidopsis thaliana* on agar plates

Sterilised seeds were placed with a distance of at least 1 cm to each other on agar plates containing ½ MS (2,2 g/l Murashige & Skoog medium including vitamins[4] (Murashige and Skoog, 1962), 0.5% (w/v) sucrose[1] and 0.8% (w/v) plant agar[4] (gel strength > 1100 g/cm²), pH 5.7-5.8). Plates were sealed with Parafilm® M[1] and kept 3-4 days at 4°C, before plants were grown for 3-4 weeks on cultivation shelves (day 16 h, 25°C; night 8 h, 20°C).

Cultivation of *Arabidopsis thaliana* in sterile hydroponic culture

Sterilized and stratified seeds were grown for four weeks in a hydroponic culture system as described in Schlesier et al. (2003). This system consists of a ¼ litre glass mould jar with lid and clamps[5], a stainless steel plantlet carrier and a light-protective pot. A simplified variant of the published protocol was applied: first, the nutritive solution was filled exactly as high as to touch the wire cloth of the plantlet carrier from below. Then, the seeds, already suspended in 0.1% agarose, were distributed evenly on the mesh with a 200µl pipette or toothpick (up to 10 seeds per jar). The nutrient solution consisted of ¼ MS (1,1 g/l Murashige & Skoog medium (Murashige and Skoog, 1962) including vitamins[4] and 0.5-3% sucrose[1] as required by the experiment (figure 56).

Figure 55: Hydroponic culture system for controlled growth conditions and easy root harvest

Cultivation of *Arabidopsis thaliana* in soil culture

Seeds were sown on a low-nutrient substrate ("Substrat 1"[6], a peat substrate for low to medium nutrient requirements, fine structured, N 140 mg/l, P 160 mg/l, K 180 mg/l, Mg 100

mg/l S 120 mg/l, with trace elements), stratified 3-4 days at 4°C, kept in a growth chamber with a 9/15 hr day/night period at 21/18°C. Level of illumination was 120-150 µmol m^{-2} s^{-1}. After 2 weeks, they were transplanted into single pots in the same substrate. Six-week-old plants were transferred to a green house with 16 h of supplemental lighting to induce flowering.

Imaging system for root fluorescence

Imaging system, version 1

Three- to four-week-old plants grown on agar plates were photographed with the gel documentation system Diana II[22]. UV light for optimum scopolin fluorescence was generated by full UV filtered by an additional 200 x 200 x 2 mm filter plate (transmittance 250-400 nm, optimum 340 nm)[23], which was placed below the agar plate. An emission filter with an optimum transmission at 460 nm (D460/50 m emission interference filter, 25 mm diameter)[22] was placed in front of the camera lens. Camera settings: Exposure Time 10sec, scan 2 times, Gamma: 0.98 (Diana/95 Version 1.6). For easier evaluation, the Diana grey scale picture was converted into a false colour image by AIDA software (Advanced Data Image Analyzer Version 2.3.007[22]).

Imaging system, version 2

Agar plates with 3-4 weeks-old plants were placed upside-down inside the dark-chamber of the fluorescence imaging system Quantum ST4[24]. Excitation was done by epi-UV at 365nm. Emission light was filtered by a 440nm-filter (F-440M58[24]). The image was taken using the camera software "Quantum-capt version 15.17".

Extraction of phenolic compounds from roots and leaves for HPLC analysis

Root material from hydroponic culture was harvested with a scalpel, dried diligently with tissue paper and frozen immediately in liquid nitrogen. Samples from soil were rinsed quickly in a three-step washing system, dried with tissue paper and frozen. Leaves were frozen immediately after harvest. All samples were kept at -80°C. Samples larger than 150 mg were pre-homogenised with mortar and pistil and only an aliquot used for analysis. Per 100 mg sample, 400 µl methanol (Lichrosolv Methanol Gradient Grade[7]) was added without thawing the sample beforehand. Homogenisation was carried out with a tissue homogenizer (Precellys 24[8]) and zirconium silicate (58%) grinding beads, Ø 1.0-1.2 mm (RIMAX ZS-R[9]) for 2 x 45 sec. at 5500 rpm in 2ml reaction tubes. Samples were centrifuged in a table top centrifuge at 16400 rpm/28500 x *g*, at 4°C for 10 min. The supernatant was transferred to a new tube and the pellet resolved in the same amount of methanol as in step 1, and the centrifugation step repeated. Both supernatants were mixed and centrifuged again for 10 min. Before injection, an aliquot of the sample was diluted to 80% with the acidic solvent from the aqueous phase of the HPLC run and centrifuged again for 5 min.

Methods used for liquid chromatography

Several HPLC methods were used because of improvements in hardware and separation techniques. The respective method for each experiment is indicated.

Preparation of standards: Scopolin, produced from scopoletin[10] by a recombinant glycosyltransferase (Matros and Mock, 2004), was quantified with a β-glucosidase assay (Maslak, 2002). Peak identification for scopolin and scopoletin in raw extracts of roots: retention time was determined by overlays with standards, further validation was done by extraction of UV spectra and comparison to the standard, by spiking the raw extract with the standard and MS analysis with a liquid chromatography- time of flight mass spectrometer (LCT premier[11]). Isoscopolin was produced from isoscopoletin[10] with the same method as scopolin.

HPLC method 1

A Waters Alliance System[11] fitted with a Jasco FP-1520 Fluorescence detector[13] was used. Separation was done on a C18 column (Aqua 5 µm; 4,6x250 mm; RP 18; 125 Å[14]). Solvent A was 98% (v/v) water (Lichrosolv[7]) and 2% (v/v) of a solution of 5% (w/v) ammonium formate[15] in formic acid[16]. Solvent B was acetonitrile[15]. Gradient: from 0min, 97% A to 20 min, 85% A, slightly convex curve. From 20min, 85% A to 50 min, 60% A, slightly convex curve. From 50 min, 60% A to 60 min, 0% A, linear curve. The flow was 1 ml per minute, column temperature was 25°C. Fluorescence was measured with an excitation wavelength of 340 nm at 430 nm emission wavelength (based on Mock et al., 1999).

HPLC method 2

A Waters ACQUITY UPLC system fitted with an ACQUITY UPLC® Photodiode Array (PDA) Detector[11] and a Jasco FP-920 Fluorescence detector[13] with a 5-µl-flow cell was used. The column used was an ACQUITY UPLC BEH C18 Column, 2.1 x 50 mm, 1.7 µm with an ACQUITY BEH Shield RP18 VanGuard Pre-column, 1.7 µm, 2.1 x 5 mm[11] and an elution gradient with solvent A (v/v: 98% water (Lichrosolv[7]) with 2% of an ammonium formate[16] -formic acid[15] solution. (5% w/v) and solvent B (acetonitrile[15]) from 0 min to 1 min, 100% A, from 1 min to 3 min to 85%A, from 3 to 6 min to 60% A, from 6 to 7 min, to 0% A, with linear curves and a flow of 0.55ml per minute (adaptation of HPLC method 1 for UPLC by A. Kothe). A 1.5 min delay was added to restore the initial conditions. Column temperature was 30°C. Fluorescence was measured with an excitation wavelength of 340 nm at 430 nm emission wavelength; UV data were extracted at 280 nm.

HPLC method 3

As HPLC method 2, but the Jasco Fluorescence Detector was replaced by an ACQUITY UPLC® Fluorescence (FL) Detector[11]. Scopolin and scopoletin were quantified by fluorescence detection at 336 nm excitation/438 nm emission wavelenght.

HPLC method 4

This separation method followed Yonekura-Sakakibara et al. (2008): The Waters Aquity UPLC system was used with a BEH phenyl column (Waters ACQUITY UPLC BEH Phenyl Column, 130Å 2.1 x 100 mm, 1.7 µm, product number 186002885) and an ACQUITY UPLC BEH Phenyl VanGuard Pre-column, 130Å, 1.7 µm, 2.1 mm x 5 mm, product number 186003979[11]. The column temperature was 35°C. The gradient was linear from 0min, 100% solvent A (0.1% (v/v) formic acid[15] in 18MΩ water (Milli-Q[17])) to 10min, 40% B (0.1% (v/v) formic acid[15] in

acetonitrile[15]). Solvent B was brought up to 100% after each run to clean the column and the initial conditions were restored in a delay of 3.5min. Samples were evaluated with UV detection at 280nm and fluorescence detection at 300/400 nm, 336/438 nm and 360/450 nm.

Isolation of plasmid DNA

Extraction of plasmid DNA from bacteria was carried out as follows, based on a protocol for alkaline lysis from Birnboim and Doly (1979): the bacterial pellet from 1.5 ml over-night culture was resolved in 100 µl lysis buffer (50 mM TRIS[1]/HCl, 50 mM EDTA[20] pH 8.0, 15% sucrose[1]). Proteins were denaturated by adding 200 µl SDS solution (200 mM NaOH, 1% (w/v) SDS[1]) and 150 µl of a cooled solution of 3 M potassium acetate[7] with 11.5% (v/v) acetic acid[1]. Samples were incubated on ice for 10 min and centrifugated at approx. 10000 x *g* at 4°C. Isopropanol (0.6 volumes) was added to the supernatant and vortexed vigorously. DNA was precipitated with 10 min of centrifugation at approx. 18000 x *g* at 4°C. The pellet was washed with 300 µl of ice-cold ethanol (70% v/v) and another centrifugation step of 5 min, 18000 x *g* and 4°C. The pellet was dried and resolved in 35 µl water in a shaker at 70°C for 30 min.

Polymerase chain reaction

Polymerase chain reactions were performed with a Taq polymerase (DreamTaq[33]) according to the manual. Primers were designed following the "Guidelines for primer design" from the Fermentas DreamTaq Polymerase manual (2008): Length 20-30bp, difference of melting temperature < 5°C, GC content 40-60%, primer should end on G or C, but avoiding of more than 3 G or C at the 3' end. Primers were checked for hairpin formation, 3' complementarity and self-annealing with OligoCalc (Kibbe, 2007) and ordered from MWG-Biotech[19].

***Agrobacterium*-mediated plant transformation**

Expression vectors with insertions of plant DNA were transferred into the *Agrobacterium* strain C58C1, containing the pGV2260 plasmid (Deblaere et al., 1985) according to Hofgen and Willmitzer (1988). *Arabidopsis* plants were transformed with the floral dipping method of Clough and Bent (1998), with minor changes: Agrobacterium was grown in 400ml YEB-medium (Vervliet et al., 1975); and a modified infiltration medium following Bechtold et al. (1993): 2.2g/l Murashige & Skoog medium including vitamins[4] and 5 % (m/v) sucrose[1], 0.0187µM benzylaminopurine[16], 0.1g/l acetosyringone[16], 0.025% Silwet L-77 ("Vac-In-Stuff"[31]), pH 5.7, was used.

SDS gel electrophoresis and blotting

SDS-Page gel electrophoresis was done after Laemmli (1970) in a vertical 20x10cm double gel system (Perfect Blue[48]) at 175 V, 300 mA and room temperature. Loading gel: 13% (v/v) of 30% acrylamid/N,N'methylenbisacrylamid (37.5:1) solution (Rotiphorese® Gel 30); 25% of 0.5M Tris-HCl; 1% of 10% (m/v) SDS solution; 0.5% of 10% (m/v) ammonium persulfate solution; 0.2% TEMED; 60.3% water. Separation gel: 32% (v/v) of 30% acrylamid/N,N'methylenbisacrylamid (37.5:1) solution (Rotiphorese® Gel 30); 24% of 1.5 M Tris-HCl; 1% of 10% (m/v) SDS solution; 0.5% of 10% (m/v) ammonium persulfate solution;

0.1% TEMED; 42.4% water). After separation, the SDS gel was equilibrated for 5 min in a solution of 25mM Tris and 20% (v/v) methanol, pH 10.4 (anode solution II). A transfer of proteins was done with a semi-dry-blotter[49] in a blot of the following assembly: on top of the anode plate were 6 layers of blotting paper (BF3[50]) soaked in a solution of 0.3 M Tris and 20% (v/v) methanol, pH 10.4 (anode solution I), then 3 layers of blotting paper soaked in anode solution II, a PVDF membrane (Immobilon-P Membrane, 0.45 µm[17]), wetted with methanol, rinsed in water for 2 min and equilibrated in anode solution II for 2 min, then the SDS gel, then nine layers of blotting paper soaked in a solution of 40 mM 6-aminohexanoic acid, 0.01% (m/v) SDS and 20% (v/v) methanol (cathode solution) and finally the weighed down cathode plate on top. The blot was run for 1.5 hours with a current of 0.8 mA/cm^2 gel area and 50 V. The aluminium-based staining protocol of Kang et al. (2002) was applied for colloidal Coomassie staining of proteins. After electrophoresis, gels were washed twice with water for 10 min and subsequently incubated in staining solution (5 % aluminium sulfate-(14-18)-hydrate, 0.02 % CBB-G250, 10 % ethanol, 2 % ortho-phosphoric acid) for 1-3 h and destaining solution (10 % ethanol, 2 % ortho-phosphoric acid) for 30 min to visualize the proteins (Hedtmann, 2012). (All chemicals from Carl Roth[1].)

Immunodetection

For immunodetection, the PVDF membrane was incubated after protein transfer overnight in a blocking solution (10mM Tris-Cl[1] pH 8.0, 150mM NaCl[1], and 0.05% (v/v) Tween20[51] (TBST) and 1% (m/v) bovine serum albumin[51]) after washing the membrane three times in TBST. The first antibody (against the His-tag of the recombinant protein = monoclonal anti-polyHistidine antibody produced in mouse[12] 1:4000 in TBST and 0.5% (m/v) bovine serum albumin or the self-produced antibody against AtTHO1 1:1000) was left on the membrane for 1 hour and excess removed with washing three times with TBST. The second antibody was an anti-mouse IgG (whole molecule)-alkaline phosphatase antibody produced in rabbit[12] or an anti –rabbit AP antibody for AtTHO1, which were applied for 30min in a 1:5000 solution in TBST with 0.5% (m/v) BSA. Excess antibody was removed by washing trice with TBST. Staining of bands was done in a NBT/BCIP[16] reaction catalyzed by the alkaline phosphatase of the second antibody according to the BCIP manual.

Statistical analyses

For comparison of mean values to detect significant differences between two or more groups, the software SigmaPlot[21] was used. Numeric data was tested for normal distribution (Shapiro-Wilk) and equality of variances. If the data set passed both tests, a t-test against the control or respectively an ANOVA with a *post-hoc* test for multiple comparisons was performed. Result graphs for normally distributed data contained the arithmetic mean value and the standard deviation. For not-normally distributed data and for non-numeric data, a Mann-Whitney-U-test against the control group, was used. For more groups, the Kruskal-Wallis test followed by a *post-hoc* test was applied. For this type of data, box plots depicting the median, the 25% and 75% percentiles, the 5% and 95% percentiles and the outliers were shown. The applied *post-hoc* tests are stated in each experiment.

Localisation of scopolin in roots

Plant material

Locus	Gene	Line	Type	Origin	Primer for PCR on insertion
Arabidopsis thaliana					
At4g34050	CCoAOMT1	SALK_151507C (NASC Stock No. N664340)	T-DNA insertion	NASC[25]	See Kai et al. (2008)
At3g13610	F6'H1	SALK_132418C (N658415), (Alonso et al., 2003);	T-DNA insertion	NASC[25]	See Kai et al. (2008)
-	-	Col-0 (N1092)	wild type	NASC[25]	
Nicotiana tabacum					
-	-	cv. SamsunNN	wild type	Bioplant, "Vereinigte Saatzuchten eG" Ebsdorf, Germany	

Homozygosis of offspring lines for *ccoaomt1* and *f6`h1* was tested by PCRs with the primers suggested by Kai et al. (2008). DNA extraction was done according to Edwards et al. (1991) using a commercial homogenizer (Precellys24[8]) and zirconium silicate (58%) grinding beads, Ø 1.0-1.2mm (RIMAX ZS-R[9]). HPLC analyses were performed using HPLC method 2.

Confocal laser scanning fluorescence microscopy

Optimum fluorescence conditions of scopolin and scopoletin were determined using dilutions of HPLC standards (see: "Methods used for liquid chromatography" page 109) in an acetic acid/Na-acetate buffer (pH 5.0) with a dual scanning micro plate spectrofluorometer (SPECTRAmax Gemini[26]). The tested range for the excitation spectra was from 300nm to 400nm and for the emission spectra from 390nm to 610nm. Fluorescence microscopy was performed by Dr. Twan Rutten (Structural Cell Biology, IPK Gatersleben, Germany) and the author with a confocal laser scanning microscope (LSM 510 Meta[27]) with the LSM software release 3.2. Samples for longitudinal views were placed untreated on slides. (Cutting was not necessary, since confocal microscopy can depict layer for layer by zooming through several planes of the sample, generating so-called "z-stacks".) The excitation wavelength was 364nm and emission wavelengths ranged from 385-450nm. Emission spectra of different root zones and of a scopoletin standard were recorded ("Lambda stacks"). Pixels belonging to different spectra were assigned different false-colours ("unmixing" Figure 56) in pictures marked as such. Cross sections were embedded in agar and hand-cut. Images were taken with a twenty-fold magnification and zoomed twice. Control samples were Col-0 (N1092) and tobacco cultivar SamsunNN (SNN).

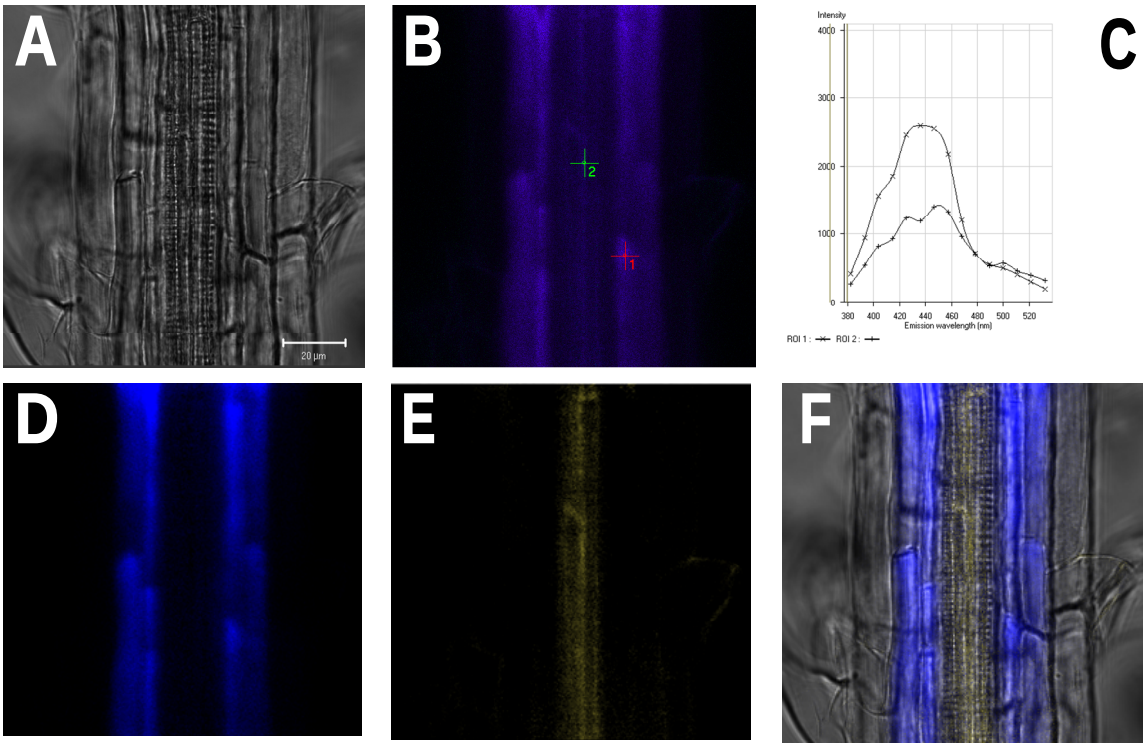

Figure 56: Generating fluorescence images, in which areas with different fluorescence properties are marked with different colours ("Unmixing"): A: A bright-field image of the sample is taken. **B** A fluorescence image is taken from the same area. Zones of interest are marked manually in the software ("1 red cross", "2 green cross"). A fluorescence spectrum of the sample is recorded ("lambda stack") (no image). **C** The fluorescence spectra of the marked areas are extracted. D+E: Pixels having the same spectra as the marked areas are assigned false-colours. (Here, for the spectrum "1" the colour blue was chosen (**D**), for spectrum "2" yellow (**E**)). **F**: The final picture is generated by overlays of images A+D+E.

Elucidation of substances missing in the profile of a root extract of the insertional mutant *f6'h1*

Extracts from roots of WT Col-0 and *f6'h1* from 50 pooled plants out of soil culture were measured using HPLC method 4. To identify the deviating peaks, chromatograms were compared to a collection of standards of secondary compounds. When a number of putative candidate compounds was gained, a preparative HPLC run was performed: the methanol (ca. 4.5ml) from 5ml of pooled root extracts (=625mg starting material) was evaporated and the liquid level refilled with solvent A (0.1% formic acid[15] in 18MΩ water (Milli-Q[17])). The sample was separated on an XBridge Prep C18 5µm column (10x150mm)[11] connected to a Waters 600 pump/controller module with the PDA 2996[11]. Solvent B was 0.1% formic acid[15] in acetonitrile[15]. The flow was 1 ml/min. The gradient was linear from 0 min, 100% A to 230 min, 40%B. Fractions (1, 4, 9 or 10 ml; relevant fractions of Col-0 were 1 ml, of *f6'h1* 4 ml) were collected with a fraction collector (Frac-100[18]). All fractions were analyzed by HPLC-FLD measurements. Peak 1 could be recovered in WT fractions 20-21 (52-53min), peak 2 in fractions 23-24 (55-56min), scopolin in fractions 25-29 (57-61min), peak 4 in fractions 36-37 (68-69min), scopoletin in fractions 46-48 (78-80min), peak 6 in fractions 62-65 (94- 97min), peak 7 in fractions 64-66 (96-98min) and peak 8 in fractions 74-77 (106-109min). These

corresponded to the *f6'h1* fractions 13-14 (peak 1), 14 (peak 2), 15-16 (scopolin), 17-18 (peak 4), 20 (scopoletin), 24-25 (peak 6 and 7) and 27-28 (peak 8). Fractions were analyzed by LC-MS measurements: separation as described in HPLC method 4 without fluorescence detection. The UPLC was coupled to an electrospray ionization-time-of-flight (ESI-ToF) mass spectrometer (LCT Premier[11]). For details of measurement see Korn et al. (2008). However, signals were close to background noise, so all fractions were evaporated completely, resolved at 30°C in 50µl methanol each, diluted to 80% with solvent A and measured again by LC-MS. The corresponding fractions of WT and *f6'h1* were compared to LC-MS runs of standards of substances they might contain considering their respective retention times. In detail, the main masses gained for the standard were explicitly searched for in the respective fractions of WT and *f6'h1* and integrated at the respective retention times. For missing peaks that matched no standard, masses of coumarins and precursors were searched and mass spectra were analyzed (appendix tables A1-A3). Theoretical masses of a collection of phenylpropanoids, with emphasis on compounds close to the pathway towards scopolin are given in the appendix, table A4.

Evaluation of the effects of a *Plasmodiophora brassicae* infection on the insertional mutant *f6'h1* and the wild type Col-0

Frozen spore suspensions of *Plasmodiophora brassicae* strain E3 (Siemens et al., 2009) were kindly provided by J. Ludwig-Müller and S. Bretschneider, Institut für Botanik, TU Dresden, Germany. Inoculation was done by injecting the soil around each plant with 2 ml of a spore suspension or in the control, with 2 ml water (Siemens et al., 2002). Plants were cultivated in a green house in April-May, June-July, September-October and February-March, respectively. Plants were grown either in large trays, divided in four parts (2x *f6'h1*, 2x WT per tray) or in single-well plates (Quickpot 96T[30], 48 plants per line) on bedding substrate 1. Disease evaluation was achieved by determining the infection rate and calculation of the disease index (DI) according to Klewer et al. (2001). For that, each single plant was sorted into one of five categories of symptom severity .The disease index is calculated as $DI = (1n_1+2n_2+3n_3+4n_4)* 100/4N_t$, where n_1-n_4 are the number of plants sorted into categories 1-4 and N_t is the total number of plants in all five categories. To make unbiased symptom evaluations, line identity was masked by the usage of neutral identifiers, which were decoded only after evaluation was finished. For HPLC analysis, roots or leaves of each category in each parallel were pooled, homogenized and measured using HPLC method 4.

Identification of genes involved in the accumulation of scopolin under high-sucrose

Expression analysis of genes in a shifting experiment from low-sucrose to high sucrose

Arabidopsis Col-0 (N1092) plants were cultivated in the hydroponic culture system described above (see p. 108). Root and leaf scopolin contents were determined from three pools from different timepoints after shifting in four independent experiments using HPLC method 4. During the fourth experiment, total protein was extracted from samples from each timepoint, desalted and used in an enzyme assay to produce scopolin out of scopoletin as

described in Maslak (2002), p. 25 f. Amounts of scopolin produced by the protein raw extract were used as a measure for SGT-activity. Northern Blot: RNA was extracted after Logemann et al. (1987) using a guanidine hydrochloride-containing buffer followed by direct extraction with phenol/chloroform. Blotting, labelling of a cDNA probe with radioactive phosphorus and hybridization also followed Maslak (2002), p. 36 f. As a probe, a 382bp long fragment from the CDS (coding sequence) of UGT73B3 (At4g34131) was used, (amplified from cDNA, primers 5'-GGCTGAGAGAGGAAAGAAAGCAAGC-3'; 5'-CAGGCCATGTCACCATTGGTAGC-3').

Micro array analysis: RNA extraction was done with a plant RNA extraction kit (RNeasy Plant Kit[28]), with an on-column DNAse digest. Labelling and hybridisation to Agilent Arabidopsis V3 Gene Expression Microarrays (4x44k)[29] was done by an external service (Array-On GmbH, Gatersleben, Germany). A Cy3-Cy5 two-colour system (Agilent.TwoColor.21169) and the Agilent Feature Extraction Software (v10.5) were used to create normalized Excel files in which fold changes for each gene out of the normalized signal were calculated.

Comparisons and replicates:

Array 1_1: time point zero/Cy3 – high sucrose/Cy5 (Pool 1)
Array 1_2: time point zero/Cy3 – control (low sucrose)/Cy5 (Pool 1)

Array 2_1: time point zero/Cy3 – high sucrose/Cy5 (Pool 2)
Array 2_2: time point zero/Cy3 – control (low sucrose)/Cy5 (Pool 2)

Data evaluation: starting point: Excel file with the normalized signal changes between each sample pair on one array as log ratio. 1. Fold changes for each feature (gene) on every array were calculated (=IF([log ratio]<0;-1/POWER(10, ([log ratio]);POWER(10, ([log ratio])). 3. For Array 1_1 and Array 2_1, ("time point zero vs. high sucrose") all features with an assigned p value > 0.01 on one or both arrays were removed. (The *p*-value is assigned to each log ratio by the extraction software based on error calculations on the raw signal.) 4. The mean value of the fold changes for Array 1_1 and Array 2_1 was calculated ("time point zero vs. high sucrose", two arrays). The mean value of the fold changes for Array 1_2 and Array 2_2 was calculated ("time point zero vs. control", two arrays). 5. The difference between the fold changes for both comparisons was calculated if the assigned *p*-value of "time point zero vs. control" was $p<0.01$ on both of its arrays to remove changes that occurred only by the application of fresh media. Features were annotated using TAIR's Bulk Data Retrieval Tool (Swarbreck et al., 2008).

Screening of activation-tagging lines for enhanced root fluorescence

Figure 57 Schematic composition of the T-DNA in the activation tagging lines: Left Border (LB) – BASTA herbicide resistance – pBstKS+ (including ampicillin resistance) – 4x 35S-Enhancer elements – Left Border (RB).

The collection of activation-tagged lines (figure 57) was delivered as a set of 86 pools containing a mixture of 100 lines per pool (NASC Stock # N21995[25], Weigel et al., 2000). An aliquot of each pool, containing 200-300 seeds, was sown on agar plates containing 0.5% sucrose[1], so that any of the 8600 lines was represented by an average of 2-3 plants in the screen. Wild type Col-0 (NASC Stock # N1092[25]) was grown on 1/3 of each plate for comparison. 3-4 week old plants were photographed with the camera system Diana II[22], see "Imaging system version 1", p. 109. Plants with higher root fluorescence than the wild type were potted into soil and protected from outcrossing and seed loss by seed harvesting devices (Aracons[31]). For the rescreen, seeds from one mother plant were grown on 2/3 of one agar plate, and wild type on the other third of each plate. Those plates were evaluated as above. Offspring lines from positive plants from the rescreen were grown in hydroponic culture and the root scopolin contents determined by HPLC method 1. Lines were selected as candidates, when they had significantly higher scopolin contents than wild type in three independent harvests.

Identification of the insertion site of the activation tag

DNA from 10 single plants from lines N21392 6/1/2 and 6/1/7 and N21368 25/1/5 was extracted using a DNA extraction kit (Invisorb® Spin Plant Mini Kit[32]). T-DNA-insertion was tested with PCR using primers for the 35S-enhancer elements (5'-GAT CCC CAA CAT GGT GGA GCA C-3', 5'-CTA GAT ATC ACA TCA ATC CAC TTG CTT TG-3'). Plasmid rescue (digest and religation of T-DNA and adjacent genomic DNA, followed by transformation in *E.coli*) was performed according to the protocol given by Weigel et al. (2000). Enzymes used for digest were EcoRI and HindIII for N21392 6/1/2 + 7, and KPNI, BamHI, NotI, HindIII or PstI[33] for N21368 25/1/5. The newly ligated vectors were transformed into *E.coli* XL1 Blue. PCR on the newly constructed plasmids was performed with the left border (LB) and right border primer (RB) of the T-DNA, for primer sequences see Weigel et al. (2000). The resulting PCR products, constituting the fragment of genomic *Arabidopsis* DNA adjacent to the insertion site were eluted from the gel with a gel extraction kit (QIAquick[28]). Samples were sequenced[19] using the primers suggested by Weigel et al. (2000). The sequence gained for N21392 6/1 was analysed using the TIGR Arabidopsis thaliana Database (Gish, 1996-2006). The sequence gained for N21368 25/1/5 was analysed with NCBI BLAST (Altschul et al., 1997). The differentiation between ACTIN7 (At5g09810) and ACTIN8 (At1g49240), the possible insertion sites in lines N21392 6/1/2 and 6/1/7, was achieved by two independent PCR approaches:

1. Primers for ACTIN7 and ACTIN8 from regions as diverse as possible within the incorporated Arabidopsis-DNA fragment in the newly constructed plasmid were used. (ACTIN7: 5'-ATT CAA TGT CCC TGC CAT G-3'; 5'-GAG GAA GAG CAT ACC CC-3'; ACTIN8: 5'-CTT TAA TTC TCC AGC TAT G-3'; 5'-GTG GAA GTG AGA AAC CC-3'). Template was the plasmid preparation from *E.coli*, which was also used for sequencing.

2. Primers binding up- and downstream of the insertion site in regions of maximum diversity were used in PCRs with genomic plant DNA from line N21392 6/1/2 which had a proven T-DNA insertion. (ACTIN7: 5'- CTTAAAGTCGTTCTCTTTTTGTAACAGG-3'; 5'-CTGAAAGTTTTAAAGAGGCGGAATTG-3'; ACTIN8: 5'- ATTGATCACAGTGAAATGATTTTCTTTA-3'; 5' GAGGAGTTGTCTACATTTAATCAAC-3'). Gene sequences were downloaded from TAIR (Rhee et al., 2003).

Identification of the gene whose over-expression caused the enhanced scopolin accumulation in line N21392 6/1

Generation and analysis of over-expression lines of the candidate genes

Over-expression lines were generated using the Gateway™[34] system combined with *Agrobacterium*-mediated plant transformation. Entry clones were produced following the Invitrogen User Manual "pENTR™-Directional-TOPO® Cloning Kits"[34]: At first, the CDS region of each candidate gene had to be made available). Several methods were applied, for details, see Table 12.

Table 12: Entry clones containing the cDNA or CDS regions of the candidate genes surrounding At5g09810 (ACTIN7)

Locus	Primer for cloning and/or control PCRs	Origin of CDS-DNA
At5g09750	5'-CACCATGAATAATTATAATATGAACCCATCTC-3' 5'-CTAGATTAATTCTCCTACTCCTCTTCC-3'	Amplification from cDNA
At5g09780	5'-CACCATGGCAAGTAACCCTGATTTTCTTTG-3' 5'-TTATGGCTTTGAGACAACTAGAAGCAGCTC-3	Gateway™ clone DQ056675[35]
At5g09800	5'- CACCATGAGGAGCGATGATC-3' 5'- TCAAAACGGCATAATATGTGTCGT-3'	pUni51 U61902[36]
At5g09805	5'-CACCATGTCTTCTCGAAGCCAC-3' 5'-TTAAGTCTTAGTACTACTTAAACGATTC-3'	Synthetic DNA in pSV-X vector[37]
At5g09830	5'- CACCATGGTGACGAAGGAGCAAGTCG-3' 5'- TCAGGCATCTTTGGTTAAGGTTGCAGAGTC-3'	Amplification from cDNA
At5g09850	5'- CACCATGGATTTGGATGATTTCCGATC-3' 5'- TTACCAGTGTCTACCACCTTGAGAAC'3	amplification from cDNA (Gateway™ clone G13994[38] 300bp at 5' too short)
At5g09860	5'- CACCATGGATGCATTTAGAGATGCTATATTG-3' 5'- TCATGAGACGGGCATAGGAGGATG -3'	amplification from cDNA, Gateway™ clone G14437[38] with point mutation)
At5g09890	5'- CACCATGGACGGCGCCGATG -3' 5'- CTATGTCTTGTGGTTTAACTCACCACCTTC -3'	Gateway™ clone G09539[38]

Production of entry clones from cDNA: RNA from *Arabidopsis thaliana* Col-0 (root and leaf) was extracted with an RNA extraction kit (RNeasy Plant Kit[28]), in which homogenized samples were treated with a lysis buffer, filtered, RNA bound to a spin column, washed and eluted (Qiagen, 2010). First-strand cDNA synthesis was performed with a reverse transcriptase (iScript™ cDNA Synthesis Kit[39]) and PCR with high-fidelity, blunt-end generating

DNA polymerases (Herculase II DNA Polymerase[40] (At5g09750 and At5g09830) or Pfu DNA polymerase[33] (At5g09850)). For AT5g09800, full-length cDNA from the pUni51 clone U61902 was used as a template for a PCR with Pfu DNA polymerase. The resulting PCR fragments were eluted from an agarose gel with a gel extraction kit (QIAquick[28]) and inserted into the vector pENTR-D-TOPO[34]. The generated entry clones were verified by sequencing[19]. Sequences were aligned with CDS sequences downloaded from TAIR (Rhee et al., 2003) using a sequence alignment programme (Lasergene 6's Megalign[41]). Sequences with less than 100% similarity were rejected. LR-clonase reactions to create destination vectors were performed with Gateway® LR Clonase® II enzyme mix[34]. The vector used was the binary vector pK2GW7 (Karimi et al., 2002)[42], the *Escherichia coli* strain was XL-1 Blue[43]. Plasmid preparations of all eight destination vectors were transformed into *Agrobacterium* and used for the transformation of *Arabidopsis* Col-7 plants (N3731[25] = background of activation-tagging lines). Seeds were selected on agar plates containing ½ MS, 1.5% (m/v) sucrose and 50 µg/ml kanamycin. The scopolin contents of the over expression lines grown in hydroponic culture were measured using HPLC method 2 (OEAt5g09750 and OEAt5g09430) or HPLC method 3.

Analyses of insertion mutation lines of the candidate genes

Insertional mutants for the candidate genes surrounding ACTIN7 were either lines from the Salk Institute Genomic Analysis Laboratory T-DNA insertion collection (Alonso et al., 2003), or JIC SM lines (John Innes Centre *Suppressor-mutator lines)* (Tissier et al., 1999), or GABI-KAT lines (Li et al., 2007), see table 13 for details. They were ordered from NASC[25] or directly from GABI-KAT[44].

Table 13: Investigated insertional mutants for the candidate genes surrounding At5g09810 (ACTIN7)

Locus	Name/ID	Segregation status
At5g09750	SALK_005294C (NASC ID: N665157)	homozygous
At5g09780	None available	
At5g09800	SALK_101434C (NASC ID: N671794)	homozygous
At5g09805	SM_3.35905 (NASC ID: 122616)	unknown
At5g09830	GABI-KAT 357C12	unknown
	SALK_013477 (R) (NASC ID: N513477)	segregating
At5g09850	GABI-KAT 772H03	unknown
	GK-332611.01 -.18 (NASC ID: N335302 – 19)	segregating
	SALK_072990 (Y) (AN) (NASC ID: N572990)	
At5g09860	GABI-KAT GK052D02	segregating
At5g09890	SALK_078846 (NASC ID: N578846)	segregating

Several offspring lines for each of the ordered insertional mutants were grown in hydroponic culture containing 0.5% or 3% of sucrose. The scopolin content of root extracts was measured using HPLC method 2 or 3 and compared to wild type Col-0.

To determine the segregation status of the GABI-Kat offspring lines GK052D02 2, 3, 6, 7, and 9, DNA was extracted from 10 single plants of each line according to Edwards et al. (1991)

using a commercial tissue homogenizer (Precellys24[8]). Each plant was tested via PCR by primers suggested in the GABI-Kat delivery papers: gene specific primer: 5'-TTGATAGGTTTACTGCTGGAAAGG-3'; T-DNA primer: 5'-CCAAAGATGGACCCCCACCCAC-3' for a T-DNA-insertion in At5g09860, and by the self-created primers 5'-CACCTTTGATGCTCAACCACTAAGCGAAGA-3' (mid-part of At5g09860) and 5'-TCATGAGACGGGCATAGGAGGATGATC-3' (end of At5g09860) for indicating a non-interrupted At5g09860.

Construction of over-expression plants for the candidate gene At5g09860 (AtTHO1)

Insertion of the CDS region of AtTHO1 into a Gateway entry vector

RNA was isolated using a plant RNA isolation kit (RNeasy Plant Kit[28]) from roots of Col-0. First-strand synthesis was successful using 300 ng total RNA, the gene specific reverse primer 5'-TCATGAGACGGGCATAGGAGGATG-3' and a reverse transcriptase with a high sensitivity (Maxima®[33]). It was performed according to the manufacturer's instructions for cDNA synthesis with gene specific primers. Second strand synthesis was achieved with Pfu polymerase[33] at 58.1°-60.7°C with the forward primer 5'-CACCATGGATGCATTTAGAGATGCTATATTG-3' and the reverse primer 5'-TCATGAGACGGGCATAGGAGGATG-3'. The integration into pENTR-D-TOPO was done according to the Invitrogen support's suggestions for long fragments: 7.5ng PCR-product, 3.7µl salt solution, 1µl vector and 12.3µl water were incubated overnight and transferred into *E.coli* TOP TEN[34]. Constructs were verified by sequencing.

Production of over-expression lines of AtTHO1

The CDS region enclosed in the produced entry clone was transferred in an LR-reaction to the over-expression vector pB2GW7 (Karimi et al., 2002)[42] according to the manual of the applied Gateway® LR Clonase® II enzyme mix[34] using the *E.coli* strain XL-1 Blue[43]. 50 Col-0 wild type plants were treated with the floral dipping method for *Agrobacterium*-mediated plant transformation described above. Seeds from T0 plants were selected on soil with a spray application of a 0.00578% (m/v) solution of ammonium glufosinate (Basta®[45]). The segregation status of T1 lines was determined by cultivation of T2 seeds on agar-plates containing 5 µg ammonium glufosinate per ml (DL-phosphinotricin[4]).

Complementation of the insertion mutation line GK052D02 of with the genomic and promoter sequence of At5g09860 – optimizing cloning procedures accommodating for an unusually large fragment

The complementation strategy followed Shibuya et al. (2007). The genomic sequence from At5g09860 and 2000bp upstream were copied out of the TAIR SeqViewer Nucleotide view (Rhee et al., 2003). A 6113bp long fragment was amplified using the primers 5'-CACCGTTTCTCCTTGATTTCAAC-3' and 5'-TTACGCAAAAGAACTTAATTGTATGATG-3' and a high fidelity DNA polymerase (PRECISOR[46]) on genomic DNA of Col-0 ((isolated with commercial DNA extraction kit (nexttec Genomic DNA isolation kit for plants[47]). The fragment was inserted into pENTR-D-TOPO[34] with variations to the manufacturer's manual: The TOPO reaction set up was 18ng PCR-Product, 1µl TOPO vector solution, 3.7µl salt solution and

14.3µl water and incubation overnight. The fragment was transferred to vector pMDC123 (CD3-747 from ABRC[36] (Curtis and Grossniklaus, 2003)) in the *E.coli* strain XL-1 Blue[43] after a LR-clonase reaction with Gateway® LR Clonase® II enzyme mix[34] according to the manufacturer's instructions. The selection of positive clones was done by EcoRI digest or colony PCR. For the colony PCR, a pipette tip was dipped into a bacterial colony, then into the PCR reaction setup and then used to inoculate a 3 ml-LB-medium overnight culture. Primers used for colony PCR were 5'- GATATTCTTGGAGTAGACGAGAGTGTC-3' (pMDC123-backbone) and 5'-CATCCTCCTATGCCCGTCTCATG-3' (insert). Fifty homozygous insertional mutant plants for At5g09860 (offspring lines GK052D02 3 and 7[44]) were transformed with the floral dipping method described above. Plants were dipped twice with a seven day interval. Seeds from T0 plants were selected on soil with a spray application of a 0.00578% (m/v) solution of ammonium glufosinate (Basta®[45]).The scopolin contents of complemented lines grown in hydroponic culture were measured using HPLC method 3. Proteins were extracted following Qi and Katagiri (2009). Immunodetection was carried out as described above with an antibody against AtTHO1.

Production of an antibody against AtTHO1

A pENTR-D-TOPO plasmid preparation containing the CDS region of AtTH01 was used in a LR clonase reaction with LR Clonase II Enzyme Mix and the protein expression vector pDest17 following the instructions in the Invitrogen manual "*E.coli* Expression System with Gateway® Technology (2010). The plasmid was transferred to *E. coli* Top10 cells and selected with ampicillin. Transformants were analysed both with PCR (primers 5'-CACCATGGATGCATTTAGAGATGCTATATTG-3' and 5'-TCATGAGACGGGCATAGGAGGATG-3') as well as with digest with EcoRI. Plasmid preparations of positive clones were transferred to the *E. coli* strain BL21-AI™ for recombinant protein expression[34]. Expression and isolation of the recombinant protein was done after "The QIAexpressionist™". The recombinant protein was purified by preparative SDS gel electrophoresis, the corresponding band cut out and eluted from the gel with an electro eluter (model 422[39]) over night with 50 mA in an elution buffer with 25 mM TRIS[1], 192 mM glycine[1] and 0.1% (m/v) SDS[1]. Identity of the protein was confirmed by MALDI-TOF mass spectrometry as described in Hedtmann (2012): After tryptic digest, 0.5 µl of the sample was deposited onto a MALDI target (MTP 384 target plate AnchorChip 800um[58]) and subsequently covered with 1 µl matrix solution (0.7 mg/ml a-cyano-4-hydroxycinnamic acid (HCCA) in 90 % ACN, 0.1 % TFA, 1 mM $(NH)_4H_2PO_4$). A MALDI tandem MS instrument (Ultraflextreme[58]) was used to acquire peptide mass fingerprints (PMF). Peptide calibration standard covering a mass range from 1,000-4,000 Dalton were used for external calibration and trypsin autolysis peaks (m/z 842.509, 2211.104) for internal calibration. PMF were subjected to protein homology identification using Mascot search engine (Matrix Science, London, United Kingdom) in Biotools 3.0 software (Bruker Daltonics) by searching for *Viridiplantae* in the NCBI non-redundant protein database. The production of the antibody in rabbit was a service of the group "Phytoantibodies", IPK Gatersleben, Germany.

Expression levels and scopolin content of other members of the putative AtTHO/TREX complex

Expression levels of the predicted members of the AtTHO/TREX complex were investigated using the array data collection of Genevestigator (Hruz et al., 2008). For scopolin analysis insertional mutants were analyzed (Table 14). The scopolin contents of roots grown in hydroponic culture with 3% sucrose were measured using HPLC method 4.

Table 14: The members of the predicted THO complex in *Arabidopsis thaliana*, based on similarity to *Drosophila* and mammalian THO/TREX (Yelina et al., 2010) and investigated insertional mutants. Primers were created with the T-DNA Primer design tool of the SALK Institute Genomic Analysis Laboratory (SIGnAL) http://signal.salk.edu/tdnaprimers.2.html

THO component	*Arabidopsis* homolog (predicted)	Gene names in *Arabidopsis*	Insertional mutant	Primers for determination of segregation status
THO1 (HPR1)	At5g09860	AtTHO1/AtHPR 1; EMU	GABI-KAT GK052D02[44]	see p. 124
THO2	At1g24706	AtTHO2	SALK_051591 (N55191)	TGCAGTTCCTAATATGTAAAGAGGGA -GTTAAAAGCTTCCTCTAAACGAGG
THO3 (TEX1)	At5g56130	AtTHO3/AtTEX 1	SALK_059869 (N559869)	CAACAATAACAGAAACGAACACAACA AGGTAAAGAAATTACATGACCGTG
			SALK_100012 (N600012)	ACTTACGGACATCCAGAATTGTAAG GAAGCGAAGCTCTGATATTCAGTAG
THO5	At5g42920	AtTHO5A; ATTHOC5A	SALK_100556 (N60556)	ATTGCTTCTAACTTCCACAACTTTC ACTTCAAGGCCTTATAAATGGGTAC
	At1g45233	AtTHO5B; ATTHOC5B	SALK_122387C (N656928)	homozygous
THO6	At2g19430	AtTHO6; AtTHOC6	SALK_021789C (N658552)	homozygous
THO7	At5g16790	AtTHO7A; AtTHOC7A	SALK_078514C (N6556338)	homozygous
	At3g02950	AtTHO7B; AtTHOC7B	SALK_052697 (N552697)	AAGTAAAATAACGGAGAAACGTGTG AGAGTGTGAGACGATAAGGAAACTG
THO4(Aly)	At5g59950 At1g66260 At5g37720 At5g02530		Not analyzed	
UAP56	Not found			

Expression analysis in the insertional mutant of AtTHO1

Plants from wild type Col-0 and GK052D02 were grown for 4 weeks in hydroponic culture with ¼ MS and 0.5% sucrose. 18 hours after replacing the medium with ¼ MS and 3% sucrose, the roots were harvested in 2 x 3 pools of 5 growth jars (=10 plants per jar) to obtain 3 biological replicates of 50 plants for each line. RNA was extracted using an RNA extraction kit with on-column DNAse digest (RNeasy Plant Kit[28]) according to the manufacturer's instructions. Affymetrix 3' Expression Profiling and bioinformatic analysis was done by ATLAS Biolabs GmbH, Berlin, Germany. Six microarrays (GeneChip Arabidopsis ATH1 Genome Arrays[52]), one per sample, were used for hybridisation. The signals were normalised over all 6 arrays. The arrays were grouped into "Col-0" and "GK052D02". The mean value for the signals for each probe set was compared between the groups with a t-

test. Level of significance was set at $p<0.05$. Fold changes between groups for each probe set were calculated. Changes smaller than twice up- or downregulated were rejected. Results were filtered by the present/absent status (Detection call) of each probe set. Probe sets that were not present in at least 50 percent of one group were rejected. Annotation of significantly regulated genes was done using TAIR database (Rhee et al., 2003); for the translated protein sequence NCBI's BLASTp (Altschul et al., 1997) and for additional expression data Genevestigator (Hruz et al., 2008).

Phenotyping of insertional mutants of genes with an altered transcription level in mutant GK052D02-3 besides AtTHO1 based on the array data

Hydroponic culture with 3% sucrose was set up as described above. Root scopolin and scopoletin content was determined with HPLC method 4 using 2 independent homozygous lines, or, if not available, two off-spring lines, in 10 pools of ten plants each. For plant material and primers for the determination of the segregation status see table 15.

Table 15: Insertional mutant lines[25] for genes with an altered expression in GK052D02-3

Locus	Gene name	Insertional mutant	Primers for determination of segregation status
At5g09870	CESA5	SALK_023353 (BA) (N523353) SALK_099008 (BN) (N599008) SALK_125535 (BZ) (N625535)	5'-GGAAGTCCAAGGGTTGAAGGAGATGAG-'3 5'-GCATCAGATATTCCCACAATGACTCCGATTAC-3'
At5g14340	MYB40	N429176 (GK-304H04 Set = GK-304H04.01-12/N719466 - N719476)	5'-CGTGCTGTGACAAAATTGGATTGAAGAGAG-3' 5'-GATTATCATCAAGATTGGCGAAGAGGTCC-3'
At1g53490	RING-U-Box superfamily protein	SALK_014624 ('E) ('R) (N514624) SALK_080613 (CG) (N580613).	5-'GGTTTGTGTCAATCTCGAATCAGGGAG-3' 5'-GACCATTAGGACTTCCACCTAGGCAG-3'
At1g43590	transposon	SALK_022212C (N667940)	5'-ATGGCTTTTGTAGTCAACGGGAATGAG-3'; 5'-TTAAGCTTGAATCGGACCAAACTTTTGCCA-3'
At3g05770	unknown protein	SALK_069840C (N669818)	5'-GATGAGATCGAGAAGAGACTTCAAGGTC-3' 5'-CGAAGATGAGATCGAGAAGAGACTTCAAG-3'
At2g22860	ATPSK2	none available	

Reaction of GK052D02 towards osmotic stress

Experiments with varying sucrose and mannitol concentrations were performed by D. Berreth and are described in her thesis, pages 69-73 (Berreth, 2011). HPLC raw data, gained with method 4, were kindly provided for further evaluation.

Morphological characteristics of GK052D02 under standard and stress conditions

Images of root tips, root middle sections and root bases of WT and GK052D02 were taken with a binocular by D. Berreth, (described in Berreth (2011), pp. 36-37). Images of root middle sections were used to count root hairs by the author. For that purpose, a paper

frame equating to 1 mm of the original root size in the image was placed on top of the image and all visible root hairs within the frame were counted. Cell layers and scopolin accumulation in WT and GK052D02 were analyzed with CLSM (D. Berreth, pp.37 and 59-61). Root length and lateral root formation was determined on three week-old plants grown on vertically placed agar plates.

Analysis of the genetic background of GK052D02

Insertion of a T-DNA into the genes At1g07260 and At5g14340 was tested via PCR with the following primers: At1g07260: 5'-CGACGGCGGCTAAAACGTTATC-3'; 5'-CTCTCTTCCTCGGCGTATCCTCAC-3' (intact gene), 5'-CGTAGATTTAAGCTCCGGCAAC-3' (gene primer forward insertion), 5'-GTTGCCGGAGCTTAAATCTACG-3' (gene primer reverse insertion), 5'-CCATATTGACCATCATACTCATTGC-3' (pAC161LB) 5'-GCAAGTGGATTGATGTGATATCTC-3'(pAC161RB). At5g14340: 5'-CGTGCTGTGACAAAATTGGATTGAAGAGAG-3', 5'-GATTATCATCAAGATTGGCGAAGAGGTCC-3' (intact gene). DNA extraction was performed following Dellaporta (1994). Number of plants: was 10-19 per line. SALK line for phenotyping: At1g07260: SALK_021979C[25]. GABI-KAT lines for phenotyping *myb40*: GK-304H04.01-12 (N7119465-72[25]). Scopolin content of roots was measured using HPLC method 4. Lateral roots were counted on 3-week-old plants vertically cultured on agar-plates.

Analysis of phenylpropanoid shifts following cold stress in *Arabidopsis thaliana*

Peak identification

Leaf extracts were separated as described in HPLC method 4. However, the UPLC was coupled to an electrospray ionization-time-of-flight (ESI-ToF) mass spectrometer (LCT Premier[11]) instead of the fluorescence detector. Details of measurement and a list of candidate masses previously identified in cold stress experiments with *Arabidopsis thaliana* were taken from the works of Korn et al. (2008).

Development of a screening system for cold stress experiments with *Arabidopsis thaliana*

Four methods for optimum peak separation for *Arabidopsis* extracts were tested: The first was the standard laboratory method (HPLC method 3, see p. 110) using a 5 cm C18 column and a 7 min gradient from 100% polar solvent to 0% polar solvent. The second method was taken from Tohge et al. (2007) with a 10 cm C18-column and a 20 min gradient from 100% polar solvent to 80% polar solvent. The third method tested was the method of Tohge et al. (2007) on the 5 cm C18 column. The fourth method was taken from Yonekura-Sakakibara et al. (2008), a method which used a phenyl column and a gradient from 100% polar solvent to 60% polar solvent within 10 minutes. The aqueous solvent was in all cases acidified water, the non-polar solvent acetonitril with or without formic acid. The same *Arabidopsis* root-shoot-mix extract was measured with all four methods and all visible peaks were counted. The highest number of peaks, 52, was gained with the method of Yonekura-Sakakibara et al., followed by the method of Tohge et al. on the long column (51peaks), the method of Tohge

et al. on the short column (44 peaks) and the laboratory standard method with 42 distinguishable peaks. In addition, the resolution R_S of each column was calculated. The highest resolution was achieved by the method of Yonekura-Sakakibara et al. with R_S = 53.62, followed by the method of Tohge et al. on the short column (R_S = 34.62), the laboratory standard method (R_S = 33.48) and the method of Tohge et al. on the long column (R_S = 32.35). For performing best in both categories, the method of Yonekura-Sakakibara et al. (2008) was selected for the screen, but it had to be extended by a washing step to clean the column and by additional fluorescence channels (for general phenylpropanoid detection: 300/400nm, for optimum scopolin detection: 336/438nm and for anthocyanin detection 340/450nm (Drabent et al., 1999)). Methanolic plant extracts were made with the standard extraction method (see p.109). HPLC runs for method comparison were performed as described in Yonekura-Sakakibara et al. (2008), Tohge et al. (2007) and HPLC method 3 (see p. 110). The resolution R_S of each column was calculated with the formula

$$R_S = \frac{2(t_{last\ peak} - t_{first\ peak})}{w_{first\ peak} + w_{last\ peak}}$$

with t=retention time and w=peak width (Lottspeich, 2005).

The following growing conditions were varied during that experiment: age of plants, substrate and growing place. Columbia 0 plants (N1092[25]) were grown for 14 days in a low-nutrient peat substrate ("Substrat 1" see p. 108) in a plant incubator (Percival[53]). (20°C/18°C, 9 hours light, 210 µmol m^{-2} s^{-1}) for seedling cultivation. They were repotted in single pots (diameter 5cm) in either the low-nutrient substrate or a high-nutrient substrate (peat, structure medium grade, N 280mg/l, P320mg/l, K 360mg/l, Mg 360mg/l, S 120mg/l ("Substrat 2"[6]). For further growth, they were placed either back in the environmental room or a plant growth chamber[54] (20°C/18°C, 9 hours light, 180 µmol m^{-2} s^{-1}). 1 week before harvest, half of the plants were transferred to 10°C into a plant incubator (Percival[53]). Time points for harvest were 5, 7, 9 and 10 weeks after sowing. Two pools of plants from each condition were analysed. Peak areas for the larger peaks were compared between 10°C plants and 20°C plants with a t-test and the relation of the areas between treatment and control was calculated. Leaf material from all conditions was analyzed via HPLC. All larger peaks at 280 nm UV detection and in the fluorescence trace at 300/400 nm were quantitatively evaluated. The percentage of increase in peak area from control to cold stress samples was taken as a measure to evaluate the influence of each condition.

Cold stress screening system for large numbers of lines (final version)

Seeds were sown on 96-well tree propagation trays (Quickpot 96T[30]) on bedding substrate 1 (see p. 108) and covered with a transparent plastic cover for propagation crates[55]. Cultivation conditions were a 12/12 hr day/night period at 21/18°C at 120-150 µmol m^{-2} s^{-1} light and 80% humidity in a walk-in growing chamber. After 14 days, 4 seedlings per line were transplanted on 96-well trays with high-level nutrient supply substrate ("Substrat 2"[6]). Twenty eight days after sowing, 4 plants per line were harvested. Then, the temperature was

lowered to 10°C. A wild type control was kept at the initial conditions. After 7 days at 10°C, the other 4 rosettes of each line were harvested into aluminium foils and frozen immediately in liquid nitrogen. Sample preparation: Homogenization of pools using mortar and pistil, followed by methanolic extraction and analysis with HPLC method 4. Data analysis: Application of a processing method for identifying the three marker peaks (kaempferol-derivatives) at 4.05 min, at 4.77 min and at 5.27 min (280 nm) and the scopolin peak at 336/438nm fluorescence detection and export of peak areas to and sorting in Excel.

Rescreen of candidates from an EMS-mutagenized Arabidopsis seed collection

46 independent or offspring-lines of an EMS-mutagenized mutant collection (*Arabidopsis thaliana* M2 Columbia (Col-0), batch no. 99A, Parental group 4 (1192 lines)[31] were subjected to a rescreen. Their behaviour under cold stress had diverged from wild type under cold stress. The original screen had been conducted by Silke Peterek 2005-2008. Every line contained an average of five point mutations. Cultivation: Plants were grown for 14 days in "Substrat 1" (see p. 108) in a plant incubator (Percival[53]) (20°C/18°C, 12 hours light, 120-150 µmol m^{-2} s^{-1}). Repotting was done in single pots (diameter 5 cm) in "Substrat 2"[6] (see p 124). 4 weeks after sowing, half of the plants were transferred to a 10°C plant incubator. 5 weeks after sowing pools of four plants were harvested. Samples were analysed with HPLC method 4. Chromatograms were sorted after the three marker peaks with the software ChromaVIIns[56].

Screen of a collection of insertional mutants of putative transcription factors in *Arabidopsis thaliana*

300 lines of the RIKEN Ds transposon seed collection[57] containing insertional mutants of putative transcription factors (TF lines) and the *Arabidopsis* wild type background Nossen-0 where screened in a slightly modified screening: Seeds were sown on 96-well tree propagation trays (Quickpot 96T[30]) on bedding substrate 1 (see page 108) and covered with a transparent plastic cover[55]. Cultivation conditions were a 12/12 hr day/night period at 21/18°C at 130-140 µmol m^{-2} s^{-} light and 80% humidity in a walk-in growing chamber. After 14 days, 8 seedlings per line were transplanted on 96-well trays with high-level nutrient supply substrate ("Substrat 2"[6], see p.124). Twenty-eight days after sowing, 4 plants per line were harvested and the temperature was lowered to 10°C. A wild type control was kept at the initial conditions. After 7 days at 10°C, the other 4 rosettes of each line were harvested. Samples were analyzed using HPLC method 4. Samples were sorted for the heights of the marker peaks with the software ChromaVIIns[56].

Cold stress screens with parts of the SALK collection

The SALK collection (Salk Institute Genomic Analysis Laboratory T-DNA insertion collection (Alonso et al., 2003)) N27941 from NASC[25] provides homozygous insertional mutants of 6868 genes of *Arabidopsis thaliana.* A pilot experiment was conducted as described in the rescreen of the EMS-mutagenized lines. 700 lines were screened with the final version of the developed system.

[1] Carl Roth GmbH + Co. KG, Karlsruhe, Germany
[2] SERVA Electrophoresis GmbH, Heidelberg, Germany
[3] Bio & Sell e.K., Feucht bei Nürnberg, Germany
[4] Duchefa Biochemie B.V., Haarlem, The Netherlands
[5] Weck Company, Wehr, Germany
[6] Klasmann-Deilmann GmbH, Geeste, Germany
[7] Merck KgaA, Darmstadt, Germany: distributor: Th. Geyer GmbH & Co. KG, Renningen, Germany
[8] Bertin Technologies, Montigny-le-Bretonneux , France; distributor: PEQLAB Biotechnologie GmbH, Erlangen, Germany
[9] Mühlmeier GmbH und Co. KG, Bärnau, Germany
[10] Extrasynthèse, Genay, France
[11] Waters GmbH, Eschborn, Germany
[12] Sigma-Aldrich Chemie GmbH, München, Germany
[13] Jasco, Groß-Umstadt, Germany
[14] Phenomenex, Aschaffenburg, Germany
[15] J.T. Baker, Deventer, the Netherlands; distributor: Th. Geyer GmbH & Co. KG, Renningen, Germany
[16] Fluka, part of Sigma-Aldrich Corporation, distributor: Sigma-Aldrich Chemie GmbH, München, Germany
[17] Millipore Schwalbach/Ts, Germany
[18] Amersham Pharmacia Biotech, now part of GE Healthcare; GE Healthcare GmbH, Solingen, Germany
[19] Eurofins MWG Operon, Ebersberg, Germany
[20] GIBCO, Life Technologies GmbH/Invitrogen, Darmstadt, Germany
[21] Systat Software GmbH, Erkrath, Germany
[22] raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany
[23] Schott Glaswerke, Mainz, Germany
[24] Vilber Lourmat, Marne-la-Vallée, France; distributor: PEQLAB Biotechnolgie GmbH, Erlangen, Germany
[25] Nottingham Arabidopsis Stock Centre, School of Biosciences, University of Nottingham, Loughborough, United Kingdom¸(Scholl et al., 2000)
[26] Molecular Devices (Germany) GmbH, Ismaning, Germany
[27] Carl Zeiss MicroImaging GmbH, Jena, Germany
[28] Qiagen GmbH, Hilden, Germany
[29] Agilent Technologies, Inc.; Life Sciences and Chemical Analysis Group, Santa Clara, CA, USA
[30] HerkuPlast Kubern GmbH, Ering am Inn, Germany; distributor: Hermann Meyer KG, Rellingen, Germany
[31] Lehle Seeds, Round Rock, Texas, USA
[32] STRATEC Molecular GmbH, Berlin, Germany
[33] Fermentas GmbH, St. Leon-Rot, Germany; now part of Thermo Fisher Scientific Inc., Waltham, MA, USA
[34] Life Technologies GmbH/Invitrogen, Darmstadt, Germany
[35] Arabidopsis Biological Resource Center, Columbus, OH, USA; Donors: Beverly A. Underwood, Yongli Xiao, Julia Redmann, Christopher D. Town
[36] Arabidopsis Biological Resource Center, Columbus, OH, USA
[37] ATG:biosynthetics GmbH, Merzhausen, Germany
[38] Arabidopsis Biological Resource Center, Columbus, OH, USA; Donor: Joe Ecker/SALK; (Yamada et al., 2003)
[39] Bio-Rad Laboratories GmbH, München, Germany
[40] Agilent Technologies Sales and Services GmbH & Co. KG, Waldbronn, Germany
[41] DNASTAR, Inc., Madison, WI, USA
[42] Department of Plant Systems Biology, Universiteit Gent, Gent, Belgium
[43] Stratagene, an Agilent Technologies Division, Agilent Technologies Sales & Services GmbH & Co. KG, Waldbronn, Germany
[44] German plant genomics research program - Kölner Arabidopsis-T-DNA lines; Bielefeld University , Chair of Genome Research, Bielefeld, Germany
[45] Aventis CropScience Deutschland GmbH, Hattersheim, Germany
[46] BioCat GmbH, Heidelberg, Germany
[47] Biozym Scientific GmbH, Hessisch Oldendorf, Germany
[48] PEQLAB Biotechnologie GmbH, Erlangen, Germany
[49] Schütt-Labortechnik GmbH, Göttingen, Germany
[50] Sartorius AG, Göttingen, Germany
[51] AppliChem GmbH, Darmstadt, Germany

[52] Affymetrix, Santa Clara, CA, USA
[53] Percival Scientific, Perry, IA, USA
[54] Heraeus Vötsch, Balingen, Germany
[55] Hermann Meyer KG, Rellingen, Germany
[56] Tobias Czauderna; IPK Gatersleben, Germany
[57] BioResource Center RIKEN Tsukuba Institute, Tsukuba, Ibaraki, Japan
[58] Bruker Daltonics, Bremen, Germany

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Publications:

Döll S, Hensen I, Schmidt-Lebuhn A N, Kessler M (2007). "Pollination ecology of *Justicia rusbyi* (Acanthaceae), a common understory plant in a tropical mountain forest in eastern Bolivia." Plant Species Biology **22**(3): 211-216.
Knüpffer H, Döll S, Vorwald J (2006): 50 Jahre Gaterslebener Sammelreisen - Vergleich des gesammelten Materials mit dem jetzigen Genbankbestand. Vortr. Pflanzenzücht. 70 (2006) 54-60.

Oral Presentations:

Screening of a collection of activation-tagged mutants for enhanced root fluorescence - Revealing genes affecting the production of scopolin, a highly fluorescent coumarin in *Arabidopsis thaliana*. - Sektionstagung „Pflanzliche Naturstoffe“of the German Botanical Society; 30. März – 1. April 2009 Fulda, Germany.

Poster presentations:

Döll S, Berreth D, Mock H-P: An efThoc1 superfamily transcription factor mediates the enhanced production of the coumarin scopolin after abiotic stress. – Institutstag IPK, Gatersleben, 04.-06.10.2011.

Döll S & Mock H-P: Identification and functional characterisation of genes involved in the biosynthesis of the coumarin scopolin. Institutstag IPK, Gatersleben, 04.-05.2010

Hedtmann C, Kaspar S, Lippmann R, Döll S, Matros A, de Jaeger G, Witzel K,Mock H-P: Analysis of Abiotic Stress Responses by Proteomic Approaches. FESPB meeting, Valencia, Spain, 4. – 9.7.2010

Döll S & Mock H-P: Coumarins in *Arabidopsis thaliana* – Identifying genes involved in secondary metabolism by activation tagging and carbon shifting experiments. 4th Plant Science Student Conference, IPK Gatersleben, 01.-04.07.2008

Czauderna T, Döll S, Peterek S, Mock H-P, Schreiber F: Advanced and automated algorithms for analysis of complex HPLC data sets. 4th Plant Science Student Conference, IPK Gatersleben, 01.-04.07.2008

Döll S, Flemming S, Knüpffer H: Enhancing the quality of IPK's genebank collection site data by georeferencing. - Institutstag IPK, Gatersleben, 10.10.2006.

Affirmation

Hereby, I declare, that all the work presented in this dissertation is my own, except the parts that were marked explicitly as a co-work with the diploma student Dorothee-Carina Berreth, whose work was supervised and completed by myself. No other aids or literature as those cited were used.

Gatersleben, September 2012

Appendix

List of families containing scopoletin and/or scopolin

Solanaceae (Geiger, 1843; Eykman, 1883)
Gelsemiaceae (Wormley, 1870)
Rutaceae (de Vry, 1864)
Poaceae (Andreae, 1952)
Araliaceae (Urban, 1958)
Caprifoliaceae (Glennie and Bohm, 1968)
Orchidaceae (Wrigley, 1960)
Meliaceae (Mahogany family) (Basak and Chakrabo.Dp, 1970)
Malvaceae ((Wakelyn et al., 1974)
Anacardiaceae (Tabulated Phytochemical Reports, 1975)
Apiaceae (Kartnig et al., 1975)
Araceae*
Liliaceae*
Apocynaceae*
Balsaminaceae*
Calicanthaceae*
Capparidaceae*
Caryophyllaceae*
Chenopodiaceae (now Amaranthaceae)*
Crassulaceae*
Dipsacaceae*
Ebenaceae*
Ericaceae*
Euphorbiaceae*
Fouquieriaceae*
Fagaceae*
Gentianaceae*
Fabaceae*
Plantaginaceae*
Loasaceae*
Loganiaceae*
Nepenthaceae*
Moraceae*
Oleaceae*
Passifloraceae*
Platanaceae*
Polemoniaceae*
Polygonaceae*
Ranunculaceae*
Rosaceae*
Rubiaceae*
Salicaceae*
Saxifragaceae*
Scrophulariaceae*
Simaroubaceae (Tree-of-heaven family)*
Thymelaeaceae*
Ulmaceae*
Violaceae*
Zygophyllaceae*
*= (Murray et al., 1982)
Hippocastanoideae (Miller et al., 1990)
Simaroubaceae (tree-of-heaven family Fo et al. (1992))
Acanthaceae (Bratoeff and Perezamador, 1994)
Lauraceae (Neville and Bohm, 1994)
Vahliaceae (Majinda et al., 1995)
Rhamnaceae (Lee et al., 1995)
Icacinaceae (polyphyletic, Wu et al. (1995))
Geraniaceae (Kayser and Kolodziej, 1995)
Bombacaceae (Paula et al., 1996)
Papaveraceae (Fliniaux et al., 1997)
Proteaceae (Erazo et al., 1997)
Urticaceae (Lichius and Muth, 1997)
Ledocarpaceae (Hnatyszyn et al., 1998)
Nyssaceae (Li et al., 2000)
Burseraceae (frankincense and myrrh family; Bandeira et al. (2002))
Brassicaceae (Rohde et al., 2004)
Zingiberaceae (Abas et al., 2005)
Adoxaceae (Mohamed et al., 2005)
Polygalaceae (Meotti et al., 2006)
Santalaceae (Zhang et al., 2006)
Sapindaceae (Lemos et al., 2006)
Dipterocarpaceae (Muhtadi et al., 2006)
Clusiaceae (Ngoupayo et al., 2007)
Quillajaceae (Ribera et al., 2008)
Connaraceae (de Oliveira et al., 2012).

Table A16: Identification of absent fluorescence peaks in *f6'h1*. Peaks were regarded as identified, if a standard matched the peak in a fluorescence chromatogram and mass and retention time in an LC-MS run. "Unknown compounds" are masses that were found at the approximated MS-retention time (FLD retention time + 0.4min) in WT and not or reduced in *f6'h1*.

Altered peaks and candidate substances found in the respective fractions	RT FLD [min]	Found in WT root (FLD)	Found in *f6'h1* root (FLD)	RT [min] (TOF MS)	Fragment masses candidate substances	Candidate substance found in WT root (exact match to standard RT and mass TOF-MS)	Found in *f6'h1* root (TOF MS)
altered peak 1	3.107	Found	N.D.				
= esculin	3.11	Found	N.D.	3.54	341 [M+H]	Found	N.D.
altered peak 2	3.496	Found	Reduced				
= isoscopolin	3.47	?	?	-	-	355 (M+H) at 3.89min	N.D.
altered peak 3	3.651	Found	N.D.				
= scopolin	3.66	Found	N.D.	4.08	355 [M+H]	Found	N.D.
altered peak 4	4.319	Found	N.D.				
unknown compound 3						M1=373 at 4.78min M2=355 at 4.78min ($M1-H_20$?)	Reduced (1/8 of WT) Reduced (1/8 of WT)
altered peak 5	4.828	Found	N.D.				
= scopoletin	4.83	Found	N.D.	5.37	193 [M+H]	Found	N.D.
altered peak 6 unknown compound 4	6.244	Found	reduced (1/3 of WT)			M=663 at 6.68min	Reduced to 1/40 of WT
altered peak 7 unknown compound 5	6.384	Found	N.D.			M=811 at 6.77min	Reduced (1/50 of WT)
altered peak 8	6.817	Found	N.D.				
7-methoxycoumarin (=herniarin)/ 3-methoxycoumarin	6.84	Found	N.D.	7.27	177 [M+H]	Found	Peak assignment unsure
or unknown compound 7						M=591 at 7.26	N.D.

Appendix

Table A17: Other substances absent in *f6'h1* that were not visible in fluorescence chromatograms.

Substance	RT FLD [min]	Found in WT root (FLD)	Found in *f6'h1* root (FLD)	RT [min] TOF MS	Fragment masses	Found in WT root (TOF MS) (exact match to standard RT and mass)	Found in *f6'h1* root (TOF MS)
unknown compound 1						M=207 at 3.45min	N.D.
sinapoylglucoside	3.93	N.D.	N.D.	4.25	M1: 207 [M-Glc-H_2O] M2: 225 (M-Glc]	Found	Reduced (1/3 of WT)
unknown compound 2						M=211 at 4.38min	N.D.
isoscopoletin	4.63	N.D.	N.D.	5.16	193 [M+H]	Found	N.D.
sinapic acid	4.913	N.D. (overlaid by scopoletin)	Traces?	5.31	M1: 207[M-H_2O +H] M2: 225 [M+H]	M1: Found M2: Traces	Traces? N.D.
unknown compound 6						321 at 7.03min	Reduced (1/5 of WT)

Table A18: Substances that were equally present or absent in *f6'h1* and WT

Standards:	RT FLD [min]	Found in WT root (FLD)	Found in *f6'h1* root (FLD)	RT [min] TOF MS	Fragment masses	Found in WT root (TOF MS) (exact match to standard RT and mass)	Found in *f6'h1* root (TOF MS)
catechin	No fluorescence	-	-	3.52	290	Found	Found
chlorogenic acid	3.33	N.D.	N.D.	3.60	M1: 355 [M+H] M2: 163 [caffeic acid -H_2O+H]	M1: Traces M2: Found	M1: Traces M2: Found
caffeic acid	3.39	N.D.	N.D.	3.87	163 [M-H_2O+H]	Traces	Traces
esculetin	3.46	?	?	3.96	179 [M+H]	N.D.	N.D.
Daphnetin	No fluorescence	-	-	4.25	179 [M+H]	ND (Traces?)	N.D. (Traces?)
p-coumaric acid	4.26	Found	Found	4.69	M1: 147 [M-H_2O+H] M2: 165 [M+H]	Traces	Traces?
umbelliferone	4.63	?	?	5.01	163 [M+H]	Traces	Traces
ferulic acid	4.77	N.D. (overlaid by scopoletin)	Traces?	5.25	M1:177 [M-H_2O +H] M2: 195 [M+H]	M1:Traces M2: N.D.	M1: Traces M2:N.D.
rutin	No fluorescence	-	-	5.36	611 [M+H]	Traces	Traces
o-coumaric acid	5.50	Traces?	Traces?	5.91	M1: 147 [M-H_2O +H] M2: 165 [M+H]	N.D.	N.D.
kaempferol-3-o-rutinoside	No fluorescence	-	-	5.91	595 [M+H]	N.D.	N.D.
quercitrin	No fluorescence	-	-	6.14	M1: 303 [quercetin+H] M2: 449 [quercetin+rha+H]	N.D.	N.D.

cyanidin 3-O-(2''-O-(xylosyl) 6''-O-(p-coumaroyl) glucoside)5-O-malonylglucoside (Tohge et al., 2005)				6.23	M=975.242		
scoparone	6.177	?	?	6.50	207 [M+H]	N.D. (Traces?)	N.D. (Traces?)
hesperidin	No fluorescence.	-	-	6.73	611 [M+H]	N.D.	N.D.
3,4-Dihydrocoumarin	No fluorescence	-	-	7.11	149 [M+H]	N.D.	N.D.
trans- 3,4-(Methylenedioxy)cinnamic acid (MDCA)	7.009	Found	Found	7.42	M1: 175 [M-H_2O +H] M2: 193 [M+H]	N.D. (Traces?)	N.D. (Traces?)
trans-cinnamic acid	No fluorescence	-	-	7.41	M1: 131 [M-H_2O +H] M2: 149 [M+H]	N.D.	N.D.

Molecular masses of some phenylpropanoids

Table A19: Molecular masses of phenolic compounds with emphasis on the coumarin derivatives pathway

Substance	Mass
trans-cinnamic acid	148
3,4-dihydrocoumarin	148
umbelliferone	162
o-coumaric acid	164
p-coumaric acid	164
3-methoxycoumarin	176
7-methoxycoumarin (herniarin)	176
coniferyl aldehyde	178
daphnetin	178
esculetin	178
caffeic acid	180
angelicin	186
psoralen	186
ayapin	190
hydrangetin	192
isoscopoletin	192
methylenedioxycinnamic acid (MDCA)	192
scopoletin	192
5-hydroxyconiferyl aldehyde	194
ferulic acid	194
2'-hydroxycaffeic acid	196
5-hydroxyconiferyl alcohol	196
scoparone	206
fraxetin	208
isofraxetin	208
2'-hydroxyferulic acid	210
bergapten	216
sphondin	216
xanthotoxin	216
sinapic acid	224
xanthyletin	228
osthol	244
isopimpinellin	246
pimpinellin	246
catechin	290
sinapoylcholine	310
skimmin	324
sinapoyl malate	339
cichoriin	340
esculin	340
daphnin	340
coniferin	342
chlorogenic acid	354
isoscopolin	354
scopolin	354
syringin	372
sinapoylglucoside	386
puberulin	443
quercitrin	448
naringin	580
kaempferol-3-*o*-rutinoside	594
hesperidin	610
rutin	610
coenzymeA	767

Genes regulated after a shift from low-sucrose to high-sucrose medium

Table A20: Top 100 genes that were upregulated in a shifting experiment from low-sucrose to high sucrose in Arabidopsis roots.

Fold Change	Systematic Name	categories	Primary Gene Symbol	Gene Model Description
	primary metabolism			
44.81499174	AT5G01320.1	pyruvate decarboxylase activity		Thiamine pyrophosphate dependent pyruvate decarboxylase family protein;
35.04139533	AT1G43800.1	fatty acid metabolic process		Plant stearoyl-acyl-carrier-protein desaturase family protein; FUNCTIONS IN: acyl-[acyl-carrier-protein] desaturase activity, oxidoreductase activity, transition metal ion binding; INVOLVED IN: oxidation reduction, fatty acid metabolic process, fatty acid biosynthetic process;
21.42810977	AT4G33070.1	pyruvate decarboxylase activity		Thiamine pyrophosphate dependent pyruvate decarboxylase family protein;
19.22020685	AT3G21720.1	carboxylic acid metabolic process	ISOCITRATE LYASE (ICL)	Encodes a glyoxylate cycle enzyme isocitrate lyase (ICL).
13.25059668	AT3G21720.1	*see above*	*see above*	*see above*
12.1313477	AT1G05650.1	carbohydrate metabolic process		Pectin lyase-like superfamily protein; FUNCTIONS IN: polygalacturonase activity; INVOLVED IN: carbohydrate metabolic process; LOCATED IN: endomembrane system;
11.61995669	AT5G65140.1	trehalose biosynthetic process	TREHALOSE-6-PHOSPHATE PHOSPHATASE J (TPPJ)	Haloacid dehalogenase-like hydrolase (HAD) superfamily protein; FUNCTIONS IN: catalytic activity, trehalose-phosphatase activity; INVOLVED IN: response to cadmium ion, trehalose biosynthetic process;
11.28479051	AT1G52700.1	carboxylesterase activity		alpha/beta-Hydrolases superfamily protein; FUNCTIONS IN: hydrolase activity, carboxylesterase activity
9.909884317	AT5G19550.1	nitrogen compound metabolic process	ASPARTATE AMINOTRANSFERASE 2 (ASP2)	Nitrogen metabolism. Major cytosolic isoenzyme controlling aspartate biosynthesis in the light.
9.416623956	AT1G52700.1	*see above*	*see above*	*see above*
7.421468149	AT3G15650.1			alpha/beta-Hydrolases superfamily protein; FUNCTIONS IN: hydrolase activity, carboxylesterase activity;
7.046798104	AT1G43800.1	*see above*		*see above*
6.902	AT1G02850.1	carbohydrate metabolic process	BETA GLUCOSIDASE 11 (BGLU11)	beta glucosidase 11 (BGLU11); FUNCTIONS IN: cation binding, hydrolase activity, hydrolyzing O-glucosyl compounds, catalytic activity; INVOLVED IN: carbohydrate metabolic process;

6.791	AT4G14690.1	regulation of chlorophyll biosynthetic process	EARLY LIGHT-INDUCIBLE PROTEIN 2 (ELIP2)	Encodes an early light-induced protein. ELIPs are thought not to be directly involved in the synthesis and assembly of specific photosynthetic complexes, but rather affect the biogenesis of all chlorophyll-binding complexes. A study (PMID 17553115) has shown that the chlorophyll synthesis pathway was downregulated as a result of constitutive ELIP2 expression, leading to decreased chlorophyll availability for the assembly of pigment-binding proteins for photosynthesis.
6.086	AT3G52720.1	one-carbon metabolic process	ALPHA CARBONIC ANHYDRASE 1 (ACA1)	Encodes a putative alpha carbonic anhydrase (CAH1) located in the chloroplast stroma. Most chloroplast proteins are encoded by the nuclear genome and imported with the help of sorting signals that are intrinsic parts of the polypeptides. CAH1 takes an alternative route through the secretory pathway, and becomes N-glycosylated before entering the chloroplast.
	stress			
133.6597248	AT5G10040.1	anaerobic respiration		unknown protein
65.61227324	AT1G77120.1	response to osmotic stress	ALCOHOL DEHYDROGENASE 1 (ADH1)	Catalyzes the reduction of acetaldehyde using NADH as reductant. Requires zinc for activity. Dimer. Anaerobic response polypeptide (ANP). Fermentation. The protein undergoes thiolation following treatment with the oxidant tert-butylhydroperoxide.
44.69307231	AT1G77120.1	*see above*	*see above*	*see above*
43.78978273	AT2G47520.1	response to anoxia, regulation of transcription	ETHYLENE RESPONSE FACTOR 71 (ERF71)	Encodes a member of the ERF (ethylene response factor) subfamily B-2 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 5 members in this subfamily including RAP2.2 AND RAP2.12.
40.73527144	AT1G69880.1	cell redox homeostasis	THIOREDOXIN H-TYPE 8 (TH8)	thioredoxin H-type 8 (TH8); INVOLVED IN: N-terminal protein myristoylation, cell redox homeostasis
37.72179725	AT3G43190.1	sucrose biosynthetic process/response to osmotic stress	SUCROSE SYNTHASE 4 (SUS4)	Encodes a protein with sucrose synthase activity (SUS4).
34.50012261	AT3G43190.1	*see above*	*see above*	*see above*
31.58295671	AT5G10040.1	*see above*		*see above*
29.85499962	AT3G43190.1	*see above*	*see above*	*see above*
26.96378754	AT5G38910.1	nutrient reservoir activity		RmlC-like cupins superfamily protein; FUNCTIONS IN: manganese ion binding, nutrient reservoir activity
23.83721548	AT3G43190.1	*see above*	*see above*	*see above*
21.52159121	AT1G05680.1	response to osmotic stress, shoot morphogenesis	URIDINE DIPHOSPHATE GLYCOSYLTRANSFERASE 74E2 (UGT74E2)	Encodes a UDP-glucosyltransferase, UGT74E2 that acts on IBA (indole-3-butyric acid) and affects auxin homeostasis. The transcript and protein levels of this enzyme are strongly induced by H2O2 and may allow integration of ROS (reactive oxygen species) and auxin signaling. This enzyme can also transfer glucosyl groups to several compounds related to the explosive TNT when this synthetic compound is taken up from the environment.
21.36183861	AT4G25200.1	response to heat	MITOCHONDRION-LOCALIZED SMALL HEAT SHOCK PROTEIN 23.6 (HSP23.6-MITO)	AtHSP23.6-mito mRNA, nuclear gene encoding mitochondrial
21.32215113	AT5G08640.1	flavonoid biosynthetic process	FLAVONOL SYNTHASE 1 (FLS1)	Encodes a flavonol synthase that catalyzes formation of flavonols from dihydroflavonols.
18.78142326	AT1G77120.1	*see above*	*see above*	*see above*

17.5817965	AT2G16060.1	Response to hypoxia/oxygen binding	HEMOGLOBIN 1 (HB1)	Encodes a class 1 nonsymbiotic hemoglobin induced by low oxygen levels with very high oxygen affinity. It is not likely to be a hemoglobin transporter because of its extremely high affinity for oxygen. Overexpression impairs cold stress-induced nitric oxide (NO) production.
14.71750876	AT5G50600.1	steroid metabolic process	HYDROXYSTEROID DEHYDROGENASE 1 (HSD1)	Encodes a hydroxysteroid dehydrogenase shown to act as a NADP+-dependent 11β-, 17β-hydroxysteroid dehydrogenase/17β-ketosteroid reductase called HSD1. Two copies of HSD1 (At5g50600 and At5g50700) exist in the Arabidopsis genome as a result of an exact 33-kb duplication on chromosome 5 encompassing seven genes. There are five homologs of HSD1 in Arabidopsis (HSD2-At3g47350, HSD3-At3g47360, HSD4-At5g50590, HSD4-At5g50690 and HSD6-At5g50770; HSD4 has two copies due to the same gene duplication event occurred to HSD1) (Plant Cell Physiology 50:1463). At5g50690 is also named as HSD7 (Plant Physiology 145:87). HSD1 is identified from the proteome of oil bodies from mature seeds. Transcription of HSD1 is specifically and highly induced in oil-accumulating tissues of mature seeds; transcript disappears during germination. To date, the endogenous substrates of this enzyme are not known.
13.80639703	AT4G10265.1	response to wounding		Wound-responsive family protein;
13.52995754	AT1G53540.1	response to heat		HSP20-like chaperones superfamily protein; CONTAINS InterPro DOMAIN/s: Heat shock protein Hsp20 (InterPro: IPR002068), HSP20-like chaperone (InterPro:IPR008978); BEST Arabidopsis thaliana protein match is: heat shock protein 17.4 (TAIR:AT3G46230.1); Has 7090 Blast hits to 7089 proteins in 1610 species: Archae - 175; Bacteria - 4287; Metazoa - 66; Fungi - 302; Plants - 1606; Viruses - 0; Other Eukaryotes - 654 (source: NCBI BLink).
12.71660064	AT3G03270.2	response to stress		
12.19103521	AT1G17180.1	toxin catabolic process	GLUTATHIONE S-TRANSFERASE TAU 25 (GSTU25)	Encodes glutathione transferase belonging to the tau class of GSTs. Naming convention according to Wagner et al. (2002).
11.98953834	AT1G07400.1	response to heat/response to oxidative stress		HSP20-like chaperones superfamily protein
11.48167517	AT4G30380.1	cellular response to hypoxia		Encodes a Plant Natriuretic Peptide (PNP). PNPs are a class of systemically mobile molecules distantly related to expansins; their biological role has remained elusive.
10.30197171	AT4G10270.1	response to wounding		Wound-responsive family protein
10.19994101	AT2G26040.1	*see above*		
10.02885383	AT3G51240.1	flavonoid biosynthetic process	FLAVANONE 3-HYDROXYLASE (F3H)	Encodes flavanone 3-hydroxylase that is coordinately expressed with chalcone synthase and chalcone isomerases. Regulates flavonoid biosynthesis.
9.510073891	AT2G16060.1	Response to hypoxia/oxygen binding		
9.337645555	AT5G20830.1	sucrose biosynthetic process/response to osmotic stress	SUCROSE SYNTHASE 1 (SUS1)	Encodes a protein with sucrose synthase activity (SUS1).
9.312342779	AT5G56080.1	nicotianamine biosynthetic process	NICOTIANAMINE SYNTHASE 2 (NAS2)	Encodes a protein with nicotianamine synthase activity. Its transcript levels rise in roots in response to zinc deficiency and rise in leaves in response to elevated levels of zinc.
8.838913263	AT5G39050.1	O-malonyltransferase activity	PHENOLIC GLUCOSIDE MALONYLTRANSFERASE 1 (PMAT1)	Encodes a malonyltransferase that may play a role in phenolic xenobiotic detoxification.
8.138045922	AT2G21640.1	response to oxidative stress		Encodes a protein of unknown function that is a marker for oxidative stress response.

8.137165503	AT1G17170.1	toxin catabolic process	GLUTATHIONE S-TRANSFERASE TAU 24 (GSTU24)	Encodes glutathione transferase belonging to the tau class of GSTs. Naming convention according to Wagner et al. (2002).
8.12316463	AT3G03270.1	response to stress		Adenine nucleotide alpha hydrolases-like superfamily protein; INVOLVED IN: response to stress
8.051482104	AT2G29500.1	response to heat/response to oxidative stress		HSP20-like chaperones superfamily protein
7.649411174	AT3G51240.1	*see above*	*see above*	*see above*
7.447940758	AT2G21640.1	response to oxidative stress	PYR1-LIKE 2 (PYL2)	Encodes a member of the PYR (pyrabactin resistance)/PYL (PYR1-like)/RCAR (regulatory components of ABA receptor) family proteins with 14 members. PYR/PYL/RCAR family proteins function as abscisic acid sensors. Mediate ABA-dependent regulation of protein phosphatase 2Cs ABI1 and ABI2.
7.296339624	AT5G20830.1	*see above*	*see above*	*see above*
6.272	AT5G49190.1	sucrose biosynthetic process/response to osmotic stress	SUCROSE SYNTHASE 2 (SUS2)	Encodes a sucrose synthase (SUS2). The activity of the enzyme could not be assayed as proved to be insoluble (PMID 17257168). However, analyses of a sus2 mutant revealed a deficiency in sucrose synthase activity 12 and 15 days after flowering. There are some reports that SUS2 transcript levels are increased in leaves specifically by O (2) deficiency whereas other reports indicate that SUS2 is expressed only in seeds. Immunlocalization shows that SUS2 is present in the cytosol of developing seeds, but, it also associated with plastids, though not located within them.
6.262	AT3G55120.1	flavonoid biosynthetic process	TRANSPARENT TESTA 5 (TT5)	Catalyzes the conversion of chalcones into flavanones. Required for the accumulation of purple anthocyanins in leaves and stems.
transcription/translation				
189.0301618	AT3G47720.1	NAD+ ADP-ribosyltransferase activity	SIMILAR TO RCD ONE 4 (SRO4)	Encodes a protein with similarity to RCD1 but without the WWE domain. The protein does have a PARP signature upstream of the C-terminal protein interaction domain. The PARP signature may bind NAD+ and attach the ADP-ribose-moiety from NAD+ to the target molecule. Its presence suggests a role for the protein in ADP ribosylation.
65.31020106	AT1G12805.1	nucleotide binding		nucleotide binding
62.45049232	AT3G47720.1	*see above*	*see above*	*see above*
31.20630654	AT1G12805.1	*see above*		*see above*
14.05315749	AT2G27535.1	cytosolic large ribosomal subunit		ribosomal protein L10A family protein
8.665058898	AT4G28811.1	regulation of transcription		basic helix-loop-helix (bHLH) DNA-binding superfamily protein; FUNCTIONS IN: transcription regulator activity
7.87081517	AT4G32950.1	dephosphorylation		Protein phosphatase 2C family protein; FUNCTIONS IN: protein serine/threonine phosphatase activity, catalytic activity
6.463	AT3G17609.1	response to karrikin/ regulation of transcription	HY5-HOMOLOG (HYH)	Encodes a homolog of HY5 (HYH). Involved in phyB signaling pathway.
transport				
22.7978632	AT2G29870.1	water transport		Aquaporin-like superfamily protein; FUNCTIONS IN: water channel activity; INVOLVED IN: transport
10.81814774	AT2G37870.1	lipid transport		Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein; FUNCTIONS IN: lipid binding; INVOLVED IN: lipid transport

10.15785917	AT5G52860.1	ATPase activity, coupled to transmembrane movement of substances	ATP-BINDING CASSETTE G8 (ABCG8)	ABC-2 type transporter family protein; FUNCTIONS IN: ATPase activity, coupled to transmembrane movement of substances
8.992584055	AT5G44110.1	transport	ATP-BINDING CASSETTE A21 (ABCI21)	Encodes a member of the NAP subfamily of ABC transporters.
8.873173878	AT2G04070.1	drug transmembrane transport		MATE efflux family protein; FUNCTIONS IN: antiporter activity, drug transmembrane transporter activity, transporter activity; INVOLVED IN: transport, drug transmembrane transport, transmembrane transport
6.377	AT4G00165.1	lipid transport		Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein; FUNCTIONS IN: lipid binding; INVOLVED IN: N-terminal protein myristoylation, lipid transport
6.138	TC303451	antiporter/ drug transporter/ transporter		tc|NM_126448 antiporter/ drug transporter/ transporter
	other			
171.4679745	AT3G29970.1	unknown		B12D protein
131.0546103	AT2G39510.1	unknown		Nodulin MtN21 /EamA-like transporter family protein
72.90182275	AT2G17850.1	ageing		Rhodanese/Cell cycle control phosphatase superfamily protein
53.55248723	AT2G17850.1	*see above*		*see above*
39.86385608	AT2G39510.1	unknown		
38.15629017	AT2G14247.1	unknown		Expressed protein
36.00384885	AT3G50610.1	unknown		unknown protein
28.10064058	AT3G21860.1	ubiquitin-dependent protein catabolic process	SKP1-LIKE 10 (SK10)	SKP1-like 10 (SK10); CONTAINS InterPro DOMAIN/s: E3 ubiquitin ligase, SCF complex, Skp subunit (InterPro:IPR016897), SKP1 component, dimerisation
25.73177312	AT4G33560.1	unknown		Wound-responsive family protein
25.20452269	AT3G20395.1	zinc ion binding		RING/U-box superfamily protein; FUNCTIONS IN: zinc ion binding
21.99348763	AT2G44460.1	catalytic activity/hydrolase activity, hydrolyzing O-glycosyl compounds	BETA GLUCOSIDASE 28 (BGLU28)	beta glucosidase 28 (BGLU28); FUNCTIONS IN: cation binding, hydrolase activity, hydrolyzing O-glycosyl compounds, catalytic activity; INVOLVED IN: response to karrikin
17.71606949	AT5G10580.2	unknown		Protein of unknown function, DUF599
16.40491946	AT2G38823.1	unknown		unknown protein
13.84217596	AT1G08090.1	lateral root development/nitrate transport	NITRATE TRANSPORTER 2:1 (NRT2:1)	High-affinity nitrate transporter. Up-regulated by nitrate. Functions as a repressor of lateral root initiation independently of nitrate uptake.

13.32026143	AT4G01630.1	plant-type cell wall modification involved in multidimensional cell growth	EXPANSIN A17 (EXPA17)	Member of Alpha-Expansin Gene Family. Naming convention from the Expansin Working Group (Kende et al, 2004. Plant Mol Bio)
13.18697099	AT3G14300.1	cell wall modification	(ATPMEPCRC)	ATPMEPCRC; FUNCTIONS IN: pectinesterase activity; INVOLVED IN: cell wall modification
12.84761981	AT3G20395.1	zinc ion binding		
11.42905828	AT3G22550.1	unknown		Protein of unknown function (DUF581)
10.51991552	AT5G61412.1	unknown		unknown protein
10.24987234	AT1G67100.1	response to gibberellin stimulus	LOB DOMAIN-CONTAINING PROTEIN 40 (LBD40)	LOB domain-containing protein 40 (LBD40); CONTAINS InterPro DOMAIN/s: Lateral organ boundaries, LOB (InterPro:IPR004883)
9.761348677	AT5G04120.1	catalytic activity		Phosphoglycerate mutase family protein; FUNCTIONS IN: catalytic activity
9.487959831	AT1G60750.1	oxidation-reduction process		NAD(P)-linked oxidoreductase superfamily protein; FUNCTIONS IN: oxidoreductase activity; INVOLVED IN: oxidation reduction
9.047490532	AT1G67270.1	unknown		Zinc-finger domain of monoamine-oxidase A repressor R1 protein
9.008340398	AT1G26290.1	unknown		Unknown protein (source: NCBI BLink).
8.82978219	AT4G19230.1	abscisic acid metabolic process/ fungus defence	CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE 1 (CYP707A1)	Encodes a protein with ABA 8'-hydroxylase activity, involved in ABA catabolism. Member of the CYP707A gene family. CYP707A1 appears to play an important role in determining the ABA levels in dry seeds. Gene involved in postgermination growth. Overexpression of CYP707A1 leads to a decrease in ABA levels and a reduction in after-ripening period to break dormancy.
8.798110289	AT5G43450.1	1-aminocyclopropane-1-carboxylate oxidase activity		encodes a protein whose sequence is similar to ACC oxidase
8.771324969	AT1G60050.1	unknown		Nodulin MtN21 /EamA-like transporter family protein
8.352842342	AT4G19230.1	AT2G21640.1		
8.321887289	AT2G14070.1	unknown		wound-responsive protein-related; CONTAINS InterPro DOMAIN/s: FBD-like (InterPro:IPR006566), Protein of unknown function wound-induced (InterPro:IPR022251); BEST Arabidopsis thaliana protein match is: Protein with RNI-like/FBD-like domains (TAIR:AT3G59180.1); Has 177 Blast hits to 175 proteins in 13 species: Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 177; Viruses - 0; Other Eukaryotes - 0 (source: NCBI BLink).
8.137767619	AT4G24110.1	response to karrikin		unknown protein
8.112079852	AT1G26770.1	plant-type cell wall modification involved in multidimensional cell growth	EXPANSIN A10 (EXPA10)	Encodes an expansin. Naming convention from the Expansin Working Group (Kende et al, Plant Mol Bio). Involved in the formation of nematode-induced syncytia in roots of Arabidopsis thaliana.
8.092580012	AT4G12735.1	unknown		unknown protein

8.019408389	AT1G05560.1	abscisic acid glucosyltransferase activity/cell wall biogenesis	UDP-GLUCOSYLTRANSFERASE 75B1 (UGT75B1)	A UDP-glucose transferase localized in the phragmoplast. It has been co-purified with the callose synthase complex and may transfer UDP-glucose from sucrose synthase to the callose synthase and thus help form a substrate channel for the synthesis of callose at the forming cell plate. Induced by salicylic acid. Independent of NPR1 for their induction by salicylic acid. UGT1 encodes a protein with glucosyltransferase activity with high sequence homology to UGT2 (AT1G05530). It belongs to an UGT subfamily that binds UDP-glucose but not UDP-glucuronate, UDP-galactose, or UDP-rhamnose as the glucosyl donor. UGT1 was shown to be able to use abscisic acid as glucosylation substrate in the presence of UDP-glucose. UGT1/UGT75B1 catalyzes the formation of the p-aminobenzoate-glucose ester in vitro and in vivo. It appears to be the enzyme predominantly responsible for pABA-Glc formation in Arabidopsis based on assays in leaves, flowers, and siliques.
7.720983199	AT4G37370.1	electron carrier activity	CYTOCHROME P450, FAMILY 81, SUBFAMILY D, POLYPEPTIDE 8 (CYP81D8)	member of CYP81D
7.317086706	AT5G50610.1	unknown		unknown protein
7.285759992	AT5G08630.1	unknown		DDT domain-containing protein
7.226778408	AT2G46420.1	unknown		Plant protein 1589 of unknown function
7.207464621	AT1G52690.1	embryo development ending in seed dormancy	LATE EMBRYOGENESIS ABUNDANT 7 (LEA7)	Late embryogenesis abundant protein (LEA) family protein; BEST Arabidopsis thaliana protein match is: Late embryogenesis abundant protein (LEA) family protein (TAIR:AT3G15670.1)
7.165372832	AT5G39890.1	cysteamine dioxygenase activity		Protein of unknown function (DUF1637); FUNCTIONS IN: cysteamine dioxygenase activity; INVOLVED IN: oxidation reduction
7.037	AT5G04120.1	catalytic activity		Phosphoglycerate mutase family protein; FUNCTIONS IN: catalytic activity; INVOLVED IN: metabolic process
6.950	AT2G46420.1	unknown		Plant protein 1589 of unknown function
6.852	AT5G03400.1	unknown		unknown protein
6.835	AT1G15415.1	unknown		The protein encoded by this gene was identified as a part of pollen proteome by mass spec analysis. It has weak homology to LEA (late embryo abundant) proteins. Encodes protein phosphatase 2A (PP2A) B'gamma subunit. Targeted to nucleus and cytosol.
6.699	TA53132_3702	unknown		tc|GB|AY144109.1|AAN08444.1 hypothetical protein [Arabidopsis thaliana] [NP537440]
6.556	AT4G18650.1	protein binding		A maternally expressed imprinted gene.
6.455	AT1G70260.1	unknown		Nodulin MtN21 /EamA-like transporter family protein
6.437	AT1G70260.1	unknown		
6.431	NP455927	unknown		tc|GB|AC004681.3|AAC25931.1 hypothetical protein [Arabidopsis thaliana] [NP455927]
6.393	AT4G26460.1	methyltransferase activity		S-adenosyl-L-methionine-dependent methyltransferases superfamily protein; FUNCTIONS IN: methyltransferase activity
6.332	BP603152	unknown		tc|ACEA_ARATH (P28297) Isocitrate lyase (Isocitrase) (Isocitratase) (ICL), partial (13%) [TC312541]
6.113	AT2G19590.1	ethylene biosynthetic process	ACC OXIDASE 1 (ACO1)	encodes a protein whose sequence is similar to 1-aminocyclopropane-1-carboxylate oxidase

Table A21: Top 100 genes that were downregulated in a shifting experiment from low-sucrose to high sucrose in *Arabidopsis* roots. (Some genes appear in replicates. Only the first entry has a description.)

Fold Change	Systematic Name	categories	Primary Gene Symbol	Gene Model Description
	primary metabolism			
-57.33151873	AT2G22980.1	proteolysis	SERINE CARBOXYPEPTIDASE-LIKE 13 (SCPL13)	serine carboxypeptidase-like 13 (SCPL13); FUNCTIONS IN: serine-type carboxypeptidase activity; INVOLVED IN: proteolysis
-27.59360349	AT3G15840.1	chlororespiration	POST-ILLUMINATION CHLOROPHYLL FLUORESCENCE INCREASE (PIFI)	Encodes a chloroplast-targeted protein localized in the stroma that is a novel component essential for NDH-mediated non-photochemical reduction of the plastoquinone pool in chlororespiratory electron transport.
-16.40155205	AT3G05950.1	nutrient reservoir activity		RmlC-like cupins superfamily protein; FUNCTIONS IN: manganese ion binding, nutrient reservoir activity
-14.49466552	AT1G80440.1	Galactose oxidase		Galactose oxidase/kelch repeat superfamily protein
-11.54801698	AT1G80440.1	*see above*		*see above*
-11.26092963	AT3G27660.1	lipid storage/response to freezing	OLEOSIN 4 (OLEO4)	Encodes oleosin4 (Plant Cell, 2006, 18:1961), a protein found in oil bodies, involved in seed lipid accumulation. Functions in freezing tolerance of seeds. Note: also referred to as OLE3 in Plant Journal 2008, 55:798.
-10.48018727	AT1G08630.1	threonine catabolic process	THREONINE ALDOLASE 1 (THA1)	Encodes a threonine aldolase, involved in threonine degradation to glycine. Primarily expressed in seeds and seedlings.
-9.808	AT5G39150.1	nutrient reservoir activity		RmlC-like cupins superfamily protein; FUNCTIONS IN: manganese ion binding, nutrient reservoir activity
-9.698878361	AT3G04300.1	unknown		RmlC-like cupins superfamily protein; CONTAINS InterPro DOMAIN/s: Cupin, RmlC-type (InterPro:IPR011051), Protein of unknown function DUF861, cupin-3 (InterPro:IPR008579), RmlC-like jelly roll fold (InterPro:IPR014710)
-9.446	AT2G22960.1	proteolysis		alpha/beta-Hydrolases superfamily protein; FUNCTIONS IN: serine-type carboxypeptidase activity; INVOLVED IN: proteolysis
-9.334	AT3G15840.3	*see above*	*see above*	*see above*
-8.712	AT1G08630.1	*see above*	*see above*	*see above*
-8.659088909	AT1G10070.1	branched chain family amino acid metabolic process	BRANCHED-CHAIN AMINO ACID TRANSAMINASE 2 (BCAT-2)	Encodes a chloroplast branched-chain amino acid aminotransferase. Complements the yeast leu/iso-leu/val auxotrophy mutant.
-8.464034346	AT1G11530.1	protein thiol-disulfide exchange	C-TERMINAL CYSTEINE RESIDUE IS CHANGED TO A SERINE 1 (CXXS1)	Encodes a monocysteinic thioredoxin, thioredoxin in which the second cysteine of the redox site is replaced by a serine, with low disulfide reductase but efficient disulfide isomerase activity.
	stress			
-43.12987024	AT5G06690.1	cell redox homeostasis	WCRKC THIOREDOXIN 1 (WCRKC1)	Encodes a thioredoxin (WCRKC1) localized in chloroplast stroma. Contains a WCRKC motif.
-32.28458463	AT2G26560.1	oxylipin biosynthetic process/HR/pathogen defence	PHOSPHOLIPASE A 2A (PLA2A)	Encodes a lipid acyl hydrolase with wide substrate specificity that accumulates upon infection by fungal and bacterial pathogens. Protein is localized in the cytoplasm in healthy leaves, and in membranes in infected cells. Plays a role in cell death and differentially affects the accumulation of oxylipins. Contributes to resistance to virus.

-31.52519487	AT1G71030.1	anthocyanin biosynthetic process	MYB-LIKE 2 (MYBL2)	Encodes a putative myb family transcription factor. In contrast to most other myb-like proteins its myb domain consists of a single repeat. A proline-rich region potentially involved in transactivation is found in the C-terminal part of the protein. Its transcript accumulates mainly in leaves.
-23.086	AT2G34315.1	avirulence/GTP binding		Avirulence induced gene (AIG1) family protein; FUNCTIONS IN: GTP binding; INVOLVED IN: biological process unknown; LOCATED IN: cellular component unknown; EXPRESSED IN: embryo, root, stamen;
-22.13267676	AT2G26560.1	*see above*	*see above*	*see above*
-17.51732829	AT2G18050.1	nucleosome assembly/response to water deprivation	HISTONE H1-3 (HIS1-3)	Encodes a structurally divergent linker histone whose gene expression is induced by dehydration and ABA.
-16.78355322	AT1G19610.1	defense response	(PDF1.4)	Predicted to encode a PR (pathogenesis-related) protein. Belongs to the plant defensin (PDF) family
-16.71247599	AT5G61590.1	response to water deprivation		Encodes a member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 18 members in this subfamily including ATERF-1, ATERF-2, AND ATERF-5.
-16.2981233	AT2G18050.1	*see above*	*see above*	*see above*
-13.94386323	AT2G41240.1	response to water deprivation	BASIC HELIX-LOOP-HELIX PROTEIN 100 (BHLH100)	Encodes a member of the basic helix-loop-helix transcription factor family protein.
-12.73733265	AT1G67980.1	lignin biosynthetic process	CAFFEOYL-COA 3-O-METHYLTRANSFERASE (CCOAMT)	Encodes S-adenosyl-L-methionine: transcaffeoyl Coenzyme A 3-O-methyltransferase.
-12.60537562	AT2G30750.1	response to bacterium	CYTOCHROME P450, FAMILY 71, SUBFAMILY A, POLYPEPTIDE 12 (CYP71A12)	putative cytochrome P450
-12.46460006	AT4G35770.1	aging/stress response	SENESCENCE 1 (SEN1)	Senescence-associated gene that is strongly induced by phosphate starvation. Transcripts are differentially regulated at the level of mRNA stability at different times of day. mRNAs are targets of the mRNA degradation pathway mediated by the downstream (DST) instability determinant.
-12.41288582	AT5G56550.1	response to oxidative stress/heavy metals	OXIDATIVE STRESS 3 (OXS3)	Encodes OXIDATIVE STRESS 3 (OXS3); involved in tolerance to heavy metals and oxidative stress.
-12.17873376	AT2G43010.1	light response	PHYTOCHROME INTERACTING FACTOR 4 (PIF4)	Isolated as a semidominant mutation defective in red -light responses. Encodes a nuclear localized bHLH protein that interacts with active PhyB protein. Negatively regulates phyB mediated red light responses. Involved in shade avoidance response. Protein abundance is negatively regulated by PhyB.
-11.12077634	AT1G76800.1	response to iron/response to ethylene		The gene encodes nodulin-like2 whose transcript abundance was repressed under conditions of Fe-deficient growth.
-10.82269606	AT2G40000.1	response to bacterium/response to oxidative stress	ORTHOLOG OF SUGAR BEET HS1 PRO-1 2 (HSPRO2)	ortholog of sugar beet HS1 PRO-1 2 (HSPRO2); CONTAINS InterPro DOMAIN/s: Hs1pro-1, C-terminal (InterPro:IPR009743), Hs1pro-1, N-terminal
-10.76499577	AT2G14560.2	defense response to fungus	LATE UPREGULATED IN RESPONSE TO HYALOPERONOSPORA PARASITICA (LURP1)	Encodes LURP1, a member of the LURP cluster (late upregulated in response to Hyaloperonospora parasitica) which exhibits a pronounced upregulation after recognition of the pathogenic oomycte H. parasitica. LURP1 is required for full basal defense to H. parasitica and resistance to this pathogen mediated by the R-proteins RPP4 and RPP5.
-10.61910414	AT2G40000.1	*see above*	*see above*	*see above*
-10.549	AT2G40000.1	*see above*	*see above*	*see above*

-10.43537398	AT2G40000.1	*see above*	*see above*	*see above*
-10.3322584	AT3G48360.1	regulation of response to stress	BTB AND TAZ DOMAIN PROTEIN 2 (bt2)	Encodes a protein (BT2) that is an essential component of the TAC1-mediated telomerase activation pathway. Acts redundantly with BT3 and BT1 during female gametophyte development and with BT3 during male gametophyte development. BT2 also mediates multiple responses to nutrients, stresses, and hormones.
-10.01897596	AT3G53770.1	response to stress		late embryogenesis abundant 3 (LEA3) family protein; FUNCTIONS IN: molecular function unknown; INVOLVED IN: response to stress
-9.840847714	AT4G18210.1	response to nematode/ purine base transport	PURINE PERMEASE 10 (PUP10)	Member of a family of proteins related to PUP1, a purine transporter. May be involved in the transport of purine and purine derivatives such as cytokinins, across the plasma membrane.
-9.739996772	AT1G78290.2	protein kinase activity/response to osmotic stress	SNF1-RELATED PROTEIN KINASE 2-8 (SNRK2-8)	Encodes a member of SNF1-related protein kinase (SnRK2) family whose activity is activated by ionic (salt) and non-ionic (mannitol) osmotic stress and dehydration.
-9.242306416	AT2G18660.1	pathogen defence	PLANT NATRIURETIC PEPTIDE A (PNP-A)	Encodes PNP-A (Plant Natriuretic Peptide A). PNPs are a class of systemically mobile molecules distantly related to expansins; their biological role has remained elusive. PNP-A contains a signal peptide domain and is secreted into the extracellular space. Co-expression analyses using microarray data suggest that PNP-A may function as a component of plant defence response and SAR in particular, and could be classified as a newly identified PR protein.
-9.101856037	AT4G34410.1	defense response to fungus	REDOX RESPONSIVE TRANSCRIPTION FACTOR 1 (RRTF1)	Encodes a member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 18 members in this subfamily including ATERF-1, ATERF-2, AND ATERF-5.
-8.907947018	AT5G63160.1	response to stress/development related	BTB AND TAZ DOMAIN PROTEIN 1 (bt1)	BTB and TAZ domain protein. Short-lived nuclear-cytoplasmic protein targeted for degradation by the 26S proteosome pathway. Acts redundantly with BT2 and BT3 during female gametophyte development.
-8.619885379	AT4G16860.1	defense response to fungus	RECOGNITION OF PERONOSPORA PARASITICA 4 (RPP4)	Confers resistance to Peronospora parasitica. RPP4 is coordinately regulated by transcriptional activation and RNA silencing.
-8.59568345	AT4G21920.1	N-terminal protein myristoylation		unknown protein; FUNCTIONS IN: molecular function unknown; INVOLVED IN: N-terminal protein myristoylation
-8.541	AT1G79700.1	pathogen response/organ morphogenesis		Integrase-type DNA-binding superfamily protein; CONTAINS InterPro DOMAIN/s: DNA-binding, integrase-type (InterPro:IPR016177), Pathogenesis-related transcriptional factor/ERF, DNA-binding (InterPro:IPR001471); BEST Arabidopsis thaliana protein match is: ARIA-interacting double AP2 domain protein (TAIR:AT1G16060.1)
-8.261352955	AT3G48360.1	*see above*	*see above*	*see above*
-8.161833179	AT4G35770.1	*see above*		*see above*
-8.095959121	AT4G33980.1	response to karrikin		BEST Arabidopsis thaliana protein match is: cold regulated gene 27 (TAIR:AT5G42900.2)
	transcription/translation			
-13.18628501	AT2G25900.1	transcription	(ATCTH)	putative Cys3His zinc finger protein (ATCTH) mRNA, complete
-12.85536174	AT5G04150.1	transcription factor activity	(BHLH101)	BHLH101; FUNCTIONS IN: DNA binding, sequence-specific DNA binding transcription factor activity; INVOLVED IN: regulation of transcription; LOCATED IN: nucleus; EXPRESSED IN: root, leaf
-9.712921887	AT1G76590.1	transcription		PLATZ transcription factor family protein; CONTAINS InterPro DOMAIN/s: Protein of unknown function DUF597
-9.702631486	AT2G25900.1			

-9.033111811	DR229968	transcription		tc|Q9LVR0_ARATH (Q9LVR0) Homeodomain transcription factor-like (At5g66700) (Homeodomain protein), partial (33%) [TC313719]
-8.51077722	AT2G21900.1	transcription	WRKY DNA-BINDING PROTEIN 59 (WRKY59)	member of WRKY Transcription Factor; Group II-c
	transport			
-28.70405708	AT4G08300.1	transport		Nodulin MtN21 /EamA-like transporter family protein; LOCATED IN: membrane
-21.927	AT5G53190.1	sugar transmembrane transporter activity	(SWEET3)	Nodulin MtN3 family protein; INVOLVED IN: biological process unknown; LOCATED IN: integral to membrane, membrane
-16.43323808	AT4G08290.1	transport		Nodulin MtN21 /EamA-like transporter family protein; LOCATED IN: endomembrane system, membrane
-9.679008051	AT3G48740.1	sugar transmembrane transporter activity	(SWEET11)	Nodulin MtN3 family protein; INVOLVED IN: biological process unknown; LOCATED IN: endomembrane system, integral to membrane, membrane
-8.110502792	AT1G29100.1	metal ion transport		Heavy metal transport/detoxification superfamily protein ; FUNCTIONS IN: copper ion binding, metal ion binding; INVOLVED IN: copper ion transport, metal ion transport; CONTAINS InterPro DOMAIN/s: Heavy metal transport/detoxification protein
	other			
-67.05014157	AT2G33830.2	unknown		Dormancy/auxin associated family protein; CONTAINS InterPro DOMAIN/s: Dormancy/auxin associated (InterPro:IPR008406)
-63.89327116	AT2G33830.2	*see above*		*see above*
-43.9885641	AT5G62360.1	negative regulation of catalytic activity		Plant invertase/pectin methylesterase inhibitor superfamily protein; FUNCTIONS IN: enzyme inhibitor activity, pectinesterase inhibitor activity, pectinesterase activity
-39.584	AT2G33830.1	*see above*		*see above*
-31.77040758	AT2G33830.1	*see above*		*see above*
-28.61890166	AT3G26740.1	unknown	CCR-LIKE (CCL)	Transcripts are differentially regulated at the level of mRNA stability at different times of day controlled by the circadian clock. mRNAs are targets of the mRNA degradation pathway mediated by the downstream (DST) instability determinant.
-24.138	AT2G05540.1	unknown		Glycine-rich protein family; LOCATED IN: endomembrane system
-23.086	AT2G34315.1	GTP binding		Avirulence induced gene (AIG1) family protein; FUNCTIONS IN: GTP binding; INVOLVED IN: biological process unknown; LOCATED IN: cellular component unknown; EXPRESSED IN: embryo, root, stamen;
-22.03284023	AT2G05540.1	*see above*		*see above*
-21.589	TC304561	unknown		unknown
-21.58177559	AT2G33830.1	*see above*		*see above*
-19.77438442	AT4G16000.1	unknown		unknown protein
-19.70954881	AT5G62360.1	*see above*		*see above*
-18.594	EF183277	unknown		ug|Arabidopsis thaliana clone asmbl_4567 unknown mRNA sequence [EF183277]

-18.1952969	AT1G61810.1	lignin biosynthetic process	BETA-GLUCOSIDASE 45 (BGLU45)	beta-glucosidase 45 (BGLU45); FUNCTIONS IN: cation binding, hydrolase activity, hydrolyzing O-glycosyl compounds, catalytic activity; INVOLVED IN: lignin biosynthetic process; LOCATED IN: endomembrane system
-18.05153209	AT4G15990.1	unknown		unknown protein
-17.0171671	AT3G23880.1	unknown		F-box and associated interaction domains-containing protein
-16.64803323	AT5G54585.1	unknown		unknown protein
-16.506	AT1G13710.1	positive regulation of organ growth	CYTOCHROME P450, FAMILY 78, SUBFAMILY A, POLYPEPTIDE 5 (CYP78A5)	Encodes the cytochrome P450 CYP78A5 monooxygenase. Contributes to the generation of a growth-stimulating signal distinct from the classical phytohormones that prevents proliferation arrest, promoting organ growth.
-16.08650559	AT2G14990.1	transposable element gene		transposable element gene; non-LTR retrotransposon family (LINE), has a 8.7e-37 P-value blast match to GB:NP_038605 L1 repeat, Tf subfamily, member 30 (LINE-element) (Mus musculus)
-15.69002305	AK222109	unknown		ug|Arabidopsis thaliana mRNA for hypothetical protein, complete cds, clone: RAFL22-91-A11 [AK222109]
-15.47046057	AT2G44220.1	unknown		Protein of Unknown Function (DUF239)
-14.549	TA29648_3702	unknown		unknown
-13.656	TA30818_3702	unknown		unknown
-12.46814199	AT3G15450.1	unknown		Aluminium induced protein with YGL and LRDR motifs; FUNCTIONS IN: molecular function unknown
-11.12966792	AT3G50560.1	oxidation-reduction process		NAD(P)-binding Rossmann-fold superfamily protein; FUNCTIONS IN: oxidoreductase activity, binding, catalytic activity; INVOLVED IN: oxidation reduction, metabolic process
-11.12644923	AT3G01175.1	unknown		Protein of unknown function (DUF1666)
-11.11166233	AT1G22160.1	unknown		Protein of unknown function (DUF581);
-11.00681255	AT2G47400.1	negative regulation of reductive pentose-phosphate cycle	CP12 DOMAIN-CONTAINING PROTEIN 1 (CP12-1)	CP12-1 encodes a small peptide found in the chloroplast stroma. It belongs to the CP12 gene family thought to be involved in the formation of a supramolecular complex with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoribulokinase (PRK) embedded in the Calvin cycle.
-10.97248679	AT2G46660.1	oxidation-reduction process	CYTOCHROME P450, FAMILY 78, SUBFAMILY A, POLYPEPTIDE 6 (CYP78A6)	member of CYP78A
-10.79532735	AT1G64160.1	lignan biosynthetic process/defence response		Disease resistance-responsive (dirigent-like protein) family protein; FUNCTIONS IN: molecular function unknown; INVOLVED IN: lignan biosynthetic process, defense response; LOCATED IN: endomembrane system; EXPRESSED IN: petal, hypocotyl, root; EXPRESSED DURING: petal differentiation and expansion stage; CONTAINS InterPro DOMAIN/s: Plant disease resistance response protein (InterPro:IPR004265)

-10.56188958	AT4G13090.1	xyloglucan:xyloglucosyl transferase activity	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 2 (XTH2)	xyloglucan endotransglucosylase/hydrolase 2 (XTH2); FUNCTIONS IN: hydrolase activity, acting on glycosyl bonds, xyloglucan:xyloglucosyl transferase activity, hydrolase activity, hydrolyzing O-glycosyl compounds; INVOLVED IN: carbohydrate metabolic process, cellular glucan metabolic process; LOCATED IN: endomembrane system, cell wall, apoplast; CONTAINS InterPro DOMAIN/s: Xyloglucan endotransglucosylase/hydrolase (InterPro:IPR016455), Beta-glucanase (InterPro:IPR008264), Xyloglucan endo-transglycosylase, C-terminal (InterPro:IPR010713), Concanavalin A-like lectin/glucanase, subgroup (InterPro:IPR013320), Concanavalin A-like lectin/glucanase (InterPro:IPR008985), Glycoside hydrolase, family 16 (InterPro:IPR000757)
-10.48077003	AT3G02390.1	unknown		unknown protein
-10.30711893	AT1G68040.1	methylation		S-adenosyl-L-methionine-dependent methyltransferases superfamily protein; FUNCTIONS IN: S-adenosylmethionine-dependent methyltransferase activity, methyltransferase activity; INVOLVED IN: biological process unknown; LOCATED IN: cellular component unknown; EXPRESSED IN: root;
-10.27182669	AT2G47400.1	*see above*	*see above*	*see above*
-10.090	BE039144	unknown		tc|Q9LE80_ARATH (Q9LE80) Arabidopsis thaliana genomic DNA, chromosome 3, P1 clone: MJK13 (AT3g15450/MJK13_11) (MJK13.11 protein), partial (75%) [TC306364]
-10.08629039	AT3G14185.1	unknown		Unknown gene
-9.967226723	AT2G28305.1	unknown	LONELY GUY 1 (LOG1)	LONELY GUY 1 (LOG1); CONTAINS InterPro DOMAIN/s: Conserved hypothetical protein CHP00730 (InterPro:IPR005269); BEST Arabidopsis thaliana protein match is: lysine decarboxylase family protein (TAIR:AT2G37210.1)
-9.931862614	AT1G58320.1	unknown		PLAC8 family protein; CONTAINS InterPro DOMAIN/s: Protein of unknown function Cys-rich (InterPro:IPR006461)
-9.925850615	AT3G15450.3	unknown		
-9.849845185	AT1G21400.1	oxidation-reduction process		Thiamin diphosphate-binding fold (THDP-binding) superfamily protein; FUNCTIONS IN: oxidoreductase activity, acting on the aldehyde or oxo group of donors, disulfide as acceptor, 3-methyl-2-oxobutanoate dehydrogenase (2-methylpropanoyl-transferring) activity; INVOLVED IN: metabolic process; EXPRESSED IN: guard cell
-9.73788155	AT1G28330.1	response to sucrose stimulus	DORMANCY-ASSOCIATED PROTEIN-LIKE 1 (DYL1)	dormancy-associated protein (DRM1)
-9.592999219	AT5G45310.1	unknown		unknown protein
-9.27750991	AT5G44440.1	oxidation-reduction process		FAD-binding Berberine family protein; FUNCTIONS IN: electron carrier activity, oxidoreductase activity, FAD binding, catalytic activity; LOCATED IN: endomembrane system
-9.209328235	AT1G30030.1	transposable element gene		transposable element gene; non-LTR retrotransposon family (LINE), has a 3.5e-30 P-value blast match to GB:AAA39398 ORF2 (Mus musculus) (LINE-element)
-9.187198904	AT5G44585.1	unknown		unknown protein;
-9.181852929	AT5G02160.1	unknown		unknown protein
-9.082768473	AT5G44582.1	unknown		unknown protein
-9.070612504	AT5G66700.1	root development	HOMEOBOX 53 (HB53)	Encodes a homeodomain protein. Member of HD-ZIP 1 family, most closely related to HB5. AtHB53 is auxin-inducible and its induction is inhibited by cytokinin, especially in roots therefore may be involved in root development.
-8.811941806	AT3G49790.1	ATP binding		Carbohydrate-binding protein; FUNCTIONS IN: ATP binding; INVOLVED IN: biological process unknown

-8.724712767	AT1G28330.2	*see above*	*see above*	*see above*
-8.689745933	AT3G15630.1	unknown		unknown protein
-8.603443004	AT4G18830.1	negative regulation of transcription, DNA-dependent	OVATE FAMILY PROTEIN 5 (OFP5)	Member of the ovate protein family. Interacts with BLH1 and KNAT3. Regulates the subcellular localization of BLH1.
-8.331	AT5G66052.1	unknown		unknown protein
-8.307	AT2G33810.1	positive regulation of flower development	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 (SPL3)	Encodes a member of the SPL (squamosa-promoter binding protein-like) gene family, a novel gene family encoding DNA binding proteins and putative transcription factors. Contains the SBP-box, which encodes the SBP-domain, required and sufficient for interaction with DNA. It binds DNA, may directly regulate AP1, and is involved in regulation of flowering and vegetative phase change. Its temporal expression is regulated by the microRNA miR156. The target site for the microRNA is in the 3'UTR.
-8.161	TA25819_3702	unknown		unknown
-8.110	AT5G44417.1	pseudogene		pseudogene, similar to CPRD2, blastp match of 42% identity and 7.0e-94 P-value to GP|13161397|dbj|BAB33033.1||AB056448 CPRD2 (Kai et al.)
-8.100	AV788815	unknown		tc|ATOLEOST4 oleosin type4 (McElver et al.) (exp=-1; wgp=0; cg=0), partial (18%) [TC311214]
-7.984882299	AT2G47400.1	*see above*	*see above*	*see above*
-7.972896593	AT1G78460.1	Heme-binding		SOUL heme-binding family protein; FUNCTIONS IN: binding;
-7.971	AT5G50590.1	oxidation-reduction process	HYDROXYSTEROID DEHYDROGENASE 4 (HSD4)	Encodes a putative hydroxysteroid dehydrogenase (HSD). Genes that encode HSD include: At5g50600 and At5g50700 (HSD1), At3g47350 (HSD2), At3g47360 (HSD3), At5g50590 and At5g50690 (HSD4), At5g50770 (HSD6) (Plant Cell Physiology 50:1463). Two copies of HSD1 and HSD4 exist due to a gene duplication event. In Plant Physiology 145:87, At5g50690 is HSD7, At4g10020 is HSD5.
-7.940256867	AT3G60270.1	electron carrier activity		Cupredoxin superfamily protein; FUNCTIONS IN: electron carrier activity, copper ion binding; LOCATED IN: anchored to membrane; CONTAINS InterPro DOMAIN/s: Plastocyanin-like (InterPro:IPR003245), Cupredoxin (InterPro:IPR008972)
-7.94020467	AT3G29240.1	unknown		Protein of unknown function (DUF179)

Appendix

Plasmid rescue and candidate genes for activation tagging line N21368 25/1/5

Plasmid rescue was also carried out for line N21368 25/1/5, a homozygous line. Sequencing returned a 169 bp fragment. An exact search with NCBI's megablast (Miller et al., 2000) of two fragments gained either by HindIII digest and sequencing via SKC12 or PST1 digest and the RB primer returned no similarity to the *Arabidopsis thaliana* genome, but 98% similarity HSP Homo sapiens v-KI-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS). Allowing less rigid search parameters (blastn, (Altschul et al., 1997) returned an insertion between AT5G27860, an unknown protein located in the chloroplast and expressed in the pollen tube; and AT5G27870, an invertase/pectin methylesterase inhibitor superfamily protein involved in cell wall modification and expressed in various stages of flowering. However, this match spanned only 28 base pairs and had 93% similarity. Some insertional mutant lines of genes around the putative insertion site were available, including some promising candidate genes. They were:

At5g27830: molecular function unknown; involved in response to oxidative stress; located in endomembrane system

At5g27840: encodes a serine/threonine protein phosphatase; expressed in roots, rosettes and flowers

At5g21860: upstream of insertion site, unknown protein, involved in pollen tube growth

At5g21870: downstream of insertion site, invertase/pectin methylesterase inhibitor superfamily protein

At5g27885: transposable element gene; gypsy-like retrotransposon family.

An analysis of the root scopolin content of the lines on 3% sucrose brought no significant difference compared to WT (Figure A58). In tendency, the insertional mutant of At5g27830 had the lowest scopolin content of the lines tested. It has the conserved domain of a folate receptor (Marchler-Bauer et al., 2011).

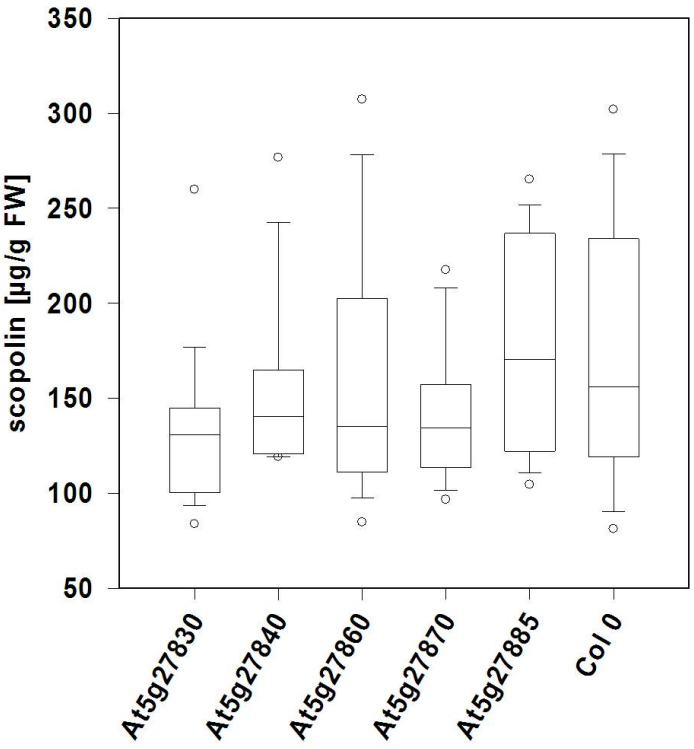

Figure A58: Root scopolin content of insertional mutants around the insertion site of the second activation-tagged line, N21368 25/1.

Genes surrounding ACT7

Table A6: Genes surrounding ACT7 (At5g09810) and their annotations. The column "Annotations up to June 2008" shows the TAIR search results from the original search in 2008 and the latest TAIR citations at that time. "Current annotation" gives the most recent gene description and citation from TAIR (Rhee et al., 2003).

Locus Name	Annotation up to June 2008	Current annotation (July 2011)
At5g09710	Magnesium transporter CorA-like family protein, involved in metal ion transport, located in membrane, has metal ion transmembrane transporter activity (Zhao et al., 2007)	Magnesium transporter CorA-like family protein; BEST Arabidopsis thaliana protein match is: magnesium transporter 7 (Wu et al., 2008)
At5g09720	Magnesium transporter CorA-like family protein, involved in metal ion transport, located in membrane, has metal ion transmembrane transporter activity	Magnesium transporter CorA-like family protein; BEST Arabidopsis thaliana protein match is: magnesium transporter 7
At5g09730 ATBX3	Member of glycosyl hydrolase family 3, similar to a beta-xylolase, involved in carbohydrate metabolic process, located in endomembrane system, has hydrolase activity, hydrolyzing O-glycosyl compounds (Iglesias et al., 2006)	Encodes a protein similar to a beta-xylosidase located in the extracellular matrix. This is a member of glycosyl hydrolase family 3 and has six other closely related members (Hanada et al., 2011)

At5g09740 HAM2	Enzyme with histone acetyltransferase activity of the MYST family 2, involved in chromatin assembly or disassembly, located in nucleus, functions in nucleic acid binding , zinc ion binding (Pikaard et al., 2007)	Encodes an enzyme with histone acetyltransferase activity. HAM2 primarily acetylate histone H4, but also display some ability to acetylate H3. Prior acetylation of lysine 5 on histone H4 reduces radioactive acetylation by either HAM2. (Latrasse et al., 2008)
At5g09750	DNA binding/transcription factor, involved in regulation of transcription, carpel formation, transmitting tissue development, located in nucleus, expressed during flower developmental stages (Yanofsky et al., 2007)	HECATE 3 (HEC3); FUNCTIONS IN: DNA binding, sequence-specific DNA binding transcription factor activity; INVOLVED IN: transmitting tissue development, carpel formation, regulation of transcription (Swain et al., 2009)
At5g09755	Pre-tRNA, tRNA-Glu, involved in translational elongation, has triplet codon-amino-acid adaptor activity (Browning, 1996)	*No updated annotation.*
At5g09760	Pectinesterase family protein, involved in cell wall modification, located in cell wall, has pectinesterase activity, expressed in flower (Culligan et al., 2006)	Plant invertase/pectin methylesterase inhibitor superfamily; FUNCTIONS IN: enzyme inhibitor activity, pectinesterase activity; INVOLVED IN: cell wall modification (Ascencio-Ibanez et al., 2008)
At5g09770	Ribosomal protein L17 family protein, involved in translation, located in ribosome, functions as structural constituent of ribosome (Bonen and Calixte, 2006)	*No updated annotation.*
At5g09780	Transcriptional factor B3 family protein, involved in regulation of transcription, cellular component unknown (Meyerowitz et al., 2007)	*No updated annotation.*
At5g09790 ATXR5	Encodes a SET-domain protein, probably involved in cell-cycle regulation (Crane and Gelvin, 2007)	Encodes a SET-domain protein, a H3K27 monomethyltransferase required for chromatin structure and gene silencing. Regulates heterochromatic DNA replication. Contains a PCNA-binding domain. ATXR5 accumulates preferentially during the late G1 or S phase, suggesting that it plays a role in cell-cycle regulation or progression. A plant line expressing an RNAi construct directed against this gene has reduced agrobacterium-mediated tumour formation. (Michaels et al., 2009; Michaels et al., 2010)
At5g09795	Pseudogene of F-Box family protein	*No updated annotation.*
At5g09800	U-Box containing protein, involved in protein ubiquitination, located in ubiquitin ligase complex, functions in binding, response to chitin (Libault et al., 2007)	ARM repeat superfamily protein; FUNCTIONS IN: ubiquitin-protein ligase activity, binding; INVOLVED IN: response to chitin; LOCATED IN: ubiquitin ligase complex (Kim et al., 2008)
At5g09805 IDL3	Similar to unknown protein At5g64667.1	Similar to Inflorescence deficient in abscission (IDA). Involved in floral organ abscission. (Aalen et al., 2008)
At5g09810 ACT7	Actin7, Member of Actin gene family. Mutants are defective in germination and root growth. involved	Member of Actin gene family. Mutants are defective in germination and root growth.

	in cytoskeleton organization and biogenesis (von Koskull-Doring et al., 2007)	(Meagher et al., 2009), (Meagher et al., 2010)
At5g09820	plastid-lipid associated protein PAP / fibrillin family protein; Identical to Probable plastid-lipid-associated protein 7, chloroplast precursor (PAP7) [Arabidopsis Thaliana], biological process unknown, located in chloroplast, structural molecule activity (Dal Bosco et al., 2004)	Plastid-lipid associated protein PAP / fibrillin family protein; FUNCTIONS IN: structural molecule activity; INVOLVED IN: biological process unknown; LOCATED IN: chloroplast; EXPRESSED IN: 23 plant structures; EXPRESSED DURING: 15 growth stages; CONTAINS InterPro DOMAIN/s: Plastid lipid-associated protein/fibrillin (InterPro:IPR006843); BEST Arabidopsis thaliana protein match is: Plastid-lipid associated protein PAP / fibrillin family protein (TAIR:AT3G26070.1); (Ascencio-Ibanez et al., 2008
At5g09830	BolA-like family protein; similar to unnamed protein product [Vitis vinifera], biological process unknown, localisation unknown, has transcription regulator activity	BolA-like family protein; CONTAINS InterPro DOMAIN/s: BolA-like protein (InterPro:IPR002634); BEST Arabidopsis thaliana protein match is: chloroplast sulphur E (Ascencio-Ibanez et al., 2008)
At5g09840	similar to unknown protein [Arabidopsis thaliana], located in mitochondrion, molecular function unknown	Putative endonuclease or glycosyl hydrolase; BEST Arabidopsis thaliana protein match is: Putative endonuclease or glycosyl hydrolase (TAIR:AT5G64710.1) (Kiba et al., 2005)
At5g09850	transcription elongation factor-related, involved in transcription, located in nucleus, functions in DNA binding, protein binding, transcription regulator activity (Green et al., 2001)	Transcription elongation factor (TFIIS) family protein; FUNCTIONS IN: transcription regulator activity, DNA binding; INVOLVED IN: transcription (van Bentem et al., 2008)
At5g09860 ATTHO1, EMU	nuclear matrix protein-related; similar to unnamed protein product [Vitis vinifera], biological process unknown, cellular component unknown, molecular function unknown	Encodes a component of the putative Arabidopsis THO/TREX complex: THO1 or HPR1 (At5g09860), THO2 (At1g24706), THO3 or TEX1 (At5g56130), THO5 (At5g42920, At1g45233), THO6 (At2g19430), and THO7 (At5g16790, At3g02950). THO/TREX complexes in animals have been implicated in the transport of mRNA precursors. Mutants of THO3/TEX1, THO1, THO6 accumulate reduced amount of small interfering (si)RNA, suggesting a role of the putative Arabidopsis THO/TREX in siRNA biosynthesis. (Furumizu et al., 2010) (Yelina et al., 2010). (Vaucheret et al., 2010)
At5g09870 CESA5	Encodes a cellulose synthase isomer, related to CESA6, involved in cell-wall biogenesis, only expressed in apical hook (Vernhettes et al., 2007)	Encodes a cellulose synthase isomer, related to CESA6. As inferred from the null role of secondary wall-type CesAs, included in a set of five primary wall-type CesAs that may support trichome cell wall thickening. (Sullivan et al., 2011)
At5g09880	RNA recognition motif (RRM)-containing protein; similar to RNA recognition motif (RRM)-containing protein [Arabidopsis thaliana] (TAIR:AT2G16940.1), involved in m-RNA processing, located in nucleus, functions in nucleic acid binding (Iida et al., 2004)	Splicing factor, CC1-like; FUNCTIONS IN: RNA binding, nucleotide binding, nucleic acid binding; INVOLVED IN: mRNA processing; LOCATED IN: nucleus; CONTAINS InterPro DOMAIN/s: RNA recognition motif, RNP-1 Splicing factor, CC1-like), Nucleotide-binding, alpha-beta plait. BEST Arabidopsis thaliana protein match is: Splicing factor, CC1-like (TAIR:AT2G16940.2) (Mahalingam

		et al., 2011)
At5g09890	protein kinase, putative; similar to protein kinase family protein [Arabidopsis thaliana] (TAIR:AT4G14350.3), involved in amino acid phosphorylation cellular component unknown, has kinase activity (Bogre et al., 2003)	Protein kinase family protein; FUNCTIONS IN: kinase activity; INVOLVED IN: protein amino acid phosphorylation; LOCATED IN: cytosol; BEST Arabidopsis thaliana protein match is: AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase family protein (Endo et al., 2011)
At5g09900 RPN5A	EMB2107/MSA/RPN5A (EMBRYO DEFECTIVE 2107); similar to 26S proteasome regulatory subunit, putative (RPN5) [Arabidopsis thaliana] (TAIR:AT5G64760.1), ubiquitin-dependent protein, catabolic process, embryonic development ending in seed dormancy, located in proteasome regulatory particle, lid subcomplex, molecular function unknown (Yang et al., 2004)	Encodes one of two isoforms for the 26S proteasome regulatory protein (RN) subunit RPN5. For many functions it acts redundantly with the paralogous gene RPN5b but also appears to exert independent effects. (Book et al., 2010)
At5g09910	ATP binding / GTP binding / transcription factor binding; similar to LIP1 (LIGHT INSENSITIVE PERIOD1), GTPase [Arabidopsis thaliana] (TAIR:AT5G64813.1), involved in protein transport, small GTPase mediated signal transduction, regulation of transcription, DNA-dependent	Ras-related small GTP-binding family protein; functions in GTP binding, transcription factor binding, ATP binding; involved in: protein transport, small GTPase mediated signal transduction, regulation of transcription, DNA-dependent; located in: intracellular (Rehrauer et al., 2010)

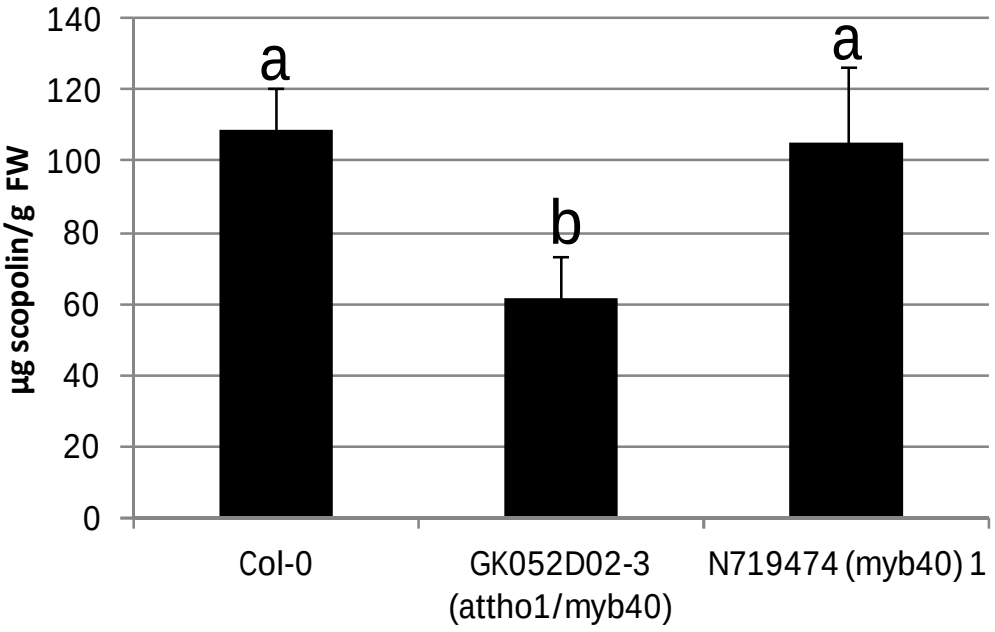

Figure A59: Scopolin contents of roots of wild type Col-0 and insertional mutation lines GK052D02-3 (*attho1*/*myb40*) and N719474_1 (homozygous). Wild type and *myb40* are not significantly different from each other ("a"; p=1.0). GK052D02_3 has a significantly lower scopolin content than the other lines ("b"; ANOVA with Bonferroni correction, p<0.001; n=10).

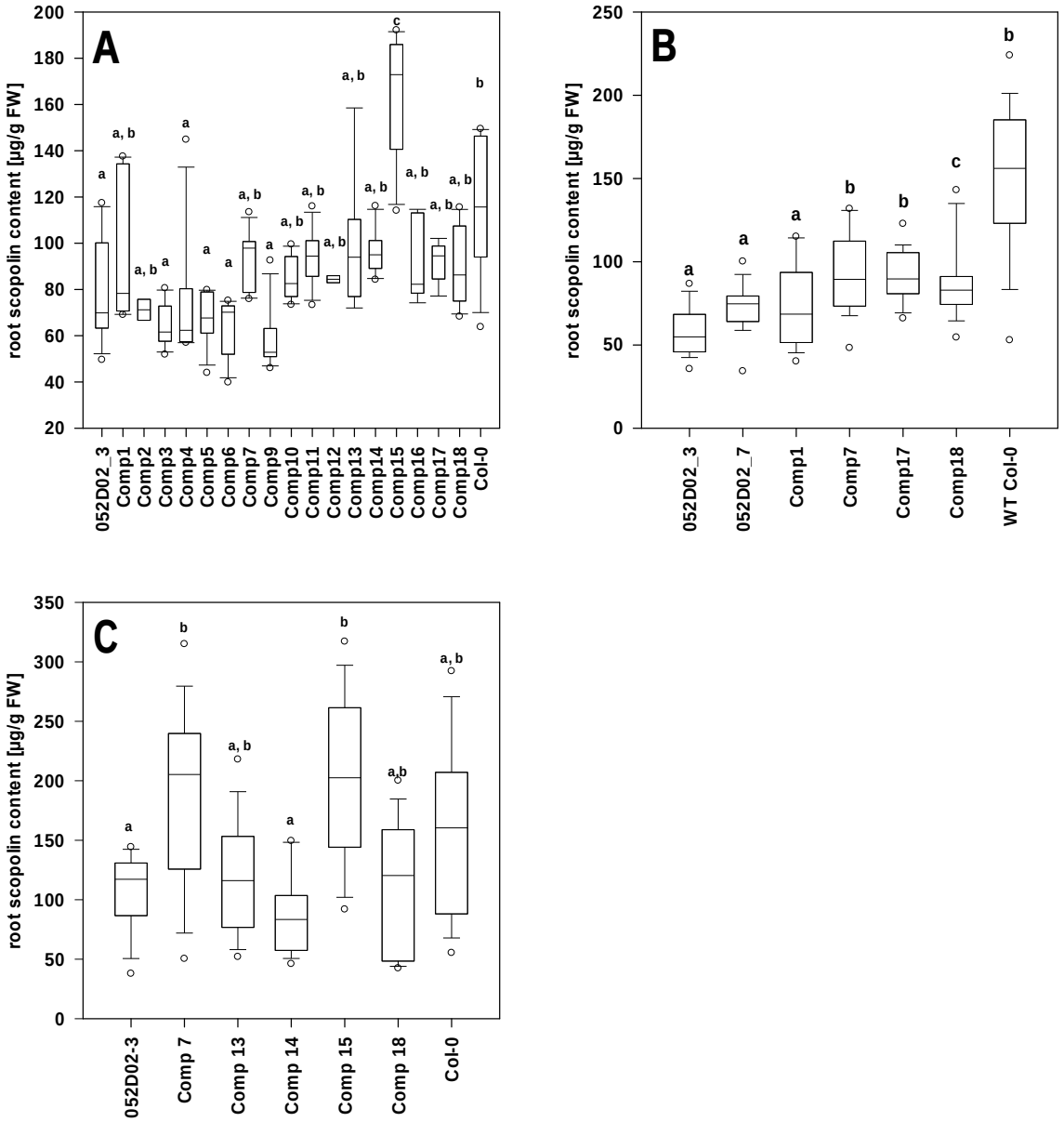

Figure A60: Scopolin contents of 17 independent complemented lines (Comp1-Comp18) of GABI-Kat line GKO52D02_3 with the promoter and genomic region of At5g09860 (AtTHO1). A-C Scopolin content of root extracts from hydroponic culture. Complementation lines with an "a" are not significantly different from the insertional mutant GK052D02; lines with a "b" are not significantly different from the WT line Col-0. Lines with a "c" are significantly different from GK052D02 and WT (t-test or u-test, $p<0.05$). A: n=2-9 (Screening) B: n = 15 C: n=10. Box Plots: Horizontal line, middle = median; upper and lower horizontal line = 25th and 75th percentile, "whiskers" = 5% and 95% percentile, circles = outliers.

Coumarin contents of tobacco cultures

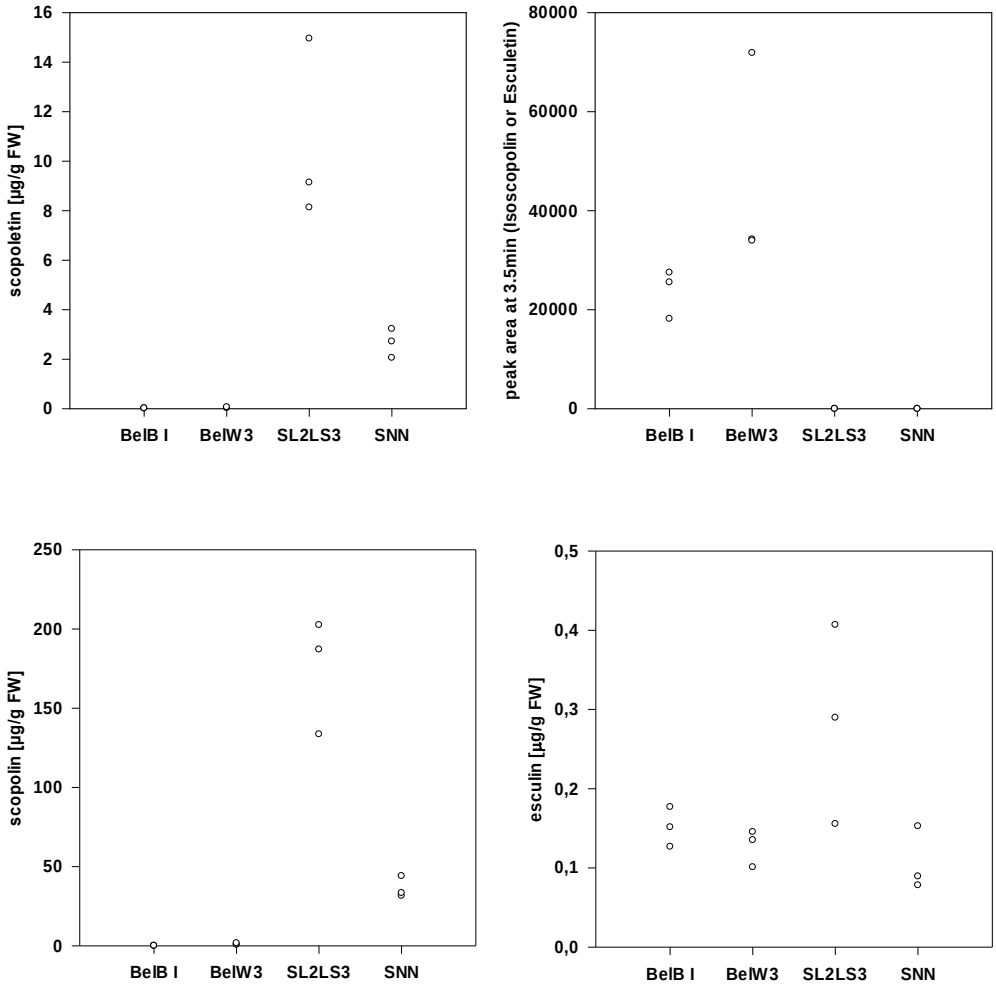

Figure A61: Coumarin contents of four tobacco callus cultures. Substance identification was done by LC-FLD only, where isoscopolin and esculetin are not distinguishable.

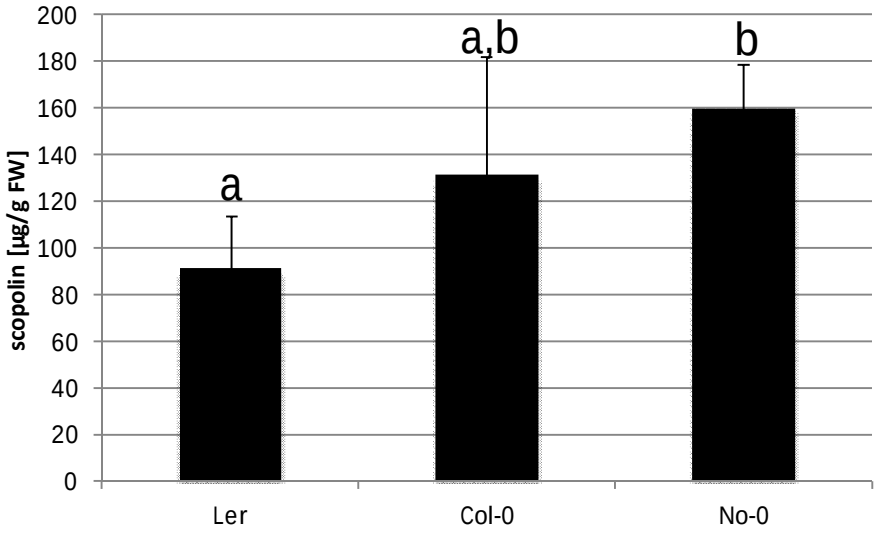

Figure A62: Scopolin contents of different ecotypes of *A. thaliana* on medium containing 3% sucrose, preliminary results. Bars with the same letters are not significantly different from each other (ANOVA, $p<0.05$, $n=4$)

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Appendix

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