

Influence of gibberellins on *Nicotiana attenuata*'s defense against insects

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I hereby declare that this master's thesis has been written by myself and without any assistance of third parties.

Furthermore, I confirm that all references and verbatim extracts have been quoted, and all sources of information have been specifically acknowledged.

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Abstract

Background: While the role of gibberellins (GAs) as growth hormones is well understood, less is known about their influence on the plant's defense against insect herbivores. By mining next generation sequencing data, we identified 15 genes involved in GA biosynthesis and signaling in *Nicotiana attenuata*. A large proportion of these genes are regulated by wounding or induction of leaves with herbivore-derived elicitors, suggesting an important role of the GA pathway in *N. attenuata*'s response to herbivore attack. To investigate the function of GA in the defense process of plants against insect herbivores, we performed feeding experiments with *Manduca sexta* larvae on *N. attenuata* plants treated with exogenously applied GA. Additionally, we silenced genes involved in GA degradation (*NaGA2ox3*) and signaling (*NaDELLA1*) using virus-induced gene silencing (VIGS). We analyzed control and simulated herbivory-induced levels of phytohormones (abscisic acid, jasmonic acid, jasmonyl-isoleucine, salicylic acid), soluble defense metabolites (nicotine, caffeoylputrescine, dicaffeoylspermidine, chlorogenic acid, diterpene glycosides, rutin) and volatile organic compounds (VOCs).

Results: The analysis of the sequencing data revealed genes, regulated after wounding and especially after perception of herbivore-derived elicitors. Compared to wild-type plants, larvae of *M. sexta* grew significantly larger on plants treated with exogenous GA when compared to mock-treated plants. The phytohormone and VOC levels were not different, neither between GA-treated and wild-type, nor between empty vector and *NaDELLA1*, *NaGA2ox3*-silenced plants, although some trends could be observed. The GA-treated plants showed decreased levels of nicotine and dicaffeoylspermidine. Some metabolites did not change, indicating that GAs regulate only specific branches of *N. attenuata*'s defense. DELLA1-silenced plants contained less nicotine as well but dicaffeoylspermidine, chlorogenic acid and cryptochlorogenic acid were increased. GA2ox3-silenced plants were mostly similar to the empty vector plants except their elevated levels of chlorogenic acid. The VIGS data was partly contradictory and difficult to interpret. A more fine-tuned regulation might have been the reason as well.

Conclusion: The present results demonstrate that GA levels and signaling regulate *N. attenuata*'s defense against *M. sexta*. Our data show that the GA pathway specifically regulates only a subset of defense against herbivores. We outline future experiments aimed to identify a role of GAs on other defense metabolites on *N. attenuata*, including protease inhibitors. Using transgenic plants will allow us elucidating the cross-talk between JA and GA signaling.

Keywords: gibberellin, jasmonic acid, *Nicotiana attenuata*, *Manduca sexta*, plant defense, herbivory, virus-induced gene silencing

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Abbreviations

#	2ODD	2-oxoglutarate-dependent dioxygenase
A:	ABA	abscisic acid
	AtGID1	gibberellin insensitive DWARF1 protein in <i>Arabidopsis thaliana</i>
B:	bp	base pairs
	BRs	brassinosteroids
C:	CA	chlorogenic acid
	CCA	cryptochlorogenic acid
	cDNA	complementary DNA
	CKs	cytokinins
	COI1	Coronatine-insensitive protein 1
	CP	caffeoylputrescine
	CPS	<i>ent</i> -copalyl diphosphate synthase
	CUL1	Cullin1 protein
	CYP714D1	elongated uppermost internode protein (EUI)
D:	d8	recombinant DELLA protein DWARF8 in maize
	DAMP's	damage-associated molecular patterns
	DCS	dicafeoylspermidine
	DELLA	protein family designated after N-terminal amino acid sequence D-E-L-L-A
	dsRNA	double stranded RNA
	DTPs	diterpene glycosides
E:	EUI	elongated uppermost internode protein
	ESI	electrospray ionization
F:	FACs	fatty acid-amino acid conjugates

G:	GA GA2ox GA3ox GA20ox GAI GAMT GC-MS GID1 GID2 GLVs	gibberellic acid gibberellin-2-oxidase gibberellin-3-oxidase gibberellin-20-oxidase gibberellin insensitive protein S-adenosyl-L-methionin-dependent methyltransferase Gas Chromatography-Mass spectrometry gibberellin insensitive DWARF1 receptor gibberellin insensitive DWARF2 receptor green leaf volatiles
H:	HAE HAMP's HGL-DTGs HPLC	herbivore-associated elicitors herbivore-associated molecular patterns 17-hydroxygeranyllinalool diterpene glycosides High Performance Liquid Chromatography
J:	JA JA-Ile JAZ1	jasmonates jasmonyl-isoleucine conjugate JA ZIM-domain 1 protein
K:	KAO KO KS	ent-kaureonic acid oxidase <i>ent</i> -kaurene oxidase <i>ent</i> -kaurene synthase
L:	LB medium LC-MS LOX3	comprised of yeast extract, sodium chloride and tryptone Liquid Chromatography-Mass Spectrometry Lipoxygenase 3
M:	<i>M. sexta</i> MYB8 MYC2	<i>Manduca sexta</i> transcription factor MYB8 basic helix-loop-helix Leu zipper protein
N:	<i>N. attenuata</i>	<i>Nicotiana attenuata</i>
O:	OD ODH ORF OS	optical density optimal defense hypothesis open reading frame oral secretions
P:	P450 pBINTRA PCR PDMS PIFs PIs PTFE PTGS pTV00 pTVGOI pTVPD	cytochrome P450 monooxygenase plasmid containing parts of the tobacco rattle virus polymerase-chain-reaction polydimethylsiloxane phytochrome interacting factors proteinase inhibitors polytetrafluorethylene post-transcriptional gene silencing empty vector plasmid plasmid containing gene of interest plasmid for silencing phytoene desaturase

R:	RBX1	RING box protein 1 (E3 ubiquitin ligase)
	RGA	DELLA protein RGA in <i>Arabidopsis thaliana</i>
	RGL	DELLA protein RGL in <i>Arabidopsis thaliana</i>
	Rht	DELLA protein in wheat
	RISC	RNA-induced silencing complex
	RNAi	RNA interference
S:	SA	salicylic acid
	SCF complex	<u>S</u> kp1, <u>C</u> ullin1 and <u>E</u> -Box protein complex
	SCR	SCARECROW protein in <i>Arabidopsis thaliana</i>
	siRNA	short interfering RNA
	Skp1	S-Phase kinase-associated protein 1
	SLN1	DELLA protein in barley
	SLR1	slender rice 1 protein
	SLY2	SLEEPY2 protein in <i>Arabidopsis thaliana</i>
	ssRNA	single stranded RNA
T:	T-DNA	transferring DNA
	Tm	melting temperature
	TPS	terpene synthase
	TRV	tobacco rattle virus
V:	VIGS	virus-induced gene silencing
	VOCs	volatile organic compounds
W:	W+OS	wounding plus oral secretions of <i>Manduca sexta</i> treatment
	W+W	wounding plus water treatment
	WT	wild-type plants of <i>Nicotiana attenuata</i>
X:	XERICO	RING-H2 zinc-finger protein
Y:	YEP medium	comprised of yeast extract, sodium hydroxide and peptone

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1 Introduction

Although plants are unable to flee biotic stress, they are not as helpless against herbivory as one would think. Over millions of years they have evolved effective defense systems maintained by specific metabolites. Plants can recognize herbivores through chemical and mechanical cues and respond with a broad spectrum of defense systems in order to secure their survival. Substances, which have repellent, antinutritive or toxic effects on herbivores but also thorns, spines and tough leaves are direct defenses against insects. But a plant can have indirect defenses too. Examples of indirect defenses are herbivore-induced plant volatiles and nectar rewards that attract natural enemies of the attacker (HOWE and JANDER, 2008). Defense metabolites are not equally distributed among different plant tissues. The optimal defense hypothesis (ODH) predicts that the limited supply of resources leads to the accumulation of defense metabolites to the most valuable, fitness relevant parts (McKEY, 1974). The accumulation of defensive metabolites in different tissues is often correlated with the probability of an attack.

As described above, biosynthesis of defense metabolites is costly and often accompanied by growth inhibition (POVEDA *et al.*, 2003; ZHANG and TURNER, 2008) and lower seed production (BALDWIN *et al.*, 1990; BALDWIN, 1998). Therefore a plant expresses not all defense systems constitutively. Plants can activate the production of defenses after perception of herbivory (ERB *et al.*, 2012). A major signaling cascade activated by perception of herbivory is the jasmonate (JA) pathway which triggers downstream metabolic pathways leading to the expression of various chemical defenses like toxins or digestion inhibitors (HOWE and JANDER, 2008). The JA pathway is activated through perception of herbivory derived cues like damage-associated molecular patterns (DAMP's), herbivore-associated molecular patterns (HAMP's) and wounding (ERB *et al.*, 2012). While the role of the JA pathway in defense against herbivores is well described, the function of growth-related hormones, including gibberellic acid (GA), cytokinins (CKs), auxins and brassinosteroids (BRs) is much less understood. However, recent data demonstrate that the signaling networks induced by GAs, CKs, auxins and brassinosteroids are cross-linked to each other and to the JA pathway and therefore possible regulate herbivory

resistance ((ERB *et al.*, 2012; MELDAU *et al.*, 2012). Among growth-related hormones, the role of GAs as regulators of plant growth and defense are well studied. When attacked, the plants release elevated levels of jasmonates, which interfere with the GA signaling cascade to prioritize its defense over growth (D. L. YANG *et al.*, 2012). However, whether GA influences responses to herbivore attack is not known.

1.1 Gibberellins

Gibberellins are diterpene (composed of four isoprene units) plant hormones synthesized from geranylgeranyl diphosphate, that control several aspects important for growth and development like seed germination, stem elongation, leaf expansion, root growth as well as flower and seed development ((RICHARDS *ET AL.*, 2001; CAO *ET AL.*, 2005; S. YAMAGUCHI, 2008; TANIMOTO, 2012). Gibberellins were first isolated from the fungus *Gibberella fujikuroi* (Sawada) Wollenw. 1931 (synonym *Fusarium moniliforme* Sheldon) by Teijiro Yabuta in 1935 (YABUTA and SUMIKI 1938). Later, gibberellins were discovered in several plants species like maize, bean or peas (RADLEY, 1956; PHINNEY *et al.*, 1957; MACMILLAN and SUTER, 1958). *G. fujikuroi* causes a disease of rice plants called “bakanae” which until today can lead to great yield loss. Infected plant seeds

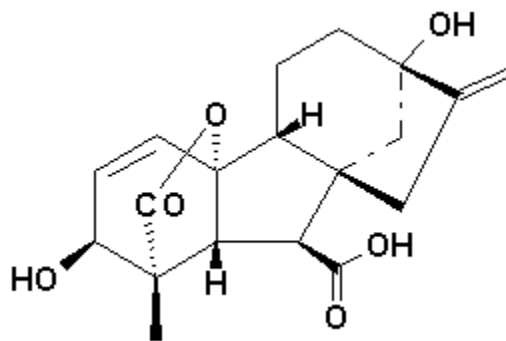


Figure 1: Gibberellin A3 (Source: <http://www.plant-hormones.info/gainfo.asp?ID=3>)

appear to be taller, have slender shapes and are slightly more chlorotic (ANDERSON *et al.* 2004). In the beginning, the infected plants grow more rapid and taller but after some time they start to senesce while healthy plants continue to grow. Most of the bakanae plants die before they reach maturity. Plants reaching the mature stage usually show empty panicles or poorly developed grains. The rapid elongation of the infected plants is mediated by GAs produced by the fungus (ANDERSON *et al.* 2004). Since the first isolation of GAs from *G. fujikuroi*, a variety of metabolites with GA-based structures were discovered. Not all of them are biologically active. Only GA₁, GA₃ and GA₄ (probably also GA₅ and GA₆) are thought to function as growth regulating hormones while hundreds of inactive gibberellins identified until today (<http://www.plant-hormones.info/ga1info.htm>) act as precursors for bioactive GA's or present

inactivation products. YAMAGUCHI (2008) suggested that bioactive gibberellins are often produced where they are needed. Especially actively growing and elongating tissues like shoot apices, young leaves and flowers contain high levels of GA's (JONES and PHILLIPS, 1966; KOBAYASHI *et al.*, 1988; ASHIKARI *et al.*, 2002).

1.1.1 Gibberellin biosynthesis and its regulation

In *Arabidopsis thaliana* and in wheat seven key-enzymes (two **terpene synthases (TPSs)**, two **cytochrome P450 monooxygenases (P450s)** and three 2-oxoglutarate-dependent dioxygenases (2ODDs) are crucial for the biosynthesis of bioactive GAs. These enzymes include **ent-copalyl diphosphate synthase (CPS)**, **ent-kaurene synthase (KS)**, **ent-kaurene oxidase (KO)**, **ent-kaureonic acid oxidase (KAO)**, GA 20-oxidase (GA20ox), GA 3-oxidase (GA3ox) and GA 2-oxidase (GA2ox). CPS, KS, KO and KAO are involved in the transformation of geranylgeranyl diphosphate to GA₁₂, which act as a precursor for the formation of other types of GA. GA₁₂ gets then transformed to the bioactive forms by the enzymes GA 20-oxidase and GA 3-oxidase. GA 2-oxidase degrades bioactive GAs to inactive forms (YAMAGUCHI, 2008) and in *Arabidopsis* also proteins like S-adenosyl-L-methionin-dependent methyltransferases (GAMT1 and GAMT2) are involved in the degradation process by methylation of the carboxyl group of bioactive GAs (VARBANOVA *et al.*, 2007). A simple scheme of the gibberellin biosynthetic pathway is shown in Figure 2. In rice bioactive GA is deactivated by GA2ox and the P450 protein EUI (elongated uppermost internode), designated as CYP714D1, which epoxidizes the 16,17 –double bond of non13-hydroxylated GAs. This epoxidation includes inactive GAs like GA₁₂ or GA₉ but also the bioactive form GA₄ (YAMAGUCHI, 2008).

Studies have shown that GA biosynthesis is modulated by the action of light and GA itself. (AIT-ALI *et al.*, 1999) demonstrated on pea seedlings that GA20ox transcript levels were increased in leaves and apical buds of light grown seedlings while the levels of GA 3 β -hydroxylase (synonym to GA3ox) were higher in etiolated seedlings. After treatment with bioactive GA₁, plants showed drastically decreased levels of both GA20ox and GA3ox transcription levels. (AIT-ALI *et al.*, 1999) concluded that the transcription of these genes is feedback inhibited by high levels of bioactive GA₁ and with different sensitivity. Similar results were revealed by other studies

(CHIANG *et al.*, 1995; PHILLIPS *et al.*, 1995; MARTIN *et al.*, 1996; CARRERA *et al.*, 1999; ROSS *et al.*, 1999; ASHIKARI *et al.*, 2002; AYELE *et al.*, 2006; GALLEG0-GIRALDO *et al.*, 2008; SUZUKI *et al.*, 2008, reviewed in HEDDEN AND PHILLIPS, 2000), although they partly show that some genes of these gene families don't underlie feedback regulation at all (YAMAGUCHI *et al.*, 1999; MATSUSHITA *et al.*, 2007).

1.1.2 Gibberellin signaling

1.1.2.1 GA receptors

In contrast to the GA biosynthesis which is well characterized (HEDDEN and PHILLIPS, 2000; YAMAGUCHI, 2008; HUANG *et al.*, 2012), the GA signaling pathway is much less understood. GA receptors play an important role in the GA signaling cascade. SASAKI *et al.* (2003) isolated and characterized the *gibberellin insensitive DWARF2* (*GID2*) gene from the rice mutant *gid2*, encoding the putative F-box protein GA insensitive dwarf 2 (*GID2*), which, as a part of the SCF protein complex, is thought to be a positive regulator for GA signaling by degrading the DELLA protein SLR1 (a repressor of GA signaling, orthologous to GAI and RGA in *Arabidopsis*, SLN1 in barley, d8 in maize and Rht in wheat) via the ubiquitin 26S proteasome pathway. When part of the SCF^{GID2} complex, *GID2* functions as a receptor, selectively recruiting target proteins into the complex which then get degraded via ubiquitination (SLR1, for example, is a target). The SCF complex is part of multi subunit RING domain E3 ligases, which is named after three of its four subunits Skp1, Cullin1 (*CUL1*) and the F-box protein. The last subunit is the RING-finger-containing protein RBX1 (WANG and DENG, 2011). In *Arabidopsis* *GID2*, which is orthologous to SLEEPY 2 (*SLY2*), is the F-box protein.

The *gibberellin insensitive DWARF 1* (*GID1*) gene, which encodes the protein gibberellin insensitive DWARF1 (*GID1*), is a soluble receptor for GA signaling. The *GID1* gene was first isolated and characterized from a GA insensitive dwarf mutant *gid1* in rice (UEGUCHI-TANAKA *et al.*, 2005). It encodes a protein which has structural similarities to the hormone-sensitive lipase (HSL) family, is localized in the nuclei and involved in the perception of bioactive GA, resulting in the interaction of *GID1* with SLR1, followed by the degradation of SLR1 through the SCF^{GID2} complex. Later, the receptor proteins SLEEPY1 (*SLY1*) and SNEEZY, executing the same DELLA

protein degrading function, were discovered in *Arabidopsis* (GRIFFITHS *et al.*, 2006; NAKAJIMA *et al.*, 2006; WILLIGE *et al.*, 2007). SLR1 stabilizes the binding of GA to the GID1 receptor (UEGUCHI-TANAKA *et al.*, 2007). It was suggested, that the stabilized GA-GID1-SLR1 complex is probably targeted by the SCF^{GID2} complex, leading to the degradation of SLR1 (UEGUCHI-TANAKA *et al.*, 2007; UEGUCHI-TANAKA *et al.*, 2007). *Arabidopsis* contains three GID1 receptor proteins, AtGID1a, AtGID1b and AtGID1c. Among them, AtGID1b has different properties than the other GID1s. It shows higher affinities for binding to GA and also interacts with DELLA proteins in presence and absence of it (NAKAJIMA *et al.*, 2006). It is likely that AtGID1b plays an elevated role for GA signaling in *Arabidopsis* since it is able to interact with the DELLA protein RGA already at concentrations ten times lower than the GA concentration needed for AtGID1a and AtGID1b (NAKAJIMA *et al.*, 2006).

1.1.2.2 DELLA proteins

DELLA proteins are involved in the GA signaling pathway and belong to the GRAS superfamily which is designated after the proteins GAI, RGA and SCR. DELLA proteins are characterized by their N-terminal DELLA domain (designated after a sequence of the amino acids aspartic acid (D), glutamic acid (E), leucine (L) and alanine (A) in their N-terminal region), which is essential for the GA-dependent GID1-DELLA interaction (PYSH *et al.*, 1999; DILL *et al.*, 2001; UEGUCHI-TANAKA *et al.*, 2007). GAs regulate the attenuation of DELLA proteins via the ubiquitin-proteasome pathway (SILVERSTONE *et al.*, 2001; FU *et al.*, 2004; SMALLE and VIERSTRA, 2004; HUSSAIN *et al.*, 2007), although it is not known if the proteasome-mediated proteolysis is the only factor degrading DELLA proteins (GALLEGO-BARTOLOME *et al.*, 2010). In principle, the target substrate is tagged by a polyubiquitin chain produced by the enzymes E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme) and E3 (ubiquitin ligase). The marked substrate is degraded by the 26S proteasome enzyme. The polyubiquitin tag can be removed by deubiquitinating enzymes (DUBs) for reuse in the next ubiquitination cycle (WANG and DENG, 2011). The ubiquitination function can be carried out either as a single subunit protein or multi-subunit protein complexes.

1.1.2.3 DELLAs link JA and GA pathways

In *Arabidopsis*, DELLAs influence the JA pathway by binding competitively to the JA ZIM domain1 (JAZ1) protein, which is a key repressor of the JA pathway (Figure 2). While bound to the DELLAs, JAZ1 can't bind to MYC2, a key transcriptional activator of the JA pathway (Hou *et al.*, 2010). Therefore, MYC2 can activate the expression of JA pathway related genes. In the absence of DELLAs, the JAZ1 protein binds to MYC2 and represses the expression of the genes involved in the JA pathway (Hou *et al.*, 2010). The degradation of DELLA proteins by GA can be seen as one crosslink between the JA and the GA pathway. Additionally, DELLAs interact with growth-promotion transcription factors like phytochrome interacting factors (PIFs) and repress the transcription of GA responsive genes (ALABADI *et al.*, 2008; DE LUCAS *et al.*, 2008; FENG *et al.*, 2008; D. L. YANG *et al.*, 2012). On the other hand, JA causes a delay in the degradation of DELLA proteins, leading to delayed transcription of genes inhibiting the JA pathway. A DELLA-deficient or non-function mutant shows less sensitivity for growth inhibition triggered by JA. Rice contains only one gene encoding a DELLA protein (*SLR1*) while *Arabidopsis* contains five of them, the repressor of *ga1-3* (*RGA*), the genes encoding the RGA-like protein 1,2 and 3 (*RGL1*, *RGL2*, *RGL3*), and the gene encoding the gibberellin acid insensitive mutant protein (*GAI*). They are thought to be nuclear localized transcriptional regulators which accumulate under low GA concentrations and act as repressors of growth and development in plants (SUN and GUBLER, 2004; CAO *et al.*, 2005; FLEET and SUN, 2005; HUSSAIN *et al.*, 2005). The proteins *GAI* and *RGA* have their overlapping function in repressing the GA biosynthesis. Mutants of *Arabidopsis* lacking the ability to produce these repressors show GA independent growth regulation. GA-deficient mutants lacking *GAI* and *RGA* do not require GA for normal stem growth (KING *et al.*, 2001).

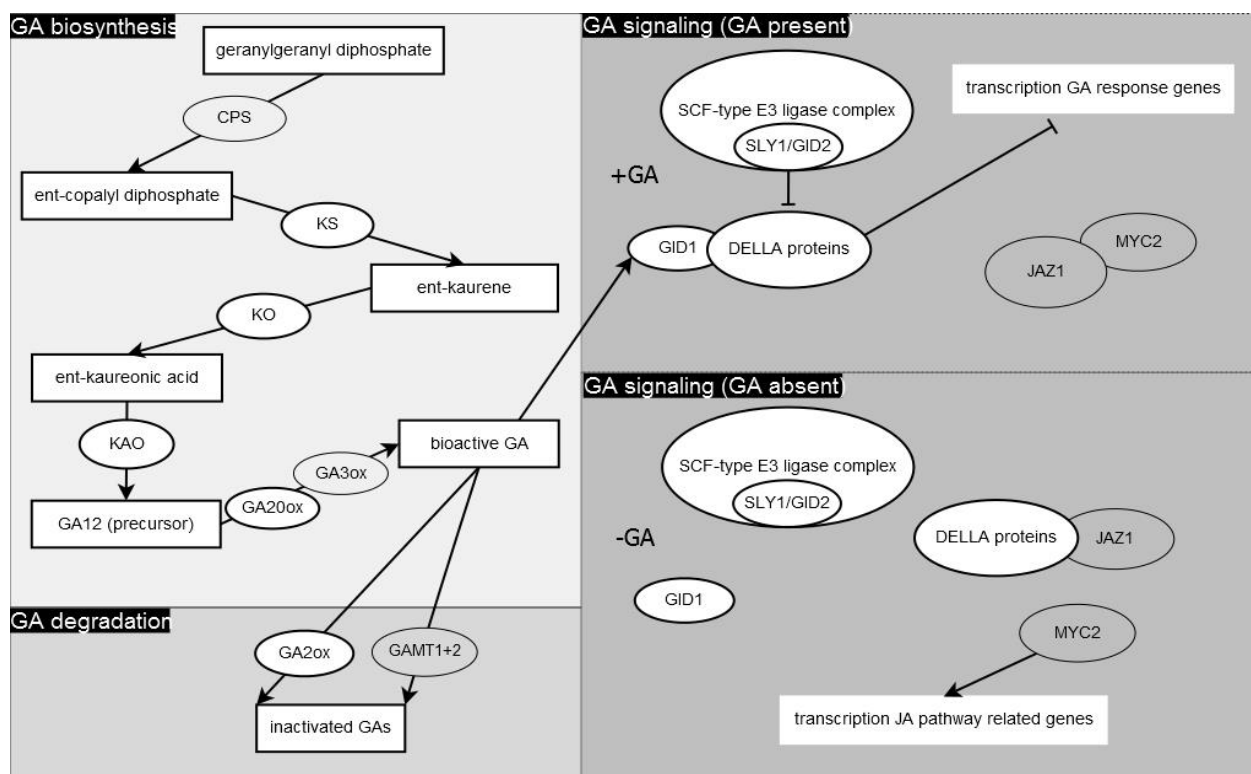


Figure 2: Simple scheme of the GA biosynthesis, degradation and signaling pathways and interaction with JA signaling.

The round shapes present proteins involved in GA-related processes. GA₁₂, which is a precursor for bioactive GAs, is synthesized from geranylgeranyl diphosphate via several intermediates through the enzymes *ent*-copalyl diphosphate synthase (CPS), *ent*-kaurene synthase (KS), *ent*-kaurene oxidase (KO) and *ent*-kaurenic acid oxidase (KAO). GA₁₂ is transformed to bioactive GAs by GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox). Bioactive GAs are degraded to inactive forms by GA 2-oxidases (GA2ox) and (in Arabidopsis) S-adenosyl-L-methionin-dependent methyltransferases (GAMT). Bioactive forms of GAs are perceived by the receptor protein gibberellin insensitive dwarf 1 (GID1), leading to the formation of a complex with the DELLA proteins, which furthermore interact with GID2 (orthologous to *Oryza* SLY1) of the SCF complex initiating the degradation of the DELLA proteins via the 26S proteasome pathway. Degraded DELLAs can't bind to the phytochrome interacting factors (PIFs), initiating the transcription of GA response genes. Degraded DELLAs also can't bind competitively to the JA ZIM domain 1 protein (JAZ1), which therefore is bound by the transcription factor basic helix-loop-helix protein (MYC2). If MYC2 is bound to the JAZ1 protein, the transcription of JA pathway response genes is inhibited (upper-right picture). In the absence of GAs, DELLAs bind to JAZ1, leading to the release of MYC2 which initiates the transcription of JA response genes. Since also the PIF transcription factors are bound by DELLA, the transcription of the GA response genes stops (lower right picture). Transparent enzymes of GA biosynthesis and degradation could not be reliably identified within the genome of *N. attenuata*.

1.1.2.4 *Nicotiana attenuata* and its defenses against insects

Nicotiana attenuata Torr. ex S. Watson, a species of wild tobacco plants, belongs to the family Solanaceae (nightshade family) and inhabits the Great Basin Desert of western North America ranging from British Columbia to Texas (<http://plants.usda.gov/java/profile?symbol=NIAT>). This annual pioneer plant occurs in areas underlying periodic disturbances, which can e.g. be caused by erosion or human activities. It grows up to 1.5 m (GOODSPEED, 1954) and is known to germinate after bushfires (BARNEY and FRISCHKNECHT, 1974; YOUNG and EVANS, 1978), which are

not uncommon in this region. After such fires the success rate of seed germination of *N. attenuata* is increased and therefore the abundance of the plant is higher (BALDWIN and MORSE, 1994; BALDWIN *et al.*, 1994). Bushfires annihilate almost the entire vegetation and form a layer of nutrient-rich ashes on the top of the soil. Seeds of *N. attenuata* can outlast for centuries in the soil until water, nutrients and chemical substances originating from burned organic matter initiate their germination (BALDWIN and MORSE, 1994; BALDWIN *et al.*, 1994). During rainfall-rich springs after years with bushfires, the conditions for germination are perfect and *N. attenuata* can partly form large monocultures on burned soils. Several thousand seeds can be produced by one plant (BALDWIN *et al.*, 1998). Most of them start their germination in the following years but during less favorable circumstances, some just fall in dormancy until the next fire provide better conditions.



Figure 3: *Nicotiana attenuata* (Photo: Danny Kessler)

Special seed germination abilities provide *N. attenuata* with great ecological benefits but its germination behavior also leads to highly unpredictable herbivore pressures. Most of the defense metabolites produced as defense responses against herbivores entail large fitness costs in the absence of herbivores. This is why this plant probably has evolved a sophisticated herbivore perception and signaling machinery. Beside the production of digestion inhibitors and toxins (BALDWIN and OHNMEISS, 1993; HALITSCHKE and BALDWIN, 2003), it is able to selectively attract natural predators of the herbivores through the release of specific volatiles (KESSLER and BALDWIN, 2001; HALITSCHKE and BALDWIN, 2003; HALITSCHKE *et al.*, 2008; DAS *et al.*, 2013). Some of these plant

volatiles are known to influence the oviposition behavior of the herbivores as well (ALLMANN *et al.*, 2013). One of the natural enemies feeding massively on *N. attenuata* are larvae of the moth

Manduca sexta, (Figure 4) also known as tobacco hornworm or goliath worm (Lepidoptera, Sphingidae), which commonly feeds on tomato, tobacco and other solanaceous plants (<http://entnemdept.ufl.edu/creatures/field/hornworm.htm>). To prevent massive damage caused by *M. sexta* herbivory, the plant releases proteinase inhibitors (PI), which slow growth of *M. sexta* (VAN DAM *et al.*, 2001; ZAVALA *et al.*, 2004). At the same time natural enemies like the big-eyed bug *Geochoris pallens* (Hemiptera, Lygaeidae), which feed on eggs and younger (smaller) stages of *M. sexta*, is attracted to infested plants of *N. attenuata* (SCHUMAN *et al.*, 2013). After its arrival it starts feeding on *M. sexta*, reducing the herbivore load and so increasing the fitness of the plant (SCHUMAN *et al.*, 2012). In an astonishing way, the plant inhibits the growth of herbivores while natural enemies are attracted to infested plants, a combination which provides a highly effective defense system for its survival. But how does *N. attenuata* get the information if herbivores are present?

The plant can distinguish herbivore damage from wounding by perceiving herbivore-associated elicitors (HAE), such as the fatty acid-amino acid conjugates (FACs) in oral secretions (OS) of *M. sexta* larvae which are introduced into wounds during feeding (HALITSCHKE *et al.*, 2001). The perception of herbivore-associated molecular patterns (HAMP) allows the plant to orchestrate specific defense responses upon attack of specific herbivores (BONAVENTURE *et al.*, 2011; ERB



Figure 4: *Manduca sexta* caterpillar

The population used in these experiments was inbred since approx. 20 years by MPI for chemical ecology.

et al., 2012). While the most valuable parts of *N. attenuata* continuously have higher concentrations of the root produced toxin nicotine, also other important parts are defended by the induction of this alkaloid. As mentioned above, the main pathway triggering these defense mechanisms is the JA pathway. In *N. attenuata*, the defense responses include defense proteins such as trypsin proteinase inhibitors (TPIs, GREEN and RYAN, 1972; VAN DAM *et al.*, 2001;

HALITSCHKE and BALDWIN, 2003), the biosynthesis of defense metabolites such as 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs), caffeoylputrescine (CP), dicaffeoylspermidine (DCS) and nicotine (STEPPUHN *et al.*, 2004; HEILING *et al.*, 2010; KAUR *et al.*, 2010; HEINRICH *et al.*, 2012), the activation of indirect defense, such as the biosynthesis and release of volatile organic compounds (VOCs) from the leaves (KESSLER and BALDWIN, 2001; SCHUMAN *et al.*, 2012).

1.2 Aim of this study

The aim of this study was to analyze the influence of gibberellins on the defense system of *N. attenuata* against insect herbivores. GAs influence the JA pathway through the destabilization of the DELLA proteins, which interact with the JAZ protein (Hou *et al.*, 2010). To analyze the role of GA in *N. attenuata*'s defense response we first mined a microarray database to identify GA-related genes that are regulated by wound or herbivore-derived cues in local and systemic tissues. Further we selected candidate genes for Virus-induced gene silencing to verify their role in plant immunity against insects. Virus-induced gene silencing (VIGS) is a virus vector technology based on RNA interference (RNAi) and presents a technique where transcript levels of target genes can be reduced to unravel their function (SCHEPERS, 2005). The principles behind this method are shown in Figure 5. Basically a fragment (ca. 300 bp) of a target gene is inserted into a viral genome containing T-DNA (transferring DNA) of a binary vector. Afterwards the plasmid is introduced into *Agrobacterium tumefaciens* by transformation and the bacteria are inoculated into the host plant. Inside the plant cell the T-DNA is implemented into the host genome and transcribed by the host's DNA-dependent RNA polymerase. Another enzyme, the RNA-dependent RNA polymerase produces double stranded RNA (dsRNA) from the viral single stranded RNA (ssRNA) transcript (BECKER and LANGE, 2010). The dsRNA induces the RNAi process. The process consists of an initiator step and an effector step. During the initiation, the long dsRNA (both strands complementary to the sequence of the target gene) is cleaved into short interfering (si) RNAs (usually around 21-24 nucleotides in length) by a double strand-specific RNase called "Dicer" (BERNSTEIN *et al.*, 2001). During the effector step, the fragments are incorporated into a protein complex and (after unwinding them) used as guiding sequence for the recognition of homologous mRNA, which gets subsequently cleaved by a RNA-induced

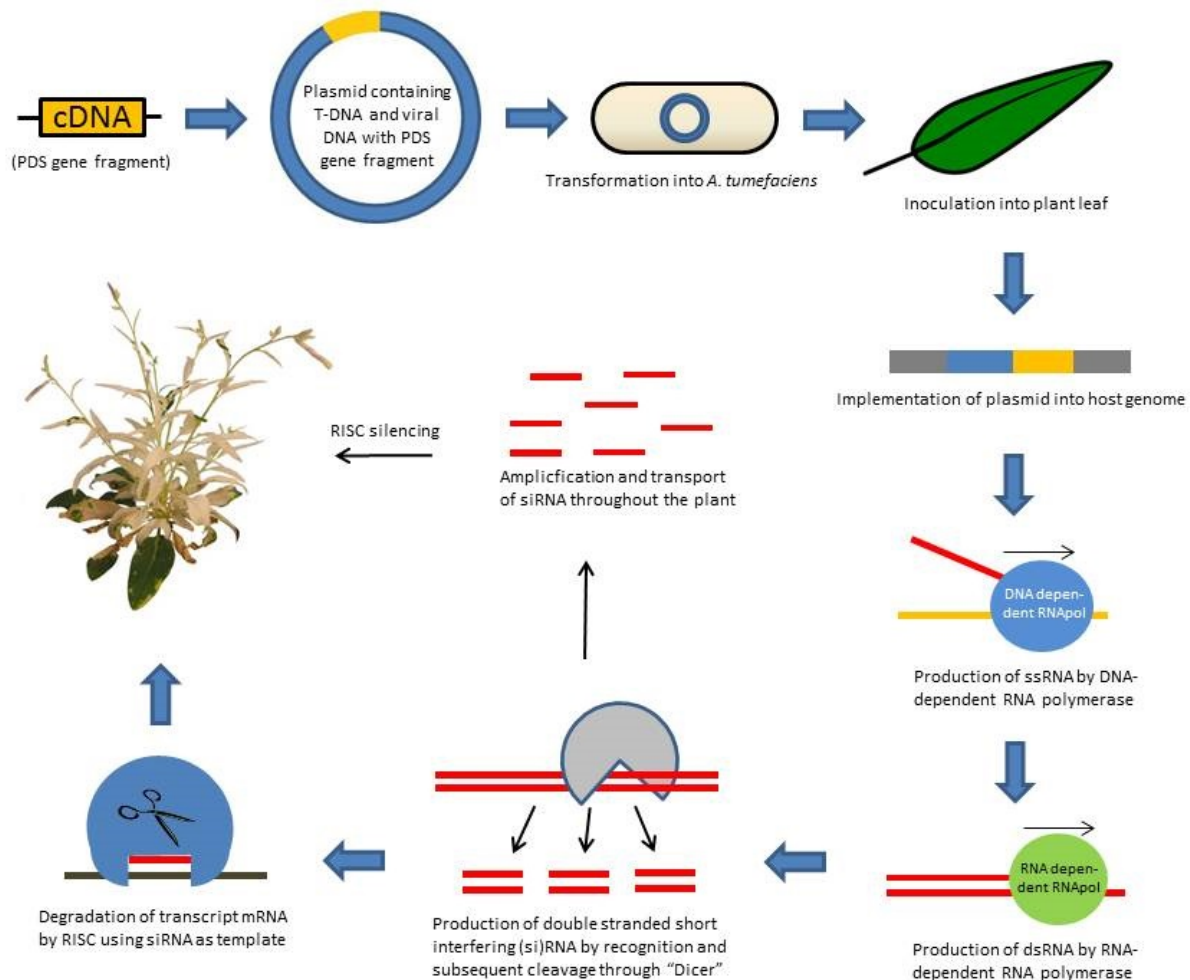


Figure 5: Simple scheme of RNAi silencing using PDS (phytoene desaturase) as an example.

Phytoene desaturase (PDS) is an enzyme involved in the chlorophyll and carotenoid biosynthesis pathway. Silencing it results in bleaching of parts of the plant (QIN et al. 2007). In the beginning a cDNA (complementary DNA) containing a partial sequence of the PDS gene is inserted into a viral genome. The viral DNA is inserted into the T-DNA (transport DNA) of a binary vector. The plasmid is transformed into *Agrobacterium tumefaciens* and the bacteria are inoculated into the leaf of the plant. Upon infection the plasmid is inserted into the host genome. During transcription DNA-dependent RNA polymerases produces single stranded RNA (ssRNA) of the T-DNA containing the target. RNA-dependent RNA polymerases uses the ssRNA as template to produce double stranded RNA (dsRNA), which is recognized by "Dicer" enzymes, cleaving the dsRNA into double stranded small interfering (si)RNAs. The RNA-induced silencing complex (RISC) incorporates the siRNA, unwind it and use it as a template for the degradation of the complementary parts of the transcript mRNA. At the same time the siRNAs are amplified and transported into other plant cells leading to silencing effects and in this case to bleaching in plant parts distant from the inoculation site.

silencing complex (RISC) leading to the degradation of the adequate mRNA (ZAMORE *et al.*, 2000). At the same time the single stranded siRNAs are amplified and spread as mobile silencing signals throughout the plant, leading to a systemic silencing effect of the target gene distant from the inoculation site (BECKER and LANGE, 2010). Naturally this effect occurs as antiviral defense system of plants, fungi and animals (SCHEPERS, 2005). The effects of gene

silencing were first discovered in plants (NAPOLI *et al.*, 1990) and the fungi *Neurospora crassa* (COGONI and MACINO, 1997) where they have antiviral-defense purposes. In 1998, FIRE *et al.* wanted to explain the unusual high silencing activity of sense control RNA found in a previous reported antisense experiment in the worm *Caenorhabditis elegans* performed by (GUO and KEMPHUES, 1995). They found that a simultaneous injection of sense- and antisense RNA lead to 10-fold higher silencing effects than antisense RNA alone. They concluded that dsRNA triggers an efficient silencing mechanism in which the levels of target mRNA are reduced by exogenous dsRNA. In conclusion, virus-induced gene silencing applies the RNAi mechanism to silence target genes with the help of a virus (LU *et al.*, 2003). The virus carries a partial sequence of the target gene. When the virus spreads, the endogenous target gene transcripts, homologous to the insert in the viral vector, are degraded by post-transcriptional gene silencing (PTGS) like described above (BAULCOMBE, 1999). The present study used the tobacco rattle virus (TRV) to silence genes involved in the GA pathway in *N. attenuata*.

To test our hypothesis, that GA can interfere with *N. attenuata*'s defense responses against *M. sexta* herbivory, we measured the phytohormones abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and its bioactive conjugate jasmonyl isoleucine (JA-ile) and compared three different kinds of treatments (untreated control plants, plants treated with wounding plus water and plants treated with wounding and oral secretions of *M. sexta*) for each transgenic line. Significant changes in phytohormones and secondary metabolites levels could support the theory of a linkage between the GA and JA pathway. The JA pathway is the main pathway for defenses against herbivores in all plants studied so far (DE GEYTER *et al.*, 2012). In *N. attenuata*, the activated JA pathway triggers the transcription of genes encoding secondary metabolites like nicotine (SAEDLER and BALDWIN, 2004), 17-hydroxygeranylinalool diterpene glycosides (HGL-DTGs, HEILING *et al.*, 2010) and phenolic compounds like caffeoylputrescine (CP), dicaffeoylspermidine (DCS), chlorogenic acid (CA, KAUR *et al.*, 2010; HEINRICH *et al.*, 2013) as well as proteinase inhibitors (PIs) like e.g. trypsin proteinase inhibitor (YANG *et al.*, 2011; HEINRICH *et al.*, 2012). Their quantitative levels and the transcript levels of genes involved into GA degradation and signaling provide information about the activity of the JA pathway. With silencing specific key modulators of the GA pathway it was therefore possible to answer the

question if GAs have an influence on the herbivory-induced accumulation of the former mentioned metabolites. In detail we tried to answer the question if silencing *DELLA1* and *GA2ox3*, both genes encoding proteins whose reduction in abundance should lead to defense symptoms similar to GA overdose, show decreased levels of nicotine, HGL-DTGs, CP, and DCS, compared to wild-type plants. Contrary to *DELLA1* and *GA2ox3*, silencing *GID1a* should lead to phenotypes with defense symptoms caused by low GA levels. The former mentioned defense metabolite levels should be increased.

Additionally some plants were treated by GA overdose. Plants with GA overdose should have weaker defense responses since the massive presence of bioactive GA lead to the degradation of DELLA proteins and therefore JAZ1 bind to MYC2 and stops the transcription of JA response genes. To test this, the levels of phytohormones and secondary metabolites were compared for plants treated with exogenous GA and plants treated without GA (mock). As a side effect the plants could grow taller and more rapid since the GA pathway is activated but if there is an inhibition effect of GA response genes to the GA biosynthesis, the plant treated with GA overdose should show no increased growth symptoms.

Another interesting question was, if the feeding behavior of *M. sexta* is influenced by plants with the exogenous application of bioactive GA. If these plants have lower defense responses, the *M. sexta* should be able to feed more intensively, which results in gaining more mass over time. To test this hypothesis, the mass of the larvae feeding on GA sprayed plants was compared to the mass of larvae feeding on mock plants. A significant increase of larval mass towards the larvae feeding on GA sprayed plants was expected.

Furthermore, volatile organic compounds like green leaf volatiles (GLVs) and terpenes are released as a direct and indirect defense against herbivores. Therefore it was interesting to measure the volatile levels of GA sprayed and VIGS plants and compare them to the levels of mock plants. If GA influences the production of volatiles, differences in their levels should be detectable in VIGS and GA overdose plants compared to the mock plants. If these differences are responses to oral secretions of *M. sexta* and not to wounding alone, the levels should be significantly different from wounding and water and the untreated control plants, respectively.

2 Material and methods

2.1 Analysis of the microarray

The protein sequences of genes involved into the GA pathway, obtained by the database NCBI (www.ncbi.nlm.nih.gov) were blasted (blasting nucleotides using a protein query) within a recently published 454-transcriptome of *N. attenuata* (ONKOKESUNG *et al.*, 2012), ITB Blast, Max Planck Institute for Chemical Ecology). Genes with similar nucleotide sequences were counterchecked by first searching for the open reading frame (ORF), translating the ORF into a protein sequence and then re-blasting the translated protein sequence in the NCBI database (blasting nucleotides using a translated protein query). Genes of the *N. attenuata* genome, encoding proteins involved into the GA pathway were then analyzed in regard to their regulation after herbivory treatment. Genes, that showed strong regulation within a short time span, were chosen as suitable candidates for VIGS (Figure 14).

2.2 Preparation of plant material

Seeds of *N. attenuata* were originally isolated from a natural population in Utah (USA) and inbred in the glasshouse for 31 generations. The preparation of the seeds was performed after KRUEGEL *et al.* (2002) with small deviations. The seeds were first washed for 5 minutes in sodium dichloroisocyanurate (DCCA 2% w/v, Sigma, St. Louis, MO, USA) mixed with tween-20 (1% v/v, Merck, Darmstadt, Germany) and distilled water to sterilize them. Afterwards they were washed with distilled water three times to remove the sterilization chemicals. Germination of the seeds was improved by the incubation of 1 h in 5 mL liquid smoke (House of Herbs, Inc.; Passaic, New Jersey, USA) containing 50 μ L of 0.1 M GA₃ additives. Before transferring them on a petri dish containing germination medium (Gamborg's B5 medium with minimal organics, Sigma, and 0.6% (w/v) phytigel, Sigma), the seeds were washed three times in distilled water again. They were incubated in a growth chamber (Percival Intellus environmental controller, Perry Iowa, USA) at 26 °C/16 h (155 μ m/s/m²) light and 24 °C/8 h dark. After 10 days the germinated seeds were transferred into TEKU communal boxes for further 10-11 days before they got potted in Glasshouse conditions.

2.3 GA overdose experiment

60 plants potted in the glasshouse were grown until elongation phase. 37 days after seed germination 30 plants were daily sprayed with 10 μ M GA₃ in 0.035% ethanol (EtOH). 30 plants were only treated with 0.035% ethanol as control plants (mock). The plants were sprayed until further measurements, which were performed during *N. attenuata*'s early flowering stage 41 days (phytohormones), 42 days (VOCs) and 43 days (secondary metabolites) after seed germination.

2.3.1 *M. sexta* larval performance experiment

After 6 days of GA/EtOH treatment (43 days after seed germination) 15 GA-treated and 15 mock plants were exposed to herbivory attack by hatched larvae of *M. sexta*. Three larvae were transferred onto one leaf of each plant. The daily GA/EtOH treatment continued during the exposure of the plants to *M. sexta*. After approximately 36 h the larvae were transferred onto a new leaf. The larvae weight was determined after 8 days, 10 days and 12 days of feeding. The experiment (feeding plus determining larval mass) was performed twice.

2.3.2 Other measurements

During the experiment some visual abnormalities of the GA sprayed plants were noted. The GA sprayed plants seemed to grow taller and showed a smaller angle of rosette leaf positions. These observations were measured with ruler and goniometer. The results are attached in the end of this document (Appendix , Figure 23, Table 2).

2.4 VIGS experiment

2.4.1 Preparation of the VIGS constructs

2.4.1.1 Standard PCR

For each of the three candidate genes (*GA2ox3*, *DELLA*, *GID1*) specific primers were designed and fragments were amplified with a polymerase-chain-reaction (PCR). The primer used consisted of a partial nucleotide sequence of the gene (ca. 300 bp) and restriction enzyme recognition sites for cloning (Table 1).

Table 1: Primers of the candidate genes for VIGS.

Light grey: promotor region, dark grey: cutting sequence for the restriction enzymes Bam HI (forward) and Sal I (reverse).

Oligo name	Sequence (5, -> 3')	Direction	Length (bp)	Tm (°C)	GC (%)
DEL1-33	GCGGCGGTCGACTTCAGATATGGCTGAAGTTGC	forward	33	82.8	57.5
DEL2-34	GCGGCGGGATCCAGCTCCACCTGGAATTGCTCTC	reverse	34	87.2	64.7
GA21-35	GCGGCGGTCGACGGTTGTCTTGTCAATACCAGCAG	forward	35	85.7	60
GA22-33	GCGGCGGGATCCAATCTTTCTATTGCCATAGCC	reverse	33	81.5	54.5
GID1-36	GCGGCGGTCGACGCTATTTCAGAAAGTGTCTGTTCTG	forward	36	83.2	55.5
GID2-34	GCGGCGGGATCCTCTCTTAGATTCGTTAGCGTTG	reverse	34	81.1	55.8

The master mix for the PCR (total volume 60 µL for each gene analyzed) was composed of 12.5 µL F-518 Phusion buffer HF (5x), 1.25 µL of each relevant primer (100 µM), 1.25 µL dNTPs (10 mM), 1.75 µL DMSO F-515 (100%), 0.6 µL polymerase and 41.4 µL Milli-Q water. Each reaction contained 2 µL of *N. attenuata* cDNA sample. The primers for each gene were tested at three different annealing temperatures using the Mastercycler gradient (Eppendorf). Afterwards the product was analyzed by gel electrophoresis in an agarose gel (1% w/v), containing EtBr (0.02 µL/mL) and performed at 100 V for 25 minutes (Figure 24). The targets showing up at ca. 300 bp were cut out (hereby the DNA of the same target gene was combined) and the DNA was extracted using NucleoSpin® Gel and PCR cleanup kit (Macherey Nagel) to a total volume of 15 µL for each gene. To test the success of the DNA extraction, 1 µL was diluted with 4 µL of Milli-Q water and analyzed in a second gel using the same concentrations and settings (Figure 25).

2.4.1.2 Restriction digestion analysis

After verifying its presence, the PCR product was restricted using the enzymes Bam HI and Sal I to isolate the targets out of the PCR product and prepare them for the ligation with the vector PTV Lox 6/1 respectively. The cutting sequences of the restriction enzymes were following:

Bam HI (NEB #R0136): 5' -G[↓]G A T C - 3'
 3' -C C T A G[↑]G- 5'

Sal I (NEB #R0138): 5' -G[↓]T C G A C- 3'
 3' -C A G C T[↑]G- 5'

The master mix for each sample used for restriction digestion was composed of 5 µL NEB Buffer B3 (10x), 5 µL of BSA (10x), 23 µL of Milli-Q water, 1,5 µL of each restriction enzyme and 14 µL of PCR product, summing up to a total volume of 50 µL. The mixture was incubated at 37 °C for 2 h. Afterwards the digested products each divided into two bags of 25 µL were analyzed by gel electrophoresis (Figure 26). Again the targets around 300 bp were isolated and extracted using the NucleoSpin® Gel and PCR cleanup kit. The total volume of restricted product was 20 µL for each target. To verify the success, 1 µL of each product, diluted with 4 µL of Milli-Q water, was analyzed on a gel (Figure 27). The settings for gel electrophoresis were the same like mentioned above.

2.4.1.3 Ligase of the target genes with the vector PTV Lox 6/1

In order to construct a plasmid containing the targets, the restricted DNA was ligated to the vector PTV Lox 6/1, which also contained a gene granting resistance against the antibiotic kanamycin (Figure 6). The master mix contained 2 µL of Fermentas T4 DNA Ligase Buffer (10x), 5 µL each of the vector and the restricted target DNA, 1 µL of Fermentas T4 DNA Ligase (#K1231) and 7 µL of Milli-Q water. For the ligation of the sample containing the *GID1* gene a different master mix, which was composed of 4 µL Invitrogen T4 Ligase Buffer (10x), 5 µL each of the vector and the *GID1* target DNA, 1 µL of Invitrogen T4 DNA Ligase and 5 µL of Milli-Q water, was used. The reason for changing the master mix was the unsuccessful ligation of *GID1* with the vector and the low ligation success

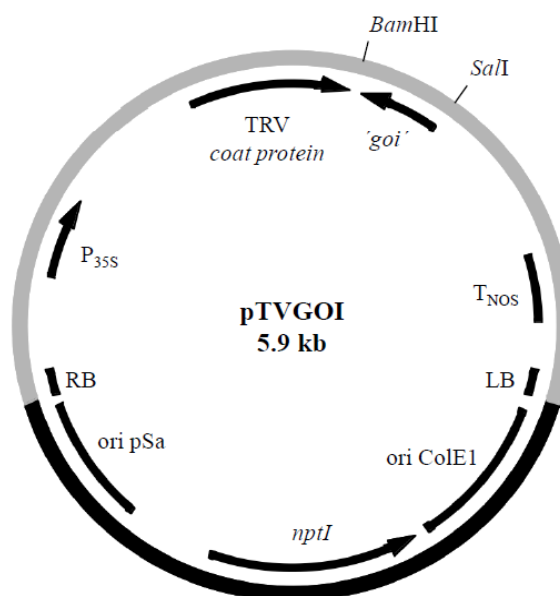


Figure 6: binary plant transformation vector pTVLox 6/1.

TRV coat protein = coat protein of the Tobacco rattle virus, *goi* = gene of interest, T_{NOS} = terminator novaline synthase gene, LB and RB = left and right border of T-DNA, ori ColE1 and ori pSa = origin of replication *E. coli* and *A. tumefaciens*, *nptII* = gene for neomycin phosphotransferase I, P_{35S} = promoter, *Bam*HI and *Sal*I=restriction enzymes.

for *DELLA* and *GA2ox*, probably caused by the dysfunction of the DNA ligase. The ligation was performed overnight at 16 °C for the Fermentas- and 14 °C for the Invitrogen ligase.

2.4.1.4 Transformation of the plasmid into *Escherichia coli* Top10 F⁻

Before performing the transformation the plasmid was purified. First 2 µL NaCl and 50 µL of EtOH (96% v/v) were mixed with 20 µL of plasmid DNA. The mixture was stored at -80 °C for 4 h to precipitate the plasmid and subsequently centrifuged at 4 °C and 13000 g for 15 minutes to divide the plasmid from the supernatant. The supernatant was carefully removed with the pipette and for each sample 100 µL of EtOH (70% v/v) was added as a second wash step. Again the DNA was centrifuged at 4 °C and 13000 g for 15 minutes before the supernatant was carefully removed. After centrifuging the sample one more time at 13000 g for 1 minute, the EtOH was evaporated completely by storing the open tube at room temperature. Subsequently the plasmid DNA was resuspended in 10 µL of Milli-Q water.

The transformation of the plasmid DNA into *E. coli* Top10 F⁻ was performed via electroporation using the MicroPulser Electroporator (BIORAD Laboratories, Germany) with the program *Ec1* (1.80 kV, 1 pulse). 5 µL of each plasmid were added into 40 µL of *E. coli* Top10 F⁻ suspension. The suspension was then transferred into an electroporation cuvette and subsequently subjected to electroporation. Immediately after transformation 700 µL LB-medium was added. The suspension was then incubated on a shaker (Innova 4000, New Brunswick Scientific) for 1 h at 28 °C, transferred on a petri dish with LB-medium containing 50 mg/L kanamycin and cultivated overnight at 37 °C.

2.4.1.5 Isolation of the plasmid from transformed *E. coli* Top10 F⁻

Pure colonies of the cultivated bacteria were picked with a sterile pipette tip and transferred into 3 mL liquid LB-medium containing 50 mg/L kanamycin. Afterwards they were incubated overnight in a shaker at 28 °C. The plasmids were extracted using the NucleoSpin® Plasmid extraction kit (Macherey Nagel) following the NucleoSpin® Plasmid/Plasmid (No Lid) protocol using 2 mL of cultivated bacterial cells as template.

To control if the resistance of the transformed *E. coli* Top10 F⁻ was caused by the insertion of the plasmid and not by spontaneous mutation, 5 µL of the extracted plasmid DNA was analyzed

via restriction digestion and subsequent gel electrophoresis as described above. The presence of partial DNA of the target genes around 300 bp were verified (Figure 28, Figure 29, Figure 30, Figure 31, Figure 32, Figure 33).

2.4.1.6 Preparation of the plasmids for sequencing

After the verification of the target sequences in the plasmid DNA the samples were prepared for sequencing. The target sequences on the plasmids were amplified by a PCR using a master mix containing labeled nucleotides. In specify the master mix (20 μ L) for one sample was composed of 13,5 μ L Milli-Q water, 4 μ L BigDye reaction mix (company, kit), 0,5 μ L either TRV1 or TRV2 primer and 2 μ L of plasmid DNA. The mix was then amplified using the Eppendorf Mastercycler gradient. Afterwards the PCR product was sequenced.

2.4.2 Transformation of the plasmid into *N. attenuata*

2.4.2.1 Preparation of electro-competent cells of *Agrobacterium tumefaciens*

The transformation of the plasmid into the plants of *N. attenuata* was performed using *Agrobacterium tumefaciens*. In order to realize a successful transformation via electroporation the *Agrobacterium* cells had to be electro-competent.

To produce electro-competent cells a pure culture of *Agrobacterium* was picked and grown overnight at 28 °C in 4 mL of autoclaved YEP medium containing yeast extract (10 g/L), sodium hydroxide (5 g/L) and tryptone/peptone from casein (10 g/L). The culture was subsequently transferred into 300 mL of fresh YEP medium and incubated on the shaker (250 rpm) at 28 °C until the optical density (OD₆₀₀) reached approximately 0.5. The OD measurement was performed using a photometer (BioPhotometer, Eppendorf, Germany). After reaching an OD₆₀₀ of 0.57, the *Agrobacteria* culture was divided into 6 falcon tubes of 50 mL each. The falcon tubes were centrifuged at 3214 g and 4 °C for 10 minutes and the supernatant was discarded. The bacterial pellet was resuspended in 25 mL of ice cold sterile distilled water. The 6 falcon tubes of 25 mL were combined to 3 tubes containing 50 mL of bacterial suspension before centrifuging it again using the same settings. The supernatant was discarded and the pellets resuspended in 25 mL of ice cold sterile distilled water. Two of the falcon tubes were combined while the third was left uncombined. After centrifuging a third time using the same settings the

supernatant was discarded and the bacterial pellet resuspended in 25 mL of ice cold water. The remaining two tubes were combined and centrifuged like described above. Again the supernatant was discarded and subsequently 50 mL of ice cold sterile glycerol (10% v/v) was added before centrifuging a last time at 4 °C for 10 minutes. The supernatant was discarded and the bacterial pellet resuspended in 2 mL of 10% glycerol. 50 µL of the purified culture was then aliquot into small cap tubes and frozen immediately on liquid nitrogen before storing them at -80 °C.

2.4.2.2 Transformation of the target plasmid into *Agrobacterium tumefaciens*

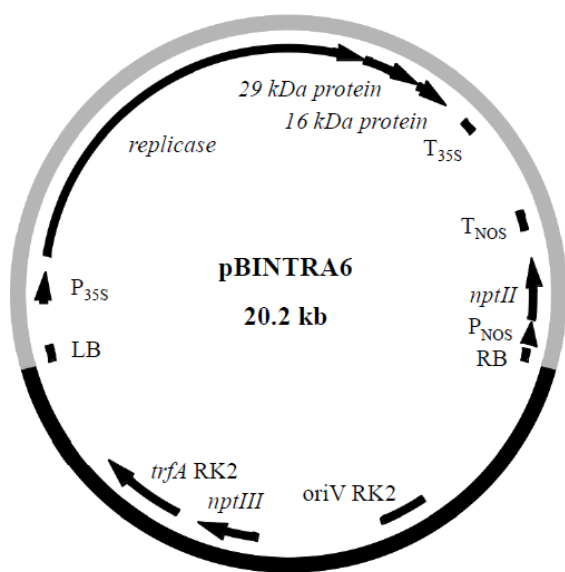


Figure 7: binary plant transformation vector pBINTRA

Replicase = RNA-dependent RNA polymerase, T35S = terminator, T_{NOS} = terminator for novaline synthase gene, nptII = kanamycin resistance gene for plants, nptIII = kanamycin resistance gene bacteria, P_{NOS} = promoter novalin synthase gene, RB and LB = right and left border of T-DNA, oriV RK2 = replication origin of plasmid RK2, trfA RK2 = gene for plasmid replication protein (binds to and activates oriV), P_{35S}, T_{35S} = promoter and terminator, 29 kDa protein = movement protein of TRV, 16 kDa protein = suppressor of RNA silencing (to prevent silencing of TRV).

The transformation of *Agrobacterium* was performed similar to the transformation of the plasmid into *E. coli* but instead of LB medium, YEP medium was used instantly after the electroporation. The transformed bacteria were transferred to Agar plates with LB medium containing 50 mg/mL kanamycin and incubated at 28 °C for 36 h in the dark until colony growth was sufficient enough to pick single colonies for further treatment. Single colonies of the transformed *A. tumefaciens* were picked from the agar plates and grown overnight in a pre-culture of 5 mL YEP medium containing 50 mg/mL kanamycin in the shaker at 28°C. Additionally pre-cultures of the strains pTV00 (containing an empty vector without the target DNA), pTVPD (silencing phytoene desaturase)

and pBINTRA were grown. The plasmid transformed into *A. tumefaciens* contained the target DNA but also one of the two genetic parts, which produce the tobacco rattle virus. The other part was produced in the *Agrobacterium tumefaciens* strain containing the plasmid pBINTRA. In order to produce the virus these cultures had to be mixed together. This procedure was

performed at a later point directly before VIGS. The pTVPD plasmid was used as a control for the successful transformation of the VIGS construct into the plant while the pTV00 plasmid was used as a negative control since the target DNA was not inserted.

After growing them overnight the pre-cultures were transferred into sterile Erlenmeyer-flasks containing new YEP medium with 50 mg/mL kanamycin. Since the cultures containing the target, the pTV00 and the pTVPD plasmid had to be mixed with the pBINTRA culture, the volume of this culture had to be four times larger. The cultures were then grown until the OD₆₀₀ reached a value between 0.4 and 0.6. The pBINTRA culture was divided into four cultures. Subsequently, the cultures were aliquot into 50 mL falcon tubes and centrifuged at 2057 g for 5 min. The bacterial pellets were resuspended in a buffer comprised of 12.5 mL 5 mM magnesium chloride (MgCl₂) and 5 mM 2-(N-morpholino)ethansulfonic acid.

2.4.3 VIGS procedure

In the VIGS chamber, a special chamber in the glasshouse reserved for VIGS experiments, the pTV00, pTVPD and the cultures containing the target plasmids were mixed with the pBINTRA cultures to inject both parts of the virus. 28 days after seed germination, approximately 1 mL of the mixed cultures was inoculated into 3 rosette leaves of each plant using a syringe. The inoculation was performed with 25 plants for *GA2ox3*, 25 plants for *DELLA1*, 25 plants for pTV00 and 5 plants for the pTVPD construct. After approximately 20 days the plants silenced in phytoene desaturase (pTVPD) showed bleaching of the newer leaves and the stem. The bleached parts gave an insight which leaves are silenced. Figure 8 shows that the older parts of the plant were not silenced. Therefore only the younger leaves of the *DELLA1* and *GA2ox3* silenced plants were used for further experiments. Although the gel electrophoresis after the restriction digestion of the GID1 plasmid showed bands around 300 bp the sequence did not contain the target (counterchecked with the GID1 primer). After several



Figure 8: Young *N. attenuata* plant silenced in phytoene desaturase approximately 20 days after VIGS.

unsuccessful attempts to get a target product it was decided to skip the silencing of the *GID1* receptor gene. Subsequently to the VIGS procedure the plants were kept in darkness for 2 days before exposing them to the light.

2.5 Measurements (GA overdose and VIGS)

2.5.1 Phytohormones

The plants of the GA overdose (41 days after seed germination) and the VIGS experiment (48 days after seed germination) were analyzed for their levels of Phytohormones 1 h and for secondary metabolites 48 h after wounding. The youngest and second youngest leaf of each plant was wounded. For the analysis of the VIGS plants the oldest (S1) and second oldest stem leaf was used. Wounding was done by using a pattern wheel and the wounded leaf was subsequently supplied with water or 1:5 diluted (with water) oral secretions of *M. sexta* (Figure



Figure 9: Simulation of herbivore attack on leaves of *N. attenuata* plants.

The plant leaves were wounded with using pattern wheel. Subsequently, 1:5 diluted oral secretions of *M. sexta* larvae were applied to the leaves. To analyze the response after wounding alone, water instead of oral secretions was used.

9). The control plants remained untreated.

To analyze the phytohormones after 1 h, each side of the mid nerve of the youngest rosette leaf of the GA overdose plants and the S1 leaf of the VIGS plants, respectively, was treated with three rows of wounding. After 1 h the mid vein was cut out and the rest of the lamina was transferred into 2 mL microfuge tubes and immediately frozen in liquid nitrogen. Prior to this the leaf outline was drawn onto a paper sheet for calculating its surface. The surface was measured with the program Photoshop Creativity Suite 5 (Adobe). The second youngest leaf (for VIGS plants the second oldest stem leaf S2) was treated only with one row of wounding. A

second row was performed after 9 h and the third row after 24 h to imitate repeated feeding. These leaves were harvested after 48 h using the same procedure than the 1 h experiment. Before harvesting them the leaf volatiles were collected. The plant material was frozen at -80 °C until further processing.

Later it was grinded to powder using a pestle. During the procedure the material was kept on liquid nitrogen to prevent degradation of the phytohormones. Approximately 0.1 g of the ground plant material was aliquot into 96 well Bio-tubes (1.1 mL individual tubes, Arctic White LLC) containing 2 steel beads. 800 µL of ethyl acetate (containing 100 ng JA and 20 ng JA-Ile, SA and ABA per sample as phytohormones standards) was added to the samples before they were extracted at 1150 strokes/min for 5 minutes (Geno Grinder 2000, Spex Sample Prep, US) to

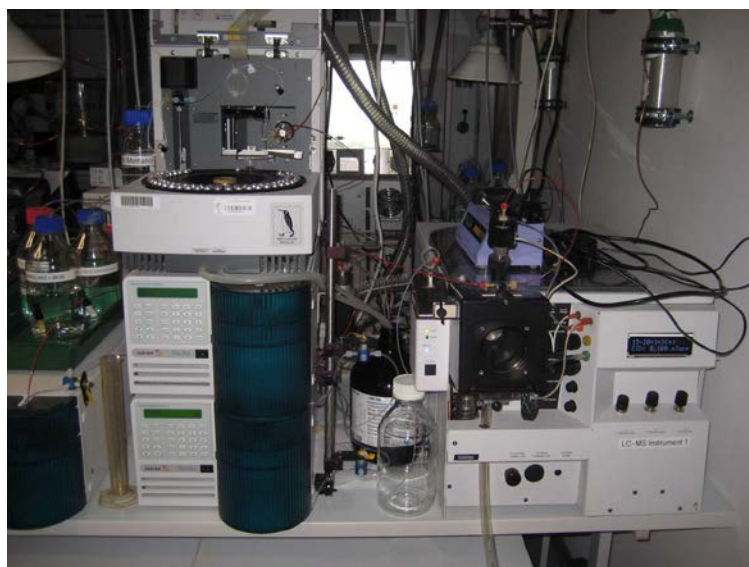


Figure 10: Varian ProStar 1200 QQQ MS (Varian, USA)

dilute the phytohormones into the liquid phase. The concentrations of the standards spiked into the samples were 10 (ng/mL) for ABA, SA and JA-ile and 50 (ng/mL) for JA. The material was centrifuged at 2057 g for 20 min. The supernatant was carefully transferred into new 1.5 mL tubes and evaporized at 45 °C (TurboVap®96, Biotage, Sweden). After evaporating the

ethyl acetate, the solid phase was resuspended in 500 µL of MeOH (70 % v/v) using the Geno Grinder at 1050 strokes per minute for 5 minutes followed by centrifugation at 2057 g for 20 minutes. 450 µL of the supernatant was transferred into glass vials and sealed with a lid. The measurements of the phytohormones was performed through liquid chromatography mass spectrometry (LC-MS) using the 1200L Triple-Quadrupol mass spectrometer (Varian, USA). The samples (V=15 µL) were injected onto a ProntoSIL column (C18, 5 µm, 50 Å – 2 mm, Bishoff, Germany) attached to pre-column (C18, 4x2 mm, Phenomenex, USA) with a flow rate of 0.1 mL/min. As mobile phases, two solvents, solvent A (0.05 % formic acid) and solvent B

(0.05 % formic acid in methanol) were used. Solvent B was injected in a gradient mode. The time/concentration for the gradient mode (min/%) for solvent B was 0:00/15, 1:30/15, 4:30/98, 12:30/98, 13:30/15 and 15:00/15 with a flow rate (mL/min) of 0:00/0.4, 1:00/0.4, 1:30/0.2, 10:00/0.2, 10:30/0.4, 12:30/0.4 and 15:00/0.4. The detection was performed with negative electrospray ionization mode (ESI). The ESI generates molecular ions from the phytohormones in the samples and the internal standards, respectively, by fragmenting the parent ions using 15 V collision energy. These specific ions were then used to create a compound chromatogram.

The compound chromatogram was adjusted and analyzed with the program Varian MS workstation version 6.6 (Varian Inc., USA). The peak areas were adjusted manually and subsequently integrated automatically by the program. The phytohormones were quantified by comparing the peak areas of the sample with the peak areas of the internal standards.

2.5.2 Secondary metabolites

For the analysis of the secondary metabolite levels (GA-overdose 43 days and VIGS 50 days after seed germination) also 0.1 g of the plant tissue was aliquot into 96 well Bio-tubes containing 2 steel beads. 800 μ L of extraction solvent (0.1 % acetic acid in 40/60 MeOH/H₂O) was added to each sample before shaking it at 1200 strokes/min for 1 minute. Afterwards the suspension was centrifuged for 20 min at 2057 g and the supernatant carefully transferred into a new Bio-tube. To ensure the absence of solid particles in the solvent the supernatant was centrifuged again using the same settings. 450 μ L of each extraction solvent was transferred into a glass vial and sealed with a lid. The analysis was performed through High Performance Liquid Chromatography (Agilent 1100-UV-Varian ELSD, Varian, USA, Figure 11) using a chromatographic column (Chromolith FastGradient RP18e, 50 x 2 mm, Merk, Germany) connected to a precolumn (Gemini NX RP18, 3 μ m, 2 x 4.6 mm, Phenomenex, USA). The temperature of the column oven was set to 40 °C. The mobile phase consisted of two solvents, solvent A (0.1 % formic acid and 0.1 % ammonium hydroxide in water, pH=3.5) and solvent B (methanol) which were used in a gradient mode (time/concentration min/% for A: 0:00/100; 0.50/100; 6.50/20; 10:00/20; 15:00/100). The flow rate was set to 0.8 mL/min. The signal was detected using PDA (Photo Diode Array) and ELSD (Evaporative Light Scatter Detector, Varian,

USA) detectors. Using these settings and components, nicotine eluted at a retention time (RT) of about 0.5 minutes and could be detected under UV light at 260 nm wavelength. Caffeoylputrescine, chlorogenic acid and dicaffeoylspermidine with RT of 2.5, 3.2 and 4.1, respectively, were detected at 320 nm and rutin (RT 4.9) at 360 nm. The 17-hydroxygeranyllinalool diterpene glycosides (RT 7.8) were detected via the ELSD detector. The data was analyzed with Chromeleon software (version 6.8, Dionex). The peak areas were integrated and adjusted to the weight of the aliquot plant material. The concentrations of

nicotine, chlorogenic acid and rutin were determined via external dilution series of standard mixtures. Caffeoylputrescine, dicaffeoylspermidine and 17-hydroxygeranyllinalool diterpene glycosides were compared only via their integrated peak areas.

2.5.3 Volatiles

After 8 days of GA/EtOH treatment (45 days of seed germination) the GLVs and terpenes were



Figure 12: Volatile trapping using plastic cups, containing PDMS tubes (encircled red).



Figure 11: Agilent 1100-UV-Varian ELSD (Varian, USA)

collected using polydimethylsiloxane (PDMS) adsorption with PDMS tubes. The VOCs of the VIGS plants were taken 49 days (GLVs) and 50 days (terpenes) after seed germination. For preparation the PDMS tubes were cut into 5 mm long pieces, stored overnight in 4:1 acetonitrile with methanol (MeOH) and (after removing the supernatant and transferring of the tubes into a 200 mL glass column containing a glass frit) heated at

210 °C for 3 h under nitrogen flow (2 L/min – PDMS tubes are sensitive to oxygen) before storing them in polytetrafluorethylene (PTFE)-sealed glass vials containing Argon (Ar) gas until further use. The collection of the volatiles was performed after the third wounding treatment during the secondary metabolite analysis. After induction the wounded leaves were enclosed in plastic cups containing the PDMS tube. After 1 h the tubes for GLV analysis were taken out of the cup and transferred into PTFE-sealed glass vials. New PDMS tubes were placed into the cups for the analysis of the terpenes. These tubes were left inside the cup for 24 h. The glass vials containing the PDMS tubes for GLVs and the terpenes were stored at -20 °C or measured directly after their collection. The same procedure was applied for the S2 leaves of the VIGS plants.

The volatile analysis was performed through gas chromatography mass spectrometry using the Ultra Thermo desorption unit TD20 connected to a quadrupole GC-MS-QP2010Ultra (Shimadzu, Figure 13). The PDMS tubes were placed in 89 mm glass TD tubes (Supelco). After desorption in



Figure 13: SHIMADZU GC-MS – QP2010 Ultra Thermo desorption unit TD20

liquid nitrogen with a flow rate of 60 mL/min at 200 °C the substances were cyro-focused onto a Tenax® adsorbent trap at -20 °C. After all substances were focused the trap was heated to 230 °C within 10 s and analytes were injected using a 1:20 split ratio onto an Rtx-5MS column with a diameter of 30 m 0.25 mm and a film thickness of 0.25 µM (Resteck). As carrier gas He was used with a constant linear velocity of 40 cm/s. The

TD-GC interface was held at 230 °C. The oven was set to 40 °C for 5 min followed by 185 °C with a thermal increase of 5 °C/min and subsequently to 280 °C raising the temperature 30 °C/min and holding it for 0.83 min. For analysis of the samples the electron impact (EI) spectra were recorded at 70 eV in scan mode from 33 to 400 m/z at a scan speed of 2000 Da/s. The transfer line was held at 240°C and the ion source at 220°C. To prevent incorrect measurements caused

by the breakdown of volatiles before they were measured, the samples were mixed during the preparation. For each analysis (GLVs/Terpenes GA overdose 1 h, GLVs/Terpenes GA overdose 24 h, GLVs/Terpenes VIGS 1 h and GLVs/Terpenes VIGS 24 h) two empty tubes, one in the beginning and one at the end of the measurement were included. Data processing was performed with the program Shimadzu GC-MS solutions software (v2.72) provided by the manufacturer converting the raw data for into computable documents format (cdf) via its export function. The results were converted into the final dataset with an MS Excel macro file. The results were filtered for values smaller than 5000 counts * second, which were considered as background noise. Afterwards the values were adjusted to the leaf surface to get the final unit (counts * second) per cm². This unit was referred to as relative emission.

2.6 Statistical analysis

The present data was analyzed with the program SPSS Statistics 17.0 (IBM, USA) using descriptive and univariate statistics. The normal distribution of the datasets was determined with the Kolmogorov Smirnov (KS) test. Before comparing the means with either Student's T-test (if data was normal distributed and homoscedastic) or Mann-Whitney U-test (for heteroscedastic datasets) the data was tested for homogeneity of variance using Levene's test and for outliers (caused by extreme values occurring in some plants) using the Grubbs test (<http://graphpad.com/quickcalcs/Grubbs1.cfm>).

3 Results

3.1 Analysis of the microarray

To understand the influence of certain environmental conditions on organisms like (in this case) *N. attenuata* plants, it is sometimes helpful to evaluate the transcriptional regulation of genes, belonging to various pathways. The microarray data of ONKOKESUNG *et al.* (2012) contained more than 100 genes, important as regulators of herbivory-induced defense responses in *N. attenuata*. We used a homology-based method to identify all known genes, involved into GA metabolism and signaling.

We were able to determine in total 21 genes, from which six belonged to GA biosynthesis (*NaGA20ox1/2*, *NaKS*, *NaKO*, *NaKAO*, *NaGA3ox1*), six to GA degradation (*NaGA2ox1/2/3/4/5/6*) and nine to GA signaling (*NaGID1a/b/c*, *NaGID2*, *NaDELLA1*, *NaDELLA2*, *NaSPY1*, *NaSPY2*, *NaSPY3*). Only few of them showed a quick regulation (within 1 hour) in their transcripts after herbivory simulation. Transcripts of *NaGA2ox3* and *NaGID1a* were strongly up-regulated while *NaDELLA1* was slightly down-regulated especially after herbivory signals. Also *NaGA20ox1* and *NaGA2ox2* showed strong and quick regulations but this regulation was no specific response to herbivory simulation, since they were regulated after wounding alone. On the other hand, the transcript levels of the genes *NaKAO*, *NaGA3ox1*, *NaGA2ox4*, *NaGID1b* and *NaGID1c* were regulated specifically in response to herbivory signals but this response was not quick (Figure 14).

To further verify their involvement in *N. attenuata*'s response to herbivory, we reduced the transcript levels of *NaGa2ox3*, *NaGID1a* and *NaDELLA1* using VIGS. We did not use other genes, since they did not respond quick or specific to herbivory simulation. Although the regulation of *DELLA1* was not quick and strong, we silenced it since it plays a key role in JA-GA signaling. Furthermore, not all of the genes involved in the GA pathway of *Arabidopsis*, *Oryza* and members of the family Solanaceae, which are studied well, could be found in the *N. attenuata* genome. For example the gene encoding CPS was not detected.

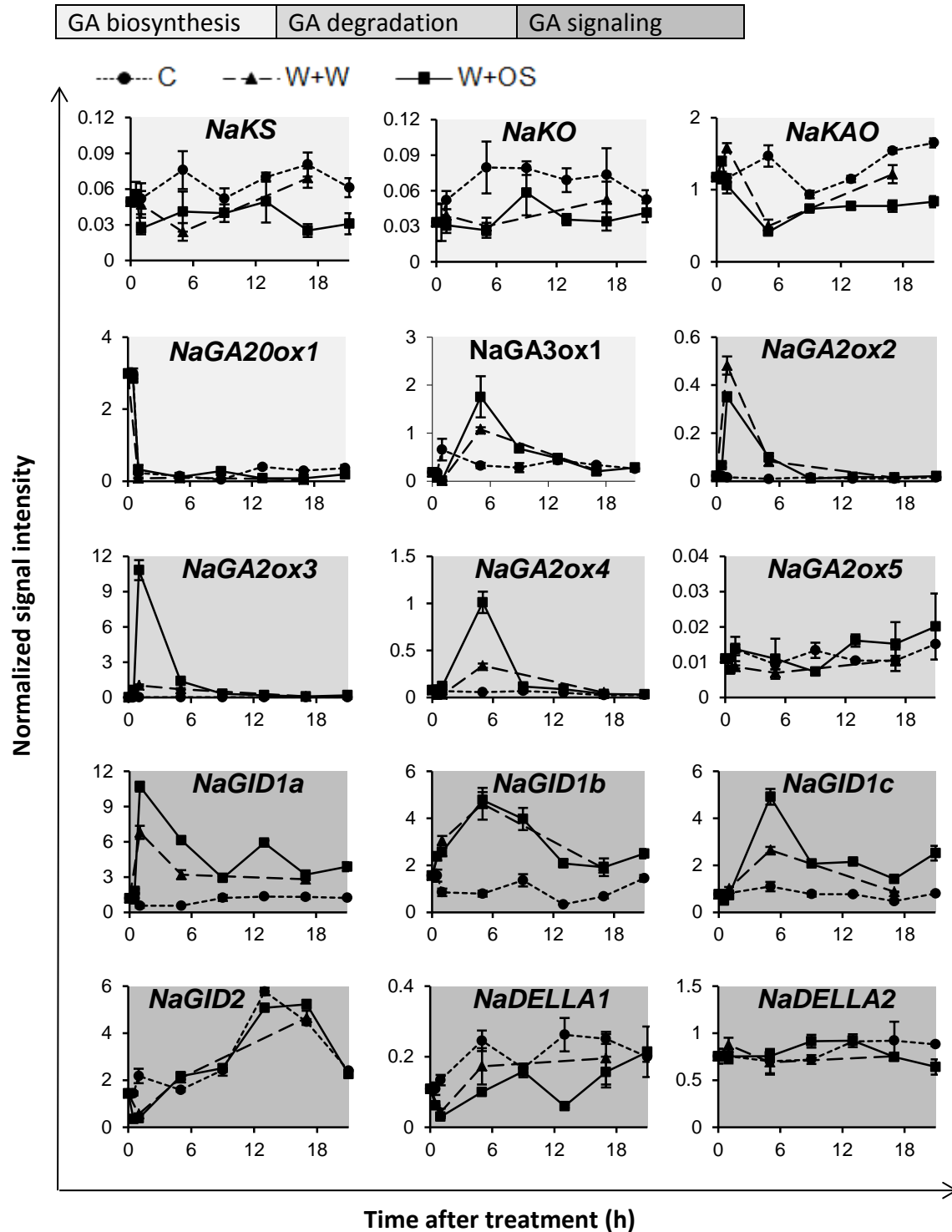


Figure 14: Herbivory-induced regulation of genes involved into the GA pathway in *Nicotiana attenuata*.

After wounding the leaves they were treated with water (W+W) or oral secretions of *Manduca sexta* (W+OS). Control leaves (C) were left untreated. The genes presented encode the following proteins: NaKS = ent-kaurene synthase, NaKO = ent-kaurene oxidase, NaKAO = ent-kaurenic acid oxidase, NaGA20ox = gibberellin 20-oxidase, NaGA3ox = gibberellin 3-oxidase, NaGA2ox = gibberellin 2-oxidase, NaGID1 = gibberellin insensitive dwarf1, NaGID2 = gibberellin insensitive dwarf2, NaDELLA = unspecified DELLA proteins.

3.2 GA-treatment influences *N. attenuata*'s resistance to the specialist herbivore *M. sexta*

To further test the role of the GA pathway we sprayed plants with GA₃, a bioactive GA that is known to trigger the germination of *N. attenuata* seeds (SCHWACHTJE and BALDWIN, 2004). After 10 days the average mass of the larvae feeding on plants sprayed with 10 µM GA₃ was highly significantly larger than the ones feeding on the control (mock) plants. *M. sexta* larval mass gain on the GA-sprayed plants (compared to the control plants) was approximately 17 % after 8 days, 50 % after 10 days and 63 % after 12 days.

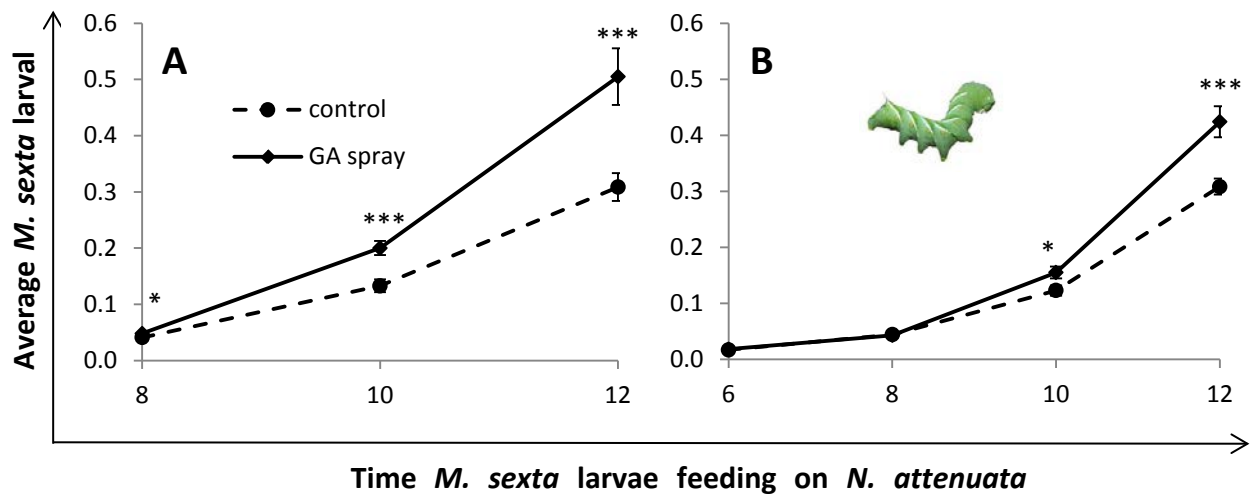


Figure 15: *M. sexta* larvae feeding on GA-treated plants of *N. attenuata* gained more mass over time.

The average mass of *M. sexta* feeding on *N. attenuata* plants treated with whether 10 µM GA₃ dissolved in 0.035 % EtOH or only treated with 0.035% EtOH as control plants significantly increased after 10 days on plants sprayed with GA₃. The black bars show the standard error, the stars above the level of significance calculated with Student's t-test. A: first experiment. B: second experiment.

The second caterpillar performance experiment showed similar results although the differences were not that strong like in the former experiment (Figure 15 B). *M. sexta* larval mass gain on GA-sprayed plants (compared to control plants) was approximately 5 % after 6 days, -2 % after 8 days, 26 % after 10 days and 37 % after 12 days.

These data demonstrate that altering GA levels influences *M. sexta* larval mass gain and suggests that the GA pathway may change the levels of plant metabolites involved in the defense against herbivores.

3.3 Metabolomics GA-overdose

3.3.1 Phytohormones

Most of the stress responses in plants are regulated via phytohormones. Therefore we analyzed phytohormones of *N. attenuata* to get a picture about possible connections between the phytohormone levels and the levels of the defense metabolites. Although some changes were observed for JA and its conjugate JA-Ile after wounding plus water (W+W) and wounding plus oral secretions of *M. sexta* (W+OS), no significant changes could be seen between the plants daily sprayed with 10 μ M GA₃ (dissolved in 0.035% EtOH) and the mock plants only sprayed with 0.035% EtOH (Figure 16). The levels of SA and ABA remained steady.

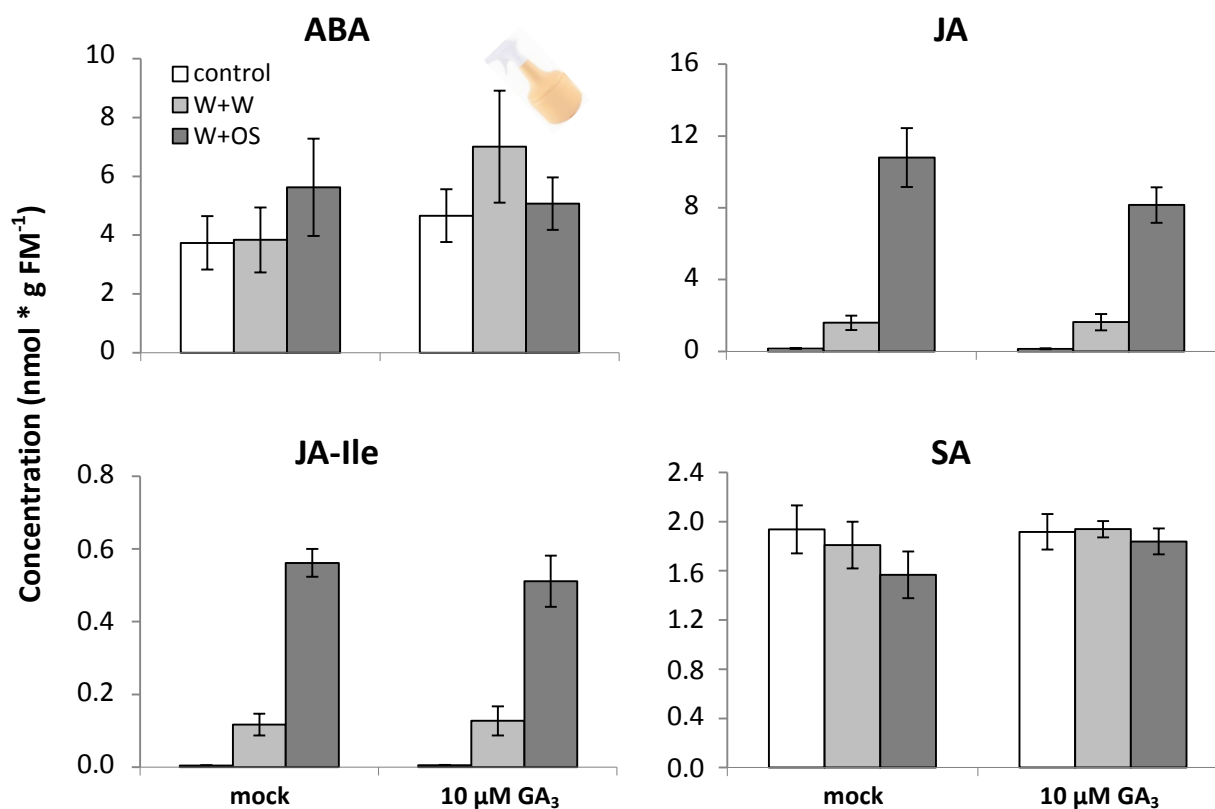


Figure 16: GA₃ application does not alter the accumulation of herbivory-induced phytohormones.

N. attenuata plants were daily sprayed either with 10 μ M GA₃ (dissolved in 0.035% EtOH) or with 0.035% EtOH only (mock) for 10 days during their elongation stage. Measurements of the local leaves were taken 1 h after wounding (using a pattern wheel) and subsequent application of water (W+W) or oral secretions of *M. sexta* (W+OS). The control leaves remained untreated. The measurements were performed during early flowering stage (41 days after seed germination). Black bars show the standard error. SA=salicylic acid, JA=jasmonic acid, JA-Ile=jasmonyl-isoleucine, ABA=abscisic acid, FM = fresh mass (leaf).

3.3.2 Secondary metabolites

The elucidation of defense metabolites like toxins or digestion inhibitors can be seen as a direct defense against herbivores. Changes in the concentration of defense metabolites give insight into the plant's answer to herbivore attack. To study the role of GAs in the defense process, it was indispensable to have a look at the "weapons" of the attacked *N. attenuata* plants. Some secondary metabolites were reduced in the plants sprayed with exogenous GA (Figure 17).

The levels of nicotine and DCS were significantly lower for plants treated with 10 μ M GA₃ compared to the plants treated only with 0.035 % EtOH. The nicotine levels of the mock plants increased after W+W and W+OS but the GA sprayed plants trended to result in a reduction of nicotine similar to the level of the untreated control plants (W+W $p=0.071$). The reduction after W+OS treatment was significant ($p=0.03$).

The levels of DCS were in general significantly lower for the GA sprayed plants independent if they were wounded or not (control, $p=0.036$, W+W, $p=0.013$, W+OS, $p=0.044$). The levels of CP and CA were higher for all plants treated by W+OS but there were no significant differences between the plants sprayed with GA and the mock plants except the W+W treatment for CP ($p=0.032$). The rutin levels remained unchanged. The HGL-DTG levels were similarly reduced in response to W+OS-treatment (wild-type plants $p=0.037$, GA-sprayed plants $p=0.047$) but no differences between mock plants and GA-sprayed plants were observed.

Taken these results together the only defense metabolites affected by GA-treatment were nicotine, DCS and the HGL-DTGs. The other metabolites did not show changes in their concentrations.

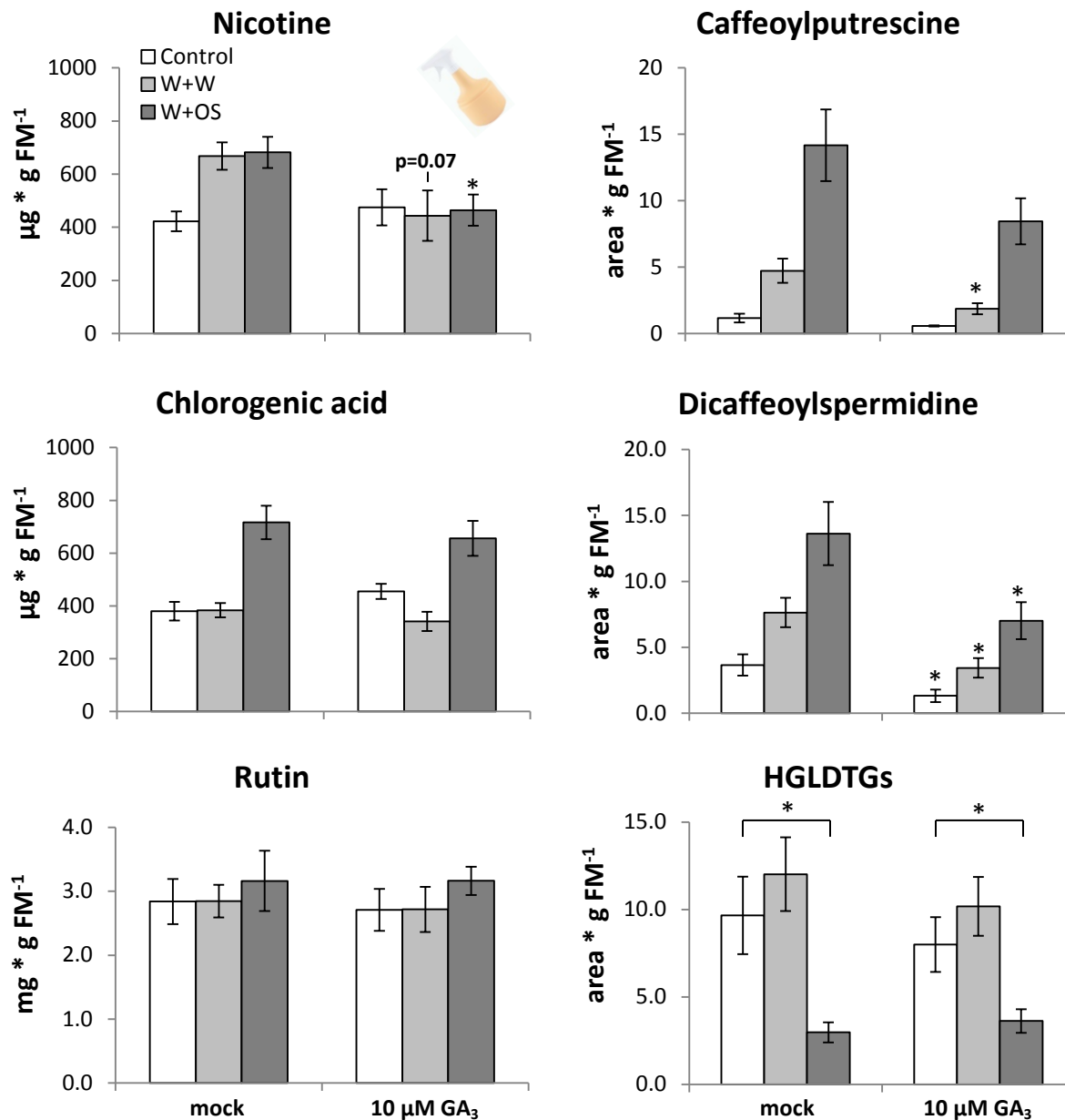


Figure 17: Nicotine and dicaFFEoylspermidine levels changed while others were not affected by GA₃ treatment.

The secondary metabolite levels were compared between plants of *N. attenuata* daily sprayed with 10 μM GA₃ (dissolved in 0.035 % EtOH) and plants only sprayed with 0.035 % EtOH (mock). The plants were sprayed for 10 days during their elongation phase. Measurements of the local leaves were taken 48 h after wounding (using a pattern wheel) and subsequent application of water (W+W) or oral secretions of *M. sexta* (W+OS). The control leaves remained untreated. The measurements were performed during early flowering stage (43 days after seed germination). Black bars show the standard error, the stars above the level of significance calculated by Student's t-test and Mann-Whitney U-test (for heteroscedastic datasets), respectively. FM = fresh mass (leaf).

3.3.3 Volatile organic compounds

As mentioned in the introduction, VOCs are considered as indirect defense metabolites of *N. attenuata*. To get a picture how GAs influence the indirect defense system of *N. attenuata* plants, we measured the GLVs emitted after 1 h and the terpenes emitted after 24 h. Although

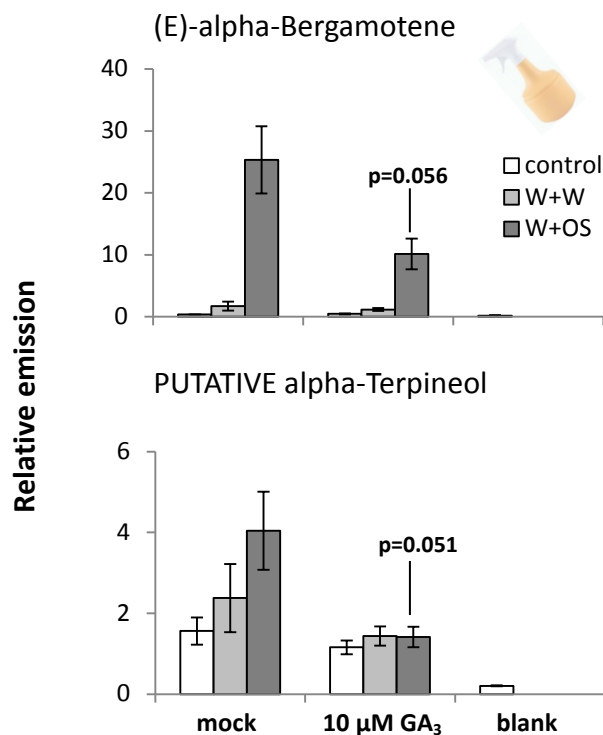


Figure 18: Volatile organic compounds after 24h.

The VOCs were compared between plants of *N. attenuata* daily sprayed with 10 µM GA₃ (dissolved in 0.035 % EtOH) and plants only sprayed with 0.035 % EtOH (mock). The plants were sprayed for 10 days during their elongation phase. Measurements of the local leaves were taken within 24 h after wounding (using a pattern wheel) and subsequent application of water (W+W) or oral secretions of *M. sexta* (W+OS). The control leaves remained untreated. The measurements were performed during early flowering stage (42 days after seed germination). The black bars are standard errors, the stars above the level of significance calculated either by Student's T-test or Mann-Whitney U-test (for heteroscedastic datasets).

GLVs were measured for the GA overdose and the VIGS experiment, the signals after one hour were too weak to meet the requirement to have at least three of five replicates with values larger than 5000 counts * second for calculating the average value of each treatment (control, W+W, W+OS). More VOCs could be detected after 24 h when terpenes were more abundant than GLVs. The levels of GLVs or terpenes showed only few differences between the wild-type plants (GA overdose experiment) and the plants treated with external GA₃. The entire dataset of the VOC analysis is shown in the appendix (Table 3).

After 24 h the plants sprayed daily with 10 µM GA₃ (dissolved in 0.035% EtOH) showed a trend towards decreased concentrations of the sesquiterpene E-α-bergamotene (only W+OS p=0.052) and (putative) α-terpineol (p=0.051). The data

are shown in Figure 18. E-alpha-bergamotene was induced strongly after the treatment with oral secretions of *M. sexta*. After 24 h, GLVs like e.g. 3-(E)-hexenyl butanoate and 3-(E)-hexenyl isobutyrate were also induced in response to W+W and W+OS but all these volatiles had no statistical differences between control and GA sprayed plants.

3.4 Metabolomics VIGS experiment

3.4.1 Phytohormones

When comparing the phytohormones levels of the GA overdose with the VIGS experiment, the concentration of ABA was much higher for the plants sprayed either with 0.035 % EtOH (control) or 10 μ M GA₃. These levels were about ten times more concentrated than the ABA levels of the VIGS plants. Also the JA-Ile levels were slightly higher. Contradicting to that the levels of JA were increased in the VIGS plants. The concentration of SA remained steady for each treatment. The only significant changes calculated was between the JA-Ile levels of the untreated pTV00 (empty vector) plants and the untreated plants silenced in *DELLA1* ($p=0.017$), as well as the untreated plants silenced in *GA2ox3* ($p=0.021$). These changes were not observed for the W+W and the W+OS treatment (Figure 19).

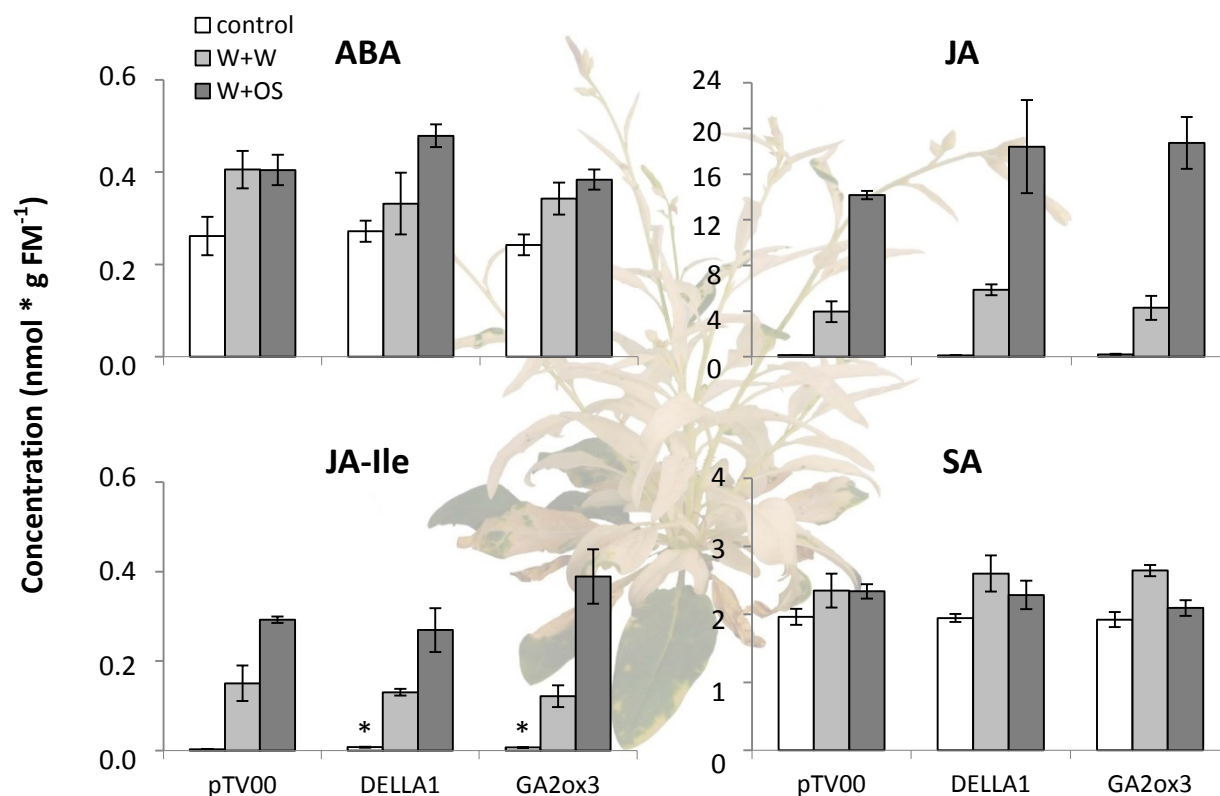


Figure 19: Only JA-Ile showed significant differences and only in the untreated control leaves.

The phytohormone levels (local leaf) of *N. attenuata* plants silenced in *DELLA1* or *GA2ox3* were compared to the empty vector plants (pTV00). Measurements were taken 1 h after wounding (using a pattern wheel) and subsequent application of water (W+W) or oral secretions of *M. sexta* (W+OS). The control leaves remained untreated. The measurements were performed during elongation phase (48 days after seed germination). Black bars show the standard error, the stars above the level of significance. FM = fresh mass (leaf).

3.4.2 Secondary metabolites

Compared to the GA overdose experiment the secondary metabolite levels of the VIGS experiment were generally lower. The most extreme difference was observed for dicaffeoylspermidine and chlorogenic acid. In some cases the *DELLA1* genotype was different from the empty vector (PTV00) and the *GA2ox3*.

The nicotine levels of the control and after W+W treatment were significantly lower ($p=0.02$ and 0.018 , respectively) compared to pTV00, although there was no difference after W+OS. The levels of chlorogenic acid (control $p=0.001$ and W+OS $p=0.016$), its isomer cryptochlorogenic acid (control $p=0.032$, W+OS $p=0.016$ and a trend after W+W $p=0.076$) and dicaffeoylspermidine (only after W+OS $p=0.032$).

Compared to pTV00 and *GA2ox3*, the *DELLA1* silenced plants showed no differences in the levels of rutin but compared to the control plants the rutin levels were already significantly reduced after W+W ($p=0.013$) while significant differences for pTV00 and *GA2ox3* were only seen between control plants and plants treated by W+OS (Figure 20). Also contradicting to pTV00 and *GA2ox3* the HGL-DTG levels in *DELLA1*-silenced plants were not suppressed after W+OS but no statistical evidence could be brought to cover this observation.

The plant genotype silenced in *GA2ox3* gene showed much less responses referring to the levels of secondary metabolites but still there were some increases in the concentration of chlorogenic acid (control $p=0.039$, W+OS $p=0.015$) and its isomer cryptochlorogenic acid (control $p=0.028$). The nicotine, rutin, dicaffeoylspermidine and 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) levels were statistically similar to the pTV00 plants (Figure 20) but rutin was significantly reduced in response to W+OS in the *GA2ox3* plants (compared to *GA2ox3* control plants).

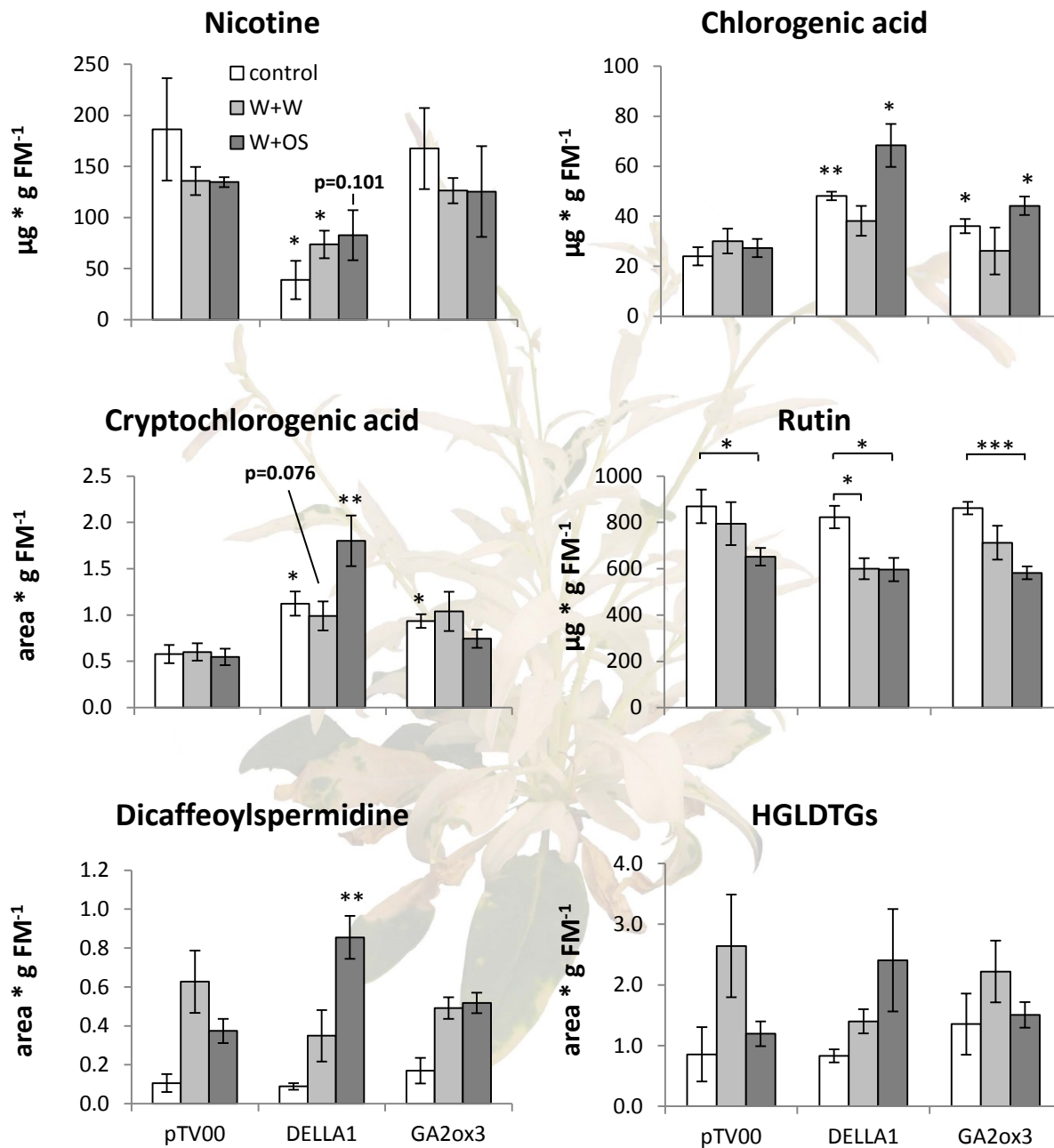


Figure 20: *DELLA1* and *GA2ox3*-silenced plants were altered in their secondary metabolite levels.

The secondary metabolite levels (local leaf) of the plants silenced in *DELLA1* and *GA2ox3* were compared to the levels of the empty vector plants (pTV00). Measurements were taken 48 h after wounding (using a pattern wheel) and subsequent application of water (W+W) or oral secretions of *M. sexta* (W+OS). The control leaves remained untreated. The measurements were performed during the elongation phase (50 days after seed germination). Black bars show the standard error, the stars above the level of significance calculated either by ANOVA or Mann-Whitney U-test (for heteroscedastic datasets). FM=fresh mass.

3.4.3 Volatile organic compounds

Like in the GA-overdose experiment the GLVs released after 1 h were too low in their concentration. The values were lower than 5000 counts * second, which we set as a baseline. Furthermore the blank levels were very high. In most cases (except nicotine) they were much higher than ten percent of the largest value, so that they were not taken into consideration (SCHUMAN M., personal information). Although there were some reductions of the emitted nicotine in *DELLA1*- and partly also in *GA2ox3*-silenced plants, nicotine was excluded from the analysis since *N. attenuata* plants have high levels of nicotine and the slightest injury of leaves or especially breaking some trichomes could have distorted the results. Therefore we could not rely on them (the nicotine emission is shown in the appendix, Figure 34). Because of the high blank levels the data are difficult to interpret. The values were summed up in the appendix (Table 3).

4 Discussion

4.1 *M. sexta* larval mass gain on plants with exogenous GA₃ treatment

In both larval performance experiments the larvae grew significantly larger on plants treated with 10 μ M GA₃ compared to the mock plants. From this result it can be concluded that gibberellins have an effect on larval growth on *M. sexta*. Also the average larval mass on control plants of about 300 mg after 12 days was similar to other studies (SON TRUONG *et al.*, 2013; YANG *et al.*, 2013) although there are also performance experiments showing much higher masses on mock plants (OH *et al.*, 2012; YANG *et al.*, 2012; HETTENHAUSEN *et al.*, 2013). Maybe the difference is caused by the disturbance of the larvae by the spray treatment, which was performed daily. Additionally, although very much diluted, all plants were sprayed with 0.035 % EtOH due to the insufficient solubility of gibberellic acid in water. Also the untreated mock plants had to be sprayed with EtOH to ensure the quality as a control plant and make them comparable with the plants treated with exogenous GA. It seems reasonable that the *M. sexta* caterpillars feeding on the plants ingested some of the ethanol. This could probably cause the lower weight compared to other studies. Our results are still interesting since all caterpillars were exposed to the same conditions except the treatment with GA.

One question arising from the experiment is how the larvae of *M. sexta*, which were cultivated since many years, perform in comparison to natural populations. The colony used for the performance experiments has their origin in the California State University and was established at the MPI for chemical ecology in 2002. Since its establishment the colony was occasionally inbred with colonies from other institutions (verbal information from KESSLER D., 2013). Recent investigations came to the result that all major colonies reared in the world derived from the same mass rearing facilities in Raleigh, North Carolina, established 1960 from field collections in Clayton, North Carolina (DIAMOND *et al.*, 2010). These colonies were reared and selected in laboratory environment for decades as well as kept on artificial diet and therefore the performance and the behavior might be different from natural populations. However, although there is no information about the behavior of *M. sexta* reared under laboratory conditions available yet, DIAMOND *et al.* (2010) tested the overall performance of *M. sexta* natural

populations versus laboratory colonies when placing them on natural host plants. They concluded that there are only slight differences between laboratory animals and animals of a natural population when it comes to performance and fitness although laboratory reared *M. sexta* animals showed slightly shorter developmental times and partly higher pupal masses. This means that the performance of the *M. sexta* used in the present study is probably not far apart from natural situations. The problem left is the behavior. The present study focused only on the question if or if not *M. sexta* caterpillars grow better on GA sprayed plants. If the larger mass gain on GA-sprayed plants is based on behavioral differences (e.g. reduced movement) requires further observation. However, one has to think about that when applying this data to natural situations. Lots of other factors beside the plant itself can influence the feeding behavior of *M. sexta* in the field. While it looks like plants with high doses of GA have ecological disadvantages compared to other plants (*M. sexta* ingests more of the plant within the same time span) there could be other factors like competitive herbivores also attracted by plants reduced in their defense responses which disturb the feeding behavior and the development of *M. sexta*. Therefore, from an ecological point of view, it is conceivable that the results might be different when compared to experiments, performed under natural environments.

4.2 Phytohormone levels remained similar in GA sprayed and VIGS plants

4.2.1 ABA levels were not affected by GA spray or silencing but generally increased in plants treated by spraying

Spraying plants of *N. attenuata* with GA did not alter the phytohormone levels compared to the mock plants but the spray treatment itself had great effects on the levels of abscisic acid. In other studies the concentrations of ABA in control plants were approximately between 120 and 400 ng/g FM (HETTENHAUSEN *et al.*, 2012; SON TRUONG *et al.*, 2013). The ABA concentrations measured in our study were much higher (approximately 1000 to 1200 ng/g FM). ABA is known to be involved into plant biotic and abiotic stress responses like drought stress response (LEUNG *et al.*, 2012). Beside this it regulates embryo and seed development, germination and vegetative development as well as general growth and reproduction (CUTLER *et al.*, 2010). The best studied function of ABA is its key role in regulating stomata closure (ROYCHOUDHURY *et al.*,

2013). High values of ABA measured during the GA overdose experiment might have been caused by spraying, leading to stomata closure. Compared to the GA overdose experiments the ABA levels of the VIGS plants with approximately 60-130 ng/g FM were much lower but the plants were not sprayed externally. Due to this it is difficult to compare the ABA levels of both experiments. Additionally the development stages of the plants were different too. While the plants of the GA overdose experiment were analyzed during early flowering stage, the VIGS plants were analyzed during the elongation stage. However, there was no significant change between the ABA levels of the empty vector plants and the plants silenced in *DELLA1* and *GA2ox3* gene. The success of the VIGS experiment has still to be proven by analyzing the transcript levels (silencing efficiency) of *DELLA1* and *GA2ox3*. If gene silencing can be confirmed, then it can be concluded that GAs probably do not affect the ABA levels of plants. On the other hand there is increasing evidence about a crosslink of ABA and GA, at least for early developmental stages of the plant. Already in the 1980ies researchers observed that the non-germinating *Arabidopsis* seedlings caused by GA deficiency partially germinated when crossed with ABA deficient mutants (KOORNNEEF *et al.*, 1982). Also other studies concluded that during imbibition and seed development of *Arabidopsis* seeds ABA is involved in the suppression of GA biosynthesis (SEO *et al.*, 2006). Furthermore, it is known that ABA-deficient mutants germinate, if the GA biosynthesis is inhibited by paclobutrazol or uniconazol treatments (NAMBARA *et al.*, 1991; NAMBARA *et al.*, 1998). It is thought that GRAS type proteins act as a linking hub (GOLLDACK *et al.*, 2013). In fact, recent studies by ARIIZUMI *et al.* (2013) found that in *Arabidopsis* DELLA proteins act as positive regulators of the RING-H2 zinc-finger protein XERICO, an inducer of ABA biosynthesis, leading to the accumulation of ABA and therefore inhibiting seed germination. These data demonstrate that GA also influences the ABA biosynthesis like ABA influences the GA biosynthesis. Taken these facts into account we expected that the ABA levels in *DELLA* silenced plants of *N. attenuata* would respond to the decreased presence of DELLA but the present results did not confirm this hypothesis. The levels within the *DELLA* silenced plant did not differ, as well as the levels of the *GA2ox3* mutants did not show differences. The unchanged levels in the *GA2ox3* mutants could be caused by feedback inhibition events as described in the introduction. In *DELLA1*-silenced plants another *DELLA* gene (*DELLA2*, which showed no

response to herbivory induction treatment, though) may have compensated for the defects of *DELLA1* in *N. attenuata*. At this point more research is necessary. However from the present results it must be concluded that silencing *DELLA1* and *GA2ox3* do not influence the ABA levels in *N. attenuata*.

4.2.2 JA and JA-Ile levels were not affected by GA spray and VIGS

The levels of JA and JA-Ile seemed to be increased especially 1 h after W+OS treatment although no statistical test was performed to prove this since it is already known that JA accumulates after wounding and perception of HAMPs (McCLOUD and BALDWIN, 1997; HALITSCHKE *et al.*, 2003; STENZEL *et al.*, 2003; HOWE and JANDER, 2008). The JA and JA-Ile levels of the -W+OS-induced GA overdose plants, which were about 2000-2500 ng/gFM and 150-200 ng/g FM, respectively, were similar to other studies (PASCHOLD *et al.*, 2008; DEMKURA *et al.*, 2010; YANG *et al.*, 2013). The levels of the VIGS plants with concentrations of about 800-1000 ng/g FM (W+W) and 3000-4000 ng/g FM (W+OS) were slightly higher although there are studies showing even higher (YANG *et al.*, 2011) or lower levels (MELDAU *et al.*, 2011; WUENSCHKE *et al.*, 2011) after induction. The fact that JA and JA-Ile concentrations are very low but increase dramatically within a short time after wounding and even stronger after wounding and treatments with oral secretions underlines the importance of JA in defense processes. However, the JA levels did not change in response to treatments with high concentrations of GA. GA is known to affect the JA-signaling it by degrading DELLA, which leads to the inhibition of the transcription of these genes since the transcriptional activator MYC2 is bound by JAZ (like explained in the introduction, Figure 2). But the transcription of *JAZ* and *MYC2* is feedback inhibited. MYC2 and JAZ proteins are JA response genes (WASTERNAK and HAUSE, 2013). The transcription (and subsequent translation) of e.g. *JAZ* leads to the accumulation of new JAZ repressors, which terminate the transcription process when sufficient concentrations are reached (KOMBRINK, 2012). The GA mediated degradation of the DELLA proteins in combination with the feedback regulation questions why the JA levels were normally increased after induction by simulated herbivory. A possible explanation is that JA is synthesized independently from the presence of GA. If GA would influence the biosynthesis of jasmonates, the levels of JA (and JA-Ile) would have been lower after induction compared to the control plants (mock and empty vector, respectively).

The same counts for the bioactive JA-isoleucine conjugate. JA-ile is crucial for the formation of the SCF^{COI1} complex acting as an E3 ubiquitin ligase, which is involved in the degradation of JAZ proteins by tagging it with ubiquitin (KOMBRINK, 2012; WASTERNAK and HAUSE, 2013). Subsequently, JAZ is degraded via the 26-S proteasome pathway. The role of JA-ile is to bind JAZ to the jasmonate receptor COI1 which is part of the SCF^{COI1} complex (KOMBRINK, 2012; WASTERNAK and HAUSE, 2013). But similar to JA, GA did not influence the levels of the bioactive JA-ile except that the levels of the untreated control plants silenced in *DELLA1* and *GA2ox3* were slightly higher. Taken together, these results suggest that GA probably has no direct influence on the biosynthesis of neither JA nor JA-ile.

4.3 Specificity in the effect of GA levels and signaling on secondary metabolites

4.3.1 Nicotine levels decreased in GA sprayed and *DELLA1* silenced plants

The nicotine levels in of the control leaves (about 400 µg/g FM) were similar between the mock plants and the plants treated with exogenous GA. These values are comparable to other studies (GILARDONI *et al.*, 2011; WUENSCHKE *et al.*, 2011). The levels in induced GA treated leaves remained close to the control leaves while the mock plants showed higher amounts of nicotine after induction (approximately 700 µg/g FM). It seemed like the nicotine production in response to induction was suppressed within the GA-treated plants. A study on plant defense metabolites in JA-deficient *lipoxigenase3* (*LOX3*)-silenced plants observed the same reduction of nicotine (HALITSCHKE and BALDWIN, 2003). These results are understandable since it is known that one protein mainly involved into the regulation of nicotine biosynthesis is NaMYC2 (WOLDEMARIAM *et al.*, 2013). As described in the introduction MYC2 is a keystone in the JA regulated defense response of *N. attenuata* (WOLDEMARIAM *et al.*, 2013), which binds competitively to the JA-ZIM domain protein if DELLA proteins are not bound to it. If DELLA proteins bind to JAZ, MYC2 is released from its bond and initiates the transcription of JA response genes (see introduction), including the genes involved in nicotine biosynthesis. A high amount of GA degrades DELLA proteins so that JAZ binds to MYC2 inhibiting the transcription of the nicotine biosynthesis. This is why the lower nicotine levels occurred not unexpected. On the other hand nicotine production is induced by JA via putrescine methyltransferase genes (WINZ and BALDWIN, 2001;

SAEDLER and BALDWIN, 2004) but interestingly no altered levels of either JA or its biologically active conjugate JA-Ile were detected. The influence of GA₃ must therefore have been downstream of JA and JA-Ile synthesis. WINZ and BALDWIN (2001) also concluded that the attack of *M. sexta* cause *N. attenuata* to produce the plant hormone ethylene which directly suppresses the biosynthesis of nicotine. This could be a reason for the low nicotine by unchanged JA levels. The cross-talk between GA and ethylene is already known and well-studied. In their review, SMALLE and VAN DER STRAETEN (1997) generally described the effect of ethylene as a growth inhibitor although they, on the other hand, mentioned the supportive role ethylene during seed germination. In *Arabidopsis*, the ethylene pathway influences the GA pathway by stabilizing the growth repressors GAI and RGA. Therefore it slows down the GA-induced degradation of these DELLA proteins (DUGARDEYN *et al.*, 2008). Due to time restrictions the ethylene levels could not be measured yet but it might be important to do this in future experiments. At this point it can only be stated that the treatment of *N. attenuata* plants with exogenous GA had an effect on nicotine production, possibly via increased ethylene production or via the degradation of DELLA proteins.

Also in the plants silenced in *DELLA1* the nicotine levels were significantly decreased compared to the empty vector plants (except in response to W+OS-treatment, which showed a trend though). This result was similar to the result of the GA-sprayed plants. The only difference was the reduced amount of nicotine in the control leaves, which could mean that the *DELLA1*-silenced plants had generally a lower nicotine production even without wounding treatments. Also the VIGS procedure itself seemed to affect the defense of the plants since the nicotine levels were lower in all VIGS plants, compared to the GA-overdose experiment.

4.3.2 GA spray partly influenced phenolamide levels

Although increased after W+W and W+OS treatment the DCS levels were significantly decreased in plants sprayed with exogenous GA independent from the treatment. Unfortunately, there were no standards to absolutely quantify the amounts of it but for the comparison between the treatments the measurement was sufficient enough. The CP levels increased after induction but no difference between GA-sprayed and mock plants was

observed. Both phenolamides are known to be JA-responsive metabolites and therefore follow the JA burst. This is because phenolamide levels in *N. attenuata* are controlled by the transcriptional activator MYB8. The activation of MYB8 in *N. attenuata* requires the activity of LOX3, which is involved into the biosynthesis of JA (ONKOKESUNG *et al.*, 2012). The JA-dependency of phenolamides could explain the increased amounts after induction (and the low amounts without induction, respectively) but since the GA treatment of the plants had no effect on the JA levels it is likely that there were also no increased phenolamide production when comparing mock and GA-treated plants. However, this was only observed for CP. ONKOKESUNG *et al.* (2012) showed that the levels of DCS are dependent on the developmental stage of the plant. While the concentrations are higher in young rosette stages, they decreased with continuing age but since in their study the expression patterns of CP were similar to DCS the stage of the plant cannot be a reason for the lower DCS levels. Additionally the mock plants and the plants treated with exogenous GA were in the same stage (early flowering). The differences in the CP and DCS levels could mean that GA probably has an influence on the biosynthesis of DCS (because the levels of all treatments were lower) but not on the biosynthesis of CP. We do not know how exactly the expression of these metabolites is influenced by GA. However, the lower DCS levels could be one reason for the higher caterpillar performance onto GA-treated plants since this effect has already been shown (KAUR *et al.*, 2010).

In contrast to the plants sprayed with exogenous GA the DCS levels of the *DELLA1*-silenced plants were significantly increased in leaves induced by oral secretions of *M. sexta*. Although the DCS levels were very low compared to the GA-overdose experiment (the concentrations were approximately 20 times lower) they were still detectable. It was interesting though, that no CP could be detected within the VIGS plants. This result is confusing since both phenolamides are regulated by JA and there was an increase in the JA levels at least after induction. The increasing DCS levels confirm the response of the phenolamides to increasing JA levels although this result is contradicting to the result of the GA-overdose experiment. In other studies CP was also found after the VIGS procedure, so that this cannot be an explanation for the low levels (ONKOKESUNG *et al.*, 2012; GAQUEREL *et al.*, 2013; SON TRUONG *et al.*, 2013). It can be

assumed that CP was produced by the plants but probably the levels were far too low for their detection via HPLC. Using more plant material or less extraction buffer might help to increase its detection. CP is also known to degrade quickly at room temperature. A longer storage of samples at RT might have limited its detection via HPLC-UV.

4.3.3 *DELLA1* silencing decreased the rutin levels after water treatment

In general the rutin levels of about 600-900 µg/g FM were comparable to another study with concentrations of about 700 µg/g FM (WOLDEMARIAM *et al.*, 2013) although the levels can also be lower in empty vector plants (YANG *et al.*, 2013). Interestingly, in the study of YANG *et al.* (2013) the rutin levels were induced after *M. sexta* herbivory while we found a reduction but different to our study they measured the levels after 15 days of continuous exposure to *M. sexta* while in our study herbivory was only simulated three times and secondary metabolites were measured after 48 h. However, in our study the only significant change was between the untreated control leaves and the W+OS leaves of the empty vector plants. The levels after W+W were slightly lower than the ones from the control leaves but still higher than the levels after W+OS. Statistical tests proved that there was no significance between them. In the *DELLA1* silenced plants the rutin levels after W+W were already knocked down to the levels after W+OS. The difference between control and W+W leaves was significant. It seemed like the rutin response after wounding alone was inhibited probably directly or indirectly by silencing the *DELLA1* gene which could mean that the rutin responses are not strongly connected to herbivory signals as they are to wounding alone. LOPEZ-GRESA *et al.* (2011) found that in tomato (*Solanum lycopersicum*) rutin (as well as chlorogenic acid) accumulated after bacterial infection with *Pseudomonas syringae* pv. *tomato*. This could lead to the hypothesis that rutin plays a more important role in the defense against pathogens than in the herbivory-induced defense. This would also explain the high levels after *M. sexta* herbivory observed by YANG *et al.* (2013) since the continuous feeding of the larvae injured the leaves and rutin probably accumulated as a defense against pathogens instead rather than a defense against *M. sexta*. Contrary to this hypothesis the rutin levels of the VIGS plants were lower than the ones from the GA overdose experiment. If rutin would play a great role in the bacterial defense, the levels of the VIGS plants should have been much higher since they were infected with *A. tumefaciens*. However,

from the present results it only can be suggested that rutin was not influenced by the application of exogenous GA.

4.3.4 *GA2ox3* and *DELLA1*-silencing increased chlorogenic acid levels

Both, the *DELLA1* and *GA2ox3*-silenced plants showed increased levels of chlorogenic acid. Interestingly, the levels were increased only in the control leaves and after W+OS-treatment. Wounding in combination with water did not show differences to the empty vector plants. The polyphenol chlorogenic acid is known to be an antioxidant, which is widely distributed among fruits and leaves. As such, it prevents biological molecules like proteins, DNA, RNA lipids and carbohydrates from damage caused by reactive oxygen species and reactive nitrogen species (Xu *et al.*, 2012). Beside this, chlorogenic acid and its isomers (including cryptochlorogenic acid) are known to have particular strong antimicrobial effects (ALMEIDA *et al.*, 2006) although this effect is controversially discussed in the literature (reviewed in LYON, 1989). Furthermore, a study investigating the effects of the tobacco rattle virus on potatoes found elevated levels of chlorogenic acid in TRV-infected plant material although it was not clear, if the accumulation was a result of the plants defense or a wound healing response (DALE *et al.*, 2000). This could explain the higher levels in the *DELLA1*- and *GA2ox3*-silenced plants, which could have been in response to the infection with *A. tumefaciens* or/and the TRV but if so, we would have expected similar levels in the empty vector plants and after W+W-treatment too. Interestingly this was not the case. However, although we cannot say what caused the elevated levels in the *DELLA1* and *GA2ox3*-silenced plants, there was definitely a response in chlorogenic acid and cryptochlorogenic acid. This response was probably not caused by accumulation of GA itself since the levels of the GA overdose experiment were similar between wild-type and GA-sprayed plants but probably by the interaction between GA pathway manipulation, herbivory and VIGS.

4.3.5 HGL-DTG levels decreased after W+OS induction in GA-sprayed plants

A study performed by HEILING *et al.* (2010) underlined the importance of HGL-DTGs as defense metabolite against herbivory attack of *M. sexta* on *N. attenuata*. In their caterpillar performance experiment the weight of *M. sexta* larvae feeding on plants silenced in geranylgeranyl diphosphate synthase (which lead to the reduction of precursors of HGL-DTGs)

was about 10 times larger compared to wild-type plants resulting in increased plant damage. Diterpene glycosides are known to be induced in response to increased jasmonate levels (KEINANEN *et al.*, 2001). Therefore we expected that the HGL-DTG levels were increased after the induction with oral secretions of *M. sexta* but lower within the plants treated with exogenous GA. However, while the levels were similar between mock and GA-sprayed plants they were not increased but instead significantly reduced after W+OS treatment. In *N. attenuata* four groups of HGL-DTGs can be found. These groups (precursors, core, malonylated and dimalonylated HGL-DTGs) are regulated in a different manner. The malonylation is the key biosynthetic step regulated by herbivory and jasmonate signaling (HEILING *et al.*, 2010). In response to herbivory the precursors, malonylated and dimalonylated groups are up-regulated while the core groups partly are negative regulated (HEILING *et al.*, 2010). We performed HPLC analysis to measure secondary metabolites. This method is not suitable to distinguish if the HGL-DTGs were precursors, core, malonylated or dimalonylated groups. Probably we measured the core groups, which are in fact down-regulated in response to herbivore attack since they become malonylated (HEILING *et al.*, 2010). To test this hypothesis we have to measure the samples again using mass spectrometry. Then we would be able to distinguish between the different HGL-DTG groups and could test if malonylated moieties change in GA-sprayed plants.

4.4 Some VOCs responded to GA-treatments and GA-related gene silencing

When it comes to indirect defense responses of plants against herbivores, some volatiles play very important roles, while others are less effective. Studies showed that volatiles like the sesquiterpene (E)- α -bergamotene and green leaf volatiles (fatty acid derived C₆ aldehydes, alcohols and esters) are involved in the attraction of *Geochoris* spp. predators (KESSLER and BALDWIN, 2001; HALITSCHKE *et al.*, 2008). It is also known that in *N. attenuata*, the attack by *M. sexta* decreases the ratio of (Z)/(E)-isomers of GLVs, resulting from the production of (E)-isomers from plant-derived (Z)-isomers. If *N. attenuata* is not attacked, (E)-isomers dominate the volatile household but within the next hours after herbivory attack the levels of (E)- and (Z)-isomers are nearly equal (ALLMANN and BALDWIN, 2010). While GLVs are immediately released from wounded leaves, terpenoids are emitted systemically and with a delay of some hours to days (DICKE, 2009). This is why they are probably involved in long-distance attraction of

carnivores (ALLMANN and BALDWIN, 2010). Therefore we took measurements after one hour and after 24 hours. However, the green leaf volatiles collected after one hour were too low concentrated and could be considered as background. Either this resulted from low concentrations within the plant leaves or the method used to measure the GLVs was not adjusted sufficiently. The GC-MS method used to analyze VOCs was still in development and the settings have to be improved. In general there were some considerations about the precision of the analysis. The VOCs were trapped with PDMS tubes for 1 h. The tubes had to be cut in pieces by hand using a cutting block. Although we took care that the pieces had approximately the same length, tiny differences in length could have influence on the total amount of VOCs trapped. The plastic cups used had two openings allowing a flow of air out of and into the cup. Especially inside the narrow VIGS chambers the VOCs of all plants are likely to emit into the air, mixing up with each other and therefore distorting the results. This could be seen on the partly high standard errors between the replicates of one treatment and the particular high blank values measured for the VIGS plants. Another possibility is that the lower temperature in the VIGS chamber (and therefore in the VIGS plants) inhibited the emission or evaporation ability of GLVs after wounding. After 24 h more VOCs were measured but only few showed significant differences between the plants compared. Nicotine was one of the VOCs showing partly reduced levels in the *DELLA1* and *GA2ox3*-silenced plants but this change was not taken into account since the slightest injury or breaking trichomes can cause additional emission of nicotine, distorting the results. However, a few changes were found. The terpenoids (putative) α -terpineol and (E)- α -bergamotene showed strong tendencies towards reduced levels after W+OS treatment of the GA-sprayed plants. Except these terpenoids the levels of the other VOCs were either not different or the blanks were too high, so that there was no significant difference to the control leaves anymore. These results suggest that GA might have an influence on the production of some terpenes. With higher sensitivity of the method, we will be able to determine the effect of GAs on GLVs.

4.5 General discussion and future perspectives

The present dataset was particularly comprehensible but although we expected similar behavior of phytohormones, volatiles and secondary metabolites between the GA-sprayed

plants and the VIGS plants silenced in *DELLA1* and *GA2ox3*, some data was partly contradicting to each other and did not match our expectations. This is why it seems necessary to analyze the transcript levels to ensure that the gene silencing was successful. Also the proteinase inhibitors could provide an answer for the heavier larval weight of *M. sexta* on GA-sprayed plants. We already have collected the plant material and will analyze the activity in future experiments.

In general the plants silenced in *GA2ox3* did not show any changes in their secondary metabolites except increased levels of chlorogenic acid in the control and W+OS leaves. This result is not surprising considering many studies which concluded that the GA biosynthesis is feedback regulated by bioactive GA itself (PHILLIPS *et al.*, 1995; AIT-ALI *et al.*, 1999; ASHIKARI *et al.*, 2002; AYELE *et al.*, 2006; GALLEG0-GIRALDO *et al.*, 2008). It is also possible that the degradation of bioactive GAs in *N. attenuata* is performed by additional enzymes and not only *GA2ox3*. GAMT-like enzymes could not be found within the genome of *N. attenuata* but probably other enzymes are involved as well. To get a clear picture it is necessary to analyze the transcript levels of genes involved into the biosynthesis of GAs as well as some GAs which could either be intermediates or degradation products of bioactive GAs or the level of bioactive GAs itself.

The caterpillar experiment, which was performed two times, showed that GA had a positive influence on *M. sexta*'s larval weight, even if the metabolomics analysis could only partly explain this difference. However, it is not clear yet that the higher larval masses on GA-sprayed plants are caused by decreased plant defense responses of *N. attenuata* or if bioactive gibberellins probably directly affect the growth of *M. sexta* caterpillars. In future experiments we will repeat the caterpillar performance using JA-deficient and wild-type plants. Because of the JA-deficiency, the caterpillars should grow larger on these plants if the difference of the weight is caused by reduced defense responses of *N. attenuata* (RAYAPURAM and BALDWIN, 2006). In a setup using two types of plants (wild-type and JA-deficient) and two treatments (control and GA sprayed) the results could look like stated in Figure 21. The caterpillars feeding on wild-type plants treated with exogenous GA could have approximately the same weight like the ones feeding on JA deficient plants and a significant increase compared to the untreated wild-type plants. If the weight of the caterpillars feeding on GA treated wild-type, JA-deficient control and

GA-treated JA-deficient plants is similar, it can be concluded that gibberellins knockdown the defense responses (at least the ones important for inhibiting *M. sexta* growth) to the level of JA-deficient plants (Figure 21 a). If the caterpillar weight differs only between the untreated

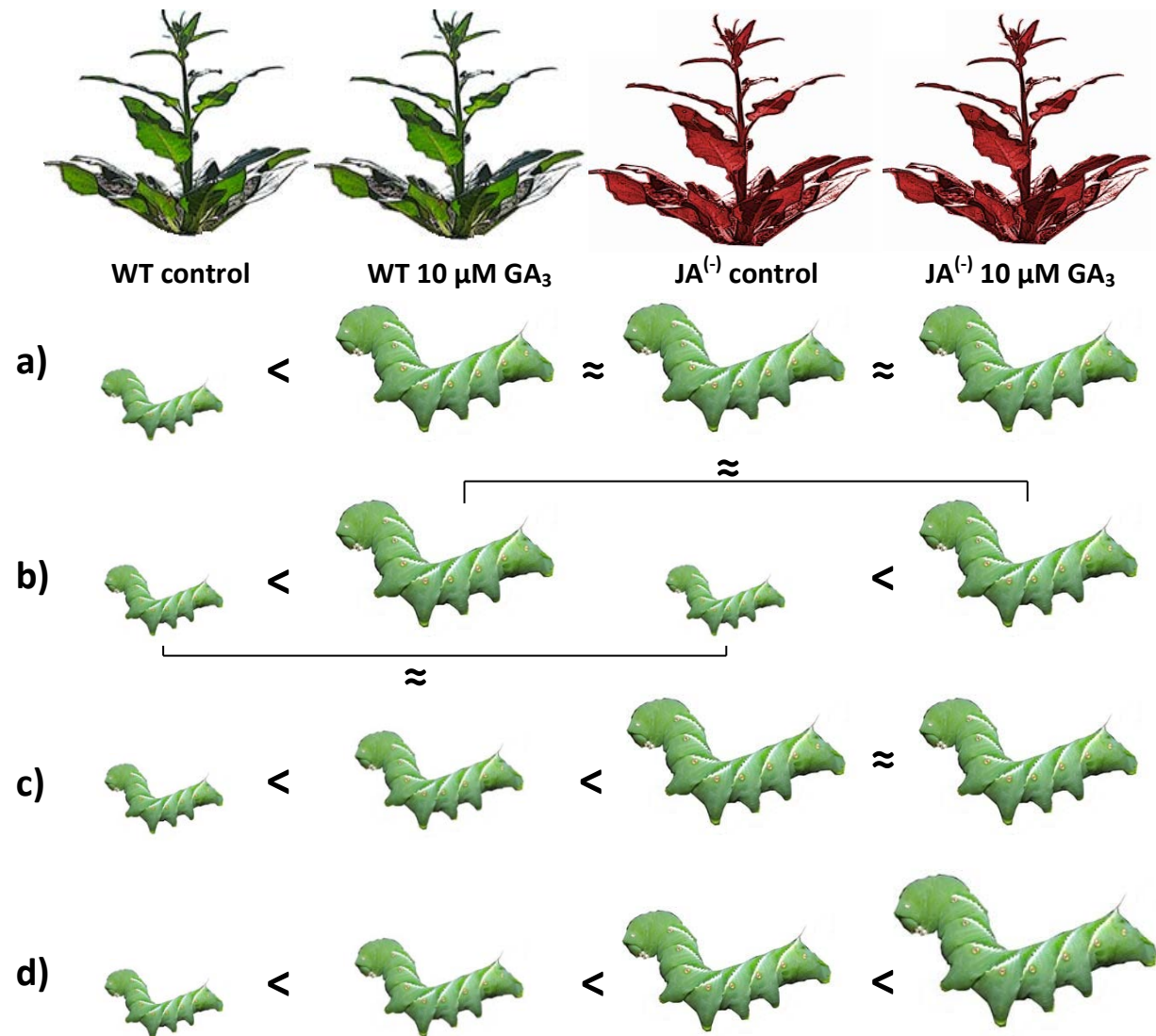


Figure 21: Possible outcomes of a caterpillar performance with wild-type (WT) and JA-deficient (JA⁻) lines.

Suggestions could be following: **a)** Spraying wild-type plants with exogenous GA knocks down the plants defense to the level of JA-deficient plants. This is why *M. sexta* larvae grow as large on GA treated wild-type plants as on JA-deficient plants while there is no difference between the treatments of the JA-deficient plants. **b)** All caterpillars feeding on GA sprayed plants grow larger but there is no difference between wild-type and JA-deficient plants. Spraying exogenous GA directly affects the caterpillar growth of *M. sexta*. **c)** The caterpillars feeding on GA-treated wild-type plants grow larger compared to the wild-type control but smaller compared to the caterpillars feeding on JA-deficient plants. This could mean spraying wild-type plants with exogenous GA reduces the plants defense but this reduction is less strong than in JA-deficient plants. **d)** Although the caterpillars feeding on GA-treated plants are larger compared to the controls they grow generally larger on JA-deficient plants but with the same effect of GA-treatment. From this it could be concluded that spraying wild-type plants with exogenous GA reduces their defense but additionally GA directly affects the growth of *M. sexta* caterpillars.

plants and the plants treated with GA but not between the JA-deficient lines and the wild-type plants, the effect of increased larvae growth is probably caused by gibberellins itself (Figure 21 b). If the caterpillars feeding on JA-deficient plants show similar, the ones feeding on GA sprayed wild-type plants slightly less and the ones feeding on the untreated wild-type plants the least weight, then it could also be a hint for a reduction of defense responses although this reduction would be less intense than in plants with JA-deficiency (Figure 21 c). Finally, if the caterpillars feeding on JA-deficient GA-treated plants grow largest, the ones feeding JA-deficient control plants second largest, the ones feeding on the GA-treated wild-type plants even smaller and the ones feeding on control wild-type plants smallest, then it could be a hint for an effect of reduced defense responses and additionally a direct effect of the GA-treatment in combination (Figure 21 d). It also seems reasonable to investigate how caterpillars perform onto the VIGS plants. We expect that *M. sexta* larvae will grow larger on plants silenced in *DELLA1* since this phenotype should be similar to GA-overexpression phenotypes.

As mentioned in the introduction, the production of defense metabolites is costly and often accompanied by growth inhibition (POVEDA *et al.*, 2003; ZHANG and TURNER, 2008). Therefore it was expected that some secondary metabolites in *N. attenuata*, like nicotine, CA, CP or DCS, were induced after simulated *M. sexta* herbivore attack. The best example is the secondary metabolite CP, which levels showed a tenfold increase after wounding and application of *M. sexta* oral secretions, compared to the untreated leaves. These results underpin the optimal defense hypotheses stated by MCKEY (1974). Although we did not focus on different plant tissues of *N. attenuata* (e.g. leaves compared to flowers or apical shoots), we were able to show that not all defense metabolites (including VOCs) were constitutively expressed among the plant. Rather than that, *N. attenuata* responded to the attack of *M. sexta* by inducing the production of defense metabolites. As described above many of *N. attenuata*'s "weapons" are regulated by the JA pathway. The analysis of the phytohormones showed a strong burst of JA and its conjugate JA-Ile already one hour after the treatments. We were also able to show, that GAs suppressed some of these weapons although others were not affected. During the GA-overdose experiment we observed the influence of exogenous applied GA on *N. attenuata*'s growth behavior. *N. attenuata* plants treated with exogenous GA were averagely about 10 cm

taller, compared to the mock plants (Figure 22, appendix Figure 23). Interestingly also the leaf-stem angle of the rosette leaves was smaller, meaning that the plants sprayed with GA “lifted” their rosette leaves away from the ground (appendix Table 2).



Figure 22: *N. attenuata* plants treated with GA were higher and showed elevated rosette leaves.

N. attenuata plants were daily treated with 10 μ M GA₃ (dissolved in 0.035 % EtOH, labeled red) during their elongation phase (start: 34 days after seed germination) and compared to mock plants (only sprayed with 0.035 % EtOH, labeled green). The picture and measurements (plant height, leaf-stem-angle) were taken during early flowering stage after 18 days of GA₃-treatment (52 days after seed germination).

These measurements show that GA had an influence on *N. attenuata*'s growth behavior. The observation, that elevated GA levels increase the height of the plant fits to the observation described by ANDERSON et al. (2004) but, as far as we know, the differences in the angle of the rosette leaves were not described before. Probably this effect is specific to *N. attenuata*.

5 Conclusion

The caterpillar performance showed that there was an influence of bioactive GA on *N. attenuata*'s defense against *M. sexta*. This was indicated by the larger larval mass of the caterpillars feeding on GA-treated plants. We also found some changes in the metabolomic household of *N. attenuata*. The most important difference we found is the reduced amount of nicotine in plants with GA-overdose treatments, which is likely to have been the main cause of the reduced growth of the caterpillars on the nicotine-rich mock plants since this result was already suggested by another study on *Nicotiana sylvestris* (VOELCKEL *et al.*, 2001). Although nicotine is a toxic alkaloid, *M. sexta* can tolerate it. But although they can tolerate higher doses of nicotine they grow less intense, from which VOELCKEL *et al.* (2001) concluded that nicotine probably decreases the palatability of the leaves. DicaFFEoylspermidine could have been another reason for the increased larval mass on GA-treated plants but since the *DELLA1*-silenced plants showed contradictory results we have to do further analysis to confirm this. All the other metabolites were not affected by GA. Although some tendencies towards reduced levels of at least α -terpineol and (E)- α -bergamotene were detected 24 h after the last wounding treatment we could not find any greater changes of the VOCs.

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7 Appendix

7.1 Additional measurements

7.1.1 Differences in plant height

The plants sprayed with GA showed significantly increased heights. Compared to the mock plants with an average size of 83.4 cm, the GA sprayed plants with an average size of 94.7 cm were approximately 10 cm taller. Student's t-test revealed a high significance level of $p=1*10^{-8}$ (Figure 23).

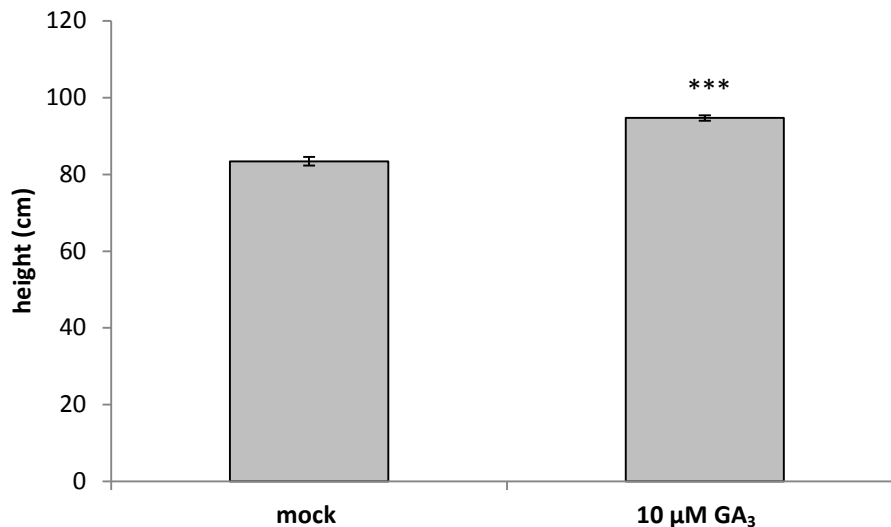


Figure 23: Spraying *N. attenuata* plants with 10 μM GA₃ (in 0.035 % EtOH) increased their height.

After 18 days of GA-treatment, the overall plant size of the GA-treated *N. attenuata* plants was higher compared to the control plants only treated with 0.035 % EtOH (mock). The measurements were performed with a folding ruler. The plants were measured from the surface of the soil to the uppermost tip. The black bars show the standard error, the stars above the level of significance calculated with Student's t-test. The number of replicates was n=29 (15 mock and 14 GA₃ sprayed plants).

7.1.2 Differences in the angle of the rosette leaves

The rosette leaves of all sprayed plants seemed to be steeper than unsprayed plants but there were also significant differences between the plants treated with GA and the mock plants. The average horizontal angle of the rosette leaves was significant larger and the leaf-stem angle (minimum and maximum) significant smaller for the plants sprayed with GA (Table 2). The

Student's t-test resulted in significant differences between the minimum horizontal leaf angel of the control and the GA treated plants ($p=5.6*10^{-6}$), as well as between the maximum horizontal leaf angle of the mock and the GA treated plants ($p=2.9*10^{-6}$). There were also significant differences between the minimal and maximal leaf-stem angles of the mock and the GA treated plants ($p=6.65*10^{-4}$ and $p=8.19*10^{-4}$ respectively).

Table 2: Measurements of the leaf-stem angle of *N. attenuata* plants.

The leaf-stem angles (measured with a goniometer) of the plants sprayed with 10 μ M GA₃ (in 0.035 % EtOH) were significantly lower compared the control plants only treated with 0.035 % EtOH (mock). For the t-test after Student the fields of the same color were compared. Before comparing the means, the data was tested for its distribution with Kolmogorov-Smirnov (KS) test. The 2-tailed asymptotic significance is abbreviated with "as Sig" and the distribution with "distr".

leaf-stem angle (°)					
Plant number	mock		10 μ M GA ₃		
	min.	max.	min.	max.	
1	50	92	19	54	
2	48	64	30	63	
3	65	80	25	71	
4	55	73	43	70	
5	47	58	36	74	
6	29	76	38	58	
7	48	75	38	57	
8	34	80	45	55	
9	44	79	45	76	
10	50	83	38	67	
11	48	80	29	70	
12	39	88	44	71	
13	55	81	35	75	
14	60	79	45	61	
15	50	81	31	78	
average	48.1	77.9	36.1	66.7	
standard error	2.4	2.2	2.1	2.1	
KS-Test (as. Sig.)	0.69	0.48	0.93	0.63	
KS-Test (distr.)	normal	normal	normal	normal	
T-test			5.31E-03	5.52E-03	

7.2 Preparation of the VIGS construct

7.2.1 Amplification of the partial target gene

After the PCR with partial target sequence specific primers (Table 1) the gel electrophoresis showed bands around 300 bp for each product (Figure 24). The annealing temperatures showing the strongest bands were 64.2 °C for *DELLA1*, 63,1 °C for *GA2ox3* and 64,2 °C for *GID1a*. The varying temperatures were applied to ensure the presence of a suitable product.

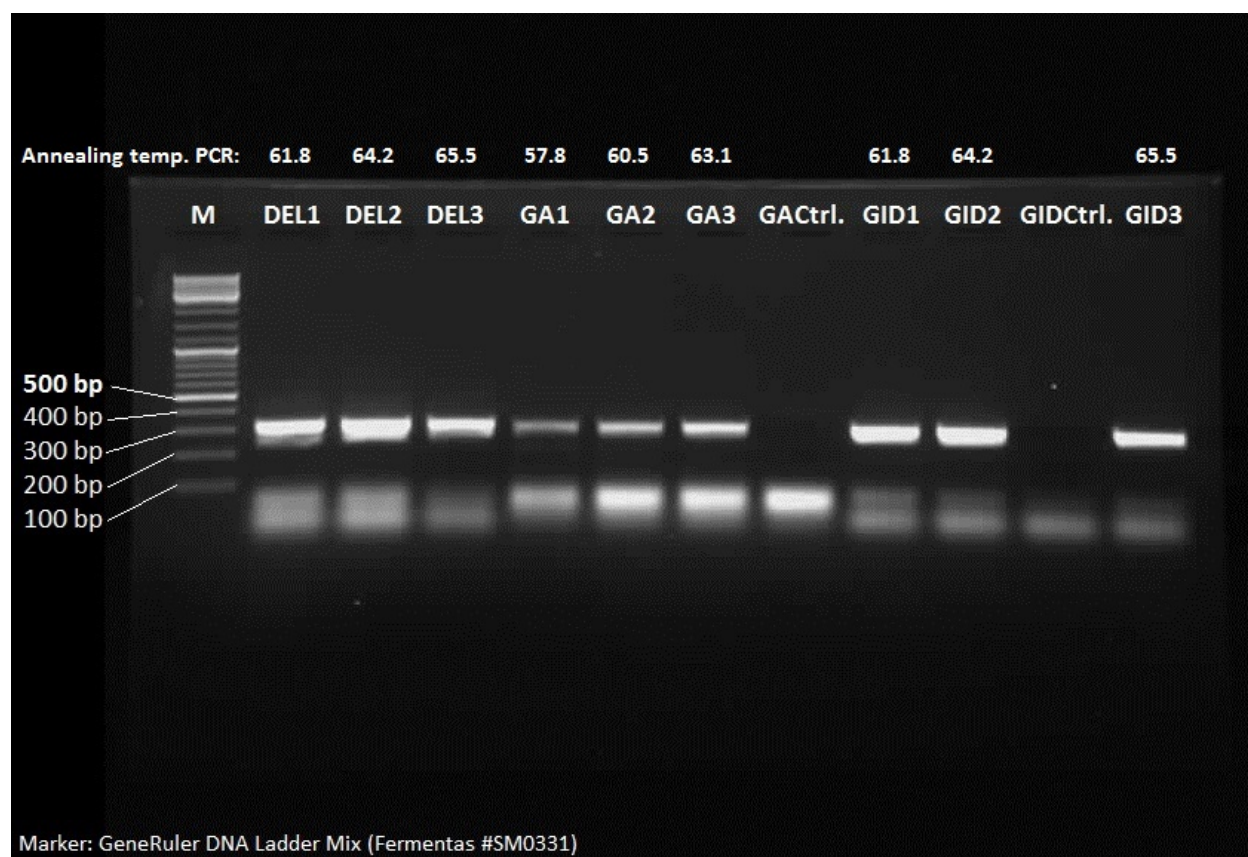


Figure 24: Gel electrophoresis after the PCR of *N. attenuata* cDNA

The primers used were specific to the partial sequence of the target gene. The strongest bands (around 300 bp) showed up at annealing temperatures of 64.2 °C for *DELLA1* (*DEL*), 63.1 °C for *GA2ox3* (*GA*) and 64.2 °C for *GID1a* (*GID*).

The extraction of the PCR product from the gel was successful. The second gel electrophoresis using 1 µL of the extracted DNA also showed the target genes at 300 bp. The concentration of the partial target DNA for *GA2ox* was lower than the ones from *DELLA1* and *GID1a* (Figure 25) but still adequate enough to continue preparing the construct.

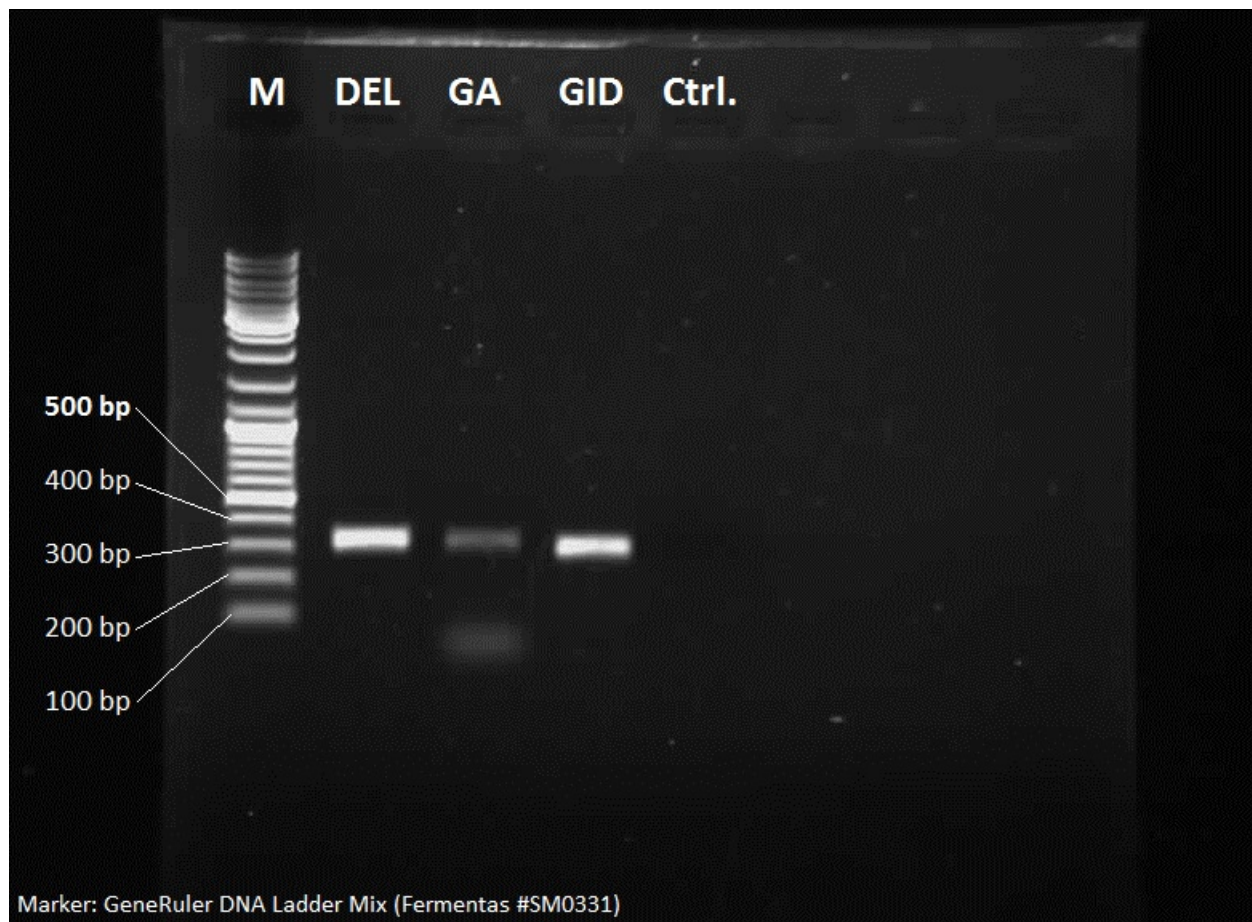


Figure 25: Gel electrophoresis of the extracted PCR products.

The products for each partial target gene incubated at varying temperatures and extracted from the gel (Figure 24) were combined (e.g. *DEL* = *DEL1*, *DEL2* and *DEL3*).

7.2.2 Restriction digestion analysis

The restriction digestion performed for each target cut the ends of the target DNA insert in order to prepare them for the ligation with the vector PTV Lox 6/1. To make sure that the restriction enzymes cut at the correct position a gel electrophoresis was performed. The product showed still bands around 300 bp, the restriction digestion worked fine (Figure 26).

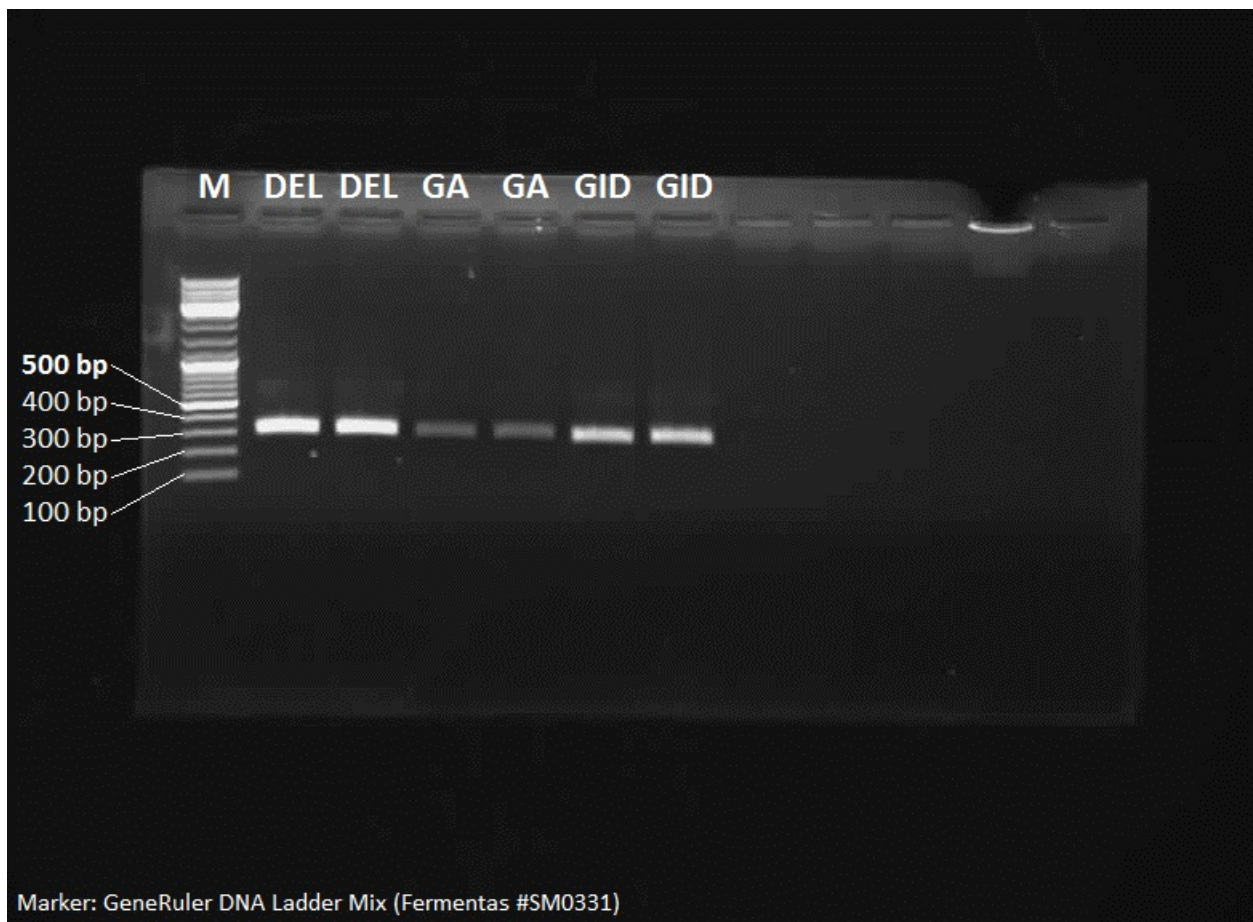


Figure 26: Gel electrophoresis after restriction digestion of the extracted target DNA.

For all target genes products showed up around 300 bp. The products after the extraction were divided into two gel bags. Therefore there are two bands for each target.

After isolating and combining the target products from the gel, another extraction was performed and the presence of the product verified via gel electrophoresis. Again the product was detectable at a length of approximately 300 bp (Figure 27).

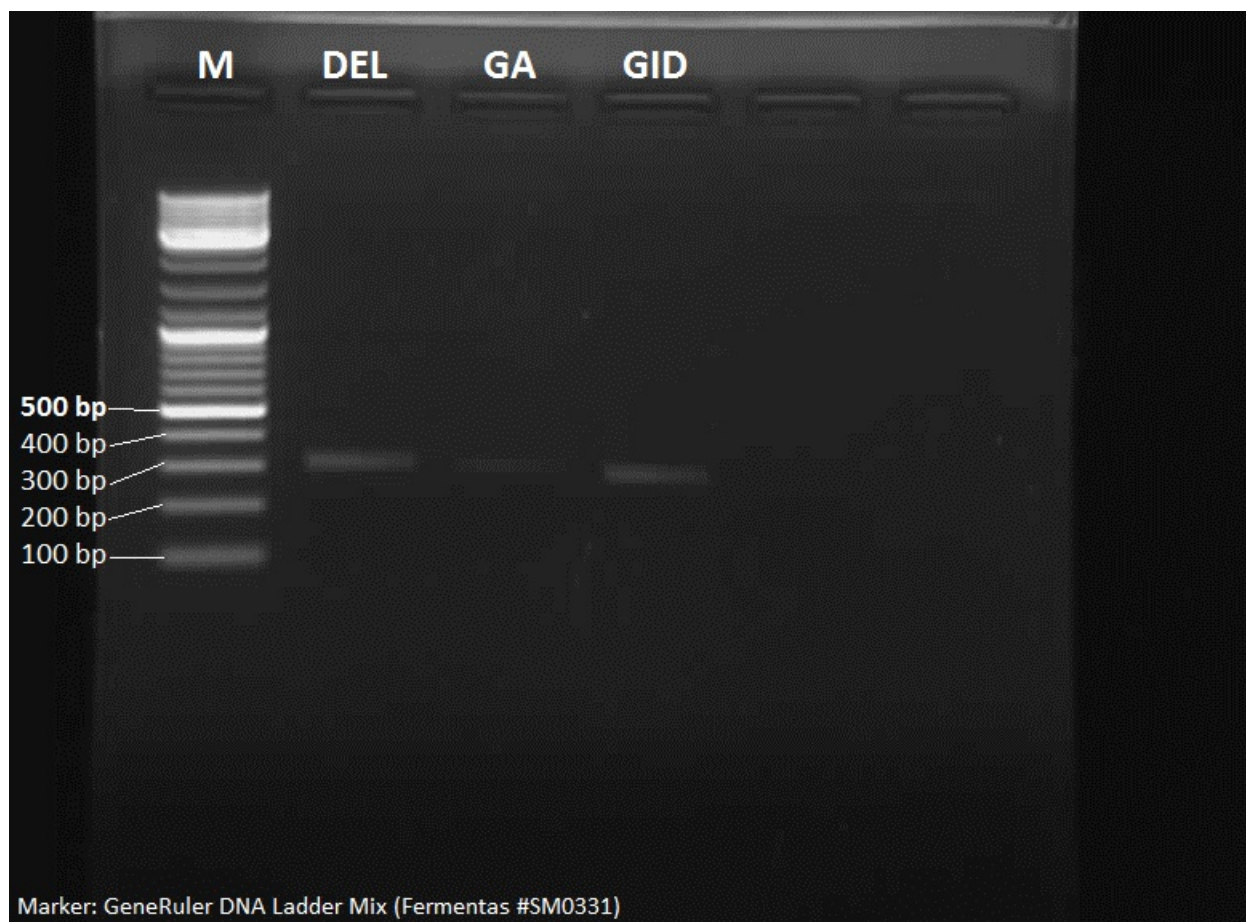


Figure 27: Gel electrophoresis of the digested target DNA.

The gel electrophoresis was performed after extracting the target DNA of the digested product from the former gel electrophoresis (Figure 26). The volume of the analyzed product was 1 μ L.

7.2.3 Isolation of the target plasmids from *E. coli* after the transformation

The ligation of the target DNAs with the vector PTV Lox 6/1 and the subsequent transformation of the plasmids into *E. coli* were successful. The gel electrophoresis performed after ligation, transformation, plasmid extraction and subsequent restriction digestion showed the target DNA fragments (at ca. 300 bp) and the vector PTV Lox 6/1 (at ca. 7000 bp) for GA2ox3 (Figure 28, Figure 29), DELLA1 (Figure 30, Figure 31) and GID1a (Figure 32, Figure 33) in a bands around 7 kbp. All three ligations and transformations performed well. The sequencing of the plasmids confirmed the presence of the target genes for *GA2ox3* and *DELLA1* but not for *GID1a*. After several unsuccessful attempts it was decided to only silence the genes *GA2ox3* and *DELLA1*.

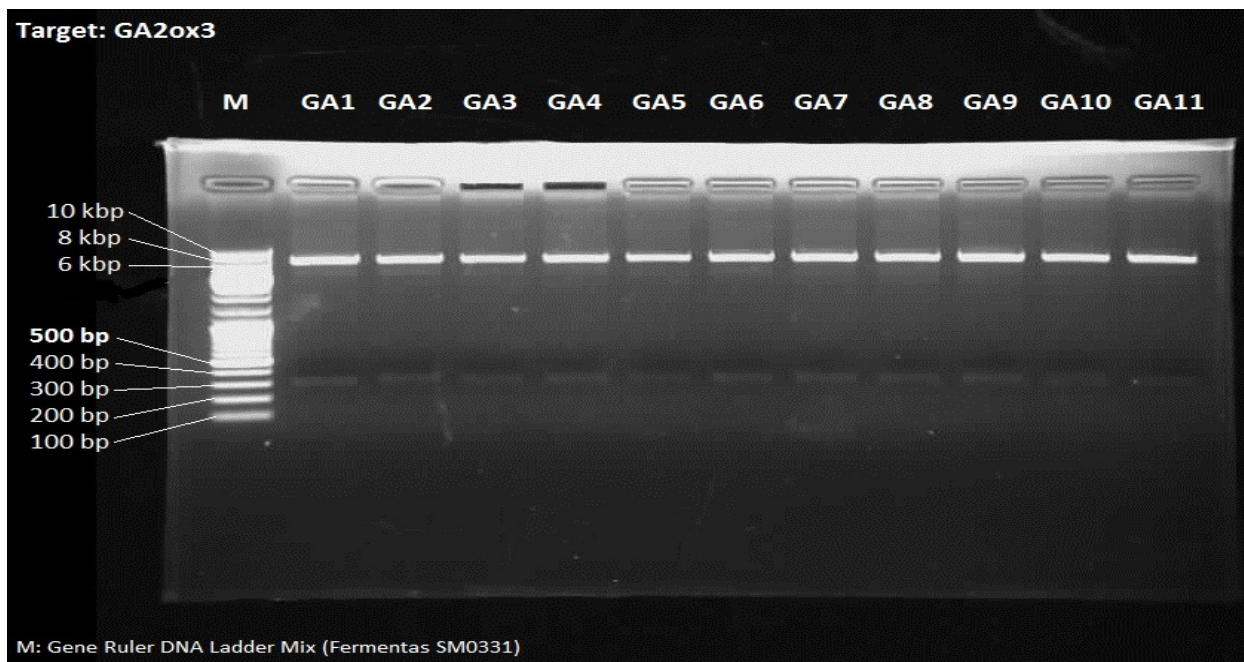


Figure 28: Gel electrophoresis performed after plasmid extraction and restriction digestion.

The plasmids of the pure colonies received after transformation of the *GA2ox3* plasmid were extracted and subsequently digested plasmid with Bam HI and Sal I. The vector could be seen at around 7000 bp and the target DNA at around 300 bp. The presence of the target DNA in the digested plasmid was taken as an evidence for successful ligation and transformation.

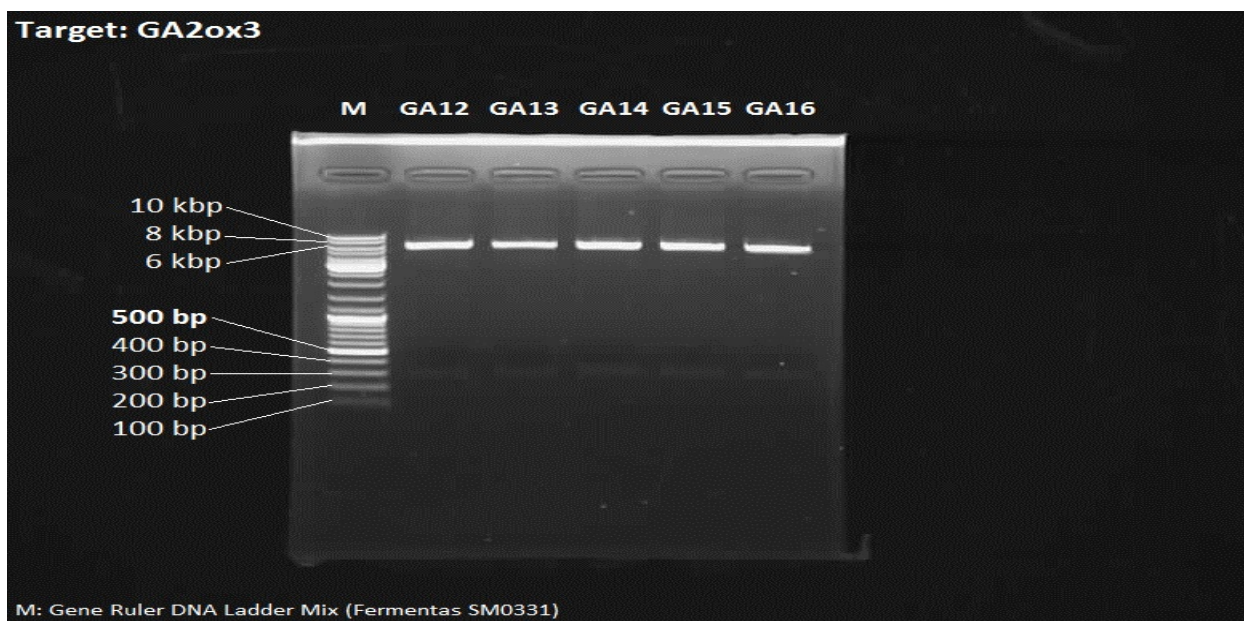


Figure 29: Gel electrophoresis performed after plasmid extraction and restriction digestion.

The plasmids of the pure colonies received after transformation of the *GA2ox3* plasmid were extracted and subsequently digested plasmid with Bam HI and Sal I. The vector could be seen at around 7000 bp and the target DNA at around 300 bp. The presence of the target DNA in the digested plasmid was taken as an evidence for successful ligation and transformation.

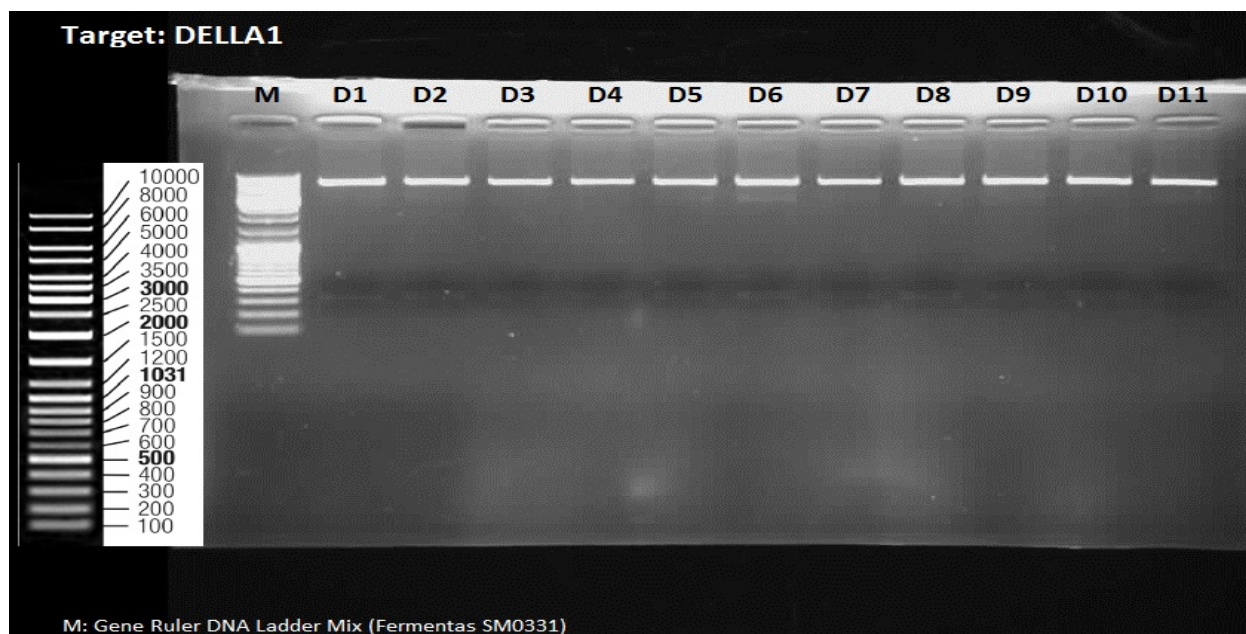


Figure 30: Gel electrophoresis performed after plasmid extraction and restriction digestion.

The plasmids of the pure colonies received after transformation of the *DELLA1* plasmid were extracted and subsequently digested plasmid with Bam HI and Sal I. The vector could be seen at around 7000 bp and the target DNA at around 300 bp. The presence of the target DNA in the digested plasmid was taken as an evidence for successful ligation and transformation.

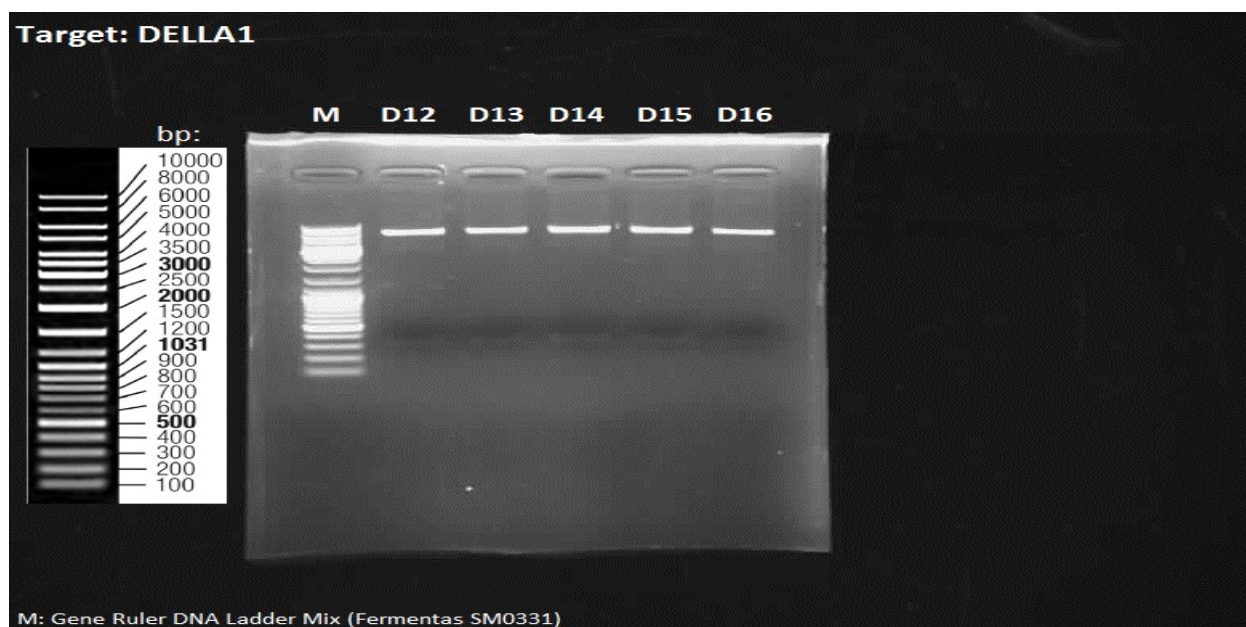


Figure 31: Gel electrophoresis performed after plasmid extraction and restriction digestion.

The plasmids of the pure colonies received after transformation of the *DELLA1* plasmid were extracted and subsequently digested plasmid with Bam HI and Sal I. The vector could be seen at around 7000 bp and the target DNA at around 300 bp. The presence of the target DNA in the digested plasmid was taken as an evidence for successful ligation and transformation.

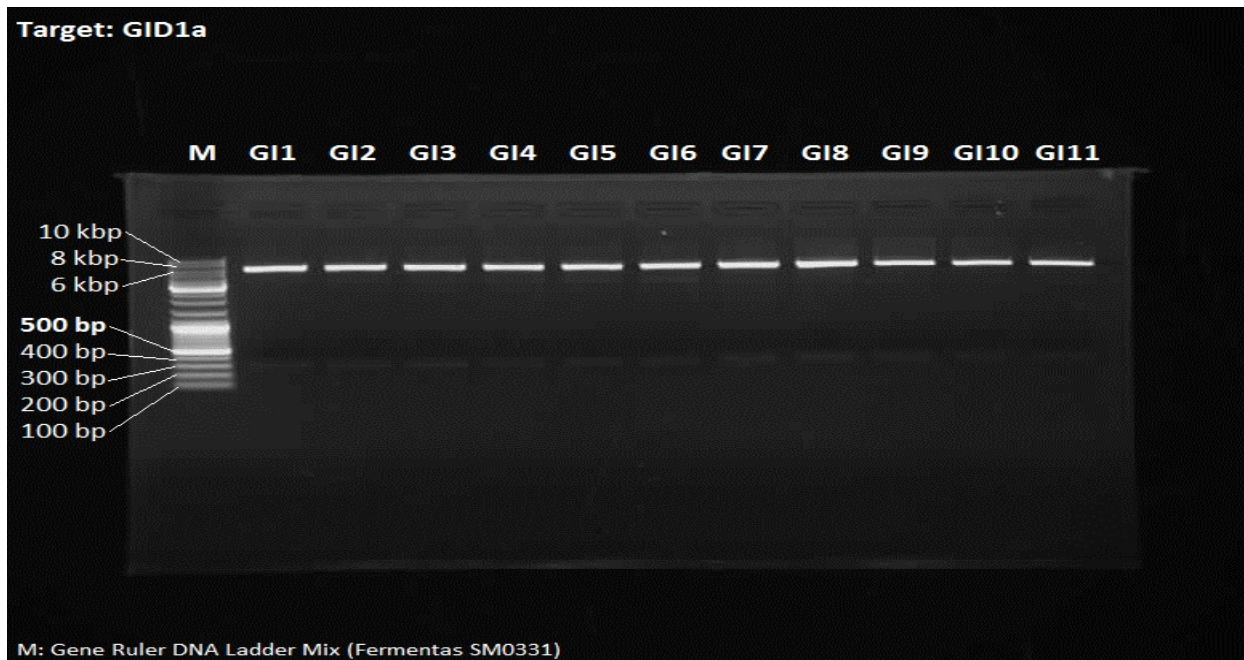


Figure 32: Gel electrophoresis performed after plasmid extraction and restriction digestion.

The plasmids of the pure colonies received after transformation of the *GID1a* plasmid were extracted and subsequently digested plasmid with Bam HI and Sal I. The vector could be seen at around 7000 bp and the target DNA at around 300 bp. The presence of the target DNA in the digested plasmid was taken as an evidence for successful ligation and transformation.



Figure 33: Gel electrophoresis performed after plasmid extraction and restriction digestion.

The plasmids of the pure colonies received after transformation of the *GID1a* plasmid were extracted and subsequently digested plasmid with Bam HI and Sal I. The vector could be seen at around 7000 bp and the target DNA at around 300 bp. The presence of the target DNA in the digested plasmid was taken as an evidence for successful ligation and transformation.

7.3 Volatile organic compounds

Table 3: Relative emission of volatile organic compounds (VOCs).

The upper part of the table contains the average values adjusted to the leaf surface and the lower part the standard errors. Only few VOCs showed differences between the plants sprayed with GA₃ (dissolved in 0.035% EtOH) and the mock plants (only sprayed with 0.035% EtOH) as well as between the VIGS plants. The samples were measured with SHIMADZU GCMS – QP2010 Ultra Thermo desorption unit TD20. The fields marked with “-” did not have the required number of three replicates with values larger than 5000 counts (which were considered not to be background noise).

Volatile Organic Compound	relative emmision (counts * second) / cm ²																	
	control						W+W					W+OS					blank	blank
	mock	GA	pTV00	DELLA	GA2ox3		mock	GA	pTV00	DELLA	GA2ox3	mock	GA	pTV00	DELLA	GA2ox3	VIGS	GA
(E)-alpha-Bergamotene	360	452	7088	10234	8763		1712	1165	10945	10316	10699	25320	10135	9093	14899	11071	7476	181
3(Z)-Hexenyl butanoate	2144	934	1666	1985	1417		6229	6021	1897	2689	2310	7579	5501	-	2306	2393	1049	-
3(Z)-Hexenyl isobutyrate	1207	917	929	1050	833		2931	3113	1029	1258	1145	2267	2260	-	1458	1294	-	205
5-epi-aristolochene	-	-	27022	39872	31357		222	-	34193	42220	38369	3209	4024	31946	43292	48130	28585	-
alpha-Duprezianene	1484	1557	13916	17339	18177		2205	1558	18190	16986	18299	1578	1375	14193	18681	18702	11810	771
Benzyl acetone	20629	22565	-	6949	-		19885	20062	-	-	-	26614	21242	-	-	-	-	19298
MW 220	2686	3477	38070	50326	46815		6515	3745	52719	44828	48545	4166	2795	37263	51914	53955	32113	1453
Nicotine	101790	132587	44594	13200	67157		65539	63039	39802	23208	34122	90430	59350	74633	26003	37047	2424	-
PUTATIVE 3(Z)-Hexenyl (E)-2-methylbut-2-enoate	2488	866	2326	-	2456		4147	4405	-	-	-	4495	3270	2098	3631	3538	2390	-
PUTATIVE 3(Z)-Hexenyl caproate	2102	1433	-	531	-		4537	3859	911	-	-	2695	2387	870	-	-	589	-
PUTATIVE 3(Z)-Hexenyl isovalerate	5530	1573	-	-	-		8270	10399	-	-	-	5155	4502	-	-	-	-	883
PUTATIVE alpha-Terpineol	1562	1157	3037	4017	3468		2377	1437	3771	4843	4378	4042	1412	3172	4297	4210	2910	208
PUTATIVE GLV ester RT:29.143	916	988	9520	12881	12456		1625	1131	13750	11784	12599	1298	878	9876	13455	14094	8616	-
PUTATIVE sesquiterpene RT:26.390	-	-	3301	5436	3826		-	-	4064	5117	4604	-	-	3731	4841	5728	3433	-
Tetradecenal	366	433	6330	9217	4782		345	398	5650	8541	4790	507	315	5834	5653	8325	5457	297
standard errors																		
(E)-alpha-Bergamotene	7	84	781	538	666		713	219	1706	375	717	5416	2467	543	2914	2702	142	7
3(Z)-Hexenyl butanoate	421	108	215	82	111		2160	1356	271	325	233	1047	1200	-	797	351	185	-
3(Z)-Hexenyl isobutyrate	258	172	131	152	152		947	613	108	231	99	300	266	-	175	76	-	20
5-epi-aristolochene	-	-	2973	2866	2424		50	-	2956	7418	2299	729	1744	938	4238	4392	1030	-
alpha-Duprezianene	183	194	854	959	1097		260	97	1316	441	1680	62	172	546	1491	1575	298	31
Benzyl acetone	2167	2009	-	441	-		1433	2960	-	-	-	2470	2532	-	-	-	-	1955
MW 220	138	564	2655	4088	3777		1020	297	3583	1148	3782	553	369	2173	5268	3342	1481	44
Nicotine	48983	50030	6963	4192	16252		23378	9296	6732	6675	6192	21947	16683	12345	2192	4952	656	-
PUTATIVE 3(Z)-Hexenyl (E)-2-methylbut-2-enoate	558	147	455	-	251		1450	885	-	-	-	1083	588	311	663	288	280	-
PUTATIVE 3(Z)-Hexenyl caproate	497	398	-	33	-		934	409	266	-	-	608	515	255	-	-	72	-
PUTATIVE 3(Z)-Hexenyl isovalerate	1525	366	-	-	-		3256	2968	-	-	-	677	623	-	-	-	-	121
PUTATIVE alpha-Terpineol	337	172	277	248	361		843	239	316	747	154	964	253	210	501	217	145	13
PUTATIVE GLV ester RT:29.143	155	174	756	859	883		253	91	762	459	784	197	261	535	1105	989	351	-
PUTATIVE sesquiterpene RT:26.390	-	-	453	554	362		-	-	295	882	359	-	-	249	729	385	174	-
Tetradecenal	43	74	1299	1246	1053		23	53	1971	1693	1149	51	61	327	1361	1797	201	40

Relating to the VIGS experiment, the only VOC showing blanks with values smaller than 10 % of the highest value was nicotine but nicotine was not taken into consideration since the smallest injury of the leaf or broken trichomes during the collection of the VOCs could have influenced the nicotine emission. Therefore it is only shown here in the appendix.

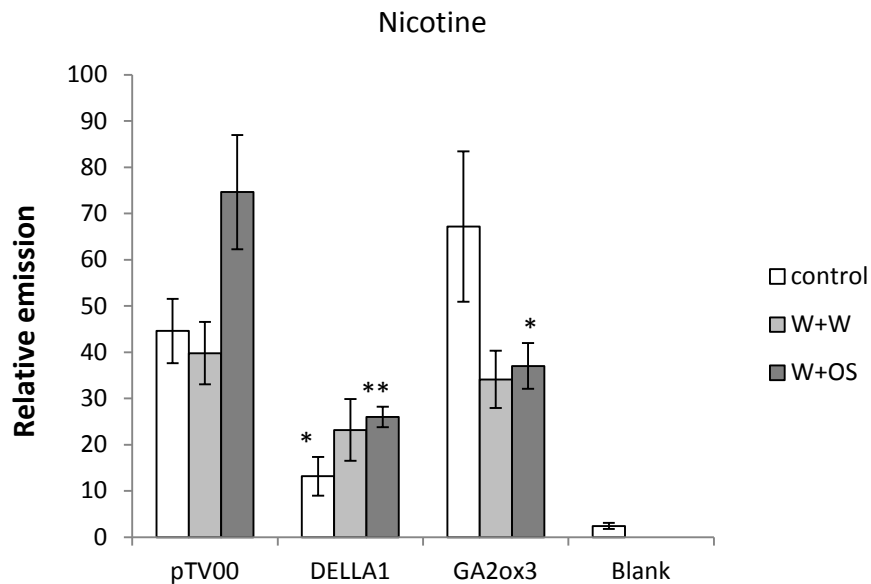


Figure 34: Emission of nicotine measured in the VIGS plants.

Nicotine as a VOC was not taken into consideration since the slightest injury of the leaves or trichomes would lead to distorted values.