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The role of a branched-chain α-keto acid dehydrogenase-encoding gene in *O*-acyl sugar biosynthesis and defense of wild tobacco (*N. attenuata)* against native herbivores and pathogens

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Table of contents

I List of tables

II List of figures

III List of abbreviations

^{32}P-dCTP	deoxycytidine-triphosphate with radioisotope of phosphor
ASH	acyl sugar hydrolase
AT	acyl transferase
BCKD	Branched-Chain α-Keto acid Dehydrogenase
BCKDE1B	β -subunit of the E1 complex of Branched-Chain α -Keto acid Dehydrogenase
Bp	base pairs
cDNA	complementary deoxyribonucleic acid
CoA	coenzyme A
CTAB	cetyl trimethylammonium bromide
DCM	dichloromethane
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleotide triphosphate

EDTA	ethylenediaminetetraacetic acid
GFP	Green Fluorescent Protein
GUS	β-Glucuronidase
HPLC	High Performance Liquid Chromatography
Kb	kilo base pairs
LC	Liquid Chromatography
Mikro-TOF	Mikro-Time-Of-Flight
NCBI	National Center for Biotechnology Information (Bethesda, Maryland)
Oligo(dT)	oligo-deoxy-thymine
PCR	polymerase chain reaction
PDA	potato dextrose agar
PVP	poly-4-vinylphenol
qPCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RSLC	Rapid Separation Liquid Chromatography
SDS	sodium-dodecyl-sulfate
SlAT2	Solanum lycopersicum acyl transferase 2
Sp	species
Spp	species pro parte
SSC	sodium chloride / sodium citrate (“standard saline citrate”)
SYBR Green I	N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine
Tris	Tris(hydroxymethyl)aminomethane
UT	Utah
UV	ultraviolet light
VIGS	virus-induced gene silencing
WT	wildtype

1 Summary

In this study, the gene encoding the E1 β-subunit of the branched-chain α-keto acid dehydrogenase (*NaBCKDE1B*), an enzyme required for *O*-acyl sugar biosynthesis, was specifically silenced in trichomes of *Nicotiana attenuata.* Using these transgenic lines, the role of *NaBCKDE1B* in controlling the trichome *O*-acyl sugar level in this plant was investigated. Moreover, the effect of reduced *O*-acyl sugar content on the susceptibility to native herbivores and pathogens was analyzed. As a result, silencing *NaBCKDE1B* in trichomes of *N. attenuata* led to a reduction of the *O*-acyl sugar content (20-30 %) and an increase in the plant susceptibility to the fungal pathogen *Fusarium brachygibbosum*. However, no significant effect on *Manduca sexta's* growth was found. These results indicated that *NaBCKDE1B* is involved in *N. attenuata´s* leaf trichome *O*-acyl sugar production and acts as an essential part of anti-pathogen defense.

1 Zusammenfassung

In dieser Studie wurde das Gen, welches für die E1 β-Untereinheit der branched-chain α-keto acid dehydrogenase (*NaBCKDE1B*) kodiert - ein notwendiges Enzym für die Sucroseesterbiosynthese – spezifisch in den Trichomen von *Nicotiana attenuata* gesilenced. Mithilfe dieser transgenen Linien wurde die Rolle dieses Gens für den Sucroseesterlevel in dieser Pflanze erforscht. Zudem wurde der Einfluss des verringerten Sucroseestergehalts auf die Anfälligkeit gegen Herbivore und auf Pathogene analysiert. Die Ergebnisse haben gezeigt, dass das Silencing von *NaBCKDE1B* in Trichomen von *N. attenuata* zu einer Verringerung des Sucroseestergehalts führt (20-30 %) sowie erhöhter Anfälligkeit der Pflanze in Bezug auf das Pilzpathogen *Fusarium brachygibbosum*. Im Gegensatz dazu wurde kein signifikanter Effekt auf das Wachstum von *Manduca sexta* nachgewiesen. Diese Ergebnisse implizieren, dass *NaBCKDE1B* in die Sucroseesterproduktion der Blatttrichome von *N. attenuata* involviert ist und einen essentiellen Teil der Verteidigung gegen Pathogene darstellt.

2 Introduction

2.1 Interaction of Nicotiana attenuata *and native herbivores and pathogens*

In its native habitat, the Great Basin Desert in the Southwest of the United States, *Nicotiana attenuata,* a wild tobacco species standing out because of its post-fire germination, has to defend against various herbivores (Long *et al.*, 2010).

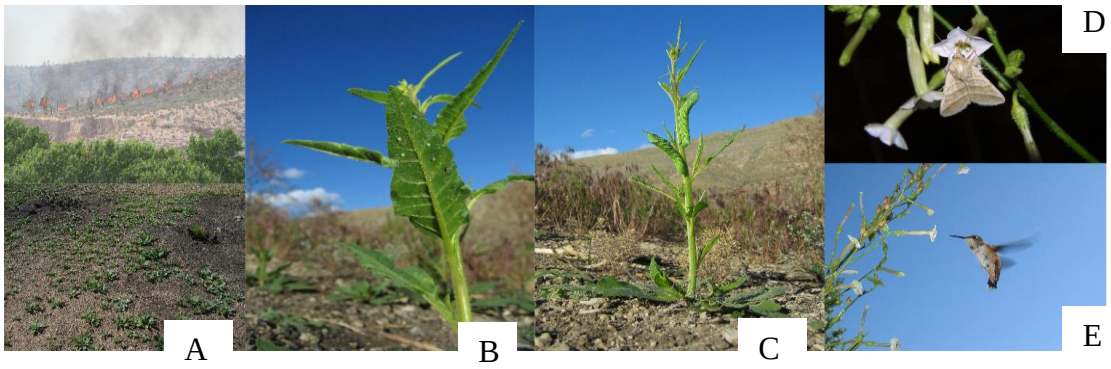

Photo credit: D. Kessler

Figure 1: *Nicotiana attenuata* in its natural habitat. Because of the heat in the Great Basin Desert wild fires can occur occasionally (A). As *N. attenuata* seeds germinate after fires, they are the first plants to grow then and therefore especially attractive to herbivores. Adult *Manduca sexta* moths place their eggs on the abaxial site of *N. attenuata* leaves (B). The caterpillars which hatch from these eggs feed on the plant (C). Adult *M. sexta* moths (D) as well as hummingbirds (E) are important pollinators.

Besides general herbivores like *Spodoptera spp.* that feed on *N. attenuata* leaves (Bandoly *et al.*, 2015), there are also specialized herbivores like *Manduca sexta*, the tobacco hornworm (Schuman *et al.*, 2012). In order to avoid too much damage of the leaves, the plant has got several mechanisms for defense. Such defense mechanisms can be mediated by phytohormones as jasmonic acid and by the release of volatiles (Wang & Wu, 2013).

In addition to herbivores, *N. attenuata* plants in their natural habitat also need to defend against phytopathogens. One example for those is the ascomycete

Fusarium brachygibbosum (Schuck *et al.*, 2014). In order to defend against infection by this fungus, *N. attenuata* has developed different mechanisms, for instance mediated by jasmonic acid signaling (Luu *et al.*, 2015). Another mechanism is to recruit beneficial bacteria that help to defend against fungal pathogens (Liu *et al.*, 2016).

2.2 O*-acyl sugars and their role in plant defense against herbivores and phytopathogens*

Except of the roots, *Nicotiana atttenuata* is fully covered by glandular trichomes, which are specialized hairs found on the surface of about 30 % of all vascular plants (Glas *et al.*, 2012).

On one hand trichomes act as physical barriers. On the other hand they are also known to produce and store a variety of secondary metabolites. *N. attenuata* trichomes contain nicotine, phaseoloidin, several phenylpropanoid polyamine conjugates, diterpene glycosides and *O*-acyl sugars (Roda *et al.*, 2003; Weinhold *et al.*, 2011). *O*-acyl sugars are the most abundant secondary metabolite which can be produced in large amounts (1.5 mg/g fresh weight, unpublished data) in *N. attenuata*.

O-acyl sugars are mainly produced by small glandular trichomes with a thin stalk and one to four secretory cells on top (Slocombe *et al.*, 2008). They consist of a glucose or sucrose backbone and two to five acyl chains esterified to different positions on the sugar (Schilmiller *et al.*, 2016). These acyl chains can be branched-chain or straight-chain fatty acids and differ in their length. *O*-acyl sugars are produced by several species among the Solanaceae. For example they have been analyzed in different tomato species as *Solanum lycopersicum* and *Solanum pennellii* (Schilmiller *et al.*, 2012), *Solanum habrochaites* (Kim *et al.*, 2012) and in tobacco species as *Nicotiana benthamiana* (Slocombe *et al.*, 2008). Among different species of the Solanaceae and even among different accessions of the same species the amount and composition of *O*-acyl sugars can vary so that

the effect of the plant's trichome exudate for defense can vary. The occurrence of *O*-acyl sugars is the reason for the stickiness of the surface of these plants.

O-acyl sugars produced by *Datura sp.* play an important role in inhibiting growth of some insects like *Manduca sexta* (Van Dam & Hare, 1998*)*. For some *Nicotiana sp.* a high toxicity of *O*-acyl sugars for *M. sexta* larvae was discovered (Thurston, 1970). The kind of damage *O*-acyl sugars cause in insects remains unclear. In *N. attenuata*, *O*-acyl sugars are shown to be involved in indirect anti-herbivore defense (Weinhold *et al*, 2011). However, it is not known whether *O*-acyl sugars are also involved in direct defense against the specialized herbivore *M. sexta* and the native pathogen *Fusarium brachygibbosum*.

2.3 O-*acyl sugar biosynthesis*

So far little is known about the pathway for *O*-acyl sugar biosynthesis in *Nicotiana attenuata.* A "one-carbon mechanism termed α-keto acid elongation" (Slocombe *et al.*, 2008) was suggested to occur in several *Nicotiana* species. It is similar to the pathway for leucine synthesis in primary metabolism. Isopropyl malate synthase, isopropyl malate dehydrogenase and isopropyl malate dehydratase take part in the production of medium branched-chain keto acids. These are converted into branched-chain acyl-CoAs by branched-chain keto acid dehydrogenase (BCKD). Branched-chain acyl-CoAs and sucrose/glucose are substrates for acyltransferase 1 (AT1), which connects an acyl chain to one site of the sugar backbone. In succession AT 2 to 4 then each add another acyl chain to the *O*-acyl sugar.

BCKD was shown to play an essential role for *O*-acyl sugar biosynthesis in *Nicotiana* and *Solanum* species (Slocombe *et al.*, 2008). This enzyme consists of the components E1 and E2. E1 has three α-subunits (α-BCKD I to α-BCKD III) and a β-subunit, as characterized in this publication. Virus-induced gene silencing of the gene encoding the BCKD subunit E1β results in 2- to 5- times reduction of total *O*-acyl sugar content in trichomes of *Nicotiana benthamiana.* This illustrates the central effect of BCKD for *O*-acyl sugar biosynthesis in *N. benthamiana*.

2.4 Aim of the study

In order to analyze the role of *NaBCKDE1B* in the trichomes of *Nicotiana attenuata* in controlling the *O*-acyl sugar level as well as its direct defense function against herbivores and pathogens, a trichome-specific, RNAi-mediated gene silencing was created. The target gene for this silencing is the gene encoding the E1β-subunit of BCKD. These lines are expected to produce less *O*-acyl sugars compared to the wildtype plants (Utah, WT). The lower *O*-acyl sugar content may then influence the growth of caterpillars as well as fungal infections. In this way it is possible to get insight into the direct defense function of *O*-acyl sugars against both herbivore and pathogen in the plant.

3 Material and Methods

3.1 General instruments

Acclaim RSLC 120C18 column	DIONEX
Analytical balance Talent TE31025	SARTORIUS
Autoclave VARIOKLAV400E	H&P
Drying oven	HERAEUS
DUO-Therm-Hybridization Oven OV5	BIOMETRA
Electrophoresis system	BIO-RAD
Eraser	RAYTEST
Gene Genius Bio Imaging System	MERCK EUROLAB
GenoGrinder 2000	SPEX SAMPLE PREP
Growth chamber	SNIJDERS SCIENTIFIC
Image Analyzer FLA3000	FUJIFILM
Inverted light microscope Axio Observer D1	ZEISS
Magnetic stirrer RH basic 2	IKA

Micro-TOF	BRUKER DALTONICS
MilliQ Advantage A10	MILLIPORE
PCR cycler Mastercycler Gradient I	EPPENDORF
pH meter type CG843	SCHOTT
Pipettes	EPPENDORF
qPCR cycler Mx3005P	STRATAGENE
RSLC System	DIONEX
Stereomicroscope Axio Zoom V16	ZEISS
Sterile work bench	HERA SAFE
Vortex Genie 2	SCIENTIFIC INDUSTRIES
Water bath	GRANT

3.2 Consumption items

1 l pots	PÖPPELMANN
96-well plates with lids	EPPENDORF
Laboratory Film	PARAFILM
Petri dishes Cellstar (16 mm Ø/20 mm Ø)	GREINER BIO-ONE
Pipette tips 2 µl, 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl	BIOZYM / NETUNE
Polypropylene Conical Tubes	FALCON
Reaction tubes (1.5 ml / 2 ml)	EPPENDORF
Whatman paper	BIO-RAD

3.3 Chemicals

10 x buffer M	CLONTECH
10 x Smart Cut B buffer	NEW ENGLAND BIOLAB

Acetate	SIGMA-ALDRICH
Acetonitrile	BAKER
Agarose	BIO-RAD
Chloroform	VWR
CTAB	SIGMA
DCM	SIGMA-ALDRICH
DEPC	SIGMA/ALDRICH
DNA loading dye	FERMENTAS
Easy Tides dCTP [alpha-^{32}P]	PERKIN ELMER LAS
EDTA	ROTH
Ethanol	SIGMA-ALDRICH
Ethidium bromide	SIGMA
Formic acid	APPLICHEM
Gamborg´s B5 medium	SIGMA
HCl	ROTH
Hickory Liquid Smoke	HOUSE OF HERBS
Hygromycin	DUCHEFA
Isopropanol	VWR-CHEMICALS
LB-Agar Lennox	ROTH
Methanol	MERCK
$MgCl_2$	ROTH
NaCl	ROTH
NaOH	SIGMA
PDA	SIGMA
Phenol/chloroform/isoamylalcohol	ROTH
Plant agar	DUCHEFA
PVP	FLUKA
SDS	ROTH
Sodium citrate	FLUKA

SSC	SIGMA-ALDRICH
Sucrose monolaureate	SIGMA
Tris	ROTH
Trizol	THERMO SCIENTIFIC
Ultrahyb	AMBION
β -Mercaptoethanol	ALDRICH

3.4 *Commercial Kits, Enzymes*

3.4.1 Commercial Kits

DNase I treatment kit	THERMO SCIENTIFIC
Amersham Rediprime II Random Prime Labeling System	GE HEALTHCARE
Revert Aid First Strand cDNA Synthesis Kit	THERMO SCIENTIFIC
Takyon qPCR Kits for SYBR assays	EUROGENTEC

3.4.2 Enzymes

Hind III	CLONTECH
RNase A	THERMO SCIENTIFIC
Xba I	NEW ENGLAND BIOLABS

3.5 General methods

Seed germination

Nicotiana attenuata seeds were germinated on Gamborg's B5 medium and kept in a growth chamber at 26 °C for 16 h light and 24 °C for 8 h dark, as recommended

for *N. attenuata (Krugel et al., 2002).* After 10 days the seedlings were transferred to Teku pots for 10 days before planting into 1 l pots. The plants were grown under greenhouse conditions of 26-28 °C for 16 h light, 22-24 °C for 8 h dark and 45-55 % relative humidity.

Trichome harvesting

Trichomes were harvested by cutting the stem and branches into small pieces with up to 10 cm length, placing them into 50 ml Falcon tubes and freezing them in liquid nitrogene. Frozen tissues were shaken thoroughly for 30 seconds and fallen trichomes were collected in 2 ml Eppendorf tubes for further usage.

Breeding conditions for *Manduca sexta*

Manduca sexta eggs were collected from an in-house colony. The eggs were kept in a growth chamber at 26 °C for 16 h light, 24 °C for 8 h dark and 65 % relative humidity until hatching. The neonates were directly used for the feeding experiment.

Phytopathogen

Fusarium brachygibbosum Padwick U4 were originally isolated from diseased plants grown in a native *Nicotiana attenuata* population in the Southwest of Utah, USA (Schuck *et al.*, 2014). They were successfully tested as a system for studies of defense of *N. attenuata* against native pathogens (Luu *et al.*, 2015). The fungus was cultured on potato dextrose agar (PDA) 14 days before infection. The pathogenicity was ensured by frequent inoculation of *N. attenuata* seedlings and re-isolation.

Statistical Analysis

Statistics were performed using Excel (Microsoft, http://www.microsoft.com). For analysis of differences in plant performance, Student´s *t*-test was used with the two-tailed distribution of two sets of samples with equal or unequal variance. The variance was determined using the F-test. The level of significance of the tests was set at $p<0,05$.

3.6 Stable transformation of Nicotiana attenuata

Trichome-specific silencing of *NaBCKDE1B* with RNAi construct

Comparing sequences at NCBI for genes encoding branched-chain α-keto acid dehydrogenase E1 β-subunit (BCKDE1B) from *Arabidopsis thaliana* and other closely related Solanaceae including *Solanum pennellii* and *Nicotiana tabacum* with the *Nicotiana attenuata* 454 transcriptome (Gase & Baldwin, 2012) and our in-house genome database, one homolog (*NIATv7_g34895)* of this gene was identified in *N. attenuata.*

The stable transformed *N attenuata* plants used in this project were silenced using an RNAi-construct for this target gene *NaBCKDE1B* by the supervisor according to a silencing method mediated by *Agrobacterium tumefaciens (Krugel et al., 2002).* As a trichome-specific promoter, SIAT2 from *Solanum lycopersicum* was used (Schilmiller *et al.*, 2012). To generate *irBCKDE1B* plants, a 351 bp fragment of *NaBCKDE1B* gene was cloned as an inverted repeat construct into the pRESC8TRCAS transformation vector containing a hygromycin (*hptII*) resistance gene as selection marker and the *S. lycopersicum* SlAT2 promoter. This transformation vector is displayed in Figure 2. The sequences of the fragments can be found in the Supplemental Data.

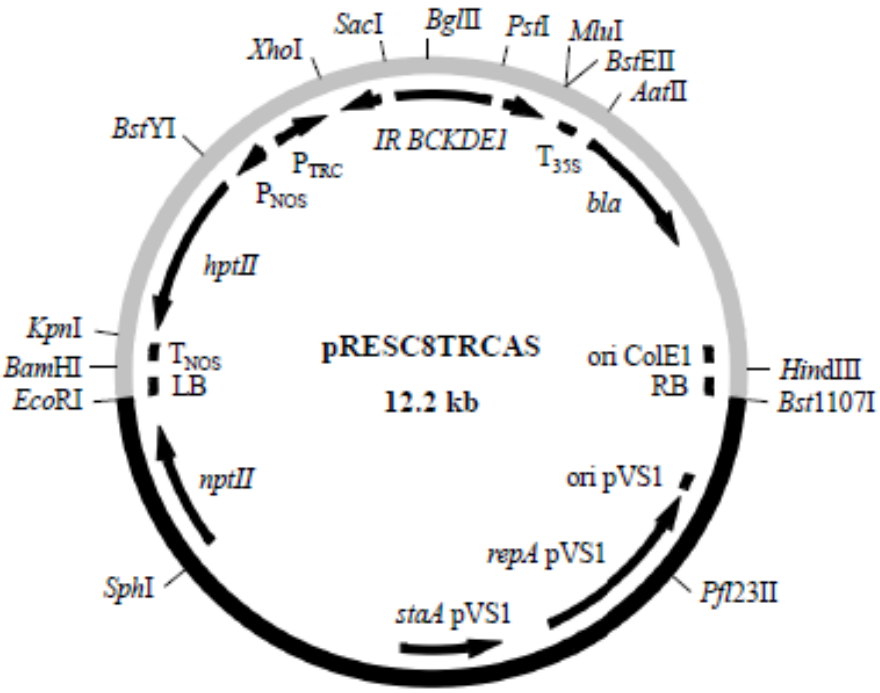

Figure 2: Transformation vector pRESC8TRCAS used for the creation of transgenic lines.

Screening for silenced plants

The screening process was performed by the supervisor according to the screening method for stable transformed *Nicotiana attenuata* plants (Gase *et al.*, 2011).

Check for the location of the expression of the SlAT2 promoter

To check whether the SlAT2 promotor can drive a trichome-specific gene expression in *Nicotiana attenuata*, this promotor is fused with a GFP-β-Glucoronidase-fusion protein as a reporter gene and expressed via stable transformation as described above. The trichome-specific GUS-expression was analyzed by stereomicroscopy with Zeiss Axio Zoom V16. Pictures of trichomes expessing β-Glucuronidase were taken with 180 x magnification.

3.7 Gene expression analysis

RNA extraction

In order to find out, how efficiently the target gene is silenced, a transcript abundance analysis for *NaBCKDE1B* was performed. RNA was extracted from trichome tissue and used for cDNA synthesis. Before cDNA synthesis, a DNAse treatment was performed to break down the DNA which was extracted together with RNA. The relative abundance of the gene product of the target gene was then measured by qPCR analysis using the cDNA.

Around 150 mg trichome tissue was ground over liquid nitrogen to powder. Then 1 ml ice-cold Trizol was added to each sample and mixed for 5 min at room temperature. Afterwards the samples were centrifuged for 10 min at 12000 g and 4 °C. The supernatant was transferred into a new tube and 200 µl chloroform was added. After shaking thoroughly and keeping at room temperature for 3 min, the samples were centrifuged again for 15 min at 10000 g and 4 °C. The upper phase was transferred into a new tube and 500 µl isopropanol was added. After mixing the samples were kept at room temperature for 10 min until the RNA precipitated. The samples were centrifuged again for 10 min at 10000 g and 4 °C. Then the supernatant was removed and the pellet was washed with 75 % ice-cold ethanol in DEPC water. In the next step, 300 µl 75% ethanol were added and samples were mixed and let stand for 2 min. The samples were centrifuged for 10 min at 7500 g and 4 °C. The ethanol was removed and the pellets were washed as described above. After removing the ethanol completely the pellets were dissolved in 50 µl DEPC water and the samples were kept at 20 °C.

DNase treatment

First, 2 µg RNA of each sample was transferred on a 96-well plate. Each well was filled up to 7.5 µl with DEPC water. Then 2.5 µl of master mix 1 were added to each sample. The plate was centrifuged briefly and incubated for 30 min at 37 °C. During this time the DNase should break down the DNA in each sample. After that step, 1 µl EDTA (50 mM) was added, the plate was centrifuged briefly again

and incubated at 65 °C for 10 min. The reason for this is to inactivate the remaining DNase.

Table 1: Composition of master mix 1

Ingredients of the DNase I treatment kit	Volume per sample [µl]
10 x Reaction Buffer with $MgCl_2$ for DNAse I, 50 mM	1
DNase I RNase-free 1 U/µl	1
RiboLock RNase Inhibitor 1 U/µl	0.5

cDNA synthesis

Into each well 9 µl of master mix 2 were added. The plate was centrifuged briefly once more and incubated at 42 °C for 1 h for the reverse transcription process. Afterwards it was heated to 70 °C for 5 min. Samples were five times diluted by adding 80 µl deionized autoclaved water (final volume 100 µl) and stored at -20 °C.

Table 2: Composition of master mix 2

Ingredients of the RevertAid First Strand cDNA Synthesis Kit	Volume per sample [µl]
5 x Reaction buffer	4
$Oligo(dT)_{18}$	1
10 mM dNTP Mix	2
RevertAid Reverse Transcriptase	0.5
DEPC water	1.5

qPCR

A master mix was produced consisting of 10.8 µl MasterMix from Takyon™ qPCR Kits for SYBR® assays with ROX, 4.8 µl water (deionized and autoclaved) and 2.4 µl of the referring primer (5 µM stem solution containing forward and reverse primer) per well. Into each well of a 96-well plate, 18 µl of the master mix and 2 µl of cDNA were transferred (total volume 20 µl per well). The plate was covered with lids and put into an Mx3005P PCR cycler.

The following program was run:

1 cycle	95 °C	3 min
40 cycles	95 °C	10 sec
	60 °C	20 sec
	72 °C	40 sec
1 cycle	95 °C	10 sec
	60 °C	20 sec
	95 °C	40 sec

The following primer pairs were used:

Actin 7	Forward: 5'-TTCTTCGTCTGGACCTTGCT-3'
	Reverse: 5'-ATCATGGATGGCTGGAAGAG-3'
BCKDE1B_34895	Forward: 5'GATTATATGTTGCCTCTCTC-3'
	Reverse: 5'-TTTAAGGTCTATCAGTTCG-3'

3.8 Control for a correct insertion of the RNAi-construct

DNA extraction

Table 3: Solutions used for DNA extraction

Solution	Ingredient	Amount
EDTA 0.5 M (292.24 g/mol, pH = 8)	EDTA Deionized water	7.31 g 50 ml
Tris-Cl 1 M (121.14 g/mol, pH = 8)	Tris Deionized water	30.29 g 250 ml
2 % CTAB buffer	CTAB NaCl EDTA 0.5 M Tris 1 M Deionized water PVP β-mercaptoethanol	5 g 20.45 g 10 ml 25 ml 250 ml 2.5 g 2.5 ml
10 % CTAB solution (w/v)	CTAB Deionized water	5 g 50 ml
High Salt TE	Tris 1 M EDTA 0.5 M NaCl	2.5 ml 0.5 ml 14.61 g
Chloroform : isoamyl alcohol 24 : 1 (v/v)		
RNase A 10 mg/ml		
Isopropanol 100 %		
80 % ethanol in deionized water		

About 1.2 g leave material was divided equally among 4 tubes (300 mg each) and ground using the GenoGrinder2000. Afterwards 800 µl preheated CTAB buffer (65 °C) were transferred into each tube and they were shaken for 5 min. Then the samples were incubated for 60 min. During this incubation, the samples were shaken every 10 min. After centrifuging briefly, 500 µl chloroform : isoamyl alcohol 24 : 1 (v/v) were added to each sample. The samples were shaken thoroughly and left untouched for 10 min at room temperature. The tubes were centrifuged for 1 min at 8000 rpm and 800 µl of the aqueous phase were transferred into new 2 ml Eppendorf tubes. To this 80 µl 10 % CTAB solution were added and the steps from adding chloroform : isoamylalcohol to centrifuging were repeated in the same way as described before. This time 650 µl of the aqueous phase were transferred into a new tube together with 650 µl 100 % ice-cold isopropanol. The samples were mixed thoroughly and kept overnight at 4 °C.

The samples were centrifuged at 4 °C for 2 min at 13600 rpm. Afterwards the supernatant was discarded and 800 µl High salt TE were added. The pellets were dissolved in the solution and the samples were incubated at 37 °C for 30 min. Then 800 µl chloroform : isoamylalcohol 24 : 1 (v/v) were added and the samples were kept at room temperature for 10 min. After this, the tubes were centrifuged for 1 min at 8000 rpm. The aqueous phase was transferred into new 2 ml tubes and 650 µl 100 % isopropanol were added, mixed and the samples were centrifuged for 2 min at 10000 rpm. The supernatant was discarded and the pellets were washed using 800 µl 80 % ethanol. After centrifuging for 1 min at 10000 rpm the supernatant was discarded. This washing step was then repeated. Then the pellets were dried and 50 µl MilliQ water were added. In order to determine the DNA quantity, 1 µl of each sample were mixed with 4 µl MilliQ water and 1 µl loading dye and run on a 0.8 % agarose gel for 45 min. The remaining DNA samples were stored at -20 °C.

Southern Blot

A Southern Blot was performed to ensure that the RNAi-construct is inserted and no double insertion has occurred. First the DNA has to be cleaved using different

restriction enzymes. Therefore the following reagents were transferred into a 1.5 ml Eppendorf tube:

Table 4: Reagents for DNA restriction

enzyme	Hind III	Xba I
reagents	8 µg DNA	8 µg DNA
	20 µl 10x buffer M	20 µl 10x Cut SmartB buffer
	2 µl Hind III (50 U/µl)	6 µl Xba I (20 U/µl)
	Filled up to 200 µl with MilliQ water	Filled up to 200 µl with MilliQ water

As the DNA concentration differed among the samples, it was estimated by loading 1 µl of the DNA samples on a 1 % agarose gel and run them in an electrophoresis for 45 min at 120 V. The DNA amount of the bands of the DNA ladder mix was applied to calculate the amount of DNA in the samples. With the resulting DNA concentration the amount needed for DNA restriction was calculated.

Table 5: Calculated DNA concentration

Sample	DNA concentration [ng/µl]	DNA volume for restriction [µl]	Water volume for restriction [µl]
UT30X	315.8	25	149
132_1	224.46	36	142
132_10	162.88	49	129
134_10	189.5	42	136
147_2	226.58	35	143
147_4	228.56	35	143

Example for calculation (sample UT30X):

C = 315.8 ng/µl

V = 8000 ng / 315.8 ng/µl

V = 25.33 µl

All samples were incubated at 37 °C for 15 h. 20 µl 5 M NaCl were added and mixed. Then 200 µl isopropanol were added and the samples were incubated at -80 °C for 3 h. The samples were centrifuged for 5 min at 4 °C and 13,200 rpm. The supernatant was discarded and the pellet was washed using 180 µl of 80 % ethanol. After mixing and centrifuging down the supernatant was removed and the pellet was dried for 45 min at 37 °C. To dissolve the pellet, 21 µl MilliQ water were added. In order to check the restriction efficiency, 1 µl DNA, 9 µl MilliQ water and 2.5 µl loading dye were transferred on a 1 % agarose gel with 6 µl ethidium bromide. The electrophoresis was run for 45 min at 120 V.

In order to separate the cleaved DNA and to transfer it on a membrane, an agarose gel electrophoresis was performed. The gel contained 1 % agarose and no ethidium bromide. For the electrophoresis 20 µl cleaved DNA and 10 µl loading dye were transferred on the gel and run for 15 h at 24 V. Afterwards the gel was washed in 800 ml 150 mM HCl solution and rinsed with distilled water. Then the gel was incubated in 500 ml denaturation buffer two times for 20 min and every time rinsed with distilled water after removing the solution. After denaturation, the gel was incubated two times in 500 ml neutralization buffer and rinsed with water as described above.

A blotting tray was filled with 10 x SSC and the fresh nylon membrane was rinsed with distilled water and soaked in 10 x SSC. Two pieces of Whatman paper were also soaked in this solution and placed on the blotting tray, touching the solution. The gel was placed on top and then the nylon membrane. Air bubbles were avoided any time. Four pieces of Whatman paper were added on top and then two packs of paper towels. Parafilm was applied between the borders of the gel and the membrane in order to avoid that the solution is sucked up by the paper towels without passing the gel and the membrane. About 500 g weight was put on the

paper towels. The whole blotting tray was covered with plastic wrap so that less of the solution could evaporate. After 20 hours of incubation the membrane was taken out, the lane positions were marked and the DNA on the membrane was crosslinked applying UV light.

The ladder was cut off. A 287 bp long, ^{32}P-labeled PCR fragment of the *hptII* gene as hybridization probe that is complementary to the gene for hygromycin resistance was synthesized by the supervisor. Approximately 10 ng of this radioactively labeled probe were aliquoted into TE buffer to a total volume of 45 µl. The membrane was wet using distilled water and rolled, avoiding that parts of the membrane were overlapping. Then the water was removed and 12 ml Ultrahyb hybridization buffer were added. The tube was rolled in the hybridization oven for 1 h at 42 °C. The labeled probe was incubated at 97 °C for 5 min and immediately transferred on ice with cold water for 5 min. The probe was added into one vial from Probe Synth GE kit including the Random Prime Labeling System as well as 5 µl ^{32}P-dCTP. After mixing the vial was incubated for 20 min at 37 °C. Then the content of the vial was transferred on a column and centrifuged for 2 min at 3000 rpm. The column was discarded and the liquid was incubated at 98 °C for 5 min. The probe was immediately transferred on ice with cold water for 5 min to denature the probe. After denaturation the probe was added to the hybridization tube with the membrane and rolled overnight at 42 °C.

The hybridization buffer was removed and 35 ml 2 x SSC, 0.1 % SDS solution, heated to 62 °C, was added. The tube was incubated for 10 to 15 min. This incubation was performed three times in the same way. Afterwards the membrane was removed and transferred in plastic that was sealed. The membrane was placed into the scanning chamber and covered with the scanning plate. The chamber was sealed with tape. After 1 h the scanning plate was erased and re-exposed overnight.

Table 6: Solutions used for Southern Blot

Solution	Ingredients
Denaturation buffer: 0.5 M NaOH, 1.5 M NaCl	20 g NaOH 87.66 g NaCl 1 l deionized water
Neutralization buffer: 0.5 M Tris-HCl, 1.5 M NaCl, 1 mM EDTA, pH 7.2	60.57 g Tris 87.66 g NaCl 2 ml 500 mM EDTA 1 l deionized water
20 x SSC : 300 mM sodium citrate, 3 M NaCl, pH 7.6	88.23 g sodium citrate 175.32 g NaCl 1 l deionized water
0.5 M EDTA pH 8	7.31 g EDTA 50 ml deionized water
2 x SSC, 0.1 % SDS: 30 mM sodium citrate, 0.3 M NaCl, 1 % (w/v) SDS, pH 7.6	100 ml 20 x SSC 1 g SDS 1 l deionized water
0.1 x SSC, 0.1 % SDS: 30 mM sodium citrate, 0.3 M NaCl, 1 % (w/v) SDS	5 ml 20 x SSC 1 g SDS 1 l deionized water

3.9 O-*Acyl sugar quantification*

In order to compare the *O*-acyl sugar amount among the different lines, secondary metabolites were extracted from leave tissue with trichomes as well as from trichome tissue. The tissue was ground over liquid nitrogen. Then 50 mg of the powdery tissue from trichomes or 100 mg from total leaves were transferred into new tubes with two steel beads and ground at 1100 strokes per min for 30 s using the GenoGrinder 2000. After grinding, 500 µl extraction buffer for trichome tissue or 1 ml for leaf tissue were added and homogenized with the tissue at 100 strokes per min for 15 min with the GenoGrinder 2000. The samples were centrifuged for 20 min at 13,200 rpm and 4 °C. Then 800 µl of the supernatant were transferred into new tubes and centrifuged again the same way. For the measurement with the MicroTOF, 100 µl of the solution were transferred into glass vials with insert.

Using a HPLC Dionex RSLC system with a Dionex Acclaim RSLC 120 C-18 column (150 x 2.1 mm, 2.2 µm), 1 µl of the solution was separated. The following binary gradient was applied: 0.5 min isocratic 90% A, 10% B; 13 min linear gradient to 80% B; isocratic for 1.5 min. The flow rate was 400 µL/min. MS detection was carried out with the Bruker MicroToF LC system operated in positive electrospray mode. The following instrument settings were applied: capillary voltage 4500 V, dry gas temperature 200 °C, dry gas flow 10 l/min. Ions were detected from m/z 50 to 1400 at a repetition rate of 2 Hz. Mass calibration was performed using sodium formate clusters (10 mM solution of NaOH in 50/50% v/v isopropanol/water containing 0.2% formic acid).

The peak areas were integrated using extracted ion traces for the sodium adduct [M+Na]+ of each individual *O*-acyl sugars in the QuantAnalysis software version 2.0 SP1 (Bruker Daltonics). The amount of each *O*-acyl sugar in plant tissue was normalized using the internal standard and the fresh weight of the tissue. Total *O*-acyl sugars were calculated by summing up the normalized peak area of all 15 *O*-acyl sugars abundant in *Nicotiana attenuata*.

Table 7: Chemicals used for *O*-acyl sugar quantification

Solution	Ingredients
Extraction buffer	50 mM acetate buffer, pH 4.8, containing 40 % methanol spiked with 10 ng/µl internal standard sucrose monolaureate
A	deionized water 0.1 % (v/v) acetonitrile 0.05 % formic acid
B	acetonitrile 0.05 % formic acid

3.10 Culturing of Fusarium brachygibbosum *spores on medium with* O-*acyl sugars*

To test the effect of *O*-acyl sugars on fungal spore germination and growth, 30 mg of extracted *O*-acyl sugars were dissolved in 1 ml DCM and added in 10 ml of spore germination medium which is comprised of 1.2% plant agar and 10 mM $MgCl_2$. Also 1ml of DCM was added as control. The medium was heated until DCM was evaporated and then diluted to get the final concentrations of 3, 1.5, 0.75 and 0.375 mg/ml for *O*-acyl sugars. Fungal spores were harvested from 14 day-old cultures and washed with 10 mM $MgCl_2$ as described previously (Luu *et al.*, 2015). For microscopy, 2.5 ml of spore germination medium was spread on microscopic slides and airdried. On top of the solid medium, 10 µl of the spore solution was transferred. The slides were placed in petri dishes with a wet Whatman filter paper (1 ml sterile water added) and kept at 25 ^{o}C in a dark chamber. The number of germinated spores was counted and the length of the germination tubes was measured after 6 and 12 hours using an inverted light microscope. Pictures were taken using AxioVision imaging software at 400-fold magnification.

3.11 Bioassay

3.11.1 *Manduca sexta* feeding assay

Newly hatched *Manduca sexta* caterpillars were placed on the largest leaf of each 30 day-old *Nicotiana attenuata* plant in the greenhouse (23 to 25 °C) from the independent lines 132-10, 134-10 and 147-2. The weight was measured after 5, 8 and 12 days.

3.11.2 Pathogen inoculation

Fusarium brachygibbosum was grown on plates with potato dextrose agar (PDA) for 2 weeks. The +1 and +2 leaf was cut from 30 day-old 15 *Nicotiana attenuata* plants each of the lines UT, 132_10, 134_10 and 147_2 and placed on a plate containing a paper towel and 20 ml water. Agar blocks with 3 mm diameter were cut off from the fungus plates and two of them placed on each leaf. The *Fusarium* plates were sealed with laboratory film and incubated at 25 °C in a chamber. The infection rate was estimated by measuring the diameter of the rings of infected, necrotic leaf tissue around the agar blocks using a caliper. As the infected area is not always round but variably formed, the diameter was measured on the longest and the shortest distance. The sum of the average longest and shortest distance of the two infected leaf regions was used for the comparison of the necrotic area on the leaves of the different lines.

4 Results

4.1 Southern blot analysis showed that irBCKDE1B plants contain single insertions

After screening for hygromycin resistance and diagnostic PCR for full insertion of the T-DNA into the plant genome, five T2 lines were selected (132_1, 132_10, 134_10, 147_2 and 147_4). A Southern blot was performed in order to check whether the RNAi-construct is correctly inserted or if a double insertion occurred.

A sample complementary to the gene for hygromycin resistance, which is also part of the RNAi-construct, was radioactively labeled and hybridized to the restricted and separated DNA on the membrane.

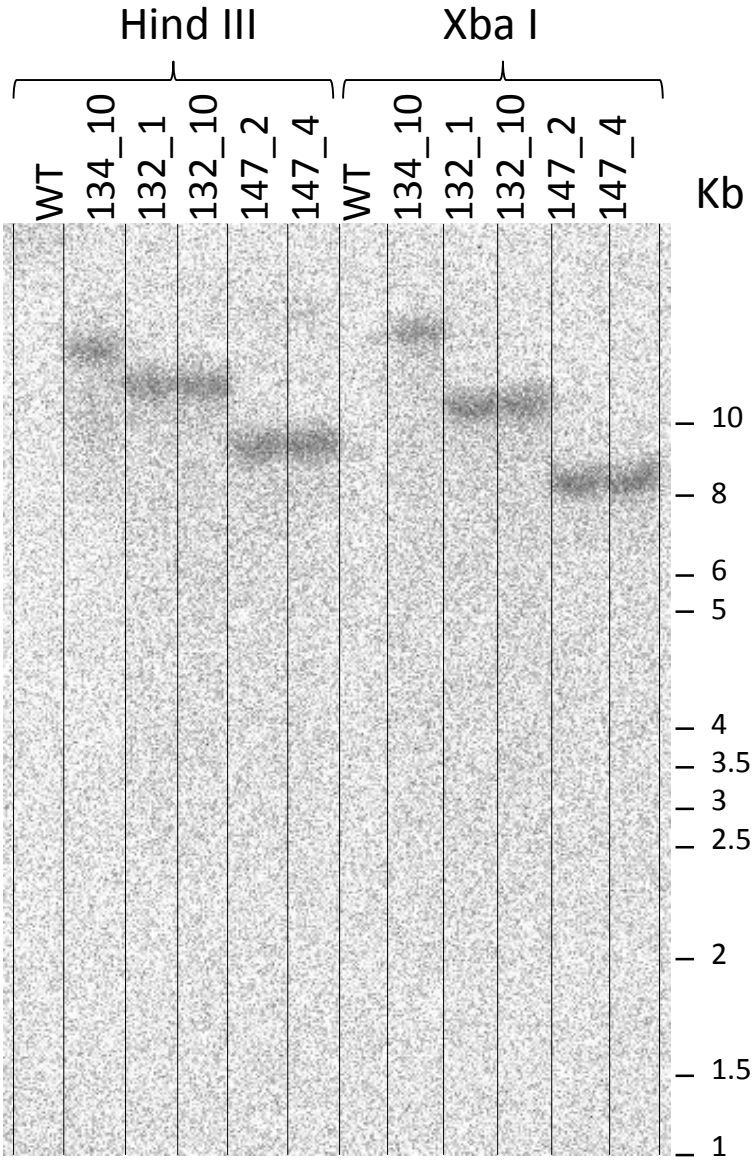

Figure 3: Picture of the Southern blot membrane. The samples on the left were cut by Hind III, the ones to the right by Xba I. WT was used as a negative control without insert.

The results (Figure 3) showed that there is no band for WT whereas there is one band each for all the transgenic lines visible for both restriction enzymes Hind III

and Xba I. WT is the untransformed wildtype, so it does not have an insertion and therefore it does not have the gene for hygromycin. For that reason the radioactively labeled DNA could not hybridize to any part of the DNA from WT. In all other lines there is one band to be seen, which means that the radioactively labeled sample could hybridize to one single part of the DNA from the lines. Therefore a single insertion has occurred. Otherwise the labeled sample would have hybridized to two locations of the DNA and there would be two bands of the same intensity on the membrane.

4.2 Trichome-specific gene silencing led to a reduction of NaBCKDE1B *expression in the trichomes and leaves*

The trichome-specific expression was ensured by stereomicroscopy after GUS-staining. The enzyme β-Glucuronidase is exclusively expressed in the tip cell of trichomes, as can be seen in Figure 4.

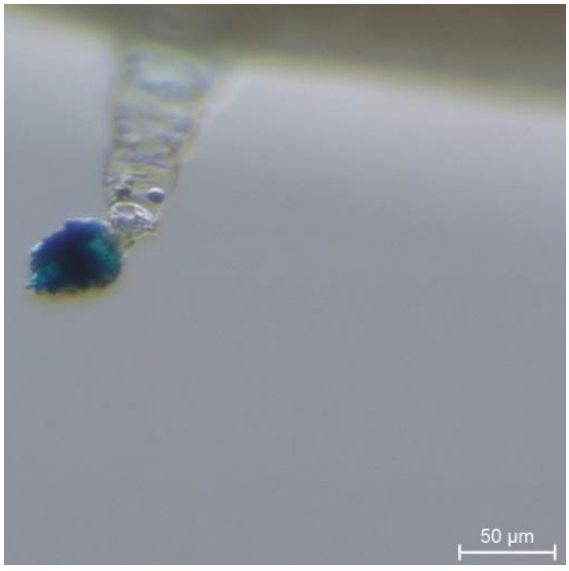

Figure 4: Expression of β-Glucuronidase at the tip cell of a leaf trichome.

A qPCR was performed in order to check whether the RNAi-construct used could efficiently silence the target gene. Since this construct was designed to silence *NaBCKDE1B* specifically in trichomes, the RNA was extracted from stem trichomes. The extracted RNA was used to create cDNA by reverse transcription.

The cDNA was amplified using primers that flank the region encoding for the target gene. For the relative quantification, the fluorescent nucleotide stain SYBR Green I was used. The relative amount of the amplified cDNA was then normalized to the total RNA in the sample by usage of the housekeeping gene Actin 7, which is highly expressed in any cell.

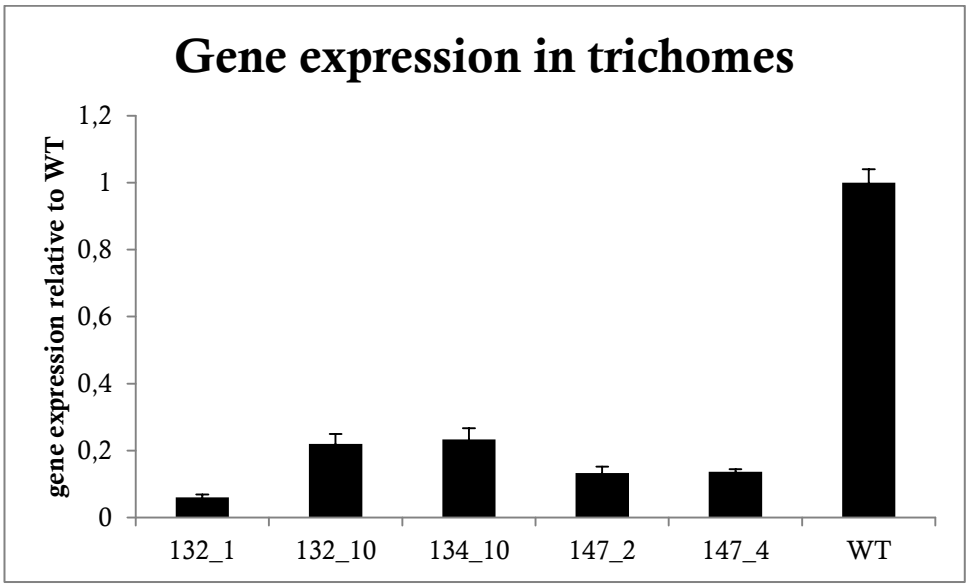

Figure 5: Relative gene expression of *NaBCKDE1B* in the trichomes of the stable transformed lines. The gene expression in trichomes harvested from the stem is compared to WT, the 30th inbred generation of *Nicotiana attenuata* plants from a natural accession in Utah.

As can be seen in Figure 5, the silencing efficiency in all transformed lines is between 75 and 95 % compared to wildtype.

NaBCKDE1B expression was also measured for total leaves to see how much a trichome-specific gene silencing contributes to the total leaf expression of this gene.

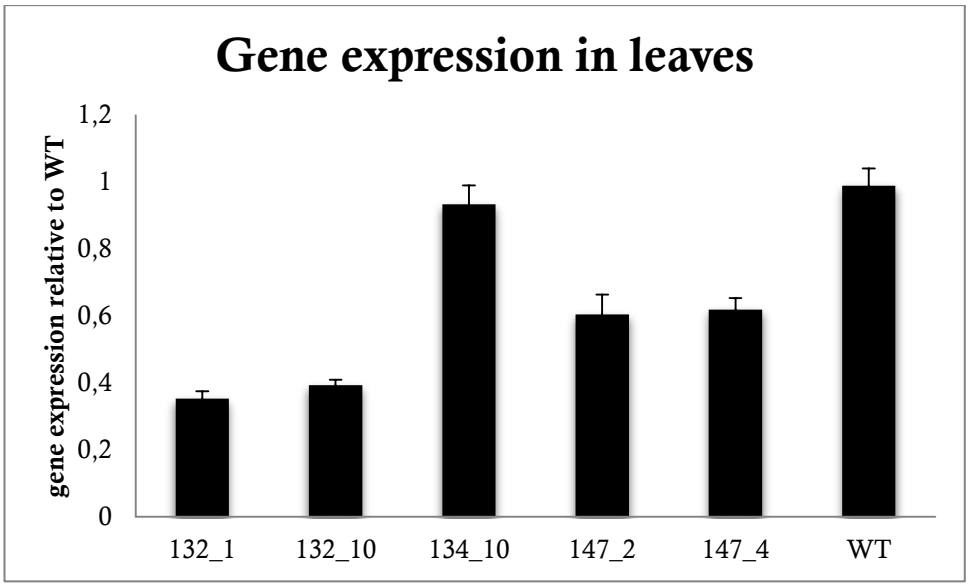

Figure 6: Relative gene expression of *NaBCKDE1B* in the total leaves of the stable transformed lines. The gene expression in total leaf extracts is compared to WT, the 30th inbred generation of *Nicotiana attenuata* plants from a natural accession in Utah.

As shown in Figure 6, the silencing efficiency in total leaf extracts is around 40 % for the lines 147_2 and 147_4 and around 60 % for the lines 132_1 and 132_10. For line 134_10 the gene expression is not different from the wildtype.

As expected, the silencing efficiency in the total leaf extract is lower than in the trichome extract, as the silencing was trichome-specific. This indicates that in the total leaf extract there was other tissue included, in which *NaBCKDE1B* was not silenced. Hence, the silencing efficiency in the total leaf was reduced by 15 to 55 % compared to the trichome. But it is unclear, why there is no silencing for line 134_10 in the total leaf extract, while it was around 75 % in the trichome.

4.3 Trichome-specific gene silencing led to a reduction of the O*-acyl sugar level*

Total secondary metabolites were extracted from trichome tissue as well as from total leaves in order to measure the *O*-acyl sugar amount in irBCKDE1B lines and to relatively compare it to WT plants. The compounds were separated by HPLC

and assessed by mass spectrometry. The resulting features were matched to the known *O*-acyl sugars of *Nicotiana attenuata* and the relative abundance was compared among the transformed lines and WT.

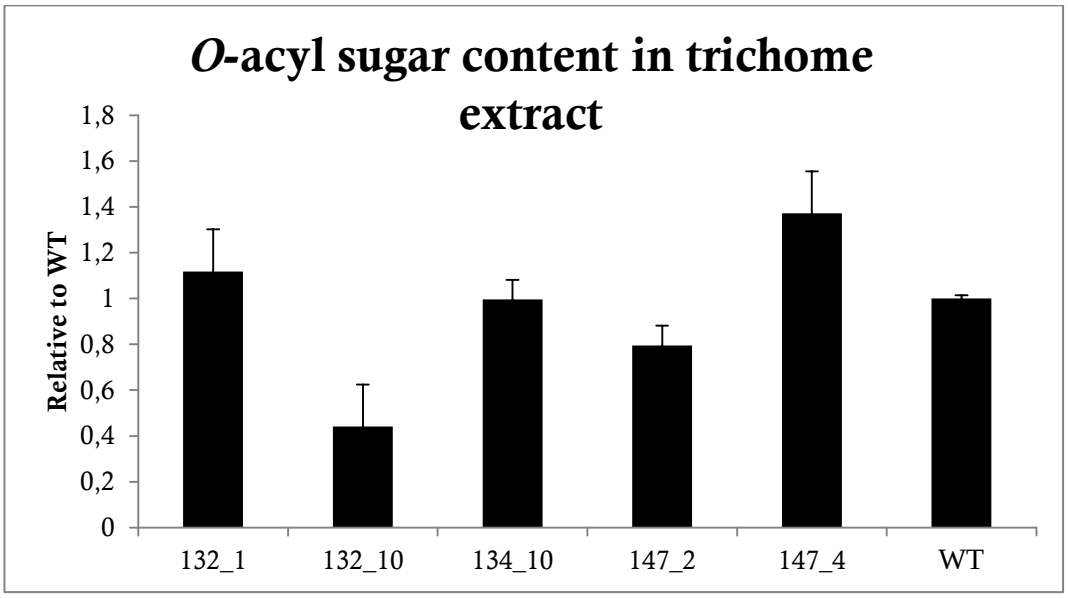

Figure 7: Relative *O*-acyl sugar abundance in the trichomes of the transformed lines compared to Utah wildtype (WT).

As can be seen in Figure 7, silencing *NaBCKDE1B* in the trichomes led to 56 % reduction of the total *O*-acyl sugar content in line 132_10; for 147_2 there is 20 % reduction. However, it did not reduce the *O*-acyl sugar content in the trichomes of the lines 132_1, 134_10 and 147_4 compared to WT.

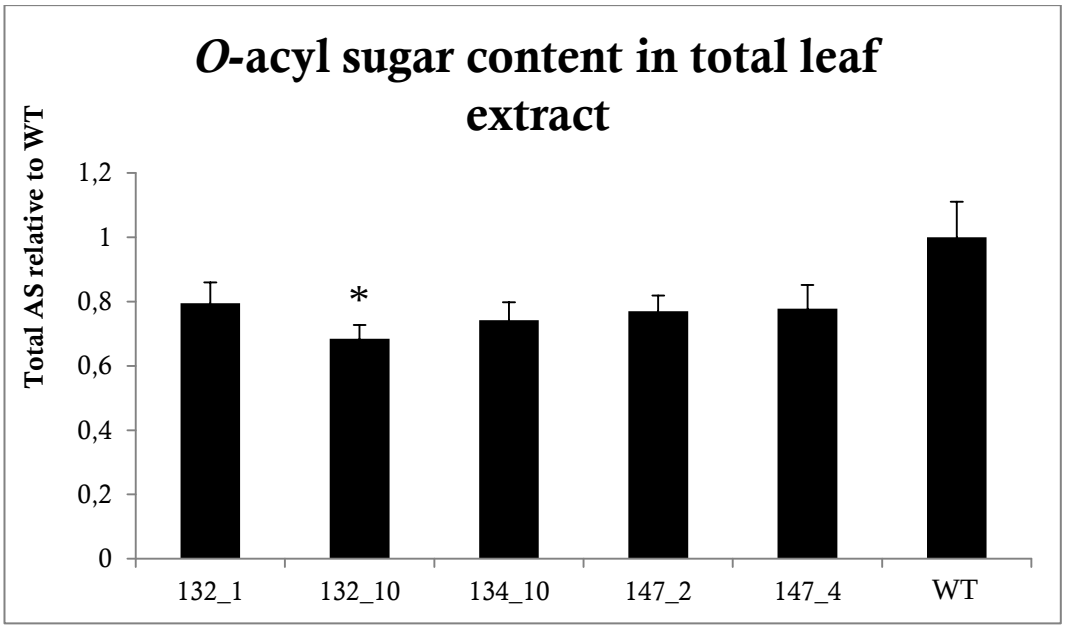

Figure 8: Relative *O*-acyl sugar abundance in the total leaf of the transformed lines compared to Utah wildtype (WT).

As shown in Figure 8, the acyl sugar content in the leaf is reduced by about 20 to 30 % in all lines compared to WT. However, the reduction is only significant for line 132_10. This is most likely due to a low sample number of 6 plants.

According to the results of the qPCR and *O*-acyl sugar measurement, the three independent lines 132_10, 134_10 and 147_2 were chosen to proceed with the bioassays, as those are the only lines to show an *O*-acyl sugar reduction in the trichome. Additionally, they also show an *O*-acyl sugar reduction in the total leaf as well as a good silencing efficiency in both trichome and leaf.

4.4 Manduca sexta *grew similarly on irBCKDE1B and WT plants*

In order to test whether silencing *NaBCDKE1B* specifically in trichomes could lead to any difference in herbivore susceptibility, a *Manduca sexta* bioassay was performed. *M. sexta* larvae were fed on the three independent irBCKDE1B lines (132_10, 134_10, 147_2) and larvae mass of those caterpillar was compared with those fed on WT plants.

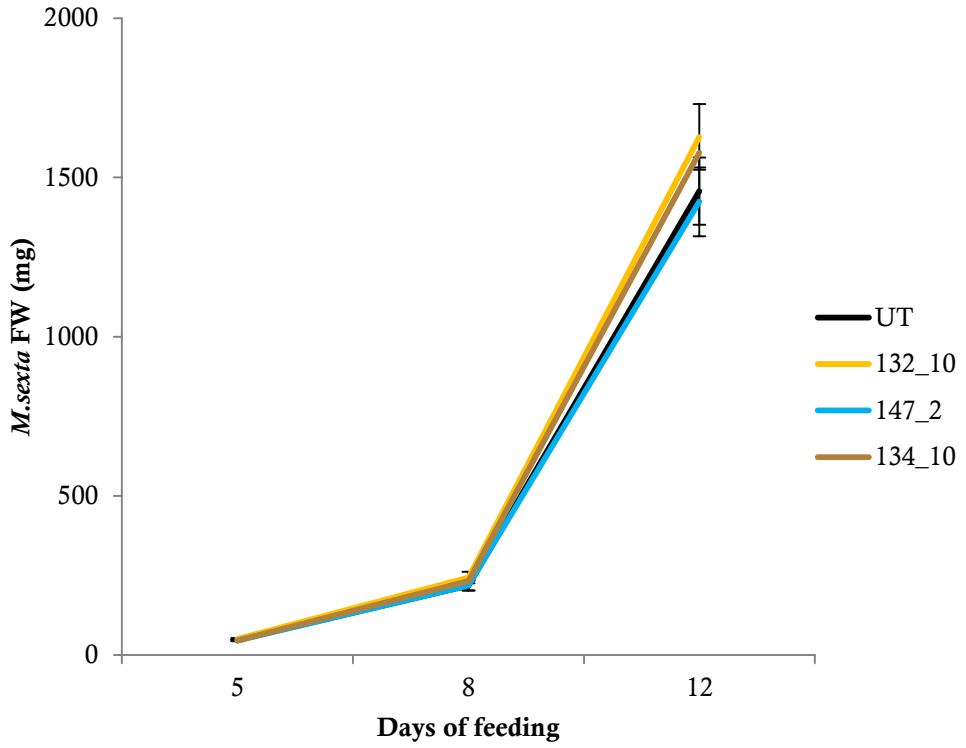

Figure 9: Weight of *Manduca sexta* caterpillars fed on plants from lines 132_10, 147_2 and WT (UT) after 5, 8 and 12 days of feeding.

As can be seen in Figure 9, the increase of the weight of the caterpillars is similar in all lines. There is no significant difference in the weight for the lines WT, 132_10, 134_10 and 147_2 after 5, 8 and 12 days of feeding.

4.5 O-*acyl sugars inhibit the germination and growth of* Fusarium brachygibbosum *on medium containing* O-*acyl sugars in vitro*

In order to evaluate the influence of *O*-acyl sugars on the germination success and hyphae growth of *Fusarium brachygibbosum*, spores of the fungus were placed on plain agar medium containing *O*-acyl sugars. The percentage of germinated spores and the length of germination tubes was evaluated using microscopy.

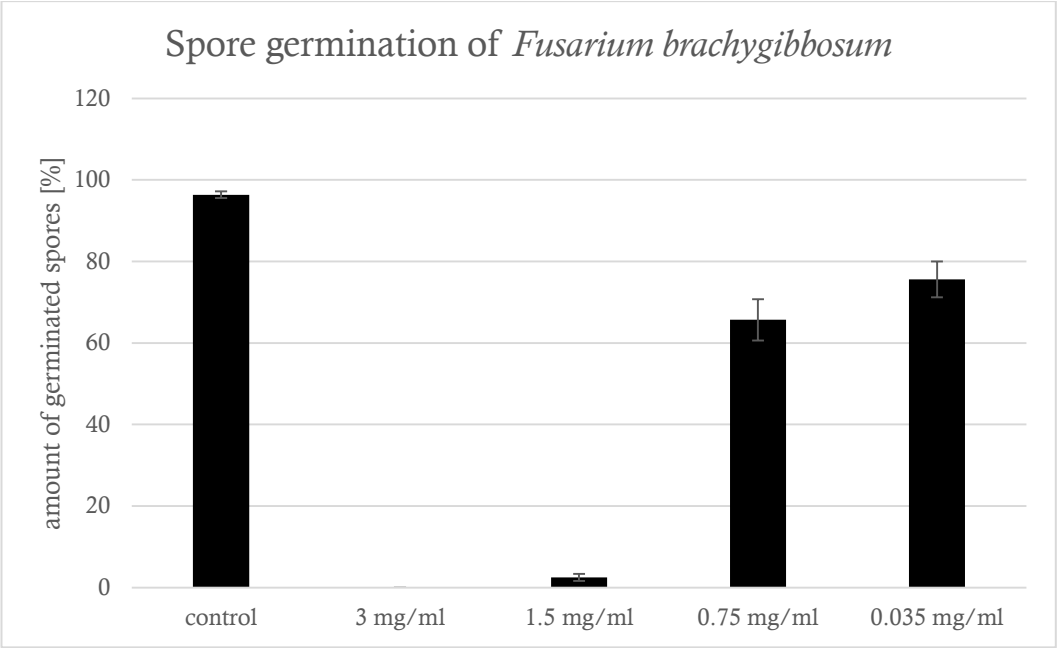

Figure 10: Amount of germinated *Fusarium brachygibbosum* spores on plain agar medium without *O*-acyl sugars (control) and with different *O*-acyl sugar concentrations (3 mg/ml, 1.5 mg/ml, 0.75 mg/ml, 0.035 mg/ml).

Figure 10 points out, how much the spore germination of *F. brachygibbosum* is hampered by *O*-acyl sugars. As mentioned before, no spores germinated at 3 mg/ml and very few at 1.5 mg/ml (about 97 % less compared to the control). Furthermore the germinated spores are reduced by around 32 % at 0.75 mg/ml and around 22 % at 0.035 mg/ml.

This results also show a strong, non-linear correlation between the amount of germinated spores and the *O*-acyl sugar concentration. The higher the *O*-acyl sugar concentration is, the less spores can germinate.

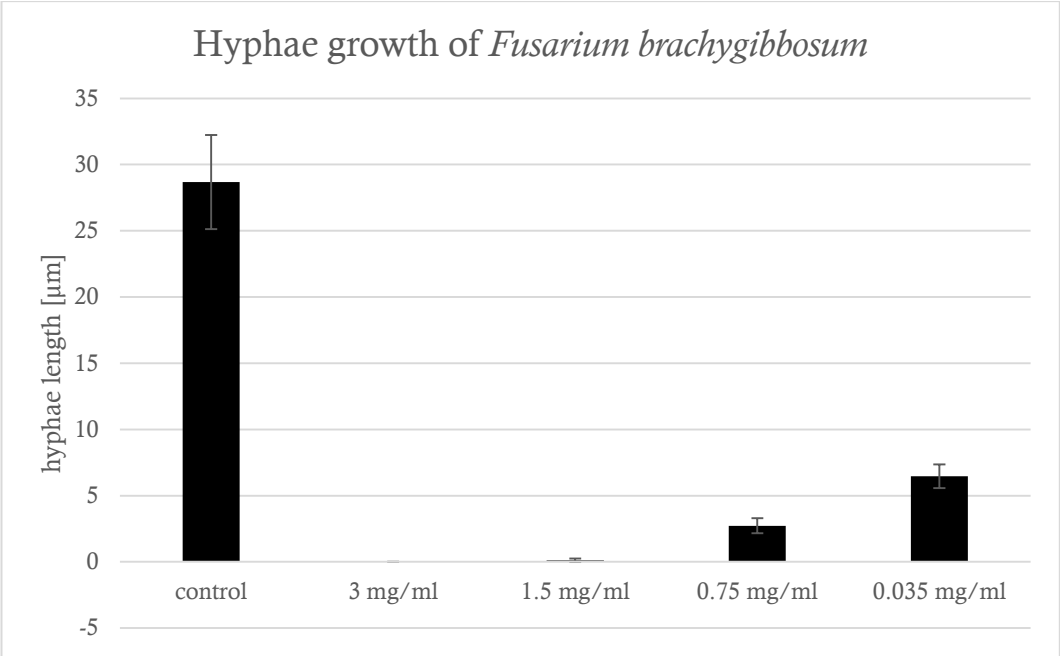

Figure 11: Length of the germination tube of *Fusarium brachygibbosum* spores on plain agar medium without *O*-acyl sugars (control) and with different *O*-acyl sugar concentrations (3 mg/ml, 1.5 mg/ml, 0.75 mg/ml, 0.035 mg/ml).

As shown in Figure 11, the growth of the germination tubes of *F. brachygibbosum* spores is highly impaired by *O*-acyl sugars. At a concentration of 3 mg/ml there is no growth possible. Similarly, at 1.5 mg/ml there is hardly any growth to be seen. In addition, the growth is reduced by about 91 % at 0.75 mg/ml and by about 77 % at 0.035 mg/ml compared to the control.

There is a strong, non-linear correlation between the length of the germination tubes and the *O*-acyl sugar concentration. These results indicate that the higher the *O*-acyl sugar concentration is, the more impaired is the fungal growth. This is in line with the effect of *O*-acyl sugars on the amount of germinated spores.

4.6 irBCKDE1B plants are more susceptible to the fungal pathogen Fusarium brachygibbosum *than WT plants*

To examine whether silencing *NaBCKDE1B* in trichomes could lead to a difference in pathogen susceptibility, a pathogen inoculation assay on this silenced plants in comparison to WT was performed. Fungal agar plugs were placed on detached leaves of three independent irBCKDE1B lines and the average diameter of necrotic lesions were measured after 8 days.

Figure 12: Picture of the average necrotic leaf area of wildtype and the lines 132_10, 134_10 and 147_2 after 8 days of inoculation.

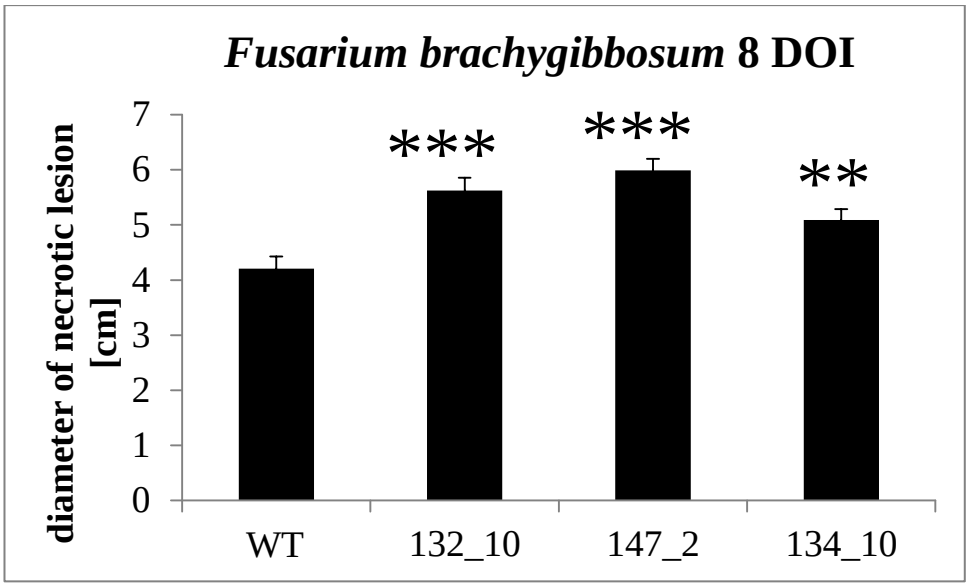

Figure 13: Necrotic lesion on leaves of the lines WT, 132_10, 147_2 and 134_10 after 8 days of inoculation with *Fusarium brachygibbosum.*

Figure 13 makes obvious, that the pathogen could infect a larger area of the leaves of all transformed lines than of wildtype after eight days of inoculation. The necrotic lesion is about 30 % higher in the silenced lines compared to WT. This result suggests that *NaBCKDE1B* plays an important role in pathogen susceptibility.

5 Discussion

This study showed that *NaBCKDE1B* takes part in *O*-acyl sugar biosynthesis in trichomes in *Nicotiana attenuata,* as silencing this gene could clearly reduce the *O*-acyl sugar content in trichomes as well as in the total leaf. Additionally, a reduced *O*-acyl sugar content enables the phytopathogen *Fusarium brachygibbosum* to grow faster on leaves. The reason for this could also be shown, as the toxicity of *O*-acyl sugars for *F. brachygibbosum* in vitro was proved. In contrast, *Manduca sexta´s* growth on intact plants was not promoted by silencing *NaBCKDE1B.*

The silencing efficiency of the target gene *NaBCKDE1B* in the trichomes was around 75 to 95 %. However, it did not cause a high reduction of *O*-acyl sugar content in both trichomes and total leaf. In the trichomes, line 132_10 had about 60 % reduction, 147_2 had around 40 % and 134_10 had no reduction at all. Despite the different amounts of *O*-acyl sugars in the trichomes of these lines, they had all about the same *O*-acyl sugar reduction for the total leaf with 20 to 30 %. The reason for this might be a transport of *O*-acyl sugars or precursors, which has not been discovered yet. For example could BCKD in the leaf tissue, where it is not silenced, create the activated branched-chain fatty acids bound to CoA, which could be transferred to the trichome cells through plasmodesmata and there used for *O*-acyl sugar biosynthesis. Another reason could be that the *O*-acyl sugar amount is not only dependent on the enzyme BCKD. It also depends on enzymes which cleave *O*-acyl sugars, like Acyl Sugar Hydrolases (ASH) (Schilmiller *et al.*, 2016). Probably they can be downregulated if less BCKD is active for *O*-acyl sugar synthesis. Also Acyl Transferases (AT) may play a role (Schilmiller *et al.*, 2012). Those enzymes exchange the acyl chains bound to the sugar backbone of *O*-acyl sugars. In this way it could be possible, that they can modify *O*-acyl sugars which are part of the cuticula in order to provide them for trichomes. Therefore, a caterpillar feeding assay on detached leaves of irBCKDE1B plants should be performed in the future.

Overall the *O*-acyl sugar reduction in the leaves of the transformed plants was slightly lower compared to the wildtype (20-30 % reduction). Therefore little or no effect on herbivores and pathogens was expected. This might explain, why the phenotype of the caterpillars fed on the transformed plants was not significantly different from wildtype. As the caterpillars eat the whole leaves or even the stem, the surface does not have so much influence on them. In addition, because we placed *M. sexta* directly on the plants for feeding, it is also possible, that the transgenic lines were able to upregulate other defense mechanisms. In this way there would be a similar stress on all caterpillars and the phenotype is the same in the end as on wildtype plants.

In contrast to the result of the feeding assay, the pathogen inoculation showed a highly significant effect of the *O*-acyl sugar amount on the leaf infection by

F. brachygibbosum. The reason for this could be that the spores of the fungus land on the surface of the plant, where there are many trichomes with the highest *O*-acyl sugar concentration on the droplet of the tip cell. As *O*-acyl sugars are toxic for the spores and impede their germination and growth, spores that come down on a droplet of a trichome might not have the chance to germinate. The higher the *O*-acyl sugar concentration, the less spores are able to germinate and grow. Consequently the growth of the fungus may highly depend on the *O*-acyl sugar content on the plant surface.

Future perspectives

Further experiments could investigate if there is any transport of *O*-acyl sugars or their precursors from the epidermal cells or other leaf tissue to the trichome cells. It would also be interesting to find out, how the *O*-acyl sugar content changes during the plant ontogenesis and if there is a diurnal change. Additionally, more enzymes should be analyzed which play an important role for the *O*-acyl sugar amount. They can be spotted by gene expression analysis and their effect could be evaluated by virus-induced gene silencing experiments.

By the discovery of the complete *O*-acyl sugar pathway, the synthesis of these defensive molecules could be promoted in species used for agriculture, like tobacco (*Nicotiana attenuata*), potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*). As those Solanacean plants are closely related to *N. attenuata* and produce *O*-acyl sugars in trichomes as well, the knowledge about the *O*-acyl sugar biosynthesis pathway in *N. attenuata* and required enzymes could be applied for increasing the plant defense of those species, even though the pathway slightly differs among them.

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B Supplemental Data

RNAi-construct sequence of *NaBCKDE1B*

GAAGATGTTGGTTTCGGTGGTGTCTTTCGTTGCACTACTGGATTAGCTGACCG
ATTTGGAAAACAGAGAGTTTTTAACACTCCTTTATGTGAGCAGGGCATAGTTG
GATTTGCTATTGGTCTGGCTGCAATGGACAATCGAGCTATAGCAGAAATTCA
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AATCTTTCTTCTGCCATGTTCCTGGTATAAAGGTG

Solanum lycopersicum SlAT2 Promoter

GTTTACACCAAATCAATACATACATACCATATATTTAATTTATAATTCATTTTA
CTTCATTAAATCACTTAATAATGTATTTTGCATTGACTTAGTGTAAACATACA
ACGCATATAAGTTTTTCCCATAAATATCTCCATGCATTATAGTTATATATTATT
AATTACAAATTAAATTATACAGAATTTTATTATTTTTCCAACTTGATTGTTAAA
GTTCTACTCTTGTTTTAAAAAATATATTTATTTAACCTGTTTTACCTATAGTAT
TTGTATGGGTGATATTATTTTCTAAACAATAAAAATTAATTATAGACAACTCT
TAAATTTTGGATGACTACTTAAAGGAATTAATTCCACCATTGAAAAATTCACT
ATATTTGAAGTAAATTCTAGAAACCAGCAAATCCTACTCCAAAAAGTTTTATA
GCTCTGAAAAATTATTAATAAGTTATGACATTTAAATCCTTCTCAACTTTTTCA
GCCGATTCACGTAGAAGAATGTTGCGTATGAGGCCCTAAAATTAATTGTCACT
TGTAGGGGTGGAAAAATAGACTAAACCTATATATATATTATATTATATGGTTA
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TAATTAAGATATTATTTTTAATAAACACTTAAAAATTCTTCAAAATAATTGGA
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CTTACTTTCAGAGTAACTTTCTTTTATCTCCAAAGGAAAAGGCTTGGTTGAAG
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CACAATAATGCTAATTATTGTAGGAATGGCTTTATCCCAAGACAAAATGAGT
CCAAATGAGTTTTCACTTTCCCAATCAAGGACATTCTAGTTCGACATGCTACT
TTCACATTCTCAATTTATTTTTAAATATTTGATAGATGAGTGAAACTTAATTTT
CGATTTTTAGTTAGTGAGTAAATTTTCTCTCAGAATAGTTTTGGTTATGAGGA
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AAGAAAATGCAACAGGTAATATATAGCGACATTTGACTGATATGATTTAAAA
TATTTACCCTGAAAATTTTGTAATTGTAAAAACTTTTAGCGTAATTAACCGCC
CAAATAAATTCTGTAGTATATACACTAGTGTTTGTTCTGAACAATGCACTTTA
TGAATGTTAAATGCAATATATTAAAGATTGACGCCATGCCAAACTATATATAT
AATAGGAATGAACATTGATAGATTTATTCAGAAACAACACTTTATTCATCCAA
AAAAAAG

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Jena, den 10.09.2016 _____

Stefanie Dreßel