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Identification and Characterization of Cytochrome P450 Monooxygenases crucial for the Biosynthesis of defensive Isoxazolin-5-one derivatives in the poplar leaf beetle, *Chrysomela populi*.

Master Thesis

To Fulfill the Requirements for the Degree of

Master of Science (M. Sc.)

Submitted by

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1. INDEX OF ABBREVIATIONS

3HAPA	3(hydroxyamino)-propanoic acid
3NPA	3-nitropropanoic acid
ACN	Acetonitrile
BLAST	basic local alignment search tool
bp	base pairs
BSA	Bovine Serum Albumin
C. populi	*Chrysomela populi*
cDNA	complementary deoxyribonucleic acid
CoA	Coenzyme A
C-terminus	Carboxyl- terminus
CYP	Cytochrome P450 Monooxygenase
Da	Dalton
DAP	Detection-of-Digoxigenin-AP
DAPI	4',6-diamidino-2-phenylindole
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxyribonucleoside triphosphate
dsRNA	double-stranded ribonucleic acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid
EF1α	Elongation Factor 1α
eIF4α	eukaryotic Initiation Factor 4α
FB	Fractionation buffer
g	gravitational force
GFP	Green fluorescent protein
GMC	Glucose-Methanol-Choline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
LB	Lysogeny broth
LC/MS	liquid chromatography/ mass spectrometry
LSM	Laser scanning microscopy
m/z	Mass-to-charge ratio
mRNA	Messenger ribonucleic acid
n	number of replicates
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
N-terminus	Amino- terminus
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pfam	Protein families
PVDF	Polyvinylidene fluoride
qPCR	quantitative Polymerase chain reaction
RB	Resuspension buffer
RNA	ribonucleic acid
RNA-FISH	RNA- fluorescent *in situ* hybridization
RNAi	RNA interference
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
Sf9	Spodopterafrugiperda 9
SIL-IS	Stable isotope labeled internal standards
SSC	Saline-sodium-citrate
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween-20
UV-light	Ultra-Violette light

2. INTRODUCTION

2.1 The leaf beetle *Chrysomela populi*

Leaf beetles (Chrysomelidae: Coleoptera) can be found nearly on every green plant on earth. They represent the most prevalent beetle group with more than 50.000 described species whereby 470 can be found in Germany (Jolivet, et al., 1995). They fulfill the role as herbivores (feeding on different host plants), as well as prey for different insectivores and are therefore incorporated into a complex food web.

The life history of many leaf beetle species is often tightly connected to a specific host and food plant. Over time, beetles have adapted to these distinct food sources and overcame the mechanism in the plant's chemical defense. This adapting process was often challenged by developing new mechanisms in the plant's protection but also by gaining resistance strategies on the beetle site. Thus, some of the today's leaf beetles are highly adaptedto their host(Zahradnik, et al., 1985).

One example of this highly specialized leaf beetle is *Chrysomela populi* (Chrysomelinae: Chrysomelidae) (Figure 1).

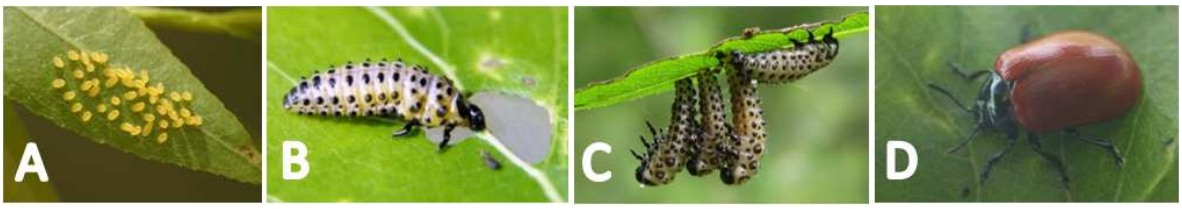


Figure 1: *Chrysomela populi* at different life stages.
The life cycle starts with ovipositedeggs (A). After eclosure of larvae (B), they will feed upon the host plant till they become prepupae (C) and pupae. After 10 days the adults (D) emerge and will produce the next generation.

Host plants of *C. populi* are poplar (*Populus sp.*) and willow (*Salix sp.*) trees, where the whole lifecycle of the insects takes place. The fertilized eggs are oviposited on the bottom side of the host plant leaf and larvae hatch after 12 days. While feeding and growing, larvae undergo three molting stages till they start to pupate after around 21 days. Approximately 10 days later, the pupae give rise to the imago which further produces the

next generation (2-3 per year). Upon mass reproduction, they can cause significant damage to salicaceous trees, especially in monocultural plantations(Brauns, 1991).

During the different life stages, the beetles and their juveniles have to face not only plant defenses but also an array of predators including birds, reptiles, spiders or ants. Additionally, they are also threatened by microbial invaders like fungi (Clarkson, et al., 1996) and bacteria (Gross, et al., 2008). Further natural enemies are tachina flies (*Tachinidae*) and ichneumon wasps (*Ichneumonidae*) which appear as parasitoids (Zvereva, et al., 2004) as well as larvae of some hoverfly species (e.g. *Xanthandruscomptus*), which feed on leaf beetles (Almohamad, et al., 2009). To affront these dangers, they have developed a wide variety of synergistic chemical and behavioral defensive mechanism.

2.2 The chemical defense of *Chrysomela populi*

2.2.1 *Chrysomela populi* can defend itself in all life stages

C. populi owns different allomones to defend themselves during all life stages via sequestered or *de novo* synthesized compounds (Pasteels, et al., 1988; Pauls, et al., 2016).

During thelarval stage, *C. populi* is capable of taking up plant derived phenol glycosides like salicin and salicortin(Bruckmann M, 2002) and use these compounds as precursors for the production of the repellent volatile salicylaldehyde, which is stored in defensive glandular reservoirs. Upon disturbance, nine pairs of defensive glands can be everted to expose this compound (Pasteels, et al., 1989).

To maintain this defensive line in *C. populi* eggs, the adults, which don't use the plant-derived glycosides for their own defense, transmit salicin in high concentrations to their offspring. Consequently, one egg can contain lethal concentrations for e.g. predatory insects like ants(Pasteels, et al., 1986). After hatching of neonate larvae, they can directly use the salicin pool to synthesize salicylaldehyde and build up their defensive secretions(Termonia, et al.).

The adult beetles have an aposematic red warning color on their elytra, to indicate their toxicity to animal predators. But as mentioned above, this toxicity is not indicating the

presence of salicin derived compounds but rather the non-volatile 3-nitropropanoic acid (3NPA). To prevent self-intoxication, this substance is stored as the ester **2** of theisoxazolin-5-one-glycoside **1**(Figure 2)in defensive glands on their elytra and can exhibit its biological activity through the release of toxic 3NPA in the predatory animals. Additionally, one to three 3NPA moieties can be stored via coupling to bare glucose and released if required (Pauls, et al., 2016).

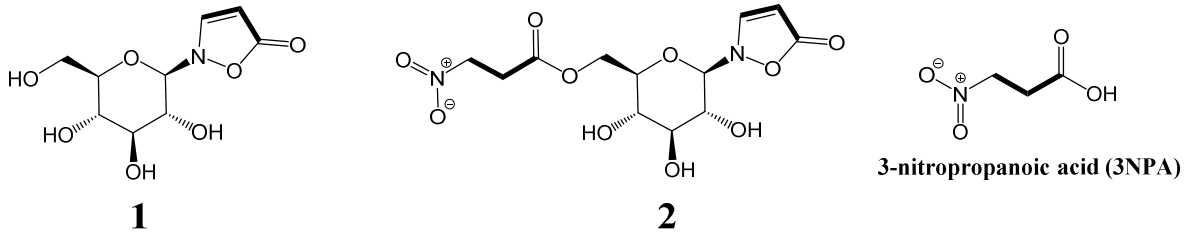


Figure 2: Compounds involved in non-volatile leaf beetle defense.
Isoxazolin-5-one glycoside (**1**) can be coupled with 3NPA to form **2**, which can be stored without any harmful effects.

These compounds, which were assumed to be only present in the adults (and as an impregnation to protect the eggs) (Pasteels, et al., 1986) had been found recently in all life stages of the leaf beetles subtribe Chrysomelina and had been identified as a taxonomic chemomarker for this tribe (Pauls, et al., 2016).

Up to date, the following defensive lines have been published regarding different life stages in *C. populi*:

Table 1: Defensive lines in different life stages of *C.populi*.
* = transmitted by the parental generation

Life Stage	Defensive line	
	based on sequestered phenol glycosides(e.g. salicin)	based on 3NPA and Isoxazolinones
Egg	(✓)*	(✓)*
Larva	✓	✓
Pupa	×	✓
Adult	×	✓

The defensive compounds are located in different organs depending on the life stage of*C. populi*. The eggs contain salicin and the 3NPA-ester is part of the shell's surface. If **2** is present inside the eggs has not yet been determined. In the larvae, the salicin based

defense is localized in the defensive glands, while compound **2** is only present in the hemolymph. Up to date, no 3NPA based defensive compounds have been detected in the secretions of Chrysomelinalarvae. In adults, the defense which relies on salicin derived compounds is abolished and **2** is now used in the defensive secretionsof the elytral glands and in the hemolymph instead of salicylaldehyde(Becker, et al., 2016; Burse, et al., 2009; Pasteels, et al., 1986; Pauls, et al., 2016)

2.2.2 Defensive mechanism in *Chrysomela populi* based on 3NPA

In all life stages, *C. populi* is capable of defending itself with 3NPA esters (**2**). Upon consumption of **2** by a predator the final toxic agent 3-nitropropanoic acid can be released through a cleavage of the ester bond and leads to suicide inhibition in mitochondrial respiration because of its high similarity to succinic acid (Alston, et al., 1977). Under physiological conditions, the similarity is much more obvious because both molecules exist in the deprotonated form (Figure 3) (Francis, et al., 2013). Thus, it can be bound by succinate - dehydrogenase (E.C. 1.3.5.1) and interferes with the enzymatic catalytic cycle leading to a covalent adduct which further leads to inhibition of the mitochondrial complex II and disruption of the citric acid cycle, an important metabolic pathway that maintains energy supply (Huang, et al., 2006).

Therefore, intoxication with 3NPA causes a reduction till depression in ATP production which affects cells with high metabolic turnover rates at first. They are especially of nerval origin like striatal cells which are involved in the animals “reward system” (Yager, et al., 2015). High doses consumed by mammals can result in symptoms similar to Huntington’s disease (Brouillet, et al., 2005). In contrast, no toxic properties of compound **1** have been proven to date, either for insect competitors or for different phyto-, entomo- or human pathogens (Pasteels, et al., 1986).

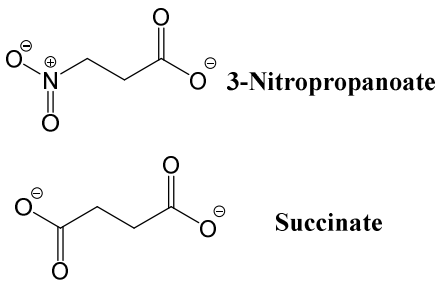


Figure 3: Structural similarities between 3-Nitropropanoate and succinate.

Giving that, the defensive compounds of Chrysomelina leaf beetles are able to wield influence on the nervous system of their predators directly. The combination of this and the aposematic warning make predators remember and to avoid further consumption of

the beetles (Ruxton, et al., 2004). Regarding larval defense and the missing red warning color, it has been suggested that olfaction of volatiles in the exudates may be the crucial component for conditioning of predators (Eisner, et al., 1981).

2.3 The Biosynthesis of Isoxazolinoneglycosides in leaf beetles

It was assumed that Chrysomelina leaf beetles produce Isoxazolinone glycosides and its 3NPA esters the same route as described in fungi, starting from aspartate as the initial precursor (Birch, et al., 1960). However, the pathway in the beetles has been revised recently (Becker, et al., 2016).

In *C. populi* and the other members of the Chrysomelina subtribe, the biosynthesis likely starts from theplant-derived β-alanine, which can be synthesized also *de novo* from valine via propanoyl - Coenzyme A, which is further oxidized to form either the 3NPA or the Isoxazolinone moiety. Both are finally coupled with glucose to form compound **2**(Figure 4).

Till date, the enzymes which are involved in the three oxidation steps (Figure 4, “?”) remain elusive but some microbial relatives belonging to the Cytochrome P450 Monooxygenase superfamily show slight similarities regarding the ability to perform oxidations of amino functions(Winkler, et al., 2007).Additionally, some similar reactions are described for Cytochrome P450 enzymes derived from rat and rabbit liver microsomes (Hlavica, 2002; Ji, et al., 2013). Therefore, the implication of CYP P450s is most likely.

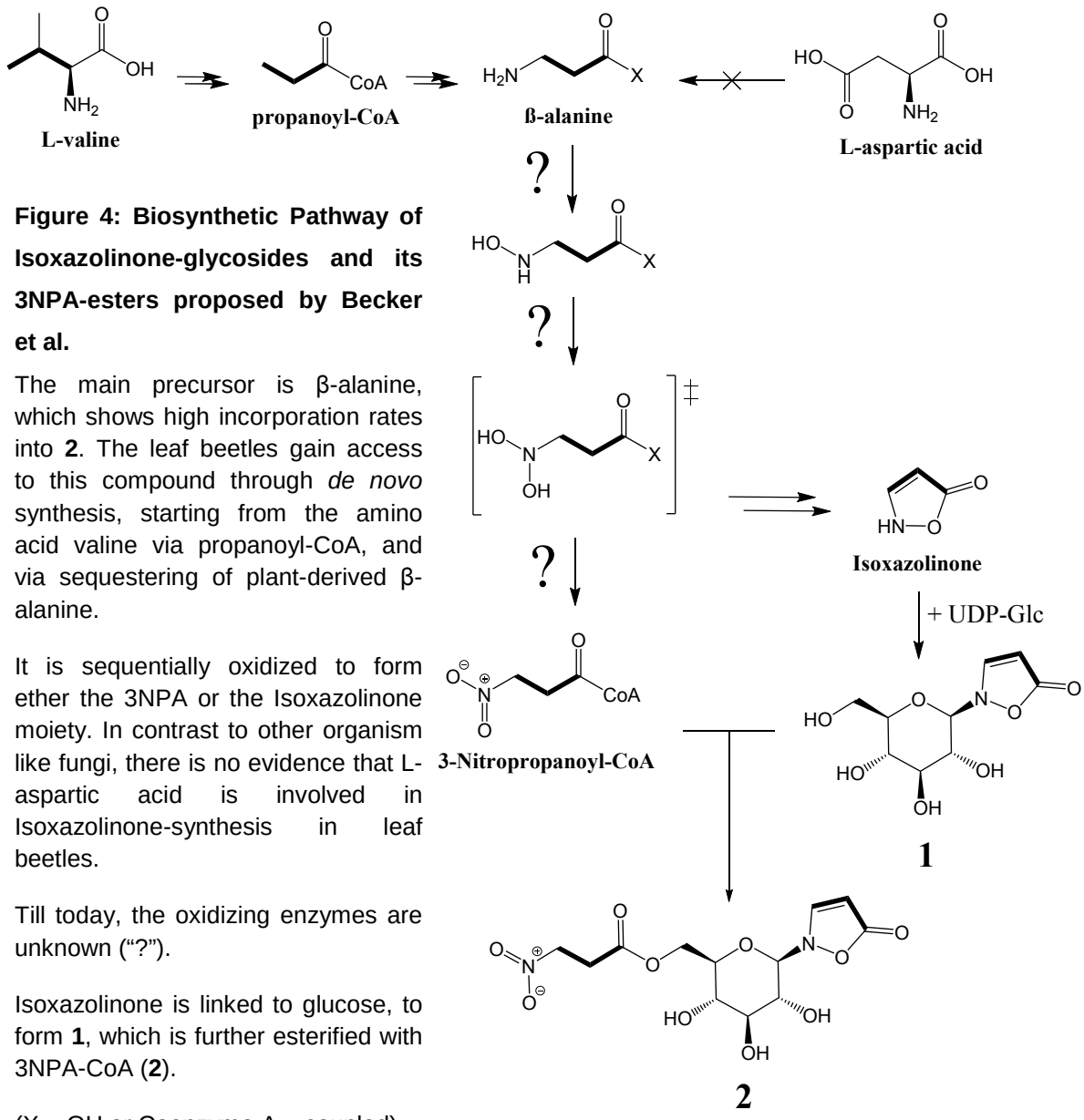


Figure 4: Biosynthetic Pathway of Isoxazolinone-glycosides and its 3NPA-esters proposed by Becker et al.

The main precursor is β-alanine, which shows high incorporation rates into **2**. The leaf beetles gain access to this compound through *de novo* synthesis, starting from the amino acid valine via propanoyl-CoA, and via sequestering of plant-derived β-alanine.

It is sequentially oxidized to form ether the 3NPA or the Isoxazolinone moiety. In contrast to other organism like fungi, there is no evidence that L-aspartic acid is involved in Isoxazolinone-synthesis in leaf beetles.

Till today, the oxidizing enzymes are unknown ("?").

Isoxazolinone is linked to glucose, to form **1**, which is further esterified with 3NPA-CoA (**2**).

(X = OH or Coenzyme A – coupled)

2.4 Cytochrome P450s may be involved in Isoxazolinone and 3NPA synthesis

Following the findingsof Becker et al. (2016), this study attempts to reveal proteins of the Cytochrome P450 family, which are involved in Isoxazolinone and 3NPA synthesis.

2.4.1 Cytochrome P450s are a member-rich protein superfamily

Cytochrome P450 Monooxygenases (CYPs; EC 1.14.-.-) are a widespread class of enzymes involved in the metabolism of endogenous and exogenous compounds and can be found in all kingdoms of life(Hodgson, 1985). In general, CYPs catalyze the following reaction by oxidizing a substrate (R) with the use of molecular oxygen:

$$\mathbf{R}H + O_2 + NADPH + H^+ \longrightarrow \mathbf{R}OH + H_2O + NADP^+$$

Due to high diversity in CYPs regarding the huge number of isoforms and broad ranging specificity, they are capable of metabolizing a myriad of different substrates(Guengerich, 1996). Most of the CYPs of animals are located in cellular membranes which derive from the endoplasmic reticulum and need an additional P450 reductase for electron supply(Peterson, et al., 1986), while bacterial CYPs are soluble and located in the cytoplasm.CYPs contain heme as an important cofactor and can be identified by their heme binding motive (commonly FXXGXXXCXG; Pfam: PF00067)(Nelson, 2006).

2.4.2 Cytochrome P450s perform different tasks in insects

In insects, CYPs are distributed in different tissues and their appearance depends on life stage, sex or diet of the organism. Therefore, the expression pattern of P450s can be totally diverse (White, et al., 1999). Typically, the highest CYP activity can be found in midgut tissue (Hodgson, 1985), but P450s are also located in thefat body, Malpighian tubules, nervous system(Korytko, et al., 1998) and antennae (Wojtasek, et al., 1999).

According to the variable distribution of CYPs, they also fulfill ahuge variety of functions and are involved in both anabolic and catabolic metabolism. Some are responsible for the production of juvenile hormones and ecdysteroidsand therefore occupy an important role in development and mating processes. Others are involved in the degradation of exogenous compounds like plant derived allelochemicals and insecticides(Feyereisen, 1999). The latter can have serious consequences for wildlife, environment, and economy(Scott, 1991).

3. AIM OF THIS WORK

Till today, the biosynthesis of Isoxazolinone-glycosides and its 3NPA esters was investigated from a chemical point of view, but no work was done trying to elucidate the biochemical aspects. In this thesis, the main aim is to find out, which enzymes are involved in the predicted three oxidation reactions (compare figure 4) in Isoxazolinone and 3NPA synthesis. Because previous studies suggesting the involvement of Cytochrome P450 Monooxygenases, this work focuses on this class of enzymes which may be incorporated in the anabolism of **2**.The proteins shall be identified and selected via a proteomics based procedure. Using a loss-of-function approach via RNA interference (RNAi), the *in vivo* importance shall be analyzed during different life stages of *C. populi* and the proteins shall be localized on acellular level using RNA-FISH. At the end, the enzymesshall be expressed heterologously and characterized regarding activity and natural substrates.

4. MATERIAL AND METHODS

4.1 Material

4.1.1 Chemicals

Table 2: Overview of Chemicals used

Substance	Manufacturer
Acetonitrile	Fisher Scientific
Agarose NEEO Ultra-Quality (for electrophoresis)	Carl Roth GmbH & Co. (Karlsruhe)
Bromophenolblue	Fluka Chemie AG (Buchs, Schweiz)
BSA	Carl Roth GmbH & Co. (Karlsruhe)
Coomassie Plus Solution for Bradford-Assay	(Pierce™, Thermo Scientific)
Co-Precipitant Pink	Bioline
DAPI	Sigma-Aldrich Co. LLC (St. Louis, USA)
Dextranesulfate	Sigma-Aldrich Co. LLC (St. Louis, USA)
Dipotassiumphosphate(K_2HPO_4)	Carl Roth GmbH & Co. (Karlsruhe)
Disodiumphosphate(Na_2HPO_4)	Sigma-Aldrich Co. LLC (St. Louis, USA)
Dithiothreitol (DTT)	Carl Roth GmbH & Co. (Karlsruhe)
EDTA	Carl Roth GmbH & Co. (Karlsruhe)
Ethanol	Sigma-Aldrich Co. LLC (St. Louis, USA)
Formaldehyde	Sigma-Aldrich Co. LLC (St. Louis, USA)
Formamide	Fluka Chemie AG (Buchs, Schweiz)
Glucose	Carl Roth GmbH & Co. (Karlsruhe)

Glycerol	Carl Roth GmbH & Co. (Karlsruhe)
HEPES	Carl Roth GmbH & Co. (Karlsruhe)
LB	Carl Roth GmbH & Co. (Karlsruhe)
LB - Agar	Carl Roth GmbH & Co. (Karlsruhe)
Magnesiumchloride($MgCl_2$)	Merck KGaA (Darmstadt)
Magnesiumsulfate($MgSO_4$)	Carl Roth GmbH & Co. (Karlsruhe)
Maleicacid	Sigma-Aldrich Co. LLC (St. Louis, USA)
Methanol	Sigma-Aldrich Co. LLC (St. Louis, USA)
Monopotassiumphosphate(KH_2PO_4)	Carl Roth GmbH & Co. (Karlsruhe)
Monosodiumphosphate(NaH_2PO_4)	Carl Roth GmbH & Co. (Karlsruhe)
Paraffin wax	Carl Roth GmbH & Co. (Karlsruhe)
Paraoxon	Sigma-Aldrich Co. LLC (St. Louis, USA)
Potassiumchloride(KCl)	Carl Roth GmbH & Co. (Karlsruhe)
Protease Inhibitor Mix	SERVA Electrophoresis GmbH (Heidelberg)
Roti®-Blue	Carl Roth GmbH & Co. (Karlsruhe)
Sodiumcitrate	Carl Roth GmbH & Co. (Karlsruhe)
Sodiumchloride(NaCl)	Carl Roth GmbH & Co. (Karlsruhe)
Sodiumdodecylsulfate(SDS)	Carl Roth GmbH & Co. (Karlsruhe)
Sucrose	Carl Roth GmbH & Co. (Karlsruhe)
Tris PUFFERAN	Carl Roth GmbH & Co. (Karlsruhe)
Triton x-100	Sigma-Aldrich Co. LLC (St. Louis, USA)
Tween 20 (10% in solution)	Bio-Rad Laboratories Inc.
Xylol	Carl Roth GmbH & Co. (Karlsruhe)
β-Mercaptoethanol	Fluka Chemie AG (Buchs, Schweiz)

4.1.2 Buffersandreactionmixtures

4.1.2.1 Differential Fractionation for proteomic analysis and microsome preparation

Name	**Ingredients**
FB (FractionationBuffer) (pH 7.4)	250 mM sucrose 20 mM HEPES 10 mMKCl 1,5 mM $MgCl_2$ 1 mM DTT (added fresh) 1X Protease Inhibitor Mix (added fresh)
Hypotonic Buffer (pH 7.5)	20 mMTris 5mM EDTA 20% (v/v) Glycerol 1 mM DTT (added fresh) 1X Protease Inhibitor Mix (added fresh)
RB (Resuspension Buffer) (pH 7.4)	100 mM potassium phosphate buffer 20% (v/v) Glycerol 1X Protease Inhibitor Mix (added fresh)

Sucrose Buffer (pH 7.5)	20 mMTris 5 mM EDTA 500 mM Sucrose 20% (v/v) Glycerol 1 mM DTT (added fresh) 1X Protease Inhibitor Mix (added fresh)

4.1.2.2 RNA – FISH

Name	**Ingredients**
Blocking solution (pH 9.5)	0.1 M maleic acid 0.15 M NaCl 10% blocking reagent (Roche)
DAP - Solution	0.1 M TRIS 0.1 M NaCl 50 mM $MgCl_2$ pH 8.0
Hybridisationbuffer	10% Dextrane sulfate 2 x SSC 0.2 mg/ml sonic. heringsperm(Invitrogen) 50% formamide
PBS (10x) (pH 7.1)	8.5% NaCl (w/v) 14 mM KH_2PO_4 80 mM Na_2HPO_4
SSC (20x)	3 M NaCl 0.3 M sodium citrate pH 7.1
TBS (5x) (pH 7.5)	100 mM TRIS 150 mM NaCl

Name	**Manufacturer**
HNPP Fluorescentdetectionset	Sigma-Aldrich Co. LLC (St. Louis, USA)
Anti-DigoxigeninAP, Fab fragments (sheep)	Sigma-Aldrich Co. LLC (St. Louis, USA)

4.1.2.3 Western Blot

Name	**Ingredients**
TBS-T (1x) (pH 7.5)	100 mM TRIS 150 mM NaCl 0.05% Tween 20

Name	Manufacturer
6x-His Tag Monoclonal Antibody (HIS.H8)	ThermoFisher Scientific

4.1.2.4 Miscellaneous

Name	Ingredients
SDS loadingbuffer (6x) (pH 6.8)	375 mM Tris-HCl 6% SDS (w/v) 48% glycerol (v/v) 1.32 M β-Mercaptoethanol 0.03% bromophenol blue (w/v)

Name	Manufacturer
10 x TBE	Bio-Rad Laboratories Inc.

4.1.3 Media

Name	Ingredients
LB Agar (Lennox) for plates	35 g/l LB Agar
LB Medium (Lennox) for liquid culture	20 g/l LB

Name	Manufacturer
Insect cell media SF 900 II	ThermoFisher Scientific

4.1.4 Antibiotics

Name	Manufacturer
Kanamycin (50 µg/ml)	Carl Roth GmbH & Co. (Karlsruhe)
Zeocin (100 µg/ml)	ThermoFisher Scientific

4.1.5 Cells

Name	Manufacturer
One Shot® TOP10 competent *E. coli*	Invitrogen
Spodopterafrugiperda (Sf)9	ThermoFisher Scientific

4.1.6 Plasmids

Name	Manufacturer
pCR™ - Blunt II – TOPO® vector	ThermoFisher Scientific
pIB/V5-His TOPO® vector	ThermoFisher Scientific

4.1.7 Enzymes

Name	Manufacturer
HF- Phusion® DNA Polymerase	New England Biolabs
GoTaq® DNA Polymerase	Promega
BamHI - FD	ThermoFisher Scientific

4.1.8 Oligonucleotides

In the following table, all Primers are listed, that had been used in this study. Oligoshave been synthesized by Eurofins Genomics which also calculated melting temperature (Tm).

Table 3: Oligonucleotides included in this study

Code	Sequence	Tm (°C)
Primers for qPCR analysis		
CL3179 fwd	AGCCTTTCAAACTGCTGCTG	57,3
CL3179 rev	ATGGTGTTGCTTTGCATGCC	57,3
CL7046 fwd	TTTCGGACCCGTTGAAGTTC	57,3
CL7046 rev	GCTCTTCATCTCCAACATTGCG	60,3
CL7715 fwd	TGCGCCCAAAACTTTGTCAG	57,3
CL7715 rev	AAGCCGTCGTAGCAATGATG	57,3
200 fwd	TGTGTTGGTCGCAAATACGC	57,3
200 rev	TGAAACCTTCCTCCCTCTTCAG	60,3
619_fwd_qPCR	ACAAAAAGGGCACAGCTGAC	57,3
619_rev_qPCR	TTTCCCTGGCGTCAAAACAC	57,3
1318_fwd_qPCR	ACGTATCTGGCTTTTGGTGGAG	60,3
1318_rev_qPCR	TTCTGATGAGGTGGGCAACTC	59,8
195 (1) fwd	TCGGACACAAGTTCGCAATG	57,3
195 (1) rev	TTCTGATTCTGAGGGGGTCAAC	60,3
195 (2) fwd	TTCACTTGCGTCACACCAAG	57,3
195 (2) rev	TTTCTCGAAGTCTGCCCTCTTC	60,3
Cp_EF1α_fwd	TCATCGGTCACGTAGATTCTGG	60,3
Cp_EF1α_rev	TTTCGATGGTACGCTTGTCG	57,3
Cp_eIF4α_fwd	TTTGTAATACCCGCCGCAAG	57,3
Cp_eIF4α_rev	TCCATGCATCGCAGAAACAG	57,3
Primers for ORF amplification		
CL3179 ORF fwd	AGTACTGGAAAACCAGGAATGTGC	61,0
CL3179 ORF rev	CTCAGCCACACATCACCTTTTTTC	61,0
CL7046 ORF fwd	AAAAATAAGAGCTCCGTTTGCCG	58,9
CL7046 ORF rev	GAGAAAATTGTCGTTCATGTGCTCC	61,3
CL7715 ORF fwd	CAAGGACTTCGACCATTTCACAGA	61,0
CL7715 ORF rev	TGACGTTCGCCTTATTCTCGTC	60,3
195 (1) ORF fwd	CAAGTTATAAGTGGTTATGGATCTTGT	58,9
195 (1) ORF rev	AGCAATCATGAACAAGATTCATTGGA	58,5
195 (2) ORF fwd	TGACATGACAATCGGTCTTCTGATT	59,7
195 (2) ORF rev	ACGGTGGTATTGAAAACAATTGAGTG	60,1

200_ORF_fwd	TGAGAATATCAAACAGCAGAATCGA	59,3
200_ORF_rev	GGATATTGAACAATGTCACAGCCA	59,3
619 ORF fwd	CGACCAAAGATCCAGACCTTTCAT	61,0
619 ORF rev	TCGAGAGACAGTCTGAGATGGTTC	62,7
1318 ORF fwd	ATTGGAAGATGGGGCAGTGTATTC	61,0
1318_ORF_rev	GGCAACATGATCAAAATGTGGTATG	62,7
2117 ORF fwd	GTTTATGTTGGTGGACGTGTTGTG	61,0
2117 ORF rev	TCAATTTGGATCATACGATTTCCCCA	60,1
Primers for amplification of RNAi fragments		
1318_RNAi_fwd	AGTTAGGAAGATGGCAGATCACT	58,9
1318_RNAi_rev	CTGCAGGGTGATCTCGATGG	61,4
CL7715_RNAi_fwd	GAGTGCGCCCAAAACTTTGT	57,3
CL7715_RNAi_rev	TAAGCATGTCATGACGCACG	57,3
195(2)_RNAi_fwd	TCATCGGATGTGCCCTTCAA	57,3
195(2)_RNAi_rev	CCAGATCTTGGTGTGACGCA	59,4
200_RNAi_fwd	TCACAGCCACAAGTGCTACC	59,4
200_RNAi_rev	ACGAGCTGATTGGCGTTTTT	55,2
Primers to add T7 sequence		
1318_T7_RNAi_fwd	TAATACGACTCACTATAGGGAGAAGTTAGGAAGATGGCAGATCACT	72,1
1318_T7_RNAi_rev	TAATACGACTCACTATAGGGAGACTGCAGGGTGATCTCGATGG	74,2
CL7715_T7_RNAi_fwd	TAATACGACTCACTATAGGGAGAGAGTGCGCCCAAAACTTTGT	72,3
CL7715_T7_RNAi_rev	TAATACGACTCACTATAGGGAGATAAGCATGTCATGACGCACG	72,3
195(2)_T7_RNAi_fwd	TAATACGACTCACTATAGGGAGATCATCGGATGTGCCCTTCAA	72,3
195(2)_T7_RNAi_rev	TAATACGACTCACTATAGGGAGACCAGATCTTGGTGTGACGCA	73,3
200_T7_RNAi_fwd	TAATACGACTCACTATAGGGAGATCACAGCCACAAGTGCTACC	73,3
200_T7_RNAi_rev	TAATACGACTCACTATAGGGAGAACGAGCTGATTGGCGTTTTT	71,3
Primers for cloning into pIB Vector		
ORFStart1318Cp	ATGTTGTTCGTTGTGATAGTGGT	65,5
1318_pET101_rv	CGGGTCTATCTTGCGGATATCC	62,1
PrimersforColony PCR		
M13_fwd_(-20)	GTAAAACGACGGCCAGT	52,8
M13_rev_(-24)	AACAGCTATGACCATG	46,6

4.1.9 Kits

Name	Manufacturer
RNAqueous® Total RNA Isolation Kit	ThermoFisher Scientific
RNAqueous® - Micro Total RNA Isolation Kit	ThermoFisher Scientific
Superscript™ III Reverse Transcriptase	Invitrogen
MEGAScript® RNAi Kit	Ambion

4.1.10 Equipment forelectrophoresis

Substance	Manufacturer
Spectra™ Multicolor Broad Range Protein Ladder	ThermoFisher Scientific
PageRuler™ Prestained Protein Ladder	ThermoFisher Scientific
6x Orange DNA loading dye	ThermoFisher Scientific
GeneRuler™ 1kb Plus DNA ladder	ThermoFisher Scientific

4.2 Methods

4.2.1 Differential Centrifugation and proteomic analysis

Eight larvae of *C. populi* were dissected to remove thehead, gut, and Malpighian tubules and each fat body tissue wasground in 150 µl Fractionation Buffer (FB) at 1200 rpm, 40 sec + 2x 30 sec, 4°C (2010 Geno/Grinder®, SPEX®SamplePrep). The lysate was removed and the remains washed with 200 µl FB. The lysates were combined, filled up with FB to 500 µl and centrifuged at 1200 x g, 10 min, 4°C to remove cellular debris. The supernatant was further centrifuged (10.000 x g, 30 min, 4°C) to pellet mitochondrial and nuclear proteins. The supernatant was ultracentrifuged (100.000 x g, 60 min, 4°C) to yield cytosolic proteins in the supernatant, which were concentrated using Ethanol precipitation. The pellet was resuspended in 500 µl Resuspension Buffer (RB) and ultracentrifuged (100.000 x g, 60 min, 4°C) to separate membrane (pellet) and membrane-associated proteins (supernatant).

Each fraction was mixed with SDS loading buffer (1x), cooked (15 min, 80°C) and loaded on a Gel (Mini-Protean® TGX™ precast Gels, any kd, Bio-Rad Laboratories)for SDS-PAGE (running conditions: 90V, 120 min). The Gel was then submitted to our Proteomic Department for analysis.

4.2.2 Bradford-Assay

To check protein concentrations prior to further approaches, a Bradford-Assay was performed.100 µl of each standard (BSA, 1 µg/ml – 50 µg/ml), blank or unknown sample was mixed with 100 µl Coomassie Plus Solution (Pierce™, Thermo Scientific) in a microplate, shook for 30 sec and stored at room temperature for 10 min. Absorbance at 595 nm was quantified with a spectrophotometer (BioPhotometer, Eppendorf)

4.2.3 Rearing of Insects

C. populi (larvae) was collected near Dornburg, Germany on *Populusmaximowiczii* × *Populusnigra* in 2015 and overwintered to 2016. The insects were propagated using a 16 h light (18 ± 2 °C) and 8 h dark (13 ± 2 °C) cycle in a Snijder chamber (Snijder Scientific, Tilburg, Netherlands). The food was applied twice a week (fresh poplar leafs).

4.2.4 Dissecting of larval and adult *C. populi*

Larvae and adult beetles were kept on ice for approximately 5 min to reduce moving speed and experience of pain. Then, they were decapitated immediately by using forceps and scientific scissors (Fine Science Tools GmbH, Heidelberg, Germany). To extract larval fat body tissue, they were cut on the ventral side from head to tail and gut as well as Malpighian tubules were removed and kept separately. The fat body was used for RNA extraction.

To collect adult elytra and wings, they were uplifted and cut at the pterathorax connection. They remainings were kept for comparing analysis. Also, from those tissues RNA was extracted and analyzed.

4.2.5 Total RNA isolation and cDNA synthesis

To get insights into the quality and quantity of total mRNA of larval and adult *C. populi* tissues, RNA was isolated and transcribed into cDNA.

RNA was isolated depending on the respective tissues. For large tissues like total larva(> 15 mg) and small tissues like Malpighian Tubules (<15 mg), the RNAqueous® Total RNA Isolation Kit (ThermoFisherScientific) and the RNAqueous®-Micro Total RNA Isolation Kit (ThermoFisherScientific) were used respectively according to the manufacturer'srecommendations.

After elution, contaminating DNA was further digested by adding 10x DNAse I Buffer to a final concentration of 1x and 1 µl DNAse I. After mixing, the reaction was carried out at 37°C for 30 min and stopped by adding DNAse Inactivation Reagent ($1/10^{th}$ of the reaction volume). After centrifugation for 2 min at 21.000 x g, the supernatant contains clean RNA ready for further applications. All components were taken from the RNAqueous®-Micro Total RNA Isolation Kit.

RNA quality was ensured by photometric measurement (NanoDrop One, ThermoFisher Scientific) and concentrations were calculated.

For cDNA synthesis, Superscript™ III Reverse Transcriptase (Invitrogen) was used. A maximum of 5 µg of RNA was mixed with 1 µl oligo$(dT)_{20}$ (50 µM), 1 µl dNTP Mix (10 mM) and ddH_2O to a final volume of 14 µl. The mixture was heated to 65°C for 5 min and chilled on ice for 1 min. 4 µl 5x First-Strand Buffer, 1 µl DTT and 1 µl of the enzyme was added and transcription was carried out at 50°C (60 min). The reaction was terminated by heat inactivation (70°C, 15 min). The resulting cDNA was used for further applications without any purification.

4.2.6 Polymerase Chain Reaction (PCR)

PCR is a genetic method based on cyclic amplification of target DNA using gene specific primers and a DNA polymerase *in vitro* (Mühlhardt, 2008). For Amplification, HF-Phusion® (NEB) or GoTaq® (Promega) polymerase was used according to the manufacturer's recommendations. Annealing temperatures and extensions times were set according to the used primers and template respectively. Normally, 35 cycles of amplification were conducted.

For PCR reactions that have been designed for adding sequence information using primer overhangs, the annealing temperature of the first 5 cycles was set according to the initial complementary part. The following 30 cycles, the annealing temperature of the whole primer was used.

The reactions were carried out in a Mastercycler® (Eppendorf).

4.2.7 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

QPCR is a polymerase-chain-reaction-based method which monitors the amplification of genes via intercalating fluorescent dyes to quantify the relative amount of a distinct mRNA-derived target compared to reference genes.

We choose a SYBR based assay (Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix, Agilent Technologies) and used it according to the manufacturers' protocol. The genes of elongation factor 1α (EF1α) and eukaryotic initiation factor 4α (eIF4α) has been used as references.

Primers were designed with Primer3Plus open source software (primer3plus.com) using qPCR server settings. The primers were further checked for potential cross-reactions using a BLASTn search linked to our *C. populi* transcriptomic library.

A complete list of the qPCR Primers used for the detection of target and housekeeping genes can be found in the materials section (table 3; 4.1.8).

The reaction was carried out in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) and the data was analyzed with Bio-Rad CFX Manager (version 3.1).

4.2.8 Purification of PCR-products and Plasmid DNA

For the purification of PCR-products, Plasmids after linearization and other applications, DiffinityRapidTips® (Sigma-Aldrich) were used according to the manufacturer's recommendations. After the isolation, DNA is ready for sensitive downstream applications like sequencing.

If thetemplatewas used for another PCR reaction, the PCR-product was cut out from a 1% agarose gel and centrifuged at 21.000 x g for 10 min. The supernatant was used as the template for the next reaction.

4.2.9 Agarose gel electrophoresis

For the separation of DNA, agarose (Carl Roth) was dissolved in 1 x TAE-buffer (1% w/v) and Midori Green Advance (Biozym Scientific GmbH) was added to visualize the nucleic acid. Running conditions: 30 min, 150 V in 1x TAE-buffer (Carl Roth). For the separation of RNA, the buffer was changed to 1 x TBE (Bio-Rad), because RNA loses its integrity in TAE-buffer. Nucleic acid was diluted with loading dye before filling into the gel slots.

For visualization, the gel was investigated under UV light using aBioDocAnalyzegel documentation station (Biometra).

4.2.10 Sequencing

PCR products and plasmids were sequenced by an external service (Eurofins Genomics).

4.2.11 Construction of a transcriptomic library by using RNA-seq

RNA-seq is a method which uses fragmented cDNA derived from cellular mRNA. The resulting pieces will be sequenced and aligned. Each fragment, which codes for a part of one target, will increase the read count of this target by 1. Due to a lot of parameters affecting the procedure like target length or sequencing biases, the resulting count cannot be easily correlated with the expression level of the target, but it is possible to compare the read counts of one target between different tissues(Conesa, et al., 2016).

Based on that data, a transcriptomic library was compiled previously to this work (Strauß, et al., 2014)

4.2.12 Production of double-stranded RNA (dsRNA) for RNA interference

For the generation of dsRNA of different targets, the MEGAScript® RNAi Kit (Ambion) was used according to the manufacturer's protocol.

The targets (CL7715, comp1318, comp195 and comp200) were amplified from fat body cDNA and the purified PCR-products were used as templates for the synthesis of the RNAi fragments. After another purification step, the T7-promotor-sequence sites were added to the fragments according to the recommendations of the kit by the use of T7-Primers in a single-tube reaction.

At the end of the procedure, the pure dsRNA was diluted in 2 volumes of 100% Ethanol, kept at -80°C overnight and centrifuged at 4°C for 30 min at 21.000 x g to pellet the nucleic acid. The supernatant was removed and the pellet dried at 35°C. It was finally redissolved in 0.9% NaCl solution to a final concentration of 1 µg/µl.

The sequences of the dsRNA-constructs are listed in the attachment (see 11.1).

4.2.13 Induction ofRNAi in larvae of *C. populi*

For the induction of RNAi, larvae of different age were injected with a total amount of 100 ng of dsRNA in two 50 nl portions using a Nano2000 injector (WPI). The injection was applied between the 2nd and 3rd leg of the larvae into the hemolymph (parasagittal between pro- and mesothorax). DsRNA of GFP (kindly provided by Lydia Schmidt), as well as untreated larvae,served as control groups.

For the analysis of RNAi during thelarval stage, larvae were injected 2-3 days after hatching and reared for 9d ± 1d. Each twotothree days food was applied (fresh poplar leafs). Additionally, larvae were weighed every day for growth control.

To check the RNAi effects in the adults, 3rd instar larvae were injected and reared until theemergenceof the beetlesafter 12 days.

The experiments were repeated several times between June and October 2016.

4.2.14 Metabolic analysis of juvenile and adult RNAi-induced *C. populi*via LC/MS

4.2.14.1 Sample preparation

Each larva was treated individually to compare the effect of the RNAi treatment on the concentration of the metabolites **1** and **2** and the mRNA levels separately. The larva was transferred into grinding tubes with 3 steel beads (2x 4mm, 1x 10mm in diameter) and 250 µl H_2O. 1 µl Paraoxon was added to prevent lipase catalyzed ester hydrolysis. As stable-isotope-labeled internal standardsof known concentration (SIL-IS), synthetic [1´,2´,3´,4´,5´,6´-$^{13}C_6$]-2-(β-D-glucopyranosyl)-3-isoxazolin-5-one; [1´,2´,3´,4´,5´,6´-$^{13}C_6$]-**1**(c = 17.33 mmol l^{-1}, V = 10 µl) as well as [1´,2´,3´,4´,5´,6´-$^{13}C_6$]-2-[6'-(3"-nitropropanoyl)-β-D-glucopyranosyl]-3-isoxazolin-5-one; [1´,2´,3´,4´,5´,6´-$^{13}C_6$]-**2** (c = 14.65 mmol l^{-1}, V = 10 µl)in acetonitrile (ACN)/H_2O 1:1 were added to each preparation (Arrivault, et al., 2015). The standard solutions were kindly provided by Tobias Becker.

The larva wasground for 1 min at 1200 rpm (Geno/Grinder) twice and centrifuged (10 min, 21.000 x g, 4°C). 100 µl of the supernatant were transferred into a solution of 200 µl H_20 and 300 µl ACN and centrifuged to remove the insoluble precipitate.The clear supernatant was used for LC/MS injection.

For adults, secretions were collected using a microcapillary by sweeping over the elytra. The harvested liquid was mixed with10 µl ACN/H_2O 1:1. For quantification, 1 µl of Paraoxon mixed withstandard solutions of compounds **1** and **2** was added (Paraoxon/**1**/**2** 1:25:10). The mixture was directly used for LC/MS analysis without further purification.

The hemolymph was collected from a scission between the head and the first thoracic segment of the beetles with a pipette. Afterward, the beetles were sacrificed

immediatelybydecapitation. The hemolymph was transferred to 50 µl ACN/H_2O 1:1 and for quantification, 4 µl of Paraoxon and standard solutionswere added (Paraoxon/**1**/**2** 1:25:10). The mixture was centrifuged and the clear supernatant was used for LC/MS injection.

The RNAi efficiency was determined by performing qPCR with the respective targets of remaining larval and adult tissue and comparison with the control group. Negative dsRNA injections, which show a downregulation of mRNA levels < 40%, were excluded.

4.2.14.2 LC/MS measurement

LC/MS analysis of compounds**1** and **2** wascarried out on an Agilent HP1100 HPLC system equipped with a column functionalized with amino groups (-NH_2), Luna (250 x 2 mm, 5 µm; Phenomenex Ltd. Germany, Aschaffenburg) connected to a Finnigan LTQ (Thermo Electron Corp., Dreieich, Germany) ion trap mass spectrometer using atmospheric pressure chemical ionization (APCI; vaporizer temperature: 500 °C, capillary temperature 300 °C).

1 µl- 3 µl of the sample volume was injected, depending on the origin of the sample (adult secretions and hemolymph = 3 µl; total larva = 1 µl). The following parameters were used: flow rate = 1 ml/min at rt: 80 % solvent A (ACN + 0.1 % v/v HCO_2H) and 20 % solvent B (H_2O + 0.1 % v/v HCO_2H) for 5 min, isocratic. The column was washed and equilibrated with A/B (4:1)prior to use.

For identification and quantification, the signals of the formic acid adducts$[M+HCO_2H-H]^-$ were analyzed (compare with figures S 3 and S 4):

Table 4: Substances analyzed via HPLC/MS and corresponding m/z ratios

Compound	**m/z $[M+HCO_2H-H]^-$**
2-(β-D-glucopyranosyl)-3-isoxazolin-5-one (**1**)	292
[1´,2´,3´,4´,5´,6´-$^{13}C_6$]-**1**	298
2-[6'-(3"-nitropropanoyl)-β-D-glucopyranosyl]-3-isoxazolin-5-one (**2**)	393
[1´,2´,3´,4´,5´,6´-$^{13}C_6$]-**2**	399

4.2.14.3 Data analysis and quantification

For quantification, the peak areas of the heavy isotope signals (m/z 298 for **1**, m/z 399 for **2**) in the chromatogram were compared to the peak areas of thenormal isotopes (m/z 292 for **1**, m/z 393 for **2**) using computational analysis (Xcalibur® 2.0.7; ThermoFisher Scientific). The molar contents $c_{m,analyte}$of compounds **1** and **2**(in nmol/mg) were calculated using the following equation (Becker, et al., 2016):

$$c_{m,analyte} = \frac{n_{analyte}}{m} = \frac{I_{analyte} \cdot c_{standard} \cdot V_{standard}}{I_{standard} \cdot m}$$

m = fresh body weight of larva or weight of adult hemolymph/secretion in mg

$n_{analyte}$ = amount of **1** or **2** in nmol

$I_{analyte}$ = area of the monoisotopic peak ([M+0]) of compound **1** or **2**

$c_{standard}$ = molar concentration of added solution of synthetic labeled compound **1** or **2** in µmol·µl^{-1}

$V_{standard}$ = volume of added solution of synthetic labeled compound **1** or **2** in µl

$I_{standard}$ = area of the isotopic peak ([M+6]) of compound **1** or **2**

4.2.15 Production of larval sections for RNA-FISH in paraffin

Larvae were embedded into paraffin wax according to Silva-Zacarin, et al., 2012.

Larvae of 1st and 2nd instar were fixed in 100 mM sodium phosphate buffered solution (pH 7.4) with 10% (v/v) Formaldehyde for 24h under agitation. The larvae were washed with water 5 times (10 min each) and dehydrated in solution with increasing ethanol concentration (30% -> 50% -> 70% -> 80% -> 90% -> 100%; 30 min each). Afterward, the larvae were kept in 100% Ethanol for additional 2.5h, transferred into Xylol (3 times, 60 min each) and incubated in paraffin wax at 60°C for 30 min and 2h. Finally, the larvae were embedded in paraffin wax by the use of metal molds, and cassettes for embedding (Carl Roth GmbH und Co.) which were stored at room temperature until use.

By the use of an automatic rotatory microtome (HM 355S; ThermoFisher Scientific), the prechilled paraffin blocks were sliced 30 µm to 40 µm and stretched in a water bath at

42°C. The flat slices were transferred to glass slides, dried overnight at 37°C and stored at 4°C until use.

4.2.16 RNA-FISH and DAPI staining of larval sections

4.2.16.1 Generation of RNA probes for detection of comp1318 mRNA

From genomic larval fat body cDNA, a 565bp DNA fragment was amplified using Primers '1318_RNAi_fwd' and '1318_RNAi_rev', which was purified and further cloned into pCR™-Blunt II- TOPO® vector (ThermoFisherScientific) containing a T7-promotor/priming site downstream of the insert. The plasmid was transformed into One Shot® TOP10 Chemically Competent *Escherichia coli* cells (Invitrogen) and positive clones were selected by plating cells on LB agar containing100µg/mlZeocin and 50 µg/ml Kanamycin. Successful transformants were screened by colony PCR, targeting the insert flanking M13 priming sites.One plasmid with the insert in normal (for antisense probe) and one with opposite direction (for sense probe as anegative control) were isolated and confirmed by sequencing. The plasmids were linearized using the BamHI-FD restriction enzyme (ThermoFisherScientific), which cuts at the respective recognition site upstream of the insert, and purified. Successful linearization was confirmed via agarose gel electrophoresis and the enzyme was deactivated by heating (5 min, 80°C). For probe labeling, the DIG RNA Labeling Mix (Sigma-Aldrich) was used. Tothe transcribed DIG-labeled RNA, 0.5 vol. Co-Precipitant Pink (Bioline) and 2.5 vol. 100% Ethanol were added. The mixture was stored overnight at -80°C, centrifuged for 30 min, 4°C, 21.000 x g, the pellet washed with 70% Ethanol and centrifuged again. After removing the supernatant, the pellet was dried and dissolved in 30 µl H_2O and analyzed on an agarose Gel. 25 µl were diluted in 225 µl hybridization buffer and stored till usage.

4.2.16.2 Staining procedure

In general, washing steps were performed with slides in a slide holder, while incubations with low volumes of reagent were performed in a humidity chamber with insect tissues in upside direction.

To remove paraffin, slides were loaded in a slide holder and washed withXylol twice (30 min each) and further rehydrated by applying several washing steps with decreasing concentrations of ethanol (100% -> 95% -> 70% -> 50%), each twice for 20 min under gentle agitation. To remove ethanol, slides were washed in turn with PBS + 0.03% Triton X-100 (1min), 0.2 M HCl + 0.03% (10 min) Triton X-100 and PBS + 1% Triton X-100 (1 min). Slides were stored in a humidity chamber and hybridization buffer was applied to each tissue till it was completely covered. After incubation for 4 days at 4°C followed by 5h in the hybridization oven at 55°C, the remaining liquid was removed and the RNA probe was applied. For that, the probe was dissolved in hybridization buffer to a final concentration of 0.5 ng/µl and added to the tissues till it was covered. The slides were incubated in the hybridization oven at 55°C for 3 days. After removing remaining liquid, the slides were washed 4 times with warm 0.1xSSC + 0.03% Triton X-100 under gentle agitation. To avoid unspecific binding of the detecting antibody, the tissues were incubated in blocking solution for 6h at 4°C. Afterward, Anti-Digoxigenin APwas applied (dissolved 1:500 in blocking solution) and the tissues were incubated for 3 days at 4°C. The slides were washed 5 times (10 min each) with TBS + 0.05% Tween 20 and after the removal of liquid, DAP - Solution was applied to every tissue. For inducing the fluorescent reaction, each tissue was incubated with the HNPP reagent for 6h at 4°C.

Because of the very light sensitive reaction, the following steps were all carried out in the dark. The slides were washed 3 times (5 min each) with TBS + 0.05% Tween 20, counterstained with DAPI (Sigma-Aldrich; 30 µM in PBS) for 30 min at room temperature and finally washed 5 times (5 min each) with PBS.

The stained tissues were embedded in VectaShield® (Vector Laboratories) and investigated.

4.2.17 Confocal Laser Scanning Microscopy (LSM)

Imaging of RNA-FISH samples was performed by using an 880 confocal microscope (Carl Zeiss) in combination with a Helium-Neon laser (543 nm), an LED lamp (405 nm) and two filter sets, ranging from 552 to 695 nm and from 415 to 500 nm. For total slice imaging, an EC Plan-Neofluar 10x/0.3 objective was used. For the collection of z-axis image scans, a C-Apochromat 40x/1.2 water immersion objective was used.

4.2.18 Maintaining of *Spodopterafrugiperda* (Sf9) insect cells

Cells were maintained in SF900II cell culture medium at 27°C andsubcultured once a week by transferring 1/5 of the original culture into fresh medium.

4.2.19 Transfection of Sf9 insect cells

Sf9 cells were transfected using the FuGENE® HD Transfection Reagent according to the manufacturer's recommendations.

Sf9 cells were grown in a 6-well plate till reaching approximately 70% confluency. 2.4 µg Plasmid-DNA (pIB vector with *comp1318* cloned in frame with a c-terminal His^6-tag) were dissolved in culture medium to a final volume of 92.8 µl, mixed with 7.2 µl FuGENE Reagent and incubated for 10 min at room temperature. Cell culture medium was replaced with 1900 µl fresh medium and 100 µl of the plasmid mixture and incubated for 72 h at 27°C.

4.2.20 Preparation of microsomes

Recombinant P450 are located in the membranes of Sf9 - microsomes and can thus be isolated using fractional centrifugation techniques (Joussen, et al., 2012).

Cells were harvested by scraping off with a culture scraper and transferred to a centrifuge tube on ice. Cells were centrifuged (500 x g, 10 min, 4°C) and the pellet was washed two times with PBS (pH 7.4). After resuspension in Hypotonic Buffer, the cells were kept on ice for 20 min to allow them to swell and then crushed using 30 strokes with a Potter (IKA Labortechnik). The same volume of sucrose buffer was added and the mixture was then centrifuged (10 min, 1.200 x g, 4°C). The supernatant was saved and the pellet homogenized like above. The pooled supernatants were centrifuged (15 min, 10.000 x g, 4°C) yielding mitochondria and nuclei in the pellet. The supernatant was ultracentrifuged (1h, 100.000 x g, 4°C) yielding cytosolic (supernatant) and microsomal proteins (pellet). Microsomes were washed with 100 µl resuspension buffer and ultracentrifuged like above and finally resuspended in 100 µl resuspension buffer. After asnap freeze, they were stored at –80°C until further analysis.

Each fraction was mixed with SDS loading buffer (1x), cooked (15 min, 90°C) and loaded on a Gel (Mini-Protean® TGX™ precast Gels, any kd, Bio-Rad Laboratories) for SDS-PAGE (running conditions: 90V, 120 min).

4.2.21 Western blot

Proteins, separated via SDS-PAGE, were transferred ontoa PVDF membrane (Trans-Blot® Turbo™ Mini PVDF Transfer Pack; Bio-Rad) using a Trans-Blot® Turbo™ Transfer System (Bio-Rad) with settings for mixed molecular weight proteins (1.3 A, 25 V, 7 min). The membrane was blocked with 5% milk powder (Carl Roth) in TBS-T for 2h and incubated with the anti-His antibody (dissolved 1:2500 in TBS-T with 2.5% milk powder) overnight at 4°C under gentle agitation. The membrane was washed 3 times with TBS-T and incubated with SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific) for protein detection. The luminescence was recorded with AmershamHyperfilm ECL (GE Healthcare).

5. RESULTS

5.1 The screening for the repertoire of CYPs in *C. populi* fat body tissue

The Isoxazolinones **1** and **2** have been detected in larval hemolymph previous to this work (Becker, et al., 2016). Based on the assumption that the fat body tissue, which is tightly associated with this larval body fluid, could be implicated in the production of CYPs, I have screened the proteome of this tissue for enzymes, which are putative candidates for further analyses. For this approach, a serial experimental set-up, consisting of transcriptomics, proteomics as well as phylogenetic and expression analysis was applied. Exploiting our transcriptomic reference library from *C. populi*, a total number of 137 putative CYP enzymes have been predicted, based on Pfam and BLAST computational analyses. By the use of a proteomics approach, this number should be reduced to the enzymes which are actually present in the living organism. The further reduction should be achieved with the use of expression analysis because the putative candidates should be highly expressed in tissues producing compound **2**.

In the following scheme, the applied workflowis shown (Figure 5). Each step is further described in the following chapters.

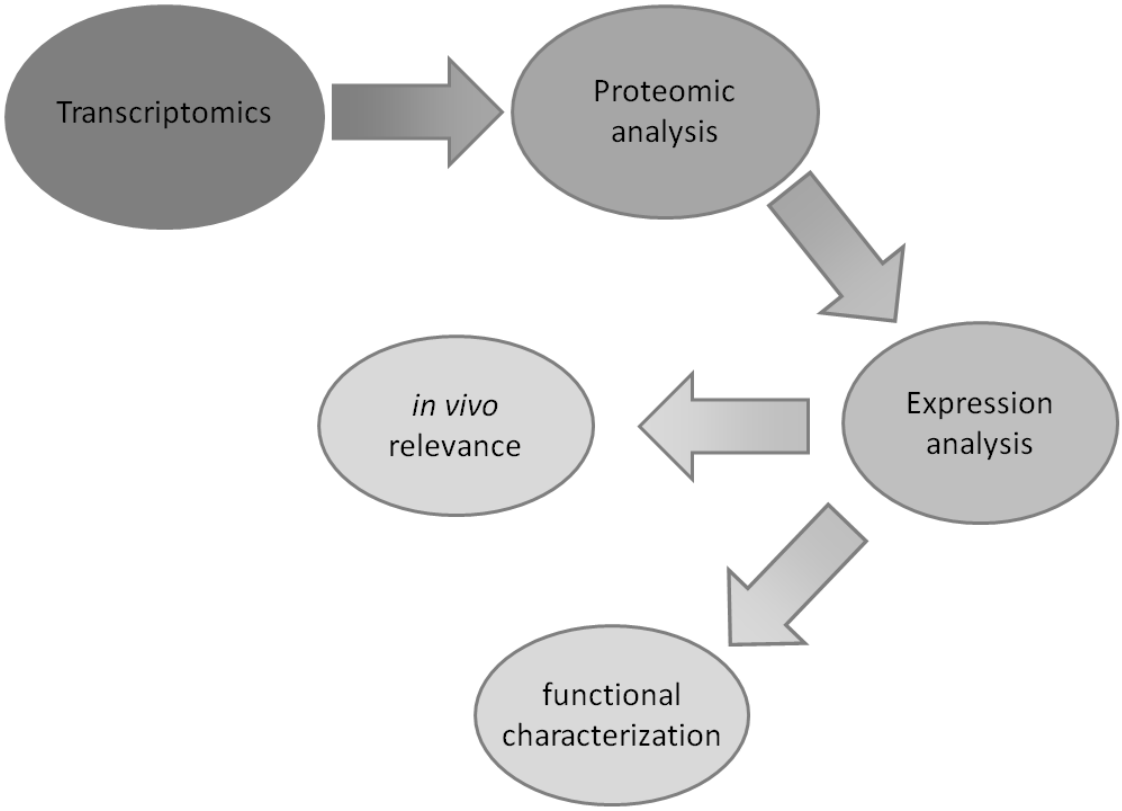


Figure 5: Methodological workflow of the screening for putative CYP candidates for RNAi. The used methods are shown in circles.

5.1.1 Proteomic analysis

To check for CYPs abundant in larval fat body tissue, a proteomic analysis approach was applied. Subcellular compartments were separated by using differential centrifugation.SDS-PAGE revealed differences in the pattern of protein bands (figure 6). A total number of 21 CYPs predicted from transcriptomics were identified,which are mostly located in the mitochondria/nucleus or membrane fraction (table 5). No enzymes were detected in the fraction of membrane-associated proteins and only one in the cytosolic fraction. However, this one candidate is also present in the membrane and mitochondrial/nucleus fraction and seems to be found in the cytosol as a contaminant. In general, the separation from cytosolic and membrane-associated protein was successful using differential centrifugation. As CYPs usually contain a membrane anchor, I chose the 19 putative enzymes, which were only present in the membrane fraction for further experiments.

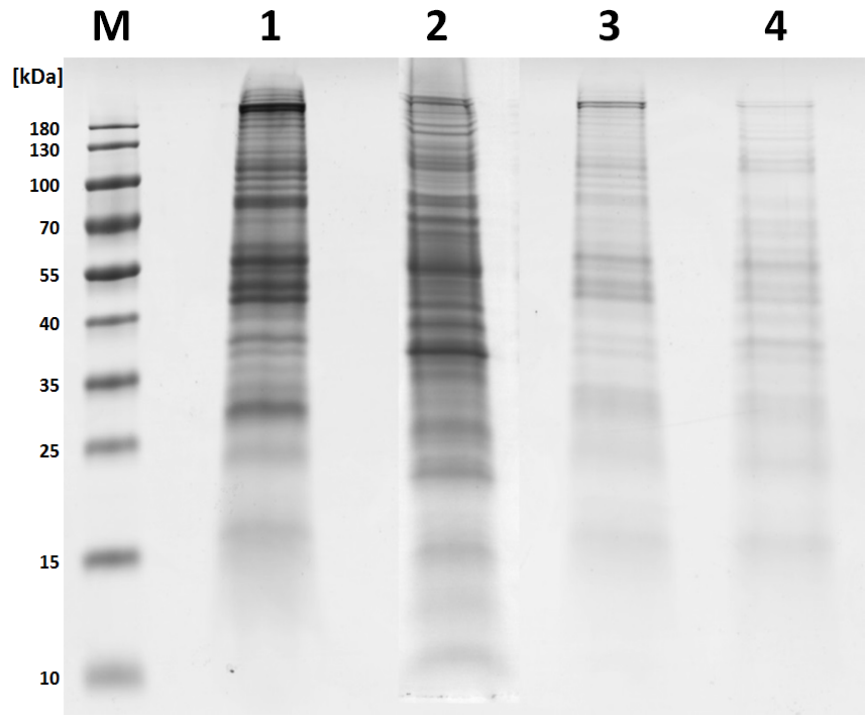


Figure 6: Results of SDS-PAGE transmitted to proteomic analysis.

Fat body tissue was separated in mitochondrial/ nuclear (1), cytosolic (2), membrane- (3) and membrane- associated proteins (4). (M = protein ladder)

Table 5: Proteins found in the proteome of *C. populi*.

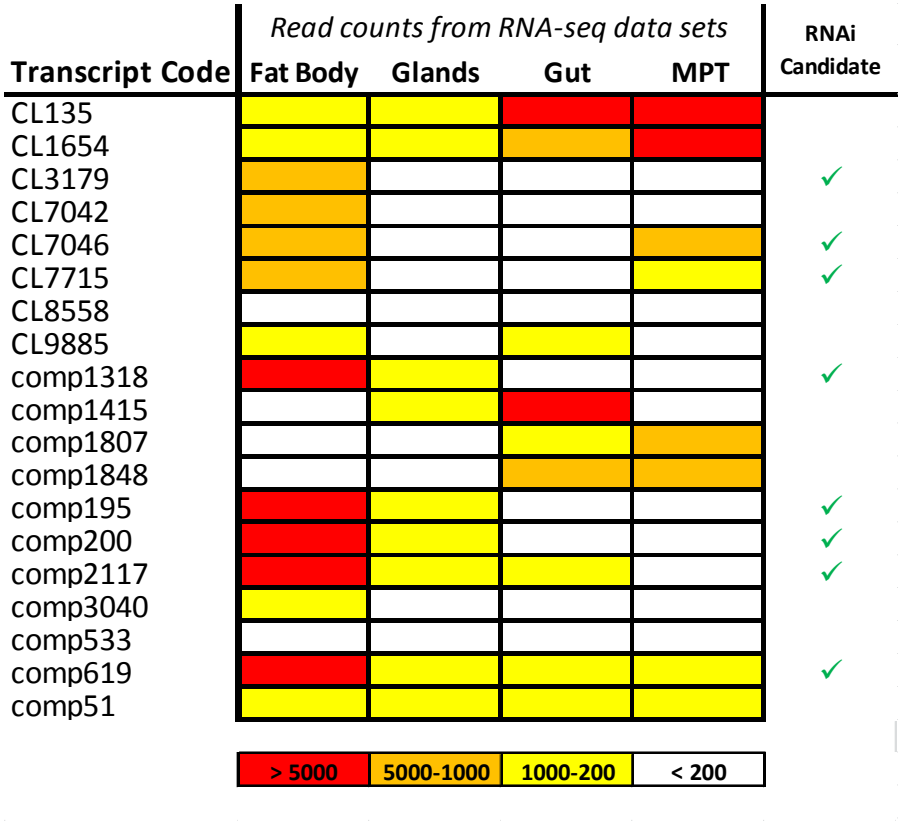
For further analysis, only candidates of the membrane fraction were selected (✓), leading to exclusion of Comp3029 and Comp3251 (gray).

Transcript Code	Protein fractions of proteomic analysis			
	mitochondrial/ nuclear	cytosolic	membrane	membrane-associated
CL135	✓	×	✓	×
CL1654	×	×	✓	×
CL3179	✓	×	✓	×
CL7042	✓	×	✓	×
CL7046	✓	×	✓	×
CL7715	✓	×	✓	×
CL8558	×	×	✓	×
CL9885	✓	×	✓	×
comp1318	✓	×	✓	×
comp1415	×	×	✓	×
comp1807	✓	×	✓	×
comp1848	✓	×	✓	×
comp195	✓	×	✓	×
comp200	✓	×	✓	×
comp2117	✓	×	✓	×
comp3040	×	×	✓	×
comp533	×	×	✓	×
comp619	✓	✓	✓	×
comp51	✓	×	✓	×
comp3029	✓	×	×	×
comp3251	✓	×	×	×
Σ	**15**	**1**	**19**	**0**

5.1.2 Transcriptomic analysis of larval tissues

For all the candidates found in the proteome, RNA-seq data sets were compiled for different larval tissues. This approach was used to correlate the proteomics data with the expression levels in the different tissues via comparing read counts of different tissues.I looked especially for targets with a high read count in thefat body as the predicted location of the biosynthesis of **2,**compared to low read counts in the others. The results are shown as a heat map in the following table 6:

Table 6: RNA-seq read counts of different targets in different tissues.
The candidates were selected by the comparison of the count in larval fat body tissue and the other tissues. Only targets which are present in distinct higher quantities in the fat body have been selected for further analysis (see '✓' in the last column).



All targets with read counts in fat body lower than in other tissues were excluded, which were CL135, CL1654, comp1415, comp1807, and comp1848. Also, candidates were sorted out where the counts in thefat body are as high as in other tissues, which are CL7042, CL8558, CL9885, comp533, and comp51. Finally, comp3040 was excluded after additional analysesbecause it only corresponds to an incomplete DNA fragment.

As a result, eight candidates remain for expression analyses, which are named CL3179, CL7046, CL7715, comp1318, comp195, comp200, comp2117, and comp619.

5.1.3 Expression analysis by qPCR

To further check for putative CYP candidates, expression analysis via qPCR was applied. Because 3NPA esters are present in the elytral glands of the adults, I screened for high expression levels in these tissues compared to others. Additionally, larvae tissue was considered to show high expression levels in the fat body. To examine, whether the eight candidates exhibit the expected expression profile, different larval (fat body, Malpighian tubules, gut) and adult tissues (elytra, gut, adult body w/o elytra and gut) were prepared and RNA was isolated and transcribed into cDNA. From all tissues, a qPCR analysis with the respective targets was performed and the expression calculated with the use of reference genes (see 4.2.7).In the following graphs, the results are shown (figure 7).

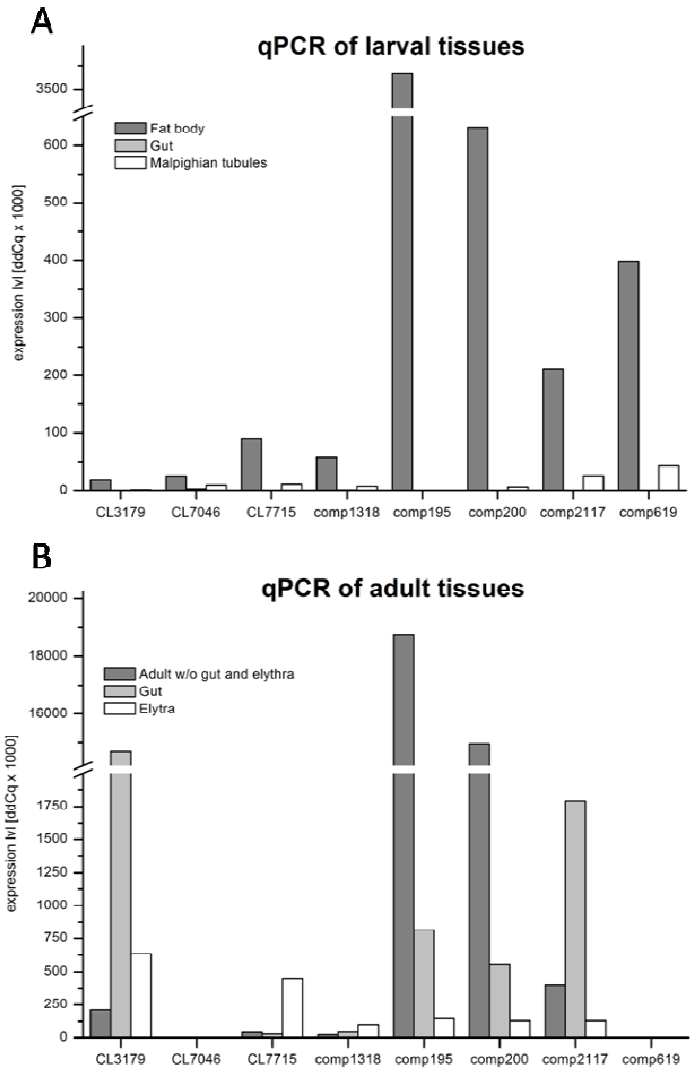


Figure 7: QPCR Results of the expression analysis of the different putative CYP targets.

In larval tissues (A), all targets show expression in the fat body, whereby in the Malpighian tubules and gut few and no expression was detected, respectively.

In adult tissues (B), the expression pattern between the targets was more diverse. Some candidates showed no expression in adults (CL7046, comp619), whereas others are primarily expressed in thegut (CL3179, comp2117), elytra (CL7715, comp1318) and the beetle without gut and elytra (comp195, comp200).

Statistical analysis was not conducted, due to low replicates involved in this screening (n = 2).

Based on this finding, the further selection of RNAi candidates was continued:

Among the tested CYP genes, CL3179 showed the lowest expression levels in the larval fat body. Its expression in the other larval tissues was even lower than in fat body tissue. In the adults, the respective RNA was mainly detected in gut tissue, indicating its main function in this organ. These results did not meet my expectations, so this candidate was excluded from further analysis. CL7046 was excluded as well because no expression in adult tissues was detected, which supports the idea that this enzyme is specific for larval metabolism. In contrast, the candidates CL7715 and comp1318 showed an expression pattern, which I expected for the involvement in the biosynthesis of **2**. Both targets were expressed in the larval fat body much higher than in the other larval tissues. Additionally, they were also highly expressed in the adult elytra, while the expression in the other adult tissues was lower. These enzymes may be incorporated in the biosynthesis of **2** and were selected as targets for RNAi. The candidatescomp195 and comp200 also shared a similar expression pattern. They were highly expressed in thelarval fat body and adult beetle w/o elytra and gut, which led to the assumption that these targets are involved in primary metabolism or other crucial pathways like degradation of xenobiotics. Because these targets seem to be of importance for larvae and beetle, they were also selected for RNAi. The candidate comp2117 showed an interesting pattern. While it is expressed in the fat body in the larval stage, in the adults the highest values are detected in gut tissue. However, this result did not fit my expectations and the candidate was excluded from further testing. The last putative CYP target named comp619 showed a promising expression pattern in the larval fat body, but no expression in adult tissues. This was taken as a hint that it is only needed for larval metabolism and was therefore excluded from subsequent studies.

In summary, the targets CL7715, comp1318, comp195 and comp200 showed promising expression patterns after qPCR analysis and were thus selected for following RNAi experiments.

5.2 RNA interference and metabolomic / expression analysis of *C. populi* larvae

Larvae of *C. populi* were injected with 100 ng dsRNA (100 nl) of each target gene as well as the GFP control two days after hatching (late June 2016) and reared for 7- 9 days till short before pupation. The used dsRNA and additional information can be found in the attachment (see 11.1).

The larvae were prepared for HPLC/MS measurement and analyzed. The peak areas of compounds and standards were used for the calculation of the specific amount of compounds **1** and **2** per mg larval fresh weight. The results for the four putative CYP candidates are shown in figure 8.

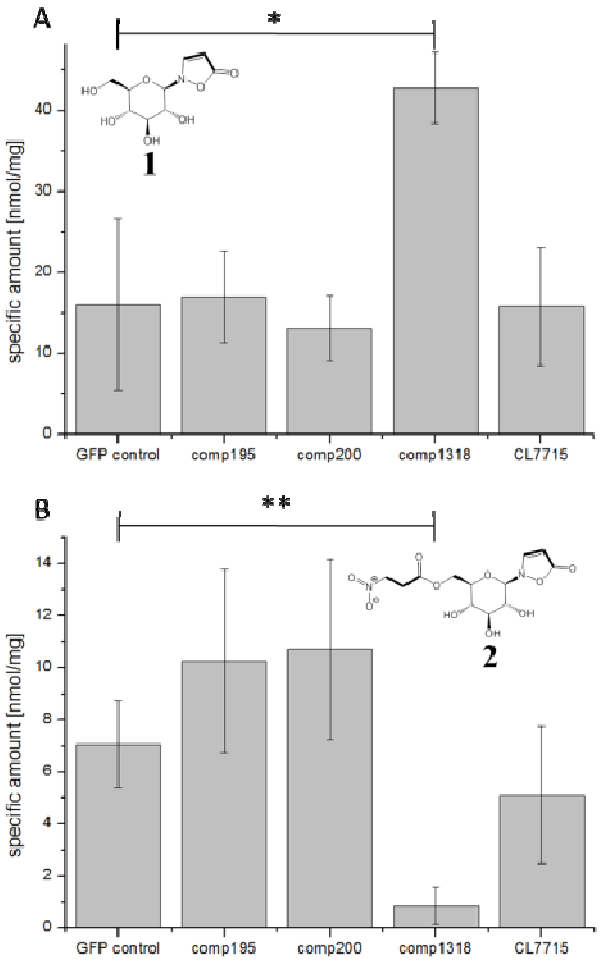


Figure 8: Specific amounts of compounds *1* and *2* in RNAi induced larvae.

(A) Compound **1** shows a significant increase in larvae treated with *comp1318* derived dsRNA compared to the GFP control group. The other candidates exhibit no differences.(*: P = 0.016)

(B) Compound **2** shows a significant decrease in RNAi induced larvae of Comp1318 compared to the GFP control group. The other candidates exhibit no significant differences.(**: P = 0.004)

Among the tested candidates, only Comp1318 revealed a significant increase of compound **1**, which exceeded a concentration of 40 nmol/mg larval fresh weight. Additionally, a significant decrease of compound **2** was observed compared to the control group. At the end, the concentration was lower than 1 nmol/mg larval fresh weight, making Comp1318 a possible target for further analysis. For all other tested targets(Comp195, Comp200 and CL7715), no differences regarding the control group were detectable, either for compound **1** or **2**. Concentrations of approximately 15 nmol/mg larval fresh weight and 6-10 nmol/mg larval fresh weight were detected for **1** and **2**, respectively.

Larvae were weighed before analysis to compare if the dsRNA had any influences on development. The results are shown in figure 9. Additionally, no obvious differences occurred regarding growth and development between the groups and the control, which indicates, that the dsRNA is not involved in vital parts of primary metabolism. If that was the case, a reduced growth or even lethal effects have been expected.

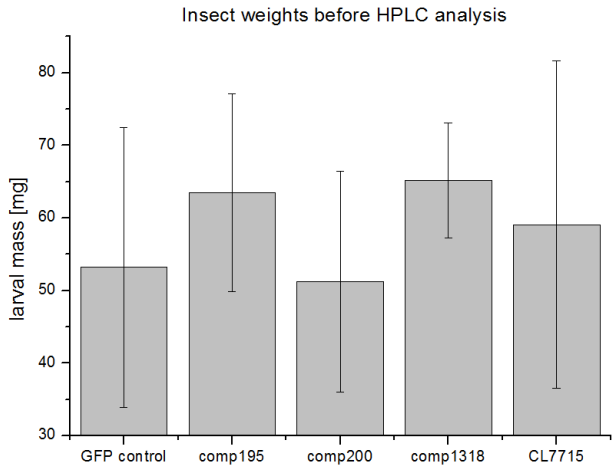


Figure 9: Weight of juvenile *C. populi* directly before HPLC analysis.
The weights represent 3rd instar larvae short before pupation and ready to use for HPLC measurement. No significant differences occur between the target and the GFP control group. n ≥ 3

To confirm, that the RNAi has led to a downregulation of expression levels, a qPCR analysis was conducted. The results are shown in the following graph (figure 10).

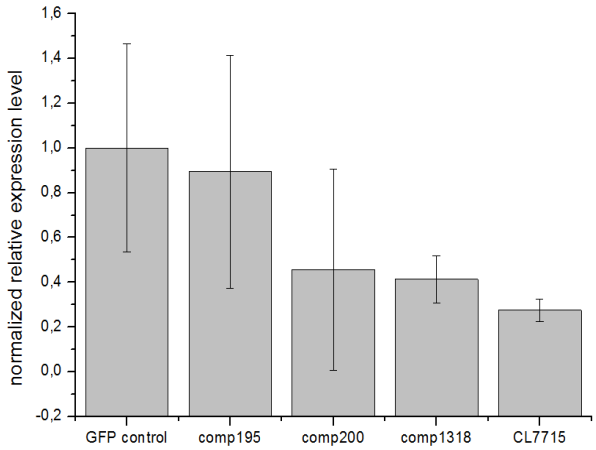


Figure 10: QPCR results of gene expression analysis of putative CYP target genes.

The relative expression was normalized according to GFP controls. No significant reduction was detected for each target, although comp1318 show nearly statistical significance (P = 0,166). Replicates: GFP control (8), comp195 (2), comp200 (2), comp1318 (3) and CL7715 (2).

In all cases, no significant downregulation in expression levels was observed, but comp1318 was nearly significant (P = 0,166). Because this candidate was also the only one which has shown a phenotypic effect regarding Isoxazolinone biosynthesis, the further studies focused on this putative CYP enzyme.

5.3 Effects of comp1318 RNAi in*C. populi* larvae

To confirm the results regarding the silencing effects of Comp1318 RNAi, the experiments mentioned above were repeated with much higher replicate numbers (GFP control: n=14; RNAi: n=17) in late September of 2016. After statistical analysis, the effect of the RNAi was significant for the reduction of **2** (P ≤ 0,001; figure 11A) as well as for the accumulation of **1**(P ≤ 0,001; figure 11B). Additionally, the qPCR experiments were repeated and showed statistical significance too (P = 0,005; figure 11C). If compared with the preliminary results, the same trends have been observed but for the compounds **1** and **2**, much lower concentrations were detected. For **1**, 15 nmol/mg larval fresh weightand 1.7 nmol/mg were found in the GFP control group of the 1st and the 2nd experiment, respectively. For the dsRNA-treated larvae, concentrations of 40 nmol/mg and 6.2 nmol/mg have been detected in the two measurements. For compound **2**, also some changes have been observed. In GFP controls, the concentrations changed from 7 nmol/mg to 2 nmol/mg between the two experiments, but in the RNAi induced larvae the concentration has not changed significantly.

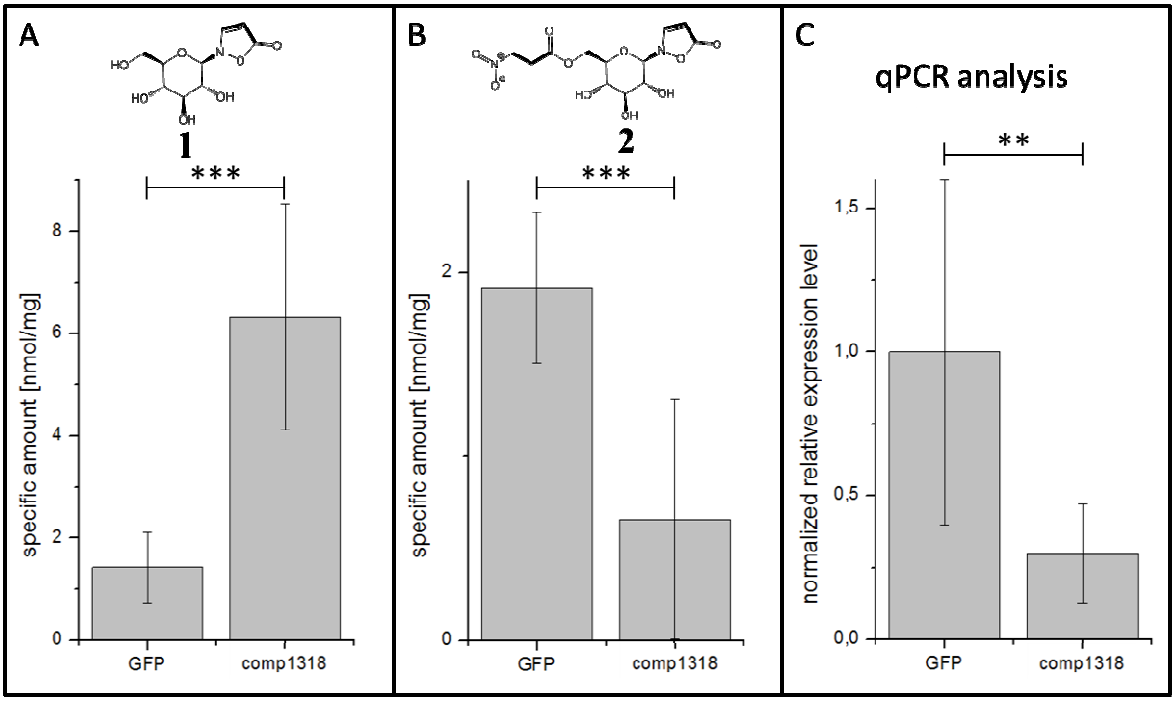


Figure 11: Results of RNAi targeting Comp1318, carried out with higher replicate numbers. Like in the preliminary results, the same trending was observed but was now confirmed with statistical significance.The analysis of **1** (A) has revealed a significant increase after dsRNA treatment. For **2** (B), a significant decrease was detected. In some larvae, the production of **2** was even totally abolished. The genetic analysis by qPCR (C) has revealed a significant reduction in expression levels of Comp1318 mRNA to approximately 25% compared to the controls.(***: P < 0,001; **: P = 0,005)

To check, if dsRNA treatment and especially Comp1318 RNAi showed any effect on development and growth of *C. populi* larvae, the weight increase, and thesurvival rate was monitored. For the growth, no differences were detected between the RNAi induced,GFP-treated as well as untreated larvae (figure S 2A). All larvae weighed approximately 7.5 mg at the day of injection and reached masses between 40 and 90 mg after 7 to 8 days.

Influences of *comp1318* deriveddsRNA regarding survival rate were monitoredas well (figure S 2B). No influences between the RNAi induced and GFP-treated larvae have been observed. However, survivability decreases dramatically if dsRNA is injected, compared to untreated controls.

5.4 Effects of Comp1318 RNAi inadult leaf beetles of *C. populi*

During this master's thesis, the first experiments were conducted which analyze the effects of Comp1318 RNAi in adult leaf beetles. In contrast to the larvae, the adults also contain compound **2** in their defensive secretions. Given that, adult secretions and hemolymph of the beetles were analyzed. Some promising, but only preliminary results have been achieved which are shown in figure 12.

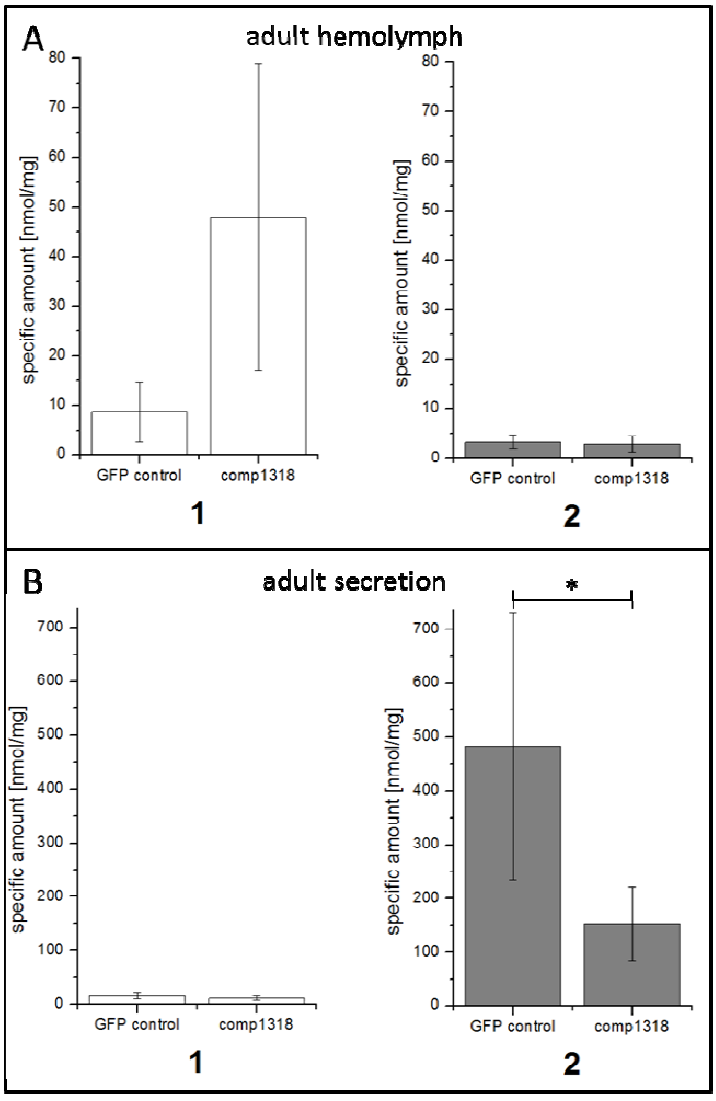


Figure 12: Abundancies of compounds *1* and *2* and adult hemolymph und secretion.
In adult hemolymph (A), compound **1** showed a strong increase (5x) after RNAi induction, which was close to statistical significance (P = 0,079). Compound **2** was present only in tiny amounts (~ 3 nmol/mg) in both groups.In adult secretions (B), compound **1** showed no changes between control and RNAi induction (~ 13 nmol/mg), but for **2** a significant decrease was detectable (*: P = 0.04).

Comparison of the two compartments showed no differences for compound **1**, but for **2** instead. In the secretion, the compound is approximately concentrated 100 times more compared to the hemolymph(P = 0,015). (n=4)

No detailed studies on growth and development were performed because of the low replicate number (n = 4), but no obvious differences were detected between RNAi induced and GFP control beetles.

5.5 Detection of Comp1318 mRNA in larval tissue

The fat body of *C. populi* is a very heterogeneous tissue, consisting of different cell types, each responsible for different functions. Next to adipocytes (energy storage and release), muscles and other specialized cells, also oenocytes are present as part of this tissue and possibly linked to the synthesis of many lipids and hydrocarbons (Arrese, et al., 2010).

To detect, if *comp1318* transcription of *C. populi* larvae takes place in one of the mentioned cell types only, I performed RNA-FISH from larval sections. I was able to identify specialized structures, where Comp1318 mRNA accumulated (figure 13B and C). These saccular compartments contain only a fewnuclei that are all attached to one side of the structure. Most of the structure seems of membranous composition, with some mRNA-free spaces in between. In a 30 µm slice of a 4-day old larva, four of these structures have been detected (figure 13A [white arrows]), which is a hint, that these compartments occur quite often in larval hemocoel. However, it is unlikely that those structures are oenocytes, which typically appear as clusters of five to eight cells (Dunkel, et al., 1968).

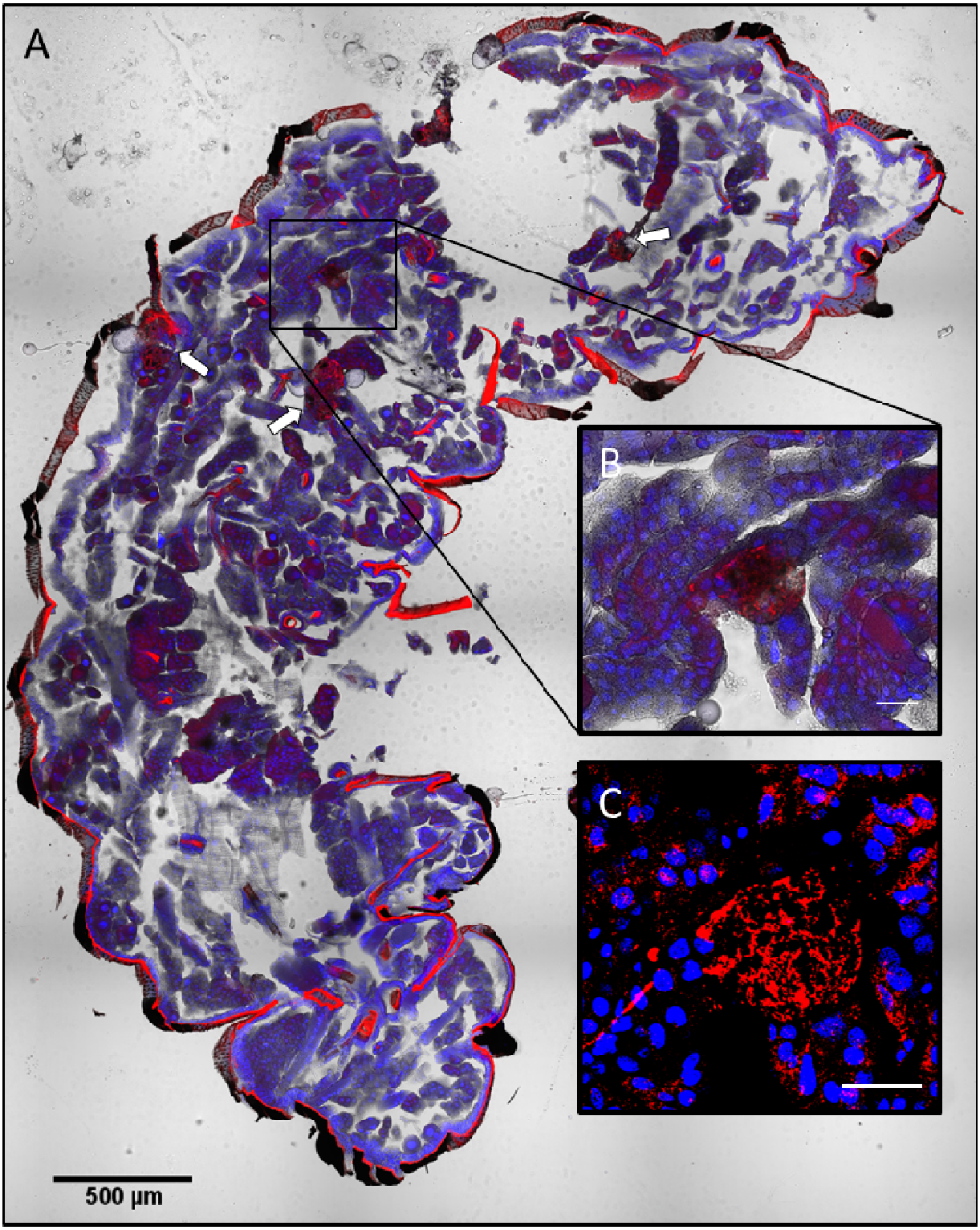


Figure 13: RNA-FISH of a 4 day old larva section of *C. populi*.

(A) Sagittal section of a 1st instar larva of *C. populi* was stained according to the RNA-FISH protocol and imaged by using fluorescent microscopy. The larva is located with the dorsum on the left and the head at the top. The target Comp1318 mRNA appears in red and nuclei in blue. The structures, where mRNA seems to be accumulating, are marked with white arrows or a black frame. (B) Zoom into one of the interesting structures (scale: 50 µm). (C) Merged focus-stacked image. Nuclei are only located on the left side of the structure, while the right part seems to be composed of membranous substructures, with spaces in between (scale: 50 µm).

5.6 Expression of Comp1318 in Sf9 insect cells

Transfected Sf9 insect cells were harvested and the different fractions analyzed by using SDS-PAGE. The gel is shown in figure 14. On the left side of the ladder, the transfected cell fractions are loaded while control,which was treated without plasmid, is loadedon the right. Comparing the pattern of each fraction, an obvious separation was achieved using the differential centrifugation, indicating that this method is suitable for purifying CYPs expressed in Sf9 insect cells. However, no expression was observed in the microsomal fraction (+3) compared to the control (-3). Additionally, no protein was detected via western blot analysis, targeting the His-tag fused to Comp1318 which makes a repetition necessary.

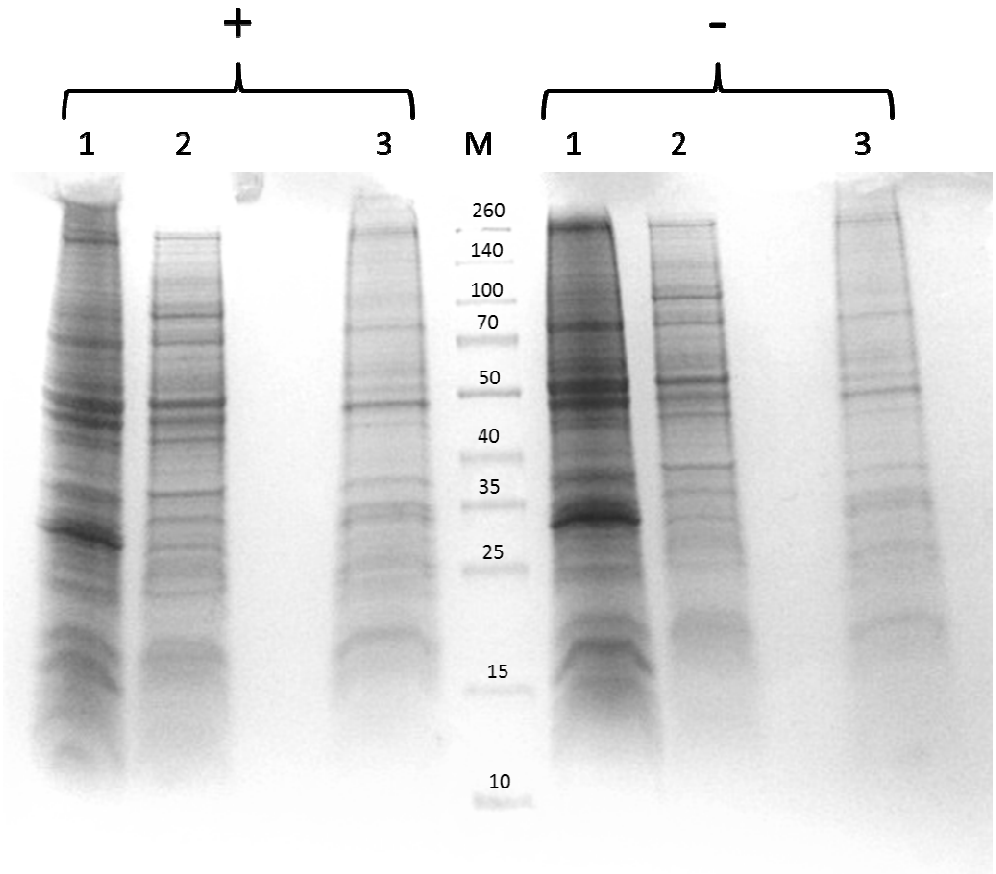


Figure 14: SDS-PAGE of different fractions derived from differential centrifugation of transfected (+) and untreated (-) Sf9 insect cells.
No difference was detected between transfected cells and the negative control.
(1: mitochondrial/nuclear proteins; 2: cytosolic proteins; 3: microsomal proteins;
M: protein marker with molecular weights in kDa)

6. DISCUSSION

To defend themselves, leaf beetles of the subtribe Chrysomelina have developed different behavioral and chemical mechanism. One strategy is the use of non-volatile 3NPA esters (**2**), which can have severe effects on the predator's nervous system. This defensive line is present in Chrysomelina exclusively and can be used as a taxonomic chemo marker. One member of this tribe, *Chrysomela populi*, was canvassed in this study to get a deeper understanding of the biosynthesis of **2**, which is most likely based on Cytochrome P450 Monooxygenases.

6.1 The search for putative CYPs was based on literature research and subjective expectations

Based on recently reported studies, it seems most likely that enzymes of the Cytochrome P450 Monooxygenase superfamily are involved in the oxidation from β-alanine to either 3NPA or Isoxazolinone (figure 2). Because the final product **2** was found recently in larval hemolymph only (no evidence for an occurrence in larval secretion)(Becker, et al., 2016), I assumed the involvement of fat body tissue in the biosynthesis. Because this tissue is very heterogenic and composed of different cell types, a distribution of roles is likely. It was found that so-called oenocytes, which are associated with the fat body, show high metabolic turnover rates compared to the cells responsible for energy storage, making them a putative location for CYP enzymes(reviewed by Martins, et al., 2012). Because these proteins usually contain a membrane anchor, I used a differential centrifugation technique to isolate membrane proteins, which were further analyzed by a proteomics approach. Regarding the separation efficiency, no CYPs were detected in the cytosolic and membrane-associated protein fraction, except one candidate (table 5). Next to proteins detected in the membrane fraction, some of them also appeared in the mitochondrial/nuclear protein fraction. Additionally, some characteristic marker proteins of mitochondria have been detected in the membrane fraction like Cytochrome c Oxidase or Heat Shock Protein 70. Thus, it must be stated, that the isolation from mitochondrial/nuclear proteins in not sufficient, which is in line with previously performed experiments(Rosentreter, 2016). The reason for that can be that CYPs located in mitochondrial membranes are separated from the organelle during the centrifugation

steps, leading to their appearance in the membrane fraction. Vice versa, microsomal membranes may associate with the mitochondrial/nuclear precipitate which leads to the detection in that fraction. In contrast, separation from cytosolic/membrane-associated and membrane proteins works fine. For the next experiments, some optimization has to be done to increase the separation efficiency of CYPs, especially regarding membrane and mitochondrial proteins.

From our transcriptomic library, 137 putative CYPs have been predicted to be present in larval fat body tissue, but only 21 have been detected using the proteomic approach. The library was based on RNA-seq analysis and the *in silico* alignment of sequenced fragments, which have been further translated into proteins using all 6 possible reading frames(Strauß, et al., 2014). By the use of this computational technique, lots of artificial proteins can be assembled, which do not occur in the living organism. But it is also worth noting, that through proteomics, possibly not all protein could have been detected. This may happen, if proteins are not expressed at the time the fat body was extracted, or if the copy number was too low. In conclusion, it can be stated, that I identified many CYPs present in fat body tissue for further testing by the use of these two methods in combination.

By conduction the read count of the RNA-seq data analysis, I expected high values in thelarval fat body as the location of Isoxazolinone biosynthesis. Eight Candidates fulfilled these requirements and were included in the further analysis. However, some candidates were excluded even if their pattern was correct like for example Comp3040. For this, only partial sequence information was stored in our database. To achieve the whole sequence, RACE-PCR or equivalent methods can be applied. The full-length product can be a putative target for future analysis. Also, the candidates, which show high read counts in thefat body, but also in one other tissue, have been excluded. If biosynthesis is not localized in fat body only, also those CYPs may be putative candidates for future experiments.

The expression analysis was performed under the expectation of high expression of CYP mRNA in larval and adult tissues, which are responsible for the production of the defensive compounds **1** and **2**. Because these substances are present in larval hemolymph as well as adult secretions, I assumed the presence of CYPs in thelarval fat body and the glandular secretion system of adult elytra, respectively. All tested enzymes show an abolished expression in larval gut tissue and a low expression in the Malpighian tubules,

but exhibit high values for thefat body. Because I was looking for ahigh expression in the latter, all tested CYPs seem to be putative candidates. After testing the adult tissues, several candidates have been excluded from further testing because they did not fulfill my requirements. Considering the expression of the different CYP candidates chosen for further experiments in the different tissues (figure 7), comp195 and comp200 show a real similar pattern, whereby comp200 is expressed less. By the use of our transcriptomic database, I checked for similarities in gene sequence. It turned out, that both genes show high overlaps. However, the primers used for qPCR analysis were designed binding in unique regions (< 50% similarity between the two genes). By the use of a phylogenetic approach, both genes show high similarities as well (see 11.2), which is a hint that the resulting enzymes may be isoforms. The other candidates Comp1318 and CL7715 didn't exhibit any special manners.

6.2 Comp1318 shows a phenotypic effect after genetic knockdownin*C. populi* larvae using RNAi

The final four candidates were used for RNAi experiments to see if knock down of their respective enzymes causes a change in phenotype. Comp1318 was the only candidate which exhibits an effect regarding Isoxazolinone biosynthesis which was characterized by an accumulation of **1** and a depletion of **2**. However, the following qPCR analysis revealed, that no significant reduction in gene expression after RNAi was observable for all candidates, which indicates that method or the design of proper qPCR primers was unsuccessful. For the candidates, where no change in the composition of the compounds was observed, the experiments have to be repeated. Additionally, it is possible that more than one enzyme is responsible for one reaction in Isoxazolinone synthesis, which is likely for Comp195 and Comp200 because both enzymes are quite similar. In that case, a double RNAi has to be applied, or the dsRNA has to be optimized the way, that the RNAi-inducing fragments can affect both targets.

Looking deeper into the involvement of Comp1318 in thesynthesis of **1** and **2**, the RNAi and qPCR analysis were repeated, causing more consistent results (figure 11) because the change in product concentration correlates with the efficiency of the RNAi (data not shown). It is noteworthy that the range of concentration of compounds **1** and **2** had

significantly changed between the two measurements performed in this study. For compound **1**, the first one, which was performed in late June, show values of 15 and 40 nmol/mg larval fresh weight for control and RNAi, respectively, while the second experiment (late September) exhibit 1.7 and 6 nmol/mg larval fresh weight for control and RNAi, respectively. This is a factor of 6.7 to 8.9 for the respective groups. For compound **2**, broadly similar observations were made for the control group. Here, the concentrations changed from 7 to 2 nmol/mg larval fresh weight between the first and second experiment respectively. The changes of compound **2**in the downregulated organism were not significant between the two experiments.The observed decrease in the defensive compoundsmay be linked to population variability caused by rearing in the laboratory. During the time in the lab, beetles haven't been exposed to their typical environmental cues. Therefore, no selection for beetles containing high amounts of defensive compounds occurred, which may lead to a downregulation till loss of this function. Because a 3-month space was in between the two measurements, a new generation was analyzed, where a possible downregulation has already happened. To receive consistent results, new animals should be collected from nature and investigated.

The comparison of the larval weights short before HPLC- measurement did not reveal any changes between the developments of the RNAi and the control group, so the applied dsRNA of *comp1318* is not affecting the general growth of the larvae. However, there are still big differences in the populations tested. Some larvaeexhibit body weights of > 100 mg, while others show< 50 mg instead. In this study, the contents of compounds **1** and **2** were normalized to the body weight but, however, it is not known how the growth is affecting the production of 3NPA esters in larval development. It is likely that larvae under bad growing conditions (e.g. poor access to a proper food source) will focus on keeping the primary metabolism functioning, before producing defensive compounds. In this work, the larvae were kept separately and were fed with poplar leafs which derive from the same tree. However, some leafs could have been affected by different environmental factors and may also be infected by fungi. This can cause the variance regarding the food source.Additionally, the rearing of the beetles in the lab may also influence the production of defensive compounds. Because they were grown since more than a year under laboratory conditions without any predators, the pressure for selecting for **2**-containing larvae was gone. Between the two experiments, at least on generation change has taken place, which may cause a reduction in expression of the needed proteins.

6.3 The Comp1318 protein can be integrated into the biosynthesis of Isoxazolinones

The main aim of this study was the identification of CYP enzymes which may be involved in the biosynthesis of compounds **1** and **2**. The results which had been achieved via RNAi and analysis of the phenotype, suggest the involvement of Comp1318 in the synthesis of the 3NPA moiety, but not in the production of **1**. By conducting the biosynthesis proposed by Becker et al. (figure 15A; "!"), a knockdown of Comp1318 will stop the production of the 3NPA moiety, but cause all precursors flow into the synthesis of **1**. The consequence, depletion in **2** and an accumulationof **1**have been observed.

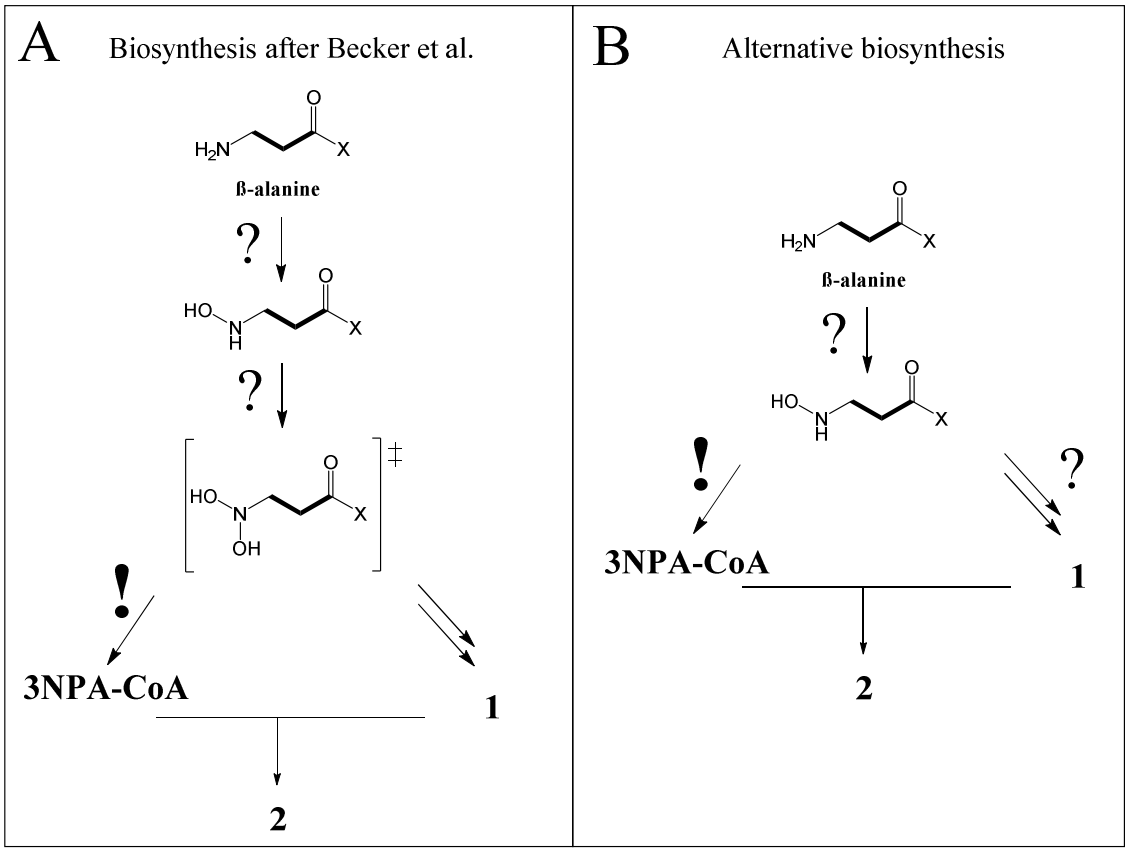


Figure 15: Two models for the biosynthesis of 1 and 2 in leaf beetles.

The biosynthesis proposed by Becker et al. (A), involves a one-step reaction, performed by two enzymes (3-(hydroxyamino)-propanoic acid ester to 3NPA), while the other (B) does not. It is more likely that one enzyme processes the reactive intermediate directly than passing it to another one. The reaction which is most likely catalyzed by the Comp1318 CYP is marked with "!".("?" = enzymes unknown; X = OH or CoA).

If the results should be linked to the biosynthesis proposed by Becker et al., the enzyme has to be connected to the conversion of the reactive intermediate to 3NPA, to be

coherent. In enzymology, such transfer reactions of a reactive intermediate from one reaction center to another are often realized in multi-enzyme complexes only. However, no reactions like this have been described for Cytochrome P450 Monooxygenases so far. It is also likely that Comp1318 is catalyzing the reaction from the 3-(hydroxyamino)-propanoic acid (3HAPA) ester to the 3NPA moiety directly (figure 15B). This leads to the idea that the 3HAPA ester can serve as the substrate for Comp1318 as well as for another, not yet identified theenzyme, which catalyzes the production of the precursor for **1**.

Up to date, the heterologous expression of this enzyme was unsuccessful. Therefore, no tests regarding substrate specificity, involved cofactors or additional requirements have been conducted to confirm the previous hypothesis. Because CYPs often need an attached P450 reductase for electron supply, it is useful to co-express the accessory enzyme directly. In some cases, the host is capable of producing their own reductases in sufficient amount, which has to be tested empirically(Joussen, et al., 2012). The use of the host's reductase is only possible if the produced CYP derives from the same genus like the host (e.g. insect CYP expressed in insect cells).

6.4 In adults, 3NPA-based chemical defense involve different compartments

In larva, compounds **1** and **2** are present in hemolymph, which seemed to be stored there. In contrast, the adults show a different distribution of these substances regarding hemolymph and glandular secretions (see figure 12). In the GFP- control beetles, compound **1** is present in hemolymph and secretion in concentrations of ~ 10 nmol/mg larval fresh weight, but **2** is nearly abolished. Instead, **2**is stored in high concentrations in the glandular secretions, with an average of 500 nmol/mg larval fresh weight. Some individuals even contained more than 700 nmol/mg larval fresh weight, which is a 100-fold increase compared to hemolymph. Given that, compound **2** seems to play a role in the adult secretions only.

After inducing RNAi in the adults, compound **1** was only accumulating in the hemolymph of the beetles (figure 12A-1), but not in the secretions (figure 12B -1).In contrast, the concentration of compound **2** only decreases in the secretions(figure 12B - 2), while no effect was observed in the hemolymph(figure 12A - 2). Because it's most likely that the underlying mechanism is the same like in larval fat body, this result indicates the

involvement of transporters and different compartments for the storage of compounds **1** and **2**.

It is likely, that the biosynthesis of **2** takes place in the adult abdomen, but the product is directly translocated into the elytral. This is based on the fact, that the concentration of **2** is always low in the hemolymph comparing RNAi and control groups. Opposed to that, the RNAi effect for **2** was only observable in the secretions. If the biosynthesis is blocked in the hemolymph, less substance is present to get transported in the glands which result in the detected decrease. The missing RNAi effect of compound **1**in the secretion supports this hypothesis. The presence of **1** in the secretion at really low amounts can be caused by a spontaneous decomposing of **2** or an unspecific translocation.

To summarize these findings, it can be stated that adult biosynthesis of **2** takes place in the abdomen, but in contrast to the larval storage location, **2** is transported into the elytral secretion system for defense (figure 16).

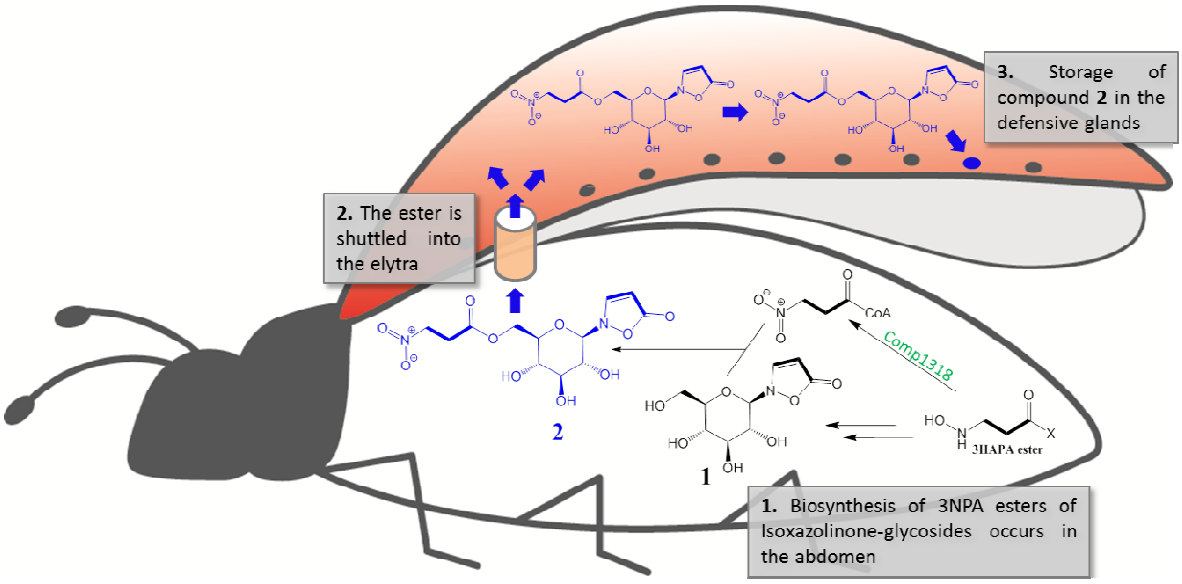


Figure 16: Proposed compartmentation of compounds *1* and *2* during adult biosynthesis of defensive substances.
After production of **2** in the abdomen, it is transported into the elytra and further to defensive secretions for storage. **1** is detected in the secretion only in traces, which may arise due to spontaneous decomposing of **2**.

6.5 The Comp1318 mRNA is present in specialized larval substructures

To localize the biosynthesis of Isoxazolinones, RNA-FISH was applied, targeting Comp1318 mRNA (figure 13). One specific structure was identified as the location of the mRNA, which appeared four times in the slice (figure 13A). Also, a lot of background staining was detected, which was of highest intensity at thecuticular tissue, but also adipocytes and muscle cells show some light emission. The interesting structure, which was not detected in negative control, exhibits a saccular shape and shows fewer nuclei, which were additionally located only at the edges of the structures (figure 13B). In the center, the most mRNA was detected, which is a hint, that also transcription will take place there. To ensure the involvement of these compartments in Isoxazolinone biosynthesis, some further analysis should be applied.

7. FUTURE PERSPECTIVES

This workpaved the way for further studies regarding the Isoxazolinone biosynthesis in*C. populi* leaf beetles through shielding light on the gene *comp1318* which codes for the protein that may transfer the 3HAPA-ester into the 3NPA moiety. The knockdown of this protein has led to significant changes in the composition of substances involved in the biosynthesis. However, I was unable to express the protein heterologously, so the real substrate was not confirmed. This is one of the main projects, which should be considered in the future. After expression, activity tests should be applied to determine substrate(s) and reaction kinetics. Additionally, it would be interesting to know which other enzymes are involved in the production of compounds **1** and **2**. If they do not belong to the CYP family, the search has to be extended to other classes of enzymes like GMC-oxidoreductases, which were also typical enzymes in insects. It is worth noting that possibly more than one enzyme can be involved in catalyzing one particular reaction. In that case, maybe also the enzymes Comp195/ Comp200 which had been mentioned in this work can be putative targets for further analysis.

Because Comp1318 is involved into a defense mechanism which has developed over a long period, it is interesting to know its ecological relevance. For that, a stable line of leaf beetles which don’t produce **2** anymoreshould be established, exposed to natural cues

and compared with untreated controls. If they were more prone to pathogens or predators, the relevance is confirmed. To establish such a line, the genetic tool CRISPR/Cas9 could be useful.

8. SUMMARY

Mechanisms of chemical defense are a widespreadfeature to protect organisms from predators. The poplar leaf beetle *Chrysomela populi* (Chrysomelidae: Coleoptera) has developed some of those defensive strategies to compete with environmental dangers and to assure survival. This work, which based on previous results of Becker et al. (2006), focuses on non-volatile 3-nitropropanoic acid (3NPA) esters of Isoxazolinone glycosides, which are involved in leaf beetle defense. Regarding biosynthesis, Cytochrome P450 Monooxygenases (CYP) were expected to be required. After selection of putative CYP candidates via transcriptomics, proteomics and expression analysis, knock-down strains of *C. populi* larvae and adults were analyzed with respect to metabolic changes by HPLC/MS. It turned out that one CYP, Comp1318, is responsible for the production of the 3NPA moiety and silencing of this enzyme leads to a massive reduction in the synthesis of the defensive ester in larval hemolymph and adult secretions. In larval sections, saccular structures have been detected by targeting Comp1318 mRNA using RNA-FISH, which seems to be thelocation for the biosynthesis in this life stage. In the adults, the production of the defensive ester is located in the abdomen, but the compound is finally stored in the defensive secretions of the elytra, indicating the involvement of transporters.

9. ZUSAMMENFASSUNG

Chemische Verteidigungsstrategien sind eine weit verbreitete Methode, um sich vor Fraßfeinden zu schützen. Der Pappelblattkäfer *Chrysomela populi* (Chrysomelidae: Coleoptera) hat im Laufe der Zeit verschiedene Mechanismen entwickelt, um Umweltgefahren zu trotzen und so sein Überleben zu sichern. Diese Arbeit, welche auf den Resultaten von Becker et al. (2006) aufbaut, beschäftigt sich mit nicht-flüchtigen 3-Nitropropionsäure (3NPA)- estern der Isoxazolinon- Glycoside, die in der Verteidigung der Blattkäfer eine wichtige Rolle spielen. Im Speziellen wurden die enzymatischen Aspekte der Biosynthese betrachtet, die eine Beteiligung von Cytochrom P450 Monooxygenasen (CYP) vermuten ließen. Mögliche CYP-Kandidaten wurden durch Kombination von Methoden aus den Bereichen Transcriptomics, Proteomics und Expressionsanalyse identifiziert und durch RNA-Interferenz in Larven und Käfern ausgeschaltet. Die resultierenden Phenotypen wurden mittelsHPLC/MS analysiert. Einer der Kandidaten, Comp1318, zeigte sich in die Biosynthese involviert und scheint für die Produktion der 3NPA-Einheit verantwortlich zu sein, da dessen Runterregulation eine starke Verringerung der Verteidigungssubstanz sowohl in der larvalen Hämolymphe, als auch im adulten Sekret zur Folge hatte. In histologischen Schnitten larvaler Gewebe konnten durch RNA-FISH sackartige Strukturen identifiziert werden, die reich an Comp1318 mRNA waren und damit möglicherweise eine übergeordnete Rolle in der Biosynthese der Verteidigungsstoffe bilden. In den adulten Käfern scheint die Synthese hauptsächlich im Abdomen abzulaufen, der 3NPA-ester wird jedoch final in die Verteidigungsdrüsen der Elytren transloziert, was wiederrum spezielle Transportmechanismen vermuten lässt

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11. ATTACHMENT

11.1 Sequence information of the candidates used for RNAi

Open reading frames and sequences used for RNAi

Comp1318:

Base pairs: 1491

Sequence: full open reading frame; fragment for RNAi (565 bp)

ATGTTGTTCGTTGTGATAGTGGTATTATTAGCTTTGTTGTATTACTTATCGAGAAGAAATCTGAATTACTGGA
GAAGCATGGGTATTCCTGGTCCCAAACCGTACCCAATCATTGGAAATATGGGGGACATTTTCATTGGAAGA
CGGGGCGCTGAGGAAATTTATGCCGACATTTATAGGAAATATGAGGGCTATCCTTTTGTTGGAGTTTACAA
AGCTGGTATACCGGTTCTACTAGTCAGAGATCCAGATCTTATCAAAAATATAGTGCAGAAGGATTTCAACAT
ATTCCACGAGAATGACATCTTCGTAGACAGGCAGATTGACCCGATATTCGGCAGGAATCCTTTTGCAGTAAA
GGGAGCAGAGTGGAAGAAAATTAGATCTCAGATGACAAGTTGCTTTACTTCTGGAAAGATGAAAGGATTG
TTCCCAGTTCTAGAAAGTAACGTCGATAAGATGATGTCGTACCTAGACAATCAAATGAAAACGAGTTCTTGT
TTCGAAGCCAAAGAGATATTCGTCAGATTCATGCTGGACAATGTTGCTGCTTGTGCCTTGGGTATCGAAGG
GAAATGCTTCGACGAACCCTATTCTGAAGTTAGGAAGATGGCAGATCACTTTTTCTTTTCAAAGGGTATCAC
TAGTCTCAAGTTCATATTAATCTTCAATCTCCCTCCGTTATCAAAATTCCTCAGGCTGAAAGTTGTTTCTCGGG
ACGTAGAAGATAAACTACTGGATATAGTGAAAACGACTCTGCAGTACAGGAGAGACAATAATATAGTTATC
AATGATATCCTGGATTCTATATCTCAAATAAGTACTACTCAAAACGTCACTGAGCTAGATATAACCTCGCATA
TTGCGACATTTTTCACCAACGCCTTTGAGACGAGTTCACTGACCATGTCATTCTTGATCTACGAATTGGCACA
GAATCCAGAAAAACAGAACAAATTGAGAAAAGAAATCAAAGAGTCCTACGAGGAAAATAACAATAGCTTC
ACGTATGAATCTATACAGGATATGGAATATCTAGATGCCTGTTTCCGAGAAAATCTGAGAAAGCACGCTACT
GTACCACTAATGCCGAAAATCTGCACTGAGACCTATAAGTACGTACCAACCAATCCAGAATTTAAACCCATC
GAGATCACCCTGCAGCCAGGTACATCGGTAATCATTCCAACAACAGCAATTCACCATGACCCCAAGTACTAT
GAAAACCCAGAAGATTTTGTGCCAGAAAGATTCTTGGGCAAAGAGAATACCAACAAATTCACGTATCTGGC
TTTTGGTGGAGGACCCAGGATGTGCATAGGTACCCGTTTTGCCTTGTCCCAAATCAAAATAGGAGTTGCCCA
CCTCATCAGAAACTATGAATTCACTGTGAACCCCAAAGGCAGGATACCTGTCAAATACGATCCCTTCTACTTC
TTGTTGACCGCCAGCGGAGGACTCTGGGTGGATATCCGCAAGATAGACCCGTGA

CL7715:

Base pairs: 1109

Sequence: incomplete open reading frame; fragment for RNAi (349 bp)

…AGATCCCGAATTGATTAAACAGATTACTGTCAAGGACTTCGACCATTTCACAGACCACAGATCTCTGGTTC
CCGAAAATATCGATCCAATTTGGGCTAACAACCTATTCGCATTGAGTGGACAAAAATGGCGAGATTTGAGA
CCTGTACTGTCTCCGTCGTTCACCAGCAAAAAAATGAAAAACATGTTTGGGCTGATGTCGGAGTGCGCCCAA
AACTTTGTCAGAAACTTTCATGAAAAGAAAGAGGACATCGTCGAAATTGAGCTGAAGGATACTTTCTCACG

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GATAAAAATGAGAGAAGAGAAAAACATCGTGCGTCATGACATGCTTAATTTGTTGATGCAAGCTAAGAAGG
GAAAACTCAAATATGAGGAAGATAAAGAAAATTTGGATGGGTTTGCTGCCGCCAAAGAATCAGATATTGG
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AATTTGCACGACACCCTATACCATCCAACCGAAGTTGCCTGGCGAAGTGCCCGTCCATTTAAAGAGAGGTG
ACGTCCTTTGGTTCCCGATAATCGGCATCCATAAGGACCCCCAGTACTTCCCCGATCCGGAACGTTTCGACCC
AGAGCGGTTCAGCGACGAGAATAAGGCGAACGTCA…

Comp195:

Base pairs: 1686

Sequence: full open reading frame; fragment for RNAi (530 bp)

ATGTCGGCCACGACCGCTAGTCCGGACCTGGTCGCTGCCAGCACCACCTTATCGGCCAGCAGTGTTTTTTAC
TTCCTCCTGATTCCAGCCACCATTTTATGGTATGCATACTGGAAAATATCCAGAAGCCACATGGTGGAATTG
GCAGACAAAATTCCTGGACCCAAAGGCCTGCCCATCATCGGATGTGCCCTTCAATTTCTCGGAACCAGTCCA
CAGATCTTCAAGAGAGTCTACGAATTATCTTATGATTATGGAAGAGTCGTAAAAATATGGGCTGGACCGAA
ACTCATCATTTTCTTGATGGACCCCAGTGATGTTGAAATCATCCTCAGCAGTCATGTTCACATTGACAAAGCC
AGTGAGTATAGATTCTTCAAGCCTTGGTTAGGAGATGGTCTTCTGATCTCCACCGGCCAGAAATGGCGTGCT
CACAGAAAACTTATCGCTCCCACTTTCCATCTGAACGTTCTCAAGTCCTTCATCGACTTATTCAACGCCAACTC
TCGTGAGGTAGTCCAGAAGCTCAAGAAAGAAATCGGTAAAGAATTTGATTGCCACGACTACATGTCTGAAG
CAACCGTAGAAATCCTCCTGGAAACTGCTATGGGTGTCAGCAAGAAGACTCAGGACCAGAGTGGATACGA
CTATGCTAAAGCTGTGATGGATATGTGCGATATCCTTCACTTGCGTCACACCAAGATCTGGCTCAGGCCGGA
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GAAGGTAATCAAGAGGAAGAGGGCAGACTTCGAGAAAGGTATCCGAGGATCAACGGCTGAGGTACCAGA
AGAAGTAAAGACTTTCTCTGCTGGAGCCGTCAGCTCGAAGACAGTTGTCGAAGGACTCTCCTACGGACAGT
CGGCAGGACTGACAGATGACTTGGACGTCGATGATGATATTGGTGAGAAGAAGAGGATGGCATTCTTGGA
CTTGATGATCGAAGCTTCGCAAAATGGAGTTGTGATTAACGACGAAGAGATCAAAGAACAAGTAGACACCA
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ACATTCAGGATAAGGTTATCCAAGAGATTGATGAGATATTCGGTGACTCAGACAGACCGGCGACATTCGCC
GACACTCTGGAGATGAAATACCTCGAGAGATGTATGATGGAAACACTAAGGATGTACCCACCAGTACCTAT
CATCGCTAGGCAATTGAGGCAAGACGTCAAACTGGCATCTGGCGACTACACGCTTCCAGCTGGTGCCACTA
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TGTCGGTCGTAAATACGCCATGCTGAAGCTCAAGATCCTCCTCTCCACCATCTTGAGAAATTATCGCATCGTC
TCCAATATTCAAGAGAAAGATTACCAGCTCCAGGCTGATATTATATTGAAGAGGGCCGAGGGATTCAAAAT
CAGGTTGGAGCCTAGGAAGAGGTTGGCAAAAGCTATGTAA

Comp200:

Base pairs: 1677

Sequence: full open reading frame; fragment for RNAi (515 bp)

ATGTCACAGCCACAAGTGCTACCACTAGGCGCCGAGAATTCTACTCTGACAAGTTCCCTCGTCACCTCCCTA
ATAGGAGTGGCTGTCGTGATAACATTGTACCACTTCTGGTTCCAAAGCCTTCGATATGTTCGGATAGCCAAA
AATATTCCGGCTCCTACAGCGCTGCCGATACTGGGTCACACCTATTTGGCATTGGGGCGGTCTCCAGCAGAT
ATCTTCAAATTCGCTATCAAAATGTACCAAGAGATCAAAAGTGACGTTGTCAAAGCCTACTTGGGTCCCAAG
CTAGTGGTTGGAGTCGCCTGTGCAGAAGACGCTGAGATTATACTGGGAAGCAATGTCCATTTGGAGAAATC
TCCAGAATATGGCCTGTTCGAACCGTGGTTAGGAAATGGATTACTCATCAGTAAAGGTGAAAAATGGCGTT
CGCATAGGAAAATGATTGCCCCCACATTCCATACTTCTATTCTGAAGTCATTTTTGCCAGTTTTTAATAAAAA
CGCCAATCAGCTCGTAGATGCATTTAGAAAAGAAAAAGGCAAGGAGTTTGATGTTCATGATTATATGAGTG
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CAAAGAGATCCAGCAGAGGGTGTTCGAAGAGCTCAGGGAGATCTTCCAGGATAACATGGACCGGCCCATC
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GAACGACAGTGATAGTGGCGCAATATCTGATTCACAGGAATCCCAAGCATTACGACAATCCGGATAAATTC
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CCTAGGAGTTGTGTTGGTCGCAAATACGCCATGTTGAAGCTGAAGGTGCTCTTAGCAGGTGTGTTGAGAAA
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Purification of dsRNA for RNAi confirmed by agarose gel electrophoresis

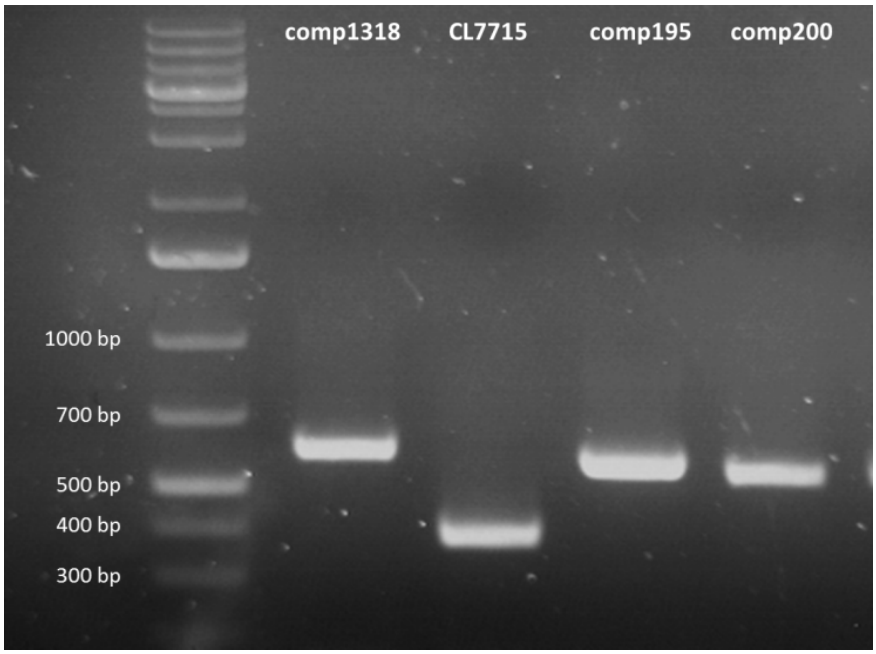
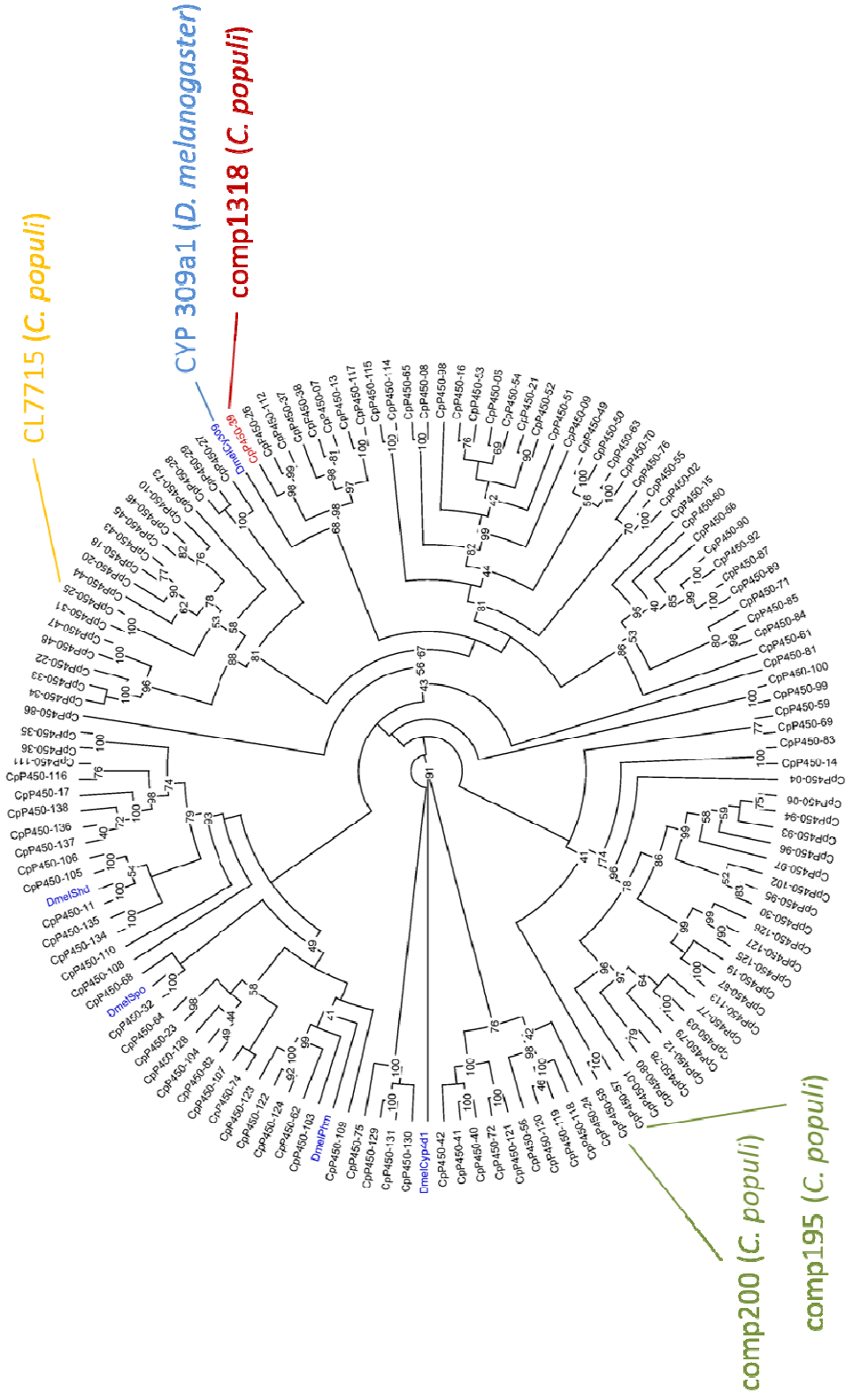


Figure S 1: Agarose gel electrophoresis after production of dsRNA for RNAi.

11.2 Phylogenetic analysis of the CYPs found in the transcriptomic library



11.3 Monitoring of larval development in *comp1318* RNAi experiment

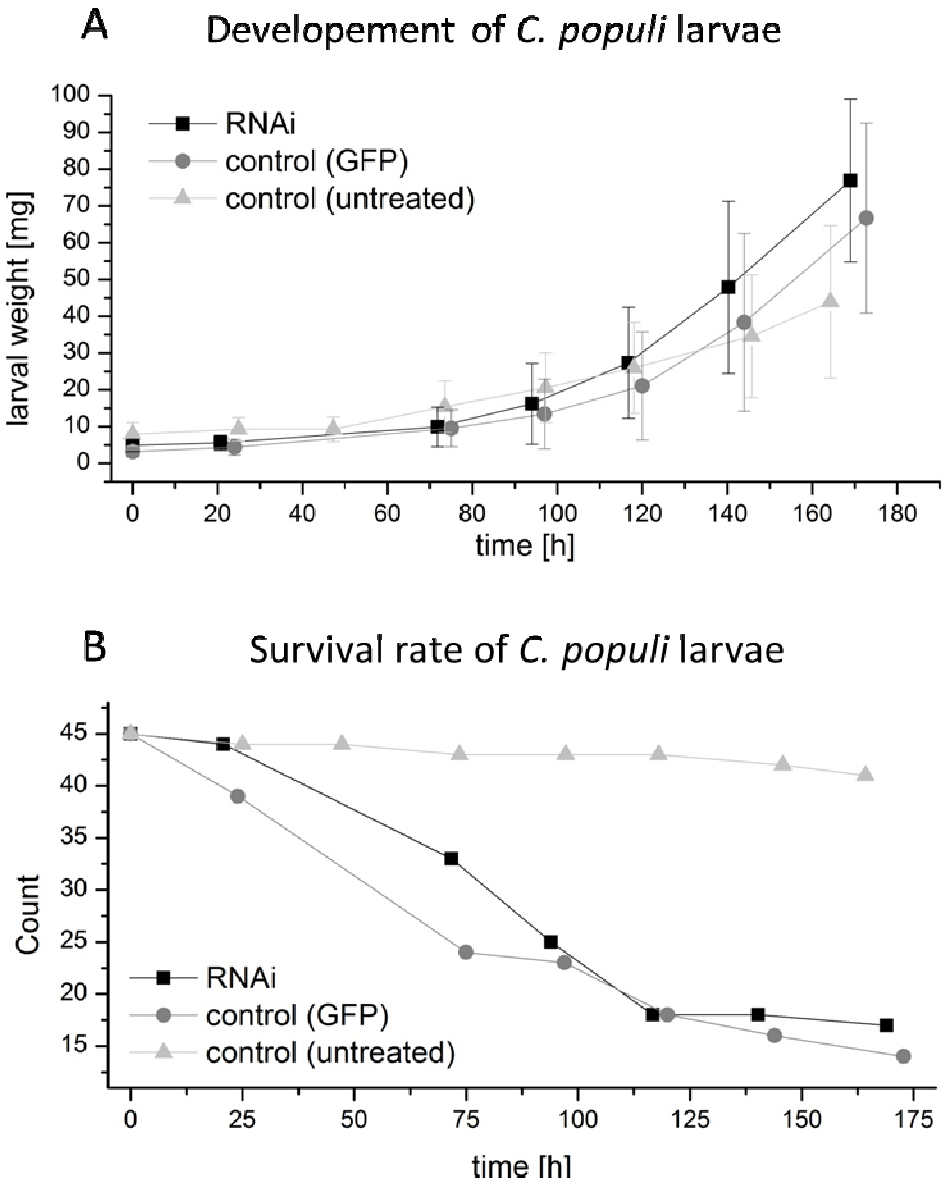


Figure S 2: Development of *C. populi* larvae in RNAi induction experiment. (A) Development based on weight increase of dsRNA-treated and untreated larvae. No obvious differences were detectable between the groups. (B) The survival rate of the larvae during the experiment. In the group of the untreated larvae, the survival was much higher compared to the dsRNA-treated groups. Severe wounding during injection causes more than 25 out of 45 dying in the GFP and RNAi groups.

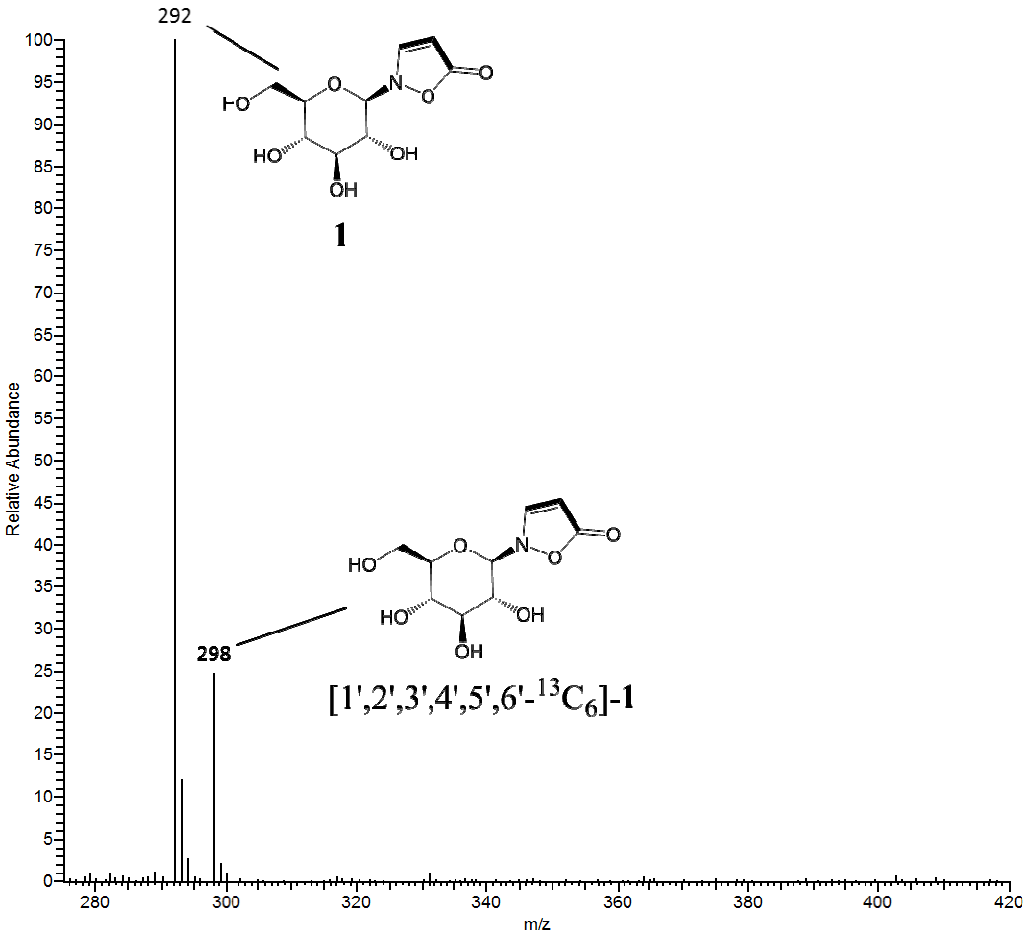


Figure S 3: Mass spectra of isoxazoline-5-one-glucoside and the heavy-isotope-labeled standards.

Marked signals correspond to $[M+HCO_2H]^-$ ions

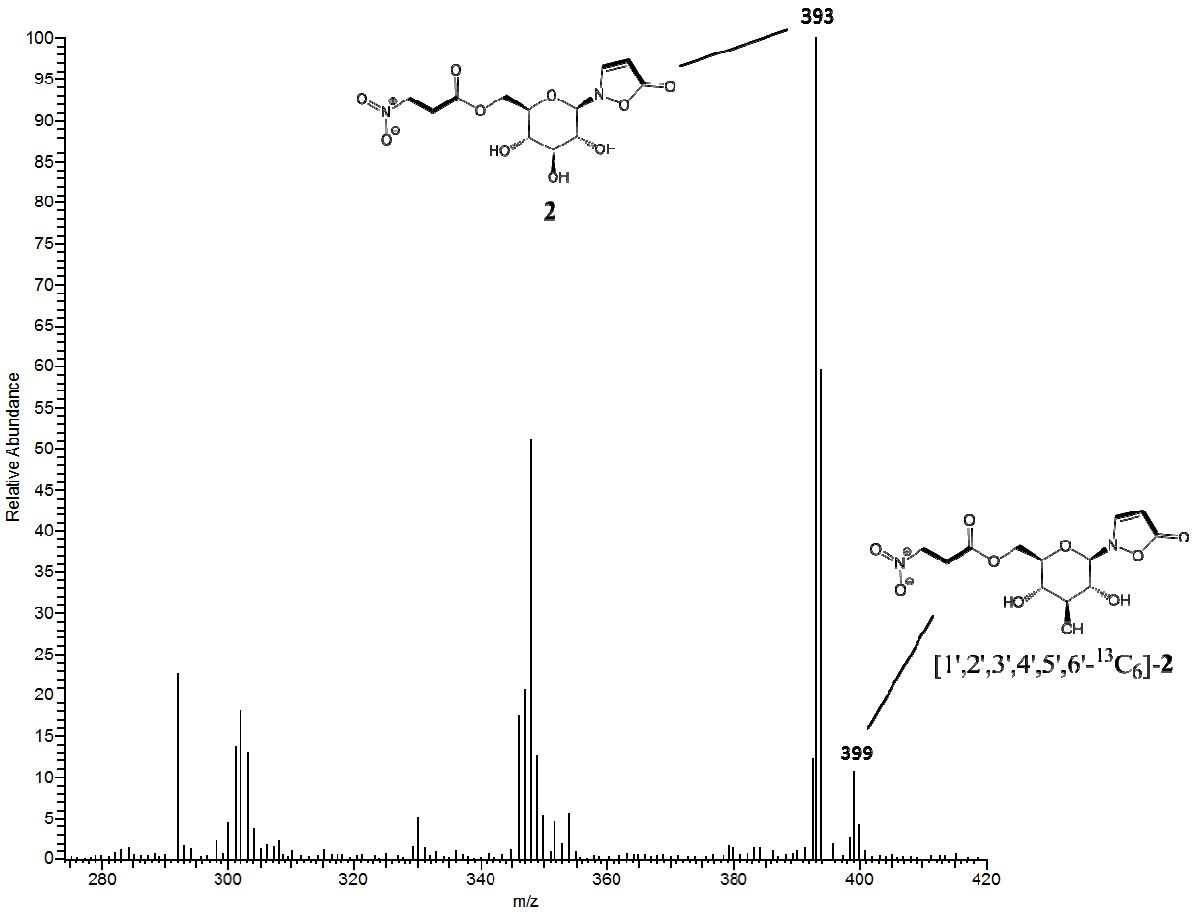


Figure S 4: Mass spectra of 3NPA ester of isoxazoline-5-one-glucoside and the heavy-isotope-labeledstandards.

Marked signals correspond to $[M+HCO_2H]^-$ ions

12. ACKNOWLEDGMENT

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Finally, I want to thank my family and friends, which always believed in me and gave me a lot of support during this work.

13. DECLARATION OF AUTHORSHIP/ SELBSTSTÄNDIGKEITSERKLÄRUNG

I hereby declare that the thesis submitted is my own unaided work. All direct or indirect sources used are acknowledged as references. This work was not previously presented to another examination board and has not been published.

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst und keine anderen Hilfsmittel als angegeben verwendet habe. Insbesondere versichere ich, dass ich alle wörtlichen und sinngemäßen Übernahmen aus anderen Werken als solche kenntlich gemacht habe.

_____

city/ date: signature: