Sequestration in leaf beetles:

Identification and characterization of ABC transporters involved in the chemical defense of Chrysomelina larvae

Dissertation

zur Erlangung des akademischen Grades

„doctor rerum naturalium“

(Dr. rer. nat.)

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät

der Friedrich-Schiller-Universität Jena

seit 1558

Anja S. Strauß (Diplom-Biochemikerin)

geboren am 24.12.1979 in Gera

24. Januar 2014

Gutachter:

1. Prof. Dr. Wilhelm Boland, Max-Planck-Institut für Chemische Ökologie, Jena

2. Prof. Dr. Stefan Heinemann, Friedrich-Schiller-Universität Jena

3. Prof. Dr. Monika Hilker, Freie Universität Berlin

Tag der öffentlichen Verteidigung: 1. Juli 2014

Anmerkung

Die nachfolgend dokumentierten Arbeiten wurden am Max-Planck-Institut für chemische Ökologie Jena in der Arbeitsgruppe für Bioorganische Chemie unter der Leitung von Prof. Dr. Wilhelm Boland angefertigt.

Meinen Eltern

Contents

1 Introduction

Insects are by far the most diverse and numerous organisms in the history of life. More than half of all described species are insects [1, 2]. Though almost one million insect species are listed, the total number is estimated to be around five million [2, 3]. Not only are they unmatched in species number but also in: the diversity of their adaptations, their biomass, the longevity of their lineage and ecological impact [3]. Various hypotheses on the reasons for the diversity and evolutionary success of insects exist; an intriguing one is based on the interactions with other organisms, especially plants, and specialization [1]. Nearly 50 % of all insect species have been using plants as a food source [4], while most of these phytophagous insects (herbivores) are specialized to their host plants [5–8]. The earliest fossil records of insect feeding damage on plants dates back to the Early Devonian - approximately 400 million years ago [9]. Their coexistence for this tremendous period of time has allowed both plants and insects to evolve an extensive network of relationships. Interactions between plants and herbivores have shaped diversification and genetic variety in both kingdoms [10,11] and are postulated to be the driving-force for the richness of species on earth [12, 13].

1.1 Plant-herbivore interactions

Plant-herbivore interactions dominate the terrestrial ecology on our planet and have attracted scientists of multidisciplinary fields since decades [14]. A multitude of plants depend on pollination by insects, but at the same time plants need to evolve strategies to fend off phytophagous insects. The array of plant defenses is to a great extend attributed to herbivores and microbes [3, 10, 11, 15]. In response to herbivores, plants have evolved several morphological and biochemical adaptations assuring their survival; one intriguing strategy is based on toxic secondary metabolite production which may be

imagined as kind of chemical warfare [16,17]. The extraordinary diversity of secondary metabolites in many plant species is thought to be the result of a biochemical arms race or reciprocal evolution between plants and herbivores [12,13,16,18–20].

Insects in turn, have evolved a number of protective measures which allowed them to adapt to plant secondary metabolites, such as selective feeding on plant parts with low doses of the toxins [21,22], the effective excretion of toxins [21,23] or the enzymatic detoxification by a variety of metabolic means [24–26] or with the help of endosymbiotic microorganisms [27,28]. Another possibility is the sequestration of plant secondary metabolites which has evolved by numerous species of all insect orders [29–31]. The sequestered substances are often toxic and various insect species use them for their own defense [29,32], allowing colonization of ecological niches [28] and thus shaping the plant world [13,33,34]. This dissertation focuses on the identification and characterization of molecular transport processes involved in these sequestration processes.

1.2 Sequestration is widely distributed in insects

Sequestration is a fundamental process of all organisms and a requisite for life. Basically, the phenomenon of sequestration is the differential organization of exogenous chemicals into other molecules, membranes, organelles, cells or tissues [29].

The sequestration of plant toxic metabolites which involves the uptake, transfer, and concentration of occasionally modified phytochemicals into the hemolymph, cuticle, specialized tissues or glands is an ingenious detoxification strategy found in most insect orders [29–31]. More than 250 insect species have been shown to sequester plant metabolites of at least 40 plant families [31]. Sequestration is prevalent in many orders, especially in Lepidoptera and Coleoptera, but also occurs frequently in the orders Heteroptera, Hymenoptera, Orthoptera and Sternorrhyncha [31]. The sequestered phytochemicals are used for defense purposes or serve as building blocks for pheromones or other toxins. Various reviews on sequestration by insect herbivores exist focusing on certain compound classes [29,35–39], or sequestering insect orders [30,40–42], or on both [43]. The latest review by Opitz et al. [31] offers a comprehensive overview of sequestration by insect herbivores when grouping the sequestered plant chemicals in different plant metabolite classes.

Up to now, the most comprehensive knowledge of sequestration processes has been obtained from juveniles of the leaf beetles belonging to the taxon Chrysomelina, which use the sequestered toxins for their own effective chemical defense [44–48].

1.3 Sequestration and chemical defense of leaf beetle larvae

Beetles are the largest insect order - they represent 20-25% of all described species [49]. Among beetles, the „Phytophaga“ are the largest and oldest radiation of herbivorous beetles. With over 135.000 species they comprise roughly 40% of all described beetle species [50]. This lineage includes the sister beetle superfamilies Curculionoidea (weevils) and Chrysomeloidea. The latter consists of the species-rich Cerambycidae (longhorn beetles) and Chrysomelidae families. Leaf beetles (Chrysomelidae) contain about 37.000 species in 19 subfamilies which mainly feed on green plant parts [8]. Their metallic and colorful appearance is reflected in the name Chrysomelidae (chrysos = gold, melolanthion = beetle). Apart from their aposematic coloration, leaf beetles, in particular, are well known for sequestering structurally different allelochemicals, such as beta-amyrin [51], cucurbitacins [52], pyrrolizidin alkaloids [53], phenolglucosides [54], cardenolides [41], glucosidically bound aliphatic alcohols [55] or iridoid glucosides [39]. The plant compounds may be slightly or profoundly modified by the beetles to become biologically active. The influence of host plants on leaf beetle defensive chemistry is often proposed by indicational studies comparing toxins of both insects and their plants, but has in some cases been confirmed by labelling experiments.

Most leaf beetles spend the whole life cycle in the foliage of their hosts and are expected to utilize secondary metabolites of their host plants for protection. While the larvae can actively sequester plant metabolites, adults do not use phytochemicals for their own benefit, though they are capable for sequestration. Pasteels *et al* [44] demonstrated that adult females can shuttle metabolites from the host plants into their eggs in amounts highly deterrent to ants. For their own defense purpose they synthesize compounds like cardenolides, glucose conjugates of 3-isoxazolin-5-one and 3-nitropropanoic acid [40]. Therefore all life stages are chemically protected within the Chrysomelina subtribe.

This thesis focuses on the chemical defense of Chrysomelina larvae which consists of compounds that are either sequestered from their host plants or synthesized *de novo*.

1.3.1 Defensive glands of Chrysomelina larvae

Larvae of the Chrysomelina subtribe possess nine pairs of specialized exocrine glands on their backs (Fig. 1.1) [56, 57].

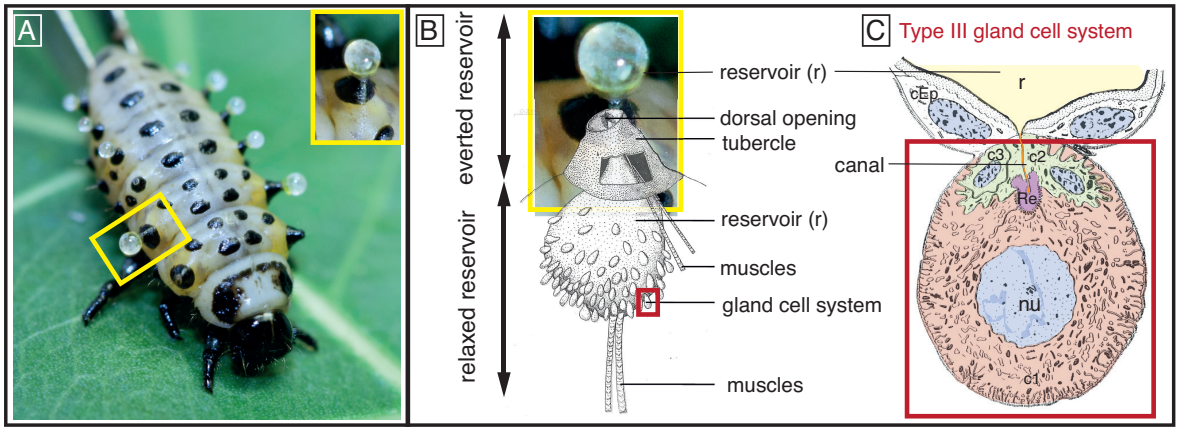


Fig. 1.1: Defensive glandular system of Chrysomelina larvae (A) *Chrysomela populi* larvae exposing defensive secretion after beeing stimulatively attacked (B-C) Morphology of defensive glands of Chrysomelina larvae (B) Drawing of dissected glandular tissue of *C. populi* according to Hinton [57] displays the relaxed reservoir in contrast to overlaid everted reservoir (C) Drawing of a single glandular cell system consisting of a enlarged secretory cell (c1), painted in red, and two canal cells (c2, c3 in green). A cuticular canal (orange) connects the extracellular storage room (Re, in magenta) adjacent to the secretory cell with the reservoir (yellow). cEp - epithel cells (white), nu - nucleus (blue).

Regardless of the origin of their defensive precursors, these compounds are transferred into their defensive glands. There, they are enzymatically converted and presented as droplets of secretions upon disturbance of the juvenile beetles (Fig. 1.1A). As soon as the attack is over, the secretions are resorbed into the so called reservoirs by retraction muscles. Beside the cuticularized reservoirs and the affiliated muscles, each defensive gland system contains of a dorsal opening and enlarged glandular cells (Fig. 1.1B). The glandular cell systems themselves are classified as type III cells [58] and share a homogenous architecture (Fig. 1.1C). Each defensive gland is composed of a number of enlarged secretory cells (varying in number among Chrysomelina species), which are in turn connected to the chitin lined reservoir. The secretory cells display an unique morphology; they are always accompanied by two canal cells that form a cuticular canal which connects the secretory cell with the reservoir [58].

1.3.2 Chemical defense of Chrysomelina larvae

The defensive compounds in the secretions of Chrysomelina larvae span four compound classes [44, 48, 59], namely iridoids (cyclopentanoid monoterpenoids), aldehydes (salicylaldehyde and benzaldehyde), esters (phenylethyl esters), naphtoquinone (juglone). Most widespread are iridoids, salicylaldehyde and phenylethyl esters [40].

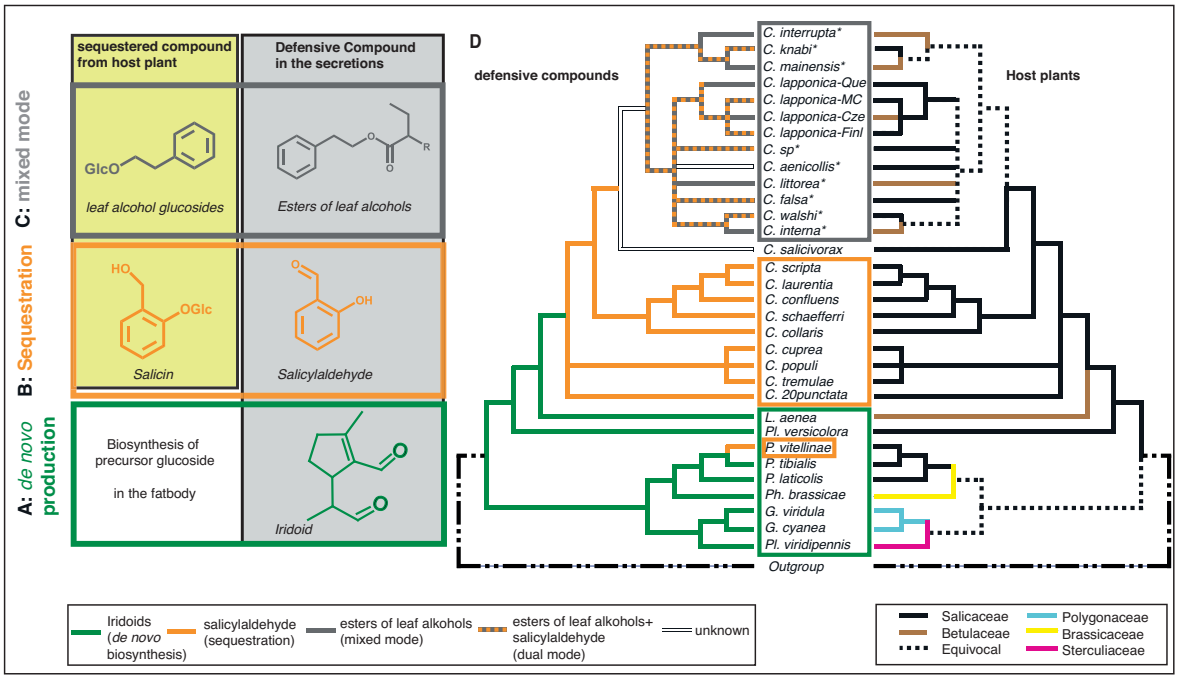


Fig. 1.2: Chemical defense strategies of Chrysomelina larvae (A-C) assigned to their phylogeny and host plant affiliation (D) based on maximum parsimony reconstruction (according to [46]). Green: autogenous monoterpene iridoid production; orange: obligate sequestration of salicylaldehyde precursors (from Salicaceae); grey: mixed mode metabolism (interrupta group). The ability to sequester plant glucosides is considered an energy-saving, monotypic adaptation within Chrysomelina. The color codes of branches are explained below the tree. Abbreviations: *C, Chrysomela; G, Gastrophysa; L, Linaeidea; Ph, Phaedon; P.: Phratora; Pl, Plagiodera.*

Phylogenetic analysis by Termonia et al. [46] revealed that the autogenous biosynthesis of iridoids is the ancestral character state in the chemical defense of chrysomeline larvae (Fig. 1.2A). More derived species sequester phytochemicals obligatorily, which is considered an energy-saving, monotypic adaptation within Chrysomelina (Fig. 1.2(A-C)). However, this caused a tight dependance on the chemistry of their hosts. Larvae of the Chrysomelina species feeding on Salicaceae sequester phenolglucosides such as salicin and salicortin [54, 60] (Fig. 1.2B). The glucosides serve as precursors for the repellent salicylaldehyde [54,61]. In contrast to the incorporation of a few plant-derived

compounds, larvae of the most evolved Chrysomelina species are able to take up a wide variety of glucosidically bound leaf alcohols. Their aglucons are further esterified with carboxylic acids derived from the insects' internal pools of amino acids, which can result in a cocktail of at least 70 deterrent esters in the defensive secretions [46, 55, 62, 63](Fig. 1.2C). These sophisticated defense strategies with different degrees of host plant dependance among Chrysomelina offers an ideal tool to study the mechanisms of adaptive evolution.

1.3.3 Host plant adaptation within the Chrysomelina subtribe

The association of allomone production strategies by leaf beetle larvae to their host plant families displays the reciprocal adaptation of Chrysomelina beetles to their hosts (Fig. 1.2D) [46,64]. Whereas the host plant range of iridoid *de-novo*-producing species is relatively broad (7 plant families [40]), salicin-adapted chemical defense restricts those larvae to feed on Salicaceae. Interestingly, a monophyletic clade within the genus Chrysomela (*interrupta*-group) evolved the biosynthesis of butyrate-esters which are defensive compounds of mixed origin, beetle and plant [55, 62]. Some of these species overcame this high degree of specialization by evolving allopatric populations which colonize Salicaceae and Betulaceae, displaying the ability to adapt dynamically to their environment despite previous investments.

In conclusion, leaf beetles of the Chrysomelina subtribe constitute an excellent model taxon for investigating sequestration processes. Their easily accessible secretions and the possibility to follow the evolution from *de-novo*-producing species to obligate sequestering species further to species which escaped the tight dependence and shifted to other host plants makes this model taxon unique.

1.4 An introduction to the investigated species

The main focus of this work is the elucidation of transport proteins involved in sequestration processes of the obligate sequestering species *Chrysomela populi*. For a comprehensive analysis, homologs of newly identified transport proteins of *C.*

populi were also investigated in *Chrysomela lapponica* and *Phaedon cochleariae* as representatives of different chemical defense strategies (Fig. 1.2).

1.4.1 *Chrysomela populi* - life cycle and occurence

The poplar leaf beetle *C. populi* is an example of an obligate sequestering species within the subtribe Chrysomelina. The larvae incorporate the phenolglucoside salicin of leaves of their food plants [54, 65]. In the reservoir of their defensive glands salicin is metabolized to the volatile deterrent salicylaldehyde [60].

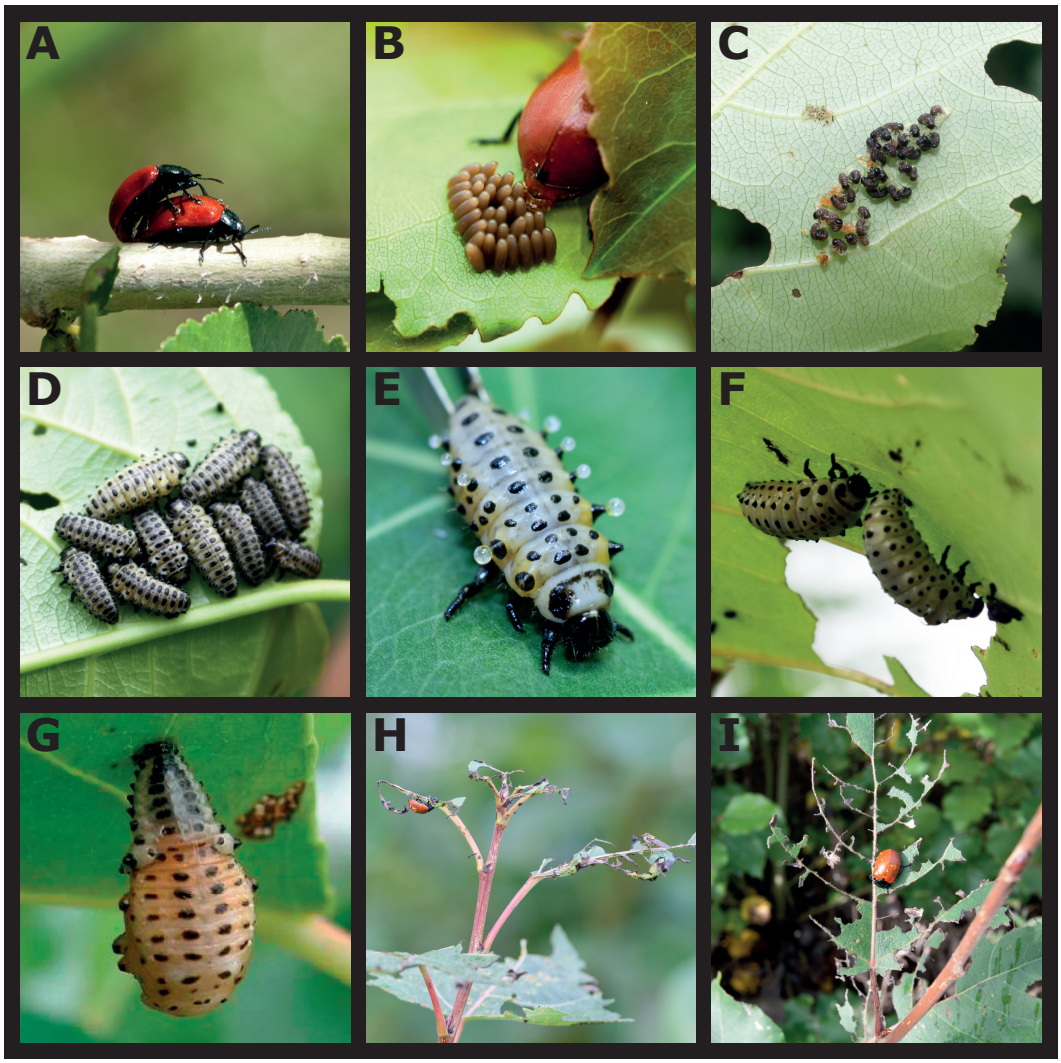


Fig. 1.3: Development of *C. populi*. (A) Mating of adult beetles, (B) Egg clutch underneath a poplar leaf (C) Hatching first instar larvae, (D) Second instar larvae, (E) Third instar larva exposing defensive secretion after beeing attacked, (F) Feeding third instar larvae, (G) Pupa, (H-I) Feeding damage of *C. populi* imago on poplar leaves.

Imagos emerge after hibernation in the ground from the end of April to the beginning of May. After mating (Fig. 1.3A) the female beetle deposits 20 to 65 eggs as one clutch (Fig. 1.3B). Under laboratory conditions, the embryonic development takes about 5 to 10 days, depending on temperature. Larvae pass through 3 instars which takes 2 to 4 weeks (Fig. 1.3C-F) before pupation and development into the second imago generation. In general, three imago generations develop during a year. Most reports on its occurrence come from Central Europe and the Mediterranean [66, 67]. Furthermore they occur for example in Sweden, India and Japan [68–70].

C. populi is considered a pest with high gradation potential from the viewpoint of forestry [71–73]. Extensive damage to even complete defoliation is observed in short-rotation plantations (Fig. 1.3H-I) [73]. Beside their high reproduction rate (roughly 500 eggs per female), the fact that all developmental stages feed on the same host plant and the occurrence of several generations per year, their effective chemical defense system is considered as an extremely beneficial trait of this species [71]. In particular, salicin-derived defensive compounds in Chrysomelina larvae have been shown to be highly efficient and of ecological relevance. In general, the defensive secretions (Fig. 1.3E) and especially salicylaldehyde act as deterrents against ants, ladybirds, spiders and wasps [62, 74–77] and possess anti-microbial and cytotoxic activity [54, 61].

1.5 Transport of glucosides in Chrysomelina larvae

The variety of metabolite classes which can be sequestered by insects has challenged chemists since decades. But concerning the transport proteins that facilitate the sequestration of secondary metabolites from plants into insects there is a gap in our knowledge.

1.5.1 Transport processes across membranes

Phospholipid bilayers form efficient barriers primarily against the free flow of polar molecules and ions. Therefore transport processes across biological membranes are essential for maintaining the homeostasis of a cell. In biological systems, fundamentally two forms of transport are distinguished: the transport via channels or via transport

proteins (carriers). Channels are pore-forming membrane proteins with an inner surface which can be either hydrophilic, hydrophobic or amphiphilic, depending on the properties of the substrate to be transported. Generally, mechanical or electrical signals or binding of a transmitter are responsible for channel opening. In contrast, carriers exhibit a stereoselective substrate recognition. Usually a conformational change within the carrier protein is triggering the translocation of substrates across the membrane. Various subgroups of channels and carriers are classified in the TC system (transporter classification system) according to their function and phylogeny [78]. The main classification criteria within carrier proteins are based on energy consumption. Carriers facilitating passive transport do not require chemical energy for channeling their substrates down an electrochemical gradient. In contrast, active transport mechanism are energy coupled and enabling them to work against a concentration gradient. Primary active transporters (e.g. ABC (ATP-binding cassette) superfamily) use chemical energy directly from e.g. the hydrolysis of adenosine triphosphate (ATP). Secondary active transport mechanisms are characterized by a „secondary“ linked transport as energy source (symport or antiport) and predominantly uses an electrochemical gradient.

1.5.2 Transport network for plant glucosides in Chrysomelina larvae

To date, transport studies using labelled substrates to provide evidence for carrier mediated sequestration of phytochemicals by insects were conducted only in the two leaf beetle subfamilies Galerucinae and Chrysomelinae [35, 53, 65, 79, 80]. These studies postulate an interplay of membrane carrier systems with different selectivity and energy requirements that support the sequestration processes of phytochemicals. Basically, the ability to sequester specific compounds out of the huge variety of chemicals occurring in the gut requires a selective carrier system. Numerous publications postulate membrane barriers with different selectivity in Chrysomelinae species [35,65,81]. There are several membrane barriers to pass in order to channel the glucoside of interest from the gut to the defensive glandular system of Chrysomelina larvae (Fig. 1.4). The first transport system has to be located in the gut membrane importing the glucosides into

the epithelial cells followed by an export system into the hemolymph. From there the glucoside is transported into the glandular cells or excreted via the Malpighian tubules.

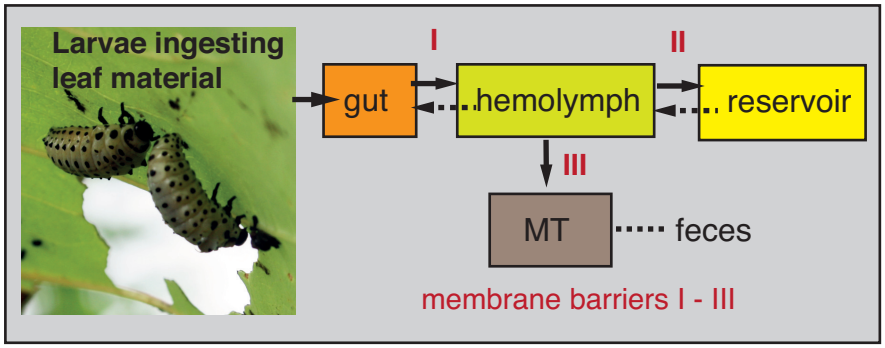


Fig. 1.4: Schematic overview of the membrane barriers (I-III) within leaf beetle larvae. Sequestering glucosides requires the passage from the gut to the glandular reservoir accompanied by the excretion of non-used glucosides via the Malpighian tubules (MT).

Physiological studies of *de novo* iridoid-producing, salicin-sequestering and ester-producing larvae were performed by using thioglucosides resembling natural glucosides to investigate selection criteria of different membrane barriers [47, 63, 65, 81]. These studies have indicated a complex influx-efflux transport network which channels the plant-derived glucosides through the insect body [65, 81]. These experiments further demonstrated that the larvae possess transport systems, which are evolutionary adapted to the glucosides of their host plants. The uptake of plant glucosides from the gut into the hemolymph allows a broad spectrum of plant precursors. In contrast, the uptake into the glandular reservoirs is a specific process that is accompanied by a non-selective excretion of the not utilized glucosides via the Malpighian tubules.

In Manuscript I we provide an overview on sequestration processes of plant-derived compounds by leaf beetles. By following the route of systematically modified strutural mimics of plant-derived glucosides, their conversion and impact on the endogenous biosynthesis, we demonstrate a highly efficient and complex transport system and discuss how to elucidate putative transport proteins as a part of the network.

1.5.3 ABC transporter as putative transport proteins involved in the sequestration of Chrysomelina larvae

Transport proteins take up a central position within the evolution of sequestration processes in insects. Though numerous publications postulate the implication of carriers in sequestration, no transport protein in any insect order has been unambiguously identified yet. To the effect that Chrysomelina larvae are able to accumulate their defensive compounds up to 500-fold in the reservoir from a hemolymph pool [47, 65], it argues for a final active transport within a complex transport network. In insects, ABC transporters are suggested to be involved in the translocation of pesticides and phytochemicals [82–85]. In addition, this transporter class has been proposed as a mechanism employed by plants to sequester plant secondary metabolites into vacuoles to prevent self-intoxication [86–88]. In focus of this thesis are ABC transporter as primary active carrier proteins and their possible role in the sequestration processes within Chrysomelina defense.

Structure and function of ABC transporters

ABC transporters constitute one of the major classes of membrane transporters. They are modular proteins harbouring specific ABC domains, also called nucleotide binding domains (NBDs) and transmembrane domains (TMDs), consisting of six membrane spanning helices [89]. The NBDs are highly conserved and comprise several characteristic sequence motifs. These intracellular domains can bind and hydrolyze ATP in a cycle that drives the translocation of substrates across the phosolipid bilayer. ABC transporters possess an impressively diverse substrate spectrum including metabolic products, lipids and sterols, heavy metals and drugs [90]. In eukaryotes ABC transporter function as exporters, transporting their substrates from the cytoplasm out of the cells or into cell organelles, such as the endoplasmic reticulum or the peroxisome, playing essential roles in various cellular processes.

Besides their physiological relevance, ABC transporters have pharmocological significance as well. Some of them can provide resistance to antibiotics and chemotherapeutic agents. A number of human genetic diseases, e.g. cystic fibrosis and macular dystrophy have been traced back to various ABC transporter dysfunctions [91].

In manuscript II, I applied the theory of an ABC transporter being involved in the accumulation of salicin in *C. populi*, our model beetle for an obligate salicin sequestering species. I demonstrated by RNAi experiments that an intracellular low-affinity ABC transporter of *C. populi* (*Cp*MRP) is a key element for sequestering plant derived defensive precursors within Chrysomelina larvae.

ABC transporter subfamilies

ABC transporters are present in all phyla of life and have been found ubiquitously in all species studied so far, constituting large protein families of 30 up to roughly 100 members [92, 93]. Based on phylogenetic analysis and amino acid sequence alignments of NBD domains, the existing eukaryotic genes have been grouped into major subfamilies, termed from A to I. Each subfamily is characterized by characteristic membrane topology patterns. Both subfamilies (H) and (I) are not present in humans, and seem to either be insect (H) or plant specific (I) [88,94,95]. The prototype and best studied ABC-transporter - MDR1/P-glycoprotein - belongs to the ABCB subfamily and is already hypothesized to function in the gut of insects regulating the absorption of plant secondary metabolites [96–98]. To date, members of the subfamilies B, C and G confer resistance to xenobiotics including drugs and plant allelochemicals (here refered as MDR-ABC transporter) [99–101].

Manuscript III gives an active inventory of ABC-Transporters of *C. populi* based on transcriptomic data. For comprehensive analysis of the presence of efflux transporters within the larva, transcript data are analyzed regarding the tissue specific distribution of each transporter Thereby, I propose a functional link to each ABC transporter class with the focus on sequestration processes. Further we investigated effects of RNAi-silencing of *Cp*MRP as predominant ABC transporter of the glandular tissue on the transcript level of others. There exists the hypothesis of compensation effects within insect subfamily C [102], which we could not confirm for *Cp*MRP as a key element in sequestration process.

2 Overview of manuscripts

Manuscript I

„Always being well prepared for defense: The production of deterrents by juvenile Chrysomelina beetles (Chrysomelidae)“ Antje Burse, Sindy Frick, Sabrina Discher, Karla Tolzin-Banasch, Roy Kirsch, Anja S. Strauss, Maritta Kunert, Wilhelm Boland

Phytochemistry, 2009, 70, 1899-1909. doi:10.1016/j.phytochem.2009.08.002.

This review comprises an overview on sequestration processes of plant-derived compounds by leaf beetles. The route of incorporated strutural mimics of plant-derived glucosides, their conversion within insects and regulatory impact on the endogenous biosynthesis is investigated on the molecular level. This study demonstrates a highly efficient and complex transport system and provides direct evidence that glandular carriers tightly control the metabolite uptake. Moreover we discuss how to elucidate putative transport proteins as a part of the network and set our results in the context of metabolite diversity in chrysomelids as well as the relevance of sequestration processes for enhancing the adaptive radiation observed in plants and beetles.

I contributed to the c-DNA library construction and microinjection experiments. Sabrina Discher and Maritta Kunert performed most of the tracer experiments. Karla Tolzin-Banasch and Roy Kirsch performed research concerning the chemical defense of *C. lapponica*. Sindy Frick and Antje Burse performed research related to the studies of HMGR and IDS. Experiments were planned and designed by S.D., A.B. and W.B., all authors contributed to the interpretation and analysis of the data. A.B. wrote first draft of the manuscript and all authors contributed substantially in terms of their research part. Wilhelm Boland supervised the work and revised the manuscript.

Manuscript II

„ABC transporter functions as a pacemaker for the sequestration of plant glucosides in leaf beetles“ Anja S. Strauss, Sven Peters, Wilhelm Boland and Antje Burse

Elife 2013, doi: 10.7554/eLife.01096

This study identifies and characterizes *Cp*MRP as an intracellular low-affinity ABC transporter (class C type) and demonstrate its key function in the glandular sequestration process of salicin by *C. populi*. RNAi-silencing experiments create a defenseless phenotype which lacks the ability to present defensive secretions on the back of the larvae. For characterization of the transporter I applied chemical analytical, protein-biochemical, molecular biological and immunohistochemical methods. The manuscript further addresses the general question how insects deal with toxic compounds produced by plants they feed on and postulates a sequestration model with ABC transporters as a key element.

I carried out all experiments and analyzed all data except for fluorescence microscopy imaging which was a joint effort with the major contribution of Sven Peters. Experiments were planned and designed by me. Antje Burse constructed the c-DNA library and designed together with Wilhelm Boland and me the study. The manuscript was drafted by me with the help of Antje Burse and Sven Peters and Wilhelm Boland participated especially in the writing process of the study.

Manuscript III

„Tissue-specific transcript profiling for ABC transporters in the sequestering larvae of the phytophagous leaf beetle *Chrysomela populi*“ Anja S. Strauss, Ding Wang, Magdalena Stock, René R. Gretscher, Marco Groth, Wilhelm Boland and Antje Burse

submitted to *PLoS ONE* (01.2014)

Manuscript III provides an active inventory of ABC transporters based on the *C. populi* transcriptomic sequences. We applied the RNA-sequencing approach for a

tissue-specific profiling of ABC transporter transcripts. On this basis and additional comparative phylogenetic analyses of human ABC transporters and other insects we propose a functional link to each ABC transporter class. Furthermore we investigated the effects of RNAi-silencing of *Cp*MRP with respect to probable complementary effects of other ABC transporters in juvenile *C. populi*. *Cp*MRP is predominantly transcribed in the glandular tissue and I showed in manuscript II its key function within the secreting process.

I conducted the RNAi- and qPCR-experiments, the sample preparation for RNA-sequencing and contributed to the bioinformatic analysis which was performed by Ding Wang and Magdalena Stock. René R. Gretscher and Antje Burse contributed to the sample preparation for sequencing of *C. populi* transcripts and Marco Groth performed the sequencing. Together with Ding Wang, Magdalena Stock, Antje Burse and Wilhelm Boland, I planned and designed the experiments and we contributed to interpretation of data. In joint work, Antje Burse, Ding Wang and me wrote the first draft of the manuscript and all authors contributed substantially in terms of their research part. Wilhelm Boland refined the manuscript.

3 Manuscripts

Manuscript I

Phytochemistry 70 (2009) 1899–1909

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Review

Always being well prepared for defense: The production of deterrents by juvenile *Chrysomelina* beetles (Chrysomelidae)

Antje Burse *, Sindy Frick, Sabrina Discher, Karla Tolzin-Banasch, Roy Kirsch, Anja Strauß, Maritta Kunert, Wilhelm Boland

Max Planck Institute for Chemical Ecology, Department of Bioorganic Chemistry, Hans-Knöll-Str. 8, D-07745 Jena, Germany

ARTICLE INFO

Article history:
Received 9 April 2009
Received in revised form 3 August 2009
Available online 4 September 2009

Keywords:
Chemical ecology
Co-evolution
Secondary metabolites
Leaf beetles
Chemical defense
Sequestration
Transport protein
Thioglucosides
Fat body
Iridoid synthesis

ABSTRACT

In response to herbivores, plants produce a variety of natural compounds. Many beetle species have developed ingenious strategies to cope with these substances, including colonizing habitats not attractive for other organisms. Leaf beetle larvae of the subtribe Chrysomelina, for example, sequester plant-derived compounds and use them for their own defense against predators. Using systematically modified structural mimics of plant-derived glucosides, we demonstrated that all tested Chrysomelina larvae channel compounds from the gut lumen into the defensive glands, where they serve as intermediates in the synthesis of deterrents. Detailed studies of the sequestration process revealed a functional network of transport processes guiding phytochemicals through the larval body. The initial uptake by the larvae's intestine seems to be fairly unspecific, which contrasts sharply with the specific import of precursors into the defensive glands. The Malpighian tubules and hind-gut organs facilitate the rapid clearing of body fluid from excess or unusable compounds. The network exists in both sequestering species and species producing deterrents *de novo*. Transport proteins are also required for *de novo* synthesis to channel intermediates from the fat body to the defensive glands for further conversion. Thus, all the tools needed to exploit host plants' chemistry by more derived Chrysomelina species are already developed by iridoid–*de novo* producers. Early intermediates from the iridoid–*de novo* synthesis which also can be sequestered are able to regulate the enzyme activity in the iridoid metabolism.

Contents

1. Introduction

Beetles have used plants as a food source for about 230 million years, which has contributed to reciprocal adaptation and the enormous biodiversity that is found today in both organism groups (Farrell, 1998). Phytophagous species account for more than double the non-herbivorous taxa. This disparity became especially pronounced with the increasing diversity of angiosperms in the Post-Cretaceous period. In response to herbivores, plants developed several morphological and biochemical adaptations which allowed them to wage a kind of chemical warfare; one strategy of this war was based on toxic secondary metabolite production, storage and eventually release (Macias et al., 2007). As some insects became adapted to these metabolites, interactions between the two organism groups occasionally led to highly specific relationships.

The sequestration of poisonous phytochemicals and their use for defense purposes or as building blocks for toxins or

* Corresponding author. Tel.: +49 3641 571265; fax: +49 3641 57120.
E-mail address: aburse@ice.mpg.de (A. Burse).

0031-9422/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2009.08.002

pheromones is a widespread phenomenon observed in Coleoptera (Duffey, 1980). Leaf beetles (Chrysomelidae) in particular are known for their ability to import structurally different allelochemicals, such as β-amyrin (Laurent et al., 2003b), cucurbitacins (Gillespie et al., 2003), pyrrolizidin alkaloids (Hartmann, 2004), phenolglucosides (Pasteels et al., 1983), naphthaleneglucoside (Pasteels et al., 1990), glucosidically bound aliphatic alcohols (Schulz et al., 1997) or iridoid glucosides (Willinger and Dobler, 2001). The sequestered compounds often have to be further transformed to become biologically active. Altering the metabolite profile of the plant may entail modifications in the deterrent pattern of the insect. Consequently, co-evolution plays a crucial role in explaining secondary metabolite diversity in plants and their grazers.

We provide an overview of our studies on sequestration processes of plant-derived compounds by leaf beetles. After we followed the route of incorporated phytochemicals and their conversion within insects on the molecular level, we explored the impact of the sequestered compounds on the endogenous biosynthesis of deterrents. Finally, we discuss our results in the context of development of metabolite diversity in chrysomelids as well as the relevance of sequestration as a process for enhancing the adaptive radiation observed in plants and beetles.

2. Chemical defense in leaf beetle larvae

Within the Chrysomelidae are many species in which not only the adults but also the larvae produce deterrents from plant-derived compounds (Pasteels et al., 1982; Blum, 1994; Schulz, 1998; Laurent et al., 2003a, 2005). In certain cases, the defensive compounds are stored in specialized structures of the body and used to repel predators, such as in the larvae of the leaf beetle subtribe Chrysomelina. The larvae possess nine pairs of defensive glands on the last two thoracic and first seven abdominal tergites (Renner, 1970; Pasteels and Rowell-Rahier, 1989). Each of the exocrine glands is composed of many secretory cells which are attached to a large reservoir. When the larvae are stimulated, they emit secretions from the tips of the glandular tubercles. As soon as the disturbance is over, the secretions are sucked back into the reservoir. The anti-predatory effect of the secretions can be attributed either to autogenously synthesized defensive compounds or to sequestered plant-derived glucosides converted within the reservoir by a few enzymatic reactions into deterrents.

According to phylogenetic analyses of Chrysomelina species by Termonia et al. (2001), the *de novo* production of deterrent iridoids (monoterpenoids with the iridane skeleton) is considered the ancestral strategy (Meinwald et al., 1977; Blum et al., 1978; Pasteels et al., 1982; Soe et al., 2004) (Fig. 1A). More derived species acquired the ability to sequester compounds, which made the biosynthesis of deterrent substances more economical. Larvae of the Chrysomelina species feeding on Salicaceae sequester phenolglucosides such as salicin and salicortin (Pasteels et al., 1983; Brueckmann et al., 2002; Michalski et al., 2008) (Fig. 1B). The glucosides serve as precursors for the odiferous and repellent salicylaldehyde. In contrast to the incorporation of a few plant-derived compounds, larvae of the most evolved Chrysomelina species are able to take up a wide variety of glucosidically bound leaf alcohols. Their aglucons are further esterified with butyric acids derived from the insects' internal pools of amino acids, which can result in a cocktail of at least 70 deterrent esters (Hilker and Schulz, 1994; Schulz et al., 1997; Termonia and Pasteels, 1999; Kuhn et al., 2007) (Fig. 1C).

A combination of the above-described strategies of allomone production with the host plant families mirrors the reciprocal adaptation of Chrysomelina beetles to their hosts (Termonia et al., 2001; Fernandez and Hilker, 2007) (Fig. 1D). Species synthesizing the deterrents *de novo* feed on different plant families, such as Brassicaceae or Polygonaceae. In contrast, Chrysomelina members whose larvae sequester salicin are adapted exclusively to Salicaceae. Larvae of *Chrysomela lapponica* sequester a blend of glucosidically bound leaf alcohols; it is remarkable that this species has developed allopatric populations which colonize salicaceous and betulaceaous plants. Populations on *Salix* spec., rich in salicin, have been reported to produce almost exclusively salicylaldehyde, whereas populations on birches synthesize a completely dissimilar pattern of defensive compounds due to the lack of or strongly reduced level of salicin in the plant (Hilker and Schulz, 1994; Schulz et al., 1997).

To sequester and use new plant-derived metabolites for self-defense, not only the transport mechanisms but also the following enzymatic reactions must be modified. Basically the same enzymatic reactions convert sequestered or *de novo* produced compounds into allomons in the larval defensive glands of all Chrysomelina species. A β-glucosidase removes the sugar moiety from the glucosides. Subsequently, an oxidase whose substrate spectrum is likely defined catalyzes the formation of (di)aldehydes (Veith et al., 1996; Brueckmann et al., 2002; Michalski et al., 2008). Pasteels et al. (1990) postulated the *de novo* synthesis of defensive compounds as the primitive state which harbors the set of enzymes that allows plant-derived glucosides to evolve the capacity to be used for defense. In many respects, the sequestration of glucosides is beneficial. The compounds are abundant in the leaves of the food plant. *A priori* they are mostly non-toxic but can be readily cleaved into deterrent aglycons or compounds that can be enzymatically converted into deterrents or even toxins. The catabolism of the glucose moiety supplies additional energy equivalents (Pasteels et al., 1983). Not least, the polar glycosides are non-diffusible through membranes unless functional transport systems mediate their passage.

3. A network of transporters mediates sequestration and the excretion of glucosides

Numerous publications postulate that selective transport systems facilitate the sequestration of secondary metabolites from plants into insects. Within the order Coleoptera, for example, the transport processes of the strongly deterrent pyrrolizidine alkaloids (PAs) are exploited by some leaf beetle species for their own predator defense; these alkaloids have been studied extensively in the last decade (for review see Hartmann (1999, 2004), Hartmann and Ober (2000, 2008)). Plants store PAs mostly as non-toxic *N*-oxides. Their reduction during the intestinal passage results in lipophilic pro-toxic free bases which can pass through membranes by diffusion. Incorporated free bases can then be converted into the toxic pyrrolic intermediates. Species of the genus *Oreina* which are adapted to feed on PA-containing plants have evolved a special sequestration mechanism to avoid self-poisoning. The beetles suppress the reduction of the PA *N*-oxides in the gut and directly absorb the polar compound (Hartmann et al., 1997, 1999). Feeding experiments using ^{14}C-labeled senecionine and its *N*-oxide, suggested carrier-mediated transport of the polar *N*-oxide from gut into hemolymph in larvae and adults and from hemolymph into exocrine glands in adults of *Oreina cacaliae* (Hartmann et al., 1999). Direct evidence for membrane transport was provided by feeding double-labeled [^{14}C]senecionine [^{18}O] *N*-oxide as a tracer to *O. cacaliae* and *Oreina speciosissima*: the ^{18}O-label was retained after passage from gut into the defensive secretions of adult beetles (Narberhaus et al., 2004a, b). The same tracer has also shown that transport proteins are responsible for incorporating polar senecionine-type *N*-oxides in adults of *Longitarsus jacobaeae*.

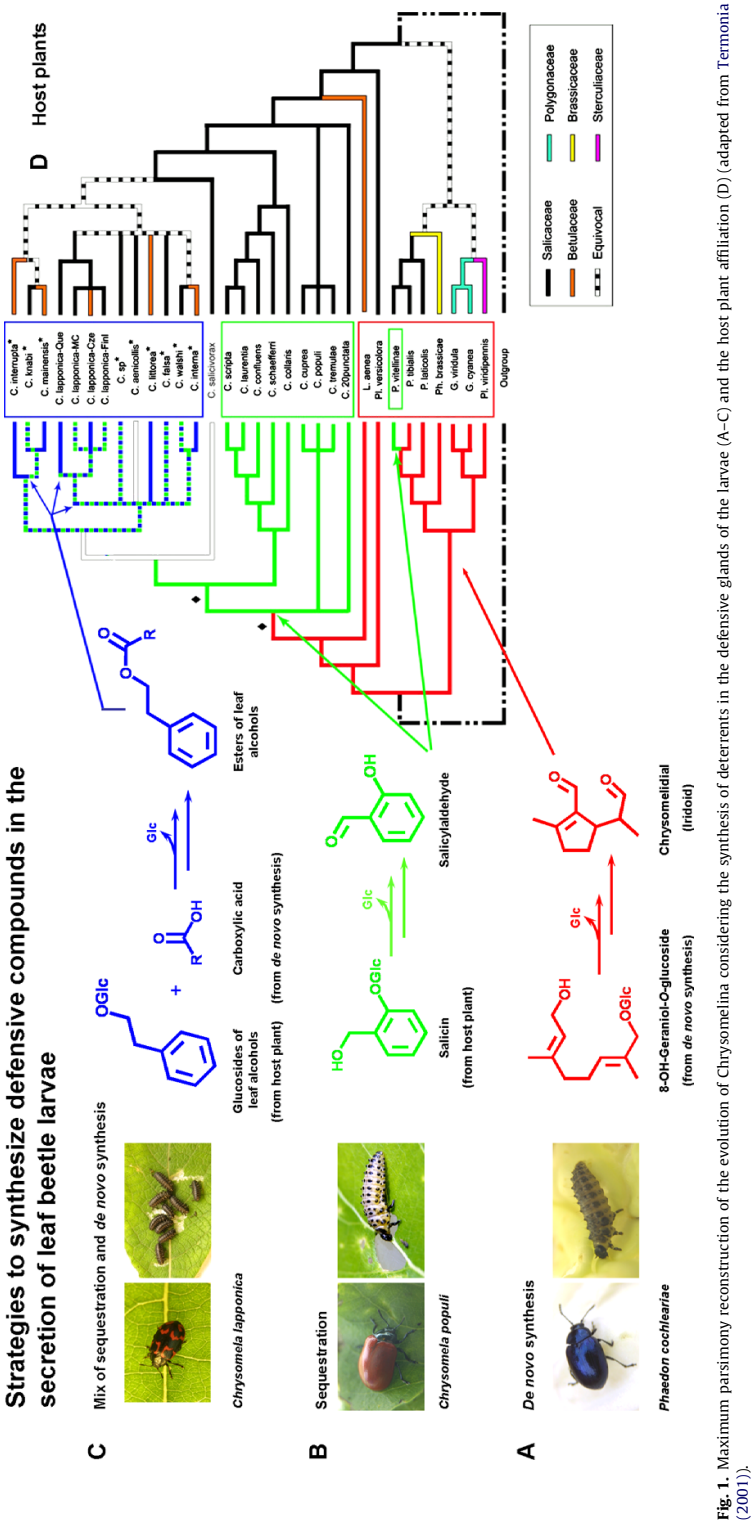


Fig. 1. Maximum parsimony reconstruction of the evolution of Chrysomelina considering the synthesis of deterrents in the defensive glands of the larvae (A–C) and the host plant affiliation (D) (adapted from Termonia et al. (2001)).

Larvae and adults of another PA-adapted species, *Platyphora boucardi*, absorb the lipophilic pro-toxic free bases and transfer them highly efficiently into the defensive glands, thus preventing them from accumulating in the hemolymph (Hartmann et al., 2001, 2003; Pasteels et al., 2001, 2003). The concentration difference of >1000 between hemolymph and the secretions of the easily diffusible lipophilic tertiary alkaloids also suggests the participation of carriers; this, however, has yet to be proven experimentally.

Radio-labeled traces are suitable to study transport processes. But in Chrysomelina species which sequester glucosides, *S*-analogs instead of the natural plant-derived *O*-glucosides were used to follow the route of incorporated compounds (Feld et al., 2001; Kuhn et al., 2004, 2007). The glycomimics are stable against the glucosidases in the gut and the gland, and they accumulate in the defensive secretions where they can be easily quantified by high-performance liquid chromatography mass-spectrometry.

To understand the selection criteria for the sequestered compounds, thioglucosides of salicin isomers, along with aliphatic and aromatic *S*-glucosides were tested (Fig. 2). The chrysomeline larvae were allowed to feed on individual compounds and assessed their passage through the intestinal and glandular system by analyzing their secretions. The feeding experiments not only revealed the existence of transport systems that channel the hydrophilic plant-derived glucosides through the gut membrane into the defensive gland *via* hemolymph transfer but also demonstrated the varied selectivity of glucoside uptake in the analyzed Chrysomelina species. For example, larvae of *Chrysomela populi* and *Phratora vitellinae* which secrete almost exclusively salicylaldehyde import predominantly the *S*-mimic (6) of the genuine precursor salicin (Kuhn et al., 2004, 2007) (Fig. 2). Iridoid-producing larvae of the four tested species *Phaedon cochleariae*, *Gastrophysa virdula*, *Hydrothassa marginella*, and *Phratora laticollis* seem to possess transporters mediating the selective uptake of the *S*-mimic of glucosidically bound 8-hydroxygeraniol (1), an early intermediate in iridoid metabolism (Feld et al., 2001; Kuhn et al., 2004) (Fig. 2). The uptake systems even differentiated between stereoisomers such as Ger-8-*S*-Glc and Ger-1-*S*-Glc; the former was favored by a factor of 10 over the 1-*S*-Glc (Feld et al., 2001). In all cases, the preference for a single glucoside corresponds to the composition of the secretions that contain one or few deterrent substances.

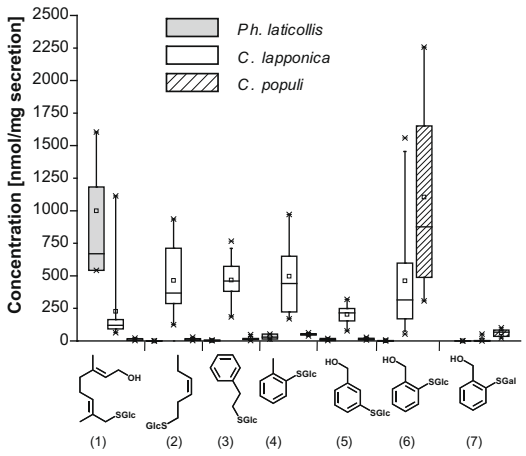


Fig. 2. Uptake of thioglucosides from gut lumen into the defensive secretions after larvae fed on the single compounds. For all experiments, the upper surfaces of host plant leafs were impregnated with methanolic solutions of the test compounds (0.5 μmol/cm leaf). Secretions samples were taken after 48 h feeding on 25–35 cm^2 leaf material. Five to seven larvae were fed for each replication (n = 3–10 depending on compound). In all experiments, post hoc multiple comparisons (Tamhane's T2 test, SPSS) were carried out to evaluate significant differences ($P < 0.05$). **1**, (2*E*,6*E*)-8-hydroxy-2,6-dimethyl-octa-2,6-dienyl-1′-thio-β-D-glucopyranoside (8-OH-Ger-*S*-glucoside); **2**, (3*Z*)-hex-3-en-1-yl-1′-thio-β-D-glucopyranoside; **3**, 2-phenylethyl-1′-thio-β-D-glucopyranoside (phenylethyl-*S*-glucoside); **4**, 2-tolyl-1′-thio-β-D-glucopyranoside; **5**, 3-hydroxymethyl-phenyl-1′-thio-β-D-glucopyranoside; **6**, 2-hydroxymethyl-phenyl-1′-thio-β-D-glucopyranoside (*S*-salicin); **7**, 2-hydroxymethyl-phenyl-1′-thio-β-D-galactopyranoside.

These results sharply contrast with those from experiments in which *C. lapponica* larvae fed on willow and birch. Regardless of the host plant, all larvae can incorporate similarly efficiently a broad range of structurally altered thioglucosides in addition to *S*-salicin (Kuhn et al., 2007) (Fig. 2). The final concentration of each tested thioglucoside in the secretions of *C. lapponica* larvae did not exceed 200–450 nmol mg^{-1}, a concentration which clearly differs from that of the secretions of *C. populi*, where *S*-salicin accumulated to about 1100 nmol mg^{-1}. But in total the glucoside content was similar to that of *C. populi*.

In the hemolymph of all studied species, only traces of the ingested thioglucosides were detected; this indicates the body fluid is rapidly cleaned from the imported substances (Feld et al., 2001; Kuhn et al., 2004, 2007). Consequently, the intestinal transport systems most likely work with a gradient from high to low substance concentrations. In contrast, the putative carriers in the defensive glands must transfer compounds against a steep gradient. From our results, it seems reasonable to assume that different transport systems facilitate the sequestration process.

Analyses carried out with isomers of *S*-salicin and *S*-cresol showed that the putative transporter seemed to select their glucosidically bound substrates by matching the orientation of the hydroxyl groups, in particular by embedding them into a network of hydrogen bonds inside the protein (Kuhn et al., 2007). For example, the transport systems of *C. populi* responded to the structural modifications of salicin, such as *para*-, and *meta*-position of the hydroxyl groups by reducing the import by about 90%. Also, *ortho*-, *para*-, and *meta*-cresol lacking only the hydroxyl group of the salicin side chain were not significantly accumulated in the secretions. A mechanistic model postulating hydrogen bonds between substituents of the glucosides and a putative carrier would also explain why the galactoside was not sequestered in lavae of either *C. populi* or *C. lapponica* (Fig. 2). Because the alglycons rely on the import of glucosidically bound compounds, their structural features and those of the sugar are important (Kuhn et al., 2007).

Apparently *C. lapponica* evolved transport mechanisms with a broad substrate spectrum, allowing changes in host plants that were even contrary to the "phytochemical bridge" postulated by Ehrlich and Raven (1964). Ecological studies carried out on the different *C. lapponica* populations revealed that willow inhabitants are frequently exposed to specialized parasitoids and predators (Zvereva and Rank, 2003, 2004; Gross et al., 2004). For example, syrphid species learned to use salicylaldehyde in the defensive secretions of larvae feeding on willow as a way to locate their prey. Grazing on birch, indeed, lowers the mortality of the larvae, suggesting that the presence of natural enemies is one of the important driving forces behind host plant shifts.

Our feeding experiments demonstrated selective glucoside incorporation into Chrysomelina larvae but did not address the localization of the selective barrier(s) and the route of glucosides within the larval body. Therefore, we carried out *in vitro* studies with dissected gut tissue and Malpighian tubules as well as *in vivo* microinjection into the larval hemolymph using equimolar mixtures of *S*-analogs; these mixtures were both similar and dissimilar to the natural glucosidic precursors of the defensive compounds (Discher et al., 2009). 8-OH-Ger-*S*-glucoside (1) mimics an early intermediate in the iridoid synthesis, phenylethyl-*S*-glucoside (3) resembles a precursor for esters produced by *C. lapponica*, and *S*-salicin (6) corresponds to the genuine precursor

for salicylaldehyde (Fig. 3). In the test series we compared larvae of two Chrysomelina species: the iridoid producer *P. cochleariae* (feeding on *Brassica rapa chinensis*) and the obligate salicin-sequestering species *C. populi* (feeding on *Populus canadensis*).

First, in feeding experiments using the thioglucoside mixture, we corroborated the results of the feeding experiments with individual thioglucosides (Discher et al., 2009). Larvae of both *P. cochleariae* and *C. populi* selectively imported the genuine precursor from food treated with the *S*-glucoside mix into the defensive secretions. In the hemolymph, only traces of all three compounds were detectable. Analysis of the feces revealed both non-precursors as well as precursors had been excreted.

Interestingly, the *in vitro* incubation of gut tissue with the thioglucoside mixture demonstrated that all compounds had been taken up into the epithelial cells regardless of the precursor function of the glucosides in either species (Discher et al., 2009) (Fig. 3A illustrates results from *P. cochleariae*). Consequently, the intestinal carriers most likely allow a broad substrate spectrum to enter the hemolymph.

In contrast, microinjecting the compound mixture into the hemocoel showed that only the genuine precursor of the deterrent compound was selectively imported into the defensive glands in both tested Chrysomelina species (Discher et al., 2009) (Fig. 3B illustrates results from *P. cochleariae*). Similar observations were made in the genus *Oreina*. After unspecific uptake of host plant PAs into the hemolymph of larvae and adults, certain macrocyclic PAs are selectively sequestered by adult beetles into their defensive glands (Hartmann et al., 1997).

Analyses of samples taken at different time points after injection showed the rapid clearance of the thioglucosides from the hemolymph (Discher et al., 2009). In both Chrysomelina species, the genuine glucoside selectively accumulated after 1 h and only traces of all three glucosides were detectable in the hemolymph at the same time point. The glucoside content within the secretions did not change significantly over a period of 48 h.

Injected thioglucosides not transferred into the defensive glands and an excess of the precursor glucoside were found in the feces after these were excreted by the Malpighian tubules and presumably also the hind gut (O'Donnell and Rheault, 2005). The *in vitro* incubation of dissected Malpighian tubules with the thioglucoside mixture also demonstrated that all compounds were taken up into this tissue, indicating their role in clearing the hemolymph from non-useful substances (Discher et al., 2009).

In contrast to the experimentally used thioglucosides, the natural *O*-glucosides entering the gut can be hydrolyzed by glucosidases. The aglucons are also able to pass through the gut membrane most likely by diffusion and reach the defensive system as demonstrated by larvae feeding of labeled d_5-8-hydroxygeraniol which was converted into the deterrent iridoid plagiodial (Kunert et al., 2008). Interestingly, after injecting the deuterated aglucon, d_5-8-hydroxygeraniol-8-*O*-β-D-glucoside was detected in the hemolymph as well as in the feces. Apparently, the compound was glucosylated by the larvae to prevent their self-poisoning. That underlines the importance of sugars as a form of transport during sequestration. Detoxification by *O*-glucosylation is a mechanism also reported from *O. cacaliae* (Hartmann et al., 1999). Typically this species incorporates the non-toxic PA *N*-oxides, but traces of the lipophilic tertiary alkaloid are also absorbed; these can be rendered innocuous by glucosylation. Although the *N*-oxidation of PAs is the most prevalent detoxification strategy known from insects, it seems inefficient in this *Oreina* species.

A complex network of different transport systems seems to control the supply of precursors that synthesize defensive compounds in both sequestering and deterrent-*de novo* producing Chrysomelina larvae (Fig. 4). First, the plant-derived metabolites have to pass through the highly polarized gut epithel cells; as transport proteins

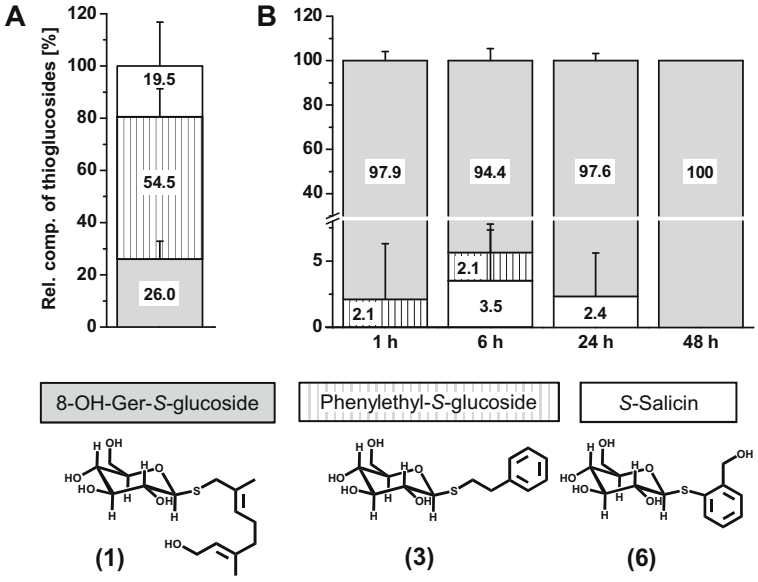


Fig. 3. Relative distribution of thioglucosides 1, 3, 6 in gut tissue and secretions of third-instar larvae of *Phaedon cochleariae*. *In vitro* uptake into gut cells was determined 1 h after incubating the dissected tissue in an equimolar mixture of thioglucosides (0.1 mM each, eight guts per sample) in physiological saline ($n = 9$). Absolute values [nmol substance/mg tissue]: **1**, 0.13 ± 0.06; **3**, 0.30 ± 0.12; **6**, 0.20 ± 0.19 (95% convidence interval) (A). Uptake into the secretions was measured at different time points after microinjecting an equimolar mixture of thioglucosides (1.47 nmol per larva, 20 larvae per sample) into the hemolymph of the larvae ($n = 5$). Absolute values [nmol substance/mg secretion]: 1 h, **1**, 2.83 ± 0.83; **3**, 0.08 ± 0.16; **6**, n.d.; 6 h, **1**, 3.32 ± 1.27; **3**, 0.10 ± 0.09; **6**, 0.30 ± 0.36; 24 h, **1**, 2.68 ± 1.11; **3**, n.d.; **6**, 0.15 ± 0.19; 48 h, **1**, 2.63 ± 0.74, **3**, n.d.; **6**, n.d. (B). n.d. = not detected (95% convidence interval) (adapted from Discher et al. (2009)).

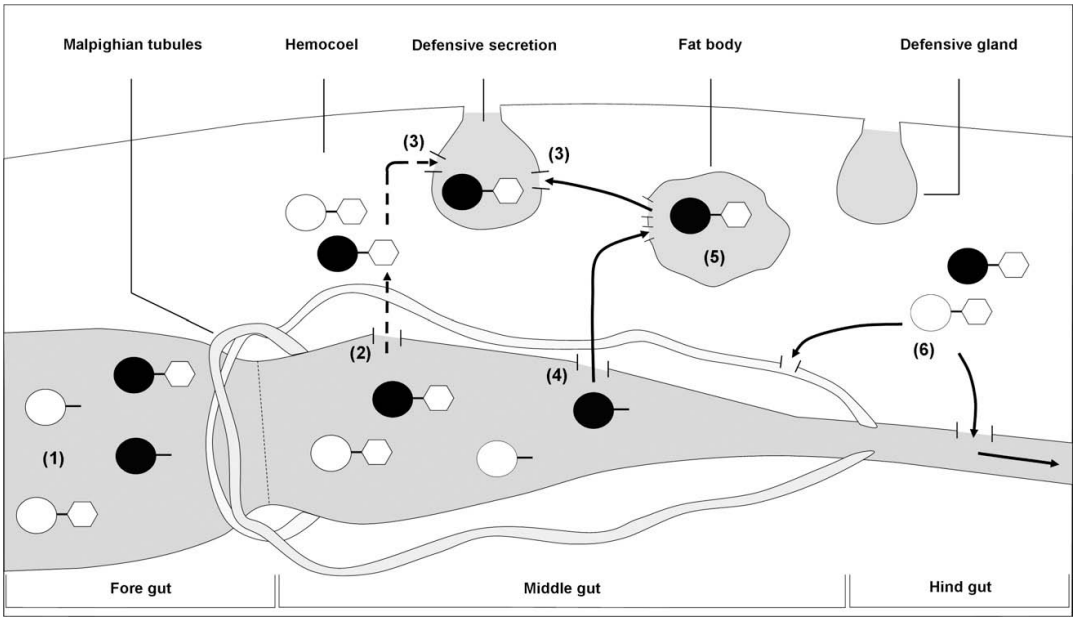


Fig. 4. A network of transport processes are implicated in the sequestration of secondary metabolites from the host plant. Glucosides and aglucons reach the gut lumen with the food (1). Carriers mediate the uptake of glucosides into the hemolymph (2). From there they are either transferred into the defensive glands, from which transporters selectively channel progenitors of deterrents into the secretions (3) or unused or excessive glucosides are excreted by the Malpighian tubules and hind-gut organs (6). Agluca may reach the hemocoel by diffusion (4) and precursors of the deterrents are most likely glucosylated in the fat body (5) from where they are channeled via the hemolymph either into the defensive secretion (3) or to the excretory tissue (6) (adapted from Kunert et al. (2008)).

are distributed in the plasma membrane somewhat unevenly, different types could mediate the trans-cellular transport of glucosides. The broad spectrum of metabolite transfer into the hemocoel allows the entrance of structurally unrelated glucosides; these circulate in the hemolymph until they reach the defensive glands via active transport or are excreted. To date it remains unknown which carrier types are implicated in the sequestration phenomenon. Most likely, the relationship between uptake into the gland and the excretion process is dynamic and depends on the capacity of the defensive system; this can change during larval development and as a result of disturbance by predators. Recently, we partly sequenced cDNA libraries generated from defensive glands and gut tissue to identify putative transporters involved in the sequestration process. Considering the broad substrate spectrum of putative carriers in the gut and the Malpighian tubules, we suggest that only minor modifications in the highly selective import mechanisms of the defensive glands and adaptations of the subsequent enzymatic reactions to the new precursors are required for beetles to use structurally different plant metabolites for chemical defense.

4. Iridoid–*de novo* biosynthesis and sequestration

The chemical defense of some beetles is often based on both the sequestration of metabolites from host plant and endogenous synthesis. In the genus *Oreina*, for example, some species produce cardenolides *de novo* and additionally take up plant-derived PAs (Dobler et al., 1996). Feeding experiments with *S*-analogs of the natural *O*-glucoside(s) demonstrated that the larvae of all tested iridoid–*de novo* producing Chrysomelina species should be able to sequester the precursor 8-hydroxygeraniol-8-*O*-*β*-D-glucoside (Feld et al., 2001; Kuhn et al., 2004). However, it has never been established that these larvae effectively take up the compound *in vivo*. This should be possible if the food plants provide metabolites matching the transport capacity and corresponding to an intermediate in the iridoid biosynthesis.

First, host plants were incubated with labeled 1-deoxy-[1-^{13}C,5,5-2H2]-D-xylulose (^{13}C-DOX), an intermediate in the methylerythriol-4-phosphate pathway which supplies precursors mainly to produce monoterpenes in plants (Kunert et al., 2008; Soe et al., 2004). Because insects cannot use ^{13}C-DOX to synthesize terpenoids, it can be used to determine the biosynthetic origin of iridoids in larval secretions. Additional treatment with jasmonic acid elicited the *de novo* biosynthesis of terpenoids and generated a broad spectrum of labeled compounds that may include precursors for iridoid biosynthesis in leaf beetle larvae. Moreover, the labeling of terpenoid volatiles emitted by the treated plants ensured the successful incorporation of ^{13}C-DOX.

The iridoid–producing larvae of *Plagiodera versicolora* (feeding on *Salix fragilis*) and *Ph. laticollis* (feeding on *P. canadensis*) fed on the ^{13}C-DOX-pretreated plant material for defined periods and then their defensive secretions were collected. The extensive incorporation of the label in the deterrent iridoid plagiodial in the two tested species indicated the uptake of iridoid precursors from the food plant (Kunert et al., 2008). In contrast, no clear evidence for the import and transformation of plant-derived precursors was found for the iridoid–*de novo* producing larvae of *P. cochleariae* (feeding on *Brassica rapa*, *Brassica oleracea* var. *gemmifera*, *Amoracia rusticana*) and *Gastrophysa viridula* (feeding on *Rumex obtusifolia*), although uptake of the 8-OH-Ger-*S*-glucoside (1) has been demonstrated for all four tested species.

In analyses of the glucosidically bound terpenoids of food plants, specifically *S. fragilis* and *P. canadensis*, we identified 8-hydroxygeraniol-8-*O*-*β*-D-glucoside, indicating that the compound can be sequestered by *Pl. versicolora* and *Ph. laticollis* larvae feeding on these plants (Table 1) (Kunert et al., 2008). The ability to sequester a plant-derived precursor represents a cost-saving strat-

Table 1
Identification of 8-hydroxygeraniol-8-*O*-*β*-D-glucoside in food plants of iridoid producing Chrysomelina larvae.

Leaf beetle species	Food plant	8-Hydroxygeraniol (μg g^{-1} FW)
Plagiodera versicolora	*Salix fragilis*	2.3 ± 0.1
Phratora laticollis	*Populus canadensis*	1.1 ± 0.03
Gastrophysa viridula	*Rumex obtusifolius*	0.08 ± 0.01
Phaedon cochleariae	*Brassica oleracae var. gemmifera*	Not detectable
	Brassica rapa	Not detectable
	Amoraciae rusticana	Not detectable

Glucosidically bound 8-hydroxygeraniol was extracted and cleaved enzymatically followed by derivatisation (Kunert et al., 2008). The aglucon was unambiguously identified by comparison with authentic references. Data represent the mean ± SE (n = 3).

egy that allows insects to reduce their metabolic investment in *de novo* biosynthesis (Pasteels et al., 1983, 1990). In contrast to the *Salicaceae*, only traces of 8-hydroxygeraniol-8-*O*-*β*-D-glucoside were found in *R. obtusifolius* and the glucoside was not detected in the tested Brassicaceae species, namely the food plants of *G. viridula* and *P. cochleariae*. Although this is consistent with the lack of labeling in the defensive secretions, it remains unclear why these species possess the capability to sequester the glucoside if the ability is not required *in vivo*.

5. Localization of the iridoid–*de novo* synthesis

After transfer from gut lumen to the glandular reservoir, glucosidically bound 8-hydroxygeraniol is processed by enzymatic reactions described for *de novo* iridoid synthesis (Pasteels et al., 1990; Lorenz et al., 1993; Daloze and Pasteels, 1994; Veith et al., 1994, 1996, 1997; Oldham et al., 1996; Laurent et al., 2003a). An unspecific *β*-glucosidase removes the sugar moiety from the glucoside, and an oxidase possessing a defined substrate spectrum subsequently catalyzes the formation of an acyclic dialdehyde (Pasteels et al., 1990; Veith et al., 1996; Brueckmann et al., 2002; Michalski et al., 2008). The final transformation is achieved by cyclization and isomerization reactions (Lorenz et al., 1993; Veith et al., 1994). Although the glucoside was detected in the secretions, the enzymes catalyzing its formation have not been identified there (Pasteels et al., 1990; Daloze and Pasteels, 1994).

Glucosidically bound 8-hydroxygeraniol is assembled from isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP) derived from the mevalonate pathway (Oldham et al., 1996; Belles et al., 2005) (Fig. 5). An important rate-limiting enzyme of this pathway is 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR; EC 1.1.1.34), which catalyzes the four-electron reduction of HMG-CoA to the carboxylic acid mevalonate using two molecules of NADPH (Friesen and Rodwell, 2004). HMGR belongs to the most highly regulated enzymes known, and it can be modulated on the transcriptional, translational and post-translational level (Goldstein and Brown, 1990). Analyses of HMGR expression and enzyme activity have been used to locate the *de novo* biosynthesis of monoterpenoids in bark beetles (Seybold and Tittiger, 2003). To localize the early steps of the iridoid synthesis in leaf beetle larvae, we compared two iridoid–*de novo* producers, *P. cochleariae* and *G. viridula*, with the salicin-sequestering *C. populi*, hoping to identify the key larval enzymes of the biosynthesis of 8-hydroxygeraniol-8-*O*-*β*-D-glucoside.

Employing quantitative real-time PCR, we found an obvious increase in transcripts of *HMGR* in the fat body of the iridoid-producing species (Burse et al., 2007). The level was approx. 1000- and 100-fold higher in *P. cochleariae* and *G. viridula*, respectively, relative to the gut tissue. In contrast, the level of *HMGR* mRNA level in the fat body of *C. populi* larvae was not significantly different from that of the gut tissue. Also, the transcript levels for *HMGR* in the Malpighian tubules, glands and head of the three examined species corresponded to the general levels observed in the gut tissue. The basal *HMGR* transcript abundance detected in all tested tissues traces back to the fact that the enzyme supplies the precursor for molecules involved in essential metabolic processes in all cells, such as dolichol, required for glycoprotein synthesis, and haem A and ubiquinone, implicated in electron transport or isopentyladenine, present in some tRNAs (Edwards and Ericsson, 1999).

Since HMGR is regulated not only on the transcriptional level but also during translation and post-translationally, the activity of the enzyme was monitored in dissected tissues of larvae of *P. cochleariae*, *G. viridula* and *C. populi*. Enzyme assays were carried out with radio-labled HMG-CoA and revealed a correlation between HMGR transcript abundance and activity (Burse et al., 2007). In the fat bodies of the iridoid producers *P. cochleariae* and *G. viridula*, HMGR activity was approx. 30 and 5 times, respectively, greater than in their gut tissue. However, the striking difference found on the transcript level could not be detected in the assays, indicating that HMGR activity is indeed modulated after transcription. The HMGR activity in the fat body of *C. populi* larvae did not differ significantly from that measured in gut tissue. Activity in guts and Malpighian tubules of all three species did not vary significantly from each other.

Whereas HMGR constitutes a key enzyme only of the early steps, isoprenyl diphosphate synthases (IDS) act as later regulatory and branch point enzymes in terpenoid biosynthesis (Liang et al., 2002). They catalyze the sequential condensation reactions of IDP and DMADP. IDS are named for their main products such as geranyl-diphosphate synthases (GDPS; EC 2.5.1.1), which catalyze the single condensation of IDP and DMADP; this single condensation results in geranyl diphosphate (GDP), the C_{10} backbone component of monoterpenes. Recently, it has been demonstrated that a GDPS from the male bark beetle *Ips pini* supplies the precursor in the *de novo* synthesis of the monoterpenoid aggregation pheromone (Gilg et al., 2005). In iridoid-producing Chrysomelina larvae, 8-hydroxygeraniol-8-*O*-*β*-D-glucoside is derived from GDP, implying that GDPS participates in the *de novo* synthesis of the deterrent compounds (Veith et al., 1994). GDP is converted into 8-hydroxygeraniol by *ω*-hydroxylation followed by glucosylation to obtain 8-hydroxygeraniol-8-*O*-*β*-D-glucoside (Daloze and Pasteels, 1994; Veith et al., 1994, 1996).

To detect the accumulation of isoprenoids which could serve as potential precursors for iridoids, we performed IDS assays where the overall product pattern differed in the fat body of the iridoid-producing larvae from that of the *C. populi* larvae (Burse et al., 2007). In the tissue of *P. cochleariae* and *G. viridula,* approx. 90% of all the identified isoprenoids was geraniol and only 10% farnesol. Geranylgeraniol was not found. In contrast, the enzymes of the fat body of *C. populi* produced only ca. 20% geraniol, 60% farnesol and 20% geranylgeraniol. Unlike assays with the fat body, assays with gut tissue of the iridoid-producing larvae showed an accumulation of 50% geraniol, 40% farnesol and 10% geranylgeraniol. The gut tissue of *C. populi* larvae produced the same compounds in the ratio of ca. 40% geraniol, 50% farnesol and 10% geranylgeraniol.

Moreover, 8-hydroxygeraniol-8-*O*-*β*-D-glucoside, the end product of the early terpenoid biosynthesis, was extracted only from the fat body tissue of the two iridoid producers (Burse et al., 2007). According to our data, it seems reasonable that the fat body – the most prominent tissue in the larvae performing myriad metabolic functions throughout insects' development – is implicated in the *de novo* production of the glucosidically bound iridoid precursor (Fig. 5). Hence, all of the required enzymes including an oxidase, which converts geraniol into 8-hydroxygeraniol, and a glucosyltransferase should be present in

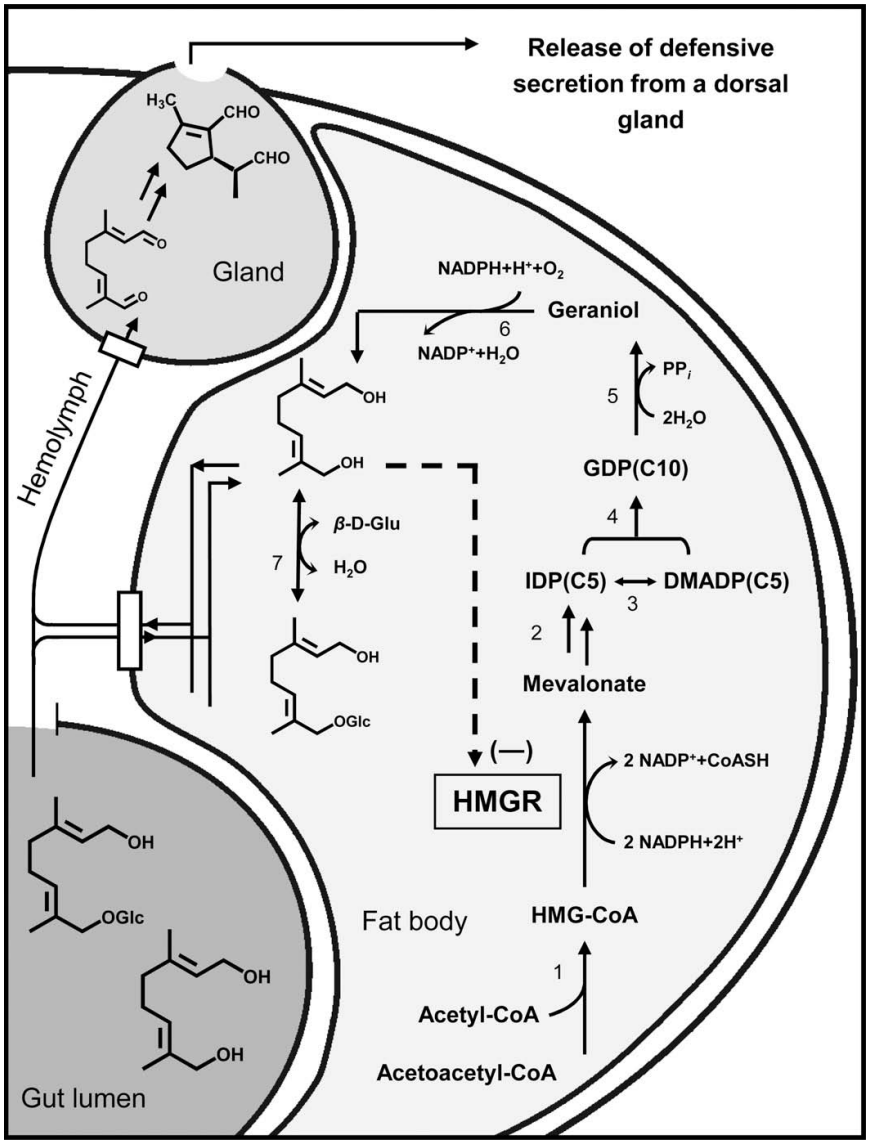


Fig. 5. Biosynthesis of deterrent iridoids in the larvae of *P. cohleariae*. (1) 3-hydroxy-3-methylglutaryl CoA synthetase; (2) mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase; (3) isopentenyl-diphosphate Δ-isomerase; (4) geranyl-diphosphate synthase; (5) phosphatase; (6) cytochrome P-450 mixed-function oxygenase; (7) β-glucosidase (adapted from Pasteels et al. (1990) and Burse et al. (2007)).

the fat body. This would correlate with observations by Veith et al. (1994), who postulated the existence of at least two different oxidative processes in iridoid-releasing larvae according to the substrate specificity of the enzymes. One enzyme type converts natural geraniol into 8-hydroxygeraniol, most likely localized in fat body tissue. The second type produces 8-oxocitral from the diol in the glandular reservoir.

The iridoid biosynthesis seems to be compartmentalized into different tissues of the larval body. According to these observations, the glucoside must be released from fat body tissue into the hemolymph before being transported into the defensive glands for further conversion. The larvae may employ endogenous or exogenous pools of the iridoid precursor, depending on needs or host plant's supply.

6. Do sequestered compounds have an impact on iridoid–*de novo* synthesis?

In our previous studies of iridoid–*de novo* synthesis, the HMGR transcript level and enzyme activity indicated that the fat body tissue is implicated in the *de novo* production of the glucosidically bound iridoid precursor (Burse et al., 2007). Furthermore, we tested whether 8-hydroxygeraniol-8-*O*-β-D-glucoside, its aglycon

or geraniol has an effect on the activity of HMGR. Because the β-D-glucoside of 8-hydroxygeraniol can be easily hydrolyzed, the stable *S*-analogon has been used. HMGR activity was assayed with the crude enzyme extract of fat body tissue from *P. cochleariae* larvae (Burse et al., 2008). Incubation with 8-hydroxygeraniol revealed that HMGR activity decreased significantly; 50% inhibition was achieved with a concentration of approx. 2 mM. geraniol (⩾5 mM) reduced the enzyme activity only by 25–35%. Almost no inhibition was detectable by incubation with the thioglucoside of 8-hydroxygeraniol. Consequently, HMGR activity can be modulated by an intermediate of the iridoid biosynthesis.

To address the inhibition site of the enzyme, we initially cloned a complete cDNA fragment that encoded the full-length HMGR from *P. cochleariae* (Burse et al., 2008). Its catalytic portion was then heterologously expressed in *Escherichia coli* cells. Purification and characterization of the recombinant protein revealed attenuated activity in enzyme assays by 8-hydroxygeraniol, whereas no effect has been observed by adding the glucoside or geraniol (Fig. 6A). The three-dimensional structure of the catalytic portion of human HMGR (PDB code 1DQ8 containing HMG and CoA) was the basis for a high-quality model of the corresponding region (E416-F887, catalytic domain) of the HMGR from *P. cochleariae* (Burse et al., 2008). The resulting most preferred docking arrangement of 8-hydroxygeraniol appeared in a position almost identical to the position of HMG in the X-ray structure of the template, directly adjacent to CoA, which defined 8-hydroxygeraniol as a competitive inhibitor. The interaction of 8-hydroxygeraniol with the protein is characterized by the formation of hydrogen bonds to S670 and D753 from chain A and to N856 from chain B (Fig. 6B). Furthermore, hydrophobic interactions with L839, L848, H852, and M643 stabilize the docking arrangement. Thus, the compound interacts directly with the catalytic domain, as do the competitive statin inhibitors (Brown et al., 1978; Istvan and Deisenhofer, 2001), rather than down-regulating the enzyme activity by lowering the enzyme mass. As a consequence, the larvae have a tool for fine-tuning HMGR activity rapidly in response to changing biosynthetic demands, such as the increased *de novo* biosynthesis of iridoids after the release of secretions or during larval development. HMGR is regulated through feedback inhibition by an intermediate in the defensive compound biosynthesis which is essential not for the cellular function but for inter-organismic communication.

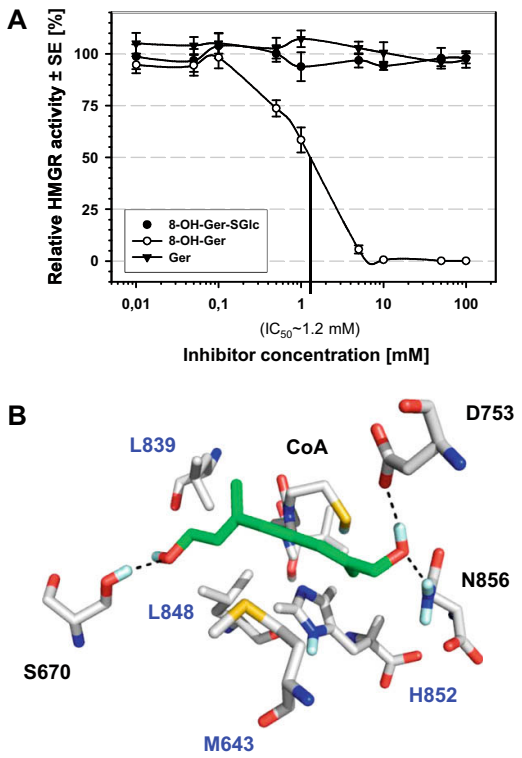


Fig. 6. Inhibition of the recombinant catalytic domain of HMGR from *P. cochleariae* by geraniol, 8-hydroxygeraniol and its thioglucoside. The enzyme was purified by nickel affinity chromatography from *E. coli* cells (A). Homology model of the interaction of 8-hydroxygeraniol with the catalytic active site of HMGR (B). Black labeled residues indicate interactions with chain A and blue labeled with chain B of the homodimeric protein. Dotted black lines represent hydrogen bonds between the ligand and the protein (adapted from Burse et al. (2008)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

HMGR activity may be affected not only by the endogenous level of 8-hydroxygeraniol but also by the sequestered glucoside. We assume that the glucose moiety could be hydrolytically cleaved off by glucosidases, and that the resulting aglycon can then attenuate HMGR activity. Due to the activity of glucosidases in the gut lumen (Pasteels et al., 1983, 1990), 8-hydroxygeraniol can be liberated from the plant-derived glucoside during the gut passage. This compound has been successfully imported through the gut membrane to the hemolymph and finally into the defensive secretion (Lorenz et al., 1993; Kunert et al., 2008). Incubating dissected fat body tissue in a solution of 8-hydroxygeraniol decreased HMGR activity, indicating that this compound had been transferred. Accordingly, HMGR represents one of the key regulators in maintaining homeostasis between metabolites that are sequestered and those that are produced *de novo* in iridoid synthesis.

7. Final remarks

We have demonstrated that the larvae of most likely all Chrysomelina species possess the ability to sequester plant-derived glucosides regardless of the extent of *de novo* synthesis of the defensive compounds. However, sequestration seems to become relevant *in vivo* for iridoid–*de novo* producers which feed on plants from the salicaceous family. The most ancestral species, those adapted, for example, to Brassicacae or Polygonaceae, are capable of incorporating glucosides but do not use the compounds for iridoid synthesis, perhaps due to the absence or very low amounts of iridoid precursors in the host plant. Interestingly, the basis for the evolution of sequestration was established in ancestral species such as *G. viridula* or *P. cochleariae*. With the distribution of the *de novo* synthesis into different tissues, they had to develop transport mechanisms into the defensive gland which can be recruited in the more evolved species for secondary metabolite uptake from the host plants. Furthermore, *de novo* producers harbor a set of enzymes in their defensive glands that can adapt to convert sequestered compounds. In fact, establishing the sequestration process did not depend on the invention of new protein functions during Chrysomelina evolution.

Although the entire sequestration process including uptake and excretion obviously requires a large number of transport mechanisms, few proteins have to be altered to adapt to a different substrate spectrum. Taking into account that import by the intestines and export by excretory tissue are fairly unspecific, only the specialized transporters and the following enzymes in the defensive glands have to be modified. Evidently, the glandular carriers representing the bottleneck tightly control the metabolite uptake. They may influence host plant affiliation as well as the diversity of deterrents in the secretions and, thus, become one of the causal

factors manipulating the co-evolutionary events between plants and Chrysomelina beetles. Finally, due to the complexity of the sequestration process emphasized in our recent studies, the Chrysomelina taxon is shown here to be a fascinating model system which provides diverse starting points for exploring the mutual reactions of beetles and plants.

Acknowledgments

This study was supported by grants from the Deutsche Forschungsgemeinschaft (SPP 1152: Evolution of metabolic diversity) and from the Max Planck Society. We wish to thank Prof. Dr. Stefan H. Heinemann, Friedrich Schiller University Jena, for the microinjection experiments, Dr. Wolfgang Brand, Leibniz Institute of Plant Biochemistry Halle/Saale, for modeling the HMGR, Dr. Axel Schmidt, MPI for Chemical Ecology Jena, for the IDS assays. Also we are very grateful to all the chemists who synthesized the compounds used in the feeding experiments. We also thank Dr. Rita Büchler for help during protein purification, Angelika Berg for taking care of the beetles, Andrea Lehr, Anja David, Marion Stäger, and Angela Roßner for technical assistance as well as Kerstin Ploss for the high-resolution MS analyses. We gratefully acknowledge Prof. Dr. Jacques M. Pasteels, Université Libre de Bruxelles, for providing leaf beetles and for his creativity and helpful advice which improved this project considerably.

References

Belles, X., Martin, D., Piulachs, M.-D., 2005. The mevalonate pathway and the synthesis of juvenile hormone in insects. Annu. Rev. Entomol. 50, 181–199.

Blum, M.S., 1994. Antipredatory devices in larvae of the *Chrysomelidae*: a unidentified synthesis for defensive electicism. In: Jolivet, P.H., Cox, M.L., Petitpierre, E. (Eds.), Novel Aspects of the Biology of the *Chrysomelidae*. Kluwer Academics Publishers, Doderecht, The Netherlands, pp. 277–288.

Blum, M.S., Wallace, J.B., Duffield, R.M., Brand, J.M., Fales, H.M., Sokoloski, E.A., 1978. Chrysomelidial in the defensive secretion of the leaf beetle *Gastrophysa cyanea* Melsheimer. J. Chem. Ecol. V4, 47–53.

Brown, M.S., Faust, J.R., Goldstein, J.L., Kaneko, I., Endo, A., 1978. Induction of 3-hydroxy-3-methyl glutaryl coenzyme A reductase activity in human fibroblasts incubated with compactin Ml-236b: a competitive inhibitor of the reductase. J. Biol. Chem. 253, 1121–1128.

Brueckmann, M., Termonia, A., Pasteels, J.M., Hartmann, T., 2002. Characterization of an extracellular salicyl alcohol oxidase from larval defensive secretions of *Chrysomela populi* and *Phratora vitellinae* (*Chrysomelina*). Insect Biochem. Mol. Biol. 32, 1517–1523.

Burse, A., Schmidt, A., Frick, S., Kuhn, J., Gershenzon, J., Boland, W., 2007. Iridoid biosynthesis in *Chrysomelina* larvae: fat body produces early terpenoid precursors. Insect Biochem. Mol. Biol. 37, 255–265.

Burse, A., Frick, S., Schmidt, A., Buechler, R., Kunert, M., Gershenzon, J., Brandt, W.G., Boland, W., 2008. Implication of HMGR in homeostasis of sequestered and *de novo* produced precursors of the iridoid biosynthesis in leaf beetle larvae. Insect Biochem. Mol. Biol. 38, 76–88.

Daloze, D., Pasteels, J.M., 1994. Isolation of 8-hydroxygeraniol-8-*O*-β-D-glucoside, a probable intermediate in biosynthesis of iridoid monoterpenes, from defensive secretions of *Plagiodera versicolora* and *Gastrophysa viridula* (*Coleoptera*: *Chrysomelidae*). J. Chem. Ecol. 20, 2089–2097.

Discher, S., Burse, A., Tolzin-Bansch, K., Heinemann, S.H., Pasteels, J.M., Boland, W., 2009. A versatile transport network for sequestering and excreting plant glycosides in leaf beetles provides an evolutionary flexible defense strategy. ChemBioChem. doi:10.1002/cbic.200900226.

Dobler, S., Mardulyn, P., Pasteels, J.M., Rowell-Rahier, M., 1996. Host-plant switches and the evolution of chemical defense and life history in the leaf beetle genus *Oreina*. Evolution 50, 2373–2386.

Duffey, S.S., 1980. Sequestration of plant natural products by insects. Annu. Rev. Entomol. 25, 447–477.

Edwards, P.A., Ericsson, J., 1999. Sterols and isoprenoids: signaling molecules derived from the cholesterol biosynthetic pathway. Annu. Rev. Biochem. 68, 157–185.

Ehrlich, P.R., Raven, P.H., 1964. Butterflies and plants – a study in coevolution. Evolution 18, 586–608.

Farrell, B.D., 1998. Inordinate fondness explained – why are there so many beetles. Science 281, 555–559.

Feld, B.K., Pasteels, J.M., Boland, W., 2001. *Phaedon cochleariae* and *Gastrophysa viridula* (*Coleoptera*: *Chrysomelidae*) produce defensive iridoid monoterpenes *de novo* and are able to sequester glycosidically bound terpenoid precursors. Chemoecology 11, 191–198.

Fernandez, P., Hilker, M., 2007. Host plant location by *Chrysomelidae*. Basic Appl. Ecol. 8, 97–116.

Friesen, J., Rodwell, V. 2004. The 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductases. Genome Biol 5, 248.1–248.7.

Gilg, A.B., Bearfield, J.C., Tittiger, C., Welch, W.H., Blomquist, G.J., 2005. Isolation and functional expression of an animal geranyl diphosphate synthase and its role in bark beetle pheromone biosynthesis. Proc. Natl. Acad. Sci. USA 102, 9760–9765.

Gillespie, J.J., Kjer, K.M., Duckett, C.N., Tallamy, D.W., 2003. Convergent evolution of cucurbitacin feeding in spatially isolated rootworm taxa (*Coleoptera*: *Chrysomelidae*; *Galerucinae*, *Luperini*). Mol. Phylogenet. Evol. 29, 161–175.

Goldstein, J.L., Brown, M.S., 1990. Regulation of the mevalonate pathway. Nature 343, 425–430.

Gross, J., Fatouros, N.E., Neuvonen, S., Hilker, M., 2004. The importance of specialist natural enemies for *Chrysomela lapponica* in pioneering a new host plant. Ecol. Entomol. 29, 584–593.

Hartmann, T., 1999. Chemical ecology of pyrrolizidine alkaloids. Planta 207, 483–495.

Hartmann, T., 2004. Plant-derived secondary metabolites as defensive chemicals in herbivorous insects: a case study in chemical ecology. Planta 219, 1–4.

Hartmann, T., Ober, D., 2000. Biosynthesis and metabolism of pyrrolizidine alkaloids in plants and specialized insect herbivores. Top. Curr. Chem. 209, 207–243.

Hartmann, T., Ober, D., 2008. Defense by pyrrolizidine alkaloids: developed by plants and recruited by insects. In: Schaller, A. (Ed.), Induced Plant Resistance to Herbivory. Springer Science+Business Media B.V., pp. 213–231.

Hartmann, T., Witte, L., Ehmke, A., Theuring, C., Rowellrahier, M., Pasteels, J.M., 1997. Selective sequestration and metabolism of plant derived pyrrolizidine alkaloids by chrysomelid leaf beetles. Phytochemistry 45, 489–497.

Hartmann, T., Theuring, C., Schmidt, J., Rahier, M., Pasteels, J.M., 1999. Biochemical strategy of sequestration of pyrrolizidine alkaloids by adults and larvae of chrysomelid leaf beetles. J. Insect Physiol. 45, 1085–1095.

Hartmann, T., Theuring, C., Witte, L., Pasteels, J.M., 2001. Sequestration, metabolism and partial synthesis of tertiary pyrrolizidine alkaloids by the neotropical leaf-beetle *Platyphora boucardi*. Insect Biochem. Mol. Biol. 31, 1041–1056.

Hartmann, T., Theuring, C., Witte, L., Schulz, S., Pasteels, J.M., 2003. Biochemical processing of plant acquired pyrrolizidine alkaloids by the neotropical leaf-beetle *Platyphora boucardi*. Insect Biochem. Mol. Biol. 33, 515–523.

Hilker, M., Schulz, S., 1994. Composition of larval secretion of *Chrysomela lapponica* (*Coleoptera*, *Chrysomelidae*) and its dependence on host plant. J.Chem. Ecol. 20, 1075–1093.

Istvan, E.S., Deisenhofer, J., 2001. Structural mechanism for statin inhibition of HMG-CoA reductase. Science 292, 1160–1164.

Kuhn, J., Pettersson, E.M., Feld, B.K., Burse, A., Termonia, A., Pasteels, J.M., Boland, W., 2004. Selective transport systems mediate sequestration of plant glucosides in leaf beetles: a molecular basis for adaptation and evolution. Proc. Natl. Acad. Sci. USA 101, 13808–13813.

Kuhn, J., Pettersson, E.M., Feld, B.K., Nie, L., Tolzin-Banasch, K., Machkour M'Rabet, S., Pasteels, J.M., Boland, W., 2007. Sequestration of plant-derived phenolglucosides by larvae of the leaf beetle *Chrysomela lapponica*: thioglucosides as mechanistic probes. J. Chem. Ecol. 33, 5–24.

Kunert, M., Soe, A., Bartram, S., Discher, S., Tolzin-Banasch, K., Nie, L., David, A., Pasteels, J.M., Boland, W., 2008. *De novo* biosynthesis versus sequestration: a network of transport systems supports in iridoid producing leaf beetle larvae both modes of defense. Insect Biochem. Mol. Biol. 38, 895–904.

Laurent, P., Braekman, J.C., Daloze, D., Pasteels, J., 2003a. Biosynthesis of defensive compounds from beetles and ants. Eur. J. Org. Chem. 15, 2733–2743.

Laurent, P., Dooms, C., Braekman, J.C., Daloze, D., Habib-Jiwan, J.L., Rozenberg, R., Termonia, A., Pasteels, J.M., 2003b. Recycling plant wax constituents for chemical defense: hemi-biosynthesis of triterpene saponins from beta-amyrin in a leaf beetle. Naturwissenschaften 90, 524–527.

Laurent, P., Braekman, J.-C., Daloze, S., 2005. Insect chemical defense. In: Schulz, S. (Ed.), Chemistry of Pheromones and Other Semi Chemicals II, vol. 240. Springer, Berlin/Heidelberg, pp. 167–229.

Liang, P.H., Ko, T.P., Wang, A.H.J., 2002. Structure, mechanism and function of prenyltransferases. Eur. J. Biochem. 269, 3339–3354.

Lorenz, M., Boland, W., Dettner, K., 1993. Biosynthesis of iridodials in the defense glands of beetle larvae (*Chrysomelinae*). Angew. Chem., Int. Ed. 32, 912–914.

Macias, F.A., Galindo, J.L.G., Galindo, J.C.G., 2007. Evolution and current status of ecological phytochemistry. Phytochemistry 68, 2917–2936.

Meinwald, J., Jones, T.H., Eisner, T., Hicks, K., 1977. New methylcyclopentanoid terpenes from the larval defensive secretion of a chrysomelid beetle (*Plagiodera versicolora*). Proc. Natl. Acad. Sci. USA 74, 2189–2193.

Michalski, C., Mohagheghi, H., Nimtz, M., Pasteels, J.M., Ober, D., 2008. Salicyl alcohol oxidase of the chemical defense secretion of two chrysomelid leaf beetles – molecular and functional characterization of two new members of the glucose–methanol–choline oxidoreductase gene family. J. Biol. Chem. 283, 19219–19228.

Narberhaus, I., Papke, U., Theuring, C., Beuerle, T., Hartmann, T., Dobler, S., 2004a. Direct evidence for membrane transport of host-plant-derived pyrrolizidine alkaloid *N*-oxides in two leaf beetle genera. J. Chem. Ecol. 30, 2003–2022.

Narberhaus, I., Theuring, C., Hartmann, T., Dobler, S., 2004b. Time course of pyrrolizidine alkaloid sequestration in *Longitarsus flea* beetles (*Coleoptera*, *Chrysomelidae*). Chemoecology 14, 17–23.

O'Donnell, M.J., Rheault, M.R., 2005. Ion-selective microelectrode analysis of salicylate transport by the Malpighian tubules and gut of *Drosophila melanogaster*. J. Exp. Biol. 208, 93–104.

Oldham, N.J., Veith, M., Boland, W., Dettner, K., 1996. Iridoid monoterpene biosynthesis in insects – evidence for a *de novo* pathway occurring in the defensive glands of *Phaedon armoraciae* (Chrysomelidae) leaf beetle larvae. Naturwissenschaften 83, 470–473.
Pasteels, J.M., Rowell-Rahier, M., 1989. Defensive glands and secretions as taxonomical tools in the Chrysomelidae. Entomography 6, 423–432.
Pasteels, J.M., Braekman, J.C., Daloze, D., Ottinger, R., 1982. Chemical defence in chrysomelid larvae and adults. Tetrahedron 38, 1891–1897.
Pasteels, J.M., Rowell-Rahier, M., Braekman, J.C., Dupont, A., 1983. Salicin from host plant as precursor of salicyl aldehyde in defensive secretion of chrysomeline larvae. Physiol. Entomol. 8, 307–314.
Pasteels, J.M., Duffey, S., Rowell-Rahier, M., 1990. Toxins in chrysomelid beetles, possible evolutionary sequence from *de novo* synthesis to derivation from food-plant chemicals. J. Chem. Ecol. 16, 211–222.
Pasteels, J.M., Termonia, A., Windsor, D.M., Witte, L., Theuring, C., Hartmann, T., 2001. Pyrrolizidine alkaloids and pentacyclic triterpene saponins in the defensive secretions of *Platyphora* leaf beetles. Chemoecology 11, 113–120.
Pasteels, J.M., Theuring, C., Witte, L., Hartmann, T., 2003. Sequestration and metabolism of protoxic pyrrolizidine alkaloids by larvae of the leaf beetle *Platyphora boucardi* and their transfer via pupae into defensive secretions of adults. J. Chem. Ecol. 29, 337–355.
Renner, K., 1970. Über die ausstülpbaren Hautblasen der Larven von *Gastroidea viridula* De Geer und ihre ökologische Bedeutung (*Coleoptera: Chrysomelidae*). Beitr. Entomol. 20, 527–533.
Schulz, S., 1998. Insect–plant interactions – metabolism of plant compounds to pheromones and allomones by lepidoptera and leaf beetles. Eur. J. Org. Chem. 1, 13–20.
Schulz, S., Gross, J., Hilker, M., 1997. Origin of the defensive secretion of the leaf beetle *Chrysomela lapponica*. Tetrahedron 53, 9203–9212.
Seybold, S.J., Tittiger, C., 2003. Biochemistry and molecular biology of *de novo* isoprenoid pheromone production in the Scolytidae. Annu. Rev. Entomol. 48, 425–453.
Soe, A.R.B., Bartram, S., Gatto, N., Boland, W., 2004. Are iridoids in leaf beetle larvae synthesized *de novo* or derived from plant precursors? A methodological approach. Isot. Environ. Health Stud. 40, 175–180.
Termonia, A., Pasteels, J.M., 1999. Larval chemical defence and evolution of host shifts in *Chrysomela* leaf beetles. Chemoecology 9, 13–23.
Termonia, A., Hsiao, T.H., Pasteels, J.M., Milinkovitch, M.C., 2001. Feeding specialization and host-derived chemical defense in Chrysomeline leaf beetles did not lead to an evolutionary dead end. Proc. Natl. Acad. Sci. USA 98, 3909–3914.
Veith, M., Lorenz, M., Boland, W., Simon, H., Dettner, K., 1994. Biosynthesis of iridoid monoterpenes in insects – defensive secretions from larvae of leaf beetles (*Coleoptera, Chrysomelidae*). Tetrahedron 50, 6859–6874.
Veith, M., Dettner, K., Boland, W., 1996. Stereochemistry of an alcohol oxidase from the defensive secretion of larvae of the leaf beetle *Phaedon armoraciae* (*Coleoptera, Chrysomelidae*). Tetrahedron 52, 6601–6612.
Veith, M., Oldham, N.J., Dettner, K., Pasteels, J.M., Boland, W., 1997. Biosynthesis of defensive allomones in leaf beetle larvae – stereochemistry of salicylalcohol oxidation in *Phratora vitellinae* and comparison of enzyme substrate and stereospecificity with alcohol oxidases from several iridoid producing leaf beetles. J. Chem. Ecol. 23, 429–443.
Willinger, G., Dobler, S., 2001. Selective sequestration of iridoid glycosides from their host plants in *Longitarsus flea* beetles. Biochem. Syst. Ecol. 29, 335–346.
Zvereva, E.L., Rank, N.E., 2003. Host plant effects on parasitoid attack on the leaf beetle *Chrysomela lapponica*. Oecologia 135, 258–267.
Zvereva, E.L., Rank, N.E., 2004. Fly parasitoid *Megaselia opacicornis* uses defensive secretions of the leaf beetle *Chrysomela lapponica* to locate its host. Oecologia 140, 516–522.

Antje Burse (born 1973, Germany) obtained her B.Sc. and M.Sc. (1999) degrees at the Friedrich Schiller University of Jena. She received her Ph.D. (2003) at the Philipps University of Marburg/Lahn. The corresponding research in the field of phytopathology was performed at the Max Planck Institute for Terrestrial Microbiology, Marburg/Lahn. She analyzed the impact of multi-drug efflux proteins on the pathogenesis of fire blight bacteria. With the studies on sequestration strategies in leaf beetle she is continuing to investigate transport processes. The current research is performed in the Prof. Dr. W. Boland's group at the Max Planck Institute for Chemical Ecology, Jena.

Manuscript II

eLIFE elife.elifesciences.org

RESEARCH ARTICLE

ABC transporter functions as a pacemaker for sequestration of plant glucosides in leaf beetles

Anja S Strauss[1]*, Sven Peters[2], Wilhelm Boland[1], Antje Burse[1]*

[1]Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Jena, Germany; [2]Department of Ophthalmology, University Hospital Jena, Jena, Germany

Abstract Plant-herbivore interactions dominate the planet's terrestrial ecology. When it comes to host–plant specialization, insects are among the most versatile evolutionary innovators, able to disarm multiple chemical plant defenses. Sequestration is a widespread strategy to detoxify noxious metabolites, frequently for the insect's own benefit against predation. In this study, we describe the broad-spectrum ATP-binding cassette transporter *Cp*MRP of the poplar leaf beetle, *Chrysomela populi* as the first candidate involved in the sequestration of phytochemicals in insects. *Cp*MRP acts in the defensive glands of the larvae as a pacemaker for the irreversible shuttling of pre-selected metabolites from the hemolymph into defensive secretions. Silencing *Cp*MRP in vivo creates a defenseless phenotype, indicating its role in the secretion process is crucial. In the defensive glands of related leaf beetle species, we identified sequences similar to *Cp*MRP and assume therefore that exocrine gland-based defensive strategies, evolved by these insects to repel their enemies, rely on ABC transporters as a key element.

DOI: 10.7554/eLife.01096.001

***For correspondence:** astrauss@ice.mpg.de (ASS); aburse@ice.mpg.de (AB)

Competing interests: The authors declare that no competing interests exist.

Funding: See page 14

Received: 17 June 2013
Accepted: 24 October 2013
Published: 03 December 2013

Reviewing editor: Marcel Dicke, Wageningen University, The Netherlands

Introduction

For millions of years, insects have relied on plants as a food source. To impede herbivory, plants have developed several morphological and biochemical traits; one of those is based on toxic secondary metabolite production. Insects, in turn, have evolved ingenious detoxification strategies, including the process of sequestration, to overcome the chemical plant defenses (***Sorensen and Dearing, 2006***; ***Li et al., 2007***; ***Opitz and Muller, 2009***; ***Boeckler et al., 2011***; ***Winde and Wittstock, 2011***; ***Dobler et al., 2012***). These counter-mechanisms thereby affect the ecology and evolution of plants (***Ehrlich and Raven, 1964***; ***Agrawal et al., 2012***; ***Hare, 2012***). The phenomenon of sequestration involves the uptake, transfer, and concentration of occasionally modified phytochemicals into the hemolymph, cuticle, specialized tissues or glands. Numerous species from almost all insect orders have evolved the ability to sequester chemicals (***Duffey, 1980***; ***Nishida, 2002***; ***Opitz and Muller, 2009***). Frequently the sequestered toxins are used by insects for their own defense, as is the case in leaf beetles (Chrysomelidae) (***Meinwald et al., 1977***; ***Pasteels et al., 1990***; ***Gillespie et al., 2003***). Up to now, the most comprehensive knowledge of sequestration processes has been obtained from juveniles of the leaf beetles belonging to the taxon Chrysomelina (***Soetens et al., 1998***; ***Termonia et al., 2001***). The chemical defenses of these larvae are made up of compounds that are either sequestered from their host plants or synthesized de novo. Regardless of their origin, these compounds are transferred into nine pairs of specialized exocrine glands that are found on the back of the larvae (***Hinton, 1951***; ***Pasteels and Rowell-Rahier, 1991***; ***Pasteels, 1993***). According to morphological studies, each defensive gland is composed of a number of enlarged secretory cells, which are in turn connected to a chitin-coated reservoir. The secretory cells are always accompanied by two canal cells that

eLife digest For millions of years, plant feeding insects have been locked in an arms race with the plants they consume. Plants have evolved defensive strategies such as the ability to produce noxious chemicals that deter insects, while many insects have evolved the means to thwart this defense and even turn it to their own advantage. The larvae of the poplar leaf beetle, *Chrysomela populi*, sequester toxic plant compounds in specialized glands on their backs and use these compounds to defend themselves against predators. The glands are lined with chemically inert chitin, the substance that makes up the insect exoskeleton, and the deterrent chemicals are released whenever the insect is threatened.

Now, Strauss et al. have identified a key transport protein used by the larvae to move toxic plant compounds to these glands. This transport protein belongs to a family of membrane proteins called ABC transporters, which help to shuttle substances out of cells or into cell organelles using energy produced by the hydrolysis of ATP molecules. The gene for this transporter is expressed in the glands of the leaf beetles at levels 7,000 times higher than elsewhere in the larvae.

Larvae that lack a functional version of the transporter gene continue to grow, but are unable to defend themselves against predators. Similar genes are found in other species of leaf beetle, suggesting that this type of transporter has been retained throughout evolution. Moreover, the transporter is not specific to a particular plant toxin; this enables leaf beetles to eat many different types of plants and boosts their chances of survival should a previous food source disappear.

DOI: 10.7554/eLife.01096.002

form a cuticular canal, which connects the secretory cell with the reservoir (***Noirot and Quennedy, 1974***). When disturbed, the juvenile beetles evert their glandular reservoirs and present droplets of secretions.

In Chrysomelina larvae, all compounds reaching the glandular reservoir via the hemolymph are glucosides that are converted enzymatically into the biologically active form within the reservoir (***Pasteels et al., 1990***). Thus, the glands also secrete enzymes for the final metabolic conversion of precursors into defensive compounds in the reservoir. The ability to sequester plant glucosides is considered an energy-saving, monotypic adaptation within Chrysomelina (***Figure 1B***), given the phylogenetic evidence that this process evolved from an ancestral autogenous biosynthesis of deterrent monoterpenes (iridoids) (***Termonia et al., 2001***).

The poplar leaf beetle *Chrysomela populi*, is an example of an obligate-sequestering species, and its larvae incorporate the phenolglucoside salicin from the leaves of their salicaceaous food plants (***Pasteels et al., 1983***; ***Kuhn et al., 2004***). In the reservoir of their defensive glands, the salicin is then metabolized into the volatile deterrent salicylaldehyde (***Michalski et al., 2008***). Additionally, in *Chrysomela lapponica* several glucosidically bound alcohols are simultaneously imported, resulting in a diversity of compounds, especially of esters, in the exudate of the larvae (***Hilker and Schulz, 1994***; ***Schulz et al., 1997***; ***Kirsch et al., 2011***; ***Tolzin-Banasch et al., 2011***). Physiological studies on de novo iridoid-producing, salicin sequestering, and ester-producing larvae using thioglucosides have indicated a complex influx–efflux transport network that guides the plant-derived glucosides through the insect body (***Discher et al., 2009***; ***Kuhn et al., 2004***). Presumed intestinal carriers in the gut epithelial cells allow a broad spectrum of secondary metabolites to enter the hemolymph, a process that is accompanied by a similar non-selective excretion via the Malpighian tubules. Furthermore, thioglucosides are being selectively accumulated, up to 500-fold, into the reservoir from a hemolymph pool, suggesting an active transport system is at work (***Feld et al., 2001***; ***Kuhn et al., 2004***). By employing the obligate salicin sequestering species *C. populi*, we focus on deciphering the transport processes involved in the sequestration of glucosides in the defensive glands of chrysomelid larvae.

The active ATP binding cassette (ABC) transporters are well-known key-components of various detoxification mechanisms in all phyla of life (***Sipos and Kuchler, 2006***; ***Leprohon et al., 2011***; ***Holland, 2011***; ***Broehan et al., 2013***). In eukaryotes, they translocate a wide variety of compounds from the cytoplasm to the extracellular space or to intracellular compartments. Their role in the sequestration of plant secondary metabolites in insect herbivores, however, has not yet been investigated (***Karnaky et al., 2000***; ***Sorensen and Dearing, 2006***).

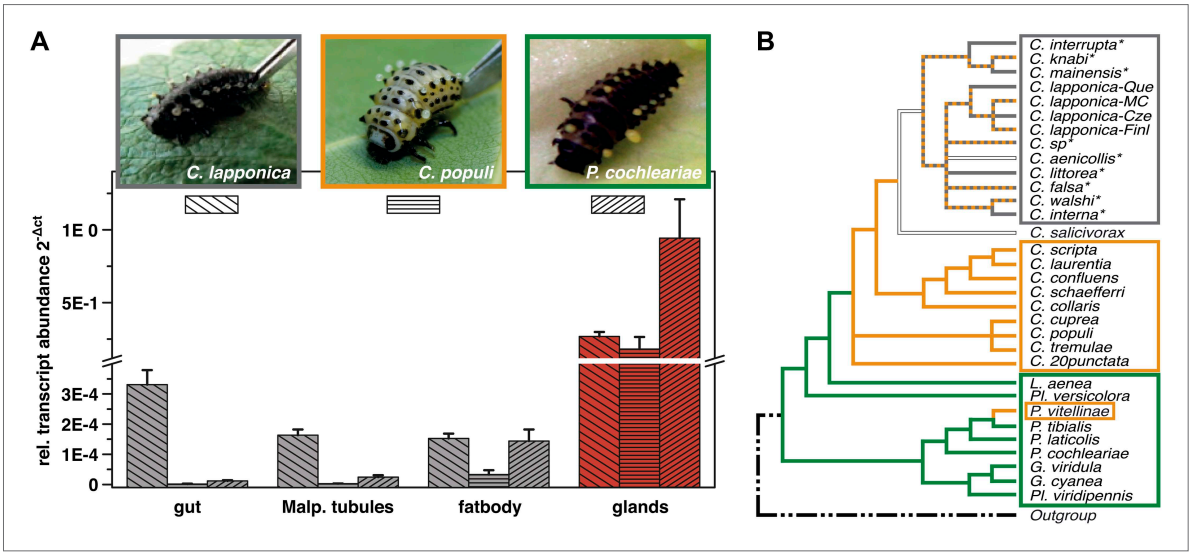


Figure 1. Glandular tissue-specific transcript level of *cpmrp* and its homologous sequences. (**A**) Relative transcript abundance of *cpmrp* (*C. populi*) and its homologous sequences from *C. lapponica* and *P. cochleariae* in different larval tissues (n = 3–4, mean ± SD) assigned to (**B**) their phylogenetic group and chemical defense strategies based on maximum parsimony reconstruction (according to ***Termonia et al., 2001***). Green, autogenous group of monoterpene iridoid producers; orange, obligate-sequestering group; gray, *interrupta* group with mixed metabolism that evolved the biosynthesis of butyrate-esters.

DOI: 10.7554/eLife.01096.003

Here we identify *Cp*MRP as a class C-ABC transporter in the defensive glands of *C. populi*. We demonstrate *Cp*MRPs transport activity for plant-derived glucoside precursors. In the absence of *Cp*MRP, larvae of *C. populi* develop normally but lack defensive secretions that assign a key role for *Cp*MRP in the process of sequestration of salicin. We also describe a sequestration model in which ABC transporters play a key role and discuss their general relevance in exocrine glands of Chrysomelina species.

Results and discussion

Screening of expression levels of transcript sequences encoding ABC transporter motifs revealed a putative candidate, referred to here as *Cp*MRP. It displayed an exceptionally high transcript level in the glandular tissue, exceeding that in the gut and Malpighian tubules by more than 7000-fold (***Figure 1A***).

Among all known and functionally characterized ABC transporters, the deduced amino acid sequence of *cpmrp*, which contained 1331 residues (154.9 kDa), shares the highest sequence similarity of 61% (41% sequence identity) to the human homologous multidrug resistance-associated protein MRP4 (ABC subfamily C) (***Lee et al., 1998***). The predicted protein of *Cp*MRP from *C. populi* possesses the typical structural elements of ABC transporters (***Zolnerciks et al., 2011***); these consist of four domains: two TMDs (transmembrane domains), harboring six proposed transmembrane spans and two NBDs (nucleotide-binding domains), containing Walker A and B boxes (sequences GPVGAGKS and VYLMD, respectively), separated by an ABC signature motif (sequence LSGGQRARINLARAI). Additionally, we conducted a 3D structure modeling of *Cp*MRP (***Figure 2D***) to support the conclusions of the sequence alignment and to illustrate the localization of characteristic sequence motifs. Both sequence alignment and structure modeling suggest that the newly identified protein *Cp*MRP is very likely an ABC transporter.

In the ancestral de novo iridoid-producing species *Phaedon cochleariae*, we have identified a sequence with 86% amino acid identity to *Cp*MRP and in the more derived species *C. lapponica* we identified a homolog of *Cp*MRP sharing 93.7% amino acid identity. Both the sequences share the same

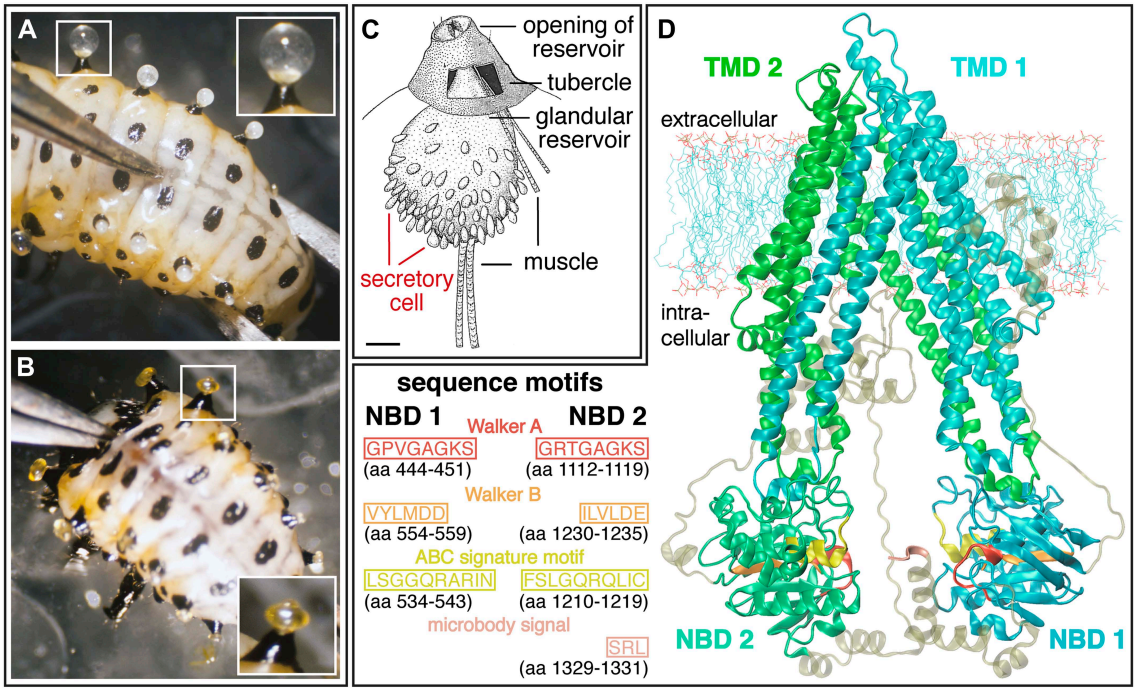


Figure 2. Silencing effect and 3D-structure model of *Cp*MRP. (**A** and **B**) Production of defensive secretions is disrupted in *Cp*MRP knockdown L3 larvae (**B**) compared to the phenotype of the control larvae (**A**). (**C**) Drawing of dissected glandular tissue of *C. populi* according to ***Hinton, 1951*** with relaxed reservoir in contrast to the everted reservoir in insets of (**A**) and (**B**). (**D**) 3D-model of *Cp*MRP, embedded in a lipid bilayer, illustrating its probable correct global topology based on I-TASSER (TM-score of 0.52 ± 0.15; C-score: −1,57) and the localization of characteristic sequence motifs.

DOI: 10.7554/eLife.01096.004

transcription pattern like *Cp*MRP, being highly expressed in the larval glands only (***Figure 1A***). Altogether this suggests that there is a highly conserved ABC transporter among Chrysomelina species that has an important ecological role. Given the uniform architecture and morphology of the defensive system (***Hinton, 1951***; ***Noirot and Quennedy, 1974***) in Chrysomelina larvae, we expect functional similarities on the molecular level. We focus in our study on *Cp*MRP as representative of an obligate-sequestering species among Chrysomelina.

To verify whether the transcript abundance of *cpmrp* is also reflected in the protein level of the defensive glands, we carried out immunohistochemical localization. The staining of full-body sections from juvenile *C. populi* showed that *Cp*MRP was exclusively localized in the defensive glands (***Figure 3—figure supplement 1***). In more detail, *Cp*MRP was present neither in the canal-forming cells (***Figure 3A—C1, C2***) nor in the canal itself (***Figure 3Bb—Cc***) but, rather in the secretory cells (***Figure 3A***). Intriguingly, mapping *Cp*MRP at subcellular magnification provided evidence for the exclusive localization within the secretory cells attached to the reservoir. The intracellular distribution of *Cp*MRP resembles a hollow sphere with a distinct reticular pattern (arrows in ***Figure 3C***, ***Figure 3E***, ***Videos 1 and 2***). Its intracellular presence in vesicular and reticular structures (***Figure 3Bc***) was corroborated by its co-localization with Bodipy-stained intracellular membranes (***Figure 3C,D***). Starting at the outside of the secretory cell, *Cp*MRP was not detected in the basal lamina or in the adjacent basal infoldings (~5 µm) (indicated with Bi in ***Figure 3Ba,C***). Instead, directly after the basal infoldings, we observed a sharp transition to a spherical zone of about 2–5 µm; here, we noted

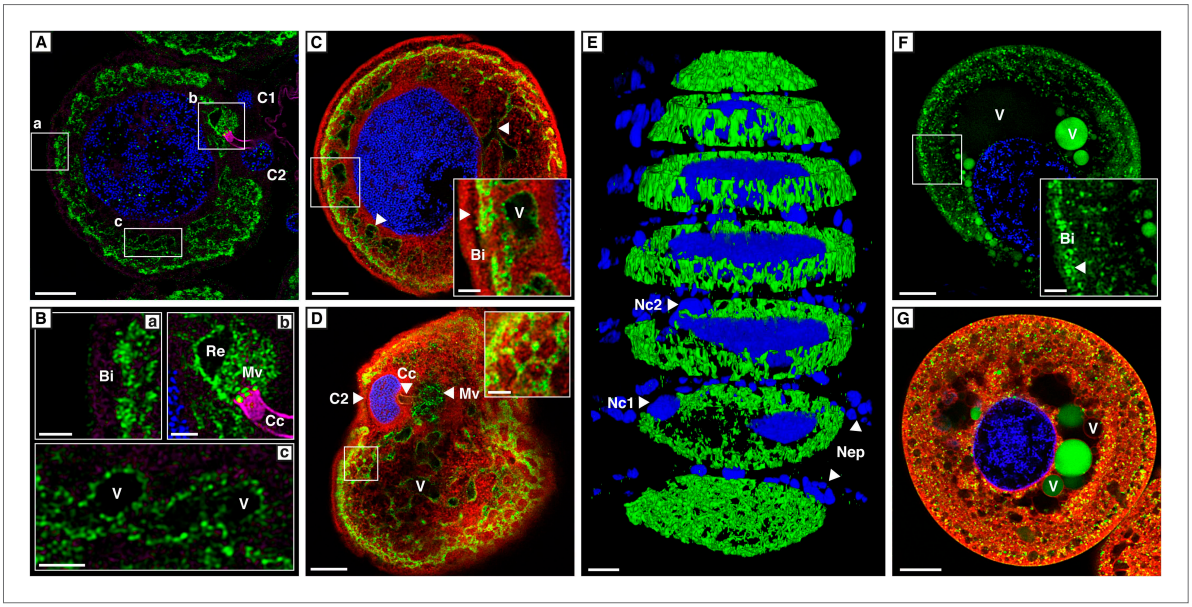


Figure 3. Localization of *Cp*MRP in secretory cells of *C. populi*. (**A**–**G**) High-magnification optical sections through secretory cells. (**A**–**E**) Immunohistochemical staining of *Cp*MRP (green) in fixed secretory cells. *Cp*MRP staining was confined to intracellular Bodipy-stained membrane structures and displayed a distinct reticular pattern. (**B**) Extracted cutouts of (**A** and **C**) optical section through the nucleus, (**D**) optical section above the nucleus of the secretory cell, (**E**) 3D stack displaying *Cp*MRPs primarily spherical distribution. (**F** and **G**) CDCFDA staining for vacuolar esterase activity (green) in live cells. A multitude of vacuoles are present that vary in their enzyme content. Bi = basal infoldings, C1 and C2 = canal cells, Cc = cuticular canal, Mv = microvilli, Nc1, Nc2 = nucleus of canal cells, Nep = nuclei of epithelium cells, Re = extracellular room, V = vacuole, Blue, nuclear staining; Red, Bodipy-stained intracellular membrane; Magenta, false color-coded autofluorescence. Scale bars, 20 μm or 5 μm (insets).

DOI: 10.7554/eLife.01096.005

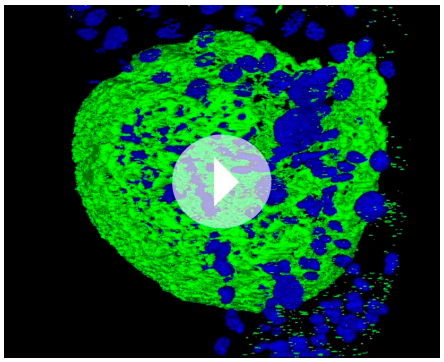
The following figure supplements are available for figure 3:

Figure supplement 1. Localization of *Cp*MRP in whole larvae cryosections of *C. populi*.

DOI: 10.7554/eLife.01096.006

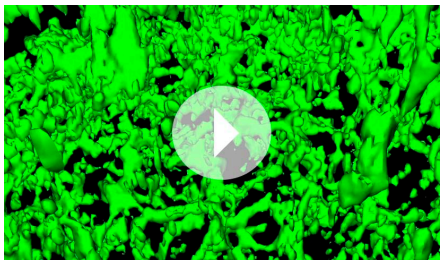
the most dense *Cp*MRP presence within the entire cell (***Figure 3A–C*** [inset arrow] and ***Figure 2D***). Moreover, according to vacuolar esterase activity, demonstrated by CDCFDA staining (***Pringle et al., 1989***), the subcellular localization of *Cp*MRP (***Figure 3F,G*** [inset arrow]) correlates with cellular storage compartments (***Figure 3A–E***).

Employing RNA interference (RNAi) to verify *Cp*MRP's relevance for salicin sequestration in vivo, we were able to demonstrate its key role in the secretion of defensive compounds. By comparing developmental traits among *C. populi* larvae after injecting *cpmrp*-dsRNA or *gfp*-dsRNA as a control, we found that silencing *cpmrp* had no influence on larval growth (***Figure 4A***). However, about 10 days post-injection, the *cpmrp* knockdown larvae completely lost their ability to respond to stimulation with droplets of defensive secretion (***Figures 4B and 2B***). The secretions began to diminish at day 8. On the basis of transcript abundance, *cpmrp* mRNA was reduced to a basal level of 15–20% within 2–3 days and persisted until the larvae pupated (***Figure 4C***). ***Figure 4D*** summarizes the immunohistochemical analysis of *Cp*MRP expression in the secretory cells. At day 3 post-injection, both the *cpmrp* knocked-down and control secretory cells displayed a similar pattern of *Cp*MRP distribution (***Figure 4Da,b***). At later time points, however, the *Cp*MRP expression in the RNAi group was strongly reduced in comparison to the *gfp* control (***Figure 4Dc—f***), which is in agreement with Western blot analysis (***Figure 4—figure supplement 1***). *Cp*MRP decayed exponentially to a relatively low basal level with a half-life of about 1 day, suggesting a degradation of *Cp*MRP that is linearly proportional to its concentration (***Figure 4—figure supplement 2***).



Video 1. 3D representation of *Cp*MRP localization within a secretory cell. Exterior view of a secretory cell of *C. populi* based on immunohistochemical staining (green, *Cp*MRP; blue, nuclei stain). The z-stack was acquired with a resolution of x = 0.146 µm; y = 0.146 µm and z = 0.500 µm. The smallest dimension of the depicted secretory cell is about 100 µm.

DOI: 10.7554/eLife.01096.007



Video 2. 3D representation of *Cp*MRP localization within a secretory cell. Interior view of ***Video 1***. The camera is centrally positioned within the nucleus and rotates within the plane of the first frame of ***Video 1***, initially pointing to the area between the nuclei of the two canal cells.

DOI: 10.7554/eLife.01096.008

This proportionality has already been reported in human hepatocytes (***Pereg et al., 2010***; ***Popov et al., 2010***; ***Nakagawa et al., 2011***). Compared to a half-life of 5 days for the ABC transporters MDR1 and MDR2 reported in hepatocytes (***Kipp and Arias, 2002***), the rate of *Cp*MRP turnover seems relatively high. Qualitative microscopic observations showed that secretory cells tended to increase the size of storage compartments, presumably vacuoles, of the *Cp*MRP knockdown larvae.

Our transport studies in *Xenopus laevis* oocytes revealed that *Cp*MRP is a transporter for salicin (***Figure 5A***), the naturally sequestered host–plant precursor of *C. populi*. In order to test the selectivity of *Cp*MRP, we chose a mixture of glucosides among plant precursors and non-precursor glucosides for comparative transport assays (***Figure 5C,D***). We applied an equimolar mixture of salicin (1), 8-hydroxygeraniol-*O*-glucoside (2), the early precursor of the iridoid monoterpene pathway found in *P. cochleariae* and phenylethyl-*S*-glucoside (3) that represents a substrate mimicking an *O*-glucoside sequestered by *C. lapponica*. This mixture was tested in feeding and hemolymph injection experiments on *C. populi* and revealed the specific transport of salicin to the reservoir (***Discher et al., 2009***). However, *Cp*MRP did not discriminate significantly between the substrates. For phenylethyl-*S*-glucoside and thiosalicin (6) the transport activity of *Cp*MRP was slightly reduced compared to salicin (***Figure 5C,D***). Moreover, the sugar moiety of the substrates (comparing salicin and its galactoside analogue (4) (***Figure 5D***), further significantly lowered the transport activity of *Cp*MRP, which is consistent with previous data obtained by feeding experiments (***Kuhn et al., 2004***). Based on the apparent Km of 5.8 (mM) for salicin, *Cp*MRP (***Figure 5B***) seems to be a low-affinity transporter. From our comparative transport assays we assume that *Cp*MRP functions as low-affinity glucoside transporter with a broad glucoside spectrum.

Taken together, these data support a sequestration model inside the secretory cells of *C. populi* in which *Cp*MRP plays a key role as a pacemaker (***Figure 6***). We assume that within the described zone of highest *Cp*MRP density (***Figure 3A,B,D***), salicin is trapped in storage vesicles as soon as it enters the secretory cells. The constant vesicular accumulation of plant glucoside precursors and further irreversible translocation into the reservoir keeps the glucoside concentration low inside the secretory cell. By this fact, we suggest a first filter for specific glucosides (salicin, in the case of *C. populi*) at the hemolymph-exposed plasma membrane of the secretory cell, which might depend on a gradient-driven, energy-independent transporter. Accordingly, *Cp*MRP does not just dictate the transport rate of this transporter in the plasma membrane, rather, it determines the effectiveness and energy coupling of the entire sequestration process as a pacemaker.

Figure 6 further illustrates the fate of storage compartments. The apical part facing the lumen of the gland is a brush border membrane where storage vesicles are secreted via exocytosis

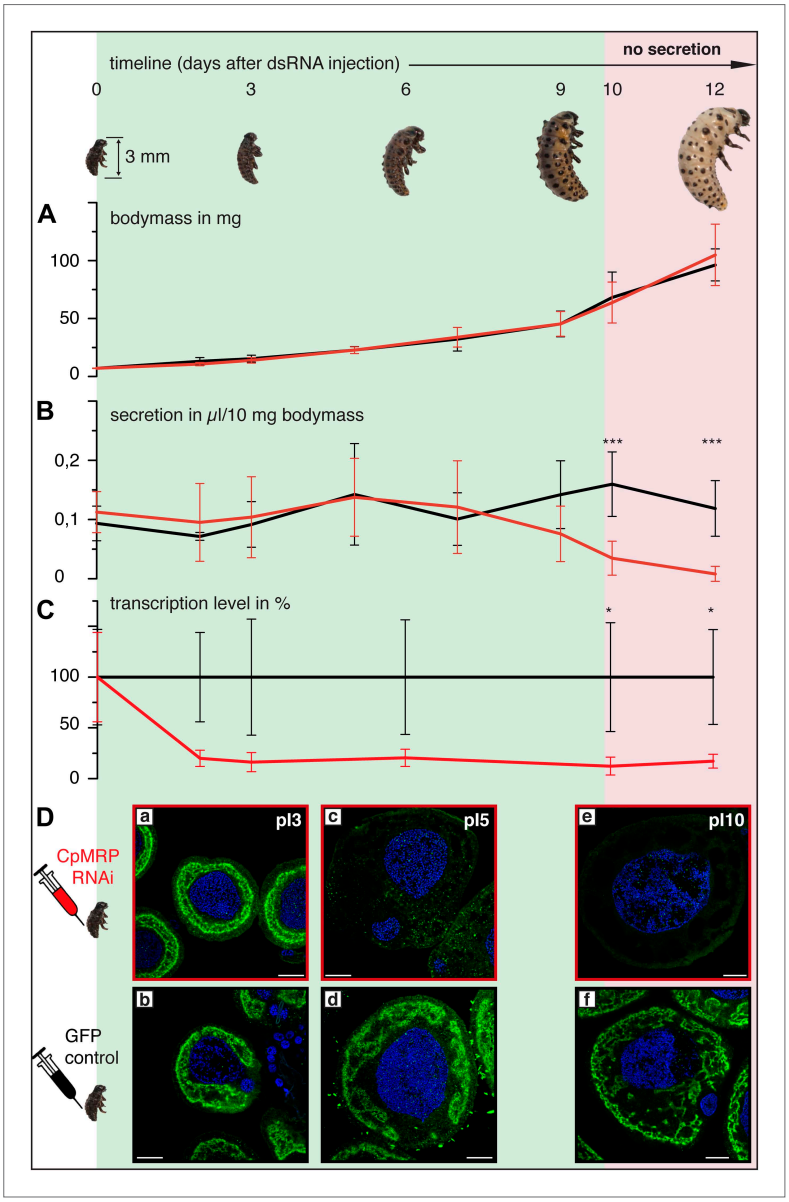


Figure 4. Timeline of different *Cp*MRP knockdown effects. (**A**–**D**) *C. populi* larvae development following *Cp*MRP knockdown by dsRNA injection into the larval hemocoel (d 0). *Cp*MRP knockdown effects; red/(**D a, c, e**) were compared to the *gfp*-injected control larvae; black/(**D b, d, f**) at different developmental stages. (**A**) Larval fitness (body mass) was not influenced by *Cp*MRP knockdown (n > 10, mean ± SD). (**B**) *Cp*MRP knockdown larvae lack defensive secretions 10 days after dsRNA injection (n > 10, mean ± SD). (**C**) Transcriptional level of *cpmrp* inside the glands (each time point contains n = 3 (biological replicates), mean ± SD). (**D**) *Cp*MRP protein level decreased after dsRNA injection—Green, *Cp*MRP; Blue, nuclear stain. Scale bars, 20 µm; pIx = x days post dsRNA-injection. Asterisks represent significant differences in *cpmrp*-silenced larvae compared to *gfp*-injected control larvae (*p≤0.05, ***p≤0.001).

DOI: 10.7554/eLife.01096.009

Figure 4. Continued on next page

Figure 4. Continued

The following figure supplements are available for figure 4:

Figure supplement 1. Effects of *cpmrp* silencing on the protein level of the glandular tissue of *C. populi*.

DOI: 10.7554/eLife.01096.010

Figure supplement 2. Degradation kinetics of *Cp*MRP in secretory cells of *C. populi*.

DOI: 10.7554/eLife.01096.011

Figure supplement 3. DNA alignment of *cpmrp*–related ABC transporter sequences in *C. populi*.

DOI: 10.7554/eLife.01096.012

Figure supplement 4. Evaluation of possible off-target effects of *Cp*MRP dsRNA in larval tissue of *C. populi*.

DOI: 10.7554/eLife.01096.013

(***Noirot and Quennedy, 1974***). It is invaginated and forms an extracellular room that is connected to the reservoir by the canal. The well-known structure of exocrine glands gives hints at exocytic processes on the basis of a microvilli membrane. That *Cp*MRP is present in the microvilli membrane (***Figure 3Bc***), indicates that this is where the exocytosis of *Cp*MRP-containing storage vesicles takes place. After

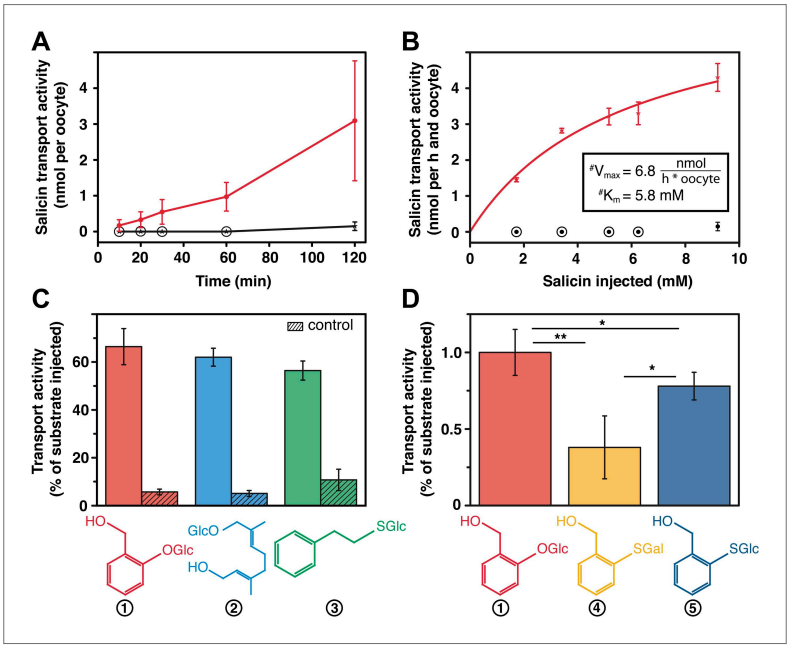


Figure 5. Salicin transport activity of *Cp*MRP in *Xenopus laevis* oocytes. (**A**–**D**) Transport activity was determined by quantifying the substrate efflux in the oocyte incubation medium of *cpmrp*-RNA compared to water-injected control oocytes via HPLC-MS. (**A**) Time course of *Cp*MRP-dependent salicin efflux after the injection of 5 nmol salicin (incubation time: 1 hr, n = 5, mean ± SD). Red, *Cp*MRP-expressing oocytes; Black, water-injected control. (**B**) Concentration dependence of *Cp*MRP-mediated salicin transport (red); water-injected control in black (n = 5, mean ± SD, #: apparent, encircled data point: not detectable). (**C**) Comparative transport assays of *Cp*MRP activity with a substrate mixture (salicin (1), 8-hydroxygeraniol-*O*-glucoside (2) and phenylethyl-S-glucoside (3)). Open bar, transport activity of *Cp*MRP-expressing oocytes. Crosshatched bar, transport activity water-injected control oocytes. (**D**) Comparative transport assays of *Cp*MRP salicin transport activity to thiosalicin (5) and its galactoside analogue (4) and thiosalicin (incubation time: 1 hr, n = 10, mean ± SD). Asterisks represent significant differences among indicated substrates (*p≤0.05, **p≤0.01). Encircled data points represent undetectable concentrations.

DOI: 10.7554/eLife.01096.014

excreting the glucoside, *Cp*MRP is most likely recycled to recover the protein in the dense packaging zone of storage vesicles/vacuoles.

Conclusion

In the present study, we show that *Cp*MRP is required to maintain defensive secretion in *C. populi*. Our results demonstrate that *cpmrp*-silenced larvae are defenseless because they lack defensive secretions. Functionally, *Cp*MRP is a transporter for plant derived glucoside precursors present in storage compartments as well as in the microvilli membrane of the secretory cell. Therefore, our results have led us to propose a functional model of sequestration based on *Cp*MRP as the key element (***Figure 6***). The identification of transporter sequences highly similar to *Cp*MRP in the larval glands of other Chrysomelina species (*P. cochleariae* and *C. lapponica*) strongly implies that broad-spectrum ABC transporters involved in the sequestration of plant-derived metabolites are commonly present in the defense mechanism among Chrysomelina.

These results, together with our published data, lead us to conclude that the sequestration of plant glucosides in Chrysomelina larvae is the result of the presence of several barriers with various degrees of selectivity: (1) those controlling the non-selective uptake of plant-derived glucosides from the gut lumen into the hemolymph and their excretion by the Malpighian tubules (together these barriers are relevant for nutrition), (2) those controlling the selective transfer from the hemolymph into the secretory cells and (3) those controlling the secretion into the reservoir where the broad-spectrum ABC transporter acts as a pacemaker. This functional arrangement of a non-selective and a selective transporter in the defensive system seems to be common to many different leaf beetles (***Discher et al., 2009***). This peculiar import system also facilitates the occasional host plant shifts of leaf beetles caused by parasite pressure (***Agosta et al., 2010***). After the shift to a new host plant only the selective transport element needs to adjust to the new metabolites; all other transport elements may remain unchanged due to their broad substrate tolerance. This assumption is supported by the observation of *Cp*MRP homologs in different leaf beetles and beyond (***Tribolium Genome Sequencing Consortium, 2008***); however, none of them has been functionally characterized or localized as yet. The identification of *Cp*MRP as a non-selective pacemaker involved in the sequestration of plant-derived glucosides highlights how insects counter plant chemical defenses to evolve new functions for the plant-derived toxins as allomones.

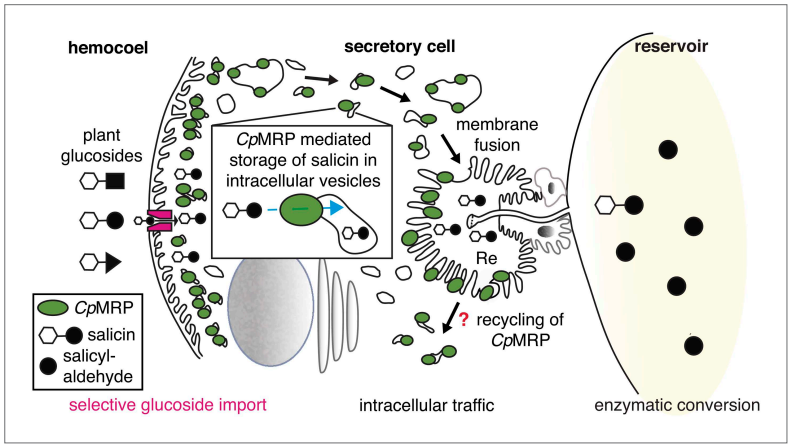


Figure 6. *Cp*MRPs pacemaker function and sequestration model. Schematic view of our sequestration model through a secretory cell (see ***Figure 2C***: overview of the defensive system, secretory cells are indicated in red). Different plant glucosides (black triangles, circles and squares joined to a glucose molecule indicated by a white hexagon) circulate in the hemocoel. *Cp*MRP dictates (pacemaker function) the transport rate of a still unknown selective, maybe gradient-driven transporter (magenta) for salicin in the plasma membrane by a constant accumulation of salicin in intracellular vesicles. These vesicles are tracked via exocytosis to the reservoir where the enzymatic conversion of salicin to salicylaldehyde takes place.
DOI: 10.7554/eLife.01096.015

Table 1. Oligonucleotide primers

Gene name	Primer name	*cpmrp* RACE
cpmrp Gene-Bank: KC112554	3′RACE	GCACGGTCCTGACTATAGCGCACAGGC
	5′RACE	CCTGCCCCCGTTCTTCCCACAATACC
	2nd 5′RACE	GGTGGAGGCCTGCATGGTCAGCTTGC
	5′nested	CGGCGTCTCGAATGGACCTTCCGTGTCG
	3′nested	GGAGAGATGGTGGGAGTATGACCACCCC
Gene name	**Primer name**	**Primer for ds RNA generation**
cpmrp Gene-Bank: KC112554	fwd	GATTAATACGACTCACTATAGGCGACTAAG TGTGAACTAGTCGGTGC
	rev	GATTAATACGACTCACTATAGGGAGACTTG TCTCCACAGCAGATAG
gfp UniProtKB:P42212.1	fwd	TAATACGACTCACTATAGGGAGATGGCTAG TAAGGGA
	rev	TAATACGACTCACTATAGGGAGATTATTTG TAGAGTTC
Gene name	**Primer name**	**qPCR Primer**
cpRP-L45 GeneBank: JX 122918	fwd	CACTGGAATCCAAAGTGGAAACTG
	rev	CTGCCTTTCAACCCATGGTC
cpActin GeneBank: JX122919	fwd	ACGTGGACATCAGGAAGGAC
	rev	ACATCTGCTGGAAGGTGGAC
pcRP-L8 Gene-Bank: JX122920	fwd	CATGCCTGAAGGTACTATAGTGTG
	rev	GCAATGACAGTGGCATAGTTACC
cpmrp Gene-Bank: KC112554	fwd	CCTGGATCCATTCGATGAGT
	rev	AGTATCGCCCTCGCTAGACA
pcmrp Gene-Bank: KF278996	fwd	CTCTAGACATCATGGTCACAGA
	rev	GGCATATCAACTGTCGTTGTC
clapmrp Gene-Bank: KF278997	fwd	CCATCTGGCAAATTTGAAGATTTC
	rev	AGTATTGCCCTCGCTAGACA
off-target_OT-1	fwd	GGAATTCGAGGACCAGTTGC
	rev	GGCATATCAACTGTCGTTGTC
off-target_OT-2	fwd	CCATCTGGCAAATTTGAAGATTTC
	rev	CAGTAACTACGAAGTCTAGAGAG
off-target_OT-3	fwd	GAATATCAGATGCCGACCTG
	rev	GGATGGCTCTGGCAAGG
off-target_OT-4	fwd	GACGACGTGCTTTATAGAGC
	rev	GTCCCACGCTATAGTTGGAC
off-target_OT-5	fwd	CAGTATTCGTTATAACTTGGACC
	rev	GTCCTGCGCTGAAATTCGATC
off-target_OT-6	fwd	GACGAATATAAGGATGAGACATTG
	rev	GCGTACTATGGCTCGAGC
off-target_OT-7	fwd	AGCCGATGATGCAACCATC
	rev	CCAACTGTCTTTGTCCAGC

All primers are listed by name, sequences and application. Forward primers are indicated as 'fwd' and reverse primers as 'rev'.

DOI: 10.7554/eLife.01096.016

Materials and methods

Identification and cloning of *cpmrp*

The full-length cDNA corresponding to the predicted open reading frame of *cpmrp* (Gen-Bank accession number KC112554) was identified from our cDNA library of *C. populi* glands, qPCR validation and subsequent rapid amplification of cDNA ends (RACE) PCR using BD SMART RACE cDNA Amplification Kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's guidelines (see ***Table 1*** for primer sequences). After confirming the fidelity of the 4-kb amplification product by sequencing, it was cloned into pIB-V5-His-TOPO (Invitrogen). Amplification of *Cp*MRP homologs of *P. cochleariae* (*Pc*MRP; Gen-Bank accession number KF278996) and *C. lapponica* (*Clap*MRP; Gen-Bank accession number KF278997) were achieved by using the SMART RACE protocol and the primer *cpmrp* 5′-RACE.

Sequence analysis and *Cp*MRP modeling

Sequence similarities were analyzed using the alignment tool BLAST (***Altschul et al., 1990***). Multiple alignment of MRP amino acid sequences was carried out with CLUSTALW using default parameters (DNASTAR Lasergene 10 Core Suite software, Madison, WI). The I-TASSER online server (***Roy et al., 2010***) was employed to predict a 3D structure model of *Cp*MRP based on its complete amino acid sequence. In a multistep modeling process, a total of about 20% of the sequence of P-glycoprotein from *Mus musculus* (***Aller et al., 2009***) and *Caenorhabditis elegans* (***Jin et al., 2012***) served as template fragments to initiate the structure modeling. Additionally, the *Cp*MRP structure model was embedded into a lipid bilayer using the CHARMM-GUI Membrane Builder (***Jo et al., 2009***). Typical sequence motifs of *Cp*MRP were identified by a sequence alignment using NCBI–Protein BLAST (***Altschul et al., 1990***). Finally, the program VMD (Visual Molecular Dynamics) was used to visualize the model (***Humphrey et al., 1996***).

Rearing, maintaining and dissecting *Chrysomela populi*

A starting culture of roughly 100 *C. populi* (L.) larvae was collected near Dornburg, Germany (+51°00′52.00″, +11°38′17.00″), on *Populus maximowiczii x Populus nigra*. The larvae/beetles were kept for 5 months in a light/dark cycle of 16 hr light and 8 hr darkness (LD 16/8) at 18°C ± 2°C in light and 13°C ± 2°C in darkness. For RNAi experiments we used 3- to 4-day-old larvae of *C. populi* that were reared separately. DNA and RNA were isolated from larvae of *C. lapponica*, which were collected from *Betula rotundifolia* in the Altai Mountains, East Kazakhstan, (2130 m altitude, +49°07′4.38″, +86°01′3.65″) and from *P. cochleariae* (F.) larvae reared in a continuous lab culture (kept in a York Chamber at 15°C (LD 16/8) on leaves of *Brassica rapa pekinensis*). Larvae were dissected for tissue preparation in saline solution and directly frozen in liquid nitrogen.

Collection of larval secretion

Larval secretions were collected and weighed in glass capillaries on an ultra-microbalance (Mettler-Toledo, Greifensee, Switzerland).

RNAi in *C. populi* larvae

Sequence-verified plasmid pIB-*Cp*MRP was used to amplify a 730 bp fragment of *cpmrp* dsRNA. As a control, a *gfp* sequence was amplified from pcDNA3.1/CT- GFP-TOPO (Invitrogen). The amplicons were subject to an in vitro-transcription assays according to instructions from the Ambion MEGAscript RNAi kit (Life Technologies, Darmstadt, Germany; see ***Table 1*** for primer sequences). The resulting dsRNA was eluted after nuclease digestion three times with 50 µl of injection buffer (3.5 mM Tris-HCl, 1 mM NaCl, 50 nM Na_2HPO_4, 20 nM KH_2PO_4, 3 mM KCl, 0.3 mM EDTA, pH 7.0). The quality of dsRNA was checked by TBE-agarose-electrophoresis.

First-instar of *C. populi* (3–4 days after hatching) with 3–4 mm body length (chilled on ice) were injected with 0.25 µg of dsRNA by using a nanoliter microinjection system (WPI Nanoliter 2000 Injector, World Precision Instruments, Berlin, Germany). Injections were made into the hemolymph next to the ventral side between the pro- and mesothorax. Relative transcript abundance was quantified by quantitative real-time PCR (qPCR) at different stages of larval development after RNAi treatment. In silico off-target prediction was done for highly specific silencing according to (***Bodemann et al., 2012***). Experimentally, we excluded off-target effects based on the analysis of co-silencing-effects on non-target genes using qPCR, SDS-PAGE and Western blot. We chose seven of the ABC transporter sequences most similar to *Cp*MRP from our cDNA library; those shared 62–76% aa-sequence identity (***Figure 4—figure supplement 3***) were seen as best potential off-targets, so we analyzed

their transcript levels in *cpmrp* dsRNA injected- and *gfp*-injected control larvae. We found no off-target effect on the transcript level when qPCR was used in different larvae tissue (***Figure 4—figure supplement 4***; see ***Table 1*** for primer sequences). On protein level, we probed off-target effects via SDS-PAGE and Western blot comparing protein samples of *cpmrp* dsRNA injected- and *gfp*-injected control larvae (***Figure 4—figure supplement 1***).

Quantitative real-time PCR (qPCR)

Total RNA was extracted from larval tissue using an RNeasy MINI kit (Qiagen, Hilden, Germany). cDNA was synthesized from DNA-digested RNA using RNAqueous micro kit (Life Technologies, Darmstadt, Germany). Realtime PCR was performed using Brilliant II SYBR Green qPCR Master Mix (Agilent) according to the manufacturer's instructions and an Mx3000P Real-Time PCR system. *CpActin* and *CpRPL45* expression was used to normalize transcript quantities for *C. populi*, *eIF4A* (***Kirsch et al., 2011***) for *C. lapponica* and *pcRP-L8* for *P. cochleariae* samples (see ***Table 1*** for primer sequences). Analyses were performed according to the MIQE-guidelines (***Bustin, 2010***; ***Bustin et al., 2010***).

Xenopus laevis oocytes isolation and RNA injection

Cpmrp RNA was generated by in vitro transcription using mMESSAGE mMACHINE kit (Ambion, Life Technologies, Darmstadt, Germany). *X. laevis* oocytes were provided by Prof Stefan H Heinemann (FSU Jena, Germany). 100–125 ng of cRNA was injected per oocyte (RNase-free water was used as a control). Oocytes expressing *Cp*MRP were maintained at 17.5°C in modified Barth's medium (MBS, in mM: 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaC1_2$, TRIS-HCL, pH 7.4) with 10 µg ml^{-1} penicillin, 10 µg ml^{-1} streptomycin and 4 µg ml^{-1} cefuroxim solution for 3 days.

Substrate efflux assay in *X. laevis* oocytes

Functional efflux studies were carried out with different glucosides in *X. laevis* oocytes at room temperature. The transport activity assay was initialized by injecting individual substrates or a substrate mixture (3 days post cRNA-injection). In comparative transport assays using a substrate mixture the control value was used to normalize the transport rates. The oocytes were immediately washed in Barth medium after substrate injection. At defined time points, the incubation medium was removed and analyzed either by HPLC-MS or UV detection at 268 nm to quantify the substrate efflux from the oocytes into the incubation medium. The calculation of the kinetic parameters was performed with GraphPad Prism (version 5.04, Graphpad Software. San Diego, CA) using the built-in enzyme kinetics module. The substrate concentration was based on the assumption of an oocyte volume of 1 µl (***Kelly et al., 1995***).

HPLC-MS analysis

The efflux of the injected substrates was monitored in the oocyte incubation medium via HPLC-MS. An Agilent HP1100 HPLC system equipped to a C18 column (Gemini 5 µ C18 110A 250 × 2.00 mm 5 µm (Phenomenex, Aschaffenburg, Germany) was used for separation; analytes were detected APCI/MS (LCQ, Thermoquest, San Jose, CA) in positive mode. Samples were analyzed by using a gradient elution at 0.35 ml min^{-1} (solvent A: H_2O+0.5% CHOOH; solvent B: MeCN+ 0.5% CHOOH) according to the following protocol: starting with 5% B, holding to 3 min, going to 20% in 12 min, going to 98% in 10 min, with subsequent washing. Peak areas from MS-chromatograms were obtained using an ICIS-algorithm (Xcalibur bundle version 2.0.7, Thermo Scientific, Waltham, MA).

Statistical analyses

Two-tailed student's *t* tests for unequal variation were used to value significance levels.

Live staining of glands

Dissected glands of *C. populi* were stained with the vital vacuolar stain 5-carboxy-2,7-dichlorofluorescein diacetate (CDCFDA). CDCFDA was added at 10 µM in the saline solution (with 50 mM sodium citrate, pH 5.0) for 20 min. The cells were co-stained with Hoechst 33342 and CellTrace BODIPY TR methylester (Image-iT LIVE Intracellular Membrane and Nuclear Labeling Kit, Invitrogen, Life Technologies, Darmstadt, Germany) for 10 min and immediately examined by two-photon imaging.

Immunolabeling

Immunolabeling was employed to specifically localize *Cp*MRP in the defensive glands or whole larvae sections, respectively. For an overview staining, entire larvae of *C. populi* were used at

second-instar stage. The larvae were anesthetized in CO_2, directly embedded and shock-frozen in optical cutting temperature compound (OCT; Sakura Finetec, Staufen, Germany). 12–20 µm vibratome sections (Microm HM560, Thermo Scientific, Waltham, MA) were prepared.

Both dissected glandular tissue and fresh frozen sections were fixed in cold 4% PFA in 0.1 M PBS (pH 7.4) at 4°C for 1 or 2 hr, respectively. After permeabilization, the samples were washed 3 × 20 min in PBS-TX (0.5%), then blocked with NGS (normal goat serum) for 2 hr at room temperature (RT) and subsequently incubated in the primary antisera for 1 hr at RT and another 12 hr at 4°C. Polyclonal rabbit anti-*Cp*MRP sera (synthetic peptide antibody against the peptide mix: C+LKDVAEKAYHKNSRL [aa 1317–1331] and SLDGNKYTNENRDFS+C [aa 760–774]) were generated by Eurogentec (Seraing, Belgium) and used as primary antiserum at a concentration of 1:1000 in PBS-TX. After incubation in the primary antisera, the tissue was washed with PBS-TX (3 × 20 min) at RT and then incubated in secondary antisera conjugated to Alexa 488 (Invitrogen, Darmstadt, Germany) at a concentration of 1:500 in PBS, overnight at RT. Finally, the tissues were washed in PBS-TX (3 × 20 min) and PBS (20 min) at RT and mounted in Vectashield fluorescence mounting medium (Vector Labs, Peterborough, UK) in spacer slides (Grace Biolabs, Bend, Oregon). Hoechst 33342 was used to co-stain the nuclei, Bodipy to stain intracellular membranes following second antibody incubation, as described.

Two-photon imaging

In order to analyze fluorescence in the glands, we employed an inverted multiphoton laser scanning microscope (Axio Observer Z.1 and LSM 710 NLO, Zeiss, Jena, Germany) in combination with a femtosecond Ti:Sapphire laser (80 MHz, 150 fs, Chameleon Ultra, Coherent Inc.) and a Plan-Apochromat 63x/1.4 objective (Zeiss, Jena Germany). An excitation wavelength of 800 nm (~2 mW) was used for glands stained with CDCFDA and 930 nm (~9 mW) in case of Alexa 488 staining. Emission wavelength detection was achieved by either integration over three spectral ranges (Hoechst 33342: 420–490 nm; Alexa 488/CDCFDA: 505–555 nm; Bodipy: 620–680 nm) or recorded spectrally resolved.

In order to separate autofluorescence from exogenous fluorophores all pixels of spectrally resolved images were assigned to individual fluorophores using a linear unmixing algorithm (Zen 2011 software; Zeiss, Jena Germany). ImageJ was used for image deconvolution (Diffraction PSF 3D; Iterative Deconvolve 3D) and 3D reconstruction (3D Viewer). *Cp*MRP degradation kinetics were estimated by integrated Alexa 488 fluorescence intensity from secretory cells normalized to autofluorescence. Spectrally resolved images were linear unmixed to separate Alexa 488 from autofluorescence. Autofluorescence images were corrected for all non-comparable contributions. The decay of Alexa 488 fluorescence was approximated by a monoexponential fit according to $N(t) = N_0*\exp(-kt)+c$.

Protein extraction and Western blot

Proteins were extracted from the dissected glandular larvae tissue by sonication in 10 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton, 1 mM dithiothreitol and protease inhibitor mix M (Serva, Heidelberg, Germany). Membrane protein fraction was roughly separated from the cytosolic fraction by centrifugation step at 20,000 × *g* at 4°C for 30 min. Proteins were separated by SDS-PAGE (Any kD Precast Gel, BioRad, Hercules, CA) and then transferred onto a PVDF membrane. The membrane was incubated first with rabbit anti-*Cp*MRP antibody (for details see 'Immunolabeling' section) and then with donkey anti-rabbit IgG-horseradish peroxidase conjugates (GE Healthcare Life Sciences, Freiburg, Germany). The proteins were detected using the SuperSignal West Dura Extended Duration Substrate Kit (Pierce Protein Natural Products, Thermo Fisher Scientific Inc. Rockford, IL).

Acknowledgements

The authors would like to express their gratitude to Stefan H Heinemann for offering *Xenopus* oocytes and equipments, Jens Haueisen for providing microscopic equipment, Angelika Berg, Franziska Eberl, Sandra Klemmer, Angela Rossner, Regina Stieber, Roy Kirsch, Anja David and Maritta Kunert for technical assistance. We wish to thank Gergely Szakacs, David Heckel and Jacques M Pasteels for advice and helpful discussions on aspects of this work and Emily Wheeler for editorial assistance. This work has been supported by the Max Planck Society.

Dedicated to Prof Dr L Jaenicke on the occasion of his 90th birthday.

Additional information

Funding

Funder	Grant reference number	Author
Max Planck Society		Anja S Strauss, Wilhelm Boland, Antje Burse
Deutsche Forschungsgesellschaft	BU1862/2-1	Antje Burse
University Hospital Jena		Sven Peters

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Author contributions

ASS, SP, AB, Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article; WB, Conception and design, Drafting or revising the article, Contributed unpublished essential data or reagents

References

Agosta SJ, Janz N, Brooks DR. 2010. How specialists can be generalists: resolving the "parasite paradox" and implications for emerging infectious disease. *Zoologia* **27**:151–62. doi: 10.1590/S1984-46702010000200001.

Agrawal AA, Hastings AP, Johnson MT, Maron JL, Salminen JP. 2012. Insect herbivores drive real-time ecological and evolutionary change in plant populations. *Science* **338**:113–6. doi: 10.1126/science.1225977.

Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, et al. 2009. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science* **323**:1718–22. doi: 10.1126/science.1168750.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J Mol Biol* **215**:403–10. doi: 10.1016/S0022-2836(05)80360-2.

Bodemann RR, Rahfeld P, Stock M, Kunert M, Wielsch N, Groth M, et al. 2012. Precise RNAi-mediated silencing of metabolically active proteins in the defence secretions of juvenile leaf beetles. *Proc Biol Sci* **279**:4126–34. doi: 10.1098/rspb.2012.1342.

Boeckler GA, Gershenzon J, Unsicker SB. 2011. Phenolic glycosides of the Salicaceae and their role as anti-herbivore defenses. *Phytochemistry* **72**:1497–509. doi: 10.1016/j.phytochem.2011.01.038.

Broehan G, Kroeger T, Lorenzen M, Merzendorfer H. 2013. Functional analysis of the ATP-binding cassette (ABC) transporter gene family of *Tribolium castaneum*. *BMC Genomics* **14**:6. doi: 10.1186/1471-2164-14-6.

Bustin SA. 2010. Why the need for qPCR publication guidelines?–The case for MIQE. *Methods* **50**:217–26. doi: 10.1016/j.ymeth.2009.12.006.

Bustin SA, Beaulieu JF, Huggett J, Jaggi R, Kibenge FS, Olsvik PA, et al. 2010. MIQE precis: practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. *BMC Mol Biol* **11**:74. doi: 10.1186/1471-2199-11-74.

Discher S, Burse A, Tolzin-Banasch K, Heinemann SH, Pasteels JM, Boland W. 2009. A versatile transport network for sequestering and excreting plant glycosides in leaf beetles provides an evolutionary flexible defense strategy. *Chembiochem* **10**:2223–9. doi: 10.1002/cbic.200900226.

Dobler S, Dalla S, Wagschal V, Agrawal AA. 2012. Community-wide convergent evolution in insect adaptation to toxic cardenolides by substitutions in the Na,K-ATPase. *Proc Natl Acad Sci USA* **109**:13040–5. doi: 10.1073/Pnas.1202111109.

Duffey SS. 1980. Sequestration of plant natural products by insects. *Annu Rev Entomol* **25**:447–77. doi: 10.1146/annurev.en.25.010180.002311.

Ehrlich PR, Raven PH. 1964. Butterflies and plants - a study in coevolution. *Evolution* **18**:586–608. doi: 10.2307/2406212.

Feld BK, Pasteels JM, Boland W. 2001. *Phaedon Cochleariae* and *Gastrophysa viridula* (*Coleoptera*: *Chrysomelidae*) produce defensive iridoid monoterpenes de novo and are able to sequester glycosidically bound terpenoid precursors. *Chemoecology* **11**:191–8. doi: 10.1007/Pl00001851.

Gillespie JJ, Kjer KM, Duckett CN, Tallamy DW. 2003. Convergent evolution of cucurbitacin feeding in spatially isolated rootworm taxa (Coleoptera: Chrysomelidae; Galerucinae, Luperini). *Mol Phylogenet Evol* **29**:161–75. doi: 10.1016/S1055-7903(03)00256-2.

Hare JD. 2012. Ecology. how insect herbivores drive the evolution of plants. *Science* **338**:50–1. doi: 10.1126/science.1228893.

Hilker M, Schulz S. 1994. Composition of larval secretion of Chrysomela-lapponica (Coleoptera, Chrysomelidae) and its dependence on host-plant. *J Chem Ecol* **20**:1075–93. doi: 10.1007/Bf02059744.

Hinton HE. 1951. On a little-known protective device of some chrysomelid pupae (Coleoptera). *Proc Biol Sci* **26**:67–73. doi: 10.1111/j.1365-3032.1951.tb00123.x.

Holland IB. 2011. ABC transporters, mechanisms and biology: an overview. *Essays Biochem* **50**:1–17. doi: 10.1042/Bse0500001.
Humphrey W, Dalke A, Schulten K. 1996. VMD: visual molecular dynamics. *J Mol Graph* **14**:33–8, 27-8. doi: 10.1016/0263-7855(96)00018-5.
Jin MS, Oldham ML, Zhang Q, Chen J. 2012. Crystal structure of the multidrug transporter P-glycoprotein from *Caenorhabditis elegans*. *Nature* **490**:566–9. doi: 10.1038/nature11448.
Jo S, Lim JB, Klauda JB, Im W. 2009. CHARMM-GUI Membrane builder for mixed bilayers and its application to yeast membranes. *Biophys J* **97**:50–8. doi: 10.1016/j.bpj.2009.04.013.
Karnaky KJ Jnr, Petzel D, Sedmerova M, Gross A, Miller DS. 2000. Mrp2-like transport of Texas red by Malpighian tubules of the common American cockroach. *Periplaneta americana*. *Bull Mt Desert Isl Biol Lab* **39**:52–53.
Kelly SM, Butler JP, Macklem PT. 1995. Control of cell volume in oocytes and eggs from *Xenopus laevis*. *Comp Biochem Physiol A Physiol* **111**:681–91. doi: 10.1016/0300-9629(95)00046-A.
Kipp H, Arias IM. 2002. Trafficking of canalicular ABC transporters in hepatocytes. *Annu Rev Physiol* **64**:595–608. doi: 10.1146/annurev.physiol.64.081501.155793.
Kirsch R, Vogel H, Muck A, Reichwald K, Pasteels JM, Boland W. 2011. Host plant shifts affect a major defense enzyme in *Chrysomela lapponica*. *Proc Natl Acad Sci USA* **108**:4897–901. doi: 10.1073/pnas.1013846108.
Kuhn J, Pettersson EM, Feld BK, Burse A, Termonia A, Pasteels JM, et al. 2004. Selective transport systems mediate sequestration of plant glucosides in leaf beetles: a molecular basis for adaptation and evolution. *Proc Natl Acad Sci USA* **101**:13808–13. doi: 10.1073/pnas.0402576101.
Lee K, Belinsky MG, Bell DW, Testa JR, Kruh GD. 1998. Isolation of MOAT-B, a widely expressed multidrug resistance-associated protein/canalicular multispecific organic anion transporter-related transporter. *Cancer Res* **58**:2741–7.
Leprohon P, Legare D, Ouellette M. 2011. ABC transporters involved in drug resistance in human parasites. *Essays Biochem* **50**:121–44. doi: 10.1042/bse0500121.
Li XC, Schuler MA, Berenbaum MR. 2007. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu Rev Entomol* **52**:231–53. doi: 10.1146/Annurev.Ento.51.110104.151104.
Meinwald J, Jones TH, Eisner T, Hicks K. 1977. New methylcyclopentanoid terpenes from larval defensive secretion of a chrysomelid beetle (*Plagiodera versicolora*). *Proc Natl Acad Sci USA* **74**:2189–93. doi: 10.1073/Pnas.74.6.2189.
Michalski C, Mohagheghi H, Nimtz M, Pasteels J, Ober D. 2008. Salicyl alcohol oxidase of the chemical defense secretion of two chrysomelid leaf beetles - molecular and functional characterization of two new members of the glucose-methanol-choline oxidoreductase gene family. *J Biol Chem* **283**:19219–28. doi: 10.1074/Jbc.M802236200.
Nakagawa H, Toyoda Y, Wakabayashi-Nakao K, Tamaki H, Osumi M, Ishikawa T. 2011. Ubiquitin-mediated proteasomal degradation of ABC transporters: a new aspect of genetic polymorphisms and clinical impacts. *J Pharm Sci* **100**:3602–19. doi: 10.1002/jps.22615.
Nishida R. 2002. Sequestration of defensive substances from plants by Lepidoptera. *Annu Rev Entomol* **47**:57–92. doi: 10.1146/annurev.ento.47.091201.145121.
Noirot C, Quennedy A. 1974. Fine structure of insect epidermal glands. *Annu Rev Entomol* **19**:61–80. doi: 10.1146/annurev.en.19.010174.000425.
Opitz SEW, Muller C. 2009. Plant chemistry and insect sequestration. *Chemoecology* **19**:117–54. doi: 10.1007/S00049-009-0018-6.
Pasteels JM. 1993. The value of defensive compounds as taxonomic characters in the classification of leaf beetles. *Biochem Syst Ecol* **21**:135–42. doi: 10.1016/0305-1978(93)90019-N.
Pasteels JM, Duffey SS, Rowell-Rahier M. 1990. Toxins in chrysomelid beetles possible evolutionary sequence from de novo synthesis to derivation from food-plant chemicals. *J Chem Ecol* 211–22. doi: 10.1007/BF01021280.
Pasteels JM, Rowell-Rahier M. 1991. Proximate and ultimate causes for host plant influence on chemical defense of leaf beetles (Coleoptera, Chrysomelidae). *Entomologia Generalis* **15**:227–35. doi: 10.1127/entom.gen/15/1991/227.
Pasteels JM, Rowell-Rahier M, Braekman JC, Dupont A. 1983. Salicin from host plant as precursor of salicylaldehyde in defensive secretion of Chrysomeline larvae. *Physiological Entomol* **8**:307–14. doi: 10.1111/J.1365-3032.1983.Tb00362.X.
Pereg Y, Liu BY, O'rourke KM, Sagolla M, Dey A, Komuves L, et al. 2010. Ubiquitin hydrolase Dub3 promotes oncogenic transformation by stabilizing Cdc25A. *Nat Cell Biol* **12**:400–6. doi: 10.1038/ncb2041.
Popov N, Schulein C, Jaenicke LA, Eilers M. 2010. Ubiquitylation of the amino terminus of Myc by SCF(beta-TrCP) antagonizes SCF(Fbw7)-mediated turnover. *Nat Cell Biol* **12**:973–81. doi: 10.1038/ncb2104.
Pringle JR, Preston RA, Adams AE, Stearns T, Drubin DG, Haarer BK, et al. 1989. Fluorescence microscopy methods for yeast. *Methods Cell Biol* **31**:357–435. doi: 10.1016/S0091-679X(08)61620-9.
Roy A, Kucukural A, Zhang Y. 2010. I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Protoc* **5**:725–38. doi: 10.1038/nprot.2010.5.
Schulz S, Gross J, Hilker M. 1997. Origin of the defensive secretion of the leaf beetle *Chrysomela lapponica*. *Tetrahedron* **53**:9203–12. doi: 10.1016/S0040-4020(97)00618-2.
Sipos G, Kuchler K. 2006. Fungal ATP-binding cassette (ABC) transporters in drug resistance & detoxification. *Curr Drug Targets* **7**:471–81. doi: 10.2174/138945006776359403.

Soetens P, Pasteels JM, Daloze D, Kaisin M. 1998. Host plant influence on the composition of the defensive secretion of *Chrysomela vigintipunctata* larvae (Coleoptera: Chrysomelidae). *Biochem Syst Ecol* **26**:703–12. doi: 10.1016/S0305-1978(98)00039-8.

Sorensen JS, Dearing MD. 2006. Efflux transporters as a novel herbivore countermechanism to plant chemical defenses. *J Chem Ecol* **32**:1181–96. doi: 10.1007/s10886-006-9079-y.

Termonia A, Hsiao TH, Pasteels JM, Milinkovitch MC. 2001. Feeding specialization and host-derived chemical defense in Chrysomeline leaf beetles did not lead to an evolutionary dead end. *Proc Natl Acad Sci USA* **98**:3909–14. doi: 10.1073/pnas.061034598.

Tolzin-Banasch K, Dagvadorj E, Sammer U, Kunert M, Kirsch R, Ploss K, et al. 2011. Glucose and glucose esters in the larval secretion of *Chrysomela lapponica*; selectivity of the glucoside import system from host plant leaves. *J Chem Ecol* **37**:195–204. doi: 10.1007/s10886-011-9913-8.

Tribolium Genome Sequencing Consortium. 2008. The genome of the model beetle and pest *Tribolium castaneum*. *Nature* **452**:949–55. doi: 10.1038/nature06784.

Winde I, Wittstock U. 2011. Insect herbivore counteradaptations to the plant glucosinolate-myrosinase system. *Phytochemistry* **72**:1566–75. doi: 10.1016/j.phytochem.2011.01.016.

Zolnerciks JK, Andress EJ, Nicolaou M, Linton KJ. 2011. Structure of ABC transporters. *Essays Biochem* **50**:43–61. doi: 10.1042/Bse0500043.

Supplementary Materials

figure supplement 1 to 5

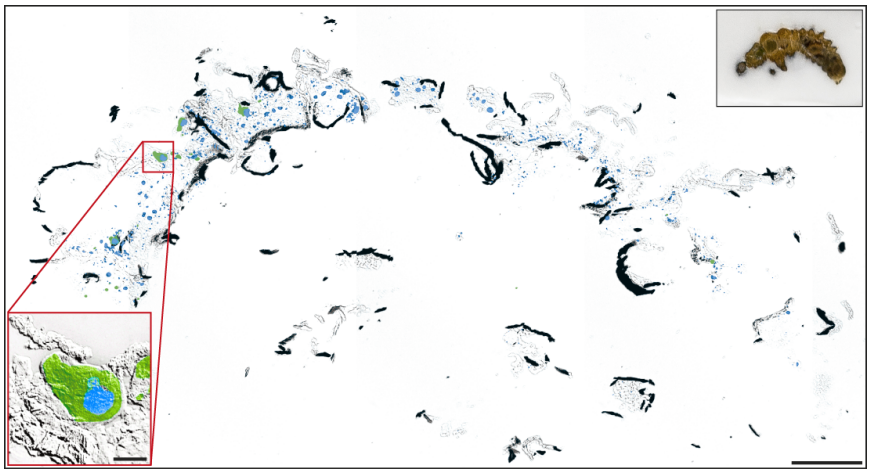


Figure 2-figure supplement 1. Localization of *Cp*MRP in whole larvae cryosections of *C. populi*

Overlay of brightfield and immunofluorescence images of lateral cryosection of an entire L2 stage larvae of *C. populi.* Green, *Cp*MRP; Blue, nuclear staining. The black inset depicts an orientation overview of the cut larvae. The red inset shows an exemplary secretory cell with higher magnification. Scale bars, 500 µm or 50 µm (red inset).

DOI: http://dx.doi.org/10.7554/eLife.01096.006

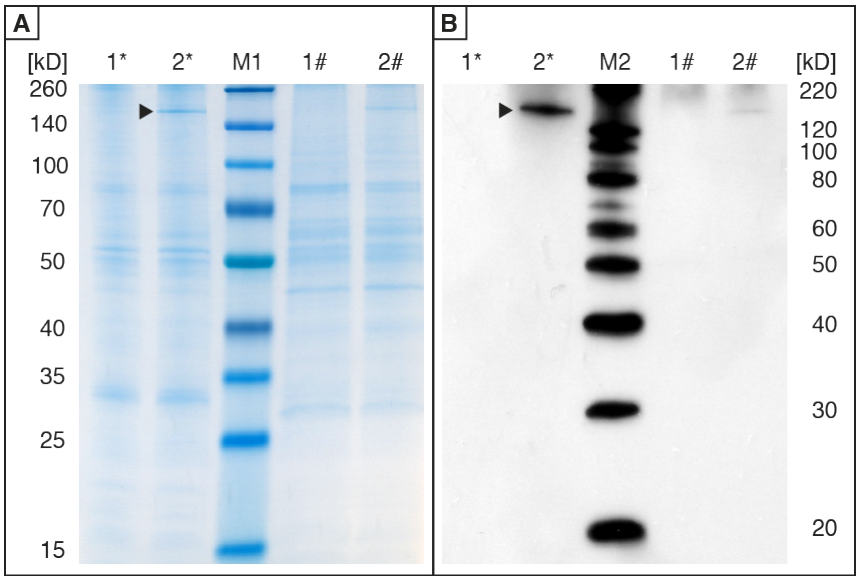


figure supplement 2. Effects of *cpmrp* silencing on the protein level of the glandular tissue of *C. populi*

(**A** and **B**) Total protein analysis of glandular tissue from *cpmrp*-knock-down (1) and *gfp*-control larvae (2) of *C. populi* (10 days post dsRNA-injection). 5 µg total protein contents of roughly separated membrane protein fraction (*) or cytosolic proteins (#) were separated by SDS-PAGE; (**A**) Coomassie staining; (**B**) Western blot with anti-*Cp*MRP. Arrows indicate *Cp*MRP in the membrane protein fraction.

DOI: http://dx.doi.org/10.7554/eLife.01096.010

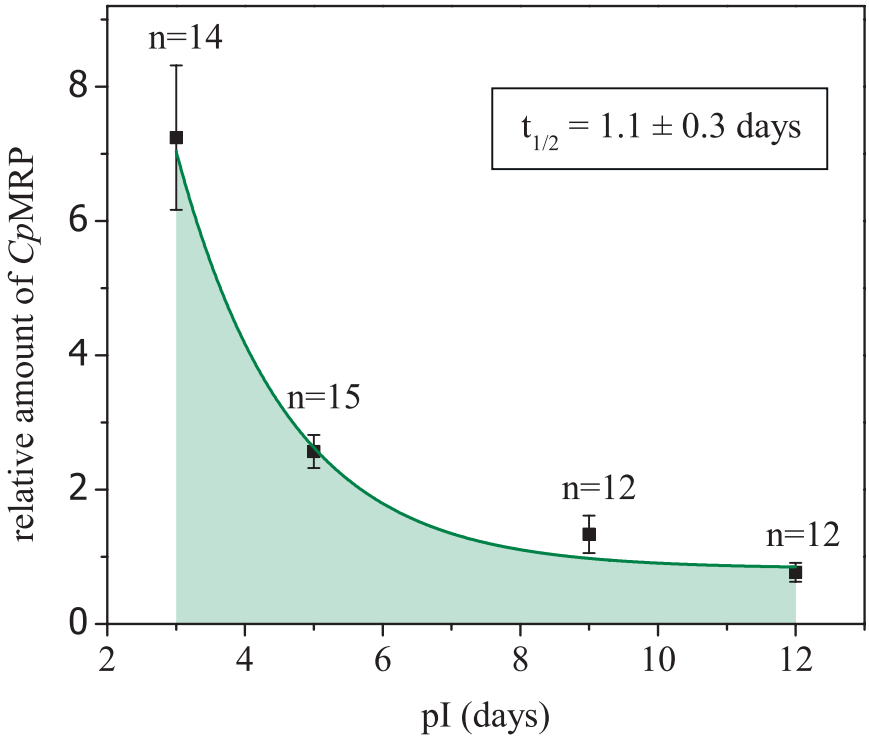


figure supplement 3. Degradation kinetics of *Cp*MRP in secretory cells of *C. populi*

Immunohistochemical staining was employed to follow the degradation kinetics of *Cp*MRP with Alexa 488 after *cpmrp* dsRNA injection (d 0). Integrated Alexa 488 fluorescence intensity from secretory cells was normalized to autofluorescence.

DOI: http://dx.doi.org/10.7554/eLife.01096.011

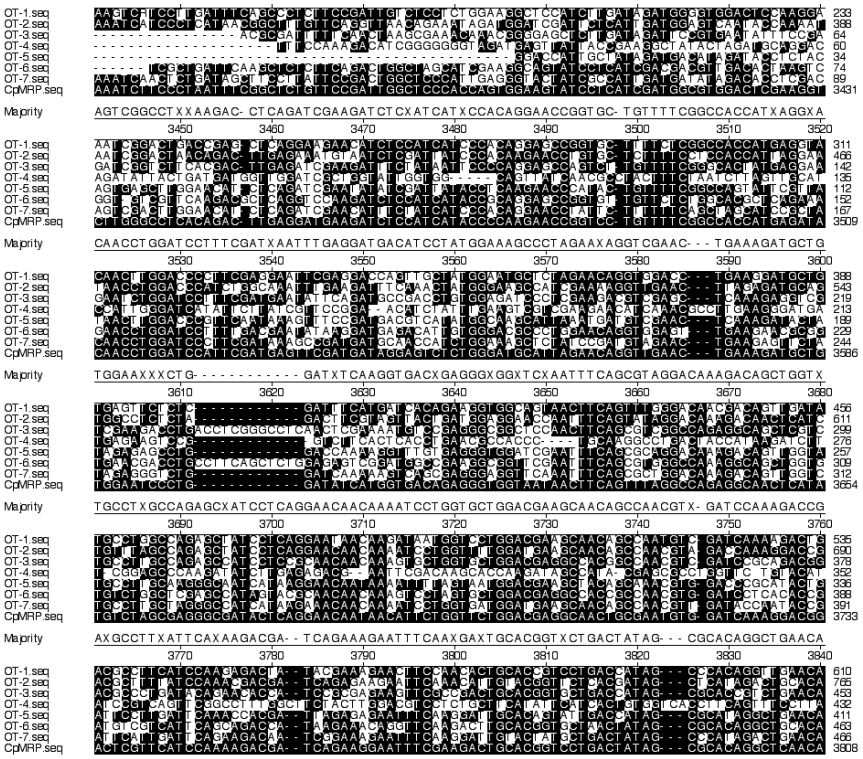


figure supplement 4. DNA alignment of *cpmrp*–related ABC transporter sequences in *C. populi*

Alignment (ClustalW) of *cpmrp* and 7 closely related ABC transporter sequences in *C. populi* (black = exact match with *cpmrp*). Amino acid sequence identity of *Cp*MRP to transporter OT-1 (76.1%), OT-2 (69.4%), OT-3 (61.6%), OT-4 (58.3%), OT-5 (63.9%), OT-6 (64.9%) and OT-7 (64.1%); OT = potential off-target.

DOI: http://dx.doi.org/10.7554/eLife.01096.012

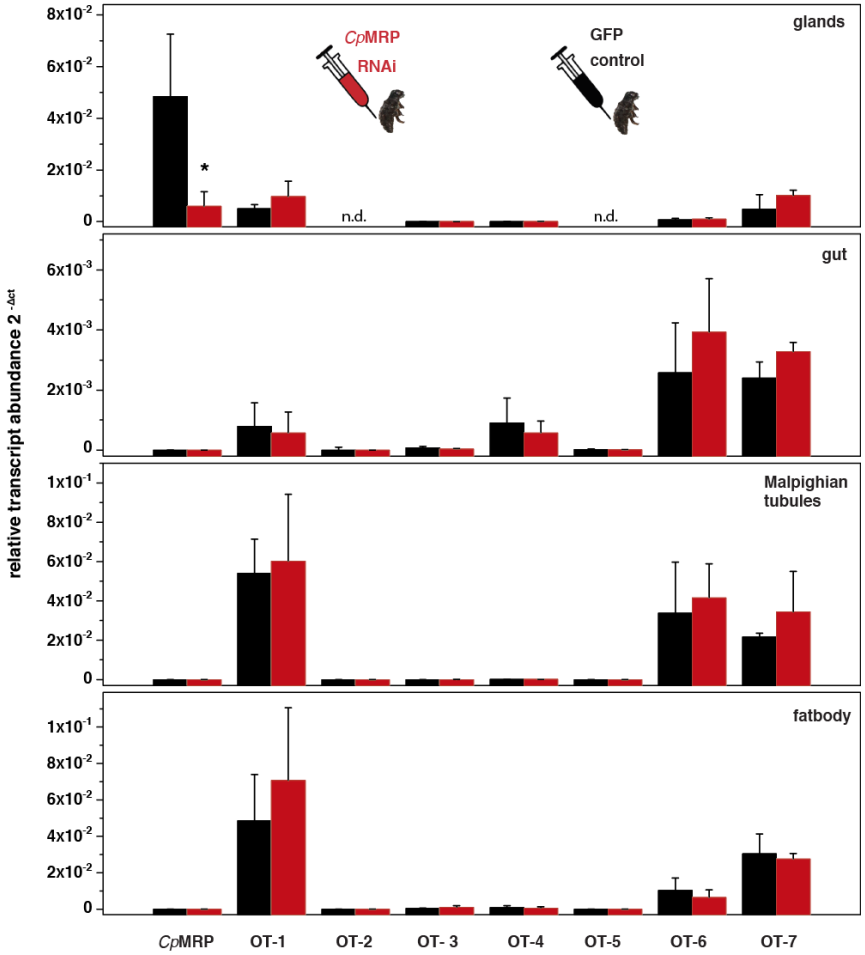


figure supplement 5. Evaluation of possible off-target effects of *CpMRP* dsRNA in larval tissue of *C. populi*

Relative transcript abundance (2ΔCt) of *cpmrp* and closely related possible off-target ABC transporter sequences (OT 1–7) in different larval tissues 10 days after dsRNA injection (n = 4, mean ± SD). Transcript abundance in *cpmrp*-dsRNA (red) was compared to *gfp*-injected control larvae (black). *CpActin* was used for normalization of transcript quantities. Asterisks indicate significant differences between *gfp*-injected control larvae and *cpmrp*-silenced larvae (*p≤0.05), n.d.=not detectable.

DOI: http://dx.doi.org/10.7554/eLife.01096.013

Manuscript III

Tissue-specific transcript profiling for ABC transporters in the sequestering larvae of the phytophagous leaf beetle *Chrysomela populi*

Anja S. Strauss[a,†], Ding Wang[a,†], Magdalena Stock[a,†], Rene R. Gretscher[a], Marco Groth[b], Wilhelm Boland[a], and Antje Burse[a*]

[a] Max Planck Institute for Chemical Ecology, Beutenberg Campus, Hans-Knoell-Str. 8, D-07745 Jena, Thuringia, Germany

[b] Leibniz Institute for Age Research – Fritz Lipmann Institute, Beutenbergstr. 11, D-07745 Jena, Thuringia, Germany

† These authors contributed equally to this study.

[*]Corresponding author. Phone: ++49(0) 3641-571265, Fax: ++49(0) 3641-571202, E-mail: aburse@ice.mpg.de

Keywords: Phytophaga; Coleoptera; leaf beetles; Chrysomela populi; Sequestration; Phytochemicals; Resistance; Chemical defense; Exocrine glands; ABC transporters; Xenobiotic; Multidrug; Phylogeny; RNA-sequencing; Transcriptome; Transcript profiling; RNA interference

Abstract

Background:

Insects evolved ingenious adaptations to use extraordinary food sources. Particularly, the diet of herbivores enriched with noxious plant secondary metabolites requires detoxification mechanisms. Sequestration, which involves the uptake, transfer, and concentration of occasionally modified phytochemicals into specialized tissues or hemolymph, is one of the most successful detoxification strategies found in most insect orders. Due to the ability of ATP-binding cassette (ABC) carriers to transport a wide range of molecules including phytochemicals and insecticides, it is highly likely that they play a role in this sequestration process. To shed light on the role of ABC proteins in sequestration, we describe an inventory of putative ABC transporters in various tissues in the sequestering juvenile poplar leaf beetle, *Chrysomela populi*.

Results: In the transcriptome of *C. populi*, we predicted 65 ABC transporters. To link the proteins with a possible function, we performed comparative phylogenetic analyses with ABC transporters of other insects and of humans. While tissue-specific profiling of each ABC transporter subfamily suggests that ABCB, D and G influence the plant metabolite absorption in the gut, ABCC with 14 members is the preferred subfamily responsible for the excretion of these metabolites *via* Malpighian tubules. Moreover, salicin, which is sequestered from poplar plants, is translocated into the defensive glands for further deterrent production. In these glands and among all identified ABC transporters, an exceptionally high transcript level was observed only for *Cpabc35* (*Cpmrp*). RNAi revealed the deficiency of other ABC pumps to compensate the function of *Cp*ABC35, demonstrating its key role during sequestration.

Conclusion: We provide the first comprehensive phylogenetic study of the ABC family in a phytophagous beetle species. RNA-seq data from different larval tissues propose the importance of ABC pumps to achieve a homeostasis of plant-derived compounds and offer a basis for future analyses of their physiological function in sequestration processes.

Introduction

Lipid bilayers form efficient barriers for cellular partitioning. The translocation across these membranous barriers is crucial for many aspects of cell physiology, including the uptake of nutrients, the elimination of waste products, or energy generation and cell signaling. The ATP-binding cassette (ABC) transporters constitute one of the largest families of membrane translocators [1]. The core functional unit of ABC proteins consists of four domains: two cytoplasmic domains containing the highly conserved nucleotide-binding domain (NBD), which is responsible for the ATP hydrolysis needed to provide energy for the transport cycle, and two transmembrane domains (TMD), each in most cases composed of six membrane-spanning helices which impart substrate specificity and translocation [2-4]. The NBD harbors several conserved sequence motifs from N- to C-terminus. These are the Walker A motif (also called P-loop) which is glycine-rich, a flexible loop with a conserved glutamine residue (Q-loop), the ABC signature (LSGGQ) motif (also called C-loop), the Walker B motif, and a conserved histidine residue (His-switch). The ABC signature motif is diagnostic for this family as it is present only in ABC transporters, while Walker A and B motifs are found in many other ATP-utilizing proteins. The domains are encoded by separate genes, either by genes encoding one NBD and one TMD whose products dimerize to form the functional transporter, or by genes encoding two NBDs and two TMDs on a single polypeptide.

In eukaryotic genomes, ABC genes are widely dispersed and highly conserved between species, indicating that most of these genes have existed since the beginning of eukaryotic evolution [5-8]. ABC transporters can be classified into subfamilies according to sequence homology and domain topology. In eukaryotes eight major subfamilies have been defined: ABCA to ABCH [9]. The eighth subfamily (H) was defined after the analysis of the genome of the fruit fly *Drosophila melanogaster* [9]. Most ABC proteins transport a wide range of compounds, either within the cell as part of a metabolic process into an intracellular compartment (e.g. endoplasmic reticulum, mitochondria, and peroxisomes) or outside the cell for transport processes to other organs. In humans, the known functions of ABC transporters include cholesterol and lipid transport, multidrug resistance, antigen presentation, mitochondrial iron homeostasis and the ATP-dependent regulation of ion channels [10-13]. Owing to the importance of ABC transporters

for cell functions, they are still extensively investigated in many eukaryotes. In insects, one of the best studied ABC proteins is White, which is crucial for pigment transfer in insect eyes [14-19]. As is known for *D. melanogaster*, ABC transporters facilitate translocation of attractants for germ cell migration [20] or participate in the modulation of the molting hormones' (ecdysteroids') signaling in insect tissues [21]. Furthermore, they seem to be frequently implicated in insecticide resistance [22,23], such as in the DTT tolerance of the *Anopheles* mosquitoes which transmit malaria agents [24] or in the tolerance against pest control toxins from *Bacillus thuringiensis* which is reported of lepidopterans [25,26].

Although ABC transporters were previously analyzed in several insect species at genome-wide level [27,28], profiles of the transcript levels of ABC transporters in non-model insects are not available to date. For this study we analyzed the transcriptomic data with regard to ABC transporters in a phytophagous leaf beetle species. Leaf beetles (Chrysomelidae *sensu lato*; including the seed beetles Bruchidae) constitute together with the Cerambycidae (longhorn beetles) and the Curculionoidea (weevils) the largest beetle radiation. These are known as "Phytophaga" and represent roughly 40% of all the 350,000 described species [29]. Leaf beetles mainly feed on green plant parts. The species of the leaf beetle taxon Chrysomelina, for example, are adapted to use host plants' leaves as a food source during their whole life cycle [30]. Therefore, they have to be protected against both, the noxious effect of plant secondary metabolites and attacks by their enemies. Some species evolved the ability to exploit the phytochemicals for their own chemical defense [31-33]. The larvae of the poplar leaf beetle *Chrysomela populi*, for example, take up the phenolglucoside salicin from salicaceaous food plants. This precursor salicin is transported into nine pairs of exocrine, dorsal glands [34,35], where the compound is converted into salicylaldehyde – a potent, volatile deterrent that repels predators and prevents fatal microbial infections [33,36,37]. This process of sequestration involves a complex influx-efflux transport network which guides plant-derived glucosides through the insect body [38].

Although sequestration is a widespread phenomenon attributed to many insect orders, we recently identified the first example of a transport protein essential for the translocation of phytochemicals in insects [39]. The transporter belongs to the ATP-binding cassette transporter family and functions in the

defensive exocrine glands of juvenile poplar leaf beetles. Thus, the comprehensive analysis of putative ABC transporters in the phytophagous *C. populi* larvae provides implications for further studies on the predicted physiological functions of this transporter class in sequestering insects, such as the incorporation and excretion mechanisms of toxic compounds. For this reason, we present a complete inventory of ABC transporters based on available *C. populi* transcriptome sequences. Detailed sequence comparisons of members of each subfamily with those of the red flour beetle *Tribolium castaneum*, the bark beetle *Dendroctonus ponderosae*, the silk worm *Bombyx mori*, *D. melanogaster* and humans reveal their correspondences. We, additionally, studied the expression profiles of ABC encoding transcripts in various tissues by using next-generation sequencing in juvenile *C. populi* and propose a function of ABC pumps in the sequestration process.

Material and Methods

Rearing, maintaining and dissecting *Chrysomela populi*

C. populi (L.) was collected near Dornburg, Germany (+51°00'52.00", +11°38'17.00") on *Populus maximowiczii x Populus nigra*. The beetles were kept in a light/dark cycle of 16 h light and 8 h darkness (LD 16/8) at 18°C ± 2°C in light and 13°C ± 2°C in darkness.

RNA isolation, library construction and sequencing

Tissue samples from five *C. populi* larvae per biological replicate were collected as described by Bodemann *et al.* [40]. Total RNA was extracted from defensive glands, fat body, Malpighian tubules and gut tissue with the RNAqueous Micro Kit (Ambion, Life Technologies) according to the manufacturers' instructions with the exception of 1% (v/v) ExpressArt NucleoGuard (Amplification Technologies, Hamburg, Germany) added to the lysis buffer. The RNA integrity was validated by electrophoresis on RNA 6000 Nano labchips on a Bioanalyzer 2100 (Agilent Technologies). RNA concentrations were determined by employing a NanoView (GE-Healthcare). Up to 5 µg of total RNA was then used for library preparation using TruSeq™ RNA Sample Prep Kit according to the manufacturer's description. RNA

sequencing (RNASeq) for three biological samples per prepared tissue was done using Illumina next-generation sequencing technique [41] on a HiSeq2000 (Illumina, Inc., San Diego, California USA) in 50-bp single read mode (two or three samples multiplexed in one lane).

***De novo* assembly of *C. populi*'s transcriptome**

The transcript catalogue of *C. populi* was generated as described in Rahfeld *et al.* [42]. Briefly, the paired-end reads were *de novo* assembled by applying the open source tool Trinity v2012-03-17 [43] with the following parameters: minimal contig length of 300 bp and the paired fragment length limited to 500 bp. In order to reconstruct full-length transcripts, we used the software TGICl (vJan.2009) [44] to reassemble the transcriptome output from Trinity with a minimum overlap length of 100 bp and sequence similarity of 90 percent. A summary of these results is given in Table S1. The raw sequence data are stored in the SRA of the National Center for Biotechnology Information (NCBI) with the accession number SRA106166. The corresponding BioProject is PRJNA212154.

Annotation of *de novo* assembled transcript library and identification of ABC transporters

We annotated the above mentioned transcriptome by translating the cDNAs of the putative transcripts into all six possible open reading frames. This was achieved by applying transeq which is part of the EMBOSS package (v6.3.1). Afterwards, the protein sequences were searched against the Pfam database (update, Jan 2013) with an e-value cut-off of 1e-5 [45,46]. 102 hits were obtained that belong to the protein family "PF00005" (ABC_tran domain). The exact NBDs of 90 ABC transporters were identified after the removal of 12 sequences highly similar to obligate intracellular Microsporida parasites found by BLASTx against the non-redundant protein sequence database (at NCBI). For the identification of NBDs, firstly, the highly conserved NBDs of the human (ABCA-ABCG, 48 amino acid sequences of NBD) and fruit fly (ABCH, 3 amino acid sequences of NBD) ABC transporters were retrieved from GenBank (NCBI) and chosen as 'homology search targets'. Then, the long coding sequence for each annotated beetle ABC transporter was determined by using getorf of the EMBOSS tools. Afterwards, these longest coding sequences and the

chosen 'homology search targets' were aligned by applying the multiple sequence alignment program MAFFT v7.01 [47] (using option E-INS-i). Transcripts containing all five motifs of NBDs with roughly 170 amino acids were kept. Secondly, the remaining ABC transporter transcripts with incomplete motifs were checked again. Their six possible protein sequences were aligned to the chosen 'homology search targets' (with the same parameter E-INS-i, MAFFT). All sequences containing at least four motifs of NBDs and having a sequence length of more than 130 amino acids were selected and added to the other sequences for further studies. After removal of isoforms, the resulting beetle sequences were deposited as Transcriptome Shotgun Assembly project at DDBJ/EMBL/GenBank under the accession GARF00000000. The version described in this paper is the first version, GARF01000000.

Calculation of phylogenetic trees

The protein sequences were aligned by the G-INS-i methods from MAFFT with default parameters. To calculate the phylogenetic tree two programs were used: MrBayes v3.2.1 [48], a program for Bayesian inference, and RAxML v7.2.8 [49], a program based on maximum-likelihood inference. In RAxML, the best fit model of protein evolution was RTREVF with gamma distribution for modeling rate heterogeneity. The best fit model was determined by the best likelihood score under GAMMA (perl script ProteinModelSelection.pl, which was downloaded from http://sco.h-its.org/exelixis/hands-On.html). The maximum-likelihood phylogenetic tree was reconstructed with a bootstrap test of 1000 replicates in RAxML. In addition, we used MrBayes with gamma-shaped rate variation and a proportion of invariable sites to check the phylogenetic tree of RAxML.

For phylogenetic analysis of the ABC transporter subfamilies, we used the same methods along with sequences of *T. castaneum*, the most closely related model species to *C. populi*. The ABC transporter protein sequences of *T. castaneum* were retrieved from Broehan *et al.* [50] with the identical designations. Further, we included homologous sequences from human, *B. mori*, *D. melanogaster*, *Apis mellifera*, *Culex quinquefasciatus*, *D. ponderosae*, and from a Microsporidia species into our calculations. If not stated in the phylogenetic trees, the accession numbers of these sequences are listed in Table S2.

Expression profiling of putative ABC transporter transcripts

Each 50-bp single-read dataset of four tissues (gut, defensive glands, fat body, Malpighian tubules) contained three biological replicate samples (Table S3 for overview of libraries). To compare the transcript expression levels of the four tissues, we mapped the RNA-seq reads onto the (*de novo* assembled) transcriptome of *C. populi* with the open source tool Bowtie v0.12.7 [51] using default parameters. Afterwards, the R package DESeq [52,53] (which is part of the Bioconductor package [54]) was used to detect differentially expressed transcripts in the four different tissues.

Based on the Lander/Waterman equation [55], the average coverage per base in each transcript of each biological replicate was separately computed. The mean values of average coverage of each replicate for each tissue, respectively, were compared to show the expression levels of tissues (see Table S4 for normalized data). To compare these results with quantitative real-time PCR measurements, we normalized the output from DESeq to the standards *Cp_elF4a* and *Cp_EF1alpha* (see Table S5 for the accession numbers of normalization genes), which were used in quantitative real-time PCR, as described by Livak *et al*. [56].

Quantitative real-time PCR (qPCR)

Total RNA was extracted from larval tissue using an RNeasy MINI kit (Qiagen). Complementary DNA was synthesized from DNA-digested RNA using the RNAqueous micro kit (Life Technologies). Real-time PCR was performed using Brilliant II SYBR Green qPCR Master Mix (Agilent) according to the manufacturer's instructions and the Mx3000P Real-Time PCR system. *Cp_elF4a* and *Cp_EF1alpha* expression were used to normalize transcript quantities (see Table S5 for primer sequences). Running conditions: 3' 94°C, 40 cycles (30" 94°C; 30" 60°C), melting curve with 1°C increase 60-95°C. Analyses were performed according to the MIQE-guidelines [57].

RNA interference of *Cpabc35* (*Cpmrp*) in *C. populi* larvae

The most abundant ABC transporter derived from the glandular tissue (*Cpabc35* (*Cpmrp*)) [39] was analyzed *via* RNAi experiments. The sequence-verified plasmid pIB-*Cp*MRP was used to amplify a 730-bp fragment of *Cpabc35* dsRNA. As control, a *gfp* sequence was amplified from pcDNA3.1/CT- GFP-TOPO (Invitrogen). The amplicons were subjected to *in vitro*-transcription assays according to the instructions of the Ambion MEGAscript RNAi kit (Life Technologies; see Table S5 for primer sequences). The resulting dsRNA was eluted three times with 50 µl of injection buffer (3.5 mM Tris-HCl, 1 mM NaCl, 50 nM Na_2HPO_4, 20 nM KH_2PO_4, 3 mM KCl, 0.3 mM EDTA, pH 7.0) after nuclease digestion. The quality of dsRNA was checked by TBE-agarose-electrophoresis.

First-instars of *C. populi* (3-4 days after hatching) with 3-5 mm body length (chilled on ice) were injected with 0.25 µg of dsRNA by using a nanoliter microinjection system (WPI Nanoliter 2000 Injector). Injections were made into the hemolymph next to the ventral side between the pro- and mesothorax. Differential expression in the glandular tissue was analysed 10 days after RNAi treatment. Therefore, we carried out RNA sequencing (RNA-Seq). Two biological replicates (pool of glandular tissue of 3 larvae, each) compared to two biological replicates of *gfp*-control samples [41] were sequenced on a HiSeq2500 (Illumina, Inc., San Diego, California USA) in 50-bp single read mode (two or three samples multiplexed in one lane). All short reads again were extracted in FastQ format for further analysis.

Analysis of differentially expressed genes in the glandular tissue of RNAi silenced *C. populi* larvae

The short reads (sequenced in 50 bp single-mode) from the glandular tissue of the RNAi-silenced (2 samples) as well as ds*gfp*-injected (2 samples) *C. populi* larvae were mapped onto *C. populi*'s transcriptome using Bowtie [51]. The mapping results for the ABC transporter transcripts were subjected to DESeq statistical analysis [52,53] by reading them into R statistics software. Transcript counts were normalized to the effective library size. Afterwards, the negative binomial testing was carried out to identify differentially expressed genes (DEGs). All those genes were determined as differentially expressed

when having an adjusted p-value less than 0.1. From all DEGs, the annotated ABC transporters were selected and checked for co-regulation.

Results and discussion

Identification of putative ABC transporters encoded in the transcript catalogue of *C. populi*

In our study, we focused on the distribution of ABC transporters in the different tissues of juvenile C. *populi* to functionally link each transcript to a certain tissue. For this purpose, we first identified potential ABC transporters in the *de novo* assembled transcript catalogue of the poplar leaf beetle. The transcriptome sequences were translated into all possible amino acid sequences and further processed as described in the method section. As a result, we predicted 65 ABC transporters for *C. populi*. This corresponds with previous studies on insects reporting, for example, 73 ABC transporter genes in the genome of *T. castaneum* [50], 44 in *Anopheles gambiae* [28], 56 in *D. melanogaster*, 43 in *Apis mellifera*, or 51 in *Bombyx mori* [27]. The *C. populi* sequences were given temporary designations as numbered series in the form of *Cp*ABCxx (Table S4).

Phylogenetic analysis of the putative ABC transporters

Based on structural and functional similarity, ABC transporters in general can be grouped into subfamilies. In order to predict the subfamilies for the 65 identified ABC transporters in *C. populi*, we used their extracted NBDs for the multiple sequence alignments and then calculated the phylogenetic tree. Similarly to other insects and eukaryotes, we were able to show a division of the predicted transporters into 8 subfamilies (A-H) (Figure 1; Table 1). Members of ABCA, ABCE/F, ABCG and ABCH form distinct branches (bootstrap value ≥75 percent). ABCH forms a sister group of ABCA. The ABCC family segregates into two groups: ABCC1 contains NBDs1 and shows a similarity to the ABCD subfamily; ABCC2 contains NBDs2 and shows a similarity to the ABCB subfamily. Among the 65 putative ABC transporters from *C. populi* we identified full, half and incomplete transporters. The distribution of

domains in the sequences is shown in detail for each subfamily in Table 2 and for each sequence in Table S6.

Next, we integrated human and other insect sequences into our phylogenetic trees. This allowed us to group the putative *C. populi* ABC transporters with functionally characterized proteins and, thus, to propose a substrate for the beetles' proteins.

In the case of **subfamily A**, its members in humans are full transporters and implicated in the transport processes of phospholipids, sterols, sphingolipids, bile salts, retinal derivatives (restricted to ABCA4) and other lipid conjugates indispensable for many biological processes [10,58-61]. In insects, both full and half transporters were identified whose physiological function, however, is not yet understood [27]. In *C. populi* we predicted five transporters. According to our phylogenetic analysis, ABCA proteins segregate into one branch containing NBD1 and one branch with NBD2 (Figure 2). Human ABCAs form three groups (I, ABCA1-4, 7; II, ABCA5, 6, 8-10; III, ABCA12 and 13) which are particularly distinguishable in the NBD2 branch with bootstrap values of ≥76 percent. Considering the beetles' homologs, the tree shows that the majority of *C. populi* and *T. castaneum* sequences seem to cluster to human ABCA3 which results in an expansion of group I.

The **ABCB** subfamily contains ABCB1 (MDR1/P-glycoprotein) which is the first characterized human ABC transporter to confer multidrug resistance (MDR) in cancer cells [62-64] and which has been intensively studied ever since the discovery of cross-resistances after selection with chemotherapeutics [65-69]. Later studies revealed additional ABCB transporters as MDR proteins. Besides xenobiotic extrusion (ABCB1, 5, 8) [70-72], ABCB members are also known in human biology for the translocation, for example, of phosphatidylcholine (ABCB4) [73], bile salt (ABCB11) [74], peptides (TAP1:TAP2 (antigen processing in the adaptive immune system), TAPL, ABCB10) [75], porphyrins (ABCB6) [76], or iron (ABCB7 and 8) [77-79]. In insects, several examples suggest the involvement of P-glycoproteins in the resistance to insecticides used for crop protection [22,23,80-88]. However, only few P-glycoprotein-like genes have been linked to a xenobiotic substrate such as *Mdr49* and *Mdr65* of *D. melanogaster* with

tolerance against colchicine and α-amanitin [89,90]. Alternatively, Mdr49 can act as transporter for a germ cell attractant in fruit flies [20].

Similar to other insects, the eight sequences from *C. populi* encode full and half transporters. Bootstrapping of the ABCB phylogenetic tree in Figure 3 and Figure S2 (together with ABCC) produced nodes weakly supporting segregation of this subfamily containing human and insect ABCB sequences. Based on our phylogenetic analysis, we found no homologs to TAP sequences (bootstrap value of 100 percent) in the insects, but insect homologs to the other human peptide transporters were identified. In accordance with the literature, we can also speculate that TAPL is the ancestor of the TAP family [91].

Full transporters of striking functional diversity are found in the **ABCC** subfamily. In humans thirteen ABCC members were identified, nine of which are referred to as multidrug resistance proteins (MRP) ('short' MRPs (ABCC4, -5, -11 and -12); 'long' MRPs (ABCC1, -2, -3, -6 and -10) [12,92,93]. Some ABCC members not considered as MRPs have unique functions. The cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7), for example, functions as an epithelial ATP-gated chloride channel [94,95]. ABCC8 and ABCC9 are assembled as sulfonylurea receptors (SUR) into ATP-sensitive K^+ channels and are coupled to the gating mechanism of the ion-conducting pore [96]. In insects, ABCC members are thought to be involved in the translocation of xenobiotics and phytochemicals [22-25,39,88,97]. As observed in the red flour beetle *T. castaneum* and the spider mite *Tetranychus urticae*, the ABCC subfamily in *C. populi* with 29 putative members has undergone an expansion (Table 1). In our phylogenetic tree, the NBDs1 and NBDs2 form distinct branches (bootstrap value of 100 percent; Figure S2). The human 'short' MRPs ABCC5, 11, and 12 are clearly separated from all other tested sequences (Figure 4). The vast majority of insect sequences cluster together with human CFTR, SURs and multidrug resistant proteins, such as 'long' MRPs and ABCC4 implying a broad substrate spectrum of these proteins (Figure 4 and S2). Into this group falls also *Cp*ABC35 (*Cp*MRP) which is known to translocate phytochemicals including salicin. A substrate for any other insect homolog in this group has not been determined to date.

Members of the **ABCD** subfamily are involved in the translocation of fatty acids into peroxisomes [98]. The ABC transporters are half-size and assemble mostly as a homodimer after posttranslational transport to peroxisomal membranes. ABCD4 is not a peroxisomal membrane protein but an ER-resident protein that mediates translocation of lipid molecules essential for lipid metabolism in the ER [99]. As in humans and other insects, *C. populi* contains two half transporters. Because they are homologous to the human peroxisomal and *T. castaneum* transporters, a similar function can be inferred in poplar leaf beetles. No orthologous insect sequence could be grouped to ABCD4 (Figure S3).

The **ABCE** and **ABCF** proteins comprise a pair of linked NBDs but lack TMDs. Therefore, they are not involved in molecule transport, but they are active in a wide range of other functions pivotal for cell viability. For example, the human ABCE1 not only acts as a ribonuclease L inhibitor, it also regulates RNA stability, viral infection, tumor cell proliferation, anti-apoptosis, translation initiation, elongation, termination, and ribosome recycling [100]. In *D. melanogaster*, the ABCE homolog Pixie plays a catalytic role in the assembly of protein complexes required for translation initiation [101]. All genomes of multicellular eukaryotes analyzed to date possess one ABCE gene [102]. In the transcript catalogue of *C. populi*, one complete ABCE protein has been predicted. The NBDs of *Cp*ABC45 are highly conserved with the respective NBDs of the human ABCE1 and *T. castaneum* ABCE-3A (Figure S3). Among the subfamily ABCF involved in translation initiation and elongation in humans [102], we found three putative members each with two NBDs that are highly similar to the transporters of human and *T. castaneum* suggesting functional proteins used in similar physiological processes in the cell.

The **ABCG** subfamily in humans is comprised of five half transporters. While the homodimer ABCG2 is a multidrug transporter with a wide substrate specificity [70], the homodimers ABCG1 and ABCG4 and the heterodimer ABCG5:ABCG8 translocate cholesterol and other sterole derivatives [103-106]. In insects, ABCG transporters are essential for the translocation of ommochromes for the pigmentation of eyes and body coloration. In *D. melanogaster*, for example, the half transporter White forms heterodimers with Scarlet or Brown, each of which is responsible for the transport of another type of ommochrome precursor to pigment granules [14-16]. In silkworms, White-orthologs (Bm-ok) are

responsible for the translocation of uric acid for accumulation in urate granules in epidermal cells, resulting in opaque white coloration of the larval skin [19,107]. In *D. melanogaster*, E23 encodes a transporter capable of modulating the ecdysone response with consequences for the circadian transcription of clock genes [21,108].

The phylogenetic analysis revealed that the majority of the chosen insect sequences, including predicted ABCG proteins from *C. populi*, cluster together with the human ABCG1 and ABCG4 (bootstrap value of 98 percent) (Figure 5). Several insect ABCG candidates form a branch with the human ABCG5:ABCG8. Also E23 from *D. melanogaster* clusters in this branch together with *Tc*ABCG-8A. Silencing of *Tc*ABCG-8A resulted in molting defects, premature compound eye development, aberrant wing development and lethality, suggesting a function in the regulation of ecdysteroid-mediated effects [50]. Because *Cp*ABC49 is homologous to *Tc*ABCG-8A and *Dm*-E23, it allows the expectation of a similar function for this protein in *C. populi*. In addition, the insect ABCG proteins (White, Brown, and Scarlet) involved in the transfer of ommochrome precursors form a separate branch (bootstrap value of 93 percent). In accordance with the observation of *T. castaneum* [50], in *C. populi* a Brown ortholog is also missing. Interestingly, not a single analyzed insect sequence clusters with the human multidrug efflux transporter ABCG2.

The transporters of the **ABCH** subfamily were observed only in insects and not in humans [6,8,109]. The ABCH subfamily of *C. populi* includes three putative ABC transporters that are highly similar to those of *T. castaneum* (Figure 2).

RNA-seq analyses reveal tissue-specific expression of ABC transporters in juvenile *C. populi*

To link the above suggested functions for the *C. populi* ABC proteins to those which are differentially expressed in the larval tissues of *C. populi*, we carried out a comprehensive transcriptome sequencing of different tissues dissected from the poplar leaf beetle. All raw sequence data (in the following called reads) are listed in Table S3 and S4. The resulting expression patterns of all identified ABC transporters in intestinal tissue, Malpighian tubules, fat body and defensive glands is depicted in Figure 6. It shows that

among the 65 predicted ABC transporters, 43 are expressed at least in one of the tested tissues with more than 25 normalized read counts per base (25-fold sequence coverage). As previously demonstrated in literature [110], evaluation of the RNA-seq data (standardized values shown in Table S7) with quantitative real-time PCR data shows also in our experiments the comparability of the two methods (Figure S1).

Five transcripts were found to be abundant in all tested tissues which suggest their essential role in cellular processes. Among them is, for example, *Cp*ABC4 which was classified as member of the ABCA subfamily. According to our phylogenetic analysis, the closest human homologs, which are involved in lipid translocation, are clustered into group I of the NBD1 branch (Figure 2). Although the NBD2 of *Cp*ABC4 clusters to ABCA12 and 13, the sequence comparison (using BLAST) of the complete sequence supported the homology of *Cp*ABC4 to human ABCA members of group I. Additionally, *Cp*ABC49 as ABCD candidate was highly expressed in all larval tissues, as well. It is homologous to the human ABCD1 and 2 and, therefore, presumably linked to the transport of very long chains of fatty acids in peroxisomes (Figure S3) [98]. Furthermore, we detected in all larval tissues abundantly expressed transcripts encoding soluble ABC proteins: *Cp*ABC52 as a member of the ABCE and *Cp*ABC57 and *Cp*ABC58 as members of the ABCF subfamily. Also, in the red flour beetle, the *Tc*ABCE-3A and *Tc*ABCF-2A transcripts were abundant throughout all life stages and highly abundant in the adult intestinal/excretory tissues and carcass [50]. Furthermore, the silencing of *Tc*ABCE-3A as well as *Tc*ABCF-2A resulted in growth arrest and mortality of the beetles. Thus, ABCE and ABCF proteins are essential for cellular functions in all insect tissues including initiation of translation [100,101] and ribosome biogenesis [111].

In the following, we describe differential expression of putative ABC transporters in the different larval tissues:

Gut tissue

We found 17 transcripts abundant in the intestinal tissue of *C. populi* encoding members of the following subfamilies: one sequence of ABCA, two of ABCB, eight of ABCC, five of ABCG (Figure 6). The existence of ABC transporters in the gut influences the absorption and bioavailability of nutrients, ions and

plant derived compounds. The predicted ABCA-similar sequence *Cp*ABC5 exhibits a high mRNA level only in the gut tissue. Its deduced protein clusters together with *Tc*ABCA-9A/B of *T. castaneum* (Figure 2). The silencing of these two red flour beetle genes resulted in high mortality and severe defects in wing and elytra development, depending on the developmental stage of treatment. This indicates an essential function for cell physiology, but a ligand has not been identified for these proteins to date [50]. The closest homolog in humans is ABCA3 which is related to phospholipid transfer but also to the modulation of cell susceptibility to chemotherapy of tumors [10,112,113]. Thus, *Cp*ABC5 may have a special function in this tissue, in addition to a role in lipid trafficking.

The highest transcript level of ABC transporters in the intestinal tissue was detected for *CpABC12* which was classified into the subfamily B. It is also expressed in Malpighian tubules but ten times less. *Cp*ABC12 is a full transporter, and possesses most likely homology to human ABCB1 (MDR1, P-glycoprotein), 4, 5 and 11 as well as the *D. melanogaster* Mdr50 (bootstrap value of 63 percent) (Figure 3). Though ABCB4 acts in humans as a transporter for phospholipids in the liver [73], it is involved in the zebrafish's cellular resistance to noxious chemicals [114]. Except for ABCB11, which is a bile salt transporter [74], all the homologous vertebrate ABCB members can confer multidrug resistance [70-72]. We hypothesize a function in the translocation of phytochemicals for *Cp*ABC12 in the gut of *C. populi*. *Cp*ABC7 is the second ABCB candidate with a high expression level in the gut, albeit not as high as *Cp*ABC12. Moreover, *Cp*ABC7 is 3 times more highly expressed in the Malpighian tubules than in the gut. Because *Cp*ABC7 is most homologous to the human mitochondrial ABCB6 (Figure 3), which facilitates porphyrin transport [76], the beetle protein could possess the specificity for structurally similar substrates.

All ABCC candidates highly expressed in the gut tissue cluster together with human CFTR, SURs, 'long' MRPs and ABCC4. This implies a broad substrate spectrum for these insect transporters which, however, cannot be specified further from our phylogenetic analysis (Figure 4, S2).

All five ABCG candidates highly expressed in the larval gut tissue cluster together with the human ABCG1 and ABCG4. These proteins are involved in sterol homeostasis. Among these *C. populi* ABCG proteins, *Cpabc55* showed the most elevated transcript level. It is homologous to *Tc*ABCG-4C whose

involvement in the transport of lipids to the cuticle has been suggested and, thus, that it is required for the formation of a waterproof barrier in the epicuticle [50]. *Cpabc55* is also highly expressed in glands and fat body tissue but not in the Malpighian tubules. The expression of *Tcabcg-4c* was higher in intestinal/excretory tissues than in carcass tissue [50]. The function of the other four ABCG transporters cannot be predicted from our analyses. However, it has been demonstrated recently that an ABCG1-homolog in the fungus *Grossmannis clavigera* confers tolerance to monoterpenes which contributes to the fungus' ability to cope with the chemical defence of its host plant [115]. Therefore, the ABCG proteins' specificity in insects may not be limited to sterols or lipids but may have a broader substrate spectrum - that is not known to date. Besides trafficking of physiological substrates, the identified ABC transporters in the gut tissue may also play a critical role in regulating the absorption of plant secondary metabolites or influence the effectiveness of pesticides in the phytophagous *C. populi*.

Malpighian tubules

Insect Malpighian tubules are critical for osmoregulation. Moreover, the tubules have the capability to excrete actively a broad range of organic solutes and xenobiotics, such as insecticides. Additionally, they play a significant role in immunity by sensing bacterial infections and mounting an effective killing response by secretion of antimicrobial peptides [116]. We found 21 transcripts abundant in the Malpighian tubules of *C. populi* encoding members of the following subfamilies known to contain multidrug resistance proteins: four of ABCB, 14 of ABCC, three of ABCG (Figure 6).

Among the four predicted ABCB members displaying a high mRNA level in the Malpighian tubules, two, *Cp*ABC7 and 12, were already described in the gut section above. The third candidate, *Cp*ABC8, is most similar to human mitochondrial ABCB10 (Figure 3). For ABCB10 different roles have been suggested, including protection against toxic reactive oxygen species, heme synthesis, or peptide transport [75,117,118]. For this tissue, we speculate that it is involved in antimicrobial peptide transfer. The forth ABCB protein, *Cp*ABC9, clusters together with the human mitochondrial ABCB7 which is involved in the iron-sulfur cluster assembly essential for multiple metabolic pathways throughout the cell

(Figure 3) [77,78]. RNAi of the homologous *Tc*ABCB-5A demonstrated the pivotal function of this gene in the red flour beetle: its down-regulation resulted in severe morphological defects and high mortality depending on the developmental stage treated [50]. Hence, the three most likely mitochondrial localized ABCB candidates, namely *Cp*ABCB7-9, are proposed to be of vital importance in the cells. However, for *Cp*ABC12, which is a full transporter and clusters to human proteins related to xenobiotic resistance, we can predict a similar function in the larval excretion system.

Most putative ABC transporter transcripts identified in *C. populi* are present at a high level in the excretion system of the juvenile beetles compared to the other tissues. Particularly, the 14 candidates belonging to the ABCC subfamily are the most highly transcribed compared to other subfamilies in this tissue. Remarkably, one of the highly expressed candidates, *Cp*ABC16, clusters in our phylogeny together with *Cp*ABC35 which is involved in the accumulation of plant-derived metabolites (Figure S2) [39]. Therefore, it is tempting to speculate a role for *Cp*ABC16 in the excretion of phytochemicals in *C. populi* larvae.

Among the three candidates of the G-subfamily, two are highly transcribed only in the Malpighian tubules: *Cp*ABC54 is a homolog of *Tc*ABCG-9B from the White group and *Cp*ABC62 is homologous to *Tc*ABCG-9A from the Scarlet group (Figure 5). RNAi targeting *Tcabcg-9a* or *b* resulted in both cases not only in white eyes but also in a whitish appearance of the Malpighian tubules due to the absence of tryptophan metabolites/kynurenine and pteridines. These eye pigment precursors are stored and processed in the larval tubules before being released for further conversion into pigments in the developing adult eyes [119-121]. In addition, in *D. melanogaster* White is expressed in intracellular vesicles in tubule principal cells, suggesting that White participates in vesicular transepithelial transport of cGMP [122]. *Cp*ABC57 is the only ABCG candidate that is also expressed in the intestine and belongs to the human ABCG1 and ABCG4 branch (Figure 5). Taken together, the conspicuous overrepresentation of drug-resistance associated proteins, including the ABCC4-like proteins together with the members of the subfamily B (*Cp*ABC12) and G (*Cp*ABC57), in the excretion system suggests a role for these candidates in the extrusion of xenobiotics or phytochemicals from the larval body.

Fat body

The fat body of insects is a polymorphic tissue. It performs a vast array of fundamental activities in the intermediary metabolism and is involved in maintaining the homeostasis of hemolymph proteins, lipids, and carbohydrates [123]. Predominantly, the storage of lipid reserves in the form of glycogen and triglycerides is essential in the life of holometabolous insects, primarily in their survival of metamorphosis [124]. In humans, members of the subfamilies A, B, D and G are known to be involved in lipid transport [10,125]. In principal, we found the expression of ABC transporters in the larval fat body of *C. populi* to be low compared to the other tested tissues (Figure 6). From the ABCB subfamily, we identified in the fat body only *Cp*ABC8 exhibiting a low transcript level comparable to that of the Malpighian tubules. As described above, it clusters with the human mitochondrial ABCB10 which is associated with different functions, also described above, but not particularly with lipid transfer.

From ABCG we found *Cp*ABC51 and *Cp*ABC55 with high expression in the fat body, both clustering to human ABCG1 and ABCG4 (Figure 5). Only one sequence was exclusively expressed in this body part, namely *Cp*ABC41, a member of the subfamily C (Figure 4). Other ABCC members which are highly expressed in this tissue are the homologous *Cp*ABC16 and *Cp*ABC35 (Figure S2). *Cp*ABC35 is known to translocate phytochemicals [39].

Noticeably, we found high expression of putative ABCH genes (*CpABC64, CpABC65)* in the fat body tissue. Up to now the function of this insect specific subfamily has been unclear. However, RNAi targeting *Tc*ABCH-9C in the flour beetle revealed a lethal, desiccated phenotype similar to the silencing of *Tc*ABCG-4C mentioned above. This ABCH member also seems to be involved directly or indirectly in the transport of lipids from epidermal cells to the cuticle [50]. Based on our data we can hypothesize a role for ABC transporters in phytochemical translocation (by members of the subfamily C and the ABCG candidate), in cuticle formation (by members of the ABCH subfamily) in the fat body, but not particularly in the lipid storage of this tissue. Transporters which are important for this function might be lowly expressed and therefore not detected in our analyses.

Defensive glands

The nine pairs of defensive glands enable larvae of *C. populi* to chemically defend themselves *via* deterrent secretions. Each of these dorsal glands is composed of several secretory cells which are attached to a large reservoir. The anti-predatory effect of the secretions can be attributed to salicylaldehyde synthesized within the reservoir by a few enzymatic reactions from the pre-toxin salicin, which is sequestered from the host plant [34,36]. Recent studies have identified *Cp*ABC35 (*Cp*MRP) which is essential for the sequestration of salicin [39]. It is associated with the accumulation of the plant-derived metabolite in intracellular storage vesicles. Intriguingly, *CpABC35* is the only predominant transcript in the defensive glands of *C. populi* (Figure 6). Its expression level lies far beyond all other ABC transporters in all tissues. There are four additional predicted ABCC proteins with high expression clustering to the human CFTR, SURs, 'long' MRPs and ABCC4, but not particularly to *Cp*ABC35 (Figure 4, S2). In *T. castaneum* another member of this group (not homologues to *Cp*ABC35) has been identified as playing a role in the production of secretions in odiferous stink glands (Figure 4) [126]. The silencing of TC015346/*Tc*ABCC-6A in *T. castaneum* resulted in a strong reduction of alkenes in the secretions produced by abdominal and prothoracic glands. Although a substrate for *Tc*ABCC-6A has not been described as yet, the hypothesis can be advanced that ABC transporters functioning in the formation of secretions seem to be a widespread phenomenon in insects.

Besides ABCC proteins, members of the subfamilies B, G and H also have elevated mRNA levels in the defensive glands. *Cp*ABC13 is a member of the B-subfamily exclusively expressed in the defensive glands. It clusters particularly with the human mitochondrial ABCB8 (Figure 3). ABCB8 is known to be responsible for iron transport and doxorubicin resistance in melanoma cells *via* the protection of mitochondrial DNA from doxorubicin-induced DNA damage [127].

Among the five candidates of the ABCG also possessing a high mRNA level in the defensive glands, *Cp*ABC56, 59 and 61 are expressed only in this tissue. *Cp*ABC59 clusters to the human ABCG5:ABCG8 that pump cholesterol and other sterol derivatives, and all of the four other proteins

cluster to human ABCG1 and 4, which may have a broader substrate spectrum including xenobiotics (Figure 5).

Remarkably, the expression of putative ABCH genes (*Cpabc64, Cpabc65)* was almost 3 times higher in the glandular tissue compared to the fat body tissue. Owing to this, the two ABCH proteins may have a special function as yet unknown in the defensive glands, but they may also be associated with the formation of the cuticle reservoir for storage of secretions. Furthermore, in the defensive glands there are also ABC candidates potentially associated with the translocation of phytochemicals or other xenobiotics.

RNAi with predominant ABC transporter – *Cpabc35* (*Cpmrp*)

Conspicuously, only one ABC gene, namely *Cpabc35,* displays an exceedingly high transcript level in the defensive glands of *C. populi*. As recently described [39], its function and key role in the sequestration of defensive compound precursors has been demonstrated. In order to test cooperative or compensation effects of other ABC genes, we performed RNAi silencing experiments for *Cpabc35*. Ten days after the injection of *Cpabc35*-dsRNA and *gfp*-dsRNA, glandular tissues were dissected and two biological replicates for each treatment were sequenced. The normalized counts of all transcripts of all samples were calculated. Thereafter, the $\log_2$ fold-changes of the silenced ABC transporter (*gfp*-injected samples as control) and adjusted p-values were determined using the DESeq package. In all samples (either in RNA-seq or quantitative real-time PCR experiments), we observed varying transcript levels corresponding to the individual biological variance and diversity despite similar developmental stage or living conditions during sample preparation.

The silencing of *Cpabc35* resulted in a significant decrease of its own transcript level (adjusted p-value (padj)=7.31E-15). One additional ABC transporter, *Cpabc50*, belonging to subfamily G, was determined as differentially expressed (slight upregulation). In non-treated larvae, *Cp*ABC50 is expressed only in the gut tissue (Figure 6). It clusters together with the human ABCG1 and ABCG4 (Figure 5). However, *Cpabc50* could not compensate the function of the salicin translocation into storage vesicles, and, hence, its function remains unclear. Overall, *Cpabc35* is an exclusive and highly specific transporter

used in the sequestration process, which explains its extraordinarily high transcript level in the defensive glands.

Conclusion

Phytophagous beetles are adapted to cope with the chemical defense of their host plant. The larvae of the poplar leaf beetle, *C. populi*, evolved the ability to sequester the plant-derived compound salicin and to use it for their own defense against their enemies. The sequestration process proceeds *via* barriers with different selectivity. While the uptake from the gut lumen into the hemolymph together with the excretion by Malpighian tubules is non-selective, the translocation into the defensive glands is selective. In these glands two barriers must be passed: a selective membrane on the hemolymph side and a non-selective membrane on the side towards the cuticle reservoir containing the defensive secretions. Based on our analyses, we predicted specific ABC proteins that are related to the translocation of plant-derived compounds in the larvae. In the gut of *C. populi*, genes of the subfamilies A, B, C and G are predominantly expressed. Almost all of these ABC candidates have been linked in our phylogenetic trees with proteins known to be associated with xenobiotic or drug resistance and which may, therefore, contribute to the non-selective translocation into the larval hemocoel. But depending on the localization of the proteins in the intestinal cells, they may also take part in the detoxification of plant metabolites or pesticides by back-exporting them into the gut lumen. The Malpighian tubules are dominated by candidates of subfamilies B, C and G. In particular, members of the multidrug-related ABCC-group are present in great numbers in this tissue, which suggests a role in the previously observed non-selective phytochemical extrusion in the excretion system. In the defensive glands the salicin-transporting ABCC protein *Cp*ABC35 (*Cp*MRP) is extraordinarily highly expressed in comparison to the other tested tissues. It is localized intracellularly in storage compartments of the gland cells and accumulates salicin in these vesicles for further exocytosis into the glandular reservoir. *Cp*ABC35 has a broad substrate spectrum of phytochemicals and controls the non-selective barrier into the reservoir. The differential expression analysis of *Cp*ABC35-silenced defensive glands in comparison to control samples corroborated the observation that the function cannot be

compensated by any other ABC transporter with overlapping substrate selectivity in this particular compartment of the glandular cells. The occurrence of other drug-resistant related ABC transporters in the defensive glands may contribute to the selectivity in the membrane of the hemolymph side of the glandular cells by extruding unused plant-derived compounds from these cells. Thus, ABC transporters are key components in the homeostasis control of phytochemicals in the sequestering poplar leaf beetle larvae.

Acknowledgments

We sincerely thank Angelika Berg for technical assistance. The authors would like to express their gratitude to Prof. Dr. Hans Merzendorfer from the University of Osnabrück, Germany for providing ABC protein sequences from *Tribolium castaneum*. Furthermore, we wish to thank Melinda Palmer Kolb for critical reading of the manuscript. This work has been supported by the Deutsche Forschungsgemeinschaft (BU 1862/2-1) and the Max Planck Society.

Author contributions

A.B., A.S.S., and M.S. designed the experiments, interpreted the results and wrote the manuscript. A.S.S. performed the quantitative real-time PCR experiments, interpreted the results and performed the RNAi experiment. D.W. performed the identification of ABC transporters and phylogenetic analyses. M.S. *de novo* assembled the transcriptome and performed and interpreted the differential expression analysis. D.W., M.S. and A.B. interpreted the phylogenetic trees. M.G. carried out the cDNA library preparation and Illumina sequencing. R.R.G. dissected the larvae and prepared the various tissues for RNA sequencing. A.B., A.S.S., M.S., and W.B. supervised the work, and all authors revised the manuscript.

Figures

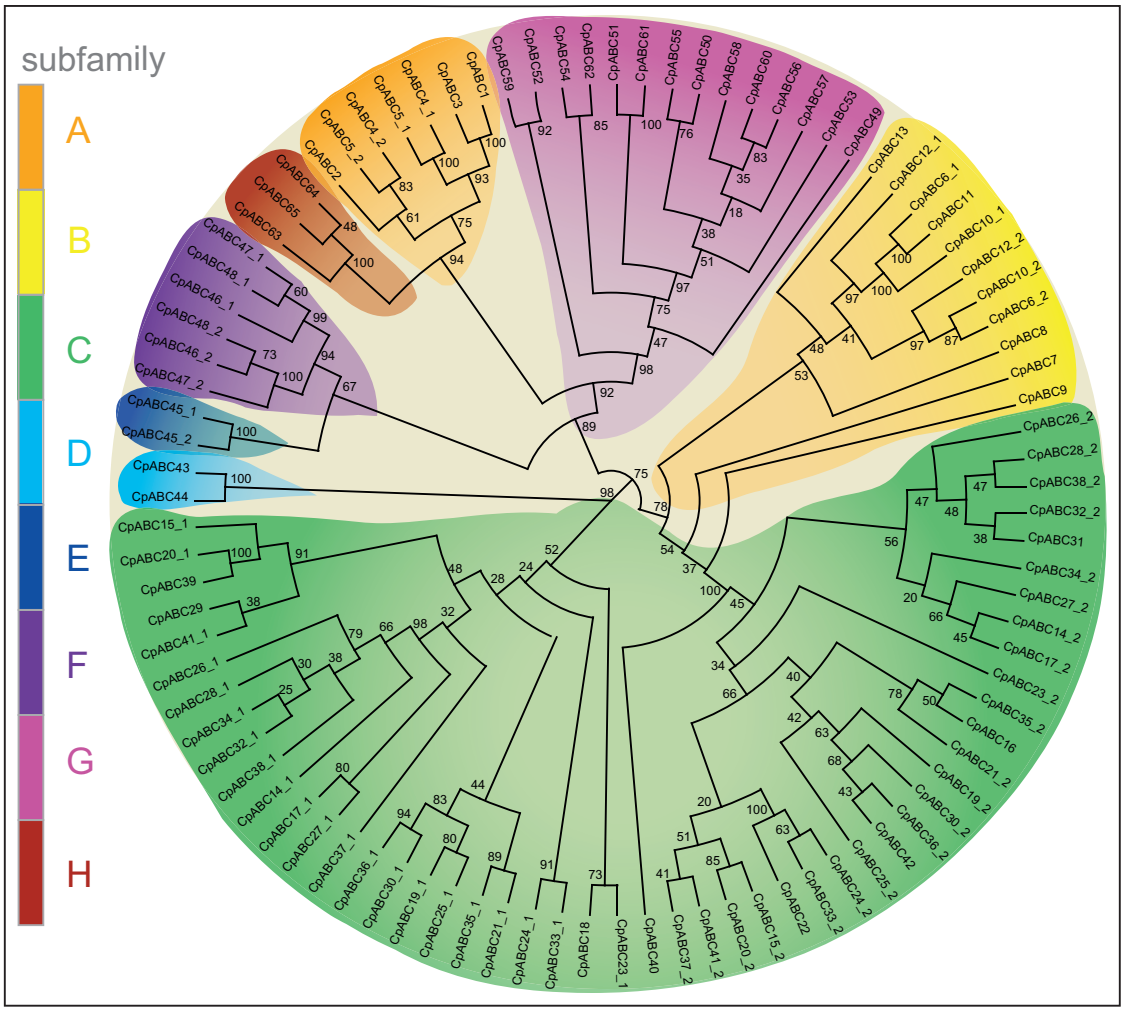


Figure 1: Eight subfamilies of 65 putative ABC transporters of *C. populi*. Some transporters contain two NBDs (NBD1 as *Cp*-ABCX_1 and NBD2 as *Cp*-ABCX_2), others contain only one NBD. Numbers at nodes represent bootstrap values. ABC gene subfamilies of *C. populi* are color-coded.

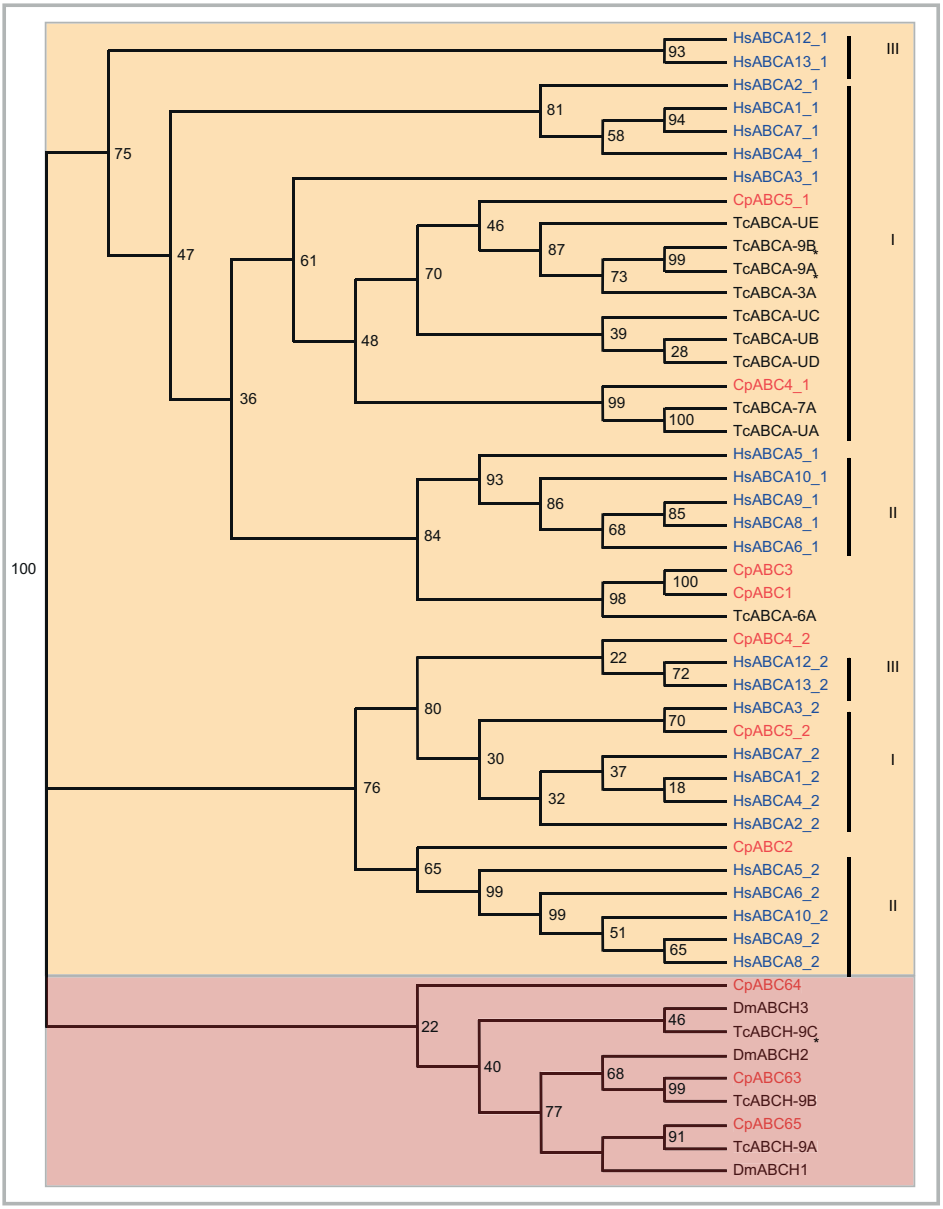


Figure 2: Phylogenetic tree of subfamilies ABCA and ABCH. Some transporters contain two NBDs (NBD1 as *Cp*-ABCX_1 and NBD2 as *Cp*-ABCX_2), others contain only one NBD. Red, *C. populi* (Cp); blue, *H. sapiens* (Hs); black, *T. castaneum* (Tc), *D. melanogaster* (Dm). *, *T. castaneum* with phenotype after RNAi. Numbers at nodes represent bootstrap values.

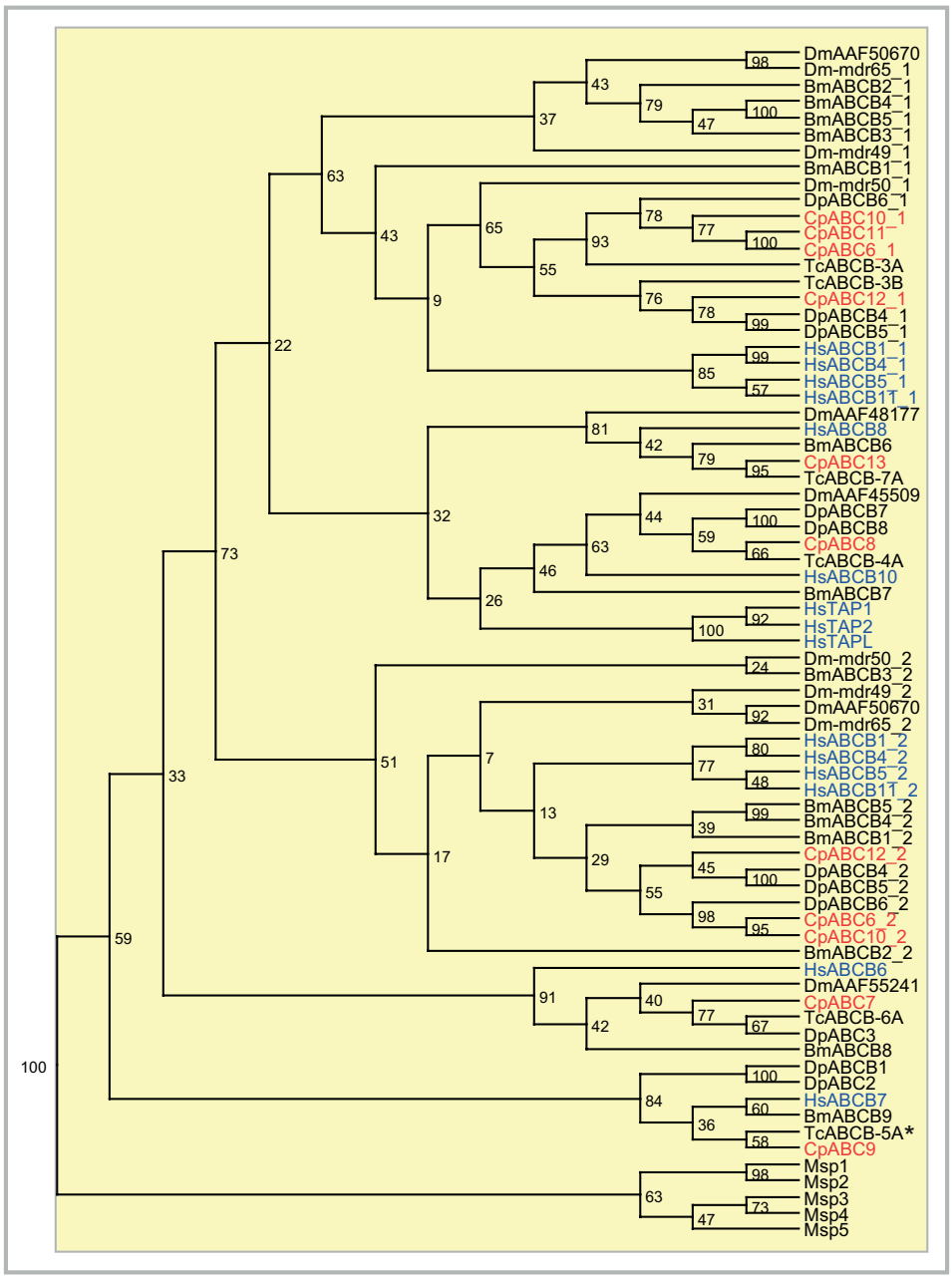


Figure 3: Phylogenetic tree of ABCB. Some proteins contain two NBDs (NBD1 as *Cp*-ABCX_1 and NBD2 as *Cp*-ABCX_2), others contain only one NBD. Red, *C. populi* (Cp); blue, *H. sapiens* (Hs); black, *T. castaneum* (Tc), *D. ponderosae* (Dp), *D. melanogaster* (Dm), *B. mori* (Bm); Microsporida (Msp). *, *T. castaneum* with phenotype after RNAi. Numbers at nodes represent bootstrap values.

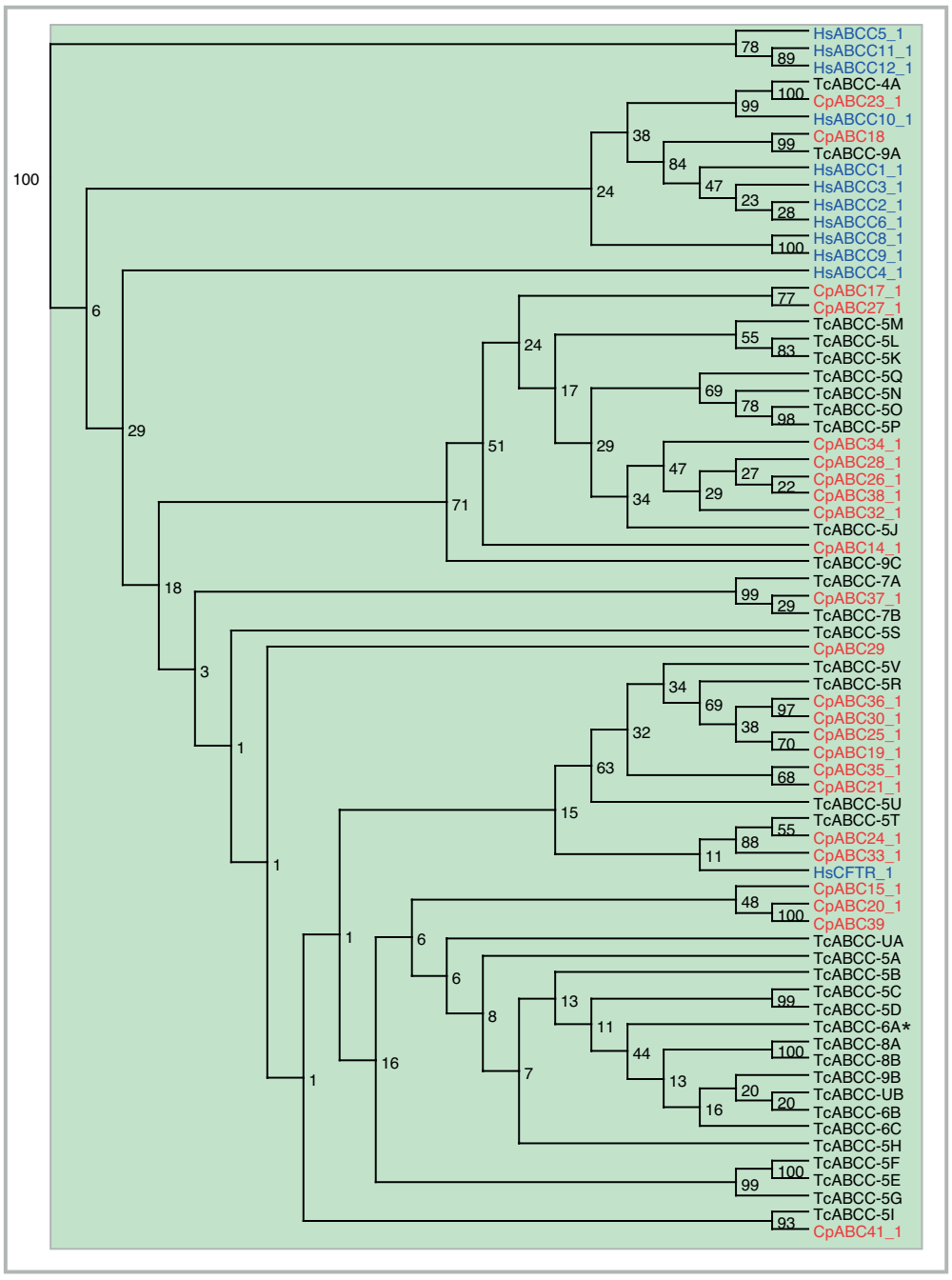


Figure 4: Phylogenetic tree of NBD1 from ABCC. Red, *C. populi* (Cp); blue, *H. sapiens* (Hs); black, *T. castaneum* (Tc). *, *T. castaneum* with phenotype after RNAi. Numbers at nodes represent bootstrap values.

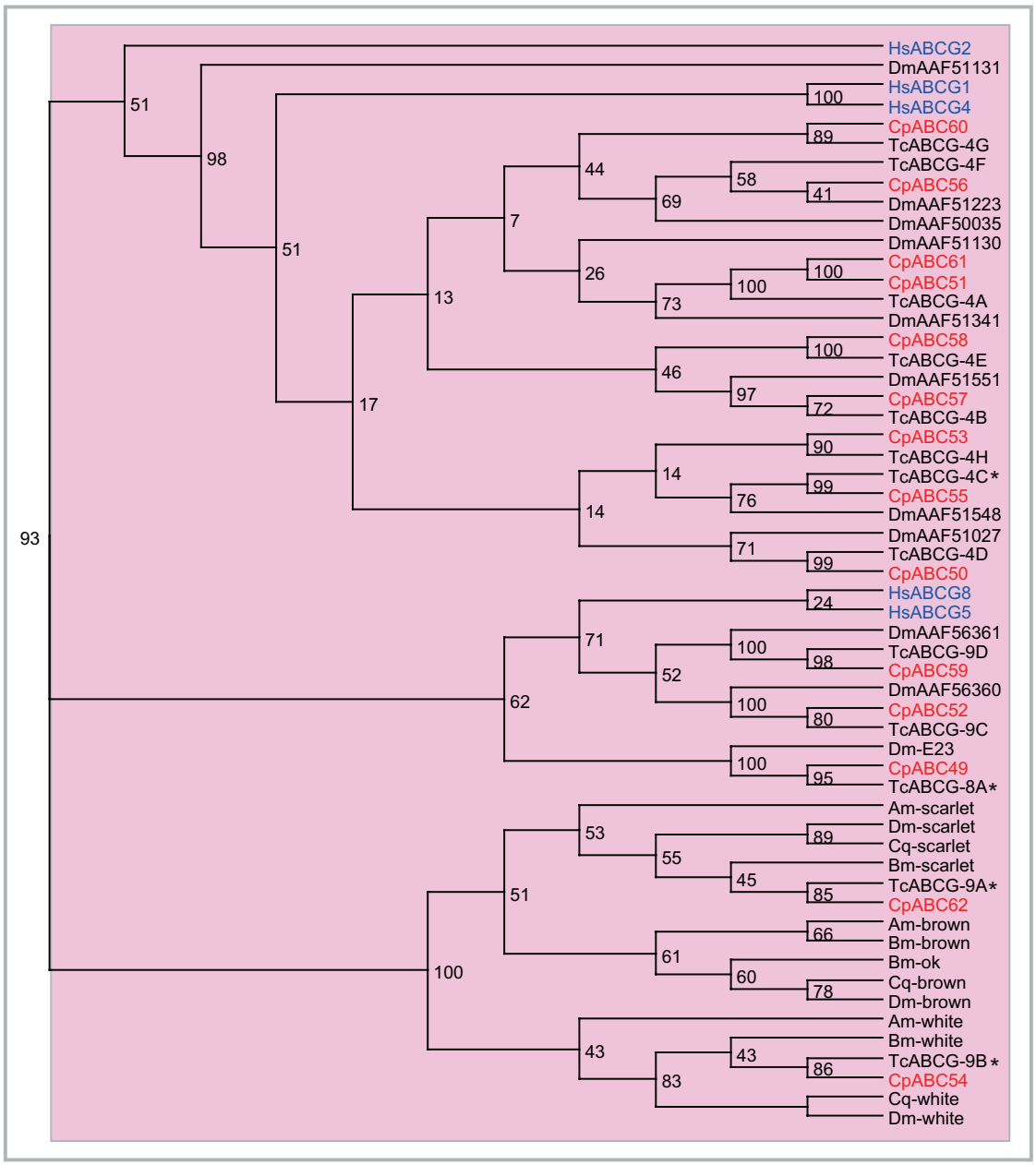


Figure 5: Phylogenetic tree of ABCG. Red, *C. populi* (Cp); blue, *H. sapiens* (Hs); black, *T. castaneum* (Tc), *D. melanogaster* (Dm), *B. mori* (Bm), *A. mellifera* (Ap), *C. quinquefasciatus* (Cq). *, *T. castaneum* with phenotype after RNAi. Numbers at nodes represent bootstrap values.

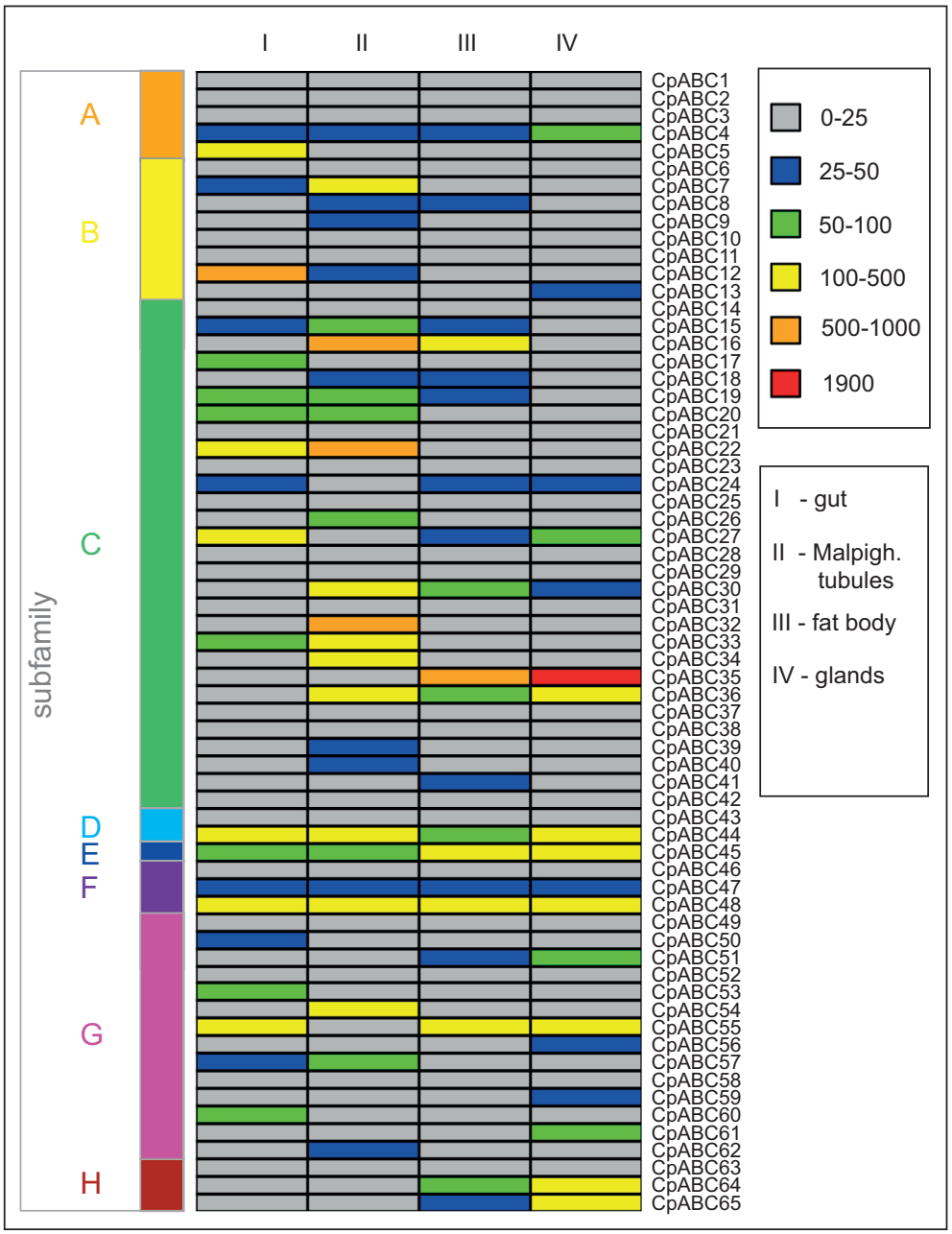


Figure 6: Heatmap of the expression profiles of the 65 putative ABC genes of *C. populi*. Values are shown for four different tissues: glands, gut, fat body and Malpighian tubules. Counts of RNA-seq reads (derived from three replicates for each tissue) normalized to the effective library size and to the length of the corresponding sequences (see Table S4 for data). Expression levels are illustrated by a six grade color

scale representing the sequence coverage for each transcript for each tissue, respectively. ABC gene subfamilies of *C. populi* are color-coded.

Tables

Table 1. Subfamilies of ABC genes in 8 species (Numbers were derived from [128]).

Species	ABCA	ABCB	ABCC	ABCD	ABCE	ABCF	ABCG	ABCH	total
S. cerevisiae	0	4	6	2	2	6	10	0	30
C. elegans	7	24	9	5	1	3	9	0	58
D. pulex	4	7	7	3	1	4	24	15	65
T. urticae	10	4	39	2	1	3	23	22	103
D. melanogaster	10	8	14	2	1	3	15	3	56
T. castaneum	10	6	35	2	1	3	13	3	73
C. populi	5	8	29	2	1	3	14	3	65
H. sapiens	12	11	12	4	1	3	5	0	48

Table 2. Distribution of the ABC transporter domains of *C. populi* in eight subfamilies. Full-trans, full transporters; Half-trans, half transporters; NBD, nucleotide-binding domain; TMD, transmembrane domain; 2*NBD+1*TMD, two NBDs and one TMD (example).

	Full-trans	**Half-trans**	**2*NBD**	**2*NBD+1*TMD**	**1*NBD**	**1*NBD+2*TMD**	**total**
ABCA	2	1	0	0	2	0	**5**
ABCB	3	5	0	0	0	0	**8**
ABCC	18	4	0	3	3	1	**29**
ABCD	0	2	0	0	0	0	**2**
ABCE	0	0	1	0	0	0	**1**
ABCF	0	0	3	0	0	0	**3**
ABCG	0	12	0	0	2	0	**14**
ABCH	0	3	0	0	0	0	**3**
total	23	27	4	3	7	1	**65**

Supporting Information

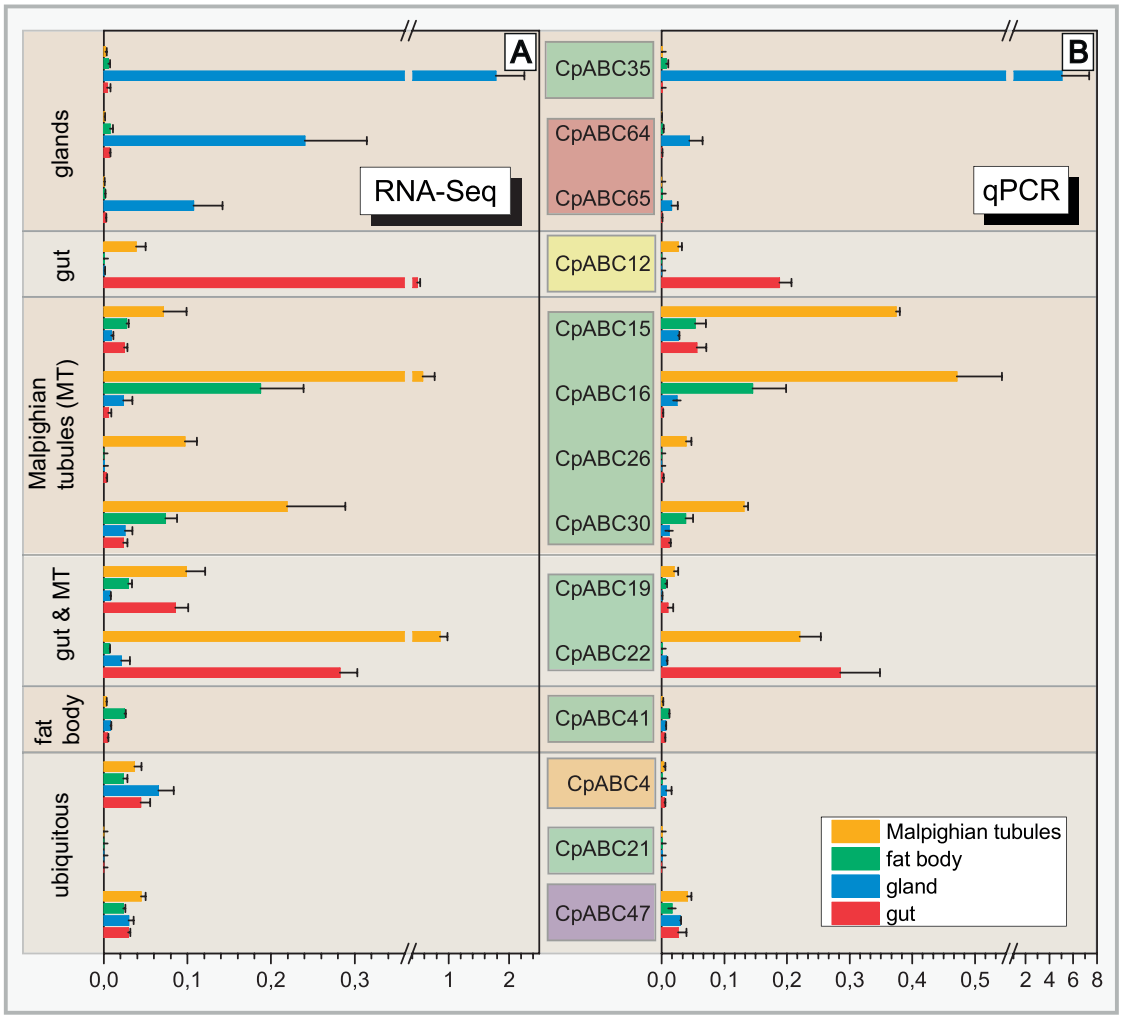


Figure S1: Relative mRNA levels of selected putative ABC transporters in the different tissues of juvenile *C. populi* determined by carrying out RNA-seq (A) and quantitative real-time PCR (B) experiments (n=3-4, mean ± SD). Green, fat body; blue, glandular tissue; red, Malpighian tubules. *Cp_EF1alpha* and *Cp_elF4a* were used for normalization of transcript quantities. ABC gene subfamilies of *C. populi* are color-coded and grouped by their tissue-specific expression level.

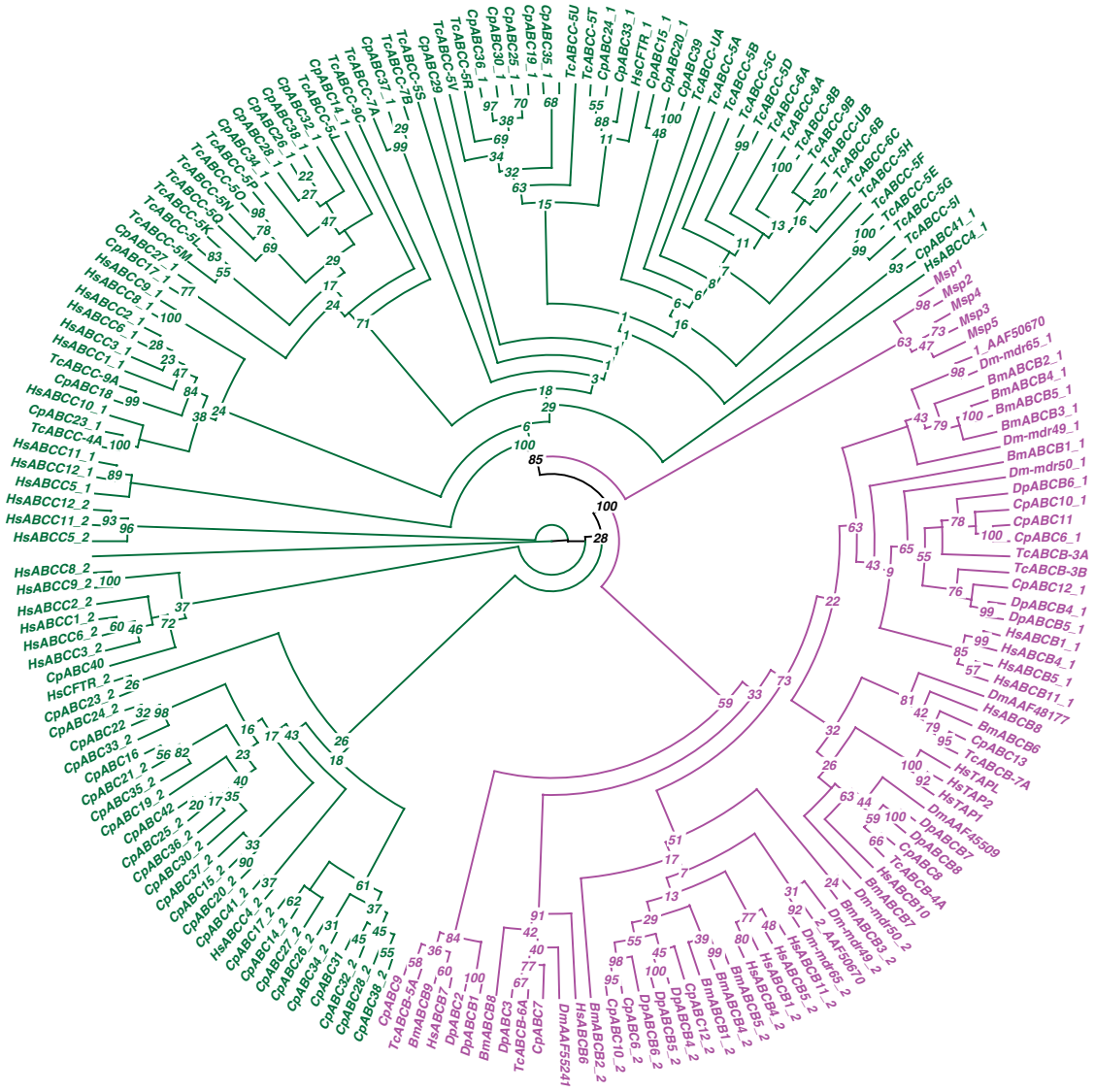


Figure S2: Phylogenetic tree of subfamilies ABCC and ABCB. Some transporters contain two NBDs (NBD1 as *Cp*ABCX_1 and NBD2 as *Cp*ABCX_2), others contain only one NBD. Numbers at nodes represent bootstrap values. *C. populi* (Cp); *H. sapiens* (Hs); *T. castaneum* (Tc)

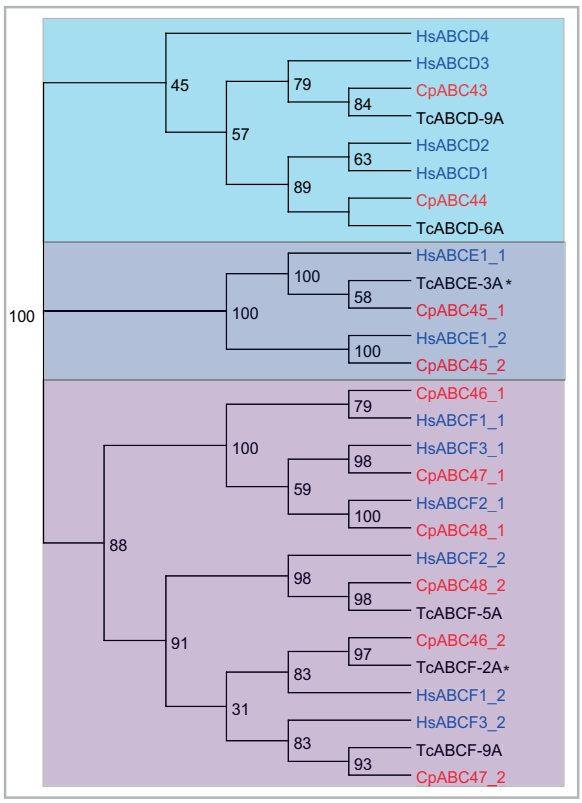


Figure S3: Phylogenetic tree of subfamilies E, F, and D. Red, *C. populi* (Cp); blue, *H. sapiens* (Hs); black, *T. castaneum* (Tc), *D. melanogaster* (Dm). *, *T. castaneum* with phenotype after RNAi. Numbers at nodes represent bootstrap values.

Table S1: *De novo* assembly of the transcript catalogue of *C. populi*. The numbers of assembled transcripts and average length after assembly and reassembly show the usefulness of reassembling.

	Number of transcripts	Sum_length (bp)	25th_pc	75th_pc	Ave_length
After Trinity assembly	74,146	73,793,128	395 bp	1135 bp	995 bp
After reassembly with TGICL	50,909	57,115,286	404 bp	1342 bp	1122 bp

Table S2: Accession numbers of sequences from different organisms added to *C. populi*'s chosen sequences to calculate phylogenetic trees.

ABC subfamily B			ABC subfamily C			ABC subfamily G		
Species	**Designation in Figure 3**	**Accession number**	**Species**	**Designation in Figure 4 and S...**	**Accession number**	**Species**	**Designation in Figure 5**	**Accession number**
Bombyx mori	BmABCB1	BGIBMGA007494-PA	Dendroctonus ponderosae	DpABCC1	gi|478260834|gb|ENN80486.1|	Bombyx mori	Bm-brown	AB780446
	BmABCB2	BGIBMGA009452-PA		DpABCC2	gi|478255664|gb|ENN75876.1|	Apis mellifera	Am-brown	XM_395665
	BmABCB3	BGIBMGA011228-PA		DpABCC3	gi|478257256|gb|ENN77419.1|	Culex quinquefasciatus	Cq-brown	XM_001862856
	BmABCB4	BGIBMGA000725-PA		DpABCC4	gi|478258345|gb|ENN78464.1|	Culex quinquefasciatus	Cq-scarlet	XP_001848966
	BmABCB5	BGIBMGA000724-PA		DpABCC5	gi|478259381|gb|ENN79278.1|	Bombyx mori	Bm-scarlet	BAK08373
	BmABCB6	BGIBMGA004142-PA		DpABCC6	gi|478260178|gb|ENN79951.1|	Apis mellifera	Am-scarlet	XP_001122240
	BmABCB7	BGIBMGA008523-PA		DpABCC7	gi|478257268|gb|ENN77431.1|	Bombyx mori	Bm-white	NP_001037034
	BmABCB8	BGIBMGA005473-PA		DpABCC8	gi|478254949|gb|ENN75182.1|	Apis mellifera	Am-white	XP_001122252
	BmABCB9	BGIBMGA012743-PA		DpABCC9	gi|478255001|gb|ENN75234.1|	Culex quinquefasciatus	Cq-white	XP_001847783
				DpABCC10	gi|478255597|gb|ENN75811.1|	Bombyx mori	Bm-ok	AB780440
Dendroctonus ponderosae	DpABCB1	DpKB741248		DpABCC11	gi|478256277|gb|ENN76467.1|			
	DpABCB2	DpKB632305		DpABCC12	gi|546684944|gb|ERL94526.1|	Drosophila melanogaster	Dm-white	gi|7290367|gb|AAF45826.1|
	DpABCB3	DpKB632394		DpABCC13	gi|478258158|gb|ENN78296.1|		DmAAF51548	gi|22945593|gb|AAF51548.2|
	DpABCB4	KB632353.1		DpABCC14	gi|478254889|gb|ENN75124.1|		DmAAF51551	gi|22945591|gb|AAF51551.2|
	DpABCB5	KB632345.1		DpABCC15	gi|478261024|gb|ENN80604.1|		DmAAF51341	gi|22945472|gb|AAF51341.3|
	DpABCB6	ERL93339.1		DpABCC16	gi|478254890|gb|ENN75125.1|		DmAAF51223	gi|7295924|gb|AAF51223.1|
	DpABCB7	BT126885.1		DpABCC17	gi|478257257|gb|ENN77420.1|		DmAAF51131	gi|7295830|gb|AAF51131.1|
	DpABCB8	BT126885.1		DpABCC18	gi|478263248|gb|ENN81636.1|		DmAAF51130	gi|28380274|gb|AAF51130.2|
							Dm-E23	gi|22945319|gb|AAF51122.2|
Drosophila melanogaster	DmAAF45509	gi|22831418|gb|AAF45509.2|	Bombyx mori	BmABCC1	BGIBMGA007735		DmAAF51027	gi|7295722|gb|AAF51027.1|
	DmAAF48177	gi|7292782|gb|AAF48177.1|		BmABCC2	BGIBMGA010332		Dm-brown	gi|21626629|gb|AAF47020.3|
	DmAAF53736	gi|44021390|gb|AAF53736.4|		BmABCC3	BGIBMGA010331		DmAAF50035	gi|23093633|gb|AAF50035.2|
	Dm-mdr49	gi|21627300|gb|AAF58437.2|		BmABCC4	BGIBMGA007793		Dm-scarlet	gi|7294101|gb|AAF49455.1|
	Dm-mdr50	gi|84795750|gb|AAF58271.3|		BmABCC5	BGIBMGA007738		DmAAF56360	gi|23172222|gb|AAF56360.2|

	DmAAF50670	gi|7295352|gb|AAF50670.1|		BmABCC6	BGIBMGA007785		DmAAF56361	gi|28381456|gb|AAF56361.2|
	Dm-mdr65	gi|7295351|gb|AAF50669.1|		BmABCC7	BGIBMGA007769			
	DmAAF55241	gi|7300071|gb|AAF55241.1|		BmABCC8	BGIBMGA003359			
				BmABCC9	BGIBMGA006882			
Mircrosporidia	Msp1			BmABCC10	BGIBMGA010636			
	Msp2			BmABCC11	BGIBMGA011220			
	Msp3			BmABCC12	BGIBMGA007784			
	Msp4			BmABCC13	BGIBMGA007792			
	Msp5			BmABCC14	BGIBMGA010849			
				BmABCC15	BGIBMGA010330			
			Drosophila melanogaster	DmAAF52866				
				DmAAN11020				
				DmAAF53736				
				DmAAS64733				
				DmAAF54656				
				DmAAF55707				
				DmAAF56869				
				DmAAF56870				
				DmAAF52639				
				DmAAF52648				
				DmAAF56312				
				DmAAF46706				
				DmAAF58947				
				DmAAF53223				

Table S3: Overview of the raw sequence data. The table exhibits the RNA derived specimens, number of reads, sequencing technology and sequencing mode.

cDNA library	Tissues for RNA isolation	Number of reads/ biological replicate	Sequencing technology
1	Tissue pool of different developmental stages	42,189,743	Solexa/Ilumina, 2x150 bp
2	Glands	35,246,691	Solexa/Ilumina, 50 bp
3		42,555,962	Solexa/Ilumina, 50 bp
4		25,106,778	Solexa/Ilumina, 50 bp
5	Gut	34,052,388	Solexa/Ilumina, 50 bp
6		40,334,391	Solexa/Ilumina, 50 bp
7		33,926,226	Solexa/Ilumina, 50 bp
8	Fat body	41,975,356	Solexa/Ilumina, 50 bp
9		45,112,201	Solexa/Ilumina, 50 bp
10		37,115,218	Solexa/Ilumina, 50 bp
11	Malphighian tubules	29,021,078	Solexa/Ilumina, 50 bp
12		40,112,105	Solexa/Ilumina, 50 bp
13		26,325,725	Solexa/Ilumina, 50 bp

Table S4: Sequence length of cDNAs encoding putative ABC transporters of *C. populi* and their corresponding read counts normalized to the effective library size as well as to the sequence length of all ABC transporters in the different larval tissues.

ABC subfam	*Chrysomela populi* Designation	Seq length in bp	Read counts normalized to the effective library size and the sequence length			
			Gut Mean Library 1-3	Malp. tubules Mean Library 4-6	Fat body Mean Library 7-9	Defensive glands Mean Library 10-12
ABCA	CpABC1	2778	0.364963201	0.197865422	0.141729434	0.858839293
ABCA	CpABC2	1884	13.96421746	10.91591836	11.32711411	12.21142278
ABCA	CpABC3	1952	0.158895158	0.056927994	0.10877338	0.32106971
ABCA	CpABC4	5775	46.85602314	35.89997028	43.51777331	69.72083745
ABCA	CpABC5	5884	278.4111328	21.79583868	13.16775607	24.24162707
ABCB	CpABC6	3804	0.022744891	0.003172382	1.055713215	0.137482974
ABCB	CpABC7	3832	37.98062474	105.3099408	19.22622997	21.21759436
ABCB	CpABC8	3704	22.64886986	48.89758099	25.42144828	22.33429043
ABCB	CpABC9	2344	14.57715035	35.02029527	22.46596801	20.8375818
ABCB	CpABC10	4411	2.875582137	19.89455623	17.51641326	2.497495317
ABCB	CpABC11	1690	0	0.007140675	0.580947155	0.080856425
ABCB	CpABC12	4240	525.3310804	38.32968794	0.700633932	1.081320292
ABCB	CpABC13	2400	15.81898377	18.37671042	18.70847331	25.65826611
ABCC	CpABC14	3028	0.112577015	13.08389334	1.287650348	0.11288119
ABCC	CpABC15	4433	26.60670457	71.68215927	26.72130465	9.558405838
ABCC	CpABC16	1031	6.275904232	568.4390736	184.0106287	24.59245679
ABCC	CpABC17	4078	85.44885056	0.033241352	0.150312152	0.179339223
ABCC	CpABC18	1843	18.7171828	45.45359843	25.69421545	21.82981758
ABCC	CpABC19	5026	94.09610638	98.17164525	30.33988767	7.874259841
ABCC	CpABC20	3305	53.67613806	83.56449006	7.285587551	5.581830293
ABCC	CpABC21	3466	0.038134177	0.016578986	0.103374253	0.040630059
ABCC	CpABC22	2421	309.9620118	853.4576221	12.39840299	21.72877345
ABCC	CpABC23	2907	14.10324861	13.66841059	6.094593778	6.956507072
ABCC	CpABC24	4614	27.07206325	22.50564549	28.68014681	29.2251931
ABCC	CpABC25	4098	0.064818043	1.91428573	0.00641891	0.011238864
ABCC	CpABC26	3832	3.196213673	96.07859274	0.107831175	0.285352127
ABCC	CpABC27	4414	211.5908346	5.129055703	39.51749618	50.23254453
ABCC	CpABC28	3620	0.025574919	0.065541449	0.101916208	0.047557395
ABCC	CpABC29	2677	0.066426802	0.090023406	0.42791625	1.055093431
ABCC	CpABC30	4385	24.70221461	217.8064156	80.18359327	26.72846951
ABCC	CpABC31	624	0	0.026704555	0.042154957	0.129350016
ABCC	CpABC32	4348	2.497213424	622.6867372	2.514387991	2.391857505
ABCC	CpABC33	5335	55.06044015	104.4150169	9.913166999	3.341841951
ABCC	CpABC34	4153	1.923523147	332.4055068	1.566539975	2.3233474
ABCC	CpABC35	4413	4.157223917	2.001115527	747.9684117	1901.886778
ABCC	CpABC36	3155	4.962055228	281.4771643	57.08562707	235.7514441
ABCC	CpABC37	4559	14.62651294	4.532187319	7.576570976	11.85851161
ABCC	CpABC38	4597	2.881540963	0.45162276	6.690344417	7.279664956
ABCC	CpABC39	2761	20.61780093	30.54894591	3.169920954	3.329220175
ABCC	CpABC40	1189	11.73779951	30.99928636	15.27567815	15.93779934

ABCC	CpABC41	4018	4.784179281	3.122187059	26.24342836	8.435712489
ABCC	CpABC42	2690	0.644785462	0.644871334	0.576353747	0.914618143
ABCD	CpABC43	2313	18.84833901	11.91046796	11.62784212	9.561143656
ABCD	CpABC44	1421	213.7650464	136.5136296	75.79983819	101.3425752
ABCE	CpABC45	2297	86.09082061	99.71627451	104.5692972	105.2432906
ABCF	CpABC46	3424	15.58232954	11.76740442	19.36333005	12.61467338
ABCF	CpABC47	2622	32.09421647	44.00514541	31.05166075	31.68853737
ABCF	CpABC48	2745	121.7057729	156.4191054	153.7906243	187.0810626
ABCG	CpABC49	3877	1.830892062	10.83204643	1.494416214	1.13606632
ABCG	CpABC50	3187	44.62199545	21.82367515	23.46336684	8.165322842
ABCG	CpABC51	3145	5.404789968	7.05557606	27.04726671	85.0424023
ABCG	CpABC52	3197	4.250303198	1.680708833	7.919395155	17.23041757
ABCG	CpABC53	3056	81.24445215	16.06796279	5.474489525	7.536245401
ABCG	CpABC54	1066	1.050823612	140.6237036	1.530135573	0.916822168
ABCG	CpABC55	2408	158.481334	16.25806384	103.7668216	143.173754
ABCG	CpABC56	2665	2.117370686	0.147759293	16.47125373	37.44537843
ABCG	CpABC57	2536	40.52733632	57.49411911	11.74539288	19.84957537
ABCG	CpABC58	2435	0.224692838	0.061862116	3.773979945	10.51598741
ABCG	CpABC59	1248	5.520873033	0.332852745	13.50590119	36.7741582
ABCG	CpABC60	2325	97.522213	1.411815889	10.52711251	10.30287674
ABCG	CpABC61	2474	4.389695347	6.588136099	22.7918988	72.58362915
ABCG	CpABC62	2265	0.726590004	43.81470262	0.066834109	0.168981859
ABCH	CpABC63	3008	1.462968954	1.020727934	3.903558146	16.09716546
ABCH	CpABC64	3508	7.192649862	1.184774309	82.85623211	254.9457149
ABCH	CpABC65	2631	2.734117225	0.624271425	40.95984893	113.6678642

Table S5: Primer sets used in quantitative real-time PCR and RNAi experiments.

Gene name	Primer name	Primer for ds RNA generation
cpmrp GenBank: KC112554	fwd	GATTAATACGACTCACTATAGGCGACTAAGTGTGAACTAGTCGGTGC
	rev	GATTAATACGACTCACTATAGGGAGACTTGTCTCCACAGCAGATAG
gfp UniProtKB: P42212.1	fwd	TAATACGACTCACTATAGGGAGATGGCTAGTAAGGGA
	rev	TAATACGACTCACTATAGGGAGATTATTTGTAGAGTTC
		qPCR Primer
Cp_EF1α GenBank:	fwd	TGGGTACTCGACAAACTGAAGG
	rev	TGATGAAATCCCTGTGTCCAG
Cp_eIF4a GenBank:	fwd	GTCGCGTGTACGACATGATAAC
	rev	CTTGAAGACGTCATGGATCTGG
Cp ABC4	fwd	CAAGGACATGCAGAACGAGA
	rev	TAGCCCCCGTACTCCTTTTT
Cp ABC15	fwd	TGG GTC TAC TGT TAT AGG
	rev	TAC TAT CGG CTA GAG TTC

Cp ABC20	fwd	GACGAATATAAGGATGAGACATTG
	rev	GCGTACTATGGCTCGAGC
Cp ABC21	fwd	GGAATTCGAGGACCAGTTGC
	rev	GGCATATCAACTGTCGTTGTC
Cp ABC24	fwd	CACTTCACTAGTTTAGGC
	rev	GAGCTTCCTGTAGATAAG
Cp ABC26	fwd	CCATCTGGCAAATTTGAAGATTTC
	rev	CAGTAACTACGAAGTCTAGAGAG
Cp ABC27	fwd	ATACTACGATGAGCACAG
	rev	TAGGAGGTATAGATGAGTG
Cp ABC31	fwd	CAAGTACTCCTACTAGCC
	rev	GTGTACTCCAGTAACCTC
Cp ABC35	fwd	AGCCGATGATGCAACCATC
	rev	CCAACTGTCTTTGTCCAGC
Cp ABC46	fwd	GAATATCAGATGCCGACCTG
	rev	GGATGGCTCTGGCAAGG
Cp ABC57	fwd	GTGCCGACTAATTTTACATC
	rev	TTGGCATTGAACCTGAAGC
Cp ABC76	fwd	GGCTACTACTTCATTGAG
	rev	GATGAAACCGTAGTAGAC
Cp ABC77	fwd	CACTACATAGAGGAATGTC
	rev	GTCAGTAATCTATCAGGAG

Table S6: Predicted domain distribution in the deduced protein sequences of all identified ABC transporters of *C. populi*.

	Chrysomela populi	**Number of domains in the deduced protein sequences**	
ABC Subfamily	**Designation**	**Nucleotid binding domains**	**Transmembrane domains**
ABCA	CpABC1	1	1
ABCA	CpABC2	1	0
ABCA	CpABC3	1	0
ABCA	CpABC4	2	2
ABCA	CpABC5	2	2
ABCB	CpABC6	2	2
ABCB	CpABC7	1	1
ABCB	CpABC8	1	1
ABCB	CpABC9	1	1
ABCB	CpABC10	2	2
ABCB	CpABC11	1	1
ABCB	CpABC12	2	2
ABCB	CpABC13	1	1
ABCC	CpABC14	2	2

ABCC	CpABC15	2	2
ABCC	CpABC16	1	0
ABCC	CpABC17	2	2
ABCC	CpABC18	1	1
ABCC	CpABC19	2	2
ABCC	CpABC20	2	2
ABCC	CpABC21	2	2
ABCC	CpABC22	1	1
ABCC	CpABC23	2	1
ABCC	CpABC24	2	1
ABCC	CpABC25	2	2
ABCC	CpABC26	2	2
ABCC	CpABC27	2	2
ABCC	CpABC28	2	2
ABCC	CpABC29	1	1
ABCC	CpABC30	2	2
ABCC	CpABC31	1	0
ABCC	CpABC32	2	2
ABCC	CpABC33	2	2
ABCC	CpABC34	2	2
ABCC	CpABC35	2	2
ABCC	CpABC36	2	1
ABCC	CpABC37	2	2
ABCC	CpABC38	2	2
ABCC	CpABC39	1	2
ABCC	CpABC40	1	0
ABCC	CpABC41	2	2
ABCC	CpABC42	1	1
ABCD	CpABC43	1	1
ABCD	CpABC44	1	1
ABCE	CpABC45	2	0
ABCF	CpABC46	2	0
ABCF	CpABC47	2	0
ABCF	CpABC48	2	0
ABCG	CpABC49	1	1
ABCG	CpABC50	1	1
ABCG	CpABC51	1	1
ABCG	CpABC52	1	1
ABCG	CpABC53	1	1
ABCG	CpABC54	1	0
ABCG	CpABC55	1	1
ABCG	CpABC56	1	1
ABCG	CpABC57	1	1
ABCG	CpABC58	1	1
ABCG	CpABC59	1	0
ABCG	CpABC60	1	1
ABCG	CpABC61	1	1
ABCG	CpABC62	1	1
ABCH	CpABC63	1	1
ABCH	CpABC64	1	1
ABCH	CpABC65	1	1

Table S7: Standardized values of RNA-seq data for comparable analysis of relative mRNA levels of putative ABC transporters in the different tissues of juvenile *C. populi*

subfamily		designation	length	daNorm-mitwert	drNorm-mitwert	fkNorm-mitwert	mpgNorm-mitwert
ABCA	CL58Contig2	CpABC1	2778	0.000339725	0.000796581	0.000100637	0.000201364
ABCA	CL8853Contig1	CpABC2	1884	0.01281027	0.01143838	0.00629388	0.01096335
ABCA	comp19862_c0_seq4	CpABC3	1952	0.000146413	0.000301204	3.16396E-05	5.74705E-05
ABCA	comp2504_c0_seq1	CpABC4	5775	0.04379806	0.0655009	0.0232536	0.03671843
ABCA	comp3378_c0_seq1	CpABC5	5884	0.2564767	0.02275788	0.004588867	0.02179625
ABCB	CL1423Contig1	CpABC6	3804	2.17726E-05	0.000129396	0.001059889	3.26986E-06
ABCB	CL2069Contig1	CpABC7	3832	0.03498858	0.01971353	0.01391713	0.1064578
ABCB	CL4892Contig1	CpABC8	3704	0.02091637	0.02077614	0.01927772	0.0492828
ABCB	CL652Contig1	CpABC9	2344	0.01332257	0.01937934	0.01664853	0.03522789
ABCB	CL735Contig1	CpABC10	4411	0.002605885	0.002283927	0.01394122	0.019931
ABCB	comp16325_c0_seq4	CpABC11	1690	0	7.61604E-05	0.000689655	7.36008E-06
ABCB	comp2567_c0_seq1	CpABC12	4240	0.4787217	0.000994146	0.00030019	0.03870405
ABCB	comp6084_c0_seq1	CpABC13	2400	0.01480838	0.02373972	0.0118018	0.01862267
ABCC	CL1556Contig1	CpABC14	3028	0.000101566	0.000107281	0.001250588	0.01315952
ABCC	CL1879Contig2	CpABC15	4433	0.02401064	0.009005777	0.02699478	0.07137637
ABCC	CL2102Contig1	CpABC16	1031	0.005472261	0.02346561	0.1873502	0.5730379
ABCC	CL2787Contig1	CpABC17	4078	0.07806521	0.000169026	6.59104E-05	3.53542E-05
ABCC	CL414Contig1	CpABC18	1843	0.01721593	0.01993184	0.01661352	0.04606902
ABCC	CL4549Contig1	CpABC19	5026	0.08578398	0.007327033	0.02912448	0.09829925
ABCC	CL621Contig1	CpABC20	3305	0.04936734	0.005232447	0.005868195	0.08420937
ABCC	CL6551Contig1	CpABC21	3466	3.66321E-05	3.77285E-05	9.90007E-05	0.000016152
ABCC	CL79Contig2	CpABC22	2421	0.2823005	0.02084096	0.006678706	0.8599617
ABCC	CL82Contig1	CpABC23	2907	0.01301352	0.006499324	0.004530207	0.01384406
ABCC	CL862Contig1	CpABC24	4614	0.02477647	0.02695525	0.02173066	0.02282663
ABCC	CL947Contig1	CpABC25	4098	6.08005E-05	1.09867E-05	0	0.001945379
ABCC	CL9675Contig1	CpABC26	3832	0.002964341	0.000288156	0.000105813	0.09699
ABCC	comp2642_c0_seq1	CpABC27	4414	0.1929992	0.04695229	0.02512859	0.005190154
ABCC	comp26598_c0_seq1	CpABC28	3620	2.30702E-05	4.18864E-05	0.000074566	6.47501E-05
ABCC	comp28697_c0_seq1	CpABC29	2677	6.39187E-05	0.000995202	7.76023E-05	9.06971E-05
ABCC	comp2929_c0_seq1	CpABC30	4385	0.02301327	0.0252689	0.07379491	0.2190657
ABCC	comp31997_c0_seq1	CpABC31	624	0	0.000117334	4.01472E-05	2.49244E-05
ABCC	comp3219_c0_seq1	CpABC32	4348	0.002233387	0.002455065	0.002102936	0.6290504
ABCC	comp3585_c0_seq1	CpABC33	5335	0.04970086	0.003116531	0.009227072	0.1056439
ABCC	comp4436_c0_seq1	CpABC34	4153	0.00171524	0.00230048	0.001276748	0.3368864
ABCC	comp5445_c0_seq1	CpABC35	4413	0.004106028	1.783494	0.005504719	0.002011229
ABCC	comp5554_c0_seq1	CpABC36	3155	0.004534622	0.2185418	0.001888176	0.2825966
ABCC	comp6005_c0_seq1	CpABC37	4559	0.01314329	0.01104639	0.003775494	0.004609019
ABCC	comp6429_c0_seq1	CpABC38	4597	0.002594283	0.00688041	0.004295215	0.000444854
ABCC	comp6704_c2_seq3	CpABC39	2761	0.01899929	0.0031344	0.002370963	0.03084387
ABCC	comp7503_c0_seq1	CpABC40	1189	0.01068827	0.01463862	0.009052064	0.03140363
ABCC	comp7689_c0_seq1	CpABC41	4018	0.004466044	0.007844111	0.02474272	0.003133248
ABCC	comp8402_c0_seq1	CpABC42	2690	0.000597978	0.000865588	0.000182574	0.000613251

ABCD	CL5636Contig1	CpABC43	2313	0.01741596	0.008942632	0.008772579	0.01202575
ABCD	CL672Contig1	CpABC44	1421	0.1939907	0.09678381	0.04509589	0.1364923
ABCE	comp973_c1_seq1	CpABC45	2297	0.07873729	0.09748655	0.07643521	0.1011434
ABCF	comp2012_c1_seq1	CpABC46	3424	0.01430881	0.01172293	0.0174971	0.01198728
ABCF	comp4930_c0_seq1	CpABC47	2622	0.02937153	0.02962993	0.02323234	0.0447192
ABCF	comp644_c1_seq1	CpABC48	2745	0.1109481	0.1730703	0.09894683	0.1580477
ABCG	CL1387Contig1	CpABC49	3877	0.001768659	0.001092904	0.00103016	0.01119367
ABCG	CL3900Contig1	CpABC50	3187	0.04071111	0.007592998	0.02229048	0.0223637
ABCG	CL4388Contig1	CpABC51	3145	0.005084693	0.07819259	0.003105495	0.007112151
ABCG	CL459Contig1	CpABC52	3197	0.003858048	0.01604181	0.001598159	0.001685164
ABCG	CL4975Contig1	CpABC53	3056	0.07533759	0.007019758	0.0029909	0.01664466
ABCG	CL8016Contig1	CpABC54	1066	0.000937451	0.000918993	0.001381351	0.1430348
ABCG	CL835Contig1	CpABC55	2408	0.1481006	0.1310699	0.06352308	0.01651501
ABCG	CL8752Contig1	CpABC56	2665	0.002058265	0.03445911	0.01196719	0.000146387
ABCG	CL9046Contig1	CpABC57	2536	0.03725408	0.0190801	0.005318518	0.05846103
ABCG	comp18280_c0_seq1	CpABC58	2435	0.000221974	0.009861331	0.000222467	6.28602E-05
ABCG	comp2726_c0_seq1	CpABC59	1248	0.005081146	0.03472196	0.001368305	0.000332343
ABCG	comp312_c3_seq8	CpABC60	2325	0.09000223	0.009720574	0.007768591	0.001432663
ABCG	comp6064_c0_seq2	CpABC61	2474	0.004151469	0.06656397	0.002574356	0.006627215
ABCG	comp9217_c0_seq1	CpABC62	2265	0.000666988	0.000177273	8.15804E-05	0.04417967
ABCH	CL3092Contig1	CpABC63	3008	0.001358556	0.01462469	0.001305997	0.001030219
ABCH	CL670Contig1	CpABC64	3508	0.00668579	0.2402539	0.007439599	0.001205931
ABCH	comp2133_c0_seq1	CpABC65	2631	0.002522675	0.107453	0.001476871	0.000634701

References

1. Dassa E (2011) Natural history of ABC systems: not only transporters. In: Sharom FJ, editor. Essays in Biochemistry: Abc Transporters. pp. 19-42.
2. Holland IB (2011) ABC transporters, mechanisms and biology: an overview. In: Sharom FJ, editor. Essays in Biochemistry: Abc Transporters. pp. 1-17.
3. Jin MS, Oldham ML, Zhang QJ, Chen J (2012) Crystal structure of the multidrug transporter P-glycoprotein from Caenorhabditis elegans. Nature 490: 566-+.
4. Al-Shawi MK (2011) Catalytic and transport cycles of ABC exporters. In: Sharom FJ, editor. Essays in Biochemistry: Abc Transporters. pp. 63-83.
5. Dassa E, Bouige P (2001) The ABC of ABCs: a phylogenetic and functional classification of ABC systems in living organisms. Res Microbiol 152: 211-229.
6. Dean M, Annilo T (2005) Evolution of the ATP-binding cassette (ABC) transporter superfamily in vertebrates. Annual Review of Genomics and Human Genetics. pp. 123-142.
7. Wang B, Dukarevich M, Sun EI, Yen MR, Saier MH (2009) Membrane Porters of ATP-Binding Cassette Transport Systems Are Polyphyletic. J Membr Biol 231: 1-10.
8. Annilo T, Chen ZQ, Shulenin S, Costantino J, Thomas L, et al. (2006) Evolution of the vertebrate ABC gene family: analysis of gene birth and death. Genomics 88: 1-11.
9. Dean M, Rzhetsky A, Allikmets R (2001) The human ATP-binding cassette (ABC) transporter superfamily. Genome Res 11: 1156-1166.

10. Quazi F, Molday RS (2011) Lipid transport by mammalian ABC proteins. In: Sharom FJ, editor. Essays in Biochemistry: Abc Transporters. pp. 265-290.
11. Sharom FJ (2011) The P-glycoprotein multidrug transporter. In: Sharom FJ, editor. Essays in Biochemistry: Abc Transporters. pp. 161-178.
12. Slot AJ, Molinski SV, Cole SPC (2011) Mammalian multidrug-resistance proteins (MRPs). In: Sharom FJ, editor. Essays in Biochemistry: Abc Transporters. pp. 179-207.
13. Tamaki A, Ierano C, Szakacs G, Robey RW, Bates SE (2011) The controversial role of ABC transporters in clinical oncology. In: Sharom FJ, editor. Essays in Biochemistry: Abc Transporters. pp. 209-232.
14. Ewart GD, Howells AJ (1998) ABC transporters involved in transport of eye pigment precursors in Drosophila melanogaster. Abc Transporters: Biochemical, Cellular, and Molecular Aspects 292: 213-224.
15. Mackenzie SM, Brooker MR, Gill TR, Cox GB, Howells AJ, et al. (1999) Mutations in the white gene of Drosophila melanogaster affecting ABC transporters that determine eye colouration. Biochimica Et Biophysica Acta-Biomembranes 1419: 173-185.
16. Ohare K, Murphy C, Levis R, Rubin GM (1984) DNA-SEQUENCE OF THE WHITE LOCUS OF DROSOPHILA-MELANOGASTER. J Mol Biol 180: 437-455.
17. Quan GX, Kanda T, Tamura T (2002) Induction of the white egg 3 mutant phenotype by injection of the double-stranded RNA of the silkworm white gene. Insect Mol Biol 11: 217-222.
18. Sumitani M, Yamamoto DS, Lee JM, Hatakeyama M (2005) Isolation of white gene orthologue of the sawfly, Athalia rosae (Hymenoptera) and its functional analysis using RNA interference. Insect Biochemistry and Molecular Biology 35: 231-240.
19. Tatematsu K-i, Yamamoto K, Uchino K, Narukawa J, Iizuka T, et al. (2011) Positional cloning of silkworm white egg 2 (w-2) locus shows functional conservation and diversification of ABC transporters for pigmentation in insects. Genes Cells 16: 331-342.
20. Ricardo S, Lehmann R (2009) An ABC Transporter Controls Export of a Drosophila Germ Cell Attractant. Science 323: 943-946.
21. Hock T, Cottrill T, Keegan J, Garza D (2000) The E23 early gene of Drosophila encodes an ecdysone-inducible ATP-binding cassette transporter capable of repressing ecdysone-mediated gene activation. Proc Natl Acad Sci U S A 97: 9519-9524.
22. Buss DS, Callaghan A (2008) Interaction of pesticides with p-glycoprotein and other ABC proteins: A survey of the possible importance to insecticide, herbicide and fungicide resistance. Pestic Biochem Physiol 90: 141-153.
23. Labbe R, Caveney S, Donly C (2011) Genetic analysis of the xenobiotic resistance-associated ABC gene subfamilies of the Lepidoptera. Insect Mol Biol 20: 243-256.
24. Jones CM, Toe HK, Sanou A, Namountougou M, Hughes A, et al. (2012) Additional Selection for Insecticide Resistance in Urban Malaria Vectors: DDT Resistance in Anopheles arabiensis from Bobo-Dioulasso, Burkina Faso. Plos One 7.
25. Gahan LJ, Pauchet Y, Vogel H, Heckel DG (2010) An ABC Transporter Mutation Is Correlated with Insect Resistance to Bacillus thuringiensis Cry1Ac Toxin. PLoS Genet 6.
26. Atsumi S, Miyamoto K, Yamamoto K, Narukawa J, Kawai S, et al. (2012) Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin Cry1Ab in the silkworm, Bombyx mori. Proc Natl Acad Sci U S A 109: E1591-E1598.
27. Liu SM, Zhou S, Tian L, Guo EN, Luan YX, et al. (2011) Genome-wide identification and characterization of ATP-binding cassette transporters in the silkworm, Bombyx mori. BMC Genomics 12.
28. Roth CW, Holm I, Graille M, Dehoux P, Rzhetsky A, et al. (2003) Identification of the Anopheles gambiae ATP-binding cassette transporter superfamily genes. Molecules and Cells 15: 150-158.

29. Gomez-Zurita J, Hunt T, Kopliku F, Vogler AP (2007) Recalibrated tree of leaf beetles (Chrysomelidae) indicates independent diversification of angiosperms and their insect herbivores. Plos One 2.

30. Termonia A, Hsiao TH, Pasteels JM, Milinkovitch MC (2001) Feeding specialization and host-derived chemical defense in Chrysomeline leaf beetles did not lead to an evolutionary dead end. Proc Natl Acad Sci U S A 98: 3909-3914.

31. Opitz SEW, Mueller C (2009) Plant chemistry and insect sequestration. Chemoecology 19: 117-154.

32. Pasteels JM, Duffey S, Rowell-Rahier M (1990) Toxins in chrysomelid beetles possible evolutionary sequence from *de novo* synthesis to derivation from food-plant chemicals. J Chem Ecol 16: 211-222.

33. Pasteels JM, Rowell-Rahier M, Braekman JC, Dupont A (1983) Salicin from host plant as precursor of salicyl aldehyde in defensive secretion of chrysomeline larvae. Physiol Entomol 8: 307-314.

34. Kuhn J, Pettersson EM, Feld BK, Burse A, Termonia A, et al. (2004) Selective transport systems mediate sequestration of plant glucosides in leaf beetles: A molecular basis for adaptation and evolution. Proc Natl Acad Sci U S A 101: 13808-13813.

35. Smiley JT, Horn JM, Rank NE (1985) ECOLOGICAL EFFECTS OF SALICIN AT 3 TROPHIC LEVELS - NEW PROBLEMS FROM OLD ADAPTATIONS. Science 229: 649-651.

36. Michalski C, Mohagheghi H, Nimtz M, Pasteels JM, Ober D (2008) Salicyl alcohol oxidase of the chemical defense secretion of two chrysomelid leaf beetles - Molecular and functional characterization of two new members of the glucose-methanol-choline oxidoreductase gene family. J Biol Chem 283: 19219-11928.

37. Gross J, Podsiadlowski L, Hilker M (2002) Antimicrobial activity of exocrine glandular secretion of Chrysomela larvae. J Chem Ecol 28: 317-331.

38. Discher S, Burse A, Tolzin-Banasch K, Heinemann SH, Pasteels JM, et al. (2009) A versatile transport network for sequestering and excreting plant glycosides in leaf beetles provides an evolutionary flexible defense strategy. ChemBioChem 10: 2223-2229.

39. Strauss AS, Peters S, Boland W, Burse A, Dicke M (2013) ABC transporter functions as a pacemaker for sequestration of plant glucosides in leaf beetles. eLife 2.

40. Bodemann RR, Rahfeld P, Stock M, Kunert M, Wielsch N, et al. (2012) Precise RNAi-mediated silencing of metabolically active proteins in the defence secretions of juvenile leaf beetles. Proceedings of the Royal Society B-Biological Sciences 279: 4126-4134.

41. Bentley DR, Balasubramanian S, Swerdlow HP, Smith GP, Milton J, et al. (2008) Accurate whole human genome sequencing using reversible terminator chemistry. Nature 456: 53-59.

42. Rahfeld Peter, Kirsch Roy , Kugel Susann , Wielsch Natalie, Stock Magdalena, et al. (2014) Independent recruitments of oxidases from the GMC oxidoreductase family enabled the evolution of iridoid defence in leaf beetle larvae (subtribe Chrysomelina). Proceedings of the Royal Society B-Biological Sciences.

43. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, et al. (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol 29: 644-652.

44. Pertea G, Huang X, Liang F, Antonescu V, Sultana R, et al. (2003) TIGR Gene Indices clustering tools (TGICL): a software system for fast clustering of large EST datasets. Bioinformatics 19: 651-652.

45. Finn RD, Tate J, Mistry J, Coggill PC, Sammut SJ, et al. (2008) The Pfam protein families database. Nucleic Acids Res 36: D281-D288.

46. Punta M, Coggill PC, Eberhardt RY, Mistry J, Tate J, et al. (2012) The Pfam protein families database. Nucleic Acids Res 40: D290-D301.

47. Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30: 3059-3066.

48. Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754-755.

49. Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688-2690.
50. Broehan G, Kroeger T, Lorenzen M, Merzendorfer H (2013) Functional analysis of the ATP-binding cassette (ABC) transporter gene family of Tribolium castaneum. BMC Genomics 14.
51. Langmead B (2010) Aligning short sequencing reads with Bowtie. Curr Protoc Bioinformatics Chapter 11: Unit 11.17.
52. Anders S, Huber W (2010) Differential expression analysis for sequence count data. Genome Biol 11: R106.
53. Anders S, McCarthy DJ, Chen Y, Okoniewski M, Smyth GK, et al. (2013) Count-based differential expression analysis of RNA sequencing data using R and Bioconductor. Nature protocols 8: 1765-1786.
54. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, et al. (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5: R80.
55. Lander ES, Waterman MS (1988) Genomic mapping by fingerprinting random clones: a mathematical analysis. Genomics 2: 231-239.
56. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-DELTADELTACT method. Methods (Orlando) 25: 402-408.
57. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, et al. (2009) The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin Chem 55: 611-622.
58. Albrecht C, Viturro E (2007) The ABCA subfamily - gene and protein structures, functions and associated hereditary diseases. Pflugers Archiv-European Journal of Physiology 453: 581-589.
59. Broccardo C, Luciani MF, Chimini G (1999) The ABCA subclass of mammalian transporters. Biochimica Et Biophysica Acta-Biomembranes 1461: 395-404.
60. Zarubica A, Trompier D, Chimini G (2007) ABCA1, from pathology to membrane function. Pflugers Archiv-European Journal of Physiology 453: 569-579.
61. Tsybovsky Y, Orban T, Molday RS, Taylor D, Palczewski K (2013) Molecular Organization and ATP-Induced Conformational Changes of ABCA4, the Photoreceptor-Specific ABC Transporter. Structure 21: 854-860.
62. Juliano RL, Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta 455: 152-162.
63. Ueda K, Cardarelli C, Gottesman MM, Pastan I (1987) Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin, and vinblastine. Proc Natl Acad Sci U S A 84: 3004-3008.
64. Gros P, Croop J, Housman D (1986) Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. Cell 47: 371-380.
65. Gottesman MM, Fojo T, Bates SE (2002) Multidrug resistance in cancer: Role of ATP-dependent transporters. Nature Reviews Cancer 2: 48-58.
66. Yu M, Ocana A, Tannock IF (2013) Reversal of ATP-binding cassette drug transporter activity to modulate chemoresistance: why has it failed to provide clinical benefit? Cancer and Metastasis Reviews 32: 211-227.
67. Zhou SF (2008) Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. Xenobiotica 38: 802-832.
68. Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, et al. (2009) Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science 323: 1718-1722.
69. Seeger MA, van Veen HW (2009) Molecular basis of multidrug transport by ABC transporters. Biochimica Et Biophysica Acta-Proteins and Proteomics 1794: 725-737.
70. Sarkadi B, Homolya L, Szakacs G, Varadi A (2006) Human multidrug resistance ABCB and ABCG transporters: Participation in a chemoimmunity defense system. Physiol Rev 86: 1179-1236.

71. Chen KG, Valencia JC, Gillet JP, Hearing VJ, Gottesman MM (2009) Involvement of ABC transporters in melanogenesis and the development of multidrug resistance of melanoma. Pigment Cell & Melanoma Research 22: 740-749.

72. Kawanobe T, Kogure S, Nakamura S, Sato M, Katayama K, et al. (2012) Expression of human ABCB5 confers resistance to taxanes and anthracyclines. Biochem Biophys Res Commun 418: 736-741.

73. Oude Elferink RP, Paulusma CC (2007) Function and pathophysiological importance of ABCB4 (MDR3 P-glycoprotein). Pflugers Archiv : European journal of physiology 453: 601-610.

74. Stieger B, Meier Y, Meier PJ (2007) The bile salt export pump. Pflugers Archiv : European journal of physiology 453: 611-620.

75. Herget M, Tampe R (2007) Intracellular peptide transporters in human - compartmentalization of the "peptidome". Pflugers Archiv-European Journal of Physiology 453: 591-600.

76. Krishnamurthy P, Xie T, Schuetz JD (2007) The role of transporters in cellular heme and porphyrin homeostasis. Pharmacol Ther 114: 345-358.

77. Pondarre C, Antiochos BB, Campagna DR, Greer EL, Deck KM, et al. (2006) The mitochondrial ATP-binding cassette transporter Abcb7 is essential in mice and participates in cytosolic iron-sulfur cluster biogenesis. Hum Mol Genet 15: 953-964.

78. Ye H, Rouault TA (2010) Human Iron-Sulfur Cluster Assembly, Cellular Iron Homeostasis, and Disease. Biochemistry 49: 4945-4956.

79. Ichikawa Y, Bayeva M, Ghanefar M, Potini V, Sun L, et al. (2012) Disruption of ATP-binding cassette B8 in mice leads to cardiomyopathy through a decrease in mitochondrial iron export. Proc Natl Acad Sci U S A 109: 4152-4157.

80. Podsiadlowski L, Matha V, Vilcinskas A (1998) Detection of a P-glycoprotein related pump in Chironomus larvae and its inhibition by verapamil and cyclosporin A. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 121: 443-450.

81. Aurade RM, Jayalakshmi SK, Sreeramulu K (2010) P-glycoprotein ATPase from the resistant pest, Helicoverpa armigera: Purification, characterization and effect of various insecticides on its transport function. Biochimica Et Biophysica Acta-Biomembranes 1798: 1135-1143.

82. Aurade RM, Jayalakshmi SK, Udikeri SS, Sreeramulu K (2012) MODULATION OF P-GLYCOPROTEIN ATPASE OF HELICOVERPA ARMIGERA BY CHOLESTEROL: EFFECTS ON ATPASE ACTIVITY AND INTERACTION OF INSECTICIDES. Arch Insect Biochem Physiol 79: 47-60.

83. Luo L, Sun YJ, Wu YJ (2013) Abamectin resistance in Drosophila is related to increased expression of P-glycoprotein via the dEGFR and dAkt pathways. Insect Biochemistry and Molecular Biology 43: 627-634.

84. Porretta D, Gargani M, Bellini R, Medici A, Punelli F, et al. (2008) Defence mechanisms against insecticides temephos and diflubenzuron in the mosquito Aedes caspius: the P-glycoprotein efflux pumps. Med Vet Entomol 22: 48-54.

85. Srinivas R, Udikeri SS, Jayalakshmi SK, Sreeramulu K (2004) Identification of factors responsible for insecticide resistance in Helicoverpa armigera. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 137: 261-269.

86. Lanning CL, Fine RL, Corcoran JJ, Ayad HM, Rose RL, et al. (1996) Tobacco budworm P-glycoprotein: Biochemical characterization and its involvement in pesticide resistance. Biochimica Et Biophysica Acta-General Subjects 1291: 155-162.

87. Murray CL, Quaglia M, Arnason JT, Morris CE (1994) A PUTATIVE NICOTINE PUMP AT THE METABOLIC BLOOD-BRAIN-BARRIER OF THE TOBACCO HORNWORM. J Neurobiol 25: 23-34.

88. Sorensen JS, Dearing MD (2006) Efflux transporters as a novel herbivore countermechanism to plant chemical defenses. J Chem Ecol 32: 1181-1196.

89. Begun DJ, Whitley P (2000) Genetics of alpha-amanitin resistance in a natural population of Drosophila melanogaster. Heredity 85: 184-190.

90. Tapadia MG, Lakhotia SC (2005) Expression of mdr49 and mdr65 multidrug resistance genes in larval tissues of Drosophila melanogaster under normal and stress conditions. Cell Stress Chaperones 10: 7-11.

91. Bangert I, Tumulka F, Abele R (2011) The lysosomal polypeptide transporter TAPL: more than a housekeeping factor? Biol Chem 392: 61-66.

92. Zhou SF, Wang LL, Di YM, Xue CC, Duan W, et al. (2008) Substrates and inhibitors of human multidrug resistance associated proteins and the implications in drug development. Curr Med Chem 15: 1981-2039.

93. Chen Z-S, Tiwari AK (2011) Multidrug resistance proteins (MRPs/ABCCs) in cancer chemotherapy and genetic diseases. FEBS J 278: 3226-3245.

94. Aleksandrov AA, Aleksandrov LA, Riordan JR (2007) CFTR (ABCC7) is a hydrolyzable-ligand-gated channel. Pflugers Archiv-European Journal of Physiology 453: 693-702.

95. Hunt JF, Wang C, Ford RC (2013) Cystic Fibrosis Transmembrane Conductance Regulator (ABCC7) Structure. Cold Spring Harbor Perspectives in Medicine 3.

96. Bryan J, Munoz A, Zhang X, Dufer M, Drews G, et al. (2007) ABCC8 and ABCC9: ABC transporters that regulate K(+) channels. Pflugers Archiv-European Journal of Physiology 453: 703-718.

97. Heckel DG (2012) Learning the ABCs of Bt: ABC transporters and insect resistance to Bacillus thuringiensis provide clues to a crucial step in toxin mode of action. Pestic Biochem Physiol 104: 103-110.

98. Morita M, Imanaka T (2012) Peroxisomal ABC transporters: Structure, function and role in disease. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1822: 1387-1396.

99. Kashiwayama Y, Seki M, Yasui A, Murasaki Y, Morita M, et al. (2009) 70-kDa peroxisomal membrane protein related protein (P70R/ABCD4) localizes to endoplasmic reticulum not peroxisomes, and NH2-terminal hydrophobic property determines the subcellular localization of ABC subfamily D proteins. Exp Cell Res 315: 190-205.

100. Tian Y, Han X, Tian D-l (2012) The biological regulation of ABCE1. IUBMB Life 64: 795-800.

101. Andersen DS, Leevers SJ (2007) The essential Drosophila ATP-binding cassette domain protein, pixie, binds the 40 S ribosome in an ATP-dependent manner and is required for translation initiation. J Biol Chem 282: 14752-14760.

102. Kerr ID (2004) Sequence analysis of twin ATP binding cassette proteins involved in translational control, antibiotic resistance, and ribonuclease L inhibition. Biochem Biophys Res Commun 315: 166-173.

103. Kerr ID, Haider AJ, Gelissen IC (2011) The ABCG family of membrane-associated transporters: you don't have to be big to be mighty. Br J Pharmacol 164: 1767-1779.

104. Moitra K, Silverton L, Limpert K, Im K, Dean M (2011) Moving out: from sterol transport to drug resistance - the ABCG subfamily of efflux pumps. Drug Metabolism and Drug Interactions 26: 105-111.

105. Tarr PT, Tarling EJ, Bojanic DD, Edwards PA, Baldan A (2009) Emerging new paradigms for ABCG transporters. Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids 1791: 584-593.

106. Woodward OM, Koettgen A, Koettgen M (2011) ABCG transporters and disease. FEBS J 278: 3215-3225.

107. Wang L, Kiuchi T, Fujii T, Daimon T, Li M, et al. (2013) Mutation of a novel ABC transporter gene is responsible for the failure to incorporate uric acid in the epidermis of ok mutants of the silkworm, Bombyx mori. Insect Biochemistry and Molecular Biology 43: 562-571.

108. Itoh TQ, Tanimura T, Matsumoto A (2011) Membrane-bound transporter controls the circadian transcription of clock genes in Drosophila. Genes Cells 16: 1159-1167.

109. Sturm A, Cunningham P, Dean M (2009) The ABC transporter gene family of Daphnia pulex. BMC Genomics 10: 170.

110. Petzold A, Reichwald K, Groth M, Taudien S, Hartmann N, et al. (2013) The transcript catalogue of the short-lived fish *Nothobranchius furzeri* provides insights into age-dependent changes of mRNA levels. BMC Genomics 14: 185.

111. Dong JS, Lai R, Jennings JL, Link AJ, Hinnebusch AG (2005) The novel ATP-binding cassette protein ARB1 is a shuttling factor that stimulates 40S and 60S ribosome biogenesis. Mol Cell Biol 25: 9859-9873.

112. Overbeck TR, Hupfeld T, Krause D, Waldmann-Beushausen R, Chapuy B, et al. (2013) Intracellular ATP-Binding Cassette Transporter A3 is Expressed in Lung Cancer Cells and Modulates Susceptibility to Cisplatin and Paclitaxel. Oncology 84: 362-370.

113. Aung T, Chapuy B, Vogel D, Wenzel D, Oppermann M, et al. (2011) Exosomal evasion of humoral immunotherapy in aggressive B-cell lymphoma modulated by ATP-binding cassette transporter A3. Proc Natl Acad Sci U S A 108: 15336-15341.

114. Fischer S, Kluver N, Burkhardt-Medicke K, Pietsch M, Schmidt AM, et al. (2013) Abcb4 acts as multixenobiotic transporter and active barrier against chemical uptake in zebrafish (Danio rerio) embryos. BMC Biol 11.

115. Wang Y, Lim L, DiGuistini S, Robertson G, Bohlmann J, et al. (2013) A specialized ABC efflux transporter GcABC-G1 confers monoterpene resistance to Grosmannia clavigera, a bark beetle-associated fungal pathogen of pine trees. New Phytol 197: 886-898.

116. Dow JAT, Davies SA (2006) The Malpighian tubule: Rapid insights from post-genomic biology. J Insect Physiol 52: 365-378.

117. Bayeva M, Khechaduri A, Wu RX, Burke MA, Wasserstrom JA, et al. (2013) ATP-Binding Cassette B10 Regulates Early Steps of Heme Synthesis. Circul Res 113: 279-287.

118. Chloupkova M, LeBard LS, Koeller DM (2003) MDL1 is a high copy suppressor of ATM1: Evidence for a role in resistance to oxidative stress. J Mol Biol 331: 155-165.

119. Sullivan DT, Sullivan MC (1975) TRANSPORT DEFECTS AS PHYSIOLOGICAL BASIS FOR EYE COLOR MUTANTS OF DROSOPHILA-MELANOGASTER. Biochem Genet 13: 603-613.

120. Ryall RL, Howells AJ (1974) OMMOCHROME BIOSYNTHETIC-PATHWAY OF DROSOPHILA-MELANOGASTER - VARIATIONS IN LEVELS OF ENZYME-ACTIVITIES AND INTERMEDIATES DURING ADULT DEVELOPMENT. Insect Biochemistry 4: 47-61.

121. Sullivan DT, Grillo SL, Kitos RJ (1974) SUBCELLULAR-LOCALIZATION OF FIRST 3 ENZYMES OF OMMOCHROME SYNTHETIC PATHWAY IN DROSOPHILA-MELANOGASTER. Journal of Experimental Zoology 188: 225-233.

122. Evans JM, Day JP, Cabrero P, Dow JAT, Davies S-A (2008) A new role for a classical gene: White transports cyclic GMP. J Exp Biol 211: 890-899.

123. Haunerland NH, Shirk PD (1995) REGIONAL AND FUNCTIONAL-DIFFERENTIATION IN THE INSECT FAT-BODY. Annual Review of Entomology 40: 121-145.

124. Arrese EL, Soulages JL (2010) Insect Fat Body: Energy, Metabolism, and Regulation. Annual Review of Entomology. pp. 207-225.

125. Tarling EJ, Vallim TQD, Edwards PA (2013) Role of ABC transporters in lipid transport and human disease. Trends Endocrinol Metab 24: 342-350.

126. Li JW, Lehmann S, Weissbecker B, Naharros IO, Schutz S, et al. (2013) Odoriferous Defensive Stink Gland Transcriptome to Identify Novel Genes Necessary for Quinone Synthesis in the Red Flour Beetle, Tribolium castaneum. PLoS Genet 9.

127. Elliott AM, Ai-Hajj MA (2009) ABCB8 Mediates Doxorubicin Resistance in Melanoma Cells by Protecting the Mitochondrial Genome. Mol Cancer Res 7: 79-87.

128. Dermauw W, Osborne EJ, Clark RM, Grbic M, Tirry L, et al. (2013) A burst of ABC genes in the genome of the polyphagous spider mite Tetranychus urticae. BMC Genomics 14.

4 General Discussion

The main aim of my thesis was to investigate transport proteins which are involved in the sequestration of phytogenic glucosides by leaf beetle larvae. To that end, we employed c-DNA library/transcriptomic analysis that enabled us to gain first comprehensive sequence information of transport proteins in our model beetle *C. populi*. Building on the overview of sequestration in leaf beetles in Manuscript I, this general discussion will summarize knowledge gained about transporter candidates from our interdisciplinary approach (4.1). Based on the newly identified ABC transporter, *Cp*MRP, functioning as a key element for the glandular defense (Manuscript II), the general importance of ABC transporters as a barrier for phytochemicals will be discussed in 4.2. In section 4.3 I will propose a sequestration model with focus on efflux processes facilitated by ABC transporters and discuss their general relevance in exocrine glands of Chrysomelina species. Additionally, cell biological parameters which might affect the secretion process influenced by *Cp*MRP are addressed in this section. Section 4.4 summarizes evolutionary aspects in the context of *Cp*MRP. Particularly, this discussion covers how Chrysomelina larvae benefit from sequestration processes and evaluates the prerequisites for and the consequences from host plant shifts. Thereby I have also considered some speculative aspects as well as future perspectives.

4.1 Elucidating transporter candidates involved in sequestration

Research in the last decades has focused on the diversity of plant natural products which can be sequestered by herbivores [29, 31, 36–38, 103]. Much progress has been made on the major chemical components of defensive secretions, the biosynthetic steps and the influence of host plants within Chrysomelina larvae [46, 48, 104, 105]. In this

subtribe a chemical approach was used to narrow down possible transporter candidates among putative passive and active transport proteins.

Systematically modified thioglucosides as structural mimics of plant-derived glucosides as well as deuterated glucosides and aglucons were applied in feeding and microinjection experiments as substrate mixtures or single compounds [47, 65, 81]. These studies address questions regarding the substrate specificity at different membrane barriers within Chrysomelina larvae. With thioglucosides as stable glycomimics, their route from the gut to the defensive system was followed. Thiolucosides are resistant to hydrolysis by glucosidases. Like natural glucosides they pass different membrane barriers and finally accumulate in the defensive reservoir without further transformation [47, 65, 81].

Manuscript I provides an overview of the results gained by the application of these labeled chemical compounds. Investigating the transport processes involved in the chemical defense of Chrysomelina larvae, the following principles can be compiled:

- Notwithstanding the mode of chemical defense, the ability to sequester glucoside precursors for the biosynthesis of their defensive secretions is a common feature.
- The transport forms of sequestered compounds are glucosides. The aglucons (demonstrated by feeding experiments applying deuterated non-glucosylated precursors) are glucosylated probably to prevent auto-intoxication [53].
- Predominantly their genuine precursor (salicin in case of *C. populi*) is selectively transported into the glandular system. Glandular carriers seem to tightly control the metabolite uptake.
- A highly efficient and complex transport system seems to exist for both obligate sequestering species and species producing deterrent *de novo*.
- Carrier proteins in the gut seem to facilitate a rather unselective translocation to the hemolymph which is rapidly cleared of the imported substances.
- The transport process is highly specific. The uptake systems discriminate between different sugars but also structure isomers such as 8-hydroxy-geraniol-8-*S*-*ß*-D-glucoside and 8-hydroxy-geraniol-1-*S*-*ß*-D-glucoside; the former was favoured by a factor of 10 over the 8-hydroxy-geraniol-1-*S*-*ß*-D-glucoside [47,106].

In general, the transport systems of different Chrysomelina larvae display similarities, irrespective of the mode of their chemical defense. Transport processes essential for

sequestration are already developed in the ancestral species [47,65,106]. It is postulated that glandular carriers at the last membrane barrier differ in substrate selectivity and thereby control the metabolite uptake depending on the aglucon properties. Typically a chemical approach as described earlier is nescessary to gain important information about substrates and substrate specificity of unknown transport proteins. Building on experimental results it is possible to forward a hypothesis about the transporter class they may belong to [35, 53, 107, 108].

Active and passive transporter classes of Chrysomelina larvae were in focus the past years. Since sequestration within the Chrysomelina subtribe tightly depend on the presence of glucose, preliminary studies on the expression of GLUT-type (Glucose transporter) and SGLT-type (sodium-glucose linked transporter) of *C. populi* and *P. cochleariae* has been performed ([109], unpublished data). Additionally, monitoring the influence of specific inhibitors on dissected tissue is a helpful tool to elucidate hypothetical transporter candidates [110–113]. However, inhibitor studies with Phlorizin and Cytochalasin B as inhibitors for glucose transporting proteins [114, 115] to identify candidates for sequestration processes were not convincing [116]. Manuscript I further addresses the question of how to elucidate putative transport proteins involved in the sequestration by leaf beetles. At this point cDNA libraries could hold essential sequence information. This approach together with quantitative realtime PCR analysis to test the overall presence and level of transporter candidates was applied in Manuscript II.

Recent advances in molecular biology and computational biology have opened new ways to complement the complex picture of mechanisms that insects have exploited to deal with a variety of plant secondary metabolites. Although genomic tools are argued to help understanding the complexity and ecological role of sequestration [16], insect genomes are still less sequenced [117, 118]. At the same time a deep understanding will require the analysis of phylogenetically diverse species that extends the current repertoire of model organisms. For non-model organisms with genomes that are yet to be sequenced, transcriptomic profiling by using the RNA-Sequencing (RNA-Seq) approach is attractive to answer a large number of biological questions. In contrast to hybridization-based techniques RNA-Seq is not limited to the existance of genomic sequences and is expected to revolutionize the field of transcriptomics

[119, 120]. Furthermore, in consideration to identify orphan genes, that are genes without homologies to genes of other species, transcriptome studies serve as an important tool [117].

In Manuscript III we use the RNA-Seq technique to identify the active inventory of ABC transporters within *C. populi*. A tissue specific profiling provides information about the level of each ABC transporter transcript and thereby its probable functional importance in the respective larval tissue. Though transcriptomic profiling suggests a physiological role of a transporter candidate in larval tissues, its biological relevance with special focus on sequestration processes remains unclear. Today the RNAi approach is usually used to investigate the function of genes and their products. Bodemann et al. [121] have demonstrated the efficient usage of the RNAi-technique to analyse unknown proteins in defensive glands of chrysomelid larvae.

In Manuscript II we modify the translocation of deterrent precursors by silencing a key ABC transporter in *C. populi* which is highly susceptible to systemic RNAi [121]. The results revealed an active transporter (ABC transporter of the subfamily C) involved in accumulating glucosides in the glandular system which is supporting earlier studies [53, 65, 81].

4.2 ABC transporter as counter mechanism to plant defense

Plant feeding insects need to cope with toxic plant secondary metabolites. The ingestion of these compounds presents a physiological challenge and can cause detrimental effects on tissues and organs [122]. In general, xenobiotic detoxification metabolism is divided into two major parts. Both phases are responsible for their biotransformation into water-soluble compounds that can be readily excreted. Phase I metabolism involves the functionalization of oxidation, reduction, hydrolysis, cyclization, decyclization reactions, respectively. Thereby, phase I is essentially based on the activity of cytochrome P-450 (CYP) enzymes [24, 100]. Subsequently in phase II reactions, the activated chemicals are conjugated with cellular glutathione, glucose, glucuronide, or other small hydrophilic molecules.

With research on multidrug resistance ABC transporters the term „chemoimmunity“ has been discussed which complements the picture of xenobiotic defense metabolisms by adding two additional, essential steps (phase zero and III) due to the role of these transporters [100,123]. This term “chemoimmunity” is preferably applied in the field of pharmacology for lipophilic ABC transporter substrates and comprises the coordinated action of multidrug restistance (MDR) transporters and enzymes that influence and interfere on the simple permeability across the membrane for lipophilic substances. Similarily, ABC transporters are discussed in insects as counter mechanisms to plant chemical defense against hydrophilic and lipophilic toxins [96]. In this context Sorensen et al. postulated the regulated absorption model. They argue that herbivores in addition to the use of passive barriers (peritrophic membranes) in the gut [124,125] limit the absorption of non used or toxic phytochemicals by actively transporting them out of enterocytes and back into the lumen of the gut. This minimizes their concentration in the hemolymph of the insect. The major digestive region of the insect gut is the midgut, which produces and secretes the digestive enzymes into the gut lumen but also absorbs part of the nutrients [126]. However, ingested phytochemicals can pass the gut although possessing chemical properties promoting their absorption [127, 128]. This concept of “preemptive pumping” (phase zero) by efflux transporters is the most effective and secure way to keep toxic phytochemicals out of the insects body. In accordance to the earlier concept of “chemoimmunity” in humans, multidrug efflux transporters would provide a general phytochemical resistance.

In the transcriptome of *C. populi*, we predicted 65 ABC transporters which group in eight subfamilies (Manuscript III). To date, members of the subfamilies B, C and G confer resistance to xenobiotics including plant allelochemicals and insecticides [83, 99–101]. Especially P-glycoprotein (P-gp) - one of the best studied proteins in the ABC superfamily, also known as ABCB1 or multidrug resistance protein 1 (MDR1) - is a strong candidate to mediate the reduced absorption of secondary metabolites in the midgut of many insects [98, 111, 113, 129, 130]. Such P-gp-like carriers present in the gut are known for their crucial regulatory function for the bioavailability, or tissue distribution of xenobiotics [100,131,132]. Recent studies showed that a P-gp-like transporter mediates the efflux of cardenolids in the nerve cord and thereby prevents interactions with the susceptible target site of Na+/K+ - ATPase [111,129]. Based on

data presented in Manuscript III, I can summarize that among the ABC subfamilies B to G known for the transport of xenobiotics, subfamily B and G are predominantly present in the gut of *C. populi* (Fig. 4.1).

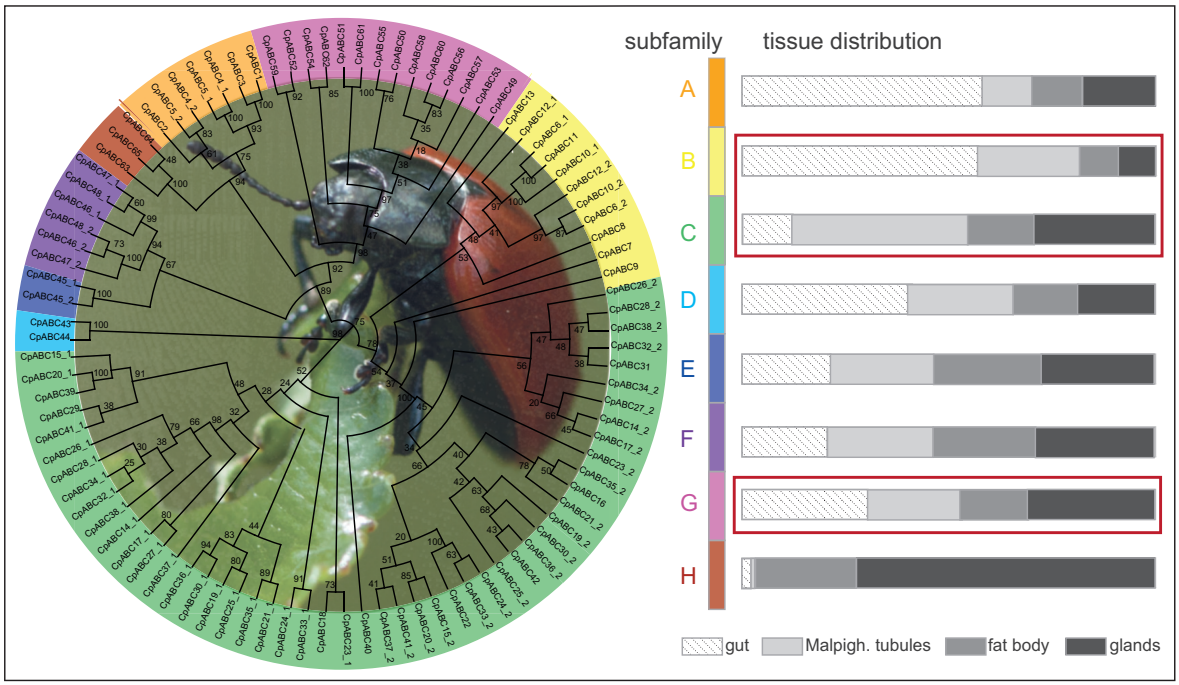


Fig. 4.1: Eight subfamilies of 65 putative ABC transporters of *C. populi*. Within each subfamily their relative tissue distribution among four different tissues (gut, Malpighian tubules, fat body and glands) was calculated based on normalized counts of RNA-Seq count reads (see Table S4 in Manuscript III for data). The red frame highlights ABC subfamilies which are know for xenobiotic transport activity (in this study referred to MDR-ABC transporters).

Subfamily B displays over 50% transcript abundance in the gut tissue. The highest transcript level of an ABC transporter in the intestinal tissue is harbouring a P-gp-like transporter of the subfamily B which implies the previously described function of P-gp. Within subfamily G, ABCG2 is the famous member for defense against xenobiotics and its apical membrane expression in epithelial cells along the gastrointestinal track suggests a major role of this protein in the first line of defense against xenobiotics. ABCG2 may be an important additional player in phase III reactions - the excretion/efflux of conjugated substrates. Interestingly, not a single analyzed insect sequence clusters with the human multidrug efflux transporter ABCG2. It is very likely that many insects avoid the toxic effects of plant secondary metabolites by excreting the compounds without absorbing them. Although, I am aware of the fact that the proposed function of ABC transporter transcripts presented in Manuscript III are only assumptions based on transcript profiling in combination with comparative

phylogenetic analyses of human and other insect ABC transporters. To state the protein level in the tissue of interest, immunohistological experiments or western blots are suitable if the hypothesized transporter is highly conserved. Moreover it would need inhibitor studies and functional assays by heterologously expression the transporter of interest.

Considering the principles of plant glucoside transport in Chrysomelina larvae which I discussed in the previous section 4.1, the phase zero function of P-gp- and ABCG2-like proteins may be negligible for sequestering plant glucosides. Previous experiments demonstrated a broad, non-selective uptake of glucosides from the gut into the larval hemolymph system (Manuscript I, [81]). The aglucons have shown to be re-glucosylated probably to prevent their detrimental effects [106, 133]. Thus, ß-glucosidases (digestive or plant origin, eventually [133]) may have forced the evolution of glucoside-adapted pathways by means of a fast non-selective carrier system in the gut and Malpighian tubules. Based on this assumption an effective toxin clearance function of ABC transporters is rather applicable within the selective sequestration of plant glucosides.

4.2.1 Effective excretion of plant glucosides in Chrysomelina larvae

Excretory processes may play a key role in allowing insects to exploit food plants that contain potentially toxic compounds [35, 80]. Typically, the toxicity of chemicals is concentration dependent. Therefore both the amount of plant secondary metabolites ingested and their absorbed quantity are essential for survival. A famous example is the tabacco hornworm *(Manduca sexta)* which excretes nicotine and other ingested alkaloids before a toxic dose can accumulate [21]. In this process P-gp like proteins are postulated as well [110]. As suggested earlier, ABC transporters can regulate the net uptake of glucosides by their active efflux activity.

Insect Malpighian tubules are critical for osmoregulation but moreover, the tubules are considered as the main site of xenobiotic excretion. Many ABC transporters are expressed in the Malpighian tubules and facilitate phase III xenobiotic detoxification reactions by eliminating conjugated substrates. The excretion via the Malpighian tubules reduces the concentration of phytochemicals in the hemolymph. Based on data

presented in Manuscript III, I can summarize that the Malpighian tubules of *C. populi* are dominated by candidates of subfamily C. Within the subfamily C of humans, nine out of twelve genes facilitate drug transport. In particular, members of the multidrug related ABCC4-group are numerously present in this tissue, which suggests a main contribution to the efflux and excretory function of this organ. In contrast to the 12 human ABCC genes, there are 29 ABCC genes in the transcriptome of *C. populi*, making this the most abundant ABC subfamily (Manuscript III). The dominance of ABCC transcripts in the Malpighian tubules is in agreement with the observed non-selective glucoside extrusion of the excretion system (Manuscript I; [81]). Compared with ABCC subfamilies in other insects, subfamily C of *C. populi* appears to have undergone an expansion as observed in the red flour beetle *T. castaneum* (35 genes) and the spider mite *Tetranychus urticae*, (39 gene). The same scenario seems to have evolved in subfamily G as well. Gene duplication appears to be a common characteristic of ABC genes of several insect species and contributes to protein diversity. One possible reason could be the intense selection pressure imposed by dietary toxins that favours a substantial diversity of transport function in insects postulated by Labbe et al [84]. These pressures may have led to the rapid evolution of numerous detoxification pumps, each with the ability to transport a particular range of chemicals.

Taken together, most putative ABC transporter transcripts identified in *C. populi* are present at a high level in the excretion system of the juvenile beetles. The overrepresentation of putative ABCC in the excretion system suggests a role of these candidates in the extrusion of xenobiotics or phytochemicals from the larval body. Despite intensive studies on the role of ABC tranporters in conferring drug resistance, little is know about their functions in insects. In order to understand their physiological role, interactions and probable contibutions of other transporter classes in the sequestration process, a lot more research needs to be done.

4.2.2 *Cp*MRP is a keyplayer in the sequestration/secretion process within Chrysomelina larvae

The principle contribution in xenobiotic resistance mechanisms of ABC proteins has generally been seen in the excretion of xenobiotics from the cell and ultimately from

the organism. The main part of the work is presented in Manuscript II which focusses on the identification and characterization of *Cp*MRP rather being involved in a well-directed accumulation of plant-derived glucosides. These precursors are used via sequestration and subsequent translocation in the defensive reservoirs of the larva to fend off predators.

The key role of *Cp*MRP has been identified via expression profiling in combination with RNAi-silencing experiments. Extraordinarily high transcript levels, like observed for *Cpmrp* expression (Manuscript II and III), pointed to a crucial function in the defensive system of *C. populi* (Manuscript II). *In-vivo* silencing of *Cp*MRP caused a defenseless phenotype incapable of secreting the defensive compounds. Therefore tissue-specific transcriptomic profiling in combination with comparative phylogenetic analysis can provide an overview of the active inventory of ABC proteins suggesting a preliminary function in a certain tissue (Manuscript III). Functionally, *Cp*MRP is a transporter for the irreversible shuttling of pre-filtered metabolites into storage compartments where it is highly present as well as in the microvilli membrane of the secretory cell. Transport studies in *Xenopus laevis* oocytes revealed *Cp*MRP as a transporter for salicin, the naturally sequestered host-plant glucoside and defensive precursor of *C. populi*. In comparative substrate assays mimicking physiological conditions (plant glucosides are being concurrently present), *Cp*MRPs transport activity displays no prevalence for salicin. These data gave us first hints for a probable similar function of *Cp*MRP homologs within Chrysomelina larvae. Moreover it is likely that *Cp*MRP provides a flexible counterdefense against plant glucosides due to its broad substrate spectrum.

For a more comprehensive understanding, further functional assays are needed. Up to 40 different glucosidic compounds could be detected in Salicaceae leaf samples (unpublished data-Pauls et al. in preparation). It would be also interesting to test glucose or diglucosides (unpublished data-Rahfeld et al. in preparation) as potential substrate for *Cp*MRP. The amount of free glucose is highly variable among Chrysomelina species. Compared to the aglucon the amount of glucose can be enriched (*C. lapponica*) [134] or lowered (*C.populi*) [54], respectively. The biological relevance of the high amounts of glucose in the larvae was speculated to function as cryoprotectant [134]. However it is clear that additional shuttle processes for glucose are going on in the secretory cells of the defensive system which might be coupled to *Cp*MRP.

Furthermore *Cp*MRPs expression level in the fat body is much higher compared to all other ABC transporter candidates, although it does not reach the magnitude of its glandular transcript level. Since the aglucons are potentially glucosylated in the fat body tissue as demonstrated by deuterated 8-hydroxygeraniol *[106]*, I suggest *Cp*MRP might facilitate the efflux process of re-glucosylated salicin and thereby regulate salicin concentrations in the hemolypmh.

In summary, ABC transporters are key elements in the complex defense system in the sequestering larvae of *C. populi*.

4.3 Sequestration model within Chrysomelina larvae

Duffey first reviewed the sequestration of plant natural products by insects with respect to its physicochemical, biophysical and kinetic aspects. He described sequestration as a kinetic phenomenon which results from a balance of gut-uptake, blood transfer, metabolism, deposition in tissue or reservoirs and excretion [29]. For example, the polarity of diverse allomones influences the absorption in the gut, body distribution and excretion. Chrysomelina larvae are able to sequester plant-derived glucosides. These polar molecules can dissolve in the digestive fluid of the larvae and thus their uptake is usually regulated by active transport or facilitative diffusion.

How do MDR-ABC transporter proteins including *Cp*MRP concert in the insect body to become part of a coordinated transport protein network for sequestering plant-derived glucosides? This complex process seems to involve transporters of different selectivity and energy coupling. Figure 4.2 illustrates an overview of the fate of plant-derived glucosides within the sequestration process of Chrysomelina larvae by highlighting the transport processes possibly involved in host plant-dependent defense of these herbivores. The model is primarily based on recent knowledge about ABC transporter function in *C. populi* (Manuscript II and III) in conjunction with previous microinjection and feeding experiments (reviewed in Manuscript I).

Ingested glucosides may be deglucosylated during their gut passage or within the hemocoel [106,133]. Nonpolar aglucons can diffuse through the gut membrane whereas glucosides cross the gut membrane only via transport proteins, potentially facilitative

passive transporters. The regulation (excretion) of excessive hemolymph glucosides can be facilitated active transporters in the Malpighian tubules [108]. ABCC transporters are most likely involved in this step as well. Further sequestration of salicin into the defensive glands depends on an interplay of active and passive transporters which might generate a decline in the concentration of salicin in the glands compared to hemolymph concentrations (detailed overview in Fig. 6 in Manuscript II), which can drive the entire sequestration process. The aglucons are potentially glucosylated in the fat body tissue [106]. Phase zero efflux reactions of MDR-ABC transporters or the "regulated absorption hypothesis", respectively was omitted in Fig. 4.2 due to the arguments described in section 4.2.

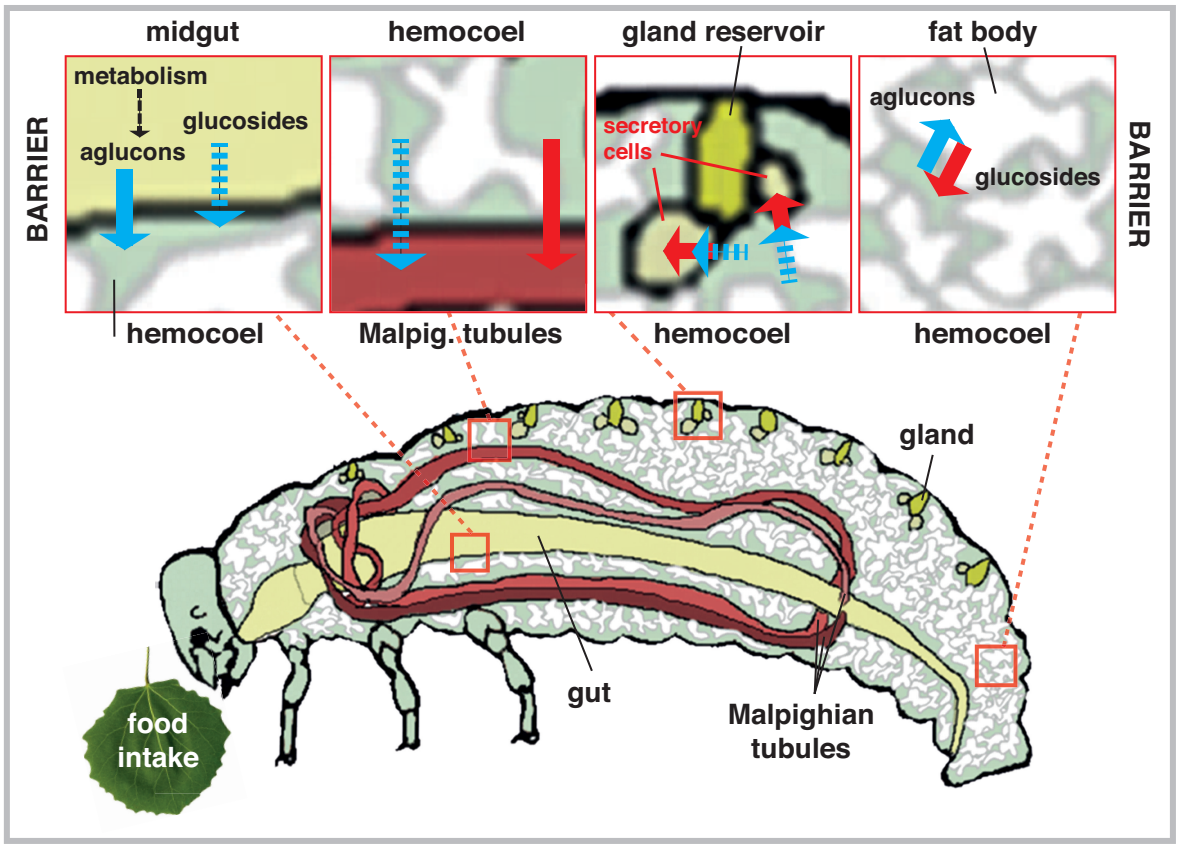


Fig. 4.2: Simplified sequestration model highlighting transport processes within Chrysomelina larvae. The model is summarizing diffusion (solid blue arrow), potentially passive (dotted blue arrows) and active transport processes (solid red arrows) across different membrane barriers in the gut, Malpighian tubules, glands and the fat body (simplified by the four insets). Ingested glucosides may be deglucosylated during their gut passage or within the hemocoel [106, 133]. Nonpolar aglucons can diffuse through the gut membrane whereas glucosides cross the gut membrane via potentially passive transport proteins. Regulation of hemolymph glucosides could be achieved by excretion via the Malpighian tubules. The sequestration of the genuine glucoside precursor into the defensive glands depends on an interplay of active and passive transporter which might generate a decline in the concentration of salicin compared to hemolymph concentrations detailed overview in Fig. 6 in Manuscript II). The fat body is considered as a dynamic tissue involved various metabolic functions, as well as postulated here: the re-glucosylation of the aglucons.

For years highly selective transport systems were postulated being responsible for the sequestration of specific metabolites in insect tissues [53, 65, 81]. In fact, we could show in Manuscript II that a probable cooperative arrangement of non-selective and selective transporters in the exocrine glands of *C. populi* are responsible for efficient sequestration of specific phytochemicals. On this basis, we suggest a first filter for specific glucosides (salicin, in the case of *C. populi*) at the hemolymph-exposed plasma membrane of the secretory cell, which might depend on a gradient-driven, energy-independent transporter.

Many herbivores shuttle ingested phytochemicals against a concentration gradient and sequester them unmetabolized in defensive glands, spezialised tissues or hemolymph [30, 53, 135, 136]. It is tempting to argue about the generality of a functioning arrangement of non-selective and a selective transporter involved in these sequestration processes. At least within Chrysomelina species, I would, for two reasons, postulate a general concept that involves *Cp*MRP-like transporters to be involved in the sequestration of plant-derived metabolites in the defense mechanism. First, I identified transporter sequences highly similar to *Cp*MRP in the larval glands of other Chrysomelina species (*P. cochleariae* and *C. lapponica*) that are similarily highly expressed in the glandular tissue (Manuscript II). Second, Chrysomelina larvae share an uniform architecture and morphology of their defensive glandular system harboring gland cells which belong to the type III exocrine gland cells [57, 58].

In *T. castaneum* another member of the ABCC4 cluster has been identified as playing a role in the production of secretions in stink glands. *In-vivo* silencing of this transporter (TcABCC-6A) resulted in a strong reduction of alkenes in the secretions produced by abdominal and prothoracic glands [137]. Thus, the hypothesis can be advanced that ABC transporters functioning in the formation of secretions seem to be a widespread phenomenon in insects. Extending the model of a principle, cooperative arrangement in insect exocrine glands harboring non-selective and selective transporters involved in the secretion/detoxification process would provide a flexible basis for the phytophagous insect to adjust to variations in the chemical composition of their hosts in evolutionary time. However, to date, this is too speculative and would be based on the uniform morphology of insect epidermal glands only (e.g., dermal glands of *Tenebrio molitor*, sternal glands of *Periplaneta americana*, pheromone gland of *Harpobittacus australis,*

defensive glands of *Agelastica alni* [58, 138]). It would need further research to identify and characterize the presence of *Cpmrp* homologs in class III gland cells of other insect species.

Based on *Cp*MRP, I postulate a more detailed sequestration model inside the secretory cells of *C. populi* (see Figure 6 in Manuscript II). According to this model, *Cp*MRP does not just dictate the transport rate of this transporter located in the plasma membrane, rather, it determines the effectiveness and energy coupling of the entire sequestration process as a pacemaker. As a consequence, there might be a continuous and selective flow of salicin molecules via yet unknown transport proteins from the larva´s body fluid into the secretory cell? Upon stimulation, refilling of the reservoir depends on membrane fusion of intracellular vesicles with the microvilli membrane encirceling the extracellular storage room (Re) which is directly connected to the glandular reservoir via a cuticular canal. Here, I suppose an osmolarity driven flux of water molecules into the extracellular storage room and the reservoir after glucoside cleavage.

This assumption is also based on the fact that qualitatively at least two types of vacuoles can be distinguished within the secretory cells (revealed by CDCFDA vacuolar esterase activity staining, see Manuscript II). Therefore it is likely that glucoside precursors and converting enzymes are stored separately in different vacuoles and are combined upon release into the extracellular storage room. Further, secretory cells of *Cpmrp*-silenced larvae tend to form enlarged vacuoles. This poses a question, which type of vacuoles swell up? What kind of cell signals are disrupted?

*Cp*MRP holds the great potential to study the entire sequestration/secretion process of Type III exocrine glands in insects. I was able to show that *Cp*MRP is required to maintain the production of defensive secretions in *C. populi*. In other words, knocking-down this specific transporter disrupts the secretion/exocytosis mechanism within the sequestration process. Interrupting the secretion process at a specific step is clearly insufficient to unravel a complete transport system applied by insects to translocate plant secondary metabolites through the insects body. But investigating it as a starting point, key processes could be elucidated. Differential expression analysis of *Cp*MRP-silenced defensive glands in comparison to control samples resulted in a total amount of 547 differentially expressed transcripts (unpublished data). Among them are a lot of interesting candidates which may influence *Cp*MRPs trafficking, its regulation and

signaling. However, ten putative transport proteins were differentially expressed as well. It is likely that the downregulation of *Cp*MRP also affects the postulated selective transporter in the hemolymph-exposed plasma membrane of the secretory cell.

Moreover, I expected an influence on the transcription level of genes involved in xenobiotic detoxification mechanisms. In general, these sequences, including genes encoding esterases, glutathione-S-transferases and cytochrome P450s as well as stress response genes, are being upregulated in *Cpmrp*-silenced larvae compared to *gfp*-injected control larvae. Among the ten differentially expressed transporter candidates were four sugar transporter, all similarily being upregulated in *Cpmrp*-silenced larvae. Sugars are assumed to be important in the glandular system for two reasons. First, they may drive the essential ATP production in this tissue. Considering the extraordinarily high expression of *Cp*MRP, I suggest an accordingly high glucose/trehalose consumption. The other important role sugars may play in the defensive glands is probably their essential role in keeping up the osmotic driving force in the defensive reservoirs. An upregulated expression level of sugar transporters implies a regulatory connection of *Cp*MRP to these above mentioned processes.

In summary, I would put forward a general concept suggesting that among Chrysomelina larvae the key physiological task of the MDR-ABC transporters is to provide a general homeostasis control and resistance to phytochemicals as well as a flexible basis for sequestration processes to set up their own defense. Our findings help to elucidate cell biological parameters in the sequestration process, but also shed light on the evolution of diverse defense systems in leaf beetles.

4.4 Evolutionary aspects on sequestration in Chrysomelina species in context of MDR-ABC transporters

The phenomenon of sequestering toxic phytochemicals by insect herbivores supports the idea of co-evolution [17]. During co-evolution insects learned to deal with plant defense responses leading to a kind of “herbivore-plant warfare”. In this context, xenobiotic detoxification mechanisms evolved by insects play a crucial role. Section 4.2 clearly

demonstrates MDR-ABC transporters as a key element in the complex defense system of the obligate sequestering leaf beetle larvae, *C. populi*.

Transport proteins take up a central position when investigating evolutionary of sequestration in insects [35, 53, 139]. In general, transport processes are considered as a bottleneck and control the selective translocation of plant-derived metabolites during sequestration [65]. Based on the arguments discussed in 4.2 and 4.3 I have postulated a general concept of MDR-ABC transporters providing resistance to phytochemicals accompanied by economically using plant glucosides for their own defense. In particular, potential evolutionary aspects in context of MDR-ABC transporters influencing the sequestration among Chrysomelina larvae will be addressed in this section.

The huge diversity of chrysomelid beetles specialised on a broad range of host plants offers an ideal tool to study the mechanisms of adaptive evolution. Within the taxon Chrysomelina, the larval defenses based on exocrine glandular are remarkable and well studied. For a variety of species, the chemical components of defensive secretions, their biosynthetic steps within the larvae as well as their efficacies and host plant origin are investigated [44, 46, 55, 61, 62, 104, 140]. Additionally, highly interesting studies are available on host plant specialization [105,141], digestive adaptation of chrysomelids to plant toxins [133], host plant location [142,143], induced plant defenses by chrysomelid oviposition [143] as well as mate recognition shaped by host plants [144]. Altoghether these data build up the fundament to comparatively address questions related to "prerequisites", constraints, adaptations, pre-adaptations, dependence and evolution of insect chemical defense in a specialist herbivore host plant interaction. However, there is still more research needed on the genetic basis of these processes to understand adaptive evolution [145].

In Chrysomelina larvae, molecular phylogenies point towards the evolution of sequestering plant glucosides being a secondary event which occurred in beetles already chemically defended [46]. The ancestral toxin stored in the reservoirs are iridoid glucosides. For the sequestration of these compounds concepts exist that a highly specific transport mechanisms most probably have evolved independently multiple times in insects [53, 139]. Such parallel evolution is strongly considered to be significantly beneficial [53].

Investigating evolutionary aspects regarding the phytogenic glucoside sequestration in leaf beetles with different biosynthesis strategies (Fig. 1.2) it is imperative to study transport processes on molecular level. Since transport proteins control the absorption, excretion and selective glandular import, they take up a key position especially when changing the diversity of deterrents in the secretions or plant defenses. Consequently, they might have a significant impact on the diversification not only of plants and herbivores itselves, but also on tritrophic interaction partners.

As summarized in section 4.1, Chrysomelina leaf beetles have evolved an efficient network of transport proteins which seems to be involved in the translocation of phytogenic or *de novo* produced precursors in their reservoirs. In general, only the glucoside precursor of the genuine chemical defense (e.g., salicylaldehyde for *C. populi*) is imported and channeled via hemolymph transport through the larval body to the glandular reservoir for the final toxification reactions. The network of MDR-ABC transporters within *C. populi* (see section 4.2) seems to represent the fundamental principle for effective host plant plant-adaptations prerequistite for sequestering phytochemicals. Studies on *Cp*MRP (see 4.2 and 4.3) further highlight the promiscuous "defense" mechanism of ABC transporter as fundamental flexible basis for sequestration processes to set up leaf beetles own defense in evolutionary time.

Host plants of the Chrysomela species significantily influence the chemical composition of the exocrine secretion of the larvae [146]. *C. lapponica* species feeding on *Betulaceae* have developed a new biosynthetic pathway for this genus. However, the glucoside transport seems to share similarities in different Chrysomelina species, irrespective of the mode of their chemical defense [40,65,81,106]. This can be corroborated by *Cp*MRP homologs in other Chrysomelina species, namely *C. lapponica* and *P. cochleariae* as representatives of different chemical defense strategies. The ancient *de-novo*-producing species *P. cochleariae* also possess the ability to selectively uptake glucosidically bound 8-hydroxygeraniol [47,106]. This strongly implicates that the larvae may employ both, endogenous and exogenous pools of the iridoid precursors. Further, I have identified sequences highly identical (96% on amino acid level) to *Cp*MRP in the larval glands of willow- and birch-feeding populations of *C. lapponica*. The amino acid sequences between the two populations were almost identical (99%) which suggests that willow-feeders are already pre-adapted to overcome the chemical constraints of a new host

plant. The host shift to birch itself is probably due to reason a complex engagement of abiotic and biotic factors or might be achieved only through the existence of a ‘‘phytochemical bridge’’ [46, 141, 147, 148].

Consequently, the involvement of broad-spectrum ABC transporters into the sequestration of plant derived metabolites enables insects a dynamic host plant use. Thereby, this peculiar import system facilitates the occasional host plant shifts of leaf beetles caused by parasite pressure [149]. After the shift to a new host plant, only the selective transport element needs to adjust to the new metabolites; all other transport elements may remain unchanged due to their broad substrate tolerance.

The diversity of ABC transcripts in *C. populi,* presented in section 4.2, may contribute to our understanding of cellular and physiological functions in its entirety. Duffey already described the sequestration as an encompassing mechanism of high complexity. Its understanding would require interdisiplinary integration of ideas [29]. To date, we are far away understanding the interplay of all factors which might play a role within the sequestration of insects and why allomonal nutritional concomitants of nutrients do not induce toxicity in the insect. The identification of *Cp*MRP as a non-selective pacemaker involved in the sequestration of plant-derived glucosides seems to hold the potential to identify at least some more crucial factors essential for sequestration processes. In particular, it highlights how insects counter plant chemical defenses to evolve new functions for the plant-derived toxins as allomones.

Research on insect ABC transporters is still in its infancy. However, due to its importance with regard to insecticide resistance it is a major challenge to pest management. In insects, MDR-ABC transporter have been implicated to be involved in the resistance to insecticides [82–85]. Insects even seem to share a tendency of expanded subfamilies more than others (Manuscript III, [84, 94, 102]). The rapid evolution of numerous MDR-ABC transporters may have contributed to the evolutionary diversity of insects by improving their resistance to toxic plant secondary metabolites [102]. Moreover, an ABC transporter was recently identified to play a role in Bt-resistance as well. The ABC protein may be crucial in the pore forming mode of action of Bt-toxins. Mutations in the transporter confer high levels of resistance to Bt, which could pose serious problems for Bt-crops [150].

Recent research on the comprehensive, integrated "interactome" of yeast ABC transporter interactions displays an unexpectedly diverse range of associated interaction partners [151]. This network promises ongoing exciting research on the fundamental cellular role and regulation of ABC transporters in the future.

5 Summary

Plant-herbivore interactions dominate life on earth. Plant secondary metabolites are keyplayers in these interactions. Studies on plant-insect co-evolution has focused primarily on the biochemical adaptations of insects to plant secondary metabolites. In particular, leaf beetles (Chrysomelidae) are known for their ability to sequester structurally different phytochemicals for their own defense purposes. During evolution, larvae of the subtribe Chrysomelina evolved nine pairs of specialized exocrine glands, from which defensive secretions are released in case of disturbance. Precursors of the main compound in the secretions are either produced *de novo* or sequestered. Chrysomelina larvae possess a sophisticated network for the translocation of plant derived glucosides from their hosts, which serve as precursors for compounds in their defensive secretions. Although the importance of carriers involved in these sequestration processes is underlined in several studies, no transport protein in any insect order has been unambiguously identified yet. The goal of this thesis was to identify and characterize transport proteins, which are involved in the complex sequestration of phytogenic glucosides by leaf beetle larvae. Larvae of the poplar leaf beetle *C. populi* were investigated and with regard to their defense strategy, they served as example for an obligate sequestering species within the Chrysomelina subtribe. Larvae transport the host plant specific glucoside salicin to its defensive glands and convert it to volatile salicylalhdehyde.

We succeded to elucidate a more detailed sequestration model based on newly identified ABC transporters in *C. populi*. This model is expecially based on the molecular characterization of an heteromeric ABC transporter *Cp*MRP. This transporter shares significant homology to the members of the ABCC branch of eukaryotic ABC transporters. Moreover, observations to *Cp*MRP open many questions regarding the secretion process in leaf beetle larvae waiting to be answered which are adressed and discussed in this dissertation. The key role of *Cp*MRP has been identified via

expression profiling in combination with RNAi-silencing experiments. *Cp*MRP is highly expressed in the glands of *C. populi*. Immunohistochemical localization studies confirmed that *Cp*MRP is exclusively localized inside the secretory cells. Silencing of *Cp*MRP *in vivo* by RNAi leads to beetles growing normally, but losing their ability to respond to stimulation with droplets of defensive secretions, clearly suggesting that this transporter plays a key role in sequestration. In addition, transport of a series of glucosides relevant to the defense mechanism of leaf beetles, could be shown by expressing *Cp*MRP in *Xenopus laevis* oocytes followed by transport experiments. *Cp*MRP did not discriminate between the glucoside as precursors or non-precursor molecules indicating broader substrate selectivity of the ABC transporter. Confirming previous data, *Cp*MRP recognizes the glucoside moiety as a key chemical element for transport. Based on the findings to *Cp*MRP, we postulate a general functional arrangement of non-selective and selective transport proteins in the exocrine glands of Chrysomelina species. This might be responsible for efficient sequestration supported by the identification of *Cp*MRP homologs in different leaf beetles (*P. cochleariae* and *C. lapponica*). With respect to the uniform architecture and morphology of Type III exocrine gland cells, this thesis further provides a comparative discussion considering possible *Cp*MRP homologs in other insects and in a general concept of the sequestration model for exocrine glands. By tissue specific profiling, we provide information about the level of all predicted ABC transporter and thereby its potential functional importance in a specific larval tissue. Especially ABC transporters of the subfamily seem to facilitate an effective toxin clearance of plant secondary metabolites via the Malpighian tubules.

Overall, the data presented in this dissertation implies a key physiological task of MDR-ABC transporters within Chrysomelina larvae. On one hand they are providing a general homeostasis control and resistance to phytochemicals, and on the other hand they generate a flexible basis for sequestration processes. Their network seems to represent the fundamental principle for effective host plant plant-adaptations as prerequistite for sequestering phytochemicals. Together, these findings not only shed light on the evolution of diverse defense systems in leaf beetles, but also might help to elucidate cell biological parameters and interactions in the sequestration/secretion process.

6 Zusammenfassung

Das Leben auf unserer Erde wird zu einem großen Teil von Wechselbeziehungen zwischen Pflanzen und Herbivoren bestimmt. In solchen Interaktionen nehmen pflanzliche Sekundärmetabolite eine Schlüsselposition ein. In bisherigen Studien zur Coevolution zwischen Pflanzen und Insekten lag der Fokus vor allem auf der biochemischen Anpassung von Insekten an die Sekundärmetabolite ihrer Wirtspflanzen. Die Blattkäfer (Chrysomelidae) sind besonders für ihre Fähigkeit bekannt, eine Vielfalt von strukturell verschiedenen Pflanzenstoffen zu sequestrieren und diese für ihre Verteidigung zu nutzen. Im Laufe der Evolution entwickelten die Larven des Blattkäfer-Subribus Chrysomelina neun paarige, hoch spezialisierte, exokrine Wehrdrüsen, mit deren Hilfe sie bei Gefahr ein Wehrsekret absondern können. Die chemischen Vorstufen ihrer Hauptabwehrsubstanzen im Sekret können entweder *de novo* produziert oder aus ihrer pflanzlichen Nahrung sequestriert werden. Chrysomelina Larven besitzen ein ausgeklügeltes Netzwerk für die Translokation von Glucosiden ihrer Wirtpflanzen, welche als Vorläufer für Verbindungen ihres Wehrsekretes dienen. Obwohl die Bedeutung von Carrier Proteinen bei derartigen Sequestrierungsprozessen in verschiedenen Studien unterstrichen wurde, sind bis heute keine Transportproteine in Insekten eindeutig identifiziert worden. Das Ziel dieser Arbeit war die Identifizierung und Charakterisierung von Transportproteinen, welche am komplexen Sequestrierungsprozess von phytogenen Glucosiden in Blattkäferlarven beteiligt sind. Hierbei wurden die Larven des Pappelblattkäfers *C. populi* untersucht, welche hinsichtlich ihrer Verteidigungsstrategie als Modell für eine obligate Sequestrierungsstrategie innerhalb des Chrysomelina-Subtibus dienten. Die Larven von *C. populi* transportieren das wirtspflanzenspezifische Glucosid Salicin, welches sie in ihren Wehrdrüsen in flüchtiges Salicylalhdehyde umwandeln.

Im Rahmen dieser Arbeit ist es uns gelungen, neue ABC-Transporter in *C. populi* zu identifizieren, auf deren Basis ein detailierteres Sequestrierungsmodell aufgestellt

werden konnte. Dieses Modell basiert insbesondere auf der molekularen Charakterisierung von *Cp*MRP - eines heteromeren ABC Transporters. Dieser Transporter teilt eine signifikante Homologie zu den Mitgliedern der ABCC Unterfamilie eukaryotischer ABC-Transporter. Darüber hinaus konnten im Rahmen dieser Arbeit viele neue Fragestellungen hinsichtlich der Sekretionsprozesse in Blattkäfern aufgeworfen und diskutiert werden. Die Schlüsselrolle von *Cp*MRP wurde mit Hilfe von Expressionsdaten in Kombination mit RNAi-Silencing-Experimenten identifiziert. *Cp*MRP ist in den Wehrdrüsen von *C. populi* hoch exprimiert. Anhand immunohistochemischer Lokalisierungsstudien wurde die exklusive, intrazelluläre Lokalisation von *Cp*MRP in den sekretorischen Zellen von *C. populi* bestätigt. Ein gezieltes Ausschalten des *Cp*MRP Gens durch die RNAi-Technik belegt die Schlüsselrolle des Transporters bei Sequestrierungsprozessen. Die Käferlarven entwickeln sich normal, verlieren jedoch ihre Fähigkeit, auf die Stimulation mit Tröpfchen von Wehrsekret zu reagieren. Zusätzlich konnte der *Cp*MRP-vermittelte Transport einer Reihe von Glucosiden, welche für die Verteidigung von Blattkäfern relevant sind, in *Xenopus laevis* Oozyten gezeigt werden. *Cp*MRP weist eine breite Substratselektivität auf und unterscheidet hinsichtlich seiner Transportaktivität nicht zwischen Vorläufer- und Nicht-Vorläuferglucosiden. Basierend auf den Erkenntnissen zu *Cp*MRP postulieren wir eine allgemeine, funktionale Anordnung von nicht-selektiven und selektiven Transportproteinen in den exokrinen Drüsen innerhalb des Chrysomelina-Subtribus. Diese Hypothese wird durch die Identifizierung von homologen Genen zu *Cp*MRP in verschiedenen Blattkäfern (*P. cochleariae* and *C. lapponica*) gestützt. Zusätzlich würde eine solche Anordnung eine effiziente Sequestrierung gewährleisten. Weiterhin stellt diese Arbeit eine vergleichende Diskussion zur Berücksichtigung von *Cp*MRP-Homologen in anderen Insekten als ein allgemeines Konzept der Sequestrierung in exokrinen Drüsen von Insekten an. Mit Hilfe eines gewebespezifischen Expressionsprofils aller für *C. populi* vorhergesagten ABC-Transporter liefert diese Arbeit erste Informationen über eine potentielle funktionelle Bedeutung in einem bestimmten Larvengewebe. Besonders ABC-Transporter der Unterfamilie C scheinen eine wirksame Exkretion toxischer Sekundärmetabolite über die Malpighischen Gefäße zu gewährleisten.

Insgesamt implizieren die in dieser Arbeit vorgestellten Daten eine physiologische Schlüsselfunktion von MDR-ABC-Transportern in Chrysomelina Larven. Auf der einen

Seite verleihen sie eine generelle Kontrolle der Homöostase und Resistenz gegenüber sekundären Pflanzenstoffe und auf der anderen Seite ermöglichen sie eine flexible Grundlage für Sequestrierungsprozesse. Ihr Netzwerk scheint sowohl ein Grundprinzip für die effektive Anpassung an Wirtspflanzen, als auch die Grundvorraussetzung für die Sequestrierung von sekundären Pflanzenstoffen zu bieten. Zusammen beleuchten die Ergebnisse dieser Arbeit nicht nur evolutionäre Aspekte der Abwehrstrategien in Blattkäfern, sondern auch zellbiologische Parameter des Sequestrierungs-/Sekretionsprozesses und könnten helfen diese Zusammenhänge aufzuklären.

List of Abbreviations

ABC transporter	ATP-binding cassette transporter
ATP	Adenosintriphosphat
BLAST	Basic Local Alignment Search Tool
C. lapponica	*Chrysomela lapponica*
C. populi	*Chrysomela populi*
*Cp*MRP	*Chrysomela populi* multidrug resistance-associated protein
CYP	cytochrome P-450
GLUT	glucose transporter
MDR	multidrug resistance protein
MRP	multidrug resistance-associated protein
NBD	nucleotide-binding domain
P. cochleariae	*Phaedon cochleariae*
P-gp	P-glycoprotein
RNAi	RNA interference
RNA-Seq	RNA-Sequencing
SLC	Solute Carrier
SGLT	sodium-glucose linked transporter
TMD	transmembrane domain
T. castaneum	*Tribolium castaneum*

List of Figures

Bibliography

[1] Mayhew P. J.. Why are there so many insect species? Perspectives from fossils and phylogenies *Biological reviews of the Cambridge Philosophical Society.* 2007;82:425–54. 1

[2] Mora C., Tittensor D. P., Adl S., Simpson A. G. B., Worm B.. How many species are there on Earth and in the ocean? *PLoS biology.* 2011;9:e1001127. 1

[3] Grimaldi D., Engel M. S.. *Evolution of the Insects (Cambridge Evolution Series).* Cambridge University Press 2005. 1, 1.1

[4] Strong D. R.. *Insects on Plants: Community Patterns and Mechanisms* . 1984. 1

[5] Bernays E. A., Graham M.. On the evolution of host specificity in phytophagous arthropods *ecology.* 1988;69:886–892. 1

[6] Bernays E. A., Chapman R. F.. *Host-plant selection by phytophagous insects.* CHapman & Hall 1994. 1

[7] Jaenike J. Host Specialization In Phytophagous Insects *Annual Review of Ecology and Systematics.* 1990;21:243–273. 1

[8] Jolivet P., Hawkeswood T. J.. *Host plants of Chrysomelidae of the world.* Backhuys Publishers, Leiden 1995. 1, 1.3

[9] Labandeira C.C.. Early history of arthropod and vascular plant associations *Annual Review of Earth and Planetary Science.* 1998;26:329–77. 1

[10] Marquis R. J.. Herbivores rule *Science (New York, N.Y.).* 2004;305:619–21. 1, 1.1

[11] Thompson J. N.. Coevolution and Alternative Hypotheses on Insect / Plant Interactions *Special Feature - Insect Host Range.* 1988;69:893–895. 1, 1.1

[12] Futuyma D. J., Agrawal A. A.. Macroevolution and the biological diversity of plants and herbivores *Proceedings of the National Academy of Sciences of the United States of America.* 2009;106:18054–18061. 1, 1.1

[13] Ehrlich P. R., Raven P. H.. Butterflies and Plants: A Study in Coevolution *Evolution.* 1964;18:586–608. 1, 1.1

[14] Jander G., Howe G.. Plant interactions with arthropod herbivores: state of the field. *Plant physiology.* 2008;146:801–3. 1.1

[15] Lewinsohn T. M., Novotny V., Basset Y.. INSECTS ON PLANTS: Diversity of Herbivore Assemblages Revisited *Annual Review of Ecology, Evolution, and Systematics.* 2005;36:597–620. 1.1

[16] Berenbaum M. R., Zangerl A. R.. Facing the Future of Plant-Insect Interaction Research: Le Retour a la "Raison d«Etre" *Plant physiology.* 2008;146:804–11. 1.1, 4.1

[17] Macías F., Galindo J. L. G., Galindo J. C. G.. Evolution and current status of ecological phytochemistry. *Phytochemistry.* 2007;68:2917–36. 1.1, 4.4

[18] Fraenkel G. S.. The Raison d'Etre of Secondary Plant Substances *Science.* 1959;129:1466–1470. 1.1

[19] Fritz R., Simms E.. *Plant Resistance to Herbivores and Pathogens: Ecology, Evolution and Genetics.* Chicago: University of Chicago Press 1992. 1.1

[20] Castellanos I., Espinosa-García F. J.. Plant secondary metabolite diversity as a resistance trait against insects: a test with Sitophilus granarius (Coleoptera: Curculionidae) and seed secondary metabolites *Biochemical Systematics and Ecology.* 1997;25:591–602. 1.1

[21] Self L.S., Guthrie F.E., Hodgson E.. Metabolism of Nicotine by tabacco feeding insects 1964;204:300–301. 1.1, 4.2.1

[22] Hesbacher S., Baur B., Baur A., Proksch P.. Sequestration of lichen compounds by three species of terrestrial snails. *Journal of chemical ecology.* 1995;21:233–46. 1.1

[23] Ruiz-Sanchez E., Van Walderveen M.C., Livingston A., O'Donnell M.J.. Transepithelial transport of salicylate by the Malpighian tubules of insects from different orders. *Journal of insect physiology.* 2007;53:1034–45. 1.1

[24] Li X., Schuler M., Berenbaum M. R.. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annual review of entomology.* 2007;52:231–53. 1.1, 4.2

[25] Wittstock U., Agerbirk N., Stauber E. J., et al. Successful herbivore attack due to metabolic diversion of a plant chemical defense. *Proceedings of the National Academy of Sciences of the United States of America.* 2004;101:4859–64. 1.1

[26] Engler H. S., Spencer K. C., Gilbert L. E.. Insect metabolism: Preventing cyanide release from leaves *Nature.* 2000;406:144–5. 1.1

[27] Engel P., Moran N. A.. The gut microbiota of insects - diversity in structure and function. *FEMS microbiology reviews.* 2013;37:699–735. 1.1

[28] Shen S. K., Dowd P. F.. Detoxification spectrum of the cigarette beetle symbiont Symbiotaphrina kochii in culture *Entomologia Experimentalis et Applicata.* 1991;60:51–59. 1.1

[29] Duffey S. S.. Sequestration of plant natural products by insects *Annual Review of Entomology.* 1980;25:447–477. 1.1, 1.2, 4.1, 4.3, 4.4

[30] Nishida R.. Sequestration of defensive substances from plants by Lepidoptera. *Annual review of entomology.* 2002;47:57–92. 1.1, 1.2, 4.3

[31] Opitz S. E. W., Müller C.. Plant chemistry and insect sequestration *Chemoecology.* 2009;19:117–154. 1.1, 1.2, 4.1

[32] Pasteels J. M., Gregoire J. C., Rowellrahier M.. The chemical ecology of defense in arthropods *Annual Review of Entomology.* 1983;28:263–289. 1.1

[33] Hare J Daniel. Ecology. How insect herbivores drive the evolution of plants. *Science (New York, N.Y.).* 2012;338:50–1. 1.1

[34] Agrawal A. A., Hastings A. P., Johnson M. T. J., Maron J. L., Salminen J-P.. Insect herbivores drive real-time ecological and evolutionary change in plant populations *Science (New York, N.Y.).* ;338:113–6. 1.1

[35] Hartmann T., Theuring C., Schmidt J., Rahier M., Pasteels J. M.. Biochemical strategy of sequestration of pyrrolizidine alkaloids by adults and larvae of chrysomelid leaf beetles. *Journal of insect physiology.* 1999;45:1085–1095. 1.2, 1.5.2, 4.1, 4.2.1, 4.4

[36] Rothschild M., Reichstein T.. *Some problems associated with the storage of cardiac glycosides by insects.* Halle: Nova Acta Leopoldina 1976. 1.2, 4.1

[37] Rimpler H.. Sequestration of iridoids by insects in *Ecological chemistry and biochemistry of plant terpenoids.* (Harborne J. B., Tomas-Barberan F. A.. , eds.):314–330Clarendon Press, Oxford 1991. 1.2, 4.1

[38] Zagrobelny M., Bak S., Mø ller B. L.. Cyanogenesis in plants and arthropods *Phytochemistry.* 2008;69:1457–68. 1.2, 4.1

[39] Baden C. U., Geier T., Franke S., Dobler S.. Sequestered iridoid glycosides - Highly effective deterrents against ant predators? *Biochemical Systematics and Ecology.* 2011;39:897–901. 1.2, 1.3

[40] Pasteels J. M., Duffey S., Rowell-Rahier M.. Toxins in chrysomelid beetles Possible evolutionary sequence from de novo synthesis to derivation from food-plant chemicals. *Journal of chemical ecology.* 1990;16:211–22. 1.2, 1.3, 1.3.2, 1.3.3, 4.4

[41] Dobler S., Daloze D., Pasteels J. M.. Sequestration of plant compounds in a leaf beetle's defensive secretion: cardenolides in Chrysochus *Chemoecology.* 1998;8:111–118. 1.2, 1.3

[42] Trigo J. R.. The chemistry of antipredator defense by secondary compounds in neotropical lepidoptera: facts, perspectives and caveats *Journal of the Brazilian Chemical Society.* 2000;11:551–561. 1.2

[43] Bowers M. D.. Recycling plant natural products for chemical defense in *Insect Defenses: Adaptive Mechanisms and Strategies of Prey and Predators* (Evans D. L., Schmidt D. O.. , eds.)ch. 13, :353–386State University of New York Press, Albany 1990. 1.2

[44] Rowell-Rahier M., Pasteels J. M.. Economics of chemical defense in chrysomelinae *Journal of chemical ecology.* 1986;12:1189–203. 1.2, 1.3, 1.3.2, 4.4

[45] Pasteels J. M., Eggenberger F., Rowell-Rahier M., Ehmke a., Hartmann T.. Chemical defense in chrysomelid leaf beetles *Naturwissenschaften.* 1992;79:521–523. 1.2

[46] Termonia A., Hsiao T. H., Pasteels J. M., Milinkovitch M. C.. Feeding specialization and host-derived chemical defense in Chrysomeline leaf beetles did not lead to an evolutionary dead end. *Proceedings of the National Academy of Sciences of the United States of America.* 2001;98:3909–14. 1.2, 1.2, 1.3.2, 1.3.3, 4.1, 4.4

[47] Feld B. K., Pasteels J. M., Boland W.. Phaedon cochleariae and Gastrophysa viridula (Coleoptera: Chrysomelidae) produce defensive iridoid monoterpenes de novo and are able to sequester glycosidically bound terpenoid precursors *Chemoecology.* 2001;198:191–198. 1.2, 1.5.2, 1.5.3, 4.1, 4.4

[48] Pasteels J. M., Rowell-Rahier M., Braekman J. C., Daloze D.. Chemical defences in leaf beetles and their larvae: The ecological, evolutionary and taxonomic significance *Biochemical Systematics and Ecology.* 1984;12:395–406. 1.2, 1.3.2, 4.1

[49] Farrell B. D.. "Inordinate Fondness" Explained: Why Are There So Many Beetles? *Science.* 1998;281:555–559. 1.3

[50] Gómez-Zurita J., Hunt T., Kopliku F., Vogler A. P.. Recalibrated tree of leaf beetles (Chrysomelidae) indicates independent diversification of angiosperms and their insect herbivores. *PloS one.* 2007;2:e360. 1.3

[51] Laurent P., Braekman J. C., Daloze D., Pasteels J. M.. Biosynthesis of Defensive Compounds from Beetles and Ants *European Journal of Organic Chemistry.* 2003:2733–2743. 1.3

[52] Gillespie J. J., Kjer K. M., Duckett C. N., Tallamy D. W.. Convergent evolution of cucurbitacin feeding in spatially isolated rootworm taxa (Coleoptera: Chrysomelidae; Galerucinae, Luperini). *Molecular phylogenetics and evolution.* 2003;29:161–75. 1.3

[53] Pasteels J. M., Hartmann T.. Sequestration of pyrrolizidine alkaloids in Oreina and Platyphora leaf beetles: physiological, ecological and evolutionary aspects in *New Developments in the Biology of Chrysomelidae*:677–691SPB Academic Publishing by The Hague, The Netherlands 2004. 1.3, 1.5.2, 4.1, 4.3, 4.4

[54] Pasteels J. M., Rowell-Rahier M., Braekman J. C., Dupont A.. Salicin from host plant as precursor of salicylaldehyde in defensive secretion of Chrysomeline larvae *Physiological Entomology.* 1983;8:307–314. 1.3, 1.3.2, 1.4.1, 1.4.1, 4.2.2

[55] Schulz S., Gross J., Hilker M.. Origin of the defensive secretion of the leaf beetle Chrysomela lapponica *Tetrahedron.* 1997;53:9203–9212. 1.3, 1.3.2, 1.3.3, 4.4

[56] Pasteels J. M., Rowell-Rahier M.. Defensive Glands and secretions as taxonomical tools in the Chrysomelidae *Entomography (2th Symposium on the Chrysomelidae).* 1989;6:423–432. 1.3.1

[57] Hinton H. E.. On a little-known protective device of some chrysomelid pupae *Proceedings of the Royal Society of London, Series B: Biological Sciences.* 1951;26:67–73. 1.3.1, 1.1, 4.3

[58] Noirot C., Quennedy A.. Fine structure of insect epidermal glands *Annual Review of Entomology.* 1974;19:61–80. 1.3.1, 4.3

[59] Dettner K.. Chemosystematics and EVOLUTION OF BEETLE CHEMICAL Defenses *Annual Review of Entomology.* 1987;32:17–48. 1.3.2

[60] Michalski C., Mohagheghi H., Nimtz M., Pasteels J. M., Ober D.. Salicyl alcohol oxidase of the chemical defense secretion of two chrysomelid leaf beetles. Molecular and functional characterization of two new members of the glucose-methanol-choline oxidoreductase gene family. *The Journal of biological chemistry.* 2008;283:19219–28. 1.3.2, 1.4.1

[61] Gross J., Podsiadlowski L., Hilker M.. Antimicrobial activity of exocrine glandular secretion of Chrysomela larvae. *Journal of chemical ecology.* 2002;28:317–31. 1.3.2, 1.4.1, 4.4

[62] Hilker M., Schulz S.. Composition of larval secretion of Chrysomela lapponica (Coleoptera, Chrysomelidae) and its dependence on host plant *Journal of chemical ecology.* 1994;20:1075–93. 1.3.2, 1.3.3, 1.4.1, 4.4

[63] Kuhn J., Pettersson E. M., Feld B. K., et al. Sequestration of plant-derived phenolglucosides by larvae of the leaf beetle Chrysomela lapponica: thioglucosides as mechanistic probes. *Journal of chemical ecology.* 2007;33:5–24. 1.3.2, 1.5.2

[64] Fernandez P., Hilker M.. Host plant location by Chrysomelidae *Basic and Applied Ecology.* 2007;8:97–116. 1.3.3

[65] Kuhn J., Pettersson E. M, Feld B. K., et al. Selective transport systems mediate sequestration of plant glucosides in leaf beetles: a molecular basis for adaptation and evolution. *Proceedings of the National Academy of Sciences of the United States of America.* 2004;101:13808–13. 1.4.1, 1.5.2, 1.5.2, 1.5.3, 4.1, 4.3, 4.4

[66] Escherich K.. *Die Forstinsekten Mitteleuropas: Ein Lehr- Und Handbuch.* Nabu Press 2011. 1.4.1

[67] Novák V. J. A.. *Atlas schädlicher Forstinsekten.* Berlin: VEB Deutscher Landwirtschaftsverlag, Berlin 1992. 1.4.1

[68] Christersson L.. Biomass production of intensively grown poplars in the southernmost part of Sweden: Observations of characters, traits and growth potential *Biomass and Bioenergy.* 2006;30:497–508. 1.4.1

[69] Gomi T., Kimura T., Hirose S.. Seasonal changes in the reproductive and life-history traits of Chrysomela populi L. (Coleoptera: Chrysomelidae) *Entomological Science.* 2008;11:31–38. 1.4.1

[70] Thakur M. L.. Insect Pest Status of Poplars in India *Indian Forester.* 1999;125:866–872. 1.4.1

[71] Urban J.. Occurrence , bionomics and harmfulness of Chrysomela populi L . (Coleoptera , Chrysomelidae) *Journal of Forest Science.* 2006;52:255–284. 1.4.1

[72] Singh A.P., Bhandari R.S., Verma T.D.. Important insect pests of poplars in agroforestry and strategies for their management in northwestern India *Agroforestry Systems.* 2004;63:15–26. 1.4.1

[73] Helbig C.. Verstärkter Käferbefall bei Pappel im Kurzumtrieb *Energieholz.* 2009:1206–1207. 1.4.1

[74] Wallace J. B., Blum M. S.. Refined Defensive Mechanisms in Chrysomela scripta *Annals of the Entomological Society of America.* 1969;62:503–506. 1.4.1

[75] Blum M. S., Brand J. M., Wallace J. B., Fales H. M.. Chemical characterization of the defensive secretion of a chrysomelid larva *Life Sciences.* 1972;11:525–531. 1.4.1

[76] Matsuda K., Sugawara F.. Defensive Secretion of Chrysomelid Larvae Chrysomela vigintipunctata costella (MARSEUL), C. populi L.and Gastrolina depressa BALY (Coleoptera : Chrysomelidae) *Applied entomology and zoology.* 1980;15:316–320. 1.4.1

[77] Denno R. F., Larsson S., Olmstead K. L.. Role of enemy free space and plant quality in host-plant selection *Ecology.* 1990;71:124–137. 1.4.1

[78] Busch W., Saier M. H.. The IUBMB-endorsed transporter classification system. *Molecular biotechnology.* 2004;27:253–62. 1.5.1

[79] Hartmann T., Theuring C., Witte L., Pasteels J. M.. Sequestration, metabolism and partial synthesis of tertiary pyrrolizidine alkaloids by the neotropical leaf-beetle Platyphora boucardi. *Insect biochemistry and molecular biology.* 2001;31:1041–56. 1.5.2

[80] Narberhaus I., Papke U., Theuring C., Beuerle T., Hartmann T., Dobler S.. Direct evidence of membrane transport of host-plant-derived Pyrrolizidine alkaloid N-Oxides in two leaf beetle genera *Journal of Chemical Ecology.* 2004;30:2003–2022. 1.5.2, 4.2.1

[81] Discher S., Burse A., Tolzin-Banasch K., Heinemann S. H., Pasteels J. M., Boland W.. A versatile transport network for sequestering and excreting plant glycosides in leaf beetles provides an evolutionary flexible defense strategy *Chembiochem.* 2009;10:2223–9. 1.5.2, 1.5.2, 4.1, 4.2, 4.2.1, 4.3, 4.4

[82] Heckel D. G.. Ecology. Insecticide resistance after Silent spring. *Science (New York, N.Y.).* 2012;337:1612–4. 1.5.3, 4.4

[83] Buss D. S., Callaghan A.. Interaction of pesticides with p-glycoprotein and other ABC proteins: A survey of the possible importance to insecticide, herbicide and fungicide resistance *Pesticide Biochemistry and Physiology.* 2008;90:141–153. 1.5.3, 4.2, 4.4

[84] Labbé R., Caveney S., Donly C.. Genetic analysis of the xenobiotic resistance-associated ABC gene subfamilies of the Lepidoptera. *Insect molecular biology.* 2011;20:243–56. 1.5.3, 4.2.1, 4.4

[85] Jones C. M., Toé H. K., Sanou A., et al. Additional selection for insecticide resistance in urban malaria vectors: DDT resistance in Anopheles arabiensis from Bobo-Dioulasso, Burkina Faso. *PloS one.* 2012;7:e45995. 1.5.3, 4.4

[86] Debeaujon I., Peeters, A. J. M., Léon-Kloosterziel K. M., Koornneef M.. The TRANSPARENT TESTA12 Gene of Arabidopsis Encodes a Multidrug Secondary Transporter-like Protein Required for Flavonoid Sequestration in Vacuoles of the Seed Coat Endothelium *THE PLANT CELL.* 2001;13:853–872. 1.5.3

[87] Sakai K., Shitan N., Sato F., Ueda K., Yazaki K.. Characterization of berberine transport into Coptis japonica cells and the involvement of ABC protein. *Journal of experimental botany.* 2002;53:1879–86. 1.5.3

[88] Kretzschmar T., Burla B., Lee Y., Martinoia E., Nagy R.. Functions of ABC transporters in plants. *Essays in biochemistry.* 2011;50:145–60. 1.5.3, 1.5.3

[89] Zolnerciks J. K., Andress E. J., Nicolaou M., Linton K. J.. Structure of ABC transporters. *Essays in biochemistry.* 2011;50:43–61. 1.5.3

[90] Higgins C. F.. ABC transporters: from microorganisms to man *Annual review of cell biology.* 1992;8:67–113. 1.5.3

[91] Dean M., Annilo T.. Evolution of the ATP-binding cassette (ABC) transporter superfamily in vertebrates *Annual review of genomics and human genetics.* 2005;6:123–42. 1.5.3

[92] Dermauw W., Osborne E. J., Clark R. M., Grbić M., Tirry L., Van Leeuwen T.. A burst of ABC genes in the genome of the polyphagous spider mite Tetranychus urticae. *BMC genomics.* 2013;14:317. 1.5.3

[93] Holland I. B., Blight M. A.. ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans *Journal of Molecular Biology.* 1999;293:381–399. 1.5.3

[94] Dean M., Rzhetsky A., Allikmets R.. The Human ATP-Binding Cassette (ABC) Transporter Superfamily *genome research.* 2001;11:1156–1166. 1.5.3, 4.4

[95] Dassa E.. Natural history of ABC systems: not only transporters. *Essays in biochemistry.* 2011;50:19–42. 1.5.3

[96] Sorensen J. S., Dearing M. D.. Efflux transporters as a novel herbivore countermechanism to plant chemical defenses. *Journal of chemical ecology.* 2006;32:1181–96. 1.5.3, 4.2

[97] Aurade R. M., Jayalakshmi S. K., Sreeramulu K.. P-glycoprotein ATPase from the resistant pest, Helicoverpa armigera: purification, characterization and effect of various insecticides on its transport function *Biochimica et biophysica acta.* 2010;1798:1135–43. 1.5.3

[98] Lanning C. L., Fine R. L., Corcoran J. J., Ayad H. M., Rose R. L., Abou-Donia M. B.. Tobacco budworm P-glycoprotein: biochemical characterization and its involvement in pesticide resistance *Biochimica et biophysica acta.* 1996;1291:155–62. 1.5.3, 4.2

[99] Slot A. J., Molinski S. V., Cole S.P. C.. Evolution of the ATP-binding cassette (ABC) transporter superfamily in vertebrates *Essays in biochemistry.* 2011;50:179–207. 1.5.3, 4.2

[100] Sarkadi B., Homolya L., Szakács G., Varadi A.. Human multidrug resistance ABCB and ABCG transporters: participation in a chemoimmunity defense system *Physiological* 2006;1:1179–1236. 1.5.3, 4.2

[101] Borst P., Evers R.. A Family of Drug Transporters: the Multidrug Resistance-Associated Proteins *Journal of the National Cancer Institute.* 2000;92:1295–1302. 1.5.3, 4.2

[102] Broehan G., Kroeger T., Lorenzen M., Merzendorfer H.. Functional analysis of the ATP-binding cassette (ABC) transporter gene family of Tribolium castaneum. *BMC genomics.* 2013;14:6. 1.5.3, 4.4

[103] Blum M. S.. *Chemical defenses of arthropods.* London: Academic Press 1981. 4.1

[104] Veith M., Lorenz M., Boland W., Simon H., Dettner K.. Biosynthesis of Iridoid Monoterpenes in Insects - Defensive Secretions from Larvae of Leaf Beetles (Coleoptera, Chrysomelidae) *Tetrahedron.* 1994;50:6859–6874. 4.1, 4.4

[105] Gross J., Fatouros N. E., Hilker M.. The significance of bottom-up effects for host plant specialization in Chrysomela leaf beetles *Oikos.* 2004;105:368–376. 4.1, 4.4

[106] Kunert M., Sø e A., Bartram S., et al. De novo biosynthesis versus sequestration: a network of transport systems supports in iridoid producing leaf beetle larvae both modes of defense. *Insect biochemistry and molecular biology.* 2008;38:895–904. 4.1, 4.2, 4.2.2, 4.3, 4.2, 4.4

[107] Frick C., Wink M.. Uptake and sequestration of ouabain and other cardiac glycosides inDanaus plexippus (Lepidoptera: Danaidae): Evidence for a carrier-mediated process *Journal of chemical ecology.* 1995;21:557–575. 4.1

[108] Torrie L. S., Radford J. C., Southall T. D., et al. Resolution of the insect ouabain paradox. *Proceedings of the National Academy of Sciences of the United States of America.* 2004;101:13689–93. 4.1, 4.3

[109] Strauss A. S.. Diploma thesis: Funktionelle Expression eines putativen Glucosetransporters aus Blattkäferlarven in Xenopus laevis Oozyten tech. rep.- Friedrich-Schiller-Universität Jena 2008. 4.1

[110] Gaertner L. S., Murray C. L., Morris C. E.. Transepithelial transport of nicotine and vinblastine in isolated malpighian tubules of the tobacco hornworm (Manduca sexta) suggests a P-glycoprotein-like mechanism. *The Journal of experimental biology.* 1998;201:2637–45. 4.1, 4.2.1

[111] Petschenka G., Pick C., Wagschal V., Dobler S.. Functional evidence for physiological mechanisms to circumvent neurotoxicity of cardenolides in an adapted and a non-adapted hawk-moth species Functional evidence for physiological mechanisms to circumvent neurotoxicity of cardenolides in an adapted and a *Proceedings of the National Academy of Sciences of the United States of America.* 2013;280:20123089. 4.1, 4.2

[112] Rheault M.R., Plaumann J.S., O'Donnell M.J.. Tetraethylammonium and nicotine transport by the Malpighian tubules of insects. *Journal of insect physiology.* 2006;52:487–98. 4.1

[113] O'Donnell M. J.. Too much of a good thing: how insects cope with excess ions or toxins in the diet. *The Journal of experimental biology.* 2009;212:363–72. 4.1, 4.2

[114] Uldry M., Thorens B.. The SLC2 family of facilitated hexose and polyol transporters. *Pflügers Archiv : European journal of physiology.* 2004;447:480–9. 4.1

[115] Wright E. M., Hirayama B. A., Loo D. F.. Active sugar transport in health and disease *Journal of internal medicine.* 2007;261:32–43. 4.1

[116] Discher S.. *Sequestrierung in Blattkäferlarven - Charakterisierung der Transportnetzwerke glucosidisch gebundener Pflanzenstoffe im Subtribus Chrysomelina.* PhD thesis-Friedrich-Schiller-Universität Jena 2011. 4.1

[117] Gibson A. K., Smith Z., Fuqua C., Clay K., Colbourne J. K.. Why so many unknown genes? Partitioning orphans from a representative transcriptome of the lone star tick Amblyomma americanum. *BMC genomics.* 2013;14:135. 4.1

[118] Wissler L., Gadau J., Simola D. F., Helmkampf M., Bornberg-Bauer E.. Mechanisms and dynamics of orphan gene emergence in insect genomes *Genome biology and evolution.* 2013;5:439–55. 4.1

[119] Maza E., Frasse P., Senin P., Bouzayen M., Zouine M.. Comparison of normalization methods for differential gene expression analysis in RNA-Seq experiments A matter of relative size of studied transcriptomes *Communicative & Integrative Biology.* 2013;6:e258491–8. 4.1

[120] Wang Z., Gerstein M., Snyder M.. RNA-Seq: a revolutionary tool for transcriptomics. *Nature reviews. Genetics.* 2009;10:57–63. 4.1

[121] Bodemann R. B., Rahfeld P., Stock M., et al. Precise RNAi-mediated silencing of metabolically active proteins in the defence secretions of juvenile leaf beetles. *Proceedings. Biological sciences / The Royal Society.* 2012;279:4126–34. 4.1

[122] Polva G.. *Biochemical Targets of Plant Bioactive Compounds.* CRC Press Book 2003. 4.2

[123] Sarkadi B., Szakács G.. Understanding transport through pharmacological barriers–are we there yet? *Nature reviews. Drug discovery.* 2010;9:897–8. 4.2

[124] Lehane M. J.. Peritrophic matrix structure and function *Annual review of entomology.* 1997;42:525–50. 4.2

[125] Barbehenn R. V.. Roles of peritrophic membranes in protecting herbivorous insects from ingested plant allelochemicals *Archives of insect biochemistry and physiology.* 2001;47:86–99. 4.2

[126] Chapman R. F.. *The Insects: Structure and Function.* Cambridge University Press 1998. 4.2

[127] Ohmart C. P., Larsson S.. Evidence for absorption of eucalypt essential oils by paropsis atomaria olivier (coleoptera: chrysomelidae) *Australian Journal of Entomology.* 1989:201–205. 4.2

[128] Southwell I. A., Maddox C. D. A., Zalucki M. P.. Metabolism of 1,8-cineole in tea tree (Melaleuca alternifolia and M. linariifolia) by pyrgo beetle (Paropsisterna tigrina). *Journal of chemical ecology.* 1995;21:439–453. 4.2

[129] Agrawal A. A., Petschenka G., Bingham R., Weber M. G., Rasmann S.. Toxic cardenolides: chemical ecology and coevolution of specialized plant-herbivore interactions *The New phytologist.* 2012;194:28–45. 4.2

[130] Murray C. L., Quaglia M., Arnason J .T., Morris C.E.. A putative nicotine pump at the metabolic blood-brain barrier of the tobacco hornworm. *Journal of neurobiology.* 1994;25:23–34. 4.2

[131] Dietrich C. G., Geier A., Oude Elferink R. P. J.. ABC of oral bioavailability: transporters as gatekeepers in the gut *Gut.* 2003;52:1788–95. 4.2

[132] Chan L. M. S., Lowes S., Hirst B. H.. The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences.* 2004;21:25–51. 4.2

[133] Dobler S., Petschenka G., Pankoke H.. Coping with toxic plant compounds-the insect's perspective on iridoid glycosides and cardenolides *Phytochemistry.* 2011;72:1593–604. 4.2, 4.3, 4.2, 4.4

[134] Tolzin-Banasch K., Dagvadorj E., Sammer U., et al. Glucose and glucose esters in the larval secretion of Chrysomela lapponica; selectivity of the glucoside import system from host plant leaves *Journal of chemical ecology.* 2011;37:195–204. 4.2.2

[135] Eisner T., Johnessee J. S., Carrel J., Hendry L. B., Meinwald J.. Defensive Use by an Insect of a Plant Resin *Science.* 1974;184:996–999. 4.3

[136] Müller C., Hilker M.. Unexpected reactions of a generalist predator towards defensive devices of cassidine larvae (Coleoptera, Chrysomelidae) *Oecologia.* 1999;118:166–172. 4.3

[137] Li J., Lehmann S., Weiß becker B., et al. Odoriferous Defensive stink gland transcriptome to identify novel genes necessary for quinone synthesis in the red flour beetle, Tribolium castaneum. *PLoS genetics.* 2013;9:e1003596. 4.3

[138] Bünnige M., Hilker M.. Larval exocrine glands in the galerucine Agelastica alni L. (Coleoptera: Chrysomelidae): their morphology and possible functions *Chemoecology.* 1999;9:55–62. 4.3

[139] Dobler S.. Evolutionary aspects of defense by recycled plant compounds in herbivorous insects *Basic and Applied Ecology.* 2001;2:15–26. 4.4

[140] Oldham N. J., Veith M., Boland W., Dettner K.. Iridoid monoterpene biosynthesis in insects: Evidence for a de novo pathway occurring in the defensive glands of Phaedon armoraciae (chrysomelidae) leaf beetle larvae *Naturwissenschaften.* 1996;83:470–473. 4.4

[141] Kirsch R., Vogel H., Muck A., Reichwald K., Pasteels J. M., Boland W.. Host plant shifts affect a major defense enzyme in Chrysomela lapponica. *Proceedings of the National Academy of Sciences of the United States of America.* 2011;108:4897–901. 4.4

[142] Matsuda K.. Feeding stimulants of leaf beetles in *Biology of Chrysomelidae* (Jolivet P., Petitpierre E., Hsiao T. H.. , eds.):pp. 41–56Dordrecht: Springer Netherlands 1988. 4.4

[143] Hilker M., Meiners T.. Early herbivore alert: insect eggs induce plant defense. *Journal of chemical ecology.* 2006;32:1379–97. 4.4

[144] Geiselhardt S., Otte T., Hilker M.. Looking for a similar partner: host plants shape mating preferences of herbivorous insects by altering their contact pheromones *Ecology letters.* 2012;15:971–7. 4.4

[145] Jong P. W., Nielsen J. K.. Host plant use of Phyllotreta nemorum: do coadapted gene complexes play a role? *Entomologia Experimentalis et Applicata.* 2002;104:207–215. 4.4

[146] Gross J., Hilker M.. Chemoecological studies of the exocrine glandular larval secretions of two chrysomelid species (Coleoptera):Phaedon cochleariae andChrysomela lapponica *Chemoecology.* 1994;5-6:185–189. 4.4

[147] Gross J., Fatouros N. E., Neuvonen S., Hilker M.. The importance of specialist natural enemies for Chrysomela lapponica in pioneering a new host plant *Ecological Entomology.* 2004;29:584–593. 4.4

[148] Kirsch R., Vogel H., Muck A., Vilcinskas A., Pasteels J. M., Boland W.. To be or not to be convergent in salicin-based defence in chrysomeline leaf beetle larvae: evidence from Phratora vitellinae salicyl alcohol oxidase *Proceedings. Biological sciences / The Royal Society.* 2011;278:3225–32. 4.4

[149] Agosta S. J., Janz N., Brooks D. R.. How specialists can be generalists: resolving the "parasite paradox" and implications for emerging infectious disease *Zoologia (Curitiba, Impresso).* 2010;27:151–162. 4.4

[150] Heckel D. G.. Learning the ABCs of Bt: ABC transporters and insect resistance to Bacillus thuringiensis provide clues to a crucial step in toxin mode of action *Pesticide Biochemistry and Physiology.* 2012;104:103–110. 4.4

[151] Snider J., Hanif A., Lee M. E., et al. Mapping the functional yeast ABC transporter interactome *Nature chemical biology.* 2013;9:565–72. 4.4

Danksagung

Ganz herzlich danken möchte ich Herrn Prof. Dr. Boland, der es mir ermöglicht hat am Max-Planck-Institut für chemische Ökologie mit seinen ausgezeichneten Forschungsmöglichkeiten promovieren zu dürfen. Insbesondere danke ich ihm für sein stetes Interesse sowie seine uneingeschränkte Unterstützung bei der Bearbeitung des Themas.

Ich bedanke mich ganz herzlich bei Dr. Antje Burse, für die wissenschaftliche Betreuung und Unterstützung während der gesamten Arbeit, vor allem für den uneingeschränkten Freiraum meine Ideen auch in ergebnislosen Zeiten weiter umzusetzen.

Sven Peters, dir danke ich für die tolle Zusammenarbeit. Ich schätze nicht nur deine Genauigkeit, Neugier und fortwährende Diskussionsbereitschaft, sondern auch als sehr guter Freund, danke ich dir von ganzem Herzen! Auf eine weitere Zusammenarbeit freue ich mich schon jetzt!

Bei Herrn Prof. Dr. Heinemann möchte ich mich für die Möglichkeit der Oozytenpräparation in seiner Arbeitsgruppe bedanken. Außerdem danke ich ihm für Ratschläge und Anregungen bei der Arbeit mit Oozyten und Transportproteinen. Mein besonderer Dank geht dabei an Angela Roßner für ihre Hilfe mit den Oozyten.

My special thanks goes to Dr. Andras Varadi for giving me the opportunity to work two weeks in his group. I am very thankful to all the members of his working group from the Institute of Enzymology in Budapest, sharing their extensive knowledge of ABC transporters. Especially, I thank Dr. Flora Szeri and Dr. Gergely Szakacs for discussions.

I am thankful to Prof. Jacques M. Pasteels for sending me several publications for fruitful discussions.

I would like to thank all members of the Bioorganic Chemistry Department and colleagues from other institute departments for their support and contribution to a fruitful working athmosphere. In particular I am grateful to the following persons:

Regina Stieber for her help with immunohistological experiments.

Astrid Groot for giving me the opportunity to work in her lab and together with Susanne Donnerhacke sharing their knowledge of the AFLP-PCR-method.

Prof. Dr. David Heckel for fruitful discussions.

Dr. Roy Kirsch for sharing cDNA samples of *C. lapponica* with me.

Dr. Andreas Habel, Dr. Maritta Kunert and Dr. Karla Tolzin-Banasch, Kerstin Ploss for helping me with chemical analytical questions.

Anja David and Dr. Maritta Kunert helping me taking samples of larvae secretions.

Dr. Anne Morgenstern, Gerhard Pauls, Hassan Salem and Holger Merker for their help in proof-reading and their friendship.

Sandra Klemmer and Franziska Eberl for their help with the project as being the best trainees or research assistents, respectively.

Angelika Berg for her help with rearing of insects and the entire beetle group for their support and discussions.

I thank the library team, the PR-office, the IT-group and the Haustechnik for their support.

Mein ganz besonderer Dank gilt all meinen Freunden in und um Jena. Darunter insbesondere Anja Mai auch für diverses Korrekturlesen. Michi, dir danke ich für unsere eine erfolgreiche Zusammenarbeit während unserer Sprecherperiode und weit darüber hinaus! Ein besonderer Dank geht auch an meinen Freund Christian für den Rückhalt und viele bereichernde Momente.

Nicht zuletzt möchte ich meinen Eltern in besonderem Maße für das Ermöglichen meines Studiums, und zusammen mit meiner Schwester für Eure unentwegte Hilfe sowie für Eure vielseitige und bedingungslose Unterstützung danken!

Danke!

Curriculum Vitae

Anja Susanne Strauß
Dipl. Biochemist

Nonnendorf 4c
07570 Harth-Pöllnitz

Curriculum Vitae

24.12.1979 born, Gera

Research Experience

2008-present **Doctorial Thesis**
Max Planck Institute for Chemical Ecology; Jena, Germany
Supervisor: Prof W. Boland
Dissertation: „Sequestration in leaf beetles:
Identi cation and characterization of ABC transporters involved in the chemical defense of Chrysomelina larvae"

05.2010 **Research stay**
Hungarian Academie of Sciences, Budapest, Hungary;
Advisor: Prof A. Varadi, Dr F. Szeri

09.2007-12.2007 **IAESTE Internship**
Institute of Medical Biology; University of Tromsø, Norway
Advisor: Ass Prof. Peik Haugen
"Identi cation of novel regulatory RNAs in *Vibrio salmonicida* using cDNA libraries and microarray"

10.2006-08.2007 **Diploma Thesis**
Max Planck Institute for Chemical Ecology/FSU Jena, Germany
Supervivor: Prof W. Boland, Prof S. H. Heinemann
„Heterologe Expression und Funktionsanalysen von GLUT1-ähnlichen Transportproteinen aus Insekten in *Xenopus laevis* Oozyten"

2004/2005 **Scienti c assistant**
Center for Molecular Biomedicine, FSU Jena, Germany
Advisor: Prof S. H. Heinemann

Education

2008-present	**PhD student** Max Planck Institute for Chemical Ecology; Jena, Germany
2001-2008	**Diploma Student** Friedrich-Schiller-University Jena; Germany Specialization: Molecular Biotechnology
2003-2004	**Biochemical Studies** University of Calgary, Canada
1998-2001	Notary assistant, Apprenticeship Notary in Weida, Germany
1994-1998	Abitur, Secondary School, Weida, Germany

Publications

2014	Salem, H.; Bauer, E.; Strauss, A. S.; Vogel, H.; Marz, M. and Kaltenpoth, M.; "Vitamin Supplementation by Gut Symbionts Ensures Metabolic Homeostasis in Firebugs"; *Cell Host & Microbe,* in prep.
2014	Strauss, A. S.; Wang, D., Stock, M., Gretscher, R. R., Groth, M., Boland, W. and Burse, A.; "Tissue-speci c transcript pro ling for ABC transporters in the sequestering larvae of the phytophagous leaf beetle *Chrysomela populi*"; submitted to *PloS ONE* (01.2014)
2013	Strauss, A. S.; Peters, S., Boland, W. and Burse, A.; "ABC transporter functions as a pacemaker for sequestration of plant glucosides in leaf beetles"; *eLIFE,* doi: 10.7554/eLife.01096
2012	Ahmad, R.; Hansen, G. Å.; Hansen, H.; Hjerde, E.; Pedersen, H. L.; Nyrud, M.L.J.; Strauss, A. S.; Willassen, N-P.; Haugen, P.; "Prediction, microarray and northern blot analyses identify new intergenic Small RNAs in *Aliivibrio salmonicida*"; *Journal of Molecular Microbiology and Biotechnology*, 22 (6), 352-360.
2009	Burse, A.; Frick, S.; Discher, S., Tolzin-Banasch, K.; Kirsch, R.; Strauss, A. S.; Kunert, M.; Boland, W.; "Always being well prepared for defense: The production of deterrents by juvenile Chrysomelina beetles (Chrysomelidae)" *Phytochemistry*, 70, 1899-1909.

Talks

2013 Strauß, A.; Peters, S.; Boland, W.; Burse, A.; "Beetle juice strategy: ABC transporter functions as a pacemaker for the sequestration of plant glucosides in leaf beetles"
International Chemical Ecology Conference, Melbourne, Australia

2013 Strauß, A.; Peters, S.; Boland, W.; Burse, A.; "Beetle juice strategy: ABC transporter functions as a pacemaker for the sequestration of plant glucosides in leaf beetles", IMPRS Evaluation Symposium, MPI for chemical ecology, IMPRS Jena, Germany

2013 Strauß, A.; Peters, S.; Boland, W.; Burse, A.; "Beetle juice strategy: ABC transporter functions as a pacemaker for the sequestration of plant glucosides in leaf beetles", 12th IMPRS Symposium, MPI for chemical ecology, IMPRS Jena, Germany

2012 Strauß, A.; Peters, S.; Burse, A.; Boland, W.; "Disarming leaf beetle larvae: The crucial role of *Cp*MRP in the defensive system"
ABC2012 - 4th FEBS Special Meeting, Innsbruck, Austria

2011 Strauß, A.; Burse, A.; Boland, W.; "Sequestration of plant glucosides by leaf beetle larvae: The ABC in the transporter network", ISCE Meeting 2011, Burnaby,Vancouver, Canada

2010 Strauß, A.; Burse, A.; Boland, W. : "Network of transport proteins involved in sequestration of plant glucosides within leaf beetles", European Congress of Entomology, Budapest, Hungary

2010 Strauß, A.; Burse, A.; Boland, W.;"How to unravel the molecular transporter network within the chemical defense of leaf beetle larvae.",
9th IMPRS Symposium, MPI for Chemical Ecology, Dornburg

2010 Strauß, A.; Burse, A.; Heinemann, S. H., Boland, W.; "Network of transport proteins involved in the sequestration of plant glucosides within leaf beetles", Postertalk, 7th Transport Colloquium, Rauischholzhausen, Germany

Poster Presentations

2013	Strauß, A.; Peters, S.; Boland, W.; Burse, A.; "Beetle juice strategy: ABC transporter acts as a gatekeeper while unifying sequestration and active defense in leaf beetle larvae" Gordon Research Konferenz - Multi-Drug E ux Systems, Ventura, CA
2012	Strauß, A.; Peters, S.; Burse, A.; Boland, W.; "The role of ABC transporter in leaf beetle larvae defense" Gordon Research Konferenz - Multi-Drug E ux Systems, Les Diablerets, Switzerland
2010	Strauß, A.; Burse, A.; Heinemann, S. H., Boland, W.; "Network of transport proteins involved in the sequestration of plant glucosides within leaf beetles", 7th Transport Colloquium, Rauischholzhausen, Germany
2009	Strauß, A.; Burse, A.; Boland, W.: "Transport of defensive precursors within leaf beetle larvae-putative transport proteins", ISCE Meeting 2009, Neuchatel, Switzerland

Awards

2013	Poster-Presentation-Award, Gordon Research Konferenz - Multi-Drug E ux Systems, Ventura, CA
2012	Young Investigator Award for the best Talk, ABC2012 - 4th FEBS Special Meeting, Innsbruck, Austria
2012	Student Fellowship, ABC2012 - 4th FEBS Special Meeting, Innsbruck, Austria
2011	Student Travel Award, ISCE Meeting 2011, Burnaby, Vancouver, Canada
2011	Poster-Presentation-Award, Gordon Research Konferenz - Multi-Drug E ux Systems, Les Diablerets, Switzerland

Januar 2014

Eigenständigkeitserklärung

Hiermit erkläre ich entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena, dass ich die vorliegende Dissertation selbständig und nur unter Zuhilfenahme der angegebenen Mittel und Literatur angefertigt habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten.

Darüber hinaus erkläre ich, dass ich mich mit der vorgelegten Arbeit an keiner anderen Hochschule um den akademischen Grad *doctor rerum naturalium* (Dr. rer. nat.) beworben und weder früher noch gegenwärtig die Eröffnung eines Verfahrens zum Erwerb des o.g. akademischen Grades an einer anderen Hochschule beantragt habe.

Jena, den 24. Januar 2014 Anja S. Strauß