Sequestration of plant-derived glycosides by leaf beetles: A model system for evolution and adaptation

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Summary Leaf beetles have developed an impressive repertoire of toxins and repellents to defend themselves against predators. Upon attack, the larvae discharge small droplets from glandular reservoirs on their back. The reservoirs are "bioreactors" performing the late reactions of the toxin-production from plant-derived or de novo synthesised glucosides. The import of the glucosides into the bioreactor relies on a complex transport system. Physiological studies revealed a functional network of transporters guiding the glucosides through the larval body into the defensive system. The first of the involved transporters has been identified and characterised concerning selectivity, tissue distribution, and regulation. The development of a well-tuned transport system, perfectly adjusted to the compounds provided by the food plants, provides the functional basis for the leaf beetle defenses and their local adaptation to their host plants.

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Introduction

Phylogenetic analyses of butterflies and plants by Ehrlich and Raven (1964) suggested that coevolution is responsible for the tremendous diversification of plant secondary substances, the huge number of angiosperm plants and the many insects that feed on them. A particular tight connection between host/food plants is manifested in the chrysomelids, with ca. 36,000 described species in 2500 genera. Especially members of the tribe Chrysomelini (ca. 3000 species) are adapted to special host plants on which they feed as monophagous or narrow oligophagous species (Wikipedia, Leaf beetle). To repel their natural enemies, many of these leaf beetles have developed a defensive system consisting of nine pairs of dorsal exocrine glands, which are inserted in the body surface and end in reservoirs containing glandular secretions. In case of predator attack, the larvae compress their glandular reservoirs and present their secretions as small droplets on their back for a few seconds followed by re-import of the precious compounds into the body. The presented toxins vary both in structure and biosynthetic origin. The major components present in the secretion of the ancestral leaf beetle group, covering the taxa Phaedon, Gastrophysa, and Phratora (except Phratora vitellinae) are iridoid monoterpenes, produced de novo via the acetate-mevalonate pathway (Oldham et al., 1996). Larvae of the more advanced Chrysomela spp. and P. vitellinae display secretions in which salicylaldehyde is the sole or major component (Pasteels et al., 1982, 1983). When feeding upon willows (Salicaceae), larvae of these species sequester phenolic glycosides (e.g., salicin) from their host plants and use these compounds as precursors to produce salicylaldehyde (Pasteels et al., 1982, 1983; Wallace and Blum, 1969; Gross et al., 2002; Meinwald et al., 1977; Blum et al., 1978; Pasteels and Rowell-Rahier, 1989). Other species are known to import cardenolides (Termonia and Pasteels, 1999), cucurbitacins (Agrawal et al., 2012), and glucosinolates (Eben et al., 1997) from their host plants. They use these compounds as such or after further processing (e.g., removal of the sugars) for their own defense. In many cases, however, biosynthetic intermediates or sequestered precursors constitute of polar glucosides that cannot pass membranes and, hence, require specialised transport systems for uptake and safe guidance of the compounds through the insect body until they reach the final target tissue. In the subtribe Chrysomelina the compounds accumulate in a large reservoir that functions as a bioreactor for the terminal modifications of the imported glucoside precursors. The basic principles underlying the import, transport and final modifications in the bioreactor to generate the defensive chemicals will be described.

The defensive system of leaf beetles

Evaluation of the transport characteristics in different leaf beetles

The defensive glands of the juvenile Chrysomelina species are arranged in nine pairs on their backs (Fig. 1A and B). According to morphological studies, each defensive gland is composed of a number of enlarged secretory cells, which are in turn connected to a chitin-lined reservoir (Fig. 1C). The secretory cells are always accompanied by two canal cells that form a cuticular canal, which connects the secretory cell with the reservoir (Beran et al., 2014). 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 (Noirot and Quennedey, 1974; Pasteels et al., 1990).

Figure 1  Larval chemical defense of the poplar leaf beetle, Chrysomela populii. (A) Non-stimulated larva of the third instar compared to (B), forceps stimulated larva demonstrating the release of droplets of secretions (black triangle) from the everted reservoirs of the defensive glands which are arranged segmentally in nine pairs along the back. (C) Drawing of a dissected defensive gland according to Hinton (1951).
Accordingly, the glands also secrete enzymes for the final metabolic conversion of the precursors into defensive compounds in the reservoir. The precursors for the biosynthesis of deterrents are either de novo produced by the insect or are sequestered from the food plant (Nishida, 2002). If the precursors are sequestered from the food plants, the glucoside precursors (e.g., phenolic glucosides from salix spp.) have first to pass the gut membrane to reach the hemolymph. Final uptake from the circulating hemolymph into the reservoir requires a second transporter system that is also essential for the uptake of de novo produced endogenous glucoside precursors (Kuhn et al., 2004).

A combination of the pattern of deterrents found in the juvenile leaf beetles with their host plant families mirrors the reciprocal adaptation of Chrysomela beetles to their hosts (Fig. 2). Ancestral species synthesising the deterrents de novo are adapted to feed on different plant families, such as Brassicaceae or Polygonaceae (Fig. 2A). In contrast, Chrysomela members whose larvae sequester selectively salicin and salicortin are adapted exclusively to Salicaceae (Fig. 2B). The non-selective sequestering members of the interrupta-group, in turn, are able to colonise Salicaceae or Betulaceae (Fig. 2C). Besides an almost identical gland anatomy and organisation, all Chrysomela larvae have the first enzymatic reactions occurring in the reservoir in common. They possess an unspecific glucosidase removing the sugar moiety from glucosides which are in turn oxidised (salicylaldehyde) or further processed by a cyclase to give iridoids (Fig. 1A–C) (Noirot and Quennedey, 1974; Kuhn et al., 2004; Soetens et al., 1993). In the highest evolved interrupta group (Chrysomela lapponica) the plant-imported glucosides are hydrolysed and subsequently converted into isobutyrate esters that also serve as repellents (Schulz et al., 1997).

Irrespective of the very different compounds and their diverse origin, their import and transport within the organism share common elements. All sequestered glucosides first enter the gastrointestinal system, where they are exposed to the action of ubiquitous glucosidases. If the ingested glucosides are cleaved, the aglucons become unpolar and may reach the glandular system simply by diffusion. However, for the import of intact glucosides dedicated transport systems are required. To obtain reliable information on the mode of import, stable and non-hydrolysable S-analogues (Nicotra, 1998) of the naturally occurring O-glucosides were painted at natural concentrations onto the leaf surface of the food plants (Kuhn et al., 2004). Then, the larvae were allowed to feed on the pre-treated leaves followed by harvesting the defensive droplets after 48 h from larvae using glass capillaries. Indeed, mass spectroscopic analysis revealed the presence of intact S-glucosides in their defensive secretions indicating that functional transport systems for glucosides are present. The use of S-glucosides provided the first information not only on the involvement of specialised transporters but also revealed their molecular preferences required for the selective uptake of e.g., salicin from Populus leaves shown for larvae of Chrysmela populi and P. vitellinae and for the processing of the iridoid precursor in Phratora laticollis and Hydrothassa marginella (Kuhn et al., 2004; Feld et al., 2001). This established that de novo producers are also able to sequester if appropriate precursors are present in the food (Soe et al., 2004).

According to Fig. 3 it is obvious, that the iridoid-producing juveniles of leaf beetles and the salicyl aldehyde
Figure 3  Survey of transport efficacies as determined for the two leaf beetle groups of iridoid-producing (H. marginella and P. laticollis) and sequestering (C. populi and P. vitellinae) species fed with different thioglucosides (Kuhn et al., 2004).

producers possess dedicated import systems that are perfectly adapted to sequester their genuine precursors, the phenolic glucoside salicin and 8-hydroxygeraniol-8-O-β-D-glucoside. Even minor stereochemical modifications (e.g., m-, p-substitution) are not tolerated and result in a reduced or no import at all. An exchange of glucose by galactose also impairs the transport, suggesting an evolutionary adaptation to the class of compounds to be sequestered. The highest evolved leaf beetles (e.g., Chrysomela interrupta, Fig. 2) are not restricted to the import of single compounds, but sequester a large number of different plant-derived glucosides. After transport to the reservoir, the sugar moiety is removed and the free alcohols are esterified to butyrates (Schulz et al., 1997; Kuhn et al., 2007). In line with the above considerations, the chemical defence in these beetles, rather than appearing constrained by inherited biochemical pathways, appears to be particularly flexible in evolutionary time. Further evaluation of the selectivity of the transport clearly revealed that the import of glucosides through the gut membrane is unselective, and only the transition from the hemolymph into the defensive glands is highly selective. Clearance of the hemolymph from circulating glucosides occurs in a non-selective fashion (Discher et al., 2009). However, released or gut-imported aglucone are reglucosylated in the fat body (Kunert et al., 2008). The advantages of a glucoside based transport network can be summarised as:

- Glucosides are polar compounds, unable to penetrate membranes without dedicated transporters.
- Specific transporters allow addressing of organs or organelles.
- Without hydrolysis glucosides can be safely circulated in the hemolymph.
- Glucosides are easily cleaved into glucose and the aglucone in the ‘‘target organ or cell’’
- Aglucons can be processed to valuable target compounds and/or defences.

Identification of transport elements and biochemical transformations

Information on the involved enzymes is obtained from mass spectroscopic analysis of the defensive secretion containing the involved enzymes along with a large number of proteins with unknown functions. 1D SDS Gel bands were de novo-sequenced by MS/MS analysis after in-gel digestion with trypsin and peptide fragment spectra were searched against the SwissProt database. Spectra unmatched by database searching were interpreted de novo to yield peptide sequences. Together with the cDNA libraries form the defensive glands of species representing de novo biosynthesis, sequestration and mixed mode biosynthesis a reliable
overview on the proteins present in the secretion of the bioreactor was obtained (Fig. 4). Similar compositions of the secretions were observed for all three evolutionary stages (Fig. 2) irrespective of the type of defensive compounds produced. Obviously linked to the production of defences are proteins belonging to the families of glyco-hydrolases and the GMC-oxidoreductases. Catalase is also essential in all cases, as the GMC oxidases generate \( \text{H}_2\text{O}_2 \) as a side product that is detoxified by the enzyme (Weibel et al., 2001; Brueckmann et al., 2002). Unknown is the role of the abundantly and generally present superoxide dismutases. A member of the JHBP (juvenile hormone binding) family 4 was identified as the cyclase required for the biosynthesis of the iridoid defences (Bodemann et al., 2012). The function of the majority of the putatively annotated enzymes is still unknown.

The screening for relevant proteins was substantially supported by the development of RNAi (Bodemann et al., 2012), which can be applied as a high throughput approach allowing to test the function of individual proteins/sequences in a minimum of time (by loss of function). The precise silencing of a target gene is triggered by injection (ca. 0.5 \( \mu \text{g} \)) of double stranded RNA (dsRNA) whose sequence (200–500 base pairs) is identical to that of the target gene (Bodemann et al., 2012; Fire et al., 1998). The result is a declined transcript and consequently also protein level. Besides embryogenesis, pattern formation, reproduction and behaviour, this loss-of-function approach allows also successful analyses of biosynthetic pathways in insects (Mito et al., 2011; Bellés, 2010; Bursei and Boland, 2014). For juvenile leaf beetles this approach facilitated the identification of all enzymes involved in the \textit{de novo} biosynthesis of iridoids (Lorenz et al., 1993), as well as of the identification of the first ABC transporter located in the glandular system of \textit{C. populii} (Strauss et al., 2013). An application is shown in Fig. 5 for the knock down of a glycoside hydrolase from the transcript catalogue of \textit{Phaedon cockerellii} identified by LC–MS in the proteome of the larval secretion. The silencing of the candidate gene revealed a \( \beta \)-glucosidase belonging to the glycoside hydrolase family 1, whose knocking down resulted in the accumulation of 8-hydroxygeraniol-\( \beta \)-D-glucoside indicating an implication of this protein in the hydrolysis of the glucoside (Häger, 2013). The lack of this enzyme leads to an enormous accumulation of the glucoside in the defensive gland and occasionally even to crystallisation (Fig. 5B).

The same protocol was applied to test all candidate enzymes of the whole sequence \textit{en route} to the iridoid chrysomelidial. The studies of the iridoid metabolism nicely illustrate the value of RNAi to disentangle biosynthetic pathways. Expression of the positively identified genes in insect cells (SF9 cells) allowed to study the function of the enzymes and to determine their kinetic constants.

**Figure 4** Protein families in the defensive secretion of \textit{C. populii} according to LC–MS/MS and MSE analysis.

**Characterisation of individual enzymes**

The silencing protocol not only works for enzymes from the defensive system, but also for enzymes in other parts of the body, for example for enzymes involved in the early terpenoid biosynthesis located in the fat body (HMG-CoA reductase, terpenoid synthases) (Bursei et al., 2008). Because the HMG is one of the most regulated enzymes known (Goldstein and Brown, 1990), its regulatory features may be also important for the biosynthesis of iridoids in the chrysomelids. The expressed enzyme was found to be negatively regulated by 8-hydroxygeraniol, an early precursor of iridoid biosynthesis. Purification of the catalytic domain revealed that inhibition by 8-hydroxygeraniol is subject to the catalytic domain which was corroborated by docking analyses on the modelled HMG catalytic centre suggesting competitive inhibition by this monoterpene (Bursei et al., 2008) (Fig. 6).

The \textit{de novo} producing larvae possess the potential to sequester glucosidically bound 8-hydroxygeraniol, if present in the food plants (Soe et al., 2004). After cleavage of the sugar moiety by the omnipresent glucosidases in the gut, the aglucone can reach the hemolymph and interfere with the HMG. In consequence the enzyme may represent a key
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regulator to maintain homeostasis of endo- and exogenous metabolites of the iridoid synthesis. Inhibition has also been found with other insect HMGRs including D. melanogaster. At 5 mM 8-hydroxygeraniol reduced HMGR activity of the two iridoid producers P. cochleariae and G. viridula by 80%, of the two sequestering Chrysomelina species C. populi and C. lapponica by 60–75%, of the Galerucinae Agelastica alni by 75% and of Drosophila melanogaster by 55%. Thus, inhibition of the HMG-CoA reductase by 8-hydroxygeraniol is most likely rather widespread across the insect kingdom (Burse et al., 2008).

Expression and kinetic analysis of an isoprenyl diphosphate synthase

The second enzyme in early terpenoid biosynthesis of the fat body catalyses the alkylation of the homoallylic isopentenyl diphosphate (C_5-IDP) by the allylic dimethylallyl diphosphate (C_5-DMADP) resulting in geranyl diphosphate (GDP), the ubiquitous C_{10}-building block of monoterpenes. This step can be controlled by the presence of several divalent cations as it has been shown by means of the IDS1 (isoprenyl diphosphate synthase) identified from P. cochleariae (Frick et al., 2013) (Fig. 7).

The enzyme PcIDS1 produces 96% C_{10}-geranyl diphosphate (GDP) and only 4% C_{15}-farnesyl diphosphate (FDP) in the presence of Co^{2+} or Mn^{2+} as co-factor, whereas it yields only 18% C_{10} GDP, but 82% C_{15} FDP in the presence of Mg^{2+}. A similar observation of such a switchable scIDSs has not been described previously. As a GDP synthase, PcIDS1 could be associated with the formation of monoterpenes, such as iridoids; as an FDP synthase with the formation of sesquiterpenes, such as juvenile hormones (Bellés et al., 2005). The identification of Co^{2+}, Mn^{2+} and Mg^{2+} in the

Figure 5  Biosynthetic sequence (Lorenz et al., 1993) and enzymes leading to the iridoid chrysomelidal (A). Glandular system with a spontaneously formed crystal of 8-hydroxygeraniol-8-O-β-D-glucoside (B). 5–7 days post-injection, the relative transcript level coding for the β-glucosidase was reduced to about 1% (C).

Figure 6  Inhibition of the purified catalytic domain of the HMGR from P. cochleariae by intermediates of the iridoid metabolism.
larvae’s fat body supports the notion that these organisms may control product specificity of PcIDS1 simply by differences in the local concentration of these metal ions. Especially in *P. cochleariae*, regulation concerning product formation is important to clearly define which metabolic pathway is served by the enzyme. The immense amount of GDP, needed for *de novo* production of iridoid defenses has to be allocated fast, highly specific and in large quantities. However, at the same time FDP dependent pathways have to be secured and should not suffer from the enormous GDP consumption due to the production of chrysomelidial. These examples clearly underline, that for understanding of the biosynthesis of chemical defences in insects, a detailed knowledge of the biochemical parameters of the involved enzymes is essential.

**ABC transporter acts as a pacemaker in the transport of glucosides to the bioreactor**

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; Kuhn et al., 2004). Furthermore, thioglucosides were selectively accumulated (Fig. 3), up to 500-fold, into the reservoir from a hemolymph pool, suggesting an active transport system is at work (Kuhn et al., 2004; Feld et al., 2001). Based on these findings the available sequence data were analysed for ABC transporters which are known to achieve high enrichments. Screening of expression levels of transcript sequences encoding ABC transporter motifs revealed a putative candidate, referred to here as CpMRP. It displayed an exceptionally high transcript level in the glandular tissue, exceeding that in the gut and Malpighian tubules by more than 7000-fold (Strauss et al., 2013). Among all known and functionally characterised ABC transporters, the amino acid sequence of CpMRP, (1331 AAs, 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 CpMRP from *C. populi* possesses the typical structural elements of ABC transporters (Zolnerciks et al., 2011); these consist of four domains: two TMDs (transmembrane domains), harbouring six proposed transmembrane spans and two NBDs (nucleotide-binding domains), containing Walker A and B boxes (sequences GPVGAGKS and YVLM, respectively), separated by an ABC signature motif (sequence LSGGQRARINLARAI) (Fig. 8D). Employing RNA interference we were able to demonstrate its key role in the secretion of defensive compounds. Silencing CpMRP had no influence on larval growth, but about 10 days post-injection, the CpMRP knockdown larvae completely lost their ability to respond to stimulation with droplets of defensive secretion (Fig. 8A and 8B). Thus, knocking down of CpMRP created a depletion of a transporter protein together with a defenceless phenotype.

In the ancestral *de novo* iridoid-producing species *P. cochleariae*, we identified a sequence with 86% amino acid identity to CpMRP and in the more derived species *C. lapponica* a homolog of CpMRP sharing 93.7% amino acid identity. Nevertheless, expression of CpMRP in frog oocytes as a test system demonstrated with a mixture of glucosides that this ABC transporter is not selective and cannot explain the highly selective import shown in Fig. 3 (Kuhn et al., 2004; Strauss et al., 2014). Thus, in combination with previous results we assume that an additional, highly selective transporter, first selects and guides the genuine glucoside of a species from the hemolymph into defensive gland. There, a micellar transport system involving CpMRP, secures an irreversible export, driven by ATP-hydrolysis, of the compounds into the reservoir (pacemaker function). From our results we conclude that the sequestration of *Chrysomelina* larvae is the result of the presence of several barriers with various degrees of selectivity: (i) those controlling the non-selective uptake of phenolglucosides from gut lumen into
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Figure 8  Silencing effect and 3D-structure model of CpMRP. (A and B) Production of defensive secretions is disrupted in CpMRP 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 (Hinton, 1951) with relaxed reservoir in contrast to the everted reservoir in insets of (A) and (B). (D) 3D-model of CpMRP, 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 localisation of characteristic sequence.

the hemolymph and excretion by Malpighian tubules (most likely also involving ABC transporters), (ii) those controlling the selective transfer from the hemolymph into the secretory cells and (iii) those where the broad-spectrum ABC transporter shuttles pre-filtered metabolites from inside the secretory cell into the defensive secretions.

The enormous complexity of the control of transport through the insect’s body becomes obvious by a

Figure 9  CpMRPs pacemaker function and sequestration model. Schematic view of our sequestration model through a secretory cell (see Fig. 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. CpMRP 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.
bioinformatic analysis of all ABC transporter motives present in transcripts from *C. populi*. We identified 65, belonging to the eight known subfamilies ABC A-H, differently distributed in the various body tissues from midgut to fat body (Strauss et al., 2014). Moreover, additional 68 members of the SLC2 sugar transporter family are present in the transcriptome of the mustard leaf beetle *P. coehleariae* (Stock et al., 2013) (Fig. 10).

It will be a herculean task to compile all the transport characteristics and kinetic data which are essential for a comprehensive understanding of the controlled transport of given compounds through the insect’s body. Especially, as there are many more transporters involved and mutual interactions between them via regulatory loops have to be anticipated.

**Evolution and adaptation of the leaf beetles defences to novel host plants**

In their environments leaf beetles often experience pressure by their natural enemies such as syrphid flies (Köpf et al., 1997), ants or parasitic wasps (Urban, 1998). Occasionally they escaped such a pressure by simply changing their host plants. The metabolites, imported from the new host, may not support the chemical communication between the leaf beetle larva and its parasite by providing a different spectrum of glucosides that ultimately leads to different volatile defence. Such a scenario might be responsible for the existence of two different strains of *C. lapponica* living either on *Salix* or on *Betula*. The salix-strain produces salicylaldehyde along with small amounts of butyrate esters (Schulz et al., 1997), while the *Betula* strain lacks salicylaldehyde and produces large amounts of butyrate esters (Tolzin et al., 2011). Careful analysis of the gene coding for the salicylaldehyde oxidase (SAO) demonstrated, that the *Betula* strain has a defect in this gene leading to an inactive oxidase (Kirsch et al., 2011). 27 amino acids are missing in the protein of the *Betula* strain. At the same time the expression level of the gene is about 100fold lower. This example demonstrates that leave beetles can escape parasite pressure by host plant change followed by adaptation to the new plant chemistry. This is facilitated by the fact that only minor modifications of the complex import/export system are necessary. The import of plant glucosides via the gut membrane into the hemolymph is non-selective (Discher et al., 2009) as it constitutes a part of the common nutritional up-take. Only the transfer of glucosides from the hemolymph into glandular system is highly selective (Fig. 9) and needs to adapt to the new spectrum of glucosides. The final export to the bioreactor is, again, non-selective. This view is strongly supported by the non-selective action, but outstanding similarity of the ABC transporters from all evolutionary stages shown in Fig. 2. Moreover, the similarity of the enzymatic transformations, carried out by glucosidas and oxidases in the bioreactor, demonstrates that already in the iridoid *de novo* producers the basic transformations are present for an evolutionary transition to sequestration. Hence, although the use of plant compounds as anti-predator defences appears to be a spectacular evolutionary innovation, it requires only a few modifications from ancestral processes. The origin of genes enabling this and other transitions is a major question to be solved in future. Also the evolutionary timeline of the transitions is a fascinating aspect. The extreme diversity of the chrysomelids providing all kind of transitions between the evolutionary stages and the continuously increasing knowledge on sequences from individual species makes them an ideal model system to study such evolutionary questions.
Conflict of interest

The author declares that there is no conflict of interest.

References


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