Identification and characterization of defense related enzymes in Chrysomelina larvae (Coleoptera: Chrysomelidae): Contributions to understand the evolutionary and molecular dynamics of chemical defense in leaf beetles

Dissertation

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1 Introduction

1.1 General Introduction: Herbivorous insect – host plant interaction

Plants and insects make up approximately half of all known species of multi-cellular organisms. Nearly 50 % of all insect species feed on living plants (Strong et al. 1984), meaning that about 400.000 herbivorous insect species (in the following mentioned as herbivores) live on approximately 300.000 vascular plant species (Schoonhoven et al. 2005). Thus, extensive relationships between phytophagous insects and their host plants do exist. Insects shape the plant world (Marquis 2004), herbivorous taxa have a higher diversification rate than non-herbivorous taxa (Thompson 1994) and plant-herbivore interactions are seen as an important driving-force of the tremendous species diversity on earth (Ehrlich and Raven, 1964). These co-evolutionary processes, enabling diversification, underlie multifaceted adaptations in both plant and herbivore.
Most herbivores are specialists and restricted to a small set of host plant species (Bernays and Graham 1988, Jaenike 1990, Bernays and Chapman 1994, Jolivet and Hawkeswood 1995). For example 35 % of beetle species (122.000) are phytophagous (Schoonhoven et al. 2005) and 75 % of them are specialists (Bernays and Chapman 1994). Host specialization of herbivores comes along with selective adaptations and has been shown to be influenced by geographical, genetic, biophysical and ecological enforcements (reviewed in Bernays and Chapman 1994, Schoonhoven et al. 2005). However, the most important factor shaping host specialization is the immense variety of secondary metabolites protecting the host plants (Ehrlich and Raven 1964, Feeny 1976, Cates 1980). Herbivores have to overcome plant chemicals by appropriate detoxification or resistance mechanisms. As a rule of thumb the specialists´ ability to detoxify ingested compounds releases them from their negative effects. Admittedly, it has been discussed that host plant specialization could lead to “evolutionary dead end” situations for the herbivores (reviewed in Thompson 1994). They may have lost the ability to react to changing environments by host plant shifts, because their specializations constrain them to shift among host plants that are chemically similar. However, specialization enables herbivores to feed on plants which are avoided or not suitable to others. This dissertation here focuses on aspects of specialized herbivores´ adaptations to plant feeding with some insights into their host plant chemistry.

1.2 Different molecular levels of host plant adaptations in leaf beetles (Chrysomelidae) of the subfamily Chrysomelinae

Chrysomelinae host plant adaptations, and how they are reflected by their chemical defense strategies, involved transport systems, and enzymatic actions are described in the following paragraphs. Within Chrysomelidae systematic relationships and nomenclature taken as a basis in this work as well as the systematic position of investigated species are depicted below.

Family: Chrysomelidae

Subfamily 1 (of 19): Chrysomelinae

Tribe 1: Timarchini (tribe/subfamily status controversially discussed)

Tribe 2: Chrysomelini

Subtribe 1 (of 8): Chrysomelina

Genus 1: *Chrysomela*

C. populi

C. lapponica

Genus 2: *Gastrophysa*

G. viridula

G. cyanea

Genus 3: *Phaedon*

P. cochleariae

Genus 4: *Phratora*

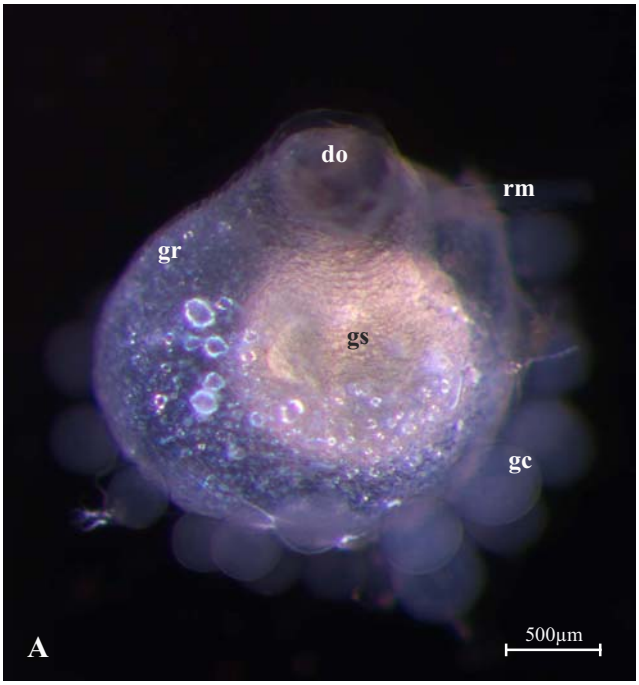
P. laticollis

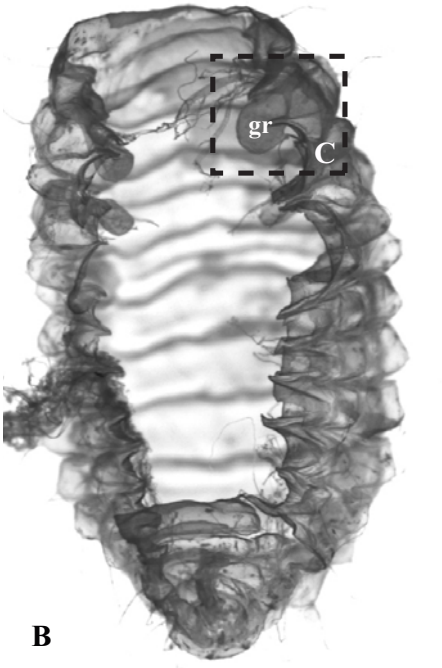
P. vitellinae

1.2.1 Defense in Chrysomelinae with special emphasis on subtribe Chrysomelina glandular chemical defense

Leaf beetles contain about 37.000 species in 19 subfamilies (according to Jolivet 1978). Almost all of them feed on plants (Jolivet and Hawkeswood 1995). Their biology, morphology, behavior and host plant spectrum varies considerably. The vast majority of the subfamily Chrysomelinae is mono- or oligophagous. Differences in the level of host plant selection are very often on a genus or even on a species level, with both adults and larvae feeding on plant leaves (Jolivet and Hawkeswood 1995. pp. 2-3). Because exposed specialized herbivores not only need to cope with host plant chemicals but also with multiple predatory attacks, a variety of defense mechanisms has been developed in different leaf beetle taxa. Reflex-immobilization or jumping (Alticinae), reflex-bleeding (Galerucinae), aposematic coloration (already implied in the name Chrysomelidae derived from the Greek *chrysos*: gold, *melolanthion*: beetle, referring to their metallic colours), protection by cases (miners and borers), enteric discharges or gregarious behavior (Cassidinae, Galerucinae) have been observed (reviewed in Dettner 1987). In the subfamily Chrysomelinae an efficient glandular chemical defense has been established. Their larval as well as adult stages are protected by those defensive glands, albeit the ultrastructure differs between these developmental stages (Claus 1861, Hollande 1909, Garb 1915, Hinton 1951, Deroe and Pasteels 1982, Pasteels et al. 1989). The adults possess elytral and pronotal glandular cells which accumulate defensive secretions in vacuoles or intercellular spaces. Those glands are also present in the Chrysomelidae subfamilies Criocerinae and some Alticinae and Galerucinae and their common origin based on morphological data has been discussed (Deroe and Pasteels 1982, Pasteels et al. 1989).

This thesis focuses on aspects of larval glandular chemical defense, which is restricted to the Chrysomelinae (absent in the tribe Timarchini, discussions for homologous structures in Galerucinae in Bünnige and Hilker 1999, 2005). Species of the subtribe Chrysomelina possess typically 9 pairs of defensive glands that are located dorsally in the meso-, metathorax and the abdominal segments 1-7. A single defensive gland contains a dorsal opening, a cuticularized reservoir, enlarged glandular cells and retractor-muscles (**Fig. 1**). When a larva is disturbed the glands are everted by hemolymph pressure and small droplets of secretion appear at the tip of the reservoirs. After a while the reservoirs are retracted by muscles.





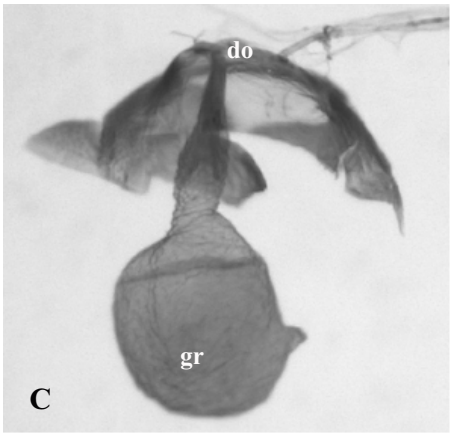


Fig. 1: Defensive glands of *Chrysomela populi* (**A**) and *Gastrophysa viridula* (**C**) 3rd instar larvae. In (**A**) a defensive gland directly after dissection is shown. An enlarged view of a defensive gland after KOH-digest of the dorsal side of the larval body (**B**) and further dissection is depicted in (**C**). After digestion with KOH only chitinous structures remain, verifying the cuticularization of the defensive glandular reservoirs and their epithelial origin during embryogenesis. Abbreviations: do: dorsal opening, gc: glandular cell, gr: glandular reservoir, gs: glandular secretion, rm: retractor muscle.

It has been shown that compounds of low molecular weight act as defensive principles against predators and microbial infestations in Chrysomelina (Wallace and Blum 1969, Blum et al. 1972, Pasteels et al. 1983, Smiley et al. 1985, Denno et al. 1992, Palokangas and Neuvonen 1992, Hilker and Schulz 1994, Gross et al. 2002). Defensive compounds of Chrysomelina larvae can be allocated to 6 chemical classes (Pasteels et al. 1984, Rowell-Rahier and Pasteels 1986, reviewed in Dettner 1987), namely cyclopentanoid monoterpenoids (iridoids), salicylaldehyde, phenylethyl esters, benzaldehyde, juglone and conjugated acetates. Most widespread are iridoids, salicylaldehyde and phenylethyl esters (Pasteels et al. 1990). Their biosynthesis inside the larvae is summarized in **Fig. 2** and explained in more detail in the following three paragraphs. However, specialized parasitoids can be attracted to these defensive compounds, enabling them a more precise detection of their insect hosts. In addition, the Chrysomelina larval secretion plays a role in intra- and interspecific interactions (Hilker 1989).

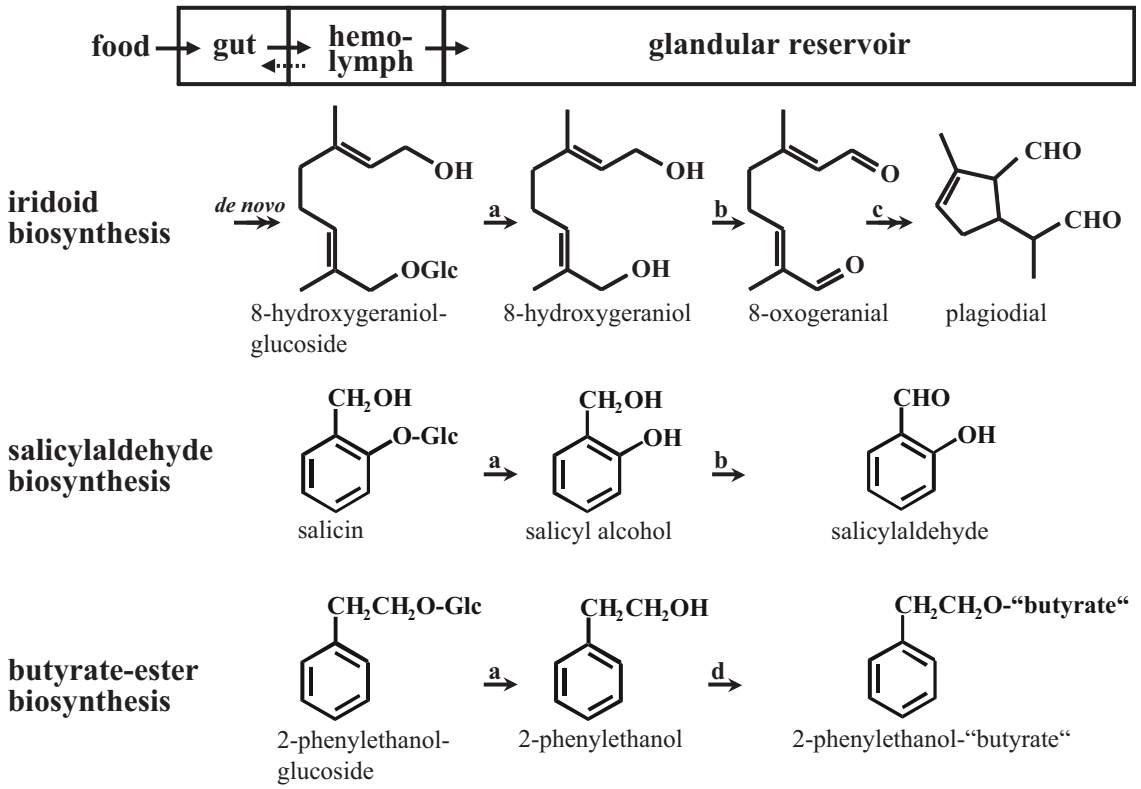


Fig. 2: Final steps of defensive compound biosynthesis in the defensive glands of Chrysomelina larvae. Iridoid biosynthesis is exemplary shown for plagiodial. In case of the esters 2-phenylethanol-"butyrate" formation is depicted, in which "butyrate" stands for isobutyric or 2-methylbutyric acid. The scheme above the chemical pathways indicates the route of glucoside precursors through the larval body. In nature iridoids are predominantly produced *de novo* (autogenous) from mevalonic acid, which is indicated by the double arrow previous to the glucoside structure. Abbreviations: a: deglucosylation, b: oxidation, c: cylization and isomerization, d: acyl transfer reaction, Glc: glucose.

1.2.2 Host plant influence and origin of Chrysomelina defensive compounds

Beside their ecological effects a lot of work has been done on the origin of chrysomeline glandular secretion. Especially the larvae exhibit different degrees of dependence of their defense on the host plant´s secondary chemistry (Pasteels et al. 1983, Pasteels et al. 1989, Veith et al. 1994, Schulz et al. 1997, Pasteels et al. 2000). Phylogenetic analyses of Termonia et al. (2001) revealed the mostly autogenous biosynthesis of iridoids as the ancestral state of chrysomeline larval chemical defense strategy (**Fig. 3**). Starting from that, two lineages (*Chrysomela* and *Phratora vitellinae*) most likely independently developed a highly efficient chemical defense by synthesizing salicylaldehyde. This made them tightly dependent on their hosts´ chemistry, because the salicylaldehyde precursor salicin is host plant derived and sequestered (Pasteels et al. 1983). Whereas the host plant range of iridoid producing

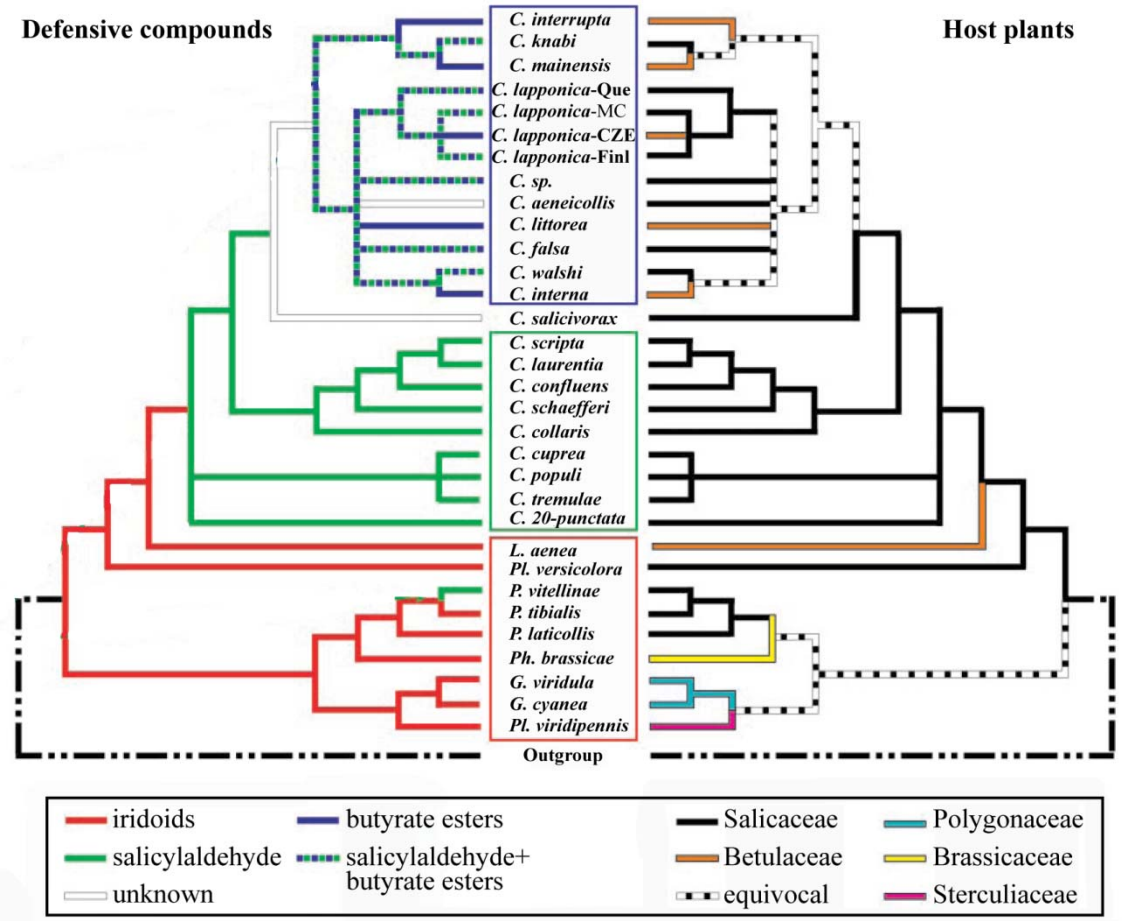


Fig. 3: Maximum parsimony reconstruction of Chrysomelina defensive compounds and host plant affiliations based on a MP strict consensus species phylogeny (modified from Termonia et al. 2001). Colour-codes of branches are explained below the tree. The originally autogenous production of iridoids, the presence of salicylaldehyde in the defensive secretion of both the genus *Chrysomela* and *Phratora* and the derived synthesis of butyrate-esters in the monophyletic interrupta-group (blue frame) is obvious. Abbreviations: *C.*: *Chrysomela*, *G.*: *Gastrophysa*, *L.*: *Linaeidea*, *Ph.*: *Phaedon*, *P.*: *Phratora*, *Pl.*: *Plagiodera*.

species is relatively broad (7 plant families (Pasteels et al. 1990)), salicin-based chemical defense restrict the larvae of *Chrysomela* and *Phratora vitellinae* to feed on the salicin containing plants. On the one hand they developed an economic way to detoxify the general feeding deterrent salicin (Ruuhola et al. 2001a) and therefore likely restrict the number of competing herbivores on their preferred host plants. On the other hand the direct effect of specialization of the salicylaldehyde protected species is obvious as they are limited in their host plant spectrum to the presence of salicin. Interestingly, a monophyletic clade within the genus *Chrysomela* (*interrupta* group) evolved the biosynthesis of butyrate-esters as defensive compounds of mixed beetle and plant origin (Blum et al. 1972, Hilker and Schulz 1994, Schulz et al. 1997). In addition, some *interrupta* species most likely independently shifted or expanded their host plant range to birch trees (Brown 1956) and therefore overcame the highly specialized,

evolutionary niche with respect to their salicaceous host affiliation (**Fig. 3**). The expansion of host range to birch is remarkable for two reasons. First, host family shifts are generally seen to be rather uncommon and occurred in less than 17 % of insect speciation events (Farrell et al. 1992). Second, the shift to birch tremendously affected the composition of the *interrupta* group species´ glandular defensive secretion. While salicaceous plant-feeders utilized a dual mode of defense accumulating butyrate esters and retaining salicylaldehyde biosynthesis, those species that shifted to birch trees lack salicylaldehyde. A dual defense strategy is discussed as a transition state between two single-defense strategies in leaf beetles in general, offering the possibility of a broader range of host plant affiliations (Termonia et al. 2002). More specifically, in the genus *Chrysomela* the evolution of the dual defense has been potentially a prerequisite to escape specialized predators and parasitoids (attracted by salicylaldehyde) by allowing host shifts to salicin-free birches without losing essential/defensive properties of the glandular secretion (Termonia et al. 2001, Gross et al. 2004). Thus, once again the intimate relationships of Chrysomelina host affiliation, their defense strategy and ecological relevance are obvious.

In a nutshell, comparative investigations of Chrysomelina larval glandular chemical defenses are excellent systems to get insights into prerequisites, mechanisms and ecological consequences of herbivorous specialists´ host plant adaptations and host plant shifts.

1.2.3 Transport mechanisms of defensive compound precursors into the Chrysomelina larval glandular reservoir

The impact of host plants on Chrysomelina larval glandular chemical defense depends on their defensive strategy. But irrespective of the degree of host plant-larval chemical defense dependence, most likely all Chrysomelina possess the ability to both deal with toxic or repellent plant metabolites and to sequester plant derived glucoside precursors for their own chemical defense. In *Phratora vitellinae* and the genus *Chrysomela* sequestration of plant-derived phenolic glucosides is compulsory for salicylaldehyde (both genera) and butyryl-ester biosynthesis (*interrupta* group only). Even iridoid producing species are able to sequester 8-hydroxygeraniol-glucoside (Kunert et al. 2008), although this iridoid precursor is usually produced *de novo* via the mevalonic

acid pathway (Oldham 1996, Soe et al. 2004, Burse et al. 2007).

Independent of the larval defensive strategy (*de novo* biosynthesis, sequestration or a mixture of both), glucosides need to be transported and further modified to bioactive principles inside the larva. The selectivity of glucoside transport from the gut into the defensive glandular reservoir has been elucidated nicely by applying thioglucosides resembling natural *O*-glucosides (Feld et al. 2001, Kuhn et al. 2004, Kuhn et al. 2007). Those experiments demonstrated that the larvae possess transport systems, which are evolutionary adapted to the glycosides of their host plants. The uptake of plant-derived glycosides from the gut into the hemolymph is not specific. However, compounds essential for the larval chemical defense are imported selectively from the glycoside pool circulating in the hemolymph into the defensive glandular reservoir (postulated in Schulz et al. 1997, Discher et al. 2009). Whereas species producing only iridoid or salicylaldehyde possess a high selectivity in precursor uptake into the glandular reservoirs, the butyryl-ester synthesizing *Chrysomela* species of the *interrupta* group sequester a broad range of structurally different glucosides, leading to a complex mixture of esters (Schulz et al. 1997, Diss. Tolzin Banasch 2009). In summary, the host plant adaptation is not only reflected by the type of chemical defense in general but in more detail also by the selectivity of the defensive compound precursor transport system. Because only glucosides (and their agluca released by glucosidases in the larval gut) are known to be used for larval glandular chemical defense, host plant influence may be restricted to those compounds (Pasteels et al. 1990).

1.2.4 Biosynthesis of defensive compounds by final enzymatic steps in the glandular reservoir

Irrespective of the quality of bioactive principles, the final steps of defensive compound biosynthesis always take place inside the glandular reservoirs of Chrysomelina larvae. The enzymes involved in defensive compound biosynthesis are beside the chemical defense strategy and the involved precursor transport system the third level to characterize the degree of Chrysomelina host plant adaptations. The modification of glucoside precursors inside the glandular reservoir is based on a consecutive action of soluble enzymes that are present in the glandular secretion (**Fig. 2**).

The first step, a deglucosylation, is common to all Chrysomelina larvae irrespective of

whether iridoid, salicylaldehyde or butyrate-ester precursors are imported into the glandular reservoir. The β-glucosidase activity has been shown to be present in iridoid and salicylaldehyde producers´ glandular secretion (Pasteels et al. 1983, Pasteels et al. 1990, Soetens et al. 1993). All glandular secretions tested possess glucosidase cross-reactivity and the ability to deglucosylate a huge variety of glucosides indicating the presence of nonspecific β-glucosidase activity.

The aglyca 8-hydroxygeraniol and salicyl alcohol are oxidized in the second step. In contrast to the β-glucosidases, the oxidases are highly substrate-specific and clearly reflect the adaptation to the larval chemical defense strategy and the precursor transport specificity. Whereas iridoid producers only have the ability to oxidize 8-hydroxygeraniol to the dialdehyde 8-oxogeranial, salicylaldehyde producers´ glandular oxidase selectively oxidize the salicyl alcohol to the respective aldehyde (Pasteels et al. 1990, Soetens 1993, Brückmann et al. 2002; exception: Veith et al. 1997). The substrate specificity of the glandular oxidase has been discussed as a key parameter affecting the mode of chemical defense (Pasteels et al. 1990).

The consecutive action of a nonspecific β-glucosidase and a highly specific oxidase is sufficient to produce salicylaldehyde but iridoid biosynthesis requires at least one more step. Species-specific cyclization of the dialdehyde 8-oxogeranial and further isomerization, leading to different iridoids (e.g. chrysomelidial, plagiodial, plagiolactone), have been postulated and finally verified (Lorenz et al. 1993, Veith et al. 1994, Kunert in preparation).

After initial deglucosylations of host-derived glucosides completely different enzymatic activities are necessary for butyrate-ester biosynthesis in the *interrupta* group of the genus *Chrysomela*. The broad spectrum of aglyca is esterified with isobutyric and 2-methylbutyric acid derivatives. Acyltransferases have been postulated to catalyze this biosynthetic step. The mode of isobutyric and 2-methylbutyric acid *de novo* production from endogenic amino acids (Schulz et al. 1997) and the acid activation prior to esterification via glucosyltransfer have been shown (Diss. Tolzin Banasch 2009).

Utilizing *in vitro* assays with glandular secretions, substrate specificity and importance for larval chemical defense has been shown for a range of enzymatic activities involved in defensive compound biosynthesis of Chrysomelina. However, little is done on a molecular and/or genetic level and neither the evolutionary origin nor the fate after host shift of the glandular enzymes is known for any Chrysomelina species.

The salicyl alcohol oxidases (SAOs) of the obligate salicin sequestering species

Chrysomela populi and *Chrysomela tremulae* are the only enzymes that have been characterized on protein level after heterologous expression (Michalski et al. 2008, in parallel in our lab). The SAOs belong to the glucose-methanol-choline (GMC) oxidoreductase superfamily. We elucidated not only glandular tissue-specific expression and gene architecture but also stereo-selectivity, substrate specificity and complex patterns of N-glycosylations for *Chrysomela populi* SAO after heterologous expression in a *Sf*9 insect cell lines (unpublished).

1.3 An introduction to the investigated species

1.3.1 Biology of *Chrysomela lapponica*

Chrysomela lapponica is a univoltine, cold climate-adapted, euro-siberian leaf beetle species (**Fig. 4**). Its patchy arctic-alpine distribution in Europe (**Fig. 5**) is most probably a relict after the last glaciations (Machkour-M´Rabet et al. 2008) and potentially also due to its general poor dispersal capability (Knoll et al. 1996). Recent investigations addressing the differences among isolated *C. lapponica* populations indicate slight reproductive constrains and local host specialization (Gross and Hilker 1995, Fatouros et al. 2006, Zvereva et al. 2010).

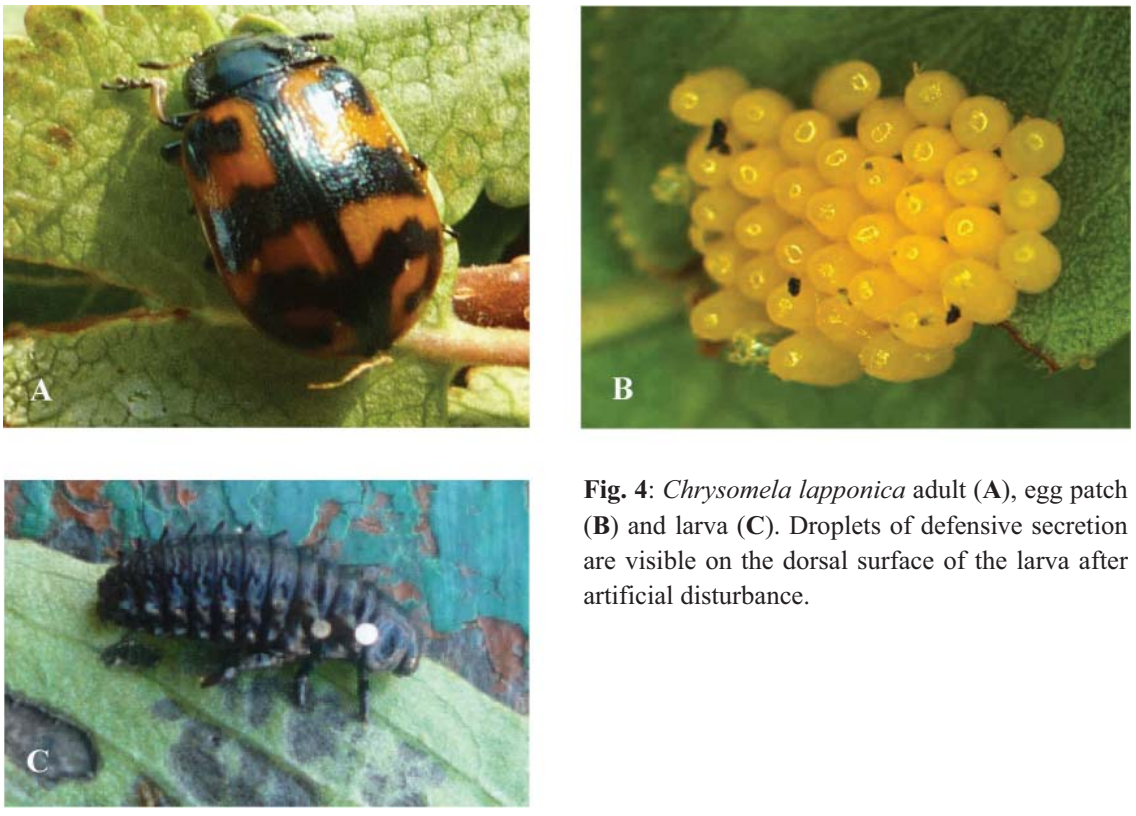


Fig. 4: *Chrysomela lapponica* adult (**A**), egg patch (**B**) and larva (**C**). Droplets of defensive secretion are visible on the dorsal surface of the larva after artificial disturbance.

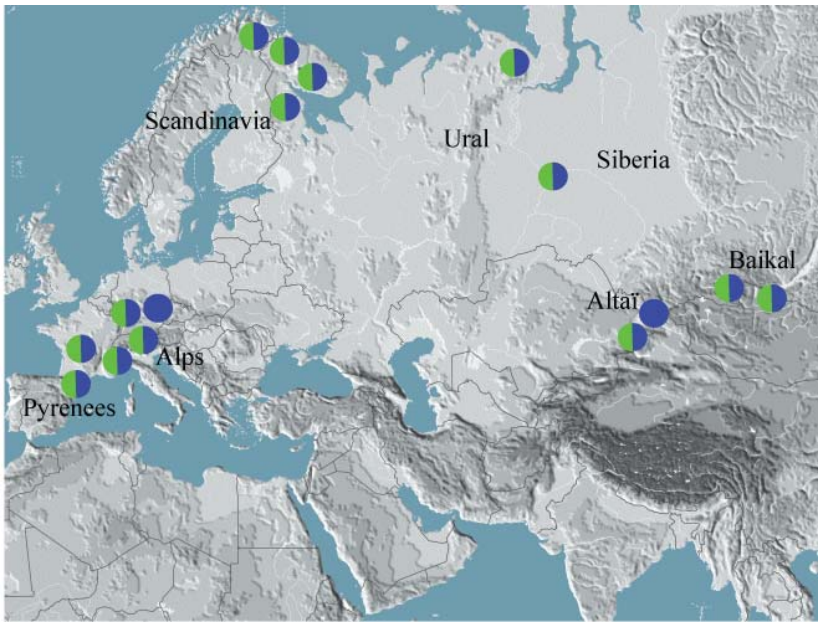


Fig. 5: Map, showing the patchy, Eurasian distribution of *Chrysomela lapponica*. Sites of populations are depicted exemplarily and their defensive compounds are indicated by halfway green and blue (salicylaldehyde and butyrate-esters) or fully blue (butyrate-esters) circles. (modified from Mardulyn et al., submitted).

However, based on genetic differentiation data, clear evidence for speciation in progress could not be found (Machkour-M´Rabet et al. 2008, Mardulyn et al. submitted).

C. lapponica belongs to the monophyletic supra-species *interrupta*-group (Brown 1956, Termonia et al. 2001) characterized by the ability to synthesize butyrate-esters as larval defensive compounds (Blum et al. 1972, Hilker and Schulz 1994, Termonia and Pasteels 1999). Although originally *Chrysomela* species are restricted to feed on willow or poplar (Salicaceae), the *interrupta*-species also colonize (in a species-specific way) willow, birch or alder (Betulaceae) (Brown 1956, reviewed in Termonia and Pasteels 1999 and Termonia et al. 2001). *C. lapponica* is unique in this group as isolated populations of this species are reported to feed either on birch or on willow (**Fig. 5, 6**) (rarely also on poplar or bird cherry: see Zvereva et al. 2010). Similar to other species of the *interrupta*-group, *C. lapponica* host plant affiliation has a tremendous impact on its chemical defense composition (Hilker and Schulz 1994, Gross et al. 2004a). Whereas willow-feeders produce salicylaldehyde and butyrate-esters, birch-feeders completely lack salicylaldehyde biosynthesis.

The shift/expansion to birch has been discussed in the context of enemy free space colonization, as the lack of salicylaldehyde prevents the attack of salicylaldehyde-specialized predators and parasitoids (**Fig. 7**) (Zvereva and Rank 2003/2004, Gross et al. 2004b).

Fig. 6: Locations and host plants of the two investigated *Chrysomela lapponica* populations. In the French Alps near St-Veran (**A**) *C. lapponica* was collected feeding on *Salix breviserrata* (**B**) and two other willow species that were not specified. In the Kazakh Altai *C. lapponica* was found near the Burkhat Pass (**C**) feeding on *Betula rotundifolia* (**D**). Both locations are about 2000 m above the sea level and the host plants are highly abundant.

Fig. 7: *Parasyrphus* sp. larvae, a syrphid predator of *Chrysomela lapponica* eggs and neonate larvae. Different larval stages (**A**) and their feeding on a *C. lapponica* egg cluster (**B**) are shown. The syrphids´ eggs and larvae were found numerously infesting about 70% of *C. lapponica* egg clusters in the French-Alps, where the beetle is a willow-feeder. But they were not present in the birch-feeder location in Kazakhstan.

1.3.2 Biology of *Phratora vitellinae*

The brassy willow beetle *Phratora vitellinae* (**Fig. 8**) is one of the most abundant leaf beetles in Europe and known as a pest insect in osier and "energy plantations" of willow

and poplar (Urban 2006). *P. vitellinae* is within the Chrysomelina the only species of the genus *Phratora* and, moreover, the only species beside the genus *Chrysomela* producing salicylaldehyde as defensive compound (Wain 1943, Pasteels et al. 1984). As known for *Chrysomela* species, *P. vitellinae* sequesters the host-derived salicin serving as the precursor of salicylaldehyde (Pasteels et al. 1983) which displays activity against generalist predators and microbial invasions (Wallace and Blum 1969, Denno et al. 1990, Palokangas and Neuvonen 1992, Hilker and Schulz 1994, Gross et al. 2002). The brassy willow beetle prefers to feed on salicin-rich willow and poplar (Rowell-Rahier 1984a/b, Denno et al. 1990, Rank et al. 1998), and this salicin-rich diet was positively correlated with better larval defense and performance (Pasteels et al. 1988b, Denno 1990, Rank et al. 1998). However, *P. vitellinae* larvae need to cope with salicylaldehyde-specialized predators (Pasteels and Gregoire 1984, Köpf et al. 1997).

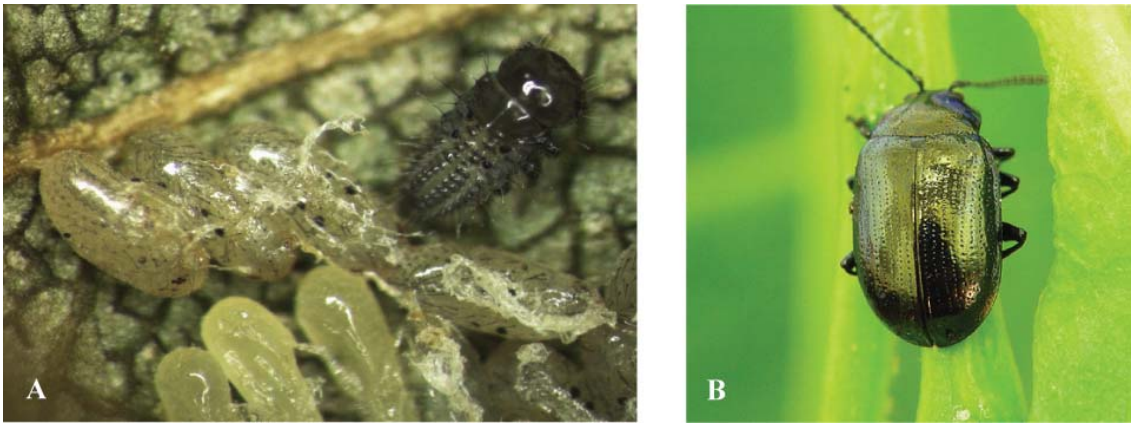


Fig. 8: A neonate larva and pre-hatching embryos (**A**) as well as an adult (**B**) of the brassy willow beetle *Phratora vitellinae* are shown. ((**B**): S. Krejcik, http://www.meloidae.com/en/pictures/6999/?s=1).

1.4 Aims and scope of the thesis

The links between host plant adaptation of Chrysomelina larvae and their chemical defense on different levels have been displayed in the previous paragraphs. These intimate relationships obviously show the suitability of investigating Chrysomelina chemical defenses to gain knowledge about mechanisms that uncover/reflect prerequisites and consequences of herbivorous specialists´ host affiliations.

Chrysomelina host plant associations and their larval glandular chemical defense compounds are well known for a variety of species. Those data served as a background to investigate the origin of defensive compound precursors and involved transport

processes. Remarkable insights into the biosynthetic pathways leading to iridoids, salicylaldehyde and butyrate-esters were achieved by the use of *in vitro* assays with glandular secretions as well as via larval feeding and injection studies.

Consequently, those findings raised a couple of questions during the last decade.

- Is the switch from "ancestral" iridoid to "derived" salicylaldehyde larval chemical defense based on a recycling (sub-functionalization) of proteins (transporter, enzymes) or an innovation (neo-functionalization)?
- Has the salicin-based defense (salicylaldehyde biosynthesis), and therefore the strong constraint to feed on salicaceous plants, evolved independently in *Phratora vitellinae* and *Chrysomela* spp.?
- What are the consequences of host shifts from willow to birch in the *Chrysomela interrupta*-group and how did those shifts affect the salicylaldehyde biosynthesis?
- How close/strict are the adaptations of the larval defensive compound precursor transport systems to the host plant, when keeping the enormous pool of host derived/offered glycosides and the specific use in Chrysomelina larvae in mind?

One way to address those questions is to elucidate identity, origin and characters of enzymes involved in defensive compound biosynthesis on a molecular-genetic level. But so far very little is known about those enzymes. The following chapters show that comparative investigations of those enzymes provide insights into prerequisites and consequences of host shifts and, moreover, are helpful to characterize host plant adaptations/specializations more precisely. In addition, in-depth analyses of the whole pool of host plant glycosides clarifies the chemical world the Chrysomelina larvae have to cope with, which at the same time they can utilize to facilitate/sustain their glandular chemical defense. The combination of knowledge of both the enzymes involved in larval glandular chemical defense and the host plants´ chemistry allows for drawing a picture of what shapes the environment whereof the biosynthetic machinery of larval defense needs to be adapted to.

The general aim of this thesis was to identify Chrysomelina defensive glandular enzymes involved in the biosynthesis of defensive compounds and to investigate which glycosides are provided from the host plants. Along these lines, the thesis had the following specific aims:

1.) *Identification of SAOs in Chrysomela spp.*

The first manuscript describes the identification of the SAO of *C. populi* and *C.*

lapponica. These data were employed in further parts of the thesis to elucidate their substrate-specificity, stereoselectivity, spatial expression, origin, genetic architecture and evolutionary dynamics. Therefore, molecular-biological techniques like qPCR, heterologous expression in insect cell lines, genomic library constructions and screenings and phylogenetic analyses were utilized.

2.) *Elucidation of the origin of salicin-based defense in Chrysomelina*

The second manuscript describes the identification and characterization of SAO in *P. vitellinae*. Herein verification of the presence of SAO in *P. vitellinae* glandular secretions, followed by SAO heterologous expression and subsequent activity assays were applied. The comparison with the SAOs of *Chrysomela spp.* provided in the first manuscript led to hypothesize a single origin of salicin-based chemical defense in Chrysomelina larvae, although *Phratora* and *Chrysomela* are not closest relatives.

3.) *Evolutionary dynamics of SAO and impact of host shift on its fate*

A comparison of a willow- and a birch-adapted population of *C. lapponica* (**Fig. 6**) with special emphasis on SAO were conducted. Based on qPCR, activity assays after heterologous expression, protein and gene sequence analyses the impact of host shift from willow to birch was shown in the first manuscript. Most likely the lack of salicin relaxed the selection pressure on SAO leading to reduced transcript abundance, alternative splicing events leading to partially truncated proteins and ultimately to loss of SAO activity in the birch-feeder.

Additionally, the investigation of the genomic background by genomic library screening showed that GMC gene duplications early in Chrysomelina evolution, followed by lineage-specific and more recent gene duplication events enabled the evolution of SAO activity. This evolutionary scenario is also supported by findings described in the second manuscript.

4.) *The link between chemical defense relying on iridoid and salicylaldehyde*

Although applying a range of molecular-biological techniques, I was not able to elucidate the oxidase of iridoid producers catalyzing the oxidation of 8-hydroxygeraniol to the dialdehyde 8-oxogeranial. However, data of the second manuscript provide evidence for an independent evolution of oxidases involved in iridoid and salicylaldehyde biosynthesis. Herein, comparative MS/MS analyses of proteins present in defensive secretions of Chrysomelina larvae were helpful.

5.) *Selectivity of host-derived defensive compound precursor uptake in C. lapponica*

The third manuscript provides a comparison of the complex pattern of glucosides

present in the host of a birch-feeding *C. lapponica* population and the aglycones present as butyrate-esters in their defensive secretion. The selectivity and efficiency of transport processes were compared to previous findings in other *C. lapponica* populations.

2 Overview of manuscripts

Manuscript 1

Roy Kirsch, Heiko Vogel, Alexander Muck, Kathrin Reichwald, Jacques M. Pasteels and Wilhelm Boland (2011)

Host plant shifts affect a major defense enzyme in *Chrysomela lapponica*

Proceedings of the National Academy of Sciences of the United States of America 108:4897-4901.

The identification and characterization of SAO in *Chrysomela lapponica* is described. Comparing two isolated populations, one feeding on willow and the other one on birch, elucidated the loss of SAO activity in the latter population due to SAO mutations causing alternative splicing and N-terminal truncation of the protein. In addition, phylogenetic analyses of SAOs and related sequences revealed their single origin in the GMC*i* subfamily.

R.K. designed and performed research, analyzed the data and wrote the manuscript to a bigger part.
H.V. and W.B. designed research as well, revised the manuscript and supervised the work.
A.M. supervised the MS/MS sample preparation of his technician and analyzed MS/MS data.
K.R. supervised the cDNA library sequencing and processed the raw-data.
J.M.P. made corrections and comments on a previous version of the manuscript.

Manuscript 2

Roy Kirsch, Heiko Vogel, Alexander Muck, Andreas Vilcinskas, Jacques M. Pasteels and Wilhelm Boland (2011)

To be or not to be convergent in salicin-based defence in chrysomeline larvae: evidence from *Phratora vitellinae* salicyl alcohol oxidase

Proceedings of the Royal Society of London B: Biological Sciences (online available).

SAO of *Phratora vitellinae* has been elucidated, characterized and compared to *Chrysomela* spp. SAOs. Phylogenetic analyses verified their SAO genes´ single origin although the genera are not closely related within the Chrysomelina. Moreover, whereas we found SAO related sequences their proteins were not detectable in the secretion of iridoid producing species indicating an independent evolution of oxidases in salicylaldehyde and iridoid producing species.

R.K. designed and performed research, analyzed the data and wrote the manuscript to a bigger part.
H.V. and W.B. designed research as well, revised the manuscript and supervised the work.
A.M. supervised the MS/MS sample preparation of his technician, analyzed MS/MS data and wrote parts of material and methods in the manuscript.
A.V. enabled the transcriptome sequencing of *Phaedon cochleariae* and made some comments on the manuscript.
J.M.P. made corrections and comments on a previous version of the manuscript and provided the insects for rearing.

Manuscript 3

Karla Tolzin-Banasch, Enkhmaa Dagvadorj, Ulrike Sammer, Maritta Kunert, Roy Kirsch, Kerstin Ploß, Jacques M. Pasteels and Wilhelm Boland (2011)

Journal of Chemical Ecology 37:195-204.

Glucose and glucose esters in the larval secretion of *Chrysomela lapponica*; selectivity of the glucoside import system from host plant leaves

Sequestration of phytogenic precursors by *C. lapponica* feeding on birch has been studied. The selectivity of larval transport mechanism has been elucidated by comparing the complex glucoside pattern of their host plant with the agluca present in the defensive secretion of the larvae. The lack of salicin but presence of salicin precursors have been described for the host, indicating together with the in principle ability of salicin sequestration by the larvae that birch affects more proteins than SAO activity.

R.K. performed research to a small proportion and contributed to writing the manuscript.
K.T.-B., E.D. and U.S. performed most of the research, analyzed the data with some contributions from the other authors.
W.B., K.T.-B. and J.M.P. designed research and wrote the manuscript with some contributions from the other authors.

3 Manuscripts

Manuscript 1

Host plant shifts affect a major defense enzyme in *Chrysomela lapponica*

Roy Kirsch[a], Heiko Vogel[a], Alexander Muck[a,1], Kathrin Reichwald[b], Jacques M. Pasteels[c], and Wilhelm Boland[a,2]

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Edited by May R. Berenbaum, University of Illinois, Urbana, IL, and approved February 16, 2011 (received for review September 22, 2010)

Chrysomelid leaf beetles use chemical defenses to overcome predatory attack and microbial infestation. Larvae of *Chrysomela lapponica* that feed on willow sequester plant-derived salicin and other leaf alcohol glucosides, which are modified in their defensive glands to bioactive compounds. Salicin is converted into salicylaldehyde by a consecutive action of a β-glucosidase and salicyl alcohol oxidase (SAO). The other leaf alcohol glucosides are not oxidized, but are deglucosylated and esterified with isobutyric- and 2-methylbutyric acid. Like some other closely related *Chrysomela* species, certain populations of *C. lapponica* shift host plants from willow to salicin-free birch. The only striking difference between willow feeders and birch feeders in terms of chemical defense is the lack of salicylaldehyde formation. To clarify the impact of host plant shifts on SAO activity, we identified and compared this enzyme by cloning, expression, and functional testing in a willow-feeding and birch-feeding population of *C. lapponica*. Although the birch feeders still demonstrated defensive gland-specific expression, their SAO mRNA levels were 1,000-fold lower, and the SAO enzyme was nonfunctional. Obviously, the loss of catalytic function of the SAO of birch-adapted larvae is fixed at the transcriptional, translational, and enzyme levels, thus avoiding costly expression of a highly abundant protein that is not required in the birch feeders.

host plant adaptation | glucose-methanol-choline oxidoreductase

Most phytophagous insects are specialized to a restricted set of host plant species (1–4). Host affiliation/specialization has been shown to be influenced by geographical, genetic, biophysical, and ecological enforcements (3, 5). But the most important barriers are toxic metabolites of the host plant (6–8), which all phytophagous insects must overcome by developing appropriate detoxification mechanisms. Adapting to plant-specific chemicals provides insects with a niche that allows them to survive, but narrows the range within which host plant shifts can occur, including only plants with similar metabolite patterns.

Chrysomelina leaf beetles are an excellent taxon for investigating host plant adaptation and relevant factors associated with host plant shifts. Most leaf beetle species are highly specialized on a single plant genus, where they spend their whole life cycle. Their well-defended larvae exhibit different degrees of dependence on the host plant's secondary metabolites (9–13).

Larvae of the genus *Chrysomela* originally feed on *Salicaceae* (e.g., willow, poplar) and sequester salicin (9). This phenolic glucoside is transported intact into the reservoirs of larvae's exocrine defensive glands (14, 15). In the reservoir, the glucoside is cleaved by a β-glucosidase to salicyl alcohol and glucose. Salicyl alcohol is further transformed to salicylaldehyde by a flavine-dependent salicyl alcohol oxidase (SAO) (16–18). Salicylaldehyde acts as a feeding deterrent against generalist predators (19–21) and has antimicrobial activity (22). The use of host-derived chemical defenses via sequestration exemplified by Chrysomelid beetles is a highly economical solution for detoxifying plant chemicals (23). The fact that de novo biosynthesis of defensive compounds is not required provides an energetic benefit while making the specialist herbivore dependent on host plant chemistry.

A monophyletic clade within the genus *Chrysomela* (*interrupta* group) evolved the biosynthesis of butyrate esters as defensive compounds (19, 21) about 1.1–2.3 Mya (24, 25). Some species of the *interrupta* group shifted host plants from willow to birch (26), which affected the composition of the insects' glandular defensive secretions. Whereas willow-feeding species retained the ability to synthesize salicylaldehyde in addition to esters as a dual defense strategy, birch-feeding specialists lack salicylaldehyde and synthesize only esters. It has been proposed that the evolution of ester biosynthesis enabled species within the *interrupta* group to shift from willow to birch. This shift altered the composition of the defensive secretions and allowed the insects to escape specialized parasitoids and predators that were attracted by the larval salicylaldehyde (25, 27).

In the present study we focus on *Chrysomela lapponica,* the only species within the *interrupta* group comprising both willow-feeding specialists with a dual strategy and birch-feeding specialists, which produce butyrate esters (25, 28). Their highly fragmented Eurasian distribution, caused by an adaptation to a cold climate and the general poor capability for dispersal of many leaf beetles (29), might have favored the isolation of populations leading to population-specific adaptations to different host plants (28, 30). Despite recent data on how shifts in host plants have influenced the composition of defensive secretions in Chrysomelinae (21, 23, 24), the impact of the biosynthetic enzymes on the defensive system is unknown. Here we focus on salicylaldehyde biosynthesis, which is the only striking difference between willow-feeders and birch-feeders. We address the evolutionary origin of SAO, how the enzyme involved in the biosynthesis of salicylaldehyde continues to evolve, and what happens to SAO after a host shift occurs. For this comparative approach, we cloned and expressed SAO from *C. lapponica* adapted to the French willow (designated SAO-W) and from birch-feeding *C. lapponica* from Kazakhstan (designated SAO-B). SAO is a member of the glucose-methanol-choline oxidoreductase (GMC) multigene family, known for its wide variety of substrates and catalytic activities (31, 32), and has been characterized at the molecular and functional levels in the obligate salicylaldehyde-producing species *Chrysomela populi* (16–18, 33). We demonstrate that a nonfunctional SAO, along with a number of paralogs, are present in the Kazakh population. We discuss the significance of the paralogs and the lack of function of SAO-B in an evolutionary context.

Author contributions: R.K., H.V., and W.B. designed research; R.K., A.M., and K.R. performed research; R.K., A.M., and K.R. analyzed data; and R.K., H.V., J.M.P., and W.B. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. HQ245144–HQ245155).

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ECOLOGY

Results

Identification and Characterization of SAO in Willow-Feeding *C. lapponica*. The 1D-SDS/PAGE gels of glandular secretions of the French population of *C. lapponica* feeding on *Salix breviserrata* displayed a highly abundant 70-kDa protein (Fig. S1) that was identified as SAO-W by comparing their de novo peptide sequences with the two known *Chrysomela* SAOs. SAO-W is a member of the GMC oxidoreductase family. Its peptide sequence was assigned to a GMC encoding EST from a cDNA library of the larval defensive glands. Full-length sequencing of the SAO cDNA showed an identity of 83% to the leaf beetle SAOs of *C. populi* and *C. tremulae* at the amino acid level. This also included the N-terminal signal peptide for the secretory pathway (Fig. 1 and Fig. S2). Enzyme assays of the putative SAO protein, heterologously expressed in Sf9 cells, revealed SAO activity (Fig. 2). The oxidation proceeded in a *Re*-specific fashion and removed the deuterium atom exclusively and yielded [1-^{1}H]-salicylaldehyde from the 1*R*-[1-^{2}H$_{1}$]-salicyl alcohol precursor. This stereochemical course is in agreement with previous studies using the glandular secretion of *Phratora vitellinae* (17, 34), confirming that the oxidation is not an autoxidative artifact (Fig. 2). After in vitro deglycosylation of the heterologously expressed SAO with PNGase F, the molecular weight was reduced by ~5–7 kDa (Fig. S3). The difference in molecular weight between the expressed protein (~77 kDa) and the PNGase F–treated sample (~70 kDa or 67 kDa, according to the amino acid sequence) suggests substantial protein glycosylation. Most importantly, the insect cell line–expressed protein is of the same size as the native SAO of the glandular secretions.

Loss of Function of the SAO from Birch-Feeding *C. lapponica*. Based on the sequence information for SAO-W, we were able to amplify an ORF encoding SAO-B from a cDNA pool; this SAO-B was constructed from the RNA of the defensive glands of *C. lapponica* from Kazakhstan feeding on *Betula rotundifolia*. After expressing this enzyme in Sf9 cells, we found no evidence for an active SAO by in vitro assays. The same result was obtained with the native secretions. The protein (SAO-B) exhibited an identity of 97% compared with the SAO-W and a predicted signal peptide for the secretory pathway (Fig. 1 and Fig. S2). The absence of catalytic activity can be attributed to a deletion of 27 amino acids next to the N-terminal signal peptide in SAO-B. Amplifying and comparing SAO-encoding genes in willow-feeding and birch-feeding populations of *C. lapponica* identified a deletion affecting the end of the second exon (Fig. S4). This is coincident with the missing amino acids and causes the translation of a truncated, nonfunctional transcript of the SAO-B by alternative splicing. Additional analysis of the splicing pattern of the SAO from *C. populi* and related genes in *C. lapponica* showed that the site for alternative splicing in the birch-feeding *C. lapponica* concerns a position that is conserved in *C. populi* and *C. lapponica* willow feeders.

SAO-Related Sequences Imply Rapid Gene Duplication Events in *Chrysomela* spp. In the willow-feeding *C. lapponica*, we identified a GMC-type protein closely related to SAO-W, termed SAO-W paralog1. Expression of this protein in Sf9 cells demonstrated that this paralog also lacked SAO activity. Like the willow feeders, the birch-feeding species expressed a SAO-B paralog1 in glandular tissue. Due to its high sequence identity of 98.8% at the nucleotide level and 99.4% at the amino acid level, willow- and birch-feeding SAO paralog1 are most likely true orthologs. cDNA library and qPCR data indicate the presence of even more SAO-W paralogs in the genome of *C. lapponica*. To identify additional SAO-W–related genes, we screened a genomic Fosmid library of the willow-feeding *C. lapponica* with probes designed from their SAO-W and SAO-W paralog1. We identified a total of four SAO-W paralogs from willow-feeding populations with an amino acid identity of the predicted and known coding regions ranging from 54% to 97%. The most similar SAO-like genes were connected pairwise in the genome (*Clap*SAO-Wp1 + p3 and p2 + p4). From birch-feeding larvae of *C. lapponica*, we also were able to amplify an SAO-B paralogous gene (*Clap*SAO-Bp2). Two additional paralogs were obtained from cDNA pools of the defensive gland (*Clap*SAO-Bp1) and the Malpighian tubules (*Clap*SAO-Bpmt). Similar paralogs were present in the genomic DNA of *C. populi* showing high sequence similarity to one another (*Cpop*SAOp and *Cpop*SAOp1).

Expression Patterns of SAO-W, SAO-B, and Their Paralogs. The gene expression levels were compared in different tissues of both larval *C. lapponica* populations. The most obvious finding is the high level of SAO-W expression in the glandular tissue, which exceeds that in the gut, fat body, and Malpighian tubules by 40,000-fold (Fig. 3*A*). This pattern is conserved in the birch-feeding population for the SAO-B but with a 1,000-fold lower transcript abundance (Fig. 3*B*). A glandular tissue–specific expression pattern was also observed for the paralog1 in both populations. However, unlike the high expression level of SAO-W, the transcript abundance for the two paralogs1 was much lower and comparable in both populations (Fig. 3).

A second SAO-B paralog (*Clap*SAO-Bpmt; see the previous paragraph) was found in the Malpighian tubules by qPCR sequence analysis, but it proved to be different from the SAO-B. Interestingly, no comparable transcript was found in the Malpighian tubules of the willow feeders, demonstrating different expression patterns of the SAO paralogs in the two populations.

Evolution of SAO Activity by Expansion and Diversification of the GMC*i* Subfamily. Phylogenetic analyses, including members of the GMC*i*, GMC*z*, and glucose dehydrogenase (GLD) subfamilies and the SAO of *C. populi*, *C. tremulae*, *C. lapponica*, and their related

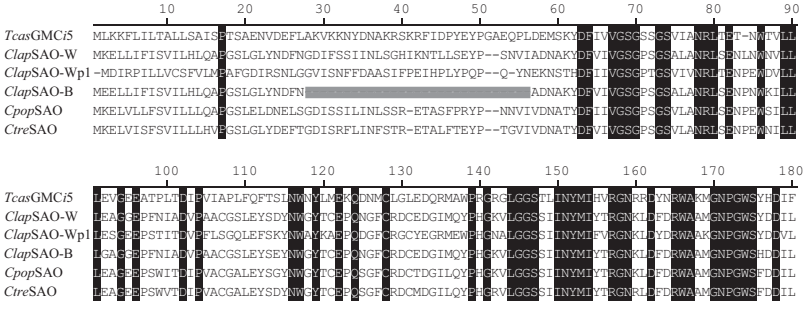

Fig. 1. Detail of an amino acid alignment of known SAOs of *C. populi* (*Cpop*SAO) and *C. tremulae* (*Ctre*SAO) with *Tribolium castaneum* GMC*i*5 (*Tcas*GMC*i*5), and the SAO of the willow (*Clap*SAO-W) and birch (*Clap*SAO-B) population as well as the SAO-W paralog1 (*Clap*SAO-Wp1) of *C. lapponica*. The truncation of *Clap*SAO-B is highlighted in gray, and overall identical amino acids are depicted in black.

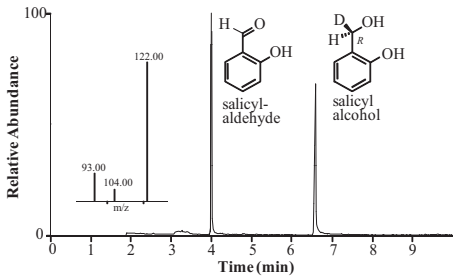

Fig. 2. GC-MS analysis of SAO-W activity assay with crude extract from Sf9 culture medium. The chromatogram shows the formation of salicylaldehyde by heterologously expressed SAO-W. The mass spectrum of the salicylaldehyde peak after an assay with deuterated salicyl alcohol in the *Re* position shows the *Re*-selective proton removal.

genes, showed the origin of all SAOs within the GMC oxidoreductases and a most recent common ancestral gene in the GMC*i* subfamily. This is indicated by their affiliation to *Tcas*GMC*i*5 supported by a high bootstrap and posterior probability value (90/1). Moreover, Fig. 4 demonstrates the SAOs in *Chrysomela* spp. have a single origin within the GMC*i* subfamily. Two true orthologous groups of SAO paralogs, supported by high probability values, are shown by identical numbers (SAOp1 and SAOp2). The presence of at least four SAO paralogs in the willow-feeding *C. lapponica*, three in the birch-feeding *C. lapponica*, and two in *C. populi* demonstrates the expansion of the GMC*i* subfamily in the genus *Chrysomela* by gene duplication events leading to the evolution of SAO activity.

The GMC*i*, GMC*z*, and GLD sequences cluster in distinct clades and within each clade according to species phylogeny. The SAO of *C. populi* and *C. tremulae* cluster tightly together and are supported by high bootstrap and probability values, but they are clearly separated from the SAO and related genes of *C. lapponica*.

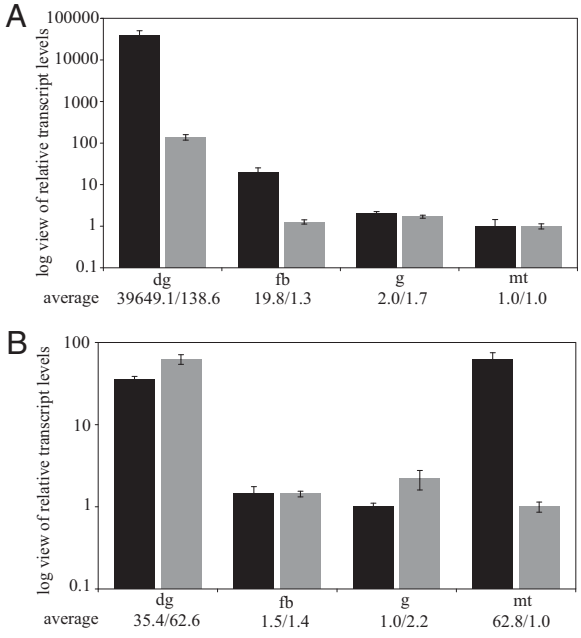

Fig. 3. Relative transcript abundance of SAO (black columns) and SAO paralog1 (gray columns) in different larval tissues. For normalization of transcript quantities, EF1α and eIF4A were used. (*A*) Willow-feeding population. (*B*) Birch-feeding population. Average transcript levels for SAO/SAO paralogs1 are shown below the graphs. Error bars indicate the SEM. dg, defensive gland; fb, fat body; g, gut; mt, Malpighian tubule.

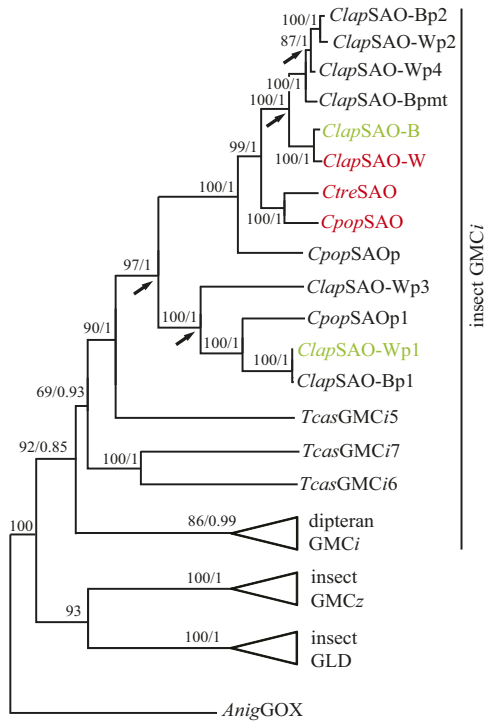

Fig. 4. Phylogeny of *Chrysomela* spp. SAO and related GMC oxidoreductases, including other insects. The phylogeny was generated using the neighbor-joining method with 1,000 bootstrap replicates. Bootstrap values are shown next to each node. The second numbers are posterior probability values based on a Bayesian phylogeny using the same set of data. (For details on the parameters and protein sequences used in this study, see *Materials and Methods* and *SI Materials and Methods*). GMC subfamilies (*i*, *z*, GLD) are well supported, as described previously (45). *Chrysomela* spp. SAO and related GMC oxidoreductases are members of the GMC*i* subfamily. Four putative gene duplication events (marked with arrows) led to an expansion of the GMC*i* subfamily in *C. lapponica*. The red highlighted proteins are those with proven SAO activity, whereas the green highlighted proteins lack SAO activity. *Clap*, *C. lapponica*; *Cpop*, *C. populi*; *Ctre*, *C. tremulae*; *Tcas*, *T. castaneum*; *Anig*, *Aspergillus niger*; GOX, glucose oxidase; p, SAO paralogs; pmt, SAO paralog Malpighian tubule–specific.

This indicates that the latter likely can be ascribed to species-specific gene duplications.

Discussion

Within the genus *Chrysomela,* the monophyletic *interrupta* group evolved the ability to biosynthesize esters from insect- and plant-derived precursors (19, 21, 24, 25, 35, 36). Whereas willow-feeding species of the *interrupta* group retained the ability to biosynthesize salicylaldehyde in addition to esters, birch-feeding species produce esters only as defensive compounds. The impact of the phytogenic precursors on the compositions of the defensive secretions has been studied intensively (16, 21, 13), but virtually nothing is known about the impact and nature of the glandular enzymes generating the defensive mixtures from the sequestered precursors.

We focused on the SAO from birch-feeding and willow-feeding larvae of *C. lapponica* because the host plant shift had a dramatic effect on the composition of the defensive secretions that could not be attributed simply to the different metabolite profiles of the food plants. For example, the presence of small amounts of alcohols [e.g., benzylalcohol (18)] in the secretions of the birch-feeding population suggests a complete lack of oxidative capacity. Therefore, we first identified and functionally expressed SAO-W from willow-feeding larvae of *C. lapponica*. This protein, a member of the GMC oxidoreductase family, consists of 625 amino acids including an N-terminal signal peptide addressing

the secretory pathway (exported from the glandular tissue to the reservoir) and is highly abundant in the defensive secretions. The enormous amount of transcript in the glandular tissue indicates an advanced state of tissue-specific expression and is consistent with the extent of oxidative capacity required to generate salicylaldehyde. In the birch-feeding population, neither SAO activity nor the SAO protein was detectable. This agrees with previous studies on birch-feeding *C. lapponica* larvae that found the presence of salicyl alcohol, but not salicylaldehyde, after larvae were transferred from birch to willow (21, 35). Glucoside transport and glucoside cleavage were not affected by the host plant shift, because this system is generally required to sequester plant-derived glucosides (37) and provides the precursors for the butyrate esters.

Although we found no SAO activity in the birch-feeding population, we were able to amplify an ORF encoding SAO-B. The transcription level of this gene is ∼1,000-fold lower in the birch-feeding population compared with the willow-feeding population. This lower (but still detectable) transcription in conjunction with the absence of SAO-B could indicate an additional post-transcriptional down-regulation of this nonfunctional enzyme. SAO-W and SAO-B demonstrated 98% amino acid sequence identity. However, in the SAO-B a truncation close to the N terminus, caused by a deletion at the second exon/intron border, leads to an alternative splicing of the SAO-B gene. By extensively sequencing SAO transcripts, we were able to identify additional splice variants of SAO-B (at lower frequency), pointing to the likelihood that mutations will accumulate in this population (Fig. S5). The structure of the SAO gene is highly conserved between the *C. lapponica* SAO-W, its paralogs, and the respective orthologs from *C. populi*, providing additional support for a specific (derived state) deletion event in the *C. lapponica* SAO-B gene. Obviously, the loss of catalytic function of SAO-B is fixed at the transcriptional, translational, and enzyme levels, thereby avoiding costly expression of a protein that is highly abundant in willow-feeding larvae but not required in the birch-feeders.

The glandular tissue–specific transcription is retained in the birch-feeding population despite the encoding of a nonfunctional SAO. This could be explained by a recent and ongoing process of SAO reversal, acquisition of a new function of the truncated SAO-B (unlikely given the variable splice pattern) and/or the cotranscription of the SAO with other genes (e.g., SAO paralog1), which demonstrates an SAO-typical expression in the glandular tissue. Polycistronic transcription is uncommon in eukaryotes, and few examples are known. One type of cotranscription is the polycistronic transcription of clustered genes that are clearly related in sequence and likely have evolved by gene duplication (38). However, we found no evidence for polycistronic mRNAs in the case of SAO, but the screening of a genomic library of willow-feeding *C. lapponica* indicates the occurrence of recent gene duplications and clustering of the duplicates with closely related GMC oxidoreductases. In total, four SAO-W paralogs with an amino acid identity of 54–97% were identified. The most similar paralogs, SAO-W paralog1+3 and 2+4, are clustered pairwise. Beside their sequence similarity, the highly conserved gene structure (Dataset S1) supports the view that these paralogs originated from recent gene duplication events. However, analogous to the SAO-W paralog1, which has no SAO activity, it is likely that none of these paralogs is a functional SAO enzyme.

Phylogenetic analyses with SAO sequences from the closely related salicylaldehyde-producing species *C. populi* and *C. tremulae* (33), *C. lapponica*, and their paralogs showed a common origin for all SAOs and their paralogs within the GMC oxidoreductase family and a most recent common ancestral gene in the GMC*i* subfamily. Furthermore, the identification of orthologs of SAO-W paralog1 and paralog2 in the birch-feeding population and another in *C. populi* shows that (*i*) the duplications arose before the evolution of the *interrupta* group, and (*ii*) the number of duplicates is comparable in willow-feeding and birch-feeding *C. lapponica*. *Tribolium castaneum* has three genes in the GMC*i* subfamily, one (*Tcas*GMC*i5*) that shares a most recent common ancestor with all of the SAOs (Fig. 4) and related genes found in the genus *Chrysomela,* providing evidence that SAO paralogs are not common to all beetles. The scenario of sub- or neo-functionalization of one gene-duplicated copy leading to SAO activity seems likely. The high degree of glycosylation of GMC proteins in general and a predicted secretory signal peptide common to all known GMC*i* members can be interpreted as preadaptations for the development of SAO activity. In addition, the lack of SAO activity of the *Clap*SAO-Wp1 protein indicates that the SAO-related genes have nonredundant functionalities, making an expansion-mediated diversification in the GMC*i* subfamily in *Chrysomela* species likely.

Our findings suggest that *C. lapponica* reflects a transition between larvae specializing on willow and birch or an ongoing speciation, which likely has occurred in some *Chrysomela* species of the *interrupta* group that are restricted to feeding on birch. The many previous comparisons of closely related phytophagous insect species and their host affiliation provide insight into the evolutionary history of host plant specialization/host shift and its impact on speciation (25, 39–41); in addition, some examples have compared different host affiliations within a species (42, 43). However, very little is known about the consequences of host shift for specific biochemical pathways and the underlying genetic background that is directly linked to host plant adaptation. SAO is an enzyme that fulfilled its function in *Chrysomela* chemical defense for millions of years; the loss of its activity in *C. lapponica* birch feeders shows which molecular mechanisms—namely, protein truncation, transcriptional down-regulation and most likely inhibition of post-transcriptional processes—can act within a short time period, especially if the complete lack of oxidative capacity also precludes the oxidation of benzyl alcohol to benzylaldehyde (18) that might be attractive to parasites as well. Therefore, to broaden our findings, SAO could be useful in addressing the evolutionary history of host plant shifts from willow to birch in the whole *interrupta* group. Comparative investigations of the fate of SAO could uncover whether the several independent shifts to birch (25) are also reflected by different, independent events of molecular changes of SAO in these species (e.g., alternative accumulation of mutations).

Most interestingly, by investigating secretory compounds of crosses between willow-feeding and birch-feeding individuals of *C. lapponica*, SAO activity can be reestablished (43). Although no speciation in progress could be identified by comparing the genetic distances among different European populations (28), the high reproductive isolation (43), population-specific host plant specialization and adaptation (30), and host-specific oviposition behavior (44), along with our results reported here, make ongoing speciation processes in *C. lapponica* caused by host plant shift likely. Although we found a variety of effects at different levels leading to the complete loss of SAO activity in the birch-feeding population of *C. lapponica*, further research is needed to elucidate the direction of the evolutionary scenario. Our findings suggest that the host plant shift from willow to birch caused the loss of SAO activity through the accumulation of mutations rather than vice versa. Comparative investigations of the selective forces that act on SAO genes in different populations feeding on different host plants will shed light on the dynamic and adaptive host plant associations of Chrysomelid beetles.

Materials and Methods

See *SI Materials and Methods* for details on population selection, identification of SAO protein in glandular secretions, and amplification of full-length GMC encoding cDNA and genes.

Heterologous Expression in Sf9 Cells. The cDNAs encoding the SAO proteins and their paralogs were amplified by PCR using gene-specific primers, including a 5′ Kozak sequence and lacking a stop codon for epitope and His-tag fusion expression after ligation into pIBV5 His TOPO TA vector (Invitrogen)

used for Sf9 insect cell expression. The correct sequence and direction of cloning were verified by sequencing. Sf9 cells were cultivated in GIBCO Sf-900 II SFM (Invitrogen) on tissue culture dishes (Falcon, 100 × 20 mm; BD) at 27 °C until 60% confluence was achieved. Transfection was performed with Insect Gene Juice (Novagen) following the manufacturer's protocol.

Genomic Library Construction and Screening. Genomic DNA, isolated from a *C. lapponica* willow population, was used for Fosmid Library construction performed with the CopyControl Fosmid Library Production Kit (Epicentre Biotechnologies), following the manufacturer's protocol. The pCC1FOS vectors were packaged with MaxPlax Lambda extracts (Epicentre Biotechnologies). The phage particles were used for EPI300-T1 cell infection. Stocks of infected cells were sent to ImaGenes (Berlin, Germany) for plating, stock library production of each clone, and duplicate colony spotting on nylon membranes. Nylon membranes were hybridized with SAO probes using the Amersham ECL Direct nucleic acid labeling and detection system (GE Healthcare) following the manufacturer's protocol for probe labeling, hybridization, and detection. Positive clones were amplified for Fosmid preparation followed by shearing on a HydroShear DNA shearing device (GeneMachines) and cloned into SmaI-digested pUC19 (Fermentas) for shotgun sequencing.

Phylogenetic Analyses. Multiple alignments of protein sequences were carried out using CLUSTALW (46). Phylogenetic relationships were inferred using a neighbor-joining algorithm (47) implemented in TREECON 1.3, taking insertions and deletions into account. A bootstrap resampling analysis with 1,000 replicates was performed to evaluate the tree topology. A model-based phylogenetic analysis using Bayesian Markov chain Monte Carlo inference, consisting of four Markov chains, was performed using MrBayes 3.1.2. The analysis was run for 1,000,000 generations, with sampling from the trees every 100 generations. The first 1,000 generations were discarded as burn-in. Trees were combined into a single summary tree. Dataset S3 provides the gene sources of all sequences used for the analyses.

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Supporting Information

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SI Materials and Methods

Leaf Beetle Larvae. Larvae of *C. lapponica* were collected from their respective host plants in the field. Willow-feeding specimens were collected from *S. breviserrata* near St Veran, France (44°20' N, 06°50' E). Birch-feeding specimens from *B. rotundifolia* were collected in the Altai Mountanins in East Kazakhstan, close to Uryl, near the Burkhat Pass (2130 m altitude, 49°07,438' N, 86°01,365' E). Larval tissues were stored in RNA*later* (Qiagen) at −20 °C until needed. *C. populi* larvae were collected in the Bavarian Forest near Furth im Wald on *Populus* sp. Willow-feeding *C. lapponica* and *C. populi* were also reared in the laboratory (20 °C, long-day conditions, 16-h/8-h light/dark period) on *Salix caprea* and *Populus* x *canadensis* for collecting glandular secretion.

Identification of SAO in Glandular Secretion. Larval glandular secretions were collected in the field using glass capillaries (Hirschmann Laborgeraete) by gently squeezing the larvae with forceps until they protrude their glands. The emerging droplets are then collected with the capillaries and stored at −20 °C. The secretion was used directly for 1D-SDS/PAGE gel runs with the Criterion XT Precast Gel System (BioRad). In brief, the separated protein bands were manually picked from deionized water using a 1- to 200-μL pipette and disposable tips cut to a 3 mm i.d. blunt inlet. The gel plugs were transferred to 96-well microtiter plates, reduced by 10 mM DTT, alkylated by 55 mM iodoacetamide (IAA), and destained in 50 mM ammonium bicarbonate/50% acetonitrile. The plugs were then air-dried and overlaid with 50 mM ammonium bicarbonate containing 70 ng of porcine trypsin (sequencing grade; Promega). The microtiter plates were covered with aluminum foil, and the proteins were digested overnight at 37 °C. The resulting peptides were extracted from the gel plugs by adding 50 μL of 50% acetonitrile in 0.1% TFA twice for 20 min each. The extracts were collected in extraction microtiter plates and vacuum-dried to remove any remaining liquid and ammonium bicarbonate. The tryptic peptides were reconstituted in 6 μL of aqueous 0.1% formic acid. The selected volume of samples (~4.5 μL) was injected on the Waters nanoACQUITY UPLC separator system. Mobile phase A (0.1% aqueous formic acid, 15 μL/min for 1 min) was used to concentrate and desalt the samples on a 20 × 0.180 mm Waters Symmetry C18 5-μm particle precolumn. The samples were then eluted on a Waters 100 mm × 75 μm i.d., 1.7-μm BEH nanoACQUITY C18 column. Phases A and B (100% MeCN in 0.1% formic acid) were linearly mixed in a gradient to 5% phase B in 0.33 min, increased to 40% B in 10 min, and finally increased to 85% B in 10.5 min, held at 85% B to 11 min, and decreased to 1% B in 11.1 min of the run. The eluted peptides were transferred to the nanoelectrospray source of a Synapt HDMS tandem mass spectrometer (Waters) equipped with metal-coated nanoelectrospray tips (Picotip, 50 × 0.36 mm, 10 μm i.d.; Waters). The source temperature was set at 80 °C, the cone gas flow was set at 20 L/h, and the nanoelectrospray voltage was 3.2 kV. The TOF analyzer was used in reflectron mode. The MS/MS spectra were collected at 1-s intervals (50–1,700 *m/z*). Infusion of 650 fmol/μL of human Glu-Fibrinopeptide B in 0.1% formic acid/acetonitrile (1:1 vol/vol) at a flow rate of 0.5 μL/min through the reference NanoLockSpray source (Waters) was performed every 30th scan to compensate for mass shifts in the MS and MS/MS fragmentation mode. The data were collected using MassLynx v4.1 MS software (Waters). ProteinLynx Global Server Browser v.2.3 software (Waters) was used for baseline subtraction and smoothing, deisotoping, de novo peptide sequence identification, and database searches. The peptide fragment spectra were searched against the Uniprot "Chrysomelidae" taxonomy-defined sub-database (downloaded on March 18, 2010, from http://www.uniprot.org/). The protein identification from MS/MS fragment spectra used a peptide mass tolerance of 15 ppm and a minimum of three peptides found, an estimated calibration error of 0.002 Da, a 0.03-Da mass deviation of de novo sequenced peptides, one possible missed cleavage, and carbamidomethylation of cysteins, possible oxidation of methionines, and possible deamidation of asparagines and glutamines.

Amplification of Full-Length GMC Encoding cDNAs and Genes. Expressed sequence tags encoding the SAO-W and SAO-W paralog1 identified in a cDNA library of willow-feeding *C. lapponica* were used for rapid amplification of cDNA ends PCR with the SMART RACE cDNA Amplification Kit (Clontech) in accordance with the manufacturer's guidelines. For full-length amplification of the SAO-B and SAO-B paralog1, sequence information of the willow-feeding population was used to design gene-specific primers. Genes were amplified with the LA Taq Polymerase (Takara), following the manufacturer's instructions for long-distance PCR. Gel-purified bands were prepared for shearing on a HydroShear DNA Shearing Device (GeneMachines) and then cloned into a SmaI-digested pUC19 vector (Fermentas) for shotgun sequencing. Positive clones were picked manually and grown overnight in DYT medium. The DNA Walking SpeedUp Premix Kit (Seegene) was used to obtain unknown flanking regions of SAO and related genes. Dataset S2 provides the complete list of primers used in this study.

Western Blot and Enzyme Assays. At 48 h after transfection, the culture medium of Sf9 cells was harvested and concentrated 20-fold with iCON concentrator (20 mL/9K; Pierce). The crude protein extract was used for Western blot analyses and enzyme assays. The heterologously expressed proteins were detected in Western blots using anti-V5 HRP antibody (Invitrogen) and the SuperSignal West HisProbe Kit (Pierce). The enzyme assay was performed in 0.5-mL plastic tubes in 50 mM potassium phosphate buffer (pH 6.0) at 30 °C for 30 min to 2 h with a final salicyl alcohol (Sigma-Aldrich) concentration of 350 μM to 3 mM in a total volume of 100 μL. Assays were stopped by freezing in liquid nitrogen. After extraction with 100 μL of ethyl acetate (Roth) and centrifugation for 5 min at 5,000 × *g*, the organic phase was directly used for quadrupole GC-MS analysis (ThermoQuest Finnigan Trace GC-MS 2000 equipped with an Alltech EC 5-column, 15 m × 0.25 mm, film thickness 0.25 μm). Compounds were eluted under programmed conditions: 45 °C for 1 min, ramped at 10 °C min^{-1} to 200 °C, followed by a ramp of 30 °C min^{-1} to 280 °C for 3 min. The He carrier gas was maintained at a flow rate of 1.5 mL min^{-1}. Eluting compounds were detected by MS and compared with authentic references. The *Re*-specificity of the enzyme was determined using chiral 1*R*-[1-$^2H_1$]-salicyl alcohol (1). The resulting salicyl aldehyde showed no deuterium labeling, demonstrating the *Re*-specificity of the oxidation.

Analysis of GMC Expression. Third larval instars were dissected. Fat body, defensive glands, gut, and Malpighian tubules were stabilized in RNAlater (Qiagen) and stored at −20 °C for further applications. For qPCR, 500 ng of total RNA pooled from the tissue of 20 individuals was reverse-transcribed with a mix of random and oligo-dT20 primers. Real-time qPCR was done in optical 96-well plates on a Stratagene MX 3000P system. All

steps were performed with the Verso SYBR Green 2-Step QRT-PCR Kit Plus ROX Vial (Thermo Scientific) following the manufacturer's instructions. Specific amplification of transcripts was verified by melting curve analysis, followed by sequencing of the PCR products. All primers were designed using Primer3 (v. 0.4.0). *Eukaryotic initiation factor 4A* and *elongation factor 1α* were used as reference genes to normalize quantities of our genes of interest. Raw data were analyzed with qBase, using a logarithmic view of relative expression level on the *y*-axis of the graphs, with the lowest transcript abundance set to 1.

1. Veith M, Oldham NJ, Dettner K, Pasteels JM, 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.

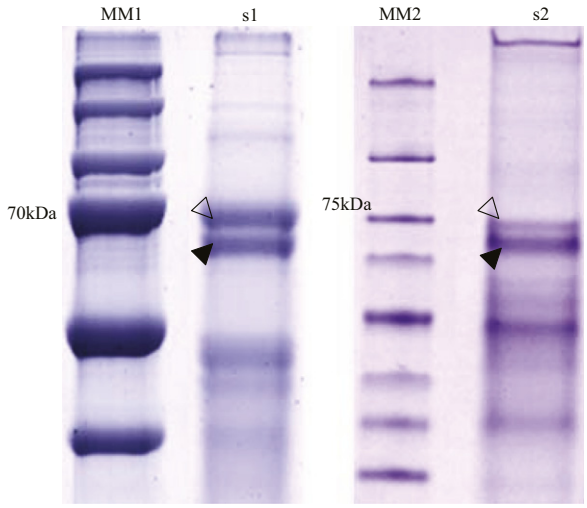

Fig. S1. A 1D-SDS gel of glandular secretion of *C. lapponica* feeding on willow (S1) and feeding on birch (S2). The open arrowhead in S1 marks the corresponding SAO-W band, whereas in S2, no SAO could be identified from the weak band of equal size. The black arrowheads denote a band in the secretion that most likely corresponds to a common protein in both populations based on MS/MS analysis. MM1 and MM2 are two different protein markers.

Percent Identity

Divergence

	1	2	3	4	5	6	
1		51.0	53.9	51.8	49.4	51.0	1 *Tcas*GMC*i5*
2	77.3		55.7	97.3	83.0	82.3	2 *Clap*SAO-W
3	70.1	65.8		56.6	56.4	57.2	3 *Clap*SAO-Wp1
4	75.3	2.7	63.8		84.1	83.2	4 *Clap*SAO-B
5	81.6	19.4	64.3	17.9		89.4	5 *Cpop*SAO
6	77.3	20.2	62.6	19.0	11.4		6 *Ctre*SAO
	1	2	3	4	5	6	

Fig. S2. GMC oxidoreductase protein sequence distances based on a CLUSTALW alignment implemented in LaserGene 8.02 (DNAStar). A detail of the alignment is shown in Fig. 1.

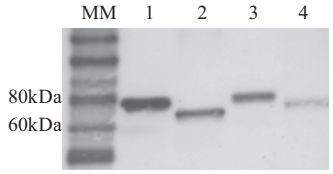

Fig. S3. Western blot analysis of heterologously expressed SAO of *C. populi* (lanes 1 and 2) and SAO-W of *C. lapponica* (lanes 3 and 4). Lane 2 and 4 show PNGase F–treated SAO with a similar shift in size compared with untreated proteins, indicating a high degree of posttranslational *N*-glycosylation.

```
                                                  v
Clapsao-B      GATCTCTTGGACTATACAATGACTTCAATGGTGACATATTCAGCTCCATAATCAA---------- 55
Clapsao-W      GATCTCTTGGACTATACAATGACTTCAATGGTGACATATTCAGCTCCATAATCAATTTATCAGGCCATATCAAGA 75
Clapsao-W E2   GATCTCTTGGACTATACAATGACTTCAATGGTGACATATTCAGCTCCATAATCAATTTATCAGGCCATATCAAGA 75
Clapsao-W E3   ---------------------------------------------------------------------- 0

Clapsao-B      ------------------------------------v---------------------------------- 55
Clapsao-W      ACACATTACTCTCTGAATATCCGTCCAATGTAATAGGTAAGTTGAAATGTATGTTCTCTCTTCAGAACGTGAATA 150
Clapsao-W E2   ACACATTACTCTCTGAATATCCGTCCAATGTAATAG                                   111
Clapsao-W E3   ---------------------------------------------------------------------- 0
                                  ▼
Clapsao-B      AGAAATGGAATATTTTCAGCTGATAATGCCAAGTACGACTTCGTGATTGTTGGGTCTGGACCCTCAGGGTC     422
Clapsao-W      AGGAATGAAATATTTTCAGCTGATAATGCCAAGTACGACTTCGTGATTGTTGGGTCTGGACCCTCAGGGTC     727
Clapsao-W E2                                                                           111
Clapsao-W E3   ------------------CTGATAATGCCAAGTACGACTTCGTGATTGTTGGGTCTGGACCCTCAGGGTC     52
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Fig. S4. A detail of the SAO alignment of willow-feeding (*Clap*sao-W) and birch-feeding (*Clap*sao-B) populations from exon2 (*Clap*sao-W E2) to exon3 (*Clap*sao-W E3), leaving out most of the intron in between. The 5′ splice site of the intron differs in birch-feeding *C. lapponica* and willow-feeding *C. lapponica* sao-W (open arrowheads), whereas the 3′ splice site of the intron is the same in both populations (closed arrowhead). Alternative splicing in the sao-B causes a truncation but no frame shift in the encoded protein.

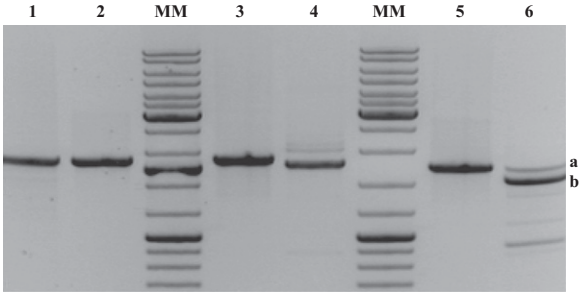

Fig. S5. Agarose gel of PCR products of different GMC transcripts of *C. lapponica* willow-feeding (lanes 1, 3, and 5) and birch-feeding (lanes 2, 4, and 6) populations. Lanes 1 and 2 show equal lengths of the SAO-W/Bp1 full-length transcript, whereas lanes 3 and 4 show a decreased length of the *Clap*SAO-B full-length transcript. In lanes 5 and 6, the forward primer binds to the region that is deleted in the birch population. Sequencing of purified PCR products of lane 6 revealed *Clap*SAO-B transcripts showing deletions at other positions (bands a and b). MM is the DNA ladder.

Dataset S1. Table of *Chrysomela* spp. GMC genes and their exon lengths in base pairs

Dataset S1

For genes highlighted in bold, full-length transcript data exist, and the gene architecture was elucidated by mRNA to genomic alignments using Spidey implemented in NCBI Toolkit. Coding regions of the other genes are based on Fgenesh, an HMM-based gene structure prediction tool implemented in Softberry, and manual searching.

Dataset S2. Table of primers used including names, sequences, and uses

Dataset S2

Dataset S3. Source of gene information of all sequences used for phylogenetic analyses

Dataset S3

Dataset S1

gene name	Exon1	Exon2	Exon3	Exon4	Exon5	Exon6	Exon7	Exon8	Exon9
***Cpop* sao**	6	154	219	335	292	174	197	217	278
***Clap* sao-W**		157	219	335	292	174	197	217	249
***Clap* sao-B**		36	219	335	292	174	197	217	249
***Clap* sao-Wp1**		154	219	335	292	174	196	218	281
Clap sao-Wp2		157	219	335	292	174	197	150	
Clap sao-Bp2		130	219	335	292	174	197	217	249
Clap sao-Wp3		162	219	335	292	177	197	217	296
Clap sao-Wp4			70	335	292	174	197	217	281
Cpop SAOp			219	335	292	174	197	218	148

Primer	Sequence	Characteristics
GDHcp_P2	5`-ACGGCTTCTGGATCGTTGTTCGGTCCCATTT-3`	*Cpop* SAO 5`RACE
GDHcl_P4	5`-CTGCATTGCTGGACTGTCATTGATCCTTTGG-3`	*Clap* SAO-Wp1 5`RACE
GDHcl_for1	5`-GATATGGATATCCGTCCAATA-3`	*Clap* SAO-B/Wp1 expression; fwd
GDHcl_rev1	5`-AACACGTTTCCAATCTTCTTTAAT-3`	*Clap* SAO-B/Wp1 expression; rev
GDHcp_for4	5`-AACATGGAGGAGTTAGTTTTACTTTTCTC-3`	*Cpop* SAO expression; fwd
GDHcp_rev2	5`-CATTCCATAATCTTTTTTAATGATGTCTGAT-3`	*Cpop* SAO expression; rev
GDHcp_P1	5`-GCGTGGTCGTACATCTTCTTACCGACTGGCAG-3`	*Clap* SAO-W 5`RACE
GDHcl2_P1	5`-ATGGGCTGCGATGGGTAATCCAGGATGGTC-3`	*Clap* SAO-W 3`RACE
GDHcl2_for1	5`-AACATGGAGGAGTTACTGATATTC-3`	*Clap* SAO-B/W expression; fwd
GDHcl2_rev1	5`-TATGCCAGTCCAATCATTCTTAATG-3`	*Clap* SAO-B/W, *Clap* SAO-Bpmt expression; rev
GDHcl_P1	5`-CTCAGCCGTGGGTCCACCACAGCATCC-3`	*Cpop* SAOp1 5`RACE
GDHcp3_P1	5`-GGTCGCTGAGGCTTATATAAAGGCAGCCCAAGA-3`	*Cpop* SAOp1 3`RACE
cp-gl14c03gen_f1	5`-TCTCAGTTATTCTGCTTCTGCAAGCTCCTGG-3`	*Cpop* SAO and SAOp gene; fwd
cp-gl14c03gen_r1	5`-GATGTCTGATGCTCTCTCTCCAACCATGTAAG-3`	*Cpop* SAO and SAOp gene; rev
cp-gl03a11gen_f2	5`-CTCCACGCATGACTTCATCATCGTTGGATCAG-3`	*Cpop* SAOp1gene; fwd
cp-gl03a11gen_r2	5`-CTTGGTGGTAGAGGCTGCTGATTATAGTTCTC-3`	*Cpop* SAOp1gene; rev
cp_sao_TSP1up	5`-CGCCTAAGAGTTCATCCAGTTAC-3`	*Cpop* SAO gene walk upstream
cp_sao_TSP2up	5`-GAGTCTAATCTGGATCAGTAGCG-3`	*Cpop* SAO gene walk upstream nested1
cp_sao_TSP3up	5`-TCAAGTTCAAGAGATCCAGGAGC-3`	*Cpop* SAO gene walk upstream nested2
cp_sao_TSP1down	5`-ACGGCTTTTGAGTCGGTTGCAG-3`	*Cpop* SAO gene walk downstream
cp_sao_TSP2down	5`-ATGGGACCGAACAACGATCCAG-3`	*Cpop* SAO gene walk downstream nested1
cp_sao_TSP3down	5`-GTTGCAGTTGCTTACATGGTTGG-3`	*Cpop* SAO gene walk downstream nested2
cp_sao_TSP1up2	5`-CGAACTCACCAAATCGATTCGTG-3`	*Cpop* SAO gene walk upstream2
cp_sao_TSP2up2	5`-GAAGAACGAAGACTATCTCCAGATG-3`	*Cpop* SAO gene walk upstream2 nested1
cp_sao_TSP3up2	5`-ATTGACGTGCAACACTGAGCTCG-3`	*Cpop* SAO gene walk upstream2 nested2
cp_sao_TSP1down2	5`-GGTTCCCAAGCTACACTTCGATAC-3`	*Cpop* SAO gene walk downstream2
cp_sao_TSP2down2	5`-CTGTCTAGAAACATCTTGGAAATGC-3`	*Cpop* SAO gene walk downstream2 nested1
cp_sao_TSP3down2	5`-ACCCGACAGGTTACAAGTGCTAC-3`	*Cpop* SAO gene walk downstream2 nested2
cl_gl14c03gen_f1	5`-CTGCATCTGCAAGCTCCTGGATCTCTTGG-3`	*Clap* SAO-B/W and SAO-B/Wp2 gene; fwd
cl_gl14c03gen_r1	5`-CTGCTGCCCTCTCTCCAACCATATAAGCTAC-3`	*Clap* SAO-B/W and SAO-B/Wp2 gene; rev
cl_gl03a11gen_f1	5`-TTGTGTTGATGCCAGCATTTGGCGATATAAGA-3`	*Clap* SAO-Wp1 gene; fwd
cl_gl03a11gen_r1	5`-CTCCAATCATGTAGGCTGCAGCAACGGTGTG-3`	*Clap* SAO-Wp1 gene; rev

Clfr_14c03_e4_fwd	5`-CCAACAAGCCCGAATACTTCAC-3`	*Clap* SAO-W Exon4; fwd: probes for genomic lib screen
Clfr_14c03_e4_rev	5`-CCAGATTCTTGACACACATTTCAG-3`	*Clap* SAO-W Exon4; rev: probes for genomic lib screen
Clfr_14c03_e5_fwd	5`-AGGTATCATATGTTCAAAGTACCAC-3`	*Clap* SAO-W Exon5; fwd: probes for genomic lib screen
Clfr_14c03_e5_rev	5`-GCCTCGTCAAGTGAGGAATTG-3`	*Clap* SAO-W Exon5; rev: probes for genomic lib screen
eIF4A_fwd	5`-ATGYTSTCYMGHGGKTTCAAGG-3`	eIF4A fishing; fwd
eIF4A_rev	5`-TTRCGACCRAARCGDCCACC-3`	eIF4A fishing; rev
EF1α_94fwd	5`-GGTGGYATYGAYAARCGTAC-3`	EF1α fishing; fwd
EF1α_470rev	5`-GAGTCCATYTTGTTSACACCRAC-3`	EF1α fishing; rev
eIF4A FWD351	5`-GGGACATGGAGCAGAGAGAG-3`	*Clap* eIF4A qPCR; fwd
eIF4A REV501	5`-TCCCTATTCGATGGCAAATC-3`	*Clap* eIF4A qPCR; rev
EF1α FWD111	5`-ACGTGGTATCACCATCGACA-3`	*Clap* EF1α qPCR; fwd
EF1α REV256	5`-CTACGATTAGCACGGCACAA-3`	*Clap* EF1α qPCR; rev
ClapFR_SAO+ClapKZ_SAODEL1 FWD1085	5`-AATTGAACGACAGCCTACCG-3`	*Clap* SAO-B/W qPCR; fwd
ClapFR_SAO+ClapKZ_SAODEL1 REV1233	5`-AGGATCCGTTGAGACATTGG-3`	*Clap* SAO-B/W qPCR; rev
ClapFR_SAOpara1+ClapKZ_SAOpara1 FWD348	5`-GGCAGAACCACAAGATGGAT-3`	*Clap* SAO-B/Wp1 qPCR; fwd
ClapFR_SAOpara1+ClapKZ_SAOpara1 REV492	5`-GGCAGCCCATCTGTCATAAT-3`	*Clap* SAO-B/Wp1 qPCR; rev
ClKzMT_Saop_fwd	5`-AACATGGAGGAGTTAGTGATACTAATC-3`	*Clap* SAO-Bpmt expression; fwd

Dataset S3

Gene name used in this study	GB no. used for our analysis ("ts", predicted manually in this study, "§[1]", taken from Iida K et al. 2007 (45))
D . melanogaster	
GMC*z* 1	AAF48398.1
GMC*i* 1	AAF48395.1
GMC*i* 2	AAF48394.2
GLD	AAF54038.1
A . gambiae	
GMC*i* 4	§[1]
GMC*i* 3	§[1]
GMC*z* 1	§[1]
GLD	§[1]
A . mellifera	
GMC*z* 1	XM_394220
GLD	ts
T . castaneum	
GMC*z* 1	EFA05531.1
GMC*i* 5	ts
GMC*i* 6	EFA05537.1
GMC*i* 7	EFA05538.1
GLD	EFA05227.1
A . niger	
Anig GOX	ACB30369.1
C . populi	
SAO	HQ245154
SAOp1	HQ245155
SAOp	HQ245148
C . tremulae	
SAO	CAQ19343
C . lapponica **(willow feeder)**	
SAO-W	HQ245149
SAO-Wp1	HQ245150
SAO-Wp2	HQ245144
SAO-Wp3	HQ245145
SAO-Wp4	HQ245146
C . lapponica **(birch feeder)**	
SAO-B	HQ245151
SAO-Bp1	HQ245152
SAO-Bp2	HQ245147
SAO-Bpmt	HQ245153

Manuscript 2

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To be or not to be convergent in salicin-based defence in chrysomeline leaf beetle larvae: evidence from *Phratora vitellinae* salicyl alcohol oxidase

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Glandular chemical defence relying on the action of salicylaldehyde is characteristic for *Chrysomela* leaf beetle larvae. The salicylaldehyde precursor salicin, sequestered from salicaceous host plants, is deglucosylated and the aglycon further oxidized by a salicyl alcohol oxidase (SAO) to the respective aldehyde. SAOs, key enzymes in salicin-based glandular chemical defence, were previously identified and shown to be of a single evolutionary origin in *Chrysomela* species. We here identified and characterized SAO of *Phratora vitellinae*, the only species outside the genus *Chrysomela* that produce salicylaldehyde as a defensive compound. Although *Chrysomela* and *Phratora* are not closest relatives, their SAOs share glucose–methanol–choline oxidoreductase (GMC) affiliation, a specific GMC*i* subfamily ancestor, glandular tissue-specific expression and almost identical gene architectures. Together, this strongly supports a single origin of SAOs of both *Chrysomela* and *Phratora*. Closely related species of *Chrysomela* and *P. vitellinae* use iridoids as defensive compounds, which are like salicylaldehyde synthesized by the consecutive action of glucosidase and oxidase. However, we elucidated SAO-like sequences but no SAO proteins in the glandular secretion of iridoid producers. These findings support a different evolutionary history of SAO, related genes and other oxidases involved in chemical defence in the glandular system of salicylaldehyde and iridoid-producing leaf beetle larvae.

Keywords: *Phratora vitellinae*; salicyl alcohol oxidase; chemical defence; Chrysomelidae

1. INTRODUCTION

Leaf beetle larvae of the subtribe Chrysomelina are efficiently protected against generalist predators (e.g. ants, wasps, ladybirds, spiders) and microbial infestation by the use of a glandular chemical defence [1–9]. When attacked by predators, the larvae release droplets of deterrent secretion through dorsal openings of eight pairs of defensive glands. The defensive secretion is partly biosynthesized and stored inside the gland reservoirs. Although a huge variety of defensive compounds in different Chrysomelina species exist, the consecutive action of a glucosidase and an oxidase is widespread to modify selectively ingested alcohol glucosides to bioactive compounds inside the glandular reservoirs [10].

Chrysomelina larvae most frequently use either iridoids or salicylaldehyde as chemical defensive compounds, the former being derived from 8-hydroxygeraniol-8-glucoside and the latter from salicin (figure 1) (reviewed in [13,14]).

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Taking the Chrysomelina phylogeny into account, the predominant de novo biosynthesis of iridoids (e.g. in the genera *Gastrophysa*, *Phaedon*, *Phratora*) is seen as the ancestral state in the evolution of deterrent compound production [12]. In comparison to this, the salicylaldehyde biosynthesis of *Chrysomela* larvae is a derived and more economic defence strategy, because the precursor salicin is sequestered from their salicaceous host plants [6]. Whether the salicylaldehyde biosynthesis is derived from the iridoid biosynthetic route (by shift of substrate specificities of the glandular reservoir oxidases) or different chemical defences in Chrysomelina evolved independently is not known so far. Furthermore, the origin of salicylaldehyde biosynthesis is not clear as beside the genus *Chrysomela*, also the more distant relative *Phratora vitellinae* produce salicylaldehyde [10,15,16] (figure 1). The salicylaldehyde precursor and biosynthesis, namely deglucosylation and oxidation, has been shown to be the same in *Chrysomela* and *P. vitellinae* larvae [6,10], but nevertheless a convergent origin of salicin-based chemical defence in both genera is discussed owing to phylogenetic analyses [12]. Therein *P. vitellinae* is placed isolated within iridoid producers without a close affiliation to the salicylaldehyde-producing genus *Chrysomela*. Moreover, *P. vitellinae* is the only species of the

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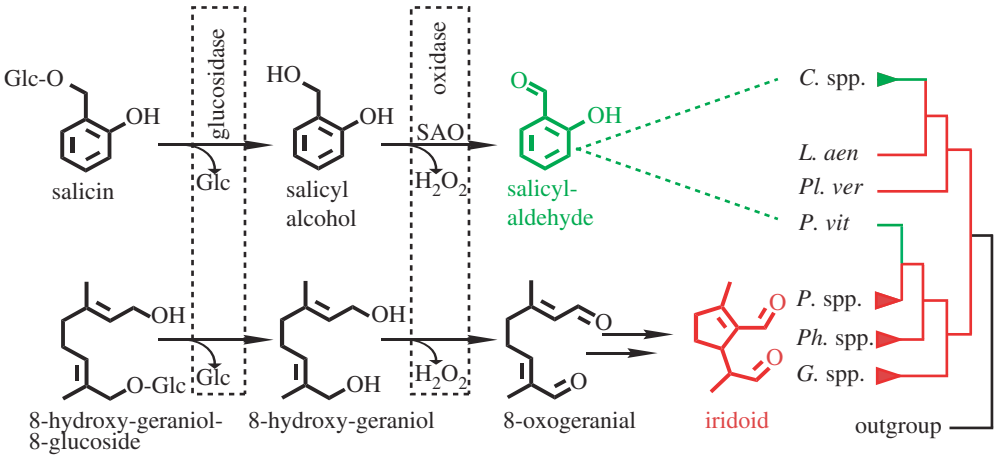

Figure 1. Schematic of salicylaldehyde and iridoid biosynthesis in glandular reservoirs highlighting common enzymatic activities with boxes. The occurrence of those defensive compounds in various chrysomeline leaf beetle genera is plotted onto their phylogeny. Therein iridoid-based chemical defence (red) is the primary state. The derived salicylaldehyde-based defence (green) is present in the genus *Chrysomela* and *P. vitellinae*. This figure is a combination and modification of Pasteels *et al.* [10], Köpf *et al.* [11] and Termonia *et al.* [12]. *C.* spp., *Chrysomela* species; *L. aen*, *Linaeidea aenea*; *Pl. ver*, *Plagiodera versicolora*; *P. vit*, *P. vitellinae*; *P.* spp., *Phratora* species; *Ph.* spp., *Phaedon* species; *G.* spp.; *Gastrophysa* species, SAO, salicyl alcohol oxidase; Glc, glucose.

genus *Phratora* synthesizing salicylaldehyde instead of iridoids [15]. Therefore, to clarify the origin of salicylaldehyde-based chemical defence, the identification, characterization and comparison of the enzymes involved in its biosynthesis are required. In case of several *Chrysomela* ssp., salicyl alcohol oxidase (SAO), which catalyses the final step in salicylaldehyde formation, has been elucidated and previous studies indicate a similar enzymatic activity and the presence of an SAO-related protein in the glandular secretion of *P. vitellinae* [17–20]. However, the enzyme itself is not known.

In the present study, we focus on the SAO of *P. vitellinae* to get insights into the evolution of salicin metabolism in conjunction with chemical defence and the origin of the SAO in chrysomelines. Here, we identified the SAO protein in the glandular secretion and used the corresponding cDNA for cloning and heterologous expression in an Sf9 insect cell line followed by enzyme assays. The SAO of *P. vitellinae* belongs to the glucose–methanol–choline oxidoreductase (GMC) family and shows high sequence similarity to *Chrysomela* ssp. SAOs. Enzymatic activity and *Re* specificity could be verified. Phylogenetic analyses indicate a common ancestor of all known SAOs, which is additionally supported by both common expression profiles and highly conserved gene architecture. The origin of chemical defence based on salicin sequestration in chrysomelines is discussed in consideration of these data and previous findings. This includes for the first time an elucidation of SAO-like sequences from transcriptome and genome screenings in iridoid-producing leaf beetle larvae.

2. MATERIAL AND METHODS

(a) *Leaf beetle larvae*

Larvae of *P. vitellinae* and *Phratora laticollis* were collected from their respective host plant in the field near Bruxelles and reared in the laboratory (20°C, long-day conditions: 16 L : 8 D period) on *S. caprea* and *Populus* × *canadensis* for collecting glandular secretion. Larval tissues were stored in RNA*later* (Qiagen) at −20°C until needed.

(b) *Identification of the salicyl alcohol oxidase in the glandular secretion*

Larval glandular secretion was collected in the laboratory using glass capillaries (Hirschmann Laborgeraete, ID: 0.28 mm, L: 100 mm) by gently squeezing the larvae with forceps until they protruded their glands. The emerging droplets are then collected with the capillaries and stored at −20°C. The secretion was directly used for one-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gel runs with the help of the Criterion XT Precast Gel System (BioRad). Briefly, the separated protein bands were manually picked from deionized water using a 1–200 µl pipette and disposable tips cut to 3 mm ID blunt inlet. The gel plugs were transferred to 96-well microtitre plates (MTPs), reduced by 10 mM dithiothreitol, alkylated by 55 mM iodoacetamide and destained in 50 mM ammonium bicarbonate/50 per cent acetonitrile. Subsequently, the plugs were air-dried and overlayed with 50 mM ammonium bicarbonate containing 70 ng porcine trypsin (Sequencing grade, Promega). The MTPs were covered with aluminium foil and the proteins were digested overnight at 37°C. The resulting peptides were extracted from the gel plugs by adding twice 50 µl of 50 per cent acetonitrile in 0.1 per cent trifluoroacetic acid for 20 min and the extracts were collected in an extraction MTP and vacuum-dried to remove any remaining liquid and ammonium bicarbonate. The tryptic peptides were reconstituted in 6 µl aqueous 0.1 per cent formic acid (FA). The selected volumes of samples (*ca* 4.5 µl) were injected on a nanoAcquity nanoUPLC system (Waters, Milford, MA, USA). Mobile phase A (0.1% aqueous FA, 15 µl min^{-1} for 1 min) was used to concentrate and desalt the samples on a 20 × 0.180 mm Symmetry C18, 5 µm particle precolumn. The samples were then eluted on a 100 mm × 75 µm ID, 1.7 µm BEH nanoAcquity C18 column (Waters). Phases A and B (100% MeCN in 0.1% FA) were linearly mixed in a gradient to 5 per cent phase B in 0.33 min, increased to 40 per cent B in 10 min and finally increased to 85 per cent B in 10.5 min, holding 85 per cent B to 11 min and decreasing to 1 per cent B in 11.1 min of the run. The eluted peptides were transferred to the nanoelectrospray source of a Synapt HDMS tandem mass spectrometer (Waters) equipped with

metal-coated nanoelectrospray tips (Picotip, 50 × 0.36 mm, 10 μm I.D, Waters). The source temperature was set to 80°C, cone gas flow at 20 l h^{-1} and the nanoelectrospray voltage was 3.2 kV. The TOF analyzer was used in a reflectron mode. The MS/MS spectra were collected at 1 s intervals (50–1700 *m*/*z*). A 650 fmol μl^{-1} human Glu-fibrinopeptide B in 0.1 per cent FA/acetonitrile (1 : 1 *v*/*v*) was infused at a flow rate of 0.5 $\mu l\ min^{-1}$ through the reference NanoLock-Spray source every 30th scan to compensate for mass shifts in the MS and MS/MS fragmentation mode. The data were collected by MassLynx v. 4.1 software. ProteinLynx Global Server Browser v. 2.3 software (both Waters) was used for baseline subtraction and smoothing, deisotoping, de novo peptide sequence identification and database searches. The peptide fragment spectra were searched against the Uniprot 'Chrysomelidae' taxonomy-defined subdatabase downloaded on 18 March 2010 from http://www.uniprot.org/. The protein identification from MS/MS fragment spectra used peptide mass tolerance 15 ppm and minimum three peptides found, estimated calibration error 0.002 Da, 0.03 Da mass deviation of de novo-sequenced peptides, one possible missed cleavage, and carbamidomethylation of cysteins, possible oxidation of methionines, and possible deamidation of asparagines and glutamines, respectively.

(c) *Amplification of full-length salicyl alcohol oxidase encoding cDNA and genes*

An expressed tag sequence (EST) encoding the *P. vitellinae* SAO identified by using *Chrysomela* SAO primers were used for RACE PCR with the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturers' guidelines. Genes of *P. vitellinae* SAO and *P. laticollis* SAO-like protein were amplified with the LA Taq Polymerase (Takara), following the recommended instructions for long-distance PCR. Gel-purified bands were prepared for shearing on a Hydro-Shear DNA Shearing Device (GeneMachines) and then cloned into *Sma*I-digested pUC19 vector (Fermentas) for shotgun sequencing. Positive clones were picked manually and grown overnight in DYT medium.

(d) *Western blot and enzyme assays*

Forty-eight hours after transfection, the culture medium of Sf9 cells was harvested, concentrated 20-fold with iCON concentrators (20 ml/9 K, PIERCE) and the crude protein extract was then used for Western blot analysis and enzyme assays. For detection of the heterologously expressed proteins in Western blots, Anti-V5-HRP antibody (Invitrogen) and Super-Signal West His Probe Kit (Pierce) were used. The enzyme assay was performed in 0.5 ml plastic tubes in 50 mM potassium phosphate buffer (pH 6.0) at 30°C for 10 min to 2 h with a final salicyl alcohol (Sigma) or 8-hydroxygeraniol [21] concentration of 350 μM to 3 mM in a total volume of 100 μl. Assays were stopped by freezing in liquid nitrogen. After extraction with 100 μl ethyl acetate (Roth) and centrifugation for 5 min at 5000*g*, the organic phase was directly used for GCMS analysis (ThermoQuest Finnigan Trace GC-MS 2000 (Quadrupole) equipped with Alltech EC 5-column, 15 m × 0.25 mm, film thickness 0.25 μm). Compounds were eluted under programmed conditions: 45°C for 1 min, ramped at 10°C min^{-1} to 200°C, followed by a 30°C min^{-1} ramp to 280°C for 3 min. Helium carrier gas was maintained at a flow rate of 1.5 ml min^{-1}. Eluting compounds were detected by mass spectrometry and compared with authentic references. The *Re* specificity of the enzyme was determined using chiral 1*R*-[1-$^2H_1$]-salicyl alcohol [20]. The resulting salicyl aldehyde showed no deuterium labelling, demonstrating the *Re* specificity of the oxidation.

(e) *Analysis of glucose–methanol–choline oxidoreductase expression*

Third larval instars were dissected. Fat bodies, defensive glands, gut and Malpighian tubules were stabilized in RNA-later (Qiagen) and stored at −20°C for further applications. For qPCR, 500 ng of total RNA pooled from the tissue of 20 individuals was reverse transcribed with a mix of random and oligo-dT20 primers. Real-time PCR was done in optical 96-well plates on an MX 3000P (Stratagene). All steps were performed with Verso SYBR Green 2-Step QRT-PCR Kit Plus ROX Vial (Thermo Scientific) following the manufacturer's instructions. Specific amplification of transcripts was verified by melting curve analysis. All primers were designed by the help of Primer 3 (v. 0.4.0.). We used eukaryotic initiation factor-4A (*eIF4A*) and elongation factor-1α (*EF1α*) as reference genes to normalize quantities of our genes of interest. For the analysis of raw data, we used qBase, choosing a logarithmic view of the relative expression level on the *y*-axis of the graphs, where the lowest transcript abundance was set to 1.

(f) *Heterologous expression in Sf9 cells*

The cDNA encoding the SAO protein was amplified by PCR using gene-specific primers, including a 5′ Kozak sequence and lacking a stop codon for epitope and His-tag fusion expression after ligation into pIBV5-His-TOPO TA vector (Invitrogen) used for Sf9 insect cell expression. The correct sequence and direction of cloning were verified by sequencing. Sf9 cells were cultivated in Sf-900 II SFM (GIBCO) on tissue culture dishes (100 × 20 mm, FALCON) at 27°C until 60 per cent confluence. Transfection was performed with Insect Gene Juice (Novagen) following the manufacturer's protocol.

(g) *Phylogenetic analyses*

Multiple alignments of protein sequences were carried out using ClustalW [22]. Phylogenetic relationships were inferred using a neighbour-joining algorithm [23] implemented in MAFFT taking insertions and deletions into account. The bootstrap re-sampling analysis with 1000 replicates was performed to evaluate the tree topology. A model-based phylogenetic analysis using Bayesian Markov Chain Monte Carlo inference was also carried out as implemented in MrBayes v. 3.1.2 consisting of four Markov chains. The analysis was run for 1 000 000 generations, sampling from the trees every 100 generations, and the first 1000 generations were discarded as the 'burn-in'. Trees were combined into a single summary tree.

3. RESULTS

(a) *Identification and sequence analysis of salicyl alcohol oxidase protein and cDNA*

One-dimensional SDS–PAGE gels of *P. vitellinae* larval glandular secretion displayed a highly abundant protein at about 75 kDa (figure 2). Through de novo MS fingerprinting, we could identify this major glandular protein as a member of the GMC oxidoreductase family. In particular, de novo peptide sequences could be assigned to known *Chrysomela* SAOs [18,19]. The corresponding cDNA was obtained using internal SAO primers designed

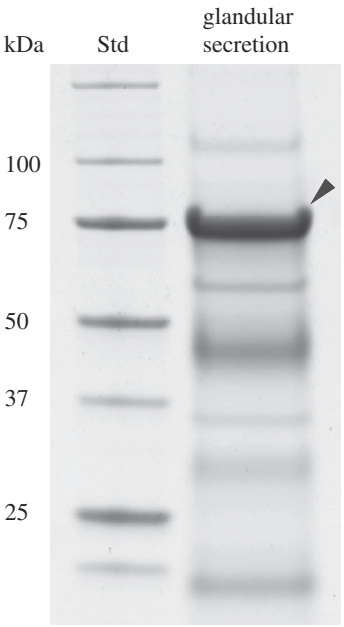

Figure 2. Coomassie Brilliant Blue-stained one-dimensional SDS protein gel of the larval glandular secretion of *P. vitellinae*. The most prominent band marked with an arrow corresponds to SAO. The size of the protein standard on the left is indicated in 10^3 Da.

to amplify a partial core fragment followed by *P. vitellinae* sequence-specific 3′- and 5′-RACE. This led to a single full-length cDNA of 2040 bp with an ORF of 1881 bp that encodes a protein of 626 amino acids. Sequence comparison showed an identity of about 72 per cent to leaf beetle SAOs of *Chrysomela lapponica*, *Chrysomela populi* and *Chrysomela tremulae* at the amino acid level, including the N-terminal signal peptide for the secretory pathway (electronic supplementary material, figure S1 and table S1). In total, 17 peptides identified by nanoLC-MS/MS of the 75 kDa protein band extract matched the full-length sequence, allowing approximately 40 per cent of total sequence coverage (electronic supplementary material, figure S2 and table S2), thus verifying that the amplified transcript corresponds to the protein present in the glandular secretion. The calculated molecular mass of *P. vitellinae* SAO-like protein with/without the signal peptide (70/68 kDa) is lower than its actual mass (approx. 75 kDa). Comparable post-translational modifications as shown for *Chrysomela* SAOs are indicated by seven predicted N-glycosylation sites in *P. vitellinae* SAO-like protein. This predicted glycosylation was confirmed by a band shift assay treating the heterologously expressed protein with PNGase F (figure 3). Interestingly, not only the total number but also four positions of predicted N-glycosylation sites are conserved in *Chrysomela* ssp. and *P. vitellinae* SAOs.

(b) *Salicyl alcohol oxidase expression and activity*

Phratora vitellinae SAO-like full-length cDNA starts with ATG AAA ATG AAG. We used a six-nucleotide shorter transcript for characterization because the methionine-encoding third triplet turned out as the most likely start codon in comparison with *Chrysomela* SAOs after multiple alignment analysis (electronic supplementary material, figure S1). The SAO-like protein was expressed in Sf9 insect cells and a protein of the predicted size was detected by Western blot analysis in the Sf9 cultural medium (figure 3). This demonstrated the functional N-terminal signal peptide for the secretory pathway and similar post-translational modifications indicated by a comparable size of Sf9 cell and beetle-expressed SAO.

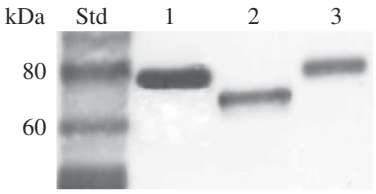

Figure 3. Western blot of heterologously expressed SAOs in Sf9 insect cells. Lanes 1 and 2 correspond to the *P. vitellinae* SAO. Size differences are due to a PNGase F treatment of probe 2 (lane 2), suggesting post-translational N-glycosylations. *Chrysomela populi* SAO is blotted (lane 3), demonstrating a similar size of the SAO in both genera after expression.

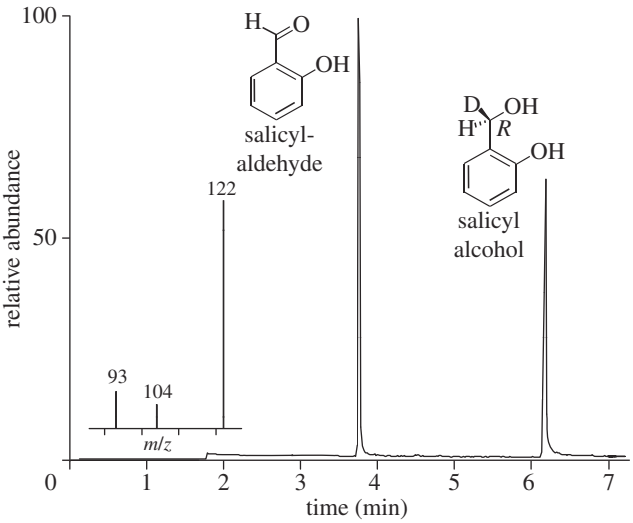

Figure 4. GC chromatogram of heterologously expressed *P. vitellinae* SAO activity assay after extraction. The formation of salicylaldehyde via a stereoselective deuterium removal of salicyl alcohol is indicated by the mass spectrum of the aldehyde.

Enzyme assays of the heterologously expressed SAO-like protein revealed SAO activity (figure 4). The oxidation proceeded *Re* specifically and removed exclusively the deuterium atom and yielded [1-^{1}H]-salicylaldehyde from the 1*R*-[1-^{2}H$_1$]-salicyl alcohol precursor. This stereochemical course is in agreement with previous studies using the glandular secretion of *P. vitellinae* [20] and confirmed that the oxidation is not an autoxidative artefact. By contrast, the previously described oxidation of 8-hydroxygeraniol, for which glandular secretion of *P. vitellinae* was used [20], was not detectable using the heterologously expressed enzyme. In addition, from MS/MS analyses, no peptides matching alternative GMC oxidoreductases, other than the SAO present in the glandular secretion of *P. vitellinae*, were identified.

(c) *Expression pattern of the* P. vitellinae *salicyl alcohol oxidase*

SAO gene expression levels were compared in different larval tissues. The SAO is specifically expressed in the

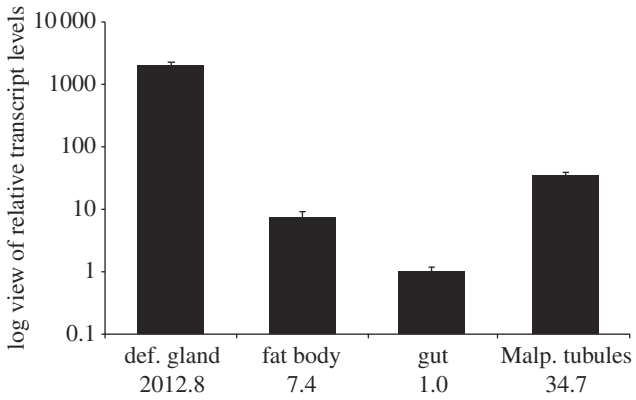

Figure 5. Expression pattern of *P. vitellinae* SAO. Relative transcript levels in different larval tissues display glandular tissue specificity of the SAO with an approximately 2000-fold higher transcript abundance compared with fat body, gut and Malpighian tubules. Transcript levels are indicated logarithmic and average values are shown below each column. For normalization of transcript quantities, *EF1α* and *eIF4A* were used. Error bars indicate the SEM.

glandular tissue (figure 5) with an approximately 2000-fold higher expression level compared with the fat body, gut and Malpighian tubules, where SAO gene expression levels are close to the background level. The large amount of protein present in the glandular secretion (§3*a* and figure 2) coincides with the high transcript level of SAO in the glandular tissue, verifying that the enzyme is expressed in a tissue-specific manner and most probably secreted into the glandular reservoir.

(d) *Salicyl alcohol oxidase-like sequences in iridoid-producing species*

Phratora vitellinae SAO is closely related to *Chrysomela* SAOs in sequence but isolated from these according to species phylogeny. To address the question of the origin of SAO, we searched for SAO-like sequences in iridoid producers first in a species that is, based on phylogenetic data, located between *Phratora* and *Chrysomela* (*Phaedon cochleariae*) and second within the genus *Phratora* (*P. laticollis*). The analysis of a BLAST search of chrysomeline SAOs against a *P. cochleariae* in-house EST library led to a single partial ORF encoding an SAO-like protein. Full-length sequencing after RACE PCR showed about 58 per cent sequence identity to known SAOs and related proteins. However, there is no indication for the presence of this protein in the glandular reservoir (see §4 for details). From genomic DNA of *P. laticollis*, we were able to amplify an 8 kb SAO-like gene fragment, using *P. vitellinae* SAO-derived primers. The predicted encoded protein possesses about 73 per cent sequence identity to *Chrysomela* and 90 per cent to *P. vitellinae* SAOs (electronic supplementary material, figure S1 and table S1).

(e) *Comparative salicyl alcohol oxidase gene architecture*

Based on the SAO cDNA sequence of *P. vitellinae*, we were able to amplify about 8 kb from genomic DNA, which covers most of the SAO-encoding gene. The alignment of cDNA and the corresponding genomic region showed that SAO possesses at least eight exons. In comparison with known *Chrysomela* SAO genes [18], both a highly similar gene length and number of exons could be identified. Most remarkably, an identical length of almost all exons in both genera was obvious. The *P. laticollis* SAO-like gene fragment (§3*d*) also possesses identical exon lengths of its predicted coding sequence. In summary, comparative SAO gene architecture indicates a common SAO origin.

Further analysis of genomic DNA sequences of GMC oxidoreductases in *Tribolium castaneum* and *Drosophila melanogaster* demonstrated one to three encoding exons of variable length. Furthermore, whereas all the fruitfly and red flour beetle GMC genes belonging to the GMC*i* subfamily possess two exons, the closest relative to the chrysomeline SAOs (*Tcas*GMC*i*5) possesses four exons (figure 6 and electronic supplementary material, figure S3). Those findings not only show an accumulation of introns common to chrysomeline GMC*i* genes, but also indicate that this increase in gene architecture complexity may have arisen in the most recent common ancestral gene.

(f) *Salicyl alcohol oxidase evolution in chrysomelines*

Phylogenetic analyses, including members of different GMC oxidoreductase subfamilies, a subset of SAO and related genes from previous work [18], showed a common origin of both *P. vitellinae* and *Chrysomela* ssp. SAOs within the GMC*i* subfamily (figure 6). This is indicated by their affiliation to *Tcas*GMC*i*5 supported by a high posterior probability value (1). Moreover, within the leaf beetle GMC*i* members, a clear separation of *P. vitellinae* SAO from *Chrysomela* SAO-related genes (paralogues 1 and 3) and the clustering of the *P. vitellinae* SAO with *Chrysomela* SAOs (posterior probability value of 1 for each node) indicate a single ancestral gene in both genera. *Phratora vitellinae* SAO is not located inside the *Chrysomela* SAO clade but represents a sister-group and clusters together with the *P. laticollis* SAO-like protein, which is reflective of the overall species phylogeny.

In addition, we included the SAO-like protein of *P. cochleariae* (§3*d*) in our analysis, which also showed unambiguously affiliation to the chrysomeline SAO clade. This generally indicates the presence of SAO-like proteins in iridoid producers and probably reflects GMC*i* subfamily expansion by gene duplications early in chrysomeline evolution.

4. DISCUSSION

Chrysomela species are known to produce salicylaldehyde for their chemical defence, a compound shown to be highly deterrent against generalist predators and microbial infestations. As the precursor salicin acts as a general feeding repellent of their salicaceous host plants (reviewed in [24]), the sequestration of this secondary compound is a remarkable example of an engaging detoxification and economical defence in leaf beetle larvae. Interestingly, beside the genus *Chrysomela*, this remarkable derived state of chemical defence strategy is only present in *P. vitellinae* [15]. This is noteworthy, because the two leaf beetle genera are not close relatives, and therefore a convergent evolution of host-derived chemical defence based

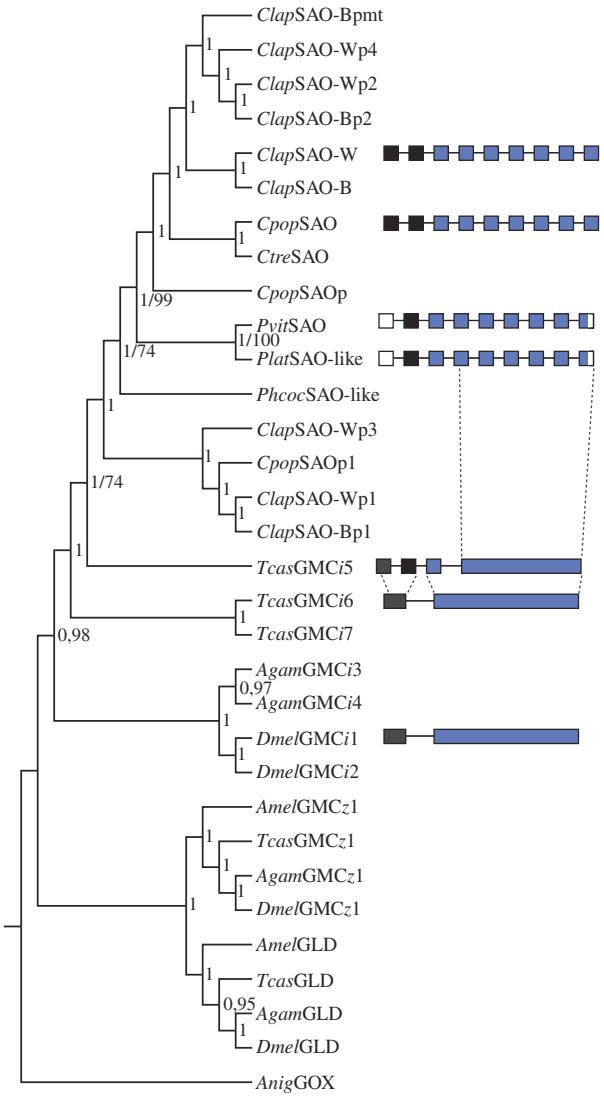

Figure 6. Phylogeny of chrysomeline SAOs and related GMC oxidoreductases, including other insects. The topology was generated using a Bayesian analysis. Posterior probability values are shown next to each node. Bootstrap values are exemplary stated as second numbers (for details of parameters and sequence information, see §2 and electronic supplementary material, table S3). Chrysomeline SAO and other insect GMC*i* gene architectures are depicted next to the phylogenetic tree. Exons are shown with boxes, the same colour coding indicates common origin among different species, empty boxes mark gaps in our dataset and the dashed lines mark intron accumulations. *Clap* (*C. lapponica*), *Cpop* (*C. populi*), *Ctre* (*C. tremulae*), *Pvit* (*P. vitellinae*), *Plat* (*P. laticollis*), *Phcoc* (*Phaedon cochleariae*), *Tcas* (*T. castaneum*), *Anig* (*Aspergillus niger*), SAO-W/B (salicyl alcohol oxidase of willow/birch-feeder), GMC (glucose–methanol–choline oxidoreductase), GLD (glucose dehydrogenase), GOX (glucose oxidase), p (SAO paralogues), pmt (SAO paralogues Malpighian tubule specific).

on salicin sequestration has been proposed [12]. However, despite the evolutionary distance between these species, the mode of sequestration as well as salicylaldehyde biosynthesis and SAO activity in particular are the same in both genera [6,10,17,20].

To shed light onto salicin-dependent chemical defence evolution, we first identified and functionally expressed the SAO of *P. vitellinae*. The SAO is the most abundant protein in larval glandular secretions, consists of 626 amino acids including the N-terminal signal peptide addressing the secretory pathway and shows a complex N-glycosylation pattern of about 7 kDa. The highly abundant transcript specific for the corresponding glandular tissue is consistent with both the proportion of oxidative capacity inside the glandular secretion and the prominent band in the one-dimensional protein gel. Sequence comparisons verified that the SAO belongs to the GMC oxidoreductase family and possess a 72 per cent amino acid identity compared with *Chrysomela* SAOs. The *Re* specificity of the heterologously expressed protein was verified for the first time, which fits to previous findings with *in vitro* assays of the glandular secretion [20]. We showed that *P. vitellinae* and *Chrysomela* SAOs not only share a highly tissue-specific expression and high amounts of protein in the glandular system, the same protein size, similar post-translational modifications and the affiliation to GMC oxidoreductases, but also a well conserved gene architecture (number and lengths of exons). These findings provide additional support for the results of our phylogenetic analyses demonstrating a single origin of *P. vitellinae* and *Chrysomela* ssp. SAO genes in the GMC*i* subfamily.

Taking leaf beetle species phylogenies into account, which all support the notion that *Chrysomela* and *Phratora* are not sister genera [12,25,26], the following evolutionary scenarios leading to SAO activity are conceivable. A single gene duplication event led to (gave birth to) the evolution of the SAO gene and activity in *Phratora* and *Chrysomela*. Whether this proceeded via one sub-/neofunctionalization in their most recent common ancestor or convergent SAO acquisitions (and additional genera-specific gene duplications of a 'precursor' gene) cannot be resolved. Therefore, we cannot exclude the possibility that *Chrysomela* and *Phratora* SAO are not true orthologues in a strict sense. However, for both scenarios, the persistence of the SAO or SAO 'precursor' gene (e.g. through retaining the original function of the SAO 'precursor' gene) in the iridoid-producing genera between *Chrysomela* and *Phratora* (e.g. *Phaedon*, *Gastrophysa*) is likely a prerequisite.

Because of a common glucosidase–oxidase pathway leading to salicylaldehyde and iridoids, shifts from iridoid to salicylaldehyde biosynthesis in leaf beetle chemical defence evolution has been proposed to take place via changing substrate specificity of the oxidase [10,12,20]. This change of substrate specificity is supported by *in vitro* oxidation of 8-hydroxygeraniol by the secretion of salicylaldehyde producing *P. vitellinae* [20], which is seen as an argument for the evolution of SAOs from oxidases of iridoid-producing ancestors.

In contrast to these findings, we were not able to verify 8-hydroxygeraniol oxidation with enzyme assays of the heterologously expressed *P. vitellinae* SAO. Furthermore, we found neither an oxidase with affiliation to the GMC*i* subfamily (e.g. *P. cochleariae* SAO-like protein: §3*d*) nor a GMC oxidoreductase of the conserved insect gene cluster at all, present in the secretion of iridoid-producing *P. cochleariae*, *Gastrophysa viridula* or *Gastrophysa cyanea* larvae (R. Kirsch 2010, unpublished data). Several chemical and biochemical properties identified (oxygen dependence and *Re* specificity) are consistent between

SAO and 8-hydroxygeraniol oxidase [27]. However, our findings strongly argue for distinct, non-SAO related oxidases converting 8-hydroxygeraniol to 8-oxocitral in iridoid-producing species and, moreover, an independent evolution of the oxidative step in salicylaldehyde and iridoid biosynthesis. In this context, the elucidation of the SAO-like EST in *P. cochleariae* and the SAO-like gene in *P. laticollis*, both iridoid-producing species, is important. The presence of SAO-like sequences in these species indicate that gene duplications in the GMC*i* subfamily started early in chrysomeline speciation followed by species specific gene duplications (shown for *C. lapponica* in [18]). Furthermore, the persistence of SAO-like genes in the iridoid producers is probably due to the acquisition of functions different from SAO activities (i.e. functions not related to chemical defence).

We clearly showed a common origin of SAOs in *Chrysomela* and *P. vitellinae* and their most likely independent evolution from iridoid biosynthesis. However, characterizations of SAO-like proteins in iridoid producers and the GMC*i*5 in *T. castaneum* are needed to resolve molecular functional origins and gene-family dynamics of SAO and related genes.

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Author contributions: R.K., H.V. and W.B designed research; R.K., H.V., A.M., J.M.P. and W.B wrote the paper; R.K., H.V., A.M. and A.V. performed research and analysed data.

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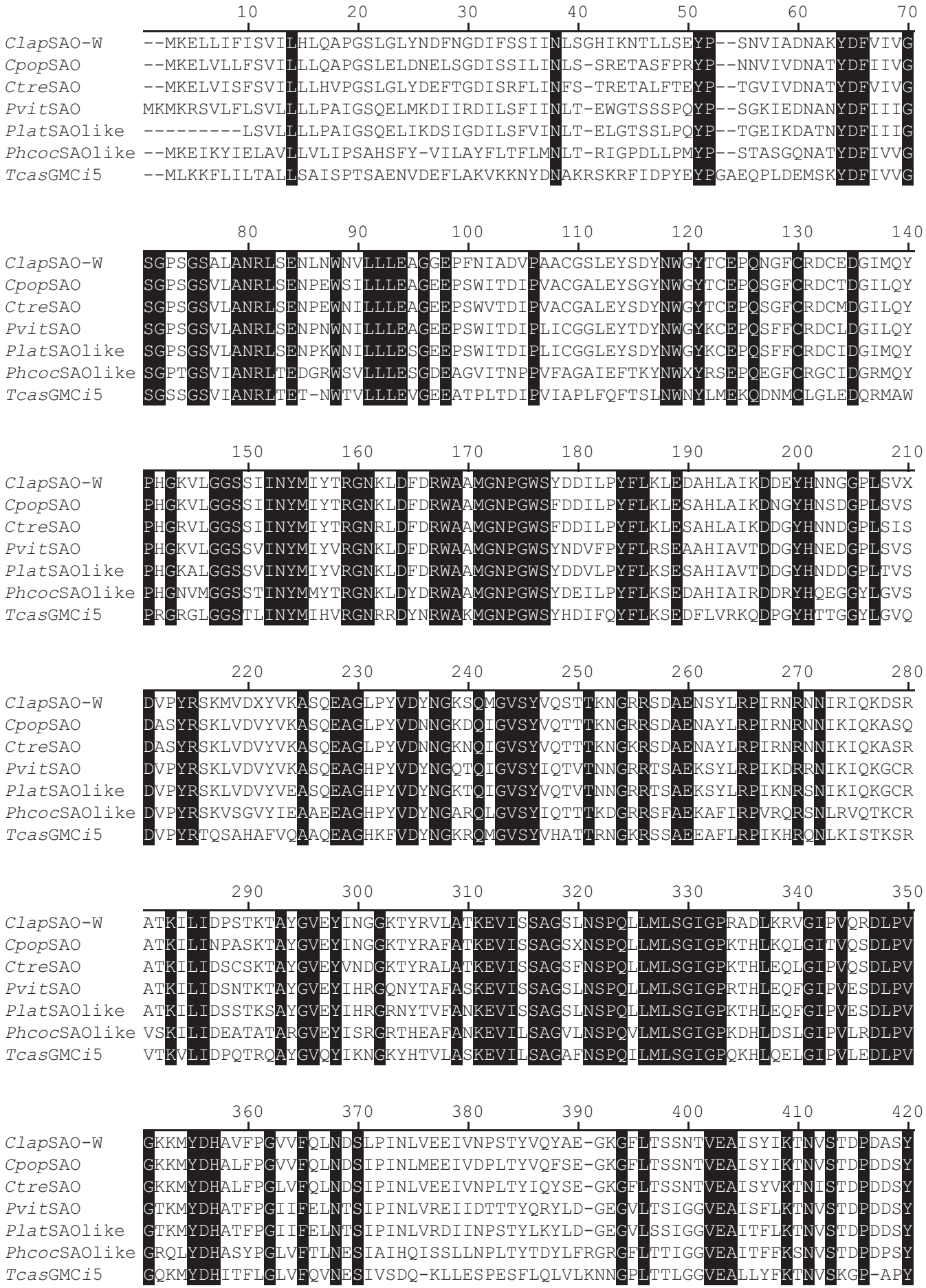
Electronic supplementary material



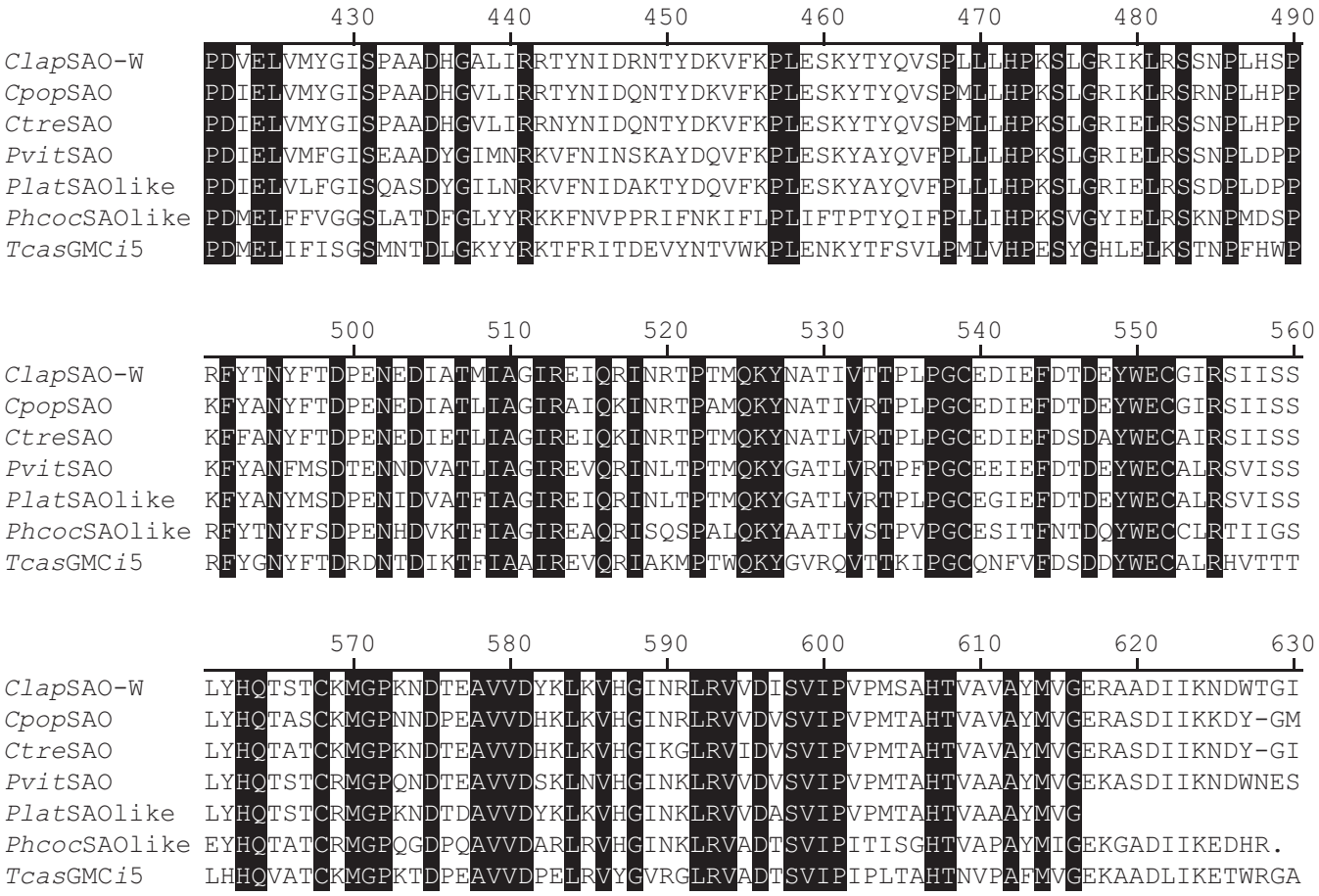


Fig. S1

Alignment of the amino acid sequences of chrysomeline SAOs and related proteins and of their closest relative in *T. castaneum* (*Tcas*GMC*i*5). Multiple alignments of protein sequences were carried out using CLUSTALW [22]. Identical amino acids in all sequences are shown in black boxes. Abbreviations: *Clap* (*Chrysomela lapponica*), *Cpop* (*C. populi*), *Ctre* (*C. tremulae*), *Pvit* (*Phratora vitellinae*), *Plat* (*P. laticollis*), *Phcoc* (*Phaedon cochleariae*), SAO (salicyl alcohol oxidase), GMC (glucose-methaol-choline oxidoreductase).

MKRSVLFLSV LLLLPAIGSQ ELMKDIIRDI LSFIINLTEW GTSSSPQYPS 50
GK**IEDNANYD FIIIGSGPSG SVLANR**LSEN PNWNILLLEA GEEPSWITDI 100
PLICGGLEYT DYNWGYKCEP QSFFCR**DCLD GILQYPHGKV LGGSSVINYM** 150
IYVRGNKLDF DRWAAMGNPG WSYNDVFPYF LR**SEAAHIAV TDDGYHNEDG** 200
PLSVSDVPYR SKLVDVYVKA SQEAGHPYVD YNGQTQIGVS YIQTVTNNGR 250
RTSAEK**SYLR PIK**DRRNIKI QKGCRATKIL IDSNTK**TAYG VEYIHR**GQNY 300
TAFASK**EVIS SAGSLNSPQL LMLSGIGPRT HLEQFGIPVE SDLPVGTK**MY 350
DHATFPGIIF ELNTSIPINL VR**EIIDTTTY QRYLDGEGVL TSIGGVEAIS** 400
FLKTNVSTDP DDSYPDIELV MFGISEAADY GIMNR**KVFNI NSKAYDQVFK** 450
PLESKYAYQV FPLLLHPKSL GRIELRSSNP LDPPKFYANF MSDTENNDVA 500
TLIAGIREVQ R**INLTPTMQK** YGATLVRTPF PGCEEIEFDT DEYWECALR**S** 550
VISSLYHQTS TCRMGPQNDT EAVVDSK**LNV HGINK**LR**VVD VSVIPVPMTA** 600
HTVAAAYMVG EKASDIIKND WNES 624

Fig. S2

P. vitellinae SAO protein sequence coverage map after nano LC-MS/MS identification of the 1D-SDS-PAGE separated extract of the band marked with an arrow (Fig. 2) reaching 40.86% of total sequence coverage (42.19% without signal peptide, which in underlined). All *P. vitellinae* SAO tryptic peptides identified from their fragment spectra are marked **bold**.

Percent Identity

Divergence	1	2	3	4	5	6	7		
1		83.0	82.3	71.5	73.1	58.7	51.0	**1**	*Clap*SAO-W
2	19.4		89.4	73.7	74.1	57.6	49.5	**2**	*Cpop*SAO
3	20.2	11.4		73.4	74.5	57.2	51.0	**3**	*Ctre*SAO
4	35.9	32.4	32.9		89.7	59.3	50.2	**4**	*Pvit*SAO
5	33.3	31.8	31.3	11.1		60.6	50.8	**5**	*Plat*SAOlike
6	59.3	61.5	62.6	58.0	55.3		52.3	**6**	*Phcoc*SAOlike
7	77.3	81.2	77.3	79.2	77.7	74.0		**7**	*Tcas*GMC*i*5
	1	**2**	**3**	**4**	**5**	**6**	**7**		

Table S1
GMC oxidoreductase protein sequence distances based on a CLUSTALW alignment implemented in DNAStar Lasergene 8.02.

Sequence	**m/z**	**Peptide MW**	**Delta (ppm)**	**Ladder Score (%)**
IEDNANYDFIIIGSGPSGSVLANR	1261.6215	2521.2446	-6.88	53.1915
IEDNANYDFIIIGSGPSGSVLANR	841.4163	2521.2446	-7.55	53.1915
DCLDGILQYPHGK	758.3685	1514.7188	1.77	78.6667
DCLDGILQYPHGK	505.9085	1514.7188	-10.96	46.6667
VLGGSSVINYMIYVR	843.9454	1685.881	-3.48	64.3678
SEAAHIAVTDDGYHNEDGPLSVSDVPYR	754.3444	3013.3687	-7.37	40
SYLRPIK	438.7672	875.5228	-4.60	25.641
TAYGVEYIHR	604.7996	1207.5984	-12.23	75.4386
EVISSAGSLNSPQLLMLSGIGPR	1163.6248	2325.2363	-1.05	51.8519
EVISSAGSLNSPQLLMLSGIGPR	776.0800	2325.2363	-8.51	49.6296
THLEQFGIPVESDLPVGTK	1034.0247	2066.0684	-16.78	56.7568
EIIDTTTYQR	620.3133	1238.6141	-2.56	56.1404
YLDGEGVLTSIGGVEAISFLK	723.3818	2167.141	-8.90	10.5691
KVFNINSK	475.7697	949.5233	0.51	60
AYDQVFKPLESK	712.8692	1423.7346	-8.32	81.1594
AYDQVFKPLESK	475.5779	1423.7346	-17.06	47.8261
YAYQVFPLLLHPK	794.9487	1587.8813	0.308	17.3333
YAYQVFPLLLHPK	530.2956	1587.8813	-11.38	41.3333
INLTPTMQK	531.2855	1060.5586	-2.30	72.549
SVISSLYHQTSTCR	819.8957	1637.7831	-4.47	74.0741
LNVHGINK	447.7617	893.5083	-0.61	53.3333
VVDVSVIPVPMTAHTVAAAYMVGEK	862.1154	2583.344	-8.22	22.449

Table S2
Bioinformatic identification overview of the MS/MS analysis of the *P. vitellinae* SAO. 17 peptides of the band marked with an arrow (Fig. 2) matching to the provided sequence using >20 ppm precursor-peak and 0.03 Da fragment ion accuracy, carbamydomethylation of cysteins and possible deamidatioin of asparagines and glutamines.

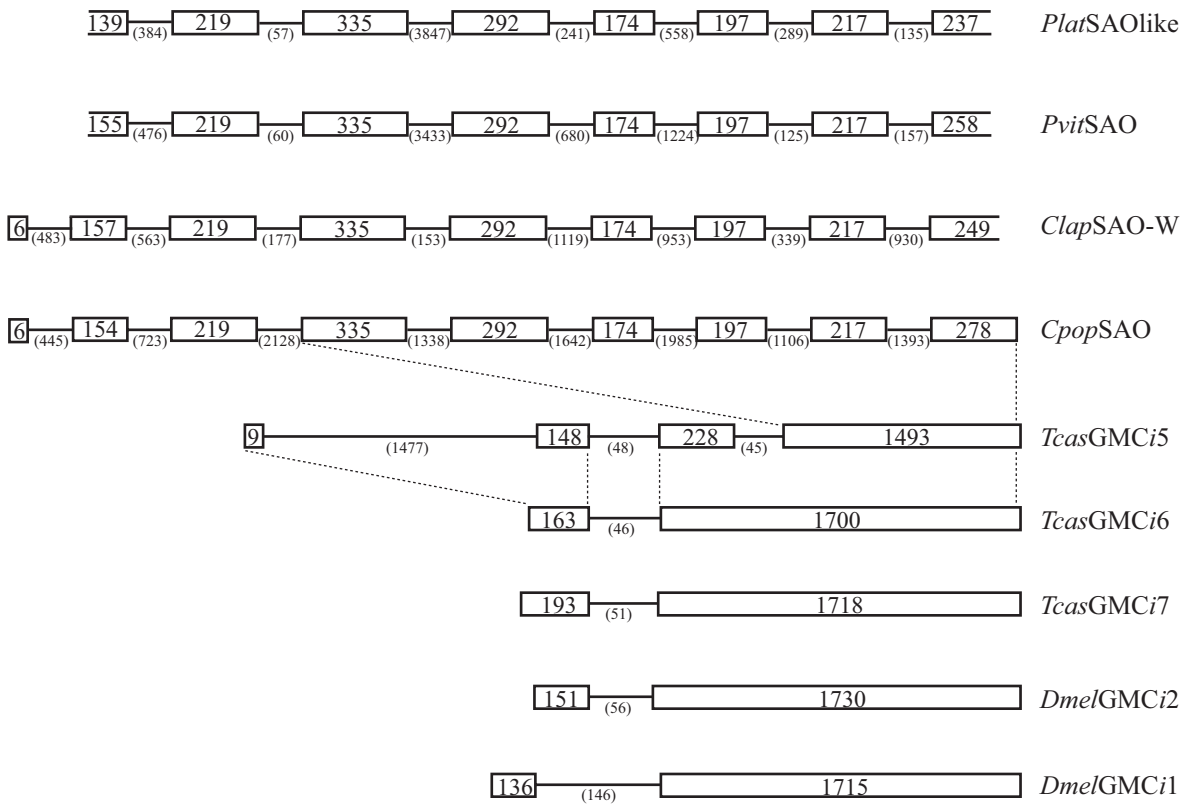


Fig. S3

GMC*i* subfamily genes and their exon (boxes) and intron lengths in base pairs. Open boxes indicate gaps in our data set. For *P. vitellinae* SAO, *T. castaneum* and *D. melanogaster* GMC*i* members gene architectures were elucidated by mRNA to genomic alignments using Spidey implemented in the NCBI Toolkit. The coding region of the *P. laticollis* SAO like gene is basedon FGENESH, a HMM based gene structure prediction implemented in Softberry, and manual searching. The data for *Chrysomela* SAO genes are taken from a previous study [18]. Dashed lines indicate intron acculations. Abbreviations: *Clap* (*Chrysomela lapponica*), *Cpop* (*Chrysomelapopuli*), *Dmel* (*Drosophila melanogaster*), *Plat* (*Phratora laticollis*), *Pvit* (*Phratora vitellinae*), GMC*i*(glucose-methanol-choline oxidoreductase i subfamily), SAO (salicyl alcohol oxidase), W (willow feeder).

Gene name used in this study	GenBank no. used for our analysis ("ps", predicted manually in previous study [18], "§¹", taken from [24])
Drosophila melanogaster	
GMC*z* 1	AAF48398.1
GMC*i* 1	AAF48395.1
GMC*i* 2	AAF48394.2
GLD	AAF54038.1
Anopheles gambiae	
GMC*i* 4	§¹
GMC*i* 3	§¹
GMC*z* 1	§¹
GLD	§¹
Apis mellifera	
GMC*z* 1	XM_394220
GLD	ps
Tribolium castaneum	
GMC*z* 1	EFA05531.1
GMC*i* 5	ps
GMC*i* 6	EFA05537.1
GMC*i* 7	EFA05538.1
GLD	EFA05227.1
Aspergillus niger	
Anig GOX	ACB30369.1
Chrysomela populi	
SAO	HQ245154
SAOp1	HQ245155
SAOp	HQ245148
Chrysomela tremulae	
SAO	CAQ19343
***Chrysomela lapponica* (willow feeder)**	
SAO-W	HQ245149
SAO-Wp1	HQ245150
SAO-Wp2	HQ245144
SAO-Wp3	HQ245145
SAO-Wp4	HQ245146
***Chrysomela lapponica* (birch feeder)**	
SAO-B	HQ245151
SAO-Bp1	HQ245152
SAO-Bp2	HQ245147
SAO-Bpmt	HQ245153
Phaedon cochleariae	
SAOlike	HQ857156
Phratora laticollis	
SAOlike	HQ857157
Phratora vitellinae	
SAO	HQ857158

Table S3
Source of gene information of all sequences used for phylogenetic analyses.

Manuscript 3

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Glucose and Glucose Esters in the Larval Secretion of *Chrysomela Lapponica*; Selectivity of the Glucoside Import System from Host Plant Leaves

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Abstract Larvae of *Chrysomela lapponica* (Coleoptera: Chrysomelidae) sequester characteristic *O*-glucosides from the leaves of their food plants, namely *Betula* and/or *Salix* The present study focuses on birch-feeding larvae of *C. lapponica* from the Altai region in East Kazakhstan. As in other sequestering leaf beetle larvae, the compounds are transported intact *via* different membrane barriers into the defensive system, followed by glucoside cleavage and subsequent transformations of the plant-derived aglycones. Unlike previous studies with model compounds, we studied the sequestration of phytogenic precursors by analyzing the complex pattern of glucosides present in food plant *Betula rotundifolia* (39 compounds) and compared this composition with the aglycones present as butyrate esters in the defensive secretion. In addition to the analytic approach, the insect's ability, to transport individual glucosides was tested by using hydrolysis-resistant thioglucoside analogs, applied onto the leaf surface. The test compounds reach the defensive system intact and without intermediate transformation. No significant difference of the transport capacity and selectivity was observed between larvae of birch-feeding population from Kazakhstan, and previous results for larvae of birch-feeding population from the Czech Republic or willow-feeding populations. Overall, the transport of the phytogenic glucosides is highly selective and highly efficient, since only minor compounds of the spectrum of phytogenic glucoside precursors contribute to the limited number of aglycones utilized in the defensive secretion. Interestingly, salicortin **44** and tremulacin **60** were found in the leaves, but no aldehyde or esters of salicylalcohol. Surprisingly, we observed large amounts of free glucose, together with small amounts of 6-*O*-butyrate esters of glucose (**27a/b** and **28a/b**).

Key Words *O*-glucosides · Butyrate esters · Salicortin · Tremulacin · Salicylaldehyde · *Betula* · *Salix* · Coleoptera · Chysomelidae · Antipredatory · Beetle defense

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Introduction

Larvae of the leaf beetles (Chrysomelina) possess nine pairs of dorsal exocrine glands from which secretions emerge as small droplets when a predator appears. As soon as the disturbance is over, the droplets are resorbed into a large reservoir into which glandular cells open. The antipredatory effect of the secretion is based either on *de novo* production of repellents or on compounds derived from sequestered, plant-derived glucosides. The major components, which are secreted by leaf beetle larvae belonging to the taxa *Phaedon*, *Gastrophysa*, *Hydrothassa*, *Prasocuris Plagiodera*, *Linaeidea*, and *Phratora* (in part) are iridoid

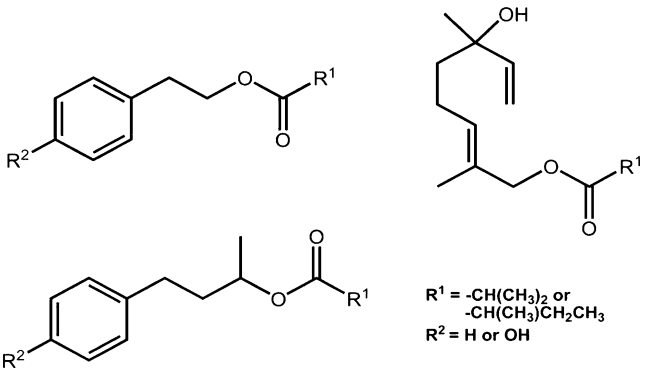
monoterpenes (Meinwald et al. 1977; Blum et al. 1978; Pasteels et al. 1982; Soe et al. 2004). According to (Termonia et al. 2001) the *de novo* production of iridoids is considered the ancestral strategy. Recent studies have demonstrated, however, that the whole group also is able to sequester early precursors from plants (Feld et al. 2001; Kuhn et al. 2004; Kunert et al. 2008). A more advanced and resource saving strategy to produce chemical defenses, is the sequestration of plant-derived precursors. The sequestration of phenol glucosides, especially salicin, has been developed and optimized by species feeding on Salicaceae, i.e., *Phratora vitellinae* and *Chrysomela* (Pavan 1953; Pasteels et al. 1983). In these species, the plant-derived glucosides serve as precursors for the repellent salicylaldehyde; a concomitant *de novo* biosynthesis does not seem to play a significant role.

The most derived Chrysomelina species, the interrupta group *sensu* (Termonia and Pasteels 1999), which includes *Chrysomela lapponica* do not sequester single glucosides, but import a broad spectrum of glucosidically bound plant-derived alcohols (Kuhn et al. 2007). After import into the reservoir, which functions as a bioreactor containing all necessary enzymes for the transformation of the sequestered precursors, the glucose moiety is removed, and the aglycones are esterified with isobutyric acid, 2-methylbutyric acid, and benzoic acid generated from the insects' internal pools of amino acids. This type of "combinatorial biosynthesis" can generate a cocktail of more than 70 secretion ingredients, as has been shown for a larval secretion of *C. lapponica* from Bavaria, Queyras, Czech Republic, and related North American species (Hilker and Schulz 1994; Schulz et al. 1997; Termonia and Pasteels 1999; Kuhn et al. 2007). Bioassays have demonstrated the protective function of these blends against predators (Hilker and Schulz 1994). For several defensive secretions of leaf beetle larvae, antimicrobial effects also have been demonstrated and discussed (Gross et al. 2002).

The sequestration of glucosides as pro-toxins is advantageous. The compounds often occur in high abundancy in the food plants. The polar glycosides are not able to pass membranes unless functional transport systems mediate their passage, which assures safe guidance of the compounds to their destination through the larval body. The leaf beetle larvae possess an interconnected network of transport systems for uptake and excretion of surplus glucosides (Discher et al. 2009).

According to model experiments with selected (thio) glucoside mixtures, the phytogenic precursors are imported via the gut membrane with low selectivity, followed by a specific import of only a few compounds from the hemolymph into the reservoir. Model studies were based on test compounds, comprising a glucoside precursor of iridoid biosynthesis, salicin for salicylaldehyde production, and phenylethanolglucoside that is converted into the corresponding (iso)butyrate (Scheme 1). Uptake into the glandular reservoir was highly specific, and only the phytogenic precursors for the genuine defenses accumulated in the reservoir. For example, only salicin was imported into the reservoir of *Chrysomela populi* and converted to salicylaldehyde. In contrast, the iridoid-producing larvae of *Phaedon cochleariae* accumulated only the glucoside of 8-hydroxy-geraniol, which is converted into the iridoid chrysomelidial (Discher et al. 2009). This is different in the case of the defensive secretion of *C. lapponica* feeding on birch. Their defensive secretion is a rather complex mixture composed of butyrate esters of very different and most likely plant-derived aglycones released from sequestered glucosides (Hilker and Schulz 1994; Schulz et al. 1997; Kuhn et al. 2007). This implies that larvae of *C. lapponica* possess either several different transport systems, each of them specifically tuned to the imported glucoside, or that the larvae possess a transport system of low selectivity that allows the import of a broad range of glucosides, as demonstrated by Kuhn et al. 2007 with *C. lapponica* from Queyras feeding on willow by using thioglucosides as probes.

In this study, we focused on birch feeding *C. lapponica* from Kazakhstan, which also produce butyrate esters, but lack the production of salicylaldehyde, as will be shown. To investigate the capacity and the "filter characteristics" of the transport systems that operate in larvae of birch feeding *C. lapponica*, we compared the spectrum of the glucosides present in the leaves of the host plant *Betula rotundifolia* with the spectrum of butyrate esters in the defensive secretion of the larvae. Here, we demonstrated that the defensive secretions and the glucoside spectrum of *B. rotundifolia* share only a small number of aglycones that are sequestered and utilized by the insect. Moreover, the defensive secretion of *C. lapponica* contains large amounts of glucose and certain glucose esters (butyrates), which may serve as cryoprotection.



Scheme 1 Butyrate esters from the defensive secretion of larvae of birch-feeding larvae of *Chrysomela lapponica* endemic to the Altai region, East Kazakhstan

Methods and Materials

Leaf Beetle Larvae and Host Plant Leaves Larvae of *C. lapponica* feeding on leaves of *Betula rotundifolia* were collected in the Altai mountains (East Kazakhstan) near the Burkhat Pass (2130 m altitude, 49°07,438‘N, 86°01,365‘E) in early August 2008. Larvae were reared for 1 week with leaves from *Betula rotundifolia* from the Burkhat Pass. Freshly harvested *Betula* twigs were kept alive for several days in tap water. Additional plant material was sun dried and stored for phytochemical analysis. Field conditions did not allow for extraction of fresh plant material.

Collection of Defensive Secretion Larvae were gently squeezed by forceps and forced to protrude their mesothoracal glands. The emerging droplets were collected with glass capillaries (Hirschmann Laborgeräte, diam: 0.28 mm, length: 100 mm). The capillaries were sealed by melting with a micro gas torch and stored at −20°C.

Analytical Procedures Analysis of defensive secretions. An aliquot of the secretion (1-3 μg) was dissolved in 20 μl dichloromethane. After vortexing and centrifuging, 1 μl was subjected to GC-EIMS analysis (ThermoQuest Finnigan Trace GC-MS 2000 (Quadrupole) equipped with Alltech EC 5-column, 15 m×0.25 mm, film thickness 0.25 μm). Substances were separated using helium as carrier (1 ml/min); conditions: 45°C (2 min), then at 10°C min^{-1} to 280°C (5 min).

Highly concentrated samples were appropriately diluted. For quantification, external standards at different concentrations were used. Polar components were analyzed after derivatization with MSTFA. The defensive secretion (5 μl solution in dichloromethane) was mixed with *N*-methyltrimethylsilyltrifluoroacetamide (MSTFA) (10 μl) and pyridine (5 μl). After heating to 70°C for 1 h, an aliquot (1 μl) of the solution was analyzed directly by GC-EIMS. Free fatty acids were derivatized with pentafluorobenzylbromide (PFBB) prior to analysis (Attygalle et al. 1991). For esterification, the secretion (0.5 μl) was dissolved in triethylamine (10 μl) containing PFBB (5 μl). The solution was kept at 25°C for 3 h and hydrolyzed with water (30 μl). After extraction with *n*-hexane (20 μl), the solution was analyzed by GC-EIMS (conditions see above). All compounds were identified by comparison with authentic references and sample chromatograms, respectively. Secretion analysis was carried out against external standards (N=3). No replicates were measured owing to the restricted availability of secretion.

Transport Analysis with Thioglucosides Leaves from *Betula pendula* (used instead of *B. rotundifolia* because of the larger surface area for the transport experiments) were painted on the upper side with 0.5 ml of MeOH/H_2O (1:1, v/v) containing the test compound at a concentration of 25 mM. After evaporation of the solvent with an air stream, third instars (four larvae per ca. 3.5 cm^{-2} leaf segment) were allowed to feed on the treated leaves for 48 h, followed by sampling of defensive secretion. The defensive secretion was withdrawn with a small capillary from the everted glands of the larvae. The secretion was stored and transported in sealed capillaries. The thioglucosides transported into the glandular system were analyzed and quantified as described (Kuhn et al. 2007).

Statistics To obtain valid data, feeding experiments with thioglucosides were repeated several times. *Chrysomela lapponica* (Kazakhstan): compounds **1** to **6** (N=6), *C. lapponica* (Czech Republic): compound **1** (N=5), compounds **2**, **4**, and **6** (N=4). Variances between groups were not homogeneous according to Levene’s test. Hence, Mann-Whitney test was used to evaluate significant differences. Statistics were calculated with SPSS Statistics 17.0.

Analysis of Leaf Glycosides Air dried leaves (3 g) of *B. rotundifolia* were pestled in liquid nitrogen and extracted with 20 ml 80% aqueous methanol using an Ultra-Turrax homogenizer for 3 min. Extraction was continued by shaking for 1 h at ambient temperature. Solids were removed by filtration and re-extracted with methanol (20 ml) for another 1 h at ambient temperature. The combined extracts were evaporated to dryness, re-dissolved in methanol (10 mg/ml), and after filtration (0.45 μm pore size) the glycosides were determined by HPLC using a Thermoquest LCQ (Thermoquest, D-63329 Egelsbach, Germany) in the APCI mode (vaporizer temperature: 560°C) connected to an Agilent HP1100 HPLC-system equipped with an RP18 column, (Purosphere 4×250 mm (5 μm). Samples (10 μl) were analyzed by using gradient elution at 0.65 ml min^{-1}. Solvent A: 0.5% acetic acid in water; solvent B: 0.5% acetic acid in acetonitrile. Compounds were eluted according to the following protocol: 0-10 min, 10% of B in A; 10–50 min 10–60% of B in A. UV-detection at 254, 266, and 360 nm prior to the mass spectrometer allowed us to gain further information on the analytes. Commercially unavailable compounds were isolated and identified based on data from HR-ESI-MS, ^{1}H- and HSQC-NMR spectroscopy, and literature data. Known compounds were compared by their retention times and UV-Vis and MS data with authentic references. Myricetin derivatives were identified after hydrolysis of the crude extract of *B. rotundifolia* (1.2 M HCl for 4 h at 100°C) by HPLC using an authentic reference. In addition, the aglycones were determined after enzymatic hydrolysis (Schulz et al. 1997) of the raw extracts followed by GC-EIMS. Compounds were identified using authentic standards.

Table 1 Identification and quantification of defensive substances in the birch-feeding population of *Chrysomela lapponica* from the Burkhat Pass, East Kazakhstan

Peak number	Substance	Compounds [nmol/mg secretion]
	Acetic acid	3.6[a]
	Isobutyric acid	1.1[a]
	2-Methylbutyric acid	0.3[a]
1	Benzyl alcohol	0.3
2	(*Z*)-3-Hexenylisobutyrate	2.2
3	Hexylisobutyrate	1.2
4	5-Hexenyl isobutyrate	Not quantified[b]
5	Benzoic acid	trace
6	(*Z*)-3-Hexenyl-2-methylbutyrate	5.3
7	Hexyl-2-methylbutyrate	1.5
8	5-Hexenyl-2-methylbutyrate	Not quantified[b]
9	Benzylisobutyrate	8.7
10	Benzyl-2-methylbutyrate	4.6
11	Phenylethylisobutyrate	1.5
12	Phenylethyl-2-methylbutyrate	1.8
13	1,3-Hexandiyl-1,3-diisobutyrate	Not quantified[b]
14	(*Z*)-3-Hexenylbenzoate	trace
15	8-OH-Geranylisobutyrate	Not quantified[b]
16	8-OH-Linalylisobutyrate	0.1
17	mixture of 1,3-Hexandiyl-1-isobutyrate-3-(2-methylbutyrate), 1,3-Hexandiyl-1-(2-methylbutyrate)-3-isobutyrate	Not quantified[b]
18	8-OH-Geranyl-2-methylbutyrate	Not quantified[b]
19	*p*-OH-Phenylethylisobutyrate	13.0
20	8-OH-Linalyl-2-methylbutyrate	0.2
21	1,3-Hexandiyl-1,3-di-2-methylbutyrate	Not quantified[b]
22	*p*-OH-Phenylethyl-2-methylbutyrate	25.0
23	Betuligenolisobutyrate	4.2
24	Betuligenol-2-methylbutyrate	4.3
25	Linolenic acid methylester	1.6
26a–e	Glucose	468.7
27a,b	Glucose-6-*O*-isobutyrate	17.2
28a,b	Glucose-6-*O*-2-methylbutyrate	26.8

Quantification was carried out using external standards (N=3), [a] quantified after derivatization with PFBB (Attygalle et al. 1991), [b] determined by spectral data comparison. Numbering in Table 1 corresponds peak numbering of Fig. 1.

Synthesis of Glucose Esters Acylation was carried out according to a modified method from Woudenberg (Woudenberg-van Oosterom et al. 1995). The procedure was conducted under an inert atmosphere. 100 mg glucose were dissolved in 10 ml *t*-butanol. 1 g molecular sieve 4 Å was added. 10 ml 12.5% ethylisobutyrate and ethyl-2-CH_3-butyrate, respectively, in *t*-butanol then were added. The reaction was started by adding 100 mg *Candida antarctica* lipase B. The suspension was stirred for 72 h at 50°C. The molecular sieve and the immobilized enzyme were separated by filtration, and the solvent was evaporated. The raw product was fractionated by column chromatography ($CHCl_3$/MeOH/H_2O=75/22/3) to yield the acylated glucoses. The described procedure produced an anomeric mixture. Hence, multiple signals were observed. Assignment of peaks was carried out using 2D-NMR-experiments (HSQC and HMBC).

Spectral Data of 6-O-isobutyryl-a/b-D-glucopyranoside ^{1}H NMR ($MeOD_4$) [ppm]: 5.01 (d, J=3.67 Hz, 0.5 H), 4.41 (d, J=7.74 Hz, 0.5 H), 4.30 (ddd, J=19.13 Hz, J=11.88 Hz, J=2.15 Hz, 1 H), 4.15-4.08 (m, 1 H), 3.89 (ddd, J=10.17 Hz, J=5.48 Hz, J=2.17 Hz, 0.5 H), 3.60 (t, J=9.29 Hz, 0.5 H), 3.43-3.38 (m, 1 H), 3.30-3.20 (m, 1 H), 3.17 (masked by

$MeOD_4$ signal, 1 H), 3.08-3.04 (t, J=8.69 Hz, 0.5 H), 2.51 (sept, J=7.00 Hz, 1 H), 1.10 (d, J=7.01 Hz, 6 H). ^{13}C NMR ($MeOD_4$) δ [ppm]: 178.8/178.7, 98.3/94.0, 78.0/73.8, 76.2/74.8, 75.4/70.8, 72.0/71.7, 64.9/64.8, 35.2/35.2, 19.4/19.3. MS (70 eV) m/z (%): 233(0.5), 219(3), 203(3), 173(5), 161 (5), 143(5), 131(18), 103(17), 89(28), 71(100), 60(19), 43 (97). ESI-HR-MS m/z $[M+H]^+$: calculated for $C_{10}H_{19}O_7$: 251.25366; found: 251.11276.

Spectral Data for 6-O-(2-methylpropanyl)-α/β-D-glucopyranoside ^1H NMR ($MeOD_4$) [ppm]: 5.03 (d, J= 3.53, 0.5 H), 4.42 (d, J=7.83, 0.5 H), 4.38-4.30 (m, 1 H), 4.16-4.09 (m, 0.5 H), 3.90 (dd, J=10.04 Hz, J=5.02 Hz, 0.5 H), 3.62 (t, J=9.42 Hz, 0.5 H), 3.44-3.39 (m, 0.5 H), 3.32-3.26 (m, 1 H), 3.24 (masked by $MeOD_4$ signal, 1 H), 3.08 (dd, J=9.62 Hz, J=7.90 Hz, 0.5 H), 2.40-2.30 (m, 1 H), 1.67-1.56 (m, 1 H), 1.48-1.38 (m, 1 H), 1.09 (td, J= 6.91 Hz, J=0.92 Hz, 3 H), 0.86 (dt, J=7.47 Hz, J=1.03 Hz, 3 H). ^{13}C NMR ($MeOD_4$) δ [ppm]: 178.4/178.3, 98.2/94.0, 78.0/73.8, 76.2/74.8, 75.5/70.8, 72.0/71.8, 64.8/64.7, 42.5/42.3, 28.0/27.9, 17.1/17.0, 12.0/11.9. MS (70 eV) m/z (%): 233(2), 217(2), 187(4), 175(4), 145(10), 103(27), 85(66), 74(37), 57(100). ESI-HR-MS m/z $[M+Na]^+$: calculated for $C_{11}H_{20}O_7Na$: 287.26207; found 287.11025.

Spectral Data of Santin-7-O-glucoside yellow amorphous powder; UV λ_{max} nm: 272, 338 (MeOH); HR ESI-FTMS *m/z* 507.14884 ($[M+H]^+$, calcd. for $C_{24}H_{27}O_{12}$, 507.14970), ^1H NMR (DMSO-d_6) δ: 12.61 (s, 1 H), 8.04 (d, J=8,8 Hz, 2 H), 714 (d, J=8.8 Hz, 2 H), 6.99 (s, 1 H), 5.12 (d, J=7.1 Hz, 1 H), 3.86 (s, 3 H), 3.79 (s, 3 H), 3.18-3.75 (sugar H), ^{13}C NMR (DMSO-d_6) δ:178.4, 161.5, 156.6, 155.9, 152.1, 151.4, 137.9, 132.3, 130.1, 122.2, 114.4, 106.3, 100.1, 94.2, 77.3, 76.7, 73.2, 69.5, 60.6, 60.4, 59.8, 55.6.

Results

The defensive secretion of the larvae of *C. lapponica* mainly comprised 2-methylbutyric and isobutyric acid esters of aglycones, which occur as glucosides in the leaves of the host plant (Table 1). Major compounds are the butyrate esters of hexanol (**3** and **7**), (*Z*)-3-hexenol (**2** and **6**), benzyl alcohol (**9** and **10**), phenylethanol (**11** and **12**), *p*-OH-phenylethanol (**19** and **22**), and betuligenol (**23** and **24**). While the concentration of free alcohols in the secretion was low, significant amounts of free 2-methylbutyric- and isobutyric acids along with acetic acid and traces of benzoic acid were found after derivatization with pentafluorobenzylbromide (Table 1). In addition, the secretion contained large amounts of free glucose (**26a-e**) along with glucose-6-*O*-butyrates (**27a/b** and **28a/b**), which are identified for the first time in leaf beetle defenses. Glucose and the two 6-*O*-glucose esters **27a/b** and **28a/b** were identified after derivatization with MSTFA by GC-EIMS using synthetic references. Identified compounds are compiled in Table 1 according to the elution order shown in Fig. 1.

The presence of large amounts of glucose was unexpected. Even more remarkable was the presence of C(6) glucose esters, indicating that the easily accessible primary hydroxy group of glucose can serve as a substrate for the acyl transferase(s) that catalyze the acylation of the plant-derived aglucones. Only the 6-*O*-butyrates **27a/b** and **28a/b** (a and b refer to α- and β-anomers of the butyrates) were detected (Scheme 2). Esters linked to a secondary hydroxyl group of the glucose moiety were not observed.

Glycosides in Leaves of B. rotundifolia We addressed the full spectrum of glycosides from dry leaves of the food

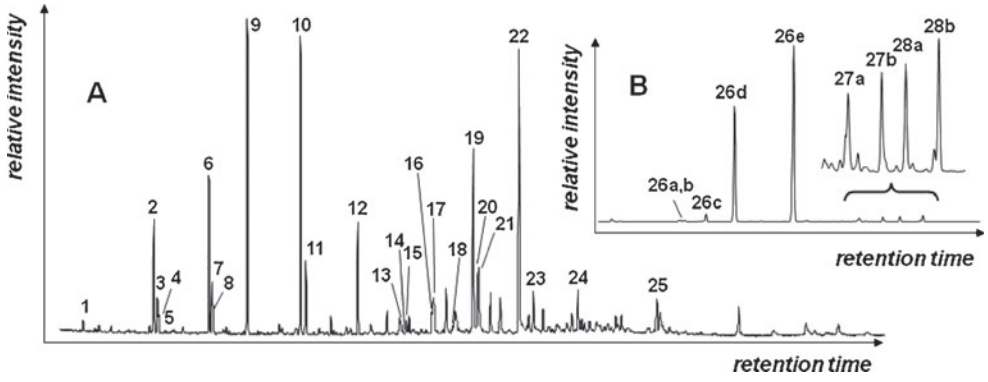
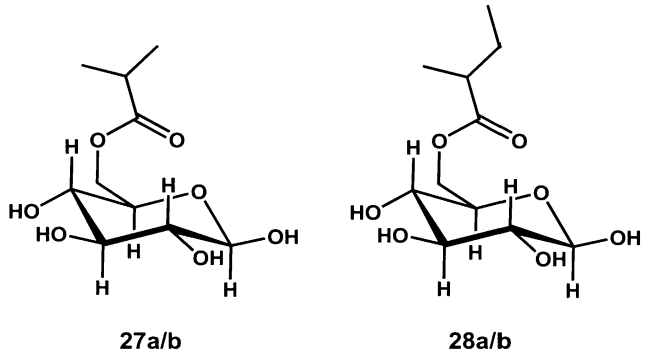


Fig. 1 Gaschromatographic profile of the defensive secretion of birch-feeding larvae of *Chrysomela lapponica* from the Altai Mountains, East Kazakhstan. Numbering and identification of the peaks corresponds to Table 1. **a** GC-EIMS analysis of the volatile esters of the secretion **b** GC-EIMS analysis of the polar constituents of the defensive secretion after derivatization with MSTFA. Low boiling silyl-derivatives are not shown. The four signals for **27a/b** and **28a/b** correspond to the α- and β-enantiomers of the silylated glucose esters. Silylated glucose is represented by the signals **26a-e**



Scheme 2 Butyrate esters (**27a/b**) and (**28a/b**) of glucose

plant *B. rotundifolia* by exhaustive extraction and analysis by HPLC-MS, LC-UV, and NMR of isolated compounds. The LC-profile (Fig. 2) of the polar extract of *B. rotundifolia* displayed a rich pattern of glycosylated flavonoids (**45**), (**46**), (**49**), (**53**) derived from quercetin (**58**), kaempferol (**62**), and the 3′,5′-trihydroxy-6,7,4′-trimethoxyflavones (**55**), and (**66**) (Table 2). To our knowledge, santin-7-*O*-glucoside (**59**) is isolated for the first time. The presence of tremulacin (**60**) and salicortin (**44**), which can ultimately serve for the production of salicylaldehyde after hydrolysis in the gut (Julkunen-Tiitto and Meier 1992), is noteworthy, since no butyrate esters of saligenin or free salicylaldehyde were found in the larval secretion. In general, comparison of the host plant chemistry with secretion revealed that the glycoside pattern of the host plant dramatically differed from the aglycone pattern found in the larval secretion. The predominant flavonoid glycosides were completely absent in the larval secretion, while the low molecular weight aromatic/phenolic esters and aliphatic hexenyl esters, which are minor or even trace components of the plant-extracted glycosides, dominated the larval secretions. Nevertheless, the aglycones of several plant-produced precursors like betuloside (**37**), phenylethyl-Glc (**42**), salidroside (**29**), and 8-OH-linaloyl-Glc (**39**) matched the aglycones present in the secretion. The identities of the 39 identified compounds are given in Table 2.

Since not all aglycones present in the defensive secretion were found as glucosides, we hydrolyzed the total extract with a mixture of glucosidases and galactosidases according to (Schulz et al. 1997). Analysis of the released compounds indeed revealed the presence of (*Z*)-3-hexenol, and benzyl-alcohol, which for unknown reasons were not found among the leaf-derived glycosides.

Hemolymph Transport of the Phytogenic Glycosides After uptake into the digestive system, the gut epithelial cells constitute the first membranes that the glycosides have to pass. Previously, we tested this passage in birch-feeding larvae from the Czech Republic and willow-feeding larvae of *C. lapponica* from Queyras by using hydrolysis-resistant thioglucosides (Kuhn et al. 2007). After application of the compounds to the leaf surface, larvae of the birch-feeding *C. lapponica* were allowed to feed on the treated leaves, and the amount of thioglucosides transported through the insect into the glandular reservoir was determined by HPLC-MS.

As shown (Fig. 3), the birch-feeding populations of *C. lapponica* from Kazakhstan and also from the Czech Republic (Kuhn 2005) show similar transport characteristics. No significant differences could be evaluated for thiosalicin (**1**), *o*-kresolthioglucoside (**2**), or 8-OH-geraniolthioglucoside (**6**). Only the uptake of phenylethylthioglucoside (**4**) differed significantly between the birch-feeding populations. Common to both population (birch and willow feeders) (Kuhn et al. 2007), is the selective import of only thioglucosides; galactosides were not taken up. Moreover, the birch-feeding

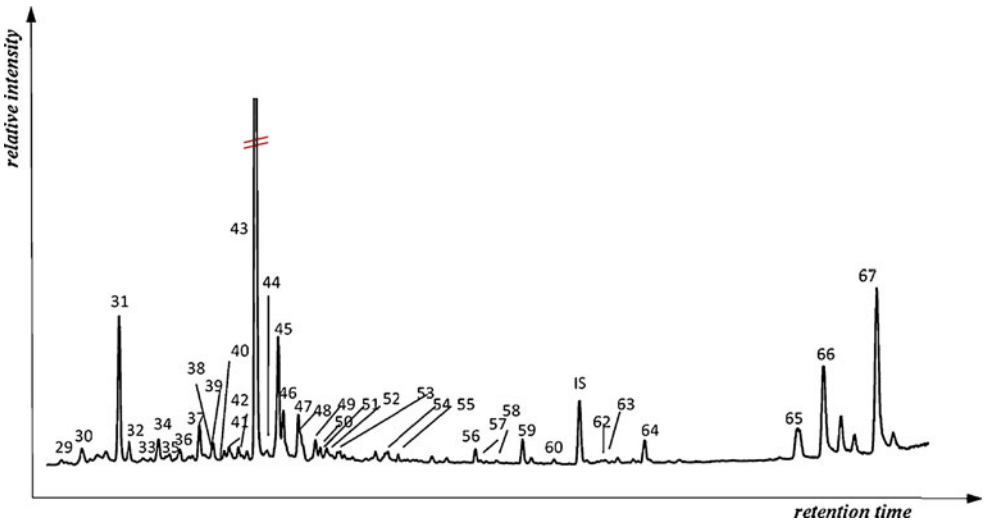


Fig. 2 HPLC-MS chromatogram of the methanolic extract of *Betula rotundifolia*. Compounds of the methanolic extract were separated and monitored by HPLC-MS on Purosphere RP18, 5 μm, 4×250 mm (Merck) using an isocratic solvent system 10% of B (10 min) and linear gradient from 10% B to 60% B (50 min). Solvent A: 0.5% CH_3COOH in H_2O, solvent B: 0.5% CH_3COOH in CH_3CN. Flow rate 0.65 ml/min. Numbering and identification of the peaks correspond to Table 2

Table 2 Identification and quantification of glucosides and other compounds from the polar extract of *Betula rotundifolia*

Peak	Molecular formula	$[M+H]^+$ *m/z*	Compound
29	$C_{14}H_{20}O_7$	301	Salidroside[a]
30	$C_{20}H_{22}O_{10}$	423	Catechin-7-0-xylopyranoside[d]
31	$C_{16}H_{18}O_9$	355	Chlorogenic acid [a]
32	$C_{15}H_{14}O_6$	291	(+)-Catechin [a]
33	$C_{19}H_{32}O_8$	389	Blumenol B – 9-O-glucoside[a]
34	$C_{19}H_{30}O_8$	387	Blumenol derivative[d]
35	$C_{16}H_{18}O_9$	355	Neochlorogenic acid[d]
36	$C_{15}H_{14}O_6$	291	(-)-Epicatechin[a]
37	$C_{16}H_{24}O_7$	329	Betuloside[b], (Smite et al. 1993)
38	$C_{27}H_{30}O_{17}$	627	Myricetin-rutinoside[c]
39	$C_{16}H_{28}O_7$	333	8-Hydroxylinaloyl-glucoside[d]
40	$C_{21}H_{20}O_{13}$	481	Myricetin-glucoside[c]
41	$C_{21}H_{20}O_{13}$	481	Myricetin-galactoside[c]
42	$C_{14}H_{20}O_6$	285	Phenethyl-glucoside[a]
43	$C_{27}H_{30}O_{16}$	611	Quercetin-3-O-rutinoside[a]
44	$C_{20}H_{24}O_{10}$	425	Salicortin[a]
45	$C_{21}H_{20}O_{12}$	465	Quercetin-3-O-glucoside[a]
46	$C_{21}H_{20}O_{12}$	465	Quercetin-3-O-galactoside[a]
47	$C_{27}H_{30}O_{15}$	595	Kaempferol-3-O-rutinoside[a]
48	$C_{28}H_{32}O_{16}$	625	Isorhamnetin-3-O-rutinoside[a]
49	$C_{20}H_{18}O_{11}$	435	Quercetin-3-O-arabinoside[b], (Hansen et al. 1999)
50	$C_{19}H_{32}O_7$	373	Blumenol C – 9-O-glucoside[a]
51	$C_{21}H_{20}O_{11}$	449	Kaempferol-3-O-glucoside[a]
52	$C_{22}H_{22}O_{12}$	479	Isorhamnetin-3-O-glucoside[a]
53	$C_{21}H_{20}O_{11}$	449	Quercetin-3-O-rhamnoside[a]
54	$C_{21}H_{20}O_{10}$	433	Kaempferol-3-O-rhamnoside[b], (Chung et al. 2004)
55	$C_{24}H_{26}O_{13}$	523	5.3′.5′-Trihydroxy-6.7.4′-trimethoxyflavone+hexose[d]
56	$C_{23}H_{24}O_{11}$	477	Pectolinaringenin-7-O-glucoside[b], (Yim et al. 2003)
57	$C_{22}H_{22}O_{10}$	447	Acacetin+hexose[d]
58	$C_{15}H_{10}O_7$	303	Quercetin[a]
59	$C_{24}H_{26}O_{12}$	507	Santin-7-O-glucoside[b,]
60	$C_{27}H_{28}O_{11}$	529	Tremulacin[a]
61	$C_{15}H_{12}O_5$	273	Naringenin (internal standard)
62	$C_{15}H_{10}O_6$	287	Kaempferol[a]
63	$C_{16}H_{12}O_7$	317	Isorhamnetin[a]
64	$C_{18}H_{16}O_8$	361	5.3′.5′-Trihydroxy-6.7.4′-trimethoxyflavone[b], (Kinoshita and Firman 1996)
65	$C_{16}H_{12}O_5$	285	Acacetin[a]
66	$C_{17}H_{14}O_6$	315	Pectolinaringenin[b], (Greenham et al. 2003; Horie et al. 1998; Vieira et al. 2003)
67	$C_{18}H_{16}O_7$	345	Santin[b], (Greenham et al. 2003; Horie et al. 1998)

Compounds were identified using authentic reference substances, by isolation, or by release of the aglycones by enzymatic hydrolysis (see Experimental). Numbering in Table 2 corresponds peak numbering of Fig. 2. [a] compared with reference substances, [b] isolated and compared ^{1}H and HSQC-NMR and UV, HR-ESI MS spectroscopic data with literature, [c] identification based on the APCI-MS and compared the aglycone with reference substances after hydrolysis, [d] identification based on the APCI-MS and HR-ESI-MS.

larvae were able to sequester salicin from the food plants. Thus, a defect in the transport system does not account for the total lack of salicylaldehyde or other aldehydes (e.g., benzaldehyde from benzylalcohol; see Table 1). Fig. 3 also demonstrates that the thioanalogs of (*Z*)-3-hexenylglucoside, phenylethylglucoside (**42**), and 8-OH-geraniolglucoside (**39**) are easily imported with a similar efficiency. In general, almost identical transport characteristics and capacities were observed for willow- and birch-feeding larvae of *C. lapponica*.

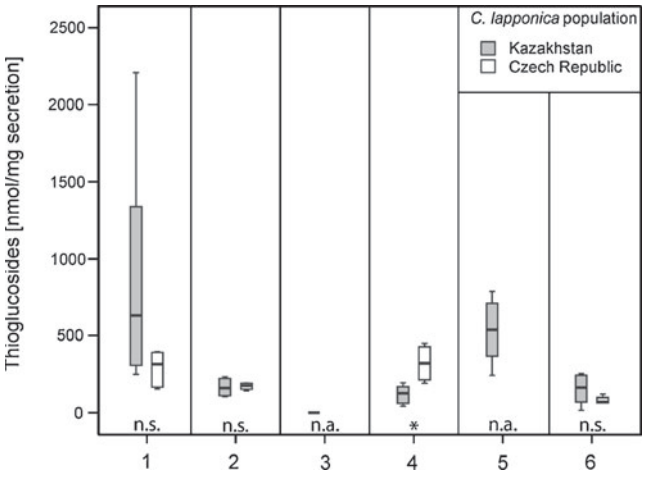


Fig. 3 Accumulation of the thioglucosides **1** to **6** in the defensive secretion of the birch-feeding larvae of *Chrysomela lapponica*. Test compounds: **1**: thiosalicin, **2**: *o*-kresolthioglucoside, **3**: salicylthiogalactoside, **4**: phenylethylthioglucoside, **5**: (*Z*)-3-hexenylthioglucoside, **6**: 8-OH-geraniolthioglucoside. Compounds were determined and quantified by HPLC-MS (see Experimental). Boxplots represent median, 25% and 75% percentile and inner fences show lowest and highest value. Data for larvae collected in the Czech Republic are taken from (Kuhn et al. 2004). According to Mann-Whitney test significant differences were only observed for **4** (P=0.019). No significant differences (n.s.) were observed for **1** (P=0.126), **2** (P=0.914) and **6** (P=0.257). Statistics were not applicable (n.a.) to **3** and **5**

Discussion

Sequestering leaf beetle larvae or those using a mixed-mode biosynthesis (sequestration and *de novo* biosynthesis) have developed an efficient network of transporters in their guts and glandular systems to channel phytogenic precursors into their reservoirs (Kunert et al. 2008; Discher et al. 2009). In general, only the glucoside precursor of the genuine chemical defense (e.g., salicylaldehyde or iridoid) is imported and channeled *via* hemolymph transport through the larval body to the glandular reservoir for the final toxification reactions. For example, larvae of *Chrysomela populi* and *Phratora vitellinae* specifically import salicin (Kuhn et al. 2004) and almost no related compounds. The *de novo* producers *Phratora laticollis*, *Hydrotassa marginella*, *Phaedon cochleariae*, *Gastrophysa viridula*, and *Plagiodera versicolora* also possess the ability to selectively uptake glucosidically bound 8-hydroxygeraniol (Feld et al. 2001; Kuhn et al. 2004) provided that the compound is present in the food plant (Kunert et al. 2008). In all cases, the imported glucosides coincided with the biosynthetic precursors of the final chemical defenses of the defensive cocktail that is based mostly on a single or only a few compounds.

In contrast, *C. lapponica* should possess transport systems of lower selectivity, thus allowing the sequestration of a range of plant-derived glucosides that is reflected in the remarkable pattern of substances within the secretion. Their principal ability to sequester structurally different glucosides was shown previously in *Salix*-feeders from Queyras using hydrolysis-resistant thioglucosides, which remain intact all the way from the leave to the defensive gland (Kuhn et al. 2007). By direct inspection of Tables 1 and 2, which compare the spectrum of glucosides present in the food plant (Fig. 2, Table 2) and the enzymatically released aglycones with the components of the defensive secretion, the selectivity of the sequestration process becomes obvious. For example, neither the dominant flavanoids, nor any of their ester derivatives, accumulate in the defensive secretion. Instead, the aglycones of (*Z*)-3-hexenol, phenylethanol, *p*-OH-phenylethanol, and betuligenol are present as major constituents in the blend of 2-methylbutyryl- and isobutyryl esters. The aglycones are imported into the reservoir as their glucosides, as demonstrated by the successful import of thioglucosides (Fig. 3, similar to the results shown for the Queyras population, (Kuhn et al. 2007). After import into the gland, the sugar moiety is hydrolyzed, followed by acylation of the resulting aglucone with the butyrates. Interestingly, all glucoside precursors of the defensive compounds were present only as minor components in the leaf extract (Fig. 2) suggesting a selective and highly efficient transport process in the larvae.

The complete lack of derivatives of salicortin (**44**) and tremulacin (**60**) in the defensive secretion is striking, although they are present in small amounts in the birch extract. Unspecific esterases, generally occurring in the enzymatic inventory of a gut, could remove the cyclohexenoic acid moiety (Lindroth 1988; Julkunen-Tiitto and Meier 1992), thus generating salicin, which would be readily sequestered into the glandular reservoir (Fig. 3). After hydrolytic cleavage in the reservoir, the released aglycone could be converted into a butyrate or oxidized to salicylaldehyde. Neither reaction was observed. However, the lack of salicylaldehyde or benzaldehyde is readily explained since the birch-feeding population of *C. lapponica* from Kazakhstan has no functional salicylalcohol oxidase (unpublished data).

Additionally, striking is the presence of high amounts of glucose (469 nmol mg^{-1} secretion) and minor amounts of the two glucose-6-*O*-butyrates **27a/b** and **28a/b** in the secretion (44 nmol mg^{-1} secretion). Apparently, glucose not only acts as a carrier for import of plant-derived aglycones into the glandular system, but also may serve as a scaffold for acylation reactions. The same compounds are known from trichomes of tomato (Neal et al. 1990), and an insecticidal activity has been demonstrated (Juvik et al. 1994).

The biological relevance of the high amounts of glucose in the larvae of the Altai populations is not clearly understood, but the sugar apparently does not interfere with the repellent function of the esters, as has been demonstrated (Hilker and Schulz 1994); probably the volatile esters form a repellent plume around the larvae, thus

preventing the necessity of a direct contact, which has been shown previously for *Phratora vitellinae* and *Phaedon cochleariae* (Gross et al. 2008; Gross and Schmidtberg 2009).

On the other hand, since the larvae experience rather low temperatures that go well beyond 0°C at night during the season (July, August), the high amount of glucose may function as a cryoprotectant (Calderon et al. 2009). Moreover, since the defensive secretion is an emulsion of organic esters and water, the actual concentration of free glucose in the aqueous phase, and hence its cryoprotective effect, might be even higher than calculated from the molar concentration (ca. 1.1°C at ca. 470 nmol mg^{-1} secretion). Since in the Altai region predators seem to be rare compared to other habitats of *C. lapponica* (personal observations), the selective pressure on adaptation to climate may be higher than to predation. The risk of infections by microorganism that utilize the "sweet secretion", particularly by entomophagous fungi (e.g., *Beauveria bassiana*) might be reduced by the presence of a few antimicrobially active constituents, such as *p*-OH-phenylethyl-isobutyrate (**19**), *p*-OH-phenylethyl-2-methylbutyrate (**22**), and betuligenoliso-butyrate (**23**) in the secretion (Tolzin-Banasch 2009).

By studying the selective import from the whole spectrum of glucosides that occur in the leaves of the food plant (*B. rotundifolia*), instead of a limited set of model compounds (Discher et al. 2009), we were able to better evaluate the selectivity and efficiency of transport. Although the larvae import several compounds (e.g., **37** or **42**), this corresponds only to a minor fraction of glycosides present in the food plants (Fig. 2). Moreover, the imported compounds do not belong to the major constituents of the plant, thus demonstrating a high enrichment capacity of the transport system. However, *de novo* biosynthesis of certain aglycones, especially phenylethanol and benzylalcohol from phenylalanine (unpublished results) in addition to sequestration cannot be excluded.

Whether or not the transport systems in the two membrane barriers (gut epithelial cells, glandular system) indeed have different selectivities (Discher et al. 2009) remains to be established.

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4 General Discussion

Most of the major published findings of my thesis have been discussed in detail individually in the previous manuscripts. For this reason it is the aim of the following general discussion to connect the results and embed them in a broader, more super-ordinate context. “Prerequisites”, origin, evolutionary dynamics and ecological significance of salicin-based chemical defense in Chrysomelina larvae are discussed. Thereby also some speculative aspects as well as future perspectives have been considered.

4.1 Evolution of salicin-based defense in Chrysomelina larvae

4.1.1 Salicylaldehyde biosynthesis

The survey of 16 ecological and entomological journals from 1969-1989 for articles concerning defenses in arthropods (354 publications) revealed that 46 % of relevant papers dealt with chemical defenses (Witz 1990). These findings reflect the impact and abundance of these anti-predator mechanisms in nature. In insects chemical defense is achieved by the release of toxins/poison through bites, stings, reflex-bleeding, regurgitate, excrement or exocrine glands (reviewed in Dettner 2007). In this context Chrysomelina leaf beetle larval exocrine glandular defense is remarkable. Not only the chemical defensive compounds themselves but also their biosynthetic steps, efficacy and especially their host plant origin are well known for a variety of species. Those data build up the fundament to comparatively address questions related to “prerequisites”, constraints, adaptation, dependence and evolution of insect chemical defense in a specialist herbivore host plant interaction.

In particular, salicin-based defense in Chrysomelina larvae has been shown to be highly efficient and of ecological relevance. The defensive secretion in general and especially the salicin-derived salicylaldehyde act as deterrents against ants, wasps, ladybirds and spiders (Wallace and Blum 1969, Blum et al. 1972, Matsuda and Sugawara 1980, Denno et al. 1990, Palokangas and Neuvonen 1992, Hilker and Schulz 1994) and possess anti-microbial and cytotoxic activity (Schildknecht et al. 1968, Gross et al. 2002).

To my knowledge beside its presence in Chrysomelina species the highly bioactive salicylaldehyde has been detected only in few other insects:

(1) In pygidial glands of ground beetles (Eisner et al. 1963, Schildknecht et al. 1968)

(2) In mandibular glands of the bee *Pithitis smaragdula* (Hefetz et al. 1979)

(3) In abdominal glands of the butterfly *Limenitis archippus* (Prudic et al. 2007)

This makes a convergent use of salicylaldehyde for defensive properties in those distantly related insect taxa likely. Moreover, it is interesting to note that the caterpillar of *Limenitis archippus* feeds on salicaceous plants and the adults possess beside the volatile salicylaldehyde also the phytogenic salicin and tremulacin in their abdominal glands. Therefore K. L. Prudic (2007) argued for a phytogenic origin of salicylaldehyde and a transfer between different developmental stages is likely. If this can be further verified not only the utilization but also origin and biosynthesis of salicylaldehyde in specialized exocrine glands convergently evolved in Chrysomelina larvae and *Limenitis archippus*.

Even within Chrysomelina the origin of salicylaldehyde biosynthesis is not clear as beside the genus *Chrysomela* also larvae of *Phratora vitellinae* produce salicylaldehyde (Wain 1943). Due to phylogenetic analyses a convergent origin of salicin-based defense in both, not closely related genera, by independent recruitments of the ancestral iridoid biosynthesis machinery for salicylaldehyde formation has been discussed (Termonia et al. 2001). The main argument for this scenario is the similarity of chemical properties of the oxidative step in both iridoid and salicylaldehyde biosynthesis (Pasteels et al. 1990, Veith et al. 1997), which favored the evolution of the more derived salicin-based defense by changes in ancestral glandular oxidase specificities of iridoid producers.

However, manuscript one and two provide data supporting a single origin of salicyl alcohol oxidase in *Phratora vitellinae* and *Chrysomela* species, although they are not closely related species. *Phratora vitellinae* and *Chrysomela* SAO genes not only share a common ancestor in the GMC oxidoreductase multi-gene family, but also display identical gene architecture, glandular tissue specific expression, SAO enzyme substrate specificity and high amount of SAO protein in the glandular secretion. Therefore, strong evidence for a single origin of *Phratora vitellinae* and *Chrysomela* SAOs is provided. However, I am aware of the fact that it is not verified yet that both species´ SAO genes are true orthologs as long as additional genomic data of more Chrysomelina species for a complete SAO phylogenetic analysis are lacking.

Regarding the scenario of iridoid biosynthesis-derived enzymatic machinery for salicylaldehyde formation I can conclude, based on manuscript two and additional unpublished data, that SAO related proteins are not involved in defensive compound formation in iridoid producing species of Chrysomelina. Although a variety of proteins of glandular secretions has been observed by MS/MS analyses, no evidence for the presence of a SAO related GMC oxidoreductase in the defensive system of the iridoid producing species *Phaedon cochleariae*, *Gastrophysa viridula* and *Gastrophysa cyanea* has been found. Contrary to previous assumptions (Pasteels et al. 1990, Veith et al. 1997, Termonia et al. 2001), this strongly supports at least the independent evolution of the oxidative step in salicylaldehyde and iridoid defended larvae.

Therefore, I suggest an alternative evolutionary scenario for SAO emergence in *Chrysomela* and *Phratora vitellinae*. The SAO paraloga found in *Chrysomela lapponica* and *Chrysomela populi* (manuscript one) as well as the SAO-like genes in the iridoid producing species *Phratora laticollis* and *Phaedon cochleariae* (manuscript two and additional unpublished data for *P. cochleariae*) indicate gene duplication events starting early in Chrysomelina speciation. The expansion of a specific GMC oxidoreductase subfamily (GMC*i*) most likely gave birth to an SAO ancestral gene followed by further lineage-specific gene duplications. The glandular-specific expression of SAO paralog1 of *C. lapponica* (manuscript one) allows for the assumption that the SAO "precursor" gene already possessed glandular tissue-specific expression even before its recruitment/selection for SAO catalytic activity. It is conceivable that the SAO ancestral protein already had a SAO side-activity, which became the main-activity through selection. Additionally, typical features of insect GMC*i* proteins like high degree of N-glycosylations and N-terminal signal peptides (personal observation), may have favored SAO functionalization.

But although a scenario of SAO gene evolution can be developed, the (gradual or fast) enzyme evolution from an ancestral catalytic activity and whether neo- or sub-functionalization of the ancestral gene took place cannot be reconstructed. This is because nothing is known about enzymatic activities of SAO-related proteins in other insect species neither within Chrysomelina nor in other beetle families of the Cucujiformia. However, knowledge about SAO-related proteins and insect GMC*i* proteins in general would be helpful to elucidate the gain of salicyl alcohol oxidase activity starting from an SAO ancestor. For Chrysomelina SAO-related proteins I can conclude that, as SAO-like genes are also present in iridoid producers but not encode

for proteins in the defensive secretion, they most likely persisted due to the acquisition of other functions than defensive glandular oxidases. Therefore, SAOs most likely originated outside the ancestral defensive compound machinery of the iridoid producers. GMC oxidoreductases in general are known to be ubiquitous present and fulfill a multiplicity of enzymatic reactions that are in most cases alcohol dehydrogenations (oxidations) leading to the respective aldehydes (Cavener 1992, Li et al. 1993, Mattevi 1998, Hallberg et al. 2002, Hallberg et al. 2004, Zamocky et al. 2004, Zamocky et al. 2006, Sarfare et al. 2005), which holds true for the SAOs, too. In insects the only well-characterized GMC oxidoreductase members are the glucose dehydrogenases (Cavener and MacIntyre 1983, Krasney et al. 1990). However, most of the insect GMC genes (including GMC*i*-subfamily) are located in a highly conserved cluster (Kunieda et al. 2006, Iida et al. 2007) but neither their affiliation with any specific biochemical pathway nor their precise catalytic activities are known. The only gene of the insect GMC cluster which is characterized so far is an ecdysone oxidase of *Drosophila melanogaster* (Takeuchi et al. 2005), leading to speculate that the whole cluster may be involved in ecdysone metabolism (Iida et al. 2007). However, this is not supported by any data so far and thus provides no hint for the catalytic activity of the SAO ancestor.

In summary, the SAO activity most likely arose from a precursor in the GMC*i*-subfamily of the insect GMC gene-cluster after gene duplication. In general, the process of duplicate gene evolution leads to one of the three following alternative fates:

i) One copy may be lost (non-functionalization) through e.g. null-mutation, leading to failure of transcription or translation into a functional protein.

ii) The acquisition of mutation(s) can also be fixed by positive selection and enable one copy to gain a new function (neo-functionalization) whereas the other copy persists by retaining the ancestral function.

iii) Both duplicates accumulate degenerative mutations and may therefore undergo loss or reduction of expression for different sub-functions (sub-functionalization *sensu* Force et al. 1998), leading to a partitioning of functionalities of one ancestral locus to two loci.

Applying those definitions to the SAO evolutionary scenario, either the ancestral gene already possessed SAO activity (sub-functionalization of one copy) or not (neo-functionalization of one copy). As already pointed out, these alternative evolutionary scenarios cannot be resolved so far.

4.1.2 “Prerequisites” for phenolic-glycoside utilization

In the previous paragraph I discussed the single origin and the evolution of SAO proteins in Chrysomelina species, which may have taken place in a step-wise manner. Starting with gene duplications in a specific GMC subfamily, glandular tissue-specific expression and selection for SAO activity of an ancestral protein with an already existing SAO side-activity may have followed. This evolutionary scenario proceeded most likely independently from the iridoid pathway at least with respect to the oxidative step in the defensive glands.

However, taking the phylogenetic analyses of A. Termonia and co-authors (2001) into account, the ancestors of recent species utilizing salicin-based defense were iridoid-protected leaf beetles. Therefore, most likely some features of the more basal iridoid-producers favored salicin-based defense. In the following I call those features prerequisites and I will focus on host plant chemistry, glycoside transport processes and defensive gland glucosidases in the larvae.

I am well aware that the recent iridoid producing species do not reflect exactly the character states of the salicylaldehyde producing species´ ancestors that produced iridoids due to their own evolutionary history after split from their common ancestor. However, by examining recent iridoid producers an underlying ground-pattern of the most recent common ancestor of iridoid and salicylaldehyde producing species can be reconstructed.

4.1.2.1 Host plant chemistry

Phenolic glycosides (PGs) e.g. salicortin, tremulacin, salicin (**Fig. 1**) have been shown to be typical secondary plant metabolites of the plant family Salicaceae (Thieme 1971, Palo 1984, Babst et al. 2010). PGs are feeding deterrents and toxic compounds acting against generalist herbivorous insects (Tahvanainen et al. 1985, Lindroth et al. 1988, Clausen et al. 1989, Denno et al. 1990, Lindroth and Hemming 1990, Ruuhola et al. 2001a, Ruuhola et al. 2001b, Ruuhola et al. 2003). Their impact in herbivore host plant interaction is most likely due to break-down products acting as toxins or inhibitors in insects As an example, in case of salicortin-degradation a mix of enzymes of both herbivore and plant origin can be assumed (Lindroth 1988, Julkunen-Tiitto and Meier

1992, Ruuhola et al. 2003, unpublished data), leading to the deleterious compounds saligenin and cyclohexenone (**Fig. 1**).

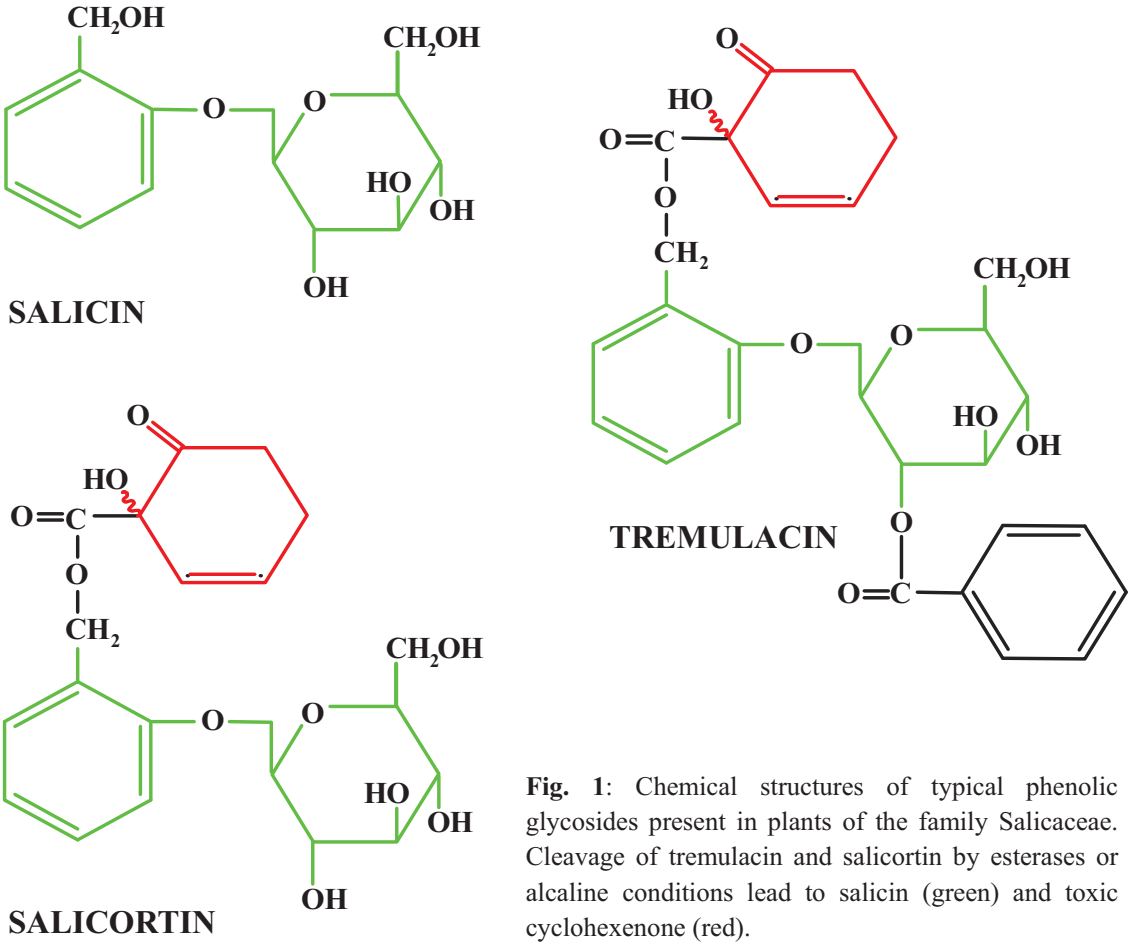

Fig. 1: Chemical structures of typical phenolic glycosides present in plants of the family Salicaceae. Cleavage of tremulacin and salicortin by esterases or alcaline conditions lead to salicin (green) and toxic cyclohexenone (red).

Additionally, salicortin itself has been shown to be a β-glucosidase inhibitor (Clausen et al. 1990, Zhu et al. 1998) and salicin possesses an inhibitory effect on human serine proteases (Jedinák et al. 2006). Moreover, salicortin and disalicortin are even systemically up-regulated by herbivory (Clausen et al. 1989, Ruuhola et al. 2001a). Altogether, salicaceous plants are efficiently protected against generalist herbivores by PGs. Thus, to benefit from those glycosides for larval defensive properties the ability to cope with the PGs is likely a prerequisite in Chrysomelina. Most European *Phratora* species and *Plagiodera versicolora* are protected by iridoids but feed on salicaceous plants and in some cases even accept moderate to high levels of PGs (Tahvanainen et al. 1985, Köpf et al. 1996, Köpf et al. 1998). Moreover, the adults of both iridoid and salicylaldehyde protected larvae feed on the same salicaceous hosts like their immature stages. In my view, this implies the evolution of an ability to cope with PG-containing

plants early in Chrysomelina radiation and most likely before and independent of salicin-based defense. To my knowledge up to now it is not known whether this ability is due to a tolerance to PGs to a certain degree or resistance mediated by detoxification mechanisms. However, as up to 70 leaf beetle species are reported to feed on salicaceous plants (Krüssmann 1962, Freude et al. 1966), most likely convergent adaptations to PGs evolved in different, distantly related chrysomelid taxa. A common origin in Chrysomelina is likely but needs to be proven. Nevertheless, salicin-based defense is strikingly economic as the PGs salicin and salicortin are both in parallel detoxified and utilized for the larval defense (Rowell-Rahier and Pasteels 1986). Salicin-based defense is an innovative strategy as it likely enabled the salicin sequestering species to feed on salicaceous plants with higher PG content than the iridoid producing species and, moreover, make the salicin-bound glucose moiety available (Pasteels et al. 1983, Pasteels et al. 1990). Interestingly, host plant PG and larval salicylaldehyde concentrations are positively correlated in salicin sequestering species (Pasteels et al. 1983, Termonia and Pasteels 1999) but above a certain amount of salicin the salicylaldehyde concentration is not significantly increased (Soetens et al. 1998). This implies that also larvae sequestering PGs for defensive properties need to cope with salicin and salicortin that are not required for their defense. Therefore, they may have retained an ancestral PG tolerance/resistance which is likely present in iridoid producing species feeding on salicaceous plants enabling them to feed on plants rich in PGs.

4.1.2.2 Phytogenic glycoside sequestration and glandular deglucosylation

Although I have shown an independent evolution of SAO enzymes of salicin sequestering species from the glandular oxidase in iridoid producers, for maintenance of chemical defense the prior glycoside transport and their glandular deglucosylation seem to be common to all Chrysomelina larvae, irrespective of the mode of their chemical defense (Pasteels et a. 1990, Kuhn et al. 2004, Discher et al. 2009). Based on the biochemical knowledge to date, both the transporter and glandular glucosidases show respective similarities in different species. However, as shown for SAO evolution, further investigations of transport proteins and glandular glucosidases on a molecular-genetic level are needed to enable formulation of hypotheses of their evolutionary

origins. Only a combination of biochemical and molecular-genetic data will clarify whether a single origin for each protein family (glycoside transporter, glandular glucosidases) in Chrysomelina and, in more detail, if a derivation from iridoid-protected ancestors during salicin-based defense evolution instead of repetitive new innovations is likely. At least for the glandular glucosidase activity I would, for two reasons, suppose a single origin in Chrysomelina larvae (β-glucosidases of the glycoside hydrolase family 1: unpublished data) instead of convergent recruitments. First, β-glucosidases of different insect orders have been shown to possess broad substrate acceptance (Chararas and Chipoulet 1982, Chararas et al. 1983, Pratviel-Sosa et al. 1987, Ferreira et al. 1998, Scharf et al. 2010). Second, *in vitro* assays with larval defensive secretions resulted in glucosidase cross-activities in different Chrysomelina species (Pasteels et al. 1990, Soetens et al. 1993). Salicin and 8-hydroxygeraniol-glucoside were comparably deglucosylated independent of adding the secretion of salicylaldehyde or iridoid producing species. Therefore, there seems to be no absolute requirement for the recruitment of different glucosidases for specific activities in the glandular secretion, but rather slight changes in substrate affinities would be sufficient to maintain glucosidase capacity, irrespective of the leaf beetles defensive strategies. However, although the enzymatic activity of a glucosidase has been shown to be responsible for deglucosylation in the glandular secretion of a variety of Chrysomelina species, I would not exclude the possibility of a spontaneous (but slow) acid hydrolysis of ingested glucosides in some species as the glandular secretions possess a pH of 4 to 5 (personal observation).

4.1.3 Fate of SAO gene and salicin-based defense

To illuminate the evolutionary dynamics of the leaf beetle SAO enzymes that are involved in host plant-dependent defense of those herbivores, not only their origin within the Chrysomelina but also the fate of *C. lapponica* SAO after host shift/expansion to birch trees has been investigated (manuscript 1). In conjunction to that the “new” host plant chemistry is important, because a changing phytochemical environment can affect the leaf beetle larval defense (Hilker and Schulz 1994, Termonia and Pasteels 1999, Termonia et al. 2002, manuscript 3) and therefore affect also the enzymes involved in deterrent compound biosynthesis (manuscript 1).

The step-wise scenario of SAO evolution that I have discussed and postulated in the previous two paragraphs includes gene duplication and the preservation of a SAO "precursor" gene as key events. Potential reasons for the subsequent loss of SAO gene functionality and enzyme activity will be addressed here. It has been shown that salicylaldehyde attracts specialist natural enemies (Pasteels and Gregoire 1984, Köpf et al. 1997, Zvereva and Rank 2003/2004, Gross et al. 2004) and therefore the lack of salicylaldehyde biosynthesis would be advantageous for larval survival. A dual defense, based on butyrate-ester biosynthesis (found in *interrupta*-species) in addition to salicylaldehyde production, may have allowed the lack of salicylaldehyde without complete loss of defensive capacity in larval glandular secretions (Termonia et al. 2001, Termonia et al. 2002). One could ask why salicylaldehyde is only absent in those *interrupta*-species feeding on birch instead of willow and why the loss of SAO activity did not evolve in willow-feeding species as well. Both questions cannot be resolved so far, but a possible explanation could be that the loss of SAO activity is somehow related to the host shift from willow to birch, maybe because salicylaldehyde formation is still essentially needed as a detoxification mechanism or energy source for willow-associated *interrupta*-species (assumed for other species in Rowell-Rahier and Pasteels 1986).

Beside the ecology driven selection pressures on the loss of salicylaldehyde biosynthetic capacity, simply a lack of selection pressure on its persistence due to ecological changes is also conceivable. As the shift from willow to salicin-free birch (manuscript 3) caused the ebbing of the phytogenic salicylaldehyde-source there is no need to retain SAO activity, which otherwise would be potential costs for maintenance of the SAO expression. On the other hand, small amounts of salicortin and tremulacin have been detected in birch (manuscript 3) which can be transformed to the salicylaldehyde precursor salicin/saligenin (**Fig. 1**) by degradation in the larval gut (Julkunen-Tiitto and Meier 1992, Ruuhola et al. 2003) and, moreover, the ability to sequester salicin is preserved in birch-feeding *C. lapponica* juveniles (manuscript 3, Kuhn 2005, Hilker and Schulz 1994). Additionally, P. Soetens (1993) has already shown salicortin as a salicylaldehyde precursor in *Phratora vitellinae*. Altogether, this argues for changes in gut enzyme specificities in birch-feeders since no salicylaldehyde or butyrate esters of saligenin were found in their secretion (manuscript 3) and/or support the hypothesis that salicylaldehyde formation is an essential detoxification mechanism/energy source for willow-associated *interrupta*-species but not for birch-associated species. Changes of

the gut enzymatic composition due to host plant shifts is supported by differential expression of glycosidases comparing birch- and willow-feeding populations of *C. lapponica* (unpublished data). In addition, although no significant differences in glucoside uptake were found comparing willow and birch-feeding larvae of *C. lapponica* (manuscript 3) I would not exclude, that changes in transport specificities beside alterations in gut enzyme composition account for the lack of saligenin-derivatives in the secretion of birch-feeding larvae.

In general, in my view the host shift to birch is the most convincing reason for loss of SAO activity. But the exciting question what favored or maybe forced the shift from willow to birch is still open. It is likely due to a complex engagement of abiotic and biotic factors (reviewed in Gross et al. 2004a/b) or, as pointed out by A. Termonia and coauthors (2001), just due to the fact that willow and birch very often co-existed during radiation and speciation of the *interrupta*-group.

4.2 Future Perspectives

In consideration of the fact that only a few transcriptome and no genome data for leaf beetles were available when I have started my thesis, simultaneous glandular protein analyses by MS/MS and the establishment of various genomic and glandular transcriptome data-sets is an appropriate approach to identify key enzymes involved in defensive compound biosynthesis of Chrysomelina larvae. The expression of candidate genes in a eukaryotic instead of a prokaryotic cell line for following characterizations is essential, as the proteins I investigated possess several post-translational modifications. However, to study comparatively the evolutionary dynamics in a multi-copy gene family, like the GMCs, whole transcriptome data-sets would improve the knowledge rapidly, as I have seen for example in case of screening the *P. cochleariae* transcriptome (manuscript 2).

Nevertheless, based on the results described herein I could imagine three specific future directions for research on evolutionary aspects of Chrysomelina host plant adaptations with special emphasis on their chemical defense-involved glandular oxidases.

First, to elucidate the origin of SAO enzymes and their activity in the Chrysomelina leaf beetles, the characterization of SAO-like proteins in iridoid producing species and related proteins in other beetles is essential. I already identified three SAO-like

transcripts in *P. cochleariae* and one SAO-like gene in *P. laticollis* (manuscript 2, unpublished data) and showed a close relationship of all SAOs and related proteins with *T. castaneum* GMC*i*5. Their spatial/temporal expression pattern, enzymatic activities and number of paraloga would provide knowledge to postulate the changes in substrate specificities and help to discriminate between sub- and neo-functionalization which most likely led to the catalytic activity of the SAO. Beside those, the reasons for SAO-like gene persistence in iridoid producers, although not involved in salicylaldehyde biosynthesis, could be addressed.

Second, to prove the hypothesis of an independent evolution of larval glandular oxidases in salicylaldehyde and iridoid producing species (manuscript 2), the elucidation and characterization of the oxidase involved in iridoid biosynthesis would be of great impact.

Third, to get more insights into host shift origin/dynamics from willow to birch in the *interrupta*-group, the SAO and related proteins could be helpful. Previous investigations indicate several independent shifts within the *interrupta*-group and also even within *C. lapponica* (Termonia et al. 2001, Mardulyn et al. submitted) in addition to population specific host adaptations (Zvereva et al. 2010). The fate of SAO gene and protein in birch-feeding species/populations compared to my results (manuscript 1), could uncover the number of host shift events from willow to birch in *C. lapponica* and moreover the entire *interrupta*-group.

Beside those specific directions, more general aspects of phenolic glycoside tolerance or detoxification in iridoid producing species should be addressed. So far very little is known about how those compounds cause feeding deterrence or toxicity in generalist herbivores and what mechanisms specialist herbivores utilize to enable them feeding on high amounts of PGs with impunity.

5 Summary

5.1 Background knowledge about Chrysomelina defense

5.1.1 Chemical defense of larvae differs in host dependency

As most leaf beetles (Chrysomelidae) spend their whole life exposed on the foliar surface, multifaceted defense mechanisms against predators are present in different taxa. In this context, the glandular chemical defense of Chrysomelina larvae is remarkable as it provides the possibility to investigate the intimate relationship between host plant adaptation and protection of specialist herbivores. The implementation of larval chemical defense is either, not, in part, or completely dependent on host derived glucosides and the degree of host dependency is strongly correlated with plasticity of host affiliation. Whereas the basal species, protected by autogenously synthesized iridoids, colonized seven plant families, the more derived salicylaldehyde producers *Chrysomela* spp. and *P. vitellinae* are adapted to sequester the phytogenic precursor salicin and therefore are restricted to feed on salicaceous hosts containing this secondary metabolite. However, the utilization of butyrate-esters of a mixed, beetle and host, origin may have enabled several species of the *interrupta*-group of the genus *Chrysomela* to broaden their host spectrum or shift from Salicaceae to Betulacea. Thus, Chrysomelina larvae and their glandular chemical defense is an excellent biological system to understand evolutionary dynamics of host plant adaptation in a clade of specialist herbivores. While a lot of work has been done on defensive compound identification and origin, and a limited amount of data is available on their precursor transport system, very little is known about the enzymes catalyzing the final steps of defensive compound formation on a molecular level. However, to understand the mechanisms of chemical defense evolution and the connected host plant adaptation in Chrysomelina, comparative molecular knowledge about these glandular enzymes is necessary.

5.1.2 Defensive gland enzymes and hypotheses on evolutionary origin of oxidases

Irrespective of the kind of defensive compound, after selective uptake of glucoside precursors into the glandular reservoir deglucosylation and further modification of the

aglucon to the bioactive principle falls into line. In case of iridoid and salicylaldehyde formation the deglucosylation is followed by a common oxidation step, which led to assume a single origin of the oxidases in those species and, moreover, would enable a shift from basal iridoid to derived salicylaldehyde biosynthesis just by slight changes in oxidase substrate specificity. If this holds true, and as the two genera are not closely related, salicylaldehyde formation common to *Chrysomela* spp. and *P. vitellinae* would imply a convergent recruitment of an ancestral oxidase of iridoid producers for salicyl alcohol oxidase (SAO) activity.

5.2 Major findings of the thesis

5.2.1 Elucidation of SAO and related sequences

SAO transcripts and genes have been identified in *C. lapponica* and *P. vitellinae*. The presence of the corresponding protein in the defensive secretion was verified by MS/MS analyses. SAO heterologous expression in a *Sf*9 insect cell line followed by *in vitro* enzyme assays revealed their SAO activity, *Re*-selectivity and a complex pattern of N-glycosylations. SAO glandular tissue-specific expression reflects the high amount of protein present in the glandular secretion. Screening of a *C. lapponica* genomic library and further gene amplification led to 4 SAO paraloga in a willow-feeding population and 3 in a birch-feeding population as well as 2 SAO paraloga in closely related *C. populi*. Additionally, SAO related sequences were found in the iridoid producing species *P. laticollis* and *P. cochleariae*. The common origin of all SAOs and related sequences in a specific GMC oxidoreductase subfamily was shown by phylogenetic analyses.

5.2.2 Establishment of a hypothesis on SAO evolution

Contrary to previous hypotheses on glandular oxidase evolution in Chrysomelina leaf beetles, I provide another step-wise scenario for SAO evolution based on molecular data. Therein, multiple gene duplications in the GMC*i* subfamily early in Chrysomelina speciation followed by lineage specific duplications took place. One of the generated copies has then been sub- or neo-functionalized to SAO activity. Thereby, a signal peptide for the secretory pathway and a high degree of N-glycosylations were most

likely already present as those are features typical for members of the insect GMC*i* subfamily. The identical expression pattern of SAO and SAO paralog1 in *C. lapponica* indicate that their ancestral gene already possessed glandular specific expression. Phylogenetic analyses revealed that SAO is of single origin in *Chrysomela* spp. and *P. vitellinae,* additionally supported by highly similar SAO gene architecture, expression patterns and post-translational modifications. Moreover, the presence of SAO-like genes but lack of their encoded proteins in the defensive secretion of iridoid producers indicate both an independent evolution of SAO from glandular oxidases in iridoid producers and the recruitment of SAO related proteins for functions other than in the defensive system of iridoid protected species. In summary, gene duplicates of the GMC*i* subfamily have undergone different paths of functionalization in iridoid and salicylaldehyde producing species during Chrysomelina evolution but SAO has most likely a single ancestral GMC*i* gene origin in both *Chrysomela* spp. and *P. vitellinae*.

5.2.3 Loss of SAO activity after host shift

Whereas specialization to feed on salicaceous plants may have favored salicin-based defense and stabilized SAO activity for millions of years in Chrysomelina species, the shift to Betulaceae within the genus *Chrysomela* negatively affected the fate of SAO. This altogether shows the impact of host plants on chemical defense and on enzymes involved in this defense system. Comparing isolated willow- and birch-adapted *C. lapponica* populations elucidated a loss of SAO activity caused by mutations, alternative splicing, massive transcript down-regulation, N-terminal protein truncation and most likely post-translational changes in the birch-feeder. Interestingly, the loss of SAO activity cannot be solely explained by the absence of salicin in birch. Salicortin and tremulacin were detected in birch, which could in principle also serve as salicyl alcohol source by degradation in the larval gut, and the ability to sequester salicin has been shown for the birch-feeder. Thus, the shift from willow to birch likely had little/no effects on the precursor transport system but may have affected - beside SAO - other enzymes as well.

5.3 Concluding remarks

In a nutshell, glandular chemical defense in Chrysomelina larvae is fascinating for chemical, biochemical, molecular-genetic, ecological and evolutionary aspects. But in my mind, and in order to understand favoring circumstances, mechanisms, constraints, dynamics and evolutionary histories of host plant adaptation in those specialized insects, the future challenge will be to look beyond their chemical defense. Thus, investigations of the Chrysomelina detoxification and digestion machinery should be included. Over and above that, research on functionalities of their larval exocrine secretion beside defensive properties may provide additional insights in selecting parameters that force or favor gain, retention and loss of chemical defensive strategies in Chrysomelina leaf beetles.

6 Zusammenfassung

6.1 Hintergrundwissen zur Verteidigung der Chrysomelina Larven

6.1.1 Die larvale chemische Abwehr unterscheidet sich hinsichtlich der Abhängigkeit von der Wirtspflanze

Aufgrund der meist exponierten Lebensweise an den Blättern ihrer Wirtspflanzen, finden sich in den unterschiedlichen Taxa der Chrysomelidae vielfältige Verteidigungsstrategien gegen Fraßfeinde. In diesem Zusammenhang stellt die glanduläre chemische Abwehr der Chrysomelina Larven eine Besonderheit dar, weil sie die Möglichkeit bietet, das enge Zusammenspiel von Verteidigungsmechanismen und Angepasstheit an die Wirtspflanze in einem phytophagen Spezialisten zu untersuchen. Die Implementierung dieser larvalen chemischen Abwehr ist entweder nicht, zum Teil, oder vollständig an die Bereitstellung phytogener Glukoside gebunden und die unterschiedliche Plastizität in Bezug auf Wirtspflanzenassoziation spiegelt den Grad der Abhängigkeit der Larven von ihren Futterpflanzen wieder. Während basale, durch autogen synthetisierte Iridoide geschützte Arten 7 Pflanzenfamilien im Laufe ihrer Evolution kolonisiert haben, sind abgeleitete, Salicylaldehyd-produzierende Spezies (*Chrysomela* spp. und *P. vitellinae*) an die Sequestrierung der phytogenen Vorstufe Salicin angepasst und daher auf salicinhaltige Wirtspflanzen der Salicaceae als Lebensraum beschränkt. Allerdings ermöglichte wahrscheinlich die Etablierung von Butyrat-Estern als Wehrsubstanzen, aus phytogenen und larvalen Vorstufen kombiniert, einigen Spezies der *interrupta*-Gruppe der Gattung *Chrysomela* ihr Wirtspflanzenspektrum um die Betulaceae zu erweitern beziehungsweise von Weiden auf Birken zu wechseln. Dies verdeutlicht, dass die Chrysomelina Larven mit ihrer glandulären chemischen Abwehr ein exzellentes biologisches System darstellen, um die evolutive Dynamik, die der Wirtspflanzenanpassung phytophager Spezialisten zu Grunde liegt, zu verstehen. Während Identität und Ursprung der Wehrsubstanzen weitgehend und zum Teil auch der Transport ihrer Vorstufen in den Larven bekannt sind, wurden die an der Biosynthese der Wehrsubstanzen beteiligten Enzyme auf molekular-biologischer Ebene kaum charakterisiert. Allerdings sind vergleichende molekulare Untersuchungen zu diesen Enzymen nötig, um die Mechanismen der Wirtspflanzenanpassung und die damit verbundene Evolution der chemischen Abwehr in Chrysomelina Larven nachvollziehen zu können.

6.1.2 Wehrdrüsenenzyme und Hypothesen zum evolutiven Ursprung der glandulären Oxidaseaktivität

Unabhängig von der Art der Wehrsubstanzklasse werden deren glukosidische Vorstufen selektiv in das Wehrdrüsenreservoir transportiert, anschließend dort deglukosyliert und das Aglukon an freigewordenen funktionellen Gruppen bis zur bioaktiven Substanz modifiziert. Die der Iridoid- und Salicylaldehyd-Synthese gemeine Oxidation des Aglukons, nach Abspaltung der Glukose, führte zur Annahme eines gemeinsamen evolutiven Ursprungs der Oxidase in beiden Produzenten. Darüber hinaus wurde ein evolutives Szenario diskutiert, in dem durch eine Änderung der Oxidase-Substratspezifität ein Übergang von basaler Iridoid- zu weiterentwickelter Salicylaldehyd-Synthese ermöglicht wurde. Wenn dieses Szenario die Realität korrekt abbildet, dann wäre zweimal unabhängig, in den nicht näher verwandten Taxa *Chrysomela* und *P. vitellinae*, eine ancestrale Oxidase von Iridoid-Produzenten zur SAO Aktivität rekrutiert worden.

6.2 Zentrale Ergebnisse der Dissertation

6.2.1 Aufdeckung der SAO und verwandter Gene

SAO Transkript und Gen wurden jeweils in *C. lapponica* und *P. vitellinae* identifiziert. Die korrespondierenden Proteine wurden mittels MS/MS im glandulären Sekret nachgewiesen. Die heterologe Expression der SAOs in einer *Sf*9 Insekten-Zelllinie und anschließender *in vitro* Inkubationsexperimente bestätigten deren katalytische Aktivität, *Re*-Selektivität und bioinformatisch vorhergesagte N-Glykosilierungen. Die Wehrdrüsen-spezifische Expression der SAO reflektiert die große Menge an SAO Protein im Wehrsekret der Larven. Das Screening einer genomischen Bibliothek und weitere Amplifikationen von *Chrysomela* Genen führte sowohl zu 4 SAO paraloga in einer Weide- und 3 in einer Birke-adaptierten Population von *C. lapponica* als auch zu 2 SAO paraloga in dem nah verwandten *C. populi*. Zusätzlich konnten SAO verwandte Sequenzen in den Iridoid-produzierenden Spezies *P. laticollis* und *P. cochleariae* nachgewiesen werden. Phylogenetische Analysen zeigen einen gemeinsamen evolutiven Ursprung aller SAOs und verwandter Sequenzen in einer spezifischen Unterfamilie der GMC Oxidoreduktasen (GMC*i*).

6.2.2 Aufstellung einer Hypothese zur Evolution der SAO

Im Gegensatz zu bestehenden Hypothesen zur Evolution der glandulären Oxidasen in den Blattkäferlarven des Subtribus Chrysomelina, schlage ich ein schrittweises Szenario zur SAO Evolution basierend auf molekularen Daten vor. Darin vollzogen sich nach multiplen Genduplikationen früh in der Chrysomelina Speziation weitere Linien-spezifische Duplikationsereignisse. Eine Genkopie wurde zur SAO Aktivität sub- oder neo-funktionalisiert. Währenddessen waren GMC*i* typische Merkmale wie ein N-terminales Signalpeptid und hochgradige N-Glykosylierung wahrscheinlich bereits vorhanden. Das identische Expressionsmuster von SAO und SAO paralog 1 in *C. lapponica* deutet auf eine bereits Wehrdrüsen-spezifische Expression des ancestralen Gens der SAO hin. Phylogenetische Analysen zeigen den gemeinsamen evolutiven Ursprung der SAO in *Chrysomela* spp. und *P. vitellinae*, was durch deren sehr ähnliche SAO-Genarchitektur, Expressionsmuster und post-translationale Modifikation zusätzlich unterstützt wird. Darüber hinaus lassen SAO-ähnliche Gene der Iridoid-Produzenten, deren kodierte Proteine allerdings nicht in deren Wehrsekret nachweisbar waren, eine unabhängige Evolution der SAO von glandulären Oxidasen der Iridoid-Produzenten vermuten. Das wiederrum impliziert eine Rekrutierung von SAO ähnlichen Genen in Iridoid-verteidigten Spezies zu anderen Aufgaben als zur Synthese der Wehrsubstanzen. Zusammenfassend lässt sich sagen, dass Genduplikate der GMC*i* Unterfamilie während der Evolution der Chrysomelina sehr wahrscheinlich unterschiedliche Wege der Funktionalisierung in Iridoid- und Salicylaldehyd-produzierenden Spezies durchlaufen haben. Allerdings besitzen alle SAOs der Gattung *Chrysomela* zusammen mit der *P. vitellinae* SAO ein gemeinsames ancestrales GMC*i* Gen.

6.2.3 Wirtswechsel in Verbindung mit dem Verlust der SAO Aktivität

Während die Spezialisierung auf Salicaceae als Nahrungsquelle die Salicin-basierte Verteidigung möglicherweise begünstigt und die SAO Aktivität für mehrere Millionen Jahre in Chrysomelina Spezies stabilisiert hat, hat der Wirtspflanzenwechsel zu den Betulaceae innerhalb der Gattung *Chrysomela* das Schicksal der SAO negativ beeinflusst. Das verdeutlicht den großen Einfluss der Wirtspflanze auf die chemische

Verteidigung der Chrysomelina Larven und darin involvierte Enzyme. Ein Vergleich isolierter Weide- und Birke-adaptierter *C. lapponica* Populationen deckte den Verlust der SAO Aktivität durch Mutationen, alternative Prozessierung des Primärtranskripts, massive Abnahme an Transkriptmenge, N-terminale Trunkierung des Proteins und wahrscheinlich auch deren post-translationale Veränderungen in der Birke-fressenden Population auf. Interessanterweise kann der Verlust der SAO Aktivität nicht allein durch das Fehlen von Salicin erklärt werden, da geringe Mengen Salicortin und Tremulacin in der Birke nachweisbar sind. Diese beiden phenolischen Glukoside sind nach Degradierung im larvalen Darm potentielle Vorstufen des Salicins und darüber hinaus besitzen die Birke-adaptierten Larven von *C. lapponica*, wie die Weide-adaptierten Populationen, die Fähigkeit Salicin zu sequestrieren. Daraus kann man schließen, dass der Wirtspflanzenwechsel von Salicaceae zu Betulaceae wahrscheinlich zwar wenige oder gar keine Effekte auf die Transportmechanismen wohl aber, neben der SAO, auch einen Einfluss auf andere larvale Enzyme gehabt hat.

6.3 Schlussbemerkung

Alles in allem ist die glanduläre chemische Abwehr der Chrysomelina Larven aufgrund chemischer, biochemischer, molekular-genetischer, ökologischer und evolutiver Aspekte faszinierend. Allerdings ist aus meiner Sicht und um die begünstigenden Umstände, Mechanismen, Bedingungen, Dynamiken und evolutionären Entstehungsgeschichten von Wirtspflanzenanpassung in diesen hoch-spezialisierten Herbivoren verstehen zu können, der Blick über deren chemische Abwehr hinaus eine zukünftige Herausforderung. Deshalb sollten Untersuchungen zu allgemeinen Entgiftungs- und Verdauungsprozessen der Chrysomelina mit eingeschlossen werden. Zusätzlich könnte die Erforschung der Funktionen der glandulären Sekrete, neben der Abwehrkomponente, Einblicke in selektive Parameter liefern, die Entstehung, Manifestierung und Verlust von chemischen Abwehrstrategien in den Chrysomelina erzwungen beziehungsweise begünstigt haben.

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9 Selbstständigkeitserklärung

Hiermit erkläre ich, entsprechend § 5 Absatz 3 der Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich Schiller Universität Jena, das mir die geltende Promotionsordnung bekannt ist. Die vorliegende Dissertation habe ich eigenständig und nur unter Verwendung angegebener Quellen und Hilfsmittel angefertigt, wobei von Dritten übernommene Textabschnitte entsprechend gekennzeichnet wurden. Alle Personen, die einen entscheidenden Beitrag zu den Manuskripten geleistet haben, sind in Kapitel 2 aufgeführt beziehungsweise in der Danksagung erwähnt. Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen noch haben Dritte geldwerte Leistungen für Arbeiten im Zusammenhang mit der vorliegenden Dissertation erhalten. Gemäß Anlage 5 zum § 8 Absatz 3 der Promotionsordnung wurde die Beschreibung des von mir geleisteten Eigenanteils von dem Betreuer der Dissertation, Prof. Dr. Wilhelm Boland, mit Unterschrift bestätigt und der Fakultät vorgelegt. Zu keinem früheren Zeitpunkt wurde diese Dissertation, eine in wesentlichen Teilen ähnliche Arbeit oder eine andere Abhandlung bei einer Hochschule als Dissertation eingereicht.

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10 Curriculum Vitae

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Publications

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Kirsch R (2010). Salicyl Alcohol Oxidase in *Chrysomela lapponica* : a key enzyme of chemical defense and its functional and evolutionary dynamics. 9. European Congress of Entomology, Budapest, HU