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

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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.

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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

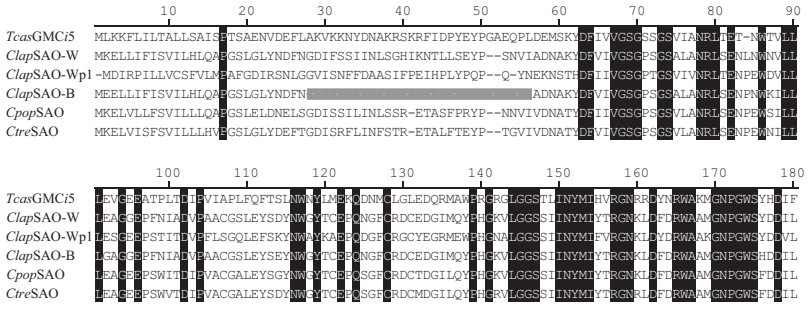

Fig. 1. Detail of an amino acid alignment of known SAOs of *C. populi* (*Cpop*SAO) and *C. tremulae* (*Ctre*SAO) with *Tribolium castaneum* GMC*i*5 (*Tcas*GMC*i*5), and the SAO of the willow (*Clap*SAO-W) and birch (*Clap*SAO-B) population as well as the SAO-W paralog1 (*Clap*SAO-Wp1) of *C. lapponica*.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

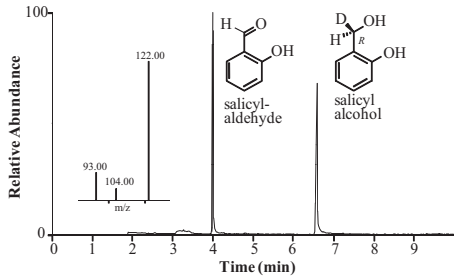

Fig. 2. GC-MS analysis of SAO-W activity assay with crude extract from Sf9 culture medium. The chromatogram shows the formation of salicylaldehyde by heterologously expressed SAO-W. The mass spectrum of the salicylaldehyde peak after an assay with deuterated salicyl alcohol in the *Re* position shows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

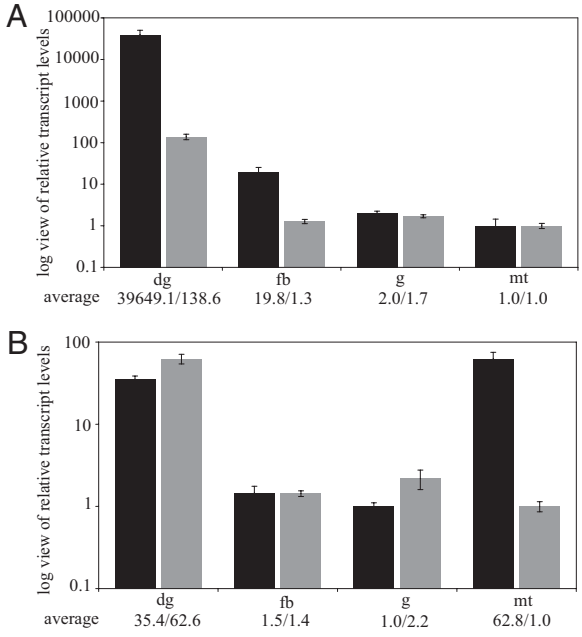

Fig. 3. Relative transcript abundance of SAO (black columns) and SAO paralog1 (gray columns) in different larval tissues. For normalization of transcript quantities, EF1α and eIF4A were used. (*A*) Willow-feeding population. (*B*) Birch-feeding population. Average transcript levels for SAO/SAO paralogs1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

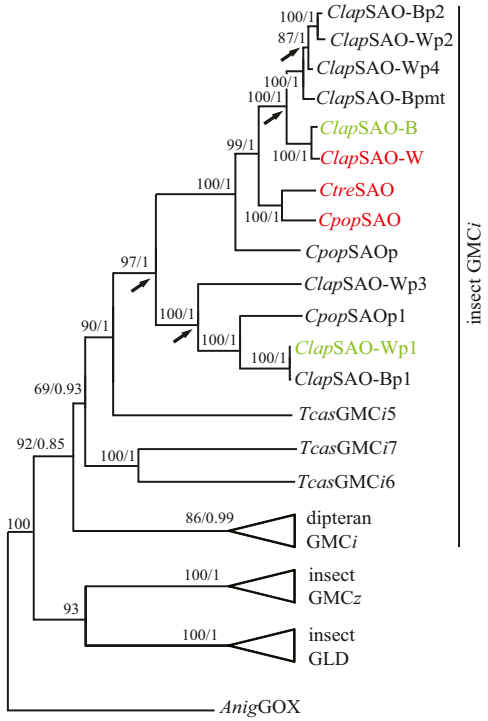

Fig. 4. Phylogeny of *Chrysomela* spp. SAO and related GMC oxidoreductases, including other insects. The phylogeny was generated using the neighbor-joining method with 1,000 bootstrap replicates. Bootstrap values are shown next to each node. The second numbers are posterior probability values based on 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

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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*i*5) 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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1. Bernays E, Graham M (1988) On the evolution of host specificity in phytophagous arthropods. *Ecology* 69:886–892.
2. Jaenike J (1990) Host specialization in phytophagous insects. *Annu Rev Ecol Syst* 21: 243–273.
3. Bernays EA, Chapman RF (1994) In *Host Plant Selection by Phytophagous Insects*, eds Miller TA, van Emden HS (Chapman & Hall, New York), pp 7–9.
4. Jolivet P, Hawkeswood TJ (1995) In *Host Plants of Chrysomelidae of the World*, ed Jolivet P (Backhuys, Leiden, The Netherlands), pp 1–281.
5. Schoonhoven LM, van Loon JJA, Dicke M (2005) *Insect–Plant Biology* (Oxford Univ Press, New York), pp 6–10.
6. Ehrlich PR, Raven PH (1964) Butterflies and plants: A study in coevolution. *Evolution* 18:586–608.
7. Feeny PP (1976) in *Biochemical Interactions Between Plants and Insects: Recent Advances in Phytochemistry*, eds Wallace JW, Mansell RL (Plenum, New York), Vol 10, pp 1–40.
8. Cates RG (1980) Feeding patterns of monophagous, oligophagous and polyphagous insect herbivores: The effect of resource abundance and plant chemistry. *Oecologia* 46: 22–31.
9. Pasteels JM, Rowell-Rahier M, Braekman JC, Dupont A (1983) Salicin from host plant as precursor of salicylaldehyde in defensive secretion of Chrysomeline larvae. *Physiol Entomol* 8:307–314.
10. Pasteels JM, Rowell-Rahier M, Braekman JC, Daloze D, Duffey S (1989) Evolution of exocrine chemical defense in leaf beetles (Coleoptera: Chrysomelidae). *Experientia* 45:295–300.
11. Pasteels JM, Termonia A, Daloze D, Windsor DM (2000) Distribution of toxins in chrysomeline leaf beetles: Possible taxonomic inferences. *Special Topics in Leaf Beetle Biology*, ed Furth DG (Pensoft Publishers, Sofia, Moscow), pp 261–275.
12. Veith M, Lorenz M, Boland W, Simon H, Dettner K (1994) Biosynthesis of iridoid monoterpenes in insects: Defensive secretion from larvae of leaf beetles (Coleoptera: Chrysomelidae). *Tetrahedron* 50:6859–6874.
13. Schulz S, Gross J, Hilker M (1997) Origin of the defensive secretion of the leaf beetle *Chrysomela lapponica*. *Tetrahedron* 53:9203–9212.
14. Feld BK, Pasteels JM, Boland W (2001) *Phaedon cochleariae* and *Gastrophysa viridula* (Coleoptera: Chrysomelidae) produce defensive iridoid monoterpenes de novo and are able to sequester glycosidically bound terpenoid precursors. *Chemoecology* 11:191–198.
15. Kuhn J, et al. (2004) Selective transport systems mediate sequestration of plant glucosides in leaf beetles: A molecular basis for adaptation and evolution. *Proc Natl Acad Sci USA* 101:13808–13813.
16. Pasteels JM, Duffey S, Rowell-Rahier M (1990) Toxins in chrysomelid beetles possible evolutionary sequence from de novo synthesis to derivation from food-plant chemicals. *J Chem Ecol* 16:211–222.
17. 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.
18. Brückmann M, Termonia A, Pasteels JM, Hartmann T (2002) Characterization of an extracellular salicyl alcohol oxidase from larval defensive secretions of *Chrysomela populi* and *Phratora vitellinae* (Chrysomelina). *Insect Biochem Mol Biol* 32:1517–1523.
19. Blum MS, Brand JM, Wallace JB, Fales HM (1972) Chemical characterization of the defensive secretion of a chrysomelid larva. *Life Sci* 11:525–531.
20. Smiley JT, Horn JM, Rank NE (1985) Ecological effects of salicin at three trophic levels: New problems from old adaptations. *Science* 229:649–651.
21. Hilker M, Schulz S (1994) Composition of larval secretion of *Chrysomela lapponica* (Coleoptera, Chrysomelidae) and its dependence on host plant. *J Chem Ecol* 20: 1075–1093.
22. Gross J, Podsiadlowski L, Hilker M (2002) Antimicrobial activity of exocrine glandular secretion of *Chrysomela* larvae. *J Chem Ecol* 28:317–331.
23. Termonia A, Pasteels JM, Windsor DM, Milinkovitch MC (2002) Dual chemical sequestration: A key mechanism in transitions among ecological specialization. *Proc R Soc Lond* B269:1–6.
24. Termonia A, Pasteels JM (1999) Larval chemical defense and evolution of host shifts in *Chrysomela* leaf beetles. *Chemoecology* 9:13–23.
25. Termonia A, Hsiao TH, Pasteels JM, Milinkovitch MC (2001) Feeding specialization and host-derived chemical defense in Chrysomeline leaf beetles did not lead to an evolutionary dead end. *Proc Natl Acad Sci USA* 98:3909–3914.
26. Brown WJ (1956) The new world species of *Chrysomela* L. (Coleoptera: Chrysomelidae). *Can Entomol* 88:1–54.
27. Gross J, Fatouros NE, Neuvonen S, Hilker M (2004) The importance of specialist natural enemies for *Chrysomela lapponica* in pioneering a new host plant. *Ecol Entomol* 29: 584–593.
28. Machkour-M'Rabet S, Mardulyn P, Pasteels JM (2008) Genetic differentiation among European samples of the arctic-alpine leaf beetle, *Chrysomela lapponica*. *Entomol Exp Appl* 129:181–188.
29. Knoll S, Rowell-Rahier M, Mardulyn P, Pasteels JM (1996) in Chrysomelidae *Biology: The Classification, Phylogeny and Genetics*, eds Jolivet P, Cox ML (SPB Academic, Amsterdam, The Netherlands), Vol 1, pp 379–388.
30. Zvereva EL, Kozlov MV, Hilker M (2010) Evolutionary variations on a theme: Host plant specialization in five geographical populations of the leaf beetle *Chrysomela lapponica*. *Popul Ecol* 52:389–396.
31. Cavener DR (1992) GMC oxidoreductases: A newly defined family of homologous proteins with diverse catalytic activities. *J Mol Biol* 223:811–814.
32. Zámocký M, Hallberg M, Ludwig R, Divne C, Haltrich D (2004) Ancestral gene fusion in cellobiose dehydrogenases reflects a specific evolution of GMC oxidoreductases in fungi. *Gene* 338:1–14.
33. Michalski C, Mohagheghi H, Nimtz M, Pasteels JM, Ober D (2008) Salicyl alcohol oxidase of the chemical defense secretion of two chrysomelid leaf beetles: Molecular and functional characterization of two new members of the glucose-methanol-choline oxidoreductase gene family. *J Biol Chem* 283:19219–19228.
34. Veith M, Dettner K, Boland W (1996) Stereochemistry of an alcohol oxidase from the defensive secretion of larvae of the leaf beetle *Phaedon armoraciae* (Coleoptera: Chrysomelidae). *Tetrahedron* 52:6601–6612.
35. Gross J, Hilker M (1994/1995) Chemoecological studies of the exocrine glandular larval secretions of two chrysomelid species (Coleoptera): *Phaedon cochleariae* and *Chrysomela lapponica*. *Chemoecology* 5/6:185–189.
36. Kuhn J, et al. (2007) Sequestration of plant-derived phenolglucosides by larvae of the leaf beetle *Chrysomela lapponica*: Thioglucosides as mechanistic probes. *J Chem Ecol* 33:5–24.
37. Discher S, et al. (2009) A versatile transport network for sequestering and excreting plant glycosides in leaf beetles provides an evolutionary flexible defense strategy. *ChemBioChem* 10:2223–2229.
38. Betrán E, Ashburner M (2000) Duplication, dicistronic transcription, and subsequent evolution of the *Alcohol dehydrogenase* and *Alcohol dehydrogenase-related* genes in *Drosophila*. *Mol Biol Evol* 17:1344–1352.
39. Köpf A, et al. (1998) The evolution of host-plant use and sequestration in the leaf beetle genus *Phratora* (Coleoptera: Chrysomelidae). *Evolution* 52:517–528.
40. Dobler S, Farrell BD (1999) Host use evolution in *Chrysochus* milkweed beetles: evidence from behaviour, population genetics and phylogeny. *Mol Ecol* 8:1297–1307.
41. Sakagami T, Saito Y, Kongchuensin M, Sahara K (2008) Molecular phylogeny of *Stigmaeopsis*, with special reference to speciation through host plant shift. *Systematics* 102:360–366.
42. Mikheev AV, Kreslavsky AG (1980) Interrelations of the willow and birch races of *Lochmaea capreae* L. (Coleoptera, Chrysomelidae) with food plants. *Zool Zh* 59:705–714.
43. Fatouros NE, Hilker M, Gross J (2006) Reproductive isolation between populations from northern and central Europe of the leaf beetle *Chrysomela lapponica* L. *Chemoecology* 16:241–251.
44. Gross J, Fatouros NE, Hilker M (2004) The significance of bottom-up effects for host plant specialization in *Chrysomela* leaf beetles. *Oikos* 105:368–376.
45. Iida K, Cox-Foster DL, Yang X, Ko W-Y, Cavener DR (2007) Expansion and evolution of insect GMC oxidoreductases. *BMC Evol Biol* 7:75, 10.1186/1471-2148-7-75.
46. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680.
47. Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425.