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Author for correspondence:
Antje Burse
e-mail: aburse@ice.mpg.de

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Independently recruited oxidases from the glucose-methanol-choline oxidoreductase family enabled chemical defences in leaf beetle larvae (subtribe Chrysomelina) to evolve

Peter Rahfeld[1], Roy Kirsch[2], Susann Kugel[1], Natalie Wielsch[3], Magdalena Stock[1], Marco Groth[4], Wilhelm Boland[1] and Antje Burse[1]

[1]Department of Bioorganic Chemistry, [2]Department of Entomology, and [3]Research Group Mass Spectrometry/Proteomics, Max Planck Institute for Chemical Ecology, Jena, Germany
[4]Genome Analysis Group, Leibniz Institute for Age Research, Fritz Lipmann Institute, Jena, Germany

Larvae of the leaf beetle subtribe Chrysomelina *sensu stricto* repel their enemies by displaying glandular secretions that contain defensive compounds. These repellents can be produced either de novo (iridoids) or by using plant-derived precursors (e.g. salicylaldehyde). The autonomous production of iridoids, as in *Phaedon cochleariae*, is the ancestral chrysomeline chemical defence and predates the evolution of salicylaldehyde-based defence. Both biosynthesis strategies include an oxidative step of an alcohol intermediate. In salicylaldehyde-producing species, this step is catalysed by salicyl alcohol oxidases (SAOs) of the glucose-methanol-choline (GMC) oxidoreductase superfamily, but the enzyme oxidizing the iridoid precursor is unknown. Here, we show by *in vitro* as well as *in vivo* experiments that *P. cochleariae* also uses an oxidase from the GMC superfamily for defensive purposes. However, our phylogenetic analysis of chrysomeline GMC oxidoreductases revealed that the oxidase of the iridoid pathway originated from a GMC clade different from that of the SAOs. Thus, the evolution of a host-independent chemical defence followed by a shift to a host-dependent chemical defence in chrysomeline beetles coincided with the utilization of genes from different GMC subfamilies. These findings illustrate the importance of the GMC multi-gene family for adaptive processes in plant–insect interactions.

1. Introduction

Beetles (Coleoptera) make up the largest order of animals with approximately 350 000 species and 40% of all insects [1]. In terms of the number of species, the family Chrysomelidae, commonly known as leaf beetles, is recognized as one of the most abundant of the coleopteran families. This success can be attributed to the long adaptive evolutionary history leaf beetles share with plants [2–4]. Several thousand species are external leaf chewers. Owing to the exposed life they lead on the surface of plants, leaf beetles are inviting targets for life-threatening predators and parasitoids. Therefore, the success of this lifestyle would have to have been based upon the development of effective defences against a variety of enemies. The use of toxins, evolved in Chrysomelidae and other insects, is one of the most potent antipredatory strategies. In the foliar feeding leaf beetles of the subtribe Chrysomelina *sensu stricto* [5,6], for example, these chemical defences ensure that all developmental stages, from egg to adult, are protected. When disturbed, the larvae display droplets of defensive secretions on their backs by everting the nine pairs of glandular reservoirs located under their dorsal cuticle [7]. The defensive droplets contain chemically diverse deterrents [8–13]. Phylogenetic analyses of Chrysomelina *sensu stricto* species revealed that the composition of their secretions reflects a

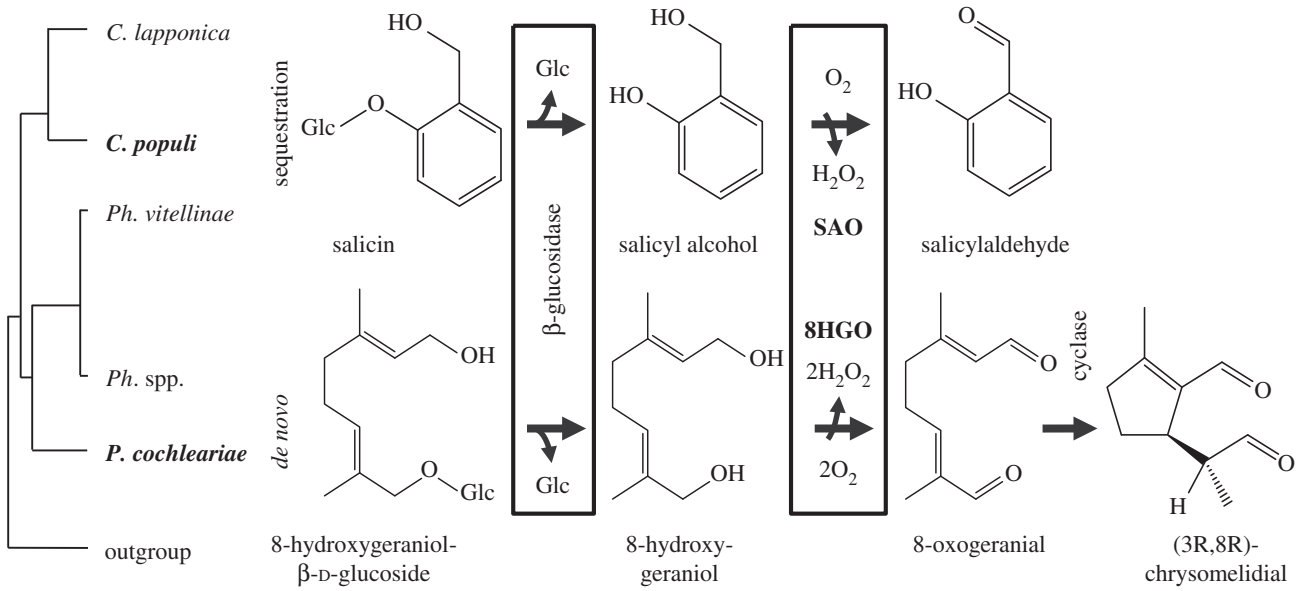

Figure 1. Steps of the deterrent biosynthesis in the larval gland reservoir of *Chrysomela populi* (sequestration) and *P. cochleariae* (de novo). The common enzymatic activities are highlighted with boxes. The occurrence of those biosynthetic pathways in various chrysomeline leaf beetle genera is plotted onto their phylogeny. SAO, salicyl alcohol oxidoreductase; 8HGO, 8-hydroxygeraniol oxidoreductase; Glc, glucose; *Ph.* spp., *Phratora* species. Adapted from [16].

stepwise scenario of host–plant adaptation [5]. The evolutionary history of the larval chemical defence started with the de novo production of deterrent iridoids (cyclopentanoid monoterpenoids) that does not rely on the secondary metabolites of their hosts [14,15]. Derived from this autonomous biosynthesis, two lineages independently developed a defensive strategy that relies on the sequestration of salicin (figure 1), a plant-derived precursor from Salicaceae used to produce the deterrent salicylaldehyde [5].

Despite the different composition and origin of the defensive compounds in the secretions of chrysomeline larvae (de novo versus sequestration), the synthesis of these defensive compounds depends on common enzymatic steps. For example, the hydrolysis of the glucosidically bound precursors that are transported into the glandular reservoir is facilitated by β-glucosidase activities [17,18] (figure 1). Subsequently, the released alcohol is oxidized in iridoid- and salicylaldehyde-producing species [19,20]. The common consecutive activity of β-glucosidase and oxidase suggested that it was the acquisition of only a few amino acid substitutions in the ancestral enzymes of the de novo iridoid-producing species that enabled sequestration-based salicylaldehyde biosynthesis to occur [7].

However, among the predicted enzymes in the Chrysomelina secretions, only the functionally characterized salicyl alcohol oxidases (SAOs) from salicin-sequestering species have been analysed with respect to their ancestry [16,20,21]. These SAOs belong to the glucose-methanol-choline (GMC) oxidoreductase multi-gene family [22,23] and convert salicyl alcohol into salicylaldehyde [20]. Comparative genome analyses show that the GMC oxidoreductase family harbours genes, most of which are in a cluster and unique with respect to their expansion in insects [24–26]. Phylogenetic analyses of the functional SAOs from salicin-sequestering Chrysomelina *sensu stricto* strongly support the SAOs' common ancestry in the GMCι clade [16] irrespective of the evolutionary affiliation of the corresponding beetle species [5].

However, so far we do not know whether the SAO of the salicylaldehyde-producing species and the predicted oxidase of the species producing iridoid defensive larval secretions share a common origin or arose independently. Early biochemical investigations of secretions in iridoid- (*Phaedon amoraciae*, *Phratora laticollis*) and salicylaldehyde- (*Phratora vitellinae*) producing species revealed side activities for the non-natural substrates salicyl alcohol and 8-hydroxygeraniol, respectively [27]. These results suggest a change in the specificity of the oxidase over the course of Chrysomelina *sensu stricto* evolution [5,20]. However, the recombinant SAO from the salicylaldehyde-producing *Ph. vitellinae* species lacks any 8-hydroxygeraniol oxidase (8HGO) activity, suggesting another enzyme is responsible for this cross-reactivity in the secretions [16]. Moreover, proteome analyses of secretions from iridoid-producing larvae did not reveal an oxidase related to the GMCι clade [16]. Hence, these recent results point to an acquisition of SAOs in salicylaldehyde-producing larvae that is independent of the 8-hydroxygeraniol-converting oxidase in the species that retained the ancestral biosynthetic pathway.

In this paper, we identified a glandular-specific oxidase from the iridoid-producing larvae of the mustard leaf beetle, *Phaedon cochleariae*. Functional characterization including the substrate specificity of this enzyme after heterologous expression revealed the selective oxidation of 8-hydroxygeraniol to 8-oxogeranial. The importance of this 8HGO activity for the formation of iridoid has been further verified by RNA interference (RNAi) *in vivo*. Phylogenetic analyses demonstrated that 8HGO originated from a beetle-specific GMC clade and not from the GMCι clade, from which SAOs arose. These findings elucidate a key event in the evolution of glandular chemical defence in chrysomeline beetles. Moreover, they provide insight into the adaptive mechanisms that enabled the transition from de novo biosynthesis to sequestration and, thus, into the underlying evolutionary dynamics of host–plant affiliation.

2. Material and methods

See the electronic supplementary material for the complete proteome analyses of the secretions by data-independent liquid chromatography/mass spectrometry detection (LC-MS^E),

cloning procedures, detailed quantitative PCR (qPCR) procedure, phylogenetic analysis, gas chromatography–mass spectrometry (GC–MS) analysis, all primer sequences (electronic supplementary material, table S1) and accession numbers (electronic supplementary material, table S2).

(a) Silencing of the *Phaedon cochleariae* 8-hydroxygeraniol oxidase (*Pc*8HGO) and the *Phaedon cochleariae* 8-hydroxygeraniol oxidase-like protein (*Pc*8HGO-like) by RNA interference

The coding sequences of *Pc8HGO* and *Pc8HGO-like* were analysed for off-target prediction according to Bodemann *et al.* [28]. This analysis revealed that *Pc8HGO* and *Pc8HGO-like* have a contiguous 26 bp fragment in common which is interrupted by only one dissimilar base at position 11 and which may be sufficient to trigger off-target effects (electronic supplementary material, figure S1). Furthermore, no putative off-target effects with other transcripts were predicted with the chosen dsRNA sequences for a critical value of at least 21 continuous nucleotides.

For the dsRNA-constructs, 200 bp fragments (electronic supplementary material, table S1) from the coding sequences of *Pc8HGO* and *Pc8HGO-like* were amplified by a Phusion high-fidelity DNA polymerase (Fisher Scientific—Germany GmbH, Schwerte, Germany). After purification with a PCR-purification kit (Roche, Basel, Switzerland), the resulting fragments were cloned into T7-promotor site free pIB/V5-HIS-TOPO vectors (Life Technologies, Carlsbad, CA, USA). For dsRNA synthesis, templates with opposite T7-promotor sites were amplified out of sequenced pIB-*200bpPc8HGO* as well as pIB-*200bpPc8HGO-like* and further processed as described in Bodemann *et al.* [28]. The concentration of dsRNA was adjusted to 1 $\mu g\ \mu l^{-1}$. Early second-instar larvae of *P. cochleariae* were used for injections. The dsRNA was delivered in the haemolymph through an injection in the thorax. They were collected 7 days after hatching and treated with 100 nl (100 ng) dsRNA of *200bpPc8HGO* or *200bpPc8HGO-like*. The dsRNA of *720bpeGFP* was used as described in Bodemann *et al.* [28] for control treatments.

(b) Heterologous expression of *Pc*8HGO in insect cells and protein purification

Heterologous expression was carried out in the insect cell line High Five (Life Technologies). The construct pIB-*Pc8HGO* was transfected with the FuGeneHD–Kit (Promega GmbH, Fitchburg, MA, USA) and MA Lipofection Enhancer (IBA GmbH, Göttingen, Germany) according to the manufacturer's instructions. After one day of incubation at 27°C, the culture was supplied with 80 $\mu g\ ml^{-1}$ blasticidin (Life Technologies) to initiate the selection of stable cell lines. The insect cells were selected over three passages. The cultivation of the stable cell lines for protein expression was carried out in six 75 cm^2 cell culture flasks with each 15 ml culture media (Express Five (Life Technologies), 20 $\mu g\ ml^{-1}$ blasticidin, 1× Protease Inhibitor HP Mix (SERVA Electrophoresis GmbH, Heidelberg, Germany)). After 3 days of growth, the supernatant was collected and the cells were discarded (4000 × *g*, 10 min, 4°C). The supernatant containing *Pc*8HGO was dialysed overnight at 4°C against 50 mM NaH_2PO_4, 10 mM imidazol (Pufferan), 5% (v/v) glycerol, pH 7.5.

The subsequent purification was done with HisPureCobalt (Life Technologies) according to the manufacturer's instructions with alterations to the elution buffer of 50 mM NaH_2PO_4, 150 mM imidazol (Pufferan), 5% (v/v) glycerol, pH 7.5. To confirm the identity of the purified protein, it was separated by any-kD gradient gels (Bio-Rad Laboratories, Munich, Germany) in one-dimensional-SDS-PAGE and then analysed *via* Nano-UPLC-MS^E as described in the electronic supplementary material.

(c) *Pc*8HGO activity assay

The purified proteins were dialysed overnight at 4°C against an assay buffer comprising 50 mM NaH_2PO_4, pH 4.5 to support the protein with the proper pH-value. To confirm the catalytic activity, 10 µl of purified protein, 10 µl 50 mM 8-hydroxygeraniol (end concentration 5 mM) or 10 µl 50 mM salicyl alcohol (end concentration 5 mM) and 80 µl assay buffer were incubated for 0, 30, 60 min at 30°C.

3. Results

(a) Identification and sequence analysis of glucose-methanol-choline oxidoreductases from the defensive secretions of *Phaedon cochleariae*

Eleven protein bands were recovered after the separation of *P. cochleariae* larval secretions by one-dimensional-SDS-PAGE (electronic supplementary material, figure S2). The resulting LC-MS^E data were searched against a *P. cochleariae* protein library derived from a *P. cochleariae* transcriptome [29]. The analysis of the band of about 70 kDa revealed two proteins (*Pc*8HGO and *Pc*8HGO-like) showing similarity to the GMC oxidoreductase family (GMC_oxred_N (PF00732)). Full-length amplification and sequencing of the corresponding transcripts led to coding sequences of 1672 bp (623 amino acids) and 1669 bp (622 amino acids) for *Pc*8HGO and *Pc*8HGO-like, respectively, with 77% sequence identity to each other on the amino acid level. Despite the sequence similarity, both proteins were unambiguously identified from the secretions as LC-MS^E-derived peptides matching *Pc*8HGO or *Pc*8HGO-like (electronic supplementary material, table S3). N-terminal signal peptides with a length of 16 amino acids (*Pc*8HGO) and 22 amino acids (*Pc*8HGO-like) were indicated by cleavage site predictions (SignalP 4.1: http://www.cbs.dtu.dk/services/SignalP/) [30].

Previous studies of larval secretions of salicin-sequestering chrysomeline species identified SAO proteins, oxidizing salicyl alcohol to the respective aldehyde, as members of the same GMC clade and of a similar molecular weight (69 kDa) [20]. But *Pc*8HGO and *Pc*8HGO-like show only a low degree of sequence identity, about 36% on the amino acid level, to these SAOs. Despite low sequence similarity, their protein alignment, including GMC oxidoreductases such as SAOs of the closely related Chrysomelina *Chrysomela populi* and *Chrysomela lapponica* as well as the aryl alcohol oxidase from the bacterium *Arthrobacter globiformis* and the glucose oxidase (GOX) from the fungus *Aspergillus niger*, illustrates, at least a few conserved regions (electronic supplementary material, figure S3). Like other GMC oxidoreductase proteins, *Pc*8HGO and *Pc*8HGO-like possess the N-terminal β-α-β dinucleotide-binding motif ($GxGxxG(x)_{18}E$) necessary to bind the flavin adenine dinucleotide cofactor [22,31,32]. Additionally, there are several blocks of conserved amino acid sequences common among GMC oxidoreductases (electronic supplementary material, figure S3) [24].

(b) Transcript localization of *Pc8HGO* and *Pc8HGO-like*

We compared the *Pc8HGO* and *Pc8HGO-like* expression levels in different larval tissues by qPCR. Both genes are

specifically expressed in the defensive glands with an at least approximately 130-fold (*Pc8HGO*) and approximately 240-fold (*Pc8HGO-like*) higher transcript abundance compared with gut, Malpighian tubules, fat body or head (electronic supplementary material, figure S4). The qPCR products were cloned and sequenced to confirm their identity. Their specific expression in the glandular tissue is in accordance with the identification of the respective proteins in the glandular secretions, revealing that both proteins possess gland-specific functions after being secreted into the corresponding reservoir.

(c) Functional importance of glandular glucose-methanol-choline oxidoreductases identified by RNA interference

RNAi was used to analyse the potential impact of *Pc*8HGO and *Pc*8HGO-like on the biosynthesis of the defensive iridoid chrysomelidial in the larval glandular secretions *in vivo*. The downregulation of the corresponding transcripts in the glandular tissue was surveyed by qPCR (electronic supplementary material, figure S5). Comparing the treatment control *eGFP* and the non-injected-control, we found no significant difference in the transcript abundance of *Pc8HGO* ($p = 0.86$) and *Pc8HGO-like* ($p = 0.74$). By contrast, 7 days after injecting the dsRNA of *Pc8HGO* or *Pc8HGO-like*, the downregulation by approximately 98% and approximately 96% of the respective transcripts compared with the *eGFP* treatment control was detected. In addition, although off-target prediction has been taken into account while designing the dsRNA fragments for RNAi [28], non-targeted transcripts were silenced. The downregulation of *Pc8HGO* led at the same time to a approximately 85% mRNA reduction of the non-targeted *Pc8HGO-like* and vice versa (electronic supplementary material, figure S5). This effect can be traced back to the high nucleotide sequence similarity of the targeted transcripts *Pc8HGO* and *Pc8HGO-like* of 83% that complicated the design of specific dsRNA-constructs (electronic supplementary material, figure S1). Nonetheless, in both cases, the downregulation of the targeted transcript was significantly more effective compared with non-targeted transcript (electronic supplementary material, table S4).

We collected glandular secretions for GC–MS analyses after silencing *Pc8HGO* or *Pc8HGO-like* to identify potential changes in the secretions' terpenoid composition. As observed in the *eGFP* larvae (electronic supplementary material, figure S6), chrysomelidial, the final product of the iridoid pathway, accumulated in the secretions when *Pc8HGO-like* was knocked-down (figure 2*a*). By contrast, in the secretions of the larvae injected with dsRNA targeting *Pc8HGO*, chrysomelidial was no longer detectable (figure 2*b*). Moreover, another substance accumulated in the secretions that could be identified as the chrysomelidial precursor 8-hydroxygeraniol [14]. Taken together, the RNAi experiments verified the importance of the *Pc*8HGO protein in the iridoid biosynthesis occurring in the glandular system of *P. cochleariae* larvae. The accumulation of 8-hydroxygeraniol indicates that this precursor is a substrate of the *Pc*8HGO enzyme, which, in turn, catalyses the oxidation to the chrysomelidial biosynthesis intermediate 8-oxogeranial (figure 1). *Pc*8HGO-like was rejected as a potential 8HGO as the glandular secretion of silenced larvae did not contain 8-hydroxygeraniol. The significance of *Pc*8HGO in the glandular context is additionally supported by a loss of the yellow

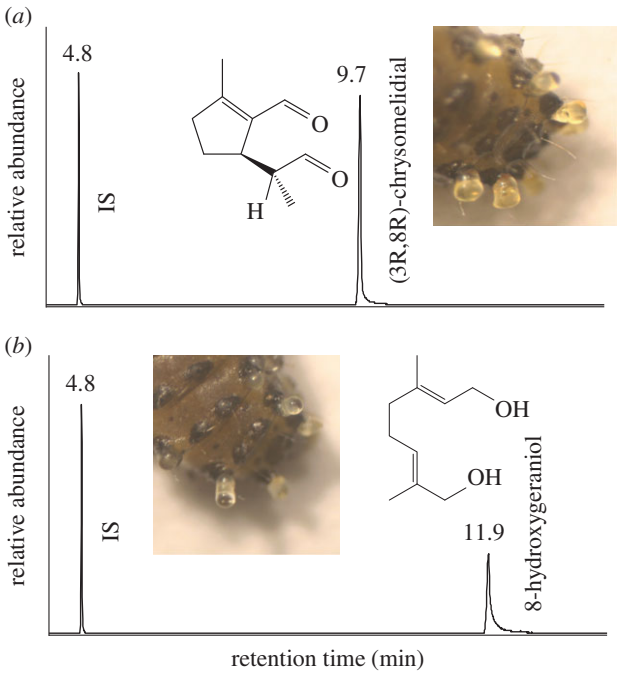

Figure 2. GC–MS analysis of larval secretions 7 days after treatment with dsRNA-*200bpPc8HGO-like* (a) and dsRNA-*200bpPc8HGO* (b). The picture shows the everted larval glands after the different treatments. Mass range (±1): 67 + 79 + 105.

colour of the secretions (figure 2*a*) that cannot be observed in any other treatment. The silencing seems to have no effect if the targeted transcript is reduced to more than 10%.

(d) Catalytic activity of the purified *Pc*8HGO

To validate the results obtained from the RNAi experiments and to test for the oxidative capacity, *Pc*8HGO was heterologously expressed. *Pc*8HGO was successfully purified with only a few impurities (electronic supplementary material, figure S7). The identity of the protein was certified through LC-MS^{E} analysis (electronic supplementary material, table S3).

The purified *Pc*8HGO was used for activity assays with 8-hydroxygeraniol as a substrate (figure 3). The reaction was stopped after 0, 30 and 60 min, and GC–MS analyses revealed that *Pc*8HGO is able to metabolize 8-hydroxygeraniol, as the corresponding peak (retention time 10.9 min) disappeared over time. Whereas in the beginning only the substrate was present, three new peaks were detectable after 30 min. Using a standard compound, one of the peaks with a retention time of 14 min could be identified as 8-oxogeranial. The other substances eluting at 12.1 and 12.7 min are most likely the semi-aldehydes 8-hydroxygeranial and 8-oxogeraniol as described in previous studies of the oxidative capacity in *P. cochleariae* secretions [19]. After 60 min, nearly all of the substrate and intermediate peaks were oxidized to 8-oxogeranial. These assays coincide with the phenotype observed after *Pc8HGO* was silenced, and the *Pc*8HGO enzyme was verified to be the oxidase in the glandular secretion of iridoid-producing *P. cochleariae* larvae converting 8-hydroxygeraniol to the respective aldehyde 8-oxogeranial.

In addition, the substrate specificity of *Pc*8HGO was tested by incubating the oxidase with salicyl alcohol, the substrate of chrysomeline SAOs. No enzyme-based conversion to salicylaldehyde could be detected (electronic supplementary material, figure S8), indicating this particular enzyme does not react with salicyl alcohol.

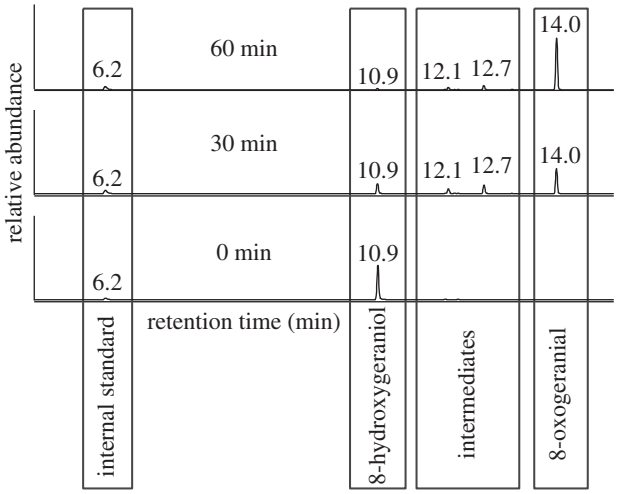

Figure 3. GC–MS analysis of activity assay with purified protein *Pc*8HGO from insect cell culture medium. The chromatogram shows the conversion of 8-hydroxygeraniol (10.9 min) to 8-oxogeranial (14.0 min) after 0, 30 and 60 min. Methyl benzoate (6.2 min) is the internal standard. Two intermediate substances, probably the semi-aldehydes (8-hydroxygeranial and 8-oxogeraniol), occur, with retention times of 12.1 and 12.7 min. Mass range (± 1): 67 + 79 + 105. The elution fraction of a similarly treated empty vector control was used as the control reaction.

(e) Evolution of glandular oxidases in Chrysomelina

To uncover the evolutionary origin of the 8HGO *Pc*8HGO from *P. cochleariae* and to test whether *Pc*8HGO and the SAO already known from *C. populi* share a common ancestral gene, among others, we combined GMC oxidoreductases from both species in a phylogenetic analysis. BLAST searches against *P. cochleariae* and *C. populi* transcriptome libraries (see the electronic supplementary material for results of the de novo assembly of *C. populi*'s transcriptome) revealed 10 and six full-length coding sequences, respectively, each showing high sequence similarity to the query sequences *Pc8HGO* and *Pc8HGO-like*. Phylogenetic analyses, including those sequences, chrysomeline SAOs and their related sequences known from previous work [16,21] as well as members of different insect GMC oxidoreductase subfamilies, showed that *Pc8*HGO and chrysomeline SAOs had independent origins (figure 4). As it has been shown earlier, SAOs and related sequences cluster in an insect GMCι clade closest to *Tribolium castaneum* GMCι5 [16,21]. By contrast, the *Pc8*HGO is affiliated with GMC oxidoreductases from *T. castaneum* (XM961538, XM961446, XM967481); according to a global insect GMC analysis [24], these cluster separately from their GMCι counterparts in the so-called beetle GMC clade. The origin of *Pc8HGO* within the beetle GMC clade is indicated by a close relationship to the *T. castaneum* GMC (XM967481) and is supported by high posterior probability and bootstrap values (1, 97, 92). The finding that *Pc8*HGO clusters with three other *P. cochleariae* GMCs (including *Pc*8HGO-like) but just with a single *C. populi* (*Cp*GMC*bl*6) and *T. castaneum* GMC (*Tcas*GMCXM967481) probably reflects gene duplications restricted to the chrysomeline iridoid-producing lineage. However, the high number of beetle GMC clade genes in *P. cochleariae* and *C. populi* in general and the presence of four strict orthologues among those indicates that the chrysomeline ancestor already possessed a diverse set of these genes. The same most likely holds true for the GMCι clade, as we found three genes of *P. cochleariae* (*Pc*SAO-like 1–3) clustering with *Chrysomela* spp. SAO counterparts and the single homologue of *T. castaneum* GMCι5. Concluding, our phylogenetic analysis supports the hypothesis that 8HGO and SAO arose from two clades of GMC oxidoreductases which started to diversify early in chrysomeline evolution.

4. Discussion

Oxidation–reduction reactions are the most prevalent and fundamental reactions in the metabolism of all organisms. Located in the defensive secretions of larvae from the subtribe Chrysomelina *sensu stricto*, these reactions are implicated in the production of deterrent compounds. Enzymes that catalyse such reactions often belong to the GMC oxidoreductase multi-gene family [16,20,21]. Here, we identified GMC oxidoreductases (*Pc*8HGO and *Pc*8HGO-like) in the secretions of the juvenile *P. cochleariae*. Based on our *in vitro* and *in vivo* experiments, we conclude that *Pc*8HGO is an indispensable enzyme for iridoid production; it converts 8-hydroxygeraniol to the corresponding dialdehyde in the secretions. By contrast, the function for *Pc*8HGO-like remains unclear, but its involvement in iridoid metabolism can be excluded (figure 2).

By identifying a GMC oxidoreductase involved in the defensive metabolism from a de novo iridoid-producing species, we gain access to phylogenetic analyses that allow us to untangle the ancestry of glandular oxidases in Chrysomelina *sensu stricto*. Although they are members of the same gene family, oxidases of the salicylaldehyde and iridoid biosynthetic pathways evolved—one from the GMCι and one from the beetle GMC clade, respectively—during chrysomeline evolution. The shift to a salicylaldehyde-based defence and also the shift to salicin-containing host plants have probably been made possible through the occurrence of a new glandular oxidase instead of the 'recycling' of an old one. The evolutionary steps towards 8HGO and SAO activity remain unknown as, for example, the functions of the respective *T. castaneum* counterparts have not been characterized. But the high copy number of GMCι and beetle GMCs in *P. cochleariae* and *Chrysomela* spp. indicates that gene duplication played a major role in the evolution of both 8HGO and SAO.

Despite comprehensive GMC gene analyses, phylogenetic relations of both clades have not yet been completely resolved [24,25]. But irrespective of whether GMCι and beetle GMCs cluster separately in two subfamilies [24] or have an intertwined evolutionary history [25], the corresponding *T. castaneum* GMCs are not close relatives. Thus, both analyses support our findings that chrysomeline SAO and 8HGO have independent origins, as do their closely related *T. castaneum* counterparts (*Tcas*GMCι5 and *Tcas*GMCXM967481 also do not cluster).

How widespread is the recruitment of GMC oxidoreductases in other insects for iridoid biosynthesis? This remains to be elucidated. The ability to biosynthesize iridoids seems to have evolved independently in different insect families and even in orders which frequently use these compounds as chemical stimuli for communication or defence [33,34]. Other beetles known to produce iridoids are the *Chloridolum loochooanum* (long-horn beetle) [35] and the carnivorous feeding *Philonthus* spp. (rove beetles) [36]. One of the first insects discovered to contain iridoids was the eponymous ant *Iridomyrmex* spp. [37]. The phasmid *Graeffea crouani* (coconut stick insect) and the pseudophasmid *Anisomorpha buprestoides*

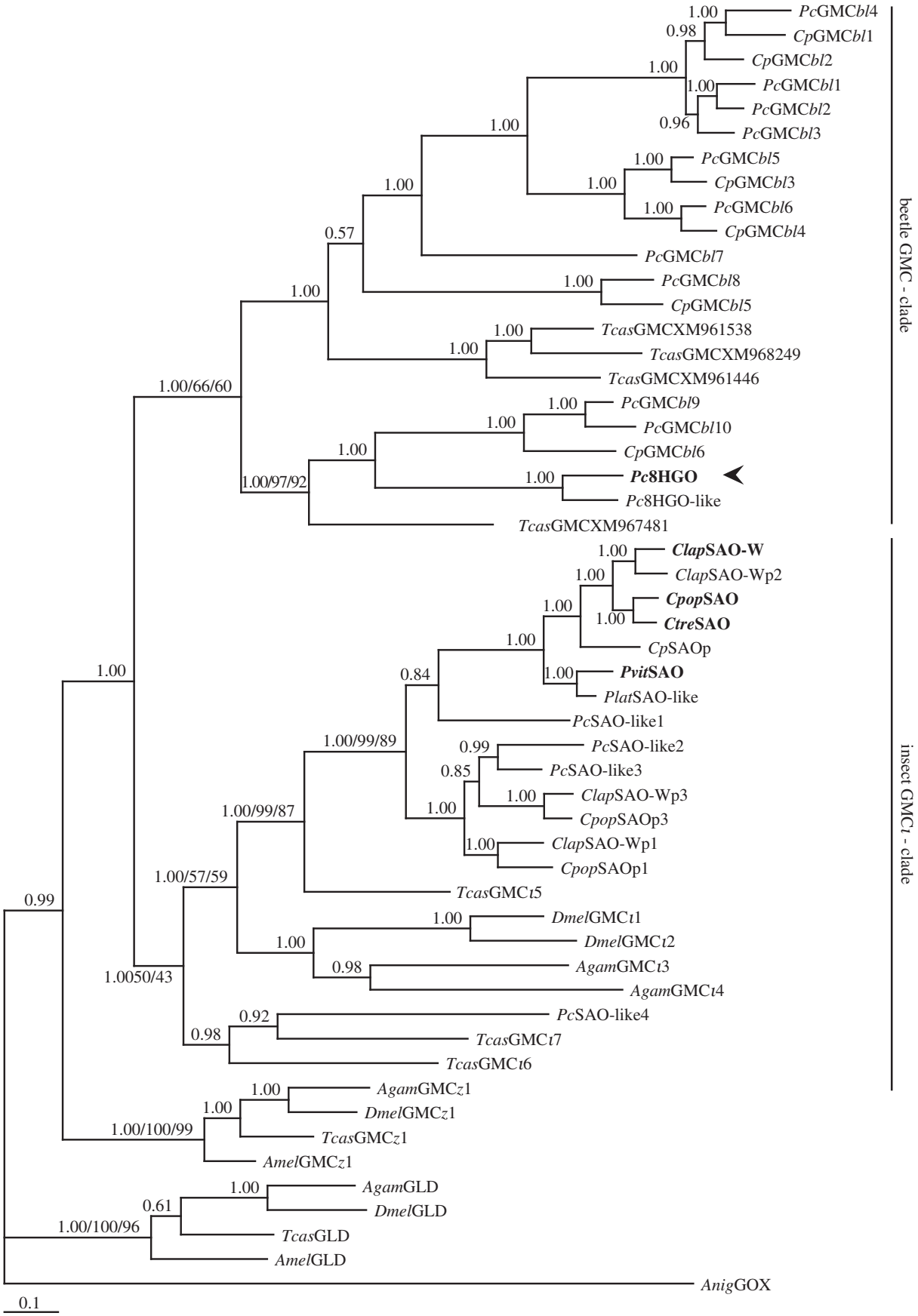

Figure 4. Phylogeny of Chrysomelina *sensu stricto* glandular oxidases and related GMC oxidoreductases including protein sequences of other insects. The phylogenetic tree was generated using a Bayesian inference method. Posterior probability values are shown next to each node. The second and third numbers, exemplarily indicated, represent bootstrap values based on a neighbour-joining algorithm and maximum-likelihood estimation, respectively, using the same set of data. *Cp* and *Cpop* (*C. populi*), *Ctre* (*C. tremulae*), *Clap* (*C. lapponica*), *Plat* (*Phratora laticollis*), *Pvit* (*Ph. vitellinae*), *Pc* (*P. cochleariae*), *Tcas* (*Tribolium castaneum*), *Agam* (*Anopheles gambiae*), *Dmel* (*Drosophila melanogaster*), *Anig* (*Aspergillus niger*), SAO-W (salicyl alcohol oxidase of willow-feeder), p (paralogous), GMC (glucose-methanol-choline oxidoreductase), *bl*1 to *bl*10 (beetle-like), GLD (glucose dehydrogenase), GOX (glucose oxidase), 8HGO (8-hydroxygeraniol oxidase) and 8HGO-like (8-hydroxygeraniol oxidase-like).

(southern walking-stick) were also found to use de novo-produced iridodials and nepetalactones [38,39]. More sequences need to be available, however, before the ancestry of oxidases implicated in insect iridoid biosynthesis can be untangled.

Compared with insects, the variety of iridoids is much higher in the plant kingdom [40–42]. One example is *Catharanthus roseus*. Here, the iridoids are precursors for secologanin, which is then processed into clinically important alkaloids such as vinblastine or vincristine [43]. Interestingly, plants use a completely different enzyme family to oxidise 8-hydroxygeraniol. In plants, a P450 enzyme (CYP76B6) [44] works as a multifunctional geraniol-8-oxidase oxidizing the geraniol first to 8-hydroxygeraniol and, subsequently, to 8-oxogeranial. The identification of a new protein family able also to produce intermediates of the iridoid biosynthesis opens the possibility of using *Pc*8HGO as an additional tool for plant engineering [45].

When discussing the development of sequestration from iridoid de novo synthesis in the Chrysomelina *sensu stricto*, the species *Ph. vitellinae* is of particular interest. *Phratora vitellinae* is a salicin-sequestering species which is evolutionarily isolated within the iridoid producers without having a close relationship to the salicylaldehyde-producing genus *Chrysomela* (figure 1). Substrate tests with secretions revealed the oxidation of both salicyl alcohol and the precursor for iridoids, 8-hydroxygeraniol [20,27]. However, the activity of the recently identified *Ph. vitellinae* SAO is restricted only to salicyl alcohol, which suggests that an enzyme other than the SAO is responsible for 8-hydroxygeraniol oxidation in larval exudates. Further, proteome analyses raised the idea of a putative oxidase that is not closely related to the SAO [21]. Based on our results of the 8HGO from iridoid-producing *P. cochleariae*, it seems reasonable to assume that this putative oxidase from *Ph. vitellinae* might also have originated from the beetle-specific GMC clade and might have been preserved in the larval secretions as a potential evolutionary relict of their host plant/deterrent shift.

Owing to its nine serial glands in the larval stage, the species *Gastrolina depressa* is also considered a member of Chrysomelina *sensu stricto* [6]. Its exact taxonomic relationship, however, has not yet been solved. *Gastrolina depressa* feeds on plants of the family Juglandaceae and the larvae produce juglone, which has been shown to be a highly effective ant repellent [46]. Pasteels *et al.* [7] suggested that glucosylated 1,5-dihydroxynaphthalene is sequestered and accumulated in the defensive exudates and, after hydrolysis to trihydroxynaphthalene, is converted into juglone by a predicted oxidase. Further study of this oxidase as well as of additional oxidases involved in the production of glandular deterrents by leaf beetles outside Chrysomelina *sensu stricto* will provide insight into the recruitment mechanisms of glandular oxidases possibly from the GMC gene pool and, thus, into the importance of the GMC multi-gene family for interactions in trophic networks.

In addition to the GMC cluster conserved in known insect genomes, which has been discussed to have a function in the ecdysone metabolism [24,47], some GMC genes exist outside of this cluster and have frequently experienced large lineage-specific expansion [24,25]. It has been suggested that these expansions of gene families may be correlated with the adaptation to different environmental issues or specific life strategies [48]. Because insects have evolved to occupy a vast diversity of habitats on the Earth, it can be hypothesized that these GMC genes have expanded further in insects in order to adapt to different environmental conditions. Besides the development of powerful antipredatory strategies, the adaptation of the immune response in insects is also a very important fitness factor. In silkworms, the knockdown of several GMC oxidoreductases, for example, reduced survival rates after treatment with *Bacillus bombyseptieus* or *Escherichia coli* [25]. This effect can be explained by the by-products arising during the oxidation reaction: GMC oxidoreductases produce H_2O_2 [49], and that H_2O_2 acts as a messenger or toxin in the immune response to microbial infections has been well described [50,51]. Interestingly, the secretions of chrysomeline larvae also have an antimicrobial effect. However, this effect is not owing entirely to the defensive compounds [52,53] but may be related to the action of the other extracellular GMC oxidoreductase, *Pc*8HGO-like. Although silencing *Pc8HGO-like* did not affect the phenotype with respect to the composition of deterrents, it may be that *Pc*8HGO-like is involved in the antimicrobial impact of the secretions.

By elucidating the catalytic activity of *Pc*8HGO, we provide the GMC oxidoreductase family with an additional functionally classified member in insects. This is the first characterized enzyme identified in insects which is involved in the late steps of iridoid production. It seems that the substrate diversity in redox reactions supplied by this multi-gene family equips insects with a toolbox that allows them to adjust to the particular biotic and abiotic conditions that may result, for example, when host plants shift. We believe that the characterization of additional GMC oxidoreductases will help clarify the role of these enzymes in the adaptation of insects to their environment.

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P.R., R.K. and A.B. designed the study. P.R. performed the identification of *Pc8HGO*, *Pc8HGO-like*, the RNAi experiment, the heterologous expression, the resulting protein assays and the interpretation of all resulting data. R.K. extracted and manually annotated GMC-encoding sequences, performed the phylogeny of larval chrysomeline glandular oxidases and related GMC oxidoreductases and made the interpretation. S.K. performed qPCR and contributed to the interpretation of output data. N.W. performed LC-MS^E analysis, collected and contributed to the interpretation of output data. M.G. and M.S. generated transcriptome libraries, applied OTP. W.B. and A.B. contributed substantially to the interpretation of all output data. P.R., R.K. and A.B. wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

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