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]).

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
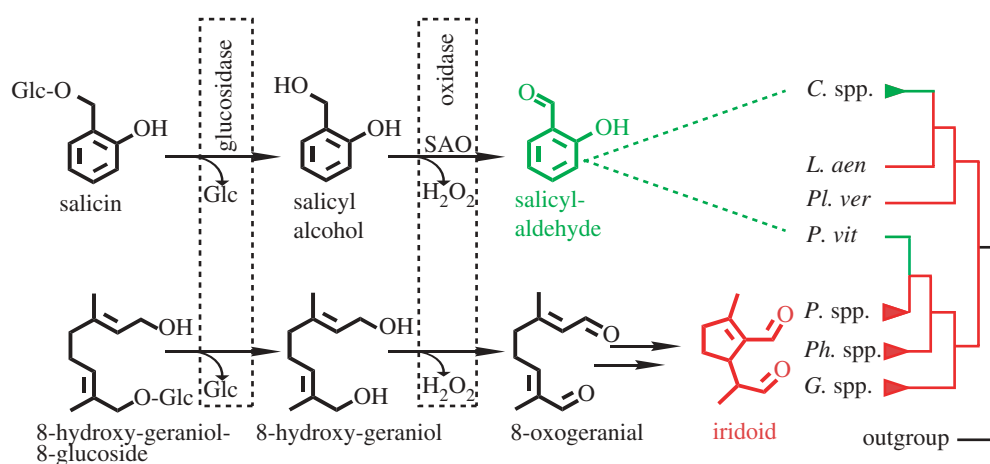
Figure 1. Schematic of salicylaldehyde and iridoid biosynthesis in glandular reservoirs highlighting common enzymatic activities with boxes. The occurrence of those defensive compounds in various chrysomeline leaf beetle genera is plotted onto their phylogeny. Therein iridoid-based chemical defence (red) 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 salicyl-aldehyde-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, 10μm 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 0.5μl 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 cysteines, 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 SuperSignal 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 350μM to 3 mM in a total volume of 100μl. Assays were stopped by freezing in liquid nitrogen. After extraction with 100μl 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 $\times 0.25$ mm, film thickness 0.25μm). 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 $1R$-[1-^{2}H$_1$]-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
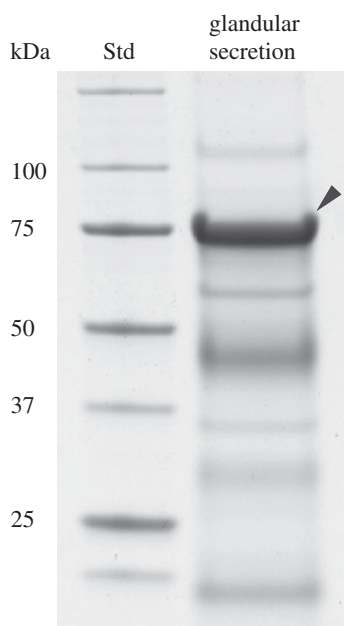
Figure 2. Coomassie Brilliant Blue-stained one-dimensional SDS protein gel of the larval glandular secretion of *P. vitellinae*. The most prominent band marked with an arrow corresponds to SAO. The size of the protein standard on the left is indicated in 10^3 Da.

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
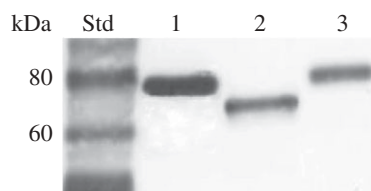
Figure 3. Western blot of heterologously expressed SAOs in Sf9 insect cells. Lanes 1 and 2 correspond to the *P. vitellinae* SAO. Size differences are due to a PNGase F treatment of probe 2 (lane 2), suggesting post-translational N-glycosylations. *Chrysomela populi* SAO is blotted (lane 3), demonstrating a similar size of the SAO in both genera after expression.
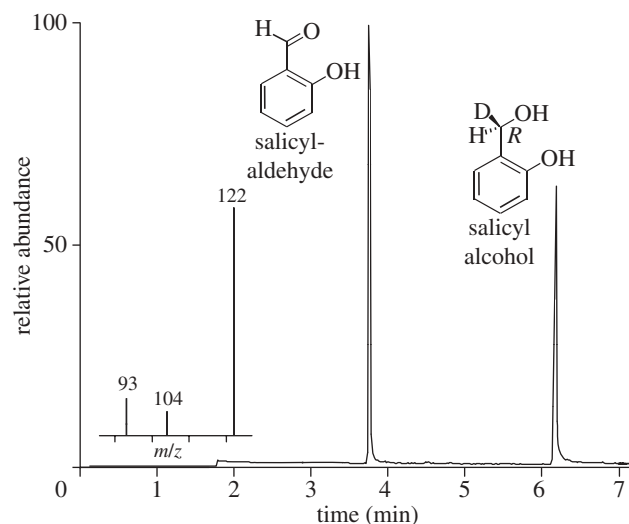
Figure 4. GC chromatogram of heterologously expressed *P. vitellinae* SAO activity assay after extraction. The formation of salicylaldehyde via a stereoselective deuterium removal of salicyl alcohol is indicated by the mass spectrum of the aldehyde.

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.

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 $1R$-[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
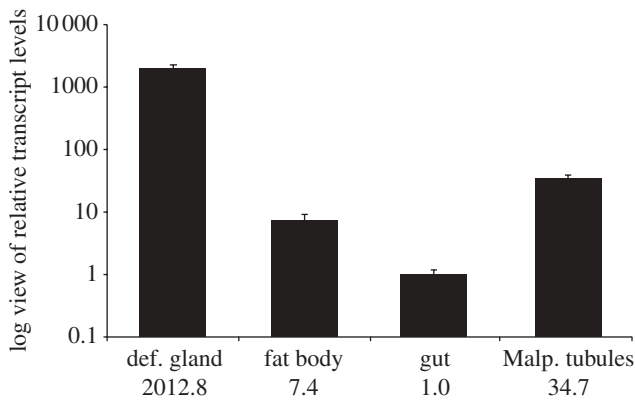
Figure 5. Expression pattern of *P. vitellinae* SAO. Relative transcript levels in different larval tissues display glandular tissue specificity of the SAO with an approximately 2000-fold higher transcript abundance compared with fat body, gut and Malpighian tubules. Transcript levels are indicated logarithmic 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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