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

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Salicyl alcohol oxidase is an extracellular enzyme that occurs in glandular reservoirs of chrysomelid leaf beetle larvae and catalyzes the formation of salicylaldehyde, a volatile deterrent used by the larvae against predators. Salicyl alcohol is the hydrolysis product of salicin, a plant-derived precursor taken up by the beetle larvae from the leaves of willow and poplar trees. The cDNA encoding salicyl alcohol oxidase from two related species Chrysomela tremulae and Chrysomela populi has been identified, cloned, and expressed in an active form in Escherichia coli. The open reading frame of 623 amino acids begins in both enzymes with an N-terminal signal peptide of 21 amino acids. Sequence comparison has revealed that salicyl alcohol oxidase belongs to the family of glucose-methanol-choline oxidoreductase-like sequences with mostly unknown function. Enzymes of this family share similar overall structure with an essentially identical FAD-binding site but possess different catalytic activities. The data suggest that salicyl alcohol oxidase, essential for the activation of the plant-derived precursor salicin, was originally recruited from an oxidase involved in the autogenous biosynthesis of iridoid monoterpenes and found in related chrysomelid leaf beetle species.

Chrysomelid leaf beetles encompass a taxon of about 40,000 mainly phytophagous species (1). Many of them possess conspicuous warning coloration, signaling unpalatability, since they release defensive chemicals from specialized glands upon attack (2). Defensive exocrine glands are found in both larval and adult leaf beetles, but these two sets of glands are not homologous (3). Larvae of species belonging to the subtribe Chrysomelina possess nine pairs of glands on the meso- and metathorax and on the first seven abdominal segments, producing mainly volatiles as irritants and repellents against predators (4). Furthermore, recent studies showed that the volatile salicylaldehyde disinfects the microenvironment of leaf beetle larvae, preventing the germination of conidia of entomopathogenic fungi on the cuticle of the host plant’s leaves (5, 6). Each gland consists of several gland cells that are attached to a glandular reservoir whose content is everted when the larvae is disturbed. The defensive secretions of larvae belonging to the subtribe Chrysomelina have been analyzed, and three strategies have been identified that reflect different degrees of specialization during evolution: first, the de novo production of iridoid monoterpenes from terpenoid precursors, providing an autogenous defense that is completely host plant-independent (7, 8); second, the biosynthesis of the aromatic compound salicylaldehyde from salicin, a phenolic glucoside taken up from the larval host plant, in a strategy that is fully dependent upon the chemistry of the host plant (9); third, the biosynthesis of esters composed of de novo-synthesized butyric acids with the alcohol moiety of leaf alcohol glucosides retrieved from the plant, representing a strategy partially dependent on the chemistry of the host plant (10).

Phylogenetic analyses have shown that the de novo synthesis of iridoid monoterpenes is the most ancestral type of chemical defense, of which the host-derived salicylaldehyde strategy has evolved at least twice during the evolution of the Chrysomeline leaf beetles. This strategy has itself been replaced in some of the more recent lineages by mixed metabolism involving the synthesis of butyric acids (11).

Larvae of Chrysomela tremulae and Chrysomela populi feed on poplar and willow leaves and produce salicylaldehyde, which has been shown to be effective against several generalist predators (9, 12). Salicylaldehyde is accumulated in large amounts in the glandular reservoir, forming an organic phase that accounts for ~15% of the total liquid volume of the secretion (13). Since the biosynthesis of salicylaldehyde requires only the hydrolysis of the plant-derived salicin (salicyl alcohol glucoside) and the oxidation of the resulting salicyl alcohol to salicylaldehyde (Fig. 1), the lower costs of this biosynthesis of defense compounds may be one of the advantages of this strategy over the autogenous biosynthesis of iridoid monoterpenes (11). Recently, sali-
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![Diagram](https://via.placeholder.com/150)

**FIGURE 1.** Final steps in the biosynthesis of deterrent iridoid monoterpenes and of salicylaldehyde in the larval glandular reservoirs of certain chrysomelid leaf beetles. Extracellular enzyme activities required for the pathways are boxed. This figure was taken and modified from Ref. 4.

Cycl alcohol oxidase (SAO)\(^3\) has been purified and characterized electrochemically from larval defense secretions of *C. populi* and *Phratora vitellinae* (13). SAO shows high substrate specificity for salicyl alcohol and has been established to use molecular oxygen as the electron acceptor, thereby yielding hydrogen peroxide. Therefore, SAO has been classified as an aryl-alcohol: oxygen oxidoreductase (EC 1.1.3.7) (14).

Pasteels et al. (4) have postulated that the enzymes involved in the biosynthesis of salicylaldehyde might have evolved from enzymes catalyzing the final steps in the production of iridoid monoterpenes. As a precursor of iridoid monoterpenes, 8-hydroxygeraniol-8-O-β-D-glucoside is synthesized in the glands from geraniol via 8-hydroxygeraniol (7, 15, 16) before it is secreted into the glandular reservoir. In the reservoir, the glycoside is cleaved by an extracellular β-glucosidase, and the aglycon is subsequently oxidized, yielding the dialdehyde 8-oxostructural. Oxidation is catalyzed by an extracellular dioxygen-dependent oxidase shown to remove stereospecifically the prop-(R)-hydrogen atoms from each of the terminal carbon atoms (17). Finally, the 8-oxostructural is cyclized and transformed to the species-specific iridoids (7, 18) (Fig. 1).

Our aim is to understand the evolution of enzymes that have evolved during the adaptation of insects to certain host-plant species and that enable the insects to use plant-derived compounds directly or as precursors for their own defense. Recently, we have been able to show that the senecionine N-oxygenase from larvae of the lepidopteran *Tyria jacobaeae* is a flavin-dependent monoxygenase that has evolved from members of this enzyme family during the adaptation to pyrrolizidine alkaloid-containing plants (19). Senecionine N-oxygenase allows these insects not only to feed on plants producing toxic pyrrolizidine alkaloids but also to sequester these plant toxins for their own defense (20). In a similar context, we show here that the extracellular SAO of two related chrysomelid leaf beetles belongs to the glucose-methanol-choline (GMC) family of oxidoreductases (21). After heterologous expression of the cDNA encoding the SAO of *C. tremulae* and *C. populi* Escherichia coli, the recombinant protein shows all of the main characteristics of the native enzyme previously characterized from *C. populi* (13). The function of SAO as an enzyme involved in the chemical defense of leaf beetle larvae against predators by the modification of plant-derived compounds to effective defensive chemicals adds further facets to the versatile GMC oxidoreductase gene family in insects, of which most members await their functional characterization.

**EXPERIMENTAL PROCEDURES**

**Collection and Rearing of Beetle Larvae**—Larvae and adults of *C. populi* were collected in Belgium and in the vicinity of Kiel, Germany, and those of *C. tremulae* were collected in France. Insects were reared in the laboratory on leaves of *Populus nigra* and *Populus balsamifera*. Ammonium sulfate-precipitated defense secretions of *Phratora vitellinae* and *Chrysomela knabi* were provided by Jacques Pasteels (Free University of Brussels, Belgium).

**Purification and Microsequencing of SAO**—SAO of *C. populi* and *C. tremulae* were purified from defense secretions by electrophoretic methods as described (13). Protein spots were cut from SDS-polycrylamide gels, washed three times with water before being digested with trypsin. The resulting peptides were subjected to electrospray ionization tandem mass spectrometry (ESI-MS/MS) as described by Zorn et al. (22). For microsequencing of the N terminus, the protein was blotted onto a polyvinylidene difluoride membrane and analyzed by automated Edman degradation in an ABI Protein Sequencer 494. The same method was used for some of the internal peptides of SAO of *C. populi*; these were digested with endoproteinase LysC before being separated by high performance liquid chromatography (HPLC) and sequenced.

**RNA Extraction and cDNA Synthesis**—Exocrine glandular secretions were collected in capillary tubes from the larvae of *C. tremulae* and *C. populi* after nudging the animals with a pair of forceps. The larvae were frozen in liquid nitrogen and used directly for the extraction of total RNA with TRIZOL reagent.
(Invitrogen). Up to 5 μg were employed as a template for oligo(T) cDNA synthesis with an oligo(dT)$_{17}$ primer (0.05 μM; Table 1) by using Superscript III reverse transcriptase (Invitrogen) at 55 °C in a total volume of 20 μl.

**Generation and Expression of Full-length cDNA Clone Coding for SAO of C. tremulae (CtSAO)**—A degenerate primer P01 was constructed according to the N-terminal peptide resulting from sequencing of the SAO of C. tremulae (trem01; Table 2). This primer in combination with the oligo(dT)$_{17}$ primer (0.8 μM each; Table 1) was used for the amplification of 1 μl of cDNA with AccuTaq LA Polymerase (Sigma) in a total volume of 25 μl. At an annealing temperature of 66 °C, a fragment of ∼1900 bp was amplified, subcloned by using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions, and sequenced. Gene-specific primers were designed against the PCR fragment for 5'-end amplification (primer P02-P04; Table 1) by using the 5'-RACE system for rapid amplification of cDNA ends (RACE; Invitrogen) according to the manufacturer's instructions. For amplification of the open reading frame (ORF) of SAO cDNA without the N-terminal signal peptide, two gene-specific primers were synthesized (P05/P06) and used (0.8 μM each) for amplification with AccuTaq in a 25-μl reaction mixture containing oligo(dT)$_{17}$-primed cDNA as the template. The resulting 1900-bp fragment was purified by electrophoresis, cloned into the pGEM-T Easy Vector System (Promega), and sequenced. The positive construct was digested with Ndel/Sall before the insert was ligated into a Ndel/Xhol-linearized pET-22b(+) vector (Novagen) for expression with the T7 polymerase system (23). After transformation in *E. coli* BL21(DE3) cells (Stratagene) previously transformed with the pREP4-groESL plasmid (24) and induction with 0.2 mM isopropyl β-D-thiogalactoside, the recombinant protein was purified by metal chelate affinity chromatography by using Ni$^{2+}$-nitrotriacetic acid-agarose (Qiagen).

**Generation and Expression of Full-length cDNA Clone Coding for SAO of C. populi (CpSAO)**—Primers P07–P09 (Table 1) were synthesized based on an alignment of the amino acid sequences of GMC oxidoreductase-related sequences of *Drosophila melanogaster*, *Anopheles gambiae*, *Apis mellifera*, and *Candida* (accession numbers NP_477503.1, NP_644677, XP_310328, XP_394220.1, AAV66467, respectively). The primer pair P08 and oligo(dT)$_{17}$ was used to amplify 1 μl of cDNA with AccuTaq LA DNA polymerase (Sigma) at an annealing temperature of 45 °C in 35 cycles. The resulting fragment of ∼1600 bp was subcloned by using the TOPO XL PCR cloning kit (Invitrogen) according to the manufacturer's instructions and sequenced. The 5'-cDNA fragment was amplified as described for CtSAO but with the gene-specific primers P10–P12 (Table 1). After amplification of the full ORF without the N-terminal signal peptide with the primer pair P13/P14 (Table 1) and at an annealing temperature of 52.1 °C, the resulting fragment was digested with VspI/BsaI, cloned into a Ndel/XhoI-linearized pET22b(+) vector, and expressed with a C-terminal hexahistidine tag in *E. coli* BL21(DE3) in the presence of the pREP4-groESL plasmid as described for CtSAO.

**Identification of a Full-length cDNA Clone Predicted to Encode a GMC Oxidoreductase of Unknown Function from C. tremulae (CtGMC)**—RNA of *C. tremulae* was used for reverse transcription-PCR in an approach identical to that used for the identification of *CpSAO* with primer pair P08 and oligo(dT)$_{17}$ but with an annealing temperature of 60.3 °C in 35 cycles. Gene-specific primers P15–P17 were designed and used for 5'-RACE.

**Sequence Analysis**—cDNA sequences were analyzed by using the SignalP3.0 server (25) for prediction of signal peptides and their cleavage sites, using the PSORT II and the TargetP 1.1 server (26, 27) for the detection of sorting signals and subcellular localizations, and using the NetNGlyc 1.0 server (28) for the prediction of N-glycosylation sites. Sequences were aligned based on their deduced amino acid sequence by using ClustalX (28) before phylogenies were built with 500 bootstrap values.

**Enzyme Assay and Salicylaldehyde Quantification and Identification by Gas Chromatography (GC) and GC-Mass Spectrometry (GC-MS)**—Standard assays for SAO were performed in a volume of 500 μl containing 50 mM potassium phosphate...
buffer (pH 6.0) and 10–250 mM salicyl alcohol (Sigma). The reactions were incubated for 2–15 min in 2-ml glass vials and stopped by the addition of 250 µl of ethyl acetate containing 0.5 mM phenol (Fluka) as an internal standard, followed by vigorous vortexing for 30 s. By centrifugation (3 min at 5000 rpm), the upper organic phase was separated from the lower aqueous phase. A Shimadzu GC-2010 capillary gas chromatograph equipped with a BP-1 column (30 m × 0.25 mm × 0.25 µm; SGE Analytical Science Pty. Ltd.) was used to analyze 1 µl of the organic phase for quantification of salicylaldehyde with a split ratio of 2:1 at 250 °C. Helium was used as carrier gas (linear velocity 40 cm s⁻¹). Compounds were separated and eluted under programmed conditions from 70 °C (3 min isothermal) to 200 °C (at 6 °C min⁻¹). Products were detected by flame ionization. As standards, salicyl alcohol and salicylaldehyde (both from Sigma) were used. For unequivocal identification of the reaction product, GC-MS was performed on an Agilent 19091N-216 gas chromatograph coupled to an Agilent MSD 5975 inert quadrupole mass spectrometer. Aliquots of 200 µl of the aqueous enzyme reaction (not treated with ethyl acetate) and of salicylaldehyde diluted with assay buffer were added to headspace vials and heated up to 70 °C for 5 min in order to generate the headspace. Subsequently, 1 ml of the gas phase was transferred to an HP-INNOWax polyethylene glycol column (60 m × 0.32 mm × 0.5 µm; Agilent) with a split ratio of 5:3:1 and an initial temperature of 260 °C. Separation was achieved under a helium flow (linear velocity 30 cm s⁻¹) with a temperature program of 1 min at 70 °C, followed by a gradient from 70 to 240 °C at 15 °C min⁻¹ with a 4-min hold time at 240 °C. Electron impact mass spectrometry values were recorded at 70 eV. To test substrate specificity of SAO enzyme with various benzyl alcohol derivatives, reactions containing 75 mM substrate were incubated for 15 and 20 min, respectively, before the reactions were stopped and prepared for GC analysis as the standard reactions. For quantification of enzyme activity, only the increase of peak areas was analyzed in comparison with identical incubations in which the enzyme was replaced by buffer. To test the recombinant enzymes for glucose dehydrogenase activity, a spectrophotometric assay was used according to Caverne and Maclntyre (31) with slight modifications. Briefly, 100 µl of enzyme preparation were added to 400 µl of dichloroindophenol reagent (0.1 mM Tris-HCl, pH 7.0, 76 mM d-glucose, 48 µM 2,6-dichloroindophenol). Reduction of absorption was monitored at 600 nm at 20 °C with an Ultraspec 3100 Pro UV-visible spectrophotometer (GE Healthcare).

RESULTS

Microsequencing of SAO and Related Enzymes Present in Larval Defense Secretions—For microsequencing, two methods were used: (i) automated Edman degradation of N termini and of HPLC-purified peptides resulting from digestion of proteins with endopeptidase LysC and (ii) ESI-MS/MS spectroscopy of peptides resulting from digestion with trypsin. Use of the latter did not allow the two amino acids Leu and Ile to be distinguished, and hence, in Table 2, these residues are given in parentheses. The purified SAO of C. tremulæae, C. populi, and P. vitellinae each showed one dominant protein band on SDS-polyacrylamide gels of ~75 kDa. These bands were subjected to microsequencing. In the defense secretion of C. knabi, a dominant protein band of comparable size was detected and also applied to further analysis. Microsequencing resulted in N-terminal peptides of 15 and 20 amino acids of SAO from C. tremulæae (trem01) and C. populi (pop01), respectively. Several further internal peptides were obtained from all proteins analyzed (Table 2).

Identification and Analysis of cDNA Coding for CtSAO—Based on the sequence information of N-terminal peptide trem01 (Table 2), a degenerate oligonucleotide was synthesized (P01; Table 1) and used for reverse transcription-PCR with total RNA of C. tremulæae larvae in combination with the oligo(dT)₁₇ primer. Initial experiments never resulted in specific fragments, although RNA degradation was not detectable by gel electrophoresis. Speculating that the aldehyde present in the defense secretions might interact with the RNA of interest during the RNA extraction procedure, we completely removed the glandular secretions before RNA extraction. Hereupon, we succeeded in amplifying a fragment of ~1900 bp of which the sequence information was used for identification of the 5’-cDNA end by the 5’-RACE technique. The resulting cDNA sequence contained an ORF for a protein of 623 amino acids that contained all four peptides sequenced from the purified SAO (Fig. 2). The 5’- and 3’-untranslated regions of CtSAO cDNA were 20 and 62 bp in length, respectively. Sequence analysis with various software tools predicted an N-terminal signal peptide for the vesicular pathway with a proposed cleavage site between amino acids 21 and 22. Endoplasmatic reticulum retention motifs were not detectable. The encoded protein had a predicted subunit size of

### Table 2

| Peptide   | Sequence |
|-----------|----------|
| C. tremulæae |
| trem01¹ | LDDEFTGDISRFLIN |
| trem02 | EV[L/I]SSAGFSNPQ[I/L][I/L]IM_{49}[I/L]SG[I/L]GPK |
| trem03 | GF[I/L]TSSNTVEA[I/L]SYVK |
| trem04 | F[I/L][I/L]NFSIR |
| C. populi |
| pop01* | LDNELSGDILLN[SS][RIE] |
| pop02 | ASQEAGI[I/L][P]YVDNDQ[GQ][I/L]GVS[VQ][TTK] |
| pop03 | Q[I/L][G][I/L][T]QVSD[I/L][PVGK |
| pop04 | GF[I/L]TSSNTVEA[I/L][Y][I/L]K |
| pop05* | YTTVQ[SPML][H] |
| pop06* | (S/I)[Q/V/I/L][H][QT] |
| pop07 | TAYV[GE][I/L] |
| pop08 | (I/L)[I/L][I/L][N][P]ASK |
| pop09* | VFKP |
| P. vitellinae |
| phrat01 | PES[I/L][A][V][T][D][G][Y][H][N][E][D][G][P][I/L][SVSDV |
| phrat02 | (I/L)[P][V][E][S][D][I/L][P][V][GT] |
| phrat03 | YAYVQF[VP][I/L][I/L][I/L][H][PK |
| phrat04 | GV[I/L][T][S][I/L][G][G][V][E][A][I/L]J |
| phrat05 | (I/L)[G][G][S][V][I/L][N][Y][F][X][H][S][I/L][Y][VR |
| C. knabi |
| knab01 | GF[I/L]TSSNTVEA[I/L][Y][I/L][K] |

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**NOTE:** The table entries include identification of the N-terminal peptides and some specific residues. The use of ESI-MS/MS spectroscopy provides a more detailed view of the peptides resulting from microsequencing starting with amino acid 22. The underlined stretch of peptides labeled by an asterisk was analyzed by N-terminal Edman degradation; the other peptides resulted from ESI MS/MS spectroscopy. The underlined stretch of amino acids in parentheses were uncertain.
69.3 and 67.1 kDa with and without signal peptide, respectively. The isoelectric point of the processed protein is predicted to be 5.2. The deduced amino acid sequence of CtSAO showed 30–51% identity in data base searches to the GMC oxidoreductases of insects (Table 3) and possessed all the characteristic sequence motifs of this family of FAD-dependent enzymes (Fig. 2).

Identification of cDNA Coding for CpSAO—Since several peptides resulting from microsequencing showed similarity to GMC oxidoreductase sequences in the data base and since the first efforts to identify an SAO-coding cDNA with degenerate primers designed according to the peptides obtained from microsequencing of SAO were unsuccessful, we decided to use a reverse genetic approach. Based on an alignment of the amino acid sequences of GMC oxidoreductases of D. melanogaster, Anopheles gambiae, Apis mellifera, and Candida, the primers P07–P09 (Table 1) were synthesized. Upon using the primer pair P08 and oligo(dT)17, a fragment of ~1600 bp was identified before the 5′-cDNA end was identified by 5′-RACE with the gene-specific primers P10–P12. The resulting cDNA encoded a protein of 602 amino acids that was predicted to contain an N-terminal signal peptide of 21 amino acids. As for CtSAO, the resulting N terminus of the native protein matched exactly the N-terminal peptide that resulted from microsequencing. A molecular mass of 69.0 and 66.7 kDa was calculated for CpSAO with and without the signal peptide, respectively; the isoelectric point of the processed protein is predicted to be 5.2. The deduced amino acid sequence of CtSAO showed 30–51% identity in data base searches to the GMC oxidoreductases of insects (Table 3) and possessed all the characteristic sequence motifs of this family of FAD-dependent enzymes (Fig. 2).

FIGURE 2. Alignment of the amino acid sequences of CtSAO and CpSAO, of CtGMC, and of ecdysone oxidase from D. melanogaster (DmEO). The sequences were aligned using ClustalX (28). Identical amino acids in all sequences are shown in black boxes, with conservative replacements according to the criteria of the BLOSUM matrix in gray boxes. Three conserved sequence motifs of GMC oxidoreductases are framed. The positions of peptides that resulted from the microsequencing of C. tremulae and C. populi are indicated by solid and dotted lines, respectively; peptides analyzed from P. vitellinae and C. knabi are shown under the sequence stretch to which they show a high degree of similarity (peptide names refer to names given in Table 2). The horizontal arrows show the position and the length of degenerate primers P07–P09 (Table 1); the vertical arrow shows the shared cleavage site of the N-terminal signal peptides of CtSAO and CpSAO. Putative N-glycosylation sites are labeled by boldface underlined letters. The asparagine residues in CtSAO and CpSAO shown to be glycosylated are labeled by an open arrowhead.

TABLE 3
Comparison of amino acid sequences of newly identified GMC-like sequences of Chrysomela with selected GMC-like sequences of insects, fungi, and bacteria

| Protein | Dm_ninaG | Dm_GLD | Dm_EO | Am_GOX | An_GOX | Ec_CHD | Tc_GMC_I5 | Tc_GMC_B5 |
|---------|----------|--------|-------|--------|--------|--------|-----------|-----------|
| CtSAO   | 89.25 (91.81) | 35.73 (46.50) | 29.83 (40.15) | 39.96 (52.23) | 31.48 (44.07) | 37.94 (46.78) | 31.83 (41.81) | 34.56 (46.33) | 51.22 (62.24) |
| CpSAO   | 36.59 (46.18) | 31.35 (42.35) | 38.70 (47.74) | 31.30 (43.90) | 38.75 (47.60) | 30.60 (39.55) | 35.27 (46.32) | 49.52 (59.55) | 35.41 (46.46) |
| CtGMC   | 32.05 (41.08) | 35.35 (46.90) | 34.85 (44.10) | 36.99 (45.49) | 30.07 (37.86) | 36.99 (45.49) | 37.12 (46.18) | 46.73 (57.62) | 47.57 (58.62) |

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point was predicted to be 6.1. Again, all peptides obtained by microsequencing were verified by the derived amino acid sequence (Fig. 2). The deduced amino acid sequence showed sequence identity of 89% and 31–50% to CtSAO and to the GMC oxidoreductases of insects, respectively (Table 3).

Identification of cDNA Encoding a GMC-like Protein of C. tremulae (CtGMC)—The same strategy as described for the identification of the cDNA encoding CpGSO was used to identify further cDNA sequences of C. tremulae that showing homology to the previously identified CtSAO. The idea was to identify a sequence that shared a common ancestor with CtSAO. With cDNA reverse transcribed from the total RNA of C. tremulae, once again, the primer combination P08 and oligo(dT)$_1$_7 resulted in the amplification of a fragment of ∼1600 bp. After 5’-RACE with the gene-specific primers P15–P17, assembly of the overlapping DNA fragments resulted in a complete cDNA sequence with an ORF encoding 619 amino acids and 5’- and 3’-untranslated regions of 23 and 118 bp, respectively. The predicted protein has a calculated mass of 68.8 kDa. By using the TargetP and PSORT II server, the encoded protein was predicted to be cytosolic without an N-terminal signal peptide. The amino acid sequence shared all sequence motifs characteristic for GMC oxidoreductases (Fig. 2).

Expression and Biochemical Characterization of Recombinant SAO—For heterologous expression of CtSAO and CpSAO, the cDNA stretch encoding the N-terminal signal peptide of SAO was replaced by an artificial ATG start codon, resulting in a recombinant SAO that differed from the native SAO only by an additional methionine at the N terminus. By using the expression vector pET22b(+), a hexahistidine tag was added to the C-terminal end of recombinant SAO, allowing metal chelate affinity chromatography. Since expression of the constructs encoding CtSAO and CpSAO in E. coli BL21(DE3) resulted in the formation of insoluble inclusion bodies, a problem that could not be solved by variation of the culture conditions, both expression constructs were co-expressed with the plasmid pREP4-groESL. This plasmid encoded chaperonine GroEL and GroES shown to stabilize the folding process of recombinant proteins with increased solubility (24). Indeed, coexpression at 15 °C for 16 h resulted, in both cases, in the formation of soluble recombinant SAO, which was purified by metal chelate affinity chromatography.

Enzymatic Activity and Identification of Reaction Products—Purified recombinant SAO of C. tremulae and C. populi were tested for their ability to catalyze the formation of salicylaldehyde under conditions previously used for the characterization of the native proteins of C. populi and P. vitellinae (13). All incubations were analyzed at increasing time intervals to ensure linearity of product formation. The identity of the product was confirmed by co-chromatography with salicylaldehyde on two different GC columns (BP-1 and polyethylene glycol columns, respectively) and by GC-MS. The retention times and the mass spectra of the product peak and of the reference compound salicylaldehyde (0.5 mM) were essentially identical (97%; data not shown). Assays with CtSAO at increasing substrate concentrations (10, 30, 75, 150, and 250 mM salicyl alcohol) revealed in independent experiments $V_{max}$ values of 87.7 and 88.4 nanokatals/mg in the presence of 75 and 150 mM salicyl alcohol, respectively. Higher substrate concentrations revealed decreasing $V_{max}$ values (i.e. 56.2 nanokatals/mg at 250 mM). The same was the case for CpSAO with $V_{max}$ of 56.8, 55.0, and 30.9 nanokatals/mg with salicyl alcohol concentrations of 75, 150, and 250 mM, respectively. Estimation of the $K_m$ values for salicyl alcohol resulted in values of 40 and 28 mM for CtSAO and CpSAO, respectively. The addition of 5 units of catalase (Applichem, Darmstadt, Germany) to the assay (0.5 ml) to remove hydrogen peroxide resulted in no increase of $V_{max}$ and had no influence on the observed substrate inhibition at salicyl alcohol concentrations of about 250 mM. Enzyme assays performed under identical conditions with enzyme preheated for 5 min at 95 °C showed no salicylaldehyde formation.

Substrate Specificity of Recombinant SAO—Various benzyl alcohol derivatives were tested as substrates for SAO in comparison with salicyl alcohol (Table 4). The data show that for recombinant and native SAO of C. populi, salicyl alcohol is by far the best substrate. Minor substrates are other benzyl alcohol derivatives with methyl or methoxy ligands in the ortho-position and those with ligands in the para-position. Substituted derivatives were not substrates or were only very weak substrates. The recombinant SAO of C. tremulae shows essentially the same specificity as the SAO of C. populi. Remarkably, 2-phenylethyl alcohol is much better accepted as substrate by the native SAO of C. populi than by the recombinant enzymes. Using a photometric assay to test for the ability of SAO to catalyze the oxidation of glucose to gluconolactone, we were unable to detect any activity. Oxidation of glucose to gluconolactone is catalyzed by glucose dehydrogenase identified from various insects (32, 33), which was also classified as a GMC oxidoreductase (21).

Posttranslational Modification of SAO—Of note, the subunit size of the native protein of defense secretions of C. tremulae and C. populi showed an apparent mass of ∼80 kDa on SDS-polyacrylamide gel electrophoresis analyses, whereas the predicted molecular mass of the processed proteins was only 67.1 and 66.7 kDa for CtSAO and CpSAO, respectively. Having the amino acid sequences of CtSAO and CpSAO on hand, we rea-
analyzed the peptide data that we had obtained from microsequencing. The amino acid sequences of CtSAO and CpSAO contained seven and six putative N-glycosylation sites of the consensus sequence NX(S/T), respectively (where X represents any amino acid except for Pro; labeled in Fig. 2 by boldface underlined letters). Because of the intensities of the asparagines observed by automated Edman degradation of the N termini of both proteins, a glycosylation of the first putative glycosylation site could be excluded. Instead, the data obtained from ESI-MS/MS suggested an N-glycosylation of the peptides YNATLVR and YNATIVR of CtSAO and CpSAO, respectively (labeled in Fig. 2 by an open arrowhead). Using peptide mapping methods, a series of signals with mass increments of 162 Da characteristic for hexose residues suggested a high mannose type structure with 6–9 mannose and two N-acetylglucosamine residues (Man$_{6–9}$GlcNAc$_2$) at this position of the CtSAO protein. In the analogous position of the CpSAO protein, a different type of N-glycan with the composition Hex$_2$HexNAc$_3$ (80%) and Hex$_4$HexNAc$_3$ (20%) was detected, suggesting the presence of hybrid type structures (GlcNAcMan$_{6–9}$GlcNAc$_2$). The fragmentation pattern obtained by MS/MS of the more abundant glycopeptide form confirmed this structural assignment (data not shown). Since the unmodified molecular ions of all peptides with further putative N-glycosylation sites were not detected, although the overall sequence coverage obtained was nearly 70%, and considering the significantly higher than expected mass found by SDS-PAGE, further N-glycosylations were likely, but they will have to be established experimentally in the future.

**DISCUSSION**

We have identified, cloned, and heterologously expressed the cDNA encoding the SAO from the defensive secretions of two related *Chrysomela* leaf beetles. The SAO cDNA contains an ORF of 623 amino acids, of which the first 21 residues act as signal peptide for the secretory pathway via which the processed protein is released from the glands into the extracellular glandular reservoir. Biochemical analyses have revealed that both enzyme proteins have essentially the same characteristics as the native proteins previously characterized from *C. populi* and *P. vitellinae* (13). The high $K_m$ values correspond nicely to the concentrations of salicyl alcohol in the defensive reservoir of the beetle larvae (13). These reservoirs can thus be interpreted as efficient reactors for the biosynthesis of large amounts of salicylaldehyde. Because of its low water solubility, salicylaldehyde readily forms an organic phase within the defensive reservoir, accounting for approximately 15% of the whole secretion and ensuring a permanent removal of reaction product from the aqueous phase and a steady oxidation of salicyl alcohol (13).

The amino acid sequences of CtSAO and CpSAO show similarity to GMC oxidoreductases from eukaryotic and prokaryotic organisms. The enzyme family of the GMC oxidoreductases was first defined by Cavener (21); these are characterized by a similar overall structure and an essentially identical binding site for the prosthetic group FAD. This binding site comprises several regions of the sequence (motifs 1–3 in Fig. 2, respectively), including a canonical ADP-binding $\beta\alpha\beta$ motif with the consensus sequence GXGXXG close to the N terminus and two further GMC oxidoreductase patterns that were suggested to be involved in binding and stabilizing the FAD (34, 35). Three-dimensional crystal structures have previously been solved for some members of this enzyme family and have shown the presence of a conserved $p$-hydroxybenzoate hydroxylase-like fold (36–40). Despite the structural and sequence similarities, the members of the GMC oxidoreductase family catalyze surprisingly diverse reactions (41), suggesting a high variability within the catalytic centers that are mainly formed by the C-terminal region of the sequence, which has been shown to contain the substrate-binding domain (34). Most of the GMC-like sequences known to date have arisen from recent genome projects, so there is no information about their natural substrates.

Within the insect genomes of *D. melanogaster* (fruit fly), *A. gambiae* (mosquito), *A. mellifera* (honey bee), and *Tribolium castaneum* (flour beetle), GMC-like genes encompass a gene family of 15, 15, 18, and 23 members, respectively (42). Only four of these GMC-like genes have been functionally characterized, all of which are within *Drosophila*: (i) glucose dehydrogenase, which is regulated by ecysynone and required for eclosion of adult fruit flies and for sperm storage in female *D. melanogaster* (43, 44), (ii) ecysynone oxidase, which is involved in ecysynone metabolism and part of the biosynthesis of unique ecysteoidietors (45), and (iii) an oxidoreductase encoded by the ninaG gene and shown to be involved in the production of visual pigment chromophore in *Drosophila* (46). In *A. mellifera*, a glucose oxidase has been characterized as being a carbohydrate-metabolizing enzyme involved in the conversion of nectar to honey (47).

In this paper, we report the identification of SAO in chrysomelid leaf beetles; the enzyme is a GMC oxidoreductase acting as an aryl-alcohol:oxygen oxidoreductase (EC 1.1.3.7) by converting an aromatic primary alcohol in the presence of molecular oxygen to an aromatic aldehyde and hydrogen peroxide. This activity is known for GMC oxidoreductases but so far only in fungi. For a third GMC-like sequence that we have identified from *C. tremulae* (CtGMC), we are unable to predict a biochemical function. Amino acid sequence comparison of these three sequences shows a high degree of identity (89%) between the two SAO sequences but a low degree of identity of only 30–40% between the SAO sequences and the functionally uncharacterized CtGMC and other GMC-like proteins of insects, fungi, and bacteria (Table 3). This low degree of sequence identity seems to be a typical feature of GMC-like proteins and has also been observed for other enzymes possessing the $p$-hydroxybenzoate hydroxylase-like structure fold (36). Thus, it is not surprising that recombinant SAO of both *Chrysomela* species did not show any activity in the glucose dehydrogenase assay. Glucose dehydrogenase from *Drosophila*, which was also described to belong to the GMC oxidoreductase enzyme family, shows only 40 and 39% amino acid sequence identity to the SAO of *C. tremulae* and *C. populi*, respectively (Table 3).

Recently, Cavener and co-workers (42) have identified, in all four insect genomes so far available (*D. melanogaster, A. gambiae, A. mellifera, and T. castaneum*), a highly conserved cluster of 10–12 GMC-like genes located within the second intron of...
the flotillin-2 gene, a non-GMC gene encoding the lipid raft protein Flotillin-2 (48). The conservation of microsynteny within this cluster in these highly diverged insect species is remarkable. Since the gene encoding ecdysone oxidase is one of the genes present in this cluster of *Drosophila*, these genes might be coordinately regulated and involved in a developmental or physiological function that is conserved among arthropods (42). Based on a neighbor-joining tree, Cavener and co-workers (42) have identified 13 monophyletic clades that have been classified as subfamilies (labeled by Greek letters), suggesting that these subfamilies encompass at least one GMC ortholog of each of the four insect species. We have used the *Tribolium* sequences of this dataset, retaining their label to construct a neighbor-joining tree, including the three *Chrysomela*-GMC oxidoreductase sequences. The resulting tree of beetle sequences (Fig. 3) shows that some of the subfamilies with only one sequence within the *Tribolium* genome cluster together (α-, δ-, γ-, ε-, and ζ-subfamilies), whereas other subfamilies, such as the κ-clade, comprise several paralog sequences. Of the 23 GMC-like genes identified within the *Tribolium* genome, 12 are part of the conserved gene cluster, whereas 11 are found outside of this cluster. Several arguments support the hypothesis that the gene cluster is the birthplace of all GMC-like genes that originate by gene duplication before they are relocated outside of the cluster (42). For example, for the TcGMC_Q5 gene of the θ-subfamily, a gene that has been shown to be one of the most conserved genes of the GMC gene cluster, a duplicate exists only ~250 kb away from the cluster (42). The phylogeny (Fig. 3) and the comparison of sequence identities (Table 3) suggest that the orthologous genes encoding the SAOs of *C. tremulae* and *C. populi*, respectively, share a common ancestor with the genes of the κ-subfamily. In *Tribolium*, all of the genes of this subfamily are part of the GMC gene cluster. On the assumption that the conserved GMC gene cluster also exists in *Chrysomela* leaf beetles, the genes encoding SAO most probably evolved by duplication of a GMC gene of the κ-subfamily present in this cluster. This gene copy was later modified and optimized with respect to the new function of the encoded oxidoreductase in the extracellular biosynthesis of salicylaldehyde as a defense chemical from plant-derived pre-
cursors in the glandular reservoirs. Work is in progress to analyze the position of the SAO-encoding genes within the *Chrysomela* genome and to identify further GMC-like genes, especially genes paralogous to the SAO-encoding genes of the β-subfamily. The GMC-like sequence of *C. tremulæ* identified in our study has been revealed to be related to the TcGMC_B5 gene product, the only member of the β-subfamily within the *Tribolium* genome, and is therefore not a likely candidate as a paralog to *CtSAO* (Fig. 3 and Table 3).

Based on a phylogeny of Chrysomelina leaf beetles on which the host plant affiliation of the beetles and their larval chemical defense strategies have been mapped, Termonia *et al.* (11) have been able to show that the host-derived defense strategy of producing salicylaldehyde from salicin taken up from the host plant has evolved from the autogenous synthesis of iridoid monoterpenes. The data suggest that this shift in defense strategy has occurred at least twice: once early in the evolution of the *Chrysomela* clade and once in the species *P. vitellinae*. Despite the differences between the autogenous and the host-derived defense strategies, the enzymes involved in the final steps of both pathways have been postulated to be related (4, 49). Both defense strategies require a glucosidase and an oxidase (Fig. 1). Thus, both enzymes might have been recruited and adapted from the autogenous defense strategy to process plant-derived phenol glucosides. If these enzymes were recruited by a change in their substrate specificity without further gene duplications, we would expect that the metabolism pathways of iridoids and salicin are mutually exclusive. Indeed, there is no example for a leaf beetle larvae using both defense strategies in parallel. The identification of the cDNA encoding SAO in the defense secretions of salicylaldehyde-producing *Chrysomela* species offers a tool to test for related enzymes in the defense secretions of beetle larvae employing the ancestral, iridoid monoterpenic-producing defense strategy. Analyzing proteins of defense secretions by microsequencing of various related beetle species, we have identified several peptides of *P. vitellinae* and one peptide of *C. knabi* showing high similarity to the SAO of *C. tremulæ* and *C. populi* (Table 2 and Fig. 2). This finding suggests that at least the SAO of *P. vitellinae* and salicylaldehyde-producing *Chrysomela* species share a common origin with the respective oxidase from the defense secretions of iridoid monoterpenic-producing beetle larvae. *C. knabi* is a derived species that uses not only the salicylaldehyde-based defense strategy but also the most recently developed strategy based on the de novo synthesis of butyric acids that are esterified with plant-derived alcohols (11). Obviously, *C. knabi* larvae have retained their SAO for the production of salicylaldehyde, whereas they have recruited a new, only partially plant-dependent, defense strategy in parallel.

Identification of the biochemical activities of more of the GMC oxidoreductase-like genes of chrysomelid leaf beetles will be a challenge for the future. Success in this area should allow a better understanding of the evolutionary processes involved in the evolution of these enzymes, which allow the beetle larvae to modify and use plant-derived compounds for their own defense.

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