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Precise RNAi-mediated silencing of metabolically active proteins in the defence secretions of juvenile leaf beetles

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Allomones are widely used by insects to impede predation. Frequently these chemical stimuli are released from specialized glands. The larvae of Chrysomelina leaf beetles produce allomones in gland reservoirs into which the required precursors and also the enzymes are secreted from attached gland cells. Hence, the reservoirs can be considered as closed bio-reactors for producing defensive secretions. We used RNA interference (RNAi) to analyse *in vivo* functions of proteins in biosynthetic pathways occurring in insect secretions. After a salicyl alcohol oxidase was silenced in juveniles of the poplar leaf beetles, *Chrysomela populi*, the precursor salicyl alcohol increased to 98 per cent, while salicyl aldehyde was reduced to 2 per cent within 5 days. By analogy, we have silenced a novel protein annotated as a member of the juvenile hormone-binding protein superfamily in the juvenile defensive glands of the related mustard leaf beetle, *Phaedon cochleariae*. The protein is associated with the cyclization of 8-oxogeranial to iridoids (methylcyclopentanoid monoterpenes) in the larval exudates made clear by the accumulation of the acylic precursor 5 days after RNAi triggering. A similar cyclization reaction produces the secologanin part of indole alkaloids in plants.

Keywords: RNAi; insects; leaf beetle; secretome; salicyl alcohol oxidase; monoterpene cyclization

1. INTRODUCTION

Insects are extraordinarily inventive when it comes to producing defensive compounds for repelling their enemies. To circumvent auto-intoxicative effects, these natural products frequently originate in the epidermis-derived exocrine glands [1]. The gland cells produce secretions that are fortified with defensive compounds [2,3]. It has been demonstrated that insects convert either intrinsic precursors or food-derived compounds into biologically active allelochemicals [4–7]. The precursors can be activated in the defensive glands or in the secretions. Immature leaf beetles of the subtribe Chrysomelina, for example, produce their deterrents in biphasic secretions, and store them in nine unique pairs of impermeable reservoirs in their backs [8,9]. The larval exudates containing salicyl aldehyde (**3**) have been of particular interest [10,11]. The hydrophobic aldehyde forms an organic layer, accounting for 15 per cent of the total discharge volume, while the aqueous phase constitutes 85 per cent [12]. The latter contains the precursor salicyl alcohol (**2**) and a flavine-dependent salicyl alcohol oxidase (SAO); the SAO uses molecular oxygen as an electron acceptor for alcohol oxidation, yielding the aldehyde and hydrogen peroxide [12–14] (figure 1). Salicyl aldehyde is considered as a potent repellent against generalist predators [11,15] and as an antimicrobial agent [16]. The larvae feed on salicaceous plants and sequester the secondary metabolite salicin (**1**) [17–19]. After shuttling salicin to the defensive glands, the glucoside is cleaved by a β-glucosidase into **2** and glucose for further metabolism [20] (figure 1). According to phylogenetic analyses, the synthesis of **3** from sequestered precursors has evolved from the de novo production of defensive iridoids (methylcyclopentanoid monoterpenes containing an iridane skeleton) [21]. Also the last steps of the iridoid pathway in the secretions are thought to be similar to those found in sequestering species [20] (figure 1). At first, the sugar moiety is cleaved from 8-hydroxygeraniol-8-*O*-β-D-glucoside (**4**), and an oxygen-dependent oxidase converts the aglucone into 8-oxogeranial (**6**) [20,22–24]. A subsequent cyclization reaction yields iridoids (**7**) [25].

Despite the many current genome- and transcriptome-sequencing projects, up to now it has only been shown for SAO sequences to be entangled in allomone production in the defensive secretions of the leaf beetle species *Chrysomela tremulae*, *Chrysomela populi*, *Chrysomela lapponica* and *Phratora vitellinae* [13,14,26]. To demonstrate the *in vivo* relevance of a target sequence, gene silencing by RNA interference (RNAi) is a suitable method. RNAi is an endogenous mechanism, derived from an anti-viral immune response [27], and can be found virtually in all eukaryotic species. It can be triggered artificially by double-stranded RNA (dsRNA), whose nucleotide sequence is identical to that of the target gene [28]. The

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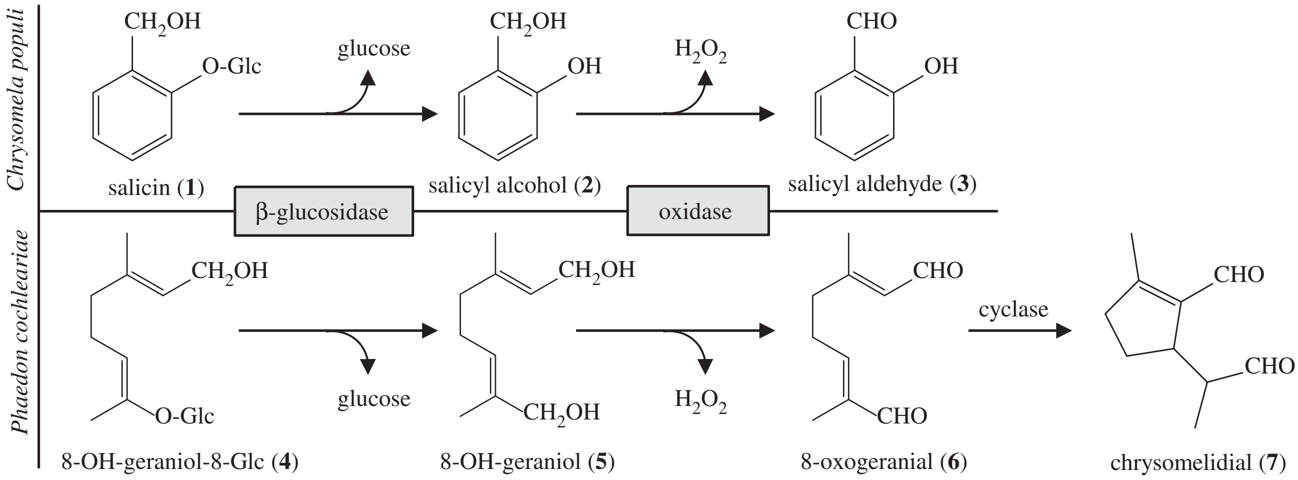

Figure 1. Enzymatic reactions in the defensive secretions of juvenile *C. populi* and *P. cochleariae* adapted from Michalski *et al.* [14]. Glc, Glucose.

RNAi effect is attended by decreased transcript and protein levels, and consequently by loss-of-function phenotypes. In addition to embryogenesis, pattern formation, reproduction and behaviour, RNAi allows biosynthetic pathways in insects to be successfully analysed [29–31].

Here, we describe how RNAi can be used to target the biosynthesis of discrete components in the defensive discharges of juvenile Chrysomelina. We first validated this technique by silencing the known *SAO* sequence in the sequestering species *C. populi* (*CpopSAO*). After knocking down the SAO, the alcohol precursor of **3** accumulated in the gland. This showed that we are able to interrupt the deterrent biosynthesis *in vivo*. Next, we extended the method to the related de novo iridoid-producing species, *P. cochleariae*. In the secretions of its larvae C_{10}-precursors are converted to the methylcyclopentanoid monoterpene chrysomelidial. Particularly, the cyclization mechanism is of importance because it occurs not only in insects but also in plants. Here, the cyclization leads ultimately to secologanin, one of the building blocks for more than 2500 indole alkaloids that have been isolated mainly from three plant families [32]. Although an enzyme with cyclase activity for secologanin biosynthesis has long been predicted, a corresponding sequence has yet to be published. In the *P. cochleariae* secretome, we identified a novel protein which is involved in the cyclization reaction of the monoterpenoid 8-oxogeranial to chrysomelidial.

2. MATERIAL AND METHODS

See electronic supplementary material for complete secretome analyses by data-independent liquid chromatography/mass spectrometry detection (LC/MS^E), cloning procedures, detailed quantitative real-time PCR procedure (qPCR), all primer sequences and accession numbers.

(a) *Beetle rearing and secretion analyses*

Chrysomela populi (L.) was collected near Dornburg, Germany (latitude 51.015, longitude 11.64), on *Populus maximowiczii* × *Populus nigra*. In the laboratory, beetles were kept in a 16 L : 8 D cycle, 18 ± 2°C in light and 13 ± 2°C in darkness. *Phaedon cochleariae* (F.) was laboratory-reared on *Brassica oleracea* convar. *capitata* var. *alba* (Gloria F1) in 16 L : 8 D cycle conditions and 15 ± 2°C. According to [33], we obtained the relative growth rate (RGR) of six biological replicates of each group of five larvae by RGR = [(final weight − weight of neonate larva)/(weight of neonate larva × developmental time (days))]. Each replicate group was weighed every 24 ± 3 h and data were compared with two-tailed *t*-test. Larval secretions were collected in glass capillaries (inner diameter, 0.28 mm; outer diameter, 0.78 mm, length 100 mm; Hirschmann, Eberstadt, Germany). Sealed capillaries containing samples were stored at −20°C until needed. Secretions were weighed in the sealed capillaries on an ultra-microbalance (Mettler-Toledo, Greifensee, Switzerland) three times; the weight of the capillaries was subtracted and the final weight was averaged.

(b) *Production of double-stranded RNA*

Sequenced plasmids pIB-*CpopSAO* (GeneBank: HQ245154.1) and pIB-*PcTo-like* (GeneBank: JQ728549) were used to amplify a 1.5 kb *CpopSAO* fragment and a 450 bp *PcTo-like* fragment, respectively. The *gfp* sequence was amplified from pcDNA3.1/CT- GFP-TOPO (Life Technology, Darmstadt, Germany). The amplicons were subject to *in vitro* transcription assays according to instructions from the Ambion MEGAscript RNAi kit (Life Technologies, Darmstadt, Germany). The resulting dsRNA was eluted after nuclease digestion three times with 50 µl of injection buffer (3.5 mM Tris–HCl, 1 mM NaCl, 50 nM Na_2HPO_4, 20 nM KH_2PO_4, 3 mM KCl, 0.3 mM EDTA, pH 7.0). The concentration of dsRNA was calculated with A = 1 = 45 mg ml^{-1} and adjusted to 1 µg μl^{-1}. The quality of dsRNA was checked by TBE-agarose-electrophoresis.

(c) *Injection of double-stranded RNA*

First instar of *C. populi* with 5 mm body length was injected with 0.1–3 µg of dsRNA approximately 10 days after hatching. *Phaedon cochleariae* second instar with 4 mm body length was injected with 0.3 µg of dsRNA approximately 5 days after hatching. Injections were accomplished with ice-chilled larvae using a Nano2000 injector (WPI, Sarasota, FL, USA) directed by a three-axis micromanipulator. The larvae were injected parasagittally between the pro- and mesothorax.

(d) *Off-target prediction*

According to the mechanism of RNAi [28], the top and bottom strands of dsRNAs of *CpopSAO*, *PcTo-like* and *gfp* were diced *in silico* into all possible 21 bp fragments [34]. The resulting siRNAs were subjected to BLASTn (stand-alone NCBI-BLAST) [35] by invoking BLASTALL v. 2.2.21 (parameters: -p blastn -e 1e-1 -G 7 -T -b 80 -v 80) searching against our in-house transcriptome databases of *C. populi* and *P. cochleariae*. Hits less than 20 nts in length were ignored and hits more than or equal to 20 nts were considered as putative off targets.

(e) CpopSAO *and* PcTo-like *transcript abundance*

Cq values of genes of interest from three biological replicates were normalized by *CpRPL45* and *CpActin* for *C. populi* and *PcRP-L8* and *PcRP-S18* for *P. cochleariae*, respectively. Real-time PCR data were acquired on an Mx3000P Real-Time PCR system using Brilliant II SYBR Green qPCR Master Mix (Agilent, Santa Clara, CA, USA).

(f) *Gas chromatography/mass spectrometry analysis of low-molecular-weight compounds in chrysomelid secretions*

Secretions of *C. populi* were diluted in 1 : 150 (w/v) ethyl acetate and secretions of *P. cochleariae* were diluted in 1 : 100 (w/v) dichloromethane. Of each diluted secretion, 1 µl was subjected to GC/EIMS analysis (ThermoQuest Finnigan Trace GC/MS 2000, Frankenhorst, Germany) equipped with Phenomenex (Aschaffenburg, Germany) ZB–5–W/Guardian–column, 25 m. Substances were separated using helium as a carrier (1.5 ml min^{-1}). Conditions for *C. populi* secretions: 50°C (1 min), 10°C per minute to 80°C, 60°C per minute to 280°C (1 min). Inlet temperature was 220°C, transfer line was 280°C. Substances were identified according to standard substances **2** and **3**. Conditions for *P. cochleariae* secretions: 50°C (2 min), 10°C per minute to 80°C, 5°C per minute to 200°C, 30°C per minute to 300°C (1 min). Inlet temperature was 220°C and transfer line was 280°C. Substances were identified according to [36] and the reference compounds 8-oxogeranial and chrysomelidial. The synthesis of 8-oxogeranial and chrysomelidial was carried out as in [25,37], respectively. Peak areas from GC-chromatograms were obtained using an ICIS-algorithm (XCALIBUR BUNDLE v. 2.0.7, Thermo Scientific).

(g) *Statistical analyses*

Two-tailed Student's *t*-tests for unequal variation were used to value the significance levels of transcript abundances and to weight differences comparing values of three different biological replicates from the non-injected control (NIC) group with those of either the RNAi group or the *gfp* control. Multi-dimensional ANOVA tests were carried out to validate significant differences in time series and between different RNAi treatments. The level of significance was reached at a *p*-value of 0.05. Calculations were done with R (http://www.r-project.org/).

3. RESULTS

(a) *Targeting the defensive glands of juvenile poplar leaf beetles by RNA interference*

Recently, a 1872-bp *CpopSAO* cDNA (Genbank/HQ245154.1) encoding a 69 kDa protein for conversion of **2** into **3** was identified from the larval defensive glands of *C. populi* [13,14] (figure 2*a*). It belongs to the glucose–methanol–choline (GMC) family of oxido-reductases [38]. Given that the expression of *CpopSAO* was detectable exclusively in glandular tissues (figure 2*b*), silencing this gene would affect only the process of glandular biosynthesis.

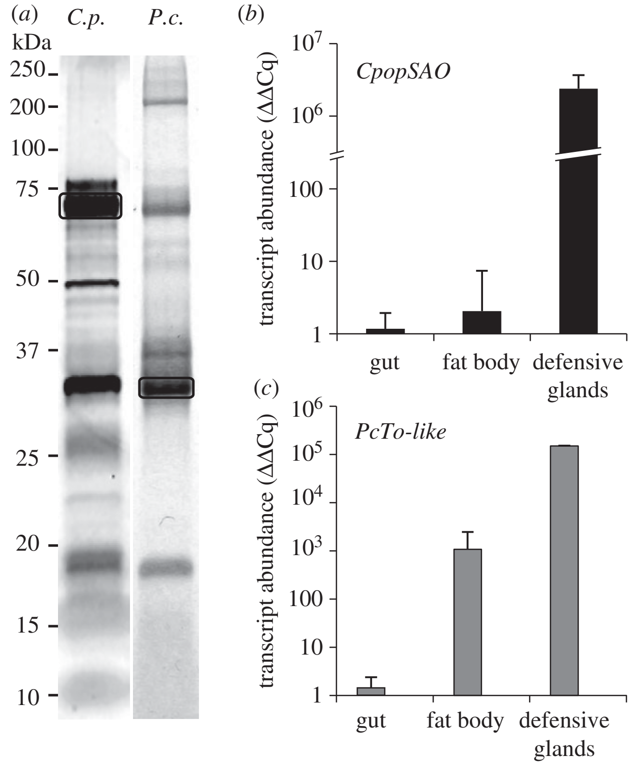

Figure 2. Protein and transcript abundance in juvenile leaf beetles. (*a*) Proteins in defensive exudates separated by one-dimensional SDS–PAGE. left: 1 mg secretions of *C. populi* (*C.p.*), silver stained, box marks *Cpop*SAO; right: 0.65 mg secretions of *P. cochleariae* (*P.c.*), Coomassie stained, box marks *Pc*To-like. (*b*) Expression pattern of *CpopSAO* ±s.d. in different *C. populi* tissues, $n = 3$. (*c*) Expression pattern of *PcTo-like* ±s.d. in different *P. cochleariae* tissues, $n = 2$. Both *y*-axes are in $\log_{10}$ scale.

To induce RNAi in *C. populi* larvae, we injected 1.0 µg of 1.5 kb *CpopSAO* dsRNA into late first instar. A 719-bp dsRNA fragment of *gfp* served as a control for effects caused by dsRNA; although the RNAi machinery will be induced, genes should not be silenced. Furthermore, we included an NIC group in our experiments. By monitoring the developmental traits and the secretion production in *C. populi* and comparing the results with those from control groups, we found that silencing *CpopSAO* did not influence either growth rate or pupae weight (see the electronic supplementary material, figure S1*a*). But the larvae treated with *CpopSAO* dsRNA produced slightly more secretions than did the larvae of the control groups (see the electronic supplementary material, figure S1*b*), which might be owing to the different osmotic characteristics between **2** and **3** [12]. Because we did not detect significant differences between NIC and *gfp* controls in any experiments delineated below, we continue showing only the data of the *gfp* controls.

Transcript abundance was measured in glandular tissue using qPCR after 1, 3 and 12 days. Comparing tissue from these samples to tissue from the *gfp* controls, we noticed significant reductions to 7.6 per cent mRNA level ($p = 0.002$) just 24 h after injection. After day 3, the transcript level was diminished to 1.6 per cent ($p = 0.004$), and after day 12, to 0.5 per cent (figure 3*a*).

In accordance with the literature, SAO corresponds to the dominant band at 70 kDa in the secretions of

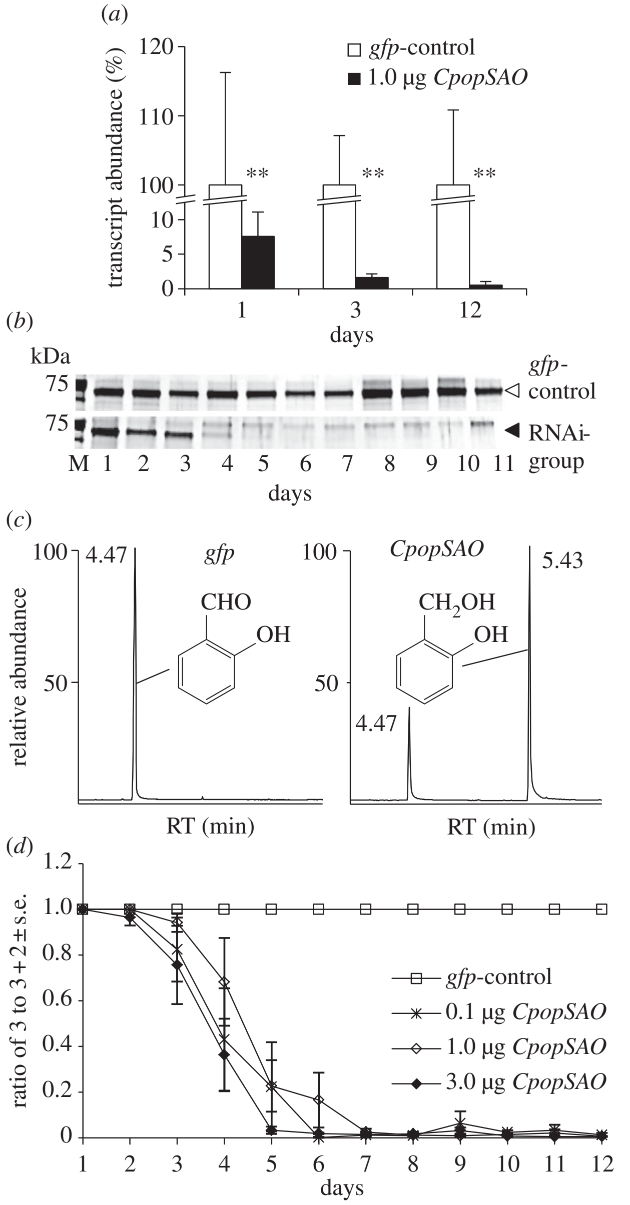

Figure 3. RNAi effects in juvenile *C. populi*. (*a*) White bars: transcript abundance of *CpopSAO* after injecting 1.0 µg *gfp* dsRNA, $n = 3 \pm$ s.d.. Black bars: transcript abundance of *CpopSAO* after injecting 1.0 µg *CpopSAO* dsRNA, $n = 3$. 100% = ΔCq of *gfp* control. Asterisks indicate level of significance: $^{**}p < 0.01$ (*b*) *Cpop*SAO protein abundance in defence secretions was monitored over time. A total of 0.85 mg secretions per lane was separated on silver-stained SDS gels. The 70-kDa band corresponds to *Cpop*SAO. Secretions originating from control treatment with 1 µg dsRNA of *gfp* (white arrowhead) and RNAi treatment with 1 µg dsRNA of *CpopSAO* (black arrowhead) are shown. (*c*) GC-chromatogram of secretions on day 5 after treatment; left: injecting 1.0 µg *gfp* dsRNA resulted in the production of **3**, right: injecting 1.0 µg *CpopSAO* dsRNA resulted in the production of **2** and **3**. (*d*) GC-chromatogram peak-area-based plot of secretions after dsRNA injection of *gfp* and different amounts of *CpopSAO* ($n = 5$).

C. populi (figure 2*a*) [12,14]. The composition of the secretome after dsRNA treatment was monitored in a time series in silver-stained one-dimensional SDS gels. Owing to the silencing effect, the quantity of *Cpop*SAO was apparently reduced just 2 days after dsRNA injection and the protein was barely visible after day 5 (figure 3*b*).

The effects on the biosynthesis of **3** in the defensive secretions were determined by GC/MS analysis. For these experiments, 0.1, 1.0 and 3.0 µg of *CpopSAO* dsRNA were injected into larvae from the same clutch. As in the protein reduction, we detected **2** in the defensive secretions just 2 days after the injection of 3.0 µg *CpopSAO* dsRNA (figure 3*c*). Compound **2** was not detectable in *gfp* control secretions. In addition, no unexpected chemical compound arose owing to the dsRNA treatments. By setting the peak area of **3** in ratio equalling the sum of the main peak areas, a diagram of the RNAi-dependent reduction of **3** can be plotted (figure 3*d*). We have tested dsRNA amounts ranging from 0.1 to 3.0 µg. After RNAi induction, significantly less aldehyde was observed for the 3.0 µg *CpopSAO* group ($p = 0.015$) on the 4th day and for all tested *CpopSAO* groups on the 5th day (0.1 µg, $p = 0.016$; 1 µg, $p = 0.002$; 3 µg, $p = \ll 0.001$). Biological variation prevented us from observing dose-dependent RNAi effects in these experiments; the amount of **2** did not differ significantly between the RNAi samples.

(b) *Off-target prediction and validation for* CpopSAO

Owing to strong sequence identities, co-silencing non-target genes can cause unintended side-effects [39,40]. Therefore, we performed off-target predictions for the desired dsRNA sequences of *CpopSAO* and *gfp*. Predicted off-target genes were validated by qPCR using cDNA derived from successful RNAi experiments. Because of a lack of genome sequences, the potential silencing effects of targeting the nucleus where fragments of the long dsRNA may bind to non-transcribed regulatory sequences [41] or introns [42] could be neither predicted nor validated.

For *gfp* dsRNA, no critical candidates were detected in the transcriptome library of *C. populi*. Off-target analyses in the *C. populi* sequence library, however, identified 25–21 bp contiguous regions of *CpopSAO* dsRNA that were identical to sequences of eight unique transcripts (see the electronic supplementary material, table ST2 for putative off-target hits). Three of them encode putative proteins having the GMC-oxidoreductase motif in the C-terminal region (*Cp*GMClike I-III) and five were annotated as hypothetical proteins (*Cp*COMP3092; *Cp*COMP6024; *Cp*COMP36289; *Cp*COMP38777; *Cp*COMP51471).

CpopSAO shares with *CpGMClike-I* two similar regions spanning 22 and 25 nucleotides (nts) each; these regions are interrupted by one mismatch (22/1 and 25/1) and, with *CpGMClike-III,* one similar sequence stretch without mismatch (24/0). *CpGMClike-II* and the five remaining transcripts possess sequence regions of 22/0 to 20/0 nts identical to *CpopSAO.* In all tissues, all putative off-target genes exhibited generally low expression levels with relative Cq values median less than 2×10^{-3}. qPCR assays were carried out for all eight targets 12 days after larvae were treated with 1.0 µg 1500-bp *CpopSAO* dsRNA; only for the *CpGMClike-I* and *CpGMClike-II* did these treatments reveal significant differences of transcript level in the gut tissue ($p = 0.049$; $p = 0.032$). No other tested transcripts showed changes of mRNA abundance in the examined tissues (see the electronic supplementary material, figure S2). Since *C. populi* larvae transport the plant-derived precursor into the defensive glands for final transformation, we assume that the off-target effects on the putative GMC-oxidoreductases in gut tissue of unknown function do not distort the RNAi effects observed in the secretions.

(c) *Identification of unknown proteins in the defence-related secretome of* Phaedon cochleariae

After successfully introducing the 'lack-of-function approach' to the defensive secretions of *C. populi* by silencing an enzyme for which we had a clear expectation of the resulting phenotype, we used the method to identify proteins in unknown secretions. For this reason, we chose the larval exudates from the related de novo iridoid-producing species *P. cochleariae*. We assigned to the abundant 35-kDa band a putative protein whose deduced sequence contains 243 amino acids and a 22 amino acid signal peptide; the existence of such a sequence suggests that the mature protein is secreted (figure 2*a*). It possesses a conserved domain (pfam06585) characteristic for the juvenile hormone-binding protein (JHBP) superfamily. Sequence comparisons using the BLAST algorithm [35] revealed that the *P. cochleariae* amino acid sequence shares only very limited identity with functionally characterized insect proteins, for example, 12 per cent identity with the JHBP from *Bombyx mori* [43] and 16 per cent with the takeout (To) 1 from *Epiphyas postvittana* [44] (figure 2*a*). Higher identities up to 25 per cent were found only with insect proteins not yet fully characterized in their functions, such as those with the To-like protein (NP_001191952) from *Acyrtosyphon pisum* or the JHBP-like (XP_001359416) from *Drosophila pseudoobscura pseudoobscura*. None of the mentioned insect species is known to produce cyclic monoterpenoids.

The JHBP superfamily combines both the To protein family and the JHBP family. There are two major differences between the families: one is the number of disulphide bonds (To proteins have one and JHBPs have two) and the second are the conserved C-terminal sequence motives that are only present in To proteins. *In silico* analyses predicted in the *P. cochleariae* sequence seven N-glycosylation sites and only one disulphide bond. Along with identifying the two To-specific motives [45] (figure 4) in the C-terminal region, we conclude that our protein can be attributed to the To family. Therefore, we named it *Pc*To-like.

Despite the generally low sequence similarity, most To proteins and JHBPs are classified as ligand-binding proteins for juvenile hormones or similar hydrophobic terpenoids [44,46–49]. Because the precursor of the cyclic iridoid is also a terpenoid, we hypothesize that the putative protein could be involved in the iridoid biosynthesis in the defensive secretions. The assumption that the putative protein has relevance in the defensive glands is corroborated by the high transcript level which has been detected mainly in the glandular tissue of juvenile mustard leaf beetles (figure 2*c*). Low mRNA levels were also detectable in the fat body tissue.

(d) *RNA interference effects in larvae of* Phaedon cochleariae

A total of 0.3 μg of dsRNA derived from a 450-bp fragment of the *PcTo-like* sequence was injected into second instars of *P. cochleariae*. Transcript quantification 5 days after dsRNA injection confirmed a significant reduction of the mRNA in glandular tissue ($p = 0.043$) down to 1.0 per cent ($\pm 0.9\%$) compared with mRNA levels in *gfp* injections (figure 5*a*).

Phenotypic analyses after injection of dsRNA on the composition of low-molecular-weight compounds in the secretions were carried out by using GC/MS. The quality of the metabolites in samples collected 1 and 2 days after *Pc*To-like RNAi induction did not vary from the quality of the metabolites in those collected from *gfp* controls. In both treatments, we detected only the end-product 7. The first deviation in the composition of the secretions was measured 3 days after dsRNA injection. Only in samples triggered by *PcTo-like* RNAi did minor amounts of the postulated intermediate **6** in addition to **7** emerge. After 5 days, however, **6** clearly accumulated in addition to **7** owing to the RNAi effect (figure 4*b*). Therefore, we conclude that the *Pc*To-like has to be involved in the cyclization of monoterpene precursors into iridoids.

Off-target effects were predicted using the method described for *CpopSAO*, and predicted off-target effects were avoided from final dsRNA sequence by choosing the template for dsRNA outside of areas of predicted off-target effects.

4. DISCUSSION

The results of our larval RNAi experiments clearly demonstrate selective excision of a component in a biosynthetic pathway. To the best of our knowledge, RNAi has never been used to target enzymes in insect defensive secretions. Owing to the silencing of *Cpop*SAO, the chemical composition in the larval exudates of *C. populi* was massively altered, starting as early as 48 h after treatment. This shows a distinct function of this enzyme *in vivo*. Before, Kirsch *et al.* [13] showed activity only in *in vitro* assays. Evidently, RNAi is a valuable technique for identifying *in vivo* relevance for unknown proteins in defensive glands. Although insects contain a large number of exocrine glands in which bioactive compounds are produced, to date few studies have relied on RNAi to provide evidence for the *in vivo* function of enzymes in insect glands. One example is the production of sex pheromones in special glands of the silkmoth *Bombyx mori*. By injecting the pupae with dsRNA, Ohnishi *et al.* [50,51] were able to dissect the components of the biosynthetic pathway as well as assign a function to a transport protein within the glands of adults. Another RNAi target was the production of pheromone in jewel wasps, *Nasonia vitripennis*. Silencing an epoxide hydrolase in these insects resulted in pheromone reduction by 55 per cent and suppressed the targeted gene transcripts by 95 per cent [52]. Freshly emerged males were injected and 2 days later levels of transcript and pheromones were analysed. As our results demonstrate, RNAi effects are easily detectable in exocrine glands. In the secretions of immature *P. cochleariae*, we were able to assign *in vivo* relevance to a cDNA encoding a protein which is important for the cyclization of iridoids. The iridoid pathway in insects was already proposed by using deuterium labelled precursors by Weibel *et al.* [53]. In his work, the stereospecifity of the cyclization was analysed and allocated to an enzymatic conversion. However, to date JHBPs and

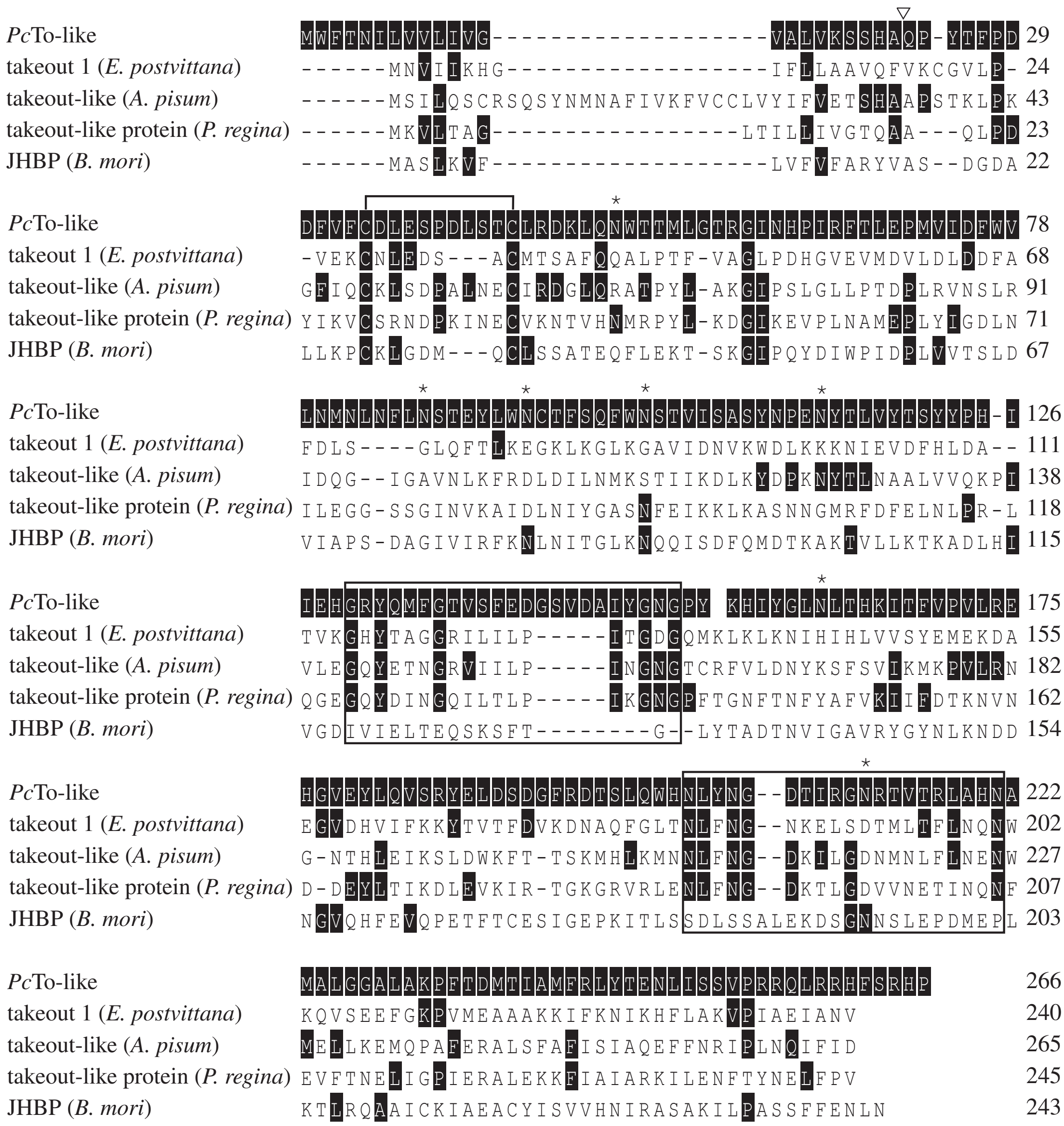

Figure 4. Amino acid alignments (ClustalW) of *Pc*To-like from *P. cochleariae* and other members from the To/JHBP family (*Epiphyas postvittana* To1: GeneBank: ACF39401; *Acyrthosiphon pisum* To-like: GeneBank: XP_001952685; *Phormia regina* To-like protein: GeneBank: BAD83405; *Bombyx mori* JHBP: GeneBank: AAF19267). Solid black shading depicts amino acids identical to *Pc*To-like sequence. White inverted triangles indicate the predicted signal peptide of *Pc*To-like. Asterisks mark the predicted N-glycosylation sites. Conserved cysteine residues that form disulphide bonds are marked with a bracket above the sequence. The two black boxes show the location of the To-typical motives.

To proteins have been established as being carriers of hydrophobic ligands [44,48]. Several lines of evidence indicate that JHBPs form complexes with juvenile hormones (JHs) which provide protection of the chemically labile JHs against nonspecific enzymatic degradation and/or adsorption to lipophilic surfaces during the delivery process from the production site to the target tissue [46,47,49]. Up to now only the crystal structure of To 1 from *E. postvittana* with ubiquinone provided direct evidence for ligand binding in To proteins [44]. Most of the putative To proteins await elucidation of their mode of action. Therefore, the actual mechanism how *Pc*To-like acts in the defensive exudates has to be analysed *in vitro* with purified recombinant protein. On-going experiments will reveal more functional enzymes in Chrysomelina and clarify the molecular machinery for the biosynthesis of deterrents in larval defence secretions.

To perform RNAi experiments, it is essential to ensure the specificity. Off-target effects can arise when siRNAs diced from long dsRNA fragments possess sufficient sequence similarity to non-target mRNA and thus triggering degradation of similar sequences [39]. For sequenced organisms, genome-scale off-target prediction programs are available [34]. These approaches are not suitable for organisms whose genomes have yet to be sequenced. In the last few years, several approaches have been used to detect off targets for those species, such as screening for target specificity by rapidly amplifying cDNA ends [54]. Another approach has used microarrays to compare the cDNAs from treated groups with those from non-treated groups; such comparisons offer proof of differentially expressed transcripts *via* qPCR [55]. Transcriptome sequences have rarely been used for approaches based on local alignment algorithms but represent an economical

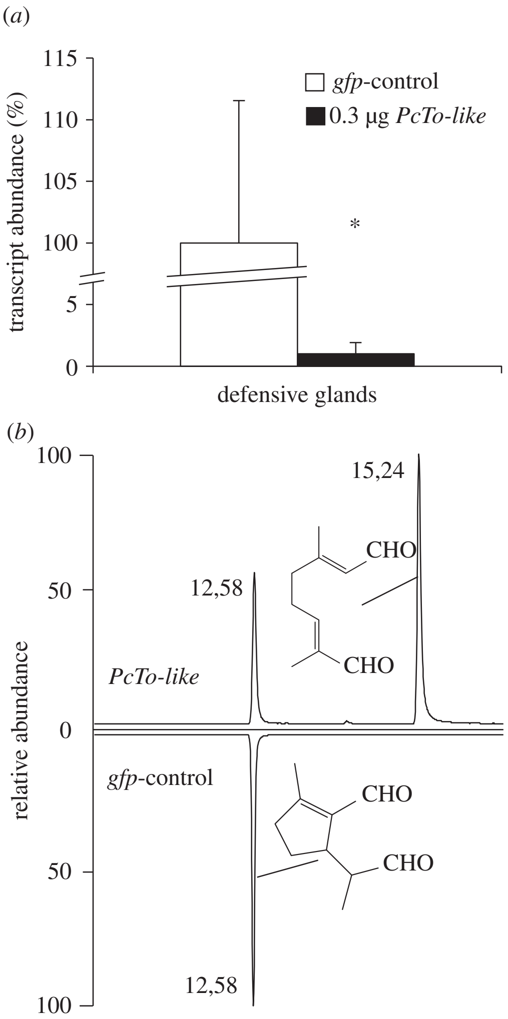

Figure 5. RNAi effects in juvenile *P. cochleariae*. (*a*) White bars: transcript abundance of *PcTo-like* after injecting 0.3 µg *gfp* dsRNA, $n = 3$ ±SD. Black bars: transcript abundance of *PcTo-like* after injecting 0.3 µg *PcTo-like* dsRNA, $n = 3$. 100% = ΔCq of *gfp*-control. Asterisks indicate level of significance: $*p < 0.05$. (*b*) GC-chromatogram of diluted secretions on day 5 after treatment, above: injecting 0.3 µg *PcTo-like* dsRNA resulted in the production of **6** and **7**; below: injecting 0.3 µg *gfp* dsRNA resulted in the production of **7**.

way to make off-target predictions [56]. In our case, we showed that *in silico* dicing of long dsRNA pieces to 21-bp fragments and subsequent BLASTn searches in our transcriptome libraries also lead to the identification of putative off-target transcripts. Subsequent qPCR analysis after successful RNAi induction revealed the co-silencing of predicted transcripts in *C. populi*. Two of eight mRNAs were significantly altered in gut tissue (see the electronic supplementary material, figure S2). But the observed off-target silencing could be assigned neither to the length of the fragments nor to the amount of pmol of the putative siRNAs (see the electronic supplementary material, table ST2). Furthermore, the composition and internal stability of the sequence fragments are supposed to have an impact on successful RNAi triggering [57] and could be included in the prediction. Although publications concerning off-target prediction have increased in the last 2 years, as yet no standard method is available. But as our results indicate, off-target validation is crucial for a realistic discussion of RNAi effects.

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R.R.B., P.R., M.S., M.K., N.W., W.B. and A.B. designed study. R.R.B. established RNAi in leaf beetles and performed RNAi treatments of *Cpop*SAO and its control treatments, performed off-target validation, collected all corresponding data except the off-target prediction and analysed output data. P.R. identified *PcTo-like*, performed RNAi treatments of *PcTo-like* and control treatments, collected all corresponding data and analysed output data. S.F., M.S. and M.G. generated transcriptome libraries. M.S. established and performed off-target prediction and contributed to interpretation of LC/MS^E output data. M.K. designed GC/MS assays, synthesized **6** and **7** and contributed to interpretation of output data. N.W. performed LC/MS^E analysis, collected and contributed to interpretation of output data. W.B. and A.B. contributed substantially to interpretation of all output data. R.R.B., P.R. and A.B. wrote first draft of the manuscript, and all authors contributed substantially to revisions.

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