Organic anion-transporting polypeptides are involved in the elimination of insecticides from the red flour beetle, *Tribolium castaneum*

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Abstract

Organic anion-transporting polypeptides (OATPs) are integral membrane transporters that mediate cellular uptake of a broad range of substrates in humans. The functions of OATPs in insects are less well investigated and only poorly understood. A large number of compounds potentially toxic to insects are organic anions that include secondary plant and insecticide-derived metabolites. Some insect *OATP* genes are expressed in metabolic, neuroprotective and excreting tissues, and they are co-expressed together with genes known to be involved in detoxification and excretion. Therefore, a role in the elimination of insecticides has been proposed for OATPs, but experimental proof was pending. The aim of this study was to identify OATPs that affect tolerance to insecticides in the red flour beetle, *Tribolium castaneum*, a genomic model species and stored product pest. We determined expression profiles of *TcOATP* genes in different tissues and developmental stages and analyzed RNAi phenotypes. We found that some *TcOATP* genes had particularly high transcript levels in relevant tissues and that knockdown of *TcOATP4-CI* led to severe developmental defects during larval–pupal molt. Then, we exposed the larvae to different chemically unrelated insecticides and analyzed transcript levels and mortalities. Some genes were specifically upregulated in response to insecticide treatment, and mortalities observed after administering certain insecticides were significantly increased when specific *TcOATPs* were silenced. By applying systemic RNAi in *T. castaneum*, we provide first evidence that OATPs are involved in the elimination of insecticides and hence may contribute to insecticide resistance, which becomes an increasingly serious problem in agriculture and forestry.

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Graphic abstract

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Key message

Insecticide resistance is largely based on target site insensitivity, metabolic detoxification and/or efflux pumps such as ATP binding cassette (ABC) transporters that excrete insecticides and/or metabolites. However, to facilitate entry into metabolic cells or transport across epithelia, influx systems are required that might contribute to insecticide resistance as well. In this study, we provide first evidence that organic anion-transporting polypeptides (OATPs) expressed in metabolic, protective and excretory tissues of insects are involved in the elimination of insecticides and/or their metabolites.

Introduction

Many insects are threatened by a multitude of potentially toxic xenobiotics such as plant secondary metabolites and pesticides. Frequently, xenobiotics comprise organic anions, including plant secondary metabolites and anthropogenic chemicals, such as indigo carmine, salicylate, acetylsalicylate and dicarboxylates as well as metabolites derived from pesticides such as 3-phenoxybenzoic acid and malathion monocarboxylic acid (Neufeld et al. 2005; O'Donnell and Rheault 2005). Organic anion transporters (OATs) and

organic anion-transporting polypeptides (OATPs) are integral membrane proteins that mediate transport of organic anions into cells, but despite their names, they can also transport cationic and neutral compounds including drugs, toxins and other xenobiotics across cellular membranes (Li et al. 2018, 2019). They belong to the solute carrier family (SLC) and hence to the major facilitator superfamily. While OATs group into subfamily SLC22A, OATPs group into subfamily SLC21A, which was renamed into SLCO in 2004 (Hagenbuch and Meier 2003). In human, OATPs are associated with the sodium-independent transport of a broad spectrum of amphipathic endogenous and exogenous organic compounds including therapeutic drugs such as statins, antibiotics, antihypertensives and chemotherapeutics like methotrexate. OATPs are less well examined with regard to their substrates in insects. However, transport of methotrexate by OATPs in the Malpighian tubules was extensively studied in *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) (Chahine and O'Donnell 2009). Other xenobiotic substrates identified to be transported by *Drosophila* OATPs are Texas Red and fluorescein (Chahine et al. 2012). Next to Malpighian tubules, OATPs are also expressed in insect's neural tissues, where they help to maintain the blood brain barrier, and in the midgut, where they prevent the entry of toxic compounds (Chahine et al. 2012; Hindle and Bainton 2014; Torrie et al. 2004). As some OATPs are co-expressed

together with genes encoding detoxification enzymes such as cytochrome P450s and glutathione-*S*-transferases or ABC transporters acting as efflux pumps such as the MDRs, it was suggested that they may play major roles in protecting from toxic xenobiotics (Dow and Davies 2006). Lepidopterans like the monarch butterfly, *Danaus plexippus* (L.) (Lepidoptera: Nymphalidae) and the large milkweed bug, *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae) for instance, feed on milkweed plants that contain Na⁺/K⁺-ATPase poisoning cardenolides (Agrawal et al. 2012). In these specialized insects, various transporters act together to regulate the balance between cardenolide efflux and sequestration to prevent poisoning (Frick and Wink 1995; Meredith et al. 1984). In the genome of *D. melanogaster*, eight *OATP* genes have been identified, of which three genes have been implicated in cardenolide binding and transport (Torrie et al. 2004). Groen et al. (2017) provided evidence that two of these OATPs are involved in protecting *Drosophila* flies from the toxic effects of cardenolides. Comparing oral toxicities of cardenolides in wild-type and mutant flies actually proved that OATPs contribute to protect against cardenolides, because cardenolide-treated *OATP* knockout mutants died more rapidly and suffered more from adverse neurological effects than wild-type flies (Groen et al. 2017).

Because insecticide resistance becomes an increasingly serious problem in agriculture and forestry, it is crucial to elucidate the underlying mechanisms. Insecticide resistance is largely based on target site insensitivity, metabolic detoxification and/or efflux pumps such as ATP binding cassette (ABC) transporters that excrete insecticides and/or metabolites (Merzendorfer 2014). However, to facilitate entry into metabolic cells or transport across epithelia also influx systems are required, which therefore could contribute to insecticide resistance. Due to their broad substrate specificity and their expression in metabolic and excretory tissues, OATPs have been suggested to be involved in the transport of synthetic insecticides, but direct proof of this hypothesis is pending. Next to other functional genomic techniques such as the GAL4/UAS system or CRISPR/Cas9 gene editing, systemic RNA interference (RNAi) has been proved to be a valuable tool for the identification of genes mediating insecticide resistance, as the knockdown of involved genes may increase susceptibility towards the insecticide analyzed (Homem and Davies 2018). For instance, RNAi-mediated silencing of genes encoding specific cytochrome P450 enzymes known to be involved in metabolic detoxification of insecticides has been shown to result in greater mortality in response to insecticide treatment, as the effective concentrations of the insecticide increase due to the lack of detoxifying enzymes (Guo et al. 2016; Wang et al. 2020).

To study potential functions of OATPs in insecticide resistance, we characterized the *OATP* gene family from the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera:

Tenebrionidae), which has developed into a powerful model for investigating the mode of action of insecticides and resistance mechanisms (Rösner et al. 2020). Particularly, *T. castaneum* shows a robust response to systemic RNA interference (RNAi), which allows efficient analysis of gene functions. First, we determined the expression profiles of four *TcOATP* genes, which we could identify in the *T. castaneum* genome and analyzed corresponding RNAi phenotypes. We found that some *TcOATP* genes had particularly high transcript levels in metabolic or excretory tissues such as the fat body or the midgut, respectively. RNAi-mediated knockdown of the expression of three *TcOATP* genes did not reveal apparent phenotypes, but RNAi for *TcOATP4-C1* led to lethal developmental defects during larval–pupal molt. Next, we exposed the larvae to different synthetic insecticides to analyze transcript levels and mortalities in response to these treatments. To test whether OATPs are involved in their elimination by metabolic and/or excretory tissues from *T. castaneum*, we have chosen four chemically unrelated insecticides, β -cyfluthrin (CF), diflubenzuron (DFB), malathion (MAL) and tebufenozide (TBF). Some *TcOATP* genes were significantly upregulated in response to insecticide treatment in metabolic or excretory tissues. Finally, we were able to show that the mortality in response to certain insecticides was significantly increased when specific *TcOATP* genes were silenced. Our study provides evidence that OATPs have a function in lowering the sensitivity to insecticides. Hence, OATPs may act in concert with other transporters such as other members of the major facilitator superfamily or ABC transporters to cope with challenges opposed by insecticides.

Material and methods

Insects

All analyses performed in this study were conducted with the *T. castaneum* strain GA-1 (Haliscak and Beeman 1983). The beetles are derived from a strain collection maintained at RW Beeman's lab (US Department of Agriculture, Manhattan, KS, USA) and are cultured in the lab of H Merzendorfer since 2009. The beetles were reared in continuous darkness on whole wheat flour supplemented with 5% (w/w) Baker's yeast at a temperature of 30 °C and a relative humidity of 70%, as described previously (Beeman and Stuart 1990).

Chemicals

Chemicals were purchased in p.a. quality from Sigma-Aldrich (Taufkirchen, Germany), Roth (Karlsruhe, Germany) and Serva (Heidelberg, Germany). Sulforhodamine 101 acid chloride (Texas Red, TR), β -cyfluthrin (CF),

diflubenzuron (DFB), malathion (MAL) and tebufenozid (TBF) were obtained from Sigma-Aldrich (Taufkirchen, Germany).

Gene expression studies by qPCR

Total RNA was isolated either from pools of three larvae, pupae and adults, or from different larval or pupal tissues (head, midgut, Malpighian tubules, elytra and fat body) with the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation and transcribed to cDNA using the SuperScript III First-Strand Synthesis System (ThermoFisher, Dreieich, Germany). qPCR was performed with the iCycler iQ Realtime PCR Detection System and iQ SYBR Green Supermix following the manufacturer's recommendations (BioRad, Hercules, USA) using pairs of gene-specific primers (Tab. S1). The specificity of the PCR was confirmed by melting curve analysis and mean normalized expression was determined according to (Simon 2003). Relative expression was calculated based on the comparison of CT values for the respective target gene and the selected reference gene *TcRPS6*. Transcript levels of *TcRPS6* vary just very little between different developmental stages and different circumstances (Arakane et al. 2009). Heat maps were generated using the Morpheus software (<https://software.broadinstitute.org/morpheus>). For this purpose, mean normalized expression values were converted into logarithmic data. Expression levels of *TcOATP* genes in response to insecticide treatments were normalized by the expression of the respective gene in untreated larvae.

Insecticide treatment

The wheat flour was supplemented with insecticides as follows: per 50 g of flour, 50 ml of acetone containing the desired amount of CF, DFB, MAL or TBF was added at the indicated concentration. The suspension was mixed for 30 min, and the acetone was evaporated under a fume hood for four days. Control diets were prepared exactly as described above but without the addition of the insecticide. Insects were treated with the insecticides at concentrations, which have low to moderate effects on mortality to allow the evaluation of RNAi effects on insecticide-induced mortality.

dsRNA synthesis and injection for RNAi experiments

Total RNA from appropriate developmental stages was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's recommendation and transcribed into cDNA with the SuperScript III First-Strand cDNA Synthesis Kit (ThermoFisher, Dreieich, Germany). *TcOATP*-cDNAs were amplified using gene-specific primers (Tab. S1) and the total cDNA as a template. PCR products were ligated into

the pGEM-T vector (Promega, Mannheim, Germany) and the resulting plasmids were used to synthesize dsRNA as described previously (Broehan et al. 2010). Briefly, the plasmids with the respective *TcOATP*-cDNA insert were used as templates in a PCR with sequence specific primers containing a T7 promoter sequence at their 5'-ends (Tab. S1). After agarose gel electrophoresis, the resulting PCR products were excised and purified using the QIAquick Gel Extraction Kit from Qiagen (Hilden, Germany). The obtained PCR products were then used as templates for dsRNA synthesis, which was performed with the Ampli-Scribe T7-Flash transcription kit according to the manufacturer's protocol (Epicentre, Madison, USA). RNAi experiments were performed with at least 50 mid-sized larvae. They were ether-anesthetized and injected with 200 nl of the respective *TcOATP*-dsRNA (1 µg/ml dsRNA in a 0.1 mM potassium phosphate buffer containing 5 mM KCl, pH 7). The insects were kept under standard conditions for monitoring RNAi phenotypes and further analyses. As a control, 200 ng of ds*TcVER* was injected to knockdown the tryptophan oxygenase encoding gene *Vermilion* (*TcVer*), which results in a white-eye phenotype. Three days after injection of dsRNA, larvae were used for functional transport assays as described above and total RNA was prepared from pools of three injected insects using the RNeasy Mini Kit (Qiagen, Hilden, Germany) to monitor RNAi efficiency by qPCR (see Fig S1).

Other methods

Measurements of TR fluorescence in ds*TcVER* and ds*TcOATP* injected larvae were performed as described previously (Rösner and Merzendorfer 2020).

Results

Phenotypes induced by RNAi for *TcOATP* genes

To investigate the functions of four *TcOATP* genes, which were identified in the genome of *T. castaneum* based on homologies of *OATP* genes from *D. melanogaster*, RNAi-mediated gene silencing experiments were performed to knockdown *TcOATP1-A6*, *TcOATP4-C1*, *TcOATP5-A1.1* and *TcOATP5-A1.2* (RefSeq and Gene IDs: Tab. S2). ds*TcVER* was injected as a positive control to knockdown the *Vermillion* gene, which results in a white-eye phenotype (Fig. 1). In this set of experiments, we found that the knockdown of three *TcOATP* genes, *TcOATP1-A6*, *TcOATP5-A1.1* and *TcOATP5-A1.2*, did not reveal apparent developmental phenotypes or RNA-induced mortalities (Fig. 1). However, RNAi to knockdown *TcOATP4-C1* caused a phenotype, which resembled known RNAi phenotypes when silencing *TcABCG-8A*, a gene encoding a ABC transporter subfamily

Fig. 1 Effects of RNAi for *TcOATP* genes on the development of *T. castaneum*. dsRNA specific for *TcVER* and *TcOATP* genes was injected into mid-sized instar larvae. **(a)** *dsTcVER*: normal development, except for the expected white-eye phenotype. **(b)** *dsTcOATP1-A6*: normal development. **(c)** *dsTcOATP4-C1*: premature compound eye in last instar larvae, developmental arrest during larval–pupal molt with a hippocampus-like phenotype **(d)** *TcOATP5-A1.1*: normal development. **(e)** *dsTcOATP5-A1.2*: normal development. **(f)** *dsTcOATP4-C1*: premature development of adult compound eye at the last larval instar

G member and *TcEcR-A* and *TcEcR-B*, genes encoding ecdysterone receptor variants (Broehan et al. 2013). Similar to these phenotypes, injection of *dsTcOATP4-C1* into mid-sized larvae led to the premature development of compound eyes. The insects arrested at the larval to pupal molt showing a hippocampus-like morphology and a final mortality of about 80% apparently due to the failed molting (Fig. 1 and Tab. S3). Silencing *TcOATP5-A1.2* by injecting dsRNA into mid-sized larvae showed a mortality of about 20%, but did not reveal apparent developmental phenotypes. RNAi for all *TcOATP* genes tested did not affect larval weight when compared to control larvae (Fig. S2).

TcOATP genes are differentially expressed in different tissues and developmental stages

To get further insight into the function of *TcOATP* genes, we analyzed the expression patterns in different tissues and in the course of development of *T. castaneum*. For this purpose, we performed qPCR using different sets of gene-specific primers (Tab. S1) for the following developmental stages and tissues: larvae, pupae, adults, larval head, larval Malpighian tubules, larval midgut, larval fat body and pupal elytra. Mean normalized expression levels (see Tab. S4) were translated into a heat map for a more vivid illustration (Fig. 2).

The analyzed *TcOATP* genes are differentially expressed during development and in the tested tissues (Fig. 2). Transcript levels were particularly high for *TcOATP1-A6* in all developmental stages and tissues except Malpighian tubules, which express this gene at more moderate levels

Fig. 2 Expression of *TcOATP* genes in different tissues and developmental stages of *T. castaneum*. Total RNA was isolated from pools of larvae and transcribed into cDNA, which was used as a template for qPCRs with primers specific to the respective genes. Mean normalized expression was determined by comparing CT values of the respective target gene and the reference gene *TcRPS6*. Values are given as means $\pm$ S.D. ($n=3$). L, larvae; P, pupae; A, adults; H, larval head; M, larval midgut; MT, larval Malpighian tubules; E, pupal elytra; F, larval fat body. White color indicates no detectable expression, and red colors indicate low to high expression levels with increasing intensities. Absolute expression values are given in Tab. S4

compared to the high levels observed in the other tissues. qPCR for *TcOATP4-C1*, *TcOATP5-A1.1* and *TcOATP5-A1.2* revealed moderate gene expression levels, with somewhat higher transcript levels in larvae, larval head and pupal elytra for *TcOATP4-C1*, in pupal elytra for *TcOATP5-A1.1* and in larvae, adults, larval Malpighian tubules and larval fat body for *TcOATP5-A1.2*. No expression or low transcript levels were detected in larval Malpighian tubules for *TcOATP4-C1* and in pupae, adults and larval fat body for *TcOATP5-A1.1*.

Expression of *TcOATP* genes in response to insecticide treatment

As it was shown that *OATP* genes are expressed in metabolic and excretory tissues and it is known that they have a broad range of transport specificities, we hypothesized that at least some *OATPs* may contribute to the elimination of insecticidal compounds. For the identification of *OATPs* that are possibly involved in the elimination of insecticides, we determined expression levels of the identified *TcOATP* genes in response to the treatment of larvae with four chemically unrelated insecticides, CF, DFB, MAL and TBF (Fig. 3 and Tab. S5). What we obtained was a mixed picture with differentiated transcriptional responses. While transcript levels for *TcOATP1-A6* and *TcOATP5-A1.2* were downregulated upon treatment with CF, MAL and TBF, they were moderately upregulated in response to DFB. In contrast, transcript levels for *TcOATP4-C1* were upregulated to various degree in response to all four insecticides. A more pronounced effect was observed for *TcOATP5-A1.1*, which was more strongly upregulated after CF, MAL and TBF exposure, but slightly downregulated in response to DFB treatment.

To examine the effects of insecticide exposure on *TcOATP* gene expression at the levels of neuronal, excretory or metabolic tissues, we isolated heads, midguts, Malpighian tubules and fat body from control larvae and from larvae which were treated with CF, DFB, MAL or TBF. The transcript levels were determined by qPCR relative to the expression of untreated larvae. As shown in Fig. 4,

Fig. 3 Expression of *TcOATP* genes in response to insecticide treatments in whole larvae from *T. castaneum*. Expression was analyzed by qPCR using mid-sized larvae treated with 10 ppm CF, 0.1 ppm DFB, 100 ppm MAL and 100 ppm TBF for 6 days. The expression levels were normalized by the expression of the respective gene in untreated larvae. Total RNA was isolated from pools of larvae and transcribed into cDNA, which was finally used as a template for qPCR with primers specific to the respective gene. Mean normalized expression was determined by comparing CT values of the respective target gene and the reference gene *TcRPS6*. White colors indicate no changes in expression, blue colors indicate downregulation of gene expression, and red colors indicate upregulation of gene expression. The more intense the color, the more pronounced are the changes in expression levels. Absolute expression values are given in Tab. S5

Fig. 4 Expression of *TcOATP* genes in response to insecticide treatment in different tissues from *T. castaneum*. Expression was analyzed by qPCR using mid-sized larvae treated with 10 ppm CF, 0.1 ppm DFB, 100 ppm MAL and 100 ppm TBF for 6 days. The expression levels were normalized by the expression of the respective gene in untreated larvae. Total RNA was isolated from tissues of head, midgut, Malpighian tubules and fat body and transcribed into cDNA, which was finally used as a template for qPCR with primers specific to the respective gene. Mean normalized expression was determined by comparing CT values of the respective target gene and the reference gene *TcRPS6*. White colors indicate no changes in expression, blue colors indicate downregulation of gene expression, and red colors indicate upregulation of gene expression. The more intense the color, the more pronounced are the changes in expression levels. Absolute expression values are given in Tab. S6

gene expression levels after CF treatment were increased for *TcOATP5-A1.1* in all tested tissues, with the strongest effect observed in Malpighian tubules (see also Tab. S6). In contrast, CF exposure led to down-regulation of transcripts for *TcOATP1-A6* in these tissues, except for Malpighian tubules where transcript levels were increased. Generally, CF treatment induced the expression of all tested *TcOATP* genes in Malpighian tubules, which may indicate that *OATPs* are involved renal excretion of CF or their metabolites. Treatment with DFB resulted in elevated transcript levels for all tested *TcOATP* genes in the head being highest for *TcOATP5-A1.1*, whose expression was also found upregulated in midgut and fat body. The transcript levels were almost unaffected for all *TcOATP* genes in Malpighian tubules in response to DFB treatment. Notably, *TcOATP1-A6* and *TcOATP5-A1.2* expression is downregulated in response to DFB treatment in fat body and exclusively in the midgut for *TcOATP1-A6*. Treatment with MAL revealed an induction of *TcOATP* gene expression to various degrees in almost all tissues. Highest transcript levels were detected for *TcOATP5-A1.1* in the head and fat body, and gene expression levels of all *TcOATP* genes were elevated in Malpighian tubules. MAL treatment resulted in decreased gene expression only in the midgut and only for *TcOATP1-A6*. An overall similar

picture was obtained for TBF treatment; however, in this case transcript levels additionally decreased in the fat body for *TcOATP1-A6* and *TcOATP5-A1.2* (Fig. 4).

TcOATP genes function in the elimination of Texas Red

Texas Red (TR) has been demonstrated to be a transport substrate of both, ABCC transporters and OATPs in insects (Chahine et al. 2012). In addition, it acts competitive to the transport of DFB by certain ABC transporters in *T. castaneum* (Rösner and Merzendorfer 2020). Therefore, we employed a previously established TR fluorescence assay in combination with RNAi, to examine whether specific OATPs are involved in TR elimination in *T. castaneum*. As shown in Fig. 5, TR is significantly excreted from larvae within 48 h after TR injection under control conditions. RNAi to knockdown *TcOATP 5-A1.2* and *TcOATP1-A6* resulted in only minor reduction in TR elimination as indicated by an increase in fluorescence by 1.6% after 24 h and 10.9% after 48 h for *TcOATP 5-A1.2* and 23.7% after 24 h and 4.7% after 48 h for *TcOATP1-A6* (Fig. 5). However, RNAi to knockdown *TcOATP4-C1* and *TcOATP5-A1.1* resulted in a more substantial reduction in TR elimination as indicated by a significant increase in fluorescence by 42.1% and 48.8% after 24 h and 36.3% and 37.6% after 48 h, respectively. Hence, we conclude that two OATPs, *TcOATP4-C1* and *TcOATP5-A1.1*, are involved in TR elimination and thus have overlapping substrate specificities.

Fig. 5 Intensity of Texas Red fluorescence in mid-sized *T. castaneum* larvae after knockdown of *TcOATP* genes. Knockdown of indicated *TcOATP* genes was performed by RNA interference and verified via qPCR. Total fluorescence was measured as intensity per mm² after 0, 24 and 48 h. Analysis was performed by Quantity One (BioRad). Values are given in means of percent ($\pm$ S.D., $n = 50$) of the fluorescence intensity measured for the control. The given p -values (***) $p < 0.001$) refer to the fluorescence intensity of the control at the corresponding time

TcOATP genes function in the elimination insecticides

As we have shown that gene expression of some *TcOATP* genes is upregulated in metabolic and excretory tissues in response to the treatment with certain insecticides, we speculated that the respective *TcOATPs* could play a role in their elimination. To test this hypothesis, we treated *dsTcOATP*-injected larvae with CF, DFB, MAL and TBF at different concentrations and compared resulting mortalities with those obtained from larvae that were injected with *dsTcVER*, which does not affect insecticide-induced mortality (Rösner and Merzendorfer, 2020). We used insecticide concentrations in the range of 1 and 10 ppm (CF), 0.1 and 1 ppm (DFB), as well as 100 and 1000 ppm (MAL, TBF), which resulted in a mortality of maximal 40% after two weeks of continued treatment, to facilitate evaluation of RNAi effects.

The observed mortalities of 5 to 10% of control larvae that were not exposed to the respective insecticide (0 ppm) is due to the control injection (Fig. 6 a, b, c and d). These larvae typically died three days post-injection, while insecticide-treated insects died later at the concentrations used. Treatment with the pyrethroid CF at concentrations of 1 and 10 ppm resulted in mortalities of about 20 and 35%, respectively (Fig. 6a). RNAi to knockdown *TcOATP* expression did not increase CF-induced mortality for any of the tested genes. Note the high mortality of *dsTcOATP4-C1* injection of about 80% in absence of CF, which is due to essential physiological or developmental functions of *TcOATP4-C1*. Treatment with the chitin synthesis inhibitor DFB at concentrations of 0.1 and 1.0 ppm resulted in higher mortalities in *dsTcOATP1-A6*-injected larvae with a significant increase of 35% at 1.0 ppm (Fig. 6b). RNAi-mediated silencing of *TcOATP4-C1* resulted in an increase in mortality from 82% at 0 ppm to 100% at 0.1 ppm and 1.0 ppm. The mortalities were slightly higher than those obtained for *dsTcOATP4-C1*-injected larvae after CF or any other insecticide treatment. Therefore, *TcOATP4-C1* may have some additional function in DFB elimination. In contrast, RNAi for *TcOATP5-A1.1* and *TcOATP5-A1.2* did not affect DFB-induced mortality. Treatments of *T. castaneum* larvae with the organophosphate MAL or the ecdysone agonist TFB were generally less efficient in *T. castaneum* than treatments with CF and DFB. MAL treatment at concentrations of 100 and 1000 ppm resulted in higher mortalities in *dsTcOATP1-A6* and *dsTcOATP5-A1.1* injected larvae (Fig. 6c). While RNAi for *dsTcOATP1-A6* led to a significant increase of 14% at 100 ppm and 23% at 1000 ppm, RNAi for *dsTcOATP5-A1.1* led to a significant increase of 39% at 100 ppm and 60% at 1000 ppm MAL concentration. RNAi for *TcOATP4-C1* and *dsTcOATP5-A1.2* did not lead to a significant increase in MAL-induced mortalities. Finally,

Fig. 6 Effects of RNAi for *TcOATP* genes on insecticide-induced mortality. Mortality of *T. castaneum* larvae in response to treatment with selected insecticides was monitored after RNAi-mediated knockdown of *TcOATP* genes at indicated concentrations observed for 14 days. Values are given as means $\pm$ S.D. ($n=50$). (a) CF

(cyfluthrin: 0.0, 1.0 and 10 ppm); (b) DFB (diflubenzuron: 0.0, 0.1 and 1.0 ppm); (c) MAL (malathion: 0.0, 100 and 1000 ppm); (d) TBF (tebufenozide: 0.0, 100 and 1000 ppm). The given p -values (** $p<0.01$, *** $p<0.001$) refer to mortalities of the control at the corresponding insecticide concentration

none of the tested knockdowns of *TcOATP* genes resulted in an increase in TBF-induced mortality (Fig. 6d).

Discussion

OATPs are members of the highly conserved SLCO superfamily of SLC transporters, encoding membrane transporters with 12 predicted transmembrane domains (Hagenbuch and Stieger 2013). They mediate sodium-independent cellular uptake of numerous endogenous substrates, as well as drugs and other xenobiotics. In insects, only a few OATPs have been recognized to transport xenobiotics such as methotrexate, fluorescein and TR and most studies were performed in *D. melanogaster* (O'Donnell et al. 2003). Although several authors suggested that specific OATPs may play a role in resistance to synthetic insecticides, experimental proof was pending. Moreover, OATPs have not been analyzed in beetles, which is the largest insect order comprising numerous economically important pest species. Therefore, we have chosen the genomic model beetle species and stored product pest, *T. castaneum*, to analyze *TcOATP* gene functions by systemic RNAi, which is highly robust and reliable in this species. Moreover, *T. castaneum* rapidly develops resistances to many classes of insecticides making this beetle a useful genomic model to

study the mode of action of insecticides and the mechanisms of resistance (Rösner et al. 2020).

We searched the *Tribolium* genome for genes that are homologous to *D. melanogaster* OATPs and identified four genes that potentially encode OATPs from *T. castaneum* (*TcOATPs*). The *TcOATP* genes are differentially expressed during development and in different tissues. The RNAi-based gene silencing revealed an interesting phenotype for *TcOATP4-C1*, which is characterized by premature adult eye development and arrest during the larval to pupal molt. The insects showed a hippocampus-like morphology and a final mortality of about 80% apparently due to failed molting. *TcOATP4-C1* is a putative orthologue of the *D. melanogaster* *OATP74D* gene, which has been described to play a role in cellular uptake of ecdysteroids (Okamoto et al. 2018). Loss of function of this *Drosophila* gene causes phenotypes that resemble those for flies deficient in ecdysteroids or ecdysteroid receptors. Comparing the RNAi phenotype for *TcOATP4-C1* and *Tribolium* genes involved in ecdysteroid signaling, such as *TcABCG-8A*, a putative ecdysteroid transporter (Broehan et al. 2013), or *TcECR-A* and *TcECR-B*, two isoforms of the ecdysteroid receptors (Broehan et al. 2013; Tan and Palli 2008), revealed striking similarities including premature adult compound eye development in the last larval instar and lethal abortive molting. Based on these findings,

we propose that *TcOATP4-C1* is a functional orthologue of *OATP74D*, acting as a cellular importer of ecdysteroids.

Next, we went on to analyze the insecticidal effects of four chemically unrelated pesticides from different classes, which are frequently used in agriculture, forestry and health protection: CF (a type-II pyrethroid), DFB (a benzoylphenyl urea), MAL (an organophosphate) and TBF (a diacylhydrazine). Particularly, we determined the expression levels of *TcOATPs* in response to treatment with these insecticides at a comparably low dose and for a short period of only six days. The analysis by qPCR showed that transcript levels of some *TcOATP* genes were altered in response to treatments with these insecticides. We knocked down their expression by RNAi and monitored mortality in the presence of the four insecticides. Global expression of *TcOATP4-C1* and *TcOATP 5A1.1* was induced in larvae in most of the insecticide treatments, except for DFB treatment, which led only to slightly increased expression for *TcOATP4-C1* and as slight decrease in expression for *TcOATP 5A1.1*. Contrarily, the expression of *TcOATP1-A6* and *TcOATP5-A1.2* was down-regulated in most insecticide treatments, except for DFB.

Except for the treatment with CF, all *TcOATP* genes were significantly upregulated in the head in response to insecticide treatments and *TcOATP4-C1* and *TcOATP5-A1.1* were the genes that were upregulated to various degree in all tested tissues, namely in the head, midgut, Malpighian tubules and fat body.

The knockdown of *TcOATP1-A6* and *TcOATP5-A1.1* led to a significant increase in DFB- and MAL-mediated mortality, suggesting they play a role in the elimination of these two insecticides. Notably, *TcOATP1-A6* was upregulated in the head and Malpighian tubules in response to DFB and *TcOATP5-A1.1* was upregulated in all tested tissues, but most pronounced in head and fat body. This may suggest that these two *TcOATP* genes have a role in protection at the blood brain barrier of *T. castaneum* next to additional functions in the Malpighian tubules and fat body. Actually, the *Drosophila* gene *Oatp58Dc* has been shown to be involved in maintaining the brain–blood barrier by preventing organic anion entry into the brain (Seabrooke and O'Donnell 2013). In particular, MAL acts on the central nervous systems by poisoning the active site of the acetylcholinesterase released into the synaptic cleft. Therefore, *TcOATP1-A6* and *TcOATP5-A1.1* expressed by cells of the blood–brain barriers may contribute to decrease toxic MAL concentrations in the brain. In addition, *TcOATP1-A6* may have a role in transepithelial transport for the renal excretion of DFB or its metabolites and *TcOATP5-A1.1* in MAL uptake into metabolic fat body cells. In case of the chitin synthesis inhibitor DFB (Merzendorfer et al. 2012), we could show previously that different ABC transporters expressed in metabolic and excretory tissues contribute to the elimination (Rösner

and Merzendorfer 2020). MAL uptake into fat body cells is supported by the previous finding that metabolic MAL resistance in *T. castaneum* is mediated by increased carboxylesterase activities (Haubruge et al. 2002), mediated by enzymes that are detected in the proteome of the insect fat body (Birner-Gruenberger et al. 2012). Notably, *TcOATP5-A1.1* may also mediate uptake of TR into fat body cells, as we have shown it previously by cryosectioning of TR injected *T. castaneum* larvae and fluorescence microscopy (Rösner and Merzendorfer 2020). This is supported by our finding that RNAi-mediated knockdown of *TcOATP5-A1.1* impairs TR elimination significantly. This is also the case for *TcOATP4-C1*, which is expressed in fat body cells.

Taken together, our study provides first insight into the functions of OATPs in *T. castaneum* and suggests that *TcOATP1-A6* and *TcOATP5 A1.1* are involved in the elimination of MAL and DFB in this species. Hence, the transport of insecticides and their metabolites across epithelia requires both, importers and exporters, being active in different tissues to mediate metabolic detoxification, excretion or sequestration. Therefore, different sets of transporters with different substrate specificities act together to prevent accumulation of toxic compounds. The identified *TcOATP* genes may further be suitable targets for transport inhibitors or gene silencing by RNAi, to increase efficiency of DFB and MAL and overcome resistance. Moreover, we could identify a *TcOATP* gene, which is required for proper molting and is potentially involved in the transport of ecdysteroids in *T. castaneum*. This transporter in turn may be a suitable candidate gene for highly species-specific RNAi-mediated pest control regimes, as the knockdown leads to a high mortality at the larval–pupal molt.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Consent for publication Not applicable.

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Code availability Not applicable.

Data Availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethical approval This is an observational study on insects. According to the German animal protection law, no ethical approval is required.

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