

Research Paper

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Aldehyde oxidases mediate plant toxicant susceptibility and fecundity in the red flour beetle, *Tribolium castaneum*

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Abstract

Aldehyde oxidases (AOXs) are a group of metabolic enzymes that play critical roles in the degradation of xenobiotics and chemicals. However, the physiological function of this enzyme in insects remains poorly understood. In this study, three *TcAOX* genes (*TcAOX1*, *TcAOX2*, *TcAOX3*) were identified and characterized from *Tribolium castaneum* genome. Spatiotemporal expression profiling showed that *TcAOX1* expression was most highly expressed at the early pupal stage and was predominantly expressed in the antennae of adults, indicating that *TcAOX1* was involved in the degradation of chemical signals; *TcAOX2* expression was most highly expressed at the late pupal stage and was mainly expressed in the fat body, epidermis of larvae and adults, respectively; and *TcAOX3* expression was in all stages and was primarily expressed in the head of adults. Moreover, the transcripts of *TcAOX2* and *TcAOX3* were significantly induced after exposure to plant oil, and RNA interference (RNAi) targeting of each of them enhanced the susceptibility of beetles to this plant toxicant, suggesting that these two genes are associated with plant toxicant detoxification. Intriguingly, knockdown of the *TcAOX1* led to reductions in female egg-laying but unchanged the hatchability and the development of genital organs, suggesting that this gene may mediate fecundity by effecting the inactivation of chemical signals in *T. castaneum*. Overall, these results shed new light on the function of AOX genes in insects, and could facilitate the development of research on pest control management.

Introduction

Aldehyde oxidases (AOXs; EC 1.2.3.1), a group of metabolic enzymes, are a kind of proteins which belong to the family of molybdo-flavoenzymes (MFE) along with the structurally related xanthine dehydrogenase (XDH) enzymes (Terao *et al.*, 2016; Xu and Liao, 2017). The structures of these enzymes are composed of a 20-kDa N-terminal 2x[2Fe-2S] domain-containing two iron-sulfur centers, a 85-kDa C-terminal molybdopterin-binding domain with the four redox centers aligned in an almost linear fashion, and a central 40-kDa FAD containing domain (Enroth *et al.*, 2000; Terao *et al.*, 2000; Garattini *et al.*, 2003). Although these domains have similar amino acid sequences in all the members of the family, these enzymes are difference in terms of substrate and inhibitor specificity as well as cofactor requirement and biochemical function (Garattini *et al.*, 2003; Hille, 2005). According to research findings, AOXs mainly catalyze the oxidation of aromatic heterocycle or aromatic aldehydes into corresponding carboxylic acids (Beedham *et al.*, 1995), while XDH play critical roles in the metabolism of some purines, and catabolizing hypoxanthine into xanthine and xanthine into uric acid (Enroth *et al.*, 2000).

AOXs widely distribute in organisms including plants and animals, and participate in a variety of physiological and biochemical functions (Huang and Ichikawa, 1994; Hemingway *et al.*, 2000). The physiological function of aldehyde oxidase was first studied in plants (Mendel and Hänsch, 2002), and AOXs are believed to participate in the regulation of plant growth, development and environmental adaptability by catalyzing the synthesis of abscisic acid (ABA) and indoleacetic acid (IAA) (Sagi *et al.*, 2002; Barrero *et al.*, 2006). Among mammals, AOXs are distinguished by broad substrate specificity, catalyzing the oxidation of various types of aldehydes, nitrogen heterocycles and oxygen heterocycles of pharmacological and toxicological significance (Schumann *et al.*, 2009; Pryde *et al.*, 2010; Garattini and Terao, 2011; Mahro *et al.*, 2011). Distinguishingly, most of the research on the function of insect AOXs has focused on the degradation of olfactory molecules, which seems to be a unique function of insect AOXs (Rybczynski *et al.*, 1989). In addition, insect AOXs also has been reported to be associated with insecticide resistance by coamplifying with insecticide resistance-associated esterase (Hemingway *et al.*, 2000; Coleman *et al.*, 2002). The initial research of insect AOXs merely

discussed its physiological activity at the biochemical detection level (Rybczynski *et al.*, 1989). But with the rapid development of modern molecular biology, the features of many AOX genes at the molecular level have been identified and characterized successively from insects such as *Bombyx mori* (Pelletier *et al.*, 2007), *Mamestra brassicae* (Merlin *et al.*, 2005), *Amyelois transitella* (Choo *et al.*, 2013), *Drosophila melanogaster* (Garattini *et al.*, 2008), *Helicoverpa armigera* (Xu and Liao, 2017). In brief, these findings greatly enriched the understanding of the functions of insect AOXs.

Currently, the role of insect AOXs in the process of olfactory becomes increasingly clear (Choo *et al.*, 2013; Xu and Liao, 2017). To maintain the fidelity and sensitivity of an insect's olfactory system, odorant-degrading enzymes (ODEs) including AOXs are deemed to inactivate odorant molecules after they convey their signals (Leal, 2013; He *et al.*, 2017). Functional research on AOXs showed that the recombinant AtraAOX2, which was the first activity characterization of a recombinant insect AOX *in vitro*, could degrade sex pheromone and plant volatile aldehydes as substrates (Choo *et al.*, 2013), which indicated that AOX did play a role in inactivating odorant molecules. Meanwhile, BmAox5 in the pheromone gland of *B. mori* participates in the degradation of aldehyde pheromone substances and odorant compounds also illustrates this point (Zhang *et al.*, 2020). Recently, the activity analysis of AOX acting on substrates *in vitro* revealed that PxyAOX3 from the antennae of *Plutella xylostella* protected olfactory neuron by inactivation of redundant odorants and detoxification of xenobiotics (Wang *et al.*, 2021). Altogether, these results revealed that AOX is potentially involved in the degradation of pheromones, plant-derived volatiles and the detoxification of xenobiotics. Of course, AOX from the silk gland of *B. mori* also could catalyze indole-3-acetaldehyde to indole-3-acetic acid (Takei *et al.*, 2019). However, these findings on insect AOX mainly in lepidopteran, especially for antennae-specific AOXs. The research of other insect orders AOXs and non-tissue specific AOXs are still rare.

The red flour beetle, *Tribolium castaneum*, is a coleoptera model pest, and the genome sequences of *T. castaneum* have been well characterized (Richards *et al.*, 2008), thus further improving research utilizing this species. However, the physiological function of AOX in coleoptera remains unclear. In light of this, our objective here was to identify and characterize TcAOX in *T. castaneum*, which, to the best of our knowledge, represents the first time AOX enzymes have been analyzed in a coleopteran. This study will help improve our understanding of the complex function of AOXs and lay the foundation for coleoptera insect defense research and development of an insect medication model.

Materials and methods

Experimental animals

The *T. castaneum* strain Georgia-1 (GA-1) was used for all experiments. The incubator conditions were as described previously (Li *et al.*, 2011). Briefly, the insects were raised in a jar, loaded with wheat flour containing 5% brewer's yeast in a growth chamber maintained at 30 °C and 40% relative humidity under a 14-h light/10-h dark cycle.

RNA purification and reverse transcription

The samples of eight developmental stages were collected as follows: early eggs (EE, 1 day old), late eggs (LE, 3 days old), early

larvae (EL, 1 day old), late larvae (LL, 20 days old), early pupae (EP, 1 day old), late pupae (LP, 5 days old), early adults (EA, 1 day old), and late adults (LA, 10 days old). The sample of various tissues including head, epidermis, fat body, gut and hemolymph were dissected from larvae, while head, epidermis, fat body, gut, ovary, testis and antennae were dissected from adults. Then all of these samples were collected in 1.5 mL RNA-free microcentrifuge tubes (EP) and immediately stored at -80 °C for later use. Total RNA was respectively extracted from these samples following the standard protocol of RNAiso™ Plus (TaKaRa, Kyoto, Japan). The purified total RNA samples were quantified and qualified by Nanodrop 2000 (Thermo Scientific, Waltham, USA) and the integrity of RNA was further evaluated by 1.0% agarose gel electrophoresis with an Agilent 2100 Bioanalyzer. Subsequently, reverse transcription was carried out with 1000 ng of the total RNA (the A₂₆₀/A₂₈₀ ratio was >1.8 and the A₂₆₀/A₂₃₀ ratio was >2.0) by using HiScript II Reverse Transcriptase (Vazyme, Nanjing, China) following manufacturer's manual. Finally, the cDNA samples were stored at -20 °C for follow-up experiment.

Bioinformatics and sequence analysis

TBtools software was used to conduct blast searches on the *T. castaneum* genome from Beetlebase (<http://www.beetlebase.org/>). The BLAST of National Center for Biotechnology Information (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to reversed comparison. ORF cDNA sequence, the deduced proteins, molecular weight (Mw) and theoretical isoelectric point (pI) were predicted by the online tool of the Expasy website (<https://www.expasy.org>). MEGA 7.0 software was used to construct the phylogenetic analysis by using the neighbor-joining (NJ) method with 1000 bootstrap tests and values lower than 50% are not shown. The identified *T. castaneum* AOX amino acid sequences were aligned using multiple alignment program ESPript 3.0 (<https://esprict.ibcp.fr/ESPript/>). The structural domains of genes were predicted was performed using SMART (<http://smart.embl-heidelberg.de/>).

Quantitative real-time PCR (qRT-PCR)

According to the manufacturer's instructions, qRT-PCR was performed to check the expression of TcAOXs in *T. castaneum* using SYBR Green Master (Vazyme, Nanjing, China) with a QuantStudio™ 6 Flex System (Applied Biosystems, Foster City, CA, United States). The reaction volume of qPCR was 10 µL, including 3.5 µL of RNase-free water, 0.25 µL of each primer, 5 µL of 2 × SYBR Mixture and 1 µL of cDNA. The reaction procedure was performed as follows: 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min; and 1 cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The *T. castaneum* ribosomal protein S3 (rpS3; GenBank accession number CB335975) was selected as a reference gene using the 2^{-ΔΔCT} method to calculate the gene expression levels (Livak and Schmittgen, 2001; Begum *et al.*, 2009). Three biological replications were performed for each experiment and all primers used are listed in table 1.

RNAi experiment and behavior analysis

RT-PCR was served as amplifying the gene sequence. The primers used to amplify dsDNAs corresponding to TcAOX are shown in table 1. The program of PCR was as follows: 94 °C, 5 min to activate advantage GC polymerase; followed by 32 cycles of 94 °C, 30

Table 1. Primers used in this study

Primers	Sequence (5-3)	Length of product (bp)	Utility
Rps3-F	TCAAATTGATCGGAGGTTTG	260	qRT-PCR
Rps3-R	GTCCACGGCAACATAATCT		
AOX1-F1	TGAACTTACATCCAAGACTTTAAAA	278	qRT-PCR
AOX1-R1	AAGCAATAGTGAACTGGACACCC		
AOX1-F2	TAATACGACTCACTATAGGG TGTAACGGTTGGAGGATTACAC	555	RNAi
AOX1-R2	TAATACGACTCACTATAGGG AAAGTCTTGGGATGTAAGTTCAGG		
AOX2-F1	GCTACATCCGAGCCCTCG	242	qRT-PCR
AOX2-R1	AACGCCAATCCCTTCTT		
AOX2-F2	TAATACGACTCACTATAGGG ACGGGGGGTGTTTACTG	790	RNAi
AOX2-R2	TAATACGACTCACTATAGGG AAGCGTTTCGCGAGATTT		
AOX3-F1	TTTTGTCTTAGGCGCTGGTACTAG	202	qRT-PCR
AOX3-R1	AACATCGATGGGAAATCGT		
AOX3-F1	TAATACGACTCACTATAGGGAGACACGCACACGAACACGT	708	RNAi
AOX3-R1	TAATACGACTCACTATAGGGAGTACTTCAGTCGCACAACTCCA		

Double stranded RNAs (dsRNAs), which primers containing the T7 polymerase promoter (TAATACGACTCACTATAGGG) at the 5'-end of both the sense primer and anti-sense primer.

s; 60 °C, 30 s and 72 °C for 30 s, and final extension was performed at 72 °C for 7 min. The integrity of PCR products was detected by 1.0% agarose gel electrophoresis. PCR products were purified and extracted using FastPure Gel DNA Extraction Mini Kit (Product code: DC301, Vazyme, Nanjing, China). The dsRNA fragment of the *TcAOX* was synthesized *in vitro* using the Thermo Scientific TranscriptAid T7 High Yield Transcription Kit (Product code: K0441, Thermo Fisher Scientific, Shanghai, China) according to the manufacturer's instructions. dsVer (*T. castaneum* vermilion, GenBank AY052390) was synthesized as a positive control to confirm that the results we observed in the subsequent RNAi analysis were due to suppression of the expression levels of the specific target genes. Subsequently, RNAi was performed by injecting the body cavity of *T. castaneum* with 2 µg µl⁻¹ dsRNA using an InjectMan 4 instrument (Eppendorf, Hamburg, Germany). After injection, the target-gene silencing efficiency was detected by qRT-PCR which reaction procedure and volume identified with the above description on the fourth day.

To further investigate the functions of *TcAOXs* in the fecundity of *T. castaneum*, the collected pupae (twenty-fifth-day-old) were separated into two groups (female and male groups), and then each group was used for non-injection (WT, negative control), dsTcVer injection (positive control), dsTcAOX injection, respectively. After the fourth day, the males and females (injected the same) were pre-mated for 3 days, and then mated formally for 3 days. The egg-laying rate was calculated after formally mated. Three biological replications with independent injections were performed for each experiment.

Induction of *TcAOXs* in response to the plant toxicant

The *Artemisia vulgaris* essential oil (*A. vulgaris* EO) was extracted and diluted as previously described by Zhang *et al.* (2021). To measure *TcAOX* expression after *A. vulgaris* EO induction, a total of 180 *T. castaneum* larvae (15-day-old) were collected and separated into three groups. Briefly, approximately 60 synchronous individuals in each group were loaded into 1.5-mL EP

tubes and exposed to 100 µl *A. vulgaris* EO or acetone (control). After soaking for 1 min, the treated larvae in each group were placed on filter paper and allowed to air dry for 2 min. Each group was then transferred to an 8-mL glass vial and maintained under standard conditions as previously described (Li *et al.*, 2011). The acetone exposure group served as the control group in this study. The induction of *TcAOXs* were determined at 12, 24, 36, 48, 60 and 72 h following *A. vulgaris* EO exposure. At each time point, three surviving beetles in each group were selected for total RNA isolation at random, and the transcript levels of *TcAOXs* were measured using qRT-PCR, which reaction procedure and volume identified with above description). Three biological replications were performed for each experiment.

Bioassay of *T. castaneum* susceptibility to plant oil

For the plant oil bioassay after RNAi, *T. castaneum* 15-day-old larvae that had been injected dsRNA for 96 h were treated with plant oil. Briefly, 30 15-day-old larvae were treated with approximately 100 µl of plant oil for 1 min and then placed on filter paper to air dry for approximately 2 min. Immediately, the treated larvae were moved to an 8-mL Drosophila Vial. After 2 h, wheat flour containing 5% brewer's yeast was added to the vials, which were maintained under standard conditions. Mortality was recorded at 24, 48, and 72 h after plant oil treatment. Larvae were considered dead if they were unable to move or show a response when disturbed with a pair of tweezers or a brush. Three biological replications with independent injections were performed for each experiment.

Statistical analysis

The gene expression data and the mean values obtained for the RNAi-treated larvae vs. the mean values obtained for the control larvae were subjected to one-way analysis of variance (ANOVA) in combination with Fisher's least significant difference (LSD) multiple comparison tests and Student's *t*-test, respectively, by

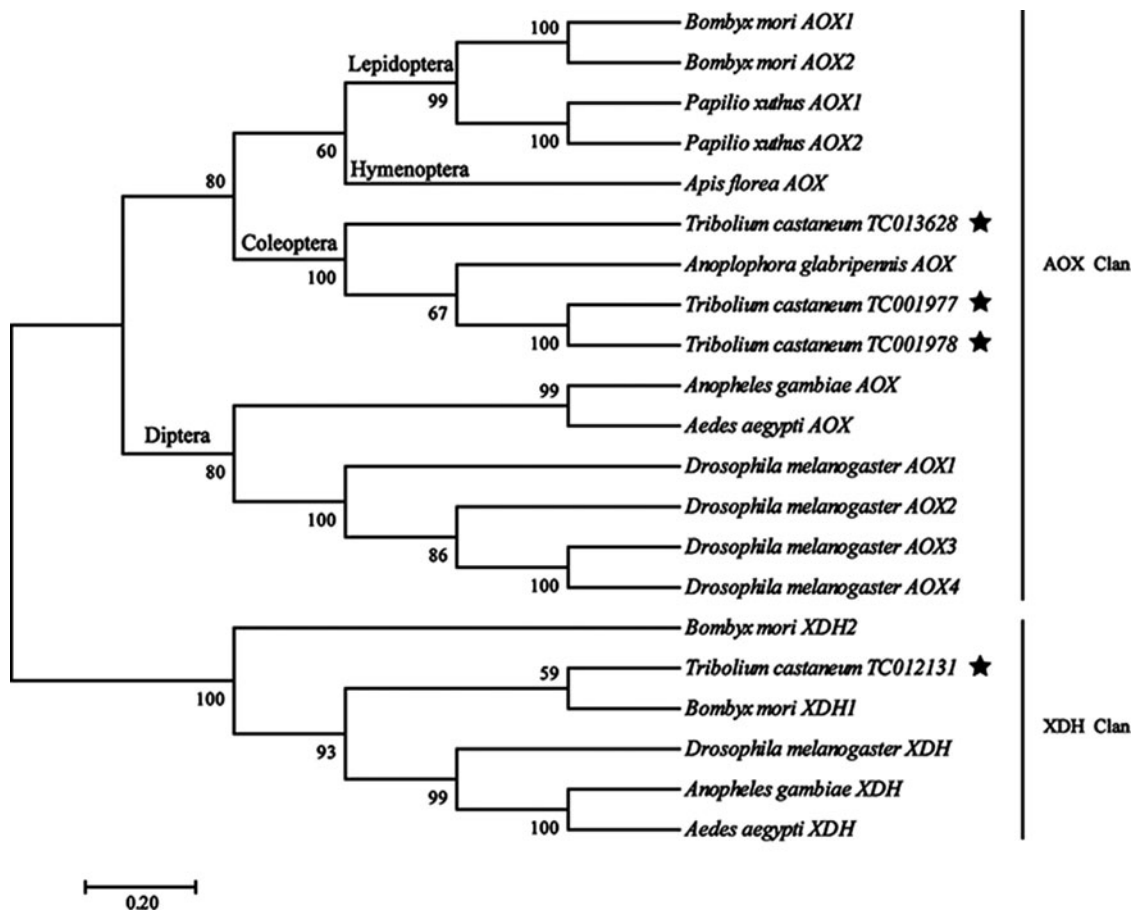


Figure 1. Phylogenetic tree of *TcAOXs* with AOXs of other insects. The phylogram was reconstructed in MEGA 7.0 using the neighbor-joining method. Bootstrap support values (in percent) based on 1000 replicates are indicated.

using the SPSS statistics program (SPSS Inc., Chicago, IL, USA). All data are expressed as the mean \pm standard error (SE); the error bars represent standard errors among three biological replications (* $P < 0.05$ was regarded as statistically significant; *** $P < 0.001$ was considered extremely significant).

Results

Identification of putative *TcAOXs* from *T. castaneum* genome

Four candidate genes encoding sequences of AOX or XDH proteins were identified by conducting TBLASTN searches on the *T. castaneum* genome using the sequence of *D. melanogaster* AOX. To further classify these genes, phylogenetic analysis with other insect AOXs and XDH genes identified from *B. mori*, *A. transitella*, *Papilio xuthus*, *D. melanogaster*, *Anopheles gambiae*, *Aedes aegypti*, *Apis florea* and *Anoplophora glabripennis* was used to identify (table S1). As shown in the fig. 1, the genes (Tc013628, Tc001977, Tc001978) were classified into AOX clan, while the gene (Tc012131) was classified into XDH clan. For being convenient to research, Tc013628, Tc001977, and Tc001978 were renamed *TcAOX1*, *TcAOX2* and *TcAOX3*, respectively, in this study.

Molecular characteristics analysis of *TcAOXs*

To further characterize the putative *TcAOXs*, we analyzed its molecular characteristics. For sequence analysis, *TcAOX1* is

3776 bp in length and has an ORF of 3711 nucleotides encoding 1236 amino acid residues with a molecular mass of 136.99 kDa and a pI of 8.01; *TcAOX2* is 4257 bp in length and has an ORF of 4038 nucleotides encoding 1345 amino acid residues with a molecular mass of 148.98 kDa and a pI of 7.11; and *TcAOX3* is 4327 bp in length and has an ORF of 3786 nucleotides encoding 1261 amino acid residues with a molecular mass of 138.91 kDa and a pI of 5.25 (table S2). Further, by comparing *T. castaneum* AOX cDNA sequences and genome sequences, we find that *TcAOX1*, *TcAOX2*, and *TcAOX3* genome DNA sequences contain 14, 17, and 14 exons, respectively (fig. 2a). In addition, the structural prediction and multiple sequence alignments for the *TcAOXs* show that each subunit contains a typical structure with a N-terminal domain containing two iron-sulfur (2Fe-2S) redox centers followed by a flavin-containing region (FAD-binding domain) and a C-terminal domain comprising the molybdenum cofactor (MoCo)-binding site within the substrate-binding pocket domain (fig. 2b, fig. S1).

Stage- and tissue-specific expression of *TcAOXs*

To get an insight in the functional and developmental significance of these three genes, the relative transcript levels of *TcAOXs* in different developmental stages and different tissues dissected from *T. castaneum* were investigated (figs 3 and 4). *TcAOXs* were transcribed throughout all developmental stages/ different

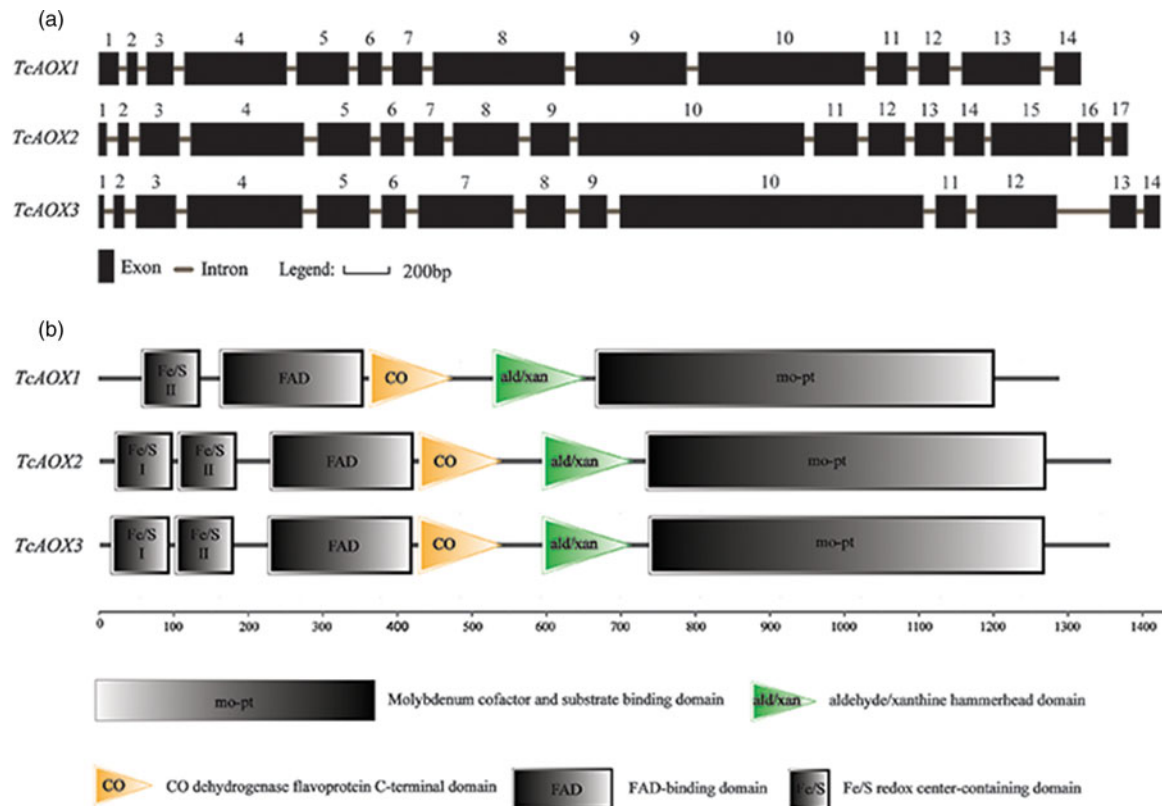


Figure 2. Schematic representation of *TcAOXs* genomic (a) and domain organizations (b). Solid blocks represent exons. Introns of known sizes are indicated by full lines. The ORFs from *T. castaneum* were compared and analysed by SMART.

tissues of *T. castaneum*, but expression levels differed significantly among the developmental stages/different tissues (figs 3 and 4). qRT-PCR revealed high levels of the transcripts in the EP stage for *TcAOX1* (fig. 3a), the LE, the EL and LP stages for *TcAOX2* (fig. 3b). In contrast, *TcAOX3* exhibited homogenous levels of the transcripts in stages from embryo to adults (fig. 3c). Further investigations with the various tissues found that the expression levels of *TcAOX1* were highest in the head and antennae dissected from larvae and adults, respectively, and lower expression levels were observed in the remaining tissues of the *T. castaneum* (fig. 4a, b). The *TcAOX2* transcript was found to be moderately expressed in larva fat body, epidermis and hemolymph, while exhibited the highest expression in adult epidermis, antennae, head (fig. 4c, d). In addition, *TcAOX3* was not highly expressed in all larva tissues, while had relatively high expression in adult head (fig. 4e, f).

Induction of *TcAOXs* by plant toxicant

To determine whether the expression of *TcAOXs* could be induced by plant toxicant, the transcript levels of these three genes were assessed by qRT-PCR after 15-day-old larvae treated with *A. vulgaris* EO at different time points (fig. 5). In comparison to the control, expression of *TcAOX1* was no significant changes from 12 to 72 h after exposure to plant toxicant (fig. 5a). Interestingly, similar expression trends were observed for *TcAOX2* and *TcAOX3* following plant toxicant treatment. *TcAOX2* and *TcAOX3* were significantly upregulated by plant toxicant treatment and reached peak levels at 36 and 48 h, respectively, which were 2.63- and 3.0-fold greater than those of the control treatment group (fig. 5b, c); subsequently, the expression

levels of these genes in the plant oil-treated groups gradually returned to lowered levels at 48 and 60 h, respectively (fig. 5b). However, the expression levels of *TcAOX3* approached normal levels at 72 h following exposure to plant oil (fig. 5c).

TcAOXs mediate plant toxicant susceptibility in the *T. castaneum*

To further demonstrate the causal role of *TcAOX2* and *TcAOX3* in plant toxicant susceptibility, RNAi of *TcAOXs* was performed to knock down its expression and the effect of this on the survival of plant toxicant-treated larvae of the ds*TcAOX* and ds*TcVer* (fig. 6). To show the interference is a specific segment of the gene, RNAi targeting of *TcAOXs* in 15-day-old larvae dramatically reduced their respective transcripts but did not significantly affect the expression of nontarget genes (fig. S2), indicating that there was no off-target effect of our RNAi in *T. castaneum*. After 96 h of injection double-stranded RNA (dsRNA) specific for *TcAOX2* and *TcAOX3*, the mRNA levels of these genes significantly decreased by about 83 and 84% as compared to controls, respectively (fig. 6a). Subsequently, susceptibility of ds*TcAOX2*- and ds*TcAOX3*-treated larvae to plant oil were evaluated. Knockdown of *TcAOX2* and *TcAOX3* significantly increased the mortality of larvae from 24 to 72 h, relative to the control, when treated with plant toxicant (fig. 6b).

TcAOXs mediate fecundity in the *T. castaneum*

To investigate the effect of *TcAOX* on fecundity, RNAi was performed using late pupa (25-day-old) that had been separated

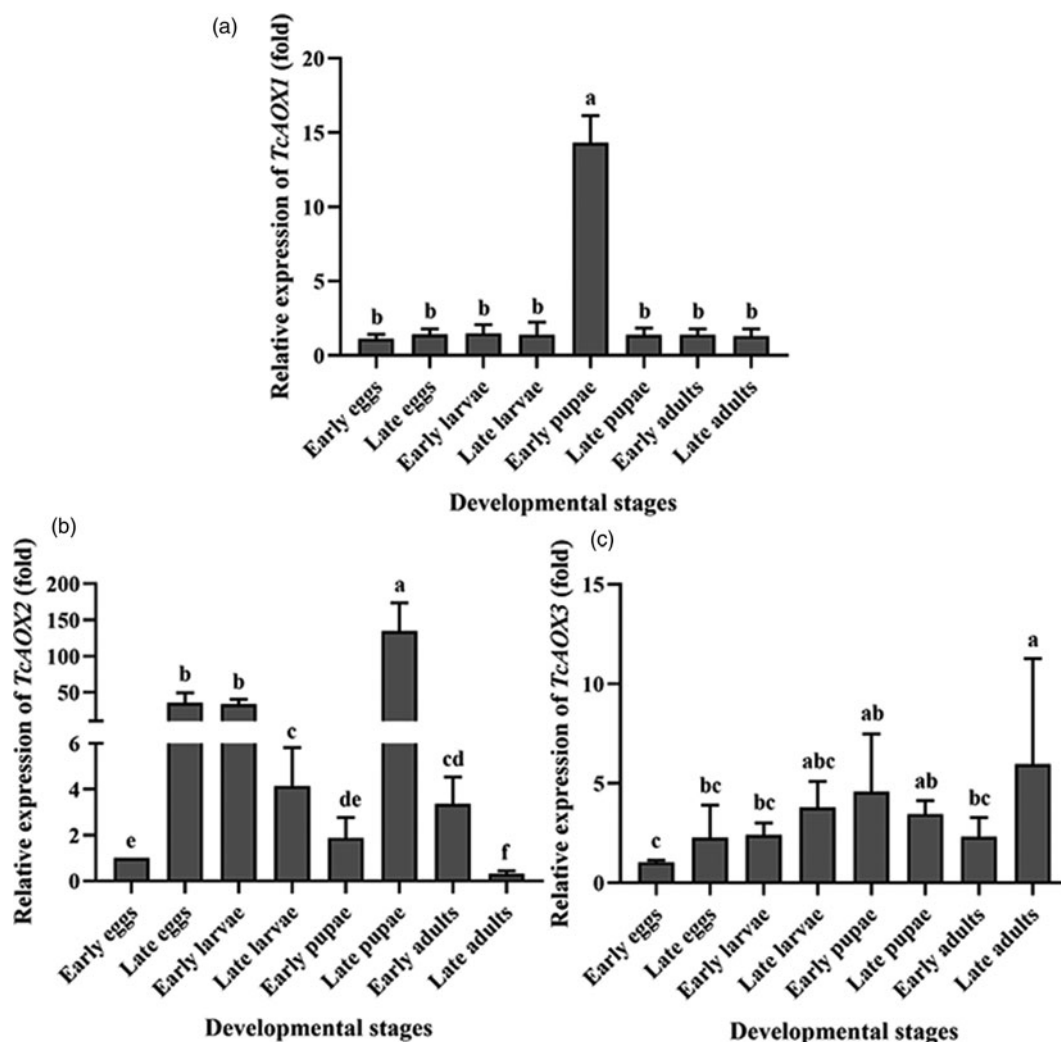


Figure 3. The expression pattern of *TcAOXs* during the eight key developmental stages of *T. castaneum*. Early eggs (1 day old); Late eggs (3 days old); Early larvae (1 day old), Late larvae (20 days old); Early pupae (1 day old); Late pupae (5 days old); Early adults (1 day old); Late adults (10 days old). Vertical bars indicate standard errors of the mean ($n = 3$) and different letters on the error bars mean significant differences among the different developmental stages at the $P < 0.05$ by one-way analysis of variance (ANOVA).

out male and female (fig. 7). After injection, the *TcAOX1* silencing efficiency was detected by qRT-PCR on the fourth day, and the transcript level of *TcAOX1*, *TcAOX2* and *TcAOX3* were reduced by about 87, 85, and 70% as compared to controls, respectively (fig. 7). After *TcAOX1* knockdown, the egg laying rate of the treatment group was reduced by 66.7% compared with the control group (fig. 8a). The egg numbers of the ds*TcAOX2* and ds*TcAOX3* knockdown group, by the contrast, were not significantly different from those of the controls (fig. 8a). Interestingly, investigating the hatching rate of eggs showed that nearly 100% of the eggs of the ds*TcAOX1*, ds*TcAOX2*, and ds*TcAOX3* knockdown groups hatched into larvae (fig. 8b), suggesting that *TcAOX1* is important for fecundity in *T. castaneum*.

Discussion

AOXs, a kind of multigene family, exist widely in plants and animals. Different species are characterized by a different complement of AOX genes. Currently, 1~4 AOX genes had been reported in *Arabidopsis thaliana* (Sekimoto *et al.*, 1998), *Mus*

musculus (Kurosaki *et al.*, 2004), *D. melanogaster* (Garattini *et al.*, 2008), *Pisum sativum* L. (Zdunek-Zastocka, 2008), *Bos taurus* (Calzi *et al.*, 1995), *Zea mays* (Sekimoto *et al.*, 1997), *Gallus* (Terao *et al.*, 2006), *Canis familiaris* (Garattini *et al.*, 2008), *Homo sapiens* (Garattini *et al.*, 2003), and *Danio rerio* (Garattini *et al.*, 2008). In addition, 8 AOX genes had identified in *B. mori* which is by far the most insects of AOX (Pelletier *et al.*, 2007; Yang *et al.*, 2010). In our study, we identified three candidate genes encoding sequences of AOX proteins in the *T. castaneum* genome sequences. Gene structure analysis shows that all candidate genes contain two [2Fe-2S] domains, one flavin adenine (FAD) containing domain, and one molybdopterin-binding domain (fig. 2), according with the structural characteristics of AOX (Garattini *et al.*, 2003; Mahro *et al.*, 2011). These conserved structural regions further confirmed that candidate genes did belong to the AOX gene family (fig. S1). Furthermore, phylogenetic analysis showed that the AOX and XDH of insect clustered with different clan (fig. 1), suggesting they have been diverged a long time ago (Marelja *et al.*, 2014; Xu and Liao, 2017; Okamura *et al.*, 2018). In fact, XDH genes

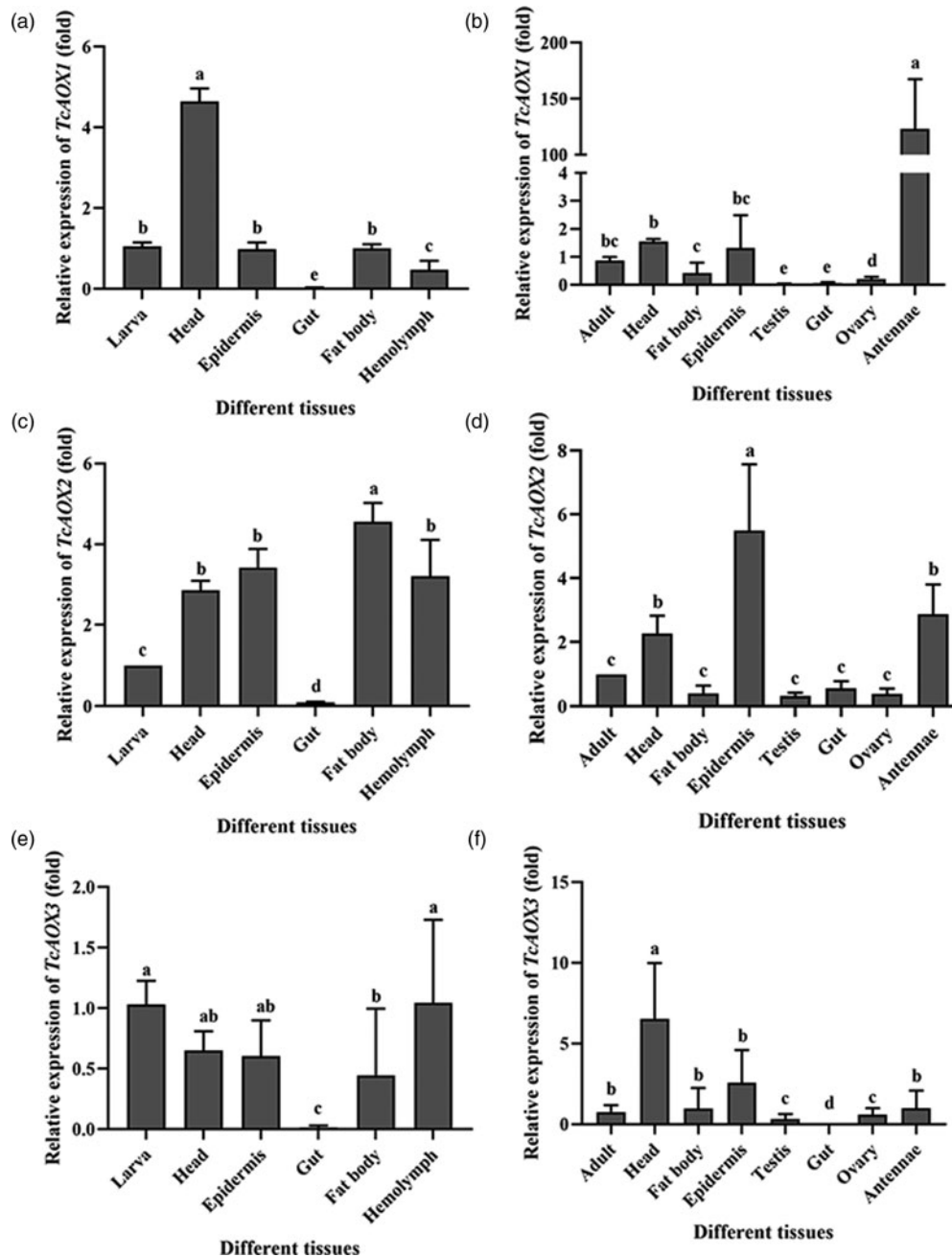


Figure 4. The expression patterns of *TcAOXs* in the different tissues of *T. castaneum*. The (a), (c) and (e) represented the tissues of *T. castaneum* larvae including head, epidermis, gut, fat body, hemolymph. The (b), (d) and (f) represented the tissues of *T. castaneum* adults including head, fat body, epidermis, testis, gut, ovary, antennae. Vertical bars indicate standard errors of the mean ($n=3$) and different letters on the error bars mean significant differences among the different tissues at the $P < 0.05$ by one-way analysis of variance (ANOVA).

is the ancestor of all AOX genes, and a series of subsequent/separate gene duplication and suppression events led to the present enzymes in plants, insects and vertebrates (Garattini *et al.*, 2003, 2008). Simultaneously, it just goes to show why different organisms have different AOX genes, and why AOXs/XDHs cluster with the different branches.

Different from previous researches (Coleman *et al.*, 2002; Merlin *et al.*, 2005; Pelletier *et al.*, 2007; Huang *et al.*, 2016; Xu and Liao, 2017; Okamura *et al.*, 2018), the candidate AOX gene sequences contained 14–17 exons (fig. 2a), the ORF was 3711–4038 bp which encoded a putative protein of 1236–1345

amino acids, the molecular weight was 137~149 kDa, and the iso-electric point was 5.28–8.01 (table S2). This may be due to the differences of AOX among species. For instance, a minor 130 kDa subunit was produced by cleavage at the N-terminal side of the 150 kDa subunit in the monkey, whereas was not detected in the mice (Asakawa *et al.*, 2008). For expression analysis of AOXs mRNAs, it is evident that *TcAOX1* and *TcAOX2* are expressed throughout development, with peaks in the early pupae and LP stages, respectively (fig. 3a, b). Similarly, *H. armigera* AOX5 and *D. melanogaster* AOX2 activity was detected only during metamorphosis too (Marelja *et al.*, 2014; Xu and Liao, 2017),

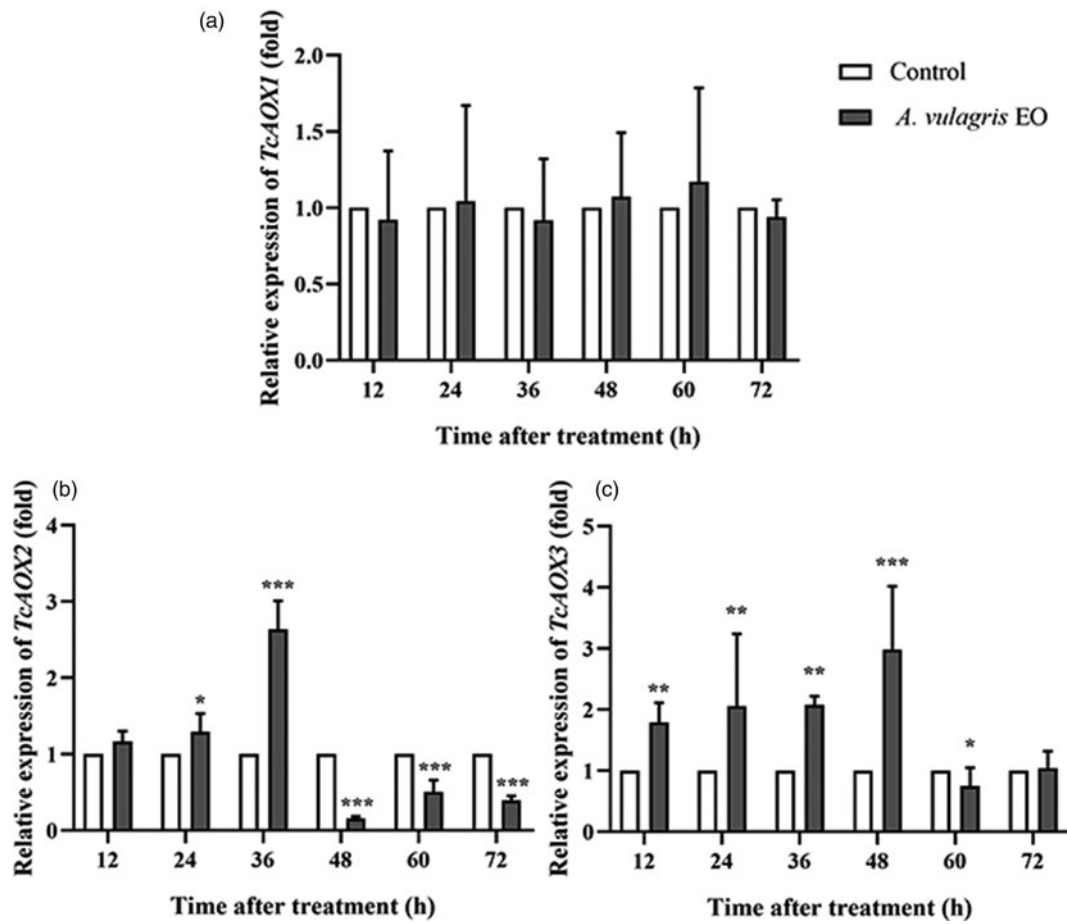


Figure 5. Transcriptional expression of *TcAOXs* after exposure to *A. vulagris*. The 15-day-old *T. castaneum* larvae were treated with 5% *A. vulagris* or acetone (negative control group, ascribed an arbitrary value of 1) at time points of 12, 24, 36, 48, 60, and 72 h. Data and error bars represent the mean and standard errors (SE) of three biological replicates. Asterisks mean significant differences between the control and the treatment at the corresponding levels (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

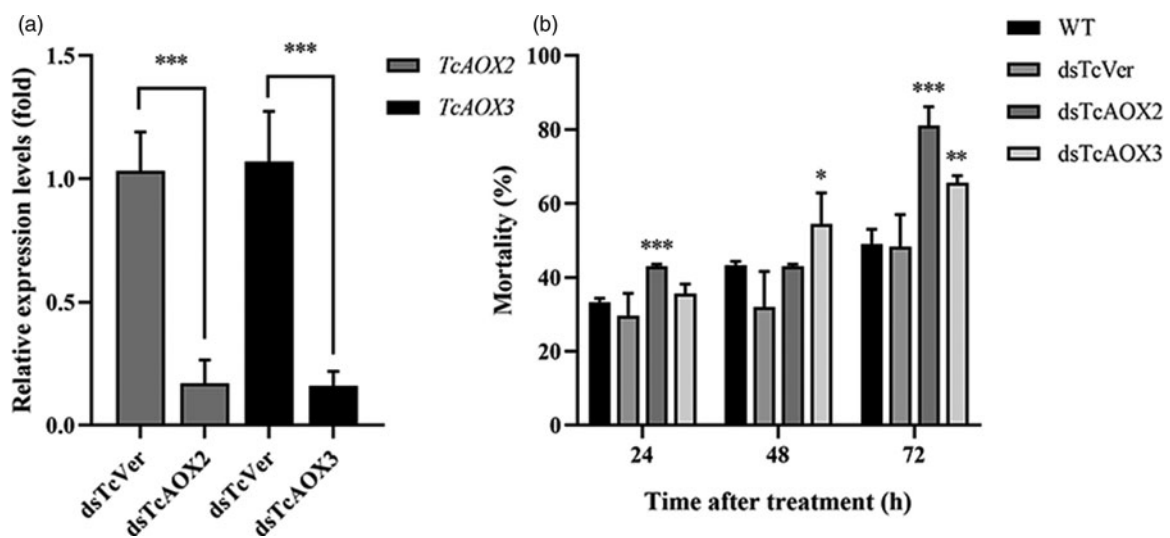


Figure 6. Effect of RNAi treatment on the transcript levels of *TcAOX2/TcAOX3* (a) and on susceptibility of *T. castaneum* larva to *A. vulagris* (b). The 15-day-old *T. castaneum* larvae were injected with 2 $\mu\text{g } \mu\text{l}^{-1}$ dsRNA. Data and error bars represent the mean and standard errors (SE) of three biological replicates. Asterisks mean significant differences between the control and the treatment at the corresponding levels (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

Figure 7. Effect of RNAi treatment on the transcript levels of *TcAOX1* (a), *TcAOX2* (b), and *TcAOX3* (c). The 25-day-old *T. castaneum* pupae were injected with $2 \mu\text{g}\mu\text{l}^{-1}$ dsRNA. Statistical comparisons were performed among the insects injected with the same amount of dsTcVer (*T. castaneum* vermilion, GenBank AY052390), WT (wild-type beetles that did not receive an injection) and dsTcAOX (beetles injected with *TcAOX* double-stranded RNA) by using the Student's *t*-test. Data and error bars represent the mean and standard errors (SE) of three biological replicates. The three asterisks indicate significant differences between the control and the treatment at $P < 0.001$ level.

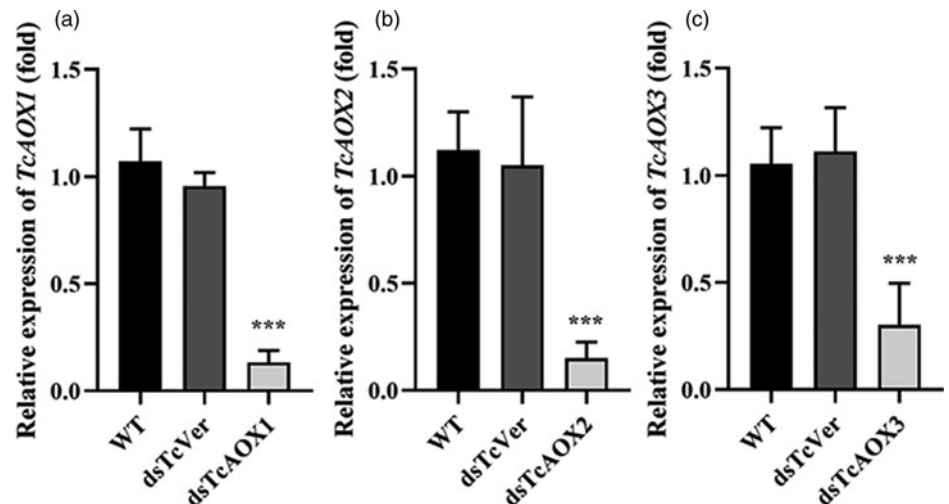
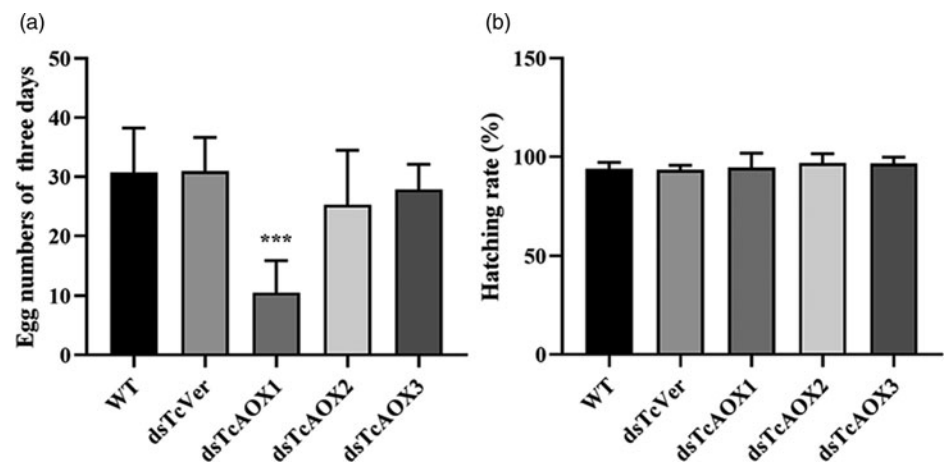


Figure 8. Statistical analysis of reproduction. (a) The number of oviposition events 3 days in each group after RNAi of *TcAOX1*, *TcAOX2* and *TcAOX3* in the late pupae stage (25-day-old). (b) The survival rate of the eggs in each group after knockdown of *TcAOX1*, *TcAOX2* and *TcAOX3* in the late pupae stage (25-day-old). Statistical comparisons were performed among the insects injected with the same amount of dsTcVer (*T. castaneum* vermilion, GenBank AY052390), WT (wild-type beetles that did not receive an injection) and dsTcAOX (beetles injected with *TcAOX* double-stranded RNA) by using the Student's *t*-test. Data and error bars represent the mean and standard errors (SE) of three biological replicates. The double asterisks and three asterisks indicate significant differences between the control and the treatment at $P < 0.01$, and $P < 0.001$ levels, respectively.



suggesting it may be involved in the metamorphosis of insect development. However, no significant phenotype was observed after knocking *TcAOX1* and *TcAOX2* down in the metamorphosis of *T. castaneum* (data not shown), which might be had something to do with the differences of AOX among different species as well. *TcAOX3*, by contrast, is expressed throughout development, suggesting it may play a general role in different stages (fig. 3c), but the specific function still needs our further research.

In insects, the chemosensory tissues including antennae play an important role in communicating the availability of food sources, habitats, and oviposition sites as well as in locating mates (van der Goes van Naters and Carlson, 2006). When sex pheromone spreading into the surrounding conditions, the odorant receptors (ORs) of antennae could combine with it by odorant-binding proteins (OBPs) or chemosensory proteins (CSPs) selectively and specifically delivering; after being detected by the ORs, these semiochemical signals will be transmitted to insect brains by a series of cascade reaction in the body to guide insect behaviors including mating, host-seeking, and oviposition; finally, degrading enzymes will inactivate these semiochemical signals and the signal inactivation step is critical in insect odorant-mediated behaviors as well (Leal, 2013). As one of the ODEs, AOXs in insect antennae is one of the key enzyme degradation of sex pheromone into inactive carboxylic acids and play an important role in insect behaviors (Rybczynski *et al.*,

1989). Further investigations with the various tissues found that the expression level of *TcAOX1* was the highest in antennae dissected from adults (fig. 4a). Moreover, it can significantly affect the fecundity of beetles after knocking *TcAOX1* down in our study (fig. 8a), indicating that *TcAOX1* may be involved in the fecundity of beetles by affecting the inactivation of sex pheromone. However, knocking *TcAOX1* down can't affect the hatching rate of eggs (fig. 8b). Interestingly, significantly low expression of *TcAOX1* in testis and ovary were observed (fig. 4b), and further dissection of dsTcAOX1-beetles discovered that the testis and ovary developed normally (fig. S3). Thus we conclude that *TcAOX1* may be involved in the fecundity of beetles by affecting the inactivation of sex pheromone between insects instead of the reproductive organ. *HarmAOX2* is a candidate ODE to inactivate the sex pheromone components (Z)-11-hexadecenal and (Z)-9-hexadecenal (Xu and Liao, 2017). Recombinant AtrAOX2, PxylAOX3, BmAOX5 from the *A. transitella*, *P. xylostella* and *B. mori*, respectively, could degrade the sex pheromone or plant-derived volatiles *in vitro* (Choo *et al.*, 2013; Zhang *et al.*, 2020; Wang *et al.*, 2021). Therefore, we further need to prove this conjecture by expressing AOX protein *in vitro* to verify whether it can bind sex pheromone substances.

The *TcAOX2* transcript was found to be moderately expressed in larval detoxification organs including fat body, epidermis and hemolymph, while exhibited the highest expression in adult

epidermis (fig. 4c, d). In addition, *TcAOX3* was not highly expressed in all larva and adult tissues (fig. 4e, f). In mammals, the role of AOXs is well known as they play a significant role in the detoxification of environmental pollutants (xenobiotics) such as pesticides (Yoshihara and Tatsumi, 1997). Acetaldehyde, one of the environmental pollutants including highly toxic, is naturally produced in leaves or fruits of plants and must be degraded into non-toxic products (Kimmerer and Macdonald, 1987; González-Mas *et al.*, 2011). Some volatile aldehydes such as propanal and (E)-2-hexenal even were reported as having insecticidal activity in fumigation assay (Hammond *et al.*, 2000; Hubert *et al.*, 2008). In fact, the involvement of AOX in insecticide resistance of *C. quinquefasciatus* had previously been reported (Coleman *et al.*, 2002). Metabolism of acetaldehyde by cytosolic oxidizing enzymes is important for the survival of *D. melanogaster* (Heinstra *et al.*, 1989; Anderson and Barnett, 1991), and *AtraAOX2* degrade the pesticide containing an aldehyde functional group (acrolein) indicate that it functions as a xenobiotic-degrading enzyme in *A. transitella* (Choo *et al.*, 2013). Furthermore, *P. xylostella* *PxylAOX3* could participate in olfactory neuron protection by inactivation of redundant odorants and xenobiotic detoxification (Wang *et al.*, 2021). With the further research of AOX, it is no wonder that AOX plays a significant role in the detoxification of insects.

In our study, we also found that the expression of *TcAOX2* and *TcAOX3* could be induced by plant volatiles (fig. 5b, c), implying that they might play a vital role during the response to plant toxicant treatment. RNA interference has become a powerful tool to study gene function in a wide range of multicellular organisms and single cells (Meister and Tuschl, 2004; Kim *et al.*, 2015; Silver *et al.*, 2021). Further, RNAi against *TcAOX2* and *TcAOX3* prior to plant toxicant exposure led to even higher mortalities (fig. 6b), indicating that *TcAOX2* and *TcAOX3* definitely performed plant toxicant susceptibility in the beetles, providing further evidence for the involvement of *TcAOX2* and *TcAOX3* in detoxication in *T. castaneum*. Of course, some shortcomings such as how AOXs participate in metabolic detoxification and whether AOXs are similar to the classic metabolic detoxification phase is still our further exploration.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S0007485322000049>

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