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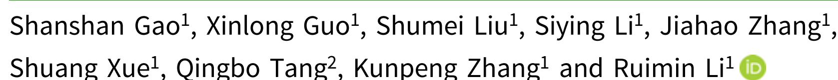
Cytochrome P450 gene *CYP6BQ8* mediates terpinen-4-ol susceptibility in the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)



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Abstract

Cytochrome P450 proteins (CYPs) in insects can encode various detoxification enzymes and catabolize heterologous substances, conferring tolerance to insecticides. This study describes the identification of a P450 gene (*CYP6BQ8*) from *Tribolium castaneum* (Herbst) and investigation of its spatiotemporal expression profile and potential role in the detoxification of terpinen-4-ol, a component of plant essential oils. The developmental expression profile showed that *TcCYP6BQ8* expression was relatively higher in early- and late-larval stages of *T. castaneum* compared with other developmental stages. Tissue expression profiles showed that *TcCYP6BQ8* was mainly expressed in the head and integument of both larvae and adults. The expression profiling of *TcCYP6BQ8* in developmental stages and tissues is closely related to the detoxification of heterologous substances. *TcCYP6BQ8* expression was significantly induced after exposure to terpinen-4-ol, and RNA interference against *TcCYP6BQ8* increased terpinen-4-ol-induced larval mortality from 47.78 to 66.67%. This indicates that *TcCYP6BQ8* may be involved in *T. castaneum*'s metabolism of terpinen-4-ol. Correlation investigation between the *CYP6BQ8* gene and terpinen-4-ol resistance in *T. castaneum* revealed that the *TcCYP6BQ8* gene was one of the factors behind *T. castaneum*'s resistance to terpinen-4-ol. This discovery may provide a new theoretical foundation for future regulation of *T. castaneum*.

Introduction

Tribolium castaneum (Herbst), commonly known as the red flour beetle, is a worldwide agricultural storage pest, and is also an important model organism for developmental, physiological, and applied entomological studies of coleopterans (Rosner *et al.*, 2020). *Tribolium castaneum* predominantly poses a significant hazard to processed and stored food crops (Golden *et al.*, 2018; Mangang *et al.*, 2020). Secretions from this beetle contain benzoquinone and other harmful substances that cause an irritating and moldy odor and affect the quality of flour as well as endangering human safety (Lis *et al.*, 2011; Saad *et al.*, 2019). Consequently, *T. castaneum* causes billions of dollars of economic losses in grain storage every year (Aronstein *et al.*, 2011; Boyer *et al.*, 2012). Currently, the main methods are used in the world to control grain storage pests, including *T. castaneum*, are the fumigant phosphine and some contact insecticides such as organophosphate and pyrethroid insecticides (Awan *et al.*, 2012; Boyer *et al.*, 2012). Nevertheless, the long-term use of such chemicals has resulted in the development of resistance and persistent health effects on humans, the non-target organisms, and environment (Awan *et al.*, 2012; Dey, 2016). Therefore, there is an urgent need to find new natural pesticides that are relatively environmentally friendly (Boukouvala *et al.*, 2016*a*, 2016*b*).

The essential oils of plants and their active ingredients can have a marked impact on target insects as well as being less polluting to the environment than chemical insecticides (Isman, 2006; Benelli, 2015). The oils from many plant species have been reported to be toxic to a wide range of pests as insecticides, ovicides, trophozoites, and food rejectors (Tunç *et al.*, 2000; Ogendo *et al.*, 2008; Islam, 2017). Among them, essential oil from *Artemisia vulgaris* (Levl. et Vant) has excellent thixotropic and fumigant activity against *T. castaneum* (Zhang *et al.*, 2020). Terpinen-4-ol is one of the active components of *A. vulgaris* essential oil (Song *et al.*, 2019). Terpineol-4 and its derivatives exhibit significant contact toxicity and inhibit Na+, K+, and ATPase in houseflies (Guo *et al.*, 2008). Furthermore, *terpinen-4-ol* displays fumigant activity that is highly toxic to *T. castaneum* (Min *et al.*, 2016; Liao *et al.*, 2018),

and is repellency active against grain storage pests, including *T. castaneum* (Suthisut *et al.*, 2011; Zhang *et al.*, 2015). Recent investigations have demonstrated that terpinen-4-ol treatment down-regulates the expression of genes involved in development (*RTKTol*, *Fz4*, *E78C*, etc) and emergency response (Attacin 1 and Defensin 1) of *T. castaneum* larvae. This shows that terpinen-4-ol stimulation may have an effect on the development and stress response of *T. castaneum*. Therefore, plant essential oils that contain terpinen-4-ol and terpinen-4-ol derivatives can be used as insecticides, especially for insect pests of stored grains.

Complex biochemical mechanisms have evolved in nature as a result of competition and co-evolution between plants and herbivorous insects (Jander, 2014). Plants produce chemicals that are toxic to phytophagous insects, this is common in nature (Heidel-Fischer and Vogel, 2015). Conversely, insects evolve and strengthen their defense mechanisms against plant toxins (Nishida, 2014; Beran *et al.*, 2019). Among them, high expression of detoxification enzymes is an important mechanism for insects to resist plant toxins (Heidel-Fischer and Vogel, 2015; Heckel, 2018). Detoxifying enzymes predominantly include carboxyl/cholinesterases (CCEs), cytochrome P450 monooxygenases (CYPs), glutathione-S-transferases (GST), UDP-glycosyltransferases (UGT), and ATP-binding cassette transporters (ABC transporters) (Ahn *et al.*, 2012; Nelson *et al.*, 2013; Li *et al.*, 2017; Tang *et al.*, 2020; Gao *et al.*, 2021). In most cases, CYPs and CCEs are participating in the first phase of metabolic detoxification of heterologous substances, while the GSTs and UGTs play a primary role in the second phase (Feyereisen, 2020). In the first phase, CYPs are essential in the metabolism of heterologous substances (Liu *et al.*, 2015; Calla, 2021).

CYPs are one of the largest and oldest supergene families found in almost all aerobic organisms (Zhu *et al.*, 2013). In insects, CYPs have a significant impact on their adaptation to different survival environments. Some CYPs are participating in the biosynthesis of endogenous compounds in insects (Helvig *et al.*, 2004*a*; Rewitz *et al.*, 2007), playing a critical role in the chemical communication, behavior, and metabolism of insects (Maïbèche-Coisne *et al.*, 2004; Helvig *et al.*, 2004*b*; Dierick and Greenspan, 2006; Wang *et al.*, 2008). Other CYPs act as genes that respond to environmental changes (Berenbaum, 2002), for example by protecting insects from chemical stress through the degradation of chemical pesticides and plant secondary metabolites (Feyereisen, 2011; Gao *et al.*, 2020). Insect P450 genes are divided into four evolutionary branches: the mitochondrial P450 evolutionary branch, the CYP2 evolutionary branch, the CYP3 evolutionary branch, and the CYP4 evolutionary branch (Feyereisen, 2006). Of these, the CYP6 gene subfamily of the CYP3 evolutionary branch is unique to insects and is extensively involved in metabolic process of exogenous toxins in Diptera and Lepidoptera (Li *et al.*, 2001; Sun *et al.*, 2019). For example, *CYP6MS1* is involved in the detoxification of tea tree oil and its major component, terpinen-4-ol, by *Sitophilus zeamais* (Motschulsky) (Huang *et al.*, 2020). *CYP6DA1*, *CYP6CY19*, and *CYP6CY22* in *Aphis gossypii* (Glover) are resistant to phytochemicals (Li *et al.*, 2019), and *CYP6AB60* in the polyphagous insect *Spodoptera litura* (Fabricius) has a potential role in the response to various toxic plant metabolites (Sun *et al.*, 2019). In summary, CYP6 subfamily genes may be associated with exogenous substance metabolism and pesticide resistance (Feyereisen, 2006; Li *et al.*, 2007).

Our RNA sequencing analysis showed that, a gene in the CYP6 gene subfamily from *T. castaneum*, *CYP6BQ8*, was significantly highly expressed during treatment with terpinen-4-ol, which is the main component of *A. vulgaris* essential oil (Gao *et al.*, 2022*b*). The objective of this study was to elucidate the physiological effects of *TcCYP6BQ8* on the metabolism of *T. castaneum* treated with terpinen-4-ol. The *CYP6BQ8* gene from *T. castaneum* was cloned and its function in the catabolism of terpinen-4-ol by *T. castaneum* was evaluated. Quantitative real-time PCR (qRT-PCR) was used to detect *TcCYP6BQ8* expression after treatment with terpinen-4-ol and to obtain a spatiotemporal expression profile. Subsequently, RNA interference (RNAi) was used to downregulate *TcCYP6BQ8* expression and explore the effect of *TcCYP6BQ8* silencing on the defense of *T. castaneum* against terpinen-4-ol.

Material and methods

Insect rearing

All experiments were conducted using stock cultures of *T. castaneum* that have been maintained in the Nanjing Normal University, originated form Kansas State University (Manhattan, KS). The strain of *T. castaneum* used was Georgia-1(GA-1); (Xie *et al.*, 2019). Wheat flour with 5% brewer's yeast was employed for the development of *T. castaneum*. Incubation is carried out at a temperature of 30°C and a relative humidity of 40% in an incubator with a 14-h day/10-h night cycle (Gao *et al.*, 2020). The study used eight insect periods; 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), and late adults (10 days old). Samples of *T. castaneum* individuals were collected directly from the stock cultures, using a sieve. Then three individuals from each developmental stage were randomly selected from the samples, were washed with 1 × PBS solution to remove the remaining flour particles and were dried by using a filter paper. Then, all individuals were put in a clean 1.5 ml centrifuge tube and were placed in a refrigerator at −20°C for RNA extraction.

Insecticidal efficacy assay of terpinen-4-ol

Terpinen-4-ol (99%, CAS: 562-74-3) was purchased from Sigma-Aldrich (Munich, Germany). The insecticidal effect of terpinen-4-ol against *T. castaneum* late larvae was measured according to the descriptions (Lu *et al.*, 2012). The experiment was divided into two groups: experimental group treated with terpinen-4-ol and control group treated with acetone. Exposure toxicity to late-stage larvae (20 days old) was assessed in a fume hood. Primarily, terpinen-4-ol was diluted to acetone. Thirty late-stage larvae in good growth condition were placed in a 1.5-ml tube with 100 μl of LC50 (median lethal concentration) terpinen-4-ol or acetone (Gao *et al.*, 2022*b*). After treating last-stage larvae with terpinen-4-ol or acetone for 1 min, dry in a fume hood. The larvae were transferred to petri dishes with a diameter of 6 cm and fed normally in an artificial incubator when they began their activity. Late-stage larvae (20 days old) were treated with terpinen-4-ol at the LC_{50} and collected at 12, 24, 36, 48, 60, and 72 h post-treatment for subsequent RNA extraction and qRT-PCR. Two technical replicates were carried out, with three biological replicates in each technical replicate.

Cloning of the CYP6BQ8 *gene in* T. castaneum

The *TcCYP6BQ8* gene's full-length open reading frame (ORF) cDNA sequence was obtained using primers intended to extract

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Table 1. Primers used for this research

Gene	Sequence (5′–3′)	Primer length (bp)	Product length (bp)	Utility
CYP6BQ8-F	CCACTGGTCTAAAAAAAGTCATGG	24	129	qRT-PCR
CYP6BQ8-R	GACTGTTACACTCAATACCGAAAGC	25		
dsCYP6BQ8-F	TAATACGACTCACTATAGGGTTCGGAAATACGGTAAAAA	39	106	RNAi
dsCYP6BQ8-R	TAATACGACTCACTATAGGGTTAAATCTGAACAATCGGAG	40		
CYP6BQ8-FF	GCCGATACCCTTCTACATC	19	1282	Cloning
CYP6BQ8-FR	CAACCAGACTCCACCTTTT	19		
CYP6BQ10-F	TCAAAGGGCTACAAACACGGT	21	156	qRT-PCR
CYP6BQ10-R	TCCACTTAATGGATCGGCATC	21		
CYP6BQ11-F	TACGAAGCGATGATGGAAATGA	22	183	qRT-PCR
CYP6BQ11-R	AGGATCGGTGTGAAGTGCTAAAA	23		
dsVER-F	TAATACGACTCACTATAGGGGTCTTGGTGGACCAAG	35		RNAi
dsVER-R	TAATACGACTCACTATAGGGCCGCCATTTCGTGATC	34		
RPS3-F	TCAAATTGATCGGAGGTTTG	20	260	qRT-PCR
RPS3-R	GTCCCACGGCAACATAATCT	20		

F denotes forward primers and R denotes reverse primers. The underlined sequence is the T7 promoter synthesized by dsRNA.

it (table 1). Total reagent was used to extract total RNA from *T. castaneum* (Invitrogen, Carlsbad, CA, USA). Using HiScript reverse transcriptase (Vazyme Biotech, Nanjing, China) in a 50-µl reaction system, 1400 ng RNA was reverse transcribed into cDNA template for *TcCYP6BQ8* cloning. For PCR amplification, TransStart FastPfu DNA polymerase (TransGen, Beijing, China) was employed. The pEASY-Blunt Zero Cloning Kit was used to subclone the purified PCR product into the Blunt Zero vector (TransGen, Beijing, China). Blue-white screening confirmed positive clones, and Sangon Biotechnology sequenced them (Beijing, China). DNAMAN was used to generate and visualize the amino acid sequence of *TcCYP6BQ8* (LynnonBiosoft, USA).

Gene structure prediction and phylogenetic analysis

TcCYP6BQ8 domains' predicted amino acid sequences were evaluated online using SMART. (http://smart.embl-heidelberg.de) and then further identified with BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis using the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). The NCBI database was utilized to extract protein sequences for the CYP6 and CYP9 genes of Coleoptera and Lepidoptera, which were then used to create a phylogeny tree. Amino acid sequence alignment was performed using the muscle method ratio in MEGA 7 software and a phylogenetic tree was reconstructed using the neighbor-joining (NJ) method of the Whelan and Goldman (WAG) model. Bootstrap analysis was conducted with 1000 resamplings and all values were above 50%.

Gene expression profile of TcCYP6BQ8

Multiple samples from each of the eight developmental periods of *T. castaneum* were collected for RNA extraction: 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), and late adults (10 days old). RNA was extracted from mixed post-mortem samples of diverse tissues from late larvae (whole larvae, head, epidermis, fat body, gut, and hemolymph) and early adults (whole adult, head, epidermis, fat body, gut, ovary, antennae, testis, and accessory gland). The extracted RNA from each experiment (developmental stage and tissue profile, respectively) was subjected to qRT-PCR and then stored at −80°C. Two technical replicates were performed for different developmental stages and different tissues, with three biological replicates in each technical replicate.

Double-strand RNA (dsRNA) synthesis and injection

For dsRNA synthesis, Primer Premier 5.0 (Premier, Canada) was used to design *TcCYP6BQ8* and *TcVER* gene-specific primers containing T7 polymerase recognition promoter sequences (table 1). The PCR was composed of 0.4 µl forward and reverse primers (10 µM), 10 µl 2 × Primer STAR Mix, 8.2 µl ddH_2O, and 1 µl plasmid DNA containing the *TcCYP6BQ8* ORF. An initial denaturation step of 5 min at 94°C was followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by a final extension step of 7 min at 72°C. With the help of a TranscriptAid T7 High Yield Transcription Kit (Fermentas, Vilnius, Lithuania), the resultant products were purified and employed as templates to produce dsRNA. An InjectMan 4 instrument (Eppendorf, Hamburg, Germany) was used to microinject the obtained dsRNA (200 ng in 150 nl) into the body cavity of late *T. castaneum* larvae. As positive and negative controls, late-stage larvae were injected with equal quantities of 200 ng ds-*VER* or buffer (IB). The positive control *VER* gene is *T. castaneum's* eye-color gene, which causes the adult beetles' eyes to turn white when it is injected. At least three biological repeats with separate injections were carried out, with each repetition including 40 larvae.

Following dsRNA injection, *T. castaneum* late larvae were normal fed normally for 1 to 5 days, with biological phenotypic changes and mortality documented. Three larvae from each group were chosen at random four days after injection for total RNA extraction and qRT-PCR analysis to determine the target gene's silencing and off-target efficiency. The expression of two

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Translation of TcCYP8BQ(1-1551)
Universal code
Total amino acid number: 516, MW=59385
Max ORF starts at AA pos 1(may be DNA pos 1) for 516 AA(1548 bases), MW=59385
       10      20      30      40      50      60      70      80      90      100     110     120     130     140     150
1      ATGCTTTTAAACAATTTTACACTTAATATACTCGCAGTGTTTATAACGGTACTAGTTGGTGTAATAGTTTATTTTAAATGGCATTTGTCGTACTGGGATCGCCTAGGTGTTCCCAGCTTAAGTCCAGTTTTATTTTTCGGAGACACGAAAAACCTT
1       M L L N N F T L N I L A V F I T V L V G V I V Y F K W H L S Y W D R L G V P S L S P V L F F G D T K N L

       166     176     186     196     206     216     226     236     246     256     266     276     286     296     306
157    ATCCTTTCAAAATGCACAATCGGAGAACAATTTAGAGTTTTTTATAACAAATTTAAATCAAAGGGTTATAAGCACGGTGGTATTTTCTTTGGGCCGATACCCTTCTACATCGCTATAGATCCAGAAATCATCAAACATATCCTACAAAAAGATTTC
53      I L S K C T I G E Q F R V F Y N K F K S K G Y K H G G I F F G P I P F Y I A I D P E I I K H I L Q K D F

       322     332     342     352     362     372     382     392     402     412     422     432     442     452     462
313    CAACATTTCATGAATCACGGATATTACATAAACGAAGAAGACGATCCTTTGACTGGCCATCTTCTCAACTTGGAAAATGTTAAATGGAAAAACATGAGAGCGAAACTAACCCCGACTTTTACTTCCGGAAAAATGAAAATAATGTTCCAAACTTTG
105     Q H F M N H G Y Y I N E E D D P L T G H L L N L E N V K W K N M R A K L T P T F T S G K M K I M F Q T L

       478     488     498     508     518     528     538     548     558     568     578     588     598     608     618
469    GCCGATTGTACCACTGGTCTAAAAAAAGTCATGGACGATTCAGCTCTAAACCACACTCCGGTAGATATCAAAGATATTTTTGGACGTTTCACGACTGATATCATCGGATCGGTTGCTTTCGGTATTGAGTGTAACAGTCTCGAAAACCCTGACGCG
157     A D C T T G L K K V M D D S A L N H T P V D I K D I F G R F T T D I I G S V A F G I E C N S L E N P D A

       634     644     654     664     674     684     694     704     714     724     734     744     754     764     774
625    GAATTTCGGAAATACGGTAAAAAAGTCTTCGAAATTGATTTTTTTGGCAGAATTAAAACGCTTTGTACGTTCGCTATTCCACACCCAATTCTCCGATTGTTCAGATTTAAATTTTACAATTCTGATGTCGCAACATTCTTCATGGACGCAATTCGT
209     E F R K Y G K K V F E I D F F G R I K T L C T F A I P H P I L R L F R F K F Y N S D V A T F F M D A I R

       790     800     810     820     830     840     850     860     870     880     890     900     910     920     930
781    GAAACCGTCAACTACCGAGAGAAAAACAATATTTACCGCAAAGATTTTATGCATTTGTTGTTGCAATTGAAAAACCGGGGTTTGGTCACAGATGATGAAAAAATAACCGGCGATAAAGATATCGTGACAGAAGCTTTAACTATGAATGAACTCGCA
261     E T V N Y R E K N N I Y R K D F M H L L L Q L K N R G L V T D D E K I T G D K D I V T E A L T M N E L A

       946     956     966     976     986     996     1006    1016    1026    1036    1046    1056    1066    1076    1086
937    GCACAAGCCTTCGTTTTCTTCTTAGCCGGTTTTGAGACGTCATCCACGGCAATGACTTGGGCTTTGTACGAACTGGCGATAAACCCAGACGTTCAACAAAAATTAAGAGCAGAAATAAACGATGTTTTGAGAAAACACAACAAATTAACTTATGAA
313     A Q A F V F F L A G F E T S S T A M T W A L Y E L A I N P D V Q Q K L R A E I N D V L R K H N K L T Y E

       1102    1112    1122    1132    1142    1152    1162    1172    1182    1192    1202    1212    1222    1232    1242
1093   GCCATGATGGACATGACTTACATGGAAAAAGTTATTTGTGAAACTCTGCGAAAGTACCCTCCAATACCAGTACTTACACGAAAGTGCACAAAAGACTACACTATCCCCAACACTTCCATTCAACTCCAACGAGGAGTTTCAGTCTCTGTTCCAGTT
365     A M M D M T Y M E K V I C E T L R K Y P P I P V L T R K C T K D Y T I P N T S I Q L Q R G V S V S V P V

       1258    1268    1278    1288    1298    1308    1318    1328    1338    1348    1358    1368    1378    1388    1398
1249   TTAGCGCTTCACACTGACCCTGAATATTATCCAAATCCAGAAAAATTCGATCCTGATCGTTTTAATGACGAGAATGTTAAAGCTAGACCCGGATTTACTTGGCTTCCGTTTGGTGAAGGACCAAGAATTTGTATTGGGTTAAGATTCGGATTGTTA
417     L A L H T D P E Y Y P N P E K F D P D R F N D E N V K A R P G F T W L P F G E G P R I C I G L R F G L L

       1414    1424    1434    1444    1454    1464    1474    1484    1494    1504    1514    1524    1534    1544
1405   CAAAGTAAAGTTGGACTGACCGCAGTTTTGAAACATTATCGAATTAAATTGAATCATAAAACGCAACTTCCTGTAACACTGAACCCACGATCGTTTATCACTTCCGCAAAAGGTGGAGTCTGGTTGGATGTGGAAAAAATAGATTAA
469     Q S K V G L T A V L K H Y R I K L N H K T Q L P V T L N P R S F I T S A K G G V W L D V E K I D *
```

Figure 1. Isolation of the *TcCYP6BQ8* nucleotide sequence and predicted amino acid sequence in *T. castaneum*. ORF, open reading frame; MW, molecular weight; AA, amino acid; *, stop codon. The ORF sequence of the *TcCYP6BQ8* transcript was obtained after translation with DNAMAN. '▲' indicates the heme-binding site. '●' indicates the chemical substrate-binding pocket.

non-target genes, *TcCYP6BQ10* and *TcCYP6BQ11*, which are substantially similar to *TcCYP6BQ8*, was tested to verify that the target gene was successfully disrupted and had no influence on non-target genes (table 1). Five days post-injection, the late larvae were treated for 1 min with terpinen-4-ol (at the LC_{50}) or acetone, followed by normal feeding to observe mortality every 12 h for 72 h.

qRT-PCR analysis

TcCYP6BQ8 expression was detected using the specific primers *CYP6BQ8-F* and *CYP6BQ8-R*. Primer Premier 5.0 was used to construct *RPS3-F* and *RPS3-R* primers for the ribosomal protein S3 (*Rps3*) gene, which has a high level of stability (Horn and Panfilio, 2016) (table 1). The amplification efficiency of the target gene and reference gene primers was similar. an ABI Q6 (CA, USA) was used to set up a 10-μl reaction system with the following settings: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 60 s, followed by 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. 0.25 μl forward and reverse primers (10 M), 5 μl 2 AceQ Universal SYBR qPCR Master Mix, 3.5 μl ddH_2O, and 1 μl cDNA made up the reaction system. At the end of each reaction, a melting curve of the amplified product was constructed to ensure that only one PCR product was amplified. Two technical replicates were carried out, with three biological replicates in each technical replicate.

Data analysis

Gene expression level were the relative mRNA levels normalized to control gene, *T. castaneum* ribosomal protein S3 (*rps3*), using $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001). The gene expression data, the mean values of the RNAi-treated vs. the mean values of the control insects were compared by Student's t-test and one-way analysis of variance in combination with a Fisher's least significant difference multiple comparison tests, respectively, by using the SPSS version 19.0 statistics program (Chicago, IL, United States). All data are presented as the mean ± standard error (SE). Differences were considered significant at *P*-value <0.05.

Results

Identification of TcCYP6BQ8

To investigate the physiological functions of *TcCYP6BQ8* in the catabolism of terpinen-4-ol, the cDNA of *TcCYP6BQ8* was cloned and analyzed. The coding region of *TcCYP6BQ8* is 1554 bp and encodes 526 amino acids in *T. castaneum* (GenBank accession number XP_015834315.1) (fig. 1). *TcCYP6BQ8*'s amino acid sequence was compared to that of other members of the CYP6 gene family and revealed that it included 25 heme-binding sites and ten substrate-binding pockets (fig. 1). Using 43 amino acid sequences from the evolutionary branches of CYP6 and CYP9 in Hymenoptera and Lepidoptera, a systematic phylogenetic tree was created (fig. 2). The phylogenetic tree revealed that the 43 amino acid sequences were divided into two evolutionary branches, with the *CYP6BQ8* gene of *T. castaneum* being significantly similar to the *CYP6BQ9* gene of the same strain of *T. castaneum*.

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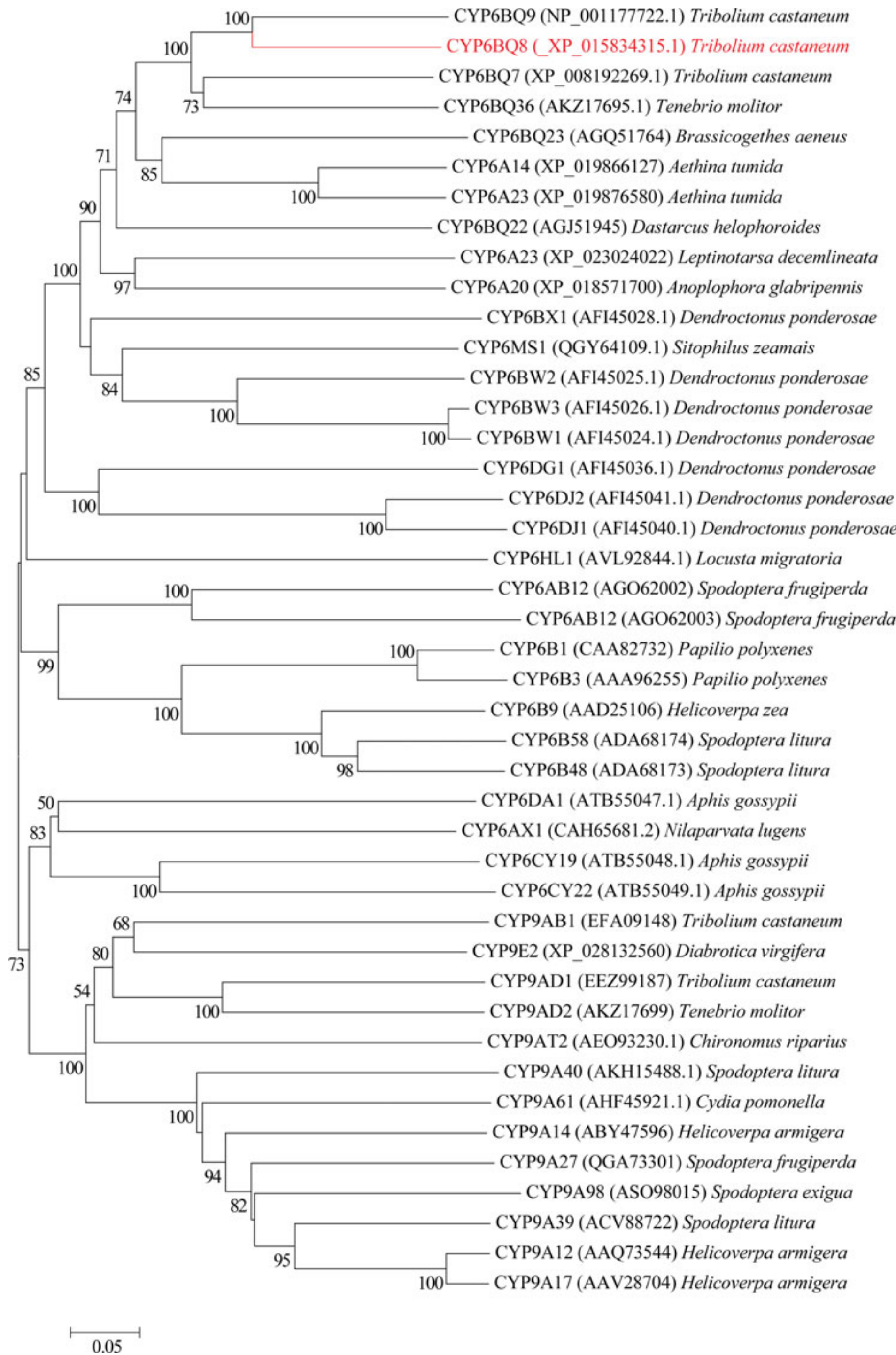

Figure 2. Phylogenetic tree constructed from the amino acid sequence of *TcCYP6BQ8* and other insect-associated P450s. Bootstrap percentage values for 1000 replications are indicated at the nodes; to remove values below 50% from the tree species. *TcCYP6BQ8* is highlighted in red. Distances are expressed on a scale. Gene name, GenBank accession number, and insect species are displayed on each branch of the tree.

Terpinen-4-ol induces TcCYP6BQ8 *expression*

The expression of *TcCYP6BQ8* in the terpinen-4-ol (LC_{50} = 62.5 mg ml^{-1}) treatment group and the acetone control group were measured by qRT-PCR at 12–72 h after treatment. Expression of *TcCYP6BQ8* was significantly higher after 36–60 h of terpinen-4-ol treatment compared with the control group (fig. 3). After treatment with the LC_{50} of terpinen-4-ol, the expression of *TcCYP6BQ8* gradually increased from 24 to 48 h, and then declined at 60 h but still showed a significant increase compared with the control group (fig. 3). The findings indicate that terpinen-4-ol may have the capacity to induce *TcCYP6BQ8* gene expression.

Expression profiles of TcCYP6BQ8 *in different developmental stages and tissues*

The qRT-PCR analysis showed that the relative expression of *TcCYP6BQ8* at different developmental stages of *T. castaneum* differed significantly (fig. 4). *TcCYP6BQ8* expression was highest in the larval stage followed by the late egg and late pupal stages

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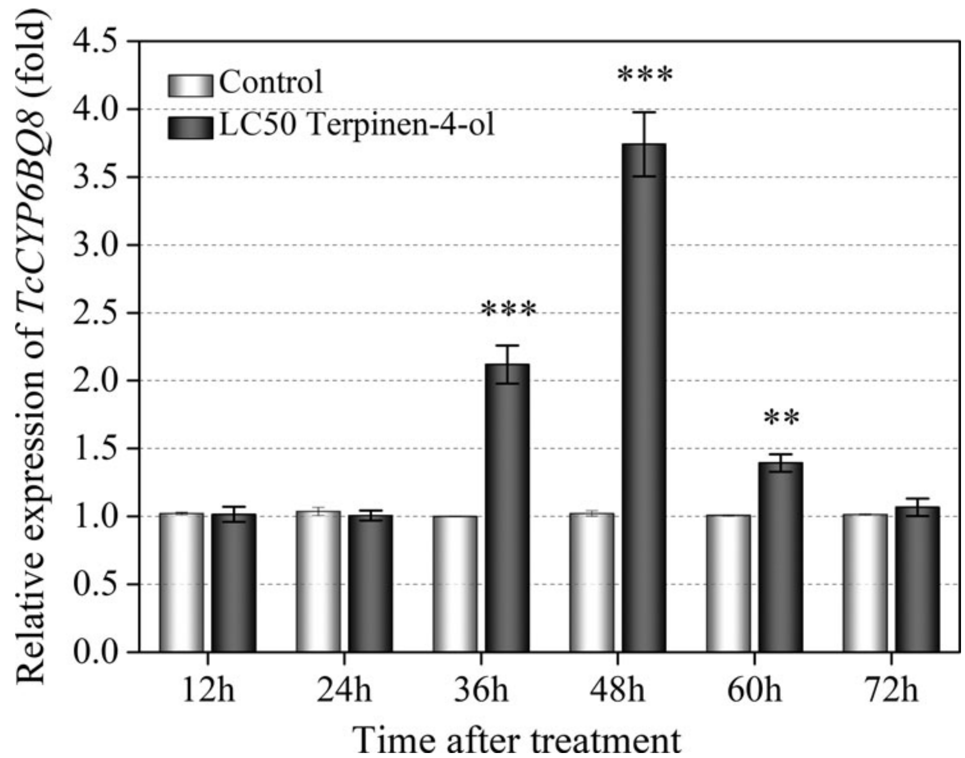

Figure 3. Transcriptional expression of *TcCYP6BQ8* after exposure to terpinen-4-ol. *T. castaneum* larvae (15 days old) were treated with 62.5 mg ml^{-1} (LC_{50}) terpinen-4-ol or equal parts acetone (The control group is arbitrarily assigned a value of 1.) for 12, 24, 36, 48, 60, and 72 h. LC_{50}, median lethal concentration. SEs ($n = 3$) are indicated by the mean of the error bars and the presence of significant differences between the control and experimental groups is indicated by an asterisk (**$P < 0.01$, ***$P < 0.001$).

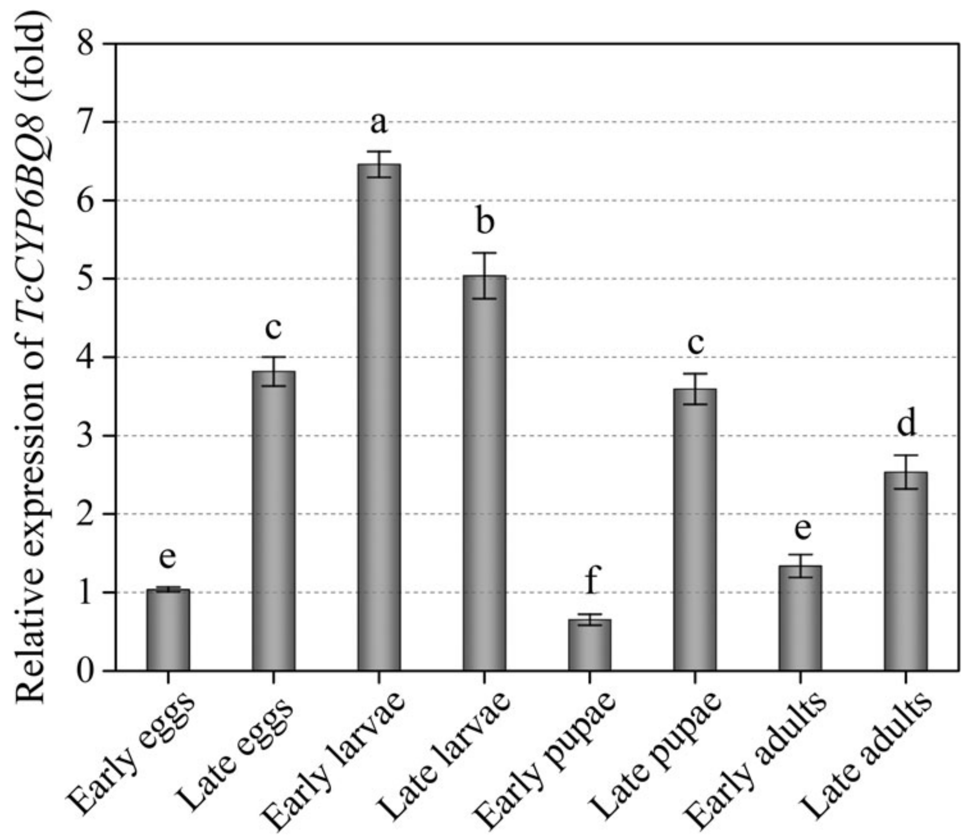

Figure 4. Expression profiles of *TcCYP6BQ8* at eight developmental stages in *T. castaneum*. Early eggs (1 day eggs), late eggs (3 days eggs), early larvae (1 day larvae), late larvae (20 days larvae), early pupae (1 day pupae), late pupae (5 days pupae), early adults (1 day adults), and late adults (10 days adults). The expression of the *Tcrps3* (*Tribolium* ribosomal protein S3) gene was used as a reference point for tissue-specific expression profiles. SEs ($n = 3$) are indicated by the mean of the error bars, and significant differences between the means of gene expression at different developmental periods at the $P < 0.05$ level are indicated by the different letters on the error bars.

and lowest in the other developmental stages (fig. 4). The expression of *TcCYP6BQ8* was further investigated utilizing a variety of *T. castaneum* tissues (late larvae and early adults). *TcCYP6BQ8* was significantly expressed in larval tissues, particularly the head and epidermis, with the greatest level of expression in the head and the lowest level in the fat body (fig. 5). *TcCYP6BQ8* expression in adult tissues was comparable to that in larvae, with the greatest levels detected in the head and epidermis,

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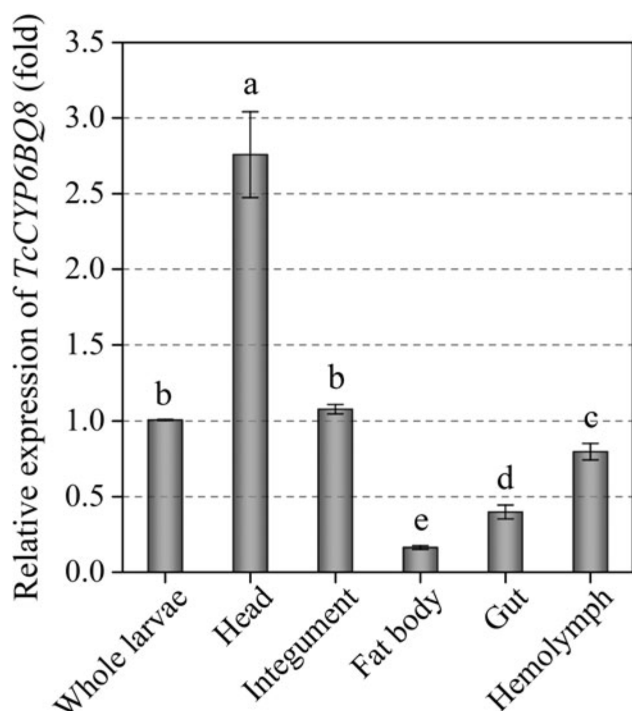

Figure 5. Expression of *TcCYP6BQ8* in *T. castaneum* larval tissues. Larval tissues include whole larvae (15 days old), head, integument, fat body, gut, and hemolymph. The expression of the *Tcrps3* (*Tribolium* ribosomal protein S3) gene was used as a reference point for tissue-specific expression profiles. SEs ($n = 3$) are indicated by the mean of the error bars, and significant differences between tissue gene expression means at the $P < 0.05$ level are indicated by the different letters on the error bars.

followed by the accessory gland and, to a lesser degree, other tissues (fig. 6). Thus, the *TcCYP6BQ8* gene is expressed in various tissues and may be involved in various physiological functions in *T. castaneum*.

Effect of TcCYP6BQ8 *RNAi on* T. castaneum *response to terpinen-4-ol*

To further analyze and understand the detoxification of terpinen-4-ol by *TcCYP6BQ8*, RNAi silencing technology was utilized. RNA silencing of *T. castaneum* larvae (20 days old) significantly reduced the expression of *TcCYP6BQ8* but had no effect on the expression of the non-target genes *TcCYP6BQ10* and *TcCYP6BQ11* (fig. 7). This indicates that RNAi experiments with *TcCYP6BQ8* successfully silenced the gene. The mortality of *T. castaneum* larvae was the same at day 5 post-injection for IB, ds-*TcCYP6BQ8*, and ds-*TcVER* (fig. 8), suggesting that ds*CYP6BQ8* injection had no effect on the physiological health of the larvae. The bioassay of *T. castaneum* larvae with terpinen-4-ol was therefore conducted on the fifth day after injection of ds*CYP6BQ8* or appropriate control. There was a cumulative increase in mortality in all groups after terpinen-4-ol treatment, but late larval mortality was significantly higher following ds*CYP6BQ8* injection compared with the IB and ds-*TcVER* groups (fig. 8). Mortality rates in the IB, ds-*TcVER*, and ds-*TcCYP6BQ8* groups were 47.78, 45.56 and 66.67%, respectively (fig. 8). Analysis of the combined results indicated that the increased mortality after terpinen-4-ol treatment was mainly due to silencing of the *TcCYP6BQ8* gene. *TcCYP6BQ8* is thought to be required for the detoxification of terpinen-4-ol.

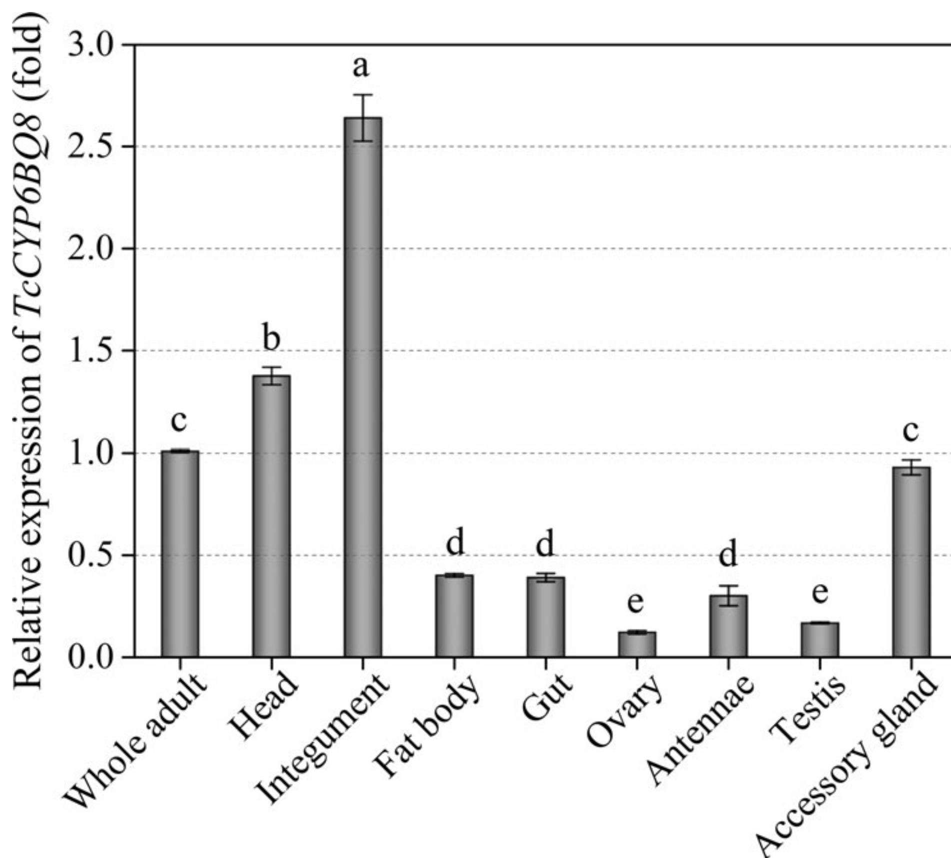

Figure 6. Expression of *TcCYP6BQ8* in *T. castaneum* adult tissues. Adult tissues include whole adult, head, integument, fat body, gut, ovary, antennae, testis, and accessory gland. The expression of the *Tcrps3* (Tribolium ribosomal protein S3) gene was used as a reference point for tissue-specific expression profiles. SEs ($n = 3$) are indicated by the mean of the error bars, and significant differences between tissue gene expression means at the $P < 0.05$ level are indicated by the different letters on the error bars.

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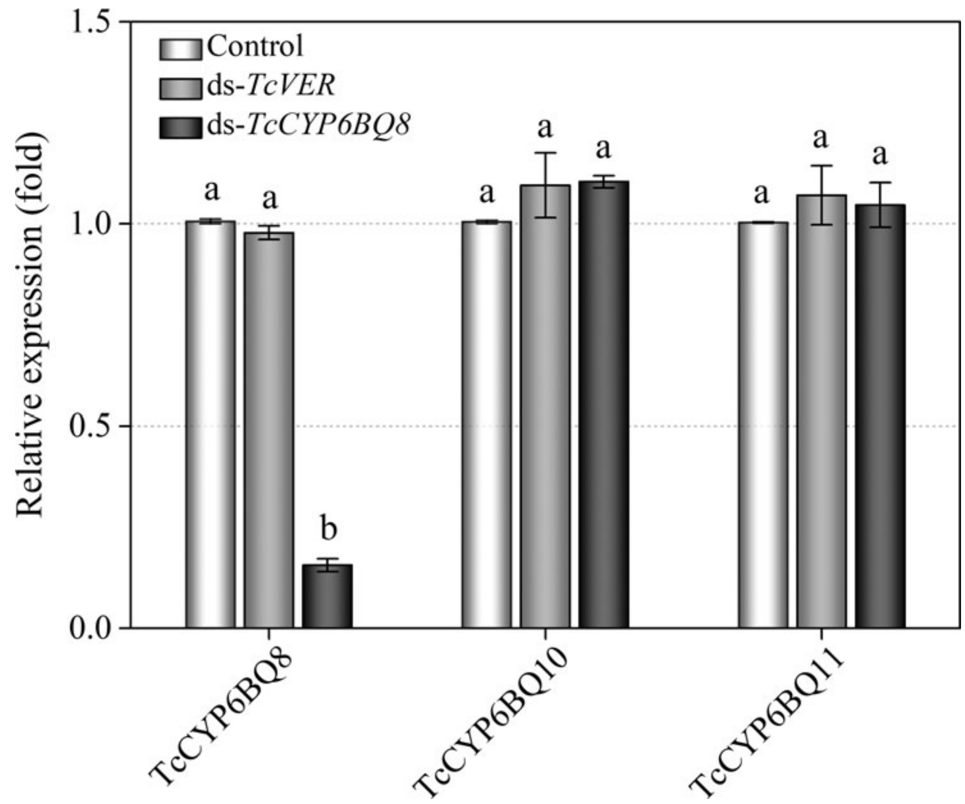

Figure 7. To detect the expression of related genes after ds-*TcCYP6BQ8* injection. IB and ds-*VER* are controls injected with the same amount of agent as the experimental group. *Tcrps3* (Tribolium ribosomal protein S3) gene expression in the IB injection group was taken as a point of reference. SEs ($n = 3$) are indicated by the mean of the error bars, and significant differences between gene means are indicated by different letters on the error bars at the $P < 0.05$ level.

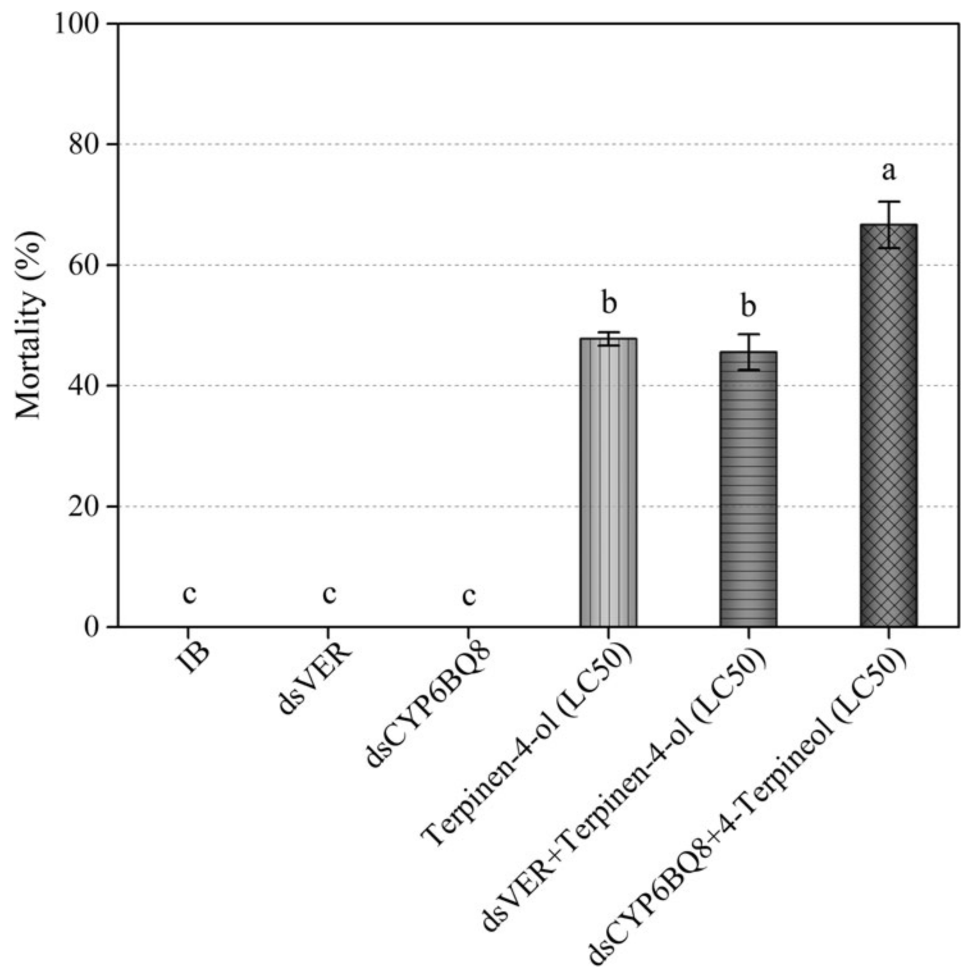

Figure 8. Effect of ds-*TcCYP6BQ8* injection on the toxicity of terpinen-4-ol to *T. castaneum* larvae. Where LC_{50} is the median lethal concentration of the experimental group of terpinen-4-ol and IB and ds*VER* are controls injected with the same amount of agent as the experimental group. Larvae are reared in an incubator under standard conditions after being microinjected. On day 5 post-injection, larvae were exposed to the LC_{50} of terpinen-4-ol to test their susceptibility to the compound. Mortality was assessed 72 h after treatment. SEs ($n = 3$) are indicated by the mean of the error bars, and significant differences between gene means are indicated by different letters on the error bars at the $P < 0.05$ level.

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Discussion

The *CYP* genes of insects are widely differentially expressed in different developmental stages and tissues, which may provide a basis for their physiological functions (Zhang *et al*., 2018, 2019, 2021). The developmental expression profile of *TcCYP6BQ8* was investigated in the present research utilizing qRT-PCR. Expression of *CYP6BQ8* occurs at all developmental stages of *T. castaneum* (fig. 4), suggesting that various physiological processes in *T. castaneum* may be impacted by *CYP6BQ8*. *TcCYP6BQ8* expression was dramatically increased in the late egg, larval, and late pupal stages compared to other developmental stages. High expression of genes during the active feeding phase of insect larvae may indicate an involvement in the detoxification of exogenous compounds. For example, *CYP6FV12* in *Bradysia odoriphaga* (Yang et Zhang), and *CYP6B50* and *CYP6AB60* in *Spodoptera. Litura* (Fabricius) (Chen *et al*., 2019; Lu *et al*., 2019; Sun *et al*., 2019).

The high expression of *TcCYP6BQ8* in late-stage pupae is reminiscent of genes that may be involved in pupae development, but the *CYP6* gene family is a currently known family of genes involved in the metabolism of exogenous substances (Bergé *et al*., 1998). It has been speculated that because insecticides enter the insects predominantly through the respiratory system and integument, the late pupae are extraordinarily sensitive to insecticides (Dai *et al*., 2014). Therefore, high expression of *TcCYP6BQ8* in late pupae may help protect *T. castaneum* from the damaging effects of endogenous siderophore metabolites and toxic substances (Xiong *et al*., 2019). The expression of *TcCYP6BQ8* was also high in late-stage eggs, which was congruent with the expression of *CYP358B1* in *Liposcelis entomophila* (Enderlein) (Li *et al*., 2016). In summary, *CYP6BQ8* may be critical in the detoxification of exogenous toxic substances for *T. castaneum* but may also be related to other physiological activities in *T. castaneum*.

To gain further clues about the physiological function of *TcCYP6BQ8*, tissue expression profiling of *TcCYP6BQ8* was performed (figs 5 and 6). *TcCYP6BQ8* expression was significantly increased in the head and integument of larvae and adults as compared to other tissues. The high expression of insect CYPs in the brain reduces the concentration of insecticides around nerve cells, thereby reducing the toxic effects of these compounds on neural tissue. It has been reported that *TcCYP6BQ9* is expressed mostly in the brain of *T. castaneum* and plays a critical role in deltamethrin resistance and degradation (Zhu *et al*., 2010). The *CYP367* gene of *Plutella xylostella* (Linnaeus) is also expressed at high levels in the head and is able to detoxify exogenous toxins (Yu *et al*., 2015). Similarly, the elevated expression of *TcCYP6BQ8* in the head of *T. castaneum* shows that this gene may be involved in exogenous hazardous chemical detoxification. The integument is crucial for adaptation of insects to the terrestrial environment (Boevé *et al*., 2004). CYPs in the insect integument are the first active barrier to insecticides that enter the insect's body (Dulbecco *et al*., 2018). In the integument of *P. xylostella*, *CYP6BG1* expression occurs during the first detoxification of foreign toxins (Bautista *et al*., 2009). Furthermore, it has been suggested that overexpression of P450s is the main cause of resistance to insecticides (Zhu *et al*., 2008). Based on the expression level of *TcCYP6BQ8* in tissues, it is postulated that this gene is required for exogenous toxin detoxification in *T. castaneum*.

In our earlier studies, we found that stimulation of *T. castaneum* by terpinen-4-ol resulted in altered expression of four classical enzymes: acetylcholinesterase, glutathione S-transferase, cytochrome P450 monooxygenases, and carboxylesterase, with a significant increase in cytochrome oxidase P450 activity (Gao *et al*., 2022*a*). In this investigation, the expression of *TcCYP6BQ8* was similarly dramatically elevated under the stimulation of terpinen-4-ol (fig. 3). To further verify the function of *TcCYP6BQ8*, we used RNAi technology. RNAi, a powerful knockout technique (Kaplanoglu *et al*., 2017; Ma *et al*., 2020), was used to explore the potential physiological functions of the *CYP6BQ8* gene on the degradation of exogenous toxins by *T. castaneum*. In the RNAi experiments, the *TcCYP6BQ8* gene was first silenced using dsRNA and the beetles were then treated with terpinen-4-ol (fig. 8). *TcCYP6BQ8* had a silencing efficiency of almost 85%. In the subsequent terpinen-4-ol treatment, the absence of *TcCYP6BQ8* resulted to a significant higher in the mortality of *T. castaneum* larvae compared with the control treatment. This is a direct indication that *TcCYP6BQ8* may be involved in the metabolic detoxification of toxic substances from plants. Similarly, mortality was significantly higher in *Nilaparvata lugens* (Stål) treated with β-asarone after silencing *CYP6AX1* compared with the control group (Xu *et al*., 2021). Silencing of *CYP4PR1* in *Triatoma infestans* (Klug) followed by treatment with two different doses of deltamethrin significantly increased mortality compared with the control treatment (Dulbecco *et al*., 2021). These experimental results provide valuable evidence to further guide the study of CYPs mediating the detoxification mechanism of phytotoxins in *T. castaneum*.

This study analyzed *CYP6BQ8* of the CYP6 subgene family of *T. castaneum*. *TcCYP6BQ8* expression was significantly induced by terpinen-4-ol. The spatiotemporal phenotype of *TcCYP6BQ8* demonstrated that expression of this gene at various developmental stages and tissues is critical for *T. castaneum's* detoxifying capabilities. Silencing of *TcCYP6BQ8* using RNAi significantly increased the sensitivity of *T. castaneum* to terpinen-4-ol, suggesting that *TcCYP6BQ8* may be involved in the detoxification of phytotoxins. The combined findings established that terpinen-4-ol was deadly to *T. castaneum* larvae and established a theoretical foundation for its use for *T. castaneum* control. This research may provide the basis for global control of grain storage pests.

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Conflict of interest. The authors declare no conflicts of interest.

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