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Characterization and Expression Analysis of the Detoxification Enzyme Gene *GSTd*3 in *Bombyx mandarina*

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Key words
***Bombyx mandarina*, Glutathione-S-transferases, *GSTd3* gene, Expression analysis, Pesticide, BmNPV**

ABSTRACT

Bombyx mandarina is one of the major pests in mulberry gardens, and because of its overlapping generations and leaf-rolling habit, it brings great difficulties to the control. At present, the control of *B. mandarina* is mainly by chemical means, but the massive use of chemical pesticides makes the *B. mandarina* resistant to drugs, and causes poisoning of silkworms and environmental pollution. Therefore, there is an urgent need to find other effective and friendly methods to control the *B. mandarina*. In this study, the gene for *GSTd*3, a detoxification enzyme of *B. mandarina*, was cloned and its physicochemical properties and structure were predicted using bioinformatics tools. Its tissue expression specificity was explored using real-time fluorescence quantitative PCR. The results showed that *GSTd*3 of *B. mandarina* was the closest relative to *BmGSTd*3 of the domestic silkworm, *Bombyx mori*. The expression pattern of *GSTd*3 in *B. mandarina* was stage-specific and tissue-specific. Further studies revealed that the expression of *GSTd*3 varied significantly in different tissues of *B. mandarina* larvae, with higher expression in the fat body and midgut. The expression levels of *BmmGSTd*3 gene in larval fat body and midgut tissues were up-regulated under all three pesticide stresses; the addition of BmNPV had an inducing effect on the expression levels of *BmmGSTd*3 gene in hemolymph and midgut of *B. mandarina* larvae. The present study reveals the expression changes of *BmmGSTd*3 in *B. mandarina*, which provides a theoretical basis for further development of biocontrol methods against *B. mandarina*. On this basis, it is expected to find more effective and environmentally friendly methods to control *B. mandarina* and to guarantee the sustainable development of the mulberry silkworm industry.

INTRODUCTION

In many developing countries, such as China and India, cultivation is the main economic source for farmers (Zhao *et al*., 2018). However, to date, pesticide poisoning and various diseases continue to cause great damage to sericulture (Wang *et al*., 2013). Pesticide contamination has become increasingly serious due to the emergence of insecticide resistance (Peng *et al*., 2011). Silkworms are non-target insects that are extremely sensitive to insecticides in their ecological environment (Peng *et al*., 2011), and pesticide exposure may affect growth, reproduction, and cocooning of silkworm, thereby causing huge economic losses to the sericulture industry (Wang *et al*., 2011).

Insect detoxification enzymes play an important role in pesticide degradation and resistance in insects (Hilliou *et al*., 2021). For example, certain insect detoxification enzymes can convert organophosphorus pesticides into non-toxic substances, thus making insects resistant to

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pesticides (Koirala *et al*., 2022). With further research, GSTs has been recognized as one of the key enzymes affecting pesticide resistance (Vaish *et al*., 2020). GSTs are a multigene-encoded, multifunctional supergene family widely distributed in organisms such as animals, plants, yeasts and bacteria (Sheehan *et al*., 2001). This enzyme family plays an important role in detoxification metabolism and antioxidant in the organism. Insect GSTs are categorized into six known families, Delta, Epsilon, Omega, Theta, Sigma, and Zeta, as well as unknown families, of which those associated with insecticide resistance are the insect-specific Delta and Epsilon families (Chelvanayagam *et al*., 2001). The main function of GSTs is to catalyze the binding of electrophilic groups of certain endogenous or exogenous hazardous substances to the sulfhydryl groups of reduced glutathione to form more soluble, non-toxic derivatives, and at the same time to make them easy to be excreted from the body or to be broken down by phase III metabolic enzymes (Coleman *et al*., 1997). In addition, GSTs also have non-catalytic functions, such as binding non-substrate ligands, acting as a phytochemical carrier, regulating signaling processes, regulating cellular redox homeostasis, and regulating cellular programmed senescence (Marrs *et al*., 1996). An important feature of the GST system is inducibility (Vaish *et al*., 2020), which is an adaptive ability of organisms to endogenous and exogenous toxic substances, and has become a hot spot in GST research.

GSTs are involved in the response of living organisms to external stresses and play an important role in the growth and development of insects. For example, the study of Zuo et al. (2007) showed that the relative expression level of the *GSTd*3 gene in the silkworm, *Bombyx mori*, increased with the prolongation of pesticide treatment for a certain period of time. GSTs also have antioxidant damage effects (Liu *et al*., 2016).

B. mandarina is a lepidopteran insect that mainly parasitizes mulberry and wolfberry trees, and is closely related to the domestic silkworm; they share a common ancestor and are distributed in most areas of China, and are one of the major pests commonly found in mulberry gardens in summer and fall. *B. mandarina* can contaminate mulberry leaves through feces, residual liquid, etc., and can indirectly transmit diseases to domestic silkworms, especially the cross-infection of BmNPV and BmCPV viruses to domestic silkworms, which will bring great harm to sericulture. Currently, there are fewer studies on the molecular mechanisms of *B. mandarina* in response to exogenous adverse stresses.

In this study, firstly, the detoxification enzyme *GSTd*3 gene of *B. mandarina* was cloned, and its physicochemical properties and structure were predicted by bioinformatics tools. The results showed that *GSTd*3 has high homology and specificity in the insect detoxification process. With further experiments, we explored the expression specificity of *BmmGSTd*3 in different tissues of *B. mandarina* using real-time fluorescence quantitative PCR. The experimental results showed that the expression of *BmmGSTd*3 was higher in the fat body and intestinal tissues of *B. mandarina*, suggesting that it plays an important role in pesticide metabolism and detoxification. By studying the expression changes of detoxification enzyme *GSTd*3, we further revealed the resistance mechanism of *B. mandarina* to pesticides. This provides a theoretical basis for finding novel and environmentally friendly control strategies for *B. mandarina*. In addition, this study provides a new research direction for the control of other lepidopteran pests.

MATERIALS AND METHODS

Experimental insects and sample preparation

B. mandarina larvae used in this study were provided by the Sericulture Research Institute of the Chinese Academy of Agricultural Sciences (CAAS) and sparsely liberated with mulberry leaves under standard conditions with a 12:12 photoperiod at 25℃±1℃and 60%-70% RH. BmNPV was provided by our laboratory at a concentration of 1×10^8 PIBs/mL (Yu *et al*., 2022). The phoxim pesticides for the experiment were obtained from Guangzhou Yinanong Biochemical Company Limited, Guangzhou City, Guangdong Province, China, and the deltamethrin pesticides were obtained from Bayer Crop Science Co (China).

RNA extraction and cDNA synthesis

RNA was extracted from the fat body tissue of *B. mandarina* larvae on the 3rd day of the 5th instar using EASYspin plus tissue/cell RNA extraction kit, and the integrity and concentration were determined by rapid electrophoresis on 1% agarose gel, and stored at -80℃. cDNA was prepared using HiScript® III RT SuperMix for qPCR (+gDNA wiper) reverse transcription kit. The cDNA was prepared using HiScript® III RT SuperMix for qPCR (+gDNA wiper) reverse transcription kit, and the prepared cDNA was stored in the refrigerator at -20℃.

Cloning of the BmmGSTd3 gene

SnapGene software was used to design the amplification primers *BmmGSTd*3-F and *BmmGSTd*3-R (Table I). The primers were synthesized by Bioengineering (Shanghai) Co. The fat body cDNA of *B. mandarina* larvae on the 3rd day of the 5th instar was used as a template to amplify the *BmmGSTd*3 gene of *B. mandarina*. The polymerase chain reaction (PCR) was performed

according to the following procedure: 94℃ for 3 min, 95℃ for 30 s, 64℃ for 1 min, 72℃ for 1 min, 35 cycles, and the last extension at 72℃ for 10 min. The PCR amplification products were detected by 1% agarose gel electrophoresis and purified, and then sent to Sangyo Bioengineering (Shanghai) Co.

Bioinformatics and phylogenetic analysis

Bioinformatics prediction and analysis of BmmGSTd3 were carried out by using online analysis tools (Table I). MEGA-X software and neighborhood connection (NJ) method were used to construct phylogenetic tree, and the guided test was repeated for 1000 times.

Quantitative real-time PCR (qRT-PCR) analysis

The primers for real-time fluorescence quantitative PCR, *BmmGSTd*3-QF and *BmmGSTd*3-QR, and the primers for the internal reference gene *Actin*3, *Actin*3-F and *Actin*3-R, were designed according to the gene sequences of *BmmGSTd*3 (Table II). The above primers were synthesized by Bioengineering (Shanghai) Co. Real-time fluorescence quantitative PCR was performed according to the instructions of ChamQ Universal SYBR qPCR master mix kit. The reaction system was 20 μL, and the program was as follows: Pre-denaturation at 95℃ for 3 min; denaturation at 95℃ for 10 s, annealing at 60℃ for 30 s, 40 cycles; 95℃ for 15 s, 60℃ for 60 s, 95℃ for 15 s. All the experiments were carried out three times. Relative mRNA levels were determined by the $2^{-\Delta\Delta CT}$ method. Experimental data were analyzed by ANOVA one-way and independent samples t-test using SPSS 26.0 software, and multiple comparisons were selected for significant difference analysis.

Patterns of spatial expression

Tissue specificity assay: Heads, hemolymph, malpighian tubule, midgut, fat body, posterior silkgland, anterior silkgland, middle silkgland and epidermis were collected from 5th instar larvae and stored at -80℃ for using.

Feed BmNPV

After the starvation treatment, BmNPV virus solution at a concentration of 1×10^8 PIBs/mL was fed orally with a pipette gun to uniformly developed 5th instar larvae, while the control group was fed orally with the same dose of sterile water, and then fed with normal mulberry leaves. At 24, 48 and 72 h after treatment, the hemolymph and midgut of *Bombyx mandarina* were processed on ice, rinsed with PBS buffer and stored in a refrigerator at -80℃. Five larvae were sampled from each treatment, and three biological replicates were set up.

Pesticide coercion

According to the results of the pre-tests, the two pesticide stock solutions were diluted to 4 μg/mL (Peng *et al*., 2011) and 0.02 mg/L (Wu *et al*., 2010), respectively, and the mulberry leaves were immersed in the solutions for 10 sec, removed and naturally dried to dry the surface moisture, and used to feed on well-developed 3rd day, 5th instar *B. mandarina* larvae, which were subsequently reared on fresh mulberry leaves under the same conditions.

Table I. Online analysis tools for bioinformatics.

Name	Website
NCBI BlastP	https://blast.ncbi.nlm.nih.gov/Blast.cgi
ProtParam tool	https://web.expasy.org/protparam/
SOPMA	https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html
SignalP-6.0 server	https://services.healthtech.dtu.dk/service.php?SignalP-6.0
TMHMM	https://services.healthtech.dtu.dk/service.php?TMHMM-2.0
Swiss-Model	https://swissmodel.expasy.org/

Table II. Primers used in this study.

Primer use	Upstream primers (5'-3')	Downstream primers (3'-5')
Clone (Loanword)		TCAAAGTTCAGTTTTGGCCTTCATT
*BmmGSTd*3	ATGGCAATAGATCTATACTTCACCG	
qRT-PCR		TCAATAGTGGAGATGGTAGC
*BmmGSTd*3	TACGGAGGAGACAGTAGC	
*Actin*3	CGGCTACTCGTTCACTACC	CCGTCGGGAAGTTCGTAAG

After 48 h of treatment, the midgut and fat body tissues were processed on ice and prepared separately. The larvae fed on mulberry leaves impregnated with distilled water were used as the control group, and five larvae were sampled from each treatment, with three experimental replicates.

RESULTS

Cloning and sequence characterization of the GSTd3 *gene of* B. mandarina

The sequence of the coding region of the *GSTd*3 gene was amplified by PCR using the cDNA of the fat body tissue of *B. mandarina* on day 3 of 5th instar as a template, and the size of the amplified bands was in accordance with the theoretical expectation. The open reading frame (ORF) of the gene was verified by sequencing to be 663 bp long, encoding 220 amino acids (Fig. 1). The predicted molecular formula of the gene was $C_{1124}H_{1754}N_{278}O_{327}S_5$, with a total atomic number of 3488, relative molecular mass of 24554.26 Da, isoelectric point of 5.00, instability coefficient of 23.44, and total average hydrophilicity of -0.025. Protein signal peptide prediction showed a probability of having a signal peptide of 4.736%, and no transmembrane region was present. The prediction of the secondary structure showed that the *BmmGSTd*3 containing 30.00% of Random coil, 50.91% of α-helix, 11.82% of extended strand and 7.27% of β-turn (Fig. 2). The 3D structure prediction of *BmmGSTd*3 protein was performed with SIWISS-MODEL and the results are shown in Figure 2.

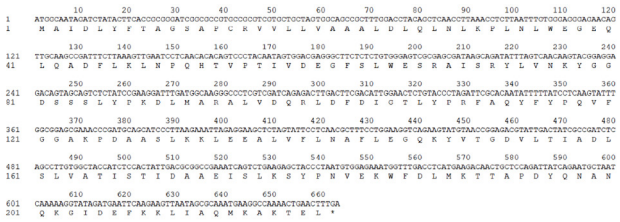

Fig. 1. CDS sequence and corresponding amino acid sequence of the *BmmGSTd*3 gene of *B. mandarina*.

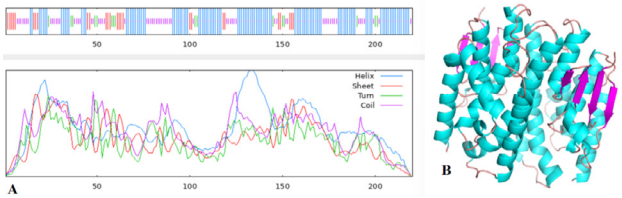

Fig. 2. *BmmGSTd*3 protein structure prediction. A, secondary structure; B, three-dimensional structure.

Multiple comparisons and phylogenetic analysis of BmmGSTd3 *in* B. mandarina

The results of base alignment showed that there were 15 base mutations in the *GSTd*3 sequence of *B. mandarina* (Fig. 3). The results of multiple sequence alignment showed that *BmmGSTd*3 has the closest relationship with *BmGSTd*3 of *Bombyx mori*, showing 98.18% amino acid sequence identity. While the sequence identity of *Galleria mellonella GSTd*3 was the lowest (70.45%). The sequence identity with *Spodoptera frugiperda GSTd*3, *Manduca sexta GSTd*3, *Pectinophora gossypiella GSTd*3, and *Helicoverpa armigera GSTd*3 was 4.21%, 74.09%, 72.73% and 71.82%, respectively (Fig. 4). The phylogenetic tree was constructed with the *BmmGSTd*3 protein and *GSTd*3 from other insects by neighbor-joining method showing that the *BmmGSTd*3 was clustered into one branch with *Bombyx mori* (Fig. 5).

Fig. 3. CDS sequence comparison of *GSTd*3 gene between *B. mandarina* and domestic silkworm.

Fig. 4. Multiple sequence comparison of *BmmGSTd*3 of *B. mandarina* with six other *GSTd*3 species.
Source species of *GSTd*3 and its GenBank accession number: *Bombyx mori* (XP 037867590.1), *Spodoptera frugiperda* (XP 035440580.2), *Manduca sexta* (XP 030035463.2), *Helicoverpa armigera* (XP 021188795.1), *Pectinophora gossypiella* (XP 049871106.1), *Galleria mellonella* (XP 026748199.1).

Expression levels of BmmGSTd3 *gene in different tissues*

The expression level of *GSTd*3 gene in different tissues varied significantly, and it was highly expressed in the head, fat body and epidermis of 5th instar larvae, with the highest expression in the fat body and relatively low expression levels in the rest of the site (Fig. 6).

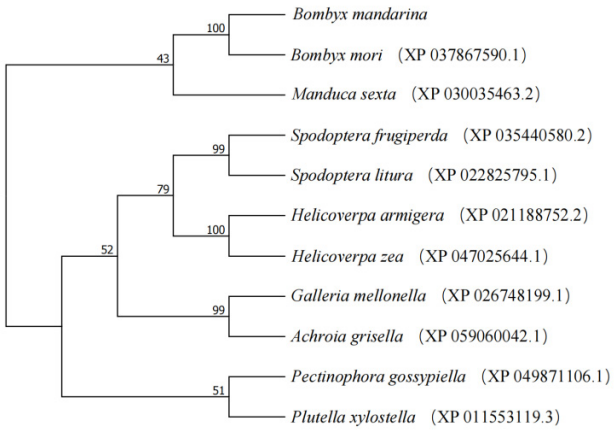

Fig. 5. Phylogenetic tree of *GSTd*3 proteins of *B. mandarina* and other species based on amino acid sequences constructed by neighbor-joining method (1000 replicates). Phylogenetic trees were generated by neighbor-joining method using MEGA-X software. Numbers on branches are bootstrap values obtained from 1000 replicates.

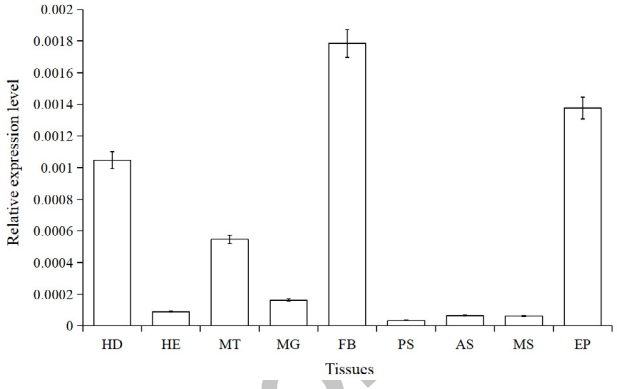

Fig. 6. Relative expression level of *BmmGSTd*3 gene in different tissues of 5th instar larvae of *B. mandarina*. The results are expressed as mean S.E. Horizontal coordinates indicate different tissues and vertical coordinates indicate relative expression levels. HD, head; HE, hemolymph; MT, malpighian tubule; MG, midgut; FB, fat body; PS, posterior silkgland; AS, anterior silkgland; MS, middle silkgland; EP, epidermis.

Expression levels of BmmGSTd3 *gene under pesticide stress*

The transcription levels of *BmmGSTd*3 gene in the midgut and fat body tissues of *B. mandarina* were determined by real-time fluorescence quantitative PCR after addition of trace amounts of the pesticides of phoxim and deltamethrin to the 5th instar larvae of *B. mandarina*, and the results are shown in Figure 7. The results showed that the expression levels of *BmmGSTd*3 gene were both up-regulated by addition of phoxim and deltamethrin in midgut and fat body of *B. mandarina,* which indicated that *BmmGSTd*3 gene may involved in response to these two pesticides in the detoxification tissues such as midgut and fat body of *B. mandarina*.

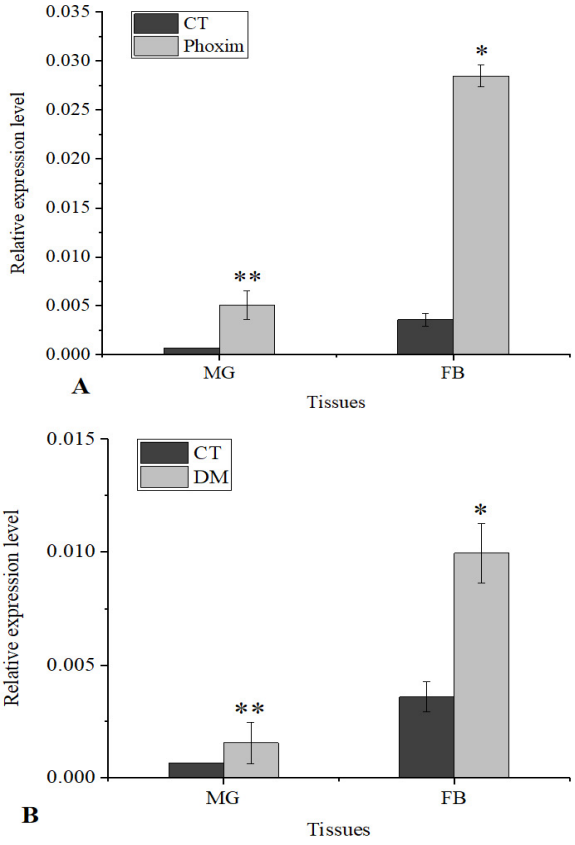

Fig. 7. Relative expression levels of *BmmGSTd*3 in the midgut and fat body of 5th instar larvae of *B. mandarina* at different times after treatments with phoxim and deltamethrin. A: phoxim, B: deltamethrin; CT: control group fed with distilled water-soaked mulberry leaves; DM, deltamethrin. Asterisks indicate significant differences between experimental and control groups, as indicated by * $P < 0.05$, and ** $P < 0.01$. The same for Figure 8.

Expression levels of the BmmGSTd3 *gene in response to BmNPV*

The expression level of *BmmGSTd*3 in hemolymph decreased significantly at 48 h after feeding BmNPV to 5th larvae of *B. mandarina*, while it was up-regulated at 72 h after feeding BmNPV, and the difference reached a highly significant level at 48 h ($P<0.01$). The relative transcription levels of *BmmGSTd*3 were up-regulated in the midgut tissues of *B. mandarina* at 24 h, 48 h and 72 h after feeding BmNPV, and the difference reached a highly

significant level at 24 h (P<0.01) (Fig. 8).

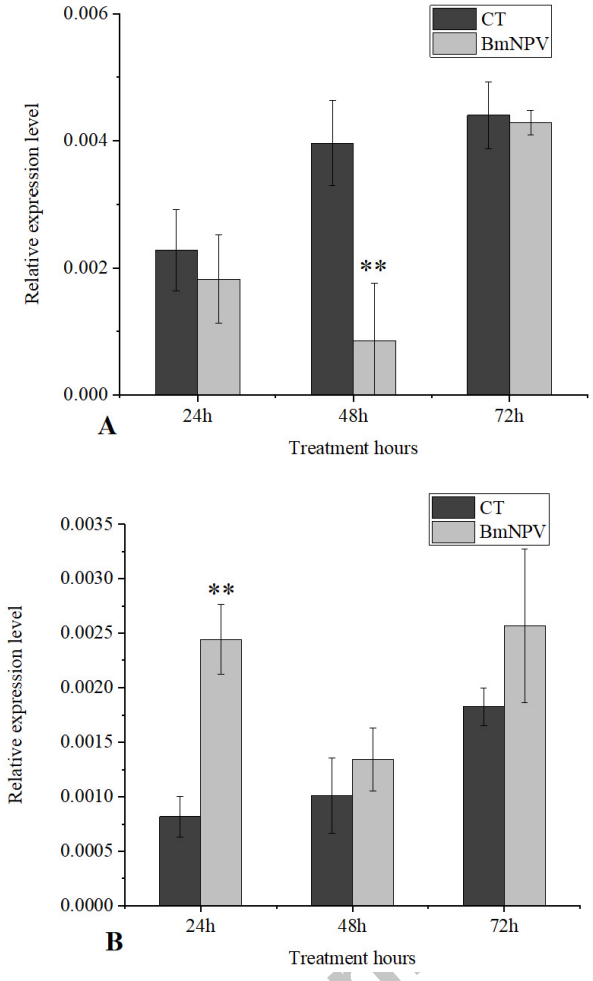

Fig. 8. Relative expression levels of *BmmGSTd3* in the hemolymph and midgut of 5th instar larvae of *B. mandarina* at different time after feeding BmNPV. A, hemolymph; B, midgut; CT, control group fed the same dose of sterile water; BmNPV, *Bombyx mori* nuclear polyhedrosis virus.

DISCUSSION

Based on the genetic information of the homologous insect *B. mandarina*, *Bombyx mori*, in the NCBI database, and using RT-PCR, we cloned the CDS sequence of the *B. mandarina* detoxification enzyme *GSTd3* gene, which is 663 bp long and encodes 220 amino acids in the open reading frame (ORF). As shown by amino acid structure prediction, the signal peptide prediction of *BmmGSTd3* protein showed a probability of 4.736% of having a signal peptide without a transmembrane region. Its secondary structure contains 7.27% of β-turning angle, which helps *BmmGSTd3* protein to form an anti-parallel structure, and then form dimers and other roles. Meanwhile, the amino acid sequences of delta members of other species have a similar secondary structure, which are composed of N-terminal and C-terminal functional domains (Zuo *et al.*, 2007). Amino acid homology analysis and phylogenetic evolutionary tree analysis showed that *BmmGSTd3* has high homology with *GSTd3* of lepidopteran insects such as the houseworm, and clusters into a single unit with the houseworm and the tobacco moth, which suggests that the *GSTd3* gene is relatively conserved in evolution, and this is in agreement with the results of the study of insects such as *Drosophila* and *Anopheles gambiae* (Gonis *et al.*, 2022; Chen and Gao, 2005).

It is generally believed that insect detoxification enzymes are a class of enzymes produced in insects that can metabolize thousands of compounds including pesticides under long-term application of pesticides, and they are expressed in various tissues in insects. In this study, the expression analysis of the *BmmGSTd3* gene revealed significant differences in its relative expression in different tissues of *B. mandarina*, which is consistent with the results of Wan *et al.* (2016) study of *Spodoptera exigua* (Wan *et al.*, 2016), and it is hypothesized that the degree of detoxification of this gene may be different in different tissues. Among them, *BmmGSTd3* had the highest expression in the fat body, which confirmed that the fat body is an important intermediate metabolic tissue involved in the detoxification process of *B. mandarina* (Shen *et al.*, 2004). And the high expression of this gene in the epidermis of *B. mandarina* also suggests that the epidermis is an important tissue in the detoxification process of *B. mandarina*, but the specific biological function in the epidermis needs to be further studied.

Both organophosphorus compounds and pyrethroids induced the expression of GSTs (Xu *et al.*, 2020). The expression of *SlGSTe8* gene in larvae of the slash-night moth was significantly up-regulated in all cases after phoxim treatment (Xu *et al.*, 2023). We further investigated the effects of pesticides on *GSTd3* gene expression in *B. mandarina*. The results showed that the transcript levels of the *B. mandarina GSTd3* gene were significantly up-regulated in both the midgut and fat body tissues of *B. mandarina* after the addition of trace amounts of the pesticides phoxim and deltamethrin. This suggests that the pesticides can induce the expression of *BmmGSTd3* gene and thus enhance its detoxification ability (Sule *et al.*, 2022).

There have been more studies on the changes in the expression of related genes after baculovirus infection of insects. The results of this study showed that 48 h after *B. mandarina* 5th instar larvae were infected with BmNPV,

the expression level of *BmmGSTd*3 gene in hemolymph decreased significantly, and then its expression was up-regulated 72 h after addition, which may be attributed to the fact that it takes time for the virus to increase in value, and its inducing effect can only be realized after a certain amount of the virus has accumulated. The expression of *BmmGST*3 increased dramatically in midgut at 24 h after BmNPV addition, which may be due to the fact that the virus was added to the midgut via the mouth, and therefore the response in the midgut was more rapid. The results of BmNPV supplementation suggest that virus infection has a regulatory effect on the expression of *BmmGSTd*3 gene, and this regulation may differ in different tissues.In addition, we found that the expression level of *BmmGSTd*3 gene in hemolymph was significantly decreased and up-regulated in midgut tissues after the addition of BmNPV. This may indicate that viral infection has a regulatory effect on the expression of the *GSTd*3 gene in *Bombyx mandarina*, and this regulation may differ in different tissues (Zhao *et al*., 2011), and it needs to be further studied.

CONCLUSION

In this study, the *GSTd*3 gene of *B. mandarina* was cloned, and its sequence characterization, homology comparison, phylogenetic evolution, and expression analysis were investigated to further reveal the resistance mechanism of *B. mandarina* to pesticides, which provides a theoretical basis for the search of novel and environmentally friendly control strategies for *B. mandarina*. In addition, this study provides a new research direction for the control of other lepidopteran pests. In future studies, we can further explore the mechanism of *B. mandarina GSTd*3 gene in different tissues, as well as the mechanism of pesticide and virus infection on the regulation of its expression.

DECLARATIONS

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Statement of conflict of interest

The authors have declared no conflict of interest.

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