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Cloning of three epsilon-class glutathione *S*-transferase genes from *Micromelalopha troglodyta* (Graeser) (Lepidoptera: Notodontidae) and their response to tannic acid

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Research Paper

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Abstract

Micromelalopha troglodyta (Graeser) is an important pest of poplar in China, and glutathione *S*-transferase (GST) is an important detoxifying enzyme in *M. troglodyta*. In this paper, three full-length GST genes from *M. troglodyta* were cloned and identified. These GST genes all belonged to the epsilon class (*MtGSTe1*, *MtGSTe2*, and *MtGSTe3*). Furthermore, the expression of these three *MtGSTe* genes in different tissues, including midguts and fat bodies, and the *MtGSTe* expression in association with different concentrations of tannic acid, including 0.001, 0.01, 0.1, 1, and 10 mg ml^{-1}, were analysed in detail. The results showed that the expression levels of *MtGSTe1*, *MtGSTe2*, and *MtGSTe3* were all the highest in the fourth instar larvae; the expression levels of *MtGSTe1* and *MtGSTe3* were the highest in fat bodies, while the expression level of *MtGSTe2* was the highest in midguts. Furthermore, the expression of *MtGSTe* mRNA was induced by tannic acid in *M. troglodyta*. These studies were helpful to clarify the interaction between plant secondary substances and herbivorous insects at a deep level and provided a theoretical foundation for controlling *M. troglodyta*.

Introduction

Glutathione *S*-transferase (EC 2.5.1.18) (GST) have a physiological role in initiating the detoxification of potential alkylating agents. They can neutralise the electrophilic sites and make the products easier to solubilise in water through the mechanism that catalyses the compounds and the –SH group of glutathione to react (Habig *et al.*, 1974). GSTs also interact with kinases and play a non-catalytic role by binding a wide range of exogenous and endogenous ligands (Morel *et al.*, 2004). With a deep understanding of GSTs, it has been demonstrated that GSTs can increase the hydrophilicity of electrophilic compounds and cause them to be easily excreted from the body. In addition, GSTs also play an important in protecting cells from oxidative damage and the intercellular transport of endogenous metabolites, exogenous compounds, hormones, and so on (Listowsky *et al.*, 1988; Clark, 1989; Rushmore and Pickett, 1993).

The well-known GSTs include microsomal, mitochondrial, and cytosolic GSTs (Fournier *et al.*, 1992). Insect cytosolic GSTs were designated as classes I and II in earlier studies according to their distinct immunological features (Fournier *et al.*, 1992; Hemingway, 2000). Later, a new class of insect GSTs was discovered and classified into class III (Ranson *et al.*, 2001). Under the new classification naming rules, class I GSTs were renamed delta GSTs; class II includes four families: sigma, zeta, theta, and omega; class III GSTs were renamed epsilon GSTs. Among these GSTs, epsilon and delta GSTs were the GSTs that were specific to insects (Board *et al.*, 1997; Chelvanayagam *et al.*, 2001; Ranson *et al.*, 2001). There were no clearly established criteria for the degree of sequence similarity required to place GSTs in a particular class. Armstrong considered that when the primary structure shows 40–60% identity, GST isoenzymes belonged to the same class, and a sequence identity less than 20% indicated that the enzyme belonged to a different class (Armstrong, 1997). Sheehan deemed that GSTs in the same class were expected to have more than 60% identity, and GSTs with less than 30% identity should be classified into different classes in general (Sheehan *et al.*, 2001). Chelvanayagam regarded GSTs as being members of the same class when they show more than 40% identity (Chelvanayagam *et al.*, 2001).

Micromelalopha troglodyta (Graeser) is a frequent and important defoliator of poplar trees with rapid growth and high fecundity. In many parts of China, they often cause huge financial losses. Our research group first cloned a *MtGSTd1* gene and detected the expression of

MtGSTd1 mRNA in the fat bodies and midguts of *M. troglodyta* (Cheng *et al*., 2015). Then, our research group cloned and characterised *MtGSTs1*, *MtGSTd2*, *MtGSTz1*, *MtGSTo1*, and *MtGSTt1* from *M. troglodyta*, and tested their responses to tannic acid stress (Tang *et al*., 2020). However, the epsilon-class GST genes of *M. troglodyta* and their characteristics have not been reported. In this study, three full-length epsilon-class GST genes were cloned from *M. troglodyta* larvae, and their response to tannic acid was evaluated. The aims of this study were to identify the GST genes and determine the roles that GSTs play in the interaction of insect and plant secondary substances, which could provide a theoretical basis for finding new ways to control *M. troglodyta*.

Materials and methods

Insect

We collected *M. troglodyta* larvae in Nanjing (31°56′17.00″N, 118°22′35.98″E), Jiangsu province, China and used them to establish a population in our laboratory. We kept the larvae at 26°C, 70–80% humidity, the photoperiod of 16 h light:8 h dark, and fed them with fresh poplar leaves. We collected samples from 1st to 5th instar larvae for follow-up experiments. In addition, 5th instar larvae were dissected on ice to obtain their heads, haemolymph, midguts, fat bodies, and body walls respectively.

We fed *M. troglodyta* larvae with the poplar leaves soaked in tannic acid solution to study the induction of tannic acid on GSTs. We used a small amount of ethanol to dissolve tannic acid (Sigma Chemical, St. Louis, MO) and diluted it to five proportional concentrations ranging from 0.001 to 10 mg ml^{-1} with distilled water. We used this solution to soak fresh poplar leaves and then dried the leaves. These leaves were fed to *M. troglodyta* larvae. Each treatment was repeated three times, and each repetition had 20 larvae. The larvae of the control groups were fed with leaves soaked in distilled water. After 96 h of feeding, *M. troglodyta* larva was dissected on ice to obtain their midguts and fat bodies.

Cloning and sequencing of GST cDNA

According to the instructions, we extracted the total RNA from individual larvae using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA), then used DNaseI (TaKaRa, China) to treat the total RNA, and synthesised cDNA by the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, China). We amplified the full-length open reading frame (ORF) of GSTs by using the cDNA as a template for polymerase chain reactions (PCRs). We used Primer Premier 5 software to design the primers and synthesised them at Shanghai Generay Biotechnology Co., Ltd (table 1). The PCR conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min; followed by 72°C for 10 min. The PCR products were cloned in pMD-19T vector (TaKaRa, China) and cloned into DH5α competent cells, and then were sequenced at the Nanjing GenScript Biotechnology Company. We used the BLASTX search program of the NCBI GenBank to search for similarities, and then analysed the complete sequences.

Sequence analysis and phylogenetic analysis

We used the cDNAs of the GSTs to deduce their amino acid sequences, and used ClustalX2 to align them. Then MEGA6 software was used to convert alignments to meg files and construct the phylogenetic tree of 92 GST proteins from 29 species with the neighbour-joining method (Saitou and Nei, 1987). The theoretical molecular weight and isoelectric point of the GSTs in *M. troglodyta* were analysed by ExPASy ProtParam. SignalP 5.0 was used to analyse signal peptide. DNAMAN software was used for multiple sequence alignment analysis.

The expression profiles of GSTs in different tissues and instars of M. troglodyta

The expression profiles of *MtGSTs* in different tissues and instars of *M. troglodyta* larvae were compared by real-time fluorescence quantitative PCR (qPCR). We extracted the total RNA from 100 mg larvae and used the Real-Time PCR Kit (Takara Biotechnology (Dalian) Co., Ltd) to perform qPCR. The mixture used for qPCR was 20 µl and consisted of the following substances: 10 µl SYBR Premix Ex Taq, 0.4 µl Rox Reference Dye (503), 1 µl cDNA, 7.8 µl double-distilled water, and 0.4 µl both sense and antisense primers of GST. We used Primer Premier 5 software to design the primers and synthesised them by Shanghai Generay Biotechnology Co., Ltd (table 1). In order to estimate whether the primers were qualified, we used LinReg PCR (Version: September 2014) software to examine the qPCR results to confirm the amplification efficiency of primers. We replaced the GST gene primers in the mixture with a pair of actin gene primers (GenBank accession no. GU262991) to serve as endogenous controls. We used a 7500 Real-Time PCR system (Applied Biosystems, Foster, CA) to perform qPCR, and set the qPCR reaction conditions as follows: the reaction mixture was kept in 95°C for 30 s, then 40 cycles of 95°C for 5 s, and 60°C for 34 s were run. The melting-curve cycles were continued under the conditions of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s to confirm the amplification of specific products. The experiment was repeated three times. We calculated the relative expression of *MtGSTe* mRNAs in different instars and tissues of larvae by the $2^{-\Delta Ct}$ method (Giulietti *et al*., 2001).

The effect of tannic acid on GST transcripts in M. troglodyta

The effects of tannic acid on *MtGSTe* mRNAs in the fat bodies and midguts of *M. troglodyta* larvae were compared by qPCR. The specific steps of qPCR were the same as in the section 'The expression profiles of GSTs in different tissues and instars of *M. troglodyta*'. According to the $2^{-\Delta\Delta Ct}$ method, we calculated the relative expression level of *MtGSTe* mRNA.

Statistical analysis

We used InStat software (GraphPad, San Diego, CA) to analyse the variance of the data collected in these experiments. Tukey's test was used for multiple comparisons with significance defined as $P < 0.05$.

Results

Cloning and identity of three epsilon-class GST genes in M. troglodyta

Three different GST transcripts were cloned and identified from *M. troglodyta* larvae, and their identities were revealed by BLASTX at NCBI. The BLASTX results showed that the three

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Table 1. Primers for PCR and qPCR of *MtGSTe* in *M. troglodyta*

Gene	Primer	Sequence (5′–3′)	Application
MtGSTe1	F	CGTATCAGGTCTCGCCA	ORF
	R	CAGCCCCTAATCTTTGC	ORF
	Q-F	TGAAAGCCACGCCATAATCC	qPCR
	Q-R	AACATTCTTCCGTCTCGCATAG	qPCR
MtGSTe2	F	ATGTCTCGCCCACTGCT	ORF
	R	TCAGCTTCTACTATTGGTGC	ORF
	Q-F	CTGACCTGTCCCTTGGATGTAC	qPCR
	Q-R	GAGGATTTCCACGGCGTTC	qPCR
MtGSTe3	F	GCAACAGTCACCTACGAATA	ORF
	R	CACTCGTTTTATTTAACCATC	ORF
	Q-F	GGACTGGATGACGCTGGC	qPCR
	Q-R	TCCGAGTCGTGTCAGCCAA	qPCR
Actin	Q-F	GCGGCGCGACTCACCGACTAC	qPCR
	Q-R	GGGAAGAGAGCCTCAGGGCAAC	qPCR

genes had high homology with the epsilon-class GSTs of *Spodoptera litura* (Fabricius) and *Spodoptera exigua* (Hübner), with identity ranging from 51 to 57%. In phylogenetic tree, these three GSTs and the epsilon-class GSTs from other species were clustered on the same branch (fig. 1). The comparison of amino acid sequence similarity and the phylogenetic tree proved that the three GSTs all belong to the epsilon class. Therefore, we named these three genes *MtGSTe1*, *MtGSTe2*, and *MtGSTe3*.

MtGSTe1, *MtGSTe2*, and *MtGSTe3* each contained an ORF of 666, 600, and 687 nucleotides encoding 221, 199, and 228 amino acids, respectively. The ORFs all contained the same start codon (ATG) and three different stop codons (TAA, TAG, TGA), indicating that these sequences contained the complete coding region. The predicted molecular weights of these three genes ranged from 22.6 to 25.3, and the theoretical pI ranged from 5.26 to 8.62. No signal peptide was predicted for these three proteins, indicating that they are all non-secretory proteins (table 2). These three *MtGSTe* cDNA sequences and corresponding amino acid sequences have been uploaded to GenBank (GenBank accession nos. KU963406, KU963407, and KU963409).

We used the GSTe amino acid sequences of *M. troglodyta* for multiple sequences alignment with the amino acid sequences from other insects (fig. 2). Our results of multiple amino acid sequence alignment showed two conserved motifs of GSTe in *M. troglodyta*. In addition, proline (P) and isoleucine (I) residues were active binding sites of glutathione, and aspartic acid (D) residue was the active binding site of the substrate in *M. troglodyta*, and serine (S) was the catalytic activity site of GSTe genes.

The expression levels of three epsilon-class MtGSTs *in different instars and tissues*

The expression levels of three *MtGSTe* mRNAs in different instars and different tissues were compared and analysed. The results showed that the expression profiles of different *MtGSTes* were different in the 1st to 5th instar larvae (fig. 3). The expression level of *MtGSTe1* increased with the increase of instar in the 1st to 4th instar, while the expression level of *MtGSTe1* decreased in the 5th instar (fig. 3a). Compared with other instars, the expression of *MtGSTe2* was the highest in the 4th instar (fig. 3b). The expression level of *MtGSTe3* in the 4th and 5th instars was higher than that in the 1st to 3rd instars (fig. 3c). Regarding the expression levels of *MtGSTes* in different tissues, the expression levels of *MtGSTe1* ranged from high to low in the fat body, body wall, midgut, haemolymph, and head, respectively (fig. 4a). The expression of *MtGSTe2* was highest in midgut, followed by head (fig. 4b). The expression level of *MtGSTe3* was higher in fat body, head, and haemolymph (fig. 4c).

The response of three epsilon-class MtGST *genes to tannic acid*

The effects of tannic acid at different concentrations on the mRNA expression of *MtGSTe* genes were compared (fig. 5). For *MtGSTe1*, the expressions of *MtGSTe1* mRNA in the midguts were increased by 0.1 mg ml^{-1} tannic acid, while the expression of *MtGSTe1* mRNA in the fat bodies were increased by 1 and 10 mg ml^{-1} tannic acid (fig. 5a). Tannic acid at 0.01 and 0.1 mg ml^{-1} increased the *MtGSTe2* mRNA expression and at 10 mg ml^{-1} decreased the expression in the midguts, while tannic acid at low concentrations did not significantly affected the gene expression in the fat bodies and 1 and 10 mg ml^{-1} tannic acid induced the gene expression in the fat bodies, and the expression was even 16 times as high as that in the control (fig. 5b). The expression of *MtGSTe3* mRNA in the fat bodies and the midguts were both increased by 1 and 10 mg ml^{-1} tannic acid (fig. 5c).

Discussion

In many insects, the GST gene sequences have been cloned and identified with the progress of molecular biology techniques and the completion of the sequencing of the *Drosophila melanogaster* Meigen and *Anopheles gambiae* Giles genomes (Aultman *et al.*, 2002; Ding *et al.*, 2003; Holt and Chaturvedi, 2003). According to the naming rules proposed by Ding *et al.* (2003), the three GSTs in this study belonged to the epsilon class,

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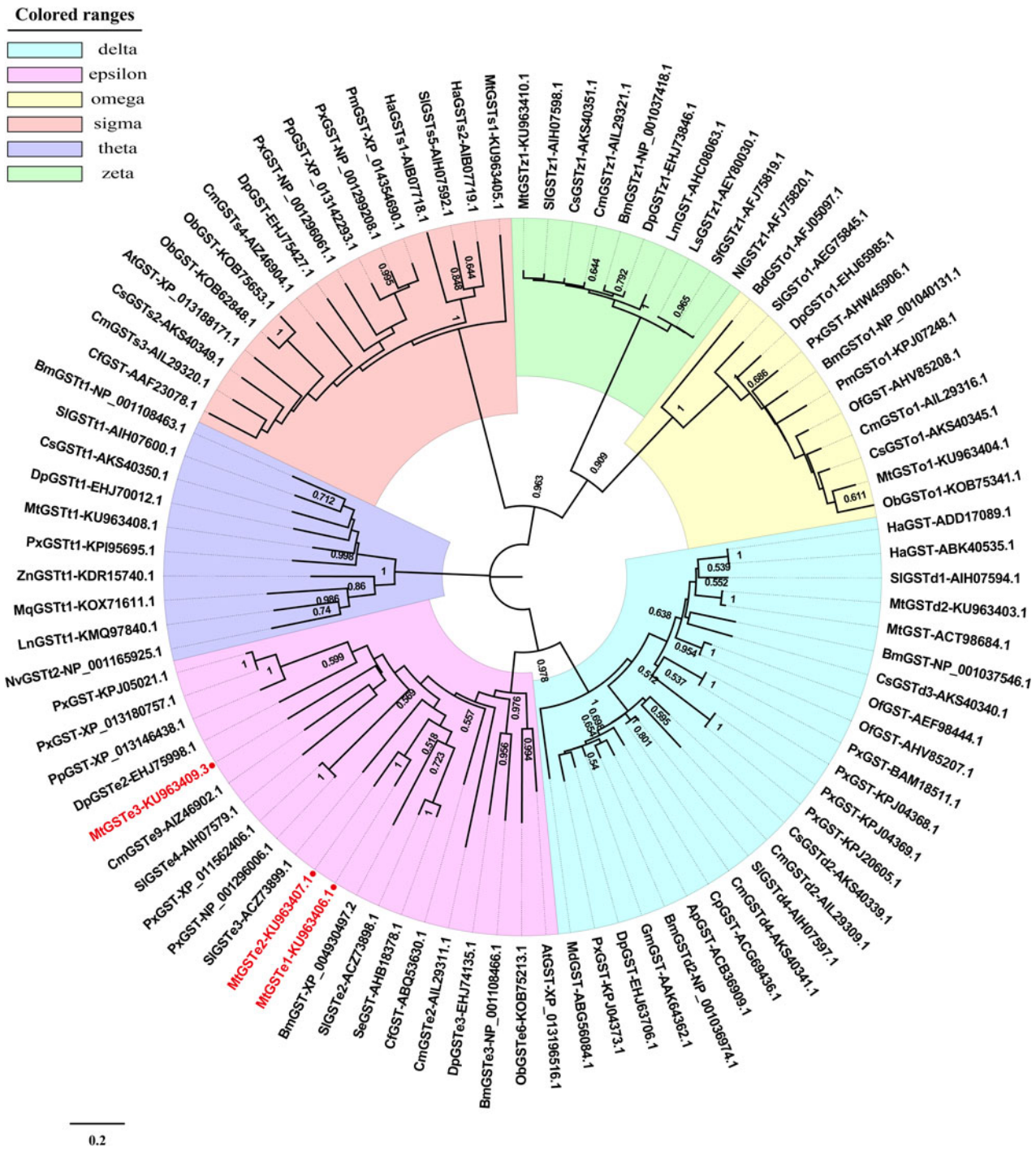

Figure 1. Neighbour-joining tree of 92 GST proteins from 29 species of insects. Mt, *Micromelalopha troglodyta*; At, *Amyelois transitella*; Pp, *Papilio polytes*; Bd, *Bactrocera dorsalis*; Ap, *Antheraea pernyi*; Cf, *Choristoneura fumiferana*; Zn, *Zootermopsis nevadensis*; Cm, *Cnaphalocrocis medinalis*; Sl, *Spodoptera litura*; Dp, *Danaus plexippus*; Gm, *Galleria mellonella*; Ha, *Helicoverpa armigera*; Sf, *Sogatella furcifera*; Ln, *Lasius niger*; Mq, *Melipona quadrifasciata*; Ls, *Laodelphax striatella*; Md, *Mayetiola destructor*; Se, *Spodoptera exigua*; Nl, *Nilaparvata lugens*; Bm, *Bombyx mori*; Nv, *Nasonia vitripennis*; Cp, *Cydia pomonella*; Ob, *Operophtera brumata*; Cs, *Chilo suppressalis*; Of, *Ostrinia furnacalis*; Lm, *Locusta migratoria*; Pm, *Papilio machaon*; Px, *Plutella xylostella* (AHW45906.1, NP-001296061.1, NP-001296006.1, XP-011562406.1), Px, *Papilio xuthus*. The nodes with distance bootstrap values (1000 replicates) are displayed.

which was unique to insects (Fournier *et al.*, 1992; Ranson *et al.*, 2001). This class of GST was closely related to insect resistance mechanisms. For instance, exposure to malathion and β-cypermethrin increased the expression of different GSTe genes in *Bactrocera dorsalis* (Hendel), respectively (Hu *et al.*, 2014). In *S. litura*, chlorpyrifos and xanthotoxin induced the expression of *SlGSTe1* and *SlGSTe3* to different degrees (Huang *et al.*, 2011). In *S. litura*, the expression of *SlGSTe2* gene was up-regulated by *Bacillus thuringiensis* Berliner, carbaryl, dichlorodiphenyltrichloroethane (DDT), deltamethrin, and tebufenozide, and the expression of *SlGSTe3* was slightly up-regulated by *B. thuringiensis*, carbaryl, and DDT (Deng *et al.*, 2009).

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Table 2. List of *M. troglodyta* GSTs

Gene	GenBank accession no.	ORF (bp/aa)	Predicted mw (kDa)	Theoretical pI
MtGSTe1	KU963406	666/221	24.7	6.91
MtGSTe2	KU963407	600/199	22.6	5.26
MtGSTe3	KU963409	687/228	25.3	8.62

The expression of GSTs in different instars and tissues is different among different insects. Sun *et al.* (2020) found that the expression levels of 18 GST genes in *Hyphantria cunea* (Drury) were higher in the 1st to 4th instar larvae and lower in the 5th to 7th instar larvae. But in *Lymantria dispar* (Linnaeus) and *S. litura*, the expression of some GST genes reached maximum at the 5th or 6th instar (Huang *et al.*, 2011; Ma *et al.*, 2021). Tissue-specific expression analysis of *B. dorsalis* showed that three GSTs were highly expressed in midgut, four GSTs were highly expressed in fat body, and six GSTs were highly expressed in malpighian tubule (Hu *et al.*, 2014). The expression levels of 25 GST genes in three larval tissues of *Cnaphalocrocis medinalis* (Güenée) were also different (Liu *et al.*, 2015). These may represent the different roles of GSTs genes in different instars and tissues of insects. In our study, we compared the expression levels of three *MtGSTes* in different instars and different tissues. Compared with other instars, the expression levels of *MtGSTe1*, *MtGSTe2*, and *MtGSTe3* were the highest in the 4th instar larvae. The expression levels of *MtGSTe1* and *MtGSTe3* in fat body were higher than those in other tissues, while the expression levels of *MtGSTe2* were highest in midgut. This indicates that these three GSTs in *M. troglodyta* might have different functions in the tissues examined.

The combination of GSTs in insects with insecticides and other toxic compounds played a vital part in detoxification metabolism and the development of resistance (Ranson *et al.*, 2002; Enayati *et al.*, 2005). There were studies that have shown the gene expression of important detoxifying enzymes in insects, such as GSTs, could be induced by various exogenous or endogenous compounds. These exogenous compounds included plant secondary substances that insects encountered when feeding. Plant secondary substances, such as alkaloids, phenols, and non-protein amino acids, were important biochemical bases for plant defence against phytophagous insects. These plant secondary substances were variously harmful to insects and other herbivores, and therefore played a key role in plants defensive response to pests (Corcuera, 1984, 1993; Duffey and Stout, 1996; Zhang *et al.*, 2013). However, during long-term evolution, insects in nature have gradually adapted. Studies have reported that the GST gene expression of insects may be induced by some plant secondary substances during the insects feeding process. In *S. litura*, xanthotoxin as a plant secondary substance induced the up-regulated expression of *SlGSTo1*, *SlGSTs1*, *SlGSTs3*, *SlGSTe1*, and *SlGSTe3* (Huang *et al.*, 2011). After feeding non-lethal doses of gramine, the expression levels of seven GST genes in *Nilaparvata lugens* (Stål) nymphs were increased (Yang *et al.*, 2021). When insect GSTs were induced to varying degrees by plant secondary substances, insect resistance was also enhanced. The overexpression of GSTs was closely related to insect resistance. There were two possible mechanisms by which GST expression increases: increased mRNA levels and gene amplification

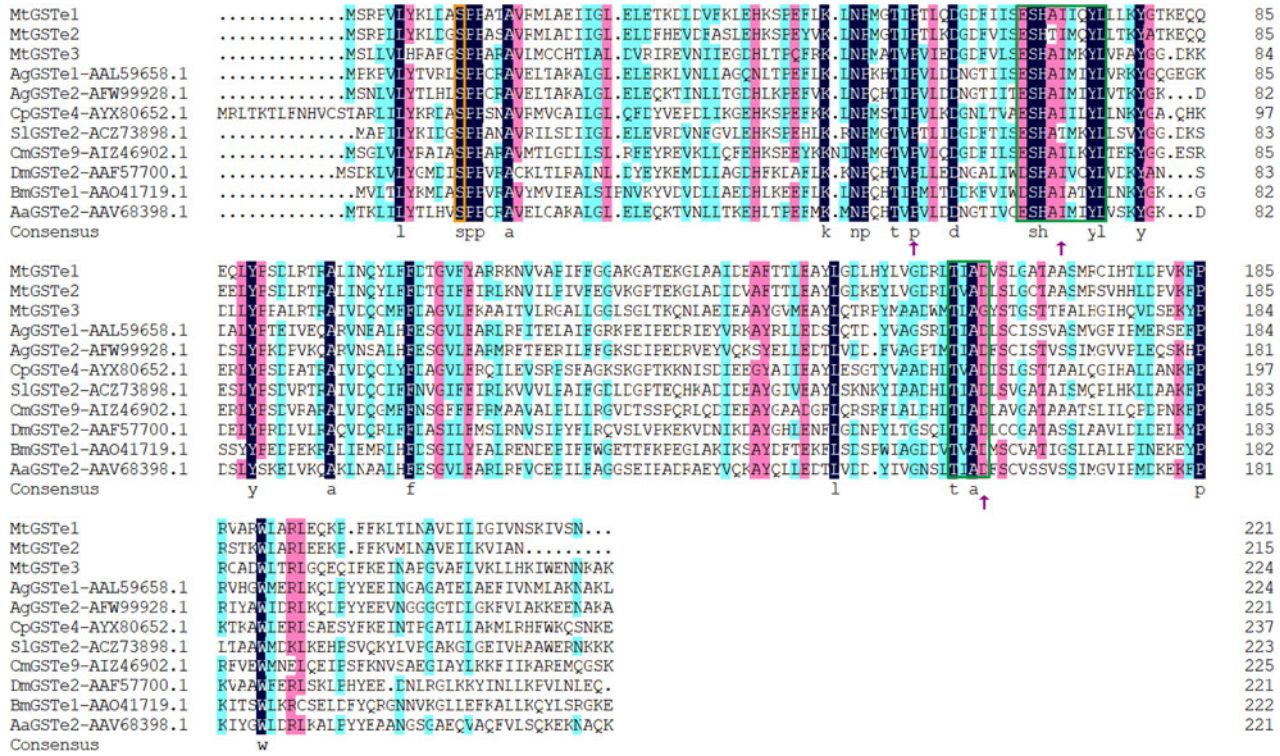

Figure 2. Amino acid sequences alignment of GSTe from *M. troglodyta* and other insects. Orange box indicated the catalytic residues; green box indicated conserved motif; purple arrows indicated active site. Mt, *Micromelalopha troglodyta*; Aa, *Aedes aegypti*; Dm, *Drosophila melanogaster*; Cp, *Cydia pomonella*; Ag, *Anopheles gambiae*; Cm, *Cnaphalocrocis medinalis*; Sl, *Spodoptera litura*; Bm, *Bombyx mori*.

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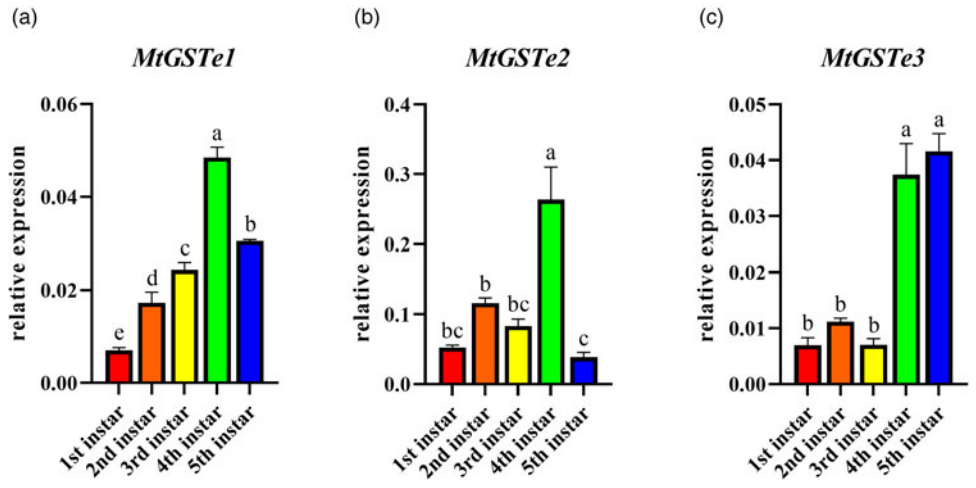

Figure 3. Expression levels of three epsilon-class *MtGSTs* in different instars. The standard errors of the means ($n = 3$) were indicated by the vertical bars. The different letters on the bars indicate the significantly difference in the means ($P < 0.05$).

(Chen and Gao, 2005). As has been reported in *Plutella xylostella* (Linnaeus), the enzyme encoded by the *PxGST3* gene could degrade organophosphorus pesticides, and its increased expression was related to resistance (Huang *et al.*, 1998). In *D. melanogaster*, phenobarbital inducted *GSTd21* and *GSTd1* mRNA (Tang and Tu 1995). In this study, our results showed that the mRNA expression levels of the three *MtGSTe* genes in the fat bodies obviously increased at the highest concentration of tannic acid, suggested that the three genes might participate in the regulation of insect resistance to plant secondary substances such as tannic acid. Tannic acid is synthesised by plants to resist attack by herbivorous insects as a major secondary substance. It is a kind of plant polyphenols which is widely distributed in plants (Cheng *et al.*, 2015). Cheng *et al.* (2015) reported that tannic acid at the concentrations of 0.001, 0.01, and 0.1 mg ml^{-1} had an induction effect on the expression of *MtGSTd1* mRNA in the fat bodies and midguts of *M. troglodyta*, while tannic acid at the concentrations of 1 and 10 mg ml^{-1} had no significant effect. Tang *et al.* (2020) reported that the expression of *MtGSTs1*, *MtGSTd2*, *MtGSTz1*, *MtGSTo1*, and *MtGSTt1* mRNA in the fat bodies and midguts of *M. troglodyta* was affected by tannic acid at five different concentrations to different degrees. However, the response of three *MtGSTe* was not exactly the same as that of other GST genes. This may mean that in association with tannic acid resistance, there are some differences in the functions of the *MtGSTe* and other GSTs found in *M. troglodyta* tissues, which needs to be determined by further research. Although these three *MtGSTe* genes were expressed in all five tissues, this paper mainly studied the response of three *MtGSTe* genes in the midgut and fat body because they are the primary detoxification organs of insects (Després *et al.*, 2007; Arrese and Soulages, 2010; Liu *et al.*, 2017).

However, it has also been found that in some cases, the expression of insects GST gene was inhibited by plant secondary substances. For example, studies found that the expression of *NlGSTd1* gene was inhibited by feeding diets containing 8.0 µg ml^{-1} ferulic acid or non-lethal doses of gramine (Yang *et al.*, 2017, 2021). Carvacrol could inhibit the expression of three detoxifying enzymes genes in *L. dispar* larvae, including GST (Chen *et al.*, 2021). These may be due to the plant secondary

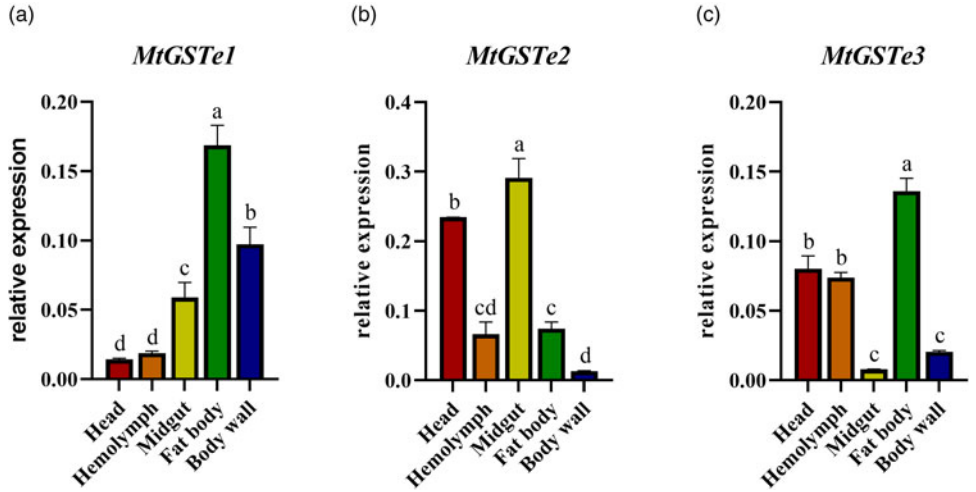

Figure 4. Expression levels of three epsilon-class *MtGSTs* in different tissues. The standard errors of the means ($n = 3$) were indicated by the vertical bars. The different letters on the bars indicate the significantly difference in the means ($P < 0.05$).

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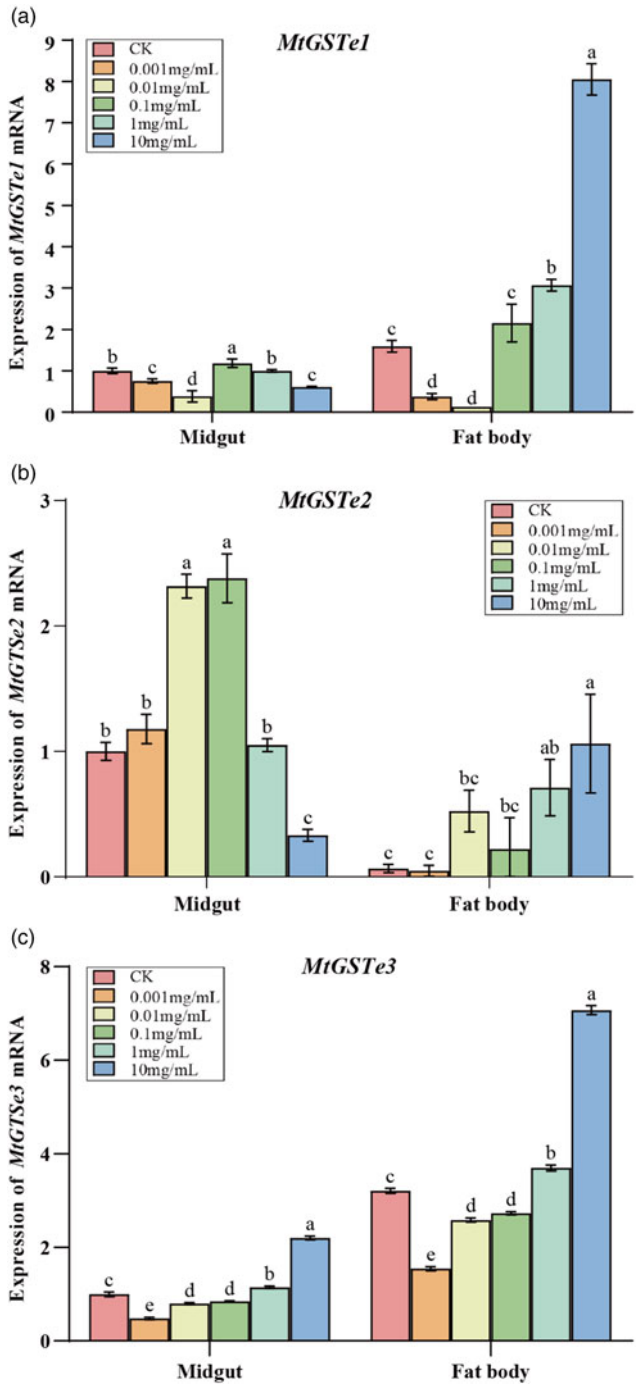

Figure 5. Response of three epsilon-class *MtGSTs* to tannic acid. The mean expression in each treatment was shown as a fold change compared with the mean expression in the control. The standard errors of the means ($n = 3$) were indicated by the vertical bars. The different letters on the bars indicate the significantly difference in the means ($P < 0.05$).

substances causing damage to phytophagous insects, as defences for the plant.

In summary, we cloned three full-length *MtGSTe* genes from *M. troglodyta* and analysed the effects of tannic acid on the mRNA expression of these three genes. Our study enhanced the understanding of the induction and the interaction between plant secondary substances and phytophagous insects at a deeper level, which provided a theoretical basis for finding new ways to control *M. troglodyta*. However, the expression regulation mechanism of GSTs needs to be further elaborated.

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Competing interests. None.

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